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Research Article

Design, Fabrication, and Evaluation of Microemulsion Based Gel of Essential Oil of Thymus Vulgaris for Superficial Fungal Infections

Jeet V. Gandhi[1]*, Disha J. Suthar[2], Hetal K. Patel[2], Pragna K. Shelat[2], Punit J. Parejiya[2]

[1]*Kadi Sarva Vishwavidyalaya, Gandhinagar-382024, Gujarat, India*
[2]*K. B. Institute of Pharmaceutical Education and Research, Gandhinagar-382023, Gujarat, India*

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ABSTRACT

Although fungus being part of the commensal skin micro-structuring, various pathogenic commensals colonize on human skin leading to superficial fungal infections. Owing to the resistance of present therapeutic treatments available, microbial resistance and serious hypoallergic reactions have been a concern to explore the phytotherapeutic nutrients for the treatment of fungal infections. One such plant essential oil-based formulation is thyme oil derived from the leaves of *Thymus vulgaris*. The aim of present work, i.e. development of thyme oil-based microemulsion for the treatment of fungal infections due to *Candida* and *Trichophyton* species. The thyme oil loaded microemulsion based gel was constructed using D-optimal design and the optimized final formulation contains 0.82% of oil, 9.22% of Smix, and 89.95% of water. The optimized microemulsions was pale yellow to amber transparent microemulsion with a globule size of 14.23 ± 0.3 nm, zeta potential of -0.69 mV and PDI value 0.00143 indicating a stable microemulsion. The microemulsion based (MBG) gel formed had a pH of 6.03, appreciable viscosity and rheological properties. The drug release of the formulation was 100.0 ± 0.22%. The % of drug permeated in skin layers was found to be 15.53 ± 0.22%. While % drug retention on the skin surface was found to be 26.32 ± 0.26% and within skin layers was found to be 58.47 ± 0.22%. The MBG was found to be safe on the dermis and efficacious than the marketed product and hence, promises its utilization as a safe and efficacious formulation for the treatment of dermal infections.

Introduction

Colonies of bacterial and fungal communities have been observed on human skin while, for decades yet, are much undermined about host-fungus fraternization within the dermal layers. At this period, different fungal microbiota, including Malassezia, Cryptococcus, Trichophyton, and *Candida* species, have been identified as genus pathogen to human skin flora.[1] It is also discovered that under conditions of primary immune deficiency disorders, these fungi, particularly to the abundance of *Candida* species has widened its spread on the dermal microflora. Although fungus has been the part of commensal skin microbiota, various species are reported to be pathogenic to human skin. It has been estimated that 20–25% of the world population is affected by fungal skin infections.[2]

Non-invasive drug delivery systems like topically formulated systems provide alternative routes of administration and enhanced delivery of drugs to localized affected sites in the body surfaces and are considered safe in the management of antifungal infections.[3] Topical antifungals exploit the difference between the host cell and fungi microstructure to destroy the fungal organism mitigating the adverse effects on human dermal flora.[4] Current clinical regimen available for the treatment of superficial fungal infections includes azole antifungals like sertaconazole, luliconazole, butoconazole, etc.[5] The development of resistance against the antifungal agents used for superficial fungal infections has been a major issue since years and is yet a concern to be addressed.[6] Owing to the long duration of therapy, repeated dose of azole antifungals has led to the microbiological as well

***Corresponding Author:** Mr. Jeet V. Gandhi

Address: Kadi Sarva Vishwavidyalaya, Gandhinagar-382024, Gujarat, India

Email ✉**:** jeetgandhi18@gmail.com

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as clinical resistance, which is actually attributed to the genomic changes in the strains of *Candida albicans* widely occurring due to mutation in genes altering the molecular mechanisms at the micron scale.[7]

The other major concern that has led to a reduction in patient compliance of current therapeutic regimens are adverse events (burning, itching, stinging, redness, skin rash, and contact dermatitis) associated with them which propagates a need to explore the agents that can overcome the issue of resistance and provide the therapeutic benefit at an equivalent or better efficacy.[8]

The potential of molecules from plant sources as a medicinal moiety is still unexplored to its full potential.[9] Amongst the estimated five lac species of plants available, only some of them are investigated phytochemically and a fraction of the same is subjected to its pharmaceutical screening and biological evaluation, of which only a few have found its place in the market.[10] Extensive research has been carried out for a past decade on the chemical constituents of plants, which serves as an active moiety for the treatment of diseases. Different parts of plants like seeds, bulbs, roots, leaves, stem, bark, resins, fruits, buds, rhizomes, and essential oils (E. oils) are studied and explored as a source of medicinal agents. E. oils derived from parts of numerous phytomes have reported inheriting antifungal activity without any side effects reported in humans and animals.[11]

One such plant essential oil-based formulation is thyme oil derived from the leaves of thymus vulgaris. In literature there are various enlisted medicinal benefits of thyme oil such as its antispasmodic action provides relief in rheumatoid arthritis, also serves as carminative, diuretic, expectorant, emmenagogue stimulant, relieves anxiety, and provides soothing action and hence, is extensively used in aromatherapy.[12] Thyme oil is also reported to have excellent anti-microbial properties against a variety of bacteria and fungus. The present work aimed to the development of thyme oil-based microemulsion for treatment of fungal infections due to *candida* and *trichophyton* species. From various microbiological studies, it was found that thyme oil containing around 10–50% of thymol which, is a powerful antifungal agent. Yet, its functionality is not explored into a patient-friendly dosage form.[13]

Marina D. Sokovik *et al*. studied the chemical phyto-constituents of various species of thyme and Mentha species and antifungal species like *Candida* and *aspergillus*. The work encompasses the study of the chemical composition of essential oils extracted via distillation using gas chromatography–mass spectrometry (GCMS) technique, including analytical estimation of analytical components against their working standards and estimation of minimum inhibitory concentration (MIC) and medical follow-up clinic (MFC) of the oils by dilution assays. *Thymus Vulgaris* constituted 48.9% of thymol and 19.0% p-cymene while (*thymus tosevii*) constituted 12.8% carvacol and 10.4% thymol, whereas, both the species inherited a strong antifungal activity against all the species under test. The study concluded with the comparative evaluation of oils with existing fungicide bifonazole that showed the superiority of essential oils inactivity than the azole, indicating the appropriateness of essential oil of thyme as well as fungicide.[14]

Eugenia Pinto *et al*. studied the antifungal activity of *T. Pulegioides* against *Candida aspergillus*, and dermatophytic species. The thyme oil was analyzed using GCMS in which the thyme and carvacol content was found to be 26.0 and 21.0%, respectively. The oil was assessed for antifungal activity against seven strain isolates of *Candida*, five isolates of *aspergillus*, and five clinical *trichophyton* strains. Flow cyclometry studies of cell membrane integrity were performed to confirm the mechanism of antifungal action of the oil. Results demonstrated strong antifungal activity of dermatophytic strains mainly by disrupting the cell membrane forming lesions in them, leading to cell death and inhibition of ergosterol synthesis. The oil showed promising results and exhibited antifungal potential for future therapy.[15]

Patricia Pozzatti *et al*. studied *the in-vitro* activity of selected essential oils of *Cinnamomum zeylanicum, Lippia gravelons, Ocimum basilicum, Organum vulgarae, Rosemarimus officinalis, Salvia officinalis, T. vulgaris and Zingibers officinalis* against various variety of *Candida* like *C. albicans, C. glabrata, etc*. The chemical composition of essential oils was characterized using gas chromatography. The results showed all the essential oils inhabited different levels of antifungal property against *Candida* species, of which oregano, along with thyme, showed high antifungal potential. All the above essential oils exhibited better antifungal action against fluconazole-resistant *Candida* species. Hence, it could be derived that all E. oils can be explored as an antifungal against *candida* infections in future therapy.[16]

M. Sokovik *et al*. evaluated the antifungal activity of essential oil of thyme vulgaris L. and thymol on externally induced dermatophytosis caused by Trichophyton rubrum and *T. tonsuran*s. This in vivo evaluation of the toxicological and antifungal activity of thyme vulgaris essential oil and its main component thymol was evaluated on 2 months old Wistar rats by inducing dermatophytosis using *T. rubrum* and *T. tonsurans*. The therapeutic effectiveness of 1% of essential oil solution and thymol were evaluated against commercial preparation of bifonazole and the study duration was 37 days. The results of the experiment confined that essential oil of thyme *vulgaris* and thymol depicted very good antifungal activity against fungal species and superior antifungal activity against commercially available bifonazole preparation.[17]

Materials and Methods

Materials

Thyme oil was procured as a from Chemical International Pvt. Ltd., Mumbai, India. Tween 80 was procured from Sulab Chemicals Pvt. Ltd., Vadodara. Isopropyl alcohol (IPA) was obtained from Finar chemicals, India. Glycerin was obtained from Sulab chemicals Pvt. Ltd., Vadodara, India. Carbopol 934 was received as a gift sample from Lubrizol, India. The test organisms (*Candida albicans ATCC 10231*) samples were purchased from the microbial type culture collection and gene bank, Chandigarh, India.

Methods

Microbial Characterization of Oil

The MIC of oil was performed via broth dilution technique using Clinical and Laboratory Standards Institute (CLSI) guideline suggested by CDC, USA. Fresh culture of *Candida albicans* was used, and subculturing of colonies of *Candida* was done on sabouraud dextrose agar, and the plate was incubated for 24 hours at 30°C. From the colonies appeared on the subculture plate, a culture of *Candida albicans* containing 2.5×10^6. *Candida albicans* were isolated and transferred to 5 mL saline solution containing 0.5% tween 80 solution. The sabouraud dextrose broth was prepared and transferred to each flask and dilutions of thyme oil were prepared in concentrations of 1000 µg to 25000 µg for initial screening which was narrowed to 3500 to 4000 µg to obtain MIC of thyme oil. 2.5×10^6 Colonies of *C. albicans* were transferred to each flask and the readings in triplicate were noted after 48 hours using UV visible spectrophotometer (UV series 1800 , M/s Shimadzu, Kyoto, Japan).[18]

The zone of inhibition (ZOI) was measured using the agar well plate method. Subculturing of *Candida albicans (C. albicans ATCC 10231)* was done on saboraud dextrose media and plate was allowed to incubate at 30°C for 48 hours. The colonies containing 2.5×10^6 *C. albicans* were isolated and stricken on plates containing SDA media. The plate was allowed to solidify and bore of 6 mm was made at the center using a suitable borer and oil was transferred to the well in the concentration of 2 X MIC concentration were subjected to study in an incubated condition of 25°C ± 2°C and the results were noted after 48 hours.[18]

Preformulation Study of API (Thyme Oil)

The density of thyme was performed using a specific gravity bottle (Borosil, India). The solubility of thyme oil was tested in various solvents as per Indian Pharmacopeia vol. III. Solvents were added per mL of thyme oil and the solubility was determined accordingly. Abbes Refractometer (Bausch & Lomb, New York, USA) was used to determine the refractive index of thyme oil. Acid value, saponification value was performed as per the method described in IP 2014. Calibration curve of thyme oil was taken preparing various concentrations of thyme oil, i.e., 2, 4, 6, 8, and 10 mcg/mL in methanol at 274 nm in a UV visible spectrophotometer (UV series 1800, M/s Shimadzu, Kyoto, Japan). Drug excipient compatibility study was conducted by physically mixing drug and excipients in 1:1 ratio and subjecting them to 40°C/75% RH for one month and then compatibility was confirmed using FTIR of the samples. Tween 80 was selected as a surfactant and Isopropyl alcohol was chosen as cosurfactant.[19]

Preparation of Thyme Oil Loaded Microemulsion (TOME)

- *Pseudo-ternary Phase Diagrams for Thyme Oil Loaded Microemulsion (TOME)*

The TOME was prepared using tween 80 as a surfactant and IPA as co-surfactant using phase titration method in which the different combinations of surfactant- co-surfactant in different ratio referred to as S_{mix} ratio, oil, and water were utilized to examine the phase behaviour utilizing a simple titrimetric method. The ratio of oil with Smix were altered at 1:1, 1:2, 1:3, 1:4, 2:1, 3:1, and 4:1 to construct a total of 7 pseudo-ternary diagrams. The construction of the pseudo-ternary phase diagram for all the Smix ratio, the mixture with oil, and Smix were prepared with volume ratios that range between 9:1 and vice versa were performed. This mixture was titrated cautiously at droplets edge with water (via continuous agitation) at room temperature till the phase starts appearing turbid. The area of the ternary phase diagram with the maximum

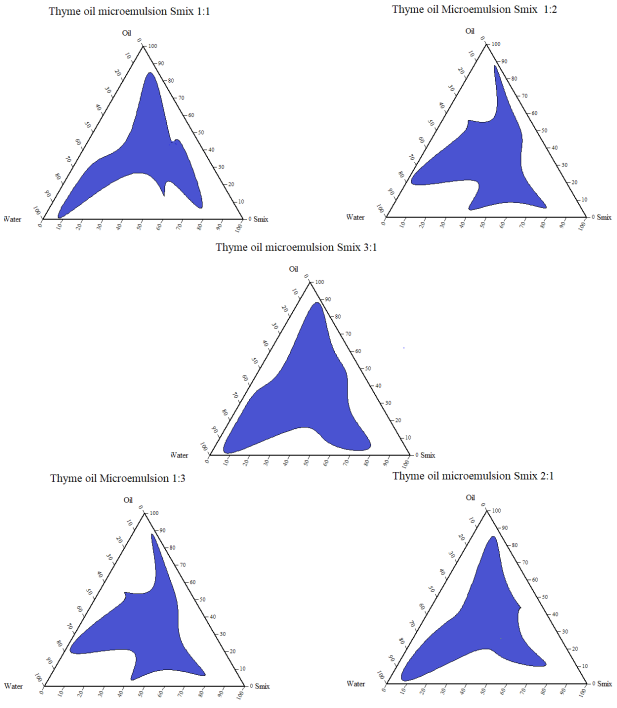


Fig. 1: Pseudo-ternary phase diagrams of thyme oil

coverage was considered as the suitable ratio of Smix, and evaluation of the optimized ratio of Smix was performed using CHEMIX software (Chemix ver. 1.1.1, India) as shown in Fig. 1.[20]

- *Optimization Using a Design of Experiment*

The D-optimal design using a Design Expert (V. 7.1, Stat Ease Inc., Minneapolis, USA) software was selected to optimize and prepare a thyme oil loaded microemulsion in which the three levels of the independent factors, i.e., oil (X_1), Smix (X_2), and water (X_3) were chosen based on pseudo ternary diagram. The range of components varied for the application of D-optimal design was X_1 from 0.3–0.9; X_2 between 5–15, and X_3 between 80–90. Hence, with the different combinations in common and 1 block design, 12 runs were obtained, including a single replicate run. The response selected were globule size, % drug retention, and % drug penetration of the drug from the skin. Table 1 below enlists of experimental runs with the responses.[21]

Variables of design of experiment (DoE) were evaluated via the software Design Expert 7.1 (V. 7.1, Stat-ease.inc, Minneapolis, USA), which fitted to a cubic model for all the three responses based on the Scheff's model. The polynomial equation was formed using the significant coefficients found by applying analysis of variance (ANOVA). The polynomial equation generated using the D-optimal design was represented in the form of the counterplots, and the optimized formulation was obtained using the polynomial regression equation or the counterplots and its superimposition called overlay plot, which helps to find best suitable formulation. A checkpoint was formulated to validate the correctness of design of experiment chosen.[21]

Preparation of Thyme Oil Microemulsion Based Gel

The microemulsion based gel was developed by the addition of carbopol to the optimized microemulsion, and addition of glycerine to the formulation was to provide the emoliency, humactancy and better texture properties to the gel formulation.

Evaluation of Microemulsion

A microemulsion is evaluated based on its physicochemical parameters like appearance, texture, odor, taste, transparency, globule size, PDI value, viscosity, and pH of a microemulsion. Appearance and texture were observed visually, while the transparency of the developed microemulsion was characterized using UV-visible spectrophotometer. A Malvern zeta-sizer obtained globule size and PDI value. The pH of microemulsion was measured using digital pH meter (Electroquip, India), while, and viscosity was obtained using Brookfield viscometer (Brookfield, USA) using LV 61 spindle. TEM was used to characterize the droplet size of microemulsion (IIT, Bombay).[22]

Evaluation of Thyme Oil Loaded Microemulsion Based Gel

- *Physicochemical Characterization of Thyme Oil Loaded Microemulsion Based Gel*

The physicochemical properties of thyme oil loaded microemulsion based gel (TOMBG) like appearance, odor, and the texture was characterized visually. The pH of TOMBG was characterized using a pH meter (Electroquip, India). Viscosity was measured using Brookfield viscometer (Brookfield CTS, USA), where 100 g of the gel was loaded in a beaker, and the spindle (S4) was plunged in the sample at 100 rpm, and the viscosity was recorded accordingly. The texture was visually observed and the spreadability test was performed by placing a weighed quantity of gel between two glass plates weighing 5 × 10 cm and measuring the distance of the spreading of the gel. The dilute ability test was performed to confirm the phase of emulsion as well as mark the phase separation, if any, was observed.

- *In-vitro Drug Release Study*

In-vitro % drug release was evaluated using a Franz diffusion apparatus (25 sq.cm.) containing a donor and a receiver compartment and a sampling port. The cellophane membrane (Thermo-fisher, USA) of 0.2 microns (which was soaked in acetate buffer pH 5.5 6 ours

Table 1: Experimental runs with results

Trial No.	*Oil*	*Smix*	*Water*	*Globule size (nm)*	*% drug permeation (%)*	*% drug retention (%)*
1	0.66	0.17	0.17	15.08 ± 0.05	16.38 ± 0.13	83.61 ± 0.17
2	1.00	0.00	0.00	18.77 ± 0.03	20.67 ± 0.17	79.36 ± 0.24
3	0.50	0.00	0.50	13.98 ± 0.04	15.71 ± 0.21	84.19 ± 0.19
4	0.16	0.17	0.67	13.83 ± 0.03	14.76 ± 0.18	85.24 ± 0.35
5	0.00	0.00	1.00	13.06 ± 0.04	13.68 ± 0.24	86.29 ± 0.21
6	0.50	0.50	0.00	13.65 ± 0.06	15.34 ± 0.31	84.67 ± 0.47
7	0.00	0.67	0.33	14.37 ± 0.04	14.73 ± 0.16	85.37± 0.23
8	1.00	0.00	0.00	18.71 ± 0.07	20.73 ± 0.19	79.24 ± 0.14
9	0.00	1.00	0.00	11.19 ± 0.03	16.1 ± 0.15	84.34 ± 0.20
10	0.34	0.33	0.33	14.19 ± 0.05	15.39 ± 0.27	84.59 ± 0.27
11	0.00	0.50	0.50	13.49 ± 0.04	15.12 ± 0.21	84.69 ± 0.35
12	0.17	0.67	0.16	12.93 ± 0.06	15.73 ± 0.11	84.19 ± 0.17

before the study) was placed on between the donor and receiver compartment and 1 g of sample was placed on the cellophane membrane which was exposed to the receptor component which contained the acetate buffer pH 5.5. A total of 1 mL of sample was performed at 15, 30, 60, 90, 120, and 180 minutes and the sink condition was maintained replacing the same of buffer in the receptor compartment and was assayed at 274 UV max using UV visible spectrophotometer (UV series 1800, M/s Shimadzu, Kyoto, Japan).[23]

- *Ex vivo Skin Permeation Study*[24]

Ex vivo skin permeation study was accomplished by using Franz diffusion apparatus. The fresh sample of skin excised from the Wistar albino rats weighing (150–250g) and the skin was made free from hair using a suitable hair removing applicator. The same was mounted above the receiver compartment and 1 g of TOMBG was applied on the skin. The receiver compartment comprised of media phosphate buffer pH 7.4. The sampling was performed at time intervals of 15, 30, 60,120, 180, 360, and 720 minutes and the sink condition was maintained, replacing the same buffer in the receptor compartment. The amount of drug transported from the donor compartment to the receptor compartment was estimated by UV visible spectrophotometer (UV series 1800, M/s Shimadzu, Kyoto, Japan) at 274 nm. Cumulative percentage, i.e., amount of thyme oil permeated through the skin, was obtained applying the below formula.

$$Q_n = C_n \times V_o + \sum_{i-1}^{n-1} C_i \times V_i$$

Where C_n is the drug concentration in the receptor medium at each sampling time, V_o is volume of receptor compartment, C_i is the concentration of drug at i^{th} sampling while V_i is sample volume. The flux values were calculated from the slope of the linear graph between the drug permeation per unit surface area versus time.

At the end of the study, the skin retained on the surface was estimated by scrapping the surface and dissolving it into the media and was estimated via a UV visible spectrophotometer (UV Ser. 1800, M/s Shimadzu, Kyoto, Japan) at 274 nm. Finally, the drug retained within the skin was found by tearing the skin into pieces macerating, and homogenizing the fraction, and analyzing it by UV visible spectrophotometer (UV ser.1800, M/s Shimadzu, Kyoto, Japan) UV_{max} 274 nm.

- *Drug Content*

The drug content of a TOMBG was measured by using UV visible spectrophotometer ((UV series 1800, M/s Shimadzu, Kyoto, Japan) at 274 nm, where the sample of formulation diluted in methanol against the drug substance as a standard compound. The absorbance was obtained spectrophotometrically, and the drug content was calculated on the equation mentioned below.

$$Cs * As = Cu * Au$$

Where Cu is a concentration of standard compound; As is the absorbance of the standard compound; Au is the absorbance of a compound under test, and Cs is the concentration of under test compound.

- *Antifungal Study Using Agar Plate Method*

The antifungal assay of the TOMBG was performed using an agar plate well method. The fresh culture of *Candida albicans ATCC 10321* is serially diluted to obtain $2.5 * 10^5$ CFU/mL of organisms and was poured in the sabouraud dextrose media. The plate was solidified hole of 6 mm was placed using a suitable borer and formulation weighing 1 g was added 1 g of DMSO and was poured into the well. The Petri plates were then incubated at 25°C for 48 hours. The zone of inhibition was measured after 48 hours and was compared with clotrimazole (1 g clotrimazole added to 1 g DMSO which was added to well) as a standard compound.

- *Skin Irritation Study*

Wistar rats that weigh about 150–250 g were chosen for the skin irritation histopathology study, whose hairs on the dorsal abdomen part were removed using a suitable hair remover clip. The skin surface was cleaned using saline solution via cotton piece 3 to 4 times and was allowed to dry. A 500 mg of microemulsion based gel was topically applied for 24 hours. Post 24 hours, the topically applied formulation was removed carefully, and then the animal was sacrificed with cervical dislocation; area of skin samples where the formulation was applied were then harvested, stained via dyes hematoxylin ink, and eosin, was then observed (under 10X) power in an optical microscope.

- *Stability Study*

The samples of thyme oil loaded MBG was subjected to stability in three climatic conditions, i.e., 25°C/60% RH, 30°C/75% Rhesus (RH), and 40°C/RH for 6 months. The samples were observed for parameters like appearance, liquefication, bleeding, drug content, pH of 10% dispersion, viscosity, and ZOI.

Results and Discussion

Excipients selection for thyme oil microemulsion was based on the solubility of the oil in surfactants and co-surfactants. Amongst all the chosen surfactants, Tween 80 showed the highest amount of miscibility (2 g/mL) then other surfactants like c EL, Span 20, Tween 20, Span 80 and Cremophor RH 40. Isopropyl alcohol was chosen as co-surfactant due to the highest solubility compared to all other co-surfactants like propylene glycol and polyethylene glycol 400. The thyme oil loaded microemulsion was clear, transparent colored pale yellow to yellow liquid with strong aromatic odor and neutral/ characteristic taste. The solubility of thyme oil is shown in Fig. 2.

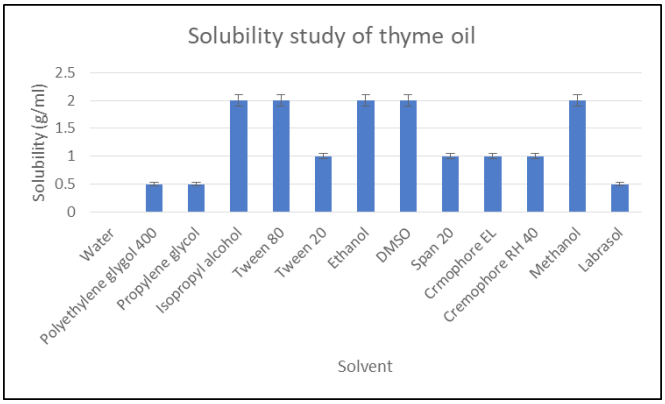


Fig.2: Solubility of thyme oil

The dilutions of 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg/mL of thyme essential oil was prepared of which 4 mg/mL of thyme oil showed complete inhibition at a transmittance of 98.7% against transmittance of reference (media with 0.5% Tween 80 solutions) observed to be 99.34%. Further dilutions of thyme oil (3.5, 3.6, 3.7, 3.8, 3.9, 4.0 mg/mL) were made to validate the MIC. The MIC of thyme oil was found to be 3.7 mg/mL. The zone of inhibition of oil at concentration twice of MIC was found to be 37 mm in diameter.

The density of thyme oil was found to be 0.914 g/mL. The refractive index of thyme oil was found to be 1.501°. The acid value and saponification value of thyme oil was found to be 2.645 and 35.79, respectively, which indicated that the oil was not rancid. The assay of thyme oil, i.e., the thymol compound, was found to be 32.01%. The calibration curve of thyme oil is shown in Fig. 3. The drug excipient compatibility study confirmed that there was no interaction between the oil and components of MBG that proves that the drug and excipients are compatible with each other.

Formulation Optimization

Table 1 showcases the set of 12 experimental runs suggested by the design of an experiment. The responses Y_1 was determined after the stabilization of microemulsion and Y_2 (% drug permeation) and Y_3 (% drug retention) were determined after the formation of microemulsion based gel. The counter plots for all the three responses for the factors are shown in Fig. 4. As it was evident from the results of the experimental runs that the least globule size was achieved with the lowest drug loading and a higher concentration of Smix, indicating the constriction of globule and its stabilization with the surfactant and cosurfactant mixture. This was supported by the work of Dr. Bhavesh Barot indicting the similar observation of oil and surfactant-cosurfactant mixture on globule size.[25] The maximum globule size achieved was 18.77 nm. Hence, the variation of globule size indicated the effect of independence on globule size of a microemulsion.

A similar observation can be derived from the experimental runs for dependent variables Y_2 (percentage drug permeation) and Y_3 (% drug retention). The higher is the amount of drug loading the higher is the amount of permeation observed. Similarly, the results of experimental runs also suggest that increase in the concentration of surfactant-cosurfactant mixture has improved the permeation of drugs in the skin layers.

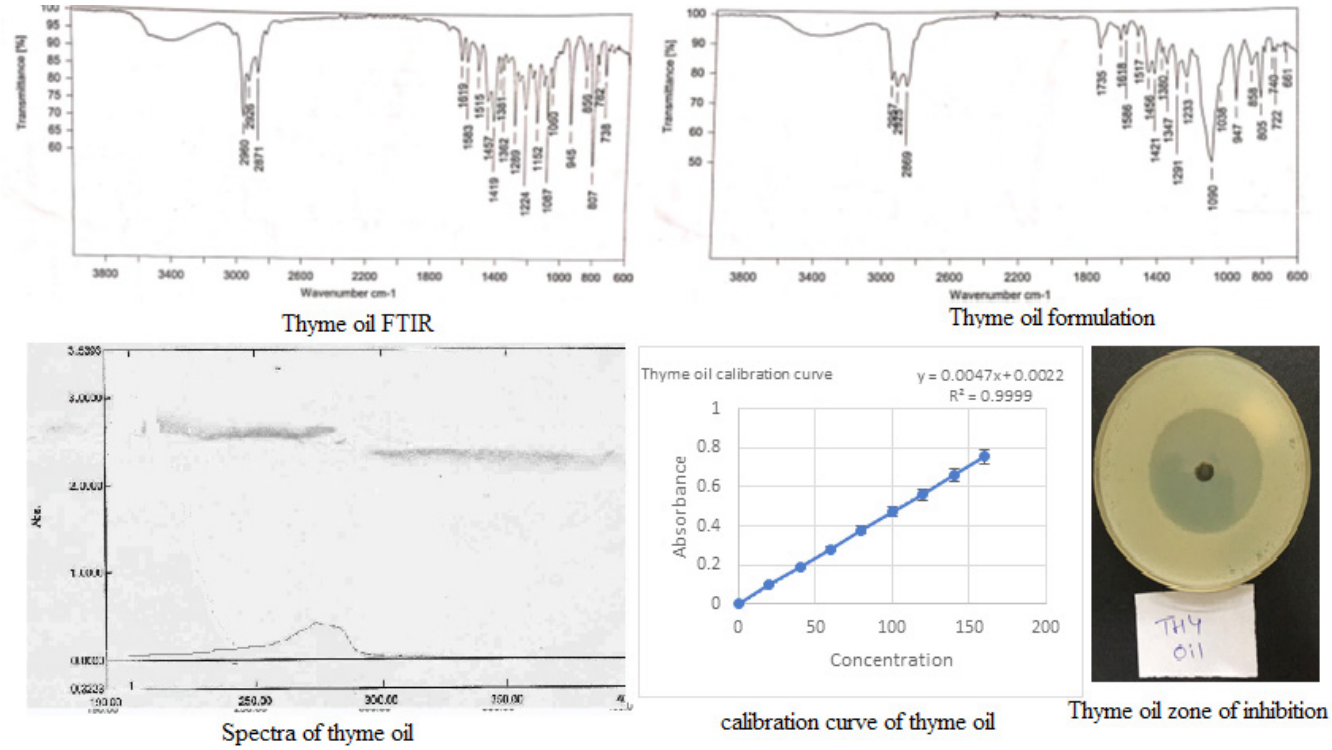


Fig. 3: Preformulation of thyme oil

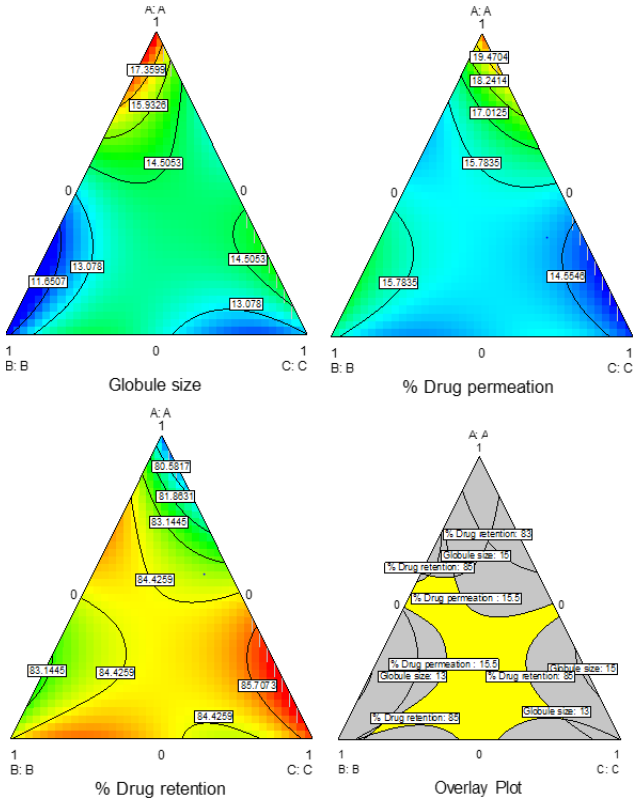


Fig. 4: Counter plots of D-optimal design for responses Y_1, Y_2 and Y_3 and overlay plot.

Along with the rise in the water concentration with the composition containing a higher amount of oil has rendered the permeation of the oil to a certain level. The vice versa effects have been observed for the variable Y3 indicating the effect of independent variables on the percentage drug retention.[26]

The relationship within the independent and the dependent variables was established by a special cubic model computed via statistical analysis provided by design expert software for the determination of appropriate microemulsion formulation. The equation that fitted the observations is described as below:

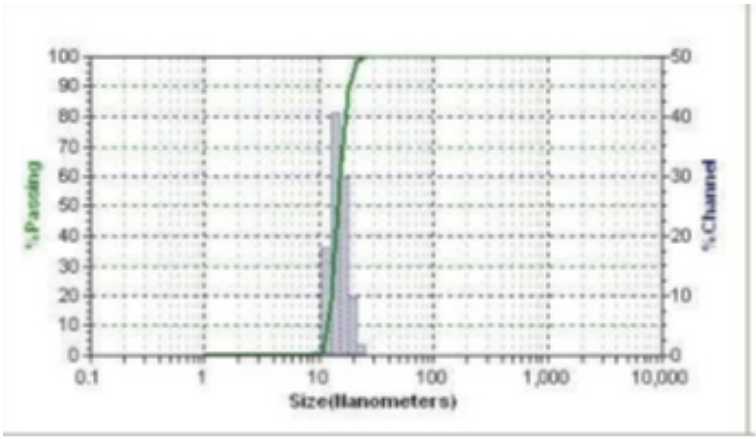
$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_1X_2 + \beta_5X_1X_3 + \beta_6X_2X_3 + \beta_7X_1X_2X_3 + \beta_8X_1X_2(X_1\text{-}X_2) + \beta_9X_1X_3(X_1\text{-}X_3) + \beta10X_2X_3(X_2\text{-}X_3)$$

Where β_1 to β_{10} are the coefficients obtained using DoE by the input of results achieved of independent variables performed practically. Coefficients with each independent variable in the equation indicate the effect of the particular factor, while the coefficients with more than one factor represent the synergistic effect within those factors. A positive sign denoted to the terms indicates synergistic effects, while the negative sign represents the antagonistic effect of the factors. Table 2 represents the model summary statistics of the dependent variables. The special cubic model shows an appropriate fit, as evident by the high R^2 and lowest PRESS values of each of the dependent factors. The p-value of all the responses was found significant (< 0.05), and the coefficient of the regression equation can be used to calculate the predicted values for formulation within design space.[24]

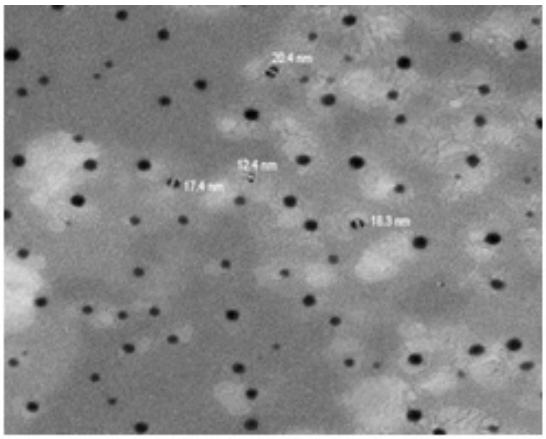
The polynomial regression equation describes interactions within the factors and its effect on dependent variables. Interaction of oil and Smix with respect to globule size is denoted as negative, indicating reduction of globule size with rise in the oil and Smix, which can be due to constriction oil globules in the presence of surfactants. The interaction of water and oil globule is negative, which contraindicates the conventional belief, but this can be attributed to an extremely low concentration of oil in comparison to water, that may constrict the globules to break apart due to collisions and repulsive forces between water and oil globules. These findings are supported by Patel *et al.*, where they have reported a reduction in the

Table 2: Model summary statistics

Coefficient code	*Y1*	*Y2*	*Y3*
Model suggested	Cubic	Cubic	Cubic
β1	18.74	20.70	79.30
β2	11.18	16.10	84.34
β3	13.05	13.68	86.29
β4	-5.29	-12.25	11.40
β5	-7.71	-5.93	5.58
β6	5.43	0.91	-2.50
β7	17.53	12.60	-8.67
β8	15.89	-18.36	23.24
β9	-23.16	7.25	-11.59
β10	18.18	10.23	12.40
R square	0.9998	1.000	0.9999
Adj. R-sq.	0.9989	0.9998	0.9993
SD	0.073	0.033	0.060
Mean	14.44	16.20	83.82
PRESS	31.28	1.34	0.34
% C.V.	0.50%	0.20%	0.072%



Globule size of TOME



TEM of TOME

Fig. 5: Globule size distribution and TEM of thyme oil

size of oil globules in the presence of water in the study of localized delivery of clobetasone propionate loaded MBG inefficacious remedy for vitiligo. The interaction of Smix and water is attributed to have the negative effect indicating the reduction in the size of oil globules.[27]

The interaction of independent variables on the drug permeation is described as follows. The interaction of oil and Smix was negative that leads to the reduction in permeation when oil and Smix are to be combined, which may be due to the reduced concentration oil and dominance of hydrophilicity due to amphiphilic surfactant. The interaction of water and Smix possess negative effect of permeation which can be attributed to an excess of water concentration that hinders the permeation of drug in the layers of skin. The interaction of Smix and water is positive, indicating the increase in permeation of drugs with increasing concentration of Smix and water. This is supported by the work by Ms. Poonam Negi on a water-based gel for enhanced delivery of lidocaine and prilocaine.[24] The vice versa effect of the interaction of independent variables on responses is observed with drug retention. The final formulation of thyme oil-based microemulsion contains 0.82% of oil, 9.22% of Smix, and 89.95% of water.

Characterization of Preparation of Thyme Oil Loaded Microemulsion (TOME) and Thyme Oil Microemulsion Based Gel (MBG)

The prepared thyme oil microemulsion was pale-yellow to faint amber-yellow colored liquid with percentage transmittance of 97.80%, which indicates the transparency of the microemulsion. The microemulsion had a zeta potential of -0.69 mV with a poly dispersibility index of 0.00143 indicates the formation of a stable microemulsion. The microemulsion had a conductivity of 114.96 µm/sec, indicating the polar nature of oil in water emulsion. The pH of thyme oil loaded microemulsion was noted to be 5.98 possessing the isotonicity with the skin pH, ensuring the non-irritant nature of microemulsion. The TEM of thyme oil loaded microemulsion depicting its globule morphology and size is shown in Fig. 5. The globule size of the formulation was 14.23 ± 0.3 nm. The globule size distribution of TOME is shown in Fig. 5.

The thyme oil loaded microemulsion based gel was pale yellow to amber gel with strong, pungent aromatic odor, and smooth texture. The pH of 10% w/v thyme oil loaded MBG was 6.03 measured by digital pH meter (Electro quip, India), indicating its isotonicity with skin pH. The viscosity of the TOMBG was viscous enough to extrude out of the tube and retain its shape on thixotropy value as 11.47 m.pas.sec^{-1} measured using Digital Brookfield Rheometer (Brookfield, U.S.A). The oil in water nature of microemulsion based gel was confirmed by dilution test. The texture properties of TOMBG, i.e., spreadability, was measured by applying the gel between glass plates to measure its spreading area. The spreading area was measured to be 4 X 5 cm for 100 mg of MBG, which was found similar to aloe-vera gel available in the market, which was observed to be 4 X 4 cm indicating an easily spreadable microemulsion based gel.

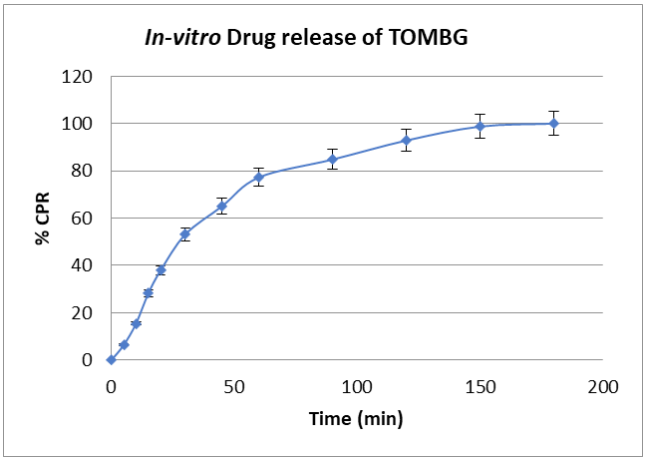


Fig. 6: *In vitro* drug release study of TOMBG

The *in vitro* drug release was performed using cellulose acetate membrane (pore size 0.22 microns, GE Healthcare, India) in acetate buffer pH 5.5 is shown in Fig. 6. And the % drug release from the formulation was 100.01 ± 0.22%. The drug content UV visible spectrophotometer found to be 102.48 ± 0.18%. The *ex-vivo* skin permeation study was conducted to observe the percentage of drug retained within the skin layers, i.e., at the site of infection where the permeation through the skin was found to be 15.53% ± 0.22%. The flux was found to be 7.19×10^{-3} mcg.cm^{-2}.hour^{-1}. The percentage of drug retained in the macerated skin fraction was found to be 58.47 ± 0.22%. The percentage of drug that found on the skin surface measured via scrapping the surface was found to be 26.32 ± 0.26% as shown in Fig. 7.

The thyme oil loaded microemulsion based gel was compared with clobet gel containing (1% clotrimazole) as a reference antifungal formulation for its efficacy testing and the results obtained supported the better efficacy of thyme oil loaded MBG compared to a marketed product as the ZOI of TOMBG was observed to be 24 mm whereas, of marketed product was found to be 16 mm as shown in Fig. 8. Also, microscopic observations of skin samples suggest no sign of irritation, observed in Fig. 9 suggested that TOMBG is non-irritant to the skin. Also, the stability study indicated the thyme oil loaded microemulsion based gel was stable in all aspects till a time span of 6 months, shown in Fig. 10. Hence, it can be derived that TOMBG is safe and efficacious formulation.

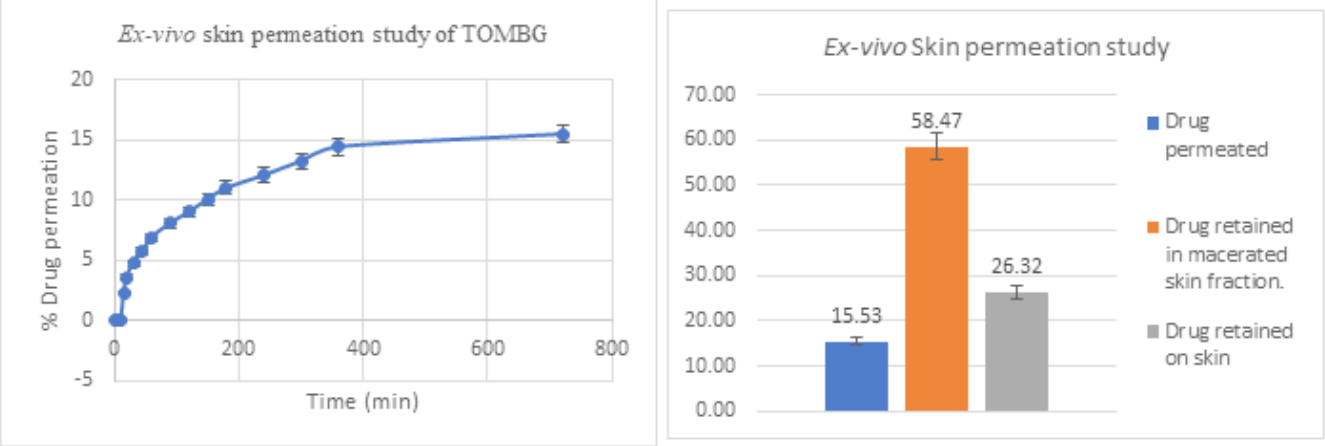
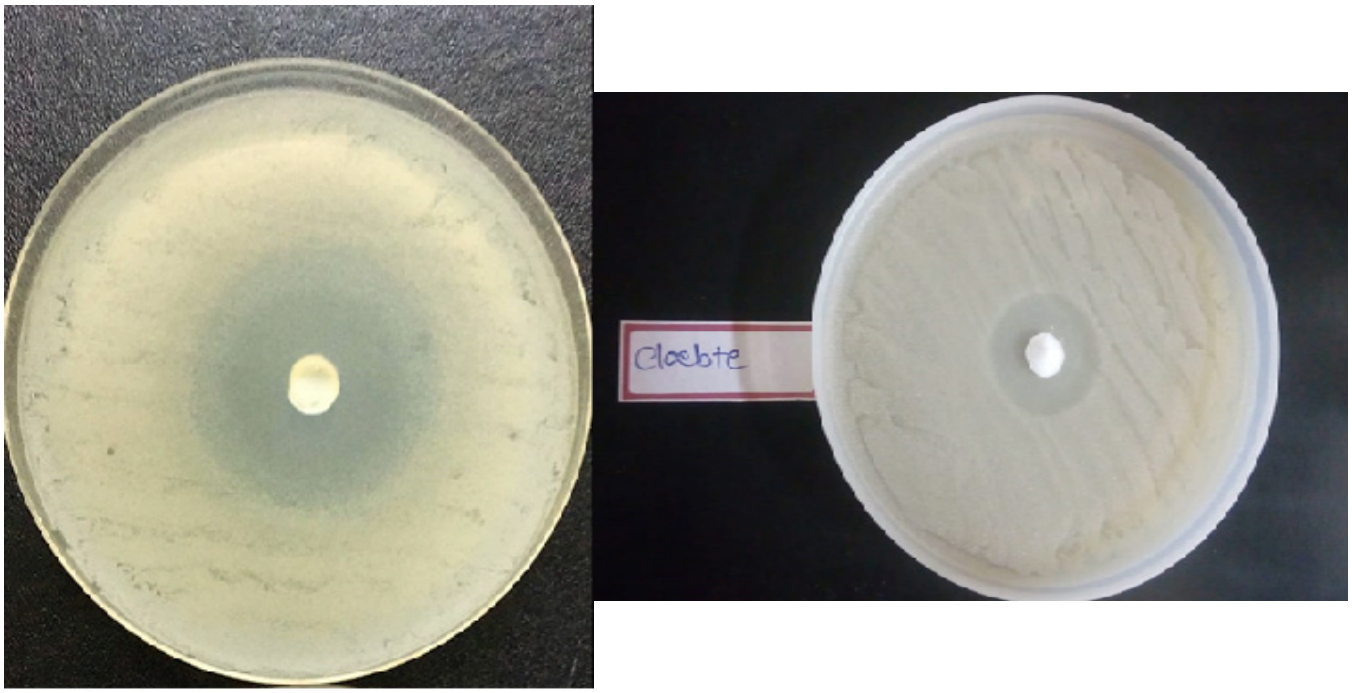


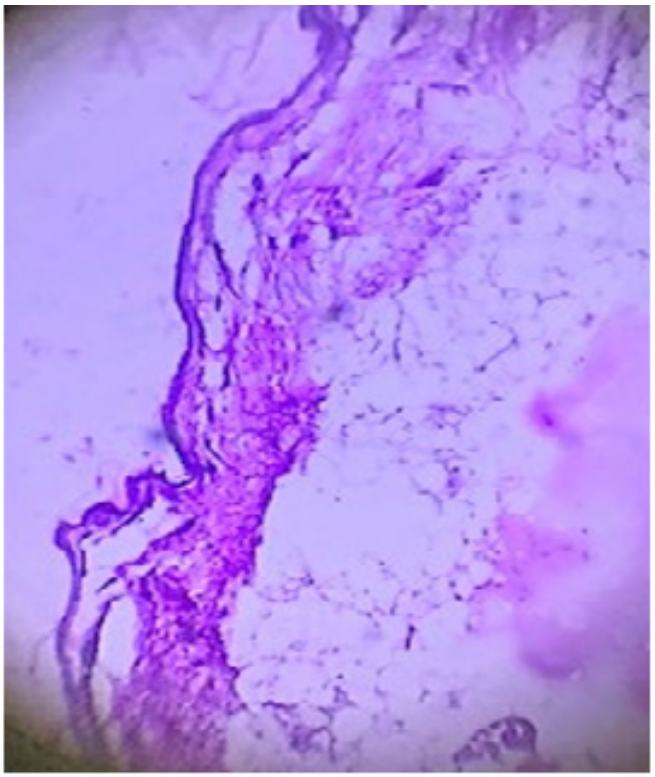
Fig. 7: *Ex vivo* skin permeation study of TOMBG



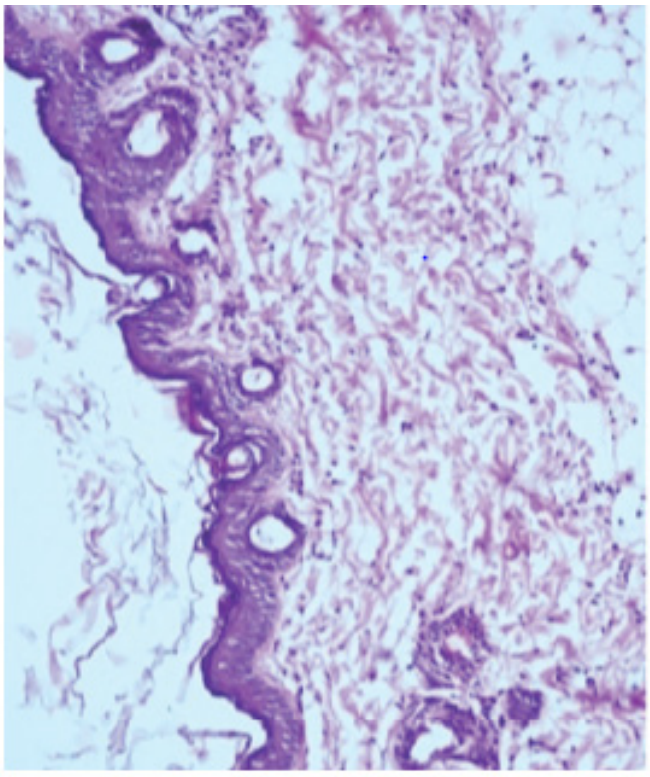
Zone of inhition of TOMBG

Zone of inhibition of cloebf gel

Fig. 8: Zone of inhibition of TOMBG and clobet gel (marketed product)



Control sample



Thyme oil loaded MBG

Fig. 9: Skin irritation study of TOMBG

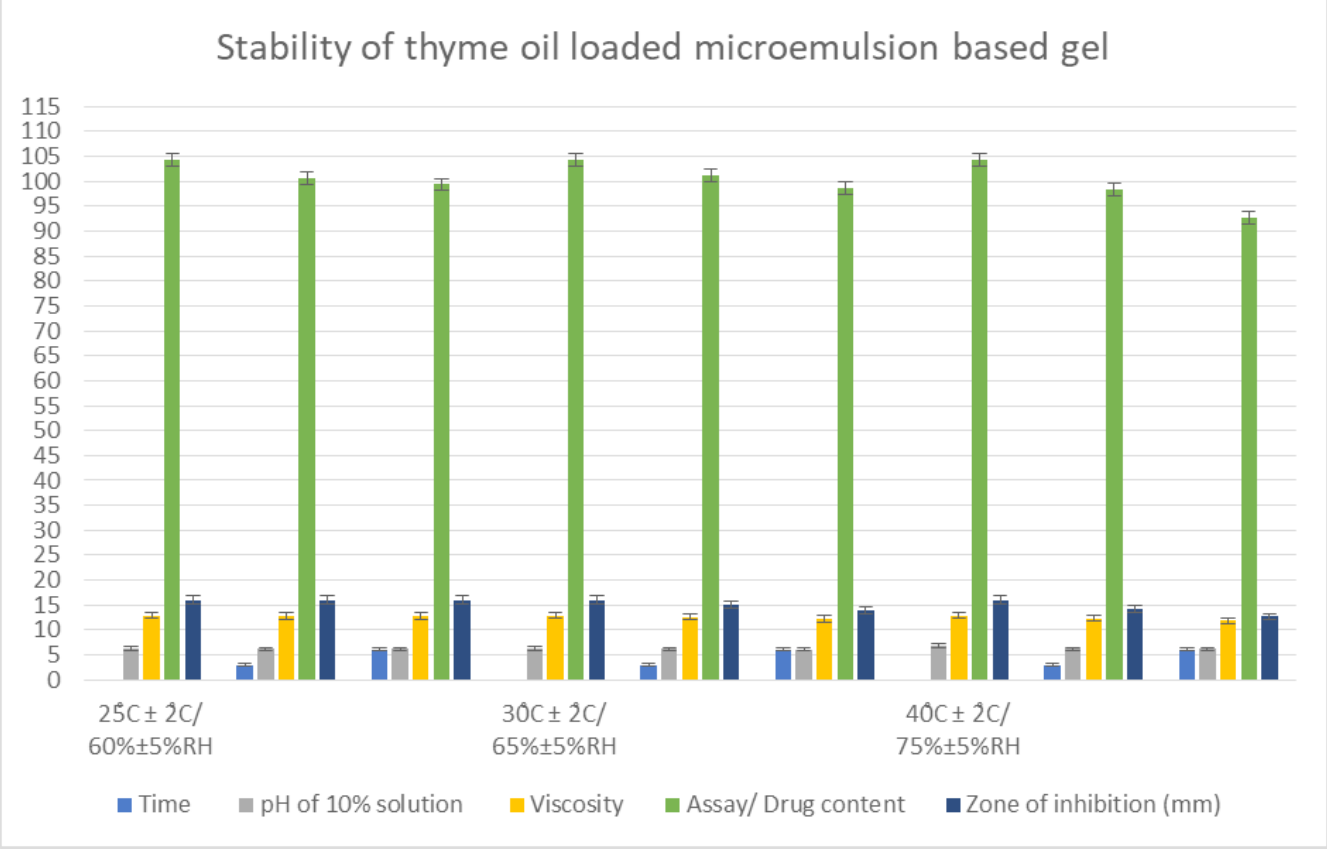


Fig. 10: Stability study of TOMBG