

Antioxidant and Prolyl Endopeptidase Inhibitory Capacities of Chromone C-glucosides from the Clove Buds (*Syzygium aromaticum*)

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Received: 30 July 2012 / Accepted: 17 August 2012 / Published Online: 30 September 2012
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Abstract Four chromone derivatives (**1–4**) were isolated from the clove buds (*Syzygium aromaticum*). Of these, two chromone C-glucosides (**1** and **2**) showed significant PEP inhibition with IC₅₀ values of 1.48±0.02 and 1.74±0.03 µM and K_i values of 0.27±0.02 and 0.50±0.05 µM, respectively. They also exhibited strong antioxidant capacities against the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt radical system with EC₅₀ values of 4.13±0.04 and 4.79±0.03 µM, respectively.

Keywords antioxidant capacity · chromone C-glucoside · clove buds · prolyl endopeptidase inhibitor · *Syzygium aromaticum*

Cloves, the dried flower buds of *Syzygium aromaticum*, have historically been used as medicine to relieve tooth pain and heal mouth and gum sores (Lee and Shibamoto, 2001). Clove extracts has antimicrobial, antiseptic, analgesic, anti-inflammatory, chemopreventive, hepatoprotective, neuroprotective, and platelet aggregation inhibition effects (Lagow, 2004). In our search for prolyl endopeptidase (PEP, EC 3.4.21.26) inhibitors as bioactive constituents against cognitive decline (Lee et al., 2004; Han and Paik, 2010; Yoon and Paik, 2010), we found that BuOH fraction of *S. aromaticum* at 8 µg/mL exhibited 98% of PEP inhibitory activity. This paper describes four chromone components (**1–4**) isolated from *S. aromaticum* (Fig. 1) that exhibit PEP inhibition abilities as well as antioxidant capacities against the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) radical system.

Dried flower buds of *S. aromaticum* (2.0 kg) were extracted twice at room temperature with 80% aqueous MeOH. After filtration and evaporation of the solvent under reduced pressure, the combined crude extract was suspended in water and then

successfully partitioned to afford hexane-, EtOAc-, and BuOH-soluble fractions. The BuOH-soluble fraction (25 g) was subjected to ODS column (190 mm × 30 mm) eluting with CH₃CN/H₂O (1:9→5:5) to afford five fractions. The fourth fraction (1.5 g) was subjected to reversed phase HPLC [10–50% CH₃CN/0.1% trifluoroacetic acid (TFA)-H₂O, ODS column; 250 mm × 10 mm, MD2010 UV PDA detector (JASCO, Japan)] to yield two chromone C-glucosides, 8-C-β-D-(6-O-galloyl)glucosylnoreugenin (**1**, 30 mg, R_t 36.7 min) and 6-C-β-D-(6'-O-galloyl)glucosylnoreugenin (**2**, 27 mg, R_t 41.1 min). Two chromones, 5-methoxyeugenin (**3**, 30 mg, R_t 54.5 min) and 5-methoxyisoeugenin (**4**, 26 mg, R_t 68.0 min), were obtained from the fifth fraction (1.7 g) by reversed phase HPLC (10–50% CH₃CN/0.1% TFA-H₂O, then 100% MeOH). **Compound 1**: R_t 36.7 min; [α]_D = -35.8 (c=0.1, in MeOH); UV (MeOH, log ε) 280 (4.14), 259 (4.16), 219 (4.41) nm; IR (KBr) 3374, 1618 cm⁻¹; Positive HRFABMS m/z 507.1141 [M+H]⁺ (calculated for C₂₃H₂₃O₁₃, 507.1139); ¹H- and ¹³C-NMR data (Table 1) were in accordance with published data (Tanaka et al., 1993).

Compound 2: R_t 41.1 min; [α]_D = -66.0 (c=0.005, in MeOH); UV (MeOH, log ε) 275 (4.05), 258 (4.13), 209 (4.28) nm; IR (KBr) 3327, 1618 cm⁻¹; Positive HRFABMS m/z 507.1136 [M+H]⁺ (calculated for C₂₃H₂₃O₁₃, 507.1139); ¹H- and ¹³C-NMR data are shown in Table 1.

Compound 3: R_t 54.5 min; UV (MeOH, log ε) 282 (3.96), 253 (4.22), 245 (4.22), 229 (4.24), 205 (4.26) nm; IR (KBr) 3432, 1661, 1607 cm⁻¹; Positive HRFABMS m/z 221.0815 [M+H]⁺ (calculated for C₁₂H₁₃O₄, 221.0814); ¹H-NMR (400 MHz, CD₃OD) δ 6.51 (s, H-8), 6.42 (s, H-6), 5.98 (s, H-3), 3.86 (s, 7-OCH₃), 3.85 (s, 5-OCH₃), 2.30 (s, 2-CH₃); ¹³C-NMR (100 MHz, CD₃OD) δ 180.06 (C-4), 166.63 (C-2), 166.33 (C-7), 162.13 (C-5), 161.77 (C-9), 111.95 (C-3), 109.04 (C-10), 97.22 (C-6), 94.12 (C-8), 56.63 (5-OCH₃), 56.59 (7-OCH₃), 19.88 (2-CH₃).

Compound 4: R_t 68.0 min; UV (MeOH, log ε) 314 (3.75), 291 (3.76), 256 (4.31), 249 (4.29), 227 (4.20), 205 (4.27) nm; IR (KBr) 3439, 1661, 1603 cm⁻¹; Positive HRFABMS m/z 235.0968 [M+H]⁺ (calculated for C₁₃H₁₅O₄, 235.0970); ¹H-NMR (400 MHz, CD₃OD) δ 6.49 (s, H-6), 5.95 (s, H-3), 3.93 (s, 7-OCH₃), 3.89 (s,

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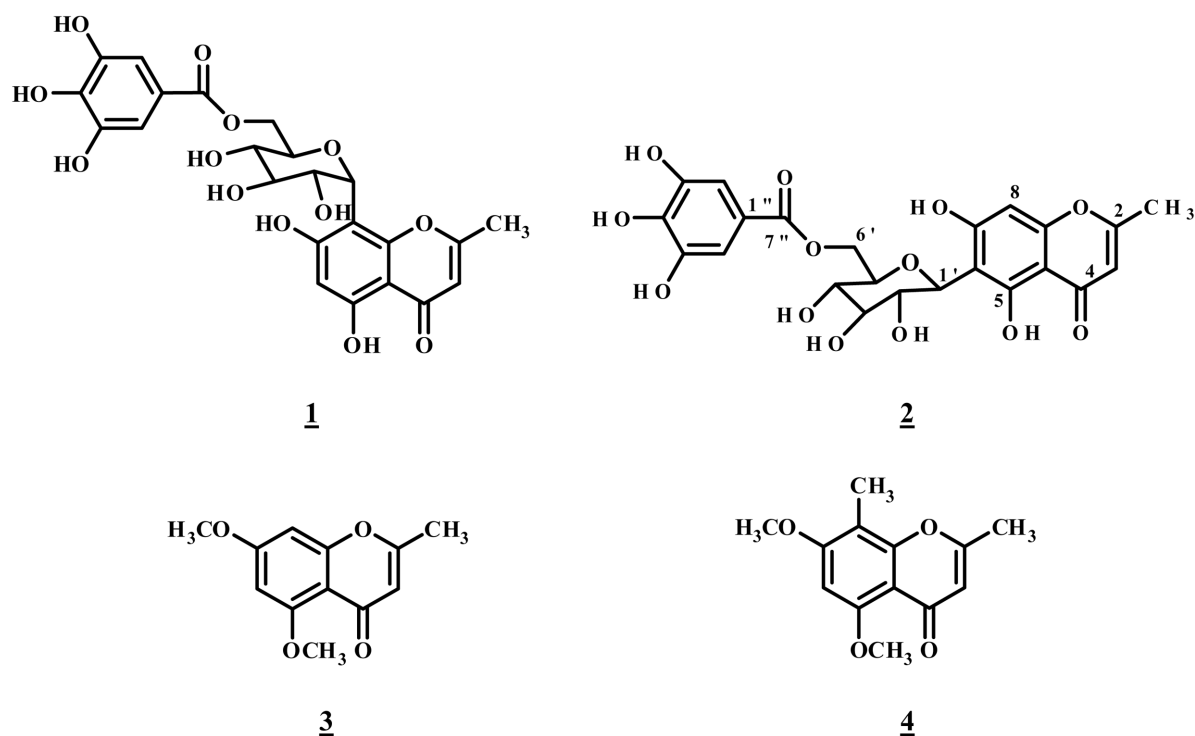


Fig. 1 Structures of compounds 1–4.

Table 1 NMR spectral data for compound 1 and 2 (in CD₃OD)

carbon	1			2		
	¹³ C	¹ H	HMBC (H→C)	¹³ C	¹ H	HMBC (H→C)
2	169.51			169.40		
3	108.89	6.03 (s)	2, 10, 2-CH ₃	109.16	6.03 (s)	2, 10, 2-CH ₃
4	188.44			184.28		
5	162.99			162.29		
6	100.01	6.23 (s)	5, 8, 10	108.86		
7	164.79			164.99		
8	104.40			95.23	6.34 (s)	6, 7, 8, 9, 10
9	158.40			159.39		
10	105.50			105.10		
2-CH ₃	20.44	2.28 (3H, s)		20.40	2.32 (3H, s)	2, 3, 2-CH ₃
1'	75.20	4.93 (d 10.0)	7, 8, 9, 2', 3'	75.55	4.91 (d 9.84)	5, 6, 7, 1', 2', 3'
2'	72.84	4.13 (m)	3'	72.64	4.20 (m)	1', 3'
3'	80.15	3.52 (m)	4'	80.06	3.50 (m)	2', 4'
4'	71.79	3.64 (m)	3', 5'	71.85	3.59 (m)	3', 5', 6'
5'	79.98	3.65 (m)	3'	80.16	3.67 (m)	4'
6'	64.84	4.63 (brd 11.8)	7''	65.24	4.57 (d 11.70)	7'', 4', 5'
		4.38 (m)			4.39 (dd 11.94, 4.86)	7''
1''	121.55			121.56		
2'', 6''	110.31	7.05 (2H, s)	1'', 2'', 3'', 4'', 5'', 6'', 7''	110.36	7.06 (2H, s)	1'', 2'', 3'', 4'', 5'', 6'', 7''
3'', 5''	146.67			146.59		
4''	140.00			139.97		
7''	168.48			168.57		

5-OCH₃), 2.32 (s, 2-CH₃), 2.09 (s, 8-CH₃); ¹³C-NMR (100 MHz, CD₃OD) δ 180.67 (C-4), 166.54 (C-2), 163.44 (C-7), 160.40 (C-5), 158.23 (C-9), 111.31 (C-3), 108.39 (C-10), 106.55 (C-8), 92.82

(C-6), 56.65 (7-OCH₃), 56.46 (5-OCH₃), 19.89 (2-CH₃), 7.93 (8-CH₃). ¹H- and ¹³C-NMR data were in accordance with published data (Kalinin and Snieckus, 1998).

Table 2 IC₅₀, K_i and EC₅₀ values of compounds 1–3

Compound	IC ₅₀ (μM) ^a	K _i (μM) ^b	EC ₅₀ (μM) ^c
1	1.48±0.02	0.27±0.02	4.13±0.04
2	1.74±0.03	0.50±0.05	4.79±0.03
3	10.96±0.02	25.4±1.2	32.7±0.5

^aIC₅₀: half maximal inhibitory concentration, ^bK_i: inhibition constant, ^cEC₅₀: half maximal effective concentration

The positive HRFABMS of compound **1** showed a [M+H]⁺ ion at 507.1141, suggesting the molecular formula of **1** as C₂₃H₂₂O₁₃. The chemical shifts of ¹H-, ¹³C-, and HMBC spectra of **1** (Table 1) were in good agreement with those of reported 8-C-β-D-(6-O-galloyl)glucosylnoeuugenin from the leaves of *S. aromaticum* (Tanaka et al., 1993). The positive HRFABMS of compound **2** showed a [M+H]⁺ ion at 507.1136, indicating the molecular formula of **2** (C₂₃H₂₂O₁₃) is the same as that of **1**. The UV spectrum pattern of compound **2** (λ_{max} at 275, 258 and 209 nm) was also very similar with that of **1** (λ_{max} at 280, 259, and 219 nm) suggesting their isomeric relationship. The chemical shifts of ¹H- and ¹³C-NMR spectra of **2** were in good agreement with those of **1**, except for the different chemical shifts at C-6 and C-8 (Table 1). In the HMBC spectrum of **2**, there is a correlation with C-8 proton signal at δ 6.34 and C-6, 7, 8, 9 and 10 carbon signals. Also, there is a correlation with glucose C-1 proton signals at δ 4.91 and C-5, 6 and 7 carbon signals, indicating the location of the C-glycosyl moiety to be at the C-6 position. These observations suggested that **2** is 6-C-β-D-(6'-O-galloyl)glucosylnoeuugenin.

The positive HRFABMS of compound **3** showed a [M+H]⁺ ion at 221.0815, suggesting the molecular formula of **3** as C₁₂H₁₂O₄. The ¹H- and ¹³C-NMR spectra of **3** were closely related with those of eugenin (Coxon et al., 1973; Han and Paik, 2010), except for

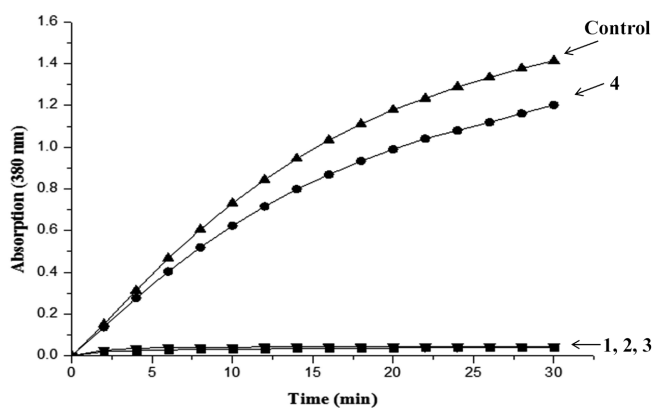


Fig. 2 Typical inhibition pattern of *p*-nitroaniline produced from the reaction of *Z*-Gly-pro-*p*NA with PEP at 380 nm in the absence and presence of compounds 1–4 (8 μg/mL).

the appearance of a methoxy group (δ_H 3.85 and δ_C 56.63). The position of this methoxy group was verified by HMBC spectrum; the methoxy proton signal at δ 3.85 is connected to δ 162.13 (C-5). These observations suggested that **3** is 5-methoxyeugenin. The ¹H- and ¹³C-NMR spectra of **4** were in accordance with published data of 5-methoxyisoeugenin (Kalinin and Snieckus, 1998). There is a possibility that compound **3** and **4** are *O*-methylated artifacts formed during extraction and isolation.

The PEP inhibitory activity of each compound was assayed using benzyloxycarbonyl-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 (Lee et al., 2004). Upon preliminary examination of 1–4 at 8 μg/mL, compounds 1–3 inhibited PEP activity almost completely (larger than 95%), whereas compound **4** showed much less PEP inhibitory activity as shown in Fig. 2.

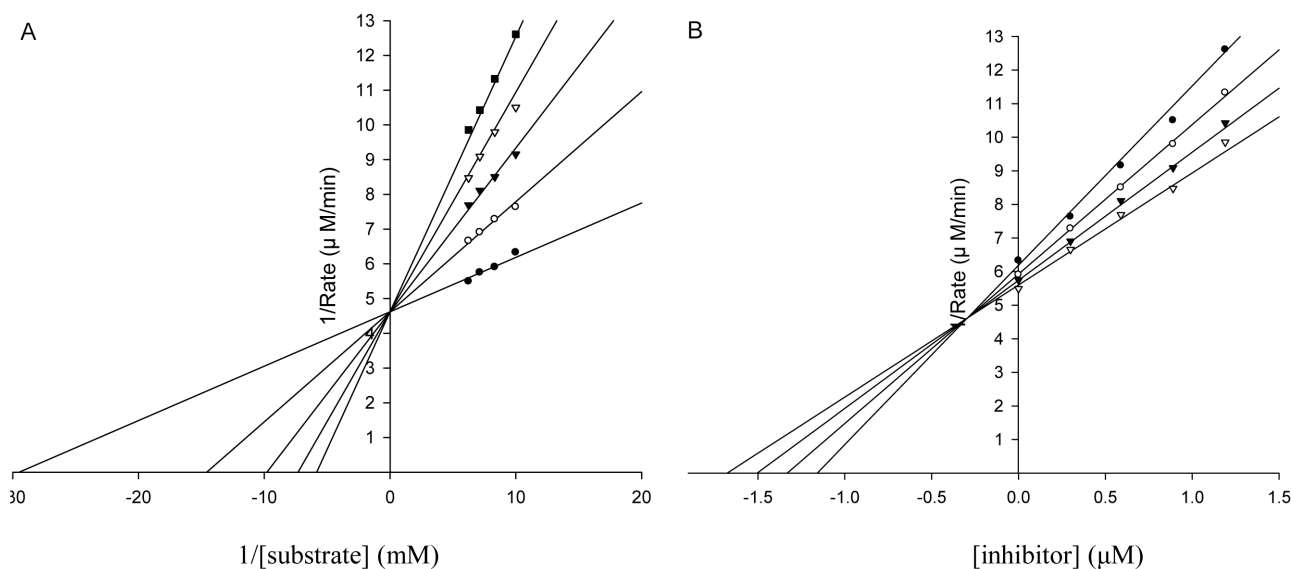


Fig. 3 Lineweaver-Burk plots and Dixon plots of PEP inhibition. (A) Lineweaver-Burk plots of PEP inhibition by **1** [in the absence (●) and presence of 0.30 (○), 0.59 (▼), 0.89 (▽), and 1.19 (■) μM]. (B) Dixon plots of PEP inhibition by **1** [S]=0.10 mM (●), 0.12 mM (○), 0.14 mM (▼), and 0.16 mM (▽).

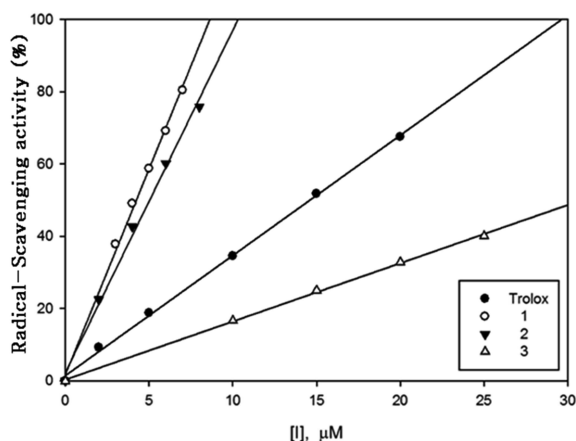


Fig. 4 ABTS radical-scavenging activities of compounds 1–3 and Trolox.

Compounds 1–3 showed dose-dependent PEP inhibitory effects with IC_{50} values of 1.48 ± 0.02 , 1.74 ± 0.03 , and 10.96 ± 0.02 μM , respectively (Table 2). The IC_{50} values of these compounds, especially 1 and 2, were strong enough and comparable to those reported for other natural inhibitors such as ginkgolic acid (0.62 μM), ursolic acid (17.2 μM), oleanolic acid (22.5 μM), and oleic acid (23.6 μM) (Lee et al., 2004; Park et al., 2005; 2006), suggesting that they may have potential use as bioactive drug against cognitive decline and prevention of memory loss.

Lineweaver-Burk and Dixon plots of the PEP inhibition by compounds 1 (Fig. 3) indicate that 1 is competitive inhibitor with K_i value of 0.27 ± 0.02 μM (Table 2). Compound 2 also shows competitive inhibition with K_i value of 0.50 ± 0.05 μM (Table 2). The exact mechanism involved in the PEP inhibition by these compounds remains to be elucidated.

Measurement of the radical-scavenging activities of compounds 1–4 was carried out using the decolorization of ABTS radical at 734 nm (Yoon and Paik, 2010). Compounds 1–3 and Trolox (standard reference compound; EC_{50} 14.4 ± 0.22 μM) showed dose-dependent effects on the ABTS radical (Fig. 4), whereas compound 4 did not show any radical-scavenging effect. EC_{50} values of 1–3 (Table 2) were 4.13 ± 0.04 , 4.79 ± 0.03 , and 32.7 ± 0.5 μM , respectively, indicating 1 and 2 have strong free radical-scavenging activities.

In summary, four chromone derivatives (1–4) were isolated from the BuOH soluble fraction of clove buds. Of these substances, 1 and 2 showed strong prolyl endopeptidase inhibitory effect with IC_{50} values of 1.48 and 1.74 μM and K_i values of 0.27 and 0.50 μM , respectively, suggesting they may have potential to use against cognitive decline. Compounds 1 and 2 also exhibited strong antioxidant capacities against the ABTS radical system with EC_{50} values of 4.13 and 4.79 μM , respectively.

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

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