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Formulation and Stability of Clove Leaf (*Syzygium aromaticum* L.) Essential Oil Microemulsion

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ABSTRACT

Clove leaf essential oil is a volatile compound, unstable to heat, light and air. Clove leaf essential oil can be prepared in microemulsion (o/w) preparations to maintain stability and aplicable in food. The purpose of this study was to obtain a microemulsion formulation of clove leaf essential oil (o/w) with good stability. The nonionic surfactants used consisted of Tween 20, Span 80 and Tween 80. This research was carried out in 2 stages. The first stage consisted of a mixture of surfactant ratios (Tween 20, Span 80 and Tween 80) with 5 treatment levels, namely (98:2:0), (98:0:2), (98:0,5:1.5), (98:1,5:0,5), and (98:1:1). The second stage consisted of the ratio of clove leaf essential oil and the ratio of surfactants selected in the first stage which consisted of 8 levels, namely (15:85), (17.5:82,5), (20:80), (22.5: 77.5), (25:75), (27.5:72,5), (30:70), and (32.5:67,5). Stability test was carried out on centrifugation force, heating, pH, and dilution with turbidity index parameters. The turbidity index value was below 1% and the transparent appearance was declared as a stable microemulsion. The results showed that the ratio of the mixture of nonionic surfactants (98: 0.5: 1.5) was the best result with the characteristic turbidity index value before testing 0.181±0.007%, after centrifugation 0.188±0.004% and after heating 0.215±0.013%. Meanwhile, the ratio of clove leaf essential oil to surfactant 22.5:77.5 was the best result with a transparent appearance, the turbidity index value before testing was 0.214±0.013%, after centrifugation was 0.225±0.013% and after heating was 0.268±0.012%. The clove essential oil microemulsion was stable at pH (3.5; 4.5; 5.5) and dilution (1:1, 1:9, 1:99). The size of the clove leaf essential oil microemulsion droplets formed 2 peaks, namely peak 1 of 93% with an average size of 35.4 nm and peak 2 of 7% with an average size of 2759.3 nm with a polydispersity index value of 0.420. The clove essential oil microemulsion had an average zeta potential value of 0.1 mV. Clove leaf essential oil was stable for 8 weeks of storage.

Introduction

Clove is widely cultivated plant in Indonesia that has aromatic flavor caused by essential oil compound. Clove leaf essential oil contains the main compound eugenol which has the ability as antibacterial, antifungi and antioxidant (Gülçin *et al.*, 2012; Hartanti *et al.*, 2020; Hossain *et al.*, 2014; Idowu *et al.*, 2021). Eugenol ($C_{10}H_{12}O_2$) is a phenylpropanoid compound which has characteristic such as slightly soluble in water, weak acid, easily soluble in organic solvents, clear to pale yellow color and aromatic aroma(Kamatou *et al.*, 2012). According to Hosseini *et al.*, (2019) clove essential oil contains eugenol (79,4%), *β-Caryophyllene* (13,36%) that able to inhibit the growth of *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* bacteria.

Clove leaf essential oil application as natural preservative has been developed. The potency of clove leaf essential oil as antimicrobe is caused by the presence of monoterpene compound that lipophilic and able to destroy the cytoplasmic microbe membrane (Cristani *et al.,* 2007). The application of clove essential oil as natural preservative in food product is still not optimal due to it was immiscible to water, so minimize contact with polar ingredients (Hamad and Hartanti, 2015; Juneja *et al.*, 2012; Van de Vel *et al.*, 2019). Clove essential oil also been widely used in food and parfum industry, but it has weakness like very volatile compound, unstable to heat, light and air (Cui *et al.*, 2015). Hence, to improve the stability, solubility and effectiveness of clove essential oil in food products, it may be prepared in microemulsion preparation (Saini *et al.*, 2019).

Microemulsions are dispersions isotropic formed spontaneously consisting of oil and water stabilized by surfactants and/or co-surfactants having droplet sizes between 5 nm – 10 nm (Zhu *et al.*, 2022). The advantages of microemulsion are high stability, small droplet size, transparent, and easy to preparation, therefore it makes suitable as carrier system on pharmaceutical and food industries (Xavier-Junior *et al.*, 2017) because toxic and causes irritation (Flanagan and Singh, 2006). Nonionic surfactants are compound that have no charge, stable to pH and non-toxic (*food grade*) (Mahdi *et al.*, 2011).

The characteristics and stability of the resulting microemulsion are influenced by type surfactant, ratio of mixed surfactant and oil, pH, dilution and temperature (Cho *et al.*, 2008). Microemulsion *o/w* that used mixed nonionic surfactants (Tween 80, Tween 20 and Span 80) are produced small droplet size and safe to consumption (*food grade*) (Permana and Suhendra, 2015). The mixed surfactant with different HLB (*Hydrophilic Lipophilic Balance*) value can increase microemulsion stability due to form small droplet and improve it solubility(Cho *et al.,* 2008). Clove leaf essential oil microemulsion produced using surfactant Tween 20 (HLB = 16.7) has better solubility because it has suitable polarity with the oil phase compared to Tween 18 (HLB = 15) (Nirmala *et al.,* 2019). Synergism of surfactant (Tween 20, Span 80 and Tween 80) with the right ratio is expected to produce a stable clove leaf essential oil microemulsion. Microemulsion stability has been also influenced by ratio oil phase and surfactant. Prasanta *et al.*, (2022) reported the best result ratio oil phase and surfactant on betel leaf microemulsion is 6 : 94. The higher oil concentration and the lower surfactant concentration causing surfactant capability decreased to dissolving oil and the solution became cloudy (Suhendra *et al.*, 2012).

Based on statement above, clove leaf essential oil has potential as an antimicrobial agent. However, clove leaf essential oil are volatile compound, unstable to heat, light and air causes difficulties in its application to food products. Clove leaf essential oil microemulsion used mixed nonionic surfactant is expected to increase solubility and have high stability. The aim of this study was to obtain a stable formulation of clove leaf essential oil microemulsion from a mixture of several mixture nonionic surfactants (Tween 20, Span 80 and Tween 80).

Materials and Methods

Materials and Tools

The materials used in this research are Tween 20, Span 80 and Tween 80 (*Merck*, Darmstadt, Germany), clove leaf essential oil was purchased in Gallery Essential Oil, Denpasar, Bali, aquadest (*Water One*). The tools used in this research are PSA (*Horiba Scientific Nano Particle Analyzer SZ-100*), *hot plate magnet stirrer* (*Companion HP-300*), analytical balance (*Shimadu AUW 220*), centrifuge (*Clements GS 150*), spectrophotometer UV-Vis (*Evolution 201*), micro pipette (*Socorex*), and beaker glass (*Pyrex*).

Research Implementation

Microemulsion Formulation Stage 1

The first stage used a completely randomized design (CRD) with the treatment being a mixture ratio formulation of nonionic surfactant (Tween 20, Span 80 and Tween 80). The treatment consisted of 5 levels with 4 repetitions to obtain 20 experimental units. The preparation started with combination of nonionic surfactants (Tween 20, Span 80 and Tween 80) with 5 levels of surfactant ratio treatment which are presented in Table 1. Clove leaf essential oil was mixed with each formulation of the surfactant mixture in a ratio of 10 : 90 and the total solution was 5 ml. Then, the stirring process was carried out with a magnetic stirrer at a speed of 700 rpm for 4 minutes at a temperature of ± 70^{o}C (Sucitawati *et al.*, 2021). Next process, 10 ml of distilled water was added dropwise so that the total clove leaf essential oil microemulsion was 15 ml. The formed clove leaf essential oil microemulsion was incubated for 24 hours.

Microemulsion Formulation Stage 2

The second stage used completely randomized design (CRD) with the treatment being a formulating of ratio clove leaf essential oil with mixture of selected surfactants. The treatment consisted 8 levels with 3 repetitions to obtain 24 experimental units. The first step started to prepared mixture selected surfactant, clove leaf essential oil and aquadest. Furthermore, clove leaf essential oil was mixed with mixture selected surfactant consisted 8 treatment levels (%), namely: F1 (15,0 : 85,0), F2 (17,5 : 82,5), F3 (20,0 : 80,0), F4 (22,5 : 77,5), F5 (25,0 : 75,0), F6 (27,5 : 72,5), F7 (30,0 : 70,0) and F8 (32,5 : 67,5). The total ratio of clove leaf essential oil and surfactant mixture was made to 5 ml. Then, the stirring process was carried out with a magnetic stirrer at a speed of 700 rpm for 4 minutes at a temperature of ± 70^{o}C (Sucitawati *et al.*, 2021). Next process, 10 ml of distilled water was added dropwise so that the total clove leaf essential oil microemulsion was 15 ml. The formed clove leaf essential oil microemulsion was incubated for 24 hours. The stable microemulsion characteristic was observed visually namely the formation of a transparent mixture, having a single phase, not forming a gel, and when shaken for 1 minute it is not cloudy (Suhendra *et al.*, 2012).

Observed Variable

The variables observed in this study were the appearance of the clove leaf essential oil microemulsion and the stability test was carried out on centrifugation, heating, pH, dilution and during storage with the turbidity index parameter referring to the Cho *et al.*, (2008) method. Microemulsion stability can be observed based on turbidity index value and the appearance of the presence or absence of gel. Microemulsion turbidity was measured using spectrophotometer UV-Visat wavelength 502 nm with the formula: turbidity (%) = 2.303 x absorbance / cuvette width (cm).

Statistical Analysis

Research data analysis of stage 1 and stage 2 was carried out using the Minitab 20 software. The data obtained in the stages 1 and 2 were analyzed using analysis of variance (ANOVA) and if there was an effect of treatment on the observed variables followed by Tukey test (Steel and Torrie, 1993).

Results and Discussion

Surfactant Mixed Formulation in Microemulsion (*o/w*)

The effect of nonionic surfactant mixture ratio formulations (Tween 20, Span 80 and Tween 80) on initial turbidity, after centrifugation and after heating on clove leaf essential oil microemulsion is shown in Table 2. The result analysis of variance showed that mixture surfactant ratio had a significant effect (P<0,05) on the clove leaf essential oil microemulsion initial turbidity index. The lowest initial turbidity index was found in the S3 formula which was 0,1808% not different from the S2, S4 and S5 however, it was different from the S1. The value turbidity index after centrifugation and after heating, analysis of variance result showed that mixture surfactant ratio had a very significant effect (P<0,01). The lowest turbidity after centrifugation was in formula S3 with an average 0,1877% or different from S2 dan S5. Meanwhile, the lowest turbidity after heating was in formula S3 with an average 0,2153% not different from S2, S4 dan S5. The turbidity value of clove leaf essential oil microemulsion after centrifugation and heating tends to increase compered to initial turbidity. However, all the formulations still produced a stable clove leaf essential oil microemulsion with a turbidity index less than 1%, transparent appearance and no separation.

Determination of the type surfactant to produced microemulsion is very important thing. The manufacture of microemulsions (*o/w*) can use surfactants with an HLB value namely 8-18(Kale and Deore, 2017). A mixture of surfactant with HLB values that match the oil phase can increase solubility and stability of the dispersion system (Mahdi *et al.,* 2011). Microemulsion that formed using mixed surfactant with different HLB is able to produced a lower surface tension than single surfactant (Ariviani *et al.,* 2015). In this study, Tween 20 was used as a surfactant, Tween 80 and Span 80 as a cosurfactant. Tween 20 (HLB = 16.7) had a better solubility with clove leaf essential oil than Tween 80 (HLB = 15) due to significant difference HLB value (Nirmala *et al.,* 2019). Tween 20 has a shorter hydrophobic tail (C12) compared to Tween 80 (C18) so that it interacts more efficiently with clove leaf essential oil in the interfacial layer which can increase the formation of microemulsions (Edris and Malone, 2012). Gupta *et al.,* (2005) reported that a stable microemulsion (*o/w*) formulation of clove essential oil as a drug carrier system used Tween 20 with a concentration 30% and clove oil 5%. A number of researchers have previously examined that the use of Tween 20 as a surfactant has a suitable polarity with clove essential oil (Hamed *et al.,* 2012; Nirmala *et al.,* 2015; Purwasena *et al.,* 2020).

The increased use of Span 80 as cosurfactant caused the turbidity index value of the clove leaf essential oil microemulsion to also increase. This due to the difference in the polarity of Span 80 (HLB = 4.3) to the oil phase that it tends to produced larger droplet sizes. The presence of cosurfactant is able to overcome the repulsion forces of oil and water phases, reduce the interfacial tension that help strengthen to resulting microemulsion structure (Yadav *et al.,* 2018). Synergism of surfactant mixtures (Tween 20, Span 80 and Tween 80) with the right concentration is able to produced high stability microemulsion (*o/w*) (Suhendra *et al.,*2012). Figure 1 is present a mixture of nonionic surfactants in the first stage.

Microemulsion stability can be determined by accelerated stability testing (abnormal conditions), namely the presence of centrifugation (4500 rpm) and heating (105^{o}C for 5 hours). Centrifugation testing was carried out to determine microemulsion stability against the influence of gravity. If the microemulsion is unstable, centrifugal force will cause dispersed microemulsion to separate from the continuous phase (Permana and Suhendra, 2015). Microemulsion testing against heating is the most critical test. High temperature causes dehydration increase in the hydrophilic part so that the emulsifier molecules aggregate and the solution became cloudy (Indirasvari *et al.,* 2018).The result of this study showed that all formulations were able to produced stable microemulsions due to turbidity index value less than 1%, transparent appearance and no separation. The best treatment in first stage is the S3 formula (98:0, 5:1, 5) because it has the lowest average turbidity, so it is continued in the second stage research.

The Ratio of Clove Leaf Essential Oil and Surfactant

The second stage was conducted to determine clove leaf essential oil and surfactant ratio using the best surfactant mixture formulation in first stage, namely Tween 20 : Span 80 : Tween 80 (98 : 0,5 : 1,5). In microemulsion (*o/w*) the oil phase functions as a solvent for non-polar bioactive compounds. The oil phase with high concentration is expected to increase ability of microemulsion (*o/w*) to carry bioactive compound compared to low concentration of oil phase. Clove leaf essential oil and mixture surfactant ratio (v/v) was carried out to determine the effect of oil phase increasing concentration on the formation of microemulsions (Table 3). The ratio of clove leaf essential oil and surfactant which is presented in Fig 2.

Initial Turbidity

The results analysis of variance showed that clove leaf essential oil and surfactant ratio had a very significant effect ($P<0.01$) on the microemulsion initial turbidity (Table 3). The clove leaf essential oil phase concentration from 15% to 30% (F1-F7) has a index turbidity value below 1% and transparent appearance. However, at a concentration of 32.5% clove leaf essential oil phase (F8) it produced cloudy solution with turbidity index value of 3.0292%.

Colloidal dispersion stability is influenced by the ratio of oil with surfactant and type oil used (Ziani *et al.*, 2012). An important factor in the formation microemulsions is the compatibility between surfactant and oil chain length (Bayrak and Iscan, 2005). The main content of phenolic compounds in clove leaf essential oil is eugenol which has a relatively low molecular volume of 0.26 nm^3 (Tchakalova *et al.*, 2008) Eugenol compound has a short unsaturated hydrophobic tail (C10) (Edris and Malone, 2012), which has a solubility that is suitable for Tween 20 (C12) surfactant because it has shorter hydrophobic tail, thereby increasing area formation of microemulsion. In a study conducted by Prasanta *et al.,* (2021) the ratio of betel leaf essential oil concentration with the maximum surfactant of forming microemulsion was 6:94. In other hand, in the manufacture of ginger essential oil microemulsion the best oil phase and surfactant ratio was 4:96 (Dwipayana *et al.,* 2022). Based on the results of this research data, clove leaf essential oil microemulsion can be formed up to the ratio of oil and surfactant that is 30:70. The increasing oil phase concentration causes the surfactant concentration to decrease, this causes decrease surfactant ability to dissolve oil so that the solution became cloudy (Suhendra *et al.,* 2012). Surfactants and oils that have the appropriate polarity cause the dissolved oil to form high microemulsions. The high oil concentration is expected to increase its ability to carry bioactive components. Surfactant concentrations above the critical micelle concentration (CMC) can form micelles that have high solubility capabilities (Zhu *et al.*, 2022).

Microemulsion Stability (*o/w*) Against Centrifugation

The results analysis of variance showed that clove leaf essential oil and surfactant ratio had a very significant effect ($P<0.01$) on the turbidity index value microemulsion after centrifugation (Table 3). Formulations F1 to F7 with oil phase concentration (15% - 30%) are able to produce microemulsions with turbidity index values below 1%, transparent appearance and no separation occurs. Oil phase concentration of 32,5%, the microemulsion was not formed because it had a turbidity index value above 1% with an average of 3.0377% and cloudy appearance. The turbidity index value (%) ratio of the highest clove leaf essential oil concentration which was still formed microemulsion was at ratio of 30% (F7) with the turbidity value after centrifugation was 0.2641%. However, the turbidity index value of F7 (30:70) was not significantly different from the formulas F3 (20:80), F4 (22.5:77.5), F5 (25:75) and F6 (27.5:72.5).

Centrifugation test is carried out to determine whether there was a phase separation due to the gravitational force. The principle of centrifugation is to separate particles based on their molecular density, with the presence of centrifugal force causing particles with smaller density to be on top and particles have larger density to go down (Listyorini *et al.,* 2018). Based on the results of this research data, almost all formulation are stable against centrifugation force because they have turbidity index value less than 1%, transparent appearance and no separation. However, the F8 formulation produces a cloudy solution and has a turbidity index value of more than 1% which is 3.0377%. The centrifugal force causes the merging of droplets to form larger droplets so that the solution becomes cloudy which causes increase turbidity index value (Permana and Suhendra, 2015). If the microemulsion is not stable to centrifugation, a particle aggregation process will occur which slowly causes the microemulsion particle size to increase (Yan *et al.,* 2021). Microemulsions have high stability are not affected by gravity and have a transparent appearance. This indicates that the F8 formula is unstable to centrifugation force.

Microemulsion Stability (*o/w*) Against Heating

The results analysis of variance showed that clove leaf essential oil and surfactant ratio had a very significant effect (P<0.01) on the turbidity index value microemulsion after heating (Table 3). The microemulsion after being heated showed transparent appearance with turbidity index still below 1% at a ratio of oil to 30%. This indicates that the resulting microemulsion has high stability against heating, except at the oil concentration of 32,5% (F8) because it produces a cloudy solution. Formula F7 (30:70) is the ratio of the highest clove leaf essential oil concentration which is still capable producing microemulsions with turbidity index value of 0.3124% not significantly different from formulas F3 (20:80, F4 (22.5:77.5), F5 (25:75) and F6 (27.5:72.5).

The most critical microemulsion test is the heating test. Based on the results of this study, the F8 formula with clove leaf essential oil and surfactant ratio (32.5:67.5) was indicated to be unstable to heat because it had turbidity index value more than 1% (3.1137%) with cloudy appearance. The high temperature can damage the micellar structure so that it affects microemulsion stability (Zhu *et al.,* 2022). Nonionic surfactant are very sensitive to temperatures. High temperatures causes dehydration to increase in the hydrophilic part which causes the emulsifier to aggregate and produce cloudy solution called the cloud point (Streck *et al.,* 2018). Large aggregates produced by high temperatures have the ability to emit light, causing the solution to became cloudy. The temperature above cloud point causes the aggregate to become large and settle due to the force of gravity (Suhendra *et al.,* 2012). Microemulsions that have high stability are not affected by high temperatures, not separate and still have transparent appearance.

Microemulsion Stability (*o/w*) Against Oil-Surfactant Ratio with pH and Dilution

Microemulsion turbidity index value on the oil-surfactant ratio treatment at pH and dilution which is presented in Table 4. The results analysis of variance showed that interaction between oil-surfactant ratio and pH had a very significant effect (P<0.01) on the microemulsion turbidity index value at a 1:1 dilution. Based on data in Table 4 showed that F4 is the most stable formula against changes in various pH values (3.5 : 4.5 : 5.5) at 1:1 dilution because it has turbidity index value below 1% with a transparent appearance. Formula F4 has turbidity index value of 0.229% (pH 3.5), 0.223% (pH 4.5) and 0.223% (pH 5.5) which are not significantly different from formulas F1, F2, and F3. However, the formulas F5, F6, F7 and F8 declared unstable to changes in various pH, because they had a turbidity index value above 1%. At a dilution 1:9, the results analysis of variance showed that interaction between oil-surfactant ratio and pH had a very significant effect ($P<0.01$) on the microemulsion turbidity index value. Based on the result data (Table 4) it showed that all formulations are stable to various pH changes. The result is similar in the 1:99 dilution, analysis of variance showed that oil-surfactant interaction and pH had a very significant effect ($P<0.01$) on the microemulsion turbidity index value. All formulations showed high stability against all pH at 1:99 dilution.

The results showed that clove leaf essential oil microemulsion was stable under acidic conditions (pH 3.5; 4.5; 5.5). This is caused by the use of nonionic surfactants in microemulsion manufacture. Nonionic surfactant are emulsifier that have no charge so they are not affected by the concentration of H^+ which makes microemulsion stable under acid conditions (Zheng *et al.*, 2022). Nonionic surfactants have good stability at low pH (acidic) compared to high pH (Esmaeili *et al.*, 2019). The turbidity index of clove leaf essential oil microemulsions at pH 4.5 and 5.5 tended to be lower that at pH 3.5 although not significant different. This is because clove leaf essential oil has an optimal pH of 5 (Hoque *et al.*, 2008).

Based on the results research above, it also shows that all formulas have good stability against 1:9 and 1:99 dilutions. However, at 1:1 dilution, the formulas F5, F6, F7 and F8 had a turbidity value more than 1% which resulted in a cloudy solution. This is thought to be caused by the increasing concentration of oil at 1:1 dilution causing contact of the oil phase to be higher with pH which causes droplet size to increase so that the stability microemulsion decreases. Therefore, based on this F4 is the most stable formula because it has high stability against various changes in pH (3.5; 4.5; 5.5) at dilution (1:1, 1:9, 1:99) and has a high oil-surfactant ratio (22.5 : 77.5).

Particle Size and Zeta Potential Testing

Based on the second stage result of this research, the stable treatment obtained was formula F4 oil-surfactant rasio (22.5 : 77,5) so that it was continued with PSA testing. Determination of the microemulsion droplet size can be tested using PSA (Particle Size Analyzer). Figure 3 showed that the particle distribution of the selected clove leaf essential oil microemulsion has 2 peaks. First peak is 93% with average size is 35.4 nm and second peak is 7% with average size is 2759.3 nm. The largest or most frequently detected droplet size distribution by PSA detectors is 22.8 nm.

Based on the PSA result test, the colloidal dispersion was a microemulsion. Microemulsion have a dispersed droplet particle size of 10 – 100 nm (Zhu *et al.*, 2022). In this study, the PSA results showed a polydispersity index (PI) value of 0.420. The polydispersity index is used to measure uniformity degree of particle size distribution. PI value below 0.5 indicates a uniform distribution (monodisperse), while PI value greater than 0.7 indicates that the sample has a wide or non-uniform particle size distribution (polydisperse) (Abbas *et al.*, 2020). The resulting droplet size can indicate stability level of system dispersion, smaller droplet size can improve microemulsion stability against sedimentation, flocculation and coalescence (Cho *et al.*, 2008). Based on the PSA test data, the selected clove leaf essential oil microemulsion showed a uniform particle size distribution because it had a polydispersity index value below 0.5 namely 0.420 and was included in microemulsion system due to it had droplet size of 22.8 nm. The particle size distribution of clove leaf essential oil microemulsion was presented in Figure 3.

Table.1 Formulation of mixed nonionic surfactant

Formulation	Tween 20 (%)	Span 80 (%)	Tween 80 (%)
S1	98	2,0	0,0
S2	98	0,0	2,0
S3	98	0,5	1,5
S4	98	1,5	0,5
S5	98	1,0	1,0

Table.2 Clove leaf essential oil microemulsion stability on surfactant mixture formulations

Formulation (T20 : S80 : T80)	Ratio of clove leaf essential oil and surfactant (15:85 v/v)					
	Incubation 24 hours		After centrifugation		After Heating	
	Turbidity (%)	Appearance	Turbidity (%)	Appearance	Turbidity (%)	Appearance
S1 (98 : 2,0 : 0,0)	0,2044[a]	Transparent	0,2171[a]	Transparent	0,2556[a]	Transparent
S2 (98 : 0,0 : 2,0)	0,1917[ab]	Transparent	0,1981[bc]	Transparent	0,2245[b]	Transparent
S3 (98 : 0,5 : 1,5)	0,1808[b]	Transparent	0,1877[c]	Transparent	0,2153[b]	Transparent
S4 (98 : 1,5 : 0,5)	0,1929[ab]	Transparent	0,2079[ab]	Transparent	0,2326[ab]	Transparent
S5 (98 : 1,0 : 1,0)	0,1889[ab]	Transparent	0,2009[bc]	Transparent	0,2211[b]	Transparent

Note : The same letter in the same column and row behind average values shows no significant difference at the 95% significant level.

Table.3 Clove leaf essential oil microemulsion stability on ratio of oil phase and surfactant

Formulation (T20:S80:T80)	Incubation 24 hours		After Centrifugation		After Heating	
	Turbidity (%)	Appearance	Turbidity (%)	Appearance	Turbidity (%)	Appearance
F1 (15,0 : 85,0)	0,1942[e]	Transparent	0,2034[c]	Transparent	0,2418[d]	Transparent
F2 (17,5 : 82,5)	0,1996[e]	Transparent	0,2088[c]	Transparent	0,2472[cd]	Transparent
F3 (20,0 : 80,0)	0,2057[e]	Transparent	0,2188[bc]	Transparent	0,2556[cd]	Transparent
F4 (22,5 : 77,5)	0,2142[de]	Transparent	0,2249[bc]	Transparent	0,2679[bcd]	Transparent
F5 (25,0 : 75,0)	0,2326[cd]	Transparent	0,2395[bc]	Transparent	0,2802[bcd]	Transparent
F6 (27,5 : 72,5)	0,2487[bc]	Transparent	0,2457[bc]	Transparent	0,3002[bc]	Transparent
F7 (30,0 : 70,0)	0,2595[b]	Transparent	0,2641[b]	Transparent	0,3124[b]	Transparent
F8 (32,5 : 67,5)	3,0292[a]	Cloudy	3,0377[a]	Cloudy	3,1137[a]	Cloudy

Note : The same letter in the same column and row behind average values shows no significant difference at the 95% significant level.

Table.4 Stability of clove leaf essential oil microemulsion on pH and dilution

Formula	**Dilution 1:1**			**Dilution 1:9**			**Dilution 1:99**		
	pH 3,5	pH 4,5	pH 5,5	pH 3,5	pH 4,5	pH 5,5	pH 3,5	pH 4,5	pH 5,5
F1	0,196^{j}	0,218^{j}	0,201^{j}	0,189^{d}	0,189^{d}	0,192^{d}	0,186^{cdefgh}	0,186^{cdefgh}	0,181^{defgh}
F2	0,208^{j}	0,194^{j}	0,192^{j}	0,178^{d}	0,177^{d}	0,171^{d}	0,164^{gh}	0,170^{efgh}	0,166^{fgh}
F3	0,212^{j}	0,202^{j}	0,230^{j}	0,193^{d}	0,194^{d}	0,206^{d}	0,179^{defgh}	0,183^{cdefgh}	0,194^{bcdefg}
F4	0,229^{j}	0,223^{j}	0,223^{j}	0,205^{d}	0,203^{d}	0,191^{d}	0,191^{bcdefgh}	0,177^{defgh}	0,180^{defgh}
F5	1,314^{f}	1,030^{i}	1,006^{i}	0,367^{ab}	0,342^{bc}	0,351^{bc}	0,204^{abcd}	0,166^{fgh}	0,167fgh
F6	3,435^{c}	1,330^{ef}	1,158^{h}	0,368^{ab}	0,350^{bc}	0,327^{c}	0,195^{bcdefg}	0,180^{defgh}	0,162^{h}
F7	4,129^{b}	1,358^{e}	1,193^{h}	0,360^{abc}	0,327^{c}	0,340^{bc}	0,213^{abc}	0,199^{bcde}	0,178^{defgh}
F8	4,413^{a}	1,744^{d}	1,267^{g}	0,395^{a}	0,361^{abc}	0,355^{bc}	0,234^{a}	0,221^{ab}	0,197^{bcdef}

Note : The same letter in the same column and row behind average values shows no significant difference at the 95% significant level.

Fig.1 Surfactant mixed formulation in clove leaf essential oil microemulsion

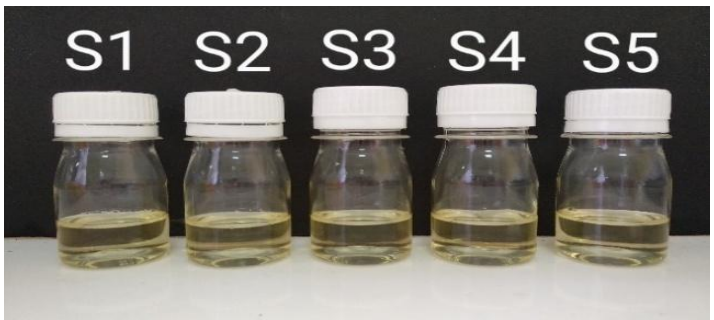


Fig.2 Ratio of clove leaf essential oil and surfactant

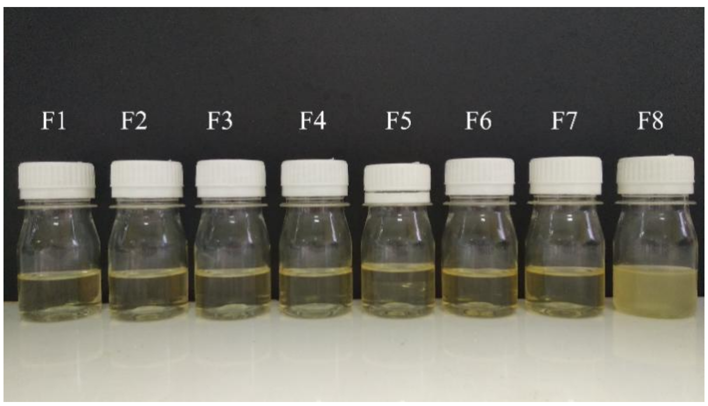


Fig.3 Particle size distribution of clove leaf essential oil microemulsion

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	0.93	35.4 nm	28.4 nm	22.8 nm
2	0.07	2759.3 nm	1902.7 nm	3015.1 nm
3	---	--- nm	--- nm	--- nm
Total	1.00	238.3 nm	884.2 nm	22.8 nm

Cumulant Operations

Z-Average : 62.8 nm
PI : 0.420

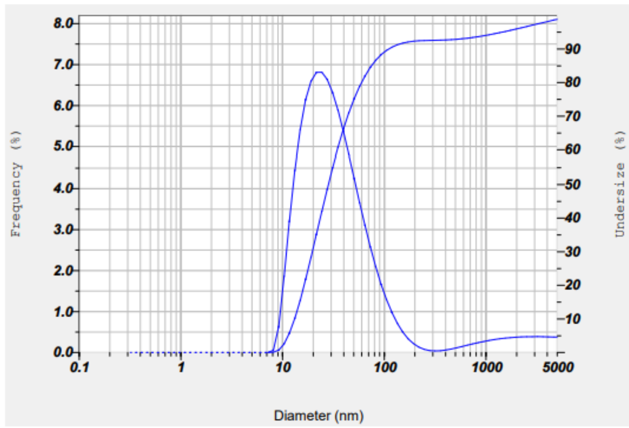


Fig.4 Zeta Potential of clove leaf essential oil microemulsion

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	0.1 mV	0.000001 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : 0.1 mV
Electrophoretic Mobility Mean : 0.000001 cm2/Vs

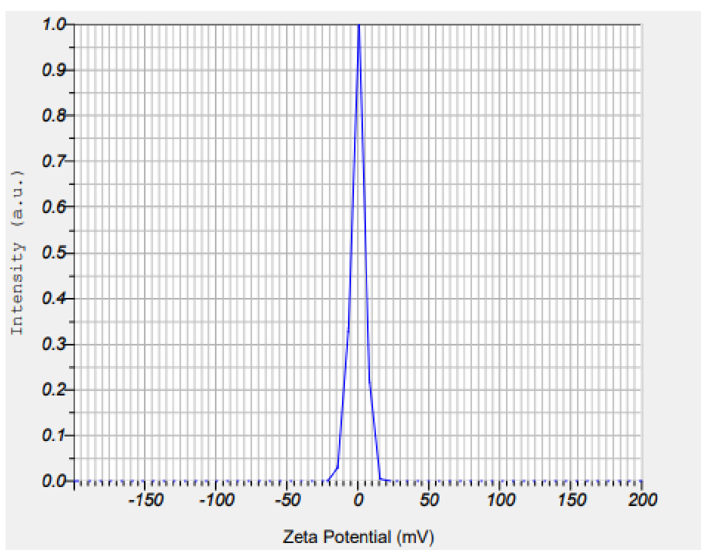
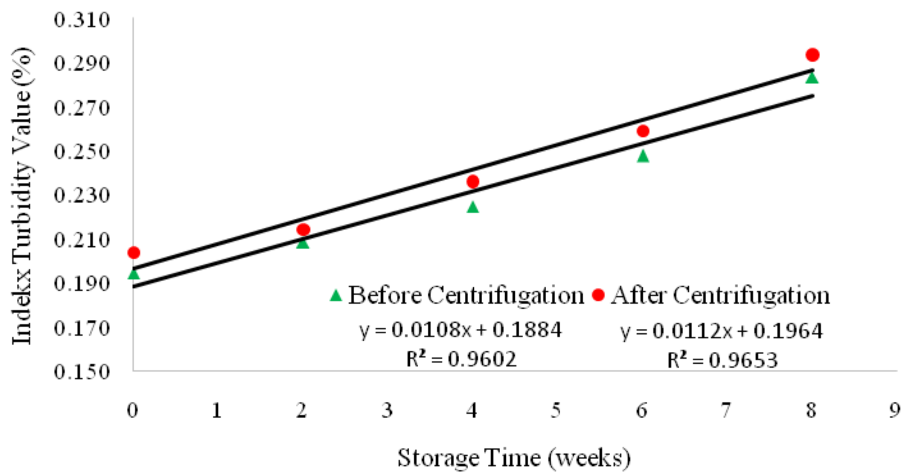


Fig.5 Stability of clove leaf essential oil microemulsion during storage



The zeta potential test is used to determine presence of dispersion system charge and measure the repulsion between particle to prevent particle aggregation. Based on this study result, the potential zeta value of the clove leaf essential oil microemulsion was 0.1 mV (Figure 4). The zeta potential value in microemulsion tends to be neutral due to the use of nonionic surfactants in there manufacture (Üstündağ Okur *et al.,* 2017). The result was similar to the research conducted by (Panomsuk *et al.,* 2020) that clove essential oil microemulsions prepared with nonionic surfactants produced a zeta potential of -0.424 to -0.207 which was close to the neutral point. A colloidal systems that have zeta potential value of -30 mV to +30 mV was said to be acceptable (Kale dan Deore, 2017). Based on this research data, clove leaf essential oil microemulsion is a stable colloid system because it has a potential zeta value of 0.1 mV.

Microemulsion Stability During Storage

The stability of clove leaf essential oil microemulsion during storage was carried out by testing the turbidity index (%) before and after centrifugation. This aims to determine the change in the level of turbidity (brakedown rate) in the clove leaf essential oil microemulsion during storage. Testing the turbidity index value of clove leaf essential oil microemulsion was carried out every 2 weeks. The following is clove leaf essential oil microemulsion during storage which is presented in Figure 5.

In Figure 5 shows that the variable coefficient of storage time (x) before centrifugation is 0.0108. Determination value before centrifugation that 96.02% of regression equation is influenced by storage time and 3.98% is influenced by other factors not examined. Meanwhile, the variable coefficient of storage time after centrifugation is 0.0112. Determination value after centrifugation that 96.53% of regression equation influenced by storage time and 3.47% is influenced by other factors not examined. Based on this, the variable coefficient values during storage before and after centrifugation had a very small differences, indicating that the clove leaf essential oil microemulsion was stable for 8 weeks of storage. Clove leaf essential oil microemulsion before centrifugation is predicted to reach a turbidity index value of 1% based on the regression equation is 82 weeks or 1.6 years.

Based on the result of this study, it was shown that the turbidity index (%) of the clove leaf essential oil microemulsion tended to increase. However, the clove leaf essential oil microemulsion was still stable, had a transparent appearance, no separation occurred and had a turbidity index value below 1%. This indicated that clove leaf essential oil microemulsion was completely dispersed during 8 weeks storage. Several studies have been conducted that examine the stability of *o/w* microemulsions during storage at room temperature and have similar results, namely an increase in the turbidity index value during storage (Ariviani *et al.,* 2015; Cho *et al.,* 2008). This is thought to be caused by an increase in the droplet size of the dispersed phase during storage. On the other hand, ginger essential oil microemulsion (Dwipayana *et al.,* 2022) and betel leaf essential oil microemulsion (Prasanta *et al.,* 2022) had similar results, namely the microemulsion (*o/w*) was able to stable for 8 weeks of storage.

Based on the research that has been done of clove leaf essential oil microemulsion formulation and stability, it can be concluded several things as follows: The ratio of the nonionic surfactant mixture (98 : 0.5 : 1.5) was the best result with characteristic turbidity index value before testing 0.181±0.007%, after centrifugation 0.188±0.004% and after heating 0.215±0.013%. The ratio of clove leaf essential oil to surfactant (22.5 : 77.5) was the best result with transparent appearance, turbidity index value before testing 0.214±0.013%, after centrifugation 0.225±0.013%, after heating 0.268±0.012%, stable at pH (3.5; 4.5; 5.5) and dilution (1:1, 1:9, 1:99).The clove leaf essential oil microemulsion has droplet size of 22.8 nm, zeta potential of 0.1 mV and stable for 8 weeks of storage.

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