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Chemical Composition and *In Vitro* Evaluation of Antioxidant Effect of Free Volatile Compounds From *Satureja montana* L.

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As a part of an investigation of natural antioxidants from Dalmatian aromatic plants, in this paper we report a study of the antioxidant activity related to the chemical composition of savory free volatile compounds. Twenty-one compounds were identified in the essential oil without fractionation, representing 97.4% of the total oil. The major compound was phenolic monoterpene thymol (45.2%). Other important compounds were monoterpenic hydrocarbons *p*-cymene (6.4%) and γ-terpinene (5.9%) and oxygen-containing compounds carvacrol methyl ether (5.8%), thymol methyl ether (5.1%), carvacrol (5.3%), geraniol (5.0%) and borneol (3.9%). The evaluation of antioxidant power was performed *in vitro* by the β-carotene bleaching and thiobarbituric acid (TBA) methods. As determined with both methods, the total savory essential oil as well as different fractions or pure constituents containing hydroxyl group exhibited relatively strong antioxidant effect. The hydrocarbons, when isolated as CH fraction, showed the poorest effectiveness in spite the fact that this fraction contained γ-terpinene, α-terpinene, *p*-cymene and terpinolene which previously were identified as potential antioxidants.

Keywords: *Satureja montana* L; Antioxidant; α-Tocopherol; Butylated hydroxytoluene; β-Carotene bleaching

INTRODUCTION

Widely used artificial antioxidants, such as butylated hydroxytoluene and butylated hydroxyanisole are very effective in their role as antioxidants.[1] However, their use in food products has been failing off due to their instability, as well as due to a suspected action as promoters of carcinogenesis.[2,3] For this reason, there is a growing interest in the studies of natural additives as potential antioxidants. Recently, Aruoma[4] and Halliwell[5] described the experimental strategies for optimization of nutritional antioxidant intake in humans. The antioxidant properties of many aromatic herbs are reported to be effective in this role.[6–8]

Shahidi *et al.*[9] reported that the antioxidant effect of aromatic plants is due to the presence of hydroxyl groups in their phenolic compounds. Lagouri *et al.*[10] studied the antioxidant activity of essential oils and they found that oregano essential oil, rich in thymol and carvacrol, has a considerable antioxidant effect on the process of lard oxidation. In our previous works, the oregano essential oil was fractionated and the fraction containing only thymol and carvacrol exhibited the strongest antioxidant power.[11] In the second study,[12] the glycosidically bound volatile compounds from oregano also showed significant antioxidant effect.

Savory (*Satureja montana* L.) is an aromatic plant which has been frequently used in local spices and as a traditional medicinal herb in Dalmatia. Because of the strong phenolic character of its essential oil it is reminiscent of the taste and fragrance of commercial oregano and thyme oils. With regard to the presence of thymol and carvacrol in essential oil,[13] savory is known to possess antibacterial activity. As a part of an investigation of natural antioxidants from Dalmatian aromatic plants, in this paper, we report a study of the antioxidant activity related to the chemical composition of savory free volatile compounds. The evaluation of antioxidant power was performed *in vitro* by the β-carotene bleaching and TBA methods.

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MATERIALS AND METHODS

Chemicals and Materials

Butylated hydroxytoluene, α-tocopherol, TBA, sodium dodecyl sulphate 2,2′-azobis (2-amidinopropane) dihydrochloride and all of the applied solvents were of pro analysis purity and were purchased from Fluka Chemie, Buchs, Switzerland. Anhydrous sodium sulfate was obtained from Merck, Darmstadt, Germany.

The plant materials of savory, sage and rosemary were collected in region of Central Dalmatia (Croatia). The voucher specimens have been deposited in the Laboratory of Organic Chemistry, Faculty of Chemical Technology, Split, Croatia.

Isolation and Fractionation of the Essential Oil

A 100 g of dried plant material were subjected to a 3-h hydrodistillation using a modified clevenger-type apparatus. The obtained essential oil was dried over anhydrous sodium sulfate and stored under nitrogen in sealed vial at −20°C until required.

The 0.5 g of the essential oil was fractionated on a silica gel column (particle size 30–60 μm). A measured quantity of 50 ml pentane was used to obtain fraction which contained only nonpolar hydrocarbons (CH fraction), and 50 ml dyethyl ether to obtain fraction of oxygenated compounds (CHO fraction). These fractions were concentrated to 0.5 ml and tested by thin layer chromatography (TLC) on silica gel plates. Different solvents were used as a mobile phase: *n*-hexane for CH fraction and *n*-hexane:ethyl acetate 85:15 (v/v) for CHO fraction. Two percent vanillin-sulphuric acid was used as a detection reagent. The obtained results were also confirmed by GC/MS analysis.

In order to obtain fraction of phenolic compounds, the 1 g of the essential oil was dissolved in 5 ml pentane and extracted with sodium hydroxide solution (20%). In this manner, the phenolic compounds were removed from the pentane layer. The effectiveness of this extraction was tested by TLC (*n*-hexane:ethyl acetate 85:15 v/v). The aqueous phase, containing dissolved phenolic compounds sodium salts, was neutralized with hydrochloric acid solution (10%). Finally, isolation of the phenolic compounds was performed by extraction with pentane (5 × 5 ml) and tested by TLC. These results were confirmed by GC/MS analysis too.

Gas Chromatography–Mass Spectrometry

The analyses of the volatile compounds were carried out on a Hewlett-Packard GC-MS system (GC 5890 series II; MSD 5971A). The fused-silica HP-20 M column (polyethylene glycol; 50 m × 0.2 mm, 0.2 μm film thickness) was directly coupled to the mass spectrometer. The carrier gas was helium (flow rate 1 ml/min). The oven temperature program was 4 min isothermal at 70°C, then 70–180°C at the rate of 4°C/min and held isothermal for 10 min. The injection port temperature was 250°C. The ionization of the sample components was performed in the EI mode (70 eV). Generally, 2 μl of 1% pentane dilution of each sample was used for GC-MS measurements

The linear retention indices for all the compounds were determined by co-injection of the sample with a solution containing the homologous series of C_8–C_{22} *n*-alkanes.[14] The individual constituents were identified by their identical retention indices referring to the compounds known from literature data,[15] and also by comparing their mass spectra with spectra of the known compounds or with those stored in the Wiley mass spectral database.

β-Carotene Bleaching Method

Antioxidant activity of the volatile compounds from the savory was determined according to slightly modified version of the β-carotene bleaching method.[16] The β-Carotene (0.1 mg) was added to a boiling flask together with linoleic acid (20 mg) and Tween 40 (100 mg), all dissolved in chloroform. After evaporation to dryness, under vacuum at 50°C with a rotary evaporator, oxygenated distilled water (50 ml, obtained by bubbling pure oxygen through the water for 15 min) was added and the mixture was emulsified for 1 min in a sonificator to form emulsion A. A measured quantity of 200 μl of ethanolic stock solution of each antioxidant was mixed with 5 ml of emulsion A in open-capped cuvettes. A control, without antioxidant, consisting of 200 μl of ethanol and 5 ml of emulsion A was prepared. A second emulsion (B) consisting of 20 mg of linoleic acid, 100 mg of Tween 40 and 50 ml of oxygenated water was also prepared. Ethanol (200 μl), to which 5 ml of emulsion B was added, was used to zero the spectrophotometer. Readings of all samples were taken immediately ($t = 0$) and at 15 min intervals for 120 min on a Perkin–Elmer Lambda EZ 201 spectrophotometer at 470 nm. The cuvettes were thermostated at 50°C between measurements. All determinations were performed in triplicate. The antioxidant activity coefficient (AAC) was calculated from the data with the formula:[17]

$$AAC = [(A_{A(120)} - A_{C(120)})/(A_{C(0)} - A_{C(120)})] \times 1000$$

where $A_{A(120)}$ is the absorbance of the antioxidant at $t = 120$ min, $A_{C(120)}$ is the absorbance of the control at $t = 120$ min and $A_{C(0)}$ is the absorbance of the control at $t = 0$ min.

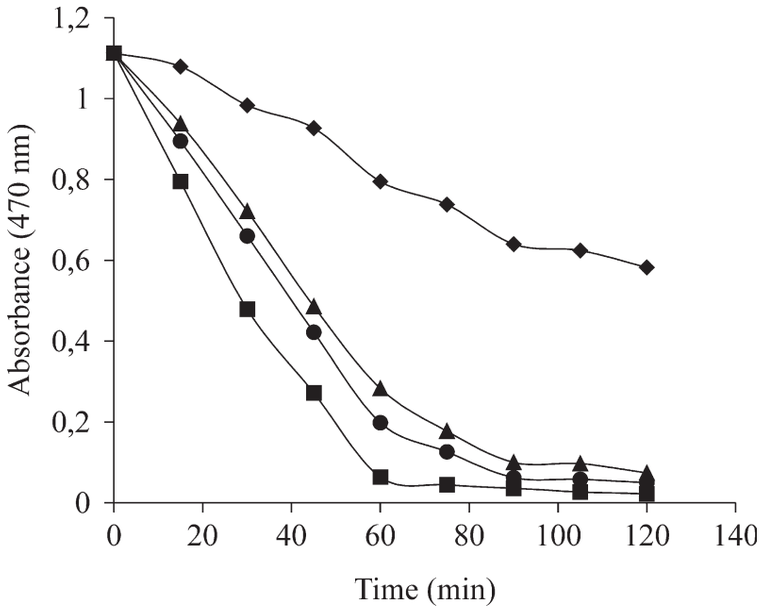

FIGURE 1 Antioxidant activity of sage, rosemary and savory essential oils as assessed with the β-carotene bleaching method. (■) Control without antioxidant, essential oil of (●) sage; (▲) rosemary, (◆) savory. The concentration of each essential oil in reacting system was 200 ppm.

Thiobarbituric Acid Method

A modified TBA reactive species (TBARS) assay[18] was used to measure the potential antioxidant capacity using egg yolk homogenates as lipid rich media. Briefly, 0.5 ml of 10% (w/v) tissue homogenate and 0.1 ml of solutions of samples to be tested in methanol, prepared immediately before use, were added to a test tube and made up to 1.0 ml with distilled water. A measured quantity of 0.05 ml of 2,2′-azobis (2-amidinopropane) dihydrochloride solution (0.07 M) in water was added to induce lipid peroxidation. 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml 0.8% (w/v) TBA in 1.1% (w/v) sodium dodecyl sulphate solution was added and the resulting mixture vortexed, and then heated at 95°C for 60 min. After cooling, 5.0 ml of butan-1-ol was added to each tube, then extensively vortexed and centrifuged at 1200*g* for 10 min. The absorbance of the organic upper layer was measured using Perkin–Elmer Lambda EZ 201 spectrophotometer, set at 532 nm.

All the values were based on the percentage antioxidant index (AI%):

$$\mathrm{AI\%} = (1 - A_T/A_C) \times 100$$

where A_C is the absorbance value of the fully oxidized control and A_T is the absorbance of the test sample.

RESULTS

Preliminary Investigation

As preliminary investigation, we examined antioxidant power of sage, rosemary and savory essential oils from sage, (Fig. 1). The savory oil demonstrated relatively high antioxidant activity, while sage and rosemary essential oils showed very poor antioxidant effect. After these results, the detailed study of chemical composition and antioxidant activity of the savory essential oil was performed in order to determine the concentration and to find the fraction or component with the highest protection against lipid oxidation according to the β-carotene bleaching method and TBA method.

Chemical Composition of Savory Essential Oil

The amount of 1.4 g of the essential oil was obtained from 100 g of dried savory leaves. As shown in Table I, 21 compounds were identified in the essential oil without fractionation, representing 97.4% of the total oil. The major compound was phenolic monoterpene thymol (45.2%). Other important compounds were monoterpenic hydrocarbons *p*-cymene (6.4%) and γ-terpinene (5.9%) and oxygen-containing compounds carvacrol methyl ether (5.8%), thymol methyl ether (5.1%), carvacrol (5.3%), geraniol (5.0%) and borneol (3.9%). After fractionation, some new compounds were identified which means that more complete analysis was obtained. In this way, 33 compounds were identified. The chemical composition of different fractions were also reported in Table I. Nineteen compounds were identified in hydrocarbons (CH) fraction with *p*-cymene (23.1%), γ-terpinene (19.8%), *trans*-caryophyllene (18.2%) and β-cubebene (8.9%) as main

TABLE I Percentage composition of *Satureja montana* L. essential oil

Number	Compound	RI*	Yield (%)	
			Total oil	Fraction
Hydrocarbons—CH fraction				
1.	α-Thujene	1031	1.0	2.7
2.	α-Pinene	1038	1.0	2.0
3.	β-pinene	1102	–	2.1
4.	α-Terpinene	1161	3.5	7.5
5.	γ-Terpinene	1231	5.9	19.8
6.	*p*-Cymene	1247	6.4	23.1
7.	Terpinolene	1262	–	0.6
8.	Alloocimene†	1351	0.6	1.8
9.	α-Copaene	1466	–	0.5
10.	β-Burbonene	1496	–	0.7
11.	β-Cubebene‡	1524	–	8.9
12.	*trans*-Caryophyllene	1578	2.3	18.2
13.	α-Humulene	1638	0.3	1.3
14.	Zingiberene	1659	–	0.6
15.	γ-Cadinene	1677	–	0.4
16.	α-Elemene	1687	0.3	0.7
17.	β-Bisabolene	1694	1.1	3.1
18.	δ-Cadinene	1729	–	2.3
19.	α-Muurolene	1735	–	0.2
				Total 96.5
Oxygen containing compounds—CHO fraction				
20.	1-Octen-3-ol	1411	0.7	0.8
21.	*trans*-Sabinene hydrate†	1423	0.2	0.3
22.	Linalool	1507	0.6	0.7
23.	Thymol methyl ether	1563	5.1	4.4
24.	Carvacrol methyl ether	1576	5.8	6.6
25.	Neral	1641	–	0.2
26.	Borneol	1653	3.9	4.7
27.	Geranial	1680	–	0.5
28.	Geranyl acetate	1729	2.1	2.2
29.	Nerol	1752	1.1	1.2
30.	Geraniol	1796	5.0	6.2
31.	3-Phenylpropanol‡	1947	–	0.2
32.	Thymol	2115	45.2	61.6
33.	Carvacrol	2140	5.3	7.1
			Total 97.4	Total 96.7
Phenolic fraction				
34.	Thymol	2115		85.5
35.	Carvacrol	2140		14.5
				Total 100.0

*Retention indices relative to C_8–C_{22} alkanes on polar HP-20M column. †Correct isomer is not identified. ‡Tentatively identification on the basis of the mass spectra (MS) only.

components. The fraction with oxygen-containing compounds, which consisted of 14 compounds, was dominated by thymol (61.6%) as major compound. Other important compounds were carvacrol (7.1%), carvacrol methyl ether (6.6%), geraniol (6.2%), borneol (4.7%) and thymol methyl ether (4.4%). The phenolic fraction contained only two compounds, thymol (85.5%) and carvacrol (14.5%).

Antioxidant Activity Determined with β-Carotene Bleaching Method

Figure 2 shows a typical profile of the decrease in absorbance of β-carotene in the presence of the total savory essential oil and its different fractions. The antioxidant power decreased in the order CHO fraction > phenolic fraction > total essential oil > CH fraction. In this series of measurements, the CHO fraction was more potent than total oil and other fractions, suggesting that synergy among minor oxygen containing compounds plays important role for antioxidant power.

The decrease in absorbance of β-carotene in the presence of different concentrations of the total savory essential oil showed that the control sample without addition of antioxidant oxidized most rapidly and descending bleaching rates were demonstrated for the increased concentration. Then, all other determinations were performed in presence of two different concentrations of potential antioxidants (100 and 1000 ppm) in triplicate and results are reported as mean ± standard deviation (Table II).

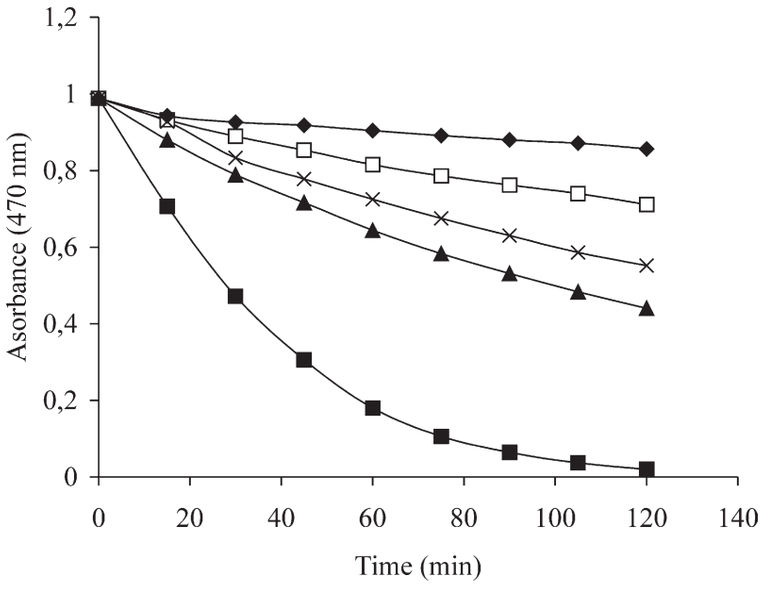

FIGURE 2 Antioxidant activity of the total essential oil and its fractions: (■) Control; (×) total essential oil; (▲) CH fraction; (◆) CHO fraction; (□) phenolic fraction. The concentrations of the total essential oil and different fractions in reacting systems were 200 ppm.

β-Carotene bleaching method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals which are formed by linoleic acid in an emulsion. As described by Koleva *et al.*[19] emulsified lipid introduces a number of variables which can influence reproducibility of results and frequently it is difficult to determine the reproducible AAC values of antioxidants for different series of measurements. However, the method can be used for comparative studies of antioxidant powers intra each separate series. In our study, the variability of AAC values was significant (standard deviations were increased) but antioxidant powers were determined always in order BHT > α-tocopherol > (the total essential oil, fractions and pure constituents containing hydroxy groups) > CH fraction.

Antioxidant Activity Determined with Thiobarbituric Acid Method

The method, known as TBARS assay, concerns the spectrophotometric measurement of the pink pigment produced through reaction of TBA with malondialdehyde (MDA) and other secondary lipid peroxidation product. The evaluation of the absorbance at 532 nm gives a measure of the extent of lipid degradation. As well as with β-carotene bleaching method, each determination was performed in

TABLE II Antioxidant activity of savory free volatile compounds measured by β-carotene bleaching and tiobarbituric acid methods

	β-Carotene bleaching*		Thiobarbituric acid†	
	AAC‡		AI%‡	
Antioxidants¶	100 ppm	1000 ppm	100 ppm	1000 ppm
Total essential oil	405.1 ± 12.4	845.5 ± 112.1	20.9 ± 1.4	58.1 ± 0.6
CHO fraction	364.1 ± 18.4	724.5 ± 40.6	19.7 ± 0.2	50.7 ± 4.7
CH fraction	71.8 ± 7.6	419.6 ± 86.3	17.3 ± 3.1	26.9 ± 1.3
Phenolic fraction	363.8 ± 18.5	736.3 ± 42.4	20.9 ± 0.6	43.3 ± 2.5
Thymol	444.9 ± 35.2	708.2 ± 33.3	24.0 ± 2.9	41.5 ± 2.2
Carvacrol	433.6 ± 42.9	724.5 ± 40.6	24.0 ± 3.7	52.8 ± 2.7
α-Tocopherol	682.4 ± 15.4	890.5 ± 95.5	72.6 ± 3.6	90.0 ± 1.8
BHT	800.0 ± 55.6	961.3 ± 15.2	37.5 ± 1.9	68.6 ± 1.1

*Decreasing of the absorbance measured at 470 nm due to the β-carotene bleaching in presence of different concentration of potential antioxidants. †Increasing of the absorbance measured at 532 nm due to the accumulation of TBARS–MDA in presence of different concentrations of potential antioxidants. ‡Antioxidant effectiveness expressed as antioxidant activity coefficient (AAC) and antioxidant index (AI%). Values represents average of three determinations with ± standard deviation. ¶Antioxidants concentration in reacting system.

triplicate and results are reported as mean ± standard deviation.

The total savory essential oil, its fractions and pure constituents were examined for their ability to act as hydroxy radical scavenging agents. As shown in Table II, two different concentrations of α-tocopherol (100 ppm and 1000 ppm) showed a very strong antioxidant effect. Thymol, carvacrol, the total essential oil and fractions with oxygen containing compounds exhibited relatively strong antioxidant effect for both concentrations. The weakest inhibition was measured for hydrocarbon fractions. The effectiveness of all antioxidants for concentration 1000 ppm were increased in comparison with their activity in the presence of 100 ppm.

DISCUSSION AND CONCLUSION

The composition of the essential oils of *S. montana*, collected from different localities was previously reported[13] and showed that thymol and carvacrol are its main constituents. If the fact that thymol and carvacrol are the major components of the savory essential oil is taken into account, our findings are identical to those reported earlier for the antioxidant activity of the plants containing such compounds.[10,20,21] As determined with both methods, β-carotene bleaching and TBA, the total savory essential oil as well as its different fractions or pure constituents containing hydroxyl group exhibited relatively strong antioxidant effect. This observation is similar with the finding of Shahidi, Janitha and Wanasundara[22] which reported that the antioxidant effect of aromatic plants is due to the presence of hydroxyl groups in their phenolic compounds. The fact that the total essential oil, phenolic fraction and CHO fraction as well as pure constituents thymol and carvacrol show similar antioxidant effects allows to conclude that there is not the synergy among minor oxygen containing compounds which sometimes can play a crucial role for antioxidant power.[23] The antioxidant effect of the savory essential oil is due probably only to the presence of elevated percentage of thymol and carvacrol.

The hydrocarbons, when isolated as CH fraction, showed the poorest effectiveness. This observation was contrary to our expectation since among 19 com pounds identified in hydrocarbons (CH) fraction γ-terpinene, α-terpinene, *p*-cymene and terpinolene were previously identified as potential antioxidants.[18]

Our results suggest that the total savory essential oil in elevated concentration were found to be potent antioxidants, comparable in activity with widely used natural antioxidant α-tocopherol and synthetic reference standard butylated hydroxytoluene. In general, the use of the savory essential oil as antioxidant additive or nutritional supplement, which acts as free radical scavenger should merit further investigation. These researches could be interesting for the pharmaceutics, phytoterapeutic and some medicinal aspect also.

Acknowledgements

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