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Prenylflavonol, acylated flavonol glycosides and related compounds from *Epimedium sagittatum*

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Abstract

Chemical examination of the *n*-BuOH extract from the aerial parts of *Epimedium sagittatum* led to isolation of three prenylated flavonol glycosides sagittasine A–C, two acylated flavonol glycosides kaempferol-3-*O*-(2″-*E*-*p*-coumaroyl,4″-*Z*-*p*-coumaroyl)-α-L-rhamnopyranoside and kaempferol-3-*O*-(3″-*Z*-*p*-coumaroyl,4″-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside, together with known flavonoids, flavonolignans, 2-phenoxychromones, a lignan, and aromatic acid derivatives. Flavonolignans were identified for the first time in this plant. The vasorelaxing properties of the *n*-BuOH extract of *E. sagittatum* and 13 isolated compounds were tested using pre-contracted rat aorta rings in an organ bath apparatus. The results indicated that the *n*-BuOH extract of *E. sagittatum* produced a partial endothelial nitric oxide-dependent vasorelaxation, with EC_{50} of 0.16 ± 0.03 mg/ml. However, the 13 compounds tested, generated only a mild or moderate relaxation, and did not possess significant vasorelaxing effect individually.

Keywords: *Epimedium sagittatum*; Berberidaceae; Prenylflavonol; Acylated flavonol glycoside; Vasorelaxation

1. Introduction

Epimedium sagittatum Maxim. (Berberidaceae) is a traditional Chinese medicine that has been used as tonic and antirheumatic agents (Juangsu New Medical, 1979). Several classes of compounds have been identified in this plant, including lignans, flavonoids, flavonol glycosides, and phenolic carboxylic acids (Hsieh et al., 2003; Mizuno et al., 1987, 1988b; Huang et al., 1993; Chen et al., 1996). Our preliminary bioassay showed that the *n*-BuOH extract of *E. sagittatum* had significant vasorelaxation effect, which attracted us to further investigate the title plant. In this paper, we report the isolation and structural elucidation of three new prenylflavonol glycosides, two new acylated flavonol glycosides and another 35 known compounds from the aerial parts of *E. sagittatum*. The effects of isolated compounds on vascular tension were also evaluated.

2. Results and discussion

2.1. Characterization of the compounds

In endothelium-intact rat aortic rings, the *n*-BuOH extract (0.04–0.40 mg/ml) of *E. sagittatum* produced concentration-dependent vasorelaxation in phenylephrine-induced contraction, as shown in Fig. 1. The average maximum relaxation (E_{max}) and median effective concentration

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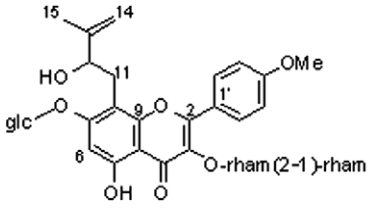
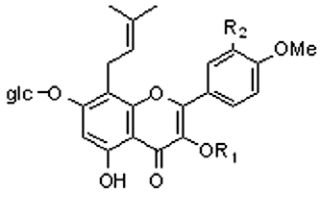
(EC_{50}) values are $100.00 \pm 0.00\%$ and 0.16 ± 0.03 mg/ml, respectively ($n = 6$–8). Pretreatment with L-NNA, a nitric oxide synthase inhibitor, partially inhibited its vasorelaxation, suggesting that nitric oxide was involved in *n*-BuOH extract-induced vasorelaxation.

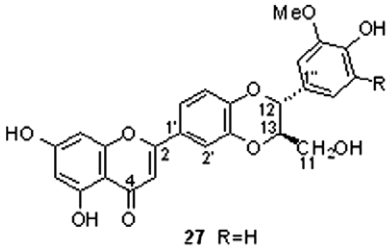
Using repeated column chromatography, compounds **1**–**40** were isolated from the bioactive fraction *n*-BuOH. Of these, compounds **4**–**26**, and **33**–**35** were identified by ^{1}H, ^{13}C NMR and mass spectroscopic data as the known flavonoid glycosides icariin (**4**) (Mizuno et al., 1987), epimedin C (**5**) (Ito et al., 1988), hexandraside F (**6**) (Mizuno et al., 1992), epimedin A (**7**) (Ito et al., 1988), epimedin B (**8**) (Ito et al., 1988), epimedoside E (**9**) (Mizuno et al., 1988a), diphylloside B (**10**) (Mizuno et al., 1988a), kaempferol-3-*O*-α-L-rhamnoside (**11**) (Lin et al., 1996), kaempferol-3-*O*-β-D-galactoside (**12**) (Lin et al., 1996), kaempferol-3-*O*-α-L-rhamnopyranosyl(1 → 2)-α-L-rhamnopyranoside (**13**), quercetin-3-*O*-α-L-rhamnoside (**14**) (Lin et al., 1996), quercetin-3-*O*-β-D-galactoside (**15**) (Lin et al., 1996), quercetin-3-*O*-α-L-rhamnopyranosyl(1 → 2)-α-L-rhamnopyranoside (**16**) (Rao et al., 1999), icariside I (**17**) (Mizuno et al., 1990), icariside II (anhydroicaritin-3-*O*-α-rhamnoside) (**18**) (Mizuno et al., 1987), and (2*R*,3*R*)-dihydrokaempferol-4′-*O*-β-D-glucopyranoside (**19**) (Dubeler et al., 1997), flavonoids: kaempferol (**20**) (Lin et al., 1996), quercetin (**21**) (Lin et al., 1996), apigenin (**22**) (Markham et al., 1982), luteolin (**23**) (Markham et al., 1982), tricetin-3′, 5′-dimethyl ether (**24**) (Markham et al., 1982), tricetin-3′-methyl ether (**25**) (Markham et al., 1982), and 5,7, 4′-trihydroxy-3′-(2-hydroxy-3-methylbut-4-enyl)flavone (**26**) (Iinuma et al., 1993), lignan (+)-dihydro-dehydrodiconiferyl alcohol-4-*O*-β-D-glucopyranoside (**33**) (Wang and Jia, 1997), and the aromatic acids, chlorogenic (**34**) and *p*-coumaric (**35**) acids.



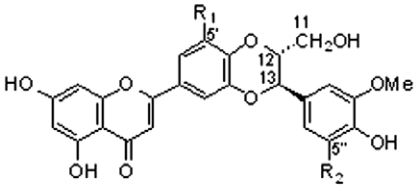
1



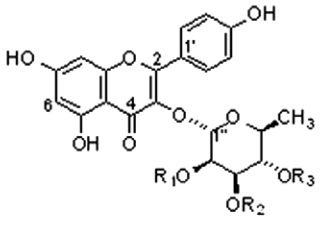
2 R_1 = rham(2-1)-rham R_2 = OH
3 R_1 = rhamnosyl R_2 = OH
4 R_1 = rhamnosyl R_2 = H
5 R_1 = rham(2-1)-rham R_2 = H

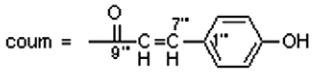


27 R=H
28 R=OMe

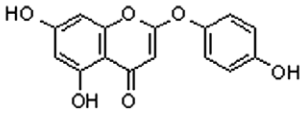


29 R_1=R_2=H
30 R_1=OMe R_2=H
31 R_1=R_2=OMe





36 R_1=R_3= E-coum R_2=H
37 R_1=E-coum R_2=H R_3= Z-coum
38 R_1=Z-coum R_2=H R_3= E-coum
39 R_1=H R_2=R_3= E-coum
40 R_1=H R_2=Z-coum R_3= E-coum



32

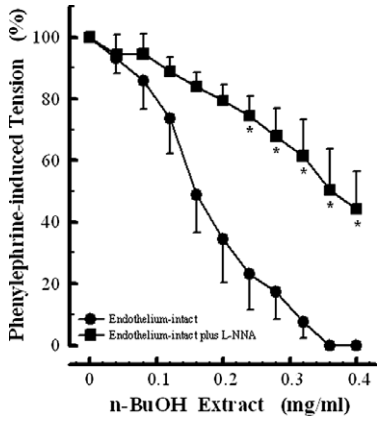


Fig. 1. Concentration–response curves of *n*-BuOH extracts of *Epimedium sagittatum* (0.04–0.40 mg/ml) on isolated rat aortic contraction induced by phenylephrine (0.3 μM). $n = 6$–8 in each group. $^{*}P < 0.05$ compared to the endothelium-intact group in the absence of L-NNA.

Compounds **1**–**3** could be flavonol glycosides, as indicated by the UV maxima absorbs at about 270, 350 nm and IR (ν_{max}) at about 3370, 1650, 1598 cm^{-1}.

Sagittasine A (**1**) had a similar UV spectrum (see Section 3) and gave the same shifts as that of icariin. The molecular formula of **1** was consistent with $C_{39}H_{50}O_{20}$ from the HR-FAB-MS spectrum *m*/*z* 839.2983 $[M+H]^+$. The ^1H NMR spectrum of **1** showed A_2B_2-type signals at δ 710/7.93 (each 2H, *d*, $J = 8.5$ Hz), an aromatic proton singlet at δ 6.71 (H-6) and an aromatic methoxy methyl at δ 3.95 (3H, *s*). In addition, the existence of a β-glucosyl and two α-rhamnosyl moieties appeared at δ 5.02 (1H, *d*, $J = 7.5$ Hz, H-1″), and 5.53 (1H, *s*, H-1‴)/0.96 (3H, *d*, $J = 6.5$ Hz, H-6‴) and 4.99 (1H, *s*, H-1⁗)/1.19 (3H, *d*, $J = 6.0$ Hz, H-6⁗). Acid hydrolysis with trifluoroacetic acid afforded D-glucose and L-rhamnose in **1** (identified by high-performance anion-exchange column chromatography). The locations of the sugar-aglycone linkage were concluded to be C(7)–C(1″), C(3)–C(1‴) and C(2‴)–C(1⁗) based on the HMBC experiments (Table 1). HMBC correlations of **1** from C-7 (δ 163.3) to H-1″ (δ 5.02) confirmed the attachment of a glucose unit to the aglycone, and the positions of the other two rhamnose units were confirmed in a similar manner by correlations from C-3 (δ 136.7) to H-1‴ (δ 5.53) and C-2‴(δ 79.1) to H-1⁗ (δ 4.99). A down-field shift of C-2‴(δ 79.1) was the other evidence for rhamnose (1 → 2) rhamnose. The ^1H and ^{13}C NMR spectra of **1** resembled those of epimedin C (**5**) (Ito et al., 1988), except for the presence of a 2-hydroxy-3-methyl-3-butenyl group instead of a 3, 3-dimethylallyl group in **5**. A set of signals at δ_H 3.03 (1H, *dd*, $J = 14.0$, 4.0Hz, H-11a), 3.10 (1H, *dd*, $J = 14.0$, 9.0Hz, H-11b), 4.36 (1H, *dd*, $J = 9.0$, 4.0 Hz, H-12), 4.80 (1H, *s*, H-14a), 4.94 (1H, *s*, H-14b), and 1.83 (3H, *s*, H-15) tied in the signals at δ_C 30.1 (*t*, C-11), 76.3 (*d*, C-12), 149.3 (*s*, C-13), 111.0 (*t*, C-14), and 18.2 (*q*, C-15) were assigned to a 2-hydroxy-3-methyl-3-butenyl group. The location of the 2-hydroxy-3-methyl-3-butenyl group at the C-8 position was supported by the chemical value of the carbon atom at the C-6 position (δ 99.5) (Markham et al., 1982). HMBC correlations of **1** from H_2-11 (δ 3.03/3.10) to C-7 (δ 163.3) and C-9 (δ 155.3) also supported this deduction. Therefore, compound **1** was identified as 4′-methoxy-5-hydroxy-8-(2-hydroxy-3-methyl-3-butenyl) flavone 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside.

Sagittasine B (**2**) was obtained as a light-yellow amorphous powder. The ^1H NMR spectrum of **2** showed an aromatic ABC-type pattern [δ 7.10 (*d*, $J = 8.5$ Hz), 7.46 (*dd*, $J = 8.5$, 2.0 Hz), and 7.39 (*d*, $J = 2.0$ Hz)], an aromatic singlet (δ 6.66), an aromatic methoxy methyl singlet (δ 3.96), and a 3,3-dimethylallyl group [δ 3.53/3.60 (H_2-11), 5.21 (H-12), 1.66/1.75 (–$CH_3 \times 2$)]. In addition, three anomeric protons at δ 5.08 (1H, *d*, $J = 7.5$ Hz), 4.99 (1H, *s*), and 5.52 (1H, *s*) suggested that a β-glucosyl and two α-rhamnosyl units were present. The ^1H and ^{13}C NMR spectra of **2** resembled that of epimedin C (**5**) (Ito et al., 1988) except for the presence of ABC-type aromatic signals instead of the A_2B_2-type aromatic signals at the B-ring position in epimedin C. The HR-FAB-MS of **2** indicated a molecular ion at *m*/*z* 839.2982 $[M+H]^+$, consistent with a molecular formula $C_{39}H_{50}O_{20}$, corresponding more to a hydroxyl group than to epimedin C. A NOESY experiment (Table 1) performed on **2** showed an interaction between the 4′-OCH_3 and H-5′, indicating that they were located on adjacent carbons. An HMBC experiment showed correlations from C-4′ to –O*C*H_3, H-2′, H-5′ and H-6′ and C-3′ to H-2′ and H-5′, indicating the placements of the 3′-OH and 4′-OCH_3 groups (Table 1). From the similarity of ^1H and ^{13}C NMR spectroscopic data of **2** and epimedin C (**5**), the linkage positions of the aglycone, glucose, and two rhamnose moieties were concluded to be epimedin C. Based on the above evidence, compound **2** was deduced to be 4′-methoxy-3′,5-dihydroxy-8-3,3-dimethylallylflavone-3-*O*-α-L-rhamnopyranosyl(1 → 2)-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside.

The ^1H and ^{13}C NMR spectra of **3** resembled those of **2**, except for the sugar protons. The ^1H NMR spectrum of the sugar region of **3** showed β-glucosyl and α-rhamnosyl moieties at δ 5.07 (1H, *d*, $J = 7.0$ Hz, H-1″), and 5.28 (1H, *br s*, H-1‴)/0.94 (3H, *d*, $J = 6.5$ Hz, H-6‴). Compound **3** also had a molecular formula of $C_{33}H_{40}O_{16}$ as derived from both the ^{13}C spectrum and HR-FAB-MS spectrum *m*/*z* 693.2391 $[M+1]^+$, indicating one less α-rhamnosyl group than **2**. HMBC correlations of **3** from C-7 (δ 162.0) to H-1″, H_2-11 and H-6, and from C-3 (δ 136.5) to H-1‴, indicated linkages of C-7 to a β-glucosyl group and C-3 to a α-rhamnosyl group in **3** (Table 1). On the basis of these spectral data, we propose structure **3** as 4′-methoxy-3′,5-dihydroxy-8-3,3-dimethylallylflavone-3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside, namely sagittasine C.

Preparative HPLC of a subfraction Fr. 2F yielded five compounds **27**–**31**. The UV absorption spectra (λ_{max} about 270, 340 nm) identified compounds **27**–**31** as flavonoids. Compounds **27**–**31** had mutually related ^1H and ^{13}C NMR spectra which showed signals arising from a flavone

Table 1
^{1}H-,^{13}C-, HMBC and NOESY NMR spectroscopic data for compounds **1**–**3** (measured in MeOH-d_4)[a]

No.	**1**				**2**				**3**			
	δ^{13}C	δ^{1}H	HMBC (^{2}J and ^{3}J)	NOESY	δ^{13}C	δ^{1}H	HMBC (^{2}J and ^{3}J)	NOESY	δ^{13}C	δ^{1}H	HMBC (^{2}J and ^{3}J)	NOESY[b]
Flavonol												
2	159.2	–	H-2′, -6′		159.4	–	H-2′, -6′		159.5	–	H-2′, -6′	
3	136.7	–	H-1‴		136.8	–	H-1‴		136.5	–	H-1‴	
4	180.1	–			180.1	–			180.2	–		
5	161.6	–	H-7		161.0	–	H-7		161.0	–	H-7	
6	99.5	6.71 (1H, *s*)		H-1″	99.3	6.66 (1H, *s*)		H-1″	99.3	6.65 (1H, *s*)		H-1″
7	163.3	–	H_2-11, H-1″, -6		162.1	–	H_2-11, H-1″		162.0	–	H_2-11, H-1″, -6	
8	108.0	–	H_2-11, H-6		110.6	–	H_2-11		110.6	–	H_2-11	
9	155.3	–	H_2-11		155.0	–	H_2-11		155.0	–	H_2-11	
10	107.6	–	H-7		107.5	–	H-7		107.5	–		
1′	123.8	–	H-3′, -5′		124.2	–			124.3	–		
2′	131.9	7.93 (2H, *d*, 8.5)	H-6′		116.8	7.39 (1H, *d*, 2.0)			116.8	7.38 (1H, *d*, 1.5)		
3′	115.3	7.10 (2H, *d*, 8.5)	H-5′	H-2′, 4′-OCH_3	147.8	–	H-2′, -5′		147.8	–		
4′	163.6	–	H-2′, -6′, 4′-OCH_3		151.8	–	H-2′, -5′, -6′, 4′-OCH_3		151.8	–	4′-OCH_3	
5′	115.3	7.10 (2H, *d*, 8.5)	H-3′	H- 6′, 4′-OCH_3	112.4	7.10 (1H, *d*, 8.5)		H-6′, 4′-OCH_3	112.4	7.08 (1H, *d*, 8.5)		4′-OCH_3
6′	131.9	7.93 (2H, *d*, 8.5)	H-2′		122.8	7.46 (1H, *dd*, 2.0, 8.5)	H-2′	H-5′	122.8	7.44 (1H, *dd*, 1.5, 8.5)		
11	30.1	3.03 (1H, *dd*, 14.0, 4.0)/ 3.10 (1H, *dd*, 14.0, 9.0)		H-12	22.7	3.53[c]/3.60[c]			22.7	3.53 (1H, *m*)/ 3.62 (1H, *m*)		H-1″
12	76.3	4.36 (1H, *dd*, 9.0, 4.5)	H-11, 15, H_2-14	H-11	123.5	5.21 (1H, triplet-like)		H-14	123.5	5.20 (1H, *t*, 6.5)	H-14, 15, H_2-11	H-14
13	149.3	–			132.8	–	H-14, 15, H_2-11		132.8	–	H-14, 15, H_2-11	
14	111.0	4.80/4.94 (each 1H, *s*)	H-12, 15		25.9	1.66 (3H, *s*)		H-12	25.9	1.65 (3H, *s*)		H-12
15	18.2	1.83 (3H, *s*)	H_2-14		18.3	1.75 (3H, *s*)			18.3	1.74 (3H, *s*)		H-11
4′-OCH_3	56.1	3.90 (3H, *s*)		H-3′, 5′	56.5	3.96 (3H, *s*)		H-5′	56.5	3.95 (3H, *s*)		H-5′
Glucosyl												
1″	102.8	5.02 (1H, *d*, 7.5)		H-6	101.9	5.08 (1H, *d*, 7.5)		H-6	101.9	5.07 (1H, *d*, 7.0)		H-6, H-11
2″	75.1	3.58[c]			74.9	3.55[c]			74.9	3.53[c]		
3″	78.5	3.52[c]			78.3	3.53[c]			78.3	3.50[c]		
4″	71.2	3.45[c]			71.2	3.46[c]			71.2	3.45[c]		
5″	77.9	3.45[c]			78.3	3.48[c]			78.3	3.50[c]		
6″	62.4	3.95 (1H, *m*)/3.76 (1H, *dd*, 5.5, 11.5)			62.4	3.93 (1H, *dd*, 2.0, 12.5)/ 3.76 (1H, *dd*, 5.5, 12.5)			62.4	3.93 (1H, *d*, 12.5)/3.73[c]		

Rhamnosyl-1									
1‴	102.5	5.53 (1H, *s*)		102.4	5.52 (1H, *s*)		103.5	5.28 (1H, *br s*)	
2‴	79.1	4.29 (1H, *s*)	H-1‴, H-1⁗	79.1	4.28 (1H, *s*)	H-1‴, H-1⁗	71.9	4.22 (1H, *s*)	
3‴	72.2	3.86 (1H, *d*, 9.5)		72.1	3.89 (1H, *dd*, 2.5, 9.5)		72.1	3.73[c]	
4‴	73.5	3.36[c]		73.5	3.35[c]		73.2	3.33[c]	
5‴	71.9	3.44 (1H, *m*)		71.9	3.46[c]		72.1	3.33[c]	
6‴	17.8	0.96 (3H, *d*, 6.5)		17.8	0.95 (3H, *d*, 6.5)		17.7	0.94 (3H, *d*, 6.5)	
Rhamnosyl-2									
1⁗	103.8	4.99 (1H, *s*)	H-2‴	103.8	4.99 (1H, *s*)	H-2‴, 2⁗			
2⁗	72.0	3.95 (1H, *s*)		72.0	3.95[c]				
3⁗	72.3	3.61[c]		72.3	3.62[c]				
4⁗	74.0	3.36[c]		74.0	3.36[c]				
5⁗	70.4	3.52[c]		70.3	3.54[c]				
6⁗	17.9	1.19 (3H, *d*, 6.0)		17.8	1.21 (3H, *d*, 6.0)				

[a] Measured on Varian Inova-500 MHz NMR spectrometer; multiplicity and coupling constant (*J* in Hz) assigned in parentheses; *br. s*, broad singlet; *d*, doublet; *dd*, double doublet; *s*, singlet; *t*, triplet.
[b] Measured on Bruker-400 MHz NMR spectrometer.
[c] Signal patterns are unclear due to overlapping.

skeleton and a coniferyl alcohol unit, corresponding to the framework of flavonolignans. By interpretation of their ^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC, and HMBC spectra followed by comparison with published data, the structures were found to be hydnocarpin (**27**) (Parthasarathy et al., 1979),5″-methoxyhydnocarpin (**28**) (Guz et al., 2001), hydnocarpin-D (**29**) (Guz and Stermitz, 2000), 5′-methoxyhydnocarpin-D (**30**) (Stermitz et al., 2000), and 5′, 5″-dimethoxyhydnocarpin-D (palstatin, **31**) (Pettit et al., 2003), respectively. In previous work, both hydnocarpin and hydnocarpin-D serial flavonolignans were, however, only partially separated (Guz and Stermitz, 2000). In this study, we successfully achieved the separation of this flavonolignan mixture by combining a C18 and C30 column using and mixtures of acetonitrile and H_2O as the mobile phase.

Compound **32** was identified as 6-demethoxycapilarisin (Kijjoa et al., 1999). The signals at δ_H 5.11 (1H, *s*), and δ_C 87.9 ppm were characteristic of H-3 and C-3 in a 2-phenoxychromone. The resonances of C-2 (δ 170.2) and C-1′ (δ 145.2) were also characteristic of a 2-phenoxychromone derivative. Although 2-phenoxychromone is a rare group of natural product, **32** and the other three 2-phenoxychromones have previously been isolated from the title plant (Huang et al., 1993).

Compounds **36**–**40** were considered to be kaempferol-3-*O*-rhamnopyranosides disubstituted with *p*-coumaroyl groups according to the NMR spectroscopic data (Tables 2 and 3), with identical quasi-molecular ions at *m*/*z*: 725 $[M+H]^+$ in the ESI MS spectra. Compounds **36**, **38** and **39** were identified as kaempferol-3-*O*-(2″,4″-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (Yada et al., 2002), kaempferol-3-*O*-(2″-*Z*-*p*-coumaroyl,4″-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (Kuo et al., 2005), and kaempferol-3-*O*-(3″,4″-di-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside (Yada et al., 2002) on the basis of spectroscopic data in agreement with the published data.

In the ^{1}H NMR spectrum of **37**, signals at δ 5.76 (1H, *d*, $J = 13.0$ Hz), 6.89 (1H, *d*, $J = 13.0$ Hz), 7.68/6.74 (each 2H, *d*, $J = 9.0$ Hz), and δ 6.41 (1H, *d*, $J = 16.0$ Hz), 7.66 (1H, *d*, $J = 16.0$ Hz), 7.49/6.80 (each 2H, *d*, $J = 8.5$ Hz), suggested the presence of *cis* and a *trans*-*p*-coumaryl moieties in the molecule. A ^{1}H–^{1}H COSY experiment showed clear coupling connections around the rhamnopyranoside ring, and assignment of protons was achieved. Down-field shifts were observed at H-2″ and H-4″, compared with those on kaempferol-3-*O*-α-L-rhamnopyranoside (**11**) (see Table 2), suggesting the acyl-substitution at these positions. HMBC correlations of **37** from C-9‴ (δ 168.2) to H-7‴ (δ 7.66), H-8‴ (δ 6.41) and H-2″ (δ 5.62), and from C-9⁗ (δ 167.4) to H-7⁗ (δ 6.89) and H-4″ (δ 4.94), indicated linkages of C-2″ to a *trans*-*p*-coumaroyl group and C-4″ to a *cis*-*p*-coumaroyl group in **37**. Therefore, **37** was deduced as kaempferol-3-*O*-(2″-*E*-*p*-coumaroyl, 4″-*Z*-*p*-coumaroyl)-α-L-rhamnopyranoside.

The ^{1}H NMR spectrum of **40** was similar to that of **37**, except for the sugar protons. The ^{1}H–^{1}H COSY

Table 2
^{1}H NMR spectroscopic data for compounds **11**, **36**–**40** (measured in MeOH-d_4)[a]

Proton	11	36	37	38	39	40
Kaempferol						
H-6	6.20 (*d*, 2.0)	6.23 (*s*)	6.21 (*s*)	6.23 (*s*)	6.23 (*s*)	6.22 (*s*)
H-8	6.37 (*d*, 2.0)	6.40 (*s*)	6.38 (*s*)	6.40 (*s*)	6.41 (*s*)	6.41 (*s*)
H-2′/6′	7.77 (*d*, 9.0)	7.82 (*d*, 8.5)	7.76 (*d*, 8.5)	7.81 (*d*, 8.5)	7.86 (*d*, 8.5)	7.86 (*d*, 9.0)
H-3′/5′	6.94 (*d*, 9.0)	7.05 (*d*, 8.5)	6.97 (*d*, 8.5)	6.84 (*d*, 8.5)	6.78 (*d*, 8.0)	6.80 (*d*, 8.5)
Rhamnosyl						
H-1″	5.38 (*br, s*)	5.78 (*s*)	5.62 (*s*)	5.70 (*s*)	5.80 (*s*)	5.78 (*s*)
H-2″	4.23 (*s*)	5.56 (*s*)	5.54 (*s*)	5.53 (*s*)	4.44 (*s*)	4.41 (*s*)
H-3″	3.72 (*dd*, 3.5, 9.0)	4.18 (*dd*, 2.5, 10.0)	4.13 (*dd*, 3.0, 10.0)	4.15 (*dd*, 3.5, 10.0)	5.39 (*dd*, 3.5, 10.0)	5.38 (*dd*, 3.0, 10.0)
H-4″	3.33[b]	4.99 (*t*, 10.0)	4.94 (*t*, 10.0)	4.86[b]	5.21 (*t*, 10.0)	5.16 (*t*, 10.0)
H-5″	3.35[b]	3.30[b]	3.37 (*dd*, 6.0, 10.0)	3.32[b]	3.28[b]	3.26 (*dd*, 6.5, 10.0)
H-6″	0.93 (*d*, 6.0)	0.87 (*d*, 6.0)	0.85 (*d*, 6.0)	0.84 (*d*, 6.0)	0.86 (*d*, 6.5)	0.84 (*d*, 6.5)
Coumaroyl-1						
H-2‴/6‴		7.51 (*d*, 8.5)	7.49 (*d*, 8.5)	7.69 (*d*, 9.0)	7.48 (*d*, 9.0)	7.68 (*d*, 8.5)
H-3‴/5‴		6.82 (*d*, 8.5)	6.80 (*d*, 9.0)	6.78 (*d*, 9.0)	6.80 (*d*, 8.0)	6.74 (*d*, 8.5)
H-7‴		7.70 (*d*, 16.0)	7.66 (*d*, 16.0)	6.92 (*d*, 13.0)	7.64 (*d*, 16.0)	6.86 (*d*, 13.0)
H-8‴		6.44 (*d*, 16.0)	6.41 (*d*, 16.0)	5.87 (*d*, 13.0)	6.31 (*d*, 16.0)	5.77 (*d*, 13.0)
Coumaroyl-2						
H-2⁗/6⁗		7.55 (*d*, 8.5)	7.68 (*d*, 9.0)	7.54 (*d*, 9.0)	7.42 (*d*, 8.5)	7.47 (*d*, 8.0)
H-3⁗/5⁗		6.85 (*d*, 8.5)	6.74 (*d*, 9.0)	7.05 (*d*, 9.0)	7.07 (*d*, 8.5)	7.07 (*d*, 8.5)
H-7⁗		7.59 (*d*, 16.0)	6.89 (*d*, 13.0)	7.58 (*d*, 16.0)	7.51 (*d*, 16.0)	7.51 (*d*, 16.0)
H-8⁗		6.32 (*d*, 16.0)	5.76 (*d*, 13.0)	6.31 (*d*, 16.0)	6.21 (*d*, 16.0)	6.20 (*d*, 16.0)

[a] Multiplicity and coupling constant (J in Hz) assigned in parentheses; *br s*, broad singlet; *d*, doublet; *dd*, double doublet; *s*, singlet; *t*, triplet.
[b] Signal patterns are unclear due to overlapping.

experiment showed down-field shifts protons of H-3″ at δ 5.38 and H-4″ at δ 5.16, compared with those on kaempferol-3-*O*-α-L-rhamnopyranoside (**11**) (see Table 2), suggesting acyl-substitution at these positions. HMBC correlations of **40** from C-9‴ (δ 167.2) to H-7‴ (δ 6.86, *d*, $J = 13.0$ Hz), and H-3″ (δ 5.38), and from C-9⁗ (δ 168.1) to H-7⁗ (δ 7.51, *d*, $J = 16.0$ Hz), H-8‴ (δ 6.20, *d*, $J = 16.0$ Hz) and H-4″ (δ 5.16), indicated the linkages of C-3″ to a *cis*-*p*-coumaroyl group and C-4″ to a *trans*-*p*-coumaroyl group in **40**. Therefore, **40** was deduced to be kaempferol-3-*O*-(3″-*Z*-*p*-coumaroyl,4″-*E*-*p*-coumaroyl)-α-L-rhamnopyranoside.

2.2. Vasorelaxation activity

In an attempt to evaluate the potentiality of these natural products as vasorelaxing agents, 13 isolated major compounds (**4**–**14**, **33** and **34**) were evaluated for their activities as rat aortic preparations. The results indicated that **11** and **14** were the most potent of the test compounds (Figs. 2 and 3), with maximal vasorelaxations of 50.7% and 53.0%, respectively. Compounds **4**–**10**, **12**, **13**, **33** and **34** produced a mild vasorelaxing effect, with EC_{50} more than 100 μM. The tested compounds seem not to be the effective component individually. According to traditional Chinese medicine concepts, the biological effect of an herbal substance may not depend on a single known active principle, However, on multiple chemicals working synergistically or cooperatively through extensive chemical interactions not yet understood (Shen et al., 2001; Wu et al., 2001; Yu et al., 2001; Ko et al., 2006). This may be one of the reasons why these isolated compounds generate only mild or moderate vasorelaxing effects when used alone.

2.3. Concluding remarks

The aerial parts of *E. sagittatum*, were found to contain large amounts of flavonol glycosides with an 8-prenyl unit, of which Kaempferol and quercetin are the major aglycones. Rare constituents flavonolignans (**27**–**31**) were also isolated for the first time from the *Epimedium* plant. Some of these metabolites also display useful bioactivity (Vasorelaxation).

3. Experimental

3.1. General experiment procedures

IR spectra were obtained on a Nicolet Avatar 320 IR spectrometer. UV spectra were measured on a Hitachi U-3200 spectrophotometer in MeOH. ^{1}H-, ^{13}C- and 2D NMR spectra were measured with a Varian Inova-500 spectrometer with deuterated solvents as internal standard. APCI-MS and HR-FAB-MS were recorded on Finnigan LCQ and Finnigan/Thermo Quest MAT spectrometers, respectively. CC was performed on Sephadex-LH-20 (Pharmacia), silica gel 60 (70–230 or 230–400 mesh, Merck; or 12–26 μm, Eurochrom, Knauer) or Cosmosil 140 C_{18} OPN (Nacalai tesque, Kyoto, Japan). Silica gel 60F_{254}

Table 3
^{13}C NMR spectroscopic data for Compounds **11**, **36**–**40** (measured in MeOH-d_4)

Carbon	11	36	37	38	39	40
Kaempferol						
2	159.3	159.4	159.4	159.4	159.3	159.3
3	136.2	134.7	135.1	134.7	134.8	134.8
4	179.6	179.2	179.3	179.3	179.3	179.3
5	163.2	163.3	163.3	163.3	163.3	163.2
6	99.8	100.0	100.0	100.1	100.2	100.2
7	165.8	166.0	166.0	166.5	166.6	166.6
8	94.8	94.8	94.8	94.9	95.0	95.0
9	158.5	158.6	158.6	158.7	158.7	158.7
10	105.9	105.9	105.9	105.8	105.8	105.7
1′	122.6	122.5	122.4	122.5	122.6	122.5
2′/6′	131.9	132.0	131.9	131.9	132.0	132.0
3′/5′	116.5	116.7	116.7	117.7	116.8	116.8
4′	161.6	161.8	161.8	161.9	161.8	161.8
Rhamnosyl						
1″	103.5	99.2	99.7	99.3	101.5	101.5
2″	71.9	73.1	73.2	72.8	69.7	69.7
3″	72.1	68.5	68.5	68.5	72.7	72.2
4″	73.2	74.6	74.4	74.6	71.7	71.7
5″	72.0	69.8	69.8	69.8	69.9	70.0
6″	17.6	17.7	17.7	17.6	17.6	17.6
Coumaroyl-1						
1‴		127.2	127.2	127.6	127.1	127.4
2‴/6‴		131.4	131.4	133.9	131.4	134.0
3‴/5‴		116.8	116.8	116.0	116.8	115.9
4‴		161.4	161.4	161.4	161.4	160.3
7‴		147.4	147.4	146.0	147.4	146.4
8‴		114.7	114.7	115.9	114.7	115.8
9‴		168.2	168.2	168.5	168.5	167.2
Coumaroyl-2						
1⁗		127.2	127.5	127.2	127.1	127.1
2⁗/6⁗		131.4	134.0	131.4	131.3	131.4
3⁗/5⁗		116.8	115.8	116.8	116.8	116.8
4⁗		161.4	160.2	160.2	161.4	161.4
7⁗		146.9	146.1	146.9	147.3	147.3
8⁗		115.0	116.1	115.0	114.5	114.5
9⁗		168.5	167.4	167.0	168.2	168.1

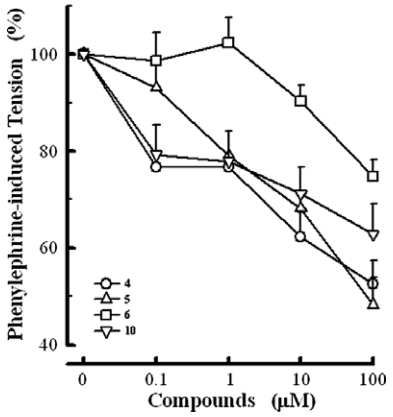


Fig. 2. Concentration–response curves of compounds **4**, **5**, **6** and **10** isolated from *Epimedium sagittatum* (0.01–100 μM) on isolated rat aortic contraction induced by phenylephrine (0.3 μM). $n = 6$–8 in each group.

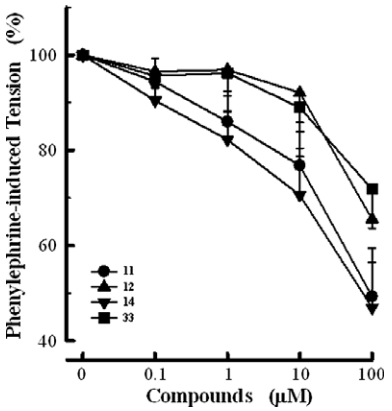


Fig. 3. Concentration–response curves of compounds **11**, **12**, **14** and **33** isolated from *Epimedium sagittatum* (0.01–100 μM) on isolated rat aortic contraction induced by phenylephrine (0.3 μM). $n = 6$–8 in each group.

(Merck, Darmstadt, Germany) was used for TLC (0.25 mm). The preparative HPLC system consisted of a chromatographic pump (LC-8A, Shimadzu, Kyoto, Japan) and a UV–Visible detector (SPD-10A vp, Shimadzu, Kyoto, Japan). A Cosmosil 5C18-AR-II column (20 × 250 mm; particle size 5 μm; Nacalai tesque, Kyoto, Japan) or Develosil C30-UG-5 column (10 × 250 mm; particle size 5 μm; Nomura chemical, Seto, Japan) were used for separations.

3.2. Plant material

The aerial parts of *E. sagittatum* were purchased from Chia-Hui Co., Inc., Taipei, Taiwan, and a voucher specimen is deposited in the National Research Institute of Chinese Medicine, Taipei, Taiwan.

3.3. Extraction and isolation

Aerial parts of *E. sagittatum* (25 kg) were crushed and extracted with EtOH (100 l × 3) under reflux. The combined extracts were concentrated to about 2 l, and extracted successively with *n*-hexane, $CHCl_3$ and *n*-BuOH (each 2 l × 3). The *n*-BuOH fraction (350 g) was subjected to CC on silica gel (10 × 120 cm), with a gradient of MeOH in $CHCl_3$ (0–10%) and six fractions (Fr. 1–6) were collected. A solid precipitate was separated from Fr. 4 and recrystallized from EtOH to give **4** (1.4 g). The filtrate of Fr. 4 (62.6 g) was repeatedly subjected to CC using both Sephadex-LH-20 (MeOH), and a silica gel column (EtOAc) to give **4** (8.4 g), **11** (15.4 mg), **12** (8.5 mg), **14** (233.7 mg) and **33** (8.4 mg). In addition, Fr. 5 (110.0 g) was applied to Sephadex-LH-20 CC with MeOH as eluent to give six subfractions 5A–5F. Fr. 5A (25.4 g) was repeatedly subjected to both Sephadex-LH-20 CC (MeOH), and preparative HPLC (column: Cosmosil 5C18-AR-II, 20 × 250 mm, 5 μm, solvent: 28%ACN/H_2O, flow rate: 15 ml/min) to give **1** (5.7 mg), **2** (4.8 mg), **3** (6.3 mg), **5** (7.8 g) **6** (4.1 mg), **7** (100.6 mg), **8** (265.2 mg), **9** (6.4 mg), and **10** (6.2 mg). Moreover, Fr. 5D (3.1 g) was repeatedly applied to

Sephadex-LH-20 CC (MeOH) and purified by preparative TLC to give **13** (1.2 g), **16** (626.5 mg), **19** (7.6 mg), and **34** (22.1 mg). A solid precipitate was separated from Fr. 5E and recrystallized from MeOH to give **15** (120.9 mg). The combined Fr. 5F and filtrate of Fr. 5E also yielded **15** (152.0 mg) after being purified by Sephadex-LH-20 (MeOH) chromatography. Fr. 2 (7.5 g) was applied to silica gel CC with $CHCl_3$ as eluent to give six subfractions 2A–2F. Fr. A solid precipitate separated from Fr. 2F (0.5 g) was subjected to preparative HPLC (column: Cosmosil 5C18-AR-II, 20 × 250 mm, 5 μm, solvent: 40%ACN/H_2O, flow rate: 17 ml/min) repeatedly to give compounds **27** (44.7 mg) and **31** (3.9 mg) and subfraction 2F-3. 2F-3 yielded **28** (3.2 mg), **29** (2.8 mg) and **30** (5.2 mg) after being purified by a preparative HPLC (column: Develosil C30-UG-5, 10 × 250 mm, 5 μm, solvent: 50%ACN/H_2O, flow rate: 3 ml/min). Fr. 2E (1.5 g) gave **20** (62.7 mg), **22** (108.6 mg), **23** (11.2 mg), **24** (40.4 mg), **27** (26.4 mg), **32** (6.9 mg), and **35** (76.9 mg) after repeated Sephadex-LH-20 (MeOH, acetone) and preparative HPLC (column: Cosmosil 5C18-AR-II, 20 × 250 mm, 5 μm, solvent: 30%ACN/H_2O, flow rate: 17 ml/min) CC. Fr. 3 (35 g) was applied to silica gel CC (4.8 × 60 cm, with a gradient of MeOH in $CHCl_3$ from 0% to 20%) to give four fractions Fr. 3A–3D. Fr. 3C (6.5 g) give **18** (758.7 mg), **21** (145.0 mg), **23** (106.8 mg), and **25** (5.5 mg) after repeated Sephadex-LH-20 (MeOH and acetone). Fr. 3D (16.5 g) was subjected to Sephadex-LH-20 CC (MeOH) to give **17** (8.3 mg), and a subfraction Fr. 3D–5, which exhibited a single spot in TLC detection. Fr. 3D–5 (0.36 g) was further purified by a preparative HPLC (column: Develosil C30-UG-5, 10 × 250 mm, 5 μm, solvent: 48%ACN/H_2O, flow rate: 2.2 ml/min) repeatedly to give compounds **36** (17.6 mg), **37** (15.7 mg), **38** (2.4 mg), **39** (17.7 mg), and **40** (3.6 mg).

3.4. Sagittasine A (*1*)

Amorphous powder; UV (MeOH) λ_{max} (log ε) 228 sh. (4.22), 270 (4.33), 317 (4.08), 348 (4.02) nm; +$AlCl_3$ 236 (4.25), 280 (4.32), 306 (4.07), 344 (4.16), 406 (3.90) nm; +NaOAc 270 (4.34), 317 (4.09), 349 (4.03) nm; IR ν_{max} (KBr) 3375, 1650, 1597, 1439, 1260, 1067 cm^{-1}; for ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectra, see Table 1; ESIMS *m/z* 837 $[M-H]^-$; HR-FAB-MS *m/z* 839.2983 $[M+H]^+$ (calc. for $C_{39}H_{51}O_{20}$, 839.2974).

3.5. Acid hydrolysis of (*1*)

CF_3CO_2H acid (0.3 ml) was added to a solution of **1** (1.2 mg) in H_2O (1.7 ml) and the solution was heated at 80 °C for 2 h. Then the solution was evaporated *in vacuo*, and re-dissolved in H_2O (2 ml). After filtration with a 0.45 μM syringe filter, the filtrate was examined by high-performance anion-exchange chromatographic analysis system (Dionex BioLC, CA, USA) [column: Carbopac PA 10, 4.6 × 250 mm, eluent: 18 mM NaOH, flow rate: 1.0 ml/min, pulsed amperometric detector (PAD-II)]. D-Glucose (t_r = 13.55 min) and L-rhamnose (t_r = 9.00 min) were detected by compared with the retention time (t_r) of authentic samples.

3.6. Sagittasine B (*2*)

Amorphous powder; UV (MeOH) λ_{max} (log ε) 258 (4.17), 269 (4.16), 350 (3.95) nm; +$AlCl_3$ 278 (4.21), 305 sh. (3.87), 354 (3.97), 411 (3.82) nm; +NaOAc 258 (4.16), 269 (4.16), 350 (3.93) nm; IR ν_{max} (KBr) 3373, 1649, 1597, 1439, 1258, 1070 cm^{-1}; for ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectra, see Table 1; ESIMS *m/z* 837 $[M-H]^-$; HR-FAB-MS *m/z* 839.2982 $[M+H]^+$ (calc. for $C_{39}H_{51}O_{20}$, 839.2974).

3.7. Sagittasine C (*3*)

Amorphous powder; UV (MeOH) λ_{max} (log ε) 258 (4.27), 269 (4.28), 345 (4.06) nm; +$AlCl_3$ 278 (4.31), 303 sh. (4.00), 349 (4.06), 404 (4.09) nm; +NaOAc 258 (4.27), 269 (4.28), 346 (4.06) nm; IR ν_{max} (KBr) 3370, 1650, 1598, 1511, 1440, 1258, 1073 cm^{-1}; for ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectra, see Table 1; EPIMS *m/z* 691 $[M-H]^-$; HR-FAB-MS *m/z* 693.2391 $[M+H]^+$ (calc. for $C_{33}H_{41}O_{16}$, 693.2395).

3.8. Kaempferol-3-O-(2″-E-p-coumaroyl,4″-Z-p-coumaroyl)-α-L-rhamnopyranoside (*37*)

UV (MeOH) λ_{max} (log ε) 313 (4.03), 299 sh. (4.00), 267 (3.86), 228 sh. (3.91) nm; +$AlCl_3$ 308 (4.07), 277 (4.01), 233 (4.07) nm; +$AlCl_3$/HCl 313 (4.04), 278 (3.98), 233 (4.03) nm; +NaOMe 367 (4.16), 273 (3.82) nm; IR ν_{max} (KBr) 3460, 1701, 1650, 1605, 1513, 1443, 1365, 1170 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; ESIMS *m/z* 725 $[M+H]^+$; HR-FAB-MS *m/z* 747.1682 $[M+Na]^+$ (calc. for $C_{39}H_{32}O_{14}Na$ 747.1690).

3.9. Kaempferol-3-O-(3″-Z-p-coumaroyl, 4″-E-p-coumaroyl)-α-L-rhamnopyranoside (*40*)

UV (MeOH) λ_{max} (log ε) 314 (4.12), 299 sh. (4.08), 267 (3.92), 231 sh. (4.00) nm; +$AlCl_3$ 315 (4.11), 307 (4.11), 278 (4.02), 230 (4.10) nm; +$AlCl_3$/HCl 316 (4.09), 307 (4.10), 279 (4.01), 229 (4.08) nm; +NaOMe 367 (4.26), 273 (3.90) nm; IR ν_{max} (KBr) 3450, 1695, 1653, 1604, 1515, 1168 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Tables 2 and 3; ESIMS *m/z* 725 $[M+H]^+$; HR-FAB-MS *m/z* 747.1692 $[M+Na]^+$ (calc. for $C_{39}H_{32}O_{14}Na$ 747.1690).

3.10. Biological studies

Adult male Sprague–Dawley rats, weighing 280–320 g (National Laboratory Animal Center, Taipei, Taiwan), were tested. The rats were allowed to become accustomed to environmentally controlled quarters with a constant

temperature of 20–22 °C, relative humidity 55% and light with 12:12 h light–dark cycles. Standard laboratory fodder (Purina Mills, Richmond, IN, USA) and drinking water were provided *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committees of National Research Institute of Chinese Medicine and were conducted in accordance with the National Institutes of Health Animal Care standards.

3.11. Vascular tension experiment

The methods were similar to our previously published method (Wang et al., 1999). Briefly, the aortic rings from male Sprague–Dawley rats were fixed in organ chambers isometrically under passive tension of 1.8 g for 60 min. The functional integrity of endothelium was confirmed by an observation of more than 90% relaxation in response to acetylcholine (3 μM) in tissues pre-contracted with phenylephrine (0.3 μM). For the evaluation of vasorelaxation, one of the following compounds was added in a cumulative manner during the tonic phase of contraction (considered as 100%) induced by phenylephrine (0.3 μM): the *n*-BuOH extract (0.04–0.40 mg/ml), vehicle (DMSO) or 13 pure compounds (0.1–100 μM) isolated from *E. sagittatum*. The samples were tested for their vasorelaxation effects on rat aortic rings with the endothelium to determine their potency. In some cases, N^{ω}-nitro-L-arginine (L-NNA), a nitric oxide synthase inhibitor, was added to determine the contribution of endothelium-derived nitric oxide in the vasorelaxing effect of specimen. Construction of concentration–response curves was based on the percent of relaxation of the phenylephrine-induced contraction. Complete relaxation was considered attained when the pre-contracted rings returned to the base line position. In each preparation only one concentration–response curve was examined.

3.12. Statistical analysis

For each experiment, data are given as mean ± SE and *n* represents the number of samples tested. All data were analyzed by an IBM-compatible statistical software package (SPSS for Windows, Ver. 10.0). The significance of the concentrations and sample treatments was determined by two-way analysis of variance (ANOVA) with repeated measures. If there were significant interactions, the simple main effect of each factor would be assessed using Kruskal–Wallis non-parametric ANOVA. Post hoc comparisons were carried out between means, according to the suitability. A P value less than 0.05 was considered to indicate a statistically significant difference.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.05.035.

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