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ISOLATION AND CHARACTERIZATION OF STIGMASTEROL AND β-SITOSTEROL FROM WOOD BARK EXTRACT OF *Baccaurea macrocarpa* Miq. Mull. Arg

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ABSTRACT

Baccaurea macrocarpa Miq. Mull. Arg. (known locally as Tampoi) is one of the edible fruit plants found in the forests of Borneo. The crude extract of wood bark of Tampoi was partitioned with *n*-hexane and ethyl acetate successively to yield respectively soluble fractions to biological activity assay. The toxicity was measured by the brine shrimp lethality test method, and the antioxidant activity was carried out by the DPPH radical scavenging method. While the isolation and purification were carried out using flash column chromatography. The results of the biological assay showed that the ethyl acetate fraction was the most active in the antioxidant activity test, with IC_{50} values 35.56 µg/ml, and none of the fractions is toxic. Isolation and purification of the ethyl acetate fraction gave white crystalline powder with a melting point 129 - 130 ^{O}C. Characterization of the compound based on FT-IR, ^{1}H, ^{13}C-NMR, NMR 2D spectra and comparison to that of the published NMR data suggested that the compound (1) was a mixture of stigmasterol and β-sitosterol.

Keywords: *Baccaurea macrocarpa*, Toxicity, Characterization, Antioxidants, Stigmasterol, β-sitosterol.

INTRODUCTION

East Kalimantan is one of the provinces in Indonesia having tropical rain forests. Diversity of tropical plants contained in it one of which is the genus of *Baccaurea.* Generally, *Baccaurea* plants have edible fruits, and some of them are traditionally used as medicine. *Baccaurea* is a reasonably large genus; around 38 species of *Baccaurea* are recognized. The distribution of this plant genus includes India, Burma, Malaysia, Borneo, Sumatra, the Philippines, Thailand, Papua New Guinea, Sulawesi (Talaud Island), Bali, and the Pacific islands[1]. Utilization of *Baccaurea* as an alternative medicine such as to treat arthritis, abdominal pain, eye pain, abscesses, constipation, facilitates urination and menstruation. Previous research results also showed that *Baccaurea* has the potential as an anticancer, antidiabetic, antioxidant, anti-inflammatory, antimicrobial, and antitrypanosomal agents[1-4]. However, based on the literature search, no one has reported secondary metabolites isolated from Tampoi. The previous studies have shown crude extracts of Tampoi wood bark is very active as an antioxidant[5]. This study is a continuation of research aimed to characterize, identify and determine the toxicity against *Artemia salina* L and antioxidant activity against DPPH radical scavenging of the compound obtained from the *Baccaurea macrocarpa* (Miq.) Mull. Arg (Tampoi) wood bark extract.

EXPERIMENTAL

Material

The sample of this research was the wood bark of *B. macrocarpa* (Mi Mull. Arg. (Tampoi) Collected from Kedang Ipil Village, Kota Bangun, Kutai Kartanegara. Methanol, ethyl acetate, and *n*-Hexane were

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used in the extraction, chromatography, and purification section. TLC Silica Gel 60 F254 (1.05554.0001) and Kieselgel 60 (1.07734.1000) were used for TLC analysis and flash column chromatography, respectively.

Instrumentation

FTIR spectrum was measured using FTIR Prestige 21 (Shimadzu Corp, Japan. Whereas the ^{1}H- and ^{13}C-NMR spectrum including NMR-2D was measured using a 500 MHz Agilent DD2 NMR Spectrometer, which operates at frequencies of 500 MHz (^{1}H) and 125 MHz (^{13}C).

General Procedure

Extraction, Isolation, and Purification

A total of 180 grams of Tampoi wood bark extract was re-dissolved into methanol then partitioned with *n*-hexane and ethyl acetate successively. After the solvent removal using a rotary evaporator, the fractions of *n*-hexane (20 g), ethyl acetate (40 g), and methanol (80 grams) were obtained. The ethyl acetate fraction (40 grams) was further fractionated using vacuum column chromatography using ethyl acetate: *n*-hexane mixture eluent (5:95 - 100: 0) and 37 vials were obtained. The fractions were combined into five fractions, E1 (346.7 mg), E2 (579.4 mg), E3 (276.3 mg), E4 (353.5 mg), and E5 (3245.5 mg) based on TLC spot profile. E2 fraction (579.4 mg) was isolated by flash column chromatography using a mixture of eluent ethyl acetate: *n*-hexane (1: 9). Fraction E2 (579 mg) was isolated by flash column chromatography using a mixture eluent ethyl acetate:*n*-hexane (1: 9) to give 5 main fractions, namely E2.1 (31 mg), E2.2 (68 mg), E2.3 (67.3 mg), E2.4 (104 mg) and E2.5 (54.3 mg). Thirty mg of white crystalline powder was obtained after recrystallization of E2.2.

The purity using thin-layer chromatography analysis on three eluent variations, showing the formation of a single spot with an Rf value of 0.27 (chloroforms: *n*-hexane = 4: 6), 0.33 (ethyl acetate: *n*-hexane = 1: 9), and 0.38 (100% chloroforms). Melting point measurement displayed that the compound (1) had m.p. 129-130 °C.

Toxicity Tests

Toxicity tests were performed using the brine shrimp lethality test method against *Artemia salina* L. The samples were dissolved into 500, 250, 125, 62.5, 31.25, 15.63, and 7.81 ppm. Each sample solution is inserted between 8-15 shrimp larvae. In the same way, blanks are made without being sampled. Both samples and blanks were repeated three times.[5-7]

Antioxidant Activity Test

The antioxidant test was performed using the DPPH free radical scavenging method refers to the previous research method. Inhibition of the sample against the DPPH free radical was calculated according to the formula: Inhibition (%) = [(A - A1) / A] X 100. Meanwhile, the determination of LC_{50} was carried out using linear regression on concentration vs inhibition (%), where, A = absorbance of blank and A1 = absorbance of the sample.[5,8-15]

Steroid Test of compound (1)

A few mg of compound (1) was put into a test tube, then a few drops of Liebermann-Burchard reagent were added (glacial acetic acid + concentrated H_2SO_4). The formation of green indicates compound 1 is a steroid.[5,8,16]

Spectroscopic Data

Spectroscopic data measurements of compound (1) were comprised of FT-IR, ^{1}H-NMR, ^{13}C-NMR and NMR-2D. IR spectrum data were recorded using a Shimadzu FTIR Prestige 21 (Shimadzu, Japan). NMR spectra were recorded using the 500 MHz NMR Agilent with DD2 console system operating at frequencies of 500 MHz (^{1}H) and 125 MHz (^{13}C) using $CDCl_3$ as a solvent in the ITB Chemistry Department. Compound (1) was obtained as a white powder with a melting point of 129-130 °C. FTIR spectrum data showed the absorption peaks at 3427.51 cm^{-1} (OH), 3050.00 cm^{-1} (CH alkene), 2866.22 cm^{-1}, 2935.66 cm^{-1}, and 1463.97 cm^{-1} (CH aliphatic), 1658.78 cm^{-1} (C=C), 1134.14 cm^{-1} (CO). The ^{1}H- and

^{13}C-NMR spectra of compound (1) were the entirety of the stigmasterol and *β*-sitosterol data as listed in Table-3.

RESULTS AND DISCUSSION

Partitions of 180 grams of crude extract of Tampoi wood bark yielded *n*-hexane, ethyl acetate, and methanol fractions of 8, 20, and 40 grams, respectively. The results of toxicity tests against larval of *Artemia salina* showed that all fractions were not toxic (LC_{50} > 1000 ppm)[6], as presented in Table-1.

Table-1: LC_{50} Value of Fractions and Compound (1) (the concentrations, total larvae, and dead larvae were the averages of three replicates).

Sample	Concentration	Log Concentration	Total Larvae	Dead Larvae	% Mortality	Probit	Linear Regression	LC_{50} (ppm)
n-hexane fraction	500	2.6989	9.7	4.7	48.4	4.95	y = 0.3773x + 3.591	5425.36
	250	2.3979	11	3	27.2	4.39		
	125	2.0969	9.7	2.3	23.7	4.26		
	62.5	1.7959	10.3	1.7	16.5	4.01		
	31.25	1.4948	10.7	2.3	21.5	4.19		
	15.63	1.1938	10	1	10	3.72		
	7.81	0.8928	10.3	2.7	26.2	4.36		
Ethyl acetate fraction	500	2.6989	10.3	7.7	74.7	5.64	y = 0.0819x + 4.6659	12005.08
	250	2.3979	8.3	2.3	27.7	4.39		
	125	2.0969	9.7	3	30.9	4.48		
	62.5	1.7959	10.7	4	37.3	4.67		
	31.25	1.4948	9.3	3.3	35.5	4.61		
	15.63	1.1938	10	4.3	43	4.82		
	7.81	0.8928	9.3	5	53.8	5.08		
Methanol fraction	500	2.6989	8.3	3.3	39.7	4.72	y = 0.2598x + 3.8505	26580.15
	250	2.3979	10.7	2	18.7	4.08		
	125	2.0969	10.3	3	29.1	4.45		
	62.5	1.7959	10.3	3.7	35.9	4.61		
	31.25	1.4948	10.7	2.3	21.5	4.19		
	15.63	1.1938	11.7	2.3	19.6	4.12		
	7.81	0.8928	10	1.7	17	4.05		
Compound (1)	500	2.6989	10	4.7	47	4.92	Y = -0.0261x + 5.114	23324.70
	250	2.3979	10	6	60	5.25		
	125	2.0969	10	4.7	47	4.92		
	62.5	1.7959	10	5.7	57	5.18		
	31.25	1.4948	10	5	50	5.00		
	15.63	1.1938	10,3	6	58,3	5.20		
	7.81	0.8928	10	5	50	5.00		

While the antioxidant test results using DPPH free radical method showed that the ethyl acetate fraction was the most active, as shown in Table-2.

Isolation and purification of ethyl acetate fraction gave compound (1) as a white powder with a melting point of 129-130 °C. FT-IR spectrum data showed that the absorption of 3427.51 cm^{-1} (hydroxyl groups) was supported by 1134.14 cm^{-1} (Secondary alcohol, C-O stretch). Absorption of stretching at 2935.66 and 2866.22 cm^{-1} indicated the presence of CH aliphatic supported by the absorption at 1463.97 cm^{-1} (for cyclic CH_2). Other absorption at 3050.00 cm^{-1} due to =CH structure and it was endorsed by 1658.78 cm^{-1} (C=C stretch). The qualitative test results against Liebermann-Burchard reagents formed in green icated the compound (1) has a steroid nucleus.

^{1}H-NMR spectrum data showed the presence of a signal at 3.52 (m, 1H) for H-3 and at 5.36 (t, 1H) for H-6. Two singlet signals 0.85 (s) and 0.10 (s) for -CH_3 at H-18 and H-19, respectively. Two methyl doublet at 1.03 (J = 7.2 Hz) (H-21) and 1.02 (d, J = 13 Hz) for stigmasterol (1)/ 0.83 (J = 11 Hz) (H-26) for β-

sitosterol (2), and one broad singlet at 0.84 (br s) (H-27). The presence of signals at 5.00, (dd, J = 1.73 Hz and 1.72 Hz) and 5.15 (dd, J = 1.75 and 1.73) are H-22 and H-23, respectively for Stigmasterol (1). ^{13}C-NMR Spectrum data shows there were 50 signals overall. The signals at 140.87 (C5), 121.84 (C6), and 140.87 (C5), 121.85 (C6) were carbon double bonds for Stigmasterol and β-sitosterol, respectively. The signal at 71.93 was one carbon oxymetin C-sp^3 for C3. The presence of carbon double bonds was shown in signals at 8.46 (C22) and 129.39 (C23) for stigmasterol (1). Stigmasterol and β-sitosterol are two types of steroids that have similar molecular formulas that differ only at C-22 and C-23. Based on NMR data, including NMR-2D and supported by literature data, compound (1) is a mixture of Stigmasterol and β-sitosterol. Stigmasterol and β-sitosterol, two plant sterols that are difficult to separate. Both of these compounds have almost the same polarity so that they are often obtained in mixed form[17-20]. The results of antioxidant tests of compounds (1) against free radical DPPH showed low antioxidant activity with an LC_{50} value of 74.33 ppm. The results of the toxicity test for compound (1) against *Artemia salina* larvae showed no toxicity with LC_{50} values above 1000 ppm[6].

Table-2. Antioxidant Activity of Fractions and Compound (1). (The concentrations and absorbances were the averages of three replicates)

Sample	Concentration (ppm)	Absorbance		% Inhibition	Linear Regression	IC_{50} (ppm)
		Sample	Blank			
n-hexane fraction	20	0.186	0.265	29.68	Y=0.6358x +18.05	50.25
	40	0.147		44.52		
	60	0.113		57.35		
	80	0.085		67.80		
Ethyl acetate fraction	20	0.153	0.265	42.26	Y = 0.6164x + 29.371	33.47
	40	0.124		53.08		
	60	0.089		66.54		
	80	0.056		78.86		
Methanol fraction	20	0.211	0.265	20.38	Y = 0.3748x + 12.516	100.01
	40	0.194		26.92		
	60	0.172		35.09		
	80	0.152		42.64		
Ascorbic acid	2	0.220	0.265	16.85	y = 9.5283x - 1.4465	5.40
	4	0.167		36.98		
	6	0.113		57.36		
	8	0.070		73.58		
Compound (1)	20	0.157	0.177	11.30	y = 0.7043x - 2.354	74.33
	40	0.131		25.80		
	60	0.104		41.24		
	80	0.083		53.11		

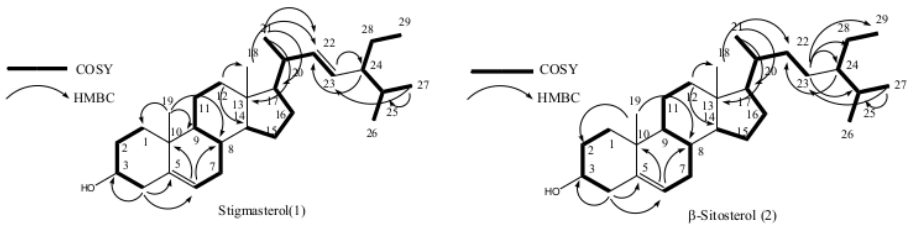

Fig.-1: Chemical Structure of Stigmasterol (1) and β-Sitosterol (2)

Table-3: ^{1}H and ^{13}C-NMR Spectrum Data for Stigmasterol (1) and β-sitosterol (2)

No	Stigmasterol (1)					β-Sitosterol (2)				
	Experimental			Literature [18]		Experimental			Literature [18]	
	^{1}H-NMR	^{13}C-NMR	HMBC correlation	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	HMBC correlation	^{1}H-NMR	^{13}C-NMR
1	1.85 (m)	37.39	C-2		37.3	1.85 (m)	37.39	C-2	-	37.3
2	1.95 (m)	32.02	C-3		31.6	1.95 (m)	32.05	C-3	-	31.6
3	3.52 (m)	71.93	-	3.52 (m)	71.8	3.52(m)	71.93	-	3.52 (m)	71.8
4	2.24 (dd, *J*= 1.44; 1.06) and 2.38, t)	42.42	C-3,5,6		42.3	2.24 (dd, *J* = 1.44; 1.06) and 2.38,1H)	42.42	C-3,5,6		42.2
5	-	140.87	-	-	140.8	-	140.87	-	-	140.8
6	5.36 (t)	121.84	C-8,10	5.357 (br s)	121.7	5.36 (t)	121.85	C-8,10	5.358 (br s)	121.7
7	1.99 (m)	31.78	C-3,8,9		31.9	1.99 (m)	31.78	C-3,8,9	-	31.9
8	2.00(m)	32.05	C-5,6,9		31.9	2.00 (m)	32.05	C-5,6,9	-	31.9
9	0.94 (m)	50.26	C-7,8,12		51.2	0.94 (m)	50.28	C-7,8,12	-	51.2
10	-	36.64	-		36.5	-	36.64	-	-	36.5
11	1.02 (m,)	21.22	C-5.8,9,13		21.1	1.02 (m)	21.22	-	-	21.1
12	1.16 (m)	39.82	C-14,18		39.8	1.16 (m)	39.91	C14,18	-	39.7
13	-	42.35	-		42.3	-	42.46	-	-	42.3
14	1.00 (m)	56.99	C-9,13,17, 22		56.8	1.00 (m)	56.90	C-9,13, 17,22	-	56.9
15	1.06 (m) and 1.58 (m)	24.45	C-8, 9,14, 16		24.3	1.06 (m) and 1.58 (m)	24.51	C-6,8, 9,14	-	24.4
16	1.66 (m) and 1.25 (m)	29.07	C-18,20, 22		28.3	1.09 (m)	28.39	C-17	-	28.4
17	1.12 (m)	56.08	C-8, 9,12, 13,18		56.0	1.12 (m)	56.18	C-15,16, 19,21,18	-	56.9
18	0.85 (s)	12.13	C-8, 22	0.680 (s)	11.0	0.85 (s)	12.00	C-8, 22	0.699 (s)	11.9
19	1,01 (s)	19.54	C-1,8,9,10	1.01 (s)	19.4	0.82 (s)	19.18	C-2,8	1.01 (s)	19.4
20	1.16 (m)	40.65	C-13,20,21, 23,24		36.2	1,35 (m)	36.30			36.2
21	1.03 (d, *J*= 7.2 Hz, 3H)	21.23	C-13,17	1.02 (d, *J*=7.5 Hz)	21.15	0.92 (d, *J*=5.12 Hz, 3H)	18.92	C-17	0.92 (d,*J*=6.4 Hz)	18.8
22	5.00 (dd, *J* =1.73 Hz and 1.72 Hz)	138.46	C-20		138.28	1,33 (m)	34.07	C-23,24, 25,29		33.9
23	5.15 (dd, j=1.75 Hz and 1.73 Hz)	129.39	C-24		129.29	1.16 (m)	26.20	C-24,25, 28,29		26.1
24	1,55 (m)	51.38	C-22		51.21	0.94 (m)	45.96	C-20,21,22 , 25,23,26		45.9
25	1.45 (m)	32.03	C-22		31.88	1.66 (m)	29.27	C-19, 23, 24, 25, 27, 28		29.2
26	1.02 (d,*J* =13 Hz)	21.21	C-29		21.06	0.83(d, *J*=11 Hz)	21.36	C-24, 27, 28, 29	0.83 (t)	19.8
27	0.84 (br s)	19.97	C-23,25	0.795 (d *J*=6.5 Hz)	19.79	0.84 (br s)	19.13	C-23,25	0.814 (d,*J*=6.5	19.3

									Hz)	
28	1.16 (m)	25.56	C-26, 29	0.846 (d, *J*=6.5 Hz)	25.38	1,25 (m)	23.20	C-22, 24,25	0.833 (d,,*J*=6.5 Hz)	23.1
29	0.81 (t)	12.41	C-25,27,28	0.845 (t , *J*=7.5 Hz)	12.22	0.85 (t)	12.19	C-23,27	0.845 (t , *J*=7.5 Hz)	12.2

Compound 1 exhibits a weak antioxidant against DPPH radicals, however, *β*-sitosterol can protect against oxidative stress through modulation of antioxidant enzymes[21] and Stigmasterol can decrease lipid peroxidation in the hepatic[22]. Also, both Stigmasterol and *β*-sitosterol are the main components of phytosteroids which will increase cholesterol excretion and reduce intestinal cholesterol absorption.[23]

CONCLUSION

Bioactivity-guided isolation of active compounds from the ethyl acetate fraction of *B. macrocarpa* wood bark extract gave compound (1). Structure elucidation based on spectral data suggested that compound (1) is a mixture of Stigmasterol and β-sitosterol. Both compounds are the first time isolated from *B. macrocarpa* (Tampoi).

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