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B.PHARM-PROGRAMME VISION & MISSION

Vision

Striving to excel in pharmaceutical education, research & innovation to develop outstanding professionals catering to the health care needs of the humankind.

Mission

The institute aims to develop employable students, researchers and entrepreneurs by inculcating critical thinking, problem solving ability, ethical values and leadership skills. Institute provides vibrant environment for continuous learning by strengthening industrial collaboration for developing competent professionals.

B.PHARM-PROGRAMME EDUCATIONAL OBJECTIVES

PEO 1: To acquire effective knowledge of pharmaceutical sciences to be able to lead the industry and health care sector

PEO 2: To attain practical training and technical expertise in pharmaceutical sciences

PEO 3: To inculcate professional and ethical standards with effective interpersonal communication skills.

PEO 4: To develop interdisciplinary & holistic approach in Pharmaceuticals for societal cause, problem solving and lifelong learning.

PEO 5: To adapt and implement best practices in the profession by enrichment of knowledge and skills in research and critical thinking.

B.PHARM-PROGRAMME OUTCOMES

No.	Programme Outcomes
PO1	**Pharmacy Knowledge:** Possess knowledge and comprehension of the core and basic knowledge associated with the profession of pharmacy, including biomedical sciences; pharmaceutical sciences; behavioral, social, and administrative pharmacy sciences; and manufacturing practices.
PO2	**Planning Abilities:** Demonstrate effective planning abilities including time management, resource management, delegation skills and organizational skills. Develop and implement plans and organize work to meet deadlines
PO3	**Problem analysis:** Utilize the principles of scientific enquiry, thinking analytically, clearly and critically, while solving problems and making decisions during daily practice. Find, analyze, evaluate and apply information systematically and shall make defensible decisions.

PO4 **Modern tool usage:** Learn, select, and apply appropriate methods and procedures, resources, and modern pharmacy-related computing tools with an understanding of the limitations

PO5 **Leadership skills:** Understand and consider the human reaction to change, motivation issues, leadership and team-building when planning changes required for fulfillment of practice, professional and societal responsibilities. Assume participatory roles as responsible citizens or leadership roles when appropriate to facilitate improvement in health and well-being.

PO6 **Professional Identity:** Understand, analyze and communicate the value of their professional roles in society (e.g. health care professionals, promoters of health, educators, managers, employers, employees).

PO7 **Pharmaceutical Ethics:** Honor personal values and apply ethical principles in professional and social contexts. Demonstrate behavior that recognizes cultural and personal variability in values, communication and lifestyles. Use ethical frameworks; apply ethical principles while making decisions and take responsibility for the outcomes associated with the decisions.

PO8 **Communication:** Communicate effectively with the pharmacy community and with society at large, such as, being able to comprehend and write effective reports, make effective presentations and documentation, and give and receive clear instructions

PO9 **The Pharmacist and society:** Apply reasoning informed by the contextual knowledge to assess societal, health, safety and legal issues and the consequent responsibilities relevant to the professional pharmacy practice

PO10 **Environment and sustainability:** Understand the impact of the professional pharmacy solutions in societal and environmental contexts, and demonstrate the knowledge of, and need for sustainable development.

PO11 **Life-long learning:** Recognize the need for, and have the preparation and ability to engage in independent and life-long learning in the broadest context of technological change. Self-assess and use feedback effectively from others to identify learning needs and to satisfy these needs on an ongoing basis

PSO1 **Drugs and diseases:** Understand different classes of drugs, their mechanism of action, dynamics, kinetics, structure activity relationships, pathophysiology and pharmacotherapeutics of various diseases.

PSO2 **Drug development:** Ability to synthesize, develop and/or evaluate various pharmaceuticals and their formulations and cosmeceuticals products

PSO3 **Analytical skills:** Develop skills in qualitative and quantitative analysis of various pharmaceuticals.

PSO4 **Training:** Acquire technical knowledge and hands on training on equipments, instruments and software used in the field of pharmaceutical sciences.

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Content

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EDITORIAL MESSAGE

There is a paradigm shift in the trends for outsourcing the testing services recently. The Contract research organizations (CROs) now are receiving testing and consulting services for every stage of the drug R&D process for commercial manufacturers such as pharmaceutical and biopharmaceutical companies and medical device firms. The CRO services market is valued at USD 36.27 Billion in 2017 and projected to reach to USD 56.34 Billion by 2023, at a CAGR of 7.6%. Worldwide, the pharmaceutical and biopharmaceutical companies are encountering changes in lieu of the patent cliff. Their main focus is on the development of new drugs for various unmet therapeutic conditions. Over the last decade, pharmaceutical and biopharmaceutical companies have increasingly outsourced most of their R&D activities to bring new products to the market cost-effectively. In recent years, due to the enormous pressure to contain fixed costs, outsourcing has evolved as a strategic alternative for pharmaceutical and biopharmaceutical companies due to lack of in-house resources required for new product development and the need for cost and time savings. Different type of testing services (clinical and laboratory) offered during the drug discovery and developmental process helps pharmaceutical and biopharmaceutical companies to mitigate risks by avoiding large investments in purchasing equipment and maintaining manpower, especially when development efforts are in the early stages. It also helps pharmaceutical companies focus on their core competencies. Thus, the development of new drug molecules is expected to propel the demand for CRO services. Companies like Pfizer, Merck, and Roche have reduced their in-house testing services to fuel their R&D pipelines in order to meet the growing demand for new drug molecules for various therapeutic areas.

There is a need for highly skilled professionals have to keep pace with the continuing changes in pharmaceutical and medical device R&D technologies and methodologies. Skilled professionals also have to provide quality services and comply with the good laboratory practices. These CROs face challenges in attracting and retaining highly skilled professionals as they compete with pharmaceutical, biotechnology, medical device, and academic and research institutions for qualified and experienced scientists.

With this issue, our enthusiasm and confidence has been slowly mounting. We are getting an annihilating response from the industrial experts, reviewers and the pharmacy community. In this issue we have invited research articles and review articles from industrial experts as well as postgraduate students spread across different facets of pharmaceutical field. We welcome contributions from all fields of research pertaining to health science. We hope the pharmacy fraternity will share our excitement and join us in this adventure.

Happy hours!!

Editorial Team, NUJPS

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ARTICLE

PARADIGM SHIFT IN PHARMACEUTICAL BUSINESS WITH RESPECT TO IMPORTANCE OF PHARMACEUTICAL DEVELOPMENT

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The pharmaceutical industry is the technology sector with the highest added-value to the industry. According to the 2018, EU/US Industrial R&D Investment Score board, the pharmaceutical and biotechnology sector amounts to 18.9 % of total business R & D expenditure worldwide. The average spending level on new products launched in 2019-2023 is expected to reach to $45.8 billion, slightly greater that the $43.4 billion observed for products launched in 2014-2018. Now a days pharmaceutical industries are developing molecules especially in the specialty segment like, orphan, biologics and oncology areas, thus confirming the trend towards an increasing role of precision medicine where a new treatment is supposed to reach fewer patients. Huge amount of efforts are also going for development of generic products by various companies. The need of common technical documents for market authorisation is also increasing even in developing markets. All these is leading to increased requirements of usage of knowledge in pharmaceutical operations.

Developing a pharmaceutical product had always been a challenge for the pharmacists, when it comes to approval from the various countries in International markets. It was always a challenge in the previous years as the documentation requirements for marketing authorisation (product registration) were largely varying (diversified) as per the individual country's requirement, not only in regulated market but also semi regulated and emerging markets.

Due to the lack of uniformity amongst the countries in framing the registration documents requirements, the task of Regulatory Pharmacist, always remains challenging. With lot of efforts by International organisations like WHO, ICH and ISO which lead to the need of

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common technical documents (CTD). Globally, most of the countries started implementing uniform platform as CTD, which forced the pharmaceutical manufacturers to implement good documentation practises, to survive in the competitive market.

For the development of finished formulation, QbD (Quality by Design) approach lays more emphasis on continuous improvement rather than end-product testing. The principles of Quality by Design have been adopted by majority of pharma industry. QbD makes certain that the product is of predictable and predefined quality. The adoption of QbD includes defining a target product quality profile; designing the manufacturing process from basic principles with a very good understanding of the mechanism involved (good Design of Experiment); identifying critical quality areas, process parameters and potential sources of variability; and finally controlling manufacturing process to achieve the most consistent quality.

QbD will become a regulatory requirement for filing dossier to international regulatory. Hence many pharma companies have now started engaging with QbD, with numerous projects now underway. Adopting QbD will increase costs and development but this would be offset by more successful launches, less loss in production, fewer deviations, and fewer recalls – so there should be an overall net gain. It applies the concept of 'First Time Right' from the manufacturing industry to the pharma.

The CTD dossier necessarily is asking for complete information about the product with respect to Product Development Report, Analytical Development Report, Complete Pharmacokinetics, efficacy and safety of the product. All these information means a lots of development work to be done by manufacturer.

Product Development can give manufacturers much more confidence in the robustness of their product, potentially increases the efficiency and quality of their development and manufacturing process as well as reduces profit leakages.

*(Source of data: EFPIA member associations (official figures))

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ARTICLE

REGULATORY EXCLUSIVITY IN THE UNITED STATES AND EUROPEAN UNION AND ITS IMPACT ON GENERIC ENTRY

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Abstract

Regulatory exclusivity is one of strategy apart from an intellectual property rights to get profit and return on investment done by the pharmaceutical industry on development of drugs. The reason of introducing regulatory exclusivity for drug products was that a lot of time would lost during approval of drug product and because of that at the time of approval of drug very little patent protection available to that drug. Pharmaceutical companies usually file patent application at respective countries at the time of an invention related to drug and it will take time more than 10 years to get approval from the authorities (FDA- Food and Drug Authority or EMEA-European Medicines Agency) and that leaves the patent holder with a much lesser duration, and sometimes none, to actually avail a return on the huge investment in R&D.

Key words: Regulatory exclusivity, Generic, Patent, Data Exclusivity, Market Exclusivity, New Chemical Entity Exclusivity, Clinical Investigation Exclusivity, Orphan Drug Exclusivity, Pediatric Exclusivity

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1. Introduction

Development of pharmaceutical medicine is an expensive process and approval from drug authority takes a lot of time because of this time consuming process very often patents protection related to pharmaceutical medicine are expired before approval or launch of medicines in the market. As a result, most pharmaceutical industries rely on the exclusivity granted under the FDA- Food and Drug Authority or EMEA-European Medicines Agency.

Regulatory exclusivity is exclusive marketing rights granted by FDA or EMEA upon approval of a drug. It may run simultaneously with a patent protection or not. It prevents submission or final approval of ANDAs or 505(b)(2) applications and therefore it is design in such way to balance between new drug innovation and generic drug competition.

Pharmaceutical companies generally file patent application in respective countries at the time of innovation of product. Approval of product will take almost 10 years or more from respective drug authority. This is because the requirement of conducting clinical trials to prove safety and efficacy of any new drug molecule is very tedious, costly and without any guarantee of favourable results. At the time of launch of product in a market, patent protection left on the product is very little. To provide pharmaceutical companies chance to recoup their investment on drug research & development and to give incentive to do an innovation, the FDA or EMEA have provided regulatory exclusivities to extend the period in which companies can market their products without any competition from generics companies.

This article is intended to provide an overview of the regulatory exclusivity provisions in the United States and European Union that pharmaceutical companies should consider while making strategy for global launch of their products and to maximize market protection of their products.

2. The United States Overview

The Hatch-Waxman Act of 1984 (The Drug Price Competition and Patent Term Restoration Act) introduced regulatory exclusivity in the United States. It provides up to five years market exclusivity to pharmaceutical companies introducing a new chemical entity to the market (NCE exclusivity), up to three years market exclusivity for conducting new clinical investigations (other than bioavailability studies) to support changes to drug products already on the market (Clinical Investigation or CI Exclusivity). Regulatory exclusivity attaches upon approval of a drug product if the statutory requirements are met. There is not any requirement to apply for these Hatch-Waxman exclusivities.

The Orphan Drug Act of 1983 provides up to seven years market exclusivity to drugs that treat diseases or conditions that affect

200,000 or fewer individuals in the United States. In addition to, The Best Pharmaceuticals for Children Act of 2002 provides 6 months market exclusivity which is attached to the other three exclusivities and the term of orange book listed patents itself.

A. New Chemical Entity Exclusivity (NCE)- 5 years

New chemical entity[1] means a drug that contains no active moiety that has been approved by FDA in any other NDA submitted under section 505(b) of the Federal Food, Drug, and Cosmetic Act. Active Moiety[2] is defined as any ion or molecule which is attributed to the drug's physiological or pharmacological action.

Since NCE exclusivity attaches to active moiety of drug, FDA cannot accept or approve generic applications of drug product contains same active moiety during the five year NCE exclusivity.[3]

For example: FDA has approved Erdafitinib on April 12, 2019. FDA has granted five years market exclusivity which is going to expire on April 12, 2023 because FDA has never approved other NDA which contains same active moiety as Erdafitinib has. Because of 5 years NCE exclusivity, FDA cannot accept any ANDA or 505(b)(2) application which contains same active moiety as Erdafitinib has until NCE expires or FDA can accept filing of ANDA or 505(b)(2) application which contains paragraph IV filing with respect to any one of orange book listed patents on NCE-1 year date. ANDA or 505(b)(2) applications can be filed after April 12, 2023 or If ANDA or 505(b)(2) applications contain paragraph IV certification with respect to at least one patent from orange book listed patents then they can be filed on April 12, 2022.

B. Clinical Investigation Exclusivity (CI)- 3 years

Pharmaceutical companies that conduct additional clinical trials on a previously-approved drug may be granted three additional years of Clinical Investigation Exclusivity[4]. Pharmaceutical companies may receive CI Exclusivity for the following changes: new dosage forms, new indications and a product's change from prescription to over-the-counter (OTC). In addition to, the clinical trials have to be new and do not have to rely on previously done clinical trials on approved drug. Unlike NCE exclusivity, ANDA or 505(b)(2) application can be filed during three years of CI exclusivity but they cannot be approved during period of CI exclusivity.[5] If generic applicants conduct own clinical trials to support approval of their application then FDA can approve their ANDA or 505(b)(2) during three years period of CI exclusivity.

C. Orphan Drug Exclusivity (ODE)- 7 years

To find a cure of rare or unusual conditions, pharmaceutical companies have to invest a lot of money and time as drug research and development cost are

really high. As patient population is very low for orphan diseases, the investment done by pharmaceutical companies to identify cure cannot be recovered even during normal exclusivity period. Therefore, FDA has given incentive of 7 years marketing exclusivity to recoup their investment. If drug product obtain seven years of orphan drug exclusivity then FDA can accept but not approve ANDA or 505(b)(2) application that contains same orphan indication during this period of exclusivity.[6] One more reason to provide longer exclusivity is to encourage pharmaceutical companies to do research on the areas where patient population is low, which otherwise get neglected and because of that the patient with such disease have to suffer a lot.

For example: Gefitinib (IRESSA) was approved by FDA on July 13, 2015 with seven years of ODE exclusivity which expires on July 13, 2022. Patent protection related to this product is expired but because of ODE, FDA cannot approve any generic version of IRESSA.

D. Pediatric Exclusivity (PED)- 6 additional months

Interaction of drug in children is different compare to adult. In order to encourage pediatric drug development and testing, congress had enacted The Best Pharmaceuticals for Children Act in 2002.[7] The act provides six additional months of exclusivity to pharmaceutical companies who conduct pediatric clinical trials on their approved and marketed drug product and generate information about the safety and efficacy of their drug product in children.

Pediatric exclusivity only attaches to orange book listed patents, NCE exclusivity, CI exclusivity and ODE exclusivity. Pediatric exclusivity runs after expiry of all other form of exclusivity. FDA generally initiate "written request"[8] to NDA applicant for a particular drug product. If NDA applicant conducts pediatric studies and submit to FDA in a response to "written request" from FDA then pediatric exclusivity will be granted to particular drug product. It is not necessary to have successful pediatric study to get pediatric exclusivity to particular drug product. There is only requirement of submission of pediatric studies to FDA in a response to "written request" is initiated by FDA. Pediatric exclusivity bar effective approval of ANDA and 505(b)(2) application.

Hypothetical case study: To understand it, consider a case where product is having NCE exclusivity expiring in Dec 2025, Orphan exclusivity for a particular indication out of multiple indication expiring in Dec 2027. The same product is also protected by product patent expiring in January 2025 and a formulation patent with specific excipient limitation expiring in Dec 2029. In such a scenario, generic player need to evaluate the blocking date before selection of product. In this case, product patent is expiring below NCE exclusivity and therefore NCE exclusivity

will be blocking instead of product patent. Formulation patent is having a specific excipient limitation, which can be easily design around; hence not considered as blocking. Orphan exclusivity will be blocking only for second indication for which orphan exclusivity is awarded but generic can market the product for first approved indication.

3. European Union overview

In 2005, the EU data exclusivity directive[9] was brought into force under which, pharmaceutical companies may receive up to 11 years of exclusivity for new drugs. In 2000, The EU orphan drug regulation[10] became effective and under which pharmaceutical companies may receive up to 12 years of exclusivity for orphan designated drugs.

A. EU Data Exclusivity "8+2+1"[11]

As per revised European Legislation, now new term of protection period is of '8+2+1' which is applicable only to new molecule entity for which the application for marketing authorisation approval has been submitted as of 30 October 2005 for MRP (Mutual Recognition Procedure), DCP (Decentralised Procedure) and national procedures and as of 20 November 2005 for centralised approval procedure.

Exclusivities of European Union which is now represented as "8+2+1" can be classified as 8 years of data exclusivity, 2 years of marketing exclusivity and additional 1 year of marketing exclusivity for approval of significant new indication if new indication approved during the first 8 years of authorisation. During first 8 years after the date of notification of the authorisation of the reference medicinal product to the Marketing Authorization Holder (MAH), no any generic or hybrid application can be filed or regulatory authority will not accept any generic or hybrid application during first 8 years. After 8 years, regulatory body will accept the generic or hybrid application, review it and provide approval after all regulatory requirements are complied. Under the provision of market exclusivity, generic or hybrid application holder cannot market their products before expiration of 10 years. Further 1 years of additional indication exclusivity will be applicable only if new indication has been approved during first 8 years and in that case, generic or hybrid applicant cannot market their product with that indication until expiry of that 1year exclusivity.

B. Orphan Drug Designation

Orphan drug designation[12] is typically for a disease category which his affecting less number of patient or a rare disease which is not commonly occurring. If any drug or medicine qualifies below criteria than it is eligible to be designated as orphan:

- Drug or medicine must be used for the treatment, prevention or diagnosis of a life-threatening disease and that prevalence of the disease in the EU must not be more than 5 in 10,000 or if drug is launched in the market then it

would generate insufficient return with respect to drug development cost.

- No satisfactory method of diagnosis, prevention or treatment of the disease is authorized in the European community or if a method exists, the drug or medicine must prove that it provides significant benefit than existing method.

Pharmaceutical companies must submit an application for orphan designation at any time prior to an application for marketing authorization. Committee for Orphan Medicinal Products (COMP) of EMA will examine the application for orphan designation. COMP must have to give its opinion within 90 days. Opinion of COMP sends to the European commission, which is responsible authority to grant orphan designation. As incentive, ten years of market exclusivity grants to orphan designated medicine by the European Union (EU).The marketing exclusivity for orphan drug can be extended by additional 2 years if pharmaceutical companies conduct specific studies in accordance with pediatric investigation plan.

The regulatory authority will not accept any generic or hybrid application and even an application to extend an existing marketing authorization until the expiry of orphan drug exclusivity that is 10 years. The authorised generic/hybrid product can only be placed on the market 10 or 12 years after expiry of the market exclusivity period applicable for the reference medicinal product.

In certain exceptional case, marketing authorisation may be granted, for the same therapeutic indication, to a similar medicinal product if:

a) consent is given by original orphan designation holder, or;

b) original orphan designation holder is not able to provide sufficient quantities of drug against the requirement of products in market, or;

c) the second application is safer, more effective or otherwise clinically superior in then the first orphan approved product.

C. Pediatric Exclusivity[13]

Pediatric exclusivity is awarded to encourage pharmaceutical companies to do clinical trials for pediatric populations. A pediatric investigation plan (PIP) is a designed and developed with aim of generating and creating data pool of clinical data in children to support an approval of medicine for children. Results of studies in children have to be included in all applications for marketing authorization for new medicines as per agreed PIP. This requirement also applicable even for a marketing authorisation holder (MAH) who wants to develop any new indication, pharmaceutical form or route of administration for an already approved pharmaceutical product and protected by intellectual property rights. Proposals by pharmaceutical companies should be

submitted for PIPs to the European medicines Agency's pediatric committee (PDCO). The committee is responsible for approval or refusal of the PIP. PDCO may grant PIP deferrals for some medicines in which an applicant can demonstrate its safety and efficacy in adults first and then can develop medicine in children. PDCO may also grants waiver for some medicines when there is no requirement to develop a medicine for children.

If pharmaceutical companies fulfil the requirement of pediatric investigation plan then applicants may benefit from following incentives:

- Product with marketing authorisation across the EU is eligible for six months of extension to their supplementary protection certificate (SPC). This extension is irrespective of the result of study means even if result is negative, six months extension is provided.
- If product is with Orphan designation, it is eligible for an additional two years of market exclusivity.
- If any drug is already approved for adult only, developing the same drug or medicine specifically for children that are already authorised but are not protected by a patent or supplementary protection certificate are eligible for a pediatric-use marketing authorisation (PUMA). If a product is approved under PUMA, such product will get 10-year period of market protection, including 8-year period of data exclusivity as an incentive.

Hypothetical case study: To understand it, consider a case where product is having Data exclusivity expiring in Dec 2025, Market exclusivity expiring in Dec 2027. The same product is also protected by product patent expiring in Jun 2028 and a formulation patent with specific excipient limitation expiring in Dec 2029. In such a scenario, generic player need to evaluate the blocking date before selection of product. In this case, product patent is expiring after Data exclusivity and Market exclusivity. Therefore Product patent blocking for market entry. Formulation patent is having a specific excipient limitation, which can be easily design around; hence not considered as blocking. However generic player can file dossier after expiry of Data exclusivity but can not market before expiry of Market exclusivity. Unlike in USA, here generic player will get approval though product patent is live and expiring in future.

4. Conclusion

It is very critical to understand the regulatory exclusivities available in the united states of America and European union to get optimum financial benefits and return of research and development cost associated with drug discovery and development. Pharmaceutical companies have to develop strategy as early as possible on regulatory exclusivities based on their intellectual property rights profile of their medicines. As early strategy

provide insight of timing of generic competition available in the markets, on that basis innovator can maximize its use of regulatory exclusivity by available additional exclusivity like CI exclusivity and pediatric exclusivity to further restrict generic competition in the markets. Hence understanding the regulatory exclusivity scenario along with patent scenario will help to take all business decisions like identification of product, starting of product development and evaluation of business case.

References:

1. See 21 CFR 314.108(a).
2. See 21 CFR 314.108
3. See 21 CFR 314.108(b)(2)
4. See 21 C.F.R. § 314.108
5. See 21 U.S.C. § 505(c)(3)(E)(iii),(iv)
6. See generally U.S.C. § 360aa-360dd; see also 21 C.F.R. Part 316.
7. Best Pharmaceuticals for Children Act of 2002 (BPCA), PUBLIC LAW 107–109
8. See https://www.fda.gov/drugs/development resources/written-requests-issued
9. Directive 2004/27/EC, amending Article 10 of Directive 2001/83/EC
10. Regulation (EC) No 141/2000 of the European Parliament

European Medicines Agency procedural advice for users of the centralised procedure for generic/hybrid applications EMEA/CHMP/225411/2006

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ARTICLE

LIPID MICROSPHERES: EMERGING TRENDS IN PHARAMCEUTICAL TECHNOLOGY

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Abstract:

Recent advances in the isolation techniques, medicinal chemistry, biotechnology and the capacity for screening new drug compounds have been able to produce several lead molecules. More than likely many potential drugs have lost because of inadequate formulation strategies during different stages of evaluation. In many cases, the loss could have been due to solubility problems. To maximize the drug potential of all molecules, there has been a renewed effort to find formulation strategies for compounds inadequately soluble in water. Many different drug delivery systems for lipid soluble compounds have been suggested, but lipid microsphere (LM) drug delivery system has great potential. The system is time tested, safe and stable at room temperature, easily mass- produced yet cheaper. The drug distribution and targeting can be achieved by manipulating size, surface charge and surface legands of LM. At present, commercially available lipid microsphere formulation are few but in future this system will find considerable share in the market.

Keyword: Lipid microsphere (LM), Solubility, surface charge and surface legands.

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Introduction

The past 30 years have seen enormous activity in drug delivery and targeting research, and in particular the use of colloidal drug delivery systems, for vascular, subcutaneous, intramuscular and oral administration. A wide range of colloidal materials have been studied, including polymeric microspheres and cross-linked proteins such as albumin, which can be used to prepare solid particles of varying sizes. The production of these particles is often complex, involving processes such as solvent evaporation, coacervation and removal of untrapped drug. In contrast, emulsion delivery systems have many advantages. They can be prepared easily in a single dispersion step; for correctly chosen drug candidates, entrapment can be high, and they can be easily administered in liquid form without the need for reconstitution by the user.

Triglyceride lipid emulsions have a number of additional advantages. They have been studied widely for parenteral feeding, and consequently their preparation, stability and biopharmaceutics are well understood [1-10]. The droplet size of triglyceride emulsions normally lies in the range 200-400 nm, and consequently they are suitable for intravenous administration. The most significant drawback of emulsion delivery systems is that they cannot easily be adapted to the delivery of water-soluble drugs. Oil-soluble drugs can be dissolved in the oil phase of the emulsion, and surface-active drugs can be adsorbed to the emulsion surface; however the only viable approach to the delivery of a hydrophilic drug is to synthesize a hydrophobic prodrug, or analogue. This approach has been used, for example, to formulate emulsions containing hydrophobic mitomycin C derivatives. [11]

Drugs such as prostaglandins [12,13], Cytokines and many active peptides show strong physiological activities, develop a local effect in the body and are rapidly metabolized. Therefore, a large dose of drug must be administered. As a result, the reaction spreads throughout the entire body, causing adverse reactions to develop [14]. Local adverse reactions, such as pain and inflammation at the administration site, are also observed. In order to overcome these problems, pharmaceutical devices such as drug delivery systems (DDS), particularly targeted delivery, need to be developed. Many studies of pharmaceutical devices have been carried out to develop drug carriers that are suitable for delivering a drug to the injured site. These pharmaceutical devices have been studied on delivery using liposome [15-17], polysaccharide-coated liposomes [18,19], micelle- forming polymers [20,21] and a virus [22]. As a result, prolongation of the effect, as well as the safety of a drug, has been improved with liposomes. However the sterilization that is essential for the injections and the stability of the emulsion on the shelf has still to be improved with liposomes. Furthermore, the safety of the raw materials used for the drug carrier

needs to be improved further, especially for toxicity problems such as the hemolytic property. Considering these problems, the lipid microsphere (LM), formed by dispersion of the LM particles in aqueous solution, appears to be a safe and excellent drug carrier [23-29].

Since LM was first used as a DDS pharmaceutical around 1970. Jeppsson applied it to barbituric acid [30], and further applications to nitroglycerin and cyclandelate [31] were examined [32]. Reviews of its use with pharmaceuticals have been reported by Collins-Gold et al. [33]; on its metabolism by Skeie et al. [34] and Illum et al. [35]; and on its clinical application by Mizushima [36,37] and by Mizushima and Hoshi [38]. Figure 1 shows morphology of LM. The effect of LM is characterized by the passive targeting of the drug into the disease site, such as inflammatory and arteriosclerotic lesions, with much clinical efficacy [39, 40], such as relieving the adverse effects of the drug. Prostaglandin E_1, (PGE_1) [41], dexamethasone palmitate (corticosteroid) [42-45] and flurbiprofen axetil (nonsteroidal sedative) are presently the only drugs available as LM pharmaceuticals on the market. Lipo-PGE_1, which is a LM formula of PGE_1, shows a stronger pharmacological effect than PGE_1 [46-48] and its clinical efficacy has been confirmed in the treatment of peripheral vascular disorders [49, 50], cold hands and feet [51], sensory disturbances, pain, and ulcers resulting from collagen diseases [52]. Lipo-PGE_1 is known to have reduced side effects, such as local irritation near the site of injection following parenteral administration. Regrettably, the pharmaceutical and pharmacokinetic factor, which is responsible for the high efficacy of Lipo-PGE_1, has not been found [53-57]. This problem must be solved if new LMs are to be developed by screening enclosed drugs, which have high targeting efficacy. This review describes, from the pharmaceutical point of view. LM as a DDS pharmaceutical for injection. [1]

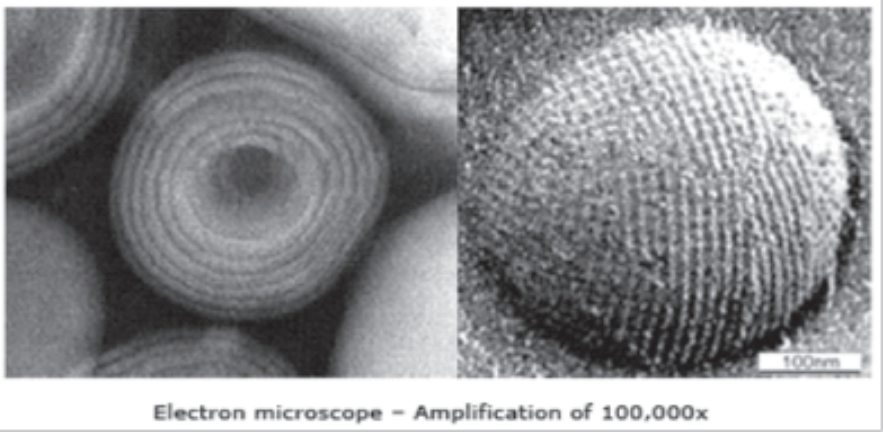

Fig. 1: Morphology of Lipid Microspheres

Advantages of lipid microspheres

- Control and/or target drug release.
- Improve stability of pharmaceuticals.
- High and enhanced drug content (compared to other carriers).
- Feasibilities of carrying both Lipophilic and hydrophilic drugs.
- Most lipids being biodegradable, SLNs have excellent
- Biocompatibility.
- Water based technology (avoid organic solvents).
- Easy to scale-up and sterilize.
- More affordable (less expensive than polymeric/surfactant based carriers).
- Easier to validate and gain regulatory approval.

Liposomes & lipid microspheres

Liposomes have been widely studied as a drug carrier of both water soluble and lipid soluble drugs, since they have both lipid and water layers. Liposomes are reported to be easily taken up by some inflammatory cells, as well as by the reticuloendothelial system (RES). Due to these characteristics, liposome-entrapped anti-inflammatory steroids show stronger activity than an equivalent dose of free steroids, both in humans and in animals. Intra-articular injection of liposomal hydrocortisone was more effective than free hydrocortisone in experimentally induced acute arthritis of rabbits [58]. De Silva et al. [59] reported a similar effect of local liposomal hydrocortisone in the treatment of rheumatoid arthritis.

The problems with liposomal treatment so far may be attributed to the instability of liposomes and a lack of clinical experience. Clinical trials of liposomes have been restricted to local application. However, liposome-entrapped amphotericin B has been developed and was recently marketed in Europe. On the other hand, lipid microspheres are very stable and easily mass-produced, as described above.

To transport a large amount of drugs, suitable carriers are needed. Liposomes are excellent drug carrier vehicles for Drug Delivery Systems (DDS) [60-62]. However, liposomes are relatively unstable and are not easily mass-produced. Lipid microspheres, with an average diameter of 0.2 μm, and consisting of soybean oil and lecithin, however, are widely used in clinical medicine for parenteral nutrition (intralipid, etc.). Lipid microspheres, themselves, are very stable and can be stored for up to two years at room temperature. They have no particular adverse effects, even at dose levels of 500 ml. Regarding their distribution in the body, lipid microspheres, like liposomes, accumulate in inflamed tissues and other lesions [63,64]. Table 1 shows comparision between liposomes and lipid microspheres and Table 2 shows generalised formulation structure.

Table 1: Comparison of lipid microspheres and liposomes3

	Lipid microspheres	Liposomes
Components		Lipid. Water
Lipid membrane	Monolayer single membrane	Mono- or multi-layer double membrane
Emulsion form	O/W	W/O/W
Incorporable drugs	Compounds soluble in soybean oil retainable in lipids.	Water soluble compounds and and compounds retainable in lipids.
Particle diameter	200 - 300 nm	Various sizes
Safety in vivo	Clinically used as intravenous	Toxic
Large-scale Large-scale	Suitable for mass production but a high pressure apparatus is necessary	A special apparatus is not necessary at the research level. An apparatus for mass has recently been developed.
Stability	nutrition in 100 ml doses Stable for 24 months at room Lipo preparations are stored at 4°C	Rather unstable temperature.

Table 2: General Formulation consideration of Lipid microspheres

CATEGORY	EXAMPLES
Oil phase	Soyabean oil, Sunflower oil, sesame oil, cotton seed oil, coconut oil,, fish oil, corn oil, Walnut oil, medium and long chain triglycerides
Emulsifiers	Soy lecithin, Egg lecithin, Phosphatidyl choline, thanolamine
Co-emulsifiers	Tween-80, Span- 60, Span-65, Myrj 52, Pluronic F-68
Stabilizers	Polyetylene glycols, poly propylene glycols, polyglycerol mono oleate.
pH adjusting agents	Oliec acid, Linoleic acid, Stearic acid, palmitic acid and their sodium and potassium salts.
Agents for adjusting isotonicity	Glycerine, sorbitol, Xylitol, Dextrose
Preservative	Benzoic acid,sorbic acid, para amino benzoic acid, methyl paraben, propyl paraben
antioxidants	Ascorbic acid, Alpha tocopherol, Butylated hydroxy anisole.

General method of preparation of Lipid microspheres

The drug to be entrapped in lipid microspheres is first dissolved in soybean oil and then emulsified with lecithin using a Manton-Gaulin homogenizer [65,66]. Since lipid microspheres are delivered preferentially to, and accumulate at, the site of inflammatory lesions as liposomes, corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) have been incorporated in them. Figure 2 shows schematic presentation of LM formulation.

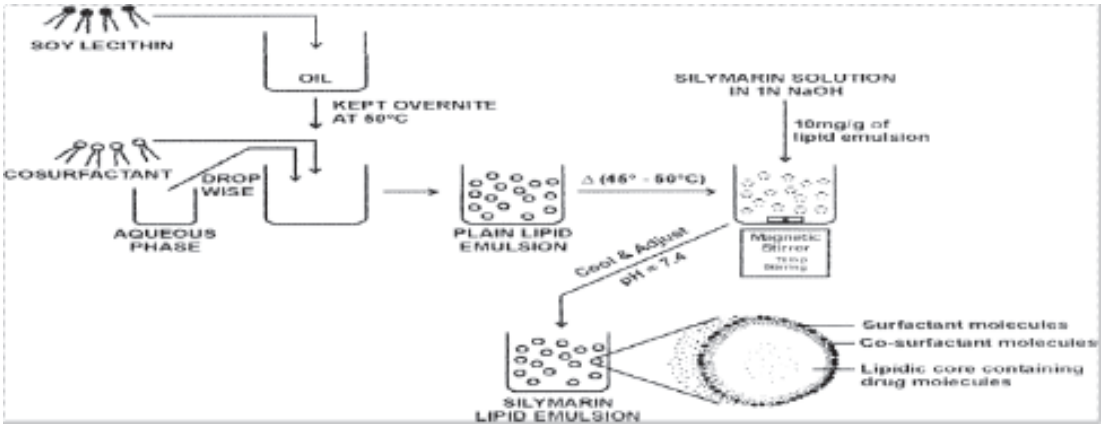

Fig. 2: Schematic presentation of lipid microspheres formulation.

Soyabean lecithin was dispersed well in soyabean oil and allowed to dissolve completely by keeping it overnight at 50^0C. The cosurfactants (hydrophilic/lipophilic) were added to the oily or aqueous phase as per their solubility. The aqueous phase was added to the oily phase dropwise with simple agitation to obtain lipid emulsion. Silymarin solution (10% w/v in 1M sodium hydroxide) was added to the prepared emulsion so as to obtain the final concentration of 10 mg/g of lipid emulsion at a temperature of 50^0C under stirring with a magnetic stirrer. After 10–15 min, the pH was adjusted to 7.4 with 1 N orthophosphoric acid [67].

The phospholipids, or lecithins, used in the preparation of most parenteral emulsions are quite heterogenous materials, being mixtures of phosphatides. A number of different factors affect the physical behaviour of these materials and these have been reviewed by Cevc and Marsh [68]

i) The saturation of the acyl chains influences the transition temperatures and the surface behaviour, both in bilayers and in monolayers. Generally, disaturated lipids have transition temperatures above room temperature (e.g. 42°C for dipalmitoyl phosphatidylcholine, DPPC), and introducing unsaturated bonds causes a reduction in transition temperature (e.g. - 8°C for dioleyl phosphatidylcholine, DOPC). These temperatures apply to the melting of

hydrated bilayers and similar phenomena are seen in lipid monolayers, which undergo solid-liquid type isotherm transitions at ambient temperatures. The extension of these phenomena to the stability of emulsions stabilized by solid- or liquid phase phospholipids is unclear; anecdotal evidence suggests that saturated phospholipids produce very stable emulsions. The majority of phospholipids from egg or soya lecithin, the commonest sources, are unsaturated, and these materials are widely used as emulsifiers for intravenous use. The degree of unsaturation is normally expressed as an iodine number; 80 is typical for egg lecithin. Figure 3 shows various types of phospholipids structures.

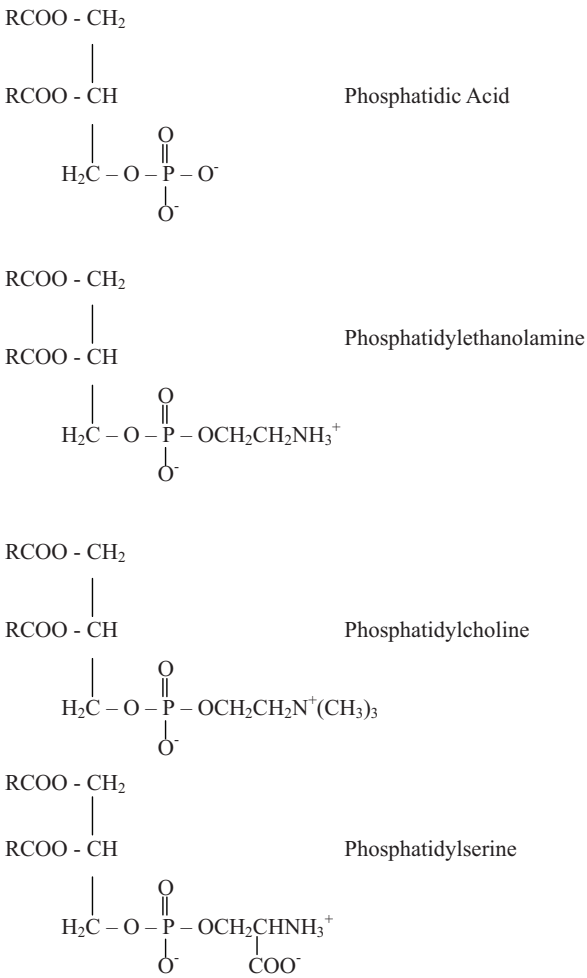

Fig. 3: Phospholipids structures; R is an alkyl chain from 8-24 units long.

ii) The length of the acyl chains influences the transition temperature, with longer chains causing higher melting temperatures (e.g. DPPC, 42°C; Distearoyl, PC 56°C). The majority of natural lecithins have chain lengths from fourteen to twenty carbons, with sixteen to eighteen being predominant.

iii) The nature of the headgroup is important in determining the ionization behaviour of the lipid. The predominant headgroups are choline and ethanolamine, which produce phosphatides (PC and PE) that are neutral at pH 7. Serine and glycerol produce acidic lipids (PS and PG), which are negatively charged at pH 7, as is the unesterified phosphatidic acid, PA. These acidic lipids are present to a much lower extent than PC and PE in natural egg and soy lecithins, and typically comprise l-2% of the total bulk. Despite this, they are of major importance in determining the stability of the emulsions prepared from them. Their concentration is sufficient to confer a surface charge of - 40 to – 60 mV on the emulsion droplets, which results in a long-term stability of several years [69-71].

Because of the extensive interest in phospholipids as emulsifiers, and for use as liposomes for drug delivery, a wide range of lecithins is commercially available. These are produced in a number of ways; crude egg or soy lecithin can be refined chromatographically, removing sterols, lysolipids and a range of other contaminants, to produce a parenteral grade of lecithin, such as Lipoid® E80 (Lipoid® AG) or Ovothin® 180 (Lucas-Meyer). These materials are almost fully unsaturated and contain approximately 80% PC; a small amount of acidic lipid leads to a high surface charge at pH 7, and hence, to excellent emulsion stability. Further chromatographic purification leads to a product which is 95-100% pure PC or PE (e.g. Lipoid® El00 or Ovothin® 200), and has very little acidic impurity; this has a low surface charge at pH 7 and thus is not per se a useful emulsifier.

To produce saturated lipids, these materials can be hydrogenated catalytically, which leads to an iodine number of 2-5, compared to 60-80 for the unsaturated lipid. Thus, Lipoid® El00-3 is a nearly pure hydrogenated PC, with a very low level of charged lipids, and Lipoid® E80-3 is a similarly hydrogenated lipid, with a higher level of acidic lipids.

These materials still have heterogenous chain lengths and some variations in headgroups. Pure individual lipids with well-defined chains cannot easily be produced by further fractionation, due to the large number of separate species in the raw feedstock. There are two possible routes to pure phospholipids; one is total synthesis and the other is the use of phospholipases to selectively transesterify chains. Phospholipase Al and A2 can be used to selectively cleave and replace the

acyl chains, while phospholipase D can modify the phosphate ester group. This process is, however, expensive, and the resulting pure lipids cost £20-80 per gram, making them of limited value for the preparation of large volume parenterals. However, they are commercially viable for the preparation of high added value drug emulsions, which normally have a small volume.

Tissue distribution/ Factors influencing the biodistribution of Lipid microspheres

Earlier studies [63,72] of lipid emulsions demonstrated that lipid microspheres had an affinity to vascular walls (including capillaries), similar to that of chylomicrons. Shaw et al. [73] reported that they had more affinity to vascular walls at inflamed sites. As described by Dr. Igarashi in this issue, we found that lipid microspheres accumulated in damaged vascular walls, such as atherosclerotic vessels and vessels of hypertensive rats.

Stability of emulsion in LM

The stability of colloidal formulations is paramount in their use as drug delivery systems. They must be sufficiently stable to be easily manufactured, sterilized (preferably by terminal autoclaving) and have a shelf life of at least a year, preferably more. Shelf life considerations are particularly important for emulsion systems. Since these are generally stored as liquids, and coalescence or aggregation can occur during storage. In contrast, microparticle colloidal systems are stored as freeze-dried solids for reconstitution, and colloid stability problems do not arise in the same way (although that is not to say that such formulations do not degrade physically during storage). There have been limited attempts to store emulsions in other forms, for example, the perfluorocarbon emulsion Fluosol [12-14] is stored frozen (a process which generally causes extensive emulsion coalescence in other systems), and there have been attempts to freeze dry emulsions.

In addition to long-term stability, it is also necessary to understand the way that colloid stability may be influenced by the various additives that may be needed in the final clinical application. The most important such interaction is that with electrolytes, which may be required to adjust tonicity. Electrolytes interact strongly with charge-stabilized colloids, causing severe stability difficulties; for this reason, the tonicity of emulsions is generally adjusted with glycerol. The interaction of electrolytes with phospholipid- stabilized emulsions is quite well understood, since it has been extensively studied due to its importance in the compounding of parenteral nutrition mixtures [15-19].

The stability of colloids depends on the forces between them; if these are repulsive, approaching droplets repel and form a stable system. If they are attractive, the droplets clump together and are unstable. The forces themselves can arise from a number of sources [22].

i) **Van der Waals forces.** These are attractive forces which exist between all particles. Their magnitude is determined by a factor called the Hamaker constant, which describes the strength of attractive interaction between the disperse and continuous phases.

ii) **Electrostatic forces.** These are repulsive forces (assuming that all the particles have the same surface charge). They can most easily be quantified using the zeta potential, which is the potential surrounding the droplet at the plane of hydrodynamic shear. The repulsive force depends on the square of the zeta potential (i.e. its sign is irrelevant). Electrolytes modify the zeta potential by adsorbing to the droplet surface and screening the droplet charges.

iii) **Solvent forces.** These are short-range repulsive forces caused by solvent-surface interactions, and are normally only important at short droplet separations of a few nanometres (i.e. the droplets are nearly "in contact").

iv) **Steric forces.** These are much less well understood than the previous forces, but are of major importance in many colloidal systems [23]. They arise when long-chain hydrophilic macromolecules are adsorbed or grafted to the colloid surface. They are repulsive in nature, since the overlap of adsorbed macromolecules is energetically unfavourable.

In general, colloids are stabilized either by steric or electrostatic repulsion, depending on the nature of the surfactant. Lecithin-stabilized emulsions are charge-stabilized, and steric forces do not play a significant role [24]. Alternately, emulsions stabilized by block copolymers, such as poloxamers, are sterically stabilized, and charge is of minor importance. Mixed electrosteric systems have also been studied; for example, the perfluorocarbon emulsion, Fluosol, uses a mixture of Pluronic F-68 and lecithin as the emulsifier [25].

Electrolyte stability

Although most lecithin-stabilized emulsions have sufficient surface charge to produce excellent long-term stability, they interact strongly with electrolytes, a phenomenon which has considerable clinical significance. The processes occurring are well-understood and are termed the DLVO theory of colloid stability, after its developers [26,27]. Electrolytes interact with charged colloids by two mechanisms; these are termed nonspecific adsorption and specific adsorption.

Nonspecific adsorption occurs when an ion is attracted to a surface solely due to electrostatic forces. There is no chemical interaction between the ion and the surface. Such an ion is sometimes called an indifferent ion for the surface. The zeta potential falls as the electrolyte concentration is increased, and asymptotes to zero at high electrolyte concentrations. This behaviour is typical of monovalent

cations (e.g. Na^+, K^+) on phospholipid surfaces. As the zeta potential falls, the repulsive force stabilizing the colloid becomes weaker, until at a critical point it is no longer able to overcome the attractive Van der Waals force. At this point the force becomes predominantly attractive, and the colloid flocculates. The concentration of electrolyte required to achieve this is termed the Critical Flocculation Concentration or CFC. Further addition of electrolyte causes the repulsive force to decline further, so the flocculation rate increases to a maximum. The CFC is approximately 0.10 M. This description of the flocculation process is qualitative, but has been put on a quantitative basis [24,28]; The actual interdroplet potentials can be calculated and from them, the flocculation rates. The agreement of the calculated and measured flocculation rates is quite good.

Specific adsorption occurs when an ion interacts with the surface chemically as well as electrostatically. Examples of this process on phospholipid surfaces are Ca^{2+} (which complexes to the lipid phosphate headgroups) and H^+ (which is involved in acid-base equilibria) [29]. Under these conditions, more ion can adsorb to the surface than is required simply to neutralize the droplet surface charge, and so the surface can acquire a charge of the opposite sign to that which it possessed in the absence of the ion. This process is called charge reversal and is characteristic of the specific adsorption process. It was observed that the effect of increasing concentrations of Ca^{2+} on the zeta potential and flocculation rate of Intralipid. Initially the charge is neutralized in a similar manner to that observed for a nonspecfically adsorbed ion, and a CFC is seen at a calcium ion concentration of 2 mM. Further addition of calcium causes all the surface charge to be neutralized at 3-4 mM, and this point is called the point of zero charge, or PZC. At still higher calcium concentrations, the zeta potential becomes positive.

These changes in the surface potential are reflected in the flocculation rate. The CFC is observed in a manner similar to a nonspecifically adsorbing ion; however, the PZC now corresponds to a well-defined maximum in the flocculation rate. As the surface becomes charge-reversed, it regains some repulsive component to the interdroplet force and as a result, the flocculation rate falls. [4]

Physicochemical properties of LM

Table 3 shows the formulae of PC99LM and PC70LM prepared with two kinds of lecithins (PC99, PC70). PC99 and PC70 are lecithins containing 99% and 70% of phosphatidylcholine (PC), respectively. PC70 contains 17% of phosphatidylethanolamine (PE). The pHs, mean particle sizes and ξ potentials for the two LMs are shown in Table 4. The pH and mean particle sizes of PC99LM and PC70LM were the same, but their ξ potentials differed. The absolute ξ potentials of both LMs increased with increasing pH. Since variation in the pH affected the charge on lecithin, it appeared

that the negative charge on the LM particle surface became larger with increasing pH [71,72]. The absolute ξ potential of PC70LM was larger than that of PC99LM. Ishii et al. [73] reported that the absolute ξ potential of LM composed of PE was larger than that of LM composed of PC. It thus appeared to influence PE content of egg yolk lecithin, which comprises the interfacial membrane.

Table 3: Formulas for LMs

Component	PC99LM	PC70LM
PC99	18 g	-
PC70	-	12 g
Soybean oil	100 g	100 g
Glycerol	23 g	23 g
NaOH	-[a]	-[a]
Water for injection[b]	1000 ml	1000 ml

[a] Adjusted to pH 6.
[b] Adjusted to 1000 ml with water for injection.

Table 4: Properties of LMs

	PC99LM	PC70LM
pH	**6.5**	**6.1**
Mean particle size (nm)	**204**	**208**
ξ potential (mV)[a]	**-8**	**-46**

[a] Each value was obtained from measurement of the mean particle electrophoretic mobillity in a 0.5 mM KCl solution at the same pH of LM.

Theoretical analysis of flocculation and coalescence of LM

It is well established that the stability and the behavior of LM in the body are related to the surfactant used and to the condition of emulsification [71-73], but many thermodynamic studies have been carried out on the change in size, flocculation or coalescence of LM particles [79-86]. It appears to be true that the flocculation process can be analyzed with DLVO theory, in a fashion similar to that for flocculation of colloid [87-90]. However, the process of increasing the particle size cannot be understood using this theory alone, since particle destruction also occurs. Thus, flocculation and coalescence of LM must be considered separately. Now, the total energy of the increase in particle size is equal to the sum of the energy of flocculation and coalescence. We analyzed the stabilities of LMs prepared with two kinds of lecithins (PC99, PC70)

taking this into consideration [91]. Both lecithins have been used in drug delivery systems and for nutrients. Only PC99 and PC70 have been used as surfactants in commercial parenteral LMs. Unstable LM particles are the first to flocculate. By the DLVO theory, the total interaction energy V_t of the flocculation of LM is the sum of contributions of the potential energy of electrostatic interaction V_R and that of the van der Waals interaction V_A:

$$V_t(H) = V_R(H) + V_A(H) \quad (1)$$

For bulk solutions containing two kinds of electrolytes, we derived an expression for V_R between two identical spheres immersed in a mixed solution of 1:1 and 2:1 electrolytes using the DLVO theory for colloid stability, as follows [92]

Consider first the electric potential, ψ, produced by a single plate with a surface potential, ψ_o, immersed in a mixed solution of electrolytes. Let n_1 and n_2 be the numberical densities (in units of m^{-3}) of 1:1 and 2:1 electrolytes in the bulk solution phase, respectively. Let the x-axis be perpendicular to the plate, with its origin 0 at the plate surface so that the region $x > 0$ is the solution phase. The Poisson-Boltzmann equation for the electric potential $\psi(x)$ at position x relative to the bulk solution phase, where $\psi(x)$ is set equal to zero, is then given by

$$\frac{d^2\psi}{dx^2} = -\frac{e}{\varepsilon_r\varepsilon_0}\left[n_1\, exp\left(-\frac{e\psi}{kT}\right) + 2n_2\, exp\left(-\frac{2e\psi}{kT}\right) - (n_1 + 2n_2)\, exp\left(\frac{e\psi}{kT}\right)\right] \quad (2)$$

where e is the electric unit charge, ε_r, is the relative permittivity of solution, ε_0 is the permittivity of vacuum, k is the Boltzmann constant, and T is the absolute temperature.

Consider next the potential energy $V_R(H)$ of the electrostatic interaction between two identical spherical particles of radius, a, at separation, H, between their surfaces. With the help of Derjaguin's approximation [93], we can obtain an expression for $V_R(H)$ from the corresponding expression for the interaction between two parallel plates. viz.,

$$V_R(H) = \pi a \int_H^x V_{pl}(h)dh = \frac{64\pi a(n_1 + 3n_2)\gamma^2 kT}{k^2}\exp(-kH) \quad (3)$$

With

$$\gamma = \frac{1}{(1-\eta/3)}\,\frac{[(1-\eta/3)\,\exp(y_0)+\eta/3]^{1/2}-1}{[(1-\eta/3)\,\exp(y_0)+\eta/3]^{1/2}+1} \quad (4)$$

$$\eta = \frac{3n_2}{n_1+3n_2} \quad (5)$$

Where y_0 is the scaled surface potential and x is the Debye-Huckel parameter.

V_A can be calculated as:

$$V_A = -\frac{Aa}{12H} \quad (6)$$

where A is the Hamaker constant.

Thus, from Eq. (3) and Eq. (6), Eq. (1) can be derived. Two LM particles are flocculated when the maximum in the potential curve $V_t = V_R + V_A$, i.e., the potential barrier, disappears.

Next, two LM particles that have undergone flocculation, become one particle by coalescence, and the volume increase. It has been reported that the following equation can be used to predict the rate of increase in LM particle volume [94]:

$$\frac{dv}{dt} = \frac{4kT}{3\eta} \boldsymbol{\phi} \exp(-E/\mathrm{R}T) \quad (7)$$

where v is the mean volume of a particle, η the viscosity of the bulk solution, $\emptyset$ the volume fraction of bulk solution and R is the gas constant. Therefore, the activation energy, E, which includes the energy for destruction of the interfacial membrane, in addition to the maximum total interaction energy (V_t^{max}) as the energy barrier, can be obtained from the plot of the rate of increase in particle volume

Influence of steam under pressure sterilization (SUPS) on mean particle size

Parenteral injections must be sterilized. We tested the sterilization of PC99LM and PC70LM by SUPS. SUPS did not change the pH of PC99LM, but did increase its mean particle size to 320 nm. On the other hand, neither the pH nor the mean particle size of PC70LM was affected by SUPS. The values of V_t^{max} at 121°C were calculated for LM using Eq. (1), by substitution of ξ potentials and mean particle sizes in Table 3 and by the Hamaker constants (PC99LM, 1.4×10^{-22}J; PC70LM, 3.1×10^{-21}J) obtained in a previous study [95]. In order to obtain the relationship between ξ potential and temperature, the ξ potentials of LMs were measured over a range of temperatures. Since the ξ potential of LM was little affected by temperature, the ξ potential at 25°C was used to calculate of V_t^{max} at other temperatures. The values of V_t^{max} for PC99LM and PC70LM at 121°C obtained in this fashion were 4.7 kT and 1.51 kT, respectively. Since the value of V_t^{max} for PC99LM was quite small, PC99LM appeared to undergo flocculation readily.

On the other hand, since the value of V_t^{max} for PC70LM was very large, the energy for flocculation was large and PC70LM did not readily undergo flocculation.

The effect of pH on change in particle size was studied using SUPS. PC99LM and PC70LM were adjusted to various pHs with 0.1 *M* KOH or 0.1 *M* HCl, and the ratios of changes in mean particle sizes, R_p, was obtained as:

$$Rp = \frac{mean\ particle\ size\ after\ SUPS}{mean\ particle\ size\ before\ SUPS} \quad (19)$$

The values of R_p, for PC99LM increased as the pH decreased below pH 8.0, but the mean particle size changed little at values of pH above 8.0. On the other hand, the value of R_p for PC70LM was approximately 1, at values of pH above 4.0, and the mean particle sizes increased very little at pH 4.0 or below. The ξ potentials of these LMs at 121°C were obtained from Fig. 6 by interpolating to pH of the LM, and the values of V_t^{max} at 121°C were calculated from Eq. (12).

Using these values, the relationships between R_p and V_t^{max} for PC99LM and PC70LM were determined. Both R_p values increased as V_t^{max} decreased, when V_t^{max} was less than 15 *kT*. The mean particle sizes of PC99LM and PC70LM increased, since values of

V_t^{max} were below 15 *kT*. These findings indicated that various LMs flocculate easily if their V_t^{max} values are less than 15 *kT*. This is "slow" flocculation, which differs from the "fast" flocculation

that occurred in the absence of the potential barrier. Values of V_t^{max} above 15 *kT* were obtained above pH 8.0 and above pH 4.0 for PC99LM and PC70LM, respectively. These findings demonstrated that the stability of various LMs could be evaluated using this method.

Reviews of lipid microspheres or drug studied for lipid microsphere drug delivery

The presence of an additional component in the emulsion can considerably complicate its behaviour. Fortunately, the effect of the drug can often be predicted (at least in terms of DLVO type emulsion stability) with knowledge of the physicochemical properties of the drug. For the purposes of emulsion formulation, we can classify drugs on the basis of their solubility characteristics and their ionization properties which, of course, are not completely independent.

A) **Class I:** drugs are largely water-soluble. In an emulsion, a significant fraction of the drug would be present in the aqueous phase, and consequently such drugs are not good candidates for emulsion formulations.

B) **Class II:** drugs are predominantly oil-soluble, and in an emulsion would be partitioned

predominantly into the oil phase. Such materials are good candidates for emulsion delivery and could normally be formulated by dissolving the drug in the oil prior to emulsification.

C) Class III: drugs are poorly soluble in both water and oil, and can only be loaded into an

emulsion by adsorbing to the droplet interface. They may require complex formulation methods due to their unusual solubility characteristics, and it is often necessary to post-load emulsions with them, in order to have a large surface area available for loading.

This is naturally an artificial classification, and boundaries between these areas are indistinct, although many drugs do fall clearly into these classes. The classification of a drug can be changed by chemical means; for example, a hydrophobic (class II) base such as chlorpromazine can be converted into its class I hydrochloride salt. Formulation chemists often do this to increase the water solubility of a drug; emulsion chemists normally want to reverse this process! The boundary between classes I and II cannot be assigned to a particular log *P* value, although we would suggest that log $P > 4$ would be a useful starting point. A particular problem can arise if the drug has a very slight water solubility and a high oil solubility, since partition can cause the water solubility to be exceeded and the drug will crystallize out into the aqueous phase. The oil phase will dump its drug load as crystals in the aqueous phase until the aqueous phase solubility is not exceeded.

The class II-III boundary is also often indistinct, since most drugs have some surface activity due to polar or ionized groups, whether strongly oil-soluble or not. Consequently we can usefully subclassify drugs into types a, b and c.

Type a drugs are weak bases and thus are positively charged at the formulation pH.

Type b drugs are weak acids and are negatively charged at the formulation pH.

Type c drugs are uncharged at the formulation PH.

The charge on type a and b drugs leads to surface activity, and so these drugs will normally adsorb, at least partly, to the droplet interface. In doing so, they will contribute some surface charge, which will influence the emulsion stability. Type c drugs may also adsorb to the interface (if, for example, they contain hydroxyl groups) but will not normally influence surface charge. Of course this classification is only valid at a particular or specified pH, which suggests that pH is a major tool to control this aspect of drug behaviour.

Evaluation of Lipid Microspheres:

Various evaluation parameters are done for Lipid Microspheres. Drug loading, % entrapment efficiency, drug content etc., are common evaluations parameters.

1. Structure and Morphology

Morphology and structure of lipid microspheres was determined using Transmission electron microscopy (TEM), and photomicrographs were taken at suitable magnifications.

The morphologies of the lipid microspheres were observed using a FEIQuanta 250 SEM (FEI Corporation, Hillsboro, OR, USA).

2. Size, PDI, and Zeta Potential of Lipid Microsphere

The average particle size, PDI, and zeta potential of the lipid microspheres were measured while using a Malvern laser particle size analyzer (Mal-vern, UK). Samples were diluted appropriately with double-steamed water for the measurements, and zeta potential measurements were detected at 25 °C.

3. Stability Studies

The stability of formulation was tested by exposing them to different storage conditions, i.e., at room temperature (20 ± 5°C), in refrigerator (4°C), and at higher temperature (40 ± 5°C) for 45 days and also subjecting to repeated centrifugation (15, 30, 45, 60, 120, 180, and 240 min) at various rpm. Sustainability of the formulations at different pH (2–12) was also determined.

4. *In Vitro* Release Rate Studies

These studies were carried out through dialysis bag using dissolution apparatus USP 1. The treated dialysis membrane was soaked in diffusion medium up to 48 hr and refrigerated till use. The membrane was washed with distilled water before use. The formulations were filled in different dialysis bags and placed in basket of dissolution apparatus USP 1. The study was run at rpm specified, and the dissolution vessel was maintained at 37 ± 0.5°C. An aliquot of 5 ml of samples was withdrawn at suitable time intervals and replaced with same amount of medium to maintain the volume of dissolution medium as 900 ml. The samples were quantitated by UV spectrophotometer.

5. Drug release mechanism and Statistical Analysis

Drug release mechanism were also studies using software and Pharmacokinetic parameters of lipid microsphere were calculated by Software version 3.3.0 (Shanghai, China).

Table 5 shows various types of drugs are taken under study for lipid microsphere formulation and Table 6 shows some commercially available LM based products.

Table 5: Drugs studied for LM formulation

Drugs	Drugs
Anti tumor drugs	Profesterone
Pregnaolone	Nifedepine
Barbituric acid	Grisofulvin
Indomethacin	Propofol
Cyclandelate and nitro glycerin	Profesterone
Biphenylacetic acid	Ketoprofen
Naproxen	Cyclosporin A and cyclosporins
Tirilazad	Lidocaine

Table 6: Commercially Available LM Drug Delivery Systems

Drug	Company	Brand Name
Dexamethasone Palmitate	Green cross	Limethasone
Flurbiprofen axetil	Green cross	Lipfen
Flurbiprufen axetil	Kaken	Ropion
Prostaglandin E_1	Green cross	Lipfe
Prostaglandin E_1	Taisho	Palux
Parenteral Nutrition	Clintec, Pharmacia, Kabipharmacia	Intralipid
Diazepam	Pharmacia, Dumex	Diazemuls
Propofol	Zeneca, ICI	Dipivan

FUTURE TRENDS:

One of the major problems of LM using as drug delivery system is its rapid uptake by liver, spleen and other RES. For drug delivery purpose, it may necessary to control and modify the uptake of the droplets. Adding block copolymers of ethylene oxide and propylene oxide to stabilize an emulsion prolongs circulation time. Preparation of small particles with negative charge also improves the circulation time of microspheres. Another stratergy for avoiding rapid clearance uses phospholipids modified with PEG or addition of surfactants. Positively charged LM may be used for drug targeting to RES. A continuing challenge is to tailor surface properties of LM to achieve the desired biodistribution. Optimizing certain

characters could endow LM with targeting properties, a line of research that has been prompted with liposomes.

CONCLUSIONS

In the early days of the 20th century, Paul Ehrlich envisioned his magic bullet concept; the idea that drugs reaches the right site in the body, at the right time, at right concentration. It should not exert side effects, neither on its way to the therapeutic target, nor at the target site, nor during the clearance process. The LMs have the potential to achieve, at least partially, these broad objectives. Apart from these, the regular objective of controlled drug delivery is aptly achieved with LMs. They are relatively young drug delivery systems, having received primary attention from the early 1990s and future holds great promise for its systematic investigation and exploitation. We can expect many patented dosage forms in the form of LMs in the future.

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ARTICLE

AN EPITOME ON TUBERCULOSIS: A PHASE OF DRUG DISCOVERY TO NANOTECHNOLOGY

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Abstract

Mycobacterium tuberculosis is a chronic infectious disease. It kills approximately 1.7 million people in the world and also has the highest risk of reactivation in the patients with latent infection. Relapse of the disease and drug resistance (MDR-TB/XDR-TB) are the key parameters of too complex tuberculosis therapy. Due to this varied failure treatment on this globe has asked the researchers to identify novel targets and to intercorrelate its mechanism of action. With an ascending growth of the disease and more specifically drug-resistance has signified its emergence for new anti-tuberculosis drugs. Thus, with the prior knowledge and applying it in the drug discovery has led a convincing opportunity for the modification of current inhibitors or to develop new drugs. With the re-arrangement of recognized scaffolds is applied in its core structure which may help in the improvement of bactericidal, pharmacokinetics and pharmacodynamics activities. Along with the surveys carried out, a large number of compounds are screened against Mycobacterium tuberculosis and can be helpful later in the computer databases. The present drug discovery should include an integrative analysis with computational databases. Nanomedicine also plays an important role in the management of the disease. The nano-sensors which help in the detection of infection rate have provoked its role for the rapid and short term diagnosis. Thus, with the current literature review it can be noted that interconnecting computational chemistry with the nanotechnology diagnosis can be helpful for short term therapy as well as lesser side-effects.

Keywords: Nanosensor; dendrimers; chemoinformatics; mycolic acid; nanotechnology

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1. Introduction

The term disease resembles to the abnormal condition or construed as a medical condition that are associated with specific symptoms and signs.[1] With large numbers of increase in bacteria/micro-organisms have caused mankind to global threats. Tuberculosis is one such kind of bacterial infectious disease that has caused a global risk to mankind/manhood.1 Remaining to be one of the most neglecting disease in the world caused by the member of MTBC (mycobacterium tuberculosis complex).3 Kills about 1.7% people in the world as rated by WHO every year. It has also been evaluated that 25% of deaths occurred in HIV-positive people are due to tuberculosis.2 Despite of renovation in sanitation and also in the living conditions has decreased its level of incidence worldwide. Mycobacterium tuberculosis being a successful pathogen dormantly infects active growth of bacteria by various processes like cell wall biogenesis, chromosome replication, etc.6 Emergence in drug-resistant tuberculosis, specifically for the patients who carry latent infection are at a risk of reactivation of tuberculosis.19 Even though devising various therapies for control of bacterial MDR-TB has its highest ratio in its area.7,20 To rule over the targets a cocktail of frontline drugs (isoniazid, rifampin, ethambutol, streptomycin, pyrazinamide) are given for the first two months. Followed by long term therapies which lasts between 6 to 9 months.6 On the other hand increase in therapy leads to patient's non-compliance. Growing at an alarming phase with rise up in statistics has led WHO declaring tuberculosis 'a global emergency'.[2]

With the past decades it has been beheld where treatment is getting more difficult along with severe side-effects. Therefore, in both cases i.e. drug-resistant and drug-sensitive strains, treatment fails because of one or more drug intolerance.[3] As a result, powerful and effective new anti-TB drugs should be given with short therapy and lesser side-effects along with low cost expenditure.11 Furthermore, it is also necessary to develop collaborative approach in drug discovery. Ruling out its development of new targets in drug design has become a main question for designing of new drugs and developing a new and potential drug. To integrate its workflow in computational approaches, chemo-informatics as well as bio-informatics plays an important role. The pathways, enzymes, databases, etc are levarging at an expanding level along with workflow for anti-tubercular in drug discovery.[5] To get the potent form for anti-TB are essentially new and effective therapies are carried out. Later, nanotechnology has increased its tremendous growth for the study of disease, diagnosis of disease, treatment of disease and design of Nano-particles. Nano medicine plays an important role along with bio-sensors for the detection of bacterial strains.[4] As a consequence of it, the present summarizes on characteristics of anti-TB agents along with computational tools and triggering

nanotechnology for advancement of new and cheaper approaches which circumvents rapid and sensitive detection of main etiologic agent.[3,4,5] Thus, the stages of tuberculosis gives the description about the phase of infection in (Figure 1).

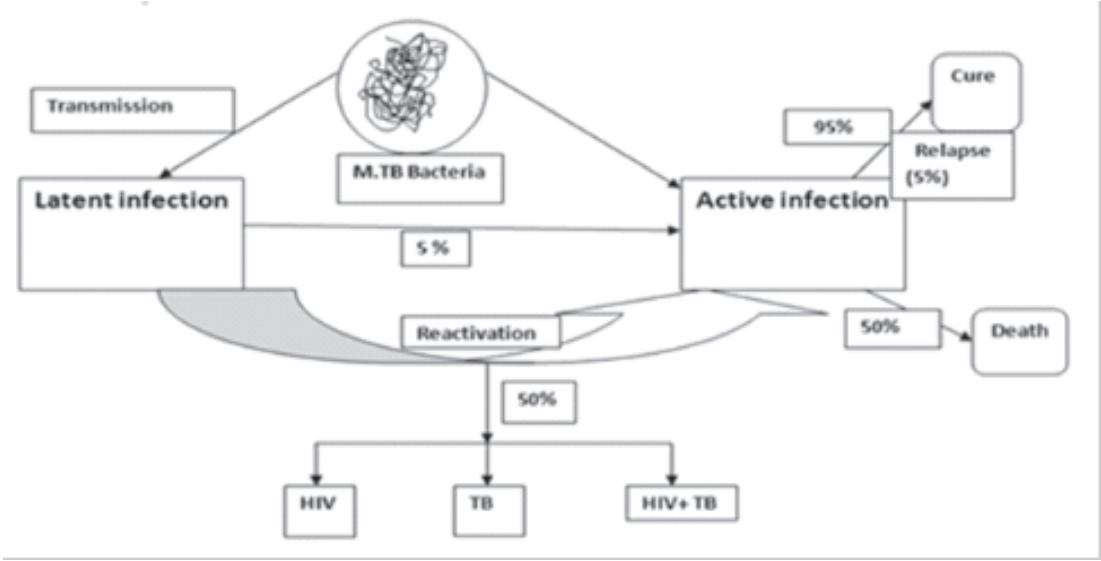


Fig. 1. Stages of Tuberculosis.[18]

2. MDR-TB and XDR-TB surveillance and its emergence

Drug resistant tuberculosis is the result of inadequate tuberculosis therapy. Due to its transmission all over the biosphere has made undetermined effects to control the global TB epidemic. With its increase in ratio on a global scale, MDR-TB as well as XDR-TB has been spread which aggrandizes its emerging level of globe.[6] The flow chart has been described in (Figure 2).

3. Current targets and its mechanism of action

The current elevation in tuberculosis cases and more potently in drug-resistant mycobacteria has indicated an emergence for an advancement of new anti-TB drugs. Because of its prolonged therapy has an upshot for persistent M.TB. They are not effectively killed by current anti-TB agents. Thus, the most happening disease has spread like a virus.5 Thus, by the use of knowledge of biology of organisms and its availability of genomic sequence has led an opportunity for the development of novel targets for drug design.5,20 Therefore, by modern approaches such as structure based drug design and combinatorial chemistry has led to development of new drugs which will not be effective in drug resistant but can also enhance short dose regimen.5,6 The chemical structures as anti-tubercular agents have been described (Figure 3). The enzymatic inhibitors are as follows:

3.1. Inhibitors of cell-wall synthesis

Isoniazid (INH) being a kind of a drug in which it requires an activation of M.TB catalase-per-oxidase (KaTG) has been aided to obtain the range of reactivate oxygen species and reactivated its organic radicals. It also helps to invade the multiple targets in tubercle bacillus. Therefore, the preliminary target is inhibition of cell wall mycolic acid synthesis pathway, where enoyl ACP reductase (Inha) was identified as a target of INH inhibition. The divergent species of Inha inhibitors are found to be isonicotinic acyl radical which reacts with NAD and forms INH-NAD complex. It also helps in inhibition of NAD metabolism. Its effect on multiple targets in tubercle bacillus is due to bactericidal activity of INH. Here, the process of mutation takes place by the involvement of INH activation, its targets and InhA and NDH-II causes INH resistance. Thus, KatG mutation plays an important role for the resistance of INH.[6]

3.2. Nucleic acid synthesis inhibition

Rifampin possessing a broad spectrum has a semi-synthetic rifamycin B derivative. Interference with RNA synthesis gets bind with bacterial DNA dependent RNA synthesis gets binds with bacterial DNA dependent RNA polymerase sub-unit encoded by rpoB. Similarly, for fluoroquinolones various derivatives were also synthesised like ofloxacin, levofloxacin, etc. And has possessed good potent activity against M.TB. Thereby, targeting DNA gyrase A and B subunits helps in inhibition. It acts on cell wall bio-synthesis and is GDP dependent process. Being a type-II DNA topoisomerase here enzymes acts as a tetramer and forms sub-units of 2A and 2B. They both gets bind to DNA molecules. The enzymes here bind to 140 base pairs, wraps at C-terminal tail domain of gyr A protein to form a positive coil. Here, sub-unit A carrier breakage reunion at active site, whereas sub-unit B promotes ATP hydrolysis. They form the A2B2 tetramer in holoenzyme and acts as a distinct function. Later, all amino acids get positively charged with two active sites tyrosine residues which are located at the centre. A region gets bind to G segment and forms DNA gate. The process of mutation takes place and leads to quinolone resistance named as QRDR (quinolone resistance determining region) gets functionally characterised by ATP hydrolysis. It gets stable reconstitute into its fragments and supercoils it by activities. Thus, M.TB develops resistance to fluoroquinolone by mutation in gyrase A and gyrase B subunit.[6]

3.2. Protein synthesis inhibition

Benzimidazole being a potent drug for an inhibition of protein on cell wall synthesis. It is ATP dependent process and is temperature sensitive mutant Z gene. Formed of cytoskeleton protein and relates to tubulin forms nucleotide exchange i.e. GDP-FtsZ monomers. The monomers are inhibited and undergoes polymerization process i.e. FtsZ-GTP monomers. These monomers get hydrolysed and

interconnected and retains its capacity forms a dynamic, restructure to fragments and anneal. Later, condense laterally developed genes.[6]

3.3. Peptide deformylase inhibition (PDF)

Bacterial peptide deformylase which belongs to mettaloproteases subfamily. Catalyses its removal of N-terminal and forms group from newly synthesised protein. Later, it was found to be reported that both the synthesis and bio-activity occurs of highly potent inhibitors. The SAR and crystallographic data clarified its required highly potent enzymes. Thus, developing potent inhibitors of H37Rv and MDR TB strains can be helpful to it.[6]

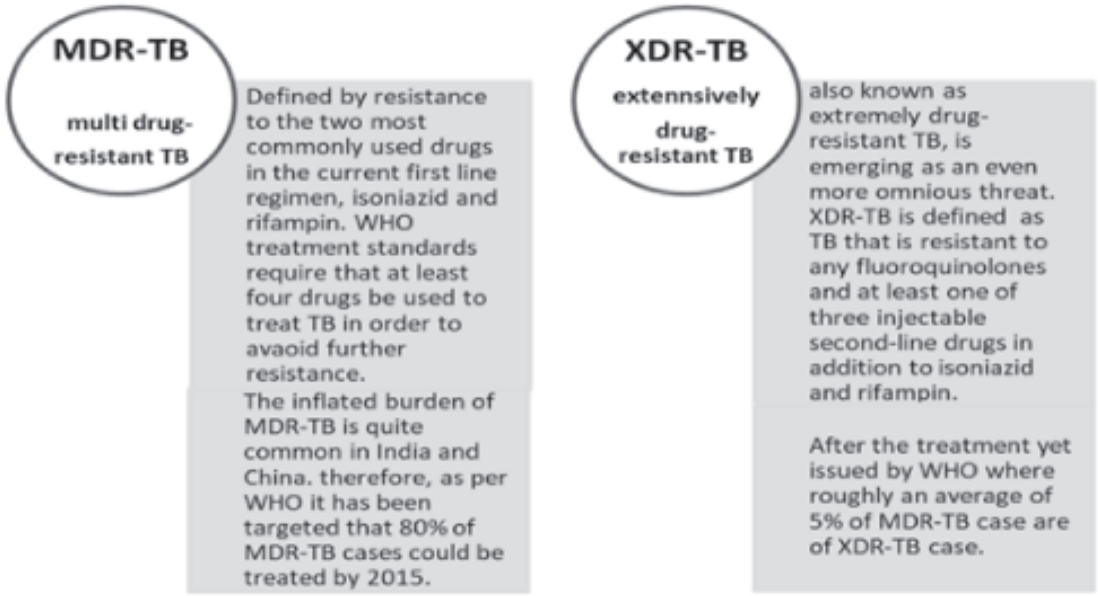
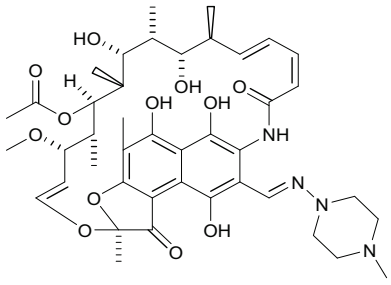
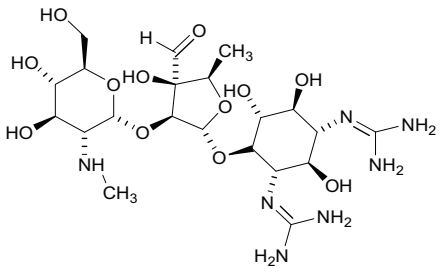


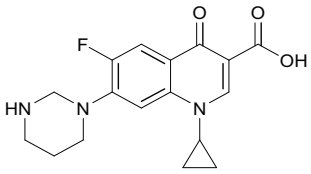
Fig. 2. Flow chart of MDR-TB and XDR-TB21



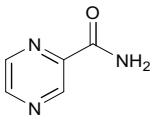
Rifampicin



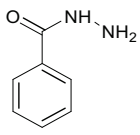
Streptomycin



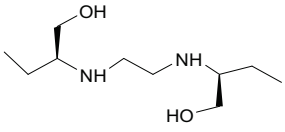
Ciprofloxacin



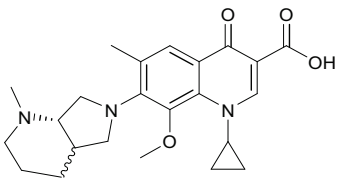
Pyrazinamide



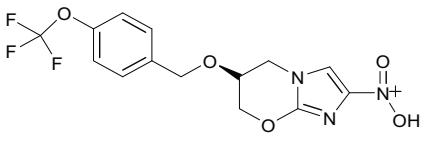
Isoniazid



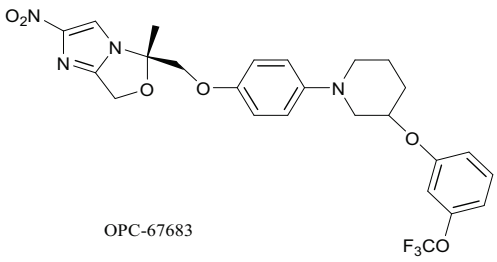
Ethambutol



Moxifloxacin



PA-824



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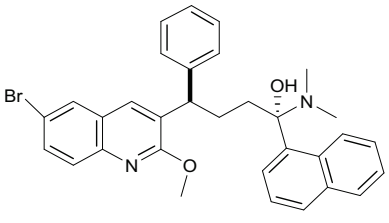

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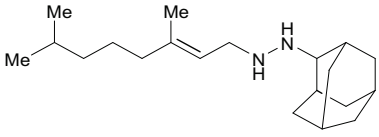

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Fig. 3. Chemical Structures of Anti-Tubercular Agents.

4. Tuberculosis: its challenge in drug discovery

Tuberculosis being a chronic disease which results into infection from M.TB is growing at a slow rate. Having its complex therapy now-a-days has become a threat for the patients acquiring with this disease. Therefore, the discovery of new targets or modifying existing targets with shorten duration of action has become a big challenge for the researchers. Earlier were the days when patients with TB and HIV where resistant strains took place. Here it included both MDR as well as HIV induced reactivation occurred. The treatment used to fail because of resistance effects. Now-a-days due to change in lifestyle, effective for both the patients (MDR-TB/ XDR-TB) and (HIV-patients) compatible with other disease, short term therapy, development of new compound attributes and effective in both cases can be helpful in tuberculosis therapy.

5. Development of new chemical scaffolds

Due to poor potency effect an identification of new tuberculosis drugs has been screened by pharmaceutical like collections which are linked to limited chemical diversity collections. Therefore, it was found that most of tuberculosis drugs don't follow lipinski's rule of five. Here, it was obtained that optimal drug-like features and pharmaceutical compound collections are found to be biased towards these properties. Due to constant development and accepting new challenges the current TB pipeline are expanding very slowly. But yet there is an inadequate development of novel regimen.[18]

6. Arrangements of new existing scaffolds

Over the past decades, it was found that many anti-biotic candidates are the chemical molecules that are re-engineered from older drug classes. Therefore, a new TB drugs from existing anti-bacterial drug classes involve re-designing of scaffolds which helps in the improvement of TB drugs that are under clinical trials. With the modification of known scaffolds, introduced into core structure aids for the advancement of bactericidal activities, good resistant action as well as pharmacodynamic and pharmacokinetic properties. For e.g. Having the modified version of oxazolidones like linezolid has led to new structures like PNU-100480 and AZD-5847 shows good activity against M.TB. The inhibition of mitochondrial protein synthesis, thrombocytopenia and myelosuppression was noted when patients were treated with oxazolidones for more than 14 days.

In case of nitroimidazoles used for the treatment of anaerobic bacteria and parasitic infections, identifies its establishment of scaffolds for synthetic modification and has been introduced for increase in anti-mycobacterial potential. Transformation of nitroimidazoles relates to unique mechanism of action, mimics its host defence strategies which can be

achieved through bio-activation and is flavin-dependent nitro-reductase. The other two candidates PA-824 and OPC-6768 are currently under clinical phase and can be helpful for shortening of the treatment. Presently, the main approach is to improve its activity its distribution in tissue and bio-availability. The current drug meropenen requires parenteral administration and thereby helpful in more serious MDR-TB/XDR-TB cases.

Thus, due to good improvement of existing scaffolds a proper statististics should be made in order to fill the drug development pipeline. It can also be helpful to improve some existing classes like fluoroquinolones, benzimidazoles and can be aided in the discovery of new chemical scaffolds for attractive approach. Hence, with proper understanding a new chemical scaffold can be facilitated along with physicochemical properties with an existing TB drugs.[18]

7. Chemo informatics and its role in Tuberculosis

Cheminformatics (also known as chemoinformatics, chemioinformatics, and chemical informatics) is the use of computer and informational techniques applied to a range of problems in the field of chemistry. It is widely used in the field of chemistry which is applied in computers and information techniques. It is largely used in the drug discovery process.[10]

8. Computational databases associated with TB

According to the surveys it was found that over 300,000 compounds were screened against M.TB in a laboratory. Therefore, it was noted that commonly millions of compounds were scanned by several numbers of the groups. The main advantage is that by gathering various data can prevent its repetition from screening by use of different groups which allows large scale of analysis of molecular properties of compounds with anti-TB activity. The database which helps for different data different aspects are developed by TB research[5] The following are the databases as below:

8.1. Bio health base: It is incorporated into PATRIC where it includes approximately 1850 to 2000 complete bacterial genomes. This website also provides some genome browser, some metabolic pathways (KEGG pathway maps), phylogenetic tree pathway, blast searches, etc.[5]

8.2. CDD TB (Collaborative drug discovery tuberculosis database): It is a kind of database which is focused mainly on small molecule libraries of compounds which acts against MTB. It is also used to find out the compounds having similar molecules to know the MTB drugs. It has also been aided in the development of novel computational machine learning and also in the identification of potent inhibitors by the development of pharmacophore models.[5]

8.3. GenoMycDB: It is also a kind of database where the tools for functional classification as well as analysis of genome structure organization and evolution can be carried out. It is also helpful in the comparative analyses of completely sequenced mycobacteria genomes.[5]

8.4. TB browses: It is a kind of a database where the resources for integrative analysis of TB genome. It is also the part of open source drug discovery.[5]

8.5. TDR target database: The database where includes all together genome sequencing, functional genomics projects, protein data. Its importance is that it includes computational evaluation of target, drug‘s ability and as well as integration of some large screening of compounds with manual data and its assembly associated with tuberculosis resistant drugs.[5]

8.6. Tuberculist: It is one of the widely recognized databases which is mainly focused on the M.TB genomes and can be aided in collating and integrating various kinds of aspects for the genomic information. It is also gives the datasets of DNA as well as protein sequences which is obtained from the strains of H37Rv. It is also connected with the annotations and functional assignments.[5]

8.7. The tuberculosis database (TBDB): It is a kind of a database where information regarding genomic data can be retrieved. It also helps the researchers to deposit their data before the publications and also helps them to carry out comparative analytical studies using genome map tools, genome synteny map or opera map browser.[5]

8.8. Web TB.org: It is a tool which contains the TB genomes, MTBreg database of proteins which is sometimes up-regulated or down-regulated in TB and also contains many more tools associated with it.[5]

9. Pathway tools for anti-TB screening

As suggested by various pathways and tools it was noted that the screening efforts for anti-TB has been facilitated by an integrative analysis of metabolic pathways, small screen and structural database. It is also helpful in the computer aided drug discovery approach. The target selection as well as drug discovery approach can be helpful in the target selection methods. The area of target selection in TB plays a difficult role in drug discovery process. Therefore, a group of researchers as well as theoreticians has been created for the collaborative approach in order to develop the transitional system of biology approach for tuberculosis.[5]

10. Applications associated with biology approaches

There are various types of databases that are helpful for identifying the stress and knowing the gene expression data. The pathways like KEGG (Kyoto Encyclopedia

of Gene and Genomes), BioCyc metabolic pathway databases and a k-shortest algorithm. Therefore, it is helpful in developing the expression related to the drug used and also for knowing the mechanism of action. Recently, an initiative was taken for a system biology program which aims to detect the regulatory and metabolic networks. It also includes the integration of profiling, high through put promoter, bioinformatics and comparative sequence analysis. Thus, provides its liberty by giving the combination in the field of chemo informatics and hereby, gives larger historical views in tuberculosis research.[5]

The databases for system biology are as follows:

10.1. BioCyc, MetaCyc (SRI):

It is a kind of database where a combination of suite tools is supported for the generation of pathways and querying of them. It consist of various organisms for specific pathway as well as genome databases. It is also specific for pathway/genome database which includes both virulent as well as drug susceptible of two MTB strains. The collection of BioCyc database also includes MetaCyc where it contains a non-redundant, exemplifies the metabolic pathways, development of experimental surveys. Largely, it was found that MetaCyc contains somewhere about 1200 pathways from more than 1600 different organisms. It is therefore, used to create the new PGDB where contains the predicted metabolic pathways of an organism, also gives interpreted genome as an input. Prediction of metabolic pathways and operons are also carried out. Thus, there is a computational analysis tool which acts as a pathway tools omics viewer.[5]

10.2. KEGG (Kyoto Encyclopedia of Genes and Genomes):

It is a database which is used as an academic resource. It consists of 16 databases which covers genome and chemical information. It is also used as a reference for many compounds and metabolites for biological pathways.[5]

11. Role of nanotechnology in the field of tuberculosis

In order to counteract its effect in the field of the bacterial world, especially in tuberculosis by applying the knowledge and giving the potentials in Nano-medicine. By aiding Nano-medicines help in the improvement of intracellular disease therapy, which offers different kinds of properties like targeting, sustained drug release and drug delivery by targeting intracellular pathogens. Thus, nanotechnology has now-a-days provoked its development for orientation of new and cheaper approaches. Hence, Nano-diagnostics will be helpful in the rapid and sensitive detection of M.TB.

12. Current Scenario

Nanotechnology gives a good chance for the detection and identification of mycobacterial strains. It also helps in the

improvement of potential drugs helpful in the treatment of tuberculosis. Nano science has given a unique and comparatively more effective drug delivery carrier, liposomal-mediated drug delivery, solid-lipid Nano particles, dendrimers, Nano suspensions, etc. Hence, the Nanoparticles, which act as drug carriers shows higher stability as well as carrier capacity with enormous improvement in drug bio-availability that later leads to reduction in dosage frequency.[3,13]

12.1. Liposomes

They are tiny spherical bubbles which are composed of the lipid bilayer membrane with an aqueous core. They act as carriers for various drugs like gentamicin, sporfloxacin, amikacin, etc. Here, it depends upon their sustainable biological compatibility. Various studies have revealed that when liposomes are encapsulated with PEG it enhances its circulatory life span in the blood stream. Thus, it is also noted that a large number of liposome-based variants if formulated it can be helpful for curing tuberculosis infection.[1] Table 1 presents liposomal-mediated drug delivery and its effect on M.TB species

Table 1 Liposomal-mediated drug delivery[4]

Drug	Liposome Formulation	M.TB Species	Effect	Animal Model
Isoniazid, Rifampicin	Multialmellar liposomes containing ePC, CH, DCP and DSPE-PEG.	Mycobacterium Tuberculosis	Controlled drug release and site directed delivery	Mouse
Pyrazinamide	Dipalmityl PC (7): CH (2) neutral and dipalmitoyl PC (7): CH (2): DCP (1) negatively charged.	Mycobacterium Tuberculosis	High therapeutic efficacy	Mouse
Clofazinamine	DMPC-DMPG (7:3) and clofazimine (drug: lipid, 1:15) in 80% tertiary butanol.	Mycobacterium Tuberculosis	Decreased cfu with no toxicity	BALB/C, Mouse

Where, DCP: dicetylphosphate, cfu: colony-forming unit, DMCP: dimyristoylphosphatidyl choline, DMPF: dimyristoylphosphatidyl glycerol

12.2. Dendrimer

They are long-chained, repeated three dimensional arrangements of a group of atoms. Being a synthetic Nano material possesses 5 to 10 nm in a diameter. In case of mycobacterium tuberculosis it was known that the cell wall of tuberculosis is similar to that of gram negative bacteria's cell wall. This cell-wall is made up of a good amount layer of mycolic acid where it renders its potency for anti-TB medicinal preparation and enters into the infected cells. Here, it acts into the latent period for curing of the disease. When a drug enters into the cell, forms complex with polymeric drug complex gets cleaved by lysosomal compartments of drug and results into drug release at high concentration in the cell. As a result of this presently mixture of drugs rifampicin, isoniazid and ethambutol is used for treatment of tuberculosis.1 Table 2 presents dendrimer-mediated drug delivery system and its effect on M.TB species:

Table 2 Dendrimer-mediated drug delivery system[4]

Drug	Formulation	M.TB Species	Effects	Animal Model
Rifampicin	Mannosylated dendrimer	Mycobacterium Tuberculosis	Biocompatibility, site-specific delivery	-

12.3. Polymeric nanoparticles

It possesses good bio-compatible and biodegradable properties for the use of drug delivery carriers. They are more stable, structured and can be synthesised with different properties like zeta potential, drug release profile, etc. These particles contain emblematic functional groups which can be transformed to structural moiety of drug or targeted ligands. The main advantage of PNP's is high stability, high loading capacity of hydrophilic and hydrophobic drugs and can be administered through different routes. The main approach of PNP's is considered to be one of the most extensively investigated with respect to anti-TB drug-loaded alginate by means of ionotropic gelatin.1 Table 3 presents Nano-Particle mediated drug delivery system and its effect on M.TB species:

Table 3. Nano-Particle mediated drug delivery system[4]

Drug	Formulation	M.TB Species	Effects	Animal Model
Moxifloxacin	Poly(butyl cyanoacrylate) Nano-particle	Mycobacterium Tuberculosis	High drug payload	-
Isoniazid	Polyactic-co-glycolic acid (PLGA) co-polymer	Mycobacterium Tuberculosis	Drug remains for prolonged period.	Rabbit
Rifampicin, Isoniazid, Pyrazinamide and Ethambutol	Alginate Nano-particle	Mycobacterium Tuberculosis	High drug payload, Improved pharmacokinetic, High therapeutic efficacy	Murine mouse
Ethionamide	PLGA Nano-particles	Mycobacterium Tuberculosis	Improved pharmacodynamics	Mouse
Rifampicin	PLGA Nano-particles dried in powdered form porous Nano-particle aggregate particle	Mycobacterium Tuberculosis	Shelf stability, Effective dispersibility and Extended release with local lung and systemic drug delivery	Guinea pig
Streptomycin	PLG Nano-particle	Mycobacterium Tuberculosis	Suitable oral dosage form	Murine mouse

12.4. Solid lipid nanoparticles

They are the promising carrier systems which are helpful for drug delivery applications. Its size ranges from 50-1000 nm. Composed of lipids and surfactants. For eg: it was noted that extra pulmonary tuberculosis considerably affects adverse immune response. These systems can be aided where it affects various conditions like they can effectively deliver drugs formulation to control lymphatic system.1 Table 4 presents Solid lipid nanoparticle-mediated drug delivery system and its effect on M.TB species:

Table 4: Solid lipid nanoparticle-mediated drug delivery system[4]

Drug	Formulation	M.TB Species	Effects	Animal Model
Rifampicin, Isoniazid, Pyrazinamide,	SLNs prepared by emulsion solvent diffusion	Mycobacterium Tuberculosis	Decreased dosing frequency	Mice
Rifabutic	Mannose coated SLNs	Mycobacterium Tuberculosis	Sustained delivery, Decreased side effects	-

13. Advantages of Nano-particles[4]

- Long shelf-life.
- Good carrier capacity.

Beneficial of both hydrophilic and hydrophobic substances.

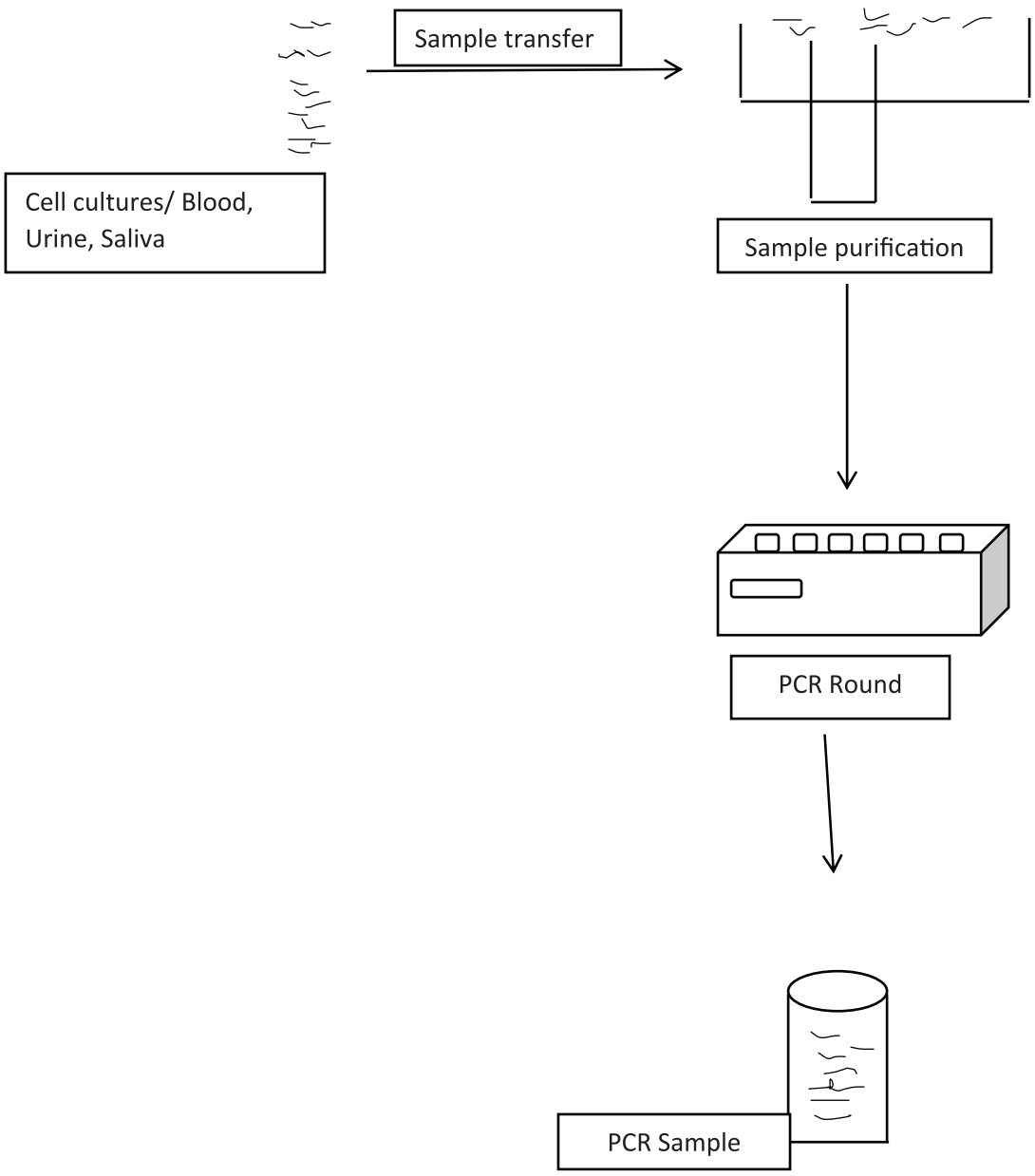
14. Drawbacks of Nano medicine in TB therapy

The main apprehension with nanoparticles as a presumed drug for tuberculosis therapy is its toxicity. The main drawback is that the physicochemical properties of nanoparticles like aggregations where the pH changes with it. The various issues like the redox potential of mitochondria, size dependent permeability of nuclear, etc. Thus, in order to remove its toxicity the carrier molecules should be treated with the utmost importance. As the size of Nano-particles are extremely small rendering its ability to cross with biological barriers like blood-brain barrier, blood-testis barrier, its regard with human health which can later cause caution and can be applied under some guidance.4

15. Nanosensors

"The term Nano sensors are defined as any biological, chemical or sensory points which are useful for passing the matter about nanoparticles in the macroscopic world." 20 Thus, the modified figure has been designed and is described in figure 4.19

a) Sample Preparation



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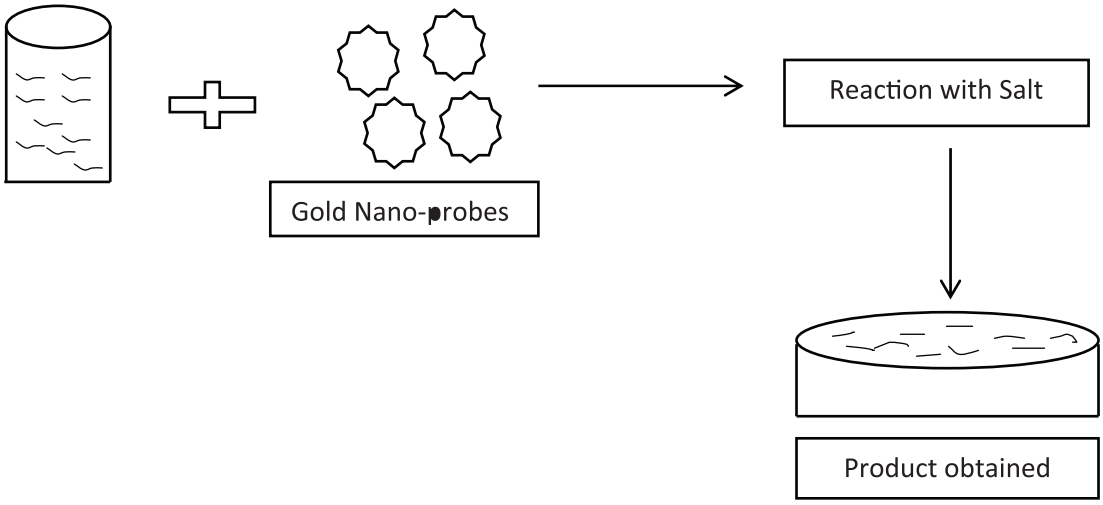

c) Data Analysis

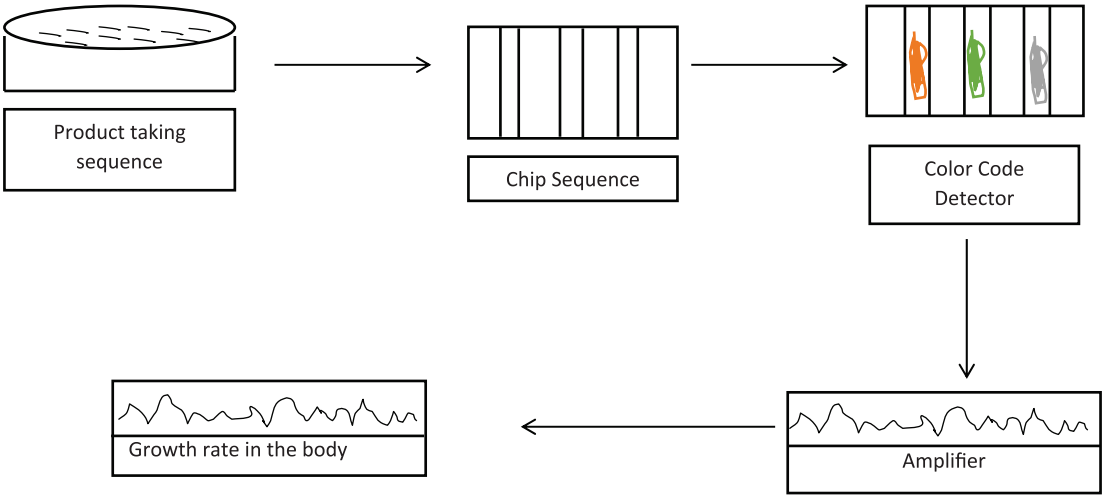

Fig. 4.Schematic representation of diagnosis using Nano sensor.[19, 20]
a) Sample Preparation, b) Sample Detection, c) Data Analysis

The following are the steps for detecting the bacterial growth rate using Nano-sensors as shown in figure 4:

1) Sample preparation.

2) Sample purification is carried out.

3) Purified sample and gold Nano-probes are taken in one plate.

4) Combined with salt and undergoes reaction.

5) The Product is obtained along with gene sequencing

6) Added to chip sequencing using Nano-sequences.

7) Detection of bacteria through color code.

8) Amplifier present, which gives a graphical growth rate of bacteria in the body.

   Note: red- indicates the major growth rate of bacteria in the body.

   Orange- indicates the average growth rate of bacteria in the body.

9) Green- indicates the minor growth rate of bacteria in the body

Anti-tuberculosis drug induce liver injury is observed in 40% of patients. Single nucleotide polymorphism and mutation account for the major contribution to drug resistance.21 The detection of resistance related mutations through the drug resistance diagnosis could improve the patient care.22 The drug resistance tuberculosis poses a serious challenges to the existing anti-TB therapies. Therefore, there is an unmet need for combination therapy.23

Conclusion

Many new anti-TB drugs have been developed in several years. But due to lack of knowledge of a novel mechanism of action, sometimes it fails to act against bacterial resistance. To reduce the duration of therapy potent drug development against M. TB should be carried out. Various dosage forms like suspension or emulsions using nanoparticle for tuberculosis can be considered for effective treatment. Taking the nanosenors by attaching it with human veins and detecting it through color code detector can be helpful in knowing its bacterial growth rate in the body. Considering all the aspects of drug discovery potential anti TB molecule can be developed with short term dose regimen with fewer side effects.

Conflicts of Interest: Authors disclose no conflicts of interest

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ARTICLE

DEVELOPMENT & VALIDATION OF HIGH PRESSURE LIQUID CHROMATOGRAPHY ANALYTICAL METHOD FOR RELATED SUBSTANCE OF ACETYLCYSTEINE EFFERVESCENT TABLET

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Abstract

The HPLC method was developed specially for the related substances of n-acetyl-l-cysteine (NAC) effervescent tablet formulation where the issue of merging of impurities peaks was observed during the development of the method. So, development was triggered with an aim of separation of all the known possible impurities peaks from that of principal peak of acetylcysteine as well as each four known impurities. Finally the analytical method for related substance of acetylcysteine with a gradient program on HPLC was developed and validated with the mandatory experiments. The related substance method was efficient to separate the co-eluting peaks of impurities and its individual known impurities from the principal peaks specifically for the effervescent formulation. The method was specific, selective and reproducible. Recovery and linearity was also found within the acceptance criteria. Solution stability was also established and evaluated during the method validation. Method was successfully evaluated for robustness using various alterations in the method parameters for its functionalities.

Keywords: Acetylcysteine, N-Acetyl-L-Cysteine, NAC, Related Substance, HPLC

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1. Introduction

N-Acetylcysteine (acetylcysteine or n-acetyl-l-cysteine or NAC or (2R)-2-acetamido-3-sulfanylpropanoic acid) acts as a mucolytic agent and used to reduce the viscosity of pulmonary secretions in respiratory diseases. It is also used as an effective antidote in the treatmentof paracetamol poisoning [1-3].

The mucolytic action of NAC is probably due to its ability to decrease the viscosity of secretions by breaking the disulphide bonds of the protein network [4].

Additionally, NAC is acting as effective antioxidant and being studied for the treatment of various diseases such as nephropathy [5], liver failure [6], chronic obstructive pulmonary disease [7] and brain disorders [8]. It has also been used as a metal chelating agent [9]and radioprotective agent [10].

Liquid chromatography (LC) methods either as a conventional method or derivatization method have been extensivelyreported for the assay of N-acetylcysteine in pharmaceuticalformulations and biological samples [11-16]. Certain LC methodsfor quantitative analysis of NAC were based on various other detectors. However, the UV detector was found as universal detector [17-21]. Majority of Related Substance method were described, but the chromatographic issues were arised during the analysis of effervescent tablets formulation due to extensive merging of the impurities peaks, which were interfering with the principal and each other among known impurities. One literature was observed which represented the method for effervescent tablets [22], however, with this method during development, the chromatography was not acceptable with the test formulation.

So, the aim was to develop the specific and selective related substances method using the universal detector (UV) using HPLC method specifically for effervescent tablets formulations without the derivatization sample preparation.

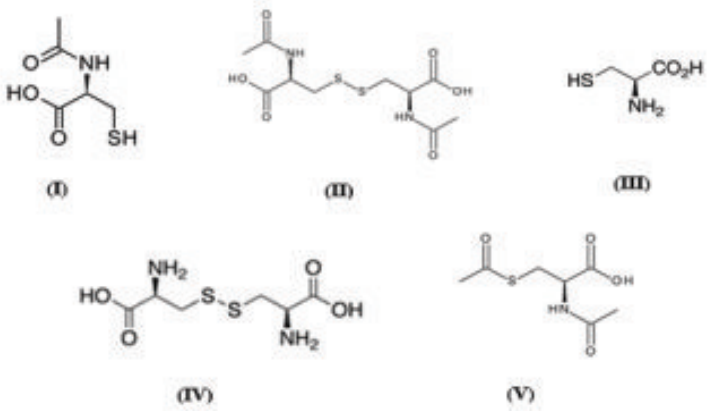

Figure-1 Chemical Structure of Acetylcysteine (I), Impurity-A (II - L-cystine), Impurity-B (III - L-cysteine), Impurity-C (IV - N,N-diacetyl-L-cystine), Impurity-D (V - N,S-diacetyl-L-cysteine)

Literature described the transformation of one polymorphic form to another dihydrate form, which is showing variable dissolution behavior of carbamazepine [11–13]. There have been several reports showing irregular dissolution [14–16], bioequivalence failures [17–19], and clinical failures of carbamazepine [20]. Certain literatures have been found which indicates the impact on polymers to make the drug release profile prolonged. But the optimization of polymer levels and combination of polymers plays a foremost role in achieving a successful extended release formulation which is correlating with the innovator formulation in each dissolution medium i.e. original medium as well as different biological pH.

In general, the enhanced dissolution pattern is depending upon either by dissolution medium pH change or by addition of the solubilizer, like surfactants and cyclodextrin derivatives in the preparation of dissolution medium [21–27]. SLS has been proven as the agent of choice because it is cost-effective and it holds good solubilizing capacity even at quite low concentrations. Already, several authors reported that SLS can be used to enhance dissolution of low water-soluble compounds [28]. Till date, many authors had published the articles on the addition and usage of SLS like sodium taurocholate or other surfactant for executing the dissolution of low soluble drug like carbamazepine using less dissolution media volume. Carbamazepine solubility was also distinctly increased in several nonionic surfactants [29]. But with the medium volume 1800mL according to USP method, very few articles were present which shows the surfactant assisted dissolution to get the higher release profile. Even the data of innovator formulation in original medium as well as in the acidic medium has not been described at a large extent.

But here, the aim of carrying out the in-vitro drug dissolution at various biological pH to partially evaluate the effect of pH on drug release and absorption during in-vivo conditions. And specifically to evaluate the effect of surfactant on drug release and to set the minimum possible optimum concentration of surfactant to achieve desired drug release with a minimum variability in the results of both – innovator as well as test formulations.

1. Experimental

2.1 Chemicals and materials

Acetylcysteine working standard (100.17%) was prepared from an API, against a standard procured from SimsonPharma, Mumbai, India.Impurities standard (Impurity – A, B, C, and D) were procured from TRC, Canada. Methanol (HPLC grade, Merck, India), Methanol (MS Grade, J. T. Baker, USA), Hydrochloric acid (GR Grade, Merck, India), Ammonium Sulfate (GR Grade, Merck, India), Sodium Pentane Sulfonate (HPLC Grade, Merck, India) were used during the development and validation experiments.

2.2 Instrumentation

A high pressure liquid chromatograph system (1260-Infinity-II, Agilent, Germany& LC-2010, Shimadzu, Japan)coupled with UV& PDA detector and quaternary pump, Micro analytical balance (MSA6-6S-000-AM, Sartorius, Japan), pH meter (Thermo Orion Star II, Thermo, USA), Membrane filter (nylon) (Pall corporations, USA), Syringe filter (Nylon, 0.22µ) (Millipore, USA), Ultrasonicator (Bioneeds scientific corporation, India), Water purification system (Milli-Q, Millipore, USA) and RO water systemwere used during method development and validation.

2.3 Chromatographic method parameters

For carrying out related substance experiment, HPLC column – XBridge C18(250 x 4.6mm, 5µ) (Waters, USA) was connected with the LC system and stabilized with the mobile phase at 30°C. Mobile phase was eluted at a 1.0mL/min follow rate with a gradient program. Gradient program was set for Mobile Phase-A as 0à3 (90%), 3à12 (10%), 12à25 (10%), 25à35 (90%). Standard and samples were injected with 100µL injection volume during analysis. The signal of eluted components will be monitored continuously using PDA detector and specifically at 205nm using UV detector using the respective chromatography softwares (LC Solution, Shimadzu, Japan and OpenLab CDS, Agilent, Germany).

2.4 Analytical Procedure

2.4.1 Mobile Phase preparation

Mobile Phase-A consists of 0.5%w/v solution of ammonium sulfate in 0.01M sodium pentane sulfonate in water. pH should be adjusted to pH 2.0 using 2M HCl.Mobile Phase-B consists of amixture of 100mL of methanol and 900mL of a 0.5%w/v solution of ammonium sulfate in 0.01M sodium pentane sulfonate in water. pH of the mixture was adjusted to pH 2.0 using 2M HCl.

2.4.2 Standard Solutions preparation

Impurity A: 5.0mg of Impurity-A standard was dissolved in 5mL of 0.1N HCl and diluted to 50mL with mobile phase-B. 3mL of the resulting solution was diluted up to 10mL with mobile phase-B.

Impurity B:5.0mg of Impurity-B standard was diluted to 50mL with mobile phase-B. 3mL of the resulting solution was diluted up to 10mL with mobile phase-B.

Impurity C: 5.0mg of Impurity-C standard was diluted to 50mL with mobile phase-B. 6mL of the resulting solution was diluted up to 10mL with mobile phase-B.

Impurity D: 5.0mg of Impurity-D standard was diluted to 50mL with mobile phase-B. 3mL of the resulting solution was diluted up to 10mL with mobile phase-B.

Acetylcysteine:5.0mg of Acetylcysteinestandard was diluted to 50mL with mobile phase-B. 6mL of the

resulting solution was dilutedup to 10mL with mobile phase-B.

Standard Solution: 1.0mL of each standard stock solution (Acetylcysteine and Impurities A, B, C, D) were transferred in to the 10mL volumetric flask and diluted with mobile phase-B.

2.4.3 Sample (Test) Solution Preparation

10 effervescent tablets were transferred in to 1000mL volumetric flask. To this, 5mL 1M HCl was added to dissolve and then diluted it with mobile phase-B. 5mL of this solution was transferred in 50mL of volumetric flask and diluted with mobile phase-B. Finally the sample was filtered through 0.22µ nylon syringe filter.

2.5 Method Development & Optimization

Initially during the development of the formulation product related substance (RS) tests, the RS method for raw material from European Pharmacopoeia (EP) was adopted. However, using this method, the issues were raised in the chromatography of the sample solution, where the excipients peaks were observed at the retention time of known impurities and principal peak with a greater intensity. Also the method was not so much efficient to separate out the impurities with a reasonable resolution.

So, ultimately the desire is to separate those known impurities with the enough resolution and the overall chromatography should be free from effervescent tablet excipients peaks. However, to develop the effervescent tablet, focus was also established for the similarity in the behavior of effervescence, appearance of the solution and taste of solution between the test formulation and innovator formulation.

To attain this goal, multiple trials were taken for the usage of mobile phase buffer, mobile phase, mobile phase pH, mobile phase ratio, mobile phase elution program, diluent, stationary phase (LC column) and various grades and types of excipients in the formulations.

Mobile Phase Buffer: Water, ammonium sulfate, Phosphate buffer, triethylamine, ammonium acetate, ammonium formate, tetrabutyl ammonium hydroxide

Mobile Phase: Acetonitrile: Buffer, Methanol: Buffer, Acetonitrile: Methanol: Buffer

Ion Pair Reagent: Pentane sulfonate, Octane sulfonate

Mobile Phase Elution: Isocratic, Gradient

Mobile Phase pH: pH 2.0, 2.5, 3.0, 4.0, 7.5

Grade of Solvent in Mobile Phase: HPLC and MS Grade

Stationary Phase (Column): GL Science Inertsil ODS C18 (3µ), Thermo Hypersil ODS (3µ), AgelaVenusil XBP Polar Phenyl (5µ), Waters X-bridge (3.5µ), Phenomenex Gemini C18 (5µ), PhenomenexSynergi Polar-RP (4µ)

In majority of above multiple combination chromatographic trials, the desired separation was not achieved, except limited number of trials.The chromatography was not powerful with respect to higher resolution and elimination of placebo interference.

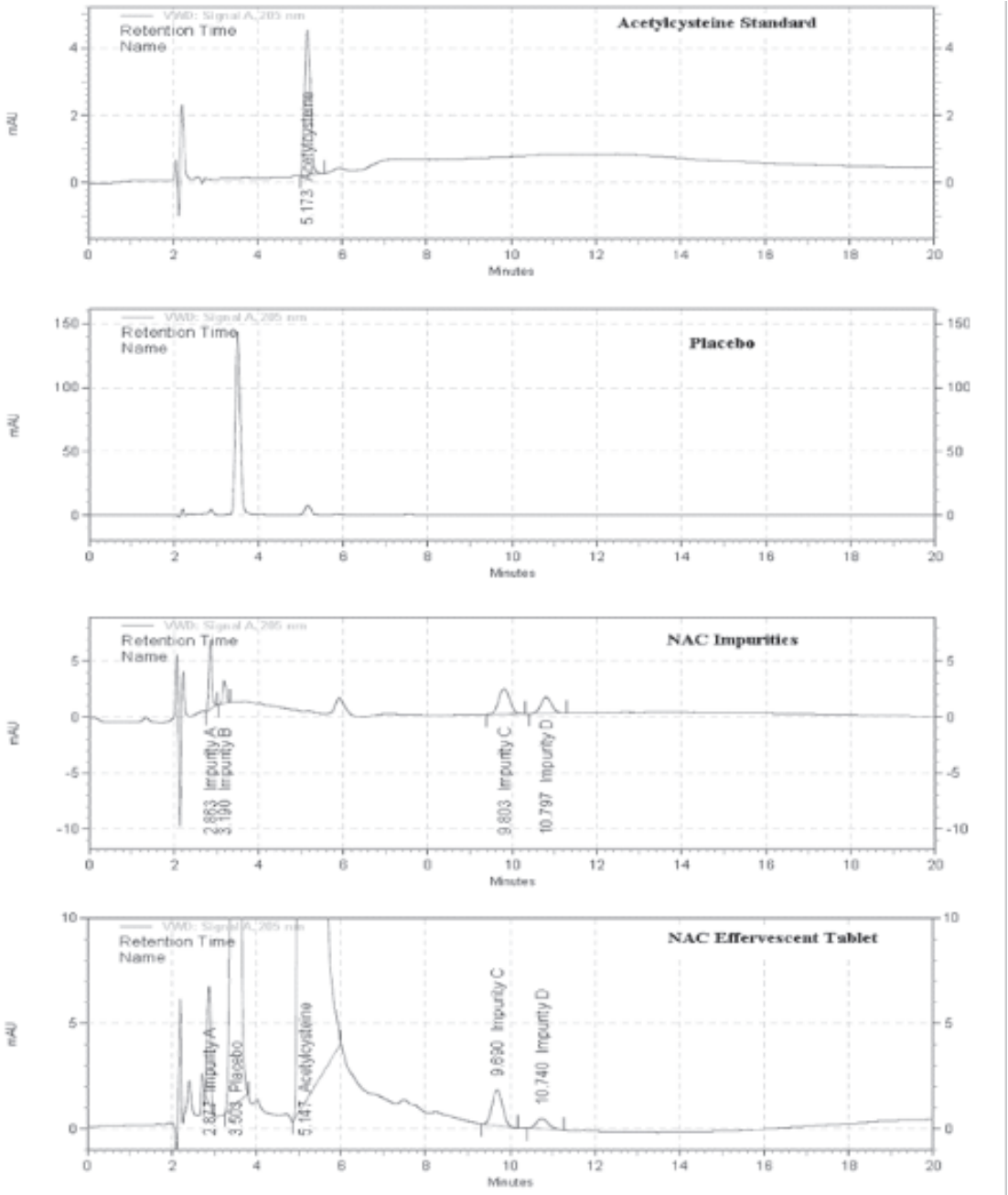

Figure-2 Excipient (Placebo) Interference and Merged Impurity Peaks

Impurity A and B are very fast eluting in almost all the condition and very difficult to separate with good resolution. Also the principal analyte is eluting just after the both impurity A and B. If the isocratic modewas selected, then impurity C and D would be eluting very late and still the difficulties in separation of Impurity A, B and principal peak. So, trials were arranged in such manner to effectively separate both the impurities A and B from each other as well as from excipient peak and principal peaks and to elute the impurities C and D to be eluted earlier after elution of principal analyte. So, overall analysis time and cost for one run can be reduced.

During development, one method was also applied using fluorescence detector to detect the impurity-A & B by derivatization method using fluorenyl-methyl-oxy-carbonyl chloride (FMOC-Cl), but still the method specificity was not achieved with respect to formulation samples. And also we could not able to analyze impurities C and D with this method. So, in that case, separate methods were needed to be developed, which was not suitable.

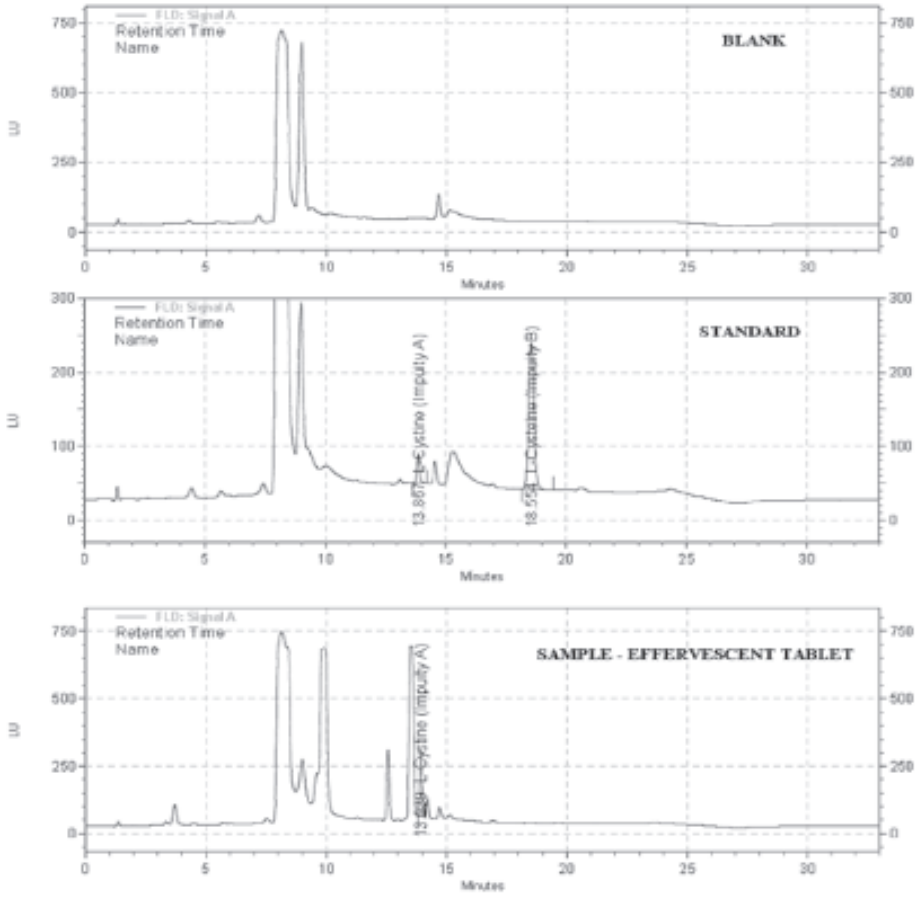

Figure-3-Chromatography with fluorescence detector after derivatization for Impurity A and B

Reasonably better chromatography was achieved with Waters X-bridge column with lower particle size and using ion pair reagent in the mobile phase with low pH and the gradient elution of mobile phase at 30°C column temperature and by using superior grade of methanol (MS grade, J.T.Baker). With this chromatography, the interference of impurities with each other was resolved. Only certain excipients within the effervescent formulation were creating a minor problem in terms of specificity compared to major issues with previous methods. So, finally to resolve the same, grade as well as replacement in the excipients were done in the effervescent formulation manufacturing process to achieve the completely smooth chromatography which can be efficient to analyze the related substances of the acetylcysteine within the product. Lastly, the formulation was developed as per the goal and evaluated for its outcome. The related substance test for this formulation was performed using as such sample solution, impurity spiked sample solution with the final optimized parameters as described above. The chromatography was well accepted with respect to routine chromatographic system suitability parameters (Resolution, Signal-to-Noise Ratio, Theoretical Plates, Tailing Factor etc.). The method specificity was also evaluated using the diluent, standard solution, placebo solution and sample solution.

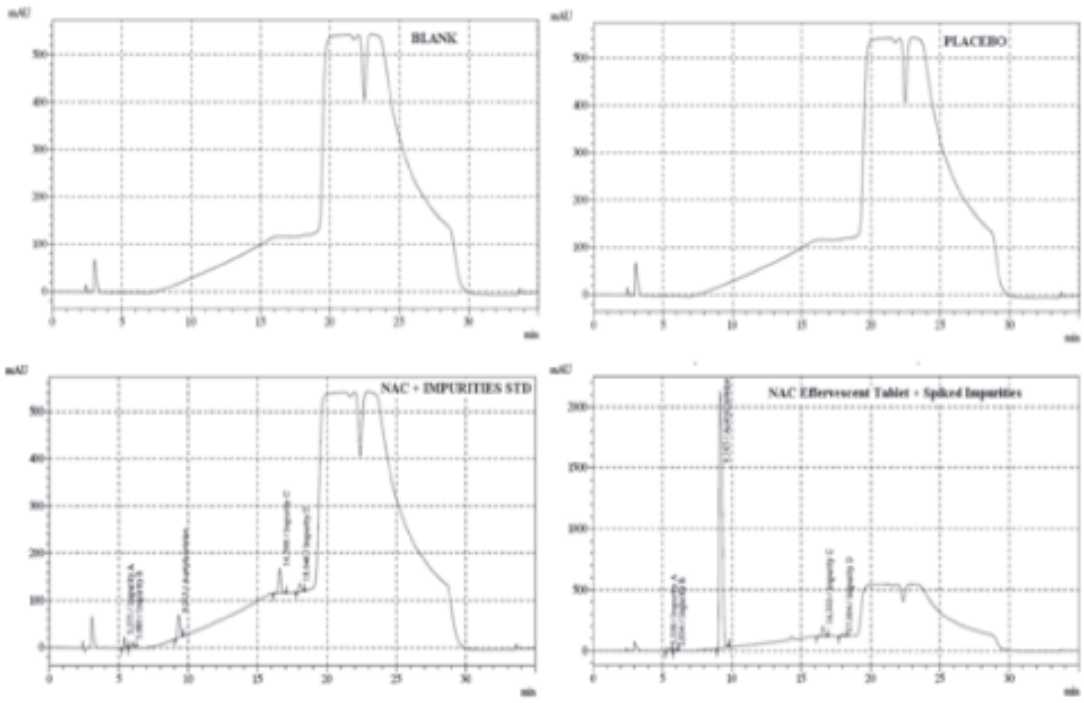

Figure-4-Final Optimized Method Chromatography

2.1 Method Validation

The related substance method for effervescent formulation was developed and optimized as in above section, was also undergone through complete validation by evaluating all the validation parameters. Specificity, accuracy, injection reproducibility, intra-assay, ruggedness using different equipment, linearity, range, solution stability at two different conditions – refrigerated and ambient temperature, robustness by change in mobile phase ratio, column oven temperature & mobile phase pH and forced degradation experiments using all degradation conditions were performed using the final method for validation. The finished product of effervescent tablets with final optimized formula and its placebo were used for the method validation purpose.Forced degradation was carried out by keeping the standard and sample at stressed condition for 24 hours. For oxidative, base and acid degradation, respectively 30% peroxide, 1N NaOH and 1N HCl solutions were used. For thermal hydrolysis, standard and sample were kept at 50°C and 80% RH conditions.

1. Results and discussion

The analytical method for RS of effervescent formulation was completely validated by evaluating all the mandatory parameters. Limit of Detection (LOD) and Limit of Quantitation (LOQ) were also found within the acceptance criteria with respect to signal to noise as well as %RSD and correlation co-efficient. Linearity and Range were accepted throughout the lowest LOD range. The results arerepresented in the following tables.The method was completely validated and it was observed that Impurity-C is more degradant via oxidative pathway and critical in terms of stability. This impurity is increased within the standard, upon storage for more time at room temperature. So, it's mandatory to prepare and inject the standard and samples solution freshly and within the short duration of time to get the exact values of impurities.

Table-1 Specificity

Sample Name	Sample ID	RT	Area	Resolution	TP	TF	Single Point Threshold
Blank	Diluent	No Additional Peak Observed					
Placebo	Placebo	No Additional Peak Observed					
Impurity A	Standard	5.4	156720	-	9602	1.4	0.988
Impurity B	Standard	6.0	90611	-	13915	1.3	0.981
Impurity C	Standard	16.6	659045	-	42949	1.1	0.998
Impurity D	Standard	18.1	237704	-	38842	1.2	0.996
Acetylcysteine+ Impurity A,B,C,D	Impurity A	5.4	184766	-	9135	1.4	0.990
	Impurity B	6.0	81194	2.8	13981	1.3	0.993
	Acetylcysteine	9.3	590698	12.5	12650	1.2	0.998
	Impurity C	16.6	619776	22.5	44244	1.2	0.998
	Impurity D	18.0	228378	4.3	39841	1.2	0.996
Acetylcysteine Tablet + Impurity A,B,C,D	Impurity A	5.3	208755	-	9825	1.4	0.993
	Impurity B	5.9	66052	2.9	14382	1.3	0.994
	Acetylcysteine	9.2	33829601	10.2	7041	1.7	1.000
	Impurity C	16.6	971725	19.6	43280	1.2	0.999
	Impurity D	18.0	225449	4.2	39258	1.2	0.995

Table-2 Recovery

Conc. Level (%)	Impurity-A		Impurity-B		Impurity-D		Impurity-C	
	Rec* (%)	RSD* (%)	Rec* (%)	RSD* (%)	Rec* (%)	RSD* (%)	Rec* (%)	RSD* (%)
10%	97.94	13.32	95.92	11.44	89.84	13.37	87.16	13.16
20%	100.64	7.15	98.82	9.48	98.88	3.97	97.39	8.43
50%	102.07	8.87	98.81	6.85	104.98	4.97	98.32	9.31
80%	101.43	8.81	98.29	7.78	103.92	6.48	96.65	9.22
100%#	99.79	9.24	97.71	7.05	103.86	5.69	109.9	6.15
150%	99.26	8.66	95.37	8.28	103.04	5.74	103.59	8.15

Values of Recovery and RSD were derived from triplicate preparation, *Rec -Recovery

#Conc. of Impurity-A, B, and D: 3ppm, Conc. of Impurity-C: 6ppm

Table-3 Reinjection Reproducibility

	Area		RT	
Standard	**Average**	**RSD (%)**	**Average**	**RSD (%)**
Impurity A	188.120	0.14	5.040	0.03
Impurity B	66.660	0.19	5.620	0.03
Acetylcysteine	543.560	0.07	8.990	0.02
Impurity C	592.420	0.20	16.490	0.01
Impurity D	188.310	0.63	17.880	0.01

Values were derived from 6 replicates injections

Table-4 Intra-Assay

	Area		RT	
	Average	**RSD (%)**	**Average**	**RSD (%)**
Standard				
Impurity A	188.12	0.14	5.04	0.03
Impurity B	66.66	0.19	5.62	0.03
Acetylcysteine	543.56	0.07	8.99	0.02
Impurity C	592.42	0.20	16.49	0.01
Impurity D	188.31	0.63	17.88	0.01
Sample + Spiked Imp.*				
Impurity A	175.119	0.42	5.05	0.05
Impurity B	76.384	0.62	5.62	0.06
Unknown 1	59.013	0.89	5.93	0.08
Unknown 2	92.16	3.85	7.81	0.03
Acetylcysteine	39667.747	0.19	8.89	0.02
Unknown 3	254.432	0.23	13.95	0.02
Impurity C	1413.205	5.81	16.48	0.03
Unknown 4	113.339	2.23	17.05	0.02
Impurity D	190.916	0.58	17.87	0.02

****Values of Average of Area & RT and RSD were derived from 6 sets of sample preparation***

Table-5 Ruggedness

	Experiment-I		Experiment-II		Experiment-III	
	Area		Area		Area	
	STANDARD					
	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
Impurity A	188.12	0.14	190074	0.24	205156	0.44
Impurity B	66.66	0.19	78520	1.25	65904	0.76
Acetylcysteine	543.56	0.07	589144	0.26	536781	0.28
Impurity C	592.42	0.20	648290	0.24	676710	0.20
Impurity D	188.31	0.63	202915	1.05	166509	0.83
	SAMPLE + Spiked Imp.*					
	Average	RSD (%)	Average	RSD (%)	Average	RSD (%)
Impurity A	175.738	0.62	187853	0.10	212299	0.22
Impurity B	76.549	0.96	83040	0.27	72916	0.29
Acetylcysteine	39740.994	0.20	38210250	0.02	37797758	0.05
Impurity C	1346.974	3.16	1153293	1.06	1161226	0.47
Impurity D	191.548	1.11	204794.5	0.30	216174.5	0.55

Values of Average of Area & RT and RSD were derived from 2 sets of sample preparation

Table-6 Linearity & Range

	Linearity			Range		
	Conc. (ppm)		Correlation coefficient (r^2)	Conc. (ppm)		Correlation coefficient (r^2)
	10%	150%		10%	150%	
NAC	0.611	9.160	0.9994	0.618	9.270	0.9991
Impurity A	0.260	3.902	0.9997	0.282	4.230	0.9996
Impurity B	0.365	5.471	0.9992	0.339	5.085	0.9987
Impurity C	0.543	8.150	0.9996	0.586	8.790	0.9992
Impurity D	0.301	4.521	0.9999	0.298	4.470	0.9995

Table-7 Solution Stability

	Initial (0 hr)		Refrigerator Condition (10 hrs)			Ambient Condition (10 hrs)		
Standard								
	Area	**% Imp.**	**Area**	**% Imp.**	**% RSD**	**Area**	**% Imp.**	**% RSD**
Impurity A	208195	0.50	206564	0.50	0.07	205415	0.50	0.47
Impurity B	66923	0.51	67260	0.51	0.29	70751	0.54	3.29
Impurity C	679661	1.01	747556	1.10	6.40	832359	1.23	13.96
Impurity D	171738	0.52	176344	0.53	1.41	175651	0.53	1.13
Sample + Spiked Imp.								
	Area	**% Imp.**	**Area**	**% Imp.**	**% RSD**	**Area**	**% Imp.**	**% RSD**
Impurity A	211529	0.51	211942	0.52	0.62	208988	0.51	0.37
Impurity B	67504	0.52	68035	0.52	0.09	72144	0.55	4.05
Impurity C	788413	1.17	879191	1.30	7.40	851114	1.26	5.11
Impurity D	171123	0.52	182789	0.55	4.20	178082	0.53	2.35

Values of Average of Area & RT and RSD were derived from 2 sets of sample preparation

Table-8 Robustness

Mobile Phase Ratio			
Condition-1: MP-B Ratio_105 ml Methanol + 895 ml Buffer			
Condition-2: MP-B Ratio_95 ml Methanol + 905 ml Buffer			
Sample + Spiked Imp.	**%Assay**		**RSD (%)**
	Condition-1	**Condition-2**	
Impurity A	107.0	97.5	6.57
Impurity B	103.9	103.7	0.14
Impurity C	89.3	92.6	2.57
Impurity D	113.4	108.2	3.32
Column Oven Temperature			
Condition-2: Column temperature 32°C			
Sample + Spiked Imp.	**%Assay**		**RSD (%)**
	Condition-1	**Condition-2**	
Impurity A	95.2	98.1	2.12
Impurity B	98.0	98.3	0.22
Impurity C	106.3	101.7	3.13
Impurity D	94.6	97.4	2.06
Mobile Phase pH			
Condition-1: Mobile Phase pH 1.8			
Condition-2: Mobile Phase pH 2.2			
Sample + Spiked Imp.	**%Assay**		**RSD (%)**
	Condition-1	**Condition-2**	
Impurity A	101.2	97.1	2.92
Impurity B	105.9	99.1	4.69
Impurity C	86.0	90.0	3.21
Impurity D	104.7	98.4	4.39

Values were derived from 2 sets of sample preparation in each experiment

Table-9 Forced Degradation

% Degradation (For 24 Hrs)					
Known and Unknown Impurity	**Oxidative**	**Base**	**Acid**	**Thermal Hydrolysis**	**Photo**
Acetylcysteine Standard					
Unknown Impurities (%)	**0.21**	**0.12**	**0.33**	**1.68**	**0.19**
Impurity A	-	-	-	-	-
Impurity B	-	-	0.48	3.85	0.09
Impurity C	5.21	2.53	0.81	3.54	2.03
Impurity D	-	-	0.18	6.57	0.11
Total Degradation (%)	**5.42**	**2.65**	**1.80**	**15.64**	**2.42**
Acetylcysteine Sample					
Unknown Impurities (%)	**0.1**	**0.12**	**0.17**	**0.23**	**0.14**
Impurity A	-	-	-	-	-
Impurity B	-	-	0.08	0.89	0.70
Impurity C	24.64	2.63	1.14	3.51	1.06
Impurity D	0.04	0.04	0.73	5.40	2.69
Total Degradation (%)	**24.78**	**2.79**	**2.12**	**10.03**	**4.59**

Table-10 LOD-LOQ

	Impurity A	**Impurity B**	**Acetylcysteine**	**Impurity C**	**Impurity D**
LOD*					
% RSD	1.44	1.22	4.83	1.35	3.36
LOQ#					
% RSD	0.39	0.05	0.13	0.29	0.25

***Values were derived from 3 replicates injections, #Values were derived from 6 replicates injections**

4. Conclusions

The principal aim of the development of this related substance method specifically for the effervescent formulation was to separate out the excipient interference from that of known impurities and principal peaks as well as to separate out impurities from individual peaks as well as principal peak using the universal UV detector of liquid chromatography and without any derivatization method. So, in the development phase, more focus was given on the analytical method aspects as well as on the excipients grade and type of excipients used which may affect the overall chromatography, as the principal analyte and impurity have the wavelength maxima at lower side (205nm), so chances of poor chromatography is more higher. Finally the optimization was done using the theoretical approach for all the chromatographic parameters to develop the robust and accurate method for acetylcysteine related substance analysis.

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ARTICLE

BIOACTIVITY GUIDED ISOLATION OF URSOLIC ACID FROM CLERODENDRUM SERRATUM ROOTS AND EVALUATION OF ITS EFFICACY AGAINST ASTHMA IN GUINEA PIGS.

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Abstract

Present study investigated anti-asthmatic activities of ursolic acid isolated from ethyl acetate fraction of crude extract of C. serratum roots along with its anti-inflammatory potential. Methanol extract (70%) was fractionated in to ethylacetate and the fraction was subjected to Gas chromatography-mass spectrum (GC-MS) in order to identify possible phytoconstituents. Column chromatography of the ethyl acetate fraction of C. serratum roots yielded ursolic acid, which was characterized by its FT-IR, mass and NMR spectral data. The effect of ursolic acid on guinea pig was assessed using inflammation of the airway caused by histamine and ovalbumin, inflammatory cell count in blood and broncho-alveolarlavage fluid, quantitative cytokine determination (IL-4, IL-5 and TNF-α) in serum and BALF, and histopathology of lung tissue. The results indicated that ursolic acid has the potential to prevent inflammatory mediators into the local lung and trachea tissues and may be useful in the management of asthma.

Key words: Asthma, Clerodendrum serratum, Cytokines, Inflammation, Ursolic Acid

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1. Introduction

Asthma, a chronic inflammatory disease of the airways,characterized by infiltration of T-lymphocytes and eosinophil, overproduction of mucus and airway hyper-responsiveness. Inflammatory mediators have a great role in the pathogenesis of chronic airway disease and facilitate the recruitment, activation, and trafficking of inflammatory cells in the airways. Inflammatory cells contribute to the generation of Th2 cytokines, chemokines, Tumour necrosis factor (TNF)-α and histamine (Liu et al., 1991), levels of which are increased in asthmatic lungs (Williams and Galli, 2000). Th2 cytokines participate in asthma pathogenesis by stimulating B-cells and induce the infiltration of eosinophils and other inflammatory cells into the airways (Hamelmann et al., 1999; Zhang et al., 1999). TNF-α, is usually associated with Th1 responses (Bazzoni and Beutler, 1996; Costa et al., 1993; Gordon and Galli, 1991 Meiler et al., 2006). Recent asthma research is focused on chronic inflammation and remodelling of the airways. Many medicinal plants are used in traditional system of medicine as an alternative to treat inflammatory diseases and have been known to provide relief from the symptoms, comparable to that obtained by allopathic medicines. Several extract/fractions along with plant derived secondary metabolites have been successfully reported to interfere directly or indirectly in the pathophysiology of inflammation. In this perspective, the crude 70% methanol extract and fractions (ethyl acetate and *n*-butanol) of roots of *Clerodendrum serratum* had been evaluated in our previous study for anti-asthmatic, anti-inflammatory and antioxidant to establish the traditional claims of roots. Our previous study concluded that ethyl acetate fraction of *C. serratum* roots (EFCSR) had potent anti-asthmatic activity that included mast cell stabilizing,bronchodilatory activities and anti-histaminic activity with inherent antioxidant and anti-inflammatory activity. The subsequent phytochemical analysis revealed the presence of polyphenols, steroids and terpenoids in EFCSR which might be responsible for the observed effects. However, the underlying mechanism and actual biologically active ingredients with antiasthmatic potential was not known. Some previous reports on flavonoids (Rogerio et al., 2007) and terpenoids (Lee et al., 2010) have shown possible efficacy against inflammatory disorders and found to significantly inhibit asthmatic responses via inhibition of chemical mediator release in broncho-alveolar lavage fluids (BALF). The increasing interest to elucidate the role of saponins and/or polyphenols for asthmatic potential has prompted scientific research into the separation and characterization of the active components. In the present investigation, EFCSR was subjected to bioassay-guided fractionation leading to the isolation of a triterpenoid, ursolic acid (UA), which was characterized and evaluated for antiasthmatic potential in animal models. The isolated compound was characterized by from its physical properties and FT-IR, EI-MS, ^{13}C-NMR and ^{1}H-NMRspectral

data. A pilot study was conducted to determine the efficacy of UA against histamine-induced bronchospasm in guinea pigs. The protective effect of UA (15 mg/kg) was investigated for anti-asthmatic activity using ovalbumin (OVA) induced airway inflammation in guinea pigs. Effects on asthmatic responses were investigated by measuring the leukocytes and differential cell count (in blood and BALF), cytokines (in serum and BALF), histamine (in BALF) and accompanying histopathological changes in lung tissues.

2. Material and methods

2.1 Drug and chemicals

Ursolic acid, histamine and ovalbumin (OVA) were purchased from Sigma Aldrich Chemical Co. (India). Ketotifen fumarate was gifted by Torrent Research Centre, Ahmedabad, India. Dexamethasone was purchased from Zydus Research Pvt. Ltd., Ahmedabad, India.Aluminium hydroxide gel and Formaldehyde solutions were obtained from S. D. Fine Chemicals, Mumbai, India. Perchloric acid was purchased from Ranbaxy Fine Chemicals Ltd., New Delhi, India. Ketamine wasobtained from Triveni Interchem Pvt. Ltd., Gujarat, India. Xylazine was obtained from Festiva Pharma, Gujarat, India. Kits for IL-4, IL-5 and TNF-α were bought from Pro LabMarketing Pvt. Ltd., New Delhi, India. All other chemicals and solvents used were of standard analytical grades.

2.2 Plant collection, authentication and identification

The roots of *Clerodendrum serratum* Linn. were collected from Government Ayurvedic Udhyan, Gandhinagar, Gujarat (India) in the month of August, 2011. Herbarium specimen (10EXTPHDP49CS11) was deposited in the Department of Pharmacognosy, Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, India. The roots were washed and dried under sun light for 15 days. The dried material was then subjected to pulverization and the powdered sample was passed through 60# sieve. Standardization of powdered sample was carried out by the usual authentication parameters as described in the standard herbal pharmacopoeia methods.

2.3 Preparation of extract and fractions:

Powdered roots of *C. serratum* (1kg) were extracted thrice with 70% methanol (5 L v/v) in soxhlet extractor at the boiling temperature for 24 h, followed by filtration. Combined filtrates were concentrated and evaporated to dryness to yield methanolic extract of *C. serratum i.e.*MECSR (yield: 12.9% w/w). The dried extract was suspended in 500 ml of water and subsequently fractionated with ethyl acetate (3×250 ml) by refluxing for 2 h followed by evaporation to dryness under reduced pressure. It was then designated as Ethyl acetatefraction of *C. serratum* (EFCSR, yield: 2.42% w/w) and was stored in labelled bottles at 4°C for further use.

Stock solution (1 mg/ml) of the fractionEFCSR was prepared by dissolving 100 mg of dried fraction in 100 ml of

methanol. Aliquots from these stock solutions were further diluted with methanol as per the concentrations required and used for quantitative estimation of phytoconstituents, anti-inflammatory and anti-asthmatic studies etc.

2.4 Determination of chemical composition of EFCSR fraction

Gas chromatography-Mass Spectrum (GC-MS) analysis of EFCSR was performed on a GCMS-QP2010 (Shimadzu) instrument fitted with Elite-1 fused silica capillary column (30 mm x 0.25 mm inner diameter x 0.25 μm film thickness) composed of 5% Diphenyl and 95% Dimethyl poly siloxane. Electron ionization system with ionizing energy of 70 eV was used for detection. Helium (99.99%) was used as carrier gas at a constant flow rate of 1.0 mL/min and an injection volume of 2 μl was employed (Split ratio of 10:1). The oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. An electron ionization (EI) Mass spectrum was acquired over a mass range 10 to 400 Da at a scan interval of 0.5 seconds. Total GC running time was 45 minutes. The identification and characterization of chemical compounds in EFCSR was based on GC retention time. The mass spectra were computer matched with those of standards available in mass spectrum libraries (NIST, 1998).

2.5 Isolation of Ursolic Acid (UA):

Preparation of the column: Glass column (60cm x 4.5cm) was packed by wet filling. The slurry of adsorbent (silica gel; 60-120 mesh) was prepared by mixing the adsorbent in the methanol and used as stationary phase. It was then poured into glass column and allowed to settle. The air entrapped was removed by stirring with glass rod. Excess of solvent was run off.

Preparation of sample and loading: EFCSR (5g) was dissolved in a minimum volume of methanol, adsorbed on silica gel (60-120 mesh), dried and applied on a column with 10 cm internal diameter and 50 cm length.

Selection of mobile phase and separation of the phytoconstituents: The combinations of solvents optimized for TLC separation were used as mobile phase for column chromatography. Alteration in the composition of the eluting solvent was achieved by adding the second solvent of more polarity gradually to a reservoir of the first. The EFCSR (5 gm) was subjected to silica gel column chromatography with a gradient of increasing polarity from total n-hexane to total ethyl acetate followed by total chloroform to total methanol as mobile phase. Mobile phase was passed with constant flow rate (5ml/min). Fractions (about 135) of 50 ml each were collected (table 1). The separation of phytoconstituents was monitored by thin layer chromatography (TLC) developed with the different solvent systems mentioned for each fractions using vanillin sulphuric acid as the detecting agent (Wagner et al., 1996). Fractions with similar TLC pattern were pooled together and evaporated to dryness under reduced pressure. Those fractions which were eluted

in considerable quantities and in pure form were characterized after recrystallization from methanol.

Table 1 Separtion of phytoconstituents from ethyl acetate fraction of C. serratum roots

Fractions (weight)	Eluting solvents and composition (% v/v)	Detection	Remarks
1 to 6 (100 mg)	*n*-hexane (100 %)	*n*-hexane: ethyl acetate (9:0.5)	It contained one UV- active spot (254 nm) with the R_f value of 0.77.
7 to 9 (150 mg)	*n*-hexane/ethyl acetate (9:1)	*n*-hexane: ethyl acetate (8:2)	Showed two violet spots at the R_f value of 0.45 and 0.50 on derivatized with vanillin sulphuric (VS) acid reagent and subsequently heating at 110 °C
10 to 15 (250 mg)	*n*-hexane/ethyl acetate (8:2)	toluene-ethyl acetate-formic acid (8:2:0.1)	Single band with R_f value of 0.52 on derivatization with VS reagent *From the non-derivatized TLC plates, the single band with R_f value of 0.52 was scraped, recrystallized for further purification from methanol and characterized. This was denoted as Compound I*
16 to 21 (220 mg)	*n*-hexane/ethyl acetate (7:3)	toluene-ethyl acetate-formic acid (8:2:0.1)	Single band with R_f value of 0.58 on derivatization with VS reagent *From the non-derivatized TLC plates, the single band with R_f value of 0.58 was scraped, recrystallized for further purification from methanol and characterized. This was denoted as Compound II*
22 to 27 (200 mg)	*n*-hexane/ethyl acetate (6:4)	*n*-hexane: ethyl acetate (8:2)	It contained one UV active spot with R*f* of 0.29 and two blue colored spots 0.13 and 0.17 on derivatization with VS reagent
37 to 48 (180 mg)	*n*-hexane/ethyl acetate (5:5)	*n*-hexane: ethyl acetate (6:3)	One blue colored spot at R*f* of 0.29 on derivatization with VS reagent

49 to 54 (200 mg)	*n*-hexane/ethyl acetate (3:7)	*n*-hexane: ethyl acetate (2:6)	Three violet colored spots at R*f* of 0.23, 0.28, 0.37 on derivatization with VS reagent
55 to 63 (220 mg)	Ethyl acetate (100%)	*n*-hexane: ethyl acetate (1:4)	Five violet colored spots at R*f* of 0.22, 0.27, 0.48, 0.56 and 0.63 on derivatization with VS reagent
64-69 (200 mg)	Chloroform (100 %)	Chloroform: methanol(4:1)	Two blue colored spots at R*f* of 0.66 and 0.61 on derivatization with VS reagent
70-84 (250 mg)	Chloroform/methanol (9:1)	chloroform/ methanol(4:1)	One UV active spot at R*f* of 0.65 and one blue colored spot at R*f* of 0.54 on derivatization with VS reagent
85-100 (340 mg)	Chloroform/methanol (8:2)	chloroform-methanol-formic acid (4:2:0.1)	The single band with R_f value of 0.53 on derivatization with VS reagent *From the non-derivatized TLC plates, the single band with R_f value of 0.53 was scraped, recrystallized for further purification from methanol and characterized. This was denoted asCompound III*
100-115 (280 mg)	Chloroform/methanol (5:5)	Chloroform: methanol(3:2)	Two blue colored spots at R*f* of 0.60 and 0.64 on derivatization with VS reagent
115-135 (420 mg)	Methanol (100 %)	-	For washing

The *sub fraction 1* was further separated by silica gel column chromatography and eluted with increasing amount of toluene to ethyl acetate (90:10 to 70:30 v/v) as eluent. Total three subtractions (subtractions 1A to 1C) were collected by monitoring the elution process with TLC using vanillin sulphuric acid as spray reagent. The *sub fraction 1C* eluted using toluene/ethyl acetate (85:15 v/v) was purified by preparative TLC using toluene-ethyl acetate-formic acid (8:2:0.1) mobile phase and recrystallized for further purification from methanol, as a pure pale yellow amorphous powder (24 mg).

2.6 Characterization of UA:

Isolated compound was subjected to different characterization techniques. The IR spectra were obtained using JASCO FT-IR 5300 (Japan) in KBr disc and absorption peaks in terms of wave numbers (cm-1) were noted for isolates (in methanol). EIMS (electron impact mass spectrum) in positive mode, were recorded on Shimadzu LCMS 2010A (Japan) instrument. NMR spectra were recorded on Brucker (200MHz) supercon multi nuclei probe spectrophotometer (West Germany) at 400 MHz (1H) and 100 MHz (13C). Chemical shifts were recorded as δ value (ppm) using TMS (tetra methyl silane) as internal standard. The spectra were observed on pyridine-d5.

2.7 Animals and drug preparation/administration

The male Dunkin-Hartley guinea pigs (350-500 g) were housed in an air-conditioned area with a light-dark period of 12 hours (temperature:22±2 ° C, humidity: 50±5 percent). Feed was given to animals with an ad-libitum free access to water by means of a regular pellet diet (certified Amrut Rotent Food, Pune, India). All studies were conducted in compliance with the protocol accepted by the Institutional Animal Ethics Committee (IAEC) in full adherence with ethic guidelines. All experiments were carried out with strict adherence to ethical guidelines and were conducted according to the protocol approved by the Institutional Animal Ethics Committee (IAEC) (Approval no: IAEC/DPS/SU/1415)and according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Ursolic acid was suspended with 0.5% sodium carboxyl methyl cellulose before oral administration. The animal dose selection of UA (Lee et al., 2010) was done on the basis of literature reports with no toxicity observed in preliminary experiment.

2.8 Bronchodilatory Effect of UA treatment on guinea pigs

The Broncho-dilatory activity of UA was evaluated in guinea pigs by exposing them to 0.5% w/v of histamine hydrochloride. Briefly, guinea pigs (250-350 g body weight) of either sex were selected and randomly divided into 05 groups each containing six animals.

Group 1 (vehicle control): 0.5% CMC-Na (1 ml/kg b.w.);

Group 2 (positive control): ketotifen fumarate (1 mg/kg b.w., *p.o.*) treated;

Groups 3-5: 7.5, 15 and 30 mg/kg b.w., *p.o.* of UA treated, respectively;

The single dose treatment was given one and half hour before challenged with histamine aerosol. Later the animals were exposed to an aerosol of 0.5% w/v of histamine hydrochloride. The time to onset of pre-convulsion dyspnoea (PCD) during challenge with histamine aerosol was

noted for each animal (Sheth et al., 1972).

2.9 Effect of UA treatment on ovalbumin (OVA)-induced airway inflammation in guinea pigs

Experimental groups and drug treatment: Animals were divided into seven groups (n=6/group).

Group I received distilled water, non-sensitized controls (2.5 ml/kg b.w.);

Group II, disease control group, sensitized to ovalbumin and supplemented with respective vehicle

Group III, the positive control group,ovalbumin and DXM was provided (5 mg/kg b.w.);

Group IV was sensitized to ovalbumin and provided UA (15 mg/kg b.w.);

All animals (except group 1) were sensitized and challenged using subcutaneous injection of 100 µg of OVA (which was adsorbed onto 100 mg of aluminium hydroxide in saline) on day 0 as the first sensitization. Two weeks later, an antigen was enhanced using the same dosage (i.e., on day 14). The daily oral doses of test and reference drugs or vehicle were conducted on day 18 and persisted until day 29.

During days 18-29, 2.5 h after receiving the appropriate drug or vehicle treatment, the animals were challenged with 0.5% w/v of aerosolized OVA for 10 min. For the challenge, conscious animals were placed into a plastic circular chamber connected to Nebulizer (Omron Medical Company Ltd., Japan). Animals from the non-sensitized group, group-I have been subjected to the same treatment of aerosolised saline (Duan et al., 2003).

2.10 Broncho alveolar lavage fluid (BALF) and serum preparation:

At 24 h after the last OVA challenge (on day 30), blood sample (3 ml) was collected from each animal under light ether anaesthesia. Then each sample was divided into two parts. A non-heparinized tube for serum separation was used for the first aliquot (2.5 ml); the isolated serum was stored at -80°C until quantitative determination of cytokines (IL-4, IL-5 and TNF-α). The second portion (0.5 ml) was kept for leukocyte count in heparinised tube. Each sample was centrifuged at 500×g for 10 min at 4°C and the cells in the pellet were washed and processed for further studies using 0.5 ml of saline. And stored for additional studies.

Broncho alveolarlavage fluid (BALF) was collected from each animal at the end of the experiment after collecting blood samples. The overdose of ketamine (30 mg / kg b.w.) and of xylazine (20 mg/ kg b.w.) in all the guinea pigs. Into the trachea a polypropylene cannula (24 G) was inserted to the lungs, then the normal saline solution (10 ml) was administered by a 10 ml syringe (0.9 percent w / v) at 37 °C and recovered after five minutes. The recovered lavage fluid (5 ml) was centrifuged at 500×g for 10 min at 4°C; for the determination of cytokine, the

corresponding supernatant has been extracted and preserved at -80 ° C. The cells in the pellet have been salinised in 0.5ml and processed in cell counts.

2.11 Inflammatory cell count in blood and BALF:

The total and differential cell counts blood and BALF were manually with a haemocytometer performed after staining with Giemsa solution. A minimum of 200 cells were counted using a compound microscope and identified as macrophages, eosinophil's, neutrophils, or lymphocytes based on normal morphologic criteria (Sanjar et al, 1990).

2.12 Cytokines in serum and BALF:

In each sample of recovered serum (400 µl) and BALF (4.5 ml), the concentrations of TNF-α, IL-4 and IL-5 were calculated using the procedure of the manufacturer's immunosorbent assay (ELISA) kits. All plates have been analyzed in an automated reader(Robonik India Pvt. Ltd., India).

2.13 Lung tissue histopathology:

Immediately after the Broncho alveolar fluid sample, lung tissue lobes were separately dissected for each animal One lobe was used in non-lavagely determined histamine, and the other for tissue histology.Tissues were washed with normal saline (5 ml) and then put in 10% v / v of formaldehyde solution for one week for the lung tissue histopathology.After fixation, tissue were blocked in paraffin wax, sectioned at 5 µm thickness and stained with haematoxylin and eosin dye to assess morphology. Tissues were subsequently mounted and cover slipped for microscopic examination.

2.14 Histamine assay on lavage lung tissue:

Lung tissue (200±20 mg) homogenate was prepared in normal saline, 2.5 ml of per chloric acid (0.4 N) was added followed by mixing and centrifugation (4000×g, 7min at 4°C), resulting supernatant was taken to a test tube having 0.25 ml of 5 N NaOH, 0.75 g NaCl and 5ml *n*-butanol. The mixture was vortexed for 5 min to partitUrsion histamine into the butanol and after centrifugation; the aqueous phase was discarded by aspiration. In order to remove residual amounts of histidine extracted to the *n*-butanol, the organic phase was further shaken with 2.5 ml salt saturated 0.1 N NaOH solution to remove any residual histamine. The mixture was re-centrifuged and the *n*-butanol layer was transferred to a test tube containing 2 ml of 0.1 N HCl and 5 ml *n*-heptane. After shaking for 5 minutes the tube was centrifuged and the organic phase was removed by aspiration. A 2 ml aliquot of the aqueous phase was transferred to a test tube and 0.4 ml of 1 N NaOH was added followed by 0.1 ml of o-phthalaldehyde reagent. After 4 minutes, 0.2 ml of 3 N HCl was added. The solution was then transferred to a cuvette and the fluorescence (excitation wavelength: 356 nm and emission wavelength: 450 nm) was measured with a spectrofluorimeter (SL-174, Elico, India) (Shore et al., 1959).

Statistical analysis

Results are reported as mean±S.E.M. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by post hoc Tukey's test; differences were considered statistically significant at $P<0.05$. All statistical analyses were performed using the Graph Pad software.

3. Results

3.1 Determination of chemical composition of EFCSR of C.

The GC-MS analysis of EFCSR enabled the presence of eighteen components (table 2) identified on the basis of retention time (RT), fragmentation patterns and data comparison with NIST mass spectral library. These compounds mainly comprised of acids, hydrocarbons, esters and alcohols. *n*-Hexadecanoic acid (19.03%) was identified as a major constituent followed by diethyl phthalate (16.44%), all trans-squalene (10.72%), dodecamethylpentasiloxane (7.53%), acetic acid hydroxy- ethyl ester (6.47%) *etc.* along with other major and minor

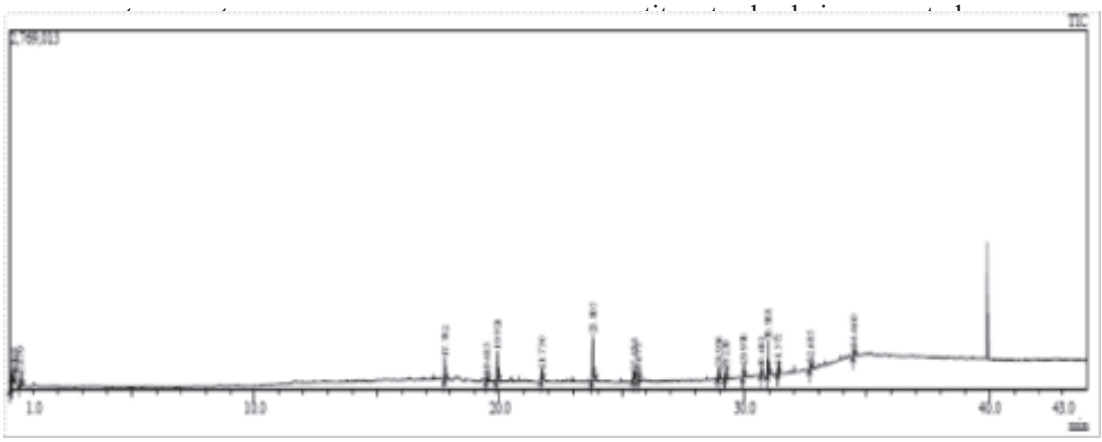

Figure 1 GC-MS analysis of EFCSR of C. serratum roots

Table 2 GC-MS analysis of EFCSR of C. serratum roots

Retention time	Area %	Compound Name
0.025	4.92	*n*-Dodecanal
0.103	6.47	Acetic acid hydroxy- ethyl ester
0.479	5.93	Acetic acid
17.782	7.53	Dodecamethylpentasiloxane
19.483	2.09	*n*-Dodecanoic acid
19.921	16.44	Diethyl Phthalate
21.730	4.11	Tetradecanoic acid
23.805	19.03	*n*-Hexadecanoic acid
25.484	3.14	Oleic acid
25.673	1.70	*n*-Octadecanoic acid
28.936	3.34	1,2-Benzenedicarboxylic acid dioctyl ester
29.230	1.74	*n*-Hexacosane
29.970	3.34	*n*-Octacosane
30.683	1.92	*n*-Nonacosane
30.968	10.72	All trans-Squalene
31.372	2.34	*n*-Tetracosane
32.685	2.34	*n*-Tritriacontane
34.460	2.91	Stigmast-5-en-3-ol

Ethyl acetate fraction of successive 70% methanol extract of C. serratum roots was subjected to silica gel column chromatography to yield compounds I,later confirmed as ursolic acid,from gradient of n-hexane/ethyl acetate eluent. The characterization of isolated compounds was performed using different spectral techniques (IR, MS, 1H-NMR and 13C-NMR) to reveal their identity. Compound I was identified as ursolic acid (Lin et al., 1987; Tundis et al., 2002) by direct comparison of the spectra with those reported in literature.

Compound I (Ursolic Acid)

TheCompound I (Ursolic Acid)was isolated as pale yellow amorphous powder (24 mg), subjected to further identification and characterization studies (table 3; Figure 2-6).

Table 3 Characteristics of isolated Ursolic Acid

State:	**Pale yellow amorphous powder**
Chemical test:	Lieberman Burchardt test
FT-IR (KBr) (cm^{-1}):	3437, 2944, 2877, 2650, 1698, 1463, 1387, 1364, 1322, 1303, 1237, 1207, 1188, 1106, 1093, 1008, 949, 825, 815, 679, 655, 539
EI-MS (*m/z*):	457 [M]+(calc. for $C_{30}H_{48}O_3$), 439, 411, 421, 391, 369, 355, 343, 331, 307, 279, 261, 247, 231, 217, 203, 191, 177, 163, 151, 137, 118, 102, 88, 82, 56
^{1}H NMR (Pyridine, 400 MHz, ppm):	δ H 3.44 (1H, t, J = 8.8 Hz, H-3), 5.46 (1H, br s, *J* = 3.6 Hz, H-12), 2.09 (1H, dt, J = 4.0, 13.1 Hz, H-16α), 2.30 (1H, dt, J = 4.8, 13.4 Hz, H-15β), 2.61 (1H, br d, J = 11.2 Hz, H-18), 1.19 (1H, *m*, H*a*-22), 1.22 (3H, *s*, Me-23), 1.02 (3H, *s*, Me-24), 0.87 (3H, *s*, Me-25), 1.00 (3H, *s*, Me-26), 1.21 (3H, *s*, Me-27), 0.98 (3H, *d*, *J* = 6.8 Hz, Me-29), 0.93 (3H, *d*, *J* = 5.8 Hz, Me-30)
^{13}C NMR (Pyridine, 100 MHz, ppm):	δ C 39.0 (C-1), 28.0 (C-2), 78.1 (C-3), 39.3 (C-4), 55.8 (C-5), 18.7 (C-6), 34.2 (C-7), 39.9 (C-8), 48.0 (C-9), 37.2 (C-10), 23.6 (C-11), 125.5 (C-12), 139.2 (C-13), 42.2 (C-14), 28.6 (C-15), 24.8 (C-16), 48.0 (C-17), 53.5 (C-18), 39.4 (C-19), 39.3 (C-20), 31.0 (C-21), 37.4 (C-22), 28.7 (C-23), 16.5 (C-24), 15.6 (C-25), 17.5 (C-26), 23.8 (C-27), 180.0 (C-28), 17.4 (C-29), 21.4 (C-30)

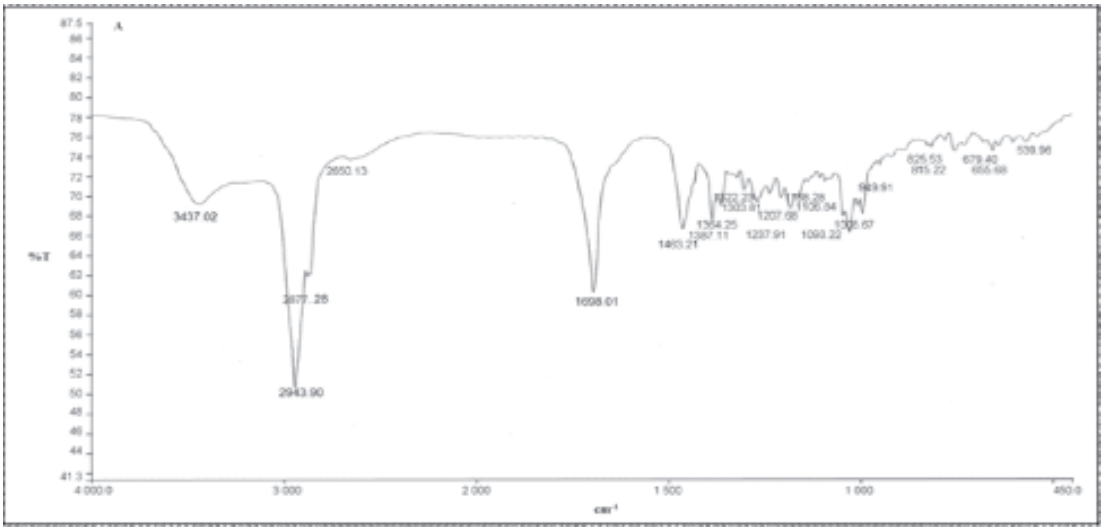

Figure 2 FT-IR spectra of isolated Ursolic Acid

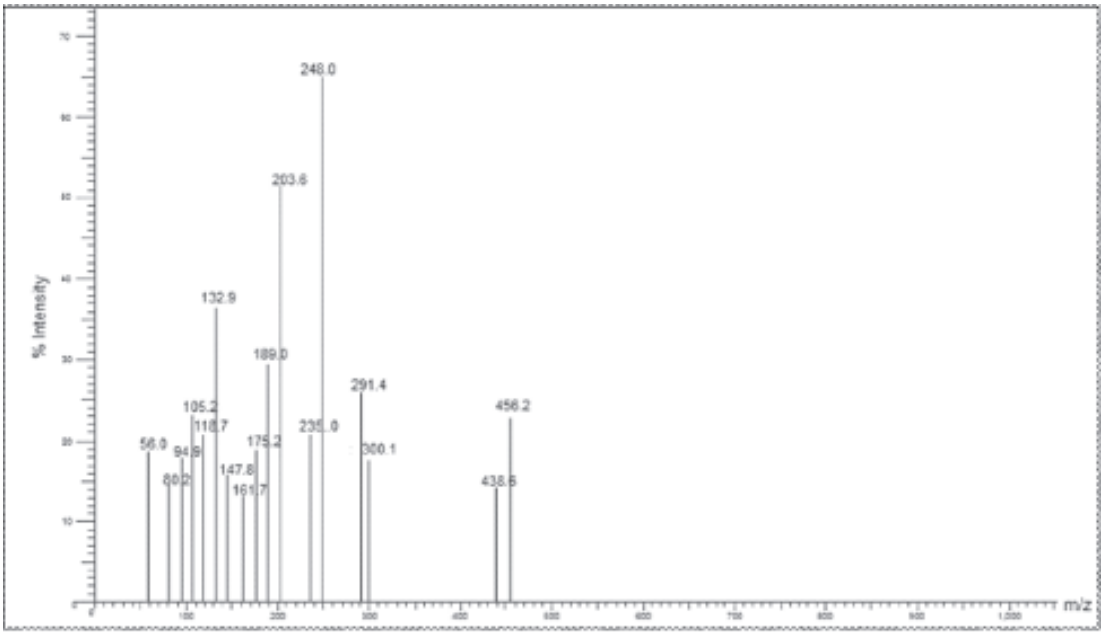

Figure 3 Mass spectrum of isolated Ursolic Acid

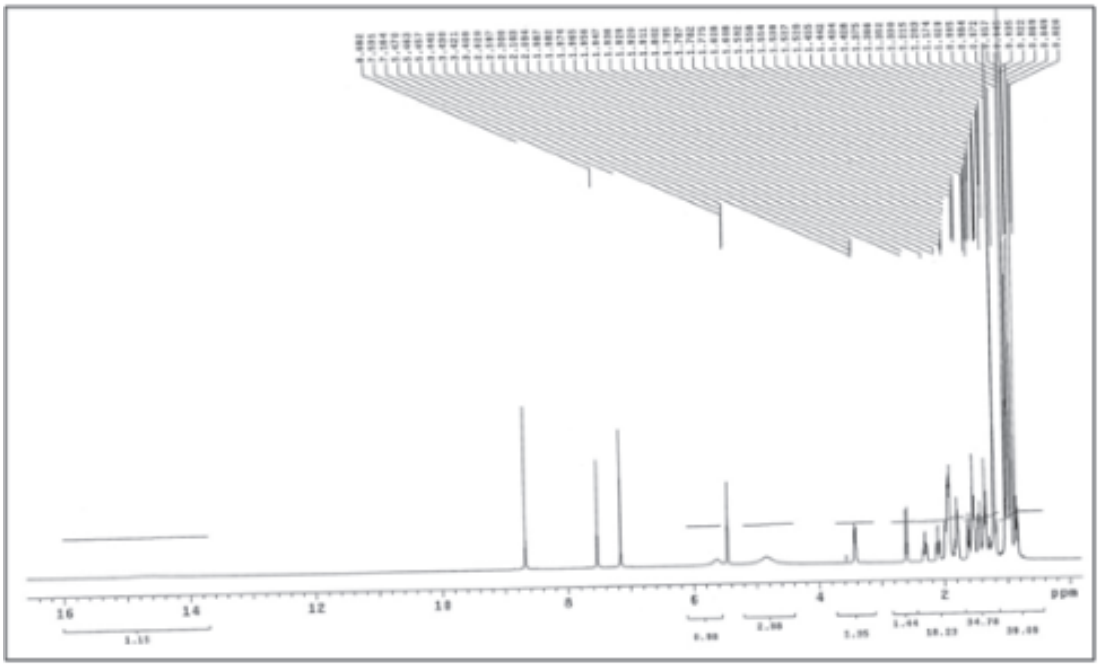

Figure 4 ^{1}H-NMR spectrum of isolated Ursolic Acid

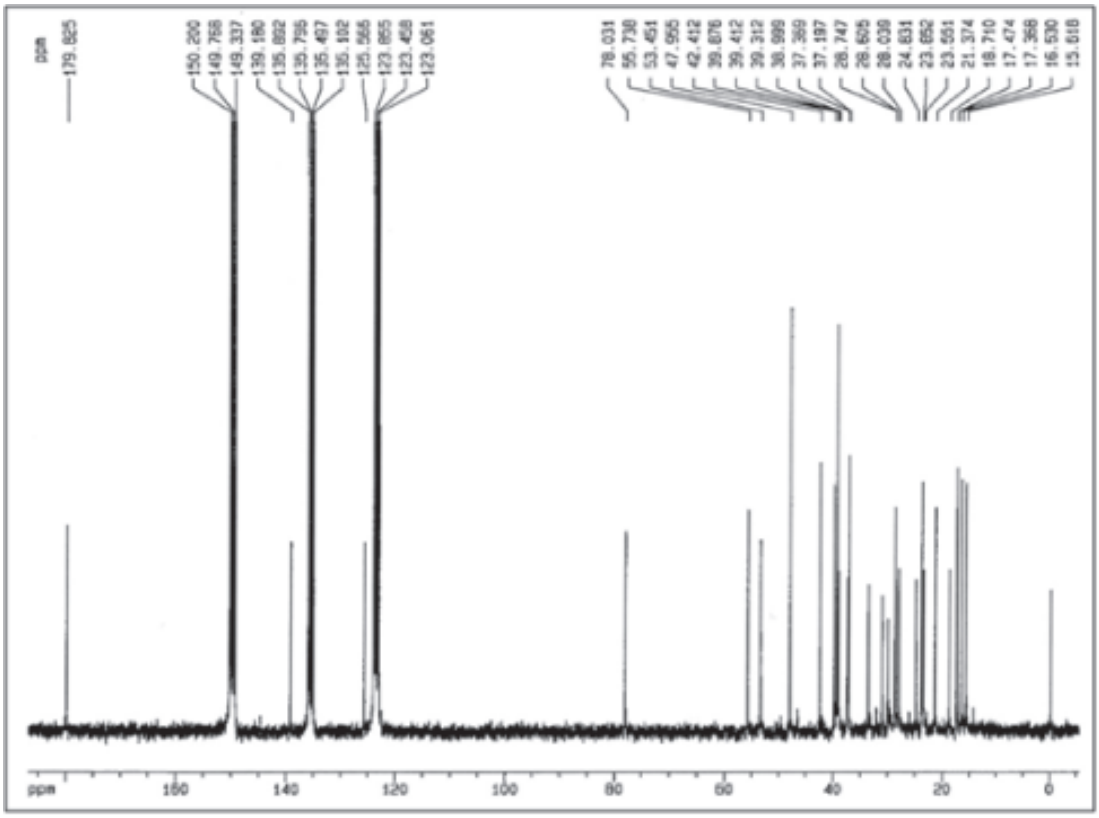

Figure 5 ^{13}C-NMR spectrum of isolated Ursolic Acid

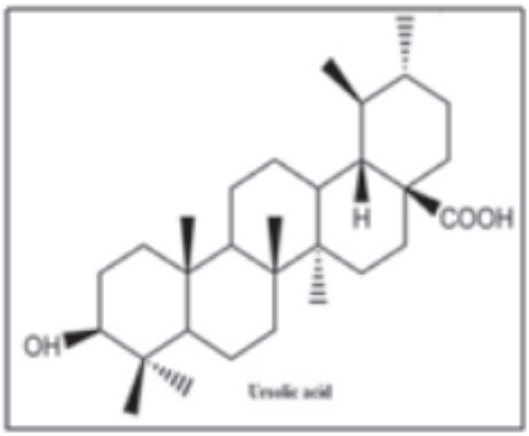

Figure 6 Structure of ursolic acid

Effect of UA treatment in guinea pigs on histamine-induced bronchospasm

3.2 Effect of UA treatment on histamine-induced bronchospasm in guinea pigs

The dose dependent and statistically significant ($p<0.001$ to $p<0.05$) broncho-dilation potential was observed with increase in preconvulsion time on pretreatment with UA isolated from EFCSR in guinea pigs on exposure of 0.5 % w/v of histamine hydrochloride aerosol (table 4). From this study, it was notable that UA post treatment at doses of 15 and 30 mg/kg significantly prolonged the pre-convulsion time compared to positive control (ketotifen fumarate, 1 mg/kg) animals. Hence, a lower dose (15 mg/kg) was selected for further studies.

Table 4 Effect of UA treatment in guinea pigs on histamine-inducing bronchospasm

Treatment	Dose (mg/kg b.w., p.o.)	% Increase in PCD time
CMC-Na (Control)	1ml/kg	4.18±0.42
Ketotifen fumarate	1	60.55±1.40***
UA	7.5	28.45±0.63*
	15	35.24±0.80*
	30	47.15±0.31**

n=6, ***P<0.001, **P<0.01, *P<0.05 compared with data of CMC-Na (control)

Effect of UA treatments on ovalbumin (OVA)-induced airway inflammation in guinea pigs

3.3 Effect of UA treatments on body weight: ***Each animal from the OVA control and experimental groups showed no significant body weight differences compared to the non-sensitized controls during the study period*** (Figure 7). Effects upon appetite/ water consumption or outward appearance (i.e. fur coat, eyes) was not observed.

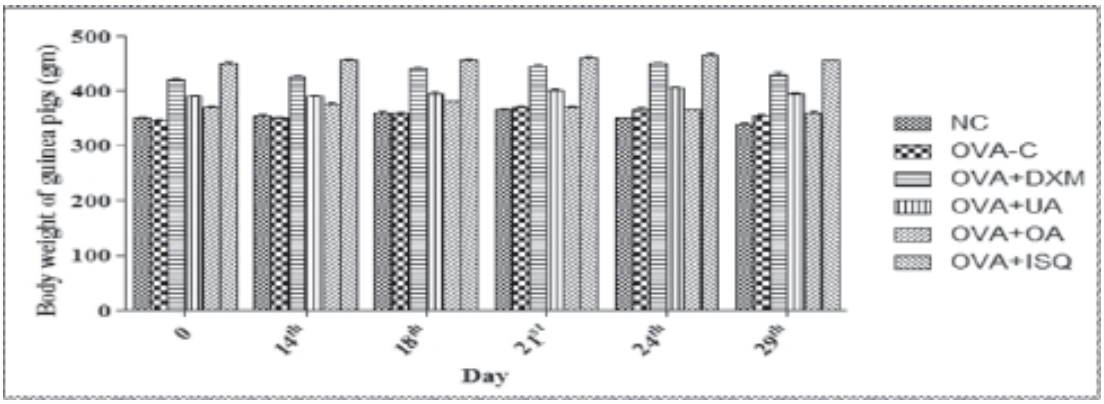

Figure 7 Effect of treatments on body weight of guinea pigs. Data are represented as mean ± SEM, n=6 guinea pigs per group. NC: Non-sensitized control, OVA-C: disease control sensitized with ovalbumin, OVA+DXM: diseased treated with dexamethasone (5 mg/kg), OVA+UA: diseased treated with ursolic acid (15 mg/kg)

3.4 Effect of UA treatment on total cells in blood and BALF:

To determine the protective effects on airway inflammation, the total cell numbers in blood and BALF were examined in disease control and experimental groups treated with UA (table 5).

The total number of leukocytes in blood samples of the disease control animals were significantly increased ($p<0.01$) compared to the non-sensitized controls. Dexamethasone ($p<0.001$), UA ($p<0.05$) treatment significantly inhibited the recruitment of leukocytes into blood as compared with disease control animals.

The study of BALF showed that compared to non-sensitized controls the number of total cells in OVA control ($p<0.001$) guinea pigs has been significantly increased. Dexamethasone ($p<0.001$) and UA ($p<0.01$) treatment significantly decreased the total number of cells in contrast with diseased control animals.

Table 5 Effect of UA treatments on total cell count in blood and in BALF

Treatment	Total cellular count ($x10^5$cells/ml)	
	Blood	BALF
NC	8.22±0.11	8.51±0.15
OVA -C	21.96±0.16**	17.64±0.26***
OVA + DXM	12.92±0.17$^{\#\#\#}$	10.74±0.14$^{\#\#\#}$
OVA + UA	17.64±0.20$^{\#}$	14.28±0.12$^{\#\#}$

n=6, Significantly different from non-sensitized control ***p<0.001, **p<0.01; significantly different from OVA-control #p< 0.05, ##p<0.01, ###p< 0.001

3.5 Effect of UA treatments on differential leukocytes in blood and BALF:

As shown in table 6, OVA (disease control) sensitized animals showed significant influx of leukocytes, eosinophil's and other inflammatory cells into blood and BALF compared to the non-sensitized control group animals. However, the numbers of circulating eosinophils, neutrophils, lymphocytes and monocytes were decreased significantly in dexamethasone and UA treated animals, respectively as compared to those numbers in OVA-control guinea pigs in blood (table 6). OVA sensitized guinea pigs treated with test samples displayed similar suppressive effects on differential cell count into BALF. The reference drug, dexamethasone ($p<0.001$) significantly decreased eosinophil's, neutrophils, lymphocytes and monocytes count upon asthma induction.

Table 6 Effect of UA treatments on differential cells count in blood and BALF (x 10^5cells/ml)

Treatments	Eosinophil		Neutrophils		Lymphocytes		Monocytes	
	Blood	BALF	Blood	BALF	Blood	BALF	Blood	BALF
NC	0.54± 0.007	0.40± 0.005	0.220± 0.005	0.240± 0.015	1.20± 0.046	1.00± 0.060	0.64± 0.010	0.40± 0.010
OVA -C	0.93± 0.012**	0.83± 0.010***	0.360± 0.009**	0.390± 0.018***	3.56± 0.045***	3.38± 0.026***	1.13± 0.027**	0.83± 0.020***
OVA + DXM	0.60± 0.010$^{\#\#\#}$	0.42± 0.011$^{\#\#\#}$	0.230± 0.007$^{\#\#\#}$	0.250± 0.009$^{\#\#\#}$	2.96± 0.010$^{\#\#\#}$	2.79± 0.012$^{\#\#\#}$	0.72± 0.015$^{\#\#\#}$	0.62± 0.018$^{\#\#\#}$
OVA + UA	0.72± 0.009$^{\#}$	0.53± 0.016$^{\#\#}$	0.251± 0.007$^{\#}$	0.285± 0.019$^{\#\#}$	3.31± 0.028$^{\#\#}$	3.28± 0.031$^{\#\#}$	0.83± 0.017$^{\#}$	0.71± 0.012$^{\#}$

n=6, significantly different from non-sensitized control $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$; significantly different from OVA-control $^{\#}p< 0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p< 0.001$

3.6 Effect of treatments on cytokines level in serum and BALF:

The level of serum IL-4, IL-5 and TNF-α in OVA-sensitized guinea pigs were significantly ($p<0.001$) greater than non-sensitized control. Dexamethasone treatment ($p<0.001$) caused significant reduction in the levels of IL-4, IL-5 and TNF-α as compared to the OVA-control. Treatment with other test samples also showed reduction in cytokines level analyzed in serum (Table 7).

To establish the mechanisms underlying the inhibitory activity of isolated compounds (OA) in airway inflammation, the levels of cytokines (IL-4, IL-5 and TNF-α) in BALF were also examined. The levels of IL-4, IL-5 and TNF-α in BALF were significantly increased in OVA-sensitized guinea pigs, compared with those in non-sensitized control animals.

Table 7 - Effect of UA treatments on cytokines level in serum and BALF

Treatment	IL-4		IL-5		TNF-a	
	Serum	BALF	Serum	BALF	Serum	BALF
NC	51.41± 0.54	20.13± 0.95	17.20± 0.67	26.61± 1.01	21.32± 0.80	18.22± 0.89
OVA-C	87.36± 1.32***	53.42± 1.07***	47.34± 0.42***	59.34± 1.21***	56.48± 1.14***	46.31± 1.29***
OVA + DXM	54.80± 1.55$^{###}$	34.86± 0.94$^{###}$	30.18± 0.88$^{###}$	33.12± 1.36$^{###}$	27.21± 1.27$^{###}$	24.40± 1.22$^{###}$
OVA + UA	60.45± 1.04$^{##}$	40.67± 0.45$^{#}$	35.26± 0.48$^{#}$	40.86± 1.03$^{#}$	39.37± 1.89$^{#}$	33.87± 0.78$^{#}$

n=6, significantly different from non-sensitized control $^{***}p< 0.001$.

Significantly different from OVA-control $^{###}p< 0.001$; $^{##}p<0.01$; $^{#}p< 0.05$

3.7 Effect of UA treatment on histopathological changes in lung tissue:

The airway inflammation effect of UA was illustrated by lung histopathological examination. Massive inflammatory cells infiltration was detected in the peribronchovascular lung areas of ovalbumin challenged guinea pig compared to non-sensitized control guinea pig, exhibiting severe airway inflammation. Treatment with dexamethasone (5 mg/kg), UA showed a significant reduction of inflammatory cells (Figure 8).

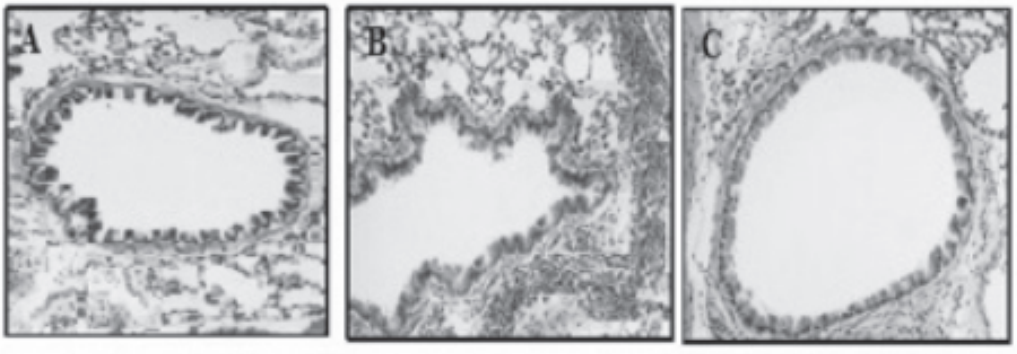

Figure 8 Effect of UAtreatment on histopathological changes in lung tissue represents haematoxylin and eosin stained lung tissue. **A**: normal lung histology (non-sensitized control), **B**: disease control (sensitized with ovalbumin), **C**: lung tissue section from OVA+DXM (5 mg/kg) treated animal, **E**: lung tissue section from OVA+UA (15 mg/kg) treated animal

3.8 Effect of treatments on histamine level in lung tissue:

Histamine content in lung tissues of the disease control guinea pigs was significantly higher ($p<0.01$) than that of the non-sensitized animals (table 8). Treatment with UA ($p<0.05$) normalized the elevated histamine level into lung tissues. However, their effects were lower than those of DXM (5 mg/kg).

Table 8 Effect of UA treatments on histamine level in lung tissue

Treatment	Concentration (mg/gm. of tissue)
NC	4.3±0.02
OVA -C	$7.9\pm0.10^{***}$
OVA + DXM	$5.1\pm0.05^{\#\#\#}$
OVA + UA	$6.1\pm0.03^{\#}$

4. Discussion

The present study was to evaluate the potential of EFCSR in asthma and to determine the chemical composition using GC-MS analysis. The EFCSR could be assigned to the presence of polyphenols including flavonoids and terpenoids, which further supports the results of phytochemical studies performed in earlier study. Based on the results of phytochemical studies performed for the EFCSR, the isolation of bioactivesresponsible for the anti-asthmatic potential were planned to separate them using column chromatography technique.

Column chromatography was performed and ursolic acid was isolated. The ursolic acid was obtained as pale yellow amorphous powder and gave positive Liebermann burchard test indicating its triterpenoid nature. The FT-IR spectrum showed characteristic absorption band at 3436 cm^{-1} and 3331cm^{-1} indicating the stretching of hydroxyl (-OH) group mainly from phenolic groups. A characteristic peak at 1697cm^{-1} appeared in compound due to carbonyl carbon (C=O).

The mass spectrum of the ursolic acidshowed base peak at *m/z* 456 and *m/z* 439 which is characteristic for ursane type of triterpenes of α- or β-amyrin series with 12-13 double bond in agreement with the molecular formula $C_{30}H_{48}O_3$. Ursane type of triperpenes contain α-amyrin skeleton while oleane type of triterpenes show its C_{30} isomer, β-amyrin skeleton. Apart from the molecular ion peak, the EI-MS showed other prominent fragment ions at *m/z* 248, 203, 177, 133, 95 and 69, which were characteristic for Δ12-amyrin skeleton (Awan et al., 2013). The prominent fragments ion peak at *m/z* 248 and 203 are characteristics of cleavage of B and C rings. Furthermore, the mass fragmentation at *m/z* 248 also indicated the location of hydroxyl group in ring A instead of in ring B, C, D or E and the ion fragment at *m/z* 203 indicated the presence of a carboxylic group in the ring D.

The 1H NMR spectrum of ursolic acid displayed signals for five methyl groups of H-22 to H-27 (δ 1.19, 1.22, 1.02, 0.87, 1.00, 1.21 ppm) together with signals corresponding to methyl protons of H-29 (δ 0.98 ppm, 3H, d, J = 6.8 Hz) and H-30 (δ 0.93 ppm, 3H, d, J = 5.8 Hz). The signal at δ 2.61 ppm attributed to H-18 (1H, d, J = 11.7 Hz) while H-3 signal observed at δ 3.44 (1H, t, J = 8.8 Hz). At δ 5.46 ppm was observed a large triplet attributed to H-12. NMR spectral data of ursolic acid showed signals corresponding to a triterpenoid skeleton of ursane type. The 1H NMR spectrum displayed

signals for five methyl groups (δ 0.73, 0.76, 0.87, 0.93 and 1.04) together with signals corresponding to methyl protons of H-30 (δ 0.82, 3H, d, 3J ¼ 6.4 Hz) and H-29 (δ 0.91, 3H, d, 3J ¼ 6.0 Hz). The signal at δ 2.14 was attributed to H-18 (1H, d, 3J ¼ 11.3 Hz) while H-3a signal was observed at δ 3.20 (1H, did). Finally, at δ 5.40 was observed a large triplet attributed to H-12. The 13C-NMR spectrum of isolated ursolic acid showed 30 signals, consisting of seven quaternary carbons, seven methines, nine methylenes and seven methyls. The most downfield signal resonated at δ180.0 is attributed to the carboxylic acid (C-28). The appearance of signals at δ125.5 (C-12) and 139.2 (C-13) indicated the presence of a double bond in urs-12-ene triterpenoid. Attribution of all resonances displayed on the 1H-NMR and 13C-NMR spectrum of isolated ursolic acid was accomplished by direct comparison with literature data (Ghosh et al., 2014).

The effects of EFCSR-isolated UA (15 mg / kg) have been observed in guinea pigs using asthma model caused by ovalbumin (OVA) to identify the mechanism of action as it shows similar effects such as human allergic asthma (Smith and Broadley, 2007). OVA challenge induces anaphylactiv reactions due to histamine, leukotrienes, prostaglandins, thromboxane A2 and platelet activating factor release. (Ogunbiyi and Eyre, 1985). Dexamethasone was used as cell migration inhibitor to the sites of inflammation (van der Velden, 1998). This study demonstrates that oral administration of UA (15 mg/kg), suppressed OVA-induced airway inflammation, accompanied by reduction of cytokine and histamine levels.

From the histamine induced bronchospasm study, it was notable that UA post treatment at all doses prolonged the pre convulsion time compared to positive control indicating the possible bronchodilatory activity. In this study, no animals showed any significant difference in body weight during the experimental period compared to the non-sensitized control animals, suggesting treatment did not interfere with the normal growth. Disease control group exhibited irritability, sneezing and hyper-rhinorrhoea, indicated the severity of disease.

Asthma is a major form of inflammatory airway disease After exposure to a specific trigger, namely eosinophil, inflammatory cells (Azzawi et al., 1990), neutrophils (Sur et al, 1993), lymphocytes (Robinson et al.,

1993), macrophages/ monocytes (Bentley et al , 1992) and mast cells infiltrate the airways (Fan and Mustafa, 2006).The main feature of allergic asthma is the eosinophilic inflammation of the airways with an increase in activated and degranulated asthma (Bousquet et al., 1990). Correlation between the eosinophil activation status in the airways and bronchial hyperrespondence development has been linked guinea pigs (Pretolani et al, 1994), non-human primates (Gundel et al., 1992) and humans (Wardlaw et al., 1988). In patients with allergic Asthma neutrophils are prominent inflammatory cells in the airways(Conese et al., 2003) and PGs, TXA2, LB4 and PAF capable is considered to be an important part of pathogenesis (Anticevich et al., 1996). Sputum analysis of patients with asthma and late-phase allergens, increasing numbers of lymphocytes expressing mRNA for IL-4 and IL-5 have been identified where they are associated with increased numbers of eosinophils (Till et al., 1995; Ying et al., 1997). Alveolar macrophages express the low-affinity receptor for IgE-FcεRII (Melewicz et al., 1982) and expression appears to be increased in asthmatic subjects compared to healthy persons (Williams et al., 1992). Inflammatory mediators (MacDermot et al., 1984) can induce endothelial cell activation, cell recruitment, and prolonged survival of eosinophil (Gosset et al, 1992). In the present study, the disease control animals showed significant increase in total and differential cellular counts in blood and in Broncho alveolarlavage fluid (BALF) directly correlate with increases in the level of cellular infiltration. Treatment with reference and test drugs alleviates broncho alveolar inflammation via decreasing the infiltration of differential inflammatory cells in blood (particularly eosinophil's and neutrophils) as well as in BALF. Thus, the results of the study suggested the possible usefulness of test drugs to control the activation of inflammatory process underlying exacerbation of allergic asthma.

Asthma treatment is now developed through a number of specific cytokine and chemical inhibitors, cytokines play a critical role in the facilitating and propagating of inflammation in asthmatic airways (Chungm and Bames, 1999). In particular, TNF-α and Th2-type cytokine interleukin (IL)-4and IL-5 (Martin et al., 2004) play central roles in initiating and sustaining an asthmatic response by regulating the recruitment and/or activation of airways mast cells and eosinophil's. IL-

4 is a critical factor for the regulation of T cell commitment to the CD4·Th2 phenotype and plays an essential role in immunoglobulin (IgE) switching in β-cells, mucus hyper-secretion, and eosinophil infiltration into lung tissue. Thus, IL-4 appears to be important in the early stages of Th2 cell development. By contrast, IL-5 regulates the growth, differentiation, and activation of eosinophil's and provides an essential signal for the recruitment of this leukocyte to the lung during allergic inflammation. IL-4 and IL-5 may also regulate eosinophil's trafficking by activating adhesion systems at the vascular endothelium. Activated eosinophils induce the release of pro-inflammatory mediators (Hogan et al., 1997). TNF-α may be an important mediator in the initiation of chronic inflammation, by activating the secretion of cytokines from a variety of cells in the airways (Hughes et al., 1995). The immune mediating activation of TNF-α can have a significant amplifying effect (Kips et al, 1992). There is evidence that asthmatic airways have increased expression of TNF-α (Bradding et al., 1994). In the present study, we have measured the level of elevated inflammatory cell markers IL-4, IL-5 and TNF-α in serum as well as in BALF. The results of our study suggest that, cytokines IL-4, IL-5 and TNF-α were increased predominantly in disease control (OVA-sensitized) animals indicating stronger anaphylactic sensitization leading to severe inflammation. Treatment with DXM and UA suppressed the level of these mediators significantly as compared to OVA-controls.

During allergic reactions the local release of histamine was recognized for many years as an important step for antigen hypersensitivity. Bartosch et al. (1932) first reported the release of histamine from the pig lung during anaphylaxis. Acute histamine release following an allergic or not allergic attack may result in bronchoconstriction, which is modulated by selective H1-receptor antagonists (Holgate and Finnerty, 1989). Further, histamine has been shown to activate eosinophils (Raible et al., 1994). In the present study, the fluorometric analysis of histamine content in lung tissues was carried out.

The existence of flavonoids and terpenoids in plants were reported to be responsible for anti-asthmatic properties along with antioxidant, anti-carcinogenic, anti-allergic and anti-inflammatory effects (Tanaka and Takahashi, 2013). These compounds contribute to their anti-asthmatic potential by inhibiting the release of

chemical mediators such as histamine and other preformed granule associated mediators by inhibiting the activation of basophils and mast cells, synthesis of Th2 type cytokines, such as IL-4 and IL-13 and CD-40 ligand expression by high-affinity immunoglobulin E (IgE) receptor-expressing cells, such as mast cells and basophils (Park, et al., 2010). Various flavonoids and terpenoids such as quercetin, apigenin, and betulinic acid have shown beneficial effects in asthmatic animal models. In support, our observation also confirmed a plausible molecular basis for the anti-asthmatic effect of UA isolated from *C. serratum* roots. In spite of the results presented in this study, it is necessary to explore the exact mechanism of the isolated compound.

5. Conclusion

From this study it can be inferred that UA isolated from *C. serratum* roots can have an anti-asthmatic potential due to inhibition of inflammatory cell infiltrations, release / synthesis of T-cell derved cytokines;IL-4,IL-5 and TNF-α. In addition, UA inhibited the releasing in the local lung tissue and trachea of mediators such as histamine, which was verified by historical pathology. These findings suggest that the *C. serratum* roots may be a valuable therapy for asthma management; however, a well-designed clinical trial is warranted, which includes persistent, mild or moderate asthmatic patients.

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