Antioxidant activity of *Ruscus* species from Serbia: Potential new sources of natural antioxidants

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Abstract

The antioxidant activity and total phenolic and flavonoid content of ethanolic, acetone and ethyl acetate extracts of *Ruscus hypoglossum* L. and *Ruscus aculeatus* L. (aerial parts) from Serbia were investigated in this paper. The best total antioxidant capacity (23.329 μg AA g^{-1}) and the highest DPPH scavenging activity (IC_{50} = 182.54 μg mL^{-1}) were found in acetone and ethyl acetate extract of *R. aculeatus* L. Ethanolic extract of *R. hypoglossum* L. showed the highest ABTS radical cation scavenging activity (IC_{50} = 3.04 μg mL^{-1}), as well as reducing power (IC_{50} = 143 μg mL^{-1}). The best inhibitory activity against lipid peroxidation (IC_{50} = 651 μg mL^{-1}) and the best ferrous ion chelating ability (IC_{50} = 110 μg mL^{-1}) were found in acetone and ethyl acetate extract of *R. hypoglossum* L. The highest total phenolic (8.569 mg GAE g^{-1}) and flavonoid contents (0.136 mg RU g^{-1}) were found in ethanolic and acetone extract of *R. hypoglossum* L. and *R. aculeatus* L, respectively.

Keywords: free radical scavenging, reducing power, chelating ability, lipid peroxidation, total phenolic and flavonoid contents.

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Different plant species are the subject of numerous studies throughout the world in order to obtain natural products with their potential application in medicine, pharmacy and food industry in recent two decades. From ancient times, the plants have been using as rich source of effective and safe medicines. About 80% of world population is still dependent on traditional medicines. Bioactive compounds and antioxidant activity are important parameters that regulate the therapeutic effects of a plant [1]. The most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, saponins, lactones and terpenoids [2]. Phenolics are widely distributed in medicinal plants and they have multiple biological effects, including antioxidant, anti-inflammatory, anti-cancer, anti-viral, anti-bacterial and cardio-protective activity [3]. An antioxidant is any substance that, when present at low concentrations, significantly delays or prevents oxidation of cell content like proteins, lipids, carbohydrates and DNA [4]. It can interfere with the oxidation process by scavenging reactive oxygen species (SOD removing O_2^-), by binding transition metal ions and preventing formation OH and/or decomposition of lipid hydroperoxides, by repairing damages (*e.g.*, *α*-tocopherol repairing peroxyl radicals and so terminating the chain reaction of lipid peroxidation) or by any combination of the above [5–7]. According to these abilities, numerous methods are applied for testing antioxidative activity of plants, such as the oxygen radical absorption capacity (ORAC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picryl-hydrazil (DPPH) radical scavenging and inhibition of the formation of thiobarbituric acid reactive substances (TBARS) [8]. Thus, antioxidant and phytochemical screening of different plants are essential to provide valuable information in the search of new pharmaceuticals.

Ruscus hypoglossum L. and *R. aculeatus* L. are two of the three species from genus *Ruscus* (fam. Liliaceae, order Asparagales) which grow in Serbia. The native range of *Ruscus hypoglossum* L. extends from Western Europe to Iran. Common names include Mouse Thorn, Spineless Butcher's Broom and Horse Tongue Lily. *Ruscus aculeatus* L. (Butcher's Broom) is originally from Mediterranean Europe and Africa [9]. The rhizomes of European species of *Ruscus* are used in herbal medicines due to their anti-inflammatory and vasoconstriction properties. Flavonoids from *Ruscus* species strengthen blood vessels, reduce capillary fragility and support healthy circulation. Many chemical constituents have been isolated from these plants. Species from *Ruscus* genus are recognizing for ruscogenin and flavonoids, especially *R. aculeatus* L. The aerial parts of these two *Ruscus* species contain several flavonoid glycosides and phenolic acids [10]. However, *R. aculeatus* L. rhizome contains *p*-coumaric acid and amides of hydroxycinnamic acids, but it has no flavonoids. Recently, Hadzifejzovic *et al.* [11] have found that ethyl acetate and butanol fraction of methanolic extract of

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herb and rhizome of *R. aculeatus* L. and herb of *R. hypoglossum* L. have antimicrobial and antioxidant activity. The authors showed, in mentioned results, that there is a relatively strong correlation between the total phenolic content and the antioxidant capacity of plants' extracts.

In literature there is no data related to the study of antioxidant activity of *Ruscus* species from the territory of Serbia. Therefore, the aim of this study was designed for the evaluation of antioxidant activity of aerial parts of two *Ruscus* species from Serbia in order to investigate the relationship between antioxidant properties and total phenolic and flavonoid content. These data will provide some useful information for healthier living, as well as the further screening of plants as potential sources of new natural antioxidants.

EXPERIMENTAL

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ascorbic acid, quercetin, butylated hydroxytoluene, ammonium molybdate, sodium phosphate, sulphuric acid, ferrous sulfate heptahydrate, Folin–Ciocalteu reagent, aluminum chloride, potassium ferricyanide, trichloroacetic acid, ferric chloride, linoleic acid, sodium acetate, Tween-20, ammonium thiocyanate, hydrochloric acid, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine--*p*,*p*'-disulfonic acid monosodium salt hydrate, 2,2--azino-bis(3-ethylbenzothiazoline6-sulfonic acid) (ABTS), sodium carbonate, potassium sodium tartrate tetrahydrate, rutine, were obtained from Sigma-Aldrich, St. Louis, MO, USA; ethanol, methanol, acetone and ethyl acetate were obtained from Fluka Chemie AG Buchs, St. Louis, MO, USA.

Plant materials

Aerial parts of *Ruscus hypoglossum* L. were collected from mountain Žeželj, near Kragujevac (Serbia), whereas aerial parts of *Ruscus aculeatus* L. were collected from Ovčar–Kablar gorge, near Čačak (Serbia), in late September, 2012. The species were identified according to Systematic key at the Institute for biology and ecology, Faculty of Science, University of Kragujevac, Serbia. Voucher specimens of *R. aculeatus* L. (17074 BEOU) and *R. hypoglossum* L. (17075 BEOU) were deposited in the Herbarium of Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade, Serbia.

Solvent extraction

The air-dried aerial parts of plant (30 g) were broken into small pieces (2–6 mm) and extracted with ethanol (96%), acetone and ethyl acetate (150 mL) using a Soxhlet apparatus. The extracts were filtered through a filter paper (Whatman No. 1) followed by evaporated on rotary vacuum evaporator at 40 °C. The resulting extracts were dried at room temperature to constant dry weight mass. The dark greenish residues were stored in a dark glass bottle at 4 °C to prevent oxidative damage until the further analysis.

Antioxidative methods

Total phenolic content

The total phenolic compounds in the extract were determined according to the Folin–Ciocalteu method by Singleton and Rossi [12] with some modifications. To 1 mL of each extract dissolved in methanol, 2 mL of 7.5% (*w*/*V*) sodium carbonate solution was added and vortexed vigorously. After 5 min, 1 mL of 1:10 diluted Folin–Ciocalteu's phenol reagent was added and vortexed again. Same procedure was repeated for the standard solution of gallic acid. All the tubes were incubated at room temperature for 30 min and then the absorbance was measured at 765 nm. The total phenolic content in the extracts was calculated from the standard curve and values are expressed as gallic acid equivalent (GAE) in mg g^{-1} of dry weight (DW) extract.

Total flavonoid content

The aluminum chloride method was used for the determination of the total flavonoids content of the sample extracts [13]. Aliquots of extract solutions were taken and made up to volume 3 mL with methanol. Then 0.1 mL $AlCl_3$ (10%), 0.1 mL potassium sodium tartrate and 2.8 mL distilled water were added sequentially. The test solution was vigorously shaken. Absorbance at 415 nm was measured after 30 min of incubation. A standard calibration plot was generated at 415 nm using known concentrations of rutine. The concentration of flavonoids in the test samples was calculated from the standard curve and values are expressed as rutine (RU) equivalent in mg g^{-1} of DW extract.

Total antioxidant capacity

The total antioxidant capacity was determined by phosphomolybdenum method according to Prieto *et al.* [14]. To 1 mL of samples or standard at different concentration performed from stock solutions (1 mg mL^{-1}), 2 mL reagent solution (ammonium molybdate 4 mM, sodium phosphate 28 mM and sulphuric acid 0.6 M) was mixed vigorously. All the reaction tubes were incubated at 95 °C for 90 min. The absorbance was measured at 695 nm against blank (methanol) after cooling at room temperature. Ascorbic acid (AA) was used as standard and total antioxidant capacity of extracts is expressed as mg AA g^{-1} of DW extract.

DPPH radical scavenging assay

DPPH radical scavenging activity was done according to the method by Takao *et al.* [15] with slight modi-

fication. Working solution of extracts was carried out by dilution stock solution (2 mg mL^{-1}) of extracts. DPPH was dissolved in methanol to obtain a concentration at 8 μg mL^{-1}. To 1 mL of DPPH solution, 1 mL of various concentrations of the extracts or the standard solution was added separately. The reaction mixtures were incubated at 37 °C for 30 min, following by absorbance measured at 517 nm using methanol as blank reference. The DPPH scavenging activity (%) of extracts and standards AA, gallic acid, butylated hydroxytoluene (BHT), *α*-tocopherol, quercetin were determined using the following equation:

$$\% \text{ Inhibition} = 100(Ac–As)/Ac \quad (1)$$

where *Ac* was absorbance of control reaction and *As* the absorbance in presence of the sample.

2,2'-Azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) decolorization assay

The $ABTS^{\bullet+}$ decolorization assay is spectrophotometric method widely use for determination of the antioxidative activity of substances. The $ABTS^{\bullet+}$ scavenging activity was measured according to the method of Re *et al.* [16]. In brief, $ABTS^{\bullet+}$ was first produced by reacting ABTS stock solution (7 mM) with potassium persulfate (2.45 mM). The mixture was then placed in the dark at room temperature for 12 to16 h before using. Under this condition, $ABTS^{\bullet+}$ can be stable in this form for more than 2 days. The $ABTS^{\bullet+}$ solution was diluted with double-distilled water to obtain an absorbance of 0.70±0.02 at 734 nm. Aliquots of 30 μL of the sample extracts of different concentrations (from 2 mg mL^{-1} to 3.91 μg mL^{-1}) were then added to 2.7 mL diluted $ABTS^{\bullet+}$ solution, and mixture was incubated at room temperature for 30 min. Absorbance was determined spectrophometrically at 734 nm. For the control, 1.0 mL of methanol was used instead of extract. AA was used as a positive control. The percentage of inhibition was calculated using the Eq. (1) and results are expressed as IC_{50} value.

Determination of inhibitory activity against lipid peroxidation

Antioxidant activity was determined by the thiocyanate method of Hsu *et al.* [17]. Serial dilutions were carried out with the stock solution (2 mg mL^{-1}) of the extracts, and 0.5 mL of each solution was added to linoleic acid emulsion (2.5 mL, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50 mL 40 mM phosphate buffer and the mixture was then homogenized. The final volume was adjusted to 5 mL with 40 mM phosphate buffer, pH 7.0. After incubation at 37 °C in the dark for 48 h, 72 and 96 h, a 0.1 mL aliquot of the reaction solution was mixed with 4.7 mL of ethanol (75 %), 0.1 mL $FeSO_4$ (20 mM $FeSO_4$ was diluted in 3.5% HCl) and 0.1 mL ammonium thiocyanate (30%). The absorbance of the mixture was measured at 500 nm and the mixture was stirred for 3 min. Ascorbic acid was used as reference compound. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compounds (AA, GA, BHT, *α*-tocopherol and quercetin) was used. Inhibition percent of linoleic acid peroxidation was also calculated using Eq. (1), and results are expressed as IC_{50} value.

Reducing power assay

The reducing power assay was determined according to the method described by Oyaizu [18]. Serial dilutions were carried out with the stock solution (1 mg mL^{-1}) of each extract. To 1 mL sample extract at different concentrations, 2.5 mL 0.2 M phosphate buffer pH 6.6, and 2.5 mL 1% potassium ferricyanide were added followed by mixed vigorously. After incubation at 50 °C for 20 min, 2.5 mL 10% trichloroacetic acid was added to mixture followed by centrifugation at 3000 rpm for 10 min. Subsequently, 2.5 mL of upper layer of mixture was added to 2.5 mL distilled water and 0.5 mL 0.1% ferric chloride, and absorbance of resulting solution was read at 700 nm against a blank. AA was used as standard. The reducing capacity of extracts was calculated using Eq. (1), and results are expressed as IC_{50} value.

Measurement of ferrous ion chelating ability

The ferrous ion chelating activity extracts was measured by the decrease in absorbance at 562 nm of the iron (II)–ferrozine complex according to Carter [19] and Yan *et al.* [20]. One mL of 0.125 mM $FeSO_4$ was added to 1 mL sample extract at different concentrations (from 2 mg mL^{-1} to 3.91 μg mL^{-1}), followed by 1 mL 0.3125 mM ferrozine. The test tubes were allowed to equilibrate at room temperature for 10 min. The absorbance was measured at 562 nm against blank. AA, BHT, *α*-tocopherol and quercetin were used as positive control. The ability of the extract to chelate ferrous ion was calculated using Eq. (1), and results are expressed as IC_{50} value.

Statistical analysis

All the results are expressed as means (MS) ± standard deviation (*SD*) of three independent measurements. For tested the normality of distribution, means and standard deviation, Student *t*-test at the level of significance 0.05 and 0.01 was used. Correlation coefficient was analyzed through Pearson's and Spearman's correlation coefficient. The IC_{50} values were calculated by nonlinear regression analysis from the sigmoidal dose-response inhibition curve. For statistical analysis, the SPSS 13.0 software program was used.

RESULTS AND DISSCUSION

According to traditional usage of these plants in herb medicine, the antioxidant capacity of different extracts of *R. hypoglossum* L. and *R. aculeatus* L. (aerial parts) from Serbia was systematically investigated by different assays. In addition, the total content of phenols and flavonoids was determined in each extracts, as well as the correlation between these phytochemicals and the antioxidant activity.

Generally, the antioxidant activity of plants is mainly associated to their bioactive compounds, such as phenolics, flavonols and flavonoids [21]. Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. The results of total phenolic content (TPC) determined in the different plants extracts are presented in Fig. 1. The TPC in all tested extracts of *R. hypoglossum* L. was higher than TPC in extracts of *R. aculeatus* L. The content of total phenolics in EtOH, EtOAc and AcOH extract of *R. hypoglossum* L. were 8.569, 8.175 and 8.036 mg GAE g^{-1}, respectively. However, the significant lesser TPC was found in extracts of *R. aculeatus* L. in following order: AcOH extract > EtOAc extract > EtOH extract. Statistically significant differences in TPC between AcOH and EtOH ($p < 0.05$, $R^2 = 0.612$), as well as between EtOAc and EtOH extracts ($p < 0.05$, $R^2 = 0.548$) of *R. aculeatus* L. were found. The highest TPC was determined in EtOH extract of *R. hypoglossum* L., whereas the highest TPC was found in AcOH extract of *R. aculeatus* L. Obviously, these plants contain phenolics by different polarity, which are involved in their antioxidant activity. The least polar solvents are generally considered to be suitable for extracting lipophilic phenols, and polar solvents are used for hydrophilic phenols [22].

Flavonoids are the most abundant polyphenols. The basic flavonoid structure is the flavan nucleus, containing 15 carbon atoms arranged in three rings (C6-C3--C6). The results of total flavonoid content (TFC) determined in the different plants extracts are also given in Fig. 1. The greatest quantity of TFC was found in AcOH extract of *R. aculeatus* L. (0.136 mg RU g^{-1}) whereas the lowest quantity was found in EtOH extract of same plant (0.113 mg RU g^{-1}). The *R. hypoglossum* L. extract with the greatest quantity of TFC was AcOH (0.129 mg RU g^{-1}), followed by EtOH (0.125 mg RU g^{-1}) and EtOAc (0.121 mg RU g^{-1}). Among all tested extracts, the AcOH extracts of both plants contain the highest quantity of TFC. However, the AcOH extract of *R. aculeatus* L. has higher flavonoid content than *R. hypoglossum* L. Our dates are in accordance with those

Plant extract or standard	Part of plant	IC_{50} (μg mL^{-1})						
		DPPH scavenging activity	Metal chelating activity	ABTS cation scavenging activity	Reducing power	Inhibitory activity against lipid peroxidation		
						48 h	72 h	96 h
RAEtOH	Aerial	502.03 ± 1.04	150 ± 0.25	3.45 ± 0.25	209 ± 0.08	1000 ± 1.26	950 ± 0.67	790 ± 0.71
RAEtOAc	Aerial	182.54 ± 0.21	165 ± 0.33	3.43 ± 0.25	223 ± 0.10	1050 ± 1.28	968 ± 0.84	810 ± 0.85
RAAcOH	Aerial	227.17 ± 0.37	170 ± 0.55	3.42 ± 0.12	239 ± 0.12	1200 ± 1.41	970 ± 0.91	840 ± 0.96
RHEtOH	Aerial	1632.33 ± 1.21	130 ± 0.17	3.04 ± 0.12	143 ± 0.05	960 ± 0.97	890 ± 0.82	765 ± 0.53
RHEtOAc	Aerial	278.37 ± 0.35	110 ± 0.09	3.09 ± 0.08	160 ± 0.10	1020 ± 0.99	1010 ± 0.95	989 ± 1.12
RHAcOH	Aerial	538.78 ± 1.18	230 ± 0.68	3.14 ± 0.10	162 ±0.10	870 ± 0.74	742 ± 0.66	651 ± 0.81
GA	-	3.97 ± 0.10	nd	nd	nd	nd	nd	255.43 ± 1.21
AA	-	6.05 ± 0.12	352.9 ± 1.25	2.85 ± 0.05	881 ± 0.15	400 ± 0.63	350 ± 0.45	250 ± 1.05
BHT	-	362 ± 0.84	>1500	nd	nd	nd	nd	1 ± 0.49
α-tocopherol	-	12 ± 0.33	>1000	nd	nd	nd	nd	3.92 ± 0.74
Quercetin	-	1.48 ± 0.29	550 ± 1.18	nd	nd	nd	nd	2.90 ± 0.25

RA-*Ruscus aculeatus* L., RH-*Ruscus hypoglossum* L, EtOH-ethanolic extract, AcOH-acetone extract, EtOAc-ethyl acetate extract, BHT-butylated hydroxytoluene, GA-gallic acid, AA-ascorbic acid, nd-not determined. Reasults are mean values ± SD from three experiments.

Fig. 1. Total antioxidant activity, total phenolic and flavonoid content of plants extracts.

reported in the literature, which revealed that acetone is the best solvent for the extraction of phenols and flavonoids [23–25].

The total antioxidant potential (TAP) of extracts from both plant species and standard (AA) was investigated (Fig. 1). The AcOH extract of *R. aculeatus* L. showed the highest antioxidant potential (23.329 μg AA g^{-1}), two fold higher than the same extract of *R. hypoglossum* L. (14.976 μg AA g^{-1}). The EtOAc extracts of *R. aculeatus* L. and *R. hypoglossum* L. showed some lesser TAP with 21.330 and 13.491 μg AA g^{-1}, whereas the EtOH extracts of *R. aculeatus* L. and *R. hypoglossum* L. were the least active extracts with 20.027 and 13.092 μg AA g^{-1}, respectively. Obviously, the aerial parts of *R. aculeatus* L. possess much stronger antioxidant potential than *R. hypoglossum* L. Results of this study showed that AcOH extract of both plants has the highest antioxidant potential followed by EtOAc and EtOH extracts. The significant differences in total antioxidant capacity between these two plants can be explained by differences in morph-physiological characteristics of plants, their natural habitat and ecological factors [26].

The results of DPPH scavenging activity of different plants extracts, as well as some synthetics antioxidants, are shown in Fig. 2. The best anti-DPPH activity was found in EtOAc extract of *R. aculeatus* L. This extract produced 50% of DPPH scavenging activity in concentrations of 182.54 μg mL^{-1}. However, slightly lower scavenger activity of *R. aculeatus* L. was found in AcOH (IC_{50} = 227.17 μg mL^{-1}), but about three times lower activity was found in EtOH extract (IC_{50} = 502.03 μg mL^{-1}). All examined extracts of *R. hypoglossum* L. showed significantly lower free radical scavenging activity, compared to *R. aculeatus* L. The extract of *R. hypoglossum* L. with the best anti-DPPH activity was also EtOAc with IC_{50} = 278.37 μg mL^{-1}, followed by AcOH and EtOH with IC_{50} = 538.78 μg mL^{-1} and IC_{50} = 1632.33 μg mL^{-1}, respectively. All tested extracts of *R. aculeatus* L. have significantly higher free radical scavenging activity, compared to *R. hypoglossum* L. The EtOAc extracts of both plants possess the best DPPH scavenging activity. This result is in accordance with report by Hadzifejzovic *et al.* [11] who found that EtOAc extract of *R. aculeatus* L. shows the best DPPH scavenging activity, with IC_{50} = 157.83 μg mL^{-1}. However, same authors investigated only DPPH activity of *R. hypoglossum* L. methanolic extract. The authors determined IC_{50} value as 518.19 μg mL^{-1}. The results of this study confirmed that this plant contains double higher free radical scavenging activity in EtOAc than in methanolic extract.

$ABTS^{\bullet+}$ scavenging capacities of extracts and reference compound (AA) were also determined in this study (Fig. 2). All examined extracts showed significant $ABTS^{\bullet+}$ scavenging activity. The EtOH extract of *R. hypoglossum L.* showed the greatest $ABTS^{\bullet+}$ scavenging activity, with IC_{50} = 3.04 μg mL^{-1}, followed by AcOH (IC_{50} = = 3.09 μg mL^{-1}) and EtOAH (IC_{50} = 3.14 μg mL^{-1}). Slightly lesser $ABTS^{\bullet+}$ scavenging activity showed the extracts of *R. aculeatus L.* The extract of *R. aculeatus L.* with the greatest $ABTS^{\bullet+}$ scavenging activity was AcOH, with IC_{50} = = 3.32 μg mL^{-1}, followed by EtOAc (IC_{50} = 3.43 μg mL^{-1}) and EtOH (IC_{50} = 3.45 μg mL^{-1}). This study shows that the aerial part of *R. hypoglossum* L. has better $ABTS^{\bullet+}$ scavenging activity than *R. aculeatus* L. The EtOH and AcOH are the solvents with best properties for the extraction of antioxidants, which are responsible for the best $ABTS^{\bullet+}$ scavenging activity of *R. hypoglossum* L. and *R. aculeatus* L, respectively. In literature, the influence type of alcohol used for extraction on the estimation of antioxidant activity of phenolic compounds in ABTS assay is well discussed. According to Oszmianski *et al.* [27], the antioxidant activity of plants against ABTS is correlated with the concentration, chemical structures, and polymerization degrees of organ antioxidants.

Plant extract	Part of plant	Total antioxidant activity μg AA g^{-1}	Total phenolic content mg GAE g^{-1}	Total flavonoid content mg RU g^{-1}
RAEtOH	Aerial	20.027 ± 1.48	5.800 ± 0.65	0.113 ± 0.02
RAEtOAc	Aerial	21.330 ± 1.39	6.275 ± 0.82	0.127 ± 0.11
RAAcOH	Aerial	23.329 ± 1.85	6.510 ± 0.53	0.136 ± 0.14
RHEtOH	Aerial	13.092 ± 1.22	8.569 ± 0.94	0.125 ± 0.18
RHEtOAc	Aerial	13.491 ± 1.35	8.175 ± 0.91	0.121 ± 0.15
RHAcOH	Aerial	14.976 ± 1.86	8.036 ± 1.03	0.129 ± 0.10

RA-*R. aculeatus* L, RH-*R. hypoglossum* L, EtOH-ethanolic extract, AcOH-acetone extract, EtOAc-ethyl acetate extract. Reasults are mean values ± SD from three experiments.

Fig. 2. The values of IC_{50} of the plants extracts and some synthetics antioxidants.

Many researchers reported that phenolic and flavonoid compounds have play an important role in stabilizing lipid oxidation in biological systems [28]. The inhibiting activity of each plant extracts against lipid peroxidation was determined at different incubation times (Fig. 2). The extract of *R. hypoglossum* L. with the greatest inhibiting activity was AcOH, with IC_{50} =651 μg mL^{-1}, followed by EtOH (IC_{50} = 765 μg mL^{-1}) and EtOAc (IC_{50} = 989 μg mL^{-1}). However, the EtOH extract of *R. aculeatus* L. showed the greatest activity in inhibiting oxidation of linoleic acid (IC_{50} = 790 μg mL^{-1}), followed by EtOAc (IC_{50} = 810 μg mL^{-1}) and AcOH (IC_{50} = 849 μg mL^{-1}). Obviously, the aerial part of *R. hypoglossum* L. exhibits better inhibitory activity than *R. aculeatus* L. The AcOH of *R. hypoglossum* L. and EtOH of *R. aculeatus* L. are the extracts with greatest activity in inhibiting oxidation of linoleic acid.

Results of reducing power of tested plants extracts and standard (AA) are presented in Fig. 2. All tested plants' extracts showed very significant reducing power with IC_{50} values in the range from 143 to 239 μg mL^{-1}. These values were more powerful than the synthetic antioxidant AA (IC_{50} = 881 μg mL^{-1}). The EtOH extract of *R. hypoglossum L.* showed the highest reducing power, with IC_{50} = 143 μg mL^{-1}, followed by EtAOc (IC_{50} = 160 μg mL^{-1}) and AcOH (IC_{50} = 162 μg mL^{-1}). The reductive potential of *R. aculeatus* L. was as follow: EtOH (IC_{50} = = 209 μg mL^{-1}), EtOAc (IC_{50} = 223 μg mL^{-1}) and AcOH (IC_{50} = 239 μg mL^{-1}). The results of this study showed that *R. hypoglossum* L. has the better reductive potential than *R. aculeatus* L. The EtOH is the best solvent for the extraction of antioxidants with the best reductive potential of both plants.

Ferrous ion chelating ability of each plant extracts was determined and results are given in Fig. 2. Among all tested extracts of *R. hypoglossum* L., the EtOAc extract showed the greatest ferrous ion chelating capacity, with the lowest IC_{50} = 110 μg mL^{-1}, followed by EtOH with IC_{50} = 130 μg mL^{-1} and AcOH with the greatest IC_{50} = 230 μg mL^{-1}. Ferrous ion chelating ability of *R. aculeatus* L. was the greatest in EtOH, with IC_{50} = = 150 μg mL^{-1}, followed by EtOAc and AcOH extracts with IC_{50} = 165 μg mL^{-1} and IC_{50} = 170 μg mL^{-1}, respectively. All tested extracts of both plants demonstrated high ferrous ion chelating capacity and much better compare to synthetic compounds (Fig. 2). Presented results showed that *R. hypoglossum* L. has better ferrous ion chelating capacity than *R. aculeatus* L.

The correlation between total phenolic and flavonoid content and antioxidant activity of plants was also evaluated, and results are given in Fig. 3. The results show very strong correlation between TPC and TOA (R^2 = 1.000), as well as between TFC and TOA (R^2 = = 1.000) in aerial parts of *R. aculeatus* L. Very strong positive correlation was also found between TPC (or TFC) and antioxidant activity by using the ABTS cation scavenging capacity (R^2 = 1.000) and reducing power (R^2 = 1.000). However, very strong negative correlation was found between TPC (or TFC) and metal chelating activity (R^2 = –1.000) of *R. aculeatus* L. These results show the importance of TPC and TFC in the antioxidant activity when measured through the methods mentioned above. For DPPH scavenging activity and inhibitory activity toward lipid peroxidation, no correlation between their IC_{50} and TPC (or TFC) was found. This finding is consistent with results of other authors [29],

	Total antioxidant activity	DPPH scavenging activity	Metal chelating activity	ABTS cation scavenging activity	Reducing power	Inhibitory activity against lipid peroxidation
R. hypoglossum L.						
TPC	-1.000[a]	-	-	-	1.000[a]	-
TFC	-	-	-1.000[a]	-	-	1.000[a]
R. aculeatus L.						
TPC	1.000[a]	-	-1.000[a]	1.000[a]	1.000[a]	-
TFC	1.000[a]	-	-1.000[a]	1.000[a]	1.000[a]	-

[a]Corelation is significant at the 0.01 level

Fig. 3. The correlation coefficient (R^2) between antioxidant activities, total phenolic and flavonoid content of plants.

and suggests on the possible presence of non-ionic compounds in extracts.

In the extracts of *R. hypoglossum* L., very strong negative correlation was found between TPC and TOA (R^2 = –1.000), but no correlation between TFC and TOA was found. These results suggest that the antioxidant activity of *R. hypoglossum* L. might be attributed to the presence of some non-phenolic compounds or some individual phenolic, which act in synergism with flavonoids. Very strong positive correlation was also found between TPC and reducing power (R^2 = 1.000) of *R. hypoglossum* L. From this result, it was clear that phenols play an important role in reducing power. This result is in accordance with results of Boulanour *et al.* [30]. For inhibitory activity toward lipid peroxidation and metal chelating activity, very strong positive (R^2 = = 1.000) and very strong negative correlation (R^2 = = –1.000) between their IC_{50} and TFC was found, respectively. Obviously, flavonoids are correlated with antioxidant activity when measured through the inhibitory activity toward lipid peroxidation. The mentioned authors [30] have also reported that flavonoids are correlated well in the protection of biological membranes. However, for DPPH scavenging activity, ABTS cation scavenging activity and reducing power, no correlation between their IC_{50} and TFC was found.

CONCLUSIONS

This is the first report about the antioxidant activity of *Ruscus hypoglossum* L. and *R. aculeatus* L. from Serbia. Acetone extract of *R. aculeatus* L. showed the best total antioxidant capacity whereas the highest DPPH scavenging activity was found in ethyl acetate extract. Ethanolic extract of *R. hypoglossum* L. showed the highest ABTS radical cation scavenging activity as well as reducing power. Acetone extract of this plant showed the best inhibitory activity against lipid peroxidation whereas the best ferrous ion chelating ability was found in ethyl acetate extract. The highest total phenolic content was found in ethanolic extract of *R. hypoglossum* L., but the highest total flavonoids content was found in acetone extract of *R. aculeatus* L. Both plant species had significant higher total phenolic than flavonoid content. Probably, some non-phenolic compounds are also involved in antioxidant activity of plants. This preliminary study provides data for supporting the use of these plant species as natural antioxidant agents, and confirms that these extracts represent a significant source of phenolic compounds. The further investigation will be focused on the chemical composition and biological activity of selected plants in order to apply them in pharmaceutical and food industry.

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REFERENCES

[1] U. Chandur, S. Shashidhar, S.B. Chandrasekar, M. Bhanumathy, T. Midhun, Phytochemical evaluation and anti-arthritic activity of root of *Saussure alappa*, Pharmacologia **2** (2011) 265–267.

[2] D. Krishnaiah, R. Sarbatly, A. Bono, Phytochemical antioxidants for health and medicine-A move towards nature, Biotechnol. Mol. Biol. Rev. **1** (2007) 97–104.

[3] R.L. Prior, X. Wu, K. Schaich, Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements, J. Agric. Food Chem. **53** (2005) 4290–4302.

[4] C.G. Nagendrappa, An appreciation of free radical chemistry-3, free radicals in diseases and health, Resonance **10** (2005) 65–73.

[5] R.S. Daniel, B.C. Mathew, D.S. Devi, K.T. Augusti, Antioxidant effect of two flavonoids from the bark of *Ficus bengalensis* Linn. in hyperlipidemic rats, Indian. J. Exp. Biol. **36** (1998) 902–906.

[6] T. Niwa, U. Doi, Y. Kato, T. Osawa, Antioxidative properties of phenolic antioxidants isolated from corn steep liquor, J. Agric. Food Chem. **49** (2001) 177–182.

[7] B. Halliwell, J.M.C. Gutteridge, Free radical in biology and medicine, 4th ed., Oxford University Press, London, 2007.

[8] T.G. Taruscio, D.L. Barney, J. Exon, Content and profile of flavonoids and phenolic acid compounds in conjunction with antioxidant capacity for a variety of Northwest *Vaccinium berries*, J. Agric. Food Chem. **52** (2004) 3169–3176.

[9] P.F. Yeo, in: T.G. Tutin, V.H. Heywood, N.A. Burges, D.M. Moore, D.H. Valentine, S.M. Walters, D.A. Webb (Eds.), Flora Europaea, Vol. 5, Cambridge University Press, Cambridge, 1980, pp. 71–73.

[10] N. Hadžifejzović, Flavonoide und hydroxyzimtsäureamide in *Ruscus aculeatus* L. und in anderen verwandten Arten. Strukturaufklärung, verteilung und physiologische bedeutung. PhD, University of Münster, 2006.

[11] N. Hadžifejzović, J. Kukić-Marković, S. Petrović, M. Soković, J. Glamočlija, D. Stojković, A. Nahrstedt, Bioactivity of the extracts and compounds of *Ruscus aculeatus* L. and *Ruscus hypoglossum* L, Ind. Crop. Prod. **49** (2013) 407–411.

[12] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, Am. J. Enol. Vitic. **16** (1965) 144–158.

[13] I.M.C. Brighente, M. Dias, L.G, Verdi, M.G. Pizzolatti, Antioxidant activity and total phenolic content of some Brazilian species, Pharm. Biol. **45** (2007) 156–161.

[14] P. Prieto, M. Pineda, M. Aguilar, Spectrophotometric quantization of antioxidant capacity through the form-

ation of a phosphomolybdenum complex: specific application to the determination of vitamin E, Anal. Biochem. **269** (1999) 337–341.

[15] T. Takao, F. Kitatani, N. Watanabe, A. Yagi, K. Sakata, A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish, Biosci. Biotechnol. Biochem. **58** (1994) 1780–1783.

[16] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. **26** (1999) 1231–1237.

[17] C.K. Hsu, B.H. Chiang, Y.S. Chen, J.H. Yang, C.L. Liu, Improving the antioxidant activity of buckwheat (*Fagopyrum tataricm* Gaertn) sprout with trace element water, Food Chem. **108** (2008) 633–641.

[18] M. Oyaizu, Studies on product of browning reaction prepared from glucose amine, Jap. J. Nut. **44** (1986) 307–315.

[19] P. Carter, Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine), Anal. Biochem. **40** (1971) 450–458.

[20] L.Y. Yan, L.T. Teng, T.J. Jhi, Antioxidant properties of Guava fruits: comparison with some local fruits, SAJ **3** (2006) 9–20.

[21] A. Kumar, P. Chaudhary, N.K. Verma, P. Kumar, TLC based phytochemical and antioxidant analysis of *Oxalis corniculata* Linn., Int. Res. J. Pharm. App. Sci. **3** (2013) 6––12.

[22] F. Medini, H. Fellah, R. Ksouri, C. Abdelly, Total phenolic, flavonoid and tannin contents and antioxidant and anti–microbial activities of organic extracts of shoots of the plant *Limonium delicatulum*, JTUSCI **8** (2014) 216–224.

[23] M. Alothman, R. Bhat, A.A. Karim, Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents, Food Chem. **115** (2009) 785–788.

[24] C.P. Anokwuru, E. Ijeoma, A. Olusola, O.A. Ayobami, Polyphenolic Content and Antioxidant Activity of *Hibiscus sabdariffa* Calyx, Research Journal of Medicinal Plant **5** (2011) 557–566.

[25] P.Z. Mašković, J.D. Mladenović, M.S. Cvijović, G. Aćamović-Đoković, S.R. Solijić, M.M. Radojković, Phenolic content, antioxidant and antifungal activities of acetonic, ethanolic and petroleum ether extracts of *Hypericum perforatum* L., Hem. Ind. **65** (2011) 159–164.

[26] J. Moor, J.G. Liu, K. Zhou, L. Yu, Effects of genotype and environment on the antioxidant properties of hard winter wheat bran, J. Agric. Food Chem. **54** (2006) 5313–5322.

[27] J. Oszmianski, A. Wojdylo, E. Lamer-Zarawska, K. Swiader, Antioxidant tannins from *Rosaceae* plant roots, Food Chem. **100** (2007) 579–583.

[28] S.J. Wu, L.T. Ng, Antioxidant and free radical scavenging activities of wild bitter melon (*Momordica charantia* Linn. var. *abbreviate* Ser.) in Taiwan, LWT-Food Sci. Technol. **41** (2008) 323–330.

[29] N. Nićiforivić, V. Mihailović, P. Mašković, S. Solujić, D. Pavlović Muratspahić, Antioxidant activity of selected plant species; potential new sources of natural antioxidants, Food Chem. Toxicol. **48** (2010) 3125–3130.

[30] B. Boulanouar, G. Abdelaziz, S. Aazza, C. Gago, M. Graca Miguel, Antioxidant activities of eight Algerian plant extracts and two essential oils, Ind. Crop Prod. **46** (2013) 85–96.

IZVOD

ANTIOKSIDATIVNA AKTIVNOST VRSTA *Ruscus* IZ SRBIJE: POTENCIJALNI IZVORI NOVIH PRIRODNIH ANTIOKSIDANATA

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(Naučni rad)

Antioksidativna aktivnost etanolnog, acetonskog i etil-acetatnog ekstrakta nadzemnih delova biljaka *Ruscus hypoglossum* L. i *Ruscus aculeatus* L. poreklom iz Srbije, kao i ukupan sadržaj fenola i flavonoida u ekstraktima, ispitivani su u ovom radu. Acetonski ekstrakt *R. aculeatus* L. pokazao je najveći ukupni antioksidativni kapacitet (23.329 μg AA g^{-1}), a etil-acetatni ekstrakt najbolju sposobnost neutralizacije slobodnih DPPH radikala (IC_{50} = 182,54 μg mL^{-1}). Etanolni ekstrakt *R. hypoglossum* L. pokazao je najbolju sposobnost neutralizacije $ABTS^{\bullet+}$ (IC_{50} = 3,04 μg mL^{-1}) i Fe^{3+}-redukujući kapacitet (IC_{50} = 143 μg mL^{-1}). Acetonski ekstrakt biljke pokazao je najbolju sposobnost inhibicije pri lipidnoj peroksidaciji (IC_{50} =651 μg mL^{-1}), a etil-acetatni ekstrakt najbolju Fe^{2+}-helatacionu aktivnost (IC_{50} = 110 μg mL^{-1}). Najveća količina ukupnih fenola (8,569 mg GAE g^{-1}) izmerena je u etanolnom ekstraktu *R. hypoglossum* L., dok je najveća količina ukupnih flavonoida (0,136 mg RU g^{-1}) izmerena u acetonskom ekstraktu *R. aculeatus* L. Na osnovu prikazanih rezultata, obe vrste *Ruscus* koje žive na teritoriji Srbije predstavljaju značajne izvore novih prirodnih antioksidanata sa mogućom primenom u farmaceutskoj i prehrambenoj industriji.

Ključne reči: Hvatanje slobodnih radikala • Redukujući kapacitet • Helataciona sposobnost • Lipidna peroksidacija • Ukupan sadržaj fenola i flavonoida