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Analytical Methods

Evaluation of antioxidant capacity of *Allium ursinum* L. volatile oil and its effect on membrane fluidity

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Abstract

A total of 20 components were identified in *Allium ursinum* volatile oil (AUVO) by GC–MS, and 10 of them are found for the first time in this plant species. The antioxidant capacity of AUVO was examined by β-carotene–linoleic acid bleaching, lipid peroxidation by Fenton reaction, EPR spin-probing assay using stable nitroxide radicals, $DPPH^{\cdot}$, and $ABTS^{\cdot+}$ scavenging assays. Reaction mechanism of the volatile oil components with nitroxide radicals, based on IR spectra analysis, is proposed. AUVO demonstrated poor scavenging ability against $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ comparing to synthetic antioxidants BHT and trolox, while in β-carotene–linoleic acid system AUVO showed an effect comparable to those for BHT. AUVO was also capable to scavenge stable nitroxide radicals such as water-soluble Tempone, and 7-DS and 12-DS, incorporated into the liposome membrane. Finally, AUVO increased membrane fluidity, which could be an important feature for further in vivo investigation of some disorders, such as hypertension and atherosclerosis.

Keywords: *Allium ursinum*; Volatile oil; Antioxidant capacity; Nitroxide radicals; Liposomes; EPR; Membrane fluidity

1. Introduction

Allium ursinum (also named wild garlic, ramson or bear's garlic) belong to the *Alliaceae* family. Wild garlic grows on fertile soil in shady, humid places and preferably under leafy trees in Europe and North Asia, and cannot be cultivated. It is extensively used for centuries in traditional medicine and as a spice or salad (Sendl, 1995).

The major flavor precursors of *A. ursinum* are *S*-allyl-L-cysteine sulfoxide (alliin) and *S*-methyl-L-cysteine sulfoxide (methiin). Traces of *S*-(trans-1-propenyl)-L-cysteine sulfoxide (isoalliin) and *S*-propyl-L-cysteine sulfoxide (propiin) are also presented (Schmitt, Schulz, Storsberg, & Keusgen, 2005). In the intact cell, the sulfoxides are found in the cytoplasm and the hydrolytic enzyme alliinase in the vacuole. Disruption of plant material results in hydrolysis of the sulfoxides, thus creating alk(en)yl-thiosulfinates, volatile, flavor compounds. These compounds are responsible for the characteristic smell and taste of *Allium* species. Aroma components can also be produced by steam distillation, where initially formed thiosulfinates are rapidly converted into their corresponding alk(en)yl(poly)sulfides (Keusgen et al., 2002). Furthermore, there is evidence that such sulfides are formed through in vivo metabolism of thiosulfinates after ingestion by humans and animals (Taucher, Hansel, Jordan, & Lindinger, 1996).

Various investigations have been carried out with volatile oils from *Allium* species and pure or fractionized alk(en)yl(poly)sulfides. Recently, it has been shown that diallyl sulfide, diallyl disulfide, and diallyl trisulfide scavenged reactive species such as superoxide and peroxynitrite radical. They were also slightly effective in scavenging

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hydroxyl radical and $ABTS^{\cdot+}$ (Kim, Chang, Kim, Chang, & Chun, 2006). In addition, alk(en)yl trisulfides exhibited antioxidant activity on human low-density lipoprotein (LDL) oxidation in vitro. Surprisingly, alk(en)yl disulfides have shown no effect in LDL oxidation (Higuchi, Tateshita, & Nishimura, 2003). Total lipids and cholesterol in rats' plasma were significantly reduced by application of a mixture of 98% diallyl disulfide and 2% diallyl trisulfide (Pushpendran, Devasagayam, Banerji, & Eapen, 1980). Epidemiological and experimental studies have revealed that alk(en)yl(poly)sulfides can suppress cancer risk and alter the biological behavior of tumors through the activation of detoxifying enzymes (thus protect against cancer (Fukao, Hosono, Misawa, Seki, & Ariga, 2004a, 2004b; Jakubowski, 2003; Lii, Tsai, & Wu, 2006; Wu et al., 2002)) and also by inducing antiproliferative effects, thus provoking apoptotic cell death (Knowles & Milner, 2001; Sakamoto, Lawson, & Milner, 1997; Sundaram & Milner, 1996a, 1996b; Takashi et al., 2005). Also, alk(en)yl(poly)sulfides were reported to modify some other biological activities, e.g. they inhibit platelet aggregation in vitro (Ariga, Oshiba, & Tamada, 1981).

In the present study, we investigated chemical composition of AUVO and evaluated its antioxidant capacity using different assays. Effects of the volatile oil on membrane fluidity have been also investigated.

2. Materials and methods

2.1. Plant material

The leaves of *A. ursinum* L. were collected in May 2006 in the vicinity of Belgrade, Serbia. A voucher specimen (AU15052006) was deposited in the Herbarium of the Faculty of Biology, University of Belgrade.

2.2. Chemicals

2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), β-carotene, linoleic acid, trolox, 1,1′-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich (Schnelldorf, Germany). 2,6-di-*tert*-butyl-4-methylphenol (BHT) and ascorbic acid were purchased from Merck (Darmstadt, Germany). Nitroxide spin probes Tempone (2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-one), 7-DS (2-(5-carboxypentyl)-2-undecyl-4,4-dimethyloxazolidine-3-oxyl), and 12-DS (2-(10-carboxydecyl)-2-hexyl-4,4-dimethyloxazolidine-3-oxyl) were purchased from Molecular Probes, (Junction City, OR, USA). The spin trap, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO), was synthesized according to a method of Clément, Fréjaville, and Tordo (2002) and purified according to a method of Jackson, Liu, Liu, and Timmins (2002). The identity of DEPMPO was confirmed by ^{1}H and ^{13}C NMR spectroscopy.

For the preparation of liposomes, a chloroform solution of L-α-phosphatidylcholine (purchased from Sigma–Aldrich) was evaporated under vacuum to dryness. Then, buffer solution (10 mM NaH_2PO_4, pH 7.4) was added to obtain final concentration of 125 mM of lipids, and the suspension was vortexed for 5 min.

2.3. Volatile oil isolation

One thousand grams of fresh leaves were mixed in blender and then subjected to hydrodistillation using Clevenger apparatus to yield 150 mg of volatile oil (0.015%). Oil was stored in a freezer (−20 °C) until further use.

2.4. Gas chromatography (GC)

A Hewlett-Packard chromatograph model HP 5890 Series II equipped with a FID and fitted with a capillary HP-5MS column with a 0.25 μm film thickness, 30 m length and inner diameter 0.25 mm. The operating conditions were as follows: injector temperature, 255 °C; split ratio, 30:1; detector temperature, 300 °C; carrier gas H_2 with flow rate, 1.0 mL/min. The temperature program was: from 40 to 150 °C (4 °C/min), from 150 to 299 °C (10 °C/min), and held at 299 °C for 10 min. One microliter of the sample dissolved in CH_2Cl_2 (1:100 v/v) was injected. The percentage composition of the oil was computed by the normalization method from the GC (FID) peak areas, calculated by means of three injections, without using correction factors.

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

Analysis was run on an Agilent 6890 GC–MS system coupled to a quadrupole mass spectrometer model Agilent 5973. The operating GC conditions were the same reported for GC analysis, and the same column was used. The mass spectrometry (MS) conditions were as follows: ionization voltage, 70 eV; ion source temperature, 200 °C; scan range, m/z 50–432. The identification of the individual components was based on computer matching with the Wiley 275 and NIST 05, software version 2.0d libraries. The constituents of the oil were also identified by comparing their retention times, the MS fragmentation patterns and GC retention indices to those of the standards. A mixture of aliphatic hydrocarbons (C_9–C_{20}) in hexane was injected under above mentioned GC conditions.

2.6. DPPH· scavenging assay

The DPPH· scavenging assay was carried out according to the procedure described by Blois (1958). Various concentrations of the samples (100 μL) were mixed with 900 μL of 0.04 mg/mL methanolic solution of DPPH. Absorbance at 517 nm was measured after 20 min. The inhibition percentage was calculated using the following equation:

$$I = [(A_c - A_s)/A_c] \times 100$$

where I was the inhibition percentage, A_c was the absorbance of the negative control (contained 100 μL of MeOH

in place of the samples), and A_s was the absorbance of the samples. Synthetic antioxidants, trolox and *tert*-butyl hydroxytoluene (BHT), were used as positive controls. The inhibition percentage was plotted against concentration of the samples, and EC_{50} values were determined by linear regression analysis.

2.7. $ABTS^{\cdot+}$ decolorization assay

The ability of the extracts to scavenge ABTS radical cation ($ABTS^{\cdot+}$) was measured by method of Re et al. (1999). The $ABTS^{\cdot+}$ was generated by reacting 7 mM aqueous ABTS with 2.45 mM $K_2S_2O_8$ (final concentration) in the dark at room temperature for 16 h. For the measurement of scavenging capacity, solution of $ABTS^{\cdot+}$ was diluted with MeOH to an absorbance of 0.7 at 734 nm. Solutions of samples in MeOH so were prepared that, after adding of a 100 μL aliquot of each dilution into the assay, they produced between 10% and 80% inhibition of the absorbance comparing to those of control. After addition of 900 μL of diluted $ABTS^{\cdot+}$ solution to 100 μL of sample, the absorbance at 734 nm was continuously recorded for 5 min. The inhibition percentage was calculated as for DPPH assay, and plotted as a function of concentration of the samples. The concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation at 734 nm as 1 mM trolox was calculated in terms of the trolox equivalent antioxidant activity at each specific time-point. To calculate TEAC the gradient of the plot of the inhibition percentage of absorbance vs. concentration plot for the sample is divided by the gradient of the plot for the trolox.

2.8. β-Carotene–linoleic acid bleaching assay

The β-carotene–linoleic acid bleaching assay was carried out according to the procedure described by Dapkevicius, Venskutonis, Van Beek, and Linssen (1998). β-Carotene (0.5 mg) was dissolved in 5 mL of $CHCl_3$, and 40 μL of linoleic acid and 300 mg of Tween 40 were added. $CHCl_3$ was removed using a rotary evaporator. Oxygenated HPLC grade water (85 mL) was added, and the flask was shaken vigorously until all materials dissolved. This test mixture was prepared fresh and used immediately. Various concentrations of methanolic volatile oil were prepared. Aliquots (2 mL) of the β-carotene–linoleic acid emulsion were mixed with 100 μL of sample solutions and incubated in a water bath, embedded in spectrophotometer, at 50 °C. Oxidation of the emulsion was monitored using spectrophotometer, continuously measuring absorbance at 470 nm over a period of 120 min. The negative control contained 100 μL of MeOH instead of the samples. BHT was used as a positive control. The antioxidant behavior of volatile oil was expressed in two different ways. In the first, antioxidant capacity was calculated as the inhibition percentage respective to bleaching rates (IBR) of β-carotene, using the following equation:

$$\mathrm{IBR} = [(R_c - R_s)/R_c] \times 100$$

where R_c and R_s were the bleaching rates of β-carotene in negative control and samples, respectively. The bleaching rates were determined from the derivative plot of the absorbance vs. time. Curve maxima were taken for the calculus.

In the second, the antioxidant capacity was calculated as the inhibition percentage respective to decrease of absorbance (IDA) during the reaction time

$$\mathrm{IDA} = [1 - (A_{s(0)} - A_{s(120)})/(A_{c(0)} - A_{c(120)})] \times 100$$

where $A_{s(0)}$ was the initial absorbance of the sample at 0 min, $A_{s(120)}$ was the absorbance of the sample at 120 min, $A_{c(0)}$ was the initial absorbance of the negative control at 0 min, and $A_{c(120)}$ was the absorbance of the negative control at 120 min.

2.9. Lipid peroxidation induced by Fenton reaction

Various amounts of AUVO were added to liposome suspension (final concentration 100 mM of phosphatidylcholine) and sonicated in sonic bath for 1 min. The suspension (80 μL) was combined with 13.5 μL of 0.03% H_2O_2 and 5 μL of 0.5 M DEPMPO spin trap. The reaction was initiated by adding 1.8 μL of 10 μmol/L $FeSO_4$. The reaction mixture was then transferred to a gas-permeable Teflon tubes, and EPR measurements were performed. The negative control contained pure water instead of volatile oil. EPR spectroscopy was used to follow the decrease of radicals in presence of AUVO, as described previously for extracts of various *Allium* species (Štajner et al., 2006). The EPR spectra were recorded using a Varian E104-A EPR spectrometer operating at X-band (9.5 GHz) adjusted to the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 200 G, during the period of 30 min at room temperature.

2.10. EPR spin-probing assay

Various concentrations of the samples were prepared in MeOH. Liposome suspension (final concentration 30 mM of phosphatidylcholine) was then mixed with spin probe Tempone, or 7-DS, or 12-DS (final concentration 50 μM, 150 μM, and 150 μM, respectively) and sonicated for 1 min. This suspension was then mixed with either methanolic volatile oil or pure MeOH (for negative control). The mixture was then transferred to a gas-permeable Teflon tubes (Zeus Industries, Raritan NJ) for EPR measurements. The EPR spectra were recorded using a Varian E104-A EPR spectrometer operating at X-band (9.5 GHz) adjusted to the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 200 G for Tempone or 100 G for doxyl stearates, during the period of 30 min at room temperature. Antioxidant capacity was evaluated by the decrease of the central peak amplitude.

2.11. EPR measurements of membrane fluidity

Liposomes (final concentration 30 mM of phosphatidylcholine) were mixed with 7-DS or 12-DS to give spin label: lipid molar ratio 1:200. Ten microliters of either methanolic volatile oil (0.65 mM) or pure MeOH were added to 50 μL of spin label-liposomes suspension. The reaction mixture was then transferred to a glass capillary, and EPR measurements were carried out using following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 100 G. The rotational correlation time was used as an indicator of the membrane fluidity, where lower values of τ stand for higher membrane fluidity. The rotational correlation time was calculated using the following equation (Koter, Franiak, Strychalska, Broncel, & Chojnowska-Jezierska, 2004):

$$\tau = K\Delta W_0 \left[\left(\frac{h_0}{h_{-1}} \right) - \left(\frac{h_0}{h_{+1}} \right) \right]$$

where ΔW_0 was the width of the central peak (in gauss), and h_0, h_{-1} and h_{+1} were the central, high and low field signal amplitudes, respectively. Constant K was taken to be 3.418×10^{-10} (Korkmaz, 2000).

2.12. Statistical analysis

The results are expressed as means ± standard deviation of three determinations. The statistical significance of differences between means was determined using one-way analysis of variance followed by Tukey's post hoc means comparison. A probability value of $p < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Volatile oil analysis

A total of 20 components were identified in the volatile oil of *A. ursinum* on the basis of comparison with MS data base spectra, retention indices, and pure reference compounds. Ten of the identified components were found for the first time in this species. The identified components and their percentage in the oil, computed from GC (FID) peak areas without using correction factors, are listed in Table 1. The identified components may be classified into the following groups: disulfides (54.7%), trisulfides (37.0%), tetrasulfides (4.7%), and the non-sulfur compounds (1.0%). In order to express molar concentrations, on the basis of qualitative and quantitative composition, average molar weight of AUVO was calculated to be 155 g/mol. It should be noted that such sulfur containing compounds were not present in intact plant, because they are mainly produced during hydrodistillation procedure. This is an important fact because these compounds could be produced during cooking of food containing *A. ursinum*.

Table 1
Chemical composition of *A. ursinum* volatile oil

No.	RI[a]	Compound	RA[b]	SDRA[c]
1	906	Methyl 2-propenyl disulfide	5.07	0.04
2	923	Methyl propyl disulfide	0.69	0.09
3	931	(*E*)-Methyl 1-propenyl disulfide	2.35	0.21
4	959	Dimethyl trisulfide	1.07	0.01
5	1004	(*E*)-Hex-3-en-1-ol	0.67	0.11
6	1071	Di-2-propenyl disulfide	24.88	0.95
7	1085	2-Propenyl propyl disulfide	5.04	0.21
8	1093	(*E*)-1-Propenyl 2-propenyl disulfide	16.65	0.14
9	1099	Nonanal[d]	0.32	0.06
10	1130	Methyl 2-propenyl trisulfide[d]	14.16	0.68
11	1143	Methyl propyl trisulfide	0.33	0.04
12	1153	(*Z*)-Methyl 1-propenyl trisulfide[d]	0.64	0.15
13	1158	(*E*)-Methyl 1-propenyl trisulfide[d]	0.95	0.18
14	1207	Dimethyl tetrasulfide[d]	0.54	0.05
15	1291	Di-2-propenyl trisulfide	16.48	0.60
16	1304	Propyl 2-propenyl trisulfide[d]	0.74	0.09
17	1309	(*Z*)-1-Propenyl 2-propenyl trisulfide[d]	1.04	0.26
18	1313	(*E*)-1-Propenyl 2-propenyl trisulfide[d]	1.63	0.27
19	1370	Methyl 2-propenyl tetrasulfide[d]	2.15	0.06
20	1530	Di-2-propenyl tetrasulfide[d]	1.97	0.08
Total			97.37	0.82

[a] Retention indices calculated against *n*-alkanes (C_9–C_{20}) on an HP-5MS column.
[b] Relative area percentage, calculated from flame ionization detector data.
[c] Standard deviation of relative area percentage, calculated from the three successive injections.
[d] Detected for the first time in *A. ursinum*.

3.2. Antioxidant capacity assays

The results of the determination of the antioxidant capacity of an extract depend greatly on which technology and which free radical generator or oxidant is used in the measurement. In some cases, individual antioxidants may act by multiple mechanisms in a single system, or by a different single mechanism depending on the reaction system. Furthermore, antioxidants may respond in a different manner to different radical or oxidant sources. No single assay will accurately reflect all of the radical sources or all antioxidants in a mixed or complex system, because multiple reaction characteristics and mechanisms as well as different phase localizations are usually involved. Matching radical source and system characteristics to antioxidant reaction mechanisms is critical in the selection of appropriate antioxidant capacity assay methods, as is consideration of the end use of the results. It is important to emphasize that there is no simple universal method which can be used to measure antioxidant capacity accurately and quantitatively. Thus, it is very useful to compare various analytical methods varying in their oxidation initiators and targets in order to understand the biological activity of an antioxidant (Prior, Wu, & Schaich, 2005).

This study relied on variety of in vitro assays, based either on the capacity to scavenge free radicals (DPPH, ABTS) or on the ability to protect artificial membrane models (β-carotene–linoleic acid bleaching, lipid peroxidation by Fenton reaction, EPR spin-probing assay).

3.3. DPPH· and ABTS·+ scavenging capacity

The ability of AUVO, trolox (water-soluble analogue of vitamin E) and BHT (synthetic antioxidant) to scavenge DPPH radical was tested, and the concentrations of the volatile oil varied between 1.3 and 25.8 mM. The scavenging effect of AUVO on DPPH radical linearly increased with concentration, reaching an EC_{50} value at 27 ± 1 mM. Synthetic antioxidants BHT and trolox demonstrated stronger scavenging effect with EC_{50} value at 0.028 ± 0.001 mM and 0.027 ± 0.001 mM, respectively.

To express the antioxidant capacity of AUVO by $ABTS^{\cdot+}$ scavenging assay, the TEAC values after 1, 3, and 5 min of reaction time were calculated (Table 2). The relatively low TEAC values of AUVO comparing to BHT and ascorbic acid showed poor ability of AUVO to neutralize $ABTS^{\cdot+}$. This is in accordance to previously reported small effectiveness of diallyl disulfide and diallyl trisulfide in scavenging $ABTS^{\cdot+}$, relative to ascorbic acid (Kim et al., 2006). The differences in TEAC values after 1, 3, and 5 min of reaction time for AUVO and for BHT suggest a slow kinetics. This was confirmed using kinetical studies (Fig. 1), which revealed that reactions of $ABTS^{\cdot+}$ with trolox and ascorbic acid were very fast, reaching steady state within first 10 s of incubation. BHT reacted slowly, reaching steady state after approximately 4 min, while the slowest reaction was one with AUVO and $ABTS^{\cdot+}$, where no measurable changes of absorbance occurred during 5 min of incubation (Fig. 1).

Generally, antioxidants can deactivate radicals by two major mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET). HAT-based methods are usually quite rapid (typically completed in seconds to minutes), and measure the classical ability of an antioxidant to quench free radicals by hydrogen donation. SET-based methods detect the potential antioxidant's ability to transfer one electron to reduce a compound, usually are slow and can require long time to reach completion (Prior et al., 2005).

Although the ABTS and DPPH assays are usually classified as SET reactions, these two indicator radicals in fact can be neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer.

Contrary to the common antioxidant constituents of the food (poliphenolic compounds, ascorbic acid, tocopherols), alk(en)yl(poly)sulfides do not posses any acid protons; hence, their ability to react with ABTS and DPPH radicals via HAT reaction mechanism is very poor. SET reaction mechanism seems to be very likely, and is supported by slow kinetics in ABTS assay.

3.4. β-Carotene–linoleic acid bleaching assay

In this system, free radical arisen from oxidation of linoleic acid, attacks the highly unsaturated β-carotene molecules, causing decrease of the absorbance at 470 nm. Lipid peroxidation assay is preferred over DPPH· and $ABTS^{\cdot+}$ assays since these synthetic stable nitrogen radicals bears no similarity to the highly reactive lipid radicals (Prior et al., 2005). The presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system (Jayaprakasha, Singh, & Sakariah, 2001). The antioxidant capacity of AUVO, measured by the β-carotene–linoleic acid model system, is presented in Fig. 2. The results are expressed as the inhibition percentage respective to bleaching rates (IBR) of β-carotene, as well as by decrease of absorbance (IDA) calculated from start of reaction to 120 min of reaction time. The results showed that AUVO was capable to reduce both the amount and the rate of the linoleic acid oxidation. Contrary to the DPPH and ABTS assays, where about 1000 times higher amount of AUVO should be used to obtain comparable activity to synthetic antioxidants, in β-carotene–linoleic acid bleaching assay they show comparable antioxidant activity.

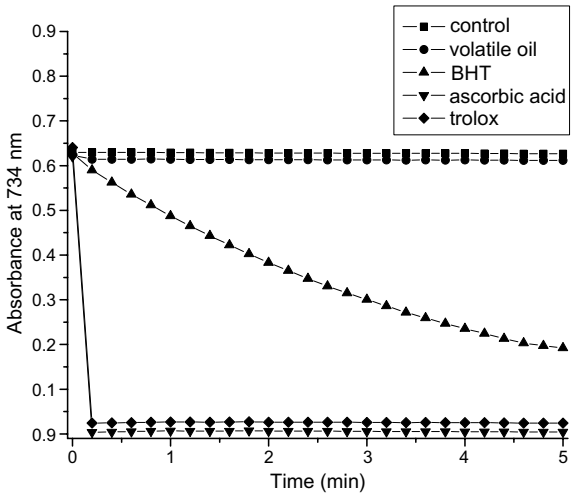

Fig. 1. Decay of absorbance of $ABTS^{\cdot+}$ in the presence of *A. ursinum* volatile oil (0.3 mM), ascorbic acid (0.3 mM), BHT (0.3 mM) and trolox (0.3 mM).

Table 2
Antioxidant capacity of *A. ursinum* volatile oil and BHT measured by the $ABTS^{\cdot+}$ decolorization assay

	TEAC (mM)		
	1 min	3 min	5 min
Volatile oil	$(1.22 \pm 0.05) \times 10^{-3}$	$(1.88 \pm 0.09) \times 10^{-3}$	$(2.10 \pm 0.10) \times 10^{-3}$
BHT	0.023 ± 0.002	0.077 ± 0.002	0.107 ± 0.003
Ascorbic acid	1.06 ± 0.03	1.06 ± 0.03	1.06 ± 0.03

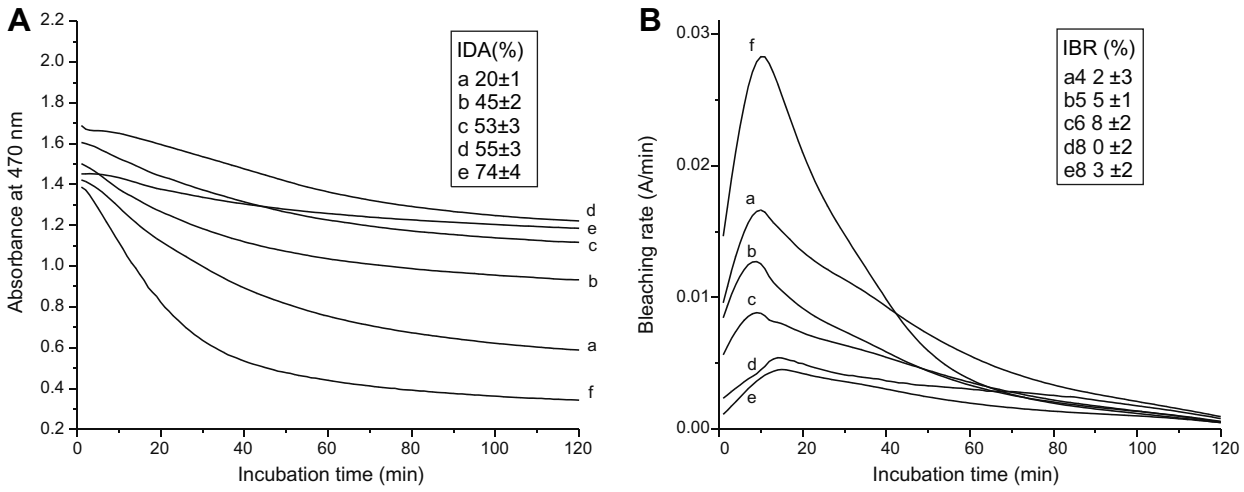

Fig. 2. Antioxidant capacity of *A. ursinum* volatile oil (a: 0.65 mM; b: 1.3 mM; c: 2.0 mM; e: 2.6 mM) and BHT (0.9 mM) as measured by the β-carotene–linoleic acid bleaching assay (changing of β-carotene absorbance (A) and bleaching rate (B) with corresponding IBR and IDA values).

Volatile oil at the maximal concentration used in this study (2.6 mM) reached the bleaching rate similar to the rate obtained for BHT (0.9 mM). Discrepancies between these assays result from physicochemical properties of AUVO, which determine their interaction with the different targets used in the different assays, e.g. the liposolubility of constituents of AUVO is important in process of their diffusion into lipophilic part of β-carotene–linoleic acid/water emulsion. However, many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH due to steric inaccessibility (Prior, Wu, & Schaich, 2005).

3.5. Lipid peroxidation induced by Fenton reaction

We investigated whether AUVO could inhibit lipid peroxidation of the membrane induced by the Fenton reaction, which is one of the main radical producing systems in biology. Lipid radicals were generated in reaction of phosphatidylcholine liposomes with radicals produced by the oxidation in Fe^{2+}/H_2O_2 system, and trapped using DEPMPO spin trap. DEPMPO adduct of lipid radical was detected and the amplitude of the characteristic signal in the obtained EPR spectra showed time-dependent decrease in the AUVO sample during 30 min of incubation. Such decrease was not observed in the negative control sample. The signal amplitude in sample with BHT was much lower than those in control sample. However, there are 2 min between starting the reaction and the acquirement of the first spectrum, which are technically inevitable. Very rapid decrease of the signal amplitude occurred during these 2 min, and DEPMPO adduct of lipid radical was stable during next 30 min of experiment (Fig. 3). On the other hand, for AUVO is difficult to determine which part of EPR signal amplitude decrease is due to the inhibition of lipid peroxidation and which is due to the removal of the DEPMPO adduct of lipid radical. Most likely, rapid decrease in first 2 min means that the AUVO and BHT inhibit production of OH radicals; hence fewer radicals are captured by the spin trap. Further decrease of the signal amplitude provoked by AUVO, is very probably due to reduction of adduct (as observed also in experiments with stable nitroxide radicals, see Fig. 4).

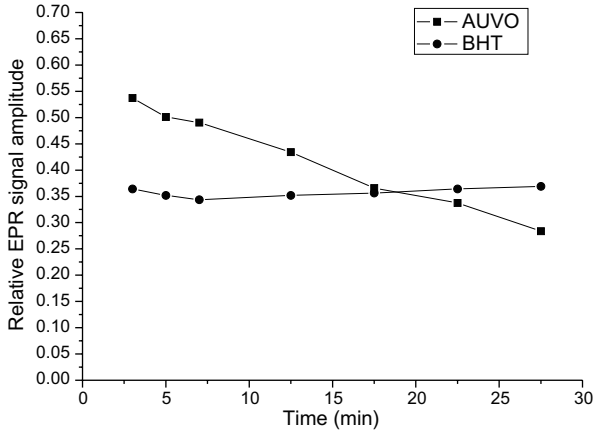

Fig. 3. EPR signal amplitude decay of DEPMPO adduct of lipid radical in presence of *A. ursinum* volatile oil (1.3 mM) and BHT (0.9 mM).

3.6. EPR spin-probing assay of antioxidant capacity of AUVO

Spin probes such as Tempone, 7-DS, and 12-DS represent stable free radicals, so their EPR spectra can be easily obtained. As reactive species, they are targets for antioxidant compounds, whose actions lead to the decrease of the signal amplitudes in the EPR spectra of probes. Therefore, we used EPR spin-probing technique to evaluate antioxidant capacity of AUVO. Stable nitroxide radicals, detectable by EPR spectroscopy, are able to accept/give electrons from/to different sources, reducing themselves

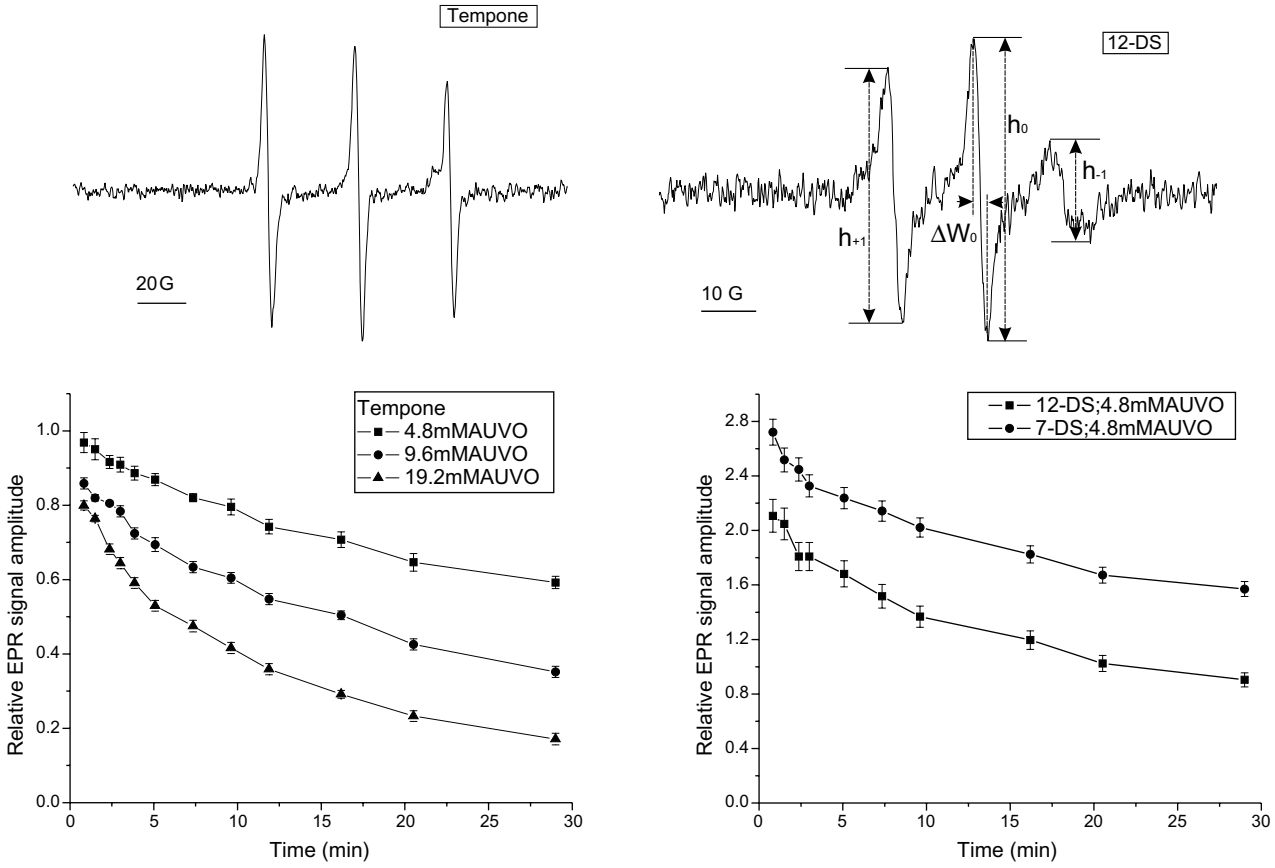

Fig. 4. EPR spectra of Tempone and 12-DS and EPR signal amplitude decay of nitroxide radical in presence of *A. ursinum* volatile oil.

to EPR-silent hydroxylamines, or oxidizing themselves to equally EPR-silent nitrones (Sentjurc, Nemec, Connor, & Abram, 2003). Depending on experimental conditions, nitroxides could act as antioxidants or pro-oxidants, due to their redox properties (Damiani, Astolfi, Benaglia, Alberti, & Greci, 2004). Tempone, which is a preferentially hydrophilic compound, was used for studying the antioxidant action of the oil in solution, while 7-DS and 12-DS, incorporating themselves into the liposome membrane, yield the information regarding such activity of the oil inside the membrane. The addition of AUVO to the Tempone solution resulted in decay of signal amplitude (Fig. 4). This effect was dose-dependent, e.g. greater concentration of the volatile oil induced faster decrease of spectral intensity. Therefore, the components of the oil acted as scavengers of stable nitroxide radicals in the solution. Ability of the oil to remove exogenous radicals present in the water indicates that it would be also capable to remove radicals produced in biosystems, many of which are produced and solvated in the aqueous environment. In order to identify a source of the decrease of EPR signal amplitude, the infrared spectra of Tempone solution, AUVO solution and mixture of these solutions are recorded. IR absorptions at 1025 and 1093 cm^{-1}, appeared only in the mixture of Tempone and the volatile oil, indicated the presence of sulfinyl functional group (data not shown). Thus, it was concluded that alk(en)yl(poly)sulfides from the volatile oil were oxidized to corresponding sulfoxides, and, consequently, Tempone must be reduced to corresponding hydroxylamine. Consequently, alk(en)yl(poly)sulfides demonstrated stronger antioxidant properties then nitroxides, under condition of the experiment.

AUVO also induced decrease in the relative amplitude of the EPR signal of 7-DS and 12-DS incorporated in the liposome membrane (Fig. 4). This shows that some compounds from the volatile oil enter the membrane easily and perform their antioxidant action inside the lipid bilayer. Similarity of the effects of AUVO on 7-DS and 12-DS indicates that antioxidant components of the oil intercalate the membrane, most likely into the "middle" layers of the membrane semileaflet.

3.7. Effects of AUVO on the membrane fluidity

In addition to the study of the antioxidant activity of AUVO inside the membrane, 7-DS and 12-DS were used to investigate effects of the oil on the fluidity of the liposome membrane. Rotational correlation time (τ), which represents a reciprocal measure of the membrane fluidity, was used. AUVO (4.8 mM) induced statistically non-significant changes of the membrane fluidity in the part of the liposome membrane close to the surface which was evaluated using 7-DS. However, in deeper layers of the membrane volatile oil induced increase of the fluidity. Values

of τ determined by using 12-DS were 6.2 ± 0.6 ($\times 10^{-10}$ s) for control samples, and 5.1 ± 0.3 ($\times 10^{-10}$ s) for liposomes treated with the oil. This verifies that antioxidant components of AUVO preferentially intercalate themselves in "deeper", more hydrophobic layers of the membrane as indicated by the results presented in Fig. 4. Such effect of the AUVO should be taken into account for applications in any specific biosystem, since changes in the cell membrane fluidity are known to affect properties and functions of the cell membrane including cell growth, signal transduction, membrane permeability, transport systems, receptor functions or enzyme activities (Cooper, 1977; Hitzemann, Hirschowitz, & Garver, 1986; Parks, Huggins, Gebre, & Burleson, 2000; Zicha, Kunes, & Devynck, 1999). Previous studies concerning EPR spin labeling method have shown that hypertension has been correlated to decreased fluidity of erythrocytes membrane. Some compounds that have hypotensive effects significantly increased membrane fluidity of erythrocytes in hypertensive patients (Tsuda, Kinoshita-Shimamoto, Mabuchi, & Nishio, 2003; Tsuda & Nishio, 2003). Hypertension is associated with an increased risk of atherosclerosis. Free radicals may be involved in the pathogenesis of accelerated atherosclerosis in hypertension (Pezeshk & Dalhouse, 2000). This study represents an in vitro experiment on the artificial membrane model, and further in vivo experiments are needed to estimate potential role of AUVO as a modulator of membrane fluidity, involved in the regulation of membrane functions in hypertension, as well as an antioxidant. These findings could justify the use of *A. ursinum* and other *Allium* species in traditional medicine for the blood pressure lowering.

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