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Preliminary Communication

Rhamnus intermedia Steud. et Hochst. – a New Source of Bioactive Phytochemicals

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Abstract. Antioxidant capacity and various classes of phenolic antioxidants were quantified in leaf and bark methanol extracts of a medicinal plant, *Rhamnus intermedia* Steud. et Hochst., whose phytochemical composition is unknown. Three well-established assays were used to determine the antioxidant potency of the extracts: the Ferric Reducing/Antioxidant Power assay (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assay, as well as oxygen radical absorbance capacity (ORAC) assay. Total phenol (TP, Folin–Ciocalteu assay), total flavonoid (TF, colorimetric assay with $AlCl_3$) and free phenolic acid (UPLC-MS/MS) content of extracts was quantified. In comparison to bark extracts, leaf extracts exhibited significantly higher TP and TF contents, as well as higher antioxidant capacity according to all four assays. Ten free phenolic acids were quantified in leaf extracts while six were detected in bark extracts. The most abundant phenolic acid in leaves was vanilic acid (1647.06 ± 79.35 μmol g^{-1} DW; DW, dry weight) while salycilic acid was the most concentrated in the bark (111.10 ± 14.14 μmol g^{-1} DW). (doi: 10.5562/cca1946)

Keywords: Rhamnus intermedia, antioxidant activity, total phenols, total flavonoids, phenolic acids

INTRODUCTION

In the past decade, much attention has been focused on the largest group of plant secondary metabolites – the polyphenolics, and their potential role in the prevetion of chronic diseases. Positive effects of plant polyphenolics on human helath have been linked to their reported *in vitro* antioxidant,[1] antimutagenic[2] and antimicrobial[3] activities. Also, current evidence strongly supports the preventive effect of plant polyphenols in development of various pathological conditions: cardiovascular diseases,[4] cancer,[5,6] osteoporosis,[7] Alzheimer's disease[8] and diabetes mellitus.[9]

It has been reported that traditional medicinal plants are especially rich sources of polyphenols[10,11] and that medicinal plant extracts are effective in treating various ailments caused by oxidative stress, bacterial and/or viral infections. Croatia harbors about 500 endemic and sub-endemic species out of the total number of 5000 registered plants. The phytochemical profiles of most of these species have not yet been described. One of them is *Rhamnus intermedia* Steud. et Hochst., an endemic Illyric-Balcanic species distributed in the Mediterranean and narrow sub-Mediterranean region that includes Croatia, Bosnia and Herzegovina, Montenegro and Albania.[12]

The bark and fruit of *Rhamnus* species have been used for centuries in folk and official medicine as purgatives and for blood detoxication.[13,14] An infusion prepared from the fruits of *R. cathartica* L. is used in Bulgarian folk medicine as an antiseptic for wounds.[15] In folk medicine in Bosnia and Herzegovina the bark of *R. fallax* is used to treat mange and skin diseases.[14] Previous chemical studies of *Rhamnus* species were mainly focused on anthranoides, due to their purgative effect. The fruit of *R. cathartica* contain anthraquinone derivates, especially frangulin and glucofrangulin and flavonols (catharticin, kaempferol and quercetin), as well as bitter substances.[13] Locatelli *et al.*[16] reported the anthraquinone profile of *R. saxatilis* and *R. alpinus* L. bark which consisted of five components (aloe-emodin, rhein, emodin, chrysophanol, and physcion). Their total

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content in analyzed bark was 0.494 mg g^{-1} (*R. saxatilis*) and 2.42 mg g^{-1} (*R. alpinus*).

This study was focused on quantifying polyphenolic constituents and antioxidant capacity of *R. intermedia* methanol extracts prepared from bark and leaf tissue. Three well-established spectrophotometric methods were used to determine the antioxidant potency of our extracts – Ferric Reducing/Antioxidant Power assay (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assay, as well as oxygen radical absorbance capacity (ORAC) assay. Also, various classes of phenolic compounds were quantified in these preparations: total phenols (TP, Folin–Ciocalteu assay), total flavonoids (TF, colorimetric assay with $AlCl_3$) and free phenolic acids (UPLC-MS/MS).

EXPERIMENTAL

Chemicals and Instruments

Except for the $FeSO_4 \times 7H_2O$ (Kemika, Croatia), $NaNO_2$ (Laphoma, Skopje) and Folin-Ciocalteu reagent (Fluka, Switzerland), all the chemicals and reagents were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, MQ, USA). Absorbance measurements were performed on a double-beam UV-VIS spectrophotometer Bio-Spec-1601 (Shimadzu Corporation, Kyoto, Japan). UPLC-MS/MS analyses were carried out using an ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) linked to a Micromass Quattro *micro*™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK).

Herbal Material and Extraction

Randomly selected samples of wild growing *Rhamnus intermedia* Steud. et Hochst. plants were collected during the blooming period in Croatia (July, 2009) on the locality Bojinac (44.3428° N; 15.4083° E; 950 m a.s.l.). Leaves and bark were separated from the stem, air-dried and saved four months until analysis. Extractions were performed with 60 mg of dried material in 2 ml of methanol (φ = 80 %). The extracts were shaken for 2 h on a rotation homogenizer at 15 rpm and room temperature, sonicated (Iskra, Zagreb, Hrvatska) for 15 min and then centrifuged on a Centrifuge 5415C (Eppendorf, Germany) at 10000 g for 15 min. The supernatant was recovered and used for analysis of phenolic compounds and antioxidant activity.

Phytochemicals Quantification

The TP content of *R. intermedia* methanol extracts was determined according to the Folin-Ciocalteu method[17] adapted for small-scale analysis and gallic acid was used as the standard The TF content of *R. intermedia* methanol extracts was determined according to the $AlCl_3$ colorimetric assay[18] adapted to small volumes and catechin was used as the standard Free phenolic acids were determined by UPLC-MS/MS as described earlier.[19] The results were expresed in μmol g^{-1} DW (DW, dry weight).

Antioxidant Activity

The FRAP assay was used to estimate the antioxidant potential of tested extracts.[20] A calibration curve was prepared, using $FeSO_4 \times 7H_2O$. The ORAC assay was carried out on a fluorescence microplate reader (Multiskan Ascent 354, Labsystems) with an excitation wavelength of 485 nm and an emission wavelength of 525 nm. The procedure was based on the ORAC method reported by Prior *et al.*[21] with small modifications. Trolox was used as the standard with concentrations ranging from 12.5 to 250 μmol dm^{-3}. Radical scavenging capacity was determined spectrophotometrically using $DPPH^{\bullet}$ radical scavenging capacity assay[22] and $ABTS^{\bullet+}$ radical cation decolorization assay.[23] A calibration curve was prepared using Trolox.

Statistical and Mathematical Analyses

All presented numeric values are means of three measurements ± standard deviation (SD). One-way ANOVA and post-hoc multiple mean comparison (Tukey's HSD test) were performed by PAST (v.1.97) software package.[24] Differences at $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Phytochemicals Content

The TP and TF contents of *R. intermedia* extracts expressed as mg galic acid equivalents per g dry weigt (mg GAE g^{-1} DW) and mg catechin equivalents per g dry weigt (mg CE g^{-1} DW), respectively, are shown in Figure 1. The average TP content of leaf extract amounted to 46.40 ± 4.12 mg GAE g^{-1} DW while the average TF content was 8.28 ± 0.50 mg CE g^{-1} DW. This is 2.4-fold and 2.3-fold higher than the TP and TF contents, respectively, of bark extracts (TP: 19.31 ± 1.60 mg GAE g^{-1} DW, TF: 3.58 ± 0.10 mg CE g^{-1} DW). Our results are in accordance with the results of Muanda *et al.*[25] who found 4.4 and 2.9-fold higher TP and TF contents, respectively, of leaf in comparison to stem barks extracts of a Malian medicinal plant *Vitex doniana* Sweet. *R. intermedia* leaf extract exhibited higher TP content than methanol extracts of 133 analyzed Indian medicinal plants[10] and 44 medicinal plants (expect *Sargentodoxa cuneata*) analyzed by Li *et al.*[11] Also, the

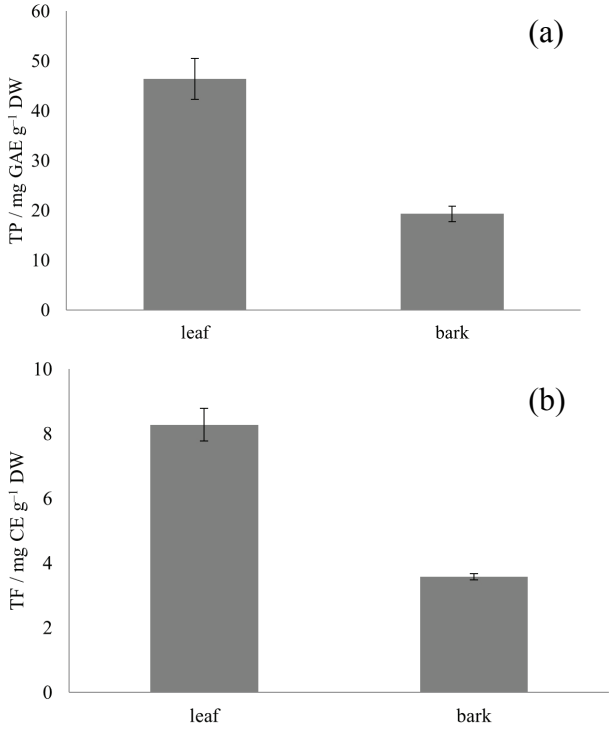

Figure 1. Total phenol (TP) (a) and total flavonoid (TF) (b) contents of leaf and bark extracts of *R. intermedia* expressed in milligrams of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW) and milligrams of catechin equivalents per gram of dry weight (mg CE g^{-1} DW), respectively.

Table 1. Content of free phenolic acids dry weight (μmol g^{-1} DW) in leaf and bark methanol extracts of *R. intermeda*

Acid	Leaf	Bark
protocatechuic	1048.78 ± 53.43	50.55 ± 2.36
4-hydroxybenzoic	161.10 ± 3.14	83.88 ± 0.79
3-hydroxybenzoic	nd	nd
gallic	261.64 ± 16.50	50.00 ± 0.00
salicylic	60.55 ± 2.36	111.10 ± 14.14
chlorogenic	351.08 ± 18.85	nd
vanillic	1647.06 ± 79.35	nd
caffeic	141.45 ± 7.07	nd
syringic	112.21 ± 6.28	nd
4-coumaric	69.44 ± 5.50	8.33 ± 0.80
ferulic	95.55 ± 1.57	56.11 ± 3.93
sinapic	nd	nd

leaf extract from *R. intermedia* has shown a 4-fold higher TP content in comparison to the aqueous leaf extracts from the Croatian endemic plant *Teucrium arduini* studied earlier.[26]

We also quantified ten free phenolic acids in leaf extracts (protocatechuic, *p*-hydroxybenzoic, gallic, salycilic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric and ferulic) and six phenolic acids in bark extracts (protocatechuic, *p*-hydroxybenzoic, gallic, salycilic, *p*-coumaric and ferulic). Significantly higher content of all detected phenolic acids, exept salicylic (leaf: 60.55 ± 2.36 μmol g^{-1} DW; bark: 111.10 ± 14.14 μmol g^{-1} DW) acid was found in leaf extracts. Salicylic acid, initially isolated from the bark of willow trees, is one of the earliest known plant-derived therapeutic compounds and is used in traditional medicine across the globe to provide relief from pain and inflammation.[27] Salycilic acid is also commonly used in dermatology in anti-acne treatments.[28,29] Abudance of salycilic acid in *R. intermedia* bark indicated that salycilic acid is probably the active component in bark of some *Rhamnus* species used in Bosnia and Herzegovina to treat mange and skin diseases.[14]

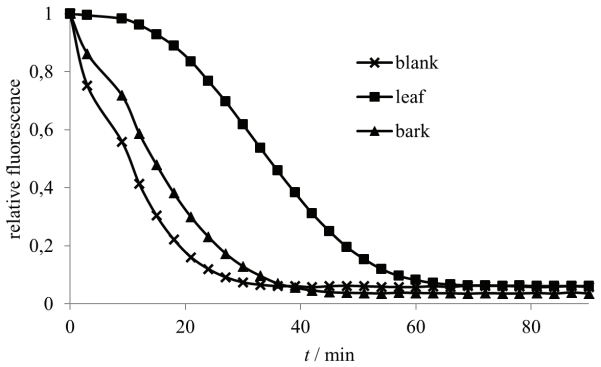

Figure 2. Fluorescence decay curves of fluorescein induced by AAPH in the presence of *R. intermedia* leaf and bark extracts diluted 1 : 300 in 75 mmol dm^{-3} phosphate buffer (pH = 7.4).

Table 2. Antioxidant capacity of *R.intermedia* methanol extracts mesaured dry weight (DW) by four different assay

	leaf	bark
FRAP / μmol Fe^{2+} g^{-1} DW	242.98 ± 12.74	92.24 ± 9.12
ABTS / μmol TE$^{(a)}$ g^{-1} DW	213.70 ± 16.11	70.72 ± 0.68
DPPH / μmol TE$^{(a)}$ g^{-1} DW	197.42 ± 11.68	57.67 ± 2.65
ORAC / μmol TE$^{(a)}$ g^{-1} DW	738.27 ± 58.58	117.65 ± 14.43

$^{(a)}$ TE-Trolox Equivalents

Antioxidant Capacity

Studies on antioxidants present in plants and foods have come to be one of the most popular topics in the area of food and agriculture research, consequently, many assays for the investigation of antioxidant activity have been developed and applied. Because agreement has not been reached about a standardized method, most studies

focused on quantifying the antioxidant capacity of plants and their components typically employ more than two different methods.[30] We employed the FRAP, DPPH, ABTS and ORAC assays to get a more comprehensive picture of the antioxidant capacity of *R. intermedia* leaf and bark extracts. Our choice was based on previous positive experience with these techniques and a high degree of reproducibility of results obtained using these methods. The results are presented in Table 2.

According to all four assays, bark extract has shown a significantly lower antioxidant capacity in comparison to the leaf extract (2.6-fold by FRAP, 3.0-fold by ABTS, 3.4-fold by DPPH and 6.3-fold by ORAC). Higher antioxidant capacity of leaf extracts in comparison to bark extracts has already been demonstrated for Amazonian plant species *Bauhinia forficata* Link, *Byrsonima crassifolia* (L.) Humb., Bonpl. et Kunth, *Cecropia palmata* Willd., *Davilla kunthii* A. St.-Hil., *Davilla rugosa* Poir. and *Inga edulis* Mart. using ABTS and ORAC assays[31] as well as *Davilla kunthii*, *Davilla rugosa* and *Inga edulis* using the ORAC and TRAP assays.[32] The antioxidant capacities obtained in the ABTS assay for *R. intermedia* leaf (213.70 ± 16.11 μmol TE g^{-1} DW) and bark (70.72 ± 0.68 μmol TE g^{-1} DW) extracts are greater than those obtained in the DPPH assay (leaf: 197.42 ± 11.68 μmol TE g^{-1} DW, bark: 57.67 ± 2.65 μmol TE g^{-1}DW). This observation was reported earlier for *Vaccinium corymbosum* leaf infusins,[33] and explained by the fact that $ABTS^{\bullet+}$ radical reacts with a wider range of compounds, including both hydrophilic and lipophilic antioxidants.[34] Furthermore, the ORAC value of *R. intermedia* methanol extracts (leaf: 738.27 ± 58.58 μmol TE g^{-1} DW , bark: 117.65 ± 14.43 μmol TE g^{-1} DW) was greater than the antioxidant capacity measured either by ABTS or DPPH assay (3.4, and 3.7-fold, respectively, for leaf and 1.6 and 2.04-fold, respectively, for bark extracts). The ORAC method is the only antioxidant assay that takes the free radical reaction to completion and uses an area-under-the-curve (AUC) for quantification, Figure 2, thereby combining both the inhibition percentage and the length of the inhibition time of the free radical's action into a single quantity.[32] It is also the only method measuring fluorescence decay, which is technically much more sensitive to small variations in concentration.[33]

CONCLUSION

The results of our experiment indicate that *R. intermedia* leaf and bark extracts exhibit high antioxidant capacity and contain appreciable concentrations of phenolic phytochemicals. We observed significantly higher antioxidant capacity, TP, and TF contents in leaf in comparison to bark extracts. With the exception of salicylic acid, significantly higher content of all free phenolic acids was found in leaf extracts. We therefore conclude that leaf and bark extracts from *R. intermedia* are important sources of phenolic phytochemicals with antioxidant properties that may be of interest in medicinal and commercial applications.

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