EVALUATION OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF XANTHONE ISOLATED FROM Orophea corymbosa LEAF

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ABSTRACT
The pharmacological properties (antibacterial and antioxidant) of the ethyl acetate extract from the leaves of Orophea corymbosa Blume Miq. the plant was evaluated and continued for obtaining bioactive compounds through the isolation process. A pure isolate from ethyl acetate extract was analyzed of structure compound using NMR Spectra, FTIR, UV-Vis, and MS (GCMS). An isolated compound was considered to be xanthone derived compound, namely 1-hydroxy-2,3-dimethoxyxanthone. Its activities against Gram-negative and positive bacteria were examined with bioindicator Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923, respectively, where the results were then compared to cephazolin; and antioxidant activity with indicator free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) compared to ascorbic acid. The xanthone compound has good activity as antibacterial and antioxidant. The plant has administrated the potential for handling bacteria and free radicals in this study.

Keywords: Annonaceae, Antibacterial, Antioxidant, Escherichia coli, Staphylococcus aureus, Xanthone.

INTRODUCTION
The application of natural products as medical objects is still in high demand. The natural product is a favorable choice with fewer side effects, safer, easy to find, and green chemistry. The discovery of natural products, especially in a tropical and subtropical country, can be simply found in plants. The plants were widely reported to have medicinal property from their metabolites, which are produced by plant or endophytic bacteria as self-defense against pathogens. The metabolites have a lot of medical activities as an antibacterial, antioxidant, antimicrobial, antifungal, wound healing, and antibiofilm agents. The use of plant parts such as a leaf, roots, and even fruit has been traditionally done by indigenous peoples since long time ago. Furthermore, to obtain the metabolite can be simply done by extraction.

The Annonaceae family is widespread in diverse habitats (forests, shrubs and grasslands) in both tropical and subtropical areas and is abundant in rainforest areas around the world. Many genera from the family were found to have medicinal properties against ulcers, fever, infection, abdominal pains, bronchitis, asthma and wounds. Moreover, they also have pharmacological activities like antifungal, antibacterial, antimalarial, antioxidant and anticancer agents. Bioactive compounds from plants (i.e xanthone) play an important role in terms of medicine.
framework (two aromatic rings and one heterocyclic ring) (Fig.-1), which can contain different substituent groups. Pharmacological activities of xanthones are widely reported, including antioxidant and antimicrobial activities. Several plant species of this family have been successfully isolated to obtain xanthone compounds. One of the species of the Annonaceae family, *Orophea corymbosa* Blume Miq., frequently find in the Gayo Lues area. The leaves of the plant have been commonly used to cure dysentery, type 2 diabetes, fever, nausea, vomiting and vertigo by the local people from generation to generation. It isn't reported on the study on the isolation of the compounds as raw material for medicines. So far, simply publications of extract activity were found via internet searching that labeled the plant has properties of antimicrobial, muscarinic receptors, lipoxygenase inhibiting, antiplasmodial, and antioxidant. For this reason, the objective of this work is to study the antibacterial and antioxidant activities of the xanthone compound isolated from *O. corymbosa* leaves.

**EXPERIMENTAL**

**Material and Instrumentation**

Leaves of *O. corymbosa* were collected from Gayo Lues District (3° 53’ 47,556” N 97° 25’ 43,526” E), Aceh Province, Indonesia. The sample was determined by the Research Center for Biology at the Indonesian Institute of Sciences (LIPI), Bogor, Indonesia. Its voucher for the specimen was stored under code 13/Jerig. It is also known as *kayu Kijang* by Indonesian. Materials were *n*-hexane 96%, ethyl acetate 96%, methanol 96%, deuterated chloroform (CDCl₃), silica gel G 60, TLC plate preparative (silica gel 60 F₂₅₄), free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), bacteria of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, methanol 99.9%, dimethylsulfoxide (DMSO) 99.9%, ascorbic acid 99.7%, reagen FeCl₃, aquadest, NaCl 0.85%, 30 µg cephalozin (disc diameter of 6 mm), Muller Hinton Agar (MHA), Lactose Broth (LB), paper disc (disc diameter of 6 mm), and Whatman filter paper grade 41. Experiments were carried out with a rotary evaporator, shaker, spectrophotometer, ultraviolet lamp, NMR, incubator, FTIR, GCMS, UV-Vis, and autoclave instruments.

**Extraction Plant**

The dried leaves of *O. corymbosa* were mashed and weighed as much as 1.5 kg. It was extracted in stages by maceration technique using either of *n*-hexane 96%, ethyl acetate 96%, and methanol 96% solvents (3 × 2.5 L). The filtrates were evaporated to yield of 34.6 g of *n*-hexane, 68.2 g of ethyl acetate, and 151.4 g of methanol extracts, respectively.

**Isolation and Elucidation of Compound**

As much as 30 grams ethyl acetate extract was further processed with compound isolation procedure using column chromatography (diameter of column = 5 cm) with 400 grams of silica gel G 60 as stationary phase and *n*-hexane: ethyl acetate as mobile phase. The polarity of the mobile phase in the chromatography column process was increased until *n*-hexane: ethyl acetate (5:5, v/v). Furthermore, the pure isolate was obtained after the purification using re-chromatography, preparative TLC, and recrystallization. The characterization of the pure isolate was carried out with ¹H, 500 MHz, and ¹³C, 125 MHz of 1D-NMR; HSQC, and HMBC of 2D-NMR; GCMS; UV-Vis; as well as FTIR spectrophotometers, which was identified as a xanthone compound (Fig.-2).

**Antioxidant Assay**

The antioxidant assay was carried out by scavenging DPPH method. A xanthone compound and positive control (ascorbic acid) were formulated at 5 various concentrations, ranging from 2 to 10 (µg/mL), in methanol solvent 99.9%, respectively. DPPH solution (0.1 mM) as much as 11 mL was prepared in
methanol solvent 99.9%. A 1 mL solution of DPPH 0.1 mM was dropped in 3 mL of varying concentrations of the xanthone compound and the positive control, respectively. A solution of DPPH 0.1 mM (1 mL) was dropped in methanol solvent 99.9% (3 mL) as a negative control and 4 mL of methanol solvent 99.9% as a blank. All various concentrations, respectively after incubation for half an hour in a dark room at room temperature was read absorbance by a spectrophotometer at 517 nm. The ability to scavenge the DPPH radical was counted by the formula: % Inhibition = [(Ao-A) /Ao] × 100. Note, Ao (absorbance of negative control), A (absorbance of a xanthone compound and positive control of varying concentrations).

Antibacterial Assay
Each bacterial strain was inoculated using the subcultures method. The bacterial suspension was taken from LB liquid media stock (0.26 gram dissolved into 20 mL aquadest) as much as 1 mL for each bacterium (E. coli and S. aureus) and added to respective LB medium (0.39 gram dissolved in 30 mL aquadest). The bacterial suspension in LB media has been shaken for a night at 37°C to get fresh bacteria. An 0.85% (10 mL) NaCl solution were prepared to suspend fresh bacteria and the absorbance was measured with a spectrophotometer at OD600 to obtain a value of 0.5-0.8 (to get 10⁸ CFU/mL).

The assay of antibacterial was through by disc diffusion on agar media method. A sterile solution of MHA 20 mL was spilled into a glass petri dish with size 100 × 15 mm and allowed to harden. In the next step, both fresh bacteria (E. coli and S. aureus) respectively, were spread on agar media in a glass petri dish. Paper discs have the substance of various concentrations of xanthone compound at 30, 60, and 120 (µg/mL); negative control discs have content of DMSO solvent; and positive control discs (cephazolin) were arranged for media overgrown with bacteria. The incubation of bacteria at 37°C has taken 24 hours. Antibacterial test results are the inhibition zone that has been measured in milliliters (mm) using a ruler.

Statistical Analysis
A simple analytical software (Microsoft Excel 2010) was employed to analyze the results from the antioxidant and antibacterial assay. The data were provided in terms of mean and standard deviation (mean±standard deviation). For representing the result of the concentration variation (µg/mL) on the inhibition (%), linear regression on the antioxidant test has been utilized to calculate the IC₅₀ value.

RESULTS AND DISCUSSION

Structural Elucidation
A pure isolate has been elucidated by UV, IR, MS and NMR spectroscopy and indicated the xanthones compound skeleton. Based on structural elucidation, the pure isolate compound isolated in the O. corymbosa plant was the same as the 1-hydroxy-2,3-dimethoxyxanthone compound (Fig.-2) isolated from the Polygala arillata plant. However, 1-hydroxy-2,3-dimethoxyxanthone compound is a compound that has been found for the first time in the O. corymbosa plant. In the previous report, the structural elucidation of a known compound of 1-hydroxy-2,3-dimethoxyxanthone using NMR was limited only to the 1H-NMR. Therefore, this study displays the results of the elucidation with additional parameters of NMR, namely 13C-NMR, HSQC, and HMBC.

The pure isolate has characteristic yellowish needles, m.p 215-218 ºC, and UV spectra have shown λmax = 201.8, 365 and 383 (in methanol). The spectrum of IR (KBr) has detected the attendance of a carbonyl group C=O (1697 cm⁻¹), hydroxy group -OH (3487 cm⁻¹) and aromatic ring C-benzene (900-650 cm⁻¹). The GCMS data have given m/z, 272, 257, 210, 181, 153 and 111.
As presented in Table-1, the ¹H NMR spectrum indicated the detected 12 protons. Two methoxy protons as singlet signals were detected with δH 4.16 ppm for OCH₃-2 and δH 4.61 ppm for OCH₃-3. Five signals of aromatic protons consisted of one singlet signal with a δH 7.14 ppm (s, 1H, H-4). Four multiplet aromatic protons were δH 9.21 ppm (m, 1H, H-5), δH 7.54 ppm (m, 1H, H-6), δH 7.55 ppm (m, 1H, H-7) and δH 7.82 ppm (m, 1H, H-8). One hydroxy (OH) group was identified at δH 8.10 ppm as a singlet signal. The ¹³C NMR spectrum (Table-1) had shown signals from 15 carbon atoms consisting of 5 aromatic carbons, 4 substituted aromatic carbons, 2 methylated phenolic carbons, 1 phenolic carbon, 2 methoxy carbons, and 1 carbonyl carbon with chemical shifts (δC, ppm), respectively C-4 (105.22), C-5 (126.75), C-6 (125.18), C-7 (126.70), C-8 (128.80) were aromatic carbon; C-4a (135.35), C-8a (116.63), C-9a (122.78), C-10a (133.60) were substituted aromatic carbons; C-2 (149.29), C-3 (147.42) were methylated phenolic carbons; C-1 (142.46) was phenolic carbon; OCH₃-2 (60.34), OCH₃-3 (63.47) were methoxy carbon and C-9 (167.11) was carbonyl carbon.

The elucidation of the structure with ¹H-NMR and ¹³C-NMR can be clarified by 2D-NMR (HSQC, and HMBC). The HSQC spectrum has shown that the proton of methoxy at δH 4.16 ppm correlates with carbon at δC 60.34 ppm and the methoxy proton at δH 4.61 ppm correlates with carbon at δC 63.47 ppm. Furthermore, the signals from the 5 protons that appear to correlate with aromatic carbon. The HMBC spectrum shows a correlation between proton and carbon (see Fig.-3). The proton of the methoxy group, namely OCH₃-2 (δH 4.16 ppm, s, 3H) and OCH₃-3 (δH 4.61 ppm, s, 3H), correlated respectively to aromatic carbon at C2 (δC 149.29 ppm) and C3 (δC 147.42 ppm). A correlation also appeared from H-4 (δH 7.14 ppm, s, 1H) to C-4a (δC 135.35), C-9a (δC 122.78); H-5 (δH 9.21 ppm, m, 1H) to C-6 (δC 125.18), C-7 (126.70); and H-8 (δH 7.82 ppm, m, 1H) to C-7 (δC 126.70).

GCMS data of this compound produced m/z 272. Its MS² fragmentation produced two peaks at m/z 257 (loss of CH₃) and 210 (loss of two OCH₃), respectively. The precursor of ion MS² m/z 210 obtained MS³ at m/z 181 and 193 suggested lost the COH and OH, respectively. The precursor at MS³ m/z 181 yielded in

**Table-1:** ¹H-NMR and ¹³C-NMR Spectra of Pure Isolate and Reference of 1-Hydroxy-2,3-dimethoxyxanthone

| Position | Pure Isolate | ¹³C-NMR Spectrum of Pure Isolate | ¹³C-NMR Spectrum of Reference ¹³C-NMR Spectrum of 1-Hydroxy-2,3-dimethoxyxanthone |
|----------|--------------|---------------------------------|----------------------------------|
|          | δH (ppm) (CDCl₃, 500 MHz) | δC (ppm) (CDCl₃, 125 MHz) | δH (ppm) (CDCl₃, 60 MHz) | δC (ppm) |
| 1        | 8.10 (1H, s, OH) | 142.46 | 12.74 (1H, s, OH) | - |
| 2        | - | 149.29 | - | - |
| 3        | - | 147.42 | - | - |
| 4        | 7.14 (1H, s) | 105.22 | 6.72 (1H, s) | - |
| 4a       | - | 135.35 | - | - |
| 5        | 9.21 (1H, m) | 126.75 | 8.28 (1H, m) | - |
| 6        | 7.54 (1H, m) | 125.18 | 7.70-7.20 (1H, m) | - |
| 7        | 7.55 (1H, m) | 126.70 | 7.70-7.20 (1H, m) | - |
| 8        | 7.82 (1H, m) | 128.80 | 7.70-7.20 (1H, m) | - |
| 8a       | - | 116.63 | - | - |
| 9        | - | 167.11 | - | - |
| 9a       | - | 122.78 | - | - |
| 10a      | - | 133.60 | - | - |
| OCH₃-2   | 4.16 (3H, s) | 60.34 | 3.95 (s) | - |
| OCH₃-3   | 4.61 (3H, s) | 63.47 | 4.02 (s) | - |
fragmentation of MS\(^4\) with a value of m/z 153 (lost CO). The precursor of the ion at MS\(^3\) m/z 193 resulted in MS\(^4\) m/z 111 (lost COH, CO, and C\(_2\)H). The possible fragmentation patterns that occur from the compound are described in Fig.-4.

![Chemical structures showing fragmentation patterns](image)

**Antioxidant Activity**

The strength of antioxidant activity was categorized in Table-2. The antioxidant activity came from the performance of its secondary metabolites, mainly from the flavonoid, alkaloid, phenol, and saponin substances. The substances worked to scavenge the free radical of DPPH by reducing DPPH to 1,1-diphenyl-2-picrylhydrazine compound. The qualitative observation confirmed that the DPPH solution color switched from purple to yellow. The intensity of the color change showed the potential for free radical capture by antioxidant agent.\(^{45}\)

| Activity       | Strength Category          | Ref. |
|----------------|---------------------------|------|
| Antioxidant    | <100 µg/mL (strong)       | 43   |
|                | 101-150 µg/mL (moderate)  |      |
|                | 151-200 µg/mL (weaker)    |      |
| Antimicrobial  | ≥20 mm (strong)           | 44   |
|                | 12 mm - 20 mm (moderate)  |      |
|                | ≤12 mm (weak)             |      |

Table-2: Category of Strong Antioxidant and Antimicrobial Activities
The antioxidant activity of xanthone compound can be determined by IC_{50} value. Linear regression analysis of the results of the concentration variation (µg/mL) on the inhibition (%) was used to calculate the IC_{50} value in the antioxidant test (Fig.-5). The xanthone compound had shown very strong antioxidant properties with the resulted of an IC_{50} value of 4.21 µg/mL. Many reports of xanthone compounds have antioxidant activity. In this study, for the first time the antioxidant property of 1-hydroxy-2,3-dimethoxyxanthone was reported.

Antibacterial Activity
The strength classification of antimicrobial activity based on the inhibition zone values has categorized in Table-2. The antibacterial action of the secondary metabolites could be assumed in several ways viz. Disturbing the function and structure of membranes, interfering with the synthesis/function of DNA/RNA, and inhibiting cell wall synthesis or protein. The xanthone compound indicated moderate activities against both used bacteria with an average inhibition zone value ranged (mean ± SD) from 14.34 ± 0.65 mm to 19.33 ± 0.58 mm (E. coli) and 11.98 ± 0.02 mm to 14.95 ± 0.09 mm (S. aureus) respectively, for concentrations ranging 30 to 120 (µg/mL). Many reports that the xanthone compounds have antibacterial activity. However, the 1-hydroxy-2,3-dimethoxyxanthone compound was first tested for antibacterial.

CONCLUSION
The xanthone compound (1-hydroxy-2,3-dimethoxyxanthone) was successfully afforded from the isolation of ethyl acetate extract of O. corymbosa. The xanthone has very strong antioxidant and moderate antibacterial activities. Hence, the O. corymbosa plant could be verified as a source of medicinal raw materials to administrate the bacteria and free radicals.

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