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Radical Scavenging Activity of Extract, Fraction and Chemical Compound from Calophyllum sclerophyllum vesq. Stembark by Using 1,1-Diphenyl-2-Picryl Hydrazil (DPPH)

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Abstract: Antioxidant activity by DPPH methods of ethanolic extract, fractions from *Calophyllum sclerophyllum* Vesq stembark, and the chemical compound from the active fraction as antioxidant has been measured in this research. Extraction was done by maceration methods with ethanol 70 % and then partition by *n*-hexane, ethyl acetate and *n*-buthanol. Then antioxidant activity assays of ethanolic extract and fractions by DPPH methods and using quercetin as positive control. The fraction that have antioxidant activity more over purified by colomn chromatography and recrystalization, and the pure compounds were elucidated by spectroscopic analysis and measured that antioxidant activity. The ethanolic extract have high antioxidant activity with IC_{50} 5,96 ppm, and also ethyl acetate fraction and *n*-buthanol extract with IC_{50} 3,03 and 3,89 ppm. The IC_{50} of quercetin value at 1,73 ppm. Two pure compounds have been isolated from ethyl acetate fraction, CSL 1 was chromanon acid (Isoapetalic acid) and CSL 2 was flavonoid (Astilbin), but only CSL 2 that showed activity as antioxidant, with IC_{50} 7, 24 ppm. Those compounds not yet reported before from this species.
Key word index: *Calophyllum sclerophyllum* Vesq, Clusiaceae, chromanon acid, Astilbin, antioxidant.

Introduction

Callophyllum is a big genus from Cluciaceae family that encompass 200 veriety of species and widely distributed in tropical rain forrest[1]. Numerous species have been used as folk medicine[2]. This genus contribute many chemical compound that have been reported such us xanthones, polyisoprenilated keton, qumarin, benzofuran, chalcone, flavonoid and triterpen[3,4]. Quite a lot of this compound have potential activity like antioxidant, anti-HIV, antiplatelet aggregation, antimicrobial and anticancer[5,6,7].

Free radicals are a molecule that got added or reduced an electron, so the electron on molecule became radical[8]. That can caused free radical that reactive and unstable, and can caused oxidative stress for body. The oxidative stress its mean unbalance between oxidant and antioxidant and can caused damage on fat, proteins, enzyme, carbohydrate and DNA in cell and tissue[9]. That damages can initiate cancer, neurogenerative desease, heart deases, diabetic and autoimune desease[9].

One of therapy that can reduced and cured cancer is by give antioxidant. Recent study about exploring antioxidant from natural product increase to replace the used of artificial antioxidant that can caused carsinogenic[1]. Polyphenolic compounds from natural product as a point of view for antioxidant activity such us radical scavenging activity and reduced fat peroxidation[1].

Calophyllum sclerophyllum Vesq (local name: bintangur jangkang) is widely distributed in Asia region such us Thailand, Malaysia (Serawak), Indonesia (Kalimantan, Sumatera, Irian Jaya, Sulawesi and Maluku)[10]. Based on chemotaxonomy this species might be also contain chemical compound that have activity as antioxidant. The chemical compounds that have been reported from *Calophyllum sclerophyllum* Vesq. were jacaerubin, 1,3,5,6 tetraoxygenated xanthones, euxanthone, but there was no study about antioxidant activity[11]. The aim of this research was to explore antioxidant activity of extract, fractions and chemical compounds from *Calophyllum sclerophyllum* Vesq stembark and elucidation of the chemical compound that isolate from active fraction by spectroscopic analysis.

Material and Methods

Material

C.sclerophyllum Vesq stembark was collected at Kalimantan island and determinated in Herbarium Bogorience, Indonesian Institute of Sciences Indonesian.

Extraction, Fractination and Isolation of Chemical Compounds

The dried and powdered stem bark of *C. sclerophyllum* Vesq (3.6 kg) was extracted by maceration methods with ethanol 70% at room temperatur. The extract was concentrated to give a 426 g ethanolic extract, then fractionated with *n*-hexane, ethyl acetate and *n*-buthanol. Yield 63.70, 74.92, 31.5 g fraction *n*-hexane, ethyl acetate and *n*-buthanol respectively.

40 g ethyl acetate fraction was purified by column chromatography, silica was used as stationary phase then eluted with *n*-hexane, ethyl acetate and methanol by 10% stepwise gradient to afford 16 fraction (fraction I – XVI). Fraction III purifed by recrystalization and afford the pure compound CSL 1. Fraction X was the highest antioxidant activity, then purified futhermore by column chromatography and recrystalization and afford the pure compound CSL 2.

Antioxidant Activity Assay by DPPH Method

Antioxidant assays was done by DPPH radical reduction methods, quercetin as positive control[12]. IC_{50} values calculated using the regression equation. Extract solution 2.0 mL added 2.0 mL methanol solution of DPPH 40 ppm, shaken until homogenous, incubated at 25-27 C for 15 minutes and measured absorption at wavelength of 517 nm. The same treatment was done for quercetinas standard. The inhibition percentage of trhe DPPH radical was calculated as the following equation[12] :

% Inhibition ((A-B)/A) x 100 %
Where : A= Absorbance of blank, B = Absorbance of sample test.

Results and Disscussion

Isolate CSL 1

CSL 1 (**I**) was obtained as an pale yellow crystall and shown to possess molecular formula of $C_{22}H_{28}O_6$ (LC-MS $[M+H]^+$ at *m/z* = 389.4159, [2M+Na] m/z = 799.8944, [M+Na] 411,4091. The IR spectrum showed the characteristic absorptions for carbonyl (1714.77 cm^{-1}), conjugated C=O (1627,97 cm^{-1}). In the ^{1}H-NMR spectrum, a chelated hydroxyl group at δ_C 12,89. The ^{13}C, DEPT, and COSY NMR spectra of compound CSL 1 (**1**) exhibited 22 carbon signals, which revealed the presence of five methyls, three methylenes, five methines, 9 quarternary carbons including two carbonyl groups (table 1).

Table 1 .^{1}H,^{13}C and HMBC spectral of CSL (**1**) (Aceton)

C	^{13}C-NMR (ppm)	^{1}H-NMR (HMQC) (m. *J* Hz)	COSY	HMBC (H-C)
2	79.78	4.28 (1H, *m*)	2.68, 1.18	C-16
3	46.29	2.68 (1H, *m*)	4.28	C-16, 4
4	200.22	-		-
5	160.10	-		-
6	116.52	5.56 (1H, *d*, *J*=10.35 Hz)	5.60	C-8, 12, 14, 5
7	126.86	5.60 (1H, *d*, *J*= 10.4 Hz)	5.56	C-8, 12
8	78.83	-		-
10	111.32	-		-
11	162.50	-		-
12	102.26	-		-
13	101.92	-		-
14	156.13	-		-
15	19.89	1.51 (3H, *d*)		C-3, 2
16	28.58	1.45 (3H, *d*)		C-3, 2, 4
17	10.09	1.18 (3H, *s*)		-
18	28.53	1.46 (3H, *s*)		C-17, 8, 7
19	31.09	3.77 (1H, *m*)		C-20, 22, 10
20	21.71	1.17 (2H, *d*)		-
21	174.29	-		-
22	36.01	1.89 and 1.57 (2H, *m*)		C-19, 20, 10, 21
23	38.78	2.75 (2H, *m*)		C-19, 20
24	14.49	0.86 (3H, *t*, *J*=7.15 Hz)		-

Extensive analysis of the ^{1}H-NMR (500 MHz) indicated that there are two protons aromatic (*d*) at δ_H 5.56 (1H, *d*, *J* = 10.35 Hz) and 5.60 (1H, *d*, *J* = 10.4 Hz) and five metils group at δ_H 0.86 (*t*, *J* = 7.15 Hz, C-24), 1.51 (*d*, C-15), 1.45 (*d*, C-16), 1.18 (*s*, C-17), and 1.46 (*s*, C-18). Three methylens (CH_2) 1.17 (*s*, C-20), 1.89 and 1.57 (*m*, C-22) and 2.75 (*m*, C-23). Five methines (CH) at 5.56 (*d*, C-6), 5.60 (*d*, C-7), 2.68 (*m*, C-3), 3.77 (*m*, C-19), 4.28 (*m*, C-2).

Analysis DEPT spectra support that this compound contain three methylens at δ_H 1.17 (*d*, C-20), 1.89 and 1.57 (*m*, C-22), 2.75 (*m*, C-23).

HMQC spectra showed direct correlation proton at δ_H 5.56, 5.60, 4.28; 2.68; 1.51; 1.45; 1.18; 1.46; 3.77; 1.17; 1.89 and 1.57; 2.75 ppm to carbon at δ_C 116.52: 126.86; 79.78; 46.29; 19.89; 28.58; 10.09; 28.35; 31.09; 21.71; 36.01; 38.78 and 14.49 ppm respectively.

HMBC spectra showed correlation proton at δ_H 5.56 ppm to carbon at δ_C 78.83; 102.26; 156.13; 160.10 ppm. Proton at δ_H 5.60 ppm to carbon at δ_C 78.83; 102.26 ppm. Proton at δ_H 4,28 ppm correlated to carbon at δ_C 28.35 ppm and also proton at δ_H 2.68 ppm correlated to carbon at δ_C 28.35 and 200.22 ppm. Proton at δ_H 1.51 ppm correlated to carbon at δ_C 79.78; 46.29 ppm. Proton signal at δ_H 1.45 ppm correlated to carbon at δ_C 79.78; 46.29; 200.22 ppm respectively. Proton at δ_H 1.46 ppm correlated to carbon at δ_C 10.09; 126.86; 78.83 ppm. Proton at δ_H 3.77 ppm correlated to carbon at δ_C 36.01; 38.78; 111.32 ppm. Proton at δ_H 2.75 ppm correlated to carbon at δ_C 31.09; 36.01 ppm. ^{13}C-NMR spectra also displayed the presence of lacton and carbonil group at δ_C 174.29 and 200.22 ppm respectively.

CSL 1(1) was obtained as pale yellow crystall, ^{1}H-NMR and 13C-NMR, Table 1; HREIMS m/z 389.4159 $[M]^{+}$(calc for $C_{22}H_{28}O_6$, 388). CSL 1 (**1**) is a chromanon acid, this compound have been published as isoapetalic acid that isolated from Calophyllum brasiliense[13].

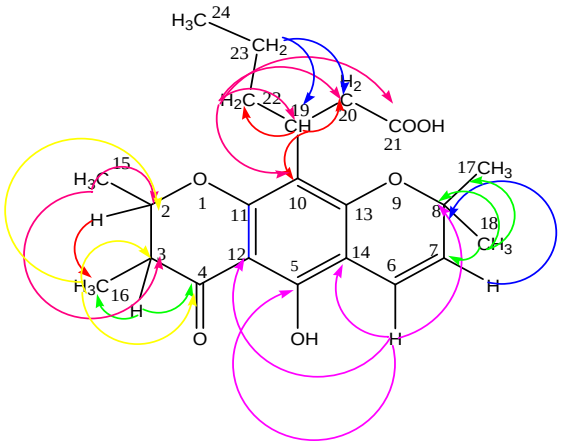

Figure 1. Structure of compound CSL **1** (isoapetalic acid) and HMBC Correlation

Isolate CSL 2 (2)

CSL 2 was isolated as a light yellow amorphous powder. Its structure was established by spectroscopic means. The molecular formula was determined as $C_{21}H_{22}O_{11}$ BM (450). CSL 2 was isolated as a light yellow amorphous powder molecular formula of $C_{21}H_{22}O_{11}$ (LC-MS $[M+H]^+$ at *m/z* = 451.4045, [2M+Na] m/z = 923.8686, [M+Na] 473,3991. The IR spectrum showed the characteristic absorptions for hydroxyl (3396.76 cm^{-1}), carbonyl (1716.70 cm^{-1}). Extensive analysis of the ^{1}H-NMR (500 MHz) and HMQC spectra indicated that there are five protons aromatic at δ_H 5.89, 5.92, 6.84, 6.80 and 6.95 in aromatic ring are ilustrated by δ_H 5.89 (1H, *d*, *J* = 2 Hz, C-6), 5.92 (1H, *d*, *J* = 2 Hz, C-8), 6.84 (1H, *dd*, *J* = 2 and 8 Hz, C-2'), 6.80 (1H, *d*, *J* = 8 Hz, C-3') and 6.95 (1H, *d*, *J* = 2 Hz, C-6'). One metil group at δ_H 1.18 (3H, *d*, C-6"). Five methinoxi (-HC-O-) group at δ_H 4.03 (1H, *d*, C-1"), 3.53 (1H, *q*, C-2"), 3.67 (1H, *dd*, C-3"), 3.32 (1H, *d*, C-4"), 4.26 (1H, *q*, C-5"), and two methin (CH) at 5.06 (1H, *d*, *J* = 10 Hz, C-2) and 4.57 (1H, *d*, *J* = 10 Hz, C-3).

HMBC spectra further showed correlation proton at δ_H 5.06 ppm to carbon at δ_C 78.63; 196.09; 129.25; 120.57 and 115.55 ppm and proton at δ_H 4.57 ppm to carbon at δ_C 84.04; 196.09; 129.25 and 115.55 ppm. Proton at δ_H 5.89 ppm correlated to carbon at δ_C 164.18 and 97.45 ppm. Proton at δ_H 5.92 ppm correlated to carbon at δ_C 168.68 and 84.04 ppm. Proton at δ_H 6.84 ppm correlated to carbon at δ_C 84.04 and 116.39 ppm. Proton signal at δ_H 6.95 ppm correlated to carbon at δ_C 120.57; 84.04 and 147.45 ppm. Proton signal at δ_H 4.03 ppm correlated to carbon at δ_C 78.63; 70.58 ppm. Proton signal at δ_H 3.53 ppm correlated to carbon at δ_C 73.87 ppm. Proton signal at δ_H 3.67 ppm correlated to carbon at δ_C 73.87 ppm. Proton signal at δ_H 3.32 ppm correlated to carbon at δ_C 71.85 and 17.94 ppm. Proton signal at δ_H 1.18 ppm correlated to carbon at δ_C 73.87 and 30.58 ppm. ^{13}C-NMR spectra also displayed the presence of carbonil group at δ_C 196.09 ppm respectively. Pattern NMR spectra of CSL **2** similar to Astilbin [14].

CSL 2, (Astilbin) from *C. sclerophyllum* Vesq was isolated as an pale yellow amorphous, melting point ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) see Table 2; LC-MS *m/z* = 451. According to these explanation sumarize the structure was Astilbin.

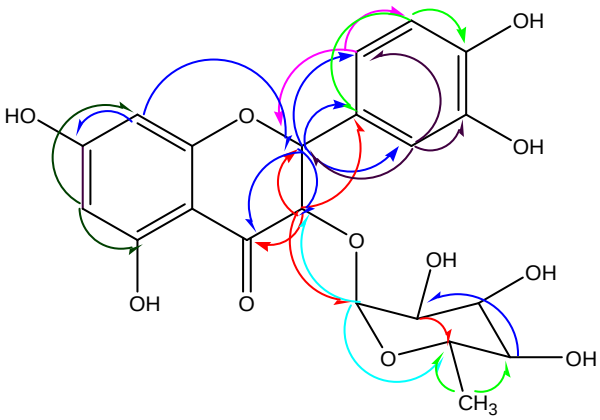

Figure 2. Structure of compound CSL **2** (Astilbin) and HMBC Correlation

Table 2 .^{1}H,^{13}C and HMBC spectral of CSL (2) (Aceton)

C	^{13}C-NMR (ppm)	^{1}H-NMR (HMQC) (m.*J* Hz)	COSY (H-H)	HMBC (H-C)
2	84.04	5.06 (1H, *d J* = 10 Hz)	4.07	C-3, 4, 1', 2', 6'
3	78.63	4.57 (1H, *d, J* = 10 Hz)	5.06	C-2, 4, 1', 1''
4	196.09	-	-	-
5	164.18	-	-	-
6	96.34	5.89 (1H, *d, J* = 2 Hz)	-	C-5, 8
7	168.68	-	-	-
8	97.45	5.92 (1H, *d, J* = 2 Hz)	-	C-7, 2
9	165.60	-	-	-
10	102.56	-	-	-
1'	129.25	-	-	-
2'	120.57	6.84 (1H, *dd J* = 2 and 8 Hz)	6.95	C-2, 3'
3'	116.39	6.80 (1H, *d J* = 8 Hz)	-	C-4', 1'
4'	146.61	-	-	-
5'	147.45	-	-	-
6'	115.55	6.95 (1H, *d J* = 2 Hz)	6.84	C-2', 2, 5'
1''	102.22	4.03 (1H, *d*)	3.53	C-3, 5''
2''	71.85	3.53 (1H, *q*)	3.67, 3.32, 4.03	C-4"
3''	72.22	3.67 (1H, *dd*)	3.53, 3.32	C-4''
4''	73.87	3.32 (1H, *d*)	-	C-2", 6"
5"	70.58	4.26 (1H, *q*)	3.32, 1.18	-
6"	17.94	1.18 (3H, *d*)	-	C-5'', C-4"

Antioxidant Assay by DPPH Methods

Table 3. describes the results of antioxidant activity by reduction od DPPH radical on ethanol extract. With IC_{50} values 5.96 ppm. The highest antioxidant by buthanol fraction, then follow by ethyl acetate fraction with IC_{50} values 3.03 and 3.89 ppm (table 4 and 5). CSL 2 that isolated from ethyl acetate fraction have antioxidant activity by reduction DPPH radical with IC_{50} values 7.24 ppm (table 6). (Quercetin as a standard with IC_{50} values 1.73 ppm (table 7)).

Table 3. Activity Antioxidant Extract Ethanol

Concentration (ppm)	Blank Absorbance	Sample Absorbance	% Inhibition	Equation Calibration Curve	IC_{50} (ppm)
5.04	0.496	0.274	44.76	y = 2.7657 + 7.9289x r = 0.9784	5.96
4.52		0.316	36.29		
3.99		0.325	34.48		
3.47		0.349	29.64		
2.52		0.379	23.59		

Table 4. Activity Antioxidant Buthanol Fraction

Concentration (ppm)	Blank Absorbance	Sample Absorbance	% Inhibition	Equation Calibration Curve	IC_{50} (ppm)
1,010	0,5005	0,3965	20,7792	y = 2,3405 + 15,7410 x	3,03
1,515		0,3805	23,9760		
2,020		0,3410	31,8681	r = 0,9912	
2,525		0,2865	42,7572		
3,030		0,2420	51,6484		
3.535		0,2120	57,6424		

Table 5. Activity Antioxidant Ethyl Acetate Fraction

Concentration (ppm)	Blank Absorbance	Sample Absorbance	% Inhibition	Equation Calibration Curve	IC_{50} (ppm)
2,057	0,5005	0,336	32,76	y = 16,3340 + 8,3436 x	3,89
2,541		0,298	40,45		
3,509		0,266	46,85	r = 0,99085	
3,993		0,252	49,65		
4,477		0,228	54,34		
5,082		0,194	61,13		

Table 6. Activity Antioxidant SCL 2

Concentration (ppm)	Blank Absorbance	Sample Absorbance	% Inhibition	Equation Calibration Curve	IC_{50} (ppm)
4,5	0,478	0,362	24,26	y = -18,832 + 9,5069 x	7,24
5,5		0,330	30,85		
6,5		0,257	46,23	r = 0,9881	
7,5		0,220	53,97		
8,5		0,201	57,94		
9.5		0,129	73,01		

Table 7. Activity Antioxidant Quercetin

Concentration (ppm)	Blank Absorbance	Sample Absorbance	% Inhibition	Equation Calibration Curve	IC_{50} (ppm)
2.50	0.546	0.136	75.09	y = -5.363 + 32.002x	1.73
2.00		0.200	63.37	r = 0.9976	
1.50		0.302	44.69		
1.00		0.401	25.56		
0.50		0.486	10.99		

Conclusion

Extract ethanol of *Calophyllum sclerophyllum* active as antioxidant with mechanism by reduction of DPPH radical. The best antioxidant activity is n-buthanol extract with mecanism reducing of DPPH radical with values 3.03 ppm, this is because the butanolic fraction contain flavonoids and phenolic compound.The chemical compound that isolated from ethyl acetate fraction (CSL 2) active as antioxidant with mechanism reduction of

DPPH radical with IC_{50} 7.24 ppm. Activity CSL 2 as antioxidant because CSL 2 have hydroxil grup that can reduced DPPH radical by donated the proton.

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