BENZOPHENONE GLUCOSIDE ISOLATED FROM THE ETHYL ACETATE EXTRACT OF THE BARK OF *MAHKOTA DEWA* [*Phaleria macrocarpa* (Scheff.) Boerl.] AND ITS INHIBITORY ACTIVITY ON LEUKEMIA L1210 CELL LINE

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ABSTRACT

*Isolation and elucidation of benzophenone glucoside from ethyl acetate extract of Phaleria macrocarpa bark and its inhibitory activity test against leukemia L1210 cell line have been done. The Phaleria macrocarpa bark were macerated using n-hexane, ethyl acetate, and ethanol, respectively. The ethyl acetate extract was then chromatographed on silica gel column and gradiently eluted by n-hexane - ethyl acetate – ethanol with the composition from 20:1:0 until 0:0:1, gave eight fractions. Separation of fraction 6 using semipreparative HPLC on reverse phase column (Capcell Pak C-18 SG120, 15 mm I.D. x 250 mm) using methanol – water (40:60, 5 mL/min) gave a brown powder, with the melting point of 182.3 ºC. Spectroscopic analysis and comparison of its physico-chemical data, this compound was clarified as 2,4'-dihydroxy-4-methoxy-benzophenone-6-O-β-D-glucopyranoside (**3**). Inhibitory activity of its compound against leukemia L1210 cell line showed that this compound exhibited inhibitory activity with IC_{50} was 5.1μg/mL.*

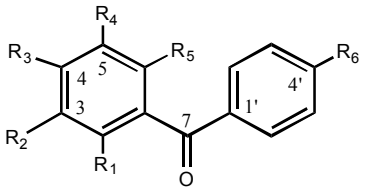
Keywords: *Phaleria macrocarpa, 2,4'-dihydroxy-4-methoxybenzophenone-6-O-β-D-glucopyranoside, cytotoxic activity, leukemia L1210*

INTRODUCTION

Mahkota dewa [*Phaleria macrocarpa* (Scheff.) Boerl] is the one of the medicinal plant which grows in Indonesia. Empirically, *Phaleria macrocarpa* are used for medical treatment. The stems are used for treatment of bone cancer, the leaves are used for impotency, blood diseases, allergies, diabetes mellitus, and tumor treatments. Egg shell of seeds are used for breast cancer, cervix cancer, lung diseases, liver and heart diseases [1]. The fruits consisting of alkaloid, saponin, flavanoid, and polyphenol [2].

Wahyuningsih, *et al.* [3] found a new benzophenone glucoside from the leaves of *Phaleria macrocarpa*, so called phalerin (4,5-dihydroxy,4'-methoxybenzophenone-3-*O*-β-D-glucoside (**1**) (Fig. 1), while another new benzophenone glucoside derivative, mahkoside A (4,4'-dihydroxy-6-methoxybenzophenone-2-*O*-β-D-glucopyranoside) (**2**) (Fig. 2) was isolated by Zhang, *et al.* [4] besides six known compounds including mangiferin, kaempferol-3-*O*-β-D-glucoside, dodecanoic acid, palmitic acid, ethyl stearate and sucrose. Biological activity test on phalerin showed that this compound was non toxic and has a potent immunostimulant [3].

Preliminary study indicated that ethyl acetate extract of the bark of *Phaleria macrocarpa* exhibited IC_{50} of 10.15 μg/mL against mouse leukemia L1210 cell as anticancer screening model [5]. Therefore, this study was aimed to isolate the mayor compound present in the ethyl acetate extract and examined its inhibitory activity against leukemia L1210 cell line.



phalerin (**1**) : R_1`= R_5 = H, R_2 = β-O-glucoside, R_3 = R_4 = OH, R_6 = OCH_3

mahkoside (**2**) : R_1 = β-O-glucoside, R_2 = R_4 = H R_3 = R_6 = OH, R_5 = OCH_3

Fig. 1. The structure of phalerin (**1**) and mahkoside A (**2**)

EXPERIMENTAL SECTION

Material

The plant of *Phaleria macrocarpa* is collected from Cibeuteung village, Parung, West Java, Indonesia in May 2006, and determined by Herbarium Bogoriensis, Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia [5]. Another materials were silica gel 60 (70–230 mesh ASTM, Merck), $Ce(SO_4)_2$ (Merck), sulfuric acid (Merck), KBr, *n*-hexane p.a., ethyl acetate p.a., ethanol p.a., methanol HPLC grade (Mallinckrodt), dimethyl sulfoxide p.a. (Merck), mouse leukemia L1210 cell line, Eagle's

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minimum essential medium, dimethyl sulfoxide-*d6*, tetramethyl silane.

Instruments

LC-MS/DIP-MS (Mariner Biospectrometry), FT-IR spectrophotometer (Shimadzu 8400) UV-Visible spectrophotometer (Shimadzu 1601), NMR spectrometer (JEOL JNM-ECA 500), melting point apparatus (Buchi B-540), HPLC (Shimadzu LC-6A equipped with UV-detector), semipreparative HPLC column: Capcell Pak C18 SG120 (15 mm i.d. x 250 mm length), analytic HPLC column: Capcell Pak C18 (4.6 mm i.d. x 250 mm length), flush column chromatography (8.0 cm i.d x 90 cm length), thin-layer chromatography chamber, UV light cabinet (254 nm wavelength), laminar air flow, microscope.

Procedure

Isolation

Amount of 746 g of the dried bark of *Phaleria macrocarpa* (3.5% of water content) was macerated with *n*-hexane, ethyl acetate, and ethanol, respectively. The maceration was done three times for each solvent. After removing of the solvent by rotary evaporator, the dried ethyl acetate extract (15.0 g, 2.0% from dried material) was then subjected into silica gel column chromatograph, gave eight fractions, i.e. Fr-1 (0.337 g, 3.1% from dried ethyl acetate extract), Fr-2 (0.298 g, 2.7%), Fr-3 (0.321 g, 2.9%), Fr-4 (0.365 g, 3.3%), Fr-5 (0.619 mg, 5.6%), Fr-6 (5.729 g, 52.1%), Fr-7 (2.017 mg, 18.3%), and Fr-8 (1.126 mg, 10.2%) [5]. Subsequently, 200.0 mg of Fr-6 (the most potent fraction) [5] was diluted in 12.5 ml methanol, and then injected into semipreparative HPLC system (50 times run, 250 μl each injection), detector: UV at 254 nm, column: Capcell Pak C18 SG120 (15 mm i.d. x 250 mm length), mobile phase: methanol – water (4:6), flow rate = 5 ml/min. The separated peaks were collected and gave mayor isolates, so called isolate A (retention time = 12,3 min) in 43.9 mg (22% from Fr-6; 0.23% from the dried bark of *Phaleria macrocarpa*), isolate B (39.9 min) in 20.2 mg, and isolate C (46.2 min) in 4.3 mg. The purity of those isolates were determined by analytic HPLC system [detector: UV at 254 nm, column: Capcell Pak C18 4.6 mm i.d. x 250 mm length, mobile phase: methanol – water (4:6), flow rate = 1.0 ml/min]. The physico-chemical data of isolate A then was taken, i.e.: mass spectra, IR spectra with KBr pellets, UV spectra, ^{1}H-, ^{13}C- and 2D-NMR spectra (solvent: dimethyl sulfoxide-*d6*, internal standard: tetramethyl silane), and melting point.

Physico-chemical data of Isolate A

Isolate A appeared as reddish-brown flakes, melting point 182.3 °C. HRESI-MS: *m/z* 422.85 $[M+H]^+$, 444.78 $[M+Na]^+$, 866.6 $[2M+Na]^+$, (calcd for $C_{20}H_{22}O_{10}$ = 421.85). UV (MeOH) λ_{max} (nm): 203, 225, 291. IR (KBr) cm^{-1}: 3316, 2921, 1597, 1512, 1280-1200. ^{1}H NMR (500 MHz, DMSO-d_6): δH 7.58 (1H, d, *J* = 8.5 Hz,), 6.79 (1H, d, *J* = 8.5 Hz), 6.29 (1H, d, *J* = 1.8 Hz), 6.13 (1H, d, *J* = 1.8 Hz), 4.8 (1H, d, *J* = 8.0 Hz), 3.74 (3H, s), 3.43 (1H, m), 3.19 (1H, dd, *J* = 9.1 and 9.1 Hz), 3.03 (1H, dd, *J* = 9.1 and 9.1 Hz), 2.94 (1H, dd, *J* = 8.0 and 8.0 Hz). ^{13}C NMR (125 MHz, DMSO-d_6): δC 110.7 (C-1), 156.2 (C-2), 92.9 (C-3), 162.2 (C-4), 95.1 (C-5), 156.4 (C-6), 192.6 (C-7), 129.7 (C-1'), 131.8 (C-2' or C-6'),115.1 C-3'or C-5'), 161.1 (C-4'),100.7 C-1"), 73.2 (C-2"), 76.7 (C-3"), 69.8 (C-4"), 77.3 (C-5"), 60.8 (C-6"), 55.1 (OCH_3).

Inhibitory activity test against mouse leukemia L1210 cell line

Exponentially growing leukemia L1210 cells at a density of 2 x 10^5 cells/mL in Eagle's minimum essential medium was incubated in a 24 well microtiter plate for 48 h (37 °C, 5% CO_2) with various concentrations of isolate A were 2.5, 5, 10, 15, and 20 μg/mL. Control consisted of exposed to fresh medium only. Duplicate wells were prepared for each concentration of isolate A and for control.

The sum of growth cells were counted using haemocytometer under the microscope. The inhibitory activity of isolate A is the ability of the isolate A to inhibit the growth of leukemia L1210 cells which calculates by comparison of live cell in the sample with live cell in control. The IC_{50} value (*inhibitory concentration fifty*) is the concentration of sample that inhibits the growth of leukemia cells in 50% can be obtained from linear regression of log sample concentration vs probit of inhibitory activity.

RESULT AND DISCUSSION

Isolation of isolate A from the Fr-6 using HPLC

In the previous work, among the Fr-1 ~ Fr-8 obtained from ethyl acetate extract, Fr-6 showed the most potent inhibitory activity against mouse leukemia L1210 with an IC_{50} 11.6 μg/mL and collected in highest yield (52.1%). Based on the fact, Fr-6 was separated by semipreparative HPLC on reverse phase column (C18;15 mm i.d. x 250 mm length) gave isolate A (retention time = 12.3 min), isolate B (39.9 min), and isolate C (46.2 min) as major peaks. Confirmation of isolates by HPLC on C18 reverse phase column showed that only isolate A was collected in high purity (43.9 mg, 87.8% from Fr-6, 0.23% from dried bark).

Mass spectrum analysis could be clarified that this isolate has molecular weight of 422 and calculated as $C_{20}H_{22}O_{10}$. Based on ^{13}C-NMR and DEPT experiment, isolate A containing 20 carbon atoms

which consisting of one CH_3, one CH_2, eleven CH, six C, and one C=O. The existing of C=O at δ_C 192,6 ppm supported by IR data at 1597 cm^{-1} and UV spectrum at λ_{max} 291 nm indicating the C=O was ketone-substituted aromatic ring [6]. From the chemical shifts of proton and carbon showed that the CH_3 was $-OCH_3$. δ_C at 100.7, 77.3, 76.7, 73.2, 69.8, and 60.8 ppm were specific for glucoside moiety and δ_C 100.7 ppm and δ_H 4,8 ppm with *J coupling* = 8 Hz showed that glucoside moiety was *O*-glycosylated at anomeric position in β-linkage (Fig. 2a).

^{1}H-NMR spectra showed the specific *meta*-coupling of aromatic ring at δ_H 6.13 (1H, *d*, *J* = 1.8 Hz) and 6.29 ppm (1H, *d*, *J* = 1.8 Hz) (Fig. 2b), and δ_H 7,57 (2H, *dd*, *J* = 8,5 Hz) and δ_H 6,78 (2H, *dd*, *J* = 8,5 Hz) was specific for *orto*-coupling (Fig. 2c). Twelve carbon atoms consisted of six =CH and six =C- was two aromatic rings, so consider with its proton and carbon chemical shifts, suggested that the backbone structure was 2,4,4',6 substituted-benzophenone. From the molecular formula, it could be decided that the substituents were 2 x OH, $-OCH_3$, and glucoside moiety.

HMBC experiment and comparison of NMR data with mahkoside A, (4,4'-dihydroxy-6-methoxybenzo phenone-2-*O*-β-D-glucopyranoside) [4] (Table 1), it was clarified that isolate A was 6,4'-dihydroxy-4-methoxy-benzophenone-2-*O*-β-D-glucopyranoside (**3**) (Fig. 3). This compound has been isolated from *Gnidia involucrata* by Ferrari, *el al.* [7]. The structure was similar with mahkoside A except the positon of 6-hydroxy and 4-methoxy in mahkoside A to be a 4-hydroxy and 6-methoxy. This position was confirmed by HMBC experiment as shown in Fig. 3. This ^{1}H- and ^{13}C-NMR data were similar with benzophenone glycoside isolated from the leave of *Phaleria macrocarpa* by Kusmardiyani *et al.* [8], but OCH_3, -OH, and gucoside positions were not elucidated, since the 2D-NMR experiment did not examinized. Similar compound, but

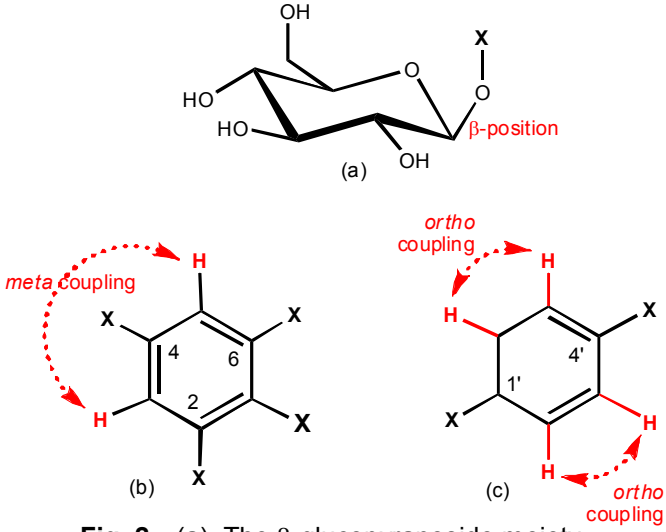

Fig. 2. (a). The β-glucopyranoside moiety
(b). Aromatic ring with *meta*-coupling
(c). Aromatic ring with *ortho*-coupling

Table 1. ^{1}H, ^{13}C, and HMBC data of Mahkoside A [5] and isolate A, both in DMSO-*d6*

Position of C	Mahkoside A			Isolate A		
	δH (mult, *J* in Hz)	δC	^{1}H-^{13}C HMBC	δH (mult, *J* in Hz)	δC	^{1}H-^{13}C HMBC
1	-	110.8		-	110.7	
2	-	156.3		-	156.2	
3	6.12 (*d*, 1.9)	95.3	C4, C2	6.13 (*d*, 1.8)	92.9	C1, C2, C4, C5
4	-	162.0		-	162.2	
5	6.30 (*d*, 1.9)	93.1	C6, C4	6.29 (*d*, 1.8)	95.1	C1, C3, C4, C6
6	-	156.5		-	156.4	
7		192.7			192.6	
OCH_3	3.74 (*s*)	53.3	C6	3.73 (*s*)	53.1	C4
1'	-	130.0		-	129.7	
2'	7.57 (*d*, 8.6)	131.9	C1', C6', C7	7.58 (*d*, 8.5)	131.8	C1', C6', C7
3'	6.60 (*d*, 8.7)	115.1	C2', C4'	6.79 (*d*, 8.5)	115.1	C1', C4', C5'
4'	-	161.2		-	161.1	
5'	6.80 (*d*, 8.7)	115.1	C4', C6'	6.79 (*d*, 8.5)	115.1	C1', C4', C3'
6'	7.57 (*d*, 8.6)	131.9	C1', C2', C7	7.58 (*d*, 8.5)	131.8	C1', C2', C7
1''	4.79 (*d*, 7.9)	100,8		4.8 (*d*, 8.0)	100.7	C6
2''	2.92 (*dd*, 7.9, 8.8)	73.4		2.94 (*dd*, 8.0, 8.0)	73.2	C1'', C3''
3''	3,18 (*dd*, 8.8, 9.2)	76.4		3.19 (*dd*, 9.1, 9.1)	76.7	C2'', C4''
4''	3.01 (*dd*, 9.2, 9.2)	70.0		3.03 (*dd*, 9.1, 9.1)	69.8	C5'', C6''
5''	3.28 (*m*)	77.4		3.29 (*m*)	77.3	C6''
6''	3.39 (*m*)	61.0		3.43 (*m*)	60.8	C5''
	3.69 (*m*)			3.68 (*m*)		

Table 2. Inhibitory activity test of isolate A against mouse leukemia L1210 cell line

Sample	Conc. (μg/mL)	Log of Conc.	% inhibition*	Probit of % inhibiton	Regr. Linier eq.	IC_{50} (μg/mL)
Isolate A	0		0,00	-	y = 3.769x + 2.35	**5.1**
	2.5	0.40	21.76	4,23		
	5	0.70	39.90	4,75		
	10	1.00	74.61	5,67		
	15	1.18	89.12	6.23		
	20	1.30	100.00	8.09		

* average from two replication

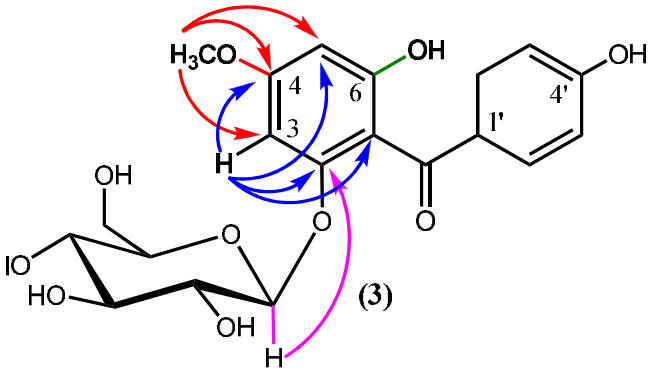

Fig. 3. 6,4'-dihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucopyranoside (**3**) (*arrow: HMBC correlation*)

the glucoside moiety was an α-anomeric, namely 6,4'-dihydroxy-4-methoxy-benzophenone-2-*O*-α-D-glucopyranoside have been found from the fruit of *Phaleria macrocarpa* by Tambunan, *et al.* [9].

Inhibitory activity test

Inhibitory activity test of isolate A against mouse leukemia L1210 cell line showed that this isolate A exhibited inhibitory activity with an IC_{50} 5.1 μg/mL (Table 2). This IC_{50} value of isolate A was lower than that its fraction, but it was still > 4.0 μg/mL. According to Swanson *et al*. [10], the isolate or compound has a potent anticancer agent if it has an IC_{50} ≤ 4.0 μg/mL in inhibitory activity test against cancer cell line *in vitro*. So, for further study, the structure and function group modifications of the isolate are needed for developing the better activity. Similar benzophenone glucoside, namely phalerin isolated by Wahyuningsih, *et al*. [3] from the leaves of *Phaleria macrocarpa* exhibited the less cytotoxic effect against myeloma NS-1 with an IC_{50} 83 μg/mL.

Isolate B (20.2 mg) and isolate C (4.3 mg) were not collected in pure, and it is not enough for structure elucidation, so the collection and purification are necessary.

CONCLUSION

From the bark of *Phaleria macrocarpa* have been isolated a benzophenone glucoside compound, namely 6,4'-dihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucopyranoside (3) and isolate B and C which were not purified yet. This benzophenone glucoside showed an inhibitory activity against mouse leukemia L1210 with an IC_{50} 5.1 μg/mL, it is higher rather than activity limit for isolate. So, the structure and function group modifications of the isolate are necessary for developing the better activity.

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