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Abstrak. Ethyl acetate extract of gandaria (*Bouea macrophylla* Griff) has very strong antioxidant activity. The aims of this study are to separate and characterize the active fraction of antioxidants and determine the antioxidant activity quantitatively. Ethyl acetate extract of *B. macrophylla* was separated by gravity column chromatography. Fraction separating was guided with qualitative testing of antioxidant activity. Active fraction result of separation was characterized by liquid chromatography-mass spectroscopy and quantitatively analyzed antioxidant activity using the 2.2-diphenyl-1- picrylhydrazyl method. Naringenin and luteolin were identified in the D-2 fraction which had very strong antioxidant activity with an IC_{50} value of 2.13 ppm. The hydroxyl group OH group of naringenin and luteolin compounds was thought to play a role in the reduction of free radicals.

1. Introduction

Gandaria (*Bouea macrophylla* Griff) including the family of Anacardiaceae which is spread in the territory of Indonesia, covering the islands of Java, Sumatera, Kalimantan, and Maluku[1]. *B. macrophylla* plants contain various secondary metabolites, such as flavonoids, phenolics, saponins, terpenoids, and alkaloids[2,3,4].

B. macrophylla seed extract had a very strong antioxidant activity with an IC_{50} value of 2.43 ppm[5]. Rajan and Bhat (2016) have tested methanol extract of *B. macrophylla* fruit on antioxidant activity with an IC_{50} value of 16.29 ppm [2]. Extract of *B. macrophylla* bark had an antioxidant activity of 20.03 ppm[6]. Stem extract of *B. macrophylla* had antioxidant activity with an IC_{50} value of 14.09 ppm[7].

Antioxidants are compounds that can prevent the immune system from weakening. Also, antioxidants can protect the body from attacks by free radicals with reducing the negative effects of free radicals[8]. Free radicals are one of the causes of various kinds of degenerative diseases such as cardiovascular disease, hypertension, stroke, liver cirrhosis, cataract, diabetes mellitus and cancer. Normally, free radicals can be suppressed by endogenous antioxidants produced by the body however, if the amount of excess free radicals in the body needs to be taken from sources of antioxidants from outside the body such as antioxidants from plant sources.

The *B. macrophylla* plant has the potential as an excellent source of antioxidants. In this study, *B. macrophylla* stem was extracted and fractionated using various organic solvents such as n-hexane, ethyl acetate, and methanol[7]. Ethyl acetate extract of *B. macrophylla* stem was separated by column chromatography. The separation fraction was tested for antioxidant activity using 2.2-diphenyl-1-

picrylhydrazyl (DPPH) method and was characterized by liquid chromatography-mass spectroscopy (LCMS/MS) to determine the active compounds.

2. Experimental

2.1. Material and methods

Solvens (n-hexane, ethyl acetate, methanol) for extraction and fractination were used technical grade (redestilation). UV spectra were measured by Shimadzu with methanol for antioxidant activity test. TLC plates with silica gel GF_{254} (Merck, 0.25 mm) and detected was achieved by spraying with 0.002% DPPH (Merck). Coloumn cromatography was conducted on silica gel 60. LCMS/MS were measured by the ACQUITY UPLC® BEH C18 column with a flow rate of 0.2 mL/minute. Mixture of methanol: aquadest was used as the mobile phase.

2.2. Separation of active fractions

Ethyl acetate extract of *B. macrophylla* stem (3 g) was separated by gravity column chromatography method using silica gel as stationary phase and n-hexane, ethyl acetate, and methanol as a mobile gradient phase to obtain fraction (A-P). D fraction (1.14 g) has antioxidant activity, so further separation is carried out using gravity column chromatography using silica gel as the stationary phase. Chromatography of the column eluted by gradient using chloroform, acetone, and methanol, so that the fraction (D1-D7) was obtained. D2 fraction (0.17 g) was analyzed for antioxidant activity using the DPPH method (Sigma Aldric) and characterized using LCMS/MS (Sciex) to determine its chemical compounds.

2.3. Antioxidant activity test

The D2 fraction was prepared with concentrations of 5, 2.5, 1.25 and 0.625 ppm using methanol solvents. At each concentration DPPH 0.002% solution was added as much as 2 mL. The solution was examined until homogeneous and incubated in the darkroom and room temperature for 30 minutes. The solution was measured for absorbance using a UV-Vis spectrophotometer (Shimadzu) at a wavelength of 515.78 nm. Calculation of antioxidant potential by calculating IC_{50} values for each sample using the linear regression equation obtained from the graph of the relationship between concentration and% of DPPH reduction[9].

The sample concentration and% inhibition were made in the sample absorbance curve and plotted on the x and y-axes in the linear regression equation, respectively. Concentration as growth x and% inhibition as y-axis so that the regression equation is obtained and calculated IC_{50} value[9].

2.4. Characterization of active fractions

D2 (1 mg) fraction was dissolved in 20 µL methanol. The solution of D2 fraction was taken as much as 10 µL and injected into LCMS/MS through the ACQUITY UPLC® BEH C18 column with a flow rate of 0.2 mL/minute. Mixture of methanol: aquadest was used as the mobile phase.

3. Results and discussion

Ethyl acetate extract of *B. macrophylla* stem has a very strong antioxidant activity[7]. Ethyl acetate extract of *B. macrophylla* stem was separated using column chromatography obtained 16 fractions (A-P). The separation fraction was tested using antioxidants qualitatively using the autography method. Fraction D showed antioxidant activity with a pale yellow color on the Thin Layer Chromatography (TLC) plate after being sprayed with DPPH 0.002% solution.

The D fraction was then reseparated using gradient column gravity chromatography so that 7 fractions are obtained. (D1-D7). The seven fractions were then tested for antioxidant activity on autography. D2 fraction shows antioxidant activity. The D2 fraction was analyzed quantitatively by antioxidant activity using the DPPH method.

3.1. Antioxidant activity

Analysis of antioxidant activity was carried out in the active fraction D2. The selection of DPPH method is due the method is simple, fast, and accurate[10]. In addition, the measured sample has conformity with the principle of DPPH radical reduction. DPPH solution as a source of free radicals will be muted by antioxidant compounds by donating protons from the OH group they have in the active fraction/compound. The more OH groups possessed by a compound/fraction the better the DPPH radical reduction[11].

The ethyl acetate extract of *B. macrophylla* stem contained a total phenolic of 22.62 mg GAE/g and contained total flavonoids of 32.28 mg quercetin/g[7]. Phenolic compounds and flavonoids have an important role in reducing free radicals, especially in the DPPH method[12]. The principle of the inhibition of free radicals by phenolic groups is to donate hydrogen to oxygen radicals so that a new radical formation cycle will occur. Also, phenolic groups can give OH groups to radical species[17].

Based on the results of the study, the active fraction IC_{50} value (D2) was 2.13 ppm. Ascorbic acid is used as a positive control with an IC_{50} value of 2.22 ppm. Comparison of IC_{50} values between the active fraction (D2) ethyl acetate extract of *B. macrophylla* and ascorbic acid stems is shown in Figure 1.

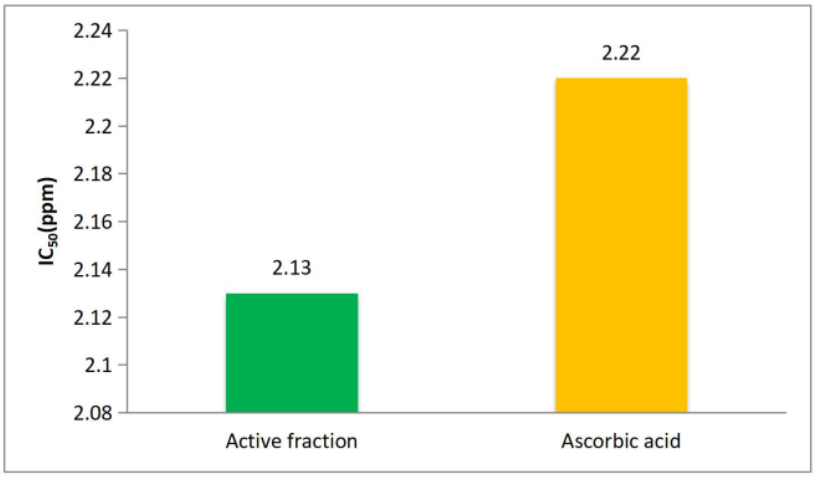

Figure 1. The IC_{50} value of ascorbic acid and active fraction D2

An ingredient can be said to be a powerful antioxidant if it has an IC_{50} value of less than 50 ppm[9] thus, *B. macrophylla* is a type of plant that has a powerful antioxidant ability because its fraction has an IC_{50} value less than 50 ppm. Antioxidant activity can be seen from the calculation of value of the Antioxidant Activity Index (AAI). AAI was obtained from a comparison between the concentration of DPPH solution and the sample IC_{50} concentration. The criteria for antioxidant activity based on AAI values are said to be weak as antioxidants if the value of AAI <0.5, moderate antioxidant activity if 0.5 <AAI <1.0, strong antioxidant activity 1.0 <AAI <2.0 and antioxidant activity is very strong if the value of AAI> 2.0[14].

Based on the index of antioxidant activity showed that the active fraction (D2) of ethyl acetate extract of *B. macrophylla* stem had potent antioxidant activity with a value of AAI 9.39. Positive control of ascorbic acid has potent antioxidant activity with a value of AAI 9.05. The active fraction (D2) ethyl acetate extract of *B. macrophylla* stem has the same antioxidant activity value or comparable to ascorbic acid. It can be said that D2 fraction of ethyl acetate extract of

*B. macrophylla*stem has the potential as a very powerful antioxidant alternative.

3.2. Characterization of active fractions with LCMS/MS

Antioxidative fraction (D2) extract of ethyl acetate *B. macrophylla*stem analyzed using LC-MS/MS. LC-MS/MS is one of the high-resolution analysis techniques that can be used in quantitative and structural analysis so that it can provide an instrumental approach in determining the profile of a metabolite. The following is the data chromatogram from the measurement of the D2 fraction presented in Figure 2.

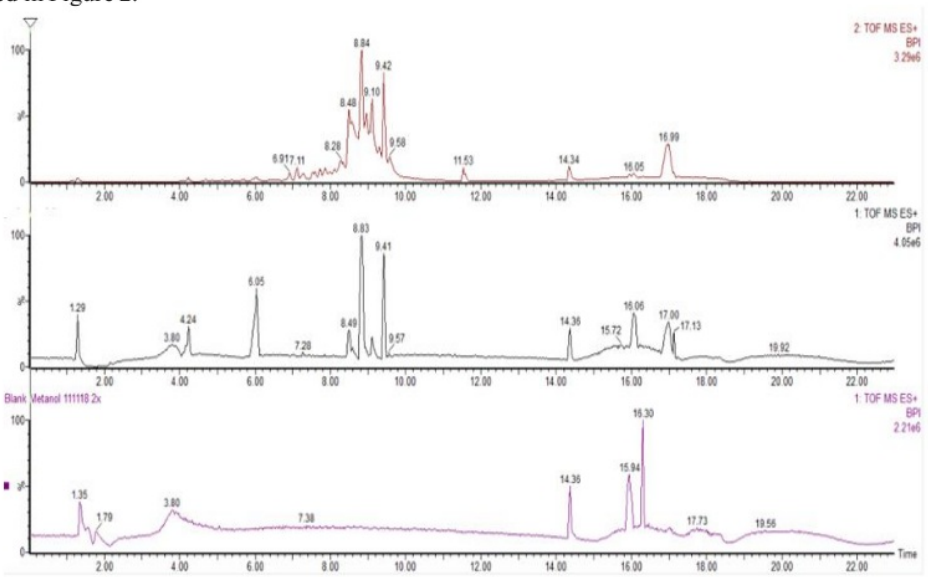

Figure 2. The D2 active fraction chromatogram

In Figure 2, it can be seen that the active fraction D2 has six peaks. LC-MS / MS chromatogram of D2 fraction of detected by mass spectroscopy produced MS spectrum. Based on the results of MS analysis at 4.24 retention times are shown in Figure 3.

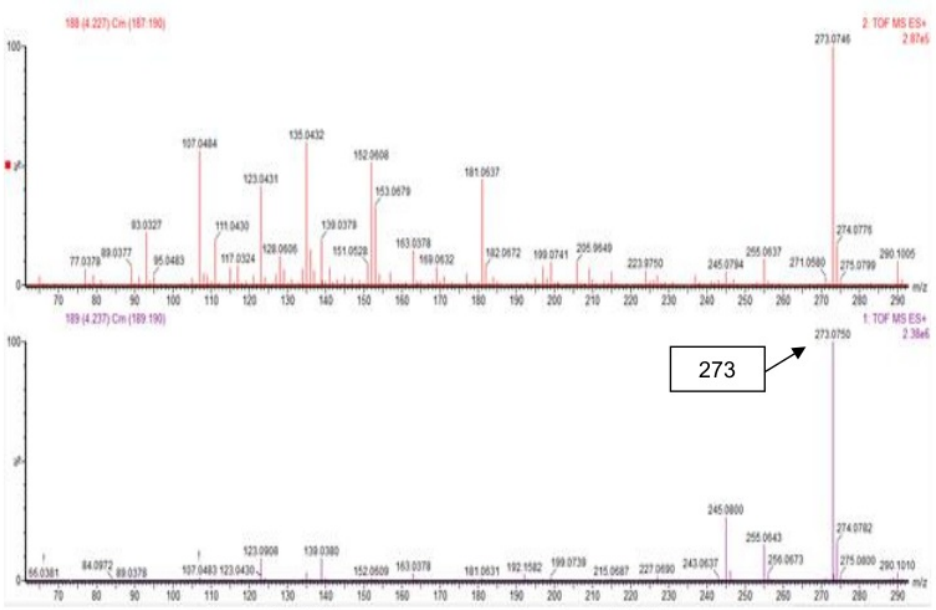

Figure 3. MS spectrum at retention time (4.24)

The MS spectrum at retention time 4.24 obtained a single mass of 273 m/z. Based on the analysis obtained the formula $C_{15}H_{13}O_5$ with Fit Conf 91.54%. Based on mass bank analysis, it is suspected that the compounds contained in the active fraction D2 with a retention time of 4.24 are naringenin compounds. MS spectrum at retention time 6.05 is shown in Figure 4.

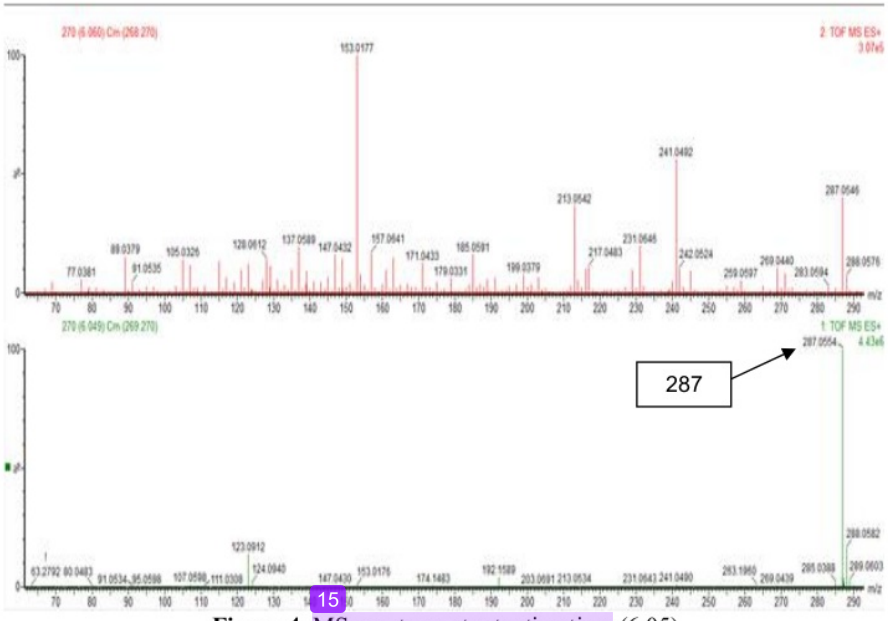

Figure 4. MS spectrum at retention time (6.05)

In the MS spectrum retention time, 6.05 obtained a single mass of 287 m/z with formula $C_{15}H_{11}O_6$ (Fit Conf 97.74%). The results on mass bank data show suspicion of luteolin compound.

Table 1. Estimated compounds in ethyl acetate stemextract *B. macrophylla*

No.	NameCompound	Formula	Retention time (minute)	Molecular mass (M/z) $[M+H]^+$	Molecular mass (m/z)
1	Naringenin	$C_{15}H_{12}O_5$	4,24	273	272
2	Luteolin	$C_{15}H_{10}O_6$	6,05	287	286

Naringenin with the chemical name 5,7,4'-Trihidoroksiflavonon (Figure 5) is a plant bioflavonoid classified as flavonoons. Naringenin has potent antioxidant activity because it has many hydroxyl groups that are numerous in the structure of compounds[15].

Naringenin was reported to be able to inhibit the secretion of apolipoprotein B from HepG2 cells stimulated by and decreasing the activity of Microsomal Triglyceride Protein Transfer (MTP). Naringenin can increased the expression of LDL receptors in HepG2 cells through an increas in PI3K-mediated SREB-l not dependent on phosphorylation of IRS-l. The risk of developing cerebrovascular disease and asthma also can be reduced by administering naringenin. Besides that, naringenin had much weaker cytotoxicity compared to 3-hydroxiflavones against TIG-1 and HUVE normal human cell cultures. Naringenin was also proven to be an antioxidant and superoxide, antiulcer, and dilatasiaorta catcher and can reduce free radicals[16].

Luteolin with the chemical name 5,7,3'4'-Tetrahidroxiflavone (Figure 5) is a flavonoid compound which belongs to the classified flavone. Luteolin is widely used as an anti-inflammatory, antioxidant, anticancer, antitumor and antiapoptotic agent, and allergic agent[17,18]. Luteolin has a potent antioxidant activity because with an IC_{50} value of 0.73 ppm[19].

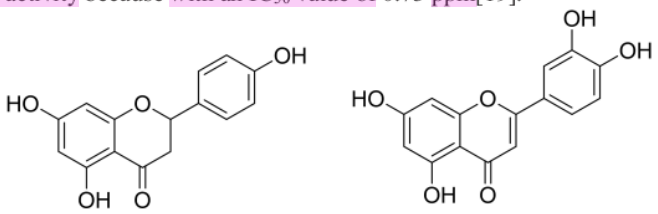

Figure 5. Structure of naringenin and luteolin compounds

4. Conclusions

Based on the results of the study, it can be concluded that the active fraction of the ethyl acetate extract of *B. macrophylla* stems has potent antioxidant activity with IC_{50} values of 2.13 ppm and AAI = 9.23 The results of the characterization of the active fraction D2 resulting from the separation of the ethyl acetate extract of the *B. macrophylla* stem with LC-MS/MS showed the presence of naringenin and luteolin compounds.

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