Antioxidant and Antibacterial Activities of Several Fractions from *Crescentia cujete* L. Stem Bark Extract

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Abstract. This research was conducted to determine antioxidant and antibacterial activities of several fractions from *Crescentia cujete* L. stem bark extract. The powdered stem bark of this plant was extracted by ethanol solvent and then separated by liquid-liquid extraction. The results of this separation were *n*-hexane, dichloromethane, ethyl acetate, and water fractions. Antioxidant compounds were identified by Thin Layer Chromatography (TLC)–autographic assay using 0.02% 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution. Antioxidant activity was analyzed using DPPH method, while antibacterial activity was evaluated using TLC-bioautographic and agar disc diffusion methods. The results showed that antioxidant compounds were found in all fractions, with the highest chemical constituents was in dichloromethane fraction. The antioxidant activity of dichloromethane fraction was the highest of all fractions with IC₅₀ value of 95.83 ± 19.64 µg/mL, while ethyl acetate fraction was the lowest activity with IC₅₀ value of 174.56 ± 21.93 µg/mL. The antibacterial assay indicated that water fraction could inhibit *Escherichia coli* growth with inhibition diameter zone (IDZ) of 2.36 ± 1.11 mm. However, dichloromethane and ethyl acetate fractions could suppress *Staphylococcus aureus* growth with IDZs of 2.72 ± 0.30 mm and 4.89 ± 0.72 mm, respectively. Further analysis with TLC-bioautography on dichloromethane fraction showed three compounds with Rf values of 0.88, 0.84, and 0.78 have antibacterial activity.

Keywords: antibacterial, antioxidant, *Crescentia cujete*, stem bark, TLC-bioautography

1. Introduction

Indonesia is one of the tropical countries that have a high level of biodiversity, especially for plants. This variety of plants contribute a lot of benefits for a human being, such as for food and health. Special for human health purposes, the use of plants for curing the diseases is considered more secure than synthetic drugs. Although the contention is still being debated, current research on plant exploration as a medicine still leads. These studies are based on the experience of Indonesian ancestors.
who used the plants as herbal medicine. These folk-medicines have been practiced for many centuries to maintain good health and to treat diseases [1].

One of the plants that have been widely used in traditional medicine is *Crescentia cujete* L. (*C. cujete* L.). In Indonesia, *C. cujete* L. has been known as ‘berenuk’ and utilized to treat various diseases, especially for diseases that caused by bacteria. This potency is probably caused by the phytochemical constituents contained in this plant. Some researchers reported that *C. cujete* L. contained flavonoids, alkaloids, saponins, tannins, and terpenoids that were potential as antibacterial and antioxidant agents [2,3]. Because of its chemical constituents, there is a significant chance to improve the health problems in Indonesia by using this plant.

However, there is a few information about its fractions by the ability as antibacterial and antioxidant agents. Some studies were still limited to the biological activity of crude extracts from this plant. For example, Parvin et al. [4] reported that ethanol extract from this bark could inhibit *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). Ethanol and water extracts also showed inhibition activity to *Mycobacterium aurum* A+ growth. This research was conducted to determine antibacterial and antioxidant activities of several fractions from *C. cujete* L. stem bark extracts. The results were expected to be scientific information to overcome infectious diseases.

2. Materials and methods

2.1. Plant collection and identification

*C. cujete* L. stem barks (4 years old) were collected from Bogor, Indonesia (6°33' 17"S/106°42'3"E). This plant was identified and authenticated by Herbarium Bogoriense, Research Center for Biology – Indonesian Institute of Sciences (LIPI), Indonesia. The collected materials were washed and cut into small pieces. After being dried in the sun and oven (50°C) for every four days respectively, these materials were milled using a grinder and then filtered into 100 mesh of powdered samples.

2.2. Moisture content determination

Moisture content was determined using AOAC method [5].

2.3. Plant extraction

The powder of *C. cujete* L. stem bark was extracted using maceration method. Fifty grams of its powder was extracted in 500 mL of 70% ethanol for 3×24 h. The obtained extracts were subjected to vacuum evaporator (N-1100, Shanghai Eyela Co. Ltd., China) to eliminate the solvents [6]. The dried extract was then used for fractionation.

Fractionations were performed using different solvents of polarity, i.e., *n*-hexane, dichloromethane, ethyl acetate, and water (figure 1). The ethanol extract (6 g) obtained from the previous step was dissolved in 225 mL of water in the separating funnel. To the solution, we added *n*-hexane at a ratio of 1:1 (v/v) to obtain *n*-hexane fraction. The remaining water fraction was fractionated by adding dichloromethane and ethyl acetate, respectively. The fractions of *n*-hexane, dichloromethane, and ethyl acetate were purified by using saturated sodium chloride. Furthermore, all fractions were purified again with sodium phosphate [7]. The yield of the fractions were calculated by comparing the obtained extract weight to the initial sample weight with the following formula.

\[
\text{yield fraction (\%)} = \frac{\text{final weight of sample}}{\text{initial weight of sample}} \times 100\% \times \text{extract yield\%}
\]
2.4. Determination of the optimum eluent
The optimum eluent was determined using various solvents, i.e., \( n\)-hexane, ethyl acetate, methanol, dichloromethane, and chloroform with \( G_{60}F_{254}\) of silica plate as a stationary phase. The ethanol extract of stem bark was dissolved using water with a concentration of 2\% (w/v). Plates (10 cm \( \times\) 1 cm) were dried in the oven (50°C) for 10 minutes, and the eluent was saturated for 20 minutes. The extract was put ten times on to the plate. After drying, the plates are then eluted with every single eluent. The resulted spot elution was observed using UV light at \( \lambda = 254\) nm and \( \lambda = 366\) nm. Eluent that produced a good spot was mixed with a specific ratio to get the optimum eluent [8].

2.5. Detection of antioxidant compounds
Antioxidant constituents were detected by thin layer chromatography (TLC) –autography DPPH method [9]. Plates that have reached the finish line were removed from the chromatographic vessel and then dried for 15 minutes. After drying, the TLC plates were sprayed with a 0.02\% DPPH solution at standard (ascorbic acid) and fractions (\( n\)-hexane, dichloromethane, ethyl acetate, and water). After 30 minutes, a pale yellow band that indicated antioxidant compound appeared with a purple plate base. For the clearer results, UV lamps with \( \lambda = 254\) nm and \( \lambda = 366\) nm were employed to observe.

2.6. Antioxidant activity determination
Antioxidant activity was determined by using DPPH method [10]. As much as 70 \( \mu\)L of 0.1 mM DPPH inserted into microplate well contained 70 \( \mu\)L Tris-HCl buffer (pH 6) and 70 \( \mu\)L of sample in methanol. Hereafter, the samples were incubated in the dark room for 20 minutes (at room temperature). The absorbance value was measured at \( \lambda = 517\) nm. Ascorbic acid was used as a

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**Figure 1.** Flow chart of ethanol extract fractionation

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70% ethanol extract
  ↓
+ water + \( n\)-hexane
  ↓
water fraction  \( n\)-hexane fraction
  ↓
+ dichloromethane
  ↓
water fraction  dichloromethane fraction
  ↓
+ ethyl acetate
  ↓
water fraction  ethyl acetate fraction
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standard, while the blank solution used 70 µL methanol, 70 µL of Tris-HCl buffer, and 70 µL of 0.1 mM DPPH. Percentage of inhibition was calculated using the following formula.

\[
inhibition(\%) = \frac{A1 - A2}{A1} \times 100\%
\]

A1= blank absorbance; A2= sample absorbance

Percentage of inhibition (x-axis) and sample concentration (y-axis) would produce an equation and the IC50 (Inhibition Concentration 50) value can be obtained. The IC50 value is a number indicating the concentration of sample test which gives 50% inhibition (can inhibit the oxidation process by 50%).

2.7. Antibacterial activity
Antibacterial activity was evaluated by TLC-bioautographic and agar diffusion methods. The fractions of n-hexane, dichloromethane, ethyl acetate, and water were dissolved in dimethyl sulfoxide (DMSO) 20% to obtain 100,000 µg/mL of each fraction. Each fraction was spotted on the silica plate three times and then eluted by the best eluent. The chromatogram was then dried and affixed to the agar medium that inoculated by bacteria with a concentration of 10^6 CFU/mL for 1 h. Then, the chromatogram was incubated at 37°C for 24 h [11]. *E. coli* and *S. aureus* obtained from IPBCC were used in this experiment.

To identify the antibacterial compounds in bioautographic method, the fractions were eluted again using TLC. Each fraction was spotted 10 times onto the silica plate and then dried. The plate was then placed into a vessel containing the eluent, started with a single eluent and then a mixture. The plates were then dried and observed under UV lamps with λ = 254 nm and λ = 366 nm [12]. The plates were then sprayed with Dragendorf reagent for detecting alkaloids, ammonia reagent for flavonoids, FeCl3 reagent for phenolics and H2SO4 reagent for terpenes [13,14].

For the agar disc diffusion method, one ose of *E. coli* or *S. aureus* cultures were dissolved into 10 mL of nutrient broth (NB, Sigma-Aldrich, Germany) and then incubated at 37°C for 24 h. The bacterial concentration that used was 10^6 CFU/mL. Bacterial concentrations were determined by OD (Optical Density) measurement and TPC (Total Plate Count) method. The suspension of bacteria was taken as much as 0.1 mL and then disseminated in a petri dish contained nutrient agar (NA, Sigma-Aldrich, Germany) medium. A paper disc (diameter = 6 mm) was prepared and dripped with 10 µL of fraction with a concentration of 100,000 µg/mL. Amoxicillin 500 µg/mL was used as positive control and 20% DMSO was used as negative control. Each paper disc was affixed to the agar surface and then incubated at 37°C for 24 h. Antibacterial activity was determined based on the clear zone appearance (inhibition diameter zone) [15].

2.8. Statistical analyses
Antioxidant and antibacterial analyses were expressed as the mean ± SD of pooled results obtained from at least three independent experiments. Statistical analysis was performed by one-way ANOVA and then followed by Duncan test. Significance level was considered as P<0.05.

3. Results

3.1. Extract and antioxidant compounds of *C. cujete* L. fractions
The moisture content of *C. cujete* L. stem bark powder was 2% (w/w). Meanwhile, stem bark extraction using 70% ethanol yielded 29.3% extract. Furthermore, fractionations of this extract using n-hexane, dichloromethane, ethyl acetate, and water resulted in fewer extract yield (table 1). From the optimum eluent, we obtained that ethyl acetate:n-hexane 9:1 was the optimum eluent that separates more compounds from the ethanol extract. TLC-DPPH autography identification resulted in a pale yellow ribbon after being sprayed with 0.02% DPPH. Before sprayed by 0.02% DPPH, the color of these compounds was purple. These results can be seen in figure 2.
Table 1. Yields of ethanol extract fractionation.

| Solvent         | Yield (% w/w) |
|-----------------|---------------|
| n-hexane        | 0.517         |
| dichloromethane | 0.619         |
| ethyl acetate   | 0.520         |
| water           | 15.2          |

Figure 2. Chromatograms on silica plates from each fraction. A) Before sprayed by DPPH; B) After sprayed by DPPH; 1. ascorbic acid, 2. n-hexane, 3. dichloromethane, 4. ethyl acetate, 5. Water.

3.2. Antioxidant activity
Antioxidant activity was determined by IC50. The results showed that the lowest IC50 values were found in dichloromethane fraction with 95.83 µg/mL, while the highest value was found on ethyl acetate fraction with 174.56 µg/mL (figure 3).

3.3. Antibacterial activity
Antibacterial analysis by agar disc diffusion method showed that dichloromethane and ethyl acetate fractions could inhibit S. aureus, and only the water fraction suppressed the growth of E. coli (table 2). Antibacterial activity of the fractions was also conducted by TLC-bioautography method. The result showed that bacterial inhibition was only found in S. aureus while not for E. coli. This inhibition was marked by the clear zone in and/or around the silica plate that contained fraction(s). The fraction having antibacterial activity of S. aureus was dichloromethane (figure 4). This fraction could inhibit S.
*aureus* growth so there was clear zone around the dichloromethane fraction. Further analysis with TLC-bioautographic on dichloromethane fraction showed that there were antibacterial activities on the compounds with Rf values of 0.88, 0.84, and 0.78.

**Figure 3.** Antioxidant activity of *C. cujete* L. fractions.

**Table 2.** Antibacterial activities of several fractions from *C. cujete* L. stem bark.

| Bacteria    | Sample/Fraction(s)       | Inhibition Diameter Zone (IDZ)* (mm) |
|-------------|--------------------------|----------------------------------|
| *E. coli*   | Amoxicillin 500 µg/mL (+) | 10.30^a                          |
|             | 20% DMSO (−)             | -                                |
|             | Water                    | 2.36^b                           |
|             | Ethyl acetate            | -                                |
|             | Dichloromethane          | -                                |
|             | *n*-hexane               | -                                |
| *S. aureus* | Amoxicillin 500 µg/mL (+) | 29.63^c                          |
|             | 20% DMSO (−)             | -                                |
|             | Water                    | -                                |
|             | Ethyl acetate            | 2.72^d                           |
|             | Dichloromethane          | 3.67^d                           |
|             | *n*-hexane               | -                                |

^*Means for homogeneous subsets are displayed (p<0.05)
+ : Positive control − : Negative control - : no inhibition
4. Discussion

Plants have been used for a wide variety of purposes for many thousands of years. In East Java, one of the provinces in Indonesia, most of the people in this area have been used *C. cujete* L. for curing the skin diseases. Some papers reported the biological activities of several parts of this plant (such as: leaves and fruit), except its stem bark. On that account, more scientific investigations are needed to confirm the claimed benefit of this plant, especially in its stem bark. To evaluate the biological activity of this stem bark, some experimental steps were done. The experiment steps were moisture content determination, stem bark extraction, optimum eluent determination, antioxidant and antibacterial activities determinations.

The moisture content determination showed that *C. cujete* L. stem bark powder was 2% (w/w). This content indicated the organic compounds that can be extracted by solvent(s). In addition, moisture content below 10% could inhibit enzymatic processes and damage that caused by microbes. It also would suppress the chemical alterations in the plant cells, so the pharmacological effects of these compounds can be maintained.

Fractionations of the ethanol extract using various solvents resulted in fewer extract yield. Every solvent that has been used in this experimental step represented types of solvent polarities. For example, *n*-hexane is a non-polar solvent, dichloromethane and ethyl acetate are semi-polar solvents (but different index of polarity), and water is polar solvent. According to the experiment, the most effective solvent that obtained more extract was water. Therefore, it can be concluded that the most compound in the extract from stem bark was a polar compound.

For chemical-compounds separation purpose, solvent mixing was performed. This mixture was used as eluent in liquid-liquid separation method. The optimum eluent to separate compounds in ethanol extract was ethyl acetate:*n*-hexane (9:1). This mixture separated more compounds from the ethanol extract than the others. The optimum eluent then applied into TLC-DPPH autography method. This method was conducted to screen antioxidant compounds and to determine antioxidant activity of the extracts.

Identification of antioxidant compounds using TLC-DPPH autography method resulted in a pale yellow ribbon after being sprayed with 0.02% DPPH. The color alteration from purple to yellow was caused by the reduction of 2,2 diphenyl-1-picrylhydrazyl into 2,2 diphenyl-1-picrylhydrazine. According to Mukti [16], the pale yellow color appeared due to the picryl group in the spots. The results also showed that ascorbic acid (positive control) could not eluted with the optimum eluent. It might be caused by the polarity of ascorbic acid. This positive control did not attract by mixed eluents of semi-polar and non-polar solvents. Based on a number of the spots on the plates, dichloromethane fraction contained the most compounds that have potency as antioxidant agents. However, this

![Figure 4. Antibacterial activity of dichloromethane fraction in S. aureus culture.](image)
experiment was qualitative analyses so it could not reveal what the specific substances contained in the fractions are. Oghuagu [2] reported that C. cujete L. contained bioactive compounds, such as quercetin, apigenin, naphthoquinone, anthraquinones, and cardenolides. Biological activities of the fractions were analysed. Antioxidant activity was determined by DPPH method that resulted IC$_{50}$ value. The lower IC$_{50}$ value indicated the higher antioxidant activity. The results indicated that dichloromethane fractions have strong antioxidant activity, the water fraction has moderate antioxidant activity, while the fraction of n-hexane and ethyl acetate have weak antioxidant activities [17]. The highest activity of the dichloromethane fraction was in accordance with by the previous step which revealed that antioxidant constituent in this fraction was the highest than the other fractions. However, there was no correlation between antioxidant activity and yield fraction. In addition, this result showed that antioxidant compounds in C. cujete L. fractions were mostly dissolved in a semi-polar solvent.

Antioxidant activity was measured from the decrease in DPPH uptake at the maximum wavelength (517 nm). The decrease in DPPH uptake is proportional to the concentration of free radical inhibitors (fractions) that added to the DPPH reagent solution [18]. The ability of the fractions to inhibit DPPH might be caused by their phytochemical compounds. In addition, the fractionated extracts were expected to have the higher antioxidant activity than the crude extract(s). Other plants and their derivate extracts were expressed different activities. For example, the antioxidant activity of the crude extract from Syzygium polyanthum was lesser than the antioxidant activity of its flavonoids extract [19,20].

Antibacterial analysis indicated that each fraction has different activity in accordance with the types of bacteria. This result confirmed that Gram-negative bacteria requires more complex inhibition because they have an outer membrane, unlike Gram-positive bacteria. The differences in antibacterial activity were also shown at the positive control treatment. Inhibition Diameter Zone (IDZ) of amoxicillin on the E. coli culture was smaller than on the S. aureus culture. However, this antibiotic could inhibit the bacterial growth by suppressing peptidoglycan formation, both in Gram-positive or Gram-negative [21]. Confirmation by TLC-bioautography method showed that only dichloromethane fraction could inhibit S. aureus growth. The principle of TLC-bioautography is that a developed TLC plate is dipped in a suspension of microorganisms growing in proper nutrient and atmosphere. Under and/or around a silica surface of the TLC where antimicrobial agents were spotted, there would be the inhibition zones of the microorganism growth [22]. In this research, agar disc diffusion method showed antibacterial activity of the water, ethyl acetate, and dichloromethane fractions. Otherwise, TLC-bioautography only showed antibacterial activity on the dichloromethane fractions. Variation of the strains might be caused differing results. Some reports showed that culture medium and strains gave different results [23].

Three compounds in dichloromethane fraction that have antibacterial activities might be similar to the constituents that have demonstrated antioxidant activities in the previous step of this report. Nevertheless, there was no correlation between antioxidant activity and antibacterial activity. It was expressed by the detectable antioxidant compounds, IC$_{50}$ values, and antibacterial activities from the same fraction. In addition, there was also different mechanisms between antioxidant and antibacterial activity. Some bioactive compounds that perform as poisons in one organism, can be used as food in another organism.

5. Conclusion
We concluded that n-hexane, dichloromethane, ethyl acetate, and water fractions of C. cujete L. stem bark have antioxidant compounds. However, there was only dichloromethane fraction that has the highest antioxidant activity of all fractions. The antibacterial assay also revealed that dichloromethane and ethyl acetate fractions could suppress S. aureus growth, while water fraction could inhibit E. coli growth. Antioxidant constituent in the dichloromethane fraction was the highest than all the fractions.
However, there was no correlation between antioxidant activity and yield fraction; as well as antioxidant activity and antibacterial activity.

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