

Formulation and characterisation of a low-surfactant microemulsion of *Perilla frutescens* leaf essential oil

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Abstract: *Perilla frutescens* (L.) Britton leaf essential oil is bioactive, but its pungent odour, volatility, poor water solubility and greasy skin feel limit topical use. This study aimed to develop an oil-in-water microemulsion of perilla essential oil and to characterise its thermodynamic stability and physicochemical properties. Pseudo-ternary phase diagrams were constructed with the oil (5–20%), Tween 20, Tween 80 or their blends ($\leq 50\%$) and water, and glycerol or PEG 400 was then added to the aqueous phase (water:additive 4:1–9:1) to enlarge the microemulsion region. Candidate formulations were screened by dilution, centrifugation, freeze–thaw and 7-day storage tests (4 °C, room temperature, 40 °C) and characterised for pH, conductivity and droplet size (dynamic light scattering). In total, 1,160 compositions were evaluated in 29 phase diagrams. Tween 20 and a Tween 20–Tween 80 (2:1) blend gave the widest microemulsion regions, and both additives enlarged them further. Of 30 dilution-stable formulations, only R1 and R4 (Tween 20–Tween 80 2:1; water–glycerol 4:1) passed all stress tests. Formulation R1 (5% oil, 5% surfactant, 90% aqueous phase) had a pH of 6.91 ± 0.04 , a conductivity consistent with a water-continuous structure and a monomodal intensity distribution peaking at 6.01 nm (PDI 0.243), whereas R4 showed a dominant micrometre-scale population. In an agar well diffusion assay, R1 (2% oil) inhibited *Staphylococcus aureus* and *Candida albicans* more strongly than the oil alone. R1 formed within 1 min in a simple low-energy 20 mL procedure and is a promising low-surfactant vehicle for perilla essential oil, pending chemical characterisation, long-term stability and skin-permeation studies.

Keywords: *Perilla frutescens*; Essential oil; Microemulsion; Pseudo-ternary phase diagram; Polysorbate; Thermodynamic stability; Droplet size

1. Introduction

Perilla frutescens (L.) Britton (Lamiaceae), known in Vietnam as "tía tô", is an aromatic annual herb cultivated throughout East and Southeast Asia and consumed both as a leafy vegetable and as a traditional remedy [1]. In traditional East Asian medicine, the leaves are used to relieve colds, headache and cough and to counteract fish and crab poisoning [2], and the plant is regarded as a medicine–food homologous resource with applications in functional foods, cosmetics and pharmaceuticals [1, 3]. The leaf essential oil, obtained by hydro- or steam distillation, is a complex mixture of monoterpenoids, sesquiterpenoids and phenylpropanoids whose composition is strongly chemotype-dependent; perillaldehyde (PA), perilla ketone (PK), elsholtzia ketone (EK), perillene (PL), citral (C) and several phenylpropanoid (PP) chemotypes have been described [3]. Vietnamese material illustrates this diversity: a PA-type oil from Thai Binh Province contained 62.13% perillaldehyde [4], another Vietnamese sample was dominated by myristicin (43.90%) and elemicin (28.79%) [5], and oils from the Mekong Delta were rich either in limonene and perillaldehyde or in 2-furyl *n*-pentyl ketone [6].

Perilla essential oil and its major constituent perillaldehyde display a broad spectrum of biological activities, many of which are relevant to dermatological use [3]. *Perilla* essential oil inhibited *Staphylococcus aureus* at minimum inhibitory concentrations (MICs) of 1–2 mg/mL by disrupting membrane

integrity [7], and perillaldehyde showed MICs of 2 and 1.67 $\mu\text{L/mL}$ against *S. aureus* and *Escherichia coli*, respectively, together with synergistic anti-biofilm activity [8]. An oil from Can Tho in the Mekong Delta produced inhibition zones of 15.33 mm against *S. aureus* and 16.33 mm against *Cutibacterium* (*Propionibacterium*) *acnes* [6], and perillaldehyde inhibited *Candida albicans* biofilm formation and hyphal transition in a murine model of oropharyngeal candidiasis [9]. Topically applied perilla essential oil (0.01–1% in petrolatum) attenuated imiquimod-induced psoriasis-like lesions and down-regulated IL-17/IL-23 and NF- κ B signalling in mice [10]. These findings support the development of perilla essential oil as a topical active ingredient for infected, inflamed or acne-prone skin.

Translating these activities into acceptable topical products is, however, hampered by the physicochemical and sensory properties of the oil. Like most essential oils, perilla oil is volatile, practically insoluble in water and prone to oxidation, and its strong, pungent odour and oily, greasy feel on the skin reduce user acceptability [11–13]. Burst exposure of the skin to free perillaldehyde may cause irritation [13], occupational allergic contact dermatitis from shiso leaves has been attributed to perillaldehyde and perillyl alcohol [14]. In addition, PK-type oils contain perilla ketone, a pulmonary toxin in livestock, and some phenylpropanoid markers raise genotoxicity concerns, which makes compositional control of the raw oil essential [3]. A delivery system that disperses the oil in water, limits its

evaporation and direct contact with the skin, and improves its skin feel is therefore needed.

Microemulsions are optically isotropic, thermodynamically stable dispersions of oil, water and surfactant, often with a co-surfactant, that form spontaneously and typically contain domains smaller than 100 nm [15–17]. Unlike kinetically stable nanoemulsions, they do not require high-energy processing and should not coarsen during storage [11, 16]. For essential oils, microemulsions solubilise the oil in a water-dispersible vehicle, reduce volatility and oxidative loss, help to mask odour and improve skin feel, while the surfactant film and large interfacial area can enhance cutaneous delivery [11]. Non-ionic polysorbates (Tween 20 and Tween 80) are the most widely used surfactants for this purpose because of their high hydrophilic–lipophilic balance (HLB) and low irritancy, and herbal microemulsions based on them have generally been non-irritating despite relatively high surfactant contents [11, 18]. Recent examples include microemulsions of clove oil [19], peppermint, lavender and eucalyptus oils [20], lemongrass oil [21], and cinnamon, thyme and tea tree oils [22], most of which were designed from pseudo-ternary phase diagrams and screened by thermodynamic stress tests. The extent of the microemulsion region depends on the surfactant type and on co-surfactants or co-solvents such as PEG 400, propylene glycol and glycerol, but these effects are system-specific [23–25].

Several carriers have been reported for perilla essential oil or perillaldehyde, including a high-pressure-homogenised nanoemulsion for psoriasis therapy [12], a lipid nanoparticle hydrogel for wound healing [13] and a hydroxypropyl- β -cyclodextrin inclusion complex [26]. These systems require high-energy processing, phospholipids or complexation. The present paper reports the phase-diagram screening, the physicochemical characterisation and the droplet-size analysis

of such a system and places the formulation in the context of essential-oil microemulsions published between 2020 and 2026. To the best of our knowledge, no low-energy, polysorbate-based microemulsion of perilla leaf essential oil designed from systematic pseudo-ternary phase diagrams has been described. The present work therefore set out (i) to map the microemulsion region of perilla essential oil with Tween 20, Tween 80 and their blends and to evaluate glycerol and PEG 400 as aqueous-phase additives for enlarging it; (ii) to screen the resulting formulations by dilution, centrifugation, freeze–thaw and temperature-storage tests; and (iii) to characterise the pH, electrical conductivity and droplet size of the most stable formulations and to establish a reproducible 20 mL laboratory-scale preparation procedure.

2. Materials and methods

2.1. Materials and equipment

Perilla frutescens (L.) Britton leaf essential oil (Perilla (Shiso) Leaf Essential Oil; INCI: Perilla Frutescens Leaf Oil; CAS 68132-21-8), steam-distilled from the leaves and twigs and originating from China, was obtained from Eden Botanicals (USA). According to the supplier's GC–MS data, the major constituents were perillaldehyde (approximately 44%), limonene (approximately 21%) and β -caryophyllene (approximately 12%), corresponding to the perillaldehyde (PA) chemotype [3]. Polysorbate 20 (Tween 20), polysorbate 80 (Tween 80), polyethylene glycol 400 (PEG 400) and glycerol were of pharmaceutical grade and complied with the manufacturer's specifications (Xilong Scientific, China). Distilled water complied with the Vietnamese Pharmacopoeia V. The equipment used is listed in Table 1. The study was carried out at the Faculty of Pharmacy, Pham Ngoc Thach University of Medicine, Ho Chi Minh City, Vietnam.

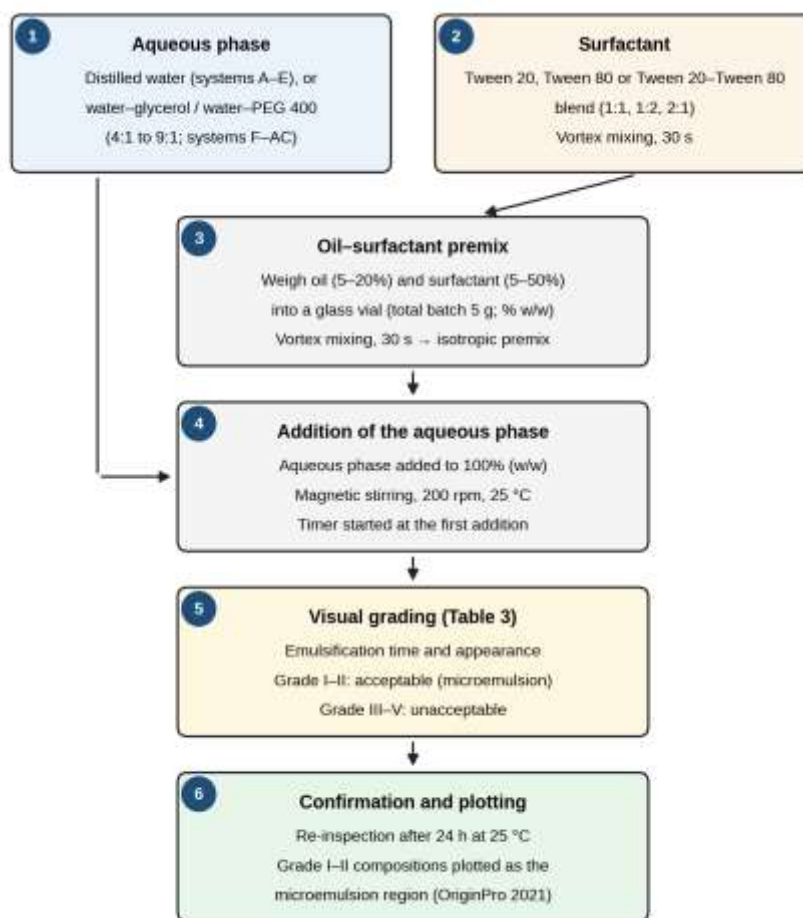
Table 1. Equipment used in the study.

No.	Equipment	Model	Manufacturer (country)
1	Analytical balance	Quintix 224-1S	Sartorius (Germany)
2	Technical balance	Quintix 3102-1S	Sartorius (Germany)
3	Heated ultrasonic bath	S10H	Elma (Germany)
4	Vortex mixer	V1 plus	Boeco (Germany)
5	Micropipette	100–1000 μ L	Vitlab GmbH (Germany)
6	Centrifuge (30-place)	Centrifuge 5420	Eppendorf (Germany)
7	Portable pH meter	pH 150	Eutech Instruments (Indonesia)
8	Conductivity meter	AD331	Adwa (Hungary)
9	Particle size analyser (DLS)	Zetasizer Nano ZS	Malvern Panalytical (UK)
10	Refrigerator	Digital Inverter 243L	Samsung (Korea)
11	Magnetic stirrer	C-MAG HS 7	IKA (Germany)
12	Drying oven (40 °C)	UN55	Memmert (Germany)

2.2. Construction of pseudo-ternary phase diagrams

The microemulsion region was mapped by constructing pseudo-ternary phase diagrams with three components: the oil phase (perilla essential oil), the surfactant (S) and the aqueous phase. To limit the pungent odour of the oil, its concentration was varied from 5% to 20%; to minimise the risk of skin irritation, the surfactant concentration was limited to $\leq 50\%$; the aqueous phase made up the balance. All percentages are expressed as % (w/w). Both variables were changed in 5% steps, giving 40 compositions per diagram. Five surfactant systems (A–E) were investigated (Table 2). Each composition (total mass 5 g) was prepared in a glass vial as outlined in

Scheme 1: the oil and the surfactant (or pre-mixed surfactant blend) were weighed and vortex-mixed for 30 s to give an isotropic premix, and the aqueous phase was then added at 25 °C under magnetic stirring at 200 rpm. The time required for emulsification and the appearance of the system were recorded, and the samples were re-inspected after 24 h at 25 °C. Pseudo-ternary phase diagrams were plotted with OriginPro 2021 (OriginLab Corporation, Northampton, MA, USA). Each composition was graded visually according to the criteria in Table 3 [27]; compositions graded I or II were regarded as lying within the microemulsion region. The system giving the widest microemulsion region was selected for further optimization



Scheme 1. Preparation and visual grading of each composition used to construct the pseudo-ternary phase diagrams (schematic).

Table 2. Composition of the pseudo-ternary systems A–E.

System	Oil phase	Surfactant (S)	Aqueous phase
A	Perilla essential oil	Tween 20	Distilled water
B	Perilla essential oil	Tween 80	Distilled water
C	Perilla essential oil	Tween 20–Tween 80 (1:1)	Distilled water
D	Perilla essential oil	Tween 20–Tween 80 (1:2)	Distilled water
E	Perilla essential oil	Tween 20–Tween 80 (2:1)	Distilled water

Table 3. Visual grading criteria for the systems formed [27].

Grade	Appearance of the system formed
I (acceptable)	Forms within 1 min; transparent or bluish
II (acceptable)	Forms within 2 min; bluish-white
III (unacceptable)	Forms within 2–3 min; milky-white liquid
IV (unacceptable)	Forms in more than 3 min; a few oil droplets visible
V (unacceptable)	Emulsifies with difficulty; oil droplets present

2.3. Enlargement of the microemulsion region with glycerol and PEG 400

Because microemulsion stability can be improved by components that increase the viscosity of the continuous phase and lower the interfacial tension between the two phases, glycerol (a water-miscible polyol that increases viscosity) and PEG 400 (a water-soluble polymer that can act as a co-surfactant) were added to the aqueous phase of the systems selected in Section 2.2. With Tween 20 as the surfactant (system A), systems F–K contained water:glycerol ratios of 4:1, 5:1, 6:1, 7:1, 8:1 and 9:1, and systems L–Q contained the same water:PEG 400 ratios. With the Tween 20–Tween 80 (2:1) blend (system E), systems R–W contained water:glycerol and systems X–AC contained water:PEG 400 at the same six ratios. Phase diagrams were constructed and graded as described in Section 2.2, giving 24 diagrams (960 compositions).

2.4. Screening of thermodynamic stability

Formulations within the enlarged microemulsion regions were subjected sequentially to the following tests [27]:

- *Dilution test*: each formulation was diluted five-fold with distilled water and inspected visually for turbidity or phase separation after 30 min.
- *Centrifugation*: formulations were centrifuged at 5,000 rpm for 5 min (Centrifuge 5420, Eppendorf) (approximately $2,300 \times g$; 25 °C); formulations showing phase separation were rejected.
- *Freeze–thaw cycling*: six cycles of 12 h, each comprising 6 h in a freezer at –20 °C followed by 6 h at room temperature. A formulation passed a cycle if it showed no turbidity or phase separation after that cycle.
- *Storage at different temperatures*: samples were stored for 7 days at 4 °C (refrigerator), at room temperature (25 °C, 50% relative humidity) and at 40 °C (drying oven) and inspected for turbidity or phase separation.

Each test was performed on three replicate samples ($n = 3$), and a formulation was considered to pass only when all three replicates showed no turbidity or phase separation. During storage, samples were inspected after 24 h, 3 days and 7 days.

2.5. Physicochemical characterisation

The pH was measured with a calibrated portable pH meter (pH 150, Eutech Instruments) at 25–28 °C ($n = 3$). Electrical conductivity ($\mu\text{S}/\text{cm}$) was measured with an AD331 conductivity meter (Adwa) at 25–28 °C to identify the

continuous phase [17] ($n = 3$). Droplet size distribution and polydispersity index (PDI) were determined by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Panalytical, UK) at the International University, Vietnam National University Ho Chi Minh City at 25–28 °C using backscatter detection at 173°. Samples were measured undiluted, and each sample was measured three times.

2.6. Laboratory-scale preparation

The selected formulation was prepared at a batch size of 20 mL. The aqueous phase (distilled water and glycerol) was mixed with the surfactants on a magnetic stirrer at 200 rpm, and the oil phase was then added dropwise at room temperature under magnetic stirring at 1,500 rpm, with stirring continued for 15 min. The preparation was evaluated for the time to formation, appearance, odour and phase separation during 7 days of storage at room temperature.

2.7. Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD) of three replicates ($n = 3$). Statistical analysis was performed with IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA). Inhibition-zone diameters of the microemulsion and the neat essential oil were compared with an independent-samples *t*-test, and differences were considered significant at $p < 0.05$.

2.8. Antimicrobial activity

The antimicrobial activity was evaluated by the agar well diffusion method against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Bacterial suspensions adjusted to 0.5 McFarland (approximately 1.5×10^8 CFU/mL) were spread on Mueller–Hinton agar, and *C. albicans* on Sabouraud dextrose agar; wells 6 mm in diameter were then cut in the agar. Each well received 50 μL of the test sample: formulation R1 diluted with distilled water to 2% (v/v) essential oil, or the neat essential oil diluted to 2% (v/v) in dimethyl sulfoxide (DMSO). Ampicillin (0.05 $\mu\text{g}/\text{mL}$; *S. aureus*), cefotaxime (50 $\mu\text{g}/\text{mL}$; *E. coli* and *P. aeruginosa*) and nystatin (100 $\mu\text{g}/\text{mL}$; *C. albicans*) served as positive controls, and DMSO and the oil-free microemulsion vehicle served as negative controls. The plates were incubated at 37 °C for 24 h (bacteria) or at 30 °C for 48 h (*C. albicans*). The diameters of the inhibition zones (including the well) were measured in millimetres ($n = 3$).

3. Results

3.1. Pseudo-ternary phase diagrams of systems A–E

A total of 200 compositions were evaluated to construct the pseudo-ternary phase diagrams of systems A–E (Figure 1).

Systems A (Tween 20) and E (Tween 20–Tween 80, 2:1) produced the widest microemulsion regions and were therefore selected for further optimisation with aqueous-phase additives

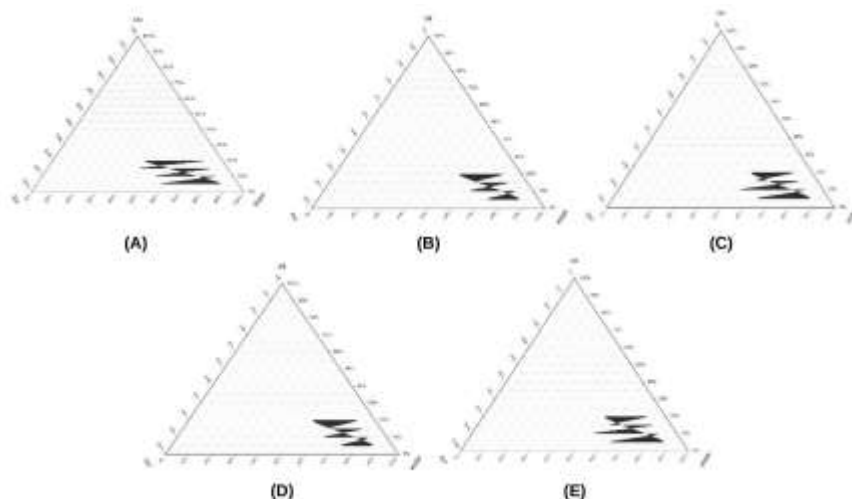


Figure 1. Pseudo-ternary phase diagrams of perilla essential oil, surfactant and water for systems A–E: (A) Tween 20; (B) Tween 80; (C) Tween 20–Tween 80 (1:1); (D) Tween 20–Tween 80 (1:2); (E) Tween 20–Tween 80 (2:1). Shaded areas indicate compositions graded I or II (microemulsion region).

3.2. Effect of glycerol and PEG 400 on the microemulsion region

A further 960 compositions were evaluated in 24 phase diagrams (systems F–AC; Figure 2). The addition of glycerol or PEG 400 to the aqueous phase markedly enlarged the

microemulsion region compared with systems A and E, and water:additive ratios of 4:1 and 5:1 gave faster emulsification than the other ratios. Systems R and X, U and AA, and Z and AB produced identical microemulsion regions. Overall, 1,160 compositions and 29 pseudo-ternary phase diagrams were evaluated.

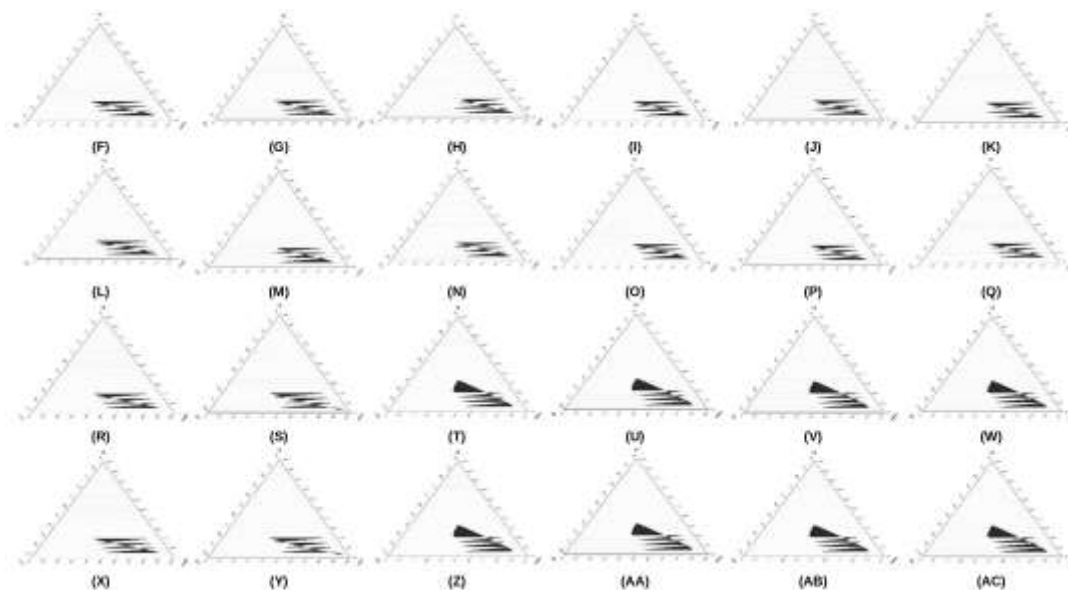


Figure 2. Pseudo-ternary phase diagrams of systems F–AC containing glycerol or PEG 400 in the aqueous phase. Tween 20 as surfactant: (F–K) water:glycerol 4:1, 5:1, 6:1, 7:1, 8:1 and 9:1; (L–Q) water:PEG 400 at the same ratios. Tween 20–Tween 80 (2:1) as surfactant: (R–W) water:glycerol and (X–AC) water:PEG 400 at the same ratios. Shaded areas indicate the microemulsion region. Systems R and X, U and AA, and Z and AB gave identical microemulsion regions.

3.3. Dilution test

The five-fold dilution test was used as a practical screen, because a topical microemulsion is diluted by water on the skin surface and may take up atmospheric moisture during storage in a hot, humid climate such as that of Vietnam. Thirty

formulations withstood dilution without turbidity or phase separation (Table 4). All of them contained 5% oil, 5–25% of the Tween 20–Tween 80 (2:1) blend and a water–glycerol aqueous phase (series R–W); none of the formulations containing PEG 400 or Tween 20 alone passed this test.

Table 4. Formulations that withstood five-fold dilution with distilled water (all with 5% perilla essential oil and Tween 20–Tween 80, 2:1).

Oil (%)	Surfactant (%)	Aqueous phase (%)	R (4:1)	S (5:1)	T (6:1)	U (7:1)	V (8:1)	W (9:1)
5	5	90	R1	S1	T1	U1	V1	W1
5	10	85	R2	S2	T2	U2	V2	W2
5	15	80	R3	S3	T3	U3	V3	W3
5	20	75	R4	S4	T4	U4	V4	W4
5	25	70	R5	S5	T5	U5	V5	W5

Column headings give the series and the water:glycerol ratio of the aqueous phase.

3.4. Thermodynamic stability

Centrifugation

Eight of the 30 formulations (R1, R3, R4 and W1–W5) showed no phase separation after centrifugation, whereas the remaining 22 separated.

Freeze–thaw cycling

R1, R3 and R4 withstood all six freeze–thaw cycles and were selected for the next test, whereas W1 failed from the second cycle and W2–W5 from the third cycle (Table 5).

Table 5. Results of the freeze–thaw cycling test.

Formulation	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
R1	Pass	Pass	Pass	Pass	Pass	Pass
R3	Pass	Pass	Pass	Pass	Pass	Pass
R4	Pass	Pass	Pass	Pass	Pass	Pass
W1	Pass	Fail	Fail	Fail	Fail	Fail
W2	Pass	Pass	Fail	Fail	Fail	Fail
W3	Pass	Pass	Fail	Fail	Fail	Fail
W4	Pass	Pass	Fail	Fail	Fail	Fail
W5	Pass	Pass	Fail	Fail	Fail	Fail

Pass, no turbidity or phase separation after the cycle; Fail, turbidity or phase separation.

Storage at different temperatures

R1 and R4 remained stable for 7 days at all three temperatures, whereas R3 became unstable within 24 h at 40 °C (Table 6). R1

and R4 were therefore selected for physicochemical characterisation.

Table 6. Stability of R1, R3 and R4 during storage at different temperatures.

Formulation	4 °C (refrigerator)	Room temperature (25 °C)	40 °C (oven)
R1	Stable after 7 days	Stable after 7 days	Stable after 7 days
R3	Stable after 7 days	Stable after 7 days	Unstable after 24 h
R4	Stable after 7 days	Stable after 7 days	Stable after 7 days

3.5. Physicochemical properties of R1 and R4

The compositions and physicochemical properties of R1 and R4 are summarised in Table 7. The pH values of R1 and R4 were 6.91 ± 0.04 and 5.39 ± 0.05 , respectively ($n = 3$), both within the range of 5.0–7.0 considered suitable for topical products. The electrical conductivities of R1 and R4 were 10.97 and 29.7 $\mu\text{S}/\text{cm}$, respectively (mean of three measurements); both formulations conducted electricity, indicating that water was the continuous (external) phase.

The DLS results are presented in Figure 3. R1 showed a single, narrow intensity-weighted population with a peak at 6.01 nm (100% of the scattered intensity), a Z-average diameter of 9.58 nm and a PDI of 0.243. The Z-average exceeds the mean

diameter of the single resolved peak, which indicates that part of the scattered intensity arises from material larger than 6 nm that the distribution fit did not resolve; the two values should therefore be read as bracketing the size of the scattering population rather than as a single droplet diameter. In contrast, R4 was bimodal: 92.6% of the scattered intensity was attributed to a population centred at 3,881 nm and 7.4% to a population at 4.47 nm. Cumulant-derived parameters (Z-average and PDI) are not reported for R4, because cumulant analysis is not valid for an intensity distribution spanning three orders of magnitude and the instrument flagged the result quality of this measurement. On the basis of these results, R1 was selected as the most suitable formulation.

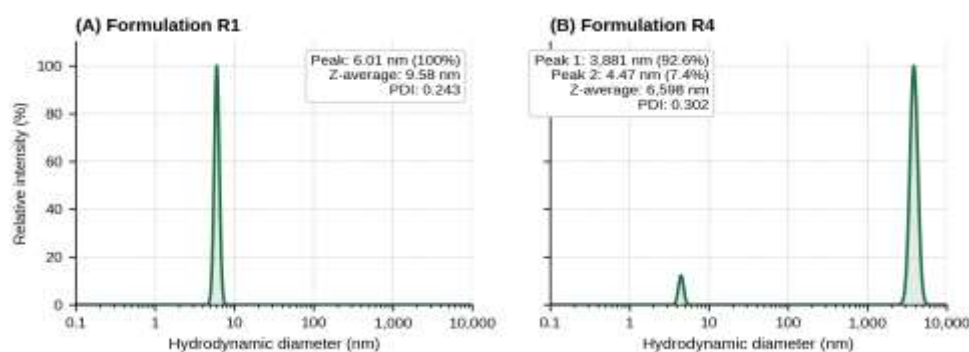


Figure 3. Intensity-weighted droplet size distributions of (A) formulation R1 and (B) formulation R4 measured by dynamic light scattering (redrawn from the peak statistics of the instrument reports: mean diameter, SD and percentage intensity of each peak).

Table 7. Composition and physicochemical properties of formulations R1 and R4.

Parameter	R1	R4
<i>Composition (% w/w)</i>		
Perilla essential oil	5	5
Tween 20	3.33	13.33
Tween 80	1.67	6.67
Distilled water	72	60
Glycerol	18	15
Surfactant:oil ratio	1:1	4:1
<i>Properties</i>		
pH (mean $\pm$ SD, $n = 3$)	6.91 ± 0.04	5.39 ± 0.05
Electrical conductivity ($\mu\text{S}/\text{cm}$)	10.97	29.7
Z-average diameter (nm)	9.58	Not reported ^b
DLS peak(s), nm (% intensity)	6.01 (100)	3,881 (92.6); 4.47 (7.4)
Polydispersity index	0.243	Not reported ^b

Composition values were calculated from the nominal formulations (oil:surfactant:aqueous phase = 5:5:90 for R1 and 5:20:75 for R4; Tween 20:Tween 80 = 2:1; water:glycerol = 4:1). ^bCumulant-derived Z-average and polydispersity index are not reported for R4: the intensity distribution spans three orders of magnitude, for which cumulant analysis is not valid, and the instrument flagged the result quality.

3.6. Laboratory-scale preparation

A 20 mL batch of R1 consisted of 1 mL of perilla essential oil (5%), 666 μ L of Tween 20 plus 334 μ L of Tween 80 (5% surfactant) and 14.4 mL of distilled water plus 3.6 mL of glycerol (90% aqueous phase); the preparation procedure is summarised in Figure 4. Preparation was simple: the

microemulsion formed within 1 min, was transparent, retained the characteristic odour of the oil and showed no phase separation during 7 days of storage at room temperature, indicating that the procedure was simple and yielded a stable preparation at laboratory scale.

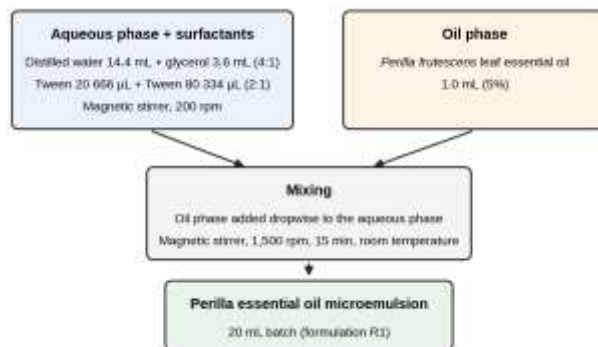


Figure 4. Flow chart of the laboratory-scale (20 mL) preparation of the perilla essential oil microemulsion (formulation R1).

3.7. Antimicrobial activity

The inhibition-zone diameters are presented in Table 8. At 2% (v/v) essential oil, formulation R1 inhibited *S. aureus* (10.3 ± 0.6 mm) and *C. albicans* (9.7 ± 0.6 mm), whereas no inhibition

of *E. coli* or *P. aeruginosa* was observed. The zones produced by R1 were larger than those of the neat oil at the same concentration (8.7 ± 0.6 mm, $p = 0.024$ and 8.3 ± 0.6 mm, $p = 0.047$, respectively). The oil-free vehicle and DMSO produced no inhibition zones, and all positive controls were active

Table 8. Inhibition-zone diameters (mm) of formulation R1, the neat perilla essential oil and the controls determined by agar well diffusion.

Sample	Concentration (volume)	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>
R1 microemulsion	2% (v/v) oil (50 μ L)	10.3 ± 0.6	0	0	9.7 ± 0.6
Perilla essential oil in DMSO	2% (v/v) (50 μ L)	8.7 ± 0.6	0	0	8.3 ± 0.6
Oil-free microemulsion vehicle	– (50 μ L)	0	0	0	0
DMSO	– (50 μ L)	0	0	0	0
Ampicillin	0.05 μ g/mL	32.3 ± 1.2	–	–	–
Cefotaxime	50 μ g/mL	–	45.7 ± 1.5	21.3 ± 1.2	–
Nystatin	100 μ g/mL	–	–	–	29.7 ± 1.2

Values are mean $\pm$ SD ($n = 3$) and include the 6-mm well; 0, no inhibition zone

4. Discussion

4.1. Surfactant selection and phase behaviour

The choice of surfactant is critical when the oil phase is a multicomponent essential oil whose terpenoid and phenylpropanoid constituents differ in polarity and molecular volume [11, 15]. In the present screening, Tween 20 alone (system A) and the Tween 20–Tween 80 (2:1) blend (system E) gave the widest microemulsion regions. Using the nominal HLB values of Tween 20 (16.7) and Tween 80 (15.0) [28], the HLB of the 2:1 blend is approximately 16.1, at the upper end of the range (about 11–16) generally used for essential-oil micro- and nanoemulsions [11]; experimentally determined required

HLB values of essential oils lie at about 10–12 [29]. The benefit of combining Tween 20 with the more lipophilic, bulkier Tween 80 is consistent with more efficient packing of mixed polysorbate films at the oil–water interface, but the direction of the effect depends on the oil. For R-(+)-limonene, the microemulsion region was largest with Tween 80 (82.8%) and smallest with Tween 20 (59.8%) [23], whereas Tween 20 gave the smallest droplets and the best stability for spearmint oil nanoemulsions [28]. The oil used here was of the PA chemotype, with the oxygenated monoterpene perillaldehyde (approximately 44%) as its main constituent; its relatively polar character may favour the more hydrophilic Tween 20, but this

remains a hypothesis to be tested with the individual constituents. Comparable sensitivity to surfactant ratios has been reported for peppermint, lavender and eucalyptus oil microemulsions [20], and increasing the surfactant-to-co-surfactant ratio from 1:1 to 3:1 enlarged the microemulsion region of *Torreya grandis* aril oil from 21.64% to 57.96% of the diagram [30]. In the present study, microemulsion regions were compared visually; reporting the area fraction of each diagram would allow a more objective comparison.

4.2. Role of glycerol and PEG 400

Incorporating glycerol or PEG 400 into the aqueous phase markedly enlarged the microemulsion region. Short-chain alcohols and polyols such as glycerol and propylene glycol can intercalate between surfactant head groups, increase the flexibility of the interfacial film and modify the solvent quality of the aqueous phase, thereby favouring the spontaneous curvature required for microemulsion formation [11, 15]. PEG 400 is widely combined with polysorbates: a Tween 80–PEG 400 ratio of 2:1 gave the widest region for clove oil [19], and a 1:1 ratio gave the largest region for peppermint oil, with no further gain at higher PEG 400 proportions [25]. These effects are nevertheless system-specific: in an alkyl polyglycoside–tea tree oil system, glycerol, propylene glycol and PEG 400 all reduced the microemulsion region at a fixed surfactant level [24]. A notable observation of the present study is that, although both additives enlarged the region, only glycerol-containing formulations (series R–W) withstood five-fold dilution. A plausible explanation is that PEG 400, like other co-surfactant-type additives, partitions between the interfacial film and the continuous phase, so that dilution depletes the interface and shifts the composition out of the single-phase region, whereas glycerol acts mainly as a co-solvent that raises the viscosity of the aqueous phase [11]. This interpretation should be verified by constructing dilution paths on the phase diagrams.

4.3. Thermodynamic stability

The stress tests progressively narrowed the 30 dilution-stable formulations to eight after centrifugation, three after freeze–thaw cycling and two (R1 and R4) after temperature storage. Only formulations containing 5% oil passed the screening, suggesting that at higher oil loads the solubilisation capacity of the surfactant film was exceeded. Bulky sesquiterpenes such as β -caryophyllene (approximately 12% of the oil) penetrate the surfactant palisade layer less readily than small monoterpenes and are therefore more difficult to solubilise [11, 15], which may explain why high-molecular-weight constituents of the oil destabilised the interfacial film under mechanical stress. The non-monotonic centrifugation outcome within series R (R1, R3 and R4 passed, whereas R2 and R5 failed) suggests that some compositions lie close to a phase boundary, where small differences in composition or preparation can determine the outcome; screening with finer composition steps around these points would clarify the boundary. The failure of the W series (water:glycerol 9:1) after one or two freeze–thaw cycles, compared with the survival of R1, R3 and R4 (4:1) over six cycles, is consistent with a cryoprotective role of glycerol,

which depresses the freezing point and limits ice-crystal growth that would otherwise dehydrate and disrupt the surfactant film. The instability of R3 at 40 °C may reflect the temperature sensitivity of polysorbate-based systems, in which progressive dehydration of the polyoxyethylene head groups on heating increases the effective lipophilicity of the surfactant and can drive a phase transition [15].

Compared with recent studies, the stress conditions applied here were relatively mild. Centrifugation for 30 min at 3,000–3,500 rpm [19, 22], six heating–cooling cycles between 4 and 45 °C of at least 48 h each [19, 21], six freeze–thaw cycles between –20 °C and room temperature combined with 12 weeks of storage at 4, 25 and 40 °C [31], and storage for 3–6 months [12, 22, 30] have been used for essential-oil micro- and nanoemulsions. Passing short stress tests does not guarantee long-term stability: a clove oil nanoemulsion that withstood centrifugation, freeze–thaw and heating–cooling cycles still showed an approximately seven-fold increase in droplet size after 30 days at 37 °C [32]. The present stability data should therefore be regarded as a preliminary screen, and long-term studies under ICH storage conditions, with monitoring of droplet size, pH and essential-oil content, are required.

4.4. pH and electrical conductivity

Although R1 and R4 contained the same components, their pH values differed by about 1.5 units (6.91 vs 5.39). The main compositional difference between them is the surfactant content (5% vs 20%), which suggests that the acidity originates mainly from the polysorbates. Polysorbates undergo autoxidation and hydrolysis, generating peroxides and short-chain acids such as formic and acetic acid, and pH decreases of more than one unit within 10 days at 25 °C under light have been observed in unbuffered polysorbate solutions [33]. The higher conductivity of R4 (29.7 vs 10.97 $\mu\text{S}/\text{cm}$) is consistent with a greater content of ionisable degradation products, in line with its lower pH. The pH should therefore be monitored during storage and, if necessary, controlled with a buffer or antioxidant. Skin surface pH ranges from about 4.1 to 5.8 depending on the body site, and topical products with a pH of about 4–6 are generally recommended to support the acid mantle [34]. Thus, R4 was closer to physiological skin pH, whereas the near-neutral pH of R1 is acceptable for a vehicle but may be adjusted when the microemulsion is incorporated into a gel. The pH of R1 is comparable to those reported for microemulsions of clove oil (7.3) [19] and peppermint oil (6.95) [25], and higher than those of lemongrass (5.4–6.2) [21], cinnamon (5.57) [35] and peppermint, lavender and eucalyptus oil (4.25–5.62) microemulsions [20] (Table 9).

The measurable conductivity of both formulations is consistent with a water-continuous structure, but single-point conductivity should be interpreted with caution. O/w microemulsions containing electrolytes typically show conductivities of the order of mS/cm, whereas w/o systems are in the $\mu\text{S}/\text{cm}$ range [17]. However, systems prepared with non-ionic surfactants in distilled water contain few charge carriers, and values of 32.4 $\mu\text{S}/\text{cm}$ for a glycerol-containing cinnamon oil microemulsion [35] and 58.27–87.90 $\mu\text{S}/\text{cm}$ for o/w peppermint, lavender and

eucalyptus oil microemulsions [20] have been reported. The values obtained here fall within the same low range, and their interpretation as o/w systems is supported by the unrestricted dilutability of both formulations with water. Conductivity titration along a water-dilution line, which reveals percolation from w/o through bicontinuous to o/w structures [17], or a dye-solubility test would provide stronger evidence of the microstructure.

4.5. Droplet size

R1 showed a monomodal distribution with a peak at 6.01 nm (Z-average 9.58 nm) and a PDI of 0.243, which is below the threshold of 0.3 commonly considered acceptable for homogeneous lipid-based carriers [36]. Comparison with other systems must be made on a like-for-like basis, because the values in Table 9 are cumulant Z-averages rather than the modes of intensity distributions. On that basis the Z-average of R1 (9.58 nm) is comparable to the 9.3 nm reported for a tea tree oil microemulsion containing 1% oil [24] and to the 10–12 nm of *Torreya grandis* aril oil [30], and smaller than the 13.07–27.20 nm reported for cinnamon, thyme and tea tree oils [22], 17.69 nm for clove oil [19], 18.98 nm for lemongrass oil [21] and 26.75–38.92 nm for peppermint, lavender and eucalyptus oils [20]. It is also much smaller than that of a high-pressure-homogenised perilla oil nanoemulsion (about 180 nm) [12] and of perillaldehyde-loaded lipid nanoparticles (122.5 nm) [13], which is consistent with the spontaneous, thermodynamically driven formation of microemulsions as opposed to the mechanically generated droplets of nanoemulsions [16].

The very small apparent size of R1 nevertheless requires careful interpretation. A peak at about 6 nm is comparable to, or smaller than, the hydrodynamic diameters of polysorbate 20 and polysorbate 80 micelles (approximately 7 and 9 nm, respectively) [37]. Moreover, the surfactant-to-oil ratio of R1 is only 1:1, at the lower limit of the range usually associated with microemulsions (typically above 2) [11]. Two technical factors

may also affect the apparent size. First, DLS derives size from the diffusion coefficient via the Stokes–Einstein equation, which requires the viscosity of the continuous phase [17]; the aqueous phase of R1 contains 20% (w/w) glycerol, for which the viscosity at 25 °C is approximately 1.5 mPa·s [38]; if the viscosity of water (0.89 mPa·s) is used instead, the size is overestimated by a factor of about 1.7. Second, in concentrated systems, interdroplet interactions alter the apparent diffusion coefficient [17]. It therefore cannot be excluded that the measured population partly reflects oil-swollen surfactant micelles. Measurement of the oil-free vehicle, measurements at several dilutions with the correct dispersant parameters, and complementary techniques such as transmission electron microscopy or small-angle scattering are recommended.

For R4, 92.6% of the scattered intensity arose from a population centred at about 3.9 µm, which is incompatible with a microemulsion. Crystallisation of oil constituents, which could increase droplet size, is unlikely for a liquid essential oil at room temperature and remains speculative. Because DLS distributions are intensity-weighted and scattering intensity rises steeply with particle size, a very small number fraction of large scatterers — dust, air bubbles, surfactant aggregates or liquid-crystalline domains formed at the higher surfactant content — can dominate the intensity distribution [36]. The visual transparency of R4 and its survival of all stress tests are consistent with such a minor population. DLS is also unreliable for micrometre-sized particles, which sediment during measurement, and the instrument software flagged the result quality of both measurements (Figure 3). Repeat measurements after filtration, together with microscopy, would clarify the nature of this population. The selection of R1 is supported not only by its monomodal distribution but also by its low surfactant content (5%), which is favourable for skin tolerability [18] and compares favourably with the approximately 15–50% surfactant used in several essential-oil microemulsions [20, 25, 35]

Table 9. Comparison of formulation R1 with essential-oil microemulsions and related carriers reported in 2020–2026.

Essential oil (system)	Surfactant / co-surfactant or co-solvent	Oil (%)	Size (nm)	PDI	pH	Preparation / stability testing	Ref.
<i>Perilla</i> leaf oil (ME, R1; this study)	Tween 20–Tween 80 (2:1); water–glycerol (4:1)	5	9.58 (Z-ave); 6.01 (peak)	0.243	6.91	Low-energy; dilution, centrifugation, 6 freeze–thaw cycles; 7 days at 4 °C/RT/40 °C	–
<i>Perilla</i> oil (NE)	PEG-40 stearyl ether (5%)	5	≈180	<0.2	NR	HPH (1,000 bar × 8); 201.9 nm after 6 months	[12]
Perillaldehyde (lipid nanoparticles)	Soy lecithin–cholesterol	NR	122.5	0.213	NR	Thin-film hydration and sonication; hydrogel	[13]
Tea tree oil (ME)	Triton CG-110–propylene glycol (1.8:1)	1	≈10	NR	NR	≈10 nm after 2 months at 25 °C	[24]

<i>Torreya grandis</i> aril oil (ME)	Tween 40–Tween 80 (9:1); 1-butanol	NR	10–12	<0.3	NR	Unchanged over 180 days at RT	[30]
Cinnamon, thyme, tea tree oils (film-forming ME)	Kolliphor RH40–Transcutol; propylene glycol	0.5–1.0	13.07–27.20	<0.2 5	4.87 – 6.81	Centrifugation; 10 cycles 5/23/40 °C; 3 months ≤25 °C	[22]
Clove oil (ME)	Tween 80–PEG 400 (2:1)	NR ^a	17.69	0.05 7	7.3	Centrifugation 3,500 rpm/30 min; six 4/45 °C cycles	[19]
Lemongrass oil (ME)	Cremophor RH40–PEG 400 (3:1)	1	18.98	0.09 5	5.4– 6.2	Centrifugation; six 4/45 °C cycles; >3 months at RT	[21]
Peppermint, lavender, eucalyptus oils (ME)	Tween 80; ethanol or DGME	7	26.75–38.92	≤0.5	4.25 – 5.62	1 month at 4/25/40 °C	[20]
Cinnamon oil (ME)	Tween 80 (50%); glycerol	13.5	44.51	NR	5.57	5,000 × g/30 min; 90 days at –5/20/45 °C	[35]
Peppermint oil (ME, carvedilol)	Tween 80–PEG 400 (1:1)	10	58	NR	6.95	Centrifugation 6,000 rpm/30 min; heating–cooling; freeze–thaw	[25]

4.8. Limitations

This study has several limitations. First, the chemical composition of the essential oil was taken from the supplier's GC–MS data and was not verified in-house; because the composition, bioactivity and safety of perilla oil vary widely between chemotypes and batches [3], the oil should be analysed by GC–MS and its content in the microemulsion quantified. Second, the phase-behaviour and stability assessments were visual, the microemulsion areas were not quantified, and storage was limited to 7 days. Third, the DLS measurements were flagged by the instrument quality check, and zeta potential, viscosity, transmittance and morphology were not determined. Fourth, the microstructure was inferred from single-point conductivity measurements. Finally, antimicrobial activity was assessed only by agar well diffusion at a single concentration, and no sensory, irritation or skin-permeation evaluation was performed. These limitations should be addressed in future studies aimed at developing a microemulsion-based gel of perilla essential oil.

5. Conclusion

A microemulsion of *Perilla frutescens* leaf essential oil was developed by systematic screening of 1,160 compositions in 29 pseudo-ternary phase diagrams. Tween 20 and a Tween 20–Tween 80 (2:1) blend produced the widest microemulsion regions, and glycerol and PEG 400 enlarged them further, but only glycerol-containing formulations tolerated dilution. After dilution, centrifugation, freeze–thaw and temperature-storage tests, formulation R1 — 5% essential oil, 5% Tween 20–Tween 80 (2:1) and 90% water–glycerol (4:1) — was selected as the most promising. R1 was water-continuous, had a pH of 6.91 ± 0.04 and showed a monomodal DLS population at 6.01 nm (PDI 0.243), and it could be prepared at a 20 mL scale by a simple low-energy procedure. In an agar well diffusion assay, R1 inhibited *S. aureus* and *C. albicans* more strongly than the neat

oil. With its low surfactant content, R1 is a promising vehicle for the topical delivery of perilla essential oil. These findings warrant chemical characterisation of the oil, long-term stability testing and the development of a microemulsion-based gel with ex vivo skin-permeation and antimicrobial evaluation.

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