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Sinensetin, eupatorin, 3′-hydroxy-5, 6, 7, 4′-tetramethoxyflavone and rosmarinic acid contents and antioxidative effect of *Orthosiphon stamineus* from Malaysia

G.A. Akowuah *, I. Zhari, I. Norhayati, A. Sadikun, S.M. Khamsah

School of Pharmaceutical Sciences, University of Sains Malaysia, Pasat Pengayian Sains Farmasi, 11800 Palau Pinang, Malaysia

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Abstract

An HPLC method was developed for the separation and determination of three methoxylated flavones:sinensetin (SEN), eupatorin (EUP) and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF) and rosmarinic acid (RA), a caffeic acid derivative in *Orthosiphon stamineus* (OS). All the compounds were separated using a reversed phase C18, Lichrosorb column with the mobile phase of methanol-water-tetrahydrofuran (45:50:5 v/v). The HPLC method was applied for the quantification SEN, EUP, TMF and RA in OS from different places in Malaysia. The results showed significant variation in the amounts of these markers in methanolic extracts of leaf samples from different parts of Malaysia. A variation in antioxidant activities, ranging from 55.5% to 84.2% and variations in total phenolics, ranging from 6.7 to 10.1 mg caffeic acid/g dry weight of the methanol extracts, were observed. Antioxidative potency of the methanol extracts was comparable to that of pure quercetin and the synthetic antioxidant butylated hydroxylanisole (BHA).
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1. Introduction

Phenolics are a class of low molecular weight secondary plant metabolites found in most land plants. Phenolics (flavonoids) protect plants against ultraviolet radiation, pathogens, and herbivores (Harborne & Willam, 2000). Although dietary intake varies considerably among geographic regions and cultures, the average daily consumption of flavonoids by humans is estimated to be 1 g (Bravo, 1998). Most of the protective effects of flavonoids in biological systems are ascribed to their antioxidant abilities, capacity to transfer electrons, free radicals and chelating abilities (Hirano, Sasamoto, Matsumoto, Itakura, Igrashi, & Kondo, 2001), activate antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases (Elliott, Scheiber, Thomas, & Pardini, 1992).

Recently, there has been considerably interest in finding naturally occurring antioxidants to replace synthetic antioxidants in foods and medicinals. Several studies have analyzed the antioxidant potential of a variety of herbs (Furuta, Nishiba, & Suda, 1997; Hertog, Hollman, & Katan, 1992). Among the different parts of plants studied, the leaves are reported to have highest antioxidant properties (Chung et al., 1999; Venkatamuru, Patel, & Rao, 1983) and the most active principle among the phytochemicals is the phenolic fraction (Nakasugi & Komai, 1998; Jung, Kim, & Kim, 1999; Pietta, Simonetti, & Mauri, 1998). The phenolics have in vivo antioxidant activities and have been used as natural antioxidants in food (Zloch, 1969; Fuhrman, Lavy, & Aviram, 1995).

Orthosiphon stamineus (OS) contains several chemically active constituents, but one of the most important classes of compounds is the phenolic group. Twenty phenolic compounds were isolated, including nine lipophilic flavones, two flavonol glycosides, and nine caffeic acid derivatives, such as rosmarinic acid and 2,3-dicaffeoyltartaric acid, were identified and quantified by HPLC (Sumaryono, Proksc, Wray, Witte, & Hartmann, 1991). Pietta et al. (1998) reported HPLC analysis of the polymethoxylated flavones, sinensetin,

* Corresponding author. Tel./fax: +60-4-6563443.
E-mail address: wuahmy@yahoo.com (G.A. Akowuah).

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tetramethylscutellarein and 3′-hydroxy-5,6,7,4′-tetramethoxyflavone, present in *Orthosiphon* leaves (Pietta, Mauri, Gardana, & Bruno, 1991). Other chemical constituents of OS include isopimarane-type diterpenes, highly oxygenated staminane-type diterpenes, pentacyclic triterpenes betulinic acid, oleanolic acid, ursolic acid and β-sitosterol (Tezuka, Stampoulis, Banskota, Awale, Kadota, & Saiki, 2000).

Though considerable work has been done on analysis of OS, a comparative study covering the regions of Malaysia has so far not been done. Malaysia has a tropical climate with high temperatures and rainfall all year, which have enabled *Orthosiphon stamineus*, Benth (belongs to the family Lamiaceae) known locally as "Misai kucing" or "Kumis kucing", to flourish extensively. In Malaysia, the plant is used for treatment of a wide range of diseases: eruptive fever, epilepsy, gallstone, hepatitis, rheumatism, hypertension, syphilis, gonorrhea and renal calculus. OS is cultivated and the leaves are used to prepare a diuretic tea, which has been reported to be active against kidney and bladder inflammation (Hegnauer, 1966; Wangner, 1982).

The aim of this work is to compare the contents of SEN, EUP, TMF and RA in leaves of cultivated OS from different regions of Malaysia and to evaluate their antioxidative properties. A rapid HPLC finger-printing of the samples from different regions was achieved by reverse-phase isocratic elution with methanol:tetrahydrofuran:water (45:50:5 v/v) with UV detection for the markers which were selected, based on the fact that they are present in the samples. The test used to evaluate the antioxidant activity of the extracts is a well-established model system, based on β-carotene-coupled reaction with autoxidized linoleic acid.

2. Materials and methods

2.1. Chemicals and reagents

Standard samples of SEN, EUP, TMF and RA were purchased from Indofine Chemical Co. (Hillsborough, NJ USA). Solvents used for chromatography were tetrahydrofuran, methanol (HPLC grade), water (HPLC grade) and ethanol (80%), obtained from Merck (Darmstadt, Germany). Folin–Ciocalteu reagent, quercetin, butylated hydroxytoluene (BHT), Tween 20, β-carotene (95%) and linoleic acid (99%) were purchased from Sigma chemical Co. (St. Louis, MO) and membrane filters (0.45-μm pore size) from Millipore were used for filtration of the mobile phase and the samples. All other chemicals were of analytical grade or HPLC grade.

2.2. Plant materials

The plants were grown in the locations sampled (Table 1) from cuttings, using standard agronomic methods. The leaves were collected in the late afternoon, from 30–45-day old white flowered plants of similar sizes and leaf area. Each specimen was labelled, numbered and annotated with the date of collection and locality. Voucher specimens of the plant material from different locations were deposited at Bilik Herba, School of Pharmaceutical Sciences, Universiti Sains Malaysia.

2.2.1. Sample preparation

1 g each of the samples from different locations was extracted with 100 ml of methanol at 40 °C for 4 h with continuous stirring. The extracts were filtered through filter paper (Whatman No. 1) with a Buchner filter un-

Table 1
Percent concentrations of marker phenolics in leaf samples of OS collected from different regions of Malaysia between 20th September 2001 and 25th July 2002[a]

Location	State	Marker concentration (% of total dry leaf weight)			
		SEN	EUP	TMF	RA
Bohor Temak	Perlis	0.34 c	0.45 d	0.10 d	5.30 f
Kepala Batas	Pulau Pinang	1.32 a	2.03 bc	0.58 a	11.10 d
Bota Kenan	Perak	1.76 a	2.27 b	0.69 a	10.07 d
Kuala Nerang	Kedah	0.38 c	0.38 d	0.14 d	7.25 e
Kuching	Sarawak	0.34 c	0.34 d	0.07 d	23.9 b
Rawang	Selangor	0.31 c	0.36 d	0.10 d	7.10 e
Semmonggok	Sarawak	0.22 c	0.72 d	0.05 d	20.15 c
Jengka	Pahang	1.13 ab	1.37 b	0.39 b	5.10 f
Gua Musang	Kelantan	0.86 b	1.28 b	0.27 c	9.35 d
Parit	Perak	0.88 b	1.05 c	0.28 bc	9.50 d
Pasir Puteh	Kelantan	0.68 b	1.30 b	0.30 bc	9.80 d
Bumbung Lima	Pulau Pinang	0.59 bc	3.37 a	0.33 bc	29.90 a

[a] Means within column with different letters indicate significantly different values ($P < 0.05$). The experiments were performed in February–March 2003.

der vacuum, and cooled to room temperature and kept in refrigerator at −20 °C until further use.

2.3. HPLC analyses

2.3.1. Preparation of samples from different regions for HPLC analyses

1 ml of the methanol extract was diluted with 5 ml of MeOH:H_2O (6:4) and the samples were filtered through 0.45 μm membrane filters prior to HPLC analysis.

2.3.2. Identification and quantification of markers in the leaf samples by HPLC

HPLC analysis was performed using an Agilent Technologies Series 1100 system equipped with an automatic injector, a column oven, and a UV detector. A LiChrosorb RP-18 column (250 mm × 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. The following reference compounds were used as markers: RA, SEN, EUP and TMF. The markers were accurately weighed and dissolved in MeOH:H_2O (6:4) to produce a series of concentrations. The markers were separated with methanol:water:tetrahydrofuran (45:50:5 v/v) at a flow rate of 1 ml/min. The pH of water was adjusted to 3.0 using phosphoric acid. The effluent was monitored at 340 nm. Standard calibration curves were established by plotting the areas of peaks against different concentrations of the reference compounds (varying from 2.0 to 1000 ng on column for SEN, RA and TMF and 2.5 to 1000 ng for EUP). The external standard method was used for quantification of the markers in the samples of the leaf extract from different places.

The system suitability of the method was evaluated by the intra- and inter-day precision and accuracy of replicates. The accuracy was evaluated through recovery studies by adding known amounts of the standard solution to the extract. Controls from all samples were prepared and analyzed. The recovery experiment was performed at three concentrations of the standards.

2.4. Determination of total phenolic and total solubles contents of methanol extracts

The concentrations of total phenols in extracts was determined by using Folin–Ciocalteu reagent and external calibration with caffeic acid. Briefly, 0.2 ml of extract solution in a test tube and 0.2 ml of Folin–Ciocalteu reagent were added and the contents mixed thoroughly. After 4 min, 1 ml of 15% Na_2CO_3 was added, and then the mixture was allowed to stand for 2 h at room temperature. The absorbance was measured at 760 nm. The concentration of the total phenolics was determined as mg of caffeic acid equivalents by using an equation obtained from the caffeic acid calibration curve. The total methanol-soluble solids content of an aliquot (20 ml) of extract was determined in triplicate (Joubert, 1988).

2.5. Antioxidant activity of extracts using β-carotene/linoleic acid system

The procedure for evaluating the antioxidant activity for the samples of the herb was modified from a method described by Taga, Miller, and Pratt (1984). One ml of β-carotene (2 mg in 20 ml of chloroform) was added to a conical flask with 40 mg of linoleic acid and 400 mg of Tween-20. Chloroform was removed with a rotary evaporator at 40 °C and the mixture was taken to dryness with nitrogen. To the resulting residue, 100 ml of oxygenated distilled water were added and mixed and aliquots (3 ml) of the oxygenated β-carotene emulsion were placed in a tube containing 0.2 ml of the extracts (0.2 mg/ml) and the absorbance measured at 470 nm immediately, against a blank consisting of the emulsion without the β-carotene. The solution was incubated in a water bath at 50 °C to induce autoxidation. Taking absorbance at every 15 min interval for 120 min, monitored oxidation of the β-carotene emulsion. A control consisted of 0.2 ml of distilled water instead of the extract. Quercetin (0.2 ml of 1.0 μM) and BHA (0.2 ml of 1.0 μM) were used as reference compounds (Makris & Rossiter, 2001). The bleaching rate (R) of β-carotene was calculated using the equation:

$$R = \ln(a/b) \times 1/t,$$

where ln = natural log, a = initial absorbance (470 nm), b = absorbance at 120 min interval and t = time (min). The antioxidant activity (AA) was determined as percent inhibition relative to control sample using the equation:

$$\text{AA}\ (\%) = [(R_{\text{control}} - R_{\text{sample}})/R_{\text{control}}] \times 100.$$

2.6. Statistical analyses

Data are expressed as means of triplicate measurements, except in the case of antioxidant activity, for which two parallel measurements were run. Correlations were obtained by Pearson correlation coefficient in bivariate correlations. Means were compared by Tukey-HSD and LSD (least significant figures). Differences at $P < 0.05$ were considered to be significant.

3. Results and discussion

3.1. Collection of the samples

Table 1 shows the locations in Malaysia where the analysis samples were collected. The leaves were

collected in the late afternoon, when leaves are less turgid and therefore less likely to be damaged and energy substrate levels are high to facilitate long storage life (Lipton, 1987; Phan, 1987).

3.2. Concentrations of the markers in methanolic leaf extracts of OS

The HPLC method applied is a modification of that reported by Pietta et al. (1991) for the analysis of polymethoxylated flavones present in *Orthosiphon* leaves. In the present study, an isocratic method was used for the simultaneous assay of the authentic markers, which were separated within a total time of 30 min (Fig. 1). All the standards were determined in a single run of HPLC. The standards were resolved and eluted at 4.9, 12.0, 15.8 and 23.1 min, with respect to RA, TMF, SEN and EUP (Fig. 2). The markers showed a good linearity in the range from 2.0 to 1000 ng in the calibration curves that were obtained by HPLC analysis. All the reference markers were present in the chromatographic profiles of the samples from various locations when the sample solution was analyzed by HPLC. The peaks of RA, EUP, TMS and SEN were confirmed by comparison of the retention times with the reference standards.

To assess the precision of these methods, standard solutions of RA, TMS, EUP and SEN were determined six times on the same day and over a six-day period. The results showed very good precision, ranging from 5 to 100 μg/ml. The accuracy of the method was evaluated through recovery studies. The recovery experiment was performed at three concentrations of the standard added to sample solutions, in which the marker content had been determined, using sample from Bohor Temak. The results for the recoveries of RA, EUP, TMS and SEN were in the range 96–103%. The limit of detection (LOD) of the HPLC method, established at signals three times that of the noise for SEN, EUP, TMF and RA, were 2.0, 2.5, 2 and 2 ng, respectively.

The HPLC procedure was applied to the determination of the markers in OS samples from different regions. As shown in Table 1, all the analyzed samples showed a wide range in the concentrations of the markers, in samples from the same region and from different regions. The variation may be ascribed to environmental conditions and variation in sample sourcing. The plants selected for this experiment were of similar height, leaf area, fresh weight and dry weight; however, the chemical composition of the leaves could be affected by the soil fertility levels and age (Taylor, Fetuga, & Oyenuga, 1983; Schimdt, 1971). The values obtained for the markers appear to fall within the range reported for the markers in OS leaves (Sumaryono et al., 1991). However, the overall levels of the marker concentration were markedly higher in samples from Sarawak (Kuching; Semonggokk), Pulau Pinang (Bumbung Lima and Kepala Batas) and Perak (Bota Kanan). RA was the main component found in OS at concentrations ranging from 5.1% to 29.9% of total dry leaf weight. Concentrations of TMF, EUP and SEN ranged from 0.05% to 0.69%, 0.34% to 3.37% and 0.22% to 1.76%, respectively (Table 1). A literature search, to identify the unkown peak at 26.9 min in the HPLC finger-print, showed that it might be tetramethylscutellarein (Pietta et al., 1991). The HPLC results showed that the relative concentrations of the markers varied considerably. Based on this observation, we evaluated the antioxidant activities of the

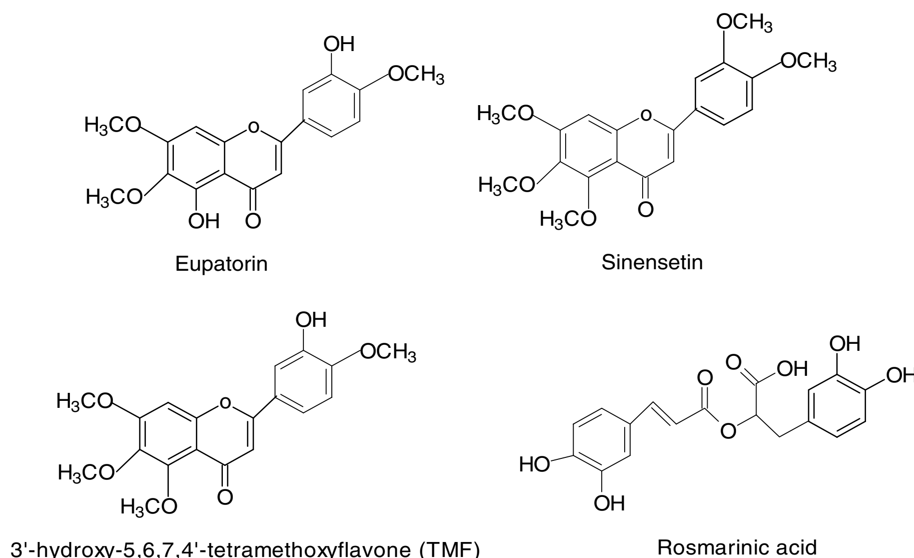

Fig. 1. Chemical structures of markers.

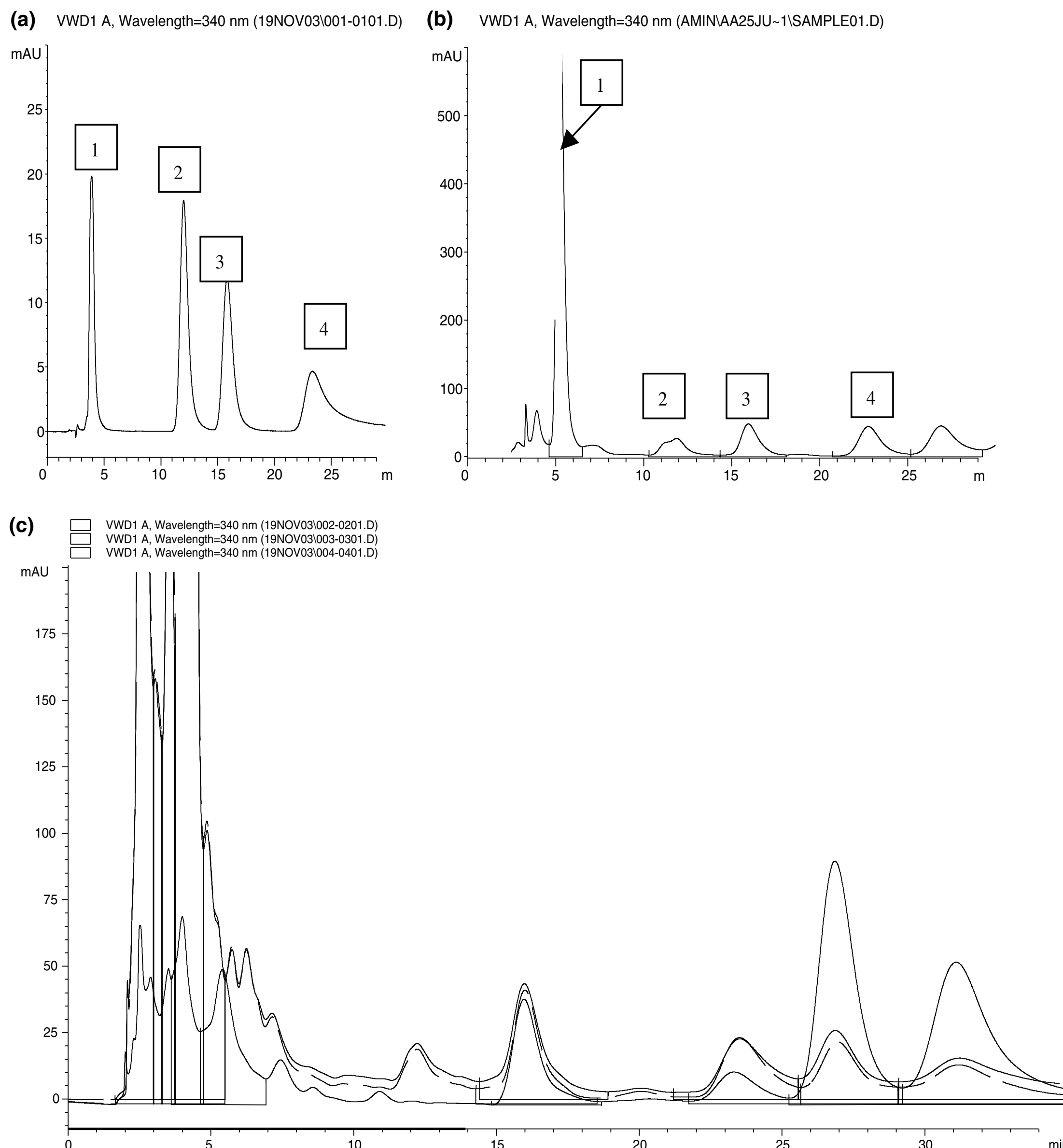

Fig. 2. (a) Chromatogram of the mixture of reference compounds, RA [1]; TMF [2]; SEN [3] and EUP [4]. (b) Typical chromatogram of *Orthosiphon stamineus* leaf extract. (c) An overlaid of chromagram from different locations. Chromatographic conditions: C18 Lichrosorb column (250 mm × 4.6 mm I.D.) eluent, methanol:H_2O (pH 3.0):Tetrahydrofuran (45:50:5 v/v); flow rate of 1 ml/min; UV detection at 320 nm; and the injection volume was 20 μl.

extracts to assess the effect of the variation of the phenolic content on the antioxidative potential.

3.3. Antioxidant activity

The methanol-soluble solids and total phenols analysis of OS leaves from different locations are given in Table 2. The results showed that the relative methanol-soluble solids of the extracts varied from 7.10% (Pasir Puteh) to 18.2% (Bohor Temak). The total phenolic content of the methanol extracts varied from 6.69 mg (Pasir Puteh) to 10.2 mg caffeic acid/g dry weight (Parit). There was a significant difference ($P < 0.05$) between the means of the methanolic extracts from different locations with respect to soluble solids and total phenolic contents, except for the sample from Bumbung Lima. The test used to evaluate the potency of the extracts as antioxidants is a well-established model system; based on β-carotene coupled with autoxidized linoleic acid, there was a gradual decrease at A_{470} with β-carotene bleaching. The decrease in absorbance of β-carotene in the presence of methanolic extracts of the OS from twelve different localities, with the oxidation of β-carotene and linoleic acid, is shown in Fig. 3. A variation in

Table 2
Antioxidant activity and total phenolics of the methanol extracts of Orthosiphon stamineus leaf collected from different regions of Malaysia between 20th September 2001 and 25th July 2002[a]

Location	State	MeOH-soluble solids (%)	Total phenolics (mg g^{-1} dry wt)	Antioxidant activity (%)
Bohor Temak	Perlis	18.2 a	9.46 a	84.2 a
Kepala Batas	Pulau Pinang	9.00 de	7.61 b	73.3 c
Bota Kenan	Perak	10.1 d	9.28 a	66.4 d
Kuala Nerang	Kedah	16.5 b	9.65 a	63.9 e
Kuching	Sarawak	11.3 d	9.59 a	62.9 e
Rawang	Selangor	13.4 c	8.86 a	51.2 g
Semmong-gok	Sarawak	15.3 b	9.77 a	73.8 c
Jengka	Pahang	14.1 c	7.36 b	71.3 c
Gua Musang	Kelantan	8.30 e	9.71 a	77.7 b
Parit	Perak	8.60 e	10.20 a	52.7 g
Pasir Puteh	Kelantan	7.10 f	6.69 b	66.3 d
Bumbung Lima	Pulau Pinang	10.6 d	10.1 a	72.8 c
BHA[b]	–	–	–	58.4 f
Quercetin[b]	–	–	–	72.3 c
Control	–	–	–	0.00

[a] Data are expressed as means of triplicate measurements, except in the case of antioxidant activity, for which two parallel measurements were run. Means within-column with different letters indicate significantly different values ($P < 0.05$). The experiments were performed in January–February 2003.
[b] Reference.

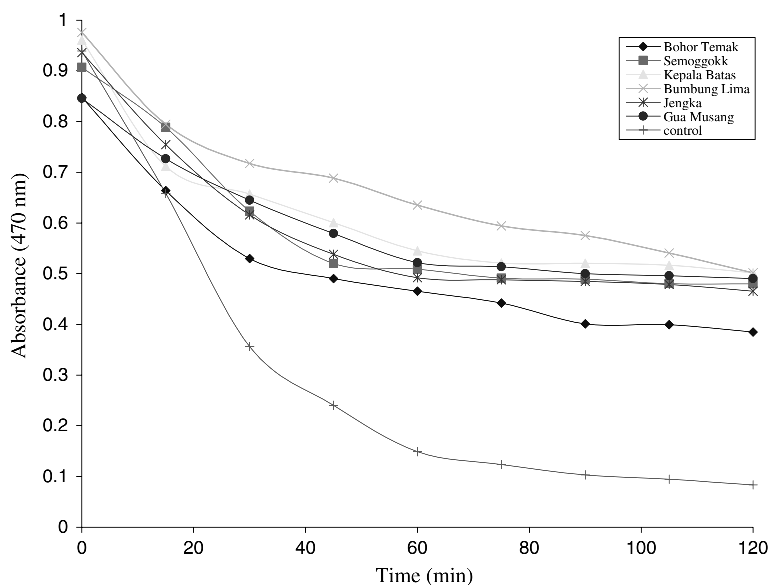

Fig. 3. Antioxidant activity of *Orthosiphon stamineus* leaf extracts (Table 3) as assessed by β-carotene bleaching method.

antioxidant activities ranging from 51.5% (Rawang) to 84.2% (Bohor Temak) was observed. The antioxidant activity, calculated from data in Fig. 3 of all the extracts tested in relation to one another, is given in Table 2. Antioxidant activities of the OS extracts tested using the β-carotene bleaching method decreased in the order Bohor Temak > Gua Musang > Semonggok > Kepala Batas > Bumbung Lima > Jengka > Bota Kanan > Pasir Puteh > Kuala Nerang > Kuching > Parit > Rawang.

Flavonoids and phenolic acids have been implicated as natural antioxidants in plants, fruits and vegetables. Lipophilic flavones and caffeic acid derivatives were identified in OS leaves and quantified by HPLC. Caffeic acid derivatives, rosmarinic acid and lipophilic flavones were predominant phenolics in the methanol extract (Table 1) and their antioxidant properties have been well documented (Brand-Williams, Curvelier, & Berset, 1995; Curvelier, Richard, & Berset, 1995). However, there was no relationship between the antioxidant activity and the total phenolic content ($r_{xy} = 0.048$), on the individual phenolics SEN ($r_{xy} = 0.002$), EUP ($r_{xy} = 0.057$), TMF ($r_{xy} = 0.048$), RA ($r_{xy} = 0.005$). The same

results on relationship between antioxidant activity of plant extracts and phenolic content have been previously reported (Maillard & Berset, 1995; Heinonen et al., 1998; Kahkonen et al., 1999). Antioxidant activity of plant extracts is not limited to phenolics; the presence of different anti-oxidant components in the extracts such as sugars and other compounds that function as hydrogen donors, may erroneously contribute to the concentration of the total phenols determined with Folin–Ciocalteau reagent (Amerine and Ough, 1980). Therefore, there is no simple relationship between the concentration of total phenol and the antioxidant activity when comparing plant extracts.

4. Conclusion

The HPLC chromatographic profiles of the OS samples from different locations were qualitatively similar but the results showed variations in the concentrations of the markers, SEN, EUP, TMF and RA. All the methanolic extracts of samples from different locations showed considerable variation in antioxidant activities, which could be ascribed to soil fertility levels, age of the plants, and variation in sample sourcing. Though the antioxidant pattern is generally complex, thus making assessment of a plant extract based on compositional data difficult, the concentration of individual antioxidants in plant extracts determined by HPLC is the preferred way to provide standardized information. The HPLC finger-printing could be used in authentication of OS samples and formulations. Radical scavenging activity and in vivo antioxidant studies of the leaf samples are being investigated in our laboratory.

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