Rhamnus intermedia Steud. et Hochst. - a new source of bioactive phytochemicals
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Repositori / Repository: Repository of Faculty of Pharmacy and Biochemistry University of Zagreb

Vrsta objekta / Object type: Paper published in journal

Verzija rada / Publication status: Published version

Naslov izvornika / Source title: Croatica Chemica Acta

Godina izdavanja / Publication year: 2012

Svezak / Volume: 85

Stranice / Pages: 125 - 129

Trajna poveznica / Permanent link: https://urn.nsk.hr/urn:nbn:hr:163:150461

DOI: https://doi.org/10.5562/cca1946

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Datum pohrane u repositoriju / Date of storage: 2020-04-17

Datum preuzimanja / Date downloaded: 2020-04-30
**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 prevention of chronic diseases. Positive effects of plant polyphenolics, and their potential role in the prevention of 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 anthraquinones, due to their purgative effect. The fruit of *R. cathartica* contain anthraquinone derivates, especially frangulin and glucofrangulin and flavonols (cathartin, 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 physochloren). Their total antimutagenic and anti-inflammatory properties, 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 vanillic 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

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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 AlCl₃) and free phenolic acids (UPLC-MS/MS).

EXPERIMENTAL

Chemicals and Instruments

Except for the FeSO₄ × 7H₂O (Kemika, Croatia), NaNO₂ (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 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 AlCl₃ colorimetric assay adapted to small volumes and catechin was used as the standard. Free phenolic acids were determined by UPLC-MS/MS as described earlier. The results were expressed in μmol g⁻¹ DW (DW, dry weight).

Antioxidant Activity

The FRAP assay was used to estimate the antioxidant potential of tested extracts. A calibration curve was prepared, using FeSO₄ × 7H₂O. 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. 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 radical scavenging capacity assay and ABTS radical cation decolorization assay. 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. 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 weight (mg GAE g⁻¹ DW) and mg catechin equivalents per g dry weight (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. 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 and 44 medicinal plants (expect Sargentodoxa cunea) analyzed by Li et al. Also, the
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.

We also quantified ten free phenolic acids in leaf extracts (protocatechuic, *p*-hydroxybenzoic, gallic, salicylic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric and ferulic) and six phenolic acids in bark extracts (protocatechuic, *p*-hydroxybenzoic, gallic, salicylic, *p*-coumaric and ferulic). Significantly higher content of all detected phenolic acids, except 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.\(^27\) Salicylic acid is also commonly used in dermatology in anti-acne treatments.\(^{28,29}\) Abundance of salicylic acid in *R. intermedia* bark indicated that salicylic acid is probably the active component in bark of some *Rhamnus* species used in Bosnia and Herzegovina to treat mange and skin diseases.\(^14\)

### 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.\textsuperscript{30} We employed the FRAP, DPPH, ABTS and ORAC assays to get a more comprehensive picture of the antioxidant capacity of \textit{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 \textit{Baubinia forficata} Link, \textit{Byronsonia crassifolia} (L.) Humb., Bonpl. et Kunth, \textit{Cecropia palmata} Willld., \textit{Davilla kunthii} A. St.-Hil., \textit{Davilla rugosa} Poir. and \textit{Inga edulis} Mart. using ABTS and ORAC assays\textsuperscript{31} as well as \textit{Davilla kunthii}, \textit{Davilla rugosa} and \textit{Inga edulis} using the ORAC and TRAP assays.\textsuperscript{32} The antioxidant capacities obtained in the ABTS assay for \textit{R. intermedia} leaf (213.70 ± 16.11 µmol TE g\textsuperscript{-1} DW) and bark (70.72 ± 0.68 µmol TE g\textsuperscript{-1} DW) extracts are greater than those obtained in the DPPH assay (leaf: 197.42 ± 11.68 µmol TE g\textsuperscript{-1} DW, bark: 57.67 ± 2.65 µmol TE g\textsuperscript{-1} DW). This observation was reported earlier for \textit{Vaccinium corymbosum} leaf infusions,\textsuperscript{33} and explained by the fact that ABTS\textsuperscript{•+} radical reacts with a wider range of compounds, including both hydrophilic and lipophilic antioxidants.\textsuperscript{34} Furthermore, the ORAC value of \textit{R. intermedia} methanol extracts (leaf: 738.27 ± 58.58 µmol TE g\textsuperscript{-1} DW, bark: 117.65 ± 14.43 µmol TE g\textsuperscript{-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 into account 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.\textsuperscript{32} It is also the only method measuring fluorescence decay, which is technically much more sensitive to small variations in concentration.\textsuperscript{33}

**CONCLUSION**

The results of our experiment indicate that \textit{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 \textit{R. intermedia} are important sources of phenolic phytochemicals with antioxidant properties that may be of interest in medicinal and commercial applications.

**Acknowledgements.** This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (projects "Molecular regulation of plant development" no. 098-0982913-2829 and "Micromorphological and chemotaxonomic research on some species of family \textit{Lamiaceae}"), as well as by the Academy of Sciences of the Czech Republic (KAN 200380801) and Czech Ministry of Education ( MSM 6198959216).

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