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Subcritical water extraction of antioxidants from mountain germander (*Teucrium montanum* L.)

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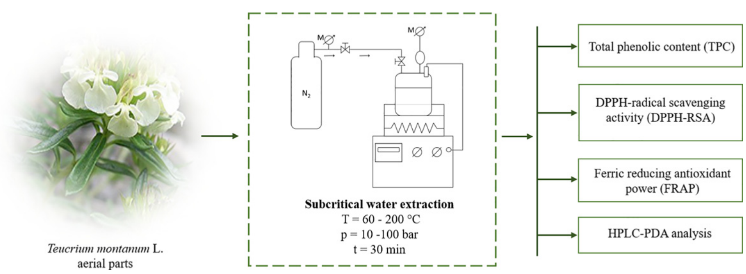
GRAPHICAL ABSTRACT



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ABSTRACT

In the present work, antioxidant compounds from *Teucrium montanum* were extracted by subcritical water. The influence of extraction temperature and pressure on antioxidant activity of extracts has been investigated in terms of extraction yield (EY), total phenolic content (TPC), and DPPH-radical scavenging activity (DPPH-RSA) and ferric reducing antioxidant power (FRAP). Additionally, the compounds responsible for the antioxidant activity were identified and quantified by high performance liquid chromatography (HPLC). The highest EY (42.63%), TPC (174.61 ± 4.09 mg GAE/g DE) and antioxidant activity by DPPH-RSA (176.23 ± 8.76 mg TE/g DE) and FRAP (141.71 ± 5.21 mg AAE/g DE) were seen in extracts obtained at temperature of 160 °C and pressure of 10 bar. HPLC analysis revealed that naringin and gallic acid were the principle antioxidant compounds in subcritical extracts. According to the results, SWE has a great potential in exploitation of natural sources of bioactive compounds and production of pharmacologically-active fractions.

1. Introduction

Naturally occurring phenolic compounds have been associated with numerous health-promoting effects such as antioxidant [1], anticarcinogenic [2], antimicrobial [3] and antiviral activity [4]. Other bioactivities which include antimutagenic [5], anti-inflammatory [6] and anti-allergic [7] have been also reported. The antioxidant activity of phenolic compounds is mainly due to their redox properties which allow them to act as reducing agents, hydrogen donors and singlet and triplet oxygen quenchers [8,9]. Phenolic compounds from natural sources are used in food industry for the prevention of lipid peroxidation which is associated with development of off-flavours and other

Abbreviations: AA, ascorbic acid; AAPH, 2,2'-azobis(2-methylpropionamide) dihydrochloride; DPPH-RSA, DPPH-radical scavenging activity; GA, gallic acid; FRAP, ferric reducing antioxidant power; HPLC, high performance liquid chromatography; HPLC-PDA, high performance liquid chromatography with photodiode array detection; SFE, supercritical fluid extraction; SWE, subcritical water extraction; TPC, total phenolic content; TPTZ, 2,4,6-tris(2-pyridyl)-s-triazine

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undesirable compounds [10].

Phenolic compounds from plants are isolated by different extraction techniques such as conventional ones, but more recently also by ultrasound extraction [11], microwave-assisted extraction [12] and pressurised liquid extraction [13]. Recently, there has been an increased interest in the use of environmentally clean and safe technologies such as supercritical fluid extraction (SFE) and subcritical water extraction (SWE) [11,14–16]. In addition to the previously mentioned advantages, extracts obtained by SWE usually show higher antioxidant activities in comparison to the ones obtained by conventional solvent [11,14,16] and SFE extraction [17,18].

In the last few decades, SWE has gained much popularity due to the replacement of toxic organic solvents with a safe and low-price solvent. This technique relying on heated and pressurized water improve extraction efficiency, among others, due to lower viscosity of the solvent and consequently better penetration into the pores of solid particles. Subcritical water enhances also mass transfer and desorption kinetics, potentiating the dissociation of the compounds from their complexes with matrix constituents [19]. Recently, a number of papers describing SWE of bioactive compounds from plants have been published [11,20–22].

Chemical profiles of SWE extracts depend on numerous factors such as sample itself, extraction mode and operational parameters. Two major operational parameters governing SWE are temperature and pressure of the extraction. In SWE, temperature has the major influence on the process because slight changes in operational temperature results in water polarity variations. In addition, temperature affects water viscosity and surface tension, as well as the interaction with the matrix [19]. The influence of temperature on bioactivity and composition of subcritical water plant extracts and extraction yields has been studied previously [21,23,24]. Number of scientific evidence evaluated the influence of pressure in SWE, as well [11,16,25]. In this work, the influence of both temperature and pressure was investigated and optimised in subcritical water extraction of phenolic compounds from *Teucrium montanum*.

Many species of *Teucrium* genus are used in ethnobotany, medicine and pharmacy due to their medicinal properties. *T. montanum* is used as a diuretic, analgesic and antispasmodic agent, as well as in the treatment of digestive disorders and pulmonary diseases. Some of the bioactive compounds previously identified in *T. montanum* include phenolic acids, mainly gentisic, chlorogenic and siringic, flavonoids, sesquiterpenes, potassium, magnesium and sodium [10,26–28]. According to Vukovic et al. [28], high antimicrobial activity of *T. montanum* could be associated with sesquiterpenes, such as δ-cadinene and α-selinene. Other authors reported that phenolic acids and flavonoids were the principal constituents of *T. montanum* extracts with antimicrobial and antioxidant activities [29]. Stanković et al. [9] indicated that phenols directly contribute to high antiproliferative and proapoptotic activities of *T. montanum* methanol extracts.

According to available literature, there are no reports on the use of subcritical water for the recovery of bioactive compounds from *T. montanum*. Thus, the aim of this study was to evaluate the efficiency of SWE for obtaining *T. montanum* extracts with high content of bioactive compounds. The influence of extraction temperature and pressure on antioxidant activity of *T. montanum* extracts has been investigated. Total content of phenolic compounds (TPC) was determined by Folin-Ciocalteau method. Antioxidant activity of the obtained extracts was essayed by DPPH-radical scavenging activity (DPPH-RSA) and ferric reducing antioxidant power (FRAP). Moreover, the bioactive compounds contributing to the antioxidant activity, namely phenolic compounds, were also identified and quantified by high performance liquid chromatography with photodiode array detection (HPLC-PDA).

2. Materials and methods

2.1. Chemicals and reagents

Folin Ciocalteau's phenol reagent, sodium carbonate (BioXtra), iron (II) chloride hexahydrate (p.a.), fluorescein sodium salt (for fluorescent tracers), TPTZ (2,4,6-tris(2-pyridyl)-s-triazine; p.a.), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid; *purum*), gallic acid monohydrate (GA; *purum*), DPPH and AAPH (2,2′-azobis(2-methylpropionamide) dihydrochloride; granular) were all acquired from Sigma-Aldrich (Steinheim, Germany). L-(+)-ascorbic acid (AA; p.a.), di-potassium hydrogen phosphate anhydrous (ultrapure) and sodium dihydrogen phosphate monohydrate (p.a.) were from Merck (Darmstadt, Germany). Sodium acetate 3-hydrate (p.a.) was purchased from PanReac AppliChem (Barcelona, Spain). Ethanol absolute anhydrous (p.a.) was acquired from Carlo Erba (Peypin, France). HPLC standards (protocatechuic acid (99.63%), (+)-catechin (≥98%), (−)-epicatechin (≥97%), vanillic acid (≥97%), β-resorcylic acid (≥97%), chlorogenic acid (> 95%), caffeic acid (≥98%), syringic acid (≥98%), *p*-coumaric acid (≥98%), ferulic acid (≥99%), sinapic acid (≥99%), rutin hydrate (≥94%), quercetin (95%), kaempferol (≥98%), naringin (≥95%), naringenin (98%) and cinnamic acid (≥99%) were purchased from Sigma-Aldrich (Sternheim, Germany) and all solvents employed were HPLC purity grade, filtrated and degassed prior to their use. All aqueous solutions were prepared using ultrapure water (18.2 MΩ cm). Nitrogen was of 99.999% purity (Messer, Germany). All other chemical and reagents were of analytical reagent grade.

2.2. Plant material

Commercially available dry *T. montanum* material was used (Adonis D.O.O., Sokobanja, Serbia). The aerial parts were grounded in a blender, providing an average particle size of 0.34 mm, and stored in dark at ambient temperature.

2.3. Subcritical water extraction

SWE was performed in a house-made subcritical water extractor. Extraction procedure and apparatus were described previously [22]. Total capacity of high-pressure stainless-steel vessel was 1.7 l. Pressurization of the vessel was performed with nitrogen to prevent possible oxidation. In all experimental runs, sample to distilled water ratio was 1:10. Extraction temperature (60–200 °C) and extraction pressure (10–100 bar) were investigated as independent variables, while all other parameters were held constant (agitation rate of 3 Hz and extraction time of 30 min). After extraction, the vessel was cooled and depressurized. Obtained extracts were filtrated and stored in a dark place at 4 °C until analysis.

2.4. Determination of extraction yield

In order to determine extraction yield (EY), certain volume of liquid extracts was evaporated under vacuum at 40 °C. Evaporated extracts were dried at 105 °C until constant mass. Further calculation of the total extraction yield was done according to the procedure described in pharmacopoeia [30].

2.5. Determination of total phenolic content

TPC was determined by a colorimetric assay based on a modified procedure initially described by [31]. The reaction mixture consisted of 25 µl of sample or standard solution, 75 µl of deionised water and 25 µl of Folin–Ciocalteús reagent diluted with water (1/1, v/v). After 6 min, 100 µl of Na_2CO_3 7.5% (w/v) were added. Absorbance was measured at 765 nm in a microplate reader (96-well plates, Nunc™ microwell, Denmark) after 90 min. Calibration curve was defined using GA as a