

Full Length Research Paper

Antioxidant property of selected *Ocimum* species and their secondary metabolite content

F. Lukmanul Hakkim, Girija Arivazhagan*, R. Boopathy

Plant Biotechnology Laboratory, School of Biotechnology and Genetic Engineering, Bharathiar University, Coimbatore, Tamilnadu, India.

Accepted 12 September 2008

In this study, chemical composition and antioxidative properties of eight selected *Ocimum* species (*Ocimum gratissimum*, *Ocimum americanum*, *Ocimum minimum*, *Ocimum citriodorum*, *Ocimum kilimandscharicum*, *Ocimum grandiflorum*, *Ocimum lamiifolium*, and *Ocimum selloi*) was investigated. Leaves of these plants were extracted using methanol. The quantitative analysis of phenolic constituents was determined using high-performance liquid chromatography. Total phenolic content was estimated using Folin-Ciocalteu reagent and antioxidant activity was assessed using iron (III) reduction, β -carotene-linoleic acid bleaching, 1,1-diphenyl-2-picrylhydrazyl and superoxide anion free radical scavenging assays. Phenolic acids, hydroxycinnamates, and flavonoids were identified and quantified within each extracts based on the area of each peaks with an external standards. The extracts of *Ocimum* species exhibited activity in all the in vitro antioxidant assays but it was not as potent as butylated hydroxyl anisole (BHA). The phytochemicals found in each extract are rich antioxidants and these extracts can be used as an effective preservative in food industry.

Key words: *Ocimum* species, antioxidant activity, free radicals, total phenols, HPLC, secondary metabolites.

INTRODUCTION

Chemical constituents with antioxidant activity present in high concentrations in plants determine their considerable role in the prevention of various degenerative diseases (Velioglu et al., 1998). Besides fruits and vegetables, certain plants are recommended at present as optimal sources of such components. The supplementation of human diet with herbs, containing especially high amounts of compounds capable of deactivating free radicals, may have beneficial effects (Lutowski, 2001). Throughout recorded history, spices and herbs have been used for flavoring foods and beverages and for medicinal purposes. The preservative effect of many plant species and herbs suggests the presence of antioxidative and antimicrobial constituents (Hirasa and Takemasa, 1998). Among the important constituents in plants, phenolic compounds are mainly involved in the cell defence system against free radicals (Szeto et al., 2002). A number of phenolic compounds with strong antioxidant and antimicrobial activities have been identified in plants, especially in those belonging to the Lamiaceae fa-

mily. They are of interest to food manufacturers as consumers move towards functional foods with specific health effects (Ozkan et al., 2003).

The genus *Ocimum*, a member of the Lamiaceae family, contains 200 species of herbs and shrubs (Simon et al., 1999). This species has a long history as culinary herbs, thanks to its foliage adding a distinctive flavor to many foods. It is also a source of aroma compounds and essential oils containing biologically active constituents that possess insecticidal and nematocidal properties (Deshpande and Tipnis, 1997; Chatterje et al., 1982). However, the antioxidative potential of herbs and spices is well correlated with the presence of phenolic compounds due to its redox properties, which permit them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Caragay, 1992). The major phenolic compounds found in plants are secondary metabolites possessing high antioxidant activity and it is wide spread in the species of Lamiaceae (Gang et al., 2001). As part of our ongoing in vitro screening programme to identify secondary metabolites from *Ocimum* species, recently we have reported the secondary metabolite content with exploitable antioxidant activity of *Ocimum sanctum* (Hakkim et al., 2007).

*Corresponding author. Email: girijabiotech@yahoo.co.in.
Telephone: (+91)- 9789501755. Fax: (91) 422 – 2422387

In this study, antioxidant property of eight selected *Ocimum* species and their secondary metabolite content has been investigated using high performance liquid chromatography coupled with photo-diode array detector. The objective of the present study is to evaluate the distribution of secondary metabolites in different *Ocimum* species and determination of their antioxidant capacity by *in vitro* assays.

MATERIALS AND METHODS

Plant materials

Seeds of eight selected *Ocimum* species (*Ocimum gratissimum*, *Ocimum americanum*, *Ocimum minimum*, *Ocimum citriodorum*, *Ocimum kilimandscharicum*, *Ocimum grandiflorum*, *Ocimum lamiifolium*, *Ocimum selloi*) were collected from small farmers and home gardens and were grown at the department of biotechnology, Bharathiar University, Coimbatore, India. Green house plants were irrigated to pot capacity daily and maintained at day/night temperatures of 27 – 33 and 20 – 25°C respectively. The leaves were harvested from the fully matured plants and kept for air-drying at room temperature for seven days for chemical analysis. Voucher specimen of each plant were also collected and deposited at the Bharathiar University Herbarium. Taxonomic identification of each plant was conducted in Botanical Survey of India, Tamilnadu Agricultural University, Coim-batore.

Extraction

Dried plant materials were pulverized using a mechanical grinder. 10 g of each powdered material was extracted twice with 500 ml of methanol continuously for 6 h using the soxhlet apparatus. Thereafter, the resulting methanolic extract was reduced in vacuo (40°C; N₂ stream), freeze-dried and stored at 4°C until further use in the experiment.

Determination of total phenolic content

Total phenol content was estimated as Gallic acid equivalents (GAE) (Singleton et al., 1999). Briefly, 100 µl aliquot of dissolved extract was transferred to a 10 ml volumetric flask, containing 6 ml ultra-pure water, to which was subsequently added 500 µl undiluted Folin-Ciocalteu reagent. After 1 min, 1.5 ml Na₂CO₃ (20%, w/v) was added and the volume was made up to 10 ml with ultra-pure water. After 30 min incubation at 25°C, the absorbance was measured at 760 nm and compared to a GA calibration curve. Values are presented as the mean ± SE of each three replicates.

High-performance liquid chromatography (HPLC) - Analysis of phenolic compounds

A HPLC system comprising of a vacuum degasser, quaternary pump, autosampler, thermostated column compartment, and photodiode array detector (PDA) was used for the quantification of individual phenolic compounds. The column, Phenomenex C₁₈ 5 µm (250 × 4.6 mm), was maintained at 26°C. Different proportions of solvents such as acetonitrile/water/acetic acid (15:84:0.85) as eluant B and methanol as eluant A were used for the separation. The multiple gradient used for chromatographic separation consisted of different proportions of eluant A/B (50:50 for 1 – 5 min, 40:60 for 5 – 10 min, 30:70 for 10 – 15 min, 15:85 for 15 – 20 min). The flow rate was 1 ml/min, the sample injection volume was 50 µl,

and the chromatogram monitored at 330 nm. The peak purity of the tested sample was determined by comparing its ultraviolet (UV) spectra to that of the reference standards. Quantification was made on the basis of the corresponding peak area recorded by chromatopac c-R6A (Shimadzu). Reference standards were used for the preparation of standard curves.

1,1-diphenyl-2-picrylhydrazyl scavenging assay

The ability of the extracts to scavenge DPPH radical was assessed spectrophotometrically (Gyamfi et al., 1999). Briefly, 50 µl aliquot of the extract (1 mg/ml) was mixed with 450 µl tris-HCl buffer (50 mmol/l, pH 7.4) and 1 ml DPPH (0.1 mmol/l, in methanol), the resultant absorbance was recorded at 517 nm after 30 min incubation at 37°C. The percentage of inhibition was calculated from the following equation.

$$\text{Percentage of inhibition} = [(A_0 - A_1) / A_0] \times 100$$

Where A₀ was the absorbance of the control (blank, without extract) and A₁ was the absorbance in the presence of the extract.

Iron (III) to Iron (II) reduction assay

The reductive capacity of the extracts were assessed using ferric to ferrous reductive activity as determined spectrophotometrically from the formation of Perl's Prussian blue coloured complex (Yildirim et al., 2000). 1 ml of each extract (1 mg/ml) was mixed with 2.5 ml phosphate buffer (0.2 M, pH 7.0) and 2.5 ml of a 1% (w/v) potassiumhexacyanoferrate [K₃Fe (CN)₆] solution. After 30 min incubation at 50°C, 2.5 ml (10%, w/v) trichloroacetic acid was added and the mixture was centrifuged for 10 min (1800 rpm). Finally, 2.5 ml of the upper layer was mixed with 2.5 ml water and 0.5 ml (0.1%, w/v) FeCl₃ and the absorbance was recorded at 700 nm.

Superoxide anion scavenging activity

Superoxide anion scavenging activity of the extracts was validated (Liu et al., 1997). Superoxide anions were generated in a non-enzymatic PMS-NADH system by the oxidation of NADH and assayed by reduction of NBT. The superoxide anion was generated in 3 ml of tris-HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 µM) solution, 1 ml of NADH (78 µM) solution and the methanolic extracts (1 mg/ml). The reaction was initiated by adding 1 ml of PMS solution (10 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance was recorded at 560 nm against blank. A lower absorbance of the reaction mixture indicated a higher superoxide anion scavenging activity. Superoxide anion scavenging activity (SASA) was calculated using the following equation.

$$\text{SASA (\%)} = [1 - (A_1 - A_2) / A_0] \times 100$$

Where A₁ was the absorbance in the presence of the extract and A₀ was the absorbance of the control (blank, without extract), A₂ was the absorbance without PMS.

β-carotene-linoleic acid bleaching

The ability of the extracts to prevent the bleaching of β-carotene was assessed (Koleva et al., 2002). In brief, 0.2 mg β-carotene in 1 ml chloroform, 20 mg of linoleic acid and 200 mg of Tween 20 were transferred into a round-bottom flask. After removal of chloroform, 50 ml distilled H₂O was added and the resulting mixture was stirred vigorously. 6 ml aliquots of the emulsion were transfer-

Table 1. Extract yield, total phenolic content of the *Ocimum* species leaves extract

Extract	EY* (mg/g of dry leaf)	TPC** (mg Gallic acid equivalents/g DE)
<i>Ocimum gratissimum</i>	110.1	168.2±3.2 ^a
<i>Ocimum americanum</i>	86.3	123.1± 2.3 ^b
<i>Ocimum minimum</i>	73.2	110.4±3.1 ^b
<i>Ocimum citriodorum</i>	69.1	96.3±3.0 ^c
<i>Ocimum kilimandscharicum</i>	58.5	82.5±2.1 ^d
<i>Ocimum grandiflorum</i>	52.3	61.2±2.3 ^e
<i>Ocimum lamiifolium</i>	41.8	54.6±2.1 ^e
<i>Ocimum selloi</i>	32.1	42.1±2.6 ^f

Values are presented as mean ± SE (n=3).

Values with lower case letters (a-f) are significantly (P < 0.05) different but values sharing a common letter are not significantly (P > 0.05) different.

* - extract yield

** – Total Phenolic content

DE – dry extract

red to tubes containing either 50 µl (1 mg/ml) extract or 50 µl (1 mg/ml) BHA. After mixing, an aliquot was transferred into a cuvette and the absorbance (Abs⁰) at 470 nm was recorded. The remaining samples were placed in a water bath at 50°C for a period of 2 h. Thereafter, the absorbance of each sample was measured at 470 nm (Abs¹²⁰). The data (n=3) are presented as antioxidant activity % (AA%) values, calculated using the equation

$$AA\% = [1 - (Ab^0_{\text{sample}} - Abs^{120}_{\text{sample}}) / (Ab^0_{\text{control}} - Abs^{120}_{\text{control}})] \times 100$$

Statistical analysis

All data were expressed as mean ± SE of the number of experiments (n = 3). The statistical significance was evaluated by one-way ANOVA, and significance of the individual comparison was obtained by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

The extract yields ranged between 32.1 (*O. selloi* extract) to 110.1 mg/g of dry leaf (*O. gratissimum* extract), and increased in the order of *O. selloi* < *O. lamiifolium* < *O. grandiflorum* < *O. kilimandscharicum* < *O. citriodorum* < *O. minimum* < *O. americanum* < *O. gratissimum* (Table 1). Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. It was reasonable to determine their total amounts of phenolics in the *Ocimum* species. The total phenolic content was estimated by the Folin-Ciocalteu reagent method, ranged between 42.1 ± 3.1 mg GA/g dry extract (*O. selloi* extract) to 168.2 ± 3.2 mg GA/g dry extract (*O. gratissimum* extract), and increased in the order as mentioned above (Table 1).

Ocimum species has been extensively reported for its essential oil content (Roberto et al., 2003), however, the antioxidant capacity of the plant extracts is mainly dependent on phenolic compounds. Phenolic compounds are an important group of secondary metabolites, which are synthesized by plants due to plant adaptation in response

to biotic and abiotic stresses (infection, water stress, cold stress, high visible light) (Pitchersky and Gang, 2000). So far, in the *Ocimum* species the *Ocimum basilicum* and *O. sanctum* have been reported for their secondary metabolite content (Javanmardi et al., 2002; Hakkim et al., 2007). In this study, for the first time, the quantification of individual phenolic compounds of *Ocimum* species (*O. gratissimum*, *O. americanum*, *O. minimum*, *O. citriodorum*, *O. kilimandscharicum*, *O. grandiflorum*, *O. lamiifolium*, *O. selloi*) leaves extracts was accomplished using high-performance liquid chromatography coupled with PDA detector, is presented in Table 2. Phenolic compounds were identified and quantified at 330 nm as phenolic acids, hydroxycinnamates and flavonoids. The components rosmarinic acid, lithospermic acid, vanillic acid, *p*-coumaric acid, hydroxybenzoic acid, syringic acid, caffeic acid, ferulic acid, cinnamic acid, dihydroxy phenyllactic acid, and sinapic acid (Figure 1) were identified by comparison to the retention time and UV spectra of authentic standards and quantitative data were calculated based on their peak area.

In this study, rosmarinic acid was the most abundant component identified in *O. gratissimum* extract in comparison with other species and other compounds (range = 0.22 to 0.06 mg/g dry wt). This is in general agreement with previously published qualitative-quantitative analyses of Lamiaceae species (Shan et al., 2005). This is also in agreement with Jamal Javanmardi and his coauthors, who reported that rosmarinic acid, is the most abundant component in *O. basilicum* (Javanmardi et al., 2002). The other components such as lithospermic acid (range = 0.02 to 0.10 mg/g dry wt), vanillic acid (range = 0.02 to 0.09 mg/g dry wt), *p*-coumaric acid (range = 0.20 to 0.12 mg/g dry wt), hydroxybenzoic acid (range = 0.11 to 0.20 mg/g dry wt), syringic acid (range = 0.03 to 0.07 mg/g dry wt), caffeic acid (range = 0.02 to 0.05 mg/g dry wt), ferulic acid (range = 0.03 to 0.12 mg/g dry wt), cinnamic acid (range = 0.02 to 0.05 mg/g dry wt), dihy-

Table 2. Extract yield, total phenolic content and quantitative analysis of secondary metabolite content of the *Ocimum* species leaves extract

Extract	RA (mg/g DE)	LA (mg/g DE)	VA (mg/g DE)	pCA (mg/g DE)	HBA (mg/g DE)	SA (mg/g DE)	CA (mg/g DE)	FA (mg/g DE)	CIA (mg/g DE)	DHPA (mg/g DE)	SPA (mg/g DE)
<i>Ocimum gratissimum</i>	0.22±0.03 ^a	0.09±0.01 ^a	0.04±0.01 ^b	0.18±0.02 ^a	0.14±0.03 ^c	ND	0.02±0.001 ^b	0.11 ±0.02 ^a	0.04±0.01 ^a	0.06±0.01 ^b	0.02±0.001 ^b
<i>Ocimum americanum</i>	0.15±0.02 ^b	0.10±0.03 ^a	0.09±0.02 ^a	0.16±0.06 ^b	0.16±0.02 ^b	0.07±0.001 ^a	0.05±0.002 ^a	0.12 ±0.03 ^a	0.05±0.02 ^a	ND	0.04±0.01 ^a
<i>Ocimum minimum</i>	0.11±0.02 ^c	0.06±0.02 ^b	0.06±0.01 ^b	0.17±0.01 ^b	0.18±0.01 ^b	ND	ND	0.08±0.01 ^b	0.03±0.001 ^b	0.09±0.002 ^a	ND
<i>Ocimum citriodorum</i>	0.08±0.02 ^c	0.03±0.01 ^c	0.05±0.02 ^b	0.19±0.03 ^a	0.20±0.05 ^a	ND	ND	0.05±0.02 ^c	0.02±0.001 ^b	ND	ND
<i>Ocimum kilimandsch aricum</i>	0.04±0.01 ^d	0.02±0.01 ^d	0.02±0.01 ^c	0.15±0.02 ^b	0.16±0.02 ^b	0.03±0.001 ^b	0.04±0.001 ^a	0.03±0.001 ^d	ND	ND	0.01±0.0008 ^b
<i>Ocimum grandiflorum</i>	0.13±0.04 ^b	0.07±0.02 ^b	0.04±0.02 ^b	0.20±0.06 ^a	0.17±0.03 ^b	0.04±0.002 ^b	0.02±0.001 ^b	ND	ND	0.04±0.001 ^c	0.02±0.001 ^b
<i>Ocimum lamiifolium</i>	0.10±0.02 ^c	0.04±0.01 ^c	0.03±0.01 ^c	0.17±0.03 ^b	0.14±0.02 ^c	ND	ND	ND	0.02±0.001 ^b	ND	ND
<i>Ocimum selloi</i>	0.06±0.01 ^c	0.03±0.02 ^c	ND	0.12±0.04 ^c	0.11±0.01 ^d	ND	ND	ND	ND	ND	ND

Values are presented as mean ± SE (n=3)

Values with lower case letters (a-d) are significantly (P < 0.05) different but values sharing a common letter are not significantly (P > 0.05) different.

DE – dry extract

ND – not detected

RA- Rosmarinic acid, LA- Lithospermic acid, VA- Vanilic acid, pCA- p-Coumaric acid, HBA- Hydroxy benzoic acid, SA-Syringic acid, CA-Caffeic acid, FA- Ferulic acid, CIA-Cinnamic acid, DHPA- Dihydroxy phenyllactic acid, SPA-Sinapic acid

dihydroxy phenyllactic acid (range = 0.04 to 0.09 mg/g dry wt), and sinapic acid (range = 0.01 to 0.04 mg/g dry wt) were found. The variation in the distribution of individual phenolic compounds in different *Ocimum* species is possibly due to (1) genotypic and environmental differences (2) choice of parts tested, (3) time of sample collection, and (4) determination methods. It could also be due to the solvent used for the extraction that is, the extraction yield, nature of the compounds and materials from which the compounds were

extracted are strongly dependent on the solvents due to the presence of different concentrations of bioactive compounds with different polarities. Similarly, in *Diospyros kaki*, the isolation of triterpene acids (barbinervic acid, rotungenic acid and 2,4-dihydroxyursolic acid) is strongly influenced by the solvent system (Fan and He, 2006).

Free radicals involved in the process of lipid peroxidation, play a cardinal role in numerous chronic diseases and are implicated in premature ageing. The DPPH· molecule contains stable free

radical which has been widely used to evaluate the radical scavenging ability of antioxidants. It is possible to determine the antiradical potential of antioxidants by measurement of the decrease in the absorbance of DPPH· at 517 nm. Thus, it was considered important to screen the *Ocimum* species extracts for antioxidant activity against DPPH radical.

As can be seen in Figure 2A, at a dose of 1 mg/ml, all the *Ocimum* species extracts were capable of scavenging DPPH free radicals. From the

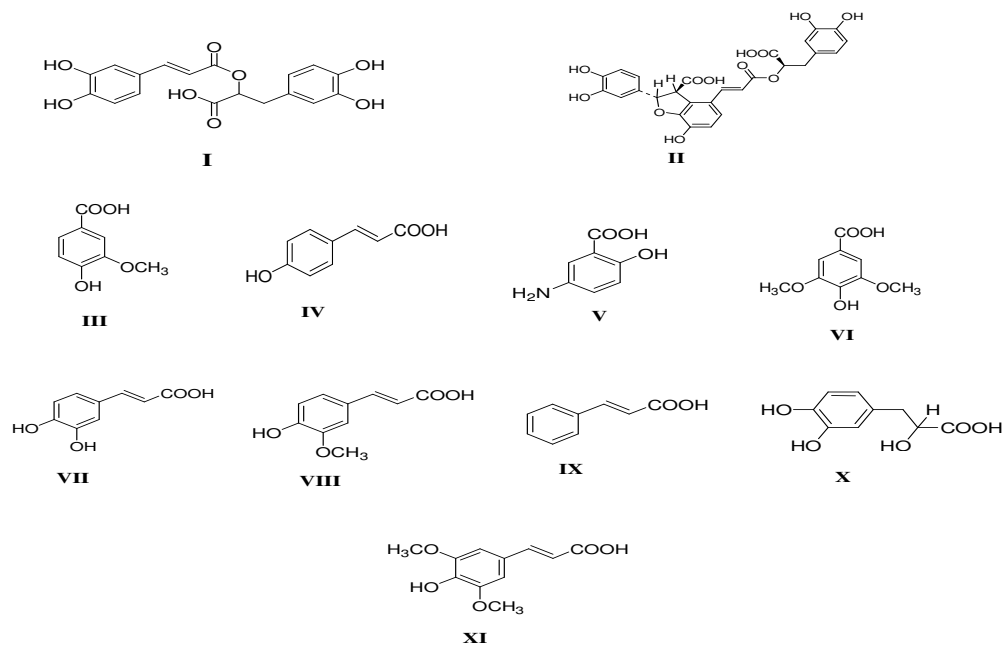


Figure 1. Structural formulae of the compounds identified in *Ocimum* species leaves extract. I. Rosmarinic acid; II. Lithospermic acid; III. Vanillic acid; IV. *p*-Coumaric acid; V. Hydroxybenzoic acid; VI. Syringic acid; VII. Caffeic acid; VIII. Ferulic acid; IX. Cinnamic acid; X. Dihydroxyphenyllactic acid; XI. Sinapic acid.

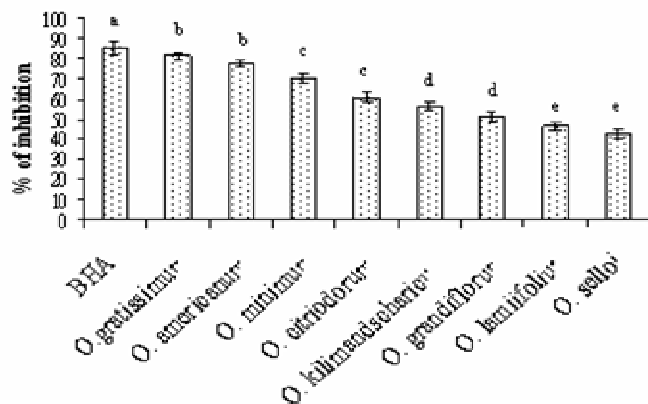


Figure 2A. The effect of *Ocimum* species leaves extracts and Butylated hydroxy anisole (BHA) on DPPH radical scavenging activity. Values are presented as mean \pm SE (n = 3). Bars with lower case letters (a-f) are significantly ($P < 0.05$) different. Values sharing a common letter are not significantly ($P > 0.05$) different.

the percentage scavenging values, it can be seen that the *O. gratissimum* extract was the most potent scavenger ($81.1 \pm 2.1\%$) followed by *O. americanum* ($77.4 \pm 1.4\%$) (statistically indistinguishable, $P > 0.05$), $> O. minimum$ ($70.1 \pm 2.2\%$) $> O. citriodorum$ ($60.6 \pm 2.5\%$) (statistically indistinguishable, $P > 0.05$), $> O. kilimandscharicum$ ($56.2 \pm 2.1\%$) $> O. grandiflorum$ ($51.3 \pm 2.3\%$) (statistically indistinguishable, $P > 0.05$), $> O. lamiiifolium$ ($46.2 \pm 2.2\%$) $> O. selloi$ ($42.4 \pm 2.4\%$) (statistically indis-

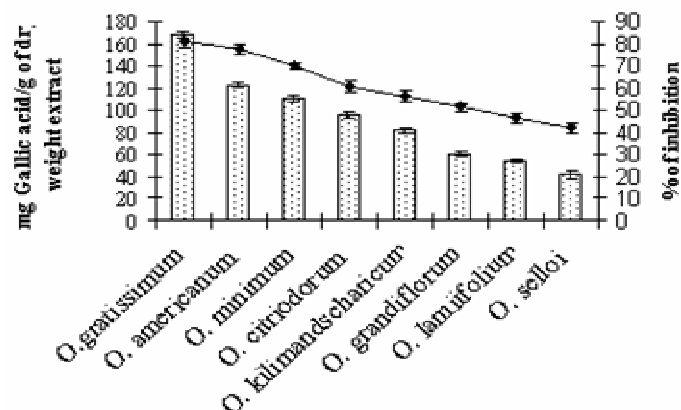


Figure 2B. Relationship of DPPH radical scavenging activity and total phenolic content of *Ocimum* species leaves extracts. Values are presented as mean \pm SE (n = 3).

tinguishable, $P > 0.05$). None of the *Ocimum* species extracts were as effective DPPH \cdot scavengers as the positive control BHA ($85.2 \pm 3.2\%$). Further in this study, a good linear correlation ($R^2 = 0.962$) was obtained between the concentration of total phenolic content and the scavenging activity of DPPH \cdot radical of each extract (Figure 2B). These results indicated that the radical scavenging capacity of each extract might be related to the concentration of phenolics. The antiradical activity of phenolic compounds depend on their molecular structure

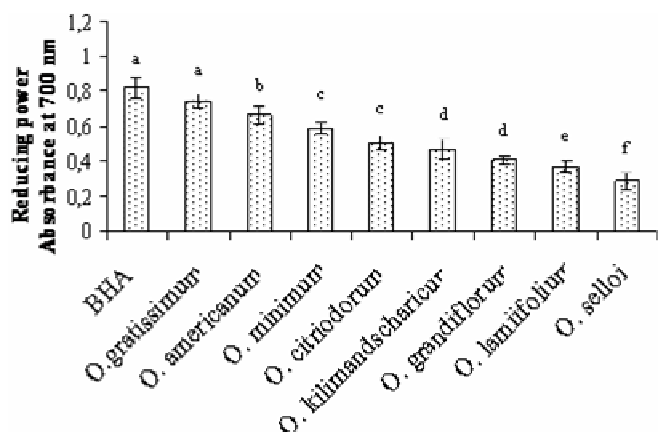


Figure 3. The effect of *Ocimum* species leaves extracts and Butylated hydroxy anisole (BHA) on iron (III) reduction. Values are presented as mean \pm SE ($n = 3$). Bars with lower case letters (a-f) are significantly ($P < 0.05$) different. Values sharing a common letter are not significantly ($P > 0.05$) different.

structure, that is, on the availability of phenolic hydrogens, which result in the formation of phenoxyl radicals due to hydrogen donation (Ramarathnam et al., 1997). A linear correlation between radical scavenging activity and phenolic concentration has been reported in an extensive range of vegetables, fruits, and beverages (Velioglu et al., 1998). The multitude of environmental factors such as mean temperature, soil, wind speed, low and high temperature, and UV radiation from the sunlight may influence the phenolics accumulation in the plants. In this study we have harvested the leaves from the matured *Ocimum* species in day time (temperature 27 – 33°C). Possibly this temperature of the day light may influence the accumulation of phenolics by inducing the plant leaves to biosynthesize more polyphenols and serves as a filtration mechanism against UV radiation.

The results of the DPPH \cdot free radical scavenging assay suggest that components within the extracts are capable of scavenging free radicals via electron or hydrogen-donating mechanisms and thus should be potent enough to prevent the initiation of deleterious free radical mediated chain reactions in susceptible matrices e.g. biological membranes.

The expression of antioxidant activity is thought to be concomitant with the development of reductones, as these species are known to be free radical chain terminators (Gordon, 1990). Therefore, it was considered important to determine the reductive capacity of the *Ocimum* species extracts as this may indicate their potential as antioxidants. The presence of reductant (antioxidants) in the tested samples would result in the reduction of Fe³⁺/ferricyanide complex to the ferrous form (Fe²⁺). The ferrous ion can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung et al., 2002).

As can be seen from Figure 3, the *O. gratissimum* ex-

tract was the most effective at reducing the iron (III), with an absorbance reading of 0.74 ± 0.04 . The hierarchy of activity for the remaining extracts can be seen as *O. americanum* (0.66 ± 0.05) > *O. minimum* (0.59 ± 0.03) > *O. citriodorum* (0.51 ± 0.04) (statistically indistinguishable, $P > 0.05$), > *O. kilimandscharicum* (0.47 ± 0.06) > *O. grandiflorum* (0.41 ± 0.02) (statistically indistinguishable, $P > 0.05$), > *O. lamiifolium* (0.37 ± 0.03) > *O. selloi* (0.29 ± 0.05) (statistically distinguishable, $P < 0.05$). None of the extracts was as effective as the positive control BHA (0.82 ± 0.12). Despite the inability of the *Ocimum* species extracts to compete with the positive control in reducing iron (III) ions, the components within the extracts did possess considerable activity and may be considered as potential preservatives where such property would be beneficial, especially in the case of the *O. gratissimum* extract.

The superoxide anion indirectly initiates lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radicals (Meyer and Isaksen, 1995). Therefore, in the present investigation, it was considered important to characterize the scavenging ability of *Ocimum* species extracts against the superoxide anion. In the PMS–NADH–NBT system, the superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction reduces NBT. The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture.

As can be seen in Figure 4, all the extracts were capable of scavenging of superoxide anions. The scavenging order was *O. gratissimum* (79.2 ± 2.4) > *O. americanum* ($72.4 \pm 3.1\%$) (statistically indistinguishable, $P > 0.05$), > *O. minimum* ($64.8 \pm 1.9\%$) > *O. citriodorum* ($57.3 \pm 1.1\%$) (statistically indistinguishable, $P > 0.05$), > *O. kilimandscharicum* ($51.9 \pm 2.2\%$) > *O. grandiflorum* ($46.4 \pm 1.8\%$) (statistically indistinguishable, $P > 0.05$), > *O. lamiifolium* ($38.9 \pm 2.2\%$) > *O. selloi* ($32.3 \pm 2.1\%$) (statistically distinguishable, $P < 0.05$). None of the extracts was as effective as the positive control BHA ($93.2 \pm 2.1\%$). The data reveals that the extracts are capable of scavenging superoxide anion radicals in this *in vitro* system. Recent studies have shown that phenolic compounds, particularly flavonoids and phenolic acids are important superoxide scavengers (Benavente-Garia et al., 1997). The observed scavenging efficacy of the extracts is reminiscent of the activity of superoxide dismutase.

Iron (III) reduction and synthetic free radical scavenging models are valuable tools to indicate the potential antioxidant activity of plant extracts, however, these systems do not use a food or biologically relevant oxidizable substrate so no direct information on an extract's protective action can be determined (Dorman et al., 2003). Therefore, it was considered important to assess the inhibitory effect of *Ocimum* species extracts on oxidation of β -carotene. In this assay, oxidation of linoleic acid produces hydroperoxide-derived free radicals, which

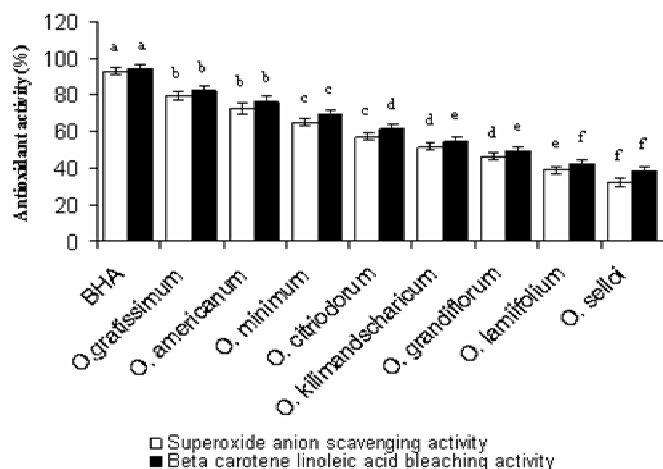


Figure 4. The effect of *Ocimum* species leaves extracts and Butylated hydroxy anisole (BHA) on superoxide anion scavenging activity and β -carotene-linoleic acid bleaching assay. Values are presented as mean \pm SE (n = 3). Bars with lower case letters (a-f) are significantly ($P < 0.05$) different. Values sharing a common letter are not significantly ($P > 0.05$) different.

attack the chromophore of β -carotene, resulting in a bleaching of the reaction emulsion. An extract capable of retarding/inhibiting the oxidation of β -carotene may be described as a free radical scavenger and primary antioxidant (Liyana-Pathirana and Shahidi, 2006).

As can be seen in Figure 4, all the extracts were capable of inhibiting the bleaching of β -carotene by scavenging linoleate-derived free radicals. The order of efficacy was *O. gratissimum* (82.4 ± 2.4) > *O. americanum* (76.5 ± 2.5) (statistically indistinguishable, $P > 0.05$), > *O. minimum* (69.2 ± 2.3) > *O. citriodorum* (61.8 ± 2.1) (statistically distinguishable, $P < 0.05$), > *O. kilimandscharicum* (54.6 ± 2.4) > *O. grandiflorum* (49.2 ± 2.6) (statistically indistinguishable, $P > 0.05$), > *O. lamiifolium* (42.4 ± 2.3) > *O. selloi* (38.7 ± 2.1) (statistically indistinguishable, $P > 0.05$). None of the extracts was as effective as the positive control BHA (94.5 ± 2.1 %).

It has been suggested that the polarity of an extract is important in water: oil emulsions, viz., non-polar extracts are more effective antioxidants than polar extracts due to a 'concentrating effect' within the lipid phase (Porter, 1986). Contrary in this work we have used methanol for extraction and appreciable inhibition of bleaching of β -carotene by scavenging linoleate-derived free radicals was observed. This phenomenon was in agreement with Koleva and his coauthor, who reported inhibition of bleaching of β -carotene by polar extract of *Sideritis* species (Koleva et al., 2003). According to the β -carotene-linoleic acid bleaching data, the extracts are capable of scavenging free radicals in a complex heterogeneous medium. This suggests that the extracts may have potential use as antioxidative preservatives in emulsion-type systems.

In conclusion, the phytochemical analysis of *Ocimum*

species extracts has led to the identification of 11 phenolic compounds (rosmarinic acid, lithospermic acid, vanillic acid, *p*-coumaric acid, hydroxybenzoic acid, syringic acid, caffeic acid, ferulic acid, cinnamic acid, hydroxyl phenyl-lactic acid, and sinapic acid), which have been described for the first time in these plants. Furthermore, all the plant extracts were found to exhibit a good anti-oxidant activity in the selected in vitro antioxidant assays. Overall, the extracts tend to possess lesser activity in comparison to positive control BHA. Potential activity of all these extracts in the tested antioxidant assays is a promising factor for their application as an effective preservative for the food and cosmetic industries. However, further research would be required before such claims could be proposed with confidence.

REFERENCES

- Benavente-Garia O, Castillo J, Marin FR, Ortuno A, Del- Rio JA (1997). Use and properties of citrus flavonoids. J. Agric. Food Chem. 45: 4505–4515
- Caragay AB (1992). Cancer preventive foods and ingredients. Food Technol. 56: 65–68
- Chatterje A, Sukul NC, Laskal S, Ghoshmajumdar S (1982). Nematicidal principles from two species of Lamiaceae. J. Nematol. 14: 118–120
- Chung YC, Chang CT, Chao WW, Lin CF, Chou ST (2002). Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. J. Agric. Food. Chem. 50: 2454–2458
- Deshpande RS, Tipnis HP (1997). Insecticidal activity of *Ocimum basilicum* L. Pesticides. 11: 1–12
- Dorman HJD, Kosar M, Kahlos K, Holm Y, Hiltunen R (2003). Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties and cultivars. J. Agric. Food Chem. 51(16):4563–4569
- Fan JP, He CH (2006). Simultaneous quantification of three major bioactive triterpene acids in the leaves of *Diospyros kaki* by high performance liquid chromatography method. J. Pharmacol. Biomedicine Anal. 41: 950–956
- Gang DR, Wang J, Dudareva N, Nam KH, Simon JE, Lewinsohn E, Pichersky E (2001). An investigation of the storage and biosynthesis of phenylpropanes in sweet basil. Plant Physiol. 125: 539–555
- Gordon MH (1990). The mechanism of antioxidant action in vitro. In: Hudson BJJ (eds) Food antioxidants: London/New York. Elsevier, pp 1–8.
- Gyamfi MA, Yonamine M, Aniya Y (1999). Free-radical scavenging action of medicinal herbs from Ghana: *Thonningia sanguinea* on experimentally-induced liver injuries. Gen. Pharmacol. 32(6): 661–667
- Hirasa K, Takemasa M (1998). Spice science technology: New York: Marcel Dekker.
- Jamal Javanmardi, Khalighi A, Kashi A, Bais HP, Vivanco JM (2002). Chemical characterization of Basil (*Ocimum basilicum* L.) found in local accessions and used in traditional medicines in Iran. J. Agric. Food Chem. 50: 5878–5883.
- Koleva II, Linssen JPH, Van Beek TA, Evstatieva LN, Kortenska V, Handjieva N (2003). Antioxidant activity screening of extracts from *Sideritis* species (Labiatae) grown in Bulgaria. J. Sci. Food Agric. 83(8): 809–819
- Koleva II, Van Beek TA, Linssen JPH, De Groot A, Evstatieva LN (2002). Screening of plant extracts for antioxidant activity: A comparative study on three testing methods. Phytochemical Anal. 13(1): 8–17.
- Liu F, Ooi VEC, Chang ST (1997). Free radical scavenging activity of mushroom polysaccharide extracts. Life Sci. 60: 763–771.
- Liyana-Pathirana CM, Shahidi F (2006). Antioxidant properties of commercial soft and hard winter wheats (*Triticum aestivum* L.) and

- their milling fractions. *J. Sci. Food Agric.* 86(3): 477–485
- Lukmanul Hakkim F, Gowri Shankar C, Girija S (2007). Chemical composition and antioxidant property of holy basil (*Ocimum sanctum* L.) leaves, stems, and inflorescence and their in vitro callus cultures. *J. Agric. Food Chem.* 55: 9109–9117
- Lutomski J (2001). Znaczenie ziół terapii i diety. *Wiadomości Zielarskie*. 10: 4–5
- Meyer AS, Isaksen A (1995). Application of enzymes as food antioxidants. *Trends Food Sci. Technol.* 6: 300–304
- Ozkan G, Sagdic O, Ozcan M (2003). Inhibition on pathogenic bacteria by essential oils at different concentrations. *Food Sci. Technol. Intel.* 9(2): 85–88
- Pitchersky E, Gang DR (2000). Genetics and biochemistry of secondary metabolites in Plants: An evolutionary perspective. *Trends Plant Sci.* 5: 459–445
- Porter WL (1986). Recent trends in food applications of antioxidants. In: Simic MG, Karel M (eds) *Autooxidation in food and biological systems*: New York. Plenum Press, pp 295–365.
- Ramarathnam N, Ochi H, Takeuchi M (1997). Antioxidant defense system in vegetable extracts. In: Shahidi F (eds). *Natural Antioxidants; Chemistry, Health Effects and Application*: Champaign IL. AOAC Press, pp 76–87.
- Roberto F, Vieira, Renee J, Grayer, Alan JP (2003). Chemical profiling of *Ocimum americanum* using external flavonoids. *Phytochemistry*. 63: 555–567
- Shan B, Cai YZ, Sun M, Corke H (2005). Antioxidant capacity of 26 spice extracts and characterisation of their phenolic constituents. *J. Agric. Food Chem.* 53(20): 7749–7759
- Simon JE, Morales MR, Phippen WB, Vieira RF, Hao Z (1999). Basil: a source of aroma compounds and a popular culinary and ornamental herb. In: Janick J (eds) *Perspectives on new crops and new uses*: Alexandria VA. ASHS Press, pp 499–505.
- Singleton VL, Orthofer R, Lamuela-Raventós RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In: Packer L (eds). *Methods in enzymology*: San Diego CA. Academic Press, pp 152–178.
- Szeto YT, Tomlinson B, Benzie IF (2002). Total Antioxidant and ascorbic acid content of fresh fruits and vegetables: Implications for Dietary Planning and Food preservation. *British J. Nutr.* 87: 55–59
- Velioglu YS, Mazza G, Gao L, Oomach BD (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *J. Agric. Food. Chem.* 46: 4113–4117
- Yildirim A, Mavi A, Oktay M, Kara AA, Algur OF, Bilaloglu V (2000). Comparison of antioxidant and antimicrobial activities of tilia (*Tilia argentea* Desf ex DC), sage (*Salvia triloba* L.), and black tea (*Camellia sinensis*) extracts. *J. Agric. Food Chem.* 48(10): 5030–5034