ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES OF SOME COMMERCIAL PLANTS FROM MACEDONIA

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ABSTRACT

The ethanolic crude extracts of *Calendula officinalis* L. (flores), *Primula officinalis* L.(flores), *Sambucus nigra* L.(flores), *Helichrysum arenarium* L.(flores), have been examined for their antioxidant and antibacterial activity. DPPH (1,1-diphenyl-2-picryl hydrazyl) radical was used for evaluation of free radical scavenging and the antibacterial activity was evaluated by using agar well diffusion assay. Among the studied plants, *Primula officinalis* L. (IC50 = 152.94 µg/ml) showed better antioxidant activity, followed by *Sambucus nigra* L (IC50 = 313.04 µg/ml) and *Helichrysum arenarium* L. (IC50 = 333.59 µg/ml). *Calendula officinalis* L. showed very weak antioxidant activity (IC50 = 1066.65 µg/ml). Plant extracts were tested against six bacterial stains: *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922, *Pseudomonus aeruginosa* ATCC 9027, *Klepsiella pneumonia* ATCC 700603, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 19433. All four extracts were able to inhibit most of the stains. The best results were given against *S. aureus*, while *K. pneumonia* was shown as the most resistant strain. The results indicated that all of the plant extracts tested in this study, could be considered promising natural antioxidant sources and great potential as natural antibacterial compounds against gram-positive and gram-negative bacteria.

KEYWORDS: antioxidant, DPPH (1,1-diphenyl-2-picryl hydrazyl) radical, antibacterial.
INTRODUCTION
There is an increasing interest in using medicinal plants as natural sources in pharmaceutical, food and cosmetic industries all over the world (Parmer, 2012). They possess many phytochemicals with various bioactivities including antioxidant and anti-inflammatory activity, also are the good sources for the discovery of new pharmaceutical compounds and medicines (Swamy et al., 2012).

Free radicals are generated due to different exogenous factors and normal metabolism of oxygen (Parajuli et al., 2012). Accumulation of free radicals can cause pathological conditions and different diseases (Kaur and Mondal, 2014). In this context, the use of plants and herbs in food, pharmaceutical and cosmetic industries as a source of natural antioxidant and biologically active compounds has attracted a great deal of scientific interest (Msaada et al., 2015). Total phenol content (TPC), and the identification of individual phenolic compounds in plant extracts have been extensively studied mainly due to their antioxidant activity, in the last decade of the 20th century (Tahirović et al., 2014).

Antimicrobial-resistant bacteria are the causes of numerous clinical problems worldwide. Infectious diseases caused by resistant microorganisms are responsible for increased health costs as well as high morbidity and mortality, particularly in developing countries (Gangoué-Piéboji et al., 2009). For this reason the search is ongoing through for new antimicrobial agents from natural sources (Bhavnani and Ballow, 2000).

The aim of this study was to determine the antioxidant activity of commercial plants used as single component teas in R. Macedonia, to find out their total phenolic and flavonoid content and to study their antibacterial activity, as well.

MATERIALS AND METHODS
Plant material
In this research, dried flowers of commercial samples obtained from the botanical departments of Macedonian manufacturers, were used (Table 1).

Table 1. Plant species used in the study

<table>
<thead>
<tr>
<th>No.</th>
<th>Common name</th>
<th>Botanical name</th>
<th>Family</th>
<th>Plant part</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Marigold</td>
<td><em>Calendula officinalis</em> L.</td>
<td>Asteraceae</td>
<td>flores</td>
</tr>
<tr>
<td>2</td>
<td>Cowslip</td>
<td><em>Primula officinalis</em> L.</td>
<td>Primulaceae</td>
<td>flores</td>
</tr>
<tr>
<td>3</td>
<td>Black elderberry</td>
<td><em>Sambucus nigra</em> L.</td>
<td>Caprifoliaceae</td>
<td>flores</td>
</tr>
<tr>
<td>4</td>
<td>Dwarf everlasting</td>
<td><em>Helichrysum arenarium</em> L.</td>
<td>Asteraceae</td>
<td>flores</td>
</tr>
</tbody>
</table>
Chemicales and reagents

- Gallic acid, 2,2-diphenyl-1 picrylhydrazyl radical (DPPH) (Aldrich Chemistry- Germany)
- Aluminium chloride (Merck KGaA- Germany)
- Folin-Ciocalteu reagent (Merck KGaA- Germany)
- Sodium carbonate (Alkaloid AD)
- Sodium hydroxide (Carlo Erba Reagents- Italy)
- Ethanol (Alkaloid AD)
- Sodium nitrite (Alkaloid AD) and deionized water

Bacterial strains

- *Escherichia coli* ATCC8739 (Liofilchem-Roseto-Italy)
- *Escherichia coli* ATCC 25922 (Liofilchem-Roseto-Italy)
- *Pseudomonus auriginosa* ATCC 9027 (Microbiologics- France)
- *Klepsiella pneumonia* ATCC 700603 (Microbiologics- France)
- *Staphylococcus aureus* ATCC 25923 (Microbiologics- France)
- *Enterococcus faecalis* ATCC 19433 (Liofilchem-Roseto-Italy)

Preparation of the extract

The dried plant flowers were minced with the laboratory grinder in order to obtain a fine powder. Each sample (0.25 g) were extracted with ethanol (15 ml) on ultrasonic bath for 15 minutes. Than the extract was filtered and the residue was extracted with 10 ml ethanol for 15 minutes. The filtrates were combined. The crude extracts were diluted using ethanol according to the assay needs.

Determination of total phenolic content

Total phenolic contents (TPC) were determined as described by Singleton, with some modification (Singleton, 1999). Plant extract (0.25 ml) was mixed with Folin–Ciocalteu reagent (0.5 ml). After 5 minutes, 4 ml sodium carbonate (7%) and 5.25 ml deionized water were added. The total phenolic content was determined after 1 h incubation at room temperature in the dark. The absorbance of the resulting blue color was measured at 765 nm with a UV–VIS spectrophotometer. Quantification was done with respect to the standard curve of gallic acid. The results were expressed as gallic acid equivalents (GAE), milligrammes per 1 g of dry weight. All determinations were performed in triplicate.
Determination of total flavonoids content

Determination of total flavonoids content (TFC) was performed as described by Swamy with some modification (Swamy et al., 2012). Sodium nitrite (5%) 100 µl was added to 0.25 ml plant extract. The mixture was shaken for 5 minutes, then was added 150 µl 10% aluminium chloride, after 6 minutes 500 µl sodium hydroxide (1M) and 1.5 ml ionized water were added. The absorbance was measured at 510 nm and results were expressed as mg quercetin equivalents (QE) per gram of dry extracts. Triplicate tests were conducted for each sample.

Determination of DPPH free radical scavenging activity

The free radical scavenging activity was determined as described by Gyamfi, with some modification (Gyamfi, 1999). DPPH was dissolved in ethanol (96%). The radical stock solution was prepared fresh daily. The DPPH solution (4 ml) was added to 200 µl of plant extract. The mixture was shaken vigorously and allowed to stand at room temperature for 10 min. The decrease in absorbance of the resulting solution was monitored at 517 nm. All determinations were performed in triplicate.

Radical scavenging activity (RSA) was calculated by using following formula:

\[ \%RSA = \frac{(Ak - Aa)}{Ak} \times 100 \]

Ak - absorbance of control, Aa - absorbance of sample

This activity was expressed as IC\(_{50}\), (IC\(_{50}\) value is the concentration of the sample required to inhibit 50% of radical). Linear regression analysis was used to calculate IC\(_{50}\) values.

Determination of the antibacterial activity

The antibacterial activity was measured by Agar well diffusion assay (Sethi et al., 2015). The plant extract were allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Petri plates containing Muller Hinton medium were seeded with the bacteria stains. Each labeled medium plate was uniformly inoculated with the test organism by using a sterile cotton swab rolled in the suspension to streack the plate surface in a form that lawn the growth can be observed. Wells were punched and 250 µl of the ethanol plant extracts were added. The plates then were incubated at 37 \(^\circ\)C for 24 hours. amikacin (250 mg/1 ml) was used as a positive control and ethanol was used as negative control. Analyses was done in triplicates. The antibacterial activity was assayed by measuring the diameter of zone of inhibition, in millimeters, formed around the well.
RESULTS

The total phenolic content

The total phenolic content for the investigated plants have been presented in Table 2. The total phenolic content was ranged in order as: *Primula officinalis* L. (155.79 ± 0.221 GAE mg/g) > *Helichrysum arenarium* L. (123.22 ± 0.7128 GAE mg/g) > *Sambucus nigra* L. (122.4 ± 0.2207 GAE mg/g) > *Calendula officinalis* L. (72.13 ± 0.2207 GAE mg/g).

The total flavonoids content

The mean values and standard deviations of total phenolic and flavonoid content of *Calendula officinalis* L., *Primula officinalis* L., *Sambucus nigra* L. and *Helichrysum arenarium* L. are presented in Table 2. In the total flavonoids content the ranking order was: *Sambucus nigra* L. (59.36 ± 2.5534QE mg/g) > *Primula officinalis* L. (53.35 ± 0.3869 QE mg/g) > *Helichrysum arenarium* L. (52.86 ± 0.5542 QE mg/g) > *Calendula officinalis* L. (14.52 ± 0.5499QE mg/g).

Antioxidant activity

The antioxidant activity of the ethanol extracts (Table 2) can be ranked in the following order: *Primula officinalis* L. (152.94 µg/ml) > *Sambucus nigra* L. (313.04 µg/ml) > *Helichrysum arenarium* L. (333.59 µg/ml) > *Calendula officinalis* L. (1066.65 µg/ml).

Table 2. Total phenolic and flavonoid contents and antioxidant activity of different flowers crude extracts (values are represented as the mean±SD of three experiments)

<table>
<thead>
<tr>
<th>Ethanol extract</th>
<th>IC₅₀ (µg/ml)</th>
<th>Total phenolic GAE mg/g</th>
<th>%Total phenolic</th>
<th>Total flavonoids QE mg/g</th>
<th>% Total flavonoids</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Calendula officinalis</em></td>
<td>1066.65</td>
<td>72.13 ± 0.2207</td>
<td>7.213</td>
<td>14.52 ± 0.5499</td>
<td>1.452</td>
</tr>
<tr>
<td><em>Primula officinalis</em></td>
<td>152.94</td>
<td>155.79 ± 0.221</td>
<td>15.579</td>
<td>53.35 ± 0.3869</td>
<td>5.335</td>
</tr>
<tr>
<td><em>Sambucus nigra</em></td>
<td>313.04</td>
<td>122.4 ± 0.2207</td>
<td>12.24</td>
<td>59.36 ± 2.5534</td>
<td>5.936</td>
</tr>
<tr>
<td><em>Helichrysum arenarium</em></td>
<td>333.59</td>
<td>123.22 ± 0.7128</td>
<td>12.322</td>
<td>52.86 ± 0.5542</td>
<td>5.286</td>
</tr>
</tbody>
</table>

In Figure 1 is presented the relationship between total phenolic content and antioxidant activity of ethanol extracts of investigated plants.
Figure 1. Linear correlation between the amount of total phenols and antioxidant activity

Antibacterial activity

The antibacterial activity of ethanol extracts of dried flowers of four different plants: *Calendula officinalis* L., *Helichrysum arenarium* L., *Primula officinalis* L. and *Sambucus nigra* L. against six different bacteria stains: *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Klepsiella pneumonia* ATCC 700603, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 19433, was qualitatively assessed by presence or absence of inhibition zone. The different concentrations as 100 mg/ml, 50 mg/ml and 10 mg/ml of the four crude extracts showed different antibacterial activity against the used bacterial stains (Table. 3).

In Table 3. the antibacterial activity is presented as a percentage compared to amikacin. It is categorized as strong (+++), moderate (++) or weak (+), if the activity of the extract is higher than 70%, between 50 - 70% or less than 50%, respectively, compared to the antibacterial activity of amikacin (Chan et al, 2007).

Antibacterial activity is categorized as strong +++ for inhibition > 70%, moderate ++ for inhibition 50 < 70%, or weak + for inhibition < 50%, compared to antibacterial activity of amikacin.
Table 3. Antibacterial activity of different crude extracts against six bacterial strains

<table>
<thead>
<tr>
<th>Ethanol extract</th>
<th>(c) mg/ml</th>
<th>S. aureus ATCC 25923 (mm)</th>
<th>E. faecalis ATCC 19433 (mm)</th>
<th>E. coli ATCC 8739 (mm)</th>
<th>P. aeruginosa ATCC 9027 (mm)</th>
<th>E. coli ATCC 25922 (mm)</th>
<th>K. pneumonia ATCC 700603 (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calendula officinalis</td>
<td>100</td>
<td>(25)+</td>
<td>(15)+</td>
<td>(13)+</td>
<td>(20)+</td>
<td>(13)+</td>
<td>(18)+</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>(20)+</td>
<td>(13)+</td>
<td>(13)+</td>
<td>(16)+</td>
<td>(12)+</td>
<td>(18)+</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>(16)+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(12)+</td>
<td>-</td>
</tr>
<tr>
<td>Primula officinalis</td>
<td>100</td>
<td>(30)+++</td>
<td>(23)+++</td>
<td>(20)+</td>
<td>(25)+++</td>
<td>(19)+</td>
<td>(15)+</td>
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<tr>
<td></td>
<td>50</td>
<td>(22)+</td>
<td>(18)+</td>
<td>(18)+</td>
<td>(20)+</td>
<td>(19)+</td>
<td>(15)+</td>
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<tr>
<td></td>
<td>10</td>
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<td>(12)+</td>
<td>(17)+</td>
<td>(14)+</td>
<td>(15)+</td>
<td>-</td>
</tr>
<tr>
<td>Sambucus nigra</td>
<td>100</td>
<td>(23)++</td>
<td>(15)+</td>
<td>(17)+</td>
<td>(23)++</td>
<td>(15)+</td>
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<td>10</td>
<td>(16)+</td>
<td>-</td>
<td>(16)+</td>
<td>(13)+</td>
<td>(16)+</td>
<td>-</td>
</tr>
<tr>
<td>Helichrysum arenarium</td>
<td>100</td>
<td>(30)+++</td>
<td>(25)+++</td>
<td>(15)+</td>
<td>(20)+</td>
<td>(18)+</td>
<td>(14)+</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>(29)+++</td>
<td>(25)+++</td>
<td>(14)+</td>
<td>(19)+</td>
<td>(14)+</td>
<td>(13)+</td>
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<tr>
<td></td>
<td>10</td>
<td>(22)+++</td>
<td>(22)+++</td>
<td>-</td>
<td>(19)+</td>
<td>(14)+</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>amikacin</td>
<td>250</td>
<td>43</td>
<td>44</td>
<td>41</td>
<td>42</td>
<td>39</td>
<td>48</td>
</tr>
</tbody>
</table>

Graphical representation of antibacterial activity of flower extracts of *Calendula officinalis* L., *Primula officinalis* L., *Helichrysum arenarium* L., *Sambucus nigra* L. is shown in Figure 2.

**Figure 2. In vitro antibacterial activity of different crude extracts**

**DISCUSSION**

Natural antioxidants that are present in herbs are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. Herbs contain free radical scavengers like phenolic compounds and flavonoids. In this study we determined the total phenolic and...
flavonoid content, antioxidant activity and antibacterial activity of ethanol extracts of dried flowers of *Calendula officinalis* L., *Primula officinalis* L., *Helichrysum arenarium* L. and *Sambucus nigra* L.

*Calendula officinalis* L. presented lower values of total phenolic and flavonoid content then *Primula officinalis* L., *Sambucus nigra* L. and *Helichrysum arenarium* L.. *Primula officinalis* L. showed the highest value of phenolic content compared to *Sambucus nigra* L. and *Helichrysum arenarium* L., both of which showed almost the same values. No significant differences were observed between *Primula officinalis* L., *Sambucus nigra* L. and *Helichrysum arenarium* L. for the total flavonoid content.

Velicković et al. (2014), evaluated total phenols and flavonoid content in ethanol extract of *Calendula officinalis* L., and reported lower values (31.86 GAE mg/g and 0.10 QE mg/g) then in the present study. Previous studies have shown that different cultivars of the same species of *Calendula officinalis* L., were markedly different in the content of their phenols and flavonoids (Ercetin et al., 2012). This could explain the different results between these two studies.

In this study, the ethanol extract of *Primula officinalis* L. showed the highest values of total phenolic content and high total flavonoid content. In addition, phytochemical analysis on various species of genus *Primula* revealed that phenolics and flavonoids are widely distributed in the genus (Aslam et al., 2014).

Studies on the chemical composition have reported that *Sambucus nigra* L. is one of the richest species in terms of total phenolic compounds, and flavonols are also important phenolic compounds present in *Sambucus nigra* L. (de Oliveira Seabra, 2010). According to this author the flower of *Sambucus nigra* L. was evaluated as a raw material suitable to obtain phenolic-rich extracts, which is in accordance with the high values of total phenolic and flavonoid contents in this study.

Gradinaru et al.(2014), investigated the phenolic content of the methanol extract from *Helichrysum arenarium* L. (160 GAE mg/g) and flavonoids were identified as major constituents. In this study the ethanol extract of *Helichrysum arenarium* L. showed lower values. This could be explained by the reports of some authors, which proved that the
methanol is the most suitable solvent for the extraction of phenolic and flavonoids compounds (Rigane et al, 2013; Yen et al., 1996).

Data contained in Table 2, shows that Primula officinalis L. presented the highest values of antioxidant activity compared to Sambucus nigra L., Helichrysum arenarium L. and Calendula officinalis L. In addition, Tunde et al (2015), suggest that the ethanol extract of Primula officinalis L. play an important role in the intonation of oxidative stress. Calendula officinalis with lowest value of total phenolic content showed ten times lower antioxidant activity than Primula officinalis L. and five times lower values compared to Sambucus nigra L. and Helichrysum arenarium L. The antioxidant activity of Sambucus nigra L. and Helichrysum arenarium L. was moderate.

Literature surveys indicated that plant phenols constitute one of the major groups of compounds acting as primary antioxidants (Rice-Evans et al., 1995; Pietta, 2000). The high antioxidant activity of Primula officinalis L., Sambucus nigra L. and Helichrysum arenarium L. may be due to their high phenolic and flavonoid content. The ethanol extracts of investigated plants in this study showed a linear correlation between the total phenolic content and antioxidative activity (Figure 1), which is in accordance with the strong relationship between total phenolic content and antioxidant activity in fruits, vegetables and grain products reported by Samarth et al., 2008.

In terms of antibacterial effects of the ethanol extracts against six bacteria strains, they showed different zones of inhibition (Figure 2). Primula officinalis L. showed the greatest inhibition against all studied bacterial strains. Pure ethanol used as a control solvent had no inhibitory effects on the tested bacteria strains.

Ali Özmenet al., 2008, also reported significant effect of ethanol extract of Primula officinalis L. against gram-positive and gram negative bacteria. Helichrysum arenarium L. showed activity against all tested bacteria, especially against S. aureus. Sambucus nigra L. showed no activity against Klepsiella pneumonia. Calendula officinalis L. showed activity against all bacterial strains.

All the ethanol plant extracts showed weak to moderate activity against Pseudomonus auriginosa. Antibacterial activity of these plants against P. auriginosa was also reported by
different authors (Chandurar et al., 2015; Orhan et al., 2012; Albayrak et al., 2010; Mohammadsadeghi et al., 2013).

CONCLUSION
The results indicated that all of the plant extracts tested in this study, could be considered promising natural antioxidant sources and great potential as natural antibacterial compounds against gram-positive and gram-negative bacteria.

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