The ethanol extract of the bastard cedar (Guazuma ulmifolia L.) as antioxidants

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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

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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 (Kaneria 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 (Duraiswamy 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 quercitin 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 (Duraiswamy 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 EE GU (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 EE GU 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 Draggendorf 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 EE GU as much as 15 µL was inserted and mixed with 10% Folin-Ciocalteu’s reagent as much as 75 µL and 7.5% sodium carbonate as much as 60 µL. The blank solution was created by mixing 135 µL of 10% DMSO with 15 µL of EE GU. 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 assumed as gallic acid equivalence (GAE) in μg/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 EE GU at 1000 µg/mL and 2000 µg/mL as much as 15 µL was used in this method. It was mixed with 75 µL 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 µg/mg. The experiment was done triplicate (Prahastuti et al., 2019b).

DPPH scavenger activity assay

A total of 200 µL of 0.0777 mmol DPPH in methanol was added to the EE GU as much as 50 µL 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 µL of DPPH, while the blank solution was contained 50 µL of the sample and 200 µL 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

H$_2$O$_2$ scavenger activity assay

The H$_2$O$_2$ scavenging was measured according to previous studies (Mukhopadhyay et al., 2016; Utami et al., 2017) with slight modification. Each sample well was contained 60 µL of EE GU, 12 µL of FeH$_2$N$_2$O$_5$S$_2$ 1 mM and 3 µL of H$_2$O$_2$ 5 mM. The mixture that containing 12 µL of FeH$_2$N$_2$O$_5$S$_2$ and 63 µL of DMSO were used as the negative control, while the mixture that containing 60 µL of EE GU and 90 µL of DMSO were used as the blank solution. After added with H$_2$O$_2$ incubated in the dark and room temperature for 5 min. The 1,10-phenanthrolines as much as 75 µL 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 H$_2$O$_2$ scavenger activity:
H$_2$O$_2$ scavenger activity (%) = \( \frac{A - B}{A} \times 100 \)

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 µg/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$_2$O$_2$ 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 27.279±0.1756 mg TAE/g DW and 0.554±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 (Duraiswamy 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 ± 1.42 µg 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±0.14 µg 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 DPH is widely used as a substrate for examining antioxidant activity. The IC\textsubscript{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\textsubscript{50} value. In this study, the IC\textsubscript{50} of EEGU was 45.70±0.7 µg/mL, while epicatechin had an IC\textsubscript{50} of 0.56±0.29 µg/mL. Epicatechin was discovered to be the most active extract in DPPH scavenging activity because it provided the lowest IC\textsubscript{50} value (Table II) and the highest DPPH scavenging activities (Figure 1), particularly at a concentration of 200 µg/mL.
Table II. The antioxidant activities (IC₅₀) of EEGU and epicatechin

| Sample    | IC₅₀ of DPPH Scavenger Activity (µg/mL) | IC₅₀ of H₂O₂ Scavenger Activity (µg/mL) | IC₅₀ 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.
**ABTS-reducing activity**

The activity of ABTS-reducing assay gauge 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).

*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*
FRAP

The FRAP method was based on the ferroin analog reduction in acidic medium, the TPTZ$^{3+}$ to the colored Fe$^{2+}$ 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](image)

*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 IC$_{50}$ 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, H$_2$O$_2$ scavenging, and FRAP assays, proves that the IC$_{50}$ values of EEGU are generally higher than those of epicatechin. For DPPH scavenging, H$_2$O$_2$ scavenging, and ABTS reduction activities, EEGU can produce IC$_{50}$ values of 45.70 μg/mL, 162.93 μg/mL, and 35.96 μg/mL. Meanwhile, for the same activities, epicatechin only yields IC$_{50}$ 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
The findings assert that EEGU and epicatechin have antioxidant activities and that EEGU is an active natural product.

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