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Isolation and Spectroscopic Analysis of Compounds from *Baccaurea racemosa* (Reinw. ex Blume) Müll. Arg. Pulp

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ABSTRACT

The pulp of *Baccaurea racemosa* (Reinw. ex Blume) Müll. Arg. is reported to have active compounds with medicinal importance, such as anti-inflammatory and antioxidant activities. However, there has been no report of any isolated compounds from *B. racemosa* pulp. The present study aims to isolate and characterize the compounds from *B. racemosa* pulp and their antioxidant activity. The powdered pulp of *B. racemosa* was extracted using methanol and was partitioned using *n*-hexane, dichloromethane and ethyl acetate to get corresponding fractions. The ethyl acetate fraction was isolated using conventional column chromatography to obtain compounds 1 and 2. Based on the spectrometric analyses (1D-NMR, 2D-NMR, infrared and GC-MS), compounds 1 and 2 were identified as 5-Hydroxymethylenefurfural (5-HMF) and tartaric acid, respectively. The compound 5-HMF showed antioxidant activity with IC_{50} > 7.94 mM. This study is the first report related to the isolation and identification of compounds from *B. racemosa* pulp, therefore 5-HMF from *B. racemosa* may be potentially used as a candidate for natural antioxidants.

Keywords: *Baccaurea racemose*, Pulp, 5-Hydroxymethylenefurfural, Tartaric acid, Natural antioxidant.

Introduction

The current lifestyle and many environmental stressors can affect the quality of human health. The degradation of physical health can be caused by free radicals which oxidize the macromolecules in the cells. The free radicals can come the environment, food, pollutants, ultraviolet radiation and cigarette smoke.[1,2] The free radicals in extensive number cannot be covered with endogenous antioxidants only, so that the exogenous antioxidants from human diet are needed. Natural products coming from pmats and fruits can be potential sources to be explored as exogenous antioxidants.

The exploration and discovery of active compounds from natural products is very important to inhibit the oxidative stress.[3] More than 90% of countries in Southeast Asia used traditional medicine from natural products.[4] The presence of secondary metabolites in natural products from plants contributed to their activity which are beneficial to human health such as anti-inflammatory, antioxidant, cytotoxic activity and antibacterial.[5–7]

Baccaurea racemosa (Reinw. Ex Blume) Mull. Arg. (Phyllanthaceae) is a plant widely found in Southeast Asia. This plant is alo found in Nusa Tenggara, Java, Sumatera, Peninsular Malaysia, Thailand, Kalimantan including Sabah, Sarawak, Brunei and Sabah. *B. racemosa* can grow in lowland until 1000 meters from sea level. The ripening fruits can be consumed freshly or being boiled.[8]

All parts of *B. racemosa* have been utilized as components in the traditional medicinal plants. Its bark was used to treat eye inflammation and to relieve the menstrual pain.[9] The pulp of this plant has been reported to have potential antioxidant activity.[10] Several compounds have been isolated from another genus of Baccaurea, such as coniferyl aldehyde, phytol, and betulinic acid from the stem bark of *Baccaurea ramiflora*.[11,12] Moreover, picrotoxane sesquiterpene was isolated from *B. ramiflora* berries.[13] However, there is no information about the phytoconstituents isolated from *B. racemosa*, especially from the pulp. Therefore, this study aims to isolate and spectroscopically analyze the structure of the compounds from *B. racemosa* pulp and their antioxidant activities.

Materials and Methods

General experiments procedures

The spectrum of ultraviolet (UV) was obtained in methanol using UV-Vis Hitachi UH5300 spectrophotometer. The infrared (IR) spectrum was measured using the Attenuated Total Reflectance Infrared (ATR IR) spectrometer Nicollet is-5. In addition, the mass spectrum of compound **1** was recorded by Gas Chromatography-mass spectrometry (GCMS) using GCMS-QP2010S SHIMADZU with Electron Impact (EI). Mass spectrum of compound **2** was recorded by Waters Xevo Q-Tof with Electrospray Ionization-Mass Spectroscopy (ESI-MS). ^{13}C (125 MHz) and ^{1}H (500 MHz) Nuclear Magnetic Resonance (NMR) spectra were identified by JEOL ECZ500R with CD_3OD (compound 1) and D_2O (compound 2). Column chromatography was applied using silica gel 60 F_{254} for column chromatography with 230-400 mesh (Sigma Aldrich). Preparative thin-layer chromatography (PLC) 60 GF_{254} was used for preparative column chromatography containing gypsum (Sigma Aldrich).

Plant materials

Baccaurea racemosa pulps were collected from Banyuwangi, East Java, Indonesia, on the 5th of April 2019. This plant was identified by I Gde Mertha (senior lecturer in Plant Taxonomy) in Biology Laboratory, Faculty of Mathematics and Science, Mataram University, West Nusa Tenggara, Indonesia, with a voucher number 01-LP.

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Extraction and isolation

B. racemosa pulps were sliced and dried in the oven at 58°C for 5x24 hours. Dried pulps (1.2 kg) were powdered and extracted with methanol using the maceration method for 3x24 hours. The extracted pulps were filtered, and the filtrate was concentrated using a vacuum rotary evaporator at 45°C to yield a methanol extract (919.9 g). The extract (800 g) was partitioned by the liquid-liquid extraction (LLE) method using *n*-hexane, dichloromethane, and ethyl acetate to get ethyl acetate fraction (6.37 g). The ethyl acetate fraction was subjected to column chromatography using silica gel F_{254} and eluted by gradient system to get fraction A – G. Fraction B was dissolved into ethyl acetate. The fraction was spotted at Preparative Thin-Layer Chromatography (PLC) plate 60 GF_{254} with the size of (20x20) cm and a thickness of 1.0 mm. The PLC was eluted with *n*-hexane: ethyl acetate (1:1). The target spot in PLC was observed in UV radiation at a wavelength of 254 nm. The target spot at R_f 0.43 was extracted with methanol: chloroform (1:1) to obtain compound 1 (32 mg). Fraction G was crystallized using methanol to get compound 2 (62 mg). The procedure of extraction and isolation compound from *B. racemosa* pulp can be seen in Figure 1.

DPPH antioxidant assay

The antioxidant activities of the 5-HMF were tested using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay. An amount of 6.4 mg of DPPH was suspended with 100 mL methanol. The isolated compound was suspended in methanol to prepared concentrations 1.59; 3.17; 4,76; 6.35; and 7.94 mM and added 100 µL DPPH solution. The absorbance was measured using an ELISA microplate reader at 515 nm.[14] The butylated hydroxytoluene (BHT) was used as positive control. The percentage (%) of radical scavenging activity was calculated using the equation;

% radical scavenging activity =

$$\frac{Absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control} x100$$

Statistical analysis

The IC_{50} of 5-HMF and its positive control were analyzed using the Statistical Package for the Social Sciences (SPSS version 16). T-test method was used for statistical analysis with significance level at $P < 0.05$.

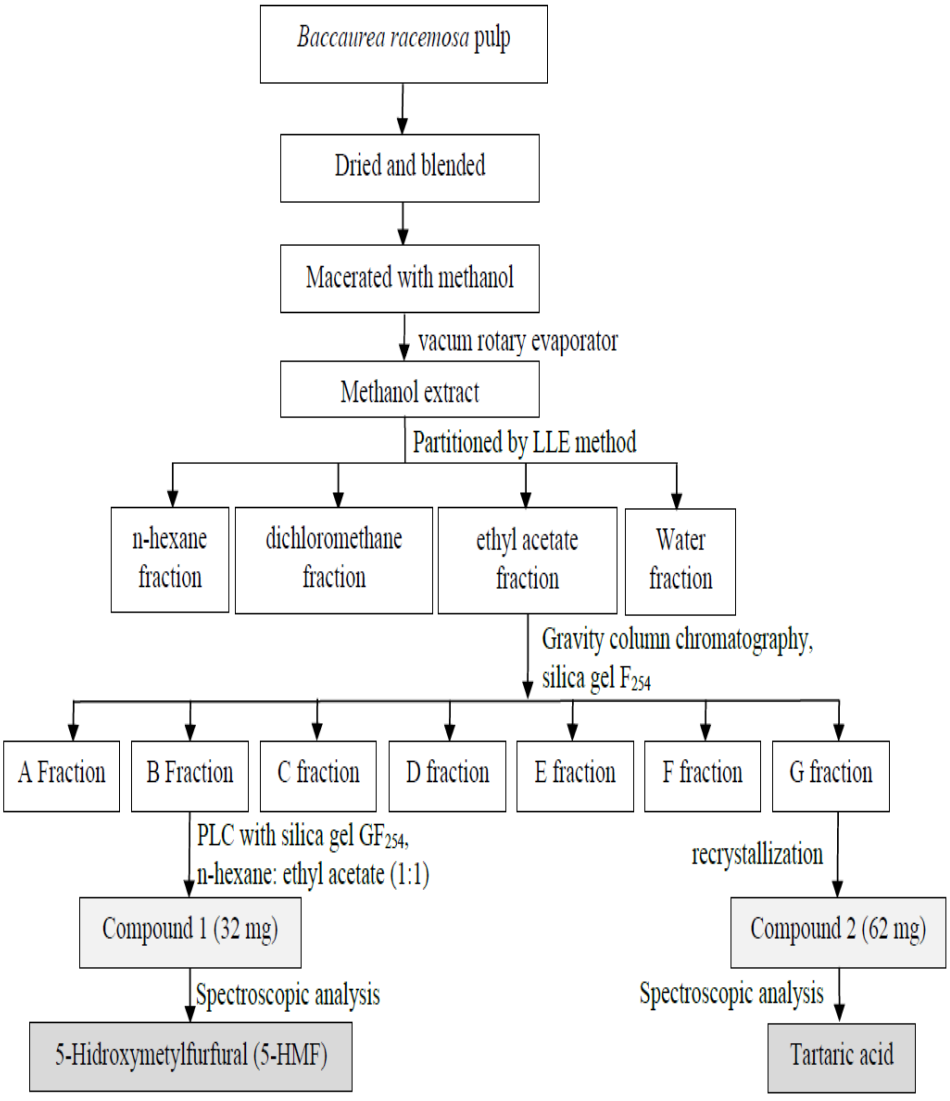

Figure 1: Extraction and isolation compound from *Baccaurea racemosa* pulp

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Results and Discussion

5-hydroxymethylenefurfural (compound 1)

Yellowish brown or tan coloured compound; 32 mg; mp 30-34^{o}C; R_f = 0.67 (n-hexane: ethyl acetate, 3:7), muffle under UV lamp (254 nm; UV-visible λ_{max}: 279 nm (log ε) 4.02; IR (ATR) ν_{max} cm^{-1}: 3368.28; 2950.25; 2855.50; 1662.14; 1397.17; 1191.79; 667.83. ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD) spectral data at table 1. EI-MS *m/z* (%intensity): 126 (M^+) ($C_6H_6O_3$), m/z 109 (10), m/z 97 (100), m/z 69 (40), m/z 41 (75). Compound (**1**) was identified as 5-hydroxymetylenefurfural (5-HMF).

Tartaric acid (compound 2)

White powder compound; 62 mg; mp 219-220^{o}C; IR (ATR) ν_{max} cm^{-1}: 3117.19; 1683.62; 1600.93; 1439.92; 1337.49; 1173.96; 669.29. ^{1}H-NMR (500 MHz, D_2O) and ^{13}C-NMR (125 MHz, D_2O) spectral data at Table 2. ESI-MS (negative ion mode): pseudo-molecular ion m/z 149 (M-H); m/z (%intensity): m/z 149 (100), m/z 131 (3), 103 (7), m/z 87 (30), m/z 73 (10), m/z 59 (14). The molecular formula was $C_4H_6O_6$ with molecular ion peak m/z 150 g/mol. Compound (2) was identified as tartaric acid.

Compound 1. The ultraviolet (UV) spectrum exhibited λ_{max} (279 nm), (log ε) 4.02, which predicted that the molecule structure consisted of 2-3 possible conjugated double bonds. The infrared (IR) spectrum showed the peak at 3368 cm^{-1} that indicated a hydroxyl group (OH). In addition, absorbance at 2950 cm^{-1} and 2855 cm^{-1} characterized the aliphatic C-H stretch. At the same time, the carbonyl (C=O) group exhibited a strong peak at 1662 cm^{-1}. The wavenumber of the carbonyl shifted to the right due to the influence of the conjugated double bond. An absorbance at 1397 cm^{-1} showed C-H bending and the absorbance at 1191 cm^{-1} informed the presence of the C – O stretch.

The ^{1}H-NMR spectrum of compound 1 showed the signals at δ 6.57 ppm (1H, H-4) and δ 7.36 ppm (1H, H-3) characterized by the presence of furan protons (Table 1). Those signals showed the same coupling constant (*J*) of 3.55 Hz, indicating neighboring protons.[15] These protons coupled to each other were supported with the COSY spectrum. Meanwhile, the chemical shift at δ 9.51 ppm (1H, H-7) was the typical chemical shift of the proton aldehyde. After that, proton δ 56.28 ppm (2H, H-6) was in a more downfield chemical shift because its carbon atom was directly attached to the oxygen atom.

The ^{13}C-NMR spectrum showed six carbon atoms (Table 1). Based on the spectral data of Distortionless Enhancement by Polarization Transfer (DEPT) 135, there were two quaternary carbons at δ 152.56 ppm (C, C-2) and δ 123.60 ppm (C, C-5). These carbons did not have a single correlation with a proton which was increasingly supported with heteronuclear single quantum coherence (HSQC) spectrum. Meanwhile, δ 123.60 (CH, C-3), 109.60 (CH, C-4) and 178.15 (CH, C-7) were tertiary carbons. In addition, DEPT 135 spectrum showed the chemical shift at 56.28 ppm (CH_2, C-6), indicating the presence of secondary carbon. The ^{13}C-NMR spectrum reported the carbonyl group that appeared at δ 178.15 ppm (CH, C-7). In addition, there was a single bond correlation between carbons at δ 178.15 (CH, C-7) with a proton at δ 9.51 (1H, H-7) at the HSQC spectrum supported the presence of the aldehyde group. The Heteronuclear Multiple Bond Correlation (HMBC) spectrum confirmed that compound 1 was 5-hydroxymethylenefurfural (Figure 2).

GC-MS spectrum showed that the molecular formula of compound 1 was $C_6H_6O_3$ and exhibited a molecular ion peak at m/z 216. The ion peak at m/z 97 indicated the release of aldehyde (–COH) groups. Meanwhile, ion peak at m/z 109 characterized the releasing of the hydroxyl group. The NMR spectrum from the previous study[16] and the spectroscopic analysis, compound 1 was identified as 5-hydroxymethylenefurfural (5-HMF).

Compound 2. The infrared spectrum of compound 2 exhibited the peak at 3117 cm^{-1} that indicated the hydroxyl group. The broad peak at 3400 - 2400 cm^{-1} identified the presence of the hydroxyl group of carboxylic acid. The carbonyl group showed a peak at 1683 cm^{-1}. The signal of the carbonyl group shifted to the right because the carbonyl group was involved in the formation of hydrogen bonds. The peaks at 1439 cm^{-1} and 1173 cm^{-1} indicated the vibration of C-H bending and the stretch vibration of C-O, respectively.

The ^{1}H-NMR spectrum of compound 2 showed a peak with signal singlet at chemical shift (δ) 4,34 ppm that indicated 2H (H-2 and H-3) (Table 2). Compound 2 has a plane of molecular symmetry that makes the H-2 was an identic chemical shift with H-3. The ^{13}C-NMR spectrum showed 2 (two) signals at δ 72.78 ppm (C-2 and C-3) and 176.39 ppm (C-1 and C-4) (Table 2). The spectrum of DEPT 135 exhibited one signal at 72.78 ppm of C-2 and C-3 that indicated there were the tertiary carbons. Meanwhile, C-1 and C-4 were C of the carbonyl group, which appeared at δ 176.39 ppm. After that, the HSQC spectrum showed a single correlation between δ 72.78 ppm (C-2/C-3) and δ 4,34 ppm (H-2/H-3), supporting the H-2 attached to C-2 and on the other hand, H-3 attached to C-3 (Table 2). Meanwhile, there was no cross peak at the COSY spectrum. The heteronuclear multiple bond correlation (HMBC) spectrum showed that compound 2 was tartaric acid (Figure 2b).

ESI-MS (negative ion mode) of compound 2 showed an ion peak at m/z 149 (M-H). The molecular formula of compound 2 was $C_4H_6O_6$. The ion peak at m/z 131 and m/z 103 showed the releasing of H_2O and CO, respectively. The NMR spectrum from the previous study[17] and the spectroscopic analysis, compound 2 was identified as tartaric acid.

Antioxidant activity

The present study showed that compound 1 was 5-Hydroxymethylenefurfural (5-HMF). The 5-HMF was the common compound found at the pulp of fruits, such as in grape berries.[18] In addition, honey contained high 5-HMF.[19] The major reactant to form the 5-HMF were fructose, sucrose, and glucose.[20] According to a previous study, 5-HMF have chemotherapeutic agents and have protected human hepatocyte cell line (LO2) damage caused by radical oxygen species (ROS) such as hydrogen peroxide and nitric oxide.[21,22] Meanwhile, 5-HMF from *Laurencia undulata* revealed antioxidant activity by scavenging intracellular ROS.[16] Several methods were used to measure *in vitro* antioxidant activity, such as DPPH radical scavenging activity. Antioxidant activity of 5-HMF from *B. racemosa* pulp was measured using DPPH free radical scavenging activity with BHT as the positive control. The study revealed that 5-HMF has antioxidant activity with IC_{50} > 7.94 mM (Table 3). The exact IC_{50} for 5-HMF could not be identified because, until the 5-HMF concentration of 7.94 mM, the inhibition of DPPH free radicals had not reached 50%. The oxygen atom in furan ring and carboxylic groups affected the antioxidant activity of 5-HMF by radical resonance.[23] It could stabilize the free radical species. Meanwhile, tartaric acid (compound 2) was the organic acid that was widely distributed at fruits.[24,25] Tartaric acid was reported to have potential antimicrobial activity.[26] The natural acidity of tartaric acid created a poor environment for microorganisms to grow and survive. Tartaric acid in grapes fruits was utilized to stabilize or balance the juice between acid and sweet flavors.[25] Antioxidant activity of tartaric acid was not identified because there was not conjugated double bond at its structure so that it cannot stabilize the free radical of DPPH.

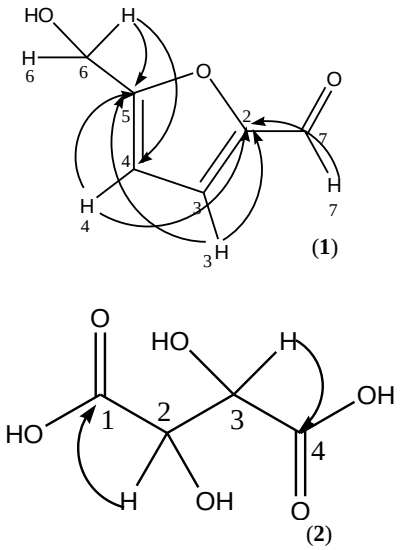

Figure 2: HMBC spectrum for 5-HMF (**1**) and tartaric acid (**2**)

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Table 1: The NMR spectra of compound 1 (5-HMF), ^{1}H-NMR: (500MHz, CD_3OD), C-NMR: (125 MHz, CD_3OD)

Position	δ^{1}H (ppm)	δ^{13}C (ppm)	literature δ^{1}H 5-HMF[16] (ppm)	Literature δ^{13}C 5-HMF[16] (ppm)	DEPT	COSY	HSQC	HMBC
1	-	-	-	-	-	-	-	-
2	152,56	-	152,4	-	C	-	-	H-3; H-4; H-7
3	123,60	7.36 (1H, d, *J*= 3.55 Hz)	122,6	7,22 (1H, d, *J=3,6 Hz*)	CH	H-4	H-3	-
4	109,60	6.57 (1H, d, *J*= 3.55 Hz)	109,9	6,52 (1H, d, *J=3,6 Hz*)	CH	H-3	H-4	H-6
5	161,89	-	160,4	-	C	-	-	H-3; H-4; H-6
6	56,28	4.56 (2H, s)	57,6	4,72 (2H, s)	CH_2	-	H-6	-
7	178,15	9.51 (1H, s)	177,6	9,60 (1H, s)	CH	-	H-7	-

Table 2: The NMR spectra of compound 2 (tartaric acid), ^{1}H-NMR: (500MHz, D_2O), C-NMR: (125 MHz, D_2O)

Position	δ^{1}H	δ^{13}C	literature δ^{1}H tartaric acid[17]	literature δ^{13}C tartaric acid[17]	DEPT	COSY	HSQC	HMBC
1	-	176.39	-	181.301	-	-	-	H-2
2	4.34 (s)	72.78	4.324(s)	76.572	CH	-	H-2	-
3	4.34 (s)	72.78	4.324(s)	76.572	CH	-	H-3	-
4	-	176.39	-	181.301	-	-	-	H-3

Table 3: Antioxidant activity of compound 2 (5-HMF)

Sample	Concentration (mM)	% DPPH free radical scavenging activity	$\bar{x} \pm IC_{50}$ (mM)
5-HMF	1.59	7.91 ± 1.95	
	3.17	8.55 ± 2.25	
	4.76	13.91 ± 1.50	>7.94[a]
	6.35	19.42 ± 1.35	
	7.94	22.81 ± 1.20	
BHT	0.02	8.57 ± 3.92	
	0.05	25.65 ±1.88	
	0.07	50.70 ± 1.57	0.0726 ± 0.0012[b]
	0.09	62.90 ± 0.16	
	0.11	69.92 ± 0.31	

The values of IC_{50} are represented as mean ± standard deviation deviation (SD). Samples with a different letter at the same column show a significant difference ($P<0.05$) measured by T-test. Butylated Hydroxy Toluene (BHT), 5-Hydroxymethylenefurfural (5-HMF).

Conclusion

Two compounds from *Baccaurea racemosa* pulp that were successfully isolated and identified were 5-hydroxymethylenefurfural (5-HMF) and tartaric acid. These were the first compounds isolated from *B. racemosa* pulp. 5-HMF from *B. racemosa* pulp has antioxidant activity with IC_{50} value > 7.94 mM. *B. racemose* pulp was potentially developed as a natural antioxidant. This present study guided the discovery and explored the other compounds from the *B. racemosa* pulp.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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