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In Vitro Antioxidant Properties and Phenolic Composition of *Salvia virgata* Jacq. from Turkey

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Antioxidant activities and phenolic compositions of the active fractions of *Salvia virgata* Jacq. (Lamiaceae) from Turkey were examined. The aerial part of *S. virgata* was extracted with different solvents in an order of increasing polarity such as hexane, ethyl acetate, methanol, and 50% aqueous methanol using a Soxhlet apparatus. Water extract was also prepared from *S. virgata* by reflux. All solvent fractions were investigated for their total phenolic contents, total flavonoids, flavonols, qualitative−quantitative compositions (by HPLC-PDA analysis), iron(III) reductive activities, free radical scavenging activities (using $DPPH^{\bullet}$), and effect upon linoleic acid peroxidation activities; also, the peroxidation level was determined by the TBA method. The results of activity tests given as IC_{50} values were estimated from nonlinear algorithm and compared with standards, viz., butylated hydroxytoluene, ascorbic acid, and gallic acid. Polar fractions were found to be more active for free radical activity whereas nonpolar fractions protected the peroxidation of linoleic acid. Rosmarinic acid was the most abundant component in the extracts, followed by caffeic acid and lutelin-7-*O*-glycoside.

KEYWORDS: *Salvia virgata*; Lamiaceae; antioxidant; $DPPH^{\bullet}$; linoleic acid peroxidation; free radical scavenging activity; HPLC; *β*-carotene assay

INTRODUCTION

Oxidation is essential to many living organisms for the production of energy to fuel biological processes (*1*) but oxygen is sometimes described as a "double-edged sword" because, as well as being a vital ingredient of energy-producing intracellular reactions, oxidative processes are also responsible for the reactive oxygen species (ROS) (*2*). ROS can be formed by different endogenous and exogenous ways: (1) aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages, and peroxyisomers are responsible for oxidation processes in the body, and (2) exogenous sources of free radicals including tobacco smoke, ionizing radiation, certain pollutants, organic solvents, and pesticides, etc. (*2*). These ROS cause oxidative changes in carbohydrate, DNA, lipid, and protein, and such changes may, in turn, lead to mutation, mitochondrial, and membrane disruption, enzyme inactivation cell damage, and tissue destruction. In addition, ROS have been implicated in more than 100 diseases including cancer, heart disease, arteriosclerosis, and diabetes (*3*).

Antioxidants can delay or inhibit the oxidation or propagation of oxidizing chain reactions (*4*) in the oxidation process. The inhibition of oxidation process is very important in foodstuff, because oxidative degradation of lipids is one of the main factors limiting their shelf life (*5*). Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *tert*-butylhydroquinone (TBHQ) have been used to protect food products against lipid peroxidation. In recent years, natural antioxidants have been focused on because of the harmful effects of synthetic antioxidants in the body (*6*).

Salvia is one of the most widespread members of the *Lamiaceae* (Labiatae) family. It features prominently in the pharmacopoeias of many countries throughout the world from the Far East, through Europe, and very different places, and several of the almost 1000 *Salvia* species have been used in many ways, e.g., essential oils used in perfumery. *Salvia* species especially *S. officinalis* are an important source of antioxidants used in food industry and have wider implications for the dietary intake of natural antioxidants (*6*).

Turkey is an important country for *Salvia* species in the world. The flora of Turkey includes 88 species of the genus *Salvia* (*7*). The decoction of *S. virgata*, a wild species for Turkey, is traditionally used for leukemia in Turkey. The objective of this work was to study *S. virgata*, hitherto not investigated for its antioxidant activity. Therefore, four extracts of different polarity and water extract of *S. virgata* were investigated for antioxidant activity in different in vitro antioxidant test systems. Furthermore, the total phenols, total flavonoids, and total flavonols were also analyzed using spectrophotometric techniques. The phenolic composition of the extracts was also identified by HPLC-PDA analysis.

MATERIAL AND METHODS

Plant Material and Reagents. Air-dried aerial plant material (*Salvia virgata* Jacq., Lamiaceae) was collected from Sivrihisar-Eskisehir on 19/6/04. Voucher specimens are kept at the Herbarium of the Faculty

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of Pharmacy of Anadolu University in Eskisehir, Turkey (ESSE 14417). Chromatographic standards were purchased from Sigma Chemical Co. Ultrapure water was used throughout and was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., MA). All remaining reagents were of the highest purity available and obtained from the Sigma Chemical Co. (St. Louis, MO).

Preparation of the Extracts. Air-dried *S. virgata* herb material (100 g) was powdered and sequentially extracted with hexane, ethyl acetate, methanol, and 50% methanol using a Soxhlet apparatus for 8 h of each. Thereafter, the extract was filtered and evaporated to dryness in vacuo at 40 °C. Separately, *S. virgata* herb material was extracted with water using reflux for 3 h. The water phase was filtered and freeze-dried. All the extracts were stored at −20 °C. Prior to analysis, an aliquot of each extract was dissolved and filtered through a 0.45 μm membrane (Whatman, UK) and used in all the methods.

Total Phenolics, Flavonoids, and Flavonols. Total phenols were estimated as gallic acid equivalents (GAE), expressed as mg of gallic acid/g extract (*8*). To ca. 6.0 mL of H_2O, 100 μL of sample was transferred in a 10.0 mL volumetric flask, to which 500 μL undiluted Folin−Ciocalteu reagent was added subsequently. After 1 min, 1.5 mL 20% (w/v) Na_2CO_3 was added and the volume was made up to 10.0 mL with H_2O. After 2 h incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. The data are presented as the average of triplicate analyses.

Total flavonoids were estimated as rutin equivalents (RE), expressed as $mg_{rutin}/g_{extract}$ (*9*). One milliliter of plant extract in methanol (10 g/L) was mixed with 1 mL of aluminum trichloride in ethanol (20 g/L) and diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min at 20 °C. Blank samples were prepared from 1 mL plant extract and 1 drop of acetic acid, and diluted to 25 mL. The rutin calibration curve was prepared in ethanolic solutions with same procedure. All determinations were carried out in quadruplicate and the mean values were used.

Total flavonols were estimated as rutin equivalents (RE), expressed as $mg_{rutin}/g_{extract}$ (*9*). The rutin calibration curve was prepared by mixing 2 mL of 0.5–0.015 mg/mL of rutin ethanolic solutions with 2 mL (20 g/L) of aluminum trichloride and 6 mL (50 g/L) of sodium acetate. The absoption at 440 nm was read after 2.5 h at 20 °C. The same procedure was carried out with 2 mL of plant extract (10 g/L) instead of rutin solution. All determinations were carried out in quadruplicate and the mean values were used.

Qualitative−Quantitative Chromatographic Analysis. The liquid chromatographic apparatus (Shimadzu LC 10AVP, Ant Ltd. Şti., Istanbul, Turkey) consisted of an in-line degasser, pump, and controller coupled to a SPD-M10AVP photo diode array detector equipped with an automatic injector interfaced to Class VP chromatography manager software (Shimadzu, Japan). Separations were performed on a 250 × 4.6 mm i.d., 5 μm particle size, reverse-phase Discovery-C18 analytical column (Supelco, PA) operating at room temperature (22 °C) at a flow rate of 1 mL/min. Detection was carried out with a sensitivity of 0.1 aufs between the wavelengths of 200−550 nm. Elution was carried out using a ternary nonlinear gradient of the solvent mixture MeOH/H_2O/CH_3COOH (10/88/2, v/v/v) (solvent A), MeOH/H_2O/CH_3COOH (90/8/2, v/v/v) (solvent B), and MeOH (solvent C). The composition of B was increased from 15% to 30% in 15 min, increased to 40% in 3 min and held for 12 min, and increased to 100% in 5 min; then the composition of C was increased to 15% in 2 min, increased to 30% in 11 min, and then returned to the initial conditions in 2 min. Components were identified by comparison of their retention times to those of authentic standards under identical analysis conditions and UV spectra using our in-house PDA library. A 10 min equilibrium time was allowed between injections. All standard and sample solutions were injected triplicate.

Iron(III) to Iron(II) Reduction Activity. The ability of the extracts to reduce iron(III) was assessed by the method of Oyaizu (*10*). One milliliter of each extract dissolved in H_2O was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of a 1% (w/v) potassium hexacyanoferrate solution. After 30 min incubation at 50 °C, 2.5 mL 10% (w/v) trichloroacetic acid (TCA) was added and the mixture was centrifuged for 10 min. Finally, 2.5 mL of the upper layer was mixed with 2.5 mL H_2O and 0.5 mL 0.1% (w/v) $FeCl_3$ and the absorbance was recorded at 700 nm. The reductive activities of the extracts are expressed as ascorbic acid equivalents (AscAE) in mmol ascorbic acid/g sample (*11*). The larger the AscAE value, the greater the reducing power of the sample. The data are presented as the average value of quadruplicate analyses.

1,1-Diphenyl-2-picrylhydrazyl (DPPH•) Radical Scavenging Activity. The ability of the extracts to scavenge DPPH• radicals was determined by the method of Gyamfi et al. (*12*). A 50 μL aliquot of each extract, in 50 mM Tris-HCl buffer (pH 7.4), was mixed with 450 μL of Tris-HCl buffer and 1.0 mL of 0.1 mM DPPH• in MeOH. After 30 min incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using eq 1. Estimated IC_{50} values are presented as the average of quadruplicate analyses.

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

Determination of Inhibition of β-Carotene/Linoleic acid Co-oxidation. Antioxidant activity of extracts of *S. virgata* was determined according to β-carotene bleaching methods (*13, 14*). Briefly, 1 mL of β-carotene (0.2 mg/mL dissolved in chloroform; Sigma Chemical Co., St. Louis, MO) was added to flask containing linoleic acid (40 mg) and Tween 80 (400 mg). Chloroform was evaporated under a stream of nitrogen. Fifty milliliters of distilled water was added and shaken vigorously. Control was prepared without sample or standard using the same procedure. Blanks of control and sample also prepared without β-carotene. Their absorbances were measured on a spectrophotometer at 470 nm. The samples were then subjected to thermal autoxidation by keeping them in a constant temperature water bath at 50 °C for 2 h. The rate of bleaching of β-carotene was monitored by taking the absorbance at 15 min intervals. Antioxidative activity was calculated according to eq 2.

$$\text{AA\%} = [1 - (\text{Ab}^0_{\text{sample}} - \text{Abs}^{120}_{\text{sample}})/(\text{Ab}^0_{\text{control}} - \text{Abs}^{120}_{\text{control}})] \times 100 \quad (2)$$

Determination of Inhibition of Linoleic Acid Peroxydation. *Iron(II) Thiocyanate Method.* The ability of the extracts to inhibit the linoleic acid peroxydation was determined by the method of Llorach et al. (*15*). All reagents were prepared fresh. Reaction mixture (2.525 mL) in screw cap bottle comprised linoleic acid (2.55) (0.25 mL) in ethanol, 50 mM sodium phosphate buffer, pH 7 (1 mL), ethanol (0.25 mL), distilled water (0.9 mL), sample solution (0.1 mL), and 1.8 mM AAPH (25 mL) for acceleration. This mixture was mixed vigorously and placed in an oven at 50 °C for 10 h incubation. Thirty microliters of the reaction mixture was taken in every 2 h added into 2910 μL of ethanol with 30 mL of ammonium thiocyanate (3.86 M) solution. Thirty microliters of iron(II) solution was added and mixed vigorously and then the absorbance at 500 nm was read after 3 min. Blank solution included all the reagents except the sample. Ascorbic acid, BHT, and gallic acid were used as positive controls. The average of quadruplicate analyses was given as result.

Measurement of MDA Value (TBA Method). The amount of malondialdehyde (MDA) formed in the reaction mixture was determined by the thiobarbituric acid (TBA) reagent (*16*). One milliliter of reaction mixture, 1 mL of trichloroacetic acid (TCA, 2.8%), and 1 mL of TBA (1%) were mixed vigorously, and then placed in a water bath at 90 °C for 20 min. After incubation, the reaction was stopped by placing in an ice bath for 10 min. Two milliliters of *n*-butanol was added into the reaction mixture and mixed vigorously. The butanol phase was separated after centrifuging at 3000 rpm for 5 min. The absorbance of butanol phase was measured at 532 nm using *n*-butanol as blank. The average of quadruplicate analyses was given as result.

Statistical Analysis. Data are presented as mean values ± standard error. All the statistical analyses were carried out using SPSS 10.0.1. (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) was performed by ANOVA procedures. Significant differences and errors between means were determined by Tukey's pairwise comparison test at a level of $p < 0.05$. IC_{50} values were estimated using a nonlinear regression algorithm.

Table 1. Extract Yield, Total Phenols, Flavonoids, and Flavonols, and HPLC Qualitative and Quantitative Data for *Salvia virgata*

		spectrophotometric results			HPLC results[e]						
					phenolic acids					flavonoids	
sample	yield[a]	total phenols[b]	total flavonoids[c]	total flavonols[d]	gallic	*p*-OH-benzoic	caffeic	*o*-coumaric	rosmarinic	luteolinglycoside	luteolin
A	21.5	28.31 ± 0.58	0.81 ± 0.09	0.02 ± 0.00	nd	nd	nd	nd	nd	nd	nd
B	12.5	64.47 ± 1.04	0.10 ± 0.02	0.02 ± 0.00	tr	0.03 ± 0.01	0.48 ± 0.04	0.83 ± 0.04	4.48 ± 0.13	0.15 ± 0.01	0.06 ± 0.00
C	45.0	133.79 ± 0.79	6.53 ± 0.11	tr	0.41 ± 0.03	tr	0.56 ± 0.02	10.78 ± 0.25	59.75 ± 1.66	0.69 ± 0.02	0.14 ± 0.01
D	119.0	212.30 ± 0.43	3.57 ± 0.05	tr	1.29 ± 0.07	0.46 ± 0.04	0.56 ± 0.04	7.63 ± 0.47	48.49 ± 2.84	0.47 ± 0.03	0.36 ± 0.02
E	152.0	116.22 ± 0.84	3.87 ± 0.06	0.01 ± 0.00	0.83 ± 0.03	0.11 ± 0.02	1.35 ± 0.11	1.34 ± 0.02	23.23 ± 0.43	0.23 ± 0.01	0.58 ± 0.02

[a] Extract yields expressed as milligrams of extract per gram (dry weight) of aerial material. [b] Total phenols expressed as gallic acid equivs milligrams of gallic acid per gram (dry weight) of extract. [c] Total flavonoids expressed as rutin equivsmilligrams of rutin per gram (dry weight) of extract. [d] Total flavonols expressed as rutin equivsmilligrams of rutin per gram (dry weight) of extract. [e] Values (mg/g) are expressed as means ± standard error. nd, not detected. tr, trace (<0.01). (A) hexane extract; (B) ethyl acetate extract; (C) methanol extract; (D) 50% methanol extract; (E) water extract.

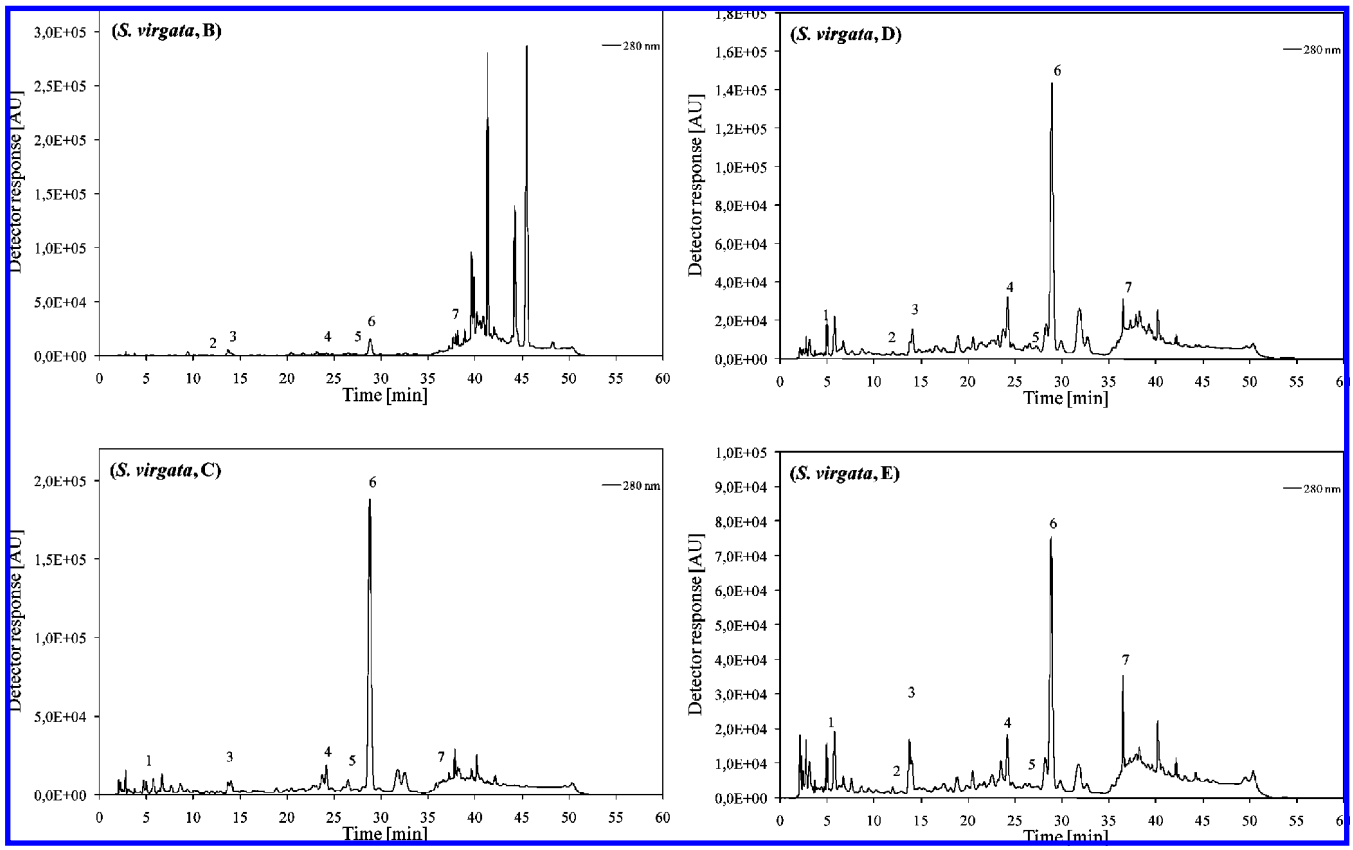

Figure 1. HPLC-PDA analysis of the *S. virgata* extracts with responses at 280: (B) ethyl acetate; (C) methanol; (D) 50% methanol; (E) water; **1**, gallic acid; **2**, *p*-OH-benzoic acid; **3**, caffeic acid; **4**, luteolin-7-*O*-glycoside; **5**, *o*-coumaric acid; **6**, rosmarinic acid; **7**, luteolin.

RESULTS AND DISCUSSION

Fraction Yields, Total Phenols, Flavonoids, Flavonols, and Compositional Analysis. *S. virgata* herb was sequentially extracted with hexane, ethyl acetate, methanol, and aqueous methanol (50%) using a Soxhlet apparatus. Water extract also obtained from *S. virgata* herb under reflux. The results of fraction yields, total phenols, total flavonoids, total flavonols, and compositional analysis of the extracts are presented in **Table 1**. According to the data presented in **Table 1**, aqueous methanol, methanol, and water extracts contained the highest amount of total phenol content, while the hexane extract had the lowest. The highest yields were obtained also from the aqueous methanol and water extracts.

The results of the qualitative−quantitative analyses of the extracts, carried out using an HPLC apparatus coupled to a PDA detector, are presented in **Table 1**, with selected chromatograms shown in **Figure 1**. Phenolic compounds were identified and quantified at 280, 320, and 360 nm as benzoates, hydroxycinnamates, and flavonoids, respectively. Gallic, *p*-OH-benzoic, caffeic, *o*-coumaric, rosmarinic acids, luteolin-7-*O*-glycoside, and luteolin were identified by comparison to the retention times and UV spectra of authentic standards, while quantitative data were calculated from their calibration curves. The aqueous methanol, methanol, and water extracts were found to be the richest for phenolics as measured by both UV spectrophotometry and HPLC. Rosmarinic acid was the main compound in all the extracts except for the hexane extract. *o*-Coumaric acid was found in the extracts as the second hydroxycinnamic acid. Methanol, aqueous methanol, and water extracts had more flavonoids among others. As well-known from the literature, rosmarinic acid was found as the principal component in sage species. Rosmarinic acid, carnosic acid, and their derivatives in sage species as main components and these compounds are responsible for their antioxidant activities (*17–24*).

Iron(III) to Iron(II) Reduction Activity. The ability of a fraction to reduce iron(III) represents its ability to donate

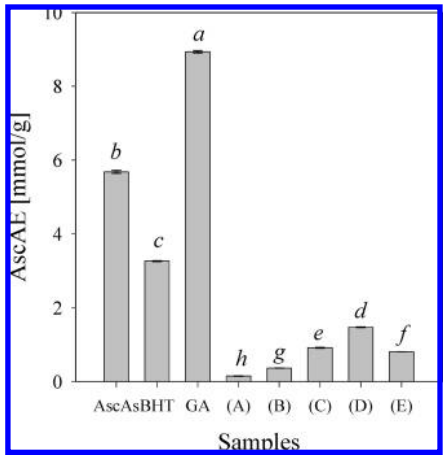

Figure 2. Effect of the extracts of *S. virgata* and positive controls on iron(III) reduction. AscAs, ascorbic acid; BHT, butylated hydroxytoluene; GA, gallic acid; (A) hexane; (B) ethyl acetate; (C) methanol; (D) 50% methanol; (E) water. Values are presented as means ± standard error. Bars with the same lowercase letter (a−f) are not significantly ($p < 0.05$) different.

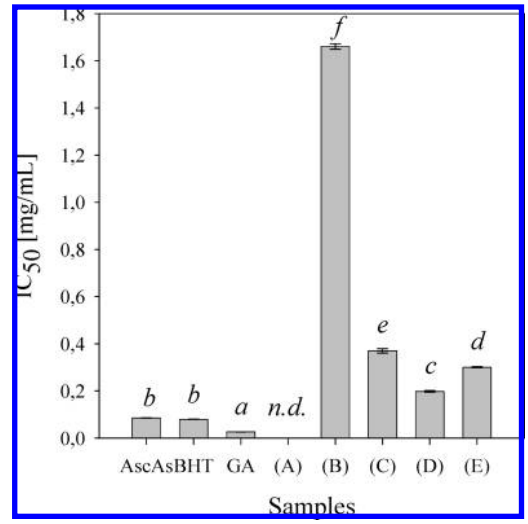

Figure 3. Effect of the extracts of *S. virgata* and positive controls on DPPH• radical scavenging. AscAs, ascorbic acid; BHT, butylated hydroxytoluene; GA, gallic acid; (A) hexane; (B) ethyl acetate; (C) methanol; (D) 50% methanol; (E) water; n.d., not detected. Values are presented as means ± standard error. Bars with the same lowercase letter (a−f) are not significantly ($p < 0.05$) different.

electrons (*3*). Reducing activity is very important in terminating radical chain reactions (*3*). The literature suggests that there is a high correlation between iron(III) to iron(II) reduction activity of aqueous extracts and antioxidant activity (*11, 25, 26*); however, this may not always be the case (*3*). In the iron(III) reduction assay, the ability of an extract to participate in redox reactions can be assessed and ranked according to its ascorbic acid equivalent value (AscAE), expressed as mmol ascorbic acid/g extract (*8, 11, 13*). The ability of all the extracts to reduce ferric iron to ferrous iron was investigated and the results are shown in **Figure 2** as AscAE. According to **Figure 2**, none of the fractions was as effective as the positive controls ascorbic acid, BHT, and gallic acid. The ability of aqueous methanol extract on the reduction of iron(III) to iron(II) was found significantly ($p < 0.05$) higher. The hierarchy of activity of the extracts was aqueous methanol > methanol > water > EtOAc > hexane.

Phenolic acids and flavonoids are well-known as natural antioxidants. Phenolic acids especially hydroxycinnamates show their antioxidant activities by a hydrogen-donating mechanism (*27, 28*). Rosmarinic acid and its derivatives, hydroxycinnamates, are reported as excellent DPPH•-radical scavengers (*29*). In this study, the solvent fractions studied were prepared with different solvents and each fraction was found to have a different compositions. Active fractions had rosmarinic acid as main compounds while some also contained flavonoids such as luteolin-7-*O*-glycoside. Both phenolic acids and flavonoids are soluble in polar solvents and show strong activity in polar test systems. Both iron(III) reduction and DPPH•-radical scavenging activities are performed in a polar media.

1,1-Diphenyl-2-picrylhydrazyl (DPPH•) Radical Scavenging Activity. The 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical is a stable radical, with an absorption maximum at 517 nm. When reduced to the hydrazine derivative by an antioxidant via electron or hydrogen atom transfer reactions, this absorption maximum decreases (*29*). All sage fractions managed to scavenge the DPPH• radicals at physiological pH and did so in a concentration-dependent fashion (data not shown). IC_{50} values, defined as the concentration required to scavenge 50% of the available free radicals, estimated by nonlinear regression for all the extracts are presented in **Figure 3**. All the extracts showed free radical scavenging activity in physiological pH except for the hexane extract. According to **Figure 3**, the aqueous methanol extract was the most active free radical scavenger of all. However, none of the fractions was as active as the positive controls, ascorbic acid, BHT, and gallic acid. However, aqueous methanol extract had twice more IC_{50} value than BHT and ascorbic acid. The order of DPPH• radical scavenging ability for the fractions was as follows: aqueous methanol > water > methanol > ethyl acetate. Hexane extracts was found to be inactive in in vitro DPPH• radical scavenging activity.

Sage is well-known as a natural source of antioxidants and widely used for purposes related to this property. The caffeic acid derivatives (caffeic acid and rosmarinic acid) and phenolic diterpenes (carnosic acid and carnosol) are principally responsible for its activity as they are very strong radical scavengers (*5, 29*). As seen in **Table 1**, the most active extract, aqueous methanol, has caffeic and rosmarinic acids and its derivatives as main components, and gallic acid as well. According to the literature, cinnamic acid derivatives were found to be more active than the benzoates, because cinnamates may be due to the presence of the conjugated unsaturation that facilitates the delocalization of the resulting free radicals. Cinnamic acids and caffeic acid derivatives had better activity than ferulic and coumaric acids (*29*). Flavonoids have relatively weak DPPH•-radical scavenging activity, and glycosylation has been reported to decrease radical scavenging activity (*21, 29*). Luteolin derivatives are more active than apigenin derivatives in DPPH• assay. This activity depends on hydroxylation, especially ortho-dihydroxylation, on the phenol ring (*29*). In a previous study using a HPLC post column derivatization method, rosmarinic acid and caffeic acid derivatives and luteolin derivatives were found to be the strong DPPH• radical scavengers (*24*).

Inhibition of β-Carotene/Linoleic Acid Co-oxidation. Food lipids and the cell membranes contained unsaturated fatty acids, linoleic and arachidonic acids, which can be oxidized easily with oxidative agents. Therefore, unsaturated fatty acid–base medium in antioxidant activity tests is important to determine the activities of test samples. The β-carotene−linoleic acid bleaching assay is such a model widely used to investigate the oxidation of unsaturated fatty acids, especially in the cell wall and food products (*13, 14*).

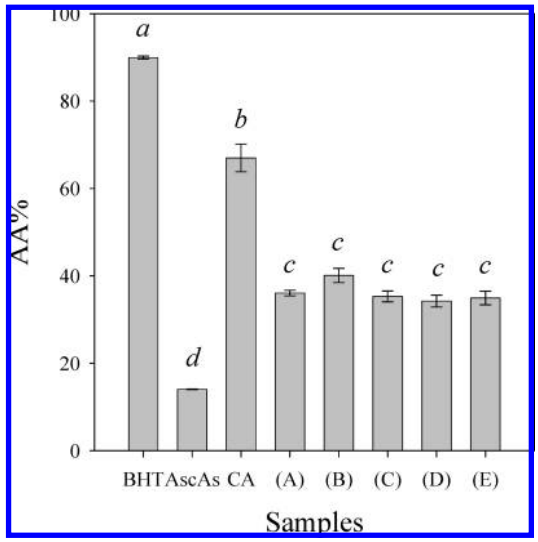

Figure 4. Effect of the extracts of *S. virgata* and positive controls on β-carotene/linoleic acid co-oxidation. AscAs, ascorbic acid; BHT, butylated hydroxytoluene; CA, caffeic acid; (A) hexane; (B) ethyl acetate; (C) methanol; (D) 50% methanol; (E) water. Values are presented as means ± standard error. Bars with the same lowercase letter (a−d) are not significantly ($p < 0.05$) different.

In β-carotene/linoleic acid co-oxidation assay, the degree of lipid peroxidation is measured. The inhibition percentages of all the extracts of *S. virgata* are given in **Figure 4**. According to **Figure 4**, all the extracts inhibited the oxidation of linoleic acid in a statistically same ($p < 0.05$) degree whereas they showed lesser activity than positive controls. Hierarchy of the extracts were BHT > CA > A ≈ B ≈ C ≈ D ≈ E > AscAs. None of the extracts was found to be as active as the positive control BHT. In this assay, ascorbic acid showed prooxidant activity and the results agreed with those of Siddhuraju and Becker (*30*). Nonpolar antioxidants are concentrated at the lipid−air interface and demonstrate high protection in emulsions against the polar antioxidants presented in aqueous phase (*30*). Koleva et al. (*31*) used the same method for *Sideritis* extracts and compared this method with some other antioxidant tests. They also reported simplicity, sensitivity, and sample-polarity dependence of this method (*31*).

Capacity of Inhibition of Linoleic Acid Peroxidation. *Iron(II) Thiocyanate Method.* Peroxidation of lipids is a principal cause of the oxidative deterioration of susceptible foodstuffs and the loss of physiological function in cellular organelles within the human body (*32*). Thus, phospholipids are a useful model substrate for the in vitro assessment of dietary components and membrane phospholipids as potential antioxidants. Furthermore, synthetic free radicals used to assess an antioxidant's reactivity to free radicals are physiologically irrelevant. The hydroxyl radical is physiologically relevant: these extremely reactive oxygen-derived species are capable of initiating deleterious in vivo chain reactions and are considered to play a role in the pathogenesis of numerous diseases (*33*).

In this method, the concentrations of 0.25% and 1% of all the extracts were used. The effects of extracts on the peroxidation of linoleic acid were determined using an accelerated oxidation method, and the results are shown in **Figure 5**. As seen in **Figure 5**, the ethyl acetate extract was found to be more active in a lipid system at both concentrations. This extract showed similar activity as the positive control BHT. The order of effects of the extracts was as follows: ethyl acetate > hexane > water ≈ aqueous methanol > methanol at the concentration

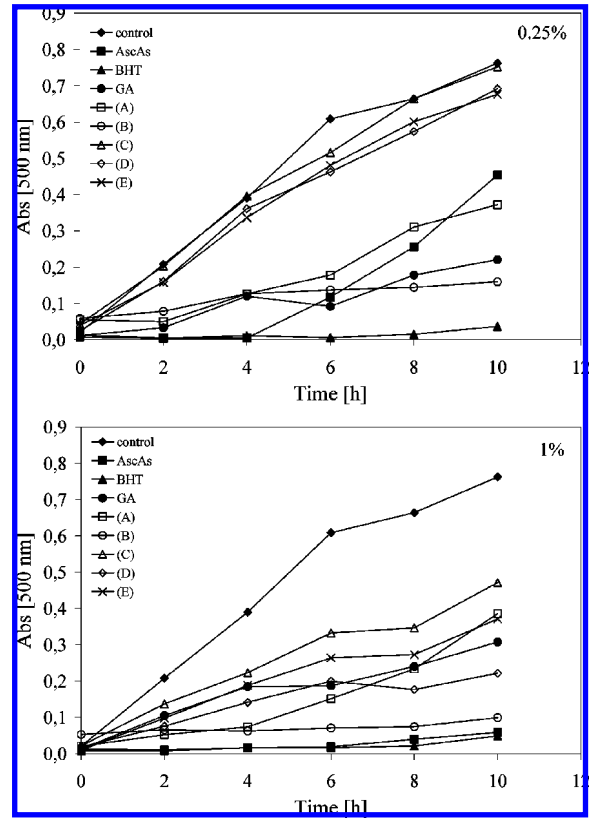

Figure 5. Effect of the extracts of *S. virgata* and positive controls on linoleic acid oxidation. AscAs, ascorbic acid; BHT, butylated hydroxytoluene; GA, gallic acid; (A) hexane; (B) ethyl acetate; (C) methanol; (D) 50% methanol; (E) water.

Table 2. Inhibition of *S. virgata* Extracts and Positive Controls on MDA Production after the Accelerated Oxidation Process

	FTC[a]		TBA[b]	
sample[e]	0.25%[c]	1%	0.25%	1%
BHT	94.93 ± 0.26[d]	93.63 ± 0.24	90.30 ± 0.73	89.73 ± 0.42
AscAs	40.84 ± 1.39	92.32 ± 0.90	20.88 ± 0.61	84.41 ± 2.31
gallic acid	70.79 ± 0.63	59.57 ± 2.35	68.17 ± 0.77	72.57 ± 0.98
A	52.07 ± 2.02	49.43 ± 1.49	81.69 ± 1.89	77.42 ± 2.30
B	79.39 ± 1.71	87.04 ± 1.21	87.86 ± 2.55	70.85 ± 2.30
C	5.95 ± 0.23	41.48 ± 1.26	34.80 ± 0.90	42.22 ± 1.94
D	15.04 ± 0.29	70.94 ± 1.73	27.05 ± 0.88	55.99 ± 2.51
E	14.49 ± 0.64	48.66 ± 1.52	44.01 ± 0.75	62.55 ± 2.03

[a] Iron(II) thiocyonate method. [b] TBA method. [c] Concentration of tested samples. [d] Values (%) are expressed as means ± standard error. [e] BHT, butylated hydroxytoluene; AscAs, ascorbic acid; (A) hexane extract; (B) ethyl acetate extract; (C) methanol extract; (D) 50% methanol extract; (E) water extract.

of 0.25% and ethyl acetate > aqueous methanol > water ≈ hexane > methanol at the concentration of 1%. It is well-known that lipophilic compounds are more active than hydrophilic compounds in the assay of lipid peroxidation (*5, 17, 18, 20*).

Measurement of MDA Value (TBA Method). In this study, MDA formed after oxidizing linoleic acid was measured using the TBA assay and the results are given in **Table 2**. Oxidized linoleic acid solutions used above were used in this assay. As seen in the table, the same results were obtained with the thiocyanate assay. Hexane and ethyl acetate extracts were found the most active extracts in this assay. Aqueous methanol and water extracts also protected linoleic acid against oxidation at the concentration of 1% in the reaction mixture.

In conclusion, sage has hydroxycinnamic acid derivatives (viz., caffeic and rosmarinic acids) as the main compounds that are

responsible for antioxidant activity as previously reported (*18, 20, 29*). The other active components in sage especially in the *S. officinalis* are the diterpenoids, carnosic acid, and carnosol derivatives. In our previous study, rosmarinic acid, caffeic acid, flavonoids, carnosic acid, carnosol, and their derivatives were identified as the free radical scavengers in sage (*S. officinalis*) using DPPH$^{\bullet}$ radical after it was separated chromatographically (*24*). Nonpolar extracts showed strong free radical scavenging activity as well as inhibition of lipids peroxidation in in vitro tests. Hydroxycinnamic acid derivatives were identified as main compounds in these extracts. Flavonoids are known to be more weakly active than diterpenoids and hydroxycinnamic acids in antioxidant activity tests. In this study, polar extracts were found to be rich in flavonoids. This can explain the weak antioxidant activity encountered in nonpolar fractions.

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