JURNAL FARMASI SAINS DAN KOMUNITAS
Journal of Pharmaceutical Sciences & Community

p-ISSN 1693-5683; e-ISSN 2527-7146

Vol. 22, No. 2, November 2025, pp. 310-318 Research Article

Optimization and Physical Characterization of Kojic Acid Nanoemulsion as a Whitening Agent

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https://dx.doi.org/10.24071/jpsc.009968

J. Pharm. Sci. Community, 2025, 22(2), 310-318

Article Info

Received: 2024-10-06
Revised: 2024-12-05
Accepted: 2024-12-06

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Keywords:
Kojic acid; Nanoemulsion; Span 80; Tween 80; VCO

ABSTRACT

Kojic acid (KA) as a topical formulation prevents hyperpigmentation through the inhibition of tyrosinase activity. However, KA has higher solubility in nonpolar solvents. Nanoparticle technology can be a novel innovation to enhance KA absorption into the skin. This study aims to improve the delivery of topically applied KA by enhancing absorption into the stratum corneum by optimizing the concentration of surfactant Tween 80 and co-surfactant Span 80. The nanoemulsion uses KA as an active ingredient and virgin coconut oil as the oil phase. The optimum area was analyzed using Design-Expert® 13 software, with a pure experimental design based on a 2x2 factorial design. The results indicate that all formulas are within the optimal region. Therefore, the determination of the optimal point can be grounded on the smallest composition within the optimal region with the objective of maximizing material efficiency, which is a formula with a concentration of Tween 80 and Span 80 of 10 grams and 3 grams, respectively. The particles are a spherical smooth shape and demonstrate an efficacy of 94.117% in inhibiting tyrosinase activity.

INTRODUCTION

Kojic acid (KA) is a natural organic compound produced by more than 58 fungal strains of the Aspergillus genus and is often used in the cosmetics industry as a topical formulation for wrinkles, melasma, and post-inflammatory hyperpigmentation treatment (Chaudhary, 2014; Gomes *et al*., 2020). KA acts as a tyrosinase inhibitor due to a copper-chelating active site (Wang *et al*., 2019). This compound prevents hyperpigmentation caused by ultraviolet (UV) exposure by inhibiting melanin production through the inhibition of tyrosinase activity (Mohiuddin, 2016; Saeedi *et al*., 2019). A study utilizing hyperspectral cameras demonstrated that KA enhanced skin brightness in roughly 75% of participants, diminished skin contrast in 83% of participants, and improved skin color uniformity in 67% of participants (Wawrzyk-Bochenek *et al*., 2023).

Kojic acid (KA) in crystal form is acicular and colorless. KA is soluble in water, and in certain organic solvents such as ethanol, and ethyl acetate. However, KA is poorly soluble in other organic solvents such as ether, alcohol-ether mixture, pyridine, and chloroform (Chaudhary, 2014; Phasha *et al*., 2022). Furthermore, KA has a higher solubility in polar protic solvents such as methanol, and ethanol compared to water, which makes it difficult for it to penetrate deeper into the skin layers (Sun *et al*., 2021). Accordingly, a topical preparation that allows for deeper deposition of KA into the skin is required to maximize the efficacy of the products. Researchers are currently working to improve the limitations of KA by developing biocompatible and effective products. One approach is to combine KA formulations with nanoparticle technology (Saeedi *et al*., 2019). Nanoparticle-based formulations containing KA represent a novel approach to enhance skin absorption.

Nanotechnology as a method of formulating pharmaceutical products, particularly topical preparations, offers numerous advantages such as enhancing drug deposition to target therapeutic areas, improving the physical and chemical stability of active

compounds, and providing more controlled drug delivery (Ghasemiyeh and Mohammadi-Samani, 2020; Patzelt *et al*., 2017). Nanoemulsions (NEs) are composed of two immiscible liquids, typically oil and water, stabilized with surfactants (Ghasemiyeh and Mohammadi-Samani, 2020). NEs are commonly used in dermal administration systems due to their small particle size, liquid properties, and ability to adsorb to the stratum corneum lipid layer. NEs are classified in two general types: oil-in-water (O/W) and water-in-oil (W/O). The O/W NEs can deliver hydrophobic substances due to their ability to increase the solubility of poorly water-soluble substances (Xue *et al*., 2021). KA is a hydrophobic compound (Wawrzyk-Bochenek *et al*., 2023). Therefore, O/W NEs can be considered for formulating pharmaceutical products containing KA to improve the solubility of active compounds in water and ensure better absorption of the formulated product.

The aim of the study is to improve the delivery of topically applied KA due to its limitations by increasing its solubility and absorption into the stratum corneum by optimizing the concentration of surfactant Tween 80 and co-surfactant Span 80.

METHODS

Kojic acid (KA) was purchased from Genero Pharmaceuticals (Indonesia). Virgin coconut oil (VCO) employed in the study was product grade purchased from Coco Milagro. Tween 80, and span 80 purchased from Merck, triethanolamine (TEA) purchased from T&T Chemical (Indonesia), and aquadest.

The instruments used were a magnetic stirrer and hotplate (Thermo Scientific), single beam UV-vis spectrophotometer (Shimadzu UV mini-1240), particle size analyzer (HORIBA Scientific SZ-100), ultrasonication (Branson 3800), Transmission Electron Microscopy (TEM JEOL JEM 1400), and pH meter.

Nanoemulsions (NEs) preparation

The preparation of KA nanoemulsions (NEs) was done by mixing the KA with VCO (mixture 1). Tween 80 and span 80 were mixed on a separated hotplate (mixture 2). Both mixtures were mixed using a magnetic stirrer at a speed of 1000 rpm and a temperature of 80°C. After homogenously mixed, mixture 1 was added to mixture 2 for 10 min with a speed of 1000 rpm and a temperature of 80°C. After adding 100 ml distilled water gradually into the homogenous system, the stirring was increased to 1250 rpm for 10 min. The final mixture was homogenized using a homogenizer for 2 min, followed by sonication for 45 min using a sonicator bath. All formulas in Table 1 were replicated three times (Yuliani *et al*., 2016).

The resulting NEs subsequently were analyzed using a pH meter to determine the pH, and dynamic light scattering (DLS) or particle size analyzer (PSA) to examine the particle size and zeta potential. The optimal formulas were observed using Transmission Electron Microscopy (TEM) to visualize the morphology of the NEs.

Particle size measurement

Particle size was determined using a PSA with a DLS type (HORIBA Scientific SZ-100). The cuvette was cleaned to avoid the analysis error. The particle size was determined by filling 10 mL of NEs sample in the cuvette. Then, the sample was inserted into the sample holder to analyze the particle size. All measurements were repeated in three replicates. Particle size was expressed as mean ($\bar{x}$) ± standard deviation (SD) (Alhasso *et al*., 2023; El-Feky *et al*., 2017).

Zeta potential

Zeta potential was measured by dissolving 10 mL of NEs sample in 10 mL of aquadest using a PSA with zeta potential measurement type. All measurements were repeated in three replicates. Zeta potential expressed as $\bar{x}$ ± SD (Alhasso *et al*., 2023).

Table 1. Design factorial formulations scheme

Formula	Kojic Acid (gram)	Tween 80 (gram)	Span 80 (gram)	VCO* (gram)	Aquadest (mL)
1	1	10	3	3	100
a	1	12	3	3	100
b	1	10	4	3	100
ab	1	12	4	3	100

*VCO-virgin coconut oil.

NEs type test

NEs type was tested by dissolving 1% sample in the water phase. If the sample is completely soluble in aquadest, then the NEs is identified as an O/W type. However, the water insoluble sample means the NEs type is a W/O type (Yuliani *et al*., 2016).

pH test

The pH value of the KA NEs was measured using a digital pH meter at room temperature (25°C) in triplicate. The measurement of pH was expressed as $\bar{x} \pm SD$ (Alhasso *et al*., 2023; Mulia *et al*., 2018).

Data analysis

Physical properties of NEs such as pH, particle size, and zeta potential were obtained in this study. Design-Expert® 13 was used to analyze the optimum area, which had a pure experimental 2x2 factorial design. Data that are homogenous and normally distributed can be examined using a two-way Analysis of Variance (ANOVA) with a 95% confidence level. A p-value < 0.05 for the physical properties of the NEs means the model has a significant difference. A computational optimizer response was utilized to identify the optimal combination of surfactants tween 80 and span 80 using the Design of Experiment (DOE) method (Sopyan *et al*., 2022; Thango, 2022).

Transmission Electron Microscopy

The size and morphology of the optimal formulation of KA nanoemulsion were determined using TEM. A drop of the optimal KA nanoemulsion was placed on a 200 mesh copper grid coated with a carbon film, left to sit for 10 minutes, stained with 3% phosphotungstic acid, and then examined under a TEM microscope (JEOL JEM-1230, Japan) (Boskabadi *et al*., 2021).

Tyrosinase Inhibitor *In Vitro Test*

The tyrosinase inhibition test is performed by adding 50 µL of the optimal KA sample and 50µL buffer as a solvent to two different microplate wells. Fifty microliters of 3.5 mM L-DOPA substrate and 50 µL of enzyme solution (500 units/mL) were added into each well. Absorbance was measured at an incubation temperature of 25°C and a wavelength of 510 nm. The absorbance was read every 2 min for 20 min (Ripaldo, 2020). The tyrosinase inhibition activity was measured by following equation:

$$\%\text{inhibition} = \frac{A-B}{A} x\ 100\%$$

With A = sample absorption; and B = sample absorption with the addition of inhibitors.

RESULTS AND DISCUSSION

Formula consideration

A well-designed formulation strategy should enhance a drug's usability by ensuring its stability and preserving its efficacy, which in turn improves safety, convenience, and patient adherence to treatment (Muralidhara and Wong, 2020). In this study, VCO is used as the oil phase due to its solvent capacity, oxidation resistance, and good stability. Besides, VCO can also moisturize, making it an ideal choice for the NEs preparation (Jusril *et al*., 2022; Romes *et al*., 2022; Umate *et al*., 2022). VCO is colorless with a coconut scent (Ma and Lee, 2016). As a fat and oil, VCO is soluble in nonpolar organic solvents due to the polarity similarity (Khairati, 2023). It has approximately 53.6% lauric acid, 18.8% myristic acid, 10.7% palmitic acid, 5.4% capric acid, 4.6% oleic acid, 3.7% butyric acid, 1.8% stearic acid, and 1.1% linoleic acid (Ströher *et al*., 2020). Tween 80 and span 80 were used as a surfactant and cosurfactants combination in this formula. An interfacial layer of the surfactant combination stabilizes the transparent dispersion of O/W in a NEs system, which is a thermodynamically stable preparation (Shaker *et al*., 2019). Tween 80 and Span 80 as a surfactants-cosurfactants combination, are nonionic surfactants known for their good safety profile due to low toxicity and their suitability for use in biological systems. (Wang *et al*., 2021).

Organoleptic

Fig. 1 illustrates the NEs formed appear transparent and yellowish. The combination of the surfactant-cosurfactant system unites the immiscible oil and water phases by forming a film layer between the particles. The HLB value of the four formulas of KA NEs falls within the range of 11.943 to 12.860.

Nanoemulsions (NEs) type test

The type of nanoemulsions (NEs) were tested by dissolving 1% sample into the water phase (Yuliani *et al*., 2016). The test results for the four KA NEs formulas explained that the NEs were found to be O/W type. This result is consistent with the estimated HLB values of the mixture, ranging from 11.943 to 12.860, which can produce an O/W type of nanoemulsion.

Figure 1. Kojic acid NEs.

Table 2. Result of physical properties of kojic acid

Formula	pH	Particle size (nm)	Zeta potential (mV)
1	5.79±0.01	50.20±2.52	-11.30±0.69
a	5.70±0.01	45.27±3.69	-10.33±0.58
b	5.80±0.01	271.03±12.99	-10.03±0.81
ab	5.76±0.02	62.20±0.76	-8.63±0.23

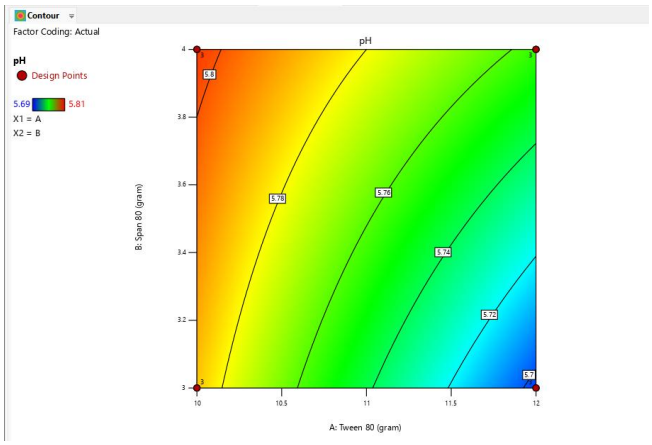

Figure 2. Contour plot response pH versus tween 80 and span 80.

HLB is a parameter of the size and strength balance of the hydrophilic and lipophilic properties of surfactants. HLBs with values in the range of 8.0 to 18.0 form emulsion systems of the O/W type (Zheng *et al*., 2015). Higher HLB values indicate a clearer nanoemulsion system (Ermawati and Jannah, 2023).

pH measurement

The skin's natural pH tends to be slightly acidic, typically ranging from 4 to 6, which helps maintain its function as a barrier against germs and prevents loss of hydration. Skin pH plays a crucial role in maintaining healthy skin by regulating the stratum corneum homeostasis and barrier permeability. The acidic pH of the skin surface is important for the formation of epidermal and corneocyte lipids, maintaining skin health, and preventing skin disturbances or diseases (Lukić *et al*., 2021).

The statistical analysis performed on the pH measurements showed a significant model due to a p-value < 0.05 (0.0001). This finding suggests that the results are statistically significant. Additionally, the analysis found that the factor that most significantly impacts pH is the Tween 80, as evidenced by the highest F-value of 420.25, as shown in Table 4.

Particle Size measurement

The particle size of the NEs sample were measured using a PSA with the DLS method. The four NEs samples were measured in triplicate. The mean size of the four NEs samples as shown

in table 3 consecutively 50.20±2.52, 45.27±3.69, 271.03±12.99, and 62.20±0.76 nm, the particle size value was acceptable to be considered as a nanoemulsion due to its size within 20-500 nm. The smaller sizes of nanoemulsion allow them to penetrate the skin relatively easily, resulting in enhanced bioavailability (Dolowy *et al*., 2017)

The Polydispersity Index (PDI), also referred to as the heterogeneity index, is a dimensionless value derived from a two-parameter fit to the correlation data using cumulants analysis. This index is scaled such that values below 0.05 typically correspond to highly monodisperse standards, indicating a narrow particle size distribution. Conversely, values greater than 0.7 suggest a very broad particle size distribution in the sample (Danaei *et al*., 2018). The PDI values ranging from 0.360 to 0.419 indicate that the NEs produced have a relatively uniform particle size distribution.

Zeta potential

The stability of nanoemulsion formulation can be predicted by the value of the zeta potential. Higher zeta potential values indicate that the nanoemulsion resists aggregation due to the highly charged particles and can be easily redispersed due to the repulsive electric forces acting upon them. Conversely, lower zeta potential values can lead to coagulation, as the reduced electric forces between particles facilitate their aggregation. Zeta potential with absolute values between ≥ 30 mV and ≤ 60 mV are considered indicative of good to excellent stability (Németh *et al*., 2022). Zeta potential ≥ ±30 mV indicates the monodisperse nanoemulsion without aggregation occurrence (Gumustas *et al*., 2017), In contrast, formulations with zeta potential around ±20 mV means a more unstable nanoemulsion system, while those with zeta potentials below 5 mV tend to aggregate rapidly. However, it is essential to note that zeta potential alone does not guarantee nanoparticle stability, since these observations are specific to electric stabilization and the use of low molecular weight surfactants (Németh *et al*., 2022). The results showed that the zeta potential values of the four NEs ranged from -8.63 mV to -11.30 mV, indicating that these values are within the acceptable range for a stable colloidal system.

The statistical analysis results of the zeta potential response show that the model form is significant by the p-value <0.05 (0.0031) as shown in Table 4. The factor that most influences the zeta potential response is Span 80, which had the highest F-value of 17.37, as shown in Table 4.

Table 3. Particle size measurement results

Formula	Particle size	PDI
1	50.20±2.52	0.360±0.02
A	45.27±3.69	0.364±0.01
B	271.03±12.99	0.419±0.01
ab	62.20±0.76	0.409±0.01

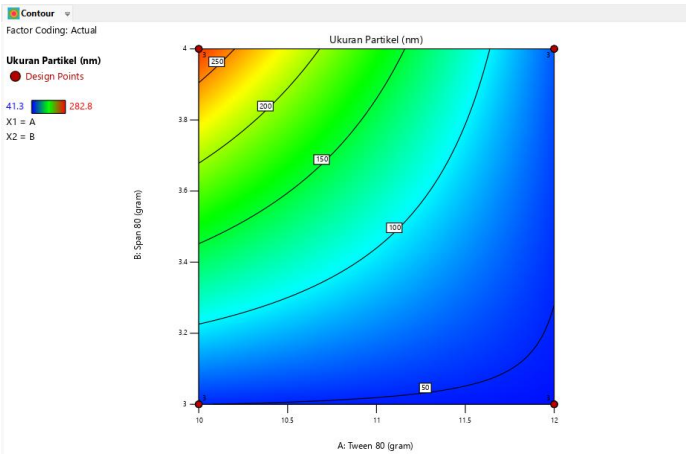

Figure 3. Contour plot response particle size versus Tween 80 and Span 80.

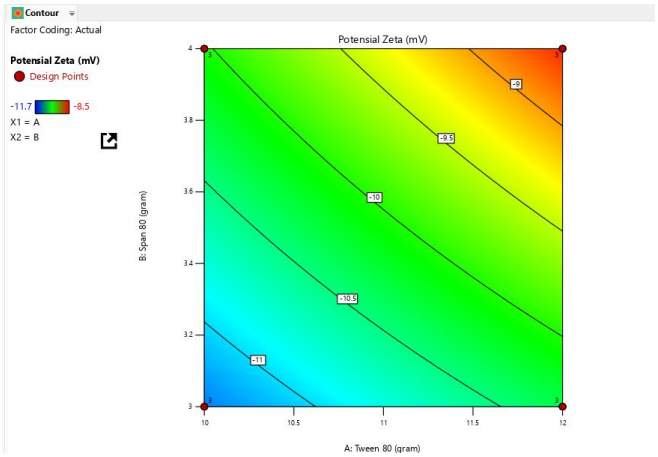

Figure 4. Contour plot response zeta potential versus Tween 80 and Span 80.

Table 4. Statistical analysis of physical properties

Formula	pH		Particle size (nm)		Zeta potential (mV)	
	[a]F value	[b]*p* value	[a]F value	[b]*p* value	[a]F value	[b]*p* value
Model	198.25	<0.0001	760.04	<0.0001	9.60	0.0050
Tween 80	420.25	<0.0001	724.54	<0.0001	11.05	0.0105
Span 80	132.25	<0.0001	896.37	<0.0001	17.37	0.0031
Tween 80*Span 80	42.25	0.0002	659.20	<0.0001	0.37	0.5596

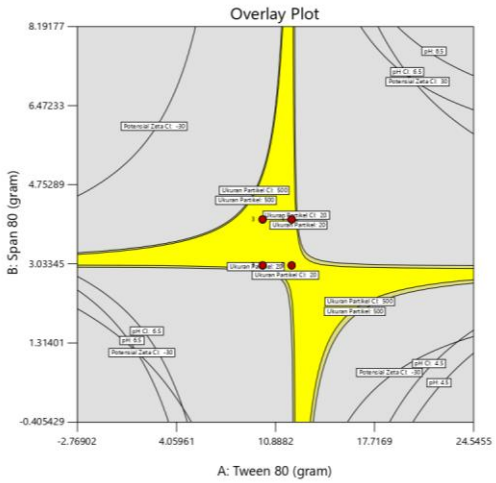

Figure 5. Overlay plot Tween 80 versus Span 80 and response pH, particle size, and zeta potential.

Formula Optimization

The kojic acid (KA) nanoemulsions (NEs) formula was optimized using a two-factor, two-level factorial design approach, which involved the variables Tween 80 at levels of 10 grams (low) and 12 grams (high), and Span 80 at levels of 3 grams (low) and 4 grams (high).

Optimization targets were set for the response of pH, particle size and zeta potential. Data optimization was analyzed using Design-Expert® 13. Figure 6 contains the overlay plot showing the areas and points recommended as the composition of Tween 80 and Span 80. The figure indicates that all formulas are within the optimal region. Therefore, the determination of the optimal point can be based on the smallest composition within the optimal region, with the objective of maximizing material efficiency. In particular, the optimal composition for Tween 80 can be characterized by a point prediction with a value of 10.80 grams, whereas the optimal composition for Span 80 is definded by a point

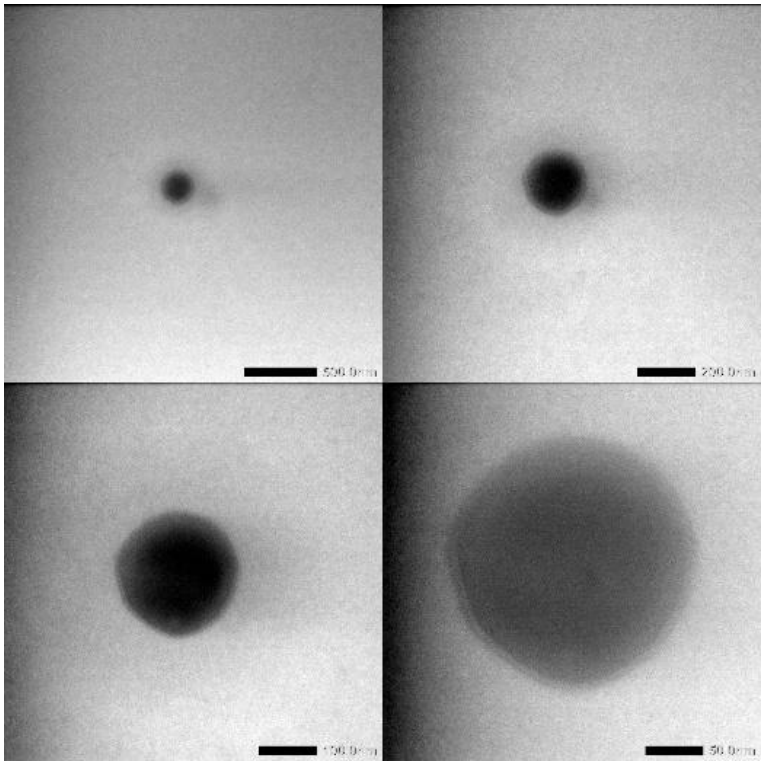

Figure 6. Transmission Electron Microscope images of the optimal kojic acid nanoemulsion.

prediction with a value of 3.35 grams.

Transmission Electron Microscopy

The detailed morphological description of the optimal KA nanoemulsions (NEs) observed using TEM is presented in Fig. 6. The morphological visualization of the particles from the KA NEs shows that the nanoemulsion produced yields particle sizes in the nanometer scale, and they have a smooth spherical shape.

Tyrosinase Inhibitor *In Vitro* Test

The effectiveness of the KA NEs in whitening was evaluated through an in vitro study assessing its tyrosinase inhibitory activity. This test aimed to determine the inhibitory potential of the active compounds within the nanoemulsion formulation (Hidayatri, 2021). The inhibition activity test is based on the ability of the sample to prevent the formation of dopachrome products from the reaction between the substrate L-DOPA and the tyrosinase enzyme (Ripaldo, 2020).

The tyrosinase enzyme inhibition activity was tested on the optimal KA formulation, the %inhibition value is 94.117%. The percentage inhibition indicates the ability of the optimal KA nanoemulsion to inhibit the activity of the tyrosinase enzyme. Higher percentage inhibition of the sample means stronger inhibitory effect on the tyrosinase enzyme. It means that the sample has a greater ability to inhibit melanin formation (Furi *et al*., 2022).

CONCLUSIONS

This study reported that the effect of surfactant (Tween 80) and cosurfactant (Span 80), with variations of concentrations, significantly impacts the pH, particle size, and zeta potential of kojic acid nanoemulsion. The results showed that Tween 80 has the greatest significant impact on pH with a p-value < 0.05. Meanwhile, Span 80 has the most significant impact on particle size and zeta potential of kojic acid nanoemulsions with a p-value < 0.05. The optimum point of tween 80 and span 80 were found at the tween 80 amount of 10 grams and the span amount of 3 grams. While in the confirmation point, the optimum point is in tween 80 with the amount of 11.92 gram and span 80 with the amount of 3.76 gram. The particles of nanoemulsion have a smooth spherical shape. This finding demonstrates an efficacy of 94.117% in inhibiting tyrosinase activity.

ACKNOWLEDGEMENTS

This research was financially funded by

Program Penelitian Tesis Doktoral Universitas Sanata Dharma 2024 No: 019 PeneI./LPPM-USD/IIL/2024 awarded to Dr. apt. Rini Dwiastuti, M.Sc.

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