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The Analysis of the Secondary Metabolite Compounds and Antioxidant Activity of Ethyl Acetate Extracts from the Stems of *Bouea macrophylla* Griff

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Abstract. Gandaria (*Bouea macrophylla* Griff) is a potential source of antioxidant compounds and this study was conducted to analyze the secondary metabolite constituents and antioxidant activity of the ethyl acetate extracted from *B. macrophylla* stem. The extracts were isolated and fractioned using chromatography techniques such as thin-layer chromatography (TLC) and column chromatography (CC). Moreover, the composition of the secondary metabolites was determined by Liquid Chromatography-Mass Spectrometry (LC-MS) while the antioxidant property of the extracts and fractions was obtained using the 1,1-diphenyl-2-picrylhidrazil (DPPH) method. The ethyl acetate extract was separated with CC and 8 subfractions represented by codes E1-E8 were obtained with E1 subfraction observed to have the most active fraction on the antioxidant assay at a moderate activity with an IC_{50} value of 232.96 ppm. E1 was further analyzed with LC-MS and two major compounds found were lauric diethanolamide and loganine with their synergy effects suggested to be the reason for the subfraction's moderate antioxidant activity. However, E1 requires purification to determine the most active antioxidant compound isolated from ethyl acetate extract of *B. macrophylla* stem.

INTRODUCTION

Free radical is one of the causes of several degenerative diseases such as cardiovascular disease, hypertension, stroke, liver cirrhosis, cataracts, diabetes mellitus, and cancer and an example is the Reactive Oxygen Species (ROS) which is a free radical commonly produced in biological systems through physiological and pathological processes [1]. It is possible to overcome free radicals normally by endogenous antioxidants but when their production exceeds the ability of exogenous antioxidants they usually accumulate in the body, thereby, requiring important substances such as antioxidants from another source. Antioxidants are obtainable from either synthetic or natural sources and an example of the natural source widely used is the *Bouea macrophylla* Griff plant which is locally known as gandaria. The chemical compounds contents of this plant including the alkaloids, terpenoids, phenolics, flavonoids, quinones, tannins, and saponins have been published by a researcher [2]. Moreover, Rajan and Bhat [2] reported the methanol extract of *B. macrophylla* fruit to have antioxidant activity with an IC_{50} value of 16.29 ppm while the ethanol extract of its bark had 20.03 ppm [3]. Previous studies have been conducted on the total phenolic content (TPC) and the flavonoid, as well as the antioxidant activity of stem extract of *B. macrophylla* [4], and the values of these properties for the n-hexane, ethyl acetate, and methanol extracts, are presented in Table 1 [4].

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TABLE 1. Total Phenolic Compounds, Total Flavonoid Compounds and Antioxidant Activity of n-Hexane, Ethyl Acetate and Methanol Extract

Extract	Total Phenolic Compounds (mg GAE/g)	Total Flavonoid Compounds (mg QE/g)	Antioxidant Activity (µg/mL)
n-Hexane	2.38	14.09	1113.59
Ethyl Acetate	22.62	32.23	4.89
Methanol	19.35	31.18	6.04

The ethyl acetate extract was separated to produce 16 fractions (A-P) while the antioxidant fraction D was analyzed with an IC_{50} value of 2.21 ppm [5] and was discovered to be containing naringenin and luteolin compounds [5]. Therefore, the extract was found to have some potentials which can be explored and this means further studies need to be conducted.

EXPERIMENTAL

Materials and Methods

The materials used in this study include *B. macrophylla* stem, redistilled technical solvents such as *n*-hexane, ethyl acetate, acetone, methanol, acetonitrile, water, and chloroform (p.a Merck), and silica gel G60 (Merck). The others are TLC plates with silica gel GF254 (Merck), 1,1-diphenyl-2-picrylhidrazil (DPPH) (Merck), ACQUITY UPLC® BEH LC-MS/MS with C18 column, and Shimadzu UV-Vis spectrophotometer.

Separation of Active Compounds

Ethyl acetate extract from the *B. macrophylla* stem was fractionated by column chromatography (CC) method using silica gel G60 as a stationary phase and *n*-hexane: ethyl acetate: methanol as a mobile phase while the gradient system was used in the elution process to obtain the 16 fractions (A-P). The antioxidant activity of Fraction E with 410 mg was determined based on the qualitative analysis using DPPH autographic method and separated by CC using chloroform: ethyl acetate: acetone: methanol with 10% gradient polarity as eluent to produce 8 subfractions, E1-E8, which were further analyzed for their antioxidant activity.

Antioxidant Activity Screening

The screening of antioxidant activity was conducted using the autography method and this involved the addition of 0.02% DPPH solution to 2 mL of samples tested after which the mixture was incubated for 30 minutes and a color change from purple to pale yellow indicated the antioxidant activity [5].

Quantitative Antioxidant Activity with DPPH

The sample solution was made in a series of concentrations of 300, 150, 75, 37.5, and 18.75 ppm, and each sample was transferred into a test tube where 2 mL of DPPH 0.002% solution was added after which the mixture was incubated in a dark room for 30 minutes at room temperature. The absorbance was determined using the UV-Vis spectrophotometer and the IC_{50} value was calculated with a linear regression equation obtained from the graph showing the relationship between the concentration and % of DPPH reduction [6] after which the most active antioxidant fraction was analyzed through the use of LC-MS/MS.

Characterization of the Active Antioxidant Fraction

The most active fraction (1 mg) B. macrophylla stem was dissolved in 20 mL methanol and 5 µL solution was injected into LC-MS/MS through C-18 column with a dimension of 1.8µm 2.1 x 50 mm and a flow rate of 0.2 mL/ min. The mobile phase used was a mixture of acetonitrile: water 0.1% while the chromatogram result was interpreted using the mass bank data.

RESULTS AND DISCUSSION

Separation of Active Fractions

The ethyl acetate extract from the *B. macrophylla* stem was separated with a thin layer chromatography (TLC) using chloroform: ethyl acetate at 6:4 as eluent and 16 Fractions, A-P, were produced as shown in Fig. 1. Meanwhile, the compounds contained in Fraction E were further separated using column chromatography and 8 combined fractions of E1-E8 were obtained.

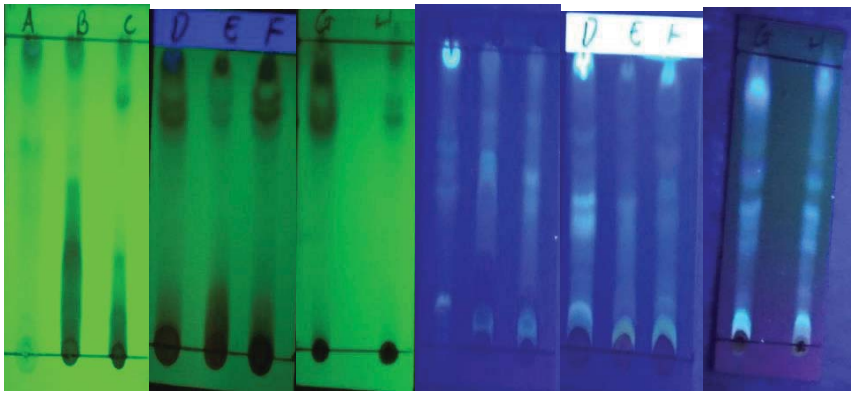

FIGURE 1. Chromatogram of E1-E8 Subfractions

Antioxidant Activity Screening by Autography

FIGURE 2. Antioxidant Qualitative Test Results with the Autographic Method

The antioxidant activity produced by the autography method is shown in Fig. 2. The autograph results in Fig. 2 show the E1 subfraction is the most active fraction on the antioxidant assay with the ability to change the color of DPPH from purple to yellow due to the redox reaction between the DPPH reagent and sample tested (E1) [6].

Antioxidant Activity Assay by DPPH

The antioxidant activity of E1 subfraction for the ethyl acetate extract from *B. macrophylla* stem was determined using the DPPH method and the results were measured with UV-Vis spectroscopy. The DPPH method involves measuring the decrease in the DPPH absorption at maximum wavelengths of 515 nm - 517 nm which is proportional to the concentration of free radical inhibitors added by the DPPH solubility [7]. The values of the antioxidant activity

obtained are, however, presented in Table 2.

DPPH free radical are paired, there is going to be a change in the color of the solution from dark purple to bright yellow (Fig. 2) [8]. Meanwhile, absorbance was measured after 30 minutes of incubation to initiate the reaction between DPPH as free radicals and the samples tested.

TABLE 2. The Antioxidant Activity Value of Subfraction E1

Sample	**IC_{50} (µg/mL)**
E1 Subfraction	232.63
Vitamin C	2.21

The results showed the IC_{50} value of the most active fraction of the ethyl acetate extract was 232.63 pp while the vitamin C used as a positive control had 2.22 ppm. This means *B. macrophylla* is a type of plant having antioxidant activity with moderate criteria due to the IC_{50} value observed to be lesser than 50 ppm in the fraction [6].

Characterization of Active Fractions of Antioxidants

E1 subfraction was characterized using LC-MS/MS which is one of the high-resolution analysis techniques usable in quantitative and structural analysis to provide a very useful approach in determining the profile of a metabolite. Moreover, according to Katajama and Oresic (2005), MZmine includes all the stages of initial data processing and LC-MS/MS chromatogram is mainly used for metabolomic purposes such as the identification of a compound in a sample [9].

LC-MS/MS chromatogram is processed into a mass array which is a three-dimensional data matrix containing accurate mass information of the detected peak, retention time, and peak intensity [10]. The metabolite was, however, identified by comparing the accurate mass value of the detected peak mass results with the accurate mass value of the compound.

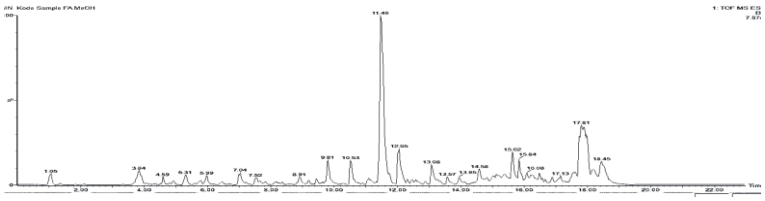

FIGURE 3. Chromatogram data samples in high pressure.

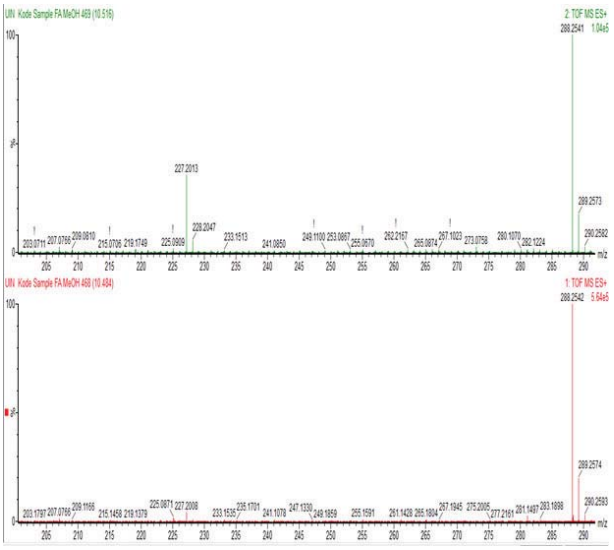

FIGURE 4. MS 1 and MS 2 data at 10.53 retention time

The results of the LC-MS / MS chromatogram for the E1 subfraction were analyzed using spectroscopy for MS 1 and MS 2 obtained at 10.53 retention time as shown in Fig. 4.

A single mass of 288.2541 (m/z) was obtained for the data and retention time with the molecular formula $C_{15}H_{33}NO_5$ while Lauric diethanolamide was discovered at the Mass bank. Moreover, the structural checking results showed the first compound in the ethyl acetate extract of *B. macrophylla* stem was predicted to be lauric diethanolamide compound (**1**).

Meanwhile, the LC-MS/MS chromatogram results of E1 subfraction were analyzed using mass spectroscopy at 17.08 retention as shown in Fig. 5.

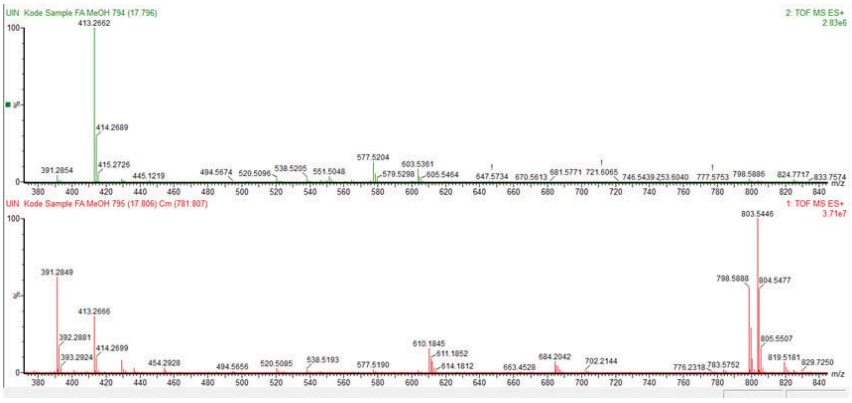

FIGURE 5. MS 1 and MS 2 data from Retention Time (17.80)

MS 1 and MS 2 data at 17.80 retention time produced a single mass at m/z 391 with the molecular formula $C_{17}H_{26}O_{10}$ and the compound from the Mass bank was predicted to be loganine (**2**). Moreover, LC-MS data showed E1 subfraction is a mixture of some major compounds containing lauryl diethanolamide and loganin as indicated in Fig. 6.

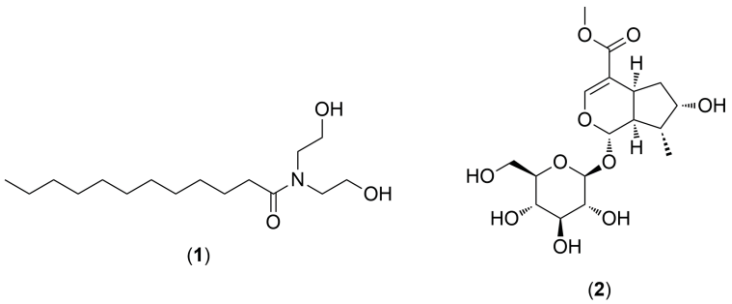

FIGURE 6. The Prediction of Compounds Structure of E1 Subfraction

Lauric dietanolamide with the IUPAC name of N, N-bis (2-hydroxyethyl) dodecanamide (**1**) belongs to the class of organic compounds known as *n*-acyl amines which contain fatty acid connected to amine groups through ester connections while loganin compound (**2**) is an antioxidant agent with very strong free radical scavenging activity and also has the potential to be used as a therapeutic agent for neurodegenerative treatment [11].

CONCLUSION

The E1 subfraction separated from the ethyl acetate extract of *B. macrophyla* stem contains lauric diethanolamide and loganin compounds and its antioxidant activity was found to be moderate with an IC_{50} value of 232.63 ppm.

ACKNOWLEDGMENTS

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