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Original Article

SAMBILOTO LEAF NANOEMULSION AS A PHOTOPROTECTIVE AGENT: OPTIMIZATION OF TWEEN 20 AND PEG-400 CONCENTRATION USING THE REGULAR TWO-LEVEL FACTORIAL DESIGN

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ABSTRACT

Objective: This study aims to determine the optimum concentration of Tween 20 and Polyethylene Glycol 400 (PEG-400) in Sambiloto leaf extract nanoemulsion.

Methods: The formula of sambiloto leaf nanoemulsion was developed using the regular two-level factorial design based on responses of pH values, density, viscosity, and transmittance percentage. The optimum formula was tested for stability and photoprotective activity by determining the Sun Protection Factor (SPF) value and antioxidant activity.

Results: Sambiloto leaf extract contains andrographolide at 3.397%. The optimum formula for nanoemulsion preparations was obtained at 10% of tween 20 and 10% of PEG-400. The optimum nanoemulsion had a distinctive green extract aroma, a transmittance percentage of 86.7±0.170, a globule size of 130.43±54.056 nm, a polydispersity index of 0.318±0.043, and a zeta potential of-26.5±0.544 mV. The optimum formula's photoprotective activity resulted in an SPF value of 42.944±0.026 and an IC_{50} of 103.611±1.085 ppm. There was no significant change in pH or transmittance percentage based on the stability test ($p<0.05$).

Conclusion: The results show that the optimum formula of sambiloto leaf nanoemulsion could be a photoprotective agent that is also stable.

Keywords: *Andrographis paniculata*, Nanoemulsion, Tween 20, Photoprotective, Polyethylene glycol 400

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INTRODUCTION

Sunlight or ultraviolet light has a wavelength of 200–400 nm, which is beneficial for the synthesis of vitamin D and the destruction of microorganisms [1, 2]. Nevertheless, the skin is susceptible to adverse effects, including skin darkening, skin thickening, premature ageing, skin inflammation, and skin cancer, as a result of excessive exposure to ultraviolet radiation [3-5]. Pharmaceutical preparations with a photoprotective effect can mitigate the risk of skin damage from ultraviolet radiation. The sambiloto plant, known scientifically as *Andrographis paniculata* (Burm f.) Wall ex Nees (*A. paniculata*), belongs to the Acanthaceae family. The primary constituents of *A. paniculata* are andrographolide compounds and their derivatives, such as 14-deoxy andrographolide, 8-methyl andrographolide, 14-deoxy-11.12-didehydroandrographolide, neo-andrographolide, deoxy andrographolide, andrographanine, and others [6-8]. Researchers have extensively studied the pharmacological effects of andrographolide compounds, which include antioxidant, anti-inflammatory, antibacterial, immunomodulatory, anti-cancer, anti-platelet, and antiviral properties [9-11]. A study by Fardiyah *et al.* [12] showed that sambiloto leaf extract can also be used as a photoprotective agent at 10 µl/ml and 20 µl/ml with an SPF value of 11.80±0.18 and 28.41±0.05, respectively. The protective mechanism of andrographolide involves various mechanisms, such as increasing antioxidant enzyme activity, downregulating Matrix Metalloproteinase (MMP) expression, and inhibiting excessive cytokine production by UV radiation [13, 14]. Photoprotective agents are frequently administered in topical dosage forms. This study formulated a nanoemulsion preparation from Sambiloto leaf extract, considering that the site of intended action affects the choice of nanoemulsion. Since UV rays can penetrate deeper into the skin, reaching the dermis [15], nanoemulsion were deemed appropriate. Nanoemulsions contain an oil phase, an aqueous phase, surfactants, and cosurfactants and have small droplet sizes between 50 and 200 nm [16]. This study selected tween 20 as a surfactant, PEG-400 as a co-surfactant, and basil oil based on its safety, physicochemical properties, and HLB value. Tween 20 has a lower irritant potential and an HLB value of 16.7, while PEG 400 has an HLB of 13.1 [17]. Basil oil was chosen because it has activity as a photoprotective agent. Based on research by Kaur and Saraf [18], basil oil at a concentration of 0.1% has a potential SPF value of 6.571, so it can work synergistically with Sambiloto leaf extract as a photoprotective agent. In addition, basil oil also has an HLB value closest to tween 20 and a PEG-400 of 13.36 [19]. The combination of the three is designed to have a mixed HLB of>10 to form an O/W nanoemulsion that is easier to penetrate. Tween 20 is used in the concentration range (10–20%) and PEG 400 in the concentration range (10–20%) to obtain safe preparations [20, 21].

The formula design was created using the Design-Expert Series 12® software and optimised using the regular two-level factorial design approach to obtain the optimum formula. The resulting optimum formula was measured by measuring the value of the sun protection factor (SPF) and testing the antioxidant activity to determine the difference between Sambiloto leaf extract and the extract that had been made into nanoemulsion preparations, as well as a stability test to determine the stability of the resulting optimum formula.

MATERIALS AND METHODS

Materials

The materials used in this study included sambiloto leaf, basil oil (Darjeeling®, Indonesia), tween 20 (Bratachem®, Indonesia), polyethylene glycol 400 (PEG-400) (Bratachem®, Indonesia), 96% ethanol (Bratachem®, Indonesia), distilled water (Smart-Lab®, Indonesia), demineral water (Smart-Lab®,Indonesia), methanol pa. (Bratachem®, Indonesia), ethanol pa. (Bratachem®, Indonesia), andrographolide standard (MarkHerb®, Indonesia), sodium acetate (Bratachem®, Indonesia), $AlCl_3$ 10% (Bratachem®, Indonesia), filter paper No. 41 (Whatman), and aluminium foil (KlinPak®, Indonesia).

Preparation of sambiloto leaf ethanolic extract

Simplisia powder from sambiloto leaves was weighed and extracted with 96% ethanol (1:10). The extraction is macerated for 72 h and filtered. The filtrate was concentrated in a vacuum rotary evaporator at 50 °C to make a thick extract [12].

Quantitative test of andrographolide compounds

A standard of andrographolide compounds was used to quantify the compounds. The solution was prepared at a concentration of 99.62 ppm and diluted to make a calibration curve by pipetting 0.2 ml; 0.4 ml; 0.6 ml; 0.8 ml; and 1.0 ml. The absorbance was measured at a maximum wavelength of 235 nm using a UV-Vis spectrophotometer. The andrographolide content in the extract was measured at 10.000 ppm using the same method as the maximum wavelength [22].

Formula design

Four formulations were created using regular two-level factorial design and design-expert series 12 software for Sambiloto leaf extract nanoemulsion. The nanoemulsion contains 1% sambiloto leaf extract and 3% basil oil. Optimised materials are surfactants and co-surfactants. Tween 20 is used as a surfactant in the concentration range of 10–20%. The co-surfactant is PEG 400, with a concentration range of 10–20%.

Preparation of sambiloto leaf nanoemulsion

Nanoemulsion was made following the research of Miksusanti, Apriani, and Bihurinin [23], with slight modifications. Sambiloto leaf extract and basil oil were in the oil phase, whereas tween 20, PEG-400, and demineral water were in the water phase. The oil phase was stirred at 5000 rpm and the water phase at 1000 rpm. The oil phase was added to the water phase drop by drop and stirred at 1500 rpm for 10 min. The two phases were combined and homogenised at 7600 rpm with an Ultra Turrax® homogenizer for 15 min to generate an M/A nanoemulsion [24].

Evaluation of sambiloto leaf nanoemulsion

Organoleptic

Organoleptic tests on the Sambiloto leaf nanoemulsion preparations were carried out by direct observation using the five senses of colour, smell, and clarity.

pH

The pH value of the nanoemulsion was measured using a pH meter. Before using the pH meter, the electrodes are calibrated with standard pH 4 and 7 buffers [25].

Density

Density was determined using a pycnometer by weighing an empty pycnometer (m), a pycnometer filled with pure water (m1), and a pycnometer filled with nanoemulsion (m2).

Viscosity

The nanoemulsion's viscosity was measured with a Brookfield viscometer. Viscosity was evaluated at 30 rpm at room temperature in a 25 ml beaker [26, 27].

Transmittance percentage

The determination of the transmittance percentage was carried out for each formula with a 100-fold dilution using distilled water. Then the transmittance was measured using a UV-Vis spectrophotometer at a wavelength of 650 nm with blank distilled water [23].

Determination of optimum formula

The reguler two-level factorial design approach finds the optimum formula using pH, viscosity, density, and transmittance percentage variables. The optimum formula meets desirability value parameters, which are close to 1 [28, 29].

Globule size, polydispersity index (PDI) and zeta potential for optimum formula

The globule size, polydispersity index, and potential zeta of nanoemulsions were measured using a particle size analyser (Malvern) at 25 °C. The test was performed by dissolving nanoemulsion in distilled water. The mixture was taken up to 3.0 ml and put into the cuvette for analysis. The test was carried out in three replications [30].

Stability test for optimum formula

The stability test was carried out on the optimum formula with the cycling test and centrifugation methods. The cycling test method was carried out at 4 °C and 40 °C for 24 h each for six cycles. The centrifugation method was carried out at 3800 rpm for 5 h. Changes in stability can be seen from organoleptic tests, pH values, density, and transmittance percentage [31].

Photoprotective activity test

Sun protection factor (SPF) value

The photoprotective test of sample preparation based on Sopyan *et al.* [32] with slight modifications. In this study, market sunscreen was used as a positive control, demineral water was used as a negative control, and 1% Sambiloto extract was used as a comparison. Absorbance values were measured at UVB wavelengths (290–320 nm) using UV-Vis spectrophotometry (Biobase®). The following equation can determine the sun protection factor (SPF) value in Equation 1.

$$SPF = CF \times \sum_{\lambda=290}^{320} EE(\lambda) \times I(\lambda) \times abs(\lambda) \ldots\ldots\ldots\ldots [1]$$

Antioxidant activity

Antioxidant activity was measured by calculating the value of inhibitory concentration 50 (IC_{50}) using the DPPH method. *Ascorbic acid* was used as a positive control, and sambiloto extract was used as a comparison. The concentrations used in this test were ascorbic acid 2–6 ppm, sambiloto extract 12.5-200 ppm, and the optimum formula 20–100 ppm, respectively. Add 2 ml of each concentration series to 3 ml of DPPH and incubate at room temperature for 30 min in the dark condition. Absorbance was measured using a UV-Vis spectrophotometer at the maximum wavelength. The absorbance obtained is then used to calculate the inhibition percentage and calculate IC_{50} value [23, 33].

Data analysis

A regular two-level factorial design with Design Expert software series 12® was used to analyse nanoemulsion preparation evaluation data to determine the effect of factor concentration on evaluation results and explain the relationship between each factor to find the optimum formula. Sun Protection Factor (SPF) stability and value were tested using the SPSS 25 Program.

RESULTS

Sambiloto leaf extract

Sambiloto leaf extract is thick, dark green, and has a distinct smell. Sambiloto leaf extract has a yield percentage of 20.207%, with an andrographolide content of 3.397%. The produced yield satisfies the requirements of the Indonesian herbal pharmacopoeia, while the yield of the sambiloto herb extract is not less than 9.6%. This result demonstrates the effectiveness of the extraction process in the study, which involved a 72-hour dark incubation period with stirring.

Sambiloto leaf nanoemulsion

Table 1 displays the evaluation results of the nanoemulsion preparations. The four formulas produce Sambiloto leaf nanoemulsion preparations that are transparent green.

Model and response analysis

Quantitative evaluation parameter values are continued for optimisation using the design-expert series 12® software. The analysis was divided into two stages: model analysis and response analysis. Table 2 displays the results of the model analysis, while table 3 and fig. 2 display the results of the response analysis.

Nanoemulsion optimum formula

The optimum formula is determined based on pH values ranging from 4.5 to 6.5, low density, and a high transmittance percentage. Based on the optimisation of the Design-Expert Series 12®, the optimum formula was obtained at a concentration of 10% tween 20 and 10% PEG-400 with desirability of 0.982. The optimum formula had a globule size of 130.43±54.056 nm, a polydispersity index of 0.318±0.043, and a zeta potential of-26.5±0.544 mV.

Stability of optimum formula

The stability test of optimum formula has been conducted. The results of stability study were described in table 4.

Table 1: Evaluation results of sambiloto leaf nanoemulsion

Evaluation	**Formula**			
	1	**2**	**3**	**4**
Organoleptic	**Dark green, transparent, and has a distinctive smell**	**Dark green, transparent, and has a distinctive smell**	**Dark green, transparent, and has a distinctive smell**	**Dark green, transparent, and has a distinctive smell**
pH	4.84±0.016	4.98±0.012	4.86±0.016	5.20±0.009
Density (g/cm^3)	1.044±0.002	1.050±0.006	1.054±0.002	1.075±0.002
Viscosity(cP)	0.98±0.209	1.02±0.167	1.14±0.039	1.28±0.199
Transmittance (%)	86.7±0.170	86.5±0.141	85.7±0.163	85.8±0.082

Note: - Data are given as mean±SD, n=3, - F1 (10% Tween 20 and 10% PEG-400), F2 (10% Tween 20 and 20% PEG-400), F3 (20% Tween 20 and 10% PEG-400), F4 (20% Tween 20 and 20% PEG-400)

Table 2: Model analysis results

Response	**Parameter**				
	Adjusted R^2	**Predicted R^2**	**Difference adjusted R^2 and adjusted R^2**	**Adequate precision**	**p-value**
pH	0.9868	0.9784	0.0084	36.1726	<0.0001*
Density	0.8895	0.8192	0.0703	12.7094	<0.0001*
Viscosity	0.0748	-0.5140	0.4392	2.5265	0.3406
Transmittance	0.8579	0.7675	0.090	9.5351	0.0003*

Note: *p<0.05 shows a significant effect

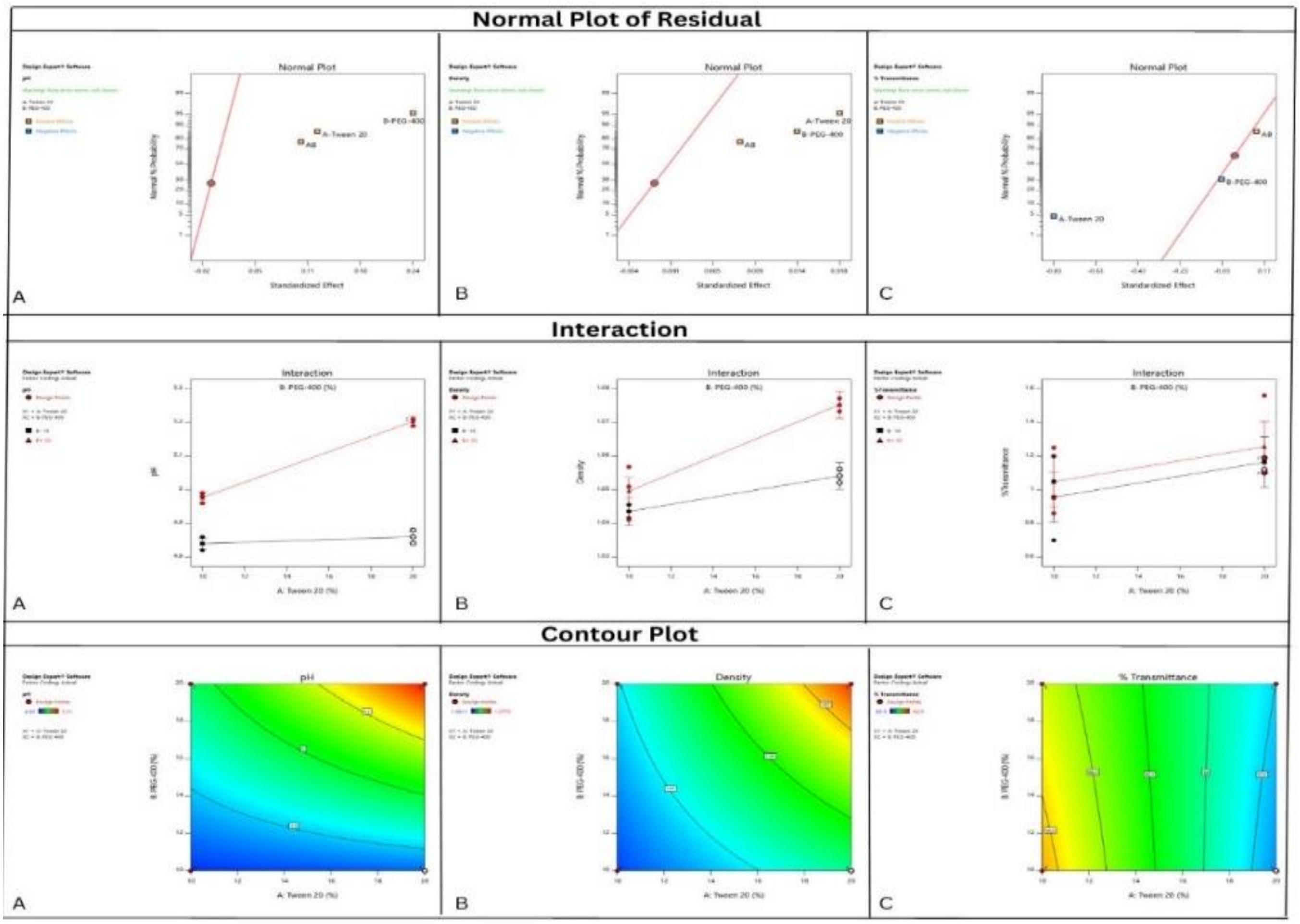

Fig. 2: Normal plot of residual, interaction and contour plot graphs of pH (A), Density (B), and transmittance percentage (C)

Table 3: Response analysis results

Parameter		Intercept	A (Tween 20)	B (PEG-400)	AB (Interaction)
pH	Coefficient	4.9683	0.0600	0.1183	0.0500
	P-value		<0.0001*	<0.0001*	<0.0001*
	% Contribution		17.7364	68.9886	12.317
Density	Coefficient	1.0556	0.0090	0.0068	0.0038
	P-value		<0.0001*	0.0006*	0.0163*
	% Contribution		52.8983	29.8468	9.2210
Transmittance	Coefficient	86.1667	-0.4167	-0.0167	0.0667
	P-value		<0.0001*	0.7508	0.2249
	% Contribution		87.2905	0.1397	2.2346

Note: *p<0.05 shows a significant effect

Table 4: Results of stability study

Parameter	Before	After	P-value
Organoleptic	Dark green, transparent, and has a distinctive smell	Dark green, transparent, and has a distinctive smell	-
Phase Separation	No	No	-
pH	4.84±0.016	4.87±0.002	0.095
Density (g/cm^3)	1.044±0.002	1.041±0.008	0.272
Transmittance (%)	86.7±0.170	86.6±0.082	0.423

Note: Data are given as mean±SD, n=3

Table 5: Results of SPF activity

Group	Sample	SPF value	Category
I	Marketed Sunscreens	24.392±0.082^c	Moderate
II	1% Sambiloto Leaf Extract	25.399±0.077^b	Moderate
III	Demineral water	0.047±0.003^d	Very Low
IV	Optimum Formula	42.944±0.026^a	High

Note: - Numbers followed by different lowercase letters indicate a significant difference (p<0.05), - Data are given as mean±SD, n=3

Table 6: Results of antioxidant activity

Group	Sample	IC50 (ppm)	Category
I	Ascorbic acid	4.617±0.029^a	Very strong
II	Sambiloto extract	419.083±8.343^c	Low
III	Optimum formula	103.611± 1.085^b	Strong

Note: - Numbers followed by different lowercase letters indicate a significant difference (p<0.05), - Data are given as mean±SD, n=3

Photoprotective activity

The photoprotective activity is described based on the results of testing the value of the SPF and antioxidants. The SPF and antioxidant IC_{50} values are shown in tables 5 and 6.

DISCUSSION

Sambiloto leaf nanoemulsion had various evaluation results due to the different concentrations of tween 20 and PEG-400 used (table 1). However, the four formulas met the requirements for nanoemulsion preparations. The pH ranged from 4.5 to 6.5, the density was more than 1 g/cm^3, the viscosity was small, and the transmittance percentage was close to 100% [34].

Quantitative evaluation parameter values are continued for optimization using the design-expert series 12® software. A model analysis was conducted to determine whether these parameters could be continued for optimization. In contrast, response analysis was carried out to see the effect of each factor and the interaction of factors on the observed parameters. The model that was obtained meets the acceptance criteria. If the adjusted R2 value is greater than 0.7, the difference between adjusted R2 and predicted R2 is less than 0.2, adequate precision is greater than 4, and the parameter p-value is<0.05 [28, 29, 35].

Table 2 shows that the parameters pH, density, and % transmittance met the requirements of a good model. The adjusted R2 values for the parameters pH, density, and % transmittance fall within the range of 0.8579–0.9868, indicating that the Tween 20 factor, PEG-400, and their interaction influence 85.79%–98.68% of the data population [36]. The difference between the resulting adjusted R2 and predicted R2 values is also less than 0.2, which means that the data predicted by the system are similar. The adequate precision value is more than 4, which means the model is quite strong. The p-value is also less than 0.05, indicating that the Tween 20 and PEG-400 factors, as well as their interaction, have a significant impact on the parameters.

Response analysis was carried out on evaluation parameters with good models: pH, density, and % transmittance. According to table 3, all factors, including Tween 20 and PEG-400, and their interactions, have a significant impact on pH and density parameters. However, only the Tween 20 factor significantly affects the transmittance parameter (p-value<0.05). According to the normal graph plot of residuals (fig. 2), the factor points lie outside the line, indicating their influence on the parameters. The coefficient data, all factors, including Tween 20, PEG-400, and their interactions, had a positive influence on the pH and density parameters. However, Tween 20 negatively affected the transmittance percentage parameter. The contour plot graph in fig. 2 can also be used to explain this statement. The interaction graph in fig. 2 also demonstrates the relationship between Tween 20 and PEG-400 interactions. The pH and density parameters have an intersect lines while the transmittance percentage parameter has no line intersection. The intersection of the lines indicates that there is an interaction between Tween 20 and PEG-400. This result is also supported by the p-value of the AB factor, where the pH and density parameters have a p-value<0.05, which means that there is an interaction effect on these parameters.

PEG-400 has the most dominant influence on the pH parameter. On the other hand, Tween 20 primarily influences the density and transmittance percentage parameters. PEG-400 has a more significant influence than tween-20 in increasing the pH of the preparation. This is because the tween-20 structure contains fewer OH groups than PEG-400 [37]. The hydrolysis reaction of PEG-400 with demineral water causes the release of the OH group, leading to an increase in the pH value [38]. Tween 20 affects increasing the density value because tween 20 has a density value of 1.1 g/cm^3, while PEG-400 has a density of 1.11–1.14 g/cm^3 [36]. Surfactants in water or solvents will form micelles, which can grow into large cylinder-shaped groups at high concentrations [39]. This means that Tween 20 can lower the transmission percentage.

The Design-Expert Series 12® optimization yielded both Tween 20 and PEG-400 has an optimum concentration at 10%. The optimum formula had a globule size of 130.43±54.056 nm. This result showed that the sambiloto leaf nanoemulsion's oil, surfactant, and cosurfactant phases are properly concentrated [16]. The optimum formula had a PDI value of 0.318±0.043. According to Bhattacharjee [40], PDI describes the intensity of light scattered by various fractions of particles of different sizes and is calculated by (width or average) for each peak, where PDI is<0.1 (high monodisperse), 0.1-0.4 (moderate polydispersion), and>0.4 (high polydispersion). The optimum formula belongs to medium polydispersionnanoemulsions. The zeta potential test for the optimum formula was-26.5±0.544 mV. Zeta potential is used to determine the stability of colloids in nanoemulsion preparations. There are four types of zeta potential values for nanoparticle dispersions: ±0–10 mV (not stable), ±10–20 mV (relatively stable), ±20–30 mV (quite stable), and ±30 mV (very stable) [40, 41]. The optimum formula has quite good stability.

The optimum formula's stability results in table 4 showed that there were no organoleptic changes or phase separations in the preparation before and after testing. The results of the quantitative stability test were analyzed using a paired sample t-test [42]. The pH, density, and transmittance percentage values were analysed and found to be significant at 0.095, 0.272, and 0.423, respectively, indicating that the pH, density, and transmittance values of the preparations did not change. The results of this stability are also supported by the potential zeta of the optimum formula of Sambiloto leaf nanoemulsion, which is quite stable, so it will prevent aggregation because the distance between the globules is far from each other [43].

The photoprotective activity is described based on the results of the sun protection factor (SPF) and antioxidants activity. The SPF value can determine photoprotective activity. SPF scoring systems range from low to high: low (SPF 2–15), medium (SPF 15–30), high (SPF 30–50), and highest (SPF>50) [44]. Based on the results of table 5, a significant difference was observed (p<0.05). Demineral water, as a negative control, has the lowest activity with an SPF value of 0.047±0.003. Sunscreens on the market and 1% Sambiloto extract have SPF activity in the medium protection category, with SPF values of 24.392±0.067 and 25.399±0.063. The optimum formula for Sambiloto leaf nanoemulsion with the same concentration is 1%, which has the highest SPF activity and is included in the category of high protection with an SPF value of 42.444±0.021. These results prove that developing Sambiloto leaf extract in nanoemulsions can increase SPF activity.

Antioxidant activity can be determined based on the value of inhibition concentration 50 (IC_{50}). The IC_{50} value describes the concentration of a substance with antioxidant activity capable of reducing 50% of free radicals. Antioxidant activity based on IC_{50} value was grouped into five groups, namely, very strong (IC_{50}<50 ppm), strong (IC_{50} 50–100 ppm), moderate (IC_{50} 110–250 ppm), weak (IC_{50} 250–500 ppm), and inactive (>500 ppm) [45]. The IC_{50} value data obtained were analyzed using SPSS 25® software. The results of Duncan's table revealed a significant difference, indicating that all groups had different notations (table 6). The IC_{50} value for ascorbic acid was 4.617±0.036 ppm, which means it had very strong activity. The Sambiloto extract had weak activity (419.083±10.217 ppm), and the nanoemulsion optimum formula had strong activity (98.791±1.234 ppm).

Sambiloto leaf nanoemulsion preparations can increase SPF value and antioxidant activity because of the small globule size and their constituent components, which have a photoprotective action. The size of globules influences the surface area; a smaller globule size results in a larger surface area, which enhances the penetration of active ingredients and boosts the SPF value of sunscreen products. This statement has been proven by Arianto and Cindy's [46], which found that testing the SPF value of nanoemulsion and crude emulsion preparations resulted in significantly different SPF results. Research from Liu *et al.* [47] also proved that making lemon essential oil nanoemulsion provides six times stronger antioxidant activity than pure lemon essential oil.

Basil oil, which has a photoprotective action, composes Sambiloto leaf nanoemulsion. According to Avetisyan *et al.* [48] and Kamelnia *et al.* [49], basil oil has essential oils like eugenol, chavicol, linalool, and a-terpineol that can be act as antioxidants to protect free radicals from UV radiation. Kaur and Saraf [18] found that 0.1% of basil oil has an SPF value of 6.571.

Sambiloto leaf extract also contains secondary metabolites with photoprotective properties. This study proves that sambiloto leaf extract contains andrographolide compounds at 33.966 mg/g. Andrographolide acts as a photoprotectant by activating the keap1/Nrf2 pathway and downregulating the NF-κB pathway in HaCaT keratinocytes, increasing antioxidant enzyme activity, downregulating MMP expression, and inhibiting excessive cytokine production by UV radiation [13, 14, 50].

CONCLUSION

The concentration of Tween 20 and PEG-400 influences the evaluation of nanoemulsion preparations, namely pH, density and viscosity. The higher the Tween 20 and PEG-400 concentration, the higher the pH and density values. Conversely, the lower the viscosity value. The optimum formula of Sambiloto leaf nanoemulsion was obtained at a concentration of Tween 20 and PEG-400 of 10%. The optimum formula in the nano charactersistic test has nano size, includes moderate polydispersion and is relatively stable. The optimum formula also shows good physical stability. The photoprotective activity of the optimum formula is in the high protection category and strong antioxidant activity.

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AUTHORS CONTRIBUTIONS

Elsa Fitria Apriani, as the first author, was responsible for the planning, conceptualization, data collection, and writing of the paper. Miksusanti, VitriAgustiarini, and Ochita Ledy Fransiska, as the second, third, and fourth authors, contributed to the review of the literature and the interpretation of data. Dwi Hardestyariki, the corresponding author, oversaw the study's concept and the design of the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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