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Formulation and Characterization of Kaffir Lime Oil Nanoemulsion

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Kaffir lime oil has many health benefits. However, an obstacle to its commercial use is oxidation during storage. Nanoemulsions (particulate colloidal systems) have been shown to be suitable carriers for lipophilic essential oil constituents due to amphipathic compounds that facilitate solubility. The objectives of this study were to formulate thermodynamically stable kaffir lime oil nanoemulsions and to investigate their physicochemical properties. Air-dried leaves of kaffir lime were subjected to steam distillation to obtain essential oil. Preparation of nanoemulsions was done using the spontaneous emulsification method. Tween 80 and propylene glycol were selected as surfactant mix components. The oil phase consisted of Miglyol 812 as a carrier oil for kaffir lime oil while double-distilled water was used in the aqueous phase. The best formula with transmittance above 95% and highest essential oil content was selected. It contained 20% of Tween 80, 10% of propylene glycol, 1.25% Miglyol 812, and 3.75% kaffir lime essential oil. This formula was then characterized and its thermodynamic stability determined. The results showed that the average size of kaffir lime oil nanoemulsionsdroplets is 18.23+0.12 nm with poly dispersity index of 0.36+0.01. The system is thermodynamically stable and robustly withstood variations in temperature, centrifugation, and long-term storage. Additionally, the nanoemulsions had low viscosity, which may facilitate its development as a pharmaceutical compound.

Keywords: nanoemulsions, kaffir lime, essential oil, spontaneous emulsification.

Kaffir lime (*Citrus hystrix* D.C.) is well known for its distinctive lemon fragrance and astringent flavor. Kaffir lime has been used for many purposes, including traditional medicines, a spice in asian cookery, and an ingredient in perfumery[1]

The efficacy of kaffir lime as a medicinal plant results from its secondary metabolite content. A number of volatile compounds found in kaffir lime are reported to have bioactive properties that may be useful in promoting health and fighting disease. Citronellal, which is the most abundant volatile compound in kaffir lime leaf, shows antibacterial activities towards *Moraxella catarrhalis*, *Haemophilus influenza*, *Staphylococcus pneumonia*, *S. aureus* and *Acinetobacter baumannii*[2]. Another major volatile compound in the leaf, [2]-citronellol, has been shown to be a potential inhibitor of HIV-1 RT and is also popular for aromatherapy[3, 4]. Limonene in kaffir lime leaf contributes to the anti-inflammatory activity of kaffir lime oil against *Propionibacterium acnes*, a skin commensal

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bacteria which causes the chronic skin disease acne vulgaris [5]**.** In addition, limonene can promote wound-healing and reduce post-acne scarring and help to remove acne blemishes[6]. All of these volatile compounds are generally obtained from essential oil extraction. Other terpenoids such as citronellyl acetate, sabinene, nerolidol, and linalool are also found in kaffir lime leaf essential oil at lower concentrations[7, 8]. Furthermore, an *in vitro* study has suggested the use of kaffir lime leaf essential oil as an anti-cancer compound. The essential oil possesses anti-proliferative activity against human mouth epidermal carcinoma and murine leukemia cell lines[9].

However, kaffir lime essential oil needs to be improved because it tends to be easily oxidized in the presence of light, oxygen, increasing acidity, heavy metals, water, and increasing heat[10]. As water makes up most of human body and oxygen is present in bloodstream, essential oil is prone to hydrolysis and oxidation when administrated to the body. Moreover, the highly lipophilic nature of essential oil constituents decreases its bioavailability in the human body. The lipophilic essential oil is nearly insoluble in blood causing its concentration to be gradually reduced prior reaching its target cells or tissues to bring the expected effects11. This limiting the clinical utility of kaffir lime-derived compounds[11].

Nanoemulsion is a type of nanometric carrier system in which particles are less than 100 nm in droplet size, consisting of oil, water, and surfactant [12, 13]. This particulate colloidal system is described as suitable carrier for lipophilic bioactive compound delivery, including essential oil constituents, due to its amphiphilic behavior. Moreover, it has several unique properties due to its small droplet size such as optical transparence, long-term stability, and enhanced bioavailability[14]. Furthermore, a nanoemulsion system is easy to prepare using the spontaneous emulsification method[12]. Various kind of essential oils have been successfully formulated into nanoemulsions such as orange oil, zedoary turmeric oil, and oregano essential oil[15, 16, 17]. As other essential oils mentioned, kaffir lime essential oil could be also formulated into nanoemulsion and this method might be a promising strategy to improve the effectiveness of the kaffir lime essential oil bioactivity for medicine and pharmaceutical use.

This is the first reported study in formulation and characterization of kaffir lime oil nanoemulsion. The objectives of this study were to formulate a thermodynamically stable kaffir lime oil nanoemulsion and to investigate its physicochemical properties.

MATERIALS AND METHODS

Essential oil preparation

Fresh kaffir lime leaves were collected from Candirejo Village, Borobudur District, Magelang Regency, Central Java. The leaves were washed with tap water and air-dried using fan for two weeks (until dry weight of the leaves are in constant level). Dried leaves then weighed and chopped prior to steam distillation. Steam distillation was done for four hours. Sodium sulfate anhydrous (e-Merck) was used to separate the obtained distillate from water.

Nanoemulsion preparation

Nanoemulsion was prepared using spontaneous emulsification method [16]. First, surfactant Tween 80 (e-Merck) and co-surfactant propylene glycol (e-Merck) were homogenized with vortex. This mixture is called surfactant mix. Oil phase, consists of Miglyol 812 (Cremer oleo) as carrier oil and kaffir lime leaf essential oil, was added into surfactant mix afterwards and homogenized with vortex. The mixture of surfactant mix and oil phase, namely organic phase, was then added drop by drop into aqueous phase (citrate buffer pH 3.5 or double distilled water) using pipette with rate at about 2 mL/minute while stirred with magnetic stirrer at 500 rpm. All steps were done at room temperature.

Screening of best nanoemulsion formula

Screening of the best formula was done based on the acquired data of percent transmittance. Each designed formula was subjected to transmittance measurement against distilled water using UV-Vis spectrophotometer at 650 nm. Formula with percent transmittance number above 95 % was selected as the best formula[18].

Physicochemical characterization.

In addition to transmittance measurement, physicochemical characteristics of the best formula were observed by viscosity measurement and particle size analysis. The viscosity of

nanoemulsion was measured using cone and plate model *Rheosys* viscometer at 50, 62.5, 75, 87.5, and 100 rpm. Particle size analysis of nanoemulsion was done with *Horiba* SZ-100 particle size analyzer.

Thermodynamic stability test

Thermodynamic stability of the nanoemulsion was examined with freeze-thaw test, followed by centrifugation test, and storage duration test. Nanoemulsion was subjected to freeze-thaw test for six cycles; each cycle consists of 24 hours storage at -20 °C and 24 hours storage at 25 °C. Physical destabilization such as creaming, cracking, aggregation, and sedimentation of nanoemulsion were observed after six cycles. Centrifugation test was done after freeze-thaw test.

Table 1. Carrier nanoemulsions formula

Number	Formula							
	Surfactant	Co-surfactant	Oil Phase	Aqueous Phase		Transmittance (%)	Physical appearance	Category
	Tween 80	Propylene Glycol	Miglyol 812	Citric acid buffer pH 3.5	Double-distilled water			
1	16.67%	8.33%	10%	65%		0.1	Cloudy	-
2	18%	9%	5%		68%	0.5	Cloudy	-
3	18.75%	6.25%	10%	65%		0.6	Cloudy	-
4	20.25%	6.75%	5%		68%	0.7	Cloudy	-
5	20%	10%	5%	65%		71.9	Transparent	-
6	**20%**	**10%**	**5%**		**65%**	**87.6**	**Transparent**	**-**
7	**20%**	**10%**	**4%**		**66%**	**97.6**	**Transparent**	**+**

Note: + Classified as nanoemulsion (percentage of transmittance > 95 %)
- Emulsion
Bold typed formula identified as most effective preparation

Table 2. Kaffir lime oil nanoemulsions formula

Number	Formula					Transmittance (%)	Physical appearance	Category
	Surfactant	Co-surfactant	Oil Phase		Aqueous Phase			
	Tween 80	Propylene Glycol	Miglyol 812	Citric acid buffer	Double-distilled water			
1	20%	10%	1%	3%	66%	99.8	Transparent	+
2	20%	10%	1.25%	3.75%	65%	97.53	Transparent	+

Note: + Classified as nanoemulsion (percentage of transmittance > 95 %)
- Emulsion
Bold typed formula identified as most effective preparation

Table 3. Freeze-thawing test results of kaffir lime oil nanoemulsions

Day 0		Day 12 (after six cycles)		Category
Transmittance (%)	Physical appearance	Transmittance (%)	Physical appearance	
95.4 ± 0.0	Yellowish, transparent	95.8 ± 0.9	Yellowish, transparent	Stable

Note:Classified as nanoemulsion if the percentage of transmittance > 95 %. The formula is stable if its transmittance remained at > 95 % after six cycles.

Nanoemulsion was centrifuged for 30 minutes at 3000 rpm. For storage duration test, nanoemulsion was stored for 28 days at room temperature. After this test, physical destabilization was observed.

RESULTS AND DISCUSSION

Previous studies suggest that the best surfactant, co-surfactant, and oil phase for oil in water nanoemulsions are Tween 80, propylene glycol, Miglyol 812, respectively[16, 19, 20]. All of these components are certified as GRASS (Generally Recognize as Safe Substances) and thus safe to be used as excipients in pharmaceutical products. Therefore we designed a carrier formula using these components as shown in Table 1.

Since the carrier formula will be used to encapsulate kaffir lime oil, Tween 80 and propylene glycol as carrier components have more advantages than other surfactants and co-surfactants considering their chemical structure and physicochemical nature. Tween 80 was selected as a surfactant in the organic phase because it is less toxic, stable under *in vivo* conditions, has biological compatibility, is not significantly affected by the potential of hydrogen, and readily available. Long alkyl chain length of Tween 80 (C18) provides greater solubilisation capacity for hydrophobic solutes than other shorter alkyl chain-length Tween such as Tween 20 (C12). Meanwhile, propylene glycol is an amphiphilic molecule that has ability to increase solubility between oil phase and aqueous phase. It also takes a role as co-surfactant when the surfactant has a low concentration in the nanoemulsions system[12, 21, 22]. Moreover, a previous study showed that disruption in the interfacial region can be minimized when the hydrocarbon chain length of surfactant is equal to the sum of co-surfactant chain length and oil chain length[23]. Propylene glycol ($C_3H_8O_2$) has a short chain length while Miglyol 812 (carrier oil) has medium-chain length[24, 25, 26]. Therefore Tween 80 ($C_{64}H_{124}O_{26}$) with long-chain length was able to entrap propylene glycol and Miglyol 812, prior to nanocarrier droplet formation[27]. In Table 1,

Table 4. Stability of kaffir lime oil nanoemulsions after 28 days of storage

Transmittance (%)			Physical appearance after 28 days storage	Category
Day 0	Day 14	Day 28		
95.4 ± 0.0	95.9 ± 0.3	96.4 ± 0.4	Yellowish, transparent	Stable

Note: Classified as nanoemulsion if the percentage of transmittance > 95 %.

Table 5. Physical destabilization of kaffir lime oil nanoemulsion after stability tests

Type of stability test	Observed destabilization				Category
	Creaming	Cracking	Sedimentation	Aggregation	
Freeze-thawing	-	-	-	-	Stable
Storage duration	-	-	-	-	Stable
Centrifugation	-	-	-	-	Stable

Note: + detected after the stability test
- not detected after the stability test
The formula is stable if creaming, cracking, sedimentation and aggregation are not detected after the stability tests.

Table 6. Physical destabilization of kaffir lime oil nanoemulsion after stability testsViscosity of kaffir lime oil nanoemulsions at various centrifugation speeds

Viscometer speed (rpm)	Viscosity (mPa.s)
50.0	146.6
62.5	142.7
75.0	135.9
82.5	130.8
100.0	124.2

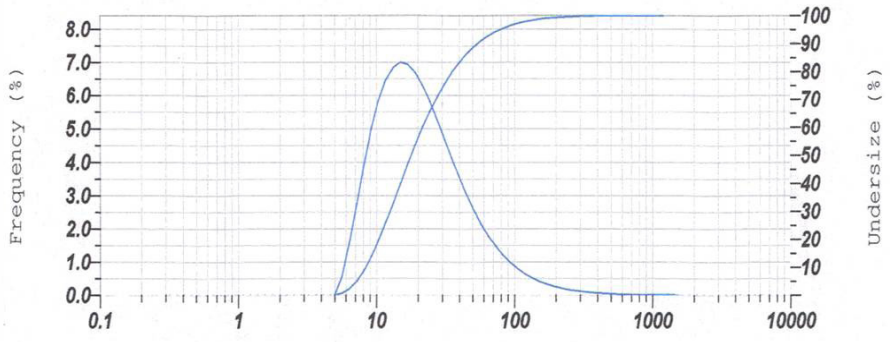

Fig.1. Particle size distribution of kaffir lime oil nanoemulsions

double-distilled water and citric acid buffer were used as the aqueous phase, for the purpose of comparing the carrier nanoemulsions' behaviors in acidic solutions and neutral solutions. The results showed that double-distilled water tends to produce more transparent nanoemulsions than citric acid buffer. Consequently, double-distilled water was chosen as the best aqueous phase as it is also more closely matches a physiological aqueous environment. Based on Table 1, we found that increasing the concentration of surfactant mix (Tween 80 and propylene glycol) has a tendency to increase the transmittance value in nanoemulsions. However, the concentration of surfactant has to be low enough to avoid any possible side effect such as hypersensitivity reactions[28]. Hence formula number 6 and 7 were chosen as the best carrier formula as its surfactant mix concentration was in an acceptable range yet maintained the highest percentage of transmittance. Formula 6 has lower transmittance than 95%, but it is still considered as a potential carrier for kaffir lime nanoemulsions because the addition of essential oils could increase the transmittance. Those carrier formulas were then incorporated with kaffir lime essential oil, showed in Table 2.

The optically transparent formula indicated that the nanoemulsions have smaller droplet size than visible light wavelength[29]. Based on transmittance measurements and essential oil content, the best formula chosen was with 20 % of Tween 80, 10% of propylene glycol, 1.25 % Miglyol 812, and 3.75% of kaffir lime essential oil. This formula has the highest essential oil content with transmittance more than 95%. Physical stability of the chosen formula then observed and the result is showed in Table 3.

Table 3 shows that the transmittance of the chosen formula has a quite constant value from day 0 to day 12, after the sixth cycle of freeze-thawing was done. Moreover, physical appearance of the nanoemulsions did not change after six cycles of freeze-thawing. The kaffir lime oil nanoemulsions still had a yellowish transparent appearance. This indicates that the chosen formula is stable from temperature changes. Yellowish color of nanoemulsions was coming from Tween 80 color and the natural color of kaffir lime oil. Table 3 also showed a slight increase in transmittance after freeze-thawing. The increasing percentage might be caused by decreasing interfacial tension during freeze and thawing. As the interfacial tension decreases, the droplet size become smaller and nanoemulsions become more transparent.

Storage stability of kaffir lime nanoemulsions was observed in room temperature at day 0, day 14 and day 28. The transmittance measurement and physical appearance of the nanoemulsions is shown in Table 4.

Based on Table 4, the transmittance was slightly increased from day 0 to day 28. This phenomenon also might be caused by decreasing interfacial tension between oil phase and aqueous phase. Furthermore, the nanoemulsions still have yellowish, transparent appearance and have transmittance > 95 % even after 28 days. This indicated that the formula is stable for a quite long duration of storage. Next, physical destabilization of the nanoemulsion from three stability tests is show in Table 5.

Creaming is movement of dispersed droplets to upper layer of aqueous phase. Conversely, dispersed droplets movement to lower layer of aqueous phase is called sedimentation. Aggregation occurs when dispersed droplets become close one to another without losing their individual intergrity. Cracking is the coalescence of nanoemulsions droplets [30]. Table 5 showed that the chosen formula has no physical destabilization after each stability test done thus the formula is physically stable.

The characteristics of each formula component contribute to the physical stability. One criteria to determine the ability of surfactant to act as an emulsifier in the oil phase is hydrophilic-lipophilic balance (HLB) value. Surfactant with a HLB value more than 11 means the surfactant is hydrophilic and fit to be used in oil-in-water system[31]. The HLB value of Tween 80 is 15 [32]. This HLB value shows that Tween 80. Moreover, high concentration of surfactant is needed to obtain an optimum formula with spontaneous emulsification. In order to stabilize the formula, it required more than 30% surfactant concentration in general[33]. Nevertheless, in this study 20% of Tween 80 is enough to make a physically stable oil-in-water nanoemulsion. This is caused by propylene glycol as co-surfactant. Co-surfactant helps in decreasing interfacial tension and increasing interfacial fluidity to keep the nanoemulsions stable[12]. The density of the oil phase also has an impact in physicochemical nature of nanoemulsions. Oil-in-water nanoemulsions with oil that has density value under 1 g/mL tend to resist creaming[34]. The components of the oil phase in this study, Miglyol 812 and kaffir lime essential oil, have density values of 0.95 g/mL and 0.86 g/mL respectively[2, 35]. This density value likely contributed to the nanoemulsions stability.

Next, physicochemical nature of the chosen formula was further observed. Viscosity measurements were conducted and the results are shown in Table 6.

In order to enable the production of nanoemulsions in liquid sachet form, the nanoemulsions need to have viscosity value of 100 to 25,000 mPa.s[36]. Based on Table 6, the viscosity of the best formulation is consistent with usage in sachet applications. This low viscosity might be caused by Miglyol 812 and kaffir lime oil which have low viscosities[22]. The viscosity of the nanoemulsions could be increased by addition of oil with long-chain fatty acid into the oil phase. Nonetheless, together with the nanoemulsion's translucent appearance, this low viscosity may be more appealing aesthetically. The low viscosity also can be improved thus the chosen formula and could be made into roll-on type formulations, sprays and gels[25].

Another criterion is the particle size distribution of the nanoemulsions droplets. The distribution graphic of nanoemulsions particle size is shown on Figure 1.

The average size of droplets in the chosen formula is 18.23 ± 0.12 nm with a polydispersity index of 0.36 ± 0.01. These results confirmed that the droplets size of obtained formula is less than 100 nm, which could be categorized as nanoemulsion[13]. Tween 80 contributed to this small droplet size. With high HLB value, Tween 80 was able to stabilize the particle in the oil-in-water system, thus decreasing droplet size. Small droplet size also decreased physical destabilization risk during storage. Therefore kaffir lime oil nanoemulsions still have transparent appearance after 28 days of storage (Table 3). The polidispersity index shows particle size distribution in a scale 0 to 1. The higher the podispersity index value of nanoemulsions, the less homogenous are its particle size distribution. Commonly, most of nanoemulsions have a polidispersity index value of 0.3. The chosen formula has polidispersity index of 0.36 and this indicated that the formula is monodispersed[22].

In conclusion, kaffir lime oil can be formulated into nanoemulsions using Tween 80, propylene glycol, Miglyol 812, and double-distilled water. Our kaffir lime oil nanoemulsions are thermodynamically stable, and robustly resist changes from variations in temperature, centrifugation, and long-term storage. The best candidate formulation was chosen from our results. This formulation had low viscosity, suggesting strong potential as a candidate compound for pharmaceutical production. Optimizing this nanoemulsion formula and increasing its kaffir lime content may permit its use as an antibacterial, anti-inflamatory, and/or aromatherapy agent.

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