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Antioxidant Activity Of Nanoparticle From Rosella (*Hibiscus sabdariffa* L) Calyx Extract Originated Indonesia And Thailand.

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ABSTRACT

Rosella (Hibiscus sabdariffa) contains flavonoids with antioxidant activity. The differences of geographical location affect secondary metabolites production of rosella calyx. Antioxidant activity determination by the thiobarbituric acid reactive substances (TBARS) assay has been widely used. Design of nanoparticles formulation is needed to improve the physical characteristic and increase the bioavailability. This study aimed to determine the antioxidant activity of nanoparticles of extracts rosella calyx from Thailand and Indonesia. Extraction on rosella were performed by maceration method using ethanol 60% and water. Extract were evaporated to get the concentrated extract and followed by freeze dryer. The preparation of nanoparticles used ionic gelation methode. The nanoparticles were tested in vitro by the thiobarbituric acid reactive substances (TBARS) assay. Results showed nanoparticles of ethanol extract rosella calyx from Thailand and Indonesia, water extract rosella calyx from Thailand and Indonesia 64.3 nm, 101 .7 nm, 102.4 nm, and 300.2 nm. Nanoparticles of four extracts have antioxidant ability as better than extract form. Nanoparticles of ethanol extract of roselle calyx from Thailand has the best antioxidant activity.

Keywords: antioxidants, *Hibiscus sabdariffa*, nanoparticles, rosella.

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INTRODUCTION

The ethanol extract rosella has efficacy as antihiperlipid [1], antidiabetic [2], antidepressants [3], and antioxidants [4,5]. The dominant compounds in rosella flowers include quercetin, sianidin, β-carotene, and vitamin C [5,6]. The content of these compounds is affected by the growing at conditions with intensity of sunlight, and nutrients in the soil. Indonesia has a region which located on the equator, so it has a different geographical area than Thailand. Such differences affect the content of the active compounds produced rosella [7]. It also affects the rosella flower extract antioxidant activity.

Compounds in the rosella flower extract that has antioxidant effects are quercetin, sianidin, β-carotene, and vitamin C has the ability absorption and bioavailability is low, and easily metabolized [8]. Drugs derived from nature can be developed into a form of nanoparticles to improve the physical properties and improves the bioavailability of the active ingredient [9].

Antioxidant was determined using the method of thiobarbituric Acid Reactive Substances (TBARS) [10]. Sheep red blood cells still contain lipid components and has similarities with human blood, so it is widely used in a study to test the antioxidant activity in vitro [11,12]. This study aimed to determine the antioxidant activity of nanoparticles of extracts rosella calyx from Thailand and Indonesia.

MATERIALS AND METHODS

Plant materials:

Plant materials used in this study is Rosella (Hibiscus sabdariffa). The rosella calyx were collected from Madiun (East Java, Indonesia) and Narathiwat (Southern Thailand).

Chemical materials:

Other ingredients used in the study was distilled (Ikapharmindo), aquabidest (Ikapharmindo), ethanol technical (Ikapharmindo), chitosan (Sigma-Aldrich), tripolifospat (Sigma-Aldrich), quercetin (Merck), aluminum chloride (Merck), acetic acid glasia (Merck), sodium acetate (Merck), sheep red blood cells (Faculty of Medicine UI), tertiary-butyl hydroksiperoxida (t-BHP) (Sigma-Aldrich), phosphate buffered saline (PBS) (Sigma-Aldrich), malondialdehida-bis-(dimetilasetal) (Merck), trichloroacetic acid (TCA) (Merck), tiobarbiturat acid (TBA) (Merck), dimethyl sulfoxide (DMSO) (Merck), ascorbic acid (vitamin C) (Merck).

Methods:

Extraction:

The extraction of dried rosella calyx were performed using maceration method with 60% ethanol and boiling method with water for one hour. After this process the ethanol extract and water extract was found. Each extract was evaporated to get the concentrated extract and followed by freeze dryer.

Total Flavonoids Contens:

50 mg quercetin dissolved in 1000 ml of ethanol. Taken then added 5 ml of 5% acetic acid (v / v) to 10 ml. Taken 1; 1.25; 1.5; 1.75; and 2 ml, and then inserted in each flask 10 ml, then each added 1 mL of 2% $AlCl_3$, next added 5% acetic acid (v / v) up to the mark. Concentrations were obtained respectively of 2.5; 3.125; 3.748; 4.378; and 5 µg / mL. Performed using a spectrophotometer absorbance reading at maximum wavelength. From these results made standard curve equation $y = bx + a$. Rosella extract 200 mg dissolved in 50 ml of 70% ethanol. Treated the same as a standard. Results absorbance incorporated into the standard curve equation, and calculated the total flavonoid content..

Preparation of Nanoparticles:

Nanoparticle formulations are based on research conducted Nurkhasanah et al (2015). Formula optimum nanoparticles on all extracts a comparison extract: chitosan: TPP of 2: 1: 0.1, the dissolution of

chitosan in acetate buffer pH 4.4. Result of preparation was characterized by particle size analyzer (PSA) DelsaTM Nano Submicron Particle Size Analyser (Beckman Coulter).

Antioxidant capability:

Testing methods based on research conducted Kusmiati (2012) were modified [13]. Sheep blood were centrifuged at 3000 rpm for 5 minutes. Blood cell layer is washed using a solution of Phosphate Buffer Saline (PBS) and then in a centrifuge at 3000 rpm at a temperature of -50 ° C for 5 minutes.

The study was carried out experimentally with a completely randomized design. Extract concentration of 3 mg / ml treatment given to treatment based on research that has been done Nisma et al (2008) using the rosella extract [14]. The sample group are shown in Table 1.

Table 1: The groups on determination of antioxidant extracts and nanoparticles rosella flower extract from Thailand and Indonesia

Group	Tretment
Normal control	1 ml SRBC
Negative control	1 ml SRBC + 1 ml t –BHP
Positive control	1 ml SRBC + 1 ml vitamin C Concentration 0,5 mg/ml + 1 ml t –BHP
Sample 1 (Ethanol Extract Thai)	1 ml SRBC + 1 ml extract rosella calyx Concentration 3 mg/ml (Ethanol Extract Thailand) + 1 ml t -BHP
Sample 2 (Ethanol Extract Indo)	1 ml SRBC + 1 ml extract rosella calyx Concentration 3 mg/ml (Ethanol Extract Indonesia) + 1 ml t -BHP
Sample 3 (Water Extract Thai)	1 ml SRBC + 1 ml extract rosella calyx Concentration 3 mg/ml (Water Extract Thailand) + 1 ml t -BHP
Sample 4 (Water Extract Indo)	1 ml SRBC + 1 ml extract rosella calyx Concentration 3 mg/ml (Water Extract Indonesia) + 1 ml t -BHP
Sample 5 (Concentration 1 Nano Ethanol Thai)	1 ml SRBC + 1 ml nanopaticle extract rosella calyx Concentration 3 mg/ml (Ethanol Extract Thailand) + 1 ml t -BHP
Sample 6 (Concentration 2 Nano Ethanol Thai)	1 ml SRBC + 1 ml nanopaticle extract rosella calyx Concentration 6 mg/ml (Ethanol Extract Thailand) + 1 ml t -BHP
Sample 7 (Concentration 1 Nano Ethanol Indo)	1 ml SRBC + 1 ml nanopaticle extract rosella calyx Concentration 3 mg/ml (Ethanol Extract Indonesia) + 1 ml t -BHP
Sample 8 (Concentration 2 Nano Ethanol Indo)	1 ml SRBC + 1 ml nanopaticle extract rosella calyx Concentration 6 mg/ml (Ethanol Extract Indonesia) + 1 ml t -BHP
Sample 9 (Concentration 1 Nano Water Thai)	1 ml SRBC + 1 ml nanopaticle extract rosella calyx Concentration 3 mg/ml (Water Extract Thailand) + 1 ml t –BHP
Sample 10 (Concentration 2 Nano Water Thai)	1 ml SRBC + 1 ml nanopaticle extract rosella calyx Concentration 6 mg/ml (Water Extract Thailand) + 1 ml t -BHP
Sample 11 (Concentration 1 Nano Water Indo)	1 ml SRBC + 1 ml nanopaticle extract rosella calyx Concentration 3 mg/ml (Water Extract Indonesia) + 1 ml t -BHP
Sample 12 (Concentration 2 Nano Water Indo)	1 ml SRBC + 1 ml nanopaticle extract rosella calyx Concentration 6 mg/ml (Water Extract Indonesia) + 1 ml t -BHP

50 mL malondialdehida bis (dimetilasetal) put in a 50 ml flask, then added to 50 ml of distilled water. Of the solution, taken 100 mL, then put in a 10 ml flask, then added distilled water to the mark. Taken respectively 50, 150, 250, 350, 450, 550, and 650 mL and then put in a 50 ml flask, then added distilled water to the mark. The levels of concentration series of consecutive .117; 0.351; 0.585; 0.818; 1,053; 1,286; and 1.520 mM.

A total of 250 mL of each series of levels inserted into a test tube, then added 1.25 ml of trichloroacetic acid (TCA) 10%, then divortex 10 seconds. Furthermore, on each of these tubes was added 0.5 ml tiobarbiturat acid (TBA) 0.67%, and divortex again for 10 seconds. The mixture was heated for 30 minutes in the water bath and cooled. All solutions series absorbance levels measured with a spectrophotometer at a wavelength of maximum. Each level of the standard solution malondialdehida bis (dimetilasetal) with absorbance calculated linear regression equation Y = a + bX.

1.0 ml of blood plasma put into a test tube, and then 1.0 ml of the test solution, and incubated at room temperature for 15 minutes. The mixture was added 1 ml solution of 10 mM t-BHP and incubated at room temperature for 15 minutes, then centrifuged for 5 minutes at a speed of 3000 rpm.

Supernatant samples taken 250 mL, then added 1.25 ml of TCA 10%, then divortex 10 seconds. Furthermore, on each of these tubes was added 0.5 ml TBA 0.67%, and divortex again for 10 seconds. The mixture was heated for 30 minutes in the water bath and cooled. The solution is measured by a spectrophotometer absorbance at the maximum wavelength. MDA levels were calculated using standard curve regression equation malondialdehida bis (dimethyl acetal).

Data Analysis

Total flavonoids were analyzed using linear regression. Antioxidant data were analyzed using the statistical analysis system SPSS.

RESULTS AND DISCUSSION

Results and Discussion

In this study using rosella flowers from Indonesia and Thailand. Indonesia conditions in the area of the equator will be different with Thailand. Where the plants grow will affect the content of secondary metabolites in rosella. Each rosella extracted using two different solvents namely ethanol and 60% water. Selection of ethanol 60% based on the high ability of the solvent in the quote of secondary metabolites in the plant. Aqueous solvent used for the empirical use of rosella flowers with boiling water use.

Extraction concentrated by evaporator. The yield of ethanol extract viscous Thailand rosella, rosella flowers ethanol Indonesia, Thailand rosella flower water, ethanol rosella Indonesia respectively for 32.73; 31.73; 33.47; and 34.27%. These results are in accordance with several studies that the yield of rosella flower extract ranged between 22-45% [15,16,17]. Concentration by evaporator aims to eliminate the time of extraction solvent used. Condensed extract is then shaped into a dry extract (powder) by using Freeze Dryer. Use of Freeze Dryer avoid damaging the active compound, rather than using the Spray Dryer. Extracts were made into a dry extract will improve the stability of secondary metabolites, compounds contained in extracts.

Determination of Total Flavonoid Content

Determination of total flavonoid using quercetin as standard. Created standard curve equation using a series of levels. The maximum wavelength of the scanning result is 412 nm. Equation standard curve obtained is Y = 0.1359 X - 0.1356 with a correlation coefficient (r) of 0.997. Furthermore, the sample was prepared and its absorbance is read, and then entered into the equation of standard curve. The results of the determination of total flavonoid levels are shown in Table 2.

Table 2: Determination of total flavonoid rosella calyx

No.	Sample	Total Flavonoid	Percen
1.	Ethanol Thailand	4,03 ± 0,14 µg/mg	0,40 %
2.	Ethanol Indonesia	3,51 ± 0,09 µg/mg	0,35 %
3.	Water Thailand	2,67 ± 0,07 µg/mg	0,26 %
4.	Water Indonesia	2,50 ± 0,08 µg/mg	0,25 %

Based on Table 2, the results of measurements showed the highest total flavonoid content of the ethanol extract of rosella flowers from Thailand amounted to 0.40%. Total flavonoid levels of the ethanol extract of rosella flowers from Indonesia is still lower (0.35%), this is likely due to the influence of the place grew. Rosella flowers from Thailand has a very red color than those originated from Indonesia. This causes the total flavonoid content is high on rosella flower extract originating from Thailand. In addition, rosella flowers from Indonesia bought from farmers already in the form of dried botanicals. The drying process is not suitable can cause the active ingredient is broken, so that the total flavonoid levels low.

In the study conducted Mun'im et al (2008), states that the total flavonoid content in rosella flower extract at 0.25% [18]. When compared with the results of these studies, the three extracts in this study had a total flavonoid content is higher. This difference grows influenced different places because rosella rosella flowers used in the study Mun'im et al (2008) came from Bogor [18]. Differences place to grow will affect the concentration of substances contained in rosella flower [7].

Total flavonoid content in the ethanol extract of rosella higher than the water extract. This is consistent with research Anokwuru et al (2011) which states that the total flavonoid content in rosella higher when used ethanol than water [4]. In another study states, ethanol has a good ability to attract flavonoids (19).

Preparation of Nanoparticles

Based on the test results by particle size analyzer (PSA), it is known that the average particle size of nanoparticles of chitosan extract ethanol from Thailand rosella, rosella flower extract ethanol from Indonesia, rosella flower extract water from Thailand, rosella flower extract water from Indonesia respectively of 64.3 nm, 101 , 7 nm, 102.4 nm, and 300.2 nm [20]. The smaller the particle size, the greater the surface area of the molecule, thereby increasing the absorption ability. Small particle size also increases the stability of the form of nanoparticles (21).

Antioxidant Activity Test

Sheep blood plasma used as widely used as in vitro studies, relatively easy to obtain, and has similarities with human blood (12,22). Plasma blood still contains lipid components, so it can be used as in vitro models to test the antioxidant activity. Blood can also be considered as a simulator of complex biological systems. Tertiary butyl hydroxy peroxide (t-BHP) is a material that is used as an organic peroxide which triggers the oxidation processes, in this study the addition of t-BHP cause lipid peroxidation in blood cells.

Tiobarbiturat acid is a reagent used in the analysis of MDA with thiobarbituric method Acid Reactive Substances (TBARS). TBARS method has the advantage that it is relatively easy to do, fast results obtained, and widely used (10). The method is based on Knoevenagel condensation reaction between acid 2-tiobarbiturat with MDA to produce chromogen. Knoevenagel condensation formation is a reaction that occurs between an aldehyde with the amine compounds (23). The reactions that occur in the TBARS method shown in Figure 1.

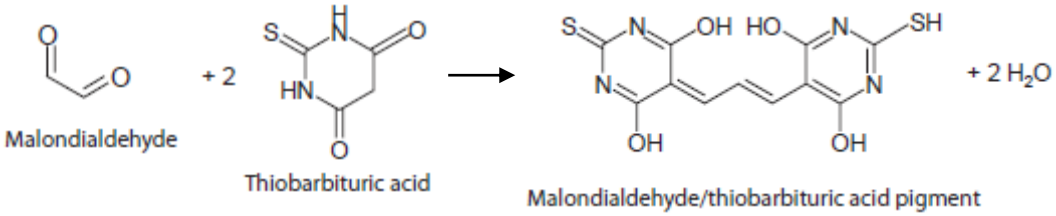

Figure 1: The reaction between the thiobarbituric acid with MDA (24)

The standard used is malondialdehida bis (dimethyl acetal). Created seven series of different levels (multiple point method), then performed with a spectrophotometer absorbance reading at maximum wavelength. The maximum wavelength of the scanning result is 532 nm. The results of standard curve shown in Figure 2.

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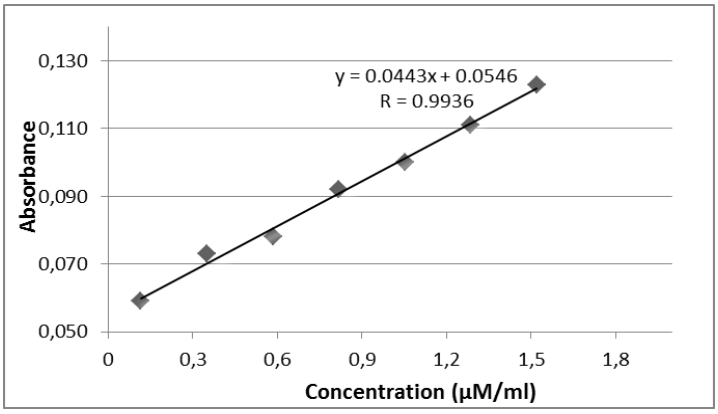

Figure 2: Standard cuve of MDA

The experimental procedure begins with the preparation of sheep red blood cells, then added a sample to be tested. In the process, the sample is a compound that will protect the red blood cells. Furthermore, added t-BHP trigger lipid peroxidation, resulting in malondialdehida (MDA). Increased levels of MDA will show high lipid peroxidation that occurs. Compounds that have antioxidant capabilities will be able to prevent damage to blood cells due to administration of t-BHP, so that MDA levels are low.

Experiments were performed in 15 groups with three replications. Antioxidant activity assay results presented in Figure 3.

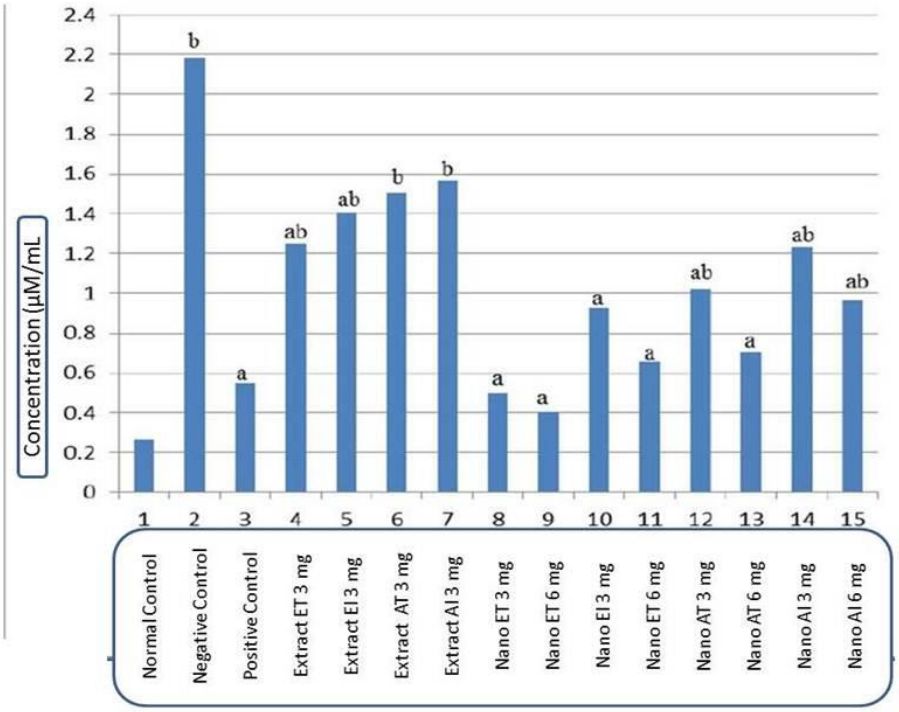

Figure 3. Antioxidant activity assay (ET= Ethanol Thailand, EI= Ethanol Indonesia, AT = Water Thailand, AI = Water Indonesia)

From Figure 3, extract or extracts nanoparticles have the ability to inhibit the formation of MDA. The results of this study demonstrate the antioxidant activity of the extract and nanoparticles. This is due to the inhibition of lipid peroxidation process, MDA formed low. Antioxidant ability of rosella flower extract in inhibiting lipid peroxidation process according to the research conducted Anokwuru et al (2011) (4). Other

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studies have also suggested that, rosella flower extract has the ability to neutralize free radicals (19). SPSS Analysis of the comparison between experimental groups are presented in Table 3.

Table 3: Comparison tests of significance between groups using SPSS with the analysis of non-parametric Mann Whitney

		Groups														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Groups	1		D	N	D	D	D	D	N	N	D	N	D	N	D	D
	2	D		D	D	D	N	N	D	D	D	D	D	D	D	D
	3	N	D		D	D	D	D	N	N	N	N	D	N	D	D
	4	D	D	D		N	N	D	D	D	N	D	N	D	N	D
	5	D	D	D	N		N	N	D	D	D	D	N	D	N	D
	6	D	N	D	N	N		N	D	D	D	D	D	D	N	D
	7	D	N	D	D	N	N		D	D	D	D	D	D	D	D
	8	N	D	N	D	D	D	D		N	N	N	N	N	D	N
	9	N	D	N	D	D	D	D	N		N	N	N	N	D	D
	10	D	D	N	N	D	D	D	N	N		N	N	N	N	N
	11	N	D	N	D	D	D	D	N	N	N		N	N	D	N
	12	D	D	D	N	N	D	D	N	N	N	N		N	N	N
	13	N	D	N	D	D	D	D	N	N	N	N	N		D	N
	14	D	D	D	N	N	N	D	D	D	N	D	N	D		N
	15	D	D	D	D	D	D	D	N	D	N	N	N	N	N	

SPSS analysis results in table 3, showed that normal controls significantly different compared to the negative control. t-BHP is a radical compounds cause increased levels of MDA significantly. This indicates that these data are valid. From table 3, it can be seen that significant differences between negative control positive control using vitamin C. This suggests that vitamin C is valid to be used as a positive control in the test.

Table 3 shows no significant difference between the negative control treatment using extracts and form nanoparticles, except the water extract rosella Thailand and Indonesia at a concentration of 3 mg / ml. This is due to the low content of total flavonoids contained in the extract water. According to Kumar et al (2012) (19), rosella extracted using a solvent water has the ability antioxidant activity lower than those extracted using ethanol. Low levels cause low total flavonoid antioxidant activity of the extract of roselle flower water.

When compared between groups extract, then the difference is meaningful only on the ethanol extract of Thailand rosella rosella flower with water extract of Indonesia. This shows the growing influence of the place and the solvent used to extract the antioxidant activity of rosella. These results are supported by research conducted Alfian and Susanti (2012) which states that a growing difference will affect the nutrients contained in the soil, so it will affect the antioxidant activity of rosella (7).

There are significant differences between the form of extracts with nanoparticles. This is consistent with research Pool et al (2012) which states nanoparticle form of quercetin has antioxidant abilities better than the free form of quercetin on in vitro assays (25). Nanoparticles have a better surface contact, thereby increasing the amount of drug to achieve the place of action (26). Form of nanoparticles also can maintain the stability of the active ingredient. The increase in affinity that causes the form of nanoparticles have the ability more powerful antioxidant activity.

When compared between groups nanoparticles on giving the same concentration, the nanoparticles are significant differences in the ethanol extract of Thailand with nanoparticles rosella rosella flower water extract of Indonesia. It is influenced by a growing interest rosella and solvent used during the extraction process. The results of research conducted in accordance with Ozdogan et al (2009) which states the place grew affect the antioxidant capacity of rosella (15). In addition, rosella extracted using ethanol has antioxidant abilities better than using water solvent (4).

In Table 3, there are no significant differences between the positive control group treated using nanoparticles rosella flower extract ethanol Thailand and Indonesia (all concentrations) and nanoparticles Thailand rosella extract water at a concentration of 6 mg / ml. This shows that the five treatment groups, have the same capabilities as a positive control, namely vitamin C concentration of 0.5 mg / ml. The fifth such treatment is a form of nanoparticles. This is according to research Kulkarni (2011) which states that an increase in the antioxidant effect of the compound quercetin were formulated in the form of nanoparticles (27). In fact, the nanoparticles of quercetin have the ability equivalent to vitamin C (28).

In Table 3 is known there is no significant difference between the normal control group with groups nanoparticles ethanol extract rosella Thailand at a concentration of 3 mg / ml and 6 mg / ml, nanoparticles extract rosella Indonesia concentration of 6 mg / ml, and nanoparticle aqueous extracts rosella Thailand concentration of 6 mg / ml. These results are supported by a study conducted Nisma et al (2008) which states that the form of extracts rosella can not restore the MDA corresponding normal conditions, while the extract formed nanoparticles have the ability of antioxidants better on tests in vitro (14,25).

CONCLUSIONS

Results showed nanoparticles of ethanol extract rosella calyx from Thailand and Indonesia, water extract rosella calyx from Thailand and Indonesia has nanoparticle size. Nanoparticles of four extracts have antioxidant ability as better than extract form. Nanoparticles of ethanol extract of rosella calyx from Thailand has the best antioxidant activity.

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