

# Fabrication, Validation, and Stability Analysis of *Melaleuca alternifolia* Oil-in-water Microemulsion for Improved Transdermal Application

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## Abstract

**Aim:** The aim of the study was to develop oil-in-water microemulsion (ME) system for *Melaleuca alternifolia* for targeting skin disorders. **Materials and Methods:** The formulation of ME system was done through the water titration method, which involved various oils, water, surfactants, and cosurfactants. The existence zones of ME were determined by constructing ternary phase diagrams, where surfactants and cosurfactants ( $S_{mix}$ ) were taken in different ratios along with different combinations of oil and  $S_{mix}$ . Quantitative estimation of tea tree oil (TTO) was performed using gas chromatography-mass spectrometry followed by total terpenoid estimation, and thereafter, solubility studies of TTO were performed using various excipients. TTO-ME was fabricated and characterized using modern characterization techniques (DLS, transmission electron microscopy, and Fourier transform infrared) along with statistical validation and *in vitro* drug release assessment. **Results and Discussion:** The optimized TTO loaded MEs (TTO ME) showed the particle size range between 32 and 160 nm with poly dispersibility index of 0.297 and zeta potential of  $-3.19$  mv with almost spherical morphology. The optimized formulation showed the sustained release of the therapeutic compound and was found to be stable at room temperature for 1 year, suggesting being a promising formulation against dermal infections. **Conclusion:** TTO was successfully loaded in thermodynamically stable ME system which enhanced its permeation through the transdermal route. Therefore, a novel drug delivery system was introduced, which can be studied on various *in vitro* and *in vivo* models further to establish its better therapeutic efficacy for various skin disorders.

**Key words:** Particle size, permeability kinetics, rheology, stability, therapeutic index

## INTRODUCTION

The use of essential oils (EOs) as therapeutics is considered as an important medicinal practice in most of the ancient civilization till present, as it indistinctly intertwines the religious and the therapeutic roles.<sup>[1]</sup> Similarly, Chinese traditional medicinal system considered EOs as the medicine which not only benefits the health but also governs the consciousness of the human mind too, with immense effects on the deepest level of the body that enhances immunity.<sup>[2]</sup> Their therapeutic benefits widely range from being a potential antifungal, antimicrobial, antidepressant, antiviral, and antiseptic agents to the elevator of many central nervous system related behaviors such as anxiety, learning, memory, attention, arousal, relaxation, sedation, and sleep. Furthermore, the effects on mood, pain, and

perception too are being influenced by the use of EOs along with giving calming and soothing effects to the nerve cells, thus helping in the treatment of epilepsy, stress, and dementia too. They are the low molecular weight hydrophobic liquids, extracted usually from plants which are rich in bioactive phytochemicals exhibiting various therapeutic properties.<sup>[3]</sup> Due to their chemical structures, EOs are listed under the class of highly volatile compounds exhibiting higher concentration of natural constituents and exclusive

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aromatherapy characteristics rich secondary metabolites ranging from antimicrobial, antioxidant, antiseptic, anti-inflammatory, and antidepressants.<sup>[4,5]</sup>

One such EO with immense therapeutic benefits, extracted from the leaves and twigs of native Australian shrub *Melaleuca alternifolia* is tea tree oil (TTO).<sup>[6,7]</sup> It is specifically used in alleviating inflammation and promotes wound healing along with enormous antimicrobial and antioxidant properties that help in treating deep-rooted infections of the skin including eczema, acne, and ringworm.<sup>[8,9]</sup> TTO has a well-defined composition and contains 40.1% of terpinene-4-ol, 23% of  $\gamma$ -terpinene, 10.4% of  $\alpha$ -terpinene, and 5.1% of 1,8-cineole as the major phytoconstituents and 3.1% of terpinolene, 2.9% of  $p$ -Cymene, 2.6% of  $\alpha$ -Pinene, 2.4% of  $\alpha$ -terpineol, and 1% of limonene as the minor ones.<sup>[6,10]</sup> It was quite interesting to know that EOs have higher abilities to penetrate easily into the dermal layers by three different dermal routes, that is, either by trans follicular, transcellular or intercellular route.<sup>[11]</sup> Although most of the EOs, including TTO, exhibit highly valuable therapeutic solutions, their efficacy is equally limited due to their certain physiological factors.<sup>[12]</sup> These factors are directly related with their structural arrangements, where most of the EOs have rich content of hydrocarbons, especially the terpenes including monoterpenes, diterpenes and sesquiterpenes, and oxygenated compounds such as carbonyls and alcohols which tends to be highly volatile and other phytoconstituents such as 1–8 cineole, reported to cause severe side effects such as redness, itching, inflammation, and rashes, if applied directly.<sup>[13,14]</sup> Thus, their direct application on skin limits their required therapeutic effect and effective resident period on skin. They are also reported to be chemically sensitivity; hence, they got decomposed easily by direct exposure to heat, light, humidity, and oxygen.

Pertaining to all the listed concerns and to enhance the overall therapeutic efficacy of *M. alternifolia* an efficient and biodegradable nanocarrier system was required since long that would eventually support in overcoming the problem of volatility, bioavailability and increase its effective resident time at the target site (skin).<sup>[15,16]</sup> However, various nano-sized carrier systems are used such as microemulsions (MEs), nanoemulsions, and nanoparticles for transdermal drug delivery. Although these carrier systems have their unique properties out of them all, MEs are reported to be the most efficacious and stable formulation for targeting the skin infections.<sup>[17]</sup> MEs are specifically noted to enhance the deeper impregnation of the EO's inside the skin, and they usually show the sustained compound release character leading to improved shelf life, and bioavailability.<sup>[18]</sup> Furthermore, ME loaded formulations show a higher degree of oil penetration into deeper dermal layers apart from showing higher spreading ability, impregnation, hydration, and skin flux.<sup>[19]</sup>

MEs are known to play a crucial role in treating skin disorders such as acne, fungal infections, psoriasis, eczema, and athlete foot as its well-defined characteristics, thermodynamic

stability, longer shelf life makes it an ideal candidate for various therapeutic application against these skin conditions.<sup>[20]</sup> Apart from this, they do exhibit a higher degree of transdermal drug delivery and enhances skin penetration due to their nanometric size range and surface polarity to seep deep down to give the desired effect.<sup>[21]</sup> Moreover, ME system altogether with its excipients is reported to reflect its antimicrobial, antioxidant properties including a synergistic effect with the drug, i.e. TTO in eliminating various skin ailments.<sup>[22,23]</sup> Therefore, in the present study, *M. alternifolia* loaded oil-in-water (O/W) ME system was designed to enhance its stability and therapeutic efficiency.

## MATERIALS

For the formulation pure *M. alternifolia* oil was purchased from Forest Glen Organics, Sydney, Australia, Eucalyptus oil from Oriental Traders, Karnataka, India. Other oils such as clove, olive, castor oils, and along with ethanol and methanol were purchased from HiMedia, India, whereas linseed oil and isopropyl myristate (IPM), sodium carbonate, Tween-20, Tween-80, span-80, span-20, and PEG-400 were procured by Sigma-Aldrich Ltd., USA. All the other chemicals used in the experiment were of analytical grade.

## METHODS

### Quantitative analysis of *M. alternifolia*

The *M. alternifolia* is known to possess a high content of terpenes including monoterpenes, diterpenes, sesquiterpenes, and oxygenated compounds such as carbonyls, alcohols, and phenols which tend to be highly volatile and other phytoconstituents like 1–8 cineole. Therefore, to determine the total quantity present in the standard extract of *M. alternifolia* oil, quantitative analysis for the same was done by both gas chromatography-mass spectrometry (GC-MS) and terpenoid estimation method.

### Quantitative estimation of *M. alternifolia* oil by GC-MS

Quantification and identification of major components of TTO were performed by GC-MS which is known to be a rapid, well-marked and most accurate quantitative determination method reported for yield and quality estimation of Melaleuca oils.<sup>[24]</sup> As reported in many studies the estimation of *M. alternifolia* exhibits a high percentage of monoterpene group, specifically by terpinene-4-ol (~48%), neo-dihydrocarveol (~7%), and 1,8-cineole (~6%). The stock sample preparation was done by mixing 1% (v/v) of TTO in acetone and injected (1 $\mu$ L) to GC-MS unit (GC-6890N with a mass selective detector 5973 (quadrupole mass analyzer) from Agilent Technologies (Thailand) Co., Ltd. Bangkok,

(Thailand). The column used for analysis was HP5-MS (0.25 mm  $\times$  30 m  $\times$  0.25  $\mu$ m). The run time of samples was estimated to be for 23 min at temperature variation of the oven between 40 and 250°C with helium as carrier gas.<sup>[25]</sup> Thereafter, GC-MS chromatogram is obtained after the run.

### Total terpenoid estimation

Terpenoids are a huge family of terpenes, diterpenes, and sesquiterpenes which are the most numerous and structurally diverse, naturally occurring phytochemicals exhibiting pharmacological activities. The presence of the total terpenoid content in the TTO was determined by the spectrophotometric method. Linalool was taken as standard and its different concentrations (10–50  $\mu$ l/ml) were prepared in methanol.<sup>[26]</sup> Then, 100  $\mu$ l of the test sample or the standard was mixed with 1.5 ml of chloroform and vortexed at 1792 g for 5 min properly. Thereafter, 100  $\mu$ l of sulfuric acid was added and incubated in the dark for 5 min at retention time (RT). The reddish-brown precipitate was formed which was further dissolved by 1.5 ml of 95% (V/V) methanol completely, and the absorbance was taken at 538 nm. Samples were analyzed in triplicates.

### Solubility studies of TTO in various excipients

Since the major therapeutic class of phytochemicals found in TTO is terpenoids such as – terpinen-4-ol,  $\alpha$ -terpinene, terpinolene,  $\alpha$ -Terpineol, and many more. Thus, suitable excipients were selected based on the highest terpenoid concentration.<sup>[27]</sup> The solubility of TTO was examined in different oils (eucalyptus oil, clove oil, olive oil, castor oil and linseed oil, and IPM), surfactants (Tween-20, Tween-80, span-80, span-20, and PEG-400), and cosurfactant (ethanol and methanol) by dissolving an excess amount of the TTO (1:1) in each of the selected oils, surfactants, and cosurfactants in a test tube and vortexed the same at room temperature in a shaker for 72 h to get to the equilibrium.<sup>[28]</sup> The equilibrated samples were then removed after 72 h, centrifuged 1008 g for 15 min and quantified spectrophotometrically at 538 nm. Thereafter, the excipients with highest terpenoid quantity were chosen for further fabrication of ME system.<sup>[29]</sup>

### Fabrication and optimization of TTO-ME (TTO loaded ME system)

After examining the solubility index of various excipients, their hydrophilic-lipophilic balance (HLB) system was also considered to attain a stable formulation. As marked in various studies that HLB system of ME excipients plays an important role in stabilizing and balancing the net blending of the same.<sup>[6]</sup> The emulsifiers that possess a large number of hydrophilic groups are suggested to have higher HLB values, displaying their hydrophilic affinity.<sup>[30]</sup> Similarly, the ones that have a lower count of hydrophilic groups, indicate

their lower HLB values; thus, imparting lipophilic character. Furthermore, the optimization and validation of different excipient blends was done to attain homogenous and clear ME colloidal suspension that ranges between the HLB value of 9-17. While selecting the excipients, it was made sure to select the ones with HLB lower than 10 and the surfactant selected must be above 12 to get an appropriate O/W ME system.<sup>[31]</sup> Then, these selected excipients (oil and  $S_{mix}$ ) were blended together by aqueous titration method by varying the surfactant and cosurfactant ratios ( $S_{mix}$ ) ranging from 1:0 to 6:1 and later optimizing the concentration of oil and aqueous phase with  $S_{mix}$  in variable ratios. The formulations were looked up to obtain clear titration zones. After the titration studies in all the above-mentioned ratios, the combinations which reflected the highest transparency with no phase separation and frothing were further plotted on pseudo ternary phase diagram to understand its phase behavior.

### Pseudoternary phase plotting

ME system topology is associated with the very low interfacial tension between the two-phase amphiphile microstructure layer (oil and water), which is equilibrated by surfactant and cosurfactants in between. Hence, to study the same phenomenon and to optimize the ratios between the  $S_{mix}$ , Oil: $S_{mix}$  and amount of aqueous medium, phase titration studies were done using the water titration method.<sup>[32]</sup> Ternary phase diagram was plotted with the help of Triplot-4.1 software, which provides an insight into the behavior and compositional structure of ME constituents. Phase behavior of ME excipients (Oil,  $S_{mix}$ , and aqueous phase) can be studied with each edge of the diagram representing 100% existence of the respective constituents. After plotting the obtained results on the ternary plot, determination of existence zone of the ME region was done.

### Characterization of microemulsions

#### Thermodynamic stability

The clear selected formulations after aqueous titration blending were further tested for their thermodynamic stability by subjecting them for the heating cooling cycle, centrifugation test (CFT), freeze-thaw cycle (FTC), and dispersibility testing (DT).<sup>[33]</sup> These testings were performed to observe any kind of phase separation, turbidity or precipitation, phase inversion or creaming in the TTO-ME samples during these experiments. In heating-cooling cycle (HCC) cycle, the selected formulations were exposed to variable temperatures such as 4°C–45°C for 48 h at each temperature, alternatively for six cycles.<sup>[34]</sup> Then, during CFT, the formulations were centrifuged at 1792 g for 30 min. Then, they were followed by FTC where formulations were subjected to –21°C–+25°C again for 48 h at each temperature. Finally, the selected microemulsions were dispersed in excess of aqueous medium (25 ml) to ensure its dispersibility ability.

### Physiological parameter evaluation

#### pH

The pH value of any sample represents the value of hydrogen ion existence in solutions, representing the logarithmic reciprocation of hydrogen ion concentration in dispersed samples.<sup>[35]</sup> The pH of all the selected TTO loaded ME combination was determined using a pH meter (WTW A061414035) at room temperature.

#### Viscosity

Viscosity is the measure of fluid friction or the internal friction of the fluid layer caused due to the movement of one on the other layer.<sup>[36]</sup> To follow the viscous behavior of the MEs, flow time was measured for drug-loaded MEs.<sup>[34]</sup> Hence, the viscosity of the sample was determined by the use of a Brookfield viscometer and measuring the time for fall of the liquid in the viscometer.

#### Density

Similarly, the density of the formulated ME was determined by Jammy *et al.*<sup>[37]</sup> using specific gravity or pycnometer bottle, and the final density was calculated by:

$$\text{Density} = \frac{\text{Weight (g)}}{\text{Volume (ml)}}$$

#### Conductivity

Electrical conductivity of the colloidal solution is a measure of the charge present on the dispersed particles in relation to its dispersion medium which was analyzed by the conductometer (CM 180, Elico, India) at constant frequency of 1 Hz by recording the charge on the optimized TTO loaded ME formulation in microsiemens (mS).

#### Optical transparency

The optical transparency of the TTO loaded ME was determined by inspecting the sample in clear and transparent container under the presence of good light against reflection into the eyes and viewed against black and white illuminated background at room temperature. The homogeneity and optical isotropy of pure and TTO loaded microemulsions were then examined by a polarimeter (ATAGO, AP-100 Automatic Polarimeter).

#### Surface tension

Surface tension is the rigidity of the surface of an emulsion caused by the attraction of the particles in the surface layer, which tends to minimize the surface area.<sup>[38]</sup> The measurements were made at  $25 \pm 0.01^\circ\text{C}$  under atmospheric pressure by Torsion Balance (White Elec. Inst. Co. Ltd.) equipped with a ring having a circumference of 4.0 cm.

#### Particle size (PS) analysis, polydispersibility index (PDI), and zeta potential (ZP) analysis

PS analysis of dispersed droplets of the colloidal solution gives an average of all the PS.<sup>[39]</sup> It is done to project the

approximate size of the formulated ME and their homogeneity in the system. PS analysis of the optimized freshly prepared diluted sample (1:50) was determined by the by photon correlation spectroscopy and ZP of the same sample was analyzed by laser Doppler anemometry, using a Malvern Zetasizer® 3000 HS. ZP analysis is done to incur the surface charge on the formulated ME.<sup>[40]</sup>

#### Transmission electron microscopy (TEM)

TEM is used to analyze and validate the morphological and structural framework of the colloidal suspension. It is used to generate the image of the sample to determine the actual size of the ME. The basic microstructure of the optimized formulation was analyzed using TECHNAI 200 Kv TEM (Fei, Electron Optics). The sample was prepared by diluting it with water in 1:100 ratios and then a drop from the same sample was placed on a pure thin bar mesh TEM grid with 2% phosphotungstic acid. The drop was blotted with filter paper until reduced to a thin film.

#### Fourier transform infrared (FT-IR) analysis

The FT-IR analysis is performed to determine the functional group present on the surface of the colloidal suspension.<sup>[25]</sup> FT-IR analysis of the optimized ME (TTO ME), TTO, and only ME was done through Perkin Elmer spectrum 1 FT-IR system. The samples were mixed thoroughly with potassium bromide (KBr), which is the commonly used alkali halide to make KBr pellets for scanning in the grid.<sup>[41]</sup> The frequency ranges were measured as wave numbers typically over the range from 4000 to 600  $\text{cm}^{-1}$ .

#### Statistical analysis

The PS analysis results of formulated ME samples were further subjected for correlation analysis and were used to study the correlation between PS and PDI with the content of O (oil), S (surfactant), and W (water).<sup>[42]</sup>

#### Permeability kinetics

*In vitro* drug release assessment assumes greater significance in serving as an indicator of product quality and performance as it is used to investigate and establish product behavior during the various stages of drug product development, as well as life cycle management providing the pattern of compound release and behavior of dosage forms, product quality, and performance.<sup>[43,44]</sup> Thus, to predict the pattern of drug permeation through dialysis membrane from TTO and TTO-ME by Franz diffusion assembly was done. The released samples of TTO and TTO – ME (1 ml) in the receptor compartment were collected after every 30 min interval till 12 h. The concentration of terpinene-4-olin both (TTO and TTO-ME) samples were further analyzed by GC-MS method and then the percentage of drug release was calculated by:

$$\text{Cumulative \% release} = \frac{A_{TS} * P(RT-1) + PT}{T_V}$$

Where,  $A_{TS}$  = Total volume of test sample withdrawn from the receptor compartment (1 ml);  $P(RT-1)$  = The percentage release of TTO prior to time “T”;  $PT$  = The percentage release of TTO at time “T”;  $T_V$  = Total volume of buffer in receptor cell (12 ml).

### Stability testing of TTO-ME

The stability testing of TTO and TTO-ME was performed by the method as reported by Singh *et al.*, 2017.<sup>[45]</sup> Since the retention of the essential terpenoid group of TTO was important for maintaining the therapeutic efficacy of the formulation; hence, the optimized formulation (TTO-ME) was tested initially after formulation and then after storing it at room temperature (37°C) for 1 year by terpenoid estimating assay as discussed above.<sup>[46]</sup> In this assay, the stability of TTO and TTO-ME at variable concentrations (100–1000 µl/ml) was tested and compared it with its terpenoid content.<sup>[47]</sup>

## RESULTS AND DISCUSSION

### Quantitative estimation of *M. alternifolia* by GC-MS method

The GC-MS estimation of TTO reflected the presence of essential therapeutic categories, as shown in Figure 1, where initial compounds such as  $\alpha$ -pinene,  $\alpha$ -terpinene, D-Limonene, and 1,8-cineole were released between 4 and 8 min (RT). These components all together form around 18–20% content

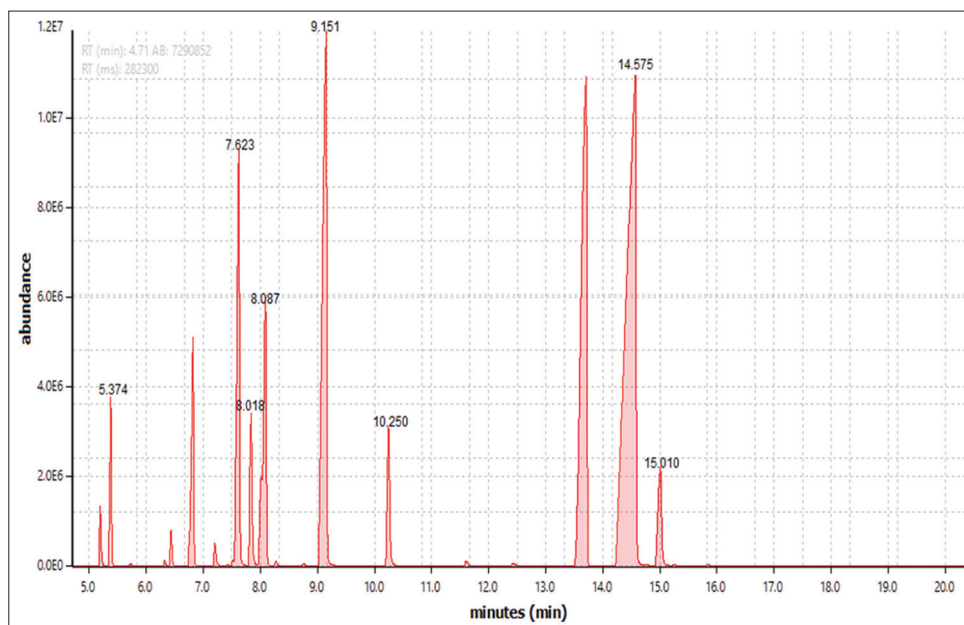
in TTO. Further, the major phytochemicals terpinene-4-ol and  $\gamma$ -terpinene, which comprises around 65% are reflected between 10 and 15 min (RT). This result scanning was further utilized for the *in vitro* phytochemical (terpinene-4-ol) release and its analysis, as depicted in Table 1.

### Solubility analysis of the ME excipients

The solubility of TTO was examined in different oils (eucalyptus oil, clove oil, olive oil, castor oil, and linseed oil, and IPM), surfactants (Tween-20, Tween-80, span-80, span-20, and PEG-400), and cosurfactants (ethanol and methanol), as shown in Table 2. After the solubility of all the excipients was analyzed by terpenoid estimation eucalyptus oil, Tween 80 and ethanol were selected as oil, surfactant, and cosurfactant phases, respectively, due to their maximum solubility and higher terpenoid content, in comparison to other excipients.

### HLB system analysis

After the solubility analysis, comparing the HLB value plays an important role in determining the longer stability of the formulation as many studies exhibited that, when a low concentration of oil taken with a higher concentration of  $S_{mix}$ , it yields a clear and stable ME system. Moreover, it has been observed that O/W ME system development requires the HLB values of excipients between 9 and 17. Therefore, the HLB value of eucalyptus oil (9.9) and Tween 80<sup>[15]</sup> were found to be required range along with the ethanol that was found to be in neutral range so not affecting the balance much and distilled water as the aqueous phase.



**Figure 1:** Representing the gas chromatography-mass spectrometry chromatogram of pure *Melaleuca alternifolia* oil showing peaks of all forms of terpenene, limonene, and cineole groups present in the same

### Preparation of microemulsions

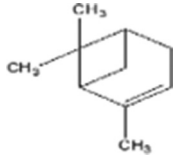
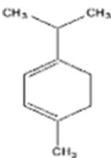
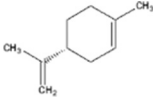
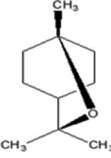
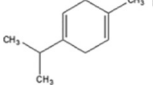
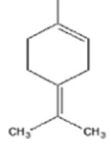
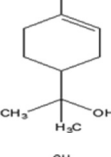
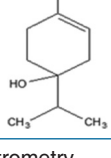
With the selected excipients (oil – eucalyptus oil, surfactant – Tween 80, and cosurfactant – ethanol) all the 6  $S_{mix}$  (1:1–6:1) combinations were formulated with varying oil: $S_{mix}$  ratios as shown in Table 3 and were further plotted on triplot for their characteristics evaluation.

### Ternary phase studies

The multicomponent system of ME was represented by three spatial dimensions illustrated on the ternary system graphical

structure where the equilibrium between all three phases is represented by a set of three lines or points which were monovariant. Since the equilibrium state was attained, when there is a minimal free energy available on each component in each phase, hence leading to optimization of different ratios of each component with another one on the basis of phase behavior, i.e. limiting coalescence, and optically isotropic phenomenon.<sup>[48,49]</sup> Hence, constructing the pseudoternary phase with all three constituents can be studied with each edge of the diagram as 100% of the respective constituents. With increasing surfactant concentration, there is a visible difference observed in two phases. The pseudoternary phase

**Table 1:** Summarization of percentage composition of *Melaleuca alternifolia* oil from GC-MS analysis

Components of <i>Melaleuca alternifolia</i>	Chemical structure	Retention time	Composition (%)
$\alpha$ -pinene		5.374	2.4
$\alpha$ -terpinene		7.623	10.5
D-Limonene		8.018	0.9
1,8-cineole		8.087	4.9
$\gamma$ -terpinene		9.151	22.6
$\alpha$ -terpinolene		10.250	2.9
4-terpineol		14.575	40.6
$\alpha$ -terpineol		15.010	2.6

GC-MS: Gas chromatography-mass spectrometry

**Table 2:** The solubility estimation of all the excipients (oil, surfactants, and cosurfactants) selected based on hydrophilic-lipophilic balance value by terpenoid estimation in pure *Melaleuca alternifolia* oil

Solubility estimation of terpenoid content ( $\mu\text{l/ml}$ ) in various excipients		
Oils	Surfactant	Co-surfactant
Eucalyptus oil=17.02 $\pm$ 1.5	Tween 20=16.34 $\pm$ 1.70	Ethanol=16.34 $\pm$ 0.87
Clove oil=7.1 $\pm$ 0.03	Tween 80=18.5 $\pm$ 1.09	Methanol=12.8 $\pm$ 1.56
Olive oil=9.63 $\pm$ 0.05	Span 20=13.92 $\pm$ 2.2	
Castor oil=3.87 $\pm$ 1.48	Span 80=8.2 $\pm$ 1.64	
Linseed oil=5.62 $\pm$ 0.07-	PEG 400=10.2 $\pm$ 1.64	
IPM=16.39 $\pm$ 0.2		

IPM: Isopropyl myristate

**Table 3:** The clear, nonturbid titration ratios of  $S_{\text{mix}}$  and oil:  $S_{\text{mix}}$  ratios of the selected combinations of excipients

S. No.	Combinations	$S_{\text{mix}}$ ratio	Clear oil: $S_{\text{mix}}$ ratios
1.	Eucalyptus oil+tween 80+ethanol (ETE-A)	1:1	(1:9), (2:8)
2.	Eucalyptus oil+tween 80+ethanol (ETE-B)	2:1	(1:9), (2:8), (3:7), (4:6)
3.	Eucalyptus oil+tween 80+ethanol (ETE-C)	3:1	(1:9), (2:8), (3:7), (4:6)
4.	Eucalyptus oil+tween 80+ethanol (ETE-D)	4:1	(1:9), (2:8), (3:7), (4:6)
5.	Eucalyptus oil+tween 80+ethanol (ETE-E)	5:1	(1:9), (2:8), (3:7)
6.	Eucalyptus oil+tween 80+ethanol (ETE-F)	6:1	(1:9), (2:8), (3:7)

diagram depicting that the clear titration zones of all the six combinations eucalyptus oil + tween 80 + ethanol (ETE) (ETE-A, ETE-B, ETE-C, ETE-D, ETE-E, and ETE-F) were plotted, as shown in Figure 2.

### Thermodynamic stability testing

Thereafter, thermodynamic stability analysis with all of the six combinations of *M. alternifolia* oil was tested for physical stability by subjecting them to four-step analytical process, they<sup>[50]</sup> underwent HCC, CFT, FTC, and DT and observed for any phase separation, creaming, cracking, turbidity, or coagulation phenomenon. After the thermodynamic stability testing, two formulations ETE-B and ETE-C passed the different stages of tests, visual inspections and attained Grade "A" and were further subjected for the next level of characterization to study their physiological, morphological, and structural concurrence.

### Characterization of *M. alternifolia* loaded ME

#### PS, PDI, and ZP analysis

PS analysis was performed to project the approximate size of the optimized formulation (ETB – C) along with its homogeneity of the particles in the ME system, as represented in Figure 3. Furthermore, ZP was done to incur the surface charge present on the same and is depicted in Figure 4.<sup>[51]</sup> The result of ETB – C exhibited the size of 149.1  $\pm$  1.34 nm with PDI score of 0.297  $\pm$  0.0056 indicating a higher degree of

uniformity of droplet size in the colloidal system, as shown in Figure 3. Moreover, the surface charge of the particles presents in the colloidal solution of ETB – C was recorded to be (-) 3.19  $\pm$  0.04 mV suggesting that the reported ZP has higher degree of stability due to the fact as stated by Honary S *et al.*, 2013<sup>[52]</sup> that particles showing ZP beyond the range of ( $\pm$ ) 30 mV are less dispersed particles and get aggregated due to Van der Waal inter particle attraction forces. The following results are represented in Table 4.

#### TEM

The size and morphology of the optimized TTO loaded ME system were in conformity with the DLS – PS results as it was showing the spherical ME structure within the range of 32–160 nm. The similar size range was exhibited with all other types of EO, like as stated by Jantrawut *et al.*, 2018, where, he developed orange oil loaded O/W ME system to enhance its antibacterial activity. The TEM micrograph showed below where analyzed at  $\times 10,000$  and  $\times 40,000$  (scale bar showing 200 nm), as shown in Figure 5.

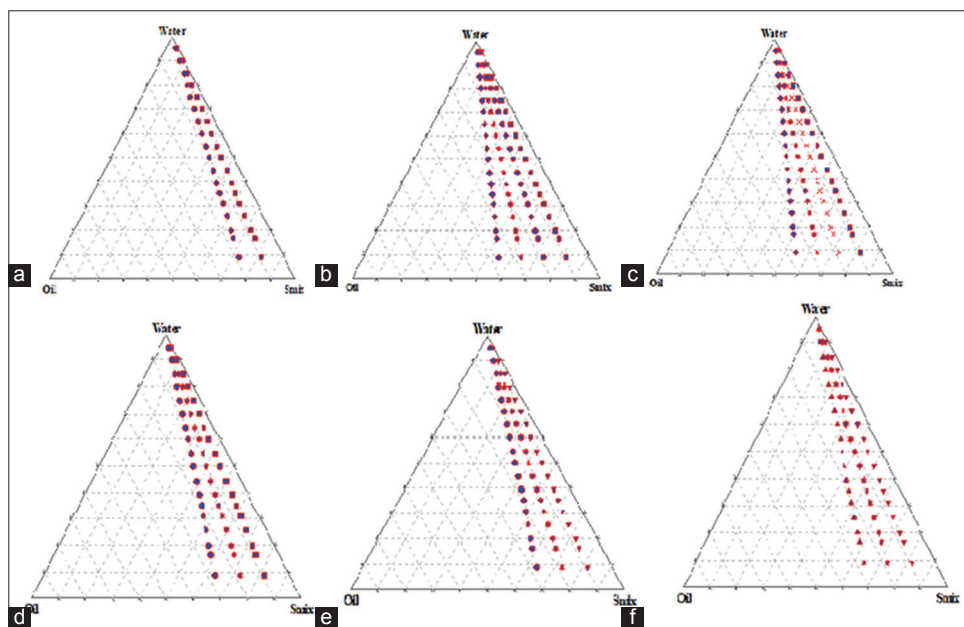
#### FT-IR analysis

The FT-IR analysis represents the functional group regions identifying the various functional groups present on the surface of the particles corresponding to the fingerprint regions. The analysis of pure TTO [Figure 6], only ME [Figure 7], and TTO – ME [Figure 8] was done which reflected the significant peaks of terpenoid groups having monoterpenes, sesquiterpenes, diterpenes, etc., in the scan of pure TTO. They were not exhibited in the scan of the TTO

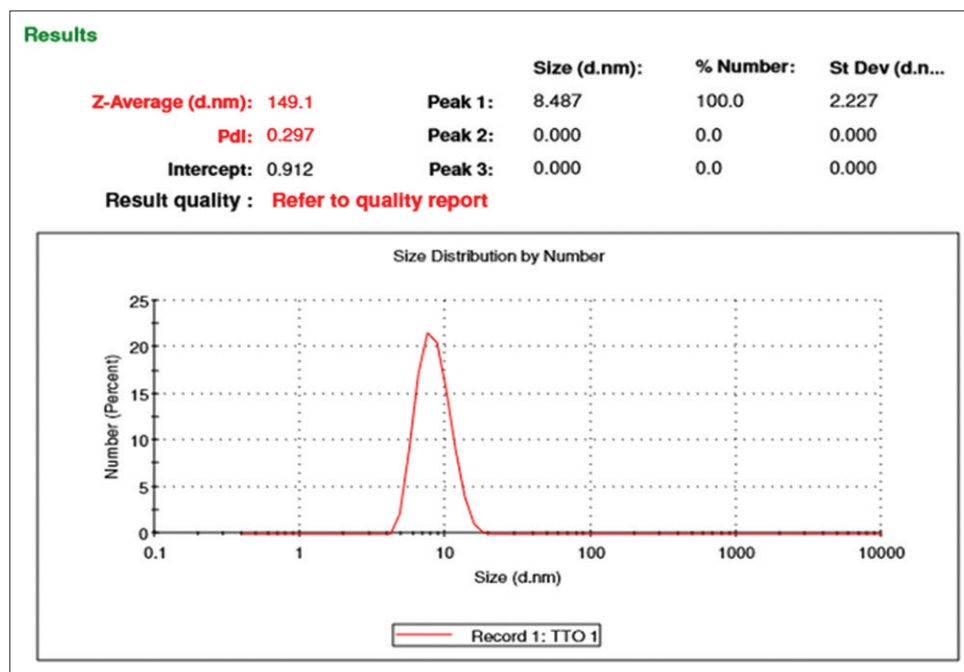
**Table 4:** Summarization of particle size, polydispersity index, and zeta potential of the optimized microemulsions ETB – C, as selected for further biological evaluation

Formulation code	Particle size (PSA)	PDI	ZP
ETE-C	149.1±1.34 nm	0.297±0.0056	(-) 3.19±0.04 mV

PDI: Polydispersity index, ZP: Zeta potential, PSA: Particle size analysis



**Figure 2:** Pseudoternary phase diagram indicating oil-in-water microemulsion using eucalyptus oil (oil), Tween 80 (surfactant), and ethanol (cosurfactant) with (a)  $S_{mix}$  ratio of 1:1 (eucalyptus oil + tween 80 + ethanol [ETE]-A), (b)  $S_{mix}$  ratio of 2:1 (ETE-B), (c)  $S_{mix}$  ratio of 3:1 (ETE-C), (d)  $S_{mix}$  ratio of 4:1 (ETE-D), (e)  $S_{mix}$  ratio of 5:1 (ETE-E), (f)  $S_{mix}$  ratio of 6:1 (ETE-F)

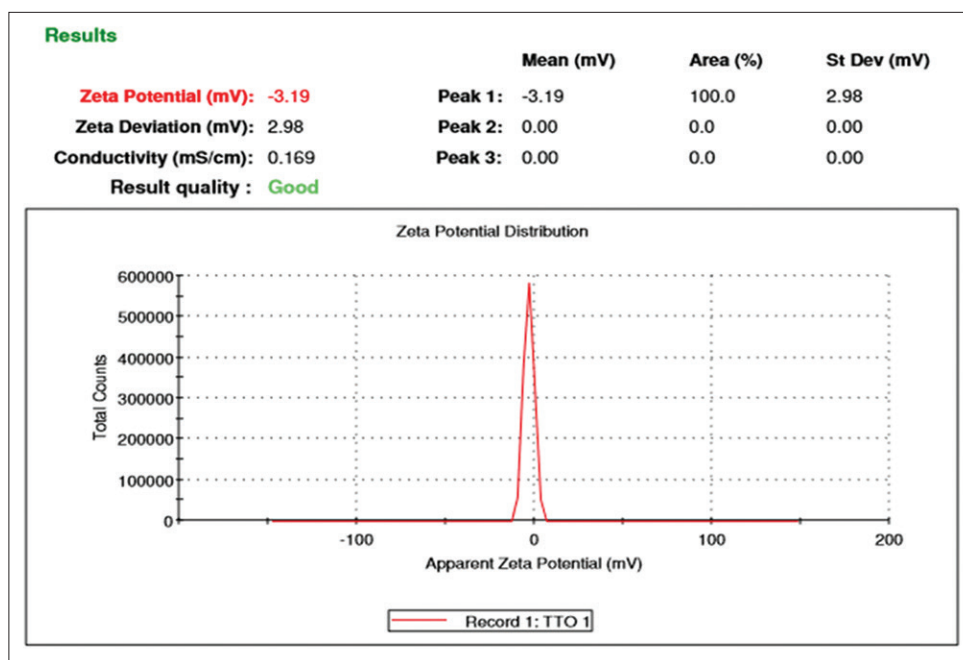


**Figure 3:** Graph representing the particle size analysis of the tea tree oil-microemulsion (eucalyptus oil + tween 80 + ethanol - C) by DLS method

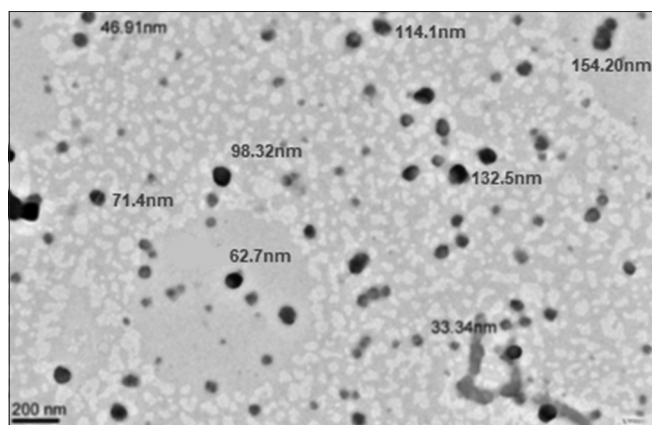
ME suggesting that the above mentioned functional groups of TTO have been masked by the excipients and therefore, the signature peaks are not significantly visible, so we can

conclude from the observation that these functional groups since not present in the outer surface may be encapsulated inside.





**Figure 4:** Graph representing the zeta potential of the tea tree oil-microemulsion (eucalyptus oil + tween 80 + ethanol - C) by DLS method



**Figure 5:** Transmission electron microscopy image of the optimized tea tree oil-microemulsion (eucalyptus oil + tween 80 + ethanol - C) at the magnification of  $\times 10,000$  (scale bar showing 200 nm)

FT-IR analysis of *M. alternifolia* shows the presence of Alkyne C-H bonds at  $3451.708\text{ cm}^{-1}$ . A peak having wave number  $2951.529\text{ cm}^{-1}$  and  $2923.074\text{ cm}^{-1}$  depicts the presence of  $\text{sp}^3$  C-H bond and an aldehyde group. Peaks ranging between  $2100$  and  $1600\text{ cm}^{-1}$  indicates the presence of ester, aldehyde, ketone, carboxylic acid or amide functional groups. The peaks below  $1600\text{ cm}^{-1}$  confirm the alkene double bonds and aromatic C-C bond. FT-IR analysis of ME and TTO-ME indicates that the functional groups present initially at TTO has been masked due to the presence of excipients and thus it can be concluded that there is no functional group present at the outer surface of the ME, and therefore, is encapsulated inside.

### Physiological parameter evaluation

The various tested physiological parameters of optimized formulation of *M. alternifolia* loaded ME (ETE-C) showed the pH of  $5.3 \pm 0.83$  which is closer to the pH of the skin which is between 5.5 and 6 depending on the type of the skin thus, determining the suitability of the mentioned formulation for transdermal application. Then, the density, viscosity, and conductivity of ETE-C were recorded as  $182.7 \pm 1.72\text{ g/ml}$ ,  $238 \pm 2.67\text{ cP}$ , and  $0.9845 \pm 0.0083\text{ mS/cm}$ , respectively, suggesting the range of all the parameters closer to water, making it most suitable formulation to impregnate the dermal layer and reach to the inner layers through transcellular route. The formulation also showed the high optical transparency when measured with the polarimeter and the surface tension measurements showed the readings of  $39.17 \pm 1.46\text{ dynes/cm}$ , suggesting the prevalence of bicontinuous ME layer between oil and aqueous phase. The result of surface tension was in concordance with the viscosity and electric conductivity of ETE-C suggesting that due to the presence of alcohol group there is a presence of more aqueous phase on the outer core of the oil shell causing increased surface tension. Therefore, the pH, density, viscosity, and conductivity measurement of the selected combination (ETE-C) are shown in Table 5.

### Statistical analysis

Table 5 shows the correlation coefficients (R) between PS and oil (O), PS and surfactant (S), PS and water (W) along with PDI with O, S, and W separately. All the correlation coefficients are significant at a 5% significance level ( $P < 0.05$ ). It can be observed that all the correlations are

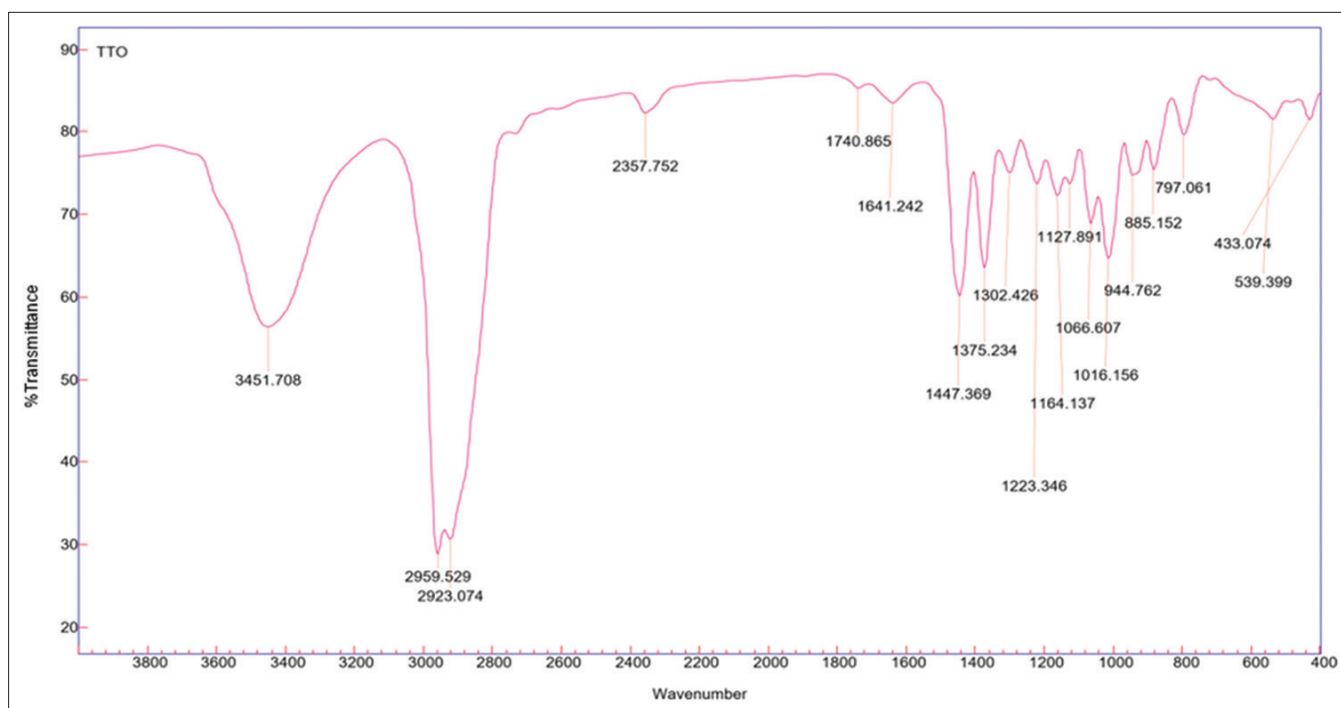


Figure 6: Graph representing Fourier transform infrared analysis of tea tree oil

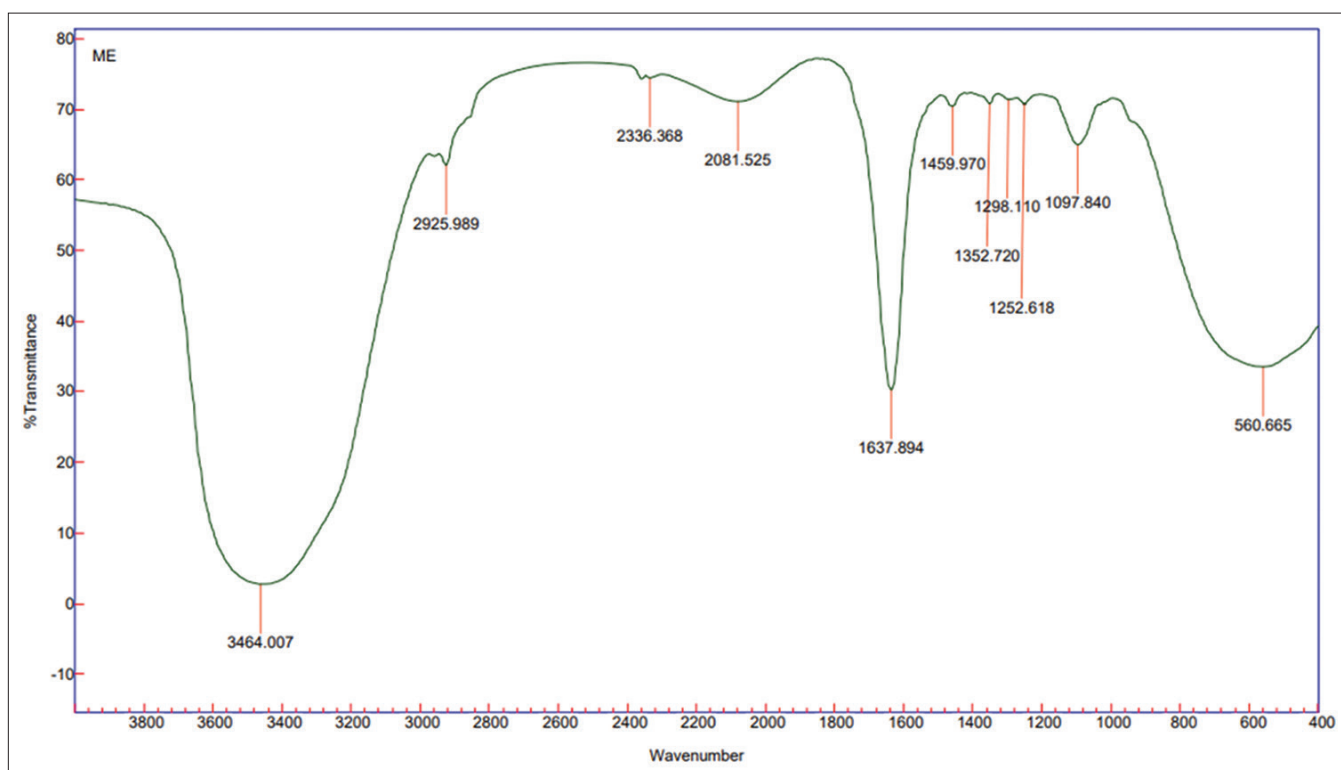
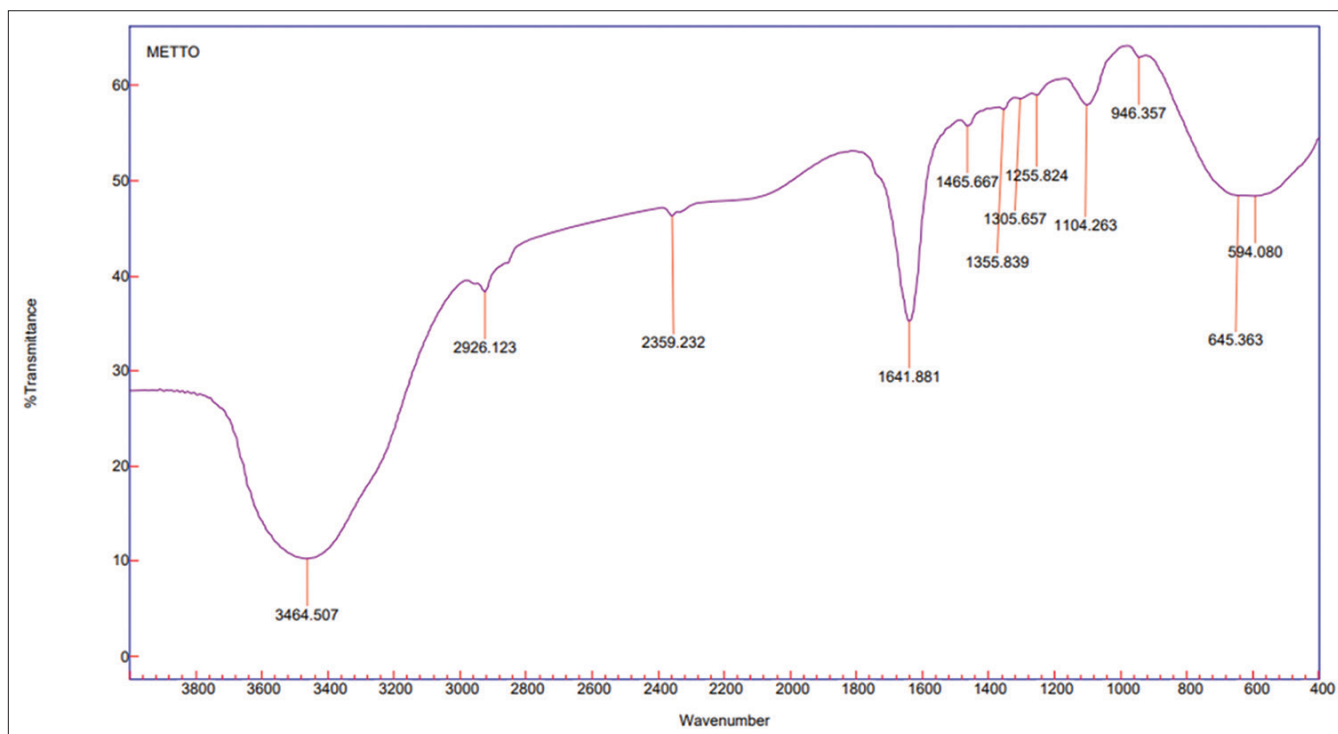


Figure 7: Graph representing Fourier transform infrared analysis of microemulsion

Table 5: The pH, density, viscosity, and conductivity of the selected combination ETE-C

Sample code	pH	Density (g/ml)	Viscosity (cP)	Conductivity (mS/cm)	Surface tension (dynes/cm)
ETE-C	5.3±0.83	182.7±1.72	238±2.67	0.9845±0.0083	39.17±1.46



**Figure 8:** Graph representing Fourier transform infrared analysis of tea tree oil-microemulsion (eucalyptus oil + tween 80 + ethanol - C)

**Table 6:** The statistical evaluation of different parameters of ETE – C was analyzed and their correlation coefficients

Parameters	Oil (%) (O)	S <sub>mix</sub> (%) (S)	Water (%) (W)
PS (d.nm)	0.929	0.928	-0.928
PDI	-0.938	-0.938	0.939

PDI: Polydispersity index, PS: Particle size

**Table 7:** The statistical evaluation of different parameters of ETE – C was analyzed and its regression coefficients for particle size and their correlation coefficients

Parameters	Coefficients	Standard error
Intercept	-16,056.10	115,082
Oil (%) (O)	346.88	1147.64
S <sub>mix</sub> (%) (S)	146.439	1151.18
Water (%) (W)	156.770	1150.97

**Table 8:** Represents the ANOVA summary of particle size for the optimized formulation (ETE – C)

Parameters	Df	SS	MS	F	P
Regression	3	812327	270776	114.3366	5.09E-11
Residual	16	37,891.7	2368.23		
Total	19	850219			

very strong ( $|R| > 0.9$ ). Moreover, the PS exhibits positive or direct correlation with O and S<sub>mix</sub> whereas negative or inverse correlation with W. On the other hand, PDI varies negatively or inversely with O and S and positively with W. The statistical evaluation of different parameters of ETE-C was analyzed and their correlation coefficients are listed in Table 6.

Further, regression analysis is applied to establish the relationships between PS, O, S, W, and PDI, O, S, W. Tables 5-7 summarize the regression coefficients and ANOVA results. The regression models are significant at 51% levels for both PS and PDI (Table 8:  $P < 0.05$  and Table 5:  $P < 0.05$ ).

The adjusted R<sup>2</sup> values for PS and PDI are obtained as 0.955 and 0.332, respectively, which indicate that oil, S<sub>mix</sub> and water can explain 95.5% variation in PS and 33.2% variation in PDI. The remaining amount of variations in both PS and PDI can be attributed to some other variables which are not considered in the experiment [Table 9].

The regression equations for PS [Table 8] and PDI [Table 10] are given by Equations 1 and 2, respectively.

$$PS = -16056.10 + 346.88(\text{oil}\%) + 146.43(S_{\text{mix}}\%) + 156.77(\text{water}\%) \quad (1)$$

$$PDI = 94.14 - 0.94(\text{oil}\%) - 0.94(S_{\text{mix}}\%) - 0.92(\text{water}\%) \quad (2)$$

### In vitro permeability studies

*In vitro* compound permeability of test samples (TTO and TTO – ME) was assessed by passing them through the semi-permeable membrane to analyze and compare the release pattern of terpenoid group from the loaded TTO and TTO – ME by GC-MS. The maximum percentage release of terpen-4-ol was recorded as  $91.5 \pm 1.23\%$  and  $97.3 \pm 2.71\%$  for TTO and TTO-ME in 24 h [Figure 9], respectively. The pattern of compound release showed a linear diffusion profile of

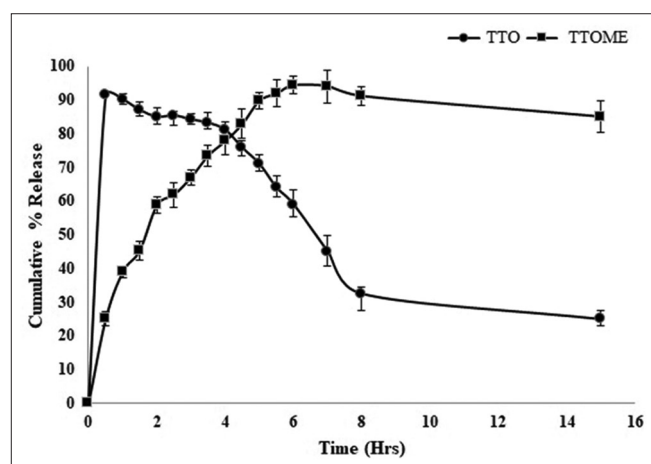


Figure 9: *In vitro* compound release pattern analysis of terpenoids from tree oil-microemulsion

Table 9: The ANOVA summary of polydispersibility index for the optimized formulation (ETE – C)

Parameters	df	SS	MS	F	P
Regression	3	0.3316	0.11053	4.160	0.023
Residual	16	0.425	0.026		
Total	19	0.756			

Table 10: The table represents the regression coefficients for polydispersibility index for the optimized formulation (ETE – C)

Parameters	Coefficients	SE
Intercept	94.146	385.4
Oil (%) (O)	-0.942	3.84
S <sub>mix</sub> (%) (S)	-0.945	3.85
Water (%) (W)	-0.926	3.85

SE: Standard error

Table 11: Summarization of compound loss percentage in TTO and TTO ME (ETE-C)

S. No.	Name of the sample	Amount present before storage (µl)	Amount present after storage (µl)	Calculated percentage loss
1.	TTO	0.87±0.002	0.075±0.011	79.5±0.009
2.	TTOME (ETE-C)	0.849±0.013	0.771±0.15	7.8±0.16

TTO: Tea tree oil, ME: Microemulsion

TTO-ME from the ME system with a consistent release rate throughout 24 h whereas, the release of only TTO showed a varying diffusion profile which reached its maximum release ( $91.5 \pm 1.23\%$ ) in first 30 min after which the release rate decreased gradually and became constant after 8 h. However, the diffusion equilibrium in case of TTO-ME was attained after 8 h hence, making the formulation suitable for transdermal application.

### Stability testing of TTO-ME (ETE-C)

Stability of TTO-ME (ETE-C) was examined after storing them at room temperature (37°C) for a year and testing the retention ability of their terpenoid content left after storing them for a mentioned time by the method of terpenoid estimating assay. The terpenoid analysis was done for the freshly prepared sample and the stored sample, and therefore it was concluded that there were no significant changes in the stored sample in comparison to the freshly prepared one which was about  $84.9 \pm 0.013\%$  and  $77.1 \pm 0.15\%$  for a freshly prepared sample and stored sample, respectively, whereas the stored sample of TTO for 1 year resulted in 80% reduction in the volume and terpenoid content since its highly volatile in nature. Therefore, summarization of compound loss percentage in TTO and TTO ME (ETE-C) is shown in Table 11.

## CONCLUSION

In the present study, *M. alternifolia* loaded O/W ME system (ETE-C) was successfully developed so, as to combat with its therapeutic limitations of being highly volatile and initiating inflammatory responses at higher doses. After characterization, the results exhibited the average PS range between 32 and 160 nm and PDI score of  $0.297 \pm 0.0056$  with thermodynamically stable ETC – C formulation. Furthermore, it showed the physicochemical parameters most suitable for the transdermal application with pH value of  $5.3 \pm 0.83$ , conductivity ( $0.98 \pm 0.083$  mS/cm), density ( $182.7 \pm 1.72$  g/ml), and viscosity ( $283 \pm 2.67$  cP) along with its surface tension of  $39.17 \pm 1.46$  dynes/cm. Then, FT-IR result analysis revealed the masking of major signature peaks of TTO, suggesting their encapsulation in the lipophilic phase. Furthermore, the stability of the prepared formulation was also checked after 1 year, and there was no significant degradation reported in the same. Therefore, it can be concluded from the study that TTO loaded (ETE-C) ME system can be studied in various *in vitro* and *in vivo* models

further to establish its better therapeutic efficacy for various skin disorders.

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