Isolation and Characterization of Compounds from the Leaves of *Pterocarpus indicus* Willd and Their Antioxidant Activity

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Abstract

The flavone glycoside was isolated from ethyl acetate fraction of ethanol extract of leaves *Pterocarpus indicus* Willd. The isolation was conducted by gravitation column chromatography and eluted successively with hexane, ethyl acetate and methanol by gradient, and purified by sephadex-LH20. The structure was elucidated base on spectroscopy data of NMR (1D and 2D), UV, LC-MS and FT-IR. Antioxidant was evaluated using 2,2-diphenyl-1-picrylhidrazyl (DPPH) radical scavenging. The isolation and identification led a stigmasterol as Compound 1 and a new flavonol-glycoside [(2R)-7-hydroxy-3-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-2-(3,4,5-trihydroxy phe nyl)chroman-4-one] or ptevon-3-*D*- glycoside as Compound 2. Antioxidant activity of Compound 2 showed IC_{50} for 18.53 µmol and blank of quercetin was 7.94 µmol and Vitamin C was 40.25 µmol. These compounds and antioxidant activities are the first time reported from this plant.

Keywords: *Pterocarpus indicus* Willd, antioxidant, flavonoid - glycoside, ptevon-3-*D*- glucoside, and quercetin

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Introduction

Pterocarpus indicus Willd. is plant belonging to the family *Papilionaceae* and is widely distributed over tropical and subtropical south Asia as Malaysia, Philippines, Brunei, Thailand, and Indonesia. It is recommended as an ornamental avenue tree and the reddish hard wood is an excellent timber in Southern Asia. A red, gum-like resin from the bark is used in folk remedies for tumours and the leaf for cancers, especially of the mouth cancer (Orwa *et al.*, 2009). The leaves significantly inhibited the growth of Ehrlich as cites carcinoma cell in mice (Orwa *et al.*, 2009). Wood contains the red colouring matters, narrin and santalin, and angolensin, narrin is a dark red amorphous powder which yields phloroglucinol and resorcinol on fusion with alkali (Duke, 1983). The physicochemical properties, elements, and amino acids have been analysed (Surowiec *et al.*, 2004). The structural analysis shows that the crystal is a macromolecular compound of tannic condensation and glucoside (Wang *et al.*, 1997). A mixture of loliolide (> 85 %) and paniculatadiol (< 15 %) was obtained from the ethyl acetate leaf extract of *P. indicus*, while the air-dried flowers afforded lupeol and phytol esters. Antimicrobial tests on a mixture of loliode and paniculatadiol indicated that it has moderate activity against *Candida albicans* and low activity against *Pseudomonas aeruginosa, Escherichia coli,* and *Aspergillus niger.* It was found inactive against *Staphylococcus aureus, Bacillus subtilis*, and *Trichophyton mentagrophytes* (Ragasa *et al*., 2005; Fatima, 2004). Hiani (2013) has reported that ethanolic extract of *P. indicus* showed reduction percentage of blood glucose level, which were 73.12 %, 72.08 %, and 67.77 % at the dosages of 250 mg/kg BW, 500 mg/kg BW, and 1000 mg/kg BW, respectively, and the standard group metformin (67.68 %). The fractions of petrol, dichloromethane, ethyl acetate, butanol and methanol of leaves, root, and stem barks of *P. indicus* were exhibited a wide spectrum of antibacterial activity. The activity was more prominence in butanol and methanol fractions (Khan & Olomoso, 2003). The ethanol leaves extract of *P. indicus* exhibited a strong

inhibition against *Staphylococcus aureus,* but weak against *Streptococcus pyrogenes* (Fatima, 2004).

As a part of our continuing search for bioactive natural product, the ethyl acetate soluble partition of methanolic extract from leaves of *P. indicus* Willd was conducted to isolate of compounds and study their antioxidant activity.

Materials and Methods

Plant material. Raw materials of *P. indicus* were collected from South Tangerang, Banten, Indonesia. The voucher specimen was identified at Research Center for Biology, Indonesian Institute of Sciences (LIPI), and specimen was deposited in Herbarium Bogoriense Research Center for Biology, LIPI.

Chemicals. Technical organic solvents, ethanol, methanol, ethyl acetate, *n*-hexane and *n*-butanol. Silica gel G_{60} (0.062-0.2000 mm) E Merck 1.07734, Silica gel G_{60} (0.2-0.5 mm) E Merck 1. 07733, Silica gel G_{60} F_{254} E Merck 1.07730, TLC silica gel 60 F_{254}, aluminium sheets E. Merck 1.05554.0001, *Sephadek* LH-20 Amersham, standard quercetin from Sigma (Q4951), and Ascorbic acid from E. Merck (611-F713827) were used.

Instruments. The melting point was determined using a micro melting point measurement Fisher Scientific. UV spectrum was measured using Agilen Technologies Carry 60 G.6860A UV-Vis. IR spectrum was taken using FT-IR Prestige-21, Shimadzu, NMR spectra of ^{1}H, ^{13}C, HMQC (Heteronuclear Multiple Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) were measured using an Inova Plus, Unity NMR 500 at 500 MHz (^{1}H) and 125 MHz (^{13}C) CD_3OD as solvent with TMS as an internal standard. LC-MS analysis was performed using Mariner biospectrometry equipped with binary pump.

Extraction and Isolation. Leaves of *P. indidus* Willd. (2.7 kg) were air dried, ground, then extracted exhaustively with 70 % aqueous of ethanol (3×10 L) at room temperature. The ethanol soluble extracts were concentrated in *vacuo* to yield a dark green semi solid mass 289 g (10.70 % w/w), 200 g of ethanol extract was suspended in aqueous (900 mL) and partitioned sequentially in three different solvents, *n*-hexane (4×900 mL), ethyl acetate (4×900 mL) and *n*-butanol (4×900 mL), to fractionate polar and non polar compounds. The organic phases were concentrated to yield residues with 0.52 g of *n*-hexane extract, 15.03 g of ethyl acetate extract, 18.42 g of *n*-butanol extract, and 114.23 g of water extract. The ethyl acetate extract (15 g) was then fractionated by gravitation of column chromatography and produced 20 fractions (1-20), white needles was found in the fifth fraction, then dissolved with methanol to solve impurities, pure crystal (15 mg) further transformed into a needle crystal to be recrystallized with hexane and chloroform. Purity test was performed by TLC using *n*-hexane and ethyl acetate solvent (4:1) and compared with standard stigmasterol. Subsequently, the sample is marked as compound 1 after determination of its melting point, FT-IR, and ^{1}H, ^{13}C NMR. The 80.57 mg of eightieth (18^{th}) fraction was purified with sephadex LH-20 column chromatography using dichloromethane : methanol (1:1) as mobile phase, and yielded 23.3 mg of pure Compound 2, which then processed further to determine its melting point, UV , FT- IR, LC-MS and ^{1}H, ^{13}C NMR one and two dimension.

Antioxidant Assay. The free radical scavenging activity of Compound 2, Vitamin C standards, and quercetin were analysed by the DPPH assay (Yen & Chen, 1995). The test compound and standards were made in concentration ranging from 1 to 25 µg/mL in methanol. Each of sample was added 500 µL DPPH (1 mM), then methanol was added to make final volume of 2.5 mL, and incubated under constant mixing at room temperature (37 ^{o}C) for 30 minutes. The absorbance was measured at 515 nm. Percent inhibitory activity was calculated from $[(A^{o}-A^{1})]/A^{o}]\times100$, where A^{o} is the Absorbance of the control, and A^{1} is the absorbance of the sample or standard.

Results and Discussion

Characterization of Compound 1.

Compound 1 was obtained as white needles with amount of 15 mg and a melting point is

160-162 ^{o}C. The IR (KBr) spectrum data showed the presence of hydroxyl group (-OH) in the absorption peak at region (3419-3294) cm^{-1} (broad) and indicated the presence of -CH, -CH_2, and -CH_3 groups in the regions absorption bands at (2937-2864) cm^{-1}. The absorption band at 1643 cm^{-1} indicated the presence of C-O- stretching. The ^{1}H-NMR spectroscopy data ($CDCl_3$, 500 MHz) showed chemical shift (δ) of methyl singlet at 1.01 ppm (3H, *s*) and 1.03 ppm (3H, *s*), it is also contained three methyl doublet at 0.84 ppm, (3H *d, j* = 3.15 Hz); 0.79 (3H, *d, j* = 5.9 Hz); 0.83 (3H, *d, j* = 5.6 Hz), one methyl doublet-doublet at δ 0.91 ppm (3H, *dd*) showed one olifinic proton substitution at δ 5.35 ppm (1H, *d. J* = 5.0 Hz, H-6) and two protons with substituted olifinic at δ 5.16 (1 H, *t, J* = 8.4 Hz, H-22) and 5.01 (1 H, *t, J* = 8,4 Hz, H-23).

Chemical shift at δ 3.52 ppm (1H, *m*) showed an axial oxymethine forward oriented (*β*) equatorial of hydroxyl group at C-3. The presence of abundant spectra at δ 1.11–2.3 ppm showed the presence of sp^3 bonds from methylen and methin groups. The ^{13}C NMR data of compound one (Table 1) show there are 29 carbons in the molecule. There are presence of three olifinic resonances at δ 121.92 ppm, 138.53 ppm and 129.43 ppm correspondent to C-6, C-22 and C-23 and a signal at δ 140.92 ppm correspondent of carbon kwartener of C-5. On the basis of ^{1}H and ^{13}C NMR spectral data and compared with authentic compound and reference data (Goad and Akihisa, 1997) concludes that Compound 1 is identified and established as a stigmasterol (Figure 1).

Characterization of Compound 2.

Compound 2 was isolated as a pale yellow powder; melting point is 180-182 ^{o}C. The UV spectrum showed absorption band at 256 and 359 nm and characterized as a flavonoid nucleus (Pinheiro *et al*., 2012). The IR spectrum showed the absorption peak in the region (3263-3541) cm^{-1} indicating the presence of hydroxyl groups, the absorption band at (2729-2958) cm^{-1} indicated the presence of asymmetric stretching of -CH groups, and the absorption band at 1658 cm^{-1} and 1606 cm^{-1} indicated the presence of quinone which have carbonyl groups in the same ring (Silverstein *et al*., 1991). Its molecular formula is $C_{21}H_{20}O_{12}$ deduced from LC-MS (m/z = 465.4712) [M+H$^+$]. 487.4825 [M+Na$^+$], 951.9893 [2M+ Na$^+$] The ^{1}H NMR spectrum of Compound 2 in CD_3OD (Table 2) exhibited 5, 6, 8-tri substituted aromatic proton in the B ring at δ_H 6.86 ppm (1H, *d, J* = 8.40 Hz, H-5), 7.58 ppm (1H, *d, d, J* = 8.40; 2.60 Hz, H-6) and 7.71 ppm (1H, *d, J* = 2.60 Hz, H-8) , there were exhibited ortho (H_5-H_6) and meta (H_6-H_8) moiety, respectively at B ring.

Table 1. ^{13}C-NMR δ data of Compound 1 compared to ^{13}C-NMR δ stigmasterol (Goad & Akihisa, 1997)

C number	δ ^{13}C-NMR ($CDCl_3$) stigmasterol (ppm)	δ ^{13}C-NMR of ($CDCl_3$) compound 1 (ppm)
1	37.2	37.43
2	31.6	31.84
3	71.8	72.01
4	42.5	42.47
5	140.9	140.92
6	121.9	121.92
7	32.8	32.08
8	31.9	31.84
9	50.2	50.32
10	36.6	36.70
11	22.7	21.40
12	39.7	39.85
13	42.3	42.45
14	56.9	57.04
15	24.3	24.55
16	28.9	29.13
17	56.0	56.10
18	12.0	12.24
19	19.3	19.59
20	40.5	40.72
21	21.3	21.30
22	138.3	138.53
23	129.3	129.43
24	51.2	51.42
25	31.8	31.84
26	18.9	19.16
27	21.1	21.32
28	25.4	24.55
29	12.2	12.24

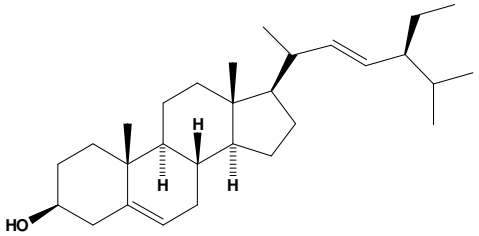

Figure 1. The chemical structure of Stigmasterol 1

This result reveals a signal aromatic proton with a meta moiety in the C ring at δ_H 6.19 ppm (1H, *d, J* = 1.95 Hz, H-2') and 6.37 ppm (1H, *d, J* = 1.95 Hz, H-6'). In addition to the a glycone signals characteristic of anomeric proton at δ_H 5.26 ppm (1H , *d, J* =7.8 Hz, H-1") for oxygen-bearing proton at δ_H 3.45 ppm (1H, t, H-2"), 3.44 ppm (1H, t, H3"), 3.35 ppm (1H, t, H-4") and 3.23 ppm (1H, *m*, H-5") a long with two aliphatic *gem*-proton at δ_H 3.58 ppm (1 H ,*dd*, *J* = 5.20, 11.70 Hz, H-6"), indicating the presence of deoxy sugar moeity (Lee *et al*., 2010). The cosy correlation on ring D indicated that the proton is axial-axial H5" to H-6" (Figure 3). The ^{13}C NMR spectra of Compound 2 reveals amounts of 21 carbon signals typical of flavonoid monoglycoside nucleus (Ahmadu *et al.*, 2007). An anomeric carbon signal at δ 104.43 ppm (C-1") indicated the presence of a single monosaccharide moiety, the four methine resonances of the β-*D*-glucopiranoside were at δ 75.87, 78.19, 71.29 and 78.47 ppm as well as methylene resonance at δ 61.10 ppm were do C-2", C-3", C4", C5" and C-6", respectively. Relationships in the bonding structure were proven through long-range correlation of ^{1}H-→ ^{13}C of HMBC spectrum are shown in Table 2.

Table 2. 1D and 2D NMR spectroscopic data for compound 2 in CD_3OD

Position	δ^{13}C (ppm)	δ^{1}H (ppm) HM QC	HMBC
2	149.97 (C)	-	
3	135.71 (C)	-	
4	179.55 (C)	-	
5	117.66 (CH)	6.86 (1H, *d, J* = 8.40 Hz)	123.29; 145.97; 149.93; 159.09
6	123.29 (CH)	7.58 (1H, *d,d, J* = 8.40; 2.60 Hz)	
7	159.09 (C)	-	99.99; 105.75; 158.55; 166.13; 179.55
8	116.09 (CH)	7.71 (1H, *d, J* = 2.60 Hz)	94.83; 105.75; 163.10; 166.13
9	149.93 (C)	-	78.19*; 135.71
10	123.14 (C)	-	
1'	105.75 (C)	-	123.29; 145.97; 149.93
2'	99.99 (C)	6.19 (1H, *d, J* = 1.95 Hz)	94.83; 105.75; 163.10; 166.13
3'	163.10 (C)	-	
4'	158.53 (C)	-	117.66; 149.93; 159.09
5'	166.13 (C)	-	
6'	94.83 (CH)	6.37 (1H, *d, J* = 1.95 Hz)	99.99;105.75; 158.55; 166.13; 179.55
1"	104.43 (CH)	5.26 (1H, *d, J* = 7.80 Hz)	78.19*; 135.71
2"	75.82 (CH)	3.45 (1 H , *t*)	71.29; 78.19; 104.43
3"	78.19 (CH)	3.44 (1H, *t*)	75.82; 78.45
4"	71.29 (CH)	3.35 (1H, *t)*	62.64; 78.19; 104.43
5"	78.45 (CH)	3.23 (1 H, *m*)	71.29
6"	62.24	3.58 (1 H, *d, d, J* = 5.20; 11.70 Hz) 3.72 (1 H, *d, d, J* = 5.20; 11.70 Hz)	71.27

On basis of IR, ^{1}H-NMR and ^{13}C- NMR (1D and 2D) spectra data and other physical properties, the Compound 2 is identified as [(2R)-7-hydroxy-3-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro -2H-pyran-2-yloxy)-2-(3,4,5-trihydroxy phe nyl) chroman-4-one] or ptevon-3-*D*- glucoside as shown in Figure 2, the correlation HMBC and cosy is shown in Figure 3. Based on database searching in http://www.chem.spider and Dictionary of Natural Product (DNP) 2006, there is no identical structure was found for Compound 2, although from those data bases (scifinder) 167 formula, we found identical result to Compound 2. Therefore, it suggests that Compound 2 is a new flovonol glycoside isolated from *P. indicus*.

Antioxidant with Free Radical Scavenger Activity of Compound 2.

DPPH is stable free radicals which disolve in methanol and their color show characteristtic absortion at wavelength 516 nm, when antioxidant scavenges the free radicals by hydrogen donation, the color in the DPPH assay solution become lighter. DPPH assay have been widely used to determine the free radical-scavenging activity of various pure compounds or extracts (Li *et al*., 2011).

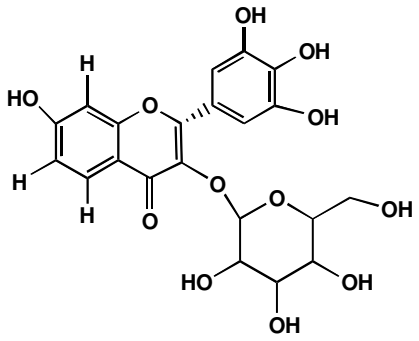

Figure 2. The chemical structure of Compound 2

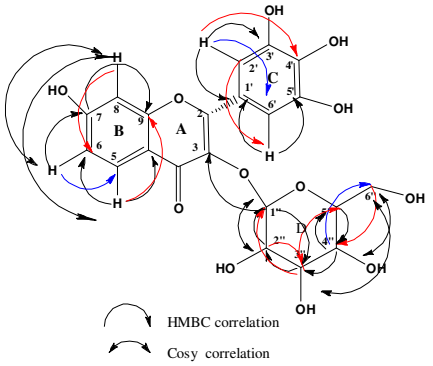

Figure 3. Correlation HMBC and Cosy of Compound 2

Free radical scavenger activity of Compound 2 was compared to ascorbic acid (vit-C) and quercetin, activity results are shown in Figure 4a. The antioxidant activity Compound 2 less active than quercetin and ascorbic acid with IC_{50} value 8.52 µg/mL, 2.40 µg/mL, 7.07 µg/mL respectively (Figure 4a). When the antioxidant activity are changed from µg/mL to µM, quercetin had antioxidant activity higher than Compound 2 (Figure 4b). Flavonoid would give antioxidant activity if it has OH in C3, oxo function in C4, double bond at C2 and C3. The OH with orto position C3' and oxo C4' has the highest influence to antioxidant activity of flavonoid. Flavonoid has OH in C3 and double bond at C2-C3 gives higher antioxidant activity than flavonoid which has only OH in C3. The flavonoid aglycones would gave higher antioxidant activity than flavonoid glicosides (Heim *et al.*, 2002). Where quercetin is flavonoid aglycon and Compound 2 is glycoside flavon.

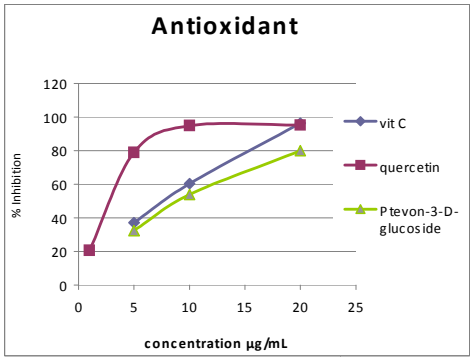

Figure 4a. Antioxidant activity of Ptevon-3-D-glycoside (Compound 2),Vit-C and Quercetin % inhibition versus concentration in µg/mL

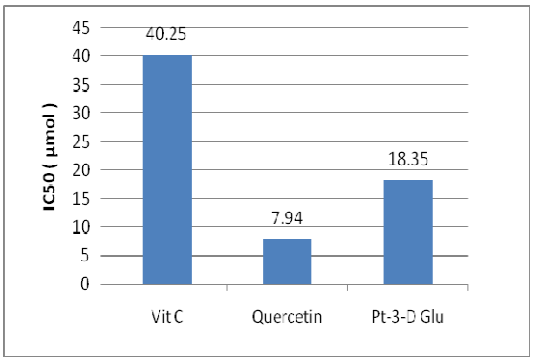

Figure 4b. Antioxidant activity of Ptevon-3-D-glycoside (Compound 2), Vitamin-C, and Quercetin (IC_{50} in µM)

Conclusion

The isolation and characterization of the compound of ethyl acetate fraction *P. indicus* Willd. were afford two compounds, which are stigmasterol and a new flavonol – glycoside [(2R)-7-hydroxy-3-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)-2-(3,4,5-trihydroxyphenyl) chroman-4-one] or ptevon-3-*D*- glucoside which is potent as a new antioxidant.

Acknowledgement

This Research was supported by LIPI Competitive Program on Drug and Molecular Farming Project funded By Indonesia Institute of Sciences (2014-2015).

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