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The effects of different extraction solvents of varying polarities on polyphenols of *Orthosiphon stamineus* and evaluation of the free radical-scavenging activity

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Abstract

Leaf powder of *Orthosiphon stamineus* was extracted with the following solvents; distilled water, 50% aqueous methanol, methanol, 70% aqueous acetone and chloroform, at 2, 4 and 8 h, respectively, on a water bath at 40 °C. The extracts were subjected to qualitative and quantitative HPLC analyses of the polyphenols, the most dominant chemical constituents in the leaf. Chloroform extraction from 4 to 8 h at 40 °C gave the highest amount of sinensetin and eupatorin. The extraction with 70% aqueous acetone extracts at 4 and 8 h gave a high yield of 3′-hydroxy-5,6,7,4′-tetramethoxyflavone. The yield of rosmarinic acid was high in 50% methanol extracts at 2, 4 and 8 h of extraction. The extracts were screened for free radical-scavenging potential, using a 1,1-diphenyl-2-picrylhydrazyl in vitro model system. The extracts exhibited significant radical-scavenging activity and the acetone extracts showed the highest activity.
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Keywords: HPLC; Lipophilic flavones; *Orthosiphon stamineus*; Rosmarinic acid

1. Introduction

Orthosiphon stamineus (OS), Benth, (Lamiaceae) is used in Southeast Asia, for the treatment of eruptive fever, epilepsy, gallstone, hepatitis, rheumatism, hypertension, syphilis and renal calculus. In Malaysia, the tea prepared from the leaves is taken as beverage to improve health and for treatment of kidney, bladder inflammation, gout and diabetes (Hegnauer, 1966; Wangner, 1982). Lipophilic flavonoids isolated from OS showed radical-scavenging activity towards the diphenylpicrylhydrazyl radical and inhibition of 15-lipoxygenase from soybeans used as a model for mammalian 15-lipoxygenase (Lyckander & Malterud, 1996). Research indicates that the flavones, sinensetin and 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone isolated from OS exhibited a diuretic activity in rats after intravenous administration of 10 mg/kg body weight and, therefore, the diuretic effect of OS extracts could be partially due to its lipophilic flavones content (Schut & Zwaving, 1993).

O. stamineus contains several chemically active constituents, such as terpenoids (diterpenes and triterpenes), polyphenols (lipophilic flavonoids and phenolic acids), and sterols (Tezuka et al., 2000). The therapeutic effects of OS have been ascribed mainly to its polyphenol, the most dominant constituent in the leaf, which has been reported to be effective in reducing oxidative stress by inhibiting the formation of lipid peroxidation products in biological systems (Hollman & Katan, 1999). Caffeic acid derivatives, including rosmarinic and 2,3-dicaffeoyltartaric acids, are the most abundant polyphenols in an aqueous methanol extract of OS leaf, which predominate over polymethoxylated flavones. The polymethoxylated

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flavones, principal flavonoid aglycones, present in OS leaf are unique, with a methoxy group at C-5, a structural feature rare in flavonoids.

The stability of different extracts from the same material depends on the extraction solvent used for removal of the polyphenolic compounds, and it is apparent that extracts from the same plant material may vary widely with respect to their antioxidant concentrations and activities. Therefore, the aim of this study was to identify the effect of different extraction solvents of varying polarities on three main polymethoxylated flavones in *O. stamineus* leaves, sinensetin (SEN), eupatorin (EUP), 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF) and the major phenolic acid, rosmarinic acid (RA) and scavenging effects of these extracts on free radical species.

2. Materials and methods

2.1. Chemicals and reagents

Standard compounds of sinensetin, eupatorin, 3′-hydroxy-5,6,7,4′-tetramethoxyflavone and rosmarinic acid were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). Solvents used for chromatography were tetrahydrofuran, methanol (HPLC grade), water (HPLC grade), ethanol (80%) obtained from Merck (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO). Membrane filters (0.45-μm pore size) from Millipore were used for filtration of the samples. All other chemicals were of analytical grade or HPLC grade.

2.2. Plant materials

Plants were grown from cuttings using standard agronomic practices at Bumbung Lima (Penang State, Malaysia). The leaves were collected in the late afternoon, from 30–45-day-old white-flowered plants. Specimen was labelled, numbered and annotated with the date of collection and deposited at Bilik Herba, School of Pharmaceutical Sciences, Universiti Sains Malaysia.

2.3. Extraction and HPLC analysis

Ten grammes of leaf powder of the plant were extracted with 100 ml of the following solvents; water, methanol, 50% aqueous methanol, 70% aqueous acetone, and chloroform, at 2, 4, and 8 h, respectively, at 40 °C on a water bath. The extracts were subjected to qualitative and quantitative HPLC analysis. HPLC analysis was performed using an Agilent Technologies 1100 system equipped with an automatic injector, a column oven, and a UV detector. A LiChrosorb RP-18 column (250 × 4.6 i.d. mm, 5 μm particle size) (Merck Darmstadt, Germany) was used. The temperature was maintained at 25 °C, with injection volume of 20 μl and flow rate of 1 ml/min. All the markers were separated with methanol–water–tetrahydrofuran (45:50:5 v/v) as mobile phase. The peaks were detected at 340 nm and identified by standard substances. The following reference compounds were used as markers: RA, SEN, EUP and TMF. The external standard method was used for the HPLC quantification. The results are reported as percent of dry leaf weight.

2.4. Free radical-scavenging activity of extracts using DPPH assay

The method for estimating free radical-scavenging activity of the extracts of OS was adapted from that of Hatano, Kagawa, Yasuhara, & Okuda (1988) with some modifications. Two ml of a methanolic solution of DPPH (0.1 mM) were mixed with 200 μl of samples of OS extract (0.05 mg/ml), made up with methanol to a final volume of 3 ml. After 60 min standing, the absorbance of the mixture was measured at 517 nm against methanol as blank using a Perkin–Elmer Lambda 45 spectrophotometer. Free radical-scavenging activity of reference compounds, quercetin and butylated hydroxylanisole (BHA) (0.05 mg/ml), and markers, RA, EUP, SEN and TMF (0.05 mg/ml) were also determined using the same procedure. The radical-scavenging activities (%) of the tested samples, were evaluated by comparison with a control (2 ml DPPH solution and 1 ml of methanol). Each sample was measured in triplicate and averaged. The free radical-scavenging activity (FRSA) was calculated using the formula:

$$\mathrm{FRSA} = [(A_c - A_s)/A_c] \times 100,$$

where A_c is the absorbance of the control and A_s is the absorbance of the tested sample after 60 min.

2.5. Statistical analysis

Experimental results were means of three parallel measurements and analysed by SPSS 10 (SPSS Inc. Chicago, IL). Differences between means were determined using Tukey multiple comparisons. Correlations were obtained by Pearson correlation coefficient in bivariate correlations. P values < 0.05 were regarded as significant.

3. Results and discussion

3.1. HPLC analysis

In the present study, all the markers (Fig. 1) were determined in a single run of HPLC by using the isocratic condition of methanol:water:tetrahydrofuran

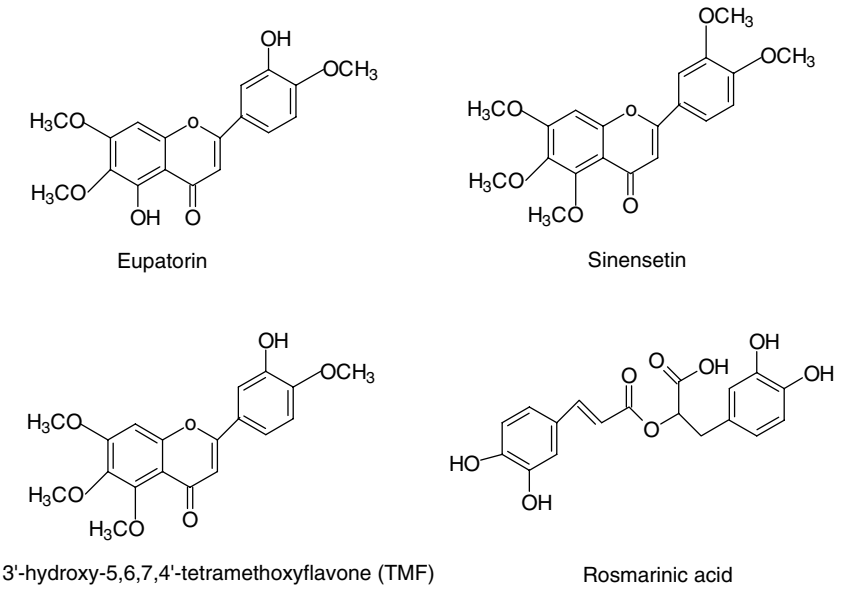

Fig. 1. Chemical structures of markers.

(45:50:5 v/v). The markers were separated within a total time of 30 min (Fig. 2). The peaks of RA, EUP, TMS and SEN were confirmed by comparison of their retention times with reference standards. The standards were resolved and eluted at 5.6, 13.3, 17.8 and 25.9 min with respect to RA, TMF, SEN and EUP (Fig. 2). The markers were shown to exhibit good linearity in the range from 2.0 to 1000 ng in the calibration curves that were obtained by HPLC analysis.

To assess the precision of the method, standard solutions of RA, TMF, EUP and SEN were determined, six times on the same day and one time, for five consecutive days. The results showed very good precision, ranging from 5 to 100 μg/ml. The accuracy of the method was evaluated through recovery studies by adding known amounts of the standard solutions to the extracts. Controls from all samples were prepared and analysed. The recovery experiment was performed at three concentrations of the standard added to sample solutions of which the marker content had been determined. The results for the recoveries of RA, TMF, SEN and EUP were in the range of 96–103%. The limits of detection (LOD), established at signal three times the noise, for SEN, EUP, TMF and RA, were 2.0, 2.5, 2.0 and 2.0 ng, respectively.

The HPLC procedure was applied to the determination of the concentration of the markers in OS extracts of different solvents of varying polarities. The quantitative analysis was performed under isocratic conditions using the external standard technique. The assay gave a separation sufficient for peak identification of the compounds of interest. As shown in Table 1, all the different solvent extraction systems showed a wide range in the concentrations of the markers. The amount of RA, the principal polyphenol in OS leaf (Akowuah, Zhari, Norhayati, Sadikun, & Khamsah, 2004) and the most polar compound among the markers, was higher in 50% aqueous methanol extract and the amount was significantly different from the other solvent extracts ($P < 0.05$) at 2, 4 and 8 h (Table 1). The order of increasing amount of RA, in different solvent extraction systems was 50% aqueous methanol > 100% methanol > 70% acetone > water > $CHCl_3$.

From our results, the $CHCl_3$ extracts gave the highest amount of EUP and SEN at 4 and 8 h of hot extraction. There was a significant difference ($P < 0.05$) of the amount of SNS and EUP in $CHCl_3$ extracts compared with the amounts in water, methanol, 50% aqueous methanol and 70% acetone extracts. TMF concentration was higher in 70% aqueous acetone extracts at 2, 4 and 8 h of extraction but the amount was not significantly different ($P > 0.05$) when compared to methanol and chloroform extracts. The *o*-methyl groups in SEN, EUP and TMF (Fig. 1) render them lipophilic. Among the various solvents used for the extraction, chloroform has the lowest polarity, therefore, the amounts of lipiphilic markers were higher, as expected. The content of the most polar marker, RA, was higher in the polar solvents but it was not detected in the chloroform extracts by our assay.

3.2. Free radical-scavenging activity of extracts using DPPH assay

The purple-coloured DPPH is a stable free radical, which is reduced to α,α-diphenyl-β-picrylhydrazine (yellow coloured) by reacting with an antioxidant. Antioxidants interrupt free radical chain oxidation by donating hydrogen from hydroxyl groups to form a stable end-product, which does not initiate or propagate further oxidation of lipids (Sherwin, 1978). The results of the

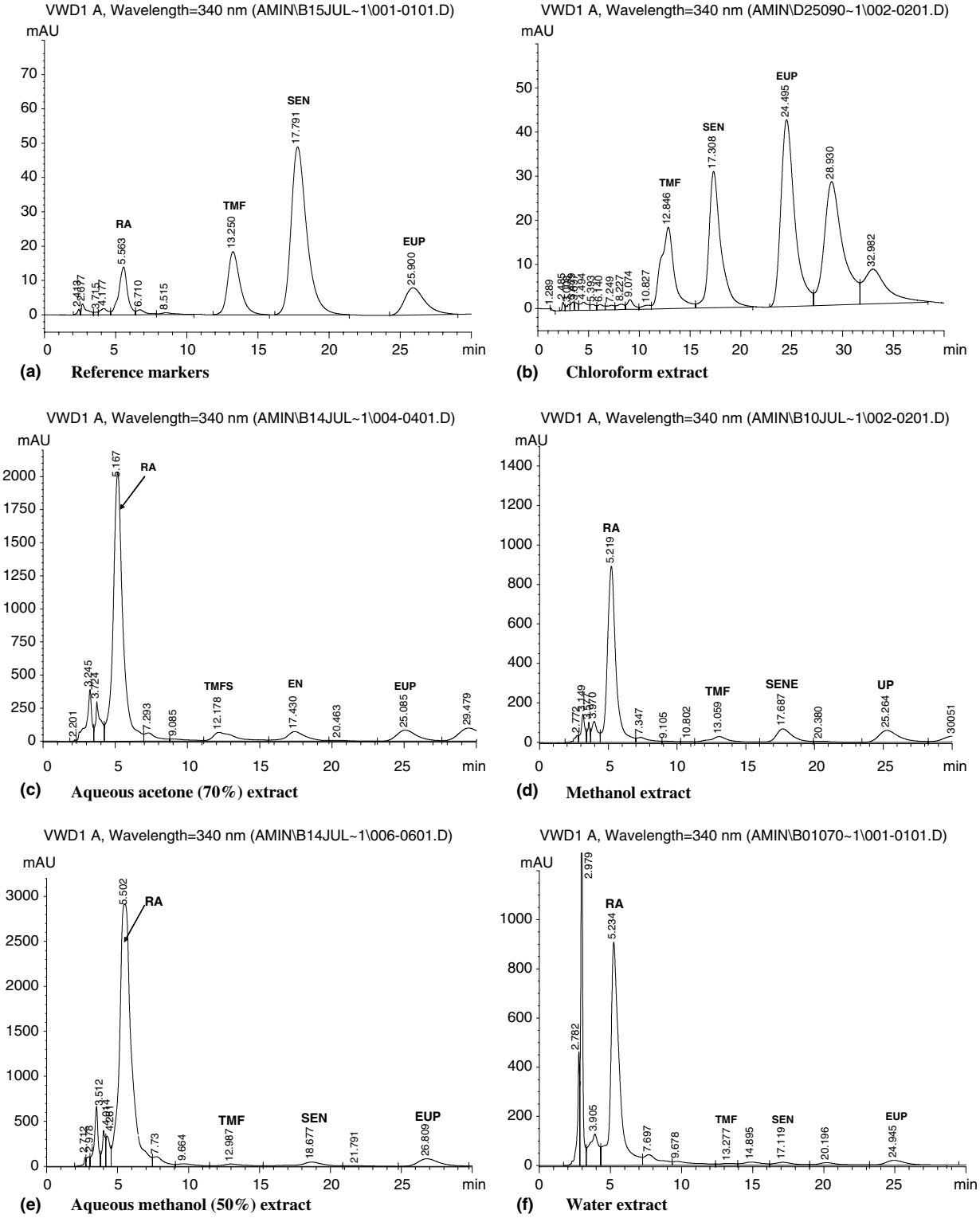

Fig. 2. HPLC chromatogram of the *Orthosiphon stamineus* leaf extracts using different solvents of varying polarities: (a) reference markers; (b) chloroform extract; (c) aqueous acetone (70%) extract; (d) methanol extract; (e) aqueous methanol (50%) extract; (f) water extract.

free radical-scavenging activity of OS extracts, using solvents of different polarities at different extraction times, are shown in Fig. 3. All extracts demonstrated a significant inhibitory activity against the DPPH radical at a final concentration of 0.05 mg/ml. The data obtained showed that the water, 50% aqueous methanol, methanol and 70% aqueous acetone extracts of OS exhibited high free radical-scavenging activity. The activities obtained for these solvent systems were not significantly different ($P = 0.5$) at 2, 4 and 8 h of extraction; however,

Table 1
Percent concentrations of marker phenolics in leaf extracts of *Orthosiphon stamineus leaf* at 2, 4 and 8 h of extraction

Marker	Extraction time (h)	Water	Methanol	Aq. methanol (50%)	Aq. acetone (70%)	Chloroform
RA	2	0.540c	0.541c	0.903a	0.735b	ND
	4	0.661c	0.648c	0.978a	0.743b	ND
	8	0.671c	0.799b	0.954a	0.772b	ND
TMF	2	0.002b	0.017a	0.003b	0.011a	0.005ab
	4	0.003b	0.010a	0.003b	0.011a	0.006ab
	8	0.003b	0.009a	0.003b	0.011a	0.006ab
SEN	2	0.030c	0.067b	0.029c	0.098b	0.365a
	4	0.033c	0.080b	0.030c	0.097b	0.433a
	8	0.030c	0.079b	0.031c	0.090b	0.344a
EUP	2	0.027c	0.099b	0.030c	0.088b	0.124a
	4	0.016c	0.093b	0.021c	0.094b	0.146a
	8	0.013c	0.094b	0.010c	0.096b	0.120a

Different letters in rows for each extract indicate significantly different values ($P < 0.05$). ND, not detected; Aq, aqueous.

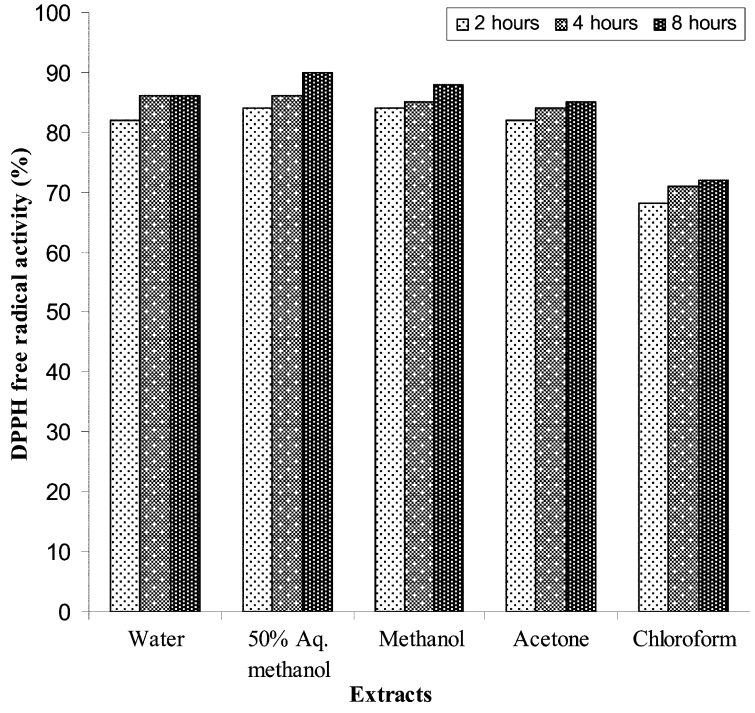

Fig. 3. Free radical-scavenging activity of the extracts at the different extraction times.

they were significantly different from the activities of chloroform extracts at the different extraction times. There were no significant differences ($P = 0.05$) between the activities exhibited by the chloroform extracts at different extraction times. The results indicated that the extracts of OS are free radical inhibitors and primary antioxidants that react with free radicals and the polar extracts have the highest free radical scavenging activity. This may be due to higher concentrations of caffeic acid derivatives present in these solvents. As shown by our data, RA concentrations were high in water, methanol, 50% aqueous methanol and 70% aqueous acetone extracts. Caffeic acid derivatives, including RA, have been reported to constitute 67% of total identified phenolics in aqueous methanol extract and about 94.6% in hot water extract (Sumaryono, Proksch, Wray, Witte, & Hartmann, 1991).

The markers (RA, EUP, TMF, SEN) and reference compounds (quercetin and BHA) were also tested against DPPH in the spectrophotometric assay at the same dosage as the extracts (0.05 mg/ml). The activities exhibited by the extracts at various hours of extraction were as strong as that of RA and quercetin, except for the chloroform extract which gave an activity lower

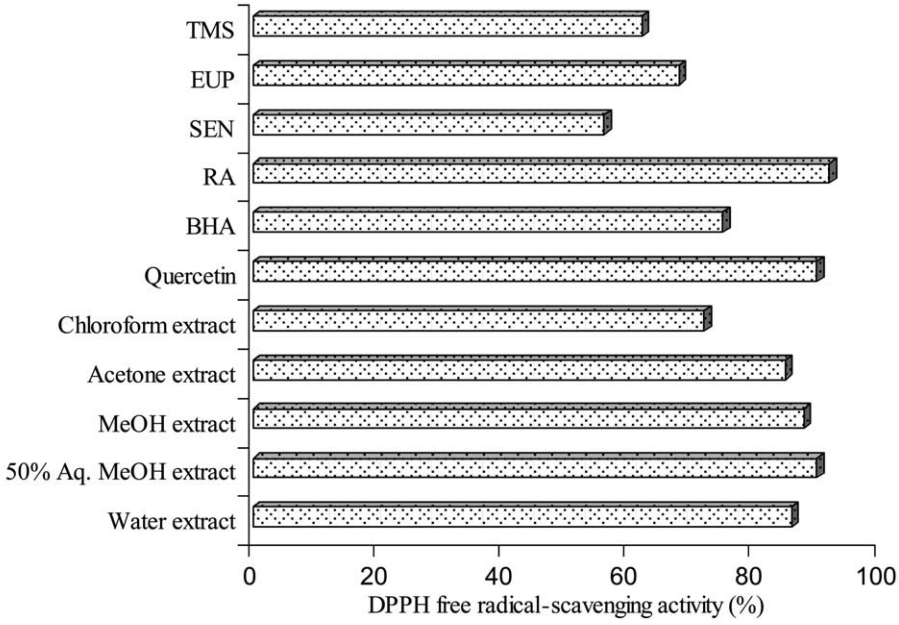

Fig. 4. Free radical-scavenging activity of samples at 8 h extraction time, markers and reference compounds.

and significantly different ($P = 0.5$) from the reference compounds. However, the activity of the chloroform was comparable to the activity of BHA, a synthetic antioxidant. EUP and TMF also exhibited activity similar to BHA but activity of SEN was significantly lower than those of BHA, the markers and the extracts (see Fig. 4).

The *o*-dihydroxy structure in the B-ring, the 2,3-double bond in conjugation with the 4-oxo function in the C ring, and the 3- and 5-OH groups with the 4-oxo function in A and C rings have been reported to be essential for effective free radical-scavenging activity (Bors, Heller, & Michel, 1990). The free radical-scavenging activity is also attributed to the high reactivities and numbers of hydroxyl substituents of the antioxidant (Rice-Evans & Packer, 1998). Quercetin satisfies the above-mentioned determinants and is a more effective antioxidant and polyphenolic. RA is also a polyphenolic antioxidant and possesses an *o*-dihydroxy structure. Therefore, RA and quercetin exhibited stronger activity than the *o*-methylated flavones (EUP, TMF and SEN), as expected. The low activity of the methoxylated flavones compared to quercetin and RA may be due to the lack of an *ortho*-dihydroxyl moiety in their B-rings (Fig. 1). The number of methoxy and hydroxyl groups in TMF, EUP and SEN may have caused the differences in their polarities and hence substantial alterations in their activities (Fig. 1).

At 0.05 mg/ml, there was poor correlation between the free radical-scavenging activity by DPPH assay ($P = 0.05$) and the concentration of the phenolics in various extracts. This may be ascribed to other secondary metabolites that functioned as hydrogen donors in the plant material, such as sugars which may have erroneously contributed to the free radical-scavenging activity (Amerine & Ough, 1980), since free radical-scavenging activities by antioxidants are due to their hydrogen-donating ability (Chen & Hu, 1995; Tang, Kerry, Sheehan, & Buckley, 2002).

4. Conclusion

Different solvent systems of varying polarities were used for the extraction of major polyphenols in OS leaves. The chloroform extraction at 2, 4 and 8 h gave the highest amount of SEN and EUP. The extraction with 70% aqueous acetone at 4 and 8 h gave a higher yield of TMF and the yield of RA was higher in 50% aqueous methanol extracts at 8 h of extraction on a water bath at 40 °C. The solvent extraction systems and the HPLC method developed in this study could be applied to analyse polymethoxylated flavones and caffeic acid derivatives in *O. stamineus* and other commercial products containing this herb. The free radical-scavenging capabilities of the extracts were comparable to those of pure quercetin and synthetic antioxidant BHA, thus presenting an alternative source of natural additives.

Acknowledgements

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