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Phenolics Composition and Antioxidant Activity of Sweet Basil (*Ocimum basilicum* L.)

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The antioxidant activity of a methanolic extract of *Ocimum basilicum* L. (sweet basil) was examined using different in vitro assay model systems. The crude extract was fractionated on a Sephadex LH-20 column, and six fractions were identified. The DPPH scavenging assay system and the oxidation of the soy phosphotidylcholin liposome model system were used to evaluate the antioxidant activity of each fraction. Fraction IV showed the strongest activity followed by fractions V and VI. Phenolic compounds responsible for the antioxidative activity of the fractions were characterized by atmospheric pressure chemical ionization liquid chromatography−mass spectrometry. The major antioxidant compound in fraction IV was confirmed as rosmarinic acid by ^{1}H NMR and characteristic fragmentations in the mass spectrum. Moreover, the native of antioxidant activity of rosmarinic acid in the liposome system was examined. The results showed that one rosmarinic acid can capture 1.52 radicals, and furthermore, the existence of a synergistic effect between α-tocopherol and rosmarinic acid was revealed.

KEYWORDS: Antioxidants; APcI; LC-MS; liposome; *Ocimum basilicum*; rosmarinic acid; sweet basil

INTRODUCTION

Antioxidants have been widely used as food additives to avoid the degradation of the foods. Also, antioxidants have an important role in preventing a variety of lifestyle-related diseases and aging because these are closely related to the active oxygen and lipid peroxidation (*1*). However, there have been concerns about synthetic antioxidants such as BHA and BHT because of their possible activity as promoters of carcinogenesis (*2*). Consequently, there has been much interest in the antioxidant activity of naturally occurring substances (*3*−*5*).

Ocimum basilicum L. (Labiatae) is a perennial crop generally known as sweet basil. It is native to Asia, Africa, South America, and the Mediterranean but widely cultivated in many countries (*6*) in natural and green house conditions in order to maximize the yield and obtain a regular supply of the material (*7*). It is a very important medicinal plant and culinary herb marketed fresh, dried, or frozen (*8*). *O. basilicum* (green) is a rich source of anthocyanins and an abundant source of acylated and glycosylated anthocyanins (*9*). Aroma compounds are also extracted from *O. basilicum* and used in a wide variety of products such as cosmetics and natural flavors (*8*). Recent interest on *O. basilicum* has resulted from its inhibitory activity against HIV-1 reverse transcriptase and platelet aggregation induced by collagen and ADP (adenosine 5′-diphosphate) (*10*, *11*). Experiments carried out using rats have shown that the leaves of *O. basilicum* markedly increased glutathione *S*-transferase activity that partly controls chemical carcinogens in the stomach, liver, and esophagus (*12*, *13*).

Various types of herbs and spices have been used in many kinds of foods, and their antioxidant properties are well-recognized (*5*). In particular, rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) in the family Labiatae have been known as herbs having strong antioxidant activity (*3*, *14*). *O. basilicum* also belongs to this family and contains phenolic compounds and flavonoids (*6*, *8*, *15*−*19*) such as cinnamic acid, caffeic acid, sinapic acid, and ferulic acid (*8*). These phenolic compounds and flavonoids are potent antioxidants, free radical scavengers, and metal chelators (*20*). However, only a few studies have been devoted to assess the antioxidant activity of *O. basilicum* leaves (*21*−*23*). Furthermore, these studies evaluated the antioxidant activity in only one system. Antioxidant activities strongly depend on the evaluation system, and a single assay may be inadequate to obtain a proper idea of the antioxidant activity of the material (*24*).

The purpose of this study was to identify the main components of *O. basilicum* leaf that act as antioxidants and evaluate their antioxidant activity in detail. Moreover, the synergistic effect of the main component in *O. basilicum* leaf against α-tocopherol was also studied. The extraction of *O. basilicum* leaf collected from the intermediate dry zone in Sri Lanka has not been used as an antioxidant commercially. Therefore, the results of this study would be beneficial for developments in the food industry.

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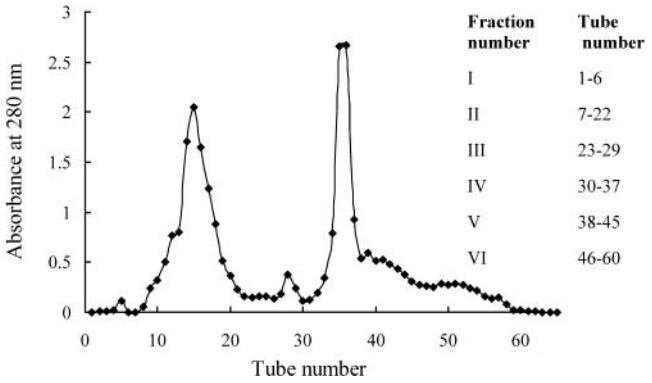

Figure 1. Sephadex LH-20 column chromatographic fraction profile of crude methanolic extract of *O. basilicum* monitored at 280 nm.

MATERIALS AND METHODS

Plant Material and Chemicals. *O. basilicum* (sweet basil) grown in an intermediate dry zone (location, Rattota, Central Province; elevation, 450 m from the sea level; average temperature, 25−26 °C; relative humidity, 85% or more; soil type, red yellow podzolic) in Sri Lanka was used for the study. All of the chemicals used in this work were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All of the chemicals and reagents were of analytical grade, and high-performance liquid chromatography (HPLC) grade was used for the HPLC analysis.

Preparation of Sample. Well-grown *O. basilicum* plants were randomly selected. Leaves were collected between the top and 50 cm down and dried in an air-draft oven (Pickstone Ovens, Thetford, U.K.) at less than 35 °C for 24−36 h. Dried leaves were packed under vacuum and kept at −18 ± 1 °C until air transport to Japan for further analysis.

Preparation of *O. basilicum* Crude Extract. Dried *O. basilicum* leaves were ground in an electric coffee grinder (Café Moulin, Philips Japan Ltd., Tokyo, Japan) for 3 min. Finely ground leaf powder was homogenized with a Nissei AM, Ace homogenizer (Nihonseik Kaisha Ltd, Tokyo, Japan) at 1500 rpm for 5 min with hexane, methanol, acetone, ethyl acetate, ethanol, or water separately at room temperature. The ratio of leaf powder and solvent was 1:5 (w/v). The resulting slurries were filtered under suction through Whatman No. 4 filter paper. This procedure was repeated twice for the residue, and the filtrates were combined. The remaining very fine particles in the filtrate were separated by centrifugation at 7000 rpm with a Himac SCR 18B centrifuge (Hitachi koki Co. Ltd., Tokyo, Japan) for 20 min. The supernatants were collected and concentrated under vacuum at 35 °C. Total extractable components in each extract were measured. The remaining crude extracts were stored at −18 ± 1 °C.

Determination of Extraction Efficiency of Phenolic Compounds by Different Solvents. Extraction efficiency of phenolic compounds by different solvents was examined using thin-layer chromatography (TLC). Each crude extract (10 mg) was dissolved in the same solution and loaded onto a 20 cm × 20 cm, 0.25 mm thick, Silica gel 60 F_{254} analytical TLC plate (Merck Co. Ltd., Darmstadt, Germany) and developed using a mixture of chloroform/methanol/water (65:35:10; v/v/v) as the developing solvent. After they were dried, the plates were sprayed with 2.5% (w/v) $FeCl_3$ in ethanol solution. Phenolic compounds with three hydroxyl and two hydroxyl groups produced blue and green colors, respectively (*25*). The colors were judged by the naked eye.

Fractionation of Crude Extracts by Column Chromatography. One gram of dried methanol extract was dissolved in 10 mL of HPLC grade methanol. It was applied to a 77 cm × 0.5 cm i.d. glass column packed with Sephadex LH-20 (Amersham Phamacia Biotech AB, Uppsala, Sweden) and eluted with methanol (*26*). Fractions of 8 mL were collected continuously in test tubes, and the content of polyphenols was monitored at 280 nm with Beckman DU 530 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). From the results, collected elutes were pooled for six fractions (I−VI) (**Figure 1**). Total solids were determined for each fraction in order to refer all of the results to the same concentration (μg/mL) and reported as percent of total (**Table 1**). The samples were stored in screw-capped glass tubes under nitrogen at −18 ± 1 °C until use.

Table 1. Percentage Recovery and Total Phenolic Contents (mg/g as Gallic Acid Equivalents) of Methanol Extract of *O. basilicum*

fraction number[a]	relative content (% of total)[b]	total phenolic contents[c]
I	4.3	7.3 ± 0.5
II	68.8	46.4 ± 8.5
III	11.7	65.6 ± 1.7
IV	12.5	227.2 ± 7.4
V	6.7	143.2 ± 8.2
VI	5.2	141.6 ± 8.4

[a] Fractions obtained after Sephadex LH-20 column chromatography. [b] Mean value of two separations. [c] Mean value ± standard deviation of each two determinations.

Determination of Amount of Total Phenolic Compounds. The total phenolic compounds of each fraction were determined (*27*) and expressed as milligrams of gallic acid equivalents per gram of methanolic extract. One milligram of each fraction was dissolved in 2 mL of deionized water. Folin-Ciocalteu's phenol reagent (2 mL) was added to the sample and kept for 3 min. Then, 2 mL of 10% aqueous sodium carbonate solution (w/v) was added and allowed to stand at ambient temperature for 1 h. Absorbance of the developed dark blue color was measured by spectrophotometer at 530 nm. Deionized water was used as a blank sample. The content of total phenolic compounds in each fraction was determined using a standard curve prepared with gallic acid.

Assay for DPPH Free Radical Scavenging Effect. The method was adapted from Germano et al. (*28*). The fractions of methanol extracts from *O. basilicum* were evaluated in terms of their hydrogen donating or radical scavenging ability using the stable DPPH radical. Ascorbic acid and α-tocopherol were used as reference materials. The samples dissolved in methanol at various concentrations (5−100 μg/mL) were introduced into 10 mL Teflon screw-capped tubes and made up to a volume of 2 mL with methanol. Three milliliters of the freshly prepared solution with 1 mM DPPH methanol was added to the sample tube and mixed vigorously for 15 s. The sample tube was then kept in a water bath at 27 °C for 30 min. The absorbance of the sample was measured at 517 nm by spectrophotometer. All tests were performed three times. The DPPH radical scavenging effect was calculated as "inhibition percentage" according to the eq 1.

$$\text{inhibition percentage (\%)} = [(A_{C(0)} - A_{A(t)})/A_{C(0)}] \times 100 \quad (1)$$

where $A_{C(0)}$ is an absorbance of control DPPH solution at 0 min and $A_{A(t)}$ is an absorbance of test samples at 30 min.

Evaluation of Antioxidant Activity Using Oxidation of the Soy Phosphatidylcholine Liposome Model. *Preparation of Soy Phosphatidylcholine Liposome.* Soy lecithin was purified using a silica gel column eluted by a mixture of chloroform and methanol. A 15 g amount of soy lecithin was dissolved in 50 mL of chloroform and introduced into a 40 cm × 6 cm i.d. glass column filled with activated silica gel of particle size 0.063−0.200 mm (Merck). The column was stepwise eluted with 3 L each of chloroform and methanol solutions mixed in ratios as follows. The initial ratio of chloroform/methanol was 8:1 (v/v), and it was gradually changed to 3:1, 2:1, 1.5:1, and 1:1 (v/v). The flow rate was 4 mL/min. Fractions (300 mL) were collected, and the presence of phosphatidylcholine in each fraction was confirmed by TLC where spots developed with a solvent mixture of chloroform/methanol/water/acetic acid (65:45:2:1; v/v/v/v). The selected fractions containing pure phosphatidylcholine were combined, and the solvent was removed. The purified soy phosphatidylcholine was adjusted to 50 mM with ethanol and kept under nitrogen at −20 °C until use.

Determination of Phosphatidylcholine Hydroperoxide. Antioxidant activity of each fraction (II−VI) was examined using soy

phosphatidylcholine liposome (50 mM) dispersed in 1 M of pH 7.4 Tris-HCl buffer. The liposome was prepared according to Batzri's method (*29*). Oxidation of the liposome was carried out with lipophilic radical initiator, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile), at 37 ± 1 °C in a shaking water bath. The synergistic effect of each fraction against α-tocopherol was also evaluated in the same system. Formation of the conjugated diene, corresponding to the amount of hydroperoxide in phosphatidylcholine, was measured at 234 nm by an LC-6A HPLC (Shimadzu Co., Kyoto, Japan) connected to the SPD-6A UV detector (Shimadzu Co.) every 15 min at room temperature. The separation was done using a 250 mm × 4.6 mm, 5 μm SUPELCOSIL LC-Si column (Supelco, Bellefonte, PA). A mixture of ethanol/2-propanol/40 mM NaH_2PO_4 (60:30:7 v/v/v) was used as the mobile phase, and the flow rate was 0.8 mL/min.

Proton and Carbon NMR (^{1}H NMR and ^{13}C NMR) Spectroscopy. The ^{1}H NMR and ^{13}C NMR spectra were used to identify the major compound (P-1) in *O. basilicum* extract. Data were recorded using a Bruker DRX 500 spectrometer (Bruker Biospin, Ibaraki, Japan). ^{1}H NMR and ^{13}C NMR (at 500 MHz) and (^{1}H−^{1}H) (^{13}C−^{13}C) correlation spectroscopy (COSY, at 500 MHz) were carried out in deuterated methanol (CD_3OD).

HPLC−PAD Analysis. Quantification and confirmation of phenolic compounds in each fraction was conducted with an HPLC Alliance 2690 with 996 PAD (Waters Co., Milford, MA) system. A 10 μL amount of each fraction (100 μg/mL) was subjected to HPLC−PAD. Data acquisition, processing, and instrument control were performed using Mass Lynx software. A 250 mm × 2.3 mm TSK gel ODS-80Ts column (TOSOH Co., Tokyo, Japan) was used, and the temperature was maintained at 30 °C. The mobile phase was a mixture of solvent A (methanol) and solvent B (acetonitrile/water with 1% acetic acid, 15:85; v/v) with a constant flow rate of 0.5 mL/min over 90 min. The gradient conditions were as follows: 0 min, 2% A; 15 min, 25% A; 30 min, 35% A; 50 min, 55% A held for 10 min, before returning to the initial conditions at 70 min. Scanning between 200 and 400 nm was performed.

APcI-MS. Phenolic compounds were identified by HPLC−PAD and APcI-MS. HPLC conditions were similar to the previous section. Mass analyses were performed on an Alliance 2690 LC-MS (Waters Co.), coupled to an Alliance micromass ZMD fitted with an APcI source, with full scan acquisition. Data acquisition, processing, and instrument control were performed using MassLynx software. The instrumental conditions were as follows: vaporizer temperature of 400 °C; gas pressure, 500 L/h; capillary voltage, 4.0 kV; and corona voltage, 15 V. Spectra were obtained over the range *m*/*z* 100−750 with a scan time of 0.5 s. Identified phenolic compounds were compared with the data reported previously.

Quantification of α-Tocopherol Presence in Liposome System. The presence of α-tocopherol in the liposome system was quantified at room temperature by HPLC consisting of an LC-3A HPLC pump (Shimadzu Co.) connected with a RF-10A_{XL} fluorescence detector (Shimadzu Co.) at an excitation wavelength of 298 nm and emission wavelength of 325 nm. A 250 mm × 4.6 mm i.d. DOCOSIL B ODS column (Senshu Scientific Co., Ltd, Tokyo, Japan) was used with methanol as the mobile phase at a flow rate of 0.8 mL/min. Quantification of α-tocopherol was carried out using a calibration curve made with pure α-tocopherol standard solution.

Quantification of Phenolic Compound Presence in the Liposome System. The presence of phenolic compound (P-1) (rosmarinic acid) in the liposome membrane system was quantified at 280 nm by an LC-10AD HPLC consisting of a DGU-14A degasser (Shimadzu Co.) connected with the SPD-10A UV detector (Shimadzu Co.). A 250 mm × 2.3 mm i.d. TSKgel ODS-80Ts (TOSOH Co.) was used with 0.025% H_3PO_4 in water/acetonitrile (2:1; v/v) as the mobile phase at a flow rate of 0.5 mL/min at room temperature. The position of the chromatogram peak corresponding to P-1, rosmarinic acid, was identified by the injection of different volumes of P-1 collected by preparative TLC as described in the previous section.

Reproducibility of Oxidation Experiments and Statistical Analysis. The oxidation experiments and LC-MS/APcI analysis were carried out repeatedly several times, and similar oxidation and fragmentation patterns were obtained. Other experiments were carried out at least three times, and mean values were recorded. HPLC analysis data were recorded and analyzed by Chromatopac manager, version 2.00 computer package (Shimadzu Co.) through a C-R3A or C-R6A chromatopac recorder (Shimadzu Co.).

RESULTS AND DISCUSSION

The phenolic compounds in *O. basilicum* were extracted by using different polar and nonpolar solvents, hexane, methanol, acetone, ethyl acetate, ethanol, and water. Methanol showed 6.12% extraction efficiency followed by ethanol (3.42%). Several dark spots that were visualized by spraying with $FeCl_3$ solution on developed TLC plates confirmed the existence of phenolic compounds in methanol and ethanol extracts (data not shown). Acetone extract (3.32%) also contained phenolic compounds, but the intensity of the spots was not as strong as that of methanol or ethanol showing that the extraction efficiency was less. Both hexane (3.14%) and ethyl acetate (3.12%) extracts did not show any spots despite similar extraction yields to ethanol in this study. Decomposition or loss of phenolic compounds during the drying process is usually reported (*8*, *16*). Apparently methanol, widely used to extract antioxidants from plant material, is the best candidate for the extraction of antioxidants from *O. basilicum*.

Figure 1 shows the fraction profile for the methanolic extract of *O. basilicum*. Six major fractions were identified and labeled as I−VI. Yields of individual fractions are presented in **Table 1** as a percentage of the crude extract introduced onto the column. All fractions contained phenolic compounds as they produced the characteristic blue color with Folin-Ciocalteu's reagent. The total phenolic contents ranged from 227.2 to 7.3 gallic acid equivalents (mg/g) in each fraction. Even though the relative content was significantly high in fraction II, the total phenolic content was comparatively low. The highest total phenolic content was found in fraction IV (227.2 gallic acid equivalents mg/g) followed by V and VI, although the relative content was less than 15%. These results suggested that the compounds in fractions IV, V, and VI would have the greatest antioxidant activity. Zheng and Wang (*23*) observed that total phenolic contents and oxygen radical capacity of an aqueous extract of *O. basilicum* were not much different from rosemary extract. These results suggest that *O. basilicum* contains many antioxidant components.

DPPH is a stable free radical that shows a maximum absorption at 517 nm in methanol. When DPPH encounters proton donating substances such as an antioxidant and a radical species, the absorbance at 517 nm disappears because the DPPH radical is scavenged (*30*). On the basis of this principle, the radical scavenging effect of each fraction was measured and the results are presented in **Figure 2**. The strongest activity was shown in fraction IV. Fractions V and VI also contained free radical scavengers, but the activity of fraction V was stronger than that of fraction VI. These results were proportionate to the total phenolic content (**Table 1**). Fraction I seemed not to contain any compounds that could act as free radical scavengers. When a high amount of fraction III was introduced to the system, the activity achieved a similar value to fractions V and VI, indicating that the radical scavengers are also present in fraction III.

Lipid peroxidation is important in food deterioration and oxidative modification of low-density lipoprotein, which is now accepted as a key initial event in the progression of arteriosclerosis (*1*). Lipid peroxidation, as shown in eqs 2−6,

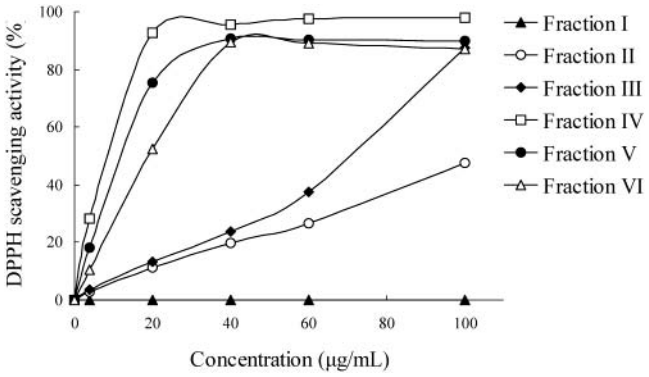

Figure 2. DPPH free radical scavenging ability of different fractions (I–VI) separated from *O. basilicum* and their effectiveness at different concentrations.

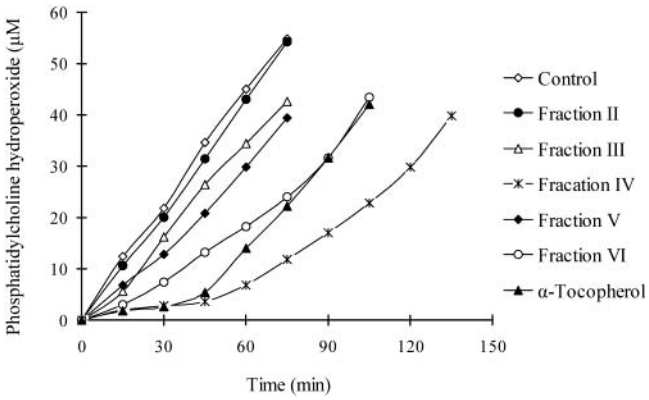

Figure 3. Inhibition activity on formation of phosphatidylcholin hydroperoxide by each fraction (II–VI) (10 μg) of *O. basilicum* and by α-tocopherol (1 μM) in the oxidation of soy phosphatidylcholin liposome.

can be inhibited by suppressing chain initiation and chain propagation and/or by enhancing chain termination.

$$\text{chain initiation} \rightarrow \text{radical } X^{\bullet} \quad (2)$$

$$X^{\bullet} + LH\ (\text{lipid}) \rightarrow XH + L^{\bullet}\ (\text{lipid radical}) \quad (3)$$

$$\text{chain propagation: } L^{\bullet} + O_2 \rightarrow LOO^{\bullet}\ (\text{lipid peroxy radical}) \quad (4)$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (5)$$

$$\text{chain termination: } 2LOO^{\bullet} \rightarrow \text{ketone} + \text{alcohol} + O_2 \quad (6)$$

The chain propagation step as shown in eq 4 is terminated when lipid radical or lipid peroxyl radical is scavenged by an antioxidant. The scavenging efficiency of the compound contained in each fraction was measured with a soy phosphatidylcholine liposome system, and the results are presented in **Figure 3**. The extracts from fraction IV suppressed the formation of phosphatidylcholine hydroperoxide strongly, and the result was the same as the DPPH experiment. Fraction VI also expressed the suppressing effect on formation of phosphatidylcholine hydroperoxide. However, other fractions did not show strong antioxidant activity. In particular, fraction V did not show strong antioxidant activity although it showed a strong radical scavenging effect in DPPH experiment. The DPPH method is a commonly used method to evaluate the antioxidant activity. This study suggests that the result of the DPPH experiment cannot predict the antioxidant activity against the formation of lipid hydroperoxide.

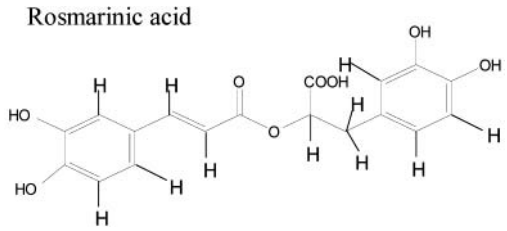

Figure 4. Chemical structure of rosmarinic acid (compound P-1).

The results of analysis of total phenolic content, the DPPH experiment, and the liposome experiment indicate that the main antioxidant components are contained in fraction IV. The main compound of fraction IV was isolated by TLC and named P-1. The structure was analyzed with ^{1}H NMR and ^{13}C NMR. The ^{1}H NMR spectrum of the compound P-1 showed two ABX systems [δ 6.63 (dd, J = 2, 8 Hz, 1H), 6.65 (d, J = 8 Hz, 1H), 6.75 (d, J = 2 Hz, 1H), 6.76 (d, J = 8 Hz, 1H), 6.91 (dd, J = 2, 8 Hz, 1H), 7.02 (d, J = 2 Hz, 1H)] attributable to a 1,3,4-trisubstituted benzene, an AB system [δ 6.27 (d, J = 16 Hz, 1H), 7.49 (d, J = 16 Hz, 1H) assigned to *trans*-olefinic protons, a 1H double-doublet [δ 5.08 (dd, J = 3, 10 Hz, 1H) assigned to a methine proton, and two 1H double-doublets [δ 2.95 (dd, J = 10, 14 Hz, 1H), 3.10 (dd, J = 3, 14 Hz, 1H)] assigned to methylene protons on a carbon adjacent to the carbon bearing the above methine proton. Proton−proton couplings were confirmed by a COSY−NMR analysis. These data suggest that compound P-1 is rosmarinic acid (**Figure 4**), previously observed by many researchers in *O. basilicum* by using different analytical techniques (*15*−*17*, *33*). Coupling constants (J) of interacting protons and chemical shifts (ppm) were in agreement with those reported previously for rosmarinic acid, extracted from *Borago officinalis* seeds (*26*) and *Lithospermum erythrorhizon* cell cultures (*32*). However, methylene protons coupling constants were different from those observed in previous studies (*26*, *32*). The results suggested by the NMR assignments for P-1 were confirmed by the mass spectrum (**Table 2**) that gave a similar fragmentation pattern to that reported for rosmarinic acid (*33*, *34*).

Other polyphenols contained in fractions II−VI were analyzed by HPLC−DAD chromatography monitored at 280 nm, specific absorbance for phenolic hydroxyl group, and the results obtained for fractions IV−VI are illustrated in **Figure 5**. The large peak at 34.69 min in the chromatogram of fraction IV appeared for rosmarinic acid. **Figure 5** reveals that the rosmarinic acid is only contained in fraction IV and is the main component of *O. basilicum*. HPLC−PAD analysis also reveals that other kinds of polyphenols with retention times of 11.21, 16.36, 23.36 (or 23.38), and 30.04 min are contained in *O. basilicum* and particularly in fractions V−VI. Their amounts in *O. basilicum* were much less than rosmarinic acid; therefore, the NMR analysis was not conducted. Instead, the LC-MS analysis was used to determine the possible structures of them. The use of either negative or positive APcI mass spectra for the determination of the flavonoids of many herbs (rosmary, sage, chives, coriander, lemon balm, cress, dill, lovage, mint, oregano, parsley, tarragon, thyme, and water cress) have been reported in early studies (*34*−*36*). In this study, both negative and positive modes (collision energy, 15 V) were used to obtain clear values for molecular masses of the compounds. When increasing the collision energy from 15 to 30 V, characteristic fragmentations in the mass spectrum appeared prominently that supported the identification of the structures

Table 2. Pseudomolecular Ions ($[M + H]^+$ and $[M - H]^-$), Along With Their Characteristic Fragment Ions in the Mass Spectrum of Polyphenols Responsible for the Antioxidative Activity of Each Fraction of *O. basilicum*[a]

fraction number	R_{time} (min)	polyphenol	$[M + H]^+$	$[M - H]^-$	$[(M + H) + 17]^+$	other fragment ions observed in positive mode	UV maxima (nm)
IV	17.44	caffeic acid	181	179	198		241, 297s, 327
	25.99	dihydrokaempferol-3-O-glucoside	467	465	484	181, 163, 152	245, 302s, 332
		luteolin acetyl-glucuronide	505	503	522		
		dihydroxykaemnipferol-glycoside	435	433	451		
	34.69	rosmarinicacid	361	359	378	163	234, 291s, 331
	39.7	caffeoyl ester	195	193		143	243, 298s, 327
	45.32	carnosic acid	333	331		687, 143	246s, 297
V	6.11	catechin	291	289	308	179	225, 250
	11.21	catechol derivatives **(NI-1)**	313	311	330	291, 240, 163	250s, 300s, 330
	16.36	cinnamyl derivatives **(NI-2)**	327	325	344	264, 215, 193, 179	300, 328, 236
	19.26	caffeic acid	181	179	198		244, 300s, 330
	23.36	caffeoyl derivatives **(NI-3)**	475	473	492	295, 181	244, 305
	24.91	**NI-4**		249		193, 179	243, 300s, 331
	30.04	ferulic acid	195	193		179, 152	243, 300s, 330
		caffeoyl -3-*O*-rutiniside		487	506	215	
		rosmarinic acid	361	359			242, 300s, 330
	38.49	apigenin	271	269			241, 300s, 331
		apigenin-glucuronide	447	445			
	39.3	caffeoyl ester	195	193		171, 163	242, 298s, 327
	52.49	**NI-5**	409	407	426	239, 181	243, 315
	52.6	chlorogenic acid	355	353	372	249, 179, 152	245, 292
	54.13	**NI-6**	311	309	328	193, 179	245, 310
		chlorogenic acid	355	353	372		
VI	11.13	catechol derivatives	313	311	330	163, 179	246s, 300s, 330
	16.13	cinnamyl derivatives	327	325	344	235, 193, 179,	328, 236
	19.25	caffeic acid	181	179	198		244, 300s, 330
	23.4	caffeoyl derivatives	475	473	492	179	244, 305
	24.91	**NI-4**		249		193, 179	243, 300s, 331

[a] The UV maxima of the polyphenols recorded by the PAD in the HPLC solvent used; s, shoulder peak.

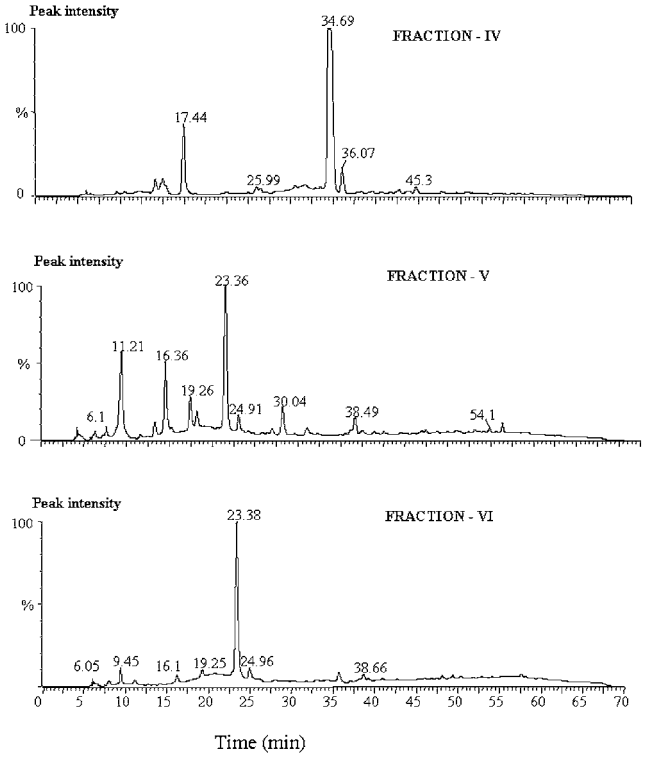

Figure 5. HPLC chromatograms for the fractions (IV–VI) of *O. basilicum*, obtained from octadecylsilane (ODS) column separation monitored at 280 nm by PAD. Peak intensities are unique for each fraction. HPLC conditions are enclosed in the text.

of compound. A similar method was applied in previous works for identification of compounds by APcI-MS (*34*, *36*). Most of the compounds commonly produced an additional ion $[(M + H) + 17]^+$ in chemical positive ionization (*37*), which supported identification of the molecular mass of the original compounds. The identified phenolic compounds that produced peaks on PAD are presented in **Table 2** with their pseudomolecular ions ($[M + H]^+$ and $[M - H]^-$) and characteristic fragmentations in the mass spectrum.

Minor constituents found were caffeic and ferulic acids as phenolic acids and carnosic acid as a diterpene in fraction IV. Dihydroxy kaempferol 3-*O*-glucoside (*m*/*z* 466), luteolin acetyl glucuronide (*m*/*z* 504), and dihydroxy kaempferol 3-*O*-glycoside (*m*/*z* 434) were the flavonoid glycosides found in fraction IV that were not reported previously in *O. basilicum* extracts but in other herbs (*38*). These three exhibited virtually the same retention times and UV and mass spectra. Interestingly, 70% of the total phenolic compounds in fraction IV was contributed by rosmarinic acid and 17% caffeic acid, with less than 15% by others.

Catechol (NI-1, *m*/*z* 312), cinnamyl (NI-2, *m*/*z* 326), and caffeoyl (NI-3, *m*/*z* 474) derivatives were the main compounds contributing 22, 15, and 33%, with respect to the rosmarinic acid in fraction IV, to fraction V, respectively. Minor amounts (<6%) of other phenolic compounds found in the same fraction were also reported as good inhibitors of oxidation in lipid systems that would increase the antioxidant function in fraction V. Antioxidant activity of fraction VI seemed mainly to depend on the caffeoyl derivative (*m*/*z* 474) (34% with respect to the rosmarinic acid in fraction IV); the presence of others was less than 2%.

The UV spectroscopic maxima of the compounds in all fractions between 300 and 400 nm indicated the presence of several flavonoids in accordance with previous observations (*6*, *19*, *37*) made on the presence of number of flavonoids in *Ocimum* spp. Unfortunately, the low concentrations existing after

Table 3. Oxidation Inhibition Rates and Inhibition Times of the Peroxidation of Soy Phospatidylcholine Liposome by Rosmarinic Acid (P-1) Separated from *O. basilicum* and Its Synergistic Effect with α-Tocopherol[a]

amount of α-tocopherol (μM)	introduced amount of rosmarinic acid (μM)	oxidation inhibition time (s)	oxidation rate × 10^{-2} (μM/s)	oxidation inhibition rate × 10^{-4} (μM/s)	S_{50} of α-tocopherol (s)	S_{50} of rosmarinic acid (s)
			1.05	0.00		
1		2880	1.13	8.68	1200	
	2	4380	0.75	14.80		1140
1	2	9240	0.71	2.48	4200	1320

[a] S_{50}, time taken to reduce 50% of total antioxidant introduced to the system.

the different extracting procedures followed in this study did not allow identification by their fragmentations in the mass spectrum.

The antioxidant activity of P-1, rosmarinic acid, was closely examined using the phosphatidylcholine liposome system. **Table 3** shows the comparison of oxidation inhibition time, oxidation inhibition rate, and oxidation rate of α-tocopherol and rosmarinic acid. The oxidation inhibition time (t_{inh}) was expressed as eq 7.

$$n \times [\mathrm{IH}] = t_{\mathrm{inh}} \times R_i \quad (7)$$

where n, [IH], and R_i indicate the radical scavenging number in an antioxidant, concentration of antioxidant, and rate of radical initiation from azo compound, respectively.

In the case of the antioxidant activity of α-tocopherol measured in this system, the oxidation inhibition time was 2880 s. The concentration of α-tocopherol was 1 μM, and α-tocopherol could scavenge two radicals ($n = 2$). Therefore, R_i is calculated as $2 \times 1/2880$ (μM/s). In the case of P-1, rosmarinic acid, analyzed in the same system, [IH] and t_{inh} were 2 μM and 4380 s, respectively. Consequently, the n value of rosmarinic acid is calculated as 1.52 ($4380 \times 2 \times 1/2880/2$). This value means that one rosmarinic acid molecule in the phosphatidylcholine liposome system can scavenge 1.52 radicals. Rosmarinic acid has four phenolic hydroxyl groups separated into two pairs located in *ortho*-positions of the benzene rings (**Figure 4**). In a theoretical sense, rosmarinic acid can capture four radicals; however, the result was 1.52. Probably, rosmarinic acid cannot act efficiently like α-tocopherol in the liposome system because rosmarinic acid is a hydrophilic compound. The formation rates of phosphatidylcholine hydroperoxides during the inhibition time for α-tocopherol and rosmarinic acid were 8.68×10^{-4} and 14.8×10^{-4} μM/s, respectively (**Table 3**). When different antioxidants were introduced to the phosphatidylcholine liposome system, the efficacy to scavenge peroxy radicals can get by calculating the ratio between "rate of oxidation" and "inhibition rate of oxidation". According to that, when 1 μM of α-tocopherol was introduced, the efficacy to scavenge peroxy radicals is calculated at 13.01 {oxidation rate/oxidation inhibition rate; 1.13×10^{-2} (μM/s)/8.68×10^{-4} (μM/s)}, and with the introduction of 2 μM of rosmarinic acid, the value became 5.06. Apparently, α-tocopherol efficacy to scavenge peroxy radicals in soy phosphatidylcholine liposome system was 2.6 times higher as compared to the rosmarinic acid. These results indicate that rosmarinic acid cannot suppress the oxidation of phosphatidylcholine to the same degree as α-tocopherol and are consistent with a low n value (stoichiometric number) for rosmarinic acid.

Vitamin E prevents the peroxidation of polyunsaturated fatty acids in membranes. Recent epidemiological studies showed that a high intake of vitamin E reduces the risk of coronary heart disease and that low levels of vitamin E seem to correlate with an increased incidence of myocardial infraction (*1*). The most active and available form of vitamin E is α-tocopherol. Vitamin E is incorporated into cell membranes of foods and has been used as a food additive to protect the oxidation of the foods.

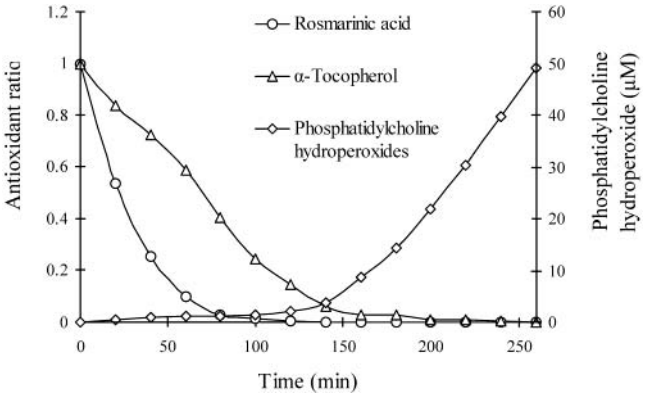

Figure 6. Inhibition of phosphatidylcholine hydroperoxides formation in the oxidation of soy phosphatidylcholine liposome by α-tocopherol (1 μM) and rosmarinic acid (2 μM) introduced to the system together. The remaining of each antioxidant in the system calculated by ratio of appropriate peak areas at "*t*" time and "0" time ($time_{(t)}/time_{(0)}$).

The synergistic effect of antioxidant activity is very important to protect food from degradation. The most familiar combination of a synergistic antioxidant effect is hydrophobic vitamin E (α-tocopherol) and hydrophilic vitamin C (ascorbic acid). This combination is now widely used for the prevention of oxidation in foods. Consequently, the interaction between α-tocopherol and each fraction (II−IV) of *O. basilicum* was examined. Results revealed that phenolic compounds contained in fractions II and III did not show synergism with α-tocopherol, while fractions IV, V, and VI had synergistic effects (data not shown). The excellent synergistic inhibition of lipid peroxidation of fraction IV with α-tocopherol in the soy phosphatidylcholine liposome system was observed. Therefore, to understand the total antioxidant potency of P-1 (rosmarinic acid) extracted from *O. basilicum* and α-tocopherol, the synergistic inhibition of lipid peroxidation was measured and results are shown in **Table 3**. The inhibition times of phosphatidylcholine hydroperoxides formation for 1 μM of α-tocopherol, 2 μM of rosmarinic acid, and a combination of the same concentrations of both antioxidants were 2880, 4380, and 9240 s, respectively. These results reveal the existence of a synergistic effect between rosmarinic acid and α-tocopherol.

Figure 6 presents the activity of antioxidants toward lipid peroxidation in soy phosphatidylcholine liposome when the radical initiator, AMVN, initiates oxidation. The data expressed that both antioxidants α-tocopherol (1 μM) and rosmarinic acid (2 μM) started to donate electrons to the system at the same time; however, the decomposition rate of rosmarinic acid is higher than that of α-tocopherol. Consequently, the S_{50} value (time taken to reduce 50% of total antioxidant introduced to the system) for α-tocopherol is 3.5 times higher than that of rosmarinic acid (**Table 3**). In the case of the combination of α-tocopherol and ascorbic acid, the decrease of α-tocopherol starts after all ascorbic acid was consumed, because ascorbic acid can donate an electron to α-tocopherol radical. Apparently, the factors affecting the function of antioxidants are dependent, and most probably, the equilibrium potential of rosmarinic acid might be higher than that of ascorbic acid.

Even though environmental differences such as growing area and climatic changes affect the composition of herbs (*37*), this study reveals that the potency of *O. basilicum* as a natural antioxidant is due to high prevalence of phenolic compounds. Rosmarinic acid, the main active component found in *O. basilicum*, has been proven to have medicinal value, and its superior antioxidant activity with vitamin E (α-tocopherol) confirms the impotence of *O. basilicum* as a culinary herb that frequently comes with our meals.

ABBREVIATIONS USED

APcI-MS, atmospheric pressure chemical ionization-mass spectroscopy; BHA, butylated hydroxy anisole; BHT, butylated hydroxy toluene; PAD, photodiode array detector; DPPH, 2,2-diphenyl-1,1-picrylhydrazyl.

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