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Total phenol, flavonoid, and anthocyanin content and antioxidant activity of *Etlingera elatior* extract nanoparticle

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ABSTRACT

Despite the long and wide application, traditional medicine is known for its minimum efficacy. Nanoparticle technology has reported to optimally address this weakness by enhancing the ability of the medicine to penetrate the biological membrane and, thereby, increasing the absorption. In this research, *Etlingera elatior* (ginger flower) extract, which has antioxiandt activity, was formulated into nanoparticles with ionotropic gelation method using chitosan (0.08, 0.09, 0.1, 0.2, and 0.3%) and 0.01% NaTPP. The nanoparticles were characterized by their particle size, polydispersity index, zeta potential, and entrapment efficiency against total phenolic compound, flavonoid, and anthocyanin. The ones with the best properties were then analyzed with Scanning Electron Microscopic (SEM) method and tested for its antioxidant activity against DPPH. The results showed that all of the formula variations produced particle size in the range of 147.0-566.2 nm with a polydispersity index of < 0.5 and zeta potential between 0.45-45.90 mV. Also, the absorption efficiencies of phenol, flavonoid, and anthocyanin were 72.62-84.24%, 55.18-92.05%, and 75.67-97.96%, respectively. Overall, the best characteristics were presented by the combination of 0.1% chitosan and 0.01% NaTPP, which produced 246.4-nm nanoparticles with a polydispersity index of 0.418, and zeta potential of 26.60 mV. These nanoparticles also contained phenol, flavonoid, and anthocyanin with good absorption efficiencies, namely 78.5186%, 92.05%, and 97.96%, respectively. SEM analysis showed that these nanoparticles were round and had a soft surface. The radical scavenging activities of the extract and the nanoparticles against DPPH, as presented by the IC_{50} values, were 19.614 ppm and 160 ppm.

Keywords: ginger flower extract, nanoparticle, antioxidant

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INTRODUCTION

The high antioxiandt capacity of plants is estimated to significantly contribute to the healing activity produced by herbal dosage forms. Various studies found that plant-based food ingredients have a much higher antioxiandt capacity than their animal-based counterparts. Among of the reasons are the active antioxiandt compounds like flavonoids and polyphenols, tannins, phenolic and lignin acids, vitamin C, vitamin E, beta-carotene, and pigments like anthocyanin and chlorophyll in plants (Carlsen *et al*., 2010; Tilak and Devasagayam, 2006).

Herbal dosage forms are often considered less effective due to their weak and slow activity. To overcome this problem, the application of formulation technology for herbal dosage forms is necessary; one of which is the use of nano-sized extract. Nanoparticle dosage form can increase the absorption of medicine and, therefore, improve its bioavailability in the blood. Particle size reduction into nanoscale widens the surface area of contact and enhances the efficiency of particle internalization into the lipid bilayer (Won *et al*., 2008).

Etlingera elatior, or known as ginger flower, is one of many plants that have the potential to be developed as an herbal dosage form. The previous study has successfully optimized the extraction of polyphenol compounds from *E. elatior*. Polyphenol compounds have better solubility in polar rather than semi-polar solvents but exhibit no reaction in non-polar ones (Lestari and Ruswanto, 2015). In a different study, they are found to have higher solubility in methanol solvent (1.114 g GAE/100 g extract) compared to ethanol (0.788 g GAE/100 g extract) and acetic acid (0.812 g GAE/100 g extract) (Munawaroh *et al*., 2014). This finding is line with another study, which proves that the methanol extract of *E. elatior* exhibits the highest antioxiandt activity (IC_{50}= 19.919 μg/mL) than ethanol extract ($_{IC50}$= 36.810 μg/mL) and acetic acid (IC_{50}= 23.233 μg/mL) (Lestari *et al*., 2015). *E. elatior* contains components that, in fact, have a great capability to scavenge free radicals to prevent oxidation (Adliani, 2012).

This research aimed to provide herbal-based medicinal active ingredients, particularly of *E. elatior*, in nano size for the manufacture of herbal dosage forms. The use of nanoparticle was to increase the therapeutic effectiveness of this dosage form.

MATERIALS AND METHODS

Tools and Materials

The tools used in this research were reflux apparatus, rotary evaporator (IKA), UV-Vis spectrophotometer (Genesis 10), magnetic stirrer (IKA RW 20 Digital with heater), Beckman Coulter Delsa ™ Nano particle analyzer, and Scanning Electron Microscope (SEM). The research materials included ginger flower, n-hexane p (Bratachem), methanol p (Bratachem), methanol pa (Bratachem), thin layer chromatography plates (Merck), 1,1-diphenyl-2-picrylhydrazyl/DPPH (Sigma), glacial acetic acid (Merck), aqua deion, chitosan (Chimultiguna), sodium tripolyphosphate (Sigma), gallic acid (Sigma), Folin-Denis reagent (Sigma), and quercetin (Sigma).

Procedure of Experiment

Sample collection and test on the standard parameter of simplicia

The samples of the ginger flower were obtained from areas in Tasikmalaya and tested for quality according to the standards issued in Farmakope Herbal Indonesia 2011.

Extraction

The simplicia was extracted with multi-stage reflux. The first extraction used n-hexane solvent. After the color of this solvent disappeared, the simplicia was re-extracted with methanol until all compounds in the samples were extracted entirely. The resultant extracts were then concentrated, and their total phenol, total flavonoid, total anthocyanin, and antioxiandt activity were examined.

Nanoparticle production

One gram of *E. elatior* extract was dissolved in 35 mL of methanol p.a and mixed with 15 mL of distilled water. It was added with 350 mL of 0.01% NaTPP gradually while stirred using a magnetic stirrer at 350 rpm for 2 hours. The characterization of the nanoparticle suspensions was based on particle size, zeta potential, and absorption efficiency. Nanoparticles with the best characteristics were freeze-dried and tested for antioxiandt activity. Furthermore, their morphological nature was identified with SEM (Scanning Electron Microscope) (Firmawan, 2012).

Identification of total phenolic content

A sample of 0.5 g was weighed and dissolved in distilled water (the distilled water was added until 10 mL). A sample of 1.0 mL of this solution was transferred with a pipet and added with 7.5 mL of distilled water. Then, it was added with 0.5 mL of Folin-Denis reactant, left for 3 minutes, and combined with 1.0 mL of saturated Na_2CO_3 solution. After 15 minutes, the absorption was read at the maximum wavelength. The total phenolic content was calculated as gallic acid using the linear regression equation y = a + bx (Mukhriani *et al*., 2014).

Identification of total flavonoid content

A sample of 50 mg was dissolved in methanol p.a. in a 50 mL volumetric flask (methanol p.a. was added until the fill line). A sample of 1.0 mL of this solution was transferred with a pipet, mixed with 3 mL of methanol, 0.2 mL of 10% aluminum chloride solution, and 0.2 mL of 1 M sodium acetate solution, and then added with distilled water until 10 mL. It was incubated at room temperature for 30 minutes. Afterward, its absorption was measured at maximum λ. The total flavonoid content was calculated as quercetin using the linear regression equation y = a + bx based on the test results (Ahmad *et al*., 2015).

Identification of total anthocyanin content

The total anthocyanin content was identified using pH differential method. The dilution factor was formed by diluting the sample with KCl-HCl buffer (pH 1.0), to obtain an absorbance smaller than 1.2 times of the maximum wavelength of the extract. To measure the total anthocyanin content, two sample solutions were prepared. For the first sample, the extract was dissolved in KCl-HCl buffer (pH 1), while for the second sample, the extract was dissolved in sodium acetate buffer (pH 4.5). Each sample was dissolved in a buffer solution based on the predetermined dilution factor. Both sample solutions were incubated at room temperature for 15 minutes. The absorbance of each solution was measured at wavelengths of 510 nm and 700 nm (Giusti and Worlstad, 2001).

The final absorbance was calculated using the following equation.

$$A = (A_{510} - A_{700})pH1 - (A_{510} - A_{700})pH4.5$$

The Total Anthocyanin Content (TAC) was calculated using the following equation.

$$TAC\ (mg/L) = \frac{A \times MW \times DF \times 1{,}000}{\varepsilon \times I}$$

where:

A = final absorbance;
ε = molar absorptivity of cyanidin-3-glucoside = 26900 L/(mol.cm);
MW = molecular weight (449.2 for cyanidin-3-glucoside);
DF = dilution factor;
I = path length (1cm); and
1000 = conversion factor from gram to mg.

Measurement of absorption efficiency

A sample of 5 mL of the nanoparticle suspension of *E. elatior* extract was added with 5 mL of borate alkaline (pH 9.7). This mixture was centrifuged at 3,500 rpm for 30 minutes. The supernatant was sampled by 1 mL, added with the appropriate reagent, and treated similarly for each compound analysis. The absorbance of the solution was measured at the proper wavelength, according to each compound. The results were then calculated as a free compound level (Iswananda, 2013).

$$Absorption\ efficiency\ (\%) = \frac{Level\ of\ compound\ in\ the\ extract - Level\ of\ free\ compound}{Level\ of\ compound\ in\ the\ extract} \times 100\%$$

Measurement of antioxiandt activity

A sample of 50 mg was weighed and dissolved in 100 mL of methanol p.a. with a concentration of 500 ppm. Six series of concentrations were created from the primary standard solution. Each of them was sampled by 1 mL with a pipet and added with 1 mL of 50 ppm DPPH. These mixtures were left for 30 minutes. Their absorbance was measured at the maximum wavelength of DPPH solution (Lestari *et al*., 2015). The antioxiandt activity of the sample was determined by calculating the %inhibition of DPPH absorbance using the following formula.

$$\%Inhibition = \frac{DPPH\ absorbance - Sample\ absorbance}{DPPH\ absorbance} \times 100\%$$

where:

DPPH Absorbance = The absorbance of 0.2 M DPPH solution

Sample Absorbance = The absorbance of the sample solution or comparative solution

Afterward, IC_{50} was calculated based on the concentration and the percentage inhibition using the linear regression equation y = a + bx.

RESULTS AND DISCUSSION

The characteristics of the standard parameter of the simplicia

The purpose of sample characterization is to obtain simplicia or herbal medicinal ingredients that meet the standards issued in applicable regulations. The test results showed that the samples meet the conditions described in Farmakope Herbal Indonesia 2011 (Table I).

Table I. The characteristics of the standard parameter of the simplicia

Parameters	Replication (%)			Mean (%)	The Standards in Farmakope Herbal 2011 (%)
	I	II	III		
Water content	5.00	5.00	5.00	5.00±0.0000	≤ 10
Total ash content	8.98	8.97	8.96	8.97±0.0100	≤ 10.6
Acid-insoluble ash content	1.54	1.38	1.29	1.40±0.1365	≤ 4.7
Loss on drying	7.72	7.75	7.78	7.75±0.0076	≤ 10
Water-soluble extract content	22.20	22.60	22.50	22.43±0.1266	≥ 11.6
Ethanol-soluble extract content	22.98	23.20	22.95	23.04±0.0300	≥ 16.5

In addition to sample characterization, macroscopic and microscopic observations were performed to identify the unique morphology and physiology of the simplicia. Regarding the morphology, ginger

flower has a shape like a spinning top inside a bouquet, a long peduncle (0.5-2.5 m x 1.5-2.5 cm), oval bracts, and crimson-pink to bright red color. The corolla and calyx are pink, ovoid-shaped, and 4 cm long with white or yellow edges. Ginger flower has a distinctive odor and slightly sour flavor.

During the microscopic observation, the fragments of the ginger flower, as seen in Figure 1, were identified.

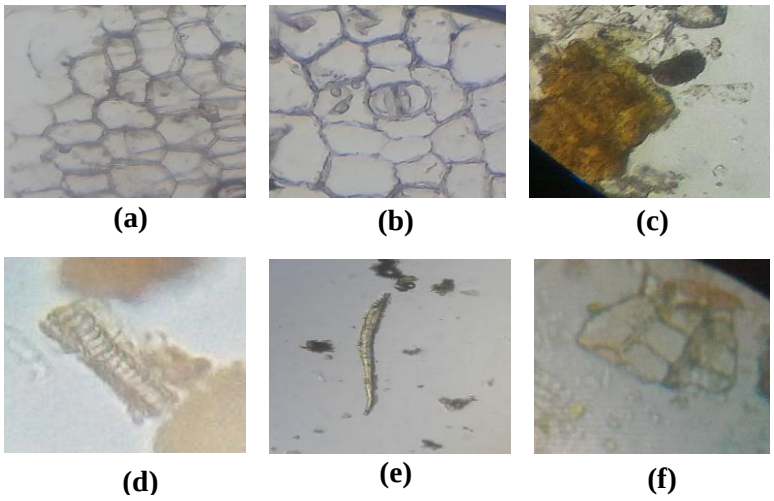


Figure 1. The results of microscopic observation: (a) Upper epidermis, (b) Stomata, (c) Oil glands, (d) Vascular tissue (transport), (e) Fragment of indumentum, (f) Epidermis of perianthium

The total phenol, flavonoid, and anthocyanin contents of the extract

There is a linear relationship between the antioxiandt activity and the phenolic content in *E. elatior* extract (Lestari *et al*., 2015; Munawaroh *et al*., 2014). Phenol, flavonoid, and anthocyanin are empirically proven to exhibit antioxiandt effects due to their ability to reduce free radicals. A higher phenolic content increases the antioxiandt activity produced by an extract (Ukieyanna, 2012).

The calculation results of the total phenol, flavonoid, and anthocyanin contents in the methanol extract of *E. elatior* are summarized in Table II.

Table II. The total phenol, flavonoid, and anthocyanin contents of *Etlingera elatior* extract

Compounds	Replications			Mean
	I	II	III	
Phenol	7.26%	7.23%	7.29%	7.26±0.0300 %
Flavonoid	8.20%	8.23%	8.27%	8.23±0.0351 %
Anthocyanin	65.30 mg/L	65.27 mg/L	65.29 mg/L	65.29±0.0153 mg/L

The production and characterization of *Etlingera elatior* nanoparticle

The nanoparticles were prepared with ionotropic gelation method using chitosan and NaTPP. This method relies on the ability of polyelectrolytes to produce a crosslink with negatively charged ions and to finally create a hydrogel. The binding of these anions with the cations of the polyelectrolyte solution forms a network that induces gelation. Hydrogels, a three-dimensional network, are formed by dripping NaTPP (anion solution) into the chitosan-based polyelectrolyte solution and the extract (Patil *et al*., 2010).

Chitosan was made in different concentrations, i.e., 0.08, 0.09, 0.1, 0.2, and 0.3%, while the concentration of NaTPP used in this research was 0.01%. In this method, the chitosan and NaTPP solutions form ionic crosslinks to trap the extract as an active ingredient (Mohanraj & Chen, 2006). Chitosan is a positively charged amino group, whereas NaTPP is negatively charged; all of which enable the occurrence of crosslinking (Chattopadhyay and Inamdar, 2010). The resultant nanoparticle suspensions were characterized by particle size, zeta potential, polydispersity index, and absorption efficiency (Table III).

Table III. The characteristics of the nanoparticle of *Etlingera elatior* extract

Chitosan (%)	NaTPP (%)	Particle Size (nm)	Zeta Potential (mV)	Polydispersity Index (pdI)	Absorption Efficiency (%)		
					Phenol	Flavonoid	Anthocyanin
0.08	0.01	147.3	3.08	0.459	79.89	64.72	75.67
0.09	0.01	231.8	15.70	0.391	84.24	55.18	80.33
0.10	**0.01**	**246.4**	**26.60**	**0.418**	**78.52**	**92.05**	**97.96**
0.20	0.01	483.4	45.90	0.491	75.46	83.39	93.85
0.30	0.01	566.2	0.457	0.470	72.62	80.58	92.83

The polymer absorptions of phenol, flavonoid, and anthocyanin were reasonably good with efficiency levels of 72.62-84.24%, 55.18-92.05%, and 75.67-97.96%, respectively. The characterization results showed that all formulations produced particles in the range of nanoscale (< 1,000 nm) and with a low polydispersity index (<0.5). Low polydispersity index represents a stable dispersion system for a long period (Gao *et al*., 2008).

As a system containing dispersed particles, the stability of nanoparticles is represented by zeta potential. It refers to the degree of the repulsive force generated by adjacent, similarly charged particles in the dispersion. If the zeta potential is greater than +30 mV or less than -30 mV, the system is deemed electrostatically stable. However, if it is greater than +20 mV or less than -20 mV, the system is sterically stable (Gao *et al*., 2008).

Based on the results of zeta potential analysis, the best stability was produced by formulas composed of 0.2% chitosan (45.9 mV). However, in general, nanoparticles with 0.1% chitosan had better characteristics because they produced smaller particle size (246.4 nm) and better absorption efficiency of the three compounds (phenol= 78.5186%, flavonoid= 92.05%, and anthocyanin= 97.96%). These nanoparticles also had a fairly good polydispersity index, i.e., 0.418 (<0.5), with a zeta potential of 26.60 mV. These parameters were the underlying reasons for the use of nanoparticles with 0.1% chitosan in further analysis. The SEM analysis results showed that these nanoparticles had round but nonuniform morphology with a smooth surface.

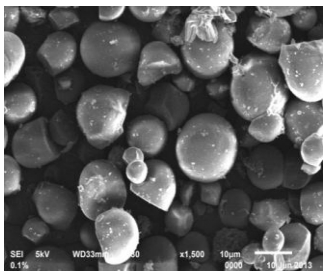


Figure 2. SEM analysis results of nanoparticles with 0.1% chitosan

The antioxidant activity of *E. elatior* extract and nanoparticles

The antioxiandt activity was examined with thin layer chromatography (TLC) and UV-Vis spectrophotometry. When the extract was sprayed with DPPH reactant, the color of the spots changed to yellow with a purple plate on the background, indicating antioxiandt activity. DPPH reacted by scavenging one hydrogen atom from the antioxiandt compounds to form an electron pair, turning the DPPH free radicals into a stable DPPH-H (Windono *et al*., 2004).

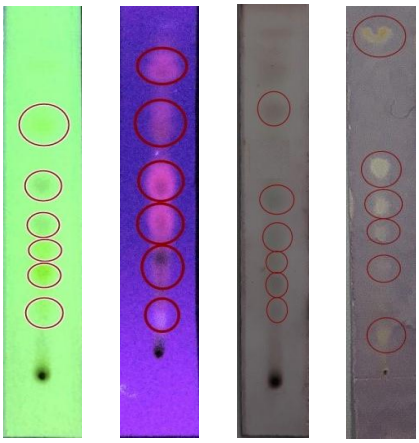
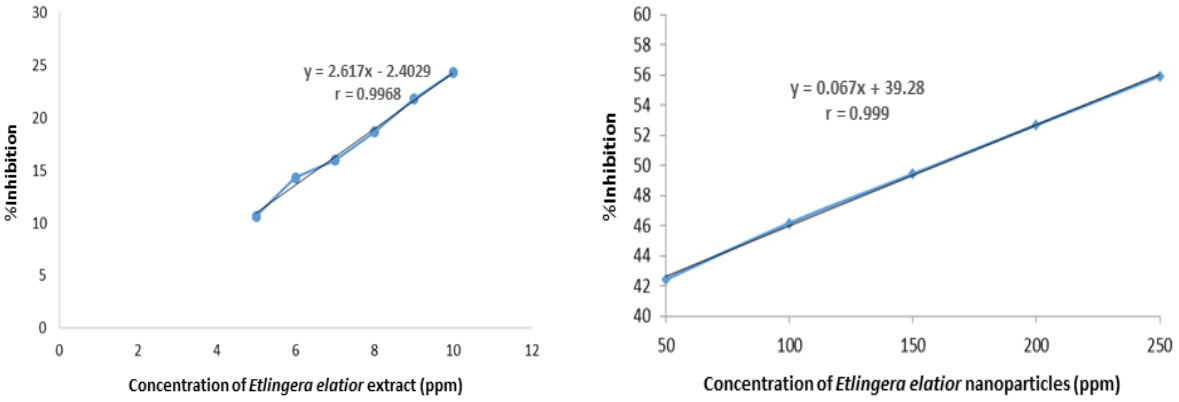


Figure 3. The thin layer chromatography of *Etlingera elatior* extract with the stationary phase "silica gel GF 254" and the mobile phase "ethyl acetate:n-hexane (7:3)": (a) observed under UV_{254} light, (b) seen under UV_{366} light, (c) sprayed with H_2SO_4 (visualization reagent) (d) sprayed with 1% DPPH

Afterward, the *E. elatior* extract and nanoparticle with 0.1% chitosan were tested for their antioxiandt activities with UV-Vis spectrophotometry. The test results showed that the antioxiandt activity of the extract decreased after the size was reduced to nanoparticles. Such decrease occurred because the amount of the extract in the nanoparticle production was considerably small (0.2%).

In this research, antioxiandt activity was defined as the capacity of a sample to inhibit 50% DPPH as free radicals (IC_{50}). The analysis showed that the IC_{50} of the extract was 19.614 ppm, while the IC_{50} of the nanoparticles was 160 ppm. Compared to the findings of other studies, these figures show better activity. For instance, the methanol extract of *Etlingera elatior* obtained with maceration method was empirically found to exhibit an antioxiandt activity of 101.84 ppm. In a different research, the dosage form produced by the nanoencapsulation of *E. elatior* contained a total phenol of 289.86 mg/100 g, and its antioxiandt activity was 32.165% (Naufalin *et al*., 2011). Another antioxiandt activity analysis was performed on the liquid *E. elatior* extract, which was able to inhibit free radical with IC_{50} of 61.6497 ppm (Hudaya, 2010).

The different results of the studies above are caused by different extraction methods and sampling locations. In this research, the samples were obtained from Taraju District, Tasikmalaya Regency, West Java, while the methanol extract was obtained from multi-stage reflux with n-hexane and methanol solvent. This method is preferable because *Etlingera elatior* contains high fat and oil that can interfere with the extraction of active antioxiandt compounds, which are polar in nature, namely phenol, flavonoid, and anthocyanin. Using n-hexane solvent, non-polar compounds like fat and oil can be removed first so that the polar and active antioxiandt compounds can be extracted optimally with methanol solvent. This process was assumed to increase the antioxiandt activity of the resultant nanoparticles.



(a) **(b)**

Figure 4. The regression curve of the antioxiandt activities of (a) *Etlingera elatior* extract and (b) nanoparticles

CONCLUSION

The simplicia used in this research has met some of the standards specified in Farmakobe Herbal Indonesia 2011. The total phenol, flavonoid, and anthocyanin contents of the methanol extract of *E. elatior* were 7.26 ± 0.0300%, 8.23 ± 0.0351%, and 65.29 ± 0. 0153 mg/L, respectively. Overall, the nanoparticles of the methanol extract of *E. elatior*, composed of chitosan and NaTPP, had a particle size in the range of 147.0-566.2 nm with a polydispersity index of <0.5. Their zeta potential varied between 0.45 mV and 45.90 mV, while the absorption efficiencies of the phenol, flavonoid, and anthocyanin contents were 72.62-84.24%, 55.18-92.05%, and 75.67-97.96%, respectively. The best characteristics were presented by the combination of 0.1% chitosan and 0.01% NaTPP. This combination produced 246.4-nm nanoparticles with a polydispersity index of 0.418, zeta potential of 26.60 mV, and absorption efficiencies of 78.5186% (phenol), 92.05% (flavonoid), and 97.96% (anthocyanin). The antioxiandt activity of the nanoparticles (IC_{50}= 160 ppm) was lower than the extract (IC_{50} = 19.614 ppm).

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