CODEN [USA]: IAJPBB ISSN : 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES
SJIF Impact Factor: 7.187

Avalable online at: http://www.iajps.com Research Article

FORMULATION AND DEVELOPMENT OF ANTIFUNGAL LOADED NANOEMULGEL FOR THE TREATMENT OF ONYCHOMYCOSIS

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Article Received: January 2021 **Accepted:** January 2021 **Published:** February 2021

Abstract:

Nanoemulgel has emerged as one of the most interesting topical delivery systems as it has dual release control system i.e nanoemulsion and hydrogel. Nanoemulgel having nanosize (below 200nm) rapidly penetrates and deliver the active substance deeper and quicker. This work was aimed to develop and evaluate nanoemulgel based transungual drug delivery system of poorly water soluble efinaconazole with better drug release for the treatment of onychomycosis. Efinaconazole has been shown to have a more potent antifungal activity in vitro than the most commonly used onychomycosis treatments. The low keratin affinity of the efinaconazole contributes to its effective delivery through nail plate and retention of its antifungal activity. Characterization of drug and excipients were performed and FTIR results confirmed the compatibility of efinaconazole with the excipients. Pseudoternary phase diagram were constructed using titration method to figure out the ratio of Smix. Nanoemulsion containing efinaconazole was prepared by spontaneous emulsification method using, Triacetin as oil, Tween 80 as surfactant and Transcutol as co surfactant. Carbopol 934 is used as the gelling agent.Thiogylcollic acid is incorporated as the penetration enhancer. Prepared nanoemulgel was evaluated for physical appearance, pH, viscosity, particle size, drug release. In vitro drug release study was performed for efinaconazole nanoemulgel and it showed better release profile compared to plain gel. It showed that 50% of the drug is released in the 8th hour. Thus more than 90% drug release will occur by 24th hour which indicate good controlled release. The kinetic modelling confirmed Non fickian or anomalous diffusion of efinaconazole from nanoemulgel.

KEYWORDS: *Nanoemulgel,Onychomycosis,Efinaconazole, Transungual drug delivery system,solubility*

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Please cite this article in press Sruthi.Sand Roma Mathew, ***Formulation And Development Of Antifungal Loaded Nanoemulgel For The Treatment Of Onychomycosis.,****Indo Am. J. P. Sci, 2021; 08(02).*

INTRODUCTION:

Onychomycosis, also known as tinea ungium, is a fungal infection of the nail [(1)]. Symptoms may include white or yellow nail discoloration, thickening of the nail, and separation of the nail from the nail bed [(2)]. Nails or fingernails may be affected, but it is more common for toe nails to be affected. Complications may include cellulitis of the lower leg. A number of different types of fungus can cause onychomycosis including dermatophytes and *Fusarium*. Risk factors include athlete's foot, other nail diseases,exposure to someone with the condition , peripheral vascular disease ,and poor immune function.The diagnosis is generally suspected based on appearance and confirmed by laboratory testing. It occurs in about 10 percent of the adult population. Older people are more frequently affected. Males are affected more often than females. Onychomycosis represents about half of the nail diseases. It was first determined to be the result of fungal infection in 1853 by Georg Meissner.

The most common symptom of a fungal nail infection is the nail becoming thickened and discoloured: white, black,yellow or green .As the infection progresses the nail can become brittle , with pieces breaking off or coming away from the toe or finger completely .If felt untreated, the skin underneath and around the nail can become inflamed and painful. There may also be white or yellow patches on the nail bed or scaly skin next to the nail and a foul smell. The causative pathogens of onychomycosis are all in the fungus kingdom and include dermatophytes, candida (yeast) and non dermatophytic moulds. Dermatophytes are the fungi most commonly responsible for onychomycosis in the temperate western countries; while *Candida* and nondermatophytic molds are more frequently involved in the tropics and subtropics with a hot and humid climate[(3)].

There are five classic types of onychomycosis [(4,5)]:
Distal subungual onychomycosis is the most common form of tinea ungium and is usually caused by Trichophyton rubrum, which invades the nail bed and the underside of the nail plate.

White superficial onychomycosis is caused by fungal invasion of the superficial layers of the nail plate to form "white island'' on the plate. It accounts for 10%of onychomycosis cases.

Proximal subungual onychomycosis
Fungal penetration of newly formed nail plate through the proximal nail fold.

Candidal Onychomycosis is candida species invasion of the fingernails.

Endonyx onychomycosis is characterized by leukonychia along with a lack of onycholysis or subungual keratolysis.

Most treatment are with antifungal medications either topically or by mouth. Topical agents include ciclopirox nail paint, amorolfine, and efinaconazole.Some of the topical treatment need to be applied daily for prolonged periods .Topical amorofine applied weekly. Topical ciclopirox results in a cure in 6% to 9% of cases; amorolfine might be more effective. Ciclopirox when used with terbinafine appears to be better than either agent alone. In trials about 17% of people using efinaconazole while cure was seen in 4% of people using placebo.

Efinaconazole inhibits fungal lanosterol 14α-demethylase involved in the biosynthesis of ergosterol, a constituent of fungal cell membranes [(6)].The accumulation of 14α-methyl sterols and subsequent loss of ergosterol in the fungi cell wall may be responsible for the fungistatic and fungicidal activity of efinaconazole. Efinaconazole is shown *in vitro* to be substantially absorbed to keratin but keratin binding is weak. Efinaconazole 's low keratin affinity is expected to result in increased availability of free drug to the nail infection site[(7)].

Nanoemulsions are nanosized emulsions, which are manufactured for improving the delivery of active pharmaceutical ingredients. These are the thermodynamically stable isotropic system in which two immiscible liquids are mixed to form a single phase by means of a emulsifying agent,i.e. surfactant and cosurfactant[(8)]. These carriers are solid spheres and their surface is amorphous and lipophilic with a negative charge. As a drug delivery system, they enhance the therapeutic efficacy of the drug and minimize adverse effect and toxic reactions. An emulsion is a biphasic system in which one phase is intimately dispersed in the other phase in the form of minute droplets ranging in diameter from 0.1 to 100 μm.

Selection of an appropriate oily phase is very important as it influences the selection of other ingredients of nanoemulsion, mainly in case O/W nanoemulsions [(9)]. Usually, the oil which has maximum solubilizing potential for selected drug candidate is selected as an oily phase for formulation of nanoemulsion. This helps to acheive maximum drug loading in the nanoemulsion.

Most of the time ,surfactant alone cannot lower the oil-water interfacial tension sufficiently to yield a nanoemulsion which necessitates the addition of an amphiphilic short chain molecules or cosurfactants to bring about the surface tension close to zero[10].Co surfactants penetrate into the surfactant monolayer providing additional fluidity to interfacial film and thus disrupting the liquid crystalline phases which are formed when surfactant film is too rigid .Usually a very low HLB cosurfactants is used with a high HLB surfactants to modify the overall HLB of the system.
The droplet size and stability of nanoemulsion is influenced by the nature of aqueous phase. Hence pH and ionic content of aqueous phase should be given due importance while designing nano emulsion. The physiological milieu has diverse pH ranges varying from 1.2(pH stomach)to 7.4 and greater (pH of blood and intestine).In addition ,the presence of various ions in the physiological milieu can also have considerable effect on the properties of nanoemulsion.Ternary phase diagram is useful for determination of best emulsification region of oil, surfactant, and co-surfactant combinations. The apex of the triangle represents ternary phase diagram of surfactant, cosurfactant and oil.

In the formulation of nanoemulsion as topical drug delivery system faces many challenges in delivering the drug effectively.Rheology property of the nanoemulsion is important. The nanoemulsion formulation, is not convenient to be used due to low viscosity and spreadability.Hence, the limitation has restrained the application of nanoemulsion clinically. Therefore, the approach of incorporation of nanoemulsion with gellying system can help in overcoming this problem. Nanoemulgels are oil in water or water in oil type nanoemulsions gelled with gelling agent. Stabilization of the new preparation occurs due to a decrease in surface tension and interfacial tension [11].

MATERIALS AND METHODS:

Efinaconazole was kindly provided by Viruj Pharma,Hyderabad.Triacetin and transcutol were purchased from TCI chemicals,Chennai. Tween 80 was purchased from Central drug house (P) Ltd., New delhi. Carbopol 934 and Thioglycollic acid were purchased from Central drug house (P) Ltd., New delhi.All the chemicals and reagents used were of analytical grade.

PREFORMULATION STUDIES

1) Preparation of calibration plot of Efinaconazole

10 mg of efinaconazole was dissolved in 20ml of methanol and make up the volume to 100ml in a standard volumetric flask using methanol (stock solution).From the stock solution 1 ml taken and made up to 10 ml to obtain 10µg/ml solution. From this 0.5 ,1,1.5,2,2.5 ml solutions were taken and made upto 10 ml and their absorbance is measured at 262 nm in UV spectrophotometer (JASCO V-630) using methanol as blank. Using the data obtained, a calibration plot of efinaconazole was plotted.

A calibration plot of efinaconazole was developed using pH 7.4 phosphate buffer using the same procedure.

2) Saturation solubility study

Saturation solubility of efinaconazole in various oils, surfactants and co surfactants were determined by shake flask method. In this study, an excesss amount of efinaconazole (approximately 100 mg) was added to 2ml of each vehicle. The mixture was mixed using magnetic stirrer to get uniform slurry. The samples were centrifuged at 4500 rpm for 10 minutes to separate the supernatant. Aliqouts of supernatant were taken, filtered through whatmann filter paper, filterate was suitably diluted with methanol and drug was quantified by measuring absorbance at 262 nm using UV spectroscopy [12].

FORMULATION AND DEVELOPMENT

Construction of Pseudo ternary phase diagram

The Pseudo ternary phase diagram with oil (Triacetin), three different ratios of surfactant: cosurfactant (Tween 80: Transcutol) and water were developed using water titration method [13]. The phase diagrams were developed with 1:3 ,2:1,and 3:1 ratios of surfactant and cosurfactants.

The procedure consists of taking specific ratio surfactant/cosurfactant and adding varying amount of oil by through mixing.Each mixture was titrated with water,observed for clarity and recorded the observation.The appearance of clear solution indicate the formation of emulsion and appearance of hazy solution indicates non emulsion. From the diagram, the maximum and minimum ratios of individual component required for getting emulsion region are noted and the same knowledge was used in the preformulation development.

Preparation of nanoemulsion

Preliminary formulation were prepared by varying triacetin and ratios of mixture of Tween 80 and Transcutol .The ratio of surfactant to cosurfactant was maintained at 1:1. Nanoemulsion is prepared by spontaneous emulsification method[14].The process used for preparation of mixture is as follows: Accurate quantity of oil and ratio of surfactant and cosurfactant were taken, distilled water was added and placed in a magnetic stirrer and stirred for few

minutes at 100 rpm .The obtained white emulsion was sonicated for 10-30 minutes to form clear nanoemulsion .To the nanoemulsion, drug was added stirred for 15 minutes. The mixture was heated to 30 to 40°C till the drug gets solubilized, if needed. The optimized formula was obtained by using Design expert Software Trial Version 12.0.9.0

Droplet Size and Poly dispersity Index

For understanding or determining the behavior of nanoemulsion, mean droplet size is very important. It was determined using Zetasizer Nano ZS90 (Malvern Instruments, Malvern UK) based on the principle of photon correlation spectroscopy, which analyses fluctuation in light scattering due to Brownian motion of particles. Light scattering was monitored at 25 °C at a scattering angle of 90°. The nanoemulsion (1-1.5ml) was transferred to a disposable polystyrene cuvette with the help of a micropipette, and the mean droplet size was measured.

Zeta Potential

The zeta potential of the diluted nanoemulsion was determined using Zetasizer Nano ZS90 .1 ml of the formulation was diluted 10 times with distilled water in a beaker.Samples were placed in clear cuvette and results were recorded. Surface charge on emulsion droplets and their zeta potential values are obtained.The magnitude of zeta potential gives an indication of potential stability of the formulation[15].

Percentage Transmittance

Transmittance was observed by using UV Spectrophotometer (Shimadzu,Japan) at 630 nm.One milliliter of nanoemulsion formulation was taken in a test tube and ethanolic dilution was analyzed at 630 nm and in triplicate.

TEM

The morphology of nanoemulsion was examined using JEOL model JSM -6390 LV and electron Transmission microscope at 70 kV[16]. After dilution with the original dispersion medium of the nanoemulsion, the samples were negatively stained with 1% (w/v) phosphotungstic acid for observation.

Entrapment Efficiency

The efinaconazole loaded nanoemulsion was centrifuged at 4500 rpm for 15 min using Remi (CFC free centrifuge CZCI -8889. The supernatant solution was diluted suitably and absorbance was determined using JASCO V-630 spectrophotometer at 261nm.

The entrapment efficiency was expressed in percentage. The percentage of drug entrapped was determined using the formula.

Percentage drug entrapped =

$$\frac{\text{Total amount of drug added-Unentrapped drug}}{\text{Total amount of drug added}} * 100$$

STABILITY STUDIES

Freeze-Defrost Cycles

The stability of prepared nanoemulsion was tested by subjecting them to Freeze-defrost cycles. The nanoemulsion were subjected to temperatures of 45 ± 5°C for 24 hours, and after, to a temperature of 4 ± 2°C also for 24 hours, thereby completing a cycle. Macroscopic evaluation was made 24 hours after nanoemulsion preparation and at the end of the cycle[17].

Centrifugation test

5 ml of optimized formulation was centrifuged at 3000rpm for time interval 10,20,30,45, and 60 minutes usingREMI R-8C centrifuge and noted the nanoemulsion for phase separation.

Thermal stress test

Samples were placed in a closed glass recipient and submitted to a range of temperature 45°C to 60°C ,30 minutes for each condition at thermostatic bath.The increment in temperature was done in an interval with 5°C.The nanoemulsion were observed for phase separation and creaming.

PREPARATION OF NANOEMULGEL

Nanoemulgel was prepared by the addition of gelling agent (1% w/v) and penetration enhancer (1.5ml). The gel base was prepared by dispersing the gelling agent (Carbopol -934 polymer) in purified water, with constant stirring at moderate speed using a magnetic stirrer, and then optimized nanoemulsion was incorporated into the gel base. Thioglycollic acid was used as the penetration enhancer. The prepared nanoemulsion was inspected visually for their colour, appearance and consistency.

CHARACTERISATION OF OPTIMISED NANOEMULGEL

Physical examination

The prepared emulgel formulation were inspected visually for their optical clarity, color, homogeneity, fluidity, and phase separation after 24 h of preparation.

pH

The pH values of 1 % aqueous solutions of prepared nanoemulgel were measured by a calibrated pH meter.

Viscosity
The viscosity was measured to determine the rheological properties of the formulation [18]. Viscosity was determined using Brookfield viscometer RV DV-E at 30°C with a CPE 01 spindle at 30 rpm. Results were taken in triplicate and the average was taken.

Spreadability [19]
The spreadability of the emulgel formulation was determined 48 h after preparation, by measuring the spreading diameter of 0.5 g emulgel which was placed within a circle of 1 cm diameter premarked on glass plate over which a second glass plate(75 cm) was placed. A weight of 100g was allowed to rest on the upper glass plate for 5 min where no more spreading was expected. The increase in the diameter due to spreading of the gel was noted. The spreadability was calculated by using the formula:

S =m.l/t,
Where :S is spreadability, m is the weight on the upper plate
l is the diameter of the spreading emulgel (cm) and t is the time taken(min)

Extrudability
It is a test to measure the force required to extrude the gel from the tube. On the application of weight,the amount of gel extruded from the aluminium tube was determined.The nanoemulgel extruded should be at least 0.5cm ribbon in 10 s. The higher the quantity of gel extruded, the better is the extrudability. The extrudability of each formulation was measured,in triplicate, and calculated by using the formula:
E=M/A
Where
E =Extrudability
M =Applied weight to extrude gel from tube
A =Area

Drug Content
For estimation of drug content 10mg of nanoemulgel was diluted to 100ml with pH 7.4 phosphate buffer. Again, 1ml was diluted to 10 ml with pH 7.4. The drug content was measured by using uv visible spectrophotometer at 261nm.

Drug Content = Theoretical yield * 100
Practical yield

***In Vitro* Diffusion Study**
The in vitro diffusion of nanoemulgel was determined by dialysis membrane method. Dialysis membrane was activated for about 1 hour in a solvent system of phosphate buffer (pH 7.4) and ethanol (3:1). About 2g of nanoemulgel were placed on artificial membrane, and the receptor compartment was filled with the solvent system (10ml).The whole assembly maintained at 37±1°C. Then aliquot of drug samples of about 2ml were withdrawn at predetermined intervals. The samples were filtered and absorbance were measured at 262 nm using Jasco V-630 UV Spectrophotometer to determine the drug content [20].

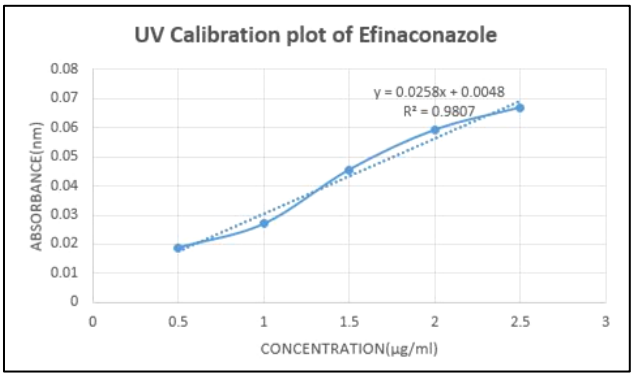
RESULTS AND DISCUSSION:

Preformulation Studies
The following table shows the data of prepared calibration curve of efinaconazole in Methanol .The UV calibration plot of efinaconazole was found to be linear.

Table 1: Data for calibration of efinaconazole in methanol

Concentration(µg/ml)	Absorbance (262 nm)
0.5	0.0187
1	0.0269
1.5	0.0456
2	0.0593
2.5	0.0670
R^2	**0.9807**

Fig 1: UV Calibration plot of Efinaconazole in methanol at 262 nm.



Calibration Curve of Efinaconazole In pH 7.4 Phosphate Buffer

The following table shows the data of the prepared calibration curve of efinaconazole in pH 7.4 phosphate buffer.

Table 2: Data of calibration curve in pH 7.4 phosphate buffer

Concentration(µg/ml)	Absorbance (262 nm)
0.5	0.0162
1	0.0247
1.5	0.0348
2	0.0469
2.5	0.0552
R^2	**0.9964**

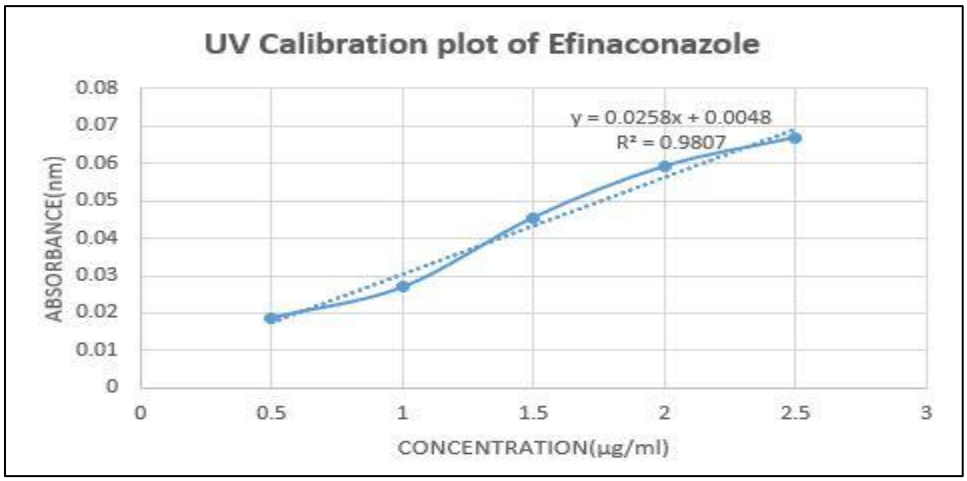


Fig 2: Calibration curve of Efinaconazole in pH 7.4 buffer.

SATURATION SOLUBILITY STUDY

Table 3: Saturation solubility study of efinaconazole in various oils, surfactants and cosurfactants.

Name of the Vehicle	Solubility in mg/ml
Labrafil	0.106mg/ml
Isopropyl myristate	0.034mg/ml
Oleic acid	0.083mg/ml
Triacetin	0.141mg/ml
Squalene	0.099mg/ml
Tween 20	0.031mg/ml
Tween 80	0.054 mg/ml
PEG 200	0.113mg/ml
PEG 400	0.130mg/ml
Transcutol	0.138mg/ml

1) **Oils-** Labrafil, Isopropyl myristate, Oleic acid, Triacetin, Squalene
2) **Surfactants –** Tween 20, Tween 80
3) **Co- Surfactant** – PEG 200, PEG 400, Transcutol

Construction of Pseudo ternary phase diagram

Three phase diagrams were obtained for three different Smix ratios (1:1,2:1,3:1).Prosim software were used.

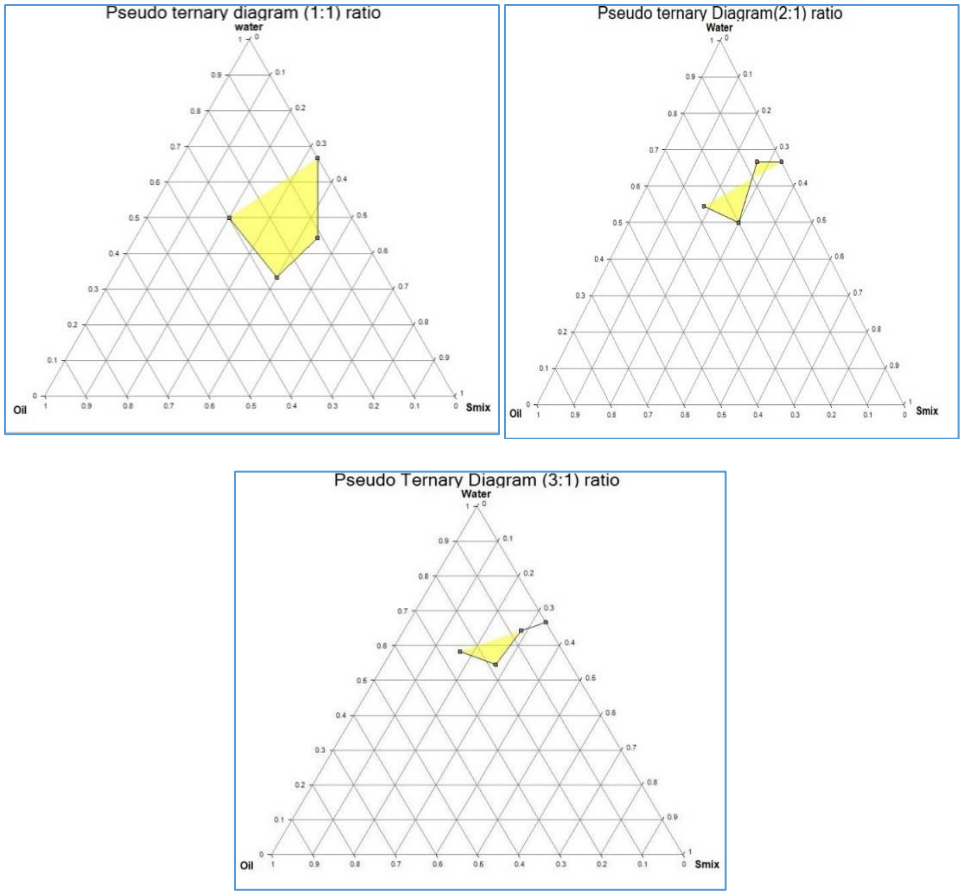


Fig 3: Pseudoternary phase diagram with Smix ratio a)1:1 b)2:1 c)3:1

The microemulsion existence region is presented in phase diagrams. The rest of the region on the phase diagram represents the biphasic, turbid and conventional emulsion based on visual examination. The pseudo ternary phase diagram prepared from Smix ratio 1:1 with higher microemulsion existence region was further utilized for selecting the optimization boundaries for different components of microemulsion.

CHARACTERISATION OF OPTIMISED NANOEMULSION

Droplet Size and Polydispersity Index

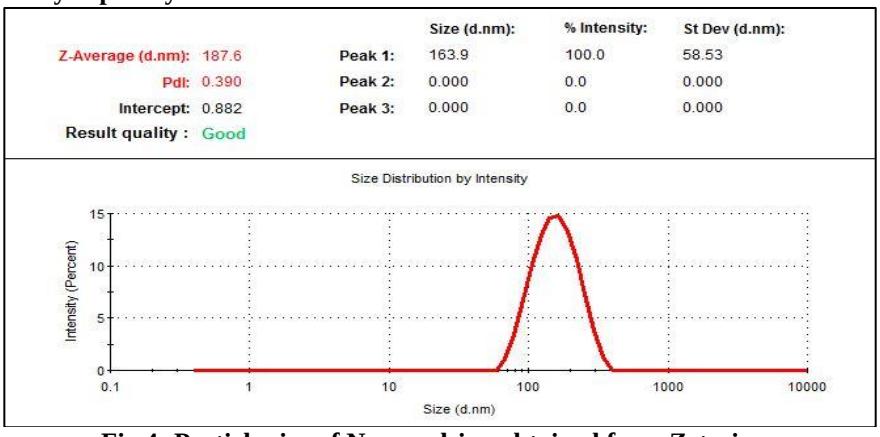


Fig 4: Particle size of Nanoemlsion obtained from Zetasizer

Particle size was measured for optimized nanoemulsion formulation by light scattering method using Malvern Zetasizer Nano-Zs90.Results showed that the particle size is 187.6 nm and is in nanometer range. The PDI value is 0.390 which is less than 1, indicates that higher stability of the nanoemulsion.

Zeta Potential Determination

Zeta potential give the type of charge present on the surface of the nanoemulsion. This gives the stability of the prepared formulation.Zeta potential of the optimized nanoemulsion was found to be -17.8mV. Large negative zeta potential value indicated good electro kinetic stability of nanoemulsion.

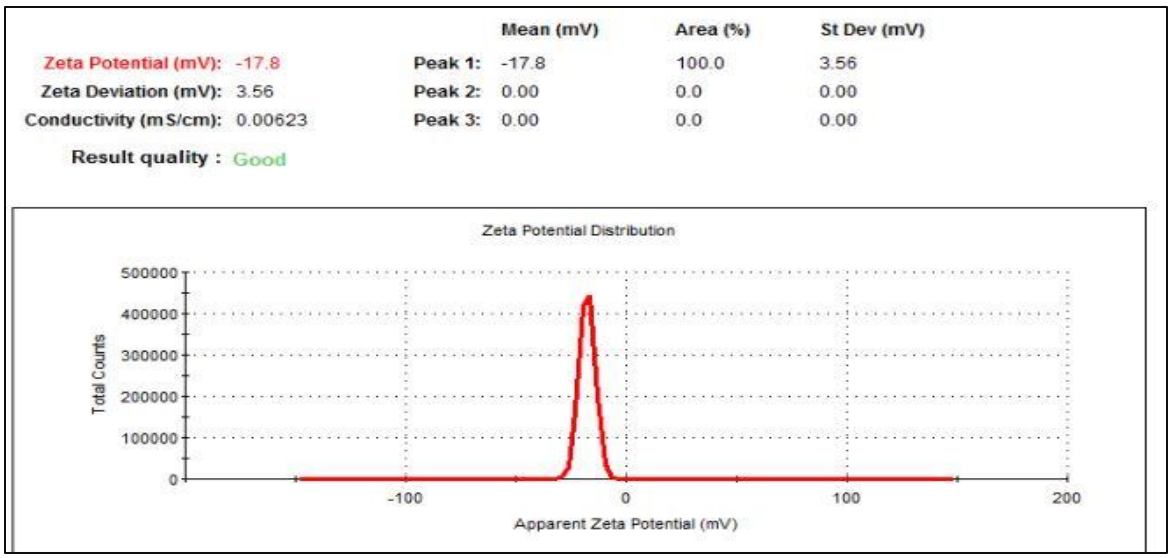


Fig 5: Zeta potential of Nanoemulsion obtained from Zetasizer

Percentage Transmittance

On 100-fold dilution, the percentage transmittance of Efinaconazole loaded nanoemulsion was found to be 97.65 ±0.46 %.This confirms the good transparent nature of Efinaconazole loaded nanoemulsion.

TEM

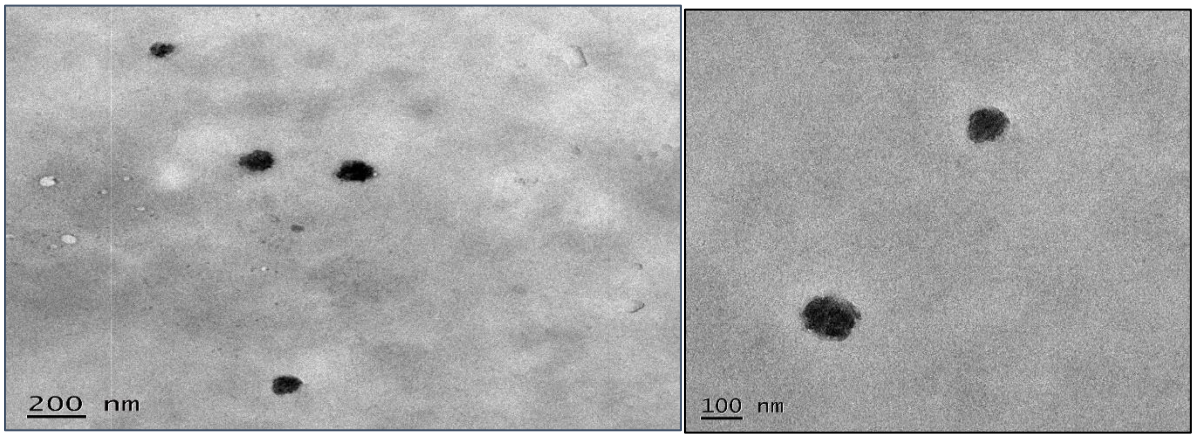


Fig 6: TEM images of efinaconazole loaded nanoemulsion

Morphology of the Efinaconazole nanoemulsion globules were examined by Hitachi, H-7650. It showed spherical globular structure.

Drug Entrapment Efficiency

The percentage drug entrapment of the formulation was found to be 83.69%

EVALUATION OF OPTIMISED NANOEMULGEL

Physical Examination

The prepared emulgel formulation were inspected visually for their optical clarity, color, homogeneity, fluidity, and phase separation after 24 h of preparation. It appeared as clear, transparent gel with good homogeneity and adequate fluidity. No phase separation was observed after 24 h of preparation.

pH

pH of the formulation was found to be 6.5±0.23 at room temperature.

Viscosity

Viscosity of the formulation was found to be 2248 ±0.21Cp at room temperature.

Spreadability

Spreadability value was found to be 2.5±0.22cm.

Extrudability

Extrudability was found to be 1.6±0.42 cm.

Drug content

The efinaconazole content in efinaconazole loaded nanoemulgel was found to be 91.00±001%

In Vitro Drug Release Study:

Table 4. Dissolution data of efinaconazole nanoemulgel

Time (Minutes)	Cumulative drug release% (pH 7.4and ethanol in 3:1 ratio)
0	0
15	1.39
30	3.00
60	9.38
120	17.35
180	24.77
240	30.24
300	35.37
360	40.47
420	44.04
480	50.40

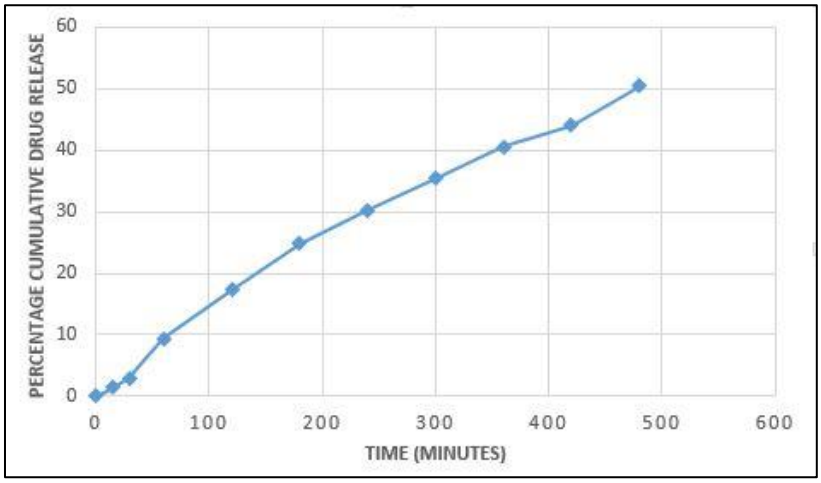


Figure 7: Dissolution data of Efinaconazole Nanoemulgel

COMPARISON OF INVITRO DRUG RELEASE OF EFINACONAZOLE NANOEMULGEL WITH PLAIN GEL

The *in vitro* release profile of efinaconazole nanoemulgel formulation together with plain gel is presented in Table no. 5 .Dissolution –accelerating components were added to the media as it results in the failure to discriminate between different *in vitro release* profiles.

Table 5. *In vitro* drug dissolution data of Efinaconazole nanoemulgel and plain gel

Time (Minutes)	Plain gel (%)	Nanoemulgel (%)
0	0	0
15	1.06	1.39
30	2.33	3.00
60	7.87	9.38
120	15.37	17.35
180	21.00	24.77
240	24.05	30.24
300	28.80	35.37
360	32.09	40.47
420	36.34	44.04
480	41.99	50.40

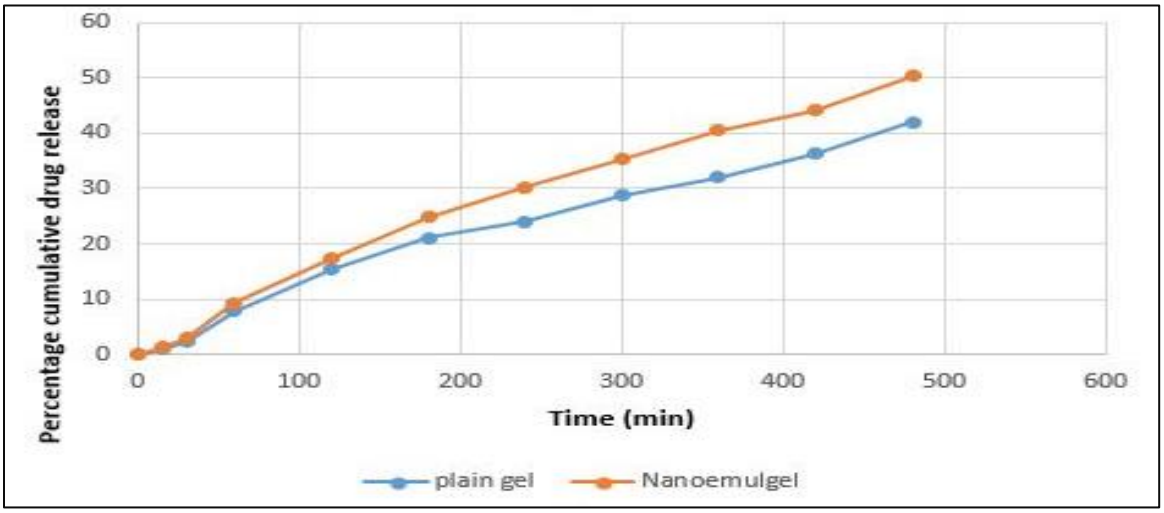


Fig. 8: Comparison graph of percentage cumulative drug release vs time of efinaconazole nanoemulgel and plain gel

Figure. 8 Signifies that *in vitro* release profiles of Efinaconazole from nanoemulgel formulation produced a constantly superior drug release rate as compared to that of plain gel .Nanoemulsion when present in nanoemulgel provide many nanosize droplets enhance the solubility of efinaconazole hydrophobic drug ,this will enhance the entrance of dissolution media to hydrophobic matrix and lower dissolution pathway for efinaconazole molecules to reach the dissolution media that make efinaconazole nanoemulgel superior and highly preferable in comparison to plain efinaconazole gel.

Stability studies of nanoemulgel

The objective of stability study was to predict the shelf life of a product at optimum temperature and relative humidity conditions.

The optimized formulation was packed in screw capped bottles and studies were carried out for 30 days by storing the sample at 4°C and, 25°C. Samples were withdrawn at 0^{th}, 15^{th} ,30^{th} day respectively were analyzed for drug content spectrophotometrically at 261nm.Results are shown in the following **Table 6**

Table 6:

Storage Condition 4°C

NO OF DAYS	DRUG CONTENT (%)
0	**91.00**
15	**90.80**
30	**89.45**

Storage Condition 25°C

NO OF DAYS	DRUG CONTENT (%)
0	**91.00**
15	**90.90**
30	**89.95**

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