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

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A B S T R A C T

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.

Keywords:
Medicinal plant
Subcritical water extraction
Antioxidant activity
Biosensor
Cytotoxic activity
HPLC-DAD analysis

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.

Table 1
Analytical parameters of the calibration curves used for HPLC-DAD quantification of phenolic compounds.

Compound	(m ± Δm)[a]	(b ± Δb)[b]	R^2	LOD[c] (mg/L)	LOQ[d] (mg/L)
gallic acid	50,706 ± 214	−6,217 ± 5,928	0.9999	0.55	1.83
protocatechuic acid	33,664 ± 661	−21,592 ± 19,863	0.998	2.77	4.24
catechin	13,105 ± 138	2,406 ± 3,573	0.9997	1.28	4.27
chlorogenic acid	56,066 ± 556	−32,375 ± 14,629	0.9997	1.23	4.09
vanillic acid	35,314 ± 321	−931 ± 777	0.9998	1.03	3.45
caffeic acid	106,608 ± 566	−16,156 ± 13,030	0.9999	0.57	1.91
epicatechin	69,040 ± 491	−25,251 ± 12,141	0.9998	0.83	2.75
syringic acid	21,490 ± 574	16,716 ± 14,077	0.998	3.08	5.26
β-resorcylic acid	12,308 ± 49	−423 ± 136	0.9999	0.52	1.74
p-coumaric acid	127,784 ± 937	−3,060 ± 2,341	0.9998	0.86	2.87
ferulic acid	107,272 ± 902	−9,853 ± 2,276	0.9998	1.00	3.32
sinapic acid	95,532 ± 1,417	−8,888 ± 3,683	0.9993	1.81	6.04
naringin	33,168 ± 49	616 ± 124	0.9999	0.18	0.59
rutin	31,543 ± 79	1,460 ± 197	0.9999	0.29	0.98
cinnamic acid	163,683 ± 393	19,094 ± 11,379	0.9999	0.33	1.09
naringenin	62,404 ± 494	12,483 ± 12,394	0.9998	0.93	3.11
quercetin	64,868 ± 1,312	−42,618 ± 31,783	0.9988	2.30	5.67
kaempferol	73,460 ± 1,244	14,914 ± 3,030	0.9991	1.94	6.46

[a] m: slope ± standard deviation (n = 5) expressed in μV min/mg L.
[b] b: intercept ± standard deviation (n = 5) expressed in μV min.
[c] LOD: limit of detection.
[d] LOQ: limit of quantification.

2.4. Subcritical water extraction

SWE was performed in a house-made subcritical water extractor. Extraction procedure and apparatus were described previously (Švarc-Gajić et al., 2017). Total capacity of high-pressure stainless steel vessel was 1.7 L. Pressurization of the vessel was performed with nitrogen in order to prevent possible oxidation. In all experimental runs, sample to distilled water ratio was 1:40. Extraction temperature (60–200 °C) was investigated as independent variables, while all other parameters were held constant comprising agitation rate of 3 Hz, extraction pressure of 10 bar and extraction time of 30 min. After extraction, extraction vessel was cooled and depressurized. Obtained extracts were filtrated and stored in a dark place at 4 °C until analysis.

2.5. Determination of total phenolic content

TPC was measured using the Folin-Ciocalteau method (Barroso et al., 2016) using GA as the standard antioxidant, and the absorbance was measured at 765 nm in a microplate reader (96-well plates, Nunc™ microwell, Denmark) after 90 min. Results were expressed as GA equivalents per gram of dry extract (mg GAE/g DE). The experiments were carried out in triplicate.

2.6. Determination of antioxidant activity

2.6.1. DPPH free radical scavenging assay

DPPH-RSA of samples was determined spectrophotometrically at 517 nm, against the stable nitrogen radical DPPH following the procedure described before (Barroso et al., 2016). Lower absorbance values of the reactive mixture indicated higher free radical scavenging activity. The calibration curve was prepared with Trolox solutions. Results were expressed as mg of Trolox equivalents per gram of dry extract (mg TE/g DE). The measurements were performed in triplicate.

2.6.2. Ferric reducing antioxidant power assay

FRAP assay was performed according to the chemical assay for reducing power developed by Barroso et al. (2016) in a microplate reader at 593 nm at 37 °C. The calibration curve was prepared with ascorbic acid solutions. The results were expressed as ascorbic acid equivalents per gram of dry extract (mg AAE/g DE). The measurements were performed in triplicate.

2.6.3. DNA-based sensor assay

Experiments using the electrochemical DNA-based sensor were conducted in four steps: DNA immobilization, damage of oligonucleotide by the immersion of DNA-CPE in the Fenton mixture, study of the effects of the antioxidants present in the system, and detection and measurement of the peak current of dA_{20} in a PBS solution at pH 7.4, following the procedure described by Barroso et al. (2016). Calibration curve was defined using GA as standard antioxidant. Results were expressed as mg GAE/g DE. Each analysis was performed in triplicate.

2.7. HPLC-DAD analysis

The identification and quantification of phenolic compounds was performed according to the method previously described by Rubilar et al. (2007) with some modifications. The separation was performed at 25 °C on a *Phenomenex* Gemini C_{18} column (250 mm × 4.6 mm, 5 μm) and a guard column with the same characteristics. Samples (20 μL) were eluted using a mobile phase consisted of methanol (solvent A) and water (solvent B) both with 0.1% formic acid. The composition of the mobile phase varied during the run according to a nonlinear gradient as follows: 85% B in 0 min, from 85% to 70% B in 20 min, from 70% to 55% B in 20 min, from 55% to 50% B in 5 min, from 50% to 45% B in 5 min, from 45% to 30% B in 15 min, from 30% to 0% B in 10 min, followed by 100% A for 5 min and back to 85% B in 10 min and 10 min of reconditioning before the next injection at a flow rate of 1.0 mL/min. Detection and quantification was performed at 280, 320 and 360 nm according to the phenolic compound maximum wavelength. Analytes in each subcritical water extract were identified by comparing their retention times and UV–vis spectra with those of standard compounds. Peak purity was checked to exclude any contribution from interfering peaks. Individual stock solutions of the above-mentioned phenolic compounds were prepared in methanol (2000 mg/L), and their mixtures to plot the calibration curves ranging from 1 to 50 mg/L were made in methanol–water (50:50, v/v). Limit of detection (LOD) and quantification (LOQ, mg/L), and the coefficient of correlation (R^2) are shown in Table 1. Results were means of triplicate injections and expressed as mg/100 g DE.

2.8. Determination of antibacterial and antifungal activities

The antibacterial activity was tested against the *Staphylococcus*

aureus ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 13883, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153 and *Bacillus subtilis* ATCC 6633. The antifungal activity was tested against the *Aspergillus niger* ATCC 16404 and *Candida albicans* ATCC 10231. Antibacterial and antifungal activities were estimated by measuring their minimum inhibitory concentrations (MICs). MICs of the extracts and cirsimarin against the test bacteria were determined by microdilution method in 96 multi-well microtiter plates (Sarker et al., 2007). All tests were performed in Muller–Hinton broth (MHB) with the exception of the yeast, in which case Sabouraud dextrose broth was used. A volume of 100 µL stock solutions of extracts (in methanol, 200 µL/mL) and cirsimarin (in 10% DMSO, 2 mg/mL) were pipetted into the first row of the plate. Fifty microliters of Mueller Hinton or Sabouraud dextrose broth (supplemented with Tween 80 to a final concentration of 0.5% (v/v)) were added to other wells. A volume of 50 µL from the first test wells was pipetted into the second well of each microtiter line, and then 50 µL of scalar dilution was transferred from the second to the twelfth well. Volume of 10 µL of resazurin indicator solution (prepared by dissolving 270-mg tablet in 40 mL of sterile distilled water) and 30 µL of nutrient broth were added to each well. Finally, 10 µL of bacterial suspension (10^6 CFU/mL) and yeast spore suspension (3×10^4 CFU/mL) were added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Standard antibiotic Amracin was used to control the sensitivity of the tested bacteria, whereas Nystatin was used as a control against the tested yeast. Plates were wrapped loosely with cling film to prevent dehydration and prepared in triplicate. The plates were placed in an incubator at 37 °C for 24 h for the bacteria and at 28 °C for 48 h for the yeast. Subsequently, color change was assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated, and the obtained value was taken as the MIC for the tested sample and a standard.

2.9. Determination of cytotoxic activity

The influence of extracts on the growth of malignantly transformed cell lines was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The following cell lines were used: RD (cell line derived from human rhabdomyosarcoma), Hep2c (cell line derived from human cervix carcinoma–HeLa derivative) and L2OB (cell line derived from murine fibroblast). Cells were seeded (2×10^5 cell/mL; 100 µL/well) in 96-well cell culture plates in nutrient medium (Minimum Essential Medium (MEM) Eagle supplemented with 5% of Hep2c, RD and L2OB) and grown at 37 °C in humidified atmosphere for 24 h. Then, corresponding extract (stock solution: 5 mg of extract dissolved in 1 mL of absolute ethanol) and control (absolute ethanol) diluted with nutrient medium to desired concentrations were added (100 µL/well) and cells were incubated at 37 °C in humidified atmosphere for 48 h. Pure nutrient medium (100 µL) represented positive control for each cell line. After incubation period, supernatants were discarded and MTT (dissolved in D-MEM (Dulbecco's modification of Eagle's medium) in concentration of 500 µg/mL) was added to each well (100 µL/well). Immediately after, all wells were incubated at 37 °C in humidified atmosphere for 4 h. Reactions were halted by adding 100 µL of sodium dodecyl sulfate (SDS) (10% in 10 mM HCl). After overnight incubation at 37 °C, absorbance was measured at 580 nm using a spectrophotometer (Ascent 6-384 [Suomi], MTX Lab Systems Inc., Vienna, VA 22182, USA). The number of viable cells per well (NVC) was calculated from a standard curve plotted as cell numbers against A_{580}. Corresponding cells (grown in flasks), after cell count by hemocytometer, were used as standards. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min and then treated with MTT/D-MEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells (*ut supra*). The intensity of absorbed light was proportional to the number of viable cells in each well, which was read in an ELISA plate reader at 580 nm. Absorbance (A) at 580 nm was measured 24 h later. Cell survival (%) was calculated by dividing the absorbance of a sample with cells grown in the presence of various concentrations of the investigated extracts with control optical density (the A of control cells grown only in nutrient medium), and multiplying by 100. The blank absorbance was always subtracted from the absorbance of the corresponding sample with target cells. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. The results of the measurements were expressed as the percentage of positive control growth taking the *cis*-diamminedichloroplatinum as a positive control (100% growth) (Baviskar et al., 2012; Mosmann, 1983). All experiments were done in triplicates.

3. Results and discussion

3.1. Optimization of the subcritical water extraction

Phenolic compounds are found both in edible and inedible plants. Increasing number of studies have shown their protective effect against various diseases likes aging (Manosroi et al., 2010), cancer (Cai et al., 2004), inflammation (Zhang et al., 2011a), cardiovascular and cerebrovascular diseases (Gan et al., 2010) by inhibiting excessive free radicals. Extractability of phenolic compounds from plant matrices depends on several factors including solvent, pH, temperature, pressure, number of steps and solvent volume. In subcritical water extraction, the most prominent operational parameter is temperature since it affects its dielectric constant (ε), viscosity and surface tension, as well as the interaction with the matrix (Švarc-Gajić, 2012). As a polar solvent at room temperature, water solubilizes well polar compounds. In subcritical conditions, higher temperatures cause the drop of water polarity, increasing its ability to solvate compounds of intermediate or low polarity. The dielectric constant of water decreases with the temperature (*e.g.*, from $\varepsilon = 80$ at ambient temperature to $\varepsilon = 27$ at 250 °C) and becomes close to that of methanol ($\varepsilon = 33.6$ at 25 °C) and ethanol ($\varepsilon = 24.5$ at 25 °C) (Akerlof, 1932). Elevated temperatures enhance also the diffusion rate and desorption kinetics, potentiating the dissociation of the compounds from their complexes with matrix constituents (Švarc-Gajić, 2012). On the other hand, too high temperatures may lead to degradation and loss of target compounds. Thus, in SWE it is very important to optimise the temperature depending on the target class of compounds. In general, in subcritical water extraction, pressure has lesser influence on solvent characteristics, selectivity and efficiency of the process (Ramos et al., 2002; Švarc-Gajić, 2012). Additionally, the influence of extraction time on the efficiency of subcritical water extraction has been previously investigated (Cvetanović et al., 2017). According to conducted study, extraction time of 30 min was adopted as optimal for the extraction of phenolic compounds. Therefore, in this study previously optimised extraction time was adopted. In this paper the optimization of the extraction temperature was performed in respect of total phenolic content and antioxidant activity.

With the aim of maximizing extraction efficiency of phenolic compounds, different temperatures (60, 100, 130, 160, 200 °C) were tested applying constant extraction pressure of 10 bar and extraction time of 30 min. The influence of extraction temperature is shown in Fig. 1.

As can be seen in Fig. 1, the temperature influenced the TPC for all studied samples; an increase in the extraction temperature resulted in higher TPC. At temperature of 130 °C, the maximum yield of phenolic compounds was obtained for *G. macrorrhizum* and *S. officinale* extracts, while the maximum phenolic content in *M. nigra* and *T. chamaedrys* extracts was reached at 160 °C. Similar findings for SWE were previously reported for other plant species (Kumar et al., 2011; Ahmadian-Kouchaksaraie et al., 2016; Cvetanović et al., 2017). Heat energy accelerates desorption of target compounds from their complexes. An increase in temperature also results in a decrease in solvent viscosity and surface tension contributing to the extraction efficiency (Švarc-

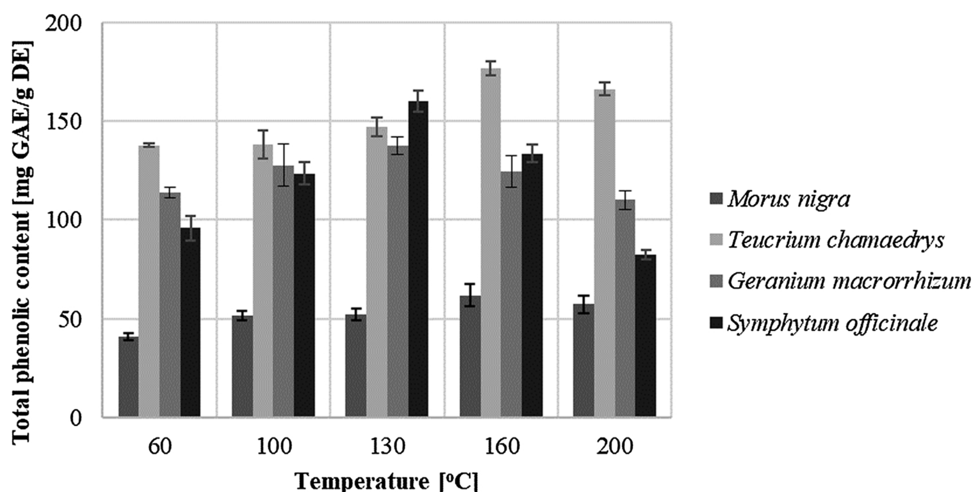

Fig. 1. The influence of the extraction temperature on total phenolic content in selected medicinal plants extracts. Operating conditions: pressure–10 bar, extraction time–30 min and agitation rate–3 Hz.

Gajić, 2012). A decrease in solvent surface tension allows better pore filling and better contact between the solvent and sample matrix. On the other hand, an extraction temperature above 160 °C caused the drop in TPC in all tested plant extracts probably due to thermal and chemical degradation. Such effects were expected and were previously reported in the SWE of other natural compounds (Kubatova et al., 2001; He et al., 2012), showing substantial degradation at higher temperatures.

At optimal temperature (160 °C), the highest TPC was obtained for *T. chamaedrys* (176.74 ± 3.41 mg GAE/g DE), while *M. nigra* (61.89 ± 5.67 mg GAE/g DE) presented the lowest content. Obtained values were comparable to those seen in extracts obtained with organic solvents and higher compared to aqueous-based extracts (Miliauskas et al., 2004; Alkan et al., 2014; Stanković et al., 2012; Žugić et al., 2014). By using methanol at room temperature, 11 mg GAE/g of total phenols was determined in *M. nigra* leaves (Žugić et al., 2014). Similar findings have been reported for *G. macrorrhizum* leaves (Miliauskas et al., 2004). A yield of 25.9 mg GAE/g extract was achieved by maceration using methanol as a solvent at room temperature. Extraction of phenolic compounds from *T. chamaedrys* aerial flowering parts by maceration using water, methanol, acetone, ethyl acetate and petroleum ether at room temperature resulted in TPC ranging from 30.39 to 169.50 mg GAE/g extract (Stanković et al., 2012), which is lower of those obtained in the present study for the same plant species. Alkan et al. (2014) determined lower levels of phenols in ethanolic fractions of *S. officinale* obtained by Soxhlet extraction and aqueous fractions obtained by decoction method at 100 °C for 30 min (116.93 and 99.49 mg GAE/g extracts, respectively). For more accurate comparison, a systematic study would be necessary because besides the extraction technique used in the process, the yield and quality of the extract can vary depending on plant sample itself. Cultivation practices, climatic conditions, morphology, soil composition, variety and other factors, can significantly affect plant composition (Pereira and Meireles, 2010).

In the present study, antioxidant activities of the subcritical water extracts of selected medicinal plant were evaluated by DPPH, FRAP and DNA-based sensor assays. The results of DPPH-RSA and FRAP assays are depicted in Fig. 2.

The obtained results showed that extraction temperature had marked effect on the antioxidant activity similarly as for TPC. By increasing the extraction temperature from 60 °C to 160 °C antioxidant activity increased, which is in agreement with previous results obtained for the content of phenolic compounds. Further increase in the extraction temperature up to 200 °C caused slight decrease in the antioxidant activity indicating possible degradation of antioxidants from medicinal plants. Similar results were obtained by Hassas-Roudsari et al. (2009) who extracted canola meal by subcritical water. According to their results, antioxidant activity increased with temperature up to 160 °C. Also, these results were in agreement with those of Kumar et al. (2011) who determined the antioxidant activity of seabuckthorn leaves extracted by subcritical water.

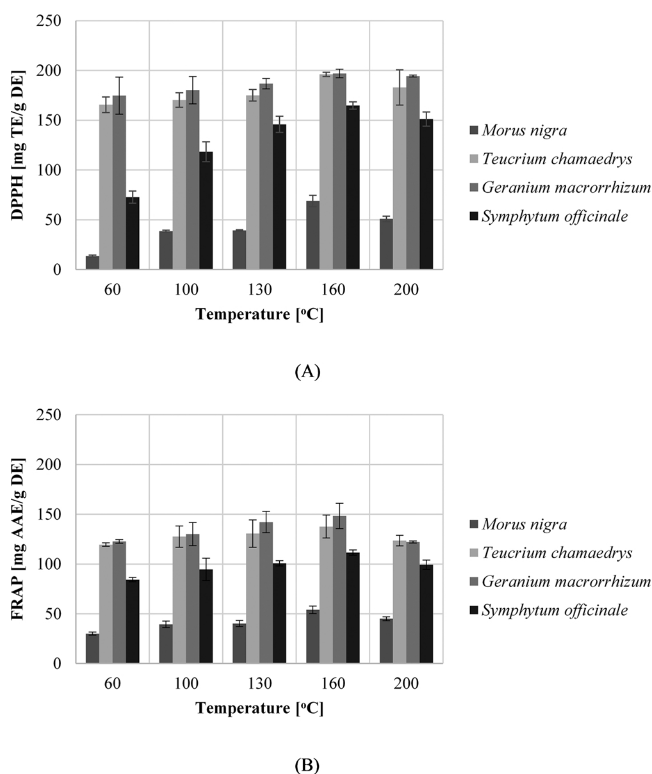

Fig. 2. The influence of the extraction temperature on antioxidant activity of medicinal plant extracts: (A) DPPH-RSA assay, and (B) FRAP assay. Operating conditions: pressure–10 bar, extraction time–30 min and agitation rate–3 Hz.

As can be seen in Fig. 2(A), the highest antioxidant activity against DPPH radicals was seen in subcritical water extracts of *G. macrorrhizum* (197.0 ± 4.3 mg TE/g DE) and *T. chamaedrys* (196.12 ± 2.12 mg TE/g DE) obtained at the temperature of 160 °C. The lowest antioxidant activity of 68.88 ± 5.73 mg TE/g DE was determined for *M. nigra* extracts. The FRAP assay also confirmed the highest activity in *G. macrorrhizum* (148.32 ± 10.75 mg AAE/g DE) and *T. chamaedrys* (137.7 ± 5.3 mg AAE/g DE) extracts.

Despite the popularity of the DPPH-RSA and FRAP assays for evaluation of antioxidant activity, they fail in effectively predict the antioxidant activity *in vivo*. According to this reasoning, the DNA-based sensor may provide a more realistic simulation of the antioxidant

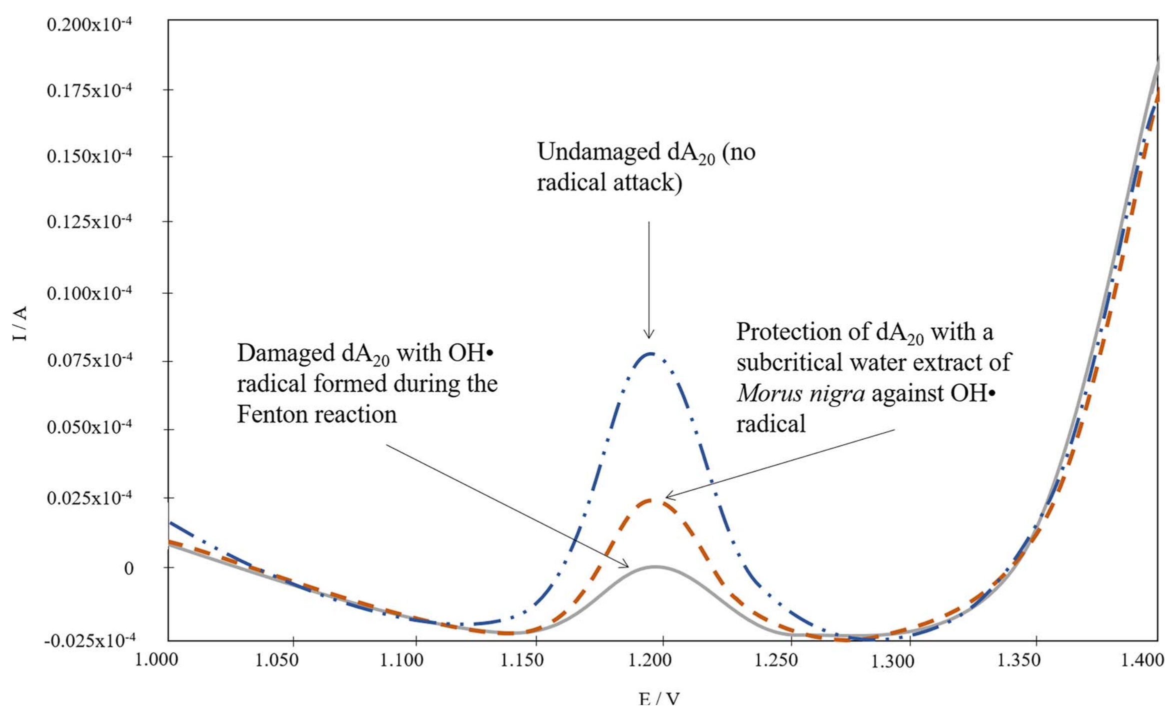

Fig. 3. Total antioxidant capacity of *Morus nigra* subcritical water extract against OH· radical generated during the Fenton reaction.

compound behavior in cellular environment through the measurement of its protection to DNA oxidative damage (Carvalho et al., 2016). Moreover, the use of electrochemical sensors for measuring antioxidant activity of plant samples has received growing interest. Their advantages include rapid detection, good sensitivity, low sample volumes, low cost and simplicity of operation (Barroso et al., 2016). In this technique, a chain of 20 adenine molecules are used in order to simulate the effect of damages provoked by the attack of OH· radicals. The protection held by the antioxidant extract against the radical attack can be related with the total antioxidant capacity (TAC) of the plant. Considering this, the antioxidant activity of the subcritical water extracts of selected medicinal plant (which generated the highest TPC levels) was also evaluated by electrochemical DNA-based sensors. An example from the obtained voltammograms of the protection given by the subcritical water plant extracts, namely from *Morus nigra,* is presented in Fig. 3.

Table 2 describes the obtained results with the DNA-based sensor. Antioxidant activity values obtained for the plants extracts, when it was used the DNA-based sensor, ranged from 11.57 ± 0.53 to 34.19 ± 7.69 mg GAE/g DE, for *Morus nigra* and *Symphytum officinale* subcritical water extracts, respectively. Actually, the antioxidant activity decreased in this way: *Symphytum officinale* > *Geranium macrorrhizum* > *Teucrium chamaedrys* > *Morus nigra.*

The antioxidant capacity of the tested extracts using different antioxidant methods varied. The antioxidant activities obtained with the DNA-based biosensor were lower than those obtained by the DPPH-RSA and FRAP methods which measured the reducing potential of the extracts. The plant extracts exibit different antioxidant power profile against a reactive hydroxyl radical (HO·) when compared to an

Table 2
Antioxidant activity results measured by a DNA-based sensor.

Samples	Extraction temperature (°C)	DNA-based sensor (mg GAE/ g DE)
Morus nigra	160	11.57 ± 0.53
Teucrium chamaedrys	160	28.01 ± 2.25
Geranium macrorrhizum	130	31.65 ± 1.22
Symphytum officinale	130	34.19 ± 7.69

artificially generated radicals such as DPPH and the Fe^{3+-} TPTZ (Mello et al., 2006). Plants contain high content of natural antioxidants acting as OH· scavengers. Sochor et al. (2013) highlighted the fact that electrochemical data must be related to standard compound with antioxidant properties and well-characterized chemical structure. In our case the standard chosen was gallic acid that was present in all extracts (Table 3). Several studies suggest that compounds with a catechol group (meta arrangement like flavonoids), multiple hydroxyl substitutions and conjugation, are oxidized below +0.4 V showing high antioxidant activity, whereas compounds with high oxidation potential (higher than +0.5 V) show little or no activity (Firuzi et al., 2005). According to these authors catechin, rutin and quercetin are the strongest antioxidants in the FRAP assay having the lowest oxidation potentials. Although gallic acid is a phenolic acid (and not a flavonoid), it presents an oxidation potential at ~+0.274 V, being a very strong antioxidant (Arteaga et al., 2012). Caffeic acid, however, produces the highest oxidation current (Sochor et al., 2013). Thus, its presence in the extract may present an antagonistic or pro-oxidant effect on the DNA protection against OH·. Considering the HPLC profile of the extracts presented in Table 3, *S. officinale* had the highest number of polyphenolic compounds, particularly flavonoids, that probably have a synergetic effect increasing the DNA protection against OH· radical. *M. nigra*, on the other hand, has the lowest content of gallic acid and the highest of caffeic acid having the lowest antioxidant capacity measured by the DNA-based biosensor. Zhang et al. (2011b) studied the electrochemical properties of 14 flavonoid standards using cyclic voltammetry and compared with the results of four spectrophotometric assays, namely, diphenyl-1-picrylhydrazyl (DPPH) method, TPC, ferric reducing ability of plasma (FRAP) method, and trolox equivalent antioxidant capacity (TEAC), to understand the varying antioxidant activities of flavonoids under different assays. They reported that flavonoids showed contrasting antioxidant activities depending on the method employed.

3.2. Phenolic profile

The phenolic compounds contributing to the antioxidant activity were identified and quantified by HPLC-DAD analysis (Fig. 4).

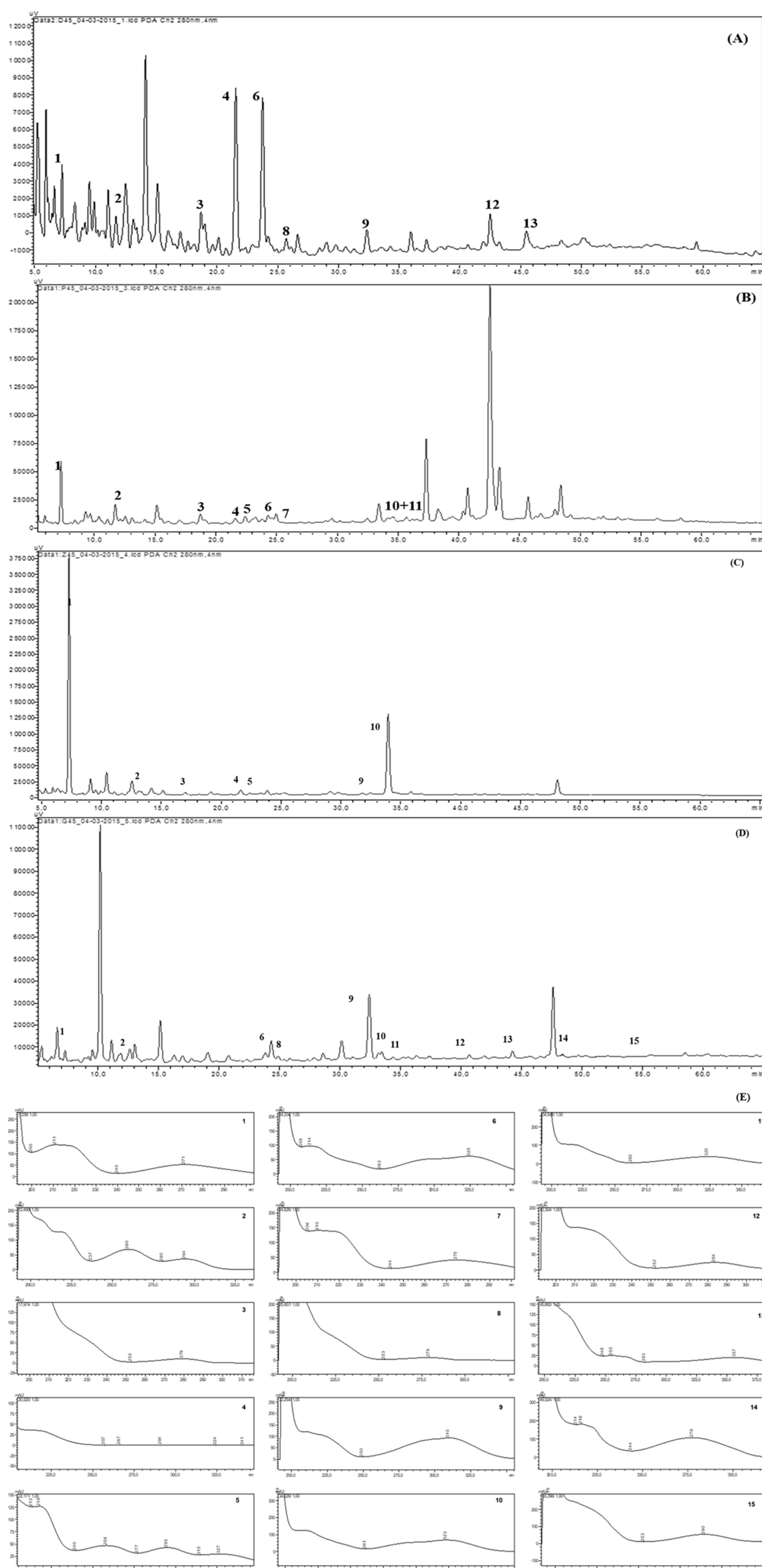

Fig. 4. HPLC chromatograms at 280 nm of subcritical water extracts for *Morus nigra* (A), *Teucrium chamaedrys* (B), *Geranium macrorrhizum* (C) and *Symphytum officinale* (D), and UV/VIS spectra of the identified phenolic compounds in subcritical water extracts of selected medicinal plants (E); gallic acid (1), protocatechuic acid (2), (+)-catechin (3), chlorogenic acid (4), vanillic acid (5), caffeic acid (6), epicatechin (7), β-resorcylic acid (8), *p*-coumaric acid (9), ferulic acid (10), sinapic acid (11), naringin (12), rutin (13), cinnamic acid (14) and naringenin (15).

The content of phenolic compounds in the subcritical water extracts was estimated from calibration curves constructed with the mixture of the selected phenolic compounds (Table 3). GA is one of the most abundant phenolic acid in the subcritical extracts, with its concentration being surprisingly higher in *Geranium macrorrhizum* plant (1,512 ± 151 mg/100 g DE). On the other hand, *p*-coumaric and sinapic acids were the phenolic compounds found in lower concentration in the four subcritical water extracts analysed. Although, it should be

Table 3
Content of the identified phenolic compounds detected by HPLC-DAD in subcritical water extracts of selected medicinal plants; results were expressed as mg/100 g DE.

Compound	Mean ± SD (mg/100 g dry extract[b]; n = 3)			
	Morus nigra	*Teucrium chamaedrys*	*Greanium macrorrhizum*	*Symphytum officinale*
gallic acid	26.3 ± 2.7	217 ± 22	1,512 ± 151	122 ± 12
protocatechuic acid	79.5 ± 7.9	73.7 ± 7.4	234 ± 23	135 ± 14
catechin	38.0 ± 3.8	101 ± 10	97.7 ± 9.8	ND
chlorogenic acid	183 ± 18	76.3 ± 7.6	106.9 ± 10.7	ND
vanillic acid	ND[a]	42.2 ± 4.2	14.3 ± 1.4	ND
caffeic acid	91.1 ± 9.1	54.2 ± 5.4	ND	89.6 ± 9.0
epicatechin	ND	38.1 ± 3.8	ND	ND
β-resorcylic acid	62.0 ± 6.2	ND	ND	30.6 ± 3.0
p-coumaric acid	9.57 ± 0.96	ND	8.64 ± 0.86	157 ± 16
ferulic acid	ND	29.1 ± 2.9	128 ± 13	14.1 ± 1.4
sinapic acid	ND	19.2 ± 1.9	ND	10.6 ± 1.0
naringin	56.8 ± 5.7	ND	ND	28.4 ± 2.8
rutin	43.7 ± 4.4	ND	ND	53.5 ± 5.3
cinnamic acid	ND	ND	ND	1.27 ± 0.12
naringenin	ND	ND	ND	3.36 ± 0.34

[a] ND: not detected.
[b] Extraction conditions: temperature of 160 °C, pressure of 10 bar, extraction time of 30 min and agitation rate of 3 Hz.

mentioned that *p*-coumaric acid was one of the main contributors to the phenolic composition of *Symphytum officinale* extract (157 ± 16 mg/100 g DE). Only few reports concerning the phenolic characterization of these type of medicinal plants by HPLC was found (Memon et al., 2010; Radulović et al., 2012; Vlase et al., 2014; Radojković et al., 2016). Memon et al. (2010) investigate the phenolic profile from *Morus nigra* leaves grown in Pakistan extracted by three different extraction techniques, namely by sonication, magnetic stirring, and homogenization, and reported that chlorogenic acid was the prominent phenolic acid in all leaves samples, with the highest content achieved using 80% aqueous methanol with sonication (104.39 ± 1.5 mg/100 g DE), which is in close agreement with the value obtained in the present study. These authors also quantified other phenolic acids, namely vanillic and syringic acids, which were not detected in the *Morus nigra* subcritical water extracts from the present study. In contrast, considerable amounts of gallic, protocatechuic, caffeic and β-resorcylic acids, as well as the flavonoids naringin and rutin, were identified and quantified in the present subcritical water extracts, demonstrating the applicability of the SWE technique for the recovery of phenolic compounds. In another report (Radojković et al., 2016), the polyphenolic profile of *Morus nigra* leaves after supercritical CO_2 extraction coupled with maceration was also investigated, and a similar composition was found. Although, it should be highlighted the significant amount of caffeic acid and rutin quantified in the obtained extracts, which was at least 22 times higher than the amount recovered by SWE. Regarding the medicinal plant *Teucrium chamaedrys,* the polyphenolic composition of the ethanolic extracts obtained by maceration technique was already reported in the literature (Vlase et al., 2014). These authors identified the presence of *p*-coumaric acid and rutin, which were not detected in the *Teucrium chamaedrys* subcritical water extracts produced. On the other hand, significant amount of gallic acid and catechin were reported in the subcritical water extracts (Table 3), demonstrating the huge variability within the same medicinal species but from different origins. The phenolic composition of methanolic extracts from *Geranium macrorrhizum* plant were also analysed by HPLC by Radulović et al. (2012), which reported that they mostly consist of glycosidilated bound phenolics, most possessing gallic and ferulic acid moieties. These findings are in close agreement with our results (Table 3).

The previously obtained results demonstrate the huge variability within the studied medicinal species, and also for the same plants but with different geographical origins. In fact, the obtained results could be used as a useful finger-printing from the studied medical plants. According to the obtained results, it was noticed that some compounds were common to all the subcritical water extracts produced, but others were characteristic from a specific medicinal plant. Chlorogenic acid and catechin were absent in *Symphytum officinale* extract, as well as the hydroxycinnamic acids caffeic, *p*-coumaric and ferulic that were not found in *Geranium macrorrhizum, Teucrium chamaedrys* and *Morus nigra* extracts, respectively. By contrast, and despite of being found in low amounts, cinnamic acid and naringenin were only detected in *Symphytum officinale* subcritical water extract, and the flavonoid epicatechin was only reported in *Teucrium chamaedrys* extract. This information concerning the phenolic profile of the studied medicinal plants, could be a useful tool to differentiate these plants.

3.3. Antimicrobial activity

A broad spectrum of plant species has demonstrated evidence of strong antimicrobial properties (Mahesh and Satish, 2008; Sharma et al., 2016; Stanković et al., 2016). Antimicrobial activity of some of medicinal plants studied in this work have previously been described (Radulović et al., 2010; Stanković et al.,2012). Gursoy and Tepe (2009) tested polar and non-polar sub-fractions of methanol extracts of *T. chamaedrys*. Polar sub-fractions of the methanol extracts exhibited the weakest antimicrobial activity when compared to the non-polar. Radulović et al. (2010) related antimicrobial effect of *G. macrorrhizum* extracts obtained by hydrodistillation mostly to germacrone. Antifungal activity against tested molds (*Aspergillus fumigatus, Aspergillus restrictus,* and *Penicillium chrysogenum*) was reported to be weak.

In the present study, the extracts of *M. nigra, T. chamaedrys, G. macrorrhizum* and *S. officinale* obtained by subcritical water at 160 °C and 10 bar were tested against a panel of microorganisms. MICs were determined for eight commercially purchased selected indicator strains. Obtained results are shown in Table 4. Calculated values were compared with those determined for Amracin and Nystatin, standard antibacterial and antifungal compounds, respectively.

Results obtained in the current study revealed strong antimicrobial activity against tested organisms. The strongest antibacterial and antifungal activities were observed for the extracts of *G. macrorrhizum* against *Staphylococcus aureus* (MIC = 19.53 μg/mL) and *Aspergillus niger* (MIC = 39.1 μg/mL). Antimicrobial activity of the leaf methanolic extract of *G. macrorrhizum* has been studied by Radulović et al. (2012). Methanol extract of *G. macrorrhizum* displayed a strong antibacterial activity, especially against *Staphylococcus aureus,* with low minimal inhibitory concentration (15.6 μg/mL). In this work, the lowest antibacterial activity was seen for the subcritical water extract of *S. officinale* against *Proteus mirabilis* (MIC = 625 μg/mL). The same plant extract demonstrated also the lowest antifungal activity against Candida

Table 4
Antimicrobial activity of subcritical water extracts of selected medicinal plants.

Microbial strain	MIC value[a] (μg/mL)					
	Morus nigra	*Teucrium chamaedrys*	*Geranium macrorrhizum*	*Symphytum officinale*	Amracin	Nystatin
Staphylococcus aureus ATCC 25923	39.1	78.125	19.53	39.1	0.97	/
Klebsiella pneumoniae ATCC 13883	78.125	78.125	156.4	156.4	0.49	/
Escherichia coli ATCC 25922	312.5	312.5	312.5	156.25	0.97	/
Proteus vulgaris ATCC 13315	78.125	156.4	312.5	312.5	0.49	/
Proteus mirabilis ATCC 14153	312.5	78.125	312.5	625	0.49	/
Bacillus subtilis ATCC 6633	312.5	156.25	78.125	78.125	0.24	/
Candida albicans ATCC 10231	156.25	156.25	156.25	312.5	/	1.95
Aspergillus niger ATCC 16404	156.25	78.125	39.1	156.4	/	0.97

[a] Statistically insignificant according to one-way analysis of variance (ANOVA). A probability value of 0.05 was considered significant.

Table 5
In vitro cytotoxic activity of subcritical water extracts of selected medicinal plants.

Cell line	IC_{50} (μg/mL)[d]				
	Morus nigra	*Teucrium chamaedrys*	*Geranium macrorrhizum*	*Symphytum officinale*	*Cis*-DDP[e]
Hep2c cells[a]	28.14 ± 0.53	36.39 ± 0.22	28.38 ± 0.64	29.88 ± 0.49	0.94 ± 0.55
RD cells[b]	11.10 ± 0.41	15.16 ± 0.36	12.22 ± 0.36	12.76 ± 0.89	1.4 ± 0.97
L2OB cells[c]	17.41 ± 0.19	18.23 ± 0.67	18.69 ± 0.49	19.39 ± 0.39	0.72 ± 0.64

[a] Cell line derived from human cervix carcinoma.
[b] Cell line derived from human rhabdomyosarcoma.
[c] Cell line derived from murine fibroblast.
[d] Mean value ± 2SD.
[e] *Cis*-diamminedichloroplatinum.

albicans with MIC value of 312.5 μg/mL. Authors that studied aqueous extracts of *S. officinale* leaves did not detect any antibacterial nor antifungal activities (Woods-Panzaru et al., 2009) indicating better activity of subcritical water extracts. Subcritical water extract of *M. nigra* was the most active against *Staphylococcus aureus* and least active against *Escherichia coli, Proteus mirabilis* and *Bacillus subtilis.* According to Yiğit and Yiğit (2008), the aqueous and methanol extracts of *M. nigra* leaves were inactive against *Proteus mirabilis*. In studies reported by other authors, methanol extract of *T. chamaedrys* showed moderate inhibition activity being the most active against *Staphylococcus aureus* and least effective against *Escherichia coli* (Stanković et al., 2012), which was in agreement with findings reported in this study.

The results of the present study suggest that subcritical water extracts of the tested medicinal plants exhibited good antibacterial and antifungal activities, superior of those reported for other extraction techniques.

Antimicrobial activity of plant extracts has been attributed to the presence of numerous bioactive secondary metabolites. Phenolic compounds identified in *M. nigra* leaves by Radojković et al. (2016), such as caffeic acid derivatives, quercetin derivatives and rutin, demonstrated strong antimicrobial activity (Saavedra et al., 2010; Daglia, 2012). The relationship between the phenolic compounds and antimicrobial activity was also stated for methanol extracts of *T. chamaedrys* (Stanković et al., 2012). Vlase et al. (2014) observed strong antimicrobial activity in ethanol extracts of *T. chamaedrys*. The authors identified following phenolic compounds in extracts: chlorogenic and *p*-coumaric acids, luteolin, isoquercitrin, rutin and quercitrin. In many studies, antimicrobial effects of plant extracts have been attributed to their flavonoid fractions (Tsao et al., 1982; Cafarchia et al., 1999). *G. macrorrhizum* extracts rich in tannins were reported to have a broad spectrum of antimicrobial activities (Radulović et al., 2012). According to literature and results obtained in this study, antimicrobial activity of extracts could be related to high content of phenolic acids and some flavonoids, but high activity of extracts may be particularly attributed to other co-extracted compounds as well as synergistic effects (Ćetković et al., 2012).

3.4. *Cytotoxic activity*

Various bioactive compounds isolated from medicinal plants have showed beneficial effects in the treatment of different ailments. A number of studies have demonstrated anticancerogenic effects of phenolic compounds found in different plants (Itharat et al., 2004; Cvetanović et al., 2015; Švarc-Gajić et al., 2017).

In the current research, cytotoxic activity of extracts of medicinal plants obtained by subcritical water was evaluated and compared with *cis*-DDP or Cisplatin, a standard chemotherapeutic agent effective in the treatment of great number of cancers and tumors. MTT assay was used in the analysis of subcritical water extracts obtained at 160 °C and 10 bar. Cytotoxic effects of extracts were determined for three different cell lines: cell line derived from human cervix carcinoma, cell line derived from human rhabdomyosarcoma, and fibroblast cell line from murine. Calculated activities of the tested extracts are summarized in Table 5.

Obtained results indicated difference in the sensitivity of different cancerous cells to tested plant extracts. In general, RD was the most sensitive to the tested extracts, while Hep2c was the most resistant. Concentrations of subcritical water extracts of all tested plants which inhibited 50% of the tested cell lines were in the range from 11.10 to 36.39 μg/mL. The activities of all tested extracts against L2OB cells were very close. *M. nigra* extracts exhibited the highest cytotoxic activity in comparison to other plant extracts despite the lowest TPC indicating cytotoxic mechanisms other for those phenolic compounds-related. The extract was the most active against RD cell line. The lowest cytotoxic activity was calculated for *T. chamaedrys* extract and Hep2c cell line (36.39 μg/mL). These findings were not supported by TPC and antioxidant activity. The highest antioxidant activity and TPC were seen in subcritical water extracts of *T. chamaedrys*, while the lowest for *M. nigra* extracts. The results presented in this work indicated much better cytotoxic properties of SWE extracts in comparison to extracts obtained by other extraction techniques. According to Radojković et al. (2016), the supercritical fluid extracts of *M. nigra* leaves were more active against Hep2c (26 μg/mL), but less active against RD (27 μg/mL)

and L2OB (30 μg/mL) cell lines.

According to the American National Cancer Institute (NCI) and the criterion for cytotoxic activity of plant extracts (IC_{50} < 30 μg/mL) (Itharat et al., 2004), all subcritical water extracts were cytotoxic for all three cell lines.

Cytotoxic activity of plant extracts can be attributed to different phytochemicals, such as saponins, triterpenes, sterols, polyphenolic compounds, and is closely related to the extraction technique. The content of phytochemicals in the extracts is determined by numerous factors, such as solvating properties of applied solvent, extraction kinetics, mass transfer, *etc*. (Švarc-Gajić et al., 2017; Cvetanović et al.,2015). According to Švarc-Gajić et al. (2017), subcritical water is able to simultaneously extract different chemical classes. By carefully balancing operational parameters of subcritical water extraction, cytotoxic activity of extracts can be enhanced. In addition, products formed in the course of hydrothermal conversion can also contribute to cytotoxic activity of extracts (Švarc-Gajić et al., 2017).

Previous studies have demonstrated that different plant extracts exhibit cytotoxic activity against various cell lines, due to different phytochemicals. Radojković et al. (2016) speculated that polar compounds from *M. nigra* leaves were mostly responsible for cytotoxic activity of extracts. Authors identified gallic acid, protocatechuic acid, catehin, vanillic acid, chlorogenic acid, epicatehin, rutin, *etc*. in *M. nigra* leaves. These compounds have been also identified in the same species in this study, and it can be assumed that they contributed to high cytotoxic activity. Literature reports cytotoxicity of catechin and epicatechin to various cell lines (Seeram et al., 2003). High cytotoxic activity of compounds identified in *G. macrorrhizum*, such as gallic acid, ferulic acid and chlorogenic acid have been previously reported in the literature (Chlopčíková et al., 2004; Gawlik-Dziki et al., 2013). In the study by Stankovic et al. (2011), *T. chamaedrys* methanol extracts showed high content of phenolic compounds and strong cytotoxic activity. According to the previous results and results obtained in this study, cytotoxic activity of extracts may be partially attributed to high content of phenolic acids, however, secondary metabolites and synergistic effects probably contribute to high cytotoxic activity as well (Ćetković et al., 2012). In addition, subcritical water extraction is specific in sense that at high extraction temperatures numerous thermochemical conversion products are formed (Švarc-Gajić et al., 2017; Simsek Kus, 2012). Neoformed compounds may affect substantially bioactivity. For some of those compounds such as Maillard reaction products, antioxidant (Plaza et al., 2010; Vhangani and Van Wyk, 2013), antibacterial (del Castillo et al., 2007) and antihypertensive (Rufián-Henares and Morales, 2007) properties have been previously reported.

4. Conclusions

The overall results of the present study clearly demonstrate that subcritical water extracts of the studied medicinal plants are promising sources of compounds with antioxidant, antimicrobial and cytotoxic properties. Extracts obtained at 160 °C and 10 bar showed the highest TPC and antioxidant activity. Moreover, the HPLC analysis reveal that gallic, protocatechuic and chlorogenic acids were the main contributors to the phenolic profile of the studied medicinal plants, especially in the case of *Geranium macrorrhizum*. The extracts were active against eight tested microbial strains. *Staphylococcus aureus* (MIC = 19.53 μg/mL) was the most sensitive to subcritical water extracts, whereas in the case of fungi the strongest activity of extracts was seen for *Aspergillus niger* (MIC = 39.1 μg/mL). Subcritical water extracts confirmed to be cytotoxic for cancerous cells derived from human cervix carcinoma, human rhabdomyosarcoma and murine fibroblast. All studied medicinal plant extracts obtained by SWE indicated better bioactivity in comparison to extracts obtained by other extraction techniques. According to the presented results it can be concluded that subcritical water extracts of studied medicinal plants may have great potential for the production of safe, pharmacologically-active fractions.

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