

STUDIES ON THE FORMULATION, PHYSICAL STABILITY, AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF TEA TREE OIL (*MELALEUCA ALTERNIFOLIA*) NANOEMULSION GEL

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ABSTRACT

Objective: This study aimed to formulate tea tree oil into a nanoemulsion gel dosage form and evaluate its physical stability and antibacterial activity.

Methods: Nanoemulsion gels were formulated with various concentrations of tea tree oil, namely, 5%, 7%, and 9%, using Tween-80 as a surfactant and propylene glycol as a cosurfactant. The tea tree oil nanoemulsion gels showed a stable physical appearance over 8 weeks of storage at low temperature (4±2°C) and room temperature (25±2°C), cycling test, and centrifugation test.

Results: The best formula was nanoemulsion gel formulation 1 (F1), which contained 5% tea tree oil, due to its good stability, smaller globule size, and greater viscosity. The results for antibacterial activity, determined by *in vitro* study, showed that the tea tree oil nanoemulsion gels exhibited antibacterial activity against *Propionibacterium acnes* through the formation of an inhibition zone.

Conclusion: Higher concentrations of tea tree oil in nanoemulsion gels (5%, 7%, and 9%) showed greater mean inhibition zones (28.33±0.88 mm, 30.33±0.33 mm, and 31.67±0.33 mm, respectively).

Keywords: Antibacterial activity, *Melaleuca alternifolia*, Nanoemulsion gel, *Propionibacterium acnes*, Physical stability, Tea tree oil.

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INTRODUCTION

Acne is a common dermatological disease caused by excessive secretion of sebum, which accumulates in the follicles so that the pores swell. Accumulated sebum in the follicle pores represents a good medium for the growth of *Propionibacterium acnes*, which will exacerbate the acne condition [1]. Over the counter acne treatments containing tea tree oil from the plant *Melaleuca alternifolia* are widely available in today's market, and it has been proven that they are a common choice for self-treating acne [2]. Tea tree oil is a natural ingredient used in antiacne products. This is an essential oil obtained from the leaves of *M. alternifolia* [3]. Tea tree oil contains more than 100 chemical compounds, especially monoterpenes and alcohol-bound sesquiterpenes [4]. The main component in this oil is terpinen-4-ol, which exhibits antibacterial activity [5,6]. However, the hydrophobic properties of tea tree oil cause solubility problems in water-based preparations.

A nanoemulsion is a dispersion of oil in water or water in oil that forms nano-sized droplets; it is stabilized by the film layer of surfactant and cosurfactant molecules. The small droplet size of a nanoemulsion allows the system to remain dispersed and prevents creaming, flocculation, or sedimentation during storage [4]. In this study, tea tree oil was formulated in nanoemulsion gel dosage form as an active ingredient for antiacne products. The nanoemulsion gel dosage form can deliver drugs with poor water solubility, and it is expected to be more stable during storage.

MATERIALS AND METHODS

Materials

Tea tree oil (Pioneer Herb Industrial, China), Tween-80 (PT. KAO Indonesia Chemicals, Indonesia), propylene glycol (Dow Chemical Pacific, Singapore), Carbopol 940 (Lubrizol, South Korea), triethanolamine, an API 20 A Kit (BioMerieux, USA), Brucella agar medium, 0.9%NaCl, and distilled water were obtained. The bacteria used in this study were a clinical isolate of *P. acnes* supplied by the Department of Microbiology, Medical Faculty, University of Indonesia.

Methods

Preliminary study

The preliminary study was conducted in advance to obtain a nanoemulsion formulation with a clear appearance (transparent), physical homogeneity, and easy flowability using the aqueous titration method. The study was conducted by varying the ratio of tea tree oil as an oil phase to a mixture of Tween-80 as a surfactant and propylene glycol as a cosurfactant to form a ternary phase diagram. The surfactant and cosurfactant (S-Kos) were mixed at two different weight ratios (1:1 and 2:1). Oil and S-Kos were combined at different weight ratios from 1:9 to 9:1 in different glass vials for each phase diagram. The phase diagrams were constructed using Chemix School software (version 3.60).

Formulation of the tea tree oil nanoemulsion gels

The nanoemulsion was prepared by dissolving Tween-80 in distilled water, and tea tree oil was added to the mixture with constant stirring at 500 rpm to form the coarse emulsion. Then, propylene glycol was added to the mixture and homogenized at 2,000 rpm for 15 minutes. A nanoemulsion with clear appearance formed after a few minutes. In a separate container, Carbopol 940 was dispersed in distilled water and allowed to swell. Triethanolamine was added, and the mixture was homogenized at 100 rpm to form a viscous gel base. The nanoemulsion was added slowly to the base gel, and the mixture was homogenized for 15 minutes to generate the nanoemulsion gel.

Evaluation of the tea tree oil nanoemulsion gels

Physical appearance

The nanoemulsion gel formulations were carefully observed in terms of their form, color, odor, and homogeneity, as well as the occurrence of phase separation or syneresis.

pH measurement

The pH of the formulations was measured using a pH meter (Eutech Instruments, Singapore) in a 1% solution of the sample formulation.

Viscosity and flow properties

The viscosity of nanoemulsion gel was determined using a Brookfield viscometer (Brookfield, USA) at 0.5, 1, 2, 5, 10, and 20 rpm. Each reading on the instrument was multiplied by a correction factor. The viscosity values at 5 rpm were selected. The obtained data were plotted against the shearing stress (dyne/cm) and rate of shear (/second) to determine the flow properties of the sample.

Globule size and zeta potential measurement

About 1 ml of diluted nanoemulsion in a cuvette was measured for globule size using the dynamic light scattering method with a zetasizer nano ZS (Malvern, UK). The nanoemulsion gel was measured for globule size using the same method. Measurement of the zeta potential was also conducted with the same instrument and the additional use of electrodes.

Globule morphology

The morphology and structure of the nanoemulsion were observed using transmission electron microscopy (TEM; JEOL JEM-1010) operating at 80 kV. To perform TEM observation, a drop of diluted nanoemulsion applied on the carbon-coated copper grid and stained with 0.2% uranyl acetate solution, then allowed to dry at room temperature. The coated grid that had already dried was observed under the microscope.

Physical stability test of the tea tree oil nanoemulsion gel

Stability testing at low temperature, room temperature, and high temperature

Samples of tea tree oil nanoemulsion gels were stored at $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 8 weeks. The samples were observed in terms of physical appearance, homogeneity, pH, phase separation, and syneresis every 2 weeks. Measurement of the viscosity and globule size was also performed before and after testing.

The cycling test

Samples were stored at $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 24 hrs and then moved to storage at $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 24 hrs. The test was repeated for six cycles. The physical conditions during the test were compared with the previous conditions and observed for phase separation and syneresis.

Mechanical testing (centrifugation)

Samples were inserted into centrifuge tubes and underwent centrifugation at 3,800 rpm for 5 hrs. The physical conditions of the samples were compared between before and after the test, and they were observed for phase separation and syneresis.

Antibacterial activity test of tea tree oil nanoemulsion gel

P. acnes was cultured in Brucella agar medium and incubated at 37°C for 24-48 hrs in an anaerobic jar. After incubation, bacteria were then identified by observing the colony morphology, Gram staining, and biochemical tests. The inoculum was prepared from the identified

bacteria equivalent to 3 McFarland (10^9 bacteria/ml). Brucella agar medium in a Petri dish was swabbed evenly on the surface with the inoculum using a sterile cotton swab. The well was carefully plugged with agar medium to form a hole. The distance between holes was arranged in such a way as to prevent the areas of observation from overlapping. Each sample was added to the hole at a volume of 50 μl . The Petri dish was then incubated at 37°C for 24-48 hrs in an anaerobic jar. The diameter of the inhibition zone was measured using calipers. The test was performed with three applications (triplicate).

RESULTS

Preliminary study

The results of the phase diagrams from the preliminary study are shown in Fig. 1. The nanoemulsion regions are marked in purple in each phase diagram. These regions were identified based on visual observation of the formulation with clear appearance (transparent), homogeneity, and easily flowability. The formulation that comprised a 2:1 ratio of Tween-80 and propylene glycol (S-Kos) produced a larger nanoemulsion region on the phase diagram and required a lower S-Kos concentration than the 1:1 ratio did. Therefore, a ratio of 2:1 was selected for use in the preparation of the nanoemulsion gel formulations.

Formulation of tea tree oil nanoemulsion gels

The concentrations of tea tree oil that were used in the formulation were 5-10%, as commonly reported in literature [7,8], while the concentrations of Tween-80 and propylene glycol (2:1) used in the formulation were selected from the nanoemulsion regions based on the phase diagrams (Table 1). Varied concentrations of tea tree oil in the formulation were used with the intention of observing the effect on the inhibition zone against *P. acnes* in the antibacterial activity *in vitro* test, while the concentrations of other ingredients remained the same.

Evaluation of tea tree oil nanoemulsion gel

Physical appearance, homogeneity, and pH

Nanoemulsion gel formulations exhibited a homogeneous physical appearance with no phase separation, viscosity, a bright yellow

Table 1: Composition of the tea tree oil nanoemulsion gel

Ingredients	Concentration % (w/v)		
	F1	F2	F3
Tea tree oil	5	7	9
Tween-80	30	30	30
Propylene glycol	15	15	15
Gel base	20	20	20
Carbopol 940 (1.0%)			
Triethanolamine (0.1%)			
Distilled water	to 100	ad 100	to 100

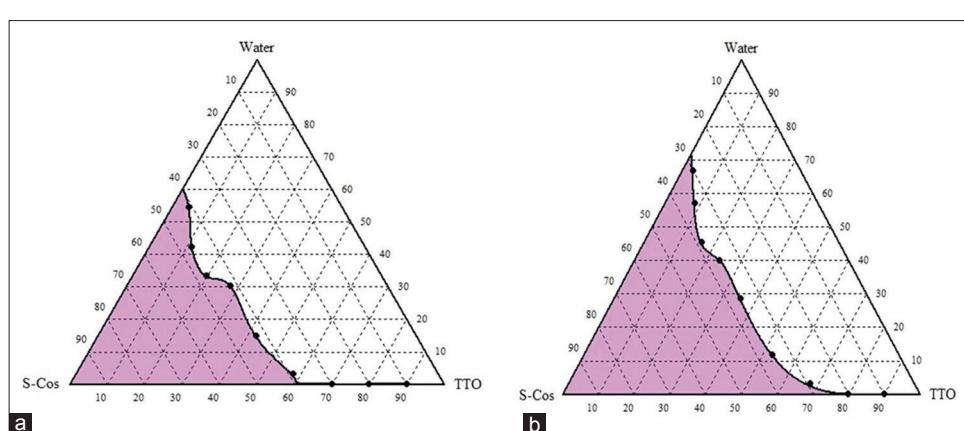


Fig. 1: (a and b) Ternary phase diagrams of tea tree oil, Tween-80/propylene glycol, and water

color (Pantone PMS 394), and the characteristic odor of tea tree oil. Nanoemulsion gel formulation 1 (F1) showed a clear physical appearance, while F2 and F3 were translucent (Fig. 2). The pH values of nanoemulsion gels F1, F2, and F3 were 6.11, 6.02, and 5.97, respectively. The pH study showed that pH tended to decrease or become more acidic with an increase in the tea tree oil content. Overall, the pH values were still within the range of skin pH (4.5–6.5).

Viscosity and flow properties

The results of the viscosity study for nanoemulsion gels F1, F2, and F3 were 8,200 centipoise, 7,100 centipoise, and 6,400 centipoise at 5 rpm. Nanoemulsion gel F1 had a slightly higher viscosity than F2 and F3 did, which may be associated with the pH value, as the carbomer gel base showed greater swelling in the context of a pH value that was close to neutral. All formulations of nanoemulsion gel were revealed to have pseudoplastic flow properties.

Globule size and zeta potential measurement

Globule size measurement was performed for all formulations of nanoemulsion and nanoemulsion gel. The results showed that the globule size of all formulations was in the nano range (100–500 nm). Globule size increased with the increased concentration of tea tree oil in the formulations. This was due to the increasing amount of the internal dispersed phase, resulting in the formation of an increasingly large globule size. A polydispersity index (PDI) value lower than 0.7 indicated the uniformity of the globule size distribution. A low PDI value was observed for all nanoemulsion formulations. Globule size and zeta potential of tea tree oil nanoemulsion are described in Table 2.

Zeta potential measurement was also conducted in this study, which is a measure of the magnitude of electrostatic potential or particles' surface charge. The zeta potential is an important parameter in determining the stability of a dispersion system and determining the possibility of flocculation or aggregation in the emulsion and suspension system [9]. Particles with zeta potential values higher than (\pm) 30 mV indicate a stable dispersion system because they have a surface charge that can prevent aggregation [10]. Zeta potential values were observed for nanoemulsions F1, F2, and F3, indicating that not all nanoemulsion formulations were completely stable (Table 3).

Globule size measurement was also performed for nanoemulsion gel preparations to compare the globule sizes of nanoemulsions before and after the addition of the gel base. The results showed an increase in the size of the globules in the nanoemulsion gels (Table 3). This may have been because the nanoemulsion globules were covered by the carbomer gel matrix, resulting in larger globule sizes.

Globule morphology

Morphological observation of nanoemulsion globules was conducted on nanoemulsion F1, which was considered an optimal formulation; it was more stable because of its smaller globule size and higher zeta potential value. The results showed that the globules in the nanoemulsion appear evenly dark with a spherical form (Fig. 3).

Physical stability testing of tea tree oil nanoemulsion gel

Stability testing at a low temperature, room temperature, and high temperature

Nanoemulsion gel formulations were stored at $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 8 weeks. After 8 weeks of storage, at the low temperature ($4^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and room temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$), all formulations showed stable physical appearance, no change in color or odor, homogeneity, and no phase separation or syneresis. Under high temperature ($40^{\circ}\text{C} \pm 2^{\circ}\text{C}$) storage, nanoemulsion gels F1 and F2 also showed stable physical appearance, while nanoemulsion F3 showed phase separation at the top of the preparation. This physical instability in nanoemulsion gel F3 was alleged to be creaming. Creaming occurs when the dispersed globules merge and rise to the top of the emulsion to form a flocculate

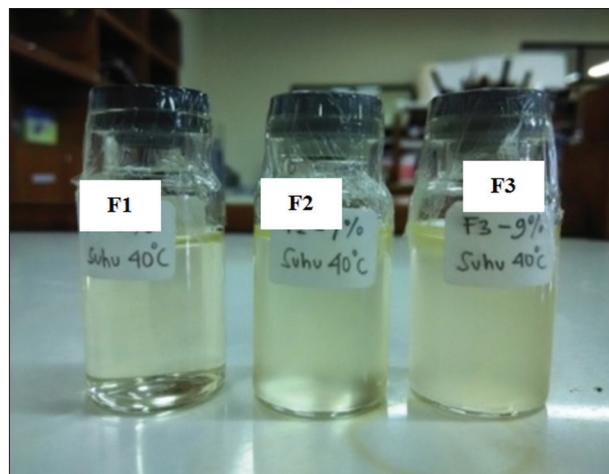


Fig. 2: Physical appearance of tea tree oil nanoemulsion gel

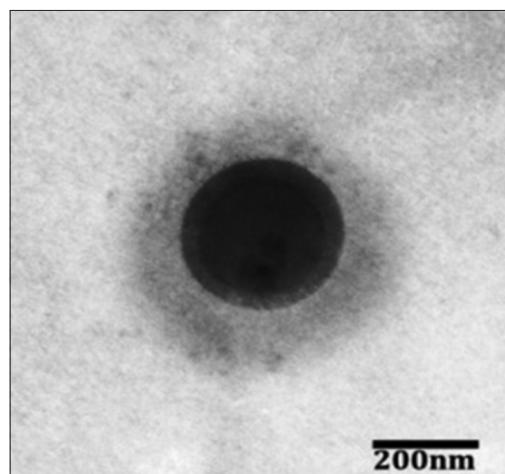


Fig. 3: Transmission electron microscopy image of a globule in nanoemulsion F1

Table 2: Globule size and zeta potential of tea tree oil nanoemulsion

Evaluation	F1	F2	F3
Globule size (nm)	166.7	169.9	188.2
PDI	0.476	0.481	0.579
Zeta potential (mV)	-24.8	-20.3	-19.2

PDI: Polydispersity index

Table 3: Comparison of nanoemulsion and nanoemulsion gel globule sizes

Sample	Globule size (nm)	
	Nanoemulsion	Nanoemulsion gel
F1	166.7	173.0
F2	169.9	201.4
F3	188.2	305.7

layer. Continuous exposure of the formulation to high temperature could alter the effectiveness of surfactant and decrease the viscosity of the continuous phase, causing oil globules to merge and form a flocculate or creaming layer. However, in this case, the formulation could be easily redispersed by mild agitation; thus, the separation was reversible, as the oil globules were still coated with the emulsifier layer.

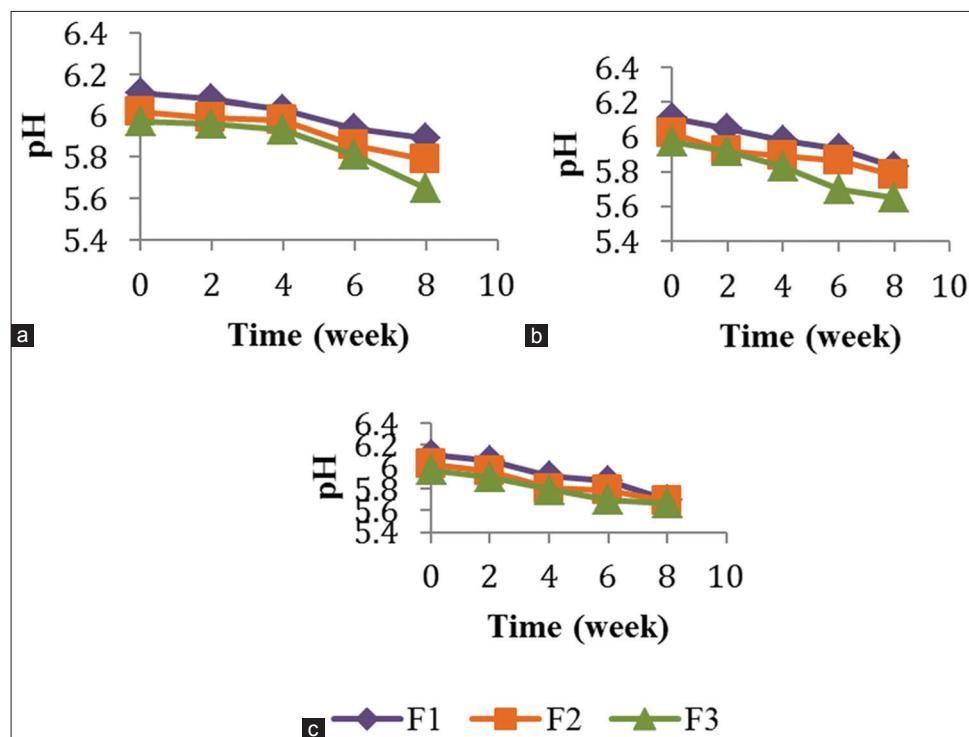


Fig. 4: pH of nanoemulsion gel formulations during 8 weeks of storage: (a) Low temperature, (b) room temperature, and (c) high temperature

In the pH study, the nanoemulsion gel formulations showed a decrease in pH during 8 weeks of storage, as illustrated in Fig. 4. This may have been due to tea tree oil being released from the gel matrix and undergoing oxidation, thereby increasing the acidity of the formulations. However, the pH values of all formulations during 8 weeks of storage were still within the range of skin pH. The result of this study is in line with previous thermal stability studies which showed that the formulations were stable for more than 8 months [11].

The viscosity study revealed that the viscosities of all formulations decreased after 8 weeks of storage at room temperature, reaching 7,000 cps, 5,500 cps, and 4,700 cps, at 5 rpm. This could be attributed to a decrease in pH (increased acidity) in the formulations that affected the viscosity of the carbomer gel; carbomer is a pH sensitive gelling agent that will decrease viscosity at a lower pH level. Meanwhile, the flow properties of all formulations remained pseudoplastic. During 8 weeks of storage, an increase in the globule size of all nanoemulsion gel formulations was observed in Table 4. This may have been due to the merging of oil globules to form a group of globules, thereby causing an increase in the size of the measured globules. The merging of these globules can also be attributed to the zeta potential values of all formulations, which were smaller than (\pm) 30 mV so that the repulsive force between globules was not strong enough to prevent merging.

The cycling test

Six cycles between temperatures of $4\pm 2^\circ\text{C}$ and $40\pm 2^\circ\text{C}$ for 48 hrs in storage were performed. In the results obtained after the cycling test, all formulations showed stable physical appearance and no phase separation, syneresis, or other forms of instability. It can be assumed that the amount of surfactant in the formulations was able to stabilize the emulsion system, and as a gelling agent, the carbomer was able to retain water in the gel matrix so that the nanoemulsion gel remained stable.

Mechanical testing (centrifugation)

Nanoemulsion gel formulations were subjected to centrifugation study, where they were made to undergo centrifugation at 3,800 rpm for 5 hrs. The results of this study showed that all formulations remained stable and did not show any phase separation or syneresis. The addition of gel

Table 4: Globule size of nanoemulsion gel formulations after 8 weeks of storage

Sample	Globule size (nm)	
	0 weeks	8 weeks
F1	173.0	191.2
F2	201.4	249.0
F3	305.7	314.9

Table 5: Inhibition zone diameter of the sample in the antibacterial activity test

Sample	Inhibition zone diameter (mm)			
	1	2	3	Mean \pm SD
F1	30	28	27	28.33 \pm 0.88
F2	31	30	30	30.33 \pm 0.33
F3	31	32	32	31.67 \pm 0.33
TTO	38	38	39	38.33 \pm 0.33
BP	13	14	14	13.67 \pm 0.33
DW	8	8	8	8.00 \pm 0.00

The inhibition zone diameter includes the hole diameter (8 mm),

F1: Nanoemulsion gel formula 1, F2: Nanoemulsion gel formula 2,

F3: Nanoemulsion gel formula 3, TTO: Tea tree oil, BP: 5% benzoyl peroxide gel (positive control), DW: Distilled water (negative control), SD: Standard deviation

base to the nanoemulsion preparation would increase the viscosity of the continuous phase so that the nanoemulsion gel became more stable. There was also no phase separation observed because of the amounts of emulsifiers used in the formulations, which were able to maintain globules inside the surfactant layer.

Antibacterial activity test of tea tree oil nanoemulsion gels

Bacterial identification was conducted to ensure that the bacteria used in the test were *P. acnes*. In observation of the colony morphology of the

bacteria, *P. acnes* growing on the Brucella agar medium formed small, circular, smooth, and yellow-white color colonies. In Gram staining and microscopic observation of bacteria, the result was purple, which means that the bacteria were Gram-positive and had a small, irregular rod shape. Identification of bacteria by the biochemical testing method was performed using the API 20 A system. Panel API 20 A consists of 20 microtubes containing various dehydrated substrates for testing of 21 biochemical reactions. The results for the identification of bacteria by biochemical testing showed that bacteria could be identified as *P. acnes* with 96% identity. Previous *in vitro* study showed that the tea tree oil was more active to Gram-positive than to Gram-negative anaerobic bacteria [12]. The results of antibacterial activity testing showed that tea tree oil and all nanoemulsion gel formulations containing tea tree oil could inhibit the growth of *P. acnes* through the formation of a clear zone around the hole. This is because of the terpinen-4-ol compound in the tea tree oil, which exhibits antibacterial activity [13]. This study showed that the diameter of the inhibition zone increased with the increase in tea tree oil concentration in the formulations. Statistical testing showed that there was a significant difference between the test group (nanoemulsion gels F1, F2, and F3) (Table 5), with a significance value of 0.041 ($p \leq 0.05$). This demonstrated that a higher concentration of tea tree oil in the formulation would have greater ability to inhibit the growth of *P. acnes*, as indicated by the increase in the inhibition zone.

CONCLUSION

Nanoemulsion gel formulations containing 5% (F1), 7% (F2), and 9% (F3) tea tree oil showed stable physical appearance over 8 weeks of storage at low temperature ($4 \pm 2^\circ\text{C}$) and room temperature ($25 \pm 2^\circ\text{C}$), cycling test, and the centrifugation test. However, under high temperature storage ($40 \pm 2^\circ\text{C}$), F3 showed instability. The best formula was nanoemulsion gel F1, as it had good stability, a smaller globule size, and greater viscosity. The results of *in vitro* testing for antibacterial activity showed that the tea tree oil nanoemulsion gel formulations exhibited antibacterial activity against *P. acnes* through the formation

of inhibition zones. Higher concentrations of tea tree oil in the nanoemulsion gel (5%, 7%, and 9%) showed greater mean inhibition zones (28.33 ± 0.88 mm, 30.33 ± 0.33 mm, and 31.67 ± 0.33 mm).

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