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Sambiloto leaves (*Andrographis paniculata* (Burm.f.) Wall. Ex. Nees) nanoemulsion preparations: Optimization of Tween 80 and PEG-400 concentrations as photoprotective agents

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ABSTRACT: Sambiloto leaf (*Andrographis paniculata* (Burm.f.) Wall. Ex Ness) has the potential to be employed as a photoprotective agent due to its high antioxidant content in the form of quercetin and andrographolide. The nanoemulsion preparations can boost the photoprotective activity of sambiloto leaf extract due to its deepest penetration. This study aims to determine the optimum Tween 80 and PEG-400 concentration in the Sambiloto leaf extract nanoemulsion preparation using the factorial 2^2 design method through Design Expert 12® on pH response, specific gravity, viscosity, and percent transmittance. The optimum formula was tested for sun protection factor (SPF) and DPPH Radical Scavenging activity (IC_{50}). The amounts of andrographolide and quercetin detected in the sambiloto leaf extract were 3.411% and 3.272%, respectively. The optimum formula for nanoemulsion preparations is at Tween 80 and PEG-400 concentrations of 15% and 25%. The optimum Sambiloto leaf extract nanoemulsion formula had a globule size of 172.433±24.312 nm, a PDI of 0.245±0.060, and a zeta potential of -18.100±0.755 mV. The optimum formula for sambiloto leaf nanoemulsion has an SPF value of 44.427 ± 0.081 and an DPPH Radical Scavenging activity (IC_{50}) of 124.863 ± 4.045 ppm. The cycling test showed no significant difference ($p>0.05$) on organoleptic parameters, pH, specific gravity, and percent transmittance. Based on the results obtained, the optimum formula of Sambiloto leaf extract nanoemulsion has high potential as a photoprotective agent with good globule characteristics and stability.

KEYWORDS: *Andrographis paniculata*; Nanoemulsion; Photoprotective; Tweens 80; PEG-400.

1. INTRODUCTION

Ultraviolet A (UVA) and ultraviolet B (UVB) are the two categories of ultraviolet (UV) radiation emitted by the sun and subsequently reaching the Earth's surface. UVA radiation spans the wavelength range of 320 to 400 nanometers, while UVB radiation spans wavelengths ranging from 280 to 320 nanometers [1]. The health of human skin might suffer from excessive sun exposure. While excessive UVB radiation can result in sunburn and an inflammatory response in the skin's outer layer, excessive UVA exposure can damage skin DNA and other macromolecules, resulting in accelerated aging [1,2]. Using photoprotective chemicals can prevent excessive UV exposure to the skin.

The sambiloto plant with the scientific name Andrographis paniculata (Burm.f.) Wall. Ex Ness contains flavonoids, diterpenoids, and phenolic compounds [3-5]. Andrographolide, a member of the diterpenoid group, is the major active ingredients of the sambiloto plant [6]. According to research by Villedieu-Percheron et al. [7], sambiloto extract includes andrographolide components in concentrations between 82 and 176 µg/mL. Sambiloto plants also have flavonoid chemicals like quercetin in their leaves [6,8]. According to research by Fardiyah et al. [9], 10 mg of the ethanol extract of sambiloto leaves has total flavonoid content of 0.002 µg/µL quercetin equivalent.

Due to its antioxidant properties, andrographolide and quercetin may have photoprotective effects [10-13]. According to Deore et al. [14] and Wadhwa et al. [15], andrographolide and quercetin exhibit photoprotective effect by neutralizing free radicals, blocking lipid peroxidase, and anti-inflammatory

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properties. Andrographolide and quercetin have anti-inflammatory effect in Human Epidermal Keratinocytes (HaCaT) [16]. UV light will increase the formation of reactive oxygen species (ROS) impacts the activation of IkB kinase, which causes NF-κB activation. The activation of this process will stimulate the release of proinflammatory cytokines, including tumour necrosis factor-α (TNF-α), interleukin-8 (IL-8), and interleukin-6 (IL-6) which exert an inhibitory influence on collagen synthesis within cells. Research conducted by Nisar et al. [17] and Wang et al. [18] proved that the administration of andrographolide and quercetin effectively reduced the expression of IL-1β, IL-6, and TNF-α due to UV induction.

Photoprotective agents can be administered through the skin in topical dosage forms such as lotions, creams, or gels. However, these conventional preparations have limitations in terms of penetration into the skin. The photoprotective mechanism is in the epidermis to the dermis. In this study, an innovation was made to make preparations capable of increasing photoprotective effectiveness in nanoemulsion preparations. Nanoemulsions are emulsions with nanoscale droplets shown in average droplet diameters ranging from a few nm to 200 nm [19]. The main components of the nanoemulsion consist of oil, surfactant, and co-surfactant [20]. Basil oil was employed as the oil phase in this study due to its synergistic photoprotective effects [21]. Natural antioxidants found in basil oil include thymol, geraniol, geranial, and eugenol [22]. Basil oil possesses antioxidant activity, according to research by Li et al. [23], with an IC50 value of 1.092 0.066 mg/mL.

In this study, Tween 80 and PEG-400 were utilized as surfactants and co-surfactants. Surfactants work to lower interfacial tension, which promotes the dispersion of all components. To create a nanoemulsion that is more stable, co-surfactant is added [20]. When the molecules reach the critical micelle concentration (CMC), which is caused by the addition of surfactant and co-surfactant concentrations to the preparation, they will assemble into micelles. A greater globule size results from the molecules producing more micelles and aggregating more readily when the concentration of surfactants and co-surfactants is higher than the CMC. Furthermore, hazardous effects, particularly for uncharged surfactants, start to show up at greater CMC concentrations [24]. Therefore, it is important to establish the ideal surfactant and co-surfactant concentration. Tween 80 is a surfactant that ranges in concentration from 15 to 25 percent. With an HLB value of 15, Tween 80 is acceptable for O/W nanoemulsions. PEG 400, with a concentration range of 15 to 25 percent, is the co-surfactant employed. According to Ahmed et al. [25], PEG 400 has the ability to create hydrogen chains, which will speed up the emulsification process and enable the development of nanoemulsion preparations.

Nanoemulsion formula was optimized by Design-Expert series 12® software using the 2^2-factorial. The resulting optimum formula will be measured against the SPF value and DPPH Radical Scavenging Assay. In addition, a stability test is also needed to determine the stability level of the optimum formula that has been produced.

2. RESULTS

2.1. Sambiloto Leaf Extract

The resulting sambiloto leaf extract is thick, dark green, and has a distinct aroma. The yield percentage for sambiloto leaf extract is 21.412%. The kind of solvent, extraction technique, temperature, and extraction duration can all have an impact on the yield outcomes [26,27]. The research from Adam et al. [28] used methanol as the solvent for extraction by maceration of sambiloto leaf, producing a yield of 5.097%. Since 96% ethanol has stronger polar qualities than methanol, it can bind more flavonoid chemicals from Sambiloto leaves. These compounds also have polar properties, which is the reason why 96% ethanol is used as a solvent. The amounts of andrographolide and quercetin detected in the sambiloto leaf extract were 3.411% and 3.272%, respectively.

2.2. Sambiloto Leaf Nanoemulsion

Organoleptic observations, pH, specific gravity, viscosity, and percent transmittance can all be used to determine the Sambiloto extract nanoemulsion's quality. The difference characteristics of each formula arise from the varying quantities of tween 80 (surfactant) and PEG-400 (cosurfactants). Table 1 shows the properties of the four tested formula nanoemulsion preparations and the organoleptic can be seen in Figure 1.

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Table 1. The Properties of Four Tested Formula Sambiloto Nanoemulsion

Evaluation	Formula			
	1	2	3	4
Organoleptic	Dark green, has a distinctive smell, transparrent	Dark green, has a distinctive smell, transparrent	Dark green, has a distinctive smell, transparrent	Dark green, has a distinctive smell, transparrent
pH	5.8 ± 0.1	5.7 ± 0.0	5.6 ± 0.0	5.8 ± 0.0
Specific Gravity (g/cm^3)	1.063 ± 0.002	1.059 ± 0.001	1.086 ± 0.004	1.078 ± 0.002
Viscosity(cP)	4.93 ± 2.21	27.57 ± 4.63	13.20 ± 0.42	18.88 ± 0.53
Transmittance (%)	85.9 ± 0.1	86.5 ± 0.1	86.4 ± 0.1	85.0 ± 0.2

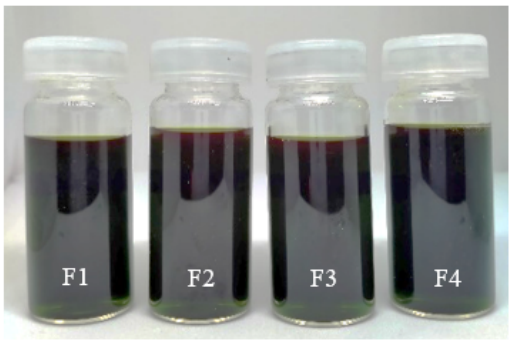

Figure 1. Four formulas of Sambiloto nanoemulsion

2.3. Model Analysis for Optimization Process

A model analysis is required to beginning the optimization process. The optimal formula was found using the observed of pH, specific gravity, viscosity, and percent transmittance. Table 2 shows the outcomes of the four responses model analyses. The model will produce good results if it satisfies the following criteria: R^2 value ≥ 0.7, p-value ≤ 0.05, difference between adjusted R2 and predicted R2 values ≤ 0.2, and adequate precision ≥ 4 [29].

Table 2. Model Analysis Result

	Response	Parameter			
		p-value	R^2	Difference Between Adjusted R^2 and Predicted R^2	Adequate precision
R1	pH	< 0.0001*	0.9375	0.0547	14.000
R2	Specific Gravity	< 0.0001*	0.9622	0.0331	17.2392
R3	Viscosity	< 0.0001*	0.9399	0.0525	15.3467
R4	Percent Transmittance	< 0.0001*	0.9618	0.0334	17.5547

Description: * p <0.05 shows a significant effect

The model analysis results are supported by half-normal plot graphs, normal plots of residuals, and predicted vs actual, as in Figure 2.

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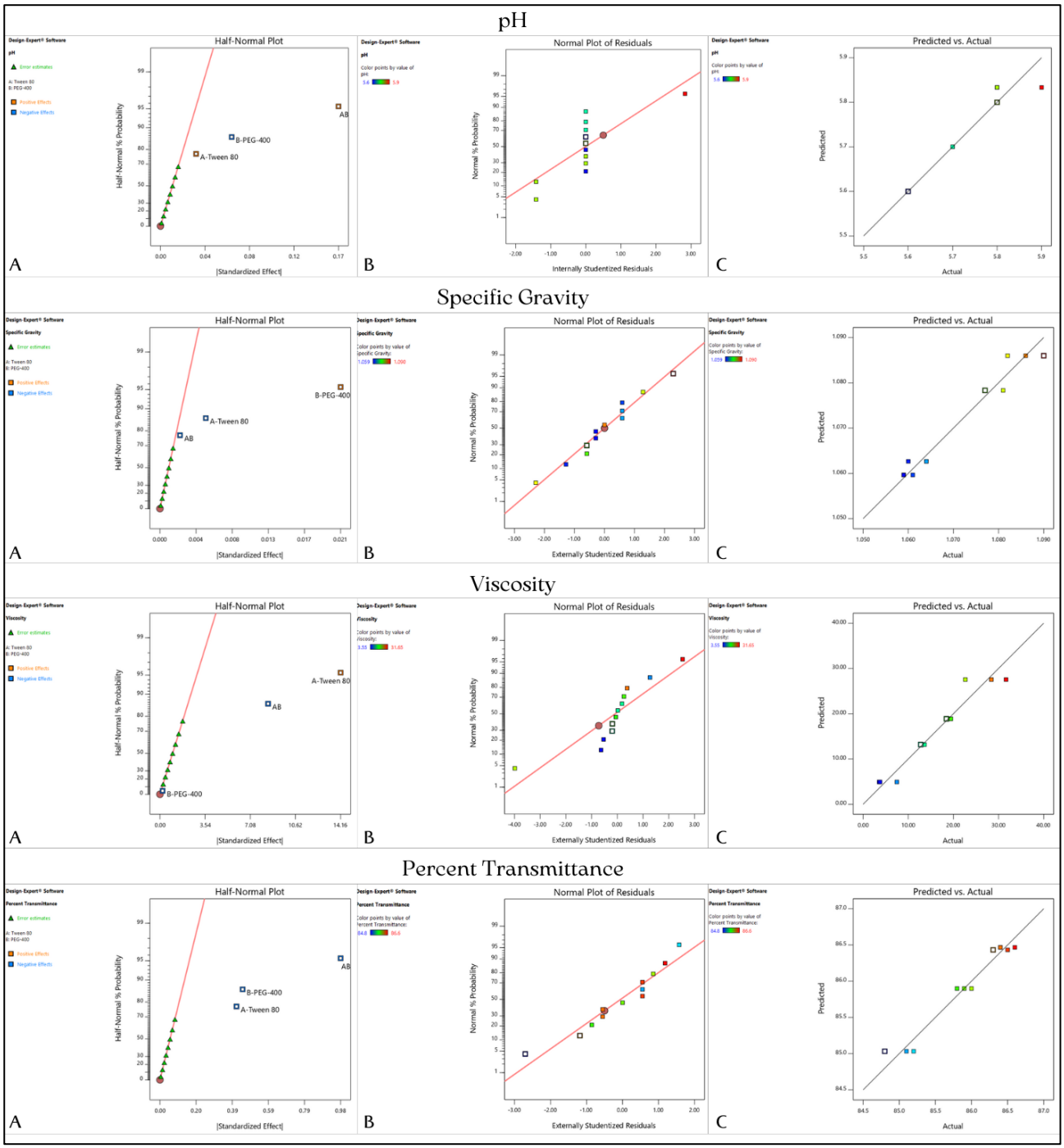

Figure 2. Graph of half normal plot (A), normal plot of residuals (B) and predicted vs actual (C) of the four responses

2.4. Response Analysis for Optimization Process

Response analysis is carried out on parameters that have a good model. By examining the coefficient value, p-value, and percent contribution from the Anova analysis. Response analysis was done to determine the influence of the Tween 80 (A), PEG-400 (B), and interaction between Tween 80 and PEG-400 (AB). Coefficients value of the factors that significantly influence will be used to create a response equation. The results of the response analysis are presented in Figure 3 and Table 3.

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Table 3. Response Analysis Result

Response	Parameter	Intercept	Tween 80 (A)	PEG-400 (B)	Tween 80 & PEG-400 (AB)
pH	Coefficient	5.733	0.017	-0.033	0.083
	% Contributions		3.125%	12.500%	78.125%
	p-value		0.0805	0.0039*	< 0.0001*
	Equation		y = 5.733 - 0.033B + 0.083AB		
Specific Gravity	Coefficient	1.072	-0.003	0.011	-0.001
	% Contributions		5.763%	89.352%	1.103%
	p-value		0.0082*	< 0.0001*	0.1651
	Equation		y = 1.072 – 0.003A + 0.011B		
Viscosity	Coefficient	16.143	7.082	-0.103	-4.238
	% Contributions		5.763%	89.352%	1.103%
	p-value		< 0.0001*	0.8921	0.0004*
	Equation		y = 16.143 + 7.082A – 4.238AB		
Percent Transmittance	Coefficient	85.958	-0.208	-0.225	-0.492
	% Contributions		12.433%	14.502%	69.246%
	p-value		0.0009*	0.0006*	< 0.0001*
	Equation		y = 85.958 – 0.208A – 0.225B – 0.492AB		

Description: * p <0.05 shows a significant effect

2.5. Optimum Sambiloto Leaf Nanoemulsion, Globul Characteristics and Stability

Low viscosity, high percent transmittance, and pH values between 4.5 and 6.5 are used to determine the optimum formula. Based on Design-Expert series 12® optimization, tween 80 at 15% and PEG-400 at 25% was selected as optimum formula with desirability value of 0.978. The optimum formula for nanoemulsion preparations has a globule size of 172.433±24.312 nm, a PDI value of 0.245±0.060, and a zeta potential of -18.100±0.755 mV.

The stability of the optimum formula can be seen in Table 4. The preparation before and after testing showed no phase separation or organoleptic alterations. A paired sample t-test was used to examine the quantitative stability test data. The pH, specific gravity, and percent transmittance values of the preparations did not change, according to the analytical results of pH, specific gravity, and percent transmittance values, which received a significance of $p > 0.05$.

Table 4. The Stability Result of Optimum Sambiloto Leaf Nanoemulsion

Parameter	Result	
	Before	After
Organoleptic	Dark green, transparent, and has a distinctive aroma of basil oil	Dark green, transparent, and has a distinctive aroma of basil oil
Phase Separation	No	No
pH	5.62 ± 0.01	5.61 ± 0.10
Specific Gravity (g/cm³)	1.086 ± 0.004	1.070 ± 0.001
Percent Transmittance (%)	86.6 ± 0.2	86.4 ± 0.1

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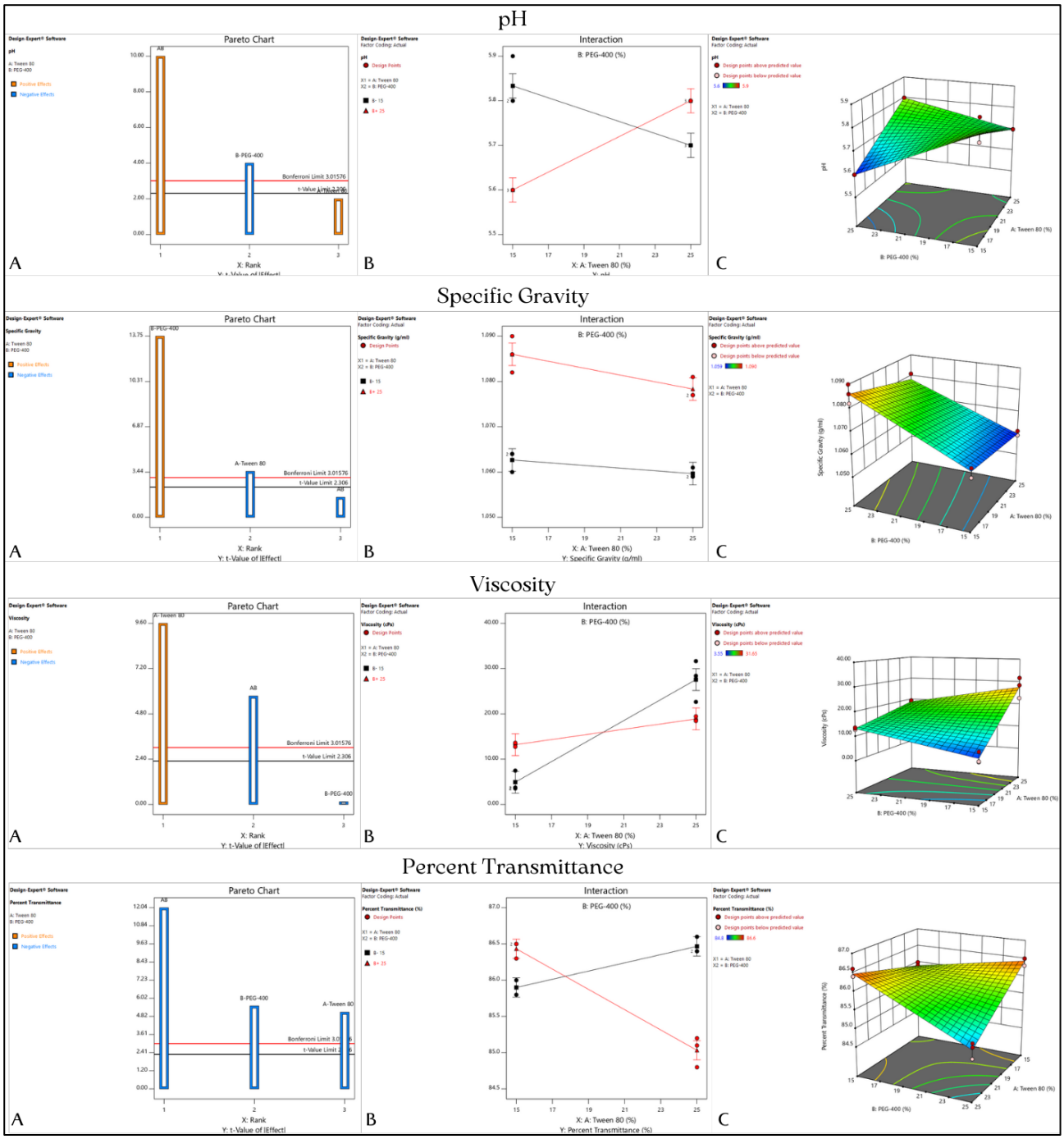

Figure 3. Graph of pareto chart (A), interaction (B) and 3D surface (C) of the four responses

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2.6. The Photoprotective Activity of Optimum Sambiloto Leaf Nanoemulsion

The photoprotective activity of the optimum Sambiloto nanoemulsion formula was observed based on the results of the Sun Protection Factor (SPF) and IC_{50}- of DPPH Radical Scavenging Assay values contained in Tables 5 and 6.

Table 5. The result of SPF Value

Sample	SPF Value	Category
Sambiloto Leaf Nanoemulsion	44.427 ± 0.081[a]	High
1% Sambiloto Extract	25.403 ± 0.081[b]	Moderate
Marketed Product	24.398 ± 0.083[c]	Moderate
Demineralized Water	0.047 ± 0.004 [d]	Very Low

Description: Different letters in a column indicate that there is a significant difference between groups ($p<0.05$)

Table 6. The result of IC_{50} of DPPH Radical Scavenging Assay

Sample	IC50 (ppm)	Category
Sambiloto Leaf Nanoemulsion	124.863 ± 4.045[b]	Moderate
1% Sambiloto Extract	419.083 ± 10.217[c]	Very Low
Ascorbic Acid	4.617 ± 0.036[a]	Very Strong

Description: Different letters in a column indicate that there is a significant difference between groups ($p<0.05$)

3. DISCUSSION

The sambiloto leaf extract used in this study was produced by the maceration method and has the characteristics of being green in color, thick in texture, and has a distinctive bitter smell. Due to the extract's high chlorophyll content, the color is acquired in a green dark. Andrographolide levels in sambiloto leaf extract are 3,411%, and flavonoids are 3,272%. It is believed that these two components have a photoprotective effect.

Sambiloto leaf nanoemulsion was made into four formulas based on variations in the concentration of Tween 80 (A) and PEG-400 (B). The characteristics of nanoemulsions, that presented in Table 1, show differences due to differences in these concentrations. The four formulas meet the requirements for a good O/W nanoemulsion, namely pH in the range of 4.5-6.5, a specific gravity of more than 1, low viscosity, and percent transmittance of more than 80% [30,31]. The optimization procedure can be utilised to characterise the impact of Tween 80 (A) and PEG-400 (B) on the nanoemulsion characteristics.

The optimization process is broadly divided into two stages: model analysis and response analysis. The model analysis is carried out to determine what responses or parameters can be used for the response analysis stage and determine the optimum formula. Based on Table 2, all parameters, namely pH, specific gravity, viscosity, and percent transmittance meet the requirements of a good model. The p-value is used to describe significance. If the p-value is <0.05, it means that the factors used, namely Tween-80 (A), PEG-400 (B) and the interaction between Tween 80 and PEG-400 (AB), have a significant influence on the observed response. The four responses have a p-value < 0.0001, meaning the factor significantly influences the response to pH, specific gravity, viscosity, and percent transmittance. The R2 value describes the size of the data population, which is affected by the Tween-80 (A), PEG-400 (B) and the interaction between Tween 80 and PEG-400 (AB). The requirement for a good R^2 is more than 0.7, meaning that 70% of the data population is influenced by the factors used and the data is normally distributed. The R^2 results from the four responses ranged from 0.9375 to 0.9622, meaning that 93.75% to 96.22% of the population of the resulting data was influenced by the factors used, while the remaining 3.78% to 6.25% was an error. This result can also be depicted in the Half Normal Plot graph in Figure 2A, where the triangle mark on the line indicates the estimation error. The depiction of data homogeneity or distribution can also be observed using the Normal Plot of the Residual graph presented in Figure 2B. When the point closer to the line, the data is normally distributed. The pH response has a normal plot of the residual graph farthest from the line, as evidenced by the R^2 value being the smallest compared to the other responses.Z

Furthermore, the difference between predicted and adjusted R^2 can illustrate the results that obtained in lab and the system predictions were similar. The difference between the four responses is no more than 0.2, meaning that the data produced based on research is similar to the system predictions. This result is also supported by the Predicted vs Actual graph in Figure 2C, where the point between predicted and actual is close to the line. The adequate precision value describes the system's resistance to disturbances. The fourth

result is a response of more than 4, which means the model resists existing disturbances, as evidenced by the low estimation error.

The four responses were then analysed to determine the effect of each factor (A, B and AB) on the observed responses. Based on the response analysis result in Table 3, the Tween 80 (A) factor has a significant influence on the parameters of specific gravity, viscosity, and percent transmittance; the PEG-400 factor (B) has a significant influence on the parameters pH, specific gravity, and percent transmittance; while the interaction factor (AB) has a significant influence on the parameters of pH, viscosity and percent transmittance based on a p-value <0.05. This conclusion is supported by the Pareto Chart in Figure 3A, where factors with t-values above the Bonferroni line are regarded as genuinely significant, factors with t-values between the t-limit line and the Bonferroni line are likely significant and factors with t-value below the t-limit line are statistically insignificant [32]. For example, the pH response factor A is below the t-limit line, and the p-value > 0.05 indicates that the Tween 80 factor has no significant effect on the pH response. The interaction of the two factors (AB) can also be observed from the interaction graph in Figure 3B. The intersecting lines on the interaction curve indicate an interaction between Tween 80 (A) and PEG-400 (B) seen in the parameters pH, viscosity, and percent transmittance. Whereas in the specific gravity response, there is no line crossing, which indicates there is no interruption between Tween 80 (A) and PEG-400 (B) as evidenced by a p-value > 0.05 and a low % contribution. Furthermore, the 3D surface graph in Figure 3C shows the possible area of the desired response value. For example, if we want a pH value of around 5.6 in the pH response, we need Tween 80 and PEG-400 of 15% and 25% according to the blue area.

The influence of each factor (A, B and AB) can be either positive or negative, which presented in the notation of the coefficients on the observed response. Positive notation denotes a linear relationship between the concentration used and the results obtained; the greater the concentration, the greater the response value obtained. Significant coefficient values will be used for response equations. The factor that most influences the response can be seen from the % contributions generated in Table 3. The Tween 80 factor (A) has the most significant % contribution to the viscosity response, which is equal to 69,195%, the PEG-400 factor (B) to the specific gravity response is 89,352 %, and the interaction factor (AB) on the pH response and percent transmittance is 78.125% and 69.246%.

Tween 80 has a pH of 6.0-8.0, while PEG-400 has a pH of 4.0-7.5 [33]. The interaction of the two will increase the pH of the nanoemulsion preparation. The pH obtained was in the range of 5.5 because the concentration of PEG-400 used was more significant than Tween 80. PEG-400 greatly influenced the specific gravity response because PEG-400 had a greater specific gravity than Tween 80, namely 1.11-1 .14 g/ml and 1.08 g/ml, respectively [33]. The viscosity of the nanoemulsion preparation was most influenced by Tween 80 with the highest concentration because Tween 80 has a greater viscosity than PEG-400. Tween 80 has a viscosity of 425 mPas, and PEG-400 has a viscosity of 105-130 mPas [33]. Therefore, as Tween 80 concentration increases, so does the viscosity of the preparation. The research by Saeedi et al. [34] also supports this result, which states that Tween 80 can increase the viscosity of preparations because it can absorb water and swelling. The interaction between Tween 80 and PEG-400 influenced the highest percent transmittance results. The interaction between the two factors negatively impacts the percent transmittance. According to Del Regno et al. [35], nonionic surfactants will produce more micelle aggregation when the concentration used is more significant because the micelle mix process occurs. The more aggregation that happens, the smaller the transmittance percentage will be.

Based on the model and response analysis results, the optimum nanoemulsion formula is at a Tween 80 concentration of 15% and PEG-400 of 25%. Tests on globule characteristics, stability and photoprotective activity followed Sambiloto leaf nanoemulsion's optimum formula. The Sambiloto leaf nanoemulsion formula has a globule size of 172.433±24.312 nm, a PDI value of 0.245±0.060, and a zeta potential of -18.100±0.755 mV. The results obtained meet good globule characteristics where the resulting globule size is less than 200 nm, the PDI value is less than 0.3, and the zeta potential is less than -10 or more than +10 mV [36,37]. The optimum formula of Sambiloto leaf nanoemulsion also provides good physical stability. The results of this stability are supported by the resulting zeta potential, where the less than -10 mV, the farther the distance between the globules will prevent the possibility of aggregation [38].

The photoprotective activity of sambiloto leaf nanoemulsion was observed based on the SPF and IC_{50} of DPPH Radical Scavenging values. Table 5 and 6 show that sambiloto leaf nanoemulsion provides better SPF and IC_{50} values than sambiloto leaf extract. Sambiloto leaf nanoemulsion gave the highest SPF value compared to other groups, including marketed products, namely 44.427 ± 0.081, in the high protection category (p <0.05). The DPPH Radical Scavenging results of the Sambiloto leaf nanoemulsion were also

better than the Sambiloto extract, namely the IC50 value of 124,863 ± 4,045 ppm (medium category) and 419,083 ± 10,217 ppm (very low category). Still, the activity was lower when compared to ascorbic acid with an IC_{50} value of 4,617 ± 0.036 ppm (very strong category) ($p<0.05$).

Sambiloto leaf nanoemulsion and Sambiloto leaf extract can provide photoprotective activity due to their andrographolide and quercetin content. The flavonoid and andrographolide content in sambiloto leaf extract was 32.72 mg/g for quercetin and 34.11 mg/g for andrographolide. Quercetin and andrographolid as photoprotective agents can neutralize free radicals or reactive oxygen species (ROS), inhibit lipid peroxidase, which causes sunburn and inhibit UVB irradiation-induced apoptosis [14,39]. In DPPH Radical Scavenging Assay, DPPH act as free radical. DPPH Radical Scavenging assay is an illustration of the action of the sambiloto leaf nanoemulsion against free radicals that found in the skin. ROS or free radicals in skin will be made more easily by UV light. This will affect the activity of IkB-kinase, which in turn activates NF-κB. When this happens, proinflammatory cytokines like tumour necrosis factor-α (TNF-α), interleukin-8 (IL-8), and interleukin-6 (IL-6) will be released. These cytokines stop cells for making collagen. Research conducted by Nisar et al. [16] and Wang et al. [17] proved that the administration of andrographolide and quercetin effectively reduced the expression of IL-1β, IL-6, and TNF-α due to UV induction. Andrographolide and quercetin also have anti-inflammatory effects in Human Epidermal Keratinocytes (HaCaT) [18]. In addition, quercetin as a flavonoid also has a chromophore group that can absorb UV radiation and reduce the intensity of UV radiation in the skin [40].

The development of nanoemulsion preparations was also proven to be able to increase the photoprotective activity of Sambiloto leaf extract, as seen from the significant difference in the SPF and IC50 results produced by Sambiloto leaf nanoemulsion and Sambiloto leaf extract ($p<0.05$). The Sambiloto leaf nanoemulsion preparation has a small globule size of 172,433 ± 24,312 nm with a large surface area so that the substances contained more easily interact with UV light or the given DPPH radicals. In addition, sambiloto leaf nanoemulsion contains basil leaf oil, which acts synergistically because it has antioxidant activity. The DPPH Radical Scavenging activity of basil leaves is obtained from the content of essential oils such as thymol, geraniol, geranial, and eugenol [22].

4. CONCLUSION

The interaction between the concentration of Tween 80 and PEG-400 in the nanoemulsion preparation of sambiloto leaf extract influenced the percent transmittance, pH, viscosity, specific gravity and stable globule size. The optimum formula was selected when tween 80 at 15% and PEG-400 at 25% with desirability value of 0.978. The optimum formula has good physical stability, a high SPF (sun protection factor) value and a moderate IC_{50} value of DPPH Radical Scavenging. The DPPH Radical Scavenging assay demonstrates in vitro the antioxidant capacity of sambiloto nanoemulsion against DPPH radical compounds. Based on the result, it can be concluded that sambiloto nanoemulsion has the potential effect to be photoprotective agent.

5. MATERIALS AND METHODS

5.1. Reagents and Materials

Sigma Aldrich (St. Louis, United States) provided standards for andrographolide and quercetin with a purity of >98%. Sambiloto leaves were obtained from Lampung, Indonesia. Tween 80, polyethylene glycol 400 (PEG-400), demineralized water, distilled water, methanol, and ethanol were purchased from Bratachem, Indonesia. 2.2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Nitra Kimia, Indonesia. Basil oil was purchased from Darjeeling, Indonesia.

5.2. Methods

5.2.1. Sambiloto Leaf Extract Preparation

Simplicia sambiloto leaf powder was extracted for 72 hours using the maceration process with 96% ethanol as the solvent in a 1:10 ratio. The filtrate was then re-macerate for 24 hours. Using a rotary evaporator at 50°C, the filtrate was concentrated until an ethanol extract of Sambiloto leaves was obtained [9].

5.2.2. Determination of Andrographolide Contents

Andrographolide compounds were tested quantitatively using andrographolide compound as standard. The concentration series used is 5 – 25 ppm to create a calibration curve by measured the absorbance at 235 nm. At a concentration of 1000 ppm, the andrographolide content in the extract was determined using the same procedures and calculate the levels of andrographolide [41].

5.2.3. Determination of Flavonoid Contents

Flavonoid compounds were tested quantitatively using quercetin compound as standard. The concentration series used is 2 – 10 ppm to create a calibration curve. Then, 0.1 ml of 1 M sodium acetate, 0.1 ml of 10% AlCl3, and 1.5 ml of each concentration series were added to a 5 ml volumetric flask containing distilled water. The flask was then left to operate for thirty minutes. The absorbance was determine at 431 nm. At 1000 ppm, the total flavonoid content of the extract was determined [42].

5.2.4. Preparation of Nanoemulsion Formula

Software from the Design-Expert series 12 was used to determine the sambiloto leaf nanoemulsion formula. Four formulas were created as a result of the optimization of the nanoemulsion formula utilizing Design-Expert 12 software and the Regular Two-Level Factorial Design approach. Both Tween 80 (surfactant) and PEG-400 (co-surfactant) have concentration ranges of 15 to 25%. Basil oil had a 3% concentration while the sambiloto leaf ethanol extract had a 1% concentration. Table 7 contains the design of the nanoemulsion formulation.

Table 7. Sambiloto leaf nanoemulsion formula

Material	Concentration (%)			
	F1	F2	F3	F4
Sambiloto Leaf Extract	1	1	1	1
Basil Oil	3	3	3	3
Tween 80	**15**	**25**	**15**	**25**
PEG-400	**15**	**15**	**25**	**25**
Demineralized water *ad* (ml)	50	50	50	50

Nanoemulsion was made by spontaneous emulsification process. In the oil phase, Sambiloto leaf extract and basil oil were combined at 5000 rpm for 15 minutes in room temperature. Water phase including Tween 80, PEG 400, and demineralized water was combined at 1500 rpm for 10 minutes in room temperature. The oil phase was added drop by drop to the water phase at 1500 rpm for 10 minutes. Ultra-turrax stirring at 7600 rpm for 15 minutes is necessary for the emulsification process [43].

5.2.5. Evaluation of Sambiloto Leaf Nanoemulsion

Evaluation parameters of the sambiloto leaf nanoemulsion were observed in the form of organoleptic, pH, specific gravity, viscosity and percent transmittance. The color, clarity, and smell of the sambiloto leaf nanoemulsion were assessed using the five senses. The pH of the nanoemulsion preparation was determined using pH meter. The specific gravity was measured using a pycnometer by weighing an empty pycnometer (m), a pycnometer filled with water (m1), and a pycnometer filled with nanoemulsion (m2). Using a Brookfield viscometer, the nanoemulsion's viscosity was calculated using a spindle at 30 rpm at room temperature. The percent transmittance was calculated using a UV-Vis spectrophotometer at 650 nm, the nanoemulsion was diluted 100 times using distilled water [43-45].

5.2.6. Determination of the Optimum Sambiloto Leaf Nanoemulsion Formula

Using the Design-Expert 12 application, the Two-Level Factorial Design approach is used to find the optimum formula. Quantitative characteristics like pH, specific gravity, viscosity, and percent transmittance are used to determine the optimum formula. Based on a desirability value near to one, the optimum formula is selected [46].

5.2.7. Determination of Optimum Sambiloto Leaf Nanoemulsion Globule Characteristics

A dynamic light scattering particle size analyzer (Zetasizer ZS90, Nanoseries, Malvern, UK) was used to evaluate the properties of nanoemulsions based on globule size, PDI, and Zeta potential parameters at a temperature and angle of 25°C and 90°, respectively. All samples were tested, and the samples were diluted

in deionized water up to 1000 times. The sample was placed into a quartz cuvette prior to testing, and measurements were made three times [29].

5.2.8. Stability Test of Optimum Sambiloto Leaf Nanoemulsion

Utilizing centrifugation and the cycle test, the stability test was performed on the ideal formula. The cycling test procedure was run six times for 24 hours each at 4°C and 40°C. 5 hours of centrifugation were completed at 3800 rpm. The stability parameters observed are organoleptic pH, specific gravity, and percent transmittance [47].

5.2.9. Calculation for the Sun Protection Factor (SPF) of Optimum Sambiloto Leaf Nanoemulsion

With a few modifications, the sample preparation for the photoprotective test was done in accordance with a study by Dutra et al. [48]. Demineralized water served as the negative control, market sunscreen served as the positive control, and 1% Sambiloto extract served as the comparator. UV-Vis spectrophotometry (Biobase®) was used to quantify absorbance values at UVB wavelengths (290-320 nm). The value of the sun protection factor (SPF) can be calculated using the equation below.

$$\text{SPF} = \text{CF} \times \sum_{\lambda=290}^{320} \mathbf{EE}(\boldsymbol{\lambda}) \mathbf{x}\, \mathbf{I}(\boldsymbol{\lambda})\ \mathbf{x}\, \mathbf{abs}(\boldsymbol{\lambda})$$

5.2.10. DPPH Radical Scavenging Assay of Optimum Sambiloto Leaf Nanoemulsion

DPPH Radical Scavenging Assay was assessed using UV-Vis Spectrophotometric to determine the Inhibitory Concentration 50 (IC50) value. Sambiloto extract served as a comparator while ascorbic acid served as a positive control. Ascorbic acid, sambiloto extract, and the optimum formula were all employed in this test at doses of 2–6 ppm, 12–200 ppm, and 20–100 ppm, respectively. To 3 mL of DPPH, up to 2 mL of each concentration series from the sample was added. In room temperature and dark condition, the mixture was incubated for 30 minutes. The absorbance at 517 nm was then used to determine the percent inhibition and carry out the IC_{50} calculation [49,50].

5.2.11. Data Analysis

Data obtained from the results of testing nanoemulsion preparations were analyzed using the factorial design 2^2 approach using Design Expert software series 12. For stability testing, the SPF value and IC_{50} values of antioxidants were analyzed using SPSS 25 for Windows software.

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