SUPPLEMENTARY MATERIAL

Phenolic compounds and bioactive properties of *Verbascum glabratum* subsp. *bosnense* (K. Malý) Murb., an endemic plant species

Erna Karalija\textsuperscript{a}, Adisa Parić\textsuperscript{a}, Sabina Dahija\textsuperscript{a}, Renata Bešta-Gajević\textsuperscript{b}, Sanja Ćavar Zeljković\textsuperscript{c,d}*  
\textsuperscript{a}Laboratory for Plant Physiology, Department of Biology, Faculty of Science, University of Sarajevo, Zmaja od Bosne 33-35, 71 000 Sarajevo, Bosnia and Herzegovina  
\textsuperscript{b}Laboratory for Microbiology, Department of Biology, Faculty of Science, University of Sarajevo, Zmaja od Bosne 33-35, 71 000 Sarajevo, Bosnia and Herzegovina  
\textsuperscript{c}Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Genetic Resources for Vegetables, Medicinal and Special Plants, Crop Research Institute, Šlechtitelů 29, 78371 Olomouc, Czech Republic  
\textsuperscript{d}Centre of the Region Haná for Biotechnological and Agricultural Research, Central Laboratories and Research Support Faculty of Science, Palacky University, Šlechtitelů 27, 78371 Olomouc, Czech Republic  

*Corresponding author. E mail: sanja.cavar@upol.cz; zeljkovic@vurv.cz; sanjacavar.sc@gmail.com.
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Abstract

Detailed analysis of phenolic composition and antioxidant and antimicrobial activities of *Verbascum glabratum* subsp. *bosnense* (K. Malý) Murb, an endemic species of southeastern Dinaric Alps was performed for the first time. The phenolic composition measured via UHPLC-MS/MS of four extract with different polarity suggested this plant species is very rich in both phenolic acids and flavonoids. Ethanol extract was chemically the most versatile containing 12 compounds with quercitrin and rosmarinic acid as the majors, while water extracts were rich in 4-hydroxybenzoic acid, salicylic acid, morin, and apigenin. All extracts showed high antioxidant potential measured spectrophotometrically with IC$_{50}$ values ranging 0.139 - 0.021 mg/mL. Antimicrobial testing using agar diffusion test showed that ethanol extract was the most potent against all tested organisms. Also, these activities are correlated with the content of phenolic compounds, which suggest they are active ingredients of the extracts.

Keywords: *Verbascum glabratum* subsp. *bosnense* (K. Malý) Murb.; phenolic compounds; UHPLC-MS/MS; antioxidant activity; antimicrobial activity.

Experimental

*Collection of samples and extract preparation*

Inflorescence samples of *Verbascum glabratum* subsp. *bosnense* (K. Malý) Murb. were collected at Mt. Jahorina in Bosnia and Herzegovina (Coordinates: 43.707445 latitude; 18.578821 longitude), during flowering stage in July 2017 (Figure S1). Specimens were authenticated by the botanist Mr.sc. Aldin Boskailo, University Dzemal Bijedic and deposited at the Herbarium of Department of Biology, Faculty of Science, University of Sarajevo under the Voucher no. 355.

Inflorescence was air dried for 7 days at room temperature (23 °C) in shaded, well ventilated laboratory. Dried samples were finely powdered in the mill and stored at +4 °C until use.
Each of 500 mg of powdered plant material was soaked in 12.5 mL of solvent (petrol ether, chloroform, ethanol, and water) and sonicated for 30 min at 23 °C. Supernatant was removed and sediment was again soaked into same solvent. Due to the high evaporation rate of petrol ether and chloroform these extracts were evaporated to dryness and re-suspended in dimethyl sulfoxide (DMSO).

**UHPLC-MS/MS analysis**

UHPLC-MS/MS was performed on UltiMate™ 3000 liquid chromatographic system consisting of binary pumps, an autosampler and a column thermostat coupled to a TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed on an Acquity BEHC18 (150 × 3.0 mm; 1.7 µm particle size) UHPLC column (Waters Corp., Milford, MA, USA) kept at 40°C. The mobile phase consisted of 10 mM formic acid in water (component A) and acetonitrile (component B). Totally 29 compounds (15 phenolic acids and 14 flavonoids) were separated using a binary gradient starting at 5% B for 0.8 min, increasing to 10% B in 0.4 min with isocratic run for 0.7 min, then increasing to 15% B for 0.5 min and isocratic run for 1.3 min, then increasing to 20% B for 0.3 min and isocratic for 1.2 min, then increasing to 25% B for 0.5 min with next increase to 35% B within 2.3 min, then increasing to 70% B for 2.5 min, then further increase to 100% B for 1 min, with isocratic run for 1 min, and then back to 5% B for 0.5 min. Finally, the equilibration to the initial conditions took 3.3 min, with total chromatographic run of 16 min. The flow rate was 0.4 mL/min and the injection volume 10 µL.

All analytes were detected in negative ionization mode ESI-. Multiple reaction monitoring (MRM) mode was used for their quantification. The MRM transitions are listed in table below, together with their optimal collision energies and retention times. The spray voltage was 3 kV, and the vaporizer the ion transfer tube temperatures were 320°C.

Standard solutions of 32 target compounds (apigenin, 2,3-dihydroxybenzoic acid, caffeic acid, carnosic acid, catechin, chlorogenic acid, chrysin, ferulic acid, galangin, gallic acid, hesperidin, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 5-hydroxyferulic acid, kaempherol, methyl p-coumarate, morin, myricetin, naringenin, naringin, p-coumaric acid, pinocembrin, quercetin, quercitrin, rosmarinic acid, rutin,
salicylic acid, salicylic acid glucoside, sinapic acid, syringic acid, trans-cinnamic acid, and vanillic acid), purchased from Sigma Aldrich Company, Germany, were firstly prepared in methanol at 1 mM concentrations, and solutions were gradually diluted in the mobile phase to the working concentrations that ranged from 0.01 to 50 µM. Quantification was performed by isotop diluting method using p-coumaric acid-d₆ and salicylic acid-d₄.

MS/MS chromatograms of each extracts are presented in Supplementary material (Figure S2-S5).

**DPPH Assay**
DPPH (2,2-diphenyl-1-picrylhydrazyl radical) antioxidant capacity was evaluated for all four extracts and standards according to Meda et al. (2005). Antioxidant potential was evaluated according to the absorbance change, and presented as percent of scavenged DPPH radical. Naringenin was used as reference substance.

**Antimicrobial assay**
Agar well diffusion method was used to evaluate antimicrobial activity of plant extracts and standards according to National Committee for Clinical Laboratory Standards (NCCLS) (Valgas et al., 2007; Magaldi et al., 2004). Each well contained 100 µL of extract or standard. Bacterial strains used in the analysis included Gram positive: *Enterococcus faecalis* ATCC® 19433™, *Staphylococcus aureus* subsp. *aureus* ATCC® 6538™ and Gram negative bacteria: *Salmonella abony* NCTC® 6017™, *Escherichia coli* ATCC® 8739™, and yeast *Candida albicans* ATCC® 10231™. Bacterial strains were used as standardised inoculum of 5x10⁵ CFU/mL using McFarland standard (McFarland, 1907).

Müller-Hinton and Sabouard medium were used for cultivation of bacterial strains and yeast, respectively. Ampicillin, was used as positive standards for bacterial strains and nystatin for *Candida albicans*. Ethanol and DMSO were used as negative controls. Antimicrobial effect was expressed as a diameter of inhibition zone in mm reduced by the inhibition zone of negative controls if appropriate.
**Statistical analysis**

All data were analysed using the STATISTICA 10.0 software (Statsoft Inc.). Experimental results were presented in tables as the mean ± standard deviation of three independent replications. Obtained data were subjected to variance analysis (ANOVA) and the Tukey post hoc test was carried out to identify significant differences between extract types. Mean values with p <0.01 were considered statistically significant. Pearson correlations were performed to observe the possible correlation between the phenolic profile, antioxidant capacity and detected antimicrobial activity. Matrix plot for the graphical abstract was created using Past 3.20 software (Hammer, 2001).

**References**

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| Extract/Sample    | Antioxidant activity (IC$_{50}$ mg/mL) | Salmonella *aboni* | Escherichia *coli* | Enterococcus *faecalis* | Staphylococcus *aureus* | Candida *albicans* |
|------------------|----------------------------------------|--------------------|--------------------|--------------------------|--------------------------|-------------------|
| Petroleum        | 0.139±0.008                            | 12.667±0.577       | 12.333±0.577       | 11.667±0.577             | 12.333±0.577             | 16.333±1.082      |
| Chloroform       | 0.175±0.010                            | n.d.               | 14.333±0.577       | 13.667±0.577             | 12.667±1.155             | 18.667±1.309      |
| Ethanol          | 0.028±0.004                            | 13.667±0.577       | 11.333±0.577       | 15.667±0.577             | 16.333±1.155             | 20.667±1.309      |
| Water            | 0.021±0.003                            | n.d.               | n.d.               | n.d.                     | n.d.                     | n.d.              |
| Salicylic acid   | 0.003±0.000                            | 13.333±0.000       | 13.000±0.000       | 13.000±0.000             | n.d.                     | 25.333±0.000      |
| Morin            | 0.005±0.000                            | n.d.               | n.d.               | 15.667±0.000             | 13.667±0.000             | 18.000±0.000      |
| Amoxicillin/Nystatin | -                         | 17.000±1.000      | 14.000±0.000      | 19.333±0.577            | 33.333±2.309            | 19.667±1.155      |

n.d. – not detected.

**Table S1.** Antimicrobial and antioxidant potential of *V. glabratum* subsp. *bosnense* extracts and standards.
Figure S1. *Verbascum glabratum* subsp. *bosnense* (K. Malý) Murb.

Figure S2. MS/MS chromatogram of petrolether extract of *V. glabratum* subsp. *bosnense*. 
Figure S3. MS/MS chromatogram of chloroform extract of *V. glabratum* subsp. *bosnense*.

Figure S4. MS/MS chromatogram of ethanol extract of *V. glabratum* subsp. *bosnense*.
Figure S5. MS/MS chromatogram of aqueous extract of *V. glabratum* subsp. *bosnense*. 