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Subcritical water extraction as an environmentally-friendly technique to recover bioactive compounds from traditional Serbian medicinal plants

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ABSTRACT

Subcritical water extraction (SWE) has become a popular green extraction technique for the isolation of different classes of compounds from natural matrices. Low price, safety and green character of water, good yields of target compounds and reduced energy consumption, make this technique favorable for potential industrial applications. The purpose of this study was to evaluate antioxidant, antimicrobial and cytotoxic activity of four medicinal plants traditionally used in folk medicine of Serbia. Black mulberry (*Morus nigra* L.), wall germander (*Teucrium chamaedrys* L.), wild geranium (*Geranium macrorrhizum* L.) and comfrey (*Symphytum officinale* L.) were extracted by subcritical water at different temperatures. Antioxidant activity of the extracts was defined by conventional spectrophotometric methods, such as the total phenolic content (TPC), DPPH-radical scavenging activity (DPPH-RSA), ferric reducing antioxidant power (FRAP) and total antioxidant capacity (TAC) assessed by a DNA-based sensor. Additionally, the main phenolic compounds contributing to the antioxidant activity of the produced extracts were also identified and quantified by high performance liquid chromatography with diode array detection (HPLC-DAD). Antimicrobial properties of extracts were evaluated against eight microbial strains. Furthermore, the cytotoxic activity was observed for two human cancer cell lines and a cell line derived from murine fibroblast.

1. Introduction

Modern medicine and pharmacy rely on the knowledge of traditional medicine. Nearly a quarter of new drugs are derived from natural sources. Plants represent insufficiently explored source of biologically-active compounds, being in the focus of many scientific researches. Identification and isolation of new bioactive compounds from plant extracts gives a significant contribution to the chemistry of natural compounds and pharmacology. Newly identified compounds are often used as models in the development of new pharmaceuticals with improved characteristics.

The Balkan Peninsula has a fortunate wide biological, ecological and landscape diversity (Stevanović et al., 1999; Radford and Odé,2009) and is known for a great number of plant species used in traditional medicine. In recent years, an increasing number of ethnobotanical studies are being conducted, elucidating the importance and traditional use of plant sources (Menković et al., 2011; Rexhepi et al., 2013; Šavikin et al., 2013; Stanković et al., 2016). The present study was focused on investigation of the bioactivity of extracts obtained by effective extraction technique, *i.e.* subcritical water, of several traditional medicinal plants from Serbia, black mulberry (*Morus nigra* L.), wall germander (*Teucrium chamaedrys* L.), wild geranium (*Geranium macrorrhizum* L.) and comfrey (*Symphytum officinale* L.). Infusion of *Morus nigra* berries are traditionally used for inflammation and to stop bleeding, the tincture of the bark for toothache, and the leaf infusion to stimulate insulin production in diabetes treatment (Volpato et al., 2011). *Teucrium chamaedrys* is usually used externally as an astringent infusion on the gums, in digestive and respiratory disorders, abscesses, gout and conjunctivitis (Stanković et al., 2012). The aerial parts of the plant have been used as antispasmodic and anti-inflammatory agent and in diabetes treatment (Vlase et al., 2014). *Geranium macrorrhizum* is a perennial herb native to the Balkans, highly valued for the treatment

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of stomach disorders in form of infusion, as well as an aphrodisiac (Radulović et al., 2010). *Symphytum officinale* leaves are traditionally used as infusion to stimulate the growth of new tissues in wounds and bone fractures, as well as for bronchitis and pneumonia (Bhat, 2014). Still, the comfrey root is mainly used by both traditional and modern herbalists, despite its potential toxic effects.

The use of environmentally-friendly technologies for the exploitation of plant potentials enjoys great scientific interest. The application of such technologies reduces or eliminates the use of organic solvents, contributing to safety, quality and applicability of plant extracts. Subcritical water extraction (SWE) is a competitive and attractive technique for the preparation of extracts rich in biologically-active compounds. In this technique, subcritical water replaces conventional organic solvents, being cheap, safe and efficient. Due to its excellent properties, subcritical water represents an excellent approach in the production of pharmacologically-active plant extracts and development of new products from supplements and cosmetic groups (Švarc-Gajić et al., 2017; Kumar et al., 2011; He et al., 2012; Koyu et al., 2017).

Very little information is available on biological activity of the studied plants. Antioxidant and cytotoxic activity of *M. nigra* extracts obtained by maceration, supercritical fluid extraction and ultrasonic-assisted extraction were reported (Memon et al., 2010; Radojković et al., 2016). According to Radojković et al. (2016), the macerates of *M. nigra* leaves exhibited higher antioxidant and cytotoxic activities than extracts obtained by supercritical fluid extraction due to higher content of polar phenolic compounds in the macerates. Also, antioxidant, cytotoxic and genotoxic activities (Miliauskas et al., 2004; Venskutonis et al., 2010) of *G. macrorrhizum* extracts obtained by maceration have been studied. The authors reported that cytotoxic and genotoxic effects were mostly attributed to quercetin and its derivatives abundant in the extracts. The volatile compounds from aerial parts and rhizomes of *G. macrorrhizum* were screened for their antimicrobial activity in disc-diffusion and microdilution assays (Radulović et al., 2010). The assays demonstrated high and selective activity of the oils against *Bacillus subtilis*. Pacifico et al. (2009) evaluated the antioxidant properties of *T. chamaedrys* leaf and root extracts obtained with petroleum ether, ethyl acetate and methanol, and reported marked radical scavenging effect for methanol extracts. Stanković et al. (2010) examined the antioxidant activity of the whole plant and different plant parts of *T. chamaedrys* extracted using different organic solvents. *In vitro* studies were also carried out to evaluate antimicrobial activity of *T. chamaedrys* demonstrating better antibacterial than antifungal activity (Stanković et al., 2012). The antioxidant and anti-proliferative effects of aqueous and ethanolic extracts of *S. officinale* leaves obtained by Soxhlet extraction and decoction method were also investigated (Alkan et al., 2014). Ethanolic extract exhibited stronger radical scavenging activity (RSA) against 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical in comparison to aqueous extract, while both extracts showed anti-proliferative activity suggesting that allantoin present in extracts of *S. officinale* had marked influence on proliferation process. However, to the best of our knowledge subcritical water extracts of selected medicinal plants have not been evaluated in terms of biological activity.

In the present work, bioactive compounds from medicinal plants from Serbia, namely *Morus nigra, Teucrium chamaedrys, Geranium macrorrhizum* and *Symphytum officinale* were extracted using subcritical water. Extraction temperature, as the most important parameter, was optimised in respect to total phenolic content (TPC) and antioxidant activity. The antioxidant activity was estimated by the following conventional spectrophotometric methods: DPPH radical scavenging activity (DPPH-RSA) assay, ferric reducing antioxidant power (FRAP) assay and by using electrochemical DNA-based biosensor. The obtained extracts were also analysed by high performance liquid chromatography with diode array detection (HPLC-DAD) to identify and quantify the main phenolic compounds contributing to their antioxidant activity. In addition, antimicrobial and cytotoxic activities of subcritical water extracts of selected medicinal plants were determined.

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, AAPH (2,2′-azobis(2-methylpropionamide) dihydrochloride (granular)), phosphate buffer (PBS) pH 7.4 and deoxyadenylic acid oligonucleotide (dA_{20}, as a desalted product) 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). Cirsimarin, resazurin, amaricin, nystatin, sabourand dextrose, Tween 80 and *cis*-diamminedichloroplatinum (*cis*-DDP) were purchased from Tedia Company (USA). 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. Instrumentation

The square wave voltammetry (SWV) was the selected voltammetric technique used to carry out the electrochemical studies. SWV was performed with an Autolab II controlled by GPES software, version 4.8 (EcoChemie, The Netherlands). A conventional three-electrode cell was used, which included a home-made carbon paste electrode (CPE) (3 mm in diameter) as a working electrode, a platinum wire counter electrode and a Ag/AgCl (KClsat.) reference electrode to which all potentials were referred. The CPE was prepared by mixing 1.8 g of paraffin oil as a pasting liquid with 5 g of spectroscopic grade graphite powder (Ultracarbon, Dicoex, Spain). The unmodified carbon paste was introduced into a teflon electrode body by a stainless-steel piston. The surface was smoothed against a plain white paper while a slight manual pressure was applied to the piston. Unless otherwise stated, after each experiment, the CPE was discarded and a new electrode surface was freshly prepared.

A Multi Mode Microplate Reader (BioTek Instruments, USA) was used to carry out the conventional optical analytical methods, such as TPC, FRAP and DPPH-RSA.

HPLC analysis were carried out on a Shimadzu Corporation system (Kyoto, Japan), equipped with a LC-20AD prominence pump, a DGU-20AS prominence degasser, a CTO-10AS VP column oven, a SIL-20A HT prominence autosampler, and a SPD-M20A diode array detector.

2.3. Plant material

In the present work, commercially available dry plant material was used (Adonis D.O.O., Sokobanja, Serbia). The following plants and their parts were used: black mulberry leaves (*Morus nigra* L.), wall germander aerial flowering parts (*Teucrium chamaedrys* L.), wild geranium leaves (*Geranium macrorrhizum* L.) and comfrey leaves (*Symphytum officinale* L.). Dry plant material was grounded in a blender before the extraction, providing an average particle size of 0.315 mm.