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ORIGINAL ARTICLE

Isolation and characterisation of flavonoids from the leaves of medicinal plant *Orthosiphon stamineus*

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KEYWORDS

Orthosiphon stamineus;
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Abstract Six flavonoid compounds were isolated from the leaves of the medicinal plant *Orthosiphon stamineus*. On the basis of chemical and spectral analyses their structures were elucidated as eupatorin, sinensetin, 5-hydroxy-6,7,3′,4′-tetramethoxyflavone, salvigenin, 6-hydroxy-5,7,4′-trimethoxyflavone and 5,6,7,3′-tetramethoxy-4′-hydroxy-8-*C*-prenylflavone. The last compound was isolated from this plant for the first time.

1. Introduction

1.1. *Orthosiphon stamineus*

Benth, known locally as ‘Misai kucing’ belongs to the Lamiaceae family. Malaysia and Indonesia have a tropical climate with high temperature and rainfall all year, which have enabled the plant to flourish extensively.

The leaves of *Orthosiphon stamineus*, Benth are commonly used as herbal tea for diuresis, to treat rheumatism, diabetes, urinary lithiasis, oedema, eruptive fever, influenza, hepatitis, jaundice, biliary lithiasis, and hypertension (Sumaryono et al., 1991; Tezuka et al., 2000; Shibuya et al., 1999).

O. stamineus is also used in Southeast Asia, for the treatment of eruptive fever, epilepsy, gallstone, hepatitis, rheumatism, hypertension, syphilis and renal calculus. In South East Asia the tea prepared from leaves is taken as beverage to improve health and for treatment of kidney disease, bladder inflammation, gout and diabetes (Hegnauer, 1996; Wangner, 1982). *O. stamin〈eus* (OS) contains several chemically active constituents such as terpenoids (diterpenes and triterpenes), polyphenols (lipophilic flavonoids and phenolic acids), and sterols (Tezuka et al., 2000). The therapeutics effects of OS have been ascribed mainly to its polyphenol, which has enzyme inhibition and antioxidant activity. Lipophilic flavonoids isolated from OS was reported to show radical-scavenging activity toward the diphenylpicryihydrazyl radical and inhibition of 15-lipoxygenase from soybeans used as model for mammalian

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15-lipoxygenase (Lyckander and Malterud, 1996). Research indicates that the isolated flavones sinensetin and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone from OS exhibited diuretic activity in rats after intravenous administration of 10 mg/kg body weight, therefore, the diuretic effect of OS extracts could be partially due to its lipophilic flavones content (Schut and Zwaving, 1993).

Living cells are constantly exposed to reactive oxygen species, some of which are capable of initiating lipid peroxidation by abstracting of allylic proton from polysaturated fatty acid. This process leads to lipid peroxidation products, which is responsible for cellular damage as a result of oxidative stress (Halliwell and Gutteridge, 1999). Xanthine oxidase is considered to be an important biological force of these superoxide radicals; hence, inhibition of XO may assist in preventing gout and kidney stones (Cos et al., 1998; Lavelli et al., 2000). The recent surge of interest in chemistry of this plant has led to the isolation of more than 50 components with different biological activities. In our recent research, methanol extract of the leaves were separated by a series of chromatographs to give six flavonoid compounds. On the basis of chemical and spectral analyses their structures were elucidated as eupatorin (Wollenweber and Mann, 1985) (**1**), sinensetin Bombardelli et al., 1972 (**2**), 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (Schneider and Tan, 1973) (**3**), salvigenin Zhong and Wu, 1984 (**4**), 6-hydroxy-5,7,3′-trimethoxyflavone (Zhong and Wu, 1984) (**5**) and 5,6,7,3′-tetramethoxy-4′-hydroxy-8-*C*-prenylflavone (Hossain et al., 2001) (**6**).

2. Experimental

2.1. General

IR spectra were recorded (KBr discs) on an FT-IR spectrometer (ν_{max} in cm^{-1}). ^1H NMR spectra were recorded on a Bruker R-32 (300 MHz) instrument in $CDCl_3$ with TMS as an internal standard (chemical shifts in δ, ppm). UV spectra were recorded on HATACHI, U-2000 spectrophotometer Ultrospeck in methanol (λ_{max} in nm). Mass spectra were recorded on a Varian 3800 mass spectrometer. TLC was performed with silica gel GF_{254}. All solvents were analytical reagent grade.

2.2. Plant material

O. stamineus Benth (Lamiaceae) leaves were collected from the Island of Penang. The plant was identified and a voucher specimen was deposited in the herbarium of the School of Biology, University Sains Malaysia.

2.3. Extraction

Dried leaves of the plant (1 kg) were milled into powder and then extracted with direct methanol (10 L) in a Soxhlet extractor for 36 h. The extract was evaporated in a rotatory evaporator and dried by vacuum pump. The methanolic extract (50 g) was suspended on water and extracted successively with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol to yield *n*-hexane (5.5 g), chloroform (11.5 g), ethyl acetate (9.3 g) and *n*-BuOH-soluble (5.2 g) fractions, respectively. Chloroform soluble fraction (10 g) was subjected to chromatography on silica gel (60–120 mesh, Merck) eluted with ethyl acetate–*n*-hexane (7:3) solvent system. By repeated chromatography to give five major fractions (Fraction-1, 0.176 g; Fraction-2, 0.216 g; Fraction-3, 1.00 g; Fraction-4, 0.074 g; Fraction-5, 0.143 g; Fraction-6, .0103 g).

2.4. Fraction-1

Fraction-1 obtained from column chromatography was further purified by preparative TLC over silica gel GF_{254} using ethyl acetate–hexane (3:2) as the developing solvent. It was a yellow crystal, m.p. 196–197 °C (lit. Wollenweber and Mann, 1985, m.p. 196 °C); MS: (M^+, 344); UV: 340, 275, 210 nm; IR (KBr): 3470, 3250, 2840, 1650, 1605, 1599, 1478, 1365, 1050 cm^{-1}; ^1H NMR (δ values, DMSO-d_6): 3.72 (s, 3H, 6-OCH_3), 3.88 (s, 3H, 4′-OCH_3), 3.96 (s, 3H, 7-OCH_3), 6.76 (s, 1H, H-3), 6.88 (s, 1H, H-8), 7.10 (d, 1H, H-5′), 7.45 (s, 1H, H-2′), 7.68 (dd, 1H, H-6′), 9.48 (s, 1H, 3′-OH), 12.55(s, 1H, 5-OH). It was characterized as 5,3′-dihydroxy-6,7,4′-trimethoxyflavone (Wollenweber and Mann, 1985) (Eupatorin, **1**).

2.5. Fraction-2

Isolated fraction was further purified by preparative TLC over silica gel GF_{254} using ethyl acetate–hexane (3:2) as the developing solvent. It was crystallized from methanol as a white powder (2, 6 mg); m.p. 175–176 °C (lit. Wangner, 1982, m.p. 175–176 °C); MS: (M^+ 373); UV: 327, 282, 213 nm; IR (KBr):2850, 1645, 1600, 1590, 1476, 1378, 1215, 1189, 870 cm^{-1}; ^1H NMR (δ values, DMSO-d_6): 3.76 (s, 3H, 5-OCH_3), 3.78 (s, 3H, 6-OCH_3), 3.84 (s, 3H, 4′-OCH_3), 3.88 (s, 3H, 3′-OCH_3), 3.96 (s, 3H, 7-OCH_3), 6.78 (s, 1H, H-3), 7.12 (d, 1H, H-5′), 7.18 (s, 1H, H-8), 7.52 (s, 1H, H-2′), 7.68 (d, 1H, H-6′). It was characterized as 5,6,7,3′,4′-pentamethoxyflavone (Bombardelli et al., 1972) (sinensetin, **2**).

2.6. Fraction-3

The isolated mixture was worked-up in the usual way and purified by preparative TLC over silica gel GF_{254} using benzene–acetone–ethyl acetate (7:5:1) as the developing solvent. It was crystallized from methanol as orange needles colour (9 mg); m.p. 102 °C; MS: (M^+, 462); UV: 228, 254, 370 nm; IR: 3415, 1645, 1600, 1590, 1375, 1365 cm^{-1}; ^1H NMR: (δ values, DMSO-d_6): 3.41 (s, 12H, –$CH_2OCH_3 \times 4$), 5.29 (s, 8H, –$CH_2OCH_3 \times 4$), 6.13 (s, 1H, H-3), 6.48 (s, 1H, H-8), 6.97 (s, 1H, H-6), 7.10 (s, 1H, H-2′), 7.29 (d, 2H, J = 9 Hz, H-5′ and H-6′), 12.75 (s,1H, 5-OH). It was characterized as 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (Schneider and Tan, 1973) (**3**).

2.7. Fraction-4

Isolated fraction from the column was further purified by preparative TLC over silica gel GF_{254} using ethyl acetate–*n*-hexane (7:3.5) as the developing solvent. It was crystallized from petroleum spirit as yellow needles colour (5 mg); m.p. 144 °C (lit. Lyckander and Malterud, 1996, m.p. 145 °C); MS: (M^+, 346); UV: 228, 254, 370 nm; IR: 3450, 1645, 1600, 1590, 1375, 1365 cm^{-1}; ^{1}H NMR: (δ values, DMSO-d_6): 3.91, 3.94 and 3.99 (3s, 9H, OCH$_3 \times 3$), 6.67 (s, 1H, H-3), 7.13 (d, 1H, J = 9.5 Hz, H-2′), 7.74 (d, 2H, J = 9.5 Hz, H-5′ and H-6′), 12.45 (s, 1H, 5-OH). It was characterized as salvigenin (Zhong and Wu, 1984) (**4**).

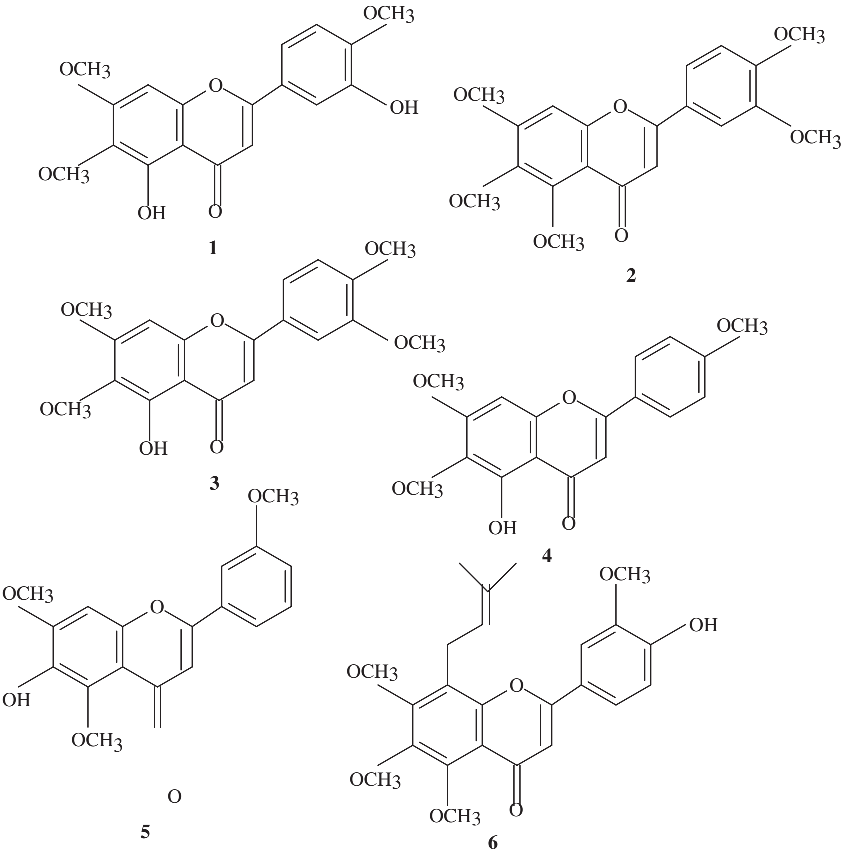
2.8. Fraction-5

It was purified by preparative TLC over silica gel GF$_{254}$ using ethyl acetate–hexane (7:3.5) as developing solvent to give compound **5** (6 mg) and compound **6** (4 mg). Compound **5**: It was crystallized from methanol-water as yellow coloured needles (4 mg); m.p. 172 °C (lit. Zhong and Wu, 1984. m.p. 172–174 °C); MS: (M^{+}, 426); UV: 232, 258, 365 nm; IR: 3470, 1642, 1595, 1590, 1375, 1365 cm^{-1}; ^{1}H NMR (δ values, DMSO-d_6): 3.91, 3.94 and 3.97 (3s, 9H, OCH$_3 \times 3$) 6.45 (s, 1H, H-3), 6. 65 (s, 1H, H-8), 7.05 9d, 1H, J = 9 Hz, H-2′), 7.45 (d, 2H, J = 9 Hz, H-5′ and H-6′), 12.75 (s, 1H, 6-OH). It was characterized as 6-hydroxy-5,7,3′-trimethoxyflavone (Zhong and Wu, 1984) (**5**). Compound **6**: It was crystallized from methanol an orange coloured needles; m.p. 134 °C (lit. Hossain et al., 2001, m.p. 134 °C); MS: (M^{+}, 426). UV: 318, 288, 213 nm. IR (KBr): 3435, 2964, 2936, 2832, 2361, 1649, 1610, 1595, 1452, 1376, 1364, 1271, 1200, 1128, 1046, 1030, 879, 832, 800 cm^{-1}. ^{1}H NMR (δ values, CDCl$_3$): 1.60 (s, 6H, $>$C(CH$_3$)$_2$, 3.51 (d, 2H, J = 7 Hz, –CH_2–CH═), 3.90, 3.94, 3.99 and 4.00 (4s, 12H, –OCH$_3 \times 4$), 5.55 (t, 1H, J = 7 Hz, –CH$_2$–CH═), 6.96 (s, 1H, H-3), 6.99 (d, 1H, J = 9 Hz, H-2′), 7.44 (m, 2H, H-5′ and H-6′), 12.76 (s, 1H, 4′-OH); ^{13}C NMR (δ values, CDCl$_3$): 153.47 (C-2), 126.27 (C-3), 183.07 (C-4), 159.16 (C-5), 90.97 (C-6), 159.16 (C-7), 104.95 (C-8), 153.47 (C-9), 111.10 (C-10), 119.50 (C-1′), 126.27 (C-2′), 112.75 (C-3′), 146.45 (C-4′), 112.75 (C-5′), 119.50 (C-6′), 77.80 (C-1″), 77.58 (C-2″), 77.38 (C-3″), 76.95 (C-4″), 77.38 (C-5″), 61.23 (5-OCH$_3$), 56.69 (7-OCH$_3$), 56.54 (6-OCH$_3$), 55.64 (3′-OCH$_3$). It was characterized as 5,6,7,3′-tetramethoxy-4′-hydroxy-8-*C*-prenylflavone (Hossain et al., 2001) (**6**).

3. Results and discussion

The methanol extract of the leaves was extracted with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. The chloroform fraction was purified and six compounds were obtained (Scheme 1). By means of spectroscopic analysis, they were characterized as eupatorin (Wollenweber and Mann, 1985) (**1**), sinensetin Bombardelli et al., 1972 (**2**), 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (Schneider and Tan, 1973) (**3**), salvigenin Zhong and Wu, 1984 (**4**), 6-hydroxy-5,7,3′-trimethoxyflavone (Zhong and Wu, 1984) (**5**) and 5,6,7,3′-tetramethoxy-4′-hydroxy-8-*C*-prenylflavone (Hossain et al., 2001) (**6**). The compound (**6**) was isolated from this plant for the first time.

3′,5,6,7-Tetramethoxy-4′-hydroxy-8-*C*-prenylflavone (**6**) was obtained as orange coloured needles crystal. High resolution mass spectrum exhibited molecular ion at m/z 426, which is consistent with the molecular formula C$_{24}$H$_{26}$O$_7$. UV spectra



Scheme 1

displayed characteristic absorption bands for cojugated double bond at 257 nm. IR spectra of compound (**6**) showed frequencies at 3435 cm^{-1} and at 2954–2832 cm^{-1} indicating the presence of hydroxyl group and C—H in conjugation, respectively and the absorption peaks at 164 9, 1610 and 1595 cm^{-1} indicated the presence of $\rangle$C=O group and unsymmetric ethylenic double bond and aromatic rings. The ^{1}H NMR spectrum of the prenylated compound (**6**) indicated the presence of C-prenyl unit. A sharp singlet at 1.60 revealed the presence of gem-dimethyl group and the presence of $-CH_2-$ and $-CH=$ protons attached to the aromatic ring was indicated by a doublet at 3.51 and a triplet at 5.55, respectively. A singlet at 6.96 indicated the presence of H-3 proton for the flavone nucleus. Four sharp singlets at 3.90, 3.94, 3.99 and 4.00 indicated the presence of four methoxy group on the aromatic rings. Also, a singlet at 12.76 indicated the presence of a hydroxyl group. All the carbons were clear in the ^{13}C NMR (Section 2) spectrum and were sorted by DEPT experiments into two $CH_{3,}$ four $OCH_{3,}$ one CH_2, five CH and twelve fully substituted carbons. Compound (**6**) was also obtained as orange needles showing a pink colour on TLC silica gel plate when heated with concentrated sulphuric acid at R_f (0.67) in chloroform–ethyl acetate (6:4). All the isolated known triterpenes were identified on the basis of UV, IR and ^{1}H NMR data and compared with the literature.

4. Conclusion

There is evidence that flavonoids scavenge free radicals and reactive oxygen species and also inhibit lipid peroxidation. The pharmacological effect of OS leaf consumption may be due the presence of its phytochemicals including flavonoids, which have antioxidant properties. Our data showed that the crude methanolic extracts of OS contains lots of flavonoids, may be important in inhibiting oxidative stress mechanisms that lead to degenerative diseases because by lipid peroxidation products and reactive oxygen species. Traditionally, OS has been used as a medicinal plant in the treatment of kidney diseases and gout; this may be linked to the ability of the extracts to inhibit xanthine oxidase and lipid peroxidation. We are currently focusing our research on the study of in vivo antioxidant activities of the leaves extract of OS.

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