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Fe(III) reductive and free radical-scavenging properties of summer savory (*Satureja hortensis* L.) extract and subfractions

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Abstract

Satureja hortensis L. (summer savory) is an annual herb belonging to the family Lamiaceae. It is used as a condiment and as a plant in traditional folk medicine to treat infectious diseases and disorders. A number of studies have suggested that the activity of *Satureja hortensis* may relate to the antioxidant properties of its secondary metabolites. Therefore, this study attempts to characterise the antioxidant properties of an acidified aqueous methanol extract from commercially available material using Fe(III) reductive and DPPH·, $ABTS^{\cdot+}$ and hydroxyl free radical-scavenging assays. The crude extract demonstrated promising in vitro activity and thus was further fractionated, by liquid–liquid partitioning against water, to determine which fraction possessed the most potent activity. The ethyl acetate-soluble fraction was the most effective fraction, with reductive activity of 2648 ± 41.4 μmol ascorbic acid/g extract, and DPPH· and hydroxyl radical-scavenging IC_{50} concentrations of 138 ± 6.0 and 45.0 ± 7.0 μg/ml, respectively. The TEAC value for this fraction was 2.59 ± 0.06 mM Trolox. The experimental results are discussed and potential applications are explored.

Keywords: *Satureja hortensis*; Antioxidant activity; Free radicals; Fe(III) reduction; Summer savory; Herbs

1. Introduction

Interest in natural antioxidants has increased dramatically in recent times due to: (1) concerns regarding the safety of the chronic consumption of synthetic antioxidants (butylated hydroxyltoluene and butylated hydroxylanisole), (2) the antioxidative efficacy of a variety of phytochemicals, (3) the consensus that foods rich in certain phytochemicals can affect the aetiology and pathology of chronic diseases and the ageing process and (4) the public's conceived belief that natural compounds are innately safer than synthetic compounds and are thus more commercially acceptable. As part of our ongoing in vitro screening programme to identify antioxidant-enriched extracts from edible herbs, spices and agricultural products for industrial applications, we have investigated plant extracts from different genera belonging to the family Lamiaceae (Labiatae), a family composed of species with exploitable antioxidant activity (Jayasinhe, Gotoh, Aoki, & Wada, 2003; José del Bano et al., 2003; Marinova & Yanishlieva, 1997).

Satureja hortensis L. is an aromatic and medicinal plant belonging to the family Lamiaceae. The aerial material has a distinctive taste and can be added to stuffing, meat pies and sausages as a seasoning. Fresh sprigs can be boiled with pulses, such as peas, beans or lentils, for flavouring or, alternatively, they can be used instead of parsley and chervil as a garnish. The leaves, flowers and stems are used for herbal tea and, in traditional medicine, to treat various ailments, such as cramps, muscle pains, nausea, indigestion, diarrhoea and infectious diseases (Güllüce et al., 2003). Extracts from *Satureja hortensis* have demonstrated a variety of activities including antibacterial, antifungal, antioxidant, antispasmodic, antidiarrhoeal and sedative properties amongst others (Deans & Svoboda, 1989; Güllüce et al., 2003; Hajhashemi, Sadraei, Ghannadi, & Mohseni, 2000; Madsen, Andersen, Christiansen, Brockhoff,

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& Bertelsen, 1996). In this study, we have characterised the antioxidant [Fe(III) reductive capacity and DPPH·, $ABTS^{\cdot+}$ and hydroxyl radical-scavenging] properties of a crude acidified aqueous methanol extract and four subfractions, based upon solubility, from commercially available *Satureja hortensis* aerial material. Furthermore, the total phenolic content of each sample was determined by the Folin-Ciocalteu method.

2. Materials and methods

2.1. Materials

Air-dried aerial parts of *Satureja hortensis* L. were obtained from Pimenta Oy (Finland). Pycnogenol was obtained from Biolandes Arômes (France). Ultra-pure water (HPLC grade) was prepared, using a Millipore Milli-RO 12 plus system (Millipore Corp., USA). All reagents used in this study were of the highest purity commercially available and were obtained from the usual sources.

2.2. Extraction and fractionation

Plant material (40 g) was macerated with 500 ml (80:20:1, v/v/v) methanol: water: acetic acid at room temperature for 12 h with constant stirring. The process was repeated twice with fresh extractant. The resulting extracts were combined, filtered, concentrated in vacuo and lyophilized. The dried crude extract was suspended in 500 ml ultra-pure water and sequentially extracted with 250 ml hexane, 300 ml ethyl acetate (EtOAc) and 350 ml *n*-butanol (*n*-BuOH). The remaining aqueous phase was filtered, concentrated in vacuo and lyophilized. The organic phases were filtered through anhydrous magnesium sulphate and the solvent was removed in vacuo. A schematic of the extraction and fractionation of the plant material is provided in Fig. 1.

2.3. Total phenol content

Total phenols were estimated as gallic acid equivalents (Singleton, Orthofer, & Lamuela-Raventós, 1999). To ca. 6.0 ml ultra-pure water, a 100 μl sample in 80% aqueous MeOH was transferred to a 10.0 ml volumetric flask, to which was subsequently added 500 μl undiluted Folin-Ciocalteu reagent. After 1 min, 1.5 ml 20% (w/v) Na_2CO_3 was added and the volume was made up to 10.0 ml with ultra-pure water. After a 30 min incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve.

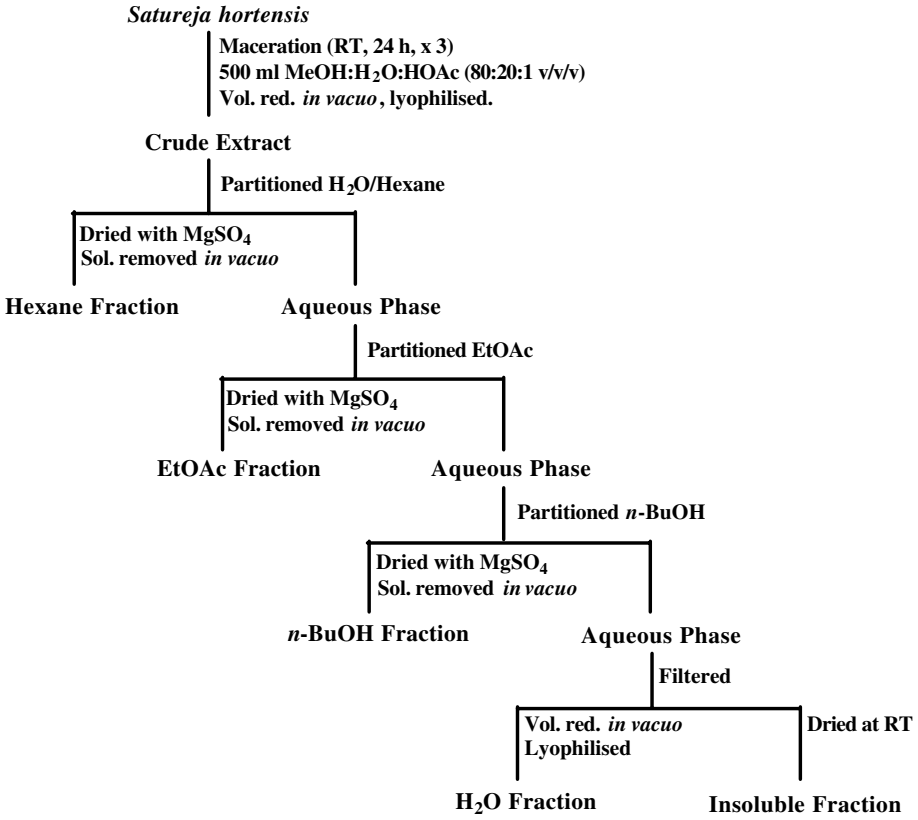

Fig. 1. Fractionation scheme for the production of the *S. hortensis* crude extract and subfractions. Scheme key: RT, room temperature; vol., volume; red., reduced; sol., solvent.

2.4. Fe(III) to Fe(II) reductive capacity

The Fe(III) reductive capacity of the extracts was assessed spectrophotometrically (Oyaizu, 1986). One ml of each extract in 80% aqueous methanol was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of a 1% (w/v) potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. After 30 min at 50 °C, 2.5 ml (10%, w/v) trichloroacetic acid (TCA) were added and the mixture was centrifuged for 10 min (2000 rpm). Finally, a 2.5 ml aliquot was mixed with 2.5 ml ultra-pure water and 0.5 ml (0.1%, w/v) $FeCl_3$ and the absorbance was recorded at 700 nm. Values are presented as ascorbic acid equivalents, AscAE (μmol ascorbic acid/g extract).

2.5. Diphenyl-2-picrylhydrazyl free radical-scavenging activity

The ability of the extracts to scavenge 1,1-diphenyl-2-picrylhydrazyl radicals was assessed spectrophotometrically (Gyamfi, Yonamine, & Aniya, 1999). A 50 μl aliquot of each extract, in 80% aqueous methanol, was mixed with 450 μl Tris–HCl buffer (50 mM, pH 7.4) and 1.0 ml 1,1-diphenyl-2-picrylhydrazyl (0.1 mM, in methanol). After a 30 min reaction period, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using equation 1 and IC_{50}s were estimated using a non-linear regression algorithm.

$$\text{Percentage inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100. \quad (1)$$

2.6. $ABTS^+$ free radical-scavenging activity

The determination of $ABTS^{\cdot +}$ radical-scavenging was carried out as described by Re et al. (1998). The $ABTS^{\cdot +}$ radical was generated by reacting an (7 mM) ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM, final concentration) in the dark for 12–16 h and adjusting the $Abs_{734\ nm}$ to 0.700 (±0.030) at ambient temperature. Extracts were diluted such that a 15 μl sample, when added to 1.485 ml $ABTS^+$ solution, resulted in a 20%–80% inhibition of the absorbance. After 15 μl extract/Trolox/standards were added to 1.485 ml $ABTS^{\cdot +}$, the absorbance at 734 nm was recorded at 1 min after initial mixing and subsequently at 1 min intervals (15 min in toto). The percentage inhibition was plotted as a function of concentration and the Trolox equivalent antioxidant capacity (TEAC) was calculated against a Trolox calibration curve.

2.7. Ascorbate-Fe(III)-catalysed phospholipid peroxidation inhibition

The ability of the extracts to scavenge hydroxyl radicals was determined by the method of Aruoma et al. (1997). Bovine brain extract (Folch type VII) was mixed with 10 mM PBS (pH 7.4) and sonicated in an ice bath until an opalescent suspension was obtained containing 5 mg/ml phospholipid liposomes. The liposomes (0.2 ml) were combined with 0.5 ml of PBS buffer, 0.1 ml of 1 mM $FeC1_3$, and 0.1 ml of extract in 80% aqueous MeOH. The peroxidation was initiated by adding 0.1 ml of 1 mM ascorbate. The mixture was incubated at 37 °C for 60 min. After incubation, 50 μl of 2% (w/v) butylated hydroxytoluene in EtOH were added to each tube, followed by 1 ml of 2.8% (w/v) trichloroacetic acid and 1 ml of 1% (w/v) 2-thiobarbituric acid (TBA) in 0.05 M NaOH. The solutions were heated in a water bath at 100 °C for 20 min. The resulting $(TBA)_2$-MDA chromogen was extracted into 2 ml of *n*-butanol and the extent of peroxidation was determined in the organic layer at 532 nm. The percentage inhibition was calculated using equation 1 and IC_{50} values were estimated using a non-linear regression algorithm.

2.8. Statistical analysis

Data are presented as mean values ±95% confidence limits. Analysis of variance was performed using ANOVA procedures. Significant differences between means were determined by Tukey's pairwise comparison test at a level of $P < 0.05$. IC_{50} values were estimated using a non-linear regression algorithm.

3. Results

3.1. Extract yield and total phenol content

The yield of the crude *Satureja hortensis* extract was 369 mg extract/g plant material and consisted of approximately 6.5% non-polar (hexane-soluble) components, 5.1% moderately polar (EtOAc-soluble) components, 56.8% polar (*n*-BuOH-soluble) components, 28.7% highly polar (H_2O-soluble) components and 2.9% insoluble components. The total extractable phenolic contents of the crude extract and hexane, ethyl acetate, *n*-butanol and water fractions were estimated by the Folin-Ciocalteu method as 166 ± 2.7, 37.1 ± 1.1, 500 ± 47.3, 27.0 ± 9.1 and 67.2 ± 25.2 mg gallic acid/g extract, respectively.

3.2. Fe(III) to Fe(II) reductive capacity

As can be seen from the AscAE values presented in Fig. 2 and the associated regression parameters (Table 1),

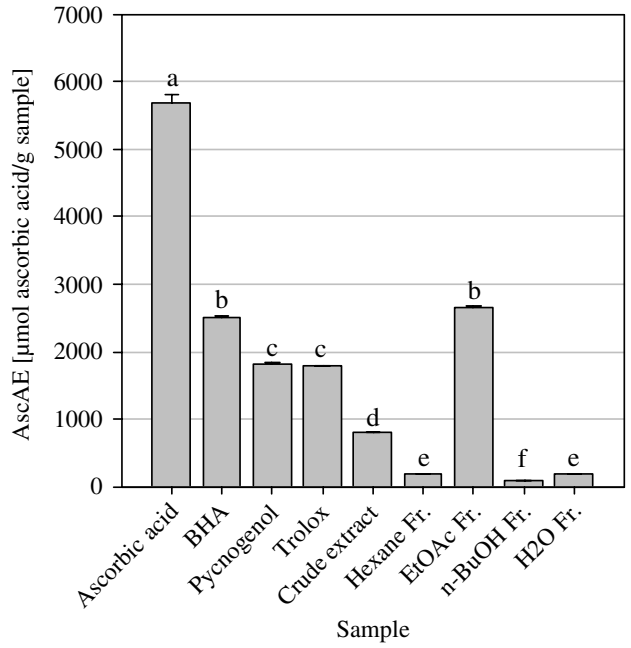

Fig. 2. AscAE values calculated for the positive controls and *S. hortensis* samples. Values are presented as mean values ±95% confidence interval. Bars with the same lower case letter (a–f) are not significantly ($P > 0.05$) different.

Table 1
Fe(III) to Fe(II) reductive capacity linear regression parameters for the test samples

Sample	Slope	Intercept ($\times 10^{-2}$)	r^2
Ascorbic acid	12.192 ± 0.316	1.2 ± 0.6	0.999
BHA	4.925 ± 0.173	10.5 ± 1.8	0.994
Pycnogenol	3.886 ± 0.263	7.0 ± 1.6	0.998
Trolox	3.800 ± 0.029	2.3 ± 0.5	0.998
Crude extract	1.682 ± 0.015	1.9 ± 0.5	1.000
Hexane Fr.	0.373 ± 0.005	0.7 ± 0.3	0.997
EtOAc Fr.	5.658 ± 0.091	2.7 ± 0.3	0.997
n-BuOH Fr.	0.115 ± 0.002	7.7 ± 1.1	0.996
H_2O Fr.	0.340 ± 0.007	6.5 ± 1.9	0.998

Values are presented as means ±95% confidence limits.

all the extracts and fractions were capable of catalysing the reduction of Fe(III) and did so in a linear dose-dependent fashion. The crude extract possessed an AscAE (ascorbic acid equivalent) of 792 ± 6.0 µmol ascorbic acid/g extract which was much lower than the value observed for the reference substance ascorbic acid or for the positive controls. This suggests that this extract is not as powerful a reducing agent as these substances. The non-polar hexane-soluble fraction possessed a very low AscAE value of 37.1 ± 1.3 µmol ascorbic acid/g extract as did the *n*-BuOH fraction (27.0 ± 9.1 µmol ascorbic acid/g extract), with the H_2O fraction containing a better reducing potency (67.2 ± 25.2 µmol ascorbic acid/g extract). The EtOAc fraction, on the other hand, possessed very potent reductive power, with an AscAE equal to 2648 ± 41.4 µmol ascorbic acid/g extract. This was significantly better than the activities calculated for the positive controls ($P < 0.05$).

3.3. Diphenyl-2-picrylhydrazyl radical-scavenging activity

The extract and fractions were capable of scavenging DPPH· free radicals in a dose-dependent manor via electron-/hydrogen-donation converting it to the non-radical hydrazine form. The concentrations of crude extract and fractions required to scavenge 50% of the DPPH· radicals, the IC_{50} values, are presented in Fig. 3. The hierarchy of ability to scavenging DPPH· radicals was determined as the EtOAc fraction > crude extract > H_2O fraction > *n*-BuOH fraction > hexane fraction. The hexane-soluble fraction required 7.12 ± 0.050 mg/ml in order to scavenge 50% of the DPPH· radicals, which suggests that the components within this fraction are extremely weak radical-scavenging components. Similarly, the components within both the *n*-BuOH and H_2O fractions were not efficient free radical scavengers, with IC_{50} values of 3.43 ± 0.082 and 2.16 ± 0.040 mg/ml, respectively.

3.4. $ABTS^+$ free radical-scavenging activity

The extract and fractions were capable of scavenging the $ABTS^{\cdot +}$ free radical and could be ranked according to their calculated TEAC values at 1, 5, 10 and 15 min time points (Table 2). The EtOAc-soluble fraction was

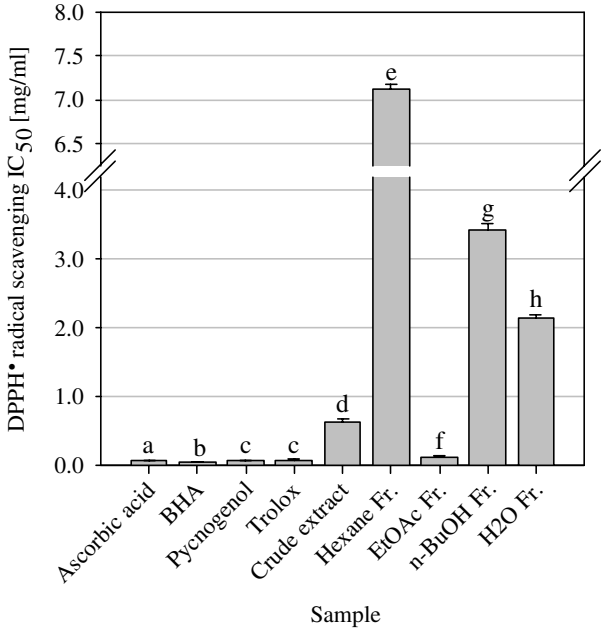

Fig. 3. The effect of the positive controls and *S. hortensis* samples upon 1,1-diphenyl-2-picrylhydrazyl scavenging. Values are presented as mean values ±95% confidence intervals. Bars with the same lower case letter (a–h) are not significantly ($P > 0.05$) different.

Table 2
Radical-scavenging activities of the test samples $ABTS^{\cdot+}$ radicals

Sample	$ABTS^{\cdot+}$ (TEAC, mM Trolox)			
	1 min	5 min	10 min	15 min
Ascorbic acid	5.60 ± 0.26a,A	5.60 ± 0.24a,A	5.59 ± 0.25a,A	5.59 ± 0.24a,A
BHA	6.39 ± 0.34b,A	6.53 ± 0.29b,A	6.56 ± 0.28b,A	6.56 ± 0.26b,A
Pycnogenol	3.34 ± 0.19c,A	3.77 ± 0.20c,A	4.00 ± 0.21c,A	4.08 ± 0.20c,A
Trolox	4.01 ± 0.12d,A	4.00 ± 0.15c,A	4.00 ± 0.15c,A	4.00 ± 0.15c,A
Crude extract	0.77 ± 0.04e,A	0.91 ± 0.04d,B	1.00 ± 0.05d,B	1.05 ± 0.05d,B
Hexane Fr.	0.15 ± 0.01f,A	0.20 ± 0.01e,B	0.23 ± 0.01e,C	0.24 ± 0.02e,C
EtOAc Fr.	2.22 ± 0.05g,A	2.50 ± 0.07f,B	2.59 ± 0.06f,B	2.64 ± 0.05f,B
n-BuOH Fr.	0.31 ± 0.08h,A	0.48 ± 0.08g,B	0.61 ± 0.08g,C	0.70 ± 0.08g,C
H_2O Fr.	0.33 ± 0.02h,A	0.37 ± 0.02g,B	0.39 ± 0.03h,B	0.41 ± 0.02h,B

Values are presented as means ±95% confidence limits. Trolox equivalent antioxidant capacity (TEAC) is defined as the concentration of Trolox having the $ABTS^{\cdot+}$ scavenging activity equal to a 1.0 mg/ml sample solution. Analysis of variance was performed by ANOVA procedures, with significant differences between means determined using Tukey's pairwise comparisons. Values with the same lowercase letter within each column are not significantly ($P > 0.05$) different. Values with the same uppercase letter within each row are not significantly ($P > 0.05$) different.

the most efficient scavenger over the reaction time followed by the crude extract. Over time, however, the potencies of the *n*-butanol and H_2O fractions changed; at 1 and 5 min these fractions did not differ significantly ($P > 0.05$) in their $ABTS^{\cdot+}$ radical-scavenging activity but, after 10 min, the *n*-BuOH fraction was a significantly ($P < 0.05$) better scavenger than H_2O fraction. The hexane fraction was the least active, with TEAC values ranging fron 0.33 ± 0.02 mM Trolox at $t = 1$ min to 0.41 ± 0.02 mM Trolox $t = 15$ min. There was no significant ($P > 0.05$) change in the ability of the crude extract or the EtOAc and H_2O fraction to reduce the $ABTS^{\cdot+}$ free radical over time after the 5 min point, which suggests that the components within these samples have fairly rapid reaction kinetics. In the case of the hexane and *n*-BuOH fractions, the reaction kinetics appeared to be a little slower, with no significant ($P > 0.05$) increase in activity observed after the 10 min reaction point.

3.5. Ascorbate-Fe(III)-catalysed phospholipid peroxidation inhibition

The crude extract and fractions were capable of preventing the formation of the malondialdehyde-thiobarbituric acid MDA-$(TBA)_2$ chromogen in a concentration-dependent fashion and, therefore, hydroxyl radical-mediated phospholipid peroxidation. The estimated IC_{50} values for the samples are presented in Fig. 4. In this assay, the EtOAc fraction was a considerably more active inhibitor of ascorbate-Fe(III)-catalysed phospholipid peroxidation than the crude extract or the *n*-BuOH and H_2O fractions. The EtOAc-soluble fraction was as effective as the positive control substances Pycnogenol and Trolox but not as effective as BHA. Ascorbic acid was not used as a positive control in this assay system as it is used as a catalyst for the production of hydroxyl radicals in this assay system. The polar and highly polar fractions were only poorly active, however, with IC_{50} values of

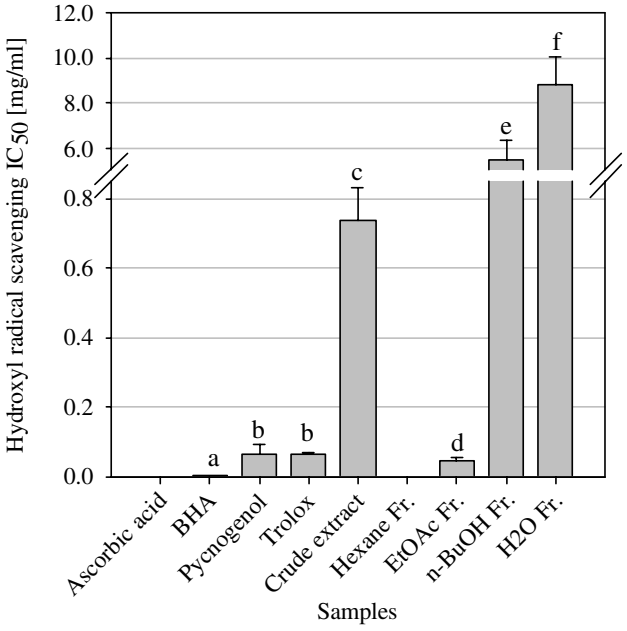

Fig. 4. The effect of the positive controls and *S. hortensis* samples upon hydroxyl radical-scavenging. Values are presented as mean values ±95% confidence intervals. Bars with the same lower case letter (a–f) are not significantly ($P > 0.05$) different.

5.49 ± 0.892 mg/ml and 8.82 ± 1.23 mg/ml, respectively. It was not possible to obtain an IC_{50} value for the hexane fraction as the biphasic dose-response curve prevented a reliable estimation by the non-linear regression algorithm used in this study.

4. Discussion

The ability of isolated compounds or plant extracts to reduce Fe(III) is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003a; Yildirim et al., 2000). The data

presented in this study suggests that components in *Satureja hortensis* which are soluble in the acidic aqueous methanol extractant are capable of electron donation and therefore should be able to donate electrons to unstable free radicals, converting them into more stable non-reactive species, with the EtOAc-soluble components being the most effective electron donators. There was no apparent association between extraction yields and Fe(III) reduction ($r^2 = 0.074$; $P = 0.658$). There was, however, a very strong association between the Folin-Ciocalteu-reactive (total phenol) content and reductive capacity ($r^2 = 0.997$; $P < 0.001$). It is known that chemicals which readily undergo redox reactions are capable of producing a high level of activity in the Folin-Ciocalteu method (Singleton et al., 1999), which may explain the high correlation between these two indices.

The cardinal mode of action of natural antioxidants is their ability to scavenge free radicals before they can initiate free radical chain reactions in cellular membranes or lipid-rich matrices in foodstuffs, cosmetics or pharmaceutical preparations. In this study, three different free radicals, were used to assess the potential free radical-scavenging activities of the *Satureja hortensis* extract and subfractions, namely the DPPH· and $ABTS^{\cdot+}$ synthetic free radicals and the hydroxyl radical. The stable nitrogen-centered free radicals, DPPH· and $ABTS^{\cdot+}$are frequently used for the estimation of free radical-scavenging ability (Dorman et al., 2003a; Re et al., 1998). The $ABTS^{\cdot+}$ free radical is commonly used when issues of solubility or interference arise and the use of DPPH· based assays become inappropriate (Arnao, 2000). However, it has been argued that these techniques are only capable of indicating potential antioxidant activity as they do not utilise a food/biologically relevant oxidizable substrate nor a relevant reactive species and, therefore, no direct information on protective performance can be determined (Dorman, Koşar, Kahlos, Holm, & Hiltunen, 2003b). Therefore, the ability of the samples to inhibit ascorbate-Fe(III)-generated hydroxyl radical-mediated peroxidation of a heterogeneous phospholipid-aqueous phosphate buffered system was determined, as phospholipids are believed to play a principal role in oxidative deterioration and off-flavour development in foodstuffs (Wu & Sheldon, 1988) and the hydroxyl radical is a highly reactive radical, capable of being generated in vivo. Recently, a number of reviews detailing the range of antioxidant methodologies currently available and describing their advantages and disadvantages have been published (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002; Frankel & Meyer, 2000; Niki & Naguchi, 2000).

The free radical-scavenging data presented in Figs. 2–4 and Table 2 suggests that the crude extract and subfractions are capable of scavenging reactive free radical species via the mechanism of electron-/hydrogen-donation. As in the case of the samples' performance in the Fe(III) reduction assay, the EtOAc-soluble components were the most active, followed by the crude extract in the three in vitro scavenging assays used. There was no association between the extraction yields and the reciprocal IC_{50} DPPH· scavenging values ($r^2 = 0.110$; $P = 0.586$). There was, however, a robust association between the reciprocal IC_{50} values and the Folin-Ciocalteu reagent-reactive content ($r^2 = 0.990$; $P < 0.001$) and Fe(III) reductive index ($r^2 = 0.992$; $P < 0.001$). There was a strong correlation between the calculated TEAC values at $t = 10$ min and the Folin-Ciocalteu reagent reactive content ($r^2 = 0.968$; $P = 0.003$). The association was stronger between the Folin-Ciocalteu reactive content and the calculated area under the curve, an index used to assess the overall antioxidant performance across the total reaction time in comparison to that of Trolox (Re et al., 1998) ($r^2 = 0.999$; $P < 0.001$). There was no association between the extraction yields and the reciprocal IC_{50} hydroxyl radical-scavenging values ($r^2 = 0.434$; $P = 0.341$) but there was a robust association between the Folin-Ciocalteu reagent reactive content ($r^2 = 0.950$; $P = 0.025$) and the Fe(III) reductive ($r^2 = 0.955$; $P = 0.023$) index and the reciprocal IC_{50} values. The crude extract and subfractions of *Satureja hortensis* may be able to protect susceptible components such as amino acids, DNA, lipoproteins, polyunsaturated fatty acids, sugars and proteins in biological and food matrices from free radical-mediated degradation (Halliwell, Aeschbach, Löliger, & Aruoma, 1995). The hexane-soluble fraction could not be reliably assessed in the hydroxyl radical-phospholipid assay, thus no conclusions can be draw about its performance.

5. Conclusions

Satureja hortensis is an annual herb belonging to the family Lamiaceae which is used as a condiment and as a traditional folk medicine especially in Mediterranean regions. For example, in the Eastern Anatolia region of Turkey, it is used to treat infectious diseases. Aerial material from commercially available plant material was macerated with acidified aqueous methanol to produce a crude extract. An aliquot of this extract was subfractioned by liquid–liquid partitioning against water with hexane, EtOAc and *n*-BuOH. These samples were screened to characterise their antioxidant properties, using a Fe(III) reduction assay and DPPH·, $ABTS^{\cdot+}$ and hydroxyl free radical-scavenging assays. The crude extract and the EtOAc fraction were the most effective samples, with the remaining subfractions demonstrating considerably weaker activities. The activity of the EtOAc-soluble components of the crude extract showed particularly strong properties and may find potential application as preservatives to retard free radical-mediated degradation of susceptible components. Furthermore, due to the role

free radicals play in the deterioration of human health, the EtOAc subfraction may potentially have beneficial effects upon human biology if incorporated in foodstuffs in appropriate quantities. However, before such claims can be made with confidence, further research on the qualitative-quantitative chemical composition and in vivo efficacy studies, including absorption and metabolism studies, should be carried out.

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