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Development and Evaluation of an Essential Oil-Based Nanoemulgel for Topical Antifungal Activity Against *Candida* Species

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Abstract:

Objective: This study aimed to develop and optimize a polymer-based nanoemulgel containing *Melaleuca alternifolia* (tea tree) and *Curcuma longa* (turmeric) essential oils and to evaluate its physicochemical properties, in-vitro release characteristics, antifungal activity against *Candida albicans* and *Candida parapsilosis*, irritation potential, and short-term stability.

Materials and Methods: A 7:3 ratio of *M. alternifolia* to *C. longa* oils was selected as the oil phase. Span 20 and Tween 80 were used at an optimized surfactant mixture (Smix) ratio of 8:2. Nanoemulsion were prepared using ultrasonication and evaluated for physical stability, droplet size, polydispersity index (PDI), zeta potential, conductivity, and morphology. The optimized nanoemulsion was incorporated into a Carbopol 934 gel base to obtain the polymeric nanoemulgel (NEG3). The formulation was characterized for physicochemical properties, essential oil content, in-vitro release, antifungal activity, and irritation potential using the Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) assay. Stability was evaluated under real-time and accelerated storage conditions for three months.

Results: The optimized nanoemulsion exhibited a mean droplet size of 155.5 nm, a PDI of 0.184, and a zeta potential of −7.36 mV. Transmission electron microscopy confirmed nanoscale droplets with predominantly elongated-to-elliptical morphology. The optimized nanoemulgel was pale yellow, smooth, homogeneous, and translucent, with a pH of 6.40 ± 0.03, viscosity of 8425 ± 35 cP, spreadability of 14.26 ± 0.41 g·cm/s, and extrudability of 8.15 ± 0.18 g/0.5 min. The measured essential oil contents were 98.84 ± 0.72% for *M. alternifolia* oil and 97.63 ± 0.65% for *C. longa* oil. At 24 h, cumulative release reached 76.99 ± 2.50% and 72.57 ± 2.90%, respectively. The Korsmeyer-Peppas model provided the best fit to the release data, with release exponents of 0.62 and 0.58, indicating non-Fickian transport. The formulation exhibited concentration-dependent antifungal activity, producing inhibition zones of 25.00 ± 1.00 mm against *C. albicans* and 20.00 ± 1.00 mm against *C. parapsilosis* at 1000 µg/mL. The minimum inhibitory concentration (MIC) was reported as 15.63 µg/mL for both strains. The HET-CAM assay yielded an irritation score of 0.00. The formulation maintained its overall physical characteristics and essential oil content during three months of real-time and accelerated storage.

Conclusion: The developed essential oil-loaded polymeric nanoemulgel demonstrated favorable physicochemical characteristics, sustained in-vitro release, and antifungal activity against both tested *Candida* species. The absence of detectable irritation in the HET-CAM assay and the maintenance of key formulation characteristics during short-term stability testing support its further investigation as a potential topical antifungal delivery system. Ex-vivo permeation, comprehensive dermal safety, in-vivo efficacy, and long-term stability studies are required before therapeutic application can be established.

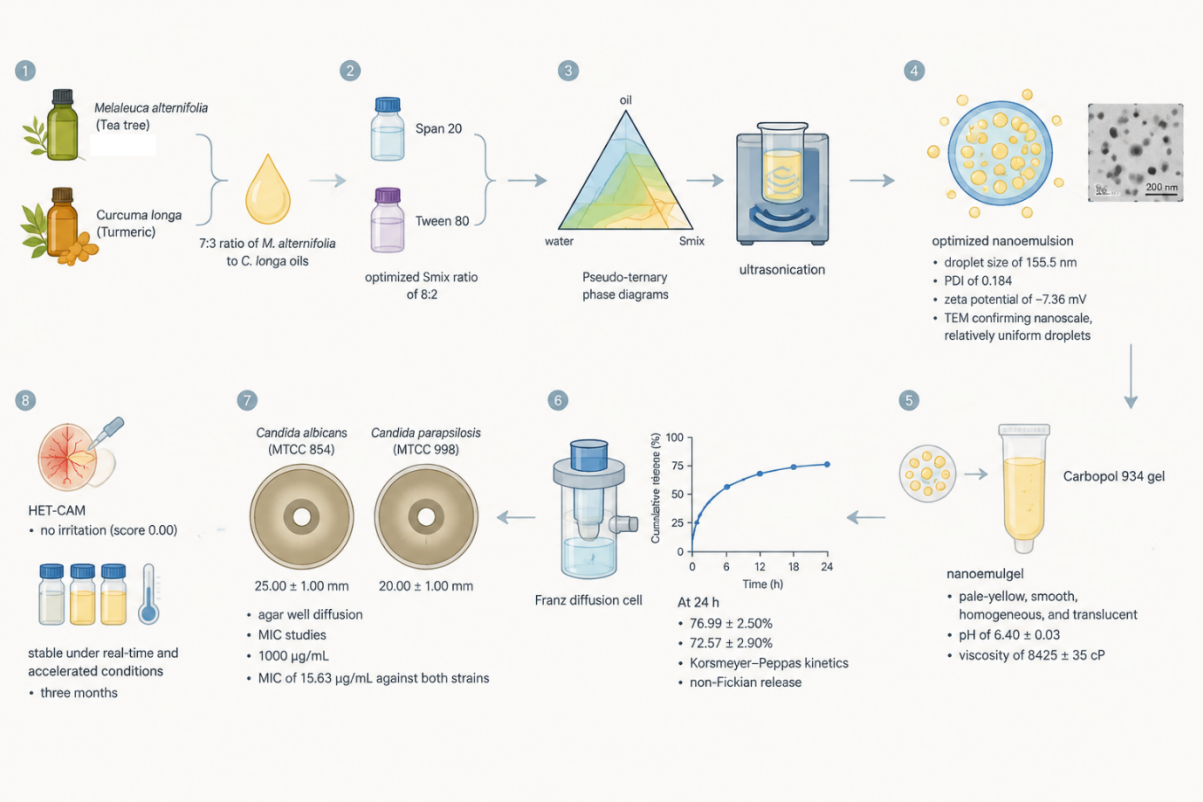
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Graphical Abstract

Introduction:

Cutaneous fungal infections constitute one of the most common dermatological concerns [1]. Research indicates that more than 40 million persons are impacted by fungal infections. Superficial fungal infections of the skin, hair, and nails are common and generally difficult to manage. A diverse array of products is accessible for treatment, including liquid, solid, and semisolid dosage forms. Gels are frequently employed in medical and cosmetic topical preparations. The use of gels has increased in both pharmaceutical formulations and cosmetic items within the principal category of semisolid preparations [2]. A fungal infection, known as mycosis, is a skin condition resulting from the presence of a fungus [3,4]. Fungal infections are among the most common dermatological issues worldwide. Currently, there is a noticeable rise in the number of patients presenting with skin fungal infections at dermatology clinics. Infections caused by *Candida* species frequently occur on the skin's surface. A compromised immune system may facilitate the spread of *Candida* into deeper tissues and the bloodstream, resulting in systemic candidiasis [5,6]. Topical antifungals are highly effective and have minimal systemic adverse effects, positioning them as the primary treatment option for superficial fungal infections [7]. The results indicate significant effectiveness in combating the *Candida* infection [8]. Recent reports from dermatology clinics indicate a troubling increase in the occurrence of skin fungal infections [9]. These cutaneous mycoses, categorized into dermatophytosis, *candida* infections, and mold infections, require careful consideration. Candidiasis, caused by *Candida* species, is recognized as a common fungal infection, affecting regions including the scalp, skin, vagina, mouth, feet, and fingers [10]. Over the past ten years, there has been a significant rise in the utilization of herbal products worldwide, encompassing food items, vitamins, and herbal formulations. The primary benefits of herbal remedies include a reduced incidence of side effects. Nonetheless, the application of herbal formulations presents certain constraints in disease treatment, particularly concerning low oral bioavailability and inadequate aqueous solubility [11]. A dosage form is a formulation that integrates an active pharmaceutical ingredient (API) with excipients to enhance the dosing, administration, and delivery of the drug to patients. Multiple dosage forms exist, differing according to the mode of administration and the site of action of the active components. Dosage forms include several options: solids (tablets, capsules, powders, granules), semisolids (creams, ointments, pastes, gels), liquids (syrups, elixirs, decoctions, lotions), and gases (aerosols) [12]. The topical administration of drugs bypasses the first-pass effect, a notable limitation of the oral route. Nonetheless, its principal limitation resides in the challenge of permeating hydrophobic chemical constituents via the dermis [13]. Nanoemulgel represent a novel drug delivery method that

integrates the characteristics of nanoemulsion and gel. They are employed for the administration of many drugs, including analgesics, anti-inflammatory agents, anti-acne treatments, and antifungal chemicals. Nanoemulgel has attributes including facile removal, excellent spreadability, absence of greasiness, prolonged shelf life, and transparency. Nanoemulgel are nanoemulsion that have been gelled, and they can be classified as either water-in-oil or oil-in-water. This vehicle is exceptionally appropriate for hydrophobic pharmaceuticals. Nanoemulgel possess the attributes of both gels and emulsions, functioning as a dual-controlled release mechanism that can administer both hydrophilic and lipophilic pharmaceuticals. This results from the existence of two separate phases: aqueous and non-aqueous [14]. Emulgel are formulated using oil-in-water systems for lipophilic drugs and water-in-oil systems for hydrophilic meds. A nanoemulgel is created by incorporating a gelling agent into a conventional nanoemulsion. The skin functions as a conduit for the delivery of drugs via topical treatment. Topical formulations are solely utilized for the delivery of locally acting drugs, including antimicrobials and antifungal agents. The topical route is selected due to the abundance of blood vessels relative to systemic preparations. Topically administered drugs can be absorbed via three main mechanisms: transcellular transport, paracellular transport, and vesicular transport. The medication absorption process from the skin involves two main stages: administration of the drug to the skin and its subsequent penetration through the skin membrane [15]. This study tackles the pressing need for effective antifungal therapies by formulating and refining a nanoemulgel comprising *Melaleuca alternifolia* (Tea tree) and *Curcuma longa* (Turmeric) oils, subsequently assessing its antifungal effectiveness against *Candida albicans (MTCC 854) and Candida parapsilosis (MTCC 998)*. The study sought to augment antifungal activity by integrating oil extract into an emulsion and embedding it within a gel matrix to optimize medication delivery to the infection site.

MATERIALS AND METHODS:

Materials:

Tea tree oil *(Melaleuca alternifolia)* and Turmeric oil *(Curcuma longa)* were sourced from reputable suppliers and supported with certificate of analysis. Surfactants, including Span 20, and co-surfactants, such as Tween 80 were employed in the formulation of the nanoemulsion. Carbopol 934 served as a gelling agent, whereas triethanolamine functioned as a neutralizing agent. Sodium Benzoate were utilized to enhance formulation stability. Distilled water was regularly employed throughout the trial. The chemicals and reagents employed for evaluation included phosphate buffer (pH 5.5/6.8), fungal culture media (Sabouraud Dextrose Agar), and a standard antifungal agent (e.g., fluconazole). All employed chemicals were of analytical grade. *Candida albicans* (MTCC 854) and *Candida parapsilosis* (MTCC 998) were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. (Table 1).

Table 1. Materials used for the formulation of the *Melaleuca alternifolia* Oil-*Curcuma longa* oil nanoemulgel and their respective sources and functions.

S. No.	Name of Ingredient	Company Name	Use in the Formulation
1	*Melaleuca alternifolia* Oil	Indus Cosmeceuticals Pvt. Ltd.	Active ingredient
2	*Curcuma longa* Oil	R V Essentials Pvt. Ltd.	Active ingredient
3	Carbopol 934	Central Drug House (P) Ltd.	Gelling agent
4	Span 20	Central Drug House (P) Ltd.	Emulsifying agent (for oil phase)
5	Tween 80	Central Drug House (P) Ltd.	Emulsifying agent (for aqueous phase)
6	Propylene Glycol	NICE Chemicals (P) Ltd.	Penetration enhancer & solvent
7	Sodium Benzoate	Central Drug House (P) Ltd.	Preservative
8	Triethanolamine (TEA)	Central Drug House (P) Ltd.	pH adjusting agent
9	Distilled Water	Laboratory Prepared	Aqueous phase medium
10	Ethanol	Changshu Hong sheng Fine Chemical Co. Ltd	Solvent
11	Phosphate Buffer	HiMedia	Buffer Solution

All chemicals and reagents used in the study were of analytical grade.

Methods:

Construction of Calibration Curve:

Standard solutions of *Melaleuca alternifolia* oil and *Curcuma longa* oil were prepared in a suitable solvent at different concentrations within the linear range. The absorbance of each solution was measured using a UV-Visible spectrophotometer at 268 nm for TTO and 235 nm for *C. longa* oil, using the corresponding solvent as blank. Calibration curves were constructed by plotting absorbance against concentration, and the slope, intercept, and

coefficient of determination (R^2) were determined by linear regression analysis [16], as sown in figure 1.

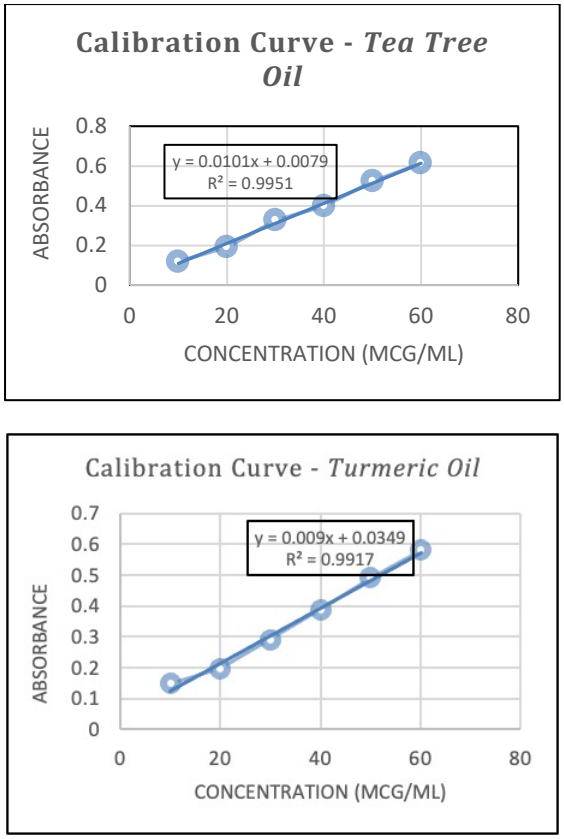


Figure 1. Calibration curves of (1A) *Melaleuca alternifolia* Oil at 268 nm and (1B) *Curcuma longa* oil at 235 nm showing the linear relationship between concentration and absorbance.

Selection of Oil Phase for Nano-emulsion:

The oil phase for the nanoemulsion formulation was created by combining Tea Tree oil and Turmeric oil in different ratios. The combinations were evaluated to identify a suitable oil phase capable of producing a stable nanoemulsion system with enhanced emulsification efficiency and therapeutic potential. The developed product was intended for topical skin application, leading to the selection of an oil-in-water (O/W) nanoemulsion system, in which the oil phase serves as the dispersed phase inside the continuous aqueous phase. [17].

Miscibility Study of Essential Oils with surfactant and co-surfactant:

The miscibility study of the selected essential oil blend, Tea Tree Oil and Turmeric Oil (7:3 v/v), was performed to select suitable surfactants, co-surfactants, and Smix components for nanoemulsion development. Equal volumes of the oil blend and selected surfactants (Span 20, Tween 20, Tween 60, Cremophor EL, and Labrasol) and co-surfactants (PEG 400, propylene glycol, ethanol, isopropyl alcohol, and Tween 80) were mixed by vortexing and visually examined for homogeneity, turbidity, precipitation, and phase separation. Homogeneous mixtures were considered miscible. Based on the screening, span 20 and Tween 80 were selected as the surfactant and co-surfactant, respectively. Different ratios of these components were evaluated to prepare the Smix system, and the optimum ratio was selected based on the required hydrophilic-lipophilic balance (HLB) [18-19].

Preparation of Smix Using Required HLB

The Smix system was prepared using Span 20 and Tween 80 based on the required hydrophilic-lipophilic balance (HLB) of the selected oil phase. The oil phase consisted of Tea Tree Oil and Turmeric Oil in a 7:3 (v/v) ratio. The required HLB values of Tea Tree Oil and Turmeric Oil were considered as 10 and 9, respectively. The required HLB of the combined oil phase was calculated using the weighted-average method [20].

Construction of Pseudo-Ternary Phase Diagram for Nanoemulsion

A pseudo ternary phase diagram was produced using a standard methodology to evaluate the phase behaviour of various formulation components. A total of 100 mL of a 4:1 mixture of surfactant (Span 20) and co-surfactant (Tween 80) was prepared, resulting in 17 distinct combinations of essential oils and the surfactant mixture (Smix) in varying volume ratios from 1:9 to 9:1 (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1). The mixture of Smix and oil was titrated with distilled water [22,23]. A visual inspection was conducted following each 5% increment of the aqueous phase added to the oil and Smix mixture, and the findings were documented. Ultimately, data were documented following the selection and formulation of the fractions of water, oil, and Smix on a phase diagram, with one axis denoting the aqueous phase, another the oil phase, and the third the Smix. Based on the subsequent criteria, specific formulations were chosen from each phase diagram generated for different Smix ratios. The oily phase was chosen at a concentration sufficient to readily dissolve the essential oil utilized as the active ingredient [21].

Formulation of Nanoemulsion

The nanoemulsion was formulated via the ultrasonication technique, utilizing Tea Tree Oil and *Curcuma longa oil* as the oil phase, Span 20 as the surfactant, and Tween 80 as the co-surfactant. The oil phase, comprising oils and Span 20, was mixed independently, while Tween 80 was solubilized in distilled water to formulate the aqueous phase [24]. The oil phase was thereafter introduced dropwise into the aqueous phase while maintaining continuous magnetic stirring at 1000-1500 rpm to create a coarse emulsion. The resultant emulsion underwent probe ultrasonication for 10 minutes at 40-60% amplitude in pulse mode (30 s ON/20 s OFF), ensuring the temperature remained below 40°C. This procedure yielded a stable, translucent nanoemulsion with nanoscale droplet size and

improved homogeneity, as presented in Table 2. [25].

Table 2. Composition of Essential oil-based Nanoemulsion.

Formulation	*Melaleuca alternifolia* oil + *Curcuma longa* oil (%)	Span 20 + Tween 80 Smix (%)	Triethanolamine (%)	Distilled Water (%)
F1	3.5	18	q.s	q.s. to 100
F2	5	22	q.s	q.s. to 100
F3	6.5	26	q.s	q.s. to 100
F4	8	30	q.s	q.s. to 100
F5	10	35	q.s	q.s. to 100
F6	11.5	38	q.s	q.s. to 100
F7	13	41	q.s	q.s. to 100
F8	14	43	q.s	q.s. to 100
F9	14.5	44	q.s	q.s. to 100

Evaluation of Nanoemulsion

Physical stability under stress conditions:

- **Heating-Cooling Cycle:** This study assessed the physical stability of nanoemulsion formulations by exposing them to alternate temperature cycles of 4°C and 40°C. Each cycle involved storing the formulations at 4°C for 48 hours, followed by 40°C for an additional 48 hours, with a total of 3 to 6 cycles conducted. Following each cycle, the formulations were visually assessed for phase separation, creaming, or turbidity. Formulations exhibiting no discernible instability were deemed physically stable [26].
- **Freeze-Thaw Cycle:** The freeze-thaw stability assessment was conducted by exposing the nanoemulsion formulations to cyclical freezing and thawing conditions. The samples were preserved at -10°C for 48 hours, thereafter thawed at 25°C for the equivalent duration, and this procedure was reiterated for a minimum of three cycles [27]. Following each cycle, the formulations were examined for phase separation, aggregation, or alterations in consistency. Formulations that maintained homogeneity without observable instability were deemed stable and resilient to temperature fluctuations [28].
- **Centrifugation Test:** The centrifugation test was conducted to assess the mechanical stability of the nanoemulsion formulations under enhanced gravity force. The samples underwent centrifugation at 3000-5000 rpm for 5 minutes, followed by a visual assessment for phase separation, creaming, or sedimentation. Formulations exhibiting no discernible instability post-centrifugation were deemed physically stable and appropriate for subsequent investigations [29].

Viscosity Measurement:

The viscosity of the formulated nanoemulsion was assessed using a Brookfield viscometer with an appropriate spindle at 25 ± 1°C. The spindle was meticulously submerged in the sample to prevent air bubble entrapment, and viscosity measurements were taken at various rotational speeds to assess the rheological properties of the formulations [30]. The viscosity of the nanoemulsion is crucial for assessing its spreadability, application ease, and skin retention, therefore affecting topical medication administration and patient adherence [31].

pH Measurement:

The pH of the nanoemulsion formulations was measured using a calibrated digital pH meter, standardized with buffer solutions at pH 4.0, 7.0, and 9.2. The electrode was submerged in the sample, and the measurement was documented following stabilization. Measurements were conducted in triplicate, and the mean value was computed. It is essential to maintain the pH within the appropriate range for topical administration (about 5.5-6.8) to guarantee skin compatibility and formulation stability [32].

Conductivity Test:

The conductivity of the nanoemulsion formulations was assessed with a calibrated digital conductivity meter to identify the emulsion type [33]. The conductivity probe was submerged in the sample at ambient temperature, and the measurements were documented in µS/cm. Elevated conductivity levels signified an oil-in-water (O/W) nanoemulsion, while diminished conductivity values indicated a water-in-oil (W/O) nanoemulsion. All measurements were conducted in triplicate, and the mean value was reported [34].

Droplet Size and Polydispersity Index (PDI)

The droplet size and polydispersity index (PDI) of the nanoemulsion formulations were assessed via Dynamic Light Scattering (DLS) utilizing a Malvern Zetasizer [35]. The samples were appropriately diluted with distilled water and examined at 25°C. The mean droplet size was quantified in nanometers (nm), and the polydispersity index (PDI) was employed to evaluate the consistency of droplet size distribution. All measurements were conducted and values were presented. Reduced droplet size improves medication release and skin penetration, whereas a PDI value under 0.3 signifies homogeneous distribution and formulation stability [36,37].

Zeta Potential

The zeta potential of the nanoemulsion formulations was assessed utilizing a zeta potential analyzer predicated on electrophoretic mobility [38]. The samples were appropriately diluted with distilled water and examined at 25°C utilizing a dedicated capillary cell. The zeta potential values, measured in millivolts (mV), were derived from the movement of charged droplets in an applied electric field. All measurements were conducted and the mean values were reported. Zeta potential is a critical measure for forecasting formulation stability, with higher absolute values (exceeding ±30 mV) signifying enhanced stability due to robust electrostatic repulsion between droplets, which inhibits aggregation and phase separation [39].

Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) was employed to examine the morphology, dimensions, and structural attributes of the nanoemulsion droplets. A diluted nanoemulsion sample was deposited on a carbon-coated copper grid, dyed with phosphotungstic acid, and permitted to dry prior to analysis. The produced grid was subsequently analysed using a transmission electron microscope at appropriate magnifications to get photographs of the droplet structure. TEM analysis yields high-resolution pictures for the direct examination of droplet morphology and dimensions, generally revealing spherical and uniformly dispersed droplets inside a stable nanoemulsion. It additionally assists in validating the droplet size derived from DLS analysis and identifying any aggregation or structural irregularities [40].

Preparation of Nanoemulgel

The nanoemulgel was prepared in two stages, involving the preparation of the gel base followed by the incorporation of the optimized nanoemulsion. For the preparation of the gel base, Carbopol 934 (2% w/w) was gradually dispersed in distilled water under continuous stirring and allowed to hydrate for 2-3 h. After complete hydration, propylene glycol (5% w/w) was added as a humectant and penetration enhancer, followed by the addition of sodium benzoate (0.2% w/w) as a preservative. The dispersion was continuously stirred, and triethanolamine was added dropwise as a neutralizing and pH-adjusting agent until a smooth and homogeneous gel was obtained. The pH of the gel base was adjusted to 5.5-6.8, and distilled water was added q.s. to 100% as the vehicle. The prepared gel base was subsequently incorporated with the optimized nanoemulsion in a 1:1 ratio. The nanoemulsion was gradually added to the gel base under continuous moderate stirring using a mechanical stirrer to ensure uniform dispersion and the formation of a smooth, homogeneous, and translucent nanoemulgel. Care was taken to minimize air entrapment during the mixing process. The prepared nanoemulgel was filled into sealed collapsible containers and stored at ambient temperature until further evaluation and characterization [41].

Evaluation of Nanoemulgel

Physical Evaluation

The nanoemulgel formulations were assessed for color, homogeneity, consistency, and phase separation using visual inspection. A limited amount of the formulation was extracted and examined under standard lighting conditions. The formulation was evaluated for consistency, smoothness, lack of grittiness, and any indications of phase separation or clumping [42].

pH Determination

The pH of the nanoemulgel was assessed utilizing a calibrated digital pH meter. The pH meter was initially calibrated with standard buffer solutions. A minimal amount of the formulation was diluted in distilled water, and the electrode was submerged in the sample. The pH was documented once the measurement stabilized.

Viscosity Measurement

The viscosity of the nanoemulgel was assessed with a Brookfield viscometer. The formulation was transferred to an appropriate container, and the correct spindle was chosen according to the gel's consistency. The experiment was conducted at a regulated temperature, and data were collected at various rotating speeds to ascertain the flow characteristics.

Spreadability

The spreadability was evaluated using the slip and drag method. A fixed mass (0.5 g) of the emulgel was placed between two glass slides, and a force (500 g) was applied for five minutes. The time taken for the upper slide to cover a specified distance was recorded. Spreadability (S) was calculated using the formula.

$$S = \frac{M \times \mathrm{L}}{T}$$

Where:

S = Spreadability (g·cm/s)
M = Weight tied to the upper slide (g)
L = Distance moved by the slide (cm)
T = Time taken (s)

Extrudability

The extrudability of the nanoemulgel was assessed to ascertain the ease of dispensing the formulation from a collapsible tube. The formulated nanoemulgel was placed into a collapsible aluminum tube, and a predetermined weight was applied to provide consistent pressure. The quantity of formulation extruded through the nozzle during a specified duration (0.5 minutes) was gathered and weighed. The test was conducted in triplicate, and the mean value was computed. Extrudability was quantified as the mass of emulgel extruded per unit time under applied pressure, reflecting the formulation's ease of application [43].

The extrudability was calculated using the following formula:

$$E = \frac{W}{T}$$

Where:

E = Extrudability

W = Weight of emulgel extruded (g)

T = Time taken for extrusion (s or min)

Drug Content Analysis

The content uniformity of the optimized nanoemulgel was determined for *Melaleuca alternifolia* and *Curcuma longa oil* using UV-Visible spectrophotometry. Ten samples (1 g each) were accurately weighed, extracted with ethanol, sonicated for 15-20 min, diluted to 100 mL, and filtered. The filtrates were suitably diluted with suitable solvent and analysed at 268 nm for Tea Tree Oil and 235 nm for *Curcuma longa oil* using their respective calibration curves. The oil content was calculated from the calibration equations and expressed as mg/g and percentage of the theoretical content, with theoretical values of 35 mg/g for Tea Tree Oil and 15 mg/g for *C. longa oil*. The results were reported as mean ± SD, and %RSD was calculated to assess the uniformity of oil distribution. [44].

In-Vitro Drug Release Profile

The in vitro drug release investigation was conducted utilizing a Franz diffusion cell with a receptor compartment capacity of 7 mL and an effective diffusion area of 0.2 cm^2. The egg membrane was immersed in phosphate buffer (pH 6.8) for 24 hours at 37 ± 1°C and subsequently positioned between the donor and receptor compartments. The receptor compartment was filled with a phosphate buffer solution (pH 6.8) to replicate physiological circumstances. Samples were extracted at specified time intervals (0.5, 1, 1.5, 2, 4, 8, 12, 16, and 24 hours) and examined by UV-Visible spectrophotometry. The cumulative percentage of drug release was computed and graphed over time to assess the release profile of Tea Tree oil and Turmeric oil from the formulation. A comparable technique was conducted utilizing pure essential oils for the purpose of comparison [45].

Bio studies

1. In Vitro Antifungal Activity

Sterile Sabouraud Dextrose Agar (SDA) plates were prepared and inoculated with *Candida albicans (MTCC 854) and Candida parapsilosis (MTCC 998)* fungal solutions using a sterile swab. Wells with a diameter of 6 mm were fabricated using a sterile cork borer. Approximately 62.5, 125, 250, 500 and 1000 µg/ml of the nanoemulgel formulation was dispensed into each well. Plates were incubated at 28 ± 2°C for 48 hours. The zone of inhibition (in mm) was measured and compared with the standard antifungal drug (Fluconazole). All tests were performed in triplicate to assure accuracy [46].

2. Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the prepared nanoemulgel was assessed using the broth dilution method against specific fungal strains. Serial dilutions of the formulation were created in Sabouraud Dextrose Broth (SDB) and subsequently inoculated with a standardized fungal suspension under aseptic conditions. The samples were incubated at 25-30°C for 24-48 hours and subsequently assessed for turbidity and fungal proliferation. The lowest concentration of the formulation that exhibited no detectable growth was designated as the Minimum Inhibitory Concentration (MIC), signifying the antifungal activity of the nanoemulgel [47].

3. Skin Irritation Study (HET-CAM)

The skin irritation potential of the prepared nanoemulgel was assessed using the Hen's Egg Test on Chorioallantoic Membrane (HET-CAM) method. Fertilized hen's eggs were incubated at 37 ± 1°C with 60-70% humidity for 9-10 days to facilitate the correct development of the chorioallantoic membrane (CAM). The eggshell was meticulously opened, and the inner membrane was extracted to reveal the CAM. A specific volume of the nanoemulgel was applied directly to the CAM surface, with normal saline and 0.1 N NaOH solution serving as negative and positive controls, respectively. The CAM was monitored for 5 minutes for indications of irritation, including bleeding, vascular lysis, and coagulation, with the onset time of each reaction documented [48].

4. Short-Term Accelerated Stability Studies

The refined nanoemulgel formulation underwent real-time stability assessment at 25 ± 2°C/60 ± 5% RH and accelerated stability evaluation at 40 ± 2°C/75 ± 5% RH, according to pertinent ICH standards. The preparation was preserved in screw-top collapsible containers and assessed at 0 days, 15 days, 1 month, 2 months, and 3 months for alterations in appearance, uniformity, grittiness, pH, viscosity, spreadability, extrudability, globule size, polydispersity index (PDI), zeta potential, and the oil content of *Melaleuca alternifolia* and *Curcuma longa* [49-51].

Result:

Selection of Oil Phase for Nano emulsion:

The oil phase was prepared by blending Tea Tree oil and Turmeric oil in different ratios and evaluating their compatibility, clarity, emulsification efficiency, and stability. Based on preliminary studies, the 7:3 ratio *of* Tea Tree oil to Turmeric oil showed optimal characteristics and was selected as the oil phase. The blend was used to develop an oil-in-water (O/W) nanoemulsion. The selected oil ratio was subsequently used for the preparation of the nanoemulsion and nanoemulgel.

Miscibility Study of Essential Oils with surfactant and co-surfactant:

The miscibility study revealed that the selected Tea Tree oil: Turmeric oil blend (7:3) exhibited satisfactory compatibility with the investigated surfactants and co-surfactants. The oil blend showed complete miscibility with Span 20, Tween 20, Tween 60, Cremophor EL, and Labrasol. Among the co-surfactants, Tween 80, PEG 400, propylene glycol, ethanol, and isopropyl alcohol demonstrated complete miscibility. Based on the observed miscibility and suitability for nanoemulsion development, span 20 and Tween 80 were selected for subsequent Smix optimization studies.

Preparation of Smix Using Required HLB:

The required HLB of the combined essential oil phase was determined based on the proportions of tea tree oil and turmeric oil. Since the oil phase consisted of 70% tea tree oil and 30% turmeric oil, having required HLB values of 10 and 9, respectively, the calculated required HLB of the combined oil phase was found to be 9.7. To achieve this required HLB, span 20 (HLB 8.6) and Tween 80 (HLB 15) were selected as the surfactants and co-surfactants. Based on the required HLB calculation, the surfactant mixture was theoretically composed of 82.81% Span 20 and 17.19% Tween 80. For practical formulation development and subsequent optimization studies, an 8:2 (Span 20: Tween 80) Smix ratio was selected as the optimized surfactant combination as shown in the table 3. This Smix composition was subsequently used for construction of Pseudo-Ternary Phase Diagram and for the preparation and evaluation of the essential oil-loaded nanoemulsion formulations.

Step 1: Calculation of Required HLB of Combined Oil:

Oil phase:

- *Melaleuca alternifolia* oil = 70%
- *Curcuma longa* oil = 30%
- Required HLB of *Melaleuca alternifolia* Oil = 10
- Required HLB of *Curcuma longa* Oil = 9

Required HLB Formula

$$\text{RHLB} = \frac{(W_1 \times HLB_1) + (W_2 \times HLB_2) + \cdots + (W_n \times HLB_n)}{W_1 + W_2 + \cdots + W_n}$$

Where:

- **RHLB** = Required Hydrophilic-Lipophilic Balance of the mixed oil phase
- $W_1, W_2, \ldots, W_n$= Weight or percentage of each oil in the oil phase
- $HLB_1, HLB_2, \ldots, HLB_n$= Required HLB value of each individual oil

$$HLB_{req} = \frac{(7 \times 10) + (3 \times 9)}{10}$$

$$HLB_{req} = \frac{70 + 27}{10} = 9.7$$

Step 2: Calculation of Surfactant Ratio

$$A = \frac{100\,(HLB_{req} - HLB_B)}{HLB_A - HLB_B}$$

Where:

- A = % of Tween 80
- HLB_a (Tween 80) = 15
- HLB_β (Span 20) = 8.6
- HLB $_{(req)}$ = 9.7

Calculation: For Tween 80:

$$A = \frac{100(9.7 - 8.6)}{15 - 8.6}$$

$$A = \frac{100(1.1)}{6.4} = 17.19\%$$

Step 3: Final Composition of Surfactant System

- Span 20 = 82.81%
- Tween 80 = 17.19%

Table 3. Optimized Smix Ratio.

Parameter	Value
Required HLB	9.7
Span 20 (%)	82.81
Tween 80 (%)	17.19
Optimized Smix Ratio Selected	8:2

Construction of Pseudo-Ternary Phase Diagram:

Pseudo-ternary phase diagrams were developed utilizing the aqueous titration approach to delineate the nanoemulsion region. This method involved the combination of the oil phase (Tea Tree oil and Turmeric oil in the optimal ratio) and Smix (a mixture of surfactant and co-surfactant) in various weight ratios from 1:9 to 9:1. Each mixture was titrated with distilled water supplied incrementally while maintaining continuous magnetic stirring at room temperature. Following each incremental water addition, the system was visually assessed for alterations in clarity, turbidity, and phase separation. The findings were systematically recorded, and the relevant compositions were plotted on a ternary phase diagram to delineate the nanoemulsion region, as shown in Figure 2.

Figure 2. Pseudo-ternary phase diagram depicting the nanoemulsion region formed by oil, Smix, and water.

Physical stability studies of Nanoemulsion:

Physical stability studies were conducted to evaluate the physical stability of the prepared nanoemulsion formulations under various stress conditions, including freeze-thaw cycles, heating-cooling

cycles, and centrifugation. The results are presented in Table 4.

Freeze-Thaw Cycle:

All the prepared nanoemulsion formulations (F1-F9) successfully passed the freeze-thaw cycle study, as indicated by the absence of any significant instability following repeated temperature stress. No visible evidence of phase separation and other major physical changes was observed. Thus, all formulations demonstrated satisfactory resistance to freeze-thaw stress.

Heating-Cooling Cycle:

The heating-cooling cycle study showed that formulations F2, F3, F4, F5, F6, and F8 successfully withstood the applied temperature cycling conditions. In contrast, formulations F1, F7, and F9 did not pass the heating-cooling cycle study, indicating comparatively lower physical stability under repeated temperature variation. Among the tested formulations, F3-F6 demonstrated satisfactory stability during heating-cooling cycles.

Centrifugation Test:

The centrifugation study demonstrated that formulations F3, F4, F5, and F6 remained stable under centrifugation stress, with no visible evidence of phase separation. In contrast, F1, F2, F7, F8, and F9 did not pass the centrifugation test, indicating comparatively lower resistance to centrifugal stress. Formulations F3-F6 demonstrated stability under all three applied stress conditions, namely freeze-thaw cycles, heating-cooling cycles, and centrifugation. Therefore, these formulations exhibited comparatively better physical stability and were considered suitable for further evaluation.

Table 4. Physical stability studies of Nanoemulsion.

Formulation	Freeze thaw cycles	Heating cooling cycles	Centrifugation studies
F1	✓	×	×
F2	✓	✓	×
F3	✓	✓	✓
F4	✓	✓	✓
F5	✓	✓	✓
F6	✓	✓	✓
F7	✓	×	×
F8	✓	✓	×
F9	✓	×	×

Following physical stability testing, formulations F3-F6 demonstrated satisfactory stability and were selected for further evaluation, while the unstable formulations were excluded. To maintain a consistent formulation coding system for subsequent characterization, F3, F4, F5, and F6 were subsequently re-designated as NE1, NE2, NE3, and NE4, respectively.

Viscosity Measurement:

The viscosity study demonstrated a gradual increase in viscosity from NE1 to NE4 with increasing concentrations of oil and Smix, as presented in Table 5. NE1 exhibited the lowest viscosity, while NE4 showed the highest due to its higher oil and surfactant content. All formulations showed acceptable viscosity suitable for topical application, indicating that Smix concentration significantly influences the rheological behaviour of the nanoemulsion.

Table 5. Viscosity of nanoemulsion.

Formulation Code	Viscosity (cP)
NE1	45.23 ± 2.01
NE2	62.47 ± 1.57
NE3	85.17 ± 1.67
NE4	92.27 ± 2.34

pH Measurement:

The pH study revealed that all nanoemulsion formulations possessed pH values within the acceptable skin-compatible range 5.5-6.8, indicating suitability for topical application. The formulations showed pH values between 5.7 and 6.2, as presented in Table 6.

Table 6. pH value of Nanoemulsion.

Formulation Code	pH Value
NE1	5.9 ± 0.13
NE2	5.8 ± 0.35
NE3	6.2 ± 0.29
NE4	5.7 ± 0.15

Conductivity Test:

The conductivity of nanoemulsion formulations (NE1-NE4) was assessed to ascertain the characteristics of the continuous phase and to verify the type of emulsion produced. Conductivity is a vital criterion for differentiating between oil-in-water (O/W) and water-in-oil (W/O) systems, as O/W nanoemulsion demonstrate elevated conductivity owing to water serving as the continuous phase, hence enhancing ion mobility. The measured conductivity values for the formulations were 169.4 ± 2.1 µS/cm (NE1), 165.7 ± 1.8 µS/cm (NE2), 140.3 ± 2.5 µS/cm (NE3), and 157.6 ± 1.9 µS/cm (NE4) as given in table 7. All formulations demonstrated comparatively elevated conductivity values, affirming that water constitutes the exterior phase, thus indicating the establishment of an oil-in-water (O/W) nanoemulsion.

Table 7. Conductivity test of Nanoemulsion.

Formulation Code	Conductivity (µS/cm)
NE1	169.4 ± 2.1
NE2	165.7 ± 1.8
NE3	140.3 ± 2.5
NE4	157.6 ± 1.9

Droplet Size and Polydispersity Index (PDI)

The droplet size and PDI analysis confirmed the successful formation of nanoemulsion with particle sizes ranging from 116.5 to 334.0 nm as shown in table 8 and figure 3. Formulation NE2 showed the smallest droplet size, while NE3 exhibited the 155.5 Droplet Size and PDI value 0.184, indicating a more uniform and homogeneous system. The NE3 demonstrated the most favourable nanoemulsion characteristics with optimum droplet size and narrow size distribution.

Table 8. Droplet Size and Polydispersity Index (PDI) determination of Nanoemulsion.

Formulation Code	Droplet Size (d. nm)	PDI
NE1	334.0	1.0
NE2	116.5	0.453
NE3	155.5	0.184
NE4	181.6	0.305

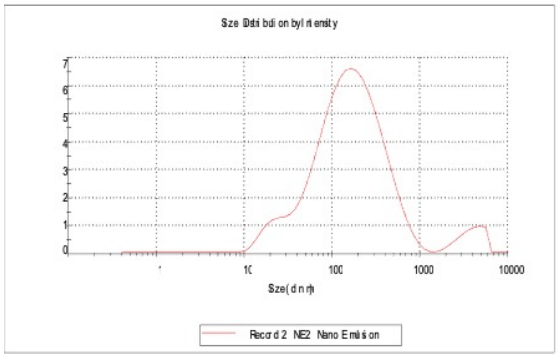


Figure 3. Droplet size distribution and polydispersity index of the optimized nanoemulsion formulations NE1, NE2, NE3, and NE4.

Zeta Potential:

The zeta potential study revealed that the formulations exhibited surface charge values ranging from -8.10 mV to +15.6 mV. Formulations NE2 and NE4 showed positive zeta potential values, while NE1 and NE3 exhibited negative values. Formulation NE3 displayed a single prominent peak with 100% intensity, confirming a uniform and homogeneous droplet distribution without significant aggregation and phase separation as present in table 9 and figure 4. Although the zeta potential values were below the conventional ±30 mV stability limit, the nanoemulsion remained stable due to steric stabilization provided by the non-ionic Tween-based Smix system.

Table 9. Determination of Zeta Potential of Nanoemulsion.

Formulation Code	Zeta Potential (mV)
NE1	−8.10
NE2	+12.9
NE3	−7.36
NE4	+15.6

Figure 4. Zeta potential distribution of the optimized nanoemulsion formulations NE1, NE2, NE3, and NE4.

Transmission Electron Microscopy (TEM)

TEM analysis confirmed the successful formation of nanosized essential oil nanoemulsion droplets in all formulations (NE1-NE4). The micrographs revealed predominantly elongated to elliptical droplets with particle sizes within the nanometric range and relatively uniform distribution. Formulation NE1 showed larger and comparatively clustered droplets, whereas NE2, NE3, and NE4 demonstrated improved dispersion and reduced aggregation as shown in the figure 5. The observed elongated morphology may be attributed to deformation of soft essential oil droplets during TEM sample preparation under vacuum conditions rather than formulation instability. Slight aggregation observed in some regions was likely due to drying and staining effects during sample preparation. The TEM findings confirmed effective emulsification, acceptable nanoscale dispersion, and good stability characteristics of the developed nanoemulsion systems suitable for topical drug delivery applications.

Figure 5. Transmission electron microscopy (TEM) image of the optimized nanoemulsion NE1, NE2, NE3 and NE4 showing the morphology and size distribution of the nanoemulsion droplets.

Based on the results of globule size analysis, polydispersity index (PDI), zeta potential measurement, and transmission electron microscopy (TEM) characterization, formulation NE3 was selected as the optimized nanoemulsion for further formulation development and evaluation. Subsequently, it was redesignated as NEG3 for subsequent studies.

Optimization of Nanoemulgel (NEG):

The optimized nanoemulsion formulation (NEG3) was further incorporated into a gel base for the development of nanoemulgel formulation. Different concentrations of gelling agents were evaluated to obtain a formulation with suitable viscosity, spreadability, homogeneity, consistency, and physical stability. The prepared nanoemulgel were subjected to various evaluation parameters including appearance, pH, viscosity, spreadability, drug content, and stability studies. Final composition of Nanoemulgel presented in table 10. Formulation NEG3 was identified as the optimal formulation for

nanoemulgel preparation based on the outcomes of globule size analysis, polydispersity index, zeta potential measurement, and Transmission Electron Microscopy (TEM) characterization.

Table 10. Final Composition of Nanfoemulgel (NEG3).

S. No.	Ingredients	Final Concentration (% w/w)
1	*Melaleuca alternifolia* Oil	3.5%
2	*Curcuma longa* Oil	1.5%
3	Span 20	14%
4	Tween 80	3.5%
5	Carbopol 934	1%
6	Propylene Glycol	2.5%
7	Sodium Benzoate	0.1%
8	Triethanolamine	Few drops
9	Distilled Water	q.s. to 100%

Physical Evaluation:

The prepared nanoemulgel formulation (NEG3) showed a pale-yellow, smooth, homogeneous, and translucent appearance with good consistency. No grittiness, lump formation, creaming, cracking, or phase separation was observed as presented in the table 11, indicating good physical stability for topical application.

Table 11. Physical Evaluation of Nanoemulgel Formulation (NEG3).

Formulation Code	Color	Homogeneity	Consistency	Phase Separation	Appearance
NEG3	Pale yellow	Homogeneous	Smooth	Absent	Translucent

pH Determination

The pH of the prepared nanoemulgel formulation (NEG3) was found to be 6.4 ± 0.03 as give in the table 12, which is within the normal skin pH range and suitable for topical application. The result indicated good skin compatibility, non-irritant nature, and uniform distribution of formulation components within the gel matrix.

Viscosity Measurement

Table 12 shows that the viscosity of nanoemulgel formulation NEG3 was 8425 ± 35 cP. The viscosity was measured using a Brookfield viscometer with spindle No. 4 at 25 ± 2°C. The formulation showed pseudoplastic (shear-thinning) behaviour, which is advantageous for application, as it allows for easy spreading of the formulation during application, while simultaneously ensuring good retention at the application site, thus demonstrating favourable rheological properties for topical application.

Spreadability

The spreadability of nanoemulgel formulation NEG3 was found to be 14.26 ± 0.41 g·cm/s as give in the table 12, indicating good spreading characteristics. The formulation spread easily with minimal force, ensuring uniform application, better patient compliance, and effective skin contact for improved therapeutic action.

Extrudability

The extrudability value of nanoemulgel formulation NEG3 was found to be 8.15 ± 0.18 g/0.5 min as give in the table 12, indicating good extrusion characteristics. The formulation showed smooth and continuous extrusion without clogging and phase separation, demonstrating suitable consistency, patient convenience, and packaging compatibility.

Table 12. Determination of pH, Viscosity, Spreadability, Extrudability of Nanoemulgel Formulation (NEG3).

Formulation Code	pH	Viscosity (cP)	Spreadability (g·cm/s)	Extrudability (g/0.5 min)
NEG3	6.4± 0.03	8425 ± 35	14.26 ± 0.41	8.15 ± 0.18

Drug Content Study of Nanoemulgel:

The essential oil content of the developed nanoemulgel (NEG3) formulation was determined by UV-Visible spectrophotometric analysis using ethanol as the extraction solvent system. The nanoemulgel samples showed satisfactory essential oil content of 98.84 ± 0.72% for Tea Tree Oil and 97.63 ± 0.65% for *Curcuma longa* oil, indicating uniform distribution of the oil components within the gel matrix, assay, expressed as percentage of theoretical oil loading. Tea Tree Oil nanoemulgel samples were analysed at 268 nm, while *Curcuma longa* oil nanoemulgel samples were analysed at 235 nm for estimation of essential oil constituents.

Drug Release Study:

The in vitro drug release study of the optimized nanoemulgel formulation (NEG3) and pure essential oils was carried out using a Franz diffusion cell to

evaluate and compare their release behavior across the egg membrane. The release profile demonstrated an initial burst release followed by a sustained and controlled release pattern from the nanoemulgel formulation. The optimized nanoemulgel formulation (NEG3) showed a controlled and sustained release pattern of both essential oils from the nanoemulgel compared with their respective pure oils. Tea Tree Oil nanoemulgel showed a gradual increase in cumulative release from 14.63 ± 1.95% at 0.5 h to 76.99 ± 2.50% at 24 h, whereas pure Tea Tree Oil reached 89.35 ± 1.59% at 8 h. *Curcuma longa Oil* nanoemulgel exhibited a gradual release from 11.30 ± 1.34% at 0.5 h to 72.57 ± 2.90% at 24 h, while pure *Curcuma longa Oil* reached 85.21 ± 3.19% at 12 h, as presented in the figure 6 and table 13. The nanoemulgel formulations showed slower and prolonged release, indicating their potential to provide sustained availability of both essential oils for topical application.

Figure 6. Cumulative release profiles of *Melaleuca alternifolia* Oil and *Curcuma longa* Oil from nanoemulgel and their respective oils.

Table 13. Drug release profile of *Melaleuca alternifolia* Oil and *Curcuma longa* oil from the optimized nanoemulgel (NEG3) and their respective pure oils at different time intervals.

Time (hrs)	*Melaleuca alternifolia* NEG (%)	*Melaleuca alternifolia* Oil (%)	*Curcuma longa Oil NEG (%)*	*Curcuma longa Oil (%)*
0.5	14.63±1.95	18.07±2.71	11.3±1.34	16.25±2.94
1	19.71±3.29	45.66±1.25	17.85±2.3	26.13±2.97
1.5	25.43±2.79	60.08±2.41	23.25±2.87	36.5±1.2
2	33.07±2.47	78.23±1.15	29.63±2.19	54.46±3.21
4	46.25±1.46	88.58±3.37	41.78±1.63	71.06±1.56
8	61.78±1.53	89.35±1.59	57.36±2.32	83.19±1.23
12	69.91±1.23	-	68.13±2.68	85.21±3.19
16	75.13±3.01	-	71.45±2.01	-
24	76.99±2.5	-	72.57±2.9	-

Mechanistic Drug Release from the Essential Oil-Loaded Nanoemulgel:

Different kinetic models including Zero-order, First-order, Higuchi, and Korsmeyer-Peppas models were applied to determine the mechanism of drug release from the optimized nanoemulgel formulation (NEG3). The *in vitro* release data obtained for NEG3 were fitted into various release kinetic equations. The results indicated that the developed nanoemulgel formulation followed the Korsmeyer-Peppas model with the highest correlation coefficient (R^2) values for both active constituents. The R^2 values were found to be 0.9876 and 0.9912 for *tea tree oil* and *Curcuma longa oil* respectively, indicating that the release of essential oil constituents from the nanoemulgel was predominantly diffusion-controlled. The release exponent (n) values were observed to be 0.62 and 0.58, which fall strictly within the range of non-Fickian diffusion ($0.45 < n < 0.89$), suggesting a combination of diffusion and matrix relaxation mechanisms governing the release as given in the table 14.

Table 14. Kinetic Models Used for Mechanistic Studies of the Optimized Nanoemulgel (NEG3).

Formulation	Zero Order (R^2)	First Order (R^2)	Higuchi Model (R^2)	Korsmeyer-Peppas (R^2)	n	Best Fit Model
Nanoemulgel (NEG3) - *Melaleuca alternifolia* oil	0.7852	0.8941	0.9523	0.9876	0.62	Korsmeyer-Peppas
Nanoemulgel (NEG3) - *Curcuma longa* oil	0.7645	0.8812	0.9415	0.9912	0.58	

Statistical Analysis:

The comparative in vitro drug release study demonstrated that the optimized nanoemulgel formulation (NEG3) provided a transition from an initial burst release to controlled, sustained delivery of *Melaleuca alternifolia* and *Curcuma longa* essential oils over 24 hours. At 0.5 hours, the differences between the nanoemulgel and pure essential oils were statistically non-significant for *M. alternifolia* (P=0.1561) and *C. longa* (P=0.0827), indicating comparable initial release behavior. However, significant differences emerged between 1.0 and 1.5 hours, with *M. alternifolia* showing a highly significant difference at 1 hour (P=0.0022), while *C. longa* exhibited significant differences at 1 hour (P=0.0210) and highly significant differences at 1.5 hours (P=0.0075). Furthermore, *C. longa* demonstrated a highly significant difference at 12

hours (P=0.0023). The most pronounced differences were observed between 2 and 8 hours, when both essential oils exhibited extremely significant differences ($P \leq 0.001$) compared with their pure counterparts. While the pure oils approached a plateau of approximately 85-89% release, the nanoemulgel maintained a more prolonged release profile, indicating the ability of the polymeric matrix to regulate essential oil diffusion. These findings are consistent with the Korsmeyer-Peppas kinetic model, which indicated anomalous non-Fickian transport involving both diffusion and polymer relaxation. The results demonstrate that formulation NEG3 effectively modulated the release of both essential oils, supporting its potential for sustained topical antifungal delivery.

Post Formulation Antifungal Activity:

The antifungal activity of the developed nanoemulgel (NEG3) was evaluated against *Candida albicans* (MTCC 854) and *Candida parapsilosis* (MTCC 998) and using the agar well diffusion method. The nanoemulgel exhibited a concentration-dependent increase in the zone of inhibition against both fungal strains. Against *C. albicans*, the zones of inhibition at concentrations of 62.5, 125, 250, 500, and 1000 µg/ml were 13.00 ± 1.00, 14.00 ± 1.00, 17.33 ± 2.08, 21.00 ± 1.00, and 25.00 ± 1.00 mm, respectively, while against *C. parapsilosis*, the corresponding zones were 9.67 ± 0.58, 12.00 ± 1.00, 14.67 ± 0.58, 18.33 ± 1.53 and 20.00 ± 1.00 mm, respectively. Fluconazole produced zones of inhibition of 26.33 ± 1.15 mm against *C. albicans* and 22.00 ± 1.00 mm against *C. parapsilosis*, whereas the negative control (Carbopol 934 plain gel base) showed no inhibition (0 ± 0 mm) for either strain. The nanoemulgel demonstrated appreciable and concentration-dependent antifungal activity against both *Candida* species, with comparatively higher inhibition observed against *C. albicans* at all tested concentrations as shown in the figure 7, 8 and table 15 and 16.

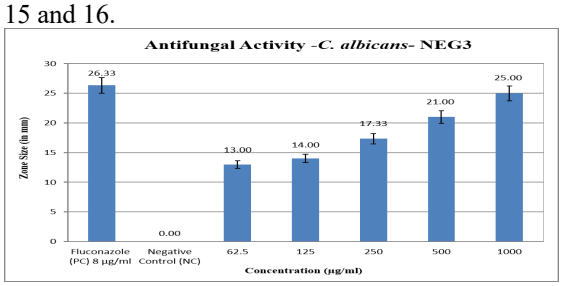


Figure 7. Antifungal activity of the optimized nanoemulgel against *Candida albicans*, Figure 7a show the plates, Figure 7b shows the corresponding graphical results.

Table 15. Antifungal activity of the optimized nanoemulgel against *Candida albicans*.

Formulation NEG3 (µg/ml)	Plate A (mm)	Plate B (mm)	Plate C (mm)	Average Zone of Inhibition
Fluconazole (PC) 8 µg/ml	27	25	27	26.33 ± 1.15
Negative Control (NC)	0	0	0	0.00 ± 0.00
62.5	12	13	14	13.00 ± 1.00
125	14	15	13	14.00 ± 1.00
250	15	18	19	17.33 ± 2.08
500	21	20	22	21.00 ± 1.00
1000	24	26	25	25.00 ± 1.00

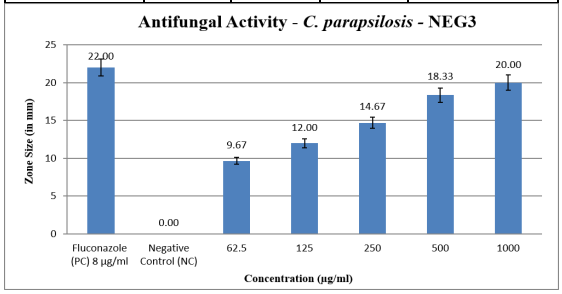


Figure 8. Antifungal activity of the optimized nanoemulgel against *Candida parapsilosis*, Figure 8a show the plates, Figure 8b shows the corresponding graphical results.

Table 16. Antifungal activity of the optimized nanoemulgel against *Candida parapsilosis.*

Formulation NEG3 (µg/ml)	Plate A (mm)	Plate B (mm)	Plate C (mm)	Average Zone of Inhibition
Fluconazole (PC) 8 µg/ml	21	22	23	22.00 ± 1.00
Negative Control (NC)	0	0	0	0.00 ± 0.00
62.5	10	9	10	9.67 ± 0.58
125	12	11	13	12.00 ± 1.00
250	15	14	15	14.67 ± 0.58
500	18	17	20	18.33 ± 1.53
1000	19	20	21	20.00 ± 1.00

Statistical Analysis: The antifungal activity of Fluconazole (8 µg/mL) and the optimized nanoemulgel (1000 µg/mL) was compared against *Candida albicans* and *Candida parapsilosis* using an unpaired Student's t-test. For *C. albicans*, no statistically significant difference was observed between the two groups ($t = 1.512$, $df = 4$, one-tailed $p = 0.1026$), with a mean difference of −1.330 and a

95% confidence interval of −3.773 to 1.113. The F-test indicated no significant difference in standard deviations (F = 1.322, p = 0.8611). Similarly, for *C. parapsilosis*, the difference in antifungal activity was not statistically significant (t(4) = 2.449, two-tailed p = 0.0705), with a 95% confidence interval of −4.267 to 0.2666. The standard deviations also did not differ significantly (F = 1.000, p > 0.9999). Overall, both comparisons showed no statistically significant difference in antifungal activity between Fluconazole and the optimized nanoemulgel at the tested concentrations (p > 0.05). However, these findings indicate a lack of statistically significant differences under the experimental conditions and do not establish therapeutic equivalence.

Minimum Inhibitory Concentration (MIC):

The optimized nanoemulgel (NEG3) demonstrated a potent, concentration-dependent inhibitory effect against both *Candida albicans* and *Candida parapsilosis*. For *C. albicans*, the percentage of organism growth inhibited systematically increased from 0.0% in the negative control 0 µg/mL (Carbopol 934 plain gel base) to 31.5%, 65.3%, and 93.6% at nanoemulgel concentrations of 1.95, 3.91, and 7.81 µg/mL, respectively. Complete growth inhibition (100.0% growth inhibited) was achieved at 15.63 µg/mL and above, establishing the MIC of NEG3 as 15.63 µg/mL (Table 17, Figure 9). Similarly, against *C. parapsilosis*, growth inhibition reached 28.6%, 62.2%, and 91.7% at 1.95, 3.91, and 7.81 µg/mL, respectively. Complete growth inhibition (100.0% growth inhibited) was also observed at 15.63 µg/mL and higher concentrations, indicating an MIC of 15.63 µg/mL (Table 18, Figure 10). The reference antifungal, Fluconazole (8 µg/mL), exhibited complete inhibition (100.0% growth inhibited) against both tested strains under identical experimental conditions. Ultimately, the optimized NEG3 nanoemulgel showed a consistent and effective MIC of 15.63 µg/mL against both tested *Candida* species.

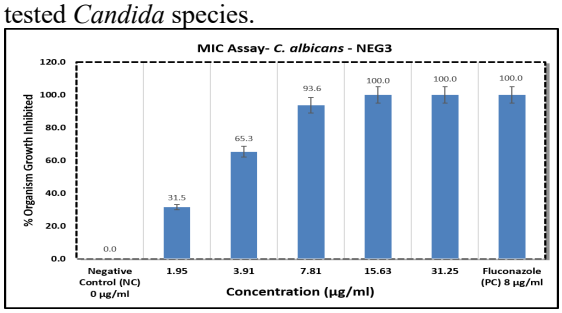


Figure 9. Minimum Inhibitory Concentration (MIC) of optimized Nanoemulgel against *Candida albicans.*

Table 17. Minimum Inhibitory Concentration (MIC) of optimized Nanoemulgel against *Candida albicans.*

S. No.	Nanoemulgel (µg/mL)	% Organism Growth	Calculated % Growth Inhibited
1	Negative control (0)	100.00%	0.00%
2	1.95	68.50%	31.5%
3	3.91	34.70%	65.3%
4	7.81	6.40%	93.6%
5	15.63	0.00%	100.00%
6	31.25	0.00%	100.00%
7	Fluconazole (PC) 8 µg/mL	0.00%	100.00%

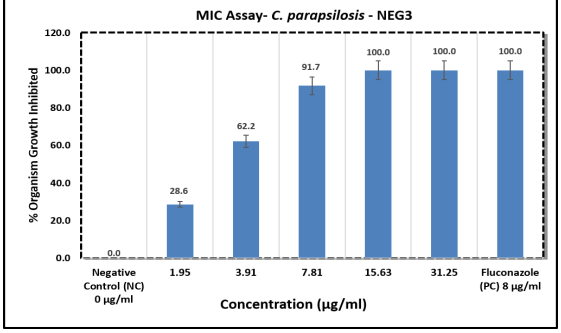


Figure 10. Minimum Inhibitory Concentration (MIC) of optimized Nanoemulgel against *Candida parapsilosis.*

Table 18. Minimum Inhibitory Concentration (MIC) of optimized Nanoemulgel against *Candida parapsilosis.*

S. No.	Nanoemulgel (µg/mL)	% Organism Growth	Calculated % Growth Inhibited
1	Negative control (0)	100.00%	0.00%
2	1.95	71.40%	28.60%
3	3.91	37.80%	62.20%
4	7.81	8.30%	91.70%
5	15.63	0.00%	100.00%
6	31.25	0.00%	100.00%
7	Fluconazole (PC) 8 µg/mL	0.00%	100.00%

Statistical Analysis: The antifungal activities of Fluconazole (8 µg/mL) and the test nanoemulgel at concentration of 15.63 µg/mL and 31.25 µg/mL were compared against *Candida albicans* and *Candida parapsilosis* using one-way analysis of variance (ANOVA), followed by an appropriate post hoc multiple-comparison test. Statistical significance was set at p < 0.05. The ANOVA and post hoc results should be reported separately for each fungal strain, with the F-statistic, degrees of freedom, exact p-values, group means, standard deviations, and 95% confidence intervals for pairwise mean differences. If the comparisons yield p > 0.05, the findings indicate that no statistically significant differences in antifungal activity were detected under the experimental conditions. However, the absence of statistical significance does not, by itself, establish therapeutic equivalence

between the test extract and Fluconazole. Equivalence can only be concluded using a predefined equivalence margin and an appropriate statistical equivalence test.

Skin Irritation Test (HET-CAM Test)

The irritating capacity of the developed nanoemulgel (NEG3) was evaluated using the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) test. The vascular responses of the chorioallantoic membrane (CAM) were observed for 300 seconds following the application of the negative control (0.9% normal saline), positive control (0.1 N NaOH), and the developed nanoemulgel. The onset durations of hemorrhage, vascular breakdown, and clotting were recorded, and the irritant score (IS) was calculated with the standard Luepke scoring methodology. The negative control (0.9% normal saline) had no observable vascular alterations throughout the 300-second observation interval. No evidence of bleeding, vascular lysis, or coagulation was seen, so confirming the integrity of the CAM and validating the experimental procedure. In contrast, the positive control (0.1 N NaOH) provoked an immediate and severe irritation response. Hemorrhage, vascular lysis, and coagulation were clearly observable at 8, 15, and 24 seconds, respectively, resulting in an irritation score of 19.67, classified as a severe irritant. The findings illustrated in figure 11 and table 19 corroborated the sensitivity and dependability of the HET-CAM assay. The prepared nanoemulgel (NEG) exhibited no signs of haemorrhagic response during the 300-second observation interval. The findings indicate that the developed nanoemulgel shows enhanced biocompatibility without any risk of skin irritation and is considered safe for external use. The irritation score in the HET-CAM assay is calculated from the time of onset of three vascular reactions: hemorrhage (H), vessel lysis (L), and coagulation (C). A commonly used formula in the literature is:

$$IS = \frac{301 - H}{300} \times 5 + \frac{301 - L}{300} \times 7 + \frac{301 - C}{300} \times 9$$

Where:

- *(H)* = time of onset of hemorrhage (seconds)
- *(L)* = time of onset of vessel lysis (seconds)
- *(C)* = time of onset of coagulation (seconds)

Figure 11. HET-CAM assay images showing the irritation response of the control samples and optimized nanoemulgel (NEG3) on the chorioallantoic membrane.

Table 19. HET-CAM irritation scores of the control samples and optimized nanoemulgel (NEG3).

Treatment	Hemorrhage (Time in Sec.)	Vessel Lysis (Time in Sec.)	Coagulation (Time in Sec.)	Irritation Score (IS)	Classification
0.9% Normal Saline (Negative Control)	Not observed (300 s)	Not observed (300 s)	Not observed (300 s)	0.00	Non-irritant
0.1 N NaOH (Positive Control)	8	15	24	19.67	Severe irritant
Nanoemulgel (NEG3)	Not observed (300 s)	Not observed (300 s)	Not observed (300 s)	0.00	Non-irritant

Stability Studies:

The optimized nanoemulgel formulation (NEG3) containing *Melaleuca alternifolia* and *Curcuma longa* essential oils was evaluated for stability under real-time storage conditions (25 ± 2°C/60 ± 5% RH) and accelerated storage conditions (40 ± 2°C/75 ± 5% RH) for three months. The formulation was assessed at 0 day, 15 days, 1 month, 2 months, and 3 months for appearance, homogeneity, grittiness, pH, viscosity, spreadability, extrudability, and essential oil content. Under real-time storage conditions, the formulation maintained its smooth, translucent, pale-yellow appearance throughout the study period. Homogeneity was preserved, and grittiness remained absent, indicating satisfactory physical stability without visible phase separation and precipitation. The pH decreased slightly from 6.40 ± 0.03 to 6.37 ± 0.04 over three months, while viscosity decreased from 8425 ± 35 cP to 8392 ± 52 cP. Spreadability showed a minor reduction from 14.26 ± 0.41 to 14.16 ± 0.43 g·cm/s, and extrudability decreased from 8.15 ± 0.18 to 8.09 ± 0.19 g/0.5 min. The essential oil content was also well maintained, with *Melaleuca alternifolia* content decreasing from 98.84 ± 0.72% to 98.42 ± 0.81% and *Curcuma longa* content decreasing from 97.63 ± 0.65% to 97.28 ± 0.73% after three months (Table 20). Under accelerated storage conditions, slightly greater changes were observed in the physicochemical parameters and essential oil content. The formulation retained its smooth, translucent, pale-yellow appearance and remained homogeneous, with no detectable grittiness

throughout the study. The pH decreased from 6.40 ± 0.03 to 6.33 ± 0.05, whereas viscosity decreased from 8425 ± 35 cP to 8340 ± 65 cP. Spreadability declined from 14.26 ± 0.41 to 14.05 ± 0.46 g·cm/s, and extrudability decreased from 8.15 ± 0.18 to 8.02 ± 0.22 g/0.5 min. Regarding drug content, *Melaleuca alternifolia* decreased from 98.84 ± 0.72% to 97.96 ± 0.85%, while *Curcuma longa* decreased from 97.63 ± 0.65% to 96.88 ± 0.77% over the three-month period (Table 21). The optimized nanoemulgel demonstrated satisfactory physical and physicochemical stability under both real-time and accelerated storage conditions over three months. The relatively small variations in pH, viscosity, spreadability, extrudability, and essential oil content, together with the maintenance of appearance, homogeneity, and absence of grittiness, indicate that the formulation retained its principal characteristics throughout the study period.

Table 20. Real-Time Stability Study of optimized nanoemulgel (NEG3).

Parameter	**0 Day**	**Real-Time Stability Study at 25 ± 2°C/60 ± 5% RH**			
		15 Days	**1 Month**	**2 Months**	**3 Months**
Appearance	Smooth, translucent, pale-yellow gel	Smooth, translucent, pale-yellow gel	Smooth, translucent, pale-yellow gel	Smooth, translucent, pale-yellow gel	Smooth, translucent, pale-yellow gel
Homogeneity	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Grittiness	Absent	Absent	Absent	Absent	Absent
pH	6.40 ± 0.03	6.39 ± 0.04	6.39 ± 0.03	6.38 ± 0.05	6.37 ± 0.04
Viscosity (cP)	8425 ± 35	8418 ± 42	8410 ± 40	8401 ± 48	8392 ± 52
Spreadability (g·cm/s)	14.26 ± 0.41	14.24 ± 0.40	14.21 ± 0.42	14.18 ± 0.45	14.16 ± 0.43
Extrudability (g/0.5 min)	8.15 ± 0.18	8.14 ± 0.20	8.12 ± 0.18	8.11 ± 0.21	8.09 ± 0.19
***Melaleuca alternifolia* Drug content (%)**	98.84 ± 0.72	98.75 ± 0.74	98.66 ± 0.78	98.53 ± 0.77	98.42 ± 0.81
***Curcuma longa* Drug content (%)**	97.63 ± 0.65	97.55 ± 0.68	97.47 ± 0.67	97.36 ± 0.70	97.28 ± 0.73

Table 21. Accelerated Stability Study of optimized nanoemulgel (NEG3)

Parameter	**0 Day**	**Accelerated Stability Study at 40 ± 2°C/75 ± 5% RH**			
		15 Days	**1 Month**	**2 Months**	**3 Months**
Appearance	Smooth, translucent, pale-yellow gel	Smooth, translucent, pale-yellow gel	Smooth, translucent, pale-yellow gel	Smooth, translucent, pale-yellow gel	Smooth, translucent, pale-yellow gel
Homogeneity	Homogeneous	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Grittiness	Absent	Absent	Absent	Absent	Absent
pH	6.40 ± 0.03	6.38 ± 0.04	6.36 ± 0.05	6.35 ± 0.04	6.33 ± 0.05
Viscosity (cP)	8425 ± 35	8408 ± 45	8388 ± 50	8365 ± 58	8340 ± 65
Spreadability (g·cm/s)	14.26 ± 0.41	14.21 ± 0.43	14.15 ± 0.47	14.09 ± 0.44	14.05 ± 0.46
Extrudability (g/0.5 min)	8.15 ± 0.18	8.12 ± 0.19	8.09 ± 0.22	8.05 ± 0.20	8.02 ± 0.22
***Melaleuca alternifolia* Drug content (%)**	98.84 ± 0.72	98.64 ± 0.75	98.42 ± 0.80	98.18 ± 0.82	97.96 ± 0.85
***Curcuma long* Drug**	97.63 ± 0.65	97.46 ± 0.69	97.28 ± 0.72	97.06 ± 0.75	96.88 ± 0.77

content (%)					

Statistical Analysis: Statistical analysis of the optimized nanoemulgel (NEG3) was performed using GraphPad InStat to evaluate changes in physicochemical characteristics and essential oil content during real-time storage at 25 ± 2°C/60 ± 5% RH and accelerated storage at 40 ± 2°C/75 ± 5% RH. One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test against the initial Day 0 control, was used to assess differences among storage intervals, with a significance level of $p < 0.05$. Under real-time conditions, no statistically significant differences were observed in pH ($F = 0.428$, $p = 0.785$), viscosity ($F = 0.388$, $p = 0.812$), spreadability ($F = 0.174$, $p = 0.946$), extrudability ($F = 0.095$, $p = 0.982$), *Melaleuca alternifolia* content ($F = 0.224$, $p = 0.918$), and *Curcuma longa* content ($F = 0.187$, $p = 0.939$). Under accelerated conditions, all evaluated parameters remained statistically non-significant, including pH ($F = 1.203$, $p = 0.368$), viscosity ($F = 0.312$, $p = 0.864$), spreadability ($F = 0.155$, $p = 0.956$), extrudability ($F = 0.254$, $p = 0.900$), *M. alternifolia* content ($F = 0.933$, $p = 0.484$), and *C. longa* content ($F = 0.941$, $p = 0.480$). All p-values exceeded the predefined significance threshold of 0.05, indicating that the observed variations were not statistically significant relative to the storage-time effect. These findings support the maintenance of the nanoemulgel physicochemical properties and essential oil content under both storage conditions during the study period. However, the absence of statistically significant differences should be interpreted as a lack of detected change rather than definitive proof of equivalence and complete protection against degradation.

Discussion:

The present study focused on the development and optimization of a polymer-based nanoemulgel containing *Melaleuca alternifolia* and *Curcuma longa* essential oils for potential topical antifungal application. The optimized formulation combined an oil-in-water (O/W) nanoemulsion with a Carbopol 934 gel matrix to facilitate the incorporation and delivery of hydrophobic essential oils. The formulation was evaluated for physicochemical properties, essential oil content, in-vitro release, antifungal activity, irritation potential, and short-term stability. The essential oil blend in a 7:3 ratio was selected based on the required hydrophilic-lipophilic balance of 9.7. Among the developed nanoemulsion, NE3 exhibited a mean droplet size of 155.5 nm and a PDI of 0.184, indicating a relatively uniform droplet distribution. Its zeta potential of −7.36 mV suggested limited electrostatic stabilization, with the non-ionic surfactant system potentially contributing to steric stabilization. Conductivity measurements confirmed the O/W nature of the system, while transmission electron microscopy revealed nanoscale droplets with predominantly elongated-to-elliptical morphology. These findings supported the selection of NE3 for incorporation into the gel matrix. The optimized nanoemulgel (NEG3) exhibited a smooth, homogeneous, translucent, pale-yellow appearance without visible phase separation. Its pH was 6.40 ± 0.03, viscosity was 8425 ± 35 cP, spreadability was 14.26 ± 0.41 g·cm/s, and extrudability was 8.15 ± 0.18 g/0.5 min, indicating satisfactory physicochemical and handling characteristics. The estimated contents of *M. alternifolia* and *C. longa* oils were 98.84 ± 0.72% and 97.63 ± 0.65% of their respective theoretical loadings, suggesting satisfactory analytical recovery. However, chromatographic analysis would provide more specific confirmation of the individual oil constituents and their stability. The in-vitro release study demonstrated prolonged release from NEG3, with 76.99 ± 2.50% of *M. alternifolia* oil and 72.57 ± 2.90% of *C. longa* oil released over 24 h. The Korsmeyer-Peppas model provided the best fit, with correlation coefficients of 0.9876 and 0.9912, respectively. Release exponents of 0.62 and 0.58 suggested anomalous, or non-Fickian, transport involving possible contributions from diffusion and polymer relaxation. These findings indicate that the nanoemulsion-gel system modulated essential oil release under the experimental conditions. However, the egg membrane-based Franz diffusion study did not establish actual skin permeation or retention. NEG3 demonstrated concentration-dependent antifungal activity against *Candida albicans* (MTCC 854) and *Candida parapsilosis* (MTCC 998). At 1000 µg/mL, inhibition zones were 25.00 ± 1.00 mm and 20.00 ± 1.00 mm, respectively. The plain Carbopol gel base showed no inhibition, supporting the association of antifungal activity with the essential oil-containing formulation. The MIC was 15.63 µg/mL against both tested strains, indicating inhibitory activity under the experimental conditions. Nevertheless, comparisons with fluconazole cannot establish equivalent potency, and further testing against a broader range of clinical isolates is necessary. The HET-CAM assay produced an irritation score of 0.00, with no detectable hemorrhage, vascular lysis, or coagulation during the 300-s observation period. This indicated no detectable irritation in the tested model but did not establish comprehensive dermal safety. During three months of real-time and accelerated storage at 25 ± 2°C/60 ± 5% RH and 40 ± 2°C/75 ± 5% RH, respectively, the formulation retained its overall appearance without visible phase separation. No statistically significant changes were reported in the evaluated parameters, supporting short-term stability under the tested conditions. Longer-term studies are required to establish chemical stability and shelf life. The study was

limited by the use of only two reference *Candida* strains, an egg membrane rather than skin for release testing, preliminary irritation assessment, and a three-month stability period. Future investigations should include ex-vivo skin permeation and retention studies, broader antifungal testing, validated chemical stability analysis, comprehensive dermal safety evaluation, and comparative assessment of individual oils, their combination, the nanoemulsion, and the final nanoemulgel. In conclusion, the optimized NEG3 formulation demonstrated satisfactory physicochemical properties, essential oil content, prolonged in-vitro release, antifungal activity against both tested *Candida* species, no detectable irritation in the HET-CAM model, and acceptable short-term stability. These findings support its potential as a topical antifungal formulation platform, warranting further investigation to establish its skin delivery, safety, efficacy, and long-term stability.

Conclusion:

The present study successfully developed and optimized a polymer-based nanoemulgel containing *Melaleuca alternifolia* and *Curcuma longa* essential oils for potential topical antifungal application. The optimized nanoemulsion, selected on the basis of droplet size, PDI, zeta potential, and TEM characterization, exhibited a mean droplet size of 155.5 nm and a PDI of 0.184. Following incorporation into a Carbopol 934 gel matrix, the resulting formulation (NEG3) demonstrated satisfactory physicochemical characteristics, acceptable handling properties, and high measured essential oil content. The formulation exhibited prolonged in-vitro release of both essential oils, with release kinetics best described by the Korsmeyer-Peppas model and release exponents consistent with non-Fickian transport. Concentration-dependent antifungal activity was observed against *C. albicans* and *C. parapsilosis*, with a reported MIC of 15.63 µg/mL for both strains. The HET-CAM assay showed no detectable irritation under the tested conditions, and the formulation maintained its principal evaluated characteristics during three months of real-time and accelerated storage. These findings support NEG3 as a promising formulation for further investigation as an essential oil-based topical antifungal delivery system. However, the current evidence is limited to formulation characterization, in-vitro release and antifungal testing, preliminary irritation screening, and short-term stability assessment. Ex-vivo skin permeation and retention studies, broader antifungal evaluation, comprehensive dermal safety testing, and long-term stability studies are required before its clinical suitability and therapeutic effectiveness can be established.

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