PREPARATION AND CHARACTERIZATION OF KETOCONAZOLE ENCAPSULATED LIPOSOME AND ETHOSOME: A COMPARATIVE STUDY

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BY

GUNJAN TIWARI

ROLL NO. 411LS2068

UNDER THE SUPERVISION OF

Dr. BISMITA NAYAK

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769 008, ODISHA, INDIA

THIS THESIS IS DEDICATED TO MY PARENTS.

For their endless love, support and encouragement.

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA

Dr. Bismita Nayak
Asst. Professor

Ref. No…..
Date……..

CERTIFICATE

This is to certify that the thesis entitled **"Preparation and Characterization of ketoconazole encapsulated liposome and ethosome: A Comparative study"** submitted to National Institute of Technology, Rourkela for the partial fulfillment of the Master degree in Life Science is a faithful record of bonafide and original research work carried out by Gunjan Tiwari under my supervision and guidance.

Dr. Bismita Nayak
Supervisor

DECLARATION

I, **Gunjan Tiwari**, hereby declare that this project report entitled **"**Preparation and characterization of ketoconazole encapsulated liposome and ethosome: A comparative study**"** is the original work carried out by me under the supervision of **Dr. Bismita Nayak**, Assistant Professor, Department of Life Science, National Institute of Technology Rourkela (NITR), Rourkela and to the best of my knowledge and belief the present work or any other part thereof has not been presented to any other University or Institution for the award of any other degree.

Gunjan Tiwari

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And to the almighty, who made all things possible……..

(GUNJAN TIWARI)

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Abstract

Skin act as a major target as well as a principle barrier for transdermal drug delivery. Vesicular system is one of the most promising approaches for transdermal delivery of active substances. Liposomes are most commonly used vesicular delivery system. But it has certain limitations such as lesser stability, reduced encapsulation efficiency, etc led to formulation of ethosomes. In the present work we encapsulated various concentration of ketoconazole (an antifungal drug) within ethosome and liposome and made a comparative evaluation of their morphology, size, potential, stability and anti-fungal efficacy. Ethosomes showed better stability, encapsulation efficiency and anti-fungal activity as compared to liposome due to its ethanolic content. So ethosomal formulation may prove as a very promising option for transdermal delivery and has potential for new opportunities for topical application of ketoconazole in the fungal infections.

Key words: drug delivery, transdermal, encapsulation efficiency, topical, ketoconazole liposome, ethosome.

Chapter 1
Introduction

Chapter 1

INTRODUCTION

Nanotechnology is a multidisciplinary field that covers a vast and diverse array of devices derived from engineering, physics, chemistry, and biology (Sahoo.S.K., et al, 2006). It is the technique of manipulating matter at the scale of atom and molecules (Drexler et al., 1986). In 1974 Norio Taniguchi used the phrase "nanotechnology" for the first time while describing an ion sputter machine. The term "nano" which means "dwarf" was originally derived from Greek word. Nanotechnology works with materials, devices, and other structures with at least one dimension sized from 1 to 100 nanometres. Nanometre is defined as one billionth of a metre that is equivalent to the length of ten hydrogen atoms. Now a day's nanotechnology has a global interest. As mentioned in Environmemt and green nano the term nanotechnology embraces various fields and specialities including green nanotechnology, wet nanotechnology, nanoengineering, nanobiotechnology. Application of nanotechnology in commercial products like nanomedicine and green technology minimizes energy consumption and enhances the environmental sustainability of processes currently producing negative externalities, thus increasing the efficiency of energy production or quantum aged atoms (QCAs) (Robert A. Freitas Jr. 1999). Nanotechnology also has numerous potential application in the fields of consumer goods, providing with products with novel functions ranging from easy to clean to scratch-resistant (Neethirajan et.al., 2009) .

The structural and functional unit of nanotechnology is nanoparticle. It is defined as a small object that behaves as a whole unit with respect to its transport and properties. According to their diameter particles are further classified into three categories.(Grangvist et al., 1976).

- Coarse particles (10,000-2,500 nm)
- Fine particles (100-2500 nm)
- Ultrafine particles (1-100 nm)

Various types of nanoparticles are present which includes Nanosphere, Nanocapsule, Dendrimer, Polymeric micelles, Liposome and SLN (solid lipid nanoparticles). Nanosphere are considered as a matrix system in which matrix is uniformly dispersed. Beside of these spherical vesicular system is known as nanocapsules. In case of polymeric nanoparticles, the polymeric membrane surrounds the drug in a matrix core. Mostly biodegradable polymers are

used in polymer nanoparticles likes polycyanoacrylate or poly (D, L-lactide) and related polymers like poly(lactic acid)PLA or poly(lactide-co-glycolide) etc. Dendrimers is a unique class of polymers which is highly branched macromolecules whose size and shape can be precisely controlled. Application of nanoparticles target drug delivery, drug bio-availability, detection of pathogen etc (Abhilash M,. 2010).

Phospholipids are the major components of all cell membranes as they form lipid layers. Phospholipid is a class of lipid mainly consisting of diglyceride and phosphate group. The structure of the phospholipid molecule consists of hydrophobic tails and a hydrophilic head. Lecithin or phosphatidylcholine from egg yolk was the first identified phospholipid. Other common phospholipids are phosphatidic acid (phosphatidate), phosphatidyethanolamine (cephalin), phosphatidylcholine (lecithin), phosphatidylserine, phosphoinositides, etc. Phospholipid synthesis occurs in the cytosol adjacent to endoplasmic reticulum (ER) (E Fahy et al., 2009).

Lecithin (phosphatidylcholine) is generic term which is used to designate yellow brownish fatty substance present in animal or plant tissue composed of phospholipids, phosphoric acid, triglyceride, glycolipids, etc. Lecithin is easily extracted from sources such as soybeans, eggs, milk, marine sources, rapeseed, cottonseed and sunflower chemically by using hexane, ethanol, etc. Lecithin has emulsification and lubricant properties. Lecithin from soybean and egg play an important role in drug delivery.

Liposome is an artificial microscopic single vesicle consisting of an aqueous core enclosed in one or more phospholipid layers used to convey vaccines, drugs, enzymes, or other substances to target cells or organs (Lawerence D, 1986). Liposomes are a form of nanoparticle prepared from lecithin. They are microscopic, concentric bilayered vesicles. Here the aqueous volume is entirely enclosed by a membraneous lipid bi-layer composed of natural or synthetic phospholipids. The major types of liposomes are multilamellar vesicles, small unilamellar vesicles, large unilamellar and cochleate vesicles (Sharma.A and Sharma U.S, 1997). Liposomes increase the efficiency, bioavailability, absorption of certain entrapped dietary and nutritional supplements and are used as topical drug delivery system. (C.George et al., 1975). But liposomal drug delivery system has certain shortcomings like the need for modification for site specific or organ specific drug delivery, high production cost and leakage and fusion of encapsulated drug/molecule. Moreover unfavourable reactions like

oxidation and hydrolysis of phospholipids reduces the half life of the formulation, decreases solubility and stability of drug in the medium (Sirisha.V.N et al., 2012). Demerits of liposome in skin delivery include a limited ability to penetrate narrow blood vessels or into skin to a significant degree. Some time the materials which one we want to deliver into skin may get entrapped in the inner layers of the liposomes while in certain conditions are virtually un- releaseable (Ralp Hill, 2011)

To overcome these shortcomings a modified form of liposome is used termed as ethosome. Ethosomes are ethanolic phospholipids vesicles, which have higher penetration rate through the skin than liposomes. It has higher penetration rate, high efficiency or bio-availability due to its ethanolic contents. Ethosome vesicles contain phospholipids and alcohol (in relatively in high concentration) (Gangwar Satyam et al., 2010).

Ethosome represents novel vesicular carrier for enhanced delivery through skin. Size of ethosome may vary from 30nm to a few microns. Ethosome has its ability to permeate intact through the human skin due to its high deformability. The physicochemical characteristics of ethosome allow this vesicular carrier to transport active substances more efficaciously through the stratum corneum into the deeper layers of the skin than conventional liposomes. Ethosome mainly used for delivery of drug through transdermal route. Advantages of ethosome such as enhanced permeation of drug molecules to and through the skin, better stability, better solubility of many drugs as compared to conventional vesicles, contrary to deformation liposomes improves skin delivery of drugs (Bharti et al., 2012).

Some common drugs administered by transdermal route include NSAIDS (Diclofenac), Acyclovir, Antibiotic, Cannabidol, Zidovudine, Ketoconazole, (Dave and Pareek, 2012). Ketoconazole is a synthetic antifungal drug which is used to treat fungal infection of skin. Chemical formula of ketoconazole is $C_{26}H_{28}Cl_2N_4O_4$ and its molecular weight is 531.43. Ketoconazole is marketed under trade name Nizoral. Some brand names are Nizoral topical, Xolegel, Kuric, Nizoral A-D. Nizoral tablet (200mg) which are pure of ketoconazole are easily available in market. Ketoconazole interferes with the fungal synthesis of ergosterol. Ergosterol is a constituent of fungal cell membrane. It also inhibits enzyme cytochrome P450 14 alpha demethylase (P450 14 DM) (Loose et al., 1983). Infections treated by ketoconazole

include blastomycosis, histoplasmosis, yeast infection (Malassezia), and Dermatophytes infections (ringworm).

OBJECTIVES

1. To prepare ethosomes and liposomes encapsulating ketoconazole in various concentrations.
2. To characterize the prepared ethosome and liposome.
3. To determine the efficacy of prepared ethosome and liposome against fungal pathogens.

Chapter 2
Review of Literature

Chapter2

2.1 NANOTECHNOLOGY:

"There's plenty of room at the bottom." -Richard Feynman.

Feynman was the first who inspired conceptual beginnings of the nanotechnology. Research and technological development of manipulating matter at the atomic, molecular or macromolecular levels, in the length scale of approximately 1-100 nanometre (nm) range in any direction. Nanotechnology has been defined in various ways according to various fields. According to the NASA's definition "Nanotechnology is the creation of functional materials, device and systems through control of matter on the nanometre length scale (1-100nanometers), and exploitation of novel phenomenon and properties (physical, chemical, biological, mechanical, electrical, biomedical...) at that length scale". The term "nano" which means "dwarf" was originally derived from Greek word. In mathematical expression nano means 10^{-9}. The term nanotechnology was first used by Professor Norio Taniguchi at the University of Tokyo, 1971 in regards to ion sputter machine. According to Professor Norio Taniguchi "Nanotechnology is the production technology to get extra high accuracy and ultrafine dimensions, i.e. the preciseness and fineness on the order of 1 nm (nanometer), 10^{-9} meter in length (Taniguchi 1974). In the 1980s, Eric Drexler authored the landmark book on nanotechnology, "Engines of Creation" (Drexler 1986), in which he described the concept of molecular manufacturing to the public at large. By the 1990s, nanotechnology was advancing rapidly.

2.1.1 Types:

The term nanotechnology according to the journal of Environment and Green Nano of 2011, embraces various fields and specialities including Green nanotechnology, Wet nanotechnology, Nanoengineering, Nanobiotechnology, etc.

Green synthesis technology: Technology which is used to enhanced environment sustainability or developed clean technology in order to minimize human health and production of new nano-products which helps to makes the environment friendlier. Green technology heightens the environment sustainability that is producing negative exteriorities. Green technology is basically about making green nano products. Two main goals of green

nanotechnology are producing nanomaterials or products without harming human health or environment, producing nano-products that provide solution for environmental problems.

Wet technology: The study of biological system that occurs primarily in water environment. The functional nanostructure of interest here are genetic material, enzyme and cellular membrane etc. Wet technology always exists in water environment (Eric Dexler, 1986).

Nanoengineering: It is the practice of engineering on the nanoscale. Nanoscale is one billionth of a metre, i.e. the length of ten hydrogen atoms. Nanoengineering is a interdisciplinary science that deals with building biochemical structure smaller than bacterium, which function like microscopic factories at atomic or molecular level.

2.1.2 Applications:

Nanotechnology is helping to considerably improve, even revolutionize, many technology and industrial sectors: energy, environmental science, information technology, medicine, homeland security, food safety, and transportation, among many other. Nanotechnology have various applications in various fields like physics, chemistry, medical, environment etc.

Application of nanotechnology in physics:

Lasers modulators for telecommunication, computer peripherals (e.g. VCSEL= Vertical Cavity Surface Emitting Laser), sunscreens with nanoparticles, nanocatalyts, "lab-on-chip" diagnostics, biomaterials, electronic displays ("intelligent ink on paper"), Intel nano transistors. It also has numerous potential application in the field of consumer goods, providing with products with novel functions ranging from easy to clean to scratch-resistant (Neethirajan et.al. 2009). Nanotechnology is already being used in numerous kinds of batteries that are less flammable, more efficient, lighter weight.

Application of nanotechnology in chemistry:

It should be bracing to chemists to realize that chemistry is playing a leading role in nanotechnology. Steel is a very important and widely used material and has major role in the construction industry. Nanotechnology helps to improve the properties of steel. Nanotechnology helps to solve out the problem which led to the structural failure of steel due to cyclic loading, such as in bridges or towers (kuennen, 2004). Current steel designs are

based on the reduction in the allowable stress and service life or regular inspection regime, etc. Advancements in this technology using nanoparticles would lead to more efficient materials free from fatigue, increased safety, less need for regular inspection regime.

Application of nanotechnology in environmental science:

Nanotechnology helps to increase the efficiency of fuel production from normal to lower grade materials. It also help meet the need for , clean drinking water through rapid, low cost detection of impurities in and filtration and purification of water. Bacterial identification and elimination in which nano carbohydrate particles bind with bacteria so they can be detected and eliminated is used to minimize and enhance the environmental sustainability of process currently producing negative externalities. Increasing the efficiency of energy production or it could lead to a strong reduction of energy consumption for illumination by nantechnology (Robert A. Freitas Jr. 1999).

Application of nanotechnology in medical science:

Nanotechnology is widely used for drug delivery, gene therapy, detection of disease causing organism, diagnosis, etc. Nanotechnology is used to treat cancer by involving targeted chemotherapy that delivers a tumour killing agent called tumour necrosis factor alpha(TNF) which is attached with gold nanoparticles, nanotechnology is also used for imaging and detection of diseases (Gunasekera A, 2009). The use of nanotechnology in these areas of medicine is one broken bones and for cell reparation can lead to better and faster healing of the body. Nanotechnology in medicine involves applications of nanoparticles currently under development, as well as longer range research that involves the use of manufactured nano-robots to make repairs at the cellular level.

2.2 NANOPARTICLES

The structural and functional unit of nanotechnology is known as nanoparticles. Michael Faraday provided the first description about properties of nanometre –scale metals in his 1857 paper. A small object that behaves as the whole unit with respect to properties and transport is called as a nanoparticles. On the basis of diameter particle are classified into three categories (Grangvist et al., 1976). Coarse particles cover a range between 10,000-2,500 nanometre, fine particles ranges in between 100-2500 nanometre and ultra fine particles range between 1-100 nanometre.

2.2.1 Types:

Any microscopic particle less than about 100 nanometers (nm) in diameter is nanoparticle. Nanoparticles can be manmade to posses highly desired and specific properties. Types of nanoparticles include Quantum Dots, Carbon Nanotubes (Buckyballs), Nanorods, Nanocrystals, Nanowires, Nanoribbons, etc.

Quantum Dot is a portion of matter whose excitation is confined in all three spatial dimensions. These materials have electronic properties (Brus L.E, 2007). Allotropes of carbons with a cylindrical nanostructure are called as carbon tubes. Nanotubes have been constructed with length-to-diameter ratio of up to 132,000,000:1 (Wang et al., 2009). Nanocrystal is also called as crystalline nanoparticles. Nanocrystal as any single crystalline nanomaterial have at least one dimension less than or equal to 100nm (Fahlman., 2007). Nano ribbons are also called as nano-graphene ribbons (GNRs). Strips of graphene are ultra – thin width (<50nm) (Fujita et al., 1996). Nanoparticles often possess unexpected optical properties as they are small enough to confine their electrons and produce quantum effects.

2.2.2 Synthesis:

There are two methods or approaches for synthesis of nanoparticles which includes physical approaches and chemical approaches.

Physical approaches: Various metal nanoparticles such as silver, gold, sulphide, lead and cadmium are synthesised by physical approaches which includes evaporation-condensation and laser ablation. Evaporation-condensation method is mostly used for synthesis of nanoparticles as compared to laser ablation methods. This method uses tube furnace at atmospheric pressure. And this furnace tube require power consumption more than several kilowatts and a preheating time of several of tens of minute to reach a stable operating temperature.(Kruis et al., 2000: Magnusson et al., 1999). Physical methods are useful as nanoparticles generators for long terms experiments.

Laser ablation methods: Nanoparticles are synthesised by laser ablation of metallic bulk materials in solution. The efficiency and characteristics of produced nanoparticles depend on wavelength of laser impinging the metallic target and duration of laser pulses (Mafune et al., 2000). Important advantage of laser ablation technique as compared to other methods of

production of metals colloids is the absence of chemical reagents in solutions. Therefore, pure and uncontaminated metals are prepared by this technique (Tsuji et al., 2003).

Chemical approaches: Most common approach for synthesis of nanoparticles is chemical reduction by organic and inorganic reducing agents. Sodium citrate, ascorbate, polyol process, tollens reagent, elemental hydrogen, etc. are the most common reducing agents. In this method it is important to use protective agent to stabilize dispersive nanoparticles during the course of metal nanoparticle preparation and protect the nanoparticles that can be absorbed on or bind onto nanoparticle surfaces and avoiding their agglomeration (Oliveira et al., 2005).

2.2.3 Uses:

Nanoparticles are mostly used in drug delivery systems like chemotherapy drugs directly to treat cancerous cell, gene delivery in gene therapy, detection of proteins, destruction of tumours with drugs or heat or biological detection of disease causing organism and diagnosis. Sometime nanoparticles are also used in probing of DNA structure. Nanoparticles of carbons are used for developing low cost electrodes for fuel cells. These electrodes may be able to replace the expensive platinum needed for fuel cell catalysts. Nanoparticles are used in labelling of bio-molecules such as antigen, antibody, DNA. Main function provided by nanoparticles is immobilisation of bio-molecules (Katz et al., 2004). Nanotechnology is also used in manipulation of cells and bio-molecules.

2.3 PHOSPHOLIPIDS

Phospholipids (a class of lipids) are the major component of all cell membrane as they form lipid layers. Phospholipids are amphipathic molecules but are soluble in water. They are also known as glycerol-phospholipids. Phospholipids are composed of phosphate, two fatty acid and glycerol backbone. They consist of two region which includes polar region and non-polar region. Polar region of phospholipid include glycerol or carbonyl of fatty acid and non polar region includes fatty acid. In former the polar region is also called as “Head region” and non-polar region is called as “Tail region”. Not all phospholipids have ester linkage as the covalent bond in between the glycerol backbone and tail region. Generally, all mammalian tissue have qualitatively same phospholipids (Ansell et al., 1973).

2.3.1 Types of phospholipids:

Some common phospholipids are phosphatidic acid (phosphatidate), phosphatidyethanolamine (cephalin), phosphatidylcholine (lecithin), phosphatidylserine, phosphoinositides, etc. Phospholipid synthesis occurs in the cytosol adjacent to endoplasmic reticulum (E Fahy et al., 2009). Common sources of phospholipids are chicken eggs, soya, sunflower, rapeseed, bovine milk, fish eggs.

2.3.2 Uses:

Phospholipid helps us to make a product that function as a second messenger in signal transduction (Crownshaw et al., 2006). Phospholipid can act as an emulsifier; it is also used as food additive and enables oils to form a colloid with water. Phospholipids are also used for drug delivery in medicine. Some phospholipids are also used as cosmetics. Example includes gold nano particles present in face cream which help in anti ageing. Omega -3 phospholipids has a tremendous health impact at cellular level. It acts as krill oil which is good for so many afflictions of body. Phospholipids stick together in unique pattern which allows to entry of oxygen and nutrient into the body but helps to keep bacteria or toxin out from the body. Omega -3 phospholipids has a tremendous health impact at cellular level. It acts as krill oil which is good for so many afflictions of body. Phospholipids stick together in unique pattern which allows to entry of oxygen and nutrient into the body but helps to keep bacteria or toxin out from the body.

2.4 LIPOSOMES

Liposomes are spherical, self closed vesicles of colloidal dimensions, in which phospho-lipid bilayer sequesters part of the solvent, in which they freely float, into their interior (Bangham et al., 1964). The name liposome is derived from two Greek words: “lipo” meaning fat and “soma” meaning body. Liposomes are simple microscopic vesicles. Liposomes are a form of nanoparticle which is formed by using lecithin. It can be prepared by disrupting biological membrane or by sonication. Liposomes can be made entirely from naturally occurring substances and are therefore nontoxic, biodegradable and non immunogenic. In 1961, liposomes were first described by British haematologist Dr. Alec D Bangham FRS, at the Babraham Institute , in Cambridge.

2.4.1 Types:

Liposomes can be classified on the basis of

i. Structural parameter,
ii. Method of preparation,
iii. Composition and
iv. Applications.

On the basis of structural parameter the liposomes are classified into 4 types- multilamellar vesicles, oligolamellar vesicles, unilamellar vesicle. Multilamellar vesicles are less than 0.5µm (>0.5µm) in size. Oligolamellar vesicles ranges between 0.1 to 1.0µm in size. Unilamellar vesicles are divided into two types which includes small unilamellar vesicles and large unilamellar vesicles. Small unilamellar vesicles range between 20 to 100nm. Large unilamellar vesicles ranges between greater than 100nm (>100nm) (Dua et al., 2012).

Liposome are classified on the basis of method of preparation which includes Dehydration-rehydration methods. Unilamellar vesicles made by reverse phase evaporation method, multilamellar vesicles made by reverse phase evaporation, vesicles made by extrusion technique, frozen and thawed multilamellar vesicles and stable pluri-lamellar vesicles(Dua et al., 2012).
On the basis of composition and application liposomes are classified into conventional liposomes these are negatively or neutral charged phospholipids and cholesterols, fusogenic liposome, pH sensitive liposome, cationic liposome, long circulatory liposome and immune-liposomes these are attached with monoclonal antibodies. (Dua et al., 2012).

2.4.2 Uses:

Liposome has its own value in different fields. Liposomes are mostly used for drug delivery to targeted gene. Liposomes are also used in mathematics for topology in two dimensional surfaces in three dimensional surface governed only by bi-lipid elasticity. In biophysics liposomes are used for checking of the aggregation behaviour and fractal strength of materials. Liposomes are also used in chemistry for micro compartilization. In pharmaceuticals it is used for studies of drug actions (Lipowsky and Sackmann, 1995). Liposomes as drug delivery vehicles in medicine, adjuvants in vaccination, signal

enhancers/carriers in medical diagnostics and analytical biochemistry, solubilizers for various ingredients as well as support matrix for various ingredients and penetration enhancer in cosmetics (Lassic et al., 1992). Liposomes containing membrane anchored chelators can be used to clean toxic or radioactive metals from solutions. (Lassic., 1993). Liposomes increase the efficiency, bioavailability, absorption of certain entrapped dietary and nutritional supplements and are used as topical drug delivery system (George et al., 1975). But liposomal drug delivery system has certain shortcomings like the need for modification for site specific or organ specific drug delivery, high production cost, leakage and fusion of encapsulated drug/molecule. Moreover unfavourable reactions like oxidation and hydrolysis of phospholipids reduces the half life of the formulation, decreases solubility and stability of drug in the medium (Sirisha.V.N et al., 2012).

2.4.3 Demerits:

They need much modification for drug delivery to special organ. Production cost is high. Leakage and fusion of encapsulated drug/molecules is a major problem. Sometimes phospholipids undergo oxidation and hydrolysis like reaction. Liposome has very short half-life, low solubility, and less stability. Liposomes in skin delivery have a limited ability to penetrate narrow blood vessels or into skin to. Sometimes the materials which we want to deliver into skin gets entrapped within the inner layers of the liposomes and in certain conditions are virtually un- releasable (Ralph hill, 2011).

2.5 ETHOSOMES

Ethosomes are soft and self closed vesicles. Ethosomes mainly consists of phospholipids, ethanol and water. These are malleable vesicles which are used for enhanced delivery of active agents. Ethosomes are non-invasive delivery carriers. Ethosome represents novel vesicular carrier for enhanced delivery through skin. Size of ethosome may vary from 30nm to a few microns. Ethosome has its ability to permeate intact through the human skin due to its high deformability. The physicochemical characteristic of ethosome allows this vesicular carrier to transport active substances more efficaciously through the stratum corneum into the deeper layers of the skin than conventional liposomes. Ethosome is mainly used for delivery of drug through transdermal route.

2.5.1 Ethosome composition:

Ethosome are spherical vesicular consisting of phospholipids, water and ethanol. Typically ethosome comprises various types of phospholipid structures like hydrogenated phosphatidylcholine, phosphatidylcholine, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl linositol, etc, with high concentration of ethanol or isopropyl alcohol. Sometimes dyes which includes amphiphilic fluroscent probes such as –D289, Rhodamine-123. Fluroscence isothiocyanate(FITC) are often added to it for characterization study (Nikalje et al., 2012).

2.5.2 Effect of high concentration of ethanol:

Alcohol or ethanol is an efficient permeation enhancer. In ethosome ethanol is present in quite high concentration i.e. 20 to 25 %. Due to its presence in high concentration ethosomes can easily disrupt the skin bi-lipid layers. Therefore, when integrated into a vesicle membrane it could give an ability to the vesicle to penetrate in skin (Dave and Pareek , 2012).

2.5.3 Uses:

Ethosomes are mainly used for delivery of drug through transdermal route. Advantages of ethosome such as enhanced permeation of drug molecules to and through the skin, better stability; better solubility of many drugs as compared to conventional vesicles, contrary to deformation liposomes improves skin delivery of drugs. (Bharti et al., 2012).

There are some common drugs administered by transdermal route like NSAIDS (Diclofenac), Acyclovir, Antibiotic, Cannabidol, Zidovudine, Ketoconazole. Ethosomal system is non-invasive, passive and available for immediate commercialization. Ethosomes provide a large platform for drug delivery, its composition is safe and the components are approved for pharmaceutical and cosmetic use (Dave and Pareek, 2012). Ethosomes are also used for pilosabeaceous targeting. Pilosabeacous is an epidermal invagination found on the most surfaces of human body and mostly composed of hair follicles and sebaceous glands. It has low risk profile (Jain et al., 2011).

2.6 KETOCONAZOLE:

Ketoconazole is one of most important synthetic antifungal drug. Ketoconazole is an imidazole antifungal agent, it has a five membered ring structure containing two nitrogen atoms. It is mostly used to treat fungal infection of skin. It belongs to azole family. Chemical formula of ketoconazole is $C_{26}H_{28}Cl_2N_4O_4$ and its molecular weight is 531.43. Ketoconazole is marketed under trade name nizoral. Nizoral tablets contain the active ingredient of ketoconazole. It is usually prescribed for topical infection such as athlete's foot, ringworm, candidiasis and jock itch. It is also used to treat fungal infection of scalp of hair i.e dandruff. Ketoconazole interferes with the fungal synthesis of ergosterols . Ergosterol and Cytochrome P450 14 alpha demethylase (P450 14 DM) enzyme is a constituent of fungal cell membrane (Loose et al., 1983). Ketoconazole inhibits p450 14 DM enzymes. This enzyme is in the sterol biosynthesis pathway that leads from lanosterol to ergosterol. The affinity of ketoconazole for fungal cell membranes is less compared to that of fluconazole an itraconazole. Ketoconazole has thus more potential to effect mammalian cell membranes and induce toxicity (Lewis et al., 1998). Nizoral tablets inhibit production of chemical called ergosterol, which is a componenet of fungal cell wall. Ketoconazole have no activity against *Aspergillus* spp., *Fusarium* spp., and zygomycetes order of fungi (Como et al., 1994). It may increase transaminase levels and hepatoxicity and may decrease testosterone and cortisol levels, resulting in gynecomastia and oligospermia in men and menstrual irregularities in women(O'connor et al., 2002). Ketoconazole also have antithyroid functions (Comby et al., 1994). It is one of the least expensive drugs used for treatment of prostate cancer.

Chapter 3
Materials & Methods

Chapter 3

3.1 MATERIALS

3.1.1 Chemicals Required:

The drug ketoconazole was obtained from local pharmaceutical store under the brand name Nizoral. Lecithin (Trade name- Leciva- S70) containing not less than 98% phosphatidyl-choline was received as a kind gift from VAV LIFE SCIENCES Pvt. Ltd, Mumbai, India. Ethanol and methanol was purchased from Hi-media Pvt. Ltd Mumbai, India. Distilled water and all other chemicals and solvents used in our work were of analytical grade and available in Department of Life Science, NIT Rourkela

3.1.2 Instruments Used:

The basic instruments used for the preparation and characterization of the samples like Weighing balance (Sartorius), Magnetic stirrer (Remi), Probe Sonicator (Plexiglas), Refrigerated centrifuge (Eppendrof), Spectrophotometer (UV Lambda 35(R)Perkin Elmer) was available in the Department of Life Science, NIT Rourkela.

Specialised facilities like Scanning Electron Microscopy, Atomic Force Microscopy, Particle Size Analyzer and FT-IR was kindly allowed by the Dept. of Metallurgical & Materials Engineering (MM), Dept. of Ceramic Engineering (CR) and Dept. of Chemistry (CY) respectively of NIT Rourkela.

3.2 PREPARATION OF STOCK SOLUTION OF KETOCONAZOLE

Ketoconazole stock solution was prepared by dissolving 1mg drug in 10 ml ethanol according to the manufacturer's instruction. Working solutions of different concentrations ranging from 0.3125µg/ml to 30 µg/ml were prepared from the stock solution.

When water was used to dissolve the drug, it resulted in immediate precipitation of the drug, so the ethanol was chosen as a solvent.

3.3 PREPARATION OF ETHOSOME AND LIPOSOME

3.3.1 Preparation of blank ethosomal particle:-

Ethosome was prepared by solvent dispersion method as described by Touitou et.al. 2000. Briefly Lecithin (up to 2-3%), was dissolved in (30-40%) of 90% ethanol by use of magnetic stirrer (Remi Motors Mumbai) for 20 minutes at 100 rpm. To this mixture warm distilled water was slowly added in a fine stream by syringe and the whole system was stirred for 30 minutes at 700 rpm. The resulting preparation was sonicated for 3 cycles of 5 minute each with 5 minute rest between the cycles.

3.3.2 Preparation of drug (Ketoconazole) encapsulated ethosomes:-

Ketoconazole (drug) encapsulated ethosomes were also prepared by solvent dispersion method following the protocol by Touitou et.al, 2000. Lecithin (2-3%) and ketoconazole was dissolved in (30-40%) of 90% ethanol by use of magnetic stirrer (Remi Motors Mumbai) for 20 minutes at 100 rpm. Then warm distilled water was added slowly in a fine stream to the ethanolic drug mixture solution and the mixture was stirred for 30 minutes at 700 rpm in a closed vessel. The resulting preparation was sonicated for 3 cycles of 5 minute each with 5 minute rest between the cycles. This method was repeated several times but by varying the drug concentration each time.

3.3.3 Preparation of blank liposomal particle:-

Liposomes were prepared by classic dispersion method as described by Touitou et.al. 2000 with certain modifications. Lecithin (2-3%) was dissolved in 6 ml distilled water. This mix was heated to 30^{o}C in a water bath. To this mixture warm distilled water was added slowly in a fine stream with continuous stirring at 700 rpm in close vessel. The resulting vesicles were sonicated for 3 cycles of 5 minute each with 5 minute rest between the cycles.

3.3.4 Preparation of drug (Ketoconazole) encapsulated liposomes:-

Ketoconazole (drug) encapsulated liposomes were prepared as described by Touitou et.al, 2000 with little modification of the classic dispersion method. Briefly lecithin (2-3%) and drug solution was taken and dissolved in 6 ml distilled water and this mix was heated to 30^{o}C in a water bath. Warm distilled water was added slowly in a fine stream to the drug-lipid

suspension with continuous stirring at 700 rpm in a closed vessel. The resulting vesicles were sonicated for 3 cycles of 5 minute each with 5 minute rest between the cycles.

3.4 Various methods of characterizations of ethosome and liposome.

3.4.1 SEM (scanning electron microscope) Analysis:

To study vesicle shape or morphology of ethosome and liposome can be done by using scanning electron microscope. Ethosome and liposomes vesicles were visualised using Jeol 6480 LVJSM electron microscope. For SEM one drop of each sample from ethosome and liposome were mounted on a stub covered with clean glass respectively. The drop was spread out on the glass homogenously. A sputter coater was used to sputter coat the samples with platinium and samples were examined under Jeol 6480 LVJSM at an accelerating voltage 20kv.

3.4.2 Vesicle size and Zeta potential Analysis:

Zeta potential is an important and useful indicator of particle surface charge, which can be used to predict and control the stability of sample. Zeta potential is a physical property which is exhibited by any particles in suspension it can be used to optimize the formulation of suspension and emulsion.

3.4.3 Determination of drug encapsulating capacity:

Determination of drug encapsulating capacity of ethosome can be determined by mini column centrifugation method or fluorescence spectrophotometer, but we utilised the fluorescence spectrophotometer method. First derivatives of UV spectrum of ketoconazole, in a range from 211nm to 295nm, presented a maximum absorption peak at 257nm,without any interference from excipients. Method was validated at the 244nm. Analytical curve was constructed in concentration range from 0.3125ug to 30ug. Spectrophotometer (UV-Lambda 35(R) was Perkin Elmer, Waltem ms USA). The % drug content of ethosomal preparation was determined by using following formula

%of drug content=sample absorbance/standard absorbance

Entrapment efficiency:

The entrapment efficiency of ketoconazole ethosome was measured by the ultracentrifugation method. Vesicular preparation containing ketoconazole was kept overnight at 4°C and centrifuge in ultra centrifugation 4°C at 30,000 rpm for 2 hrs. Ketoconazole was assayed in both sediment and supernatant. The entrapment efficiency was calculated using following formula

%entrapment efficiency= Amount of drug added –amount of drug non encapsulated *100/amount of drug loaded.

3.5 Antifungal activity:

Fungal culture was inoculated into test tube containing PDB (potato dextrose broth) and incubated at 28°C for 2-3 days. 100 µl of the broth culture was added onto a slide and the slide was put in the prepared PDA plates. Wells were punctured onto the agar plates and 100µl of various concentrations of suspensions were loaded into the wells and incubated for 3-4 days. After 3-4 days, the area in the plate with no fungal growth was measured.

Table 3.1 Composition of working solutions:

Concentration	0.1M HCL	Drug Stock Solution
30 µg/ml	1400 µl	600 µl
25 µg/ml	1500 µl	500 µl
20 µg/ml	1600 µl	400 µl
15 µg/ml	1700 µl	300 µl
10 µg/ml	1800 µl	200 µl
5 µg/ml	1900 µl	100 µl
2.5 µg/ml	1950 µl	50 µl
1.25 µg/ml	1975 µl	25 µl
0.625 µg/ml	1998.75 µl	12.5 µl
0.3125 µg/ml	1993.75 µl	6.25 µl

Table 3.2 Composition of blank liposome:

Lecithin	Distilled water	Distilled water	Total volume
0.6ml	6ml	13.4ml	20ml

Table 3.3 Composition of drug encapsulated liposomes:

Drug Concentration	Lecithin	Distilled water	Drug solution	Total volume
30 µg/ml	0.6ml	17.4ml	2ml	20ml
20 µg/ml	0.6ml	17.4ml	2ml	20ml
10 µg/ml	0.6ml	17.4ml	2ml	20ml
5 µg/ml	0.6ml	17.4ml	2ml	20ml

Table 3.4 composition of blank ethosome:

Lecithin	Ethanol	Distilled water	Total volume
0.6ml	6ml	13.4ml	20ml

Table 3.5 Composition of drug encapsulated ethosome:

Drug Concentration	Lecithin	Ethanol	Drug solution	Distilled water	Total volume
30 µg/ml	0.6ml	6ml	2ml	11.4ml	20ml
20 µg/ml	0.6ml	6ml	2ml	11.4ml	20ml
10 µg/ml	0.6ml	6ml	2ml	11.4ml	20ml
5 µg/ml	0.6ml	6ml	2ml	11.4ml	20ml

Chapter 4
Results

Chapter 4

RESULTS

The various formulations of liposomes and ethosomes were characterized and their results shown below.

4.1 Vesicle Morphology:

The various drug loaded formulations of ethosomes and liposomes (including the blank particles) appeared more or less spherical when observed by SEM (Fig. 1).

4.2 Particle size and particle size distribution:

The particle size, zeta potential and size distribution of the various suspensions are shown in Table 4.1. The zeta potential of all the ethosomal vesicles was of higher magnitude than liposomal vesicles (measured in milivolts). The poly index measured in PDI and particle size measured in nanometre.

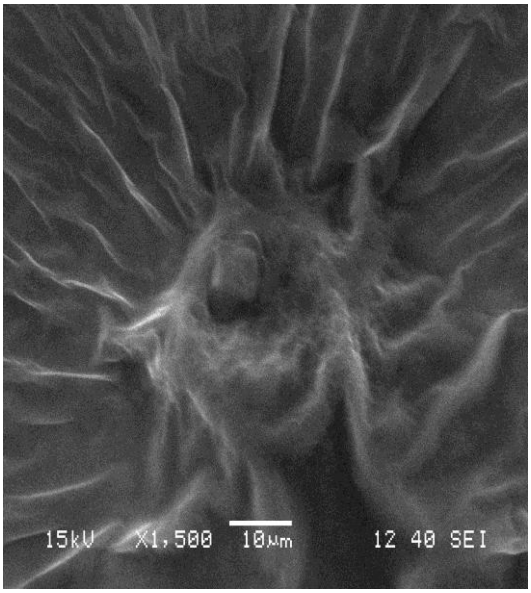
4.3 Drug Entrapment Efficiency:

The entrapment efficiency was calculated as mentioned in Chapter 3. The quantity of drug entrapped is more in case of ethosome as compared to liposome. Among the various formulations when drug concentration is around 20-30 µg/ml, the quantity of drug entrapped is more. The high ethanol concentration favoured better encapsulation. The values of encapsulation efficiency is shown in Table 4.4.

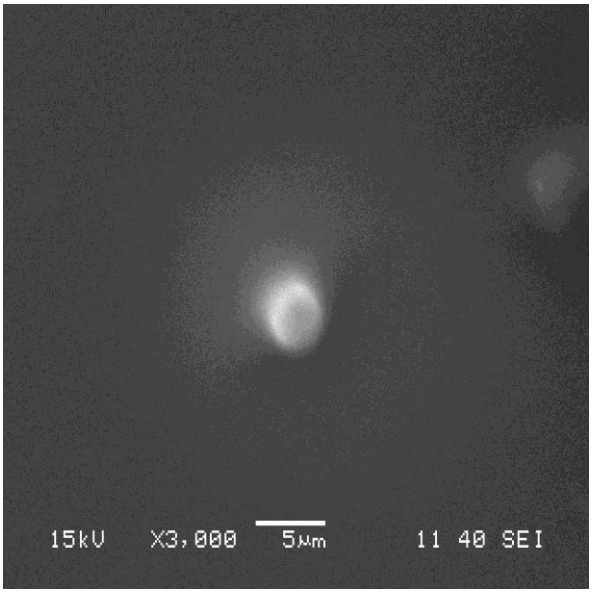
4.4 Antifungal Studies:

The in-vitro antifungal studies qualitatively showed the better efficacy of drug loaded ethosomes against the drug loaded liposomes. The blank formulations did not show any activity against the fungal pathogens. The results are shown in Fig.4.13.

Fig 4.1.Concentration of drug 5µg/ml

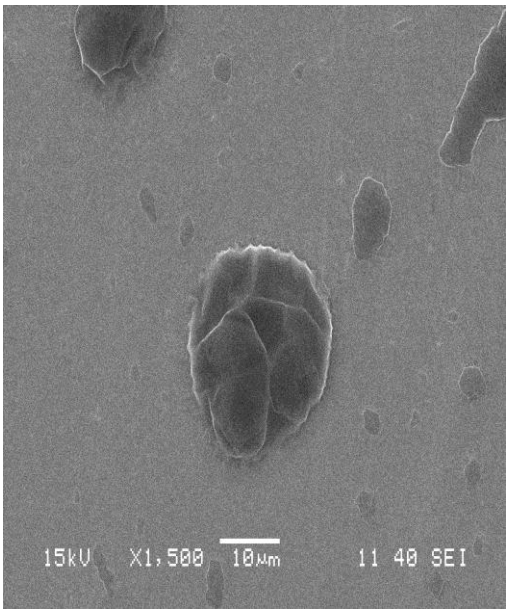


Liposome

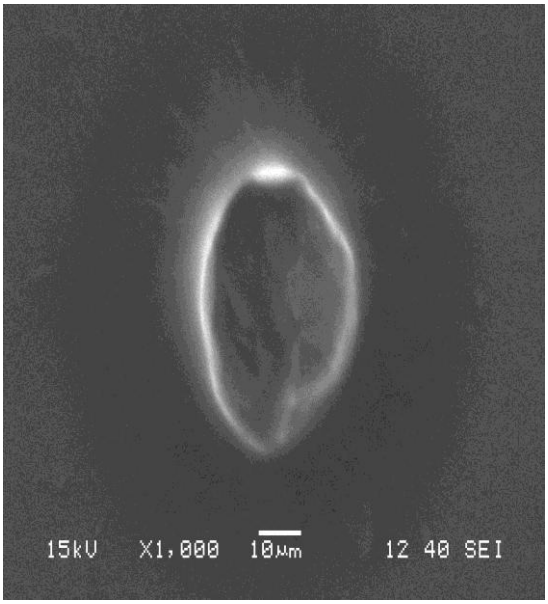


Ethosome

Fig 4.2. Concentration of drug 10µg/ml

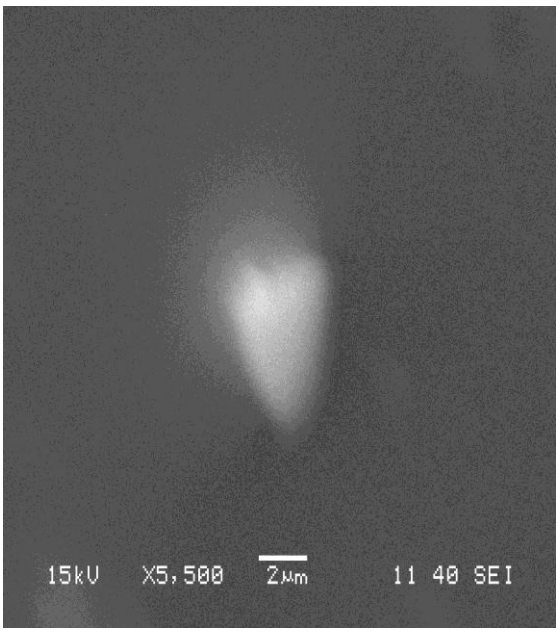


Liposome

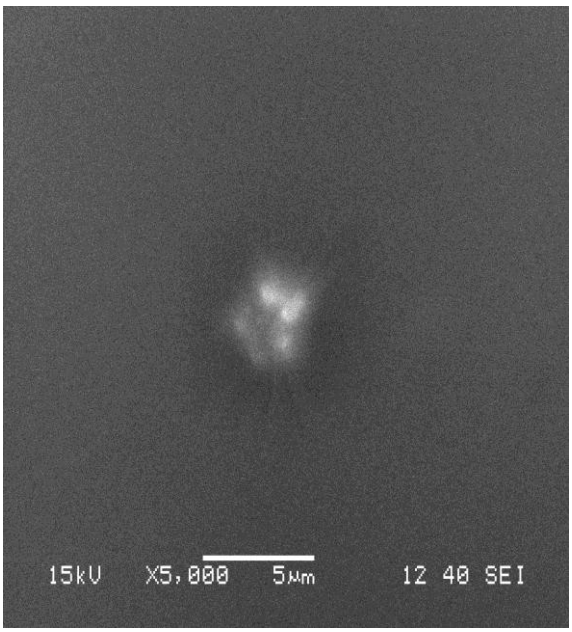


Ethosome

Fig 4.3.Concentration of drug 20µg/ml

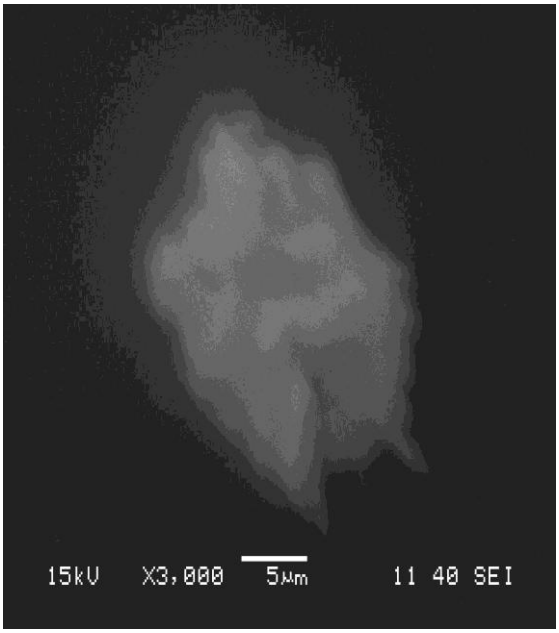


Liposome

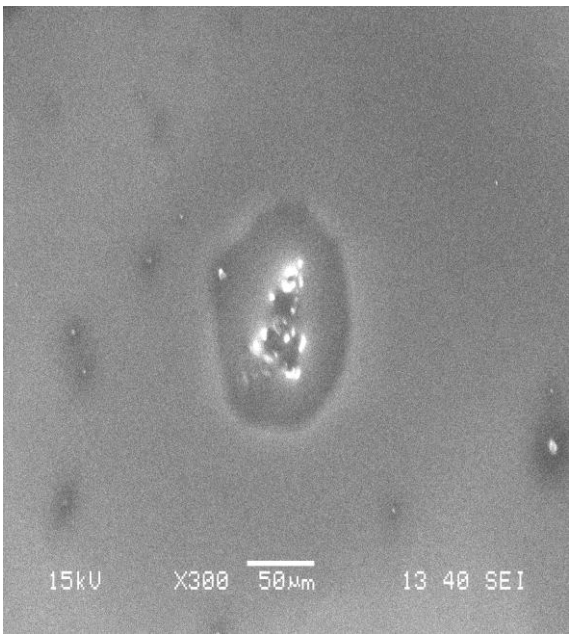


Ethosome

Fig 4.4. Concentration of drug 30µg/ml



Liposome



Ethosome

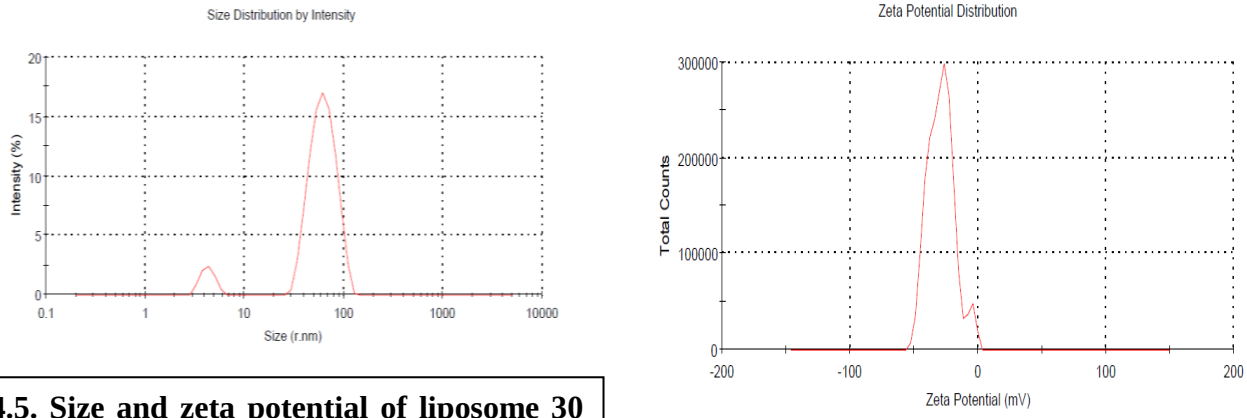


Fig 4.5. Size and zeta potential of liposome 30

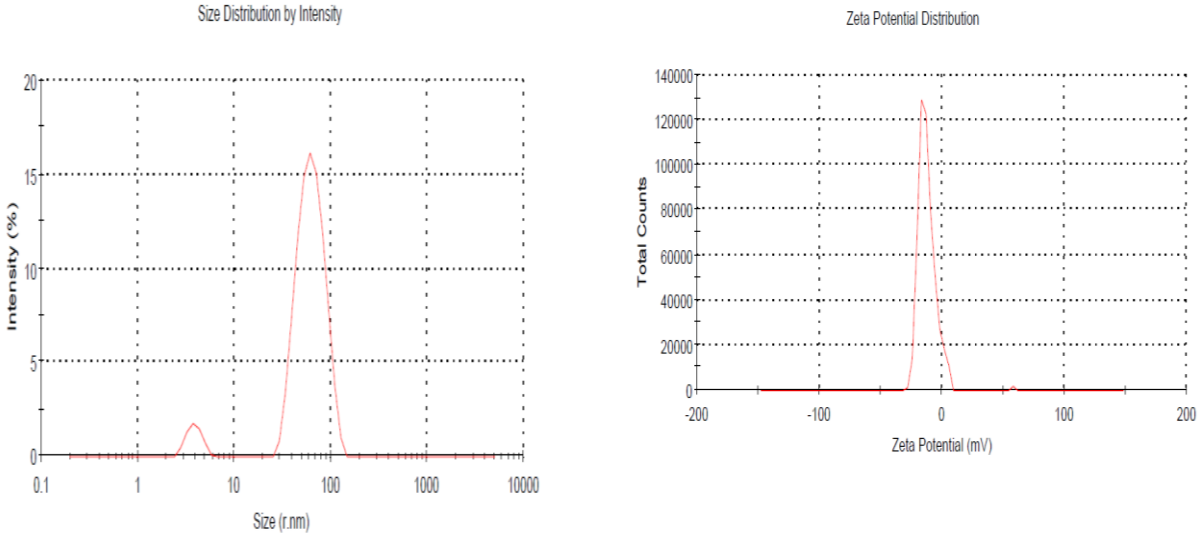


Fig 4.6. Size and zeta potential of liposome 20

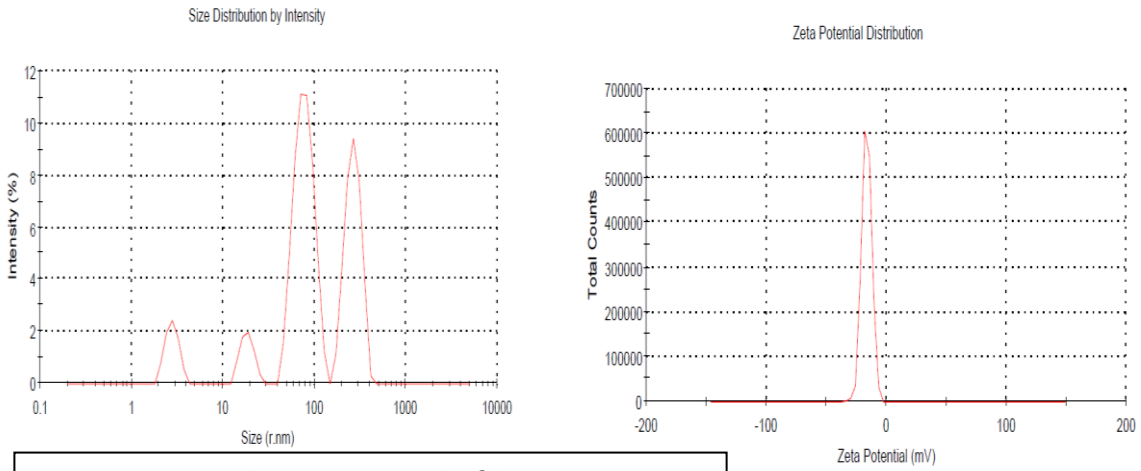


Fig 4.7. Size and zeta potential of liposome 10

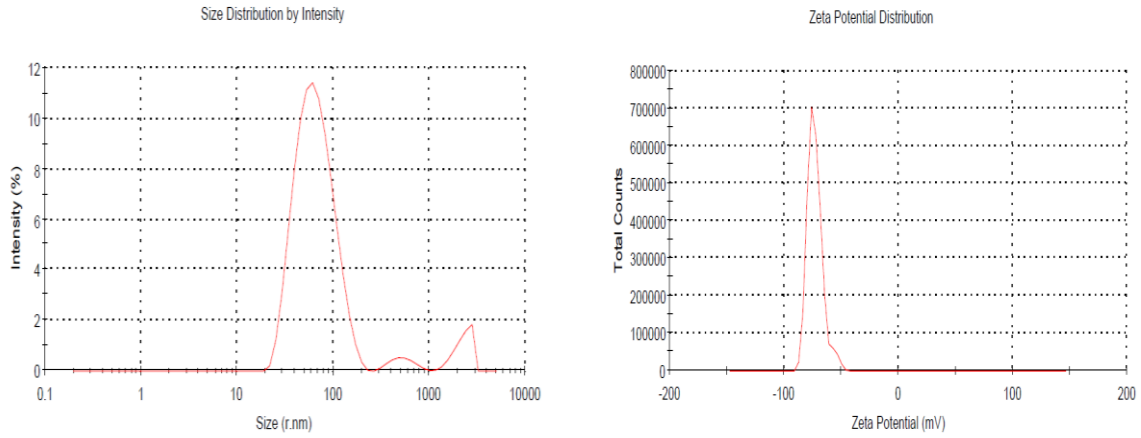


Fig 4.8. Size and zeta potential of liposome 5

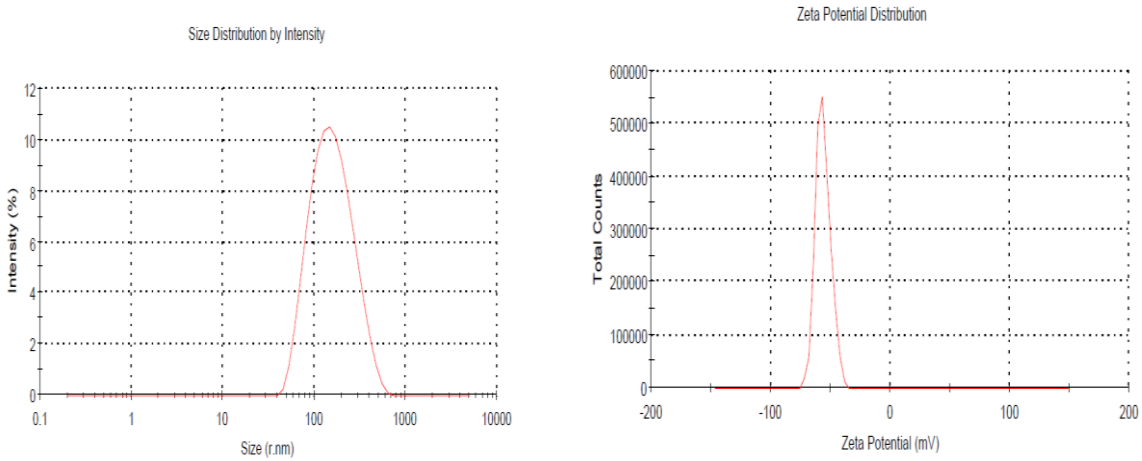


Fig 4.9. Size and zeta potential of ethosome 30

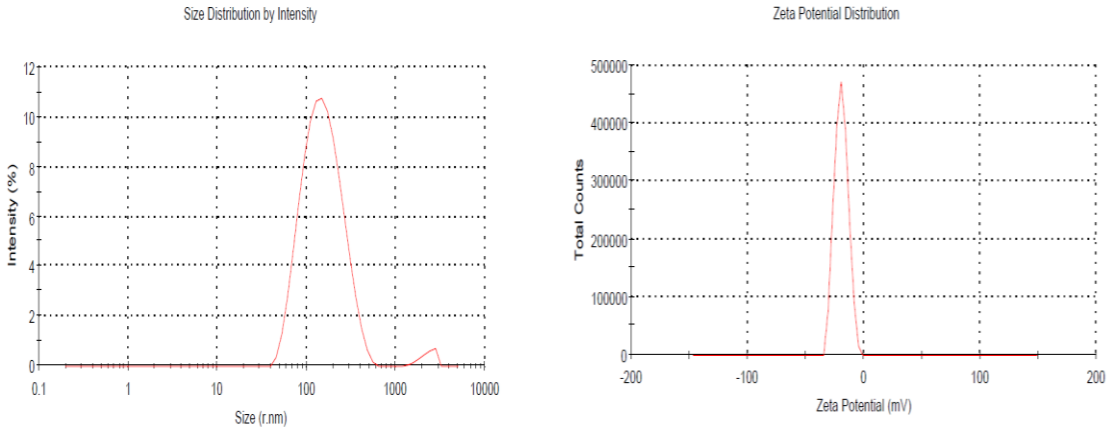


Fig 4.10. Size and zeta potential of ethosome 20

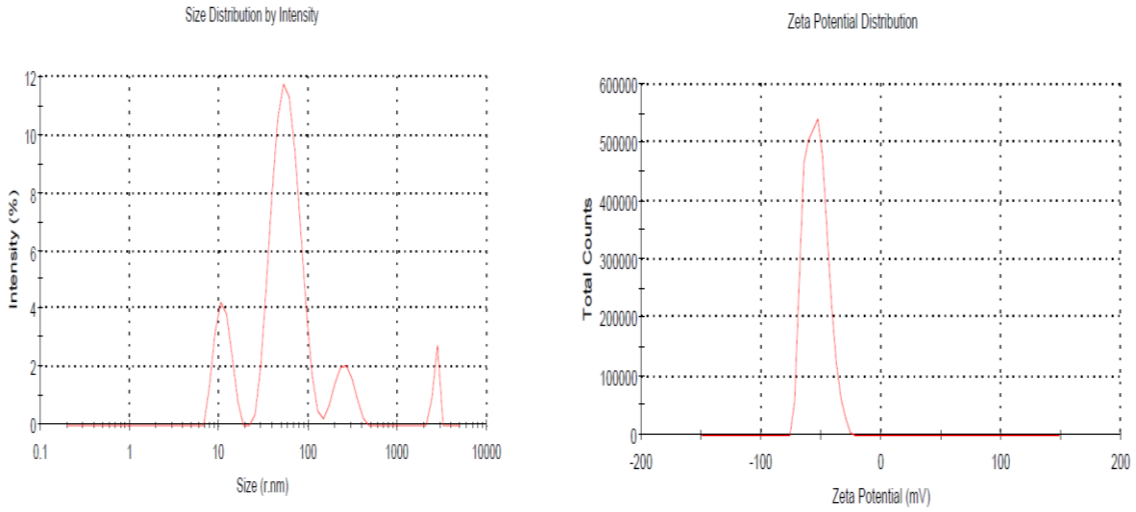


Fig 4.11. Size and zeta potential of ethosome 10

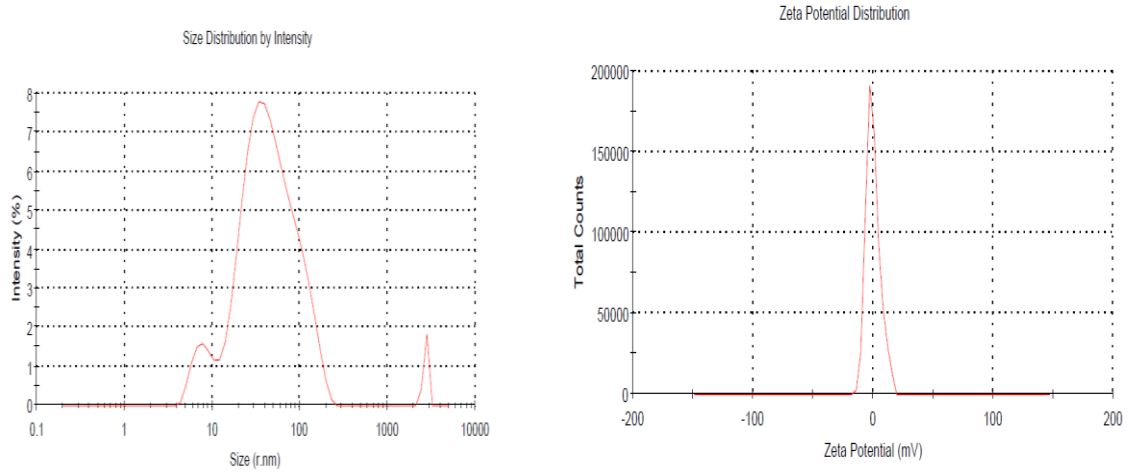


Fig 4.12. Size and zeta potential of ethosome 5

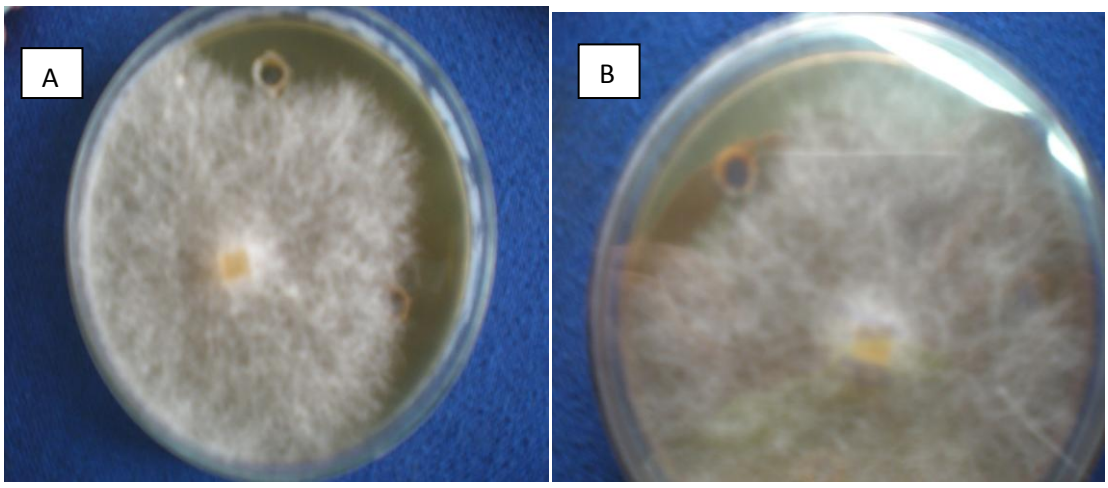


Fig 4.13 Antifungal activity shown by liposome(A) and ethosome(B)

Table 4.1 Vesicle size and zeta potential of the formulations

Drug encapsulated sample(liposome and ethosome)	Particle size(nm)	Zeta potential (mV)	Poly dispersity Index (PdI)	Charges
Liposome30µg/ml	61.23	-29.0	0.351	Negative
Liposome 20 µg/ml	52.34	-12.2	0.418	Negative
Liposome 10 µg/ml	187.1	-16.5	0.673	Negative
Liposome 5 µg/ml	58.65	-72.8	0.396	Negative
Ethosome 30 µg/ml	133.9	-56.2	0.212	Negative
Ethosome 20 µg/ml	131.5	-19.4	0.249	Negative
Ethosome 10 µg/ml	50.61	-52.85	0.530	Negative
Ethosome 5 µg/ml	42.86	0.00508	0.383	Negative

Table 4.2 Absorbance of the different conc. of drug (ketoconazole) at 224 nm

Concentration	**OD1**	**OD2**	**Mean of OD1 and OD2**
30 µg/ml	1.836	1.786	1.811
25 µg/ml	1.512	1.501	1.506
20 µg/ml	1.206	1.202	1.204
15 µg/ml	0.917	0.903	0.910
10 µg/ml	0.578	0.579	0.578
5 µg/ml	0.300	0.280	0.290
2.5 µg/ml	0.129	0.119	0.124
1.25 µg/ml	0.050	0.047	0.0485
0.625 µg/ml	0.010	0.009	0.0095
0.3125 µg/ml	-0.001	-0.008	-0.0045

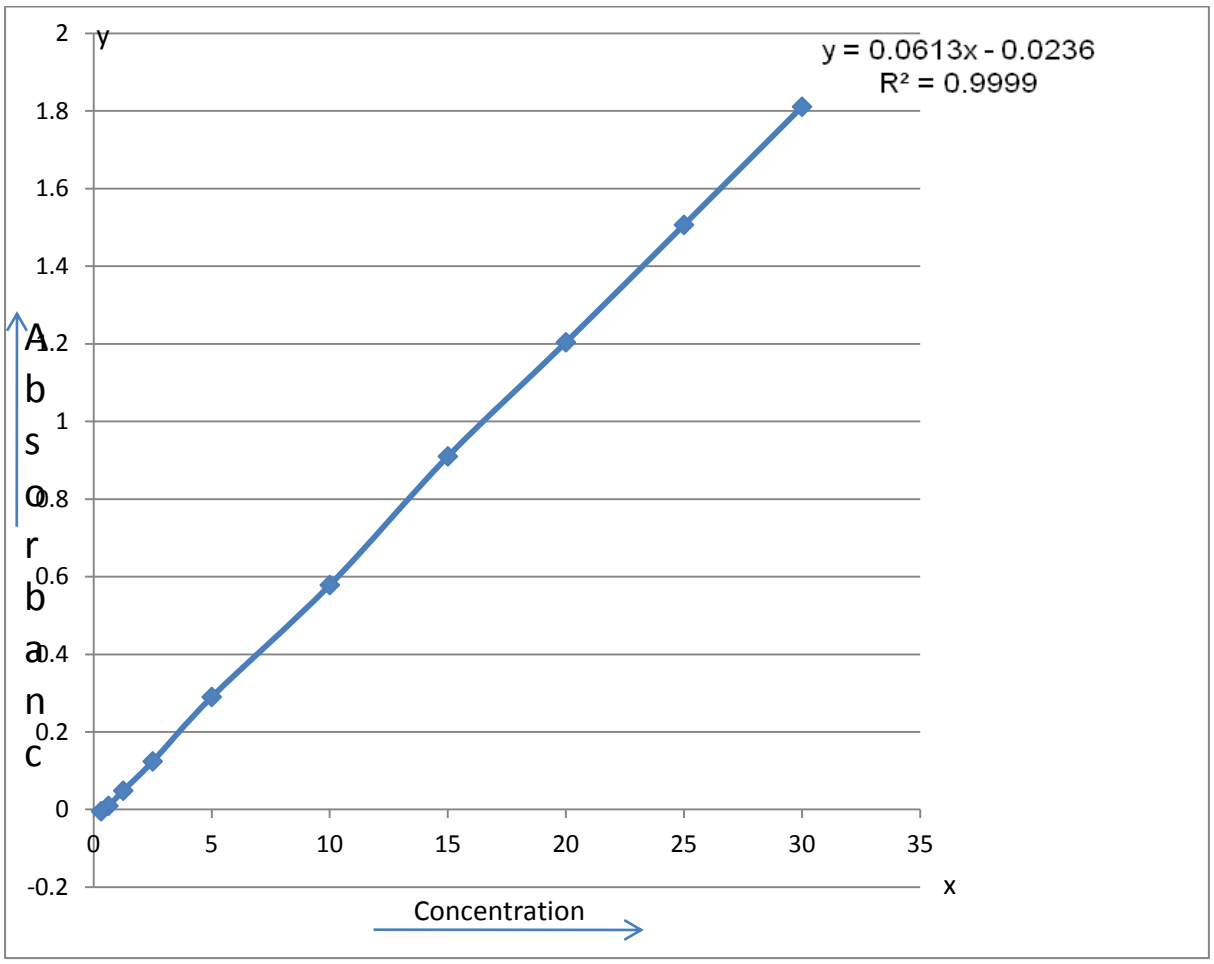


Fig 4.14. Standard Curve

Table 4.3 OD of supernatant of emulsions

Drug Concentration in µg/ml	O.D of Liposome	O.D of Ethosome
30 µg/ml	0.413	0.433
20 µg/ml	0.392	0.395
10 µg/ml	0.257	0.280
5 µg/ml	0.25	0.233

Table 4.4 Concentration of drug in supernatant and sediment and determination of encapsulation efficiency

Sample Name	Conc. of drug in supernatant µg/ml	Conc. of drug in sediment µg/ml	Encapsulation efficiency(%)
Liposome30 µg/ml	7.4	22.6	75.33
Ethosome 30 µg/ml	7.1	22.9	76.33
Liposome 20 µg/ml	6.828	13.17	65.85
Ethosome 20 µg/ml	6.77	13.23	66.15
Liposome10 µg/ml	4.95	5.05	50.5
Ethosome 10 µg/ml	4.577	5.423	54.23
Liposome 5 µg/ml	4.18	0.82	16.4
Ethosome 5 µg/ml	4.055	0.94	18.8

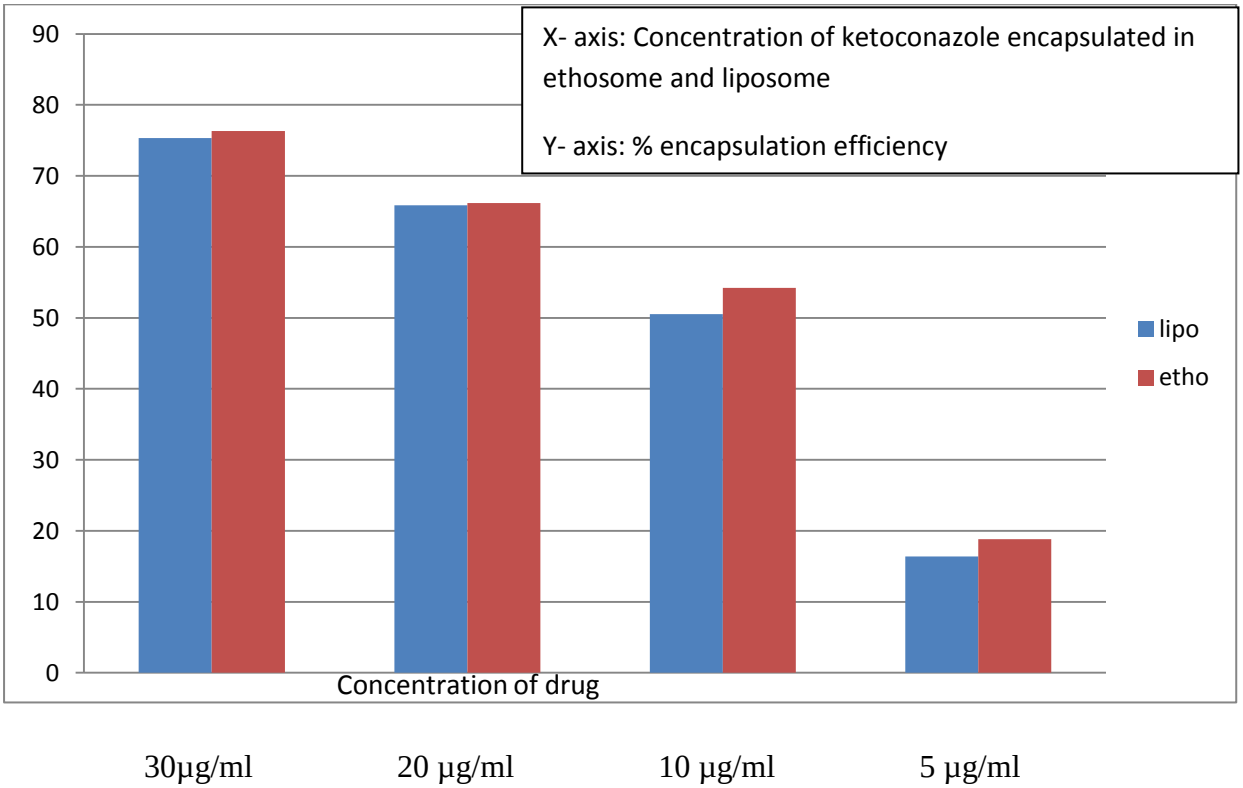


Fig 4.15. Graph showing comparative encapsulation efficiency of ethosome and liposome at various drug concentrations

Chapter 5
Discussion

Chapter 5

DISCUSSION

Development of novel drug delivery carriers are a necessity to deliver the drugs to target site at a faster rate to overcome the drawbacks of multi-dose therapy and increase patient compliance and improve their safety. For drug delivery via dermal and transdermal routes ethosomes have emerged as a non-invasive mean.

5.1 Vesicle morphology:

The SEM morphology shows the formation of uniform, more or less spherical vesicular structures in case of ethosome. Encapsulation of ketoconazole can be clearly observed within the vesicles. Better encapsulation has been observed in case of ethosomes with higher concentration of drug i.e. 20-30µg/ml. Liposomal suspension also showed spherical morphology in the SEM micrograph. But mostly the particles were found in an aggregated manner. Hence the SEM Micrograph show better formation and encapsulation by ethosome as compared to liposome.

It can also be observed that high ethanol concentration is responsible for stable vesicles. This finding is supported by Lasic et al., who proposed that ethanol causes a modification of net charge of the vesicular system and confers on it some degree of steric stabilization.

5.2 Particle size and particle potential:

The zeta potential of the ethosomal suspensions were mostly in the range of -43.4 mV to 59.4 mV which indicated good stability. But the zeta potential values of liposomes ranged between -12.2 mV to -29.0 mV indicating incipient stability. Similarly the PDI values of ethosome and liposome lie in the range 0.212- 0.383 and 0.351- 0.673 respectively. This value helps to determine whether the suspension is mono-dispersed or poly-dispersed. Generally if the PDI value lies between 0.00-0.5. the suspension is considered mono-dispersed, while PDI values greater than 0.5 indicates poly-dispersed suspension. So from the above values we observe the ethosomal suspensions are mono-dispersed while the liposomal formulations may be monodispersed or poly-dispersed.

Stable and smaller ethosomes may be due to formation of hydrocarbon phase with inter-penetrating properties (touitou et al., 2000).

The high negative charge on ethosomes is dependent on two factors: (i) ethanol, which provides a net negative surface charge thus avoiding aggregation of vesicles due to electrostatic repulsion; and (ii) lecithin, which provides a greater rigidity to the layers and reduced likelihood of vesicles fusion, as well as a greater resistance to the high rotational energy exerted by sonication, resulting in a high negative charge (Verma P et al.,2011).

5.3 Drug entrapment efficiency:

The graph showed better encapsulation by ethosomes as compared to liposomes in each concentration range. This may be due to better solubility of the drug in ethanol. Moreover higher the concentration of drug solution used higher was the encapsulation efficiency.

Our results showing higher encapsulation efficiency and stability in case of Ethosomal formulations is in compliance with results of other research groups (Dubey et al., 2007). High encapsulation efficiency and stability is due to better solubility and retentivity if the drug Ketoconazole in ethanol (Touitou et al., 2000).

5.4 Antifungal activity:

Area inhibited was more in case of ethosomal suspension as compared to liposomal formulation. This may be due to the extra potential of ethanol to kill organisms by denaturing their proteins and dissolving their lipids, apart from skin fluidization and penetration McDonnell and Russell, 1999).

From this analysis we can assume that ethanol containing formulation will prove as a better carrier system and a therapeutically promising candidate for the efficient treatment of fungal infection over surfactant based formulations (liposome).

Chapter 6 Conclusion

Chapter 6

CONCLUSION

The ethosomal formulations were more spherical with stable zeta potential and mono-disperse with no clumping. Although the liposomes showed spherical morphology but were less stable and usually poly-dispersed in nature. The antifungal activity of liposome was less as compared to ethosomes.

So from the study it was confirmed that ethosomal formulation of ketoconazole showed a good entrapment efficiency and better stability profile as compared to liposomes. Thus it is concluded that ethosomal formulation is a very promising option for transdermal delivery and has potential for new opportunities for topical application of ketoconazole in the fungal infections.

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Chapter 7

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