Short Communication:

Pseudoternary Phase Diagram and Antibacterial Activity of Microemulsion-Based Citronella Oil

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Abstract: *Citronella oil (CTO) is extracted from citronella leaves by maceration or steam distillation process, which has antibacterial and insect-repellent activities. However, the use of CTO is limited and requires modification in other formulations, such as microemulsion (ME), to increase its bioactivities. ME consists of oil, water, surfactant and/or cosurfactant and is commonly applied in food and beverages, cosmetics, and carrier for drug delivery applications. CTO was used as the oil phase for ME with nonionic surfactant and ethanol as a cosurfactant for lowering interfacial tension between oil and water phase. Subsequent observations regarding stability and antibacterial tests were carried out on ME formulations with surfactant/cosurfactant mixture of 2 due to its largest ME area. A hydrodynamic diameter analysis was also carried out to see the stability of the ME within a period of 50 d. ME with 10% CTO, 30% surfactant mixture, and 60% water showed the best formulation observed from the consistent hydrodynamic diameter measurement. In addition, ME with different formulations could inhibit the growth of* Escherichia coli *and* Staphylococcus aureus *by more than 90%. From this research, CTO-based ME potentially improve and develop drug carrier applications, especially via topical route.*

Keywords: *microemulsion; citronella oil; Tween 80; pseudoternary phase diagram; antibacterial*

■ INTRODUCTION

Microemulsion (ME) is a transparent mixture, homogeneous, and has low interfacial tension between oil and the aqueous phase. The hydrodynamic diameter of ME is varied and is commonly less than 300 nm [1-2]. ME is thermodynamically stable and contains three main components: oil, surfactant and/or cosurfactant, and aqueous phase which mostly consists of water. The oil phase of ME is mostly vegetable and essential oils, such as soybean oil [3], sunflower oil [4], olive oil [5], castor oil [6], and citronella oil (CTO) [7]. The application of ME varies from enhanced oil recovery to a carrier for drug delivery.

Citronella plants grow abundantly in tropical climates in America, Asia, and Africa [8]. Indonesia is known as one of the largest producers of CTO in the world, which can fulfill ~40% of the world's CTO needs. CTO mainly consists of geraniol, citronellal, citronellol, and eucalypt-*p*-menthane-3,8-diol [9-11]. The demand

and consumption of CTO continue to increase because it has various advantages, such as insect repellent [10], antioxidant [12], antimicrobial [13-14], and antiaging [15]. CTO has been proven as an antibacterial agent against *Staphylococcus aureus* to reduce and inhibit up to 12.5% growth of bacteria [13]. In addition, CTO oil was able to inhibit growth and kill fungi *Fusarium subglutinans* [16], *Pseudocercospora*, *Streptomyces acidus*, and *Solanaceae Ralstonia* [13]. However, the physical properties of CTO, such as volatility and solubility are undesirable thus, improvement is required to enhance its activities [12]. A study by Oh et al. [17] demonstrated the combination of steam phases of citronella and lemongrass oil, resulted in the slowest growth rate of *S. aureus* compared to other essential oils, such as cinnamon oil, oregano oil, thyme oil, and carrot seed oil. The application of CTO in steam phases is intractable compared to liquid phase formulation. Therefore, a combination of CTO with another liquid component has been conducted to enhance its stability.

Nanocomposite formulated from zinc oxide and CTO resulted in higher antimicrobial against both *Escherichia coli* and *S. aureus* compared to the CTO alone [18]. Another study by Gharsan et al. [19] showed modification of CTO with Tween 80 and water that enhanced the effectiveness as a chemical pesticide to protect stored products. The development and application of ME-based CTO have been investigated in recent years. Physical properties from the oil phase, surfactant, cosurfactant, and aqueous phase induced the thermal stability of ME [20]. The usage of CTO as an oil phase in ME was studied by Sieniawska et al. [21] with the combination of soybean oil and Tween 80 as surfactant. This formulation had higher antioxidant activity and was stable for 7 d compared to mint oil and eucalyptus as oil phase [21].

In earlier work in formulating CTO oil modification with Tween 80 and water, only one formulation was chosen in the application of pesticides towards the sawtoothed grain beetle *Oryzaephilus surinamensis* [19]. However, different formulations with the various compositions of CTO, surfactant mixture, and water have not been explored. To provide the development of CTO-based ME, the pseudoternary phase diagram between CTO, surfactant mixture, and the aqueous phase was investigated in this study. The pseudoternary phase diagram aims to classify the phase synthesized at each point of formulation. The antibacterial activities toward two most common bacteria, *E. coli* and *S. aureus* were conducted. In addition, this study also investigated the stability of formulated ME at 30 °C for 50 d for further application as drug delivery.

■ EXPERIMENTAL SECTION

Materials

CTO was obtained from an essential oil supplier in West Java, Indonesia. Tween 80 and Tween 20 purchased from Sigma-Aldrich (USA) were used as surfactants, and ethanol (99.9%) supplied by Merck (Germany) was used as cosurfactant in the formulations. Nutrient broth (NB) obtained from Merck (Darmstadt, Germany) was used as the growth media of tested bacterial strains.

Instrumentation

The hydrodynamic diameter, polydispersity index (PDI), and zeta potential values of various formulations were determined by Zetasizer Nano Series Nano-ZS, Malvern at 25 °C at a fixed angle of 90°, and the data processed by Zetasizer 7.01 software. To prevent multiple scattering, the samples were diluted 100-fold with DI water. For antimicrobial activity, UV-vis spectrophotometer, (Shimadzu UV-1700) was used to determine the concentration of microbes.

Procedure

Construction of pseudoternary phase diagram

Either Tween 80 or Tween 20 as surfactant was mixed with ethanol as cosurfactant in three different weight ratios (S_{mix}, wt.%) of 1:2, 2:1, and 3:1. CTO as oil phase was added into the S_{mix} at predetermined weight ratios (wt.%; 10:1, 8:1, 6:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:6, 1:8, 1:10, and 1:50) and continued by titration of DI water as the aqueous phase (AP) based on previous study [22]. The pseudoternary phase diagrams were plotted and the percentage of ME area could be calculated by Eq. (1).

The ME was determined based on the physical appearance, which is one phase, transparent, and homogeneous;

$$\text{Area } (\%) = \frac{A}{0.5} \times 100 \quad (1)$$

where A is the ME area obtained from Origin software.

Stability of citronella oil-based ME

The S_{mix} with the largest ME area in pseudoternary phase diagram was selected for further stability analysis. Four formulations were selected and stored at 30 °C for 50 d. The hydrodynamic diameter, PDI, and zeta potential of MEs were measured on day 0, 20, and 50.

Antibacterial of formulated MEs

Effects of antibacterial activity on ME-based CTO were evaluated against two bacterial strains, *E. coli* and *S. aureus*. Each strain was inoculated in NB medium, containing peptone and meat extract at 37 °C for 24 h. The final concentration of the cultured medium was adjusted to obtain absorbance of 0.5 at 600 nm by UV-vis spectrophotometer. A sterile Erlenmeyer flask was prepared for the assay. The antibacterial was done by dilution of each formulated MEs in NB medium to reach a concentration of 75 µL/mL, then added 10% (v/v) of bacterial suspension and incubated for another 24 h. The absorbance of final mixtures, containing diluted ME formulation and bacterial suspension were observed by UV-vis spectrophotometer, and inhibition of bacterial growth from each MEs formulation was calculated and plotted.

■ RESULTS AND DISCUSSION

Pseudoternary Phase Diagram of Citronella Based ME

The physical appearance of formulations was observed to determine and plot the pseudoternary phase diagram. A transparent, one-phase system and homogeneous mixture is classified as ME. Three different S_{mix} (1:2, 2:1, and 3:1) were carried out to observe the effect of Tween 80 or Tween 20 and ethanol

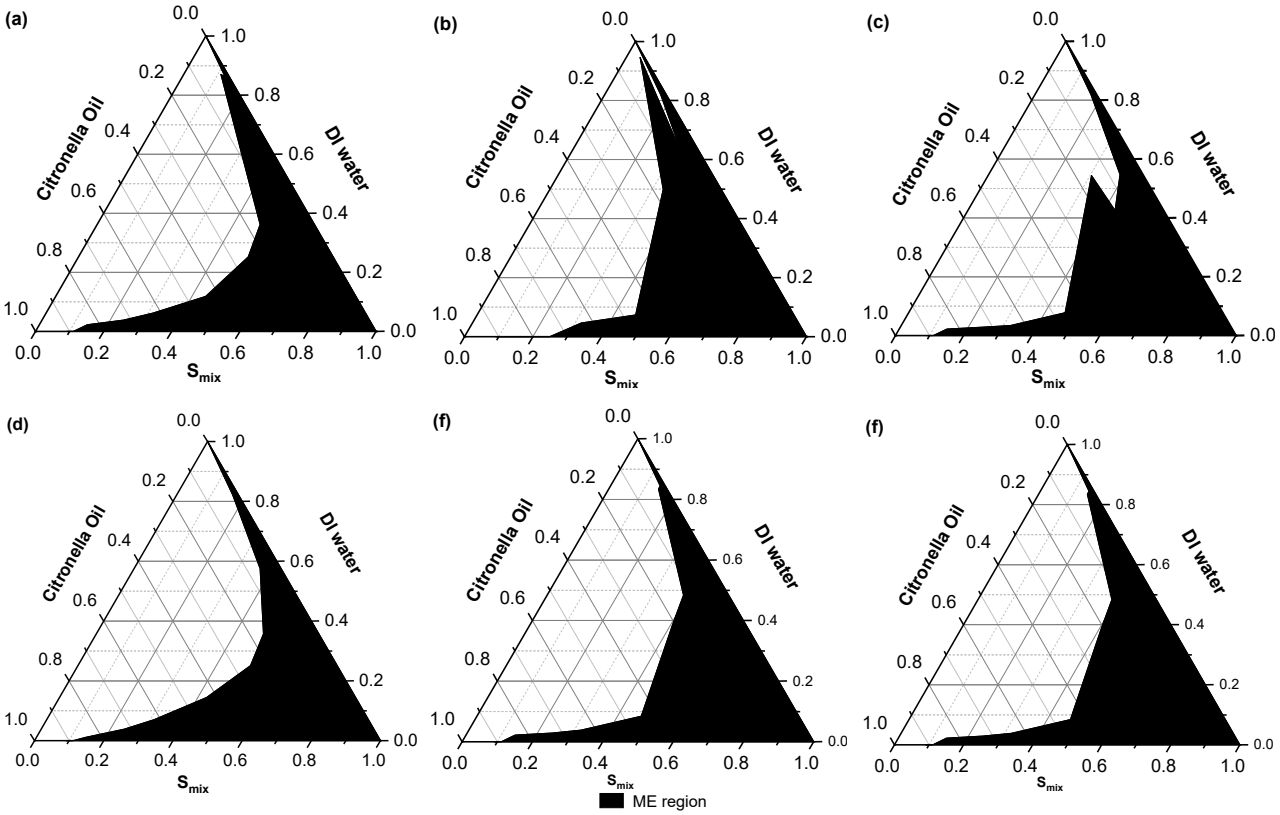

Fig 1. Pseudoternary phase diagram of CTO, Tween 80 as surfactant, ethanol as cosurfactant, and DI water as AP with (a) S_{mix} 0.5, (b) S_{mix} 2, and (c) S_{mix} 3, and pseudoternary phase diagram of CTO, Tween 20 as surfactant, ethanol as cosurfactant, and DI water as AP with (d) S_{mix} 0.5, (e) S_{mix} 2, and (f) S_{mix} 3

composition on ME area. The pseudoternary phase diagram of CTO as oil phase, Tween 80 or Tween 20/ethanol as S_{mix}, and DI water as AP have been plotted in Fig. 1. The black region inside the pseudoternary phase diagram indicates the ME area for each S_{mix}. Microemulsion area for S_{mix} with Tween 80 as surfactant of 1:2, 2:1, and 3:1 (Fig. 1(a-c)) was 38.50 ± 0.08%, 43.98 ± 0.81%, and 40.71 ± 0.95%, respectively. However, the substitution of Tween 20 as surfactant in S_{mix} ratio of 1:2, 2:1, and 3:1 (Fig. 1(d-f)) resulted in ME area as follows: 36.84 ± 0.43%, 42.34 ± 0.23%, and 39.90 ± 0.18%, respectively. The ME region became larger with a higher amount of surfactant (S_{mix} 2). However, excessive surfactant amount reduced the ME area as seen in the psudoternary phase diagram with S_{mix} of 3 for both Tween 80 and Tween 20 as surfactant. As reported previously, usage of Tween 80/ethanol has larger monophasic area of up to 16.92% with the S_{mix} of 2 [22]. ME formulated from olive oil with T85 as surfactant and propylene glycol or ethanol with S_{mix} of 2 had ME region of 5.13% and 10.8%, respectively [23]. The addition of ethanol as cosurfactant reached twice higher ME region in pseudoternary phase diagram. Higher ME is favorable to generate a wider range of alternate compositions for ME formulation. Larger ME area with Tween 80 as surfactant compared to Tween 20 with CTO as oil phase due to lower hydrophobicity of Tween 80 [22,24]. Therefore, Tween 80 as surfactant with S_{mix} of 2 was selected for further stability and antibacterial activity.

Stability of CTO-based ME

Five different formulations from pseudoternary phase diagram of CTO, Tween 80/ethanol = 2, and DI water were indicated in Fig. 2(a), and the weight percent composition of each formulation was listed in Table 1. Point A, CTO10-SM10-AP80 formulation was located in white colored area, which is not the ME region, validated from the hydrodynamic diameter value was 330.80 ± 6.73 nm. Formulation CTO10-SM10-AP80 demonstrated that pseudoternary phase diagram is correlated to hydrodynamic diameter to determine the ME. In addition, the physical appearance of formulation

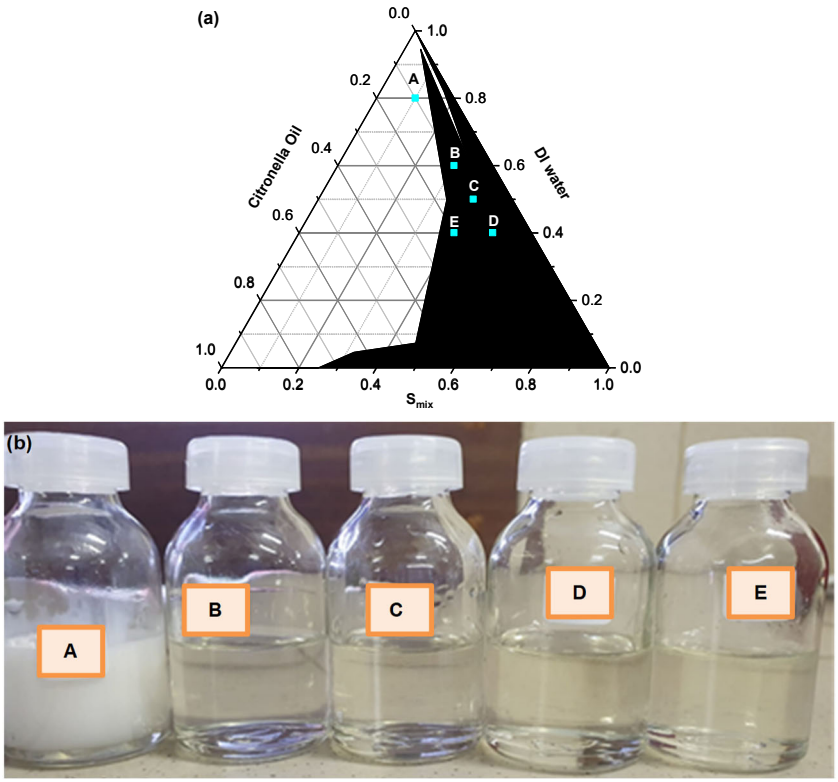

Fig 2. Points of different formulations in pseudoternary phase diagram of CTO as oil phase, S_{mix} = 2, and DI water as AP, ME region showed by the (a) black-colored area and (b) physical appearance of each formulation

Table 1. Composition of CTO, S_{mix}, and AP of each formulation

	Formulation	Composition (wt.%)		
		CTO	S_{mix}	AP
A	CTO10-SM10-AP80	10	10	80
B	CTO10-SM30-AP60	5	80	15
C	CTO10-SM40-AP50	5	75	20
D	CTO10-SM50-AP40	10	75	15
E	CTO20-SM40-AP40	15	75	10

CTO10-SM10-AP80 was turbid and white-colored, in contrast with the other four formulations as shown in Fig. 2(b). Thus, the stability of ME was investigated only for CTO10-SM30-AP60, CTO10-SM40-AP50, CTO10-SM50-AP40, and CTO20-SM40-AP40 formulations.

Formulation CTO10-SM30-AP60, CTO10-SM40-AP50, CTO10-SM50-AP40, and CTO20-SM40-AP40 were prepared and kept for 50 d to examine the stability of formulated CTO-based ME (Fig. 3). These formulations had S_{mix} no more than 50% to maintain the amount of ethanol as cosurfactant in the system. The four ME formulations were analyzed for the hydrodynamic diameter on day 1, 20, and 50 with storage conditions at 30 °C. Previous study of castor oil based ME showed insignificant changes in hydrodynamic diameter in various temperatures (−20, 4, 25, and 50 °C) [25].

During the storage process, the formulations CTO10-SM40-AP50, CTO10-SM50-AP40, and CTO20-SM40-AP40 showed an increase in hydrodynamic diameter and ranged from 30.03 to 205.57 nm. Meanwhile, ME with 10 wt.% of CTO, 30 wt.% of S_{mix}, and 60 wt.% of DI water tends to have a consistent hydrodynamic diameter between 17.93 to 24.93 nm from the first day to 50 d of storage (Fig. 3). Formulation with higher CTO composition (20 wt.%) and identical S_{mix} composition (40 wt.%) resulted in bigger hydrodynamic diameter of ME. This result is in agreement with Tandel et al. [26] with composition of 10 wt.% Capryol 90 as oil phase in ME had higher hydrodynamic diameter (55.74–67.19 nm) compared to ME containing 6 wt.% oil (30.32–38.95 nm) [26]. In addition, higher AP composition (60%) is preferable to prevent the toxicity possibility caused by composition of ME. However, all these formulations are considered stable MEs although had risen in hydrodynamic diameter since the limitation for ME is < 300 nm [1].

Table 2 shows the results of the PDI and zeta potential of the four formulated MEs on the first day to 50. From the table, it can be seen that the PDI of these formulations has a value of less than 0.5 during storage of 50 d, indicating that the formulations were homogeneous, based on one of the criteria of a stable ME [27]. The zeta potential values of the ME formulations in between −14.97 to −20.22 mV on the first day, −19.19 to −24.48 mV after being stored for 20 d, and −20.31 to −23.95 mV after being kept for 50 d. A higher magnitude of zeta potential implies higher electrostatic repulsion, thus preventing the phase separation in the system resulting in more stable formulations [28].

Antibacterial Activity of Citronella Oil-based Microemulsions

Based on the insignificant differences in hydrodynamic diameter for formulations with the same CTO amount (10%), formulations CTO10-SM30-AP60, CTO10-SM40-AP50, and CTO20-SM40-AP40, were selected and assayed for their antibacterial activity. Three ME formulations were tested for antibacterial against two types of bacteria (*E. coli* and *S. aureus*) for 24 h incubation at 37 °C. From Fig. 4, all three ME formulations strongly inhibited between 98.64% to 99.39%

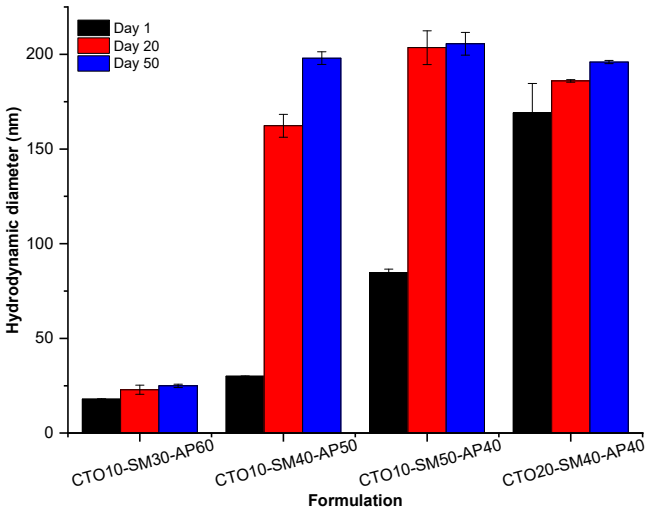

Fig 3. The hydrodynamic diameter of various CTO-based ME formulations at initial storage condition, day 20 and 50 at 30 °C

Table 2. The PDI and zeta potential values in various CTO-based ME formulations during a storage period at 30 °C

Formulation	Characterization	Day		
		1	20	50
CTO10-SM30-AP60	PDI	0.3340 ± 0.0185	0.3573 ± 0.0135	0.3113 ± 0.0064
	Zeta potential (mV)	−16.63 ± 2.32	−24.48 ± 3.02	−21.24 ± 1.18
CTO10-SM40-AP50	PDI	0.3657 ± 0.0101	0.3400 ± 0.0118	0.3770 ± 0.0070
	Zeta potential (mV)	−15.63 ± 0.74	−23.12 ± 1.43	−23.95 ± 3.53
CTO10-SM50-AP40	PDI	0.3417 ± 0.0155	0.3300 ± 0.0062	0.3180 ± 0.0090
	Zeta potential (mV)	−14.97 ± 0.35	−23.01 ± 1.50	−20.31 ± 0.26
CTO20-SM40-AP40	PDI	0.2950 ± 0.0384	0.2183 ± 0.0122	0.2147 ± 0.0332
	Zeta potential (mV)	−20.22 ± 1.87	−19.19 ± 1.80	−20.65 ± 1.06

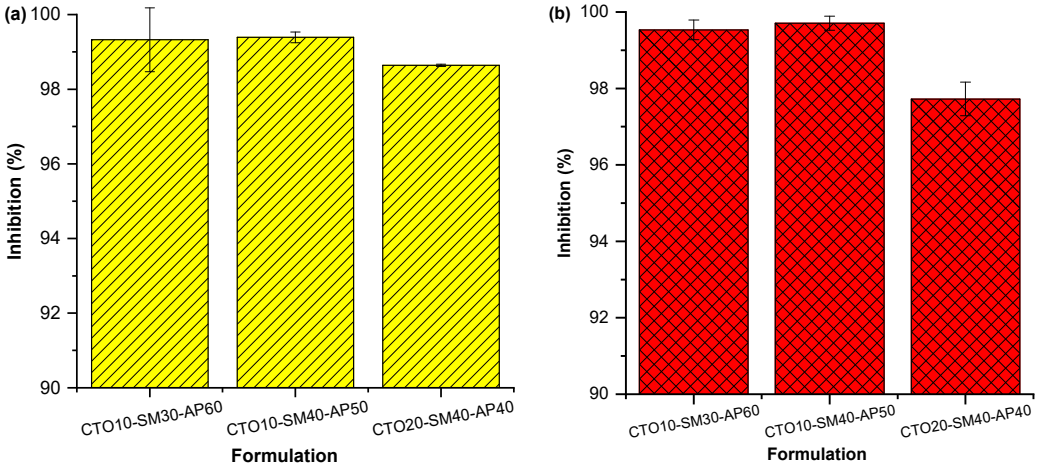

Fig 4. Antibacterial activity of formulated CTO-based MEs against bacteria (a) *E. coli* and (b) *S. aureus*

and 97.72% to 99.71% growth of *E. coli* and *S. aureus*, respectively. Percent inhibition of both bacteria by CTO10-SM30-AP60, CTO10-SM40-AP50, and CTO20-SM40-AP40 did not show a significant difference. This result indicated that CTO-based MEs are able to inhibit bacterial growth although the CTO composition was only 10–20%. Comparison of S_{mix} amount in the formulation of MEs showed no effect on *E. coli* and *S. aureus* growth. All CTO10-SM30-AP60, CTO10-SM40-AP50, and CTO20-SM40-AP40 formulations have antibacterial activity, and the amount of CTO, S_{mix}, and DI water did not affect significantly. Therefore, citronella oil-based ME formulations have antibacterial activity and potential as a carrier for topical drug delivery.

■ CONCLUSION

The CTO-based MEs were successfully formulated using CTO, Tween 80 as surfactant, ethanol as cosurfactant, and DI water. Several ME formulations with different compositions were selected based on the largest ME area observed in the pseudoternary phase diagram with S_{mix} of 2 and Tween 80 as surfactant. All formulations showed hydrodynamic diameters lower than 300 nm, indicating that the formulations were stable in the form of MEs. The CTO10-SM30-AP60 formulation has a hydrodynamic diameter between 17.93 to 24.93 nm during storage for 50 d at 30 °C. ME with higher S_{mix} compositions resulted in higher hydrodynamic diameters after being kept for 20 and 50 d. From the antibacterial activity, three different formulations of the ME could inhibit the growth of bacteria by more than 90%. This shows that the ME from CTO as the oil phase can be utilized for antibacterial applications and is independent of the composition of the ME.

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■ CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

■ AUTHOR CONTRIBUTIONS

Chintya Gunarto: data curation, formal analysis, investigation, methodology, visualization, validation, conceptualization, writing – original draft, funding acquisition. Alchris Woo Go: conceptualization, resources, formal analysis, visualization. Artik Elisa Angkawijaya: conceptualization, resources, formal analysis, visualization. Jenni Lie: formal analysis, investigation. Felycia Edi Soetaredjo: supervision, visualization. Suryadi Ismadji: supervision, validation, conceptualization. Nathania Puspitasari: formal analysis, investigation, writing – original draft. Jindrayani Nyoo Putro: formal analysis, investigation, writing – original draft. Chandra Risdian: formal analysis, investigation, writing – original draft. All authors agreed to the final version of this manuscript.

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