

ISOLATION AND CHARACTERIZATION OF ANTIOXIDANT COMPOUNDS FROM ETHYL ACETATE AND METHANOL EXTRACTS OF *GARCINIA DAEDALANTHERA* PIERRE LEAVES

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ABSTRACT

Objective: This study aimed to isolate and characterize the compounds responsible for the high antioxidant activities of the ethyl acetate and methanol extracts of *Garcinia daedalanthera* Pierre leaves.

Methods: In this study, the ethyl acetate extract was obtained by column chromatography, and the methanol extract was obtained by vacuum column chromatography. The mobile phase comprised *n*-hexane, ethyl acetate, and methanol with increased polarity. Antioxidant activity was examined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The fraction with the highest antioxidant activity was purified through column chromatography, recrystallization, and preparative thin-layer chromatography. This fraction, termed the isolate of B, was identified using DPPH and $AlCl_3$, and its antioxidant activity was quantitatively tested.

Results: From this research, 21.7 mg of the isolate of B were obtained with an IC_{50} of 5.82 μ g/mL. Identification using an $AlCl_3$ sprayer produced yellow phosphorescent spots under UV light. UV-Vis spectrum analysis revealed the presence of an aromatic compound and conjugated double bonds. Infrared spectrum analysis revealed the presence of -OH, C-H alkane, C=C aromatic, C=O, and C-O-C groups.

Conclusion: Based on this research, 21.7 mg of the isolate of B was derived through fractionation of the methanol extract, and this isolate exhibited antioxidant activity with an IC_{50} of 5.82 μ g/mL. The isolate of B was considered to be a flavonoid, as it was fluorescent under UV light (366 nm) after being sprayed with $AlCl_3$ reagent.

Keywords: Antioxidant, 1,1-diphenyl-2-picrylhydrazyl, Isolation, *Garcinia daedalanthera* Pierre.

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INTRODUCTION

Herbs have been widely used as alternative or adjunctive therapies in recent years. The use of natural medicines has increased due to their fewer side effects than that of synthetic drugs [1]. *Garcinia* is one of the genera that are widely studied. This plant is an essential component of forests, and it is widely distributed among all islands of Indonesia. Of the approximately 200 *Garcinia* species found worldwide, nearly 100 are found in Indonesia, primarily in Kalimantan followed by Sumatra and Sulawesi [2,3].

Prior studies have identified several compounds in *Garcinia* with biological and pharmacological activities such as anti-inflammatory, antimicrobial, and antifungal activities. Moreover, the compounds also inhibited the activities of xanthine oxidase and monoamine oxidase enzymes and induced antioxidant effects [4]. Antioxidants are compounds which, when present in smaller amounts than other oxidizable substances, could prevent or delay the oxidation of the substance [5]. It is hazardous when the oxidation occurs because it might result in various diseases such as atherosclerosis, diabetes, tumors, heart disease, and accelerate the aging process [6]. One of the causes of oxidation is free radicals. Free radicals are molecules with one or more unpaired electrons that attack nearby stable molecules nearby for acquiring its electrons [7].

Garcinia daedalanthera Pierre is one of the *Garcinia* species found in Indonesia. Based on previous research on *G. daedalanthera* Pierre leaves, the extract of *n*-hexane contains alkaloid compounds, whereas that of ethyl acetate has alkaloids, terpenes, saponins, and flavonoids. Moreover, the methanol extract contained alkaloid compounds, tannins, flavonoids, and saponins. In prior research, the *n*-hexane, ethyl acetate,

and methanol extracts had antioxidant activity with IC_{50} values of 56.78, 9.04, and 12.83 μ g/mL, respectively [8-11].

Based on these data, this study aimed to isolate and characterize the compounds responsible for the high antioxidant activities of the ethyl acetate and methanol extracts of *G. daedalanthera* Pierre leaves. The results of this study are expected to increase knowledge regarding the antioxidant activity of *G. daedalanthera* Pierre considering the limited number of reports on this plant, and the data should be useful for developing traditional medicines.

METHODS

Tools

The instruments used in this research included a vacuum rotary evaporator (Janke and Kunkel IKA, Germany), water bath, aluminum thin-layer chromatography (TLC) and preparative TLC glass plates (Merck, TLC Silica Gel 60 F₂₅₄, Germany), column chromatography equipment, UV-Vis spectrophotometer (T80 + UV/VIS Spectrometer, PG Instruments Ltd., China), analytical scales (A and D Company, Japan), vials and container bottles of various sizes, micropipettes (Eppendorf), sonicator (Elmasonic S 60 H), quartz cuvettes (Merck, Germany), incubator, vortex mixer (VM-2000 models), melting point apparatus (Stuart, Scientific), infrared spectrophotometer (Fourier transform infrared, Prestige-21, Shimadzu, Japan), reaction tubes of various sizes, and volumetric flasks of various size (Pyrex, Japan).

Materials

Concentrated extracts of ethyl acetate and methanol of *G. daedalanthera* Pierre were obtained from the Phytochemistry Laboratory of the Faculty of Pharmacy, Universitas Indonesia. The other materials included

n-hexane, ethyl acetate, and technical methanol (Brataco) that had been distilled; 1,1-diphenyl-2-picrylhydrazyl (DPPH, Wako, Japan), silica gel 60 (70–230 mesh, E. Merck 1.07734, Germany), silica gel 60H (E. Merck 1.07736, Germany), Celite (Merck, Germany), and concentrated sulfuric acid (Merck, Germany).

Fractionation

Fractionation by column chromatography was performed using 25 g of the ethyl acetate extract, whereas 25 g of the methanol extract of *G. daedalanthera* Pierre leaves was fractionated using vacuum column chromatography. The stationary phase was silica gel, whereas the mobile phase comprised n-hexane, ethyl acetate, and methanol with increased polarity. The separation profile of the obtained fractions was observed using TLC. Further, fractions with similar profiles were merged.

Antioxidant activity assay

The antioxidant activity of each fraction was assessed qualitatively and quantitatively. In the qualitative assay, DPPH spray reagent was prepared at a concentration of 100 µg/mL. Meanwhile, the sample solution was prepared by dissolving 10 mg of each fraction into methanol in a 10 mL volumetric flask. Further, approximately 5 µL of each solution was dripped onto chromatography paper and sprayed on using DPPH. The ability to remove the purple color from the DPPH solution and produce a yellow color on the dripped area indicated high antioxidant activity [12].

Antioxidant activity was quantitatively assayed using fractions with antioxidant activity in the qualitative assay. The solution of each fraction was prepared at the same final concentration of 6.25 µg/mL. Nearly 1 mL of the solution was added to a reaction tube that had been covered with aluminum foil. Then, 1 mL of DPPH solution and 2 mL of methanol were added to the reaction tube. The mixture was homogenized using vortex for 10 s and incubated at 37°C for 30 min. Then, the absorption of the mixture was measured at a wavelength of 516 nm. Further, the percentage of inhibition was calculated by dividing (Absorbance of blank – Absorbance of sample) with absorbance of blank, then times 100%.

Purification

The fraction with the highest percentage of inhibition at the final concentration was then purified. A total of 3.65 g of the fraction were purified through advanced column chromatography. The stationary phase was silica gel, and the mobile phase comprised ethyl acetate and methanol with increased polarity. Then, TLC was performed on the obtained subfractions using an ethyl acetate:methanol eluent ratio of 90:10. Subfractions with the same profile were then mixed.

The qualitative and quantitative assays were performed using the mixed subfractions. The working process was the same as the antioxidant activity assay. The subfraction with the highest percentage of inhibition was purified through recrystallization and preparative TLC. In recrystallization, n-hexane and ethyl acetate were used as solvents. The purity of crystals produced through recrystallization was examined using TLC with an ethyl acetate: methanol eluent ratio of 90:10.

Then, purification continued through preparative TLC performed using the Semwal, Rawat, and Singh method. In total, 25 mg of impure crystals were dissolved in 5 mL of ethyl acetate and then dripped onto the TLC plate for elution using ethyl acetate and methanol at a ratio of 90:10. After the elution, the preparative TLC glass plate was dried and observed, and the edge of the plate was sprayed using DPPH solution to identify the spot that was positive for antioxidant activity. Spots with antioxidant activity were scrapped and filtered using Celite.

Purity test and characterization of isolates

The obtained isolates were then tested for purity using two-dimensional TLC and melting point test. In two-dimensional TLC, a single spot indicated purity. The isolate was completely dissolved in methanol. The isolate solution was dripped onto a TLC plate and then eluted in a reaction vessel that had been previously saturated using ethyl acetate-methanol eluent

(ratio=90:10). After the elution process was complete, the plates were dried, and the spots were examined under UV light at wavelengths of 254 and 366 nm. The spots were marked with a pencil, and the plates were rotated 90° and reeluted in a reaction vessel that contained n-hexane-ethyl acetate eluent (ratio=1:99). After elution, the plates were dried and sprayed using AlCl₃ reagent. Further, the spots were reexamined under UV light at 254 and 366 nm, and the R_f value was also calculated. The melting point test was performed to determine whether the purity of the obtained isolates. Pure isolates have a narrow melting range of 2°C.

Characterization of the obtained isolates was performed using UV-Vis and infrared spectrophotometer. The UV-Vis spectrum was examined by weighing 1 mg of the isolate, dissolving it in methanol, and performing spectrometry at wavelengths of 200–600 nm. The UV-Vis spectrum was examined to determine the maximum wavelength and the absorption spectrum. Meanwhile, the infrared spectrum was examined by grinding 1 mg of isolate with 49 mg of KBr until it became homogeneous and measured its infrared absorption.

RESULTS AND DISCUSSION

Fractionation and antioxidant activity assay

A total of 10 compound fractions were obtained through fractionation of the ethyl acetate extract (E1–E10). Meanwhile, eight compound fractions were obtained from the fractionation of the methanol extract (M1–M8).

Among the ethyl acetate fractions, E1, E2, E3, E5, E9, and E10 exhibited scavenging activity in response to DPPH radicals, whereas all methanol fractions excluding M3 displayed scavenging activity. M6 exhibited the greatest antioxidant activity (42.62% inhibition). This compound was selected for purification using advanced column chromatography.

Purification

A total of 10 compound subfractions were obtained through purification of the M6 fraction (A–J). Based on the qualitative antioxidant activity assay, all of the subfractions exhibited a scavenging response to DPPH radicals. Furthermore, subfractions A, B, and C exhibited similar antioxidant activity (43.96, 46.49, and 46.13% inhibition, respectively). Using TLC, three spots were observed for subfractions A and C, whereas two spots were observed for B. Based on the TLC result, purification was continued using subfraction B.

Purification of subfraction B was performed using n-hexane-ethyl acetate solvent, and purity of the crystal was assessed using TLC. TLC revealed that the crystal was not pure because two spots were produced. Both spots were then sprayed using DPPH solution to determine which spot was positive for antioxidant activity. Only one spot exhibited the ability to scavenge DPPH radicals. The spot with antioxidant activity was scrapped, dissolved in ethyl acetate, and filtered using Celite. In total, 21.7 mg of an odorless and yellowish-white isolate was produced through preparative TLC. The isolate was termed isolate of B.

Purity test of isolate

The isolate of B was found to have a melting range of 205–207°C. The result indicated that the isolate was pure due to its narrow melting range of 2°C [13]. Then, a single spot was obtained using two-dimensional TLC (Fig. 1a). The result indicated that the isolate of B was extremely pure. Next, spraying using AlCl₃ reagent revealed that the isolate was a flavonoid because a yellow spot formed and yellow phosphorescent areas were visible on exposure to UV light at a wavelength of 366 nm (Fig. 1b). In the first elution process using ethyl acetate-methanol eluent (90:10), the R_f value was 0.6, whereas the second elution using n-hexane-ethyl acetate (1:99) resulted in an R_f value of 0.3.

A quantitative antioxidant activity assay using the isolate of B revealed an IC₅₀ of 5.82 µg/mL (Table 1).

Characterization of isolates

The UV-visible spectrum of the isolate of B revealed absorption peak of 3.487 at 233 nm, 3.178 at 290 nm, and 2.259 at 345 nm. These three

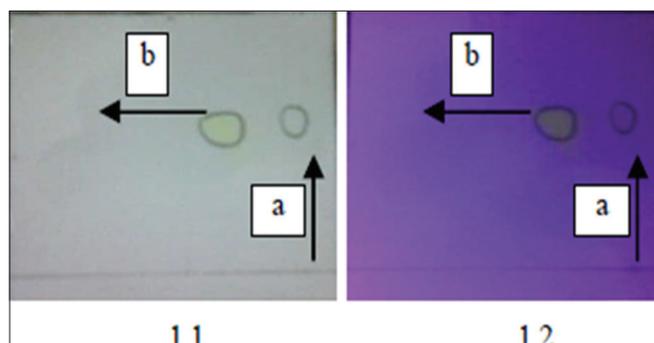


Fig. 1: (a and b) The result of two-dimensional thin-layer chromatography. (a) The direction of elution I using ethyl acetate-methanol eluent at a ratio of 90:10 (Rf = 0.6). (b) The direction of elution II using n-hexane-ethyl acetate eluent at a ratio of 1:99 (Rf = 0.3)

Table 1: The IC₅₀ of the isolate of B

Sample	Concentration (µg/mL)	% inhibition	IC ₅₀ (µg/mL)
The isolate of B	0.25	9.692	5.82
	1.25	17.385	
	2.5	25.077	
	3.75	35.231	
	5	42.415	
	6.25	54.462	

peaks were characteristic of aromatic compounds and conjugated double bonds in the structure of the isolate [14].

In the infrared spectrum, the isolate of B produced an absorption band with a width of 3595–3203 cm⁻¹, which was the vibration range of hydroxyl groups (–OH) bound by aromatic rings. At wavelength of 2937 and 2854 cm⁻¹, strong absorption bands denoting the bending vibration of an alkane C-H group were identified. The sharp and moderate absorption band at a 1647 cm⁻¹ and the sharp and strong absorption band at 1458 cm⁻¹ indicated the presence of a C=C aromatic group [14].

CONCLUSION

Based on this research, 21.7 mg of the isolate of B was derived through fractionation of the methanol extract, and this isolate exhibited

antioxidant activity with an IC₅₀ of 5.82 µg/mL. The isolate of B was considered to be a flavonoid, as it was fluorescent under UV light (366 nm) after being sprayed with AlCl₃ reagent.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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