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Flavonol 3-*O*-robinobiosides and 3-*O*-(2″-*O*-α-rhamnopyranosyl)-robinobiosides from *Sesuvium portulacastrum*

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ABSTRACT

Six new flavonol 3-*O*-robinobiosides and 3-*O*-(2″-*O*-α-L-rhamnopyranosyl)-robinobiosides, sesuviosides A–F, were isolated from the aerial portion of *Sesuvium portulacastrum* together with ecdysterone, adenosine, 2′-*O*-methyladenosine, and L-tryptophan. The structure elucidations were based on analyses of chemical and spectroscopic data including 1D and 2D-NMR. Sesuviosides A–F and their aglycones exhibited radical scavenging activity using DPPH and ORAC assays.

1. Introduction

Sesuvium portulacastrum (L.) L. (Aizoaceae, Thai name: Phak-bia-ta-le) is a sprawling perennial herb, and has been found to grow naturally by the ocean side in the tropical and subtropical regions throughout the world. It is reported as a salt tolerant plant and utilized for the bio-remediation of saline soil in arid and semiarid areas.[1,2] In southern Thailand, the aerial part is used as vegetable for cooking purposes. There is no mention of its medicinal uses in Thai traditional medicine; however it has been used in African folk medicine for treatment of several diseases, such as scurvy, infections, and kidney disorders.[3] The essential oil extracted from the fresh leaves, collected in Zimbabwe, showed significant antibacterial, antifungal, and antioxidant activities.[3] There are a few reports on the phytochemical investigation of this species. In previous studies, ecdysterone and α-ecdysone along with the 3-*O*-glucopyranoside and the 3-*O*-rutinoside of 3,5,4′-trihydroxy-6,7-dimethoxyflavone have been reported from plant sources of India,[4–6] while *trans*-4-hydroxy-prolinebetaine and prolinebetaine were detected from a plant source of Venezuela.[7]

In our ongoing study of Thai plants, we investigated the polar constituents of *S. portulacastrum* collected from southern Thailand. Ten compounds were isolated, including six new flavonol glycosides (**1**–**6**), one phytoecdysteroid, two nucleosides, and one amino acid, from the aerial part of this plant. The present paper deals with the isolation and structure elucidation of these compounds. In addition, all new flavonol glycosides and their aglycones were also evaluated for their radical scavenging activity using DPPH and ORAC assays.

2. Results and discussion

2.1. Structure elucidation of new flavonol glycosides

The aqueous soluble fraction of a methanol extract of *S. portulacastrum* was subjected to column chromatography over HP-20 using H_2O, MeOH, and Me_2CO as eluants, successively. The portion eluted with MeOH was repeatedly subjected to silica gel and RP-18, as well as preparative HPLC-ODS chromatography to afford six new flavonol glycosides, namely sesuviosides A–F (**1**–**6**) (Fig. 1), and four known compounds.

Sesuvioside A (**1**) was isolated as a yellow amorphous powder, and its molecular formula was determined as $C_{29}H_{34}O_{16}$ by high-resolution atmospheric pressure chemical ionization time-of-flight

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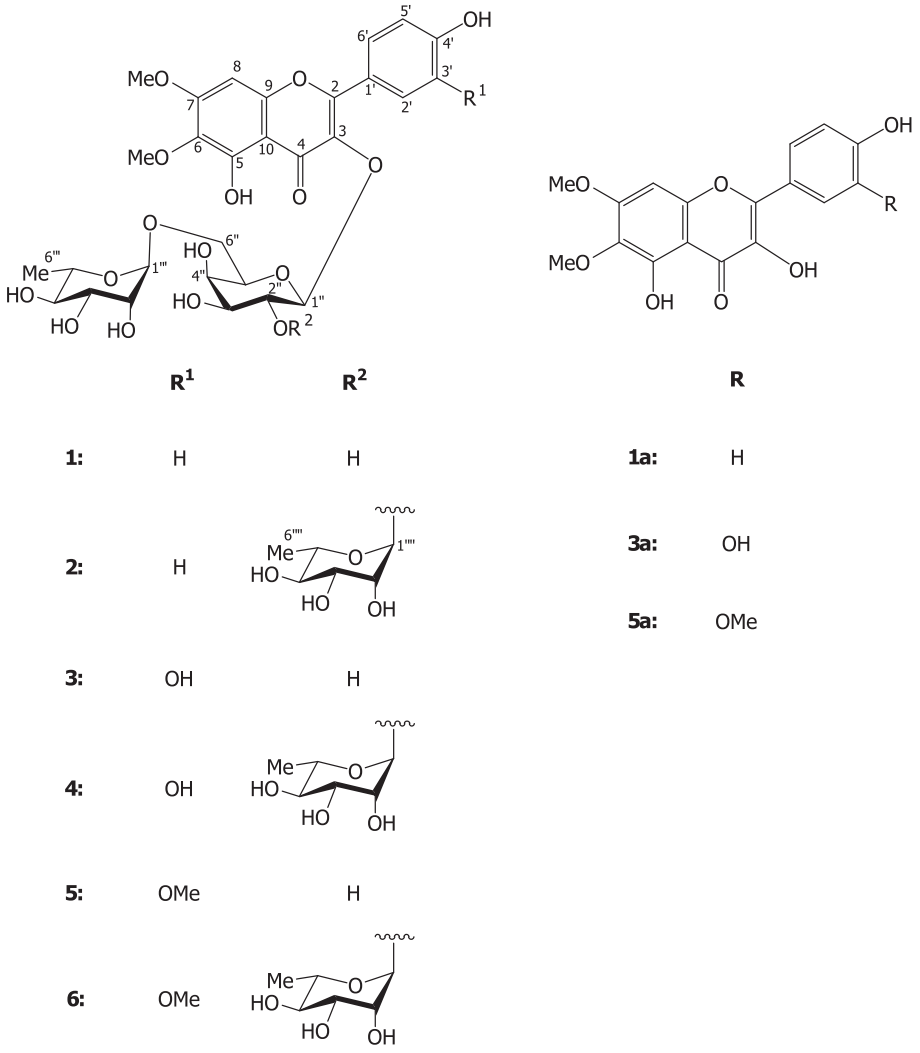

Fig. 1. Structures of sesuviosides A–F (**1**–**6**) and their aglycones **1a**, **3a**, and **5a**.

(HRAPCI-TOF) mass spectrometric analysis. The ^{1}H NMR spectrum (Table 1) revealed the presence of a *para*-disubstituted aromatic ring from the chemical shifts at δ_H 6.87 and 8.10 (each 2H, d, J=8.7 Hz), a downfield singlet signal at δ_H 6.86 (s), and two methoxyl singlet signals at δ_H 3.73 and 3.92 (each 3H) for the aglycone moiety, in addition to two anomeric protons of sugar moieties at δ_H 5.35 (d, J=7.7 Hz) and 4.39 (br s). One sugar unit was suggested to be rhamnose based on the characteristic methyl doublet signal observed at δ_H 1.05 (H_3-6‴). In the ^{13}C NMR spectrum (Table 2), 12 signals belonging to the sugar part could be identified as α-rhamnopyranosyl-(1→6)-β-galactopyranosyl unit (robinobioside) by comparing chemical shifts with the reported data.[8] The methyl signals at δ_C 56.5 and 60.1 were assignable to two methoxyl groups. The remaining 15 carbon atoms were consistent with a flavonol skeleton, having a carbonyl carbon atom at δ_C 177.8 (C-4), a *para*-disubstituted aromatic ring, and a penta-substituted aromatic ring due to the appearance of only one methine carbon at δ_C 91.4, in accordance with C-8 of a flavonoid skeleton (Table 2).[9] In the ^{1}H NMR spectrum (measured in DMSO-d_6), a broad signal at δ_H 12.55 (br s) was observed, which was assigned as the chelated hydroxyl group located at C-5 of the flavonoid. The upfield shift observed for the methine signal at δ_C 91.4 suggested that a methoxyl group (δ_C 56.5) rather than a hydroxyl group should be connected to the neighboring carbon atom (C-7), whereas the second methoxyl group was found to be attached to C-6 on the basis of its downfield shift at δ_C 60.1.[10] This was confirmed by the respective HMBC correlations (Fig. 2). From these spectral data, the aglycone of this compound was established as 3,5,4′-trihydroxy-6,7-dimethoxyflavone, which was also consistent with the COSY, HMQC, and HMBC experiments (Fig. 2). The attachment of the galactose moiety to C-3 of the aglycone was evident from the HMBC correlation of its anomeric proton (H-1″) to C-3. Rhamnose was the terminal sugar attached to C-6″ of galactose based on the correlation between its anomeric proton (H-1‴) and C-6″. Moreover, acid hydrolysis provided further confirmation of the aglycone moiety as 3,5,4′-trihydroxy-6,7-dimethoxyflavone (**1a**, eupalitin),[11] and the absolute configurations of galactose and rhamnose were determined to be D and L, respectively, by comparison of their optical rotations with those of authentic samples (see Experimental section). Accordingly, compound **1** was identified as 3,5,4′-trihydroxy-6,7-dimethoxyflavone 3-*O*-robinobioside, representing a new natural product for which the name sesuvioside A is suggested.

Sesuvioside B (**2**) was obtained as a yellow amorphous powder, and its molecular formula was determined as $C_{35}H_{44}O_{20}$ by HRAPCI-TOF MS. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) were very similar to those of sesuvioside A (**1**), except for an additional set of signals arising from a second α-rhamnopyranosyl unit. This additional sugar was assigned to be attached at C-2″ (δ_C 75.0) of the β-galactopyranosyl moiety due to the downfield shift of C-2″ by 3.9 ppm and the upfield shift of C-1″ by 2.9 ppm in comparison to

Table 1
^{1}H NMR spectroscopic data of sesuviosides A–F (**1**–**6**) (measured in DMSO-d_6, 400 MHz)

Position	**1**	**2**	**3**
8	6.86 (s)	6.83 (s)	6.84 (s)
2′/6′	8.10 (d, *J*=8.7 Hz)	8.08 (d, *J*=8.4 Hz)	
3′/5′	6.87 (d, *J*=8.7 Hz)	6.86 (d, *J*=8.4 Hz)	
2′			7.59 (d, *J*=1.7 Hz)
5′			6.83 (d, *J*=8.4 Hz)
6′			7.68 (dd, *J*=8.4, 1.7 Hz)
5-OH	12.55 (br s)	12.60 (br s)	12.60 (br s)
6-OCH$_3$	3.73 (3H, s)	3.72 (3H, s)	3.73 (s)
7-OCH$_3$	3.92 (3H, s)	3.89 (3H, s)	3.92 (s)
Gal-1″	5.35 (d, *J*=7.7 Hz)	5.56 (d, *J*=7.7 Hz)	5.35 (d, *J*=7.7 Hz)
2″	3.34[a]	3.78 (dd, *J*=9.7, 7.7 Hz)	3.33[a]
3″	3.41[a]	3.57[a]	3.43[a]
4″	3.62[a]	3.59[a]	3.62[a]
5″	3.60[a]	3.62[a]	3.58[a]
6″	3.24 (dd, *J*=10.9, 4.5 Hz)	3.24[a]	3.26 (dd, *J*=10.9, 3.2 Hz)
	3.56 (br d, *J*=10.9 Hz)	3.57[a]	3.57 (br d, *J*=10.9 Hz)
Rha-1‴	4.39 (br s)	4.34 (br s)	4.40 (br s)
2‴	3.37[a]	3.32[a]	3.39[a]
3‴	3.29 (dd, *J*=9.3, 3.2 Hz)	3.26 (dd, *J*=9.3, 3.2 Hz)	3.28 (dd, *J*=9.3, 2.8 Hz)
4‴	3.09 (dd, *J*=9.3, 9.3 Hz)	3.06 (dd, *J*=9.3, 9.3 Hz)	3.09 (dd, *J*=9.3, 9.3 Hz)
5‴	3.60[a]	3.31[a]	3.62[a]
6‴	1.05 (3H, d, *J*=6.1 Hz)	1.05 (3H, d, *J*=6.1 Hz)	1.06 (3H, d, *J*=6.1 Hz)
Rha-1⁗		5.05 (br s)	
2⁗		3.32[a]	
3⁗		3.50 (dd, *J*=9.4, 3.0 Hz)	
4⁗		3.14 (dd, *J*=9.4, 9.4 Hz)	
5⁗		3.75[a]	
6⁗		0.79 (3H, d, *J*=6.1 Hz)	

Position	**4**	**5**	**6**
8	6.82 (s)	6.86 (s)	6.86 (s)
2′	7.56 (br s)	8.01 (d, *J*=1.7 Hz)	8.05 (br s)
5′	6.81 (d, *J*=8.3 Hz)	6.90 (d, *J*=8.3 Hz)	6.90 (d, *J*=8.3 Hz)
6′	7.71 (br d, *J*=8.3 Hz)	7.56 (dd, *J*=8.3, 1.7 Hz)	7.57 (br d, *J*=8.3 Hz)
5-OH	12.60 (br s)	12.56 (br s)	12.60 (br s)
6-OCH$_3$	3.72 (3H, s)	3.72 (3H, s)	3.73 (3H, s)
7-OCH$_3$	3.91 (3H, s)	3.90 (3H, s)	3.91 (3H, s)
3′-OCH$_3$		3.85 (3H, s)	3.89 (3H, s)
Gal-1″	5.58 (d, *J*=7.7 Hz)	5.46 (d, *J*=7.7 Hz)	5.69 (d, *J*=7.7 Hz)
2″	3.83 (dd, *J*=9.4, 7.7 Hz)	3.32[a]	3.82 (dd, *J*=9.4, 7.7 Hz)
3″	3.57[a]	3.43 (dd, *J*=9.4, 3.3 Hz)	3.61[a]
4″	3.59[a]	3.62[a]	3.61[a]
5″	3.61[a]	3.60[a]	3.63[a]
6″	3.28[a]	3.34[a]	3.32[a]
	3.57[a]	3.60[a]	3.59[a]
Rha-1‴	4.37 (br s)	4.41 (br s)	4.39 (br s)
2‴	3.34[a]	3.37[a]	3.34[a]
3‴	3.27[a]	3.29 (dd, *J*=9.3, 3.0 Hz)	3.28[a]
4‴	3.10 (dd, *J*=9.3, 9.3 Hz)	3.07 (dd, *J*=9.3, 9.3 Hz)	3.07 (dd, *J*=9.6, 9.3 Hz)
5‴	3.34[a]	3.60[a]	3.31[a]
6‴	1.04 (3H, d, *J*=5.8 Hz)	1.04 (3H, d, *J*=6.1 Hz)	1.04 (3H, d, *J*=6.2 Hz)
Rha-1⁗	5.06 (br s)		5.00 (br s)
2⁗	3.34[a]		3.34[a]
3⁗	3.46[a]		3.49[a]
4⁗	3.14 (dd, *J*=9.3, 9.0 Hz)		3.12 (dd, *J*=9.6, 9.4 Hz)
5⁗	3.75[a]		3.76[a]
6⁗	0.82 (3H, d, *J*=5.6 Hz)		0.72 (3H, d, *J*=6.1 Hz)

[a] Chemical shifts assigned from COSY and HMQC spectra.

Table 2
^{13}C NMR spectroscopic data of sesuviosides A–F (**1**–**6**) (measured in DMSO-d_6, 100 MHz)

Position	**1**	**2**	**3**	**4**	**5**	**6**
2	157.0	156.9	157.0	156.9	157.0	156.7
3	133.3	132.8	133.6	132.8	133.2	132.7
4	177.8	177.7	177.8	177.5	177.7	177.6
5	151.9[a]	151.9[a]	151.9[a]	151.8	152.0[a]	151.9[a]
6	131.7	131.8	131.8	131.7	131.8	131.8
7	158.7	158.7	158.8	158.6	158.8	158.8
8	91.4	91.4	91.4	91.2	91.5	91.4
9	151.7[a]	151.8[a]	151.8[a]	151.8	151.8[a]	151.8[a]
10	105.3	105.4	105.4	105.3	105.4	105.4
1′	120.7	120.9	121.1	120.5	120.8	121.0
2′	131.1	131.0	116.3	115.8	113.5	113.5
3′	115.2	115.2	145.1	145.3	147.2	147.1
4′	160.3	160.2	148.9	149.5	150.1	149.7
5′	115.2	115.2	115.4	115.3	115.3	115.2
6′	131.1	131.0	122.1	122.2	122.3	122.0
6-OMe	60.1	60.2	60.2	60.1	60.2	60.2
7-OMe	56.5	56.6	56.6	56.5	56.6	56.6
3′-OMe					56.0	56.0
Gal-1″	101.9	99.0	102.0	99.0	101.8	99.1
2″	71.1	75.0	71.3	75.0	71.2	75.3
3″	73.0	73.6[b]	73.2	73.5[b]	73.1	73.6[b]
4″	68.1	68.3	68.2	68.2	68.1	68.2
5″	73.7	73.9[b]	73.8	74.0[b]	73.8	73.7[b]
6″	65.4	65.4	65.4	65.2	65.4	65.3
Rha-1‴	100.1	100.2	100.2	100.1	100.2	100.2
2‴	70.5	70.5[c]	70.5	70.5[c]	70.5	70.5[c]
3‴	70.7	70.7[c]	70.8	70.7[c]	70.7	70.7[c]
4‴	71.9	72.0	72.1	72.0	72.0	72.0
5‴	68.3	68.4	68.4	68.3	68.4	68.4
6‴	17.9	18.0	18.0	18.0	18.0	18.0
Rha-1⁗		100.7		100.6		101.0
2⁗		70.7[c]		70.7[c]		70.8[c]
3⁗		70.8[c]		70.7[c]		70.8[c]
4⁗		72.0		72.0		71.9
5⁗		68.7		68.7		68.6
6⁗		17.4		17.3		17.2

[a–c] Assignments with the same superscript may be reversed.

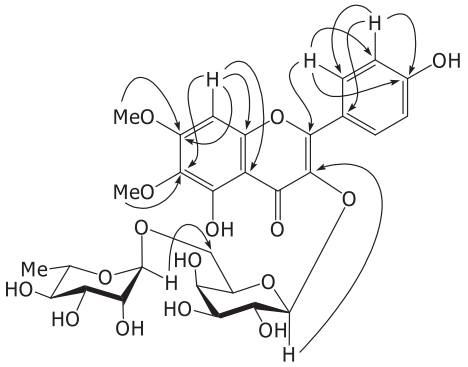

Fig. 2. Significant HMBC correlations of sesuvioside A (**1**).

sesuvioside A (**1**). This assignment was supported by HMBC correlations observed between H-1″ and C-3, H-1‴ and C-6″, and H-1⁗ and C-2″. Therefore, the structure of compound **2** was determined to be 3,5,4′-trihydroxy-6,7-dimethoxyflavone 3-*O*-(2″-*O*-α-rhamnopyranosyl)-robinobioside.

Sesuvioside C (**3**) was isolated as a yellow amorphous powder, and its molecular formula was established as $C_{29}H_{34}O_{17}$ by HRAPCI-TOF MS. Inspection of the ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) indicated that this compound was closely related to sesuvioside A (**1**) differing by the presence of one additional oxygen atom. In addition, the ^{1}H NMR spectrum showed a set of resonances corresponding to an ABX aromatic ring system comprising signals at δ_H 6.83 (d, *J*=8.4 Hz), δ_H 7.59 (d, *J*=1.7 Hz), and δ_H 7.68 (dd, *J*=8.4, 1.7 Hz) instead of the AA′BB′ aromatic ring system of sesuvioside A

(**1**). Thus, sesuvioside C (**3**) was identified as the 3′-hydroxy derivative of sesuvioside A (**1**), which was confirmed by HMBC correlations between δ_H 7.59 (H-2′) and δ_C 157.0 (C-3) and δ_H 7.68 (H-6′) and δ_C 157.0 (C-3).

Sesuvioside D (**4**) was obtained as a yellow amorphous powder. Its molecular formula was determined as $C_{35}H_{44}O_{21}$ by HRAPCI-TOF MS. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) indicated that this compound had the same aglycone, 3,5,3′,4′-tetrahydroxy-6,7-dimethoxyflavone, as sesuvioside C (**3**), while the sugar moieties were identical to those of sesuvioside B (**2**). Therefore, this compound was elucidated as 3,5,3′,4′-tetrahydroxy-6,7-dimethoxyflavone 3-*O*-(2″-*O*-α-rhamnopyranosyl)-robinobioside.

Sesuvioside E (**5**) was isolated as a yellow amorphous powder with a molecular formula $C_{30}H_{36}O_{17}$ as determined by HRAPCI-TOF MS. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) indicated that this compound was an *O*-methylated derivative of sesuvioside C (**3**), as evident from the appearance of an additional methoxyl singlet signal at δ_H 3.85. This extra methoxyl group was suggested to be located on the B-ring since the chemical shifts of this ring were changed by the substituent effect.[10] A complete assignment was achieved by inspection of the HMBC spectrum (Fig. 3) and nOe difference experiments. Upon irradiation of the methoxyl signal at δ_H 3.85, the intensity of δ_H 8.01 (H-2′) was enhanced, indicating that this methoxyl group was linked to C-3′ (δ_C 147.5). Accordingly, sesuvioside E (**5**) was elucidated to be 3,5,4′-trihydroxy-6,7,3′-trimethoxyflavone 3-*O*-robinobioside.

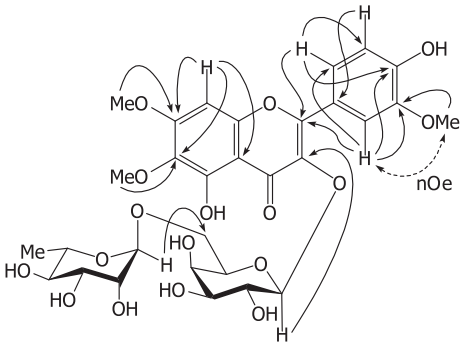

Fig. 3. Significant HMBC correlations and nOe difference experiments of sesuvioside E (**5**).

Sesuvioside F (**6**) was isolated as a yellow amorphous powder, and its molecular formula was determined as $C_{36}H_{46}O_{21}$ by HRAPCI-TOF MS. Inspection of the ^{1}H and ^{13}C NMR spectra (Tables 1 and 2), indicated that it was the 2″-*O*-α-rhamnopyranosyl derivative of sesuvioside E (**5**), since the chemical shifts of the sugar moieties were virtually identical to those of sesuviosides B (**2**) and D (**4**). Consequently, sesuvioside F (**6**) was established to be 3,5,4′-trihydroxy-6,7,3′-trimethoxyflavone 3-*O*-(2″-*O*-α-rhamnopyranosyl)-robinobioside.

The absolute stereochemistries of the sugar moieties in sesuviosides B–F (**2**–**6**) were not experimentally determined, but assumed to be identical to the ones in sesuvioside A (**1**), because they were derived from the same plant material.

Four known compounds were elucidated as ecdysterone,[12] adenosine,[13] 2′-*O*-methyl adenosine,[14] and L-tryptophan by comparison of theirs ^{1}H and ^{13}C NMR spectroscopic data.

2.2. Free radical scavenging activity

In this study, sesuviosides A–F (**1**–**6**) and their aglycones; 3,5,4′-trihydroxy-6,7-dimethoxyflavone (**1a**), 3,5,3′,4′-tetrahydroxy-6,7-dimethoxyflavone (**3a**), and 3,5,4′-trihydroxy-6,7,3′-trimethoxyflavone (**5a**) were evaluated for their radical scavenging activity using DPPH and ORAC assays.[15–17] The aglycones **1a**, **3a**, and **5a** were obtained by acid hydrolysis from sesuviosides A (**1**), C (**3**), and E (**5**), respectively (see Experimental section). In the DPPH assay, compounds **3**, **4**, **1a**, **3a**, and **5a** displayed scavenging activity with SC_{50} values of 13.1, 35.1, 20.4, 9.1, and 21.5 μM, respectively, comparable with the activity of ascorbic acid used as positive control, while compounds **1**, **2**, **5**, and **6** were inactive. In the ORAC assay, the unit values of all new compounds and their aglycones were about 2–6 fold more potent than the positive control, Trolox (Table 3).

Table 3
Radical scavenging activity of sesuviosides A–F (**1**–**6**) and their aglycones (**1a**, **3a**, and **6a**)

Compound	DPPH assay[a] (SC_{50}, μM)	ORAC[b] (ROO•, unit)
1	>250 (16%)[c]	6.1±0.4
2	206.8±6.4	6.2±0.6
3	13.1±4.1	3.1±0.6
4	35.1±2.1	2.8±0.4
5	>250 (24%)[c]	3.5±0.6
6	>250 (23%)[c]	3.1±0.6
1a	20.4±0.6	2.4±0.5
3a	9.1±0.6	2.5±0.5
5a	21.5±1.2	1.8±0.4
L-Ascorbic acid	21.2±1.4	—[d]
Trolox	—[d]	1

[a] SC_{50} is half-maximal scavenging concentration.
[b] 1 ORAC unit equals the net protection of fluorescein produced by 1 μM Trolox.
[c] Numbers in parentheses indicate the percentage of scavenging.
[d] Not determined.

3. Conclusion

S. portulacastrum is well known to contain ecdysterone and 3,5,4′-trihydroxy-6,7-dimethoxyflavone 3-*O*-glucoside as major constituents.[4–6] In the present study, ecdysterone was also isolated as a major constituent. This compound acts as an important role of insect molting hormone in the sericulture industry.[1] The occurrence of 3,5,4′-trihydroxy-6,7-dimethoxyflavone glycoside derivatives (**1**–**6**) from the plant source of Thailand is related to flavonoid compounds, isolated from the other plant sources.[4–6] The significant difference is found only in the sugar moiety. The presence of 3,5,4′-trihydroxy-6,7-dimethoxyflavone or its derivatives is important and expected to be a characteristic flavonoid from this plant, and may serve as useful for chemotaxonomic point of view. Furthermore, *S. portulacastrum* contains flavonoids exhibiting antioxidant properties, which could be classified as food additive for health beneficial effects.

4. Experimental section

4.1. General procedures

^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 using a Bruker AV-400 spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR). Mass spectra were obtained on a Bruker Micro TOF-LC mass spectrometer. Optical rotations were measured with a Jasco P-1020 digital polarimeter. For column chromatography, Diaion HP-20 (Mitsubishi Chemical Industries Co. Ltd.), silica gel 60 (70–230 mesh, Merck), and RP-18 (50 μm, YMC) were used. HPLC (Jasco PU-980 pump) was carried out on ODS columns (20×150 mm i.d., YMC, column A; and 21.2×250 mm i.d., Vertisep™ AQS, column B) with a Jasco UV-970 detector at 254 nm. The UV–vis and fluorescence measurements were performed using Spectramax 384 Plus with Softmax Pro 4.0 software (Molecular Devices; Sunnyvale, CA) and Spectramax GeminiXS with Softmax Pro 4.3.1 LS software (Molecular Devices; Sunnyvale, CA), respectively. 96-Well microplates were purchased from Greiner (Frickenhausen, Germany).

For HPLC analysis, the flow rates were 6 mL/min for column A and 8 mL/min for column B. The solvent systems were: (I) EtOAc–MeOH

(9:1); (II) EtOAc–MeOH–H_2O (40:10:1); (III) EtOAc–MeOH–H_2O (70:30:3); (IV) EtOAc–MeOH–H_2O (6:4:1); (V) 10–80% aqueous MeOH; (VI) 5% aqueous MeCN; and (VII) 20% aqueous MeCN. The spraying reagent used for TLC was 10% H_2SO_4 in 50% EtOH.

4.2. Plant material

The aerial portion of *S. portulacastrum* (L.) L. was collected from southern coastal area in November 2007, Pattani province, Thailand. The identification of the plant was done by Mr. Nopporn Nontapa of Department of Pharmaceutical Botany and Pharmacognosy, Faculty of Pharmaceutical Sciences, Khon Kaen University. A voucher specimen (TK-PSKKU-0062) is on file in the Herbarium of the Faculty of Pharmaceutical Sciences, Khon Kaen University.

4.3. Extraction and isolation

The aerial portion of *S. portulacastrum* (2.4 kg) was macerated three times with MeOH (12 L for each extraction) at room temperature. The MeOH extract was concentrated in vacuo to dryness. This residue (556.0 g) was suspended in H_2O, and partitioned with Et_2O (each 1.0 L, three times). The aqueous soluble fraction (345.4 g) was subjected to a Diaion HP-20 column, and eluted with H_2O, MeOH, and $(CH_3)_2CO$, successively. The fraction eluted with MeOH (29.7 g) was subjected to a silica gel column using solvent systems I (4.0 L), II (4.0 L), III (6.0 L), and IV (12.0 L) affording six fractions (fractions A–F), monitored by TLC. Fraction B (3.2 g), which showed the presence of the one major compound, was applied to an RP-18 column using solvent system V to give ecdysterone (1.8 g) by precipitation. Fraction C (4.7 g) was subjected to an RP-18 column using solvent system V, providing five fractions (C-1 to C-5). Fraction C-3 was purified by preparative HPLC-ODS (column A) with solvent system V to afford compounds **1** (1.3 g), **3** (56 mg), and **5** (77 mg). Fraction E (5.5 g) was repeatedly separated on an RP-18 column using solvent system V, giving seven fractions (E-1 to E-7). Fraction E-7 was further purified by preparative HPLC-ODS (column B) with solvent system VII to afford compounds **2** (236 mg), **4** (58 mg), and **6** (192 mg).

*4.3.1. Sesuvioside A (**1**).* Yellow amorphous powder, $[\alpha]_D^{27}$ −27.0 (H_2O, *c* 1.14); ^{1}H NMR (DMSO-d_6): Table 1 and ^{13}C NMR (DMSO-d_6): Table 2; negative HRMS (APCI-TOF): $[M+Cl]^-$, found 673.1531. $C_{29}H_{34}ClO_{16}$ requires 673.1541.

*4.3.2. Sesuvioside B (**2**).* Yellow amorphous powder, $[\alpha]_D^{26}$ −40.6 (H_2O, *c* 1.26); ^{1}H NMR (DMSO-d_6): Table 1 and ^{13}C NMR (DMSO-d_6): Table 2; negative HRMS (APCI-TOF): $[M+Cl]^-$, found 819.2116. $C_{35}H_{44}ClO_{20}$ requires 819.2120.

*4.3.3. Sesuvioside C (**3**).* Yellow amorphous powder, $[\alpha]_D^{26}$ −18.7 (H_2O, *c* 1.06); ^{1}H NMR (DMSO-d_6): Table 1 and ^{13}C NMR (DMSO-d_6): Table 2; negative HRMS (APCI-TOF): $[M+Cl]^-$, found 689.1473. $C_{29}H_{34}ClO_{17}$ requires 689.1490.

*4.3.4. Sesuvioside D (**4**).* Yellow amorphous powder, $[\alpha]_D^{26}$ −31.0 (H_2O, *c* 1.00); ^{1}H NMR (DMSO-d_6): Table 1 and ^{13}C NMR (DMSO-d_6): Table 2; negative HRMS (APCI-TOF): $[M+Cl]^-$, found 835.2084. $C_{35}H_{44}ClO_{21}$ requires 835.2069.

*4.3.5. Sesuvioside E (**5**).* Yellow amorphous powder, $[\alpha]_D^{27}$ −44.4 (MeOH, *c* 0.22); ^{1}H NMR (DMSO-d_6): Table 1 and ^{13}C NMR (DMSO-d_6): Table 2; negative HRMS (APCI-TOF): $[M+Cl]^-$, found 703.1641. $C_{30}H_{36}ClO_{17}$ requires 703.1647.

*4.3.6. Sesuvioside F (**6**).* Yellow amorphous powder, $[\alpha]_D^{26}$ −28.5 (MeOH, *c* 1.16); ^{1}H NMR (DMSO-d_6): Table 1 and ^{13}C NMR (DMSO-d_6): Table 2; negative HRMS (APCI-TOF): $[M+Cl]^-$, found 849.2259. $C_{36}H_{46}ClO_{21}$ requires 849.2226.

*4.3.7. Acid hydrolysis of sesuvioside A (**1**).* A solution of sesuvioside A (149 mg) in 1,4-dioxane (0.5 mL) and 2 M HCl (4.5 mL) was heated at 80 °C for 4 h. After cooling, H_2O (5 mL) was added and neutralized with 2 M KOH. The reaction was extracted with EtOAc (30 mL×3) and the combined organic part was concentrated in vacuo to yield an aglycone **1a** (69.5 mg) after re-crystallization from MeOH. The structure of **1a** was identified to be 3,5,4′-trihydroxy-6,7-dimethoxyflavone (eupalitin) by NMR spectral analysis.

The aqueous layer was concentrated to dryness, providing the sugar fraction. This fraction was subjected to a silica gel column and eluted with increasing polarity mixtures of EtOAc–MeOH as solvent system to give L-rhamnose (25.2 mg, $[\alpha]_D^{26}$ +5.3; H_2O, *c* 1.26) and D-galactose (23.9 mg, $[\alpha]_D^{27}$ +65.7; H_2O, *c* 1.20) in comparison with authentic samples. Aglycones of sesuvioside B–F, compounds **3a** and **5a**, were also obtained in an analogous manner.

*4.3.8. 3,5,4′-Trihydroxy-6,7-dimethoxyflavone (eupalitin, **1a**).* Yellow amorphous powder; ^{1}H NMR (DMSO-d_6): δ_H 12.42 (1H, br s, 5-OH), 8.08 (2H, d, *J*=8.9 Hz, H-2′/6′), 6.93 (2H, d, *J*=8.9 Hz, H-3′/5′), 6.83 (1H, s, H-8), 3.90 (3H, s, 7-OMe), 3.73 (3H, s, 6-OMe); ^{13}C NMR (DMSO-d_6): δ_C 176.1 (C-4), 159.3 (C-4′), 158.5 (C-7), 151.5 (C-5), 151.0 (C-9), 147.3 (C-2), 135.8 (C-3), 131.2 (C-6), 129.6 (C-2′/6′), 121.6 (C-1′), 115.4 (C-3′/5′), 104.3 (C-10), 91.1 (C-8), 60.1 (6-OMe), 56.4 (7-OMe).

*4.3.9. 3,5,3′,4′-Tetrahydroxy-6,7-dimethoxyflavone (eupatolitin, **3a**).* Yellow amorphous powder; ^{1}H NMR (DMSO-d_6): δ_H 12.44 (1H, br s, 5-OH), 7.72 (1H, d, *J*=1.9 Hz, H-2′), 7.57 (1H, dd, *J*=8.5, 1.9 Hz, H-6′), 6.89 (1H, d, *J*=8.5 Hz, H-5′), 6.81 (1H, s, H-8), 3.90 (3H, s, 7-OMe), 3.72 (3H, s, 6-OMe); ^{13}C NMR (DMSO-d_6): δ_C 176.1 (C-4), 158.5 (C-7), 151.5 (C-5), 151.1 (C-9), 147.8 (C-4′), 147.4 (C-2), 145.1 (C-3′), 135.8 (C-3), 131.2 (C-6), 121.9 (C-6′), 120.0 (C-1′), 115.6 (C-2′), 115.3 (C-5′), 104.3 (C-10), 91.1 (C-8), 60.1 (6-OMe), 56.4 (7-OMe).

*4.3.10. 3,5,4′-Trihydroxy-6,7,3′-trimethoxyflavone (**5a**).* Yellow amorphous powder; ^{1}H NMR (DMSO-d_6): δ_H 12.43 (1H, br s, 5-OH), 7.79 (1H, d, *J*=1.9 Hz, H-2′), 7.75 (1H, dd, *J*=8.5, 1.9 Hz, H-6′), 6.94 (1H, d, *J*=8.5 Hz, H-5′), 6.90 (1H, s, H-8), 3.92 (3H, s, 7-OMe), 3.86 (3H, s, 3′-OMe), 3.74 (3H, s, 6-OMe); ^{13}C NMR (DMSO-d_6): δ_C 176.1 (C-4), 158.6 (C-7), 151.6 (C-5), 151.0 (C-9), 149.0 (C-4′), 147.4 (C-2), 147.1 (C-3′), 136.0 (C-3), 131.3 (C-6), 121.9 (C-6′), 121.9 (C-1′), 115.5 (C-5′), 111.7 (C-2′), 104.3 (C-10), 91.3 (C-8), 60.1 (6-OMe), 56.4 (7-OMe), 55.9 (3′-OMe).

4.4. Assay for radical scavenging activity

4.4.1. Scavenging of diphenyl-picrylhydrazyl (DPPH) radicals. This assay was performed using a previously reported method with some minor modifications.[15,16] The mixtures containing test samples (in DMSO, 5 μL) and DPPH ethanolic solution (100 μM, 195 μL) were allowed to react in a 96-well microplate. The plate was incubated at 37 °C for 30 min. Reduction of the DPPH free radical was measured by the absorbance at 515 nm using a UV–vis microplate reader. L-Ascorbic acid (10 mM) was used as a positive control. The scavenging activity was expressed in terms of the concentration of samples, which scavenged free radical by 50% (SC_{50}).

4.4.2. Measurement of oxygen radical absorbance capacity (ORAC). The peroxyl radical absorbance capacity of test samples was determined using a previously reported method.[16,17] The reaction mixture containing fluorescein solution (7×10^{-5} mM, 175 μL) in phosphate buffer (75 mM, pH 7.4) was added to either test sample (10 μL) or DMSO (10 μL) (as a blank) diluted in phosphate buffer and pre-incubated at 37 °C for 10 min. The reaction was initiated by addition of 2,2′-azobis-(2-amidinopropane) dihydrochloride

(AAPH) (255 mM, 15 μL). Changes in intensity of the fluorescent probe caused by free radicals was then monitored at 37 °C every 2 min for 1 h by using a fluorescent microplate reader at wavelengths of 485 and 530 nm. Trolox (20 μM, 10 μL) was used as a standard with phosphate buffer as a blank. The relative ORAC unit was calculated using the following equation.

$$\text{Relative ORAC unit} = \left[\left(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}\right) \Big/ \text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{blank}}\right]$$

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