Antioxidant and Antibacterial Assay Against Fish Pathogen Bacteria of *Kjellbergiodendron celebicum* (Koord.) Merr. Leaf Extract

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Abstract

Introduction: *Kjellbergiodendron celebicum* (Koord.) Merr. (local name: *tombe uwa*) is a plant endemic to Sulawesi, Indonesia, and grows around lakes or aquatic environments where fish live. Based on phytochemical screening in previous studies, i.e. methanol extract and ethyl acetate fraction from the leaves of *Kjellbergiodendron celebicum* (Koord.) Merr., the methanol extract gives positive results containing polyphenol compounds in the flavonoid group which have been known to have strong antioxidant and antibacterial properties. Objective: To test the effectiveness of the comparison of the natural content in the compounds (antibacterial and antioxidant properties) and the total content of phenol in *Kjellbergiodendron celebicum* (Koord.) Merr., which was extracted using two methods, i.e. maceration and Ultrasonic-Assisted Extraction (UAE), in fish-disease bacteria. Method: The leaves were separated to be extracted with two different methods: maceration and Ultrasound-Assisted Extraction (UAE). Extracts were first screened qualitatively for antioxidant activity and then quantified with respect to *in vitro* antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the ferric-reducing antioxidant power (FRAP) assay. Antibacterial activity was determined by the paper disc diffusion method and microdilution. Results: 70% Ethanol in leaves extract of *Kjellbergiodendron celebicum* (Koord.) Merr. The extract which has the highest activity based on the DPPH test and FRAP test is the extract from UAE extraction with IC₅₀ value of 9.81512 µg/mL and ferrous equivalent antioxidant capacity (FeEAC) value of 1.661.3 µmol/gr. UAE method also has a higher potential in antibacterial activity based on the diffusion method of paper discs and microdilution with the MIC obtained as much as 390.6 µg/mL. Conclusion: The UAE extraction method is better at scanning polyphenol compounds compared to the conventional maceration extraction method. Therefore, the results of the antioxidant and antibacterial activity using the UAE method are better than the maceration method.

Key words: *Aeromonas hydrophila*, *Edwardsiella ictaluri*, *Flavobacterium columnare*, Maceration, Phytochemical compound, Ultrasonic-Assisted Extraction.
Kjellbergiodendron celebicum (Koord.) Merr. Plant extraction uses two different methods, namely maceration and Ultrasound-Assisted Extraction (UAE) to see whether there are differences in extraction results obtained.

**MATERIALS AND METHODS**

**Chemicals**

Standards used were ascorbic acid (Sigma-Aldrich, A5960; city, state [abbreviation], USA), quercetin (Sigma-Aldrich, Q4951; city, state [abbreviation], USA), gallic acid (Sigma-Aldrich, USA), and chloramphenicol.

**Fish pathogen**

The pathogenic bacteria that will be used in this study are pathogenic isolates Aeromonas hydrophila, Edwardsiella ictaluri, and Flavobacterium columnare, which are the collections of the laboratories for fish disease research and development of the Ministry of Marine and Fisheries of the Republic of Indonesia.

**Sample preparation**

The leaves of *Kjellbergiodendron celebicum* (Koord.) Merr. from Lake Towuti, South Sulawesi, were freshly picked, collected, sorted, and dried (collection and drying were carried out by the Indonesian Research Centre for Ornamental Fish). The leaves were then crushed until they became smaller.

**Microscopic observations by scanning electron microscope (SEM) and light microscope**

Microscopic testing of leaf powder *Kjellbergiodendron celebicum* (Koord.) Merr. using SEM Model: JSM – IT 200 was conducted by the Zoology Field of the Biology Research Centre – Indonesian Institute of Sciences (LIPI), Cibinong, and the observations were also made using a light microscope.

**Extraction**

Extraction was done using maceration and Ultrasound-Assisted Extraction (UAE). Simplisia powder extraction was carried out by the maceration method using 70% ethanol with the ratio of simplisia to solvent 1:10. Extraction was done by soaking the powder for 24 hours in a closed vessel with occasional stirring. This maceration stage was repeated three times. All maceration results were filtered and then collected. Simplicial powder was also extracted using UAE with 70% solvent ethanol with a ratio of 1:10 at 50°C. Both extracted results from maceration and UAE were evaporated with a rotary vacuum evaporator and water bath at 50°C. Calculation of the extraction yield was carried out by dividing the thick extract with the initial simplicial weight (grams), the result of which was expressed in percentage.

\[
\text{Rendement} = \frac{\text{Weight of extract}}{\text{Weight of simplicia}} \times 100\%
\]

**Qualitative antioxidant activity test with DPPH solution**

A qualitative test was carried out by spraying DPPH solution into the sample solution which was bottled on the TLC plate and incubated for 30 minutes. The TLC profile in UV rays was observed with a wavelength of 254 nm.

**Quantitative antioxidant activity test with the DPPH assay**

This assay was carried out using the method of, with minor modifications. The antioxidant activity test was performed on extracts from maceration and UAE (dry matter adjusted to a working solution of 1000 μg/mL in methanol, then diluted to 5 concentrations: 60; 80; 100; 120; and 140 μg/mL) and standards (100 μg/mL quercetin stock solution in methanol). The DPPH solution absorption was measured using a UV-Vis spectrophotometer first. A total of 20 μL of quercetin solution, negative control or sample, was added with 180 μL of DPPH 150 mmol/L solution. The mixture was shaken for 60 seconds in the well, and then the solution was incubated at room temperature for 40 minutes in dark conditions. Absorbance of the test solution was measured at the wavelength obtained in the DPPH maximum wavelength test. The percentage of inhibition of extracts against DPPH was calculated by the following formula:

\[
\text{Percentage inhibition} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100\%
\]

After the inhibition percentage of each concentration was obtained, linear regression was made so that the equation \( y = a + bx \) was obtained, where \( x \) is the concentration (μg/mL) and \( y \) is the percentage of inhibition (%). Antioxidant activity is expressed by 50% Inhibition Concentration or IC50, which is the concentration of the sample which can reduce DPPH radicals by 50% from the initial concentration.

**Quantitative antioxidant activity test with the FRAP assay**

This test was based on the microplate reader method described by with minor modifications, using ammonium ferrous sulphate (AFS) as the standard. Antioxidant activity in the FRAP method was calculated as ferrous equivalent antioxidant capacity (FeEAC) in μmol/L/g, using the equation:

\[
\text{FeEAC (μmol/g)} = \frac{\Delta A}{\text{GRAD}} \times \frac{A v}{\text{Spv}} \times D \times \frac{1}{C} \times 10^5
\]

Where \( \Delta A = \text{pathlength correction value} \), GRAD is the gradient of the AFS calibration curve, \( A v = \text{aliquot volume} \) (300 μL), \( \text{Spv} = \text{test sample volume} \) (20 μL), \( C = \text{sample concentration} \), and \( D = 1 \).

**Antibacterial test with the paper disc diffusion method**

In the antibacterial test, bacterial culture and bacterial suspension of *Aeromonas hydrophila*, *Edwardsiella ictaluri*, and *Flavobacterium columnare* were carried out. Chloramphenicol was used as the positive control. 0.2 g leaf extract of *Kjellbergiodendron celebicum* (Koord.) Merr. from maceration and UAE was dissolved in a tube with 1 mL 5% DMSO solvent. The Mueller Hinton Agar (MHA) media which already contained the inoculum in the test tube was poured into a Petri dish containing 10 ml of MHA media, then homogenized by shaking, and waited until the media solidified. After sterile paper discs were prepared, the extract was dropped onto the paper discs. Negative control using extract sample solvent, DMSO, was dropped on paper discs by 20 μL. Positive control of chloramphenicol 30 μg/mL was also placed on the plate agar. The discs were then incubated for 24 hours at 30°C. The antimicrobial agent diffused into the agar and inhibited the growth of the tested microorganism, and then the diameters of the inhibition growth zones were measured with units of millimetres (mm).

**Antibacterial test with the microdilution method**

Each type of bacteria was tested triply using 96-well microplates. A multilevel dilution of extract solution was carried out in 5% DMSO. Antibiotic control (KB) consisted of 50 μL dilution of chloramphenicol antibiotics with a medium and 50 μL bacterial suspension was also carried out. The negative controls (KN), media control (KM), and germ control (KK) were also carried out, as well as negative control consisting of 50 μL medium and 50 μL 5% DMSO, KM consisting of 100 μL medium, and KK consisting of 50 μL medium and 50 μL bacterial suspension.
Each microplate was covered with a microplate cover and then incubated at 30°C for 24 hours. Bacterial growth could be seen from the turbidity in the well that would be determined using a microplate reader to determine the value of Optical Density (OD) at a wavelength of 600 nm. MIC was determined in units of µg/mL.

**Phytochemical screening**

Phytochemical screening was performed in this study to identify the compound classes present in the extract. The compound classes tested for were terpenoid, alkaloid, anthraquinone, flavonoid, tannin, saponin, and glycosides.

**Total phenolic content determination**

Total phenolic content in the leaf extracts of *Kjellbergiodendron celebicum* (Koord.) Merr. was determined by the Folin–Ciocalteau colorimetric method. The microplate used in the TPC method was based on the 96-well microplate the Folin–Ciocalteau method given by with some modifications. A total of 25 µL of the sample solution or the standard solution was mixed with 100 µL of 1:4 diluted Folin–Ciocalteu reagent and shaken for 60s in a 96-well microplate and incubated for 4 minutes. Then the solution was added with 75 µL of sodium carbonate solution (1%) and shaken for 60s. The solution was incubated within two hours at room temperature. The absorbance was measured at λ 750 nm using a microplate reader 96-well (Versa Max ELISA Microplate Reader, USA). The calibration curve of standards (gallic acid) was measured by the absorbance from the microplate reader instrument and was calculated. The total phenolic content was derived from the calibration curve. The total phenolic contents were expressed as mg gallic acid equivalents (GAE)/g extract.

**RESULTS**

**Sample preparation**

600 grams of simplicial powder of *Kjellbergiodendron celebicum* (Koord.) Merr. leaves were derived from the leaves obtained from Danau Towuti, South Sulawesi, Indonesia. The fresh leaves of *Kjellbergiodendron celebicum* (Koord.) Merr. were processed by wet sorting and washing to separate impurities. After drying, the simplicial powder was sorted dry to remove the remaining impurities or separate the simplicial powder that does not meet the requirements. The simplicial powder was then pollinatated to reduce its size which can enlarge the surface area of simplicia, so that it will facilitate the penetration of solvents during the extraction process, and the extraction of compounds during extraction can give maximum results.

**Microscopic observations by scanning electron microscope (SEM) and light microscope**

Microscopic observations of leaf powder *Kjellbergiodendron celebicum* (Koord.) Merr. were carried out using the Scanning Electron Microscope (SEM) by the Zoology Field of the Biology Research Centre- Indonesia Institute of Science (LIPI), Cibinong, using a light microscope. The use of SEM aims to provide a 3-dimensional picture of the object being observed. The results of the microscopic identification of the dry powder from the simplicial leaf of *Kjellbergiodendron celebicum* (Koord.) Merr. are shown in Figure 1.

**Extraction**

The extraction process of the leaves of *Kjellbergiodendron celebicum* (Koord.) Merr. was completed using the two methods, i.e. maceration and Ultrasound-Assisted Extraction (UAE). The selection of 2 extraction methods was carried out to see whether there was any correlation between the extraction method and the antioxidant and antibacterial activity. The maceration and UAE methods are extraction methods that are inexpensive and easy to do. In the UAE method, extraction of phenolic components can be carried out at frequencies between 20-60 kHz. The extraction was carried out using 70% ethanol. Water and ethanol and their mixtures can be used as solvent fluids in making extracts because they are not toxic. The UAE method of extracting phenolic components uses ethanol with concentrations between 35% and 70%, which will increase with the increasing ethanol concentration. The percentage of the yield of 70% ethanol leaves extract *Kjellbergiodendron celebicum* (Koord.) Merr. is shown in Table 1.

**Qualitative antioxidant activity test with DPPH solution**

The leaf extract of *Kjellbergiodendron celebicum* (Koord.) Merr. tested positive for antioxidant activity. Quantification of the antioxidant activity was then determined using both the DPPH and FRAP assay methods.

**Quantitative antioxidant activity test with the DPPH assay**

Quercetin has been known to have significant antioxidant activity, so it can be used as a positive control to ensure that the chosen method is valid and can be used. IC₅₀ obtained from the linear regression equation was 2.9758 µg/mL. The calibration curve of quercetin for the DPPH antioxidant assay is shown in Tables 2 and 3.

The percentage inhibition of both maceration and UAE extractions can be seen in Table 3. From the linear regression equation, the IC₅₀ obtained from the maceration sample is 11.48336 µg/mL and 9.81512 µg/mL from the UAE sample. The higher IC₅₀ value from the UAE extraction...
The antibacterial activity test using the paper disc diffusion method was based on the principle that antimicrobial agents diffuse into the agar and inhibit the growth of microorganisms tested, and then the diameter of inhibition area is measured.9 The average measurement of the zone inhibition of the maceration and UAE extraction methods for the three microbial assays are presented in Table 4 and Figure 2. Tests were also carried out using positive control of chloramphenicol with a concentration of 30 µg/mL and 5% DMSO solvent as a negative control.

Antibacterial test with the microdilution method

A determination of the Minimum Inhibitory Level (MIC) by the microdilution technique was carried out by diluting the test solution serially on the microplate. The concentrations made were 3.125; 1,562.5; 781.25; 390.6; and 195.3 µg/mL. A positive control of chloramphenicol with a concentration of 30 µg/mL was also determinate. MIC values were determined based on the turbidity measurements which were based on the Optical Density (OD) at 600 nm wavelength which approached the OD measurement results from the chloramphenicol control, assuming that at certain concentrations where the OD values of the test samples were similar to the antibiotics in the test samples which had inhibitory activity against bacterial growth. The MIC data for each microbe are in Table 5. From the MIC data, the extract from the UAE method shows a lower MIC value than the maceration method. This may relate to the total phenolic content present in each extract, which will be discussed further in the next section.

Phytochemical screening

The results of identification can be seen in Table 6.

Total phenolic content determination

Polyphenol compounds have the potential as good antioxidants and antibacterial. Therefore, the determination of total phenolic compounds is important. The measurement of total phenolic content in leaf extract of Kjellbergiodendron celebicum (Koord.) Merr. was performed using the Folin-Ciocalteu method with a microplate reader. The Folin-Ciocalteu method works based on the principle of the tungstate-molybdate complex which is reduced by phenolic compounds,
forming a blue complex that increases absorbance and can be detected spectrophotometry at a wavelength of 750 nm.\textsuperscript{10} The total phenolic content is then expressed in GAE (Gallic Acid Equivalent), which is the amount of equivalence of milligrams of gallic acid contained in 1 gram of the sample (extract) tested.

From the absorption measurements of gallic acid, a linear regression equation $y = 0.2038 + 0.066x$ and the correlation coefficient ($r$) of 0.996544 were obtained. The results of the gallic acid calibration curve are shown in Figure 3. Leaf extract of Kjellbergiodendron celebicum (Koord.) Merr. from the maceration and UAE extraction methods was then examined by the same method as the gallic acid standard. Based on the results of the tests, it shows that the maceration extract contained 224.84 mgEAG/gr extract, while the UAE extracts contained 313.57 mgEAG/gr extract.

The UAE yield extract containing 313.57 mgGAE per gram of extract gave a stronger antioxidant activity than the maceration extract with 224.84 mgGAE per gram of extract. The IC\textsubscript{50} value of the maceration sample obtained in the DPPH antioxidant activity test was 11.48336 µg/mL, whereas from the UAE sample it was 9.81512 µg/mL. The smaller the IC\textsubscript{50} value of a sample was, the stronger the antioxidant activity became. In the antioxidant activity test using the FRAP method, extracts from the UAE method also had a higher reduction capacity than the macerated extracts with samples from the UAE extracts which were 1.661,3 µmol/gr, and 1.581,6 µmol/gr FeEAC from macerated extracts.

In the antibacterial test of the paper disc diffusion method against *Aeromonas hydrophila*, *Edwardsiella ictaluri*, and *Flavobacterium columnare*, the average diameter of the inhibition zone obtained from the UAE method extract was greater than the one from the maceration method extract. The MIC from the UAE method extract was also smaller than the MIC from the maceration method. The UAE extraction method is one of the modern extraction methods recommended for extracting polyphenol compounds. In previous studies, the UAE method had the ability to extract polyphenol components better than the conventional methods such as maceration\textsuperscript{7}. However, to extract

### Table 5: Average MIC of each bacterial tested.

| Bacterial Name       | Extraction Method | Chloramphenicol (30 µg/mL) | Average OD |
|----------------------|-------------------|---------------------------|------------|
|                      | Maceration        | UAE                       |            |
| *Aeromonas hydrophila* | 0,879             | 0,833                     | 390,6      | 0,818 |
| *Edwardsiella ictaluri* | 0,401             | 0,442                     | 390,6      | 0,463 |
| *Flavobacterium columnare* | 0,514         | 0,413                     | 390,6      | 0,486 |
|                      | Average OD        | MIC (µg/mL)               |            |
| Maceration           | 781,25            | 390,6                     |            |
| UAE                  | 781,25            | 390,6                     |            |

### Table 6: Phytochemical screening result.

| Compound     | Reagent                  | Result                  | Conclusion |
|--------------|--------------------------|-------------------------|------------|
| Alkaloid     | Bouchardat               | No precipitation       | (-)        |
|              | Mayer                    | No precipitation       | (-)        |
|              | Drageendorff             | No precipitation       | (-)        |
| Tannin       | Gelatin 10%              | Precipitation formed   | (+)        |
|              | FeCl\textsubscript{3}    | Green                   | (+)        |
| Saponin      | Hot water + HCl          | Froth formed            | (+)        |
|              | UV & AI\textsubscript{3} | Yellow-green fluorescence | (+) |
| Flavonoid    | HCl + Mg                 | Red                     | (+)        |
|              | HCl + Zn                 | Red                     | (+)        |
| Terpenoid    | Liebermann-Burchard      | Green                   | (+)        |
| Anthraquinon | Sulfuric acid + Benzene + NaOH | Red layer            | (+)        |
| Glycoside    | Anhydrous acetic acid & sulfuric acid | Purple ring | (+)        |
|              | Molisch                  | Green                   | (+)        |

Figure 3: Gallic acid calibration curve.
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Polyphenol compounds using the UAE method, we must pay attention to several factors, such as solvents, frequencies used, and temperature at extraction.

**CONCLUSION**

70% ethanol leaf extract of *Kjellbergiodendron celebicum* (Koord.) Merr. which has the highest activity based on the DPPH and FRAP tests is an extract from the UAE extraction with IC$_{50}$ value of 9.81512 µg/mL and FeEAC value of 1.661.3 µmol/gr. The UAE method also has a higher potential as antibacterial activity based on the diffusion method of paper discs and microdilution with the MIC obtained 390.6 µg/mL. The UAE extraction method is better at scanning polyphenol compounds compared to conventional maceration extraction methods. Therefore, the results of the antioxidant and antibacterial activity of the UAE method are better than those of the maceration method. Leaf extract of *Kjellbergiodendron celebicum* (Koord.) Merr has strong antioxidant activity and has strong antibacterial activity against fish pathogenic bacteria *Aeromonas hydrophila*, *Edwardsiella ictaluri*, *Flavobacterium columnare*.

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**CONFLICTS OF INTEREST**

The authors declare that there are no conflicts of interest in this study.

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