Food Chemistry 116 (2009) 470–479

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant, anticholinesterase and antimicrobial constituents from the essential oil and ethanol extract of *Salvia potentillifolia*

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ARTICLE INFO

Article history:
Received 12 August 2008
Received in revised form 11 December 2008
Accepted 23 February 2009

Keywords:
Lamiaceae
Salvia potentillifolia
Antioxidant activity
Antimicrobial activity
Anticholinesterase activity
Essential oil
GC–MS

ABSTRACT

The essential oil of *Salvia potentillifolia* was analysed by GC and GC–MS. Totally, 123 components were detected in both hydrodistilled and steam-distilled oils, α- and β-pinenes being major compounds. The antioxidant activities were determined by using complementary tests, namely, DPPH radical-scavenging, β-carotene-linoleic acid and reducing power assays. The ethanol extract also showed better activity (IC_{50} = 69.4 ± 0.99 μg/ml) than that of BHT in the DPPH system, and showed great lipid peroxidation inhibition in the β-carotene-linoleic acid system (IC_{50} = 30.4 ± 0.50 μg/ml). The essential oil showed meaningful butyrylcholinesterase activity (65.7 ± 0.21%), and α-pinene showed high acetylcholinesterase inhibitory activity (IC_{50} = 86.2 ± 0.96 μM) while β-pinene was inactive. Antimicrobial activity was also investigated on several microorganisms, and the essential oil showed high activity against *Bacillus subtilis* and *B. cereus*. It also exhibited remarkable anticandidal activity against *Candida albicans* and *C. tropicalis* with MIC values of 18.5 and 15.5 μg/ml, respectively, while α- and β-pinenes showed moderate activity.

1. Introduction

Salvia potentillifolia Boiss. and Heldr. ex Bentham (Lamiaceae) is an endemic species and grows in open habitats in the eastern Mediterranean. *S. potentillifolia* is known as "Adaçayı" or "Şalba" in Mugla, Turkey and has been used as a folkloric drug, rarely consumed as a tea. *Salvia* genus has about 900 species, and is widespread throughout the world. This genus is represented in Turkish flora, by 89 species and 97 taxa, 45 of which are endemic (Güner, Özhatay, Ekim, & Başer, 2000). *Salvia* species have also been used for medicinal purposes in several regions of the world (Ulubelen & Topçu, 1992). Several *Salvia* species are also used against stomach ache, headache, wounds and skin infections and the most common use of *Salvia* species is against colds. Some members of this genus have economic importance as flavouring agents in perfumery and cosmetics. *Salvia* species have been sold commercially, not only for use in therapy, but also as a spice to flavour meats, such as pork, sausage and poultry (Gali-Muhtasib, Hilan, & Khater, 2000). Sage (*S. officinalis*) has been credited with a long list of medicinal uses, e.g., spasmolytic, antiseptic and astringent (Newall, Anderson, & Philipson, 1996). *Salvia* species are used as traditional medicines all around the world, possessing antibacterial, antioxidant, antidiabetic and antitumor (Ulubelen & Topçu, 1992) properties. *Salvia* species (*S. officinalis* L. and *S. lavandulaefolia* Vahl., *S. miltiorrhiza* Bunge.) are also promising for their reputed beneficial effects on memory disorders, depression and cerebral ischaemia (Howes, Perry, & Houghton, 2003). Several studies have now been undertaken to investigate the traditional (CNS-related) use of *S. lavandulaefolia* relevant to the treatment of Alzheimer's disease; these have centered on the activity of the essential oil (Perry, Houghton, Jenner, Keith, & Perry, 2002). From Turkish *Salvia* species many antibacterial (Ulubelen & Topçu, 1992), cytotoxic (Topçu, Tan, Kökdil, & Ulubelen, 1997), antioxidant (Topçu, Ertaş, Kolak, Öztürk, & Ulubelen, 2007) and antituberculous (Ulubelen, Topçu, & Bozok-Johansson, 1997) compounds, as well as cardioactive terpenoids, have been isolated. Some Turkish *Salvia* extracts have also been investigated for their AChE and BChE inhibitory activities (Orhan et al., 2007).

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Spices are well known for their antioxidant capacities

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doi:10.1016/j.foodchem.2009.02.069

(Madsen & Bertelsen, 1995). In recent decades, the essential oils and various extracts of plants have been of great interest as sources of natural products. In order to prolong the storage stability of foods, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ) have been widely used in the food industry to prevent oxidative deterioration, but BHA and BHT are suspected of being responsible for liver damage and carcinogenesis (Grice, 1988). Some secondary metobolites of the plants have begun to receive much attention as safe antioxidants since they have been consumed by people and animals for years. Therefore, the development and utilisation of more effective antioxidants of natural origin are desired.

So far, a few studies on *S. potentillifolia* have been carried out. However, no anticholinesterase, antimicrobial or antioxidant activities of *S. potentillifolia* have previously been published. There is a report on the essential oil of *S. potentillifolia* collected from Antalya, Turkey (Sarer, 1990), but it has not been investigated in detail. We aimed to investigate the constituents of the ethanol extract and detailed essential oil composition of *S. potentillifolia* plants with antimicrobial, anticholinesterase and antioxidant activities. The objective of this study is to compare antioxidant, anticholinesterase and antimicrobial activities of the extracts, including major constituents, with those of commercial and synthetic antioxidants and antibiotics which are commonly used in the food and pharmaceutical industries.

2. Materials and methods

2.1. Plant material

Whole plant material of *S. potentillifolia* Boiss. and Heldr. ex Bentham (Lamiaceae) was collected from the Gölhisar-Altınyayla region (Burdur), Turkey in July, 2004. The voucher specimen (No.: S-1003), identified by Dr. Tuncay DIRMENCI, has been deposited in the Herbarium of the Department of Biology, Faculty of Arts and Science, Muğla University, Turkey.

2.2. Spectral measurements and chemicals

The spectra were recorded with the following instruments: IR: Perkin–Elmer 1615 in $CHCl_3$; NMR: Varian-Innova-500 'Defne', 500 MHz and 125 MHz for ^1H and ^{13}C NMR, in $CDCl_3$, respectively, only for compounds **2**, **5** and **6**, Varian Mercury-Vx 400, 400 MHz and 100 MHz for ^1H and ^{13}C NMR, respectively. UV–VIS measurements were recorded on a Shimadzu UV-1601 (Kyoto, Japan) including antioxidant activity tests, UV spectral analyses of flavonoids (**2**, **3**) and flavonoid glycosides (**5**, **6**) were carried out according to Mabry, Markham, and Thomas (1970). Anticholinesterase activity measurements were performed with a SpectraLab 340PC, Molecular Devices (NY, USA).

Silicagel 60, used for column chromatography, and Kieselgel $60F_{254}$ (E. Merck), used for preparative TLC as precoated plates, potassium ferricyanide, ferric chloride, chloroform, methanol, pyrocatechol, quercetin, sodium acetate, boric acid, aluminium chlorate, metallic sodium and trichloroacetic acid (TCA) were obtained from E. Merck (Darmstadt, Germany). Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide (AcI) and butyrylthiocholine chloride (BuCl), Folin–Ciocalteu's reagent (FCR), β-carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), 1,1-diphenyl-2-picrylhydrazyl (DPPH·), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and α-tocopherol (TOC) were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were of analytical grade.

2.3. Isolation and analysis of the essential oil

2.3.1. Oil preparation

The essential oil of dried aerial parts of *S. potentillifolia* was obtained via hydrodistillation by using a Clevenger type apparatus for 4 h and by steam-distillation for 5 h. The oils were dried over anhydrous sodium sulphate and stored under nitrogen until required.

2.3.2. Gas chromatography (GC)

GC analyses of the essential oil were performed using a Shimadzu GC-17 AAF, V3, 230V series gas chromatograph equipped with a FID and a DB-1 fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm); the initial oven temperature was held at 60 °C for 5 min, then increased up to 220 °C with 2 °C/min increments and held at this temperature for 10 min; injector and detector temperatures were 250 and 270 °C, respectively; carrier gas was He at a flow rate of 1.4 ml/min; sample size, 1.0 μl; split ratio, 50:1. The percentage composition of the essential oil was determined with a Class-GC computer programme. The relative percentages of the oil constituents were expressed as percentages by peak area normalisation.

2.3.3. Gas chromatography–mass spectrometry (GC–MS)

The analysis of the essential oil was performed using a Varian Saturn 2100, (E.I. Quadrapole) equipped with a DB-1 MS fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm). For GC–MS detection, an electron ionisation system with ionisation energy of 70 eV was used. Carrier gas was helium at a flow rate of 1.7 ml/min. Injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. The oven temperature was held at 60 °C for 5 min, then increased up to 220 °C with 2 °C/min increments and held at this temperature for 10 min. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 μl were injected manually in the splitless mode.

Identification of components of the essential oil was based on GC retention indices and computer matching with the Wiley, Nists and TRLIB libraries, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature and, whenever possible, by co-injection with authentic compounds.

2.4. Extraction, isolation and identification

The dried and powdered aerial parts of *S. potentillifolia* (20 g) were extracted with 100 ml of ethanol, five times, at room temperature. After filtration, the solvent was evaporated to dryness *in vacuo*. The crude extract (2.312 g) was fractionated on a silica gel column (1.0 × 10 cm). The column was eluted with petroleum ether (40–60°), followed by a gradient of dichloromethane up to 100%, and then 2% gradient of methanol up to 100%. The similar fractions were combined by using thin-layer chromatography. Antioxidant fractions were determined, on TLC, by DPPH· and β-carotene-linoleic acid assays. The last purification of the compounds of antioxidant-active fractions on TLC was done using preparative thin-layer chromatographic methods. The compounds (**1**–**6**) were isolated by using the following solvent systems: β-sitosterol (7.1 mg) (**1**) (hexane:CH_2Cl_2, 1:9) and 6-methoxyapigenin (3.0 mg) (**2**) and salvigenin (4.5 mg) (**3**) from fractions 13 to 17; ursolic acid (9.0 mg) (**4**) (hexane:ethyl acetate, 3:2) from fractions 19 to 23; luteolin 7-*O*-α-D-glucoside (3.2 mg) (**5**) (chloroform:methanol, 9:1) and quercetin 3-*O*-β-D-galactoside (0.9 mg) (**6**) from fractions 41 to 45.

By comparison of the spectral data (UV, IR, 1D- and 2D-NMR, MS) of purified compounds with those of standard samples, compounds (**1**–**6**) were identified as β-sitosterol (**1**), 6-methoxyapige-

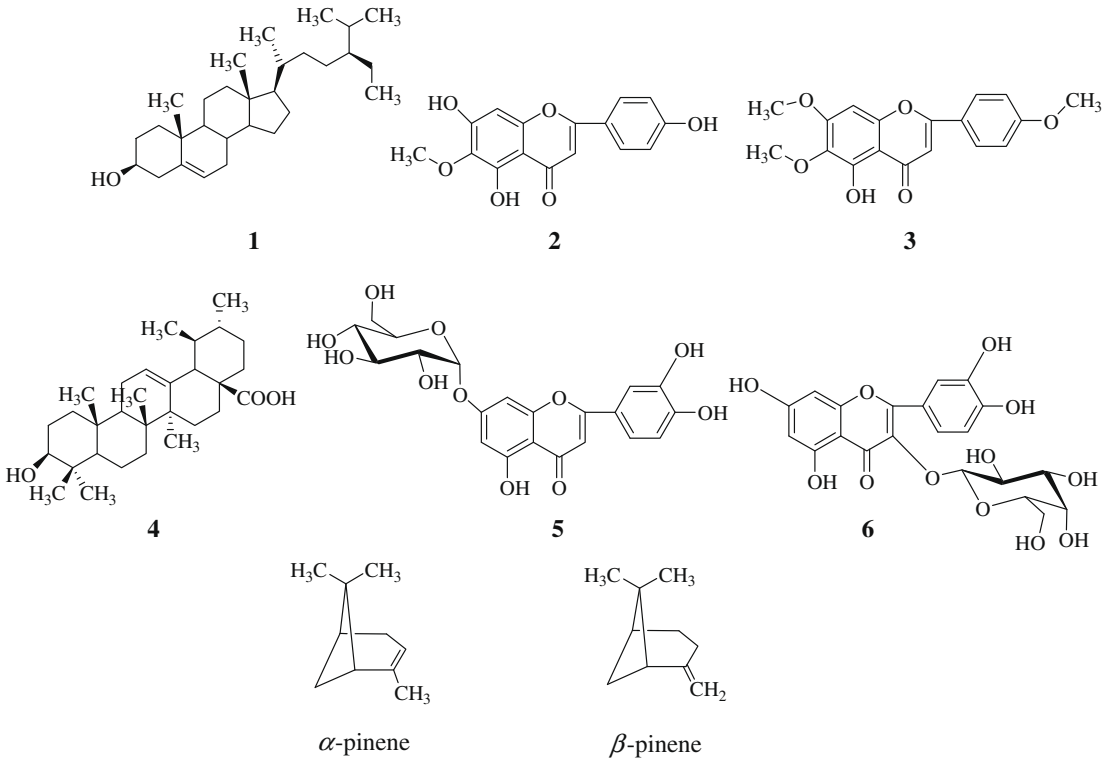

Fig. 1. Chemical structures of the compounds identified from *S. potentillifolia*.

nin (**2**), salvigenin (**3**), ursolic acid (**4**), luteolin 7-*O*-α-D-glucoside (**5**), and quercetin 3-*O*-β-D-galactoside (**6**) (Fig. 1).

2.5. Bioassays

2.5.1. Antimicrobial activity

2.5.1.1. Inhibitory effect by disc diffusion method. The following strains of bacteria were used: *Pseudomonas aeruginosa* NRRL B-23, *Salmonella enteritidis* RSKK 171, *Escherichia coli* ATCC 25922, *Morganella morganii* (clinical isolate), *Yersinia enterecolitica* RSKK 1501, *Klebsiella pneumoniae* ATCC 27736, *Proteus vulgaris* RSKK 96026, *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* NRRL B-4375, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* RSKK 863, *Candida albicans* ATCC 10231 and *C. tropicalis* (clinical isolate). The bacteria were obtained from the culture collection of the Microbiology Department of Pamukkale University and Ankara University. The antimicrobial activity of the essential oil of *S. potentillifolia* was assayed by the standard disc diffusion method (Murray, Baron, Pfaller, Tenover, & Yolke, 1995). Empty sterilised discs of 6 mm (Schleicher and Schuell, No. 2668, Germany) were each impregnated with 25 μl of essential oils. All the microorganisms mentioned above were incubated at 37 ± 0.1 °C (30 ± 0.1 °C for *M. luteus* NRRL B-4375) for 24 h by inoculation into nutrient broth and the yeast cultures were incubated in YEPD broth at 28 ± 0.1 °C for 48 h. The culture suspensions were prepared and adjusted by comparing against 0.5 Mac-Farland turbidity standard tubes. Nutrient Agar (NA) and YEPD Agar (15 ml) were poured into each sterile Petri dish (100 mm diameter) after injecting cultures (0.1 ml) of bacteria and yeast and distributing medium into Petri dishes homogeneously. The discs, injected with samples, were placed on the inoculated agar by pressing slightly. Petri dishes were kept at 4 °C for 2 h; plates injected with the yeast cultures were incubated at 28 °C for 48 h, and the bacteria were incubated at 37 °C (30 °C for *M. luteus* NRRL B-4375) for 24 h. At the end of the period, inhibition zones formed on the medium were evaluated in millimetre. Studies performed in duplicate and the inhibition zones were compared with those of reference discs. Reference discs used were as follows: Ketoconazole (50 μg), Ampicillin (10 μg), Penicillin (10 U) and Gentamicin (10 μg).

2.5.1.2. Determination of minimum inhibitory concentrations (MIC) of essential oil, α- and β-pinenes. Micro-dilution broth susceptibility assay was used (NCCLS (National Committee for Clinical Laboratory Standards), 1999). Stock solution of the essential oil and crude extract, as well as of α- and β-pinenes, was prepared in 10% DMSO. Serial dilutions, ranging from 2.5 to 85.00 μg/ml, of the essential oil were prepared. Test strains were prepared using 24 h cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The plates incubated at 37 °C for bacteria, and at 28 °C for yeasts. α- and β-pinenes were tested for the anticandidal activity. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms.

2.5.2. Anticholinesterase activity

Acetylcholinesterase and butyrylcholinesterase inhibiting activities of the essential oil, ethanol extract and the essential oil major compounds were measured by slightly modifying the spectrophotometric method developed by Ellman, Courtney, Andres, and Featherston (1961). Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates of the reaction and DTNB was used for the measurement of the cholinesterase activity. One-fifty microlitres of 100 mM sodium phosphate buffer (pH 8.0), 10 μl of test compound solution and 20 μl of AChE or BChE solution were mixed and incubated for 15 min at 25 °C, and 10 μl of 0.5 mM DTNB was added. The reaction was then initiated by the addition of 10 μl of acetylthiocholine iodide (0.71 mM) or butyrylthiocholine chloride (0.2 mM), in that order. The hydrolyses of these substrates were monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine, released by the enzymatic

hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, respectively, at a wavelength of 412 nm. MeOH was used as a solvent to dissolve test compounds and the controls.

2.5.3. Antioxidant activity

2.5.3.1. Free radical-scavenging activity (DPPH· assay). The free radical-scavenging activity was determined by the DPPH· assay described by Blois (1958). In its radical form, DPPH absorbs at 517 nm but, upon reduction by an antioxidant or a radical species, its absorption decreases. Briefly, a 0.1 mM solution of DPPH· in methanol was prepared and 4 ml of this solution was added to 1 ml of sample solution in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH-scavenging effect } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Extract concentration providing IC_{50} was calculated from the graph plotting inhibition percentage against extract concentration. Tests were carried out in triplicate.

2.5.3.2. DPPH· assay on TLC. The ethanol extract (100 mg) was spotted onto a silica TLC plate and chloroform–methanol (9:1) mixture was used as developer. The plate was sprayed with a 0.2% DPPH· reagent in methanol and left at room temperature for 30 min. Yellow spots, formed from bleaching of the purple colour of the DPPH· reagent, were interpreted as positive antioxidant activity (Cuendet, Hostettmann, Potterat, & Dyatmiko, 1997). Quercetin, luteolin, apigenin, (+)-catechin and rutin as positive controls, and the isolated compounds **1**–**6** were tested.

2.5.3.3. β-Carotene-linoleic acid assay. The antioxidant activity was evaluated using the β-carotene-linoleic acid model system (Miller, 1971). β-Carotene (0.5 mg) in 1 ml of chloroform was added to 25 μl of linoleic acid, and 200 mg of Tween 40 emulsifier mixture. After evaporation of chloroform in a vacuum, 100 ml of distilled water saturated with oxygen, were added by vigorous shaking. Four thousand microlitres of this mixture were transferred into different test tubes containing different concentrations of the sample. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometre. The emulsion system was incubated for 2 h at 50 °C. A blank, devoid of β-carotene, was prepared for background subtraction. BHA and α-tocopherol were used as standards.

2.5.3.4. β-Carotene-linoleic acid assay on TLC. The ethanol extract (100 mg) was spotted onto a silica TLC plate and chloroform–methanol (9:1) mixture was used as developer. After migration of the sample, the plate was thoroughly dried by means of a hair dryer and sprayed with the mixture of linoleic acid (Sigma, two drops) in EtOH (60 ml) and β-carotene (12 mg) in $CHCl_3$ (30 ml). Orange spots remained after exposing the plate to sunlight, indicative of antioxidant activity (Whittern, Miller, & Pratt, 1984). Quercetin, luteolin, apigenin, (+)-catechin and rutin as positive controls, and the isolated compounds **1**–**6** were tested.

2.5.3.5. Ferric reducing assay. The reducing power of was determined according to the method of Oyaizu (1986). Sample solutions at different concentrations were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%). After the mixture was incubated at 50 °C for 20 min, 2.5 ml of TCA (10%) were added and the mixture was centrifuged at 1000 *g* (MSE Mistral 2000, London, UK) for 10 min. Supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of ferric chloride (0.1%), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates greater reducing power.

2.5.4. Determination of total phenolic compounds

The concentrations of phenolic content in ethanol extract were expressed as microgrammes of pyrocatechol equivalents (PEs), determined with FCR, according to the method of Slinkard and Singleton (1977). One millilitre of the solution (contains 1 mg) of the extract in methanol was added to 46 ml of distilled water and 1 ml of FCR, and mixed thoroughly. After 3 min, 3 ml of sodium carbonate (2%) were added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was read at 760 nm. The concentration of phenolic compounds was calculated according to the following equation that was obtained from the standard pyrocatechol graph:

$$\text{Absorbance} = 0.00246\ \mu\text{g pyrocatechol} + 0.00325 \quad (r^2 : 0.9996)$$

2.5.5. Determination of total flavonoid concentration

Measurement of flavonoid concentration of the extract was based on the method described by Park, Koo, Ikegaki, and Contado (1997) with a slight modification and results were expressed as quercetin equivalents. An aliquot of 1 ml of the solution (contains 1 mg of extract in methanol) was added to test tubes containing 0.1 ml of 10% aluminium nitrate, 0.1 ml of 1 M potassium acetate and 3.8 ml of ethanol. After 40 min at room temperature, the absorbance was determined at 415 nm. Quercetin was used as a standard. The concentrations of flavonoid compounds were calculated according to following equation that was obtained from the standard quercetin graph:

$$\text{Absorbance} = 0.002108\ \mu\text{g quercetin} - 0.01089 \quad (r^2 : 0.9999)$$

2.6. Statistical analysis

All data, for both anticholinesterase and antioxidant activity tests, are the average of triplicate analyses. The data were recorded as means ± standard deviation. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by the Student's *t*-test; *p*-values <0.05 were regarded as significant; *p*-values <0.01 were regarded as very significant.

3. Results and discussion

Yields of essential oils obtained by hydrodistillation and steam-distillation were found to be 0.88% and 0.55%, respectively, based on the dry weight of aerial parts of the plant. One-hundred and twenty-three components were detected in essential oils obtained from *S. potentillifolia* by both methods, and 121 of them were determined by using GC, GC–MS analytical methods and literature comparison. Determined ratios of the essential oils obtained by hydrodistillation and steam-distillation were 99.32% and 99.05%, respectively. Major components of both essential oils were α-pinene (29.2% and 31.3%), β-pinene (14.9% and 14.6%), 1,8-cineole (7.44% and 7.27%), terpinen-4-ol (3.53% and 1.76%) β-myrcene (2.83% and 3.13%), limonene (2.64% and 2.77%), sabinene (2.53% and 2.78%), caryophyllene oxide (2.44% and 2.47%) and camphor (2.34% and 1.67%) (Table 1). Other components were present at less than 2.2% of the total yield. In another study, α-terpineol terpinen-4-ol, camphor and borneol were found as major components (Sarer, 1990). To the best of our knowledge, there are many reports on the chemical composition of the oils isolated from the plants belonging to the genus *Salvia* (Esmaeili et al., 2008). Most of these

Table 1
The essential oil constituents of *S. potentillifolia*.

Peak number	Compound	Rt[c]	A[a] (%)	B[b] (%)	Identification methods
1	(E)-2-Hexenal	771	0.06	0.03	1, 2
2	(Z)-3-Hexen-1-ol	787	0.08	tr	1, 2, 3
3	1-Heptenal	806	0.03	tr	1, 2
4	Santolina triene	888	0.12	0.14	2
5	Triyclene	895	0.08	0.09	1, 2, 3
6	α-Thujene	901	1.54	1.58	1, 2, 3
7	α-Pinene	914	29.30	31.25	1, 2, 3
8	2-Methylpropyl benzene	917	0.14	0.16	2
9	Camphene	925	1.91	1.93	1, 2, 3
10	6,6-Dimethylbicyclo (3,1) hepta-2(8),3-diene	929	0.14	0.16	2
11	1-Octen-3-ol	942	0.04	0.02	1, 2, 3
12	Sabinene	954	2.53	2.78	1, 2, 3
13	β-Pinene	960	14.85	14.56	1, 2, 3
14	2,3-Dehydro-1,8-cineole	969	0.06	0.03	2
15	(E)-2,4-Nonadienal	974	0.02	tr	1, 2
16	β-Myrcene	977	2.83	3.13	1, 2, 3
17	(Z)-4-Hexen-1-ol acetate	982	0.03	0.02	2
18	2,6-Dimethyl (E)(E)-1,3,5,7-octatetraene	987	0.04	0.04	2
19	α-Phellandrene	989	0.04	0.03	1, 2, 3
20	4-Acethyl-1-methyl-cyclohexene	1000	tr	tr	2
21	Benzene acetaldehyde	1000	0.06	0.02	1, 2
22	α-Terpinene	1003	0.81	0.62	1, 2, 3
23	*p*-Cymene	1007	2.08	2.23	1, 2, 3
24	*trans*-2-Caren-4-ol	1009	0.12	0.07	2
25	1,8-Cineole	1015	7.44	7.27	1, 2, 3
26	Limonene	1018	2.64	2.77	1, 2, 3
27	*cis*-Ocimene	1026	0.13	0.12	1, 2, 3
28	Thujol	1029	0.07	0.04	1, 2
29	*trans*-Ocimen	1037	0.08	0.08	1, 2, 3
30	γ-Terpinene	1047	1.89	1.55	1, 2, 3
31	*cis*-*p*-Menth-2-en-1-ol	1049	0.56	1.09	1, 2, 3
32	*cis*-Linalool oxide	1055	0.03	0.02	1, 2
33	(Z)-5-Andecene	1059	0.03	0.02	2
34	Eucarvone	1060	0.05	0.04	1, 2
35	Fenchone	1064	0.08	0.07	1, 2
36	3-Camphenol	1068	0.06	0.03	2
37	2,5-Dimethyl phenylethane	1073	0.03	0.04	2
38	Terpinolene	1074	0.44	0.33	1, 2, 3
39	*cis*-Sabinene hydrate	1076	0.34	0.43	1, 2, 3
40	*trans*-4,5-Epoxy-caran	1078	0.05	0.04	2
41	*cis*-*p*-Mentha-2,8-dien-1-ol	1080	0.18	0.10	1, 2, 3
42	Linalool	1082	0.43	0.32	1, 2, 3
43	*cis*-Thujone	1088	0.10	0.95	1, 2, 3
44	*trans*-Thujone	1090	0.07	0.56	1, 2, 3
45	α-Campholenal	1094	0.76	0.63	2
46	*trans*-Sabinene hydrate	1098	0.23	0.14	1, 2, 3
47	Camphor	1106	2.34	1.67	1, 2, 3
48	7-Methyl-4-octanol acetate	1108	tr	tr	2
49	*cis*-Pinocamphone	1110	0.85	0.59	2, 3
50	*cis*-Verbenol	1112	0.48	0.25	1, 2
51	*trans*-Verbenol	1116	0.96	0.60	1, 2
52	Pinocarvone	1122	0.37	0.35	1, 2
53	2,6-Dimethyl-1,5,7-octatriene-3-ol	1128	0.13	0.10	2
54	Borneol	1132	2.26	1.29	1, 2, 3
55	*trans*-Pinocamphone	1133	0.09	0.05	2, 3
56	Terpinen-4-ol	1142	3.53	1.76	1, 2, 3
57	Myrtenal	1145	0.37	0.29	1, 2, 3
58	α-Terpineol	1150	1.74	0.97	1, 2, 3
59	Verbenone	1153	0.23	0.10	1, 2
60	Myrtenol	1154	0.57	0.35	1, 2, 3
61	*trans*-Piperitol	1162	0.08	0.45	2
62	*trans*-Carveol	1168	0.24	0.12	1, 2
63	Cuminaldehyde	1175	0.06	0.07	1, 2
64	Carvone	1177	0.08	0.06	1, 2
65	Phenylethyl acetate	1186	0.06	0.06	2
66	*cis*-Verbenyl acetate	1188	0.04	tr	1, 2
67	*p*-Mentha-1,3-diene-7-al	1194	0.05	0.04	2
68	Cuminol	1208	0.03	tr	1, 2
69	Bornyl acetate	1211	1.18	1.11	1, 2, 3
70	Sabinyl acetate	1214	0.10	0.07	1, 2, 3
71	Carvacrol	1216	0.03	0.03	1, 2, 3
72	*trans*-Carvyl acetate	1218	0.04	0.16	2, 3
73	Thymol acetate	1222	0.02	0.02	2
74	*cis*-Carvyl acetate	1229	0.03	0.02	2, 3
75	Eugenol	1241	0.07	0.06	1, 2

(*continued on next page*)

Table 1 (*continued*)

Peak number	Compound	Rt[c]	A[a] (%)	B[b] (%)	Identification methods
76	α-Terpinenyl acetate	1244	0.51	0.57	1, 2, 3
77	α-Cubebene	1250	0.10	0.19	1, 2, 3
78	Geranyl acetate	1251	0.04	0.03	1, 2
79	*trans*-Jasmone	1253	0.01	tr	1, 2
80	*cis*-Jasmone	1257	0.03	0.03	1, 2
81	α-Copaene	1261	0.11	0.20	1, 2, 3
82	β-Bourbonene	1263	0.59	0.93	2, 3
83	α-Bourbonene	1264	0.04	0.06	2, 3
84	İsoledene	1265	tr	0.01	2
85	β-Elemene	1267	0.05	0.09	2, 3
86	α-Gurjunene	1271	tr	tr	2
87	β-Caryophyllene	1277	1.66	2.86	1, 2, 3
88	β-Panasinsene	1281	0.07	0.12	2
89	β-Gurjunene	1282	0.22	0.46	2
90	α-Cadinene	1286	0.07	0.09	1, 2, 3
91	Epi-bicyclosesquiphellandren	1287	0.02	0.03	2
92	α-Caryophyllene (α-humulene)	1289	0.32	0.56	1, 2, 3
93	β-Cadinene	1292	0.06	0.20	2
94	γ-Muurolene	1295	tr	0.01	1, 2, 3
95	Germacrene D	1299	0.85	1.75	1, 2, 3
96	Valencene	1300	0.01	tr	2
97	Bicyclogermacrene	1301	0.05	0.07	2, 3
98	1,7-Dimethyl-4-isopropyl-2,7-cyclodecadienol	1303	0.13	0.17	2
99	γ-Elemene	1304	0.04	0.08	2, 3
100	$C_{15}H_{24}$	1306	0.03	0.03	2
101	Not identified	1309	0.36	0.66	–
102	Not identified	1313	0.32	0.29	–
103	Elemol	1319	0.10	0.09	2, 3
104	Spathulenol	1326	0.17	0.14	1, 2, 3
105	Caryophyllene oxide	1328	2.44	2.47	1, 2, 3
106	Aristolene epoxyde	1330	0.10	0.10	2
107	8-Hydroxy-endo-cycloisolongifolen	1332	0.03	0.03	2
108	γ-Gurjunen epoxide-I	1335	0.35	0.40	2
109	Selina-4,11-dien-2-ol	1336	0.12	0.10	2
110	Cubenol	1342	0.15	0.12	2, 3
111	*tau*-Cadinol	1344	0.13	0.10	2, 3
112	α-Eudesmol	1346	0.16	0.12	2, 3
113	7,8-Dehydro-8α-hydroxy isolongifolene	1347	0.14	0.12	2
114	α-Cadinol	1348	0.07	0.06	2, 3
115	Alloaromadendrene oxide-I	1349	0.03	0.02	2, 3
116	Ledene oxide-II	1354	0.21	0.15	2
117	Patchouli alcohol	1356	0.08	0.09	2
118	Benzyl benzoate	1362	tr	tr	1, 2
119	Dibutyl phtalate	1370	tr	0.03	1, 2
120	Kaur-16-ene	1415	tr	tr	1, 2
121	Manoyl oxide	1417	0.03	0.04	1, 2
122	Dehydroabietol	1422	0.03	0.03	2
123	8,13-Epoxy-labd-14-en-2-one	1445	0.14	0.14	2
	Class of compounds percentage		%	%	
	Monoterpene hydrocarbons		61.5	63.6	
	Monoterpenoids		27.3	23.3	
	Sesquiterpene hydrocarbons		4.96	7.50	
	Sesquiterpenoids		4.34	4.19	
	Diterpeneoids		0.36	0.43	
	Others		0.27	0.03	
	Total		99.5	100	

1, Co-injection with authentic compounds; 2, MS; 3, literature comparison; tr < 0.02.
[a] Essential oil obtained by hydrodistillation.
[b] Essential oil obtained by steam-distillation.
[c] In DB-1 fused silica capillary column.

reports indicate that 1,8-cineole (eucalyptol) and borneol are the main and characteristic constituents of *Salvia* essential oils. Components of *S. potentillifolia* essential oil found in this study are in accordance with the components of other *Salvia* species.

Most of the essential oil consists of monoterpene hydrocarbons, represented by 61.5% in the essential oil which was obtained by hydrodistillation, and 63.6% by steam-distillation. Both hydrodistilled and steam-distilled essential oils also contain monoterpenoids (27.3% and 23.3%, respectively), sesquiterpene hydrocarbons (4.96% and 7.50%) and sesquiterpenoids (4.34% and 4.19%). Both essential oils also contain diterpenoids (0.36% and 0.43%). In fact, *Salvia* species are rich in diterpenoids, particularly in their roots. While the components of the oils obtained from *S. potentillifolia*, by both methods, are qualitatively same, there are some quantitative differences. While amounts of terpinen-4-ol, α-terpineol, camphor and borneol significantly decreased in the essential oil obtained via the steam-distillation method, increases in the amount of β-caryophyllene, germacrene D, *trans*-piperitol, *trans*-carvyl acetate, and *cis*-*p*-menth-2-en-1-ol were observed (Table 1).

Antimicrobial activity of the *S. potentillifolia* evaluated herein was determined by the application of disc diffusion and MIC tests against some pathogenic bacteria and yeasts. As can be seen from Table 2, the essential oil of *S. potentillifolia* was inhibitory to the

Table 2
Antimicrobial activity of *S. potentillifolia*, α- and β-pinenes.

Test bacteria	Disc diffusion (mm)		MIC (μg/ml)			
	Essential oil	Ethanol extract	Essential oil	Ethanol extract	α-Pinene	β-Pinene
Pseudomonas aeruginosa NRRL B-23	6	10	77.5	85	NT	NT
Salmonella enteritidis RSKK 171	14	8	38.5	45	NT	NT
Escherichia coli ATCC 25922	12	10	47.5	67.5	NT	NT
Morganella morganii (clinical isolate)	8	10	65	67.5	NT	NT
Yersinia enterecolitica RSKK 1501	10	19	52.5	26.5	NT	NT
Klebsiella pneumoniae ATCC 27736	18	10	25	36	NT	NT
Proteus vulgaris RSKK 96026	16	12	30	40	NT	NT
Staphylococcus aureus ATCC 25923	15	20	27.5	38.5	NT	NT
Micrococcus luteus NRRL B-4375	28	22	20	28	NT	NT
Bacillus subtilis ATCC 6633	22	20	17	27.5	NT	NT
Bacillus cereus RSKK 863	21	16	17.5	28.5	NT	NT
Candida albicans ATCC 10231	33	–	18.5	27.5	32.5	38.5
Candida tropicalis (clinical isolate)	40	4	15.5	26.5	28.0	36.5

NT, Not tested and –, no inhibition.

growth of all the bacteria and yeasts under test. The inhibition zones of disc and MIC values for strains, sensitive to the essential oil, were in the ranges 6.0–40.0 mm and 15.5–77.5 μg/ml, respectively. There are many reports on the chemical composition and antimicrobial activity of the oils isolated from the plants belonging to the genus *Salvia*. For example, essential oil of *S. officinalis* exhibited a broad antifungal spectrum, with higher activity against dermatophyte strains (Pinto, Salgueiro, Cavaleiro, Palmeira, & Gonçalves 2007). In the present study, according to disc diffusion method, the essential oil exhibited high anticandidal activity on *C. albicans* and *C. tropicalis* (inhibition zones 33 and 40 mm, respectively). From Tables 2 and 3, it can be seen that the oil possessed greater antifungal potential than did the commercial drug, ketoconazole which was used as a positive control. The results of an *in vitro* study provided evidence that the plant is a potential anticandidal agent against *C. albicans* and *C. tropicalis*. In our study, MIC values of anticandidal activity were found to be 32.5 μg/ml against *C. albicans* and 28.0 μg/ml against *C. tropicalis* for α-pinene. However, β-pinene showed weaker activity than did α-pinene against *C. albicans* and *C. tropicalis* (38.5 and 36.5 μg/ml, respectively). As observed from these results, α- and β-pinenes were not very effective anticandidal agents when used alone, but when combined with other components in essential oil, a synergism should be occurred.

Generally, the essential oil showed better antimicrobial activity against the Gram-positive bacteria than the Gram-negative bacteria. Gram-positive bacteria, *S. aureus*, *M. luteus*, *B. subtilis* and *B. cereus*, were most susceptible to this oil, with inhibition zones of 15–28 mm, and with MIC values of 17.0–27.5 μg/ml. Gram-negative bacteria were most resistant to this oil with inhibition zones between 6 and 18 mm, and with MIC values of 25–77.5 μg/ml. These results are similar to those of previous reports in the literature, indicating that Gram-negative bacteria are more resistant to essential oils than Gram-negative bacteria (Mangena & Muyima, 1999). The essential oil of *S. potentillifolia* was also found to inhibit the growth of medically important Gram-negative bacteria, such as *K. pneumoniae*, *P. vulgaris*, *S. enteritidis* and *E. coli* (inhibition zones of 18, 16, 14, and 12 mm, respectively).

As it is well known, *S. aureus*, *E. coli* and *Bacillus* species, especially *B. cereus*, are agents of food poisoning. The most interesting area of application for plant extracts is the inhibition of growth and reduction in numbers of the more serious food-borne pathogens (Burt, 2004). In the present study, MIC values of the essential oil of *S. potentillifolia* against *S. aureus*, *E. coli* and *B. cereus* were 27.5, 47.5, and 17.5 μg/ml, respectively. From these results, *S. potentillifolia* may be considered as a natural preservative against food-borne pathogens for the food production industry. The antibacterial activities were comparable with those of commonly used antibiotics against these microorganisms (Table 3).

In general, the essential oil was more active than those of the reference discs. Although *K. pneumoniae* and *P. vulgaris* are resistant to Ampicillin, the antimicrobial effect of essential oil was higher than that of Ampicillin on these bacteria. The above results indicate that the essential oil of *S. potentillifolia* may be used in the treatment of diseases caused by microorganisms tested. When comparing the antimicrobial activity of the tested samples to those

Table 3
Antimicrobial activity of reference discs.

Test microorganisms	Disc diffusion (mm)				MIC (μg/ml)			
	Ketaconazole	Ampicillin	Penicillin	Gentamicin	Ketaconazole	Ampicillin	Penicillin	Gentamicin
Pseudomonas aeruginosa NRRL B-23	NT	NT	NT	16	NT	NT	NT	9.5
Salmonella enteritidis RSKK 171	NT	–	NT	NT	NT	R	NT	NT
Escherichia coli ATCC 25922	NT	18	18	NT	NT	18	NT	NT
Morganella morganii (clinical isolate)	NT	NT	NT	–	NT	NT	NT	R
Yersinia enterecolitica RSKK 1501	NT	20	18	NT	NT	20	18	NT
Klebsiella pneumoniae ATCC 27736	NT	–	NT	NT	NT	R	NT	NT
Proteus vulgaris RSKK 96026	NT	–	NT	NT	NT	R	NT	NT
Staphylococcus aureus ATCC 25923	NT	NT	30	NT	NT	NT	8.75	NT
Micrococcus luteus NRRL B-4375	NT	28	31	NT	NT	8	15.5	NT
Bacillus subtilis ATCC 6633	NT	NT	12	NT	NT	NT	7.5	NT
Bacillus cereus RSKK 863	NT	NT	22	NT	NT	NT	7.5	NT
Candida albicans ATCC 10231	16	NT	NT	NT	9.75	NT	NT	NT
Candida tropicalis (clinical isolate)	18	NT	NT	NT	9.75	NT	NT	NT

NT, Not tested; R, resistance; and –, no inhibition.

Table 4
Acetyl- and butyrylcholinesterase inhibitory activities of *S. potentillifolia*[a].

Extract	AChE		BChE	
	Inhibition (%) (200 μM)	IC_{50} (μM)	Inhibition (%) (200 μM)	IC_{50} (μM)
Essential oil[c]	21.9 ± 1.40	>200	65.7 ± 0.21	105 ± 1.01
α-Pinene	87.2 ± 0.50	81.7 ± 0.96	17.5 ± 1.18	>200
β-Pinene	NA	>200	NA	>200
Ethanol extract[c]	2.12 ± 1.04	>200	46.3 ± 3.31	>200
Galanthamine[b]	81.4 ± 1.03	5.01 ± 0.09	75.5 ± 1.05	53.9 ± 0.56

[a] Values expressed are means ± SD of three parallel measurements ($p < 0.05$).
[b] Standard drug.
[c] At 200 μg/ml concentration.

of reference antibiotics, the inhibitory potency of the tested extracts were found to be remarkable. This is due to the fact that medicinal plants are of natural origin, which means more safety for consumers, and they might present low risk of resistance development by pathogenic microorganisms.

Table 4 shows the anticholinesterase activity of the essential oil, α-, β-pinenes and the ethanol extract, compared with galanthamine at the same concentration. The essential oil and the ethanol extract indicated moderate butyrylcholinesterase activity at 200 μg/ml concentration, while they showed almost no activity against acetylcholinesterase. In contrast, the major compound of the essential oil, α-pinene, demonstrated acetylcholinesterase inhibitory activity with an IC_{50} value of 81.7 ± 0.96 μM, and showed almost no activity against butyrylcholinesterase. Interestingly, β-pinene displayed no inhibitory activity on either enzyme at a concentration of 200 μM. The inhibitions of acetylcholinesterase by synthetic bicyclic monoterpenoids, including α- and β-pinenes, were studied and α-pinene was found to be more active than β-pinene (Miyazawa & Yamafuji, 2005).

The essential oil and the ethanol extract of *S. potentillifolia* were screened for their possible antioxidant activity using three complementary test systems, namely DPPH·-scavenging activity, β-carotene-linoleic acid and ferric reducing power capacity assays. In addition, total phenolic and total flavonoid contents of ethanol extract were determined as pyrocatechol and quercetin equivalents, respectively. DPPH·-scavenging and β-carotene bleaching assays were also carried out on TLC plates, spraying on the four isolated flavonoids, a terpenoid, a steroid and the ethanol extract. These six compounds (**1**–**6**) were found to be active in the β-carotene-linoleic acid assay while only the flavonoids (**2**, **3**, **5** and **6**) appeared to be active in the DPPH·-scavenging activity assay.

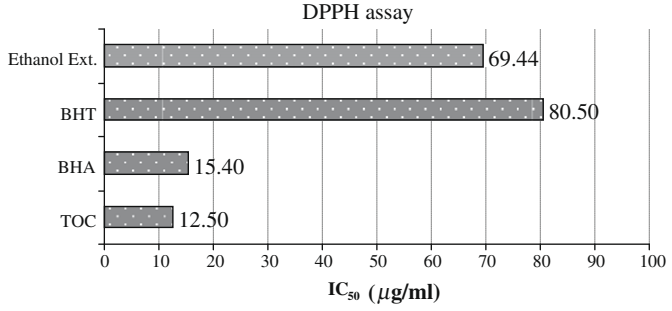

Fig. 2. Free radical-scavenging activities of essential oil and ethanol extract of *S. potentillifolia* compared to those of standards BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) and TOC (α-tocopherol) by DPPH·. Since, IC_{50} value of essential oil >100 μg/ml, it was not shown herein. Values are means ± SD, $n = 3$; $p < 0.05$, significantly different with Student's *t*-test.

Fig. 2 shows the DPPH free radical-scavenging activity of the essential oil and the extract of *S. potentillifolia*. The difference between the extracts and control was statistically significant ($p < 0.05$) when compared with the control. Radical-scavenging activity increased with increasing amount of the extracts. Only the ethanol extract was found to be better than the well-known standard BHT, but not BHA. The IC_{50} values of BHT, BHA and ethanol extract were 80.5 ± 0.50, 15.2 ± 0.66 and 69.4 ± 0.99 μg/ml, respectively. The IC_{50} value of the essential oil was more than 250 μg/ml. Thus, it was not mentioned in Fig. 2.

Fig. 3 shows the total antioxidant activity of the essential oil and the extract of *S. potentillifolia*, compared with TOC and BHA, determined by the β-carotene bleaching method. Total antioxidant activity increased with increasing amounts of the extracts. The results were statistically significant ($p < 0.05$) when compared with

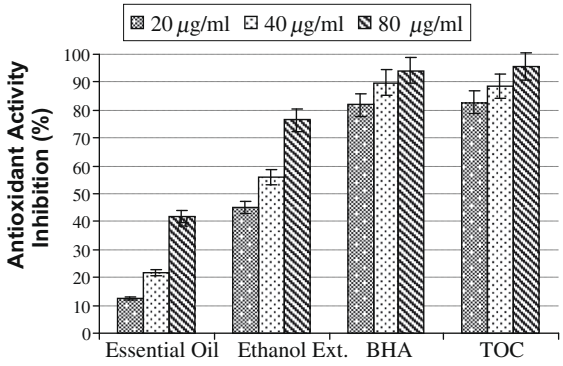

Fig. 3. Inhibition (%) of lipid peroxidation of the essential oil and ethanol extract of *S. potentillifolia* compared to those of standards BHA and TOC by the β-carotene bleaching method. Values are means ± SD, $n = 3$; $p < 0.05$, significantly different with Student's *t*-test.

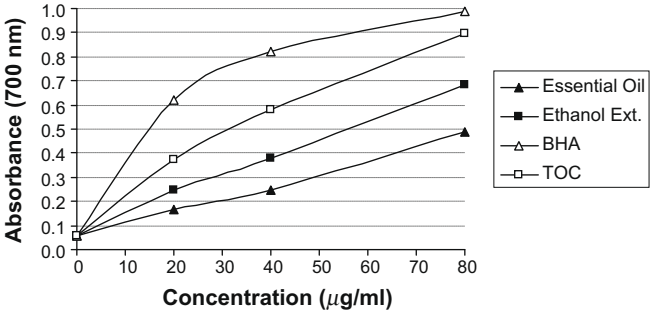

Fig. 4. Reducing powers of essential oil and ethanol extract of *S. potentillifolia* compared to those of standards BHA and TOC. Values are means ± SD, $n = 3$; $p < 0.01$, significantly different with Student's *t*-test.

the control. None of the tested extracts showed greater antioxidant activity than BHA or TOC. Inhibition was observed in the ethanol extract (75.4 ± 0.80%), and a lower inhibition value was found in the essential oil (36.2 ± 1.10%) at 80 μg/ml concentration.

Fig. 4 shows the reducing powers of the essential oil and the extract of *S. potentillifolia* and standards, such as α-tocopherol and BHA, using the potassium ferricyanide reduction method. The reducing power of the oil and the extract increased with increasing concentration. The oil and the extract, at all concentrations, exhibited higher activities than the control; the differences were significant ($p < 0.01$). The ethanol extract showed stronger reducing power than did the essential oil, but not stronger than the standards.

Polyphenolic compounds have an important role in stabilising lipid oxidation and are associated with antioxidant activity and phenolic compounds are also known as powerful chain breaking antioxidants (Shahidi & Wanasundara, 1992). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily is ingested from a diet rich in stems and vegetables (Tanaka, Kuei, Nagashima, & Taguchi, 1998). The concentration of phenolics and flavonoids in the extract was expressed as micrograms of pyrocatechol and micrograms of quercetin equivalents per milligrams of the extract, respectively. The ethanol extract had 214 ± 0.02 μg pyrocatechol equivalents as its phenolic content, and demonstrated 54.2 ± 0.15 μg quercetin equivalents as its flavonoid content.

Several abietane diterpenoids, such as horminone, 7α-acetylhorminone, ferruginol and cryptanol (11,12,14-trihydroxy-abietae-6,8,11,13-tetraene), along with triterpenoids, sterols and sugars, have previously been isolated from *S. potentillifolia* (Ulubelen & Tuzlacı, 1987). The antioxidant activities of these abietane diterpenoids were studied and they were found to be highly active (Kabouche, Kabouche, Öztürk, Kolak, & Topçu, 2007).

In this study, carried out with *S. potentillifolia*, the high free radical-scavenging and lipid peroxidation inhibition activities may be related to the presence of flavonoid-type compounds and other phenolics.

4. Conclusions

The results presented in this study are the first information on the antioxidant, anticholinesterase and antimicrobial activities of *S. potentillifolia*. Among the three tested methods, the highest activity was observed for inhibition of lipid peroxidation in the β-carotene-linoleic acid system by the ethanol extract of the plant. The ethanol extract also showed high DPPH·-scavenging activity, even higher than those of the standards BHA and BHT. As known, there is a significant linear correlation between phenolic concentration and free radical-scavenging activity, especially on the DPPH radical. The ethanol extract also showed strong ferric reducing power.

In this study, the essential oil showed, not only antibacterial activity, but also very significant anticandidal activity against *C. albicans* and *C. tropicalis*. Therefore, the results obtained in this study proved that the plant is potentially a good antimicrobial, (specifically anticandidal) agent. Hence, the essential oil of *S. potentillifolia* may be useful as an alternative anticandidal agent, as a natural medicinal drug, as well as a moderate anticholinesterase agent, particularly against BChE.

In conclusion, the results showed the antioxidant importance of *S. potentillifolia*, which is one of the commonly used medicinal plants in Anatolia. Thus, the plant may protect people against lipid peroxidation and free radical damage, and its extracts will probably be of use for the development of safe food products and additives.

Acknowledgement

The authors would like to thank The Scientific and Technological Research Council of Turkey (TUBITAK-TBAG-106T095) for financial support.

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