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Editorial

Vikas Jain

Department of Pharmaceutics, JSS College of Pharmacy, JSS Academy of Higher Education & Research, Mysuru, Karnataka, INDIA.

25th Annual National Convention of Association of Pharmaceutical Teachers of India (APTI) was organized by JSS College of Pharmacy, JSS Academy of Higher Education & Research, Mysuru from 2nd September to 4th September 2022 at JSS Medical Institutions Campus, Mysuru. The theme of the conference was **"Empowering Academia for Advancing Pharmacy Education"**. About two thousand pharmacy professionals, students, industrialists, scientists, and leaders from different states of the country took part in this three-day event. Among the participants, we received more than 60 full text manuscript from multiple streams to be considered for publication in **APTICON 2022: Special Issue of Indian Journal of Pharmaceutical Education and Research (IJPER),** which is an official journal of Association of Pharmaceutical Teachers of India **(APTI)**. Around 150 reviewers were invited to voluntarily participate in the peer review process and after multiple rounds of revisions and addressing reviewer's comments, 22 articles were accepted for publication. The shortlisted 22 articles were a mix of original research, review manuscripts, clinical reports, case studies and review of guidelines. We accepted 7 manuscripts from pharmaceutics and technology stream, 4 from pharmaceutical chemistry and analysis, 3 from pharmacognosy and biotechnology, 6 from pharmacy practice and 2 from regulatory affairs. On behalf of local organizing committee and editorial board, I congratulate all authors for successfully publishing their research work.

Correspondence:
Dr. Vikas Jain
Guest Editor, Department of Pharmaceutics, JSS College of Pharmacy, JSS Academy of Higher Education & Research, Mysuru-570015, Karnataka, INDIA.
Email: vikasjain@jssuni.edu.in

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Review Article

Orphan Drug Pricing and Cost Trends in USA: An Analysis of Impact of Orphan Drug ACT

Sangita Mishra[1], Venkatesh Madhugiri Prakash[1,2,*]

[1]Department of Pharmaceutics, JSS College of Pharmacy, JSS Academy of Higher Education and Research, SS Nagar, Mysuru, Karnataka, INDIA.
[2]Guest Assistant Professor, Faculty of Pharmaceutical Sciences, UCSI University, MALAYSIA.

ABSTRACT

Objectives: The study analyses the impact of incentives in Orphan Drug Act on Orphan Drug revenue strategies of pharmaceutical and biotechnology companies in USA and proposes policies and steps to address the same. **Results:** There are 389 orphan drugs in circulation as of 2019 with average price of $32,000; prices ranging between $6,000 till more than $500,000. 39% of the marketed drugs costing more than $100,000 treats 23% of patient population. Out of 1.8 million treated patients in 2019, only 0.1% of patients received treatment with drugs having cost greater than $500,000. Orphan drug research and development spending by pharmaceutical and biotechnology companies was 11% of total expenditure of the companies in 2019. The high cost of orphan drugs remains an issue as overall 10% of impacted patient population receiving treatment. **Conclusion:** The Orphan Drug Act of 1983 has introduced various incentives for pharmaceutical companies to invest more in orphan drug research. It has been observed that there is increase in investment as well as orphan drug approvals because of the incentives and grants. Orphan Drug development is not aligning with the provided incentives and overall cost overall remains high, and availability of treatment is not as expected due to the high costs. Incentives to manufacturers needs to be balanced with treatment availability based on affordable pricing to ensure increased research and higher therapeutic coverage.

Keywords: Orphan Drug Act, Orphan drug, Rare Disease, Volume Based Contract, Outcome Based Contract, Value Based Pricing.

Correspondence:
Dr. Venkatesh Madhugiri Prakash[1,2]
[1]Associate Professor, Department of Pharmaceutics, JSS College of Pharmacy, JSS Academy of Higher Education and Research, SS Nagar, Mysuru-570015, Karnataka, INDIA.
[2]Guest Assistant Professor, Faculty of Pharmaceutical Sciences, UCSI University, MALAYSIA.
Email: venkateshmpv@jssuni.edu.in

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INTRODUCTION

Any disease that affects less than 200,000 people in USA or any disease for which research and development costs are not expected to be recovered are generally regarded as "rare disease." There has been substantial progress in development and approval of drugs required for treating rare diseases in the last 40 years. These drugs are referred to as "Orphan Drugs". But significant unmet medical need still exists not only in USA but around the globe and almost 7000 rare diseases effecting millions of people remain to be treated with proper therapeutics.[1]

As there are unknown risks associated with development of orphan drugs; due to low patient population the economic benefits of cost recovery are difficult due to low revenue potential, as a consequence various incentives are provided by the Federal Government to promote research on rare diseases.[2] As mentioned in the provisions of The Orphan Drug Act (ODA) of 1983, US Government provides incentives for research and marketing of orphan drugs which includes research and clinical trial grants for academic and corporate sponsors of rare disease research, 25% tax credit on clinical trial expenditures, Prescription Drug User Fee Act (1992) marketing application fees waiver, access to FDA fast track approval, access to FDA's Investigational New Drug Program and pre-approval mechanism of drugs under development, and a seven-year market exclusivity provided by FDA for designated orphan drugs. During the seven-year period, FDA will not approve any new or generic drug application for the same disease or same product.[3]

The economic incentives are not sufficient to promote innovation in the long run and are also or unable to address the orphan drug pricing and treatment cost for patients and insurance companies.[4-6] From a value point of view, patients end up paying indirectly for orphan drug development through taxes and directly for purchasing the treatment.[7] Lack of significant development in orphan drug research area despite economic benefits to sponsors and increasing financial burden to all stakeholders,[8] due to rising cost of treatment is a major concern in the society. It is important to address both the treatment affordability of patients to increase therapeutic coverage as well as required incentives to promote research and development of orphan drugs.[9,10]

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MATERIALS AND METHODS

Analysis was performed on Orphan Drug designations and approvals obtained from Orphan Drug Database and Orange Book Database of FDA. Patient population and revenue information were taken from IQVIA reports. The information gave a view of orphan drug approval and pricing landscape in USA.

RESULTS

There has always been a lag between patient needs and available treatment. Along with that, rising cost of treatment, lack of adequate insurance coverage and premium pricing of drugs are contributing to bigger issues.[11] The primary health care cost factors associated with the expense of treating rare indications are inpatient care and cost of prescription medications. A small population of patients who require a lot of treatment resources often cause the average treatment cost to be skewed.[12] In addition, the significant degree of variation in healthcare needs, as well as the kind and cost of therapy, makes it difficult to conclude the incurred cost per patient or as per rare indication.[13]

Orphan Drug Cost Trends

As illustrated in Figure 1, in 2019, treatments ranged in cost from $6,000 to $500,000 per year, with an average yearly cost of $32,000 for each patient who received an orphan drug treatment. 389 drugs were in circulation as in 2019.[2,4,14]

As per insurance or payer data, there has been increase in patient population receiving treatment with annual cost greater than $1 million. 77% of patients with population of about 1.4 million, received treatment with annual cost up to $100,000 out of which 52% of patients with a population of 0.94 million, received treatment with annual costing less than $50,000.[15]

Annual cost of treatment with 39% or 152 of listed orphan drugs cost higher than $100,000 but covers only 23% or 0.4 million of patient population. Hence, treatments with high costs are prescribed to a smaller number of patients. Specialty treatments involving gene and cell therapies are costlier with pricing starting from several thousand dollars. Treatments with annual cost more than $500,000 are extended to less than 0.08% or 0.014 million of treated patient population.

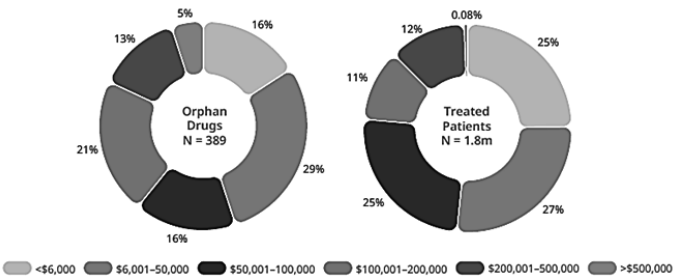

Figure 1: Orphan Drugs and Patients Treated by drugs with an orphan indication in 2019 by Annual Drug Cost Bands. Adapted from Orphan Drugs in the United States: Rare Disease Innovation and Cost Trends through 2019: IQVIA Institute for Human Data Science. Dec 2020.

An orphan drug becomes a blockbuster if it costs $100,000 annually covering 10,000 patients and has the capability of generating $1 billion revenue annually. Few examples are Neurontin (Gabapentin) from Pfizer, Gleevec (Imatinib) from Novartis, Alimta (Pemetrexed) from Eli Lilly and Spiriva (Tiotropium) from Boehringer Ingelheim.[13,15]

Orphan Drug Price Growth Trend (1993-2017)

When comparing orphan drug price trend of 25 years, as illustrated in Figure 2, it is observed that increase in orphan drug price increases have been slower than non-orphan drug price for much of the decade of 2010, while being higher than the market in the 1990s.[2,15] The higher price growth is noteworthy considering that many orphan medications licenced in the 1990s were older generic drugs that were repurposed, and their pricing were adjusted to the new targeted patient demographics.[16] Drug prices have fluctuated across the market, but those that gain an orphan designation (whether novel or repurposed) have continuously increased at a slower rate than the market.[17]

DISCUSSION

Factors contributing to high price of Orphan Drugs

Concentration of research on specific therapeutic class

Oncology contributed to 32% of orphan designations while other individual therapeutic classes hardly crossed 10%. As a result, only oncology has shown growth in orphan drug designation which caused overall growth in orphan drug market.[18] The concentration of research in the field of oncology makes it the rapidly growing and highly lucrative therapeutic class. As a result of this, other areas of rare diseases are not given much attention. This results in low availability of treatment for certain disorders thereby raising the overall cost due to demand of drugs.[19]

Less Payer coverage and increasing pressure on insurance companies for increasing cover

Due to recent scientific advances and regulatory flexibility, there are significant number of orphan drugs awaiting approval and more are in the budgetary pipeline of pharmaceutical companies.[20] Orphan Drugs being good revenue generator segment, there is increased focus of manufacturers and sponsors to invest more. Previously, rare disease treatment coverage by payers was not significant and there was no clear roadmap for the payer companies for addressing rare disease treatment coverage.[21] Payers are now confronted with additional issues in the management of orphan pharmaceuticals as a result of demand from patient advocacy organizations, clinicians, and plan sponsors to increase access and reduce healthcare spending.[22] There has been no new policy brought in by the payer companies

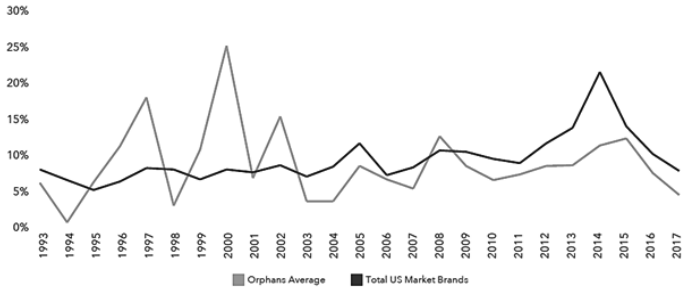

Figure 2: Average price growth trend, orphan versus branded drug market, 1993-2017. Adapted from Orphan Drugs in the United States: Exclusivity, Pricing and Treated Populations. IQVIA Institute for Human Data Science. Dec 2018.

to address rare disease coverage and it is expected to follow pathways similar to that of non-orphan and specialty drugs. The covered benefits may not be able to address needs as per specific orphan conditions which may not reflect as an efficient price control or treatment benefit outcome.[23]

Lack of competition due to Orphan Drug Exclusivity

The FDA is not permitted to approve a new brand name or generic medicine application for the same product and for the same rare disease indication once a product has been granted orphan drug exclusivity of seven years.[24] However, a medicine can be approved for use in more than one indication, and no restriction is there on drugs that can be authorized for a given disease. Patients may profit from the availability of more therapy alternatives and price reductions brought about by increased competition if more medicines are allowed for certain rare diseases.[24] But non availability of branded alternatives still causes monopoly of specific brands thereby reducing the perceived benefit of therapy alternatives. But removing the exclusivity clause may remove a key incentive for investment in orphan drug research.[24]

Higher cost of perceived value of treatment

Individual cost of treatment is highly dynamic since many biologic agents, and expensive orphan drugs, fall under the category of "tier 4" drugs,[25] for which patients are required to cover between 20 and 33 percent of total costs under the increasingly prevalent private coinsurance-like plans in the United States.[26] Although more orphan pharmaceuticals enhance patients' health and quality of life, the expense of newer drugs restricts access of patients to quality healthcare.[27]

Non-alignment of incentives with perceived societal benefits

With new diagnostic techniques and understanding of specific causes of disease there have been rise in the number of orphan drug patients.[28,29] Whereas supply of effective treatment is still not as expected. The high cost of available treatments causes significant financial burden on patients, government aided healthcare programs and private insurers.[30] Lack of alignment of research grants and incentives with societal demographic and economic aspect has caused benefits to be skewed in favor of companies and neglected patient priorities. It is important to address every aspect of regulatory, clinical, and economic landscape to ensure rare disease patients get access to quality, affordable and effective treatments.[31]

Lack of clinical data to assess cost benefit effectiveness

There is lack of information about overall health status of a patient and pre-market cost strategy of a company related to Orphan Drugs. Due to this proper cost-benefit effectiveness assessment is not of proper quality.[32] Thus, it is still crucial to weigh the costs of economic incentives on orphan development initiatives against their overall advantages and enhancements in treatment outcomes. If not, there is a chance of exacerbating market imperfections and maintaining inefficiencies. Given the diversity of the medications authorized for orphan indications, study results should be evaluated with caution.[32]

Freedom of pricing and minimal competition

Drug manufacturers in the US negotiate with patient organizations and insurance companies but can set own initial prices as US market allows "free pricing". Additionally, competition between manufacturers has little impact on regulatory standards compared to price caps.[32] Due to the challenges in proving therapeutic equivalence and the rarity of large-scale trials by generic manufacturers, many orphan medications, particularly biotechnology products, have minimal competition even when exclusivity restrictions expire.[33] Hence these generics are still expensive with prices being only 20-25% less expensive than branded biologics.[34]

Proposals to address pricing issue

Adopt best practices from other regulated markets

Initiatives from Japan could be taken into consideration to solve the issue of commercial pharmaceuticals that have benefited from various incentives for orphan drug development (such as research and development support funds, fee waivers, and tax credits). Pharmaceutical companies in Japan are required to charge a 1% sales tax on orphan drugs with yearly gains of more than 100 million yen until the obtained government subsidies have been returned.[35] This provision has not caused any roadblock in orphan drug development and there has been approval of almost 100 orphan pharmaceuticals in the twelve years since the policy's enactment in 1993. Incentives given to one R&D company is returned by marketing company. This tax clause might potentially be transferrable. Profitable orphan pharmaceuticals would no longer receive government funding in this fashion, but less successful orphan drugs would continue to receive incentives.

Redefine Rare Disease and Orphan Drugs

Senators Metzenbaum and Kassebaum planned to introduce legislation that would clarify the definition of an orphan drug at the start of the 1990s.[36] The terms "orphan medication" and "rare disease" need to be redefined given the current state of medical technology, which heralds the advent of customized therapy, and the ageing of the population, which causes the emergence of new rare diseases By pursuing this goal, it would be possible to limit the therapeutic market for orphan pharmaceuticals, generate more uniform investment distribution among different therapeutic areas of rare diseases, and manage the number of indications for each condition.

Development of patient registry funded by Federal Government

In order to address the needs of patients, doctors, and payers and to effectively analyze the cost effectiveness of therapies, more sophisticated systems for collecting and analyzing observational data are required These data systems ought to be created to be able to record patient-reported outcomes that represent therapeutic effects on the patient and their families. By supporting rare condition registries, the federal government may speed up efforts to improve evidence generation with the aid of illness and patient groups.[24,36]

Orphan Drug price reduction through price control

Spending on orphan medications has risen with the expansion of orphan drug development. Increases in orphan medication volume and their greater cost are clearly visible in available data published by companies and market research agencies. The average annual orphan medication cost in 2017 was 25 times higher than that of non-orphan drugs.[37] An analysis by Institute for Clinical and Economic Review (ICER) revealed that there was huge gap in pricing of top 100 medicines by sales based on treated indications (Cost of therapy for orphan drugs was $150,854 which was 4.5 times that of nonorphan drugs which was $33,654 per patient). This gap needs to be addressed to effective price control measures.[37]

Prevalence and adoption of contracts based on supplied drug volume

Through guaranteed coverage, by encouraging participation and education of patient and payer, by ensuring fair market access and cost utilization, and by simplifying contractual clauses, contracts based on drug volume can be another strategy that could help the commercialization of rare drug products. While the government has historically utilised this type of contract to buy huge quantities of pharmaceuticals (such as vaccines), a similar strategy might be used in the orphan drug market. The government or a consortium of private payers might actively bargain to buy adequate orphan drugs to cover most or all of qualified patients with a specific rare ailment under this scenario. This will provide a clear picture of effected population and data around treatment coverage. A price specific to the concerned orphan indication could be established by the government or a private body consortium under the contract. A simple and understandable contract, better patient accessibility, and utilisation that can be predicted would be advantageous to manufacturers as this will help in better price forecast and cost planning.[36] The adaptability of this policy by manufacturers needs to be seen as it is indirectly taking out price fixing authority.[37]

Patient Assistance Programs (PAP's)

Many patients find great value in patient assistance programmes, which are often the cornerstone of orphan medication company marketing campaigns. The level of financial aid varies depending on the patient's specific income and could involve other organizations. These initiatives provide eligible patients with a limited supply of free medications. Initiatives provided and managed by National Organization of Rare Diseases (NORD), help covered patients with their insurance premiums and co-payments. 34 patient support initiatives that NORD manages on behalf of orphan medicine producers are listed on its website.

CONCLUSION AND SUMMARY

Although multiple factors like scientific advances, regulatory flexibility, lack of competition and accelerated FDA approval pathways are facilitating orphan drug development and increasing the orphan drug market attractiveness to sponsors, still high pricing remains an issue. An analysis of orphan drug landscape throws conflicting results about lack of treatment availability for majority of rare diseases despite increasing innovation and market approvals. Additionally, attention is being redirected away from economic incentives that were implemented to encourage innovation and toward other elements that will encourage long-term and economically viable investments. By lowering treatment and insurance affordability and accessibility, increasing financial strain threatens to undermine the anticipated benefits of orphan drug innovation and exacerbate the already existing problem with premium pricing. Despite these difficulties, patients and their families frequently have no other option but to rely on orphan medications. As a result, it is crucial to continue developing and promoting safe and efficient medicines to meet the health demands of rare disease patients. Adoption of proposed policies may be able to address the high price concern. Price restriction would not impede the research and development of orphan treatments because it would affect all pharmaceuticals,

not just those that target orphan diseases. Additionally, concerns over profitable and exorbitantly priced orphan medications would be addressed. The fundamental inducement for the development of orphan pharmaceuticals, the seven-year exclusivity period for orphan drugs, would be unaffected. Finally, producers would continue to have the opportunity to generate significant profits, enabling returns on investment and economic expansion.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

JSSCP: JSS College of Pharmacy; **JSS AHER:** JSS Academy of Higher Education and Research.

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Review Article

Transmissible Spongiform Encephalopathy and its Regulations

Monika S[1], Balamuralidhara V[2,*]

[1]Department of Pharmaceutics, Regulatory Affairs Group, JSS College of Pharmacy, Mysuru, JSS Academy of Higher Education and Research Mysuru, Karnataka, INDIA.
[2]Department of Pharmaceutics, JSS College of Pharmacy, Mysuru, JSS Academy of Higher Education and Research Mysuru, Karnataka, INDIA.

ABSTRACT

Bovine Spongiform Encephalopathy (BSE) has a place with the uncommon cluster of continuously progressive neurological infections identified as Transmissible Spongiform Encephalopathies (TSEs). TSE sicknesses are described by long incubation periods ranging from a while for transmissible mink encephalopathy, to several years for BSE. TSE consistence testaments are a sort of CEP (Certificate of Suitability). During the 1980s, when the principal TSE pandemic happened, researchers started centering a greater amount of their time and work to figure out these circumstances. By 1996, a connection between the human type of mad cow illness, Creutzfeldt-Jakob infection, and BSE from ingestion of meat was found. With the connection between BSE and Creutzfeldt-Jakob being found, researchers affirmed that level transmission of TSEs from animals to people can happen. This is of extraordinary concern while working with specific animal-derived reagents in the lab since there is right now no fix or treatment for TSEs. Research recommends that TSEs are brought about by a strange variant of a protein called a prion (prion is short for proteinaceous irresistible molecule). Prion isoforms of the Prion Protein (PrP), are conjectured as the reason for transmissible spongiform encephalopathies, including scrapie, Chronic Wasting Disease (CWD), bovine spongiform encephalopathy and Creutzfeldt-Jakob Infection (CJD). Numerous materials utilized in labs are engineered or gotten from creature tissues that don't represent a gamble of getting a prion illness so not all items will be TSE ensured.

Keywords: Degenerative disorder, Prion, Transmissible, Bovine, Spongiform, Regulation.

Correspondence:
Dr. Balamuralidhara V
Associate Professor and Head, Department of Pharmaceutics, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Mysuru-570015, Karnataka, INDIA.
Email id: baligowda@jssuni.edu.in

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INTRODUCTION

BSE is a dynamic neurological problem of cattle that outcomes from disease by an unusual contagious agent called a Prion. The idea of the contagious specialist isn't surely known. As of now, the most acknowledged hypothesis is that the agent is a changed type of a typical protein known as prion protein. Because of reasons that are not yet perceived, the typical prion protein changes into a pathogenic (unsafe) structure that then harms the focal sensory system of cattle.[1]

Prion infections, also known as TSEs, are a group of fascinating degenerative brain disorders characterized by microscopic holes that give the mind a "spongy" look (Figure 1). When viewing cerebrum tissue using a magnifying glass, these apertures ought to be apparent.[2] Of all the human TSEs, Creutzfeldt-Jakob Disease (CJD) is the most well-known. A rare form of dementia affects around 1 in 1,000,000 people annually. Kuru, Fatal Familial Insomnia (FFI), and Gerstmann-Straussler-Scheinker (GSS) disease are examples of further human TSEs. In Papua New Guinea, Kuru was recognized in members of a split clan, but it has since all but disappeared. FFI and GSS are extremely rare hereditary diseases that have only been linked to a small number of families worldwide. Variant CJD (vCJD), a different type of CJD, was first described in 1996 and has since been discovered in Extraordinary England and a few other European countries.[2]

Causative agent

It is well acknowledged that prions are the TSEs' causal agents. The term "prions" refers to aberrant, infectious pathogens that can cause the odd collapse of specific, routine cell proteins known as prion proteins, which are found most abundantly in the mind. These typical prion proteins' components are still not fully understood. The odd collapse of the prion proteins causes damage to the brain as well as the disease's recognizable symptoms. The majority of prions are consistently, swiftly, and somewhat fatal.[3]

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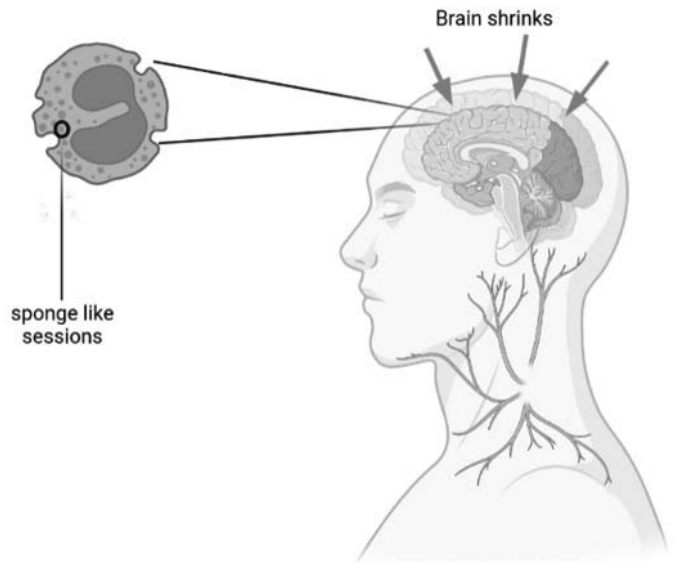

Figure 1: Prion protein affecting brain causing sponge like appearance in brain.

Kinds of prion illnesses include

Creutzfeldt-Jakob disease (CJD)

A degenerative mental ailment called Creutzfeldt-Jakob disease (CJD) causes dementia and finally results in death. Creutzfeldt-Jakob disease symptoms can match those of other mental illnesses that resemble dementia, such Alzheimer's disease. However, the disease Creutzfeldt-Jakob generally advances far more quickly.[4] Process of formation of Creutzfeldt-Jakob disease is shown in Figure 2.

When some people in the Unified Realm developed variation CJD (vCJD) after consuming meat from sick cattle, the disease gained widespread attention in the 1990s. Nevertheless, contaminated meat hasn't been linked to "exemplary" Creutzfeldt-Jakob disease. Many CJDs are serious but at the same time extremely fascinating. Every year, approximately one to two cases of CJD per million people are discovered around the globe, most usually in older adults.[4]

Variation Creutzfeldt-Jakob disease

Variation A highly uncommon, fatal virus known as variant Creutzfeldt-Jakob disease, or vCJD, can infect a person for a very long time before damaging synapses and rendering them incapacitated. The main cause of vCJD is consuming excessive amounts of hamburger products contaminated with the intoxicating specialist of cow-like spongiform encephalopathy (BSE).[5] The disease known as variation CJD (vCJD) is not the same as example CJD (frequently just called CJD). It differs from typical CJD in terms of clinical and pathologic characteristics. The quality of the prion protein has a unique genetic characteristic for each disease as well. The two issues are perpetually fatal cerebrum illnesses with oddly protracted brooding times that are measured in years.[5,6]

Gerstmann-Straussler-Scheinker disease

One of these is the prion infection known as Gerstmann-Straussler-Scheinker disease (GSS). The sensory system is impacted by a class of disorders known as prion diseases. The core elements of GSS include the cerebellum, an area of the brain that controls muscular tone, coordination, equilibrium, harmony, and different phases of dementia. Changes in the PRNP's quality are the cause, and autosomal inheritance predominates.[7] The primary symptoms of the Gerstmann-Sträussler-Scheinker sickness are a modest lack of coordination and little difficulty speaking (dysarthria). Shakiness, difficulty walking, and clumsiness can all be signs of loss of coordination. Planning deliberate developments may be difficult for those who are affected (ataxia). Slurred speech can be the beginning of discourse issues, which can progress to severe dysarthria, in which people have difficulty speaking and other people have difficulty understanding what they are trying to say. Dysphagia, or issues gulping, may be brought on by an inability of the gulping muscles to coordinate.[8]

Origin of Bovine Spongiform Encephalopathy

The reason for the first case or instances of BSE stays a puzzle. Sheep scrapie or a formerly undetected inconsistent TSE have for quite some time been considered as competitors, however no persuading proof to help these recommendations has become known. We present another hypothesis, with three related speculations:

(1) That BSE was obtained from a human TSE (prion illness);

(2) That the course of disease was oral, through creature feed containing imported mammalian unrefined components tainted with human remaining parts; and

(3) That the beginning was the Indian subcontinent, from which a lot of mammalian material were imported during the significant time span. Human remaining parts are known to be integrated into feast made locally, and may in any case enter sent out material.

Further examinations are required into the wellsprings of creature results utilized in creature feed produce, and into the contagiousness of human TSEs to steers.

Risk of TSE and BSE in Pharmaceutical Products

There is a chance that the completed pharmaceutical dosage form for human consumption contaminated with infected animal derived goods could spread TSE or BSE to people. The use of goods or materials derived from animals is occasionally used in pharmaceutical preparations such as finished dosage forms, active pharmaceutical ingredients and their starting components, and primary packaging materials. For instance, the manufacture

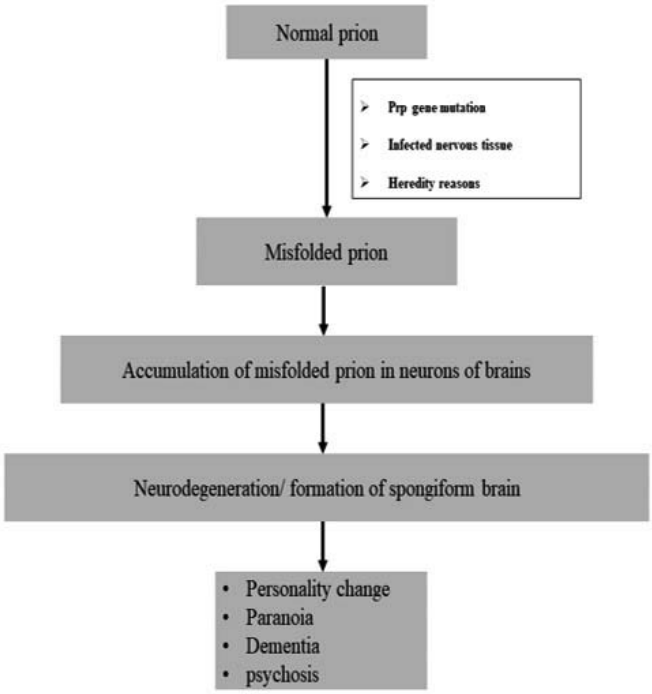

Figure 2: Creutzfeldt-Jakob disease formation process.

of API and the raw ingredients for API use animal proteins, enzymes, and amino acids.[9,10]

The likelihood of TSE/BSE transmission is also increased by major packaging materials such gelatin capsules made from animal fat. When the source material for a biotechnological product comes from animals or goods obtained from animals, such as serums, blood products, and vaccines, there is a considerable risk involved.[10] Additionally, there is a chance that TSE or BSE could spread through the tools or resources used to handle items with biological origins or those made from animals. For instance, culture media employed in media fill research in reactors.[10,11]

TSE/BSE Regulation in Us

The FDA issued a final directive in 1997 banning the use of the majority of mammalian protein in the preparation of animal feed for ruminant animals like cows, sheep, and goats. The standard does not forbid the use of mammalian protein as an ingredient in feed for non-ruminants, but it does call for management and control systems to ensure that such use does not contaminate feed for ruminants during manufacturing or transportation. In 2008, the FDA strengthened the 1997 rule by limiting the use of the riskiest dairy bovine tissues in ALL animal feed. These high-risk cow materials are the brains and spinal cords of dairy cattle that are 30 months old or older, as well as the entire remains of steers that have not been examined and approved for human use, unless the cadavers can be proven to be those of steers that are younger than 30 months old or the brains and spinal cords have been removed.[12]

Figure 3: Member state classification based on TSE risk.

FDA has made Laws/Regulations to suppress the chances of development of TSE and BSE. Following are the CFR made to regulate animal related process and products in US.

9 CFR 94.18: Restrictions on importation of meat and edible products (USDA)

21 CFR Part 589.2000: BSE/Ruminant Feed Regulations.

21 CFR Part 589.2001: BSE/Substances Prohibited from Use in Animal Food or Feed.[12]

9 CFR 94.18: restrictions on importation of meat and edible products

Importation of meat and edible products are strictly regulated in US. Certain edible products can get into US market if they are proved to meet all the requirements of the Food and Drug Administration. Given that all essential requirements are satisfied, the following products made from cattle may be brought into the US without fear of contracting BSE:

Milk and milk products

(Except for perfectly isolated meat) Boneless skeletal muscular meat that:

- Is derived from cows that underwent posthumous review and postmortem examination and were not subjected to a pitching cycle or dazzling using a device that performed butchery before injecting compressed air or gas into the skull orifice;
- Has been set up to prevent contamination by SRMs; and[13]

Import of meat and meat derived products from a negligible BSE risk regions are also regulated. Though the risk of infection of BSE/TSE is negligible, there is a door for infection if it is not properly regulated. US permits import of meat and meat derived product from a negligible BSE risk region only if the product meets certain requirements. The requirements are as follows;[14]

A. The export of the goods came from an area with a very low BSE risk.

B. If BSE has been found in one or more native cows in the area of negligible risk, the commodities were made from cows that were not allowed to be fed meat-and-bone meal or greaves made from ruminants.

APHIS has designated the exporting region as one with a low risk of BSE, and the original certificate confirming these designations is included with the commodities. A full-time, salaried veterinarian employed by the exporting region's national government, or a person who has been given this power by the veterinary services of that government, must issue and sign the certificate.[14]

21 CFR PART 589.2000: BSE/ruminant feed regulations

Any component of a mammalian species that contains protein is considered to be derived from mammalian tissues, with the exception of endless blood products, gelatin, fat containing about 0.15 percent insoluble pollutants, and fat subsidiaries. examined meat products that have been prepared for human consumption and then intensely handled for feed (such as used cellulosic food containers and plate scraps); milk products (constant milk proteins); and any product whose primary mammalian protein is totally made up of porcine or equine protein.[15,16]

The following steps must be taken by producers of goods intended for use in animal feed that contain or may contain protein obtained from mammalian tissues in order to prevent materials from being used in the diet of ruminants:

- "Do not forage to livestock or other ruminants" should be written on the supplies.
- Keep sufficient records to follow the materials from the time they are received, processed, and distributed, and make the copies available for the Food and Drug Administration to examine and copy.[16]

21 CFR PART 589.2001: BSE/substances forbidden from use in animal food or feed

The intent of this regulation is to further reduce the risk of the spread of bovine spongiform encephalopathy (BSE) within the United States by outlawing the use of certain components of cattle origin in the food or feed of all animals.[17]

Materials that should not be used in animal feed include

To generate mechanically separated beef, an excellently minced meat food item, the majority of the bone from attached skeletal muscle of dairy cattle corpses and sections of cadavers is mechanically separated.[18]

A steer's entire corpse that has BSE

Steers that are at least 30 months old and have developed minds and spinal lines;

- The entire corpses of cows older than 30 months, declared unfit for human consumption, and whose minds and spinal cords were either not properly removed or were not allowed to be fed to animals;[19]
- The existence of the BSE specialist in tissues is often not proven in stone by delivering testing to animals, frequently mice, and then watching the mice to see if they die and acquire the recognized brain tissue abnormalities.[20,21] Negative results (i.e., no alterations in the mental tissue in the infused mice) would just suggest that there was too much of the irresistible specialist present to have unwanted consequences, not that the material was entirely freed from the irresistible specialist. For up to 700 days, mouse vaccination focuses on eating most of the day to identify the specialist. Although this method does not precisely assess the infectivity of the infected material, special staining techniques can be employed to detect the presence of the abnormal prion protein in tissues (such as the brain).[21]

When a medical organization, like the ACMA, issues a BSE free Certificate, it certifies that the products mentioned have no prohibited ingredients and that the manufacturing and packaging processes were likewise pollution-free. The BSE Free Certificate can then be confirmed, validated, and legitimized for use in a foreign country after being guaranteed and apostilled.[22]

TSE/BSE Regulation in Europe

Through Scrapie in the middle of the 1980s, Europe was the first region to be victimized by the TSE/BSE situation. The guidelines were then initially evaluated from Europe. In any case, WHO also warned the globe about the epidemic in 1997. In light of this, WHO also entered the scene with the aim of educating and guiding people all around the world. Canada and the USFDA also distributed their rules.[23]

The European Parliament and Council passed Regulation (EC) No. 999/2001 on May 22, 2001, which established guidelines for the avoidance, reduction, and abolition of specific transmissible spongiform encephalopathies (Figure 3).[24] It lays out guidelines for the European Association to shun, exterminate, and govern Transmissible spongiform encephalopathies (TSEs). Additionally, it deals with the manufacture, distribution, and occasionally the

sale of creatures and creature-related goods.[24,25] According to their level of risk of BSE, EU Member States or areas are ranked by the European Commission as follows:[24]

As per European Pharmacopeia, General Part 5.2.8., Chance Appraisal shows "Risk minimization as opposed to take a chance with disposal" as underneath. The total end of chance at source is seldom conceivable, proper measures and contemplations ought to be taken to deal with the gamble of communicating creature TSEs by means of restorative items imply the danger minimization instead of the gamble disposal.[23] The well-known cow illness, BSE, which has killed a sizable number of cows in the UK and the rest of Europe, raised the level of concern. As a result, actions have been taken across Europe, and the EU has issued early orders to restrict TSE/BSE. The EU administrative consistency mandates' Add-on I principles have given the note for direction the power to regulate throughout Europe.[23,26]

Among CEPs, TSE compliance certificates are one type (Certificate of Suitability to the European Pharmacopoeia). When handling products that might be contaminated with TSE, they are employed to maximize safety. The European Directorate for the Quality of Medicines has validated that any item that carries a TSE CEP is appropriately controlled under the pertinent monographs produced by the European Pharmacopoeia.[27]

The guidelines for combating, controlling, and eliminating TSEs in bovine and caprine wildlife are outlined in Regulation (EC) No 999/2001. It covers the development of living things, their release into the world, and in some circumstances, their trade. The Regulation also provides a legal basis for the classification of Part States and third-party nations or territories into those having a negligible BSE risk, a controlled BSE risk, and a subverted BSE risk based on their BSE infection status, as stated in Commission Decision 2007/453/EC3.[28]

The conditions for introducing live animals, embryos, eggs, and other products of animal conception into the Association are set forth in Annex IX of Regulation (EC) No 999/2001. The conditions for the imports of bovine animals are expressly stated in Chapter B of the Annex, which takes into account the BSE status of the third nations or areas. Furthermore, Chapter D of that Annex outlines the specifications for the creation of a verification regarding the TSE related risk in the health certification necessary for the importation into the Association of specific animal by-products and inferred items, including, among other things, handled animal protein.[28]

TSE RISK Assessment Strategies

Since there is no way to completely eradicate TSE/BSE agents, the European community has established sound risk assessment techniques. Additionally, because the TSE/BSE agents remain unaffected to heat besides additional methods of elimination, the only option is to minimize the danger. The European community has therefore created the following risk assessment techniques. Only if any product or material made from living or butchered animals is employed, then are the risk assessment methodologies applicable.[23]

The Scientific Steering Committee and its TSE/BSE ad hoc Group provided scientific guidance and risk assessments regarding transmissible sponge-like encephalopathies (TSE) in general and bovine sponge-like encephalopathies (BSE) in particular to the European Commission between 1997 and the beginning of 2003. This paper's main objective is to provide an easily understandable summary of six years' worth of BSE risk assessments to all relevant parties. As a result, it is planned to help preserve the EU-level BSE risk assessment procedure now that the SSC and the TSE/BSE ad hoc committee have finished their work. Additionally, the paper will give risk managers and other interested parties a clear introduction to BSE and every comprehensive SSC opinion adopted since 1997.[29]

CONCLUSION

The neurological condition known as Bovine Spongiform Encephalopathy (BSE), also referred to as mad cow disease, is lethal and incurable in cattle. The rare degenerative brain disorders known as Transmissible Spongiform Encephalopathies (TSEs), commonly referred to as prion diseases, are categorized by microscopic pits that give the brain a "spongy" presence. When brain tissue is examined, these holes are visible.

As soon as the health care sectors realized the importance of controlling the spread of this disorder there were many regulations which are made mandatory to be followed by the pharmaceutical sector manufacturers and distributors, as it is one of the ways of TSE infection. When raw materials from animals are used for the purpose of drug manufacturing, there are chances of those raw materials being infected by the unfolded Prion Protein. Hence it is mandatory to regulated and monitor the animal related raw material. Since then, there are certificates that the country provides to indicate the TSE free product and guidelines which must be met by the manufacturer in order to allow the product into the market. As such, because of all these stringent rules and regulations, TSE cases through pharmaceutical products have been reduced.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ACMA: Accreditation Council for Medical Affairs; **APHIS:** Animal and Plant Health Inspection Service; **API:** Active Pharmaceutical Ingredients; **BSE:** Bovine Spongiform Encephalopathy; **CEP:** Certificate of Suitability; **CJD:** Creutzfeldt-Jakob infection; **CWD:** Chronic Wasting Disease; **EC:** European Council; **EU:** European Union; **FDA:** Food and Drug Administration; **FFI:** Fatal Familial Insomnia; **GSS:** Gerstmann-Straussler-Scheinker; **PrP:** Prion Protein; **TSE:** Transmissible Spongiform Encephalopathy; **vCJD:** Variant Creutzfeldt-Jakob infection.

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Original Article

Assessment of Solid-State Behaviour and *in vitro* Release of Artemether from Liquisolid Compact Using Mesoporous Material as an Excipient

Archana Manjunath, Sayani Bhattacharyya*

Department of Pharmaceutics, Krupanidhi College of Pharmacy, Bangalore, Karnataka, INDIA.

ABSTRACT

Background: Artemether is a potent antimalarial drug used in the first-line treatment of multi-drug-resistant malaria. It belongs to BCS II, exist in different polymorphic forms, and exhibits incomplete absorption and low oral bioavailability owing to poor dissolution. **Aim:** The present study evaluates the effect of different mesoporous materials in the liquisolid compact for the augmentation of dissolution of the drug, and polymorphic stability. **Materials and Methods:** In the liquisolid compact Tween 80 was used as a non-volatile solvent for the dispersion of the drug, microcrystalline cellulose as carrier, and Syloid244FP and SyloidXDP as coating materials at different coating and carrier ratios. Eight such formulations were prepared. The formulated liquisolid compact was evaluated for precompression parameters, followed by direct compression process. The tablets thus prepared were assessed for hardness, friability, wetting time, *in-vitro* dissolution, and stability studies. Physico-chemical characterization was done to study drug excipient interaction, thermal behaviour, and surface characteristics. **Results and Discussion:** The study revealed that an increasing quantity of mesoporous material exhibited a better release profile compared to the pure drug. Good compressibility and tablet ability were observed at a carrier and coating materials ratio of 1:5. Compatibility between the drug and excipients was established from the FTIR study. Noteworthy findings of PXRD and DSC suggested the presence of artemether in metastable β form in the formulations. Short-term stability of the formulations was established. **Conclusion:** Hence, the approach of liquisolid compact using mesoporous silicas at a suitable excipient ratio could be an optimistic approach for the enhancement of dissolution and stability of artemether.

Keywords: Artemether, Liquisolid compact, Solid-state behaviour, Dissolution, Mesoporous silica.

Correspondence:
Dr. Sayani Bhattacharyya
Department of Pharmaceutics,
Krupanidhi College of Pharmacy,
Bangalore 560035, Karnataka, INDIA.
Email id: sayanibh@gmail.com

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INTRODUCTION

Artemether is an effective and rapidly acting antimalarial drug used in the first-line treatment of multi-drug-resistant malaria. It is methyl ether derivative of artemisinin. It is enlisted in the WHO list of essential medicines.[1] It has shown its efficacy against the resistant strains of *Plasmodium vivax* and *P. falciparum*.

Artemether belongs to BCS II and shows incomplete absorption and low oral bioavailability due to poor dissolution. It acts by interacting with the ferrous ion or ferriprotoporphyrin IX in the parasitic blood and generates cytotoxic radicals and acts as gametocidal agent. The clinical efficacy of artemether is greatly affected by its poor aqueous solubility which is a major limiting factor.[2]

Artemether exhibits two polymorphic forms – Alpha(α) and Beta(β). The β form has higher antimalarial activity due to the presence of endoperoxide linkage. But this linkage affects the stability of the molecule and makes its unstable to heat resulting in polymorphic changes.[3] Hence only solubility enhancement of artemether may not be beneficial to improve its bioavailability, it also requires preservation of the bioactive polymorphic form of the molecule.

Therefore, the present study proposes the formation of a liquisolid compact to preserve the bioactive form of artemether, and the use of different grades of mesoporous silica as coating material in the compact to increase the dissolution of the drug by improving the specific surface area. Formulation of liquisolid compact is a unique process that uses a non-volatile water-miscible solvent, coating, and carrier material to yield a free-flowing, readily compressible powder mixture of the drug. The non-volatile water-miscible solvent ensures molecular dispersion of the drug and is included as a drug solution in the preparation. Hence, the

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excipient used for liquisolid compact should have high porosity and liquid absorption capacity. Mesoporous silica has been widely used as a coating material in liquisolid formulations for its large specific surface area, great loading ability, good flow, and tableting properties with nontoxic and inert characteristics.[4]

Hence, the present investigation is based on the use of ordered mesoporous silica as adsorbent material for liquisolid compact of the model drug for the study, artemether, with an aim to achieve polymorphic retention and observe the effect on the dissolution profile of the drug.

MATERIALS AND METHODS

Artemether was procured from Strides Pvt. Ltd., Bangalore. Syloid 244FP was gifted from Grace Pharmaceuticals, Mumbai. Syloid XDP was obtained as a gift sample from Syngene, Bangalore. Rest all the analytical grade chemicals were purchased from SD Fine Chemicals, Bangalore.

Preformulation studies in liquisolid compacts of artemether

Solubility studies

The solubility studies of artemether in different non-volatile solvents like, PEG 400, PEG 600, tween 20, tween 80, propylene glycol, and glycerine were estimated. Excess of drug was added in the non-aqueous vehicle kept on a mechanical shaker for 24 h at room temperature.[5] A specific volume was withdrawn and diluted suitably in a mixture of 1N hydrochloric acid and water at a ratio of 1:3 and was estimated spectrophotometrically at 248 nm.[6] The process was carried out in triplicate.

Fourier Transform Infrared Spectroscopic Studies (FTIR)

The sample was sprinkled on a zinc solenoid crystal plate of Bruker ATR alpha instrument, the samples were scanned in region of 4000-400cm^{-1} and the spectra were recorded. The experimentation was carried out at 25.0 ± 0.5°C.[7] FTIR was performed for the pure drug and mixture of drug and mesoporous silica (Syloid 244FP and Syloid XDP) to detect any sign of interaction.

Estimation of liquid load factor for carrier and coating materials

Liquid-retention potential (Φ-value) is termed as the capability of a powder material to hold maximum amount of liquid per unit weight with the perseverance of its flow properties.[8]

An increasing volume (0.25 mL) of non-volatile solvent was added to a certain amount of carrier or coating material separately and thoroughly mixed in glass mortar. The amount of liquid that has been retained by the powder material with acceptable flow property was noted by measuring the angle of slide.

The following equation was used to determine the corresponding Φ-value

$$\Phi \text{ value} = \frac{\text{Weight of liquid}}{\text{Weight of Carrier}}$$

The liquid Load Factor (Lf) of the compact is obtained from the following equation, where Φca and Φco represents liquid retention potential of carrier and coating material respectively and R represents excipient ratio.

$$Lf = \Phi ca + \Phi co(1/R)$$

The excipient ratio (R) represents the ratio of the weight of the carrier (Q) and the coating (q) material.[9,10]

$$R = \frac{Q}{q}$$

Method of preparation of liquisolid compact

To prepare the liquisolid compact, a concentration of 8%w/v of artemether was dissolved in Tween 80. Microcrystalline cellulose (MCC) was used as carrier and whereas Syloid 244FP and Syloid XDP were used as a coating material. The estimation of the liquid loading factor was considered in the identification of the excipient ratio and the excipients were varied accordingly to maintain a constant tablet weight (250 mg) for all the experimental trials. The excipient ratio was varied from 2.5 to 15 and 8 such formulations were prepared as per Table 1, where the Syloid 244FP and Syloid XDP combinations are referred as LSCSYL and LSCXDP respectively and the different excipients ratio is indicated with a number between 1 to 4. Croscarmellose sodium was used as super disintegrant at a concentration of 2.5% w/w and magnesium stearate was used as lubricant at a concentration 1% w/w for all the formulations and the weight of the tablet was maintained at 250 mg. Excipients were taken into sufficient amount to punch 40 tablets for each coded formulation. The solution of drug in tween 80 was blended with the excipients in the mortar and pestle. The mixture was evaluated for flowability and was compressed into tablet using tablet punching machine (Rimek Mini ress, Gujarat, India).[10]

Evaluation of Liquisolid compact

Precompression evaluation of liquisolid compact of artemether

Pre-compression studies were determined for the prepared liquisolid compact by estimation of the following parameters and each experimentation was carried out in triplicate.

Bulk density

Bulk density of powder is the ratio of the mass of untapped powder sample to its bulk volume of the powder[11] and is calculated by the following formula,

$$\text{Bulk density} = \frac{\text{Weight of powder}}{\text{Bulk volume}}$$

Table 1: Preparation of liquisolid compact with syloid 244FP and syloid XDP as coating material.

Formulation code	LSCSYL1	LSCSYL2	LSCSYL3	LSCSYL4	LSCXDP1	LSCXDP2	LSCXDP3	LSCXDP4
Artemether (mg)	80	80	80	80	80	80	80	80
MCC (mg)	151.17	146.59	134.3	115.17	151.17	146.59	134.3	115.17
Syloid 244FP (mg)	10.08	14.66	26.95	46.08	-	-	-	-
Syloid XDP (mg)	-	-	-	-	10.08	14.66	26.95	46.08
Excipient ratio	15	10	5	2.5	15	10	5	2.5
Loading factor (Lf)	0.011	0.013	0.020	0.034	0.009	0.010	0.014	0.021
Croscarmellose Na (mg)	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25
Mg stearate (mg)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Total weight (mg)	250	250	250	250	250	250	250	250

Tapped density

The tapped density is obtained by mechanically tapping 100 times of a specific weight of sample in a graduated measuring cylinder in the bulk density apparatus.

The tapped density is expressed in grams per millilitres (g/mL) and calculated using the following formula and measured in the tap density apparatus (Tap density tester (USP), Electrolab),[12]

$$\text{Tapped density} = \frac{\text{Weight of powder}}{\text{Tapped volume}}$$

Angle of repose

Fixed funnel method was used to determine the angle of repose (θ) of the powder admixture.[13] The powder was allowed to flow through a funnel to form a conical pile, mean radius and height of the pile were measured and the angle of repose was computed from the following equation:

$$\text{Angle of repose } \tan\theta = h/r$$

h = height of the pile

r = radius of the pile

Hausner's Ratio and Compressibility index or Carr's index (%)

Hausner's ratio and Carr's index were calculated as follows:[14]

$$\text{Hausner"s Ratio} = \frac{\text{Tapped density}}{\text{Bulk density}}$$

$$\text{Carr"s index (\%)} = \frac{[(\text{Tapped density} - \text{Bulk density}) \times 100]}{\text{Tapped density}}$$

Post compression evaluation of liquisolid compact of artemether

Post-compression studies were determined for the prepared tablets by performing the following estimations.

Weight variation

Twenty tablets were selected randomly, collective and individual weight were estimated. Average weight was calculated. The % weight variation was determined as follows.[15]

$$\text{\% Weight variation} = \frac{\text{Average weight-weight of each tablet}}{\text{Average weight}} * 100$$

Hardness

Monsanto hardness tester was used to perform this test. From each formulation six tablets were selected randomly for the test and the average hardness was calculated and expressed in kg/cm^2.[16]

Friability

Roche friabilator was employed to assess the tablet strength during transport. From each formulation 20 tablets were selected randomly, and weighed prior experimentation. The tablets were rotated for 100 rotations at 25 rpm. Tablets were re-weighed. The % friability was computed.[17]

$$\text{\% Friability} = \frac{\text{Initial weight of tablets} - \text{Final weight of tablets}}{\text{Initial weight of tablets}} \times 100$$

Table 2: Solubility of artemether in various non-aqueous solvent.

Sl. No	Non-volatile solvent	Solubility (mg/mL) ± SD
1	Tween 20	0.998 ± 0.04
2	Tween 80	2.610 ± 0.05
3	PEG 400	0.035 ± 0.01
4	PEG 600	0.107 ± 0.15
5	Propylene Glycol	0.037 ± 0.007
6	Glycerine	0.340 ± 0.33

*All values are mean± SD.

Wetting time

For the estimation of wetting time, a piece of sponge was soaked initially in a small petri dish containing amaranth solution on which the tablet was placed carefully. The time required for the dye solution to colour the entire surface of the tablet was recorded as a wetting time. The study was performed in triplicate.[18,19]

In-vitro ***dissolution testing***

USP type II apparatus was used for the drug release study. The dissolution was carried out as per the standard of Indian Pharmacopoeia.[20] The dissolution media used was 1000 mL of phosphate buffer pH 7.2 containing 2% w/v solution of sodium lauryl sulphate. The basket was set to rotate at 100 rpm for 60 min at 37±0.5°C. At an appropriate intervals sample was withdrawn and replaced with equal volume of fresh buffer solution. The withdrawn samples were diluted with 1N hydrochloric acid and water mixture at a ratio of 1:3 and were analysed at 248 nm by UV-visible spectrophotometer. An average of three trials was considered to quantify the drug release.[21]

A comparative dissolution was carried out with a tablet of pure drug made with diluent MCC, croscarmellose sodium and magnesium stearate.

Dissolution efficiency was calculated for the formulations showing the maximum release using the following equation,[22]

$$DE = \frac{AUC_{0\text{-}60min}}{Q_{100.T}} 100$$

Statistical analysis

Dunnett's Multiple comparison test was carried out on the dissolution profile at a significance level $p<0.05$ to observe the efficacy of the selected formulations (LSCSYL3 and LSCXDP3) on dissolution over the pure drug.

Differential Scanning Calorimetry (DSC)

DSC analysis was performed using Perkin-Elmer- 4000 series. Samples of formulation were placed in aluminium pans. The thermal process under nitrogen purging was carried out at a heating rate of 20°C per minute over a range of 30°C to 300°C.[23] The pure drug and the formulations (LSCSYL3 and LSCXDP3) were subjected for thermal study.

Powder X-ray diffraction (PXRD) analysis

The surface characteristics of the drug and the formulations (LSCSYL3 and LSCXDP3) using Ni filtered Cu Kα radiation were studied using Simens D5000 X-ray diffractometer (wavelength 1.540 Å) at 25°C. The scanning was carried out at 2θ range from 2° to 80° and the diffraction patterns were recorded.[13]

Stability study

Stability study of the tablets for the formulations LSCSYL3 and LSCXDP3 was carried out as per ICH Q1A guidelines.[24] The 30 tablets of each formulation were placed in an airtight sealed glass container and stored at 40±2°C/75±5% RH for 90 days. The samples were tested after 90 days to observe the effect on friability, hardness, wetting time and drug release study. The data was compared with the fresh batch of the preparations.[25]

RESULTS AND DISCUSSION

Solubility studies

The solubility studies of artemether in different nonaqueous solvents unveiled the variations in solubility as shown in Table 2. The maximum solubility of drug was observed in tween 80. Hence tween 80 was selected as the non-aqueous vehicle for the preparation of liquisolid compact of artemether.

FTIR

The compatibility study between the drug and the different grades of mesoporous silica were carried out by ATR technique. The IR spectrum of artemether exhibits characteristic peaks at 2914 cm(C-H stretching), 1121 cm^{-1} (CO-C bending), 1023 cm^{-1} 1277 cm^{-1} (C-O-C stretching), 1451 cm^{-1} (C-H bending), 3198 cm^{-1}(O-H stretching), 857 cm^{-1}(O-O-C stretching), and 746 cm^{-1}(O-O stretching).

The sustenance of these peaks in the physical mixtures of drug with Syloid 244FP, and Syloid XDP stipulated the absence of interactions between the drug and silicas. Therefore, it can be concluded that the drug and the novel carriers are compatible. The FTIR results are shown in Figure 1.

Liquid load factor for carrier and coating materials

The ΦCA-value (carrier) and ΦCO-value (coating material) quantified the amount of carrier and coating materials respectively required to adsorb a given amount of liquid medication to yield a dry, free flowing and compressible powder admixture.

The Φ value and angle of slide of MCC, syloid 244FP and syloid XDP in Tween 80 were listed in Table 3. The efficacy of mesoporous silicas to hold liquified drug in their porous network structure and exhibit good flow characteristic were established

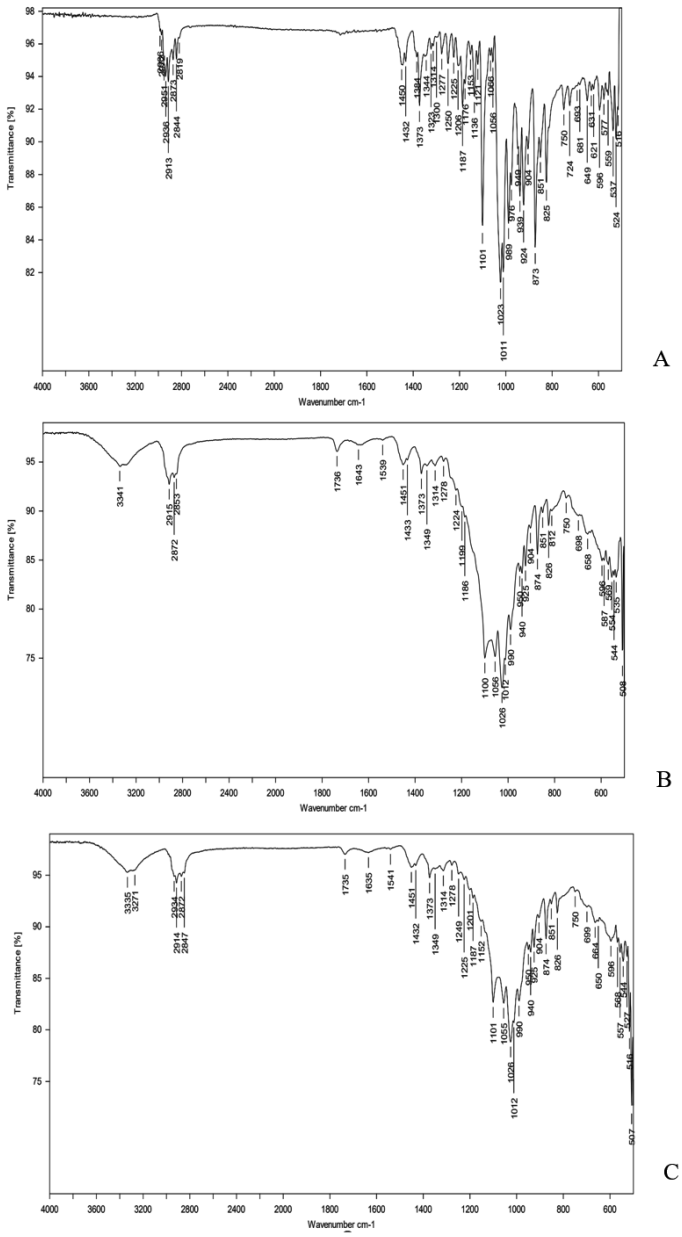

Figure 1: FTIR Spectrum of Pure drug (A), Pure drug with Syloid244FP (B) and Pure drug with Syloid XDP(C).

and proved the suitability as coating materials in the preparation of liquisolid compact.

Evaluation of liquisolid compact

Precompression evaluation

The bulk density of the compacts with syloid 244FP was varied in the range of 0.253 gm/mL to 0.388 gm/mL and tapped density was varied in the range of 0.314 gm/mL to 0.411gm/mL as presented in Table 4.

Bulk density of the powder blends with syloid XDP was in the range of 0.301 gm/mL to 0.327 gm/mL and tapped density was in the range of 0.351 gm/mL to 0.423 gm/mL.

The angle of repose of all the formulations revealed a good flow characteristic of the compact.

It was observed that as the proportion of silica was increased, the flow ability of the compact was increased, whereas a fall in compressibility (Carr's index) of the compact was observed. The formulations LSCSYL3 and LSCXDP3 showed comparatively good flow characters and compressibility compared to other formulations as presented in Table 4.

Post compression evaluation

The different formulations of liquisolid compact were compressed into tablets and subjected to post compression studies, as shown in Table 5. It was observed that hardness and friability of the tablets were markedly affected by the increment of mesoporous silica in the formulations. At an excipient ratio of 2.5 the formulations (LSCSYL4 and LSCXDP4) were found to be more friable and lack of storage and transportability. Hence LSCSYL4 and LSCXDP4 were not subjected for further evaluation.

The wetting time of the tablets were reduced as the proportion of silica was increased. The wicking effect of mesoporous silica is responsible for water permeation through its highly porous network structure, thereby resulted in fast wetting and rapid dissolution of artemether from its liquisolid compact.

The weight variation of each formulation was measured. The average weight variation of each formulation was found to be within the agreement of Pharmacopeial specifications.[26]

In vitro dissolution studies

The drug release study revealed that the liquisolid compact of artemether exhibited higher drug release compare to the pure drug. The dissolution graphs (Figure 2) represented that among all other different formulations, LSCSYL3 and LSCXDP3 showed 80% and 75% release in 60 min. A drastic improvement in drug release was observed in the formulations compared to the pure drug which was observed from the calculated values of dissolution efficiencies. The improvement on dissolution of LSCSYL3 and LSCXDP3 was found to be 5 times and 4 times respectively over the pure drug as shown in Table 6. The high dissolution of the formulation might be due to the wicking effect of the mesoporous silica to absorb water, and enhancement of the specific surface area. The molecular dispersion of drug in tween 80 also resulted in high wetting of the drug and hence remarkable improvement in dissolution.

Statistical analysis

The Dunnett's multiple comparison test on dissolution profile also Revealed the remarkable improvement in solubility of artemether in its liquisolid compact form for the formulations LSCSYL3 and LSCXDP3 over the pure drug as shown in Table 7.

Table 3: Liquid load factor for carrier and coating material.

Carrier/Coating	Φ value		Angle of slide
	ΦCA	ΦCO	
MCC	0.0064±0.01	-	35±0.51
Syloid 244FP	-	0.068±0.05	23±0.11
Syloid XDP	-	0.037±0.01	24±0.32

*All values are mean± SD.

Table 4: Precompression evaluation of powder compact with syloid 244FP and syloid XDP.

Formulation code	Bulk density(g/mL)	Tapped density(g/mL)	Hausner's ratio	Carr's index	Angle of repose
LSCSYL1	0.253 ± 0.6	0.314 ± 0.2	1.24	19.42	32.34 ± 0.6
LSCSYL2	0.290 ± 0.2	0.367 ± 0.5	1.26	20.98	31.50 ± 0.9
LSCSYL3	0.388 ± 0.2	0.441 ± 0.2	1.13	12.01	26.63 ± 0.5
LSCSYL4	0.237 ± 0.5	0.316 ± 0.3	1.33	25	24± 0.5
LSCXDP1	0.327 ± 0.01	0.423 ± 0.01	1.29	22.6	31.14 ± 0.5
LSCXDP2	0.321 ± 0.01	0.378 ± 0.01	1.17	15.07	28.21 ± 0.6
LSCXDP3	0.301 ± 0.009	0.351 ± 0.01	1.16	14.2	25.79 ± 0.7
LSCXDP4	0.321 ± 0.02	0.435 ± 0.02	1.35	26.2	23.12 ± 0.2

*All values are mean± SD.

Table 5: Post compression evaluation of tablets prepared with syloid 244FP and syloid XDP.

Formulation code	Weight (mg)	Hardness(kg/cm^2)	Friability (%)	Wetting time (min)
LSCSYL1	250.5±0.05	3 ± 0.6	0.2 ± 0.6	14 ± 0.7
LSCSYL2	249±0.03	3.6 ± 0.5	0.15± 0.7	10 ± 0.5
LSCSYL3	249.5±0.15	3.5 ± 0.2	0.3 ± 0.3	8 ± 0.4
LSCSYL4	250.1±0.23	2 ± 0.3	1.5 ± 0.2	5 ± 0.5
LSCXDP1	248±0.05	3 ± 0.3	0.4 ± 0.3	12 ± 0.2
LSCXDP2	249.2±0.3	3.5 ± 0.2	0.3± 0.4	9 ± 0.1
LSCXDP3	249.6±0.05	3.5 ± 0.2	0.5 ± 0.3	6 ±0.2
LSCXDP4	250.34±0.65	2 ± 0.3	1.3 ± 0.4	2 ± 0.1

*All values are mean± SD.

Table 6: Determination of dissolution efficiency on dissolution over the pure drug.

Formulation code	Dissolution efficiency
LYCSYL3	55.25
LSCXDP3	44.14
Pure drug	11.48

Table 7: Dunnett's Multiple Comparison Test.

Comparison Test	Mean Diff.	q	Significant? $p < 0.05$?	Summary
Pure drug vs LSCSYL3	-41.77	3.849	Yes	**
Pure drug vs LSCXDP3	-32.52	2.997	Yes	*

Mean diff.: Mean difference; q: difference between two means divided by standard error

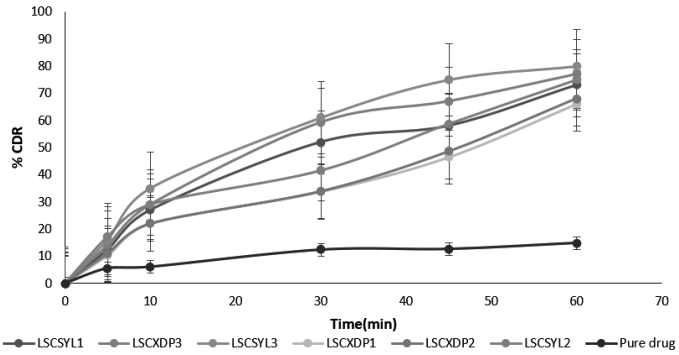

Figure 2: % Cumulative drug release of compact with syloid 244FP, syloid XDP and pure drug.

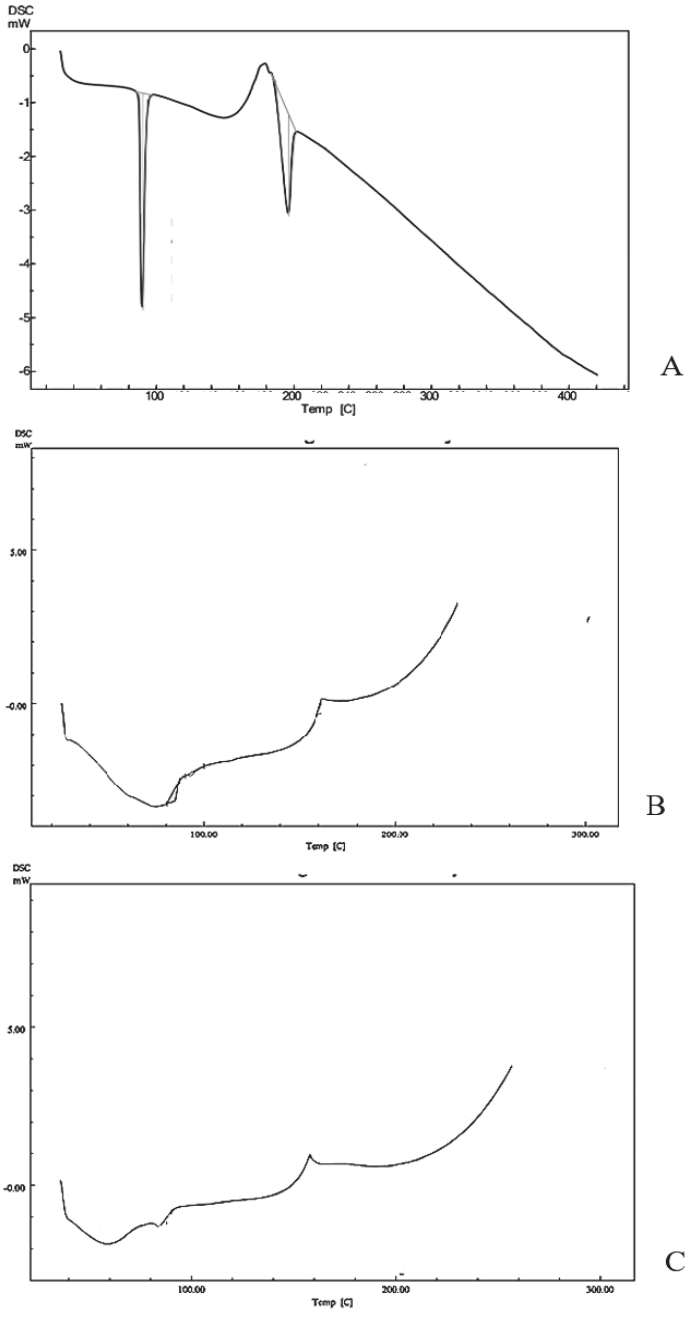

Figure 3: DSC Thermograms of Pure drug (A), LSCSYL3 (B), and LSCXDP3 (C).

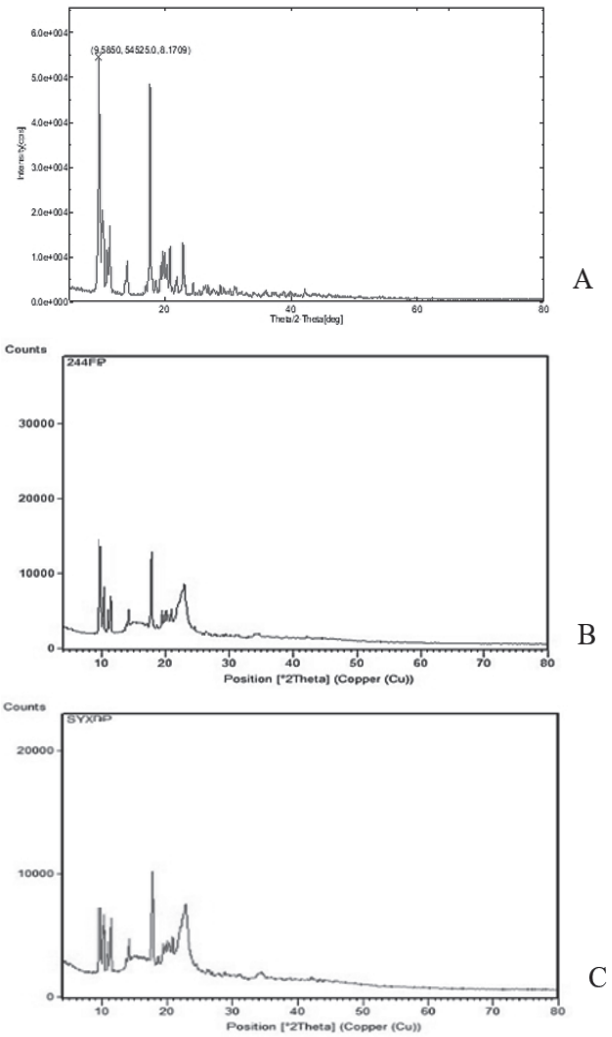

Figure 4: PXRD of Pure drug (A), LSCSYL3 (B), and LSCXDP3 (C).

DSC

The pure drug showed a broad endothermic peak at 86℃ corresponding to the melting point of the beta(β) polymorph.[27] The peak area of the drug is drastically reduced in the formulations (LSCSYL3 and LSCXDP3) as shown in Figure 3, absence of sharp peak of the drug indicated complete amorphization due to molecular dispersion of the drug in the non-volatile solvent (Tween 80) and adsorption onto the powder admixture. This might be responsible for high dissolution of the drug. These observations further indicated the retention of the drug in β-polymorphic form and resistance of transition to other polymorphic forms during storage, hence ensures the stability of the β form of artemether in the formulation.

PXRD

The XRD patterns of artemether showed high intensity peaks at 2θ diffraction angle at 9°, 10°, 11°, 17°, 22°, 25° etc confirming the crystallinity of β artemether. The peak intensity was markedly reduced for the formulations (LSCSYL3 and LSCXDP3) at same diffraction angles revealing the conversion of crystalline to

Table 8: Stability studies of LSCSYL3 and LSCXDP3.

Sample	Hardness (kg/cm²)	Friability (%)	Wetting time (min)	Dissolution (%)
Freshly prepared LSCSYL3	3.5 ± 0.2	0.3 ± 0.3	8 ± 0.4	80±2.38
LSCSYL3 after 3 months	3.45 ± 0.32	0.312 ± 0.15	8.3 ± 0.54	80.53±3.81
Freshly prepared LSCXDP3	3.5 ± 0.2	0.5 ± 0.3	6 ±0.2	75.4±4.10
LSCXDP3 after 3 months	3.35 ± 0.52	0.45 ± 0.26	5.7 ±0.25	74.9±3.03

*All values are mean± SD.

amorphous form (Figure 4). The amorphization of the drug was further confirmed with the enhancement in dissolution of the drug.

Stability studies

The samples after short term stability studies have revealed that the tablets were capable to meet their integrity, not much variations were observed in friability, hardness, wetting time and dissolution as presented in Table 8.

The stability study indicated that the formulations could retain their properties at the storage conditions, no major changes in the wetting time, friability and hardness stipulated that moisture absorption was minimum during storage, and the drug release was not affected. Hence the formulations LSCSYL3 and LSCXDP3 can be a promising composition for polymorphic stability and improvement of dissolution behaviour of artemether.

CONCLUSION

The present research is focused on improving the solid-state stability and dissolution of an antimalarial drug, artemether by employing liquisolid compact technique using mesoporous silica as novel coating material. The perseverance of the metastable form of artemether (β form) was achieved through molecular dispersion of drug in Tween 80 followed by adsorption into a carrier and porous coating material. Both the coating materials syloid 244FP and syloid XDP were found to be effective in improving the dissolution of the drug. Tableting property was found to be best in the formulations LSCSYL3 and LSCXDP3 at a carrier to coating ratio 5. The DSC and PXRD study demonstrated the conservation of the preferred polymorphic form in the amorphous state. The short-term stability study also manifested the safeguarding of the formulation characteristics and the firmness of both the varieties of mesoporous silica in the formulations. Hence it can be concluded that the liquisolid compact of artemether using tween 80 as non-volatile solvent and mesoporous silicas as coating material can be an optimistic way to preserve the solid-state behaviour of the β polymorphic form and enhance the dissolution of the drug.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

BCS: Biopharmaceutics Classification System; **DSC:** Differential scanning calorimetry; **FTIR:** Fourier-transform infrared spectroscopy; **ICH:** International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; **PXRD:** Powder X-ray diffraction analysis; **PEG:** Polyethylene glycol; **SD:** Standard Deviation; **RH:** Relative Humidity; **WHO:** World health organization.

SUMMARY

The liquisolid compact of artemether was prepared with a novel mesoporous material with an aim to improve the solubility and retention of bioactive polymorphic form of the drug. Syloid 244FP and Syloid XDP were established as a good coating material. The surface characteristics study, dissolution, and short-term stability study revealed the preservation of the β polymorphic form of artemether and enhancement of the dissolution of the drug.

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Original Article

Production of L-tyrosinase from Novel Variants of *Streptomyces cellulosae*

G Sowjanya*, G Girijasankar

A.U. College of Pharmaceutical Sciences, Pharmaceutical Biotechnology Division, Andhra University, Visakhapatnam, Andhra Pradesh, INDIA.

ABSTRACT

Background: Recently, a lot of interest has been focused on the discovery of high L-tyrosinase-yielding microorganisms that catalyze the production of enantiomerically pure L-DOPA, which has been recognized as the conventional treatment for Parkinson's disease. **Objectives:** The current work primarily focused on isolating novel, promising tyrosinase-producing isolates and performing taxonomical characterization on them. **Materials and Methods:** Initially six various actinomycetes isolates were used in present study. Primary screening and secondary screening of selected isolates were performed. By evaluating the morphological, cultural, physiological, biochemical and molecular characterization, promising isolates were identified. Using 16S rRNA gene sequencing, the isolates' Phylogenetic investigations were conducted. **Results:** *Streptomyces cellulosae* variants, isolates PD18 and PD26 were identified to be the potential actinomycete isolates and their respective maximal tyrosinase yields were reported to be 21.20 IU/mL and 24.19 IU/mL. **Conclusion:** According to the observations of the experiment, SS (Soluble starch) medium with 1mg/mL copper sulphate was most suitable medium for tyrosinase production from our promising isolates PD18 and PD26. The maximum yield was noticed after 168 hr of incubation. This work emphasizes the significance of finding novel variants of *Streptomyces cellulosae*, which were first used to produce tyrosinase as well as promising sources for potentially bioactive substances like antimicrobial metabolites, biosurfactants and enzymes like amylase, lipase, cellulase, uricase, urease, β- galactosidase and L- asparaginase.

Keywords: Tyrosinase, L- tyrosine, L-DOPA, SS medium, copper sulphate, *Streptomyces cellulosae.*

Correspondence:
Mrs. G Sowjanya
A.U. College of Pharmaceutical Sciences, Pharmaceutical Biotechnology Division, Andhra University, Visakhapatnam-530003, Andhra Pradesh, INDIA.
Email id: gsowjanyachakravarthy@gmail.com

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INTRODUCTION

Actinomycetes have virtually completely different characteristics from bacteria and fungi, which is why they were given their own class. Branching spore-forming, Gram positive, aerobic unicellular organisms with aerial mycelia growth and substrate are found in the order Actinomycetales. Fission or unique spores or conidia are the two ways that actinomycetes reproduce. They have DNA that has a high (G+C) concentration of more than 55 mol%. The 16s ribosomal cataloguing and DNA evidence studies using rRNA pairing showed a Phylogenetic link between the actinomycete group.[1] Actinomycetes, one of the 18 fundamental lineages of the domain bacteria is a huge taxonomic category.[2] *Streptomyces* species generate a wide range of extracellular enzymes including glucose isomerase, amylase, cellulase, protease, lipase, xylanase, pectinase, glucose oxidase, keratinase and serratiopeptidase as well as antibiotics, pigment and other bioactive molecules. There has been less investigation on their capacity to manufacture tyrosinase.[3] Copper-containing tyrosinase (EC 1.14.18.1) is a diphenolase (catecholase) and monophenolase (cresolase) enzyme. When monophenols are o-hydroxylated to produce equivalent catechols, tyrosinase's monophenolase (cresolase) and diphenolase activities are active (catecholase). This enzyme is present in many different species, ranging from prokaryotes to mammals. Tyrosinase can play a significant impact in the biosynthetic pathway of skin pigment. Its monophenolase (cresolase) activity can catalyze the transformation of the material tyrosine into the o-diphenol 3,4-dihydroxyphenylalanine (DOPA) and its diphenolase (catecholase) activity can catalyse the transformation of the formed product DOPA into the quinone (o-quinone) through oxidization.[4,5] The significant capability of this enzyme for the food, medical (melanoma patients, pro drug activation), agricultural, analytical and environmental industries is supported by a wealth of data.[6-8]

The standard treatment for Parkinson's disease until 1967 was L-DOPA.[9] In cases of neurogenic damage, it is also employed to control the myocardium.[10] Each year roughly 250 tonnes of L-DOPA are sold worldwide.[11] The majority of L-DOPA used in commercial products is made from vanillin and hydantoin using an eight-step chemical procedure, one of which is an optical resolution.[12]

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Many investigations have been conducted to develop an alternative enzyme-based technique using free or immobilised tyrosinase to the chemical synthesis that L-DOPA is currently manufactured at industrial scale.[14-16]

MATERIALS AND METHODS

Primary screening

Initially, six plates of skim milk agar medium, which comprises peptone (1%), sodium chloride (0.5%), yeast extract (0.3%), agar (2%), and skim milk (10%) were streaked with five different actinomycetes isolates from kapuluppada plastic waste dumping yard, close to kommadi, Visakhapatnam, A.P and one actionomycetes isolate from Korangi mangrove forest, Korangi, Kakinada. All the plates were incubated at 27°C for 7days. Positive outcomes were seen as being very clear and negative outcomes as having no zone. Additionally, all six isolates were streaked on six Melanin medium plates that included yeast extract (0.1%), L-tyrosine (0.1%), NaCl (0.8%) and agar powder (1.5%) with an adjusted pH of 7. In contrast, control plates were created by producing screening media devoid of L- tyrosine. These plates served as test plates. At 27°C, all test and control plates were incubated for 7 days.[17]

Secondary Screening

The selected isolates were further tested for its ability to produce tyrosinase extracellular by submerged fermentation technique e using tyrosine medium arbitrarily. After 7 days of incubation at 27°C, the fermented flask was centrifuged at 4°C, 4000 rpm for 20 min. The obtained clear supernatant was used to check quantity of tyrosinase produced during fermentation by means of tyrosinase assay and protein assay.[19,20]

Tyrosinase enzyme Assay

Activity of tyrosinase as a monophenolase

Tyrosine was used as a substrate to assess the production of tyrosinase utilizing monophenolase activity.[21-25] The quantity of extracellular tyrosinase was determined by modified dopachrome method using molar extinction co-efficient.[26] The test solution was prepared by mixing 3mL of L- tyrosine (0.02M) substrate and 0.1mL of clear enzyme supernatant in a test tube where as in the blank the reaction mixture was prepared by mixing 3mL of L-tyrosine (0.02M) and 0.1mL of 0.05M sodium phosphate buffer. Both test and blank were incubated at 37°C for 5 min then absorbance of test is noted against blank using UV visible spectrophotometer at 475 nm.

An international unit (IU) of extracellular enzyme activity was established as the enzyme molecule required to catalyze the oxidation of 1Mmol of L-DOPA for each minute under the aforementioned circumstances. The dopachrome's $3600 M^{-1}cm^{-1}$ molar extinction co-efficient was used for calculation.[26]

Following equation was used to calculate enzyme activity in terms of IU/mL

$$\text{IU/mL Enzyme} = \frac{\text{absorption / min x assay volume x DF}}{\epsilon_{nm}\ (M^{-1}\ cm^{-1}.\ \text{cm.enzyme volume})} \times 10{,}000$$

ϵ_{nm} = absorption coefficient value 3600 M^{-1} cm^{-1}

DF= dilution factor

Lowry technique

The protein content was determined using the Lowry technique. 0.1mL of the sample or standard is mixed with 2N NaOH. Undergo hydrolysis at 100°C for ten min in a boiling water bath or heating chamber. Bring the hydrolyzate to ambient temperature before adding 1mL of newly prepared complex-forming reagent. Let the solution sit at ambient temperature for ten minutes. Folin reagent should be added using a mechanical stirrer and the mixture should be allowed to rest for 30 to 60 min at ambient temperature (do not exceed 60 min). If the protein concentration becomes less than 100 g/mL, read the optical density at 750 nm; if it was between 100 and 2000 g/mL, read the optical density at 550 nm.[20]

Preliminary screening of tyrosinase production medium

The suitable production medium composition was determined by adding the 1mL inoculums medium (Absorbance 0.9 OD) in different production media such as tyrosine broth [medium1-Peptone (0.5%), Beef extract (0.3), L-tyrosine (0.1%), NaCl (0.8%), pH 7.0],[22] tyrosine production medium [medium2 - Soluble starch (0.2%), Polypeptone (casein peptone (0.05%) + meat peptone (0.05%-0.1%), NaCl (0.1%), K_2HPO_4 (0.1%), $MgSO_4$ (0.005%), $FeSO_4$ (0.005%), $CuSO_4$ (0.005%), pH 7.0],[18] SS Medium (Soluble starch medium) with copper [medium3 - Soluble starch (2.5%), Glucose (1.0%), Yeast extract (0.2%), $CaCO_3$(0.3%), trace salt solution-100µL (0.005% of $FeSO_4$, Cu SO_4, $ZnSO_4$ and $MgSO_4$ were mixed in 100mL distilled water), L- tyrosine (0.1%), $CuSO_4$ (1mg/ mL), pH 7.0][22] and SS Medium Composition without copper (medium 4), ISP-7 [medium 5- Glycerol (1.5%), L-Tyrosine (0.05%), L-Asparagine(0.1%), K_2HPO_4 (0.05%), $MgSO_4.7H_2O$ (0.05%), $FeSO_4.7H_2O$ (0.001%), NaCl (0.005%), Trace salt solution (100µL), p^H (7-7.4) and distilled water upto 100 mL], ISP-5 [Medium 6- L- Asparagine (0.1%), Glycerol (1%), K_2HPO_4 (0.1%), Trace salt solution (100µL), p^H (7-7.4) and distilled water upto 100mL], ISP-4 [Medium 7- Soluble starch (1%), K_2HPO_4 (0.1%), $MgSO_4.7H_2O$ (0.1%), NaCl (0.1%), $(NH4)_2SO_4$ (0.2%), Ca_2CO_3 (0.1%), $FeSO_4.7H_2O$ (0.0001%), $MnCl_2.7H_2O$ (0.0001%), $ZnSO_4.7H_2O$ (0.0001%), p^H (7.0-7.4)] respectively and allowed to incubate at 27°C for 7 days. After incubation, enzyme was centrifuged and clear supernatant was used. Tyrosinase production was assayed in terms of monophenolase activity.[21-25]

Effect of copper concentration on production medium

In each flask, the specified medium was supplemented with different amounts of copper sulphate (1μM, 3.5 μM, 10 μM) as well as 1 mg, 2.5 mg, 5 mg and 10 mg of copper sulphate. The selected promising isolates were then added to the mixture in the appropriate amounts. Tyrosinase test was carried out using the clear enzyme supernatant following incubation.[21-25]

Polyphasic method for the identification of the promising isolate

Isolate features in terms of morphological, cultural, physiological, and biochemical

Cultural features, morphology, physiology, biochemical, and genetic analyses were employed to identify the potent actinobacterial strain. The type of the mycelium, colour and spore arrangement were taken into consideration during the microscopic evaluation using the direct method, inclined coverslip technique and slide culture technique.[27-29] To examine the strain's cultural traits such as the colour of the upper mycelium, the colour of the basal mycelium, the generation of pigment and the creation of spores, the strain was cultivated on seven International Streptomyces Project (ISP) media and four non-ISP media.[30] By growing the strain on tyrosine agar (ISP-7) media and screening medium, the synthesis of melanin pigment was evaluated.[31] Utilizing established procedures, biochemical characterization was done.[32-34] The growth of the isolates' physiological features, such as the impact of pH (5-10), temperature (25-40°C) and salinity were examined.[33,34]

Molecular Characterization and Phylogenetic analysis of the selected actinomycete isolates

The screened actinomycete isolated strains were identified by 16S rRNA gene sequencing (Sanger method). The sequencing was carried out at NCIM- Pune.

The evolutionary process was derived using a neighbor-joining technique.[35] An ideal tree has a total branch length of 0.15528769. The percentages of duplicate trees where related taxa were bootstrapped together (1000 repetitions) are shown beside the branches.[36] Using the same evolutionary distance measure that was used to estimate the tree, the branch lengths are calculated. Changes were computed after removing all places with holes and missing data. The final dataset contained 1428 sites altogether. At MEGA 6, an evolutionary study was conducted. It is determined using the Kimura two-parameter approach[37] and expressed as base substitutions per site. 18 nucleotide sequences were being investigated.[38]

Submission of nucleotide sequence to the National Center for Biotechnology Information (NCBI)

The isolates PD18 and PD26 have their nucleotide gene sequence [16S ribosomal RNA (rRNA)] data deposited in the Gene Bank database (NCBI).

Preliminary screening of actinomycetes for bioactive metabolites

Amylase activity

The starch agar medium which contains beef extract (0.3%), peptone (0.5%), soluble starch (0.2%) and agar (1.5%) was utilized for the amylase enzyme plate assay. The selected actinomycetes were streaked on a plate of starch agar and grown for seven days at 27°C. Following the incubation time 1% iodine solution was given to the starch agar plates and the presence of a clear zone of starch breakdown served as a marker for the presence of an amylase.[39]

Cellulase activity

For the cellulase enzyme plate assay, carboxymethylcellulose (CMC) agar was composed of yeast extract (0.005%) agar (1.5%), $NaNO_3$ (0.01%), K_2HPO_4 (0.01%), $MgSO_4$ (0.05%) and CMC (0.05%). On prepared media, promising actinomycetes were streaked and cultivated for seven days at 27°C. Congo red was applied to the plate after the incubation period, which was followed by 30 min rest period. The plate was then drained of additional Congo red, flooded with 1M NaCl and stand for 30 min. By obtaining a clean zone around the isolate, cellulolytic activity could be observed.[40,41]

Protease activity

The selected Actinomycete isolate was streaked on Skim Milk Agar (SMA), which comprises yeast extract (0.3%), NaCl (0.5%), agar (1.5%) and skim milk (1%). Plates were incubated for seven days at 30°C. A microorganism which produces proteases was observed in such a clear zone of hydrolyzed skim milk around the growth of microbe.[39,42]

Lipase activity

An modified version of the methodology used by Gopinath *et al*. For the purpose of detecting lipase enzyme plate assay, the selected actinomycetes were streaked on Tween-20/ Tween-80 agar which is composed of agar (2%), Tween-20/Tween-80 (1%), Peptone (1%), NaCl (0.5%) and $CaCl_2$ (0.01%). On tween-20 agar, selected actinomycetes were inoculated and the results were analyzed after seven days at room temperature. Around the colony, calcium salt precipitation revealed the presence of lipase enzyme.[43]

L-Asparaginase activity

On L-asparagines-glucose agar medium, the isolates were cultivated and incubated for 5-7 days. When an isolate produced positive results, pink colour media replaced yellow colour media and the colonies produced distinct pink zones.[44]

Gelatinase activity

Actinomycetes isolates were streaked on a gelatin agar medium made up of gelatin (12%), beef extract (0.3%), peptone (0.5%), agar (1.5%) in petri plates and the mixture was then incubated at 27°C for seven days. After seven days of incubation, the petri plates were spread with 10 mL of mercuric chloride solution and the area of hydrolysis around the isolates growth as well as their zone of growth zone's breadth were determined. From these measurements, the ratio between the hydrolyzing zone and growth zone was determined.[32-34]

Nitrate reductase activity

The promising isolates were put to 10 mL of nitrate broth and cultured for 5-7 days at 27°C as a control with nitrate broth that had not been inoculated. When 4- amino benzene sulfonic acid and α-naphthylamine are combined after incubation, an instantaneous cherry red colour results which is regarded as positive for such nitrate reductase enzyme assay. A little amount of zinc dust was also applied to the nitrate broth under investigation to further validate the existence or absence of nitrate reductase.[32-34]

ß- galactosidase activity

In comparison to lactose which is a synthetic substrate, O-nitrophenyl-beta-D-galactopyranoside (ONPG) has glucose replaced with an ortho-nitrophenyl group. The substrate ortho- nitrophenyl-beta-D-galactopyranoside (ONPG) able to penetrate into the microbe without the aid of permease unlike lactose. The organism is obtained from a medium with a high lactose concentration and inoculated into the ONPG medium using the broth technique of screening. If the organism has β-galactosidase, the enzyme will break down the β-galactosidase linkage and release the yellow chemical o- nitrophenol. This denotes a successful evaluation.[45]

Uricase Activity

By seeding the test organisms into agar plates with uric acid medium comprising uric acid (0.3%), beef extract (0.3%), peptone (0.5%), NaCl (0.2%) and agar (2.3%) at pH 7.5 incubation at 37°C, the primary screening of uricase from promising isolates was done. The development of clear zones surrounding the culture colonies is proof that uricase enzyme is being produced.[46]

Biosurfactant production

CTAB / Methylene blue agar plate technique

The CTAB agar plate assay is a semi-quantitative technique for detecting extracellular glycolipids and other anionic surfactants. Our isolates' crude extracts were put to cups of CTAB agar medium which contains the following: $NaNO_3$ (1.5%), KCl (0.11%), NaCl (0.11%), $FeSO_4$ (0.02%), KH_2PO_4 (0.34%), K_2HPO_4 (0.44%), $MgSO_4$ (0.05%), yeast extract (0.05%), glucose (2%), p^H (7) and others. Diffusion was permitted to occur for 24 hr at 28°C. The cups create a deep blue colour that denotes the presence of biosurfactants.[47]

Antimicrobial activity

Cross-streak method

The cross-streaking technique was used to assess the antibacterial spectrum of each actinomycete isolate against Gram-positive, Gram-negative and fungal bacteria. To incubate for seven days at 27°C, each isolate was streaked individually or separately along the middle of a plate containing starch casein agar (SCA) medium. An actinomycetes isolate that had fully matured and generated spores was streaked perpendicularly with a 48-hr-old fungus test organism and a 24-hr-old bacterium test organism. It was demonstrated that the actinomycete isolate might prevent the test organisms from growing.[48,49]

Agar overlay method

Isolates which exhibited a broad spectrum of antimicrobial activity were selected for agar overlay method. Selected isolates were centre stabbed on the starch casein media plates and incubated for 7 days at 28°C followed by overlay with nutrient agar inoculated with 24 hr fresh test bacterial culture or Sabouraud's dextrose agar containing 48 hr fresh fungal culture on the surface of foster medium having rich antimicrobial metabolites production with less agar content of 0.75%. In case of bacteria they are incubated for 18-24 hr at 37°C and for fungi 72-120 hr.[48,49]

RESULTS AND DISCUSSION

A copper metalloproteinase tyrosinase was selected for this study because of its potential applications in diverse fields. Basic structure of tyrosinase was conserved in various species and we attempted to isolate novel strains in order to produce the enzyme the industrially in a cost efficient manner. Therefore screening and isolation of tyrosinase producing actinomycetes were performed. As tyrosinase is a protease, so both proteolytic activity and melanin production test were performed with six cultures PD4, PD9, PD18, PD26, PD66, KMFA1 isolated earlier in our laboratory. Among them five strains PD4, PD9, PD18, PD26, KMFA1 were identified as protease producers, two isolate PD18 and PD26 were recognized as tyrosinase producers. The results of primary screening were shown in Figure 1. Screening of

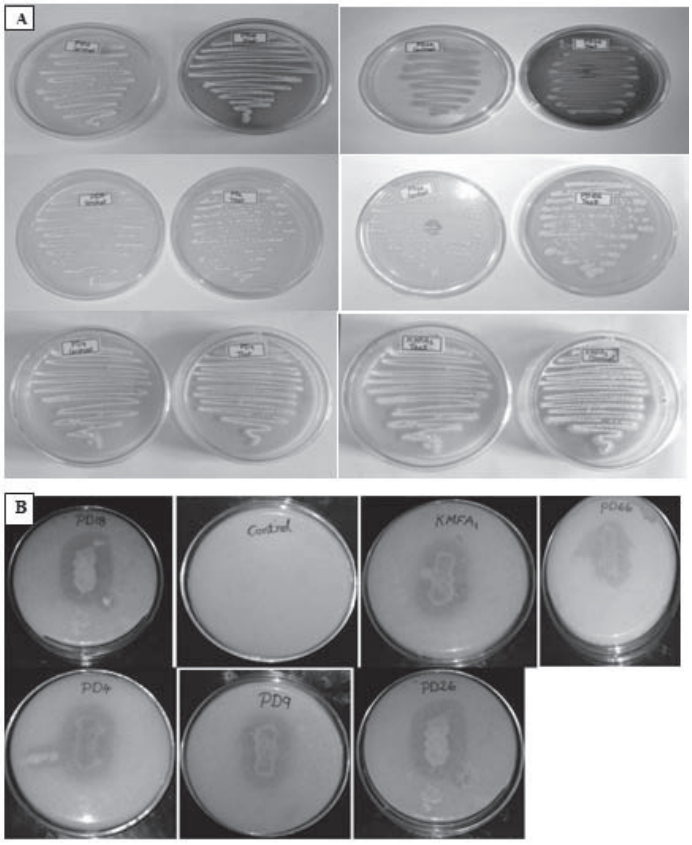

Figure 1: Preliminary screening A- melanin medium, B -skimmed milk agar medium.

suitable medium composition is a crucial step for the industrial production of metabolites so the selected isolates PD18 and PD26 were grow in different growth media such as tyrosine broth medium, tyrosine production medium, SS medium with copper and SS medium without copper. Among them SS medium with copper (medium 3) showed maximum growth and activity of enzyme. The results of the study were shown in Figure 2, similar results were also reported by Roy *et al*[16] and Bhaskara Rao *et al.*,[51] where extracellular tyrosinase was produced by *Streptomyces espinosus* strain LK4 and *Streptomyces aureofasciculus* respectively using SS medium with copper sulphate. Whereas, Subhash *et al*,[52] used peptone yeast extract iron broth for the production of tyrosinase where copper sulphate was omitted. By studying the impact of incubation period it was found tyrosinase production was started after 144 hr of incubation of culture broth of PD18 and PD26 respectively and reached maximum level after 168 hr and subsequently gradually decreased (Figure 3). The impact of copper on production medium was determined by supplementing different concentration of copper sulphate to production medium (medium 3) and it was found that PD18 and PD26 showed maximum monophenolase activity 21.20 IU/mL and 24.19 IU/mL respectively at a concentration of 1 mg/ mL of copper sulphate (Figure 4). Protein assay revealed that amount of protein concentration of PD18 and PD26 were found to be 264µg and 325µg after extrapolating the absorbance from standard graph of BSA (Figure 5). Our results revealed that SS medium with 1mg/mL copper was optimum for production of tyrosinase from cultures PD18 and P26 respectively whereas Roy *et al*[16] and Bhaskara *et al*[51] reported that addition of 10 mg/ mL of copper sulphate supplement to SS medium showed maximum tyrosinase yield from the cultures *Streptomyces espinosus* strain LK4 and *Streptomyces aureofasciculus* respectively. Study highlights the fact that new strains PD18 and PD26 required less supplement of copper sulphate for their growth to produce tyrosinase molecule. The morphology of aerial and substrate mycelium of promising isolates were shown in the Figure 6. The cultural characteristics of the strain are represented Table 1. The isolates PD18 and PD26 exhibited good growth on Tryptone yeast extract agar (ISP-1), YMD agar (ISP-2), Glycerol asparagine agar (ISP-5), Inorganic

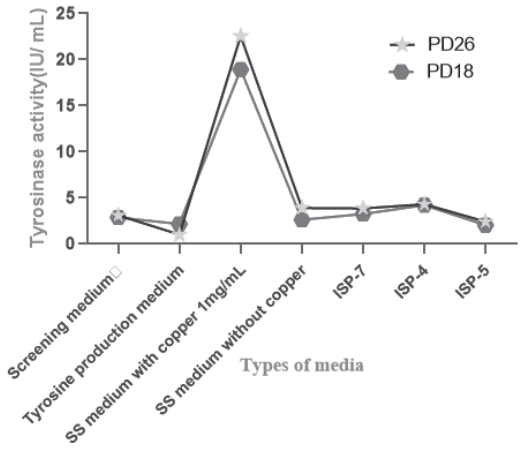

Figure 2: Effect of various media on tyrosinase activity.

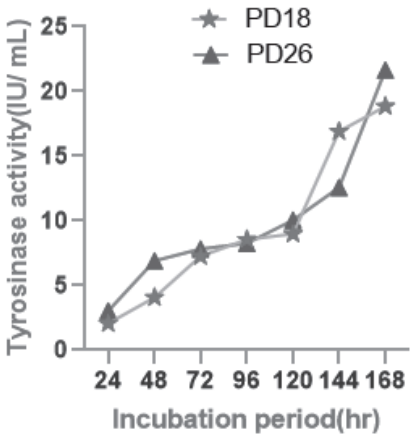

Figure 3: Effect of incubation period on tyrosinase activity.

Table 1: Cultural characteristics of the isolates PD18 and PD26.

Name of medium	Growth (PD26)	AM* (PD26)	SM** (PD26)	Pigmentation (PD26)	Growth (PD18)	AM* (PD18)	SM** (PD18)	Pigmentation (PD18)
Tryptone yeast -extract agar (ISP-1)	Good	White	Brown	Yes	Good	White	Brown	Yes
Yeast extract malt extract (ISP-2)	Good	White	White (Pale brown)	Yes	Good	Brown	Brown	Yes
Potato dextrose agar (ISP-3)	Poor	White	White	No	Poor	White	White	No
Inorganic salt starch agar (ISP-4)	Good	White	White	No	Poor	White	White	No
Glycerol Asparagine agar (ISP-5)	Good	White	Yellowish-brown	Yes	Good	White	Brown	Yes
Tyrosine agar (ISP-7)	Good	Brownish	Brown	Yes	Good	White	Brown	Yes
SAB	Moderate	White	White	No	Moderate	-	-	-
Nutrient agar	Good	White	White	No	Good	White	Pale Brown	No
Starch casein salts agar	Good	White	Brownish	Yes	Good	White	Brown	Yes (Light Brown)
Glucose Tryptone agar	Good	White	Yellowish-brown	No	Good	White	Yellow	No
Soluble Starch medium	Good	Grayish-brown	Brown	Yes	Good	Grayish-white	Pale Brown	Yes
Screening medium (melanin medium)	Good	Grayish-brown	Dark brown	Yes	Good	Grayish	Brown	yes

AM*-Aerial mycelium, SM**-Substrate mycelium and SAB-Sabouraud's dextrose agar medium.

salt starch agar (ISP-4) Tyrosine agar (ISP-7), Starch casein salts agar, Soluble Starch medium, Nutrient agar, Glucose tryptone agar and screening media. The growths were moderate on SAB medium. The poor growth or no growth was observed on Potato dextrose agar (ISP-3) medium. The colour of aerial mycelium was white and substrate mycelium were different on the different media tested. Soluble pigment production was observed on the Tryptone yeast - extract agar (ISP-1), Yeast extract malt extract (ISP-2), Glycerol Asparagine agar (ISP-5), Tyrosine agar (ISP-7), Starch casein salts agar, Soluble Starch and screening media tested. Isolate PD26 (*Streptomyces cellulosae)* also have capable to produce L- asparaginase, β- galactosidase, uricase, urease. Isolate PD18 (*Streptomyces cellulosae)* also have capable to produce L- asparaginase, β- galactosidase, urease but in less quantity. Actinomycetes have a reputation for marked nutritional versatility which is supported by the results of our analysis. In present study, the isolates were able to utilize and grow on variety of carbon and nitrogen sources (Table 2). The results of present study are correlated to previous results of carbohydrate utilization studies reported by Pridham and Gottlieb (1948) for the species differentiation of actinomycetes, hence the selected actinomycete strains were classified under the genera of *Streptomyces.* The 16S rRNA gene sequences of 1493 and 1495 nucleotides were generated for isolates PD18 and PD26 respectively after

Table 2: Physiological characteristics of the isolates PD18 and PD26.

Physiological Characterization		
Production of melanin pigment	+++	+++
Range of temperature for growth	25-40°C	25-40°C
Optimum temperature for growth	30°C	30°C
pH 5	+	+
pH 6	++	++
pH 7	+++	+++
pH 8	+++	+++
pH 9	++	++
pH 10	+	+
Optimum pH for growth Gram reaction	7	7
Utilization of Carbon Source		
Source Type	Isolates PD18	Isolates PD26
Starch	+++	+++
Glycerol	++	++
CMC	+	+
Lactose	++	++
Glucose	+++	+++
Arabinose	+	+
Mannitol	+	++
Sucrose	+	+
Utilization of Nitrogen Source		
Source Type	Isolates PD18	Isolates PD26
Casein	+++	+++
Gelatin	+	+
Tryptone	++	++
Meat extract	+	+
Beef extract	++	++
Malt extract	+	+
Yeast extract	+++	+++
Peptone	++	++

+++: Strong activity, ++: Moderate activity, +: Fair

removal of primer sequence. The BLAST similarity search and Phylogenetic analysis of the 16S rRNAgene revealed that the isolate PD18 shared 99.98% sequence similarity with the strain *Streptomyces cellulosae,* the isolate PD26 shared 100% sequence similarity with the strain *Streptomyces cellulosae.* The phylogenic

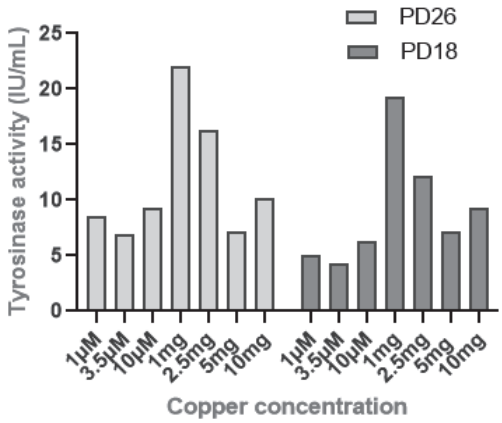

Figure 4: Effect of copper concentarion tyrosinase activity.

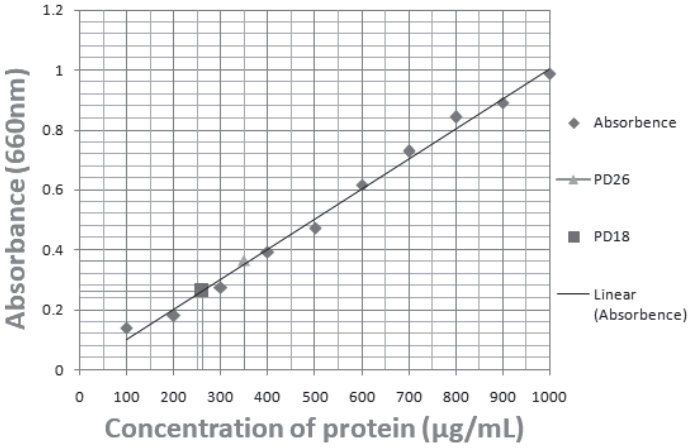

Figure 5: Standard graph of protein bovine serum albumin (BSA).

tree also suggested that the isolates were clustered with the other well characterized *Streptomyces* sp. In terms of sequence similarity in the range of 99.1% to 99.5%. The nucleotide sequences (16S rRNA) of both isolates PD18 and PD26 were submitted to Gene bank (NCBI) with Accession numbers OK652588.1 and OK655680.1. Consequently, the isolates PD18 and PD26 were designated as new variants of *Streptomyces cellulosae* strain PD18 and *Streptomyces cellulosae* strain PD26 respectively. According to earlier studies, the *Streptomyces cellulosae* strain was exclusively used to make antibiotics, biosurfactants and enzymes like lipase, amylase, cellulase, chitinase, and protease up until this point. This study highlights the contribution of discovering novel strains of *Streptomyces cellulosae* which were first used to produce tyrosinase as well as promising sources for potentially bioactive molecules such as antibiotics, antifungal metabolites, biosurfactants, and enzymes such as amylase, lipase, cellulase, uricase, urease, beta galactosidase and L- asparaginase (Figure 7, Table 3).

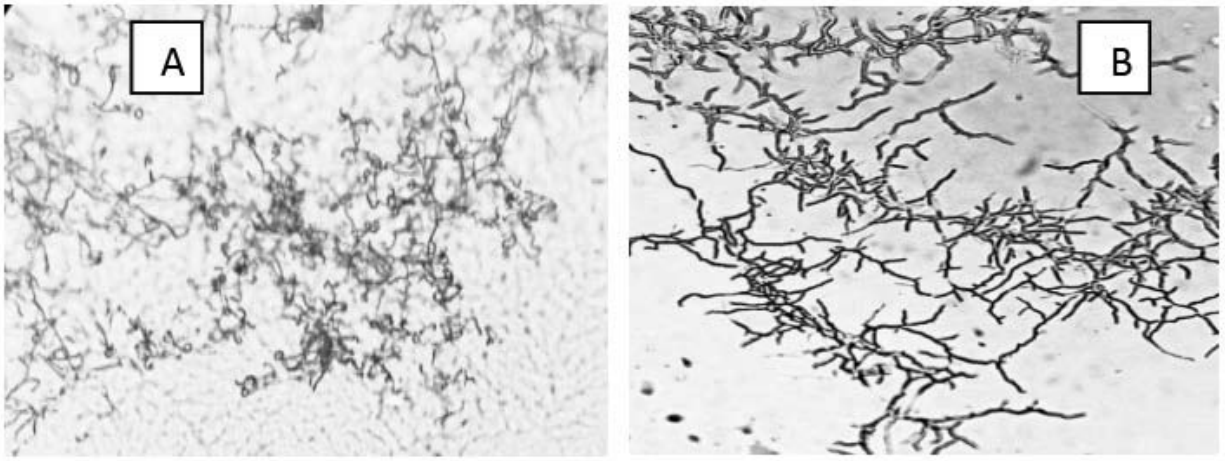

Figure 6: Aerial and substrate morphology of isolates (400X). A-Isolate PD18 and B- Isolate PD26.

Table 3: Morphological and biochemical characteristics of the isolates PD18 and PD26.

Character	Isolate PD 18	Isolate PD 26
Morphological characterization		
Sporophore morphology	Rectiflexible and short branched	Rectinaculiperti and long branched
Color of aerial mycelium	Greyish-white	Greyish-Brown
Color of substrate mycelium	Pale yellow to brown	Brown
Biochemical Characterization		
Amylase activity	+	+
Gelatin liquefaction	+	+
Lipase activity	+	+
Cellulase activity	+	+
Protease activity	+	+
Nitrate reduction	+	+
β-galactosidase activity	+	+
Carbohydrate fermentation	+	+
Indole test	+	+
Methyl red test	+	+
Voges Prosker test	-	-
Citrate utilization	+	+
Uriase activity	+	+
Uricase activity	-	+
Bio surfactant	+	+
Antibiotics	+	+
Antifungal metabolite	+	+

+: Positive and -: Negative

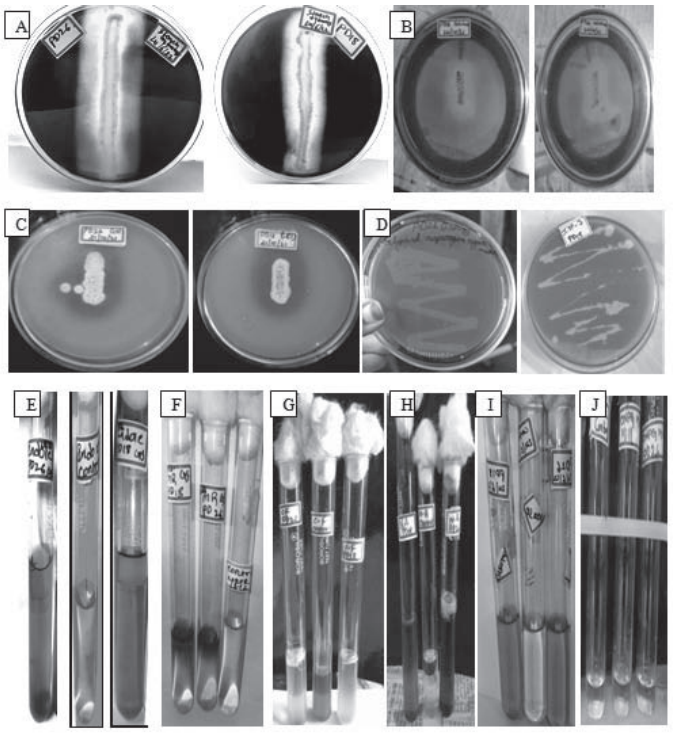

Figure 7: Biochemical characterization of isolates.
A- Amylase activity, B- Gelatinase activity, C- Cellulase activity, D- L-aspariginase activity, E- Nitrate reductase activity, F-Indole test, G- Methy red test, H- Carbohydrate fermentation test, I-uriase activity and J- β – galactosidase activity.

CONCLUSION

An attempt was made to isolate a tyrosinase producing actinomycetes from pre-existed cultures of our laboratory. Among six cultures, two strains PD18 and PD26 were found to be a novel source for tyrosinase production. Further, secondary screening revealed that SS medium was best for growth of isolates and tyrosinase production and maximum yield was noticed after 7 days (168 hr) of incubation. It was also found that presence of copper ion showed positive influence on the yield of tyrosinase

when compared to previous reports very less concentration (1mg/mL) is enough for the maximizing tyrosinase yield were found to be 21.20 IU/mL and 24.19 IU/mL by the cultures PD18 and PD26 respectively. The protein concentration of PD18 and PD26 were found to be 264µg and 325µg after extrapolating the absorbance from standard graph of BSA. As per previous research, the *Streptomyces cellulosae* strain was exclusively used to produce antibiotics, biosurfactants and enzymes like lipase, amylase, cellulase, chitinase, and protease up until this point. This work highlights the importance of discovering novel variants of *Streptomyces cellulosae* which were initially employed to produce tyrosinase, in addition to attractive sources for potentially bioactive compounds like antibiotics, antifungal metabolites, biosurfactants and enzymes like amylase, lipase, cellulase, uricase, urease, beta galactosidase and L- asparaginase. The BLAST similarity search and Phylogenetic analysis of the 16S rRNAgene revealed that the isolate PD18 shared 99.98% sequence similarity with the strain *Streptomyces cellulosae,* the isolate PD26 shared 100% sequence similarity with the strain *Streptomyces cellulosae.* Consequently, the isolates PD18 and PD26 were designated as new variants of *Streptomyces cellulosae* strain PD18 and *Streptomyces cellulosae* strain PD26 respectively.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool; **cm:** Centimeter; **mg:** Milligram; **µL:** Microliter; **µM:** Micromolar; **M:** Molar; **mL:** Milliliter; **rpm:** Revolution per minute; **hr:** Hours; **°C:** Degree Centigrade; **nm:** Nanometer; **IU:** International unit; **%:** Percentage; **NCIM:** National Collection of Industrial Microorganisms; **NCBI:** National Center for Biotechnological Information; **rRNA:** Ribosomal Ribonuclic acid.

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Original Article

Effect of Process Variables on the Development and Characterization of Nanocellulose as Novel Biopolymer

Pradeep Heregangur Keshavamurthysetty*, Dipti Hiteshkumar Patel

Department of Pharmaceutics, Institute of Pharmaceutical Sciences, Faculty of Pharmacy, Parul University, Limda, Waghodia, Gujarat , INDIA.

ABSTRACT

Nanocellulose has excellent mechanical, physical, and biological properties; it is an ideal material for many applications. The present study aimed to develop nanocellulose from cellulose by hydrolyzing with sulphuric acid to yield nanocellulose. In order to prepare nanocellulose, two factors were considered: the concentration of sulphuric acid and the variation in temperature (AHNC1 to AHNC5). Optimized NC was characterized by its size, zeta potential, TEM, XRD, and FT-IR. NCs had a size range between 134 to 644 nm. AHNC4 and AHNC5 showed respective Zeta potentials of -40.6 and -37.4. XRD diffractograms showed crystallinity indices in the 55-66% range. The TEM showed that the nanocellulose was semicrystalline. FT-IR measurement showed a peak at 1177 cm^{-1} due to O-H association bond of nanocellulose, which is similar to cellulose's peak. This work analyzed that the nanocellulose preparation involves the optimization process to get the desired size of particles by altering process parameters in the acid hydrolysis method like concentration of acid and temperature maintained during the reaction, which depend on the source of cellulose used in the development of nanocellulose.

Keywords: Nanocellulose, Acid hydrolysis, CNC, CNF, Nanocrystals.

Correspondence:
Mr. Pradeep Heregangur Keshavamurthysetty
Department of Pharmaceutics, Institute of Pharmaceutical Sciences, Faculty of Pharmacy, Parul University, Limda, Waghodia, Vadodara- 391760, Gujarat, INDIA.
Email id: pradeephkgmips@gmail.com

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INTRODUCTION

Among the natural polymers, cellulose is most abundantly available as a polysaccharide as it is a significant component of all plant materials.[1] Because of its chemical nature and intramolecular or intermolecular hydrogen bonding, it is insoluble in water and many other organic solvents.[2] Most cellulose comprises large polymeric chains with varying molecular weights with the formula $(C_6H_5O_5)n$, where n denotes the degree of polymerization.[3] The degree of polymerization of the cellulose mainly depends on the source of plant material and the methodology adopted during manufacture. Data reveals the degree of polymerization between 1500 to 3500.[4]

Several types of nanocellulose were found, and they found their high significance in many aspects. Nanocellulose (NC) or Cellulose Nanocrystals (CNC) are in whisker–shaped and rod-like structures. These are found in crystalline regions of cellulose. These crystalline cellulose provide a high aspect ratio.[4] Nanocellulosic fibrils, NCF, are thread-like polymers containing hydrogen bonding between the cellulose and OH groups that interact intra-molecularly. They can be prepared easily by a mechanical method.[5] Reducing the size of cellulose to nano level yields nanosize particles and provides high crystallinity. This can be achieved by various methods like acid hydrolysis, tempo oxidation, and ionic liquid.[6]

In the present evolving trend, nanocellulose is the tool for various branches like pharmacy, medical field, engineering, tissue engineering, chemical engineering, etc. Nanocellulose is one of the best carriers in the pharmaceutical field because it can be used as natural super disintegrants,[7] drug delivery carrier,[8] thickening agent in the hydrogel,[9] aerogels,[10] wound healing,[11] transdermal films,[12] and ophthalmic drug delivery.[13]

Several types of research are being found nowadays on developing nanocellulose from various sources of plant materials, bio waste, and agricultural residues. This study focuses on the acid hydrolysis process parameters in developing nanocellulose and, for this purpose, compares results of acid hydrolysis and size of nanocellulose by available literature as tabulated in Table 1. The particle size of nanocellulose is significantly influenced by the acid content, reaction temperature, and reaction duration as per the observation from Table 1. In addition to this method of approach, knowledge of the source of cellulose employed for the investigation is necessary.

MATERIALS AND METHODS

Materials

Cellulose and sulphuric acid (98%) were purchased from SD Fine Chem. All chemicals are 98.0% pure and used without

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Table 1: Influence of methodologic factors and source of cellulose on particle size of nanocellulose.

Sl. No	Research Papers	Source of Cellulose	Concentration of acid	Temperature	Particle Size
1	Nanocellulose prepared by acid hydrolysis of isolated cellulose from sugarcane bagasse.[14]	Sugar Cane bagasse– CNC	50% V/V H_2SO_4	40°C 30 min	111 nm
2	Acid Hydrolysis-Mediated preparation of Nanocrystalline Cellulose from Rice Straw.[15]	Rice straw –CNC	64% V/V H_2SO_4	45°C for 60 min	129.9 to 292.0 nm length
3	Preparation and Characterization of Cellulose Nanocrystal Extraction From Pennisetum hydridum Fertilized by Municipal Sewage Sludge via Sulfuric Acid Hydrolysis.[16]	Pennisetum hydridum– CNC	65% V/V H_2SO_4	37°C 120 min	272.5 nm
4	Isolation and Characterization of Nanocellulose Obtained from Industrial Crop Waste Resources by Using Mild Acid Hydrolysis.[17]	Pineapple leaf	30% V/V H_2SO_4	50°C for 6 hr	250 nm
			60% V/V H_2SO_4	50°C for 30 min	160 nm
		Banana rachis	30% V/V H_2SO_4	50°C for 6 hr	459 nm
			60% V/V H_2SO_4	50°C for 30 min	296 nm
		Sugarcane bagasse	30% V/V H2SO4	50°C for 6 hr	730 nm
			60% V/V H_2SO_4	50°C for 30 min	334 nm
5	Preparation and characterization of cellulose nanocrystal extracted from ramie fibers by sulfuric acid hydrolysis.[18]	Ramie fibers CNC	58% V/V H_2SO_4	45°C for 30 min	145.61 nm length and 6.67 nm Width
6	Isolation and Characterization of Nanocrystalline Cellulose Isolated from Pineapple Crown Leaf Fiber Agricultural Wastes Using Acid Hydrolysis.[19]	Pineapple crown leaf fiber	1 M H_2SO_4	45°C for 60 min for 1 to 3 hr	11.55 to 20.21 μm
7	Production of Nanocellulose Crystal Derived from Enset Fiber Using Acid Hydrolysis Coupled with Ultrasonication, Isolation, Statistical Modeling, Optimization, and Characterizations.[20]	Enset Fiber	51.6% H_2SO_4 V/V	47°C for 66.5 min	66 nm
8	Physicochemical Properties of Nanocellulose Isolated from Cotton Stalk Waste.[21]	Cotton Stalk	65% V/V	50°C	
				40 min	90.5 nm
				50 min	270.4 nm
				60 min	664.9 nm
9	Facile synthesis and characterization of nanocellulose from *Zea mays* husk.[22]	*Zea mays* husk	60% W/V H_2SO_4	45°C for 60 min	14 to 29 nm

purification. GEM Laboratories was kind enough to provide the lab-grade water for research.

Methods

The acid hydrolyzed nanocellulose (AHNC) was prepared by keeping the temperature and concentration of the acid as the independent parameters. To study the effect of acid on the development of nanocellulose,35,41,44,47 and 50% v/v sulphuric acid concentrations were chosen to study. NC was prepared according to the general acid hydrolysis method. Briefly, 1g of cellulose sample was dispersed slowly in the sulphuric acid at different concentrations. The temperatures that were maintained for the various concentrations are shown in Table 2. The reaction was carried out under constant stirring with the aid of a magnetic stirrer for 2.5-3 hr. The reaction was quenched by the addition of 10 folds of ice-cold water. The change of colour of mixture

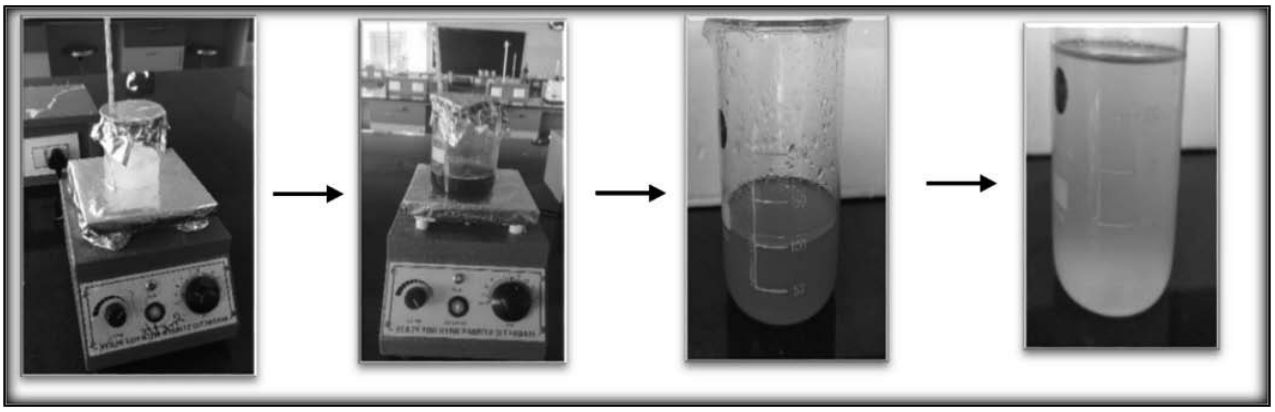

Figure 1: Change of colour during the process of reaction.

Table 2: Parameters considered for development of NC and results.

Formulation Code	Concentration of Sulfuric Acid v/v	Temperature in °C
AHNC1	35%	30
AHNC2	41%	35
AHNC3	44%	36.5
AHNC4	47%	40
AHNC5	50%	43.5

during the reaction shown in Figure 1. The obtained product was washed until the supernatant reached a neutral pH. The filtrate was checked for sulphate ions through a barium chloride test. The suspension was centrifuged and collected. The sample was stored in the refrigerator for future use (Figure 2). Methodology is graphically represented as shown in Figure 3.

Characterization of Nanocellulose

Fourier transform infrared spectroscopy

The presence of functional groups in cellulose and nanocellulose was determined using Fourier Transform Infrared Spectroscopy and spectrum obtained from Bruker at poornayu research lab, Bangalore, Karnataka, India. The range obtained from 500 to 4000 cm^{-1}, with the KBr pelletization method, is adopted during analysis.

Dynamic light scattering

Dynamic light scattering analysis is one of the well-known techniques to determine a nanoparticle's average particle size and intensity size distribution. Through the various treatments, the cellulosic polymeric chains break down into smaller units, changing the size of cellulose to nanocellulose. The samples were diluted with purified water and shaken for 15 min in a shaker bath to remove any clumps formed during the transit. This was followed by filtration to analyze the sample using a glass cuvette and disposal of zeta cell for zeta potential by Malvern zeta sizer.

X-Ray Diffractometer

The crystallinity index of the sample was analyzed for the samples for a desired size range. The X-Ray Diffractograms (P-XRD) were recorded with MiniFlex 300/600. The nanosuspensions were dried using the solvent exchange method by using acetone as a solvent. The Crystallinity index calculated by using following formula,

$$\text{Crystallinity Index } (\%) = \frac{\text{Area of crystalline peaks}}{\text{Total area of peaks}} \text{ x } 100 \ldots\ldots.(1)$$

Transmission electron microscopy (TEM)

The morphological studies of optimized nanocellulose suspension were observed by TEM analysis. TEM images were recorded using the FEI TECNAI G2 TEM @200KV, a Field Emission Gun (FEG), and a +/-80-degree tilted computer-controlled LiN cryostage.

RESULTS AND DISCUSSION

Fourier transform infrared spectroscopy (FTIR)

The FT IR spectra of nanocellulose, as shown in Figure 4, reveals that the presence of a strong, broad peak at 3334.93 cm^{-1} is due to the -OH stretching present in cellulose. The peak absorption at 1629.73 cm^{-1} was reported as H-O-H and conjugated C=O stretching. The minor peak at 2105 cm^{-1} represents superimposed OH peaks due to intermolecular hydrogen bonding. The peak at 1197.40 cm^{-1} is due to the O-H association bond of cellulose. The peak around 1046.76 cm^{-1} (due to the β-glycosidic linkage) was attributed to the O-C-O stretching during the C-H deformation of cellulose.[22]

Dynamic light scattering method

The DLS method was used to determine the particle size and polydispersity index of nanocellulose produced by the acid hydrolysis method.

Particle size and particle size distribution were examined by DLS, and findings were recorded in Figure 5 and Figure 6. In Figure 7, AHNC 1 and 2 illustrates the tri-modal peaks size of particle ranging from 100 to 10000 nm, respectively. The Z average mean

Figure 2: Nanocellulose suspensions.

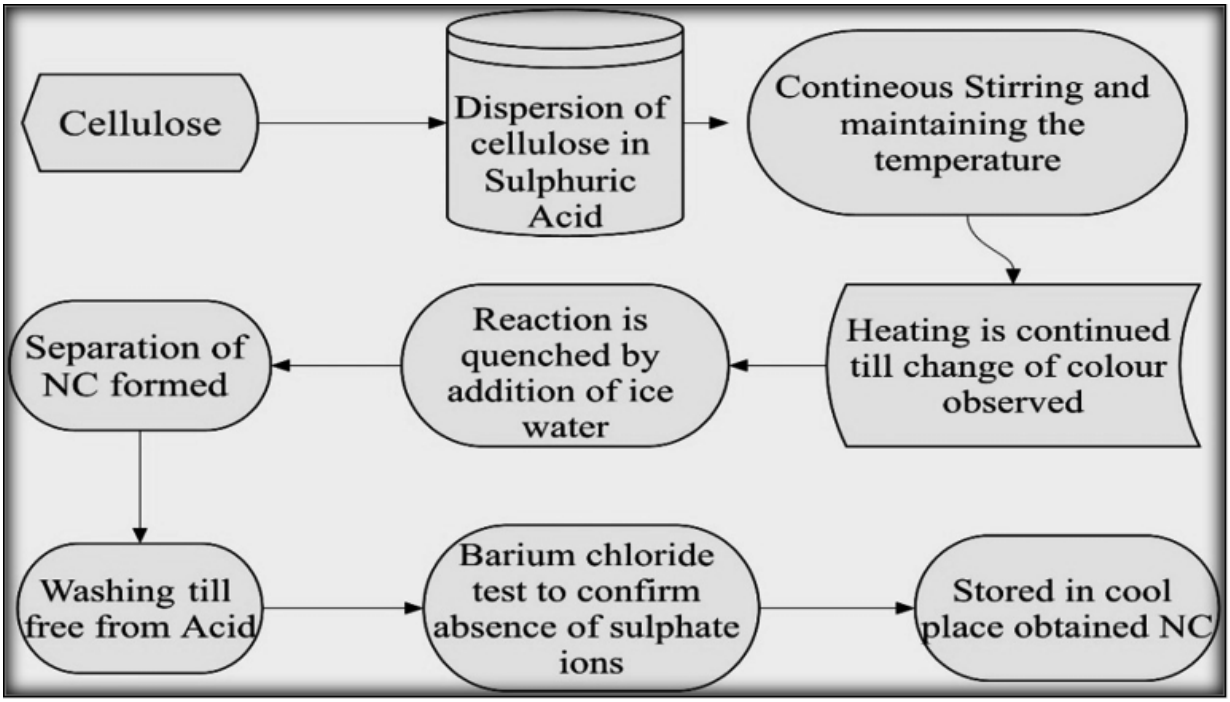

Figure 3: Steps involved in the process of nanocellulose.

recorded by the instrument is 644.5 and 522.5 nm. The results are not in the acceptable range as % the intensity of size distribution varies with tri-modal peaks. Further acid hydrolysis of cellulose continued with increasing the concentration of sulphuric acid and temperature, as mentioned in Table 2. From Figure 7, it was observed that AHNC4 showed a better particle range between 50 to 600 nm with uniform distribution, and its measured Z average mean documented 134 nm for nanocellulose suspension prepared from 47% v/v and 40°C temperature, which is in nanometer scale and desired for the study.

On the other hand, AHNC 3 has shown bimodal peaks with particle sizes ranging from 50 to 400 nm, even though it is within the nanometer scale; because of the nonuniform distribution of particles, it was not considered. Similarly, AHNC 5 has shown particle size between 70 to 900 nm with Z average particle size of 208.3 nm within the nanometer scale. Based on the particle size AHNC4 is considered an optimized formula for further studies.

The intensity of particle size distribution determines the Z average. The polydispersity index (PDI) explains the homogeneity of the sample. As the sample's PDI increases, the sample's dispersibility decreases. Typically, the samples having PDI less than 0.4 with unimodal peak are said to be monodisperse. In the case of more than 0.4, PDI bimodal and trimodal peaks are observed.

Table 3: Z-Average particle size from DLS instrument.

Formulation code	Formulation Code		Size (d. nm)	% Intensity	Zeta Potential
AHNC1	Z-average (d.nm): 644 PDI: 1.0 Result quality: Refer to quality report	Peak 1	1067	64.5	NA
		Peak 2	175.2	20.7	
		Peak 3	4998	14.8	
AHNC2	Z-average (d.nm): 522.5 PDI: 0.826 Result Quality: Refer to quality report	Peak 1	624.9	62.2	NA
		Peak 2	4675	21.3	
		Peak 3	122.7	16.5	
AHNC3	Z-average (d.nm): 255.6 PDI: 0.467 Result Quality: Refer to quality report	Peak 1	235.4	66.4	NA
		Peak 2	85.93	33.6	
		Peak 3	0.0	0.0	
AHNC4	Z-average (d.nm): 134 PDI: 0.341 Result Quality: Good	Peak 1	159.	94.4	- 40.6
		Peak 2	0.0	0.0	
		Peak 3	0.0	0.0	
AHNC5	Z-average (d.nm):208.3 PDI: 0.212 Result Quality: Good	Peak 1	268.0	100	-37.4
		Peak 2	0.0	0.0	
		Peak 3	0.0	0.0	

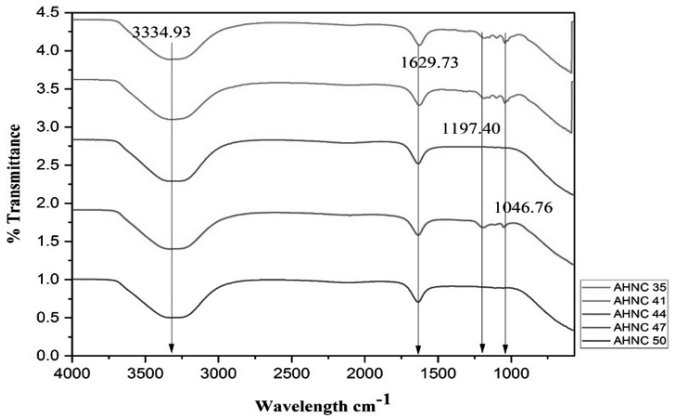

Figure 4: FTIR spectrum of AHNC 1 to 5.

The PDI of the AHNC4 and AHNC 5 prepared from experimental work is found to be 0.341 and 0.212, which confirms the uniformity of the nanocellulose with a single peak.

Zeta potential is used to estimate the long-term stability of the particles. The particles which show the zeta potential of more than ± 30 mV are said to be good as they are less prone to form aggregates.[23]

The Zeta potential of acid hydrolyzed nanocellulose suspension of AHNC4 and AHNC5 is found to be -40.6 and -37.4, which confirms that prepared nanocellulose is more stable in long-term storage if proper storage conditions are maintained. The zeta potential of AHNC 4 and AHNC5 is shown in Figure 7. The average particle size from DLS instrument shown in Table 3.

From Table 1 it was observed the behavior of sulphuric acid and temperature were analyzed for the preparation of nanocellulose by acid hydrolysis method. The temperature was varied in the range of 37°C to 50°C with varying concentrations of sulphuric acid from 30 to 65% v/v. As a result, the particle size of NC has been obtained in the range of 14 nm to 730 nm.

The reason for varying particle size can be explained by understanding the mechanism of acid hydrolysis on cellulose. Cellulose consists of amorphous and crystalline zones alternatively. Amorphous areas in cellulose are considered as structural flaws or chaotic areas. Enzymatic and acid hydrolysis are preferential methods for removing those flaws.[24-28] Comparatively speaking, the amorphous sections hydrolyze more quickly than the crystalline segments.[29] Thus, the concept of controlled hydrolysis was applied to the disruption of the amorphous areas to produce crystalline nanocellulose. Hence, the particle size mainly varies or depends on the source of cellulose and how much it contains amorphous regions. Hence the rate of reaction depends on the concentration of acid and temperature which can be observed from Table 1.

X-Ray Diffractometer

AHNC 4 and AHNC 5 were taken for further studies as they have shown better results. To understand the crystallinity of the sample after chemical treatment was analyzed by XRD. Figure 8(a) and (b) shows intense high peaks at 22.5° and 22.6°, explaining the crystalline nature of the nanocellulose. In both other peaks at 16°, 15.3°, and 34°, 34.7° is observed about an amorphous form so that it may be considered as developed nanocellulose is in semicrystalline. Similar results were obtained by comparing commercial NC, having a peak at 22.66 at 2θ.[22]

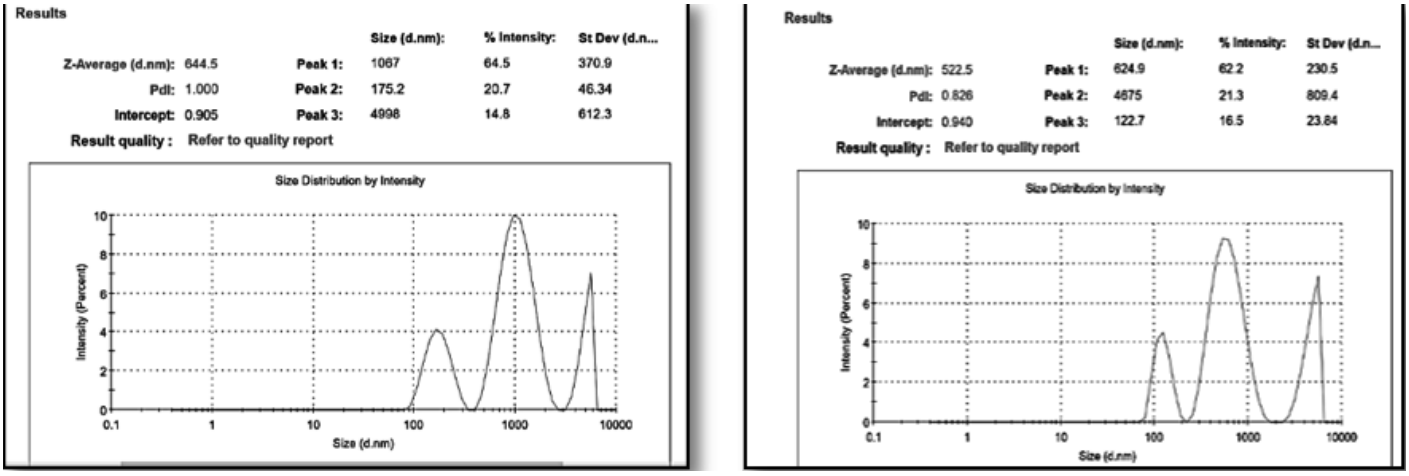

Figure 5: Particle size distribution by DLS method for nanocellulose of AHNC 1 and 2.

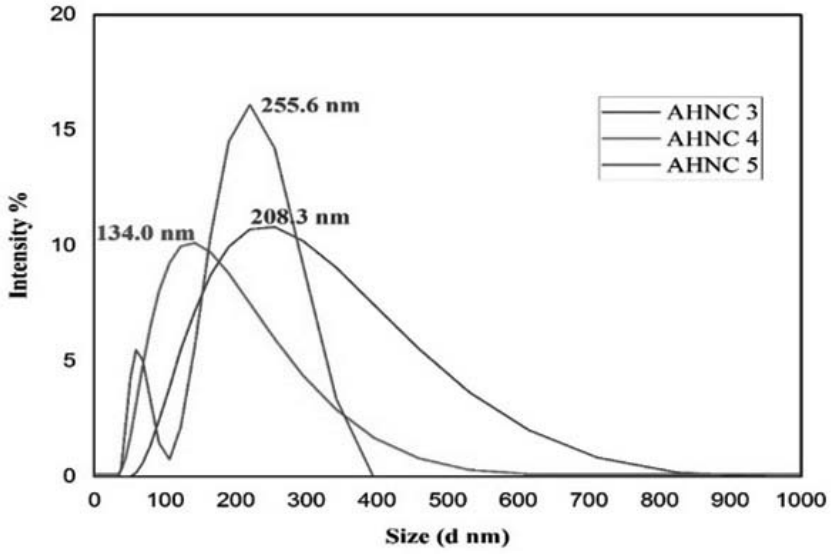

Figure 6: Particle size distribution by DLS method for nanocellulose of AHNC 3 to 5 and varied with temperature and concentration.

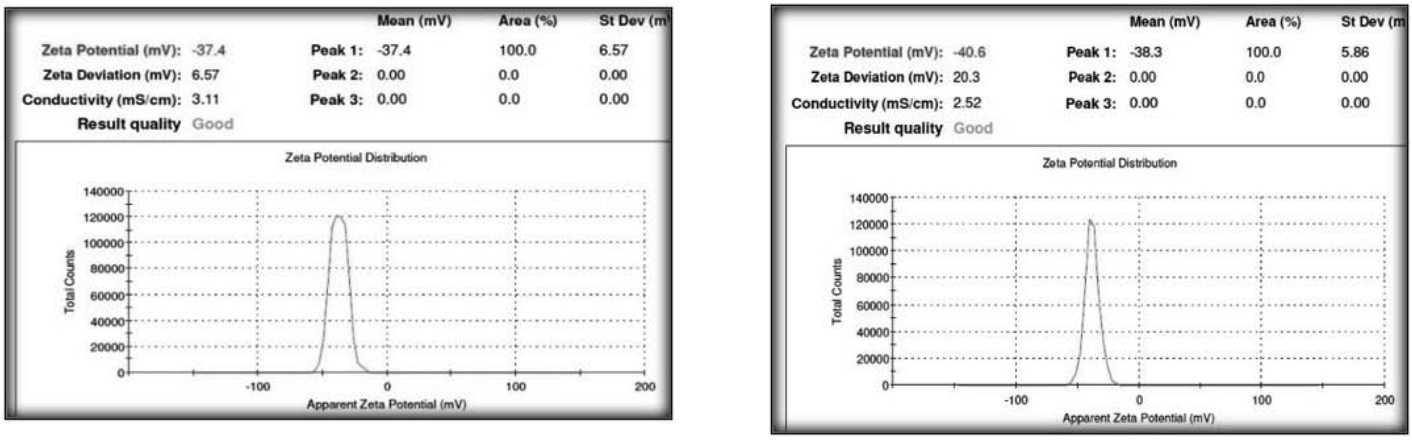

Figure 7: Zeta potential of AHNC 4 and AHNC 5.

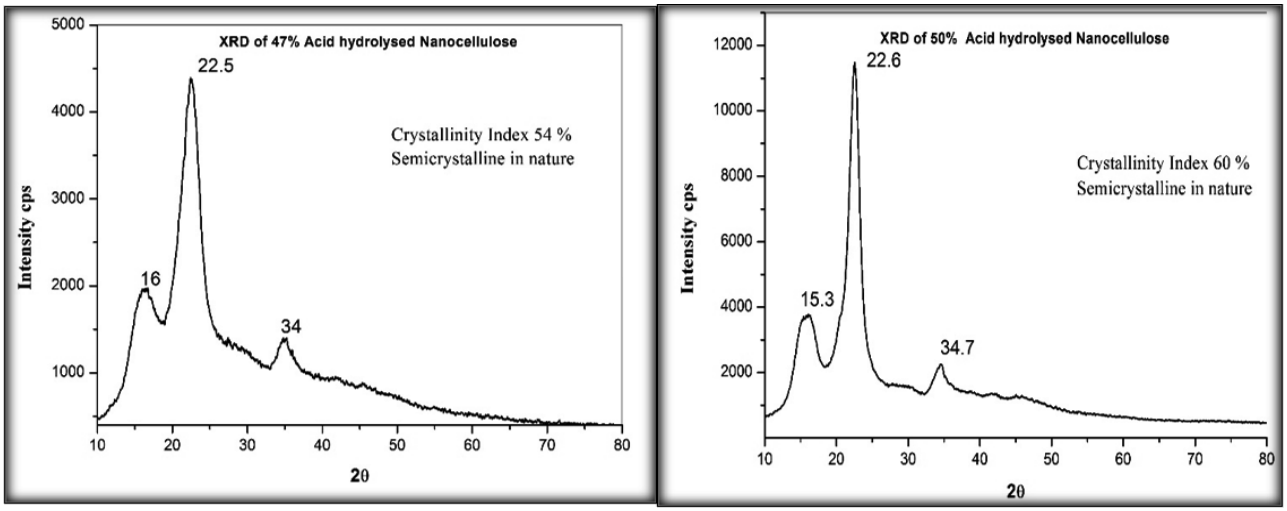

Figure 8: XRD of (a) AHNC 4 and (b) AHNC 5.

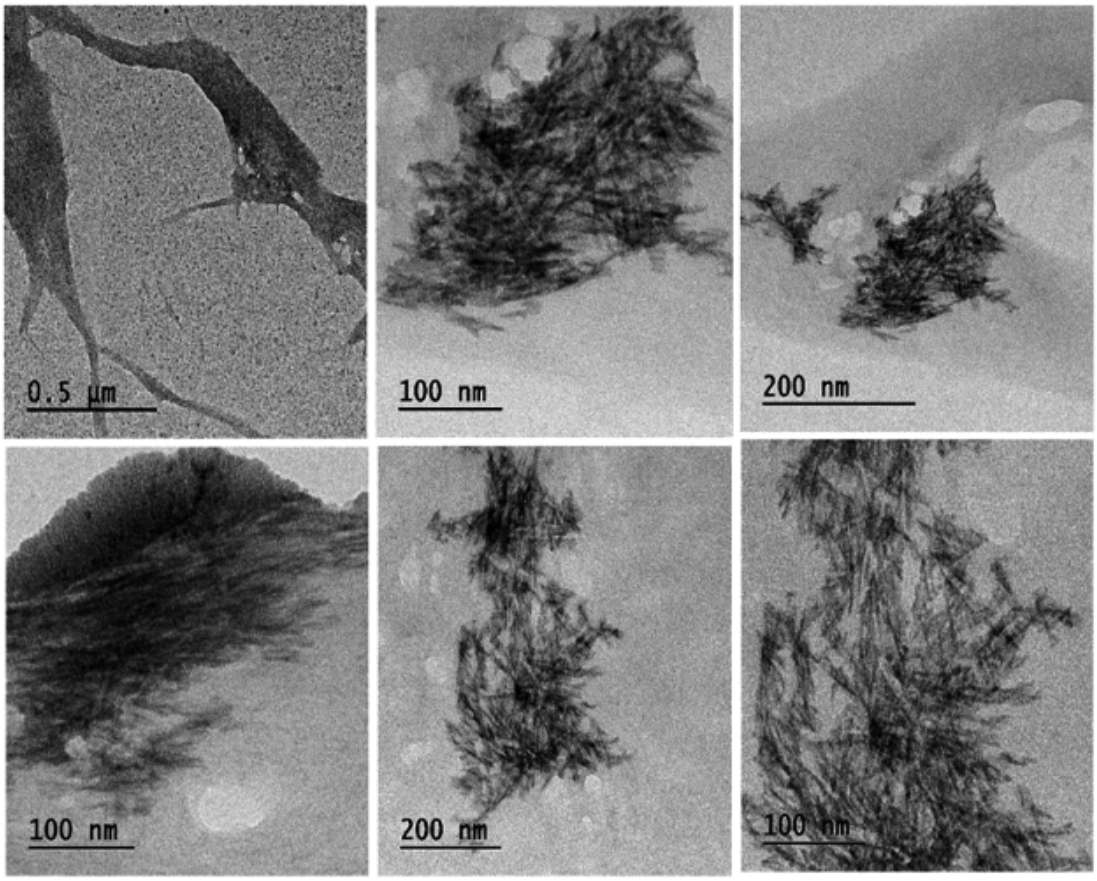

Figure 9: TEM images of AHNC4.

The crystallinity index was calculated by using equation 1 (as discussed in methodology) by using Origin 2019b 64-bit software. It was found that 54 and 60%, respectively, for AHNC4 and AHNC5.

Transmission electron microscopy

The structure and internal morphology of prepared nanocellulose were examined using TEM analysis. The prepared cellulose has a nanofibrous morphology with fibers, as shown in Figure 9. It was simple to see that the nanofibers were clearly separated from one another. The lengths of prepared nanocellulose range from 50 to 300 nm. It can be observed in the fibrous aggregation images. It is inferred that the harshness of the acid hydrolysis process results in the production of rod-like nanocellulose.

CONCLUSION

In this study, commercial cellulose was acid hydrolyzed to produce nanocellulose. The impact of changing acid concentration and temperature on acid hydrolysis was thoroughly investigated. From this work, it was established that an optimal temperature and acid concentration were needed to start the reaction and produce the desired particle size. For any source of cellulose, it is necessary to optimize the factors to obtain the desired size. FTIR measurements confirm the existence of functional groups similar to those found in cellulose. According to PXRD data and referred data, manufactured nanocellulose has the same crystallinity as commercial nanocellulose, and the nature of semicrystalline was seen in TEM images. The presence of fibrous nature with diameters ranging from 50 to 300 nm is confirmed by TEM images. Thus obtained, nanocellulose was further studied for its applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

FTIR: Fourier transform infrared spectroscopy; **PXRD:** Powder X- Ray Diffraction; **TEM:** Transmission electron microscopy; **AHNC:** Acid hydrolysed nanocellulose; **NC:** Nanocellulose; **CI:** Crystallinity Index; **CNC:** Cellulose nanocrystals; **CNF:** Cellulose nanofibrils.

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Original Article

Development and Evaluation of Fast Dissolving Oral Films of Mefenamic Acid for the Management of Fever

Krishnameera Sajayan[1,*], Swathy KK[1], Sarath Chandran C[1], Jafna MC[1], Rajesh Sreedharan Nair[2], Sourav K[1], Shijina Kappally[3], Sreejith KR[1], Jim Joseph[4]

[1]College of Pharmaceutical Sciences, Government Medical College Kannur, Pariyaram, Kerala, INDIA.
[2]School of Pharmacy, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, Selangor, MALAYSIA.
[3]College of Pharmacy, Sharjah University, Sharjah, UNITED ARAB EMIRATES.
[4]Sance Laboratories Pvt. Ltd., Pala, Kottayam, Kerala, INDIA.

ABSTRACT

The marketed formulations of Mefenamic Acid (MA) used for the treatment of fever in the paediatric population are reported to have several drawbacks. This study aimed to develop and evaluate the mefenamic acid–loaded oral dispersible films which may be a better alternative than the existing formulations. The solubility of mefenamic acid was enhanced by forming inclusion complexes with β cyclodextrin. The best ratio for the mefenamic acid-β cyclodextrin inclusion complex, 1:0.5 was selected based on the drug content and *in vitro* drug release. Fourier transform Infrared spectroscopy and scanning electron microscopy analysis was performed on the complex. The oral dispersible films were developed by solvent casting method and evaluated for parameters such as average weight, thickness, pH, folding endurance, percentage moisture uptake and loss, drug content, *in vitro* disintegration time and drug release. The statistical analysis of the data suggested that oral dispersible films with 4% w/v of crospovidone (coded P3) as the best. Drug content (95.46±0.93%), disintegration time (28.6±2.0 s), cumulative percentage drug release (97.41±0.68% in 180 s), and all other investigated parameters of P3 were well within the acceptable limit. The *in vitro* dissolution profile of P3 showed no significant difference from the marketed mefenamic acid suspension and has a good stability profile at in-house testing conditions. The data obtained from this investigation revealed that mefenamic acid oral dispersible films could act as an excellent alternative to existing marketed paediatric formulations.

Keywords: Oral dispersible films, Mefenamic acid, β Cyclodextrin, Paediatric, Kneading method, Solvent casting.

Correspondence:
Ms. Krishnameera Sajayan
College of Pharmaceutical Sciences, Government Medical College Kannur, Pariyaram–670 503, Kerala, INDIA.
Email: krishnameera1697@gmail.com

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INTRODUCTION

Mefenamic Acid (MA) is a Non-Steroidal Anti-Inflammatory Drug (NSAID), that belongs to the Biopharmaceutical Classification System II (BCS II).[1] It is a registered NSAID used in the paediatric population from 6 months of age for the management of fever and may be an effective alternative to ibuprofen. The MA is recommended to administer not more than three times a day, in a dose of 6.5 mg/kg of body weight.[2] The suspensions, syrups, capsules and tablets are the most commonly available dosage forms of MA for paediatrics. But the solid oral dosage form of MA has less acceptance in the paediatric population due to fear of choking and the bitter taste of the drug. Moreover, the MA tablet needs splitting to get an accurate dose, which may lead to the loss of the drug. Nowadays, oral liquid dosage forms like syrups and suspensions of MA are commonly prescribed to the paediatric population. The taste–related concerns and viscous consistency may also lead to poor acceptance of paediatric MA liquid dosage forms. The associated problems of liquid dosage forms such as the possibility for inaccurate dosing, microbial contamination, stability issues and accidental breakage of containers reduce the popularity of MA liquid dosage forms.[3]

To overcome the drawbacks associated with existing MA oral dosage forms, alternative delivery approaches such as Oral Disintegrating Tablets (ODT) and Oral Dispersible Film (ODF) may be considered. ODT had superiority over the existing oral solid dosage forms, such as no swallowing difficulty, consumption without water, reduction in drug loss, and rapid onset of action.[4] But the fear of choking may keep some sections of the patient population away from using the ODT.[5] The ODT were fragile and friable, which required special packaging for safe storage and transportation. The higher cost of the formulation and specialised packaging made the ODT more expensive in

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comparison with standard oral tablets.[6] The oral dispersible film (ODF) may be considered as an alternative for the conventional solid and liquid dosage forms as well as ODT. The ODF is a thin film placed in the oral cavity, hydrated with saliva, rapidly dissolved and release medicament/s for quick absorption. The ODF gained popularity with its impressive ability to deliver active pharmaceutical ingredients.[7] The ODF had advantages over other oral solid formulations, which included no difficulty in swallowing, disintegrating instantaneously, avoiding a situation of splitting large tablets and water not required for consumption.[3,8] When compared with popular oral liquid dosage forms, the advantages of ODF included, accuracy in dose, minimal contact with mouth while administration, convenience in storage as well as transportation, no additional measuring cups or devices required and improved stability and taste.[9,10] The large surface area for quick disintegration, increased flexibility, resistance to mechanical stress and improved patient compliance may provide superiority for the ODF over ODT.[8,9] The possibilities of ODF were exclusively explored in the paediatric and geriatric populations to administer drugs effortlessly. This study aimed to develop and evaluate the MA–loaded ODF which may be a better alternative than the existing paediatric formulations of MA. The objectives of the study included solubility enhancement of MA by forming inclusion complexes with β Cyclodextrin (βCD) and comparing the *in vitro* release profile with the marketed product of MA.

MATERIALS AND METHODS

Materials

MA, β Cyclodextrin (βCD) and Orange flavour were procured from Yarrow chemicals (Mumbai, India), Hydroxypropyl Methylcellulose E5 (HPMC E5) was collected from Balaji chemicals (Gujarat, India), Polyvinyl Alcohol (PVA), Polyethylene Glycol–400 (PEG–400), Crospovidone (CP), Citric Acid (CA) and Saccharin sodium were purchased from Nice Chemicals (Kochi, India).

Drug–excipient compatibility studies

The chemical interactions between the pure MA, as well as the physical mixture of MA and βCD and MA with selected excipients, were analysed by Fourier Transform Infrared (FT–IR) spectroscopy. Dried pellets were formed by mixing the samples with a sufficient quantity of potassium bromide, and were then scanned from 4000 cm^{-1} to 400 cm^{-1} using a Bruker alpha II FT–IR spectrophotometer.[11]

Preparation of MA-βCD inclusion complexes

The solubility of MA was enhanced by forming inclusion complexes with βCD using the kneading method. The MA-βCD inclusion complexes were developed at different molar ratios such as 1:0.5, 1:1, and 1:2. The weighed quantity of βCD was taken in a glass mortar and added a sufficient volume of distilled water to obtain a homogenous paste. The paste was dried at 40°±2°C for 6hr. The dried complex was kept in a desiccator and used for further investigation.[12]

Evaluation of MA-βCD inclusion complexes

Drug content estimation

MA-βCD inclusion complexes equivalent to 10 mg of MA were weighed and dispersed in 100 mL of Phosphate Buffered Saline (PBS) of pH 6.8. The solution was shaken well and allowed to settle. 1mL of the supernatant solution was pipetted out from this stock solution and made up to 10mL by using PBS of pH 6.8. Drug content was measured using a UV–visible spectrophotometer at 285 nm using PBS of pH 6.8 as a blank.[13]

In vitro drug release study

The *in vitro* dissolution study was performed in USP type–II apparatus. MA-βCD inclusion complexes equivalent to 65 mg of MA were dispersed in 900 mL PBS of pH 6.8 taken in a vessel. The temperature was maintained at 37°±0.5°C. The dissolution was performed at 100 rpm and 10 mL of sample was withdrawn at 0, 30, 60, 90, 120, 150, 180, 210, and 240th ses and replaced with an equal volume of fresh medium. The absorbance of the resulting solutions was measured using a UV–visible spectrophotometer at 285 nm.[12]

Selection and analysis of the best ratio of MA-βCD inclusion complex

The best ratio for the MA-βCD inclusion complex was selected after the evaluation of complexes. The selected ratio was analysed using Scanning Electron Microscopy (SEM) and FT–IR to confirm the formation of the inclusion complex.[12]

Preparation of MA oral dispersible films

The solvent casting method with necessary modifications was used for the development of ODF based on the composition given in Table 1. The weighed quantity of HPMC E5 was dissolved in a one-by-the–fourth volume of distilled water taken in a beaker. The solution was continuously stirred using a magnetic stirrer at 600 rpm. MA-βCD inclusion complexes equivalent to 65 mg of MA were dissolved in a sufficient volume of distilled water and vortexed. The drug solution was added drop by drop to the polymer solution, which was kept under continuous stirring. In a separate beaker, weighed quantity of CP, CA, PEG–400, and saccharin sodium was dissolved in the remaining volume of distilled water, which was stirred for 30 m using a magnetic stirrer at 600 rpm. The resultant solution was added to the drug–polymer blend, followed by the addition of orange flavour, and continued stirring for the next 1 hr. The solution was kept aside and allowed for the removal of any entrapped air bubbles. The solution was poured into a previously designed glass mould of

Table 1: Composition of developed oral dispersible films.

Ingredients	Formulation Code							
	H1	H2	H3	H4	P1	P2	P3	P4
MA-βCD complex equivalent to (mg)	65	65	65	65	65	65	65	65
HPMC E5 (mg)	390	390	390	390	-	-	-	-
PVA (mg)	-	-	-	-	260	260	260	260
PEG–400 (mL)	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39
Crospovidone (%w/v)	-	2	4	6	-	2	4	6
Citric acid (mg)	103	103	103	103	103	103	103	103
Saccharin sodium (mg)	103	103	103	103	103	103	103	103
Orange flavour (mL)	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Distilled water (mL)	13	13	13	13	13	13	13	13

7.5cmx5.5cm (W x L) dimension and allowed for drying at room temperature (30°±2°C) for the next 24–48 hr. The dried film was cut into strips of 2cmx2cm size, wrapped in aluminium foil, and stored in a desiccator until used for further studies. A similar procedure was followed for ODF based on PVA and ODF without CP.

Evaluation of MA oral dispersible film strips

To determine the average weight, three ODF strips (2cm x 2cm) were randomly selected from each developed batch of formulations. The individual weight of each strip was measured in a previously calibrated digital weighing balance and the average weight was calculated (n=3).[14] The thickness of the strip (2cmx2cm) at three different spots was measured using a previously calibrated screw gauge and the mean average thickness was calculated (n=3).[15] The folding endurance for the MA oral dispersible film strips was measured by folding the film strip repeatedly at the same point, till it was broken.[14] To determine the pH of the oral dispersible film, the strips were allowed to dissolve in a petri dish containing 2 mL of distilled water and the pH of the resulting solution was measured at room temperature (30°C±2°C) using a digital pH metre.[16] The percentage moisture loss from ODF strips (2cmx2cm) was analysed by measuring the initial weight of each selected strip using a digital weighing balance followed by storage in a desiccator containing calcium carbonate for three days and reweighed. The percentage moisture loss for the developed ODF strip was calculated using equation 1. To identify the percentage moisture uptake the initial weight of each selected strip was weighed using a digital weighing balance followed by exposure to a relative humidity of 75%±5% at room temperature (30°±2°C) for 7 days and reweighed. The percentage moisture uptake of the ODF strips was calculated using equation 2.[15]

$$\text{Percentage moisture loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \ldots\ldots (1)$$

$$\text{Percentage moisture uptake} = \frac{\text{Final weight-Initial weight}}{\text{Initial weight}} \times 100 \quad (2)$$

Drug content estimation for MA oral dispersible film strips

The drug content estimation was performed over three randomly selected ODF strips from each batch. The strips were dissolved in 100 mL PBS of pH 6.8. The solution was filtered, and 1 mL was diluted to 10 mL by using PBS pH 6.8. The drug content was measured using a UV–visible spectrophotometer at 285 nm with PBS pH 6.8 as a blank. The average drug content was calculated for each of the developed batches of ODF strips and reported.[14]

In vitro disintegration time of oral dispersible film strips

The disintegration study was carried out by randomly selecting, three ODF strips (2cmx2cm) from each batch and were placed in a beaker containing 10 mL PBS of pH 6.8. The time taken to disintegrate into tiny particles was measured. The average time in seconds was analysed in triplicate.[17]

In vitro drug release study of oral dispersible film strips

The *in vitro* dissolution of MA from ODF strips was performed in a 50 mL beaker containing 30 mL of PBS of pH 6.8 maintained at 37°C±0.5°C. Three ODF strips (2cmx2cm) were randomly selected from each batch of the developed formulations and placed into three different beakers containing PBS solution. 5 mL of sample solution was withdrawn at 0, 30, 60, 90, 120, 150, 180, 210, 240, and 300th sec and replaced with an equal volume

of fresh medium. The beaker was intermittently shaken using the mechanical beaker shaker. The absorbance for the withdrawn samples was measured using a UV–visible spectrophotometer at 285 nm.[18]

Statistical optimization of developed MA oral dispersible film strips

The data from *in vitro* disintegration and *in vitro* release studies were statistically analysed using GraphPad software, Prism version 9.0 to select the best MA ODF developed during the investigation.[19]

Drug release kinetics

The drug release kinetics of MA ODF was studied by fitting the release of the optimised batch to various kinetic models such as zero–order, first–order, Higuchi, Korsmeyer–Peppas and Hixson–Crowell models. Criteria for selecting an appropriate model were based on the best goodness of fit.[20,21]

Comparison of *in vitro* drug release profile against a marketed suspension

The *in vitro* drug release profile of the best formulation was compared with the available marketed product of MA i.e., Suspension (MS). The study was conducted using USP type–II dissolution apparatus. The selected MA ODF and suspension equivalent to 65 mg of MA were placed in dissolution vessels containing 900 mL of PBS pH 6.8 maintained at 37°C±0.5°C and 50 rpm. 5mL of sample solution was withdrawn at 0, 1, 3, 5, 10, 20, and 45 m and replaced with an equal volume of fresh medium. The absorbance for the withdrawn samples was measured using a UV–visible spectrophotometer at 285 nm.[20,22]

Stability studies

The stability study of the optimised batch was performed under in-house testing conditions. The samples were stored at different conditions 4°C±2°C–8°C±2°C (Refrigerated temperature), 30°C±2°C/70%RH±5%RH (Room temperature), 40°C±2°C (Elevated temperature) for 30 days in two primary packaging materials i.e., aluminium foil and transparent polyethene ziplock. The samples were evaluated for folding endurance, percentage moisture loss, percentage moisture uptake, *in vitro* disintegration time, drug content and cumulative percentage drug release.[23]

Statistical analysis

The measurements were taken in triplicates and the corresponding results were reported as averages with standard deviations. All the data were statistically analysed by one-way ANOVA followed by Tukey's *post hoc* analysis using GraphPad Prism software 9.0.

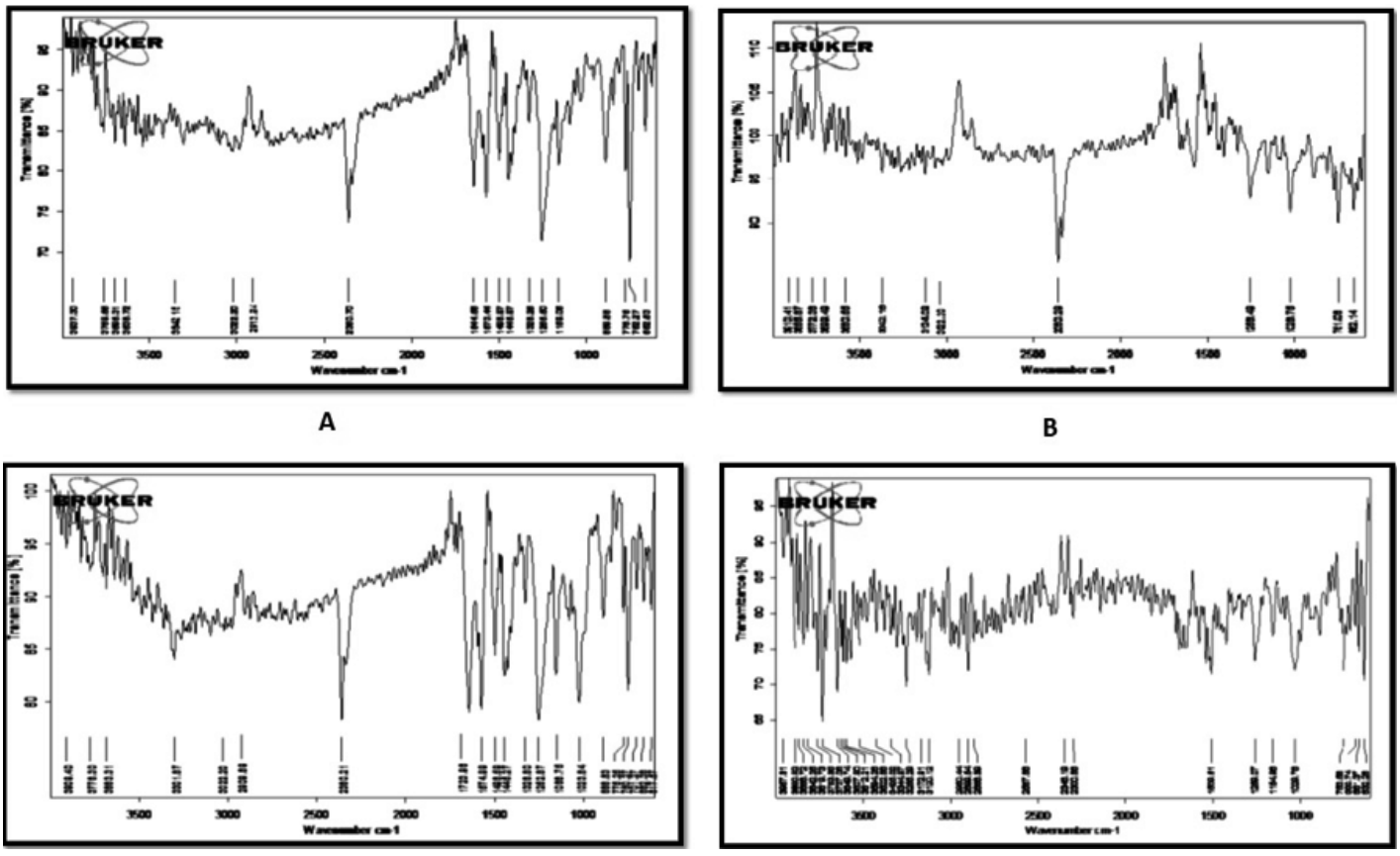

Figure 1: FT–IR spectrum 1A MA, 1B MA & βCD, 1C final physical mixture and 1D MA-βCD inclusion complexes at 1:0.5 ratio.

Table 2: *In vitro* dissolution study of selected ratios of MA-βCD inclusion complexes.

Time (s)	*Cumulative % drug release		
	1:0.5	1:1	1:2
0	2.39±1.12	2.23±0.08	3.14±0.61
30	7.88±1.69	7.96±0.54	7.99±0.35
60	25.15±1.17	26.10±0.46	27.11±0.67
90	28.13±2.07	28.28±2.86	29.30±1.18
120	55.98±0.04	56.12±1.27	57.01±1.24
150	79.09±0.21	80.26±1.57	82.19±1.96
180	88.22±1.18	89.15±0.94	90.52±1.30
210	90.21±0.43	90.66±1.18	91.88±2.10
240	92.25±0.38	92.99±1.16	93.01±1.28

*Values SD. Samples taken in triplicates (n=3), p>0.05.

RESULTS AND DISCUSSION

Drug–excipient compatibility studies

The MA showed characteristic peaks at 2500–3400 cm^{-1} for O–H stretching of COOH, C–H stretching of aromatic C–H bond at 3022 cm^{-1} and 2900–3000 cm^{-1} for C–H stretching of aliphatic C–H bond, N–H stretching of secondary amine showed at 3310–3350 cm^{-1} and 1644 cm^{-1} for C=0 stretching of carboxylic acid as well as C–N stretching showed at 1255 cm^{-1} (Figure 1A). The FT–IR spectrum of the MA and βCD blend (Figure 1B) and the final physical mixture (Figure 1C) suggested that all the characteristic peaks reported in MA were present in the MA and βCD blend and the final physical mixture. There were no new or missing significant peaks in the final physical mixture indicating that MA and selected excipients are compatible with each other in the final formulation.

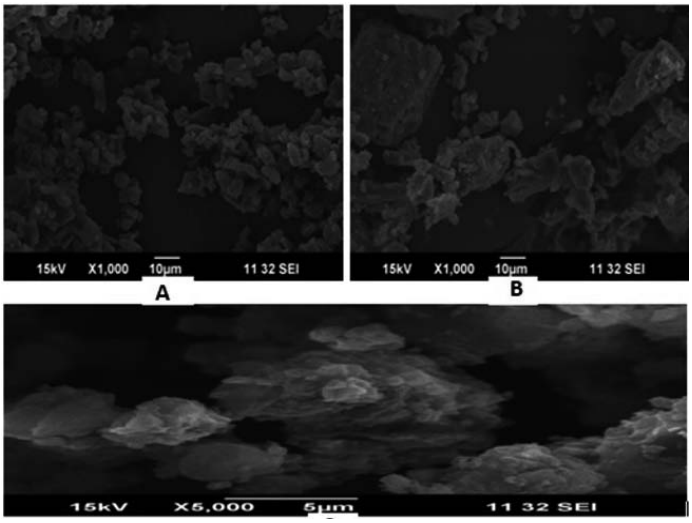

Figure 2: SEM photomicrograph 2A MA, 2B βCD, 2C MA-βCD inclusion complexes (1:0.5).

Evaluation of MA-βCD inclusion complexes

Drug content estimation

More than 90% MA was available in the developed inclusion complexes of MA-βCD. The highest percentage of drug content was observed in the 1:0.5 MA-βCD inclusion complex i.e., 97.00±1.55 and 93.13±1.12 for 1:1 as well as 93.07±1.23 for the 1:2 complex. A statistically significant difference was observed in the percentage drug content for the complex at the molar ratio of 1:0.5 and the other two complexes whereas, no significant difference was reported between the 1:1 and 1:2 molar ratio by one-way ANOVA followed by Tukey's *post hoc* test.

In vitro drug release study

All three complexes showed more than 90% of drug release within 240 sec (Table 2). When compared to the drug release from MA-βCD inclusion complexes, less than 30% release was reported for the pure MA suggesting the significant improvement in drug release from the MA-βCD inclusion complexes. Statistical assessment by One–way ANOVA indicated that no statistically significant difference was observed between the three (1:0.5, 1:1, and 1:2) molar ratios of MA-βCD in terms of the cumulative percentage drug release. Based on the literature, no further improvement in drug release may be possible from drug-βCD complexes, beyond a certain level of molar ratio.[12] Hence, beyond the 1:0.5 molar ratio, a significant improvement in drug release may not be achieved. The MA-βCD inclusion complex at 1:0.5 was selected as the best molar ratio for the development of an ODF which had the highest drug content of 97.00±1.55% and greater than 90% drug release, and later it was subjected to SEM analysis and FT–IR spectroscopy.

SEM and FT–IR analysis of MA-βCD inclusion complexes

The photomicrograph of pure MA and βCD were loose aggregates of heterogeneously distributed particles with irregular shapes and rough surfaces which also suggested a crystalline nature for both (Figure 2A and 2B). But the photomicrograph of MA-βCD at 1:0.5 suggested compact solids with smooth surfaces as well

Table 3: Average weight, thickness, folding endurance, pH, percentage moisture loss and percentage moisture uptake of ODF strips containing MA.

Formulation Code	*#Average weight (mg)	*#Thickness (mm)	*#Folding endurance	*pH	*Percentage moisture loss	*Percentage moisture uptake	*Percentage Drug content	*#Disintegration time (s)
H1	0.094±0.004	0.062±0.0005	121±1	6.56±0.11	1.28±0.20	1.51±0.30	88.85±1.11	120±2.64
H2	0.120±0.010	0.092±0.001	84.3±1.52	6.56±0.15	1.21±0.11	1.62±0.13	89.67±1.54	80.33±0.57
H3	0.148±0.008	0.113±0.006	66.6±1.52	6.56±0.18	1.15±0.75	1.85±0.17	93.35±1.25	45.33±0.57
H4	0.174±0.001	0.140±0.003	54.6±0.57	6.55±0.20	1.06±0.21	1.99±0.15	90.70±0.97	48.0±1
P1	0.087±0.007	0.048±0.0005	299±1	6.65±0.01	1.90±0.18	1.21±0.11	94.05±1.64	45.0±1
P2	0.113±0.016	0.068±0.0005	293.6±0.33	6.71±0.03	1.85±0.39	1.25±0.11	92.44±2.15	37.0±1
P3	0.139±0.009	0.092±0.001	253.3±0.33	6.67±0.01	1.39±0.27	1.36±0.09	95.46±0.93	28.6±2.0
P4	0.165±0.006	0.103±0.005	150.6±0.33	6.63±0.04	1.35±0.15	1.38±0.14	94.45±0.76	28.0±1.52

*Values SD. Samples taken in triplicates (n=3), # p<0.05.

as a possible change of crystalline structure to amorphous, which may be the reason behind the enhanced solubility and dissolution profile for the inclusion complexes (Figure 2C). The FT–IR spectrum of MA-βCD inclusion complexes at 1:0.5 molar ratio (Figure 1D) showed all the peaks present in the individual spectrum of MA and βCD inclusion complexes. However, the peak at 1644 cm^{-1} corresponding to C=0 stretching of carboxylic acid disappeared in the FT–IR spectrum of MA-βCD inclusion complexes. It indicates that the chemical interaction may have occurred between the MA and a polar cavity of βCD. Similar results were reported in the literature on drug-βCD complexes prepared by the kneading method.[12] The SEM analysis and FT–IR spectroscopy of the MA-βCD inclusion complex at a 1:0.5 molar ratio suggested a modification in physicochemical parameters, which was supporting the reason laid down for the improvement of solubility and successful formation of the complexes.

Evaluation of MA oral dispersible films strips

Based upon the proposed composition, eight formulations were developed i.e., four each for HPMC E5 and PVA. The formulations with HPMC E5 were coded between H1–H4 and PVA were P1–P4. The average weight of the developed ODF strips ranged from 0.087±0.007 mg to 0.174±0.001 mg. The ODF strips with HPMC E5 i.e., H1–H4 had a slightly higher average weight in comparison with ODF strips developed using PVA (P1–P4) (Table 3). Since the molecular weight of HPMC E5 is higher than PVA, which may be one of the possible reasons for the slightly higher average weight for the ODFs based on HPMC E5. The ODFs based on HPMC E5 and PVA without crospovidone in their composition were recorded with the lowest average weight of 0.094±0.004 mg and 0.087±0.007 mg respectively, whereas the highest average weight of 0.174±0.001 mg and 0.165±0.006 mg was recorded for H4 and P4 which had a maximum concentration of crospovidone i.e., 6% w/v. To analyse the effect of crospovidone concentration in the developed ODF strips of HPMC E5 and PVA i.e., H1–H4 and P1–P4 one-way ANOVA followed by Tukey's multiple comparison test was applied. The data indicated that the increase in the concentration of crospovidone significantly increased the weight of developed ODF strips (p<0.05), as well as the ODF strips without crospovidone i.e., both H1 and P1, showed statistically significant differences in average weight from the rest of the formulations containing crospovidone (Figure 3A and 3B).

The average thickness for the developed ODF strips of HPMC E5 and PVA were recorded between 0.062±0.0005 mm to 0.140±0.003 mm and 0.048±0.0005 mm to 0.103±0.005 mm respectively (Table 3). The statistical assessment of the data by one-way ANOVA followed by Tukey's multiple comparison test confirmed that an increase in the concentration of crospovidone from 2% w/v to 6% w/v was producing a statistically significant change in the thickness of developed ODFs in both HPMC E5 and PVA polymers (Figure 3C and 3D). It also confirmed that

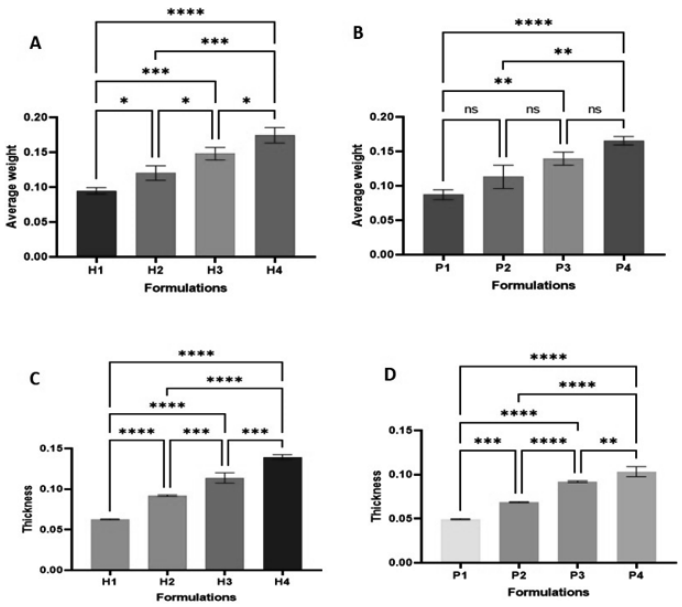

Figure 3: Comparison of the average weight 4A (H1–H4), 4B (P1–P4); thickness 4C (H1–H4), 4D (P1–P4); and folding endurance 4E (H1–H4), 4F (P1–P4) of the developed ODF strips. (*p*-value; 0.0332[*], 0.002[**], 0.0001[***], <0.0001[****]).

there was a statistically significant difference between ODFs without super disintegrant (H1 and P1) and ODFs containing superdisintegrant, which strongly suggested the influence of crospovidone concentration over the thickness of MA-βCD ODF strips. A similar trend was observed in a study conducted by Heer D *et al.*,[24] The folding endurance of developed ODF i.e., both HPMC E5 and PVA ranged between 54.6±0.57 to 299±1 (Table 3). The ODF with PVA i.e., P1–P4 was having comparatively higher folding endurance than the HPMC E5–based ODFs. The ODFs H1 and P1 i.e., without superdisintegrant were reported with higher folding endurance. The influence of crospovidone on the folding endurance was statistically analysed using the one-way ANOVA with Tukey's *post hoc* test indicated that an increase in the concentration of crospovidone significantly reduced the folding endurance (HPMC E5–based ODF reduced from 121±1 to 54.6±0.57 and for PVA based ODF, it was reduced from 299±1 to 150.6±0.33) (Figure 3E and 3F). The previous investigations also suggested that the thickness of ODF has an indirect relationship with folding endurance and results from the study supported a similar observation.[24] This further proved that the influence of crospovidone was producing a significant effect on the folding endurance of ODFs developed during the investigation.

The pH of the developed ODFs i.e., both HPMC E5 and PVA based (H1–H4 and P1–P4) ranged between 6.55±0.20 to 6.71±0.03 (Table 3). The pH obtained for the developed ODFs was closer to the pH of the oral cavity i.e., 6.8. This suggested that all the developed ODF may be safer for usage in the paediatric population in terms of pH. The statistical assessment of the data by one-way ANOVA reveals that the change in concentration of superdisintegrant could not produce any influence on the pH of the developed ODF ($p>0.05$).

The percentage moisture loss and percentage moisture uptake for the developed ODFs of MA (H1–H4 and P1–P4) were measured and reported between 1.06±0.21 to 1.90±0.18 and 1.21±0.11 to 1.99±0.15 respectively (Table 3). Since both parameters indicate the overall stability of ODFs, the data obtained during the investigation were within acceptable limits. The statistical analysis of the data by one-way ANOVA reported with $p>0.05$ suggested that the change in percentage moisture loss and percentage moisture uptake was not statistically significant, which further confirmed that crospovidone concentration was not producing any significant influence over percentage moisture loss and percentage moisture uptake in any of the developed batches i.e., H1–H4 and P1–P4.

Drug content estimation for oral dispersible film strips

The developed ODF strips i.e., H1 to H4 and P1 to P4 had MA ranging between 88.85±1.11% to 93.35±1.25% and 92.44±2.15% to 95.46±0.93% respectively (Table 3). The data obtained during the investigation suggested that the measured drug content between the batches was within the acceptable limit and they were uniform (85%-115% with a standard deviation ≤ of 6%).[25] To further confirm, the data was statistically analysed using the one-way ANOVA method, the $p>0.05$ indicated no statistically significant difference observed in drug content between the developed batches. This indicated that the methodology and conditions adopted for the formulation development were appropriate, and developed ODFs maintained consistency.

In vitro disintegration time of oral dispersible film strips

The *in vitro* disintegration time for the developed ODF strips of both HPMC E5 and PVA i.e., H1–H4 and P1–P4 ranged between 26.3±1.52 sec to 120±2.64 sec (Table 3). The data indicated that the addition of crospovidone showed improvement in the *in vitro* disintegration of the developed ODF. This was further supported by the data obtained for H1 and P1 formulations (without superdisintegrant) which took a maximum disintegration time of 120±2.64 sec and 45.0±1 sec in comparison with ODF strips containing superdisintegrant (Figure 4). The data also suggested an improvement in the disintegration process with an increase in the concentration of crospovidone in the formulation i.e., 2% w/v to 6% w/v. A similar trend was reported in peer–reviewed literature published on similar formulations.[25] The ideal disintegration time recommended for ODF is less than 60 ses.[26] When the *in vitro* disintegration data for the developed ODF strips were investigated, the formulation with PVA i.e., P1–P4 falls under the prescribed limit, whereas HPMC E5–based formulation H1, as well as H2, had *in vitro* disintegration time beyond the prescribed limit of 60 sec. The presence of crospovidone at 4%w/v and 6% w/v could reduce the *in vitro* disintegration time

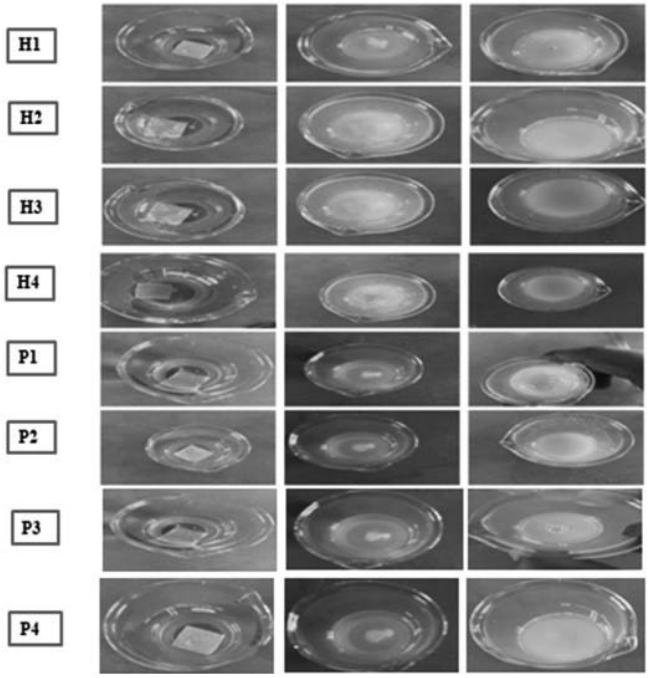

Figure 4: *In vitro* disintegration study of the developed ODF strips.

considerably to 45.33±0.57 sec and 48±1 sec respectively for H3 and H4. This suggested the importance of superdisintegrant in the MA ODF prepared using HPMC E5.

The PVA–based ODF formulations P1–P4 were recorded with a disintegration time of fewer than 60 sec. The crospovidone could further improve the disintegration time and bring it down to as low as 28 sec. When the comparison was made between ODFs of HPMC E5 and PVA, all PVA–based ODFs were having a faster *in vitro* disintegration process. The P1 without any superdisintegrant was able to disintegrate at almost the same time duration as that of the HPMC E5–based H3 formulation containing 4% w/v of crospovidone. Hence, polymer selection may be an important parameter along with the superdisintegrant for the development of ODF.

The statistical analysis by one-way ANOVA followed by *post hoc* analysis indicated that there was a statistically significant difference ($p<0.05$) in disintegration time exist between formulations without crospovidone i.e., H1 and P1 and those with crospovidone (H2–H4 and P2–P4) (Figure 5A and 5B). Further interpretation of the data suggested that formulations containing 2% w/v of crospovidone i.e., H2 and P2 were having a statistically significant difference in their disintegration time when compared to formulations containing 4% w/v and 6% w/v of crospovidone (H3–H4 and P3–P4). The interpretation revealed that the formulation H3–H4 and P3–P4 with 4% w/v and 6% w/v of crospovidone did not show any statistically significant difference in the *in vitro* disintegration time. This indicated that increasing the crospovidone concentration did not result in a substantial improvement in the *in-vitro* disintegration time. Hence it may be concluded that beyond 4% w/v crospovidone may not influence

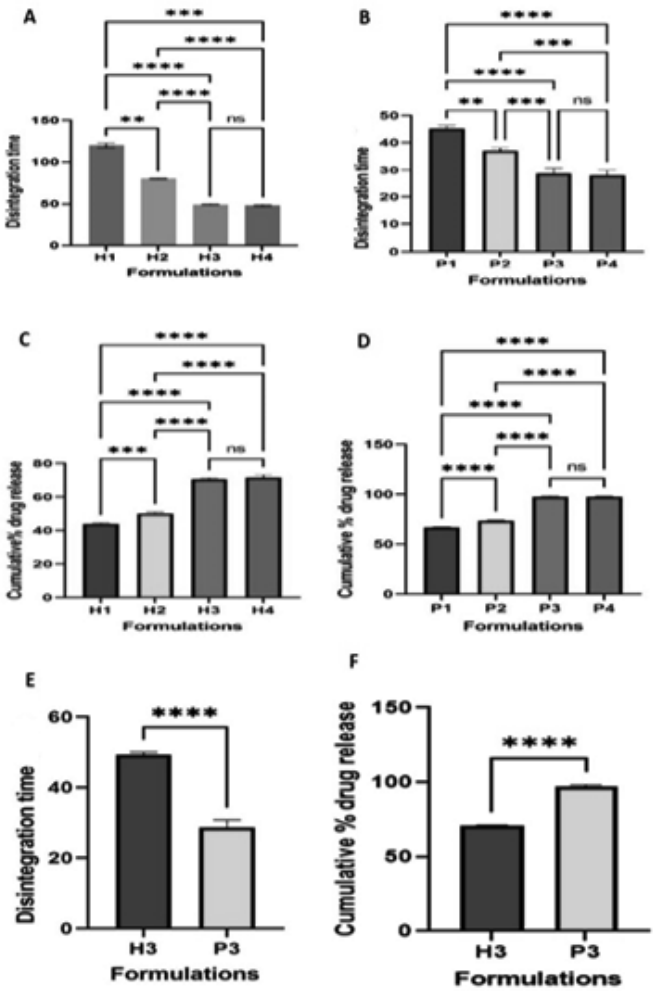

Figure 5: Comparison of the *in vitro* disintegration time 6A (H1–H4), 6B (P1–P4); cumulative percentage drug release 6C (H1–H4), 6D (P1–P4); *in vitro* disintegration time 6E (H3 and P3), cumulative percentage drug release 6F (H3 and P3) of the developed ODF strips (*p*-value; not significant[ns], 0.002[**], 0.0001[***], <0.0001[****]).

the disintegration process of an MA ODF prepared using HPMC E5 and PVA.

In vitro drug release study of the oral dispersible film strips

The percentage cumulative drug release obtained for MA ODF with HPMC E5 (H1–H4) and PVA (P1–P4) was illustrated in Figure 6. The MA ODF without superdisintegrant (H1) released 78.58±0.14% of MA after the completion of 300 sec. The H2 to H4 ODF strips developed using HPMC E5 i.e., 2% w/v to 6% w/v crospovidone could release MA at a higher percentage after 300 s i.e., 91.80±0.30%, 97.85±0.52%, and 98.31±0.31% respectively. The formulations H2, H3, and H4 could produce much faster drug release than H1 throughout *in vitro* drug release study. More than 50% of the loaded MA was released from H2, H3, and H4 within 180 sec, whereas H1 (without superdisintegrant) took almost 240 sec to cross 50% drug release. But at a similar time interval, formulations H3 and H4 could release more than 95% of loaded MA. This improved drug release profile H2–H4 indicated

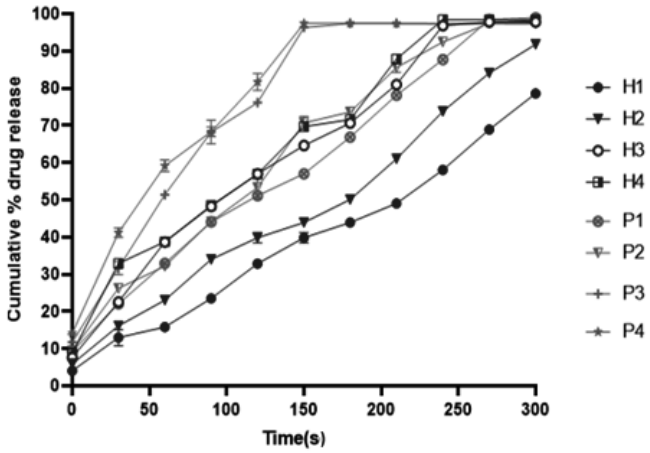

Figure 6: *In vitro* drug release study of the developed ODF strips.

the influence of crospovidone as a superdisintegrant selected for the formulation.

The ODFs with PVA (P1–P4) had a much faster drug release profile when compared with H1–H4 at all measured time intervals. At the 300th sec, the formulation of PVA (P1–P4) could release more than 95% of loaded MA. Formulation P3 and P4 with 4% w/v and 6% w/v crospovidone released more than 50% of loaded MA within 60 sec whereas formulation P1 without any super disintegrant took almost 120 sec to cross 50% drug release. This was a trend observed even with the ODFs developed using HPMC E5. This further supported the inclusion of superdisintegrant in ODFs. The previous study reports supported the claim made during this investigation.[24,25]

The comparison between ODFs of HPMC E5 and PVA in percentage Cumulative drug release indicated a difference i.e., P1–P4 was showing a superior drug release profile than H1–H4 at any measured time interval. The reason for the improved drug release from the PVA–based ODF may be due to its high moisture–holding capacity as compared to HPMC E5.[27] The drug release is also affected by the polymer concentration to develop a stable ODF. As the concentration of polymer increases, the drug release from the ODF may decrease.[18] The polymer concentration in PVA–based ODF strips (2% w/v) is less as compared to HPMC E5–based ODF strips (3% w/v). This may be a reason behind the enhanced drug release from the PVA–based ODF as compared to HPMC E5–based formulation. The faster disintegration time reported for PVA–based ODFs was further substantiated by the faster drug release profile observed in ODFs P1–P4.

The statistical analysis of the data at 180 sec by one-way ANOVA followed by Tukey's multiple comparison test reveals the statistically significant difference in the data ($p<0.05$), which may be due to the influence of superdisintegrant in the formulation, as well as the formulations without superdisintegrant i.e., both H1 and P1, were having a drug release profile which had a statistically significant difference from the rest of the developed formulations (H2–H4 and P2–P4) (Figure 5C and 5D). It also revealed that the ODF formulations H3–H4 and P3–P4 (4% w/v and 6% w/v crospovidone) were having a drug release profile that had a statistically significant difference from H2 and P2 with 2% w/v crospovidone. But when the drug release profile was compared between the formulations with 4% w/v and 6% w/v crospovidone (H3–H4 and P3–P4), there was no statistical difference identified. Irrespective of polymer change, this trend was similar in HPMC E5 and PVA. This indicated a possibility that crospovidone concentration beyond 4% w/v may not produce a significant change in the drug release profile for a MA ODF prepared using either HPMC E5 or PVA. The findings of the *in vitro* drug release study supported the observation from the *in vitro* disintegration study. Hence 4% w/v may be the ideal concentration of crospovidone as a super disintegrant to provide an enhanced release profile for MA ODF prepared using HPMC E5 or PVA.

Statistical optimization of developed MA oral dispersible film strips

Based on the above discussion, increasing the concentration of crospovidone beyond 4% w/v may not produce a significant effect on the *in vitro* disintegration time and *in vitro* drug release of the developed MA ODF. This trend was common for the ODFs developed using HPMC E5 and PVA. Hence, H3 and P3 i.e., HPMC E5 and PVA–based MA ODF with 4% w/v crospovidone may be an optimised formulation among the selected polymers. For the further comparison between H3 and P3, the *in vitro* disintegration and *in vitro* drug release data were subjected to one-way ANOVA, which suggested that change in the polymer had a statistically significant effect on disintegration time and drug release($p<0.05$). The *post hoc* analysis using Tukey's test confirmed that the ODF formulation P3 had a statistically highly significant ($p<0.001$) difference from the H3 formulation based on *in vitro* disintegration time and percentage cumulative drug release (Figure 5E and 5F). The statistical analysis of the data demonstrated that a PVA–based formulation with 4% w/v of crospovidone may be the best MA ODF developed during the investigation.

Drug release kinetics

To determine the mechanism of MA release from the ODF formulation P3, the *in vitro* MA release data were fitted into zero–order, first–order, Higuchi, Korsmeyer–Peppas, and Hixson–Crowell models. It was observed that the R^2 value for zero–order was 0.794 and the first order was 0.854, which indicated that the drug release data for P3 formulation showed the best fit in first–order kinetics. The data plot of the Higuchi model showed an R^2 value of 0.925, and the release exponent (n) value in the Peppas model for P3 formulation was found to be 0.465. The regression value obtained in the Hixson–Crowell model was higher (0.855).

Table 4: Stability study of P3 ODF packed in aluminium foil.

Parameters	Initial	After 30 days		
		18°C±2°C	30°C±2°C/ 70%RH±5%RH	40°C±2°C
Physical appearance	Light orange, Smooth, opaque, and homogenous	No change		
Folding endurance	255±0.33	253.6±0.56	251±0.89	250±0.89
Percentage moisture uptake	1.36±0.09%	1.30±0.25%	1.34±0.51%	1.32±0.41%
Percentage moisture loss	1.39±0.27%	1.35±0.23%	1.36±0.33%	1.38±0.56%
In vitro disintegration time	28.10±2.01sec	27.99±1.99sec	28.95±1.68sec	28.05±1.68sec
Drug content	95.66±1.96%	95.00±2.01%	94.99±1.80%	92.99±1.80%
Cumulative percentage drug release (After 3 min)	97.41±2.06	97.02±1.35	97.58±1.58	98.58±1.58

*Values SD. Samples taken in triplicates (n=3), p>0.05.

Table 5: Stability study of P3 ODF packed in polyethylene ziplock.

Parameters	Initial	After 30 days		
		18°C±2°C	30°C±2°C/ 70%RH±5%RH	40°C±2°C
Physical appearance	Light orange, Smooth, opaque, and homogenous	No change		
Folding endurance	255±0.33	253.6±0.56	251±0.89	250±0.89
Percentage moisture uptake	1.36±0.09%	1.30±0.25%	1.34±0.51%	1.32±0.41%
Percentage moisture loss	1.39±0.27%	1.35±0.23%	1.36±0.33%	1.38±0.56%
In vitro disintegration time	28.10±2.01sec	28.99±1.99sec	28.95±1.68sec	28.05±1.68sec
Drug content	95.66±1.96%	94.99±2.01%	93.90±1.80%	92.89%
Cumulative % drug release (After 3 min)	97.41±2.06	97.02±1.35	96.99±1.58	96.58±1.58

*Values SD. Samples taken in triplicates (n=3), p>0.05.

Based on drug release kinetics, it may be concluded that the P3 formulation followed the first–order, Quasi–Fickian diffusion–controlled release profile and the study also revealed that change in surface area of developed MA ODF with the progressive dissolution of a matrix was with respect to time.[10,16]

Comparison of in vitro drug release profile against a marketed suspension

The percentage cumulative drug release obtained for optimised MA ODF i.e., P3 and MS Figure 6 depicted that, P3 released 97.14±0.12% of MA and MS could release 96.60±0.21% of MA after the completion of 3 min. The MA-βCD inclusion complex, crospovidone and inherent property of PVA might have played a significant role in enhancing the drug release from the MA ODF, which had comparable results as that of available marketed products. The statistical assessment by one-way ANOVA at a 95% confidence interval revealed that optimised MA ODF i.e., P3 have a dissolution profile similar to marketed MS (p>0.05). Hence the ODF of MA may be an effective alternative formulation to marketed oral liquid dosage forms in the paediatric population for the management of fever.

Stability studies

The stability study of the best MA ODF strips i.e., P3 was performed under in-house testing conditions. The samples were evaluated for physical appearance, folding endurance, percentage moisture uptake, percentage moisture loss, *in vitro* disintegration time, drug content and cumulative percentage drug release after 30 days (Table 4, 5). The data collected indicated that the samples packed in aluminium foil and polyethylene ziplock stored at different temperature conditions had no major stability issues after the completion of 30 days. The colour and texture of the product remained unchanged throughout the study period at different temperature conditions. The data were subjected to one-way ANOVA, and when compared with the initial readings, there was no statistically significant change after 30 days in the folding endurance, percentage moisture uptake, percentage moisture loss, *in vitro* disintegration time, drug content and cumulative percentage drug release (after 3 m) in all testing conditions. The stability study indicated that the developed MA ODF using polyvinyl alcohol i.e., P3 was a stable product when packaged in both aluminium foil and polyethylene ziplock.

CONCLUSION

The study suggested the significance of inclusion complexes of βCD in enhancing the solubility profile of MA. The MA-βCD inclusion complexes may be ideal for loading fast–dissolving oral films with an optimised composition. The film–forming polymer PVA at 3% w/v with 4% w/v crospovidone as a superdisintegrant was recommended for the improved release profile for MA from oral dispersible films. Based on the findings, it is concluded that the MA oral dispersible films are an excellent alternative to existing marketed paediatric formulations. However, further *in vivo* evaluations may be required to substantiate the findings reported during the *in vitro* studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Original Article

Formulation and Evaluation of Aspirin-PLGA Microsphere for the Dental Stem Cell Stimulation

Litha Thomas[1,4], Preeti Karwa[2,*], V Kusum Devi[3]

[1]Department of Pharmaceutics, Krupanidhi College of Pharmacy, Bengaluru, Karnataka, INDIA.
[2]Department of Pharmaceutics, Al-Ameen College of Pharmacy, Bengaluru, Karnataka, INDIA.
[3]Department of Pharmaceutics, NITTE College of Pharmaceutical Sciences, Bengaluru, Karnataka, INDIA.
[4]Part-time Research Scholar, Department of Pharmaceutics, Al-Ameen College of Pharmacy, Bengaluru, Karnataka, INDIA.

ABSTRACT

Aim: According to WHO, dental caries is the most prevalent oral disease, and its progression leads to tooth loss. Clinical management of caries focuses on the severity and extent of disease with the main aim, i.e., the 'art' of creating a good restoration. Recently, it has been reported that aspirin can stimulate existing stem cells and regenerate damaged teeth. But, the therapeutic effectiveness of a drug depends on developing a suitable novel drug delivery system, to retain at the site and suitably release the drug to produce effective therapy. Therefore, the present investigation intends to develop Aspirin-Poly lactic-co-glycolic acid microspheres for the restoration of dentin. **Materials and Methods:** Aspirin- Poly lactic-co-glycolic acid microsphere was formulated by the double emulsion technique and evaluated for particle size, encapsulation efficiency, characterization (differential scanning calorimetry, X-ray powder diffraction), *in vitro* release, as well as irritation testing using the Hen's egg test-chorioallantoic membrane method. **Results:** The formulation exhibited good encapsulation efficiency (87.31±1.52%) and a particle size of 7.52 µm by Scaning Electron Micrscopy. *In vitro* release study exhibited sustained release (98.76±0.49%) for 16 days and triphasic release. This confirms that release is due to polymer erosion, swelling, and degradation. The *ex vivo* permeation study also confirmed sustained permeation and showed the significant partition and accumulation of the drug in the tissue. Further, the prepared formulation showed significantly low irritation compared to positive control by Hen's Egg Test-Chorioallantoic Membrane method. **Conclusion:** Thus, the above finding suggests that the formulation can stimulate stem cells for the regeneration of dental tissue.

Keywords: Stem cell, Dental caries, Sustained release, Aspirin, Dentinogenesis, Microsphere.

Correspondence:
Dr. Preeti Karwa
Professor and Head of Department,
Department of Pharmaceutics,
Al-Ameen College of Pharmacy,
Nr. Lalbaug Main Gate, Hosur Road,
Bangalore - 590027, Karnataka, INDIA.
Email id: aacp012018@gmail.com

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INTRODUCTION

According to World Health Organization, oral health plays a crucial role in good health and well-being.[1] Improper oral care may lead to a severe threat to the oral cavity causing cavity to cancer and systemic diseases.[1] Dental caries is a multifarious infection that develops due to bacterial biofilm (dental plaque), leading to the demineralization of hard dental tissues.[2,3] If the disease is not treated properly, it will lead to the formation of a dental cavity and even permanent loss of the tooth. The main aim of the management of present diseases is to prevent the progression of the disease by pharmacological agents and also some non-therapeutic approaches like surgical intervention, and mechanical therapy, depending on the stage of the disease and person to person.[4] Although these therapeutic and non-therapeutically approaches provide satisfactory clinical efficacy in the control of infection, pain and seal the space in the cavity. But, these treatment modalities, may lead to tooth fracture, loss of the tooth, or even reinfection. However, till now no treatment is developed for the restoration of the decayed tooth. Hence, this remains a thrust area for pharmaceutical scientists to explore and develop a suitable method for the complete restoration of teeth.

Aspirin (Acetyl Salicylic Acid, ASA) most widely used non-steroidal anti-inflammatory drug (NSAIDs), is used to modulate a variety of disease conditions like pain[5] (toothaches, and headaches), cardiovascular disease,[5,6] arthritis[5,7] and anti-inflammatory.[8] Recent studies reported that Aspirin is capable of stimulating the various dental stem cells, this promotes the migration, proliferation, and differentiation of odontoblast cells responsible for the enhance the regeneration of dentin.[9-11] These findings strongly suggest that Aspirin has excellent potential in the management of several dental problems for

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which there is no clear-cut treatment strategy. Hence, Aspirin's therapeutic effectiveness depends on the design and development of a suitable novel drug delivery system for Aspirin.

MATERIALS AND METHODS

Materials

Aspirin was a gift from Alta Labs, India, also PLGA (75:25) was a gift sample from Evonik, India, and ethyl acetate was procured from S.D. fine chemicals. India, Poly (vinyl alcohol) (MW 1,30,000, 18-88% hydrolyzed) was obtained from Sigma-Aldrich. Analytical grade chemicals and solvents were used.

Methods

Formulation and optimization of Aspirin loaded microsphere

Aspirin-PLGA microspheres were formulated by the double-emulsion-solvent extraction method (w/o/w).[12,13] Aspirin in the aqueous solution (different volume) was dispersed into the ethyl acetate-ethanol solvent system containing PLGA (different concentration) resulting in a primary emulsion. For stabilization, the emulsion was homogenized using a probe homogenizer (Kinematica Polytron™ PT2100) and to the aqueous phase of polyvinyl alcohol (PVA, different ratios) the primary emulsion was injected and stirred continuously to form a W/O/W emulsion. Microspheres formed were hardened by pouring into a defined volume of water,[12] stirred overnight at room temperature to remove the solvent, separated by centrifugation (Remi Equipment Pvt. Ltd., India), and washed continuously. Schematic diagram of the formulation is represented in Figure 1.

Box-Behnken (BB) design (3-factor 3-level) was adopted to optimize the concentration of PLGA (X_1), Volume of the internal phase of primary emulsion (mL) (X_2), PVA concentration (%) (X_3) as the independent variable and encapsulation efficiency (Y_1, %), and mean particle size (Y_2, μm) as dependent variable. The level of the selected factors were fixed based on the preliminary trials conducted and applied in BB design (Table 1).

Characterization of Aspirin PLGA Microsphere

Powder X-ray Diffraction (PXRD)[14,15]

PXRD for the drug, polymer, physical mixture, and formulations were carried out using a D8 Advance BRUKER diffractometer. Samples were mounted on aluminum plates and measured at 2θ diffraction angle from 0 to 40° with a source of nickel-filtered and Cu-anode ceramic[14,15]

Differential Scanning Calorimetry Analysis

The thermograms of pure drugs, polymers, and formulations were recorded (Perkin Elmer Corporation, Mississippi, MA, USA). A aliquot amount sample was heated in a closed aluminum pan. Measurements were done at a scanning rate of 10°C/min between 30 and 350°C.[14,15]

Particle size and Polydispersity Index

The mean particle size of the formulated microspheres were calculated by using an optical microscope equipped with a camera. The measured mean particle size was used to study the Polydispersity Index (PDI).[16]

Surface morphology

The shape and the surface morphology of the optimized microsphere were examined with scanning electron microscopy (SEM, Joel JSM-6490la, Japan).

Encapsulation Efficiency

The amount of Encapsulated (EE) was calculated by the ratio of the difference between the amount of Aspirin used for microsphere formulation and the amount of non-entrapped Aspirin remaining in the external phase after microsphere formation to the amount of Aspirin used for the formulation.[17,18]

$$EE(\%) = \frac{(\text{mass ofdrug loaded in MS})^{*}100}{\text{mass of drug processed}}$$

In vitro release study

An aliquot amount of microsphere is filled into the dialysis tube (21 mm, Mw: 8000–14400 Da), fastened on both sides. The bag is suspended into 50 mL of the simulated salivary fluid at 50 rpm 37°C. At specified intervals of time, the sample was withdrawn, filtered, and analyzed at 265 nm to find out the amount of drug release.[19]

The release data were further analyzed to study the release mechanism by fitting into the five kinetic models: zero-order, first-order, Higuchi and Korsmeyer– Peppas, and Hixson-Crowell equation was evaluated by DD solver.

Ex vivo Permeation Studies

Ex vivo permeation studies were conducted using modified Franz diffusion cells with an excised porcine buccal mucosa.[20] The tissue was mounted on the cell and the microsphere was placed on the donor compartment. The receptor compartment was filled with simulated salivary fluid at 37±0.5°C and 50 rpm. The sample was withdrawn at a set time and were analyzed.[21,22]

Drug Deposition studies in Mucosa

After permeation studies, the buccal mucosal was sonicated with a known amount of ethanol and analyzed.[21]

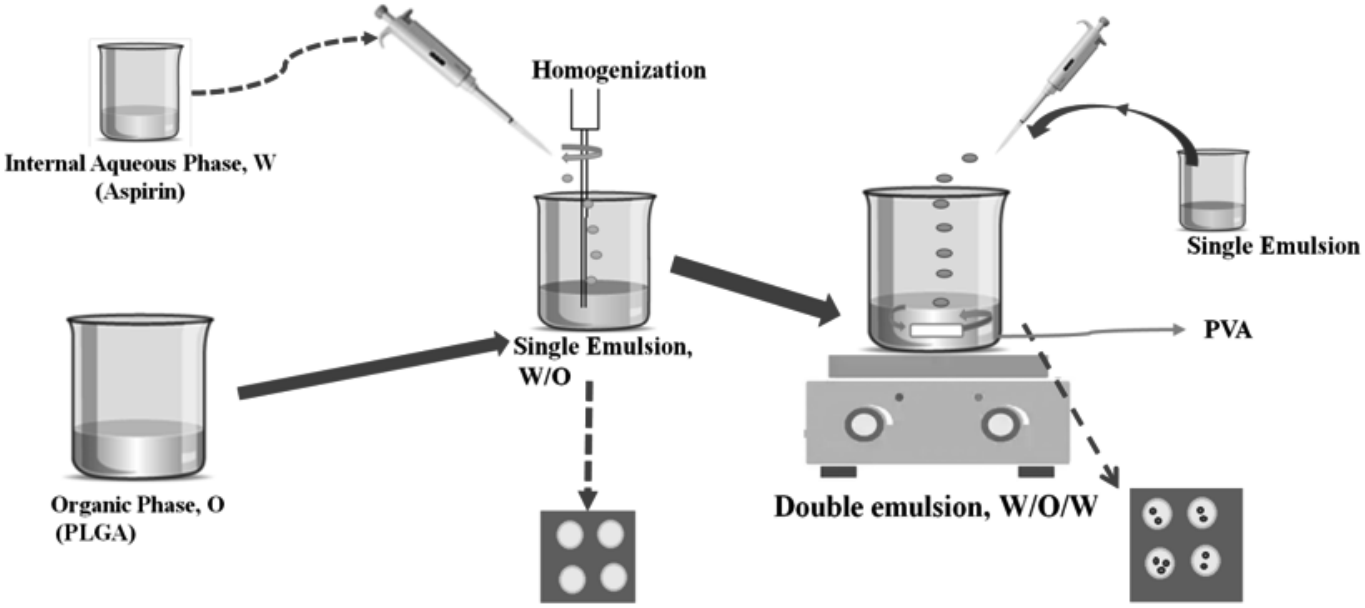


Figure 1: Schematic representation of formulation of Aspirin PLGA microsphere by double emulsion method.

Table 1: Independent and dependent Variable Levels in Box-Behnken design.

Independent Variables	Levels		
	Low (-1)	Center point (0)	High (+1)
X_1-PLGA(mg)	250	500	750
X_2-Volume of internal phase of primary emulsion(mL)	0.75	1.00	1.25
X_3 - PVA concentration(%)	0.5	1	2
Dependent Variables	Constraints		
Y_1- encapsulation efficiency (%)	Maximize		
Y_2-Particle size(μm)	Minimize		

Irritation testing

The optimized formulation was used to study the irritative reaction on the gingival tissue, which was performed on the hen egg using HET-CAM (Hen's egg test-chorioallantoic membrane) technique. The procured eggs were incubated at 37±0.5°C for ten days and rotated every day.[23,24] On the 10th day of incubation, non-viable eggs were discarded by the candling method. Selected egg shells were moistened with 0.9% sodium chloride and carefully shells were removed, and the Chorioallantoic Membrane (CAM) was exposed.

The eggs were divided into the following category

Positive control: Sodium Hydroxide (0.1 N)

Negative control: 0.9% sodium chloride

Test formulation(T1): Optimized aspirin PLGA microspheres

0.3 mL of the above formulations were applied on the CAM and was observed for the endpoint for 300s. Time for the Hemorrhage (H), Vascular Lysis (V), and Coagulation of Protein (C) to appear was recorded as the endpoint. The irritancy potential is calculated and expressed as an Irritation Score (IS), ranging from 0-21(Luepke scale).[24]

$$IS = \frac{5(301 - H)}{300} + \frac{7(301 - V)}{300} + \frac{9(301 - C)}{300}$$

The average score IS is classified from non-irritant to strongly irritant.

RESULTS AND DISCUSSION

According to the recent study, aspirin aids in the enhancement of dental tissue regeneration by stimulating the stem cells.[10] So, a suitable drug delivery system is necessary to deliver the drug to the site of action and efficiently release the drug to increase its efficacy. PLGA polymer was selected due to its potential role in dental tissue regeneration, by inhibiting the early degeneration of gingival epithelium and connective tissues and enhancing regeneration by repopulating the denuded root surface with cells. This can step towards achieving mechanical stability and three-dimensional niches for the growth of new tissue.[25] Therefore,

Table 2: Results of process variables on dependent variable.

Formulation	Concentration of PLGA(mg)	Volume of internal phase(mL)	PVA concentration(%)	Encapsulation Efficiency(%)	Particle size(μm)
1	250	0.75	1	48.75±2.31	39.45±1.24
2	500	1	1	60.87±1.32	40.51±2.34
3	500	0.75	0.5	66.67±0.67	22.75±0.56
4	250	1	2	62.24±1.56	19.73±1.25
5	500	0.75	2	81.3±2.3	15.7±1.34
6	750	1	2	89.97±1.34	30.23±2.3
7	750	0.75	1	85.56±1.25	41.38±0.78
8	500	1.25	2	83.09±1.34	34.41±0.89
9	750	1	0.5	67.25±1.56	38.44±1.23
10	250	1	0.5	42.17±2.34	39.89±2.3
11	250	1.25	1	47.16±1.67	68.2±2.5
12	500	1.25	0.5	68.71±1.56	58.24±2.3
13	750	1.25	1	74.78±2.78	75.61±2.67

*Mean±SD, n=3

PLGA is selected for the formulation of the aspirin microsphere, to achieve collective synergistic regeneration of the dental tissue in dental conditions like dental caries. Aspirin-loaded PLGA microsphere was formulated by the double emulsion-solvent extraction method, and preliminary screening revealed that concentration of PLGA, volume of internal phase and PVA concentration had significant effect on the entrapment efficiency and hence to optimize and examine the effect and the interaction of various factors, the Box Behnken design was ultilized. The variables are concentration of PLGA (X_1), Volume of the internal phase of primary emulsion (mL) (X_2), PVA concentration(%) (X_3) at constant homogenization time, stirring speed for double emulsion, emulsification and flow rate, the experimental runs were employed to maximize encapsulation efficiency (Y1) and minimize the mean particle size (Y2). The effect of independent variables on dependent variables was evaluated and contour plots were developed and represented in Table 2.

Effect on Encapsulation efficiency

The encapsulation efficiency of developed microspheres was found to be between 42.17±2.34% -89.97±1.34%.

The given polynomial equation was projected by the model for the effect of independent variables on the encapsulation efficiency

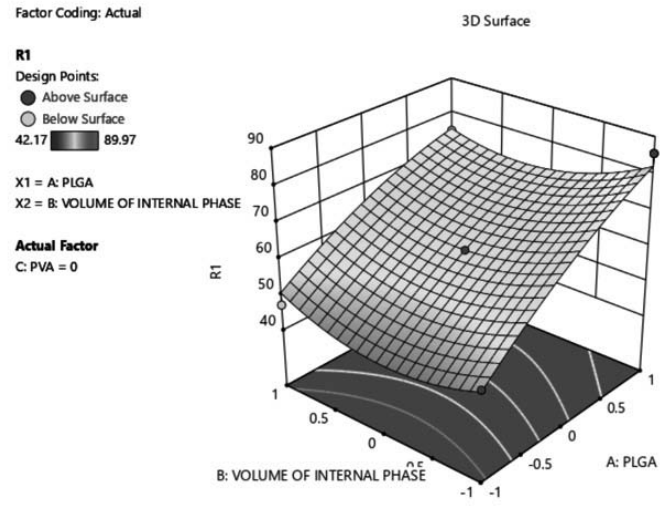
$$\text{Encapsulation Efficiency} = 60.87+14.65X_1-1.0675X_2+8.975X_3-2.2975X_1{*}X_2+0.6625X_1{*}X_3 -0.062497X_2{*}X_3-3.17125X_1^2+6.36375X_2^2+7.70875X_3^2$$

The model was considered significance with F-value = 12.37, $p<0.05$. The developed model's R^2 value of 0.9976 demonstrated strong correlation between experimental and predicted values. Analyzing the model, PLGA concentration (X1), PVA concentration (X3) had positive effect on the entrapment efficiency and was significant, whereas the volume of internal phase (X2) had negative effect and exhibited no significant effect (Figure 2).

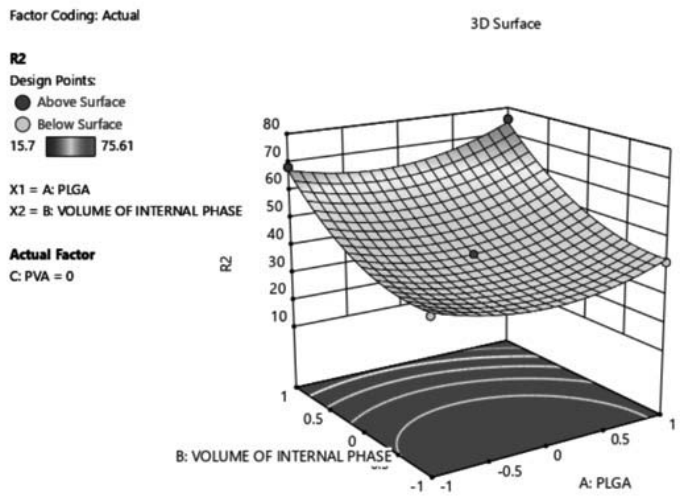
It is clear from the results that the encapsulation of aspirin significantly depends on PLGA and PVA concentration, indicating increase in PLGA and PVA concentration enhances the encapsulation efficiency. The increased PLGA concentration will lead to increased polymer concentration in the orangic phase, thereby enhances rate of polymer precipitation and solidification at constant temperature and homogenization. This prevents the drug from diffusing from the organic phase, increasing encapsulation efficiency.[12,26]

The second parameter that direct relation on encapsulation efficiency is PVA. This can be accounted for by the fact that viscosity increases with PVA concentrations. Furthermore, PVA stabilizes the double emulsion, contributing to higher resistance to aspirin diffusion out of the polymeric phase, resulting in higher encapsulation efficiency in microspheres prepared with more PVA.[12]

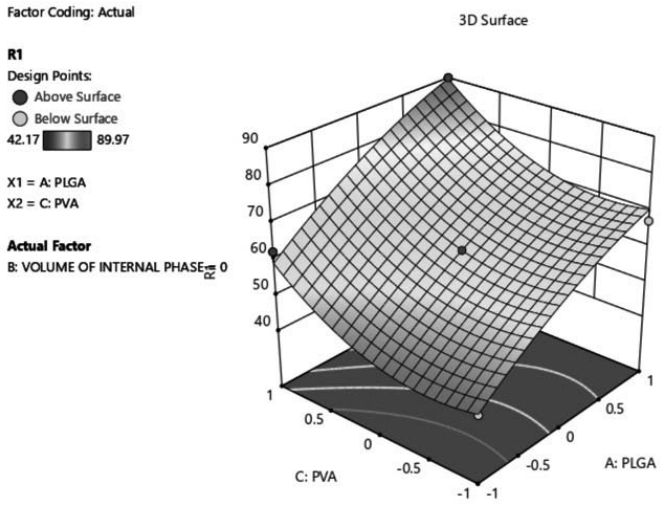
Whereas the volume of the internal phase exhibited negative encapsulation efficiency, this may be to the fact that an increase in the concentration of the aqueous phase may lead to a higher rate of leaching, physical instability of the primary emulsion, and hence leads to a decrease in encapsulation efficiency.[27,28]



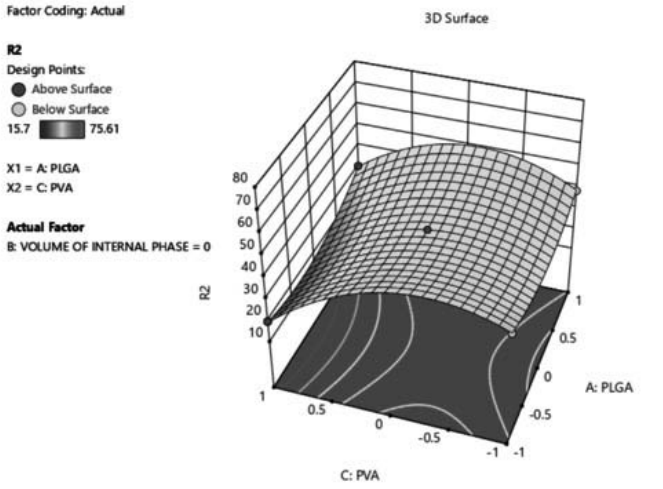
A. PLGA and volume of the internal phase



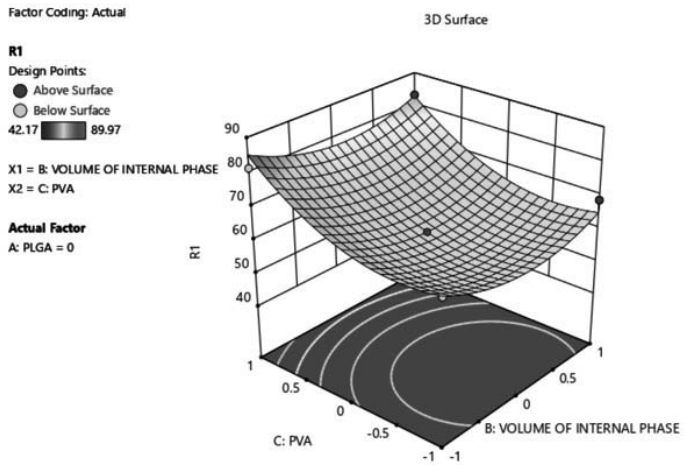
A. PLGA and volume of internal phase



B. PLGA and PVA concentration

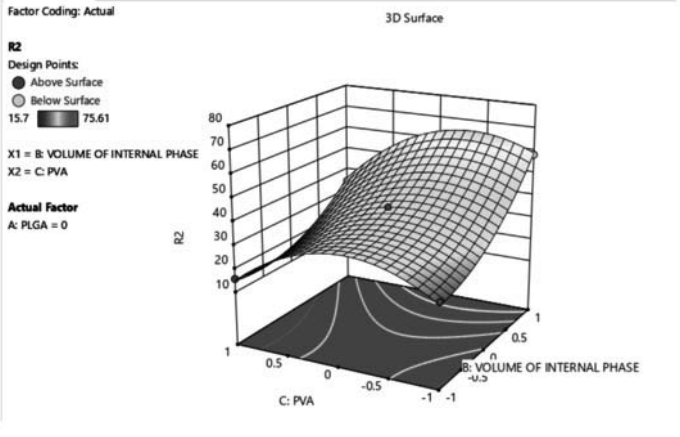


B. PLGA and PVA concentration



C. Volume of internal phase and PVA concentration

Figure 2: 3D surface plot for the effect of process parameter on encapsulation efficiency.



C. Volume of internal phase and PVA concentration

Figure 3: 3D surface plot for the effect of process parameter on particle size.

Effect on Particle size

The effect PLGA concentration (X1), the volume of internal phase (X2), and PVA concentration (X3) on particle size was analyzed and found between 15.75 µm and 75.61 µm.

$$\text{Particle Size} = 40.51 + 2.29875X1 + 14.6475X2 - 7.40625X3 + 1.37X1*X2 + 2.9875X1*X3 - 4.195X2*X3 + 7.47375X12 + 8.17625X22 - 15.91125X32$$

The model was considered significance with *F*-value = 121.86, $p<0.05$. The developed model's R^2 value of 0.9973 demonstrated strong correlation between experimental and predicted values. Analyzing the model linear terms of PLGA (X1), volume of internal phase(X2), PVA (X3), and interaction between PLGA and PVA concentration (X_1*X_3) and volume of internal phase*PVA concentration (X_2*X_3) are significant in 95% confidence level, whereas PLGA concentration and volume of internal phase (X_1*X_2) is non-significant (Figure 3).

The results shows that as PVA concentration increases, the particle size decreases. This accounts that PVA (stabilizer) can orient at the interface of organic and the aqueous phase. Therefore, reduces the interfacial tension by enhancing the net shear. Thus, results in smaller particle sized microsphere.[12,29]

Whereas, as mentioned PLGA concentration and volume of the internal phase positive impact. This could be as a result of the fact that when the volume of the internal phase increases, the droplet size raises and results in increased size of microspheres.[26-28] Furthermore, when PLGA concentration increases, viscosity increases. This decreases the shear force and forms a larger globule size.[29]

Optimization of process parameter

The independent variables were optimized by employing Design-Expert software, after identifying the relationship between the main effects (PLGA, Volume of internal phase, and PVA concentration) on both encapsulation efficiency and particle size. The levels of independent variables that spontaneously produced the highest enacapsulation efficiency and smallest particle size was established using desirability function. Based on the generated model, microspheres mean 22.51 µm sized with 89.43% encapsulation efficiency were calculated with minimum errors, 3.8% and 1.9%, respectively represented in Table 3 and Figure 4.

The optimized microspheres were evaluated for particle size, polydispersity index, and SEM. Obtained results reveal that the particle size is 4.49±0.8 µm, the polydispersity index is 1.0025, and SEM results indicate that the microsphere is smooth and has spherical surface morphology. Particle size, particle size distribution, and SEM of the optimized microsphere were given in Figure 5.

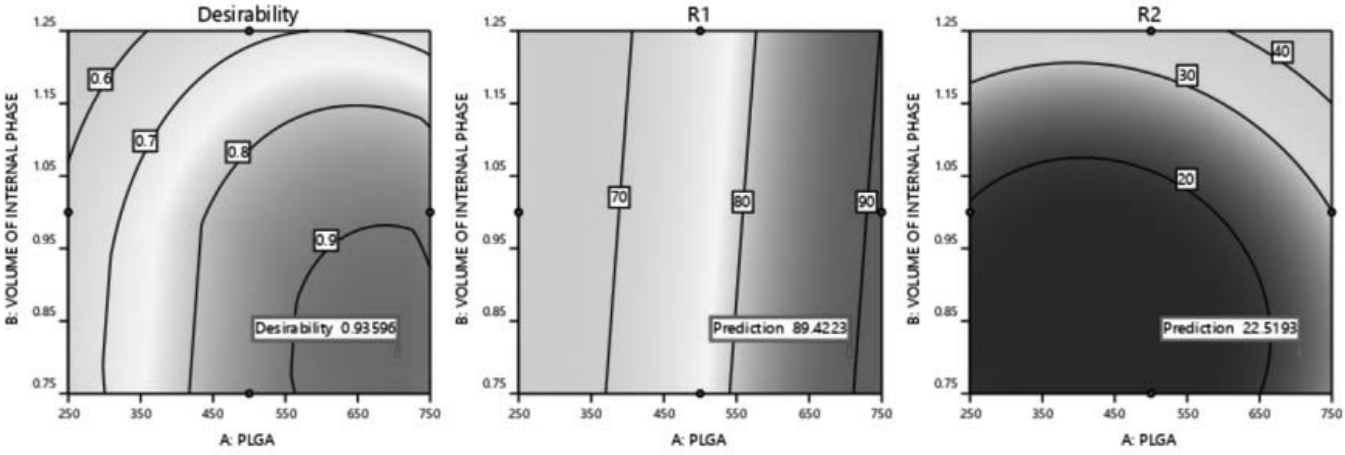


Figure 4: Desirability plot of optimized formulation.

Table 3: Predicted and experimental response of optimized formulation.

Factors			Desirability	Model		Experiment		PDI
Concentration of PLGA(mg)	Volume of internal phase(mL)	PVA concentration(5)		EE(%)	Particle size (µm)	EE(%)	Particle size(µm)	
705.29	0.8	2.00	0.935	89.43	22.51	87.65±1.8	4.49±0.8	1.0025

*Mean±SD, n=3

Table 4: *In vitro* Release study of the optimized formulation and drug.

Time(hr)	% Cumulative drug release (optimized formulation)	% Cumulative drug release (Aspirin)[a]
0	0.00	0
0.25	0.31±0.11	12.9±1.23
0.50	1.66±0.35	22.7±0.87
0.75	2.33±0.08	46.6±1.09
1	3.01±0.13	56.9±0.98
2	3.73±0.4	69.9±1.34
3	4.79±0.46	79.9±1.87
4	5.79±0.39	98.7±0.98
5	6.97±0.49	
6	7.96±0.73	
24	10.57±0.32	
48	13.44±1.35	
72	15.69±2.69	
96	18.36±4.2	
120	21.06±5.35	
132	26.65±4.89	
144	33.83±5.84	
168	39.44±5.96	
192	46.11±4.90	
216	53.42±5.18	
240	62.97±5.2	
264	70.85±4.21	
288	77.44±4.42	
312	84.12±4.36	
336	90.66±3.5	
360	96.10±2.97	
384	98.76±0.49	

*Mean±SD, *n*=3

Characterization of Optimized Microsphere

The raw materials and optimized formulation was characterized by XRD and DSC thermograms. The XRD patterns of Aspirin, PLGA, and Aspirin-loaded PLGA microspheres were recorded and aspirin exhibited a sharp and well defined peaks at 15.9◦, 17.1◦, 21.3◦, 22.8◦, 27.3◦ indicates crystalline nature (Figure 6). Similar peaks were reported in the literature.[14,15] Whereas, no distinct and intense peak in the diffractogram of PLGA was observed demonstrating the amphorous nature. Aspirin peaks were not observed in the XRD pattern of the Aspirin microsphere, exhibiting that aspirin is uniformly dispersed in the polymer matrix, revealing the amorphous form.

Similarly, the DSC studies are an important thermoanalytical tool to confirm the physical nature and the stability of the drug during in the formulation. In the present study, Aspirin exhibited endothermic peak at 144. 82°C (sharp) with 170.98 J/g of enthalpy of fusion (H)[14,15] represents its melting point (Figure 7). Another endothermic peak was also observed at 172.24°C, which may be partly superimposed to the melting one and is ascribable to the thermal degradation of aspirin, this confirms the crystalline nature of the drug. In PLGA (75:25), no peak observed for pure PLGA polymer, confirming its amorphous nature. Whereas, the drug's peak in the thermogram of aspirin PLGA microsphere has disappeared, while a wide peak was observed near 36.76°C.

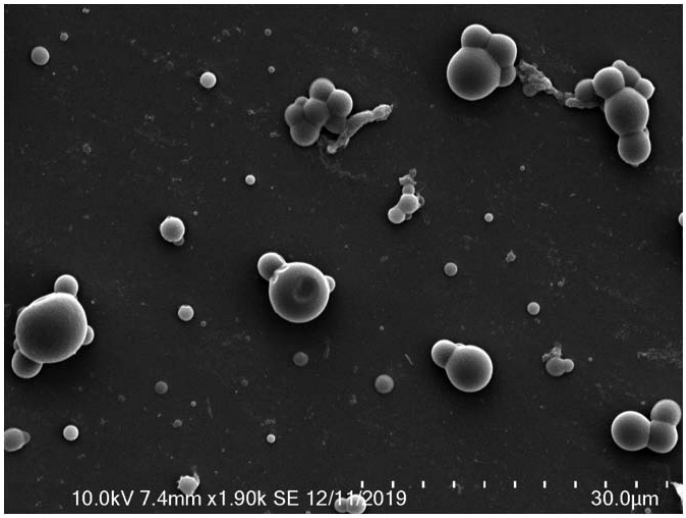


Figure 5: Scanning electron microscopy of optimized Aspirin PLGA microsphere.

In vitro release study

The release study PLGA microsphere is represented in Figure 8 and Table 4, it is clear that aspirin tended to release a lesser amount of drug ~10.57±0.32% initial 24 hr, with relatively 98.76±0.49% drug being released within 16 days. The release pattern indicates a triphasic release profile[30,31] i.e. an initial slight burst phase (0-1d), subsequently steady lag phase (1-5d), and a rapid burst release phase(5d ~ 16d). Literature reveals that release is due to diffusion, hydrolysis, and erosion. The initial burst release may account for the diffusion of drug molecules trapped on the surface of the microsphere, followed by a second phase (lag phase), which may be controlled by polymeric erosion. Further, the second burst effect is due to the degradation of polymeric matrix.[31,32]

The above-mentioned drug release data was used to determine the kinetics necessary to know the pattern and mechanism of release and is recorded in Table 5. Formulation followed the zero-order release model as compared to Hixson Crowell followed by the Higuchi model. The '*n*' values from the Korsmeyer-Peppas model were found to be 0.619 which follows between $0.45 < n = 0.89$, indicates the release in non-Fickian transport. Hence the study confirms that release is due to polymer erosion, swelling, and degradation.[32]

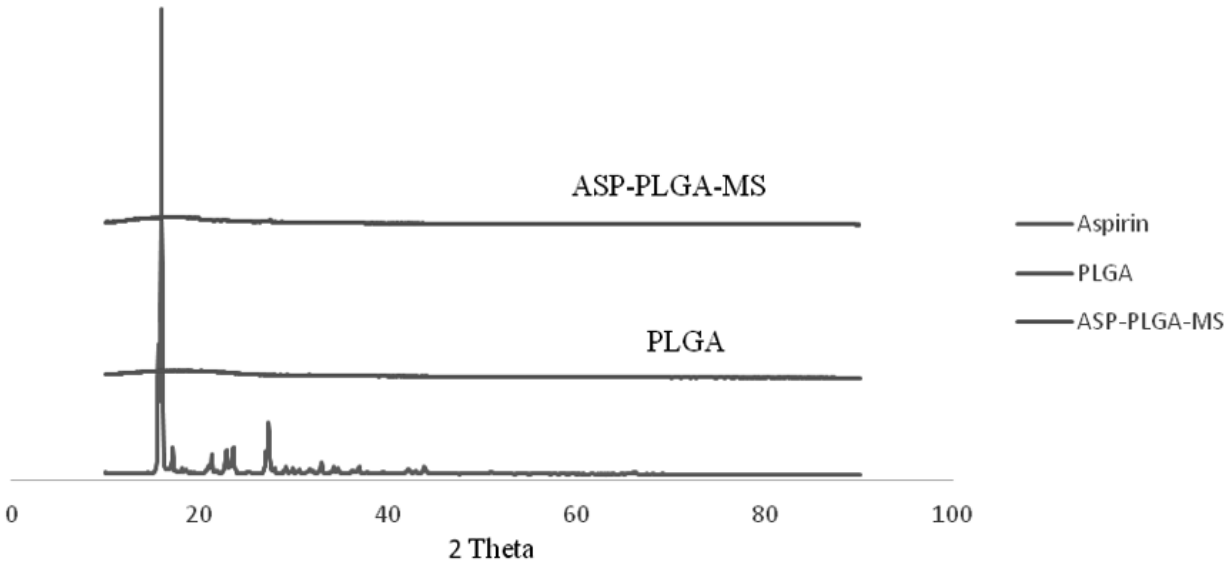


Figure 6: X-ray Diffractation pattern of Aspirin, PLGA, and Aspirin-loaded PLGA microspheres.

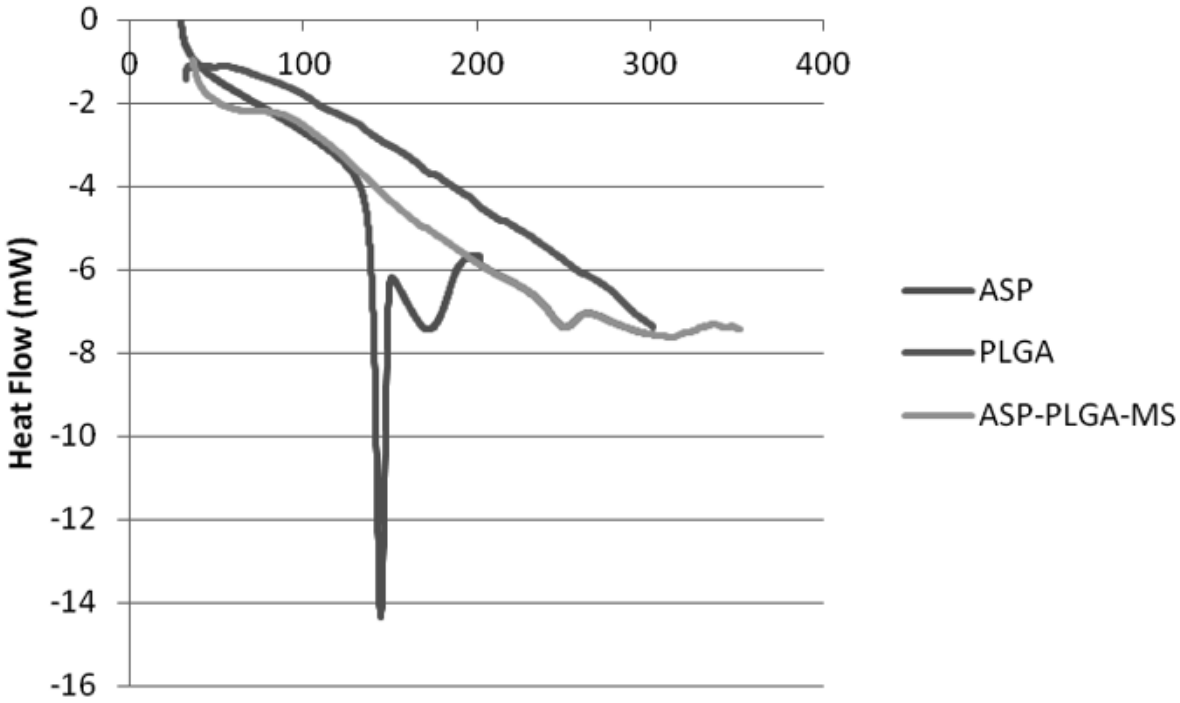


Figure 7: DSC thermogram of Aspirin, PLGA, and Aspirin-loaded PLGA microspheres.

Table 5: *R*²' values of various kinetic models and value of 'n'.

Kinetic model	Zero order R^2	First order R^2	Higuchi R^2	Hixson Crowell R^2	Korsmeyers-peppas	
					R^2	n
Optimized microsphere	0.984	0.780	0.889	0.901	0.939	0.619

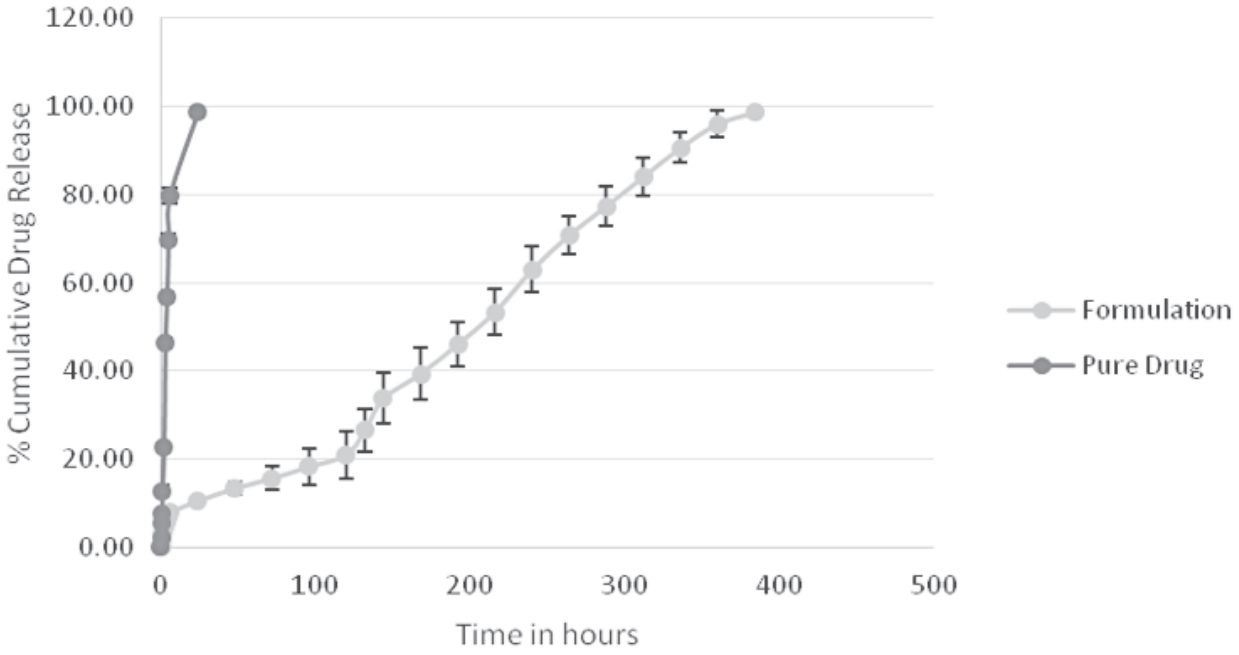


Figure 8: *In vitro* release pattern of Aspirin from PLGA Microsphere.

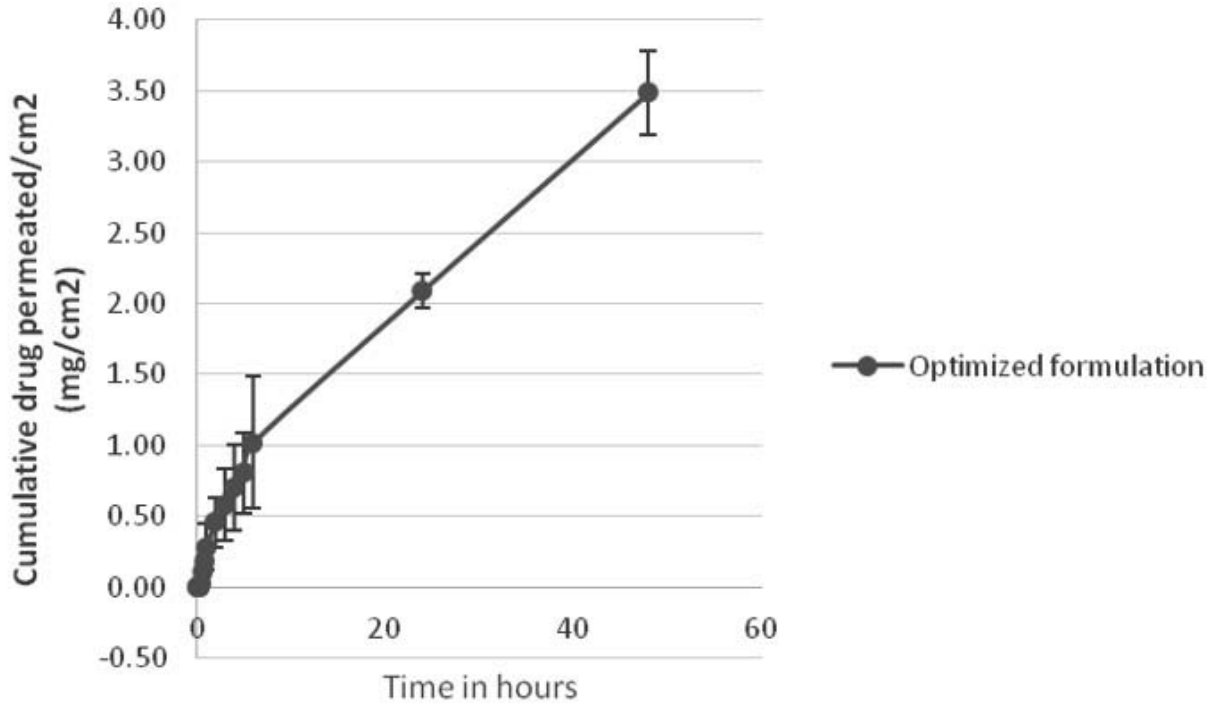


Figure 9: *Ex vivo* permeation studies of Aspirin PLGA Microsphere.

Ex vivo permeation study

Ex vivo permeation study was conducted through porcine buccal mucosa due to the following reason: human and porcine buccal mucosa were non-keratinized,[21] with the cell nucleus observed in the superficial layers, permeation behavior through porcine mucosa was significantly higher compared to that of bovine mucosa, and also the thickness buccal mucosa of porcine mucosal was almost same to human.[33] The permeation study was carried out for 48 hr with 26.05±2.21% drug release and illustrated in Figure 9. Permeation study reveals that permeation flux, permeability coefficient (Kp), diffusion coefficient and Partition coefficient are calculated and recorded in Table 6. Also, the study shows that the microspheres are partitioned into the

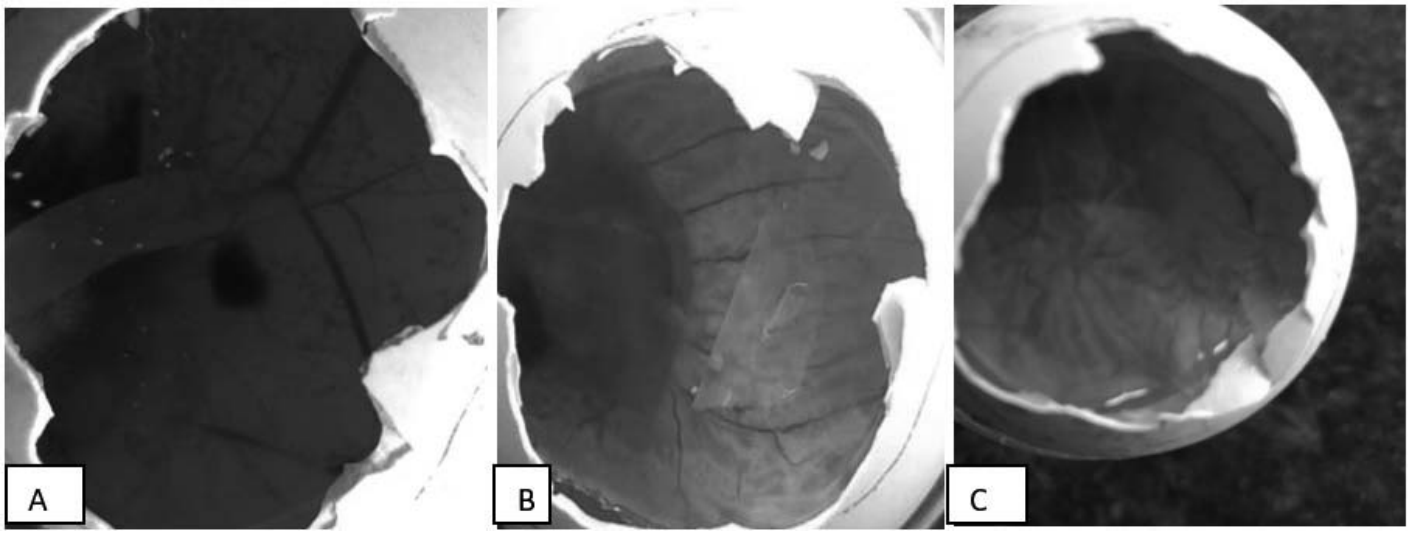


Figure 10: HET-CAM Method for A: Positive control, B: Negative Control, C: Optimized PLGA microsphere.

Table 6: Permeation Parameter of *ex vivo* permeation studies.

Parameters	Optimized formulation
Time (days)	2
% Cumulative drug permeated(%)	26.05±2.21
Flux (mg/cm^2/h)	0.073
Permeability coefficient, Kp cm/h x10^{-3}	1.7
Lag Time(Hr)	0.33
Diffusion coefficient(D)	2.02
Partition coefficient(Km)	1.68

Table 7: Irritation study by HET-CAM study.

	Time in seconds									Irritation Score
	Hemorrhage			vascular lysis			coagulation			
Positive Control	80	100	165	94	110	145	83	105	151	12.58±0.103
Negative Control	295	297	300	295	298	300	294	299	300	0.205±0.012
Test Formulation(T1)	286	283	300	273	288	300	278	283	300	0.778±0.056

*Mean±SD, *n*=3

mucosal lining and release the drug in a sustained manner. So, the result support that aspirin accumulates in the layer below the site of application, and releases the drug in the defined manner. This property can account for the activation of stem cells of the dental cavity and hence regenerate the tooth structure.

Irritation Study

The irritation study results are recorded in Table 7 and Figure 10. The irritation potential of aspirin PLGA microsphere was assessed using the *in vitro* HET-CAM method. The irritation score for PLGA microsphere was 0.778±0.056. The study revealed that the formulation didn't irritate mucous membranes. The isotonic sodium chloride and NaOH(0.1N) solution scores were 0.205±0.012 and 12.58±0.103, respectively. The outcomes demonstrated that the formulation has a much lower irritation value than the positive control, hence appropriate to retain in the oral cavity for a longer duration of time.

CONCLUSION

Aspirin loaded PLGA microsphere was prepared by double emulsion technique using PLGA (75:25) and ethyl acetate-ethanol as solvent system and optimized using Box-Behnken method. Synthesized microsphere with maximum encapsulation efficiency (87.65±1.8%) and minimum particle size (4.49±0.8µm) at optimum formulation conditions; 705.29 mg PLGA concentration, 0.8mL of Volume of internal phase and 2.00% PVA concentration were obtained. Aspirin loaded PLGA microsphere

released the drug in the sustained manner for a period of 16 days. Drug release mechanism showed triphasic release, with initial burst release phase subsequently lag phase and nearly complete release at the end of this time course. This confirm that release is due to polymer erosion, swelling and degradation. The drug release kinetic of *in vitro* release and permeation showed zero order kinetics. *Ex vivo* permeation study through porcine mucosa confirmed sustained permeation and also shows the significant partition and accumulation of drug in the mucosa. This property signifies that the formulation has the potential to stimulate the stem cells, hence it can help in the regeneration of dental tissues. Hence, further study needs to be carried out in dental stem cells to find the mechanism of regeneration.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PLGA: Poly Lactic-co-Glycolic Acid; **PVA:** Polyvinyl Alcohol; **XRD:** X-ray Diffraction Study; **DSC:** Differential Scanning Calorimetry; **SEM:** Scanning Electron Microscopy; **HET-CAM:** Hen's egg test-chorioallantoic membrane; **IS:** irritation score.

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Original Article

Formulation and Evaluation of Niosomal Gel Loaded with *Asparagus racemosus* Extract for Anti-inflammatory Activity

Nikhilkumar Yeshwanth Hegdekar[1], Sneh Priya[1,*], Suchith S Shetty[1], Divya Jyothi[2]

[1]Department of Pharmaceutics, NITTE (Deemed to be University), NGSM Institute of Pharmaceutical Sciences, Deralakatte, Mangalore, Karnataka, INDIA.
[2]Department of Pharmacognosy, NITTE (Deemed to be University), NGSM Institute of Pharmaceutical Sciences, Derelakatte, Mangaluru, Karnataka, INDIA.

ABSTRACT

Background/Aim: Inflammation typically occurs when infectious microorganisms enter the body, settle in specific tissues, and/or circulate in the blood. *Asparagus racemosus* extract contains various saponins and flavonoids and plant-origin drugs have fewer side effects and toxicity. The phytoconstituents have less permeability through the skin; to enhance their permeability and effectiveness it was loaded in niosomes. Therefore, the study aimed to formulate and characterize the niosomal gel loaded with *Asparagus racemosus* extract for anti-inflammatory activity. **Materials and Methods:** The niosomes containing saponins in the extract were prepared using thin film hydration method and 2^3 full factorial design was employed to assess the influence of independent variables span 60 and cholesterol on vesicle size, PDI, zeta potential, and percentage entrapment efficiency. 10% of niosomal and conventional gels were prepared by incorporating optimized niosomes and extract containing total saponin in 1% carbopol gel. *Ex vivo* permeability studies of prepared gels were performed through goat skin using Franz diffusion cell. An anti-inflammatory study was conducted on albino rat. **Results:** The statistical analysis revealed the significant effect of independent variables on vesicle size, PDI, entrapment efficiency and zeta potential. SEM image shows vesicles are spherical in shape and uniform in size. The niosomal gel provided a significantly higher amount of steady-state flux and permeability coefficient into the skin than conventional gel. The animal model proved that niosomal gel loaded with total saponins in extract showed significant anti-inflammatory compared to the control. **Conclusion:** It was concluded that the niosomal gel had better efficacy than the conventional gel.

Keywords: *Asparagus racemosus*, Niosomes, Span 60, Cholesterol, Factorial design, Carbopol 934.

Correspondence:
Dr. Sneh Priya
Assistant Professor, Department of Pharmaceutics, NGSM Institute of Pharmaceutical Sciences, Deralakatte, Mangaluru-575018, Karnataka, INDIA.
Email id: snehpriya123@nitte.edu.in

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INTRODUCTION

Inflammation is a protective response involving immune cells, immune blood vessels, and molecular mediators that is a part of the intricate biological response of body tissues to harmful stimuli such as pathogens, damaged cells, and irritants. The main criteria of anti-inflammatory are to discard the initial cause of cell injury and to discard necrotic cells and damaged tissues and initiate the repair. Plenty of drugs such as non-steroidal anti-inflammatory, and corticosteroids are used to decrease joint pain and swelling.[1] The anti-inflammatories are available in different forms such as gel ointments sterile preparation etc. Anti-inflammatories are used in combination for their differing effects. Synthetic drugs such as NSAID drugs may cause gastric haemorrhage, stomach burn, ulcers etc.[2]

Herbal extracts are considered to have fewer side effects when compared to conventional dosage forms.[3] Plant-origin drugs are safer to use because of their fewer side effects and promising therapeutic effect.[4] Novel drug delivery systems such as (niosomes,[5] liposomes,[6] ethosomes,[7] nanoparticles) loaded with herbal extract tend to decrease dose dumping, and drug degradation, and enhance the permeation of drugs through the skin.

Transdermal therapeutic systems are safe and non-interfering when compared to other routes of administration. Even though the *stratum corneum* layer of the skin is a found to be a barrier to most drug absorption, this system provides a larger surface area (1-2m^2) for drug diffusion and in order to prolong drug release, it can be incorporated into formulations like gel, patch etc. Liposomes, niosomes, ethosomes, proniosomes etc., are the other sustained release formulations. Topical route usually causes local

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irritation effect and itching whereas it also avoids the first pass effect, prolongs duration of action, provides flexibility in dosing, reduces other side effects and also ensures uniform plasma levels.[8] One of the semisolid topical drug delivery systems is gels, which are transparent and opaque preparations. Gels contain transparent gelling agents which help form three dimensional colloidal structures. They are of two types; aqueous gels that are water based and organogels that are organic solvent based. Gels have features like easily spreadable, water soluble, non-staining, less greasy and emollient. During formulation, gels require higher aesthetic value and lower energy.[9]

Niosomes are vesicular systems that, like liposomes, are used to transport lipophilic and amphiphilic drugs. These are non-ionic surfactant vesicles. They are obtained on the hydration of synthetic non-ionic surfactants that can be done with or without use of systemic circulation cholesterol or their lipids.[10]

Encapsulation of drug in the vesicular system has shown to prolong the presence of the drug in, improve penetration into target tissues and this may also reduce toxicity if selective uptake is achieved. *Asparagus racemosus*, commonly known as Shatavari, belongs to the family *Liliaceae*. It contains a major class of steroidal saponins like Shatavarin I to IV along with minor steroids and their glycosides, flavonoids such as rutin and alkaloids. Shatavari root is used to treat nervous disorders, diarrhoea, dyspepsia, tumors and inflammation.[11] The same extracts have shown to possess antiulcer, antioxidant, immunomodulatory, anti-diabetic, phytoestrogenic, anti-ageing and adaptogenic properties. Aqueous extract of *Asparagus racemosus* acts as an immunomodulator. It increases the activity of macrophages, thereby contributing to its anti-inflammatory activity.[12]

MATERIALS AND METHODS

Materials

Asparagus racemosus (Shatavri) powder was procured from Dr. Jain's Forest Herbals Pvt. Ltd., Mumbai. Cholesterol was purchased from Merck, Mumbai, India. Chloroform, span 60, disodium hydrogen phosphate, potassium dihydrogen orthophosphate, carbopol 934 and triethanolamine was obtained from Lobachemie, Mumbai, India. Ethanol was purchased from Nice chemicals, Kerala, India. All the chemicals used were of analytical grade.

Extraction of plant material (Soxhlet extraction)

Dried powder of *Asparagus racemosus* was collected from localities. Weigh approximately 150 g of powder and was kept in the thimble. Then 500ml of ethanol was added to the round bottom flask the solution was heated at 50°C for 6-7 hr to complete 5 cycles per day. The apparatus was switched off until 50 cycles were completed. The collected extract was then dried at 90°C in a water bath for concentration.[13] To determine the various phytoconstituents, the prepared extract was subjected to various chemical tests according to standard procedure.[14]

Fourier transform infrared spectroscopy (FTIR) study

FTIR spectroscopy of the extract was carried out to find the principal peak and compare with the reference standard. It was also performed to check the compatibility between constituents present in the extract, span 60 and cholesterol in the case of niosomes. The FTIR spectra of the extract and optimized niosomal formulation were recorded on an Alpha Bruker spectrometer. After obtaining the FTIR spectra for the samples, the reference peak was compared and analysed for any incompatibilities.

Determination of λ_{max} and calibration curve of *Asparagus racemosus* extract

Accurately 20 mg of extract was dissolved in ethanol and made up to 10 mL with the same solvent to get 2000µg/mL. 0.5 mL of the above stock solution was transferred into another volumetric flask. It was made up to 10 mL with the phosphate buffer pH 5.5. This solution was then scanned between 200-600 nm. The extract showed maximum absorbance at 274 and 365 nm which showed the presence of total saponins and rutin respectively. The absorbance of the 5-25 µg/mL samples was measured at the $\lambda_{max,}$ and a standard calibration curve was constructed by plotting concentration versus absorbance.

Preparation and characterization of niosomes

Design of experiment

From the literature, the factors that influenced the formation of niosomes were identified as the concentration of span 60 and cholesterol. A 3^2 factorial design was selected to analyse the influence of these factors as shown in Table 1, on responses such as vesicle size, polydispersity index (PDI), zeta potential, and entrapment efficiency by using Design Expert Software (version 11.0.3.0 64-bit, Stat-Ease, Inc. Minneapolis, MN, U.S.A). The following equation is obtained from regression analysis:

$$Yi= \beta_0+ \beta_1A+ \beta_2B+\beta_3C+\beta_4AB+ \beta_5AC +\beta_6BC+ \beta_7ABC$$

Where, β_0- $\beta_{7:}$ standardized beta coefficients for monitored experimental values of $Y_{i,}$ ABC, AB, BC and AC are the interaction term, and present the polynomial term of the independent variables. After a data analysis of the negative and positive signs of coefficient values in equations, the antagonist and agonist of independent variables can be identified. The magnitude of the beta coefficient represents the extent of the impact. Polynomial terms were included to account for the curvature effect of the independent variable. The model's suitability was determined by the statistically significant F ratio (p<0.05) and modified coefficients of determination (adjusted R^2) between 0.8 and 1.0.[15]

Table 1: Factors and their values.

Variables	Levels			Dependent variables	Goal
	-1	0	+1		
Independent variables	250	375	500	Vesicle size Y1	Minimum
Span 60	150	200	250	PDI Y2	Minimum
Cholesterol				Zeta potential Y3	Maximum
				Entrapment efficiency Y4	Maximum

Table 2: Formulation composition batches as per 2^3 full factorial design.

Formulation	Span 60 (mg)	Chloroform (ml)	Cholesterol (mg)	Extract (mg)	Phosphate buffer pH 5.5
F1	250	10	150	500	10
F2	375	10	150	500	10
F3	500	10	150	500	10
F4	250	10	200	500	10
F5	375	10	200	500	10
F6	500	10	200	500	10
F7	250	10	250	500	10
F8	375	10	250	500	10
F9	500	10	250	500	10
F10	375	10	200	500	10
F11	375	10	200	500	10

Preparation of niosomes

Niosomes were formulated using the thin film hydration method as given in Table 2. An accurate amount of surfactant that is span 60 and cholesterol was dissolved in 10ml chloroform. Then the following mixture was subjected to thin film hydration at a temperature of 50°C until the thin film was found. The film was hydrated with 10ml phosphate buffer of pH 5.5 containing extract for 3 hr with gentle shaking.[16]

Characterization of niosomes

Vesicle size, PDI and zeta potential

The vesicle size, PDI and zeta potential of prepared niosomes was used using Malvern zeta sizer which employs dynamic light scattering. Zeta potential is defined as the charge particle obtains when it is present in the medium. It helps in determining the stability of the formulation.

Entrapment efficiency

By using the centrifugation method, entrapment capabilities of niosomal formulations were assessed. At 3°C, the niosomal suspension was centrifuged for 10 min at 8000 rpm. After that, the solid mass was isolated from the supernatant, and PB was used to create the appropriate dilutions (pH 5.5). A UV-visible spectrophotometer technique was used to measure the drug concentration at 274 nm. The following equation was used to compute the drug entrapment efficiency.[16]

$$\text{Entrapment efficiency (\%)} = [(Ct - Cf)/ Ct] \times 100$$

Where Ct is the amount of total drug and Cf is the concentration of unentrapped drug

Formulation and characterization of optimized formulation

The optimized formulation was prepared as per the solution given by the software that is 355.619 mg of span 60 and 231.912 mg of cholesterol was used for the formulation. Vesicle size, size distribution and zeta potential was performed as per the factorial batches. Optimized formulation was further evaluated for the parameters like optical high-resolution microscopy, scanning electronic microscopy and *in-vitro* drug release study.

Optical high-resolution microscopy

A drop of prepared niosomes was diluted with water and placed on a glass slide and then viewed under a high-power microscope (Biovis Particle Analyser-Carl Zeiss Microscopy, NGSMIPS Advanced research centre) for the appearance of vesicles.[17]

Scanning electron microscopy

The obtained niosomes were centrifuged at 10,000 rpm for 15 min then the sediment obtained from centrifugation was subjected to freeze drying. The obtained solid residue was viewed under SEM.[18]

Preparation and characterization of niosomal gel

Preparation of gel

The extract-loaded niosomal gel (10% w/v) was prepared by adding niosomal suspension (equivalent to 1 g of total saponins in the extract) to 10 g of 1% Carbopol 934 gels. Whereas conventional gel (10% w/v) was prepared by incorporating extract equivalent to 1 g of total saponins into 10 g of 1% Carbopol 934 gel. Triethanolamine was introduced and it was stirred rapidly to achieve the gel-like consistency.[19]

Drug content

500 mg gel was added and dissolved in 100 mL phosphate buffer pH 5.5. The gel solution filled in the volumetric flask was shaken continuously with the help of a mechanical shaker for 2 hr to enhance the solubility of the drug. Then 1ml of gel solution was made up to 10 mL phosphate buffer pH 5.5 and was scanned under UV-Visual spectroscopy at λ_{max} 274 nm using phosphate buffer pH 5.5 as blank.[20]

Spreadability, Viscosity and pH measurement

The spreadability of niosomal gel was determined using two glass slides having a similar length. To one glass slide, 1 g of gel was added to the other glass slide weights were added. Time taken by the second glass slide to slip off from the first was determined.[21] The viscosity of the gel was measured using a Brookfield viscometer (LV Model D 220 NGSMIPS Advanced research centre). A small amount of gel was taken and then rotated at 10 rpm using T bar spindle number 96 and the values were noted.[21]

pH of the gel was measured using a digital pH meter.

Ex-vivo drug permeation studies

From the slaughterhouse, goat ear skin was collected; the hair which was removed from the skin and immersed in the phosphate buffer pH 5.5. By using a Franz Diffusion cell containing two compartments the *ex vivo* studies of the skin were carried. The donor compartment involves two open ends where one end of the donor compartment is covered with animal skin which was soaked previously with phosphate buffer pH 5.5. Add 500 mg of gel (equivalent to 50 mg of total saponin) on the dermal side of the skin in the donor compartment. 50 ml of pH 5.5 phosphate buffer that included a tiny magnetic bead and swirled at a fixed speed of 50 rpm was placed in the reservoir compartment. For eight hours, the investigation was conducted at 37±0.5°C. At regular intervals, 5 mL of samples were taken from the reservoir compartment, and absorbance was calculated spectrophotometrically at 274 nm. To keep the sink state constant, the reservoir compartment was consistently refilled with the same amount of new 5.5 pH phosphate buffer.[22]

Calculation of Skin Permeation Parameters

As a function of time, the cumulative amount of drug permeated by unit area was calculated. The flux was determined from the linear portion of the slope. The extract permeability coefficient (*Kp*) through goat skin was determined using the relationship established from the first law of Fick's diffusion, represented in the following equation:

$$Kp=J/C$$

Where *J* is the flux and *C* is the drug concentration in the donor compartment.[23]

Anti-inflammatory activity

Using a method to cause oedema in a rat's paw using carrageenan, anti-inflammatory activity was assessed. After receiving proper consent from the Institutional Animal Ethical Committee (IAEC) of the NGSM Institute of Pharmaceutical Sciences, Deralakatte, Mangalore, albino rats of the Wister strain (150–200g) were utilised (Approval No: NGSMIPS/IAEC/MARCH-2019/144). The animals were housed in plastic cages with plush bedding and six to a cage under the usual lighting and darkness schedules. They were given free access to food (a typical pellet diet) and tap water. Before the test, the rats were given a week to acclimate. 12 hr before the trial and throughout it, food was removed. Three animals were each made up of the three groups from which the animals were separated. The first was used as a control and received a gel base (500 mg/kg) without medication. The second group received 1% diclofenac gel (10 mg/kg) as standard, and the third group received 1% niosomal gel (500 mg/kg). All gels were applied on the dorsal surface of the paw with 50 times gentle rubbing. Before 30 min of gel application, a carrageenan solution (1% w/v in normal saline) was employed to cause inflammation. To reduce any stress-related behavioural changes, the animals were kept in isolation in observation chambers for 10 min. Paw volume was measured before injecting the carrageenan solution and after 30, 60, 120 and 180 min using of Plethysmograph.[24-26]

$$\% \text{ inhibition}=\frac{V_c - V_t}{V_c}\times 100$$

Where, Vc and Vt represents the average volume of the paw of the control and the treated animals respectively

RESULTS AND DISCUSSION

Extraction and phytochemical testing

Ethanolic extract of *Asparagus racemosus* was a yellowish-brown colour, and the percentage yield was 7.56%, respectively. The preliminary phytochemical investigation revealed that the plant extract included flavonoids and saponins and the results are shown in Table 3.

FTIR study

An FTIR spectrum of the extract was shown in Figure 1a. The peak observed at 3319.50 cm^{-1} corresponds to the hydroxyl group of the Shatavari extract. The peak at 1634 cm^{-1} is attributed to the carboxyl group. Aromatic unsaturation (C=C) peak is observed at 1416 cm^{-1} and 1016.99 cm^{-1} corresponds to C-O-C. CH_3 can be observed at 1372.90 cm^{-1} and asymmetric CH stretch at 2936.62 cm^{-1}. FTIR spectrum of optimized niosomal formulation was shown in Figure 1b, it was found that the important peaks observed in the FTIR of the extract are retained in the formulation, which indicates that there are not many considerable interactions of the extract with the components of the formulation.

Determination of λ_{max} and calibration curve of *Asparagus racemosus* extract

The U.V absorption spectra of *Asparagus racemosus* extract showed two maximum peaks at 274 and 365nm as shown in Figure 2. The 274 nm peak may be due to total saponins and 365 nm may be due to flavonoids as said rutin. The λ_{max} 274nm was selected for further investigation.[26] The calibration curve of plant extract in phosphate buffer pH 5.5 follows beers –lamberts in a given concentration range between 5-25 µg/ml with a regression coefficient of 0.9994 as given in Figure 3.

Table 3: Phytochemical testing.

Tests	Observation	Inference
Saponins	Appearance of froth	The presence of saponins confirmed
Flavonoids	Yellow colour produced in organic layer	Flavonoids present
Shinoda test	Pink colour	The presence of flavonoids confirmed

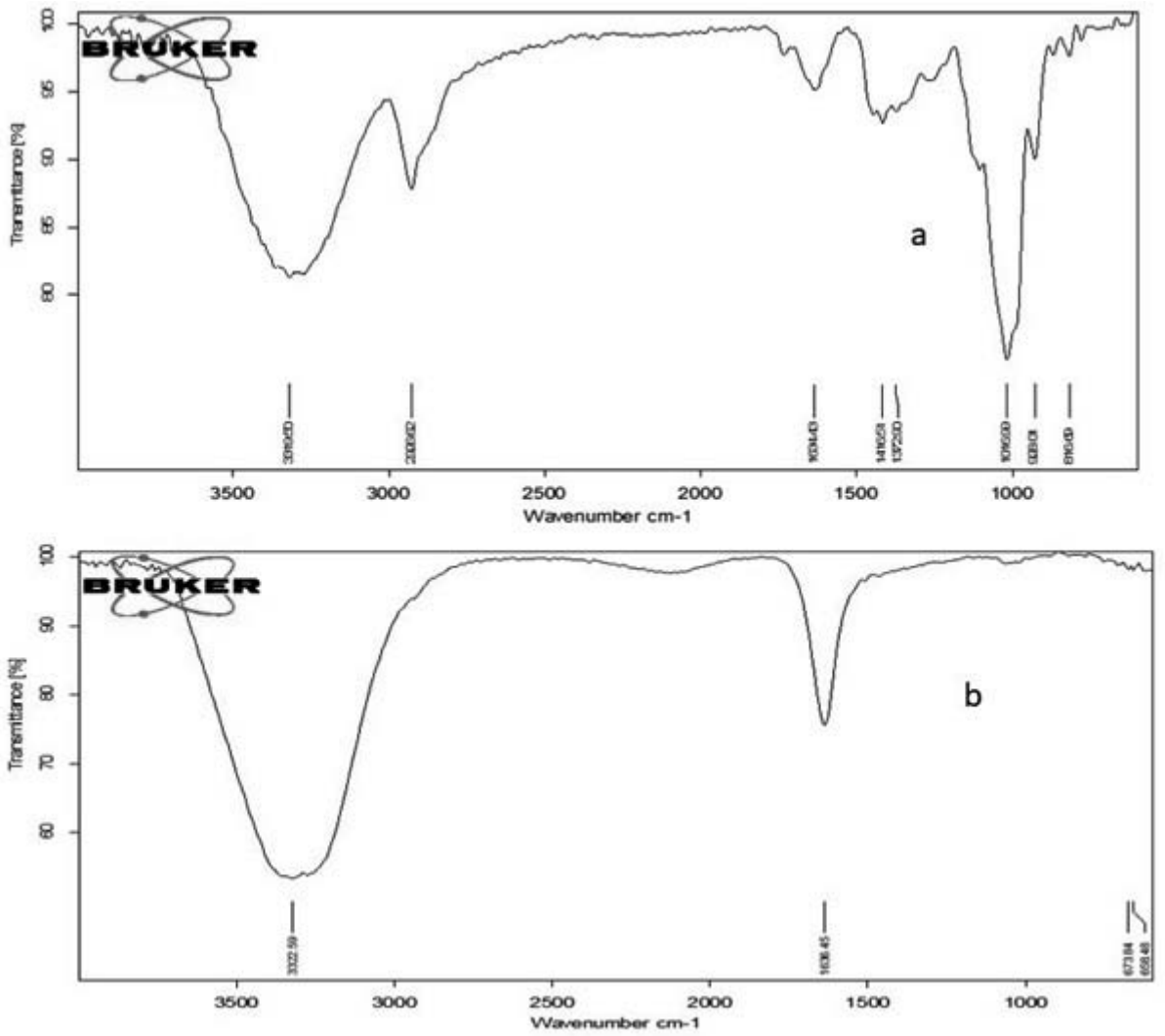

Figure 1: FTIR spectra of a) *Asparagus racemosus* root extract b) Niosomes loaded with extract.

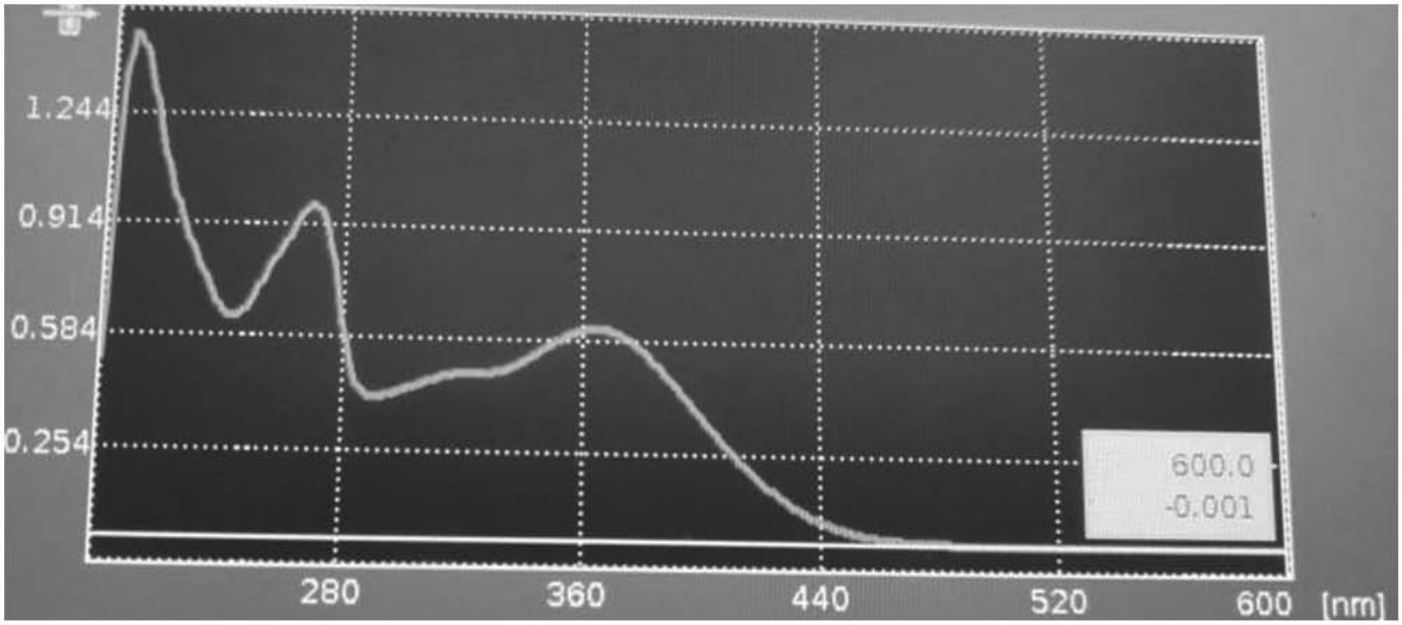

Figure 2: UV spectra of *Asparagus racemosus* root extract in phosphate buffer pH 5.5.

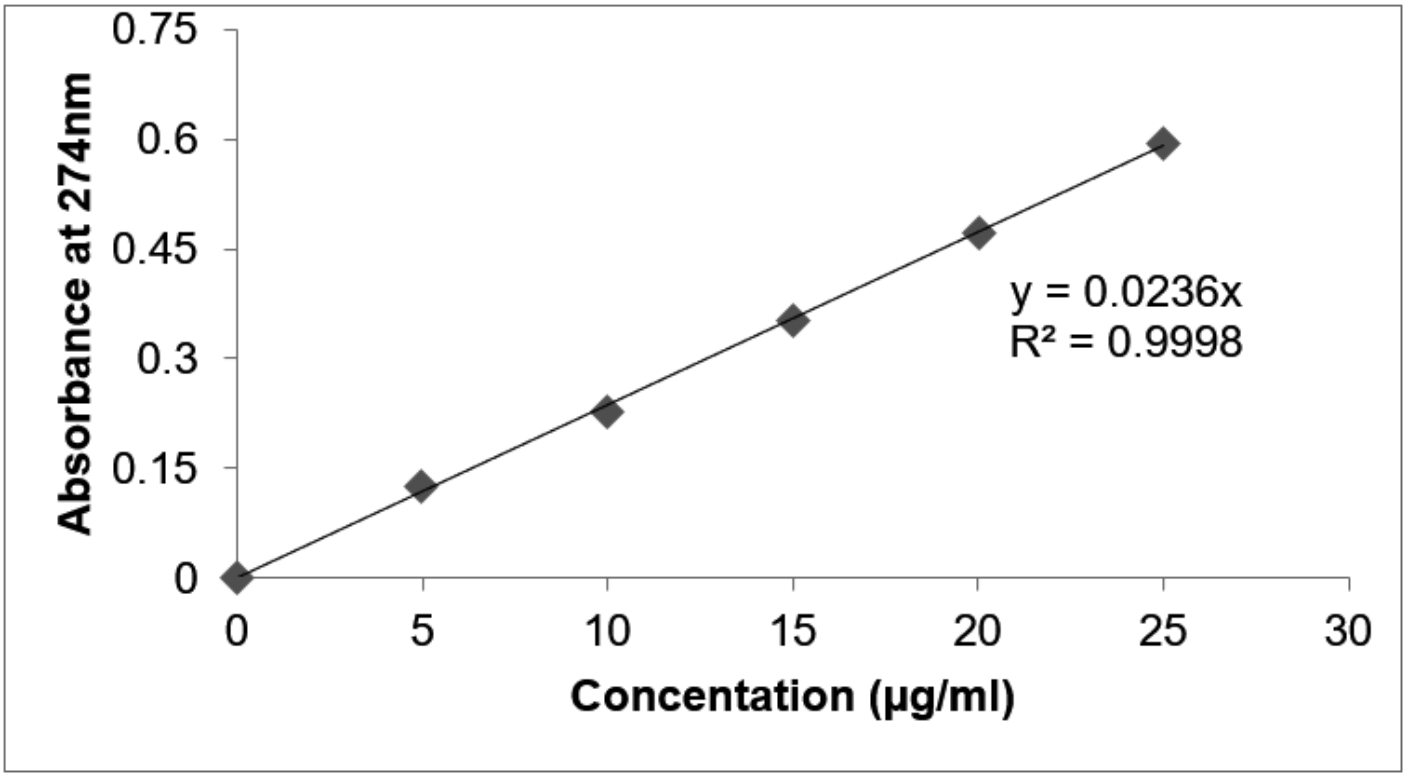

Figure 3: Calibration curve of *Asparagus racemosus* extract in phosphate buffer of pH.

Formulation and characterization of niosomes

Statistical analysis of experimental design

The extract containing total saponin loaded with niosomes was successfully formulated employing 3^2 factorial designs to comprehend how cholesterol and the nisomal component span 60 affect its characteristics.

Vesicle size

Vesicle size plays a important role in the permeation of niosomes through the skin as per literature. The impact of span 60 and cholesterol on vesicle size was shown in Table 4 and Figure 4. It was found that as the concentration of surfactant was increased, vesicle sizes were increased may be higher contraction led to the aggregation of vesicles. As shown in Table 5, the model created

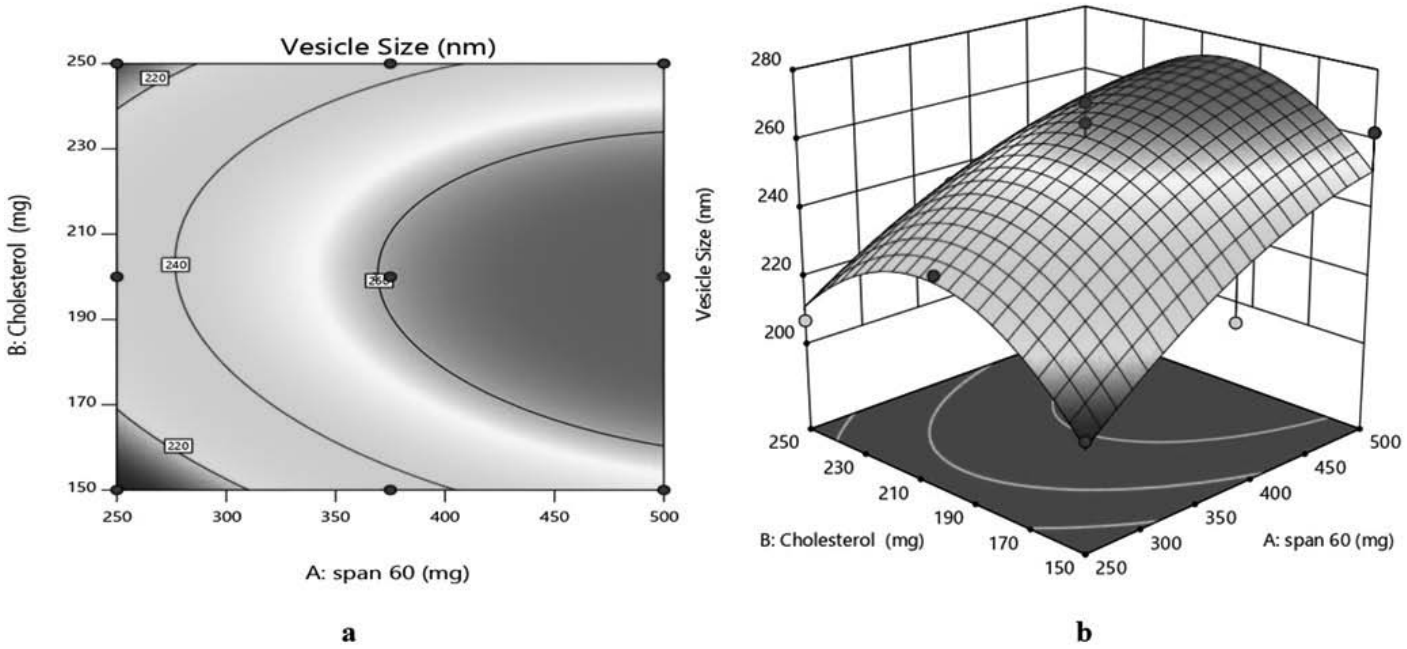

Figure 4: Responses surface curve representing a) Contour and b) 3D effect of span 60 and cholesterol on vesicle size.

Table 4: Results of responses of niosomes as per full 3^2 factorial.

Form. No.	Vesicle size nm	PDI	Zeta potential mV	Entrapment Efficiency %
F1	204.8±2.30	0.36±0.020	-30.8±2.3	45± 3.3
F2	221.4±1.59	0.39±0.028	-29.8±2.6	60± 2.4
F3	262.2±2.60	0.485±0.015	-25.9±1.8	48± 4.1
F4	234.6±2.35	0.372±0.019	-24.6±1.5	56± 2.7
F5	270.8±3.95	0.395±0.025	-29.3±2.10	65± 3.1
F6	258.4 ±2.55	0.42±0.023	-28.6±1.65	56±3
F7	206.9±4.78	0.386±0.016	-26.1±2.35	50± 4.1
F8	235.5±2.78	0.41±0.024	-28.5±2.10	55± 2.7
F9	250±3.45	0.398±0.022	-31.5±1.98	34± 3.6
F10	260.8±3.90	0.398±0.013	-28.4±2.30	63± 3.2
F11	265.9±5.86	0.3870.019	-29.6±2.24	62± 2.3

for the vesicle size has a *p*-value of less than 0.05 and a F value of 6.83 and model was significant. The value of 8.75 indicates a non-significant lack of fit, indicating that the model can be used to determine and calculate the particle size. The adjusted R^2 value (0.5541) and anticipated R^2 value (0.3565) differed by less than 0.2, showing fair agreement between the two.

The analysis's findings led to the following quadratic equation

$$\text{Vesicle Size} = +260.95 + 20.65A^{*} + 0.6667B - 3.58AB - 7.78A^{2*} - 25.63B^{2*}$$

Where A is the concentration of span 60 and B is the cholesterol concentration, the co-efficient in this equation represents the stranded beta co-efficient and the asterisk sign indicates the significance of the variables. With high adjusted R^2 values of 0.5541, the derived regression model was proven to be statistically significant ($p<0.05$).

PDI

PDI show the homogeneity of vesicle size. The lower value of PDI (<0.5) shows formulation is more homogeneous in nature. Results show that as the cholesterol concentration was increased PDI value decreased as shown in Table 4 and Figure 5. The Polynomial model implied to be significance with a model having *F*-value of 14.97. The *p*-value of 0.1092 indicates that the model is a non-significant lack of fit, and was significant to calculate the

Table 5: Summary of regression analysis of niosomes.

Factor	Vesicle size (Adjusted R^2=0.9174)		PDI (Adjusted R^2=0.8074)		Zeta potential (Adjusted R^2=0.5096)		Entrapment efficiency (Adjusted R^2=0.9030)	
	Estimated Beta Co-Efficient	*P* value	Estimated Beta Co-Efficient	*P* value	Estimated Beta Co-Efficient	*P* value	Estimated Beta Co-Efficient	*P* value
Intercept	260.95	0.0275	0.4001	0.0020	-28.46	0.0472	64.58	0.0027
A-Span 60	20.65	0.0082	0.0308	0.0011	-0.7500	0.2588	-2.17	0.1228
A-cholesterol	0.6667	0.8965	-0.0068	0.2799	0.0667	0.9161	-2.33	0.1023
AB	-3.58	0.5752	-0.0282	0.0055	-2.58	0.0108	-4.75	0.0210
A^2	-7.78	0.3469	-	-	-	-	-10.45	0.0021
B^2	-25.63	0.0189	-	-	-	-	-8.95	0.0042
Lack of Fit	-	0.1043	-	0.1092	-	0.1200	-	0.1660

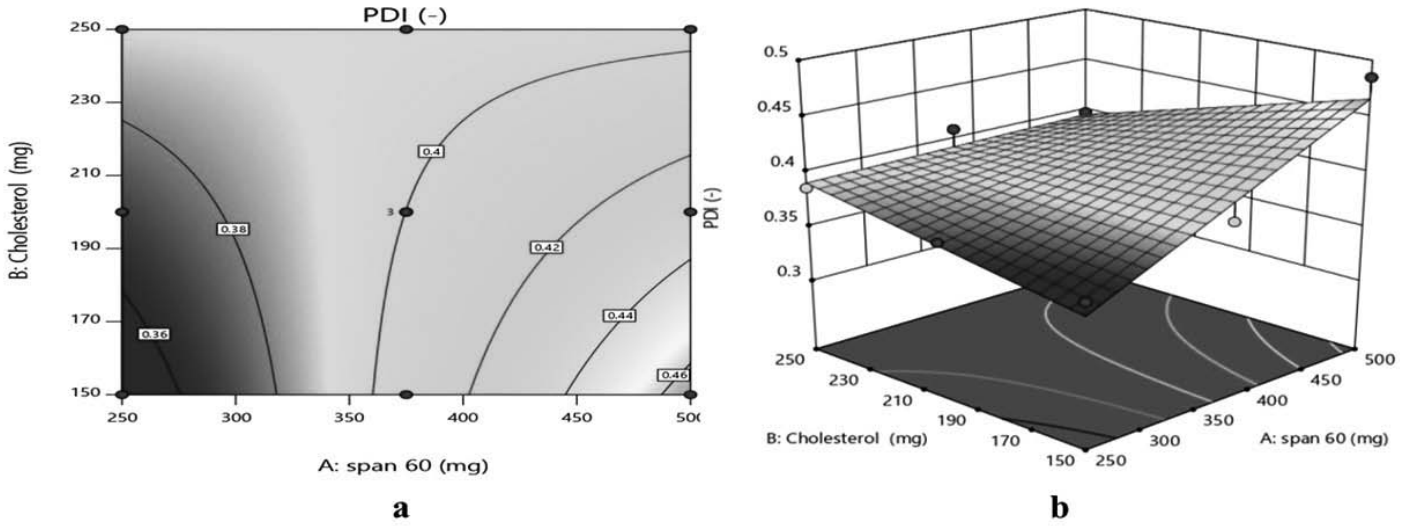

Figure 5: Responses surface curve representing a) Contour and b) 3D effect of span 60 and cholesterol on PDI.

size of the particle. The equations for the response i.e., PDI based upon the 2FI (interactive effect) show the equation as;

$$PDI=0.4001 + 0.0308 (A^*) -0.0068 (B) -0.0283 (AB^*)$$

Where A is the concentration of span and B is the concentration of cholesterol the co-efficient in this equation represents standardized beta co-efficient and the asterisk sign indicates the significance of the variables The obtained regression model was proven to be statistically significant (p<0.05) with high adjusted R^2 values of 0.8074 as shown in Table 5.

Zeta potential

Zeta potential can affect the stability of the vesicles. During storage, the aggregation of vesicles is stopped by the electrostatic repulsive force produced by charged particles. The cholesterol at all SPC concentrations prevents or at least delays the formation of electrostatic repulsions cause vesicle clump to develop as shown in Table 4 and Figure 6. A p-value of <0.05 and a F Value of 4.46 in the model developed for particle size indicated that the model was significant. Since the value of 7.62 shows that the lack of fit is not statistically significant, the model can be used to determine the particle size. The polynomial equations for the response i.e., Zeta potential based upon the 2FI (interactive effect) are

$$Zeta\ potential= -28.46 - 0.750\ A^* +0.0667) - 2.58\ AB^*$$

Table 5 shows that the generated regression model had high adjusted R^2 values of 0.5096 and was statistically significant (p<0.05).

Entrapment efficiency

As the concentration of span, 60 increases at all concentrations of cholesterol, the percentage efficiency of the entrapment was marginally decreased as shown in Table 4 and Figure 7. It may be chances of vesicle aggregation rising at higher concentrations of span 60 which often reduces the potential to form a stable film surface. As a consequence, drug leaching happens, and thus the efficiency of entrapment reduces. As can be seen in Table 5, the model developed for the particle size had a significant p-value of

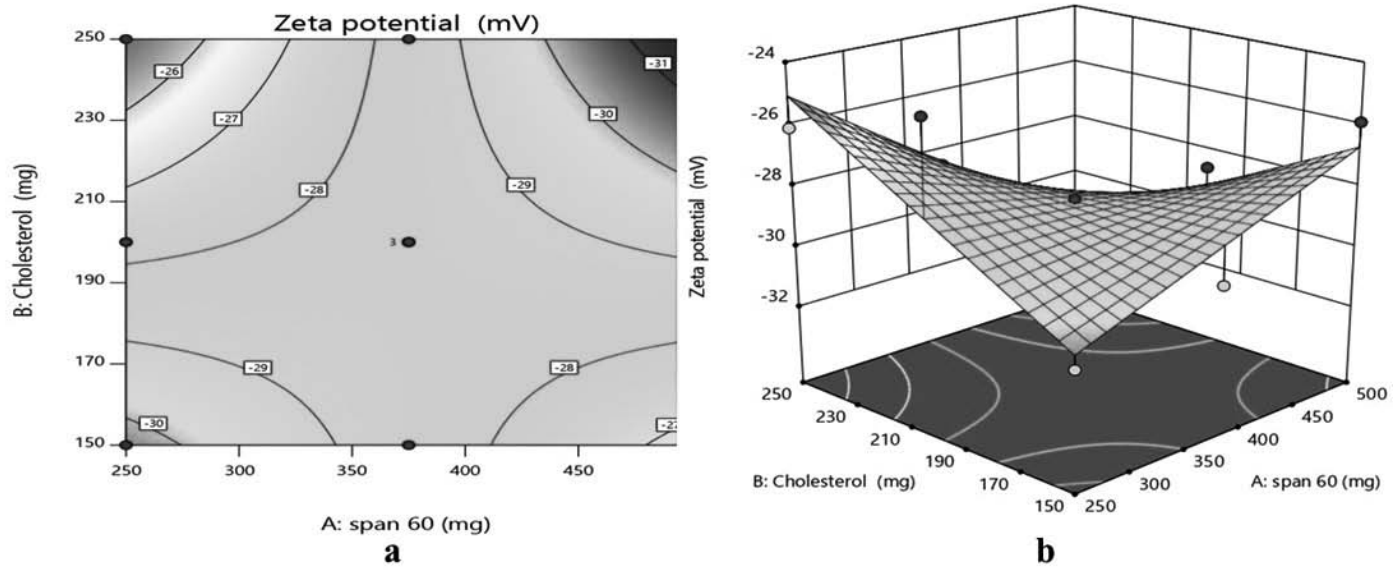

Figure 6: Responses surface curve representing a) Contour and b) 3D effect of span60 and cholesterol on zeta potential.

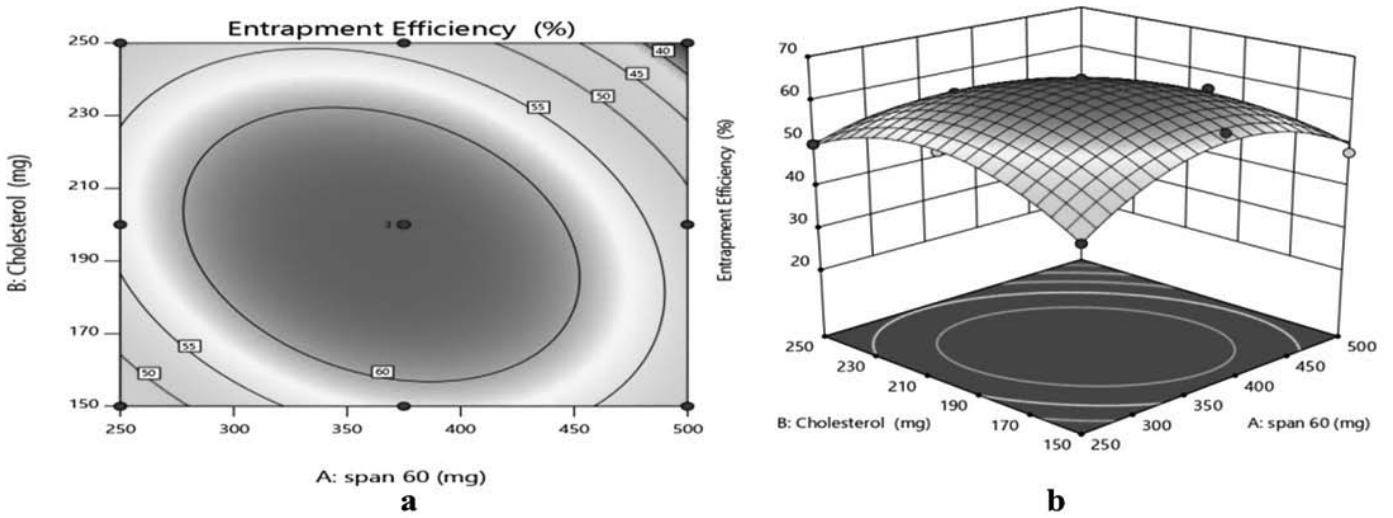

Figure 7: Responses surface curve representing a) Contour and b) 3D effect of span60 and cholesterol on entrapment efficiency.

<0.05 and a F value of 19.61. The difference between the adjusted (R^2 0.9030) and the predicted (R^2 0.7357) model R-squared value was less than 0.2 showing fair agreement between the two. The polynomial equation which was obtained from the results of the analysis as shown below:

$$\text{Entrapment Efficiency} = 64.58 - 2.17\,(A) - 2.33\,(B) - 4.75\,(AB^*) - 10.45(A^{2*}) - 8.95\,(B^{2*})$$

Where A is the concentration of span and B is the concentration of cholesterol the co-efficient in this equation represents standardized eta co-efficient and the asterisk sign indicates the significance of variables The obtained model for regression was found to be statistically significant (p<0.05) having a high adjusted R^2 values of 0.9030.

Optimization of niosomes loaded with extract

Niosomes were optimized based on constraints such as minimum vesicle size and PDI, maximum zeta potential and entrapment efficiency. Based on a desirability value greater than 0.8, the optimal formula was chosen. The formulation which was desirable and selected was prepared according to the solution given by the software containing 355.619 mg of span 60 and 231.912 mg of cholesterol. The vesicle size, PDI, zeta potential, and entrapment efficiency values given by the software were 245.90 nm, 0.394, -28.05 mV, and 60.8% respectively, whereas the experimental value was found to be 248.8 nm, 0.3734, -28.4 mV, and 62.3% as shown in Figure 8. The observed values were found to be within ± 5% error of the predicted value which is acceptable.

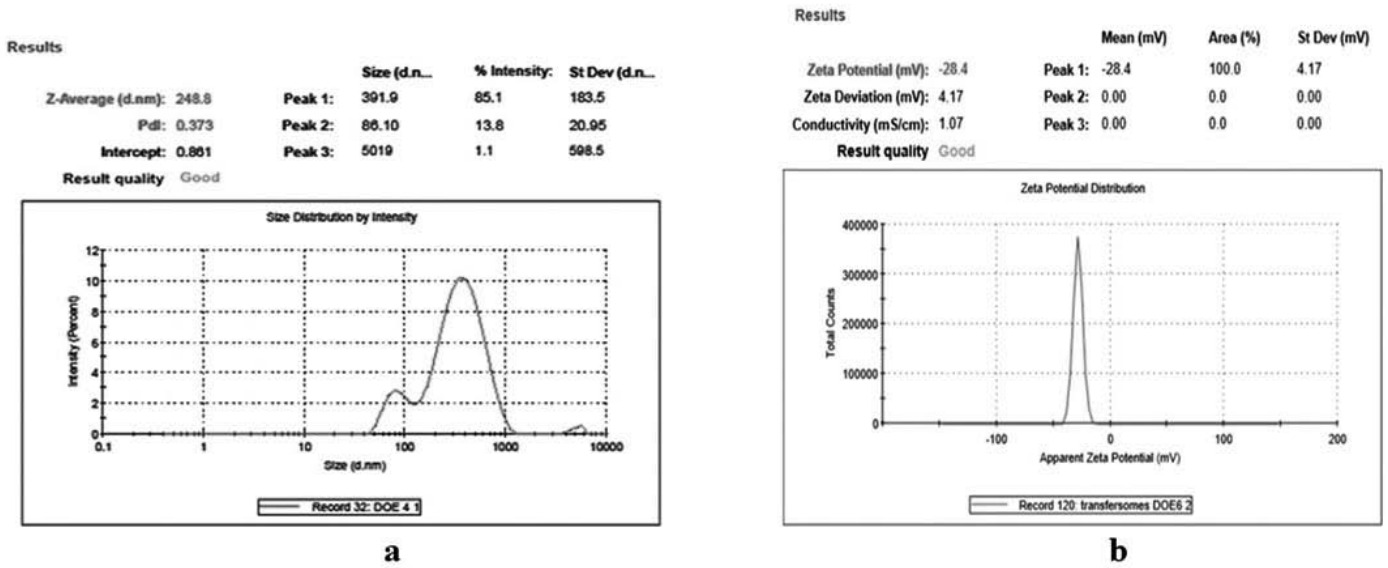

Figure 8: Optimized niosomal formulation a) Vesicle size b) Zeta potential.

Table 6: Result of pH, spreadability, drug content and viscosity of gels.

Form. code	Measurement of pH	Spreadability (g/cm^2)	% Drug content	Viscosity (cps)
NGL	6.4	15.26	85.61±1.63	4968.4 ± 12.61
CGL	6.2	14.94	87.56±1.34	4297.7 ± 10.92

Table 7: The permeated amount of extract at 480 min, flux, and permeability coefficient.

Form. Code	Permeated amount at 480 min (µg/cm^2)	Flux (µg/cm^2).min	Permeability constant (K_p) × 10^{-3} (cm/hr)
Conventional gel	2800 ±6.23	5.283	0.5283
Niosomal gel	4200±7.95	8.119	0.8119

Table 8: Effect of gels on carrageenan-induced rat paw edema.

Sl. No	Treatment Group	Mean Volume of Edema Induced By Carrageenan (Ml)			
		30 min	60 min	120 min	180 min
1	Control	0.36 ± 0.05	0.47± 0.09	0.52± 0.06	0.57± 0.10
2	1% Diclofenac Gel	0.25 ± 0.02	0.20 ± 0.03	0.18 ± 0.04	0.16 ± 0.03
3	Niosomal Gel (1%)	0.31± 0.06	0.27± 0.04	021± 0.06	0.20 ± 0.05

(Mean±SD, *n*=3)

Scanning electron microscopy

The vesicle shape and the surface morphology of the formulations was determined by Scanning electron microscopy (SEM) as shown in Figure 9. The vesicles observed were spherical and uniform. The surface of the vesicles was found to be smooth and also the SEM analysis also supported that the vesicle size was less than 500 nm.

Formulation and characterization of Niosomal gel

The niosomal (NGL) and conventional (CGL) gels that were created were uniform, off-white in colour, smooth, and gritty-free. The findings of measuring the pH, spreadability, medication content, and viscosity of both gel formulations are displayed in Table 6.

For topical formulations, pH evaluation is a crucial factor because if it differs from the pH of normal skin, it could irritate the skin. The pH of the niosomal and conventional gel was observed to be 6.4 and 6.2 respectively shown in Table 6, which are closer to the skin pH. The mechanical and physical characteristics of the preparation, such as its spreadability, consistency, and hardness, which in turn are connected to how easily the product can be removed from the container, are all influenced by viscosity, an

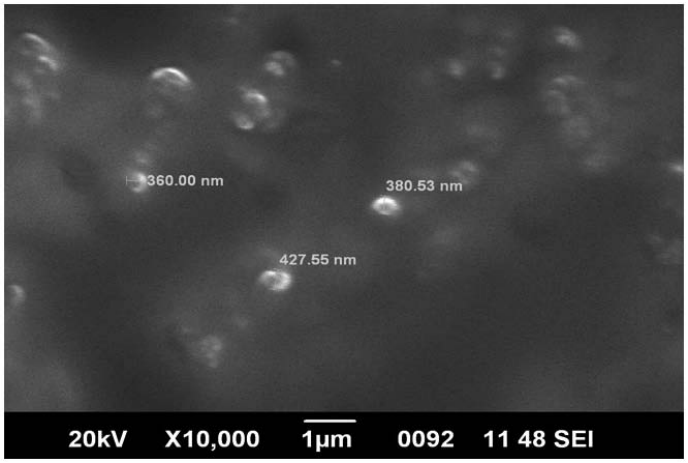

Figure 9: SEM image of optimized niosomal formulation.

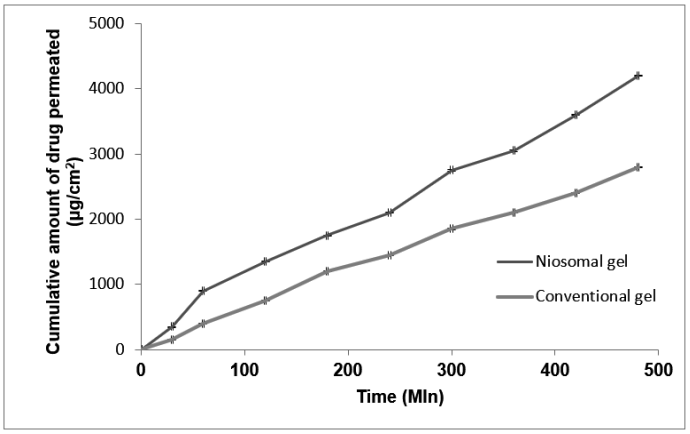

Figure 10: Comparative e*x vivo* drug permeability study of niosomal gels with conventional gel.

important rheological parameter. Both the gels showed required viscosity in order to ease the application of the gel. The drug content of the niosomal gel and conventional gel was found to be 85.61±1.63% and 87.56±1.34% respectively as shown in Table 6. It showed that the drug was uniformly distributed throughout the gel. Drug content uniformity is essential for semi-solid preparation to confirm the homogeneity of the dispersed drug throughout the formulation.

Ex-vivo drug permeation studies

An *ex vivo* permeation study was conducted to determine the amount of drug released through the goat skin. The permeation profile of total saponins present in an extract from niosomal and conventional gel formulation is shown in Figure 10. The total quantity of saponins delivered from niosomal gel was 4200 µg; this was far more than the amount supplied by conventional gel which was substantially higher, which was 2800 µg ($p< 0.0001$). The inclusion of surfactant, which aids in the solubilization of the lipid in the stratum corneum and permits high vesicle penetration, may have contributed to the improved permeation of the niosomal gel extract. Parameter of permeability, as shown in Table 7, in the case of niosomal gel the steady-state flux was

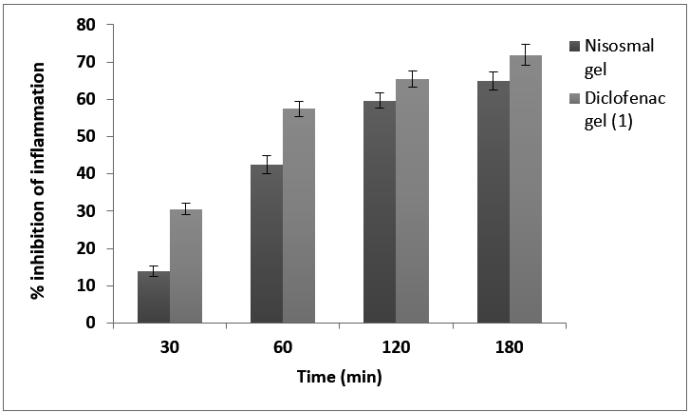

Figure 11: % inhibition of inflammation by niosomal gel (test) and diclofenac gel (Standard) on carrageenan-induced rat paw edema (*n*=3).

found to be greater than the conventional gel. The steady-state flux and permeability coefficient of niosomal gel was 8.119 µg/cm^2. min and 0.8119 cm/min respectively whereas conventional gel is shown to be 5.283 µg/cm^2.min and 0.5283 cm/min respectively after 480 min (8 hr). It was discovered that steady-state flow and permeability coefficient are directly related. Results indicated that the flux and permeability coefficient of niosomal gel was 1.55-fold higher than conventional gel.

Anti-inflammatory activity

The niosomal gel of *Asparagus racemosus* significantly reduced the inflammation brought on by the carrageenan-induced paw edema model. After 30 min of induction of edema niosomal gel caused a significant anti-inflammatory effect comparable to control group at the respective time. The extract eventually achieved a maximum inhibitory effect as time went on. 1% Diclofenac gel was used as a standard drug and it produced a significant reduction of paw edema compared to that of the control group as shown in Table 8 and Figure 11. As previously discovered in research, the niosomal gel present in *Asparagus racemosus* extract displayed an anti-inflammatory activity that may have resulted from the inhibition of the production of inflammatory mediators such as histamine, serotonin, cytokines, and prostaglandins.[27]

CONCLUSION

From the results, it concluded that the 3^2 factorial design was capable of obtaining an optimized niosomal formula, with small vesicle size and high EE. The gel-containing niosomes performed through goat skin showed increased effectiveness in penetrating the drug through the skin. The animal model proved that the niosomal gel loaded with an extract containing total saponin showed a significant anti-inflammatory effect.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

SEM: Scanning electron microscopy; **µg:** Microgram; **FT-IR:** Fourier transform infrared spectroscopy; **PDI:** Polydispersity index.

SUMMARY

Asparagus racemosus extract contains various types of saponins and flavonoids. The phytoconstituents of the extract have less permeability through the skin; so to enhance their permeability and effectiveness it was loaded in nisomes. The main objectives of the study were to design, formulate and characterize the niosomal gel loaded with *Asparagus racemosus* extract for anti-inflammatory activity. The niosomes containing total saponins in the extract were prepared by using 3^2 factorial designs using the thin film hydration method. The span 60 and cholesterol were selected as the independent variable. Vesicle size, PDI, zeta potential, and percentage entrapment efficiency were considered for responses. The prepared gel was evaluated for *ex vivo* studies on goat skin using a modified version Franz diffusion cell. When compared to conventional gel, the niosomal gel provided significantly more steady-state flux as well as permeability coefficient into the skin. The anti-inflammatory study proved that niosomal gel loaded with total saponins in extract showed significant anti-inflammatory compared to the control.

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Ind. J. Pharm. Edu. Res, 2023; 57(1s):s75-s84.
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Original Article

Formulation and Evaluation of Castor Oil Containing Self-emulsifying Pellets by Using Design of Experiment

Shubhangi V Shekade[1,*], Sanjeevani S Deshkar[2], Satish V Shirolkar[3]

Department of Pharmaceutics, Dr. D.Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, Maharashtra, INDIA.

ABSTRACT

Background/Objectives: The objective of the study was to examine the feasibility of extrusion/spheronization techniques for formulation of castor oil loaded self-emulsifying pellets. The study was also aimed at optimization of the self-emulsifying pellet system with design of experiments. **Materials and Methods:** Various surfactants and co-surfactants were tested for their ability to emulsify castor oil. The proportion of components (castor oil, surfactant and co-surfactant) in self-emulsifying pellets was optimized using a D-optimal mixture design. During optimization, the effect of composition of oil(X_1), surfactant(X_2) and cosurfactant(X_3) blend on responses like dispersion time (Y_1), resultant globule size (Y_2) and polydispersity index (Y_3) was studied. The developed self-emulsifying mixture was further loaded on pellet formulation using extrusion spheronization technique. **Results:** The application of D-optimal mixture design resulted in 16 batches, out of which the optimized batch consists of castor oil (30.92%), Cremophore EL (58.56%) and PEG 400(10.51%) having dispersion time of 53 sec, globule size of 242 nm and polydispersity index of 0.243 with desirability value of 1. All the formulation components affected the performance of pellets significantly. Free flowing pellets were obtained with 30% of oil loading. **Conclusion:** The self-emulsifying pellets of castor oil were successfully optimized with mixture design. The developed system containing castor oil can be used for orally for conditions like arthritis or can also serve as a carrier system for any drug or herbal active.

Keywords: Solid self-emulsifying drug delivery system, Castor oil, Self-emulsifying pellets, Extrusion spheronization, Dispersion time.

Correspondence:
Ms. Shubhangi V Shekade
PhD Scholar, Department of Pharmaceutics, Dr. D.Y Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune-411018, Maharashtra, INDIA.
Email id: shubhangishekade@gmail.com

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INTRODUCTION

Self-emulsifying drug delivery system (SEDDS) or self-micro emulsifying drug delivery system (SMEDDS) is defined as an isotropic combination of oil, surfactant, co surfactant and lipophilic drug. In the GI tract, when diluted with aqueous medium and gently agitated, they form fine oil in water emulsions or microemulsions.[1] They enhance the interfacial area and distribution of drugs in the GI tract.[2,3] SEDDS typically produce opaque emulsion with globule size range between 100 and 300 nm,[4] whereas the globule size produced by SMEDDS is below 100nm. Microemulsions form spontaneously and deliver the drug in a solubilized form. Furthermore, the drug is rapidly dissolved and absorbed across the intestinal membrane due to its large surface area and rapid dissolution.[1]

Since fine oil droplets of emulsion can pass immediately from the stomach and distribute uniformly throughout the GI tract, so they minimize irritation typically experienced during prolonged contact with bulk drugs. Compared to simple oily solutions, SEDDS provide a large surface area between oil and water, which promotes partitioning of the drug between the two phases. These systems may therefore provide more reproducible plasma concentration profiles and improvement in absorption rates for lipophilic drugs whose oral absorption is dissolution limited. SEDDS formulations have also attracted attention for improving the bioavailability of compounds classified as Class II under the Biopharmaceutical Classification System (BCS).[5] The self-emulsification ability of the formulation is determined by various formulation parameters including, concentration of surfactant in mixture, relative proportion of oil to surfactant, polarity of both oil the phases in emulsion, resulting globule size, and charge. Self-emulsification ability in turn determines the efficiency of formulation regarding oral absorption.

For oral administration, generally SEDDS are prepared in the liquid state and then filled in either soft or hard gelatin capsules. In terms of production costs, drug stability, compatibility with capsule shells, leakage, and precipitation, these liquid dosage forms often have some drawbacks.[6] The creation of several solid self-emulsifying (SE) dosage forms, such as self-emulsifying tablets and pellets, have been investigated in order to address

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these problems, including the desirable inclusion of liquid SEDDS into solid dosage form.[7,8]

In pharmaceutical industries, formulation scientists are very interested in pellets due to their advantages over traditional solid dose forms. These benefits include improved bioactive safety and efficacy as well as versatility in developing and optimizing the dosage form. It is the fact that pellets disperse/distribute readily and equally throughout the gastrointestinal tract. As a result, there is an increase in drug absorption, which lowers the fluctuations in plasma levels and reduces the incidence of side effects. Because of this, drug absorption is increased; resulting in decrease in peak plasma fluctuations reducing potential side effects. Pellets, on the other hand, diminish intra- and inter-subject variability of plasma profiles, which increases the safety and effectiveness of the medication. They also reduce differences in gastric emptying rates and overall transit duration.[9] Additionally, taking pellets prevents the dose dumping effect, which is a major factor in high local drug concentrations, as well as discomfort brought on by some active ingredients.[10]

The most widely used techniques for manufacturing pellets in the pharmaceutical industry are extrusion/spheronization, solution-suspension layering, and powder layering. Recent years have seen the preparation of pellets containing self-emulsifying mixture with microcrystalline cellulose and lactose through extrusion/spheronization or wet granulation in high-shear mixers.[11,12] Controlling the *in vitro* release of drugs from such pellets could be achieved by film coating of the pellets with an appropriate polymer.[13] Research on the development of solid self-emulsifying systems is expanding, but there are very few publications on the topic.[11,14,15] Compared to other approaches, the extrusion/spheronization technology has significant technological advantages. Extrusion/spheronization technique results in spherical, uniformly packed pellets with a narrower size distribution, good flow characteristics, and minimal friability.[16] Extrusion/spheronization technique is the preferred way for creating pellet-based dosage forms because of these benefits, and this technology can be successfully used to combine the benefits of SEDDS with pellets. High lipid loads usually make it challenging for formation of self-emulsifying pellets.

The effective development of an ideal SEDDS formulation depends on the selection of the constituents, including oil, surfactant, and cosurfactant, and a proportion of these constituents that is well-balanced. The experimental designs have been extensively utilized for developing effective formulations.[17] Based on conventional one-factor-at-a-time methods, the best proportions of SEDDS components have been determined. But these techniques are not only labor-intensive, time-consuming, and ineffective; they frequently also give insufficient information to examine the impact of each component and their potential correlations.[18,19] Statistical optimization tools based on experimental designs including, factorial, Box-Behnken, central composite and mixture designs have been widely used to evaluate the effects of individual variable and their interactive effect on a product or process.[20-22] The D-optimal mixture design is one of the preferred response surface methodologies for optimizing the relative proportion of components within a mixture as it reduces the variance associated with the evaluation of model coefficients and produces the effectively subset by accounting for the criteria for enhancing information matrix determinants.[1,23] Additionally, D-optimal mixture design considers the individual and interactive effect of proportion of components within a mixture keeping fixed weight of total mixture.[1]

The objective of this study was to formulate and optimize the castor oil containing self-emulsifying pellets. The relative proportion of oil, surfactant, and cosurfactant in a self-emulsifying pellet formulation was optimized using D-Optimal Mixture Design. The resulting batches were evaluated for dispersion time, globule size and polydispersity index and design space was obtained to understand the range of critical variables.

MATERIALS AND METHODS

Materials

Castor oil was procured from Research Lab Fine Chem industries, Mumbai. Cremophore EL was received from BASF (Ludwigshanfen, Germany). Lactose, starch and HPMC (E50 LV) was provided from Loba Chemie Pvt. Ltd., Mumbai, India. All other chemicals used were of analytical grade.

Pre-formulation studies

Selection of surfactants

Selection of surfactant was done based on its ability to emulsify the castor oil. Castor oil was added dropwise to 15% aqueous solution of tween 80, tween 20 and Cremophor EL by vortexing until turbidity was obtained. The amount of oil required to make the surfactant solution cloudy was compared and the surfactant that can emulsify maximum concentration of castor oil was selected.[24]

Screening of co-surfactant

For the selection of co-surfactant, a method developed in our laboratory was used. Briefly, each co-surfactant (Propylene glycol and polyethylene glycol 400) was mixed with selected surfactant in the ratio of 1:1, 1:2 and 2:1 (Smix). This Smix was blended with oil in ratio 9:1 (Smix: oil). The mixture (0.5 g) was then diluted by 20 ml distilled water using vortexing.Globule size of resulting dispersion was then recorded. The co-surfactant showing lowest globule size (less than 100 nm) was selected for further study.

Table 1: Composition of solid self-emulsifying pellet.

Name of ingredient	Amount (%w/w)	Role
Liquid SEDDS	30	Self-emulsification system
Corn starch	5	Disintegrating agent
Microcrystalline cellulose	37.33	Pelletization aid
Lactose	18.67	Diluent
Hydroxy propyl methyl cellulose	5	Binder
Isopropyl alcohol	q.s.	Solvent

Preparation of castor oil containing self-emulsifying pellet

Preparation of Self Emulsifying Pellets by Extrusion/ Spheronization

Considering the emulsification ability, Castor oil, Cremophore EL, and PEG 400 were selected for preparation of liquid SEDDS. The preparation of liquid SEDDS involved mixing of castor oil (Oil), Cremophore EL (Surfactant) and PEG 400 (Co-surfactant) with magnetic stirring until formation of isotropic mixture. The pellets were prepared by the extrusion spheronization technique. The pellets were formed by mixing fixed proportion of liquid SMEDDS (30%) with other excipients (70%) which were mixture of cornstarch, lactose and Microcrystalline cellulose. The lactose, cornstarch and microcrystalline cellulose were mixed in a mortar and the liquid SMEDDS formulation was adsorbed on this powder blend. Hydroxy Propyl Methyl Cellulose in isopropyl alcohol was used as binder solution for formation of wet mass. The wet mass was passed through sieve no. #16 to obtain extrudates which were spheronized using a cross hatch geometry frictional plate (2mm size) in Shakti Lab Spheronizer (India) at rotation speed of 850 rpm for 5 min.[6] The moist pellets were dried in vacuum oven (Being, DZF-6032) at 50°C for 3 hr. The composition is shown in Table 1.

Experimental design for optimizing castor oil containing self-emulsifying pellets

In a classical mixture design, random selection of levels is not done since it helps to optimize the relative proportion of constituents from all the possible proportion of component within the constraint. The total of all the component in a mixture is one. A computer-aided design method known as D-optimal design increases the definite information matrix while reducing the generalized variance. The levels of constraints of component in mixture were chosen through preliminary tests, which evaluated the mixtures capacity to construct self-emulsifying systems.

For the optimization of composition of Self-emulsifying pellet formulation D-optimal mixture design was used. Three different components were used as independent variables in this design. The concentrations of different component of formulation were set within range viz, 30%–70%, of Castor oil (oil; X_1), 10%–60% of Cremophore EL (surfactant; X_2), and 10%–60% of PEG 400 (cosurfactant; X_3) respectively. In every experiment, the concentrations of these three components added up to 100%. The optimization was carried out based on the responses viz., Dispersion time (Y_1), Globule size (Y_2) and polydispersity index (Y_3). To develop and evaluate experimental run, Design-Expert Software version 10 (Stat-Ease Inc, Minneapolis, MN, USA) was used. Sixteen experimental runs were suggested by design, which included factorial points (high and low level from the constraints on each factor), centers of edges, and an overall center point. Mathematical models were obtained after data processing by application of ANOVA.[1]

Characterization of castor oil containing self-emulsifying pellets

Micromeritic properties

According to USP, standard procedure, different micromeritic characteristics such as bulk density, tapped density, Carr's index, Hausner ratio, and angle of repose of pellet were calculated.[25]

Dispersion time

The time required to disperse 0.5 g pellets in 100 ml HCl at 37 ± 0.5°C to form a solution using magnetic stirrer was noted as dispersion time. The resulting emulsion was further evaluated for globule size and polydispersity index.

Emulsion globule size analysis and polydispersity index

Analysis of globule size and polydispersity of nanoemulsion was performed using particle size analyzer (Horiba, SZ 100, Japan) based on dynamic light scattering. The sample was placed in cuvette and light scattering was observed at a 90° angle at 25°C temperature. The mean globule size and size distribution was obtained along with polydispersity index.

Field Emission scanning electron Microscopy

The morphology of solid pellets for the optimized castor oil containing SEDDS formulation was observed using a field emission scanning electron microscope (Nova NanoSEM NPEP303, Germany) with 10 kV acceleration voltage.

RESULTS AND DISCUSSION

Selection of surfactant

Surfactants play a major role in performance of SEDDS. The formulation should immediately disperse upon dilution by gastrointestinal fluid just by gentle mixing. Considering the non-toxicity of nonionic surfactants and their GRAS status (generally recognized as safe), Cremophore EL, Tween 20 and

Tween 80 were screened. Castor oil was soluble in Cremophore EL at 231±0.83 mg/mL, in Tween 20 at 189±0.650 mg/mL and in Tween 80 at 14.6±0.650 mg/mL as shown in (Figure 1). The results demonstrated greater emulsification ability of Cremophore EL compared to Tween 20 and Tween 80 for the castor oil and hence was selected for the formulation.

Selection of cosurfactant

Selection of cosurfactant was based on its ability to yield a lower globule size. From (Figure 2) it is evident that propylene glycol as cosurfactant in different ratio with surfactant resulted in globule size of 830nm (1:1), 917 nm (1:2) and 1013 nm (2:1) respectively. Polyethylene glycol 400 resulted in globule size 85.8nm (1:1), 356.2 nm (1:2) and 361.9 nm (2:1) respectively. Considering the lower globule size with PEG 400 at 1:1 ratio (S: CoS), it was selected as co-surfactant over polyethylene glycol.

Optimization by D-optimal mixture design

Table 2 indicates various independent variables *viz.* Castor oil (X_1), Cremophore EL (X_2), and PEG 400 (X_3) selected for study. To evaluate performance of self-emulsifying pellets, various responses such as the dispersion time (Y_1), globule size (Y_2) and polydispersity index (Y_3) were selected. Table 3 indicates result of 16 batches from design wherein, the dispersion time (Y_1) ranged from 14 sec to 122 sec, globule size (Y_2) ranged from 94.06 nm-839.73 nm and polydispersity index (Y_3) ranged from 0.2323 to 1.5826. All the responses were fitted to suggested mathematical models for linear regression analysis. Various statistical parameters, including the squared correlation coefficient (R^2), adjusted R^2 values, sequential *p*-value, and lack of fit *p*-value, suggested that the linear model was the most appropriate mathematical model for the dependent variables Y_1 and Y_2, while a cubic model was recommended for the response Y_3. Nearby values of R^2 and adjusted R^2 (difference less than 0.2) suggested good model fit. For Y_1, Y_2, and Y_3, the sequential *p*-values were 0.0594, 0.0351, and 0.0300, respectively. The model terms are considered significant when the sequential *p*-value is less than 0.05. An adequate model fit is indicated by a lack of fit *p*-value >0.1. The responses Y_1, Y_2, and Y_3 had a lack of fit *P*-value was 0.0011, 0.5269, and 0.8340, respectively. In this experiment for dispersion time (Y_1) a sequential *p*-value was greater than 0.05 and lack of fit value was less than 0.1 indicating model terms were not significant. For globule size (Y_2) and polydispersity index (Y_3) model terms were significant. Additionally, the suggested model's suitability was verified and resulted in normal plots of residuals (Figures 3A, 3B and 3C).

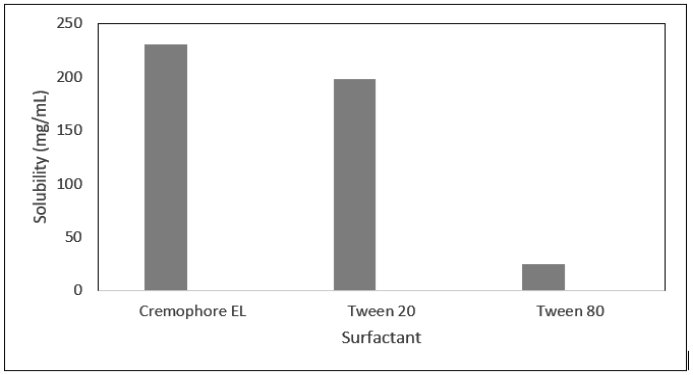

Figure 1: Solubility of castor oil in various surfactant.

Influence of independent variables on dispersion time

Equation 1 was obtained from multiple regression analysis and indicates the relationship between proportions of components and dispersion time (Y_1). Table 4 shows the findings of the analysis of variance for dispersion time (Y_1).

$$\textbf{Dispersion Time } (Y_1) = +23.19*X_1+53.46*X_2+84.24*X_3 \text{ (Eq. 1)}$$

As shown in (Figure 4 A Y_1; left panel) a 3D response surface plot explained the effect of each component and its mixture on dispersion time extrapolated using the plot. As a *p*-value for X_1 and X_2 component was greater than 0.1, it indicated non-significance of effect on dispersion time whereas for X_3, *p*-value less than 0.05 indicated significant increase in dispersion with increase in co surfactant concentration.

The two component mixture graphs in (Figure 4 B; left panel) illustrate the results of altering the ratio of X_1 and X_2 with a constant amount of X_3. With an increase in X_1 (oil) and a reduction in X_2, the dispersion time decreased (surfactant).

A value of desirability 0 denotes an undesired response value, while 1 denotes the response value that is desirable as shown in (Figure 5 A).

Table 2: Variable used in D-optimal Mixture design.

Component	Range (%)	
	Minimum	Maximum
Castor oil	30	70
Cremophore EL	10	60
PEG 400	10	60

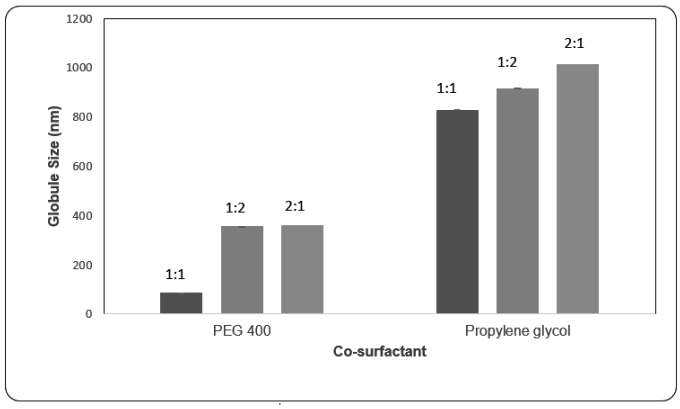

Figure 2: Selection of different co-surfactant on the basis of globule size.

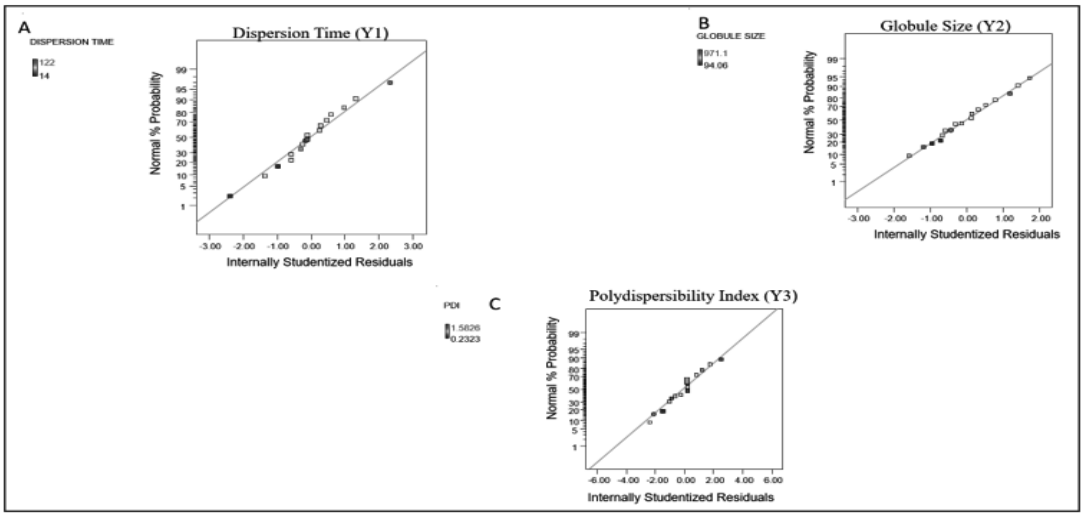

Figure 3: Adequacy of the model for checking the normality of residuals and outliers of the responses dispersion time (A), globule size (B) and polydispersity index (C).

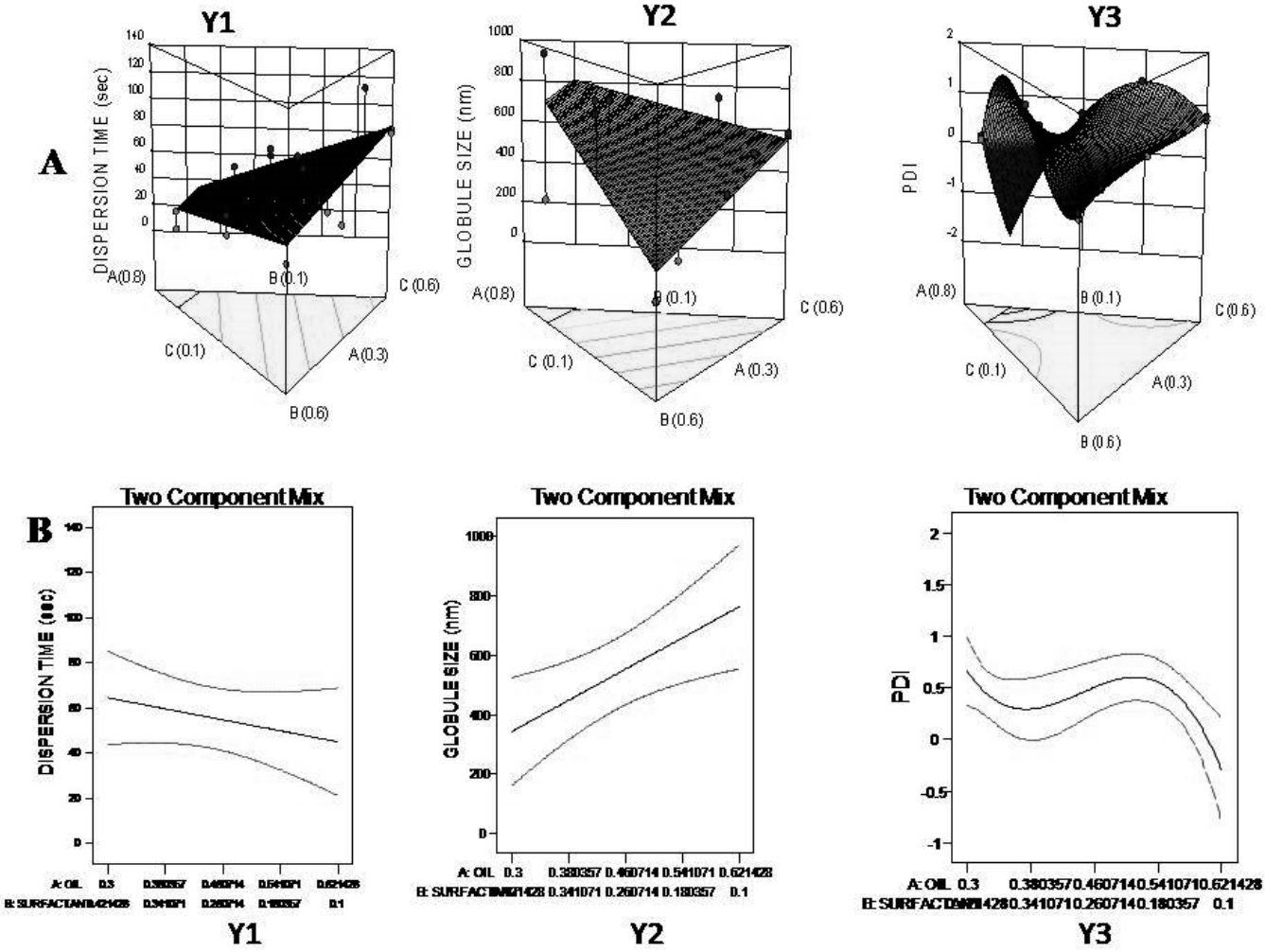

Figure 4: Effect of SEDDS component on response Y1, Y2 and Y3.

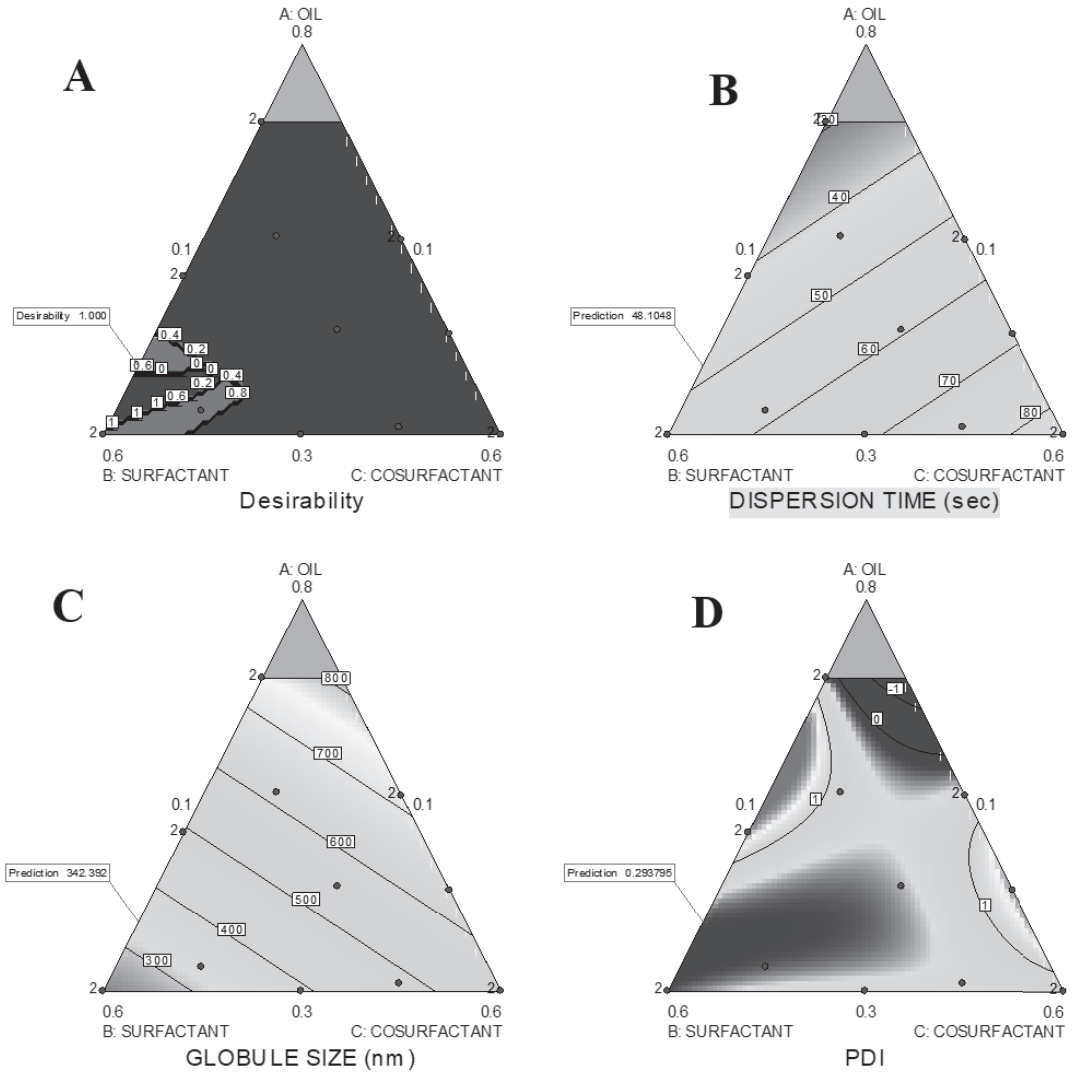

Figure 5: Contour plots for the effect of oil, surfactant and co surfactant on responses. (A-desirability; B-Dispersion time (sec); C-globule size (nm) and D-polydispersity index).

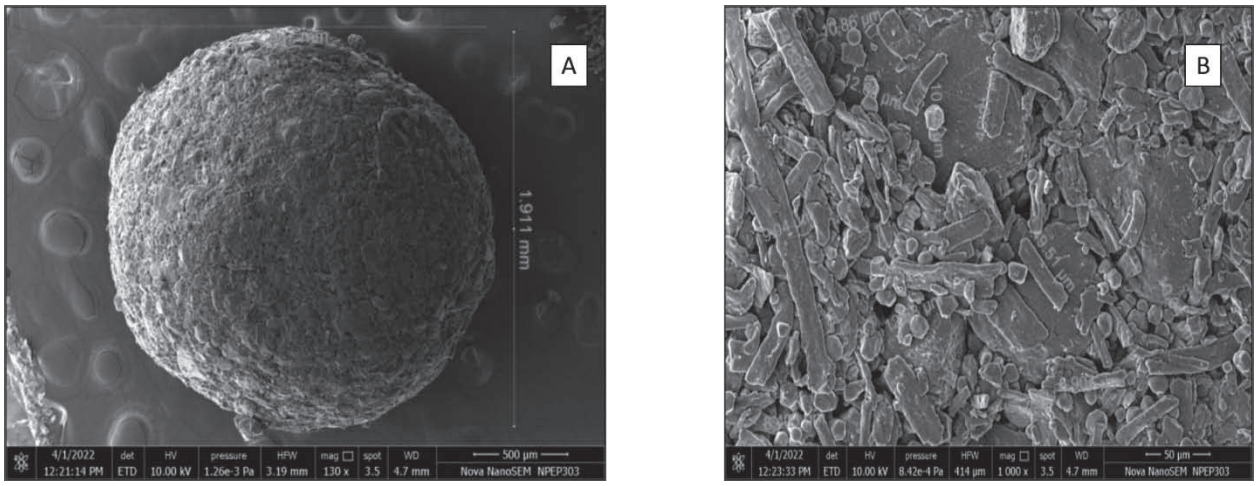

Figure 6: FE-SEM micrographs of the surfaces and cross-section of the castor oil self-emulsifying pellet formulation. (A) (130x) and (B) (1000x).

Table 3: Experimental Matrix for the D-Optimal Design and Results.

Run	Variable			Results		
	Castor oil (%;X1)	Cremophore EL (%;X2)	PEG 400 (%;X3)	Dispersion Time (Sec;Y1)	Globule size (nm;Y2)	Polydispersity index (Y3)
1	42.89	10	47.11	18.9±0.1	745.5±3.2	1.405±0.23
2	30	10	60	79.1±3.1	582.9±4.6	0.782±0.07
3	43.40	23.79	32.79	74.2±2.5	426.3±2.8	0.514±0.13
4	30.97	22.29	46.73	122.1±8.3	544.9±3.8	0.671±0.08
5	70	20	10	14.2±0.1	971.3±9.3	0.447±0.02
6	30	10	60	82.4±0.3	570.1±4.2	0.717±0.18
7	33.05	46.09	20.84	88.2±1.2	180.3±2.3	0.341±0.17
8	54.95	10	35.04	60.1±2.6	582.5±2.9	0.648±0.09
9	30	35.09	34.90	38.2±0.7	418.0±2.7	0.661±0.26
10	70	20	10	27.1±2.1	275.2±6.3	0.380±0.46
11	50.27	39.72	10	34.2±0.2	839.7±8.9	1.582±0.19
12	55.38	25.46	19.15	67.1±1.4	781.1±3.5	0.852±0.45
13	30	60	10	41.3±0.3	94.1±0.9	0.232±0.07
14	30	60	10	41.2±0.8	98.6±1.3	0.353±0.39
15	50.27	39.72	10	48.4±0.2	780.3±2.3	1.213±1.23
16	54.95	10	35.04	65.5±2.3	598.6±4.2	0.726±0.60

Table 4: Analysis of variance for model of the measured responses.

Source	Y1(Dispersion time)			Y2 (Globule size)			Y3 (Polydispersity index)		
	SS	F	*p*-value	SS	F	*p*-value	SS	F	*p*-value
Model	4354.06	3.48	0.00435	4.327	4.47	0.033	2.15	17.13	0.0013
X_1	-	-	0.0649	-	-	0.0287	-	-	-
X_2	-	-	0.8512	-	-	0.0145	-	-	-
X_3	-	-	0.0275	-	-	0.8170	-	-	-
X_1X_2	-	-	-	-	-	-	0.14	33.44	0.0012
X_1X_3,	-	-	-	-	-	-	0.47	22.25	0.0033
X_2X_3	-	-	-	-	-	-	0.31	1.55	0.2593
$X_1X_2X_3$	-	-	-	-	-	-	0.022	24.72	0.0025
X_1X_2 (X_1 - X_2),	-	-	-	-	-	-	0.35	11.14	0.0157
$X_1X_3(X_1-X_3)$-	-	-	-	-	-	-	0.16	0.16	0.7073
$X_2X_3(X_2-X_3)$	-	-	-	-	-	-	2.167	0.68	0.4424
Residual	68.83	-	-	6.293	-	-	9.443	-	-
Lack of Fit	7944.19	24.89	0.132	3.850	0.99	0.5322	0.084	0.049	0.8340

Notes: X_1- oil; X_2-surfactant; X_3-cosurfactant.
Abbreviations: SS, sum of squares.Effect of independent variable on globule size

In (Figure 5B) shows the contour plot for the dispersion time which indicates lower dispersion time (quick dispersion) at higher concentration of oil, lower concentration of co surfactant and medium concentration of surfactant within a mixture.

Notes: (A) Three-dimensional response surface plot for the effect of the component. (B)Two-component mixture plot for the effect of varying ratio of two components with a fixed amount of the other component.

Table 5 shows the findings of the analysis of variance for globule size (Y_2). Globule size (Y_2) was affected by each component of self-emulsifying pellets formulation. Three-dimensional response surface plots show the relationship between the independent variables schematically (Figure 4 A Y2; middle panel). These

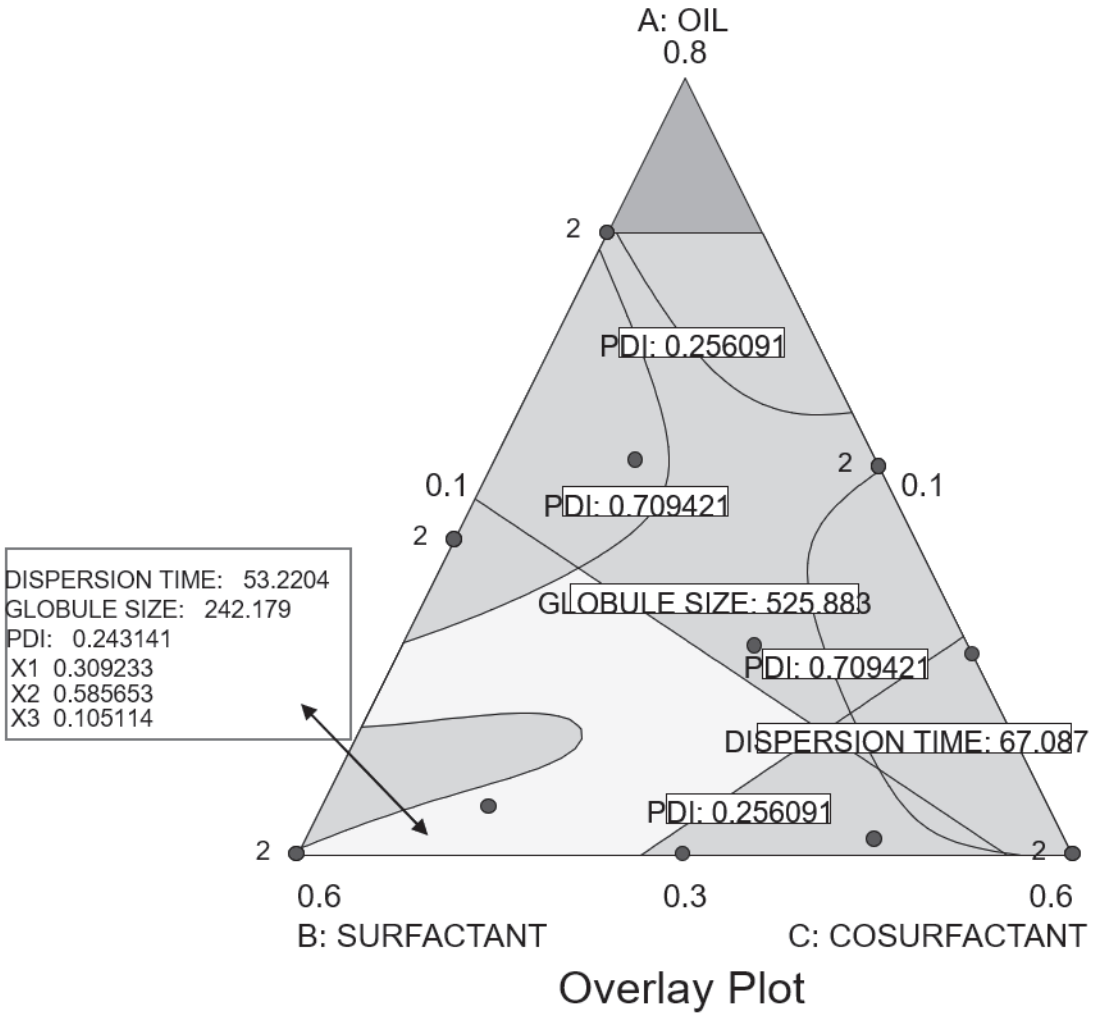

Figure 7: Overlay plot of the optimized castor oil containing self-emulsifying pellets.

findings led to the development of the following linear equation for the globule size: (Y_2).

$$\textbf{Globule Size } (Y_2) = +879.73.4*X_1+226.78*X_2+553.99*X_3 \text{ (Eq.2)}$$

Equation 2 demonstrated a significant effect of all the components of mixture on globule size (Y_2) as evident from *p* value (Table 4). From the contour plot (Figure 5C) and two-component mixture plot (Figure 2B; middle panel) it is evident that showed that the globule size decreased with increase in surfactant concentration and with decrease in oil and co surfactant concentration.

Effect of independent variable on polydispersity index

Table 4 shows analysis of variance for polydispersity index (Y_3). For polydispersityindex, cubic model was found to be the best fit. Three-dimensional response surface plots (Figure 4 A Y_3; right panel) shows the relationship between independent variables. In order to verify the relationship between the independent variables and Y_3, following equation was created on the basis of the analysis of variance.

$$\text{Polydispersity Index } (Y_3) = 4.31*X_1+0.29*X_2+0.75*X_3+15.26*X_1*X_2+10.00*X_1*X_3+0.61*X_2*X_3 33.65*X_1*X_2*X_3+14.83*X_1*X_2*(X_1-X_2)-0.65*X_1*X_3*(X_1-X_3)+0.93*X_2*X_3*(X_2-X_3) \text{ (Eq. 3)}$$

p-value (Prob>F) less than 0.05 indicate significance of model and individual model terms (Table 4). Two component mixture plot (Figure 4B, right panel) and counter plot (Figure 3 D) demonstrated lower polydispersity index value at higher surfactant and oil concentration and lower co surfactant concentration in a mixture.

Characterization of castor oil containing self-emulsifying pellet

Micromeritic Properties of the Pellets

Table 5 indicates micromeritic properties of pellet formulations. The Hausner ratio and compressibility index showed that all

Table 5: Values of micromeritics properties of castor oil containing self-emulsifying pellets.

Run	Bulk Density (g/ml)	Tapped Density (g/ml)	Carr's Index (%)	Housner Ratio	Angle of Repose (°)
1	0.65±0.01	0.714±0.03	12.46	1.14	26.01
2	0.63±0.02	0.71±0.02	10	1.11	27.31
3	0.56±0.07	0.618±0.02	9.38	1.10	28.32
4	0.55±0.03	0.666±0.01	16.66	1.23	28.27
5	0.58±0.01	0.612±0.02	4.91	1.05	26.45
6	0.62±0.03	0.714±0.04	13.16	1.15	30.65
7	0.58±0.02	0.714±0.03	17.64	1.21	28.31
8	0.52±0.01	0.625±0.02	15.84	1.18	31.12
9	0.58±0.06	0.666±0.02	11.81	1.83	28.13
10	0.51±0.01	0.55±0.01	7.27	1.07	26.31
11	0.67±0.03	0.732±0.02	8.46	1.09	25.23
12	0.62± 0.09	0.769±0.01	12.75	1.23	30.89
13	0.62± 0.03	0.714±0.02	12.46	1.14	26.23
14	0.53±0.06	0.632±0.01	13.52	1.36	28.36
15	0.58±0.01	0.666±0.03	11.81	1.13	28.23
16	0.58±0.2	0.714±0.02	17.64	1.21	29.13

Table 6: Experimental and predicted values for the optimized self-emulsifying drug delivery system.

Response	Experimental Value	Predicted Value	Prediction error (%)
Dispersion time (Sec;Y_1)	51.1±1.96	53.2	-4.35
Globule size (nm;Y_2)	263.2±1.99	242.1	7.92
Polydispersity index (Y_3)	0.251±1.67	0.243	3.18

pellet formulations had good flow properties and compressibility characteristics.

The Field emission scanning electron microscopy

The Field emission scanning electron microscopy analysis for castor oil containing pellets is shown in (Figure 6). The pellets were spherical in shape with rough surface.

Selection of optimized self-emulsifying pellet formulation using desirability function

The criteria of selection of optimized formulation, minimum dispersion time, less globule size and less polydispersity index. The optimization was carried out for Y_1, Y_2, and Y_3 after obtaining the polynomial equations showing the relationship between the responses and independent variables. The overlay plot for the effect of various variables on the three responses is shown in (Figure 7). Overlay plot revealed design space indicating working concentration of independent variables to obtain desired design response. The optimized batch was selected from design space with concentration of 30.92% of X_1, 58.56% of X_2, and 10.51% of X_3 respectively, with the desirability value of 1. The validation of the model was performed by formulating the optimized batch and comparing experimental results with predicted values. To evaluate accuracy and reliability, prediction error values were calculated from predicted and experimental values. The prediction error of less than 10% confirmed model validation (Table 6). Thus, this result demonstrated the accuracy and reliability of the D-optimal mixture design approach, which was used to optimize the castor oil containing self-emulsifying pellet.

CONCLUSION

The D-optimal mixture design was successfully used to develop and optimize castor oil containing self-emulsifying pellet formulation. The optimized castor oil containing SEDDS included 30.92% castor oil (oil; X_1), 58.56% cremophore EL (surfactant; X_2) and 10.51% PEG 400 (co-surfactant; X_3). Model prediction and experimental value showed good agreement with various responses such as, dispersion time (Y_1), globule size (Y_2) and Polydispersity index (Y_3). The optimized formulation resulted in low dispersion time (53 sec), globule size (242 nm) and polydispersity index (0.243). The validation of model confirmed the robustness of formulation to achieve desired result. Extrusion spheronization process was effectively utilized to load up to 30% of self-emulsifying formulation on pellets. Thus, this study provides the basis for further studies wherein the developed system can effectively utilized for delivery of actives.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PEG 400: Polyethylene glycol 400.

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Original Article

Terbinafine HCl Film-Forming Spray for the Treatment of Topical Fungal Infections

Nidamanuri Bala Sai Soujith, Natarajan Jawahar*

Department of Pharmaceutics, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Ooty, Tamil Nadu, INDIA.

ABSTRACT

Terbinafine HCl is an allylamine used to treat fungal infections. It has substantial side effects that can be mitigated by using a topical semisolid dose form. The objective of this study was to develop a 1% Terbinafine HCl film-forming spray formulation to treat topical fungal infections. The formulation was developed by combining polymers, penetration enhancer, plasticizer, and a suitable solvent system. The central composite design with 3 independent variables and 2 dependent variables is implemented to optimize the formulation. The film-forming spray was put through its tests to assess formulation and container-related parameters such as pH, spray angle, spray pattern, density, volume delivered for one actuation and evaporation time. From the study, it was observed that the concentration of ethyl cellulose and Eudragit RSPO has a greater influence on the viscosity of the spray solution, whereas the eutectic mixture has a greater influence on the drug permeation followed by the polymers. It also tested with various fungi, and it was found that formulation showed better fungicidal activity than the marketed product achieved by triple action. The stability studies have shown that the optimized formulation was stable with temperature of 25 ± 2°C and RH 60 ± 5(six months) on the pH, viscosity and visual appearance of the formulation. The study has concluded that the formulated film-forming spray formulation is highly efficient in treating topical and transdermal fungal infections when compared to the traditional dosage forms.

Keywords: Terbinafine HCl, Formulation, Fungal infection, Solid dosage forms, Ethyl cellulose.

Correspondence:
Dr. Jawahar Natarajan
Associate Professor, Department of Pharmaceutics, JSS College of Pharmacy, Ooty-643001, Tamil Nadu, INDIA.
Email id: jawahar.n@jssuni.edu.in
ORCID: 0000-0003-0737-1237

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INTRODUCTION

The skin is the most readily accessible organ of the body and acts as a barrier against the micro and macromolecules of the environment because of its low permeability to such substances. A normal individual's skin has a surface area of roughly $2m^2$ and gets about one-third of the entire blood running throughout the body.[1] Fungal infections are becoming more common at an alarming rate, posing a significant challenge to hospitals and doctors. Fungal infections involving the skin, nails, hair, and mucous membranes are all examples of topical fungal infections. Onychomycoses, or nail infections, are considered to account for about 33% of all fungal skin infections and 50% of all nail problems. Fungal infections of the toenails and feet can act as a reservoir for organisms that can spread to other parts of the body or to other people.[2] Direct contact with infected individuals, animals, soil, or fomites causes fungal transmission. Dermatophytes such as Trichophyton, Microspores and *Epidermophyton* are responsible for most topical fungal infections. Other fungi like Candida and Aspergillus are also known to cause various fungal infections.[3] The symptoms of topical fungal infections are usually minor and not life-threatening, but they can have a significant influence on the patient's quality of life. These infections can lead to systemic when untreated and should be concerned immediately with proper medication and treatment.

There are many formulations available for treating topical fungal infections, such as creams, powders, patches, ointments and gels. Patches have several drawbacks, including skin irritation caused by their occlusive qualities, which block sweat ducts, prevent water vapour loss from the skin surface, difficulties placing on curved surfaces, discomfort while peeling off, and a lack of aesthetic appeal. Moreover, patches have restricted size and shape that cannot fit the area of the application, which stands as the major drawback.[4] Semisolids, which have been important dosage forms for various diseases, had their own limitations. They do not maintain long-term touch with the skin and are quickly wiped away by the patient's clothing. As a result, chronic disorders such as athlete's foot, ringworm, and candidiasis require frequent application. Furthermore, they leave a sticky, oily remnant on the skin after application, resulting in poor patient compliance.[5] The film-forming spray (FFS) is a revolutionary technique that can replace traditional topical and transdermal formulations. It's

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Table 1: Factor Level for Central Composite Design.

Factors	Coded Levels	
Independent Variables	Low Level (-1)	High Level (+1)
A= Eudragit	5	15
B = Ethyl cellulose	2.5	7.5
C = Eutectic mixture	7.5	12.5
Dependent Variables	Goals	
Viscosity (cps)	Minimize	
Drug permeation (%)	Maximize	

a non-solid dosage form that forms a film *in situ* or after being applied to the skin or any other body surface. These systems contain the drug and film-forming excipients in a solvent that evaporates when comes in contact with the skin, leaving a film of excipients and the drug behind. The produced film can be a solid polymeric material that works as a matrix for drug release to the skin over time or a residual liquid film that is quickly absorbed in the stratum corneum.[1]

Terbinafine HCl, also called Lamisil, is a widely used drug that is proven to have broad-spectrum antifungal activity. Squalene epoxidase, also called squalene monooxygenase, is a fungal enzyme that converts the squalene to 2,3-oxydosqualene, a stage in ergosterol synthesis pathway. Terbinafine HCl inhibits the squalene epoxidase, thus preventing the conversion of squalene to 2,3-oxydosqualene, which in turn prevents the synthesis of the ergosterol that will reduce the fungal membrane integrity. As there is no membrane integrity is observed for the fungi, thus fungicidal activity can be achieved. Terbinafine HCl has demonstrated excellent fungicidal activity against the dermatophytes and variable activity against yeasts and non-dermatophyte moulds *in vitro*.[6,7] The objective of this study is to formulate, optimize and characterize Terbinafine HCl Film-forming spray formulation (FFS).

MATERIALS AND METHODS

Materials

Terbinafine Hydrochloride was purchased from BDL Pharmatech Pvt. Ltd., (Hyderabad, India), DL-Camphor, Ethyl cellulose, Polyethylene glycol 4000 (PEG 4000) were purchased from S.D.Fine Chemicals Ltd. (Mumbai, India), Menthol crystals was obtained from Micro Fine Chemicals (India), Eudragit RSPO was purchased from Evonik Industries Pvt Ltd., (Mumbai, India), Ethanol and ethyl acetate used were of analytical lab grade.

Selection of drugs by Docking studies

The protein squalene epoxidase of PDB ID 6C6N, plays a major role in synthesis of ergosterol in fungi. Inhibiting this enzyme induces fungicidal activity. Allylamine drugs were proven to be efficient inhibitors of the squalene epoxidase/ squalene monooxygenase. The drug selection among all the allylamines for the antifungal activity was done by the docking using Pyrx virtual screening tool. Depending on the binding energy, the drug among the allylamines was selected.

FTIR- Compatibility studies

The drug and excipients have been subjected to FTIR studies for conforming the compatibility between them.

Preparation of the formulation

The Terbinafine HCl Film-forming spray solution were prepared by a simple solvent dissolving method. To prepare the camphor-menthol eutectic mixture, equal quantities of the camphor and menthol were weighed and then kept in a bath sonicator for 10 min to liquify. In another beaker Ethanol and ethyl cellulose blend was taken to this drug (Terbinafine HCl) and plasticizer (Polyethylene glycol 4000) was added and stirred for 30 min on a magnetic stirrer at room temperature to get a clear solution. Then the liquified camphor-menthol eutectic mixture was added to the solvent mixture under stirring. After that, the polymers (Eudragit RSPO, Ethylcellulose) were weighed and added to the above mixture. Then, this was kept under mechanical stirring for 20 min to get dispersed homogeneously. The prepared solution was kept in a bath sonicator to disperse if there are any polymer aggregates. This final solution was transferred into a refillable suitable spray container.

Experimental Design

A response surface technique based on a Three-factor; three-level Central Composite Design (CCD) was used to optimize the formulation parameters of Terbinafine HCl FFS with the least number of tests possible. The primary elements impacting the formulation's physicochemical qualities, namely the polymer Eudragit RSPO (X_1), the concentration of polymer ethyl cellulose (X_2), and the concentration of camphor-menthol eutectic mixture (X_3), were chosen and examined at two distinct levels designated as -1 and +1. The dependent response variables to optimize were viscosity (Y_1) and Drug permeation (Y_2). In the Table 1 lists the dependent and independent variables and their actual values at their high and low levels. A total of 15 runs were needed based on the CCD matrix provided by the Design-Expert® program (Version 13, M/s Stat-Ease, Minneapolis, USA) in Table 2.

Table 2: Central Composite Experimental Design.

Std	Run	Eudragit	Ethylcellulose	Eutectic mixture
5	1	5	2.5	12.5
7	2	5	7.5	12.5
8	3	15	7.5	12.5
1	4	5	2.5	7.5
15	5	10	5	10
13	6	10	5	5.79552
12	7	10	9.20448	10
11	8	10	0.795518	10
2	9	15	2.5	7.5
4	10	15	7.5	7.5
14	11	10	5	14.2045
9	12	1.59104	5	10
10	13	18.409	5	10
3	14	5	7.5	7.5
6	15	15	2.5	12.5

Evaluation studies

Formulation-related evaluation

Viscosity

The viscosity plays a major role in deciding the spray pattern and spray ability of the solution. The more viscosity, the less will be the area of spray ability. A Digital Brookfield viscometer was used to determine the viscosity of the formulation at room temperature. The ULA S00 spindle was set at 4 rpm, and the sample size was in the ULA cylinder. The torque level was set at ten while measuring the viscosity.

pH

The pH of the formulation was evaluated to omit skin irritation. The formulation with a similar pH of the skin will have less chance of showing skin irritation. The pH of the optimized formulation is evaluated by the use of calibrated digital pH meter 335 (Systronics, Pvt. Ltd., India). The pH meter's rod is dipped in the Film-forming solution being tested, and the meter's pH were recorded.

Density

The density plays a major role in the spray angle, amount of delivery per actuation and the spray pattern, and the spraying area. The density of the spray solution can be determined by using the specific gravity bottle (density bottle). The empty weight of the 25 mL capacity-specific gravity bottle was recorded. Then it is filled with the optimized batch solution and then reweighed. Then the density was calculated by the formula.

Density (D) = (Weight of bottle filled with sample solution-Weight of empty bottle) / 25

In-vitro Drug permeation studies

The *in-vitro* drug permeation was carried out using the cellophane membrane arranged on the Franz diffusion cell. The membrane used for the permeation studies was cut with the shape and size to fit the size of the diffusion cell. The 10 mL of the 5.5 pH phosphate buffer were filled in the receptor compartment of the diffusion cell. The cellophane membrane with a 2.4 cm diameter were arranged in between the donor and receptor compartments. 1 mL (equivalent to 10mg of drug) of the film-forming solution was taken and added to the receptor compartment. The circulation of 37± 2°C water in the outer jacket of the diffusion cell was performed using a water pump to mimic the body temperature. Then the stirrer was set at the rpm of 200. Exactly, 0.5 mL of sample were withdrawn at periodic intervals of time from the receptor chamber for up to 7 hr. These samples were made up to 10 mL in a volumetric flask, and absorbance is recorded using a U.V spectrophotometer at 283 nm. The concentration of the drug was calculated using the absorbance recorded and the calibration curve line equation.[8] Then the release kinetics of the optimized formulation for evaluating the release model were performed for Zero-order, first-order, Higuchi and Korsmeyer-Peppas. The regression (R^2) of all the models was calculated.

Evaporation time

The evaporation time is also termed drying time. To detect how rapidly the film develops after the solution is sprayed, the evaporation of the film is determined. The optimized batch film-forming solution was sprayed on the clean petri dish to

determine the drying time. Then, a glass slide was laid on the film without stress after a certain period. The film is said to be dry if no moisture remains observed on the glass slide. The drying time defines the rate of film formation. This process is repeated 3 times, and the average evaporation time was calculated.

Anti-fungal studies

The anti-fungal activity of the formulations and excipients were determined by using the cup-plate method for six common skin infection-causing fungi, namely *Candida albicans, Candida krusei, Aspergillus fumigatus, Epidermophyton, Trichophyton mentagrophytes* and *Cryptococcus neoformans*. The plates were prepared with the Sabouraud's dextrose agar; the medium is sterilized using an autoclave at 121°C, 15 pounds per inch pressure for 20 min. Then the sterilized medium was poured into the agar in the laminar airflow and allowed to solidify. Then 100 µL of fungal culture was poured on the plates and spread on the agar medium. This is repeated for all the fungal species mentioned above, making 5 sets of plates for each fungal species labelled sets A, B, C, D, E. One set of the plates is labelled as the control (set A) and immediately kept for incubation. Another set of 6 fungi plates (set B) were inoculated with fungi immediately after spraying the optimized formulation. Then sets A and B were compared with the fungal growth. If no colonies are observed in set B, then it is stated that formulation prevents the growth of fungi.

In set C, the plates were kept for incubation for 2 days, when the growth is observed, the formulation was sprayed once on each plate and kept for incubation again. After two days, then set C and set A plates were compared for the growth. If the growth is seized, that indicates that the fungi are killed, and no replication is going on, and it proves that the formulation has fungicidal activity.

In set D, after spreading the fungi culture on the plates, then 2 wells were made on each plate and naming one as the MC (marketed cream) and another as the TF (test formulation). 100 mg of marketed cream was weighed and placed in the MC wells of 6 plates, and 100µl of formulation solution was poured into the TF wells of 6 plates. Then all 6 plates of set D were incubated for some days, and inhibition of fungal growth around the wells is observed and recorded.

Four wells were made on each plate after spreading fungi on the 6 plates of set E. Wells were named Sol for solvent alone, EM for eutectic mixture, E+S for Eudragit dissolved in the solvent, and EC+S for ethyl cellulose dissolved in the solvent. Then 100 µL of each sample was poured into the wells according to the naming on all the plates of set E. These plates were then incubated for 3 days to check the anti-fungal activity of the excipients.

Container-related evaluations

Volume of spray solution delivered per each actuation

The volume of spray solution released can be used to calculate the amount of drug-delivering for one actuation. To calculate the amount of spray solution released for each actuation, the initial weight of the spray container with spray solution is checked (W1). Then the solution was sprayed once with a single actuation, and then it is again reweighed (W2). The amount of spray solution delivered for each actuation was calculated by the formula

$$\text{Amount delivered for one actuation (A)} = (W1-W2) / D.$$

Where,

D = density of the spray solution

Spray pattern

The spraying pattern in the area and pattern of the spray solution falls when sprayed. This was determined by spraying the solution horizontal on a white sheet held at a distance of 10cm. Then, the droplets of the spray solution wet the sheet and make it visible. Immediately, a dotted line was drawn around the wet region of the sheet. Then the diameters of the circle formed in three directions were measured with a ruler. The average of the 3 diameters was calculated.

Spray angle

The solution was sprayed horizontally onto a white sheet held at a distance of 10 cm (d). The diameter of the circle drawn on the paper was measured three times from different angles. From the diameter, radius (r) is calculated. The spray angle (θ) is calculated by the formula

$$\text{Spray angle } (\theta) = \tan^{-1} (L/r)$$

Where,

L = distance between sheet and spray nozzle

R = radius of spray region

Short-term stability studies

The optimized batch's short-term stability was tested using a photostability chamber for two months at 25±2°C and RH 60±5%. The goal of stability studies was to see how the quality of a formulation changes over time as a result of a variety of factors like pH, viscosity, the volume of solution released for each actuation and homogeneity of the optimized batch stayed constant throughout the experiment. If nothing has changed before and after, then the formulation is said to be stable.

Table 3: Binding energy of antifungal allylamine drugs with squalene epoxidase.

Drug	Binding energy (kcal/mol)
Terbinafine HCl	-10.3
Naftifine	-10
Amoralfine	-9.2
Butenafine	-8.5
Co-crystal	-7.9

RESULTS AND DISCUSSION

Selection of drug by docking

By the docking results, it was found that the Terbinafine HCl had a good affinity with the enzyme squalene epoxidase of PDB ID 6C6N. The binding affinity was seen better for the terbinafine HCl compared to that of the co-crystal. The binding energy values of antifungal allylamine drugs with the protein of interest are given in Table 3.

FTIR- Compatibility studies

The FTIR results for the drug and the physical mixture has been performed, and no addition or deletion of the major functional groups such as C-H–3040.23, C=C-H–2967.91, C=C–2222.56, C-N–1132.97 was observed, which confirms no compatibility between drug and excipients. The data has been given in Figure 1.

Formulation and optimization of Terbinafine HCl Film-forming spray

The general preparation of the Terbinafine HCl Film-forming spray formulation is by dissolving the excipients in the blend of solvents and sonicating it until a homogenous solution forms. The formulation optimization was performed using the statistical tool, namely the design of experiments. The 3-factorial Central composite design was chosen for the optimization by selecting the formulation factors Eudragit, Ethyl cellulose and Eutectic mixture and the response variables Drug permeation and viscosity. The total number of the batches is 15 batches, which includes 8 corner point runs, 6 axial point runs and 1 center point run, which includes a total of 15 non-repeating combinations of excipients. The effect of the different concentrations of the excipients used on the drug permeation and viscosity can be determined by the 15-run study. In Table 4 summarizes all experimental runs of 3-a factorial central composite design. The values of Y_1 response (Viscosity) were found to be in the range of 9.81 cps to 27.14cps, and Y_2 response (Drug permeation) in the range of 73.53% to 97.19%, respectively.

To demonstrate the effects and relationships between the excipients, computer design tools were used to create polynomial equations and interactive charts. The sequential model sum of squares, the lack of fit test, and model summary statistics were used to choose the response analysis models. ANOVA was used to verify the polynomial equations statistically, with model terms deemed significant when 'Prob>F' 0.0500 and non-significant when 'Prob>F'>0.1000. For the observed data, three-dimensional response surface charts were constructed to determine the effect of the chosen independent factors on the responses.

ANOVA analysis for each model selected for each response

ANOVA for Linear model for response 1

The 44.01 Model *F*-value shows that the model is valid. *F*-value of this value has a 0.01 percent probability of happening due to noise (Table 5). Model terms with *P*-values less than 0.0500 are valid. A is a crucial model term in this situation. The model terms are not useful if the value is more than 0.1000. Model reduction may improve the model if there are numerous insignificant model terms.

ANOVA for Linear model for response 2

The *F*-value of 10.55 for the model shows that it is valid. *F*-value of this level has a 0.14 percent probability of happening due to noise (Table 6). Model terms with *P*-values less than 0.0500 are significant. A is a crucial model term in this situation. The model terms are not useful if the value is more than 0.1000. Model reduction may improve the model if there are numerous insignificant model terms.

Response surface plot

The response surface plots, such as the 3D response graphs, are a great tool for evaluating the interactions and influence of the independent variables. From the interpretation of the 3D plots in Figures 2 and 3, it is also observed that the viscosity has increased with the increase in the polymer concentration, particularly ethyl cellulose. It was seen that the drug permeation had increased with the increased eutectic mixture from Figures 4 and 5.

Perturbation plots

The perturbation plot is useful for comparing the effects of all variables at a certain point in the design space. Over the response's range, just one variable was modified, while all other parameters stay constant. More the deviation of a factor from the centre point indicates more influence of factor on the response and vice-versa. In Figure 6 it was observed that both factor A (Eudragit) and factor B (Ethyl cellulose) has an influence on the viscosity when compared to factor C (Eutectic mixture). In both factors A and B, it is observed that Factor B has more effect on the viscosity. In Figure 7 it is observed that factor C (eutectic mixture) has more influence on the drug permeation.

Desirability Criteria and overlay plot

The desirability factor generated by the software was 0.912, demonstrating the rationale for selecting the optimized formula.

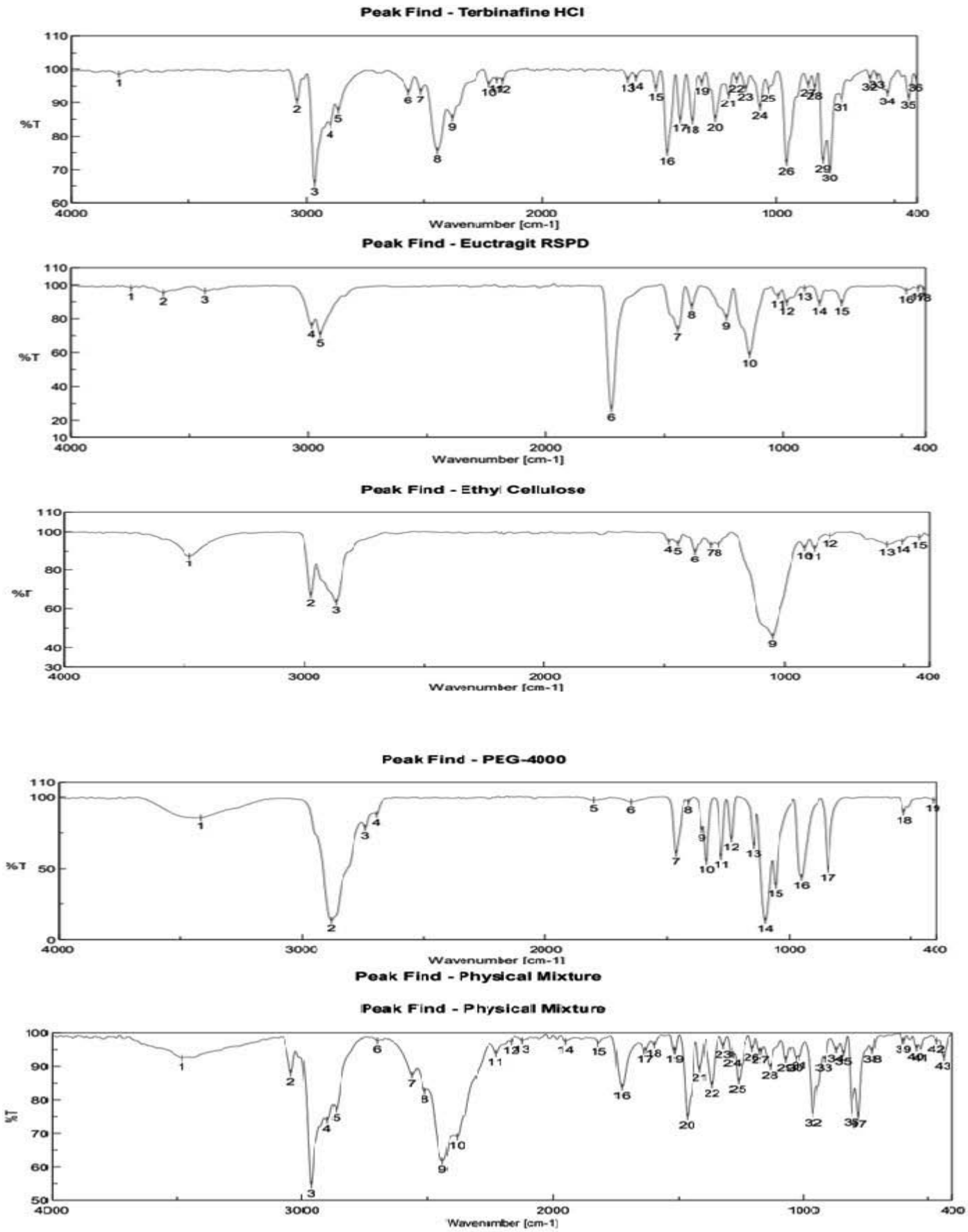

Figure 1: FTIR compatibility studies of drug and excipients.

Table 4: Experimental runs using 3-factorial Central composite design.

Run	Eudragit	Ethylcellulose	Eutectic mixture	Viscosity (cps)	Drug permeation (%)
1	5	2.5	12.5	11.94	96.66
2	5	7.5	12.5	21.04	92.46
3	15	7.5	12.5	27.14	80.37
4	5	2.5	7.5	9.81	92.98
5	10	5	10	18.07	86.125
6	10	5	5.79552	16.5	73.53
7	10	9.20448	10	26.1	81.42
8	10	0.795518	10	9.34	88.255
9	15	2.5	7.5	15.12	79.31
10	15	7.5	7.5	25	79.84
11	10	5	14.2045	19.52	97.19
12	1.59104	5	10	14.01	94.56
13	18.409	5	10	16.2	84.5
14	5	7.5	7.5	19.8	89.83
15	15	2.5	12.5	17.27	81.94

Table 5: ANOVA for Response 1 (Viscosity)

Source	Sum of squares	d_f	Mean square	*F*-value	*P*- value	
Model	388.92	3	129.64	44.01	< 0.0001	significant
A-Eudragit	48.07	1	48.07	16.32	0.0019	
B-Ethyl cellulose	328.96	1	328.96	111.68	< 0.0001	
C-Eutectic mixture	11.88	1	11.88	4.03	0.0698	
Residual	32.40	11	2.95			
Cor Total	421.32	14				

Table 6: ANOVA for Response 2 (Drug permeation).

Source	Sum of squares	d_f	Mean square	*F*-value	*P*- value	
Model	539.17	3	179.72	10.55	0.0014	significant
A-Eudragit	332.53	1	332.53	19.52	0.0010	
B-Ethyl cellulose	28.95	1	28.95	1.70	0.2190	
C-Eutectic mixture	177.69	1	177.69	10.43	0.0080	
Residual	187.42	11	17.04			
Cor Total	726.59	14				

The predicted response provided by the software for the selected optimized formula was 11.93 Cps of viscosity and 96.59% of drug permeation for the factors using 5gm of Eudragit and 2.5gm of ethyl cellulose and 12.5gm of the eutectic mixture. Terbinafine HCl film-forming spray formulation was prepared using these values in run 1 of the design, and the actual responses obtained were 11.94 Cps viscosity and 96.665 drug permeation after 7 hr as shown in Table 7. This shows that the Formulation prepared is in very close accordance with the predicted values. Figure 8 shows the overlay plot for the optimized variables for the formulation.

Evaluation studies

Formulation-related evaluation studies

Viscosity

The viscosity was determined for all the 15 batches using the Brookfield digital viscometer at the torque of 10. Then the results are found to be highest for batch 3 (27.14), which was formulated with 15gm Eudragit, 7.5 gm of ethyl cellulose and 12.5gm of the eutectic mixture. Similarly, the lowest viscosity was found for batch 5 (9.81), formulated with Eudragit 5gm, ethyl cellulose 2.5gm and eutectic mixture 7.5gm. This indicates

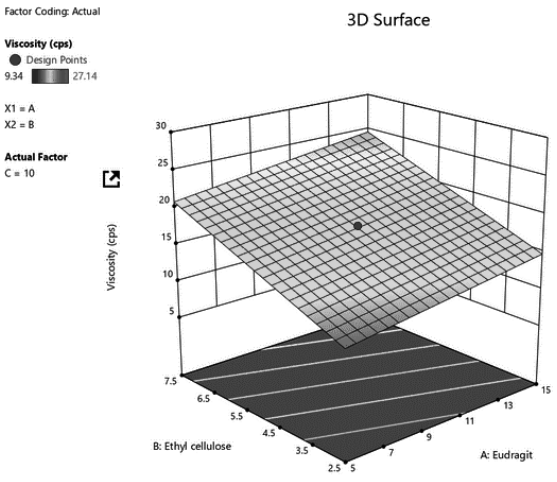

Figure 2: 3D response surface plot showing the influence of the Eudragit, ethylcellulose on Viscosity.

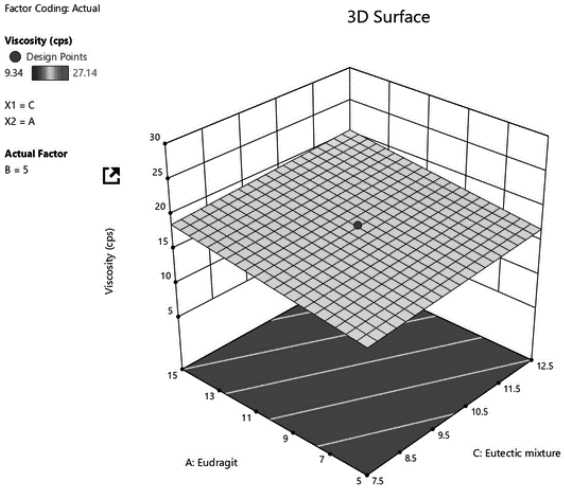

Figure 3: 3D response surface plot showing the influence of the Eudragit, Eutectic mixture on Viscosity.

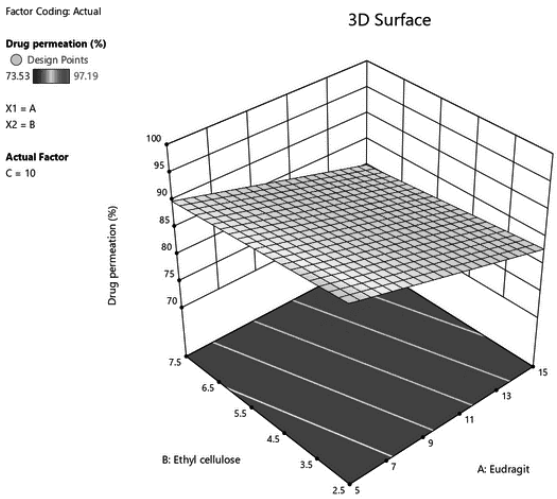

Figure 4: 3D response surface plot showing the influence of Eudragit, ethylcellulose on Drug permeation.

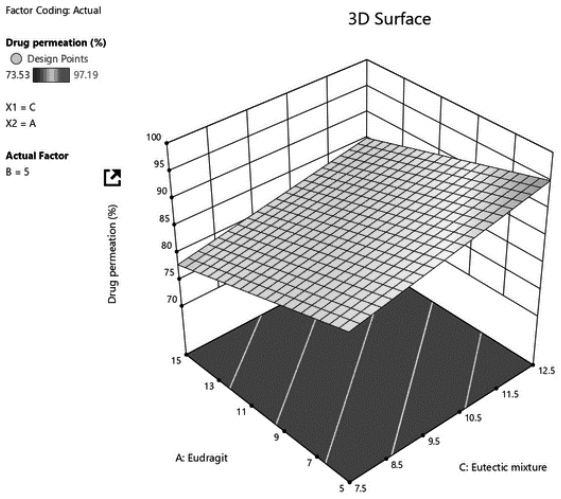

Figure 5: 3D response surface plot showing the influence of Eudragit, Eutectic mixture on Drug permeation.

that the polymer concentration is influencing the viscosity of the formulation, particularly ethyl cellulose. The viscosity of all the batches is presented in Table 8 as follows.

pH

The pH of the optimized batch formulation was determined by analyzing the 20 mL of the optimized spray solution with the calibrated pH meter at room temperature. The pH of the solution was found to be 5.16, which is safe for topical application.

Density

The density of the optimized batch was determined using the 25 mL capacity-specific gravity bottle of weight 20. 519 gm. The weight of the density bottle filled with a solution is found to be 42.298 gm. The density of the spray solution was found to be 0.87116 gm/mL. The following formula was used to calculate the density.

Density (D) = (Weight of bottle filled with sample solution-Weight of empty bottle) / 25

Evaporation time

The evaporation time is the time taken for the spray solution to form a film by solvent evaporation. The evaporation time is calculated by keeping the glass slide on the film; if no wetness sticks to the slide, it is said to be dry. The evaporation time of the optimized formulation was calculated three times, and it is found to be 75 sec, 80 sec, and 75 sec respectively. As the solvent systems used are volatile in nature, the drying time or film-forming time is low comparatively. The average evaporation time is found to be 76.66 sec.

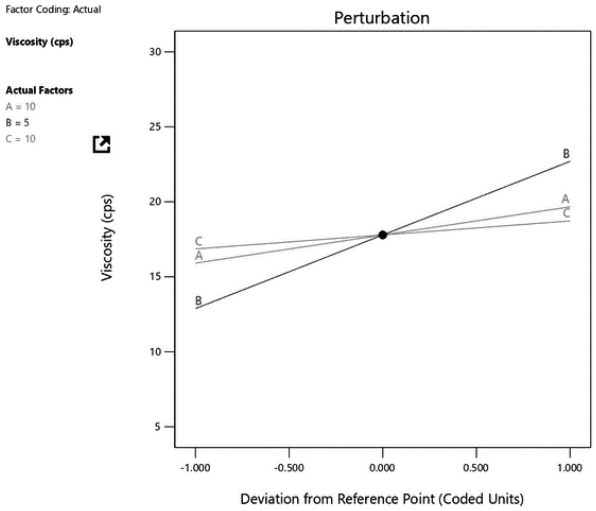

Figure 6: Perturbation plot showing the influence of Eudragit, Eutectic mixture, and ethyl cellulose on Viscosity.

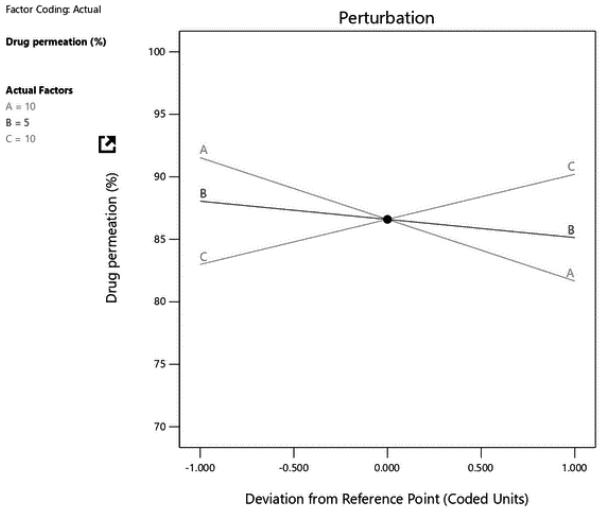

Figure 7: Perturbation plot showing the influence of Eudragit, Eutectic mixture, and ethyl cellulose on Drug permeation.

In-vitro drug permeation studies

The drug permeation studies are conducted using the Franz diffusion cell with the cellophane membrane. The permeation studies of all 15 batches were performed using the 5.5 pH phosphate buffer. As the fungal infections spread up to the deeper layers of the skin, more dug permeation is needed. Batch 10 was found to have the greater permeability with 97.19% in 7 hr, while batch 5 got the lowest permeability with 73.53% drug permeation after 7 hr. From the results, it was observed that the drug permeation has increased with the permeation enhancer (eutectic mixture) concentration and reduced with the increase in polymer concentration. The cumulative drug permeation results are given in Table 9, and the drug release profile of the optimized batch is given in Table 10. The R^2 (regression) value is found to be highest for the zero-order model; thus, the drug permeation from the optimized batch is found to follow the zero-order kinetics,

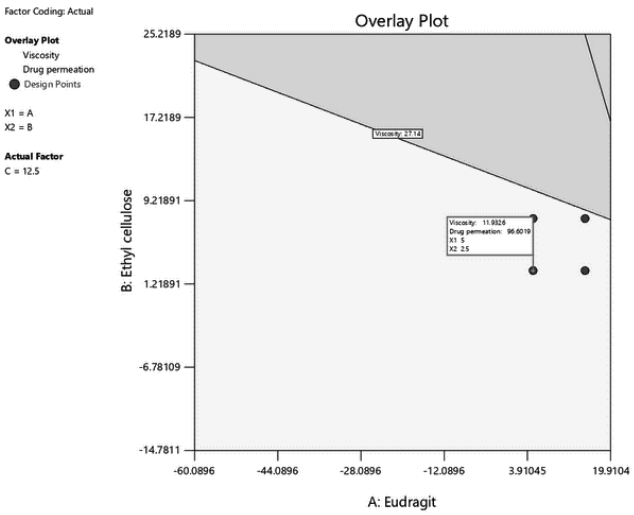

Figure 8: Overlay plot for the optimized variables for the formulation.

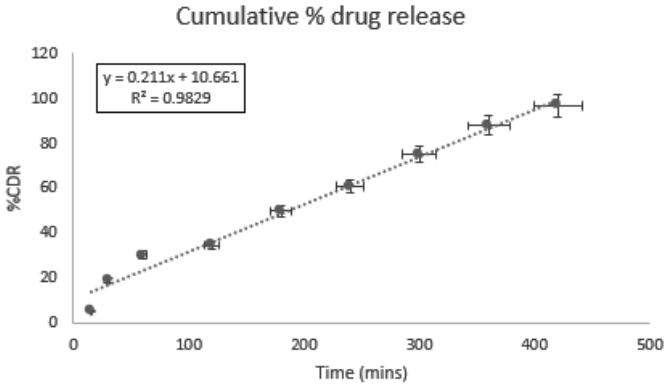

Figure 9: *In-vitro* drug permeation profile.

Table 7: Predicted and actual responses for Terbinafine HCl film-forming spray formulation.

Response	Viscosity (Cps)	Drug permeation (%)
Predicted	11.93	96.59 ± 0.07
Actual	11.94	96.66 ± 0.43

as shown in Figure 9. The R^2 values of all the models are given in Table 11.

Anti-fungal studies

The antifungal studies of the optimized Terbinafine film-forming spray formulation were performed in different phases for the various topical infection-causing fungi such as *Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus, Candida krusei, Trichophyton mentagrophytes* and *Epidermophyton*.

The anti-fungal activity of the formulation was evaluated by inoculating the fungi immediately after spraying the formulation. There is no single colony of the fungi observed after incubation for 4 days. This proved that the prior administration of the

Table 8: Viscosities of all the batches.

Run	Eudragit	Ethylcellulose	Eutectic mixture	Viscosity (cps)
1	5	2.5	12.5	11.94 ± 1.33
2	5	7.5	12.5	21.04 ± 0.44
3	15	7.5	12.5	27.14 ± 3.21
4	5	2.5	7.5	9.81 ± 1.86
5	10	5	10	18.07 ± 0.54
6	10	5	5.79552	16.5 ± 3.42
7	10	9.20448	10	26.1 ± 2.21
8	10	0.795518	10	9.34 ± 2.41
9	15	2.5	7.5	15.12 ± 0.48
10	15	7.5	7.5	25 ± 1.44
11	10	5	14.2045	19.52 ± 2.54
12	1.59104	5	10	14.01 ± 2.09
13	18.409	5	10	16.2 ± 1.76
14	5	7.5	7.5	19.8 ± 2.76
15	15	2.5	12.5	17.27 ± 0.55

Table 9: Cumulative % drug permeation profile of terbinafine HCl of all batches.

Run	Eudragit	Ethylcellulose	Eutectic mixture	Drug permeation (%)
1	5	2.5	12.5	96.66 ± 2.55
2	5	7.5	12.5	92.46 ± 1.87
3	15	7.5	12.5	80.37 ± 0.75
4	5	2.5	7.5	92.98 ± 1.54
5	10	5	10	86.125 ± 3.21
6	10	5	5.79552	73.53 ± 2.22
7	10	9.20448	10	81.42 ± 0.89
8	10	0.795518	10	88.255 ± 0.74
9	15	2.5	7.5	79.31 ± 1.32
10	15	7.5	7.5	79.84 ± 1.66
11	10	5	14.2045	97.19 ± 2.21
12	1.59104	5	10	94.56 ± 2.86
13	18.409	5	10	84.5 ± 3.21
14	5	7.5	7.5	89.83 ± 2.09
15	15	2.5	12.5	81.94 ± 3.21

film-forming solution prevents fungal infections. This was compared with the control group, which was not administered with any formulation. Figure 10 (a-e) shows that there was no growth of the fungi on the sprayed plates in comparison with the control groups.

The fungal cultures of the 6 species mentioned above spread on the agar plates and incubated for 2 days along with the control plates. Then the 6 test plates which show the growth is sprayed with the formulation and incubated for another 2 days. The control group is kept undisturbed after being spread with cultures in an incubator. The cessation of the growth in the sprayed plates proved that the formulation had fungicidal activity (as the dead fungi can't replicate in incubation). Figure 11 (a-e) shows the growth of fungi in the control group and test group at the time of spraying the formulation on the test group.

The control group continued to grow, but the test group didn't show any growth even after incubation indicating fungicidal

Table 10: Cumulative % drug permeation profile of Terbinafine HCl from optimized formulation.

Sl. No.	Time (mins)	% Drug Release
1	15	5.58974359 ± 0.05
2	30	18.87051282 ± 0.65
3	60	30.43461538 ± 1.55
4	120	34.63974359 ± 1.76
5	180	49.88333333 ± 2.11
6	240	60.92179487 ± 1.86
7	300	75.11410256 ± 2.13
8	360	87.72948718 ± 0.66
9	420	96.66538462 ± 3.22

Table 11: Models and the regression values of the optimized formulation.

Sl. No	Model	Regression value (R^2)
1	Zero-order	0.9829
2	First-Order	0.7655
3	Higuchi	0.9752
4	Korsmeyer-Peppas	0.9477

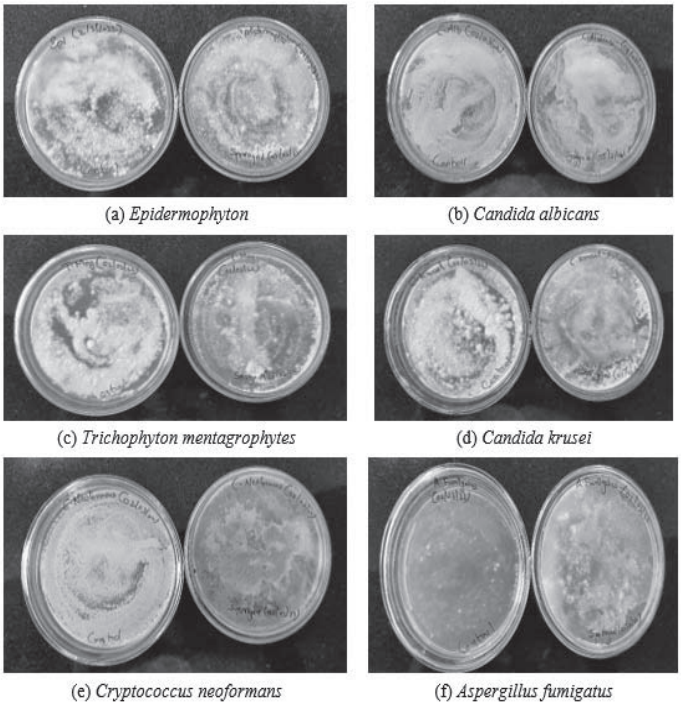

Figure 11: Comparison of control and test group fungi after at the time of spraying the formulation.

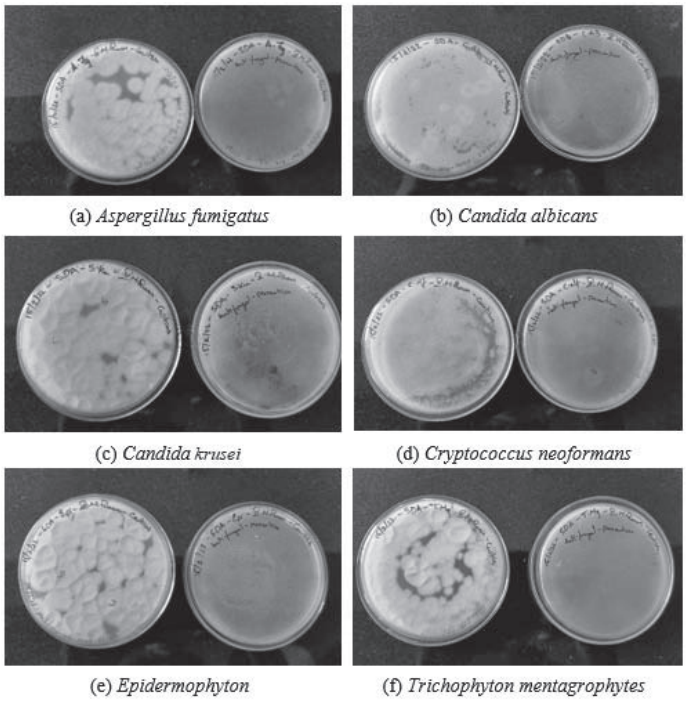

Figure 10: Anti-fungal activity of the optimized Terbinafine HCl film-forming formulation.

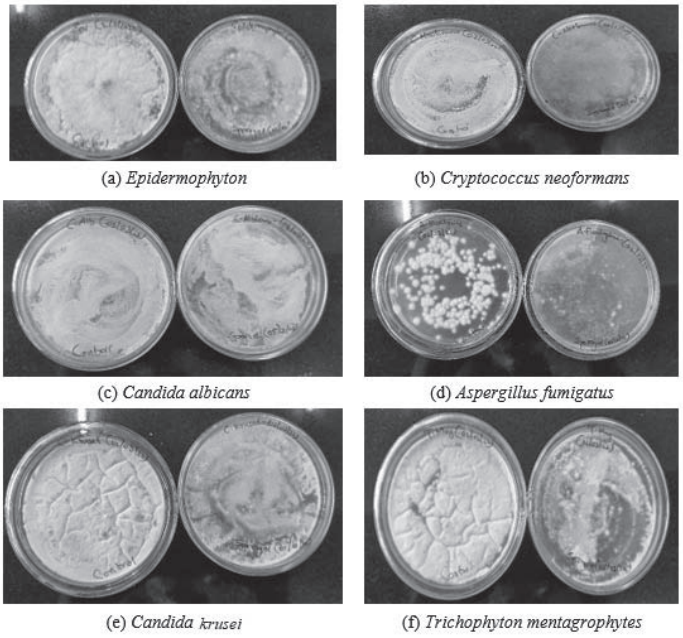

Figure 12: Comparison of control and test group fungi after two days of spraying the formulation.

activity. The fungicidal activity of the optimized formulation is illustrated in Figure 12 (a-e).

The anti-fungal activity of the excipients and solvents is evaluated by the cup-plate method. Only the Eutectic mixture has restricted the growth of the fungi. The rest of the excipients and the solvent has shown no anti-fungal activity. The anti-fungal activity of excipients and the solvent is illustrated in Figure 13 (a-d).

A comparative study among the marketed semi-solid formulation and the Optimized film-forming spray formulation for the anti-fungal activity has been performed, and the results have shown that the optimized Terbinafine HCl film-forming spray solution has greater anti-fungal activity when compared to that of

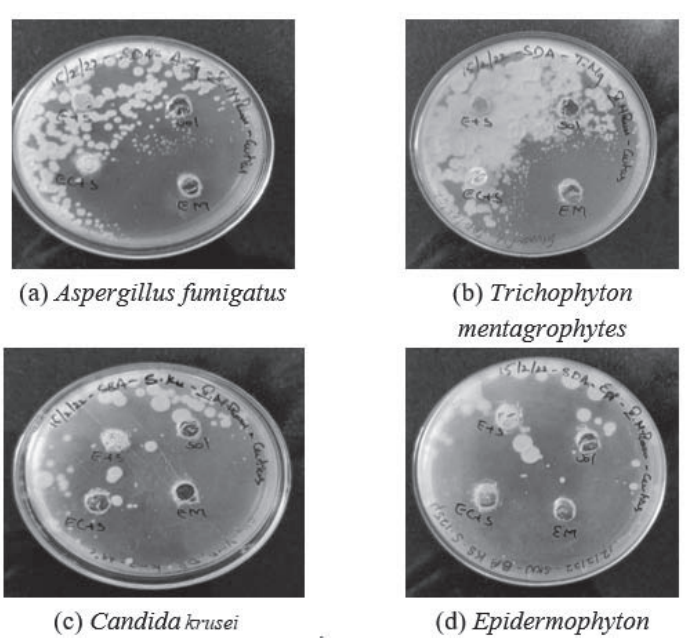

Figure 13: Anti-fungal activity of solvent and excipients.

Table 12: Parameters before and after the stability studies.

Parameter	Before stability testing	After stability testing
pH	5.16	5.19
Viscosity	11.94 Cps	12.07 Cps
Visual appearance	No floccules/ aggregates of polymer were observed	No floccules/ aggregates of polymer were observed

the marketed formulation. The antifungal activity comparison of the marketed antifungal cream and the test formulation is shown in Figure 14 (a-e).

Container-related evaluation

The volume of spray solution delivered per each actuation

The initial weight of the container and the spray solution was found to be 201.527. The weight of the container and the formulation after one actuation was found to be 201.342. From the above studies, the density of the optimized batch solution is found to be 0.87116 gm/mL.

Amount delivered for one actuation (A) = (W1-W2) / D.

The volume of the solution delivered per actuation is found to be 0.212 mL.

Spray pattern

The average diameter of the spray pattern formed by the spraying of optimized batch formulation on the sheet at a distance of 10cm from the nozzle was found to be 15.41 cm.

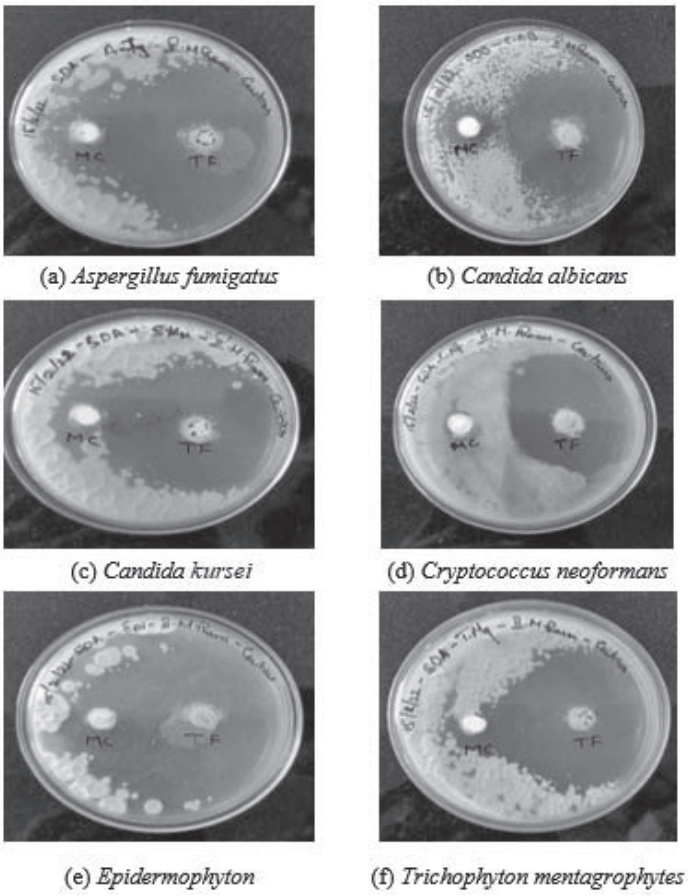

Figure 14: Comparison of anti-fungal activity of marketed cream and test formulation.

Spray angle

The average diameter of the spray pattern was found to be 15.41 cm from the spray pattern analysis, and then the radius (r) is calculated to be 7.705cm. The length (L) from the nozzle to the sheet is taken as 10 cm. Then the spray angle (θ) is found to be

The spray angle for the optimized batch is found to be 52.38°.

Stability studies

After two months of stability studies of the optimized Terbinafine HCl film-forming formulation at the temperature of 25±2°C and RH 60±5%, it is observed that there were no significant changes in the pH, viscosity and the appearance of the formulation. The parameters evaluated before and after the stability studies are shown the Table 12.

CONCLUSION

The Terbinafine HCl film-forming spray was optimized using the Central composite design. The optimized formulation containing 5% Eudragit RSPO, 2.5% Ethyl cellulose and 12.5% camphor menthol eutectic mixture has good spray ability, viscosity and drug permeation. The viscosity of the optimized batch was found to be 11.94cps and *in-vitro* drug permeation after 7 hr was found

to be 96.66%. The pH was found to be 5.16 with a spray angle of 58.38° and a good spray pattern. The stability studies at 25±2°C and 60±5% RH for 2 months has revealed that the formulation is stable with no change in pH, viscosity and appearance. Thus, the prepared optimized formulation may be considered a promising approach for the treatment of topical fungal infections.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Original Article

Synthesis, Antidiabetic Evaluation and Molecular Docking Studies of Thiazolidine-2,4-Dione Analogues

Dolly R Pardeshi, Vithal M Kulkarni, Sandeep S Pathare*

Department of Pharmaceutical Chemistry, Poona College of Pharmacy, Bharati Vidyapeeth (Deemed to be University), Pune, Maharashtra, INDIA.

ABSTRACT

Introduction: Diabetes Mellitus is a disorder of metabolism described by high glucose levels. The disorder kills a larger number of individuals consistently than of malignant growth and AIDS combined. Presently available drugs have several drawbacks forcing to withdraw from treatment. The potent side effect i.e., hepatotoxicity and cardiovascular toxicity limits the use of thiazolidine-2,4-dione derivative as safe drugs. Our aim is towards the development of synthetic compounds as potential antidiabetic agents, particularly preparation and screening of new analogues of thiazolidine-2,4-dione (TZD) which are well established as oral insulin sensitizing agents that improve insulin resistance and are agonists of Peroxisome Proliferator Activated Receptor–γ (PPAR-γ). **Materials and Methods:** Proper substitution at C-5 position of thiazolidine-2,4-dione could produce better and potential antidiabetics with improved pharmacological properties, including toxicity. Our aim of this research work is towards this. **Results:** A series of C-5 substituted thiazolidine-2,4-dione analogues were synthesized. Structures of these new analogues were confirmed by IR, ^{1}H-NMR and MASS spectroscopy. Among the synthesized compounds, three compounds: 5-(2-pyridinylbenzylidene) thiazolidine-2,4-dione, 5-(3,4-dimethoxybenzylidene) thiazolidine-2,4-dione and 5-(2,3,4-trifluorobenzylidene) thiazolidine-2,4-dione showed significant antidiabetic activity in streptozotocin induced diabetic mice comparable with Pioglitazone drug. The molecular docking studies of these compounds performed using protein target showed amino acid interactions with Leu270, Gln283 and Arg288 similar with that of Rosiglitazone and Pioglitazone. The compounds did not show any toxic effect in mice even at 2000 mg/kg of dose. Therefore, synthesis of modified and better thiazolidine-2,4-dione containing drugs other than the currently available drugs is of importance in antidiabetic drug research.

Keywords: Thiazolidine-2,4-dione, Molecular docking, Antidiabetic evaluation, *Swiss albino* mice.

Correspondence:
Dr. Sandeep S Pathare
Department of Pharmaceutical Chemistry, Bharati Vidyapeeth (Deemed to be University), Poona College of Pharmacy, Pune-411038, Maharashtra, INDIA.
Email id: sandeep.pathare@bharatividyapeeth.edu

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INTRODUCTION

Diabetes is an incredibly complex disease, influencing different age groups over the world.[1] Among several complications of the disease, the most predominant are kidney infections, visual impairment, hypertension, hypoglycemia, dyslipidemia and respiratory failure or stroke. More commonly used drugs for the treatment are belonging to the class of biguanide, sulfonylurea, thiazolidine-2,4-dione. Presently accessible medications have improper activity profile and pharmacokinetic properties.[2] Recent reports suggested that cardiovascular toxicity with Rosiglitazone and development of bladder cancer with Pioglitazone are of significant.[3] Other drugs such as troglitazone, englitazone are not currently used for the treatment.[4] Because of these drawbacks associated with some of such glitazone drugs, new drugs with better activity profile are necessary for the treatment of diabetes, particularly type-2 disease. Among the variously C-5 substituted benzylidene-2,4-thiazolidine (TZD) compounds, Pioglitazone and Rosiglitazone are not drugs of choice, though rarely used clinically. Therefore, we report here the synthesis of variously C-5 substituted 2,4-thiazolidine (TZD) analogues and *in vivo* antidiabetic activity determined using *swiss albino* mice.

MATERIALS AND METHODS

Materials

The chemicals and reagents used for the synthesis were obtained from Sigma-Aldrich, Merck and Loba Chemicals and were used after their characterization. Melting points were taken in open capillary tube on Campbell Melting-point apparatus. The progress of reaction was monitored by TLC and purity of all the compounds were assessed by thin layer chromatography (Silica Gel G coated TLC plates). Fourier Transform Infrared (FT-IR)

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spectra (cm^{-1}) were recorded in potassium bromide (KBr) disk on "Jasco FTIR 4100". Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded in DMSO-d6 using JEOL (500 MHz) with tetramethylsilane (TMS) as an internal standard. The mass spectra of compounds were recorded on Agilent 6460 Triple Quadrupole LC/MS System with Jet stream ESI ion source. Log *p* values were calculated using ChemBioDraw Ultra vs 14.0 software.

Chemistry

General Method of Synthesis of Thiazolidine-2,4-Dione

A solution containing chloroacetic acid (9.45 gm, 0.1 Mol) and thiourea (7.61 gm, 0.1 Mol) taken in excess of water was placed in round bottom flask under stirring. The mixture was stirred at low temperature for 15 min or until a white precipitate is formed. To the contents of the flask was then added slowly 3ml of conc. HCl through a dropping funnel, the mixture was then refluxed until a clear solution is obtained (10 -13 hr). Progress of reaction was monitored by TLC. On cooling the contents, needle shaped solid product resulted and was filtered, washed several times with water and dried. It was purified by dichloromethane (DCM, 3 x 100 ml). Melting Point (M.P.) and spectral characteristics are recorded (Figure 1).[5,6]

General Method of Synthesis of 5-Substituted Benzylidene Thiazolidine-2,4-Diones

To an equimolar ethanolic solution of thiazolidine-2,4-dione as obtained above, variously substituted benzaldehydes were added (m/m). A catalytic amount of piperidine (0.2 ml) was added. The mixture was stirred and refluxed for 4-6 hr.[7] The completion of reaction was assessed by TLC ($ClCH_3$: CH_3OH; 9:1) and then, the reaction mixture was poured on to crushed ice. Few drops of glacial acetic acid were added to effect precipitation of product.[8,9] The products thus obtained were filtered, washed with sufficient cold water and recrystallized from ethanol. Melting Point (M.P.) and spectral characteristics are recorded (Figure 2).

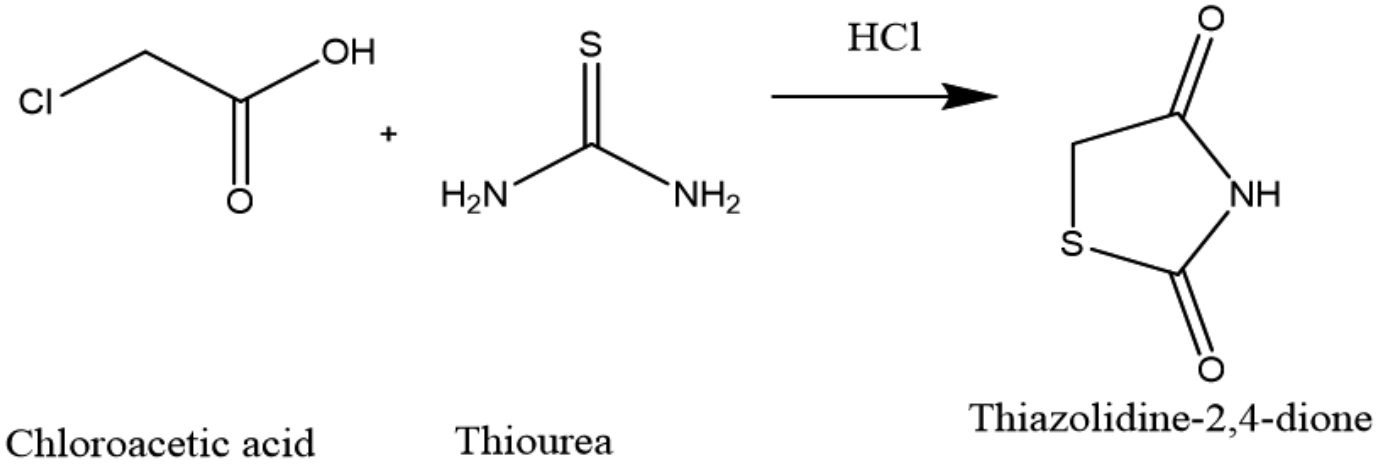

Figure 1: Synthesis of thiazolidine-2,4-dione.

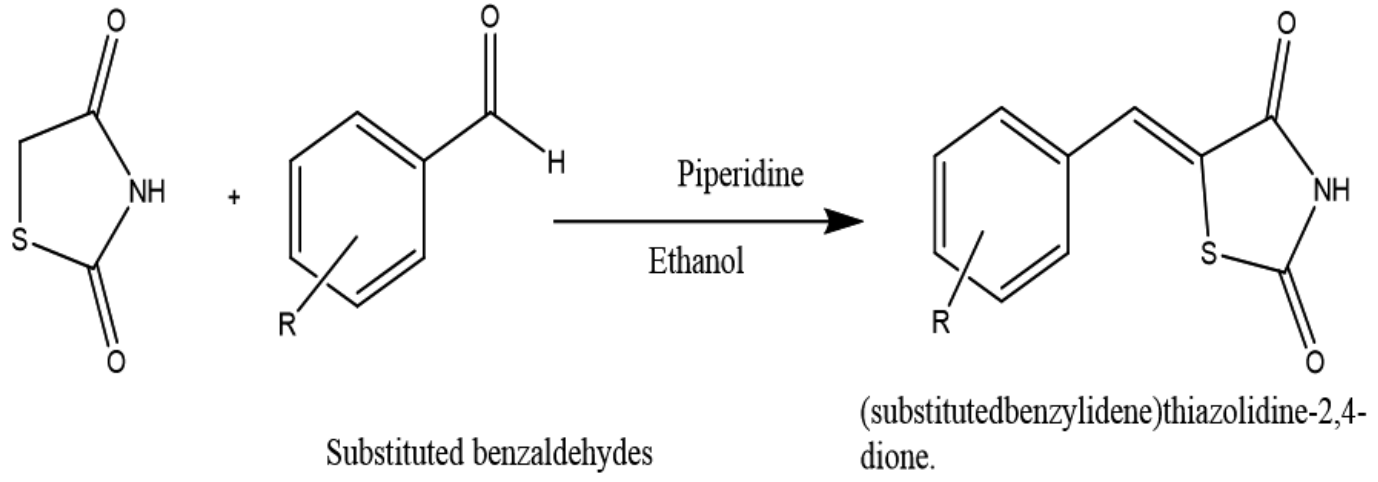

Figure 2: Synthesis of (substitutedbenzylidene)thiazolidine-2,4-dione.

Male Swiss Albino Mice with an average weight of 33±5 gm were used. Animals were housed in propylene cages at an ambient temperature of 25±2°C and 45-55% relative humidity with standard 12 hr light and dark cycle. They had a free access of feed and water. The guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA) Govt. of India were followed and earlier consent was observed from the institutional animal ethics committee.[9] (IAEC approval no-PCP/IAEC/2021-22/ "1-11".

Acute Oral Toxicity Test

Oral acute toxicity test of the compounds was performed. The mice were fasted for 4 hr prior to administration of the synthesized compounds. After checking the solubility of the compounds at different dose levels, distilled water was used as a vehicle. The final compounds were given orally in sequential in the form of suspension which is prepared with 2% of acacia gum and water at a dose of 2000 mg/kg. The mice were observed for any signs of toxicity for 4 hr, with special attention. After that up to 48 hr periodically. Then daily thereafter, for 14 days for sign of toxicity and motility.[10]

Induction of Diabetes

On the basis of results of molecular docking study, three compounds were selected for *in vivo* antidiabetic activity. Diabetes was induced with streptozotocin (200 mg/kg), dissolved in 0.1M citrate buffer solution, i.p. after 15 min of injecting the nicotinamide (NIC) solution (110 mg/kg) for injection. The vehicle used for the nicotinamide solution is saline solution and for citrate buffer solution water was used as a vehicle. After injecting the streptozotocin, animals were allowed for free access to food and water. After 72 hr the blood glucose level of animals was determined using glucometer. The mice having blood glucose level beyond 250 mg/dL were considered for further investigation.[11,12]

Molecular Docking Study

For this study, 3D structures of the compounds (Table 1) converted to pdbqt coordinate as per procedure. Before arrangement of auto dock configuration of protein, the water particles and the inhibitors were removed from it. Then, at that point utilizing polar hydrogen's were added, Koullman charges were assigned, and the obtained compound design was utilized as an information file for the AUTOGRID program. Each atom type in the ligand, maps were calculated with 0.375 Å spacing between grid points and

Table 1: 5-substituted benzylidene-2,4-thiazolidinedione.

Sl. No	Compound Name	Amino acid Interactions	Binding energy (Kcal/mol)	Log P
1	Rosiglitazone	Ile281, Gln283, Leu270.	-7.5	3.21
2	Pioglitazone	Met348, Ile341, Glu259, Cys285, Leu330, Arg288, Tyr327, Ile326, Met364.	-7.6	3.58
	R substitution			
SD-1	Pyridin-2-yl	Ser342, Ile262, Phe247, Lys265, Ly261.	-7.2	1.83
SD-2	3-Br,4-F	Leu333, Leu330, Arg288, Gys285, His449, Met304.	-6.8	2.48
SD-3	3,4-$(OCH_3)_2$	Phe287, Leu270, Gly204, Arg288, Ser342, Gln283, Arg280.	-6.6	1.24
SD-4	4-Cl	His323, Tyr327, Cys285, Leu330, Met365, Arg288, Ile341.	-6.5	2.05
SD-5	2-OH, 3-OCH_3.	Glu259, Ile262, Leu255, Ile249, Ile341, Phe247, Gln271, Arg280.	-6.0	0.98
SD-6	4-OH	Gln271, Leu270, Arg288, Ser342, Phe287.	-6.4	1.1
SD-7	4-F.	Gln271, Leu270, Arg288, Ser342, Phe287.	-6.4	1.65
SD-8	3-Br.	Val339, Met394, Cys282, Gly284, Ile341, Argln288, Ser345.	-6.4	2.32
SD-9	2,3,4-$(F)_3$.	Gln470, Tyr473, Leu453.	-6.4	1.97
SD-10	2-NO_2	Val339, Arg288, Gln47.	-5.2	1.98

Note: Log *P* value is calculated by using chem bio draw ultra vs 14.0 software.

the center of the grid box was placed at x =49.390, y = -34.847, and z =14.992. The components of the dynamic site box were set at 50 × 50 × 50 Å. Flexible ligand dockings were achieved for the selected compounds. The best pose of each ligand was selected for analyzing the interactions between PPAR-γ and the inhibitor. The results were visualized using Discovery Studio 4.0 Client.

RESULTS AND DISCUSSION

A series of thiazolidinedione analogues were synthesized by reacting thiazolidine-2,4-dione (0.2 Mol) and various aromatic benzaldehydes (0.2 Mol). The structures of these compounds were confirmed by spectroscopy, IR, MASS and ^{1}H-NMR. The title compounds (SD-1 to SD-9) were docked with the PPAR-γ receptor PDB ID: 2PRG. Then depending on the binding energy and amino acid interactions, three compounds (SD-1, SD-3 and SD-9) were selected for antidiabetic activity screening using streptozotocin induced diabetic mice.

Physical and Spectral Properties

Thiazolidine-2,4-dione (Scheme 1); White solid, yield: 89%, M.P 124-128°C; FT-IR (KBr) cm^{-1}: 3309 (N-H), 1746 (C=O), 1337 (C-N), 621 (C-S). ^{1}H-NMR (δ-ppm) (500 MHz, DMSO): 12.01 (s,1H, NH), 41 (s,1H,CH). MS: m/z 115.96 (Exp) [M^{-1}],117 (cal). R_f: 0.9.

5-(2-pyridynylbenzylidene) thiazolidine-2,4-dione (Scheme 2); (SD-1); Yellow solid, Yield:84%, M.P 262-266°C; FT-IR (KBr) cm^{-1}: 3045 (Ar-CH), 3309 (N-H), 1746 (C=O), 1337 (C-N), 621 (C-S). ^{1}H-NMR (δ-ppm) (500 MHz, DMSO): 12.47 (s,1H, NH), 8.0 (d, 1H Alkene CH), 8.2 (s, 1H Alkene CH), 8.26 (d, 1H Alkene CH), 8.08 (s, 1H Alkene CH). MS: m/z 281.11 (Exp) [M^{-1}], 282.32 (cal). R_f: 0.6.

5-(3-bromo4-fluorobenzylidene) thiazolidine-2,4-dione (SD-2); Yellow solid, Yield:92%, M.P 202-206°C; FT-IR (KBr) cm^{-1}: 3332 (N-H), 3066 (ArC-H), 1720 (C=O), 1 309(C-N), 756 (C-F), 699 (C-Br), 657 (C-S). ^{1}H-NMR (δ-ppm) (500 MHz, DMSO): 12.47 (s, 1H, NH), 8.0 (d, 1H Alkene CH), 8.2 (s, 1H Alkene CH), 8.26 (d, 1H Alkene CH), 8.08 (s 1H Alkene CH). MS: m/z 301 (Exp) [M^{-1}], 302 (cal). R_f: 0.7.

5-(3,4-dimethoxybenzylidene) thiazolidine-2,4-dione (SD-3); Yellow solid, Yield:84%, M.P 206-210°C; FT-IR (KBr) cm^{-1} : 3323 (N-H), 3229 (Ar-CH)1746 (C=O),1317 (C-N), 618 (C-S), 1033 (Ar-O-C). ^{1}H-NMR: (δ-ppm) (500MHZ DMSO):12.5 (s,1H,NH), 7.19 (d,2H,Ar CH) 7.19 (s,1H Ar CH)7.7 (s,1H alkene CH), 3.81 (s,6H,OCH3),. MS: m/z 264 (Exp) [M^{-1}], 265 (cal). R$_f$0.5.

5-(4-chlorobenzylidene) thiazolidine-2,4-dione (SD-4); Yellow solid, Yield:72%, M.P 184-188°C; FT-IR (KBr) cm^{-1}: 3323 (N-H), 3202 (ArC-H), 1717(C=O), 1331 (C-N), 764 (C-Cl), 635 (C-S). ^{1}H-NMR: (δ-ppm) (500MHZ DMSO): 12.6 (s,1H,NH),7.6 (d,4H Ar-CH), 7.8 (s,1H alkene CH). MS: m/z220 (Exp) [M^{-1}], 219 (cal). R_f: 0.5.

5-(2-hydroxy,3-mehoxybenzylidene) thiazolidine-2,4-dione (SD-5); Pale green solid, Yield:86%, M.P146-148°C; FT-IR (KBr) cm^{-1} : 3410 (O-H), 3121 (N-H), 3000 (Ar C-H), 1740 (C=O), 621 (C-S). ^{1}H-NMR: (δ-ppm) (500MHZ DMSO): 12.4 (s,1H,NH), 13.80 (s,1H,OH), 7.17 (d,Ar CH) . 6.61 (d,Ar CH). 7.09 (t,Ar-CH). 8.34 (s,alkene CH), 3.81 (s,3H,OCH3). MS: m/z 250 (Exp) [M^{-1}], 251 (cal). R : 0.5.

5-(4-hydroxybenzylidene) thiazolidine-2,4-dione (SD-6);Yellow solid, Yield: 94%, M.P 172-176°C; FT-IR (KBr) cm^{-1}: 3410 (O-H), 3121 (N-H), 1724 (C=O), 621 (C-S). ^{1}H-NMR (δ-ppm) (500MHz, DMSO): 12.45 (s,1H,NH), 10.30 (t,1H,OH), 6.95 (d,2H Ar CH), 7.45 (m,2H,Ar CH), 7.61 (s,alkene CH). MS: m/z 220 (Exp) [M^{-1}], 221 (cal). R_f:0.7.

5-(4-fluorobenzylidene) thiazolidine-2,4-dione (SD-7); Brown sticky, Yield:76%, M.P 206-210°C; FT-IR (KBr) cm^{-1}: 3428 (N-H), 2934 (Ar-CH), 1736 (C=O), 1516 (C-F), 621 (C-S). ^{1}H-NMR (δ-ppm) (500MHz,DMSO): 12.47 (s,1H,NH), 7.72 (d,2H Ar CH), 7.76 (s,1H alkene CH), 7.40 (d,2H Ar CH). MS: m/z 222 (Exp) [M^{-1}],223 (cal). R_f:0.8.

5-(3-bromobenzylidene) thiazolidine-2,4-dione (SD-8); White solid, Yield:92%, M.P 216-220°C; FT-IR (KBr) cm^{-1} : 3400(N-H), 3200 (Ar C-H), 1730 (C=O), 1343 (C-N), 698 (C-Br), 628 (C-S). ^{1}H-NMR (δ-ppm) (500MHz,DMSO): 12.47 (s,1H,NH), 7.56 (s,1H Ar CH), 7.46 (d,1H Ar CH), 7.32 (m,1H Ar CH), 7.60 (d1H Ar CH), 8.6 (s,1H Alkene CH). MS: m/z 283.13 (EXP) [M^{-}]. 284.14 (cal). R_f:0.6.

5-(2,3,4-trifluorobenzylidene) thiazolidine-2,4-dione (SD-9); Yellow solid, Yield:89%, M.P 230-234; FT-IR (KBr) cm^{-1}: 3462 (N-H), 3127 (Ar C-H), 1745 (C=O), 792 (C-F), 620 (C-S). ^{1}H-NMR (δ-ppm) (500MHz, DMSO): 12.79 (s,1H,NH), 7.80 (d,1H alkene CH), 7.67 (s,1H Ar CH), 7.59 (d,1H Ar CH). MS: m/z 258 (Exp) [M^{-1}], 259 (cal). R_f:0.6.

5-(4-nitrobenzylidene) thiazolidine-2,4-dione (SD-10); Brown solid, Yield:60%, M.P172-174; FT-IR (KBr) cm^{-1}: 3397(N-H), 2968 (Ar-CH), 1703 (C=O), 1526 (N-O), 1314 (C-N), 621 (C-S). ^{1}H-NMR (δ-ppm) (500MHz, DMSO): 12.47 (s,1H,NH), 8.37 (d,2H Ar-CH), 8.03 (d,2H Ar –CH), 7.61 (s,alkene CH).MS: m/z 249.07 (Exp) [M^{-1}], 250(Cal). R_f:0.7

Molecular Docking

To study the interaction of synthesized compounds in the active site of PPAR-γ, a docking study was performed using crystal structure complexed with Rosiglitazone which has major interactions with Ile 281, Gln283 and Leu270. The most potent compounds: SD-1, SD-3 and SD-ss9 have amino acid interactions. The benzyl ring of the compound SD-1 shows the

π-π cation with Phe247 and Tyr265, van der Waals interaction are with N of pyridynyl ring. Lys261 shows the π-cation interaction with thiazolidinedione ring. In addition, the phenyl ring of the compound SD-3, shows the π–sigma interaction with Leu270. The substituted methoxy group shows van der Waals interaction with the Gln 283 and alkyl interaction with the Arg280. The π–sulphur interaction is observed with Phe287 and π –sigma interaction with Arg 288. Finally, the carbonyl function of thiazolidinedione compound shows conventional hydrogen bond interaction with the Arg288. These interactions can be observed in Figures 3 to 6. Other interactions are given in Table 1.

Acute Oral Toxicity Test

The acute oral toxicity test of molecules SD-1, SD-3 and SD-9 were performed and found no toxic effect even at 2000 mg/kg. It is confirmed thus that our compounds do not show any side effect even at the higher dose.

Antidiabetic Activity

Three compounds: SD-1, SD-3 and SD-9 exhibited significant reduction in fasting blood glucose levels after 8 days of treatment compared to standard drug Pioglitazone. (Table 2).

Antidiabetic Activity in Mice

Further, to understand structure activity relationship log *p* values were calculated by the One-Way ANOVA (Dunnet hoc test) test

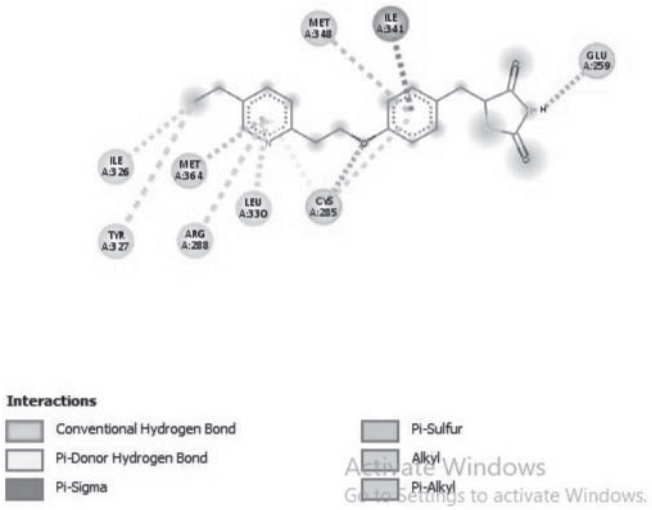

Figure 3: Amino acid interactions of Pioglitazone.

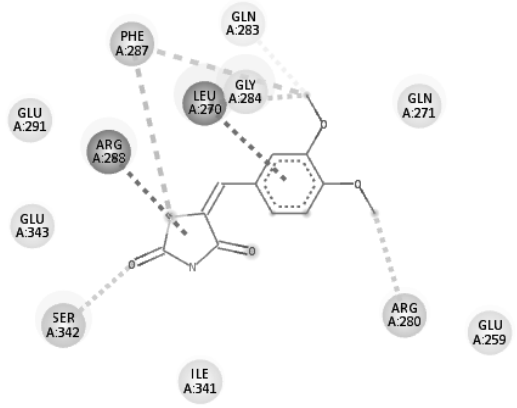

Figure 5: Amino acid interactions of 5-(3,4-dimethoxybenzylidene)-2,4-thiazolidinedione. (SD-3).

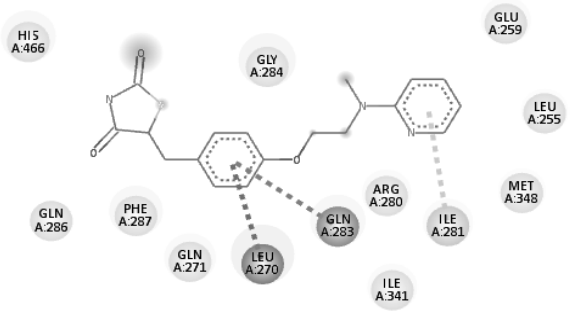

Figure 4: Amino acid interactions of Rosiglitazone.

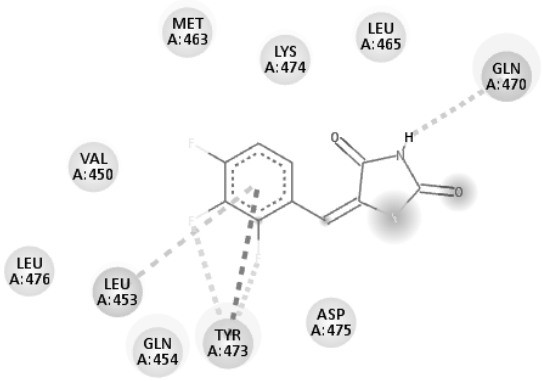

Figure 6: Amino acid interactions of 5-(2,3,4- trifluorobenzylidene)-2,4-thiazolidinedione (SD -9).

Table 2: Effect of Test Compounds on Streptozotocin Induced Diabetic Mice.

Group	Blood Glucose level(mg/dl) 1st day	Blood Glucose level(mg/dl) after 72 hr of STZ administration	Blood Glucose level(mg/dl) after 8 days of treatment
NC	160	----	154
DC	170	238	245
SD-1	158	236	133
SD-3	152	230	124
SD-9	160	231	125
Pioglitazone	165	222	127

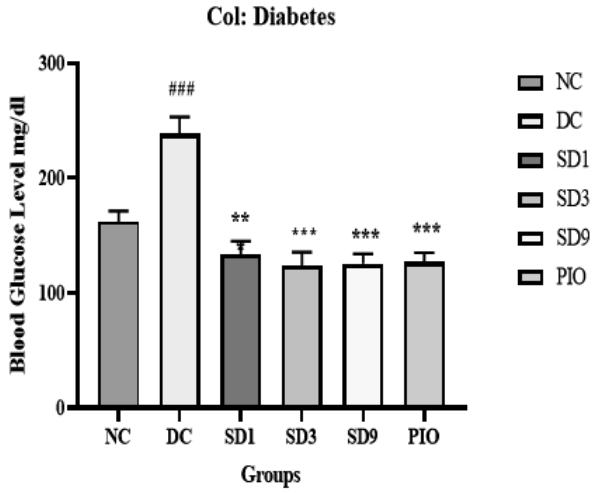

Figure 7: Effect of SD-1, SD-3, SD-9 and Standard Drug Blood Glucose Level in Streptozotocin Induced Diabetic Mice. $p<0.0001$ Which is Significant and Indicated in Terms of ***. NC= Normal control, DC= Diabetic control, PIO= Std drug Pioglitazone.

using Prism Software. Significant *p* value of all three compounds is <0.001. The results are shown in Figure 7. The compounds having Log *p* value between 1-2 show good antidiabetic activity in mice.

CONCLUSION

Recent studies have indicated that cardiovascular toxicity with Rosiglitazone and increase in bladder cancer with Pioglitazone. These drugs are no longer drugs of choice. To obtain better and safe drugs containing thiazolidine-2,4-dione moiety, we have attempted modification by introducing suitable substituents at C-5 of thiazolidine-2,4-dione nucleus.[13] A novel series of thiazolidine-2,4-dione analogues has been thus synthesized. The structures of these compounds were established by IR, ^{1}H-NMR and MASS spectrometry. The acute oral toxicity test of these molecules were performed and found no toxic effect even at 2000mg/kg of drug. Three compounds were tested for antidiabetic activity in swiss albino mice. The synthesized compounds were then docked with the PPAR-γ (PDB ID: 2PRG) using Auto Dock Vina software. The results indicated interaction with Gly283 and Leu270 comparable with Rosiglitazone. Among all the synthesized compounds, three derivatives; SD-1, SD-3 and SD-9 have similar amino acid interactions as of Rosiglitazone and Pioglitazone. These compounds also showed better glucose lowering effect.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Original Article

Analog-based Design and Development of Novel Molecules as Anti-tubercular Agents

Satheeshkumar Sellamuthu[1,*], Mohan Sellapan[1], Kandasamy CS[1], Dileep Kumar[2, 3, 4], Kavitha Kasimayan[1], Sasikala Manickavasagam[1]

[1]Karpagam College of Pharmacy, Othakkalmandapam, Coimbatore, Tamil Nadu, INDIA.
[2]Poona College of Pharmacy, Bharati Vidyapeeth (Deemed to be University), Pune Maharashtra, INDIA.
[3]Department of Entomology, University of California, Davis, One Shields Ave, Davis, CA, USA.
[4]UC Davis Comprehensive Cancer Centre, University of California, Davis, One Shields Ave, Davis, CA, USA.

ABSTRACT

Background: The burden of *tuberculosis* is immense as most of the drugs developed resistance to *Mycobacterium tuberculosis* (Mtb). This issue shall be addressed by developing new drugs acting through novel mechanisms. GSK 2556286 (GSK-286) is a phase 1 clinical candidate with a novel mechanism of action related to cholesterol catabolism, hence it was selected as template/ parent molecules for our analogue-based drug design strategy. **Materials and Methods:** The novel-designed molecules were initially checked to be drug-like using 'Lipinski's rule of five and synthesized with good yields. The obtained compounds were evaluated for anti-tubercular activity against Mtb *H37Rv,* antibacterial activity against *S. aureus* and *E. coli*, cytotoxicity screening against mammalian VERO cells and BBB permeability against Porcine Brain Lipid. Docking was also performed against HsaA monooxygenase (PDB ID: 3AFF) to understand the possible mechanism of action. **Results and Conclusion:** The compounds 3a and 7a exhibited promising anti-tubercular activity at MIC of 3.13 µg/mL. Further, the compounds proved to be effective towards *S. aureus* at 0.98 µg/mL and *E. coli* at 7.81 µg/mL. The molecules 3a and 7a produced good docking scores of -9.2 and -9.3 kcal/mole respectively. The association between docking results and anti-tubercular activity has postulated that the molecules could act through novel mechanisms by inhibiting HsaA monooxygenase. Moreover, all the compounds in the study produced very less cytotoxicity at CC_{50} > 190 µg/mL against mammalian VERO cells and also showed minimal BBB permeability in PAMPA assay. The effective anti-tubercular activity of the lead molecules with additional safety against mammalian VERO cells with reduced BBB permeability could provide a new standpoint in anti-tubercular drug discovery.

Keywords: Analog-based Design, Anti-tubercular activity, BBB permeability, GSK 2556286, VERO cells.

Correspondence:
Dr. Satheeshkumar Sellamuthu
Associate Professor, Karpagam College of Pharmacy, Othakkalmandapam, Coimbatore-641032, Tamil Nadu, INDIA.
Email id: satheeshpharma6@gmail.com

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INTRODUCTION

Tuberculosis (TB) was the foremost cause of death until the emergence of COVID-19 due to a single infectious agent.[1] The challenges in controlling TB were attributed to the rapid development of resistant strains and comorbidity of HIV along with TB.[2] WHO estimated that 10 million people get affected by TB in 2020. Among them, men accounted for 5.6 million cases, women 3.3 million and children 1.1 million cases.[3] An effective method to control any infectious disease is vaccination.[4] The BCG vaccine is used as a preventive measure in the control of TB, but *Mycobacterium tuberculosis* (Mtb) masks the protective efficacy of the vaccine by inducing heterologous immunity.[5,6] The severe cases of drug-resistant TB viz. MDR-TB and XDR-TB are reported which pose a serious threat to the control of TB. Hence, new treatments are the need of the hour to combat the deadly infectious disease. Several drug candidates are in the pipeline with efforts on developing new drugs for treating TB. After the approval of Rifapentine in 1998 to treat TB, Bedaquiline, Pretomanid and Delamanid were the only three new drugs that got US-FDA approval in recent years.[7,8] Therefore, there is a huge necessity for the discovery of novel mechanism-acting TB drugs to battle the different resistant strains of TB. GlaxoSmithKline, Melinda Gates Foundation and TB Drug Accelerator groups are working together on the development of GSK2556286 as a novel drug with a new mechanism of action in treating TB. GSK2556286 acts by inhibiting a novel enzyme target, HsaA monooxygenase involved in cholesterol metabolism.[9] The objective of this study is to develop lead molecules homologous to GSK2556286, a phase 1 clinical candidate and acting through the novel target HsaA monooxygenase. The approach of drug design is to retain and

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modify certain parts of pharmacophore and linker in an attempt to attain novel molecules analogues to GSK2556286. Thereby, the designed molecules could act through a novel mechanism of action by inhibiting cholesterol metabolism. During the design of molecules, we retained the phenoxy group from GSK2556286, modified the piperidine to benzene and uracil to a substituted-phenyl ring from GSK2556286 to obtain a series of novel molecules (Figure 1) and the novelty of these molecules was affirmed through ZINC database search with all subsets. The molecules were further checked against Lipinski's rule of five to affirm the drug-likeness and later taken for synthesis and biological evaluation for antitubercular activity, antibacterial activity, cytotoxicity and BBB permeability.

MATERIALS AND METHODS

Procedure for Synthesis of 1-bromo-4-phenoxybenzene (2)

Diphenyl ether (1.7g, 0.01M) was dissolved in carbon tetrachloride (100 mL) and bromine solution (1.6g, 0.02M) was added slowly with occasional stirring for 1 hr. Hydrogen bromide evolved during the reaction. The solvent was distilled to obtain the crude product. The byproducts viz dibromo diphenyl ether and other higher brominated derivatives were removed in petroleum ether.[10]

Procedure for the Synthesis of 1-(4-phenoxyphenyl) urea (3)

1-bromo-4-phenoxybenzene (2.5g, 0.01M) and urea (1.2g, 0.02M) were dissolved in Dichloromethane (DCM). Then, 1mL (0.01M) of triethylamine was added and refluxed for 2 hr.[11] The solvent in the reaction mixture was removed through distillation. The residue obtained was purified through an acid-base workup to get pure 1-(4-phenoxyphenyl)urea.[11]

Procedure involved in the Synthesis of diphenyl ether tethered urea derivatives (1a-15a)

1-(4-phenoxyphenyl)urea (3) (2.3g, 0.01M), obtained in the previous step appropriate was added with corresponding chloro-benzenes (0.02M), K_2CO_3 (0.005M) and KI (0.05M) and refluxed using THF as a solvent for 3 hr at 80°C.[12] The progression of the chemical reaction was checked by performing TLC. After the completion of the reaction, the crude reaction mixture was transferred into crushed ice with stirring. Precipitates of different diphenylether-tethered urea derivatives were obtained, then filtered and washed with a brine solution and water. Further on the requirement, impure products were introduced in column chromatography using SiO_2 as the stationary phase and ethyl acetate: hexane as the mobile phase at different ratios (Figure 2).

1-(4-phenoxyphenyl)-3-phenylurea (1a)

Yield: 84%; MP: 143-146°C; IR (KBr, cm^{-1}): 3427 (N-H Stretch), 3097 (=CH Stretch), 1742 (C=O Stretch), 1606 (C=C Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm); 7.395-7.259 (m, 9H, Biphenyl-CH)); 7.224-7.208 (t, 2H, J=8, C3, C5-phenyl), 7.125-7.110 (t, 2H, J=7.5, C2, C6-phenyl), 7.096-7.080 (t, 1H, J=8, C4-phenyl), 3.954 (s, 1H, NH) 3.828 (s, 1H, NH); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 167.323 (C=O), 142.012, 141.884, 130.624, 130.108, 129.337, 128.670, 127.215, 126.843, 126.197 (Aromatic carbons), ESI-MASS (*m/z)*: calculated-304.35, found-305.5 (M+1, 45.2%).

1-(4-nitrophenyl)-3-(4-phenoxyphenyl)urea (2a)

Yield: 79%; MP: 154-156°C; IR (KBr, cm^{-1}): 3422 (N-H Stretch), 3105 (=CH Stretch), 1746 (C=O Stretch), 1606 (C=C Stretch), 1543 (NO_2 asymmetric stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm); 7.381-7.293 (m, 9H, Biphenyl-CH), 7.058-7.044, (dd, J=9, 1.5 Hz, 2H, C3-phenyl, C5-phenyl), 6.714, 6.697 ((dd, J=8.0, 1.5 Hz, 2H, C2-phenyl, C6-phenyl), 4.004 (s, 1H, NH) 3.894 (s, 1H, NH); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.312 (C=O), 143.084, 141.952, 129.991, 129.683, 129.472, 129.235, 128.857, 128.639, 128.274, 127.715, 126.763, 126.266, 125.778, 119.333, 119.165, 119.028, 116.002 (Aromatic carbons), ESI-MASS (*m/z)*: calculated-349.35, found- 350.7 (M+1, 74%).

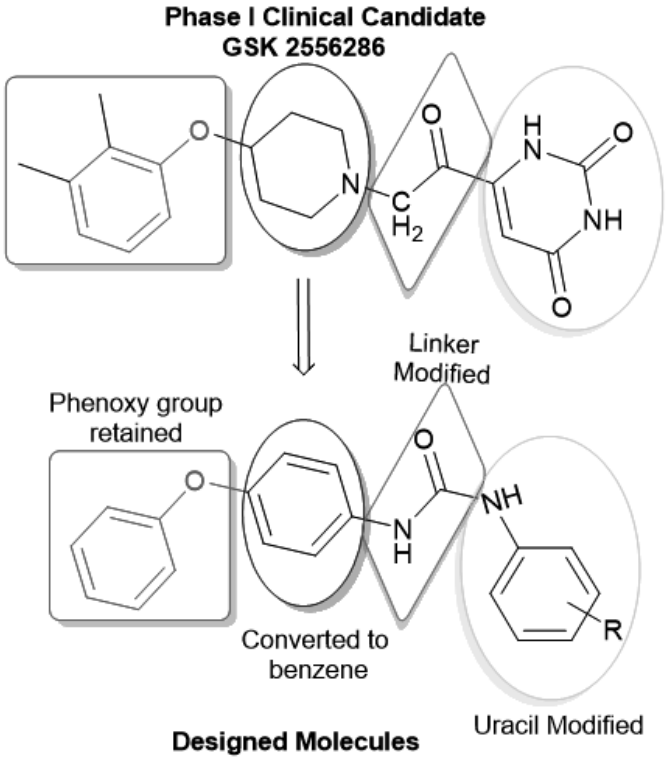

Figure 1: Strategy used in the design of new molecules.

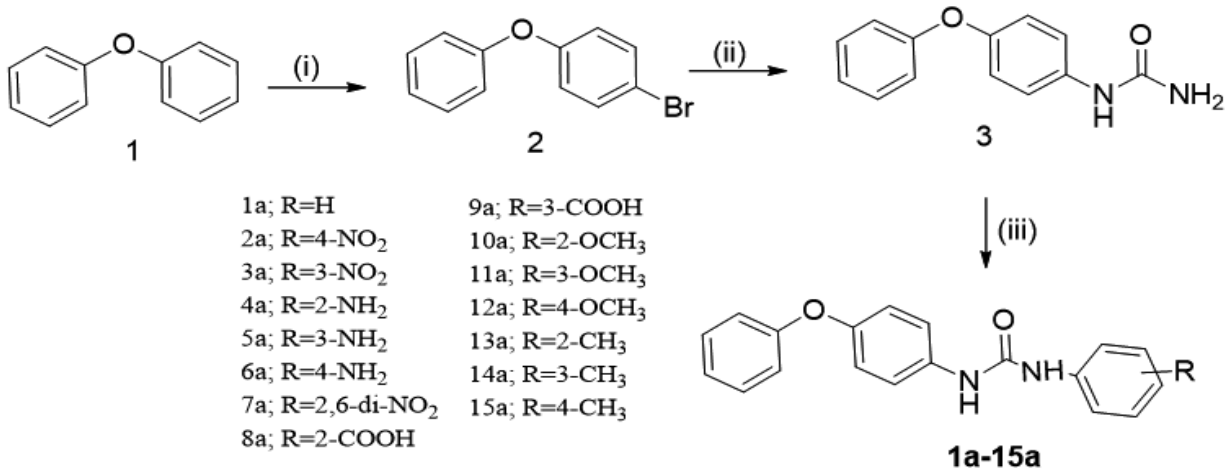

Figure 2: Synthetic scheme of the designed molecules.

1-(3-nitrophenyl)-3-(4-phenoxyphenyl)urea (3a)

Yield: 75%; MP: 153-155°C; IR (KBr, cm^{-1}): 3425 (N-H Stretch), 3089 (=CH Stretch), 1747 (C=O Stretch), 1604 (C=C Stretch), 1536 (NO_2 asymmetric stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm); 7.377-7.290 (m, 9H, Biphenyl-CH), 7.253-7.220 (t, 1H, J=7, C5-phenyl), 7.153-7.140 (d, 1H, J=8.5, C4-phenyl), 6.872-6.822 (m, 2H, C4, C6-phenyl) 3.991 (s, 1H, NH), 3.892 (s, 1H, NH); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.308 (C=O), 143.079, 141.946, 129.986, 129.679, 129.468, 129.228, 128.854, 128.633, 128.268, 127.711, 126.761, 126.262, 125.772, 119.332, 119.159, 119.025, 115.991 (Aromatic carbons); ESI-MASS (*m/z)*: calculated-349.35, found- 350.7 (M+1, 74%).

1-(2-aminophenyl)-3-(4-phenoxyphenyl)urea (4a)

Yield: 70%; MP: 145-148°C; IR (KBr, cm^{-1}): 3460, 3412 (NH_2 Stretch), 3094 (=CH Stretch), 1753 (C=O Stretch), 1608 (C=C Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm); 7.383-7.248 (m, 9H, Biphenyl-CH), 7.091-7.068 (t, 1H, J=7, C5-phenyl), 6.816-6.887 (d, 1H, J=8.5, C4-phenyl), 6.605-6.576 (m, 2H, C4-phenyl, C6-phenyl), 3.925 (s, 1H, NH) 3.868 (s, 1H, NH) 3.397 (2H, NH_2); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.289 (C=O), 145.018, 141.889, 141.668, 130.999, 130.236, 130.025, 129.727, 129.410, 129.190, 128.575, 128.364, 128.115, 126.819, 126.579, 126.291, 126.003, 123.248, 116.337 (Aromatic carbons), ESI-MASS (*m/z)*: calculated-319.36, found- 320.8 (M+1, 75%).

1-(3-aminophenyl)-3-(4-phenoxyphenyl)urea (5a)

Yield: 69%; MP: 148-150°C, IR (KBr, cm^{-1}): 3508, 3455 (NH_2 Stretch), 3095 (=CH Stretch), 1743 (C=O Stretch), 1601 (C=C Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm); 7.384-7.296 (m, 9H, Biphenyl-CH), 7.052-7.038, (t, 1H, J=7, C5-phenyl), 6.710, 6.693 (d, 1H, J=8.5, C4-phenyl), 6.559-6.508 (m, 2H, C2-phenyl, C6-phenyl), 4.081 (s, 1H, NH), 4.906 (s, 1H, NH), 3.293 (s, 2H, NH_2); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.298 (C=O), 147.725, 141.937, 134.910, 130.370, 130.063, 129.689, 129.410, 129.199, 128.700, 128.124, 126.809, 126.300, 126.070, 125.859, 125.542, 118.506, 115.051, 113.323 (Aromatic carbons); ESI-MASS (*m/z)*: calculated-319.36, found- 320.5 (M+1, 72%).

1-(4-aminophenyl)-3-(4-phenoxyphenyl)urea (6a)

Yield: 66%; MP: 152-155°C; IR (KBr, cm^{-1}): 3502, 3448 (NH_2 Stretch), 3089 (=CH Stretch), 1739 (C=O Stretch), 1600 (C=C Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm); 7.384, 7.308 (m, 9H, Biphenyl-CH), 7.250, 7.235 (d, 2H, J=7.5, C3-phenyl, C5-phenyl), 6.855, 6.838 (d, 2H, J=8.5, C2, C6-phenyl), 3.982 (s, 1H, NH), 3.802 (s, 1H, NH), 3.173 (s, 2H, NH_2); 125 MHz-125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.289 (C=O), 143.770, 142.455, 141.908, 130.102, 129.785, 129.401, 129.055, 128.700, 126.560, 126.195, 125.792, 116.164, 115.799, 115.627, 113.985, 113.928 (Aromatic carbons); ESI-MASS (*m/z)*: calculated-319.36, found- 320.3 (M+1, 74%).

1-(2,6-dinitrophenyl)-3-(4-phenoxyphenyl)urea (7a)

Yield: 75%; MP: 160-162°C, IR (KBr, cm^{-1}): 3431 (N-H Stretch), 3091 (=CH Stretch), 1748 (C=O Stretch), 1614 (C=C Stretch), 1549 (NO_2 asymmetric stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm): 7.384, 7.296 (m, 9H, Biphenyl-CH), 7.048-7.034, (d, 2H, J=7, C3-phenyl and C5-phenyl), 6.811, 6.794 (m, 1H, C4-phenyl), 3.999 (s, 1H, NH), 3.864 (s, 1H, NH); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.298 (C=O), 147.926, 141.937, 130.658, 130.543, 130.044, 129.766, 129.410, 128.959, 128.585, 128.115, 127.040, 126.665, 126.195, 125.734, 123.104,

121.386, 117.921, 113.736 (Aromatic carbons); ESI-MASS (*m/z)*: calculated-394.34, found- 395.4 (M+1, 70%).

2-(3-(4-phenoxyphenyl)ureido)benzoic acid (8a)

Yield: 60%; MP: 158-160°C, IR (KBr, cm^{-1}): 3456 (O-H Stretch), 3228 (N-H Stretch), 3096 (=CH Stretch), 1737 (C=O Stretch-carbamide), 1694 (C=O Stretch-carboxylic), 1599 (C=C Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm): 12.203 (s, 1H, OH), 7.814, 7.798 (d, J=8 Hz, 1H, C3-phenyl), 7.389-7.308 (m, 9H, Biphenyl-CH), 7.013, 6.988 (m, 2H, C4-phenyl, C6-phenyl, 6.794-6.768 (t, J=7 Hz, 1H, C5-phenyl), 3.551 (s, 1H, NH) 3.411 (s, 1H, NH); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.289 (C=O), 145.546, 141.937, 132.079, 130.159, 129.977, 129.785, 129.410, 129.209, 128.662, 128.537, 128.297, 127.942, 127.385, 126.636, 126.291, 126.108, 125.888, 116.798, 110.242 (Aromatic carbons); ESI-MASS (*m/z)*: calculated-348.36, found-349.8 (M+1, 70%).

3-(3-(4-phenoxyphenyl)ureido)benzoic acid (9a)

Yield: 62%; MP: 162-165°C, IR (KBr, cm^{-1}): 3461 (O-H Stretch), 3235 (N-H Stretch), 3089 (=CH Stretch), 1758 (C=O Stretch-carbamide), 1710 (C=O Stretch-carboxylic), 1599 (C=C Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm): 12.218 (s, 1H, OH), 7.535-7.498 (m, 2H, C2, C4-phenyl) 7.392-7.258 (m, 9H, Biphenyl-CH), 7.112-7.098 (t, J=7.5 Hz, 1H, C5-phenyl), 7.003-6.892 (d, 1H, C6-phenyl), 3.524 (s, 1H, NH); 3.434 (s, 1H, NH); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.289 (C=O), 148.329, 142.157, 141.927, 135.477, 130.236, 129.977, 129.410, 128.969, 128.220, 128.009, 127.855, 127.510, 126.876, 126.233, 125.830, 118.372, 113.294 (Aromatic carbons); ESI-MASS (*m/z)*: calculated- 348.36, found- 349.8 (M+1, 75%).

1-(2-methoxyphenyl)-3-(4-phenoxyphenyl)urea (10a)

Yield: 68%; MP: 170-172°C, IR (KBr, cm^{-1}): 3436 (N-H Stretch), 3087 (=C-H Stretch), 2923 (-C-H Stretch), 1755 (C=O Stretch-carbamide), 1607 (C=C Stretch), 1220 (C-O Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm): 7.392-7.301 (m, 9H, Biphenyl-CH), 7.375-7.256 (m, 3H, C3, C4, C6-phenyl) 6.989 (t, 1H, C5-phenyl), 3.538 (s, 1H, NH), 3.691 (s, 1H, NH), 3.291 (s, 3H, CH_3); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.289 (C=O), 148.771, 141.927, 141.860, 130.015, 129.823, 129.458, 129.055, 128.710, 128.355, 128.057, 126.963, 126.694, 126.243, 126.003, 125.619, 123.709, 123.402, 116.587 (Aromatic carbons), 32.725 (methyl carbon); ESI-MASS (*m/z)*: calculated- 334.38, found- 335.4 (M+1, 70%).

1-(3-methoxyphenyl)-3-(4-phenoxyphenyl)urea (11a)

Yield: 65%; MP: 168-170°C; IR (KBr, cm^{-1}): 3433 (N-H Stretch), 3086 (=C-H Stretch), 2941 (-C-H Stretch), 1746 (C=O Stretch-carbamide), 1606 (C=C Stretch), 1158 (C-O Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm); 7.394-7.258 (m, 9H, Biphenyl-CH), 7.252-7.188, (t, 1H, J=8, C5-phenyl), 6.846, 6.789 (d, 1H, J=8.0, C6-phenyl), 6.648-6.507 (m, 2H, C2, C4-phenyl), 3.328 (s, 1H, NH), 3.198 (s, 3H, CH_3); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.289 (C=O), 152.649, 150.748, 143.204, 141.908, 130.169, 129.257, 128.595, 128.259, 126.761, 126.204, 125.926, 121.117, 120.973, 116.999, 116.827, 116.491, 114.312 (Aromatic carbons), 38.165 (methyl carbon); ESI-MASS (*m/z)*: calculated- 334.38, found- 335.9 (M+1, 70%).

1-(4-methoxyphenyl)-3-(4-phenoxyphenyl)urea (12a)

Yield: 64%; MP: 172-174°C, IR (KBr, cm^{-1}): 3427 (N-H Stretch), 3079 (=C-H Stretch), 2950 (-C-H Stretch), 1738 (C=O Stretch-carbamide), 1586 (C=C Stretch), 1162 (C-O Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm): 7.391-7.256 (m, 9H, Biphenyl-CH), 7.157-7.036 (dd, J=9, 1.4 Hz, 2H, C3-phenyl, C5-phenyl), 6.925, 6.917 (dd, J=8, 1.5 Hz, 2H, C2, C6-phenyl), 3.671 (s, 1H, NH), 3.546 (s, 1H, NH), 3.154 (s, 3H, CH_3); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.289 (C=O), 141.917, 141.754, 130.505, 130.082, 129.506, 129.410, 128.930, 128.575, 128.335, 127.155, 126.876, 126.550, 126.070, 125.696, 119.389, 119.188, 116.107, 115.416, 115.233 (Aromatic carbons), 38.744 (methyl carbon); ESI-MASS (*m/z)*: calculated- 334.38, found- 335.7 (M+1, 70%).

1-(4-phenoxyphenyl)-3-(o-tolyl)urea (13a)

Yield: 70%; MP: 151-152°C, IR (KBr, cm^{-1}): 3425 (N-H Stretch), 3095 (=C-H Stretch), 2975 (-C-H Stretch), 1746 (C=O Stretch-carbamide), 1603 (C=C Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm); 7.375-7.258 (m, 9H, Biphenyl-CH), 7.098-7.016, m, 3H, C3, C4, C6-phenyl, 6.891-6.875 (t, 1H, J=8, C5-phenyl), 3.562 (s, 1H, NH) 3.419 (s, 1H, NH), 2.792 (s, 3H, CH3; 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.356 (C=O), 152.754, 142.071, 141.879, 141.754, 139.086, 130.284, 130.140, 129.689, 129.410, 128.921, 128.633, 128.326, 127.155, 126.742, 126.425, 126.137, 113.429 (Aromatic carbons), 31.763 (methyl carbon); ESI-MASS (*m/z)*: calculated- 318.38, found- 319.1 (M+1, 70%).

1-(4-phenoxyphenyl)-3-(m-tolyl)urea (14a)

Yield: 72%; MP: 152-154°C, IR (KBr, cm^{-1}): 3424 (N-H Stretch), 3097 (=C-H Stretch), 2994 (-C-H Stretch), 1739 (C=O Stretch-carbamide), 1599 (C=C Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm); 7.371-7.253 (m, 9H, Biphenyl-CH), 7.151-7.137 (m, 1H, J=8.5, C5-phenyl), 6.852-6.836 (m, 2H, J=8, C2, C4, C6-phenyl), 3.569 (s, 1H, NH), 3.496 (s, 1H, NH), 2.842 (s, 3H, CH_3); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.356 (C=O), 152.753, 142.102, 141.881, 141.754, 139.095, 130.285, 130.141, 129.691, 129.464, 128.936, 128.630, 128.321, 127.251, 126.447, 126.421, 126.034, 113.668 (Aromatic carbons), 33.510

(methyl carbon); ESI-MASS (*m/z)*: calculated- 318.38, found- 319.2 (M+1, 70%).

1-(4-phenoxyphenyl)-3-(p-tolyl)urea (15a)

Yield: 75%; MP: 156-158°C, IR (KBr, cm^{-1}): 3427 (N-H Stretch), 3095 (=C-H Stretch), 2994 (-C-H Stretch), 1740 (C=O Stretch-carbamide), 1587 (C=C Stretch); 500 MHz- ^{1}H-NMR, Solvent-$CDCl_3$ δ (ppm): 7.389-7.252 (m, 9H, Biphenyl-CH), 7.151-7.079 (dd, J=9, 1.6 Hz, 2H, C3-phenyl, C5-phenyl), 6.895-6.881 (d, J=8 Hz, 1H, C2, C6-phenyl), 4.645 (s, 1H, NH), 4.583 (s, 1H, NH), 2.979 (s, 3H-CH_3); 125 MHz- ^{13}C-NMR, Solvent-$CDCl_3$ δ: 166.317 (C=O), 149.059, 141.937, 141.678, 138.798, 129.353, 128.710, 128.489, 128.143, 127.932, 126.867, 126.329, 126.032, 125.868, 125.475, 124.390, 117.690, 113.803 (Aromatic carbons), 32.681 (methyl carbon); ESI-MASS (*m/z)*: calculated- 318.38, found- 319.4 (M+1, 75%);

Antitubercular screening

The Minimum Inhibitory Concentration (MIC) of the study compounds was evaluated against H37Rv strain of *Mycobacterium tuberculosis* by performing a Microplate Alamar-Blue assay (MABA).[13] The lowest concentration of the drug that prevented the growth of the Mtb was marked as the MIC. All the wells of 96 well plates were added with Middlebrook 7H9 broth media (100 µL) and the synthesized compounds were added at 100 to 0.2 µg/mL. All the wells except the blank well were then added with Mtb inoculum (100 µL). The parafilm wrapping was done and incubated at 37°C for continuous 5 days. The Alamar-blue reagent mixed with 10% tween 80 at a 1:1 ratio was added to each well (25 µL) and once again incubated for another twenty-four hours. The observation of pink colour in the well was recorded as the growth of the Mtb cells, whereas the blue colour was manifested as the absence of growth. The study was performed thrice to nullify the chance of error in interpretation.

Antibacterial screening

The antibacterial activity of the study compounds was performed against *E. coli* (ATCC 35218) and *S. aureus* (ATCC 25323) to understand the spectrum of activity exerted by the compounds. The Agar-plate disc-diffusion method was adopted for the study.[11,14] 20 mL of Mueller Hinton Agar was spread over the petri-dish. Then the inoculum of *E. coli* or *S. aureus* was prepared by mixing with sterile physiological saline solution by adjusting the turbidity to ~10^6 colony-forming units per millilitre. The prepared inoculum was then evenly streaked over the agar media in a petri dish. The synthesized compounds/ standards were saturated to Whatman paper discs of 6 mm diameter and positioned over the inoculated agar plate. Dimethyl sulfoxide was used as the negative control and Ciprofloxacin as the positive control. The treated agar plates were incubated for twenty-four hours. The lowest concentration of the drug that prevented the growth of the Mtb was recorded as the MIC against *S. aureus* and *E. coli*. The study was repeated thrice to obtain data in triplicate.

Cytotoxicity screening

The cytotoxicity of study compounds was evaluated against mammalian VERO cells to understand the safety of synthesized compounds against normal human cells. VERO Cells (1x 10^5 / well) were plated in 96-well plates containing 100µl of the medium and incubated in 5% CO_2 for twenty-four hours at 37°C. The cells were then incubated with test compounds at different concentrations (7.8 µg/mL to 1000 µg/mL) for another seventy-two hours. Samples were washed with pH 7.4 phosphate-buffered saline. MTT dye was introduced and once again incubated for four hours. DMSO (1mL) was added and absorbance was measured at 570nm to determine the percentage of cell viability.[15]

$$\% \text{ Cell Viability} = \frac{\text{Absorption recorded in test sample}}{\text{Absorption recorded in control}} \times 10$$

BBB permeability screening (PAMPA assay)

A membrane-permeability assay (PAMPA) was carried out to assess the Blood Brain Barrier (BBB) permeability of the study compounds.[16] The assay required an acceptor and a donor well plate. The acceptor plate was first moisturized with pH 7.4 buffer for 30 min. The buffer was then removed and the wells were coated with Porcine Brain Lipid (4 µL) having dodecane at 20 mg/mL concentration. The donor plate was transferred with 200 µL of test samples at 25 µg/mL concentration. BBB of Verapamil, Diazepam, Atenolol and Levofloxacin was performed for comparative study. Both the plates were then sandwiched and incubated for eighteen hours at room temperature. The sandwiched plates were then separated and recorded the absorbance in the acceptor plate as well as in the donor plate using an ELISA plate reader. The concentration of the drug that infused across the membrane was determined based on recorded absorbance by applying the following equation;

$$Pe\ (cm/s) = \{-\ln[1-CA(t)/Ceq]\}/[A*(1/VD+ 1/VA)*t]$$

Where, Pe is the measured permeability in cm sec^{-1}; A is filter area (i.e., 0.3 cm^2); VD is the volume of donor well (i.e 0.3 mL); VA is the volume of acceptor well (i.e., 0.2 mL); t is the duration of incubation (sec); CA(t) is the concentration of drug in acceptor well at particular time duration; CD(t) = concentration of drug in donor well at particular time duration.

$$Ceq = [CD(t)*VD + CA(t)*VA]/(VD+VA)$$

RESULTS AND DISCUSSION

Chemistry

As shown in Figure 2, the molecules were synthesized. It involved the synthesis of 1-bromo-4-phenoxy benzene (2) followed by 1-(4-phenoxy phenyl) urea (3). Different chlorobenzenes were reacted with 1-(4-phenoxyphenyl) urea to obtain the final derivatives. Concomitantly TLC was performed to monitor the progress/completion of the reaction. Crude products obtained at the end of the reaction were purified by performing column chromatography to get pure components. The final pure compounds were obtained with yields ranging from 45 to 79%. Melting point and TLC were performed to confirm the purity. ^{1}H and ^{13}C NMR and Mass Spectral data were obtained and structures of the synthesized compounds were interpreted.[16,17]

Antitubercular Activity

The antitubercular activity was determined by performing Micro Plate Alamar-Blue Assay on the synthesized compounds against the virulent strains of Mtb H37Rv.[13] Minimum Inhibitory Concentration (MIC) was calculated based on the presence or absence of the growth of Mtb and the result was recorded in Table 1. The structures of the study compounds were related to the anti-tubercular activity and found that the compounds containing nitro groups (3a, 7a) were found most active among all the compounds screened for antitubercular activity at MIC of 3.13μg/mL. The anti-tubercular activity of lead compounds 3a and 7a was found comparable to the standard Pyrazinamide (PYZ). Compounds containing the carboxylic group (8a, 9a) produced antitubercular activity at MIC of 6.25μg/mL. The electron-withdrawing nitro and carboxylic groups would have positively influenced the antitubercular activity. Further, the compounds containing the electron-donating methyl group (13a, 14a, 15a) have shown a drastic reduction in antitubercular activity. Varying the substitutions on the phenyl ring showed diverse results based on the nature of the substituent groups, while the basic core moiety (1a) retained the activity at MIC of 6.25μg/mL. It can be inferred that molecules designed in analogy to the parent molecule GSK 2556286 were found to exhibit a positive effect against *Mycobacterium tuberculosis* and they shall be further optimized for improved activity.

Antibacterial Activity

The synthesized compounds were further evaluated against *E. coli* and *S. aureus* to determine the spectrum of activity. The study was performed through the disc diffusion method.[18] Compounds 3a, 7a, 8a, and 9a have shown good activity against *S. aureus* at the concentration of 0.98 μg/mL in comparison to the standard Ciprofloxacin (CPFX) whereas against *E. coli*, compounds 2a, 3a, 7a-12a have produced activity only the concentration of 7.81μg/mL (Table 1). The compounds have shown comparatively better against *S. aureus* than *E. coli.* The difference in the activity between the gram-positive and gram-negative organisms may be related to the permeability of the molecules through the cell wall.[19] Compounds containing nitro and carboxylic groups were found to show better activity over other substituents against both *E. coli* and *S. aureus.*

Table 1: Antibacterial and Anti-tubercular effects of the study compounds.

Code	*S. aureus* MIC in μg/mL	*E. coli* MIC in μg/mL	Mtb MIC in μg/mL
1a	3.91	15.63	12.5
2a	1.95	7.81	6.25
3a	0.98	7.81	3.13
4a	3.91	15.63	>25
5a	3.91	15.63	12.5
6a	3.91	15.63	12.5
7a	0.98	7.81	3.13
8a	0.98	7.81	6.25
9a	0.98	7.81	6.25
10a	3.91	7.81	12.5
11a	1.95	7.81	12.5
12a	1.95	7.81	12.5
13a	15.63	31.25	>25
14a	15.63	31.25	>25
15a	15.63	31.25	>25
CPFX	1.95	3.91	3.13
PZA	Not Determined	Not Determined	3.13

Table 2: Cytotoxicity and selectivity index of the study compounds.

Code	CC_{50} in µg/mL [a]	Selectivity Index [b]
1a	255.5±0.64	20.4
2a	198.1±0.82	31.7
3a	190.3±0.37	60.8
4a	225.8±4.54	9.0
5a	232.4±0.83	18.6
6a	195.7±0.43	15.7
7a	190.2±0.66	60.8
8a	209.8±1.26	33.6
9a	220.4±0.83	35.3
10a	225.8±1.21	18.1
11a	222.5±2.55	17.8
12a	218.1±1.49	17.4
13a	232.8±0.81	9.3
14a	240.2±0.43	9.6
15a	244.2±0.45	9.8

[a] Data are provided as mean ± SEM (n =3)[b] Selectivity index- calculated by taking ratio of cytotoxicity (CC_{50}) to Mtb MIC

Table 3: Effective BBB permeability estimation of the study compounds.

Compound Code	P_e x10^{-6} cm/s	Classification
Verapamil (Peripheral drug)	0.0	Low permeability (CNS-)
Diazepam (CNS drug)	12.4	High permeability (CNS+)
Levofloxacin (Peripheral drug)	0.0	Low permeability (CNS-)
1a	2.3	Permeability Uncertain (CNS+/-)
2a	2.2	
3a	2.1	
4a	2.5	
5a	2.2	
6a	2.1	
7a	2.0	
8a	2.6	
9a	2.6	
10a	2.6	
11a	2.6	
12a	2.6	
13a	3.0	
14a	3.2	
15a	3.0	

Cytotoxicity Screening and Selectivity index

For a drug to be safe, it should not produce any toxicity towards normal cells. To evaluate the cytotoxicity, the study compounds were screened against the kidney epithelial cell line of an African green monkey. The concentration required to produce 50% cytotoxicity (CC_{50}) was determined and all the compounds exhibited CC_{50} >180 µg/mL, which falls in the safe range and proved the study compounds are non-toxic towards normal mammalian cells (Table 2). The selectivity of the molecules towards the desired activity will avoid the undesired side effects. The antitubercular drugs should show selectivity towards *Mycobacterium tuberculosis* rather than normal human cells. Therefore, the selectivity index of the synthesized compounds was calculated by taking the ratio of the obtained cytotoxicity (CC_{50}) values and the obtained antitubercular MIC values. The lead compounds from the anti-tubercular study (3a to 7a) showed a good selectivity index > 57 and the result of the compounds showed nominal selectivity >9. The lead compounds are selective towards Mtb and safe towards normal mammalian cells.

Blood Brain Barrier (BBB) Permeability

BBB permeability is the main cause for neurotoxicity of any peripherally acting drugs, therefore PAMPA assay needs to be performed to study the BBB permeability. The study provides important information about CNS+/- effects of the synthesized compounds. The BBB permeability of our study compounds was equated with drugs in the market and our compounds are classified into three categories viz low permeable (CNS-) having Pe= < 2.0 * 10^{-6} cm s^{-1}, high permeable (CNS+) having Pe= > 4.0 * 10^{-6} cm s^{-1}, and permeable uncertain (CNS+/-) having Pe =4.0 - 2.0 *10^{-6} cm s^{-1}. Diazepam is a highly permeable drug with Pe=12.4 x10^{-6} cm/s, whereas levofloxacin and verapamil are low permeable drugs with Pe=0.0 cm/s. The lead compounds of anti-tubercular study (3a and 7a) produced Pe of 2.1 * 10^{-6} cm s^{-1} and 2.0 * 10^{-6} cm s^{-1} respectively and classified under permeable uncertain (CNS+/-) (Table 3). The positive point is that the Pe is close to the border to be classified as low permeable (CNS-). Therefore, the lead compounds can be easily optimized to get into the low permeable (CNS-) class by introducing more polar groups as substituents in the phenyl ring.

Molecular Docking

The synthesized molecules were docked against HsaA monooxygenase (PDB ID: 3AFF) using a web-based molecular docking program 1-Click Mcule.[20] All the molecules entered the binding site of the substrate[21] and produced critical interactions with the active site residues Trp84, Ile88, Tyr120, Trp141, Ser143, Trp182, Leu187 and His368, which are presented in Table 4. The molecules 3a and 7a produced interaction/binding energies of -9.2 and -9.3 kcal/mol respectively, which are close to -10.1 kcal/mol of the parent molecule GSK 2556286. The docking poses of 3a and GSK 2556286 are shown in Figure 3. The molecules 2a, 8a

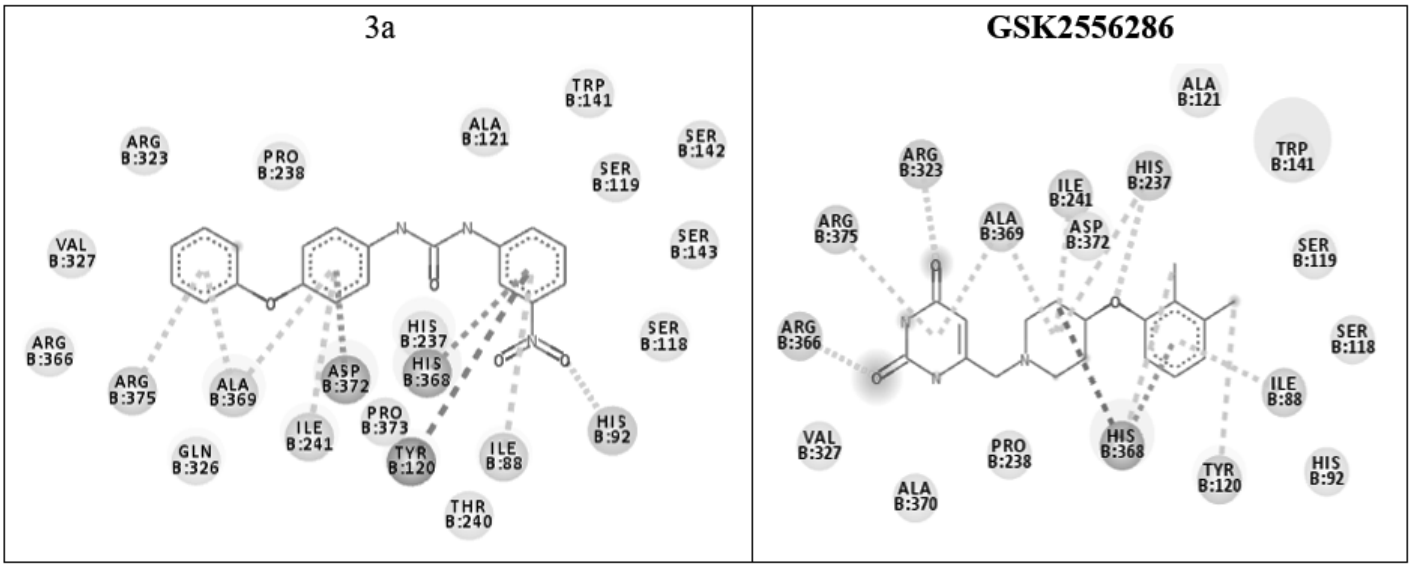

Figure 3: Docking poses of 3a and GSK 2556286 against HsaA monooxygenase.

Table 4: Binding energies and the interacting residues of HsaA monooxygenase with the ligand molecules.

Code	Binding/ Interaction energy (Kcal/mol)	Interacting residues of HsaA monooxygenase with the ligand molecules (Active site residues Trp84, Ile88, Tyr120, Trp141, Ser143, Trp182, Leu187 and His368)
1a	-7.5	His368, Ile88
2a	-8.8	His368, Ile88
3a	-9.2	His368, Ile88, Tyr120
4a	-7.3	His368, Ile88
5a	-7.5	His368, Ile88
6a	-7.3	His368, Ile88
7a	-9.3	His368, Ile88, Tyr120
8a	-7.8	His368, Ile88
9a	-7.9	His368, Ile88
10a	-6.9	His368, Tyr120
11a	-6.7	His368, Tyr120
12a	-6.0	His368, Tyr120
13a	-5.9	His368, Tyr120
14a	-5.8	His368, Tyr120
15a	-5.1	His368, Tyr120
GSK 2556286	-10.1	His368, Ile88, Tyr120

and 9a produced binding energies at -8.8 -7.8 and -7.9 Kcal/mol respectively, while the rest of the molecules produced binding energies below -7.5 kcal/mol. The results of the docking study proved apparent that there could be a correlation between the inhibition of HsaA monooxygenase and antitubercular activity. The parent molecule GSK 2556286 produced a docking score of -10.1 kcal/mol which is better than our study molecules. This indicates that GSK 2556286 could produce a better affinity towards the target HsaA monooxygenase. The study molecules must be further optimized for even better affinity towards the target.

CONCLUSION

A series of fifteen molecules were designed with drug-likeness using GSK 2556286 as the template molecule. They were then synthesized and evaluated for anti-tubercular activity and found compounds 3a and 7a were found to be effective and comparable to the standard drug Pyrazinamide. The same compounds were also proved to be effective against both gram-positive and gram-negative bacterial strains. Moreover, the lead compounds produced only negligible toxicity against the mammalian cell and exhibited reduced BBB permeability. The docking study had shown a correlation with the antitubercular activity, therefore it is postulated that the effective antitubercular activity from the lead compounds would have resulted from the novel mechanism of inhibiting HsaA monooxygenase involved in cholesterol metabolism. TB Alliance is working with GlaxoSmithKline, Bill and Melinda Gates Foundation and TB Drug Accelerator groups in developing a novel drug (GSK2556286) acting through a new mechanism. Contemporary to the research happening at the industry level, we attempted to develop such drug candidates at the academic institutional level. We believe that the lead molecules obtained from our study could provide a new vista for further drug discovery research against TB.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Original Article

An Alternative Excipient from Vegetable Source for Oral Drug Dosage Forms to Regulate Drug Delivery

Gatla Venkata Amruthavalli, Arumugam Vijayalakshmi*

Department of Pharmacognosy, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Chennai, Tamil Nadu, INDIA.

ABSTRACT

Introduction: Tablet is the preferred drug dosage form. Cellulose fiber (Micro Crystalline Cellulose) is the major excipient used. For chronic diseases, frequent dosage is required resulting in cumulative excipient load and tachyphylaxis due to drug. We intent to invent excipients from vegetable sources to mitigate above challenges and reduce dosage as chronic sufferers (Diabetes mellitus) requires lifelong treatment. **Aims and Objectives:** To develop an excipient from *Brassica oleracea* and *Vigna radiata* sprouting which can regulate drug delivery, avoid excipient led toxicity and tachyphylaxis. **Materials and Methods:** Alkali and acid based depolymerization method was followed and cellulose fibers were studied for safety, physical, chemical and molecular parameters. With the developed cellulose fibers two drugs were formulated using Active Pharmaceutical Ingredient- Metformin and Miglitol. Efficacy was assayed using cell lines-Neuroblastoma, Kidney HK-2, L6 Myoblasts, 3T3-L1 Preadipocytes, INS-1 and HEPG 2 hepatocytes and compared with conventional micro crystalline cellulose- based formulations. **Results:** Characterization of developed excipients was done in comparison with conventional cellulose, invented excipients had long fibers, higher bulk density, flow, even particle size compared to conventional cellulose. Dissolution and disintegration of Metformin from *Brassica oleracea* fiber was slow and Miglitol showed quick release from *Vigna radiata* compared to conventional excipient. Therapeutic effect of both drugs from developed excipients was higher compared to conventional excipient-based drug by cell culture assay. Both developed excipients did not show mutagenic effect. **Conclusion:** *Brassica oleracea* fiber slows release of metformin; may reduce drug dosage, tachyphylaxis, release of Miglitol from *Vigna radiata* was rapid, may compliment metformin therapy.

Keywords: Herbal excipients, Cellulose fibers, Microcrystalline cellulose, Tachyphylaxis, Vegetable source excipient, *Brassica oleracea*, *Vigna radiata*, Metformin, Miglitol.

Correspondence:
Dr. Arumugam Vijayalakshmi
Department of Pharmacognosy, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Chennai-600 117, Tamil Nadu, INDIA.
Email id: amrutha.valli4@gmail.com

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INTRODUCTION

Among various dosage forms of drugs in the allopathic system of medicine, tablet dosage form is the most preferred form of drug because of portability comfort and high degree of compliance scope by the patient.[1] Chronic diseases like psoriasis, diabetes mellitus, arthritis etc., requires lifelong medication with high frequency of drug intervention. Such kind of medication often would result in drug resistance by the system called as tachyphylaxis.[2] Besides the above, the excipient(s) of the tablet due to frequent drug usage also would produce their share of side-effects at cumulative level.[3] If time-release of the drug is achieved, the enhancement of the therapeutic value as well as dosage reduction, both can be achieved.[4] Thereby the problem of tachyphylaxis and excipient lead toxicity can be avoided.

Metformin is the most sought-after prescription drug for treating type 2 diabetes mellitus. Metformin exerts an array of pharmacological effects resulting in improved sensitivity of cells towards insulin. The frequency and high dosage of metformin is required when the blood glucose level is too high which often results in various metformin related complications, especially Vitamin B_{12} depletion.[5] Sustained release metformin of 850 mg is already available which helps to reduce the daily dosage of 1000 mg, wherever such high dosage is required. If metformin is engineered to release slow from the base (excipient) in a programmed/ sustained manner, the efficacy potentiation can be easily achieved along with dosage reduction. But as on date, a backbone excipient with 'extra' nutritional value that can replace the conventional microcrystalline cellulose (MCC) is far from reach.

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Other pharmaceutical agents used for the treatment of type II DM are combination of Miglitol and Metformin which essentially inhibit the enzyme that is responsible for the conversion of carbohydrate to glucose (α- glucosidase). With reference to the release per se of the above two drugs, we presume slow release of metformin and rapid release of miglitol may offer better therapeutic benefit to the patient.

In the present study, we have explored the scope of developing an excipient, alternative to MCC from *Brassica oleracea* (Bracoli) and *Vigna radiata* (moongdal) sprouts. We chose the above source due to rich digestible fiber contents presence, nutritional value and known safety. We have adopted the conventional acid and alkali based depolymerization method, followed by purification with H_2O_2 for obtaining cellulose fibers from the above plants.

Using the cellulose fiber thus obtained, both Metformin and Miglitol were formulated and tested by cell culture method using Neuroblastoma, Kidney HK-2, L6 Myoblasts, 3T3-L1 Preadipocytes, INS-1 and HepG2 hepatocytes. The formulation of the above drugs of the same dosage was also made with MCC and compared the activity. Sustained release metformin solid dosage form of the same strength was used for comparison.

The physio-chemical characteristics of the depolymerized cellulose fibre obtained from *Brassica oleracea* and *Vigna radiata* sprouts were studied in comparison with MCC using standard method.[6-10] Details of the findings are presented in the article.

MATERIALS AND METHODS

Preparation of depolymerized cellulose fiber from *Brassica oleracea* (Broccoli)

Fresh, healthy *Brassica oleracea* (Broccoli) was obtained and then was washed thrice, cut into small pieces to remove the stem portions and then used. Then the plant material was then treated with 50-70% NaOH at 70°C for 2 hr and then the fiber was cleaned thoroughly using water. The dried fiber was then bleached with 40% H_2O_2 three times and then washed with water. The thus obtained fiber was neutralized with 10N HCl and washed with water to remove the residual acid, air dried and stored in airtight container until use.

Preparation of depolymerized cellulose fiber from *Vigna radiata* (Mung bean) sprout

The *Vigna radiata* (Mung bean) were soaked overnight in distilled water to imbibe sufficient water and then were allowed to sprout in a cloth bag stored inside an aerated, temperature and humidity adjusted, dark chamber. Adequate care was taken to moisten the seeds regularly to facilitate sprouting. On day 7, the sprouts were separated from cotyledon shell and subjected depolymerization process as described above.

Test sample preparation for cell culture assay

We followed the method described by Aruna *et al*., 2022 for the present study.[11] In brief, Metformin 500 mg sustained release tablet (uncoated) available in the market was used as reference test sample for the present study. 500 mg metformin active pure compound was formulated as tablet using partially depolymerized cellulose fiber of *Brassica oleracea* and *Vigna radiata* sprouts separately without any other additional excipient.

Similarly, 500 metformin was also formulated with MCC without the addition of any other excipient. The tablet formulations of Metformin 500 mg were dipped into 50 mL of Phosphate buffer at different time intervals such as 0 hr, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr, 7 hr and 9 hr. After which, the mixture was centrifuged and the 10μL of the sample from each set was used for cell culture assay where the objective was to study the effective release time versus efficacy. For cytotoxic study, 10, 50 and 100 μg/mL of the test sample was used.

Similarly, the release of metformin from different formulations was measured using a spectrophotometer at 532 nm and concentration was plotted against standard curve of metformin (pure compound) in phosphate buffer.

Cell culture assay

Minimal essential medium (MEM) was used for the study. Supplements such as 1mM non- essential amino acids, 0.5 mM L-glutamine, 0.1 mM sodium pyruvate, FBS 10% were used. For the present study SK N SH human neuroblastoma cells were used. The above cells in the said medium was incubated in 5% CO_2 chamber at 37°C to allow the cells to reach 80% confluence.

Cytotoxicity assay

MTT assay was performed to study the cytotoxic effect of vegetable based cellulose fiber.[12] In brief, the cultured SK N SH cells ($0.2X10^6$ cells per well) were seeded into 96-wellplate containing in 200 mL of medium supplemented with 10% FBS and then exposed to varying concentrations of the test samples ranging from 10 to 100 μg/mL. After 24 hr of treatment, MTT reagent (20μL) was added and incubated for further 4 hr period. The formazan thus formed was dissolved in 200 μL of 0.1 N acidic isopropyl alcohol and read at 570nm.

Neuroprotection assay

The method described by Nistico *et al.*, 2008 was followed in the present study; where the neural cell - SK N SH was subjected to stress.[13] For this purpose 1.0 mM of H_2O_2 was used. One set of cells were treated with the test samples at concentration ranging from 10 to 100 μL/mL and then incubated with MTT for 3hr in a humidified CO_2 incubator with 5% CO_2. The test samples pre-treated cells after 24hr, were treated with 1.0 mM H_2O_2 to induce cell death, cell incubated for 24 hr. After incubation,

MTT assay was performed and results of test samples treated and untreated were compared.

Study on Kidney HK-2 cells

The present study was performed in line with the earlier work of Ryan *et al*., 1994.[14] In brief, Keratinocyte SFM medium with 5% FBS along with rhEGF 0.005g/mL, bovine pituitary extract 0.05 mg/mL. the above setup was incubated in 5% CO_2 chamber at 37°C. Tryphan blue dye exclusion assay was performed over $2X10^4$ cells. The cells were treated with the test samples as described above. MTT assay was performed to understand the cytotoxic effect of the test samples.

Triglyceride (TG) measurement in HepG2 cells

Triglyceride measurement in HepG2 cells was performed by adopting the method of Xiaopeng Zhu *et al*., 2018.[15] One group of HepG2 cells were treated with 30 mM of glucose and 100 nM of Insulin. To the other set of cells, in addition to the above treatment, 10 µL of test samples was also added.

TG was evaluated by digesting the PBS washed cells in trypsin (300 µL) for 2 min and then 700 µL of fresh medium was added to stop further digestion. From the total of 1000µL sample, 100 µL was used for protein quantification and rest of the 900 µL was used for TG measurement. The portion meant for TG measurement was centrifuged at 800 rpm for 3 min, then 1 mL of chloroform/ methanol (2:1 v/v) was added and vortexed for 2 hr. Then 500 µL of 0.1 M Nacl was added, centrifuged at 3700 rpm and then the lower layer was transformed, dried, treated with 40 µL of 1% Triton X, and TG was measured using the TG reagent kit.

Assay of Glucose utilization in HepG2 cells

We have adopted the method of Kerimi *et al*., 2015 for the study.[16] In brief, HepG2 cells were dislodged from tissue culture flask using 0.25% trypsin phosphate buffered saline, the cell density of 6000 cells per well was loaded in 96 well culture plate and incubated as described elsewhere. The media was changed on day 3, then the test material was added. After 2 days the medium was removed and replaced with 25 µL incubation buffer which is RPMI with PBS 0.1% BSA and 8 mM glucose. The setup was again incubated for 3 hr under the same condition followed for the cell culture.

1,1-Dimethylbiguanide hydrochloride (0.1 gm/mL) was used as positive control and without the same used as negative control. Subsequently, 10 µL of medium was transferred and incubated with 200 µL of glucose oxidase reagent to measure the concentration of glucose in the medium. After 15 min, absorbance value was taken at 492 nm using micro plate reader, the glucose utilized by the cells was calculated.

Glucose utilization by L6 Myoblasts

The method of Yap *et al*., 2007 was followed for the present study.[17] In brief, L6 Myoblasts 3000 cells/well were plated into 96 well culture plate with DMEM containing 2% FBS and incubated for 5 days. 48 hr prior to the incubation period, the medium was replaced and insulin 4g/mL instead of test sample in one row, and along with test sample was added wherein he incubation buffer was RPMI with PBS, 0.1% BSA and 8 mM glucose. Incubation of the same for 3 hr at the said condition was followed, after which 5 µL of incubation buffer was loaded into 96 well plate and then reacted with 200 µL of glucose oxidase reagent. Absorbance after 15 min incubation was done at 520 nm using micro titre plate reader. The glucose utilized by the control cells, test drug treated cells and insulin treated cells were calculated.

Lipid Accumulation in 3T3-L1 Preadipocytes

The methodology of Park and Sung, 2015 was followed in the present study.[18] 3T3-L1 Preadipocytes cells at the density of 6000 cells /well was plated into 48 well culture plate. After 2 days the cells were treated by the test samples and Rosiglitazone 0.4 gm/ mL, used as positive control. Then the cells were grown in DMEM with 10% FBS for 10 days with media replacement followed at every 2-3 days. After 10 days the cells were washed with PBS and allowed to fix at room temperature with 500 µL per well 10% formaldehyde in PBS. The fixative solution was removed and cells were stained with 200 µL pre-warmed oil red solution and incubated for 15 min. After which excess dye was washed and dried at 37°C. Then the dye was extracted in iso-proponal and then 200 µL of the solution was read at 520 nm using micro titre plate reader.

Glucose metabolism by INS-1 cells

This test was performed to determine insulin secreting ability of INS-1 cells which was done using standard procedure.[19] RPMI medium with 5% FCS was used. 8000 cells per well was seeded and MTT assay was performed. The INS-1 cells were cultured in RPMI containing 5% FCS. The cells were seeded into 96-well plates at a density of 8000 cells per well, with a volume of 100 µL. The cells were left to attach overnight and treated with the test drugs prepared as described above or PBS (which serve as a control) in the presence or absence of glucose (20 mM). After 48 hr of incubation at 37°C, medium was removed from the cells and 100 µL of DMEM medium containing 10% FCS and 0.5 mg/mL M was added and incubated for additional 30 min at 37°C. The medium was later aspirated and MTT crystal (purple formazan) was dissolved in DMSO (200 µL/well). The absorbance was read at 540 nm using a microplate reader.

Alpha glucosidase assay

According to the method described in the work of Amruthavalli *et al*., 2019 was followed in the present study.[20] In a 96- well plate reader, a reaction mixture containing 50 µL of phosphate buffer

(50 mM; pH 6.8), 10 µL of alpha-glucosidase (1 IU/mL) and 20µL of test sample (miglitol) was incubated for 5 min at 37°C, and then 20 µL of 1 mM PNPG was added to the mixture as a substrate. After further incubation at 37°C for 30 min, the reaction was stopped by adding 50 µL of sodium carbonate (0.1 M). The enzyme, inhibitor and substrate solutions were made using the same buffer. Voglibose was used as a positive control and distilled water as negative control. The yellow colour produced (due to pnitrophenol formation) was quantitated by colorimetric analysis and reading the absorbance at 405 nm.

The % inhibition calculated using the formula: % inhibition = {Absorbance (control) –Absorbance (sample)}/ Absorbance (control) IC_{50} value is defined as the concentration of extract inhibiting 50% of alpha-glucosidase activity under the stated assay conditions.

RESULTS

Yield

Two hundred gram of broccoli of two different batches were taken for partial depolymerization. After reducing the moisture level to 5% by drying, we got the yield of 120/200 and 141/200 g respectively for two batches. After subjecting partial depolymerisation process on the above fiber, the final yield obtained was 102/120 and 121/141 gm with the percentage final yield of 85 and 86 respectively for the two batches Table 1.

Two hundred gram of Mung bean sprouting of two different batches were taken for partial depolymerization. After drying to attain 5% moisture level, we got the yield of 76/200 and 81/200 gm respectively for the two batches. After subjecting the above fiber for partial depolymerisation process, the final yield obtained was 65/76 and 67/81 gm with the percentage final yield of 85 and 83 respectively for the two batches Table 2.

The broccoli fiber after first cycle of treatment for de-polymerization appeared deep yellow in colour and on third cycle of treatment appeared near white with increased fineness, high flow property and powdery-ness. In the case of Mung bean sprouting also, three cycles of treatment for partial de-polymerization has distinctly reduced the colour from deep yellow to near white with significant improvement in flow property, fineness and powdery-ness Table 3.

Powder microscopy

Microscopic appearance of partially depolymerized cellulose fiber -Figure 1, 2 –*Vigna radiata* **(Mung bean) Sprouting***

Varying degrees of length, breadth and orientation of the fibres of Mung bean sprouting was observed after third cycle of treatment as in Figure 1 and 2.

Microscopic appearance of partially polymerized cellulose fiber -Figure 3, 4 –*Brassica oleracea* (Broccoli)

Three cycles of treatment of Braccoli fibres for partial depolymerization, we obtained flat, globoid, irregularly shaped, deep with hyaline receptacles and occasionally elongated fibres as in Figure 3 and 4. Highly clustered, clumsy, irregularly shaped fiber with enlarged, flat deeply coloured fibres were observed when MCC was examined under a microscope as in Figure 5 and 6.

Microscopic characterization of depolymerized cellulose fiber–Micro crystalline cellulose (MCC)

FTIR spectra

The FTRI spectra of the partially de-polymerized Braccoli fibres showed a distinct pattern with a few comparable peaks with that of MCC between 1000 to 3500 wave number as shown in Figure 7. The FTIR spectra of Mung bean sprouting showed highly comparable pattern with that of MCC where MCC showed a strong band at 3390 cm^{-1} and a band at 1636 cm^{-1} corresponding to the stretching and bending modes of the surface hydroxyls of MCC. The peak at 2905 cm^{-1} belongs to the asymmetrically stretching vibration of C-H in a paranoid ring, and broad absorption peak 1059 cm^{-1} is attributed to the C-O of cellulose, mung bean showed similar FTIR patter of MCC as in Figure 8. The FTIR pattern of MCC showed a strong band at 3390 cm^{-1} and a band at 1636 cm^{-1} corresponding to the stretching and bending modes of the surface hydroxyls of MCC. The peak at 2905 cm^{-1} belongs to the asymmetrically stretching vibration of C-H in a paranoid ring, and broad absorption peak 1059 cm^{-1} is attributed to the C-O of cellulose as in Figure 9.

Ash content analysis

The ash content analysis has revealed insignificant presence of ash content in the partially depolymerized fibres of both Broccoli and Mung bean sprouting when compared to the significantly high level of ash in MCC, Table 4.

Particle density test

Thebulk density and tapped density of the partially depolymerized fibres of Broccoli and Mung bean sprouting were relatively higher than that of MCC and so was the Hausner's ratio of the above in comparison with MCC, Table 5.

Angle of repose

The evaluation of angle of repose study of the partially depolymerized fibres of Broccoli and Mung bean sprouting were highly comparable with the angle of repose of MCC meeting similar flow property, Table 6.

Table 1: Yield of *Brassica oleracea* (Broccoli).

Batch details	Initial weight of wet *Brassica oleracea* (Broccoli) (g)	After air drying (moisture level 5%)	After partial de-polymerization gm. and (%)
1	200	120	102 (85)
2	200	141	121 (86)

Table 2: Yield of *Vigna radiata* (Mung bean).

Batch details	Initial weight of wet *Vigna radiata* (Mung bean) (g)	After shred drying (moisture level 5%)	After partial de-polymerization gm. and (%)
1	200	76	65 (85.5)
2	200	81	67 (82.71)

Table 3: Physical characteristics of partially de-polymerization.

Herb	Parameters	First cycle	Second cycle	Third cycle
Brassica oleracea (Broccoli)	Colour	Deep yellow	Pale yellow	Near white
	Fineness	Corse	Corse	Very fine
	Powderyness	Fibrous	Less fibrous	Powdery
	Flow property	Poor	Poor	High
Vigna radiata (Mung bean) Sprouting	Colour	Pale yellow	Pale yellow	Near white
	Fineness	Corse	Corse	Very fine
	Powdery ness	Fibrous	Less fibrous	Powdery
	Flow property	Poor	Poor	High

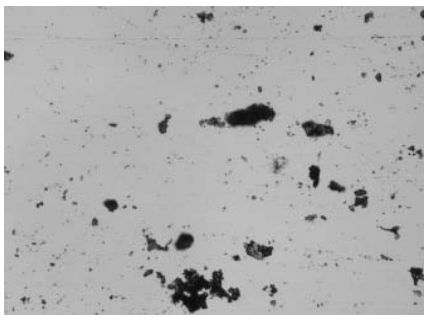

Figure 1: *Vigna radiata* fibers in 4X.

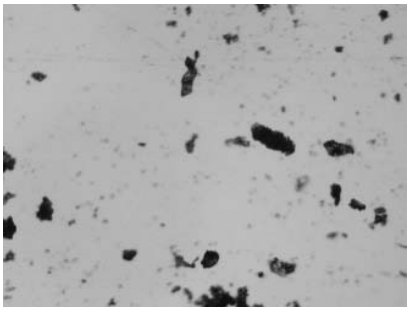

Figure 2: *Vigna radiata* fibers in 10X.

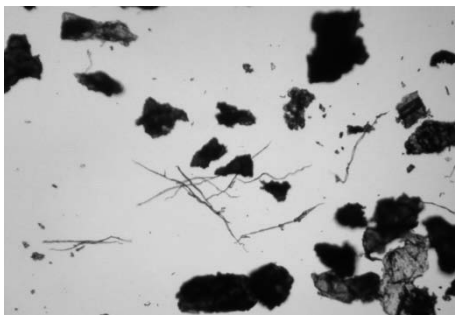

Figure 3: *Brassica oleracea* fibers in 4X.

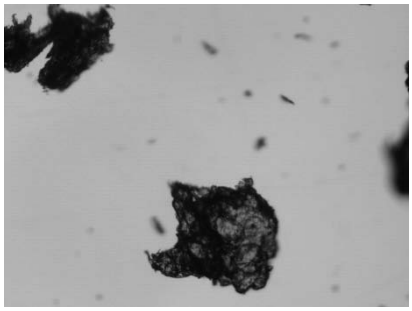

Figure 4: *Brassica oleracea* fibers in 10X.

Formulation development

Five tablet formulations were made with the active pharmaceutical drugs such as metformin and Miglitol. Metformin 500 mg tablet was formulated separately with 50 mg each of MCC, the partially depolymerized Broccoli fibres and partially depolymerized Mung bean sprouting. Similarly, 20 mg of Miglitol was made into tablet using 50mg of MCC, partially depolymerized fibres of Broccoli and Mung bean sprouting, Table 7.

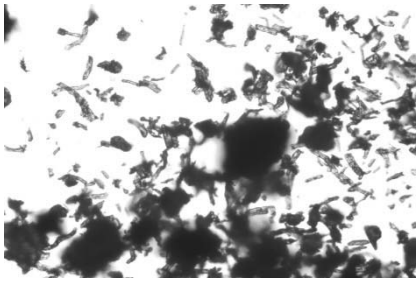

Figure 5: MCC in 4 x.

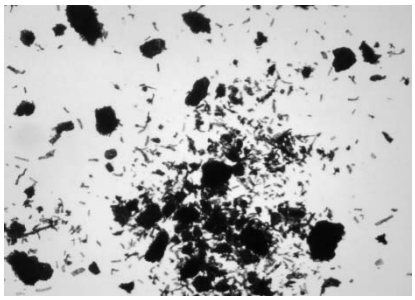

Figure 6: MCC in 10X.

Evaluation of tablet formulations

The tablet formulations of 500 mg Metformin and 20 mg Miglitol using 50 mg each of partially depolymerized fibres of Broccoli and Mung bean sprouting showed highly comparable physical characteristics with reference to surface finish of the tablet, hardness, friability and disintegration time with that of the tablet formulated with MCC, Table 8.

Cell line studies

None of the four formulations of Metformin exhibited strong cytotoxic effect on SKNSH human neuroblastoma cells from the concentration ranging from 10 to 100 µL. However, the Metformin sustained release and Metformin formulated in MCC showed a slight increase in cytotoxic effect on SKNSH human neuroblastoma cells vis-à-vis concentration when compared to the formulation of Metformin in partially depolymerized fibres of Braccoli and Mung dal sprouting suggesting faster release of drug from MCC and sustained release formulation Table 9.

Study on Neuroprotection effect of the four formulations of Metformin after H_2O_2 induction, the cells showed that Metformin formulated in depolymerized fibres of Broccoli offered significant protection (63%) at 100 µL whereas all the other three formulations did not offer such protection vis-à-vis concentration. When compared to control, all the four formulations of Metformin offered good degree of Neuroprotection, Table 10.

The cytotoxic study on kidney cells HK2 of the four different formulations of Metformin showed that the formulation in partially depolymerized fibres of Broccoli from 10 to 100 µL did not cause any cytotoxic effect whereas other three formulations showed a small linearity in cytotoxic effect, however the effect was not significant, Table 11.

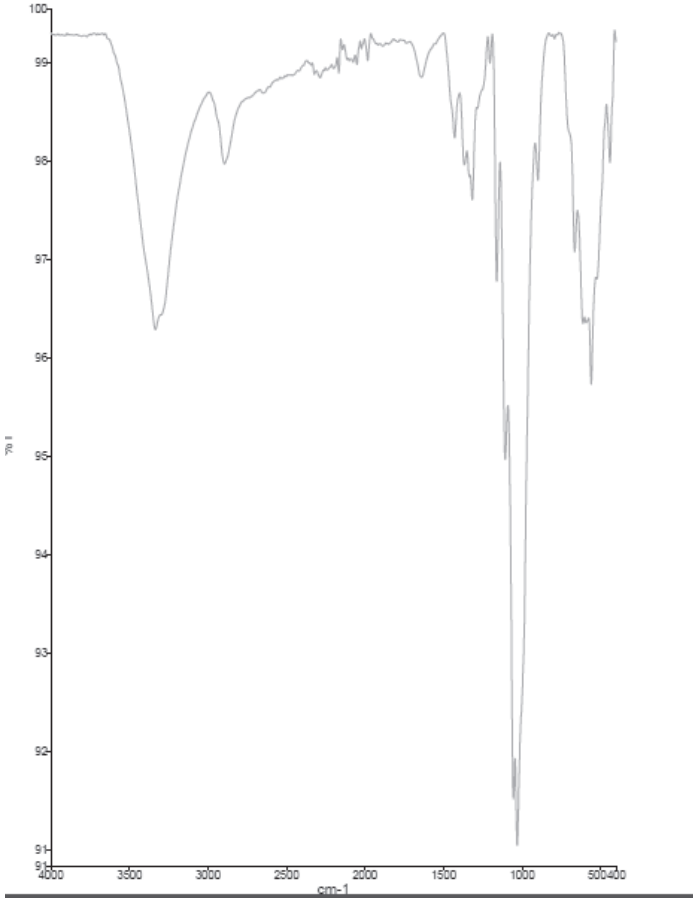

Figure 7: FTIR spectra of *B. oleracea* fibers.

Triglyceride accumulation study showed that the cells treated with high glucose and high insulin showed TG accumulation of 62 µg. The Metformin formulated in partially depolymerized Braccoli fiber did not induce TG accumulation vis-à-vis the release time of 9 hr. Whereas the other two formulations of Metformin although showed a slight increase in TG accumulation in HepG2 cells vis-à-vis time of drug release, however the TG accumulation was not significant, Table 12.

Glucose utilization by HepG2 cells treated with the four different formulations of Metformin showed that the drug formulated in partially depolymerized Braccoli fiber augured greater glucose utilization by cells vis-à-vis release time suggesting sustained higher release of drug over time may be responsible for the above effect when compared to other three formulations. Appreciable linearity between level of glucose utilization and time of drug release from all the formulations was also observed, Table 13.

The glucose utilization by L6 Myoblast cells treated with the four formulations of Metformin showed that Metformin formulated with partially depolymerized fiber of Braccoli significantly accelerated glucose utilization by the cells when compared to other three formulations of Metformin. However, other three formulations also showed positive effect on the cells with reference to glucose utilization. Release time vis-a-vis activity linearity was also observed in the case of Metformin formulated with partially

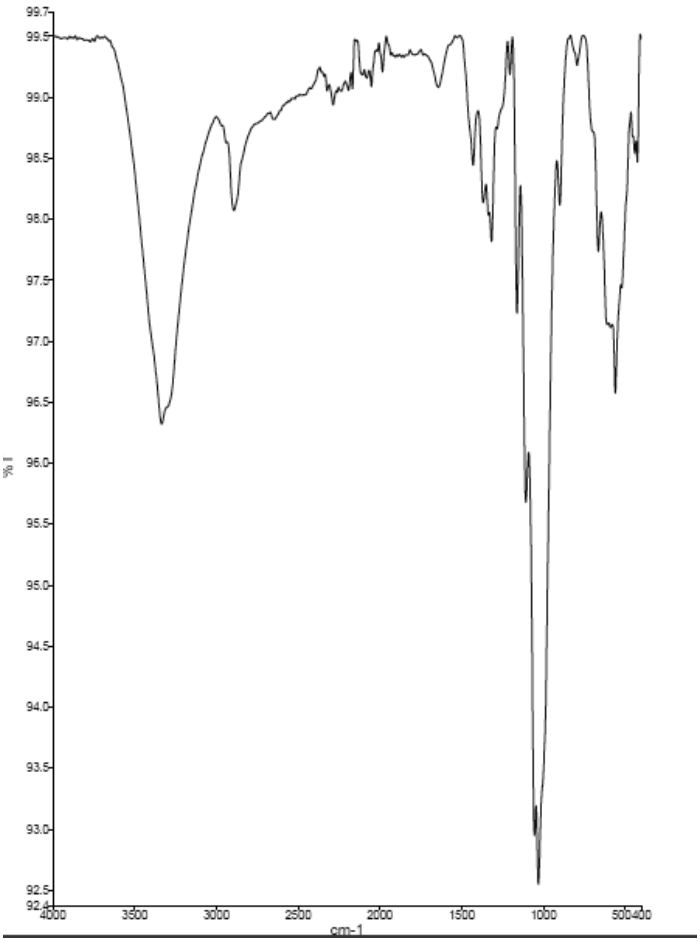

Figure 8: FTIR spectra of *V. radiata.*

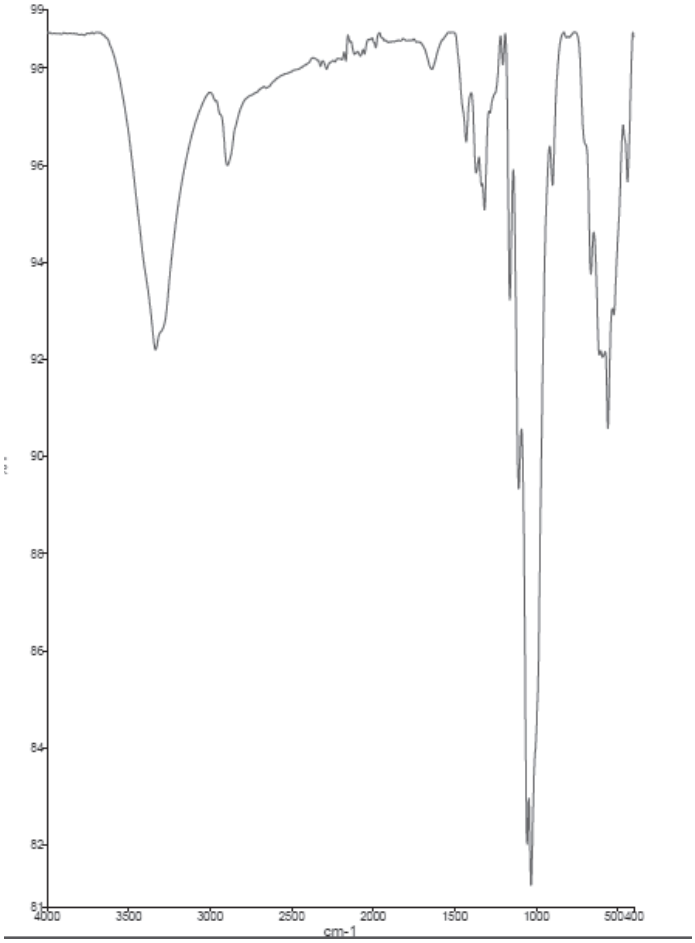

Figure 9: FTIR spectra of MCC.

depolymerized Braccoli fiber suggesting the relative slow release of the drug, Table 14.

The lipid accumulation study in 3T3 L1 preadipocytes showed that Metformin formulated in partially depolymerized Braccoli fiber showed greater effect when compared to other formulations and correlation between release time and high activity suggests the possible slow release of the drug. Other three formulations also however showed similar positive effect, Table 15.

The Metformin formulated in partially depolymerized Braccoli fiber resulted in significant increase in the glucose metabolism capability of INS-1 cells with or without glucose and the activity showed higher correlation with time suggesting slow release of the drug. The activity of the formulation in partially depolymerized Braccoli fiber was higher than the sustained release Metformin formulation. The formulation made with MCC showed least effect when compared to other three formulations Table 16.

Miglitol formulated with partially depolymerized Mung bean fiber (*Vigna radiata*) inhibited the enzyme at low concentration. Whereas, Miglitol formulated in partially depolymerized Braccoli fiber (B.o) showed activity only at higher concentration and also has taken longer time suggesting the possible poor release of the drug as in Table 17.

DISCUSSION

Our present investigation has revealed that a value added, safe alternative to conventional excipient – MCC is possible from the most commonly used vegetable like *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean). The advantage of using such alternate depolymerized cellulose fiber is the additional nutritional value, safety and also such fiber being digestible.[21,22] Besides the above benefits, such fiber also would help in the treatment effectively by avoiding tachyphylaxis and reduced drug dosage if the same could delay the drug release.

The multifactorial disease like diabetes mellitus require lifelong medication, monitoring and lifestyle changes.[23] More often, the drug dosage required to bring down high blood glucose burden is also quite high where the scope for drug induced tachyphylaxis and the excipient led toxic effect, both are quite possible. Besides all the above, prolonged high dosage of metformin is also known to deplete B_{12}, an essential vitamin for our body function besides many other side effects.[24]

Our experiment has shown that metformin binds well with the fiber of *Brassica oleracea* (Broccoli) than with *Vigna radiata* (Mung bean) fiber. As a result of such firm binding, the release of metformin was observed to be relatively slow. However, such

Table 4: Ash values of *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean) Sprouting and MCC.

Sl. No	Herb	Ash value (% w/w)
1	*Brassica oleracea* (Broccoli)	0.02
2	*Vigna radiata* (Mung bean) sprouting	0.05
3	MCC	0.17

Table 5: Bulk density, tap density and Hausner's ratio of *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean) Sprouting and MCC.

Parameters	Partially depolymerized *Brassica oleracea* (Broccoli) fibers	*Vigna radiata* (Mung bean) Sprouting	MCC
Bulk density (g/cm^3)	0.41	0.425	0.29
Tapped density (g/cm^3)	0.47	0.48	0.34
Hausner's ratio	1.1	1.12	1.17

Table 6: Angle of repose of *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean) Sprouting and MCC.

Sl. No	Herb	Angle of repose(degrees)
1	*Brassica oleracea* (Broccoli) fiber	51.1
2	*Vigna radiata* (Mung bean) sprouting fiber	50.9
3	MCC	49.7

Table 7: Tablet formulation with MCC, *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean) Sprouting.

Details	Proportion in mg	Formulation details					
		1	2	3	4	5	6
MCC	50	+	+	-	-	-	-
B.o fiber	50	-	-	+	+	-	-
V.r fiber	50	-	-	-		+	+
Metformin	500	+	-	+	-	+	-
Miglitol	20	-	+	-	+		+

Table 8: Surface finish, Capping, Hardness, friability and disintegration test for formulations made of MCC, *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean) Sprouting.

Sl. No.	Parameters	Formulation details					
		1	2	3	4	5	6
1	Surface finish	S	s	s	s	s	S
2	Capping	Nil	Nil	Nil	Nil	Nil	Nil
3	Hardness (N)	4	4.2	4.5	4.5	4.7	4.3
4	Friability (%)	1	0.8	0.7	0.7	08	0.7
5	Disintegration (min)	6.3	6.0	6.3	6.7	6.5	6.6

S= smooth

Table 9: Cytotoxic effect of test samples on SK N SH human neuroblastoma cells.

Test samples	% Of cell death/concentration in µL		
	10	50	100
Sustained release Metformin (500 mg)	11	15	20
Metformin in B.o (500 mg)	8	10	13
Metformin in *Vigna radiata* (500 mg)	10	12	15
Metformin in MCC (500 mg)	9	14	16

Table 10: Neuroprotection effect of test samples against H_2O_2 induced damage.

Test samples	% Of viable cells/concentration in µL		
	10	50	100
Sustained release Metformin (500 mg)	44	38	23
Metformin in B.o (500 mg)	38	50	63
Metformin in *Vigna radiata* (500 mg)	19	32	35
Metformin in MCC (500 mg)	11	16	26
H_2O_2 – 1.0 Mm Concentration	12		

Table 11: Cytotoxic effect of test samples on kidney cells HK2.

Test samples	% Of cell death/concentration in µL		
	10	50	100
Sustained release Metformin (500 mg)	28	18	17
Metformin in B.o (500 mg)	18	9	9
Metformin in *Vigna radiata* (500 mg)	25	16	11
Metformin in MCC (500 mg)	39	24	21

Table 12: TG accumulation in HepG2 cells.

Test samples	Cellular TG in µg/mg protein vis-à-vis release time of test samples (hr)							
	0	1	2	3	4	5	7	9
NC	30							
HGHin	62							
Met B.o	-	24	25	20	11	9	8	6
Met V.r	-	11	25	22	23	25	23	28
Met.MCC	-	18	27	29	31	29	30	31
SR Met	-	21	14	11	17	11	10	11

Table 13: Glucose utilization assay by HepG2 cells.

Test samples	Glucose utilization (% control) vis-à-vis release time of test samples (hr)							
	0	1	2	3	4	5	7	9
Control	100							
1,1-Dimethylbiguanide hydrochloride (0.1 µg/mL)	183							
Met B.o	-	38	42	44	61	77	78	91
Met V.r	-	54	59	77	81	81	81	84
Met.MCC	-	56	57	60	78	77	80	81
SR Met	-	28	32	34	54	65	76	78

Table 14: Glucose utilization by L6 Myoblasts.

Test samples	Glucose utilization (% control) vis-à-vis release time of test samples (hr)							
	0	1	2	3	4	5	7	9
Control	100							
Insulin (6µg/mL)	165							
Met B.o	-	44	67	77	87	89	90	109
Met V.r	-	44	59	76	77	78	82	85
Met.MCC	-	68	77	78	77	78	81	83
SR Met	-	22	38	39	78	79	80	88

Table 15: Lipid accumulation in 3T3-L1 Preadipocytes.

Test samples	Lipid accumulation (% control) vis-à-vis release time of test samples (hr)							
	0	1	2	3	4	5	7	9
Control	100							
Rosiglitazone (0.4 µg/mL)	161							
Met B.o	-	67	88	91	92	98	101	107
Met V.r	-	64	69	79	88	91	93	96
Met.MCC	-	66	69	70	88	87	89	88
SR Met	-	67	76	79	84	85	88	90

Table 16: Glucose metabolism by INS-1 cells.

Test samples	% viable cells vis-à-vis release time of test samples (hr)							
	0	1	2	3	4	5	7	9
Control w/ glucose	28							
Control w/o glucose	11							
Met B.o w/	-	33	39	45	52	78	79	82
Met b.o w/o	-	22	28	31	38	43	44	55
Met V.r w/	-	61	79	72	80	81	81	82
Met V.r /w/o	-	44	45	45	48	52	55	67
Met.MCC w/	-	33	39	34	41	45	48	53
Met. MCC w/o	-	11	11	12	13	17	18	21
SR Met w/	-	63	64	69	70	71	72	70
SR Met w/o	-	23	21	26	28	29	30	32

Table 17: µ-glucosidase assay.

Test samples	IC_{50} value (µg/mL) vis-à-vis release time of Miglitol (minutes)			
	10	20	30	35
Miglitol in *Vigna radiata* fiber	13	18	20	22
Miglitol in B.o fiber	27	39	51	62

binding does not seem to affect the functional aspect of the drug. Further the formulation can be made just with the fiber without any other excipients such as diluent, flow enhancer etc. Slow-release metformin is known to help to reduce the daily drug dosage from 1000 mg to 850. But such formulation has been made with conventional excipient Microcrystalline Cellulose (MCC).

We have performed a series of experiments with metformin formulation made with *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean) fiber and the formulation with MCC. The sustained release, uncoated metformin tablet available in the market was used for comparison.

Our initial evaluation on the safety of both the fiber yielded positive result with the reference to neuroblastoma cells and kidney cells. The safety profile of both *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean) are known for ages but for the want of scientific proof, we performed cytotoxic study with the above fiber.

Our research work on how the metformin from the excipients such as *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean) would exhibit the therapeutic effect in comparison with the drug from MCC and sustained release tablet. For this purpose, we subjected the drug to passive dissolution in phosphate butter at different time intervals such as 1,2,3,4,5, 7 and 9 hrs and then a fixed quantity of sample from all the above experiment was tested on various cell lines. Initially we studied the cellular accumulation of TG in Hep G2 cells showed that the metformin from *Brassica oleracea* (Broccoli) fiber than *Vigna radiata* (Mung bean) fiber affected significantly the TG accumulation. The effective release time of metformin from the above formulation was 4 hrs whereas the metformin got released much earlier from sustained release tablet. So was with the formulation made with MCC. Our above findings led us to two possible affirmations such as

Metformin may be binding firmly with *Brassica oleracea* (Broccoli) fiber

The release of metformin from such fiber is near total

We went further to confirm the above findings through a series of experiments using various cell lines. The glucose utilization pattern of HepG2 and L6 Myoblst cells post exposure to the test samples showed that metformin from *Brassica oleracea* (Broccoli) fiber significantly increased glucose utilization ability of the cells with late time release. The lipid accumulation in 3T3 preadiposites and glucose metabolism ability of INS 1 cells also yielded same result.

On the contrary, the depolymerized fiber of *Vigna radiata* (Mung bean) was not having any such selective binding preference for metformin and instead miglitol got released faster from the above excipient.

For treating type 2 diabetes mellitus, the combination of metformin and miglitol or vildagliptin are given where the later drug would hinder the surge and rush of glucose into blood post meal by hindering glucose conversion from carbohydrate. The above combination of drugs when administered, the release time of both drugs needs to be adjusted or timed through formulation engineering otherwise the alpha reductase inhibitor miglitol by delaying the glucose surge into blood stream would provide no benefit as the release and excretion of metformin would have happened prior to the above event. If metformin release is delayed, then the delayed import of glucose will be well metabolized by the cells where the timing of metformin empowering the cells would perfectly sync with glucose surge.[25]

The depolymerized fiber from *Brassica oleracea* (Broccoli) and *Vigna radiata* (Mung bean) met all most all characteristics of MCC with reference to physical and tablet formulating behaviour. With reference to colour, odour, taste, starch content, ash content, bulk density, tap density, angle of repose, all respect, the fiber could match MCC. The tablet made using the above fiber also showed high compressibility, smooth finish over the surface of the tablet, hardness, friability and disintegration time.

The alpha glucosidase inhibitor from *Vigna radiata* (Mung bean) fiber showed faster release whereas the metformin showed very slow but complete release from *Brassica oleracea* (Broccoli) fiber, complementing the treatment requirement of type 2 diabetes mellitus, perfectly.

Relatively poor response that we obtained from sustained release tablet of metformin may be due to the presence of several other excipients in the formulation. We, in the present study used the fiber or MCC without the addition of any other excipient. Therefore, the result obtained from sustained release tablet of metformin may be due to poor release or due to interference of other excipient in cell culture system, we could not elucidate clearly. All we know is that cell culture system is quite sensitive to artefacts.[26]

Findings of our present investigation may be just a beginning, but nevertheless it gives elaborate scope and confidence for further research to develop a value-added excipient, especially to reduce excipient accumulation led toxic side effects, reduce the dosage of the drug and to avoid tachyphylaxis due to prolonged drug exposure.[1]

CONCLUSION

The micro cellulose fibers from *Brassica oleracea* and V*igna radiata* are the best alternatives for conventional cellulose fibers. The fibers isolated from *Brassica oleracea* showed slow release of metformin thus reduces drug dosage, tachyphylaxis. The release of Miglitol from *Vigna radiata* fibers was rapid and may compliment metformin therapy.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MCC: Micro crystalline cellulose; **NaOH:** Sodium hydroxide; **H_2O_2:** Hydrogen peroxide; **HCl:** Hydrogen chloride; **FBS:** Fetal bovine serum; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; **PBS:** Phosphate buffered saline; **TG:** Triglyceride; **CO_2:** Corbon dioxide; **DMEM:** Dulbecco's Modified Eagle Medium; **RPMI:** Roswell Park Memorial Institute Medium; **BSA:** Bovine Serum Albumin; **FCS:** Fetal calf serum; **DMSO:** Dimethyl Sulfoxide; **B.o:** *Brassica oleracea;* **V.r:** *Vigna radiata.*

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Original Article

Preparation and *in-vitro* and *in-vivo* Evaluation of Ayurvedic Formulation "Amruthotharam" Formulated by Classical and Modern Technique

Ketaki Dhane[1,*], Manish Kumar Gupta[2], Supriya Hyam[3], Abhinandan Patil[4]

[1]Department of Pharmaceutical Chemistry, PSPS Indira Institute of Pharmacy, Sadavali, Maharashtra, INDIA.
[2]Department of Pharmacy, Faculty of Pharmaceutical Science and Nursing, Vivekananda Global University, Jaipur, Rajasthan, INDIA.
[3]Department of Pharmaceutical Chemistry, Shree Saraswati Institute of Pharmacy, Tondavali, Kankavali, Maharashtra, INDIA.
[4]Department of Pharmaceutics, D.Y. Patil Education Society (Deemed to be University), Kolhapur, Maharashtra, INDIA.

ABSTRACT

Glucose intolerance, central obesity, hypertension, and dyslipidemia are all part of the metabolic syndrome. Thus, a number of theories have been put out to explain the development of the metabolic syndrome, including an initial condition of insulin resistance that progressed to the other elements, with obesity serving as the primary initiator of the metabolic syndrome. Thus, it can be understood that metabolic syndrome is multi related and the basic cause is inflammation. Thus, while treating the metabolic syndrome the root cause needs to be targeted and for this the ayurvedic formulation is the best solution. Amruthotharam is one of such preparation which takes care of metabolic syndrome through inflammation. "Amruthotharam" is the combination is known as Kashayam was made from three main herbs whose efficacy in treating indigestion and other stomach-related issues has been demonstrated in several cases. Three parts of *Tinospora cordifolia*, two parts of *Terminalia chebula*, and one part of *Zingiber officinale* are combined to create the mixture. The present study is focusing on the comparisons between the Amruthotharam a kerealian Ayurvedic medicine formulated by classical and modern technique which can be evaluated analytically and pharmacologically.

Keywords: Metabolic syndrome, Amruthotharam, Immunomodulatory.

Correspondence:
Prof. Ketaki Dhane
Assistant Professor, PSPS, Indira Institute of Pharmacy, Sadavali-415804, Maharashtra, INDIA.
Email id: archupharma21@gmail.com

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INTRODUCTION

The multiple theories and hypothetical explanations have been given by the modern medical science, which helps to recognize the origin of metabolic disorders and the research is continued further till date. Few say that an advent of insulin resistance progresses to the subsequent factors, according to some generalized systemic obesity is the main precursor of the total syndrome.

Current recent data establishes that, low-grade inflammatory status that stay for a longer duration that often accompanies impaired metabolism and therefore is deemed as important causative factor for onset and establishment of the metabolic syndrome along with the subsequent Patho-physiological changes. The other causative factors for development of metabolic syndromes are diabetes and oxidative damage. Diabetes mellitus, a prominent and rapidly spreading illness in group of people all over the globe.[1]

Numerous factors that developed diabetes, includes glucose auto-oxidation resulting from formation of free radicals, tyrosine kinases receptor, like epidermal growth factor receptor, the enzyme glucosidase as well as insulin receptor may be regarded as a primary target to monitor for antioxidant also anti-inflammatory agents.[2]

Antioxidants have been widely acknowledged as having a significant impact on the biochemistry of living organisms in the last decade. An antioxidant is a chemical which inhibits oxidative damage of a target molecule.[3]

Metabolism is an example of a reaction that may produce free radicals. Although metabolism is an unavoidable process and oxygen is a need for living, the paradox is that oxygen is a highly reactive molecule that may be damaging when reactive oxygen species are produced. Free radicals have an unpaired electron, which makes them very reactive species. They may remove an electron from a stable molecule, making it unstable and starting a chain reaction. This is where antioxidants come into play,

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acting as a defense factor and reducing the negative effects of free radicals.[4]

Thus, it can be understood that in metabolic disorders is multi related and the basic cause is inflammation which is well discussed and addressed along with detailed solutions in the science of Indian Medicine, Ayurveda. While treating the metabolic disorders the root cause needs to be targeted and for this the Ayurvedic science is the best solution.

Ayurveda with new approach of disease management

Since ancient times India is practicing *Ayurveda*, approximately dates back to three thousand years. *Ayurveda, the science of life*, involves the incorporation of universal 5 elemental forces-namely Jala (Water), Prithvi (Earth), Vayu (Air), Aakash (space, ether) and Agni (Fire).

The medication is basically in the forms of various herbal, herbo-mineral, mineral drug formulations aimed at elimination of the root pathology of any kind of disorder in the body and by restoring balance, simultaneously creating apro-healthy life-style, thereby preventing the recur of any disorder. Cure and prevention is the two folded aim of Ayurveda.[5]

"Ama" may show to be a valid marker of early stage of inflammation in persons at risk for metabolic disorder or malignancies and thus management of these disorders will become easier even before the pathologies progress further. As in Ayurveda diagnoses of the Ama condition is laid down in elaborate and specific details and the process of physical examination can bypass the chemical examination of blood or urine samples thereby reducing the time and other investments in the diagnosis of early pathologies of metabolic syndrome.[6]

There are many treatment modalities in Ayurveda, to correct the status of Agni and metabolize or neutralize AMA based on the state and the stage of the disease. Dietary corrections or stipulated nutritional regimens work in congregation with carious oral formulations, Amrutottaram being one of them, which is the subject of study here.

Ayurvedic drug formulations like 'Amruttottaram', are extremely potential in reversing metabolical pathologies if given in intended dose and stage but they are still plagued by some loopholes in this era, affecting their ability and efficacy in treatments.

Clinical studies of individual components of *Amruttottaram Kashayam*

Zingiber officinalis (shunthi)

Studies related to Hepato protective and curative actions of ginger also gave promising results. The overall studies showed that rhizome of *Zingiber officinale* extracted in water displayed potent anti-inflammatory activity, hepato-protective and anti-viral activity.[7]

Another study to assess the anti-obesity action of ginger was conducted with significant positive findings. The ethanolic constituents, of *Zingiber officinale* in 'Shunthi' extract is known to have various properties like antidiabetic, antioxidant and antihyperlipidemic potential in animals under experimentation. Furthermore, there was improved HDL-C level was also observed, emphasizing the anti-obesity potential of Ginger.[8]

This study evaluated cancer opposing activity of ginger by studying its effect on 'Cholangiocarcinoma' (CCA) an adenocarcinoma of bile ducts. The results were very encouraging to discover further prospects about anti-carcinogenic actions of *Amrutottaram*.[9]

Tinospora cordifolia (Amruta)

This study showed Guduchi (*Tinospora cordifolia*) as a natural antioxidant, fibre source, and nutritional content in nutrition, fodder, also pharmaceutical sectors.[10]

Anti-inflammatory, antipyretic, antioxidant, anti-leprotic, anti-stress, antimalarial, anti-allergic, adaptogenic, anti-rheumatic, immunomodulatory, hepatoprotective, hypoglycemic, and anticancer characteristics have been observed.

The overall results of this study of guduchi bark imply that phytochemical evaluation may be liable for the high therapeutic efficacy.[11]

Tinospora cordifolia extracts were tested for *in vitro* phagocytosis immune system activation. Assay findings confirm this plant's immunomodulatory properties.[12]

This particular study was proven to give insightful information in determining the therapeutic efficiency of guduchi, for the controlling of depressive diseases.[13]

Terminalia chebula (Haritaki)

The researchers analyzed *Terminalia chebula* extracted in methanol for anti-ulcerogenic efficacy. The investigated extract inhibited gastrointestinal injuries generated by Pylorus ligation induced ulcer as well as Ethanol induced gastric ulcer, determining anti-ulcer also lesion healing capabilities of haritaki in the upper digestive tract also in stomach area.[14]

The previously reported analysis revealed that dried fruit of Haritaki shows antimicrobial activity on broad spectrum microbial flora and a prospective opportunity to examine active component required for various strains of bacteria.[15]

This study proved the immune-modulatory activity of fruits of Haritaki or *T. chebula* fruits depicted by increased concentrations of antioxidants, GSH, T and B cells, establishing its importance in effective immune response. This improves the melatonin concentration in the pineal gland as well as showed improved

cytokines level that play important roles in stress management and a normal immunity.[16]

Amrutottaram Kashayam

Amruthotharam/ Amrutottaram Kashayam a form of formulation used in metabolic disorder like inflammation. "Amruthotharam" Kashayam is the decoction, prepared from three herbal drugs that have proven to be extremely beneficial in many pathologies of ranging from indigestion to deeper cellular inflammation wherein cellular neutralization of AMA is expected.

The three ingredients are; Guduchi (*Tinospora cordifolia*),Haritaki (*Terminalia chebula*) and Shunthi (*Zingiber officinale*).[17]

Traditional Ayurvedic texts including Chikitsa Manjari and Sahasrayogam mention in it. It composed of three medicaments with the ratio 3:2:1. In terms of active ingredients, Amruta or Guduchi (*Tinospora cordifolia*) contains Tinosporaside, Tinsosporic acid, caridioside etc. Haritaki (*Terminalia chebula*) contains tannins, gallic acid, chebulagic acid, ellagicacid while Shunthi (*Zingiber officinale*) contains volatile oil 1%, 6-gingerol, 8-gingerol, 10-gingerol and shagol etc.[18]

MATERIALS AND METHODS

Drugs

The crude forms of herbal drugs *Tinospora cordifolia, Terminalia chebula, Zingiber officinale* were procured from local market of Ratnagiri, Maharashtra (India).

The aqueous extracts *Terminalia chebula* (Hareda), *Tinospora cordifolia* (Guduchi) and *Zingiber officinalis* (Ginger) were procured from authentic supplier, Amsar Pvt. Ltd., Goa with COA of each sample extract.

Methods

Methods for Preparation of amruthotharam Formulations (Classical methods)

The formulation F1 was prepared by mixing raw drugs such as guduchi, hareda and ginger in 3:2:1 by traditional techniques. 48 g of crude mixture of all the three herbs is taken and 768 mL (16 times to that of the churna) of water is added to it. The kashayam is then boiled on medium flame until the water quantity is reduced to 96 mL (1/ 8th of the original quantity). This is the standard method of Kashay preparation.[19]

Modern Method

The formulation F2 was prepared by mixing aqueous extracts of *Terminalia chebula* (Hareda), *Tinospora cordifolia* (Guduchi) and *Zingiber officinalis* (Ginger) in 3:2:1 by modern technique.

In-vitro antioxidant study

The antioxidant action of herbs was measured by various techniques. The methods are popular because of their ease, speed and sensitivity. In the present study antioxidant action was measured through DPPH, ABTS and lipid peroxidation inhibition assay, xanthine oxidase and superoxide radical scavenging assay.[20,21]

Calculation of 50% inhibition concentration (IC_{50})

The optical density at each concentration of the specified test chemicals and Butylated Hydroxyl Toluene (BHT). Percentage inhibition was estimated using equation.

$$\% \text{ Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

The IC_{50} value was determined by plotting graph taking concentration on X- axis and % inhibition on Y-axis.[22]

In- vivo studies

The experiment was done out on both sexes of Wister albino rats. The rats weighing 200-250 g were chosen with the committee's agreement. The experimental protocol was authorized by the Institutional Animal Ethics Committee (IAEC) of Indira Institute of Pharmacy, Sadavali, Maharashtra, (IIP/IAEC/07/2019-20) in accordance with the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

The animals were kept in a well-ventilated animal unit with a 12-hr light/dark cycle. For 28 days, the six experimental groups of six animals each were given a customized high cholesterol diet. There were 48 rats given a high cholesterol diet and 6 rats provided a regular diet. All administrations were done orally.[23]

Collection and Analysis of Blood Sample

After the induction of diabetic on 5th, 7th, 9rd week blood samples were collected and evaluated for blood glucose fasting, postprandial glucose level and blood lipid profile.

Histopathological study

The purpose of histopathology on the harvested pancreas and liver part was to see the effect of the formulations (F1 and F2). After completion of treatment i.e. after 4 weeks, the animals were sacrificed. The pancreas was collected from the group of animals I, III, IV, VII, VIII to evaluated for diabetic condition. The liver of animals (group I, II, V, VI) were collected and evaluated for liver condition due to high fat diet. This was done in accordance with established procedures. The staining of the kidney segment was done according to normal histological techniques. The examination of sections was carried out using microscopic techniques.[24]

Table 1: IC_{50} in µg/mL for each activity.

Sl. No.	Activity	IC_{50} value in µg/mL	
		F1	F2
1	DPPH	271.99	269.84
2	ABTS	293.07	225.04
3	Lipid Peroxidation	231.63	232.44

Table 2: Change in body weight during treatment (g).

Groups	Change in body weight during treatment (gm)			
	1st Week	2nd Week	3rd Week	4th Week
F1.1	254.52±5.90	247.58±5.35	240.07±4.66	230.77±4.30
F1.2	254.55±7.07	245.88±3.73	237.93±2.18	224.68±2.51
F2.1	254.03±1.67	243.58±2.96	236.53±2.68	223.72±3.10
F2.2	252.87±1.61	239.68±1.33	232.22±1.67	220.05±0.94
Standard	254.10±1.80	241.67±1.01	233.67±1.28	221.55±1.66
Standard	171.50±0.84	165.00±2.28	161.17±3.60	155.33±3.33

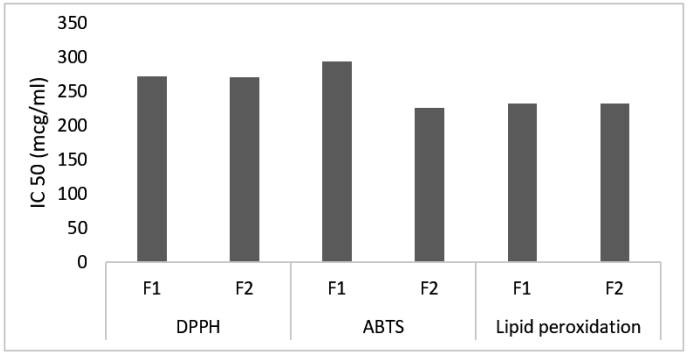

Figure 1: Determination of IC_{50}.

RESULTS AND DISCUSSION

In-vitro antioxidant study

The *in vitro* antioxidant study was carried out to evaluate the antioxidant potential of *Amruthotharam Kashayam* (F1) and Kashaya mixture (F2). The overall antioxidant activity was found to be good in both formulations. The formulation F2 showed better antioxidant action shown in Table 1 and Figure 1.

Pharmacological Activity

Obesity and its accompanying diseases, such as diabetes, provide significant problems for fundamental science and clinical research.

The various studies were discovered that the disorders caused by high-fat feeding were similar to the human metabolic syndrome, which is indicated by a rise in body weight (obesity), moderate hyperglycemia, hypertriglyceridemia, hypercholesterolemia, and compensatory hyperinsulinemiaas well as a decreased glucose disappearance rate, and this may extend to cardiovascular complications.[25,26] A rat model of high fat density (HFD) diet-induced obesity is well-controlled and has many characteristics with human obesity. Obesity-related cardiovascular problems can be studied using a mouse model of obesity based on HFD diet.[27]

The present work investigates the antiobesity action of Amruthotharam Kashaya formulations prepared by classical method and modern method on high fat diet induced obesity in Wistar rats. This study will help us to understand whether changing the formulation method, will there be any change in pharmacological effect.

The animals were grouped in the eight groups containing 6 rats in each group. The obesity was induced using high fat diet. The animals were fed with high fat diet for four week of period. Furthermore the animals were treated with alloxan to induced diabetes. The process was carried out for four week of time period for determination of fasting glucose level and postprandial glucose level in blood. The treatment was carried out to evaluate the change in body weight and glucose level in blood after 4 week of treatment with formulation F1 and F2.

Body weight changes

The body weights (g) of all the animals were measured at 4 weeks of the study. After the induction of high fat diet to the rats, weight was increased in the weight was observed in all rats selected in each group. After the treatment with formulations F1 and F2 on animals with HFD and HFD-DIA, there was decreased in the body weight observed from 254.52- 230.7 g (group F1.1), 184.48- 252.25 (group F1.2), 254.03- 223.72 gm (group F2.1), 252.87-220.05 g (group F2.2), 254.10- 221.55 g in standard group. The results concluded that both formulations F1 and F2 reduced increased body weight of rats. However, the formulation F2

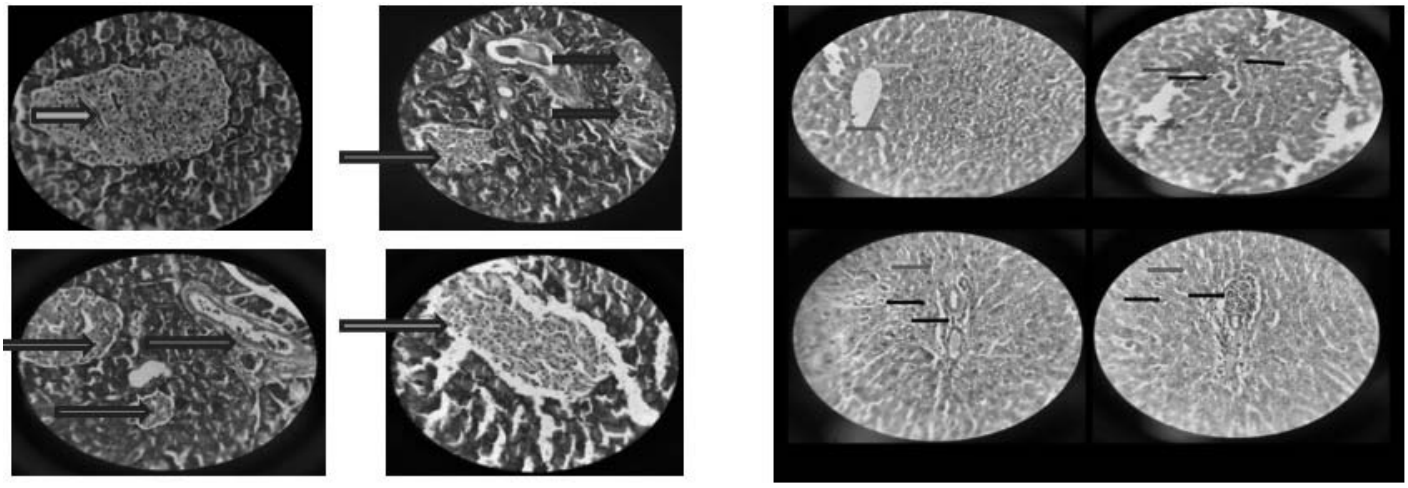

Figure 2: Histopathology of Liver and Pancreas.

showed better action in decreasing body weight compared to the formulation F1 prepared by classical method, shown in Table 2.

Change in blood glucose level

The fasting glucose level and postprandial glucose level in blood was measured and given in Table 3 and 4. After the induction of diabetes in groups normal, HFD- DIA, F2.1, F2.2 and standard there was increased in fasting glucose level and postprandial sugar level in blood observed. After the treatment with formulations F1 and F2 on animals with HFD- DIA, there was decreased in the fasting glucose level observed from 177.00- 161.33 mg/dL (group F2.1), 173.00- 156.83mg/dL (group F2.2), 171.50- 155.33mg/dL in standard group. Also decreased in the postprandial sugar level in the blood was observed ranges from 171.33- 137.33 mg/dL (group F2.1), 169.33- 132.33 mg/dL (group F2.2), 164.33- 130.17 mg/dL in standard group. Both the formulation successfully reduced increase fasting and postprandial sugar level in blood. It was concluded that formulation F2 reduced increase fasting and postprandial sugar level in blood more efficiently shown in Table 3 and Table 4.

Effect on Serum Biochemical Parameters

The rats with high fat diet and alloxan induced diabetes solution (i.e. HFD + DIA) when treated with formulation F1 (i.e., F2.1 group VI) and formulation F2 (i.e., F2.2 group VII), there was a significant decreased in the total cholesterol level observed in group VII (155.42 mg/dL) when compared with group VI (175.5 mg/mL) on the 9th week after treatment shown in Table 5 All the studied on comparison with standard group treated with standard drug (Metformin group VIII) concluded that Amruthotharam Kashaya prepared by modern method (F2) was found to be effective in reducing total cholesterol level in serum.

Effect on serum triglycerides levels (mg/dL)

The rats with high fat diet and alloxan induced diabetes solution (i.e. HFD + DIA) when treated with formulation F1 (i.e. F2.1 group VI) and formulation F2 (i.e. F2.2 group VII), there was a greater reduction in the triglyceride level observed in group VII (35.17 mg/dL) when compared with group VI (43.72 mg/dL) on the 9th week after treatment. All the studies on comparison with standard group treated with standard drug (Metformin group VIII) in Table 6 concluded that *Amruthotharam kashaya* prepared by modern method (F2) was found to be effective in reducing triglyceride level in serum.

Effect on serum HDL levels (mg/dL)

The rats with high fat diet and alloxan induced diabetes solution (i.e. HFD + DIA) when treated with formulation F1 (i.e. F2.1 group VI) and formulation F2 (i.e. F2.2 group VII), the increased in HDL level was observed in group VII (38.78 mg/dL) when compared with group VI (33.83 mg/dL) on the 9th week after treatment. All the studies on comparison with standard group treated with standard drug (metformin group VIII) in Table 7, concluded that *Amruthotharam kashaya* prepared by modern method (F2) was found to be effective in significantly improving HDL level in serum.

Effect on serum LDL levels (mg/dL)

The rats with high fat diet and alloxan induced diabetes solution (i.e. HFD + DIA) when treated with formulation F1 (i.e. F2.1 group VI) and formulation F2 (i.e. F2.2 group VII), there was a greater reduction in the LDL level observed in group VII (55.68 mg/dL) when compared with group VI (66.26 mg/dL) on the 9th week after treatment. All the studies on comparison with standard group treated with standard drug (metformin group VIII) in Table 8 concluded that Amruthotharam kashaya prepared by modern method (F2) was found to be effective in reducing LDL level in serum.

Effect on serum VLDL levels (mg/dL)

the rats with high fat diet and alloxan induced diabetes solution (i.e. HFD + DIA) when treated with formulation F1 (i.e. F2.1

Table 3: Change in the fasting glucose level in blood after treatment with formulations.

Groups	Change in fasting glucose level in blood (mg/dL)			
	1st Week	2nd Week	3rd Week	4th Week
Normal	91.33±0.82	92.33±1.03	93.33±1.37	94.17±1.33
HFD-DIA	194.67±3.33	201.00±3.03	207.50±3.73	214.83±2.86
F2.1	177.00±2.37	173.50±2.43	165.50±1.97	161.33±2.07
F2.2	173.00±1.55	167.33±1.63	162.50±1.87	156.83±1.17

Table 4: Change in the postprandial glucose level in blood after treatment with formulations.

Groups	Change in postprandial glucose level in blood (mg/dL)			
	1st Week	2nd Week	3rd Week	4th Week
Normal	117.67±0.52	118.83±1.17	120.00±0.89	120.33±0.52
HFD-DIA	193.67±3.20	202.17±1.60	212.50±1.38	222.17±2.14
F2.1	171.33±2.50	161.33±1.51	150.00±1.41	137.33±1.97
F2.2	169.33±2.16	159.00±1.26	145.67±2.42	132.33±2.07
Standard	164.33±2.42	155.17±1.94	143.50±2.26	130.17±1.17

Table 5: Effect of F1 and F2 formulations on serum total cholesterol level.

Group	Serum total cholesterol level (mg/dL)		
	0 week	4th week	9th week
F1.1	104.53± 1.30	243.87± 1.54	190.1± 1.28
F1.2	103.95± 0.94	243.95± 0.94	185.8± 1.96
F2.1	105.52± 0.70	244.28± 1.39	175.58± 1.70
F2.2	104.68± 1.09	243.85± 1.72	155.42± 1.70
Standard	104.78± 1.12	244.95± 1.37	173.5± 1.60

Table 6: Effect of F1 and F2 formulations on serum triglycerides level.

Group	Serum triglycerides level (mg/dL)		
	0 week	4th week	9th week
F1.1	23.27±0.69	89.17±0.67	65.02±1.34
F1.2	23±1.04	87.6±0.56	54.75±1.90
F2.1	23.15±0.66	87.82±1.39	43.72±1.48
F2.2	23.07±0.46	88.02±0.96	35.17±1.88
Standard	23.08±0.64	88.3±1.01	44.93±1.31

Table 7: Effect of F1 and F2 formulations on serum HDL level.

Group	Serum HDL level (mg/dL)		
	0 week	4th week	9th week
Normal	42.88±1.22	46.92±0.75	47.53±0.85
HFD	42.53±1.37	20.17±0.90	18.55±0.80
HFD-DIA	43.42±0.35	20.4±0.74	19.62±0.52
F1.1	42.98±1.10	20.72±1.05	25.62±1.12
F1.2	42.78±0.77	20.77±1.23	30.98±1.47
F2.1	42.18±1.55	20.05±0.55	33.83±1.00
F2.2	42.33±1.00	20.7±0.75	38.78±0.74
Standard	42.67±1.35	20.85±1.10	32.67±1.04

Table 8: Effect of F1 and F2 formulations on serum LDL levels (mg/dL).

Group	Serum LDL level (mg/dL)		
	0 week	4th week	9th week
F1.1	50.18±1.03	95±0.99	94.78±1.89
F1.2	51.58±1.14	95.2±0.95	85.57±1.59
F2.1	51.1±1.09	94.57±1.52	66.26±1.62
F2.2	51.38±1.32	95.25±1.88	55.68±2.05
Standard	51.28±0.77	95.72±0.78	65.25±2.10

Table 9: Effect of F1 and F2 formulations on serum VLDL level.

Group	Serum VLDL level (mg/dL)		
	0 week	4th week	9th week
F1.1	14.41±0.75	50.13±0.85	45.58±1.07
F1.2	14.75±0.89	50.53±1.46	37.43±1.16
F2.1	14.58±0.66	50.97±1.97	32.65±1.76
F2.2	14.7±0.92	50±1.24	26.13±1.49
Standard	14.58±0.70	50.22±1.68	33.02±0.97

group VI) and formulation F2 (i.e. F2.2 group VII), there was a significant reduction in the VLDL level observed in group VII (26.13 mg/dL) when compared with group VI (32.65 mg/dL) on the 9th week after treatment. All the studies on comparison with standard group treated with standard drug (metformin group VIII) in Table 9 concluded that Amruthotharam kashaya prepared by modern method (F2) was found to be effective in reducing VLDL level in serum.

Histopathological Evaluation

Histopathology of liver

Histopathology of liver tissue of rats treated with high fat diet and HFD- alloxan induced diabetes showed infiltered cells (Figure 2B, 2C) when compared to normal healthy control rats liver tissue which showed normal hepatocyte and normal sinusoids (Figure 2A). The rats with high fat diet and HFD-alloxan induced diabetes, when treated with formulation F1 and F2 showed decreased number of infiltered cells (Figure 2D, E, F, G). While comparing the formulation F1 and F2, F2 showed better action with no infiltered cells as compared to the rats treated with metformin (Figure 2).

Pancreatic slice of HFD-alloxan-induced diabetic rats treated with Formulation -1 (Kasaya) at 1.5gm/kg body weight demonstrating a modest number of islets of langerhans (orange arrows) and a normal islet of langerhans with many beta cells (green arrows) (Figure 2D). PF2: Pancreatic slice of HFD-alloxan-induced diabetic rats treated with Formulation -2 (extract combination) at 1.5gm/kg body weight revealing a limited number of islets of langerhans (orange arrows) and normal islets of langerhans with abundant beta cells (green arrows) (Figure 2E). PMET: pancreatic slice of diabetic rats treated with Metformin demonstrating normal pancreatic islet of langerhans with increased beta cell population (blue arrows), shown in Figure 2.

The formulation contains guduchi *(Tinospora cordifolia)*, haritaki (*Terminalia chebula*), and shunthi (*Zingiber officinale*). Because natural medicines are derived from wild sources, maintaining consistent product quality is challenging owing to external variables such as well as geographic location, nutrient availability, temperature variations, light and water availability, and soil conditions. The two well-known and widely used medical systems in India are called Ayurveda and Unani Medicine respectively. In India's AYUSH systems, about 8,000 different herbal treatments have been given official status. Tabatabaei M *et al*., 2016[28] One of these medicines is called *Amruthotharam/Amrutottaram Kashayam*, and it treats metabolic issues by treating inflammation as its primary mechanism of action. You LB *et al*., 2019 *Tinospora cordifolia, Terminalia chebula*, and *Zingiber officinale* are used in the composition these natural medicines are derived from wild sources, Singh MP *et al*., 2009 it can be difficult to maintain a consistent product quality due to external factors and climatic conditions. Mukherjee PK *et al*., 2002,[20] Both of the formulations were found to possess an amount of antioxidant activity that was sufficient after being examined as a whole. The F2 formulation has a stronger antioxidant activity than the F1 formulation. Bahmani M, *et al*., 2012[21] The purpose of the pharmacological study was to ascertain whether or not the formulation was successful in combating diabetes and obesity. The evaluation took into account changes in both the fasting glucose level and the postprandial sugar level in the blood. Body weight change was also taken into account. It has been demonstrated that the contemporary method formulation F2 is successful in lowering both excess sugar levels and additional body weight. The total cholesterol level, triglyceride level, HDL level, LDL level, and VLDL level are some of the blood

biochemical indicators that are investigated in this part of the research. It has been demonstrated that consumption of F2 can assist in the lowering of total cholesterol, triglyceride levels, LDL levels, and VLDL levels in the blood, as well as the elevation of HDL levels**.** Ighodaro OM *et al*., 2017[23] Histopathology of liver tissue treated with high fat diet and HFD-alloxan induced diabetes demonstrated infiltered cells as compared to normal healthy control rats liver tissue, which displayed normal hepatocytes and sinusoids. The number of infiltered cells was decreased in rats with high fat diet and HFD-alloxan induced diabetes who were treated with formulations F1 and F2.

CONCLUSION

The populace of developing nations uses medicinal herbs as an alternate form of treatment. Recent years have seen an increase in the scientific foundation of herbal treatment. Studies leading to the scientific explanation of plant medicinal qualities are enabling this approach to achieve increased credibility and acceptability within the medical community in response to requests from both the public and medical authorities. Antioxidant activity of *Amruthotharam kashayam* (F1) and the Kashaya mixture (F2) was examined. It was revealed that both formulations had high antioxidant activity. The F2 formulation was shown to be more effective in protecting against oxidative stress. The pharmacological study was carried out to evaluate the action of formulation against diabetes and obesity. The evaluation was carried out based on parameters such as change in body weight and change in fasting glucose level and postprandial sugar level in blood. The formulation F2 prepared by modern technique was found to be effective in reducing increased body weight and sugar level. The study also evaluates the effect of formulations (F1 and F2) on serum biochemical parameters such as total cholesterol level, triglyceride level, HDL level, LDL level, VLDL level. The formulation F2 was found to be effective in reducing total cholesterol level, triglyceride level, LDL level, VLDL level and improving HDL level in the serum.

Histopathology of liver tissue of rats treated with high fat diet and HFD- alloxan induced diabetes showed infiltered cells when compared to normal healthy control rats liver tissue which showed normal hepatocyte and normal sinusoids. The rats with high fat diet and HFD- alloxan induced diabetes, when treated with formulation F1 and F2 showed decreased number of infiltered cells. The *Amruthotharam* is indeed a very potent medicine which treats overall metabolic disorder as it alters the cellular mishaps.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Original Article

Evaluation of Immunomodulatory Effect of Aqueous Extract of *Bauhinia variegata* L. Leaves

Santanu Saha[1,*], EVS Subrahmanyam[2]

[1]Department of Pharmacognosy, NGSM Institute of Pharmaceutical Sciences, NITTE (Deemed to be University), Mangaluru, Karnataka, INDIA.
[2]Department of Pharmaceutical Chemistry, Srinivas College of Pharmacy, Mangaluru, Karnataka, INDIA.

ABSTRACT

Natural products have the potential to modulate the immunity of human beings either by stimulating or depressing it and the approach was being used over time by medicinal practitioners to treat ailments. This present study was planned to ascertain the immunomodulating potential of one of the Indian traditional medicinal plants *Bauhinia variegata* L. The aqueous extract of powdered leaves of *Bauhinia variegata* L. was prepared by using the decoction method and the extract was then dried and made free from solvent. The extract of *Bauhinia variegata* L. (BVAE) was screened for immunomodulating activity by hemagglutination reaction for humoral and delayed-type hypersensitivity study for cellular immune responses. The sheep RBC ($5X10^9$ cells/mL) was used as antigen and levamisole was used as the standard drug in both studies. The extract of plant material (BVAE) showed a dose-dependent response in these studies. BVAE at 400 mg/kg dose among all the doses of plant material showed significantly ($p<0.05$) high antibody titer 7.13±0.17) in humoral immune response and potential delayed-type hypersensitivity response (0.96±0.06) in the cellular immune study. Whereas, the standard drug levamisole showed the most significant ($p<0.01$) higher antibody titer (7.87±0.19) and delayed-type hypersensitivity response (1.24±0.04). Though the standard drug showed a better response the plant material also showed the potential immunomodulatory effect in the present study. Hence, the findings of this study proposed the immunomodulatory effect of the aqueous extract of *Bauhinia variegata* L., and 400 mg/kg dose of the extract was found to be potent among other doses.

Keywords: *Bauhinia variegata*, Cellular immune response, Haemagglutination, Humoral study, Immunomodulation.

Correspondence:
Dr. Santanu Saha
Assistant Professor, Department of Pharmacognosy, NGSM Institute of Pharmaceutical Sciences,
NITTE (Deemed to be University),
Deralakatte, Mangaluru-575 018,
Karnataka, INDIA.
Email id: santanusaha@nitte.edu.in

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INTRODUCTION

The immune system is a defense mechanism for all living organisms. Innate and adaptive, two types of immune systems are observed. The innate immune system is a natural non-specific immune mechanism initiated during the entry of any pathogens into the host body. Unlike the innate system, adaptive immunity is an antigen-specific immune mechanism that provides an immunologic memory to strengthen the defense system of the host.[1,2] Further, adaptive immune systems are two types, humoral and cell-mediated immune responses which are facilitated by B and T lymphocytes, respectively.[3,4] In healthy conditions, the homeostasis of the immune system is maintained but during pathological conditions cells and chemokines trigger the innate and/or adaptive immune system to control the disorders. Nevertheless, in several disease conditions, like viral infections, cancer, HIV, etc., the natural body defense system failed to fulfill the desired purposes.[5] In such conditions additional immunomodulators are required.

Immunomodulators are natural or synthetic agents which modulate the immune system either by stimulation or suppression to treat disorders.[6] The adaptive immune system plays an important role in immunomodulation as it provides antigen-specific immunological memory which is found to be useful in the treatment process. Though, there are several immunomodulator drugs are available, natural immunomodulating drugs are found to be safe and have the potential for the reason of their synergistic effect.[7] Additionally, the search for effective botanical immunomodulators is always an interest to the scientist.

India has age-old traditional healthcare systems which provide great knowledge about local natural products for the treatment of various diseases. Ayurveda, the ancient Indian medicinal system provides information about several natural immunomodulatory drugs under the Rasayana treatment process.[8] *Bauhinia variegata* L., a traditional Indian plant is usually called "Raktakanchana".[9] Previous studies reported anti-inflammatory, anticarcinogenic, cytotoxic antimicrobial, antioxidant and hepatoprotective

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activities of the plant.[10-14] Authors of the present study have also reported their previous works on the antioxidant and anti-inflammatory activities of the plant.[15,16] Hence, this study was designed to ascertain the immunomodulatory activity of the aqueous extract of leaves of *Bauhinia variegata* L., an Indian traditional medicinal plant.

MATERIALS AND METHODS

Chemicals and drugs

Analytical-grade materials were used in this experiment. Standard immunostimulant drug, Levamisole tablets "DICARIS" was procured from Encore Healthcare Pvt. Ltd, Paithan. 0.9% NaCl, was procured from MERCK Ltd, Mumbai. Ether for Anesthesia was procured from Qualigens Fine Chemicals, Mumbai. Carboxymethyl cellulose (CMC) was obtained from Loba Chemie Pvt. Ltd., Mumbai.

Collection of plant material

Leaves of *Bauhinia variegata* were collected from Paneer, Deralakatte, Karnataka, India and the identity of the plant was authenticated by Dr. Noeline J. Pinto, Head of Botany, Department St. Agnes College, Mangaluru. A specimen (no. 324 b) was submitted to the institute.

Preparation of aqueous extract

Collected leaves were dried under shade and powdered. The powdered leaves were extracted by boiling them with distilled water and then filtered. Four times the process was repeated. The resultant extract was concentrated in the water bath and further made into a dry extract. This aqueous extract (BVAE) was further used in the study to screen for the immunomodulatory effect of the plant material.

Pharmacological activity studies

Animals

Swiss albino mice of weight between 15-20g and of male and female both sex were obtained from KSHEMA (K. S. Hegde Medical Academy), Deralakatte Mangaluru. Acute toxicity and immunomodulatory activity studies of the plant drug were carried out by using these animals. Animals were acclimatized for one week in cages in 12 hr light and dark cycle at room temperature and 60 ± 5% relative humidity. Animals of the present study were fed with pellets which were supplied by Hindustan Lever Co., Mumbai, and water *ad libitum* during the period of the study. Experiments of the present study were conducted as per the institutional animal ethics committee guidelines, KSHEMA, Deralakatte, Mangaluru (KSHEMA /AEC/077/2008).

Acute toxicity study

The toxicity study of BVAE was carried out in adult female albino mice by the "up and down" method of OECD guidelines 425 and the test drug was found to be safe till a higher dose.[17] Based on the toxicity study, 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight, per oral doses of plant material (BVAE) were decided for the immunomodulatory activity studies.

Immunomodulatory effect of aqueous extract of Bauhinia variegata L. (BVAE)

The immunomodulatory activity of BVAE was evaluated by *in vivo* models, humoral study, and delayed-type hypersensitivity study models in mice.

Experimental antigen

The antigen of the present study sheep red blood cells/sheep RBC (SRBC) was collected as per the requirement freshly in the container containing Alsever's solution. The SRBC was centrifuged three times with normal saline (0.9% NaCl w/v) at 2000 rpm for 10 min at 20°C. Phosphate buffer saline (pH 7.4) was mixed with SRBC to adjust the final volume. 20 microliters of SRBC ($5X10^9$ cells/mL) were administered intraperitoneally (i.p) to animals for immunization and challenge as per the schedule.

Group design

For each study, five groups of animals (Swiss albino mice) were made and each group contains six animals (n=6) as follows:

Sensitized control group: Immunized animals of this group received only 1mL of 2% solution of CMC (vehicle).

BVAE 100, 200, 400 mg/kg group: Immunized animals of these groups received respectively 100 mg/kg, 200 mg/kg, 400 mg/kg b.w., p.o. dose of BVAE as a suspension in the vehicle.

Standard group: Immunized animals of this group were treated per day with 2.5 mg/kg b.w., p.o dose of suspension of standard drug Levamisole in the vehicle.

Humoral study: Hemagglutination reaction model

Animals of all groups were immunized with 0.2mL of $5X10^9$ SRBC/mL SRBC by injecting intraperitoneally (i.p) on day 0. From day 1 to day 14 animals of the individual group as explained in the group study received oral treatment of respective drugs and blood was collected from those animals on the 15th day of the study to check primary humoral immune responses. On the same day, all animals were further received 0.2mL SRBC intraperitoneally (i.p) and kept under observation till day 21 of the study. To check secondary humoral immune responses blood was collected from animals of all groups on day 21. In the present study, the hemagglutination reaction method i.e., anti-SRBC antibody titer,

was used to measure humoral response as described by Nelson and Midenhall (1967).[18]

Cellular immune response study: Delayed type hypersensitivity (DTH) model

A delayed-type hypersensitivity model in the present study was carried out as described by Doherty, to evaluate the cellular immune response or protective immune response of BVAE.[19] On day 0, $5X10^9$ cells of SRBC/mL and saline respectively were injected subcutaneously (S.C.) in the footpad of the left hind paw and the right hind paw of animals of all groups. From day 1 to day 14 animals of the individual group as explained in the group study received oral treatment of respective drugs and on day 15, animals were challenged with SRBC. After 24 hr of the challenge, the paw thickness of animals was measured by using digital vernier calipers.

Statistical Analysis

All results were expressed as mean ± S.E.M. ANOVA, followed by Dunnet's *t*-test was used to determine statistical significance and the results with *p*-value <0.05 were considered statistically significant.

RESULTS

The acute oral toxicity study of aqueous extract of *Bauhinia variegata* L. leaves was carried out for up to 2000 mg/kg dose as per OECD guidelines. The test drug was found to be safe in the study up to a higher dose. Hence, 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight, per oral dose of the plant drug were chosen for further studies. The immunomodulatory activity of BVAE was evaluated by humoral and cell-mediated immune response study models.

Humoral study

BVAE showed a dose-dependent immune response in the present study. The 400 mg/kg dose of BVAE showed a significant (p<0.05) and the most potent humoral response (7.13±0.17) among the test materials (Table 1 and Figure 1). Standard drug levamisole showed a significant (p<0.01) effect (7.87±0.19) in the study. BVAE at 100 and 200 mg/kg doses also showed humoral response but the result was found to be insignificant. Concerning the percentage of immunomodulatory effect, the standard drug showed the highest 37.83% effect (Table 1) but the response of the BVAE with 400 mg/kg dose was also found to be nearby (24.87%).

Cellular immune response study

As shown in Table 1, in the present study, the standard drug, levamisole showed a potent delayed-type hypersensitivity response (p<0.01, 1.24±0.04) with 58.97% immunomodulatory effect (Figure 2). A dose-dependent response for BVAE was observed in this study also and 400 mg/kg dose showed a higher response (p<0.05, 0.96±0.06) with 23.07% immunomodulatory effect among all the test materials (Table 1 and Figure 2).

DISCUSSION

Bauhinia variegata L. is a widely available Indian traditional medicinal plant. As we discussed in the introduction part of this article, the anti-inflammatory, antidiabetic, antioxidant, anti-carcinogenic, and hepatoprotective activity of the plant was reported earlier in several studies. The present study was designed to evaluate the immunomodulatory activity of the plant as there are no studies that have reported so far, the above-mentioned activity of the leaves of *Bauhinia variegata* L.

Adaptive immunity is a common phenomenon for the defense mechanism of the human body against antigens. The immune mechanism either triggers antibody development and/or apoptosis as a cell-mediated response to defend the body against foreign pathogens.[20,21] The natural adaptive system can be modified and improved by immunomodulating agents. In this context, a natural product was evaluated for immunomodulating activity in this study.

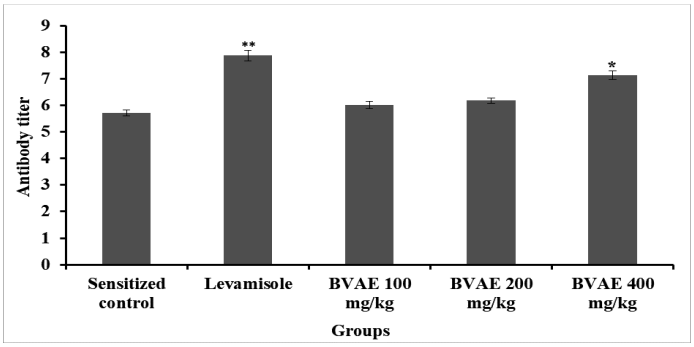

Figure 1: Immunomodulatory effect of aqueous extract of leaves of *Bauhinia variegata* L. by *in vivo* models for humoral immune response study. Values are mean ± S.E.M for each group containing six animals (n=6); *indicates the *p*-value is <0.05 and ** indicates the *p*-value <0.01in comparison with the sensitized control group. BVAE: aqueous extract of leaves of *Bauhinia variegata* L.

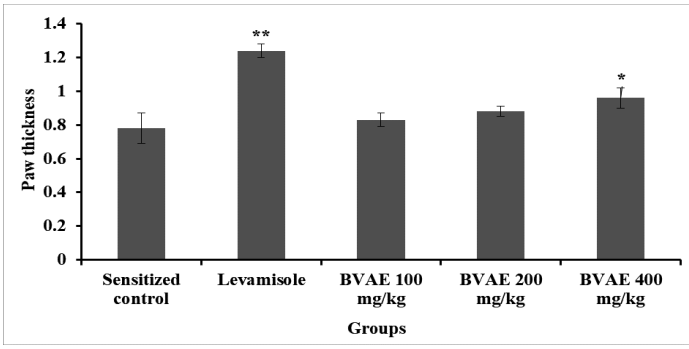

Figure 2: Immunomodulatory effect of aqueous extract of leaves of *Bauhinia variegata* L. by *in vivo* models for delayed-type hypersensitivity study. Values are mean ± S.E.M for each group containing six animals (n=6); *indicates the *p*-value is <0.05 and ** indicates the *p*-value <0.01in comparison with the sensitized control group. BVAE: aqueous extract of leaves of *Bauhinia variegata* L.

Table 1: Immunomodulatory activity of aqueous extract of leaves of *Bauhinia variegata* L. by *in vivo* models for humoral and cellular immune response study.

Group	Humoral study		Cellular immune response	
	Antibody titer	Immunomodulatory activity (%)	Paw thickness	Immunomodulatory activity (%)
Sensitized control	5.71±0.12	-	0.78±0.09	-
Levamisole	7.87±0.19	37.83**	1.24±0.04	58.97**
BVAE 100 mg/kg	6.02±0.14	5.42	0.83±0.04	6.41
BVAE 200 mg/kg	6.18±0.10	8.23	0.88±0.03	12.82
BVAE 400 mg/kg	7.13±0.17	24.87*	0.96±0.06	23.07*

Values are mean ± S.E.M for each group containing six animals (n=6); *indicates the p-value is <0.05 and ** indicates the p-value <0.01in comparison with the sensitized control group. *BVAE:* aqueous extract of leaves of *Bauhinia variegata* L.

The formation of antigen-specific antibodies takes place in B-lymphocyte cells. This humoral immune response takes certain days to develop, hence the study period used to be kept for 21 days to assess the response of the test drugs.[22] The humoral immune response of the test drugs is evaluated by the antigen-antibody interactions. In the *in vivo* immunomodulatory evaluation study, the haemagglutination reaction is being used for assessing antigen and antibody interactions and the titer value of the reaction is considered to be the humoral response.[23] In the present study, the aqueous extract of leaves of *Bauhinia variegata* L. (BVAE) at 400 mg/kg dose showed a 24.87% humoral immunomodulatory effect with the titer value of 7.13±0.17 and the result were found to be most potent among all the other doses of the test material.

The immune response of the test material in the humoral study was further corroborated by the protective immune response study. The protective or cellular immune response of the drug was assessed by a delayed-type hypersensitivity study. T lymphocytes are the marker for cellular immune response and the previous studies reported its correlation with B cells for humoral responses.[24] BVAE at 400 mg/kg dose showed a 23.07% immunomodulatory effect in the cell-mediated immune response study, whereas, levamisole showed a 58.97% response in the study. Hence the present study showed the immunomodulatory effect of the aqueous extract of leaves of *Bauhinia variegata* L.

CONCLUSION

The present study was planned to evaluate the immunomodulatory effect of leaves of *Bauhinia variegata*. The results of the study showed the potent effect of plant material in humoral and cellular immune response studies. Doses of aqueous extract of leaves of *Bauhinia variegata* were decided by performing an acute toxicity study as per the standard guideline and the test material with all those doses was used for further immunomodulatory activity studies. The test material showed a dose-dependent immunomodulatory response in studies and 400mg/kg dose of BVAE was found to be the most potent among all the test materials. Hence the finding suggested the immunomodulatory activity of leaves of *Bauhinia variegata* L. which also supported the hypotheses of the present study. Nevertheless, further in-depth studies are required to be carried out to find the possible mechanism of action and phytoconstituent of the drug which is responsible for the above activity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

ABBREVIATIONS

b.w.: Body weight; **p.o.:** Per oral.

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Original Article

Cost-Minimization Analysis of Medications Used in the Management of End-stage Renal Disease

Jarupala Gangadhar Naik[1], Sreedhar Dharmagadda[1], Pradeep Manohar Muragundi[1,*], Virendra Ligade[1], Shankar Prasad Nagaraju[2], Manjunath Kulkarni[3]

[1]Department of Pharmacy Management, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal, Karnataka, INDIA.
[2]Department of Nephrology, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, Karnataka, INDIA.
[3]Department of Nephrology, Father Muller Medical College, Rajiv Gandhi University of Health Sciences, Mangalore, Karnataka, INDIA.

ABSTRACT

Aim/Background: Several branded pharmaceuticals and generic medicines are available in the market for the management of End-Stage Renal Disease (ESRD) as a supportive care, and clinicians are unaware of the cost minimization and cost consequences aspects of these medications. Thus, this study aimed to compare the prices of branded versus generic medicines for ESRD treatment and to present the cost savings with a generic alternative. **Materials and Methods:** A prospective observational study was conducted among ESRD patients from three different tertiary care teaching hospitals in South India. The cost of branded pharmaceuticals were determined using the most recent current index of medical specialties, while the prices of generic medicines were accessed using the Pradhan Mantri Bhartiya Jan Aushadhi Pariyojana scheme, 2022. **Results:** The data were collected from 385 patients with ESRD. All Jan Aushadhi generic medicines were less expensive when compared to the branded medicines. The cost of ESRD medicines accessible in India varies greatly. The greatest difference in percentage cost savings were noted with amlodipine 5 mg tab (93.03%), voglibose 0.2mg/tab (88.10%), calcium carbonate + Vitamin D_3 supplements 500 mg tab (80.27%), torsemide 10 mg tab (78.01%), and hematopoietic agent, erythropoietin 2000 U/inj (75.38%). **Conclusion:** The haematopoietic medicines and antidiabetic insulin injections were the most expensive medicines among the study population. Our research indicates that replacing the generic medicines with the branded medicines could help in reducing the cost burden. Healthcare professionals may consider prescribing generic medicines for cost-savings.

Keywords: Pharmacoeconomics, Cost minimization, End stage renal disease, Antibiotics, Insulin, Jan Aushadhi.

Correspondence:
Dr. Pradeep Manohar Muragundi
Department of Pharmacy Management, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal-576104, Karnataka, INDIA.
Email id: pradeep.mm@manipal.edu

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INTRODUCTION

End-Stage Renal Disease (ESRD) is a severe medical condition which contribute to the serious health consequences and financial burden. As per the recent statistics, the global prevalence of Chronic Kidney Disease (CKD) is estimated to be 13.4% (11.7-15.1%) with 4.902 to 7.083 million people required Renal Replacement Therapy (RRT) with ESRD.[1] In India, 152 individuals in a million population have ESRD and the most frequent cause of ESRD is diabetic renal disease.[2] The global variation in prevalence and incidence of ESRD is based on each nation's specific genetic, environmental, lifestyle, and sociodemographic behaviors.[3] Though there are many RRT are available, the choice and adherence are based on the patient's financial background and affordability.[4]

The term Pharmacoeconomics (PE) defined as "the studies that evaluates the behavior of individuals, firms, and markets relevant to the use of pharmaceutical products, services, and programs, and which majorly focuses on the costs (inputs) and consequences (outcomes) of that use.[5,6] The PE evaluations includes the Cost Minimization Analysis (CMA), Cost Effectiveness Analysis (CEA), Cost Utility Analysis (CUA), and Cost Benefit Analyses (CBA). CMA is an excellent PE method for assessing the drug costs and highlights the least costly drug or therapeutic modality between the similarly efficacious drugs or procedures.[7]

It is well documented that, ESRD being advanced stage of CKD, burden the patient and patient relatives.[8,9] The higher costs of hemodialysis and the associated medicines itself forces the patient and patient party to non-adhere the treatment pattern in many ways like, cut down on the number of dialysis procedures

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prescribed, not procuring the medicines as prescribed or incomplete duration of the treatment. There was a significant association between the ESRD and non-adherence of therapy. Also, the evidences indicate the influence of poor financial background in discontinuation or non-adherence of treatment.[10,11] The cost of therapy is one of the most important components of medical management and price of medicines is the most significant cost that the patients or patient party must endure it. The trademark or branded drug prescribing nature of the physician burdens the patients and may lead to the patient non-compliance or non-adherence.[10,12] Hence, CMA are very much important to give a clear insight to reduce the economic burden, enhance the treatment adherence, and better patient outcomes. Moreover, the CMA compares and measures input costs under the assumption that the outcome is equivalent between two alternatives which are clinically equivalent.[13,14]

Table 1: Socio-demographic and medical outline of hemodialysis sufferers at tertiary care Hospital (*n*= 385).

Parameters	Total number and percentage (*n*=385)
Gender	
Male	283 (73.50%)
Female	102 (26.49%)
Family annual gross income	
Less than 1 lakh	136 (35.32)
1-5 lakh	168 (43.63)
5-10 lakh	63 (16.36)
More than 10 lakhs	18 (4.67%)
Residence area	
Urban	147 (38.18)
Rural	238 (61.81)
Social History	
Smoking	49 (12.72)
Alcohol	32 (8.31)
None	304 (78.96)
Number of Hemodialysis (sessions / week)	
One / week	14 (3.63)
Two/ week	301 (78.18)
Three /week	70 (18.18)
Total number of medications	
<5	36 (9.35)
5 to 10	251 (65.19)
11 to 15	90 (23.37)
16 to 20	8 (2.07)

Pharmaceuticals used as supplementary systematic therapy are manufactured by numerous drug companies and sold under variety of brands at high prices that are unaffordable to the patients, particularly in developing countries like India. Whereas the generic medicines are accessible in the Indian market at a lower cost which are having the equal efficacy and safety.[7] To address these issues of accessibility of medicine to a huge proportion of the community, the government of India has launched many initiatives through different programs and schemes intended to increase public awareness of generic medicines. One such initiative is Jan Aushadhi.

Many supplementary medications for hemodialysis at ESRD are now available at low prices as generic medicines under the Pradhan Mantri Bhartiya Jan Aushadhi Pariyojana (PMBJP) scheme, which are marketed and made public through the Jan Aushadhi Kendra drugstores to offer various generic medicines to the populace.[15] However, no studies have been conducted to provide information on the cost differences for each therapeutic dose, for a given period, as well as operating costs when using generic medicines for ESRD patients. Hence, we used CMA method to analyze the proportion of price fluctuation as well as evaluated price reductions through prescribing the generic alternative of the branded medicines in the overall length of therapeutic interventions among the patients with ESRD which will help to give a clear picture of the current scenario and assist to make further policies in future.

MATERIALS AND METHODS

Ethical approval

The ethical clearance to conduct this research is obtained from the respective institutional ethics committees of the study sites included. The study is conducted in accordance with the terms of the 1995 Helsinki Declaration (as revised in Edinburgh 2000), ensuring the confidentiality of patient data. Also, the study protocol is registered in Clinical Trial Registration-India (CTRI) with a registration number CTRI/2019/08/020874.

Study setting and design

This research was designed and conducted as a prospective CMA among the ESRD patients at three prominent tertiary care teaching hospitals in Karnataka, India, during the six months (November 2021 to April 2022).

Study participants

The non-probability convenience sampling method was used for the selection of patients. The ESRD patients who are undergoing the hemodialysis with an age of 18 or above were considered in this study. The AKI or CKD patients without hemodialysis or with peritoneal dialysis, pregnant woman, and patients with hemodialysis for less than three months were excluded from the

study. Patients who were undergoing therapy on the day of the visit and who refused to give permission were also not included in the study. Participation in the research was voluntary and written consent was obtained from patients who accepted to be a part of the research.

Data collection

The drug utilization pattern of various medications was collected from the patient's medication chart. The information such as name of the brand, generic name, dose, route of administration, frequency and duration of treatment were noted on a well-defined data collection form. The data was classified in accordance with the category of medication.

Cost data

The online CIMS was utilized to obtain price information for each branded medicine, and the generic version available in the Indian market was obtained through the Jan Aushadhi scheme portal. The variation in the percentage of cost and the cost-saving potential for haemodialysis regimens was then calculated by incorporating the available generic drug and forecasting it using the standard formula. The medicine prices were expressed in Indian Rupees (INR).

Cost-minimization analysis

All the drug prices were mentioned in Indian rupees, and each drug's price was compared to the equivalent generic version. The cost difference between the branded drug to the generic drug was calculated, and consecutive data was entered into the excel sheet. To calculate the overall cost of therapy, the cost of each drug was determined. The individual medicine cost was compared, and the calculated amount was denoted as percentage variation. Cost savings was presented by drug therapeutic category wise. The percentage cost variation was calculated using the formula given by the previous literature.[7,14]

$$\text{Percentage cost savings} = \frac{\text{Difference in the cost between branded and generic drugs}}{\text{Cost of branded drug}} \times 100$$

Statistical analysis

The information was entered into Microsoft Excel (version 2016) and presented in tabular format. A descriptive statistics were used to compute the demographic data and presented in frequency in percentage. All the cost minimization results were presented in percentage.

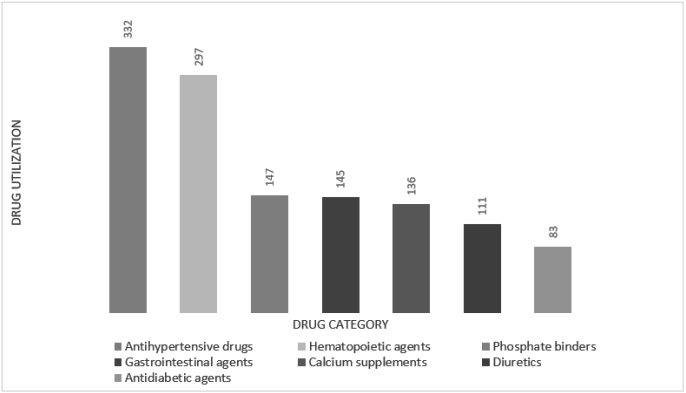

Figure 1: Drug utilization pattern among the ESRD patients.

RESULTS

Demographics of the included patients

A total of 385 patients with a mean age of 53.79 (13.61) years undergoing ESRD treatment at three different study sites were enrolled. The majority (73.50%; *n*=283) of the population were male and remaining 26.49% (*n*=102) were female. The major proportion of the included participants were receiving dialysis at the general dialysis units (87.27%; *n*=336), followed by the isolation room (6.49%; *n*=25), and special dialysis units (6.23%; *n*=24). The demographic characteristics of patients is provided in Table 1.

Drug utilization pattern

Out of 385 patients included, 332 (86.23%) patients used antihypertensive drugs, 297 (77.14%) used hematopoietic agents, 147 (38.18%) used phosphate binders, 145 (37.66%) used gastrointestinal agents, 136 (35.32%) used calcium supplements, 111 (28.83%) used diuretics, and 83 (21.55%) used antidiabetic agents. The drug utilization pattern among the participants is given in Figure 1.

Cost-minimization analysis

The monthly expenditure may be saved if hemodialysis patients used generic medicines. Antihypertensive (HTN) agents such as S (-) Amlodipine 5 mg/tab (93.03%), Cilnidipine 20 mg/tab (87.98%), Amlodipine 10 mg/tab (81.81%), Prazosin HCl 2.5 mg/tab (74.52%), Carvedilol 6.25 mg/tab (89.06%), Metoprolol 50 mg/tab (77.32%), Atenolol 50 mg/tab (79%), Nebivolol 5 mg/tab (70.67%), Telmisartan 40 mg/tab (83.37%), Enalapril 5mg/tab (86.11%), and Labetalol (74.70%) are depicted in Table 2. HTN medications have the lowest chance of saving the cost for HD patients e.g., Clonidine 100 mcg/tab (34.30%), Nifedipine 10 mg tab (16.66%). Hematopoietic agents Erythropoietin 4,000 U/inj (66.21%) and 2000 U/inj (75.41%). Cresp (Darbepoetin alfa) and Venofer (Iron Sucrose) injections are not provided under the Jan Aushadhi scheme. Phosphate binders, such as calcium acetate 667 mg/tab, can save 33.80% of the amount. The

Table 2: Cost-minimization analysis for antihypertensive, hematopoietic, and phosphate binders.

Brand name (Generic name)	Dose	Monthly consumption	Unit cost of branded drugs (In rupees INR)	Monthly cost of branded drugs (In rupees INR)	Unit cost of generic drugs (In rupees INR)	Monthly cost of generic drugs (In rupees INR)	Monthly cost minimization (Difference between branded and generic drugs) (In rupees INR)	Percentage cost Savings
Anti-hypertensives								
Arkamin (Clonidine)	100 mcg/ tab	60	1.37	82.2	0.9	54	28.2	34.30
Nicardia (Nifedipine)	5 mg/cap	90	1.6	144	-	-	-	-
	10 mg/cap		0.96	86.4	0.8	72	14.4	16.66
Cilacar (Cilnidipine)	5 mg/tab	60	5.95	357	1	60	297	83.19
	10 mg /tab		9.88	592.8	1.5	90	502.8	84.81
	20 mg/tab		14.98	898.8	1.8	108	790.8	87.98
Amlodac (Amlodipine)	5 mg/tab	60	2.74	164.4	0.5	30	134.4	81.75
	10 mg/tab		5.5	330	1	60	270	81.81
Prazopress XL (Prazosin HCl)	2.5 mg/tab	60	7.85	471	2	120	351	74.52
Cardivas (Carvedilol)	3.125 mg/ tab	60	4	240	0.7	42	198	82.5
	6.25 mg/tab		6.4	384	0.7	42	342	89.06
Metolar (Metoprolol)	25 mg/tab	60	2.4	144	0.6	36	108	75
	50 mg/tab		3.97	238.2	0.9	54	184.2	77.32
Asomex (S (-) Amlodipine)	2.5 mg/Tab	60	4.59	275.4	1.1	66	209.4	76.03
	5 mg/tab		9.47	568.2	0.66	39.6	528.6	93.03
Aten (Atenolol)	25 mg/tab	60	1.99	119.4	0.42	25.2	94.2	78.89
	50 mg/tab		2	120	0.42	25.2	94.8	79
Nebicard (Nebivolol)	2.5 mg/tab	60	8.48	508.8	2.6	156	352.8	69.33
	5 mg/tab		13.64	818.4	4	240	578.4	70.67
Telma (Telmisartan)	20 mg/tab	60	3.99	239.4	1	60	179.4	74.93
	40 mg/tab		7.22	433.2	1.2	72	361.2	83.37
	80 mg/tab		11	660	2.2	132	528	80
Envas (Enalapril)	5 mg/tab	60	3.61	216.6	0.5	30	186.6	86.14
Lobet (Labetalol)	100 mg/tab	60	17.79	1067.4	4.5	270	797.4	74.70
Haematopoietic agents								
Eryprosafe (Erythropoietin)	4,000 U/inj	4	879.2	3516.8	297	1188	2328.8	66.21
	2,000 U/inj	8	853.32	6,826	210	1680	5,146	75.38
Cresp (Darbepoetin alfa)	60 mcg/inj	4	4900	19600	-	-	-	-
Venofer (Iron (Sucrose)	100 mg/inj	8	360	2880	-	-	-	-

Brand name (Generic name)	Dose	Monthly consumption	Unit cost of branded drugs (In rupees INR)	Monthly cost of branded drugs (In rupees INR)	Unit cost of generic drugs (In rupees INR)	Monthly cost of generic drugs (In rupees INR)	Monthly cost minimization (Difference between branded and generic drugs) (In rupees INR)	Percentage cost Savings
Phosphate binders								
Phostat (Calcium acetate)	667 mg/tab	90	4.23	380.7	2.8	252	128.7	33.80
Revlamer (Sevelamer carbonate)	400 mg/tab	90	8.6	774	-	-	-	-
Fosbait (Lanthanum carbonate)	250mg/tab	90	13.61	1,224.9	-	-	-	-
	500 mg/tab		29.68	2,671.2	-	-	-	-
Dynulta (Sucroferric oxyhydroxide)	2500mg/tab	90	-	-	-	-	-	-

phosphate binders that are not available for haemodialysis under the Jan Aushadhi scheme, are Revlamer (Sevelamer carbonate), Fosbait (Lanthanum carbonate), and Dynulta (Sucroferric Oxyhydroxide). Haematopoietic medicines and phosphate binders are very expensive for HD patients.

DISCUSSION

Indian population suffers numerous issues when it comes to paying for medicines. This is due to their lack of knowledge about various health regulations and government schemes, as well as the fact that they must pay the bills out of their own pockets.[16] Health care professionals should be aware to offer them the best drug with least price. The cost analysis study become particularly crucial to provide better medical care to more patients at a lower cost while maintaining efficacy and safety to the greatest extent possible.[14] The economic situation in India, particularly in hemodialysis treatment, makes it difficult to pay for 80-90% of the treatment costs.[17] In this case, the findings of our study will be useful in the selection of low-cost drugs. The results presented in this study provide more clarity to understand the individual drug users based on their therapy. As a result of this, the overall cost savings can be demonstrated in the study concerned with the field category of each drug. The outlay opportunities from branded drugs to generic drugs, which are commonly used in haemodialysis therapy could be beneficial to the patients to reduce the cost burden especially in a country like India.The PMBJP scheme in India has played a significant role in lowering miscellaneous costs and offering better medicines at a reasonable cost. Moreover, this plan offers various economic benefits, such as medication price control, tax reductions, and the regular updating of critical drug information.[18] As per a medication audit, about 80% of all pharmaceuticals in India are promoted like trademarked compounds, which are costly than their non-branded generic counterparts.[19] Nevertheless, different investigations have demonstrated that unbranded medications are equally efficacious as trademarked pharmaceuticals as well as meet the quality criteria of the Indian Pharmacopoeia.[20] Furthermore, comparative studies have confirmed that substituting non-branded medications can result in a 15% reduction in overall medication costs.[21]Our findings indicate that pricing of various drugs used during the ESRD management vary widely in Indian healthcare market. The medicines with the highest cost-benefit ratios in INR per month are antihypertensive agents, such as labetalol (100mg/tab), cilnidipine (20 mg/tab), nebivolol (5 mg/tab), S (-) amlodipine, and telmisartan (80 mg/tab). The haematopoietic agents such as erythropoietin (2,000 U/inj), phosphate binders are calcium acetate (667 mg/tab), gastrointestinal agents, pantoprazole + domperidone (40 mg cap), rabeprazole (20 mg/tab), and esomeprazole (40 mg/tab). The calcium supplements like calcium carbonate + Vitamin D_3 (500 mg/tab), and diuretics torsemide (10 mg/tab). Antidiabetic agents including Insulin human (recombinant; 40 IU/inj), Insulin human (soluble; 40IU/inj), vildagliptin (50 mg/tab) and voglibose (0.3 mg/tab).The medicines with the lowest cost-benefit ratios in INR per month are antihypertensive drugs such as amlodipine (5mg/tab), atenolol (25 mg/tab), metoprolol (25 mg/tab), clonidine (100 mcg/tab), and nifedipine (10 mg/cap). The haematopoietic agents like erythropoietin (2,000 U/inj). The gastrointestinal agents are omeprazole 20 mg/cap, ranitidine 150 mg/tab, and famotidine 20 mg/tab. Calcium supplements like calcium with Vitamins (0.25 mcg/tab), and diuretics frusemide (40 mg/tab). Also, the antidiabetic agents like glipizide (5 mg/tab), metformin (500 mg/

Table 3: Cost-minimization analysis for gastrointestinal agents, calcium supplements, diuretics, and antidiabetic agents.

Brand name (Generic name)	Dose	Monthly consumption	Unit cost of branded drugs (In rupees INR)	Monthly cost of branded drugs (In rupees INR)	Unit cost of generic drugs (In rupees INR)	Monthly cost of generic drugs (In rupees INR)	Monthly cost minimization (Difference between branded and generic drugs) (In rupees INR)	Percentage cost Savings
Gastrointestinal agents								
Pan D (Pantoprazole + Domperidone)	40 mg/ cap	60	11.86	711.6	2.2	132	579.6	81.45
Omez (Omeprazole)	20 mg/ cap	60	3.68	220.8	0.9	54	166.8	75.54
Nexpro (Esomeprazole)	20 mg / tab	60	5.12	307.2	1	60	247.2	80.46
	40 mg/ tab		9.10	546	1.9	114	432	79.12
Rantac (Ranitidine)	150 mg/ tab	60	1.22	73.2	0.5	30	43.2	59.01
Rabicip (Rabeprazole)	20 mg/ tab	60	9.75	585	0.8	48	537	91.79
Acilo (Famotidine)	20 mg/ tab	60	0.32	19.2	0.21	12.6	6.6	34.37
	40 mg/ tab		0.56	33.6	0.28	16.8	16.8	50
Calcium supplements								
Shelcal (Calcium carbonate + Vitamin D_3)	500 mg/ tab	60	6.59	395.4	1.3	78	317.4	80.27
Calcit (Calcium with Vitamins)	0.25 mcg/tab	30	6.4	192	1.3	39	153	79.68
Diuretics								
Frusenex (Frusemide)	40 mg/ tab	60	0.52	31.2	0.5	30	1.2	3.84
Dytor (Torsemide)	10 mg/ tab	60	5.14	308.4	1.13	67.8	240.6	78.01
Antidiabetic agents								
Ten 20 (Teneligliptin)	20 mg/ tab	60	11.95	717	5	300	417	58.15
Reclide – XR (Gliclazide)	60 mg/ tab	60	11.75	705	4	240	465	65.95
Volibo (Voglibose)	0.2 mg/ tab	60	9.25	555	1.1	66	489	88.10
	0.3 mg/ tab	60	12.5	750	1.4	84	666	88.8
Obimet (Metformin)	500 mg/ tab	60	1.64	98.4	0.6	36	62.4	63.41
Galvus (Vildagliptin)	50 mg/ tab	60	19.93	1,195.8	4	240	955.8	79.92

Brand name (Generic name)	Dose	Monthly consumption	Unit cost of branded drugs (In rupees INR)	Monthly cost of branded drugs (In rupees INR)	Unit cost of generic drugs (In rupees INR)	Monthly cost of generic drugs (In rupees INR)	Monthly cost minimization (Difference between branded and generic drugs) (In rupees INR)	Percentage cost Savings
Glynase (Glipizide)	5 mg/tab	60	0.61	36.6	0.5	30	6.6	18.03
Human actrapid (Insulin human, Recombinant)	40 IU/inj	45	154.66	6,959.7	80	3600	3359.7	48.28
Human mixtard (Insulin human, soluble)	40 IU/inj	45	158.42	7,128.9	90	4050	3078.9	43.18

tab), teneligliptin (20 mg/tab), and gliclazide (60 mg/tab) are depicted in Table 3. Interestingly, Erythropoiesis-Stimulating Agents (ESAs) have a significant impact on the health of patients suffering from renal anemia. ESAs are extremely expensive, and access to them is likely to be limited.

Results of the study clearly indicate that switching from branded medicines to generic equivalents can be an important strategy to reduce the economic burden in a condition like ESRD and in a country like India, where health is completely an out-of-pocket expenditure. Similarly, other CMA on medications like chemotherapeutic agents and proton pump inhibitors from South Indian states also indicates a cost savings from generic alternative replacements. Venkataraman R *et al*., indicated a potential cost saving of INR 41582 by replacing the branded medicines with its generic alternatives in proton pump inhibitors. Also, they observed a percentage cost variation from 135% to 490% with esomeprazole and pantoprazole, respectively.[14] Similarly, the study by Kashyap *et al*., recorded that, cyclophosphamide and 5-Fluorouracil observed to have a 25% to 606.11% cost variation, respectively. A lowest variation was observed with cyclophosphamide (71.42%-114.28%) and highest with gemcitabine (373.68%-990.78%) between its different brands compared with the generic alternatives.[7]Our results were also in line with the findings of the previous evidences and an extensive cost minimization and other pharmacoeconomic evaluations are needed to reduce the cost burden among the patients especially in a developing country like India. These evidences could help in the development of policy making by the respective authorities and stress the need to be implemented in the healthcare system of India.

CONCLUSION

The study findings indicate that substituting the branded medication with a generic alternatives could help the patients to reduce the economic burden, especially in patients with ESRD. This will help in a better patient compliance, treatment adherence and treatment outcomes. Healthcare practitioner may adopt prescribing the generic alternatives with least price without compromising the efficacy of the drug for the benefit of the ESRD patients.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CMA: Cost minimization analysis; **PMBI:** Pharmaceuticals and Medical Devices Bureau of India; **PMBJP:** Pradhan Mantri Bhartiya Jan Aaushadhi Pariyojana; **ESRD:** End stage renal disease; **HD:** Haemodialysis; **ISPOR:** International Society for Pharmacoeconomics and outcomes Research. **OOPEs:** Out-of-pocket expenditures; **API:** Active Pharmaceutical Ingredient; **INN:** International Nonproprietary Names; **CBA:** Cost Benefit analysis; **CEA:** Cost effectiveness analysis; **CUA:** Cost utility analysis; **CCA:** Cost consequence analysis; **COI:** Cost of Illness.

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Original Article

Prevalence of Drug-Related Problems in Elderly Cancer Patients: A Prospective Observational Study in a Cancer Specialty Hospital

Acsah Annie Paul[1], Madhan Ramesh[1,*], KG Srinivas[2]

[1]Department of Pharmacy Practice, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Mysuru, Karnataka, INDIA.
[2]Department of Medical Oncology, Bharat Hospital and Institute of Oncology, Mysuru, Karnataka, INDIA.

ABSTRACT

Background: The geriatric population comprises the majority of patients with oncological disorders. They are highly vulnerable to drug-related problems (DRPs) due to multiple comorbidities, polypharmacy, altered pharmacokinetics and pharmacodynamics. This study aimed to determine the rate and pattern of drug-related problems in elderly cancer patients. **Materials and Methods:** A observational study was conducted prospectively for a period of 4 months, where cancer patients of any gender above 60 years were enrolled and followed daily. All necessary data like patient demographics, past and current medication history were obtained from various data sources including medical records, treatment charts, patient interviews etc. DRPs were identified and assessed. **Results:** A total of 50 patients were enrolled into the study. From which, 96 DRPs were identified among 74% of the patients. Commonly observed DRPs were Adverse Drug Reactions (40%) and Drug-Drug Interactions (20.8%). More than half of the patients who developed DRPs (67.6%) had comorbidities, 86% were currently on chemotherapy alone or in combination and majorly (81%) were in the advanced cancer stage (Stage III and stage IV). Hyper polypharmacy was observed in 67.6% of the patients with DRPs. **Conclusion:** Our study revealed that DRPs are highly prevalent amongst elderly cancer patients and geriatric cancer patients need careful follow-up to identify DRPs and reduce adverse outcomes.

Keywords: Drug-related problems, Geriatric oncology, Cancer, Polypharmacy.

Correspondence:
Dr. Madhan Ramesh
Professor, Department of Pharmacy Practice, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Mysuru-570015, Karnataka, INDIA.
Email: mramesh@jssuni.edu.in

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INTRODUCTION

Oncological disorders are a considerable cause of morbidity as well as mortality globally majorly in the geriatric subpopulation.[1,2] Based on the worldwide statistics by the international agency for research on cancer, the risk of cancer development in this population increases 12-36 fold.[3] Elderly patients are also routinely associated with DRPs due to multiple factors including pharmacokinetic and pharmacodynamic alterations, comorbidities and polypharmacy.[4-6] DRP is defined as "an event or circumstance involving drug therapy that actually or potentially interferes with desired health outcomes" according to the Pharmaceutical Care Network of Europe (PCNE).[7] Thus, DRPs are considered a major safety concern worldwide. Geriatric patients with cancer are at a higher risk for these problems because of the intricacy of the disease and its treatment which can consequently lead to substantial health and economic burden.[8,9] Since, the elderly are a rapidly growing population, proper and safe use of medications is of paramount importance for which identification and categorization of DRPs is the primary step.[4,10,11] In our study we explore this initial step of appropriate use of medications in aged cancer patients by determining the rate and pattern of DRPs.

MATERIALS AND METHODS

This was a prospective observational study conducted at Bharat Hospital and Institute of Oncology, Mysuru, Karnataka for a period of four months. Inpatients who were treated for any type of cancer, patients of any gender aged 60 years and receiving either standardized treatments or supportive care were included in the study. Outpatients except day-care patients were excluded. The approval from the institutional ethics committee of HCG-Bharat Hospital and Institute of Oncology was obtained prior to the commencement of the study (Clearance number ECM/31/22).

All admitted and day-care patients were followed on a daily basis. Eligible patients meeting the study criteria were enrolled into the study. All requisite data including demographics of the patient, clinical characteristics, type of cancer, comorbidities, past

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medications and current medications were sourced from various data sources including inpatient case notes, current treatment chart, laboratory tests and direct patient/caretaker (s) interviews. This information was then recorded in a well-designed data collection form and later transcribed into an Excel sheet. The clinical pharmacist obtained a clear past medical and medication history of the patient's adherence pattern and reassessed the treatment chart for DRP identification, if any and categorized them according to Hepler and Strand Classification.[12]

RESULTS

Overall, 50 geriatric oncology patients were enrolled into the study. The patient's clinical and demographic details are as mentioned in Table 1. 56% of the study patients were males and 44 % were female. Patients belonging to the age group of 60-70 years were in majority (66%). 70% of the subjects were presently married, 26% were widowed and 4% were single. A larger part of the patients were illiterate (46%) or completed primary education (40%). The majority (64%) of the patients followed a non-vegetarian diet. 20 patients (40%) who were enrolled into the study were smokers, 30% were alcoholics and 16% were tobacco chewers. The performance status (PS) of the individual subjects was assessed using the Eastern Cooperative Oncology Group (ECOG) performance scale according to which, 44% had a PS-0, 34% had a PS-1, 18% had PS-2 and 4% had PS-3. Half of the study, patient's treatment expenses were covered by the government-organized Suvarna Arogya Suraksha Trust (SAST) scheme, whereas 26% of the patients had to pay the treatment expenses personally. Other schemes through which patient expenses were covered in this study were private insurance companies (8%), Ex-servicemen Contributory Health Scheme (ECHS) (6%), Employees State Insurance Corporation (ESIC) (4%) and Arogya Bhagya Yojana Scheme (ABY) (4%).

About 61% of the elderly cancer patients had comorbid conditions as mentioned in Table 2. Of which hypertension (40%) was the most common followed by type 2 diabetes (30%) and ischaemic heart diseases (10%). Other comorbities included asthma (6%), stroke (2%), anaemia (2%), tuberculosis (2%), gastritis (2%), chronic liver disease (2%), arrhythmias (2%), benign prostate hypertrophy (2%), osteoarthritis (2%), hypotension (2%) and aortic valve disease (2%) Almost all the study patients (96%) had polypharmacy which is defined as the use of 5 medications at the same time. Amongst which, more than half of the geriatric subjects (56%) had hyper polypharmacy which is the administration of 10 medications per day.

The diagnosis, stage of cancer and current type of treatment given to the study participants are as illustrated in Table 3. The most common types of cancer seen among enrolled geriatric patients were gastrointestinal cancers (34%) followed by head and neck cancers (14%), liquid tumours (10%) and lung cancers (10%). Other types of carcinoma like cancer of the breast (6%), cervix (6%), ovary (4%), urothelial (4%), endometrium (4%), smooth muscle (4%), prostate (2%), penis (2%) and skin (2%) were observed.

A greater number of aged patients (38%) were diagnosed at the end stage (Stage IV) of cancer followed by locally advanced (30%) stage (Stage III). Only 30% of the patients were diagnosed at an early stage of cancer (Stage I and II).

About 78% (*n*=39) of the study patients were currently on chemotherapy, 8% (*n*=4) were on combination treatment with radiotherapy and chemotherapy, 4% (*n*=2) of the patients were on post-operative treatment or immunotherapy and 2% (*n*=1) were on radiotherapy alone, supportive care or on hormonal therapy.

Table 1: Demographic and Clinical status of the study subjects.

Total Number of Patients = 50		
Demographics		**N (%)**
Gender	Male	28 (56)
	Female	22 (44)
Age (years)	60-70	33 (66)
	71-80	13 (26)
	80	4 (8)
Marital Status	Married	35 (70)
	Widowed	13 (26)
	Single	2 (4)
Education	Illiterate	23 (46)
	Middle/High school/ Pre-university	20 (40)
	Degree	7 (14)
Diet	Vegetarian	18 (36)
	Non-vegetarian	32 (64)
Social Habits	Smoking	20 (40)
	Alcoholic	15 (30)
	Tobacco chewing	8 (16)
ECOG Performance Scale (PS)	PS 0	22 (44)
	PS 1	17 (34)
	PS 2	9 (18)
	PS 3	2 (4)
	PS 4	0 (0)
Payment Scheme	SAST	25 (50)
	Cash	13 (26)
	Private Insurance	4 (8)
	ECHS	3 (6)
	ESIC	2 (4)
	ABY	2 (4)

Note: ECOG: Eastern Cooperative Oncology Group, SAST: Suvarna Arogya Suraksha Trust, ECHS: Ex-servicemen Contributory Health Scheme, ESIC: Employees State Insurance Corporation, ABY: Arogya Bhagya Yojana

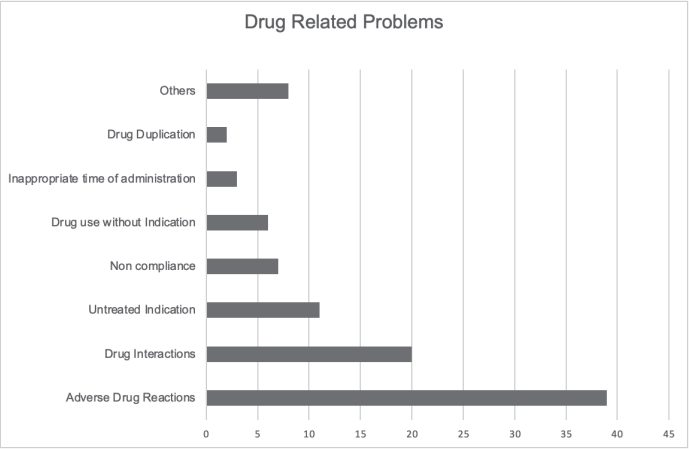

Figure 1: Types of Drug-related problems identified in the study.

Table 2: Comorbidities and Polypharmacy.

Comorbidities and Polypharmacy		N(%)
Number of comorbidities	Nil	19 (38)
	1	12 (24)
	2	14 (28)
	3	4 (8)
	4	1(2)
Type of comorbidity	Hypertension	22 (44)
	Type 2 Diabetes Mellitus	15 (30)
	Ischaemic Heart Disease	5 (10)
	Others	14 (28)
Total number of medications	Polypharmacy (5 medications)	48 (96)
	Hyper polypharmacy (10 medications)	28 (56)

Out of 50 patients, 37 (74%) were identified to have DRPs. A total of 96 DRPs were recognized. Common DRPs identified were ADRs (40%), drug interactions (20.8%), untreated indication (11%), medication non-adherence (7%), drug use without indication (6%), inappropriate time of administration (3.1%) and drug duplication (2%) as illustrated in Figure 1. Other medication-related problems included the wrong drug dispensed (2%), wrong dose dispensed (2%), wrong drug mentioned in the discharge summary (2%) and wrong dose mentioned in the discharge summary (2%).

All patients with DRPs had a history of social vices like smoking, alcoholism and/or tobacco chewing. 86% of the patients were currently undergoing chemotherapy and 81% were in the advanced stage of the disease. Amongst the 37 patients who developed DRPs, 26 patients were of the age group between 60 to 70 years. Hyper polypharmacy was observed in 67.6% of the patients and an equal number of patients had comorbidities as shown in Table 4.

Table 3: Types of Cancer diagnosed along with Stages.

Type of Cancer	N (%)	Stage of Cancer: N (%)
Gastrointestinal cancer	17 (34)	Early Cancer: 3 (6)
		Locally Advanced: 7 (14)
		Metastatic:7 (14)
Head and Neck cancer	7 (14)	Early Cancer: 3 (6)
		Locally Advanced: 4 (8)
Liquid tumours	5 (10)	Early Cancer: 2 (4)
		Locally Advanced: 1 (2)
		Metastatic: 1 (2)
Lung cancer	5 (10)	Locally Advanced: 2 (4)
		Metastatic: 3 (6)
Breast cancer	3 (6)	Early Cancer: 2 (4)
		Metastatic: 1 (2)
Cervical cancer	3 (6)	Locally Advanced: 2 (4)
		Metastatic: 1 (2)
Ovarian cancer	2 (4)	Early Cancer: 1 (2)
		Locally Advanced: 1 (2)
Urothelial cancer	2 (4)	Early Cancer: 1 (2)
		Metastatic: 1 (2)
Endometrial cancer	2 (4)	Early Cancer: 1 (2)
		Metastatic: 1 (2)
Sarcoma	2 (4)	Early Cancer: 1 (2)
		Metastatic: 1 (2)
Prostate cancer	1 (2)	Metastatic: 1 (2)
Penis carcinoma	1 (2)	Metastatic: 1 (2)
Melanoma	1 (2)	Metastatic: 1 (2)

DISCUSSION

Cancer is a common disease of geriatrics, where drug-related problems (DRPs) are a crucial issue in safety.[2,8] Our study on the prevalence of DRPs in geriatric oncology, even though limited is to the best of our knowledge, the earliest study done in India. According to our study, 74% of aged cancer patients developed DRPs. Various studies done in geriatric settings demonstrated the prevalence of developing DRPs was between the range of 15-81%, our study prevalence rate fits well into that range.[5,13,14-16] An average of 1.9 DRPs were observed per patient which is consistent with a study conducted by Halu *et al.* on drug-related problems in a geriatric setting.[5] However, in a study done on elderly cancer patients by Yeoh *et al.* the average DRPs per patient was 3.[13] This is higher when compared to our study because both inpatients and outpatients were included in the former whereas the latter only enrolled inpatients. Ageing develops a powerful cocktail

Table 4: Patterns of DRPs observed.

Total number of patients with Drug-related problems *n*=37 (%)	
History of social vices (Smoking, alcoholism, tobacco chewing)	37 (100)
Current treatment: Chemotherapy	32 (86.4)
Advanced stage of cancer (Stage III and IV)	30 (81)
Age (60-70 years)	26 (70.2)
Hyper polypharmacy (10 medications/day)	25 (67.6)
Patients with comorbidities	25 (67.6)

of comorbidity, polypharmacy or functional loss for developing DRPs.

The most common DRPs identified were ADRs which accounted for 40% of the total DRPs. Elderly patients have a negative association with ADRs due to age-related changes in their bodies. This risk further increases with cancer due to the perplexity of the disease and treatment. All the patients who developed ADRs were currently on chemotherapy. This can be explained by the fact that the toxicity of chemotherapeutic agents is amplified in older adults due to a decline in the functional and protective capacity of multiple organ systems. Our study results are similar with a study done by Yeol and colleagues, where the ADRs were observed in about 81.5% of geriatric patients who were currently on chemotherapeutic agents.[13]

Polypharmacy is universally defined as the use of 5 medications.[17,1] In addition, the use of more than 10 medications is usually termed as hyper polypharmacy. Various studies have analysed the prevalence of polypharmacy in elderly cancer patients to be between 20% to 96%.[18-20] In a study done by Nightingale *et al*, the prevalence of polypharmacy and hyper polypharmacy was found to be 84% and 43% respectively.[20] Similar results were obtained from our study where polypharmacy was observed in 96% and hyper polypharmacy was observed in 56% of our patients. One of the main reasons for increased rates of polypharmacy can be multiple comorbid conditions in the concerned population. According to our data, more than half of the study subjects had one or more comorbid conditions and the most common among them were hypertension and type 2 diabetes mellitus. 67% of the patients who developed DRPs had comorbidities. Another probable cause can be that most of the cancer patients in our study were in the advanced stage of disease (Stages III and IV) and mostly on chemotherapy. Treatment and supportive management for advanced cancers are highly complex, difficult and often accompanied by the use of an increased number of medications. It is important to note that 89% of the patients with hyper polypharmacy developed DRPs. Polypharmacy also increased the chances of drug interactions which was the common secondary DRP (20.8%) observed in our study. This is similar to an interventional study in Singapore where drug-drug interactions were observed in 30% of the patients.[12] Most of the drug interactions identified were significant in nature. While many of the drugs given to elderly cancer patients are inappropriate, numerous patient-related issues remain untreated as reflected in our study where about 11% of the indications were untreated.

This study had various limitations. Firstly, it was conducted in a solitary institution with limited sample size. Secondly, outpatients were excluded from the study, which resulted in the loss of critical outpatient-based data. Thirdly, the study did not collect information on Complementary Alternative Medicine (CAM) which can be potentially crucial in the assessment of DRPs in vulnerable cancer populations. Another important disadvantage of this study was the lack of interventions for the identified DRPs. When identified Medication-related problems are appropriately intervened and resolved, it can prevent potential harm from occurring and improve health and economic outcomes in the concerned subject.

A clinical pharmacist can play a key role in this setting by identifying a DRP, assessing the significance, appropriately intervening, resolving the problem if possible and providing alternative solutions.

The field of geriatric oncology and the impact of clinical pharmacists in improving care and preventing DRPs are yet to be rigorously explored and remain a grey area. More studies with a large sample size are warranted and needed for the fastest-growing demographic fighting a nasty disease.

CONCLUSION

In conclusion, DRPs are substantially prevalent among geriatric patients with cancer. Multiple comorbidities, age, polypharmacy, and cancer stage increase the susceptibility to developing DRPs. Adverse drug reactions and drug interactions are commonly identified. Elderly cancer patients need careful follow-up and clinical pharmacists have a window of opportunities in this area to improve and optimise patient care outcomes.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Original Article

Medication Reconciliation Practices in Two Multispeciality Hospitals

Allwin Kunjachen Syju[1], Chelsiya P Abraham[1], Princy Sara Korath[1], Reenu Sara Jacob[1], Savitha R Sanathan[1,*], Anjali Arun[2]

[1]Department of Pharmacy Practice, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Sri Shivarathreeshwara Nagar, Mysuru, Karnataka, INDIA.
[2]DNB and Academic Co-ordinator, HOD- LAB and Blood Bank, Apollo BGS Hospitals, Mysore, Karnataka, INDIA.

ABSTRACT

Background: Medication errors are common and can compromise patient safety. Commonly seen at discharge, they can be identified and resolved even during admission. Medication reconciliation is recommended to prevent errors arising from medication discrepancies. Aim: To conduct medication reconciliation in two multispeciality hospitals and classify the identified medication discrepancies according to their potential to cause harm. **Materials and Methods:** This prospective interventional study was carried out in medicine and surgery departments of two urban hospitals over a period of six months. Patients who satisfied the criteria were enrolled and medication reconciliation was performed. Interventions were provided whenever necessary. The identified discrepancies were then given to an expert panel for classifying them based on their potential to cause harm. **Results:** 580 medication discrepancies were identified from a total 372 patients, drug interaction (*n*=345, 61.6%) was the most commonly observed discrepancy, followed by omission error (*n*=127, 12.9%). The medication discrepancies observed from both the hospitals were found to be statistically not significant (*p*=0.246). From a total of 580 discrepancies, 454 (78.27%) discrepancies were Significant, 80 (13.79%) Serious and 46 (7.93%) Not Significant. **Conclusion:** The results of our study show that there are discrepancies in medication use when the patient transitions in a hospital. It is recommended that medication reconciliation practices be performed by clinical pharmacist during the hospital stay to ensure continuity of healthcare and for patient safety. An electronic medical record capable of capturing and continuously updating medication information may be a long-term solution. To achieve this, professional development of clinical pharmacists is of paramount importance.

Keywords: Medication reconciliation, Clinical pharmacist, Medication discrepancies, Medication errors.

Correspondence:
Dr. Savitha R S
Department of Pharmacy Practice, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Sri Shivarathreeshwara Nagar, Mysuru-570 015, Karnataka, INDIA.
Email id: rssavitha@jssuni.edu.in

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INTRODUCTION

The process of continuous medication management is an important patient safety concern across the world. It is also a complex process that requires information-sharing, good communication among the providers, patients, and families across different settings.[1] Since drug prescribing and reconciliation relies upon an accurate medication history, proper history taking can avoid medication errors; it is estimated that about 20% of medication errors result in patient harm.[2,3] Differences in treatment regimens in different healthcare settings can lead to extended hospitalization, readmissions and even death.[1] Medication reconciliation is reported to be the most important intervention to minimize the occurrence of unintended medication discrepancies at points of transition in healthcare.[4] It is purported to be a robust process to spot and put right any discrepancies before they do any harm and increase healthcare costs.[5]

Medication reconciliation has been available since 2005.[6] Several international patient safety organizations such as the World Health Organization (WHO), the Joint Commission International (JCI) and Institute for Health Care Improvement (IHI) have encouraged the establishing of medication reconciliation processes at each transition of patient in the hospital to improve patient safety.[7,8]

In line with The Joint Commission on Accreditation of Healthcare Organizations (JCAHO), the process of reconciliation of medications is defined as "the process of comparing the medications a patient is taking (and should be taking) with newly ordered medications" in order to straighten out disparities or potential problems.[9] It is recommended that the reconciliation process be carried out when patients are transferred between

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health care settings or whenever a medication is discontinued or changed.[5]

Discrepancies occur more commonly at the time of discharge and medications like cardiovascular drugs, drugs that act on the central nervous system, Other drugs like antibiotics, opioid analgesics, anti-hypertensives, anticoagulants, antidiabetic drugs, antipsychotics, and immunosuppressives are likely to cause potential damage to patients.[10] The main reasons for a high number of reconciliation errors are aging, lack of understanding of treatment with medications, variable health literacy, low recall ability, difficulties in communicating a particular language, gaps in the drug history, multiple medical records for each patient, increased length of hospital stay, comorbidities, therapy with multiple drugs, complexity of medication names, doses and frequencies.[11-13]

Pharmacists, by their knowledge and training, are ideal individuals who can identify wrong doses and routes of administration, and therapeutic duplications by obtaining medication histories and thereby assist in the reconciliation process. There are reports from literature stating that in comparison with physicians working alone, physicians working along with pharmacists during the admission process can significantly improve the accuracy of a medication history.[4,14]

The objective of this study was to perform medication reconciliation, using the developed medication reconciliation forms and to classify identified medication discrepancies based on their potential to cause patient harm.

MATERIALS AND METHODS

The study was conducted in in patient wards (Medicine, Surgery) in Hospital 1 and Hospital 2 located in Mysuru, Karnataka, India. This was a prospective interventional study. The study was carried out for a period of six months from September 2019 to February 2020. Patients with at least one comorbid condition staying for at least 24 hr were included. Patients with mental instability and/or cognitive impairment, and those unwilling to participate were excluded from the study. Using Cochran's formula and keeping a margin of error of 5% and 95% confidence interval, we estimated the sample size to be 385. However, we followed a non-probability sampling method (convenience sampling) and did not fix a number for Hospital 1 and Hospital 2.

All in-patients who met the inclusive criteria were reviewed at the time of admission and discharge. On admission, data relating to patient demographics, past medical conditions, past medication history, allergic status, family and social histories were extracted within 24-48 hr. Self-reported medication adherence was assessed using the Morisky's 4-item adherence scale and those scoring below 3 were considered to have adherence issues. We modified the questionnaire to also include the reasons for non-adherence. Any reported medication adherence issues were addressed and patient counselling was performed. Past medications noted were compared with the present medications prescribed and discrepancies were sorted. Similarly, on discharge, medication reconciliation was performed and documented. Interventions were identified and documented.

The clinical significance of the medication discrepancies was determined through a consensus of an expert panel of 4 clinicians (2 specialists in internal medicine, 1 surgeon, 1 GP) and 3 hospital pharmacists. The panelists were asked to rate the significance of the discrepancies using the modified Pippins J *et al.* scale (I = not significant, II = significant, III = serious, IV = life threatening), then an average score was calculated and classified.[15] Patients were grouped gender wise with different age groups and the respective percentage proportion was calculated. Study patients were categorized and expressed in numbers and percentages based on the hospital and department to which they were admitted, number of comorbid conditions, family history, social history, use of Over The Counter (OTC)/herbal medications, their length of stay and the number of medications received before admission, during hospital stay and at discharge. Reasons for medication non-adherence were categorized and expressed in numbers and percentages. The number of medication reconciliations performed at admission and discharge and discrepancies identified were categorized.

The statistical analysis (Chi-square test) was performed by using Statistical Presentation System Software (IBM-SPSS) version 22.0. A p value <0.05 was considered as statistically significant. Discrepancies with potential to cause harm were classified appropriately and reported.

RESULTS

Out of 385 subjects selected for inclusion, 3 of them declined to participate and 10 subjects were transferred to other healthcare facilities within 24 hr of admission. A total of 372 patients formed the study sample, of which 148 (39.7%) patients were from Hospital 1 and 224 (60.3%) from Hospital 2.

The department wise distribution of patients from Hospital 1 was found to be 95 (64%) from medicine and 53 (36%) from surgery. Similarly, 168 (75%) patients were enrolled from the medicine department of Hospital 2 whereas 56 (25%) patients were from the surgery department. The majority of the participants from both Hospital 1 as well as 2 belonged to the age group of 61-80. The mean hospital stay for patients in Hospital 1 was 4 days and for those in Hospital 2 was 8 days (Table 1).

Among the 372 patients enrolled, the number of patients with a positive family history for comorbid conditions was 137 (37%). It was found that 143 (38.4%) patients had one comorbid condition, followed by 138 (37%) patients with two, 73 (19.6%) patients with three, 16 (4.3%) patients with four comorbid conditions and highest number of comorbid conditions- five were seen in

2 (0.5%) patients. The most common comorbid condition in the family histories of the patients was found to be Type 2 Diabetes Mellitus. 28 patients (38.8%) were found to have a history of alcohol and tobacco use followed by 27 patients (37.5%) with a history of tobacco use and 17 patients (23.6%) with a history of alcohol intake. 17 patients (5%) out of the total of 372 were found to have a history of OTC/Herbal drug use within the past one month. The most commonly used OTC medications were Ayurvedic drugs (29%), followed by Analgesics (17%), Antipyretics (12%), Cough syrups (12%) and Antidiarrhoeals (12%), whereas the least commonly used were Homeopathic drugs (6%), Antihistamines (6%) and Home remedies (6%). It was found that the average length of stay of patients in Hospital 1 was shorter than when compared with patients in Hospital 2. This may be because of differences in the severity of the disease at admission of the study population and hospital policies. A total of 580 discrepancies were identified from Hospital 1 and Hospital 2 from a total of 372 subjects. Discrepancies that were identified are as shown in Table 2.

Table 1: Patient demographics and clinical characteristics.

	No. (%) of patients	
	Hospital 1	Hospital 2
Age (in years)		
21-40	5 (3.3%)	23 (10.26%)
41-60	62 (41.8%)	90 (40.17%)
61-80	75 (50.6%)	97 (43.3%)
81-100	6 (4.05%)	14 (6.25%)
Gender		
Male	108 (73%)	137 (61%)
Female	40 (27%)	87 (39%)
Mean hospital length of stay, days [SD]	4 [±2.35]	8 [±4.87]
Mean medication use before admission, number [SD]	2.96 [±1.68]	2.49 [±1.5]
Mean medication use at admission, number [SD]	5.41 [±2.18]	6.12 [±1.83]
Mean medication use at discharge, number [SD]	4.91 [±2.08]	5.46 [±2.01]
Discrepancies identified	231 (39.82%)	349 (60.17%)

Drug interactions (345 cases, 59.5%) accounted for maximum discrepancies identified, followed by omission errors (127 cases, 21.9%). Among the drug interactions, 122 cases (35.36%) were reported from Hospital 1, whereas 223 cases (64.63%) were from Hospital 2. The drug interactions were found to be higher in Hospital 2 as a majority of the subjects were from rural background and were found to consult multiple physicians and hence were prescribed with multiple medications, resulting in drug-drug interactions. This was not the case in Hospital 1, as a majority of the subjects were from urban settings and would consult only a single physician. Among the drug interactions identified, 193 (55.94%) were major, 142 (41.15%) were moderate and 10 (2.89%) were minor interactions.

Omission errors were the second most common discrepancies identified. 66 cases (51.96%) were reported from Hospital 1, whereas 61 cases (48.03%) were from Hospital 2. The drugs that were observed to be commonly omitted were anti hypertensives and anti diabetic drugs followed by IHD, Thyroid, Dyslipidemic, Parkinson's and CVA drugs. Subtherapeutic dosage and improper drug selection were the least commonly found discrepancies, each with 1 case from Hospital 2. An additional category was added to

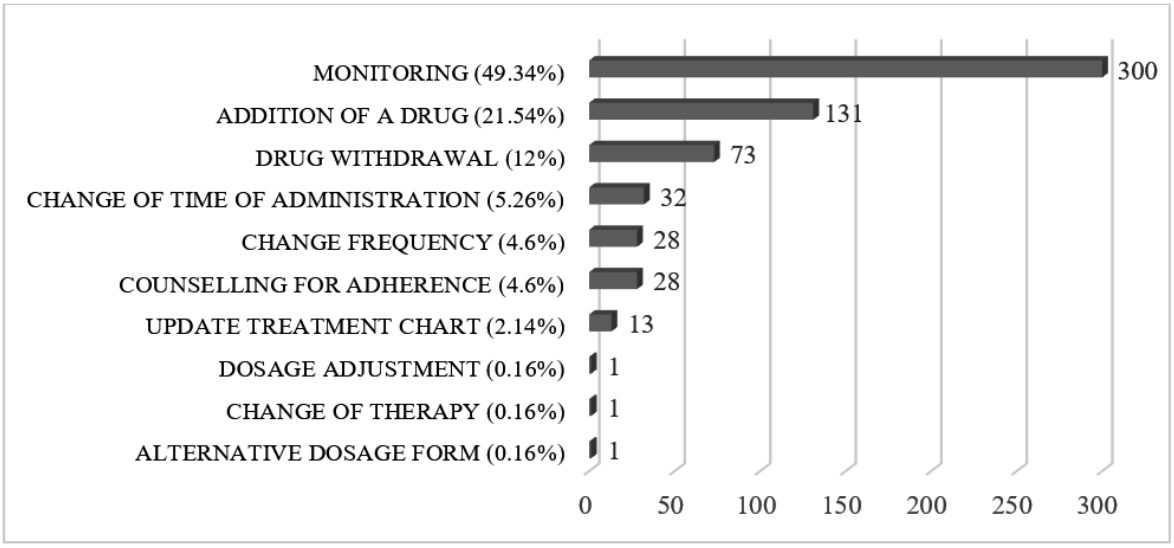

Figure 1: Interventions provided by the clinical pharmacists.

Table 2: Discrepancies Identified in Hospital 1 and Hospital 2.

Discrepancies Identified	Hospital 1	Hospital 2	Total
Omission Error (21.89%)	66 (51.96%)	61 (48.03%)	127
Drug Duplication (1.72%)	4 (40%)	6 (60%)	10
Drug Interaction (59.4%)	122 (35.36%)	223 (64.63%)	345
Adverse Drug Reaction (2.06%)	1 (8.33%)	11 (91.66%)	12
Untreated Indication (0.68%)	1 (25%)	3 (75%)	4
Drug use Without Indication (1.03%)	0	6 (100%)	6
Improper Drug Selection (0.17%)	0	1(100%)	1
Improper Frequency (4.82%)	16 (57.14%)	12 (42.85%)	28
Documentation Error (2.24%)	8 (61.53%)	5 (38.46%)	13
Subtherapeutic Dosage (0.17%)	0	1 (100%)	1
OTHERS (5.68%) (Improper time of administration, alternative Dosage Form)	13 (39.39%)	20 (60.60%)	33
Total	231 (39.82%)	349 (60.17%)	580

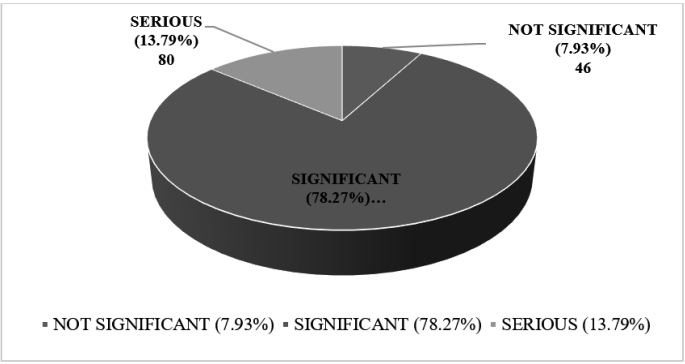

Figure 2: Classification of Identified 580 discrepancies.

the list of identified discrepancies, to include errors that did not come under the ones mentioned above. This category included improper time of administration with cases of Tab. Aspirin which was administered in the afternoon but shows better action when administered at night and alternative dosage form with a case of an 83 year old female subject who was discharged with an inhaler and a spacer, whereas nebulization therapy is more preferred in the elderly. The medication discrepancies observed from both Hospital 1 and Hospital 2 at admission as well as at discharge were found to be statistically not significant (p=0.246). At admission, the discrepancies identified from Medicine and Surgery departments of Hospital 1 and Hospital 2 were not significant statistically (p=0.443). Similarly, at discharge, the discrepancies identified from Medicine and Surgery departments of both hospitals were not significant statistically (p=0.225).

Assessment of Medication Adherence

Some common reasons for non-adherence in both the hospitals were found to be forgetfulness, purposeful omission, fear of needle, pain at injection site, and relatively high cost of drugs for patients from low economic backgrounds. Non-adherence in Hospital 2 (12%) patients was observed to be higher when compared to that in Hospital 1 (1%), as patients were unaware of their disease conditions and the importance of medication adherence. Due to the identification of medication adherence issues in the study population, patient counselling was performed for 28 patients.

Table 3: Description of the 30 identified discrepancies.

Not-Significant	1) Improper frequency of Proton Pump Inhibitors (2) 2) Drug Duplication at admission (1) 3) Drug Use Without Indication (1) 4) Others (Improper Time of Administration) (1)
Significant	1) Omission Error at admission (3) 2) Drug Duplication both at admission and at discharge (2) 3) Improper Frequency of Antibiotic (1) 4) ADRs (2) 5) Untreated Indication (4) 6) Drug Use Without Indication (2) 7) Documentation Error (2) 8) Drug Interaction-Major & Moderate (5) 9) Others (Improper Time of Administration) (1)
Serious	1) Omission Error at discharge (2) 2) Drug Interactions- Major (1)

Interventions made by Clinical Pharmacist

Among the identified 580 discrepancies that were assigned interventions, monitoring the interaction (300, 49.34%) was the most common intervention, followed by addition of a drug (131, 21.54%) for omission error and untreated indication. Interventions provided are depicted in Figure 1.

Acceptance of interventions at Hospital 2 was found to be 94% whereas at Hospital 1 it was 66%. This difference in acceptance of interventions was because at Hospital 2, there is an established clinical pharmacy service since 20 years and pharmacists and pharmacy interns are in close contact with HCPs, discussing treatment charts and any other patient medication problems openly. So, prescribers are more likely to accept interventions because of the rapport built with them.

Out of the identified 580 discrepancies, clinical significances of commonly occurring 30 medication discrepancies were determined through a consensus of an expert panel. According to the ratings provided by the panelists, 23 (77%) discrepancies were categorized as Significant, 4 (13%) were Serious and 3 (10%) were Not Significant. A description of the 30 identified discrepancies are given in Table 3.

Based on the rating by the panellists, the remaining 550 discrepancies were classified in a similar manner. 454 (78.27%) discrepancies were classified as Significant, 80 (13.79%) were classified as Serious and 46 (7.93%) were classified as Not Significant, as shown in Figure 2.

DISCUSSION

Our study results are similar to other published studies involving the process of medication reconciliation. A previous study showed that patients were directly admitted to medical or surgical units[7] hence medical and surgical units were chosen for our study as 80% of the patients are admitted in these departments in the study setting. In the current study the main purposes were to develop a medication reconciliation form which was used to record various data, to perform medication reconciliation process, prevent medication discrepancies by intervening whenever necessary and classifying the errors according to their potential to cause harm. During the study pharmacists interviewed the patients and collected the medication histories, clarified treatment orders and suggested alternatives to prevent medication discrepancies. As the study progressed, various discrepancies were found such as drug interactions (345 cases, 59.5%) that accounted for maximum discrepancies identified. Omission errors were the second most common discrepancies identified. 66 cases (51.96%) were reported from Hospital 1, whereas 61 cases (48.03%) were from Hospital 2. Subtherapeutic dosage and improper drug selection were the least commonly found discrepancies, with 1 case in Hospital 2. In other medication reconciliation studies conducted, omission errors dominates as the most common discrepancy rather than drug interactions. A study conducted by Rey and his colleagues, showed the chief discrepancy was omission error (54.5%), followed by subtherapeutic dosage (5.9%), drug used without indication (1.2%), drug duplication (0.4%), documentation error (0.2%) and drug interactions (0.2%).[8]

Among the drug interactions that were identified, 193 (55.94%) were major, 142 (41.15%) were moderate and 10 (2.89%) were minor interactions. In a study conducted by Babu *et al.*, 77 interactions were identified; among them there were a high percent of significant interactions (62.33%).[16]

A total of 127 omission errors were identified from both the hospitals with 66 (51.9%) from Hospital 1 and 61 (48%) from Hospital 2. The drugs that were omitted were anti hypertensives and anti diabetic drugs, IHD, Thyroid, Dyslipidemic, Parkinson's, CVA and CKD drugs. A study conducted by Mazar *et al.* showed the most frequent reconciliation error was omission and the commonly omitted drugs were Lipid-lowering (12.4%) and antihypertensive agents.[7] Drug duplication (10 cases) accounted for 2.07% of the total discrepancies identified, out of which (4) 40% were from Hospital 1 and (6) 60% were from Hospital 2. As per the study by Wong *et al.*, out of 105 unintentional discrepancies 2.9% of the errors were drug duplication.[17]

ADRs constituted 5.6% (12) of the total discrepancies, out of which 1 (8.33%) was from Hospital 1, and from Hospital 2, 11(91.66%). The most commonly occurred ADRs was the Insulin induced hypoglycemia followed by Piperacillin induced rashes and Furosemide induced hyponatremia. According to a study by Pfister *et al.*, 41 cases of ADR were reported. Some of them were furosemide induced hypokalemia, amlodipine induced edema.[18]

Out of the identified discrepancies, 4 (0.69%) were untreated indications, with 1 (25%) from Hospital 1 and 3 (75%) from Hospital 2. The study conducted by Ashjian *et al.*, showed 58 or 11.4% of patients with medication-related problems among whom untreated indications were seen in 9 patients.[19]

Drug use without indication was found only in Hospital 2, with 6 cases (2.5%). In a prospective, single-centre pilot study by Mazhar *et al.*, drug used without indication were 2.3% from medicine and 4.1% from surgery.[7] Improper drug selection was found only in Hospital 2, with 1 case (0.5%). Khalil *et al.* conducted a prospective parallel study, in which improper drug selection was one of the most common type of error (intervention group=14, control group=34).[20]

Improper frequency (28 cases) accounted for 4.8% of the total discrepancies identified, out of which 16 (57.14%) were from Hospital 1 and 12 (42.85%) were from Hospital 2. According to study by Wong *et al.*, at least one actual or potential unintentional discrepancy existed in 70.7% patients, among them 60 (8.6%) were improper frequency.[17]

Documentation errors found in both hospitals were 13 (2.24%) with 8 (61.53%) in Hospital 1 and 5 (38.46%) in Hospital 2. The documentation error was that the treatment chart had not been updated. In a study conducted by Babu *et al.* that consisted of two test groups and one control group, 34.72% documentation errors were found in the control group while, test groups 1 and 2 included 6.81% and 19.17% respectively.[16]

Subtherapeutic dosage was observed only in Hospital 2 with only 1 case (0.5%). According to a study by Pfister *et al.*, among 66% of the participants, 14 subtherapeutic dosage errors were identified.[18] The Others category included 33 (5.6%) cases, out of which 13 (39.39%) were from Hospital 1 and 20 (60.60%) were from Hospital 2. This category included improper time of administration.

As medication adherence was assessed, most of patients in Hospital 1 were aware about their disease conditions and medications, only 1% of the cases were non adherent due to two reasons, one case was due to forgetfulness and the other patient was purposely omitting the drugs. In Hospital 2, non-adherence to past medications was found to be 12% and more compared to Hospital 1 since most of the patients were unaware of their disease conditions and medications. Aside from forgetfulness and purposely omitting drugs, fear of needle (2 cases), pain at injection site (1 case) and relatively high costs of drugs (4 cases) for patients from low economic backgrounds were some of the reasons. Westberg and his colleagues conducted a study that showed medication adherence as a drug therapy problem, and found 147 cases of medication non adherence among 408 patients.[21]

Interventions were made for the discrepancies. Monitoring the interactions was the most common intervention, followed by addition of a drug. Acceptance of interventions were more in Hospital 1 (94%) than Hospital 2 (66%) due to the established clinical pharmacy services as well as rapport with HCPs and pharmacists in Hospital 2. Lau conducted a retrospective chart review, in which patient education was the most performed intervention (58%), followed by ordering new prescriptions or refill requests (49.9%), and provision of supportive care (32.6%).[22]

According to the ratings provided by the panellists 454 (78.27%) discrepancies were classified as Significant, 80 (13.79%) were classified as Serious and 46 (7.93%) were classified as Not Significant. Similar to ours, a study was conducted by Knez *et al.*, where an expert panel provided the clinical significance of medication errors. The rating of the errors was classified using a four-point scale (0 - not important to 3 - potentially fatal). More than half of the evaluable errors were rated as clinically important by the panel.[23]

This study had a few limitations, firstly the study was limited to conduction of Medication Reconciliation in medicine and surgery departments and consequently the type of problems identified may not be duplicated in other departments. Secondly unavailability of pharmacists at all points of transitions of the patient and thirdly the effectiveness of medication reconciliation and any interventions performed by pharmacists entirely depends on the accuracy of the information provided by the patient.

CONCLUSION

This study provides evidence that there exists discontinuity in drug therapy during patients' transitions within the hospital that can affect the safety of the patient. Discrepancies during patient transitions can be prevented by implementing medication reconciliation practice by clinical pharmacists in hospital settings. The presented results highlight the need for implementation of pharmacist provided medication reconciliation during the patient's stay in the hospital to ensure continuous provision of healthcare and improved patient safety. An electronic medical record capable of capturing, continuously updating medication information as well as providing pop-up reminders may help the pharmacist and the health care professionals as a long-term solution.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Original Article

Perceptions of Indian Physicians towards Deprescribing of Medications for Chronic Diseases in Elderly: A Questionnaire-based Study

Shribhavana Jawahar, Lakshmipriya Selvaraj, Kailash Muruganantham, Iniya Kumar, Vanitha Rani Nagasubramanian*

Department of Pharmacy Practice, Sri Ramachandra Faculty of Pharmacy, Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, INDIA.

ABSTRACT

Deprescribing is the process of reconstructing multiple medications use by review and analysis, which concludes with the modification, replacement or elimination of drugs. Medications that were once appropriate may become inappropriate due to age-related physiological changes that increase the risk of harm from medications eliminated by the liver and kidneys and other co-morbidities. In India, with a steadfast rise in the elderly population paralleled with an augmented necessity for chronic disease management, the essence of deprescribing is rarely known and less practiced by physicians. Hence, a questionnaire-based interventional study was conducted to assess their perception and attitude towards implementing the deprescribing process. The study included 75 physicians from the departments of General Medicine and Nephrology, who were initially educated on deprescribing through a self-developed and validated educational video of three minutes duration, following which they were asked to fill the Perceptions, Attitudes and Challenges of Physicians towards Deprescribing Questionnaire. About 76% of the physicians felt that deprescribing was needed to reduce medication-related problems in older patients. Involvement of multiple prescribers (54.6%) was the main challenge stated by physicians; 92% stated deprescribing to be beneficial in current clinical scenario, and 85.3% showed a positive attitude towards integrating deprescribing into their day-to-day clinical practice. Physicians below 40 years with an experience of 0 to 10 years had a significantly positive perception and attitude towards deprescribing. In view of reducing potential medication-related adverse events in the elderly, daily practice of the deprescribing process should become a norm, thereby improving the quality of life.

Keywords: Comorbidities, Polypharmacy, Deprescribing, Elderly patients.

Correspondence:
Dr. Vanitha Rani Nagasubramanian
Former Faculty, Department of Pharmacy Practice, Sri Ramachandra Faculty of Pharmacy, Sri Ramachandra Institute of Higher Education and Research (DU), Chennai- 600116, Tamil Nadu, INDIA.
Email is: reachvanitha99@gmail.com

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INTRODUCTION

Elderly patient characteristics

The World Health Organisation predicts that by 2050, the number of people aged more than 60 would increase by 22% and an unrestrained lifestyle will implicitly indicate more chances of the development of multimorbidity.[1] It is also projected that the proportion of Indians aged 60 and older will rise from 7.5% in 2010 to 11.1% in 2025, of which southern states (Andhra Pradesh, Karnataka, Kerala, and Tamil Nadu) may hold a larger proportion followed by other north Indian states.[2] Chronic illnesses (cardiovascular diseases, metabolic disorders, renal dysfunction, hepatic dysfunction, etcetera) are found to be of higher incidence in India, in around 60% of the elderly population, according to the Centre for Disease Control and Prevention.[3] Being an aged person, and taking lots of medications itself can become a burden. Under chronic co-morbid conditions, the functioning of an elderly patient's body is much more complex than an adult's and is more prone to experience drug-related problems (like drug interactions, adverse events, additional medicines to treat adverse events, etc). Medications that were once appropriate may become inappropriate due to old-age-related physiological changes that increase the risk of harm from medications metabolized by the liver and kidneys and other co-morbidities.[4] Moreover, the safety and efficacy profile of a particular medication in an elderly person is not extensively available as more geriatrics are not included in drug clinical trials.

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Potentially inappropriate medication

Prescribing multiple medications, more than or equal to 5 drugs per day is termed polypharmacy. The overall prevalence of polypharmacy in India from 2002 to 2020, was found to be 49% which was most prevalent in the north Indian states (65%).[5] Multiple medications for chronic illnesses might be a real necessity but at the same time can lead to an increased risk of falls, reduced physical or cognitive function, poor medication adherence, adverse drug reactions and hospitalization.[6] Not prescribing medications that are potentially beneficial for the patient, just for the sake of reducing the number of medications prescribed is also considered inappropriate. Finding a balance between under-prescribing and overprescribing is essential to provide optimal therapy for patients.

Around 45% of elderly patients in an Indian population are prescribed at least one potentially inappropriate medication (PIM).[7] There are tools and algorithms like Beers criteria 2019, Screening tool of older people's prescriptions/screening tool to alert to right treatment (STOPP/START) Criteria, and Anti-cholinergic burden scale, to identify PIMs. Rather than overruling the clinical assessment and intervention made by the physicians, they tend to guide and help physicians to precisely choose the best treatment option available, providing physician's a confidence to reduce medication, thereby improving their quality of life.[4]

Deprescribing

Deprescribing is the structured approach towards tapering or stopping drugs when potential harm exceeds the benefit, which is carried out by the healthcare professional in view of reducing PIMs, keeping in mind the feasibility of the process and taking into account the individual's medical and social condition, treatment care plan, quality of life and their preferences.[8] Optimizing appropriate medication use while minimizing the risk is the essence of deprescribing.[9] There is a generalised process to guide physicians in deprescribing a medication.[10]

Concerns in deprescribing

Potential adverse drug withdrawal reactions, pharmacokinetic and pharmacodynamic alterations to the withdrawal of medication, and return of the medical condition are some of the risks that may be faced while withdrawing a medication. Hence, tapering of doses, close monitoring of the patient's condition, and finding an appropriate alternative is essential to avoid these risks.[4] The limited availability of an evidence-based approach for deprescribing a medication is a major concern for acceptance of the process. Patients are generally wired to think more medications are required for their chronic condition and that it is formidable to remove any medication that they have been taking for a long time. A patient's willingness to stop a medication plays a pivotal role in deprescribing.[10]

Barriers to deprescribing

Some physicians are not in favour of deprescribing due to some of the constraints that they face during consultation. Lack of time, lack of insight about PIMs, fear of unknown consequences, lack of data on discontinuing and monitoring medication, and patients' unwillingness towards deprescribing.[1,4,9]

Advantages of deprescribing

Deprescribing ultimately improves the medication outcome by overcoming the challenges of non-adherence, polypharmacy, risk of potentially inappropriate medication use, increased cost and pill burden, and disability in patients and promotes rational use of prescription.[4] A better understanding of how elderly patients and prescribing physicians perceive the problems related to deprescribing, can make us find effective ways to overcome those potential issues, thereby implementing them in daily practice.[8]

Rationality of the study

Widespread adoption of deprescribing is becoming a trend worldwide to rationalize the prescription and reduce PIM. At the same time, avoiding medications that are known to be problematic or those considered inappropriate is a challenge for physicians, especially for those physicians involved in chronic disease management in the elderly. A major hurdle faced by Indian physicians is the less evidence available about the various guidelines and algorithms for deprescribing medication in elderly patients.[4,11]

Deprescribing is a very new and less familiar concept among Indian physicians, hence, this study focused on assessing their perception, identifying various enablers and barriers towards deprescribing and making physicians aware which is the first big step towards implementing the deprescribing process.

MATERIALS AND METHODS

Research design

A questionnaire-based interventional study was conducted in the departments of General medicine and Nephrology of Sri Ramachandra Medical Center and hospital, Chennai, Tamil Nadu, India, for a period of 6 months (from November 2021 – April 2022).

Sample size

The sample size for enrolling physicians was based on the literature,[11] with a 95% confidence interval and a relative perception of 20%, the sample size needed for the study was calculated as 75.

Study subjects

75 physicians who have at least completed a Bachelor of Medicine and Bachelor of Surgery (MBBS) degree from the

above-mentioned clinical departments and gave their voluntary consent were included in the study. The physicians who provide consultations in the mentioned departments on an appointment basis were excluded from the study due to their time constraints.

Materials used

The study was conducted after obtaining approval from the Institutional Ethics Committee (IEC) (CSP/21/NOV/102/592). A self-developed and validated educational video explaining the deprescribing process for three minutes was shown to physicians individually with the intention of creating awareness or furthering their knowledge on deprescribing. The video was developed and validated in four steps. The first step was the construction of the script for the development of the video on deprescribing, the second step was the validation of the script for its content by five subject experts and the third step was the recording of the video. The final step was the validation of the video by eight subject experts for its creativity, relevance to the educational goal, audio-visual quality; language clarity, content adequacy and learning potential for the target audience, with each criterion being evaluated using a four-point scale of 1 to 4 ranging from not relevant to somewhat relevant, quite relevant and highly relevant to the measured criterion. The overall relevance of the educational video was calculated using a content validation index which met the satisfactory level.

Data on the demographic details were obtained from the Physicians, which included their age, gender, educational qualification, and years of clinical experience. Physicians were assessed for their perception of deprescribing in the elderly using a validated instrument "Perceptions, Attitudes and Challenges of Physicians towards Deprescribing (PACPD-12) Questionnaire" which consists of 12 questions.[12] This questionnaire covers the physicians' benefits, preferred age, preferred drugs, reasons, specific criteria, views, enabling factors, barriers, and implementing factors for deprescribing a medication. It also focuses on the positivity, attitude and perception towards deprescribing a medication. Necessary approval was obtained from the author for the use of this questionnaire.

Statistical analysis

The obtained data were entered into a Microsoft Excel spread sheet. Results were analyzed and expressed using descriptive statistics. Categorical variables were expressed as frequency and percentage. Continuous variables were expressed as mean and standard deviation. The association between the baseline characteristics (age, gender, educational qualification and years of experience) and the perception and attitude of the physicians towards deprescribing was assessed using chi square test. The question 1 (Deprescribing is beneficial in the current clinical scenario) was taken to assess the perception of the physicians. Responses strongly agree and agree were taken as positive perception and responses disagree and strongly disagree were taken as negative perception. Likewise, question 11 (Positivity towards deprescribing on a scale of 0-5) was taken to assess the extent of positive attitude of the physicians towards deprescribing. Responses at the scale of 0 to 3 were taken as poor attitude and 4 and 5 were taken as positive attitude towards deprescribing. A p value of <0.05 was considered statistically significant.

RESULTS

Table 1 explains the baseline demographic characteristics of the 75 physicians with a mean age of 32.1 ± 9.44 years. The majority of the physicians (92%) agreed that deprescribing is beneficial in the current clinical scenario. Around 60% of physicians preferred to deprescribe in the geriatric population. Antibiotics (61.3%) followed by Benzodiazepines (56%), Antidepressant drugs (56%) and Antipsychotic drugs (53.3%) were majorly considered by the physicians for deprescribing. The cardinal reason for deprescribing is to reduce harm to patients in view of adverse drug reactions (76%) and to reduce the pill burden in patients (73.3%). Around 90% of the physicians disagreed with not making deprescribing a point in daily practice and 78.7% of them do have an approach towards deprescribing a medication. 45.3% of physicians chose AGS Beers Criteria over STOPP-START Criteria (26.6%) for identifying PIM. Most of the physicians (84%) perceived that deprescribing will do more good than harm to the patients. Among the factors that might allow physicians to deprescribe, 65.3% believed that learning how to deprescribe a specific medication and 49.3% felt that complete focus on deprescribing in their respective departments would help them. With concerns to various challenges faced by the physicians while deprescribing, 54.6% perceived that the physician who is about to

Table 1: Baseline demographics of the physicians.

Baseline characteristics	No. of participants =75 n (%)
Gender	
Male	39 (52)
Female	36 (48)
Age (years)	
<40	57 (76)
41-50	14 (18.6)
51-65	4 (5.4)
Mean (SD)	32.1± 9.44
Highest educational degree	
MBBS	18 (24)
MD	57 (76)
Years of practice	
0-10	55 (73.3)
11-20	8 (10.6)
21-30	9 (12)
>31	3 (4)

deprescribe is unsure of the rationale with which the particular medication was prescribed by another physician; 46.6% felt they would have less time to properly deprescribe and 42.6% have concerns about the less evidence available on the risk/benefit assessment on deprescribing a medication. A huge number of medications (76%), lower economic status of the patient (74.6%) and advanced age (64%) were some of the apparent situations that might make physicians more likely to deprescribe. On the whole, 85.3% of physicians showed a strong positive attitude towards deprescribing (Table 2).

Table 3 depicts that age (p=0.037) and years of experience (p=0.005) had a significant association with positive perception towards deprescribing. Likewise, age (p=0.039) and years of experience (p=0.024) also had a significant association with the positive attitude of the physicians towards deprescribing (Table 4).

DISCUSSION

This study was conducted to assess the attitude and perceptions of physicians towards the deprescribing of medications in the elderly population. This study included 75 physicians from the General Medicine and Nephrology departments of which 39 (52%) physicians were males and 36 (48%) physicians were females with a mean age of 32.1 ± 9.44 years.

The majority of the physicians (92%) in our study agreed that deprescribing is beneficial which is also found high in the study conducted by Sweta, *et al.*[12] The study conducted by *Farrell, et al.,* found that 92%, 88% and 77% of physicians agreed that Benzodiazepines, Antipsychotics and Antidepressants, respectively, were the most preferred drug for deprescribing.[13] In another study conducted by Sweta, *et al.*, only 34.6%, 32.5% and 33.2% of physicians agreed that these drugs were most preferred for deprescribing, respectively.[12] Both these studies showed different responses than our study.

The present study found that only half of the physicians agreed towards deprescribing Benzodiazepines, Antipsychotics and Antidepressants. The study found that 45.3% of physicians used AGS-Beers criteria while the study conducted by Sweta, *et, al.,* reported only 6.6% of physicians used this criterion.[12] This study identified that majority of the physicians considered deprescribing to reduce harm to the patients which is in accordance with the study conducted by Sweta, *et al.*[12]

Among the barriers faced by physicians, this study showed that 54.6% and another study conducted by Sweta, *et al.*, showed that 55.2% of physicians felt that prescriptions provided by another physician were a major barrier.[12] The study conducted by the same author found that 53.8% and 51.7% of physicians would consider the existence of PIMs and acute symptoms related to medications, respectively, as the main indication for deprescribing a medication, whereas, our study showed that 76% and 74.6% of physicians would consider a large number of prescription medications and lower socioeconomic status of the patient, respectively, as the main indicator for deprescribing a medication.[12]

The present study had attempted to assess the association of age, gender, educational qualification and years of experience of the physicians with their perception and positive attitude towards deprescribing by taking question 1 and question 11 of the study questionnaire respectively as they were found to be direct and more suitable to obtain the desired outcome. The study identified that the physicians in the age group of less than 40 years and those with the experience of 0-10 years had agreed and strongly agreed as deprescribing to be beneficial in clinical practice and also expressed more positive attitude towards deprescribing

In India, to our knowledge, there are very few studies to assess the practice of deprescribing in the elderly from Physicians' perspectives. There are studies[14] reported on the benefits of

Table 2: Responses to the Perceptions, Attitudes and Challenges of Physicians towards Deprescribing (PACPD-12) Questionnaire.

Sl. No.	Questions	Responses n (%)
1.	Deprescribing is beneficial in the current clinical scenario	
	Strongly agree	45(60)
	Agree	24(32)
	Neutral	6(8)
	Disagree	0(0)
	Strongly disagree	0(0)
2.	Not making deprescribing a point in daily practice	
	Strongly agree	1(1.3)
	Agree	7(9.3)
	Neutral	20(26.6)
	Disagree	36(48)
	Strongly disagree	11(14.6)
3.	Have an approach to deprescribe a medication	
	Strongly agree	31(41.3)
	Agree	28(37.3)
	Neutral	15(20)
	Disagree	1(1.3)
	Strongly disagree	0(0)
4.	Preferred Age*	
	All	27(36)
	Paediatrics	1(1.3)
	Adults	12((16)
	Geriatrics	46(61.3)
	None	1(1.3)

Sl. No.	Questions	Responses n (%)
5.	Preferred drugs for deprescribing*	
	Benzodiazepines	42(56)
	Antidepressant drugs	42(56)
	Antipsychotic drugs	40(53.3)
	Anticonvulsant drug	27(36)
	Antiplatelet drugs	18(24)
	Antihypertensive drugs	15(20)
	Antibiotics	46(61.3)
	Opioids	36(48)
	Proton pump inhibitor	25(33.3)
	Cholinesterase inhibitors	2(2.6)
	Vitamin supplement	23(30.6)
	Bisphosphonates	9(12)
	Statins	14(18.6)
	Antiarrhythmic drug	3(4)
	Steroids	35(46.6)
	Analgesics	31(41.3)
	Glucocorticoids	26(34.6)
	Diuretics	13(17.3)
	Bronchodilators	5(6.6)
	Based on patient profile count	27(36)
6.	Reason for Deprescribing*	
	To reduce harm to patient in view of adverse drug reaction	57(76)
	Based on latest guidelines, the medication is not indicated	26(34.6)
	To reduce cost of treatment	51(68)
	To reduce pill burden	55(73.3)
	Because medication has minimal benefit for patient in view of age and comorbidities	19(25.3)
7.	Specific criteria for deprescribing*	
	STOPP-START Criteria	20(26.6)
	AGS-Beers Criteria	34(45.3)
	No criteria used	10(13.3)
	Others	13(17.3)
8.	Statement that best expresses view on deprescribing	
	Does more harm than good	63(84)
	Does neither good nor harm	2(2.6)
	Does more harm than good	7(9.3)
	Not sure	3(4)
9.	Enabling factors*	
	Flags by pharmacist to deprescribe medications in a patient-centered approach	21(28)
	Training on de prescribing specific medications	49(65.3)
	Strong department focus on deprescribing medication	37(49.3)
	Having a pharmacist in your team	18(24)
	Others	7(9.3)
10.	Barriers to deprescribing*	
	Medication usually prescribed by another doctor and the current doctor is unsure of the rationale	41(54.6)
	Concerned about adverse events after deprescribing medication	27(36)
	Damaging relationship with original doctor who prescribed medication	19(25.3)
	Resistance from patient/family	25(33.3)
	Lack of benefit/risk information about deprescribing	32(42.6)
	Lack of time to consider deprescribing	35(46.6)
	Pressurized to prescribe according to guidelines	30(40)
	Patients' belief that you are giving up on them	16(21.3)
	Lack of experience	28(37.3)
11.	Positivity towards deprescribing on a scale of 0-5	
	0	1(1.3)
	1	0(0)
	2	1(1.3)
	3	9(12)
	4	19(25.3)
	5	45(60)
12.	Factors that make physicians more likely to deprescribe*	
	Existence of potentially inappropriate medication listed in Beers criteria	27 (36)
	Large number of prescription medication	57 (76)
	Advanced age	48 (64)
	Lower economic status of patient	56 (74.6)
	Concomitant co-morbidities likely hepatic/renal dysfunction that affects drug metabolism	36 (48)
	Existence of chronic condition	27 (36)
	Concomitant ethanol abuse	11 (14.6)

*Multiple responses were given for the question; hence, the addition of n will not yield a total of 75.STOPP-START Criteria - Screening Tool of Older Person's Prescriptions and Screening Tool to Alert to Right Treatment, AGS Beers Criteria - American Geriatrics Society Beers Criteria.

Table 3: Association between baseline demographics and perception towards deprescribing.

Question	Baseline characteristics	No. of Responses		Significance *P*
		Strongly agree/agree	Disagree/ Strongly disagree	
Q. 1. Deprescribing is beneficial in the current clinical scenario	Age (years)			
	<40	55	2	0.037*
	40-50	11	3	
	51-65	8	1	
	Gender			
	Male	34	5	0.109
	Female	35	1	
	Highest education degree			
	MBBS	18	0	0.151
	MD	51	6	
	Years of practice			
	0-10	53	2	0.005*
	11-20	8	0	
	21-30	6	3	
	>30	2	1	

*p<0.05 - Significant value

Table 4: Association between baseline characteristics and attitude towards deprescribing.

Question	Baseline characteristics	No. of Responses		Significance *P*
		0-3	4 and 5	
Q. 11. Positivity towards deprescribing on a scale of 0-5	Age (years)			
	<40	9	47	0.039*
	40-50	1	14	
	51-65	0	4	
	Gender			
	Male	3	36	0.075
	Female	8	28	
	Highest education degree			
	MBBS	2	16	0.642
	MD	9	48	
	Years of practice			
	0-10	10	45	0.024*
	11-20	0	8	
	21-30	0	8	
	>30	0	4	

*p<0.05 - Significant value

deprescribing in older patients and the role of general physicians and primary care doctors in educating the elderly towards the discontinuation of PIMs. However, it is also reported that deprescribing should be patient-centric and the prescribers should involve the patients in the medication management process for successful implementation of deprescribing.

Limitations

The major limitations of this study were the smaller sample size and the presence of recall bias observed when the questionnaire was answered by the physicians.

CONCLUSION

The present study was conducted to assess the attitude, perception, willingness and challenges towards deprescribing medications. Even though there were various challenges perceived by the physicians, the majority of them expressed a positive attitude towards deprescribing. In a developing country like India, where there is an increase in the elderly patient population, implementing deprescribing in daily clinical practice will reduce potential adverse events related to medication use, increase patients' medication adherence and thus increase the clinical outcome. Future studies can implement deprescribing in any particular class of drug and find their exact effect on reducing the cost, pill burden and adverse effects.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PIM: Potentially Inappropriate Medications; **PACPD 12:** Perceptions, Attitudes and Challenges of Physicians towards Deprescribing questionnaire; **STOPP/START:** Screening tool of older people's prescriptions/screening tool to alert to right treatment; **MBBS:** Bachelor of Medicine and Bachelor of Surgery; **IEC:** Institutional Ethics Committee.

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Original Article

A Meta-Analysis on Misuse of Prescription/OTC Drugs: How Pharmacist Can Prevent and Manage Drug Abuse

Mohammed Mustafa G, Chandana C*

Department of Pharmacy Practice, Sree Siddaganga College of Pharmacy, Rajiv Gandhi University of Health Sciences, Tumkur, Karnataka, INDIA.

ABSTRACT

Background: Drug misuse is a critical issue related to both physical and psychological health associated with growing threats across the world. The role of pharmacist in preventing drug abuse is crucial to address prescription/OTC medication misuse. **Aim and Objectives:** To synthesize the research on misuse of prescription/OTC drugs, role of pharmacist in preventing and managing prescription/OTC medications induced health conditions and to provide methodological guidance for further research. **Materials and Methods:** Systematically searched epidemiological research on the topics relevant to the study through PubMed and EMBASE from 2017 to 2022 (n = 3022), studies are screened by title/abstract (n = 981) and full text articles are assessed (n = 153). The studies included for meta-analysis (n = 12) are selected by applying inclusion and exclusion criteria. STATA Version 17.0 is used to analyse the data. **Results:** Out of 12 research articles, 6 were conducted on misuse of prescription/OTC medications and another 6 were about prevention and management of the drug abuse by pharmacist. The researchers analysed data through odds ratio, 95% CI at p-value of 0.001* by plotting forest plot. The overall effect size is found to be 1.96 (1.21, 2.71) with 95% CI that shows association between drugs misuse and pharmacist role in drug abuse. Publication bias and Heterogeneity are graphically represented through Funnel plot and Galbraith plot respectively. **Conclusion:** The researchers concluded that the drug misuse is the global burden and pharmacist can endeavour to offer the management and prevention of drug abuse.

Keywords: Prescription drug misuse, OTC medication abuse, Opioid abuse, Meta-analysis.

Correspondence:
Ms. Chandana C, Pharm D
Department of Pharmacy Practice,
Sree Siddaganga College of Pharmacy,
Rajiv Gandhi University of Health Sciences, Shivakumara Swamiji Circle,
Tumkur-572102, Karnataka, INDIA.
Email id: chandanac2100@gmail.com

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INTRODUCTION

Drug misuse is the term which defines as intentional use of medicinal product by inappropriately and not in accordance with the terms the marketing authorisation such as incorrect dose, taken at incorrect time, indication or schedule.[1] Drug abuse is an insistent or erratic, intentional excessive use of medicinal product, taken to get high, inflict self-harm complemented by harmful physical and psychological effects.[2] The key difference between drug misuse and drug abuse is consumer's intent as treatment versus getting high/self-harm respectively.[3] Drug misuse is a critical issue related to both physical and psychological health associated with growing threats across the world.[4] Drug misuse is accompanying with significant global morbidity, mortality, monetary expenses and community expenses.[5] The role of pharmacist in preventing drug abuse is crucial to address the prescription/OTC (Over-The-Counter) medication misuse.[6,7]

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Globally along with Asian countries drug abuse, drug misuse, drug dependence and drug addiction, including consumption of non-medical, legal, and illegal drugs is increased and became the most critical clinical issue which impact seriously on public health.[8] Around 70 million people were diagnosed with a drug use disorder throughout the world.[9] Irrational use of antibiotic due to prescription drug misuse leads to medication discrepancies like antibiotic resistance in the community.[10] Self-medication of OTC drugs leads to OTC drugs misuse and physician prescription, pharmacist lack of knowledge leads to prescription misuse.[11] Many problems confronted by a patient can be easily resolved by pharmacists, including product selection, OTC brand name confusion, appropriate product use, and when to take medications.[12] However, relatively diminutive is acknowledged about those affected individuals, particularly in relation to their management at drug dependence treatment centres.[13] Hence, Pharmacists have focused information about both prescription and OTC drugs and are trained best to communicate their potential and expected harms and benefits to patients.[14] The phenomenon involving the non-medical use and misuse of prescription and OTC medications is called as Pharming.[15]

Table 1: Characteristics of included studies.

Sl. No	Authors, years	Study Design	Sample size	Country	Gender (F=Female; M=Male)	Age (years)	Study conducted year	Follow up in years	Outcome
1.	Vi T. Le, *et al*. 2017	Cross sectional survey	939	U.S.A.	F: 561 M: 361 Missed: 17	18-19: 223 20-21: 275 >22: 433 Missed: 8	2011	N/A	OTC misuse has greater odds than prescription drug misuse.
2.	T.Tran *et al*. 2017	Interventional study	661	Australia	F: 561 M: 361 Missed: 17	Not mentioned	-	0.3	Decrease of oxycodone prescription and median of dose from 100mg per patient to 50mg per patient after post-intervention.
3.	Saul Shiffman *et al*. 2018	Cross sectional survey	14481	U.S.A.	Not mentioned	Not mentioned	2011	5	Odds of misuse of Paracetamol has greater than OTC/Prescription drugs misuse in prescribed days.
4.	Penelope wood *et al*. 2018	Cross sectional survey	904	Australia	Not mentioned	<30: 242 30-39: 288 40-49: 151 50<: 223	March 2016- June 2017	1	Pharmacist had assessed the clinical tool via The Pharmacy guild, myCPD website, online PDF version to evaluating inappropriate use of OTC combination analgesics containing codeine.
5.	Scholz Irene et.al. 2019	Cohort study	904	Switzerland	F: 126 M: 218	16-36: 166 36-56: 147 >56: 30	2012-2017	5	Misuse of more 1 Prescription/OTC drugs or additional illicit drugs co-use had greater odds than misuse of Ethanol co-use.
6.	Richard H et.al. 2019	Interventional study	4630	U.S.A.	F: 126 M: 218	Mean: 60.65	2011-13 to 2014-16	3	Analgesics use for pain management service has greater odds than opioid misuse.
7.	Desmond cariveau et.al. 2019	Cohort study	234	U.S.A.	F: 153 M: 81	Mean: 60.65	2016	1	The percentage of naloxone prescribing increased from 3.4% for high-risk patients treated with chronic opioid therapy for pain.
8.	Wui Ling Chan et.al. 2020	Cross sectional survey	999	Singapore	F: 498 M: 500 Transgender: 1	Median= 35	2015	N/A	Prescription drug misuse associated with lifetime and past year misuse in this study.
9.	Abubaker *et al*. 2020	Cross sectional survey	98	Nigeria	F: 31 M: 64	<30: 31 31-40: 34 41-50: 16 51<: 15	2019	-	Community pharmacist's confidence in dispensing antibiotics without prescription is the main reason for prescription antibiotic misuse.

Sl. No	Authors, years	Study Design	Sample size	Country	Gender (F=Female; M=Male)	Age (years)	Study conducted year	Follow up in years	Outcome
10.	Anette De Santigo *et al.* 2021	Cross sectional survey	11	U.S.A.	Not mentioned	Not mentioned	Dec 2019-Jan 2020	N/A	Pharmacist role in preventing OTC misuse had greater odds than preventing opioids abuse.
11.	Shu-wei liu *et al.* 2021	Cross sectional survey	2126	India	F: 1078 M: 1048	15-24: 301 25-44: 745 45-64: 728 >65: 352	2020	N/A	Higher suicidal ideation has greater odds with prescription drug use.
12.	Kotwani. A, Joshi. J., *et al.* 2021	Cross sectional survey	72	India	F: 31 M: 41	15-25: 15 26-34: 16 35-45: 14 46-55: 14 >55: 13	2020	N/A	Antibiotic misuse has greater odds than OTC misuse.

Training programmes increase the knowledge of pharmacist about pharming.[16,17]

Meta-analysis provides more robust results that can help researchers better understand the magnitude of an effect.[18] A meta-analysis can make available of a single summary statistic of the strength of an association across a huge number of studies and can assess connects of effect size.[19,20] Drug misuse is associated with both prescription and OTC drugs that results in many severe medical conditions, increased casualty visits, overdose deaths and admissions for addiction treatment. The impact of pharmacy directed management services and prevention programme are essential to prevent the drug abuse.[21] In this study, the researchers conduct a statistical analysis of misuse of drugs and how pharmacist prevent and manage the drug abuse in which the results of several studies are combined and then analysed.

The aims of present study are to statistically summarize the misuse of prescription/OTC drugs with the role of pharmacist in preventing and managing the prescription/OTC medications induced health conditions; and to provide methodological guidance for further research.

MATERIALS AND METHODS

Primary study inclusion

The researchers applied inclusion criteria for the process of literature search includes studies must be published in English language, it should be scientific peer reviewed article which published in the period of 2017-2022, the reason for restricting the search period illuminates how knowledge has changed within the field and time as every year a new drug therapy is introduced and prescribed. Also, as it is a Meta-analysis it represents a novel approach for further drug therapy design therefore newer cases needed to be analysed in this study. The study population aged 15 years and above, the study must be Epidemiological study.

Literature search and search strategy

The researchers used to search scientific literature through database sources like EMBASE, PubMed and Google Scholar. Search strategy includes the searching terms "Misuse of drugs", "Misuse of prescriptions drugs", "Misuse of OTC and prescription drugs", "Prevention of misuse of drugs", "Preventing misuse of prescription drugs", "Preventing prescription drug abuse", "Role of Pharmacist in misuse of prescription and OTC drugs", "Role clinical pharmacist in preventing drug abuse", "Preventing drug abuse", The researchers collected the studies which shown under these searching terms and screened by applying inclusion and exclusion criteria.

Criteria for inclusion and exclusion

The researchers design the inclusion criteria which classifies literatures into cross-sectional study, cohort study or case-control studies. The study conducted on the misuse of OTC or prescription

drug and about the role of pharmacist in drug abuse. The effects of drug misuse occurred in only human. The Study outcome must represent the report of Prescription/OTC misuse and role of pharmacist in drug abuse. The exclusion criteria determine that the study which are published only as an Abstract, Review literature, which contains not enough data, Case studies, sample size less than 10, used inappropriate statistics.

Data Extraction

Data abstraction is conducted through Microsoft Excel Version: 1808. The researchers extract the data by using headings such as author names, years of published, study design, sample size, country names, gender, age in years, study conducted year, follow up in years, outcome, source of outcome, and calculated odds ratio (95% CI) with respect to the outcome (Table 1).

Statistical Analysis

The researchers calculated the Odds ratio (95% CI), heterogeneity by applying Random-effect Der Simonian- laird model. Because of the heterogeneity in the included studies, the researchers applied random effect model. If the study results Homogeneity, then the researchers can apply fixed effect model. Heterogeneity was identified by using I^2. I^2 is reported that from 0% to 40% represents might not be significant heterogeneity; from 30% to 60% might represent reasonable heterogeneity; from 50% to 90% might represent substantial heterogeneity; from 75% to 100% shows significant heterogeneity at the *p*-value <0.001*, the researchers used to test publication bias by plotting Funnel plot. The Statistical analysis of included studies is conducted through the Statistical software STATA Version 17.0.

RESULTS

The researchers identified 12 studies out of 3024 studies which can be included for the meta-analysis, that consists of 25,499 individuals with drug misuse and the included population were assessed about the role of pharmacist in preventing managing drug abuse. The selection criteria were reported through PRISMA flow diagram (Figure 1).

Characteristics of included studies

The (Table 1) descriptions of involved studies were extracted and presented in which there are 6 studies showing outcome as prescription/OTC drugs misuse,[22-27] and other 6 studies reports the role of pharmacist in preventing and managing drug abuse.[28-33] The 5 studies were conducted at U.S.A. (n=20, 295, 79.59%),[26-28,30,32] 2 studies from Australia (*n* = 1565, 6.14%),[31,33] 1 each studies were conducted at Taiwan (*n*=2126, 8.34%)[22], Singapore (*n*=999, 3.92%),[24] Switzerland (*n* = 344, 1.35%),[25] Nigeria (*n* = 98, 0.38%),[29] and India (*n*=72, 0.28%).[23] The 10 studies were Cross-sectional studies and 2 were Cohort Studies. The Commonly misused class of drugs are graphically presented in the (Figure 2) in that The researchers presented through Bar graph of number of studies mentioned about the class of drugs misuse in which Pain medications misuse are most mentioned in the included studies.[34]

Data and analysis

The researchers represent the data analysis of this meta-analysis through forest plot (Figure 3), Galbraith plot (Figure 4), Funnel plot (Figure 5).

Forest plot (Figure 3) is the graph, individual study resembles to square indicates effect size and weightage of study centred at the point with horizontal line (whiskers) represents effect size at Confidence interval. The green diamond estimates the complete effect size with horizontal lines on both sides of overall CI.

Heterogeneity

It is a natural for effect sizes of studies collected in meta-analysis, shows variations between studies of sampling variability. In this study, the heterogeneity measures I^2=74.12%, H^2=3.86, τ^2=0.98 and homogeneity shows Q statistic of 42.5. There are several strategies to address the heterogeneity such as subgroup analysis, Meta-regression, performing random effect analysis, exclude studies. The researchers consider random effect meta-analysis that shows the result (Figure 3). There are no significant changes with the subgroup analysis. Henceforth, the researchers excluded 3 studies due to large sample size and other 3 studies due to variance in the outcome. Then, the researchers found the Heterogeneity

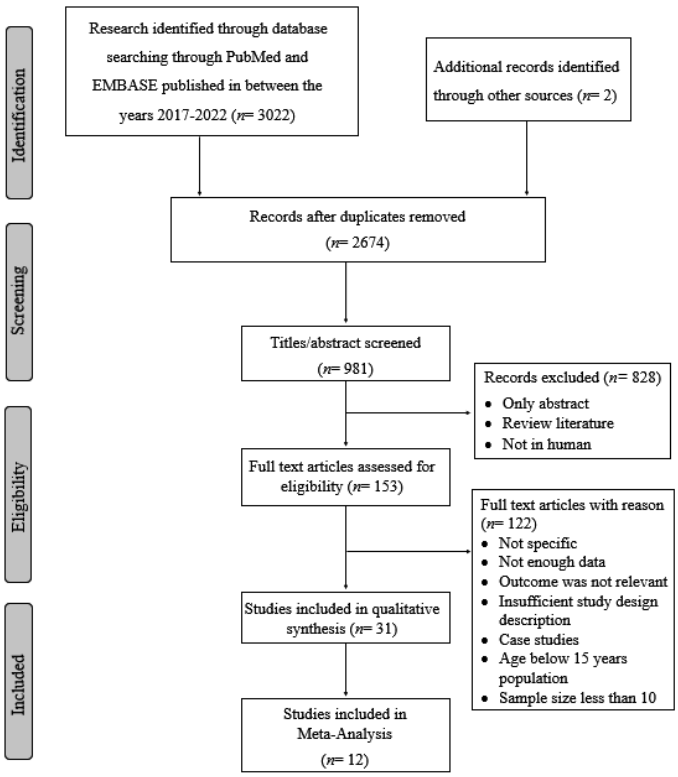

Figure 1: The PRISMA flow diagram of inclusion in this meta-analysis; *n*=number of articles.

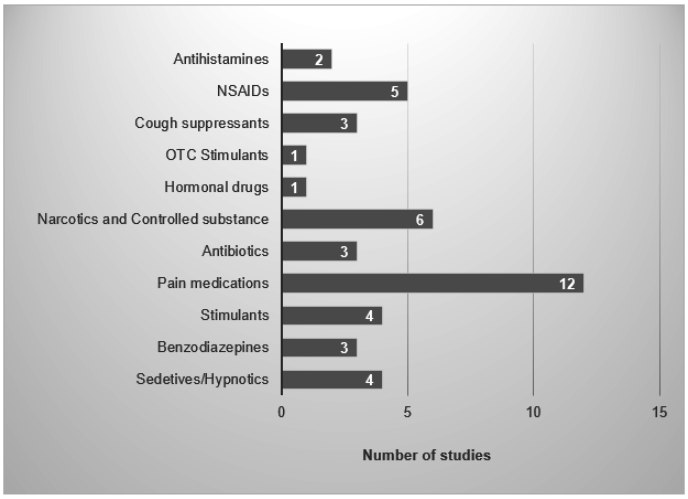

Figure 2: Number of studies mentioned about the misuse of class of drugs.

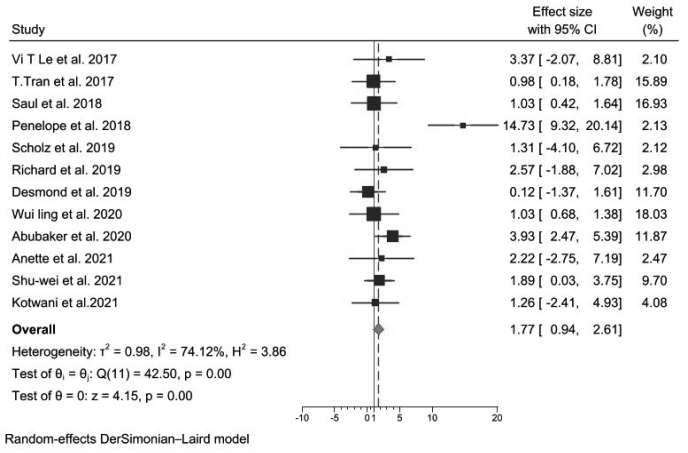

Figure 3: Forest plot of included studies.[34]

of 41.07% that can be acceptable and shows association between misuse of prescription/OTC drugs and the role of pharmacist in prevention and management of drug abuse with the overall effect size of 1.96 (at 95% CI 1.21, 2.71) (Figure 6).

Galbraith plot is the magnificent of forest plot which consists of studies close to y-axis have low precision. The regression line represents the effect size. In the absence of significant heterogeneity. The researchers can assume around 95% of the studies to remain within the 95% CI section (shaded area). The funnel plot is asymmetric with smaller, less precise studies. This might suggest the existence of publication bias.

The objective of this study is to statistically summarise the drugs misuse and role of pharmacist in prevent and managing drug abuse. A meta-analysis is conducted according to the PRISMA 2009 guidelines and statistical analysis is conducted through the STATA Version 17.0. The result is reported through the PRISMA flow diagram, forest plot, galbraith plot, funnel plot and presented the characteristics of the included studies. This study concluded

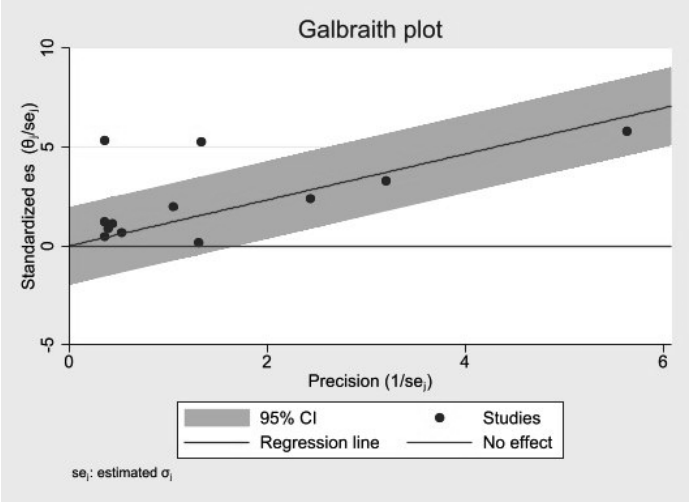

Figure 4: Galbraith plot which represents the heterogeneity of the studies.[34]

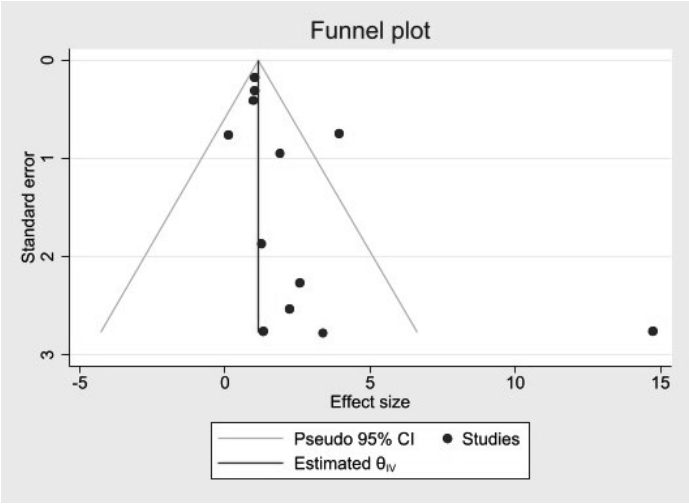

Figure 5: Funnel plot which represents the Publication bias.[34]

that the drug misuse is the global burden and pharmacist can endeavour to offer the management and prevention of drug abuse.

DISCUSSION

Main findings

Out of 12 included studies, total sample size of 25,499 for assessment of prescription/OTC drug misuse and role of pharmacist in prevention and management of drug abuse. There is an relation between these two outcome variables.[2] There is a substantial association between Non-medical use of OTC drugs and Non-medical use of prescription drugs.[27] Risk of hospitalization or death is higher in those individuals who simultaneously misuse prescription benzodiazepines with opioids and alcohol co-use.[27] The pharmacist vigilance in the doctor's prescription and providing proper counselling to the patients limits the drug misuse.[31,33] There is an decrease in the number of Emergency visits, rapid response team activity and code blue events related to prescription and OTC drugs abuse.[25,30]

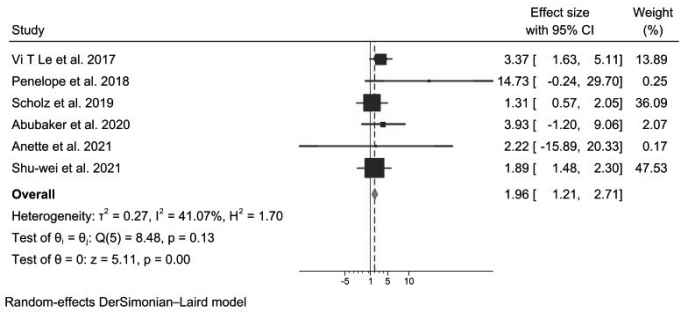

Figure 6: Forest plot showing association between drugs misuse and role of pharmacist in prevention and management of drug abuse after exploring the heterogeneity.[34]

Implications

Dextromethorphan, cough suppressant found in about 140 OTC cold medications, when it is misused it shows the effect of euphoria, dissociative and stimulant effects that results psychosis, dependence, and tolerance.[27] The Benzodiazepines which commonly prescribed for insomnia and anxiety disorders. Patients may continue these types of drugs after resolving the condition due to improper awareness about the misuse of prescription drugs. Benzodiazepines, the most common misuse of prescription drugs.[22] There are 6.7% population who misuse the prescription drugs in their lifetime and 4.8% population who have past year misuse of prescription drug misuse.[24] The 8.5% population who misused prescription drugs had a prevalence of lower levels of self-rated health, psychological distress, and suicidality.[22] The involvement of pharmacist in the opioid stewardship decreases the opioid prescription for unintended uses from 57.24% to 8.79%[30] and increase in the higher NSAID naloxone co-prescribing by 3.4%. to 37.2%.[32] The main reasons for misuse of drugs includes lack of awareness, insufficient knowledge in pharmacists about the dispensing of medicines,[23] patient's perceptions and believes, prescriber's practice and conditions.[28,35] The 39.7% of pharmacists dispense the antibiotics and other prescription drugs without proper prescription to the consumers.[23]

Strengths, limitations, and future directions

This study has a number of strengths. First, this study inclusion criteria include only scientific studies with statistical analysis and the researchers excluded the studies that shows inappropriate outcome and irrelevant study outcome. Second, the researchers explored the heterogeneity through the exclusion of number of studies and proved that there is an association between the misuse of prescription/OTC drugs and role of pharmacist in prevention and management of abuse. Third, the researchers exclude the studies which shows generalised results. However, this study has some limitations. First, most of included studies were conducted in U.S.A. The researchers did not select those studies intentionally but the researchers primarily focused on the studies which are significant to this study inclusion and which are having appropriate outcome. Second, the studies included were not conducted on same class of drug misuse. Future studies can interpret the meta-analysis by selecting the same class of drugs misuse and their effects. Furthermore, this study can be considered as base for the assessment of relation between the importance of pharmacy profession in the preventing and Managing drug abuse.

CONCLUSION

With this study, the researchers confirmed that there is huge number of prescription/OTC drugs misuse occurs with the substance abuse. The misuse of drugs is associated with additional illicit drugs co-use and ethanol co-use. Individuals who misused drugs had a greater prevalence of psychological distress and suicidality as well as lower levels of self-rated health. Confirms the Pharmacists' involvement in the prevention of drug abuse is crucial. The malpractice was associate with the lack of awareness about the medicinal discrepancies. Determines the potential benefits of clinical pharmacy services includes medication use, prescribing pattern and their potential effects on patient outcomes in the management of drug abuse. The researchers concluded that the drug misuse is the global burden and pharmacist can endeavour to offer the management and prevention of drug abuse.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

OTC: Over-the-Counter; **U.S.A.:** United States of America; **CI:** Confidence Interval.

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Original Article

Pharmacophore based High Throughput Virtual Screening towards the Discovery of Novel BLK (B-lymphocyte kinase)-tyrosine Kinase Inhibitors

Sana Sumera*, Sanjai Srinivasan, Harshitha BV, Sharanagoud Biradar, Shankarrao Patil

Department of Pharmacy, Aditya Bangalore Institute of Pharmacy Education and Research, Bengaluru, Karnataka, INDIA.

ABSTRACT

Aim: LK (B-lymphocyte kinase) is a protein from the family SRC (Proto-oncogene tyrosine-protein kinase), an important cell signaling molecule that influences cellular response. The BLK tyrosine-protein kinase has been a potential target for cancer therapy. As a result, this could be an initial step toward the development of novel inhibitors to fight cancer. **Materials and Methods:** A homology model of human BLK tyrosine kinase was constructed using Phyre2. Active site prediction was done for the model using the CASTp server. High Throughput virtual screening was performed with the help of a ligand-based pharmacophore model of FDA (Food and Drug Administration) approved SRC tyrosine family kinase inhibitors using the PharmaGist and ZINCPharmer servers. The 250 novel compounds obtained were docked by a Python script-based method with Autodock Vina. To ensure drug safety, ADME/Tox (Absorption, Distribution, Metabolism, Elimination, Toxicity) analysis was performed for the molecules with the lowest binding energy. Six compounds that passed ADME/Tox analysis were again utilized to perform molecular docking with Autodock4. The active residues were then identified using PLIP [protein ligand interaction profiler]. **Results and Conclusion:** Six compounds passed ADME/Tox analysis. Based on the molecular docking analysis, the compound ZINC57306994 showed an increased binding affinity with the target BLK tyrosine kinase. The compound ZINC57306994 may serve as a lead molecule that could be developed into a potent BLK tyrosine kinase inhibitor.

Keywords: Pharmacophore modeling, Molecular docking, BLK tyrosine kinase, Cancer.

Correspondence:
Ms. Sana Sumera
Department of Pharmacy, Aditya Bangalore Institute of Pharmacy Education and Research, Bengaluru-560064, Karnataka, INDIA.
Email id: drsumera4@gmail.com

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INTRODUCTION

Cancer is a serious disease caused by cells dividing uncontrollably and spreading into surrounding tissues and distant organs. If the tumor has progressed to the stage where it cannot be treated, it eventually leads to the death of the affected patient. Cancer is caused due to changes in our genetic material, DNA. The most common risk factors for cancer include aging, tobacco chewing, exposure to radiation, chemicals, some bacteria and viruses, family history of cancer, certain hormones, alcohol, an unhealthy diet, lack of physical activity, and obesity. An estimated 19.3 million new cancer cases were found worldwide, and almost 10.0 million cancer deaths occurred in 2020.[1] Female breast cancer is the most diagnosed cancer in 2020. About 2.3 million cases were estimated. One way to overcome cancer is through the identification of drugs against potential protein targets. Tyrosine kinase are a family of enzymes that catalyze phosphorylation reactions by using ATP and are involved in cell proliferation, differentiation, migration, metabolism, and programmed cell death. Recent studies have revealed that tyrosine kinase is involved in the pathophysiology of cancer.[2,3] BLK is a non-receptor tyrosine kinase belonging to the SRC kinase family.[4] The SRC family kinase consists of proteins SRC, YES1, HCK, FYN, FGR, LCK, LYN, and BLK that are present in humans, all of which are characterized by the presence of an SH3 and SH2 domain N-terminal to the catalytic kinase domain. BLK is functionally involved in B-cell signaling and B-cell development and is ectopically involved in hematological and multiple non-hematological malignancies, including breast, kidney, and lung cancer, indicating that BLK could be a new potential target for the therapy of cancer.

MATERIALS AND METHODS

Collection of data

PubChem contains data on a wide variety of chemical entities, including small molecules, lipids, carbohydrates, amino acids, and nucleic acids.[5] The chemical structures of the FDA-approved drugs, Dasatinib, Ponatinib and Saracatinib were downloaded from PubChem.[6-9] Uniprot is a knowledge base where over 120

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million proteins from all branches of life were collected, with their sequences and annotations. The amino acid sequence of various protein tyrosine kinase from the SRC kinase family was taken and its amino acid sequence was retrieved from Uniprot.[10] The multiple sequence alignment was performed using the fasta sequence that we obtained from Uniprot.[11] The Protein Data Bank (PDB) contains more than 134,000 structures of biomolecules that have been identified by crystallography, NMR spectroscopy, and 3D electron microscopy.[12] Here we used the PDB web server to download protein structures in PDB format. Open Babel software which was used to convert different file formats used in this study.[13] The ZINC database is a collection of chemical compounds that are commercially available and specially prepared for virtual screening.[14] The ligands downloaded from here were used in the virtual screening process. An extendable application called chimera allows for the interactive viewing and analyzing of molecular structures and associated data.[15]

Homology modeling and structural validation of protein

Analysis of conserved sequences throughout the protein SRC tyrosine kinase family of proteins, SRC, YES1, LCK, HCK, and BLK was done using Clustal Omega.[16] It gives the alignment between two or more sequences by using seeded guide trees and the HMM profile technique. The BLK tyrosine kinase protein was homology modeled using the PHYRE2 server.[17] The amino acid sequence of BLK was subjected to PHYRE2 to recognize the most acceptable crystal structure in the database as a template for modeling BLK. Sequence identity, resolution, domain coverage, and cognate ligand binding give the best template selection. And the structure validation was done by using the servers QMEAN4, ProSA, PROCHECK and verify3D.[18-20]

Active site prediction of protein model

Active site residues of the modeled BLK tyrosine kinase were found using the CASTp server.[21] The CASTp server recognizes the surface of all the pockets and their interior cavities throughout the channels in the structure of a protein and gives detailed information about all the atoms taking part in the formation. The CASTp server also measures the exact area and volume of the pocket. The structure of PDB ID 2C0I was retrieved from the protein data bank and the active residues were identified by using the PLIP [protein ligand interaction profiler] server. The Plip server detects and visualizes the target and ligand interactions. PLIP identifies the halogen bonds, water bridges, salt bridges, hydrogen bonds, hydrophobic contacts, pi-stacking, and pi-cation interactions between the target and ligand.[22]

Molecular docking of FDA-approved BLK inhibitors

Molecular docking was done using Autodock 4.2.6 software.[23] It is used to fit a ligand into a 3D structure binding site and undergoes two steps. The first is the search for conformational space for binding, and the second is binding by releasing free binding energy. At first, the macromolecule modeled BLK tyrosine kinase was checked for missing amino acid residues, and the missing residues were added with the help of the misc option. Polar hydrogen and partial Kollman charges were assigned. The torque bonds of the inhibitors were selected and defined. A Lamarckian genetic algorithm was used to explore flexible inhibitors. Grid maps of the interaction energy between atoms and proteins present in inhibitors have been pre-calculated using the Autogrid program. The three-dimensional grid box with a 60-grid size (x, y, z) with a spacing of 0.500 and a grid center (x, y, z) (-0.06844, 65.4316, 20.9191) was created. Active site residues of the docked complex of FDA drugs and modeled BLK tyrosine kinase were found using the PLIP server.

Pharmacophore modeling

Ligand-based pharmacophore modeling using FDA-approved drugs Dasatinib, Ponatinib, and Saracatinib was done using the PharmaGist server.[24] It is an ensemble of steric and electronic features that are important to recognize the optimal supramolecular interactions with the protein and trigger its biological response. By this definition, the interactions between the proteins and the bioactive molecules can be represented through a 3D arrangement. The interaction types include hydrogen bonds, metal interactions, charged interactions, and hydrophobic and aromatic contacts. High-throughput virtual screening of BLK tyrosine kinase inhibitors was done using the ZincPharmer server.[25] It gives us a database of conformations that has been calculated from the zinc database of purchasable compounds. The filters used were as follows: Molecular weights ranging from 450 to 550 and rotatable bonds ranging from 6 to 9 were used to identify 250 zinc-purchasable compounds.

Virtual screening

Here, virtual screening was employed to help with lead optimization and hit detection. A python script-based approach using Autodock vina was used to do virtual screening on 250 active ligands taken from the top hits of the zinc purchasable database on the target.[26] The macromolecule was prepared the same as in Autodock 4.2.6. The three-dimensional grid box with a 60-grid size (x, y, z) with a spacing of 0.500 and a grid center (x, y, z) (-0.06844, 65.4316, 20.9191) was created, and virtual screening was performed.

ADMET/Tox prediction

The top six ligands with the lowest binding energy from 250 hits were taken and ADMET was analyzed. Drug ADME prediction and drug-likeness prediction were done using the Swiss ADME server.[27] In the discovery and development of a drug, chemical absorption, distribution, metabolism, excretion, and toxicity (ADMET) play an important role. For drug likeliness, Lipinski's Rule of Five was considered because a molecule will not be orally

active if it violates two of the four Lipinski's Rule of Five.[28] The drug toxicity analysis was done using OSIRIS Property Explorer and the Pro-Tox II.[29]

Molecular docking of ADME passed novel drugs

Molecular docking analysis of modeled BLK tyrosine kinase against the drugs that passed ADME was done. The six ligands that passed the ADME test, were docked uniformly by using Autodock4. The macromolecule was prepared and grid preparations were done as previously. And the binding free energy and inhibition constant of the macromolecule-ligand complexes were obtained.

RESULTS AND DISCUSSION

Homology modeling and structural validation of protein

By performing conserved sequence analysis, it was found that the HCK kinase protein has the highest sequence similarity to our protein of interest, BLK tyrosine kinase. The homology model was constructed from the phyre2 server using template PDB ID 2C0I (HCK tyrosine kinase) with a sequence identity of 69% with BLK. Structure validation of the model was done by using many servers like ProSA-web, PROCHECK, QMEAN4, and verify3D, and the results are shown in (Table 1). The Ramachandran plot and its statistics for the model BLK tyrosine kinase were obtained using the PROCHECK server.[30] Ramachandran plot statistics implied that 87.60% of residues are present in the favored region. The ProSA - webserver was used to find the Z-score. It indicates the quality of overall protein model. Here we obtained a Z-score value of -9.4 for our homology model. A high Z score indicates high quality. Ramachandran plot and Z-score plot shown in (Figure 1). QMEAN4 [qualitative model energy analysis] is the composite scoring function that describes the geometrical aspects of protein structures. Here we got a QMEAN4 score of 0.73 for our model. Furthermore, the model was subjected to the Verify3D algorithm for a more thorough evaluation, which measures protein model compatibility with its amino acid sequence. It was found that the residue had an average 3d-1d score>= 0.2 and gave a value of 99.10%.

Active site prediction of protein model

The active site residues of template PDB ID 2C0I were found on the PLIP server. The template residues are Leu-247, Val-255, Ala-267, Phe-281, Met-288, Ile-310, Leu-367, Leu-367, Ala-377, and Leu-381. Their distances are 3.81 Å, 3.82 Å, 3.97 Å, 3.68 Å, 3.95 Å, 3.58 Å,3.70 Å,3.98 Å, 3.88 Å, 3.83 Å respectively. An active pocket of the BLK model was identified using the CASTp server it gives pockets of the binding sites for the ligand. We finalized pocket 2, which has a volume of (723.546). And this pocket had active site residues matching the template PDB ID 2COI.

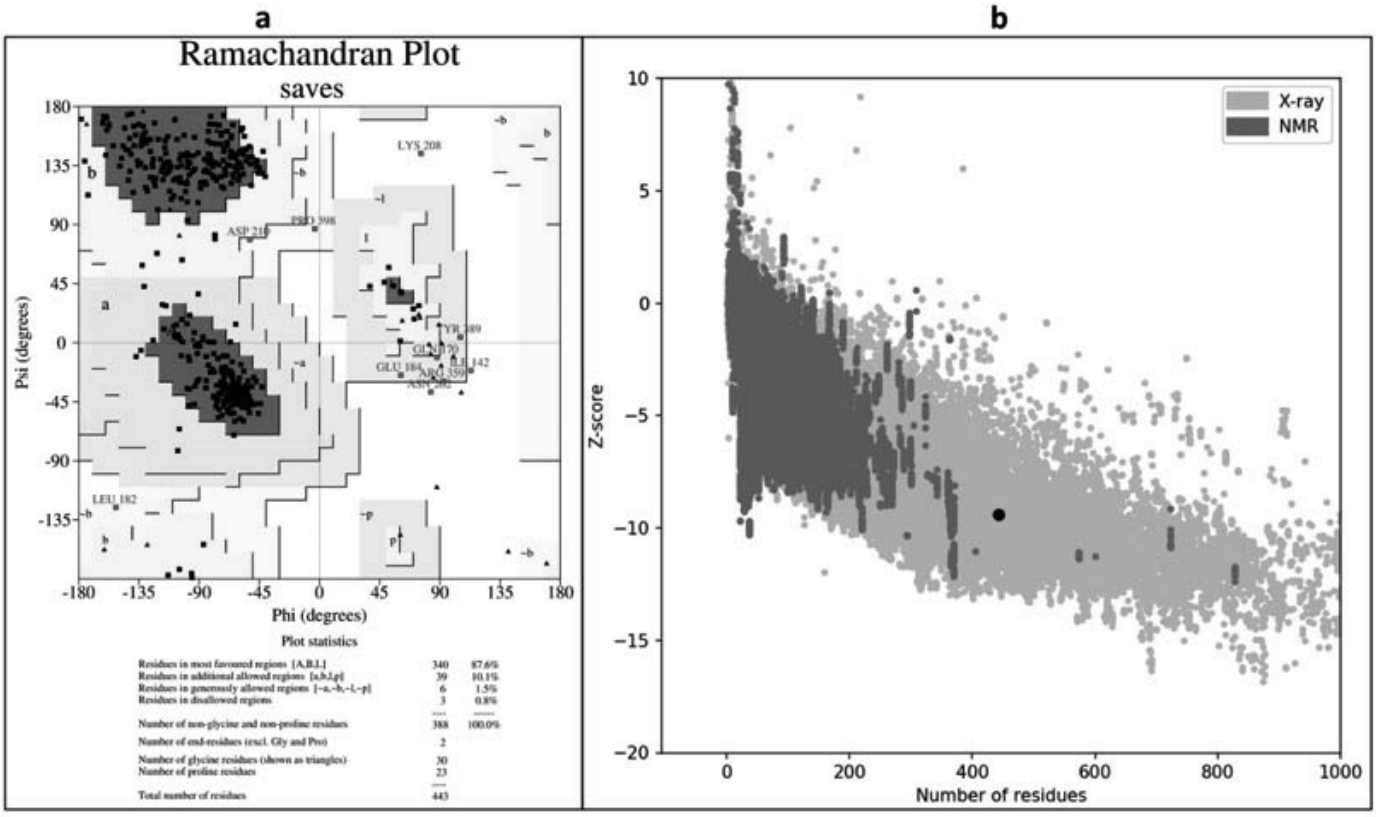

Figure 1: Structure validation results of homology model by (a) Ramachandran plot obtained from PROCHECK, (b) Z-score graph obtained from PROSA.

Table 1: Structure validation results of homology model performed with various servers.

Sl. No	Server	Parameters	Values
1	PROSA	Z-SCORE	-9.4
2	PROCHECK	Residues in most favored region	87.60%
3	Qmean4	Q-mean score	0.73
4	VERIFY 3D	The residues that have averaged 3d-1d score>=0.2	99.10%

Table 2: Molecular docking analysis results of known FDA approved drugs used for pharmacophore model with modelled BLK tyrosine kinase.

Sl. No	Drugs	MOLECULAR FORMULA	Binding energy (kcal/mol)	Inhibition constant (nM)
1	Dasatinib	$C_{22}H_{26}C_lN_7O_2S$	-9.38	132.62
2	Ponatinib	$C_{29}H_{27}F_3N_6O$	-10.22	32.33
3	Saracatinib	$C_{27}H_{32}C_lN_5O_5$	-8.59	507.81

Table 3: Active site residues of modelled BLK tyrosine kinase interacting with FDA approved inhibitors.

Sl. No	Drugs	Hydrogen-bond	Residual hydrophobic interactions
1	Dasatinib	Gly-253, Val-255, Lys-269, Asp-378, Phe-379	Lys-269, Leu-271, Phe-281, Met-288, Lys-269, Val-297, Leu-299, Ile-310, Phe-379. Leu-381
2	Ponatinib	Val-255	Val-255, Lys-269, Leu-271, Phe-281, Met-288, Ile-310, Leu-381
3	Saracatinib	Gly-253, Val-255	Gln-251, Phe-252, Val-255, Leu-381. Ile-385

Table 4: ADME prediction results of novel shortlisted compounds based on best binding energy and other physiochemical properties.

Physiochemical Properties	ZINC57306994	ZINC33263215	ZINC02709773	ZINC11696138	ZINC19166011	ZINC12637348
Molecular weight	476.55	481.54	484.57	489.59	474.96	500.61
Num. rotatable bonds	6	9	6	6	6	7
H-bond acceptors	6	6	6	5	6	5
H-bond donors	1	2	1	1	1	1
TPSA[1](A°[2])	111.56	132.53	111.02	105.57	111.56	102.33
Lipophilicity (LogP)	3.01	3.21	3.27	2.85	3.04	3.21
Metabolism						
CYP1A2*	No	No	No	No	Yes	No
CYP2C19*	Yes	Yes	No	Yes	Yes	Yes
CYP2C9*	Yes	Yes	No	Yes	Yes	Yes
CYP2D6*	No	Yes	No	No	Yes	Yes
CYP3A4*	Yes	Yes	Yes	Yes	Yes	Yes
Excretion						
P-Gp[2] substrate	Yes	Yes	No	Yes	No	No

Abbreviations [1]Total polar surface area, [2]P-glycoprotein, * Cytochrome P450 inhibitors

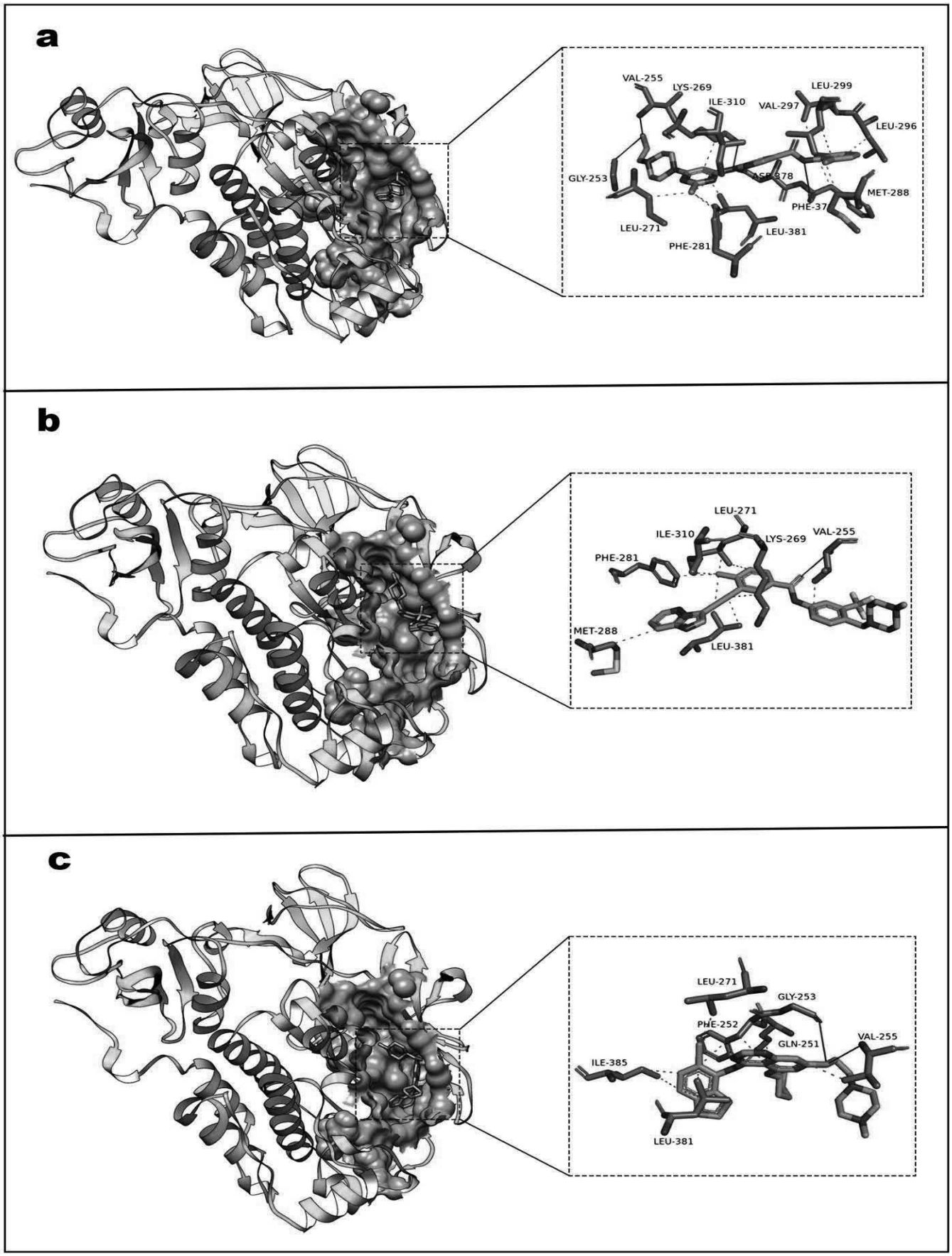

Figure 2: Active site residues of a Docked complex of modeled BLK tyrosine kinase and FDA approved drugs (a) Dasatinib, (b) Ponatinib, (c) Saracatinib.

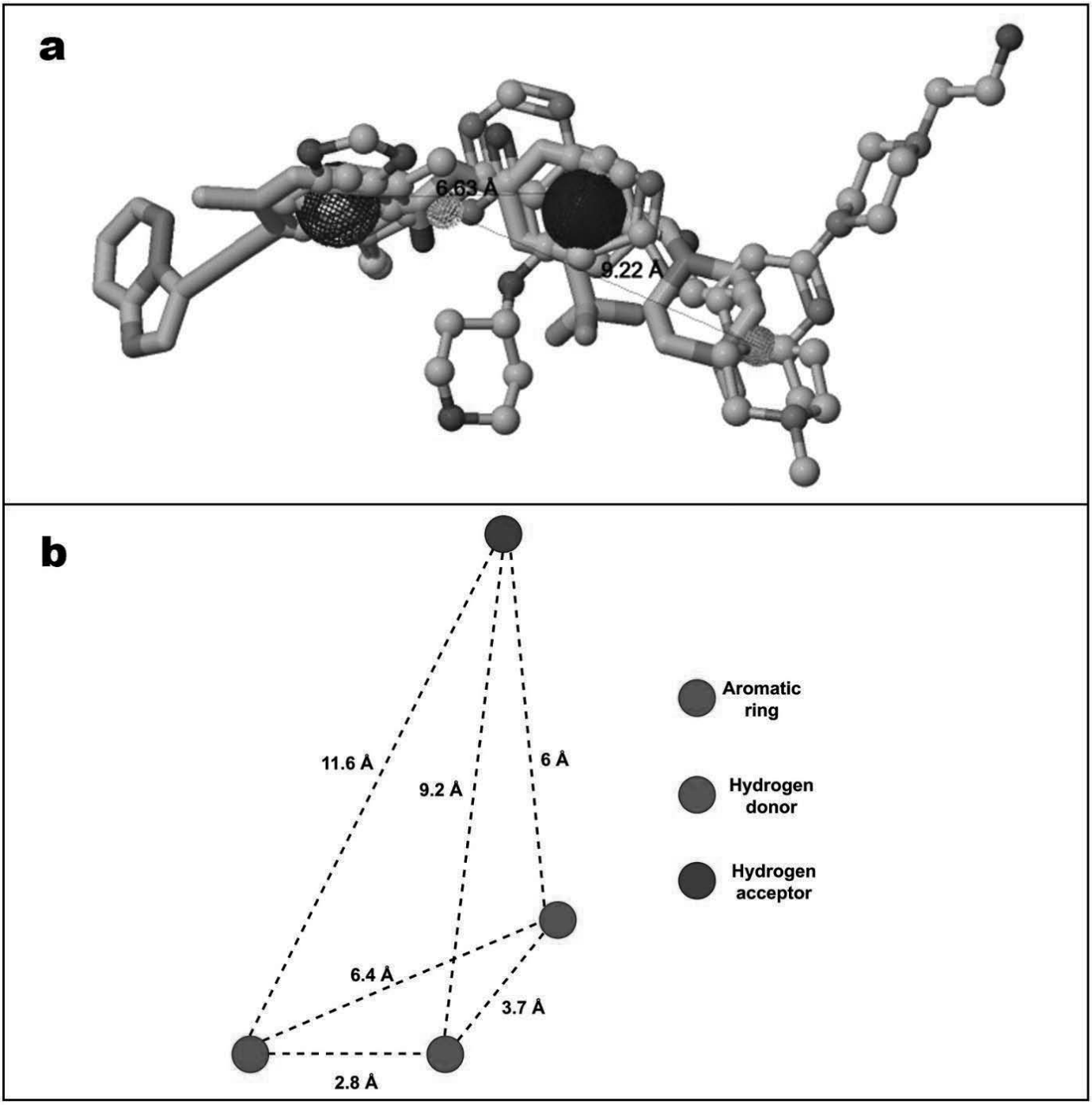

Figure 3: (a) Pharmacophore model of FDA-approved SRC tyrosine kinase inhibitors, (b) Four-point model of pharmacophore with the distances between molecular descriptors.

Molecular docking analysis of FDA-approved BLK inhibitors

Molecular docking analysis was done by using Autodock4 for BLK tyrosine kinase and FDA-approved SRC tyrosine kinase inhibitors, the binding energies and inhibition constants are listed in (Table 2). The binding energies of Dasatinib, Ponatinib, and Saracatinib were -9.38 kcal/mol, -10.22 kcal/mol, and -8.59 kcal/mol, respectively. The inhibition constant wavelengths are -132.62nm, 32.33nm, and -507.81nm, respectively. The active site residues of all docked complexes were noted by PLIP, and the results are shown in (Table 3) and (Figure 2).

Pharmacophore modeling

Ligand-based Pharmacophore modeling of FDA-approved drugs was done using the PharmaGist server. The drugs in complex

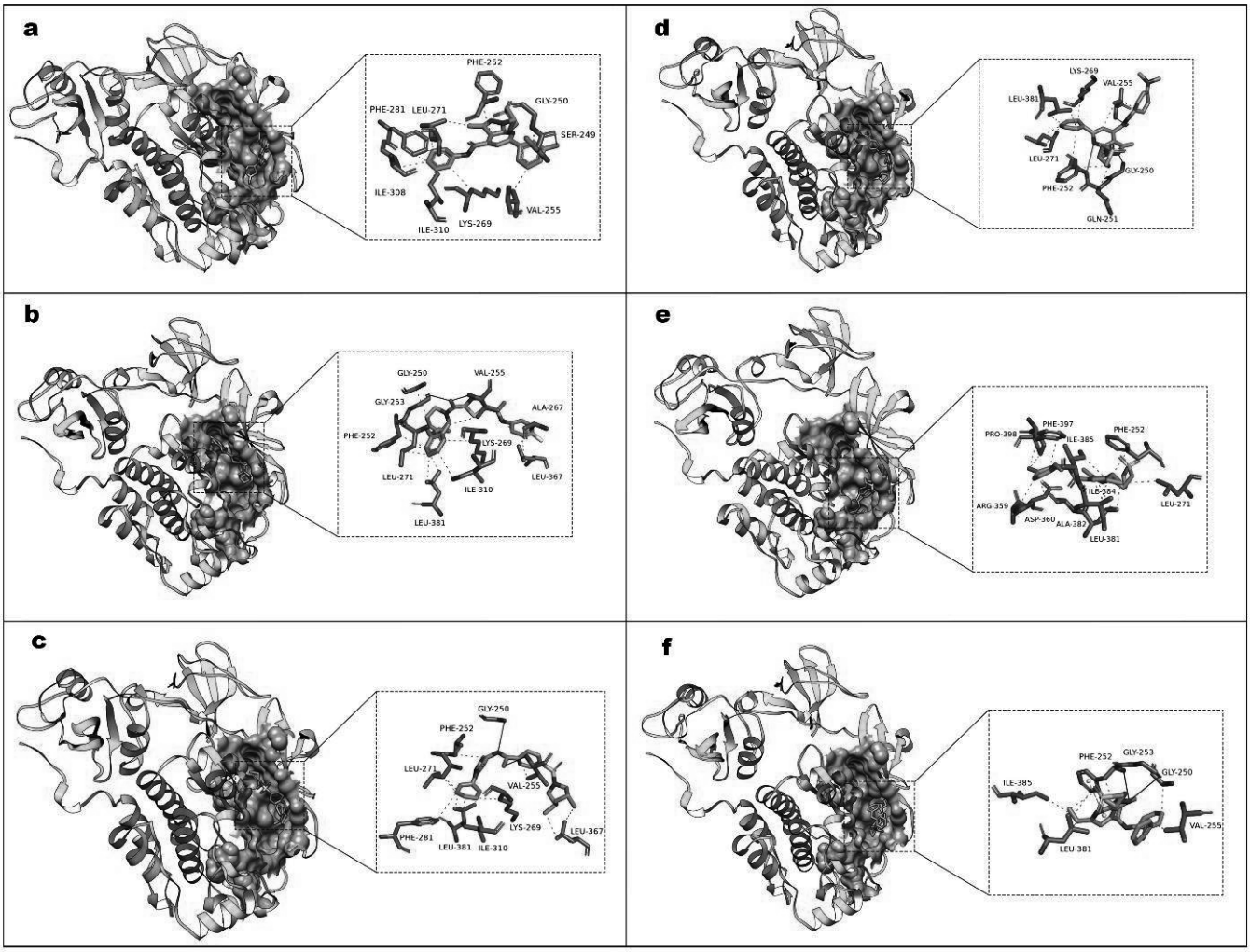

Figure 4: Active site residues of Docked complex of modelled BLK tyrosine kinase and its Novel inhibitors (a) ZINC57306994, (b) ZINC33263215, (c) ZINC02709773, (d) ZINC11696138, (e) ZINC19166011, (f) ZINC12637348.

Table 5: Toxicity prediction results of novel shortlisted compounds.

Toxicity	ZINC57306994	ZINC33263215	ZINC02709773	ZINC11696138	ZINC19166011	ZINC12637348
Toxicity Class	4	4	5	4	4	4
Hepatotoxicity	Inactive	Inactive	Active	Inactive	Active	Inactive
Carcinogenicity	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Immunotoxicity	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Mutagenicity	Inactive	Inactive	Active	Active	Inactive	Inactive
Cytotoxicity	Inactive	Inactive	Inactive	Active	Inactive	Inactive
Irritant	No	No	No	No	No	No

with the protein model generated a total of four pharmacophore features and their molecular descriptors two aromatic rings, one hydrogen donor, and one hydrogen acceptor. The Pharmacophore model having a Jmol score of 22.405 was selected and it's shown in (Figure 3). ZINCPharmer server was used for High-Throughput Virtual screening of BLK tyrosine kinase inhibitors. Although it can automatically extract a pharmacophore from the interactions the user further increases the applicability and specificity of the results by editing query properties or by applying filters to get high-quality hits. Each hit shows a unique orientation and conformation to the query. Finally, the best 250 hits of Zinc purchasable compounds were selected.

Virtual screening

Virtual screening of the top 250 hits was obtained using the Autodock Vina software. AutoDock Vina is a program for

Table 6: Molecular docking analysis results of ADMET passed drugs with BLK.

Sl. No	Compound name	Molecular formula	Binding energy (kcal/mol)	Inhibition constant (nM)
1	ZINC57306994	$C_{25}H_{24}N_4O_4S$	-11.23	5.89
2	ZINC33263215	$C_{24}H_{24}FN_5O_3S$	-11.17	6.47
3	ZINC02709773	$C_{24}H_{28}N_4O_5S$	-10.98	8.88
4	ZINC11696138	$C_{26}H_{27}N_5O_3S$	-10.92	9.88
5	ZINC19166011	$C_{22}H_{23}ClN_4O_4S$	-9.39	130.39
6	ZINC12637348	$C_{28}H_{28}N_4O_3S$	-8.8	414.3

Table 7: Active pocket residues of shortlisted compounds against modelled BLK tyrosine kinase.

Sl. No	Compound name	Hydrogen-bond	Residual hydrophobic interactions
1	ZINC57306994	Ser-249	Gly-250, **Phe-252**, **Val-255**, **Lys-269**, **Leu-271**, **Phe-281**, Ile-308, **Ile-310**
2	ZINC33263215	Gly-250, Gly-253, **Val-255**	Gly-250, **Phe-252**, **Val-255**, Ala-267, **Lys-269**, **Leu-271**, **Ile-310**, Leu-367, **Leu-381**
3	ZINC02709773	Gly-250, **Val-255**	**Phe-252**, **Val-255**, Lys-**269**, **Leu-271**, **Leu-381**, **Ile-310**, Leu-367, **Leu-381**
4	ZINC11696138	Gly-250, Phe-252, **Val-255**	**Gln-251**, **Phe-252**, **Val-25**5, **Lys-269**, **Leu-271**, **Leu-381**
5	ZINC19166011	Gln-251, Phe252	**Phe-252**, **Leu-271**, Arg-359, Asp-360, **Leu-381**, Ala-382, Ile-384, **Ile-385**, Phe-397, Pro-398
6	ZINC12637348	Phe-252, **Gly-253**	Gly-250, **Phe-252**, **Val-255**, **Leu-381**, **Ile-385**

*Bold Amino acid residues coincide with the active residues of FDA approved BLK inhibitors

virtual screening. It uses a sophisticated gradient optimization method in its local optimization procedure. From among the top 250 hits obtained, we chose the best 50 ligands that have free binding energies of 14 to -8 kcal/mol and were further utilized for ADMET analysis.

ADMET/Tox prediction

ADME properties were predicted using SwissADME server, all the parameters predicted are shown in (Table 4). First, drug likeliness was seen where the Lipinski's Rule of Five Violations was analyzed, and compound 6 had one Lipinski's Rule violation of having a molecular weight greater than 500g/mol. Then the properties like skin permeation value, polar surface area, gastrointestinal absorption, and blood-brain barrier permeability were obtained, and all six compounds have high gastrointestinal absorption, but there is no blood-brain barrier permeability. Then P-gp(P-glycoprotein) substrates and cytochrome P450 inhibitors were obtained, and it was found that all compounds were P-gp substrates. P-gp involved in the efflux function. It extrudes toxins and xenobiotics out of the cell. The measurement of toxicity is an important step towards the selection of a better compound. Electronic toxicity measurement shows more accuracy and accessibility, which is why it is widely used and can show information about any natural or synthetic compound. By using the Pro-Tox II server and OSIRIS Property Explorer software toxicity prediction was performed, all the parameters predicted are shown in (Table 5). At first, Toxicity Class and irritant effect were predicted and all the compounds didn't have any irritant effect. Finally, toxicity prediction was performed using the Pro-Tox II server include hepatotoxicity, i.e., liver toxicity; carcinogenicity, i.e., the ability to cause cancer; mutagenicity, i.e., the ability to induce mutation, and cytotoxicity, i.e., cell toxicity. All six compounds do not show any carcinogenicity or immunotoxicity.

Molecular docking analysis of ADME passed novel drugs

Among all the 250 hits, 6 ligands that passed the ADME were taken for molecular docking analysis. It was done using Autodock4. (Table 6) gives information about the molecular docking analysis of ADMET-passed drugs. Here we note the binding energy in kcal/mol (ranges from -11.23 to -8.8) and inhibition constant in nM. Among all the six ligands, ZINC57306994 has the best binding energy of -11.23 kcal/mol and an inhibition constant of 5.89 nM. (Table 7) and (Figure 4) gives the active site residues obtained from the PLIP server.

CONCLUSION

The study design is based on a bioinformatics approach that has successfully established a novel drug, ZINC57306994, as a potential lead molecule against BLK protein tyrosine kinase using

homology modeling, ligand-based pharmacophore modeling, high-throughput virtual screening, ADME/Tox prediction, and molecular docking. The compound ZINC57306994 was found to have the least binding energy of -11.23 kcal/mol with an inhibition constant of 5.89 nM (*in-silico*). This compound may act as potent inhibitor against BLK protein tyrosine kinase. Further studies including *in-vitro* and *in-vivo* have to be carried out for checking the efficacy of the novel compound. This study is an attempt to screen and shortlist candidate molecule as a potent BLK inhibitor.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Original Article

Assessment of Adherence and Common Non-adherence Factors for Inhaled Medications in Asthma and Chronic Obstructive Pulmonary Disease (COPD) Patients

Ashita Maria Nazareth, Rohit O Agarwal, Sujit Kumar Sah, Neethu Reji, Minju Biju, Shilpa Palaksha*

Department of Pharmacy Practice, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Mysuru, Karnataka, INDIA.

ABSTRACT

Background: Inhaled medications are the cornerstone for the treatment of asthma and chronic obstructive pulmonary disease (COPD). Adherence of inhaled medications can be influenced by many factors such as patient preferences, education and awareness. Therefore, it is fundamental to identify and address the factors that helps to improve adherence to inhaled medications. Hence, this study aims to assess the adherence and common non-adherence factors for inhaled medications in asthma and COPD patients. **Materials and Methods:** A prospective observational study was conducted at the department of respiratory medicine for 6 months in a tertiary care teaching hospital. A total of 150 patients who were diagnosed with asthma and COPD, and used inhaled medications were enrolled. Patients were interviewed and administered with Inhaled Medication Adherence Questionnaire (IMAQ). The identified factors for non-adherence were classified based on the five dimensions of adherence instrumented by the World Health Organization (WHO). **Results:** A total of 97/150 (64.7%) patients [asthma: 42 (64.2%) and COPD: 55 (68.7%)] were found non-adherent to inhaled medications. Between them, 54% were attributable to socioeconomic reasons, 38% were patient-related, 4.6% therapy-related and 3.3% healthcare system-related. According to the study results majority of patients showed non-adherence due to socioeconomic-related factors followed by patient-related factors. **Conclusion:** This study concluded that non-adherence to inhaled medication was high in asthma and COPD patients. Low economic status, social stigma, lack of knowledge, inappropriate usage technique and complexity of treatment were the most common factors influencing non-adherence to inhaled medications in asthma and COPD.

Keywords: Asthma, Chronic obstructive pulmonary disease (COPD), Medication adherence, Medication adherence questionnaire, Non-adherence factors.

Correspondence:
Ms. Shilpa Palaksha
Department of Pharmacy Practice, JSS College of Pharmacy, JSS Academy of Higher Education and Research, SS Nagar, Mysuru-570015, Karnataka, INDIA.
Email id: shilpapalaksha@jssuni.edu.in

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INTRODUCTION

According to World Health Organization (WHO), chronic respiratory disorders account for 5% disease burden globally, resulting in more than 4 million deaths per year.[1] Asthma and COPD are two most common prevalent chronic respiratory disorders with increased morbidity, mortality and economic burden globally.[2] According to the disability adjusted life years (DALY), asthma was 16th leading cause of disability. It affected around 300 million people worldwide and expects an additional 100 million to be affected by 2025. Prevalence of asthma is high in developed countries while mortality was high in developing countries.[3] In case of chronic obstructive pulmonary disease (COPD), it is the 3rd leading cause of death that accounted for 3.23 million death by 2019.[1] Asthma and COPD are the non-communicable diseases that can lead to episodic or persistent damaged airways resulting in shortness of breath, chest pain, wheezing, crackles, airway thickening and restricted or limited airflow.[2,4] Both diseases pose complex mechanism and heterogeneous pathophysiology, progression triggered by various risk factors such as genetic predisposition, age, allergic rhinitis, smoking, exposure to environmental hazards, obesity and other respiratory infections like tuberculosis and pneumonia infections.[5,6] Asthma and COPD exacerbations are known as major causes of mortality, morbidity, increased treatment cost and loss of lung function. The frequency of exacerbation has been decreased in asthma or can be fully prevented while in patients' with COPD exacerbation has still not decreased due to irreversible and has progressed with time resulting in higher mortality than asthma.[7,8]

Over the past decades, inhaled bronchodilators, glucocorticosteroids, antibiotics, mucolytic agents and

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antitussives are commonly prescribed for the treatment of asthma and COPD. The single use or fixed-dose combination of inhaled bronchodilators and glucocorticosteroids are the mainstay in the reduction of airway inflammation and enhancing the airway follow.[9,10] However, due to the chronic nature of the diseases, patients may require complex drug regimens for longer duration that increases the risk of non-adherence to medications. Especially, medication non-adherence associated with poor disease control, exacerbate the disease, increases the risk of emergency department visit, hospitalization, economic burden and decreased patients' quality of life.[11,12] Previous studies have shown 22-78% of medication adherence rates in patients with asthma and COPD.[13,14] However, limited studies are published on the assessment of non-adherence to inhaled medications. This study shows poor adherence to the use of inhaled medications.[15,16] The use of multiple inhalers presented a higher non-adherence rate compared to the single inhaler in asthma and COPD patients. The complexity of inhaled medication regimen increases the chances of non-adherence. The non-adherence to inhaled medications has been dependent mainly on a poor understanding of techniques to use inhaler devices, disease characteristics (such as age, breathing pattern, disease severity and airway diameter), aerosol characteristics (characteristics of inhaled drugs), pharmacokinetics and pharmacodynamics characteristics and patient behavior towards inhaled medications. Further, various type of inhalers introduced in markets increases the risk of poor knowledge about new inhalers and also increases the risk for poor adherence.[17-19] Therefore, to enhance patients' inhaled medications adherence, patients' may be prescribed with friendly inhaler and provided with adequate education about their usage. The frequent assessment of adherence rate of inhaled medications during patient care process become essential to avoid non-adherence. To measure non-adherence various techniques can be used such as maintaining self-report/asthma diaries, self-report questionnaires, semi-structured interviews and medication measurement.[20] However, literature search reveals that the limited studies were published in the assessment of medication adherence and factors associated with non-adherence of inhaled medications among asthma and COPD patients.[15,16] Hence, we developed a medication adherence questionnaire to assess medication adherence specific to inhaled medications, validated and administered to the patients.

MATERIALS AND METHODS

A prospective observational study was conducted at the department of respiratory medicine in a tertiary care teaching hospital for 6 months from September 2021 to February 2022. Patients aged above ≥18 years and of either gender who was diagnosed with Asthma and COPD and received at least one inhaled medication was included in this study. The study protocol was approved by Institutional Human Ethics Committee (JSSMC/IEC/17112021/14 NCT/2021-2022). Inhaled medication adherence questionnaire (IMAQ) a 10-item questionnaire was used to assess medication adherence. The questionnaire was developed, validated and used to assess medication adherence among study patients.

Development of questionnaires

Study researchers developed the questionnaire following an adherence scale like the Medication Adherence rating scale (MARS). A total of 10 questions in English were developed suitable to assess medication adherence for inhaled medications. The response was recorded in 'Yes' and 'No'. Question number eight had 2 subdomains. The total score ranged from 0-11 and the overall response was categorized into low (score: 0-4), medium (5-8), and high (9-11) adherence. Patients falling in low and medium adherence were considered non-adherent towards medications and high were considered adherent. Questionnaire was translated into the local vernacular language.

Validation of questionnaire

Each question content was validated using the content validity index (CVI) with the help of four-point criteria 'relevance', 'clarity', 'simplicity', and 'ambiguity'. Each question was distributed to ten experts including two pulmonologists, one postgraduate student, five pharmacist professionals, and two resident pharmacists. The experts were asked to rate each question based on four-point criteria. Each question scored more than 94%. Overall obtained content validity score and the I-CVI of the prepared questionnaire were 3.8% and 95.5% respectively. Simplicity was high among all the parameters (96.8%) in the scale content validity index (S-CVI). The statistical analysis was done using Microsoft excel and SPSS v 21. Cronbach's alpha was used to estimate the internal consistency/ reliability of the questions in the questionnaire. The reliability test was done using the chi-square test.

Data collection

A suitable data collection form was prepared in two sections. The first section was to collect patient information such as demographic details and medication history while in the second section validated IMAQ was incorporated to record patients' medication adherence status. Clinical pharmacists visited the ambulatory care of the respiratory department on a daily basis to assess patients for study inclusion. Patients who met the study criteria were enrolled and study-relevant data was collected in the data collection form. To assess medication adherence IMAQ was asked to enroll patients after reviewing inhaled medications. The collected data were assessed for medication adherence and categorized into adherence status based on obtained score. Five dimensions of adherence instrumented by the World Health Organization (WHO) were used to identify the determinants for medication non-adherence. It includes socioeconomic factors, patient-related factors, therapy-related factors, healthcare

system-related factors, and condition-related factors. Descriptive statistics were used to analyze the data.

RESULTS

A total of 150 patients [asthma: 70 (46.7%) and COPD: 80 (53.3%)] were enrolled for this study. Mean age of study patients was 54.7 ± 13.5 years [asthma (mean ± SD): 43.98 ± 17.52 years and COPD (mean ± SD): 65.53 ± 9.55 years] and 68 (45.3%) [asthma: 27/70 (38.5%) and COPD: 41/80 (51.25%)] patients belonged to age group of 41-60 years. Majority of patients were from upper lower class [*n*=32 (45.2%)] in asthma and upper middle [*n*=31 (38.7%)] in COPD. Demographic details of study patients are presented in Table 1.

Between the 150 patients, who responded to IMAQ questionnaire, majority [*n*= 42 (60%)] of patients with asthma answered 'Yes' to Q4 and 'No' to Q8.a. [*n*=45 (64.2%)] while in COPD patients, majority answers for 'Yes' was 55 (68.7%) and 'No' was Q8.b. 62 (77.5%). The details of the patient responses to IMAQ are presented in Table 2.

A total of 97/150 (64.7%) patients [asthma: 42 (64.2%) and COPD: 55 (68.7%)] were found non-adherent to inhaled medications and 53/150 (35.3%) patients [asthma: 28 patients and COPD: 25 patients) were found adherent to inhaled medications. Of the 70 asthma patients, majority [*n*=28 (40%)] of patients were high adherent to inhaled medications followed by low adherent [*n*=24 (34.3%)] and medium adherent [18 (25.7%)] while in COPD patients, majority were medium adherent [*n*=37 (46.2%)] followed by high adherent [25 (31.2%)] and low [*n*= 18 (22.5%)]. The details of the categorization of medication adherence of inhaled medication are presented in Table 3.

Among all the dimensions, Socioeconomic factors were reported as the most common non-adherent factor (54%) followed by patient-related factors (38%). In socioeconomic factors, the lower socioeconomic class was the most prevalent factor for non-adherence while in patient-related factors and therapy-related factors, social stigma (14%) and complexity of treatment (2.8%) were the most common factors for non-adherence respectively. The details of common non-adherence factors of inhaled medication based on WHO is presented in Table 4.

DISCUSSION

In this study, we observed 64.7% (asthma: 64.2% and COPD: 68.7%) patients were found to be non-adherent to inhaled medications. A similar study conducted in asthmatic patients by Ayele AA *et al.*, reported 50.6% patients were non-adherent to inhaled medications.[21] The prevalence of non-adherence was comparatively high since majority of patients had poor economic status and low literacy rate. Another study conducted in COPD patients by Davis JR *et al.*, reported 71.7% non-adherence to the inhaled corticosteroids and long acting ß2 adrenergic agonists.[22] These findings indicate that the non-adherence is high for inhaled medications mostly due to lack of adequate

Table 1: Demographic details of study patients.

Categories	Variables	Asthma [(*n*=70 (%)]	COPD [(*n*=80 (%)]
Gender	Male	28 (40%)	50 (62.5%)
	Female	42 (60%)	30 (37.5%)
Age group distribution (In years)	≤20	7 (10%)	0 (00%)
	21-40	24 (34.2%)	2 (2.5%)
	41-60	27 (38.5%)	41 (51.25%)
	61-80	11 (15.7%)	28 (35%)
	≥81	1 (1.4%)	9 (11.25%)
Residency	Urban	46 (65.7%)	25 (45.4%)
	Rural	24 (34.2%)	30 (54.5%)
Social History	Smokers	28 (40%)	25 (45.4%)
	Alcoholic	9 (12.8%)	11 (20%)
Socio-economic Class	Upper	7 (10%)	12 (15%)
	Upper- Middle	10 (14.2%)	18 (22.5%)
	Lower- Middle	20 (28.5%)	15 (18.7%)
	Upper-Lower	18 (25.7%)	20 (25%)
	Lower	15 (21.4%)	15 (18.7%)
Educational Status	Literate	32 (45.7%)	32 (40%)
	Illiterate	38 (54.2%)	48 (60%)

Table 2: Patient responses to IMAQ.

Questionnaires	Items		Asthma [*n*=70 (%)]		COPD [*n*=80 (%)]	
			Yes	No	Yes	No
Q1	Do you take your inhaled medication regularly?		27 (38.5%)	43 (61.4%)	24 (30%)	56 (70%)
Q2	Do you take your inhaled medication only when you have symptoms?		40 (57.1%)	30 (42.8%)	54 (67.5%)	26 (32.5%)
Q3	When you use DPI/MDI/MDI with a spacer do you feel difficulty remembering the steps?		41 (58.5%)	29 (41.4%)	55 (68.7%)	25 (31.2%)
Q4	Have you ever stopped taking your inhaled medication because of fear of side effects?		42 (60%)	28 (40%)	51 (63.7%)	29 (36.2%)
Q5	Do you have difficulty remembering the name/dosage/frequency of your inhaled medications?		40 (57.1%)	30 (42.8%)	50 (62.5%)	30 (37.5%)
Q6	Do you forget to carry your medication when you travel?		38 (54.2%)	32 (45.7%)	49 (61.2%)	31 (38.7%)
Q7	Do you stop taking your inhaled medications because you have difficulties buying them?		36 (51.4%)	34 (48.5%)	58 (72.5%)	22 (27.5%)
Q8	a.	Do you follow your physician's advice	25 (35.7%)	45 (64.2%)	20 (25%)	60 (75%)
	b.	Visit your healthcare professional as per the schedule	27 (38.5%)	43 (61.4%)	18 (22.5%)	62 (77.5%)
Q9	Do you know the difference between? a. Reliever b. Preventer		26 (37.1%)	44 (62.8%)	24 (30%)	56 (70%)
Q10	Are you involved in self-medication?		37 (52.8%)	33 (47.1%)	54 (67.5%)	26 (32.5%)

Table 3: Categorization of medication adherence of inhaled medication based on obtained IMAQ assessment score.

Overall medication adherence status	Categories of adherence based on obtained scored	Asthma n (%)	COPD n (%)	Total n (%)
Adherence	High (Score: 9-11)	28 (40%)	25 (31.2%)	53 (35.3%)
Non-adherence	Medium (Score: 5-8)	18 (25.7%)	37 (46.2%)	97 (64.6%)
	Low (Score: 0-4)	24 (34.3%)	18 (22.5%)	
Total patients		70 (100%)	80 (100%)	150 (100%)

knowledge about inhalers and longer duration of disease. Thus, the prescribers, nurses and clinical pharmacists frequently need to assess non-adherence and provide adequate education in the appropriate use of inhaler and related medications.

Further, in this study it was observed that socio-economic factors and patient related factors were the most common determinants for non-adherence of inhaled medications among asthma and COPD patients. In socio-economic factors, the most persistant determinant for non-adherence was lower economic class. This indicates that our study patients were not able to buy the prescribed inhalers and related medications or buy few and/or a single inhaler instead of multiple inhalers prescribed. A cross-sectional study conducted in retroviral therapy by Alvi Y *et al.*, found no association between socioeconomic factors and non-adherence of retroviral medications.[23] However, these findings suggest the prescribers and pharmacists to look into the possible socio-economic risk factors associated with patients and prescribe pocket-friendly inhalers to patients.

Another most common factor for non-adherence with inhaled medications were patient related factors. Under which, social stigma includes hesitancy in administrating inhaled medications in a public setting and fear of side effects. These were most commonly identified risk factors for non-adherence. A study conducted by Simoni AD *et al.*, also revealed that adults and adolescents with asthma presented social stigma – embarrassed using inhalers in public.[24] These finding reveals that social stigma need to be addressed and patients need to be educated. Other most observed patient related factors for non-adherence

Table 4: Determinants of non-adherence factors of inhaled medication based on WHO.

Dimensions of WHO	Factors		n = 97 (%)	Total
Socio-economic Factors	Socio-economic class	Upper	4 (4.1%)	52 (53.6%)
		Upper- Middle	4 (4.1%)	
		Lower-Middle	9 (9.2%)	
		Upper-Lower	15 (15.4%)	
		Lower	20 (20.6%)	
Patient-related	Social stigma		13 (13.4%)	36 (37%)
	Lack of knowledge about inhaled medication		9 (9.2%)	
	Inappropriate technique to use inhaled medication		8 (8.2%)	
	Lack of follow-up		6 (6.1%)	
Therapy-related	Duration of therapy		1 (1%)	5 (5.1%)
	Complexity of treatment		3 (3%)	
	Fixing dose		1 (1%)	
Healthcare system related	Duration of travel		2 (2%)	4 (4.1%)
	Long waiting hours for the appointment		1 (1%)	
	Inadequate time for consultation		1 (1%)	
	Doctor-patient relationship		0 (0%)	
Condition Related	Acute attacks		0 (0%)	0 (0%)
	Comorbidities		0 (0%)	

were lack of knowledge about inhaler/inhaled medications and inappropriate techniques to use inhaler/inhaled medications. This indicates that patients need education about proper usage techniques of inhaler/inhaled mediation and their importance.

Among therapy related factors, 'complexity of treatment' was the most prominent factor for non-adherence of inhaled medications. Prescribing multiple drug regimens and/or multiple inhalers had higher risk for non-adherence due to complexity of treatment. Thus, prescribers must counsel the patients regarding the appropriate use of inhaled medications and benefits of completion of the therapy.[25] The limitation of this study is that it only identified the non-adherent factors influencing inhaled medication adherence but did not resolve it.

CONCLUSION

This study concluded that non-adherence to inhaled medication was high in asthma and COPD patients. Low economic status, social stigma, lack of knowledge, inappropriate technique to use inhaled medications and complexity of treatment have been identified as most common factors for non-adherence of inhaled medications in asthma and COPD. Therefore, frequently measuring medication adherence, promoting appropriate use and educating patients about disease and inhaler use/inhaled medications in each patients helps to enhance medication adherence.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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