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DEVELOPMENT AND EVALUATION OF NANOEMULSION LOADED GEL OF TERBINAFINE HYDROCHLORIDE FOR THE TREATMENT OF FUNGAL INFECTIONS

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ABSTRACT

Objective of study was to develop and optimize nanoemulsion gel of terbinafine using *Melaleuca alternifolia* (tea tree) oil for synergistic antifungal activity against Candida albicans. Permeation coefficient of nanoemulsion gel and marketed cream was found to be 2.54×10^{-3} cm/h and 1.64×10^{-3} cm/h,respectively. $C_{skin\ max}$, $AUC_{0–5h}$, $AUC_{0-\infty}$, and penetration constant (K_p) were found to be significantly more in the skin treated with nanoemulsion gel ($p < 0.0001$). Confocal studies depicted deeper penetration of terbinafine from nanoemulsion gel. Moreover, FTIR and DSC analysis indicated lipid bilayer fluidization mechanism of epidermal drug deposition enhancement. Zone of inhibition for tea tree oil, nanoemulsion gel and marketed cream of terbinafine hydrochloride was found to be 10.00 ± 1.12 mm, 43.20 ± 1.88 mm, and 34.70 ± 1.59 mm, respectively. These findings demonstrated that nanoemulsion gel of terbinafine containing tea tree oil could be used as promising approach to treat skin fungal infection.

KEYWORDS: Skin Permeation, Nanoemulsion, Nanoemulsion, Dermatokinetic.

1. INTRODUCTION

Terbinafine Hcl (TB) is a synthetic allyl amine derivative with antifungal activity. TB exerts its effect through inhibition of squalene epoxidase, thereby blocking the biosynthesis of ergosterol, an important component of fungal cell membranes. As a result, this agent disrupts the fungal cell membrane synthesis and inhibits the fungal growth. It is used topically for

superficial skin infections such as jock itch (tinea cruris), athlete's foot (tinea pedis), and other types of ringworm (tinea corporis) and Candida species.[3] The objectives of the present study are improving solubility and antifungal activity of TB through incorporation in nanoemulsion based gel.

Terbinafine Hcl (TB) is a poorly water soluble antifungal drug. Topical nanoemulsion based gel containingTB was prepared with a view to improve its solubility and antifungal activity. In preparation of the nanoemulsion (NE), excipients were selected based on the solubility study. Peceol was optimized as the oil phase. Tween 80 and propanol were optimized as the surfactant and co-solvent respectively, and were mixed (Smix) in different weight ratios (1:1, 1:2, 1:3, 1:4, 4:1, 3:1 and 2:1, respectively). Pseudoternary phase diagrams were developed and Pecol and Smix were mixed in different weight ratios ranging from 1:9 to 9:1. Based on the NE region of each diagram, the formulae were selected. The formulated nanoemulsions were characterized and evaluated for in vitro drug release and thermodynamic stability. The optimum nanoemulsion formulae containing 10 or 15% w/w oil, 45% w/w Smix (1:2/1:3) and 45-40% w/w aqueous phase) were incorporated into Carbopol 940 gel bases forming three different TB nanoemulsion based emulgel formulae (F1-F3) which were examined for ex vivo drug permeation and in vivo antifungal activity compared to the marketed product; Lamisil® emulgel. The results showed that TB skin permeation from all the prepared nanoemulsion based gel formulae was significantly ($p < 0.05$) improved in relation to the commercial emulgel. F3 exhibited a superior in vivo antifungal activity over the marketed emulgel for the treatment of Candida infection.

Skin

Skin is the largest organ of the body which covers approximately 1.8 m2 a total surface area of a mature person. Its chief function is concerned with protection, temperature regulation, control of water output, and sensation. There are three important layers in human skin namely epidermis, dermis and hypodermis.

Epidermis

Epidermis is the outer most region of the skin, which is in permanent contact with the external environment. Epidermis can be further subdivided into following layers.

a) Stratum corneum: - The stratum corneum (or the horny layer) is the outermost layer of epidermis. It varies in thickness from approximately 10 microns to several hundred microns,

depending on the region of the body. The stratum corneum is the major barrier to transdermal drug molecules. It is quiet dry and is best suited for the purpose of permeability barrier (**Prausnitz MR *et al*., 2008**). It consists of one to two dozen flat and partly overlapping dead cells which called as corneocytes. The Corneocytes is organized in column or columnar clusters and coupled together with numerous desmosomes. They are devoid of lipids or organelles, but are filled with structural proteins (keratin filaments) and osmotically active small molecules. Lipid material between the corneocytes is not only ample but also is highly organized and thus acts as extra intercellular _glue'sealing the spaces between the cells in the skin (**Cevc G *et al*., 1996**). The intercellular lipids in the horny layer mainly encompass a non polar substances such as the free fatty acids, cholesterol and cholesteryl esters which are more than ceramides. Due to low overall lipid polarity in the skin, the inter corneocytes lipid are tightly packed and locally appear as the lipid multi lamellae. All these factors contribute to the tightness and impermeability of the intact skin.

b) Stratum granulosum: - Below the stratum corneum is the granular layers in which each keratinocyte contains basophilic keratohyalin granules. The major component of these granules is the protein called filaggrin, which bind to the keratin filaments.

c) Stratum Spinosum: - Below the granular layer lies the spinous or pricky cell layer. At high magnification, when observed it has prickly appearance due to fine cell processes containingdesmosomes attaching one polyhedral shaped cell to another. Active protein synthesis occurs in this layer producing a fibrillar protein keratin which aggregates to form a tonofibrils. These tonofibrils migrate to granular layer to become part of the keratin complex.

d) Stratum Basale: - Stratum Basale consists of one cell layer of cuboidal cells attached by hemi-desmosomes to a thin basement membrane which separates it from underlying dermis.

II. Dermis

The dermis is the layer of skin beneath the epidermis that consists of epithelial tissue and it cushions the body from stress and strain. It is tightly connected to the epidermis by a basement membrane. The dermis contains small vessels that distribute the drugs into systemic circulation and regulates the body temperature by a system known as the skin's microcirculation. It also contains sensory neurons and a lymphatic network. The blood vessels in the dermis provide nourishment to the cell and remove waste from its own cells as

well as from the Stratum basale of the epidermis. The dermis is structurally divided into two areas, mention below-

(a) A superficial area adjacent to the epidermis called the papillary region.

The papillary region is composed of loose areolar connective tissue. It is named for its finger like projections called papillae, that extend toward the epidermis. The papillae provide the dermis with a "bumpy" surface that interconnect with the epidermis, strengthening the connection between the two layers of skin.

(b) A deep thicker area known as the reticular region

The reticular region lies deep in the papillary region and it is composed of dense irregular connective tissue. It is much thicker than any other layer. These protein fibers give the dermis its properties of strength, extensibility, and elasticity. It is also present in reticular region like the roots of the hair, sebaceous glands, sweat glands, receptors, nails. Tattoo ink is held in thedermis. Stretch marks from pregnancy are also located in the dermis.

III. Hypodermis

It is made up of fats, helps body to stay warm and absorb shock. The subcutaneous tissue or hypodermis is not actually considered a true part of the structure of the skin. It is composed of loose textured, white, fibrous connective tissue in which fat and elastic libers are intermingled. It contains blood and lymph vessels, the base of hair follicles, the secretory portion of sweat glands and cutaneous nerves. Most of the studies support that drugs permeating through the skin enters directly to the systemic circulation before reaching the hypodermis.

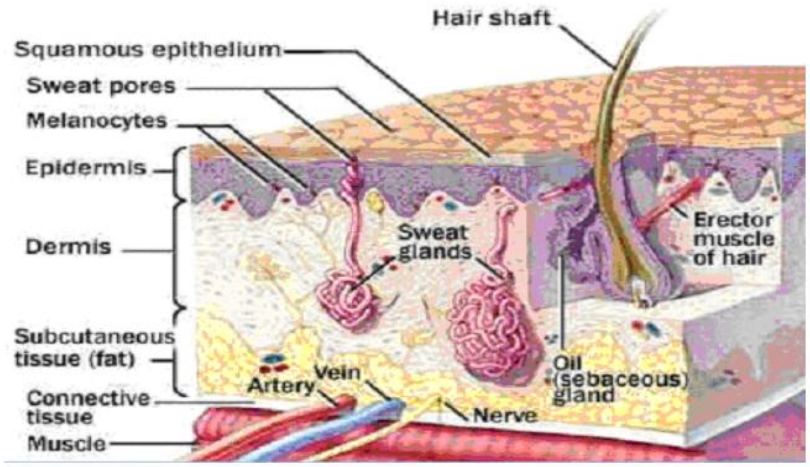


Figure 1.1: Cross section of skin ***(Uchechi O et al., 2014)***

1.2 Routes of drug permeation through the skin

a) Transepidermal route: - Transepidermal route is also known as intercellular pathway. Intercellular pathway is predominantly used pathway through the skin, especially when the steady state in the stratum corneum are reached. In this route of permeation, the drug passes through small spaces between the of the skin

b) Transcellular route: - Transcellular route is also known as intracellular pathway. This nvolves permeation through the corneocytes followed by the intercellular lipids. Compounds permeating through this route utilize the imperfections in the corneocytes that create openings comprised of water. This route is therefore believed to prefer hydrophilic compounds for delivery.

c) Transappendageal route: - Passage of molecules *via* sweat glands, hair follicles.

1.3 Fungal Infection

Superficial cutaneous fungal infections are one of the most common infectious diseases affecting 20–25% of the general human population worldwide which are caused by yeasts (e.g., *Candida* species and *Malassezia* species), dermatophytes and non–dermatophyte species of filamentous fungi (dermatomycoses). Such Dermatological problems are challenging worldwide for human health which yet are unresolved.

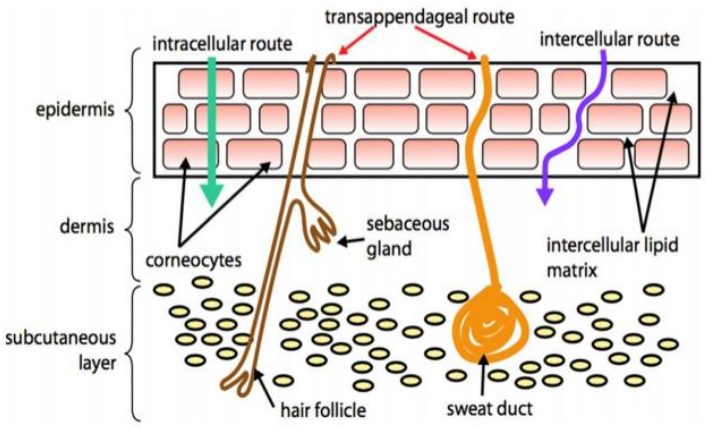


Figure 1.2: *Schematic showing different routes of drug permeation through*

Fungal infections are caused primarily by a group of *Filamentous keratinophilic* fungi known as dermatophytes. Dermatophytes use keratin as a source of nutrient during skin, hair and nail

infection in both immunocompetent as well as immunocompromised individuals **(Peres NT *et al.*, 2010).** Fungal infections can be an economical burden with approximately $1.67 billion spent on treatments **(Bickers DR *et al.*, 2006).** There are many antifungal agents for the treatment of fungal infections. Although, it does not cause mortality, however, causes significant morbidity and possess a major public health problem especially in the tropical countries like India, due to the hot and humid climate. *Candida albicans* is responsible for candidiasis and cause either acute or chronic infections. However, infections due to non–*albicans Candida* species have emerged over the past two decades and a shift from *Candida albicans* to other species such as *Candida parapsilosis, Candida glabrata, Candida guilliermondii* and *Candida tropicalis* has occurred.

1.4 Treatment of fungal infections

All commonly used topical antifungal treatments seek to disrupt ergosterol synthesis, which is a vital component of fungal cell membranes. Changes in the cell membrane cause inhibition of fungal growth. Ergosterol synthesis is a complex process involving the formation of several intermediate stages; different treatments affect different parts of the pathway. Azole antifungals, such as miconazole, possess mainly fungistatic (inhibit fungal cell growth) activity; at high concentrations they may also be fungicidal (kill fungal cells) Their principal effect is selective inhibition of the fungal Cytochrome P450 (CYP450) 14 α-demethylase enzyme. This prevents conversion of lanosterol to ergosterol, thus disrupting membrane integrity and preventing growth. Terbinafine, an allylamine, acts by blocking the conversion of squalene into squalene-2,3-epoxide, a precursor of ergosterol formation. This exerts both a fungistatic effect by prevention of cell growth as well as a fungicidal effect via the accumulation of squalene. Other treatments are involved in the disruption of the fungal cell membrane by other means. Treatments that include a corticosteroid component are used in people troubled by itch in the hope of rapidly reducing the inflammatory response. Systemic absorption of topical corticosteroids is negligible meaning that serious side-effects are extremely unlikely; to cause problems, such as striae, papule formation, and scarrin.

1.5 Topical Drug Delivery

The fundamentals of successful formulation are to deliver the active substance to the target organ with minimal discomfort and side effects. In this respect, topical route excels because of the following advantages:

- There is no hepatic first pass metabolism, salivary metabolism and intestinal metabolism.

The ease of usage makes it possible for patients to self-medication.

- Since the composition of skin structurally and biologically is the same in almost all the humans, there is minimal inter and intra patient variation.
- Direct site application

1.6 Kinetics of Skin Permeation

Knowledge of skin permeation kinetics is vital to the successful development of dermal therapeutic systems. Transdermal permeation of a drug involves the following steps: Sorption by stratum corneum. Penetration of drug through viable epidermis.

- Uptake of the drug by the capillary network in the dermal papillary layer.

This permeation can be possible only if the drug possesses certain physiochemical properties. The rate of permeation across the skin is given by the following equation.

$$dQ/dt = PS\ (Cd - Cr) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ (i)$$

Where,

Cd = concentration of the skin penetrant in the donor compartment i.e. on the surface of stratum corneum.

Cr = concentration of the skin penetrant in the receptor compartment i.e.

body.

PS = overall permeability coefficient of the skin tissue to the penetrant.

dQ/dt = rate of permeation across the skin.

This permeability coefficient is given by the equation given below-

$$\mathbf{Ps = Ks.Dss/hs} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ (ii)$$

Where,

KS = the partition coefficient.

DSS = the apparent diffusivity for the steady state diffusion of the penetrant molecule

PS = overall permeability coefficient of the skin tissue to the penetrant

hS =overall thickness of skin tissues.

From equation (i) it is clear that rate constant of drug permeation can be obtained only when Cd>> Cr .

From equation (i) & (ii)

Methods used to increase skin permeation

Many approaches are available to increase the drug permeation across the skin. These are discussed below (**Wu XM *et al*., 2007**).

A. Use of penetration enhancers

Penetration enhancers (also called sorption promoters) are substances which penetrate into the skin, reversibly by decreasing the barrier resistance caused due to stratum corneum. Many potential sites and modes of action have been identified for skin permeation enhancers, i.e. the intercellular lipid matrix in which the accelerants may disrupt the packing or by increasing the drug partition into the tissue by acting as a solvent for permeation within the membrane. Further potential mechanism of penetration enhancers takes place by acting on desmosome connection between corneocytes or by altering metabolic activity within the skin or exerting an influence on the thermodynamic activity.

The following substances act by these mechanisms

- Sulphoxides (dimethyl sulphoxide)
- Azones (laurocaprom)
- Pyrrolidones (2- pyrrolidone)
- Surfactants (sodium lauryl sulfate, polysorbates like span and tween)
- Alcohols (ethanol, isopropanol)
- Fatty acids (oleic acid, capric acid)
- Terpenes (limonene, 1,8- cineole)

B. Iontophoresis

Iontophoresis deliver ions and charged molecules across the skin into systemic circulation at an increased rate in a controllable manner by the use of electric current. An electrode patch loaded with drug is placed on the skin which acts as the working electrode. That can be either positive or negative depending upon the characteristics of the drug. Another electrode is placed elsewhere to complete the electric circuit. The small current applied on the patches 0.5 A/cm^2 are imperceptible to patients. The iontophoretic technique is highly desirable to improve the transdermal delivery of peptide and proteins using a low current intensity with a short time period. The phoresor iontophoretic drug delivery system is FDA approved for the production of local dermal anesthesia and the transdermal delivery of certain drugs.

C. Electroporation

In this method, intense electric charge creates small pores in the phospholipids bilayer of cell membrane. These pores provide pathways for drug penetration that travel through the horny layer. High voltages (2100 V) and short duration treatment (milliseconds) are most frequently employed. The technology has been successfully used to enhance the skin permeability of molecules with different lipophilicity and size (i.e. small molecules, proteins, peptides and oligonucleotides) including biopharmaceuticals with molecular weights greater than 7kDA. As electroporation improves the diffusion of such a wide range of compounds. It depicts that the pores created in the superficial layers of the skin are directly responsible for the increase in skin permeability.

D. Ultrasound (Sonophoresis, phonophoresis)

This technique was originally used in physiotherapy and sports medicine by applying the preparation topically and massaging at the site with an ultrasound source. This procedure was extended to transdermal drug delivery. This involves the use of ultrasonic energy to enhance the transdermal delivery of solutes either simultaneously or by pre-treatment. The ultrasound energy (at low frequency) disturbs the lipid packing in stratum corneum by cavitation. Shock waves of collapsing vacuum cavities increase free volume space in bimolecular leaflets and thus enhance drug penetration int o the tissue.

E. Microporation

Microporation have a small area which is covered by hundreds of microneedles, that pierce only the stratum corneum thus, allowing the drug to bypass. The microneedles are usually the drug coated projections of solid or hollow silicion or drug-filled metal needles. Example ―Alza‘s technology Macroflux can deliver bolus of drug transdermally by this mechanism‖ **(Barry BW, 2001)**.

F. Needleless jet injectors

This combines the advantages of transdermal and parentral drug delivery system. Over the years, there have been numerous examples of both liquid (Ped-O-Jet, Iject, Biojector 2000, Medi-jector and Intraject) and powderMED‘s (PMED device formerly known as Powderjectinjector)systems. The latter device has been reported to successfully deliver testosterone, lidocaine hydrochloride and macromolecules such as calcitonin and insulin. This method of administering drugs circumvents issues of safety, fear and pain associated with the use of hypodermic needles. Transdermal delivery is achieved by firing the liquid or solid

particles at supersonic speeds through the outer layers of the skin using a suitable energy source. The PMED device consists of a helium gas cylinder, drug powder sealed in a cassette made of plastic membrane, a specially designed convergent-divergent supersonic nozzle and a silencer to reduce the noise associated with the rupturing of the membrane when particles are fired.

G. Novel vesicular systems

Vesicles are water filled colloidal particles. The wall of the vesicles consists of amphiphilic molecules in a bilayer conformation. In an excess of water these amphiphilic molecules can form one (unilamellar vesicles) or more (multilamellar vesicles) concentric bilayers. Hydrophilic drugs can be entrapped into the internal aqueous compartment, whereas amphiphilic, lipophilic and charged hydrophilic drugs can be associated with the vesicle bilayer by hydrophobic and/or electrostatic interactions.

Lipid based carriers

To improve the permeation and bioavailability of poor water soluble drugs, lipid based carriers have gained focus in last few decades **(Uchechi O *et al.*, 2014**). Lipid based delivery system are accepted, proved and commercially viable to formulate topical, oral, parenteral delivery. Since, lipids are one of the basic component or building blocks of biological membranes **(Pouton CW, 2006).** Thus, the incorporation of active lipophilic drug in an inert lipid vehicle such as oil, blend of surfactant includes formulation such as liposomes, microemulsion, nanoemulsion, self-micro or self-nano emulsifying drug delivery system can increase permeability through skin. They act by increasing the surface area of drug by use of surfactant, which improves solubilization, permeation and bioavailability of drugs **(Pouton CW., 2000)**.

A. Nanoemulsion

Nanoemulsions are defined as transparent or translucent dispersion of oil and water. Itisstabilized by an interfacial film of surfactant molecule with a small droplet size in the range of 20–200 nm **(Jannin V *et al.*, 2008).** They can be prepared by spontaneous emulsification such as PIT emulsification or phase inversion composition, or by using a high shear device, which allows a better control of the droplet size and a large choice of compositions (**Chellapa P *et al.*, 2016**). The definition of nanoemulsion is different according to different authors. According to **Graves and associates —**Nanoemulsion‖ are dispersions of submicron droplets that have significant surface tension, which form only

when extreme shear is applied to fragment droplets strongly, and are kinetically inhibited against recombining by repulsive interfacial stabilization due to surfactant **(Grave S *et al*., 2005)**. They can exist as oil-in-water and water- in-oil forms, where the core of the particle is either oil or water, respectively. They are frequently called as mini emulsion, finely dispersed emulsion, submicron emulsion and are characterized by greater stability due to fine particle size. The potential benefits of nanoemulsion include optical clarity, good stability against separation, flocculation and coalescence. It also improves absorption and bioavailability of functional compounds (**Gadhave AD., 2014)**. Nanoemulsion is a promising alternative to increase drug penetration and targeting poorly soluble drugs, by increasing its absorption through the skin, better retention time of drug in the target area and eventually result in less side effects **(Chellapa P *et al*., 2015)**

Mechanism of permeation enhancement *via* nanoemulsion

The nanoemulsion alters both the lipid and the polar pathways. The lipophilic domain of nanoemulsion interact with stratum corneum in many ways. The drug dissolved in the lipid domain can directly partition into lipids of stratum corneum or lipid vesicles themselves. It can intercalate between the lipid chains of the stratum corneum, thereby, destabilizing its bilayer structure, leading to increased permeability of the lipid pathways to the drug. On the other hand, the hydrophilic domain of the nanoemulsion can hydrate the stratum corneum to the greater extent which plays an important role in the percutaneous uptake of the drug. Since some lipid chains are covalently attached to the corneocytes, hydration of these proteins will also lead to the disorder of the lipid bilayers. The greater drug penetration enhancing efficacy of the nanoemulsions as compared to other vehicles can be attributed to the combined effect of both the lipophilic and hydrophilic domains of the nanoemulsions (**Shakeel F *et al*., 2008**). The droplet size of the nanoemulsion may also affect its efficiency in promoting the percutaneous absorption of drugs. When the droplet size is small, the number of vesicles interact on a fixed area of stratum corneum increases. Due to small size of the droplet of the nanoemulsion permeation into the skin will also be more. Hence, efficiency in the percutaneous uptake of drugs increased (**Thacharodi D *et al*., 1993**). Nanoemulsions appear to have the ability to deliver large amount of water and topically applied agents into the skin than the water alone or other traditional vehicles (lotions, creams) because they provide a better reservoir for a poorly soluble drug through their capacity for enhanced solubilization. It has also been said that the nanoemulsion may also have the ability to lower the interfacial energy between the skin and the vehicle simultaneously upon its intimate contact with skin

lipids and water, further enhancing the drug delivery **(Eccleston PA., 1992**). Several factors affecting topical drug delivery from nanoemulsions include the affinity of the drug to the internal phase in nanoemulsion, ingredients of nanoemulsion reducing the barrier properties of stratum corneum, increased concentration gradient toward the skin and the dispersed phase acting as a reservoir, which makes it possible to maintain a constant concentration in the continuous phase (**Baroli B *et al.*, 2000)**A topically applied nanoemulsion is expected to penetrate the stratum corneum. Once it enters into the stratum corneum,

Merits of nanoemulsions as a topical drug delivery system

- Excellent kinetic stability that ensure its long shelf life **(McClements DJ., 2012)**.
- Ease of manufacturing and scale up.
- High solubilization capacity enabling efficient delivery of hydrophobic drugs

(Gupta A *et al*., 2016).

- Optical transparency for aesthetic appeal.
- Versatility offered in delivery of hydrophobic as well as hydrophilic drugs.
- Improved percutaneous penetration by fluidizing the lamellar structure of intercellular skin lipids and by reducing the interfacial tension between skin an dvehicle.

Formulation and Preparation of Nanoemulsion

The formulation of nanoemulsion usually involves components namely oil phase, aqueous phase and a primary surfactant and in many case a secondary surfactant (cosurfactant). All components are discussed below.

a) Oils Phase

Oils are any neutral, non-polar chemical substance, that is viscous liquid at ambient temperature, and are immiscible with water but soluble in alcohol or ether. Selecting an appropriate oily phase is very important as its influences the selection of other ingredients of nanoemulsion. Formulation of nanoemulsion with oil of low drug solubility requires incorporation of more oil to solubilize the amount of drug which in turn requires higher concentration of surfactant that can cause toxicity (**Azeem A *et al.*, 2009)**. Thus, the oil which has maximum solubilizing potential for the given drug candidate is selected as oils phase for the formulation of nanoemulsion which help to achieve maximum drug loading in nanoemulsion (**Sonneville-Aubrun O *et al.*, 2004**). Natural occurring oils and fats are comprised of mixtures of triglycerides which contain fatty acid of varying chain lengths and

degree of unsaturation. Triglycerides are classified as short or long chain and may be synthetically hydrogenated to decrease the degree of unsaturation thus conferring resistance to oxidative degradation. The choice of oily phase is often a compromise between its ability to solubilize the drugs and its ability to facilitate formulation of nanoemulsion of desired characteristic. Some of the examples of oils that has been incorporated in the formulation are tea tree oil, capmul PG-8NF, castrol oil, olive oil, corn oil, sunflower oil, coconut oil, sefsol 218 (propylene glycol monocaprylic ester), oleic acid, capryol 90 (polyglycolysed glycerides) almond oil, grape seed oil.

- **Choice of components**

Although there are no strict rules for choosing the appropriate nanoemulsion components, still choosing surfactant and co-surfactant is a crucial step. The surfactant(s) selected must have -

- Lower interfacial tension to a very small value to aid dispersion.
- Provide a flexible film that can readily deform round small droplets.
- Be of appropriate HLB character to provide the correct curvature at the interfacial region for the desired nanoemulsion type.

b) Surfactant

The substances having both hydrophilic and hydrophobic regions in their molecular structures are called surfactants or surface active agents, when added to a liquid, reduces interfacial tension between two liquids thereby increasing its spreading and wetting properties. These are soluble in both water and oil. On addition, of surfactants into the dispersed system, the hydrophilic (polar) and hydrophobic (non-polar) groups orient themselves in a monomolecular layer facing the polar (i.e. water) and non-polar (i.e. oil) solvents, respectively. As the concentration of surfactant in an aqueous solution is increased, the interfacial tension is appreciably lowered. The appropriate selection of surfactant and determining its minimum concentration in any nanoemulsion formulation is crucial (**Azeem A *et al*.,2009**). The surfactant should favor emulsification of the oily phase and should also possess good solubilizing potential for the hydrophobic drug compounds. Surfactant which have HLB value <10 are generally used to form w/o nanoemulsion and are hydrophobic in nature, whereas surfactant having high HLB value form o/w nanoemulsion. However, mixture of high and low HLB value surfactant is required for the formulation of

nanoemulsion. The surfactant used in nanoemulsion is either ionic or non-ionic but ionic surfactants are not preferred due to toxicological effect.

c) Co-surfactant

In most of the cases, single chain surfactants alone are unable to reduce the oil water interfacial tension sufficiently to form a stable nanoemulsion. Thus, a co-surfactant which is usually a medium chain fatty alcohol, acid or amine is taken along with the surfactant to lower the interfacial tension to a very small or even transient negative value. At this value fine droplets get formed due to the interface expansion and more of surfactant/cosurfactant get adsorbed on the surface until the bulk condition is depleted enough to make the interfacial tension positive again. This process is called the spontaneous emulsification. Co-surfactant had an effect of further reducing the interfacial tension by the _dilution effect‘ while increasing the fluidity of the interface by decreasing the constant. Thereby, increasing the entropy of the system. They also increase the mobility of the hydrocarbon tail and allow greater penetration of the oil into this region.

E. Characterization of Nanoemulsion

Nanoemulsion have been evaluated using a wide range of techniques. They are generally characterized for ceratin parameter like **(Chime SA et al., 2014).**

a) Viscosity: The viscosity of nanoemulsion with several compositions can be measured at different shear rates at different temperatures using Brookfield type rotary viscometer.

b) Conductivity: Conductivity measurement using conductometers provide a means of determining whether the nanoemulsion is oil-continuous or water-continuous.

c) Morphology: The morphology of nanoemulsions can be determined by transmission electron microscopy (TEM). In TEM, higher resolution images of the disperse phase are obtained. Thesample is negatively stained with 1% aqueous solution of phosphotungstic acid or by addingdrop of 2 % uranyl acetate solution onto a 200 μm mesh size copper grid usingmicropipette and the sample examined under a transmission electron microscope at appropriate voltage. Qualitative measurements of sizes and size distribution of TEM micrographs can be performed using a digital image processing programme. More sophisticated techniques, such as x-ray or neutron scattering, atomic force microscopy, or

cryo-electron microscopy are typically required to explore the structure and behaviour of nanoemulsions.

d) Droplet size, polydispersity and zeta potential: Dynamic light scattering (DLS) otherwise called photon correlation spectroscopy (PCS) is used to analyze the fluctuations in the intensity of scattering by droplets/particles due to Brownian motion. This instrument also measures polydispersity index, which is a measure of the broadness of the size distribution derived from the cumulative analysis of dynamic light scattering, indicates the quality or homogeneity of the dispersion. The polydispersity index indicates the quality or homogeneity of nanoemulsion. Zeta potential of formulation for better physical stability should be ±30 mV as at this charge value droplets repeal each other which avoids coalescence.

Techniques used to assess the permeation of drugs through skin

There has been increased interest not only in cutaneous and percutaneous nanoemulsion for local therapeutic effect on diseased skin. But also quantifying permeation of drugs in different layers of skin as it is essential to know amount of drugs specifically present in each layer of skin. Penetration through stratum corneum is the rate limiting step in topical drug formulations as it forms a barrier which restricts the entry of foreign substances into body and excretion of exogenous substances. Therefore, many dermatological studies are focused on investigation of this skin layer. In past decades a number of techniques have been developed to quantify permeation of drug through skin layers. Earlier invasive methods like surface skin biopsy and deep skin biopsy were used to determine amount of drug permeated through skin (**Touitou E, 1998**). These methods were then replaced with tape stripping technique, microscopical techniques (TEM, SEM, etc) and thermal methods (DSC), which are less painful and more precise. Now-a-days, many other new techniques have been developed to determine amount of drug permeated through skin. These include ATR-FTIR, Confocal laser scanning microscopy (CLSM), Gammascintigraphy.

OBJECTIVE OF RESEARCH

The objective of the present study was to develop, optimize and characterize topical nanoemulsion gel of Terbinafine hydrochloride for the treatment of fungal infections. The topical treatment of fungal diseases is often limited by the poor percutaneous permeation through the human skin. Due to this reason, the formulation should be designed in such a way that it can improve the percutaneous permeation at the required concentration and achieves its

therapeutic action to the targeted site. It is anticipated that the developed formulation will serve the following purposes:

- Improvement in the overall managements of fungal infections.
- Better topical penetration through the stratum corneum
- Better patient compliance.

The objectives of the present study were

- To develop a kinetically stable nanoemulsion using a blend of surfactant and cosurfactants.
- To formulate the developed nanoemulsion system into sustained release gel for the treatment of fungal infection.
- To check the release and permeation of the developed nanoemulsion system.
- To determine the drug disposition in the skin after topical application of gel.
- To test the prepared formulations for their antifungal activity

Drug Profile

Name: Terbinafine hydrochloride

Category: Antifungal drug. Use in treatment of onychomycosis

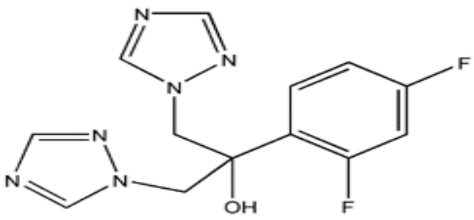
CAS Number: 78628-80-5

Molecular weight: 327.9g/mol

Molecular Formula : C21H25N•HCl

Chemical name :(E)-N-(6,6-dimethyl-2-hepten-4-ynyl)-N-methyl-1-naphthalenemethanamine hydrochloride

Molecular structure



fluconazole

Physical properties

Solubility: Freely soluble in methanol, chloroform, ethanol and slightly soluble in water.

Appearance: White to off white fine crystalline powder

Partition coefficient: 3.3

Acidity or Alkalinity: pH of 1% w/v solution of methanol was found to be 3.4

Melting point: 204-208oC

Mechanism of action

Terbinafine is an allylamine antifungal agent and acts by inhibiting squalene epoxidase, thus blocking the biosynthesis of ergosterol, an essential component of fungal cell membranes. This inhibition also results in an accumulation of squalene, which is a substrate catalyzed to 2,3-oxydo squalene by squalene monooxygenase. The resultant high concentration of squalene and decreased amount of ergosterol are both thought to contribute to antifungal activity of terbinafine hydrochloride.

Plan of Work

- Literature review
- Selection and procurement of drug and excipients
- Physical characterization and identification of the drug
- Color
- Solubility
- Melting point
- UV spectral analysis
- FTIR spectral analysis
- Differential scanning calorimetric (DSC)

- Analytical Methodology
  - Determination of λmax
  - Calibration curve in methanol
  - Calibration curve in 6.8 phosphate buffer
- Formulation, optimization and evaluation of Nano emulsion
  - Screening and selection of oil
  - Screening and selection of surfactant and co-surfactant
  - Construction of pseudoternary phase diagram

Optimization used central composite rotable design (CCRD)

- Selection of optimized formulation.

- Physical stability study of nanoemulsion

a. Freeze thaw cycle

b. Centrifugation studies

c. Heating cooling cycle

- Characterization of the optimized nanoemulsion

- Droplet size and droplet size distribution (polydispersity index)
- % Transmittance
- Zeta potential
- Surface morphology and size using Transmission electron microscopy (TEM) studies

MATERIALS AND METHODS

Table: List of Equipments.

S. No.	Equipment	Model/Manufacture
1	Double Beam UV Spectrophotometer	Shimadzu, Kyoto, Japan
2	Differential Scanning Calorimetry	Perkin Elmer, Mumbai, India
3	Electronic Weighing Balance	Metler Toledo, Langancher, Switzerland
4	FTIR Spectrophotometer	Shimadzu, Kyoto, Japan
5	Melting Point Apparatus	Adarsh Scientific, Ambala, India
6	Micropipettes	Eppendorf micropipette, Chennai, India
7	pH Meter	Mettler Toledo, Langacher, Switzerland
8	Vortex mixture	Nirmal International, Delhi, India
9	Ultra Sonicator	Bio Technics, Mumbai, India
10	Biological Shaker	Nirmal International, Delhi, India
11	Refractometer	Abbes, India
12	Magnetic Stirrer	Mertex, Delhi, India
13	Viscometer	Brookfield Engineering Laboratories, USA
14	Centrifuge Apparatus	Remi equipment, Delhi, India
15	Oven	Thermo Scientific, Delhi, India
16	pH Meter	Mettler Toledo, Langacher, Switzerland
17	Water Bath	Grover Enterprises, Delhi, India
18	Zetasizer	Malvern Instrument, Worcestershire, UK
19	Deep Freezer	Vestfrost, Delhi, India
20	Conductivity/TDS/ ºC Analyzer	Analab Scientific Instruments Pvt. Ltd,India
21	Transmission Electron Microscopy	TECNAI 200 Kv TEM (Fei, Electron Optics)

Table 5.2: List of Chemicals and Materials.

S.No.	Name of Chemicals	Manufacturer/ Supplier
1	Terbinafine hydrochloride	Mascot,India
2	Methanol	Merck, Mumbai, India
3	Solutol HS	Signet, Mumbai, India
4	PlurolOleque CC 497	Gattefosse, Saint Priest, Cedex, France
5	Transcutol P	Gattefosse, Saint Priest, Cedex, France
6	Tween 20	S.D Fine Chemicals, New Delhi, India
7	Tween 80	S.D Fine Chemicals, New Delhi, India
8	Polyethylene glycol 400	Thomas baker (Chemicals) Pvt Ltd,Mumbai, India
9	Potassium dihydrogen phosphate	S.D Fine Chemicals, New Delhi, India
10	Sodium chloride	S.D Fine Chemicals, New Delhi, India
11	Carbopol 940	Merck, Mumbai, India
12	Sodium hydroxide pellet	S.D Fine Chemicals, New Delhi, India

Physical Characterization and Identification of Terbinafine Hydrochloride

A. Physical Properties

Physical properties of terbinafine hydrochloride such as nature, color and odor were determined. The solubility of terbinafine hydrochloride in methanol was determined. The melting point was determined with the help of the melting point apparatus (scientific apparatus, India) using capillary tube method and by using differential scanning Calorimetry (DSC) to check the impurity /foreign matter in the drug and to authenticate It.

B. Solubility Studies

For crude solubility, equivalent amount of the drug was taken in different Eppendorf tubes containing 1ml of different solvents. Eppendorf tubes were shaken vigorously with the help of vortex shaker after the addition of each portion of solvent and then crude solubility was observed by visual inspection.

Identification Test

A.UV Spectral Analysis

Accurately weighed quantity (10 mg) of terbinafine hydrochloride was dissolved in methanol in the volumetric flask and volume was made up to 10 ml (1000 µg/ml). One milliliter of above solution was diluted to 10ml with methanol in 10 ml volumetric flask (100 µg/ml). The

resulting solution was then scanned for UV-Vis absorption using UV.Vis spectrophotometer in the range of 200-400 nm and λmax was determined.

B. Fourier Transform Infrared Spectrophotometry (FTIR) Analysis

FTIR spectrum of terbinafine hydrochloride was carried out using Shimadzu Borad FTIR system (Kyoto, Japan) by potassium bromide (KBr) pellet technique. Terbinafine hydrochloride was dispersed and triturated with dry KBr (5% wt. of sample), grinded well in motor and pestle andKBr disk was prepared at a pressure of 1000psig. The disk was placed in the FTIR sample holder and IR spectra in absorbance mode were obtained in the spectral region 400 to 4000 per cm.

C. Differential Scanning Calorimetry (DSC) Analysis

Thermal investigation of terbinafine hydrochloride was carried out by using Perkin Elmer Pyris 6 DSC (MA, USA). About 5 mg of sample was placed in aluminium pan and crimped with a lid containing a pin hole which was kept in the DSC unit along with a similar pan as a reference. The following conditions were used:

Sample quantity: 5 mg in crimped pan

- Reference: blank pan
- Heating rate: 10oC
- Nitrogen gas flow: 15-20 ml/min
- Heating range: 40-400o C

5.3 Analytical Methodology (UV method)

A. Preparation of working solution

Different working solution were prepared according to USP and IP 2007

- 0.2 M potassium dihydrogen phosphate solution: 27.218 gm of potassium dihydrogen phosphate (M.W. 136.09) was dissolved in 1000ml of distilled water to give 0.2M potassium dihydrogen phosphate.
- 0.2 M sodium hydroxide solution: 8 gm of NaOH (M.W. 40) was dissolved in 1000 ml of distilled water to give 0.2 M sodium hydroxide solution.
- Phosphate buffer (pH 6.8):

50 ml of 0.2 M potassium dihydrogen phosphate was added 22.4 ml of 0.2 M sodium hydroxide solution in a 200 ml volumetric flask and volume was made up to 200 ml with distilled water.

B. Calibration plot of Terbinafine hydrochloride in methanol

An accurately weighed quantity of terbinafine hydrochloride (10 mg) was dissolved in methanol in 10ml volumetric flask and kept in sonicator for about 15 minutes and volume was made to 10 ml with methanol to make concentration of 1000 µg /ml. One milliliter of above stock solution was diluted to 10 ml in the volumetric flask. Further dilution was done with methanol to obtain the required concentration in the range 2-30 µg /ml. The absorbance values were measured at λ max 223.5 nm spectrophotometrically against the blank medium. The regression analysis was made.

C. Calibration plot of terbinafine hydrochloride in phosphate buffer (pH6.8)

Accurately weighed quantity (1mg) terbinafine hydrochloride was dissolved in 3 ml of methanol in 10 ml volumetric flask and volume was made up to 10 ml with phosphate buffer and kept insonicator for 15 minutes. Further dilution was done with phosphate buffer to obtain the required concentration in the range 0.6-4.8 µg/ml. The absorbance values were measured at λ max 222.5 nm spectrophotometrically against the blank medium. The regression analysis was made.

Formulation, Optimization and Evaluation of Terbinafine hydrochloride Nanoemulsion

A. Screening and selection of oil

The solubility of terbinafine hydrochloride was determined in different oils by adding excess amount of terbinafine hydrochloride in 1 ml of the selected oil in 5 ml stopper vials which was allowed to mix in a vortex mixer. After mixing it was kept at 25˚C in an isothermal shaker for about 72 hours to reach to equilibrium. Then samples were centrifuged at 3000 rpm for 15 min. The supernatant was taken and the concentration of terbinafine hydrochloride was determined using UV method.

Table 5.3: List of oils used for solubility study.

S.No.	Name of Oil	HLB
1	Tea tree oil	-
2	Wheat germ oil	7
3	Olive oil	7
4	Vitamin E	6

B. Screening and selection of surfactant and co-surfactant

The surfactant used in nanoemulsion should be non-ionic. Combination of surfactant of high HLB value with low HLB value co-surfactant at optimum temperature is generally used for the formulation of stable nanoemulsion. One of the criteria used for the selection of surfactant was the HLB value. Since, the formulation is o/w nanoemulsion. Therefore, only those surfactants which had HLB value in the range of 8 to 16 were selected. The different surfactant and co-surfactant used in the study are listed in ***table 5.4***. The second selection criteria used for the selection criteria used for the selection of cosurfactant was the miscibility with the selected oil and surfactant. Miscibility studies with oil were carried out by mixing them in 1:1 ratio and vortexing for about 5 minutes after which they were left to stand for 24 hours at room temperature. After 24 hours, the sample was evaluated for phase separation and visible color change. Of surfactant was drug solubility and miscibility with the selected oil. On other hand selection.

Table 5.4: List of surfactant and co-surfactant.

S.No.	Name of surfactant (S)/Co surfactant (C)	HLB
1	Tween 20 (S)	16.7
2	Tween 80 (S)	15
3	Solutol HS 15 (S)	14
4	Unitop FFT-40 (S)	14.3
5	Polyethylene glycol 400 (C)	11.6
6	PlurolOleque CC 497 (C)	3
7	Lauroglycol 90 (C)	5
8	Transcutol P (C)	4

Preparation of nanoemulsion

Nanoemulsion was formulated using tea tree oil as oil phase and distilled water as the continuous aqueous phase. Terbinafine hydrochloride (1% w/w) was dissolved in the oil phase with continuous stirring at high speed using a magnetic stirrer at room temperature. Tween 20 an d PEG 400 were added after the drug was completely solubilized in the oil. The preparation was continued by adding the required amount of water drop wise to the oily solution under continuous vortexing.

➢ **Physical stability study of nanoemulsion**

a) Freeze thaw cycle: Nanoemulsion was kept in deep freezer (at -20 °C) for 24 h.

After 24 h the nanoemulsion was removed and kept at room temperature. Three such cycles were repeated**.**

b) **Centrifugation studies:** Nanoemulsion after freeze thaw cycle were subjected to centrifugation studies where it was made to undergo centrifugation for 30 min at 5,000 rpm in a centrifuge.

c) **Heating cooling cycle:** Six cycles between refrigerator temperature (4 °C) and 40 °C with storage of 48 h were performed.

Characterization of nanoemulsion

a) Globule size and PDI

Globule size and size distribution of selected nanoemulsion formulation was determined using photon correlation spectrometer (PCS, Zetasizer 1000 HAS, Malvern instruments, Worcestershire, UK). One millitre sample of nanoemulsion was taken and added into the sample cell to measure the average globule diameter. Light scattering was measured at 25˚C at 900. Polydispersity index, a measure of globule size distribution in the sample was measured in triplicate.

b) Surface Morphology

Transmission electron microscopy (TEM) analysis was carried out to determine the shape and morphology of nanoemulsion through which real diameter and shape of droplet can be obtained. Terbinafine hydrochloride nanoemulsion was studied for surface morphology with the help of TEM operating at 200 KV, which was capable of point to point resolution (Nanoemulsion sample was deposited directly on the circular copper film grid of 400 mesh and stained with 2% (w/v) phosphotungstic acid for 30sec and placed for air drying. The excess of phosphotungstic acid was removed by absorbing on filter paper. The dried grid was analyzed for form and size of nanoemulsion.

c) Percentage transmittance

The percentage transmittance of the nanoemulsion was determined using UVVisible double beam spectrophotometer at 630 nm by using distilled water blank.

d) Refractive index

Refractive index (RI) of the optimized formulation was determined using Abbe‘s type refractometer. Few drops of the formulation were placed on the slide and RI was measured thrice.

e) pH

Calibrated PH meter (Eutech instruments, Singapore) was used to determine PH of nanoemulsion. The tip of glass electrode of PH meter was firstly cleaned with distilled water and then dipped in nanoemulsion to observe the PH in triplicate.

f) Conductivity

Conductivity of formulation was determined by conductivity meter (Conductivity Analyzer µCONCAL5, Analab, India). A 20 ml sample was used for measurement. The temperature at the time of measurement was 25±2°C.

Formulation of nanoemulsion gel

Carbopol-940 (2gm) was dissolved in distilled water (98 ml). After complete dispersion, solution was then kept in dark for 24 h for complete swelling of Carbopol-940. Then, terbinafine hydrochloride (1% w/w) nanoemulsion was added slowly into the aqueous solution of Carbopol-940. An appropriate quantity of triethanolamine (TEA) was added to obtain a homogeneous dispersion of the gel.

Evaluation of nanoemulsion gel

A. Viscosity

The method for measurement of viscosity involves the determination of the time required for a given volume of liquid to flow through a capillary. Viscosity of nanoemulsion gel was measured without dilution using a Brookfield DV III ultra V6.0 RV cone and plate viscometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) measurement was carried out at a temperature of 25 ± 0.5ºC using Rheocale V2.6 software.

Table: Specification set in Viscometer for determining viscosity.

Parameter	Specification
Sample	0.5 gm
Probe speed (rpm)	30
Data interval (sec)	60
Loop start (sec)	60
Temperature	25 ± 0.5ºC
Shear rate (/sec)	60

B. pH of nanoemulsion gel

pH of optimized nanoemulsion gel was observed using digital pH meter (Eutech instruments, Singapore) in triplicate.

C. Homogenicity

A small quantity of nanoemulsion gel was pressed between the thumb and index finger and the consistency and homogenicity of gel was noticed i.e. whether homogenous or not for the presence of any particle on the finger.

D. Spreadibility

A weighed quantity (350 mg) of nanoemulsion gel was taken on a glass plate (10 * 5 cm). Another glass plate (10 * 5 cm and 5.8± 1 g) was dropped from a distance of 5 cm. The diameter of the circle of spread was measured after 1 min.

E. Extrudibility

The quantity (g/cm2) of nanoemulsion gel extruded from lacquered aluminum collapsible tube on application of weight (in grams) required to extrude at least 0.5 cm ribbon of nanoemulsion gel in 10 s was determined (**Vijaya BP *et al.*, 2011**). The measurement of extrudability of each formulation was done in triplicates.

F. Drug content

A specific quantity (100mg) of optimized gel were taken and dissolved in 100ml of methanol. The volumetric flask containing gel solution was kept in mechanical shaker for 3 h in order to solubilize the drug. This solution was filtered and absorbance was measured at 223.5 nm against methanol as blank.

G. Drug permeation studies

To evaluate the terbinafine hydrochloride nanoemulsion gel and marketed cream permeation, *ex vivo* skin permeation studies was carried out using Franz diffusion cell. Franz diffusion cell consisted of a donor and receiver compartment with water jacket to maintain the temperature of assembly at 37 ± 0.5ºC. The capacity of receiver compartment was 15.2 ml. The area of opening of donor compartment was 0.65 cm2 . The receiver chamber was stirred continuously with magnetic stirrer. The skin sample was placed between two compartments with stratum corneum facing the donor compartment.

A. Preparation of skin for permeation studies

Male wistar rats weighing approx 150-250 gm were obtained from central animal facility at Jamia Hamdard (protocol number 1320) which was used for the preparation of skin sample. The skin excised from the abdomen region was stored at -21 ºC until use. The hair was removed with electric clipper and subcutaneous fat was removed by isopropyl alcohol. At last the skin was washed with distilled water and observed physically for any damage. The skin was wrapped in aluminum foil and kept overnight at 4 ºC.

B. Stabilization of the skin

The skin was cut in to appropriate size and mounted between two half of cell with the stratum corneum facing towards the donor compartment. The receptor compartment consisted of phosphate buffer pH 6.8 was stirred properly at constant speed. The whole system was equilibrated at 37 ± 0.5ºC. The buffer solution was replaced every hour with the fresh one and absorbance was taken every hour up to 6 hours and UV spectrum was taken. No absorption band in the UV indicated that skin was stabilized.

C. Permeation studies

Permeation studies were carried out in Franz diffusion cell in which donor compartment was filled with formulations (terbinafine loaded nanoemulsion gel and commercial Skin formulation), each containing terbinafine equivalent to 1% w/w. The receptor media was stirred approx at 200 rpm for proper mixing. Sample (1 ml) were withdrawn at regular interval (0, 1, 2, 3, 4, 5, 6hours) and replaced with the same volume of media. The samples were filtered using 0.45µmmembrane filter and amount of drug content was analyzed using UV spectrophotometer. The permeation flux values were calculated for all of the formulations by plotting the amount of drug permeated per unit surface area *vs* time. The slope of the regressed line of the straight portion of the graph was reported as the permeation flux value

Permeation coefficient (Papp) was calculated by using following formula:

$$P_{app} = \left(\frac{dQ}{dt}\right) / SA_o$$

Where,

dQ/dt = rate of drug permeation

S = cross-sectional area of tissue and

Ao = initial concentration of drug in donor compartment at t=0, respectively.

Determination of antifungal activity of formulation

In vitro antifungal activity of nanoemulsion gel of terbinafine was studied against *Candida albicans* using Sabouraud dextrose agar (SDA) media agar diffusion method. This media is acidic having pH about 5 and inhibits the growth of bacteria but permits the growth of yeasts and most filamentous fungi. Media was prepared and sterilized (by autoclaving at 15 psig pressure, 121°C for 15 min). The spores of *C. albicans* were added to agar plate and incubated at 37 ± 2°C for 48 h in dark condition. Spherical ditch was made in sterilized agar plate with a sterile borer in an aseptic area. Each of the formulation (nanoemulsion gel, placebo gel and marketed formulation) was mixed thoroughly with the medium and was poured in the ditch made on agar plate under sterile conditions. The plates were dried and incubated at 37 ± 2°C for 48 h. Zone of inhibition was measured at the end of incubation.

Histopathology examination of untreated and treated skin

The hair from the back side of the rats were removed using a hair removal cream. One group was treated with the optimized TBH loaded nanoemulsion gel and the second group with the marketed formulation (1% w/w). Sample was applied on the skin to the area of 4 cm2 once-a-day for 7 days. At the end of seven days the animals were sacrificed and the skin samples were fixed in 10% buffered formalin solution. The section slides of skin were prepared and stained with hematoxylin and eosin. Stained slides were observed under light microscope (Motic Japan) at 10X for histopathological changes. The results were compared with the skin sections of untreated rats.

RESULTS AND DISCUSSION

Physical Characterization and Identification of Terbinafine Hydrochloride

A. Physical properties

The obtained drug was characterized for various physical properties like nature, colour, odour, melting point and solubility.

Table: Physicochemical properties of terbinafine hydrochloride.

S.No.	Parameter	Inference	
1	Nature	Crystalline powder	
2	Colour	White powder	
3	Odour	Odourless	
4	Melting point	DSC method	209.85°C
		Capillary method	206.5°C
		Reported M.P	204-208°C **(USP Convention., 2009).**
5	Solubility	Methanol	48mg/ml

Conclusion: The physical properties of the drug sample were found to be similar as mentioned in reference **(USP Convention., 2009)**

B. Identification Test

a) UV Spectral Analysis

A UV-Vis spectrum of Terbinafine hydrochloride in methanol was taken at 100 µg/ml in the range of 200-400 nm. The λ max of drug in methanol was found to be 223.5 nm.

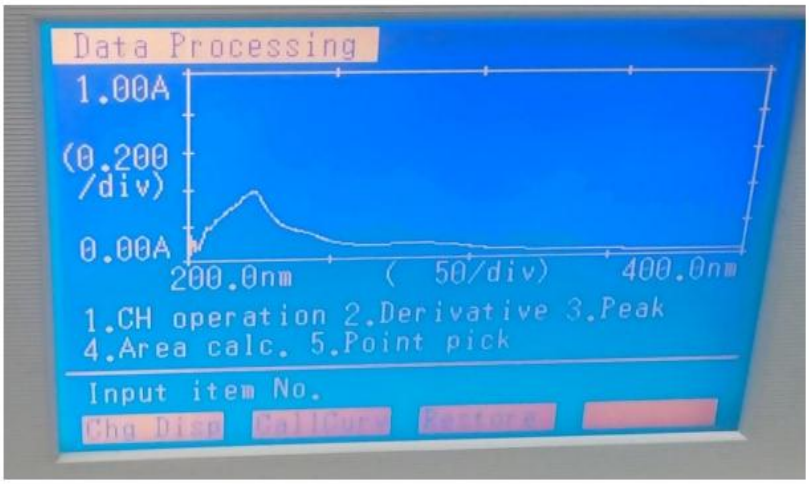


Figure 6.1: *UV Spectrum of Terbinafine Hydrochloride in methanol*

b) FTIR spectrum analysis

The different characteristics peaks of drug are listed in table 6.2. The spectrum was found to have the peaks in the similar region as that of the reference (Fig 6.2 and 6.3) confirming that the drug sample was authentic.

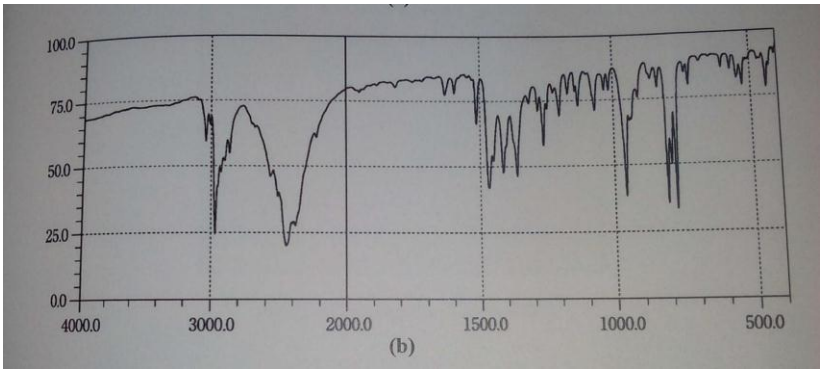


Figure 6.2: Reference FTIR Spectra of terbinafine hydrochloride.

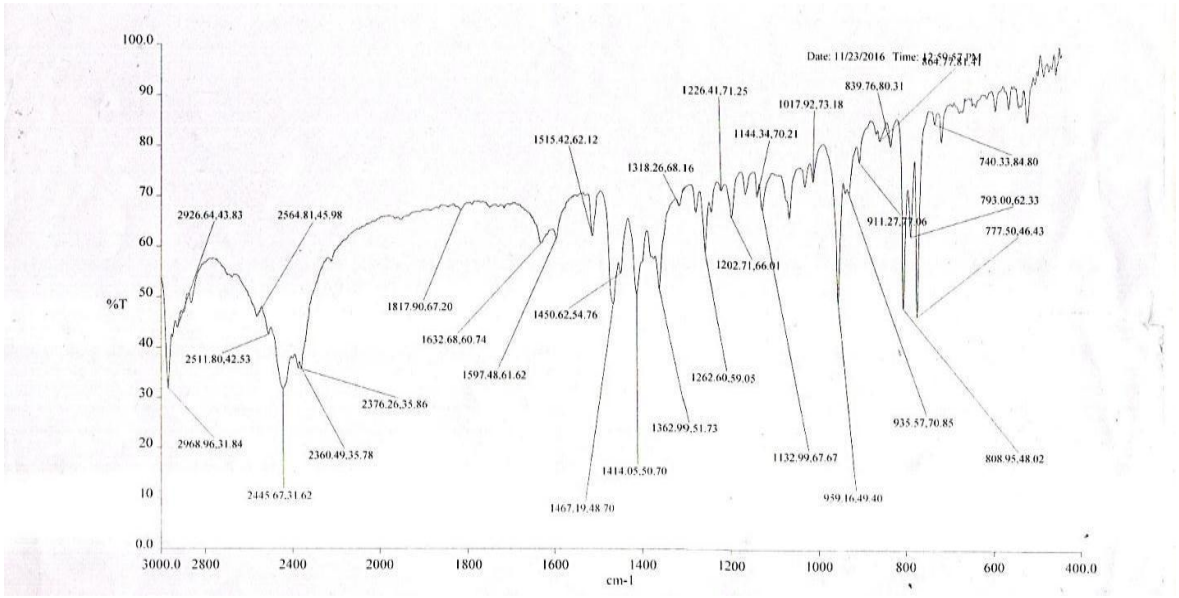


Figure: FTIR Spectra of terbinafine hydrochloride sample.

Table: IR Spectra having wave number range which shows signal assignment of drug.

S.No.	Functional group	Standard frequency range (cm^{-1})	Observation (cm^{-1})
1	OH streching	3200-2800	2968.96
2	SH streching	2800-2400	2445.67
3	CN streching	2400-2000	2376.26, 2360.49
4	C=O streching	1800-1600	1632
5	CH bending	1600-1400	1467.19
6	COOH streching	1400-1200	1362.99
7	S=O streching	1200-1000	959.16
8	CH bending	1000-800	808.95
9	C-Cl streching	800-600	777.50

c) DSC analysis

The melting point of terbinafine hydrochloride was determined by DSC method which showed an endothermic peak at 209.850ºC. The reported melting point lies in the range of 204-208ºC (**USP Convention., 2009**).

Conclusion: The results of the physical analysis and identification tests proved that the sample was pure and authentic.

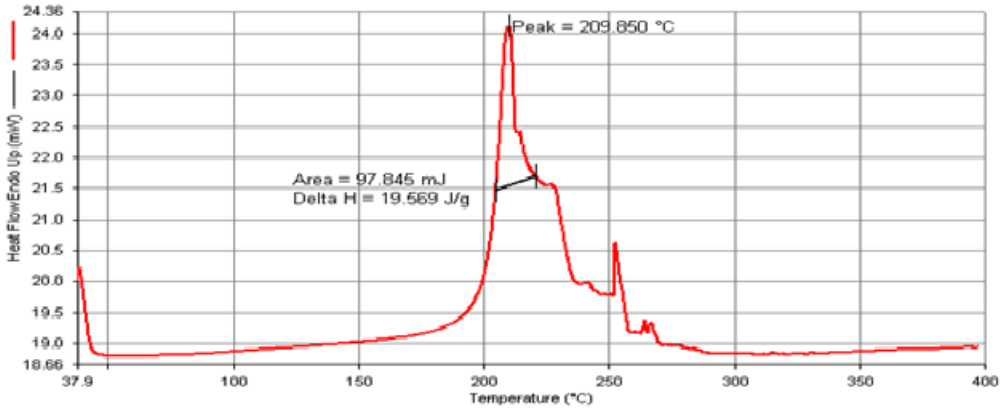


Figure 6.4: DSC thermogram of Terbinafine hydrochloride sample.

6.2 Analytical methodology

UV-spectrometric method was used for the estimation of terbinafine hydrochloride in *invitro* samples.

A. Calibration curve of Terbinafine Hydrochloride in Methanol

Sample of terbinafine hydrochloride was scanned using Shimadzu Double Beam Spectrophotometer between 200-400 nm. The solution showed absorption maxima at 223.5 nm. The calibration curve was found to be linear over the concentration range of 0.2-3.0µg/ml with regression equation y=0.03071x-0.0148 and correlation coefficient (R2) of 0.9996 (Table 6.3 and Fig.

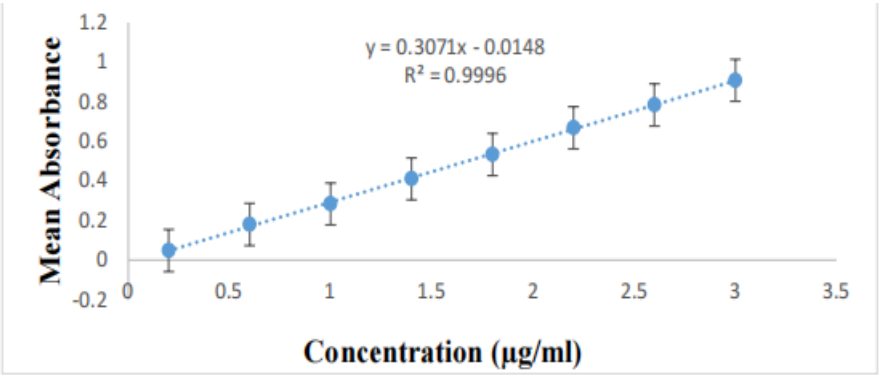


Figure 6.5: Calibration plot of terbinafine hydrochloride in methanol at λ max 223.5nm.

Table 6.3: Absorbance values of terbinafine hydrochloride in methan.

Conc. (µg/ml)	Mean absorbance ± SD (n=3)	Regressed absorbance
0.2	0.048 ± 0.001	0.0466
0.6	0.179± 0.003	0.1694
1.0	0.283± 0.004	0.2682
1.4	0.410± 0.006	0.4151
1.8	0.533± 0.0055	0.5379
2.2	0.668± 0.006	0.6608
2.6	0.783± 0.008	0.7836
3.0	0.908± 0.014	0.9065

B. Calibration curve of Terbinafine Hydrochloride in phosphate buffer (pH 6.8).

Sample of terbinafine hydrochloride was scanned using Shimadzu Double Beam Spectrophotometer between 200-400 nm. The solution showed absorption maxima at 222.5 nm. The calibration curve found to be linear over the concentration range of 0.6-4.8 µg/ml with regression equation y = 0.1477x + 0.0325 and correlation coefficient (R2) of 0.9935 (Table 6.4 and Fig.

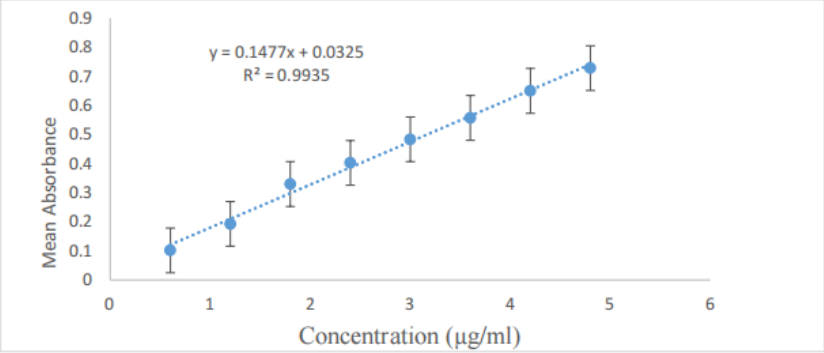


Table 6.4: Absorbance values of terbinafine hydrochloride in phosphate buffer (PH 6.8).

Conc. (µg/ml)	Mean absorbance ± SD (n=3)	Regressed absorbance
0.6	0.102± 0.001	0.1211
1.2	0.193± 0.003	0.2097
1.8	0.330± 0.004	0.2983
2.4	0.403± 0.006	0.3869
3.0	0.484± 0.007	0.4756
3.6	0.558± 0.008	0.5642
4.2	0.651± 0.009	0.6528
4.8	0.729± 0.011	0.7414

6.3 Formulation, Optimization and Evaluation of Terbinafine Hydrochloride Nanoemulsion

A. Screening and selection of oils

Oil was selected based on the solubility of required drug dose in the formulation. The oil selected should be capable of dissolving the required quantity of the drug freely as forced solubility with the incorporation of surfactants results in the precipitation of the drug over a period of time. The calculated solubility of terbinafine hydrochloride in Tea tree oil, vitamin E, wheat germ oil, and olive oil were found to be 18.00 ± 1.82, 9.21 ± 0.50, 10.19 ± 0.44 and 7.05 ± 0.87 mg/ml respectively (Table 6.5) and (Figure 6.7). The highest solubility in Tea tree oil can be attributed due to the non-polarity of terbinafine hydrochloride. Tea tree oil was selected as oil in the formulation as it had the potential to solubilize the maximum amount of drug. Tea tree oil is composed of terpene hydrocarbons, mainly monoterpenes, sesquiterpenes, and their associated alcohols. Terpenes are volatile, aromatic hydrocarbons and may be considered polymers of isoprene, which has the formula C5H8. According to many research it has been found that the membrane fluidity of *C. albicans* cells treated with 0.25% tea tree oil substantially altered. It has also been reported that glucose-induced medium acidification by *C. albicans*, *C. glabrata*, and *Saccharomyces cerevisiae* is also inhibited by tea tree oil. Medium acidification occurs largely by the expulsion of protons by the plasma membrane ATPase, which is fuelled by ATP derived from the mitochondria. Fungicidal action of tea tree oil is due to changes or damage to the functioning of fungal membranes. This proposed mechanism is further supported by work showing that the terpene eugenol inhibits mitochondrial respiration and energy production.

Table 6.5: Solubility of terbinafine hydrochloride in different oils.

S.No.	Oil	Solubility(mg/ml) ± SD (n=3)
1	Tea tree oil	18.00 ± 1.82
2	Vitamin E	9.21 ± 0.50
3	Wheat germ oil	10.19 ± 0.44
4	Olive oil	7.05 ± 0.87

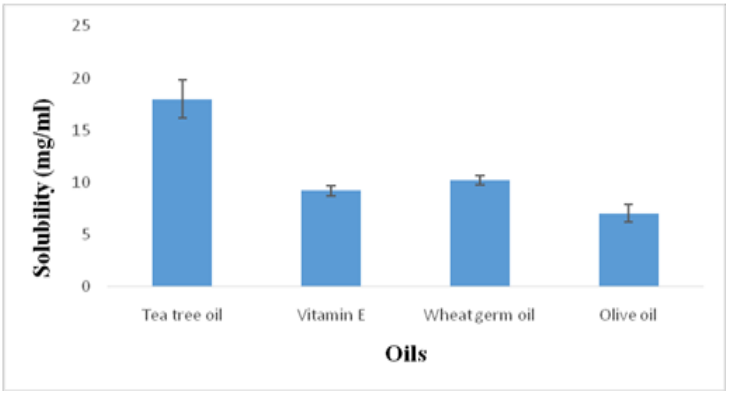


Figure: Solubility profile of terbinafine hydrochloride in different oils.

B. Screening and selection of surfactant and co-surfactant

Surfactants play an important role in the formation of nanoemulsion. Selection of surfactant is also critical as the selected surfactant should not only lower the interfacial tension but should be safe at the concentration used. Generally non-ionic surfactants are considered to be less toxic than ionic surfactants. Surfactant selection was based on HLB value, solubility and miscibility studies. The solubility in different surfactant along with their HLB value are given in table 6.6. Amongst surfactants, drug showed highest solubility in tween 20 (18.11 ± 2.50 mg/ml) in comparison to tween 80 (15.83 ± 2.04 mg/ml). Drug exhibited 12.58±1.69 mg/ml solubility in Solutol HS 15 and 14.26±1.98 mg/ml solubility in Unitop FFT-40.

Table: Solubility and miscibility of terbinafine hydrochloride in different surfactants.

S. No.	Surfactant	HLB	Solubility(mg/ml) ± SD (n=3)	Miscibility with Tea tree oil
1	Tween 80	15	15.83± 2.04	Clear
2	Tween 20	16.7	18.11 ± 2.50	Clear
3	Solutol HS 15	14	12.58±1.69	Turbid
4	Unitop FFT-40	14.3	14.26±1.98	Turbid

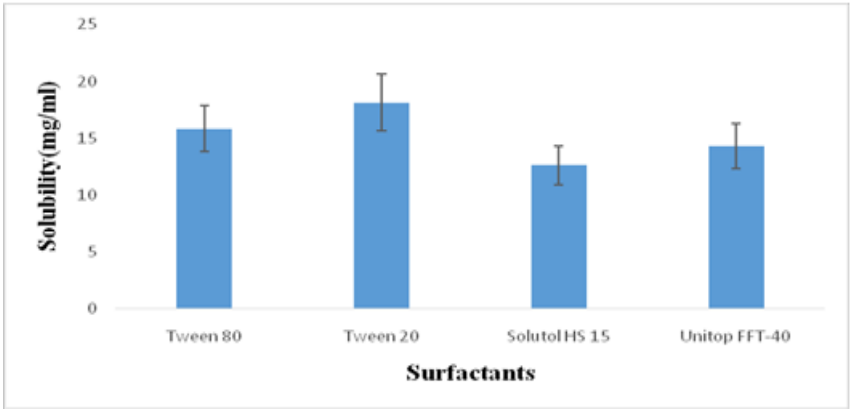


Figure 6.8: Solubility profile of terbinafine hydrochloride in different surfactants.

Co-surfactant plays a very important role in reducing the interfacial tension as they fit themselves into void space of surfactant molecule. Selection of co-surfactant was done on the basis of miscibility studies with selected oil and surfactant. Table 6.7 lists the miscibility data of co-surfactant with selected oil and surfactant. Polyethylene glycol 400 exhibited miscibility with the selected oil as well as surfactant hence it was selected on the basis of above criteria. PEG 400 (polyethylene glycol 400) is a low-molecular-weight grade of polyethylene glycol. It is hydrophilic, clear, colorless, viscous liquid. Due to its low toxicity, PEG 400 is widely used in a variety of pharmaceutical formulations. It improves drug solubility in vesicle dispersion and avoid precipitation of drug. It has good penetration properties, which makes it an excellent component as drug carrier for skin delivery.

Table 6.7: Miscibility of co-surfactant with oil and surfactant.

S. No.	Co-surfactant	Miscibility with Tea tree oil	Miscibility with Tween 20
1	Plurol Oleque CC 497	Phase separation	Phase separation
2	Lauroglycol 90	Turbid	Turbid
3	Polyethylene glycol 400	Clear	Clear
4	Transcutol P	Clear	Turbid

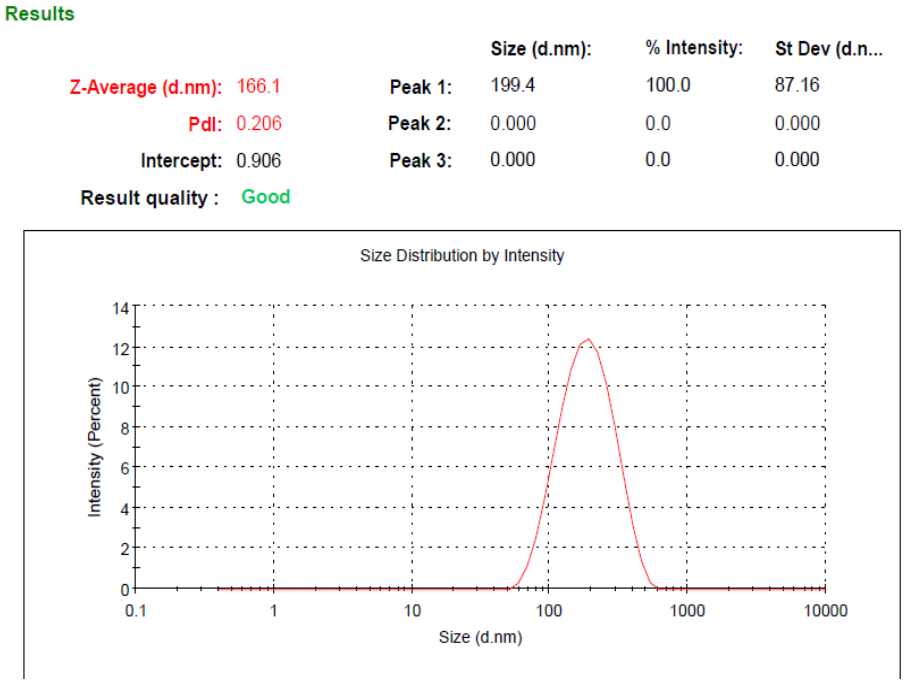


Figure 6.11: D roplet size of optimized nanoemulsion.

B. Percentage transmittance

Percentage transmittance value of 100% indicates the isotropicity of formulations. The optimized nanoemulsion exhibited percentage transmittance of 98.60 ± 0.20 which indicated that the formulation was isotropic in nature.

C. Zeta potential

The magnitude of charge between droplets is represented by zeta potential. Evaluation of zeta potential was helpful to calculate the surface charge of droplet which was responsible for the stability of colloidal dispersions. Moreover, it provides information about degree of repulsion between adjacent similarly charged droplets present in dispersion. Zeta potential values for the optimized nanoemulsion was 10.60 ± 3.83 mV (figure 6.12). The physical stability of colloidal system depends on zeta potential and minimum ±25mV is required for stable nanoemulsion (**Samson S *et al.*, 2016**). Small value of zeta potential is due to nonionic surfactant used in formulation. Moreover, formulation is physically stable at 10.60 ± 3.83 mV because of steric hindrance exerted by Tween 20 molecules.

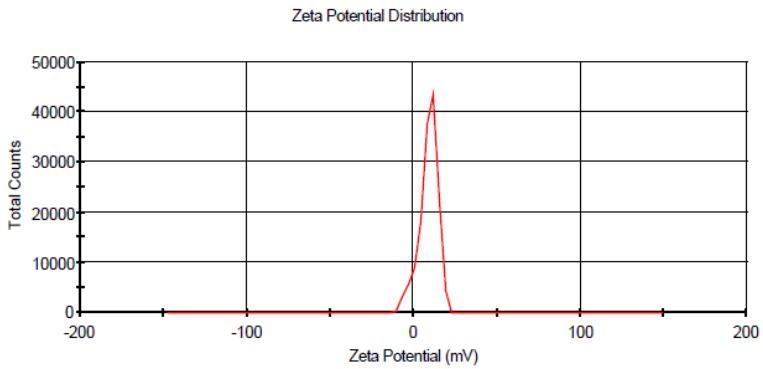


Figure 6.12: Zeta potential of optimized nanoemulsion.

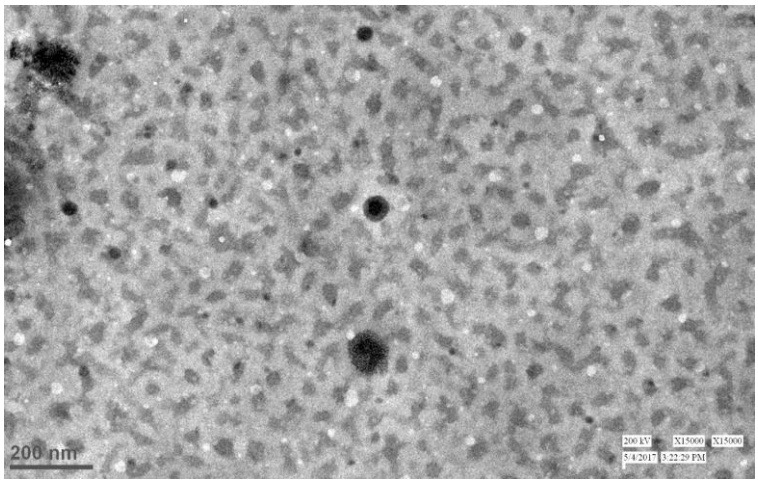


Figure 6.13: TEM image of nanoemulsion.

E. Refractive index (RI)

Similarly, to percentage transmittance, RI is helpful to analyse the isotropicity of system. The interactions between the ingredients were also evaluated by RI value. RI of formulation was found to be 1.395±0.002. The obtained RI data bring to a view that the developed formulation was chemically stable and was isotropic in nature. The refractive index of water is 1.333.

F. Conductivity

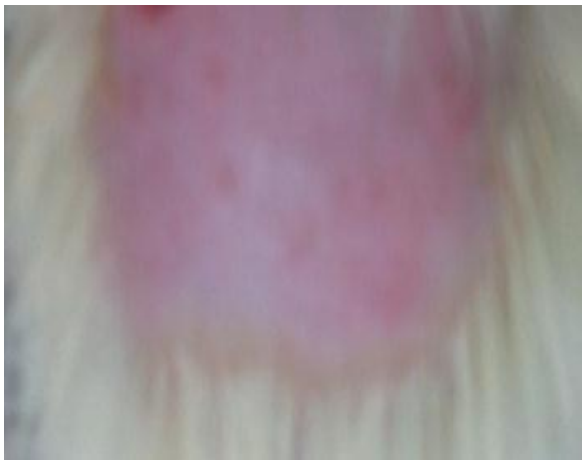
The parameter conductivity is used to evaluate the nature of the emulsions (oil/water or water/oil type). Water/oil type emulsions have low conductivity (lower than 1 µS/cm) but o/w type show more conductivity. Optimized Terbinafine hydrochloride nanoemulsion (1% w/w) showed conductivity of 523.46± 4.73 µS/cm. Thus, conforming that the formulation is oil/water type.

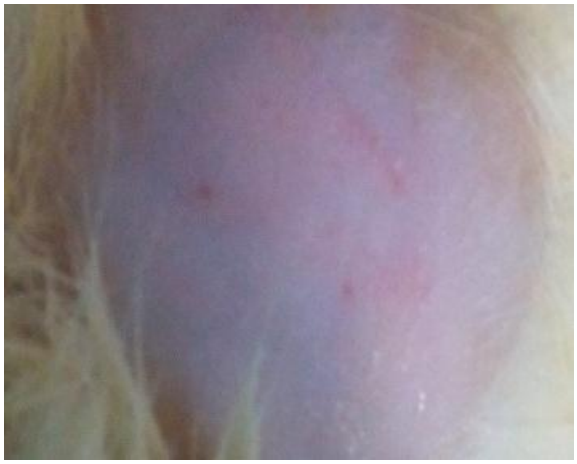
Table 6.12: Drug content uniformity of optimized terbinafine hydrochloride nanoemulsion gel.

Drug Content uniformity (mean ± SD) in percentage		
Portion 1	Portion 2	Portion 3
98.48%±1.31	99.33%± 0.43	99.78%± 0.86
Mean	99.19%	

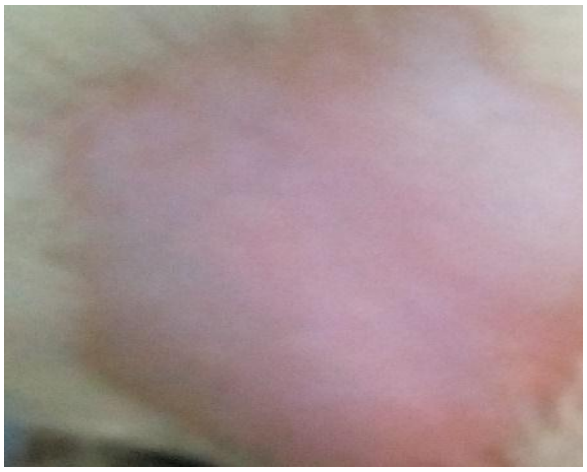
Skin irritation studies for optimized formulation

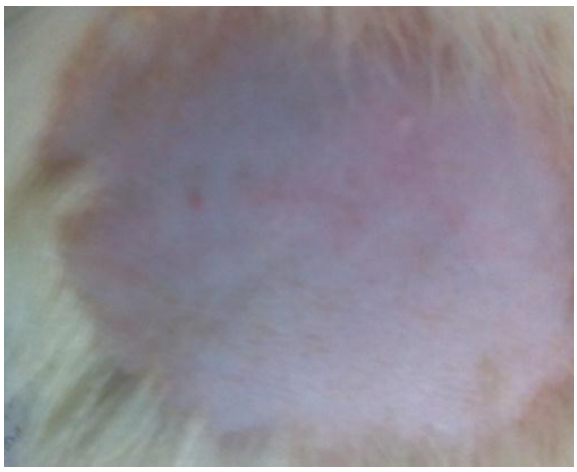
The skin irritation test was performed to confirm the safety of the optimized nanoemulsion gel of terbinafine as compared to the marketed formulation. The skin of wistar rat after treatment with nanoemulsion gel and marketed cream of terbinafine (1% w/w) for 7 days are shown in figure 6.15. The skin was observed for the presence of erythema, edema, rashes on the test site and scored as 0, 0.1-0.4, 0.41-1.9, 2.0-4.9 and 5.0-8.0 for non-irritation negligible irritation, slight irritation, moderate irritation and severe irritation, respectively. Optimized nanoemulsion gel and marketed cream were scored zero that suggesting that formulations were non-irritant to skin.





Before treatment (Day 1) **After treatment (Day 7)**





Before treatment (Day 1) **After treatment (Day 7)**

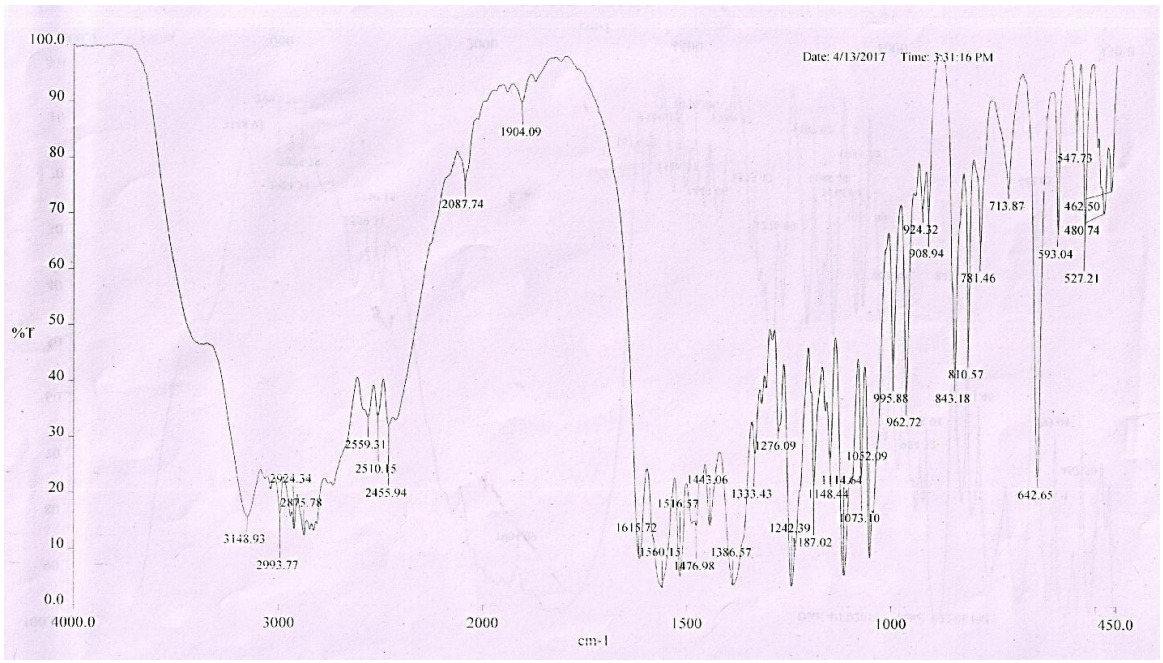


Figure 6.17: FTIR spectrum of normal skin.

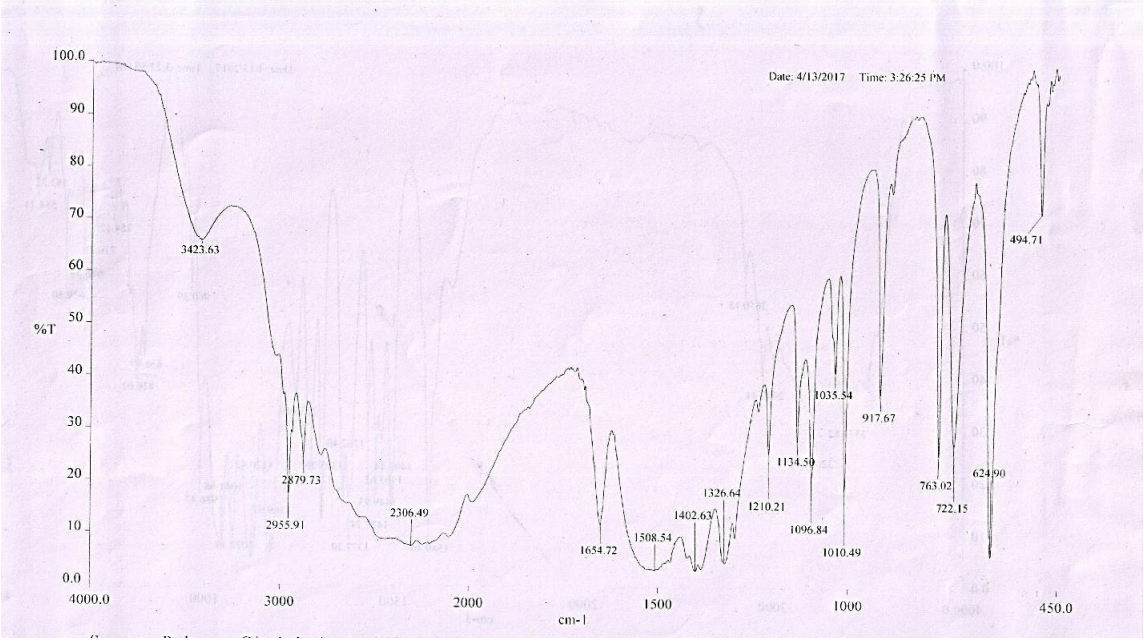


Figure 6.18: FTIR spectra of skin treated with nanoemulsion gel.

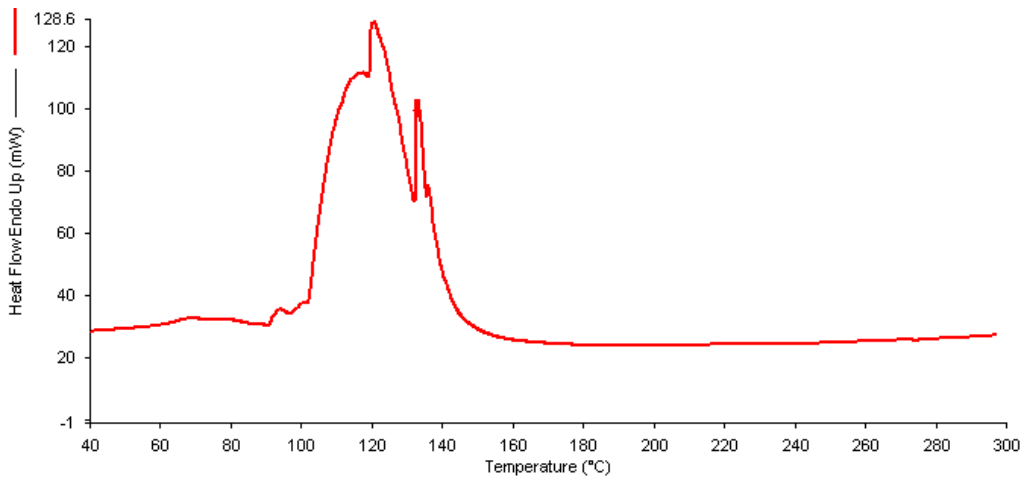


Figure 6.19: DSC thermogram of untreated skin.

Table 6.14: Zone of inhibition of nanoemulsion gel, placebo gel and marketed cream of terbinafine hydrochloride.

Formulation	Zone of inhibition (mm)
Placebo gel	0.00
Nanoemulsion gel	43.2
Marketed cream	34.7

Figure 6.22: Zone of inhibition of different treatment (nanoemulsion gel, marketed cream and placebo gel).

Summary and Conclusion

The aim of the present study was to develop, optimize and characterize topical nanoemulsion gel of Terbinafine hydrochloride for the treatment of fungal infections. Terbinafine hydrochloride is a synthetic allylamine derivative, which exerts its antifungal effect by inhibiting squalene epoxidase, a key enzyme in sterol biosynthesis in fungi. This action results in deficiency in ergosterol and a corresponding accumulation of squalene within the fungal cell and cause fungal cell death. It can be used topically or orally. This drug has broad spectrum activity against yeast, dimorphic fungi, dematiaceous fungi, moulds and dermatophytes. The topical treatment of fungal diseases is often limited by the poor percutaneous permeation designed in such a way that it can improve the percutaneous permeation at the required concentration and achieves its therapeutic action to the targeted site. Therefore, it is imperative to design a formulation which can enhance the permeation of terbinafine hydrochloride through skin. Nanoemulsions are isotropic, kinetically stable, transparent system having a particle size range between 20-200nm. They can solubilise lipophillic drugs and because of the presence of surfactants and small size also increase the permeability through the skin. The nanoemulsion formulation, as such is not convenient to be used topically because of its low viscosity and poor spreadability which leads to short duration of contact at site of action. Nanoemulsion gel offers several advantages as drug delivery system over nanoemulsion topical drug delivery systems. It decreases surface and interfacial tension which enhanceS the viscosity of the aqueous phase. Further, emulsifiers and thickeners are added to strengthen the gelling capability of hydrogel which serves to provide a better stability, permeation and suitable viscosity for the delivery of topical drug-

loaded nanoemulsion. The stability of nanoemulsion is enhanced by the distribution of oily droplets in gel network which act as carrier for lipophilic drugs .Nanoemulsion gel have a good adhesion property on the skin together with high solubilizing capacity. These properties lead to larger concentration gradient towards the skin that influence further skin penetration of drug. Such formulations also improve patient compliance as they are not sticky and easily spread as compared to other topical delivery system such as ointment and cream. Therefore, it is imperative to design a formulation which can enhance the permeation of terbinafine hydrochloride through the skin. So, it is proposed to develop Terbinafine hydrochloride loaded nanoemulsion gel that have better delivery as a result of increased permeation. Therefore, Terbinafine hydrochloride loaded nanoemulsion gel was formulated to have better delivery as a result of increased permeation. The following results were obtained from the present study and are concluded as below:

- The physical properties of the obtained drug and identification (UV, FTIR, DSC) test assured that the obtained drug sample was pure and authentic.
- The solubility of terbinafine hydrochloride was performed in different oils. It was found that terbinafine hydrochloride exhibited maximum solubility in Tea tree oil (18.00±1.82mg/ml) and therefore it was selected as the oil phase. Moreover, this solubility was sufficient to administer the required dose of terbinafine hydrochloride through dermal route.
- Similarly, surfactant was selected based on the solubility and miscibility studies whereas co-surfactant was selected based on miscibility studies. Data obtained from these studies were analyzed and Tween 20 and polyethylene glycol 400 were selected as surfactant and co-surfactant respectively.
- Pseudo ternary diagram were prepared using oil, Smix, and water with different concentration ratio with the aim to determine the ratio of surfactant and co-surfactant which was capable to formulate maximum nanoemulsion area. From the study it was concluded that maximum nanoemulsion area was formed in the ratio of 4:1 thus this ratio of Smix was selected for nanoemulsion preparation.

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