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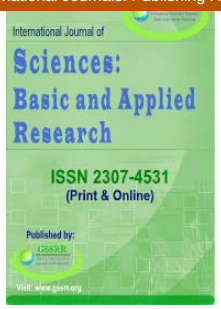

The Ethanol Extract Nanoemulsion Production of *Abelmoschus Manihot* L by the Combination of Homogenization and Solvent Displacement Technique

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Abstract

The ethanol extract of *Abelmoschus Manihot* L. has a compound of flavonoid glycosides having the potential as a source of antioxidants, but the bioavailability of these antioxidants tends to be low on form of large molecular size. The aim of this study was to obtain the process conditions, which is able to produce nano emulsion and has the best antioxidant characteristics, as well as to obtain the indicators of the stability of the nano emulsion. The method used was the combination of homogenization and evaporation techniques. The results showed that the best process conditions obtained in the homogenization speed of 20,000 rpm for 10 minute, with the particle size resulted was 100 ± 4 nm, while the conductivity and pH values were 259.55 ± 0.59 µS / cm and 6.73 ± 0.00.

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The particle size of nano emulsion in the temperature of 25 ± 2°C, for 14 days was not significantly different, however, the other parameters such as conductivity, pH, total flavonoid content and antioxidant activity tend not yet stable.

Keywords: Nanoemulsion; Abelmoschus manihot; Homogenizatio; Evaporation.

1. Introduction

The characterization of ethanol extractted from *Abelmocus Manihot* L has a flavonoid glycosides compound, which could be a potential source of antioxidants [1,2,3]. Furthermore, ethanol extract derived from the leaves of *Abelmocus Manihot* L has the highest antioxidant activity with IC_{50} values of 575 ppm [4]. Another study showed that the leaves of *Abelmocus Manihot* L extracted by steaming method had a total antioxidant content of 100 mg / 100 g of material, quercetin of 8 mg / 100 g material and beta-carotene of 28 mg / 100 g of material [5].

On the other hand, the antioxidant activity of flavonoids tend to be low on the condition which a large molecular size [6]. Therefore, to increase the antioxidant activity of *Abelmocus Manihot* L, modifications is needed to be done in the form of Nano emulsion. Nano emulsion is one of the optimal delivery system, because it can be formulated with all natural ingredients, and be able to improve its bioavailability [7].

Nano emulsions can be produced by two approaches, high and low energy method [8,9,10], by high-energy approach (high pressure homogenizer), it has a very low efficiency, which energy is earmarked to form nano emulsion only 0.1%, whereas 99.9% is diverted to increase the solution temperature [10]. Therefore, in this study, it was important to modify the method by combining high and low energy method called homogenization and solvent displacement. Nano emulsion parameters measured include particle size, pH, and conductivity. These parameters eventually reflect the stability of the emulsion during storage [11,12]. Furthermore, the aim of this study were to obtain the best process conditions based on the particle size and the antioxidant activity, and to determine nano emulsion stability to the particle size, conductivity, pH, total flavonoids and antioxidant activity of 14 days.

2. Material and Method

2.1.Material

Plant material collected from Cianjur West Java Province and identified by determination team from Purwodadi Botanical Garden Indonesian Institute of Science. Extraction of flavonoid from *Abelmocus Manihot* L leaves were extracted using maceration method with ethanol 96%. Ethanol extracts yielded total flavonoid following solvent removal under vacuum. Tween® 80, distilled water and ethanol 96% obtain from CV. Makmur Sejati Malang, East Java Province. Quercetin and 2,2-Diphenyl-1-picrylhydrazyl manufactured by sigma aldrich.

2.2. Method

Nano-emulsion Production

This research was carried out by combining the two approaches, namely high energy (homogenization) and low energy (solvent displacement method). The method used was based on the previous research which some modified [13]. Ethanol extract of *Abelmocus Manihot* L was dissolved by ethanol in the ratio between *Abelmocus Manihot* L extract and ethanol were 1:99, which will be called the oil phase. On the other hand, the water phase was dissolved Tween 80 as much as 6% and 94% distilled water. Both the oil and water phase was then mixed in the ratio between oil and water phase ratio of 1: 9. This solution was then homogenized by Ultra Turrax T25 and varied with a rotation speed between 5,000 rpm - 20,000 rpm and homogenizing time between 5 -10 minutes.

The selection of these two factors was based on previous research [13] states that the nano particle size and particle size stability was strongly influenced by the rotation speed homogenizer and homogenization time. After homogenization, the next step was solvent or ethanol removal in the low pressure. Furthermore, it was important to know nano emulsion properties, such as nano particle size, pH, and conductivity, levels of flavonoids in emulsions, nano size resilience and the antioxidant activity of nano emulsion generated.

Particle Size Distribution Analysis

Nano emulsion size distribution of the ethanol extract of *Abelmocus Manihot* L leaves was measured by gauges nano particles (Particle Size Distribution CILAS 1090). The Nano emulsion then was measured by particle distribution which formed (in units of nm). An amount of 2 ml solution of nano-emulsion was added into cuvette, then inserted into the tool and measured by particle distribution. Sampling was done on days of 0, 5, 9 and 4. The distribution of the particle size distribution was mapped in the form of a graph.

pH Value

pH measurement was conducted by taking a nano-emulsion sample, then tested its pH value by means of a pH meter (C861 multi-parameter analyzer Consort United Kingdom) in the temperature of 25 ± 2 ^{o}C. PH measurements are conducted periodically on the days of 0, 5, 9 and 14.

Conductivity Analysis

The conductivity analysis is used to determine the amount of ion contained in a liquid or solution. The electrical conductivity is measured directly by using a conductivity meter (C861 multi-parameter analyzer Consort United Kingdom) at a temperature of 25±2^{o}C. The conductivity measurement was done periodically on the day of 0, 5, 9 and 14.

The Total Flavonoids Determination of Abelmocus Manihot* L *Leaf Extract

The determination of the total flavonoid content was based on the method used previous research with slight modifications [14]. The extract solution of *Abelmocus Manihot* L, in amount of (0.5 mL, which contain

flavonoids, was mixed with 0.5 mL of $NaNO_2$ 5% (w/w) and allowed to stand for 6 minutes. The solution was then added AlCl3 0.5 mL of 10% (w / w), after 6 minutes of the result of the mixed solution was added 5 mL of NaOH 1 mol/L. After 15 minutes, the solution absorbance was measured using a spectrophotometer UV/VIS with a wavelength of 510 nm. The range of the calibration curve using the standard of 5.00 to 50.00 mg of quercetin was the function of y = 0,0125x -0.01613 (R = 0.9993) where y is the value of the absorbance and x were the value of quercetin (mg/g). Furthermore, the determination of total flavonoid value is based on a formula described as bellow [15]:

$$Flavonoid\ Total\ \left(\frac{mg}{g}\right) = \frac{Y \times N \times V}{W} \quad (1)$$

Y is the concentration of flavonoids example calculated using the standard curve equation (mg/g), N is the value of dilution, V is the volume extracted (mL) and w is the weight of leaf powder (g)

***Antioxidant Activity Test of Ethanol Extract Nano Emulsion Of Abelmocus Manihot* L**

The antioxidant activity of *Abelmocus manihot* L's leaf extract was determined using the method from previous research with slight modifications [16]. *Abelmocus manihot* L's leaf extract was produced in form of solution with varying concentrations, which ranged between 200-800 ppm with methanol solvent. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution to be used was prepared by dissolving DPPH in methanol with a concentration of 1mm. a number of 4.5 ml of the test solution or the comparison reacted with 500µl 1mM DPPH solution in a test tube. The blending solvent was stirred and incubated in the temperature of 37 °C under conditions of glasses for 30 minutes. Furthermore, the absorbance was then measured on a spectrophotometer with a wavelength of 517 nm. The antioxidant activity of each sample and quercetin expressed in percent inhibition are calculated by the formula:

$$Persentese\ Inhibisi\ (I\ \%) = \frac{(A-B)}{A} \times 100 \quad (2)$$

A is the absorbance of control (DPPH solution in ethanol) and B is the absorbance of the sample (DPPH solution in the extract solution and quercetin). The relationship between each concentration and free radical scavenging activity is plotted and IC_{50} values are calculated. The IC_{50} values declared the concentration of the sample solution (extract and quercetin) which is needed to reduce the free radical DPPH by 50% [17]

Experimental Design

This research was conducted by using a completely randomized factorial design and statistically evaluated using ANOVA $\alpha = 0.05$ with software MINITAB Ver. 17 Trial Version. There were two treatment variables used in the production process of nano emulsion, namely speed homogenization (A), and the time of homogenization (B). Meanwhile, homogenization speed was expressed in 5 levels (A1 = 5.000 rpm, A2: 10.000 rpm, A3: 15.000 rpm, and A4: 20.000 rpm) and the time of homogenization was expressed in two levels (B1: 5 minutes and B2: 10 minutes). Each combination treatment was repeated three times, and the mathematical model used was analysis using this formula [18]:

$$Y_{ij} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ijk} \quad (3)$$

where:

Y_{ij}	=	Response variable, due to effect of *i*th level of A factor, and *j*th level of B factor
μ	=	The effect of the real average
A_i	=	The effect of the *i*th level of the A factor
B_j	=	The effect of the *j*th level of the B factor
AB_{ij}	=	The effect of error factor A and B level I to j
I	=	Level total A = 6
J	=	Level total B = 2
ε_{ijk}	=	a random error component

3. Result and Discussion

3.1. The Particle Size of Ethanol Extract Nano Emulsion of Abelmocus Manihot L

The nano emulsion manufacture of *Abelmocus Manihot* L leaf extract, with two stages of homogenization and solvent displacement, had successfully formed a particle size in the range of 100 ± 4 nm to 385 ± 7 nm. The smallest particle size (100 ± 4 nm) was obtained on homogenization treatment by the speed of 20,000 nm for 10 minutes, whereas the most large particle size (385 ± 7 nm) was obtained in the homogenization process by the speed of 5,000 rpm for 5 minutes.

Generally, emulsions are homogenized by high speeds, will have a particle size ranging from 2,000 - 10,000 nm [19], while if using Ultra Turrax, homogenization process is only able to produce a particle size of 1μm [20, 21]. It indicated that the combination method, between homogenization and solvent displacement technique, was able to facilitate the diffusion of the oil phase into the aqueous phase to form nano emulsion.

Homogenization, carried out for 5 minutes, had a larger particle size compared to the size of the particle, which is homogenized for 10 minutes. On the other hand, the speed of homogenization influenced on the particle size reduction of nano emulsion manufacture of *Abelmocus Manihot* L leaf extract, It also occurs in the production of beta-carotene nano emulsion with high pressure homogenizer, the most dominant factors are the time and speed of homogenization [13].

Analysis of ANOVA with a P-Value parameter values, showed that the speed of homogenization, the time of homogenization and both interaction had P-Value < 0.05. These results indicated that the interaction between the time and speed of homogenization factor had significantly effect on the particle size. Furthermore, the results of a Tukey test (Table 1) showed that the best condition was obtained on condition of homogenization speed of 20,000 rpm, the homogenization time of 10 minutes, and the particle size resulted was 100 ± 4 nm. The particle size was smaller when compared with nano emulsion extracted from *Phyllantus Amarus* with a particle size of 213 nm [22].

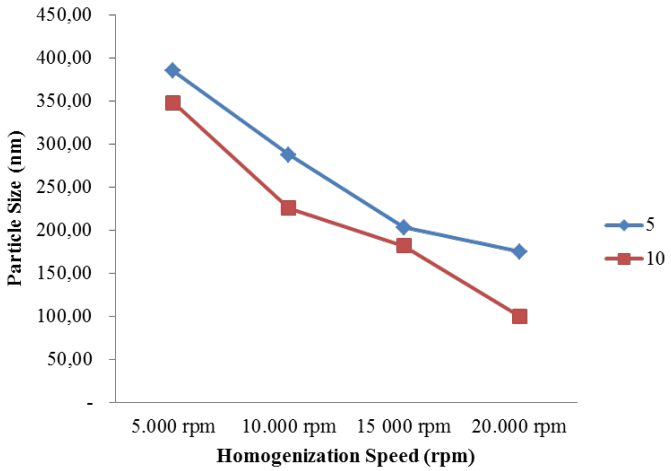

Figure 1: The average of particle size of ethanol extract nano emulsion of *Abelmocus manihot L*, on the time and speed combination of homogenization

3.2. Conductivity Value of Ethanol Extract Nano Emulsion of Abelmocus Manihot L

Before the process of homogenization and solvent displacement, *Abelmocus manihot L* leaf extract had conductivity value of 325 mS/cm, but after the homogenization and solvent transfer, the value is changed in the range of 259.55 ± 0.59 µS/cm to 265.32 ± 0.71. It indicated that the solution have physically changed the number of ions and total soluble solid in it. Furthermore, the change is due to the influence of particle size [23] and the surfactant tween 80 [24].

Analysis of variance indicated that the speed of homogenization had P-Value <0.05 and the time of homogenization and the interaction between these factors had P values > 0.05. It meant that the factor affecting the value of conductivity was the speed of homogenization. On the other hand, Tukey test (Table 1) showed that the best conditions, with the lowest conductivity value that was equal to 259.41 µS / cm, obtained in the process operated by homogenization speed of 20,000 rpm.

Table 1: Tukey Test against nano emulsion of ethanol extract nano emulsion of *Abelmocus manihot L*, at various speeds of homogenization

Homogenization Speed (rpm)	The Average of Conductivity (µS/cm)
5.000	264,838[a]
10.000	263,737[ab]
15.000	262,362[b]
20.000	259,417[c]

Note: The different superscript letters in the same column indicate significantly different values (P-Value <0.05)

3.3. pH Value of Ethanol Extract Nano Emulsion of Abelmocus Manihot L

The emulsion's pH value in food and drinks varies depending on the nature of the product [25]. pH value of nano emulsion extremely determine the interaction in environment, particularly the mechanisms used are Self nano emulsifying Drug Delivery Systems (SNEDDS) on the dosage of nano emulsion [26]. The results showed the pH value was from 6.67 ± 0.01 to 6.86 ± 0.01. The lowest the pH was obtained on the operating conditions of homogenization speed of 5,000 rpm for 10 minutes. On the other hand, the highest pH value was obtained condition of homogenization speed of 10,000 rpm for 5 minutes

Analysis of ANOVA indicated that the speed of homogenization, the time of homogenization time and interaction between these factors provide P-Value < 0.05, so that it could be concluded that the interaction between the speed and the time of homogenization significantly made significant effect. Furthermore, Tukey test (Table 2) indicated that the treatment was divided into six groups which was significantly different, namely a, b, c, cd, d and e. The group having pH smallest value was group E (5.000 rpm, 10 minutes) with a pH value of 6.67 and most of it was group "A" with a pH value of 6.86 (10,000 rpm, 5 minutes).

Table 2: Tukey Test on the pH value of of ethanol extract nano emulsion of *Abelmocus manihot L,* at various treatments

Homogenization Speed (rpm)	Homogenization's Time (menit)	pH
5.000	5	$6,77 \pm 0,01^{c}$
5.000	10	$6,67 \pm 0,01^{e}$
10.000	5	$6,86 \pm 0,01^{a}$
10.000	10	$6,77 \pm 0,01^{c}$
15.000	5	$6,83 \pm 0,01^{b}$
15.000	10	$6,78 \pm 0,00^{c}$
20.000	5	$6,75 \pm 0,01^{cd}$
20.000	10	$6,73 \pm 0,00^{d}$

Note: The different superscript letters in the same column indicate significantly different values (P-Value <0.05)

3.4. The Total Flavonoids and Antioxidant Activity Value of Ethanol Extract Nano Emulsion of Abelmocus Manihot L

The results of total flavonoids analysis showed that the increased speed and time of homogenizing had an impact on the total flavonoid content contained in solution (Figure 2a). ANOVA analysis showed that the interaction between speed and time of homogenization had significant effect on the total flavonoid levels of *Abelmocus Manihot L* extract nano emulsion. It was in accordance with production topical nanoemulsion from *Achyrocline satureioides* extract that the use of homogenization at high speed will cause temperature increasing of the solution and have an impact on the flavonoid damage contained in the solution [27].

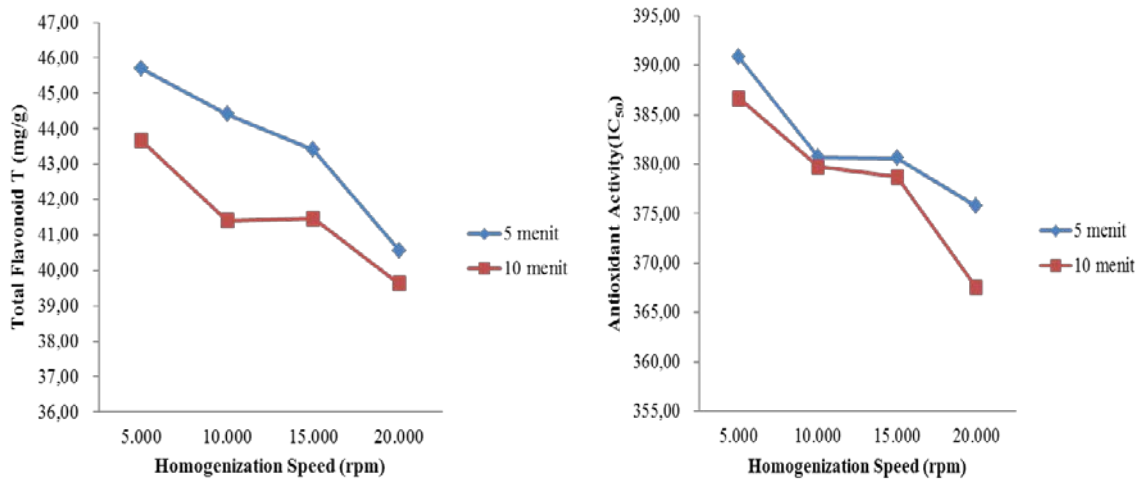

Figure 2: The average levels of total flavonoids (a) Antioxidant activity IC_{50} (b) ethanol extract nano emulsion of *Abelmocus Manihot* L leaf on the combination of speed and time homogenization

It was different from total flavonoid content; the antioxidant activity would increase accordance with the increasing of the speed and time of homogenization (Figure 2b). It was due to a smaller particle size (100.07 nm). The particle size of nano emulsion will affect the antioxidant activity which is consistent with the increase of the free radical DPPH solubility [7].

3.5. The Stability of Ethanol Extract Nano Emulsion of Abelmocus Manihot L

The observation of the results of 14 days of storage at room temperature (Figure 3) indicated that all treatments tend to increase the particle size. The largest increase occurred in treatments were stored for 14 days under the conditions of homogenization speed of 10,000 rpm for 5 minutes by size increase of 7.33 nm, while the lowest particle size was about 4.33 nm, resulted from the process conditions of homogenization speed of 20,000 rpm for 10 minutes. On the other hand, the analysis of variance, with the value of P-Value > 0.05, indicated that the storage time did not have no significant effect. Some studies about nano emulsion of also indicated that the increase of particle size but it is not significantly different [28, 29, 30].

The stability of the total flavonoids and the antioxidant activity is one of the most important components in the production of ethanol extract nano emulsion of *Abelmocus Manihot* L. The measurement of the total flavonoid levels (Table 3) showed the decrease of the total flavonoid. The analysis variance to the total flavonoid content during storage showed that storage time significantly influenced the decrease of the total flavonoids and the antioxidant activity, because the value of the P-Value < 0.005.

The average of the total flavonoids decreased value was 1.06 mg / g, whereas the greatest decline occurred in the process conditions by homogenization speed of 20,000 rpm for 10 minutes. On the other hand, the lowest decreased value of total flavonoids was occurred in the operating conditions of homogenization speed of 5,000 rpm for 10 minutes. This decrease was due to high temperature storage conditions (25 ± 1 ° C) and the influence of light. It also occurs in the production of catechin nano emulsion, where the decrease of total flavonoid levels

was caused by high storage temperatures and the light effect [28].

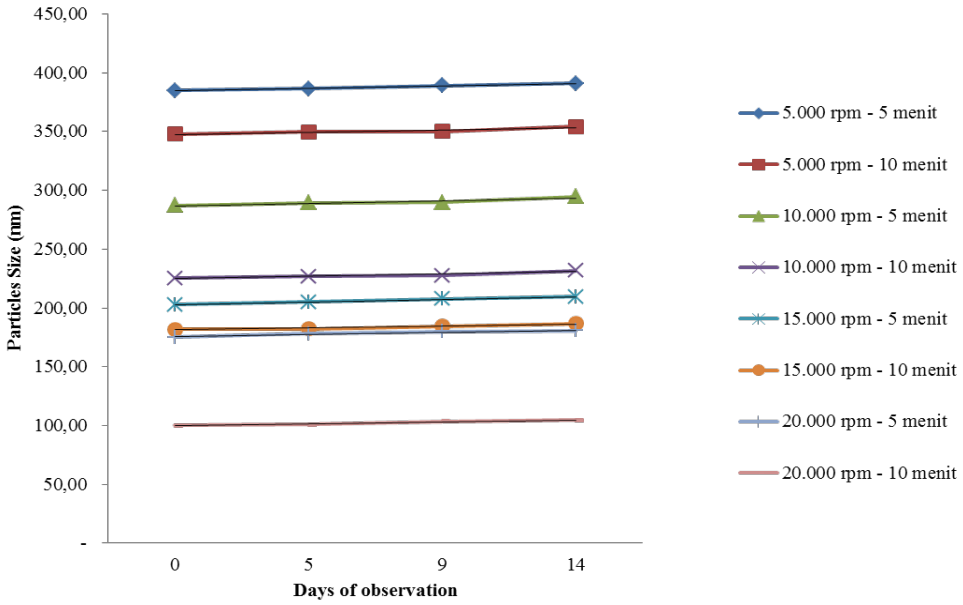

Figure 3: The particle size average value of ethanol extract nano emulsion of *Abelmocus Manihot* L leaf on the various treatment conditions for 14 days

Table 3: Comparison of the value of flavonoids and antioxidant activity during the storage process

Homogenization Speed (rpm)	Time of Homogenization's (minutes)	Total Flavonoids (mg/g)		Antioxidant Activity (IC_{50})	
		0 hari	14 hari	0 hari	14 hari
5.000	5	45,70 ± 0,40	44,38 ± 0,35	390,82 ± 0,40	392,68 ± 0,32
5.000	10	43,67 ± 0,31	43,20 ± 0,14	386,66 ± 0,41	399,79 ± 0,36
10.000	5	44,41 ± 0,18	43,20 ± 0,07	380,71 ± 0,28	383,52 ± 0,29
10.000	10	41,40 ± 0,12	40,60 ± 0,26	379,74 ± 0,35	380,92 ± 0,30
15.000	5	43,40 ± 0,13	42,52 ± 0,22	380,61 ± 0,45	382,71 ± 0,51
15.000	10	41,47 ± 0,17	40,20 ± 0,06	378,72 ± 0,41	380,45 ± 0,39
20.000	5	40,56 ± 0,27	39,47 ± 0,09	375,79 ± 0,40	378,71 ± 0,43
20.000	10	39,63 ± 0,10	38,23 ± 0,08	367,55 ± 0,36	370,66 ± 0,41

The antioxidant activity during storage also decreased (Table 3), although the magnitude of decrease in antioxidant activity is varied in each process conditions. The analysis of variance showed that the amount of time giving real effect to the IC_{50} value, which means that a significant decline due to the value of P-Value <0.05. The decrease of antioxidant activity was due to the total value decrease of flavonoids caused by environmental influences during storage.

4. Conclusion

The best result of ethanol extract nano emulsion of *Abelmocus Manihot* L was obtained under the conditions of homogenization speed of 20,000 rpm for 10, with the resulting particle size was 100 ± 4 nm, while the conductivity and pH value were 259.55 ± 0.59 and 5.73 ± 0, 00. On the other hand, the total flavonoids achieved in the best process conditions was smaller than the ethanol extract of *Abelmocus manihot L*, but it had a higher antioxidant activity (IC_{50} = 367.55 ± 0.36). Furthermore, the particle size stability of *Abelmocus manihot L* leaf extract nano emulsion was not significantly different for 14 days, but it was different for the other parameters such as conductivity, pH, total flavonoid content and antioxidant activity tends to be unstable.

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