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The Potency of *Alpinia galanga* as Natural Antioxidant

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ABSTRACT

An antioxidant is a chemical compound that can bind free radicals in the body. Reactive oxidative species (ROS) is a reaction that involves oxygen. ROS consists of free radicals and non-radical ones. The imbalance between ROS and antioxidants can cause oxidative stress, one of the factors contributing to the development of numerous diseases. This study aims to evaluate the possible antioxidant activity of lengkuas extract (LE), which may be employed as a medicine component to reduce ROS. The method that used in this research were total phenolic content, total flavonoid content, 2,2 diphenyl 1 picrylhydrazyl (DPPH) scavenging, hydrogen peroxide (H2O2), NO scavenging, 2,2′-Azinobis(3-Ethylbenzthiazoline-6-Sulfonate) (ABTS), and ferric reducing antioxidant power (FRAP). The result shows that the TPC of LE was 6.80 ± 0.34 (GAE) µg/mg with gallic acid standard and the TFC was 3.39 ± 0.06 µg/mg by quercetin standard. The IC_{50} value of LE were 121.20; 87.65; 139.94; 181.09 µg/ml by ABTS, DPPH, NO, and H_2O_2 assay respectively. The scavenging activity of LE was increased with a higher concentration in every method. In conclusion, *Alpinia galanga* has the potential as an antioxidant. Thus, it can be widely consumed or used as a mixture in medicine to reduce ROS.

Keywords: *Alpinia galanga*; Antioxidant; Free radicals; Reactive Oxidative Species

INTRODUCTION

A chemical known as an antioxidant can bind free radicals in the body. Atoms or molecules with unpaired electrons are known as free radicals. Thus, it is radical which means unstable and very reactive to react with another molecule (Lobo et al., 2010). Free radicals in the human body were obtained from endogenous and exogenous sources (Phaniendra, Jestadi, and Periyasamy, 2015). Free radicals in the body react with bind another molecule. Thus, the molecules become radical themselves and bind to another molecule. This chain reaction caused the cell didn't work properly. In enzyme-substrate binding, binding the wrong substrate can affect the function of the enzyme and leads to another disease.

Reactive oxidative species (ROS) is a reaction that involves oxygen. ROS of consists free radical and non-radical. The imbalance between ROS and antioxidants can cause stress oxidative which is one of the causes of various diseases. Previous research has demonstrated that oxidative stress contributes to the development of several diseases, such as cancer, diabetes, heart diseases, inflammation, atherosclerosis, high blood pressure, and neurological disorders. (Hajhashemi et al., 2010).

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Consumption of antioxidants is one of the ways to prevent various diseases. Antioxidants are classified into primary and secondary antioxidants, based on their mechanism (Hermund, 2018). An antioxidant that directly reacts with free radicals is called the primary antioxidant. It is also known as a chain-breaking antioxidant. By directly reacting with free radicals, the antioxidant converts them into stable molecules and becomes a non-radical compound. Thus, it is important in the lipid oxidation process to generate lipid radicals and preventing in lipid disintegration. Secondary antioxidants, also known as preventive antioxidants, act in a different way to reduce lipid oxidation. The secondary antioxidants used processes including singlet-oxygen quenching (in photooxidation), transition metal chelation, and oxygen scavenging to stabilize the free radical molecules instead of directly reacting with them (Decker, 2002).

Based on the formation and origin of antioxidants in the body, there are two types of antioxidants, endogenous and exogenous antioxidants. Endogenous antioxidants are antioxidants found both in intracellular and extracellular. Exogenous antioxidants are those that are added from the outside. Antioxidants are frequently added to food products to prevent oxidative damage (Widowati, 2011). However, synthetic antioxidants such as BHA (Butylated

hydroxy anisole), TBHQ (ter-butyl hydroquinone), PG (Propyl gallate), and BHT (butylated hydroxytoluene) give some negative effects including skin allergies, gastrointestinal tract, and even cancer (Wang and Kannan, 2019; Lourenço, Moldão-Martins, and Alves, 2019).

Gingers, which belong to the Zingiberaceae family, are perennial herbs with aromatic rhizomes. Ginger plants are a popular spice, condiment, and traditional medicine. Ginger rhizomes are used to flavor meals and are consumed raw or cooked as vegetables. Species that are widely cultivated are *Alpinia galanga* (lengkuas), Curcuma longa, and Etlingera elatior. *Alpinia galanga* rhizomes are used as a spice for meat dishes (Chan et al., 2011). In Indonesia, the consumption of lengkuas is still rare. In contrast to other rhizome groups that can be consumed directly in the form of food or drink, lengkuas is only used as a spice in cooking. Lengkuas is rich in polyphenols thus it can act as antioxidants in the body. Polyphenols are sources of antioxidants that function as natural scavengers for toxic substances in the body. Thus, a lower incidence of numerous liver diseases, especially hepatocellular carcinoma in humans, has been connected to their consumption (Turati et al., 2014).

This study aims to examine the possible antioxidant activity of lengkuas extract (LE), which may be employed as a medicine component to reduce ROS. This study employed standardized extracts that adhered to the NA-DFC (Herbal Good Manufacturing Practices of the Republic of Indonesia's National Agency of Drug and Food Control). This study was conducted to develop a novel Obat Herbal Terstandar (Herbal Drug Standardization), or known as OHT for hepatoprotective properties based on the potential of the extract as an antioxidant.

METHODOLOGY

Preparation of The Samples

Lengkuas extract (LE) was produced by The Indonesian Food and Drug Authority standards. The LE was obtained from PT. FAST (Fathonah Shidiq Tabligh Amanah) with CoA no. Batch 00103211074. The LE was extracted from the rhizome of *lengkuas* with ethanol 70% and the additional substance lactose.

Total Phenolic Content

The solution of gallic acid in 6 concentrations and sample LE in 3 concentrations were prepared. Standard and sample solutions in various concentrations were blended with 10% Fiolin-Ciocalteu reagent for a total of 75ul (1.090.010.500, Merck) and Na_2CO_3 7.5% 60 ul (Merck A87992745). Then, each solution was incubated at 50℃ for 10 minutes. A microplate reader or spectrophotometer was used to test the absorbance with a wavelength of 760nm (Multiscan Go Reader, Thermo Fisher Scientific 1510). The standard used for this assay was gallic acid (Sigma Aldrich, G7384) and the linear regression equation was (Utami et al., 2018; Prahastuti et al., 2020) :

$$y = 0.0429x + 0.152$$

Total Content of Flavonoid

To determine the total flavonoid content (TFC) of lengkuas, the $AlCl_3$ colorimetric test was performed. The standard solution used for this assay was quercetin (Sigma Q4951) in various concentrations (500.00; 250.00; 125.00; 62.50; 31.25; 15.60; 7.80 ug/ml). The samples were SEE in 2000 and 1000 ug/ml. Both standard and sample as much as 75 ul were mixed with 75 ul of $AlCl_3$ 2% (Merck 449598) in a microplate, then measured at 415 nm. The quercetin standard (Sigma Aldrich, Q4951)wase used as a linear regression equation (Prahastuti et al., 2020) :

$$y = 0.0095 + 0.037$$

DPPH Scavenging Assay

The mixture of 50 ul samples and 200ul DPPH solution was put in 96 well microplates. The microplate was then incubated for 30 minutes at room temperature in the dark. The sample absorbance was then measured at wavelength 517 nm. The equation of scavenging activity is as follows (Widowati et al., 2018) :

$$\%\ \text{scavenging activity} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}}\ x\ 100$$

FRAP Assay

The determination of FRAP used a modification method. The mixture of FRAP reagent (142.50 ul) and 7.50 ul of samples in 96 well plates were incubated for 30 minutes in dark conditions. Then, the absorbance of the mixture was measured by a microplate reader at 539 nm (Widowati et al., 2018).

ABTS Assay

The ABTS•+ [Sigma Aldrich A1888-2G] solution was made by mixing potassium persulfate [Merck EM105091] at 4.9 mM and ABTS at 14 mM in a 1:1 volume ratio. Then, for 16 hours, the solution was incubated at 25°C in the dark. Then, PBS 5.5 mM (pH 7.4) was added to the mixed solution to dilute it until the absorbance at 745 nm was 0.70 + 0.02. In a 96-well plate, samples (2 ul) and ABTS solution (98 ul) were added. Incubated

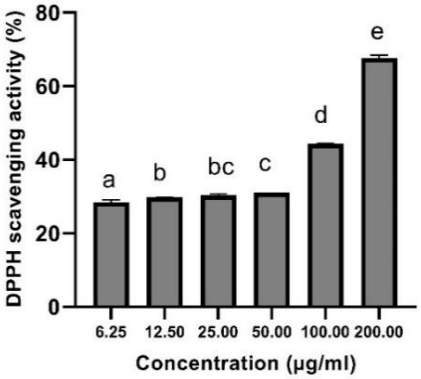

Figure 1. DPPH Scavenging Activity of *Lengkuas* Extract

* Data are displayed as means and standard deviation, and the differences letter for LE demonstrates significant differencs in concentrations at P <0.05 (Tukey HSD post hoc test)

the well for 6 minutes at 30 C, then measured the absorbance using a spectrophotometer (Multiscan Go Reader, Thermo Fisher Scientific 1510). The following formula is used to obtain the IC50 calculation from the scavenging activity (Widowati et al., 2018; Ginting et al., 2020) :

$$\%\ \text{scavenging activity} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}}\ x\ 100$$

H_2O_2 Assay

In the sample and control well, 60 ul samples were added with 12 µL of Ferrous ammonium sulfate (215406, Merck) 1mM. Then, 63 ul of DMSO (Supelco, 1.02952.100) was added. A total of 3 µL of H_2O_2 (1.08597.1000, Merck) with a concentration of 5mM were added to the well. The mixtures were incubated for five minutes in the absence of light. Then 75 µL of 1,10-phenanthroline (131377, Merck) was added to the mixtures after they were incubated. The mixtures were then incubated again in dark conditions for 10 minutes. A wavelength of 510 nm was used to measure the absorbance by a microplate reader (Thermo Scientific Multiscan GO). The scavenging activity was calculated by equation (Ginting et al., 2020) :

$$\%\ \text{scavenging activity} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}}\ x\ 100$$

NO Assay

The variation concentration of the samples with sodium nitroprusside 10 mM (106541, Merck, Germany) in phosphate-buffered saline (PBS)) (1740576, Gibco) were added to the well. Then, Griess reagent made with 0.1% N-(1-napththyl) ethylenediamine dihydrochloride (Sigma 22248), 2% H3 PO4 [Merck 100573], and 1% sulfanilamide [Merck 111799], were mix in the microplate and incubated for two hours at 25°C. A microplate at a wavelength of 546 nm was used to measure the production of chromophore absorbance caused by the coupling of naphthyl ethylenediamine dihydrochloride and diazotization of nitrite with sulfanilamide. To calculate the scavenging capacity of NO, the following equation was employed (Utami et al., 2018):

$$\%\ \text{scavenging activity} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}}\ x\ 100$$

RESULT AND DISCUSSION

In this research, the activity of antioxidants in *lengkuas* extract was determined by TPC, TFC, DPPH, ABTS, FRAP, NO, and H_2O_2 assay. The *lengkuas* was extracted in ethanol solvent. Ethanol is used as a solvent because it can penetrate cell membranes compared to aqueous extract. So polyphenol compounds are more soluble in ethanol solvent. (Valko, Morris and Cronin, 2005).

The principle of TPC was the reaction between phenolic compound with Folin-Ciocalteu reagent. The gallic acid standard was used to calculate the total phenol (Prahastuti et al., 2020). This technique was chosen to quantify phenolics because of it's accuracy and quickness (Ahmed and Iqbal, 2018). The flavonoid content (TFC) measurement was conducted by Aluminium chloride ($AlCl_3$) colorimetric assay (Pontis et al., 2014). The basic concept of this technique is that aluminum chloride binds to the C-4 keto group as well as the C-3 or C-5 hydroxyl groups of flavones and flavonols to create acid-stable complexes. Moreover, it creates the ortho-dihydroxyl A or B rings of flavonoids to form acid-labile compounds (Ahmed and Iqbal, 2018). Based on the research

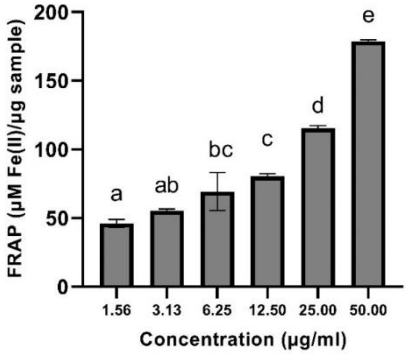

Figure 2. FRAP Reducing Activity of *Lengkuas* Extract

* Data are displayed as means and standard deviation, and the differences letter for LE demonstrates significant differencs in concentrations at P <0.05 (Tukey HSD post hoc test)

that has been done, the phenol compound in LE was greater compared with the flavonoid compound. The total phenolic content of *Lengkuas* extract (LE) was 6.80 ± 0.34 µg/mg extract with gallic acid as standard. The total flavonoid content of LE were 3.39 ± 0.06 µg/mg extract with quercetin as standard. This result corresponds to another research about antioxidants of LE that showed the TPC of LE was 480.13 µg/mg and was expressed as gallic acid equivalent. Meanwhile, the total phenolic compound of LE was 67.68 µg/mg and expressed as quercetin equivalent (Melanathuru, Rengarajan, and Thangavel, 2017).

Flavonoids are a class of organic substances that are present in a variety of foods, including fruits, wine, vegetables, tea, cereals, and flowers (Panche, Diwan, and Chandra, 2016). The phenolic compound is the main component that plays a role as an antioxidant. The structure of the phenolic compound has -OH groups because it is reactive. Thus, it can bind free radical molecules. There are 3 mechanisms of polyphenol compounds as an antioxidant. 1. Hydrogen donor 2. Electron transfer 3. Chelation formation (Das, Ramani, and Suraju, 2016).

The phenolic components in lengkuas that have an antioxidant effect are galangin, 1,8-cineole, galangal acetate, and kaempferol. It has been discovered to have several positive impacts on human health, particularly for preventing oxidative stress by removing free radicals and having antibacterial capabilities, which are also known for it's antimicrobial and antioxidant activities (Ekundayo, Adeboye and Ekundayo, 2011; Ghosh and Rangan, 2013).

The antioxidant activity of LE was assessed using the DPPH technique. The DPPH reagent (2, 2 Diphenyl 1-1-hydrazyl) is a stable radical that can be converted into a diamagnetic molecule by taking an electron or hydrogen radical. The decrease of DPPH in the presence of an antioxidant that donates hydrogen is the foundation of the DPPH technique. Extracts have a strong hydrogen-donating ability, which helps them to lighten DPPH's color. One of the substances that has a proton free radical with a distinctive absorption is DPPH, which is greatly reduced when exposed to substances that scavenge proton radicals (Ahmed and Iqbal, 2018). The DPPH scavenging activity of LE was shown in Fig.1. The DPPH scavenging activity was directly correlated with the concentration of the extract. The IC_{50} of LE was 121.20 µg/ml with the equation of linear regression was y= 0.002049x + 25.145. Another research has shown that the DPPH scavenging activity of LE was 69.5±1.375 µg/ml (Srividya and Dhanabal, 2011).

An intense bright blue Fe2+-TPTZ is created when a potential antioxidant interacts with a colorless Fe3+-TPTZ (tripyridyltriazine) complex. This process is known as the ferric-reducing antioxidant power test (FRAP). This technique worked well for reasonably priced compared with the efficacy of various compounds and screening of antioxidant capacities. As a result, in this study, the FRAP technique was used to examine the antioxidant capacity of chosen phenolic acids compounds (Spiegel et al., 2020). The reducing activity of LE by FRAP assay was shown in Figure 2. The highest reducing activity by FRAP assay was shown at the highest concentration. The highest concentration produced the maximum reducing activity as measured by the FRAP technique. This is due to the high antioxidant activity that might be associated with the various active chemical compounds in the plant (Rusmana et al., 2017). As

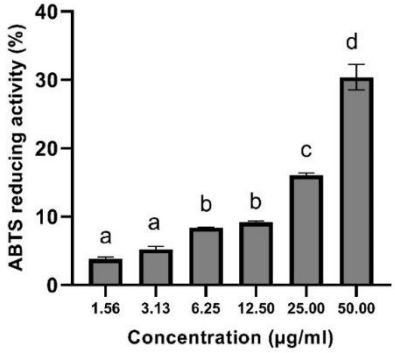

Figure 3. ABTS Reducing Activity of *Lengkuas* Extract

* Data are displayed as means and standard deviation, and the differences letter for LE demonstrates significant differencs in concentrations at P <0.05 (Tukey HSD post hoc test)

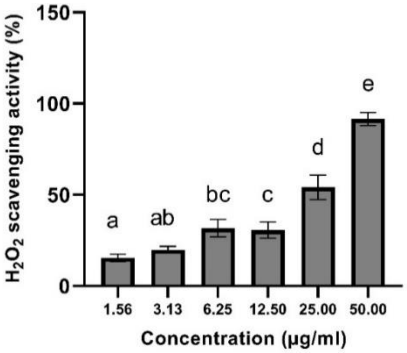

Figure 4. H_2O_2 Scavenging Activity of *Lengkuas* Extract

* Data are displayed as means and standard deviation, and the differences letter for LE demonstrates significant differencs in concentrations at P <0.05 (Tukey HSD post hoc test)

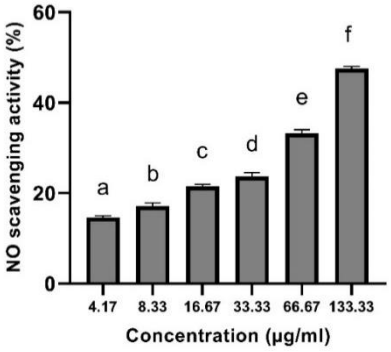

Figure 5. NO Reducing Activity of *Lengkuas* Extract

* Data are displayed as means and standard deviation, and the differences letter for LE demonstrates significant differencs in concentrations at P <0.05 (Tukey HSD post hoc test)

a result, the extract's antioxidant activity increases with extract concentration.

ABTS method mechanism: radical formation between ABTS salt with oxidator (Potassium permanganate or Potassium sulfate) (Shalaby and Shanab, 2013). The ABTS radical which is a blue-green color was reduced because of the hydrogen-donating from antioxidants. Then the

characteristic long-wave absorption spectrum that was given by this reaction was measured (Ratnavathi and Komala, 2016). Figure 3 shows LE's scavenging activities for ABTS. The highest LE concentration exhibits the strongest ABTS reduction action. The IC_{50} value of LE was 87.65 µg/ml based on the calculation of the linear regression equation (y= 0.531x + 3.5474). Previous studies have shown the potent scavenging activity by the ABTS method with an IC_{50} value of 0.086±1.10 µg/ml (Srividya and Dhanabal, 2011).

The H_2O_2 scavenging activity principle was based on the reaction between H_2O_2 as a radical and LE as an antioxidant. The presence of H_2O_2 inhibited the ferrous ammonium and phenanthroline reaction. Thus, the determination of the sample's antioxidant capacity was discovered by the reaction between samples against H_2O_2 (Stevenie et al., 2019). Figure 4 shows the LE's scavenging activity while using the H2O2 assay. with IC_{50} value 181.09 µg/ml with linear regression 0.1897x + 15.648. The previous study shows that the IC 50 value of *lengkuas* was 55±1.59 µg/ml by its ability to scavenge hydrogen peroxide radicals (Srividya and Dhanabal, 2011).

Sodium nitroprusside produces nitrite when reacts with oxygen, which is then converted into nitric oxide. The nitrite ions pair with naphthyl ethylenediamine and diazotize with sulphanilamide acid, resulting in a pink color at 546 nm. When the antioxidants add protons to the nitrite radical, it causes the absorbance to decrease. The amount of nitrite radical scavenging was measured by the decrease in absorbance (Boora, Chirisa, and Mukanganyama, 2014). According to the research, Figure 5 shows the scavenging activity of LE using the NO technique. The IC_{50} value was 139.94 µg/ml with the linear regression 0.2464x+15.52. Another research about *lengkuas* ethanolic extract showed that the IC_{50} value by NO assay was 0.69 ± 0.61 mg/ml (Devi et al., 2018).

The phenolic component in LE, as mentioned above, shows its antioxidant properties. 1'-acetoxychavicol acetate, 1,8-cineol, β-bisabolene, β-farnesene, β-pinene, α-fenchyl-acetate, and α-bergamotene are the main chemical substances present in lengkuas. Most research on *Alpinia galanga* found that 1,8-cineole was the most abundant compound present in a lengkuas. (Subramanian and Nishan, 2015). The previous study also showed that flavonoid compounds in *lengkuas* are catechin, kaemferide, kaemferol, quercetin 3-methyl ether, and quercetin (Tungmunnithum et al., 2020).

CONCLUSION

In conclusion, *Alpinia galanga* has the potential as an antioxidant, thus it can be widely consumed or used as a mixture in medicine to reduce ROS.

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