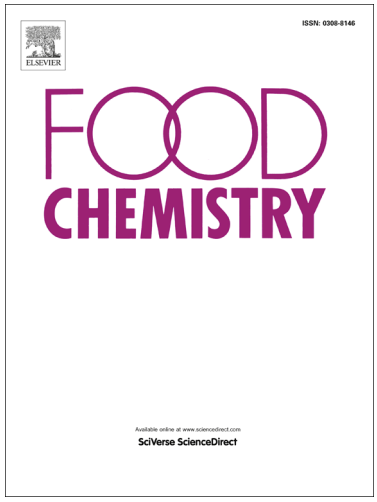

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The influence of the extraction temperature on polyphenolic profiles and bioactivity of chamomile (*Matricaria chamomilla*L.) subcritical water extracts

Aleksandra Cvetanović[1*], Jaroslava Švarc-Gajić[1], Zoran Zeković[1], Jelena Jerković[1], Gokhan Zengin[2], Uroš Gašić[3], Živoslav Tešić[3], Pavle Mašković[4], Cristina Soares[5], M. Fatima Barroso[5], Cristina Delerue-Matos[5], Saša Đurović[1,6]

[1]Faculty of Technology, University of Novi Sad, Bulevar Cara Lazara 1, 21000 Novi Sad, Serbia

[2]Department of Biology, Faculty of Science, Selcuk University, Campus, Konya, Turkey

[3]Faculty of Chemistry, University of Belgrade, P.O. Box 51, 11158 Belgrade, Serbia

[4]Faculty of Agronomy, University of Kragujevac, Cara Dušana 34, 32000 Čačak, Serbia

[5]REQUIMTE/LAQV, Instituto Superior de Engenharia do Instituto Politécnico do Porto, Rua Dr. António Bernardino deAlmeida, 431, 4249-015 Porto, Portugal

[6]Institute of General and Physic Chemistry, Studentski trg 12/V, 11158 Belgrade, Serbia

[*]Corresponding author: Tel.: +381641590019; Fax: +38121450413

E-mail address: a.c.istrazivac@gmail.com (Dr. Aleksandra Cvetanović)

Abstract

The main goal of this research was to determine the relationship among chemical structure, bioactivity and temperature of chamomile during subcritical water extraction in isobaric conditions (45 bar) at seven different temperatures (65-210°C). The influence of temperature on phenolic profiles was defined by UHPLC-HESI-MS/MS. The overall results indicate that the presence of conjugated double bonds, side chains, glucose moiety or ether moiety in molecules influence the efficiency of polyphenols' extraction in subcritical water. In terms of antioxidant activity, the extracts were the most active towards ABTS radicals (IC_{50}=7.3-16.8 µg/mL), whereby temperature of 150°C was optimal. On the other hand, the extracts obtained at 115°C showed highest cytotoxicity. Inhibition of α-amylase and α-glucosidase was the highest at 65 and 85°C, i.e. 0.51 and 4.13 mmolAE/g, respectively. Activity against tyrosinase was the highest at 210°C (17.92 mgKAE/g). The data showed that different non-phenolic compounds may also participate in bio-activities of the extracts.

Keywords: Subcritical water extraction, chamomile, UHPLC-HESI-MS/MS, DNA-based sensor, bioactivity.

1. INTRODUCTION

Chamomile (*Matricaria chamomilla*L., syn: *M. recutita*) is a medicinal plant mostly used in the form of infusions, the consumption of which is estimated to be over a million cups per day (Srivastava and Gupta, 2010). Its pleasant, aromatic taste, delicate sedative effect, and a range of beneficial health effects are the main reasons for its widespread use. Numerous studies have shown that chamomile can be used for various purposes due to its beneficial activities such as: anti-inflammatory, antiphlogistic (Srivastava, Shankar and Gupta, 2010), antiallergic (Nemecz, 1998), antibacterial (Lis-Balchin, Deans, and Eaglesham, 1998), antispasmodic, antiseptic (Sahebkar and Emami, 2013), antioxidant (Lee and Shibamoto, 2002) and anti-tumor (Shukla and Gupta, 2004). Because of its numerous health benefits and frequent use, the composition of chamomile has been studied extensively and many therapeutically-attractive compounds have been identified. Among others, chamomile phenolic compounds have been well studied and different classes of phenolic compounds were identified in chamomile, such as: phenolic acids (chlorogenic, caffeic, vanillic, syringic, anisic, coumaric acid, etc), flavonoids (aglycones and/or glycosides form: isorhamnetin, luteolin, quercetin, apigenin, patuletin and some others), coumarins (umbelliferone and herniarin) (Atoui, Mansouri, Boskou, and Kefalas, 2005), etc.

It is believed that polyphenols, namely the subfamily of flavonoids, are the most responsible for high antioxidant activity of chamomile (Pinto, 2013). The most common extraction approaches for the isolation of phenolic compounds from chamomile include conventional extraction techniques using ethanol as a solvent (Pinto, 2013). Lately, non-conventional extraction approaches, such as microwave or ultrasound extraction, have been reported for chamomile extraction (Cvetanović, Švarc-Gajić, Mašković, Savić and Nikolić, 2015). During the last 20 years, the focus of the research has been put on the non-conventional

extraction approaches. For instance, water in its subcritical state offers numerous advantages as a completely green and economically viable solvent, the selectivity of which can be varied by changing temperature and pressure. To the best of our knowledge, only few reports have dealt with subcritical water extraction (SWE) of chamomile (Cvetanović et al., 2015; Cvetanović et al., 2017).

SWE offers the possibility to fine tune water polarity by varying operational parameters, so slight changes in operational parameters may have significant impact on chemical composition of extracts. By changing two principal operational parameters, temperature and pressure, dielectric constant of subcritical water can be varied from 13 (in critical point) to 80 (ambient water). Moreover, pH value of water decreases three orders of magnitude with a temperature increase to 250 °C, thus providing more H_3O^+ ions for acid-catalyzed reactions (Cvjetko Bubalo, Vidović, Radojčić Redovniković and Jokić, 2015). Carefully balanced, moderate hydrolytic potential of superheated water may be exploited when using this solvent for the extraction of bound forms of bioactive molecules. Within the last 10 years, this solvent attracts attention for the extraction of bioactive compounds such as polyphenols and due to its green character it is particularly interesting for obtaining extracts with improved bioactivity. High bioactivity of SCW extracts is a complex function of desired but also other co-extracted compounds. Moreover, there are indications that different chemical reactions, such as Maillard reaction, can occur in this medium (Plaza, Amigo-Benavent, Del Castilo, Ibáñez and Herrero, 2010). Such a reaction produces beneficial bioactivities, such as antioxidant, antimicrobial or antiproliferative. Thus, high bioactivity of SCW extracts is also influenced by neo-formed product. From this point of view, it would be extremely difficult to ascribe certain bioactivity to some specific compounds. Although numerous findings have demonstrated correlation between

polyphenols and bioactivity of plant extracts, the overall activity of the extracts is also a consequence of non-phenolic compounds. The occurrence of different reactions in SCW medium depends on the extraction conditions, and particularly on the temperature. Different extraction conditions cause differences in chemical composition, further reflecting their bioactivities and functionalities.

There is no data on the influence of the extraction parameters on the bioactivity of SWE chamomile extracts. Thus, the main goal of this study was to define the influence of temperature, as a dominant parameter in SWE, on phenolic profiles and, antioxidant, cytotoxic and enzyme-inhibitory activity of chamomile extracts. The following analytical procedures were used: UV/V spectrometry, DNA-based biosensor and UHPLC-HESI-MS/MS. Possible influence of other co-extracted non-phenolic compounds on bioactivity of observed extracts was also considered. We analyzed the data to obtain full profiles of biological activity.

2. MATERIALS AND METHODS

2.1. Chemicals

The following chemicals and compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA): kojic acid, α-amylase solution (ex-porcine pancreas, EC 3.2.1.1), α-glucosidase solution (from *Saccharomyces cerevisiae*, EC 3.2.1.20), L-glutathione, tyrosinase, 3,4-dihydroxy-L-phenylalanine (L-DOPA), acarbos, deoxyadenylic acid oligonucleotide (dA_{20}, as a desalted product), concentrated saline sodium phosphate EDTA (20x SSPE; 0.2 mol/L sodium phosphate, 2 mol/L, NaCl, 0.02 mol/L EDTA), phosphate buffer (PBS) pH 7.4, iron (II) sulphate heptahydrate, hydrogen peroxide (30 %, w/v), Folin Ciocalteu reagent, 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS),

paraffin oil and polyphenolic standards - analytical grade and purity ≥ 99% (apigenin-7-*O*-glucoside, apigenin, kaempferol-3-*O*-glucoside, kaempferol, luteolin-7-*O*-glucoside, luteolin, naringenin-7-*O*-neohesperidoside, naringenin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, catechin, galangin, phloretin, resveratrol, aesculin, ellagic acid, *p*-hydroxybenzoic acid, protocatechuic acid, caffeic acid, sinapic acid, cinnamic acid, 5-*O*-caffeoylquinic acid, *p*-coumaric acid and coniferyl aldehyde). Aluminium chloride hexahydrate, sodium carbonate, PNPG (4-N-trophenyl-α-D-glucopyranoside), sodium acetate trihydrate, acetonitrile and acetic acid (both of MS grade), methanol (HPLC grade) were purchased from Merck Co (Darmstadt, Germany). Potassium ferricyanide and ferric chloride were obtained from Zorka (Šabac, Serbia). Ultrapure water (Thermofisher Scientific, Bremen, Germany) was used to prepare standard solutions and blanks. Syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA). Graphite powder was obtained from Ultracarbon (Dicoex, Spain). All other chemicals and reagents were of analytical reagent grade.

2.2. Plant material

Plant material was produced by the Institute of Field and Vegetable Crops, Bački Petrovac, Serbia. Since phenolic compounds are mainly contained in chamomile ligulate flowers, the extractions were done only with this part of Matricria flos. Matricria flos was collected at the end of April and dried at 40 °C in a solar dryer until the moisture content reached 12%. Chamomile ligulate flowers were then separated from the tubular parts with a sieve. The flowers were packed in paper bags and stored in a dark place for future use.

2.3. Subcritical water extraction

Subcritical water extraction was performed using a homemade subcritical water reactor as described in Cvetanović (Cvetanović et al., 2017). Dry plant material (10.0 g) was placed in a reaction vessel and 300 mL of double distilled water were added. The process of extraction was performed within 30 minutes upon reaching the desired temperature at 45 bar in a temperature range between 65 to 210 °C. The mass transfer during extraction was potentiated by vibrational movements of the platform housing the extraction vessel. After filtration, the obtained extracts were evaporated by the use of vacuum evaporator (Devarot, Slovenia) and dried at 40 °C. The obtained dry extracts were stored in a dark place at 4°C until analysis.

2.4. UHPLC–DAD MS/MS analysis of polyphenolic compounds

Qualitative and quantitative analyses of phenolic compounds in SWE extracts were performed using a Dionex Ultimate 3000 UHPLC system equipped with a diode array detector (DAD) that was connected to TSQ Quantum Access Max triple-quadrupole mass spectrometer (ThermoFisher Scientific, Basel, Switzerland). Separation process was performed at 40°C on a Syncronis C18 column (100 × 2.1 mm, 1.7 µm particle size) from ThermoFisher Scientific. The mobile phase consisted of water + 0.01% acetic acid (A) and acetonitrile (B), that were used in the following gradient elution: 5% B in the first 2.0 minutes, 2.0^{nd}–12.0^{th} minutes 5–95% B, 12.0^{th}–13.0^{th} minutes from 95% to 5% B, and 5 % B until the 20^{th} minutes. The flow rate was set to 0.3 mL/min and the detection wavelengths to 254 and 280 nm. The injection volume was 5 µL.

Stock methanolic solutions of polyphenolics in the concentration of 1000 mg/L were prepared. The stock solutions were mixed and diluted with water in order to obtain working solutions (concentrations of 0.01, 0.05, 0.10, 0.25, 0.50, 0.75, and 1.00 mg/L).

A TSQ Quantum Access Max triple-quadrupole mass spectrometer equipped with heated electrospray ionization (HESI) source was used with the vaporizer temperature kept at 250°C, and ion source settings as follows: spray voltage 4500 V, sheet gas (N_2) pressure 27 AU, ion sweep gas pressure 0 AU and auxiliary gas (N_2) pressure 7 AU, capillary temperature 275°C, skimmer offset 0 V, and capillary offset -35 V. The mass spectrometry data were acquired in the negative ionization mode, in *m*/*z* range from 100 to 1000. Multiple mass spectrometric scanning modes, including full scanning (FS), and product ion scanning (PIS), were conducted for a qualitative analysis of the targeted compounds. The collision-induced fragmentation experiments were performed using argon as collision gas, while the collision energy varied, depending on the compound. The time-selected reaction monitoring (tSRM) experiments for quantitative analysis were performed using two MS^2 fragments for each compound that was previously defined as dominant in the PIS experiments (Gašić et al., 2015). Xcalibur software (version 2.2) was used for instrument control. Phenolics were identified and quantified according to the corresponding spectral characteristics: molecular ion, mass spectra, characteristic fragmentation, and characteristic retention time. The limits of detection (LOD) and quantification (LOQ) were calculated using standard deviations (SD) of the responses and the slopes of the calibration curves (S) according to: LOD = 3(SD/S) and LOQ = 10(SD/S). The values of standard deviations and slopes were obtained from the calibration curves created in MS Excel. The total amount of each compound was calculated based on the peak areas and was expressed in mg/kg. SRM transitions and calibration ranges for each compound, as well as correlation coefficients, LOD and LOQ are given in Supplementary Data (Tables S1).

2.5. Biological activity

2.5.1. Antioxidant and antiradical activity

Dry extracts were dissolved in water to the final concentration of 1 mg/mL. This solution was further used for measuring antioxidant and antiradical activities. The activities were measured by both spectrophotometrical (inhibition of lipid peroxidation, $DPPH^{\bullet}$, $OH^{\bullet}$ and $ABTS^{\bullet+}$ tests) and electrochemical DNA-biosensor analysis. All applied methods rely on different mechanisms, and by their simultaneous application, we obtained a comprehensive view of the antioxidant potential of the investigated extracts. All tests were performed in triplicate and the results were expressed as IC_{50} (μg/mL). Spectrophotometrical measurements were performed on Yenway 6300 Spectrophotometer (Barloworld Scientific Ltd, Dunmow, Essex, UK).

2.5.1.1. Inhibitory activity against lipid peroxidation

Inhibitory activity against lipid peroxidation was evaluated using the thiocyanate method (Misuda, Yasumoto and Iwami, 1966). The applied method actually measures the amount of peroxide produced during the initial stages of oxidation, which is the primary product of lipid oxidation. Briefly, the obtained extracts were diluted to serial dilutions (20 - 40 μg/mL), and 0.5 mL of each concentration was added to linoleic acid emulsion (2.5 mL, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid and 0.2804 g of Tween-20 in 50 mL of 40 mM phosphate buffer. The mixture was incubated at 37 °C for 72 hours. After that, 0.1 mL of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL of $FeCl_2$ (20 mM), and 0.1 mL of ammonium thiocyanate (30%). The mixture was stirred for 3 minutes and absorbance was measured at 500 nm. Ascorbic acid, gallic acid, α-tocopherol and BHT were used as reference compounds. All tests were performed in triplicate, and the results were expressed as IC_{50} values (the concentration of the test solution for inhibiting 50% oxidation inhibition).

2.5.1.2. $DPPH^{\bullet}$ radical-scavenging assay

DPPH$^{\bullet}$ radical-scavenging assay was conducted following Espín, Soler-Rivas and Wichers method (2000). DPPH is a stable free radical with an unpaired electron that is delocalized over the entire molecule. During the assay, its purple color turned yellow, due to pairing of the odd electron of the DPPH radical with hydrogen to form the reduced DPPH-H form. The resulting discoloration, which is stoichiometric, is proportional to the number of captured electrons. In a nutshell, the start solutions of the extracts were mixed with methanol (96%) and 90 µM DPPH to give final concentrations of 0.01; 0.02; 0.05; 0.1 and 0.2 mg/mL of dry extract. After incubation at a room temperature for 60 minutes, the absorption of the reaction mixture was measured at 515 nm.

2.5.1.3. Determination of hydroxyl radical scavenging activity

Determination of hydroxyl radical scavenging activity was carried out following the method described in the literature (Halliwell, Gutteridge and Aruoma, 1987). Inhibition activity of the obtained extracts against hydroxyl radicals was determined by measuring the level of oxidation of 2-deoxy-D-ribose by $^{\bullet}$OH with subsequent measurement of the products by their reaction with thiobarbituric acid (TBA). The reaction of deoxyribose and $^{\bullet}$OH has been discussed extensively in the literature (Aruoma, 1993.). The extract was mixed with 500 µL of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4–NaOH buffer (50 mM, pH 7.4), 200 µL of premixed 100 µM $FeCl_3$ and 104 mM EDTA (1:1, v/v) solution, 100 µL of 1.0 mM H_2O_2, and 100 µL of 1.0 mM aqueous ascorbic acid. Reaction mixture was incubated at 50 °C for 30 minutes. 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were then added to each tube. The samples were vortexed and heated in a water bath at 50 °C for 30 minutes. The degree of 2-deoxyribose oxidation was estimated from the absorbance of the solution at 532 nm.

2.5.1.4. ABTS$^{\bullet+}$ radical scavenging activity

$ABTS^{\bullet+}$ radical scavenging activity was determined following Jiménez-Escrig, Dragsted, Daneshvar, Pulido and Saura-Calixto method (2003). In this assay, ABTS is converted into its radical cation ($ABTS^{\bullet+}$). The ABTS assay utilizes a free radical which is generated when ABTS substrate is oxidized with potassium persulfate. $ABTS^{\bullet+}$ has a blue/green color with maximum absorption spectra at 734 nm, in water. The $ABTS^{\bullet+}$, is decolorized when reduced in the presence of the test sample. This indicates the extent of relative radical scavenging activity which is expressed as a percent of inhibition. In this study, $ABTS^{\bullet+}$ was generated by 7 mM ABTS stock solution and 2.45 mM potassium persulfate. The mixture was left in a dark place at room temperature for 12–16 hours. The $ABTS^{\bullet+}$solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) to the absorbance at 730 nm of 0.70±0.02. Upon addition of 10 µL of the sample to 4 mL of diluted $ABTS^{\bullet+}$ solution, the absorbance was measured after 30 minutes. Gallic acid, ascorbic acid and butylated hydroxytoluene (BHT) were used as reference antioxidants.

2.5.1.5. Electrochemical DNA-based biosensor

The protective effect of antioxidants at a cellular level could be achieved by monitoring the DNA integrity. So, an electrocatalytic voltammetric method was performed to assess total antioxidant capacity of SWE chamomile extracts using DNA-modified carbon paste electrodes (CPE). The oxidative lesions were generated after immersion of the DNA-CPE in the Fenton mixture. The lesions were indirectly quantified after the electrochemical oxidation of the adenines that remained unoxidized on the electrode surface (Barroso et al., 2016). The increase of the electrocatalytic current in the presence of antioxidants from chamomile was evaluated.

Stock solutions of deoxyadenylic acid oligonucleotide (dA_{20}, as a desalted product, 1 g/L), were prepared and stored at 4ºC and diluted with 2x SSPE buffer solution prior to use. Fe^{2+},

EDTA and H_2O_2 (1 µmol/L : 2 µmol/L : 40 µmol/L) were mixed to prepare the Fenton solution (generation of hydroxyl radical). All solutions were prepared with ultra-pure water produced with a Simplicity 185 system manufactured by Millipore (Molsheim, France). Square wave voltammetry (SWV) was performed in an Autolab PSTAT 10 controlled by GPES software, version 4.8 (EcoChemie, The Netherlands). A conventional three electrode cell was used, which includes a homemade carbon paste electrode (CPE) (3 mm in diameter) as a working electrode, a platinum wire counter electrode and an Ag|AgCl|KClsat reference electrode (Barroso et al., 2016).

The biosensor experiments were conducted in three steps: DNA immobilization, damage of oligonucleotide (dA_{20}) by immersion of the dA_{20}-CPE on the Fenton solution ($HO^{\bullet}$ generation) in the absence/presence of antioxidants or real samples, and detection and measurement of the peak current of dA_{20} in a PBS solution at pH 7.4.

DNA immobilization was performed by dry adsorption placing a 4 µL of 100 mg/L of dA_{20} on the CPE surface and evaporating it to dryness under a stream of nitrogen. DNA damage was carried out by immersing the DNA-based CPE in a freshly prepared Fenton solution in the absence or in the presence of antioxidant standard or chamomile extract in 2x SSPE buffer. After a fixed period of 30 seconds of reaction time, the DNA-based CPE was washed with water and immediately immersed in PBS (pH 7.4). SWV was then conducted between + 0.7 to + 1.5 V and the obtained oxidation peak current of dA_{20} was used as a detection signal. For the electrochemical studies, the maximum signal current obtained was for the dA_{20} electrochemical signal, with no damage or antioxidant effect.

2.5.2. Cytotoxic activity

The influence of the 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). The activity of the chamomile extracts was determined by the method described elsewhere (Mosmann, 1983). All experiments were done in triplicate.

2.5.3. Enzyme-inhibitory activity

The enzyme inhibitory effects were tested against α-amylase (Caraway-Somogyi iodine/potassium iodide (IKI) method), α-glucosidase (PNPG (4-N-trophenyl-α-D-glucopyranoside) method) and tyrosinase (L-DOPA (3,4-dihydroxy-L-phenylalanine) method by using microplate reader. The procedures were explained in previous papers (Orhan, Senol, Gulpinar, Sekeroglu, Kartal and Sener, 2012; Zengin, Sarikurkcu, Aktumsek, Ceylan and Ceylan, 2014). The inhibitory effects were evaluated and expressed as equivalents of standard drugs (acarbose for amylase and glucosidase; kojic acid for tyrosinase).

2.6. Statistical analysis

All analyses were performed in triplicate and they were expressed as means ± standard deviation (SD). Mean values were considered significantly different at $p < 0.05$ confidence level, after the performance of the ANOVA single/double factor statistical analysis followed by Tuckey test.

3. RESULTS AND DISCUSSION

3.1. UHPLC-HESI-MS/MS analysis of polyphenolic compounds

In SWE the optimal extraction temperature depends on the target compounds. The increase of extraction temperature produces a series of effects, including improvement of the mass transfer, desorption kinetics and solubility. However, the most important effects of the temperature in SWE are related to the weakening of hydrogen bonds, resulting in polarity changes. Elevated temperatures may cause degradation of compounds via numerous reaction paths (Plaza and Turner, 2015). Thus, a comprehensive study is needed to maximize the yield of target components in the final extract.

In the current study, the relationship among the extraction temperature, the yield of phenolic compounds from chamomile and biological activity of extracts was observed. In the examined extracts, 24 different polyphenolic compounds (Tables 1 and 2) were identified and their solubility in subcritical water was influenced by the temperature which could be related to their structures.

Regarding the flavonoids and their glucosides, the results showed that lower temperatures favored the extraction of glycosides (Table 1). This could be explained by the fact that sugar moiety increases the polarity of molecules. With heating, the polarity of water decreases and subcritical water becomes a moderately polar solvent. Thus, glycoside forms were better extracted at lower temperatures. For example, the highest yield of apigenin, luteolin, and naringin glucosides was achieved at 85 °C. Their concentration dropped at higher temperatures, probably as a consequence of hydrolysis and degradation in subcritical water. Glucosides, except the apigenin-7-O-glucoside, were not extracted at the temperatures above 150 °C. In case of apigenin-7-O-glucosides, the yields at the temperatures above 150 °C were very low, and insignificant differences among them were noticed ($p > 0.05$). For the extraction of aglycones apigenin, luteolin, naringenin and kaempferol, 115°C was determined to be the optimal

temperature. Temperatures higher than 130 °C resulted in the decrease of their yields indicating their degradation. When aglycones are to be extracted, the use of subcritical water at carefully optimized temperature allows convenient simultaneous extraction and hydrolysis. Hydrolysis of glycosides in subcritical water did not result in approximately equal sum of aglycones and its glycosides. It could be assumed that high hydrolytical potential of subcritical water also caused hydrolysis of glycosides derivatives. Other apigenin-glycoside derivatives, mainly mono- and diacetylated derivatives, were converted to apigenin-7-glucoside. It could be speculated that during SWE, a fraction of apigenin-7-*O*-glucoside was hydrolyzed to apigenin, while other derivatives of apigenin-7-glucoside were simultaneously transformed to apigenin-7-*O*-glucoside.

In case of aglycones, it was noticed that applying high temperature (210 °C) is unjustified, due to the fact that similar yields were obtained at both 65 and 210 °C. For example, in case of luteolin, the yield was insignificantly changed (p = 0.058060) with increasing the temperature from 65 to 210 °C.

Quercetin was detected only in its bound forms as quercetin-3-*O*-galactoside and quercetin-3-O-rutinoside. In case of both compounds, temperature rise from 85 to 150 °C resulted in significant differences in their yields ($p < 0.05$). Maximal yields of quercetin bound forms were achieved at the temperature of 115 ºC. At this temperature the yield of kaempferol 3-glucoside peaked, as well.

Catechin structurally belongs to the group of flavan-3-olswithout a keto-group in position 4 in C-ring. The structural difference between flavanols and above discussed flavonols, between flavones and flavanones also influences the solubility of catechin, which was detected only in extract obtained at 130 °C.

The influence of hydroxyl groups in phenyl ring on solubility in subcritical water could be clearly seen in the case of galangin. The compound belongs to the class of flavonols without hydroxyl substituent in B-ring. Galangin was extracted only at 210 °C because it has higher hydrophobicity than other aglycones.

Apart from flavonoids, other polyphenolics were detected as well, including various phenolic acids, one coumarin and one stilbene derivative. Compounds, belonging to different chemical classes, showed different solubility in subcritical water, which could be linked to their structure, the presence of double bonds, and different substituents in their structures (Table 2).

Phloretin, which belongs to the class of dihydrochalcone, was detected only in extracts obtained at 210 °C. The conjugation of phenyl ring with keto-group in the molecule affected the stability, polarity and solubility in subcritical water. Resveratrol (stilbene class) has conjugated system of double bonds and this compound was detected only in extracts obtained at 180 °C and 210 °C with the higher yield at 210 °C. Significant differences in its yield at 180 and 210 °C were noticed ($p = 0.00017$). For aesculin, a coumarin glucoside, as in the case of other flavonoid glucoside, glucose moiety had big influence on solubility in subcritical water; this led to the maximal yield at 85 °C. As the temperature is further increased, its yield decreased significantly ($p < 0.05$).

The influence of temperature was also obvious in the case of phenolic acids (Table 2). Ellagic acid contains two condensed benzopyran rings with keto- and hydroxyl-groups oriented towards the exterior of the molecule. High potential for hydrogen bond formation suggests that this compound is better dissolved in SWE at lower temperature, i.e. in water with higher dielectric constant. In accordance with this, the maximum yield of the compound was achieved at the temperature of 85 °C.

Strong influence of polar groups on solubility in SWE was noticeable in the case of *p*-hydroxybenzoic and protocatechuic acids. Hydroxyl group in *meta* position in protecatechuic acid contributed to better solubility of this compound in SWE at lower temperature compared to *p*-hydroxybenzoic acid.

Caffeic acid, 5-*O*-caffeoylquinic acid, *p*-coumaric acid, sinapic acid, cinnamic acid and coniferyl aldehyde share the same structural unit consisting of 3-phenyl-2-propenoic acid (cinnamic acid). The solubility of these compounds in SWE depended on the position, type and number of substituents in the molecule.

Cinnamic acid (3-phenyl-2-propenoic acid) has a system of conjugated double bonds in hydrocarbon chain extending to phenyl ring which indicates better solubility at higher temperatures. The results confirmed this assumption with the maximum yield of the phenolic acid at 210 °C. On the other hand, maximum yields of caffeic acid, 5-*O*-caffeoylquinic acid, and *p*-coumaric acid, were obtained at 85 °C. Such results might be explained by structural similarity of caffeic and *p*-coumaric acids, whereas 5-*O*-caffeoylquinic acid represents an ester of caffeic and quinic acids. The presence of hydroxyl and carboxyl groups in those molecules led to their maximal solubility in SWE at lower temperature.

On the other hand, the maximum yield of cinnamic acid, sinapic acid, and coniferyl aldehyde was achieved at the temperature of 210 °C. Unlike caffeic and *p*-coumaric acids, coniferyl aldehyde and sinapic acid are ethers containing methoxy groups attached to phenyl ring, while cinnamic acid does not contain hydroxyl groups linked to aromatic ring. Such structural diversity affected solubility in SWE. By substituting polar hydroxyl groups with ether groups and carboxyl group with aldehyde group, the solubility of the compounds increased in

water at higher temperatures. Generally, in case of phenolic acids, an increase in temperature produces significant differences in its yields ($p < 0.05$).

The influence of the extraction temperature on the efficiency of the extraction of phenolic compounds was in accordance with the existing data, indicating that compounds that are not able to form hydrogen bonds, and compounds with double bonds in their structure, are better solubilized in water at higher temperatures (Ko, Cheigh, Chung, 2014). Performed research also show that the system of conjugated double bonds, polar groups (hydroxyl, carboxyl, etc.) and glycoside moieties increase the solubility of compounds in subcritical water at lower temperatures (Ko et al., 2014). On the other hand, the substitution of these groups with ether moieties or hydrogen atom shifts the maximum solubility to higher extraction temperatures.

3.2. Antioxidant activity

In this paper, the relationship between extraction temperature and antioxidant activity of extracts was studied by different methods (Table 3) which were based on different mechanisms. Therefore, a more thorough insight in the antioxidant potential of extracts was provided.

Hydroxyl radicals ($OH^{\bullet}$) are among the most reactive free radical species associated with tissue damage, degradation of proteins, insoluble lipids, carbohydrates, nucleic acids and other important biomolecules (Calderon and Robertfroid, 1988). Because of their negative impact on living systems, the capacity of their neutralization by natural molecules is of great importance. The capacity of chamomile extracts obtained by SWE to neutralize $OH^{\bullet}$ radicals was determined by measuring the degree of oxidation of 2-deoxy-D-ribose by hydroxyl radicals and subsequent measurement of the product with thiobarbituric acid. All tested extracts showed high neutralization capacity against $OH^{\bullet}$ radicals. The most potent in neutralizing $OH^{\bullet}$ radicals was the extract obtained at the temperature of 150 ºC, while the lowest activity was measured for the

sample obtained at 65 ºC. A slight increase in antiradical activity with temperature up to 150 °C was noticed, although there were no significant differences among the extracts obtained at temperatures from 65 to 130 ºC ($p > 0.05$). Insignificant difference was also noticed between the extracts obtained at 150 and 180 °C ($p = 0.567869$). Activity decreased at the highest temperature (IC_{50} = 41.50 µg/mL) and became similar to the activity of the extracts obtained at lower temperatures (65, 85, 115, 130 °C). Insignificant differences among extracts obtained at lower temperatures and extracts obtained at 210 °C were noticed ($p > 0.05$). The activity of all extracts was higher than antioxidant activity of standard compounds, ascorbic and gallic acids.

The temperature of 150 °C showed to be the optimal in the case of $ABTS^{\bullet+}$ radicals, as well. Generally, the influence of the extraction temperature on the scavenging ability towards long-living $ABTS^{\bullet+}$ radical species was similar to the previous case. Extraction temperatures up to 150 °C produced extracts with increased activity. This implies that at this temperature the extraction of antioxidant compounds was the most efficient. Thermochemical process and reactions might have contributed to the overall antioxidant activity. Neo-formed compounds could have been produced during the Maillard reaction or caramelization. These reactions are particularly favored at the temperatures between 140 °C and 160 °C (Plaza, Amigo-Benavent, Del Castilo, Ibáñez and Herrero, 2010). Furthermore, different constituents may appear in SWE extracts because of their formation during the hydrothermal conversion or because of rearrangement reactions. This can explain the fact that significantly higher activity of extract was obtained at 150 °C in comparison to all others ($p < 0.05$). The ability of extracts to neutralize $ABTS^{\bullet+}$ radicals was comparable with BHT, a well-known synthetic antioxidant.

Slightly different situation was observed in the case of $DPPH^{\bullet}$ radicals where maximum antiradical activity was seen in the extract obtained at 210 °C. The obtained inhibitory

concentrations were in the range from 10 to 45 μg/mL, indicating a strong antiradical potential. The extracts obtained at the temperatures below the boiling point showed significantly lower activity ($p < 0.05$). With the temperature increase, the extracts started to be more active towards DPPH radicals. However, significant differences among the extracts obtained at the temperature above the boiling point were not noticed ($p > 0.05$). The exceptions were extracts obtained at 115 and 210 °C, which showed significantly different activities among each other ($p = 0.00381$). As in the previous case, the activity was comparable with BHT (Table 3).

The peroxidation of unsaturated fatty acids is the main cause of oxidative damage of cell membranes, as well as other biological systems, containing lipids (Halliwell and Gutteridge, 1985). The ability of extracts to inhibit lipid peroxidation was evaluated by the thiocyanate assay. SWE extracts demonstrated high activity against inhibition of lipid peroxidation (Table 3). The results showed that all tested extracts inhibited the formation of hydroperoxide. The analyzed extracts showed different degrees of effectiveness (IC_{50} = 28.67 - 36.60 μg/mL). Increasing the temperature from 65 to 85 °C resulted in extracts which expressed significant differences in terms of their ability to inhibit the process of lipid peroxidation ($p = 0.038656$). Also, significant differences were observed with increasing the temperature from 130 to 150 °C ($p = 0.0000627$) and from 150 to 210 °C ($p = 0.010463$). Temperature of 150 °C resulted in the extract with the highest potential to inhibit lipid peroxidation (IC_{50} = 28.67 μg/mL). The activity of all extracts in inhibiting lipid peroxidation was much higher in comparison to ascorbic and gallic acids.

Antioxidant activity assays indicated strong influence of the extraction temperature. However, clear relationship between polyphenolic composition and antioxidant activity could not be defined. This implies that other co-extracted compounds of non-phenolic structure

contributed to antioxidant activity of the extracts. In addition, antioxidant compounds may be formed during the SWE process. Furthermore, synergistic effects between phenolic compounds and other co-extracted and neo-formed compounds influence the overall antioxidant and antiradical activities.

The use of electrochemical DNA-based biosensors for the antioxidant activity assessment is useful because the principle of measurement is closer to the activity of antioxidants in biological systems by simulating the *in vivo* damage caused by free radicals (Barroso et al., 2016). Basically, these biosensors are based on the DNA-based materials immobilization onto CPE (by adsorptive processes) which is then exposed to radicals and antioxidant compounds. Using SWV, the DNA damage (induced by radicals) can be monitored by analyzing the dramatic decreases of the electrochemical oxidation currents of the DNA nucleobases when compared with the DNA native electroactivity. The effects of antioxidants can be estimated by measuring an increase of the electrochemical current attributed to the scavenging activity of the antioxidants. In this work, the ability of chamomile SWE extracts to protect DNA was tested. The obtained results showed that all extracts expressed protective effects. As shown in Table 3, when the DNA-based sensor was used, all extracts presented antioxidant activity from 28 to 218 µg GAE/mL. The highest antioxidant activity (218 µg GAE/mL) was determined in the extract obtained at 210 ºC, which was in accordance with results obtained in DPPH assay. The obtained results showed that the temperature rise from 65 to 85 °C led to the extracts which possess similar ability to protect DNA molecules, and the differences between these two extracts were insignificant ($p > 0.05$). Further temperature increases led to obtaining the extracts which have significantly different ability ($p < 0.05$).

The results show that the temperature has a direct influence on antioxidant activity of SWE chamomile extracts. Fine tuning of the temperature during SWE process causes makes changes in the ability of chamomile extracts to act as antioxidants. Taking into account overall results, it could be concluded that extractions at the temperatures above 150 °C resulted in obtaining extracts with improved antioxidant ability in comparison to the extracts obtained at lower temperatures. The data could be vital to the food industry. Due to the well-known unwanted effects of synthetic antioxidants, there is a huge tendency to use natural antioxidants as food additives in foods. Among good antioxidant characteristics, chamomile extracts prepared by SWE technique are characterized by their "green" character. Such extracts do not contain residue of organic/toxic solvents and there is no need for their further purification. From this point, such extracts can be directly used or incorporated in myriad of food products. Furthermore, avoiding purification makes this process economically acceptable from the industrial point of view.

3.3. Cytotoxic activity

Cytotoxic activity of the extracts was evaluated against three cell lines and compared with standard cytotoxic compound (cis-diamminedichloroplatinum - cis-DDP). Both extracts and the standard caused considerable dose-dependent inhibition of the cell growth. Strong influence of the extraction temperature on the cytotoxic activity was noticed (Table 4). Based on the criterion for cytotoxic activity for plant extracts (IC_{50}< 30 µg/mL) (Itharat, Houghton, Eno-Amooquaye, Burke, Sampson, Raman, 2004), the extracts obtained at temperatures above 115 °C may be considered as potent cytotoxic agents.

For all tested cell lines, extracts obtained at 65 and 85 ºC showed the lowest activity against growth inhibition. Further increase in the extraction temperature resulted in better activity. Maximum activity was seen in extracts obtained at 115 ºC for all tested cell lines.

Measured IC_{50} values for extracts obtained at this temperature in the case of Hep2c, RD and L2OB were 13.44, 16.44 and 30.52 mg/mL, respectively. Further temperature increase led to higher IC_{50} values, but samples still retained their high activity. Generally, it was noticed that temperatures below water boiling point did not result in high activity of the extracts. According to these findings, temperature of 115 °C is preferable if the aim is to obtain extracts with maximum cytotoxic activity. The increase in temperature from 65 to 85 °C resulted in significant differences among the extracts ($p < 0.05$). In case of Hep2C and RD cell lines, insignificant differences were noticed among the extracts obtained at 150 and 180 °C, 150 and 210 °C, 180 and 210 °C ($p > 0.05$). In the case of L2OB, insignificant differences were observed between the extracts obtained at 130 and 150 °C ($p = 0.104325$), and between the extracts obtained at 150 and 210 °C ($p = 0.962278$).

The highest activity of extract obtained at 115 ºC may be linked with its composition. Based on previous findings described in this work, extracts obtained at 115 ºC, had the highest concentration of flavonoid aglycones (apigenin, luteolin, naringenin, kaempferol) but also other phenolic compounds (quercetin-3-*O*-galactoside, kaempferol-3-*O*-glucoside, rutin). Antitumor effects of these compounds have been well documented in the literature (Chen et al., 2013; Ganeshpurkar and Saluja, 2017; Lee, Han, Yun and Kim, 2015; Sudan and Rupasinghe, 2014), thus their presence in extracts probably contributed to cytotoxicity of the extracts. In addition, high concentration of other phenols, the specificity of the composition of subcritical water extracts and synergism could have been responsible for such high activity of the extracts. This is especially important because different unknown processes can occur in subcritical water and produce beneficial bioactivities of obtained extracts. It was already reported in the literature that chamomile extracts obtained by SWE exhibit higher cytotoxic potential in comparison to

chamomile extracts obtained by other modern techniques (Cvetanović et al., 2015). When it comes to cytotoxicity of SWE extracts, the results obtained in this study showed higher activity in comparison to those in the literature. This confirms the statement that balancing in water temperature and thus in its polarity could have multiple influence on extracts. Such results could be valuable for fine tuning the extracts composition and their activities.

3.4. Enzyme-inhibitory activity

The inhibition of α-amylase and α-glucosidase, the main enzymes in carbohydrates metabolism, plays a crucial role in control of blood glucose level and diabetes. The potential of chamomile to inhibit these enzymes has already been shown previously (Cvetanović et al., 2017). However, the influence of the extraction temperature on the activity has not yet been discussed.

The activity of SWE chamomile extracts towards inhibition of *α*-amylase and *α*-glucosidase were expressed as the equivalents of acarbose per gram of dry extract. The analyzed extracts were more active against inhibition of α-glucosidase (Lazarova et al., 2015).

The extract obtained at 85ºC exhibited the highest activity against *α*-glucosidase (4.13 mmol AE/g) followed by the extract obtained at the temperature of 65 ºC (3.98 mmol AE/g). Temperatures above 85 ºC caused the decrease in activity. However, significant differences between these two extracts were not noticed (p = 0.498565). Noticeably lower activity was observed for the samples obtained at the temperature above 130 ºC. Insignificant differences among the extracts obtained at: 115 and 130 °C (p = 0.228223), 150 and 180 °C (p = 0.017105) as well as between 180 and 210 °C (p = 0.986179) were noticed. Among all other extracts, the differences in activity were significant ($p < 0.05$). The highest activity of the extract obtained at 85 ºC could be a consequence of its specific composition. This extract had the highest content of

p-coumaric, 5-*O*-caffeoylquinic, caffeic, ellagic, protocatechuic acid, followed by aesculin (coumarin glycoside), and apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside and naringin (naringenin-7-*O*-neohesperidoside). Research suggest that glycosides are more active molecules in the inhibition of α-amylase and α-glucosidase than aglycones (Grussu, Stewart, McDougall, 2011, Dou et al., 2012). Extraction of these components was more efficient at lower temperatures, which might have been related to the better activity of the samples.The activity against α-amylase was in the range from 0.39 mmol AE/g to 0.51 mmol AE/g. The highest activity was noticed at the lowest extraction temperature, while further temperature increase led to the decline in activity with lowest activity seen at 150 °C (Figure 1). The extract obtained at the lowest temperature showed significantly higher activity in comparison to the extracts obtained at 130 °C (p = 0.046115), 150 °C (p = 0.005776), 180 °C (p = 0.023075) and 210 °C (p = 0.046210). The differences in the activity were insignificant for all other extracts ($p > 0.05$).

Tyrosinase is the key enzyme in melanin synthesis and has been linked to melanoma and different pigmentation disorders. Synthetic tyrosinase inhibitors exhibit side-effects, such as high cytotoxicity and dermatitis (Chiari, Vera, Palacios, Carpinella, 2011). The first natural tyrosinase inhibitor glabridin was isolated from the roots and seeds of *Glycyrrhiza* species. The compound is 15 times more potent in comparison to kojic acid and also it also possesses higher activity than arbutin (Yokota, Nishio, Kubota and Mizoguchi, 1998). In traditional cosmetics, chamomile is known as natural skin lightener for its constituents such as endothelin inhibitor. Tyrosinase inhibitory activity of chamomile has not been described well in literature. Our previous study gave the first information on the ability of SWE chamomile extracts to inhibit tyrosine (Cvetanović et al., 2017).

Inhibitory activity of chamomile extracts obtained at different temperatures is illustrated in Figure 2 and is expressed as the kojic acid equivalents per gram of dried extract (KAE mg/g). The lowest activity was noticed for the extract obtained at 65 ºC (5.37 mg KAE/g). The activity of this extract was significantly lower in comparison to all other extracts ($p < 0.05$). On the other hand, extracts obtained at 210 ºC showed significantly higher ability (17.92 mg KAE/g) to inhibit tyrosinase ($p < 0.05$). This could be a consequence of its composition. Unlike the other extracts, in the sample obtained at 210 °C galangin, phloretin, and resveratrol were detected. The first one – galangin, has been identified as a potent tyrosinase inhibitor (Li, Chen, Huang, Wang, Zhang, 2003). It was also proven that phloretin can fade melanin stains, making the skin whiter, with proven effects superior to kojic acid and arbutin. Phloretin is used as a new type of whitening agent in cosmetics (Zuo et al., 2014). Apart from these two, research suggests that resveratrol can be used as an original substrate for tyrosinase and that it has very promising cosmetic perspectives (Bernard and Berthon, 2000). Furthermore, high activity towards tyrosinase inhibition could be a consequence of the presence of non-flavonoid structures which also possess tendency to inhibit the tyrosinase-α (e.g., alkaloids). The plant extracts have very complex matrix and the interactions of several bioactive phytochemicals (synergetic or antagonistic actions) may be effective on the observed enzyme inhibitory effects. Taking into consideration these aspects, the most effective extraction condition is one of the most important steps for designing valuable ingredients. With respect to this fact, the presented results could open new avenues for developing functional products from chamomile.

4. CONCLUSION

The chamomile extracts obtained by subcritical water at different temperatures were compared in respect to their chemical profiles and biological activity. Chemical profiles of the

tested extracts defined by UHPLC-DAD-HESI-MS/MS analysis showed rich phenolic profiles and 24 compounds were identified and quantified. The analysis confirmed the presence of different flavonoids, glycosides, phenolic acids, and other compounds of polyphenolic structure. Apigenin was the dominant compound and its yield varied from 230.98 to 1501.25 mg/kg depending on the extraction temperature. Its maximal concentration was measured in extracts obtained at 115 °C. Phenolics with conjugated double bonds and glucose moiety were better solubilized by subcritical water at lower temperatures, whereas substitution with H-atoms or ether moiety led to better solubility in subcritical water of higher temperatures. Biological activity of extracts was highly influenced by the extraction temperature. It was demonstrated that the extraction temperature of 150 °C was optimal for obtaining extracts with maximal antioxidant activity. The extracts obtained at 115 °C had the highest yield of apigenin and demonstrated the highest cytotoxic activity towards three different cell lines. Lower extraction temperatures (65-85 °C) produced extracts with higher anti-diabetic activity, in contrast to the anti-tyrosinase activity, where higher extraction temperature (210 °C) was recommended for the maximum activity.

Author contribution

Conceptualization: Aleksandra Cvetanović.

Investigation: Aleksandra Cvetanović, Gokhan Zengin, Uroš Gašić, Živoslav Tešić, Pavle Mašković, Cristina Soares, M. Fatima Barroso, Cristina Delerue-Matos, Saša Đurović

Supervision: Zoran Zeković.

Writing – original draft: Aleksandra Cvetanović.

Writing – review & editing: Aleksandra Cvetanović, Jaroslava Švarc-Gajić, Jelena Jerković.

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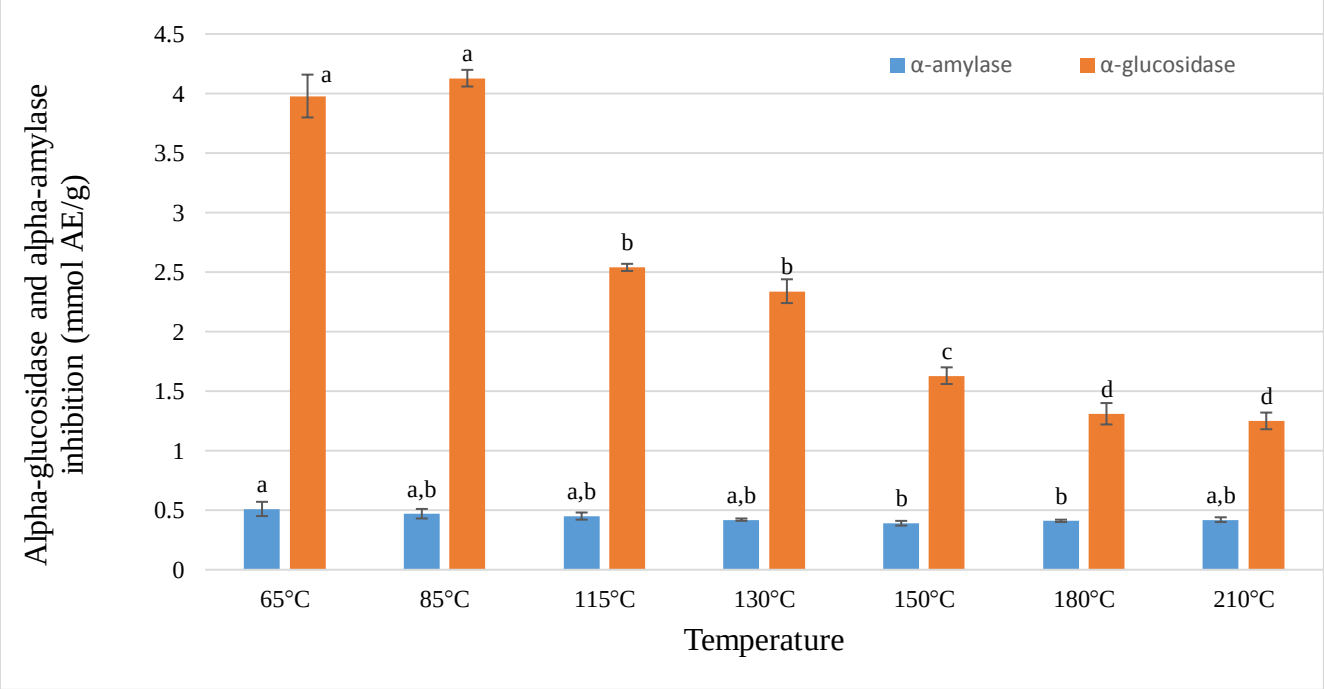
Figure Captions

Figure 1. The activity of chamomile extracts obtained at different temperatures against α-glucosidase and α-amylase inhibition; Significant differences among the samples obtained at different temperatures are indicated by different letters ($P < 0.05$).). Error bars represent standard error of the mean for 3 replicates.

Figure 2. The activity of chamomile extracts obtained at different temperatures against tyrosinase inhibition; Significant differences among the samples obtained at different temperatures are indicated by different letters ($P < 0.05$).). Error bars represent standard error of the mean for 3 replicates.



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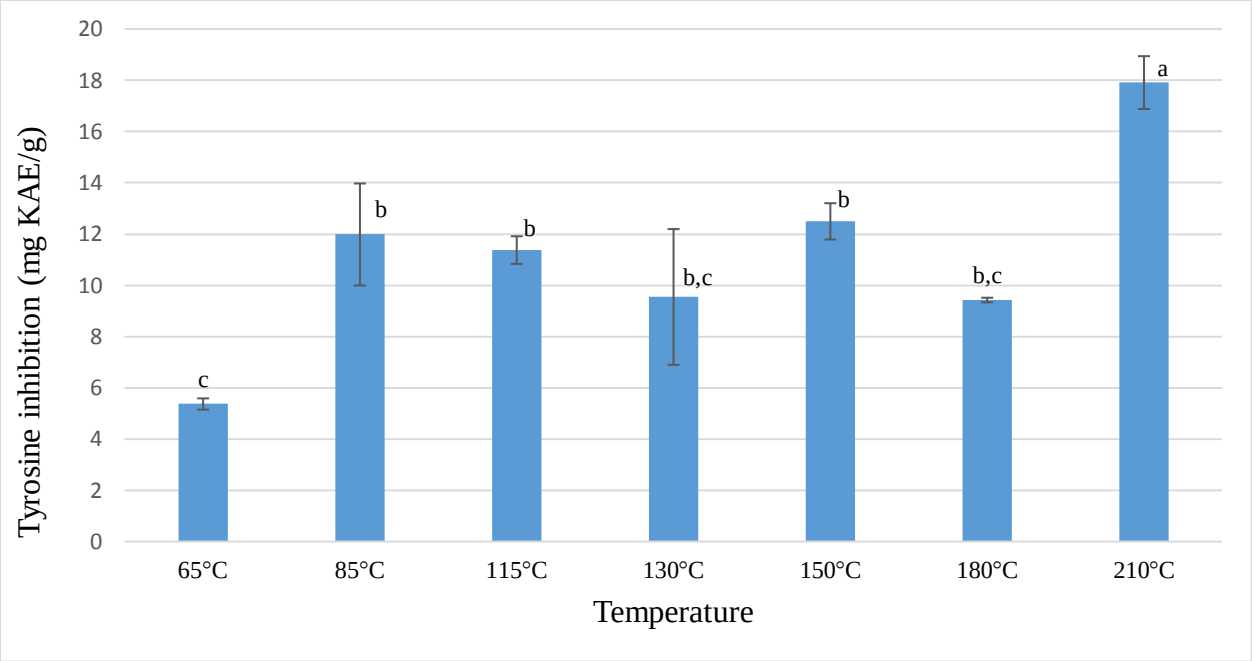

Figure 2. The activity of chamomile extracts obtained at different temperatures against tyrosinase inhibition; Significant differences among the samples obtained at different temperatures are indicated by different letters ($P < 0.05$). Error bars represent standard error of the mean for 3 replicates.

Table 1. The influence of the extraction temperature on yields of flavonoids and their glycosides

Compounds	Yield (mg/kg*)						
	65°C	**85°C**	**115°C**	**130°C**	**150°C**	**180°C**	**210°C**
Apigenin-7-*O*-glucoside	47.5d	158a	69.9b	53.4c	2.58e	0.09f	-
Apigenin	231g	740c	1501b	1297a	634d	283f	298e
Kaempferol-3-*O*-glucoside	-	-	24.5a	-	-	-	-
Kaempferol	10.5e	29.2b	60.4a	58.7a	27.1b	14.5d	19.8c
Luteolin-7-*O*-glucoside	351b	1101a	278c	166d	-	-	-
Luteolin	24.3e	55.2c	97.2a	83.2b	36.3d	20.7f	27.0e
Naringin (Naringenin-7-*O*-neohesperoside)	1.44bc	4.08a	2.32ab	-	-	-	-
Naringenin	1.62d	4.63bc	7.87a	6.64ab	3.37cd	1.55d	1.52d
Rutin (Quercetin-3-*O*-rutinoside)	-	-	5.35a	2.15b	-	-	-
Quercetin-3-*O*-galactoside	-	-	4.69a	3.01b	-	-	-
Catechin	-	-	-	46.1a	-	-	-

Galangin	-	-	-	-	-	-	2.22a

* mg of compound per kg of dry extract.

Significant differences between the contents of samples obtained at different temperatures are indicated by different letters ($P < 0.05$).

Table 2. The influence of the extraction temperature on yields of polyphenolic compounds

Compounds	**Yield (mg/kg*)**						
	65°C	**85°C**	**115°C**	**130°C**	**150°C**	**180°C**	**210°C**
Phloretin	-	-	-	-	-	-	1.26a
Resveratrol	-	-	-	-	-	15.9b	30.4a
Aesculin	212b	326a	85.1c	56.9d	4.84e	-	-
Ellagic acid	14.1f	30.2a	18.4cd	20.8bc	22.7b	15.4ef	17.0de
***p*-Hydroxybenzoic acid**	-	259d	497c	535b	558a	208e	184f
Protocatechuic acid	2.44b	34.4a	-	-	-	-	-
Caffeic acid	-	134a	-	-	-	-	-
Sinapic acid	14.2g	39.2f	69.1d	55.5e	120b	113c	150a
Cinnamic acid	-	-	-	-	-	4.55b	6.80a
5-O-caffeoylquinic acid	148b	188a	24.3c	18.1d	6.56e	-	8.35e

p-Coumaric acid	55.4b	79.3a	13.6c	9.70d	8.42d	7.51d	10.2d
Coniferyl aldehyde	-	-	-	-	-	-	7.62a

* mg of compound per kg of dry extract.

Significant differences between the contents of samples obtained at different temperatures are indicated by different letters ($P < 0.05$).

Table 3. Antioxidant activity of chamomile extracts obtained at different temperatures.

Extraction temperature	IC_{50} values (μg/mL)*				DNA-based sensor (μGAE/mL)*
	OH scavenging capacity	ABTS scavenging capacity	DPPH scavenging capacity	Inhibition of lipid peroxidation	
65 °C	43.1±0.9a	16.8±0.6a	45.0±1.4a	32.6±1.1a	57.0 ± 4.1d,e
85 °C	42.7±0.4a	15.5±0.3b	28.3±2.0b	35.0±0.9a	40.5 ± 3.2e,f
115 °C	42.7±0.2a	14.6±0.5b	15.3±0.5c	34.9±0.8a	147 ± 13b
130 °C	42.1±1.1a	12.5±0.3c	13.6±0.6c,d	32.8±0.7a	107 ± 10c
150 °C	38.1±0.6a	7.3±0.1d	12.5±0.0c,d	28.7±0.6b	78.8 ± 1.4d
180 °C	39.2±0.7b	14.9±0.5b	13.5±2.3c,d	29.7±0.6b	28.9 ± 4.6f
210 °C	41.5±0.9b	15.3±0.2b	10.0±0.6d	32.6±1.1a	219 ± 9.0a
Gallic acid	59.1±1.1	2.00±0.41	3.79±0.69	255±12	
Ascorbic acid	160.5±2.3	11.0±0.9	6.05±0.34	>1000	
BHT	33.9±0.8	7.23±0.87	15.61±1.26	1.00±0.23	
α-tocopherol				0.48±0.05	

*±2SD; BHT- Butylated hydroxy toluol; GAE-Galic acid equivalent

Significant differences between the activities of samples obtained at different temperatures are indicated by different letters ($P < 0.05$) within a column.

Table 4. Cytotoxic activity of SCW chamomile extracts obtained at different temperatures.

Cell line	IC_{50} Values (µg/mL)						
	65 °C	**85 °C**	**115 °C**	**130 °C**	**150 °C**	**180 °C**	**210 °C**
Hep2C cells[A]	90.9± 0.9a	34.4 ± 0.6b	13.4±1.4e	17.5±0.5d	24.0 ± 1.8c	23.5±1.1c	25.9±1.0c
RD cells[B]	96.2±0.5a	47.3±0.5b	16.4±0.2d	19.6±1.6d	25.1±1.2c	26.8±0.7c	26.4±1.9c
L2OB cells[C]	132±0.41a	110±0.52b	30.5±0.9e	44.4±1.1d	42.0±0.5d	61.5±2.0c	41.2±0.5d

[*] Mean value ± 2SD; Significant differences between the activity of samples obtained at different temperature are indicated by different letters ($P < 0.05$).

[A]cell line derived from human cervix carcinoma;

[B]cell line derived from human rhabdomyosarcoma;

[C]cell line derived from murine fibroblast.

Highlights

- Extraction of chamomile by subcritical water
- Influence of temperature on chemical composition of obtained extracts
- Biosensor DNA assays for antioxidant potential of extracts
- Influence of temperature on biological activity of the extracts