

Full Research Paper

## Antioxidant Potential, Lipid Peroxidation Inhibition and Antimicrobial Activities of *Satureja montana* L. subsp. *kitaibelii* Extracts

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**Abstract:** The antioxidant activity of different *Satureja montana* L. subsp. *kitaibelii* extracts was tested by measuring their ability to scavenge reactive hydroxyl radical during the Fenton reaction, using ESR spectroscopy. Also, the influence of these extracts on lipid peroxy radicals obtained during lipid peroxidation of: (I) sunflower oil (37 °C, 3h) induced by 4,4'-azobis(4-cyanovaleric acid) (ACVA) and (II) liposomes induced by 2,2'-azobis(2-amidino-propane)dihydrochloride (AAPH) was studied. *n*-Butanol extract had the best antioxidant activity (100% at 0.5 mg/mL in Fenton reaction system; 89.21% at 5 mg/mL in system I; 83.38% at 5 mg/mL in system II). The antioxidant activities of the extracts significantly correlated with total phenolic content. The antimicrobial activity of *Satureja montana* L. subsp. *kitaibelii* extracts was investigated. Petroleum ether, chloroform and ethyl acetate extracts expressed a wide range of inhibiting activity against both gram-positive and gram-negative bacteria.

**Keywords:** *Satureja montana* L. subsp. *kitaibelii* extracts, antioxidant, lipid peroxidation, antimicrobial activity

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## 1. Introduction

Phenolic compounds, important constituents in many plants, have received considerable attention as potentially protective factors against cancer and heart diseases because of their antioxidant potency and their ubiquity in a wide range of commonly consumed foods of plants origin [1-4]. The presence of phenolic compounds in plants is important for normal growth development and defence against infection and injury. Also, phenolics may have an important effect on the oxidative stability and microbial safety in injured plants. Phenolic compounds possess one or more aromatic ring-bearing a hydroxyl substituents, including their functional derivatives such as esters, methoxy compounds and glycosides. Approximately 8000 naturally occurring compounds are known which include simple phenolic, phenolic acids (benzoic and cinnamic acid derivatives), lignans, lignins, coumarins, styrylpyrones, flavonoids, isoflavonoids, stilbenes, flavonolignans and tannins [5,6]. It is generally accepted that phenolic compounds behave as antioxidants as a result of the reactivity of the phenolic moiety. There are several mechanisms of antioxidant activity but it is believed that radical scavenging via hydrogen atom donation is the predominant mode. Other established antioxidant mechanisms involve radical complexing of prooxidant metals as well as quenching through electron donation and singlet oxygen quenching. Many studies have reported that phenolic compounds possess other biological activities such as antiinflammatory, antiulcer, antispasmodic, antisecretory, antiviral, antidiarrhoeal, antitumor, etc. [7].

Herbal and especially herbal extracts are very attractive not only in modern phytotherapy but also for food industry as spices and additives [8]. Winter savory (*Satureja montana* L., Lamiaceae) is native to the Mediterranean region, but is now found all over Europe, Russia and Turkey. In the Mediterranean region, it has been used as a culinary herb since antiquity. The leaves, flowers, and stems are used for herbal tea and, in traditional medicine homeopathic, to treat various ailments (with bactericidal, carminative, digestive, expectorant, fungicidal, laxative, antidiuretic, etc. activities) [9]. This plant contains various biologically active constituents such as essential oil [10,11], triterpenes [12] and phenolic compounds [13,14]. Essential oil as well as ethanolic extract of winter savory possesses antioxidant [15] and antimicrobial properties (antibacterial and antifungal) [16,17].

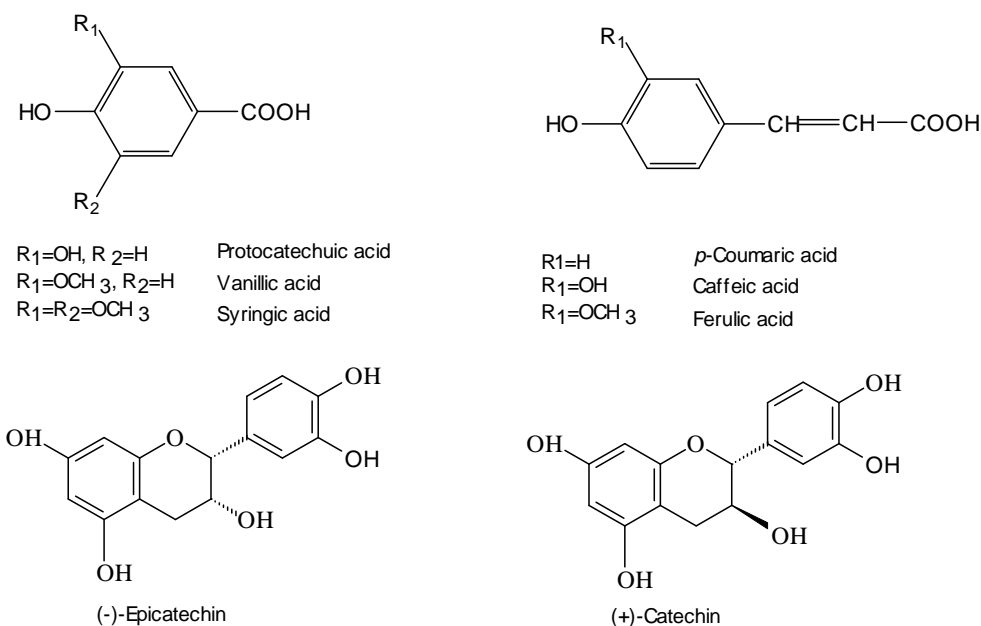
The aim of the present study was to examine the antioxidative activities of the different winter savory (*Satureja montana* L. subsp. *kitaibelii*) extracts by electron spin resonance (ESR) spectroscopy using three different reaction systems: the Fenton model system, the lipid system consisting of sunflower oil and the 4,4'-azobis(4-cyanovaleric acid) (ACVA; system I), and the liposomal model system with 2,2'-azobis(2-amidino-propane)dihydrochloride (AAPH; system II). Also, the relationship between phenolic contents and antioxidant activities of winter savory extracts was evaluated.

## 2. Results and Discussion

Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating free radicals or preventing decomposition of hydroperoxides into free radicals. In our previous work, the total phenolic content in winter savory was determined by the Folin Ciocalteu method (expressed as a chlorogenic acid equivalent per g dry weight of plant), while phenolic compounds were identified and quantified by the HPLC method [14]. The results obtained by the Folin

Ciocalteu method showed that the quantity of phenolic compounds in ethyl acetate (969.43  $\mu\text{g/g}$ ) and *n*-butanol (1358.14  $\mu\text{g/g}$ ) extracts were significantly higher ( $p < 0.001$ ) than in other investigated extracts. HPLC analysis proved that the ethyl acetate and *n*-butanol extracts contained hydroxybenzoic acid derivatives (protocatechuic, syringic and vanillic acids), hydroxycinnamic acid derivatives (caffeic, *p*-coumaric and ferulic acids), and flavanols (( $\pm$ )-catechin and (-)-epicatechin) (Figure 1).

**Figure 1.** Chemical structures of phenolic acids and flavanols in extracts



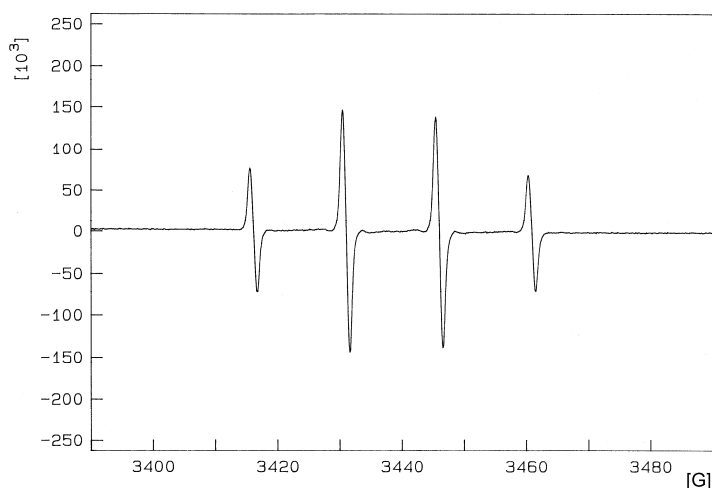
According to HPLC analysis, total content of phenolic acids in ethyl acetate and *n*-butanol extracts was 47.59 and 96.70  $\mu\text{g/g}$ , respectively, while flavanols were present in ethyl acetate and *n*-butanol extracts in the extent of 638.76 and 438.90  $\mu\text{g/g}$ , respectively. The chromatograms of petroleum ether, chloroform and water extracts showed that these extracts were poor in phenolic compounds. This fact is in accordance with the determined low value of total phenolic content in these three extracts (0  $\mu\text{g/g}$  for petroleum extract, 8.36  $\mu\text{g/g}$  for chloroform extract and 96.36  $\mu\text{g/g}$  for water extract).

Since the main mechanism of antioxidant action in foods is radical scavenging, many methods have been developed in which the antioxidant activity is evaluated by the scavenging of synthetic radicals in polar organic solvents at room temperature. The antioxidant activity of extracts was tested by measuring their ability to scavenge the reactive hydroxyl radical during the Fenton reaction, using ESR spectroscopy. Also, the influence of these extracts on lipid peroxy radicals obtained during lipid peroxidation of sunflower oil induced by ACVA and in the liposomal model system was studied.

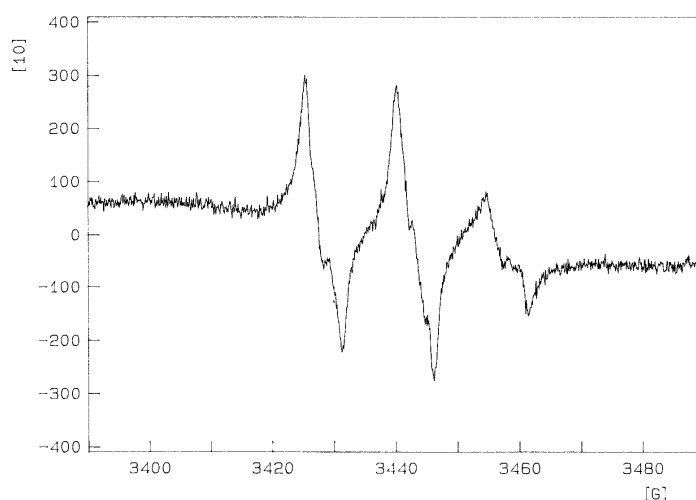
Hydroxyl radicals generated by the Fenton reaction ( $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ ) in the presence of the spin trapping agent 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) generated a 1:2:2:1 quartet of lines with hyperfine coupling parameters ( $a_{\text{N}}=a_{\text{H}}=14.9$  G) (Figure 2a). The intensity of the ESR signal, corresponding to the concentration of formed the DMPO-OH spin adducts, was changed in a dose-

depend manner in the presence of different amounts of *Satureja montana* L. subsp. *kitaibelii* extracts. This activity could be due to a direct scavenging effect and/or to the inhibition of hydroxyl generation. The second mechanism occurs by ferrous ion chelation. The antioxidant activity ( $AA_{OH}$ ) of the different concentrations of investigated extracts on the hydroxyl radical is shown in Figure 3. The *n*-butanol extract showed a very high  $AA_{OH}$ , and in the investigated range of concentration (0.1-0.5 mg/mL) it changed from 42.67 to 100%. The abilities of all samples to scavenge hydroxyl radicals decreased in the order: *n*-butanol>ethyl acetate> water>chloroform. The petroleum ether extract did not exert any  $AA_{OH}$ .

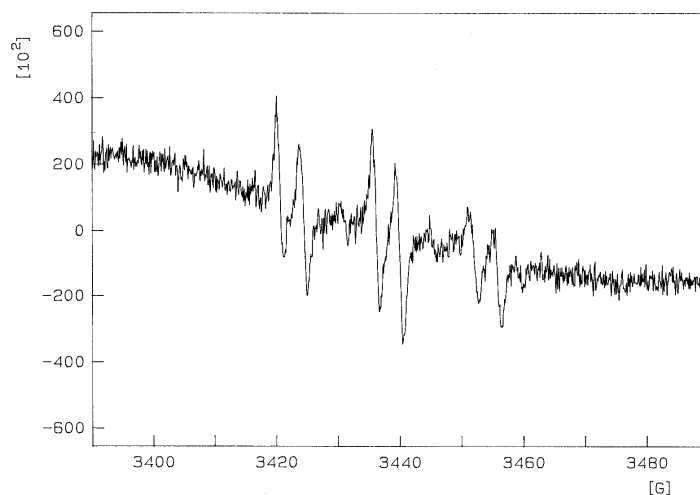
**Figure 2.** ESR spectra of a) DMPO-OH spin adduct obtained by Fenton reaction, b) PBN-OOL spin adduct formed during ACVA-induced peroxidation of sunflower oil and c) PBN-OOL spin adduct formed during AAPH-induced peroxidation of liposomes.



(a)

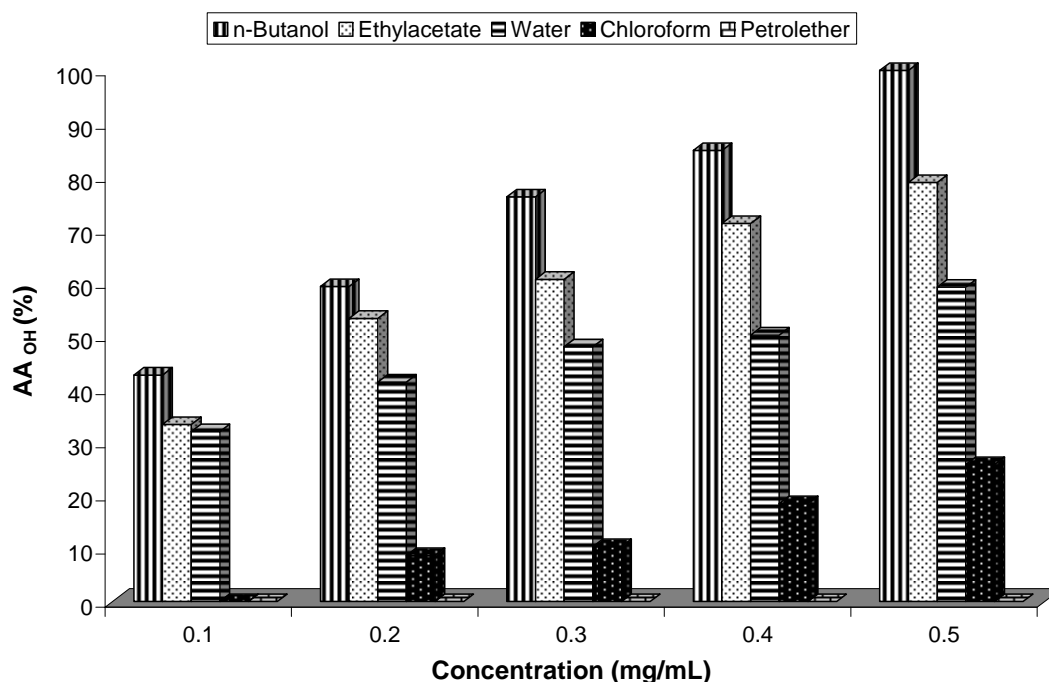


(b)



(c)

**Figure 3.** The effect of different concentrations of *n*-butanol, ethyl acetate, water, chloroform and petroleum ether extracts of *Satureja montana* L. subsp. *kitaibelii* on hydroxyl radical.

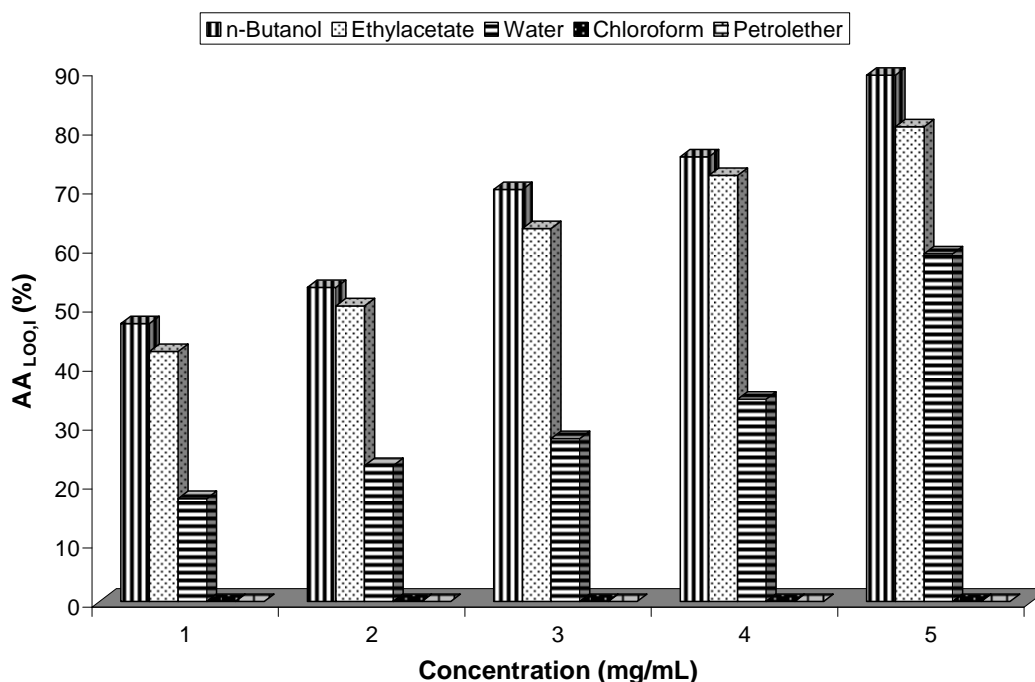


The azo-initiators, such as ACVA and AAPH, used in our study, generate the alkyl or/and peroxy radical by their thermal cleavage [18]. These radicals induce lipid peroxidation and the formation of lipid peroxy radicals. The ability of different extracts to scavenge lipid peroxy radicals was investigated by the ESR – spin trapping method using  $\alpha$ -phenyl-*N*-*tert*-butylnitron (PBN) as the spin trap.

Typical ESR spectra of the PBN-peroxy radical (PBN-OOL) spin adduct were detected during the peroxidation of sunflower oil induced by ACVA (Figure 2b) with hyperfine coupling parameters,  $a_N=14.75$  G and  $a_H=2.8$  G. The antioxidant activity ( $AA_{LOO,1}$ ) of different concentrations of the extracts

of *Satureja montana* L. subsp. *kitaibelii* on the peroxy radical obtained during ACVA-induced peroxidation of sunflower oil is presented in Figure 4.

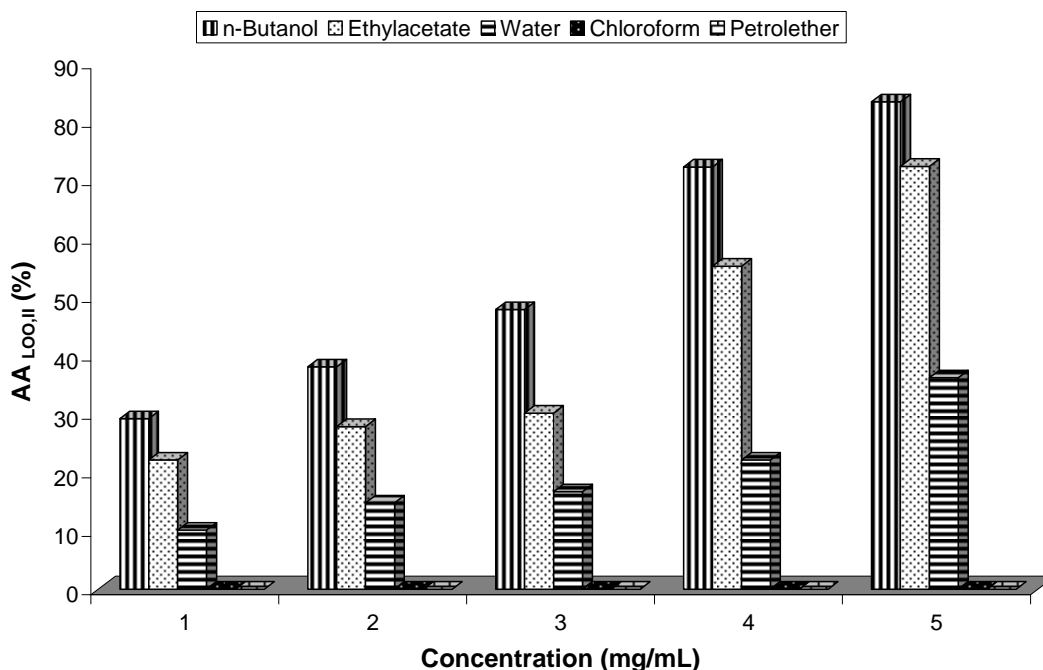
**Figure 4.** Antioxidant activity of different concentrations of *n*-butanol, ethyl acetate, water, chloroform and petroleum ether extracts of *Satureja montana* L. subsp. *kitaibelii* on PBN-OOL spin adduct during ACVA-induced peroxidation of sunflower oil (Data are represented as inhibition percentage of PBN-OOL spin adduct peak height measured by ESR).



The antioxidant activity of ethyl acetate, *n*-butanol and water extracts increased dose-dependently at mass concentrations ranging from 1 to 5 mg/mL. The highest antioxidant activity on the peroxy radical ( $AA_{LOO,I} = 89.21\%$ ) was observed for the *n*-butanol extract of *Satureja montana* L. subsp. *kitaibelii* at 5 mg/mL (Figure 4), while the same concentration of ethyl acetate extract exhibited somewhat lower activity ( $AA_{LOO,I} = 80.47\%$ ). Butylated hydroxyanisole (BHA) eliminated 57.69% of the peroxy radicals at 5 mg/mL (data not shown). Petroleum ether and chloroform extracts did not show any antioxidant activity.

The high lipid content of cell membranes make them one of the main targets of the reactive oxygen species that can be generated *in vivo* by both enzymatically and non enzymatically. The incubation of liposomes with hydrophilic AAPH induced lipid peroxidation, as measured by the accumulation of the very reactive lipid peroxy radical and resulting PBN-OOL spin adduct exhibits a characteristic signal (Figure 2c). The influence of different concentrations of the extracts of *Satureja montana* L. subsp. *kitaibelii* on peroxy radical formation during the AAPH-induced lipid peroxidation of liposomes is presented in Figure 5.

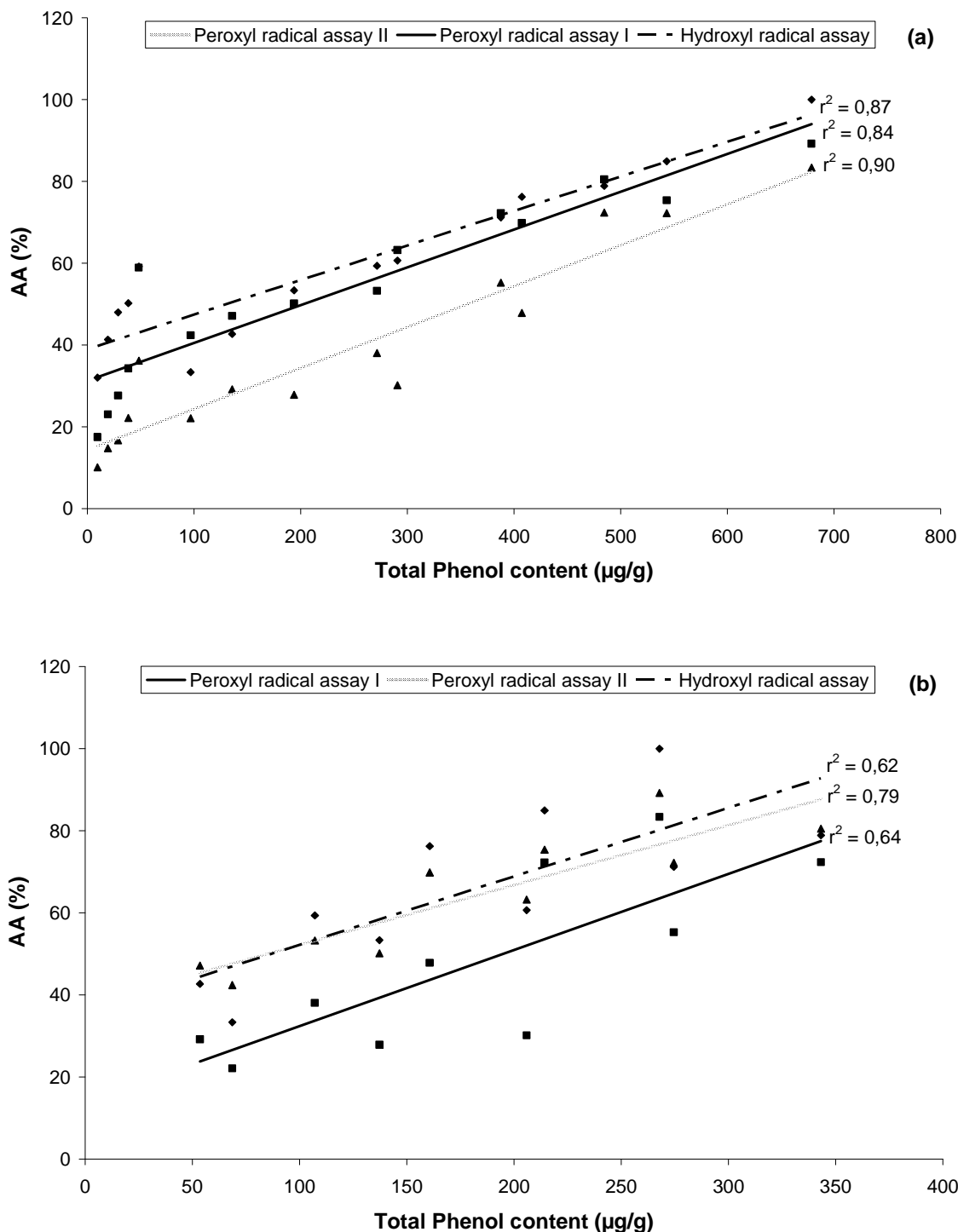
**Figure 5.** Antioxidant activity of different concentrations of *n*-butanol, ethyl acetate, water, chloroform and petroleum ether extracts of *Satureja montana* L. subsp. *kitaibelii* on PBN-OOL spin adduct during AAPH-induced peroxidation of liposomes (Data are represented as inhibition percentage of PBN-OOL spin adduct peak height measured by ESR).



It is evident that the antioxidant activity ( $AA_{LOO,II}$ ) on peroxy radicals during the AAPH-induced lipid peroxidation of liposomes depends on the type and concentration of the investigated extracts. Only at higher concentrations of applied ethyl acetate, *n*-butanol and water extracts, the scavenging effects on peroxy radicals in the investigated system were similar to the results of the scavenging effects on hydroxyl radicals. Generally, the antioxidant activity of ethyl acetate, *n*-butanol and water extracts increased dose-dependently at mass concentrations ranging from 1 to 5 mg/mL. The highest antioxidant activity on the peroxy radical ( $AA_{LOO,II} = 83.38\%$ ) was observed for the *n*-butanol extract of *Satureja montana* L. subsp. *kitaibelii* at 5 mg/mL (Figure 5), while the same concentration of ethyl acetate extract exhibited somewhat lower activity ( $AA_{LOO,II} = 72.35\%$ ). BHA eliminated 57.69% of peroxy radicals at 5 mg/mL (data not shown). Petroleum ether and chloroform extracts did not show any antioxidant activity.

The relationship between the total content of phenolic compounds obtained by the Folin-Ciocalteu method and the HPLC method were correlated with antioxidant activities (Figure 6).

**Figure 6.** Relationship between radical scavenging activities and total phenolic content obtained by Folin-Ciocalteu (a) and HPLC method (b).



The phenolic content, obtained by the Folin-Ciocalteu method and the HPLC method, and antioxidant activities are very closely correlated for the all cases. The correlation coefficient between total phenolic content, determined by the Folin Ciocalteu method, and AA was very good (in the case of hydroxyl radicals  $r^2 = 0.87$ ; in the case of peroxy radicals in system I and system II,  $r^2 = 0.84$  and  $r^2 = 0.9$ , respectively). Lower ones were obtained between the total phenolic content obtained by HPLC



and AA (in the case of hydroxyl radicals  $r^2 = 0.62$ ; in the case of peroxy radicals in system I and system II,  $r^2 = 0.79$  and  $r^2 = 0.64$ , respectively).

The relationship between structure and activity can be proposed from this series of the phenolics. Generally, phenolic compounds with *o*-dihydroxyl group in aromatic ring possess stronger antioxidant activity than monophenolics [19]. For example, caffeic acid reacted better with oxygen centered radicals than *p*-coumaric acid. Also, methoxy substitution of the hydroxyl group in the *ortho* position of the diphenolics, as in ferulic or vanillic acid, resulted in to a decrease in the free radical scavenging reaction [20]. According to literature data, (+)-catechin and (-)-epicatechin possess marked free radical scavenging activity [21]. In both compounds the presence of *o*-dihydroxyl group in B-ring is fundamental for their scavenging properties, but *m*-hydroxyl groups at C<sub>5</sub> and C<sub>7</sub> in ring A also contribute to scavenging activity.

The values of antimicrobial activity of petroleum ether, chloroform, ethyl acetate, *n*-butanol and water extracts of *Satureja montana* L. subsp. *kitaibelii* and standards are shown in Tables 1 and 2.

**Table 1.** Antimicrobial activity of different *Satureja montana* L. subsp. *kitaibelii* extracts and referent standards.

Microorganism <sup>a</sup>	<i>Satureja montana</i> L. subsp. <i>kitaibelii</i> extracts (100 mg/mL) <sup>b</sup>				Standards			
	Petroleum ether	Chloroform	Ethyl acetate	<i>n</i> -Butanol	P <sup>c</sup>	Ac <sup>d</sup>	Kt <sup>e</sup>	Ns <sup>f</sup>
<i>P. aeruginosa</i>	-	16.0±0.0	16.7±0.52	-	23.0±0.79	22.7±1.51	-	-
<i>E. coli</i>	-	-	+/-	+/-	10.0±0.0	21.3±1.00	-	-
<i>S. aureus</i>	16.3±0.60	-	15.3±0.58	10.0±0.0	30.3±2.25	27.3±1.15	-	-
<i>S. lutea</i>	13.0±0.0	13.3±0.39	10.0±0.0	-	37.7±0.49	35.0±1.00	-	-
<i>B. cereus</i>	11.3±0.0	14.7±0.58	11.0±0.0	10.0±0.0	33.7±1.67	29.7±1.91	-	-
<i>S. cerevisiae</i>	8.67±0.39	-	9.33±0.58	-	-	-	15.0±0.0	30.3±1.5
<i>C.pseudotropicalis</i>	8.0±0.0	-	8.0±0.0	-	-	-	19.0±0.0	21.0±0.0

Values (mean of three replicates) are: diameter of zone of inhibition (mm) ±SD (standard deviation); -, no inhibition; +/-, the zone of inhibition < 6mm.

<sup>a</sup> Cell suspensions contain  $1 \times 10^7$  cfu/mL.

<sup>b</sup> 10 µl/disc.

<sup>c</sup> P; Penicillin (10 IU/disc).

<sup>d</sup> Ac; Amoxicillin (25 µg/disc).

<sup>e</sup> Kt; Ketoconazole (10 µg/disc).

<sup>f</sup> Ns; Nystatin (100 IU/disc).

**Table 2.** Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of different *Satureja montana* L. subsp. *kitaibelii* extracts

Microorganisms	Satureja montana L. subsp. Kitaibelii L. extracts (mg/mL)							
	Petroleum ether		Chloroform		Ethyl acetate		<i>n</i> -Butanol	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>P. aeruginosa</i>	>100	>100	10	25	10	25	>100	>100
<i>E. coli</i>	>100	>100	>100	>100	>100	>100	>100	>100
<i>S. aureus</i>	10	25	>100	>100	10	50	50	100
<i>S. lutea</i>	25	50	25	50	50	75	>100	>100
<i>B. cereus</i>	25	75	10	50	50	75	50	100
<i>S. cerevisiae</i>	100	>100	>100	>100	100	>100	>100	>100
<i>C. pseudotropicalis</i>	100	>100	>100	>100	100	>100	>100	>100

MIC of standards. Amoxicillin: *Pseudomonas aeruginosa* 1.0 µg/mL; *Escherichia coli* 0.12 µg/mL; *Staphylococcus aureus* 0.5 µg/mL; *Sarcina lutea* 0.12 µg/mL; *Bacillus cereus* 0.5 µg/mL. Nystatin: *Saccharomyces cerevisiae* 20 µg/mL; *Candida pseudotropicalis* 16 µg/mL.

The maximum antibacterial activity (MIC=10 mg/mL) was shown by the ethyl acetate extract against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and by the chloroform extract against *Pseudomonas aeruginosa* and *Bacillus cereus*. The petroleum ether extract remarkably inhibited the growth of *Staphylococcus aureus*; *Sarcina lutea* and *Bacillus cereus*, while *n*-butanol were weakly active against *Staphylococcus aureus* and *Bacillus cereus*. On the other hand, a very low antimicrobial activity (MIC ≥100 mg/mL) of all extracts was found against the tested yeasts.

Phenolic compounds are known to be synthesized by plants in response to microbial infection. It is therefore logical that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms [22]. The structure-activity relationships of the antimicrobial activity of phenolic compounds are contradictory. For example, Chabot *et al.* [23] reported that less polar compounds, i.e. flavonoids lacking hydroxyl groups on their B ring, are more active against microorganisms than those with OH groups. On the other hand, Mori *et al.* [24] found that a free 3',4',5'-trihydroxy B-ring and a free 3-OH were necessary for antibacterial activity. Based on this, it can be concluded that the antimicrobial activity of investigated extracts depend not only on phenolic compounds. The presence of different secondary metabolites also contributed to the antimicrobial activity. An additional research on chemical composition of each extract is required for comprehensive assessment of individual compounds exhibiting antimicrobial activity.

### 3. Conclusion

The results of ESR analysis indicate that ethyl acetate, *n*-butanol and water extracts have significant antioxidant activities against hydroxyl and peroxy (obtained in two different systems) radicals, which is in correlation with the contents of total phenolic compounds. The following order of antioxidant activities (AA<sub>OH</sub>, AA<sub>LOO,I</sub> and AA<sub>LOO,II</sub>) has been established: *n*-butanol > ethyl acetate > water. *Satureja montana* L. subsp. *kitaibelii* extracts were also observed to possess antibacterial activity depending on the solvent used and microorganisms tested. All of these results show that the *Satureja*

*montana* L. subsp. *kitaibelii* extracts can be used accessible sources of natural antioxidants, as a possible food supplements and as a phytochemicals.

#### 4. Experimental Section

##### 4.1. Plant material and chemicals

Plant material was purchased from a local herbal drugstore. Aerial parts of *Satureja montana* L. subsp. *kitaibelii* (winter savory) were collected in the period May-June, 2005, in the region of mountain Zlatibor, Serbia, as labelled. The voucher specimen of the collected plant material was confirmed by Biljana Božin and deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Medicine, University of Novi Sad. All chemicals and reagents were of analytical reagent grade and were purchased from Sigma Chemical Co. (St Louis, MO, USA), Aldrich Chemical Co. (Steineheim, Germany) and "Zorka" (Šabac, Serbia). Sunflower oil was from A.D. "Vital", Oil and Vegetable fat factory (Vrbas, Serbia). Commercial preparation of liposomes (PRO-LIPO S, with 30% soybean phosphatidylcholine) was obtained from Lucas-Meyer, Hamburg, Germany.

##### 4.2. Preparation of Extracts

The dried aerial parts (moisture content 3.72%) of winter savory were ground in a grinder (DLFU Bühler Miag Laboratory Disc Mill, Germany) with a 2 mm in diameter mesh. This material (100 g) was macerated with 70 % methanol in water (2 x 2500 mL) at room temperature for 2 x 24 h. The obtained extract was concentrated under reduced pressure to remove methanol, and then extracted sequentially with petroleum ether (2 x 200 mL), chloroform (2 x 200 mL), ethyl acetate (2 x 200 mL) and *n*-butanol (2 x 200 mL). The solvent extractions were performed in separation funnels, shaking for 10 min. The petroleum ether, chloroform, ethyl acetate, *n*-butanol and remaining water extract were evaporated to dryness under reduced pressure at 40°C with a water bath.

The yields, average of triplicate analysis, of the extracts were: petroleum ether,  $m = 0.58 \pm 0.029$  g; chloroform,  $m = 0.49 \pm 0.024$  g; ethyl acetate,  $m = 0.55 \pm 0.028$  g; *n*-butanol,  $m = 2.13 \pm 0.106$  g and water,  $m = 6.03 \pm 0.30$  g.

##### 4.3. Hydroxyl radical assay

Hydroxyl radicals were obtained during a Fenton reaction by mixing 200  $\mu$ L 0.3 M 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 200  $\mu$ L 10 mM H<sub>2</sub>O<sub>2</sub>, 200  $\mu$ L 10 mM Fe<sup>2+</sup> and 10  $\mu$ L of methanol (blank). The influence of different types of extract on the formation and transformation of hydroxyl radicals was investigated by adding the petroleum ether, chloroform, ethyl acetate, *n*-butanol and water extracts in the Fenton reaction system at the range of concentrations 0.1-0.5 mg/mL (probe). The ESR spectra were recorded on a Bruker 300E, after 5 min, with the following spectrometer settings: field modulation 100 kHz, modulation amplitude 0.512 G, receiver gain  $2 \cdot 10^5$ , time constant 81.92 ms, conversion time 163.84 ms, centre field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23°C. The antioxidant activity (AA<sub>OH</sub>) of the extracts was defined as:  $AA_{OH} = 100 \cdot (h_o - h_x) / h_o$  [%] where  $h_o$  and  $h_x$  are the height of the second peak in the ESR spectrum of the DMPO-OH spin adduct of the blank and probe, respectively.

#### 4.4. Oxidation of Sunflower Oil Induced by Azo-initiator ACVA

Oxidation was investigated in a solution containing 1 mL of sunflower oil, 0.0018g ACVA, 0.0274 g of  $\alpha$ -phenyl-N-*tert*-butylnitron (PBN) and 380  $\mu$ L of various concentrations of methanolic solutions of the investigated extracts (final concentrations were in range 1-5 mg/mL). The solutions were incubated at 37°C for 3 h under aerobic conditions.

#### 4.5. Oxidation of the PC Liposomes Induced by Azo-initiator AAPH

Liposome suspension was prepared by adding 1 g of commercial liposomes to 10 mL water and incubated with stirring at 37°C for 1h. Oxidation was investigated in the solution containing 1 mL of liposome suspension, 0.0019 g AAPH, 0.0274 g of PBN and 380  $\mu$ L of various concentrations of methanolic solutions of the investigated extracts (final concentrations were in the range 1-5 mg/mL). The solutions were incubated at 37°C for 3 h under aerobic conditions.

Lipid oxidation in both systems was measured by following the formation of peroxy radicals with ESR spin trapping method. The ESR spectra of the formed PBN-peroxy radical spin adducts were recorded with the following spectrometer settings: field modulation 100 kHz, modulation amplitude 1.021 G, receiver gain  $5 \cdot 10^5$ , time constant 20.48 ms, conversion time 327.68 ms, centre field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23°C.

The antioxidant activity value ( $AA_{LOO}$ ) of the extract was defined as:  $AA_{LOO} = 100 \cdot (h_o - h_x) / h_o$  [%], where  $h_o$  and  $h_x$  are the height of the second peak in the ESR spectrum of the PBN-OOL spin adduct obtained in the absence and in presence of the investigated extracts, respectively.

Magnetic field scanning determinations were calibrated using Fremy's salt. Splitting constants were calculated from computer-generated second derivatives of the spectra, after optimizing signal-to-noise ratios, and were verified by computer simulations.

#### 4.6. Antimicrobial activity test

For these investigations the disc diffusion method and microbroth dilution methods were applied. From the primary isolation medium 2-3 colonies of investigated bacteria were taken by flamed loop, suspended in Mueller-Hinton or Sabouraud broth, and they were incubated at 37°C. The suspension for inoculation was prepared from the broth cultures. The number of cells in 1 mL of suspension for inoculation measured by the McFarland nephelometer was  $1 \times 10^7$  cfu/mL. A volume of 1 mL of this suspension was homogenized with 9 mL of melted (45°C) Mueller-Hinton or Sabouraud dextrose agar and poured into Petri dishes.

For screening, sterile 6 mm discs (HiMedia, Mumbai, India) were impregnated with 10  $\mu$ L of 100 mg/mL of *Satureja montana* L. subsp. *kitaibelii* extracts. After incubation for 24-48 hours in the thermostat, inhibition zone diameters, ZI, (including disc) were measured and expressed in mm. The presence of the inhibition zone indicates the activity of tested extracts against bacteria or yeasts. The minimum inhibitory concentration (MIC) was reported as the lowest concentration of the extracts capable of inhibiting the growth of the bacterium tested. The MIC was determined by the broth macrodilution test [25] using an inoculum of  $1 \times 10^7$  cfu/mL. Final concentrations of the investigated extracts were 6.25, 12.5, 25, 50, 75 and 100 mg/mL. Both tests were done in three replications.

*Pseudomonas aeruginosa* (ATCC 27853, gram negative), *Escherichia coli* (ATCC 25922, gram negative), *Staphylococcus aureus* (ATCC 25923, gram positive), *Sarcina lutea* (ATCC 9341, gram positive), *Bacillus cereus* (ATCC 10707, gram positive), *Saccharomyces cerevisiae* (112, Hefebank Weihenstephan) and *Candida pseudotropicalis* (clinical isolate) microorganism strains were employed for the determination of antimicrobial activity. Penicillin (10 IU/disc) and amoxicillin (25 µg/disc) were used as reference standards as obtained from Bioanalyse Co., Ltd., Ankara, Turkey.

In parallel with the antibacterial investigation of *Satureja montana* L. subsp. *kitaibelii* extracts, pure solvent was tested too, and it did not exhibit any antibacterial activity (data are not shown). Bacteria were obtained from the stock cultures of the Microbiology Laboratory, Faculty of Technology, University of Novi Sad.

#### 4.7. Statistical analysis

All measurements were carried out in triplicate, and presented as mean ± SME or ± SD. Regression analyses and significance of differences were carried out using a SPSS Statistical Software package (SPSS for Windows, 8.0, 1997, SPSS Inc., Chicago, IL, USA).

#### Acknowledgements

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