ABSTRACT: In this work were examined aqueous, methanol, ethanol and acetone leaf extracts of *Rubus discolor*, wild growing blackberry, for their antioxidant properties and total phenol and flavonoid content. The total phenol content (TPC) varied from 250.05 to 446.61 mg GAE/g of dry extract, while total flavonoid content (TFC) was in range between 22.44 and 61.15 mg QE/g of dry extract. Aqueous extracts were the richest in phenols, as well as in flavonoids. *In vitro* antioxidant capacity of leaf extracts was evaluated by 2,2-diphenyl-1-picrylhydrazil (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) free radical scavenging procedures and ferric reducing ability of plasma (FRAP) assay. Aqueous extracts were the most effective through all antioxidant tests. The total phenol content highly correlated with antioxidant activity of extracts. Moreover, weak correlation was established between total phenol and total flavonoid content. The results presented in this work indicate that phenol compounds contribute to antioxidant ability of extracts.

KEYWORDS: *Rubus discolor*, extracts, flavonoids, phenols, antioxidant activity

INTRODUCTION

The Rosaceae family is a large and diverse family which includes over 3,000 economically important fruits and ornamental species (Potter *et al.*, 2002). The genus *Rubus* counts about 750 species native to all continents except Antarctica (Alice and Campbell 1999). Many *Rubus* species are globally consumed as fresh and frozen fruits or processed in juices, jams and jellies (Kaume *et al.*, 2012). On the other hand, some of them are important not only as ornamental species, but also as invasive weeds, and in early forest succession. Consequently, that gives immense economical and ecological importance to the genus *Rubus* (Alice and Campbell 1999).

*Rubus* species have been globally very appreciated in traditional medicine due to their therapeutic and healing properties (Hummer 2010). *R. discolor* fruits,
leaves and roots are used as traditional remedy for nephritis and prostatitis; leaves are also used as antidiarrheals and for wound healing (Kültür 2007).

Blackberries are rich in phenols, particularly in anthocyanins and ellagitannins that are considered to possess antioxidant properties and, therefore, provide many health benefits (Reyes-Carmona et al., 2005; Ivanovic et al., 2014; Keser et al., 2015). Daily consumption of fresh vegetables and fruits, including blackberries, is directly connected with decreased occurrence of cancer, coronary heart diseases and many other disorders (Vendemiale et al., 1999; Reyes-Carmona et al., 2005).

*R. discolor* is a shrubby plant with high and strong angled brown to purple stems. Leaves consist of 5 leaflets from which terminal is ovate or suborbicular with truncated base, while basal are on short petioles. Flowers have about 30 mm in diameter and are grouped in pyramidal-truncate and large inflorescence. Fruits are large (Heslop-Harrison 1968). It is widespread in Europe, from France to Mediterranean and Balkan region. In Serbia, it is distributed in hills and mountains in the western part of the country (Tatić 1972).

*R. discolor* fruits possess higher amounts of bioactive compounds, such as anthocyanins, phenols, and non-flavonoid phenolics, compared to cultivated blackberries and even *R. idaeus* (Dujmović Purgar et al., 2012). *R. discolor* extracts exhibit notable antioxidant activity, particularly flower and leaf extracts. Additionally, flower extract has effect on the quantity of antioxidant enzymes, lipophylic vitamins (A and E), cholesterol, glutathion, total protein, and malondialdehyde (Keser et al., 2015). Despite the fact that fruits of various *Rubus* species have been the subject of different studies, information about antioxidant properties of blackberry leaves is still scarce. Therefore, the aim of the present study was evaluation of antioxidant properties of leaves collected from natural populations of *R. discolor*, as well as estimation of total phenol and flavonoid content.

**MATERIALS**

**Plant material**

Leaves were collected during summer 2012 at two locations: in Belgrade and in Cer Mountain (near the village of Čokešina). Leaves were taken from natural populations during fruiting stage of plants. Voucher specimens have been deposited in the Herbarium of the Institute of Botany and Botanical Garden “Jevremovac”, Faculty of Biology, University of Belgrade BEOU; vouchers no. 17084 and 17081.

**Chemicals**

Organic solvents (methanol, ethanol, acetone) and acids − HCl (concentrated hydrochloric acid) and CH$_3$COOH (glacial acetic acid) − were purchased
from Zorka Pharma, Šabac (Serbia). Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron (III) chloride (FeCl$_3$×6H$_2$O), iron (II) sulphate heptahydrate (FeSO$_4$×7H$_2$O), and sodium acetate (CH$_3$COONa×3H$_2$O) were obtained from Sigma Chemicals Co., St. Louis, MO (USA). Folin-Ciocalteu phenol reagent was purchased from Merck, Darmstadt (Germany). Sodium carbonate anhydrous (Na$_2$CO$_3$) and L(+)-ascorbic acid (vitamin C) were purchased from AnalR Normapur, VWR, Geldenaaksebaan, Leuven (Belgium). Aluminum nitrate nonahydrate (Al(NO$_3$)$_3$×9H$_2$O) and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Fluka Chemie AG, Buchs, (Switzerland). Quercetin hydrate was obtained from TCI Europe NV, Boereveldsweg (Belgium). All chemicals and reagents used in experiments were of analytical grade.

**METHODS**

**Extract preparation, total phenol and flavonoid content**

Extracts were prepared identically, by dissolving 5 g of dried powdered leaves in 50 ml of appropriate solvent and sonication in ultrasonic bath for 1 hour. After 24 hours samples were again sonicated for 1 hour, then filtered through Whatman no. 1 filter paper and evaporated by rotatory vacuum evaporator. Samples were left in the fridge at +4 °C till use.

The total phenol (TPC) content was determined spectrophotometrically as suggested by Singleton and Rossi (1965). In brief, 10% Folin-Ciocalteu reagent was added to 0.2 mL aliquots of sample solution. Then, 7.5% sodium-carbonate solution was added to this mixture and left for 2h at room temperature to react. The results were expressed as mg of gallic acid equivalents (GAE) per g of dry extract.

The total flavonoid content (TFC) in *R. discolor* leaves was estimated by JENWAY 6306 UV/VIS spectrophotometer according to the procedure previously described by Park *et al.* (1997) and expressed as mg of quercetin equivalents (QE) per g of dry extract.

All measurements were carried out in triplicate.

**Evaluation of antioxidant properties**

Antioxidant properties were evaluated spectrophotometrically by three different, previously described, *in vitro* procedures: DPPH (Blois 1958), ABTS (Miller and Rice-Evans 1997), and FRAP (Benzie and Strain 1996).

DPPH is a procedure based on the ability of plant extracts to neutralise 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. The DPPH solution in methanol (0.04 mg/mL) was added to the aliquots of sample of different concentrations. After 30 minutes of incubation in the dark at room temperature,
the absorbance was read at 517 nm against the control which contained methanol instead of sample. The results were expressed as IC$_{50}$ values (µg/mL).

ABTS is another antioxidant method which involves ABTS free radicals to estimate radical scavenging abilities of plant samples. The results were derived from triplicate measurements using JENWAY 6306 UV/VIS spectrophotometer and expressed as IC$_{50}$ values (µg/mL).

FRAP method was used to test capability of samples to reduce iron in complex with tripyridyl-s-triazine (TPTZ) from ferric to ferrous form which is measured by PERKIN ELMER LAMBDA BIO UV/VIS spectrophotometer at 595 nm. The results were calculated from calibration curve of aqueous solution of FeSO$_4$·x7H$_2$O and expressed as µmol Fe$^{2+}$ equivalents per mg of dry extract.

L-ascorbic acid and BHA, as well-known antioxidants, were used as a control.

RESULTS AND DISCUSSION

Yields, total phenol and flavonoid content

The extraction yields of R. discolor leaf extracts, as well as TPC and TFC, are presented in Table 1. The highest yield was found for aqueous and methanol extracts. TPC varied between 250.05 and 446.61 mg GAE/g of dry extract. Aqueous extracts from both localities were the richest in phenols. Despite the fact that samples from Cer had lower yield, TPC was higher in comparison to those from Belgrade.

TFC was in range from 22.44 to 61.15 mg QE/g of dry extract. The highest amount of flavonoids was in acetone (61.15 mg QE/g and 45.35 mg QE/g in sample from Belgrade and Cer, respectively), while the lowest were in aqueous and ethanol extracts.

The total phenol and flavonoid content in leaves of different Rubus species was previously investigated by several authors. R. ulmifolius extract was the richest in phenols, among 11 tested Sardinian species (Dall’Acqua et al., 2008). Similarly, Conforti et al. (2011) investigated bioactive compounds of 70% aqueous ethanol extracts and found the highest amount of phenols in R. caesius leaf extract. According to Keser et al. (2015), who previously examined variations in TPC among different plant parts of R. discolor, flower and leaf extracts were the richest in phenols. These researchers identified several flavonoid compounds such as rutin, apigenin, naringin, naringenin, myricetin and quercetin. Particularly abundant in flower extracts were myricetin and naringin, while rutin and naringin were abundant in leaf extracts.
Table 1. The yield, total phenol and total flavonoid content and antioxidant properties of *R. discolor* leaf extracts

<table>
<thead>
<tr>
<th></th>
<th>Yield¹</th>
<th>Total phenol content²</th>
<th>Total flavonoid content³</th>
<th>DPPH⁴</th>
<th>ABTS⁵</th>
<th>FRAP⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgrade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aqueous</td>
<td>8.67</td>
<td>359.19±±9.51</td>
<td>35.63±±0.37</td>
<td>17.31±±0.11</td>
<td>8.00±±0.78</td>
<td>2.24±±0.04</td>
</tr>
<tr>
<td>methanol</td>
<td>10.62</td>
<td>277.19±±2.04</td>
<td>36.74±±0.47</td>
<td>22.46±±0.09</td>
<td>10.10±±0.13</td>
<td>1.03±±0.06</td>
</tr>
<tr>
<td>ethanol</td>
<td>5.23</td>
<td>250.05±±3.90</td>
<td>24.49±±0.27</td>
<td>26.54±±0.25</td>
<td>13.32±±0.42</td>
<td>0.74±±0.56</td>
</tr>
<tr>
<td>acetone</td>
<td>2.78</td>
<td>289.46±±5.80</td>
<td>61.15±±0.60</td>
<td>29.46±±0.80</td>
<td>14.36±±0.22</td>
<td>2.01±±0.01</td>
</tr>
<tr>
<td>Cer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aqueous</td>
<td>11.03</td>
<td>446.61±±6.01</td>
<td>22.44±±0.44</td>
<td>15.76±±0.06</td>
<td>4.76±±0.36</td>
<td>2.96±±0.06</td>
</tr>
<tr>
<td>methanol</td>
<td>8.80</td>
<td>341.14±±3.01</td>
<td>30.97±±0.76</td>
<td>17.61±±0.14</td>
<td>7.02±±0.17</td>
<td>1.51±±0.04</td>
</tr>
<tr>
<td>ethanol</td>
<td>3.77</td>
<td>414.05±±1.00</td>
<td>28.95±±0.31</td>
<td>16.65±±0.41</td>
<td>8.03±±0.12</td>
<td>1.90±±0.06</td>
</tr>
<tr>
<td>acetone</td>
<td>1.92</td>
<td>407.68±±27.03</td>
<td>45.35±±1.74</td>
<td>15.86±±0.71</td>
<td>5.36±±0.06</td>
<td>1.44±±0.04</td>
</tr>
</tbody>
</table>

Results were expressed as mean ±± standard deviation

¹%  
²mg GAE/g d.w.  
³mg QE/g d.w.  
⁴IC₅₀ (µg/ml)  
⁵IC₅₀ (µg/ml)  
⁶µmol Fe²⁺/mg d.w.

Evaluation of antioxidant properties

Antioxidant characteristics of *R. discolor* leaf extracts were examined through three *in vitro* assays and the results are presented in Table 1. IC₅₀ values for DPPH varied from 15.76 µg/ml for aqueous sample from Cer to 29.46 µg/ml for acetone extract of the sample from Belgrade. The most powerful in neutralisation of DPPH free radicals were aqueous extracts with IC₅₀ values 15.76 µg/ml and 17.31 µg/ml for samples from Cer and Belgrade, respectively. IC₅₀ values for ABTS assay were between 4.76 µg/ml and 14.36 µg/ml. Similarly, aqueous extracts showed the strongest ability to scavenge ABTS free radicals. Possible explanation for lower IC₅₀ values obtained in ABTS in comparison to DPPH test could be the ability of ABTS radicals to react with both hydrophilic and lipophilic compounds. Additionally, approach to DPPH free radicals is restricted only to smaller molecules (Magalhães et al., 2008; Badarinath et al., 2010; Nur Alam et al., 2013).

Values obtained for FRAP procedure were in range from 0.74 to 2.96 µmol Fe²⁺/mg of dry weight. Aqueous extracts exhibited the most promising antioxidant properties among tested samples with values 2.24 and 2.96 µmol Fe²⁺/mg of dry weight, for samples from Belgrade and Cer, respectively.

Samples from Cer exhibited stronger antioxidant activity through all three used methods, which was probably the consequence of greater amount of phenols present in those samples. The differences among samples could be ascribed
to different environmental conditions and consequently different phytochemical composition of examined extracts.

Antioxidant activity of leaf extracts of related blackberry species was previously reported by some researchers (Dall’Acqua et al., 2008; Martini et al., 2009; Conforti et al., 2011; Keser et al., 2015; Veličković et al., 2015). The results of this research corresponded to these findings.

The correlation between secondary metabolites content in leaf extract of *R. discolor* and antioxidant properties, expressed by Pearson’s correlation coefficient, was also examined. A strong and negative correlation was found between total phenol content and radical scavenging abilities tested by DPPH (r = −− 0.8532) and ABTS (r = −− 0.8631), which was a consequence of the usage of IC\textsubscript{50} values to express antioxidant properties. In addition, strong positive correlation was established between total phenol content and FRAP values (r = 0.7147), which was expectable because both methods were based on reducing power of extracts. That was also the reason why method used for evaluation of TPC was not so sensitive and specific. On the other hand, total flavonoid content moderately correlated with DPPH and ABTS, and weakly with FRAP values. Weak correlation was established between total phenol and flavonoid content. The results of this study suggest that antioxidant properties of tested samples could be ascribed not only to flavonoid group of phenolic compounds, but also to some other compounds.

CONCLUSIONS

The presented results demonstrate that *R. discolor* leaves possess notable antioxidant activity which is strongly correlated to phenolic compounds. Therefore, *R. discolor* should be considered as a rich source of phytochemicals which could be potentially implemented in food and pharmaceutical industry. Consequently, there is need for further examinations of phytochemical composition of *R. discolor* leaves and their biological activity, particularly through *in vivo* tests.

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REFERENCES


АНТИОКСИДАТИВНА СВОЈСТВА ЕКСТРАКТА ЛИСТОВА Rubus discolor
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РЕЗИМЕ: У овом раду испитивана су антиоксидативна својства и присуство фенола и флавоноида у воденим, метанолним, етанолним и ацетонским екстрактима листова самоникле купине Rubus discolor. Укупан садржај фенола је варирало између 250,05 и 446,61 mg GAE/g сувог екстракта, док се укупан садржај флавоноида кретао у опсегу од 22,44 до 61,15 mg QE/g. Водени екстракти су били најбогатији фенолима и флавоноидима. In vitro антиоксидативни капацитет екстраката листова је одређен DPPH, ABTS и FRAP методама, при чему су најефикаснији били водени екстракти. За разлику од флавоноида, феноли су били у јакој корелацији са антиоксидативном активношћу екстраката. Међутим, установљена је слаба корелација између укупне количине фенола и флавоноида. Презентовани резултати указују да је антиоксидативна активност екстраката последица присуства фенолних једињења.
КЉУЧНЕ РЕЧИ: Rubus discolor, екстракти, флавоноиди, феноли, антиоксидативна активност