

Antioxidant property of leaves and calluses extracts of *in-vitro* grown 5 different *Ocimum* species

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Received: 7 September 2012 / Accepted: 14 September 2012

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Abstract In this study, the antioxidant property of leaf and callus extracts of five selected *in vitro* grown *Ocimum* species (*Ocimum sanctum*, *Ocimum kilimandscharicum*, *Ocimum gratissimum*, *Ocimum basilicum*, and *Ocimum americanum*) and their respective callus extracts was investigated. The callus cultures were successfully initiated on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) (1 mg·L) combined with different concentrations (0.1–0.4 mg·L) of kinetin as plant growth regulators. Total phenolic contents were estimated using the Folin-Ciocalteu reagent. 1,1-diphenyl-2-

picrylhydrazyl (DPPH) radical scavenging, ferric reducing antioxidant power, Fe²⁺ chelating activity, and β-carotene-linoleic acid bleaching assays were used to determine the biological effects of the extracts. Interestingly, all the callus extracts exhibited significant (p<0.05) increase in phenolic contents and antioxidant activity. Furthermore, a liner correlation was obtained between the total phenolic contents and free radical scavenging activity (R² = 0.783). The extracts of leaves and calluses of *Ocimum* species exhibited activity in all the *in vitro* antioxidant assays, but its extent was less potent than the positive controls butylated hydroxyl anisole (BHA) and ascorbic acid. A higher accumulation of phenolics in the callus extracts suggests that isolation of high-concentration materials with antioxidant activity is possible from *in vitro* callus cultures rather than field-grown plant organs. Furthermore, these extracts may be used as an effective preservative in the food industry.

Keywords DPPH, *Ocimum kilimandscharicum*, *Ocimum gratissimum*, *Ocimum basilicum*, *Ocimum americanum*, phenolics

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Introduction

Chemical constituents that have antioxidant properties in high concentrations in plants can play a role in the prevention of various degenerative diseases (Velioglu et al. 1998). In recent years, research efforts have been focused on the possibilities utilizing natural sources of bioactive compounds for the dietary management of certain chronic diseases such as diabetes, obesity, cardiovascular diseases, and cancer (Vadivel and Biesalski 2011). Throughout the past history, spices and herbs have been used to flavor foods and beverages, as well as for medicinal purposes. The preservative effect of many plant species and herbs

suggests the presence of antioxidative and antimicrobial constituents (Hirasa and Takemasa 1998). Besides fruits and vegetables, herb or medicinal plants are recommended at present as optimal sources of such components. The supplementation of the human diet with herbs, containing especially high amounts of compounds capable of deactivating free radicals, may have beneficial effects (Lutomski 2002). Among the important constituents in plants, phenolic compounds have been reported to be involved in cell defence 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 family. They are rising in popularity and in interest among food manufacturers, as consumers increasingly seek after functional foods with specific health effects (Ozkan et al., 2003). Generally, antioxidant activity is assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), Fe^{2+} chelating activity assay, and β -carotene-linoleic acid bleaching methods (Gyamfi et al. 1999; Benzie and Strain 1996; Dinis et al. 1994; Koleva et al. 2002). DPPH method uses a free radical scavenging ability of chemicals or materials using in animal and plant cells, and FRAP method assessed the total reducing power of the electron donating substances from the chemicals or tested materials in the cultured cell. Fe^{2+} chelating activity can be measured the activity of Fe^{2+} -ferrozine complex formation, indicates oxidation of molecules, and β -carotene-linoleic acid bleaching method can determine the colour bleaching level of the tested samples on β -carotene. These methods are widely used to determine the ability of antioxidant.

The genus *Ocimum*, a member of the Lamiaceae family, contains 200 species of herbs and shrubs (Simon et al., 1999). This species boasts of long history as culinary herbs, due to its foliage adding a distinctive flavour to many foods. It is also a source of aromatic compounds and essential oils containing biologically active constituents that possess insecticidal and nematicidal properties (Chatterjee et al. 1982; Deshpande and Tipins 1977). In our previous study, we selected the *ocimum* species such as *Ocimum sanctum*, *Ocimum kilimandscharicum*, *Ocimum gratissimum*, *Ocimum basilicum*, and *Ocimum americanum* to test their antioxidant ability and found significant free radical scavenging ability (Hakkim et al. 2008). Generally, the callus biomass accumulates high amounts of phenolics compared to their *in vivo* counter parts. We demonstrated this from a previous study, in which *Ocimum sanctum* leaf callus cultures showed an accumulation of maximum level of phenolics

than *in vivo* leaves (Hakkim et al. 2007).

Although we showed that leaves of *Ocimum kilimandscharicum*, *Ocimum gratissimum*, *Ocimum basilicum*, and *Ocimum americanum* have antioxidant (AO) ability, whether their respective callus biomass contains AA remains elusive. In this study, we included the *O. Sanctum* callus cultures also as a positive control. To the best of our knowledge, our study is the first to comparatively analyze the *in vivo* and *in vitro* AO ability of different *Ocimum* species. Since the *Ocimum* species extract is one of the important herbal formulations in ayurvedic medicine as a tonic for treating many diseases, this study adds further value for the possible use of these plants, especially the *in vitro*-induced callus extract as a food additive.

Materials and methods

Plant source

The plant materials of *Ocimum sanctum*, *Ocimum americanum*, *Ocimum basilicum*, *Ocimum kilimandscharicum*, *Ocimum gratissimum* were collected from local farms and were grown at the department of Biotechnology, Bharathiar University, and Coimbatore, India. Green houseplants were irrigated to pot daily and maintained at day/night temperatures of 27-33 and 20-25°C respectively. The leaves was harvested from fully matured plants and kept for air-drying at room temperature for 7 days for chemical analysis.

Callus induction

Leaf explants from 1-year-old *Ocimum sanctum*, *Ocimum americanum*, *Ocimum basilicum*, *Ocimum kilimandscharicum*, *Ocimum gratissimum* plants were surface-sterilized for 3-5 min in Tween 80 and for 3 min with 0.1% (w/v) HgCl and then rinsed three times with sterilized distilled water. Sterilized explants were cut into 1 cm-long pieces and cultured on Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 3 % sucrose (analytical grade) as a carbon source and 0.8% agar (Himedia Laboratories, Mumbai, India) for gelling, and growth regulators such as 2,4-D (1 mg·L) combined with different concentrations (0.1-0.4 mg·L) of kinetin were used individually. The cultures were maintained at 25±2°C under 12 hr alternative dark and light (1,000-2,000 lux) cycles. Culture responses of each explant for callus induction were recorded after 8 weeks of culture.

Extraction (*In Vivo*)

The five varieties of *Ocimum species* plant leaves were collected, shade dried for a period of 10 days, grounded into powder. 10 mg 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.

Extraction (*In vitro*)

The callus induced on MS medium fortified with 2,4-D (1 mg·L) combined individually with varying concentrations (0.1–0.4 mg·L) of kinetin were harvested, dried, and the dry weights were determined (Szabo et al. 1999). Extraction was performed as described above.

Determination of total phenolic Content

Total phenolic contents were determined as Gallic acid equivalents (GAE) (Singleton et al. 1999). The dry extract (30 mg) was diluted in 1 ml of methanol, and 100 µl of the solution was transferred to a 10 ml volumetric flask, to which 0.5 ml undiluted Folin-Ciocalteu reagent was added. After a minute, 1.5 ml of 20% (w/v) Na₂CO₃ was added and the volume reconstituted in 10 ml of water. After one hour incubation at 25°C, the absorbance was measured at 760 nm and compared to a pre-prepared Gallic acid calibration curve.

DPPH radical scavenging assay

The ability of the extracts to scavenge the 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. Butylated hydroxy anisole (BHA) (0.2 mg·ml) and ascorbic acid (0.2 mg·ml) served as control.

Ferric reducing antioxidant power (FRAP) assay

The FRAP, a method for measuring the total reducing power of the electron donating substances, was assessed according to Benzie and Strain (1996). Briefly, 6 ml of freshly prepared working FRAP reagent (0.1 M acetate buffer: 0.02 M FeCl₃:0.01 M TPTZ =10:1:1) was mixed with 20 µl of extract sample (1mg·ml). The absorbance at 593 nm was recorded after a 30-min incubation at 37°C. (BHA) (0.2 mg·ml) and ascorbic acid (0.2 mg·ml) served as control.

Fe²⁺ chelating activity assay

The chelating activity of extracts for ferrous ions Fe²⁺ was determined (Dinis et al., 1994). Briefly, to 0.5 ml of the extract (1 mg·ml), 1.6 ml of distilled water and 0.05 ml of FeCl₂ (2 mM) was added and after 30 s, 0.1 ml ferrozine (5 mM) was added. Then the reaction mixture was incubated for 10 min at room temperature, and the absorbance of the Fe²⁺-Ferrozine complex was measured at 562 nm. A lower absorbance indicates a higher chelating power. The chelating activity of the extracts on Fe²⁺ was compared with that of EDTA (0.01 mM) and Citric acid (0.025 M).

The chelating activity was calculated according to the following equation:

$$\text{Chelating activity (\%)} = [(A_0 - A_1) / A_0] \times 100$$

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

β-carotene-linoleic acid bleaching

The ability of the extracts to prevent the bleaching of β-carotene was assessed (Koleva et al. 2002). Briefly, 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 transferred to tubes containing either 50 µl (1 mg·ml) extract or 50 µl (0.2 mg·ml) BHA or 50 µl (0.2 mg·ml) ascorbic acid. 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 (Abs^{120}). The data ($n=3$) are presented as antioxidant activity % (AA%) values, calculated using the equation:

$$AA\% = \left[\frac{1 - (Ab_{\text{sample}}^0 - Abs_{\text{sample}}^{120})}{(Ab_{\text{control}}^0 - Abs_{\text{control}}^{120})} \right] \times 100$$

Statistical Analysis

All data were expressed as mean \pm SD of the number of experiments ($n=3$). Two-way analysis of variance (ANOVA) was performed using SPSS software to analyze the statistical significance. The level of significance was considered at $p<0.05$.

Results and discussion

Callus Induction

Most naturally-occurring antioxidants are secondary metabolites which include alkaloids, flavonoids, and phenolic acids. The antioxidant activity of these compounds is predominantly determined by their structures, particularly the presence of hydroxyl groups over the aromatic nucleus. While the intact plant organs contain secondary metabolites, the culture of undifferentiated cells (*calli*) are a potent source for the production of wide variety of secondary metabolites (Verpoorte and Alferman, 2000). In this study, the callus cultures were successfully induced from 5 different *Ocimum* species of leaves, grown on MS medium containing 2,4-D (1 mg·L) combined with kinetin (0.1 - 0.4 mg·L), to assess its chemical composition and antioxidant ability. Interestingly, the culture response was found to vary according the concentration of kinetin supplementation (Table 1). *O. sanctum* showed maximum response with supplementation of 2,4-D (1 mg·L) and

kinetin (0.1 mg·L), while *O. basilicum* showed maximum response with supplementation of 2,4-D (1 mg·L) and kinetin (0.2 mg·L). Furthermore, *O. americanum*, *O. gratissimum*, and *O. kilimandscharicum* exhibited maximum response with supplementation of 2,4-D (1 mg·L) and kinetin (0.3 mg·L). This variation in the culture response may be due to the difference in inter-species metabolism upon kinetin treatment.

Total phenolic content

In the present study, we evaluated the total phenolic content of the methanolic extract of the five varieties of *Ocimum* species in leaves and their respective callus cultures induced from each explants. Phenolic contents ranged from 66.4 to 112.3 mg GAE·gm of dry extract in leaf extracts and from 84.6 to 153.4 mg GAE·gm of dry extract in leaf callus culture extracts (Fig. 1). This demonstrates that callus cultures have the capacity to retain significant amounts of phenolics rather than their *in vivo* counter parts. As the callus growing in a nutrient-rich culture medium is undoubtedly exposed to a more carbon influx than the field-grown plant parts, it may influence the metabolic flux for the biosynthesis of elevated levels of phenolics. Similarly, the accumulation of phenolics was reported in *Salvia officinalis* callus cultures, where the level of phenolics in callus is based on the abundance of carbon sources in the medium (Paula et al. 2002).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity

Free radicals (DPPH) are well known to cause auto oxidation of unsaturated lipids in foods (Kaur and Perkins 1991). Determination of scavenging stable DPPH was a very fast method to evaluate the antioxidant activity of the extracts. With this method, it was possible to determine

Table 1 Effect of various concentrations of auxins on callus induction using leaves of 5 different *Ocimum* species

Growth regulators (mg/l)		% of culture response				
		<i>O. sanctum</i>	<i>O. basilicum</i>	<i>O. americanum</i>	<i>O. gratissimum</i>	<i>O. kilimandcharicum</i>
2,4D	+ kinetin					
1.0	0.1	99.4 \pm 2.3*	70.1 \pm 2.1	80.5 \pm 2.1	73.5 \pm 2.4	70.1 \pm 2.7
1.0	0.2	75 \pm 2.4	99.5 \pm 3.4*	75 \pm 2.3	70 \pm 2.2	78 \pm 3.2
1.0	0.3	98.5 \pm 2.6	70 \pm 3.2	99.1 \pm 2.6*	96 \pm 3.1*	99.4 \pm 3.4*
1.0	0.4	50 \pm 2.8	55 \pm 2.4	58 \pm 3.1	50 \pm 2.6	53 \pm 2.6

Data were collected after 8 weeks of culture. Values are given as mean \pm SD for each three replicates. * indicates the level of significance at $p<0.05$ compare to other auxin combination supplementation.

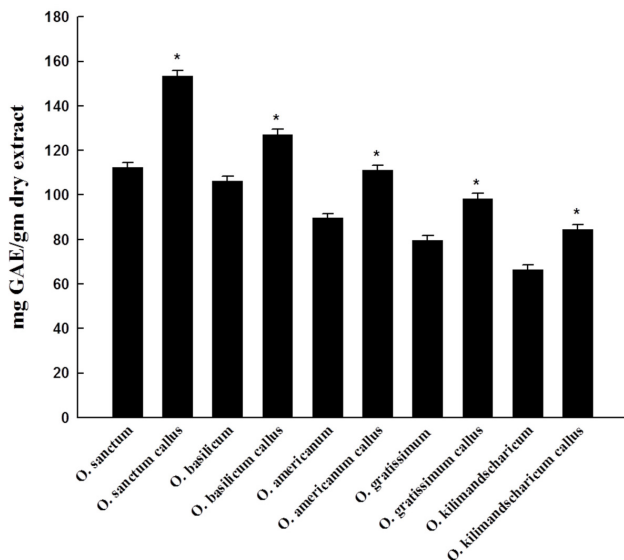


Fig. 1 Total phenolic contents of five different *Ocimum* species leaves and their *in vitro* leaf callus extracts. Data are presented as mean \pm SD of each of three replicates (n=3). * indicates a significant ($p < 0.05$) increase in callus extract compare to their respective *in vivo* leaf extracts

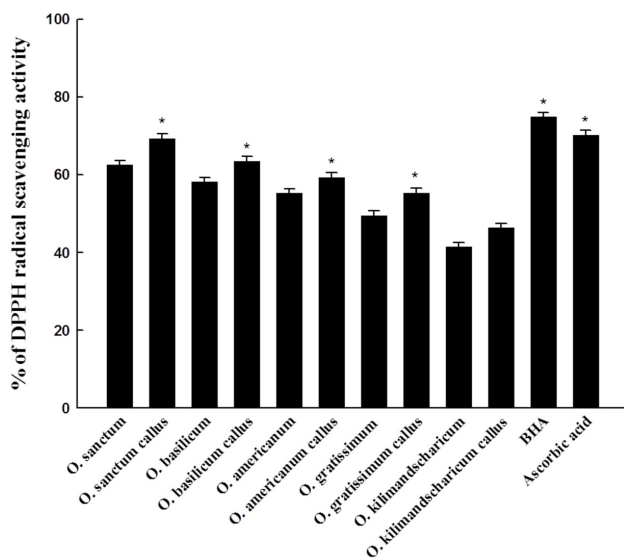


Fig. 2 DPPH radical scavenging activities of five different *Ocimum* species leaves and their *in vitro* leaf callus extracts. Data are presented as mean \pm SD of each of three replicates (n=3). * indicates a significant ($p < 0.05$) increase in callus extract compare to their respective *in vivo* leaf extracts along with positive controls. BHA - Butylated hydroxyl anisole

the antiradical power of the antioxidant activity by measuring the decrease in absorbance of DPPH at 517 nm.

As can be seen in Fig. 2, all the *Ocimum* species extracts at a dose of 1.0 mg/ml were capable of scavenging DPPH \cdot free radicals. From the percentage scavenging values, it can be seen that the *O. sanctum* leaf extract was

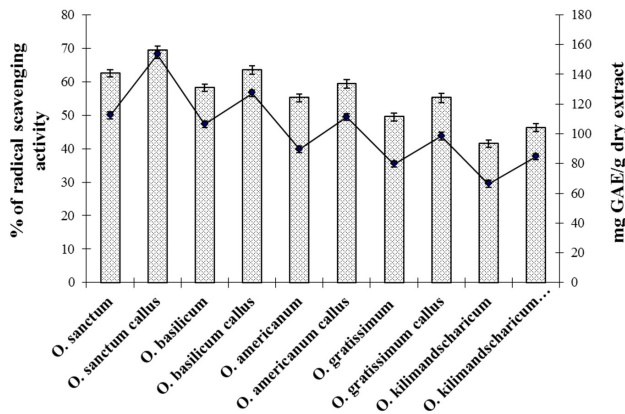


Fig. 3 Relationship between DPPH radical scavenging activity and total phenolic contents of five different *Ocimum* species leaves and their *in vitro* leaf callus extracts. Data are presented as mean \pm SD of each of three replicates (n=3)

the most potent scavenger (62.5%) followed by *O. basilicum* (58.2%), $>$ *O. americanum* (55.2%) $>$ *O. gratissimum* (49.5%), $>$ *O. kilimandscharicum* (41.5%). Further, the callus extracts from all the *Ocimum* species showed more scavenging activity when compared to the leaf extracts. *Ocimum sanctum* leaf callus showed highest scavenging activity at 69.3%, followed by *Ocimum basilicum* 63.5%, *Ocimum americanum* (59.3%), *Ocimum gratissimum* (55.2%), and *Ocimum kilimandscharicum* (46.3%). None of the *Ocimum* species extracts were as effective DPPH \cdot scavengers as the positive controls BHA (74.87%) and ascorbic acid (70.2%). Further in this study, a good linear correlation ($R^2 = 0.783$) was obtained between the concentration of the total phenolic content and the scavenging activity of the DPPH \cdot radical of each extract (Fig. 3). These results indicate that the radical scavenging capacity of each extract may be related to the concentration of phenolics. The anti-radical activity of phenolic compounds depends on their molecular structure, i.e. on the availability of the phenolic hydrogens that can form phenoxyl radicals via 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).

Effect of extracts on FRAP assay

Determination of ferric/antioxidant power FRAP is a direct and simple test for measuring the antioxidant capacity, and the reaction is reproducible and linearly related to the molar concentration of the antioxidant present. This method was initially developed to assay the plasma antioxidant capacity but can be used for plant extracts as well. It is based on

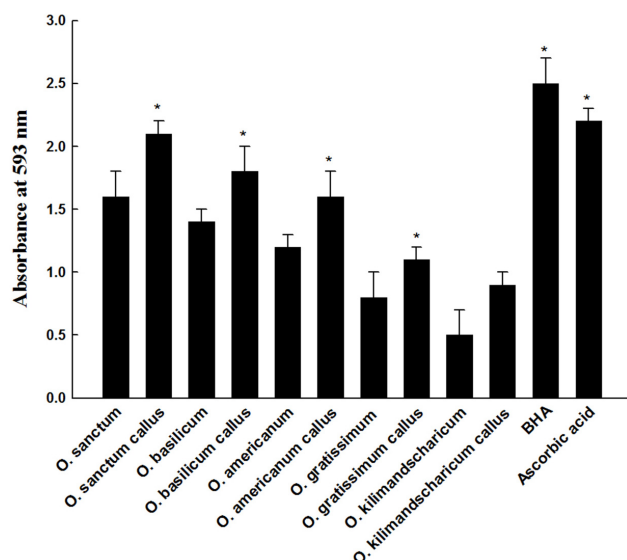


Fig. 4 Reducing power ability of five different *Ocimum* species leaves and their *in vitro* leaf callus extracts. Data are presented as mean \pm SD of each of three replicates (n=3). * indicates a significant ($p < 0.05$) increase in callus extract compare to their respective *in vivo* leaf extracts along with positive controls. BHA - Butylated hydroxyl anisole

comparison of the total amount of antioxidant to the reducing capacity of the sample. The total antioxidant potential of the sample was determined using the ferric reducing ability (FRAP) assay (Benzie and Strain 1996) as a measure of the “antioxidant power”. This assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{2+} -tri-pyridyltriazine compound from the colorless oxidized Fe^{3+} form by electron donating antioxidants.

A hierarchy in the reducing capacity of samples could be observed in the order of *O. sanctum* > *O. basilicum* > *O. americanum* > *O. gratissimum* > *O. kilimandscharicum* (Fig. 4). Interestingly, all callus extracts showed significantly increased activities compared to their respective *in vivo* leaves. This could be due to a higher accumulation of phenolics in the callus extracts.

Effect of Extracts on Fe^{2+} Chelating Activity

The ability to chelate transition metals can be considered an imperative antioxidant mode of action. The ferrous state of iron accelerates lipid oxidation by breaking down the hydrogen and lipid peroxidases to reactive free radicals via the fenton type reaction. The Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of the Fe^{2+} ion (Miller and Denis 1998). The Fe^{2+} ion is the most powerful pro-oxidant among various

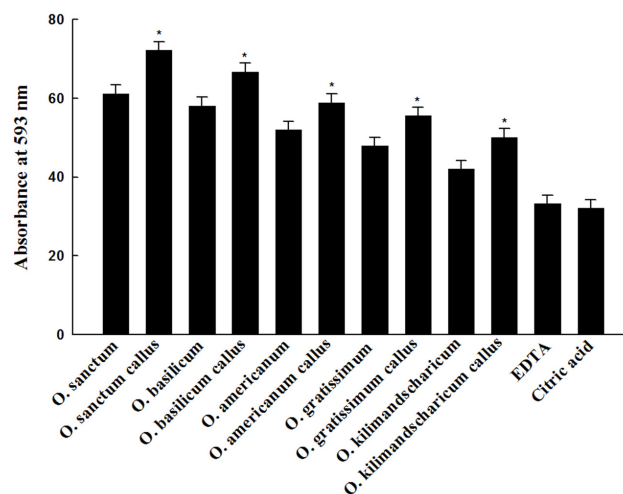


Fig. 5 Fe^{2+} chelating activities of five different *Ocimum* species leaves and their *in vitro* leaf callus extracts. Data are presented as mean \pm SD of each of three replicates (n=3). * indicates a significant ($p < 0.05$) increase in callus extract compare to their respective *in vivo* leaf extracts along with positive controls. EDTA - Ethylenediamine tetra acetic acid

species of metal ions (Halliwell and Gutteridge 1984). Ferrozine is a chelating reagent used to indicate the presence of a chelator in the reactionsystem. Ferrozine forms a complex with free Fe^{2+} ions but not with Fe^{2+} bound to electron donating molecules. In the presence of chelating agents, the complex formation of ferrous and ferrozine is disrupted, resulting in a decrease in the red color of the complex. Measuring the color reduction, therefore, allows the estimation of the metal chelating activity of the coexisting chelator (Yamaguchi et al. 2000).

Therefore, this study was conducted to determine the ability of five different *Ocimum* leaf extracts and *in vitro* callus (induced from each explants) extracts to chelate iron (II) ions. The chelating activity of all the extracts of 1 mg·ml on Fe^{2+} was determined. The chelating activity of the methanol extract of all the *Ocimum* species with the concentration of 1 mg·ml was higher than that of EDTA at 0.01 mM and citric acid at 0.025 mM.

It was reported that the chelating activity of a particular extract is mainly due to the presence of chelated metal ions or suppressed reactivity, by occupying all coordination sites of metal ion, by the antioxidants present in the extract (Mahoney and Graf 1986). Similarly, in the present study, the increased chelating activity of the callus extract could be due to the abundant presence of compounds with antioxidant activity. Therefore, it may be used as an effective agent in delaying the Fe^{2+} -catalyzed lipid oxidation.

β -Carotene-linoleic acid bleaching

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 foods or biologically relevant oxidizable substrates, so a direct protective activity of an extract can be determined (Dorman et al. 2003). Therefore, it was 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 attack the chromophore of β -carotene, resulting in a bleaching of the reaction emulsion. An extract capable of hindering the oxidation of β -carotene may be described as a free radical (Liyana-Pathirana and Shahidi 2006).

As can be seen in Fig. 6, all the extracts were able to inhibit the bleaching of β -carotene by scavenging linoleate-derived free radicals. The order of efficacy was *O. sanctum* (leaf - 76.4, callus - 81.2%) > *O. basilicum* (leaf - 68.4, callus - 76.2%), > *O. americanum* (leaf - 61.2, callus - 68.3%) > *O. gratissimum* (leaf - 56.3, callus - 65.2%) > *O. kilimandscharicum* (leaf - 48.2, callus - 55.3 %). None of the extracts were as effective as the positive control BHA (93.5 \pm 2.1%) and ascorbic acid (84.6 \pm 2.1%). It has been suggested that the polarity of an extract is important in generating 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). In contrary, we used in this work methanol for

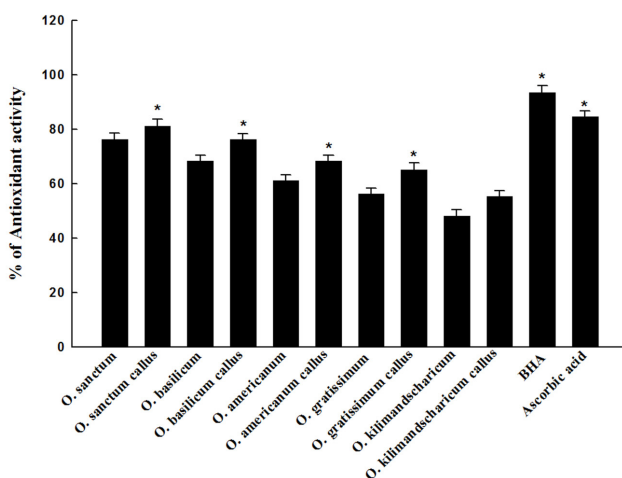


Fig. 6 Antioxidant ability of five different *Ocimum* species leaves and their *in vitro* leaf callus extracts by inhibiting the bleaching of β -carotene. Data are presented as mean \pm SD of each of three replicates (n=3). * indicates a significant (p<0.05) increase in callus extract compare to their respective *in vivo* leaf extracts along with positive controls. BHA - Butylated hydroxyl anisole

extraction, which demonstrated appreciable inhibition of β -carotene bleaching by scavenging linoleate-derived free radicals. This phenomenon is in agreement with Koleva et al. (2002), who reported inhibition of β -carotene bleaching by the polar extract of the *Sideritis* species. 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 a potential use as antioxidative preservatives in emulsion-type systems.

In conclusion the observed the observed results suggest that callus extracts contains more significant amounts of phenolics, compared to other parts of *Ocimum* species which tested in this study. In particular, the *O. sanctum* callus extract showed more significant antioxidant ability than other callus extracts, consistent with previous observations. The enhanced antioxidant ability of callus extracts is due to the electron donating ability of the accumulated phenolics. Furthermore, these extracts can be utilized as potential food preservatives against oxidative deterioration, as demonstrated by the β -Carotene-linoleic acid bleaching assay. Consumption of these extracts may lead to benefits in human health, namely protection against excessive oxidative stress.

Acknowledgment

This work was carried out with the support of the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ008460022012), Rural Development Administration, Republic of Korea.

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