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Methodological aspects of biologically active compounds quantification in the genus *Hypericum*

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ABSTRACT

Accumulation of selected secondary metabolites in two *Hypericum* species (*H. perforatum* and *H. annulatum*) was compared in their vegetative parts (stems and leaves) and in terms of the extraction solvent (80% aq. methanol or 60% aq. ethanol). The presence of chlorogenic acid and quercitrin was not detected in stem of both species. Almost all metabolites were more accumulated in the leaves than in the stems (rutin, hyperoside, quercetin and hypericin) but epicatechin showed the opposite in both species and hyperforin in *H. annulatum*. Extraction solvents showed rather species-specific differences with EtOH being more suitable for the extraction of hypericin, quercetin, quercitrin, and hyperoside (on average, for both the leaves and stems, extraction increased by approximately 130, 30, 25, and 15%, respectively) while MeOH for the extraction of epicatechin, rutin, and hyperforin (increased extraction by approximately 50, 40, and 35%, respectively). On the other hand, content of total soluble phenols did not differ in relation to solvent in any organ or species. Various ages of *H. annulatum* plants did not show dramatic impact on the amount of metabolites. Subsequently, the usefulness of capillary electrophoresis (CE) as an alternative to HPLC for the quantification of metabolites in *H. perforatum* was tested and results showed non-significant differences between CE and HPLC with the methods we developed (the difference did not exceed 10%).

1. Introduction

The *Hypericum* genus includes almost 500 species of herbs, shrubs and trees with numerous health positive effects [1]. Despite the very large number of species in the genus *Hypericum*, mainly *Hypericum perforatum* has been widely studied with regard to the phytochemistry and pharmacological activity [2]. However, several *Hypericum* species are used in traditional medicine for the treatment of burns or skin injuries and for its antidepressant, antimicrobial, and anti-inflammatory activity [1]. Therapeutic effect of *Hypericum* plants is related to the presence of various bioactive compounds, including flavonoids (e.g. rutin, hyperoside, quercetin, and quercitrin), phloroglucinols (e.g. hyperforin), naphthodianthrones (e.g. hypericins), phenolic acids (such as chlorogenic acid) and constituents of essential oil [2]. However, biochemical composition strongly differ between species [3–5] and detailed profiling of some of them as well as physiological significance *in planta* have only rarely been studied [6,7].

Wide range of secondary metabolites produced by the genus *Hypericum* indicates that they can only hardly be monitored by a single method or extracted by a single solvent. Liquid chromatography is the most widely used for quantitative analyses [8–10] and qualitative approaches have also been tested for *Hypericum* [11] or other matrices [12]. Earlier report concluded that capillary electrophoresis (CE) technique was not sufficiently sensitive for some *Hypericum* metabolites but flavonoids were not considered [13]. For the extraction of *Hypericum* metabolites, pure ethanol or methanol were frequently used [3,9,14] but tissue-specific localization of individual metabolites has only rarely been studied [14].

The aim of the present work was to study the variation pattern in the content of major flavonoids, chlorogenic acid, hypericin and hyperforin in the vegetative tissue (leaves and stems) of two *Hypericum* species (*H. perforatum* and *H. annulatum*) along with the comparison of various age of *H. annulatum* plants. We also compared two extraction solvents: 80% aq. methanol (MeOH) was used as first choice owing to its wide used in the extraction of phe-

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nolics also from *Hypericum* [7] and 60% aq. ethanol (EtOH) was subsequently used owing to values comparable with 80% MeOH in various matrices [12]. Additionally, we verified the usefulness of capillary electrophoresis (CE) technique as an alternative to high-performance liquid chromatography (HPLC) for the analysis of secondary metabolites in the leaves of *H. perforatum*. Data are compared with available studies and practical significance is also suggested.

2. Materials and methods

2.1. Plant material and statistics

The seeds of the studies species (*H. perforatum* and *H. annulatum*) were obtained from the botanical gardens and pre-cultured under *in vitro* conditions during 2016 as reported previously [3]: thereafter plants were planted to soil (experimental field in the Botanical garden of P.J. Safarik University in Košice, Slovakia) and collected in November 2016. Plants marked as "*H. annulatum* 2015" in results were pre-cultured *in vitro* in 2015, adapted to soil in the same year and planted outdoor in 2016 as mentioned above. All samples were therefore collected in November 2016 and separated to leaves and stems. Air-dried samples were kindly provided by Prof. Eva Čellárová (Department of Genetics, Institute of Biology and Ecology, P.J. Šafárik University in Košice, Slovakia).

The plant material was dried for 3 h at 60 °C to remove residual humidity and then milled into fine powder using a laboratory grinder IKA A11 (IKA-Werke, Staufen, Germany). The powder of 250 mg of dry material was extracted three times with a fresh portion of 80% aq. methanol p.a. (MeOH in the subsequent text) or 60% aq. ethanol p. a. (EtOH in the subsequent text) in an ultrasonic bath for 30 min at a temperature below 40 °C. The extracts obtained were centrifuged at 10000 × *g* for 5 min and the mixture of the three supernatants was filtered through a 0.22 μm membrane filter prior to the analysis in HPLC and CE (the final volume of each sample was 250 mg DW/5 mL of 80% MeOH or 60% EtOH).

Significance of differences was evaluated using Student's *t*-test. Additionally, principal component analysis (PCA) of the secondary metabolites concentration was carried out. The data were analyzed using Statistica ver. 12 (StatSoft., Inc. 2014). Three individual plants and two technical repetitions were tested for each species and/or organ.

2.2. Assay of total soluble phenols

The EtOH or MeOH extracts mentioned above were assayed for total soluble phenols using standard protocol with the Folin-Ciocalteu reagent and gallic acid as a standard for the calibration curve as reported earlier [12]. Primary extracts were diluted 5-times with respective solvent prior to measurement (i.e. 250 mg DW/25 mL). Reaction mixture (final volume 1.5 mL) consisted of 30 μL of the extract, 470 μL of redistilled water, 975 μL 2% Na_2CO_3 and 25 μL (2 N) Folin-Ciocalteu reagent (Sigma-Aldrich, Germany).

2.3. HPLC analysis

The chromatographic analyses were performed on a VWR Hitachi Elite LaChrom HPLC equipped with a diode-array detector (UV-VIS, DAD) (190–700 nm) and EZChrom Elite software (Merck, Darmstadt, Germany). The chromatographic system for analysis of chlorogenic acid and flavonoids was used as follows: an RP18 reversed-phase column Kinetex (Phenomenex, Torrance, CA, USA) (10 cm × 4.6 mm i.d., 2.6 μm particle size) at 25 °C. A mixture of acetonitrile with 0.025% of trifluoroacetic acid (solvent A) and water with 0.025% of trifluoroacetic acid (solvent B) were used as the mobile phase. The compounds were separated by gradient elution with program: 0–8.5 min A 7%, B 93%; 8.5–15 min A 7–12%, B 93–88%; 15–29 min A 7–12%, B 93–88%; 29–40 min A 12–20%, B 88–80%; 40–50 min A 20–25%, B 80-75%. Flow rate was 1.1 mL/min.

The isocratic chromatographic system for analysis of hyperforin and hypericin was used as follows: an RP18 reversed-phase column Kinetex (Phenomenex, Torrance, CA, USA) (10 cm × 4.6 mm i.d., 2.6 μm particle size), a mixture of acetonitrile with 0.025% of trifluoroacetic acid–200 mM ammonium acetate aqueous solution (70:30 v/v) as a mobile phase, flow rate 1.0 mL/min, temperature of 20 °C.

2.4. Capillary electrophoresis (CE)

The analyses were performed on an Agilent 7100 capillary electrophoresis system (CE) equipped with a diode-array detector (UV-VIS, DAD, 190–600 nm, Agilent Technologies, Santa Clara, CA). The flavonoids and chlorogenic acid were separated in bare fused-silica 50 μm i.d. capillaries (Agilent Technologies, Santa Clara, CA, USA) with an effective total length of 64.5 cm. The samples were injected by pressure (50 mbar) for 4 s followed by BGE (background electrolyte) injection for 4 s (50 mbar). The flavonoids-BGE contained 40 mM Borax (POCH, Gliwice, Poland), 50 mM SDS (sodium dodecyl sulphate, Sigma-Aldrich, St. Louis, MO, USA), and 12% acetonitrile (Sigma-Aldrich, St. Louis, MO, USA). A voltage of 30 kV was applied during the analysis.

The analysis of the lipophilic compounds in *Hypericum* extracts – hypericin and hyperforin was performed with the non-aqueous capillary electrophoresis method described by Jensen and Hansen [15] with minor modification. The separation of hypericin and hyperforin was conducted using bare fused-silica 75 μm i.d. capillaries (Agilent Technologies, Santa Clara, CA, USA) with an effective total length of 80.0 cm. The non-aqueous BGE consisted of a mixture of methanol (JT Baker, Phillipsburg, NJ, USA), dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA), *N*-methyl formamide (Sigma-Aldrich, St. Louis, MO, USA) (3:2:1 v/v/v) as a solvent, with 50 mM ammonium acetate (Sigma-Aldrich, St. Louis, MO, USA) 150 mM sodium acetate (Sigma-Aldrich, St. Louis, MO, USA), and 0.02% (w/v) of cationic polymer hexadimethrine bromide (Sigma-Aldrich, St. Louis, MO, USA) to reverse the flow. The samples were introduced by pressure (50 mbar) for 12 s followed by BGE injection for 4 s (50 mbar). The voltage was set at −20 kV.

2.5. Identification and quantification of metabolites

In order to identify and chose the maximum absorbance wavelength, spectral analyses between 190 and 600 nm were performed (see supplementary Fig. S1). Based on the retention times and spectra absorption of the standards, 8 compounds were identified in the *Hypericum* extracts (chlorogenic acid, epicatechin, hyperoside, rutin, quercitrin, quercetin, hyperforin, hypericin). As the standards showed different maximum absorbance wavelength in the HPLC and CE, the maximum absorbance wavelength for determination of each compound was established individually for HPLC and CE (supplementary Table S1). All standards were purchased from Sigma-Aldrich (Sigma-Aldrich St. Louis, MO, USA). The flavonoids (rutin, hyperoside, quercitrin, quercetin, epicatechin) and chlorogenic acid were prepared in 80% MeOH. Hypericin and hyperforin were dissolved in anhydrous 99.8% EtOH and anhydrous 99.8% MeOH, respectively. The standards were prepared at a 1000-ppm concentration and stored at 4 °C.