SUPPLEMENTARY MATERIAL

Biophenols and antioxidant activity in wild and cultivated heather

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Abstract

Over the last decade there has been an increasing interest to study the antioxidants from natural sources which can be used to replace the synthetic compounds commonly used in food or as cosmetic ingredients. *Calluna vulgaris* (L.) Hull (heather), a plant grown in most parts of Europe and Northern America, has been used in ethnopharmacology. The content of selected biophenols and the antioxidant capacity of the extracts of wild heather collected from natural environmental localities of central Poland has been assessed in this study and compared with cultivated plant. Chlorogenic acid was the major biophenolic compound present in the extracts, followed by a high amount of catechins. The reducing power of the extracts evaluated by Folin-Ciocalteu assay were in the range of 75.7 - 89.1 mg GA/g dry flowers. The cultivated plant extract showed the largest radical scavenging on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals.

Keywords: *Calluna vulgaris*; Biophenols; Antioxidant properties

Plant materials and their extraction

C. vulgaris flowers were collected during September of 2014, in three different locations in central Poland (the Mazovia plain). The sample 1 (S1) was from 12-years old pine forest (N 52° 41', E 21° 29'), the sample 2 (S2) from 25-year old pine forest (N 52° 49', E 21° 45') and the sample 3(S3) from 77-years old pine wood (N 52° 01', E 21° 06'). The voucher specimen was authenticated by Dr Catherina Fyalkowska from Forest Ecology Department of Forest Research Institute, Poland. We selected a limited area, central part of Poland, in order to have uniform climatic conditions. The sample of the cultivated garden *C. vulgaris* plant (S4) was obtained from the specialist garden store (PNOS Warszawa, Poland). The collected plant material was air dried at ambient temperature (~20°C) and stored in paper bags until performing analysis.

The dry plant material (700 mg) was mixed with 25 mL of ethanol-water solution (60:40, v/v) for 20 min at room temperature. Then, the extracts were filtered through Whatman No.1 filter paper. For a given sample three independent extractions using appropriate solvent were carried out.

Biophenols quantification

Chromatographic analysis was performed using a Shimadzu high performance liquid chromatography system equipment with a binary pump, degasser, autosampler and connected to 3200 QTRAP Mass spectrometer (Applied Biosystem/MDS SCIEX). A MS system (3200 QTRAP, Applied Biosystem/MDS SCIEX) was equipped with an electrospray ionization source (ESI) operated in negative-ion mode and a quadrupole mass analyser in a scan mode from 50 to 1500 m/z. Nitrogen was used as curtain and auxiliary gas at 0.3 MPa.

Chromatographic separations were evaluated on a Kinetex™ C-18 column (100 x 2.1 mm, 2,6 µm) from Phenomenex with formic acid (2 mmol/L, pH 2.8) as eluent A and acetonitrile as eluent B. The mobile phase was delivered at 0.2 mL/min in the following gradient mode: 0-5 min. 20% B, 10 – 15 min 25 % B, 20-25 min 30% B, 30-31 min 90% B, 32 min 20% B. Compounds were identified by comparing retention time and m/z values obtained by MS and MS² with the mass spectra from standards tested under the same conditions. Quantification of compounds was done from the calibration curves obtained in Multiple Reaction Mode (MRM).

The total flavonoid content

1 mL of a sample was mixed with 0.3 mL of NaNO₂ (5%, w/v) and after 5 min 0.5 mL of AlCl₃ (2%, w/v) was added. A sample was mixed and six minutes later was neutralized with 0.5 mL of 1 M NaOH solution. The mixture was left for 10 min at room temperature and then subjected to spectral analysis. Catechin (in the 50-500 µM concentration range) was the standard of choice for the expression of results at 510 nm.

The reducing power

The reducing power of *C. vulgaris* extracts was evaluated using Folin-Ciocalteu (FC) assay. 0.1 mL of sample was mixed with 0.1 mL of FC reagent (from Merck) and 0.9 mL of water. After 5 min, 1 mL of 7% (w/v) Na₂CO₃ and 0.4 mL of water were added. The mixture was incubate for 30 min. The absorbance was measured at 765 nm against the reagent blank. The results were expressed as mg of gallic acid per gram of dry sample. Each analysis was done in three repetitions.

2.5 The scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The DPPH assay was applied to estimate the radical-scavenging ability of the heather extracts. 0.1 mL of a given extract was mixed with 2.4 mL DPPH solution (9×10^{-5} M) in methanol and immediately the change of absorbance at 518 nm was recorded over time against the blank. Trolox, a vitamin E analogue, solution (in the concentration range of 1-10 μ M) was used to calibrate the standard curve. The mean $\pm$ SD results of triplicate analyses were expressed in mg of trolox per gram of dry sample.