Extraction and Formulation of Quersetin Nanoemulation from Kenikir Leaves (Cosmos Caudatus Kunt) in the Phase of RBO Oil as Antioxidant

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ABSTRACT
This study was an effort to obtain high yield of quercetin extraction from kenikir leaves employing several extraction methods, which were maceration, percolation, soxhlet, and reflux, using methanol as solvent followed by quercetin incorporation into a Nanoemulsion system with Rice bran oil (RBO) as the oil phase. The resulting Nanoemulsion was tested with antioxidant activity analyses using DPPH (1,1-diphenyl-2-picrylhydrazil) reagent. The nanoemulsion preparation was produced from kenikir leaf powder at 20% concentration. The characterizations of formula consisted of organoleptic tests, analyses of pH, particle size, emulsion type, and determination of quercetin content absorbed in the nanoemulsion system, as well as antioxidant activity testing. The results showed that the highest quercetin yield of 14.26 ppm was produced from the soxhlet method for two hours. The Nanoemulsion preparation with 20% kenikir leaf extract and RBO oil phase was good and stable. The formulations also exhibited 6.3 pH, O/W emulsion type, and 176.8 nm particle size. The absorption of quercetin in the nanoemulsion showed an %EE value of 99.99%, indicating the formulation's high capacity to absorb quercetin. The antioxidant activity testing using DPPH assay produced an IC50 value of 106.805 ppm.

Keywords: *Kenikir (Cosmos Caudatus Kunth), Nanoemulsion, Quercetin, RBO (Rice Bran Oil), Antioxidant*.

1. INTRODUCTION

Kenikir (Cosmos Caudatus Kunth) is one of the many herbal plants found in Indonesia. In Malay this plant called suring or ulam raja. This plant has a unique aroma and is usually consumed directly as fresh vegetables [14]. The Chemical content in kenikir leaves includes flavonoids, polyphenols, terpenoids, saponins, steroids and essential oils, while the roots are known to contain coniferil alcohol and hydroxyugenol. Quercetin is one of the chemical ingredients including the flavonoid class which can have an ant oxidative, antifungal, antibacterial, anti-inflammatory, antitumor, anticancer, etc. [12]. Therefore, it would be to our benefits to be able to extract quercetin from natural sources, such as plants, based on these health potentials. From these extractions, quercetin can be further studied and applied.

Quercetin compounds found in plants have very low solubility in water, causing limitations in the adsorption process and affecting their bioavailability in the body [15]. Quercetin solubility can be increased through the formation of Nanoemulsions so that it can increase bioavailability in the body. Solubility of quercetin can be improved by incorporating it in a Nanoemulsion system. Oils that can dissolve active compounds are important ingredients in the formulation of Nanoemulsion, and one of these is rice bran oil (RBO). RBO is nutritious containing good fatty acids, biologically active compounds, and various antioxidants such as oryzanol, tocopherol, tocotrienol, phytosterol, polyphenol, and squalene [2].

The first objective of this study was to determine the optimal method(s) of quercetin extraction from kenikir leaves. A Nanoemulsion formulation has been previously conducted for kenikir leaf extract generated with ultrasonic extraction method and dissolved in virgin coconut oil (VCO) at the ratio of 1:20 (m/v) [17]. Due to limitations in this study, another extraction method was

chosen to obtain high quercetin levels by reducing the amount of solvent because the ratio of 1:20 was not economical. The extraction methods tested in this study included maceration, percolation, soxhlet, and reflux. The next objective of this study was to generate Nanoemulsion system using RBO instead of VCO, like in the previous study, and using 20% sample. Lastly, this study aimed to characterized the resulting Nanoemulsion system by performing organoleptic testing, pH monitoring, particle size analyses, and emulsion type determination. Antioxidant activity was also determined using DPPH (1,1-diphenyl-2-picrylhydrazil) reagent to verify the effects of kenikir leaf powder concentration on the quercetin content in the nanoemulsion formulation.

2. PROCEDURES

2.1. Materials and Equipment

The devices used comprised of oven, desiccator, blender, vacuum rotary evaporator, Erlenmeyer flasks 250 ml, funnels, vials, test tubes, stirring rods, digital scales, hotplates, drop pipettes, magnetic stirrer, volumetric flasks 100 ml, measuring cups, Whatman 1 paper, centrifuge, pH meter, UV-VIS Spectrophotometer, and particle size analyzer. The materials consisted of fresh kenikir leaves, 96% methanol, RBO, Tween 80, Span 80, Propylene glycol, oleic acid, phosphate buffer (pH 6), pure quercetin, 10% AlCl3, 4% NaOH, 5% NaNO2, pure DPPH, and distilled water.

2.2. Extraction Preparation and Measurement for Kenikir Leaf Powder

Fresh kenikir leaves were dried in an oven set at 50oC for five hours. The dried leaves were put in a blender to reduce the size and the resulting powder was sieved with 30 mesh. The water content of 10% was required for the simplicial to be appropriate for the study [4]. Gravimetric analyses were thusly conducted by drying five grams of powder sample in an oven set at 105oC, then cooled in a desiccator, and weighed until a constant mass was reached. The analyses were conducted three times to obtain an accurate value. The extraction of kenikir leaf powder was conducted using 96% methanol as the solvent in four separate methodologies, which were maceration, percolation, soxhlet, and reflux. The complete procedures are summarized in Figure 1. After obtaining the extract, it is tested Qualitative and Quantitative Analyses of Flavonoid (Quercetin)

2.2.1. Qualitative Analyses of Quercetin

To test the presence of flavonoids, one mL of kenikir leaf extract standard solution was added with four mL distilled water and 0.15 mL 5% NaNO2, then incubated for six minutes. The addition of 0.15 mL 10% AlCl3 and another six-minute incubation followed. Finally, two mL 4% NaOH and 15-minute incubation ended the process. The changing of color into light red or red signified the presence of flavonoids in the sample [18].

2.2.2. Quantitative Analyses of Quercetin

A quercetin standard solution with the concentration of 100 ppm was generated by dissolving 10 mg quercetin with methanol p.a. up to the 100 mL mark in a 100-mL volumetric flask. Working samples were produced by diluting the 100 µg/mL quercetin standard solution with methanol p.a. to obtain concentrations of 0.5, 1, 2, 5, 10, 15, and 20 mg/L. The absorbance of these samples were measured in UV-Vis Spectrophotometer set at the wavelength of 371 nm. A standard curve was calculated by plotting the quercetin concentrations (mg/L) against the absorbance. The resulting regression formula was used to determine the quercetin content in the extraction yields of kenikir leaf powder extracted with various methods.

To determine the quercetin content in the kenikir leaf powder extract, 10 mg filtrate was dissolved in methanol p.a. up to the 100 mL mark in a 100-mL volumetric flask. The absorbance of this solution was measured in UV-Vis Spectrophotometer set at the wavelength of 371 nm. The resulting values were used in the formula obtained in the previous step.

The analyses on the kenikir leaf extract in the absence of methanol were conducted using esterification method [13]. First, extract was dissolved in H2SO4 in a test tube, then added with CH3COOH. The tube was closed with cotton and heated to boil. The presence of ester odour on the cotton plug indicated the presence of methanol in the extract. Figure 1 shows the flowchart of Quercetin extraction from Kenikir leaf.

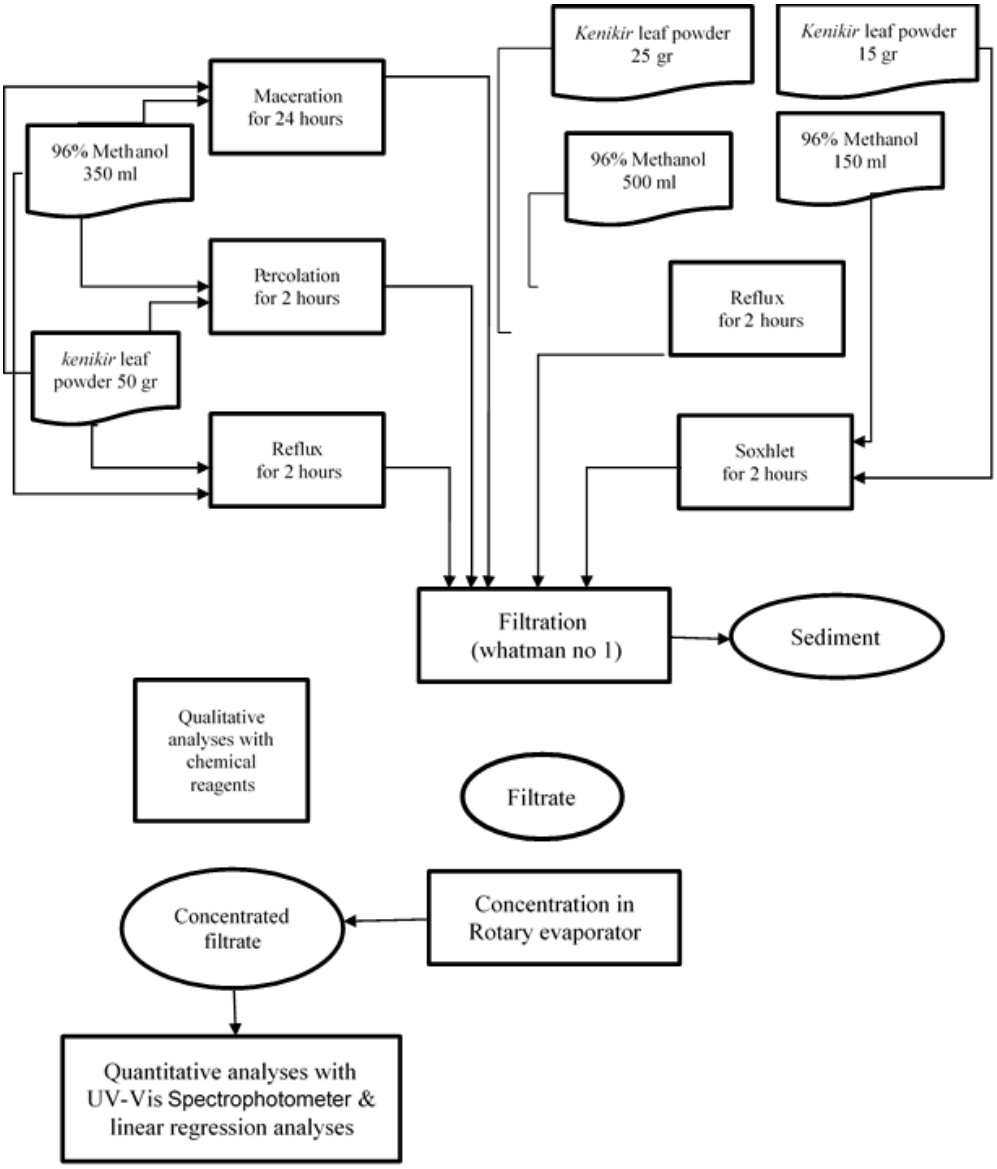

Figure 1 The flowchart of Quercetin extraction from Kenikir leaf

2.3. Nanoemulsion Formulation

Nanoemulsion system formulation was performed in 60-mL duplicates using the sample from the highest yielding extraction method. Span 80, RBO, and oleic were sequentially added to the *kenikir* leaf extract. The mixture was homogenized using magnetic stirrer at 1000 rpm for 30 minutes and at temperature of 37^0C to produce the oil phase. The water phase consisted of Tween 80, propylene glycol, and phosphate buffer (pH 6) homogenized at 50^oC and 1000 rpm. The oil phase was slowly added to the water phase as the mixture was homogenized. The composition of nanoemulsion formula consist 20% extract *kenikir*, Tween 80 and Span 80 as surfactant non-ionic each 46% and 4%, Oil Phase RBO 5%, Oleic Acid as enhancer 5%, Propylene glycol as Co-Surfactant 5% and Fit the volume up to 60 ml with a phosphate buffer.

2.4. Product Analysis Methods

Nanoemulsion product testing consists of ***Organoleptic Test,*** these test involved visual observations on the colors and forms as well as olfactory detections on the odor emitted by the preparations [7]**.,** ***pH measurement,*** these test were important to ensure that products would not induce adverse reaction on skin. The recommended pH values for skin products are in the range of 4.5-6.6 [4]. ***Emulsion type Identifications,*** these test According to [5] there are three types of emulsion based on the composition of Nanoemulsions, namely:

- Oil in water Nanoemulsion (O/W) wherein the oil droplets are dispersed in the water phase
- Water-in-oil Nanoemulsion (W/O) in which water droplets are dispersed in the oil phase

- Bi continuous Nanoemulsion in which micro domains of oil and water are dispersed in the system.

Meanwhile, there are several methods for testing the emulsion type of Nanoemulsion, namely dye solubility test and dilution test. Dye solubility tests use water-soluble dyes such as methylene blue or brilliant CFC blue which are dropped on the emulsion surface. If the dye dissolves and diffuses homogeneously in the external phase which is water, then the emulsion type is O/W. If the dye appears as droplets in the internal phase, then the emulsion type is W/O. Meanwhile, the dilution test was carried out by diluting the emulsion with water, if the emulsion was well mixed with water, the emulsion type was M / A, whereas if it was not well mixed with water, the emulsion type was A / M [9]. ***Particle Size Measurements,*** these test measurements of Nanoemulsion particle size were conducted using Particle Size Analyzer, from Malvern Analytical. This apparatus is capable of measuring particle sizes at nanoparticle, colloid, protein, zeta potential, and molecular mass ranging in 0.15 nm – 10 µm with sensitivity of 3-10.000 nm [8].

For active compound testing is carried out ***Determination of Quercetin Content Absorbed*** in ***the Nanoemulsion,*** one gram of Nanoemulsion containing *kenikir* leaf powder extract was dissolved into methanol to the final volume of 10 ml. The mixture was centrifuge at 2500 rpm for 45 minutes. The pellet should contain the entrapped quercetin, while the supernatant would contain quercetin that was not absorbed by the nanoemulsion. The amount of quercetin in supernatant was measured with UV-Vis Spectrophotometer. This test is also called entrapment efficiency, expressed in percentage (% EE), and calculated with the following formula:

$$\% \text{ EE} = \left[\frac{TD-FD}{TD} x\ 100\ \%\right] \quad (1)$$

Notes: EE= Percentage of Entrapment Efficiency

TD= amount of drug added in the system

FD= amount of drug not adsorbed

% The entrapment efficiency (% EE) is according to the standard and is said to be good with a yield between 80-100%. The greater the entrapment efficiency, the better the resulting nanoemulsion system [10].

Antioxidant testing was carried out using the DPPH method where DPPH assay is a conventional method to quantitatively determine the antioxidant activity of a chemical compound [16]. DPPH is a stable radical containing nitrogen with a strong absorbance at λmax of 517 nm and exhibits dark purple coloration in its pure form. DPPH undergoes reduction when it reacts with antioxidant compounds and changes its colour into yellow. Antioxidant activity is expressed as an IC50 value which can be derived from the regression analyses of % Inhibition against the concentration of samples.

2.4.1. Preparation of DPPH reagent

DPPH reagent was prepared by dissolving two mg of pure DPPH with methanol to the 100 mL mark of a 100-mL volumetric flask to obtain a final concentration of 20 ppm. The flask containing the working DPPH reagent was covered with a piece of aluminium foil and kept in a dark place.

2.4.2. Measurement of Antioxidant Activity of Nanoemulsion Formula

The resulting nanoemulsion was prepared in tubes at concentrations of 50, 90, 110,130, and 170 ppm. Two mL of 20 ppm DPPH reagent was added to each tube. Absorbance was measured in UV-Vis Spectrophotometer set at wavelength of 517 nm from zero minute and every following five minutes until there was no significant difference in the absorbance reading. It was determined that 30 minutes was sufficient time for absorbance reading for each sample.

Antioxidant activity of a sample is the percent inhibition of DPPH reagent radicals and calculated with the following formula [16]:

$$\% \text{ Inhibition} = \left[\frac{Blank\ Abs - Sample\ Abs}{Blank\ Abs} x\ 100\ \%\right] \quad (2)$$

Notes: Blank Abs = Absorbance at 0 minute of DPPH reagent addition

Sample Abs = Absorbance at 30 minutes after DPPH reagent addition

3. RESULT AND DISCUSSION

3.1. Qualitative and Quantitative Analyses of Quercetin Content in Kenikir Leaf Powder Extract

3.1.1. Qualitative Analyses for Flavonoid

There was a change of colours from yellow to light red. Based on this result, flavonoid compounds, such as quercetin, were detected in the sample [18].

3.1.2. Quantitative Analyses for Quercetin

The extraction processes were conducted with five different methods, which were maceration, percolation, soxhlet, and reflux with two different sample concentrations. The highest extraction yield was obtained from the soxhlet method with the value of 26.93% Among the extraction methods tested, 2-hour soxhlet procedure produced the highest yield of quercetin content at 14.26 ppm. A previous study also demonstrated that

soxhlet generated high extraction yield [6]. Based on these results, the production of nanoemulsion formulas in the subsequent steps utilized the extract of *Kenikir* leaf powder generated with the soxhlet method as presented at Table 2.

3.2. Production and Testing of Nanoemulsion Formulas

3.2.1. Organoleptic Testing

The organoleptic testing involved ten respondents and was conducted in Week 0 and Week 6. The preparations were stored at room temperature. The observations consisted of color, odor, and homogeneity. The results are presented in Table 3.

Based on Table 3, after storage until week 6, it can be observed that there is no change in the colour, taste and texture of the resulting formulation, this means that the resulting formula is stable. Meanwhile, the criteria for a good nanoemulsion are transparent and have a viscosity that is not too high so that the extract concentration of 20% with the RBO oil phase meets the criteria for good nanoemulsion where the active substance, the oil phase and the water phase have the right composition to produce a transparent and stable nanoemulsion formula visually [7].

Table 2. Concentration Quercetin in Various Method

No	Extraction Method	Extraction Time	Solvent Vol (ml)	Initial Weight (g)	Initial Weight (g)	Yield (%)	Absorbance	Concentration (ppm)
1	**Reflux I**	2 Hour	500	25	3.12	12.48	0.373	7.62
2	**Reflux II**	2 Hour	350	50	7.65	15.30	0.608	12.48
3	**Soxhlet**	2 Hour	175	15	4.04	**26.93**	**0.694**	**14.26**
4	**Percolation**	2 Hour	350	50	1.8	3.60	0.316	6.45
5	**Maceration**	1 Day	350	50	5.73	11.46	0.387	7.91

Table 3. Organoleptic Tests on Nanoemulsion Formulas in Week 0 and Week 6

No	*Test Component*	*Week 0 Observations*	*Week 6 Observations*
1	**Color**	Dark Yellow (Transparent)	Dark Yellow (Transparent)
2	**Odor**	Moderate Odor Tween 80	Moderate Odor Tween 80
3	**Homogeneity**	Homogeneous and Slightly Thick Solution	Homogeneous and Slightly Thick Solution

3.2.2. pH Measurement

After measuring with a pH meter, the pH formula is 6.3. Based on the results of this pH measurement, the nanoemulsion formula still meets the standards of a preparation to be formulated for skin with a skin pH range of 4.5 - 6.5 [4].

3.2.3. Emulsion Type Identification

After the dilution test was carried out, it was observed that the emulsion type of this nanoemulsion formula was the O/W emulsion type because after adding water, the emulsion and water were mixed well. In the nanoemulsion formula, a combination of surfactants tween 80 and span 80 is used, where Tween 80 is hydrophilic with a greater amount than span 80 which has lipophilic properties which results in the preparation tends to be O/W, besides the concentration of rice bran oil used in a higher amount. lower than phosphate buffer pH 6 and propylenglycol as a co surfactant which is polar will further strengthen it to form an Oil in Water (O/W) nanoemulsion type [9].

3.2.4. Particle Size Measurement

From the test results, the particle size is known to be 176.8 nm. The results obtained were that the nanoemulsion met the requirements of the nanoemulsion particle size requirements, namely 10-200 nm [8].

3.2.5. Determination of Quercetin Content Absorbed in the Nanoemulsion System

Table 4 indicating that the nanoemulsion system possessed high absorption efficiency for quercetin extracted from *Kenikir* leaf powder.

Table 4. The Quercetin Content Absorbed in the Nanoemulsion System

Oil	Sample	Initial concentration (ppm)	Finally concentration (ppm)	%ee
RBO	**I**	171,07	0,971	99.9943
	II	171,07	1.012	99.9941

The% EE of 99.99% means that the resulting nanoemulsion is good because the active compound can be maximally absorbed or trapped in the nanoemulsion system and in accordance with the expected standards for% EE [10].

3.2.6. Antioxidant Activity Testing on the Nanoemulsion System using DPPH method

From the results of sample measurements with some extract concentration (x) is obtained percent Inhibition (y) and its regression equation of y = 0.2479x + 23.523 with R^2 = 0.949. Figure 4 the shows that Curve for % Inhibition against the Concentration of Nanoemulsion Formulation.

Analyses of antioxidant activity showed that higher concentrations of the nanoemulsion containing quercetin from Kenikir leaf powder in RBO oil phase produced higher %Inhibition. This result corresponds with that of a previous study [1]. The IC50 of the nanoemulsion system generated in this study amounted to 106.805 ppm. This value falls into the category of moderate antioxidant activity, which is IC50 in the range of 101-250 ppm. Table 5 shows an Antioxidant Activity Tests on Nanoemulsion.

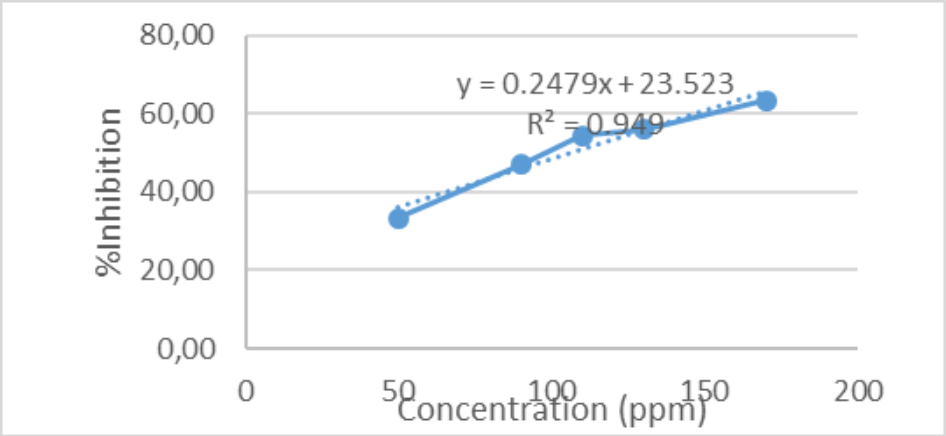

Figure 4 The Curve for % Inhibition against the Concentration of Nanoemulsion Formulation

Table 5. Antioxidant Activity Tests on Nanoemulsion

Concentration (ppm)	Abs at 0 minute	Abs at 30 minutes	%Inhibition	IC_{50}(ppm)
50	1.651	1.101	33.31	
90	1.679	0.891	46.93	
110	1.904	0.868	54.41	
130	1.612	0.71	55.96	106.805
170	1.665	0.61	63.36	

4. CONCLUSION

The conclusions from these results are soxhlet extraction method produced the highest concentration of quercetin extracted from *Kenikir* leaf powder at 14.26 ppm, with a sample to solvent ratio of 1:10 (m/v). Nanoemulsion system containing quercetin extracted from *Kenikir* leaf powder in RBO oil phase exhibited a moderate antioxidant activity with IC_{50} of 106.805.

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