Antioxidative and antimicrobial properties of different extracts from *Sideritis montana* L

Received for publication, May 12 2011
Accepted, April 20, 2012

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Abstract

The antimicrobial, antioxidant activity, total phenol and flavonoid content of methanolic, acetone and ethyl-acetate extracts from *Sideritis montana* from the region of Stara Planina Mt. in eastern Serbia, were evaluated. Total phenol contents were determined by Folin-Ciocalteu’s reagent and their amounts ranged from 49.05 to 97.85 mg GA g⁻¹. The amount of flavonoids in plant extracts were in range from 106.62 to 206.43 mg Ru g⁻¹. Antioxidant activity was determined in vitro using the DPPH reagent and was expressed as IC₅₀ values that ranged from 527.96 to 31.37 µg ml⁻¹. The methanolic extract contained the highest amount of phenols (97.85 mg GA g⁻¹) and showed strong antioxidant activity (IC₅₀ = 31.37 µg ml⁻¹). In vitro antimicrobial activity is investigated by microdilution method. Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) have been determined. Testing was conducted against 23 microorganisms, including fifteen strains of bacteria (standard and clinical strains), five species of fungi and three yeast species. All statistical analyses were performed using SPSS package. Statistical analysis showed that there are no significant differences in the activity of different extracts. The tested extracts showed significant antibacterial activity against *G*+ bacteria and weak to moderate activity against other microorganisms.

Keywords: antioxidant activity; total phenol content; total flavonoid content; antimicrobial activity;

Introduction

Plants are a large source of new bioactive molecules. Many research studies have demonstrated that medicinal plants, fruits and vegetables contain various components with antioxidant activity, which are responsible for their beneficial health effects. In addition to vitamin C, vitamin E and carotenoids, polyphenols (a wide class of components including phenolic acids, catechins, flavonols and anthocyanins) have also shown strong antioxidant capacity [1]. Due to their natural origin, the antioxidants obtained from plants are of greater benefit in comparison to synthetic ones. The use of natural antioxidants from plants usually does not induce side effects, while synthetic antioxidants were found to have negative side effects. Most frequently used synthetic antioxidants in food industry at high doses, such as BHA, exhibit genotoxic and carcinogenic effects, while BHT was proven to cause hemorrhaging [2, 3, 4]. Negative effects of free radicals can be largely prevented by intake of antioxidant substances. Antioxidants act by donating an electron to a free radical and converting it to a nonradical form [5, 6].

Regardless the numerous previous studies most species of higher plants have never been described, much less surveyed for chemically or biologically active constituents and new sources of commercially valuable materials remain to be discovered [7].
Antioxidative and antimicrobial properties of different extracts from *Sideritis montana* L.

*S. montana* (Lamiaceae) is an annual species with low branched trunk, up to 40 cm high. Inhabits sand arid meadows in the Europe and the Mediterranean [8]. *S. montana* is very rich in phenolic compounds with very strong biological activity. It is known in traditional medicine as Ironwort and was used for the antispasmodic, carminative [9], antimicrobial [10, 11] effects. Medicinal active substances in *S. montana* are flavonoid and phenylpropanoic glycosides and different phenolic acids [12].

The present study is prompted by the fact that no data on antimicrobial, antioxidant activity, phenol and flavonoid content of *S. montana* from Serbia, have been provided so far. The basic aim of this research was to determine the total phenol and flavonoid contents of various extracts made from *S. montana* species, that grows in Stara Planina Mt. region, eastern Serbia, using spectrophotometric methods, as well as to examine the antimicrobial and antioxidant activity of the same extracts *in vitro*.

**Materials and methods**

**Preparation of plant extracts**

**Plant material**

*S. montana* was collected in July 2008, from the region of Stara Planina Mt. in eastern Serbia. The voucher specimen was confirmed and deposited in Herbarium at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant material was air-dried in darkness at room temperature (20 °C). Dried plant parts were cut up and stored in tight-seal dark containers until needed.

**Chemicals**

Acetone, methanol, ethyl acetate and sodium hydrogen carbonate were purchased from „Zorka pharma“ Šabac, Serbia. Gallic acid, rutin hydrate, chlorogenic acid and 2,2-Dyphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. Folin-Ciocalteu phenol reagent and aluminium chloride hexahydrate were purchased from Fluka Chemie AG, Buchs, Switzerland. All other solvents and chemicals were of analytical grade.

**Extraction**

Prepared plant material (10 g) was transferred to dark-coloured flasks and mixed with 200 ml of methanol and stored at room temperature. After 24 h, infusions were filtered through Whatman No. 1 filter paper and residue was re-extracted with equal volume of solvents. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40 °C using Rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4 °C.

**Determination of total phenol content**

Total soluble phenolic compound in the different extracts of *S. montana* were determined with Folin-Ciocalteu reagent [13] using gallic acid as a standard. Methanol extract was diluted to the concentration of 1 mg/ml and 0.5 ml of the soluted extract was mixed with 2.5 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of NaHCO₃ (7.5%). After 15 min of staying at the 45°C, the absorbance was measured at 765 nm versus blank sample on spectrophotometerc (ISKRA, MA9523-SPEKOL 211). Content of phenolics in extracts was expressed in terms of gallic acid equivalent (mgGA g⁻¹ extract). Values were uniformly expressed as the corresponding dry weight of plant extract (1 g). All measures were repeated three times.
Determination of total flavonoid content
The total flavonoid contents were determined spectrophotometrically [14]. Briefly, 0.5 ml of 2% solution of AlCl₃ in methanol was mixed with the same volume of extract (1 mg ml⁻¹). Absorption readings at 415 nm were taken after 1 h against a blank (methanol). The total flavonoid content was determined using a standard curve with rutin (0-50 mg l⁻¹). Values were uniformly expressed as the corresponding dry weight of plant extract (1 g). All measures were repeated three times.

Evaluation of antioxidant activity
The ability of the plant extract to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was assessed by the standard method adopted with suitable modifications [15]. DPPH (20 mg) was dissolved in methanol (250 ml) to obtain a concentration of 80 mg mL⁻¹. The stock solution of plant extract was prepared in methanol to achieve the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99, 0.97 µg ml⁻¹. Diluted solutions (1 ml each) were mixed with DPPH (1 ml). After 30 min in darkness at room temperature (23 ºC), the absorbance was recorded at 517 nm. Samples contained all the reagents except the extract. Percentage inhibition was calculated using Eq. (1), whilst IC₅₀ values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values ± standard deviation (n = 3).

\[
\% \text{ inhibition} = \left( \frac{A \text{ of control} - A \text{ of sample}}{A \text{ of control}} \right) \times 100
\]

Eq. (1)

In vitro antimicrobial assay
Test substances
The tested compounds were dissolved in DMSO and then diluted into nutrient liquid medium to achieve a concentration of 10% DMSO. An antibiotic, doxycycline (Galenika A.D., Belgrade) was dissolved in nutrient liquid medium, a Mueller–Hinton broth (Torlak, Beograd), while an antifungal, fluconazole (Pfizer Inc., USA) was dissolved in Sabouraud dextrose broth (Torlak, Belgrade).

Test microorganisms
Antimicrobial activity of acetone, ethyl acetate and methanol extract was tested against 23 microorganisms including fifteen strains of bacteria (standard strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Bacillus pumilus* NCTC 8241 and clinical strains: *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Sarcina lutea*, *Salmonella enterica*, *Bacillus subtilis* and *Bacillus cereus*); five species of fungi: *Penicillium italicum* PMFKG-F29, *Penicillium digitatum* PMFKG-F30, *Penicillium chrysogenum* PMFKG-F31, *Trichothecium roseum* PMFKG-F32, *Botrytis cinerea* PMFKG-F33, and three yeast species *Candida albicans* (clinical isolate); *Rhodotorula sp.* PMFKG-F27 and *Saccharomyces boulardii* PMFKG-P34. All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Suspension preparation
Bacterial and yeast suspensions were prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland’s standard [16].
Initial bacterial suspension contains about $10^8$ colony forming units (CFU) mL$^{-1}$ and suspension of yeast contains $10^6$ CFU mL$^{-1}$. 1:100 dilutions of initial suspension were additionally prepared into sterile 0.85% saline. The suspensions of fungal spores were prepared by gentle stripping of spore from slopes with growing aspergilli. The resulting suspensions were 1:1000 diluted in sterile 0.85% saline.

**Microdilution method**

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) using microdilution method with resazurin [17]. The 96-well plates were prepared by dispensing 100 μL of nutrient broth, Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for fungi and yeasts, into each well. A 100 μL from the stock solution of tested compound (concentration of 80 mg mL$^{-1}$) was added into the first row of the plate. Then, twofold, serial dilutions were performed by using a multichannel pipette. The obtained concentration range was from 40 to 0.156 mg mL$^{-1}$. The method is described in detail in previous paper [18].

Doxycycline and fluconazole were used as a positive control. Solvent control test was performed to study an effect of 10% DMSO on the growth of microorganism. It was observed that 10% DMSO did not inhibit the growth of microorganism. Also, in the experiment, the concentration of DMSO was additionally decreased because of the twofold serial dilution assay (the working concentration was 5% and lower). Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

**Statistical analysis**

Presented data averaged means ± standard deviations where appropriate. All statistical analyses were performed using SPSS package. Mean differences were established by Student’s $t$-test. Data were analyzed using one-way analysis of variance (ANOVA). In all cases $P$ values <0.05 were considered statistically significant.

**Results and discussion**

**The total phenol content, total flavonoid content and antioxidant activity**

The total phenol content in the examined plant extracts determined using the Folin-Ciocalteu reagent was expressed in terms of gallic acid equivalent, GAE as mg of GA g$^{-1}$ of extract (Table 1). The concentrations of phenols in the examined extracts ranged between 49.05 and 97.85 mgGa g$^{-1}$.

Various solvents with diverse polarity were used to achieve extraction of active substances. Methanolic and acetone extract had higher total phenol content. Values obtained for total phenol content indicate that the polar solvents are more efficient for extraction of phenolic compounds from *S. montana*. Methanol has already been proven to be a good extraction solvent in earlier studies [19].

<table>
<thead>
<tr>
<th>type of extract</th>
<th>total phenolic content$^1$</th>
<th>flavonoid concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic</td>
<td>97.85±0.98</td>
<td>159.54±1.63</td>
</tr>
<tr>
<td>Acetone</td>
<td>84.55±1.29</td>
<td>206.43±1.78</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>49.05±0.81</td>
<td>106.62±1.49</td>
</tr>
</tbody>
</table>

$^1$Each value in the table was obtained by calculating the average of three analysis ± standard deviation.
The content of flavonoids in plant extracts from *S. montana* was determined using spectrophotometric method with AlCl₃ and was expressed as rutin equivalent (RuE) as mg of Ru g⁻¹ of extract (Table 1). The concentrations of flavonoids in plant extracts ranged from 106.62 to 206.43 mgRu g⁻¹, highest being one measured in acetone extract. Based on these results it was found that the highest concentration of these compounds was in the extracts obtained using moderate polar and polar solvents.

The results obtained for the concentration of flavonoids show that *S. montana* has a significant content of flavonoids.

Flavonoids are very useful natural compounds which are present in most plants species. The structural components common to these molecules include two benzene rings on either side of a 3-carbon ring. Multiple combinations of hydroxyl groups, sugars, oxygens, and methyl groups attached to these structures create the many different classes of flavonoids: flavanols, flavanones, flavones, flavan-3-ols (catechins), anthocyanins, and isoflavones [20]. Flavonoid glycosides and flavones are most abundant groups of flavonoids in *Sideritis* extracts. Detailed quantitative analysis of extracts confirmed the presence of apigenine, luteoline, kaempferol, very important flavonoids that exhibit strong antioxidant activity [21, 22, 23].

The antioxidant activity of different plant extracts of *S. montana* was determined using methanol solution of DPPH. DPPH method has also been used to quantify antioxidants in complex biological systems in recent years and based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. The scavenging activity was measured as the decrease in absorbance of the samples versus DPPH standard solution [24].

The antioxidant activity of *S. montana* extracts was expressed in terms of IC₅₀ (µg ml⁻¹) values (Table 2). Parallel to the examination of the antioxidant activity of the plant extracts, the values for chlorogenic acid as standard substance (Table 2) were obtained and compared to the values of the extract antioxidant activity.

Table 2. Antioxidant (DPPH scavenging activity) of investigated plant extracts and standard substance presented as IC₅₀ values (µg ml⁻¹).

<table>
<thead>
<tr>
<th>type of extract</th>
<th>IC₅₀ values (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic</td>
<td>31.37±2.56</td>
</tr>
<tr>
<td>Acetone</td>
<td>64.55±3.61</td>
</tr>
<tr>
<td>Ethyl cetate</td>
<td>527.96±4.31</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>11.65±0.52</td>
</tr>
</tbody>
</table>

¹Each value in the table were obtained by calculating the average of three analysis ± standard deviation.

The obtained values for antioxidant activity examined by DPPH radical scavenging activity ranged from 31.37 to 527.96 µg ml⁻¹. The largest capacity to neutralize DPPH radical was measured for methanolic extract with IC₅₀ value of 31.37 µg ml⁻¹.

Comparing the concentration of phenolic compounds and antioxidant activity we found that extracts with higher phenol content also have stronger antioxidant activity, which is in a good correlation with other studies of antioxidant activity of plant extracts which have confirmed a high linear correlation between the values of phenol content and antioxidant activity [25].

Antimicrobial activity

The results of in vitro testing antibacterial and antifungal activities of acetone, ethyl acetate and methanol extracts of *S. montana* are shown in Table 3. and Table 4. For comparison, MIC and MMC values for doxycycline and fluconazole are also listed in Table 3. and Table 4. The solvent (10% DMSO) did not inhibit the growth of the tested microorganisms.
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Antimicrobial activity of tested extracts was evaluated by determining MICs and MMCs in relation to the 23 species of microorganisms. MICs and MMCs values were in range from 0.625 mg ml\(^{-1}\) to 40 mg ml\(^{-1}\). The tested extracts showed different degree of antimicrobial activity in relation to the tested species. The intensity of antimicrobial action varied depending on the groups of microorganisms and on the type of the extracts.

In general, the tested extracts demonstrated low to moderate antimicrobial activity, while showing more potent inhibitory effects on the growth of G+ bacteria than to other tested microorganisms. Statistically significant difference in activity between the extracts of *S. montana* was not observed. All three extracts demonstrated approximately similar activity in relation to the tested standard and clinical strains of bacteria.

The tested extracts showed high antibacterial activity against G+ bacteria, especially for species of the genus *Bacillus* (clinical isolates and standard strains). MICs values were in range from 0.625 mg mL\(^{-1}\) to 1.25 mg mL\(^{-1}\), and MMCs values were from 0.625 mg mL\(^{-1}\) to 2.5 mg mL\(^{-1}\). Significant effect extracts showed in other G+ bacteria including food spoilage isolates *Sarcina lutea* and *Staphylococcus aureus* (see Table 3.). Based on this information, plant extracts from this plant could be used as natural sources of preservatives substances with high importance in food industry.

**Table 3.** Antibacterial activities of acetone, ethyl acetate and methanol extracts of *S. montana* against tested microorganisms based on microdilution method.

<table>
<thead>
<tr>
<th>Species</th>
<th>Acetone extract MIC</th>
<th>Ethyl acetate extract MIC</th>
<th>Methanol extract MIC</th>
<th>Doxycycline</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>20</td>
<td>40</td>
<td>40</td>
<td>7.81</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 2592</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>15.625</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1.25</td>
<td>2.5</td>
<td>1.25</td>
<td>0.448</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 29212</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>0.224</td>
</tr>
<tr>
<td><em>Enter. faecalis</em></td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>&lt;0.448</td>
</tr>
<tr>
<td><em>Enter. faecalis</em> ATCC 29212</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>7.81</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>10</td>
<td>20</td>
<td>2.5</td>
<td>250</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 27853</td>
<td>5</td>
<td>20</td>
<td>2.5</td>
<td>&gt;250</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>1.25</td>
<td>1.25</td>
<td>0.625</td>
<td>0.112</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC 6633</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.953</td>
</tr>
<tr>
<td><em>Bacillus pumilus</em> NCTC 8241</td>
<td>0.625</td>
<td>0.625</td>
<td>0.625</td>
<td>0.112</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>0.625</td>
<td>0.625</td>
<td>0.625</td>
<td>0.977</td>
</tr>
<tr>
<td><em>Sarcina lutea</em></td>
<td>1.25</td>
<td>1.25</td>
<td>2.5</td>
<td>&lt;0.448</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>15.625</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>&gt;250</td>
</tr>
</tbody>
</table>

*Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values are given as mg ml\(^{-1}\) for plant extract and μg ml\(^{-1}\) for antibiotics. Antibiotic: Doxycycline.*

The gram-positive bacteria were more sensitive than the gram-negative bacteria [11]. If we compare the effect of extracts of among G- and G+ bacteria there is statistically significant difference at the level of MIC and MMC in acetone and ethyl acetate extract (p <0.05). The tested extracts did not affect the growth of clinical isolates and standard strains of G- bacteria or their activities were very low (MIC and MMC ranged from 1.25 mg mL⁻¹ to 40 mg mL⁻¹). The exception is the methanol extract of the species *Proteus mirabilis*, where the MIC and MMC at 1.25 mg mL⁻¹.

**Table 4.** Antifungal activities of acetone, ethyl acetate and methanol extracts of *S. montana* against tested microorganisms based on microdilution method.

<table>
<thead>
<tr>
<th>Species</th>
<th>Acetone extract</th>
<th>Ethyl acetate extract</th>
<th>Methanol extract</th>
<th>Fluconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC*</td>
<td>MMC</td>
<td>MIC*</td>
<td>MMC</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Rhodotorula sp.</em></td>
<td>5</td>
<td>10</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td><em>Saccharomyces boulardii</em></td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Penicillium italicum</em></td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><em>Penicillium digitatum</em></td>
<td>10</td>
<td>20</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>2.5</td>
<td>20</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td><em>Trichothecium roseum</em></td>
<td>10</td>
<td>20</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><em>Botrytis cinerea</em></td>
<td>10</td>
<td>20</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>ATCC 16404</td>
<td>2.5</td>
<td>20</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values are given as mg ml⁻¹ for plant extract and μg ml⁻¹ for antibiotics. Antibiotics: Fluconazole.*

The tested extracts showed low to moderate antifungal activity. For fungi, statistically significant difference in the activity (at MMC) is noticed when comparing the concentration of the acetone and ethyl acetate extract (p <0.05). Methanol extract showed a significant effect on species *Aspergillus niger* ATCC 16404, where the MIC 0.625 mg mL⁻¹, and MMC was 1.25 mg mL⁻¹. All three extracts inhibited the growth of *Penicillium chrysogenum* at concentration of 2.5 mg mL⁻¹.

MICs and MMCs for yeasts ranging from 10 mg ml⁻¹ to 20 mg ml⁻¹ for *C. albicans* and *Saccharomyces boulardii*. Slightly better effect of extracts can be seen in *Rhodotorula sp*. where the value range from 2.5 mg ml⁻¹ to 5 mg ml⁻¹.

**Conclusions**

According to the obtained results, *S. montana* can be considered as a rich natural source of polyphenolic compounds with very good antioxidant activity. Investigation of antioxidant activity of extracts and comparison with the activity of reference substances revealed intense antioxidant activity of plant extracts, which was related to high concentration...
of phenolics in the examined extracts. The obtained results indicate that the use of polar solvents allows very effective extraction of phenolic compounds. The results of antimicrobial activity indicate that tested extracts showed different degree of antimicrobial activity in relation to the tested species. In general, there was no difference in activities between different extracts. Extracts of *S. montana* demonstrated more potent inhibitory effects on the growth of G+ bacteria than to the other tested microorganisms. Results of our study suggest the great value of the species *S. montana* for use in phytotherapy and food industry.

**Acknowledgments**

This study was supported by the Ministry of Science and Technological Development of the Republic of Serbia (grant No. 41010 and 173032).

**References**