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The formulation of ginger oil nanoemulsions of three varieties of ginger (*Zingiber officinale* Rosc.) as natural antioxidant

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ABSTRACT: One of traditional plants in Indonesia that could be potentially developed as antioxidant is ginger (*Zingiber officinale* Rosc.). There are three varieties, namely elephant ginger, yellow ginger, and red ginger. Its contents mainly essential oil consisted of many antioxidant compounds. Nevertheless, the use of ginger oil in topical dosage form formula is less effective, unstable, and volatile which affects its bioavailability. In this study, nanoemulsion formulas of ginger oil were optimized according to the ratio of tween 80 as surfactant and ethanol as cosurfactant using simplex lattice design (SLD) method. The optimum nanoemulsion formulas of each varieties were characterized and the result showed that nanoemulsion of yellow ginger oil had the highest percentage transmittance (99.676 ± 0.096%) and half-maximal inhibitory concentration (IC_{50}) value (3.010 ± 0.008 mg/mL). Furthermore, all optimum formulas had complied the other nanoemulsion specifications. The study indicated that nanoemulsion formulas of three varieties of ginger essential oils could be developed as potential antioxidant agent.

KEYWORDS: Ginger oil; nanoemulsion; tween 80; ethanol; antioxidant.

1. INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) is widely used as spice and medicinal plant, mainly its rhizomes. Traditionally, ginger is used to treat digestive problems, appetite disorders, blood purifying, aphrodisiac, anti-hemorrhoid, and sex stimulants [1]. The content of ginger rhizomes are volatile oil (2-3%), fatty oils (3-6%), crude fiber (3-8%), ash (8%), proteins (9%), water (9-12%), and carbohydrates (60-70%) [2]. Ginger oil of the rhizomes varies in range from 1.0 to 3% depending on its growing location [3]. Unpeeled rhizomes cultivars from India yielded 2.22-4.17% oil containing zingiberene (10.5-16.6%), β-sesquiphellandrene (5.8-7.2%), e-citral (7.4-10.5%), z-citral (5.3-7%), ar-curcumene (2.9-9.8%), limonene (1.3-6.4%), camphene (0.9-7.6%), and o-cymene (0.9-6.5%) [4]. Previous study showed that ginger oil had several pharmacological effects such as antioxidant [5-7], antimicrobial [7-10], anti-inflammatory, analgesic [6,11-15], anticancer [16], anti-ulcer [17], and immuno-modulatory effect [18].

In Indonesia there are three varieties of ginger, namely elephant ginger (*Zingiber officinale* var. Officınarum), yellow ginger (*Zingiber officinale* var. Amarum), and red ginger (*Zingiber officinale* var. Rubrum). The rhizome of elephant ginger is larger and fatter, but it has less aroma and flavor. Elephant ginger oil (EGO) contained 0.82-2.8% of essential oil. Yellow ginger which was also called as emprit or white ginger had sharper aroma and hotter taste. Its rhizome is flat with whitish yellow colour and soft fiber. Yellow ginger oil (YGO) contained 1.50-3.50% of essential oil. Meanwhile, red ginger had very sharp aroma and very hot taste. The rhizome is small with reddish yellow colour and rough fiber. Red ginger oil (RGO) contained 2.58-3.90% of essential oil [19]. Morphological differences among three varieties of ginger was shown in Figure 1.

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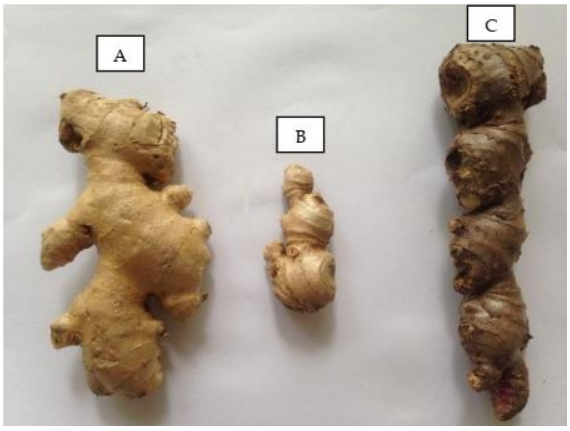

Figure 1. Morphological differences of three varieties of ginger: A. Elephant ginger, B. Yellow ginger, C. Red ginger.

One of main bioactivities of ginger oil is as antioxidant. It could scavenge several free radicals, such as superoxide, hydroxide radicals, and inhibited lipid peroxidation of tissue. Moreover, it increased antioxidant enzymes in bloods such as catalase, super oxide dismutase, glutathione, and glutathione reductase [6]. Free radicals as unstable reactive molecules have unpaired electrons which react with various substances such as lipids, proteins, and DNA. The radicals have a role in cell damage which result in oxidative stress and several chronic diseases, and accelerate the aging process [20-21]. Several antioxidant products are applied on the skin as the largest organ of the human body that functions as a barrier between internal and external environment [22]. Healthy skin acts as a protector effectively and has a good aesthetic to support human appearance. Antioxidants involve in maintaining the skin health. They affect intracellular signaling pathways involved in skin damage and protect against photodamage, as well as prevent wrinkles and inflammation [23]. Unfortunately, the use of essential oil such as ginger oil directly as antioxidant is less effective, unstable, volatile, susceptible to light, air, and heat. Hence, it can decrease the bioavailability [24]. An adequate delivery system is needed to deliver antioxidant essential oils into the skin because it have to penetrate the main barrier, the stratum corneum. A topical delivery system that can increase the absorption and stability of essential oils is nanoemulsion. The preparation can increase their penetration into the skin [25].

Nanoemulsion is a stable and translucent emulsion system with droplet sizes between 20-600 nm [26]. The most important components of nanoemulsion is surfactant and cosurfactant, which stabilize the system by decreasing fluid interface tension [27]. The aim of this research was to determine optimum nanoemulsion formulas of three varieties of ginger oil through optimization of tween 80 and ethanol composition as surfactant and cosurfactant factors and characterize it. Responses observed in this study were percentage transmittance, pH, and antioxidant activity. Selection of optimum nanoemulsion formulas was carried out using the two factors SLD method.

2. RESULTS AND DISCUSSION

2.1. Preparation of nanoemulsions

Before preparing nanoemulsion, surfactant and cosurfactant used in the formula were determined. According to [28], the use of tween 80 as a surfactant produces nanoemulsion with droplet sizes less than 100 nm compared with tween 21 and tween 85. The use of tween 80 in topical preparations is safe, non-irritating, and non-toxic [29]. Nevertheless, [30] reported that the use of single surfactant was not able to reduce the surface tension between oil and water phase. Whereas, the combination of surfactant and cosurfactant in appropriate concentration would produce a stable nanoemulsion system [27].

According to Figure 2, it was known that ethanol was the best cosurfactant in producing ginger oil nanoemulsion due to its translucent visualization and highest percentage transmittance. Furthermore, the higher the percentage transmittance, the smaller the particle size [31-32]. Combination of tween 80 and propylene glycol resulted a turbid nanoemulsion with percentage transmittance less than 90%. Nanoemulsion with combination of tween 80 and PEG 400 resulted percentage transmittance less than 80% and higher turbidity. [30] reported that short chain alcohols such as ethanol produced a translucent and stable nanoemulsion. In this study, combination of tween 80 and ethanol in the ratio of 1:3 showed the highest percentage transmittance characterized by good emulsification (99.567% for EGO, 99.863% for YGO, and 99.785% for RGO).

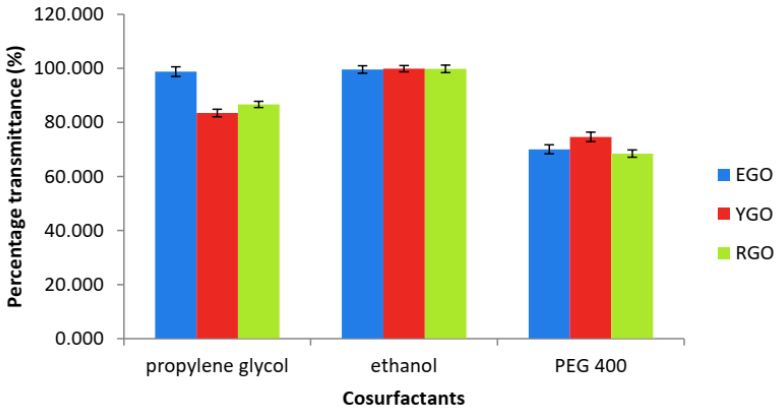

Figure 2. Cosurfactant determination in combination with tween 80 as surfactant of ginger oil nanoemulsion formulas.

2.2. Response evaluation of nanoemulsion formulas

The study used SLD of quadratic model to design the best nanoemulsion compositions of ginger oil effectively. The design of ginger oil nanoemulsion optimization with response values was presented in Table 1. Based on One way analysis of variance (ANOVA) test, it was known that the p-value of the each response was 0.0001 which showed that each factors had significant effect to all responses. The effect was also described by equation of TRC (Terms of Real Component) and TAC (Terms of Actual Component) as shown in Table 2.

Table 1. The design of ginger oil nanoemulsion formulas optimization based on responses.

Ginger varieties	Composition (mL)		Responses		
	Tween 80	Ethanol	Percentage transmittance (%)	pH	IC_{50} (mg/mL)
EGO	3	1	99.722 ± 0.090	6.310 ± 0.032	12.001 ± 0.018
YGO			99.759 ± 0.403	6.160 ± 0.015	2.486 ± 0.007
RGO			99.386 ± 0.047	6.140 ± 0.021	12.863 ± 0.060
EGO	2	2	98.631 ± 0.596	6.120 ± 0.051	10.096 ± 0.056
YGO			98.471 ± 0.280	6.040 ± 0.036	2.646 ± 0.008
RGO			96.658 ± 0.296	5.900 ± 0.016	13.611 ± 0.375
EGO	1	3	94.323 ± 0.262	5.800 ± 0.005	12.677 ± 0.019
YGO			95.405 ± 0.102	5.800 ± 0.015	2.944 ± 0.004
RGO			94.817 ± 0.168	5.990 ± 0.021	13.538 ± 0.060

From the equations in Table 2, it was known that tween 80 had greater effect to percentage transmittance and pH responses than ethanol. But, it showed opposite effect to IC_{50} value. In Table 1, it was known that increase of tween 80 composition caused increase of percentage transmittance, pH responses, and antioxidant capacity. The use of the same concentration of tween 80 and ethanol resulted reduction of percentage transmittance, pH, and IC_{50} value. Based on data in Table 2, an increase in the amount of tween 80, ethanol, and combination of both produced higher percentage transmittance and pH responses of EGO and YGO nanoemulsions. Moreover, addition of tween 80 and ethanol individually increased IC_{50} value of EGO and YGO nanoemulsions, but combination of both decreased IC_{50} value. Tween 80 and ethanol also increased percentage transmittance and pH responses of RGO nanoemulsion, but utilization of both decreased the responses. The use of tween 80, ethanol, and combination of both increased IC_{50} value of RGO nanoemulsion.

The effect of two components (surfactant and cosurfactant) to responses variable of ginger oil nanoemulsion was shown in Figure 3-5. Percentage transmittance of all ginger oil nanoemulsion was more than 90%. The response of percentage transmittance indicated that an increase in the amount of tween 80 and ethanol resulted in a percentage transmittance significant increase. Higher concentration of tween 80 caused

a large reduction in the interfacial tension, which might lead interfaces that spontaneously generated interfacial turbulence droplets [33]. Moreover, an increased concentration of tween 80 produced a stabil interface between oil and water and smaller particle size [34]. Ethanol as cosurfactant reduced the interfacial tension [35]. Because of its effect, concentration of surfactant in nanoemulsion formulas could be higher to obtain higher percentage transmittance. In contrast, a high level of cosurfactant resulted in nanoemulsion with lower percentage transmittance. Furthermore, the response of pH was also useful for determining nanoemulsion formulas with a suitable pH for the skin. Therefore, pH of all nanoemulsion components used was in the same range, namely 4.5 to 6.5 [36]. The response of antioxidant activity was useful in predicting the capacity of all nanoemulsion formulas. İn this study, the antioxidant activity could increase due to increasing concentration of tween 80 as surfactant. The result was similar to previous study reporting that increase of tween 80 level could inhibit the formation of malondialdehyde (MDA), a secondary metabolite resulted in degradation of polyunsaturated fatty acid from oxidation process of commercial milk [37]. The reducing effect of antioxidant occured when ethanol concentration was increased. Nevertheless, the use of tween 80 and ethanol combination caused the increasing effect of antioxidant. The effect may be caused by phenolic group of ethanol which acts as antioxidant. Hydroxyl group will donate hydrogen atoms when reacts with radical compounds, thus the oxidation process is inhibited [38].

Table 2. Terms of Real Component (TRC) and Terms of Actual Component (TAC) of ginger oil nanoemulsion.

Ginger varieties	Responses	TRC*	TAC*
EGO	Percentage transmittance	Y = +97.59733A +86.80000B +25.72800AB	Y = +24.39933 A +21.70000B +1.60800AB
	pH	Y = +6.38333A +5.36333B +0.986667AB	Y = +1.59583A +1.34083B +0.0061667AB
	IC_{50}	Y = +18.40967A +19.74833B - 35.93067AB	Y = +4.60242A +4.93708B - 2.24567AB
YGO	Percentage transmittance	Y = +99.26833A +90.56167B +14.22400AB	Y = +24.81708A +22.64042B +0.899000AB
	pH	Y = +6.16667A +5.43333B +0.960000AB	Y = +1.54167A +1.35833B +0.060000AB
	IC_{50}	Y = +2.43257A +3.34877B - 0.938933AB	Y = +0.608142A +0.837192B - 0.058683AB
RGO	Percentage transmittance	Y = +103.00133A +93.86333B - 7.09600AB	Y = +25.75033A +23.46583B - 0.443500AB
	pH	Y = +6.72333A +6.42333B - 2.69333AB	Y = +1.68083A +1.60583B - 0.168333AB
	IC_{50}	Y = +11.29383A +12.64370B +6.56693AB	Y = +2.82346A +3.16092B +0.410433AB

*Y = Percentage transmittance (%) or pH or IC_{50} (mg/mL), A = proportion of tween 80, B = proportion of ethanol.

2.3. Formula optimization of nanoemulsion

The nanoemulsion formulas of ginger oil were selected based on its desirability. The response variables criteria was determined to obtain nanoemulsion formulas with an appropriate properties as topical dosage forms. The desirability was shown in the value of 0 to 1. The value of 1 represented a good response value, but 0 represented a completely undesirable value. The optimized formula of EGO nanoemulsion consisted of 2.203 mL tween 80 and 1.797 mL ethanol with desirability value of 0.940. The predicted response of percentage transmittance value was 99.112%, pH value was 6.169, and IC_{50} value was 10.121 mg/mL. The optimized formulation of YGO and RGO nanoemulsion consisted of 3 mL tween 80 and 1 mL ethanol with desirability value of 0.939 and 0.980. The predicted response of YGO nanoemulsion showed percentage transmittance of 99.759%, pH of 6.163, and IC_{50} of 2.486 mg/mL. Meanwhile, the predicted reponse of RGO nanoemulsion was shown at percentage transmittance of 99.386%, pH of 6.143, and IC_{50} of 12.863 mg/mL.

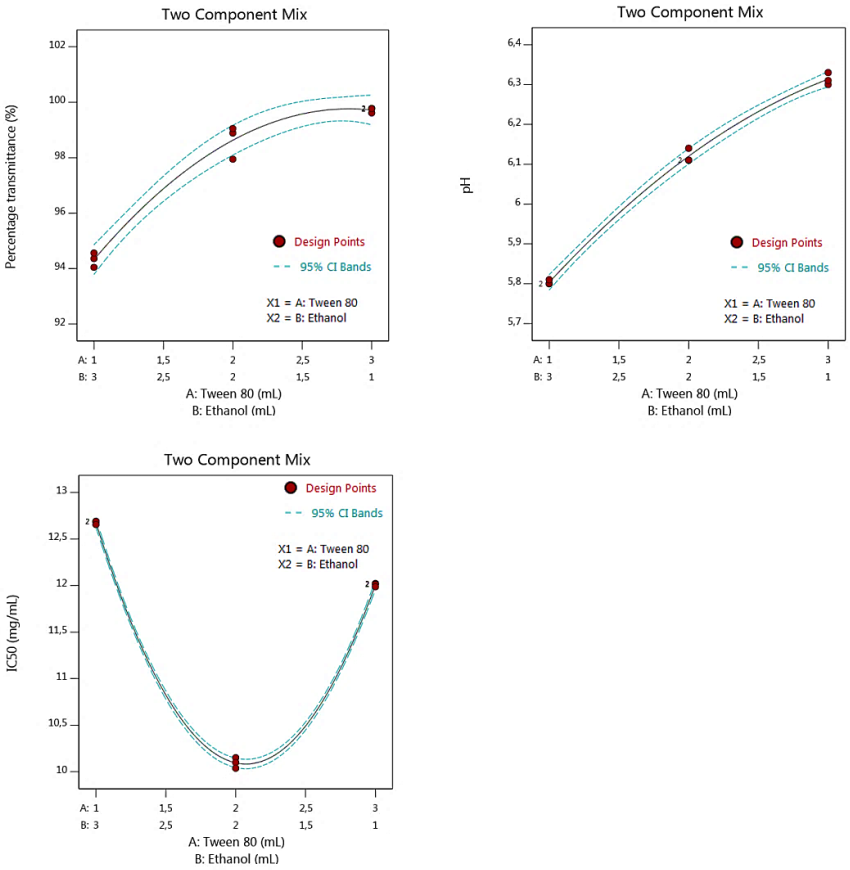

Figure 3. Contour plot response of tween 80 and ethanol for EGO nanoemulsion.

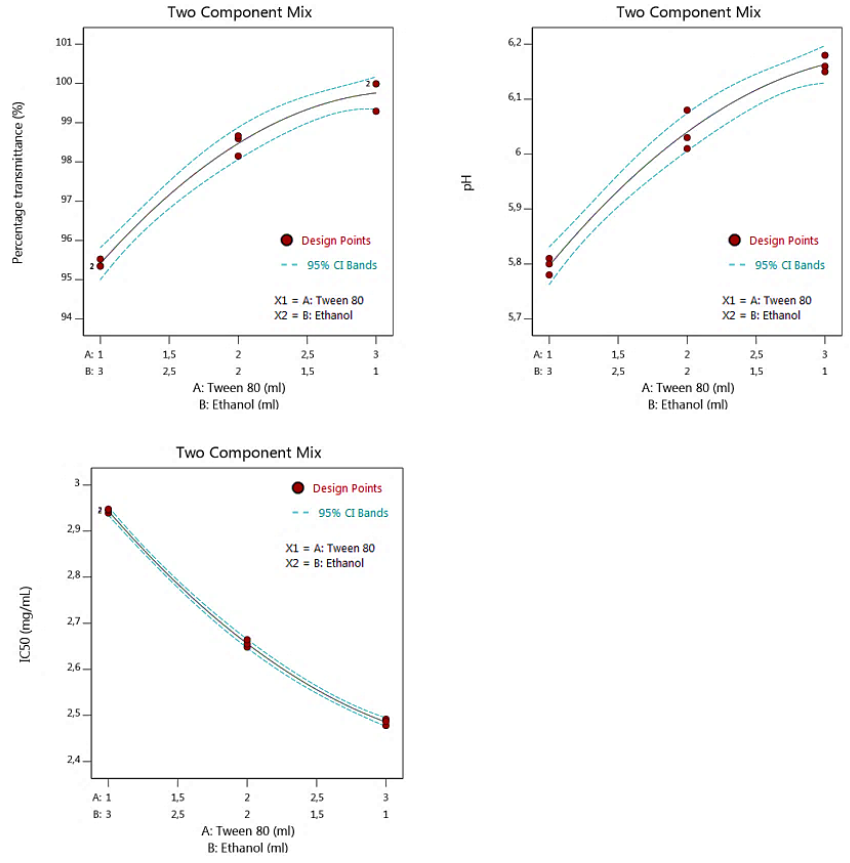

Figure 4. Contour plot response of tween 80 and ethanol for YGO nanoemulsion.

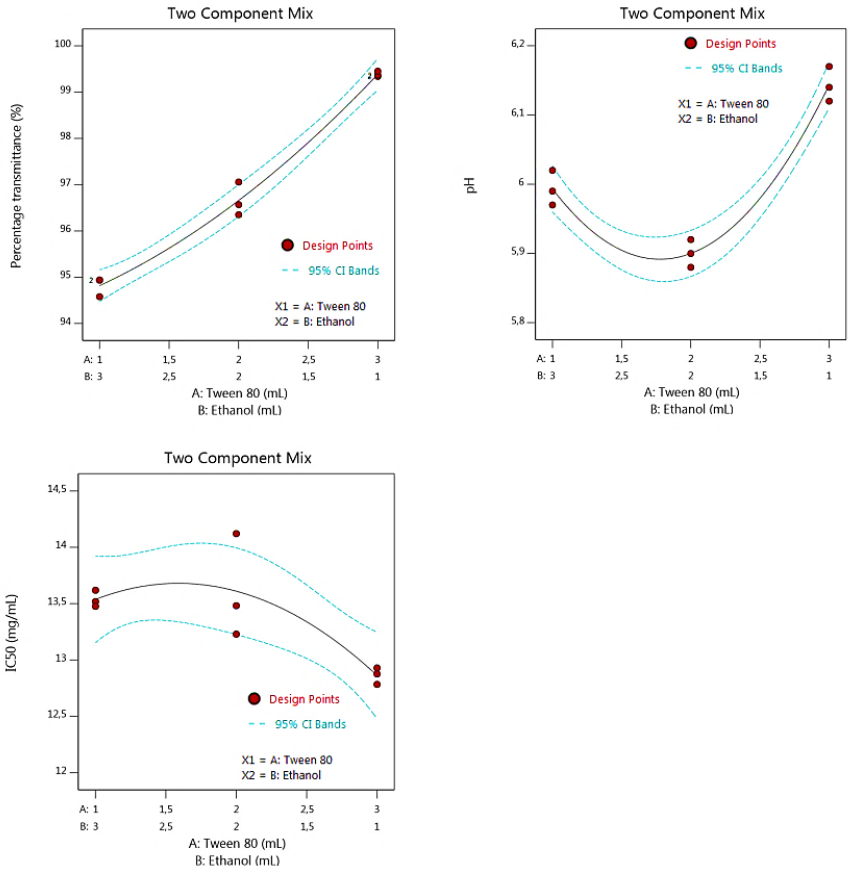

Figure 5. Contour plot response of tween 80 and ethanol for RGO nanoemulsion.

2.4. Characterization of optimum nanoemulsion formulas

The result of characterization test for optimum nanoemulsion formulas of ginger oil was shown in Table 3. Organoleptic properties of all ginger oils had complied color, odor, clarity, and viscosity requirement as nanoemulsion preparations. From specific gravity test, it was known that YGO nanoemulsion exhibited the highest value. The result of viscosity test showed that EGO nanoemulsion had the lowest value, but all ginger oil nanoemulsions had complied specification of 1-200 mPas for nanoemulsion dosage form [39]. Viscosity affects the release of active substances because a high value of viscosity causes difficulty in releasing active substances. Hence, nanoemulsion bioactivity will decrease [40].

Stability test performed using centrifugation test and freeze–thaw cycle showed that all ginger oil nanoemulsions were stable. There were not separation between oil and water phase, cracking, creaming, and turbidity. The thermodynamic stability of nanoemulsions provided a long shelf life compared to convensional emulsions [41]. The surface charge of nanoemulsion with nonionic surfactant comes from the adsorption of ions in the water phase or due to droplets friction with its dispersing medium. The adsorbed ions on the droplets surface produce an electric double layer resulting a repulsive force among the particles, thus it prevents aggregation [42]. Moreover, dye test showed that all ginger oil nanoemulsions type were O/W (Figure 6). It was characterized by uniform dispersion of methylene blue mainly in water phase. O/W nanoemulsion with nonionic surfactant results a film layer on the droplets surface preventing droplets integration in the dispersing medium [42].

RGO nanoemulsion had the lowest average particle size. Meanwhile, polydispersion index of EGO and RGO nanoemulsion was lower than YGO nanoemulsion. All ginger oil nanoemulsions complied particle size specification of 5-200 nm [43]. Polydispersity index described particle size distribution. The smaller the polydispersity index value, the greater the uniformity of particle size of dosage form. In this study, category of polydispersity index values of optimum nanoemulsions formula from all ginger oil was monodispersion (0.01-0.7) [44]. The category produced nanoemulsion with homogeneous particle size and narrow particle size distribution. Percentage transmittance of optimum nanoemulsion formulas had complied specification, more than 90%. YGO nanoemulsion with the highest value of percentage transmittance showed that it had more

translucent visualization than the other nanoemulsions. pH of optimum nanoemulsion formulas was in a range of 4.5-6.5 which was suitable for topical dosage forms [45]. YGO nanoemulsion exhibited the highest antioxidant activity. Nevertheless, antioxidant activity of all nanoemulsions were weak. It might be caused by the low dose of ginger oil in the formulas. Hence, dose enhancement was needed in increasing the activity.

Table 3. Characterization of optimum nanoemulsion formulas.

Characteristics	Nanoemulsion of three varieties of ginger oils		
	EGO	YGO	RGO
Organoleptic test:			
Color	Yellow	Yellow	Yellow
Odor	Spesific for EGO	Spesific for YGO	Spesific for RGO
Clarity	Clear	Clear	Clear
Viscosity	Viscous	Viscous	Viscous
Specific gravity (g/mL)	1.0086 ± 0.0000^{a}	1.0197 ± 0.0001^{b}	0.9824 ± 0.0001^{c}
Viscosity (mPas)	29.892 ± 0.419^{a}	62.170 ± 1.759^{b}	88.483 ± 0.694^{c}
Stability	Stable	Stable	Stable
Dye test	O/W	O/W	O/W
Average of particle size (nm)	13.5 ± 0.4^{a}	24.1 ± 1.1^{b}	11.5 ± 0.1^{c}
Polydispersity index	0.195 ± 0.025^{a}	0.429 ± 0.029^{b}	0.277 ± 0.069^{a}
Percentage transmittance (%)	99.112 ± 0.082^{a}	99.676 ± 0.096^{b}	99.473 ± 0.020^{c}
pH	6.153 ± 0.005^{a}	6.100 ± 0.032^{ab}	6.033 ± 0.075^{b}
IC_{50} (mg/mL)	9.836 ± 0.0213^{a}	3.010 ± 0.008^{b}	12.365 ± 0.079^{c}

Data were mean of all samples ± SD (n=3). A different superscript letter in the same row showed a significant differences according to ANOVA test ($p<0.05$).

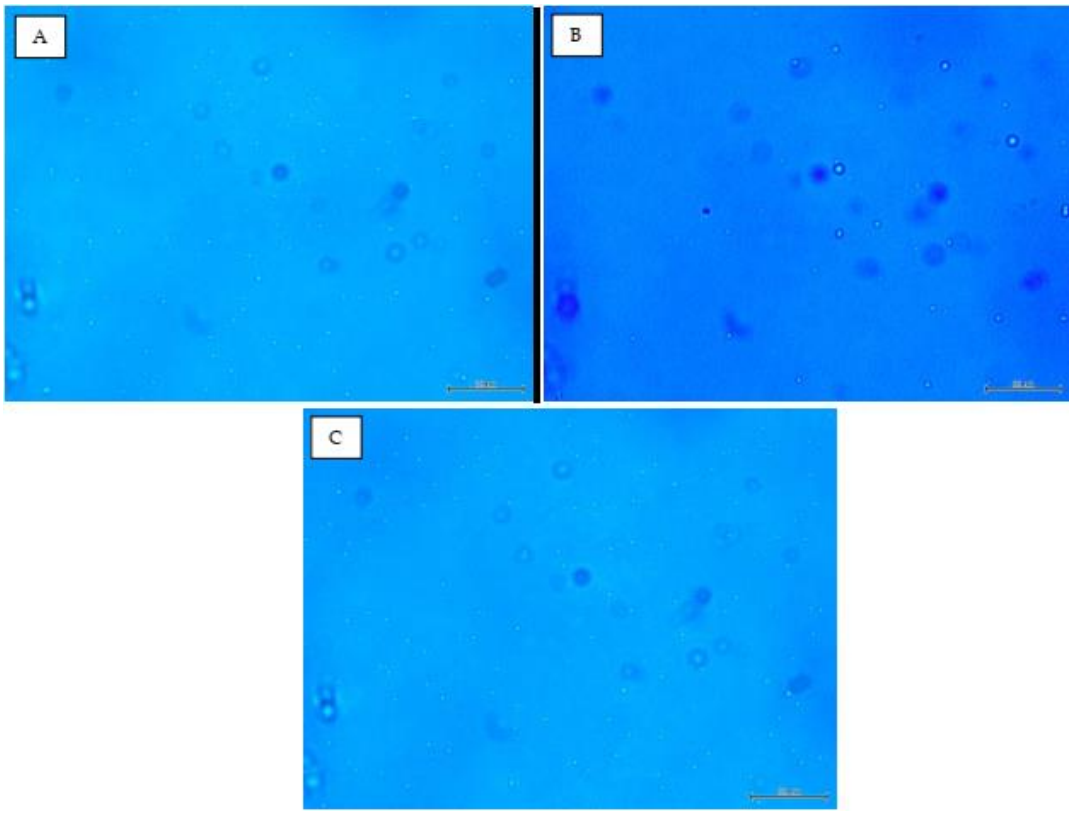

Figure 6. Dye test result of optimum nanoemulsion formulas of three varieties of ginger oil using electron microscope in 1000x magnification: A. EGO, B. YGO, C. RGO.

3. CONCLUSION

Tween 80 and ethanol could be used as surfactant and cosurfactant of three varieties of ginger oil nanoemulsions to increase its bioavailability as topical dosage form. Based on desirability data from SLD method of Design-Expert 11.0 software, it was obtained optimum nanoemulsion formulas for each varieties with expected percentage transmittance, pH and IC_{50} value. Characterization of the optimum formulas showed that nanoemulsion from three varieties of ginger oil had fulfilled all specification as a good, stable, and effective antioxidant dosage form for topical administration. The study exhibited that antioxidant activity of optimum formula of YGO nanoemulsion was higher than EGO and RGO nanoemulsion.

4. MATERIALS AND METHODS

4.1. Materials

Essential oils from three varieties of ginger rhizome were purchased from PT. Eteris Nusantara (Yogyakarta, Indonesia). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, methylene blue, ethanol were supplied by Sigma-Aldrich (St. Louis, MO, USA). Tween 80 and trietanolamin were purchased from Brataco Chemical (Jakarta, Indonesia). Design-Expert 11.0 software was used to facilitate computation and evaluation of SLD method.

4.2. Preparation of nanoemulsion

In this study, tween 80 was chosen as a surfactant and combined with several cosurfactants such as ethanol, propylene glycol, and PEG 400 in the ratio of 1:1, 1:2, and 1:3. All formulations were observed for its percentage transmittance to obtain the best combination of surfactant and cosurfactant. Different ratios of selected surfactant and cosurfactant (1:1, 1:2, and 1:3), ginger oil as oil phase, and distilled water as water phase were utilized to compose three formulas of water-based (O/W) nanoemulsions. Preparation of nanoemulsions was carried out using spontaneous emulsification method [45]. Surfactant, cosurfactant, and ginger oil were mixed completely to form a homogenous solution for 5 min at room temperature and 250 rpm. Moreover, water was added dropwise and mixed using magnetic stirrer (C-MAG HS 7, IKA, China) for 5 min at 250 rpm.

4.3. Response evaluation of nanoemulsion formulas

4.3.1. Determination of percentage transmittance

Percentage transmittance of nanoemulsions was determined using UV-Vis spectrophotometer (Genesys 10S, Thermo Fisher Scientific, America). One milliliter of the formulations was analyzed at 650 nm against distilled water as blank. A transparent nanoemulsion had percent transmittance of greater than 99% [45].

4.3.2. pH measurement

pH measurement of ginger oil nanoemulsions was carried out by using digital pH-meter (Elmetron CP-502, Zabrze, Poland).

4.3.3. Determination of antioxidant activity

The free radical scavenging activity of three varieties of ginger oil was determined by using DPPH method according to [46] with slight modification. The DPPH solution (0.1 mmol) was prepared in 96% ethanol immediately before the assay. Several concentrations of each nanoemulsion sample (1, 2, 4, 6, 8, 10, 12, 14, 16 mg/mL) were added to DPPH solution (1:1). The reaction mixtures were incubated for 50 min in dark condition at room temperature. The absorbance of the samples was measured by using UV-Vis spectrophotometer at 517 nm. Gallic acid was used as a standard antioxidant and inhibition percentage was calculated according to equation 1. Abs control was absorbance of DPPH in 96% ethanol (1:1), and abs sample was absorbance of sample in ethanol (1:1). IC_{50} was calculated according to regression analysis of inhibition percentage from all samples. The experiment was repeated triplicate.

$$\% \text{ inhibition} = \frac{(abs\ control - abs\ sample)}{abs\ control} \times 100\% \qquad \text{[Eq. 1]}$$

4.4. Formula optimization of nanoemulsion

Optimum formulation of nanoemulsion was determined based on desirability resulted from SLD method using Design Expert 11 software. In this study, the factors as independent variables were proportion of surfactant and cosurfactant. Meanwhile, the responses of percentage transmittance, pH, and IC_{50} value as dependent variables were optimized simultaneously (Table 2). The value of Ba, Bb, and Bab coefficient in equation 2 obtained from the software was used to determine the main effect of the factors to the responses, and the effect of the factors combination to the responses. Y described responses of percentage transmittance, pH, and IC_{50} value. The selected optimum formula of ginger oil nanoemulsions was the formula with the highest desirability index.

$$Y = Ba(A) + Bb(B) + Bab(A)(B) \quad \text{[Eq. 2]}$$

4.5. Characterization of optimum nanoemulsion formulas

The optimum nanoemulsion formulas of each ginger oil was observed for its color, odor, and clarity. A stable nanoemulsion has homogenous, clear appearance, and no phase separation. The viscosity of the nanoemulsion was determined by using Oswald viscometer. Specific gravity was measured using pycnometer (Brand, Wertheim, Germany). The stability test of ginger oil nanoemulsion was performed using centrifugation test and freeze thaw cycle test. In centrifugation test, the optimum nanoemulsion formulas were centrifuged at 3,500 rpm for 30 minutes and observed to evaluate its potential metastable conditions, creaming, phase separation, and its precipitation [39]. Freeze thaw cycle test was carried out using 3 cycles between -21°C and +25°C for not less than 48 hours [30]. Dye solubility test was conducted by dropping methylene blue to the nanoemulsions on object glass and mixing it properly. Then, the formulation was observed using microscope (Olympus, Tokyo, Japan). Particle size of optimum nanoemulsion formulas was determined using PSA (*Particle Size Analyzer*) (SZ 100 HORIBA) with type of dynamic light scattering. 10 mL of sample was placed into cuvettes and measurements were recorded at 25 °C and 90 °C.

4.6. Statistical analysis

To optimize nanoemulsion compositions of ginger oil, the study applied SLD of quadratic model. All of the responses of ginger oil nanoemulsion formulas were analyzed using ANOVA of Design-Expert 11.0 software. Furthermore, several properties of optimum nanoemulsion formulas from three varieties of ginger oil such as specific gravity, viscosity, average of particle size, polydispersity index, percentage transmittance, pH, and IC_{50} were analyzed using ANOVA with Least Significance Different (LSD) method to test any significant differences among samples. Value of $p < 0.05$ were considered to be significantly different ($\alpha = 0.05$).

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