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Research Article

Formulation and Evaluation of Nanoemulsion Kelakai (*Stenochlaena palustris*) Herbs with Composition of Smix (Tween 80 and Glycerin) and Pine Oil

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Abstract

Kelakai (*Stenochlaena palustris*) herb is a typical South Kalimantan plant containing bioactive substances that can serve as antioxidants. It can be formulated into nanoemulsions to increase bioavailability in the skin. This study aimed to determine the ratio of pine oil and Smix (tween 80 and glycerin) as the optimal base for nanoemulsion based on the highest percent transmittance and to determine the physical characteristics of the *S. palustris* herb extract nanoemulsion with variations in the amount of extract. Bases of nanoemulsion formula were optimized using a ternary phase diagram and D-Optimal Mixture Design. Nanoemulsion contained three extract concentrations: 0.1; 0.25; and 0.5%, respectively, and nanoemulsion was tested for physical characteristics. The results of this study were a light yellow to light brown, clear, and transparent, with a characteristic weak-strong odor, forming an O/W nanoemulsion. Increasing the concentration of the extract significantly increased the viscosity and decreased the pH and percent transmittance. This study concluded that variations in extract concentration affected the percent transmittance, organoleptic, pH, viscosity, and the optimal ratio of nanoemulsion components was 1% pine oil, 9% Smix, and 90% water.

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INTRODUCTION

Kelakai (*Stenochlaena palustris*) herb is a typical Kalimantan plant that grows in peat or swamp soil and is used in traditional medicine[1]—secondary metabolites of *S. palustris* herb: alkaloids, steroids and flavonoids[2]. *Stenochlaena palustris* also contains vitamin C, β-carotene, protein, folic acid, potassium, phosphorus, calcium, manganese, and zinc[3]. Antioxidants contained in *S. palustris* can be used to repair damaged skin cells and ward off free radicals. The IC_{50} value of *S. palustris* root ethanol extract are between 19.06 to 24.4 ppm and belongs to the category of strong antioxidants[4]. The high phenolic content indicated by the IC_{50} value in the ethanol extract of *S. palustris* herb, leaves, stem, and root are 126.0715, 24.24, 89.96, and 19.06 µg/mL respectively[4-6].

Antioxidants are compounds that can neutralize and prevent cell damage caused by free radicals[7,8]. Antioxidants from *S. palustris* herbs are hydrophilic and lipophilic. However, lipophilic antioxidants have a problem of large molecular size and low solubility in water[9-11], and hydrophilic antioxidants have difficulty penetrating the lipophilic stratum corneum[12]. In addition, the extract has a large molecular weight, causing its bioavailability to be low[13]. This affects the route of administration and stability of the preparation. Based on these problems, preparations are made with a better delivery system: nanoemulsion.

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Nanoemulsion is a colloidal dispersion with a diameter of less than 500 nm that can increase the solubility and bioavailability of active substances. Nanoemulsion also does not irritate the skin, so it is suitable for topical preparation[14]. The component in nanoemulsion is pine oil as an oil phase—tween 80 as a surfactant with a safe and stable HLB 15 value for O/W nanoemulsion. Glycerin is a cosurfactant that helps surfactants work and is not susceptible to oxidation in storage[15]. Therefore, this study aims to establish the ratio of pine oil and smix (tween 80 and glycerin) as the optimal nanoemulsion base with the highest % transmittance and determine physical characteristics by the presence of variations in the number of extracts so that the best formula can be determined as a natural antioxidant nanoemulsion made from *S. palustris* herbs as a development of natural ingredients of Kalimantan.

MATERIALS AND METHODS

Materials

The tools used in this research were glassware (Iwaki Pyrex), oven (Memmert), hot plate stirrer (VELP AM4), analytical scale (Ohauss Pioneer PA123), spectrophotometer UV-Vis (APEL PD-303S), LV-type Brookfield viscometer, mesh sieve 20 (Retsch AS 200), pH meter (Hanna Instruments), magnetic bar, Buchner funnel, macerator, rotary evaporator (Heidolph Labourite 4000 Efficient), blender (Miyako), water bath (SMIC), software Design Expert 13.0 (State-Ease), and SPSS 21 (IBM Statistic). The materials used in this study were *S. palustris* herb, pine oil (Pine Oil®, pharmaceutical grade, Fern & Petal), tween 80 (Sorbilene®, pharmaceutical grade, Lamberti S.P.A), glycerin (Moon K Glycerin®, pharmaceutical grade, P&G Chemical), distilled water, 70% ethanol (cosmetic grade), Whatman filter paper number 41, methylene blue, as well as phosphate buffer pH 4.0 and 6.8.

Methods

Plant collection and determination

Stenochlaena palustris herb (**Figure 1**) was taken from Manarap Baru, South Kalimantan, in December 2021. The selected herbs were green mature leaves in good condition, without holes and traces of insect bites. Determination was carried out to identify and ascertain the plant species' identity. Determination was accomplished using all plant parts at the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Lambung Mangkurat, Banjarbaru, with certificate number 108/LB.LABDASAR/IV/2021.


a **b**

Figure 1. *Stenochlaena palustris* herb (**a**) and simplicia (**b**).

Preparation of simplicia

Stenochlaena palustris herb was sorted in wet sorting and washed thoroughly with running water so that dirt does not stick. It was stretched and dried using an oven with a 55 ± 2°C temperature to dry[16]. Simplisia was then preoccupied and sifted with a mesh sieve 20. The herbaceous powder is stored at room temperature and in a tightly sealed container. *Stenochlaena palustris* herb powder of 150 g was macerated using 70% ethanol as much as 1 : 10 for 3 x 24 hours. Every 24 hours, the solvent was replaced with a new one as much as half of the first solvent. The liquid extract was filtered using a Buchner funnel with Whatman filter paper number 41, then concentrated with a rotary evaporator at a temperature of 50 ± 2°C, then evaporated in the water bath at a temperature of 50 ± 2°C until a thick extract with a constant weight was formed, then stored in a tightly sealed container[17].

Formula optimization

Optimization was done by creating a ternary diagram to map the area of the optimal composition range for excipients to form a nanoemulsion and show the effect of phase changes. Ternary diagrams were created by mixing nanoemulsion bases: oil : smix (surfactant and cosurfactant) as much as 4 : 1 and water[18]. Smix was prepared with the 4 : 1 weight ratio of tween 80 and glycerin. The oil phase and smix were mixed with the ratio can be seen in **Table I**.

Table I. Comparison of pine oil and smix.

Component	Concentration ratio								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Pine oil	1	2	3	4	5	6	7	8	9
Smix	9	8	7	6	5	4	3	2	1

The oil and smix phases were weighed and then stirred using a magnetic bar for 5 minutes at a speed of 250 rpm, then titrated with drip after a drop of water while stirring. The amount of water phase titration varies to 90% of the total volume, and its physical appearance was visually observed with each addition of the water phase. Based on visual observations, the results were grouped into four categories:

1. O/W nanoemulsion with transparent and easy-flowing characteristics
2. Nanoemulsion gel with viscous and transparent physical features
3. Emulsions that were milky or cloudy
4. Emulgels with characteristics such as milk but thick texture

The percentage of each material from visual observation results was subsequently plotted on a ternary phase diagram graph created with the help of Prosim Ternary software to visualize the area of formation of nanoemulsion[19].

Optimization was continued with the Design-Expert 13 D-optimal mixture design menu. Independent variables were filled with oil, smix, and water; dependent variables were the desired response filled with % transmittance. Each variable's upper and lower limits were obtained from the ternary diagram. It was also incorporated into the formula design. Furthermore, the desired optimization criteria were included to obtain an optimal nanoemulsion formula. Then, the formula was tested in the laboratory to get the actual % transmittance[20]. The formula of the nanoemulsion of *S. palustris* herbs extract can be seen in **Table II**.

Table II. *Stenochlaena palustris* extract nanoemulsion formula.

Material	Function	Concentration (%w/w)
Stenochlaena palustris herb extract	Active substance	0.1; 0.25; 0.5
Pine oil	Oil phase	1
Smix	Surfactant : cosurfactant	9
Distilled water	Water phase	Ad 100 mL

Nanoemulsion was made with an optimal formula and three variations of the concentration of extracts. For nanoemulsion, all materials were weighed according to their concentration. The extract and tween 80 were mixed into the glass and stirred with a magnetic bar for 10 minutes at 250 rpm; then pine oil was added and stirred back for 10 minutes; then glycerin was added and stirred again for 10 minutes. Next, the distilled water was titrated until the final volume of 100 mL was obtained and stirred with a magnetic bar at 500 rpm for 10 minutes[21].

Physical evaluation of preparations

Organoleptic test: Organoleptic tests were performed by observing the preparation's color, smell, and clarity using the five senses[22]. Clear preparations were an indicator that the preparation had a small droplet size[23].

Nanoemulsion type test: This test was carried out by dripping a methylene blue on the surface of the nanoemulsion placed on the glass of the object. Nanoemulsion type O/W will make methylene blue dissolve and spread evenly throughout the preparation, and type W/O will make methylene blue clump on the surface of the nanoemulsion[24].

Percent transmittance test: The value of the % transmittance determined the clarity of the nanoemulsion. A sample of 0.1 mL was dissolved in a 10 mL measuring flask using distilled water. Percent transmittance was measured at wavelengths of 650 nm by UV-Vis spectrophotometry, with distilled water as blank. An absorbance close to 100% indicates that the droplet size has reached nanometers, and the visual appearance was transparent[25].

pH test: pH meters were calibrated with a solution of pH 4.0 and 6.8. Following, the electrodes were rinsed using aquadest, and then the pH was dipped in the preparation and waited until the pH value on the pH meter screen was constant[26]. The pH limit of the preparation that the skin can tolerate was 4.2-7.0[27].

Viscosity test: Viscosity was measured by an LV-type Brookfield viscometer at room temperature. Nanoemulsion was included in a 100 ml glass. The spindle number was set at 2, and the speed was set at 3 rpm. Viscosity values in centipoise (cps) were obtained from the result of the multiplication of dial readings with correction factors[22].

Data analysis

The data were analyzed using normality and variance tests (Kolmogorov-Smirnov test and homogeneity of variances). For normally distributed data, it was continued to the one-way ANOVA test with a 95% confidence level to determine the significant effect of *S. palustris* herb in various concentrations. If the results of the ANOVA test were $p < 0.05$, then analysis of post hoc Least Significance Difference (LSD) to determine the significant differences in each treatment group was performed.

RESULTS AND DISCUSSION

The extract yield obtained was 25.38 g, with a yield of 16.92%. This result differs from study of Syamsul *et al.*[28], which reports that the yield of *S. palustris* leaves extract using maceration with 70% ethanol was 22.92%. This difference is due to the difference in the number of samples with the solvent used; the more solvents used, the more extracts obtained[29]. The characteristics of the resulting extract are thick, brownish-green, and strongly scented extracts. Powders and thick extracts of *S. palustris* can be seen in **Figure 2**.

a **b**

Figure 2. *Stenochlaena palustris* powder (**a**) and thick extract (**b**).

Optimization is carried out in two stages: creating ternary diagrams and D-optimal Mixture Design. Ternary diagrams map the range of optimal composition of excipients so that they can form a nanoemulsion and show the effect of phase changes. The base is made by mixing the oil phase and water[18]. The optimization results can be seen in **Figure 3**. Based on the results, only F1 produces clear visuals with no phase separation from the beginning to the end of adding water. Meanwhile, F2 to F9 produces cloudy visuals with a milky white color. Based on the results of visual observations for each addition of the water phase, four phases were formed: the nanoemulsion phase, gel phase, emulgel phase, and emulsion phase. The nanoemulsion phase was formed in 1-4% pine oil, 9-36% smix, and 60-90% water. The resulting nanoemulsion phase was visually clear, transparent, and easy to flow. Cho *et al.*[30] showed that nanoemulsions were formed when the oil phase had a concentration of less than 6%, more than 8% surfactants, and more than 60% water.

Figure 3. Nanoemulsion formula optimization results.

The percentage of each material from the observations was plotted on a ternary phase diagram graph with Prosim Ternary software to visualize the nanoemulsion formation area. The nanoemulsion formed is O/W type because it has a large concentration of water and little oil, is visually clear and transparent, has a low viscosity or is easy to flow, and the concentration of smix used can reduce the surface tension between the oil phase and the water phase[30]. The graph of the ternary phase diagram obtained can be seen in **Figure 4**. The results of the ternary phase diagram are obtained from data on the upper and lower limits of the concentration of oil, smix, and water in the nanoemulsion area, shown in red. The upper and lower limit values for nanoemulsions can be seen in **Table III**.

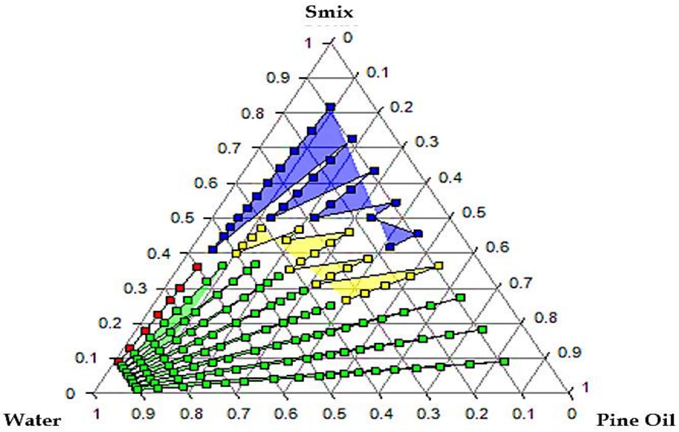


Figure 4. Ternary phase diagram.

Table III. The lower and upper limits of nanoemulsion.

Parameter	Lower limit (%)	Upper limit (%)
Pine oil	1	4
Smix	9	36
Water	60	90
% transmittance	90	100

Optimization continued with the software Design-Expert 13 menu D-optimal mixture design. The material component is entered into the software as an independent variable with a total concentration of 100%, and then the dependent variable, the % transmittance, is entered. The lower and upper limit values obtained are entered into the design as a reference in the formulation. The D-optimal mixture design will provide several formula designs, which are then tested for the % transmittance value in the laboratory[31]. The optimal nanoemulsion formula based on research by Handayani *et al.*[32] has a composition of oil between 0.95 and 18.18%, Smix between 3.81 and 72.73%, and water between 15.7 and 95.24%. The results of the formula design and % transmittance can be seen in **Table IV**.

Table IV. Nanoemulsion base formula design and response.

Run	Pine oil (%)	Smix (%)	Water (%)	% transmittance
1	1.6	9	89.39	98.4
2	1	25.27	73.72	99.4
3	4	9.6	86.4	95.2
4	4	25.73	70.26	95.9
5	4	9.6	86.4	95.5
6	1	32.36	66.63	99.9
7	1	13.82	85.17	99.1
8	2.72	21.04	76.22	97.3
9	2.72	21.04	76.22	97.6
10	1	29.24	69.76	99.6
11	1.6	9	89.39	98.6
12	2.72	21.04	76.22	97.5
13	3.09	16.57	80.32	96.8
14	4	32.85	63.15	96.3
15	1.99	36	62	98.1
16	1.99	36	62	98.3

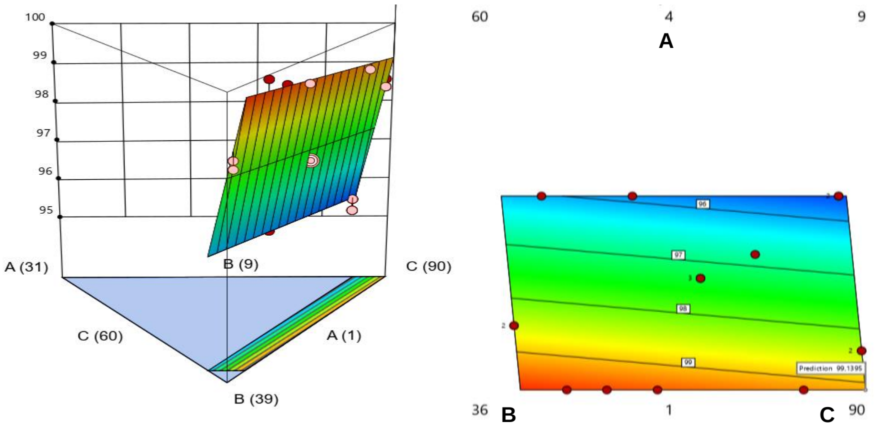
After all the data is entered, it is processed and statistically analyzed with a D-optimal Mixture Design. The results are that the % transmittance response has a significant value with a p-value <0.05, which follows a linear model. The lack of fit value shows insignificant results, and the f value >0.05 means that the model is following the response data. The results of the ANOVA test indicate that the % transmittance obtained is influenced by the components of the material included. Statistical results can be seen in **Table V**. The final equation of the model showing the similarities between the components and the response data can be written as shown in **Equation 1**, with A is pine oil, B is smix, and C is distilled water. This equation explains the significant relationship between the % transmittance response and the percentage of material components.

Table V. ANOVA statistical test.

ANOVA parameter	Response transmittance	Parameter
Linear model	Significant	Significant
R^2	0.9797	≤1
p-value	<0.0001	<0.05
f-value	313.78	>0.05
Lack-of-fit	Not significant	Not significant

$$Y_1 = -0.201276\ (A) + 1.02264\ (B) + 1.00152\ (C) \qquad \textbf{[1]}$$

The relationship between the components and the % transmittance response that has been plotted in the 3D image can be seen in **Figure 5**. Pine oil is antagonistic, which means that the higher the concentration of pine oil (A), the cloudier the nanoemulsion will form; this is because the size of the particles formed will increase, resulting in the formation of unstable nanoemulsions. After all, the smix is not strong enough to lower the interfacial tension. Meanwhile, the addition of smix (B) and water (C) has a synergistic effect, which means that increasing the concentration will form a better nanoemulsion[30].



a **b**

Figure 5. 3D surface (**a**) and contour (**b**) of the relationship of components to the % transmittance response.

Based on the statistical results, criteria were made for the optimal formula. The oil criterion was chosen to "minimize" because the lower the oil concentration, the clearer the visual appearance, and the smaller the particle size. Smix was chosen to "minimize" to reduce its toxicity. Water was chosen with the criteria of "is in range" 60-90% because the nanoemulsion made was of the O/W type. The % transmittance was chosen to "maximize" 90-100% because, in this range, it is estimated that the particle size reaches nanometers, so it is expected to produce nanoemulsions with clear, transparent visuals and high % transmittance. The optimal formula design criteria can be seen in **Table VI**.

Table VI. Optimal criteria design formula D-optimal Mixture Design.

Parameter	Criteria	Lower limit (%)	Upper limit (%)
Pine oil	Minimize	1	4
Smix	Minimize	9	36
Water	Is in range	60	90
% transmittance	Maximize	90	100

D-Optimal will provide the optimal nanoemulsion formula that has been adjusted to the criteria. Furthermore, laboratory manufacturing with the optimal formula was done to see the actual % transmittance value. The results met the requirements for the % transmittance of 90-100% nanoemulsions with clear and transparent visualization[33]. The desirability value obtained is close to 1, which is 0.970, indicating that the confidence level in the formula made by the D-optimal is good. The results of the optimal formula and % transmittance can be seen in **Table VII**.

Table VII. Optimal formula for nanoemulsion base and % transmittance value.

Pine oil (%)	Smix (%)	Water (%)	Predicted % transmittance	Actual % transmittance (from three replications)	% bias
1	9	90	99.139	99.633 ± 0.152	0.498

The nanoemulsion (**Figure 6**) was made with an optimal formula with three extract concentrations: 0.1, 0.25, and 0.5%. The extract was weighed according to the concentration, then added tween 80, pine oil, and glycerin each time the addition of the material was stirred with a magnetic bar for 10 minutes at a speed of 250 rpm, and distilled water was added little by a little while stirring at a speed of 500 rpm for 10 minutes until the volume of the preparation was 100 mL. Stirring reduces the interfacial tension to reduce the particle size, but if the stirring is too fast, the globules in the nanoemulsion will collide more easily and can make the nanoemulsion cloudy[34].

Figure 6. Nanoemulsion of *S. palustris* herb.

Table VIII. Physical evaluation of nanoemulsion.

Physical evaluation	Formula (Concentration of the extract)		
	F1 (0.1%)	F2 (0.25%)	F3 (0.5%)
Organoleptic			
- Color	Light yellow	Yellow	Brownish yellow
- Odor	Weak	Medium	Strong
- Clarity	+++	++	+
Nanoemulsion type	O/W	O/W	O/W
% transmittance	99.333 ± 0.152	98.833 ± 0.115	97.466 ± 0.115
pH	5.923 ± 0.005	5.806 ± 0.005	5.753 ± 0.011
Viscosity (cps)	10.666 ± 0.176	11.8 ± 0.176	12.844 ± 0.203

The result of the physical evaluation can be seen in **Table VIII**. The results of the organoleptic test show that the variation in the concentration of the extract affects the color, odor, and clarity; the higher the concentration of the extract, the more the intensity of the color and odor produced increases[35]. The results of the nanoemulsion type test were the three nanoemulsion formulas of oil in water (O/W) type. These results are based on research by Maha *et al.*[22], in which O/W type nanoemulsions are formed due to methylene blue being soluble and spread evenly throughout the preparation. This type was chosen because the skin more easily absorbs it and is easily washed off with water.

The results of the % transmittance show that the three formulas have a % transmittance value close to 100%. Meanwhile, from the research of Syukri *et al.*[36], the transmittance value obtained is 96-100%. This value has met the requirements for nanoemulsions, around 90-100%, with clear and transparent visuals[33]. Adding the extract causes the percent transmittance to decrease due to an increase in the droplet molecular size[31]. The results of statistical tests showed a p-value of 0.000 (<0.05), which means that the variation in the concentration of the extract significantly affects the % transmittance value of the nanoemulsion.

The pH test results show a pH value of around 5.7-5.9. The pH results meet the pH that can be tolerated by the skin, about 4.2-7.0[27]. A pH value that is too acidic can irritate the skin, while a pH value that is too alkaline can make the skin dry and scaly[37]. The increase in the concentration of the extract resulted in a decrease in the pH value; this was due to the release of the H^+ group, so the pH became more acidic[38]. The statistical test results showed a p-value of 0.000 (<0.05), which means that the variation in the extract's concentration significantly affected the nanoemulsion's pH value.

The viscosity results showed that the greater the extract concentration, the more viscosity increased. This is because of the increase in particle size, which causes the friction between particles to get higher[39]. The viscosity results have met the nanoemulsion viscosity range between 1-100 cP[40]. The results of statistical tests showed a p-value of 0.000 (<0.05), which means that the variation in the extract's concentration significantly affected the nanoemulsion's viscosity.

CONCLUSION

The optimal comparison of the nanoemulsion base of the extract of *S. palustris* herb with the highest percentage transmittance value is 1% pine oil, 9% smix, and 90% water. Variations in extract concentration affect the physical characteristics of nanoemulsion preparations in the test of % transmittance, organoleptic, pH, and viscosity.

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AUTHORS’ CONTRIBUTION

All authors have an equal contribution in carrying out this study.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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