J. Korean Soc. Appl. Biol. Chem. **53**(6), 847-851 (2010)

Short Communication

Identification and PEP Inhibitory Activity of Acetophenone Glucosides from the Clove Buds (*Syzygium aromaticum*)

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Received August 9, 2010; Accepted September 16, 2010

Two new acetophenone glucosides, 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-glucoside (1) and 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-(6'-*O*-galloyl)glucoside (2), together with three known compounds, eugenin (3), 8-*C*-glucosylnoreugenin (4), and gallic acid (5) were isolated from the EtOAc soluble fraction of the MeOH extract of clove buds (*Syzygium aromaticum*). Of these, compound 2 showed significant prolyl endopeptidase inhibitory activity with IC_{50} value of 17.2±1.1 μM, suggesting it may have potential use against memory impairment and cognitive decline.

Key words: acetophenone glucoside, prolyl endopeptidase inhibitor, *Syzygium aromaticum*

Cloves, the dried flower buds of *Syzygium aromaticum*, are the sources of antimicrobial agents against oral bacteria associated with dental caries and periodontal disease [Lee and Shibamoto, 2001]. Clove extracts has analgesic, anti-inflamatory, antifungal, antiviral, chemopreventive, hepato-protective, neuroprotective, and platelet aggregation inhibition effects [Lagow, 2004]. Upon our search for bioactive constituents against memory impairment and cognitive decline from herbal medicine, EtOAc fraction of the methanol extract of clove buds exhibited 91% of prolyl endopeptidase (PEP, EC 3.4.21.26) inhibitory activity at 8 μg/mL. From the EtOAc fraction of clove buds, two new acetophenone glucosides, 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-glucoside (**1**) and 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-(6'-*O*-galloyl) glucoside (**2**), together with three known compounds, eugenin (**3**), 8-*C*-glucosylnoreugenin (**4**), and gallic acid (**5**), were isolated and identified (Fig. 1). Among them, compound **2** showed significant PEP inhibitory activity with the IC_{50} value of 17.2±1.1 μM. The present paper deals with the isolation and structural elucidation of these compounds, and examination for their PEP inhibitory effect.

The cloves (2.0 kg) were extracted twice at room temperature with 80% aqueous methanol. After filtration and evaporation of the solvent under reduced pressure, the combined crude extract was suspended in water and then successfully partitioned with hexane and EtOAc to afford hexane- and EtOAc-soluble fractions. EtOAc-soluble fraction (135 g), which showed 91% of PEP inhibitory activity at 8 μg/mL, was chromatographed on the silica gel column eluting with $CHCl_3$/MeOH (30:1 →1:1) to afford nine fractions (EA1-EA9). Activity-guided fractionation indicated that the ninth fraction (EA9) inhibited PEP most potently (98% inhibition at 8 μg/mL). EA9 was further chromatographed on the silica gel column eluting with $CHCl_3$/MeOH/H_2O (6:5:1) to afford three fractions. The second fraction (EA9-2) was subjected to reversed phase HPLC [20~30% CH_3CN/ 0.1% TFA-H_2O; ODS column, 250×10 mm; flow rate, 1 mL/min; MD2010 UV PDA detector (JASCO, Tokyo, Japan)] to afford 8-*C*-glucosylnoreugenin (**4**, R_t 14.8 min, 10 mg), gallic acid (**5**, R_t 16.3 min, 9.7 mg), 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-glucoside (**1**, R_t 18.2 min, 15 mg), and 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-(6'-*O*-galloyl)glucoside (**2**, R_t 35.0 min, 8.5 mg). Eugenin (**3**, R_t 58.6 min, 240 mg) was obtained from EA3 by the silica gel column eluting with $CHCl_3$/MeOH (30:1 →1:1).

Compound **1**: mp 136.7°C; $[\alpha]_D$ +0.36 (*c*=0.1, in MeOH); UV (MeOH, log ε) 231 (3.93), 285 (3.88) nm; IR (KBr) 3456, 1630 cm^{-1}; Negative HRFABMS *m/z* 343.1025 $[M-H]^+$ (calculated for $C_{15}H_{19}O_9$, 343.1029); ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) spectral data are shown in Table 1.

Compound **2**: yellow syrup; $[\alpha]_D$ +0.53 (*c*=0.1, in MeOH); UV (MeOH, log ε) 218 (3.97), 283 (3.79) nm;

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doi:10.3839/jksabc.2010.129

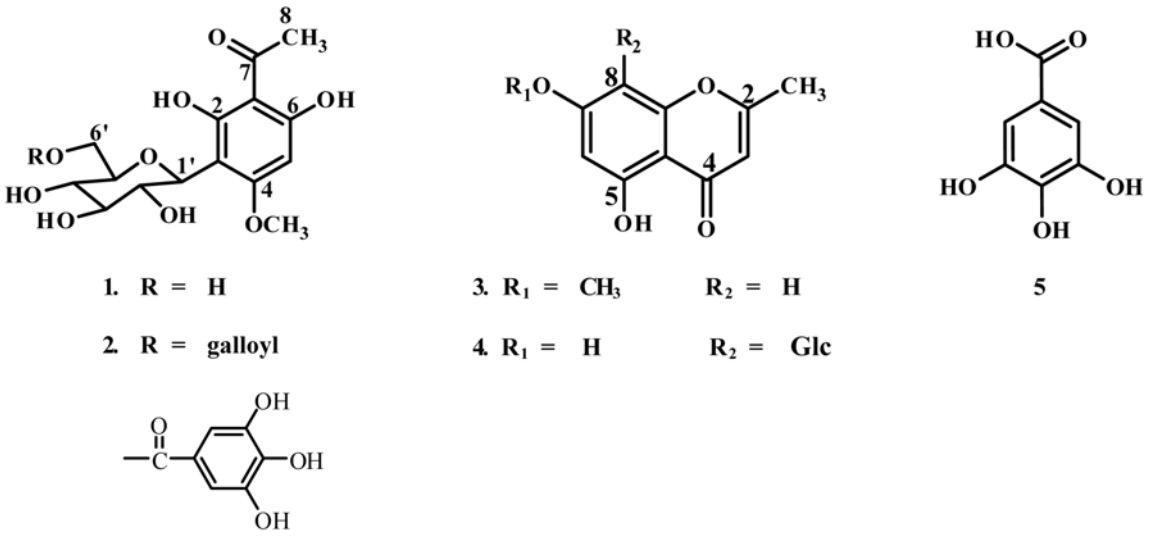


Fig. 1. Structures of compounds 1-5.

IR (KBr) 3567, 1658 cm^{-1}; Negative HRFABMS *m/z* 495.1133 $[M-H]^+$ (calculated for $C_{22}H_{23}O_{13}$, 495.1139); ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) spectral data are shown in Table 1.

Compound **3**: EIMS *m/z* 206 $[M]^+$; ^1H NMR (400 MHz, CD_3OD) δ 6.31 (1H, d, *J*=2.0 Hz, H-8), 6.29 (1H, d, *J*=2.0 Hz, H-6), 5.99 (1H, s, H-3), 3.82 (3H, s, -OCH_3), 2.32 (3H, s, -CH_3); ^{13}C NMR (100 MHz, CD_3OD); δ 182.26 (C-4), 166.64 (C-2), 165.11 (C-7), 161.95 (C-5), 157.89 (C-9), 108.68 (C-3), 105.10 (C-10), 97.82 (C-6), 92.36 (C-8), 55.75 (-OCH_3), 20.56 (-CH_3). ^1H-NMR and ^{13}C-NMR data were in accordance with published data [Coxon *et al.*, 1973; Tuntiwachwuttikul *et al.*, 2006].

Compound **4**: FABMS *m/z* 377.1 $[M+Na]^+$; ^1H NMR (600 MHz, CD_3OD) δ 6.24 (1H, s, H-6), 6.08 (1H, s, H-3), 4.89 (1H, d, *J*=9.9 Hz, H-G1), 4.08 (1H, m, H-G2), 3.86 (1H, br d, *J*=11.3 Hz, H-G6), 3.70 (1H, m, H-G6), 3.47 (1H, m, H-G3), 3.46 (1H, m, H-G4), 3.40 (1H, m, H-G5), 2.40 (3H, s, -CH_3); ^{13}C NMR (150 MHz, CD_3OD); δ 184.50 (C-4), 169.53 (C-2), 164.79 (C-7), 162.97 (C-5), 158.40 (C-9), 108.92 (C-3), 105.48 (C-10), 104.90 (C-8), 100.37 (C-6), 82.59 (C-G5), 80.18 (C-G3), 75.49 (C-G1), 73.01 (C-G2), 71.98 (C-G4), 63.06 (C-G6), 20.44 (-CH_3). ^1H-NMR and ^{13}C-NMR data were in accordance with published data [Tanaka *et al*., 1993; Okamura *et al*., 1998].

Compound **5**: EIMS *m/z* 170 $[M]^+$; ^1H NMR (400 MHz, CD_3OD) δ 7.06 (s, H-2,6); ^{13}C NMR (100 MHz, CD_3OD); δ 170.64 (C-7), 146.53 (C-3,5), 139.72 (C-4), 122.21 (C-1), 110.49 (C-2,6). ^1H-NMR and ^{13}C-NMR data were in accordance with authentic sample.

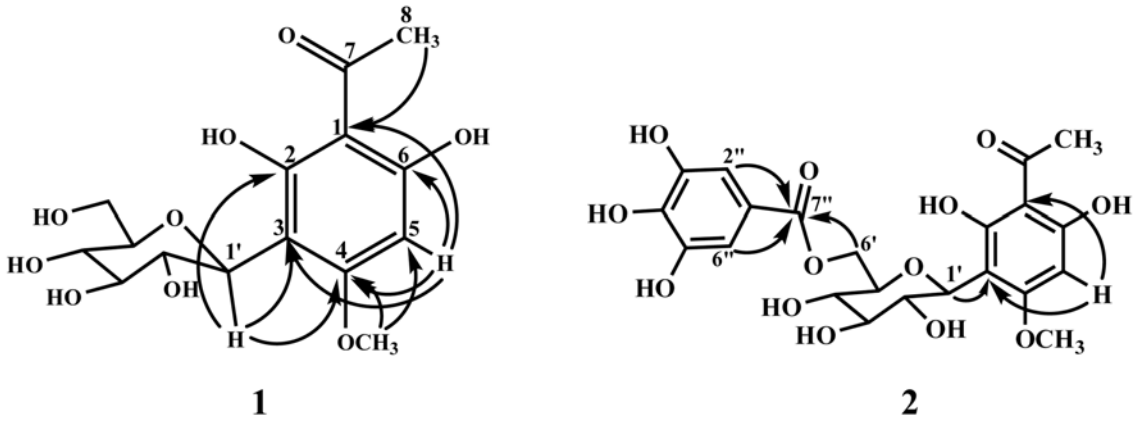
Compound **1** showed UV absorption maxima at 231 and 285 nm. The negative HRFABMS of compound **1** showed a $[M-H]^+$ ion at 343.1025, suggesting the molecular formula of **1** as $C_{15}H_{20}O_9$. The ^1H NMR spectrum (Table 1) indicated the presence of one aromatic proton signal at δ 6.05, a methoxy signal at δ 3.81, an acetyl signal at δ 2.63, and signals attributable to a *C*-glycosidically linked hexose residue at δ 4.80 (*d*, *J*=9.8 Hz), 4.05, 3.84, 3.70, 3.42, 3.41, and 3.35, suggesting the β configuration of the glycosidic linkage. The chemical shifts of these proton signals of **1** were in good agreement with those of the reported 2,4,6-trihydroxyacetophenone 3-*C*-β-D-glucoside ($C_{14}H_{18}O_9$) from the leaves of *S. aromaticum* [Tanaka *et al*., 1993], except for the appearance of a methoxy singlet at δ 3.81. In the ^{13}C NMR spectrum (Table 1), compound **1** showed signals due to a phloroglucinol-type ring (δ 166.34, 165.59, 164.42, 106.51, 105.30, and 92.11), a methoxy group (δ 56.36), and an acetyl group (δ 205.52 and 33.25), along with signals attributable to a *C*-glucoside residue [δ 82.67, 80.29, 75.77, 72.81, 71.96, and 62.96]. The chemical shifts of these carbon signals were also in good agreement with those of the reported 2,4,6-trihydroxyacetophenone 3-*C*-β-D-glucoside [Tanaka *et al*., 1993], except for the appearance of a methoxy group (δ_H 3.81 and δ_C 56.36). The position of this methoxy group was verified by COSY and HMBC spectra: COSY spectrum shows that methoxy proton signal at δ 3.81 is connected to δ 6.05 (H-5) in phloroglucinol ring. In the HMBC spectrum (Table 1 and Fig. 2), there is a correlation with methoxy proton signal at δ 3.81 and carbon signals at δ 166.34 (C-4) and δ 92.11 (C-5), indicating the position of methoxy group to be at C-4. These observations suggested that **1** is 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-glucoside.

The UV spectrum pattern of compound **2** (λ_{max} at 218 and 283 nm) was very similar with that of compound **1**. The negative HRFABMS of compound **2** showed a [M-

Table 1. NMR spectral data for compounds 1 and 2 (in CD_3OD)

Carbon	1			2		
	^{13}C	^{1}H	HMBC (H→C)	^{13}C	^{1}H	HMBC (H→C)
1	106.51			106.47		
2	164.42			162.88[a]		
3	105.30			105.02		
4	166.34			163.37[a]		
5	92.11	6.05 (s)	1, 3, 4, 6	92.12	6.03 (s)	1, 3
6	165.59			163.20[a]		
7	205.52			205.52		
8	33.25	2.63 (s)	1	33.30	2.59 (s)	
1'	75.77	4.80 (d 9.8)	2, 3, 4, 2', 3', 5'	75.93	4.80 (d 10.0)	3, 2', 3'
2'	72.81	4.05 (m)		72.85	4.06 (m)	
3'	80.29	3.41 (m)	1', 2', 4'	79.90	3.46 (m)	2', 4'
4'	71.96	3.42 (m)	5'	71.73	3.58 (m)	
5'	82.67	3.35 (m)	4'	79.90	3.61 (m)	
6'	62.96	3.84 (dd 12.2, 1.2)	4', 5'	64.81	4.57 (dd 11.9, 1.6)	4', 5', 7"
		3.70 (m)			4.38 (m)	
OCH_3	56.36	3.81 (s)	4, 5	56.32	3.77 (s)	
1"				121.58		
2", 6"				110.40	7.08 (s)	1", 2", 3", 4", 5", 6", 7"
3", 5"				146.66		
4"				140.01		
7"				168.51		

[a]interchangeable



1 **2**

Fig. 2. Selected HMBC (H→C) correlations for 1 and 2

H]$^+$ ion at 495.1133, suggesting the molecular formula of **2** as $C_{22}H_{24}O_{13}$. The ^1H and ^{13}C NMR spectra of **2** were in accordance with the published data of 2,4,6-trihydroxyacetophenone 3-*C*-β-D-(6'-*O*-galloyl)glucoside ($C_{21}H_{22}O_{13}$) [Tanaka *et al*., 1993], except for the appearance of a methoxy group (δ_H 3.77; δ_C 56.32). Besides the common structural framework resemblance of **2** and 2,4,6-trihydroxyacetophenone 3-*C*-β-D-(6'-*O*-galloyl)glucoside, the ^1H and ^{13}C NMR spectra of **2** were also in good agreement with those of **1** ($C_{15}H_{20}O_9$), except for the appearance of a typical galloyl group. The characteristic galloyl proton signal at δ 7.08 (2H, s) and the downfield shift of the glucose C-6 proton signals at δ 4.57 and 4.38 indicated that the location of galloyl group was at C-6 of glucose. The location of galloyl group at C-6 of glucose was also verified by the HMBC spectrum (Table 1 and Fig. 2). These observations suggested that **2** is 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-(6'-*O*-galloyl) glucoside.

The PEP inhibitory activity was assayed using benzyloxy-

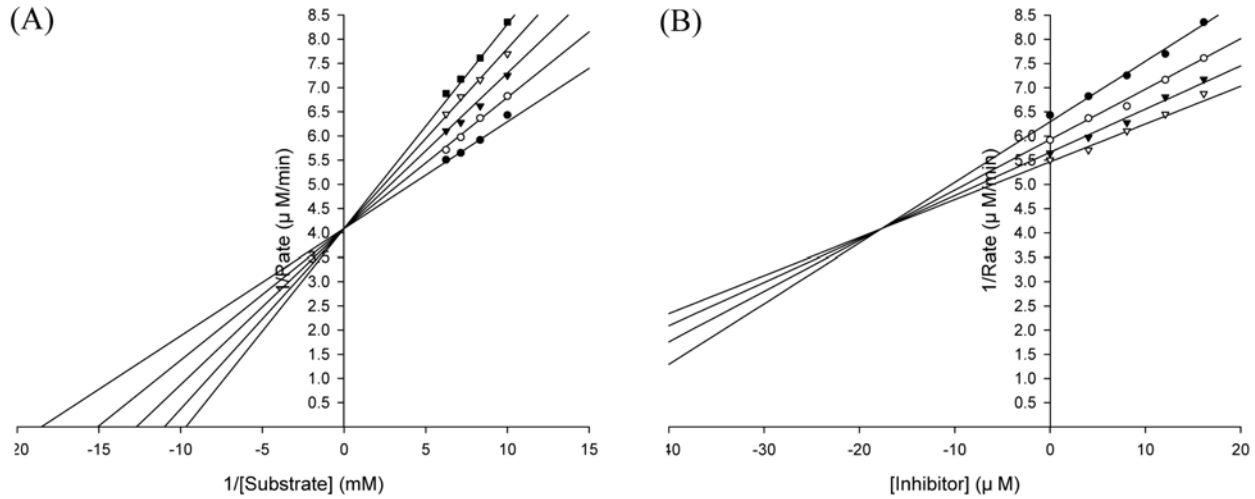


Fig. 3. (A) Lineweaver-Burk plots of PEP inhibition by 2 [in the absence (●) and presence of 4.03 (○), 8.06 (▼), 12.09 (▽), and 16.13 (■) μM]. (B) Dixon plots of PEP inhibition by 2. [S]=0.10 mM (●), 0.12 mM (○), 0.14 mM (▼) and 0.16 mM (▽).

carbonyl-glycyl-L-prolyl-*p*-nitroanilide (*Z*-Gly-Pro-*p*NA) as a substrate, and the amount of released *p*-nitroaniline was determined at 380 nm [Yoshimoto, 1980; Lee, 2004]. Upon preliminary examination of **1-5** for the PEP inhibitory activity, **1** and **2** showed 11.9 and 50.4% of PEP inhibition at 8 μg/mL, whereas **3-5** showed less than 10% inhibitory activity. Compounds **1** and **2** were further investigated at various concentrations to evaluate IC_{50} values. They showed dose-dependent PEP inhibitory effect with IC_{50} values of 218.9±5.7 μM (compound **1**) and 17.2±1.1 μM (compound **2**). Compound **1**, 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-glucoside, showed weak PEP inhibition. However, compound **2** containing galloyl group in its structure, showed significant PEP inhibitory activity, indicating that additional galloyl group may function synergistically on the PEP inhibition. The IC_{50} value for **2** was similar to those of other reported natural inhibitors such as ursolic acid (17.2 μM), oleanolic acid (22.5 μM), and oleic acid (23.6 μM) [Park *et al.*, 2005; 2006]. Lineweaver-Burk and Dixon plots of the PEP inhibition by compound **2** indicate that it is a competitive inhibitor with K_i value of 16.4±1.3 μM (Fig. 3). The exact mechanism involved in the PEP inhibition by compound **2** remains to be elucidated.

In summary, two new acetophenone glucosides, 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-glucoside (**1**) and 2,6-dihydroxy-4-methoxyacetophenone 3-*C*-β-D-(6'-*O*-galloyl)glucoside (**2**) were isolated from the EtOAc soluble fraction of the MeOH extract of clove buds (*Syzygium aromaticum*), although there is possibility that both compounds were *O*-methylated artifacts formed during extraction and isolation. Compound **2** showed significant prolyl endopeptidase inhibition with IC_{50} and K_i values of 17.2±1.1 and 16.4±1.3 μM, respectively, suggesting that it may have potential use for the prevention of memory loss.

Acknowledgment. This work was supported by Plant Metabolism Research Center, Kyung Hee University.

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