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Chemical Composition and Antioxidant Property of Holy Basil (*Ocimum sanctum* L.) Leaves, Stems, and Inflorescence and Their in Vitro Callus Cultures

F. LUKMANUL HAKKIM, C. GOWRI SHANKAR, AND S. GIRIJA*

Plant Biotechnology Division, Department of Biotechnology, Bharathiar University, Coimbatore, Tamilnadu, India

In this study, the chemical constituents and antioxidant property of holy basil (*Ocimum sanctum* Linn.) field-grown plant parts (leaves, stems, and inflorescence) were compared with those of respective callus cultures induced from each explant in in vitro. 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 (0.1–0.5 mg/L) of kinetin as plant growth regulators. The distribution of phenolic compounds in these extracts was analyzed using reverse phase high-performance liquid chromatography with reference standards. Interestingly, rosmarinic acid (RA) was found to be the predominant phenolic acid in all callus extracts in comparison with field-grown plant parts. In this study, the antioxidant activity of the extracts was evaluated with six different in vitro antioxidant-testing systems. Their activities of scavenging superoxide anion radicals, 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH), hydroxyl radicals, hydrogen peroxide, chelating ferrous iron, and ferric ion reducing potential were assessed. The antioxidant activity was increased in all testing systems with increasing amounts of extract. However, at the same concentration, the callus extracts exhibited higher antioxidant activity in all of the testing systems than the extract obtained from field-grown plant parts. The data obtained from this study suggested the possibility of the isolation of a high content of RA from in vitro callus cultures rather than field-grown plant organs of holy basil.

KEYWORDS: ***Ocimum sanctum*; holy basil; callus cultures; antioxidant activity; rosmarinic acid**

INTRODUCTION

Oxidative deterioration of fat components in foods is responsible for the rancid odors and flavors that decrease nutritional quality. The addition of antioxidants is essential to preserve food quality. Synthetic compounds such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), and propyl gallate (PG) are broadly used as antioxidants in the food industry. Their safety, however, is still questionable. At high doses, BHT may cause internal and external hemorrhaging, which contributes to death in some strains of mice and guinea pigs. This effect is attributed to the ability of BHT to reduce vitamin K-dependent blood clotting factor (*1*). Therefore, the importance of replacing synthetic antioxidants by natural resources has risen globally. Principal candidates in this discovery of natural antioxidants process have been herbs, spices, and medicinal plants. It is well-known that natural antioxidants extracted from herbs and spices such as rosemary, oregano, and thyme have enormous antioxidant activity and are used in numerous food applications (*2, 3*). Most of the antioxidative potential of herbs and spices is due to the redox properties of their phenolic compounds, which permit them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (*4*). Plant phenolic compounds are mostly secondary metabolites possessing high antioxidant activity and are widespread in the species of Lamiaceae (*5, 6*). Advances in the area of tissue culture for the production of secondary metabolites by callus culture have made it possible for the increased yield of a wide variety of pharmaceuticals such as alkaloids, terpenoids, steroids, saponins, phenolics, and flavonoids (*7*).

Holy basil is an annual herbaceous plant belonging to the family Lamiaceae. Its slightly hairy, pale green leaves are widely used as a flavoring agent in Southeast Asian cuisine, especially in Thai stir-fries. Holy basil leaves are spicy and have lemony notes (*8*), and many Indians consume small quantities of the young leaves either as an offering after divine worship in temples or as a food additive (*9*). Traditional use has attributed a number of properties to holy basil. These include rejuvenating, tonic, and vitalizing properties that would contribute to longevity and a healthy life, as well as antiseptic, antiallergic, and anticancer effects (*10, 11*).

Although antioxidant properties of ethanol extract of holy basil leaves have already been reported (*12*), in the present study, we have attempted for the first time to compare the antioxidant properties between holy basil plant parts (leaves, stem, and

* Corresponding author [telephone (91) 422-2424575; fax (91) 422-2422387; e-mail girijabiotech@yahoo.co.in].

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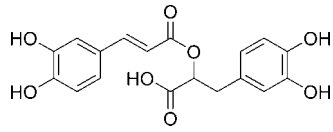

Figure 1. Structure of rosmarinic acid.

inflorescence) and in vitro callus cultures induced from each explant including total phenolic content (TPC) (qualitative–quantitative composition), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, iron reducing power, Fe^{2+} chelating activity, superoxide anion scavenging activity, hydroxyl radical scavenging activity, and hydrogen peroxide scavenging activity. We have found significantly higher levels of antioxidant activity in the callus extracts than in the field-grown plant parts of holy basil. Since holy basil 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 this plant, especially the in vitro-induced callus extract, as a food additive.

MATERIALS AND METHODS

Plant Material. The seeds of *Ocimum sanctum* were purchased from a local market and the plants grown in the field of Bharathiar University, Coimbatore, Tamilnadu, India. Taxonomical identification of this plant was done in the Botanical Survey of India, Tamilnadu Agricultural University, Coimbatore. A voucher specimen (no. 231) was deposited in the Department of Botany, Bharathiar University.

Chemicals. 2,4-Dichlorophenoxyacetic acid (2,4-D), kinetin, nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine), trichloroacetic acid (TCA), hydrogen peroxide, 1,10-phenanthroline, and L-ascorbic acid used were of analytical grade, available commercially. The high-performance liquid chromatography (HPLC) standards isothymusin, carnosic acid (CA), ursolic acid, eugenol, sinapic acid, and rosmarinic acid (RA; **Figure 1**) were purchased from Sigma-Aldrich (St. Louis, MO). The solvents used for extraction and quantification of phenolics were of HPLC grade.

Callus Induction. Leaves, stems, and inflorescence explants from 1-year-old *O. sanctum* plants were surface sterilized for 3–5 min in Tween 80 and for 3 min with 0.1% (w/v) $HgCl_2$ 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) (*13*) medium containing 3.0% 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 (0.1–0.5 mg/L) of kinetin were used individually. The cultures were maintained at 25 ± 2 °C under alternative dark and light (1000–2000 lx) for 12 h photoperiods. Culture response of each explant for callus induction was recorded after 8 weeks.

Extraction Procedure for Field-Grown Plant Parts. Leaves, stems, and inflorescences were harvested from field-grown *O. sanctum* plants and shade-dried for 7 days. The dried materials were powdered using a mechanical grinder. Ten grams of each powdered material was extracted twice with 500 mL of methanol continuously. Thereafter, the resulting methanolic extract was reduced in vacuo (40 °C; N_2 stream), freeze-dried, and stored at 4 °C until further use in the experiment.

Extraction Procedure for in Vitro Callus. Callus induced on MS medium was harvested and dried, and the dry weight was determined (*14*). One gram (dry weight) of callus was soaked in 10 mL of 80% methanol for 3 h and sonicated in an Ultrasonic Sonifier (Branson Sonifier) at 20 pulses for 20 min. The extract was centrifuged at 10000 rpm for 10 min. Then the supernatant was concentrated under vacuum (40 °C; N_2 stream), freeze-dried, and stored at 4 °C until further use in the experiment.

Separation of Polyphenols from Methanol Extract. Sample preseparation was done for the separation of polyphenols from the extract (*15*). Briefly, 0.5 mL of methanolic extract and 0.2 mL of tetrachloromethane were added to give a clear solution. Adding a further 0.3 mL of distilled water to the solution resulted in the formation of a two-phase layer. After thorough shaking, the tube was centrifuged at 10000 rpm for 10 min to obtain a sharp interface. The aqueous methanolic phase was separated, dried, and dissolved in methanol. The resulting extract was freeze-dried and stored at 4 °C until further use in the experiment.

Determination of Total Phenolic Content (TPC). To determine the TPC, 2.5 mL of Folin–Ciocalteu reagent was added to both 40 μL of extract and 40 μL of phenol standard solution. After 8 min of reaction time, saturated sodium carbonate solution (7.5 mL) was added, and the test solution was made up to 100 mL with distilled water and mixed thoroughly. After 2 h, the solutions were transferred into a cuvette, and the absorbance was measured at 765 nm (*16*). Phenol was used as standard in different concentrations (0.10, 0.5, 1.5, 2.0, and 5 mg/mL), and the content of total phenolics in the extracts was calculated using linear regression (*17*).

High-Performance Liquid Chromatography (HPLC) Analysis of Phenolic Compounds. A HPLC system comprising 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 C_{18} 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 1.0 mL/min, the sample injection volume 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 (DPPH) Radical Scavenging Assay. The ability of the extracts to scavenge the DPPH radical was assessed spectrophotometrically (*18*). Briefly, a 50 μL aliquot of the extract was mixed with 450 μL of Tris-HCl buffer (50 mmol/L, pH 7.4) and 1.0 mL of DPPH (0.1 mmol/L, in methanol), and the resultant absorbance was recorded at 517 nm after 30 min of incubation at 37 °C. The percentage of inhibition was calculated from the equation

$$\text{percentage of inhibition} = [(A_0 - A_1)/A_0] \times 100 \qquad (1)$$

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract. IC_{50} values of the extracts were obtained from best linear fit using Origin 7.0 software.

Reducing Power Assay. The reducing power of the extracts was measured (*19*). Briefly, 0.5 mL of extracts was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. Then 2.5 mL of TCA (10%) was added. The mixture was centrifuged at 650*g* for 10 min. The supernatant (2.5 mL) was mixed with an equal volume of distilled water and 0.5 mL of ferric chloride (0.1%). Absorbance was measured at 700 nm in a spectrophotometer (Shimadzu). Higher absorbance of the reaction mixture indicated greater reducing power. The reducing power of the extracts was compared with 0.2 mg/mL of standard ascorbic acid solution.

Fe^{2+} Chelating Assay. The chelating activity of the extracts for ferrous ions Fe^{2+} was determined (*20*). Briefly, to 0.5 mL of extract, 1.6 mL of distilled water and 0.05 mL of $FeCl_2$ (2 mM) were added, and after 30 s, 0.1 mL of Ferrozine (5 mM) was added. Then the reaction mixture was incubated for 10 min at room temperature, and the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. A lower absorbance indicates a higher chelating power. The chelating activity of the extracts on Fe^{2+} was compared with that of EDTA (0.01 mM) and citric acid (0.025 M)

$$\text{chelating activity (\%)} = [(A_0 - A_1)/A_0] \times 100 \qquad (2)$$

where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Superoxide Anion Scavenging Assay. Superoxide anion scavenging activity of the extracts was validated (*21*). Superoxide anions were generated in a nonenzymatic 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 1 mL of extracts at various concentrations (0.10, 0.25, 0.50, 0.75, and 1.0 mg/mL) of the methanolic extracts. 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 equation

$$\text{SASA (\%)} = [1 - (A_1 - A_2)/A_0] \times 100 \quad (3)$$

where A_1 was the absorbance in the presence of the extract, A_0 was the absorbance of the control (blank, without extract), and A_2 was the absorbance without PMS.

Hydroxyl Radical Scavenging Assay. The scavenging activity of extracts on hydroxyl radicals was measured with the Fenton reaction (*22*). Reaction mixture contained 60 μL of $FeCl_2$ (1.0 mM), 90 μL of 1,10-phenanthroline (1 mM), 2.4 mL of phosphate buffer (0.2 M, pH 7.8), 150 μL of H_2O_2 (0.17 M), and 1.5 mL of extracts at various concentrations (0.10, 0.25, 0.50, 0.75, and 1.0 mg/mL). The reaction was initiated by adding H_2O_2. After incubation at room temperature for 5 min, the absorbance was recorded at 560 nm. The hydroxyl radicals scavenging activity of the extracts was calculated according to the equation

$$\text{scavenging activity (\%)} = [1 - (A_1 - A_2/A_0)] \times 100 \quad (4)$$

where A_0 was the absorbance of the control (blank, without extract), A_1 was the absorbance in the presence of the extract, and A_2 was the absorbance without 1,10-phenanthroline.

Hydrogen Peroxide Scavenging Assay. Hydrogen peroxide scavenging activity of the extracts was estimated by replacement titration method (*23*). An aliquot of 1.0 mL of H_2O_2 (0.1 mM) and 1.0 mL of various concentrations (0.1, 0.25, 0.50, 0.75, and 1.0 mg/mL) of extracts were mixed, followed by 2 drops of 3% ammonium molybdate, 10 mL of H_2SO_4 (2 M), and 7.0 mL of KI (1.8 M). The resulting solution was titrated with NaS_2O_3 (5.09 mM) until the yellow color disappeared. Percentage of scavenging of hydrogen peroxide was calculated as

$$\text{scavenging activity (\%)} = [(V_0 - V_1)/V_0] \times 100 \quad (5)$$

where V_0 was the volume of NaS_2O_3 solution used to titrate the control sample in the presence of hydrogen peroxide (without extract) and V_1 was the volume of NaS_2O_3 solution used in the presence of the extract.

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

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, in particular the presence of hydroxyl groups over the aromatic nucleus, in those based on phenolic structure. Despite the intact plant organs contains secondary metabolites, the culture of undifferentiated cells (*calli*) is a potent source for the production of a wide variety of secondary metabolites (*24*). In this study, the callus cultures were successfully induced from holy basil leaves, stems, and inflorescence on MS medium containing 2,4-D (1 mg/L) with the combination of kinetin (0.1–0.5 mg/L) to assess its chemical composition and antioxidant ability. It was reported that 2,4-D with the combination of kinetin favors the growth of callus and production of secondary metabolites in *O. basilicum* (*25*) and *Zataria multiflora* (*26*). In the present investigation, the percentage of callus induction from leaves, stems, and inflorescence was found to be dependent on the concentration and combination of 2,4-D with kinetin used (**Table 1**). Leaf explants of *Coleus blumei* on MS medium supplemented with 2,4-D (1 mg/L) with kinetin (0.1 mg/L) showed the maximum callus induction, but the same hormonal combination was insufficient for the induction of callus tissues from internodal explants of this plant (*27*). Similarly in the present study, the holy basil leaf explants showed maximum (99.1%) culture response in the cultures supplemented with the combination of 2,4-D (1 mg/L) and kinetin 0.1 (mg/L), but the same combination was insufficient for maximum culture response from the stem and inflorescence explants. In contrast to leaf explants, the stem and inflorescence explants of holy basil showed the maximum (96.1 and 94.6%) culture response on MS medium supplemented with the combination of 2,4-D (1 mg/L) and kinetin (0.2 mg/L). This result was consistent with an earlier report on *Z. multiflora*, where the similar combination was found to be suitable for inducing the callus from stem and inflorescence explants (*26*).

Table 1. Effect of Various Concentrations of Auxins on Callus Induction Using Leaf, Stem, and Inflorescence Explants of Holy Basil[a]

hormone concn (mg/L)		% of culture response		
2,4-D	kinetin	leaf	stem	inflorescence
1.0	0.1	99.1 ± 1.4a	89.2 ± 1.3a	84.7 ± 1.6b
1.0	0.2	75.1 ± 2.4b	96.1 ± 2.1a	94.6 ± 1.1a
1.0	0.3	69.9 ± 1.9d	77.3 ± 1.2b	74.2 ± 1.9c
1.0	0.4	72.3 ± 1.1c	64.1 ± 1.1c	63.1 ± 2.2d
1.0	0.5	56.0 ± 1.6e	52.9 ± 1.4d	51.2 ± 1.7e

[a] Data were collected after 8 weeks of culture. Values are given as mean ± SD for each three triplicates. Values not sharing a common letter differ significantly at $p < 0.05$ (DMRT).

Qualitative and Quantitative Analysis of TPC. In the present study, we evaluated the TPC of methanolic extract of holy basil leaves, stems, and inflorescence and the callus cultures induced from each explant. The phenolic content of leaves was 3.0 mg/mL of extract, and leaf callus culture posesses 4.0 mg/mL of extract, whereas stems and inflorescence consist of 2.5 and 2.1 mg/mL of extract and their callus cultures constitute 3.1 and 2.6 mg/mL extract, respectively (**Table 2**). A significant ($p < 0.05$) increase in the level of total phenolics was observed in leaf, stem, and inflorescence callus cultures in comparison with their respective field-grown plant parts. As callus growing in a nutrient-rich culture medium is undoubtedly exposed to 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 source in the medium (*28*).

The presence of phenolic compounds (isothymusin, CA, ursolic acid, eugenol, RA, and sinapic acid) in holy basil plant has been documented previously (*29*), but the distribution of these compounds in each organ, such as leaves stems and inflorescence of *O. sanctum*, has not yet been reported. In this study, the quantitative analysis of the individual phenolic compounds in the extracts performed using HPLC is given in **Table 2** and representative chromatograms are shown in **Figures 2** and **3**. The components isothymusin, CA, ursolic acid, eugenol, RA, and sinapic acid were identified in holy basil plant

Table 2. Content of Isothymusin, Ursolic Acid, Carnosic Acid, Eugenol, Sinapic Acid, Rosmarinic Acid, and Total Phenolics of Holy Basil Field-Grown Plant Parts (Leaves, Stems, and Inflorescence) and Its in Vitro Callus Extracts[a]

extract	TPC (mg/mL of extract)	IT (mg/g of dry matter)	UA (mg/g of dry matter)	CA (mg/g of dry matter)	EU (mg/g of dry matter)	SA (mg/g of dry matter)	RA (mg/g of dry matter)
leaves	3.05 ± 0.12b	0.14 ± 0.09a	nd	0.19 ± 0.04d	0.70 ± 0.21a	0.54 ± 0.01a	0.25 ± 0.05d
stems	2.5 ± 0.13c	0.13 ± 0.06a	0.06 ± 0.04a	0.12 ± 0.03e	0.44 ± 0.02b	nd	0.21 ± 0.13d
inflorescence	2.1 ± 0.16d	0.09 ± 0.04b	0.02 ± 0.01b	0.11 ± 0.01e	nd	0.05 ± 0.05b	0.12 ± 0.04e
leaf callus	4.0 ± 0.12a	nd	nd	0.38 ± 0.02a	nd	nd	2.7 ± 0.09a
stem callus	3.1 ± 0.12b	nd	nd	0.27 ± 0.03b	nd	nd	2.2 ± 0.11b
inflorescence callus	2.6 ± 0.14c	nd	nd	0.22 ± 0.02c	nd	nd	1.4 ± 0.02c

[a] Data represented as mean ± SD of each three replicates ($n = 3$) at a concentration of mg/mL. Values not sharing a common letter differ significantly at $p < 0.05$ (DMRT). TPC, total phenolic content; IT, isothymusin; UA, ursolic acid; CA, carnosic acid; EU, eugenol; SA, sinapic acid; RA, rosmarinic acid; nd. not detected.

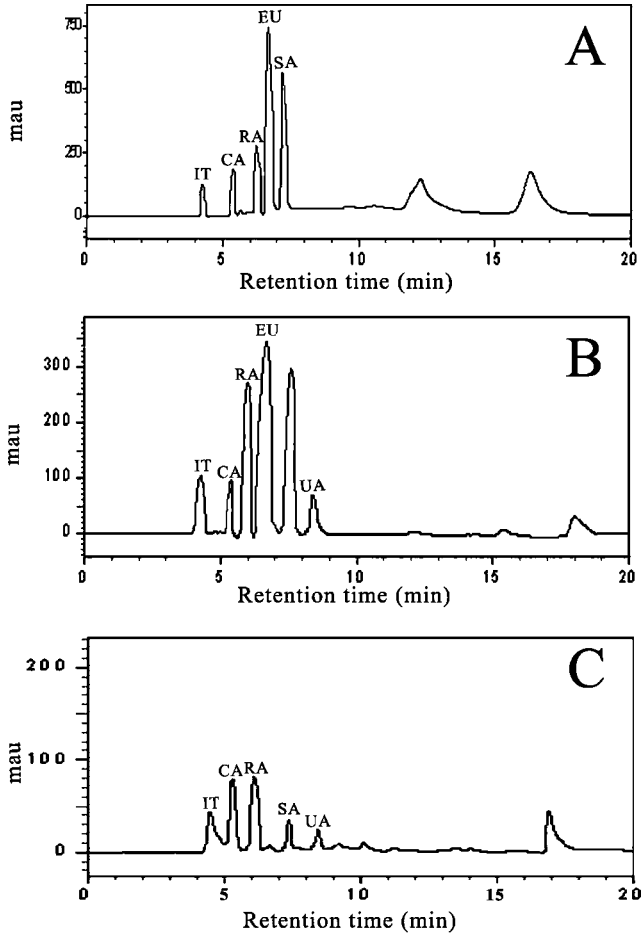

Figure 2. HPLC chromatogram of polyphenols extracted from holy basil leaves (**A**), stems (**B**), and inflorescence (**C**). IT, isothymusin; UA, ursolic acid; CA, carnosic acid; EU, eugenol; SA, sinapic acid; RA, rosmarinic acid.

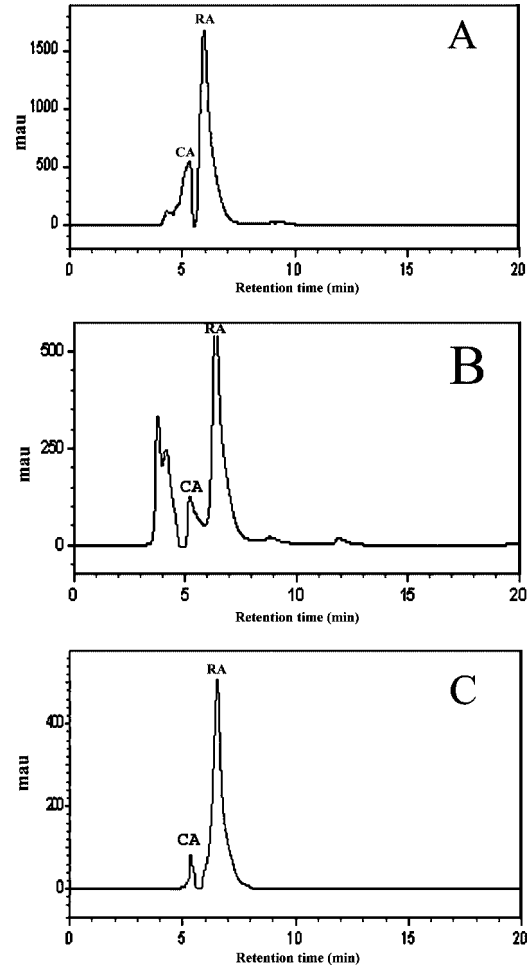

Figure 3. HPLC chromatogram of polyphenols extracted from holy basil leaf callus (**A**), stem callus (**B**), and inflorescence callus (**C**). CA, carnosic acid; RA, rosmarinic acid.

parts by comparison with the retention time, and the UV spectra of authentic standards and quantitative data were calculated on the basis of the peak area of each compound. The variation in the distribution of individual phenolic compounds in the plant parts remains unclear. The ursolic acid was not detected in the leaves, whereas sinapic acid and eugenol were not detected in stems and inflorescence, respectively. This may be due to the solvent used for the extraction. The extraction yields, 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 (*30*). In this study, the concentration of each polyphenol was found to be higher in the leaves among the plant parts, confirming that the biosynthesis of polyphenols is accelerated by light exposure and serves as a filtration mechanism against UV-B radiation (*31*). In accordance, Young et al. (*32*) reported that *Beta vulgaris* (subspecies *cycla*) leaves constitute significantly superior phenolic content than the other parts of the plant.

Further in this study, it was found that the leaf callus contains high TPC among the callus extracts. Obviously the choice of original plant material having high yields of the phytochemical may be important in establishing high-yielding callus cultures (*33*). It has become apparent from the study of Tadhani et al. (*34*), who also found that *Stevia rebaudiana* leaf callus contains significantly higher TPC than its leaves. **Figure 3** shows that RA (**Figure 1**) is a principal component in all three callus

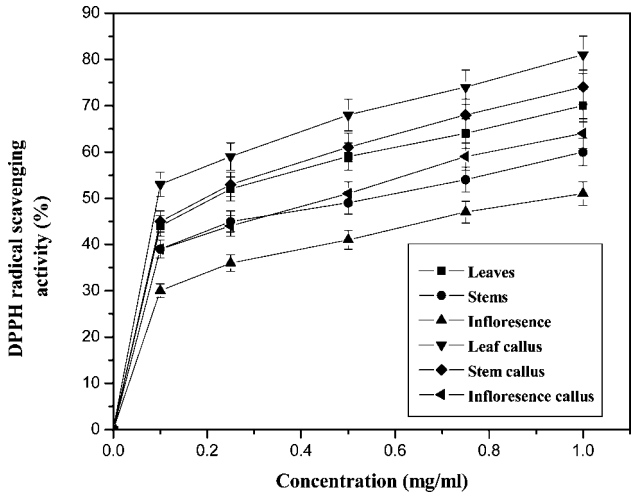

Figure 4. DPPH radical scavenging activity of field-grown plant parts and in vitro callus extracts of holy basil at different concentration. Data are presented as mean ± SD of each of three replicates ($n = 3$).

extracts (range = 1.4–2.7 mg/g of dry matter); a low quantity of CA (range = 0.22–0.38 mg/g of dry matter) was also detected. This is in agreement with the results of the previous studies, whereby caffeic acid derivatives (i.e., caffeic acid and RA) were identified as a major component in *Dracocephalum moldavica* extracts (*35*). The accumulation of RA in holy basil callus cultures was 10.8-fold higher than the field-grown plant parts. Our results are supported by the earlier report of Spiridon et al. (*25*), who found a high accumulation of RA in *O. basillicum* callus cultures. In general, to obtain maximum biomass yield with high secondary metabolite content, it is necessary to fine-tune the type and concentration of auxin and cytokinin, either alone or in combination with nutrients (macro and micro) in the culture medium, which has been known to profoundly influence growth as well as product formation in cultured cells (*36*). In this study, 2,4-D with kinetin was used as a growth regulator for callus induction. This combination with nutrients may influence the RA synthesis in holy basil callus cultures. A similar tendency has been shown with callus cultures of *Aralia cordata*, for which the highest anthocyanin yield was obtained in 2,4-D with kinetin-supplemented callus cultures along with nutrients (*37*). In our work, we also study the influence of other growth regulators such as BAP, NAA, and IAA alone and in combination on growth and accumulation of RA in holy basil callus cultures (data not shown). However, the appreciable response was obtained only with the culture supplemented with 2,4-D and kinetin. RA has an antimicrobial, antiviral effect, which makes it a valuable product for the pharmacological, food (as additive), and cosmetic industries, and it is also an efficient natural antioxidant (*38*).

Effect of Extracts on DPPH Radical Scavenging Activity. The antioxidant activity of natural antioxidants has been shown to be involved in the termination of free radical reactions and reducing power (*39*, *40*). The DPPH molecule that contains stable free radical 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. In this study, extracts of both holy basil field-grown plant parts (leaves, stems, and inflorescence) and in vitro callus induced from each explant were able to scavenge DPPH radicals in a concentration-dependent manner. The methanol extract of each callus extract showed better scavenging activity than its respective external plant parts at each concentration tested (**Figure 4**). IC_{50} is the concentration of extract required to quench 50% DPPH under the chosen experimental conditions. In this study, the IC_{50} value of the extracts of holy basil leaves was 0.46 mg/mL, whereas the extracts of stems and inflorescence showed 0.63 and 0.83 mg/mL, respectively. Likewise, the IC_{50} value of the extract of in vitro leaf callus was 0.34 mg/mL, whereas the extract of stem and inflorescence calluses exhibited 0.42 and 0.56 mg/mL, respectively (**Table 3**). The amount of each in vitro callus culture extract required to quench 50% DPPH is significantly ($p < 0.05$) less than the extract of its respective field-grown plant parts. This property could be due to more accumulation of active principles (phenolic compounds with one or more hydroxyl groups) in the callus extracts. The data obtained reveal that the extracts of holy basil plant parts and their in vitro callus cultures act as free radical inhibitors and thus as primary antioxidants that react with free radicals (*12*). Further in this study, a good linear correlation ($R^2 = 0.971$) was obtained between the concentration of TPC and the scavenging activity of DPPH radical in each extract (**Figure 5**). These results indicated that the radical scavenging capacity of each extract might be mostly related to their concentration of phenolics. The antiradical activity of phenolic compounds depends on their molecular structure, that is, on the availability of phenolic hydrogens and on the possibility for stabilization of the resulting phenoxyl radicals formed by hydrogen donation (*41*). A linear correlation between radical scavenging activity and phenolic concentration has been reported in an extensive range of vegetables, fruits, and beverages (*42*). Using the DPPH radical scavenging method, the antioxidant activities of holy basil plant parts and their callus extracts were ranked in the order leaf callus > stem callus > leaves > infloresence callus > stems > inflorescence. Therefore, the total phenolic compounds in each extract are likely to be responsible for the antioxidant properties studied.

Table 3. IC_{50} Values of Field-Grown Plant Parts and in Vitro Callus Extracts of Holy Basil against DPPH Radical[a]

extract	IC_{50} (mg of extract/mL)
leaves	0.46 ± 0.02b
stems	0.63 ± 0.03d
inflorescence	0.83 ± 0.04e
leaf callus	0.34 ± 0.01a
stem callus	0.42 ± 0.02b
inflorescence callus	0.56 ± 0.02c

[a] Data represented as mean ± SD of each three replicates ($n = 3$). Values not sharing a common letter differ significantly at $p < 0.05$ (DMRT).

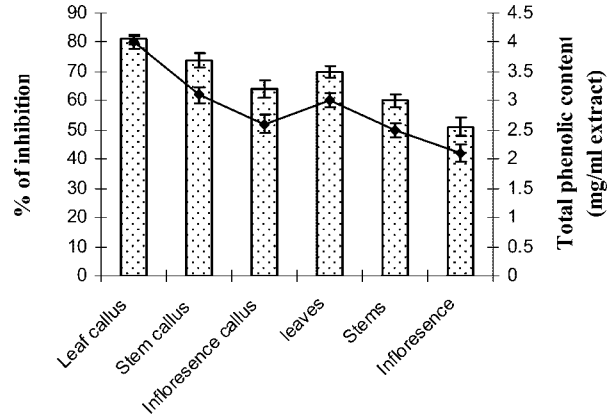

Figure 5. Relationship of DPPH radical scavenging activity and total phenolic content of field-grown plant parts and in vitro callus extracts of holy basil. Data are presented as mean ± SD of each of three replicates ($n = 3$).

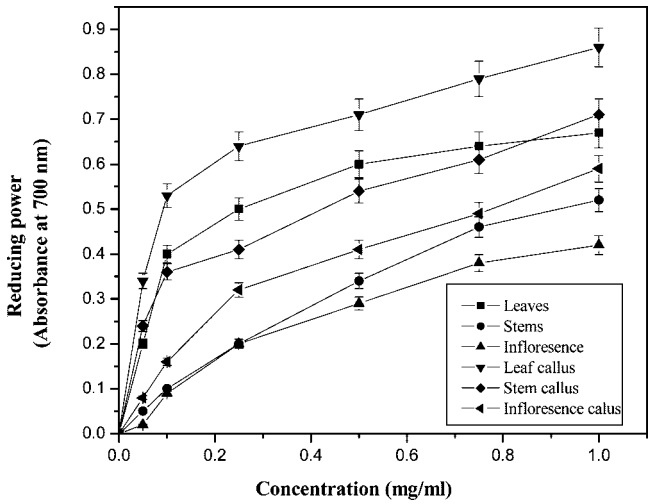

Figure 6. Reducing power of field-grown plant parts and in vitro callus extracts of holy basil at different concentrations. Data are presented as mean ± SD of each of three replicates ($n = 3$).

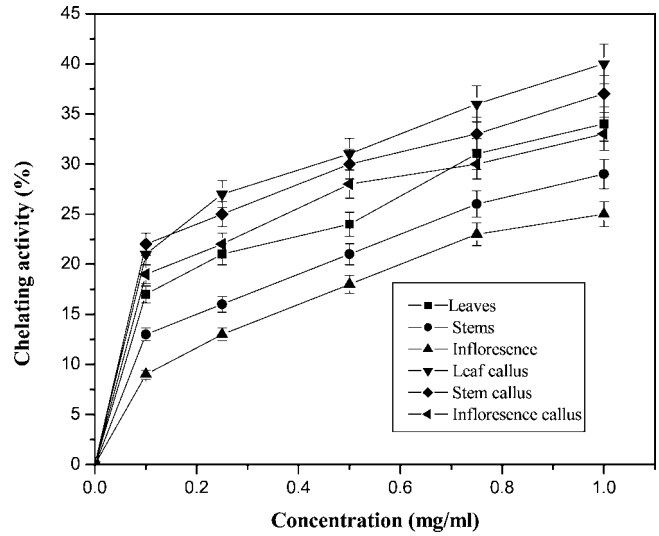

Figure 7. Fe^{2+} chelating activity of field-grown plant parts and in vitro callus extracts of holy basil at different concentrations. Data are presented as mean ± SD of each of three replicates ($n = 3$).

Effect of Extracts on Reducing Power. To examine the reducing power of extracts, the reduction of Fe^{3+} to Fe^{2+} was investigated in the presence of extracts. The presence of reductant (antioxidants) in the tested samples would result in the reduction of Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The ferrous ion can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm (*43*). The reducing power of methanol extracts of holy basil field-grown plant parts (leaves, stems, and inflorescence) and the extracts of in vitro calluses induced from each explant has been recorded (**Figure 6**). The reducing power (as indicated by the absorbance at 700 nm) of all extracts arose with an increasing concentration of sample. Holy basil leaves, stems, and inflorescence extracts showed absorbance readings of 0.67, 0.52, and 0.42 at 1.0 mg/mL, whereas at the same concentration callus culture extracts of the respective explants showed 0.86, 0.71, and 0.59, respectively. In contrast, the same concentration of leaf, stem, and inflorescence callus extracts exhibited significantly higher levels of reducing power than the extracts obtained from respective external sources. In addition, the reducing power of leaf and stem callus extracts (1.0 mg/mL) was higher than that of L-ascorbic acid (0.2 mg/mL; reducing power 0.69), whereas the reducing power of holy basil leaves, stems, and inflorescence extracts (1 mg/mL) was less than that of L -ascorbic acid (0.2 mg/mL), which indicates the abundant presence of antioxidant active compounds in less amount of callus extracts.

Effect of Extracts on Fe^{2+} Chelating Activity. The ability to chelate transition metals can be considered as an imperative antioxidant mode of action. Among the transition metals, iron is known as the most important pro-oxidant metal, which induces lipid oxidation due to its high reactivity. Therefore, in the present study, an attempt was made to determine the ability of holy basil leaves, stems, and inflorescence and in vitro callus (induced from each explant) extracts to chelate iron(II) ions. The chelating activity of all six extracts ranging from 0.10 to 1.0 mg/mL on Fe^{2+} was tested. All of the extracts showed chelating activity on Fe^{2+} in a concentration-dependent manner (**Figure 7**). The chelating activity of methanol extract of holy basil leaves, stems, and inflorescence was higher than those of EDTA (0.1 mM) and citric acid (0.025 M) at concentrations of 0.75 and 1.0 mg/mL, whereas leaf, stem, and inflorescence callus extracts showed higher chelating activity than that of EDTA (0.1 mM) and citric acid (0.025 M) at 0.50, 0.75, and 1.0 mg/mL. Results reveal that callus extracts begin to chelate Fe^{2+} ion better than EDTA at a concentration of 0.5 mg/mL, whereas field-grown plant part extracts showed better chelating activity at a concentration of 0.75 mg/mL. This implies that low concentrations of callus extracts are sufficient to chelate Fe^{2+} ions better than the extracts obtained from field-grown plant organs. It was reported that chelating activity of a particular extract is mainly due to either the presence of chelated metal ions or suppressed reactivity, by occupying all coordination sites of metal ion by the antioxidants present in the extract (*12*, *44*). Similarly in the present study, the increased chelating activity of the callus extract could be due to the abundant presence of antioxidant active compounds. Therefore, it may be used as an effective agent in retarding Fe^{2+}-catalyzed lipid oxidation.

Effect of Extracts on Superoxide Anion Scavenging Activity. The superoxide anion indirectly initiates lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radicals (*45*). Therefore, in the present investigation, it was considered important to characterize the scavenging ability of extract of holy basil leaves, stems, and inflorescence and extracts of in vitro calluses induced from each explant 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. In this study, superoxide anion scavenging activity of all extracts had increased with an increasing amount of extracts (**Figure 8**). This result was in coherence with the previous results on *Cetraria islandica* L., for which superoxide anion radical scavenging activity depends on the concentration and increased with increased amount of sample (*46*). In the present study, superoxide anion scavenging activity of holy basil leaves, stems, and inflorescence extracts at an amount of 1.0 mg/mL was 70, 61, and 49%, whereas the scavenging activity of leaf, stem, and inflorescence callus extracts at the same concentration was 87, 76, and 60%, respectively. The results revealed that the superoxide radical scavenging activity of each callus extract was higher than that of its respective field-grown plant part extract at similar concentrations tested. Recent studies have shown that phenolic compounds, particularly flavonoids and phenolic acids, are

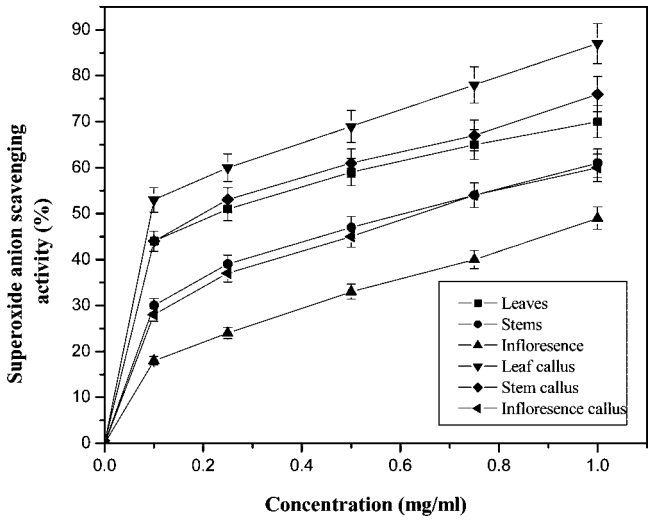

Figure 8. Superoxide anion scavenging activity of field-grown plant parts and in vitro callus extracts of holy basil at different concentrations. Data are presented as mean ± SD of each of three replicates ($n = 3$).

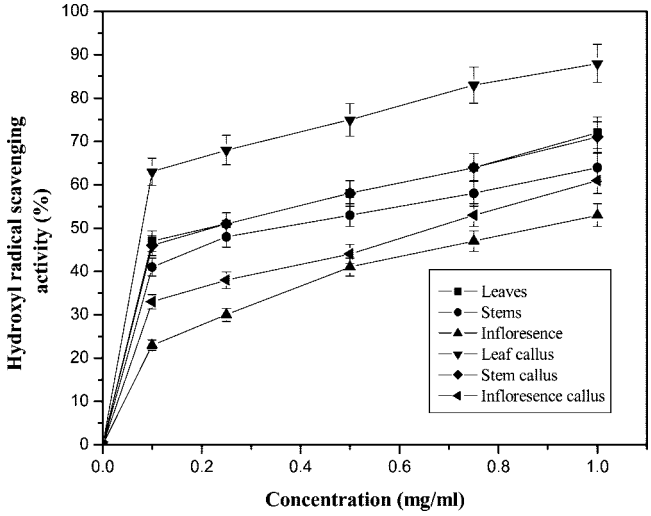

Figure 9. Hydroxyl radical ion scavenging activity of field-grown plant parts and in vitro callus extracts of holy basil at different concentrations. Data are presented as mean ± SD of each of three replicates ($n = 3$).

important antioxidants and superoxide scavengers (*47*). The observed scavenging efficiency of these extracts depends on the presence of concentration of phenolics.

Effect of Extracts on Hydroxyl Radical and Hydrogen Peroxide Scavenging Activity. Unsaturated lipids in cell membrane are susceptible to peroxidation. Consequently, this chain reaction is initiated by hydroxyl radical attacking lipids and extended by the generated lipid hydroperoxide free radicals. Despite the low reactivity of hydrogen peroxide, the high penetrability of cellular membrane leads to hydroxyl radical formation when it reacts with ferrous ion or superoxide anion radical in the cell leading to oxidative stress (*48*). Therefore, in this study, in order to consider the antioxidant property of methanol extracts of holy basil plant parts and the in vitro callus induced from each explant, it is necessary to determine the scavenging activity of hydroxyl radical and hydrogen peroxide by these extracts. All six extracts exhibited concentration-dependent scavenging abilities against the hydroxyl radical and hydrogen peroxide (**Figures 9** and **10**). However, at the same concentration, the callus extracts showed higher scavenging activity on hydroxyl radical and hydrogen peroxide than the

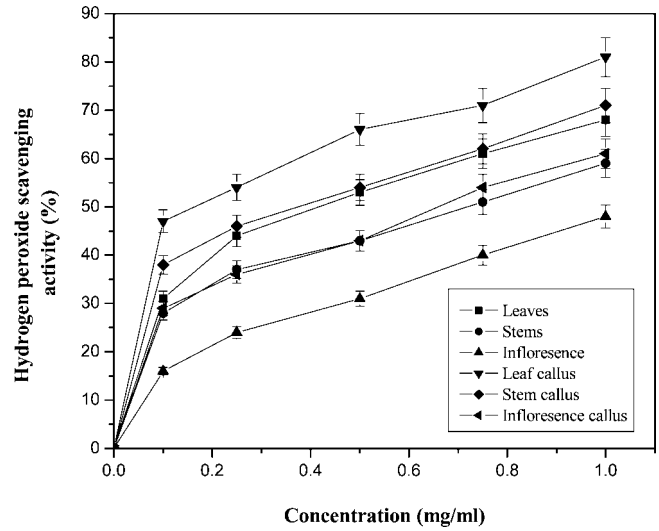

Figure 10. Hydrogen peroxide radical ion scavenging activity of field-grown plant parts and in vitro callus extracts of holy basil at different concentrations. Data are presented as mean ± SD of each of three replicates ($n = 3$).

extracts obtained from field-grown plant parts. The scavenging activity exhibited by holy basil plant part extracts (at 1.0 mg/mL) on hydroxyl radicals was 72% (leaves), 64% (stems), and 53% (inflorescence), whereas the scavenging activity of the same concentration of extracts on hydrogen peroxide was 68% (leaves), 59% (stems), and 48% (inflorescence). At the same time, the scavenging activity of in vitro callus extracts (at 1.0 mg/mL) on hydroxyl radicals was 88% (leaf callus), 71% (stem callus), and 61% (inflorescence callus), and scavenging activity of callus extracts at the same concentration on hydrogen peroxide was 81% (leaf callus), 73% (stem callus), and 64% (inflorescence callus), respectively. These activities are reminiscent of the activities of antioxidant enzymes superoxide dismutase and catalase.

In conclusion, the antioxidant activity of methanol extracts of field-grown plant parts (leaves, stems, and inflorescence) and extracts of callus induced from each explant of holy basil was evaluated with six different in vitro antioxidant-testing systems. Our in vitro assays showed that extracts of leaf, stem, and inflorescence callus cultures have stronger reducing power, more effective Fe^{2+} chelating activity, and higher scavenging activities for superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, and DPPH radicals than extracts of respective field-grown plant parts at each concentration tested. The major contribution to the observed antioxidant activity by callus extracts could be explained by the abundant presence of phenolics with one or more hydroxyl groups such as RA. The accumulation of RA in the callus extracts accounts for >60% of the TPC. As RA is known to be an effective antioxidant, this could act as an antioxidant active compound in the callus extract. The presence of RA in the extracts was in the order of leaf callus > stem callus > inflorescence callus > leaves > stems > inflorescence. From this, it can be inferred that the yield of desired phytochemical in callus is based on the original concentration of compound in external sources from which the callus derived (*33*). The observed efficacy suggests not only that these extracts have potential use as a preservative in foods against oxidative deterioration but also that consumption of these extracts may positively modulate human biology under conditions of excessive oxidative stress. Furthermore, the production protocol for RA can be developed by utilizing the holy basil callus cultures. As callus can grow indefinitely and culture

conditions for its growth can be easily controlled, the method developed in this study has great potential for producing RA on an industrial scale.

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