ISSN 1996-0700

Asian Journal of Biotechnology

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Asian Journal of Biotechnology 3 (2): 200-205, 2011
ISSN 1996-0700 / DOI: 10.3923/ajbkr.2011.200.205

New Prenylated Flavonoids of *Orthosiphon stamineus* Grown in Malaysia

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ABSTRACT

The present study was carried out aiming to isolate the flavonoidal components of the plant and test them cytotoxicity. The leaves were extracted with methanol and than fractionated with hexane, chloroform, ethyl acetate and finally with butanol. The crude extracts were purified by using different chromatographic techniques. Two prenylated flavonoid derivatives; 5,7,3',5'-tetramethoxy-8-C-prenyl-flavone, 5,7,3',5'-tetramethoxy-6-C-prenylflavone, together with four known flavonoids 5,6,7,3',4'-pentamethoxyflavone,5-hydroxy-6,7,3',4'-tetramethoxyflavone, ladanein and 6-hydroxy-5,7,4'-trimethoxyflavone isolated from the leaves extracts of *Orthosiphon stamineus*. The structures of these compounds have been established by spectroscopic methods. All the isolated compounds were tested for their cytotoxicity towards highly liver metasttatic murine colon 26-L5 carcinoma cells and the new prenylated flavones and flavonoids (5,6,7,3',4'-pentamethoxyflavone, 5-hydroxy-6,7,3',4'-tetramethoxy lavone, ladanein and 6-hydroxy-5,7,4'-trimethoxyflavone) showed cytotoxicity with an ED_{50} value between 10-90 μg mL^{-1}.

Key words: *Orthosiphon stamineus*, prenylated flavonoids

INTRODUCTION

Flavonoids are a diverse group of phytochemicals which are produced by various plants in high quantities. More than 8000 compounds of flavonoid structure have been identified (Hossain and Rahman, 2003). The large number of compounds arise from various combinations of multiple hydroxyl and methoxyl groups substituting the basic flavonoids skeleton (Hossain *et al*., 2004).

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. It is one of the popular traditional folk medicine extensively used in Southeast Asia for the treatment of wide range of diseases in Indonesia and Vietnam for rheumatism, diabetes, urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice, biliary lithiasis and hypertension etc. (WHO, 2001; Eisai, 1995; Hossain *et al*., 2004, 2007, 2008, 2009; Hossain and Ismail, 2005) and in Malaysia to alleviate diabetes and kidney stone disease (Hossain *et al*., 2008, 2009). Owing to its beneficial pharmaceutical utility, it is under systematic cultivation in Malaysia and is locally known as Misai kucing meaning Cats whisker and consumed as a healthy Java tea to facilitate the body detoxification. In particular, extracts of *Orthosiphon stamineus* are now widely used in Malaysia

as drugs for the treatment of diabetes and kidney stone disease. The recent surge of interest in chemistry of this plant has led to the isolation of more than 50 components including flavonoids (Hossain *et al*., 2008, 2009; Sumaryono *et al*., 1991), terpenoids (Hossain *et al*., 2008, 2009; Awal *et al*., 2002; Sumaryono *et al*., 1991) and caffeic acid derivatives (Awal *et al*., 2002; Sumaryono *et al*., 1991) with different biological activities. They are also known to be antihypertensive and to have β_1-adrenergic inhibition and antimicrobial activities (Hossain, 2006; Eisai, 1995; Hossain *et al*., 2008, 2009; Sumaryono *et al*., 1991). In particular, extracts of *Orthosiphon stamineus* are now widely used in Malaysia as drugs for the treatment of diabetes and kidney stone diseaes. Prenlated flavonoids have been found to display a variety of biological activies such as behavioral depression and muscle relaxation; they are also known to be antihypertensive and to have β_1-adrenergic inhibition and antimicrobial activities (Awal *et al*., 2002).

The present study was carried out aiming to isolate the flavonoidal components of the plant and test them cytotoxicity.

MATERIALS AND METHODS

General: Melting points were determined using an electrothermal melting point apparatus (Gallenkamp). IR spectra were recorded (KBr discs) on a FT-IR spectrophotometer, validation (ν_{max} in cm^{-1}). ^{1}H-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). TLC was performed with silica gel GF_{254}. All solvents were analytical reagent grade.

Plant material: *Orthosiphon stamineus* Benth (Lamiaceae) leaves were collected from the Island of Penang. The plant was identified and voucher specimen (Number 027) was deposited in the herbarium of the School of Biology, University Sains Malaysia. This study was conducted from 2006 to 2008.

Extraction: The dried and milled plant leaves (1500 g) was 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 (100 g) was suspended on water and extracted successively with hexane, chloroform, ethyl acetate and butanol to yield hexane (4 g), chloroform (10.5 g), ethyl acetate (7.4 g) and BuOH-soluble (4.23 g) fractions, respectively. Chloroform soluble fraction (8 g) was subjected to chromatography on silica gel (60-120 mesh, Merck) eluted with ethyl acetate-hexane (7:3) solvent system. Repeated chromatography of the chloroform fraction to give five major fractions (Fraction-1, 0.110 g; Fraction-2, 0.143 g; Fraction-3, 1.229 g; Fraction-4, 0.059 g; Fraction-5, 0.125 g).

Fraction-1: Obtained from column chromatography was further purified by preparative TLC over silica gel GF_{254} using benzene-methanol (95:5) as developing solvent to give 5,6,7,3',4'-pentamethoxyflavone (3 mg) (Awal *et al*., 2002).

Fraction 2: It was further purified by preparative TLC over silica gel GF_{254} using ethyl acetate-chloroform (60:40) as developing solvent to give 5-hydroxy-6,7,3',4'-tetramethoxyflavone (5 mg) (Sumaryono *et al*., 1991).

Fraction 3: It was also rechromatographed over silica gel with a methanol-hexane solvent system to give two prenylated compounds (1) and (2).

Compound 1: 5,7,3',5'-Tetramethoxy-8-C-prenylflavone (1): It was crystallized from methanol. It was an orange needles colour. m. p. 134°C; R_f 0.42 (methanol-hexane; 95:5); MS: (M^+, 426). UV: 378, 288, 213 nm. IR (KBr): 2950, 2936, 2812, 2361, 1645, 1605, 1595, 1452, 1376, 1364, 1271, 1200, 1128, 1046, 1030, 879, 832, 800 cm^{-1} (^{1}H-NMR and ^{13}C-NMR; Table 1).

Compound 2: 5,7,3',5'-Tetramethoxy-6-C-prenylflavone (2): It was crystallized from methanol. It was an orange needles colour. m. p. 101°C; R_f 0.42 (methanol-hexan-water; 65: 5; 30); MS: (M^+, 426). UV : 368, 281, 213 nm. IR (KBr): 2965, 2936, 2832, 2361, 1642, 1600, 1590, 1472, 1376, 1364, 1271, 1200, 1128, 1046, 1030, 879, 832, 800 cm^{-1} (^{1}H-NMR and ^{13}C-NMR; Table 1).

Faction 4: Obtained from column chromatography was further purified by preparative TLC over silica gel GF_{254} using benzene-methanol (95:5) as developing solvent to give ladanein[5] (2.5 mg) and 6-hydroxy-5,7,4'-trimethoxyflavone (6 mg) (Sumaryono *et al.*, 1991).

Cytoxic assay: A cytotoxic assay was done using the standard 3-(4, 5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide (MTT) assay was described previously (Sumaryono *et al.*, 1991) with slight modification. In brief, exponentially growing cells were harvest and 100 µL of cell suspension

Table 1: Spectral data for compound 1 and 2

	Compound 1		Compound 2	
Position	^{1}H NMR	^{13}CNMR	^{1}HNMR	^{13}CNMR
2		150.47		151.47
3	6.82 (s, 1H)	126.07	6.86 (s, 1H)	126.03
4		183.07		182.05
5		159.16		160.10
6	6.99 (s,1H)	90.97		101.77
7		158.11		159.16
8		104.95	6.34 (s,1H)	71.95
9		153.47		152.40
10		111.10		115.12
1'		119.55		120.44
2'	7.12 (d, 2H, J = 9 Hz, 2' and 6')	126.22	7.15 (d, 1H, J = 9 Hz, 2' and 6')	125.29
3'		112.75		115.75
4'	7.32 (s,1H)	146.40	7.28 (s,1H)	146.45
5'		112.75		112.70
6'		119.55		119.55
1"		77.80		77.70
2"	3.51 (d, 2H, -CH_2-)	77.58	3.55 (d, 2H, -CH_2-)	77.88
3"	5.55 (t, 1H, -CH=)	77.38	5.53 (t, 1H, -CH=)	77.12
4"		76.95		76.23
5"	1.67 (s, 6H, >CH_3x2)	77.38	1.65 (s, 6H, >CH_3x2)	77.99
5-OCH_3	3.95 (s, 3H)	61.23	3.92 (s, 3H)	61.49
7-OCH_3	4.00 (s, 3H)	56.69	3.99 (s, 3H)	56.32
6'-OCH_3	3.98 (s, 3H)	56.54	3.94 (s, 3H)	56.55
3'- OCH_3	3.97 (s, 3H)	55.64	3.97 (s, 3H)	55.63

Assignments made by combination of COSY DEPT 45, 90, 135 and HBQC experiments

containing 2000 cells was plated in 96-well plates. After 24 h incubation at 37°C under 5% CO_2, the cells were treated with varying concentrations of test specimens in 100 μL medium and incubated for 3d under the same conditions. At 3 h after adding an MTT solution, UV absorption was measure at 590 nm. Test specimens were dissolved in dimethyl sulphoxide and then diluted by the medium. 5-Flurouraci; was used as a positive control and ED_{50} values were calculated from the mean values of data from three wells. The following compounds showed cytotoxicity with an ED_{50} values less then 100 μg mL^{-1}. 5,7,3',5'-tetramethoxy-8-C-prenyl-flavone (1), 45 μg mL^{-1}; 5,7,3',5'-tetramethoxy-6-C-prenylflavone, 55 μg mL^{-1}; 5,6,7,3',4'-pentamethoxyflavone, 89 μg mL^{-1}; 5-hydroxy-6,7,3',4'-tetramethoxyflavone, 92 μg mL^{-1}; ladanein, 34 μg mL^{-1}; 6-hydroxy-5,7,4'-trimethoxyflavone 42 μg mL^{-1}; 5-Flurouraci. 0.24 μg mL^{-1}.

RESULTS AND DISCUSSION

The methanol extract of the leaves was extracted with n-hexane, chloroform, ethyl acetate and n-butanol. The fractions were subjected to a series of chromatographic separation and purified by preparative TLC to afford six flavonoids. On the basis of chemical and spectral analyses, their structures were elucidated as 5,7,3',5'-tetramethoxy-8-C-prenyl-flavone, 5,7,3',5'-tetramethoxy-6-C-prenylflavone, 5,6,7,3',4'-pentamethoxy flavone, 5-hydroxy-6,7,3',4'-tetramethoxyflavone, ladanein and 6-hydroxy-5,7,4'-trimethoxyflavone. (1) 5,7,3',5'-Tetramethoxy-8-C-prenyl-flavone and (2) 5,7,3',5'-tetramethoxy -6-C-prenylflavone (Fig. 1) were isolated from this plant for the first time.

The compound (1) was obtained as an orange needles and gave a pink spot on TLC sprayed with conc. H_2SO_4. The mass spectrum exhibited molecular ion at m/z 426, which was consistent with the molecular formula $C_{24}H_{26}O_7$. IR spectrum showed absorpyion bands at 2950-2812 cm^{-1} indicating the presence of -CH_2- and -CH_3 groups. The absorption peaks at 1645, 1605 and 1595 cm^{-1} indicated the presence of >C=O group and ethylenic double bond and aromatic rings, respectively. The ^{1}H-NMR spectrum of the compound (1) indicated the presence of C-prenyl unit. A sharp singlet at δ 1.67 (6H, 2 x CH_3) revealed the presence of gem-dimethyl group whereas the presence of -CH_2- and -CH= protons attached to the aromatic ring was indicated by a doublet at δ 3.51 (J = 7 Hz) and a triplet at δ 5.55 (J = 7Hz) respectively. A singlet at δ 6.86 indicated the presence of H-3 for the flavone nucleus. Four sharp singlets of three protons each at δ 3.95, 3.97, 3.98 and 4.00 indicated the presence of four methoxy group on the aromatic rings. Two singlets at δ 6.99 and δ 7.32 indicated the presence of H-6 and H-4' protons. A doublets at δ 7.12 (J = 9 Hz) indicated the presence of H-2' and H-6' protons. The ^{13}C-NMR and DEPT spectra showed 24 carbon atoms for the molecules consisting of two -CH_3, four -OCH_3, one -CH_2-, six CH and sixteen fully

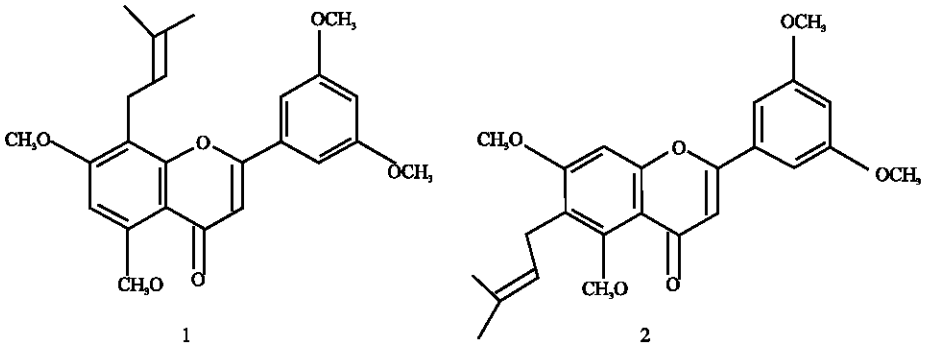

Fig. 1: The prenylated flavonoid derivatives. (1) 5,7,3',5'-Tetramethoxy-8-C-prenyl-flavone and (2) 5,7,3',5'-tetramethoxy-6-C-prenylflavone

substituted carbons. Thus on the basis of above studies the structure of compound (1) was established as 5,7,3',5'-tetramethoxy-8-C-prenylflavone.

The compound 5,7,3',5'-tetramethoxy-6-C-prenylflavone (2) is isomeric to 5,7,3',5'-tetramethoxy-8-C-prenylflavone (1) in which the prenyl unit has migrated to C-6 of the same aromatic ring. The prenylated flavone (2) was also obtained as orange crystals showing a dark pink colour on TLC silica gel plate when heated with concentrated sulphuric acid at R_f (0.42) in chloroform-benzene (1:4). High resolution mass spectrum exhibited molecular ion at m/z 426, which is consistent with the molecular formula $C_{24}H_{26}O_7$ and its ^{1}H-NMR and ^{13}C-NMR data are closely resembled to compound (1). IR spectrum showed absorpyion bands at 2965-2812 cm^{-1} indicating the presence of -CH_2- and -CH_3 groups. The absorption peaks at 1642, 1600 and 1590 cm^{-1} indicated the presence of >C=O group and ethylenic double bond and aromatic rings, respectively. The ^{1}H-NMR spectrum of the compound (1) also indicated the presence of C-prenyl unit. A sharp singlet at δ 1.65 (6H, 2 x CH_3) revealed the presence of gem-dimethyl group whereas the presence of -CH_2- and -CH= protons attached to the aromatic ring was indicated by a doublet at δ 3.55 (J = 7 Hz) and a triplet at δ 5.53 (J = 7 Hz), respectively (Hossain and Islam, 1993). A singlet at δ 6.82 indicated the presence of H-3 for the flavone nucleus. Four sharp singlets of three protons each at δ 3.92, 3.94, 3.97 and 3.99 indicated the presence of four methoxy group on the aromatic rings. Two singlets at δ 6.34 and δ 7.28 indicated the presence of H-8 and H-4' protons. A doublets at δ 7.15 (J = 9 Hz) indicated the presence of H-2' and H-6' protons. The ^{13}C-NMR and DEPT spectra also showed 24 carbon atoms for the molecules consisting of two -CH_3, four -OCH_3, one -CH_2-, six CH and sixteen fully substituted carbons (Hossain *et al*., 1999, 2002). Thus on the basis of above studies the structure of compound (2) was established as 5,7,3',5'-tetramethoxy-6-C-prenylflavone.

To the best of our knowledge, the isolation of prenylated flavone from *Orthosiphon stamineus* or other plant has not been reported elsewhere.

The known flavonoids 5,6,7,3',4'-pentamethoxyflavone (Sumaryono *et al*., 1991), 5-hydroxy-6,7,3',4'-tetramethoxyflavone, ladanein and 6-hydroxy-5,7,4'-trimethoxyflavone (Sumaryono *et al*., 1991) were identified by compairing their spectral data with those in the literature. A search in the literature has revealed related structures in which a biflavonoid containing a similar C-prenyl moiety has been identified from different sources (Ngadjui, *et al*., 1998, 1999; Abegaz *et al*., 1998). Interestingly, spectroscopic studies have revealed that this compound exists as a mixture of tautomers.

CONCLUSION

More than 23 flavonoids were isolated from the leaves of *Orthosiphon stamineus*. These are aglycones and glycosides thereof. The prenylated flavones and isoflavones are represented by six compounds. In general, the plants of *Orthosiphon stamineus* are rich of these isoprenylated metabolites. The majority of the said isolated flavonoids were obtained from leaves of *Orthosiphon stamineu*. Most of these pigments were concentrated in *Orthosiphon stamineus* and the majority of them are prenylated derivatives. It could be observed that chalcones and isoflavans very often co-occur together with prenylated flavones and isoflavones.

ACKNOWLEDGMENTS

The authors are grateful to Mr. Khoo Kay Hock and Mr. Yee Chin Leng of the School of Chemistry, Universiti Sains Malaysia, Malaysia for their help in connection with ^{1}H-NMR and mass spectra. One of the authors (M. Amzad Hossain) is grateful to Universiti Sains Malaysia, Malaysia for providing a fellowship.

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