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Antioxidant and biological activity of chamomile extracts obtained by different techniques: perspective of using superheated water for isolation of biologically active compounds

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ABSTRACT

In this work, extracts of chamomile (*Matricaria chamomilla* L.) ligulate flowers obtained by Soxhlet, microwave-assisted, ultrasound-assisted and subcritical water extraction were compared in respect to their bioactivity, more specifically antioxidant, antimicrobial and cytotoxic activity. In all obtained extracts, the content of total phenols and flavonoids was determined, indicating significant total extraction yields (49.70%) and total phenol content (151.45 mg ECA/ml) in water extracts obtained under moderate subcritical conditions. By applying DPPH and Reducing Power Test subcritical water extraction showed superior free-radical scavenging ability in comparison to other investigated extraction techniques. Microbial properties of extracts were examined using eight selected indicator strains and for all extracts minimum inhibitory concentrations were in the range between 19.53 and 312.50 μg/ml. Subcritical water extracts showed the highest activity against *Escherichia coli* (MIC = 39.10 μg/ml) and *Aspergillus niger* (MIC = 39.10 μg/ml). Cytotoxic effects of extracts were examined on human rhabdomyosarcoma cells (RD), cell line derived from human cervix carcinoma Hep2c (HeLa) and cell line derived from murine fibroblast (L2OB). On all three tested cell lines, subcritical water extracts demonstrated much better antitumor properties in comparison to extracts obtained by other techniques, with IC_{50} values for Hep2c, RD and L2OB cells 30.54, 20.54 and 19.65 μg/ml, respectively.

1. Introduction

All over the world, there is an increasing interest in medicinal plants. Researchers, as well as the public, in general, recognize that natural products, predominantly those derived from plants, exhibit benefits for human health (Petronilho et al., 2012). For centuries, traditional medicine provided a crucial health support for millions of people around the globe. In some countries, less than 20% of population has access to basic generic medicines or healthcare products (Hogerzeil and Mirza, 2011). Even in industrialized nations, over 40% of population uses complementary or alternative medicines because of insufficient therapeutic outcomes of pharmacotherapy, as well as its adverse effects (Barnes et al., 2008; Robinson and Zhang, 2011). Phytochemicals are bioactive secondary plant metabolites (Ghani, 1990; Dobelis, 1993) that often exhibit synergistic effects.

Throughout the history, chamomile (*Matricaria chamomilla* L. synonym: *M. recutita*) has been one of the most popular and frequently used medicinal plants due to the richness in therapeutically active compounds. Its inflorescences (so-called anthodia) contain over 120 constituents, and owing to this wide range of biologically active compounds, this plant has many beneficial health effects such as antioxidant (Hernández-Ceruelos et al., 2010), neuroprotective (Ranpariya et al., 2011), anti-allergic (Chandrashekhar et al., 2011), anti-inflammatory (Bulgari et al., 2012), anti-microbial (Silva et al., 2012) and anticancer (Matić et al., 2013). Although the composition of chamomile has been extensively studied, along with its effects, the exact mechanisms of bioactivity have not been entirely clarified, due to complexity of extracts composition. However, it is well known that depending on the plant origin and manner of preparation, chamomile extracts may produce different effects. Extraction efficiency depends on the extraction method

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used, the sample particle size, the solvent used, as well as the presence of interfering substances. Moreover, the extraction yield depends on the solvent's selectivity, pH, temperature, extraction time and the composition of a particular sample matrix. Therefore, it is very important to find an optimal extraction method for obtaining extracts with the highest content of biologically active compounds and the lowest content of interfering substances.

Heated or pressurized solvents allow more abundant release of solutes from solid matrices due to lower viscosity and consequently better penetration into the pores of solid particles, improvement of mass transfer kinetics and better solubility of solutes. This approach is applied in many modern extraction techniques (Švarc-Gajić, 2011). Significant advantages of microwave-assisted extraction are the reduction of extraction time and the amount of used solvent (Li et al., 2004a). According to Pare et al. (1994), microwaves induce a sudden increase in temperature inside the cellular structure, which might result in an eventual rupturing of cell walls and rapid release of plant constituents into surrounding medium. The success of ultrasound-assisted extraction (UAE) can be attributed to cavitation phenomenon (Paniwnyk et al., 2001; Yang and Zhang, 2008) which, on the other hand, can also provoke a degradation of analytes due to free-radical formation (Švarc-Gajić, 2011).

Nowadays, from the point of view of green chemistry, sub- and supercritical fluid extraction has gained much popularity. These methods replace toxic organic solvents with benign fluids, such as carbon-dioxide or water. Water as an extraction fluid is still not being used to expected degree due to the lack of data describing physical and chemical properties of super/subcritical water (Švarc-Gajić, 2011). Subcritical water (SCW) or compressed hot water is water that remains in its liquid form in the temperature range between 100 and 374 °C under conditions of elevated pressure (Adachi, 2009). Such fluid has unique characteristics such as dramatically decreased dielectric constant, surface tension and viscosity. Dielectric constant, which drops by temperature increase, is one of the most important factors when using water as an extraction solvent; it decreases from 80 (at room temperature) to 27 (at 250 °C) which is almost equal to that of ethanol at ambient temperature (Galkin and Lunin, 2005; Herrero et al., 2006). Early works with subcritical water demonstrated its ability to selectively extract different classes of compounds, with more polar organic compounds being extracted at lower temperatures and less polar organic compounds being extracted at higher temperatures (Švarc-Gajić, 2011). On the other hand, high water reactivity at elevated temperatures must be considered when applying this emerging technique for isolation of biologically active compounds from natural sources.

The objective of this study was to compare the efficiency of subcritical water extraction with ultrasound-assisted, Soxhlet and microwave-assisted extractions, in terms of the recovery of bioactive compounds with antioxidant, anti-microbial and cytotoxic activity from chamomile ligulate flowers.

2. Methods and materials

2.1. Chemicals and reagents

Folin-Ciocalteu reagent, trichloroacetic acid, 1,1-diphenyl-2-picryl-hydrazyl-hydrate (DPPH), chlorogenic acid, apigenin, apigenin-7-*O*-glucoside and rutin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Aluminum chloride hexahydrate, sodium carbonate and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Potassium ferricyanide and ferric chloride were obtained from Zorka (Šabac, Serbia). Cirsimarin, resazurin, amaricin, nystatin, sabourand dextrose, Tween 80 and *cis*-diamminedichloroplatinum (*cis*-DDP) were purchased from Tedia Company (USA). Acetonitrile and water were purchased from Fisher Chemical (LC-MS and HPLC grade, respectively). Formic acid was purchased from Carlo Erba (Italy). All other chemicals and reagents were of analytical reagent grade.

2.2. Plant material

Chamomile ligulate flowers (CLFs) used in this study were produced by the Institute of Field and Vegetable Crops, Bački Petrovac, Serbia, in a spring of 2012. Chamomile flos were dried at the temperature of 40 °C in a solar dryer. The layer thickness of plant material was 5 cm. Drying was performed until the moisture content of 12%. After drying, the CLFs were separated from the tubulare parts by sieving. Separated CLFs were packed in paper bags and stored in the dark place until further use.

2.3. Extraction of the plant material

Soxhlet extraction (SE) was done in a conventional laboratory apparatus. A sample (5 g) was placed in a sample thimble and 250 mL of solvent (70% ethanol) was added. After 40 min, the extraction process was stopped and the obtained extracts were filtrated.

The same extraction time and solvents were used for microwave-assisted extraction (MAE). The MAE was performed in an open system by using a modified domestic microwave previously described by Švarc-Gajić et al. (2013). The extraction procedure program was as follows: 1 min pre-heating at 160 W; 1 min preheating at 320 W; 40 min extraction at 480 W.

The ultrasound-assisted extraction (UAE) was performed in ultrasonic water bath (Branson, USA). A sample (5 g) was placed in a volumetric flask and 250 mL of solvent (70% ethanol) was added. The mixture was sonicated for 40 min.

Subcritical water extraction (SCW) was performed in commercially available high-pressure reaction vessels lined with polytetrafluoroethylene. The total volume of the vessel was 12 mL. The extraction was performed with pure water at the temperature of 200 °C and the pressure of 1.6 bar for 40 min, in static conditions. The sample:solvent ratio was 1:50.

All liquid extracts were evaporated by using a vacuum evaporator (Devarot, Elektromedicina, Slovenia) and dried at 40 °C. Obtained dry extracts were stored in a dark place at 4 °C until analysis.

2.4. Determination of the total extraction yield

In order to determine the total extraction yield, certain volume of liquid extracts was evaporated under vacuum. Evaporated extracts were dried at 105 °C until a constant mass. Further calculation of the total extraction yield was done according to the procedure described in pharmacopoeia (Ph. Jug. V, 1984).

2.5. Determinations of phenolic and flavonoid compounds

Dried extracts were dissolved in water and 70% ethanol to the final concentration of 10 mg/mL of dried extract. This solution was further used for the determination of total phenols and flavonoids, as well as for measuring antioxidant power of the obtained extracts.

The Folin-Ciocalteu method (Singleton and Rossi, 1965; Kähkönen et al., 1999) was used to determine the total phenolics content. The reaction mixture was prepared by mixing 0.1 mL of the extract solution (10 mg/mL), 7.9 mL of distilled water, 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of sodium carbonate (20%, w/w). After incubation at room temperature for 1 h, absorbance was measured at 750 nm. The blank was prepared by replacing the extracts with distilled water. Triplicate measurements were made for each

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