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Isolation of Phenolic compounds from ethyl acetate soluble fraction of the *Piper betle*

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***Abstract*—Four phenolics including pinoresinol-4-O-β-D-glucopyranoside (1), tachioside (2), 3,5-dimethoxy-4-hydroxyphenyl-O-β-D-glucopyranoside (3) and 3,4-dihydroxyallylbenzene (4) were isolated from the ethyl acetate soluble fraction of *Piper betle*. Their chemical structures were determined from ESI-MS, 1D-NMR, 2D-NMR spectral data and comparison to previous literature. With the exception of compound 4, this is the first report on the identification of compounds 1-3 from *P. betle*.**

Keywords—Piper betle; phenolic; lignan; allylbenzene derivative;

I. INTRODUCTION

Piper betle L., a species of Piperaceae family, is a well-known herb and medicinal plant distributed widespread in tropical countries in the southern and southeastern Asia such as India, Sri Lanka, Malaysia, Thailand, Vietnam [1]. The leaves and roots of *P. betle* have a long history of use in traditional medicine for treatment of infectious diseases, sores, inflammation, flatulence, and cold [1, 2]. In term of the chemical constituents, several terpenoids, phenolics, alkaloids, steroids and aliphatic acids have been isolated from various parts of *P. betle* [3-5]. Its methanolic extract, essential oil, and some isolated compounds were investigated and showed a lot of valuable bioactivities such as anti-microbial activity, antiseptic, antidote, anti-inflammation, and anti-oxidant. In the aim to clarify chemical components of *P. betle* and support for explanation of its biological effects, herein, we report the isolation and structural elucidation of four phenolic compounds from ethyl acetate soluble fraction of the whole plants of *P. betle*

II. MATERIAL AND METHODS

A. General experimental procedures

NMR spectra were recorded on Jeol FT-NMR spectrometer (400 and 600 MHz). Column chromatography was performed using a silica gel (Kieselgel 60, 70 - 230 mesh and 230 - 400 mesh, Merck) or reversed phase C18 resins (150 µm, YMC). Thin layer chromatography (TLC) was performed using a pre-coated silica gel 60 F_{254} and/or RP-18 F_{254S} plates. The compounds were visualized by UV radiation (254 and 365 nm) and spraying with aqueous solution of sulfuric acid (C = 5%) followed by heating with a hot plate.

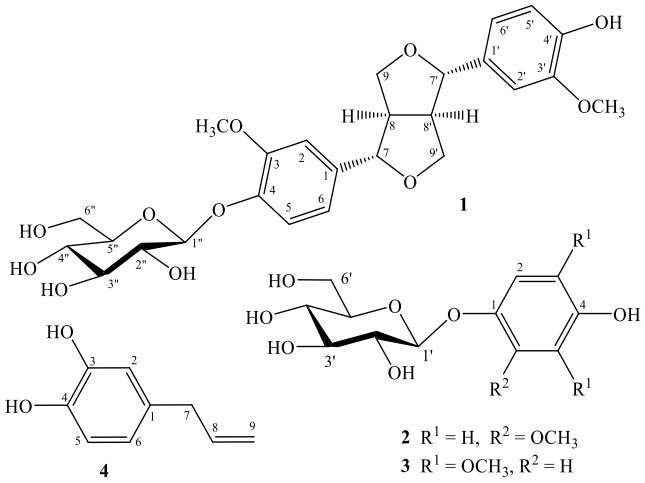

Fig. 1. *Chemical structure of compounds **1-4***

B. Extraction and Isolation

The dried and powdered whole plants of *P. betle* (3 kg) was macerated in MeOH for three times (each 6 L, 4 hrs in ultrasonic bath). Filtration and evaporation of the solvent under reduced pressure gave methanolic residue (170 g). This crude extract was then suspended in water (2 L) and successively partitioned with n-hexane, dichloromethane, and ethyl acetate The ethyl acetate soluble extract (18 g) was fractionated on a silica gel column chromatography (CC) eluting with gradient solvent systems of CH_2Cl_2/MeOH (0-100% MeOH) to obtain five fractions (E1A - E1E). Fraction E1A was then purified by silica gel CC eluting with solvent system of EtOAc:CH_2Cl_2 (2/1, v/v) to obtain four smaller fractions (E1A1 - E1A4). Fraction E1A2 was continuously purified by silica gel CC using CH_2Cl_2/MeOH (7/1, v/v) to yield compound **4** (107 mg). Fraction E1D was purified by CC using solvent system of CH_2Cl_2/MeOH (5/1, v/v) to get four smaller fractions (E1D1 - E1D4). Fraction E1D1 was further purified by reversed phase C18 column chromatography with MeOH/H_2O (1/1, v/v) as eluent to afford compounds **2** (14 mg) and **3** (25 mg). Compound **1** (19 mg) was obtained from the fraction E1D3 on a reversed phase C18 column chromatography eluting with MeOH/H_2O (2/3, v/v).

- Pinoresinol-4-*O*-*β*-D-glucopyranoside (**1**): Pale yellow amorphous powder; ESI-MS *m/z*: 543 $[M+Na]^+$;

^{1}H-NMR (600 MHz, CD_3OD) and ^{13}C-NMR (150 MHz, CD_3OD) are given in the Table 1.

- Tachioside (**2**): White amorphous powder; ESI-MS *m/z*: 325 $[M+Na]^+$; ^{1}H-NMR (600 MHz, DMSO-d_6) and ^{13}C-NMR (150 MHz, DMSO-d_6) are given in the Table 2.

- 3,5-dimethoxy-4-hydroxyphenyl-*O*-*β*-D-gluco-pyranoside (**3**): White amorphous powder; ESI-MS *m/z*: 355 $[M+Na]^+$; ^{1}H-NMR (600 MHz, DMSO-d_6) and ^{13}C-NMR (150 MHz, DMSO-d_6) are given in the Table 2.

- 3,4-dihydroxyallylbenzene (**4**): Pale yellow amorphous powder; ESI-MS *m/z*: 151 $[M+H]^+$; ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H ppm: 3.26 (1H, d, *J* = 6.8 Hz, H-7), 5.09 (2H, m, H-9), 5.93 (1H, m, H-8), 6.65 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), 6.73 (1H, d, *J* = 2.0 Hz, H-2), 6.81 (1H, d, *J* = 8.0 Hz, H-5); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C ppm: 39.3 (C-7), 115.6 (C-9), 115.7 (C-2), 116.0 (C-5), 121.3 (C-6), 133.5 (C-1), 137.5 (C-8), 141.4 (C-3), 143.2 (C-4).

III. Results and Discussion

Compound **1** was obtained as pale-yellow amorphous powder. The ^{1}H-NMR spectrum of **1** exhibited six signals of protons at aromatic area which belongs to two ABX spin coupled systems at δ_H 6.98 (1H, d, *J* = 1.2 Hz), 7.11 (1H, d, *J* = 8.4 Hz), 6.76 (1H, dd, *J* = 1.2, 8.4 Hz) and δ_H 6.91 (1H, d, *J* = 1.2 Hz), 6.74 (1H, d, *J* = 8.4 Hz), 6.88 (1H, dd, *J* = 1.2, 8.4 Hz); two oxygenated methines at δ_H 4.70 (1H, d, *J* = 4.2 Hz) và 4.67 (1H, d, *J* = 4.2 Hz); anomeric proton at δ_H 4.86 (1H, d, *J* = 7.8 Hz); and two singlet signals of methoxy groups at δ_H 3.81 và 3.82 (each 3H, s). The ^{13}C-NMR spectrum revealed 26 carbon signals. Among them, the signals at δ_C 102.7, 74.8, 77.8, 71.3, 78.1, 62.5 were characterized for a glucopyranosyl group. At the same time, the spin-spin coupling constant of anomeric proton *J* = 7.8 Hz indicated the *β*-glucopyranosyl linkage. Two carbon signals at δ_C 56.7, 56.4 were assigned to methoxy groups. Other carbon signals comprising twelve aromatic carbons in region δ_C 110.9 - 150.9 and six others at δ_C 87.4, 87.0, 72.7, 72.6, 55.5, 55.3 suggested for a lignan compounds. The chemical structure and NMR data assignment of compound **1** were further clarified by analyses of 2D – NMR experiments (Figure 1 and Table 1). Diagnostic HMBC correlations (Figure 2) were observed between H-2 (δ_H 6.98), H-6 (δ_H 6.76) with C-4 (δ_C 149.0); anomeric proton H-1″ (δ_H 4.87) and C-4 (δ_C 149.0) indicating the *O*-*β*-glucopyranosyl group at C-4. The location of methoxy group at C-3 were clarified by HMBC correlations between H-5 (δ_H 7.11) with C-1 (δ_C 137.4)/ C-3 (δ_C 147.2), and methoxy proton (δ_H 3.82) with C-3 (δ_C 147.2). HMBC correlations of H-7 (δ_H 4.70) and C-1 (δ_C 137.4)/ C-2 (δ_C 111.5)/ C-6 (δ_C 119.7) were confirmed the linkage of benzene ring at C-7. Alternatively, in the HMBC spectrum, it can be observed the correlation of H-7 (δ_H 4.70) and C-9′ (δ_C 72.6), which was proved the formation of ether-cyclic between C-7 and C-9′. Similarly, the HMBC correlations of H-2′ (δ_H 6.91), H-6′ (δ_H 6.88) to C-4′ (δ_C 150.9) also allowed to assign NMR data of C-2′, C-4′, and C-6′. The HMBC correlations of H-5′ (δ_H 6.74) to C-3′ (δ_C 147.4), C-1′ (δ_C 133.7) and methoxy protons (δ_H 3.81) to C-3′ (δ_C 147.4) suggested for the position of other methoxy group at C-3′. Another benzene ring was linked at C-7′ confirming by HMBC correlations between H-7′ (δ_H 4.67) and C-1′ (δ_C 133.7), C-2′ (δ_C 110.9), C-6′ (δ_C 120.0). Also, the HMBC correlation of H-7′ (δ_H 4.67) to C-9 (δ_C 72.7) allowed to determine ether linkage between C-7′ and C-9′. Therefore, chemical structure of compound **1** was determined to be pinoresinol-4-O-β-D-glucopyranoside. The ^{13}C-NMR spectrum of compound **1** was also similar to that of pinoresinol-4-O-β-D-glucopyranoside in the literature [6]. The ESI-MS of **1** revealed a quasi-molecular ion peak at m/z 543 which was well agreed with its molecular formula of $C_{26}H_{32}O_{11}$. Recently, this compound was found in some plants such as *Forsythia suspense* [7], *Lippia triphylla* [8], *Nepenthes mirabilis* [9]. It was reported exhibiting anti-inflammatory effect, anti-oxidation, and protection of nerve cells. However, until now, there have not been any reports on the identification of this compound from *P. betle*

Compound **2** was isolated as a white amorphous powder. The ^{1}H-NMR analysis of **2** showed three aromatic protons with ABX spin-spin coupled system [δ_H 6.68 (1H, d, *J* = 2.4 Hz), 6.45 (1H, dd, *J* = 2.4, 8.4 Hz), and 6.64 (1H, d, *J* = 8.4 Hz)], an anomeric proton

TABLE I. ^{1}H- AND ^{13}C-NMR SPECTRAL DATA OF COMPOUND **1**

No.	δ_C [a,b]	δ_H [a,c] (mult., *J* in Hz)
1	137.4	-
2	111.5	6.98 (d, 1.2)
3	147.2	-
4	149.0	-
5	117.9	7.11 (d, 8.4)
6	119.7	6.76 (dd, 1.2, 8.4)
7	87.0	4.70 (d, 4.2)
8	55.3	3.07 (m)
9	72.7	4.19 (m)/ 3.82 (m)
1'	133.7	-
2'	110.9	6.91 (d, 1.2)
3'	147.4	-
4'	150.9	-
5'	116.1	6.74 (d, 8.4)
6'	120.0	6.88 (dd, 1.2, 8.4)
7'	87.4	4.67 (d, 4.2)
8'	55.5	3.07 (m)
9'	72.6	4.19 (m)/ 3.82 (m)
OGlc		
1"	102.7	4.87 (d, 7.8)
2"	74.8	3.48 (dd, 7.8, 9.6)
3"	77.8	3.45 (dd, 9.9, 9.6)
4"	71.3	3.37 (m)
5"	78.1	3.37 (m)
6"	62.5	3.84 (br d, 12.0)
		3.66 (dd, 4.8, 12.0)
3-OCH3	56.7	3.82 (s)
3′-OCH3	56.4	3.81 (s)

Measured in [a] CD_3OD, [b] 150MHz, [c] 600MHz

TABLE II. ^{1}H- AND ^{13}C-NMR SPECTRAL DATA OF COMPOUNDS **2** AND **3**

No.	Comp. 2		Comp. 3	
	δ_C [a,b]	δ_H [a,c] (mult., *J* in Hz)	δ_C [a,b]	δ_H [a,c] (mult., *J* in Hz)
1	151.3	-	150.8	-
2	148.3	-	95.9	6.35 (s)
3	103.0	6.65 (d, 2.4)	148.7	-
4	141.8	-	130.8	-
5	108.4	6.42 (dd, 2.4; 8.4)	148.7	-
6	115.7	6.62 (d, 8.4)	95.9	6.35 (s)
OGlc				
1'	102.2	4.63 (d, 7.2)	102.2	4.65 (d, 7.6)
2'	73.8	3.15 (dd, 7.2, 9.0)	73.8	3.16 (dd, 7.6, 9.0)
3'	77.6	3.23 (t, 9.0)	77.7	3.19 (t, 9.0)
4'	70.5	3.08 (t, 9.0)	70.6	3.05 (t, 9.0)
5'	77.2	3.21 (m)	77.3	3.24 (m)
6'	61.4	3.67 (br d, 12.0) 3.41 (dd, 4.2, 12.0)	61.4	3.65 (br d, 12.0) 3.38 (dd, 4.2, 12.0)
2-OCH_3	56.0	3.70 (s)		
3,5-OCH_3			56.3	3.68 (s)

Measured in [a]DMSO-d_6, [b]150MHz, [c]600MHz

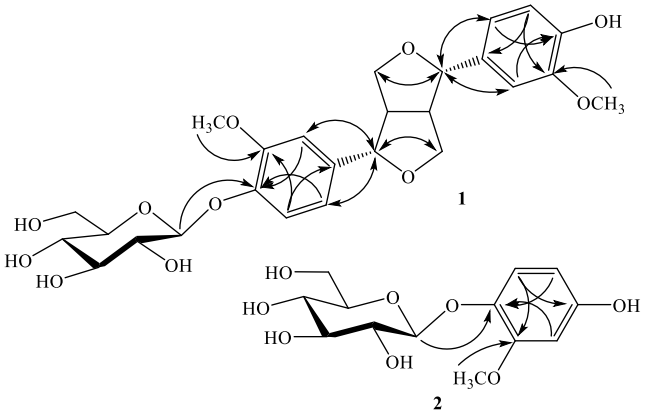

Fig. 2. Key HMBC correlations of representative compounds **1** and **2**

[δ_H 4.66 (1H, d, *J* = 7.2 Hz)], and a methoxy group [δ_H 3.71 (3H, s)]. The ^{13}C-NMR spectrum of **2** showed the signals of thirteen carbons. Similar to compound **1**, six carbinol carbon signals (δ_C 102.2, 73.8, 77.6, 70.5, 77.2, 61.4) and J coupled constant of anomeric proton (*J* = 7.2 Hz) suggested the present of a β-glucopyranosyl group. The signal at δ_C 56.0 was assigned to a methoxy group. Six remaining carbons at aromatic carbon area [δ_C 151.3, 148.3, 141.8, 115.7, 108.4, 103.0] were indicated for the presence of a benzene ring. Therefore, compound **2** was a phenyl glucoside analog. Analyses of HMBC of **2** revealed the correlations between H-3 (δ_H 6.68)/ H-5 (δ_H 6.45) and C-1 (δ_C 151.3), anomeric proton H-1′ (δ_H 4.66) and C-1 indicating O-glucopyranosyl group at C-1. On the other hand, HMBC correlations between H-6 (δ_H 6.64) and C-2 (δ_C 148.3)/ C-4 (δ_C 141.8), methoxy proton (δ_H 3.71) and C-2 (δ_C 148.3), and de-shielded signals of C-2/ C-4 led to the elucidation of a methoxy group at C-2 and hydroxy group at C-4. Consequently, compound **2** was determined to be 4-hydroxy-2-methoxyphenyl glucopyranoside. The ^{13}C-NMR analyses was also agreed to the reported data in literature [10]. Common name of this compound was tachioside. It was found in some plants such as *Saprosma merrillii* [11], *Polygonum capitatum* [12] and reported as the constituent exhibiting anti-inflammatory, anti-oxidation activities of them. However, this compound was reported for the first time from *P. betle*.

Compound **3** was obtained as a white amorphous powder. The ^{1}H- and ^{13}C-NMR spectrum of **3** was similar with that of compound **2** except signals corresponding to benzene ring. The appearance of aromatic proton signals at δ_H 6.35 (2H, s) suggested the presence of 1,3,4,5-tetrasubtituted benzene ring. Moreover, signals of methoxy groups at δ_H 3.68 (6H, s) and δ_C 56.3 indicated for two methoxy group at C-3 and C-5, forming symmetric structure. HMBC correlation between aromatic proton H-1' (δ_H 4.65) and C-1 (δ_C 150.8), chemical shift of C-4 (δ_C 130.8) indicated a structure as 3,5-dimethoxy-4-hydroxyphenyl-*O*-*β*-D-glucopyranoside. The NMR data of compound **3** was found to match with those in the literature [13]. Similar with compounds **1** and **2**, this is the first finding of compound **3** from *P. betle*

Compound **4** was isolated as pale-yellow amorphous powder. The ^{1}H-NMR spectrum of compound **4** showed ABX coupled aromatic protons [δ_H 6.73 (1H, d, *J* = 2.0 Hz), 6.81 (1H, d, *J* = 8.0 Hz), 6.65 (1H, dd, *J* = 2.0, 8.0 Hz)], a vinyl group (CH_2=CH) group [δ_H 5.09 (2H) and 5.93 (1H)], and a methylene group [δ_H 3.26 (2H, d, *J* = 6.8 Hz)]. The ^{13}C-NMR spectrum of compound **4** exhibited the signals of nine carbons and divided by DEPT as three non-protonated carbons (δ_C 133.5, 141.4, 143.2), four methine (δ_C 115.7, 116.0, 121.3, 137.5), and two methylene (δ_C 115.6, 39.3). Among them, two de-shielded signals (δC 141.4, 143.2) suggested that they linked to hydroxyl groups. From above evidences compound **4** was predicted to be 3,4-dihydroxyallylbenzene. This deduction was strongly confirmed by good consistence between NMR spectral data of compound **4** to that of 3,4-dihydroxyallylbenzene in the literature [14] and its ESI-MS data showing quasi-molecular ion peak at *m/z*

151 $[M+H]^+$). This compound is a common constituent and reported as one of the main ingredients of *Piper* species [15]

In conclusion, four phenolic compounds including pinoresinol-4-O-β-D-glucopyranoside (**1**), tachioside (**2**), 3,5-dimethoxy-4-hydroxyphenyl-O-β-D-glucopyranoside (**3**), and 3,4-dihydroxyallylbenzene (**4**) were isolated from ethyl acetate soluble fraction of the whole plants of *P. betle*. Their structures were determined by ESI-MS, NMR spectroscopic methods and well consisted with those reported in the literature. To the best of our knowledge, this is the first report of compounds **1**-**3** from *P. betle*.

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