FORMULATION AND IN-VITRO EVALUATION OF

ETHOSOMEL GEL OF TOLNAFTATE

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Submitted by

SUTHISH S
(REG. NO: 261711453)

Under the guidance of

Dr.D.SAKTHIVEL M.Pharm., Ph.D.,

Professor, Department of Pharmaceutics.

PGP COLLEGE OF PHARMACEUTICAL SCIENCE

AND RESEARCH INSTITUTE

NH-7, Karur Main Road, Namakkal-637207

May 2019

Prof. Dr. G. ARUNACHALAM. M. Pharm., Ph.D. FIC.,
Principal
PGP College of Pharmaceutical Science and
Research Institute, Namakkal-637207.

CERTIFICATE

This is to certify that the dissertation entitled **"FORMULATION AND *IN-VITRO* EVALUATION OF ETHOSOMEL GEL OF TOLNAFTATE"** was carried out by **SUTHISH.S** (Reg. No: 261711453), under the guidance of **Dr.D.Sakthivel M.Pharm., Ph.D.,** Professor in the Department of Pharmaceutics, PGP College of Pharmaceutical Science and Research Institute, Namakkal, Affiliated to The Tamilnadu Dr. M.G.R Medical University, Chennai - 32. .

Place: Namakkal
Date:

Dr. D.Sakthivel, M.Pharm., Ph.D.,
Professor
Department of Pharmaceutics
PGP College of Pharmaceutical Science and Research Institute, Namakkal-637207.

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This is to certify that the dissertation entitled **"FORMULATION AND *IN-VITRO* EVALUATION OF ETHOSOMEL GEL OF TOLNAFTATE"** was carried out by **SUTHISH.S** (Reg. No: 261711453) in the Department **of Pharmaceutics, PGP College of Pharmaceutical Science and Research Institute, Namakkal-637207, Affiliated to "The Tamilnadu Dr. M.G.R Medical University." Chennai - 32** under my direct supervision and guidance to my fullest satisfaction.

.

Place: Namakkal

Date:

Prof. Dr. G. ARUNACHALAM. M. Pharm., Ph.D. FIC.,

Principal

PGP College of Pharmaceutical Science and Research Institute, Namakkal-637207.

DECLARATION

I hereby declare that the matter embodied in the dissertation entitled **"FORMULATION AND *IN-VITRO* EVALUATION OF ETHOSOMEL GEL OF TOLNAFTATE"** is a bonafide and genuine research work carried by us under the guidance of **Dr. D.SAKTHIVEL M.Pharm., Ph.D.,** Professor, Department of Pharmaceutics, PGP College of Pharmaceutical Science and Research Institute, NH-7, Karur Main Road, Namakkal-637207.

SUTHISH.S

(Reg. No: 261711453)

Place: Namakkal
Date:

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By

S.SUTHISH

CONTENT

1. INTRODUCTION

Vesicles are the structures that are small in size having a bilayer arrangement similar to the natural lipid bilayer structure of our body membrane. They are highly efficient in encapsulating drugs having varied physico-chemical properties. Stratum corneum is regarded as the major hindrance in attaining a good penetration of drugs transdermally and is easily overcome by these vesicular structures [1-3]. Amphiphillic nature of vesicles helps to deliver both hydrophilic as well as lipophilic drugs to their respective targets with relative ease[4-6].Liposomes were developed earlier as pioneer model in vesicular delivery system. Vesicles contribute greatly in cellular communication as well as particle transport. Researchers have revealed their conclusion that the vesicular morphology helps them to deliver drugs in efficient manner and vesicles can be tagged for cell specificity, thus producing a targeted action. The liposomes were further modified for better features which lead to the discovery of ethosomes which is considered by many as one of the major advancement and advantage in vesicular research [7,8].

Ethosome was developed in the first place by Touitou and her colleagues' in 1997 [9, 10].They are highly malleable, soft lipid vesicles and has an enhanced chance to attain deeper penetration into the skin as well as systemic circulation. Their size can vary from 10 nanometres to a very few microns and contain a very high concentration of ethanol (20-45%) and a lower content of water[6].They are considered as the modified successors to classical liposome with increased ethanol content[11].The action of ethosomal permeation is mainly by lipid bilayer disturbance in skin caused by the ethanol which enhances their penetrability[3,12].Fluidized lipids in the membrane and high flexibility of the vesicular membrane together help them to even squeeze through the pores in stratum corneum which are smaller than them[13].The major constituents are phospholipids(phosphatidylcholine,phosphatidyl serine,phosphatidic acid)generally used at 0.5-10% concentration along with high concentration of alcohol(ethanol or isopropyl alcohol),glycols such as transcutol and propylene glycol enhance permeation or act as edge activators. Cholesterol is generally used at 0.1-1% range. These advancements have helped in producing several marketed formulations as well as newer types of ethosomes such as transethosomes for enhanced therapy.

1.1. Vesicular systems

1.1.1. Liposomes

They are microscopic sized water containing vesicles similar in structure to skin phospholipid bilayer structure .The phospholipid chain from soya or egg yolk and cholesterol in some cases [18].Mezei was the pioneer in using liposomes as carrier agents for delivery. It only helped the drug to reach the reservoir in the upper layer of skin and no percutaneous absorption was achieved thus addressing the need of newer methods. Certain studies showed enhanced deposition of miconazole nitrate in upper skin strata with minimum penetrability when liposomes were used [19].

1.1.2. Niosomes

They are similar to classical liposomes in their composition except for the use of non ionic surfactants thus giving them higher stability and lesser cost. The mechanisms depends on physico-chemical properties of drug, vesicle type as well as lipids used [12]. Fluconazole niosomes with span 60, span 40 and brij 72 prepared by thin film hydration method exhibited sustained drug release and higher cutaneous retention [13].Cyclopirox is another drug delivered with better efficiency in a different study.

1.1.3. Transferosomes

They are also known as ultra deformable vesicles or liposomes due to greater elasticity and deformability. Phospholipids along with surfactants of different types provide the flexibility and is used as an efficient delivery system for transdermal as well as topical delivery of drugs, genetic materials and vaccines study of clotrimazole loaded ethosomes lead to the finding that the drug flux was found to be more in the system than normal transferosomes which ultimately proved the higher efficiency of ethosomes as a vesicular delivery system.

1.1.4. Spingosomes

They are concentric bilayered vesicles where the aqueous compartment is completely enclosed by bilayered membrane composed of natural or synthetic

spingolipids and has a size range of 0.05 to 0.45 microns. They are more stable and have increased circulation time compared to normal vesicular systems because they are made of only amide and ether linkages with lesser number of double bonds than lecithin. They are ideal in targeting tumours and gene delivery and immunology studies. It was used in cancer therapy according to saraf et al in 2001.Spingosomes are prepared using spingomyelin based cholesterol that impart them characteristics like resistance against oxidation and acid hydrolysis giving them greater stability in plasma as well as increased circulation time leading to better bioavailability. The oral as well as transdermal mode of use was coined by Webb et al in 1996.Major limitations of the system are limited use because of highly expensive spingolipids and also decreased entrapment efficiency.

1.1.5. Pharmacosomes

Pharmacosomes are the potential alternatives for conventional vesicular systems. Pharamcon means drug and some means carrier and thus they constitute of colloidal dispersion of drug covalently bound to lipids and may exhibit ultra fine vesicular, micellar or hexagonal aggregate forms depending drug- lipid complex structure. Certain drugs with a carboxyl group or an active hydrogen group can be esterified producing an ampiphilic prodrug containing system. It is a self assembled nanoparticle system and can be used to load more amount of drug and can have a low interfacial tension, higher contact area leading to enhanced bioavailability.

1.1.6. Virosomes

Virosomes are spherical, unilamellar phospholipid bilayered vesicles incorporating virus derived proteins so that it can fuse with target cells. The nucleocapsid and genetic material of source virus is incorporated within the envelope and for influenza virus resistance, lipids are intercalated with membrane proteins like haemagglutinin and neuraminidase thus enabling them to transfer the drug to target cell cytoplasm. The viral surface glycoprotein is contained in the vesicles and size ranges from 120-180 nanometres.

1.1.7. Colloidosomes

Colloidosomes are hollow shelled microcapsules consisting of coagulated or fused particles at the interface of the emulsion droplets. They have highly versatile and flexible application as their membrane offers greater potential in controlling permeability of entrapped species ensuring selective and timed drug release. They system is only at its developmental stage as wider utility is not prevalent.

1.1.8. Aquasomes

It is a three layered self assembled nanoparticle system with ceramic carbon nanocrystalline particulate core coated with glassy cellobiose that helps in specific targeting and molecular shielding.

1.1.9. Cubosomes

These are systems which have been experimentally put into use for herbal medicine delivery for the KIOM-MA 128 drug used in atopic dermatitis treatment. The permeation feature of M-A 128 was enhanced using cubosomes compared to suspension form.

1.1.10. Ethosome

Ethosome is another novel lipid carrier, recently developed by **Touitou et al. (2000)**, showing enhanced skin delivery. The ethosomal system is composed of phospholipid, ethanol and water. The size of ethosomes varies from few nanometres' to micrometres depending on method of preparation and application of techniques like sonication

Several studies investigated the effect of ethanol on physicochemical characteristics of the ethosomal vesicles. One reported characteristic of ethosomes is their small size relative to liposomes, when both are obtained by preparation methods not involving any size reduction steps. This reduction in vesicle size could be explained as a result of incorporation of high ethanol concentration. Ethanol confers a surface negative net charge to the liposome which causes the size of vesicles to

decrease. The size of ethosomal vesicles was reported to increase with decreasing ethanol concentration in the ethanol concentration range of 20–45%. The effect of phospholipid concentration on the size of ethosomal vesicles was also investigated [20].

Ethosomes have been shown to exhibit high encapsulation efficiency for a wide range of molecules including lipophilic drugs. This could be explained by multilamellarity of ethosomal vesicles as well as by the presence of ethanol in ethosomes, which allows for better solubility of many drugs. Encapsulation experiments showed that ethosomes are able to entrap both hydrophilic and hydrophobic drugs

1.2. Major types of ethosomes based on composition

1.2.1. Classical ethosomes

They are actually modification of classical liposomes with high alcohol content(45%w/w).they have enhanced entrapment efficiency and higher negative zeta potential compared to classical ethosomes. Molecular weight ranges from 130.07Da to 24kDa.Thus have greater stability as well as increased permeation.

1.2.2. Binary ethosomes

They were introduced first by zhou et al .They are binary because they are made by adding another alcohol in to the formulation for enhancement of ideal properties. The commonly added alcohols include propylene glycol (PG) and isopropyl alcohol (IPA).

1.2.3. Transethosomes

The new generation of vesicular systems developed by song et al in 2012.They are similar to classical preparations but contains an additional component in the form of an edge activator(surfactant mostly) and/or penetration enhancer. The novel delivery system combines the ideal properties of classical ethosomes as well as the elasticity and deformability of transferosomes as in one formulation known as transethosomes. They were reported to have superior and beneficial characteristics compared to classical

ethosomes. They are capable of entrapping drug which have a molecular weight ranging from 130.077Da to 200-235kDa.

Table.1. Differentiating the ethosomes based on their characteristic

Parameter	**Classical ethosomes**	**Binary ethosomes**	**Transethosomes**
Composition	1. Phospholipids 2. Ethanol 3. Stabilizer 4. Charge inducer 5. 6. Drug/agent	1. Phospholipids 2. Ethanol 3. Propylene glycol other alcohol 4. Charge inducer 5. 6. Drug/agent	1. Phospholipids 2. Ethanol 3. Edge activator (surfactant) penetration enhancer 4. Charge inducer 5. Water 6. Drug/agent
Skin permeation	Higher than classical liposomes	Equal to or higher than classical ethosomes	Typically higher than classical ethosomes
Entrapment efficiency	Higher than classical liposomes	Usually higher than classical ethosomes	Mostly higher than classical ethosomes

Potential	Negatively charged	Negatively charged	Positively or negatively charged
Size	Smaller than the classical liposomes	Equal to or smaller than classical ethosomes	Based on edge activator/ permeation enhancer concentration

1.3. Impact of major constituents on ethosomal properties

1.3.1. Ethanol

Ethanol is considered to be of great use as an ideal penetration enhancer. The amount of ethanol used was found to range between the concentrations of 10%to50%in most of the studies Various researches over the years have given enough evidence stating that increasing the ethanol concentration has an impact on vesicular size thus the size of vesicles are decreased to some extend with increased ethanol concentration. It should be noted that the increase in concentration above a specific limit which is considered as optimum after which further increase may lead to a slight increase in the vesicular size.

This case also causes a very high reduction in the entrapment efficiency also. The actual reason behind the decreasing vesicular size is due to the interpenetration of the ethanol hydrocarbon chain that leads to a reduction in vesicular membrane thickness that reduces the size of vesicles significantly .But the very high content may not give fruitful results as it causes the solubilisation of the vesicle as such and the ideal process of drug delivery is not attained .The net charge on the system is also affected by ethanol as the

surface charge which is expressed as the zeta potential helps to build some steric stability due to negative charge given by the ethanol. Bendas and Tadros are two researchers who in their studies revealed that the vesicular size decreased around 44.6% compared to classical liposomes when ethanol concentration of 40% was used in the ethosomal preparations [21].

The vesicular zeta potential has a great impact on the properties of the system. It produces effects on stability as well as skin interaction of the vesicle. Dayan and Touitou found that net negative charge was imparted to the vesicles by ethanol. Thus this further was found to enhance the vesicle stability as it makes use of the principle of electrostatic repulsion between the components. Increasing ethanol content increases the entrapment efficiency. Another important aspect that is greatly improved by ethanol is the solubility of lipophilic as well as amphiphillic drugs which shows a greater dissolution profile or solubility when the ideal concentration of ethanol which is 20-40% is made use of in the formulation. Entrapment efficiency shows an increasing trend until a very high concentration of alcohol is reached where it predominantly leads to dissolution of the vesicles itself where the entrapment efficiency is diminished [22, 23].

1.3.2. Phospholipids

The amount as well as the type of phospholipids used can have an influence on the vesicular structure and other properties .Soya and egg phosphatidyl choline mostly lecithin are used in general. The variations arising in the source can also affect the vesicular size, zeta potential, stability as well as penetration power. Phospholipon 90H ,Phospholipon 80H and soya phosphatidyl choline were used by Prasanthi Lakshmi et al so as to reach the conclusion that revealed the major impact of source of the phospholipids on ethosomal size as well as vesicular entrapment efficiency[24]. Shen in his research found out that higher phospholipid content enhanced vesicular stability greatly. The optimal range of the component is found to be 0.5% to5% [25].It was found that there was a moderate or slight increase in the size with increased concentration .This trend of increasing size is only observed till a point is reached. Entrapment efficiency increases with increase in phospholipid concentration till a particular level after which no more effect is observed on the entrapment efficiency [26].

1.3.3. Cholesterol

The component finds lesser use in the preparation process and is steroidal moiety having a very complex structure on the whole. It is capable of imparting good rigidity which further leads to reduced leakage and higher stability of the system. It also enhances entrapment efficiency and is used at concentrations less than 3% [27].It can also have a very high concentration in certain cases that is around 70%.most studies reveal that they cause a slight or moderate increment in the vesicular size. The above mentioned idea is made clear by the results of a certain study wherein increase in the concentration from 0% to0.15% caused a great increment of the vesicular size from 102±13 nanometres to 152±12 nanometres.

Very high concentrations of cholesterol may often have a negative impact as the rigidity exceeds the required limit and the deformability of the vesicles may be reduced greatly further causing a hindrance to penetration through stratum corneum as it is commonly seen in case of certain multi lamellar vesicles.33nn.Such rigidity related problems were addressed by various other researchers [25].

1.3.4. Propylene glycol

The use of propylene glycol as the component in a binary ethosomes showed a significant decrease in particle size from 103.7±0.9 nm to 76.3±0.5 nm when 0% to 20% v/v of propylene glycol was used. Many researches also identified propylene glycol as an agent that greatly improves drug stability as well as drug distribution. Higher entrapment efficiency was also noted in many cases. Enhancement of drug permeation is based on the relative ratios of ethanol and propylene glycol. Terbinafine hydrochloride showed a higher skin deposition when ratio used was 7:3.They increased stability by enhancing viscosity as well as by preventing hydrolysis [28,29] .Classical ethosomes have lower stability than binary ethosomes when stored at 4^0C[30].

1.3.5. Isopropyl alcohol

It was clear from the studies conducted by Dave et al that isopropyl alcohol had an influence over enhancing drug entrapment efficiency in the system. The study was based on finding out various properties of 3 different formulations namely a) classical ethosomes containing 40% alcohol in the form of ethanol, b) binary ethosome containing both ethanol and isopropyl alcohol in 20% each concentration, c) vesicular system which contain 40% of isopropyl alcohol. Further the comparison studies revealed that the vesicular system had the highest entrapment efficiency at 95% .The in vitro release studies carried out showed the vesicular system showed the lowest release rate in 8hrs.It also had least transdermal flux than other preparations. It was concluded that isopropyl alcohol had an enhanced effect on entrapment efficiency but a reduced effect on drug release.[31]

1.3.6. Dicetyl phosphate

This component was found to have minimal influence on the prevention of aggregation of the ethosomes and thus increases stability of system. It is usually used at a range of 8-10% of the total phospholipids used in the formulation. They are capable of producing vesicles with sharp negative zeta potential [31].

1.3.7. Penetration enhancers/edge activators

Penetrability of drug is an important aspect of the topical drug delivery systems. Several ethosomal systems are available but it is the transethosomal preparation which has a very high success rate in such delivery .They makes use of certain extra components compared to the classical ethosomes called penetration enhancers or edge activators.

1.3.8. Tweens and spans

Tween 80 was generally used at 10-50% concentration of the total phospholipids used. It was attributed to have a positive impact on the system by reducing the vesicular size while increasing stability and skin permeation characteristics. The major action has been explained as a direct outcome of their solubilising property as well as their innate

ability to prevent fusion of the vesicles. A study where Tween 20 was found to be used to prepare transethosomes produced ethosomes with smaller size, higher entrapment efficiency and enhanced ex vivo skin permeation through human skin than Tween 80[32-34].Span 20 was found as ideal component in preparing transethosomes containing caffeine and vitamin E [35].

1.3.9. Oleic acid

Vesicle size, elasticity, zeta potential and skin permeability are enhanced by oleic acid as it alters the stratum corneum properties to improve the fluidity of the layer. It is usually a penetration enhancer when used at a low concentration of 0.5%.The oleic acid containing transethosomes have a negative zeta potential, higher skin permeation and greater drug disposition in rat dermis/epidermis under the studies conducted [17,36,37].

1.3.10. Polyethylene glycol 4000

The studies that were conducted using transethosomes containing mycophenolic acid revealed a major result that they have a very positive impact on essentially increasing the vesicular size while it produced no visible effect on other properties like entrapment efficiency, permeability and vesicular stability [38].

1.3.11. L-menthol

They are penetration enhances that were reportedly used in transethosomes containing 5%ascorpic acid .It enhanced the release rate and showed increased release pattern of the drug through human skin cadaver .A higher release of (36.5%) was obtained which was higher than that observed in case of classical ethosomes after 24 hrs. The process that is responsible for the higher release is the formation eutectic mixture of drug and L-menthol leading to enhanced solubility of the drug and also leads to alteration of the barrier characteristics of stratum corneum layer [39]

1.4. Ethosome Preparation techniques

The first two techniques that are most widely used are relatively simpler than others as it lacks the use of highly sophisticated technology.

1.4.1. Hot method

The phospholipids are dispersed on the whole into water taken and the colloidal solution is formed by heating at 40^0C.ethanol and propylene glycol is heated up to 40^0C in a separate vessel. The organic phase is added to aqueous phase .further the drug is dissolved either in alcohol or water based on its hydrophilicity/lipophilicity, which in turn determines its solubility in either of the solvents. Size of the obtained vesicles is reduced by subjecting to probe sonicator or by extrusion technique· The storage was properly done afterwards [40].

1.4.2. Cold method

This is the method having the greatest popularity among all the methods, a covered vessel is used to dissolve phospholipids, drug and other lipid constituents in alcohol mostly ethanol at room temperature, this process is followed by vigorous stirring .Polyols like poly ethylene glycol is added during the stirring process at 40°C.The arrangement is allowed to be heated in a water bath at 30^0C.Water that is separately heated at 30^0C. Is added and mixed for 5 minutes in a covered vessel.

The process of size reduction of vesicles to the desirable levels done by sonication [22].and extrusion [41].The use of proper temperature that fulfils the demands of the preparation is essential. It implies the necessity of a refrigerated storage.The aqueous phase used can be water [42], buffer solution [10, 43] or normal saline [44]

1.4.3. Mechanical dispersion or thin film hydration method

Phospholipids namely soya phosphatidyl choline and organic solvents namely chloroform and methanol in the ratio 3:1 is taken in a round bottom flask. The organic solvents are further removed from the contents by use of a rotary evaporator above the transition temperature of the lipid molecules present. This leads to the formation of a thin lipid film on the walls .It is further kept in vacuum overnight for the removal of the traces

of solvent remaining .The hydration of the film by a hydro ethanolic solution of drug at an ambient temperature is done with or without the use of sonicator for sonication process [22].The product is cooled to room temperature and the suspension is stored under refrigeration. The lipid is heated and rotated during hydration process and these parameters are set depending on the properties of phospholipids.30 minutes process .1hr or 6hr [45].

1.4.4. Classic method

The phospholipids, drug and ethanol were mixed till the drug dissolution at a temperature of $30^{O}C$ using a water bath .The fine stream of double distilled water were added to the mixture with continuous stirring at 700 rpm in a closed vessel. Homogenization was carried out by passing the resultant suspension through polycarbonate membrane with a hand extruder for 3 cycles [46].

1.4.5. Reverse phase evaporation technique

This is the rarest method and finds very less use .Production of large unilamellar vesicles are its major use. Diethyl ether is used as the organic phase to dissolve the phospholipids. It is mixed with aqueous phase at3:1 ratio in ultrasonic bath at $0^{0}C$ for 5 minutes which leads to the formation of water in oil emulsion. The pressure is reduced to a minimal to bring about the removal of the organic phase which in turn leads to the formation of colloidal dispersion on mechanical agitation in a vigorous manner [46].

1.4.6. Ethanol injection-sonication method

Organic phase made use of is the ethanolic solution of phospholipids which is further injected into the aqueous phase by using a syringe system. The flow rate is maintained at 200 µL/minutes. Homogenization is further done using ultra sonic probe in 5 minutes [47].

1.5. Ethosomal drug penetration mechanism

The mechanism of absorption of drug from ethosomal vesicles is still not clear in idea, but the proposed mechanism of penetration involves 2 steps in concise.
Ethanol effect and the ethosomal effect together bring about the high level penetration of the system into deeper skin layers.

1.5.1. Ethanol effect

Ethanol is an important constituent that improves penetrability of the drug to a great extent. Generally it acts by interacting with the lipid bilayer membrane by increasing its fluidity and subsequently decreasing the thickness and density of the multilayer membrane. Further stratum corneum is the major layer that hinders the permeation but in this case a much greater permeability and reduced blockage due to ethanol interaction is guaranteed [48].

1.5.2. Ethosomal effect

Ethosomes as such has its own merits and acts by fusing with the skin lipid bilayer structurally and due to already increased membrane fluidity are delivered into deeper layers of the skin and their ability to fuse with skin lipids enables them to achieve better release characteristic [48-51].

Permeation studies were conducted using fluorescent probes with different physicochemical properties like rhodamine red ,rhodamine B,B-carotene ,rhodamine 6G for vesicle filling[52,27].On the whole the vesicular softness depends on the transition temperature of lipids in the system. The amount of ethanol as well as phospholipids play an important part in determining the drug entrapment efficiency and particle size which is pivotal in determining the leakage parameters as well as penetration.

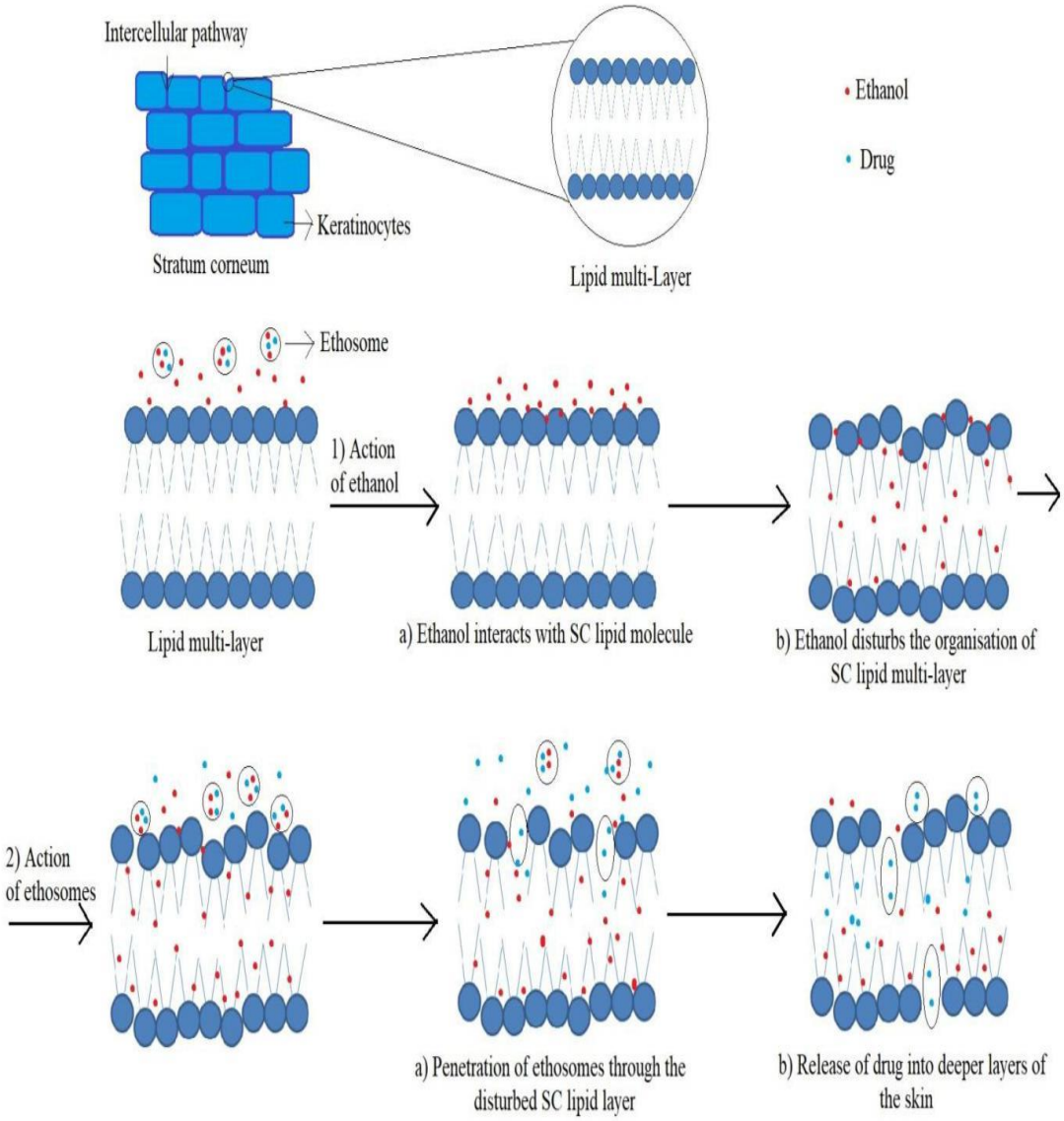

Figure 1.Proposed mechanism of ethosomal drug delivery

1.6. Advantages of ethosomal drug delivery

Ethosomes have a greater impact on enhancing the permeability through skin which is ideal in transdermal and intercellular drug delivery. They help in better and efficient delivery of larger constituent groups(proteins and peptides..The technique is relatively simpler compared to other physical methods like iontophoresis and sonophoresis. The use of non toxic raw materials has its own benefits and being a non

invasive and passive technique it has immediate marketability and commercial utility. It provides a better patient compliance along with greater stability and solubility than many other systems.

Particle size is also smaller to acceptable limits. Ease of industrial scale up is another important feature as large quantities of ethosomes can be easily prepared without the use of rather sophisticated technology or equipments, Thus proprietary technology boosts market attractiveness on the whole .They thus find wider domain of usefulness in the fields of biotechnology, pharmaceutical, cosmetics and veterinary fields. Lipophilic and hydrophilic drugs can be incorporated into the vesicle so that most of the drugs that lack permeation and solubility can be easily delivered .As the vesicle mimics the skin and its phospholipid bilayer structure, it can further enhance the availability of potent drug at the site of action and thus bring about targeted and better therapy.

1.7. Limitations of ethosomal drug delivery

The yield from ethosomal formulations can be poor at times; if not properly prepared. The loss of drug while transfer from organic to aqueous media is of considerable importance Ineffective shell locking in certain cases may lead to coalescence as well as loss of much needed stability. Molecular size of the drug to be loaded should be reasonable so all types of drugs cannot be delivered by the method .In certain cases it may be uncomfortable to wear or irritation causing rendering them less useful. The uneconomical aspect involved in their pricing is also a great deal of hindrance.

1.8. Characterization Studies of Ethosomes

1.8.1. SEM/TEM Imaging:

Visualization of ethosomes can be done using transmission electron microscopy (TEM) and by scanning electron microscopy (SEM). This provides visual information about the size, shape, surface morphology, lammelarity etc. Different lipid types might influence the surface morphology or shape of the particles.

1.8.2. Vesicle size distribution and Zeta potential

Particle size and zeta potential can be determined by a zeta-sizer or dynamic light scattering (DLS) using a computerized inspection system and photon correlation spectroscopy (PCS). The size of ethosomes ranges between tens of nanometers to microns and is influenced by the composition of the formulation.

Zeta potential is an important and useful indicator of particle surface charge, which can be used to predict and control the stability. In general, particles could be dispersed stably when the absolute value of zeta potential was above 30 mV due to the electric repulsion between particles.

1.8.3. Entrapment Efficiency

The entrapment efficiency of drug by ethosomes can be measured by the ultracentrifugation technique. The vesicles are separated in a high speed cooling centrifuge at temperature maintained at 4°C. The sediment and supernatant liquids are separated; amount of drug in the supernatant is determined by UV-Visible spectrophotometry. From this, the entrapment efficiency is determined by the following equation,

$$\text{Entrapment Efficiency (EE \%)} = \frac{\quad}{\quad}$$

Where, T- Total amount of drug in system

C- Amount of drug in supernatant only

Also T- (Supernatant + Sediment)

1.8.4. *In-Vitro* Skin Permeation Study

In-vitro skin permeation of drugs is studied using Franz diffusion cell. The excised skin from abdomen of male nude rats (***Touitou et. al., 2000, Mustafa M.A. Elsayed et. al., 2005***), rabbit pinnae (***Giuseppe Lucania et. al. 2005***), human cadaver

(***Zhen Zhang et. al. 2011***) etc. is separated from the adhering fat and/or subcutaneous tissue. The skin is mounted between donor and receptor compartment with the stratum corneum side facing upward into the donor compartment. Phosphate buffer saline pH 7.4 was taken in the receptor compartment. The formulation was applied on the skin in donor compartment which was then covered with aluminum foil to avoid any evaporation process. Samples were withdrawn at predetermined time intervals over 12/24 hours, and suitably diluted to analyze the drug content. The receptor medium was immediately replenished with equal volume of fresh medium to maintain the sink conditions throughout the experiment. The percentage of drug release was plotted against time to find the drug release pattern.

1.8.5. Skin Retention Study:

The amount of drug retained in the skin is determined at the end of the 12 hours in-vitro permeation studies. The formulation remain in the in-vitro permeation experiment is removed by washing with distilled water. The receptor content is replaced by 50% v/v ethanol and kept for further 12 hours with stirring and the drug content was estimated. This receiver solution diffused through the skin, disrupting any liposome and ethosome structure and extracting deposited drug from the skin.

1.8.6. Vesicular stability

The abilities of the formulations to retain the drug content and shape are analyzed at different temperatures I.E., 25 ± 2°C (Room Temperature, Rt), 37 ± 2°C And 45 ± 2°C For Different Periods Of Time (1, 20, 40, 60, 80 And 120 Days).Nitrogen gas was flushed and was kept in sealed vials. Stability can be found by analyzing the size and structure of vesicles over time and the mean size is measured by DLS while structural changes are estimated by making use of transmission electron microscopy [53].

Table 2. Marketed ethosomal formulations

Product Name	**Drug Name**	**Company Name**	**Use**
Noicellux	Methylxanthine – Caffeine	Novel Therapeutic Technologies ,Israel	Topically Applied Anti Cellulite Cream
Cellotight	Powerful Combination Of Ingredients To Increase Metabolism And Break Down Fat	Hampden Health, USA.	Applied Anti Cellulite Cream
Lipoduction	Pure Grape Seed Extracts	Osmotics, Israel	Anti Cellulite Cream, Antioxidant
Skin Genuity	Caffeine/ Retinol Or The Antioxidant Di methylaminoethanol	Physonics Nottingham, Uk	Anticellulite Gel

	In General		
Nanominox	Minoxidil At 4% Concentration.	Sinere, Germany	Hair Growth Promoter
Decorin Cream	Decorin Proteoglycan	Genome Cosmetics, Pennsylvania, Usa	Anti-Aging Cream
Supravir Cream	Acyclovir	Trima ,Israel	Against Retroviral Disease Like Herpes Virus.

1.9. Studies Conducted Based on Antivirals Incorporated in Ethosomal Carriers

Herpes labialis was treated using synthetic nucleoside analog of acyclovir.The comercial cream is zovirax produced by Glaxo smith kline. In this randomized double-blind clinical study, the efficiency of an ethosomal formulation, a commercial acyclovir cream (Zovirax®, GlaxoSmithKline S.p.A.) and a solution of the free drug was compared in fourty participants, experiencing 61 assessable episodes. The parallel arm consisted of 31 participants of whom 12 received ethosomal acyclovir (EA), 10 Zovirax® cream (ZC) and 9 vehicle (V). In the crossover arm, 8 participants were treated with EA followed by ZC and 7 participants were treated with Zovirax® cream followed by ethosomal acyclovir .Time (in days) to crust formation, time (in days) to loss of crust, the proportion of abortive lesions of all assessable lesions, time (in days) to first reduction of reported pain intensity, time (in days) to absolute resolution of pain and the proportion of lesions in which reported pain intensity was reduced from day 1 to day 2 and from day 1 to day 3 were assessed in this study.

The marketed formulation when compared to ethosomal delivery using a double blind randomised clinical study lead crusting of 80 percent lesions compared to 10 percent for zovirax. Supravir is a cream based on ethosomes developed by trima, Israel.

Transcutaneous immunisation was done using antigen loaded ethosomes for hepatitis B treatment done as per Mishra et al. It showed higher entrapment efficiency, size range, and efficient uptake with improved characteristics. Ethosomes in murine dentritic cells was carried out in vitro and greater permeation was also obtained in human cadaver. Spectral bio imaging and flow cytometric studies showed an efficient uptake of ethosomes by murine dendritic cells *in vitro*, reaching a peak by 180 minutes. This protective immune response was helpful in delivery of transcutaneous hepatitis B vaccine

1.10. The Skin: A Barrier to Drug Permeation

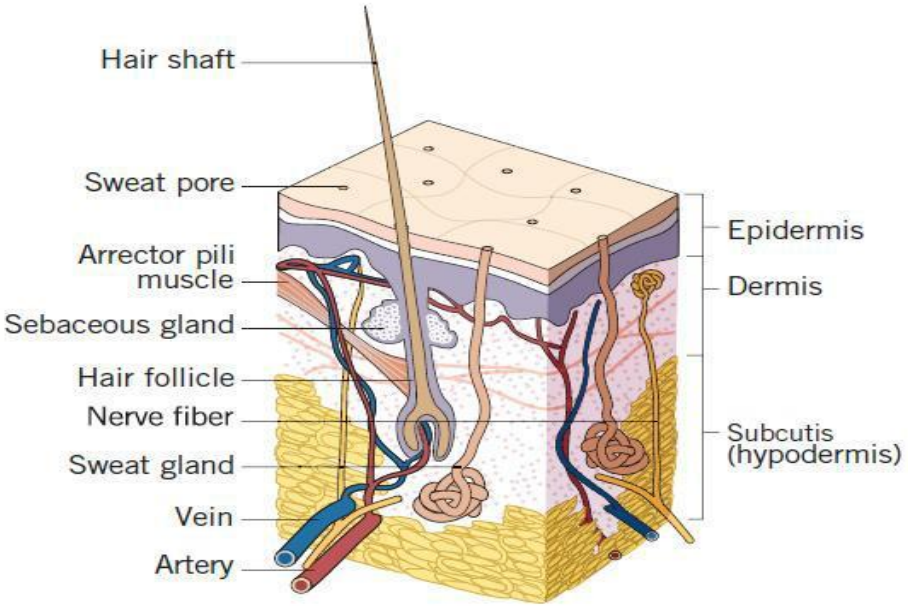

Figure.2 Structure of Skin

Skin accounts for the most wide spread body organ extending all over the body and thus provides a very large surface area for drug delivery. These favourable

reasons make it an ideal candidate to deliver drugs. Skin constitute of multiple layers namely epidermis, dermis and subcutaneous. The delivery of potent drug through skin offers a great deal of advantages over the other conventional methods. Drugs delivered transdermally are capable to attain a fixed or consistent level in the plasma which is of great therapeutic value. Hence they reduce the requirement of frequent and multiple dosing. They help in reducing toxicity problems, diminishing gastrointestinal irritation by avoiding the first pass metabolism are also useful attributes of transdermal drug delivery. Drugs which have a very short half life can also be delivered. The skin permits the easy passage of the lipophilic as well as low molecular weight drugs without many complications. It is the hydrophilic and high molecular weight drugs that are difficult to pass through.

1.11. Brief outline on structure and functions of skin

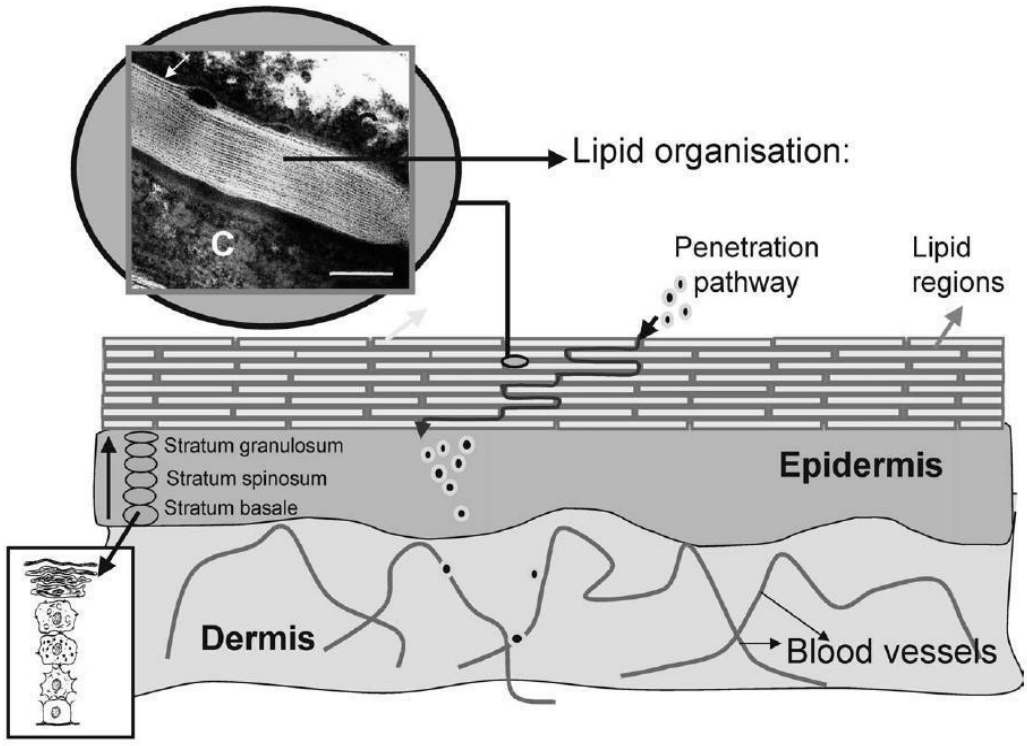

Figure 3. A schematic drawing of a skin cross-section. The corneocytes are embedded in lipid lamellar regions. Substances permeate mainly along the tortuous pathway in the intercellular lamellar regions also known as stratum corneum

A major challenge for dermal drug delivery is to overcome the tightly packed and relatively impermeable stratum corneum (SC). The physicochemical properties of the drugs are highly decisive for their ability to pass the SC, and passive drug permeation across the SC generally requires drugs of a relatively low molecular weight (less than 500 g/mol) and a log P value of 1-3.Composite structure of skin is attributed to its component layers namely epidermis, dermis and hypodermis also called as subcutaneous adipose tissue

1.11.1. Hypodermis

It actually serves as the food reserve protective component and heat insulator, lies beneath dermis connecting to lower skin structures. Certain hair shafts, sebaceous and sweat glands have a major role in molecular transport and are together referred to as pilosebaceous glands [14].

1.11.2. Dermis

It consists of mainly connective tissues and cells such as mast cells, endothelial cells, blood cells and nerve cells. The thickness ranges from 3-5mm.the molecules have greater chance of reaching the central nervous system as the layer constitute of blood capillaries, lymphatic systems and sensory nerves[15].

1.11.3. Epidermis

It has 2 layers namely the inner viable epidermis as well as the stratum corneum layer [34-38].Cells are called keratinocytes. Melanocytes, langharhan cells, migrant macrophages and lymphocytes are present in the viable epidermis.[16].Stratum corneum provide main barrier for percutaneous absorption constituting of layer of corneocytes cells which are hexagonal and anucleate and are made of keratin surrounded by cross linked protein and is present within lipids. They are highly vascularised and it acts as a rate limiting step for most penetrants. They have a brick(corneocytes) and mortar(intercellular lipids).Formation of stratum corneum is due to cell death of outermost horny layer(stratum granulosm)[17].the layer lack phospholipids unlike most bio membranes and the intercellular lipids include ceramids ,free fatty acids, cholesterol and its esters.

The three major routes for deep layer penetration through skin are intercellular, transcellular and transappendagial route .most molecules pass through the difficult intercellular route while some electrolytes and larger molecules with low partition coefficient prefer transappendagial route.

1.12. Fungal Infections

Fungal infection of the skin, hair or nails affects 25% of the world's population. A **mycosis** (plural: **'mycoses**) is a fungal infection of animals, including humans. Mycoses are common, and a variety of environmental and physiological conditions can contribute to the development of fungal diseases. Inhalation of fungal spores or localized colonization of the skin may initiate persistent infections; therefore, mycoses often start in the lungs or on the skin.

1.12.1. Causes

People are at risk of fungal infections when they are taking strong antibiotics for a long period of time because antibiotics kill not only damaging bacteria, but healthy bacteria as well. This alters the balance of microorganisms in the mouth, vagina, intestines and other places in the body, and results in an overgrowth of fungus. Individuals with weakened immune systems are also at risk of developing fungal infections. This is the case of people with HIV/AIDS, people under steroid treatments, and people taking chemotherapy. People with diabetes also tend to develop fungal infections. Very young and very old people, also, are groups at risk.

1.13. Classification

Mycoses are classified according to the tissue levels initially colonized.

1.13.1. Superficial mycoses

Superficial mycoses are limited to the outermost layers of the skin and hair. An example of such a fungal infection is Tinea versicolor, a fungus infection that commonly affects the skin of young people, especially the chest, back, and upper arms and legs. Tinea versicolor is caused by a fungus that lives in the skin of some

adults. It does not usually affect the face. This fungus produces spots that are either lighter than the skin or a reddish-brown. Mycoses to cause the fungus to become more visible include high humidity, as well as immune or hormone abnormalities. However, almost all people with this very common condition are healthy. Clinically these superficial mycoses are labeled according to the region involved. These are as follows

- **Tinea capitis** occurring on the scalp, especially in children
- **Tinea barbae** affecting the region of beard in adult males
- **Tinea corporis** involving the body surface at all ages
- **Tinea cruris** occurs most frequently in the region of groin in obese men
- **Tinea pedis** or **athlete foot** is located in the web spaces between the toes
- **Onychomycoses** shows disintegration of nails substance
- **Tinea versicolor** caused by malassezia furfur generally affects the upper trunk.

1.13.2. Cutaneous mycoses

Cutaneous mycoses extend deeper into the epidermis, and also include invasive hair and nail diseases. These diseases are restricted to the keratinized layers of the skin, hair, and nails. Unlike the superficial mycoses, host immune responses may be evoked, resulting in pathologic changes expressed in the deeper layers of the skin. The organisms that cause these diseases are called dermatophytes. The resulting diseases are often called ringworm (even though there is no worm involved) or tinea. Cutaneous mycoses are caused by *Microsporum*, *Trichophyton*, and *Epidermophyton* fungi, which together comprise 41 species. One common disease is the athlete's foot which most commonly affects men and children before puberty. It is divided in three categories: chronic interdigital athlete's foot, chronic scaly athlete's foot, and acute vesicular athlete's foot.

1.13.3. Subcutaneous mycoses

Subcutaneous mycoses involve the dermis, subcutaneous tissues, muscle, and fascia. These infections are chronic and can be initiated by piercing trauma to the skin, which allows the fungi to enter. These infections are difficult to treat and may require surgical interventions such as debridement.

1.13.4. Systemic mycoses due to primary pathogens

Systemic mycoses due to primary pathogens originate primarily in the lungs and may spread too many organ systems. Organisms that cause systemic mycoses are inherently virulent. Generally, primary pathogens that cause systemic mycoses are dimorphic.

1.13.5. Systemic mycoses due to opportunistic pathogens

Systemic mycoses due to opportunistic pathogens are infections of patients with immune deficiencies who would otherwise not be infected. Examples of immunocompromised conditions include AIDS, alteration of normal flora by antibiotics, immunosuppressive therapy, and metastatic cancer. Examples of opportunistic mycoses include Candidiasis, Cryptococcosis and Aspergillosis.

1.14. Treatment

Antifungal drugs are used to treat mycoses. Depending on the nature of the infection, a topical or systemic agent may be used. Photo chemotherapy or photopheresis is a technique used at medical centers for the treatment of mycosis fungoides. An example of antifungal is fluconazole or Diflucan, which is the basis of many over-the-counter antifungal treatments. Another example is amphotericin B (the A form being toxic) which is more potent. It is used in the treatment of the most severe fungal infections that show resistance to other forms of treatment and it is administered intravenously. Drugs to treat skin infections are Tolnaftate (Tinactin), an over the counter topical; Ketoconazole, especially used to treat tinea versicolor and other dermatophytes; Itraconazole; Terbinafine (Lamisil); Echinocandins (caspofungin); Griseofulvin, commonly used for infections involving the scalp and nails. Yeast infections in the vagina, caused by candida albicans, can be treated with medicated suppositories and pessaries whereas skin yeast infections are treated with medicated ointments.

1.15. Fungal Infections Commonly Treated Using Terbinafine

1.15.1. Athlete's foot

Tinea pedis or athlete's foot is a common fungal infection that affects the foot. Athlete's foot is commonly associated with sports and athletes because the fungus grows perfectly in warm, moist environments, such as socks and shoes, sports equipment, and locker rooms. In reality, anyone may be affected by athlete's foot. It is most common in warmer climates and summer months, where it can quickly multiply.

Symptoms The symptoms of athlete's foot may vary slightly from person to person. Classic symptoms include:

- redness or blisters on the affected area
- the infected skin may be soft, or layers may start to break down
- peeling or cracking skin
- the skin may scale and peel away
- itching, stinging, or burning sensations in the infected area

1.15.2. Jock itch

Tinea cruris, commonly known as jock itch, is another common fungal skin infection. These fungi love warm and damp environments, and thrive in moist areas of the body, such as the groin, buttocks, and inner thighs. Jock itch may be more common in summer or in warm, humid areas of the world. Jock itch is mildly contagious and is often spread through direct contact with an infected person or an object that is carrying the fungus. Jock itch appears on the body as an itchy, red rash that often has a circular shape to it.

Symptoms

- redness in the groin, buttocks, or thighs
- chafing, irritation, itching, or burning in the infected area
- a red rash with a circular shape and raised edges

- cracking, flaking, or dry peeling of the skin in the infected area

1.15.3. Ringworm

Ringworm is a skin infection that causes jock itch and athlete's foot.Tinea corporis or ringworm is a skin infection caused by a fungus that lives on dead tissues, such as the skin, hair, and nails. Ringworm is the fungus that causes both jock itch and athlete's foot. When it appears anywhere else on the body, the infection is just called ringworm.

Symptoms

Ringworm is usually easy to notice because of its shape. A red patch that may itch or be scaly will often turn into a raised, ring-shaped patch of skin over time. It may even spread out into several rings.The outside of this ring is red and may appear raised or bumpy, while the inside of the ring will remain clear or become scaly. Ringworm is highly contagious, and it can be transmitted by skin-to-skin contact, or from contact with pets, such as dogs. The fungus may also survive on objects, such as towels, clothes, and brushes. The ringworm fungus also infects soil and mud, so people who play or work in infected dirt may catch ringworm as well.

1.16. Topical Antifungal Therapy

Many types of fungi are the causative agents of infections like Candida albicans species, Dermatophytes and Tinea species. A wide variety of antifungal agents are available for either topical or systemic use. However, their use is associated with prolonged duration of treatment, low therapeutic value, local and systemic side effects. The main two obstacles in the treatment of skin fungal infections are the skin barrier nature against drug molecules and the low penetration power deeper into skin layers

Topical treatment of fungal infections has several superiorities including, targeting the site of infection, reduction of the risk of systemic side effects, enhancement of the efficacy of treatment and, high patient compliance. Different type

of topical effective antifungal compounds has been used in the treatment of a variety of dermatological skin infections. The main classes of topical antifungals are polyenes, azoles, and allylamine/benzylamines. Cyclopirox is an antifungal agent also used topically. Currently, these antifungal drugs are commercially available in conventional dosage forms such as creams, gels, lotions and sprays.

Allylamines work through inhibition of squalene epoxidase, which is an essential enzyme in the ergosterol biosynthesis pathway of fungal cell membrane formation. Alterations in fungal cellular membranes result in increased cellular permeability and growth inhibition. **The fungicidal action of Terbinafine hydrochloride** is closely associated with the development of high intracellular squalene concentrations, which are believed to interfere with fungal membrane function and cell wall synthesis. In the case of Candida albicans, growth inhibition with terbinafine appears to result from the ergosterol deficiency. The mechanism of terbinafine has a very good scope for systemic and also transdermal delivery.

1.17. Future prospects of ethosomes

The topical route was largely disregarded as an ideal means of drug delivery due to the practical difficulties that arise in evading the stratum corneum layer which leads to reduced access to deeper layers .Thus attaining effective concentration at target site was very difficult. Novel delivery systems like ethosomes make use of vesicular systems to efficiently increase the penetration of hydrophilic and high molecular weight drugs.

They have even showed good delivery characteristics for cationic drugs, proteins and peptides which are much larger in size and are very difficult to attain by conventional methods. They can deliver the required amounts or doses of drugs in a controlled manner and also boosts patient compliance as well as therapeutic effectiveness. Insulin, testosterone, salbutamol and minoxidil are some of the major drugs whose topical and transdermal delivery has been enhanced by the above method. Pilosebaceous delivery, topical delivery of DNA, delivery of viral drugs, anti arthritis, hormone replacement therapy, and cardiovascular treatments and anti Parkinsonism drugs and topical delivery of

hormones are some of the latest advances in the field of ethosomal therapy. Simplicity, efficiency and better control over drug resistance are certain ideal characteristics. Novel therapeutic technology inc (NTT) is a biopharmaceutical company producing treatment for alopecia, deep skin infection, herpes, hormone deficiency, inflammation, post operative nausea, atopic dermatitis and erectile dysfunction using the novel delivery methods. With the advent of newer vesicular technologies such as transethosomes which are considered as a great push in the field, it sure promises to have a future of wider possibilities in making several therapies less costly and more fruitful eliminating various complications.

2. SCOPE &

OBJECTIVE SCOPE

Terbinafine hydrochloride (a potent allylamine antifungal) being a BCS class II drug is prone to solubility problems and is causative of gastric problems when given orally. The current study is so designed to bypass the GI so as to deliver the drug through skin by attaining greater penetration and longer resident time so as to enhance bioavailability using ethosomes as the nanocarriers (novel drug delivery systems).

MAIN OBJECTIVE

The main objective of the current study was to develop and evaluate an ethosomal topical gel using Terbinafine hydrochloride as the antifungal agent (novel vesicular drug delivery system).

FURTHER OBJECTIVES

- To develop Nano ethosomal formulation using Terbinafine hydrochloride, phospholipids along with ethanol for effective therapy against fungal infections such as ringworm, athlete's foot(tinea pedis), and jock itch (tinea cruris).
- To formulate Ethosomes those are able to improve skin delivery of drugs both under occlusive and non-occlusive conditions.
- To assess and evaluate ethosomal as well as gel characteristics.
- To develop effective, cost efficient and safe formulation against onchomycosos.

3. PLAN OF WORK

STAGE I: Review of literature.

- ✓ Literature survey was carried out from various international, national journals, books and internet sources.

STAGE II: Preformulation studies.

- ✓ Compatibility studies using FT-IR
- ✓ Preparation of calibration curve of Terbinafine hydrochloride
- ✓ Determination of Melting point

STAGE III: Optimization of formulation of Ethosomal vesicles.

STAGE IV: Characterization of Ethosomes.

- ✓ Visualization of vesicles and its morphology by SEM imaging.
- ✓ Measurement of zeta potential and vesicular size distribution.
- ✓ Study of drug entrapment efficiency of vesicles.
- ✓ Stability studies.

STAGE V: Preparation of Gel using Optimised Batch of Ethosomes

STAGE VI: Characterization of the Gel

- ✓ Physical examination and homogeneity
- ✓ Determination of spreadability

STAGE VII: *In-Vitro* permeation study using Franz-diffusion cell.

- ✓ Determination of the drug diffusion across the cellophane membrane

4. LITERATURE REVIEW

E. Touitou *et. al* (2000) [5] in this research article they have described a novel carrier for enhanced skin delivery, the ethosomal system, which is composed of phospholipid, ethanol and water. Ethosomal systems were much more efficient at delivering a fluorescent probe to the skin in terms of quantity and depth, than either liposomes or hydroalcoholic solution. Ethosomal systems composed of soy phosphatidylcholine 2%, ethanol 30% and water were shown by electron microscopy to contain multilamellar vesicles. P-NMR studies confirmed the bilayer configuration of the lipids. Calorimetry and fluorescence measurements suggested that the vesicular bilayers are flexible, having a relatively low T_m and fluorescence anisotropy compared with liposomes obtained in the absence of ethanol. Dynamic light scattering measurements indicated that ethanol imparted a negative charge to the vesicles. The average vesicle size, as measured by dynamic light scattering, was modulated by altering the ethosome composition. Experiments using fluorescent probes and ultracentrifugation showed that the ethosomes had a high entrapment capacity for molecules of various lyophilicities.

Cevc, Gregor *et. al* (2004) [2] the study is based on Lipid vesicles and other colloids as drug carriers on the skin. Th**e** study explains that Colloids from an aqueous suspension can cross the skin barrier only through hydrophilic pathways. For a 100 nm colloid trespassing the skin this means at least 5-fold deformation/elongation. (Lipid) Bilayer vesicles are normally more adaptable than the comparably large (lipid coated) fluid droplets. One of the reasons for this, and an essential condition for achieving a high bilayer adaptability and pore penetration, is high bilayer membrane elasticity. It can modulate drug transport through the barrier. In contrast, the adaptability-and stability-optimised mixed lipid vesicles (Transfersomes®, a trademark of IDEA AG) can trespass much narrower pathways between most cells in the skin; such highly adaptable colloids thus *mediate* drug transport through the skin. Sufficiently stable ultra-adaptable carriers, therefore, can ensure targeted drug delivery deep below the application site. This has already been shown in numerous preclinical tests and several phase I and phase II clinical studies.Sustained drug

release through the skin into systemic blood circulation is another field of ultra deformable drug carrier application.

Paolino D *et. al* (2005) [10] The aim of this work was the evaluation of various ethosomal suspensions made up of water, phospholipids and ethanol at various concentrations for their potential application in dermal administration of ammonium glycyrrhizinate, a useful drug for the treatment of various inflammatory-based skin diseases. Physicochemical characterization of ethosomes was carried out by photon correlation spectroscopy and freeze fracture electron microscopy. The percutaneous permeation of ammonium glycyrrhizinate/ethosomes was evaluated in vitro through human stratum corneum and epidermis membranes by using Franz's cells and compared with the permeation profiles of drug solutions either in water or in a water–ethanol mixture. Reflectance spectrophotometry was used as a non-invasive technique to evaluate the carrier toxicity, the drug permeation and the anti-inflammatory activity of ammonium glycyrrhizinate in a model of skin erythema in vivo on human volunteers. Ethosomal suspensions had mean sizes ranging from 350 nm to 100 nm as a function of ethanol and lecithin quantities, i.e., high amounts of ethanol and a low lecithin concentration provided ethosome suspensions with a mean size of ~ 100 nm and a narrow size distribution. The ethosome suspension showed a very good skin tolerability in human volunteers, also when applied for a long period (48 h). Ethosomes elicited an increase of the in vitro percutaneous permeation of both methylnicotinate and ammonium glycyrrhizinate. Some in vivo experiments also showed the ability of ethosome to ensure a skin accumulation and a sustained release of the ammonium glycyrrhizinate.

Elsayed *et. al* (2007) [21] the study focuses on liposomes which were first shown to be of potential value for topical therapy by Mezei and Gulasekharam in 1980, and studies continued towards further investigation and development of lipid vesicles as carriers for skin delivery of drugs. Despite this long history of intensive research, lipid vesicles are still considered as a controversial class of dermal and transdermal carriers. Accordingly, this article provides an overview of the development of lipid vesicles for skin delivery of drugs, with special emphasis on recent advances in this field, including the development of deformable liposomes and ethosome.

Fang YP *et. al* (2008) [22] it is based on Topical photodynamic therapy (PDT) with 5-aminolevulinic acid (ALA) as an alternative therapy for many non-melanoma skin cancers. The major limitation of this therapy, however, is the low permeability of ALA through the stratum corneum (SC) of the skin. The objective of the present work was to characterize ethosomes containing ALA and to enhance the skin production of protoporphyrin IX (PpIX), compared to traditional liposomes. Results showed that the average particle sizes of the ethosomes were less than those of liposomes. Moreover, the entrapment efficiency of ALA in the ethosome formulations was 8–66% depending on the surfactant added. The particle size of the ethosomes was still approximately <200 nm after 32 days of storage. An *in vivo* animal study observed the presence of PpIX in the skin by confocal laser scanning microscopy (CLSM). The results indicated that the penetration ability of ethosomes was greater than that of liposomes. The enhancements of all the formulations were ranging from 11- to 15-fold in contrast to that of control (ALA in an aqueous solution) in terms of PpIX intensity. In addition, colorimetry detected no erythema in the irradiated skin. The results demonstrated that the enhancement ratio of ethosome formulations did not significantly differ between the non-irradiated and irradiated groups except for PE/CH/SS, which may have been due to a photobleaching effect of the PDT-irradiation process.

Liu X, *et. al* (2011) [27] The purpose of this study was to develop a transdermal ligustrazine patch containing a stable formulation and with good entrapment efficiency, release rate, and transdermal absorptionLigustrazine ethosomes were prepared by ethanol injection-sonication, with entrapment efficiency as an indicator. Using acrylic resin as the primary constituent, the ligustrazine ethosome patch was prepared by adding succinic acid as a crosslinking agent and triethyl citrate as a plasticizer. In vitro release and transdermal permeation studies were carried out. Finally, a pharmacokinetic study was carried out in rats to explore relative bioavailability. The formulations of ligustrazine ethosome were 1% (w/v) phospholipid, 0.4% (w/v) cholesterol, and 45% (v/v) ethanol. Ligustrazine ethosomes were obtained with an average particle size of 78.71 ± 1.23 nm and an average entrapment efficiency of 86.42% ± 1.50%. In vitro transdermal testing of the ligustrazine ethosome patches showed that the cumulative 24-hour amount of ligustrazine was up to 183 ± 18 μg/cm^2. The pharmacokinetic results revealed that the

relative bioavailability was 209.45%.Compared with conventional ligustrazine administration; ligustrazine ethosome patches could promote better drug absorption and increase bioavailability. This study demonstrates that the transdermal action of the ligustrazine ethosome patch was comparatively good.

Zhang JP *et al* (2012) [29] the aim of this study was to compare the skin permeation of ethosomes, binary ethosomes and transfersomes of Terbinafine Hydrochloride (TH) under non-occlusive conditions. These lipid vesicles were prepared and characterized for shape, size, zeta-potential and entrapment efficiency. Franz diffusion cells and confocal laser scanning microscopy (CLSM) were used for the percutaneous absorption studies. The quantity of drug in the skin from ethosomes, binary ethosomes (the weight ratio of ethanol to propylene glycol 7:3, ethanol-PG = 7:3, w/w), and transfersomes was 1.26, 1.51 (p <0.05), 1.56 (p <0.01) times higher than that of TH from traditional liposomes (control). The skin deposition of the applied dose (DD%) of TH from ethosomes, binary ethosomes, and transfersomes was 3.34 (p < 0.05), 9.88 (p < 0.01), 2.52 times higher than that of TH from control. The results of CLSM experiments showed that penetration depth and fluorescence intensity of Rhodamine B from binary ethosomes was much greater than that from ethosomes and transfersomes. These results indicated the binary ethosomes (ethanol-PG = 7:3, w/w) most effectively permitted drug penetration through skin; transfersomes made drug easiest to accumulate in the skin. Ethosomes improved drug delivery with greater improvement in skin permeation than improvement in skin deposition.

Zhu X *et. al* (2013) [28] the aim of this study was to prepare Lidocaine base ethosomes using the injection-sonication-filter method. Size, loading efficiency, encapsulation efficiency, and stability were evaluated using a Zetasizer and high performance liquid chromatography. Formulation was determined by measuring the maximum encapsulation efficiency in the orthogonal test. Percutaneous penetration efficiency in vitro was analyzed using a Franz-type diffusion cell experiment. In vivo effectiveness was analyzed using the pinprick test. Cutaneous irritancy tests were performed on white guinea pigs, followed by histopathologic analysis. The results were compared with lidocaine liposomes as well as lidocaine delivered in a hydroethanolic solution. Lidocaine base ethosomes composed of 5% (w/w) egg phosphatidyl choline, 35% (w/w) ethanol, 0.2% (w/w) cholesterol, 5% (w/w)

lidocaine base, and ultrapure water had a mean maximum encapsulation of 51% ± 4%, a mean particle size of 31 ± 3 nm, and a mean loading efficiency of 95.0% ± 0.1%. The transdermal flux of lidocaine base differed significantly for the 3 preparations (F= 120, P < 0.001), being significantly greater from ethosomes than from liposomes (95% corrected CI, 1129–1818 μg/(cm^2·h); P < 0.001), and from hydroethanolic solution (95% corrected CI, 1468–2157 μg/(cm^2·h); P < 0.001). Lidocaine base ethosomes had a shorter onset time and longer duration in vivo than did lidocaine base liposomes or lidocaine delivered in a hydroethanolic solution. Lidocaine base ethosomes showed no evidence of dermal irritation in guinea pigs. Ethosomes are potential carriers of local anesthetics across the skin and may have applicability for other percutaneous drugs that require rapid onset.

Tomar S *et. al* (2015) [54] the study is based on Azithromycin which is a semi-synthetic macrolide antibiotic drug, effective against a wide variety of bacteria. It is primarily used to treat the bacterial infections associated with weaker immune system. Prednisolone is a synthetic corticosteroid, used for suppressing the immune system and inflammation. When used in combination, both the drugs are very much effective in the management of inflammatory conditions or diseases in which the immune system plays an important role. The aim is to study the preformulation parameters for niosomal gel for topical use. The objective of Preformulation study is to generic information useful to the formulator in developing stable and bioavailable dosage form. The use of Preformulation parameter maximize the chances of getting a formulation which is safe, efficacious and stable product and at the same time provide optimization of the drug product quality. Administration of conventional tablets of prednisolone has been reported to exhibit delayed release and unwanted side effects so prednisolone loaded niosomes were developed and azithromycin which tend to cause allergic reaction was incorporated into gel base provide rapid penetration through skin , improve therapeutic performance, restrict action to the target cell and improve patient compliance, hence the objective of the study was made to develop sustained release gel containing azithromycin and niosomal vesicles of prednisolone using Carbopol as a polymer which will controlled the release of drug, increasing the bioavailability of the drug and thus decreasing the dosing frequency of the drug. The Preformulation studies were carried out for identification (physical appearance, melting point, and uv spectrophotometer), solubility profile, TLC, FTIR,

compatibility studies, simultaneous estimation. All the observation and results showed that the azithromycin and prednisolone serve as suitable candidate for Topical drug delivery system that may improve the bioavailability.

Shen S *et. al* (2015) [34] this study focus to develop a novel antimalarial agent. The objectives of this study were to construct a novel compound antimalarial transdermal nanosystem–ethosomal cataplasm, to investigate its characteristics and efficiency, and to systematically explore the penetration-enhancing mechanisms of ethosomal cataplasm. Artesunate-loaded ethosomes and febrifugine-loaded ethosomes were prepared, and their characteristics were evaluated. Drug-loaded ethosomes were incorporated in the matrix of cataplasm to form the compound antimalarial ethosomal cataplasm. With the help of ethosomal technology, the accumulated permeation quantity of artesunate significantly increased at 8 hours after administration, which was 1.57 times as much as that of conventional cataplasm. Soon after administration, the ethosomal cataplasm could make a large quantity of antimalarial drug quickly penetrate through skin, then the remaining drug in the ethosomal cataplasm could be steadily released. These characteristics of ethosomal cataplasm are favorable for antimalarial drugs to kill *Plasmodium* spp. quickly and prevent the resurgence of *Plasmodium* spp. As expected, the ethosomal cataplasm showed good antimalarial efficiency in this experiment. The negative conversion rates were 100% and the recurrence rates were 0% at all dosages. The mechanism of penetration enhancement of the ethosomal cataplasm was systematically explored using an optics microscope, polarization microscope, and transmission electron microscopy. The microstructure, ultrastructure, and birefringent structure in skin were observed. Data obtained in this study showed that the application of ethosomal technology to antimalarial cataplasm could improve the transdermal delivery of drug, enhance the efficacy, and facilitate practical application in clinic

5. MATERIALS AND METHODS

5.1. Materials

Table No 3. List of Materials

SL NO.	MATERIAL	MANUFACTURER
1.	Terbinafine Hydrochloride	Complimentary pack
2.	Lecithin ex. Soya, 30% Phospholipon® 90H	Sisco Research Laboratories Pvt.Ltd.
3.	Propylene glycol	Sisco Research Laboratories Pvt .Ltd.
4.	HPMC	S.D.Fine Chemicals
5.	Absolute Ethanol (99.9%)	Merck, India
6.	Cholesterol	Sisco Research Laboratories Pvt .Ltd
7.	Methanol (HPLC grade)	Merck, India
8.	Dipotassium Hydrogen Phosphate Dihydrate	S.D.Fine Chemicals
9.	Potassium Dihydrogen Phosphate	S.D.Fine Chemicals
10	Triethanolamine	Sisco Research Laboratories Pvt .Ltd

5.2Equipments and Instruments

Table No. 4. List of Equipments/Instrument

SL NO.	**Equipment/Instrument**	**Manufacturer**
1.	Magnetic stirrer	Remi Motors
2.	Electronic Balance	
3.	FT-IR Spectrophotometer	Shimadzu
4.	Zetasizer	Malvern Instruments
5.	SEM	Motic
6.	UV-Spectroscopy	
7.	Triple blade stirrer	Remi Motors,
8.	Sonicator	Bandelin RK 100H
9.	Digital Melting Point Apparatus	
10.	Research Centrifuge	Remi Motors

5.3. Preformulation studies

5.3.1. Determination of Melting Point

The melting point of the drug was determined by capillary method. In this drug was filled in the capillary tube sealed at one end to a height of 3mm from closed end and capillary was introduced into digital melting point apparatus. The temperature range at which drug melts was noted.

5.3.2. Compatibility studies using FTIR spectroscopy

FT-IR can be used to investigate and predict any physico-chemical interactions or incompatibilities between different components in a formulation by matching of IR spectrum peaks of pure ingredients and physical mixture of the drug and excipients. Therefore it can be applied to the selection of suitable, compatible excipients. While selecting ingredients, we chose excipients which are stable, compatible and cosmetically and therapeutically acceptable.

5.3.3. Analytical method development

The drug is firstly subjected to wavelength scan for determination of absorbance maxima (λmax). A stock solution (1000µg/ml) of drug was prepared by dissolving 10 mg drug in 10 ml water in a volumetric flask .From this solution 10 ml is taken out and add 100 ml of water. At last from that solution 10, 20, 30, 40, 50 ml of solution was taken out and final volume was made up to 100 ml with respective dissolution media (water). So the concentration became 10, 20, 30, 40, 50 µg/ml. Then the samples were scanned between ranges of 200 - 400 nm by using UV - Visible spectrophotometer. The wavelength at which maximum absorbance observed was selected as the analytical wavelength of the drug for that particular media [54].

5.4. Method of Preparation of Ethosomal Vesicles

Ethosomes were prepared as reported by ***Touitou et. al.*** with some modifications. The ethosomal systems investigated here were composed of 1-2% w/v soybean phosphatidyl choline (Phospholipon 90H (PL)), 10–30% v/v ethanol, 5% w/v terbinafine hydrochloride and water to 100% v/v. Phospholipid and drug were dissolved in ethanol. *Mili-pore* water was added slowly in a fine stream (flow rate ~ 1.5 ml/min) with constant mixing at 850±15 rpm on magnetic stirrer in a well-sealed container. Mixing was continued for an additional 15 min at 1200±15 rpm. The system was maintained at 30°±2°C throughout the preparation and was then left to cool at room temperature. The preparations were further extruded through a filter of pore size < 0.45 µm to reduce the vesicular size distribution. The final ethosomal systems were stored at 4°C until required for characterization and in-vitro studies.

Table 5. Formulation of the ethosomes

Formula code	**Terbinafine hydrochloride (%)**	**Soybean phosphatidylcholine (%)**	**Ethanol (%)**	**Propylene glycol (%)**
EF1	5	1	10	10
EF2	5	1	20	10
EF3	5	1	30	10
EF4	5	2	10	10
EF5	5	2	20	10
EF6	5	2	30	10

5.5. Characterization of ethosomes

5.5.1. Size analysis of vesicular systems

The mean size of ethosomal colloidal suspension was analyzed by dynamic light scattering technique with a Zeta sizer 3000HSA. The sample was placed in quartz cuvette and size measurements were carried out at a scattering angle of 90º. All observations were recorded in triplicate for each formulation.

5.5.2. Entrapment efficacy

One milli litre of the formulation was centrifuged at 20,000 rpm for 1 h at 10^0C in a centrifuge tube. The supernatant was collected and the amount of drug was determined by ultraviolet spectroscopy at 282 nm. Entrapment efficiency was calculated using the following equation. After further dilutions it was analyzed for drug content using UV Spectrophotometer at 282nm [56]. From this, the entrapment efficiency was determined by the following equation,

Entrapment Efficiency (EE %) =

$$(T-C)/T \times 100$$

Where,

T- Total amount of drug in system (Supernatant + Sediment)

C- Amount of drug in supernatant only

5.5.3. Stability Study

Stability studies were carried out by storing the ethosomal formulations at two different temperatures 4°C and 25±2°C (Ambient room temperature). The drug content/ %entrapment efficiency was estimated for every 15 days for 2 months to identify any change in the entrapment efficiency of ethosomal.

5.6. Preparation of Ethosomal gel

Ethosomal gel (1%) was prepared my mixing the sufficient amount of the optimised batch of formulation (EF3) in a previously prepared aqueous solution of

HPMC (1g in 100ml).The aqueous solution of HPMC was stirred until a clear solution was obtained at 300 rpm for sufficient time using a magnetic stirrer followed by adding small drops of triethanolamine.It was then stirred in an uniform continuous manner until sufficiently acceptable consistency of gel was obtained.

5.7. Characterization of Ethosomal Gel

5.7.1. Physical examination and homogeneity.

The prepared ethosomes and ethosomal gel formulations were inspected visually for their color intensity difference. All developed gels were tested for homogeneity by visual inspection after the gels have been set in the container .They were also tested for their appearance and presence of any aggregates.

5.7.2. Determination of spreadability

Spreadability was determined by apparatus which was suitably modified in the laboratory and used for the study. It consists of a wooden block provided by a pulley at one end .By this method, spreadability is measured on the basis of 'Slip' and 'Drag' characteristics of ethosomal gels. A ground glass slide is fixed on the block. An excess of gel (about 2gm) under study was placed on the ground slide. The gel was then sandwiched between ground slide and another glass slide having the dimension of fixed ground slide, provided with the hook. A 1kg weight was placed on the top of the two slides for 5 minutes to expel air and to provide a uniform film of the liposomal gel between the slides. Excess of the liposomal gel was scrapped from the edges. The top plate was then subjected to weight of 100gms with the help of string attached to the hook and the time (in seconds) required by the top slide to cover a distance of 7.5cm was noted. A shorter interval indicates better spreadability. Spreading coefficient is determined by using the formula:

S= M×L/T

Where,

S = Spreadability, L = Length of glass slides, M = Weight tied to upper slide T = time taken to separate the slides completely from each other

5.8. *In vitro* drug release (Franz diffusion study)

The synthetic membrane (cellophane membrane) samples were mounted on Franz diffusion cells, which have an effective diffusion surface of 1 cm^2.The ethosomal gel was kept in the donor chamber; the blank gel (without drug) was treated as a control. The receptor chamber was filled with phosphate buffer pH 7.4 to ensure sink condition and Thermostat at $32^0C \pm 0.5\ ^0C$ with continuously stirring at 300 rpm. Sampling was done at predetermined time points; 1mL solution was drawn from the receptor and replaced with fresh receptor medium [57].

Inorder to elucidate the mode of drug release and mechanism the in-vitro data was transformed and interpreted at graphical interface constructed using various kinetic models. Zero order release is characteristic of several modified release dosage forms like in transdermal delivery systems and osmotic systems etc.Here the drug dissolution and hence release is concentration independent

$$Qt=Q_0+K_0t$$

Where, Qt is amount of drug released in time t,

Q_0 is the initial amount of drug in the solution,

K_0 is the zero order rate constant

Table 6. Interpretation of release mechanism based on release exponent ("n")

Release exponent (n)	Drug transport mechanism	Rate as function of time
0.45	Fickian diffusion	$t^{-0.5}$
0.45<n=0.89	Non fickian transport	t^{n-1}
0.89	Case ii transport	Zero order release
n>0.89	Super case ii transport	t^{n-1}

6. RESULTS & DISCUSSIONS

6.1. Preformulation studies.

6.1.1. Melting point Determination

The melting point of the drug was determined by capillary method where it is introduced into digital melting point apparatus the temperature range at which drug melts was found to be 195^0C.

6.1.2. Compatibility studies using Fourier-transform infrared spectroscopy.

The experiment was performed to practice working with the Fourier-transform infrared spectroscopy with different ingredients (Terbinafine HCl, SPC, HPMC). The FTIR data reveals that the drug and excipients are compatible with each other and no interaction was observed between drug and excipients and hence proceeded for formulation development. The spectra obtained from IR studies of a range of 4000cm1_400cm1 are depicted in figures 6-8

7. Functional groups present in the IR spectrum

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Sl. no	Group	**Functional Group**	**Wave no. in std Drug spectra**	**Wave no. in Mixture spectra**	**Observation**
1	Carboxylic acid	(C-0) (0-H)	1615 1798	1615 1794	No major shifting No major shifting
2	Primary amine	(N-H)	2860	2849	No major shifting
3	Cyclic	(C-N)	1300	1300	No shifting
4		(C-C)	1455	1455	No shifting

6.1.3. Analytical method development

Table 8. Standard Calibration curve of TBH

Concentration (µg / ml)	Absorbance
0	0
10	0.244
20	0.489
30	0.740
40	1.001
50	1.250

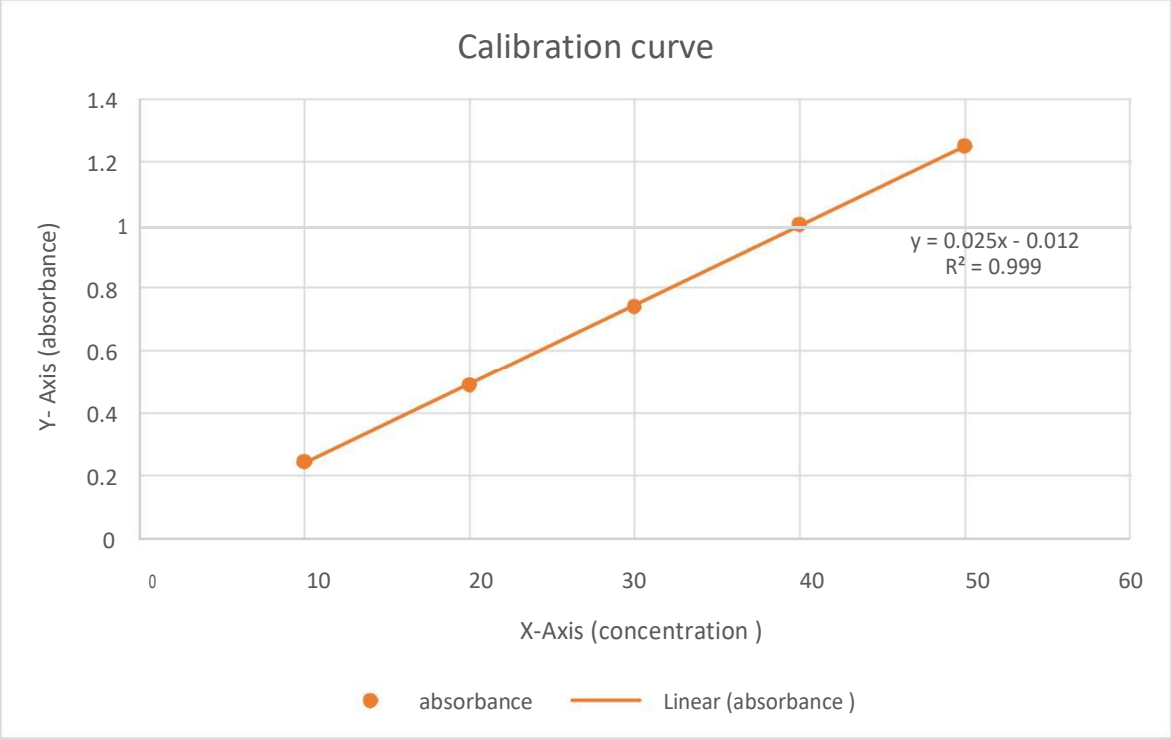

Fig 9. Standard Calibration graph of TBH

6.2. Ethosome characterization

Table 9. Particle size, PDI, zeta potential and entrapment efficiency of prepared ethosomes.

Formula code	Particle size (nm)	PDI	Zeta Potential	Entrapment efficiency
EF1	185.7 ±4.32	0.08 ± 0.02	-6.85 ± 0.72	79.2 ± 1.23
EF2	138.9 ± 3.40	0.30 ± 0.11	-6.85 ± 1.01	82.6 ± 2.25
EF3	**84.57 ± 2.50**	**0.34 ± 0.16**	**-13.2 ± 1.23**	**86.0 ± 3.26**
EF4	192.2 ± 6.92	0.30 ± 0.09	-4.10 ± 0.84	64.8 ± 2.15
EF5	172.8 ± 2.67	0.310 ± 0.09	-5.94 ± 0.35	68.8 ± 3.49
EF6	94.02 ± 4.06	0.261 ± 0.1	-6.50 ± 1.38	72.5 ± 1.36

Optimized batch of formulation was EF3 which had the highest entrapment efficiency ensuring that the formulation can provide high therapeutic value due to effective dose made available in the system. The formulation also has a good negative zeta potential so that the consistent formulation is not prone to aggregation and easy loss of viability

Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 185.7	Peak 1:	190.2	100.0	39.75
PdI: 0.088	Peak 2:	0.000	0.0	0.000
Intercept: 0.671	Peak 3:	0.000	0.0	0.000

Result quality : Good

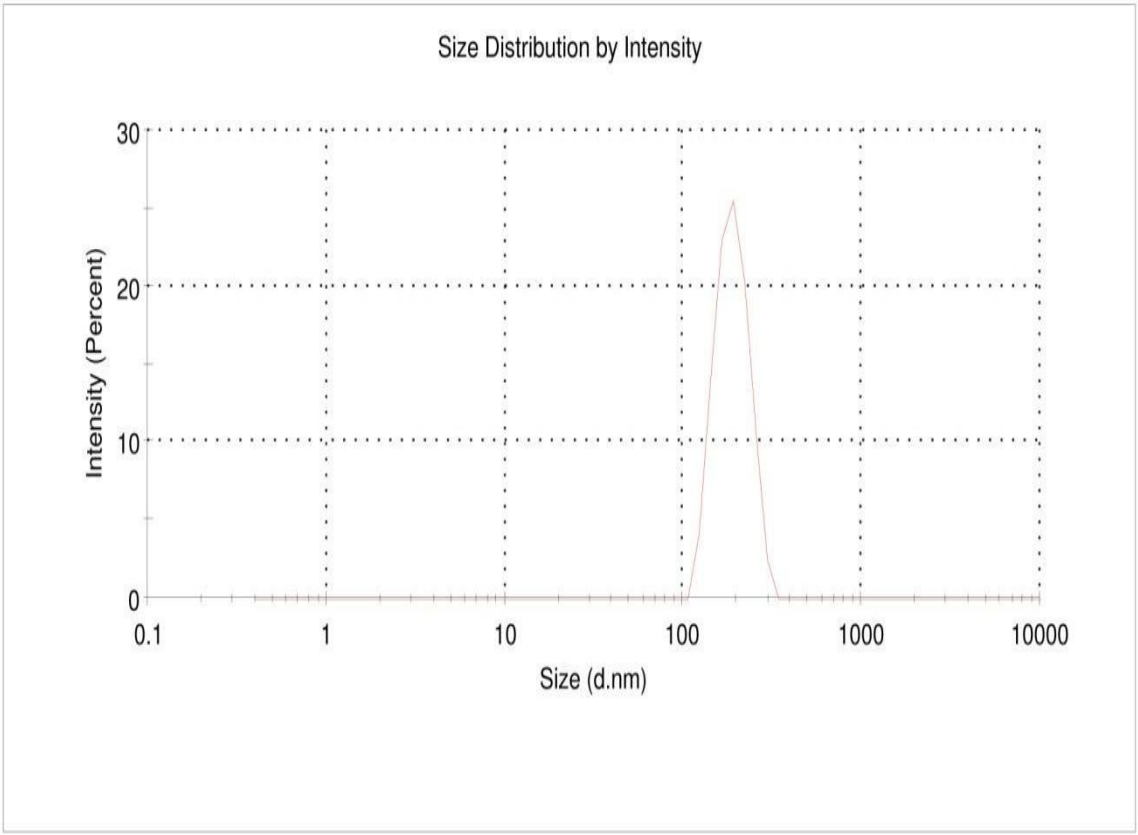

Fig 10. Particle size of EF1

Results

			Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV):	-6.85	**Peak 1:**	8.64	17.1	7.28
Zeta Deviation (mV):	106	**Peak 2:**	-49.0	16.6	15.8
Conductivity (mS/cm):	0.111	**Peak 3:**	49.7	14.5	9.50

Result quality : See result quality report

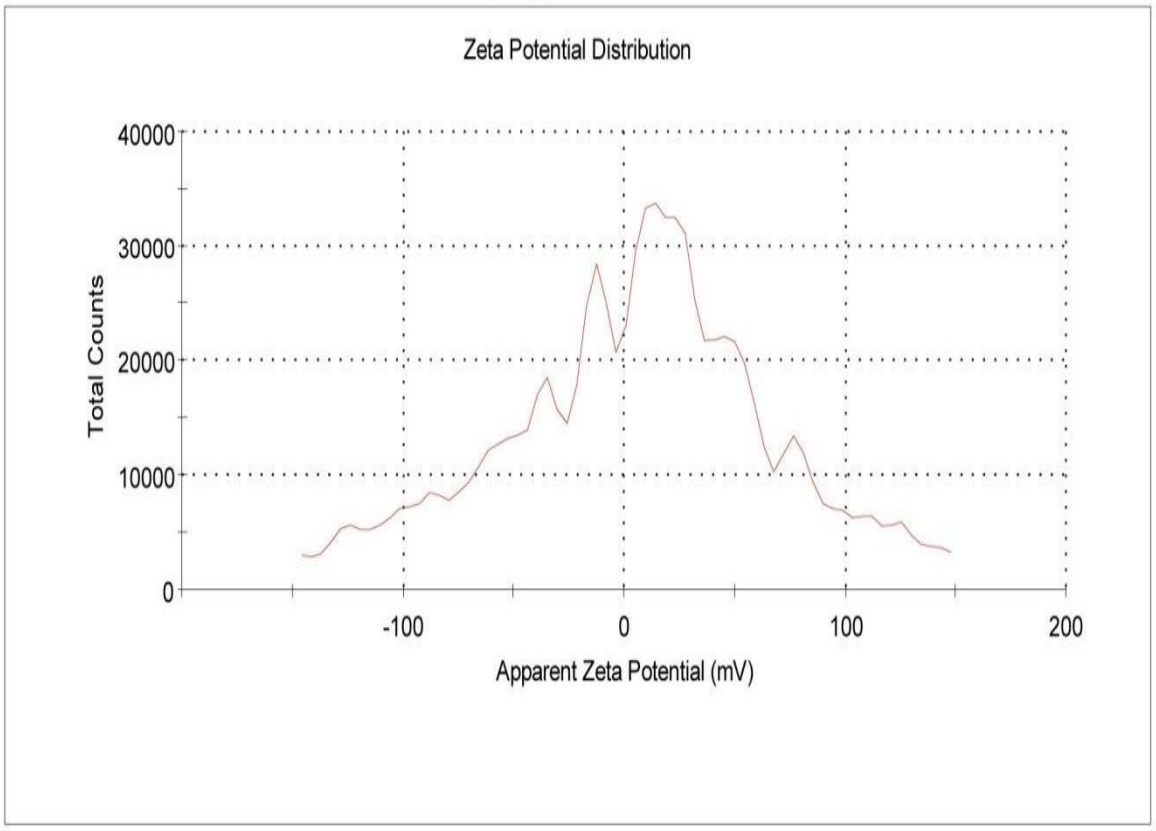

Fig 11. Zetapotential for EF1

Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 138.9	Peak 1:	214.2	88.6	83.10
PdI: 0.305	Peak 2:	37.45	11.4	8.105
Intercept: 0.857	Peak 3:	0.000	0.0	0.000

Result quality : Good

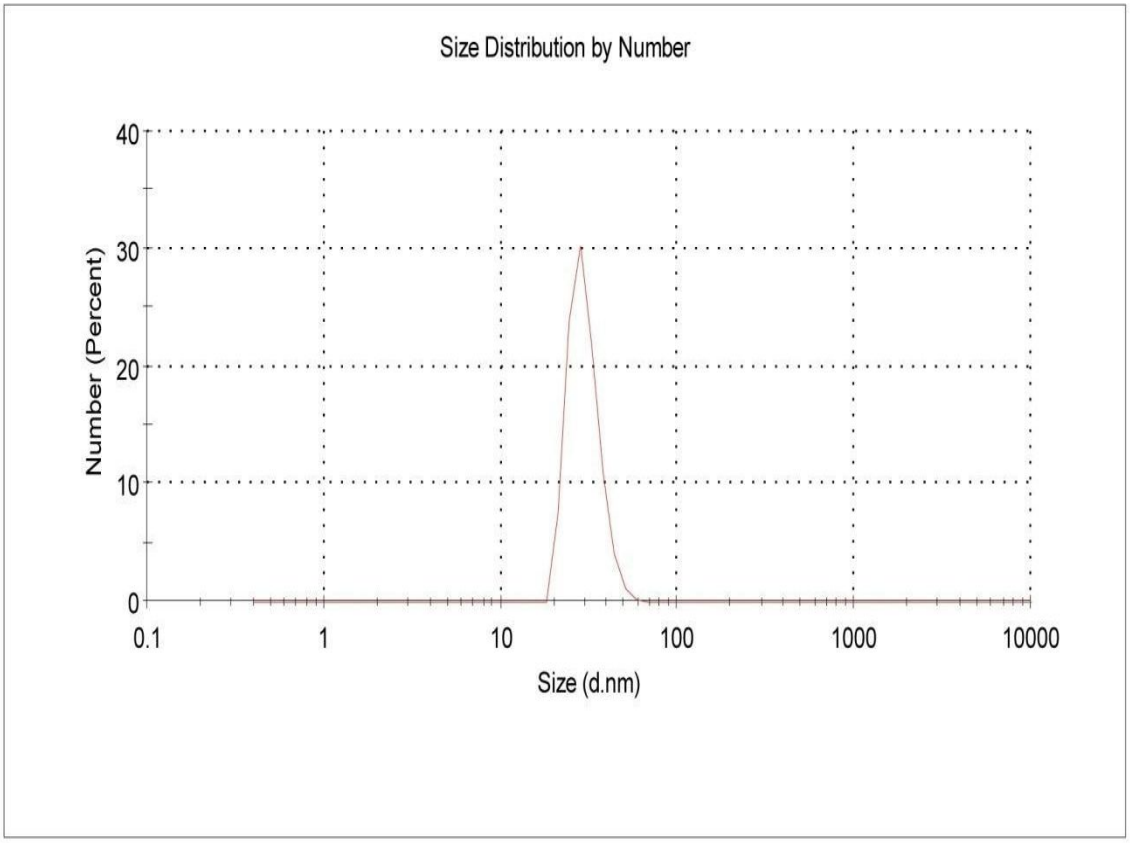

Fig 12. Particle size of EF2

Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -6.85	Peak 1:	8.64	17.1	7.28
Zeta Deviation (mV): 106	Peak 2:	-49.0	16.6	15.8
Conductivity (mS/cm): 0.111	Peak 3:	49.7	14.5	9.50

Result quality : See result quality report

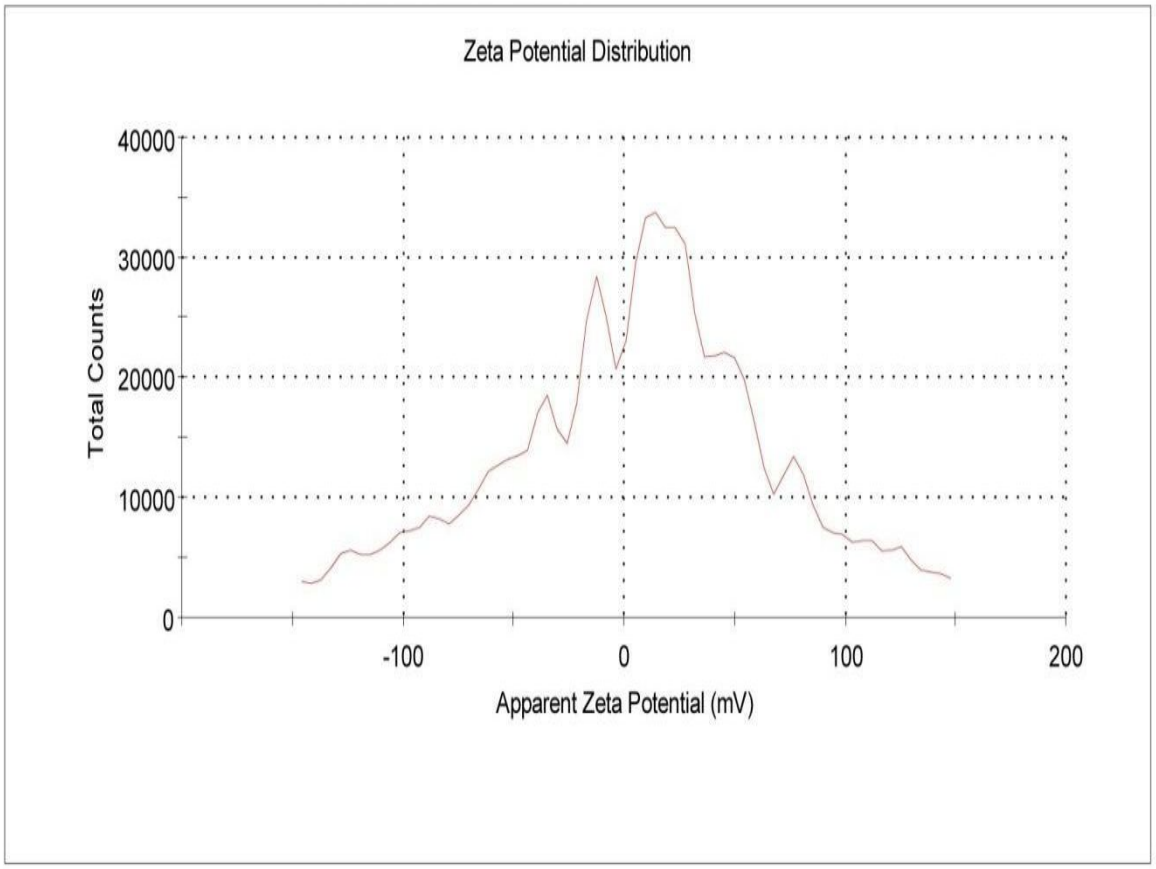

Fig 13. Zetapotential for EF2

Results

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	84.57	Peak 1:	149.3	86.4	81.33
PdI:	0.342	Peak 2:	25.83	13.6	7.293
Intercept:	0.835	Peak 3:	0.000	0.0	0.000

Result quality : Good

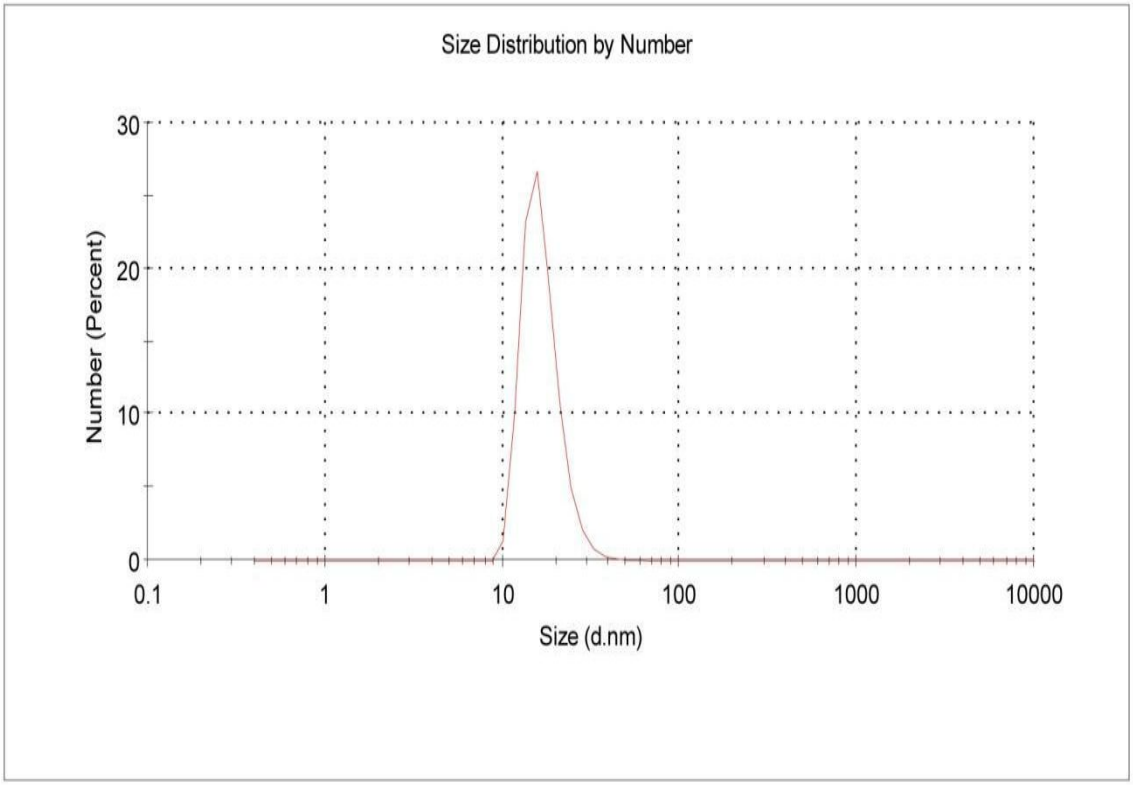

Fig 14. Particle size of EF3

Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -13.2	**Peak 1:**	-13.2	100.0	6.73
Zeta Deviation (mV): 6.73	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm): 0.558	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

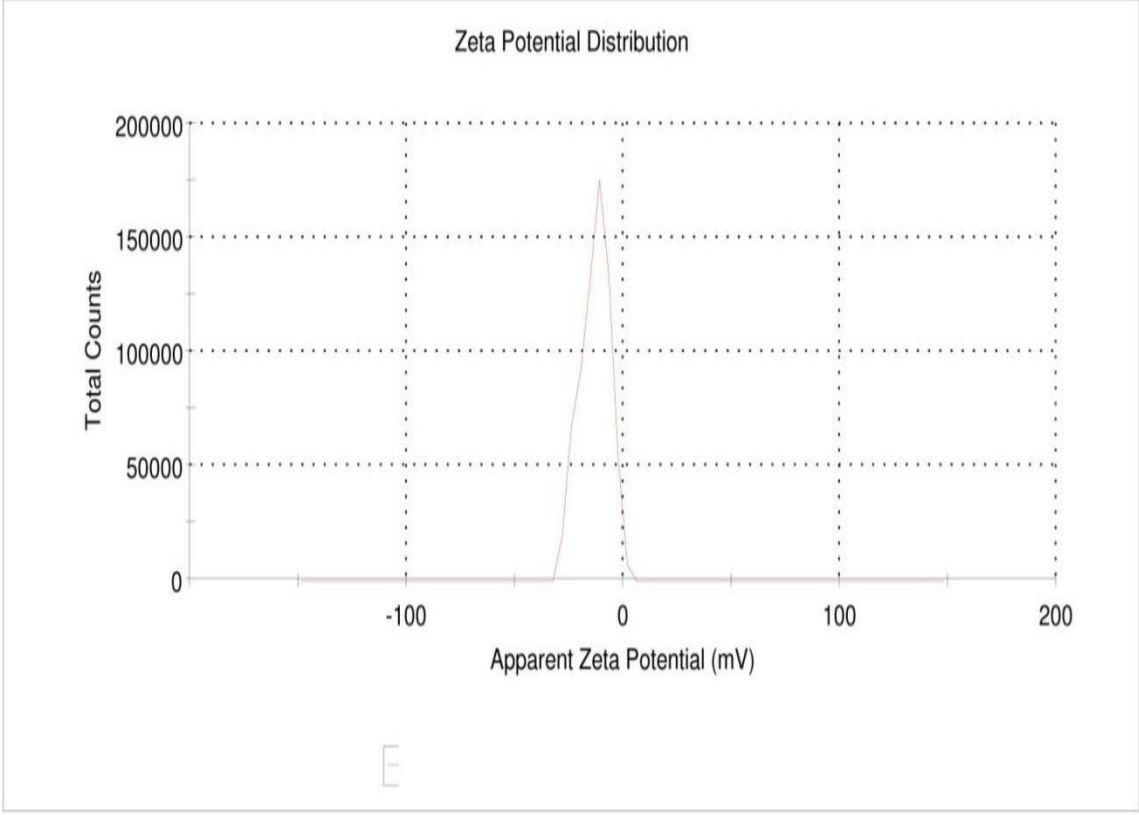

Fig 15. Zetapotential for EF3

Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 192.2	Peak 1:	189.4	100.0	35.70
PdI: 0.302	Peak 2:	0.000	0.0	0.000
Intercept: 0.668	Peak 3:	0.000	0.0	0.000

Result quality : Good

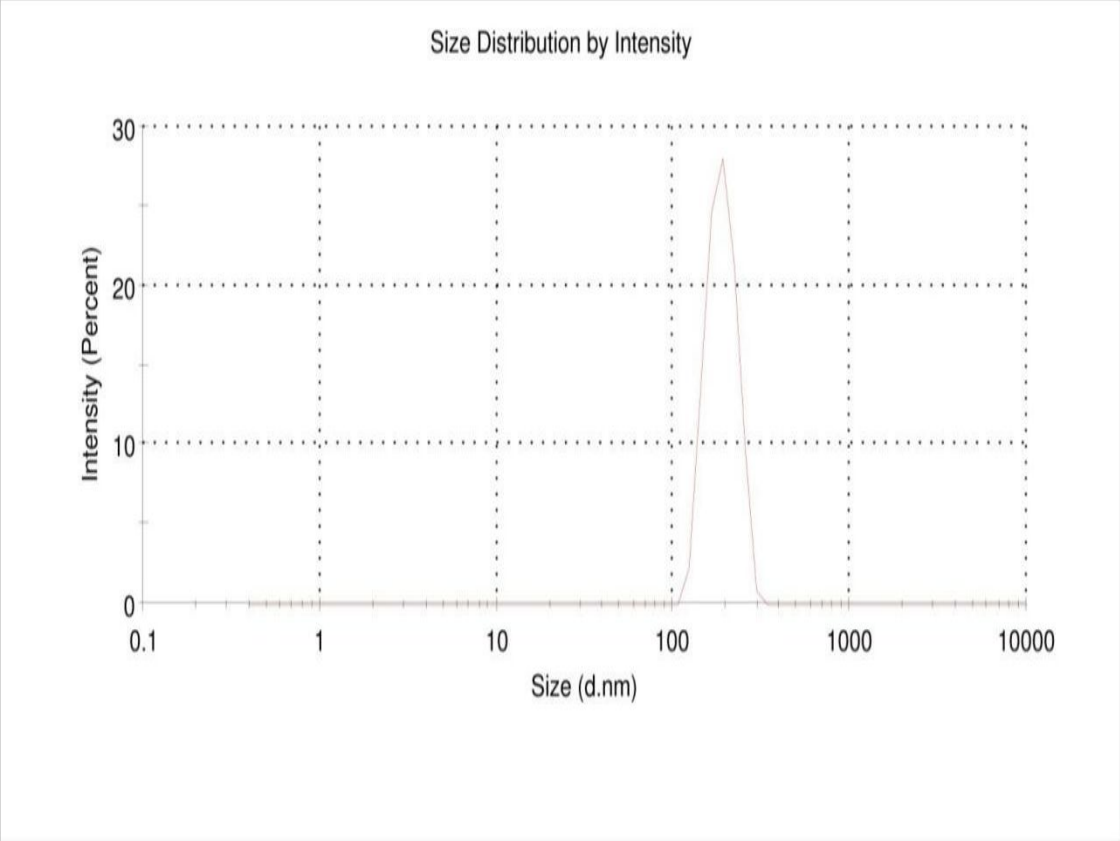

Fig 16. Particle size of EF4

Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -4.10	**Peak 1:**	55.9	11.3	9.30
Zeta Deviation (mV): 172	**Peak 2:**	27.6	10.6	7.99
Conductivity (mS/cm): 0.386	**Peak 3:**	-91.6	10.0	11.2

Result quality : See result quality report

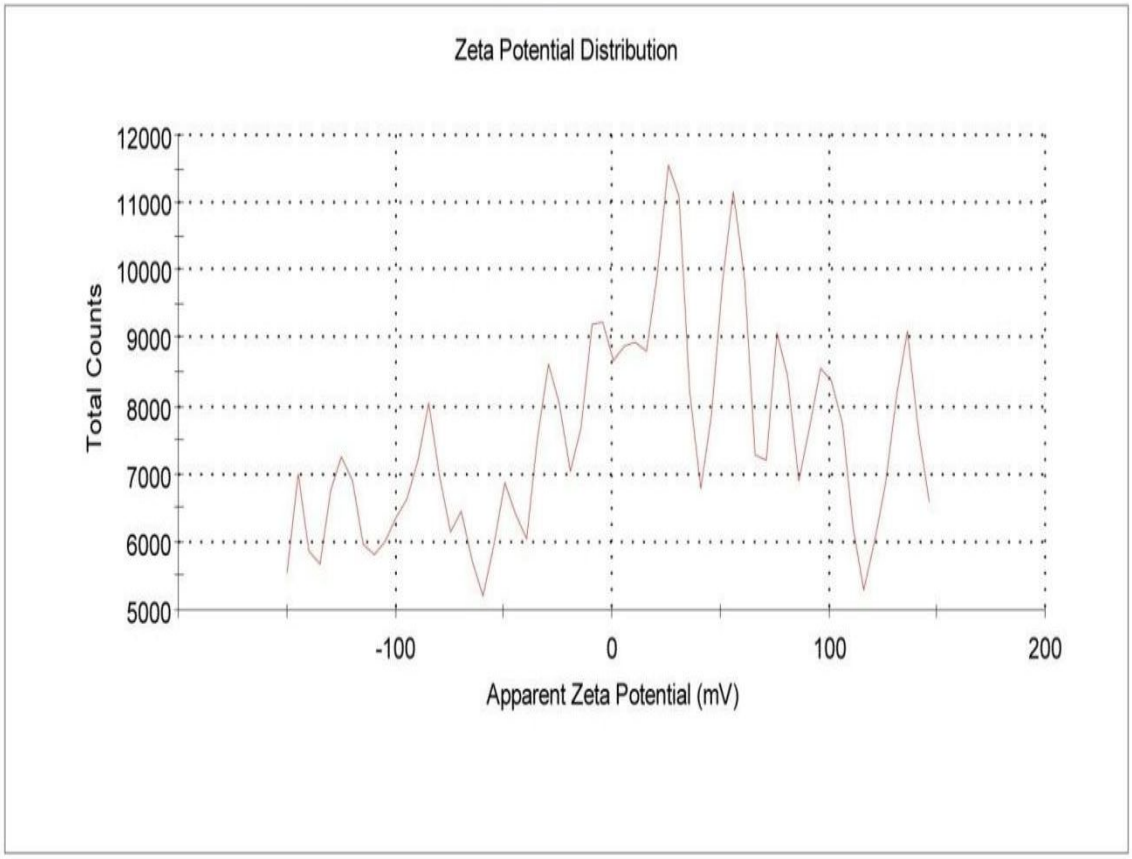

Fig 17. Zetapotential for EF4

Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 172.8	Peak 1:	266.9	92.7	154.1
PdI: 0.310	Peak 2:	39.53	7.3	10.86
Intercept: 0.873	Peak 3:	0.000	0.0	0.000

Result quality : Good

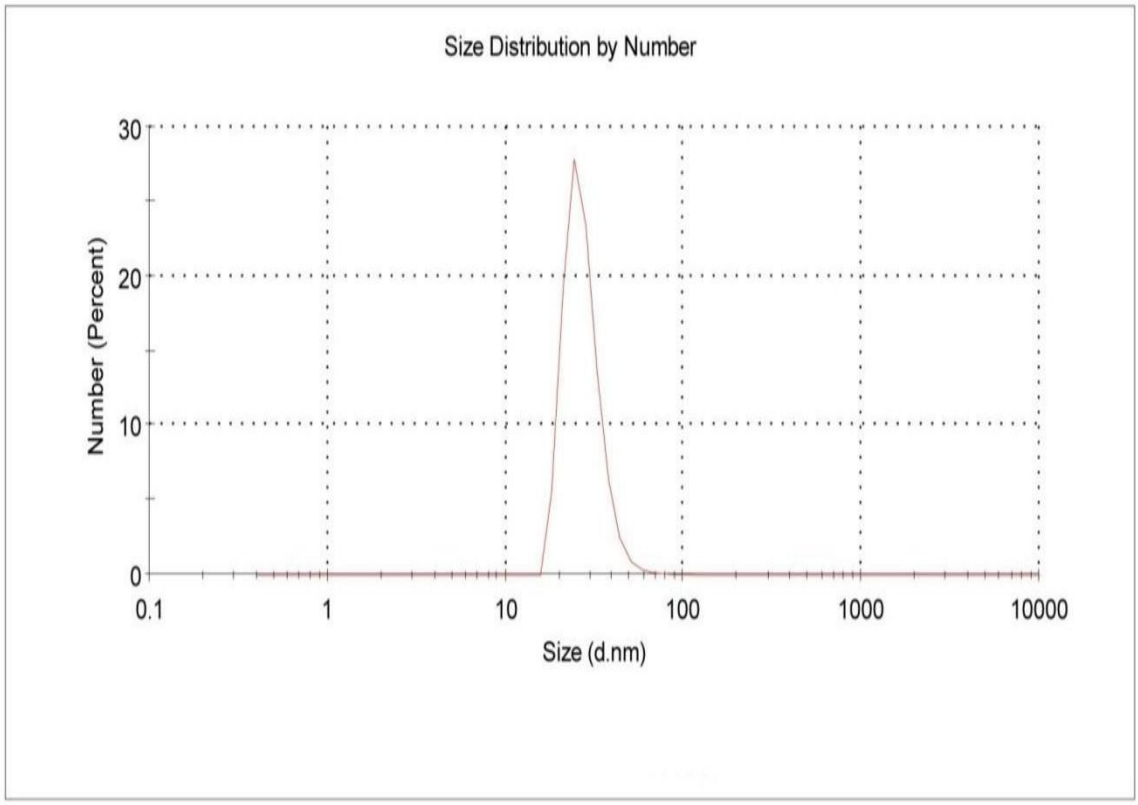

Fig 18. Particle size of EF5

Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -5.94	**Peak 1:**	-5.94	100.0	8.63
Zeta Deviation (mV): 8.63	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm): 0.0263	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

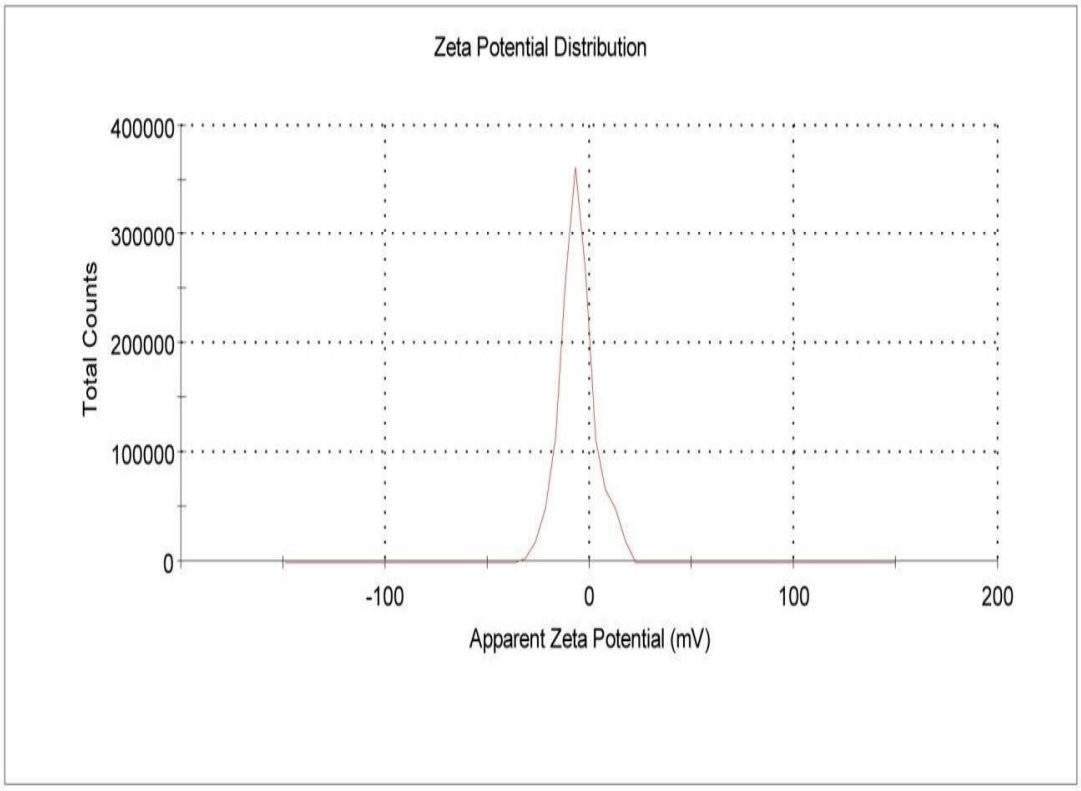

Fig 19. Zetapotential for EF5

Results

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 94.02	Peak 1:	172.0	100.0	223.8
PdI: 0.261	Peak 2:	0.000	0.0	0.000
Intercept: 0.683	Peak 3:	0.000	0.0	0.000

Result quality : Good

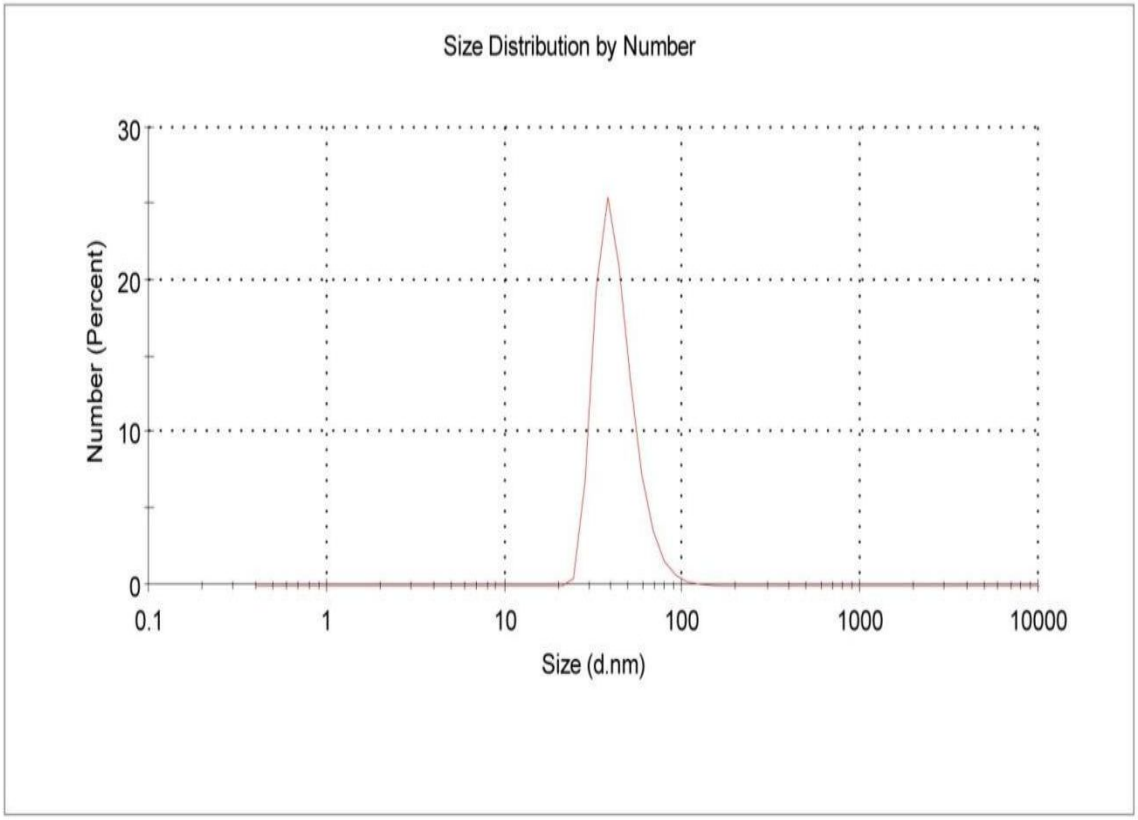

Fig 20. Particle size of EF6

Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -6.50	Peak 1:	-12.9	78.6	16.6
Zeta Deviation (mV): 25.5	Peak 2:	31.7	18.5	13.1
Conductivity (mS/cm): 0.0700	Peak 3:	-68.2	2.9	5.92

Result quality : See result quality report

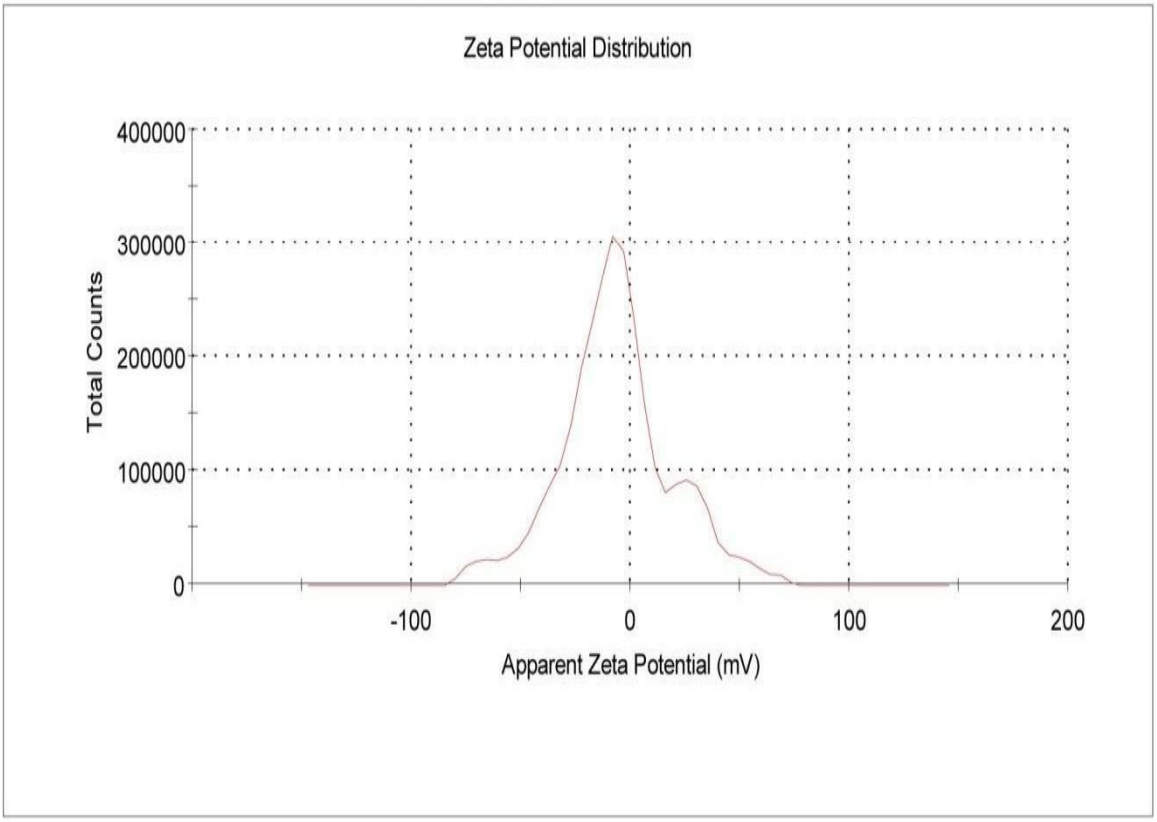

Fig 21. Zetapotential for EF6

6.2.1. Vesicular size analysis & zeta potential

The particle size of ethosomes prepared ranged from 84.57 ± 2.50 to 192.2.4 ± 6.92nm (Table 7). The results of the particle size analysis indicated that the particle size of ethosomes was dependent upon the amount of ethanol and amount of SPC added in the formulation. Ethanol was used as a penetration enhancer in the preparation of ethosomes to rupture the lipid vesicles present in the skin and to facilitate the entry of drug across the stratum corneum. The results of the particle size analysis depicted in Table shows that, when the amount of SPC was constant, an increase in the amount of ethanol from 10 to 30% significantly decreased the particle.. An increase in the amount of ethanol will enhance the solubility of drug and SPC, which in turn will consequently reduce the viscosity of the ethanol and will lead to better dispersion of the formed ethosomes. Additionally, high concentration of ethanol will change the surface characteristics of the vesicles, which will also reduce the particle size. Thus, high amounts ethanol is necessary for the formation of small sized ethosomes. Similarly, the amount of SPC also affected the particle size of the FES. When the amount of ethanol was constant, an increase in the amount of SPC significantly increased the particle size of ethosomes. With an increase in the amount of SPC, the viscosity of the system increases, leading to reduced ability of the ethosomes to get dispersed in the system. Hence, a low amount of SPC is favorable to obtain smaller particles.

The zeta potential of ethosomes were almost neutral the values. Since SPC, a non-ionic surfactant, was used in the preparation, the zeta potential of the formulations remained neutral. Presence of high amounts of ethanol confers the surface a mild negative charge of the vesicles. Confirming to the above discussion, a smallest size of 84.57 ± 2.50nm of the nanoparticles was observed for formulation EF6 containing highest amount of ethanol (30%) and lowest amount of SPC (2%).

6.2.2. Percentage of Entrapment efficiency

The percentages of entrapment efficiency in ethosomes were found to range from 64% to 86% in EF3 respectively as per table 7. The entrapment efficiency is usually enhanced when the amount of SPC is enhanced till an optimum level. The excess ethanol content may sometimes lead to leaky vesicles as stabilizers such as cholesterol are not used in notable concentrations. Cholesterols may be used as the vesicle stabilizer

6.2.3. Stability Studies

The results revealed that the drug retention capacity (entrapment efficiency) was more with ethosomal formulation (EF3) stored at 4°C than at 25±2°C. The decrease in entrapment efficiency may be due to drug leakage from the ethosomes at higher temperature. Hence increase in temperature decreased the drug retention capacity of ethosomes

Table No. 10. Entrapment efficiency of ethosomal formulation EF3 when stored at various temperatures for 2 months

Time of testing	**(EE %) At 4°C**	**(EE %) At 25°C**
Immediately after formulation (within 24 hrs)	86.0±1.05	86±1.05
After 15 days	83. 95±0.57	81.65±0.68
After 30 days	81. 27±0.45	77.03±0.97
After 60 days	74.97±0.72	69.39±0.38

6.3. Characterization of gel

6.3.1. Physical examination and homogeneity

The prepared ethosomal gel formulation was inspected visually for their color, homogeneity, consistency and phase separation. The gel formulation was found to be homogenous without any color intensity differences and aggregates.

6.3.2. Spreading coefficient

The spreadability of the gel containing optimized formulation was found to be 35.Acceptability and clinical efficacy of topical preparations require to possess optimal mechanical properties (ease of removal from the container, spredability on the substrate), rheological properties (viscosity, elasticity, thixotropy, flow ability) and other desired properties such as bio adhesion, desired drug release and absorption. The efficacy of topical therapy depends on the patient spreading the formulation is an even layer to deliver a standard dose. The optimum consistency of such a formulation helps ensure that a suitable dose is applied or delivered to the target site. The delivery of the correct dose of the drug depends highly on the spreadability of the formulation. So spreadability is directly proportional to efficacy.

Table 11. *In vitro* Release kinetics and model fitting

	RELEASE KINEITCS				
	ZERO	**HIGUCHI**	**PEPPAS**	**FIRST**	**Hixson Crowell**
	1	**2**	**3**	**4**	**5**
	R(CvT)	**R(CvRoot(T))**	**Log T vs Log C**	**TIME vs LOG % REMAINING**	**TIME Vs (Q1/3-Qt1/3)**
Slope	13.200	40.268	0.701	-0.014	0.450
Correlation	0.9250	0.9854	0.9884	-0.9806	0.9774
R^2	0.8557	0.9710	0.9769	0.9616	0.9552

6.4. *In vitro* drug release (Franz diffusion study)

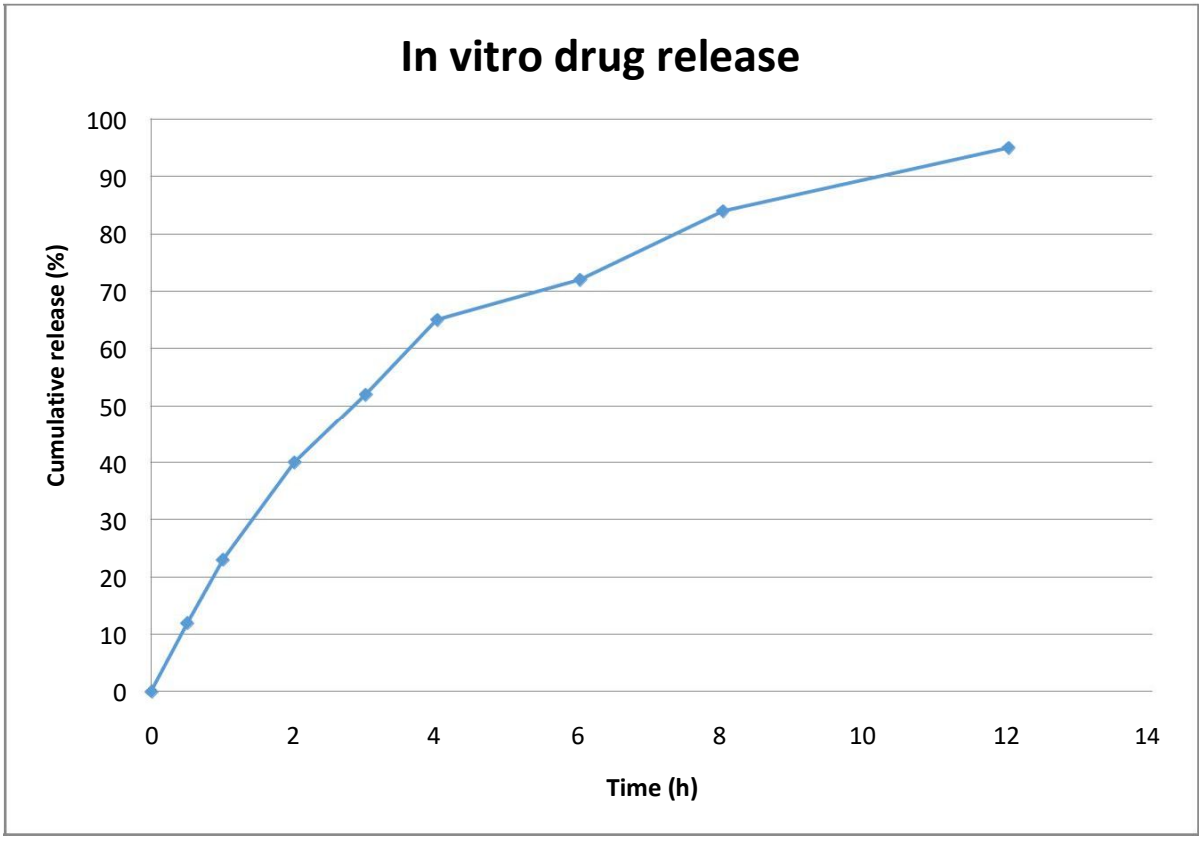

Fig 23. *In vitro* drug release of EF3

In vitro drug release studies were performed in pH 7.4 PBS using synthetic dialysis membrane (cellophane) which only allows transport of free drug across its membrane. The release pattern in Figure shows a biphasic release pattern, where almost 25% of drug was released in the initial phase. This accounts for the burst phase where almost all the unentrapped drug would be released. During the final phase, a comparatively sustained pattern of release of drug from ethosomes was observed for about 8 h.

This sustained phase accounts for the release of drug from the lipid vesicles of the ethosomes and the fluidity provided by the ethanolic content. The obtained data was analyzed for release kinetics to understand the mechanism behind the release of drug from ethosomes. According to the R^2 values for the release pattern of drug from TDDS, a best fit was obtained for Korsmeyer–Peppas mechanism of release. All the formulations showed a non-Fickian pattern of release as the n values are greater than 0.5 according to table. This indicates that the release of drug followed diffusion and erosion controlled model.

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