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A new 4',6-dihydroxy,4-methoxybenzophenone-2-*O*- -*D*-gentiobioside and 4',6-dihydroxy,4 methoxybenzophenone-2-*O*- -*D*-glucoside isolated from methanolic extract of *Phaleriamacrocarpa* (Scheff.) Boerl leaves

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Abstract

A new 4',6-dihydroxy,4-methoxybenzophenone-2-*O*- -*D*-gentiobioside(1) was obtained from the leaves of *Phaleriamacrocarpa* (Scheff.) Boerl along with known 4',6-dihydroxy,4-methoxybenzophenone-2-*O*- -*D*-glucoside (2). Bioactivity assays indicated that compound (1) and (2) have antioxidant activity and enhancement on SR-B1 genes expressionin vitro. Their structures compounds were established by the interpretation of their spectroscopic data and comparisonwith known compounds.

Keywords: *Phaleriamacrocarpa*, methoxybenzophenonegentiobioside, SR-B1

Introduction

Plants are still the main source in search for the new medicine resources due to their advantages such as cheap, abundance, low toxicity, and low side effect if used with the right dosage. One of this example is MahkotaDewa(*Phaleriamacrocarpa* (Scheff.) Boerl). The fruits of *P. macrocarpa* have been used as a traditional medicinein two forms. In freshly prepared or it consume as it is, like guava or as a mixture together with chili in fruit salad. Consumption of fresh fruit is very dangerous as it is toxic, causing serious effect such as mouth ulcer, numbness of the tongue, and drunk. Secondly, it has already processed into dried powder. It also can be combined with dried powder from other medicine plants to use as a traditional medicine (it's called " jamu"). *P. macrocarpa* traditionally can be used to cure many diseases like cancer, hepatitis, heart, diabetes, skin diseases, rheumatism, bacterial and viral infection, hypercholesterolemia, and hypertension [1]. Some medicine products of *P. macrocarpa* fruits have already been marketed, i.e. for cancer or tumor treatment, stroke, hypertention, diabetes, uric acid, allergy, influenza, and brain supplement. Recently, *P. macrocarpa* fruit has been used in cosmetic products, such as shampoo, beauty powder, beauty soap, scrubs, hair spa, message oil, and balsam. While, leaves from *P. macrocarpa*has been used as tea for leaves tumor or cancer treatment.

The therapeutic effect of natural material is related to the bioactive compounds. For instant, in *P. macrocarpa* fruit mesocarp, there are many alkaloid, saponin, flavonoid and tannin compounds. While it leaves contains alkaloid, flavonoid, saponindanpolifenol [2]. Subsequently, *P. macrocarpa* seed contains some compounds such as alkaloid, flavonoid, polifenol, and tannin which are found in abundance in its leaves [3]. The present of those compounds caused the anti- cancer, antioxidant and antibacterial activities [3, 4]. There are several compounds from *P. macrocarpa* which are alreadypublished and reported. Phalerin(4,5-dihydroxy-4'-methoxybenzophenone-3-*O*- -*D*-glucoside)was isolated from *P. macrocarpa* leaves [5].Icariside, phalerin, mangiferin[6], gallic acid [7], Isomer of phalerin [8, 9, 10]were isolated from *P. macrocarpa* fruit. Moreover,

desacetylfevicordin, together with it derivatives were also isolated from seeds of *P. macrocarpa*[11]. All compounds above have already published as anticancer agent.

Previous study reported that anticancer activity of the *P. macrocarpa* fruits and leaves were detected in Hela cells [12]. Its fruit was also showed anticancer activity againts leukemia cells [13], cervical cancer [14], and breast cancer ([15]. Meanwhile, the *P. macrocarpa* bark has anticancer activity against L1210 cells [16]. Moreover, anti-hypercholesterolemia activities from *P. macrocarpa* fruit extract in white rats has been studied by others researchers[17, 18].

Research on *P. macrocarpa* fruit and leaves merit have been done and published. However, there are no products and research related to the ability of compounds from *P. macrocarpa* leaves to lower the cholesterol substance (hypocholesterolemia). Antioxidant is one kind of the mechanisms which has a role in lowering cholesterol level. Antioxidant compound have been increased the expression of scavenger receptor class B type I (SR-B1) [19]. Antioxidant potencies of *P. macrocarpa* leaves extract was studied by Andrianiet al.[20]. They were reported the potential of ethyl acetate and methanol extractsof *P. macrocarpa* leaves as antioxidant agent.Thus, the isolated compoundsfrom *P. macrocarpa* leaves extract might also have an antioxidant activity and ability in increasing of expression of key genes in cholesterol metabolism such as SR-B1.

Materials and methods

General Experimental Procedure

Isolation of crude extracts were separated using gravity column chromatography, using silica gel 60 PF_{254} Art No. 7734 of particle size 0.063 – 0.200 mm (Merck, Germany). For further separations of fractions were carried out by using vacuum column chromatography by using silica gel 60 PF_{254} Art No. 9385 of particle size 0.040 – 0.063 mm. AR grade methanol, n-hexane, chloroform, ethyl acetate and methanol (Merck, Germany) were used in extraction and chromatographic analysis. The 1H and 13C NMR spectra were recorded on a FT-NMR Bruker Ultra Shield™ 400 and 500 MHz spectrometers, as well as a Unity Inova Varian 500 MHz spectrometer using tetramethylsilane (TMS) as internal standard. And EI-MS spectra spectra was measured on ThermoFinnigan MAT 95 XL spectrometer..Melting point was determined by using instrument Model Perkin Elmer Pyris 6 Disc (Disc Scanning Calorimeter). IR spectra were recorded on spectrometer model Perkin Elmer Spectrum 100 FTIR Spectrometer using KBr disc. The adsorption bands were measured in cm^{-1}. NMR Spectra were recorded on Varian Unity Inova 400 M*Hz* (with Pulsed Field Gradient) instruments. Meanwhile, mass spectrum was measured on instrument model Trace GC Brand Thermo Finnigan. TMS was used as internal standard. Thin layer chromatography (TLC) was used to identify the number of component in extract and fraction mixture while obtained the appropriate solvent system for the column chromatography isolation. TLC profiling of HMD, CMD and EMD crude extracts were performed by using TLC plastic sheets (Merck 1.05735.0001) which were pre-coated with silica gel $60F_{254}$, and MMD extract on TLC Aluminum sheets (Merck 1.05559.0001) which were pre-coated with RP-$18F_{254S}$. The TLC visualization was carried out by using UV-254nm, iodine vapour, DPPH TLC bioautographic assay (antioxidant potency tested), reagent spray with vanillin and ferric chloride ($FeCl_3$). Then, compounds purification was carried out by recrystalization, and gravity column chromatography for non-polar or semi polar compound, meanwhile by C-18 packing column combined with VLC for very polar compound..

Plant Material

Sample of *P.macrocarpa* leaves was collected from the same location of Bengkulu city, Indonesia, in 2008. The voucher specimen (SA1611/2008) has been deposited in the Herbarium of Bogoriense, Bogor, Indonesia.

Extraction and Isolation

Subsequently, the dried sample (500g) was soaked in solvent by successive extraction method [20] usinghexane, chloroform, ethyl acetate and methanol. The dried powder of leaves was extracted five times at room temperature for 24 hours. The extract were evaporated under reduced pressure to give 5% hexane (HMD), 6% chloroform (CMD), 2.5% ethyl acetate (EMD), and 3.3% methanol (MMD). Based on antioxidant assay, MMD was used to the next step. MMD (35 g) was subjected to open column chromatography (23 cm x 8 cm) on Si gel 60 (230-400 mesh, Merck) gravity chromatography. The column was packed by Chloroform/MeOH (8:2) and then followed by gradient elution of sequential chloroform and methanol. The fractions were collected 200 mL each, evaporated, labeled and monitored by TLC using water: acetonitril (6:4) as a solvent system. The fractions showed same TLC pattern were combined resulting in 10 fractions namely CF1-CF7. The active fractions (CF.3, 4, and 5) which was crystal in form were continued for further purification. Each them (CF.3=4.3g, CF.4=5.6g, CF.5=3.7g) were subjected to different sephadex LH-20 column chromatography (25cm x 4.5cm). The column was packed by chloroform/methanol (1:2) and then follows by chloroform and methanol (1:1) until finish. The fractions were collected 100 mL each, evaporated, labeled and monitored by TLC precoated C-18 using water: Acetonitril (6:4) as a solvent system. CF.4 was yielded one active fraction (F4). Meanwhile CF.5 was yielded two active fractions (F5 and F6). Then, each fractions were subjected to medium pressure liquid chromatography (MPLC) glass column with reverse phase C-18 and using water:acetonitril as solvent system, and follow by gradient elution of sequential water and acetonitril. Fraction F4 was yielded yellowish brown solid compound (MMD 2, 55mg).

Furthermore, F5 was yielded yellowish cotton compound (MMD4, 30mg). Yellowish cotton compound (MMD5, 40mg) was obtained from fraction F6. Then, each compound was freeze dried to evaporate the water solvent. The purity were checked by TLC aluminium pre-coated sheets with reverse phase-18F_{254S} to show one spot. The TLC visualization was carried out by using UV-254nm, iodine vapour, reagent spray with DPPH, vanillin and ferric chloride ($FeCl_3$). TLC visualization of MMD2 (55mg) and MMD4 (30mg) were showed the same Rf value (0.55) with water/acetate solvent system 6/4 (v/v) migrated, then they were combined as compound (**1**). Subsequently, MMD5 was showed Rf value at 0.45 as compound (**2**).

4',6-Dihydroxy,4-methoxybenzophenone -2-O-β-D-gentiobioside

Yellowish solid; m.p. 165–167; IR Vmax (KBr) (cm^{-1}): 3374 (O–H stretching), 2935 (C-H stretching), 1614 (C=O stretching), 1512, 1436,(C=C aromatics). For ^{1}H NMR (DMSO-d_6, 500 M*Hz*) and APT ^{13}C NMR (,DMSO-d_6, 100 M*Hz*) spectroscopic data, see Table 1. EIMS m/z 259 [M]+, 166, 121.

4',6-dihydroxy,4-methoxybenzophenone -2-O-β-D-glucoside

Yellowish brown solid; m.p. 180–181; IR Vmax (KBr) (cm^{-1}): 3369 (O–H stretching), 2937 (C-H stretching), 1616 (C=O stretching), 1511, 1436,(C=C aromatics). For ^{1}H NMR (MEOD, 400 M*Hz*) and ^{13}C NMR (MeOD, 100 M*Hz*) spectroscopic data, see Table 2. EIMS m/z 259 [M]+, 166, 121.

Bioassay

(1). Determination of antioxidant activity by DPPH· Stable Free Radical Scavenging Activity.

The ability of extracts and pure compounds to scavenge the α, α-diphenyl-β-picrylhydrazyl (DPPH) radical was measured as reported by Gadovet al.[21].

(2). The effect of the isolated compounds on SR-B1 genes expression.

Extraction of the Reporter Plasmid DNA.

Two recombinant reports plasmid were used in this experiment, namely pGL-3 SR-B1 and pRL-CMV. In order to purify the recombinant plasmid used in transfection luciferase assay experiment, plasmid midi kit (Qiagen 100 protocol, cat no 12145) was used. Briefly 1 mL of recombinant vector combining pGL-3 SR-B1 and pRL-CMV, respectively were transferred into 25mL LB broth in erlenmeyer 50mL and incubated at 37^{0}C with shaking at 200 rpm for 16 hours. Then, the culture was transferred into backman tube and centrifuged at 6000 rcf for 15 minutes at 4^{0}C. Supernatant was removed and the pellet was air dried. Subsequently 4 mL Buffer P1 was transferred into the tube and mixed with pellet by vortexing 4 mL buffer P2 was then added, mixed and incubated at room temperature for 5 minutes. Next, 4 mL buffer P3 was added and centrifuged at 12,000 rcffor 30 minutes at 4^{0}C. The supernatant was transferred to another backman tube and re-centrifuged at 12,000 rcf for 15 minutes at 4^{0}C. The supernatant was then applied into Qiagen-tip and allowed to enter the resin by gravity flow. The Qiagen-tip was washed with 2x10mL QC and the coagulant plasmid DNA was eluted with 5 mL QF by grafity flow. Subsequently DNA solution was mixed with 3.5 mL of isopropanol, mixed gently, and centrifuged at 15,000 g for 10 minutes. The supernatant was carefully removed and pellet was air dried for 5-10 minutes. Plasmid DNA was redissolved in a suitable volume (100-200µL) ddH_2O. Then proceed to calibrate concentration by spectramax and double digestion of pGL3-SR-B1 plasmid to confirm the inserts using XhoI and Hind III. Distilled water was use as a blank. Then it was run by electrophoresis to make sure the plasmid was inexistence.

Lipofectin® Transfection. Transient transfection was carried out using Lipofectin® (Invitrogen) as described by the manufacturer. Prior to the day of transfection, 4-5 x 10^4 cells/mL were seeded in 96-well plates in a volume 200µL complete medium each well. The cell were incubated overnight (18-24 hours) at 37^{0}C in a CO_2 incubator until the cells were 60-80% confluent. On the day transfection, two solution were prepared. In solution A, 24µL of Lifofectin was diluted in 600µL of MEM serum free medium and incubated for 10-20 minutes at room temperature. Solution B was prepared by mixing 4.5 µg pRL-TK and 18 µg pGL3-SR-B1 in the final volume of 600 µL MEM serum free medium and left at room temperature for 10-20 minutes. pRL-TK contains the cDNA encoding *Renila* luciferase driven by thymidine kinase minimal promoter element reporter gene was normalized against for transfection efficiency. Then, both solution A and B was mix gently and incubated at room temperature for 10-15 minutes. Subsequently, 12 mL of fresh MEM serum free medium was added and mix well. For transfection, the growth medium was removed from the cells and wash once with PBS. Subsequently, 100 µL of transfection medium was added into each well and incubated at 37^{0}C for 12 hours. Then the cell were washed with PBS twice and treated with variousconcentrations (3,1; 6,3; 12,5; 25,0; 50,0; 100µg/mL) of compounds/extracts in 100 µL volume of fresh medium and incubated at 37^{0}C for 24 hours. The cells were then lysed and promoter activity was assayed.

Assay of Luciferase Activity.

Luciferase activity was measured using Dual-Luciferase® Reporter Assay System (Promega) where the activities of firefly (*Photinuspyralis*) and *Renilla* (*Renilla*reniformis) luciferases were measured sequentially from a single sample. Following treatment, the cells were subjected to luciferase assay. Briefly, 25 µL of medium was removed from each well and 75 µL dual glo stop and glo luciferase reagent was added to each well, incubated for 10 minutes and *Renilla* luciferase activity (as the control for

transfection efficiency) measured. All the luminescence reading were then substrated against the untreated sample. The SR-B1 promoter luminescences reading were then normalized against pRL-TK luminescence reading for each respective concentration. Luminescence reading taken from transfected cells treated with rosiglitazone (positive control) was assigned as 100% and the readings from treated cells were then calculated as relative to the positive control (Result calculated as ratio of SR-B1 promoter expression of samples compared to control x 100%). It was called as relative induction (% of control).

Result and Discussion

Structure elucidation

IR spectrum of compound (1) showed absorption bands at 3374 cm^{-1} indicating the presence of aromatic -OH group. Absorption band at 2935 cm^{-1} indicated the presence of saturated C-H group. Absorption bands at 1512 cm^{-1} and 1436 cm^{-1} represented as conjugated C=C aromatic. Absorption band at 1163 cm^{-1} and 1075 came from C-O stretching. Characteristic absorption band at 1614 cm^{-1} and 1280 cm^{-1} strongly correlated with C=O functional group4',6-Dihydroxy-4-methoxybenzophenone-2-*O*- -*D*-gentiobioside or 4',6-di hydroxy-4methoxybenzophenone-2-*O*-[-*D*-glucopyranosyl-(1 6)- -*D*-glucopyranoside] was isolated from MMD extract from *P. macrocarpa* leaves. The compound physically is a yellowish solid. The ^{1}H NMR data showed the hydroxy protons (-OH) as two singlets at 6.70 (1H, *s*) and 10.30 (1H, *s*) substituted at carbon 4' (160.8) and 6 (161.3) which correlated to benzophenone. The existing of -OH supported by IR data at 3374 cm^{-1}.The assignment of carbon and proton were established by using the HSQC and HMBC correlation (Figure 1). The presence of the same para-substituted symmetric phenol ring as appeared as deshielded signals at 7.936 (2H, *J*=7.2 *Hz*) and 6.932 (2H, *J* =9.0 *Hz*). The aromatic protons of the other ring displayed two doublets at 6.87 (1H, *d*, *J*=2.4 *Hz*) and 7.03 (1H, *d*, *J*=2.4 *Hz*), characteristic for a meta coupling and these correlation established by COSY experiment data, and it also showed the specific ortho-coupling between proton 7.93 (2H, *d*, *J*=7.2 *Hz*) and 6.93 (2H, *d*, *J*=9.0 *Hz*).

From APT ^{13}C NMR spectrum data, compound (1) carbon atoms consisted of one=CH_3 (56.0), one=C=O (176.8), two=CH_2 (68.6 and 65.6), six =C- and others appeared as C-H (data showed in Table 1). The APT ^{13}C NMRand ^{1}H NMR spectrums suggested the presence of a methoxy group (^{1}H: 3.90; ^{13}C: 56.08), where Its position was assigned in C-4 to an HMBC correlation experiment (Figure 1). A signal at 176.8, typical for the carbonyl group of a benzophenone.The existing of C=O supported by IR data at 1614 cm-1 indicating the C=O was ketone-substituted aromatic ring (Silverstein *et al.*, 1991). Carbon signals which appeared at 104.7 - 65.6 were specific for gentiobiosidemoeity and other characterize signals of aromatic carbons appeared at 96.6 – 163.5. ^{1}H and ^{13}C assignments for compound (1) showed on Table 1. The presence of an *O*- -*D*-gentiobioside was confirmed by the characteristic ^{1}H signal for the anomeric proton at 4.78 (H-1"), its linkage position at C-2 of benzophenone was ascertained by HMBC correlations (Figure 1). The presence of an *O*- -*D*-glucopyranoside was confirmed by other ^{1}H signal for an anomeric proton at 4.20 (H-1'") with linkage position at C-6" (1 6) which is showed correlation between two glucoses in gentiobiose. An -anomeric protons both of them was confirmed by appear as doublets with *J* = 7.8 *Hz*. This is agreement with Agrawal[22] and Ferrrari *et al.* [23], which they reported that -anomeric protons appear as doublets with $^3J_{1,2}$ in the range 6.0 - 7.8 *Hz*. Moreover, hydroxy signals were observed as a singlet signal at 6.70 ppm and 10.30 ppm as they was shown by the absence of carbon directly attached to –H signal on the ^{13}C-^{1}H 2D-NMR (HSQC) spectra. The MS spectrum suggested the molecular formula $C_{26}H_{32}O_{15}$. The molecular formula of compound (1) which has relative molecular mass 584 g/mol was determined on the basis of EI-MS. The EI-MS analysis confirmed the loss of this sugar moiety with a *base peak* at *m/z* 259 $[M\text{-Gentiobiose-H}]^+$. The fragmentation ion of compound (1) at *m/z* 166 and 121 are due to the fission of ring. Therefore, a chemical IUPAC name of compound (1) is 4',6-dihydroxy-4-methoxybenzophenone-2-*O*-[-*D*-glucopyranosyl-(1 6)- -*D*-glucopyranoside] or 4',6-dihydroxy-4-methoxybenzophenone-2-*O*- -*D*-gentiobioside (Figure 2). Confirm that compound (1) is a new compound as it has not found yet both in leaves, fruits and other part of *P. macrocarpa* and also not published yet elsewhere.

Compound (2), namely 4',6-Dihydroxy,4-methoxybenzophenone-2-*O*- -*D*-glucopyranoside was isolated from MMD extract from *P. macrocarpa* leaves. The compound (2) appeared as a yellowish brown solid with melting point 180-181 ^{0}CandR$_f$ valueis0.35. The IR spectrum data indicated the presence of aromatic hydroxyl group (3369 cm^{-1}, broad), C-H stretching at 2937 cm^{-1}, and conjugated carbon-carbon bonds (C=C) at 1616 cm^{-1}. Moreover, Bands at 1511 cm^{-1} and 1436 cm^{-1} showed the present of aromatic ring due to C=C group. The signal of ^{1}H NMR (400 M*Hz*, MeOD) data indicated that the presence of aromatic proton group which correlated to benzophenone skeleton hydroxy group (-OH) at 4.9 substituted at carbon 4' and 6. The existing of -OH supported by IR data at 3369 cm^{-1}. Deshielded signals appeared at 7.72 (2H, *d*, *J*=8.8 *Hz*) and 6.82 (2H, *d*, *J* =8.8 *Hz*) showing the presence of the same para-substituted symmetric phenol ring. The aromatic protons of the other ring displayed two doublets at 6.19, *J*=2.4 *Hz*) and 6.42, *J*=2.4 *Hz*), characteristic for a meta coupling, it was established by COSY experiment. The ^{1}H-^{1}H COSY spectrum showed the characteristic ortho-coupling correlations between proton 6.82 (2H, *d*, *J*=8.8 *Hz*) and 7.72 (2H, *d*, *J*=8.8 *Hz*), and the specific meta-coupling correlation between proton 6.19 (1H, *d*, *J*=2.4 *Hz*) and 6.42 (1H, *d*, *J*=2.4 *Hz*).

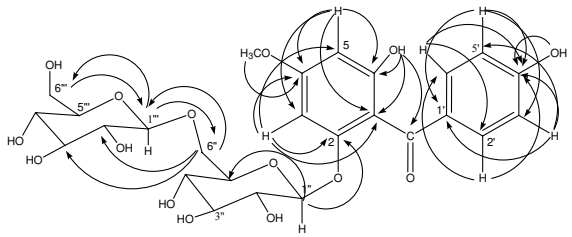
Table 1: 1D-NMR (^{1}H and ^{13}C NMR) and 2D-NMR assignments (500M*Hz,* DMSO-d_6) for compound (1)

Compound (1)						
Position -C-	^{13}C-NMR (, ppm)	Position -H-	^{1}H-NMR (, ppm, *J*=Hz)	HMBC Correlation		
				1*J*	2*J*	3*J*
-C=O	176.8	-	-		C-6	
C-4	163.5	-	-			
C-6	161.3	-	-			
C-4'	160.8	-	-			
C-2	158.1	-	-			
C-2'		H-2'	7.93(2H, *d*, 7.2)			C-4', C-6'
C-6'	128.0	H-6'				C-2', C-4'
C-1'	115.8	-	-			
C-3'		H-3'	6.93(2H, *d*, 9.0)			C-1', C-5'
C-5'	115.8	H-5'				C-2', C-3'
C-1	109.1	-	-			
C-1"	104.7	H-1"	4.78(1H, *d*, 7.8)		C-2	C-5"
C-1"'	103.7	H-1"'	4.20(1H, *d*, 7.8)		C-6"	C-6"'
C-3	102.9	H-3	6.87(1H, *d*, 2.4)	C-3	C-2, C-4	C-1, C-5
C-5	96.6	H-5	7.03(1H, *d*, 2.4)		C-4	C-1, C-3
C-3"'	76.5	H-3"'	3.12(1H, *m*)			
C-5"	75.9	H-5"	3.57(1H, *m*)			
C-3"	75.6	H-3"	3.30(1H, *m*)			
C-2"	73.4	H-2"	2.99(1H, *m*)			
C-4"'		H-4"'	3.38(1H, *d*.1.8)			
C-5"'	73.3	H-5"'	3.36(1H, *d*.1.8)			
C-4"	69.7	H-4"	3.26(1H, *m*)			
C-2"'	69.5	H-2"'	3.28(1H, *m*)			
C-6"'	68.6	H-6"'a	3.99(1H, *d*, 10.2)			C-1"'
-	-	H-6"'b	3.65(1H, *dd*, 4.8;11.4)			C-1"'
C-6"	65.6	H-6"a	3.70(1H, *dd*, 5.4;10.8)		C-1"'	C-2"'
-	-	H-6"b	3.04(1H, *d*,10.8)		C-1"'	C-2"'
OCH_3	56.0	$-OCH_3$	3.90(3H,*s*)			
		-OH	6.70(*s*)	C-6	C-1	C=O
		-OH	10.30(*s*)	C-4'		

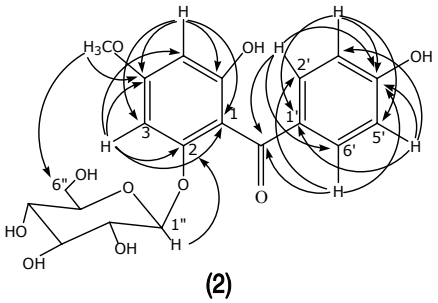
Table 2: 1D-NMR (^{1}H and ^{13}C NMR) and 2D-NMR assignments (400M*Hz*, MeOD) for compound (2)

Compound (2)					
Position -C-	^{13}C-NMR (, ppm)	Position -H-	^{1}H-NMR (, ppm, *J*=Hz)	HMBC Correlation	
				2*J*	3*J*
-C=O	195.6	-	-		
C-4	162.8	-	-		
C-4'	162.4	-	-		
C-6	157.6	-	-		
C-2	157.0	-	-		
C-2'	132.1	H-2'	7.72(2H,*d*,8.8)	C=O	C-4'
C-6'		H-6'		C=O	C-2', C-4'
C-1'	130.4	-	-		
C-3'	114.5	H-3'	6.82(2H,*d*,8.8)	C-4'	C-1',C-5'
C-5'		H-5'			C-1', C-3'
C-1	110.3	-	-		
C-1"	101.1	H-1"	4.88(1H, *s*)	C-2	
C-5	95.3	H-5	6.19(1H,*d*, 2.4)	C-4,C-6	C-1,C-3
C-3	93.4	H-3	6.42(1H,*d*, 2.4)	C-2,C-4	C-1,C-5
C-5"	76.9	H-5"	3.42(1H,*m*)	C-4'	
C-3"	76.4	H-3"	3.39(1H,*m*)	C-2"	
C-2"	73.3	H-2"	3.15(1H,*t*, 7.6;9.2)	C-1",C-3"	
C-4"	69.8	H-4"	3.27(1H,*m*)	C-5"	C-6"
C-6"	61.2	H-6"	3.67(2H,*dd*,6.0;12.0)	C-5"	
-OCH_3	54.5	OCH_3-	3.82(3H, *s*)	C-4	
		-OH	4.90		

The ^{13}C NMR spectrum data showeda signal at 195.6, typical for the carbonyl group of a benzophenone, and 12 carbons assignment of this structure gives in Table 2. The existing of C=O supported by IR data at 1616 cm^{-1} indicating the C=O was ketone-substituted aromatic ring [24]. ^{13}C NMR signals which correlated to doublets ^{1}H NMR signal showed at 132.1 (C-2' and C-6') and 114.5 (C-3' and C-5'). Carbon signals at chemical shift 101.1 (C-1"), 76.9 (C-5"), 76.4 (C-3"), 73.3 (C-2"), 69.8 (C-4"), and 61.2 (C-6") were specific for glucoside moiety. Others carbon signals of aromatic carbons `of benzophenone at chemical shift 162.8 (C-4), 162.4 (C-4'), 157.6(C-6), 157.0 (C-2), 130.4 (C-1'), 95.3 (C-5) and 93.4 (C-3), confirmed the structure hypothesis. Spectrum data of HMQC, HMBC and COSY experiments allowed complete assignment and identification of the aglycone. ^{1}H and ^{13}C NMR suggested the presence of a methoxy group (^{1}H: 3.82; ^{13}C: 54.5) of benzophenone structure. Its position was assigned in C-4 (162.8) according to the HMBC (Figure 1) correlation experiment. The presence of an O- -D-glucose was also confirmed by the characteristic ^{1}H signal for the anomeric proton at 4.88 [22]. Its linkage position at C-2 was ascertained by HMBC (Figure 1). Usually, -anomeric protons appear as doublets with $^{3}J_{1,2}$ in the range 6.0 - 6.8 *Hz* [22] and around 7.8 *Hz*[23]. Meanwhile, there was not shown double peak in this study, it could be caused by compound (2) has an anomeric proton chemical shift quite same with proton chemical shift of the solvent (overlapping between MeOD and compound (2), those why the double peak was not appear. Moreover, hydroxy signal was observed as a broad signal at 4.90 ppm corresponded to –OH sugar as it was shown by the absence of carbon directly attached to –H signal (4.90 ppm) on the ^{13}C-^{1}H 2D-NMR (HMQC) spectra data. The Hydroxyl signal at 4.90ppm could be also effect on the appearance of anomeric proton.



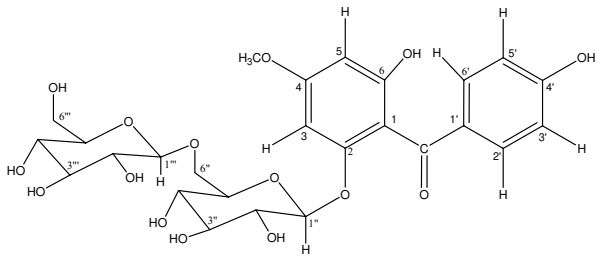
(1)



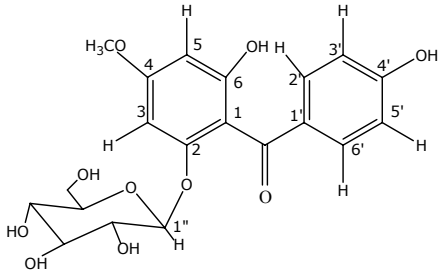
(2)

Figure1: Selected HMBC (H C) correlations exhibited by compound (1) and (2).

The EI-MS spectrum pattern together with ^{1}H and ^{13}C NMR spectra suggested that molecular formula for compound (2) was $C_{20}H_{22}O_{10}$ (Figure 2). The EI-MS analysis confirmed the loss of this sugar moiety with a *base peak* at *m/z* 259 $[M\text{-}Glc\text{-}H]^{+}$. The fragmentation ion at *m/z* 166 and 121 are due to the fission of ring based on fragmentation ion pattern from Aripin[9]. Therefore, a chemical IUPAC name of compound (2) is 4',6-dihydroxy-4 methoxybenzophenone-2-*O*- -*D*-glucopyranoside (Figure 2). The spectrum pattern of this compound is similar to 4',6-dihydroxy-4-methoxybenzophenone-2-*O*- -*D*-glucopyranosidewhich has been isolatedfrom *P. macrocarpa*fruit [6, 9] and bark [16]. Similar compound, but the glucoside moiety was an -anomeric, namely4',6-dihydroxy-4-methoxybenzophenone-2-*O*- -*D*-glucopyranosidewas also obtained from *P.macrocarpa* fruits [8].



(1)



(2)

Figure 2: Structure of 4',6-Dihydroxy-4-methoxybenzophenone-2-*O*- -*D*-gentiobioside or 4',6-dihydroxy-4-methoxybenzophenone-2-*O*-[-*D*-glucopyranosyl-(1 6)- -*D*-glucopyranoside] (1) and 4',6-dihydroxy-4-methoxybenzophenone-2-*O*- -*D*-glucoside (2)

Antioxidant activity (DPPH free radical scavenging ability)

Evaluation of antioxidant activity of *P.macrocarpa* leaves extracts was adapted from Gadov*et al.* [21] with some modifications. Crude extract of MMD and EMD exhibited a strong and moderate free radical scavenging with the inhibition percentages are 79 and 76%, respectively. Crude extract of HMD and CMD exhibited low and moderate inhibition with percentages ranging from 59-69% (data not shown). Meanwhile, 4',6-dihydroxy,4 methoxybenzophenone -2-*O*- -*D*-gentiobioside (1) and 4',6-dihydroxy,4 methoxybenzophenone -2-*O*- -*D*-glucopyranoside (2)

have moderate free radical activity with inhibition percentages are 66% and 69%, respectively (Table 3). All extracts have less activity as compared to Quercetin. Quercetin was used as a positive control in this screening. Phenolic compounds such as quercetin, rutin, narigin,catechins, caffeic acid, gallic acid and chlorogenic acid in plants were reported to play important role in antioxidant activities [25].

Table 3: DPPH free radical scavenging activity of *P.macrocarpa* leaves

Type of sample	Sample	Free radical scavenging activity (%)	IC_{50} (mg/mL)
Compound (1mg/mL)	(**1**)	66.25±0.06	4.9
	(**2**)	69.45±0.03	4.2
Positive control mg/mL)	Quercetin	85.59±0.06	0.34

*Each value is presented as mean ± SD (n = 3).

The Effect of *P. macrocarpa* Leaves on the Transcriptional Activity of SR-B1 Promoter

The methanolcrude extract of *P. macrocarpa*leaves (MMD produced significant effects on the SR-B1 promoter luciferase activity (Figure 3). Generally, the active crude extract of *P. macrocarpa*leaves MMD produced higher transcriptional activity of SR-B1 promoter as compared to the untreated samples. In the MMD and P1 cases, 12.5 µg/mL produced highest transcriptional activity with MMD increased the promoter activity of SR-B1 gene to 40% of control and its compounds (P1) to 30%. Only in P2 case, 25 µg/mL produced highest transcriptionalto 32% of control. The result clearly demonstrated the potential role of MMDand its isolated compounds in increasing the expression of SR-B1 gene and subsequently the number of receptor present on the surface of liver cells, which in turn, suggested the potential of the extract and compounds in reducing the cholesterol levels in bloodstream by increasing the free HDL levels. Some studies reported, the hepatic over expression of SR-B1 is associated with decreased of plasma cholesterol levels transport into the bile and increased of plasma levels of free HDL [26, 27, 28]. It was well established that HDL plays an important role in reverse cholesterol transport (RCT) by removing plasma cholesteryl ester (CE) as well as accumulated CE along the lining of blood vessels to the liver, thus, reducing the risk of atherosclerosis [29, 30].

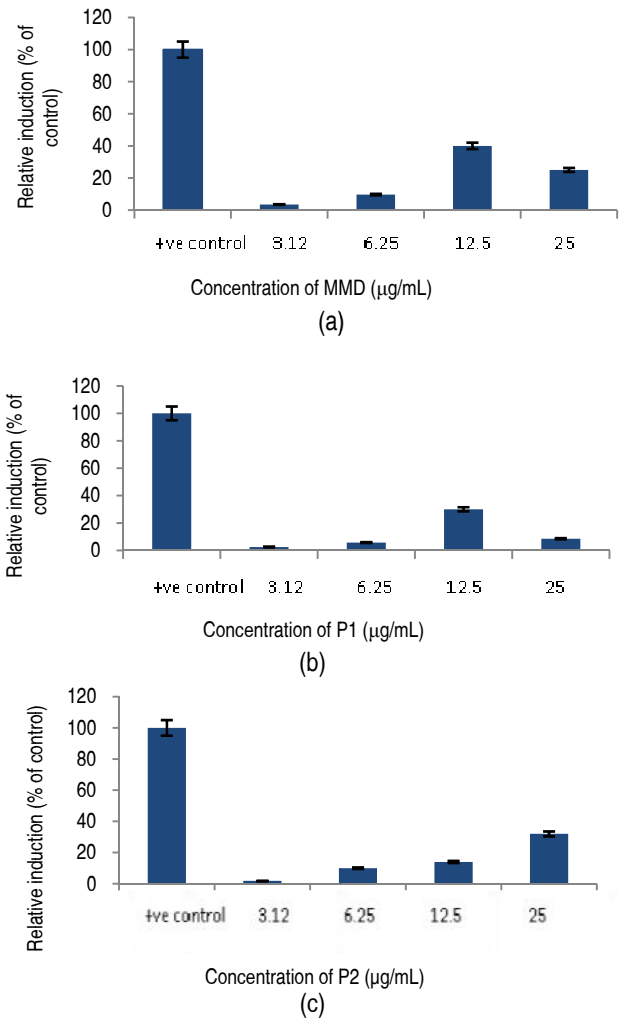


Figure 3: The SR-B1 expression of active fraction of MMD (a), P1 (b),and P2 (c) from *P. macrocarpa* leaves. Values is presented as mean ± SD.

Conclusion

In summary, a new benzophenonegentiobioside (1) along with known benzophenoneglucoside (2) were isolated from the leaves of *Phaleriamacrocarpa* (Scheff.) Boerl. Antioxidant assay was indicated the compound (1) and (2) have antioxidant activity by 66 and 69%, respectively. In addition, MMD and compounds (1 and 2) were also increased the SR-B1 gene expressions with activity by 40%, 30% and 32%, respectively compared to the positive control (rosiglitazone) at 12.5µg/mL.

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