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77

**The ethanol extract of the bastard cedar (*Guazuma ulmifolia* L.)
as antioxidants****Sijani Prahastuti¹, Meilinah Hidayat¹, Stella Tinia Hasiana¹,
Wahyu Widowati*¹, Wahyu Setia Widodo², Rr. Anisa Siwianti Handayani²,
Rizal Rizal², Hanna Sari Widya Kusuma²**¹Medical Research Center, Faculty of Medicine, Maranatha Christian University
Jl. Prof. Drg. Surya Sumantri 65, Bandung, West Java, Indonesia²Aretha Medika Utama, Biomolecular and Biomedical Research Center,
Jl. Babakan Jeruk II No. 9, Bandung, West Java, Indonesia

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ABSTRACT

Guazuma ulmifolia, or commonly known as the bastard cedar, has many pharmaceutical activities. Therefore, it is claimed as a source of various plant-based medicines. This research was purposed to identify the antioxidant activities of the ethanolic extract of *G. ulmifolia* (EEGU) by phytochemical screening assay, total flavonoid and total phenolic testing, and comparative analysis between the antioxidant activities of EEGU and epicatechin. The qualitative phytochemical screening assay of EEGU detected the availability of phenols, flavonoids, alkaloids, tannins, and terpenoids, but not saponins and triterpenoids. Meanwhile, the total phenolic content was 32.24 µg GAE/mg extract, and the total flavonoid content was 6.48 µg QE/mg extract. The role of antioxidants examined by FRAP, DPPH, H₂O₂, and ABTS assays. These assays are proved that the IC₅₀ values of EEGU are higher than epicatechin. For DPPH scavenging, H₂O₂ scavenging, and ABTS reduction activities, EEGU resulted IC₅₀ 45.70 µg/mL, 162.93 µg/mL, and 35.96 µg/mL, while epicatechin only yielded IC₅₀ 0.56 µg/mL, 57.91 µg/mL, and 16.74 µg/mL respectively. Otherwise, the highest reduction in FRAP activities were shown at 50 µg/mL concentration of epicatechin and EEGU were 236.33 and 202.71 µM Fe (II)/µg respectively. Based on these results, EEGU is concluded as an active natural product because it exhibited antioxidant activities.

Keywords: antioxidant, *Guazuma ulmifolia*, phytochemical, total flavonoid, total phenolic

***Corresponding author:**

Wahyu Widowati

Medical Research Center, Faculty of Medicine, Maranatha Christian University

Jl Prof. Drg. Surya Sumantri 65, Bandung, West Java, Indonesia

E-mail: wahyu_w60@yahoo.com

INTRODUCTION

Excess of free radicals and oxidants increases oxidative stress, which have a negative impact on a variety of cellular structures, including, proteins, lipids, membranes, and DNA (Pizzino *et al.*, 2017). Antioxidants are known to be potential protective agents to reduce oxidative damages to the human body. Most importantly, they are naturally abundant in fruits and can neutralize free radicals by giving an electron and transforming it into harmless molecules. Also, with the reactive free radical quenching properties, these substances can prevent the oxidation of other molecules so that they have the effect of improving health in the prevention of degenerative diseases (Kanerla *et al.*, 2009).

Guazuma ulmifolia belongs to the family Sterculiaceae, commonly named as a Guacimo or Mutamba. *G. ulmifolia* is commonly known in Caribbean, South America, Mexico, Central America, and some parts of India. In India, this tree was introduced more than 100 years ago and, today, it thrives along roadsides and in wasteland. Based on many previous studies, *G. ulmifolia* had been used as antiobesity (Iswantini *et al.*, 2011), antioxidant (Feltrin *et al.*, 2012), photocatalyst, antimicrobial and anticancer agents (Karthika *et al.*, 2017), and hepatoprotective drugs (Sharma *et al.*, 2013). Some of which are associated with oxidative stress. The fruit was chosen for analysis because of its excellence in treating different ailments (Duraishwamy *et al.*, 2018). Blood lipid level was decreased and the occurrence of atherosclerosis in animals with hyperlipidemia was inhibited by *G. Ulmifolia* (Sukandar *et al.*, 2012). In case of progression of atherosclerosis, the relevance of apoptosis regulation has been discussed recently (Tabas, 2005).

One of the main compounds for the antioxidant activity of *G. ulmifolia* is flavonoids and its derivative products. At the same time, rutin and quercitrin which are phenolic compounds also relate to the antioxidant effects of *G. ulmifolia* extract (Morais *et al.*, 2017). This present study aims to analyze the antioxidant activity, secondary metabolite constituents (qualitatively), and total phenolics and flavonoids of *G. ulmifolia*.

Prahastuti *et al.* (2019a) confirmed that *G. ulmifolia* extract (6.25 µg/mL) decreased reactive oxygen species (ROS) in glucose-induced mesangial cells in chronic kidney disease. After comparing several conventional testing methods of antioxidant activities, Amorati and Valgimigli (2015) identify several limitations in them, namely, specialized instrumentation requirement, cannot differentiate between stoichiometry and kinetics reaction, fluorescent low reactivity against ROO• radicals that generate the yield for best antioxidants, the necessity of multiple chromatographic analysis, and long reaction time for each experiment, lack of initiation reproducibility, and incapability to present specific for the superoxide dismutation (SOD).

Various methods used in this research were to evaluate the antioxidant activities, namely, DPPH (2,2-diphenyl-1-picrylhydrazyl), H₂O₂ (hydroperoxide), and ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) free radical scavenging test and FRAP (ferric reducing antioxidant power). Phytochemical investigations can produce valuable information about the different phytoconstituents present in *G. ulmifolia* leaves. The plant defense mechanism for countering ROS and phenol synthesizes phenolic compounds in response to ecological pressures (Duraishwamy *et al.*, 2018). This research was purposed to evaluate the antioxidant potential of the ethanol extract of *G. ulmifolia* (EEGU) by phytochemical screening assay, total flavonoid and total phenolic tests, and comparative analysis between the antioxidant activities of EEGU and epicatechin.

MATERIALS AND METHODS

Materials

The bastard cedar (*G. ulmifolia*) leaves were obtained from Bumi Herbal plantation, Dago, Bandung, Province of Jawa Barat, Indonesia and determined at Biology Department, School of Life Science and Technology, Bandung Institute of Technology (the Province of Jawa Barat, Indonesia) by the herbarium staff.

Methods

***G. ulmifolia* extract preparation**

Bastard cedar leaves were crushed and macerated using 70% distilled ethanol for 24 hours, the filtrate was collected and the residue was soaked until colorless filtrate. Then, the collected filtrate was concentrated using an evaporator at a temperature of 50°C to obtain EEGU (Widowati *et al.*, 2017; Widowati *et al.*, 2018; Prahastuti *et al.*, 2019a).

Qualitative phytochemical screening assay

The *G. ulmifolia* leaves extract (EEGU) phytochemical screening was conducted by using Farnsworth method with a slight modification to identify flavonoids, phenols, alkaloids, saponins, tannins, terpenoids, and steroids/triterpenoids qualitatively (Bera *et al.*, 2015; Adnyana *et al.*, 2016; Widowati *et al.*, 2018). The procedures for each compound detection test are as follows.

Phenols identification

About 10 mg of EEGU was placed on spotting plates and added with 1% FeCl₃. The phenols availability was shown by forming of black, red, blue, purple, or green color (Bera *et al.*, 2015; Adnyana *et al.*, 2016; Widowati *et al.*, 2018; Prahastuti *et al.*, 2019b).

Steroids/triterpenoids identification

About 10 mg of EEGU was placed on spotting plates and fully immersed in acetic acid for 10-15 mins. One mL of absolute H₂SO₄ was added to the mixture. Steroids were detected when blue or green color is formed, while triterpenoids were shown by orange or red sediment formation (Bera *et al.*, 2015; Adnyana *et al.*, 2016; Widowati *et al.*, 2018; Prahastuti *et al.*, 2019b).

Tannins identification

In the test tube was added by 10 mg of EEGU and 2 mL of HCl 2N, then heated for 30 mins in water bath. The mixture was chilled and the filtrate was collected. The filtrate was added by pentyl alcohol. If purple color was formed exhibited the sample positive containing tannins (Bera *et al.*, 2015; Adnyana *et al.*, 2016; Widowati *et al.*, 2018; Prahastuti *et al.*, 2019b).

Saponins identification

The tube containing water, 10 mg of EEGU was added and boiled for 5 mins. Then it was shaken tremendously. After that, 1 N HCl was dripped into the test tube. The stable foam presence on the surface of the solution indicated saponin content (Bera *et al.*, 2015; Adnyana *et al.*, 2016; Widowati *et al.*, 2018; Prahastuti *et al.*, 2019b).

Terpenoids identification

EEGU as much as 10 mg was placed on spotting plates and mixed with vanillin and H₂SO₄. The reaction was positive if the color changed into purple (Bera *et al.*, 2015; Adnyana *et al.*, 2016; Widowati *et al.*, 2018; Prahastuti *et al.*, 2019b).

Flavonoids identification

In the test tube, EEGU as much as 10 mg was inserted and added with the Mg/Zn powder and HCl 2 N. The mixture was heated for 5 – 10 mins and then allowed to cool and filtered. Afterward, the pentyl alcohol and filtrate were mixed. Red or orange color indicated positive reaction and sample contained flavonoid (Bera *et al.*, 2015; Adnyana *et al.*, 2016; Widowati *et al.*, 2018; Prahastuti *et al.*, 2019b).

Alkaloids identification

About 10 mg EEGU was added ammonia 10% then vaporized with chloroform in water bath up to two layers. The base layer collected in first tube and added with 3 drops HCl 1N, the residue as second tube was dissolved in HCl 1N. The residue as upper layer was added with 2 drops Dragendorff solution. The positive reaction if the sample changed to red or yellow (Bera *et al.*, 2015; Adnyana *et al.*, 2016; Widowati *et al.*, 2018; Prahastuti *et al.*, 2019b).

Phenolic content assay

Phenolic content was assayed with minor modification of Folin-Ciocalteu's method. In the sample well, the EEGU as much as 15 μ L was inserted and mixed with 10% Folin-Ciocalteu's reagent as much as 75 μ L and 7.5% sodium carbonate as much as 60 μ L. The blank solution was created by mixing 135 μ L of 10% DMSO with 15 μ L of EEGU. Then, it was heated for 10 mins at 50°C. Furthermore, the absorbance was determined using a microplate reader at 760 nm. Total phenolic content was asserted as gallic acid equivalence (GAE) in μ g/mg sample. This test was done triplicate (Nurhayati *et al.*, 2018; Rusmana *et al.*, 2017; Widowati *et al.*, 2015; 2017; 2018; Prahastuti *et al.*, 2019b).

Flavonoid content assay

Flavonoid content was conducted using the altered previous method (Kalita *et al.*, 2013; Prahastuti *et al.*, 2019b). Briefly, standard solution in 7 concentration level of epicatechin and EEGU at 1000 μ g/mL and 2000 μ g/mL as much as 15 μ L was used in this method. It was mixed with 75 μ L AlCl₃ 2%. The absorbance was quantified in 415 nm of wavelength. The linear regression equation ($y = ax + b$) was made based on the standard (quercetin) absorbance value. The analysis of sample flavonoid content was performed based standard linear regression equation. The flavonoid content was presented as quercetin equivalence (QE) in μ g/mg. The experiment was done triplicate (Prahastuti *et al.*, 2019b).

DPPH scavenger activity assay

A total of 200 μ L of 0.0777 mmol DPPH in methanol was added to the EEGU as much as 50 μ L with various concentrations in the 96-well microplate. Then, it was incubated for 30 min at room temperature. After that, the absorbance value was quantified using microplate reader at a wavelength of 517 nm. The negative control was contained 250 μ L of DPPH, while the blank solution was contained 50 μ L of the sample and 200 μ L of DMSO (Widowati *et al.*, 2015; 2016; 2017; 2018). The subsequent formula was used to measure the DPPH scavenging activity.

$$\text{DPPH scavenging activity (\%)} = \frac{A-B}{A} \times 100$$

A: control solutions absorbance

B: sample absorbance

H₂O₂ scavenger activity assay

The H₂O₂ scavenging was measured according to previous studies (Mukhopadhyay *et al.*, 2016; Utami *et al.*, 2017) with slight modification. Each sample well was contained 60 μ L of EEGU, 12 μ L of FeH₈N₂O₈S₂ 1 mM and 3 μ L of H₂O₂ 5 mM. The mixture that containing 12 μ L of FeH₈N₂O₈S₂ and 63 μ L of DMSO were used as the negative control, while the mixture that containing 60 μ L of EEGU and 90 μ L of DMSO were used as the blank solution. After added with H₂O₂, incubated in the dark and room temperature for 5 min. The 1,10-phenanthrolines as much as 75 μ L was added into the sample and control well and incubated again for 10 min in the dark and room temperature. The 510 nm wavelength was used to measure the absorbance. The subsequent formula was used to calculate the H₂O₂ scavenger activity:

$$\text{H}_2\text{O}_2 \text{ scavenger activity (\%)} = \frac{A}{B} \times 100$$

A: control solutions absorbance

B: sample absorbance

ABTS reduction activity assay

Briefly, the EEGU as much as 2 μL at various concentrations was added into the well. Then, it was mixed with 198 μL of ABTS work. As for the control wells, each of them was added with 198 μL of ABTS work and 2 μL of DMSO. Meanwhile, the blank well was added with 198 μL of DMSO and 2 μL of the EEGU. The microplate was closed, then incubated for 6 min at 37°C. The absorbance value was determined using microplate reader at a wavelength of 745 nm (Widowati *et al.*, 2016; 2017; 2018). The ABTS reduction percentage was calculated using the subsequent formula:

$$\text{ABTS reduction activity (\%)} = \frac{A-B}{A} \times 100$$

A: control solutions absorbance

B: sample absorbance

FRAP

The acetate buffer (acetic acid was added to create conformation at pH 3.6) at 300 mM of concentration as much as 10 mL, dissoluble ferric chloride hexahydrate in distilled water at 20 mM of concentration as much as 1 mL, and dissoluble TPTZ in 40 mM HCl at 10 mM of concentration as much as 1 mL was mixed to create the FRAP reagent. A total of 7.5 μL of the sample was mixed with the FRAP reagent as much as 142.5 μL in a 96-well microplate, then mixed and incubated for 30 min at 37°C. The wavelength at 593 nm was used to measure the absorbance value using the microplate reader. Afterward, a FeSO_4 standard curve with varying concentrations, starting from 0.019 to 95 $\mu\text{g}/\text{mL}$ was created. The results were presented as μM Fe (II)/ μg extract (Widowati *et al.*, 2017; 2018).

Statistical analysis

The SPSS program (version 20.0) was used to carry out the statistical analysis. The DPPH scavenger, H_2O_2 scavenger, and ABTS reduction assays results were analyzed using linear regression analysis. Then, the linear regression equation generated in this process was used to determine the Median Inhibitory Concentration 50 (IC_{50}).

RESULTS AND DISCUSSION

There have been previous studies documenting the natural compounds of *G. ulmifolia* extract. Both its fruits and leaves contain tannins, saponins, flavonoids, terpenoids, cardiac glycoside, and alkaloids (Patil, 2013). However, EEGU has been reported to have high cardiac glycosides, moderate levels of tannin, phenol, terpenoids, and coumarin, and lower concentrations of sterol, quinone, and acid, but not flavonoids and saponins (Duraiswamy *et al.*, 2018).

Duraiswamy *et al.* (2018) have declared that the total phenolic and flavonoid contents of *G. ulmifolia* are 27.2797 ± 0.1756 mg TAE/g DW and 0.5544 ± 0.00018 mg QE/g DW. Meanwhile, the present study yielded higher values, which were 32.24 ± 1.42 μg GAE/mg EEGU and 6.48 ± 0.14 μg QE/mg EEGU.

Phenolic compounds are a very good antioxidant or have a broad number of biochemical activities such as antimutagen, anticarcinogenic and modify gene expression for curing various oxidative stress diseases. A phenolic compounds antioxidant activity reflects its composition, the hydroxyl groups location also number, and the nature of its substitution on the aromatic ring. Flavonoids are excellent biological reaction modifications as the yeomen's function in antihistamine, antimicrobial, memory, and even mood-enhancing properties. The plants thus find

their medicinal values due to the presence of respective phytochemical constituents (Duraishwamy *et al.*, 2018). Flavonoids induce mechanism that inhibit tumor invasion, kill the cancer cell, apart from this show anti-inflammatory, antiallergic, anticancer and antimicrobial (Sharma *et al.*, 2015).

Phytochemical screening

The *G. ulmifolia* leaves extract was subjected to preliminary phytochemical analysis, and the results are shown in Table I. This research showed that the extract contains flavonoids, phenols, tannins, steroids, terpenoids, and alkaloids while saponins and triterpenoids were not detected.

Table I. The EEGU qualitative phytochemical screening assay

Phytochemical assay	Results
Tannins	+
Saponins	-
Flavonoids	+
Phenols	+
Alkaloids	+
Terpenoids	+
Steroids/Triterpenoids	+/-

(+): present; (-): not detected

Phenolic content

Total phenolics were gauged by a colorimetric method modified from a previous study (Widowati *et al.*, 2015; 2016; 2017; 2018). It was based on entangle phenolic compounds reduce Folin-Ciocalteu's reagent with a blue complex generated unanimously. The total polyphenols were measured using the calibration curve for gallic acid. The experiments revealed the presence of a high quantity of phenolics in the extract, which was $32.24 \pm 1.42 \mu\text{g GAE/mg EEGU}$.

Flavonoid content

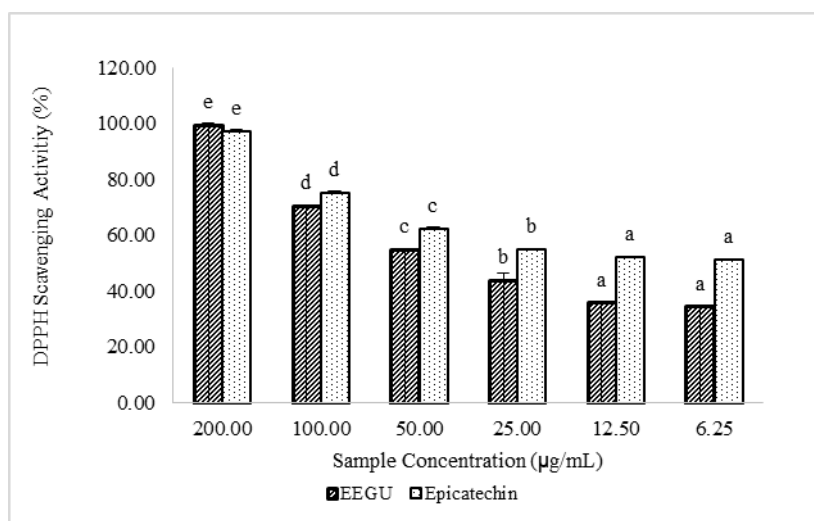
The total flavonoid content of EEGU was measured by the aluminum chloride method. Its quantification revealed the presence of a high quantity of flavonoids in EEGU, which was $6.48 \pm 0.14 \mu\text{g QE/mg EEGU}$.

DPPH scavenger activity

Various assay have been used to test antioxidant activity, but the most commonly used methods are the forming of free radical species and being neutralized with antioxidant compounds. Unpaired electron caused a stable free radical DPPH. DPPH is a stable free radical that centered on nitrogen, usually used to assign the compounds or plant extracts radical scavenging activity (Kedare and Singh, 2011; Sasikumar and Kalaisezhiyen, 2014). In the presence of hydrogen donor, it becomes paired and reduced the absorption at 517 nm (Widowati *et al.*, 2015). The extracts reduced stable DPPH radicals to yellow-colored diphenyl picrylhydrazine (DPPH-H) during DPPH test. The radical DPPH is widely used as a substrate for examining antioxidant activity. The IC_{50} value is the concentration allowed by an antioxidant to scavenge 50% of the DPPH free radical; higher antioxidant activity is defined by a smaller IC_{50} value. In this study, the IC_{50} of EEGU was $45.70 \pm 0.7 \mu\text{g/mL}$, while epicatechin had an IC_{50} of $0.56 \pm 0.29 \mu\text{g/mL}$. Epicatechin was discovered to be the most active extract in DPPH scavenging activity because it provided the lowest IC_{50} value (Table II) and the highest DPPH scavenging activities (Figure 1), particularly at a concentration of $200 \mu\text{g/mL}$.

Table II. The antioxidant activities (IC₅₀) of EEGU and epicatechin

Sample	IC ₅₀ of DPPH Scavenger Activity (µg/mL)	IC ₅₀ of H ₂ O ₂ Scavenger Activity (µg/mL)	IC ₅₀ of ABTS-reducing Activity (µg/mL)	FRAP-reducing highest Activity (µM Fe (II)/µg extract)
EEGU	45.70	162.93	35.96	202.71
Epicatechin	0.56	57.91	16.74	236.33

**Figure 1. The DPPH scavenging activities of EEGU and epicatechin**

*Each sample was diluted in 10% DMSO to create the final concentrations: 200, 100, 50, 25, 12.50, and 6.25 µg/mL. This experiment was done triplicate for each sample concentration

H₂O₂ scavenger activity

Hydrogen peroxide plays a role in producing energy in various in vivo systems, phagocytosis, intercellular signal transfer, cell growth control, and the synthesis of essential biological compounds (Packer *et al.*, 2008). As byproducts of normal aerobic metabolism H₂O₂ is generated and increased during infections, workouts, and stress conditions (Mukhopadhyay *et al.*, 2016). The Median Inhibitory Concentrations (IC₅₀) of epicatechin and EEGU in H₂O₂ radical scavenging activity was shown in Table II. The IC₅₀ values of EEGU and epicatechin were 162.93 µg/mL and 57.91 µg/mL, respectively. Figure 2 also shows that epicatechin had higher activities than EEGU, particularly at 300 µg/mL.

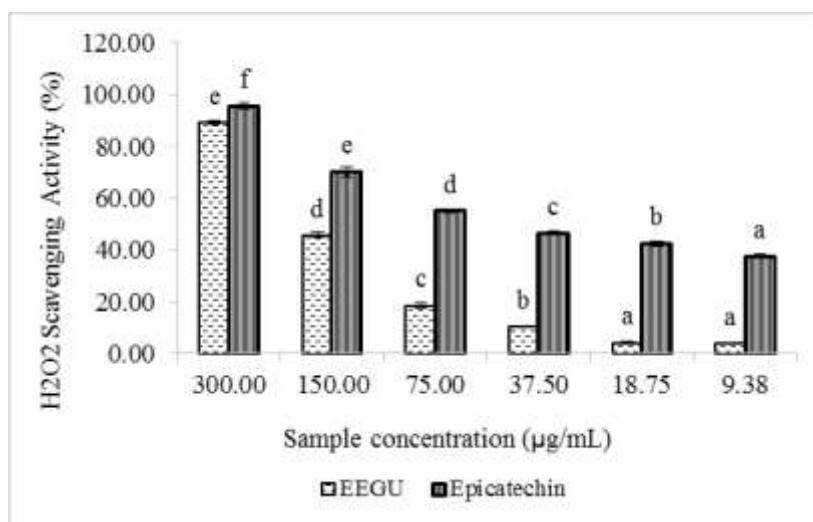


Figure 2. The H₂O₂ scavenging activities of EEGU and epicatechin

*Each sample was diluted in 10% DMSO to create the final concentrations: 300, 100, 150, 75, 37.5, 18.75, and 9.38 µg/mL. This experiment was done triplicate for each sample concentration.

ABTS-reducing activity

The activity of ABTS-reducing assay gauges the antioxidant's relative potential to scavenge the ABTS generated. In this study, ABTS was produced by reaction between a powerful oxidizer and ABTS salt. The ABTS radical blue-green-colored solution was reduced by hydrogen-donating antioxidant (Widowati *et al.*, 2016) and analyzed at spectrum of a long-wave absorption. The percentages of ABTS-reducing activity of EEGU and epicatechin were compared, with epicatechin having the highest activity at 50 µg/mL, as shown in Figure 3. Also, their IC₅₀ values were 35.96 µg/mL and 16.74 µg/mL, respectively (Table II).

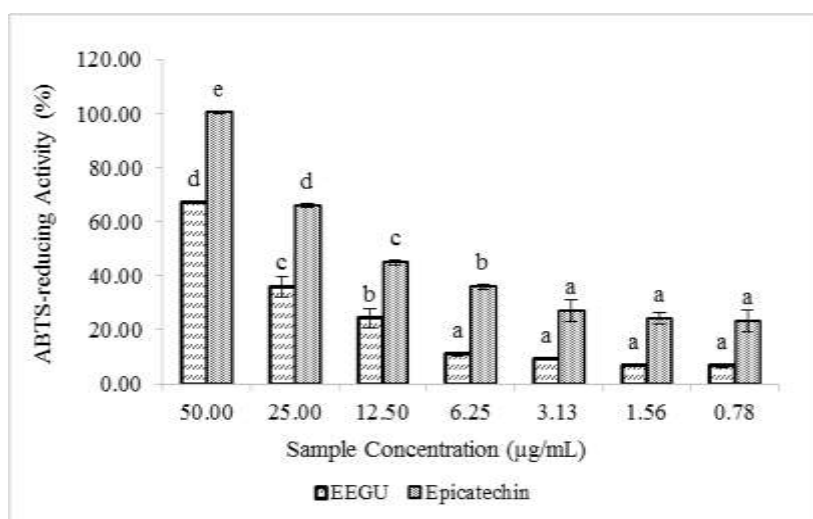


Figure 3. The ABTS-reducing activities of EEGU and epicatechin

*Each sample was diluted in 10% DMSO to reach final concentrations of 50, 25, 12.50, 6.25, 3.13, 1.56, and 0.78 µg/mL. This experiment was done triplicate for each sample concentration.

FRAP

The FRAP method was based on the ferrioxalate reduction in acidic medium, the TPTZ³⁺ to the colored Fe²⁺ complex of Fe(TPTZ)²⁺ (greatly blue) by antioxidant (Widowati *et al.*, 2018). A reduction in the corresponding tripyridyltriazine Fe(III) complex at 593 nm results from the absorbance of Fe(II) complex.

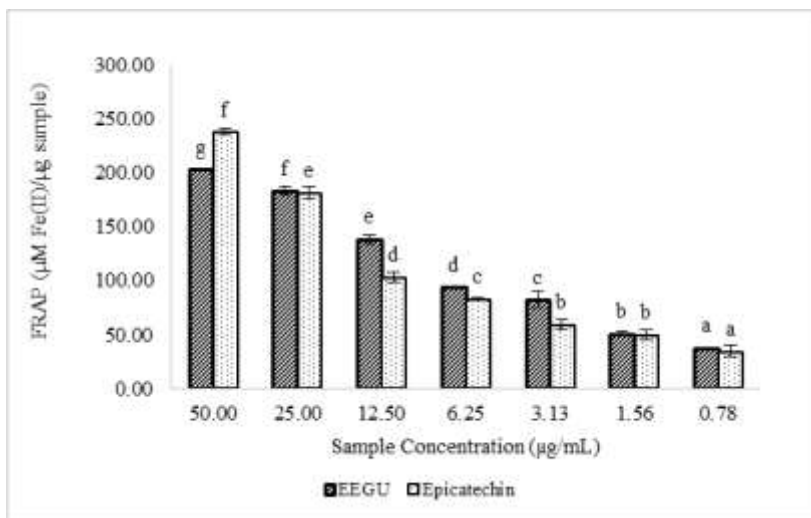


Figure 4. The FRAP of EEGU and epicatechin

*Each sample was diluted in 10% DMSO to create the final concentrations: 50, 25, 12.50, 6.25, 3.13, 1.56, and 0.78 µg/mL. This experiment was done triplicate for each sample concentration

The highest FRAP activities of EEGU and epicatechin were 202.71 µg/mL and 236.33 µg/mL, respectively (Table II, Figure 4). Duraiswamy *et al.* (2018) claim that the most significant inhibition of FRAP activity is 40%, and this is produced by the fruit extract of *G. ulmifolia* at 500 µg/mL. Meanwhile, in this research, the highest FRAP activity was 202.71 µM Fe(II), which was generated by EEGU at 50 µg/mL.

High correlations between antioxidant activities and phytochemical content of polyphenols were observed. The antioxidant activities potentially contribute to the polyphenol contents found in the plant extracts. *G. ulmifolia* stem bark and leaves extracts exhibited DPPH scavenger activity at IC₅₀ of 25.2 µg/mL and 39.3 µg/mL (dos Santos *et al.*, 2018). There have been some previous studies examining the antioxidant activities of EEGU using different assays, namely, FRAP, DPPH, MDA dosage, human erythrocyte suspension, hemolytic activity, and inhibition of oxidative hemolysis in human erythrocytes by 2,2'-Azobis(2-amidinopropane) dihydrochloride-induced (AAPH) or DOX (Duraiswamy *et al.*, 2018; dos Santos *et al.*, 2018; Kaneria *et al.*, 2009).

CONCLUSIONS

Based on the phytochemical screening, EEGU has been confirmed to contain flavonoids, phenols, tannins, alkaloids, steroids, and terpenoids but not saponins and triterpenoids. The flavonoid contents and total phenolic of EEGU are 32.24 µg extract/mg GAE and 6.48 µg QE/mg extract, respectively. Comparison between EEGU and epicatechin using different tests for antioxidant activities, namely DPPH scavenging, ABTS reduction, H₂O₂ scavenging, and FRAP assays, proves that the IC₅₀ values of EEGU are generally higher than those of epicatechin. For DPPH scavenging, H₂O₂ scavenging, and ABTS reduction activities, EEGU can produce IC₅₀ values of 45.70 µg/mL, 162.93 µg/mL, and 35.96 µg/mL. Meanwhile, for the same activities, epicatechin only yields IC₅₀ values of 0.56 µg/mL, 57.91 µg/mL, and 16.74 µg/mL. At the same time, EEGU has the highest FRAP reduction properties at 50 µg/mL or equal to 202.71 µM Fe

(II)/ μg . These findings assert that EEGU and epicatechin have antioxidant activities and that EEGU is an active natural product.

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