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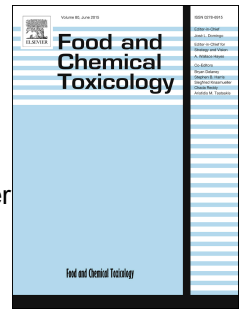

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Comparative *in vitro* studies of the biological potential and chemical composition of stems, leaves and berries *Aronia melanocarpa's* extracts obtained by subcritical water extraction

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Abstract

Preparation of functional products as well as natural-based products requires non-toxic but effective extraction techniques. In this study, subcritical water extraction was used for the extraction of different aronia parts in order to explore their potential. Stems, leaves and berries of *Aronia melanocarpa* were extracted under the following conditions: temperature 130 °C; pressure 35 bar; time 20 min. The total phenols and flavonoid contents of the produced extracts were evaluated by conventional spectrophotometric methods. Additionally, the main phenolic compounds were also identified and quantified by high performance liquid chromatography with diode array detection (HPLC-DAD). The biological potential of the extracts was evaluated by determining their antioxidant (DPPH, ABTS and lipid peroxidation assays), antimicrobial, enzyme inhibitory (cholinesterase and elastase) and cytotoxic effects (HeLa, A-549, LS-174T, MRC-5 cell lines). The results indicate that leaves and berries extracts exhibited stronger antioxidant action when compared with stems. The strongest cholinesterase and elastase inhibitory activity was also found in berries extract. Similarly, the extracts obtained from leaves and berries showed considerable cytotoxic effects against tested cell lines. A moderate antimicrobial effects was observed too. Demonstrated biological potential of all three aronia parts can trace a new road map for developing newly designed functional products.

Keywords: Subcritical water extraction, aronia, HPLC, antioxidants, enzyme inhibitors, cytotoxicity, antimicrobial activity.

1. Introduction

Natural products possess enormous structural and chemical diversity that is unsurpassed by any synthetic libraries. The products obtained from plants represent a rich source of potent bio-molecules with strong biological activity, such as: antioxidant, antibacterial, antifungal, antiviral, cytotoxic, anti-inflammatory and anti-cancer activity (Raj Narayana et al., 2001, Mayaud et al., 2008; Friedman et al., 2003, Cvetanović et al., 2015a). Novel scientific trends imply the usage of natural products (such as plants extract, pure compounds or as standardized extracts) in the therapy but also in the prevention of numerous diseases. Moreover, it was reported that a diet rich in plants offers some benefits for human health and protection against cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases. (Graf et al., 2005; Arts and Hollman, 2005). From this reason, the interest for investigation new bioactive molecules and their sources has been increasing. This interest is particular increased in the food industry which turned to implementation of different natural products in food and foodstuffs in order to improve their functionality. The start point in the process of designing new products is extraction and isolation of biologically active components from natural sources. For this purpose numerous techniques are available. Unfortunately, the most commonly applied extraction approaches rely on the organic solvents utilization. In addition, a negative impact of large amounts of organic solvents on human health and environment as well as low yields and large amounts of plant material are drawbacks too. During the last 20 years, researchers' efforts have been focused on environmentally-benign technologies, in accordance with main principles of green chemistry and in that sense, water is the first-choice "green" solvent. However, the negligible solubility of many organic compounds in water and high energy demands for water removal upon completion of process are limitations (Cvjetko Bubalo et al., 2015). Nevertheless,

under subcritical conditions water becomes an excellent solvent able to dissolve even those compounds hardly to very slightly soluble in water at room temperature. On the other hand, the solubility of the compounds which are well soluble in water at room temperature (polar and moderately polar compounds) does not change significantly. Thus, subcritical water could be used for the extraction of non-polar and moderately polar compounds under the controlled experimental conditions. The temperature can be tuned very easily to fit the solubility of target compounds. With heating, the polarity of water decreases enabling the dissolution of moderately polar compounds. At the same time the viscosity, density and surface tension decrease further contributing to improved efficiency of the extraction. So far, subcritical water extraction (SWE) has been used for extraction of different molecules from different matrices and numerous advantages of this technique over traditional were observed (Mašković et al., 2017; Švarc-Gajić et al., 2017). Many advantages of SWE over other modern extraction techniques, such as ultrasound and microwave extraction are confirmed and reported as well (Cvetanović et al., 2015b; Cvetanović et al., 2017a; Zeković et al., 2017).

Aronia melanocarpa often called aronia or black chokeberry is one of the plants which gained much attention due to its richness in biologically active ingredients. It is believed that high concentration of procyanidins, anthocyanins and flavonols but also phenolic acids can contribute to health benefits of this plant (Taheri, et al., 2013; Xie et al., 2017). A series of papers reported on antioxidant potential of this plant as well as its products (Denev et al., 2012). Other studies demonstrated an anti-diabetic activity of aronia (Simeonov et al., 2002; Valcheva-Kuzmanova et al. 2007), indicating its influence on possible prevention of diabetes. Different researchers reported on its anti-proliferative effects against cancer of colon (Bermúdez-Soto et al., 2007; Malik et al., 2003), hepatoprotective effects (Kowalczyk et al., 2003; Valcheva-

Kuzmanova et al., 2006) as well as antimicrobial activity (Valcheva-Kuzmanova et al., 2006). It was observed that consumption of 300 mg aronia extract per day during the period of 2 months decreases blood pressure (BP), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and total triglyceride (TG), and increases antioxidant enzyme activity in adults with metabolic syndrome (Xie et al., 2017; Broncel et al., 2009). In order to ensure maximal recovery of bioactive molecules from this plant different conventional extraction approaches have been employed (Brazdauskas et al., 2016; Ćujić et al., 2016; D'Alessandro et al., 2012; Simić et al., 2016; Slimestad, et al., 2005; Rugină et al., 2012). So far, only the berries of this plant have been utilized. However, other aronia parts could also be rich sources of novel bioactive molecules as well.

The main goal of this research was oriented towards valorisation and exploitation of different aronia parts (stems, leaves and berries) by using cutting edge technology, *i.e.* SWE. Biological potential of such extracts was confirmed by applying *in vitro* assays, including: antioxidant, enzyme inhibitory, cytotoxic, and antimicrobial tests. In addition, the chemical characterization of studied extracts was done by HPLC analysis.

2. MATERIALS AND METHODS

2.1. Plant material

Aronia (*Aronia melanocarpa)* was collected in the area of southern Serbia (territory of Leskovac, Latitude: 42° 59' 46.9536" N; Longitude: 21° 56' 38.5224" E) in August 2015. Voucher specimens for the plant material (*Aronia melanocarpa* L. 1753 *„Nero"* No 2-1485, determinator: Goran Anačkov) were confirmed and deposited at the Herbarium of the Department of Biology and Ecology - BUNS Herbarium, Faculty of Natural Sciences, University of Novi Sad

(Holmgren and Holmgren, 2003). Aerial parts of the plant (berries, leaves and stems) were stacked in a crate with perforated bottom, in order to ensure air flow. Drying was performed naturally on draft in dark until moisture content of 10%. Dry plant material were packed in glass jars and stored in the dark until use.

2.3. Preparation of SWE extracts

The extraction of berries, leaves and stems of aronia was performed in a home-made batch-type extractor described elsewhere (Cvetanović et al., 2017b). Extractions were performed at the temperature of 130 °C for 20 min at the pressure of 35 bar maintaining sample-to-solvent ratio 1:20. Obtained extracts were filtrated and stored in a refrigerator for further analysis.

2.4. Determinations of total polyphenolic content

The Folin-Ciocalteu method (Singleton and Rossi, 1965; Kahkonen et al., 1999) was used to determine the total phenolics content. The reaction mixture was prepared by mixing 0.1 mL of the extract, 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. The measurements were done in triplicates for each sample. Total phenols content in obtained extracts was calculated by interpolating the measured sample absorbance into calibration curve defined with standard solutions of chlorogenic acid, defined for the concentration range 0.02–0.1 mg/mL ($Y = 4.91C - 0.00833$, $R^2 = 0.998$). The results were expressed as chlorogenic acid equivalents per gram of dry plant material (mg CAE/g).

The total flavonoid content was determined by colorimetric assay (Markham, 1989). SCW extracts of aronia (1 mL) were mixed with 5% $NaNO_2$ solution (0.3 mL). After 5 min

aluminium choride hexahydrate (10%, 0.3 mL) was added and allowed to stand for 6 min. Sodium-hidroxide (1 mol/dm^3, 1 mL) was added to the mixture. Immediately, distilled water was added to the final volume of 10 mL. The blank was prepared by replacing the extract with distilled water. Immediately after mixing, absorbance was measured at 510 nm. Total flavonoid content in obtained extracts was calculated by interpolating the measured sample absorbance into calibration curve defined with standard solutions of rutin, defined for the concentration range 0.02–0.1 mg/mL ($A = 1.88727C - 0.0096$, $R^2 = 0.997$). The results were expressed as rutin equivalents (RE) per gram of dry plant material (mg RE/g).

2.5. Determination of antioxidant and antiradical activity

In order to determine the antioxidant and antiradical activity, liquid extracts were evaporated by using a vacuum evaporator (Devarot, Elektromedicina, Slovenia) and dried at 40°C. The obtained dry extracts were dissolved in water to the final concentration of 10 mg/mL. This solution was further used for the determination of the ability of the extracts to act as scavengers of DPPH and ABTS free radicals as well as for the measuring their ability to inhibit the process of lipid peroxidation. Spectrophotometric measurements were performed on Yenway 6300 Spectrophotometer (Barloworld Scientific Ltd, Dunmow, Essex, UK). All tests were performed in triplicates and the results were expressed as IC_{50} values (µg/mL).

2.5.1. Inhibitory activity against lipid peroxidation

The ability of the extracts to inhibit the process of lipid peroxidation was measured by using thiocyanate method (Hsu et al., 2008). The starting solution of the extracts (10 mg/mL) was diluted with water and series of dilutions were made (0.01-0.03 µg/mL). Diluted extracts were mixed with linoleic acid emulsion, which was consisted of linoleic acid (0.2804 g), Tween

(0.2804g) and 50 mL of 40 mM phosphate buffer. After mixing, the period of incubation of 72 h at the temperature of 37 °C, ethanol (4.7 mL; 75%), iron(II) chloride (0.1 mL; 20 mM) and of ammonium thiocyanate (0.1 mL; 30%) were mixed with 0.1 mL of the reaction solution. The absorbance of this mixture was measured at λ = 500 nm. All measurements were carried out in triplicates, and results were expressed as IC_{50} values (µg/mL). Simultaneously, the assay was performed with ascorbic acid (10-1000 µg/mL) and BHT (0.5-5 µg/mL) and α-tocopherol (0.1-1 µg/mL) as standard antioxidant substances and the results were compared.

2.5.2. ABTS test

$ABTS^{\bullet+}$ radical scavenging activity was determined following the procedure previously described in the literature (Delgado-Andrade, et al., 2005). In order to generate the $ABTS^{\bullet+}$ radicals, solution of ABTS (7 mM) was mixed with potassium persulfate (2.45 mM) and left for 14h. After this period, phosphate-buffer (5 mM) was added to the $ABTS^{\bullet+}$solution. That solution (4 mL) was mixed with 10 µL of aronia extracts (10-50 µg/mL), and absorbance was measured at 730 nm. Ascorbic acid (1-10 µg/mL) and BHT (5-20 µg/mL) were used as reference antioxidants. The results were expressed as IC_{50} values (µg/mL).

2.5.3. DPPH scavenging activity

In order to determine the ability of the extracts to neutralize DPPH radicals, the method previously described by Espin et al., (2000) was used. In a nutshell, the extracts were mixed with methanol (96%) and 90 mM DPPH to give final concentrations of 10, 20, 50, 100 and 200 µg/mL of dry extract. The obtained mixture was incubated at room temperatures in a dark for 60 minutes. Thereafter, the absorbance was measured at 515 nm and IC_{50} (µg/mL) value was

calculated. Ascorbic acid (1-10 µg/mL) and BHT (5-20 µg/mL) were used as reference antioxidants.

2.6. Enzyme inhibitory activity

2.6.1. Cholinesterase inhibition

Ability of the extracts to inhibit certain enzymes was explored by using cholinesterase and elcatase. In case of cholinesterase, the Ellman's method with slight modification (Zengin, 2016) was applied. Sample solution (50 µL, 1-5 mg/mL) was mixed with DTNB (125 µL) and AChE (or BChE) solution (25 µL) in Tris–HCl buffer (pH 8.0) in a 96-well microplate and incubated for 15 min at 25° C. The reaction was then initiated with the addition of acetylthiocholine iodide (ATCI) or butyrylthiocholine chloride (BTCl) (25 µL). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (AChE (acetylcholinesterase) or BChE (butyrlcholinesterase)) solution. The absorbances of sample and blank were measured at 405 nm after a 10 min incubation at 25° C. Galanthamine was used as positive control (0.1-0.5 mg/mL). The cholinesterase inhibitory activity was expressed as equivalents of galanthamine per gram of dry extract (mgGALAEs/g extract).

Ability of the samples to inhibit elastase was determined by using previously\ described procedure (Wittenauer et al., 2015). Samples (50 µL, in the concentration range 1-5 mg/mL, were mixed with the substrate (25 µL, N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide in Tris-HCl buffer, 10 mM, pH 8). The mixture was incubated for 15 min at room temperature. Then, 25 µL of enzyme solution (0.3 units/mL in 10 mM Tris-HCl buffer, pH 8) was added and the obtained mixture was incubated for 15 min at room temperature. Absorbances were measured at 410 nm. Catechin was used as the positive control (1000 − 5000 µg/mL). The results were expressed as milligrams of catechin equivalents per gram of dry extract (CEs/g extract).

2.7. Cytotoxic potential

The cell lines of human cervical adenocarcinoma (HeLa), human lung adenocarcinoma (A549), human colorectal adenocarcinoma (LS 174T) and normal, human embryonic lung fibroblast (MRC-5) were maintained in complete nutrient medium RPMI-1640 at 37°C in humidified atmosphere with 5% CO_2 (Matić et al., 2013). All cell lines were obtained from American Type Culture Collection (Manassas, VA, USA).

The cells were seeded into 96-well microtitar plates at a density of 3000 cells/well for HeLa, 5000 cells/well for A549 and MRC-5 and 7000 cells/well for LS 174T . After 24h, they were treated with different extract concentrations, while control cells were grown in culture medium only. The used range of concentration of the extracts in case of HeLa line was from 0.52 to 8.33 µg/mL, while in case of A549, LS174T and MRC-5 from 1.38 to 33.33 µg/mL. The positive control (cisplatin) was used in the concentration range from 10 µg/mL to 0.62 µg/mL.

After the additional 72 h of incubation, the cell survival was determined by MTT test (Mosmann 1983; Ohno and Abe 1991; Matić et al., 2013; Pantelić et al., 2013). The absorbance was measured at 570 nm using Multiskan EX reader (Thermo Labsystems Beverly, MA, USA). Experiments were performed in triplicate and the data are presented as mean ± standard deviation (SD).

2.8. Antimicrobial activity

The antimicrobial potential of the investigated aronia extracts was determined by measuring their antibacterial and antifungal activities according to the method previously described in the literature (Sarker et al., 2007; Cvetanović,et al., 2015b). For antibacterial test five different bacterial strains were used, while antifungal activity was measured by using two fungal species. Simultaneously, the activity of standard antimicrobial compounds (amracin and

nystatin) was measured as well. Minimum inhibitory concentrations (MIC) of the extracts against the test bacteria were determined by microdilution method in 96-multi-well microtiter plates. All tests were performed in Muller–Hinton broth (MHB). Extracts were dissolved and seven different concentration (19.53-625 µg/mL) were used for determination of their antibacterial activity. In case of amracin and nystatin the concentration range used for the test was 19.53-156.25 µg/mL. The plates were placed in an incubated for 24 h. Subsequently, color change was assessed visually. Any colour change from purple to pink or colourless was recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value. The average of three values was calculated, and the obtained value was taken as the MIC.

2.9. HPLC-DAD analysis

Detection and quantification of individual phenolic compounds was performed using reversed phase HPLC analysis. The equipment used was an HPLC Agilent-1200 series with UV–Vis DAD detector for multi wavelength detection. After injecting 5 µL of sample, the separation was performed in an Agilent-Eclipse XDB C-18 column (4.6 × 150 mm) thermostated at 25 °C. Two solvents were used for the gradient elution: A (H_2O + 2% HCOOH) and B (80% CAN + 2% HCOOH + H_2O). The elution program was as follows: from 0 to 10 min 0% B, from 10 to 28 min gradually increased 0–25% B, from 28 to 30 min 25% B, from 30 to 35 min gradually increased 25–50% B, from 35 to 40 min gradually increased 50–80% B, and finally for the last 5 min gradually decreased 80–0% B. Phenolic compounds in the samples were identified by comparing their retention times with those obtained for standards, for each component. Quantitative data were calculated from the calibration curves. Calibration curve, coefficient of correlation (R^2), limit of detection (LOD) and limit of quantification (LOQ) are shown in Table 1. The content of phenolic compounds was expressed in micrograms per milliliter of extract (µg/mL).

Table 1

2.10. Statistical analysis

All analysis were run in triplicate and 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. Polyphenolics profile

Due to the remarkable biological effects of polyphenols, determination of their content in extracts is of utmost importance. It has been suggested that the phenolic compounds are the most abundant constituents in aronia, and responsible for many of its putative medical properties (Kowalczyk et al., 2003; Kulling and Rawel, 2008).

At this point, both total and individual phenolics of aronia SWE extracts were determined. In terms of total phenolic content, obtained results (Table 2) show that all extracts were rich in phenolics but certain differences among the extracts were noticed. The leaves of aronia contain the highest concentration of total phenolic (131.53 mg CAE/g) and flavonoid (88.64 mg RE/g) compounds, followed by stems and berries (Table 2). Notable difference was noticed between extracts made from berries and leaves. More precisely, the concentration of total phenols in leaves extracts was 10 times higher than its concentration in berries. In case of leaves and stems, more than 50% of total phenolic content were actually flavonoids, while in case of berries more that 70% of total phenols were flavonoids. This can be explained with the fact that aronia berries are usually characterized by the high level of specific phenolic groups, *i.e.*, anthocyanins and proanthocyanidins and their biosynthesis is usually higher in berries than is

other part of plant. Stress factors such as temperature, moisture and soil nutrients might be responsible for the dissimilarity in phenols content among different plant parts (de Matos Nunes et al., 2014; Nantongo et al., 2018). Differences in phenols content among the different plant part was reported previously. For example, Eftekhari et al (2017) tested different vegetative parts (leaves and stems) of *Vitis vinifera* and the highest content of phenolic components was recorded in the leaves extracts. Also, another study performed by Senica et al (2017) highlighted different levels of phenolics accumulated in leaves, flowers and berries of *Sambucus nigra* in dependence on sampling location. However, in recent study published by D'Urso et al (2017), LC-MS metabolomics of different parts of myrtle berries were detected and the seed extracts contain more metabolomics than berries and peel. In addition, Gouveia-Figueira et al (2015) recorded that the considerable levels of phenolic in the berries of *Rubus grandifolius,* than in leaves and flowers. Amri et al., 2017 reported total phenolics concentration in extracts of different pomegranate organs in two pomegranate varieties whereby notable differences were noticed. Further, Kolniak-Ostek (2016) determined differences in polyphenols profile of different anatomical pear parts.

The differences between polyphenolics in analysed extracts can be explained by different binding strengths of molecules in various plant parts causing different bioavailability. For example, water insoluble phenolic compounds (condensed tannins or phenolic acids etc.) are bounded to plant cell walls components, such as polysaccharides or proteins providing a poor bioavailability. On the other hand, simple phenols and flavonoids (insoluble in water) are not bounded (Giada et al., 2013). Based on the available literature (Zengin et al., 2015; Mena et al., 2018; Mollica et al., 2018; Cai et al., 2018), the chemical and biological approaches on different

plant parts become important in selection the plant parts with high level of bioactive compounds.

Table 2.

The content of individual phenolic components was determined by HPLC-DAD and the results are given in Table 3. Rutin was found to be the predominant compound in all aronia parts, with the highest concentration in berries (5.544 mg/g extract). Furthermore, the aronia extracts contained remarkable levels of sinapic acid, quercetin and luteolin. The presence of these compounds in aronia was reported in the several studies (Worsztynowicz et al., 2014; Teleszko and Wojdyło, 2015; Tian et al., 2017). All of the above mentioned compounds exhibited a broad spectrum of biological activities such as antioxidant, antimicrobial, antimutagenic and anti-cytotoxic effects (López-Lázaro, 2009; Nićiforović and Abramovič, 2014; David et al., 2016; Ganeshpurkar and Saluja, 2017).

Table 3.

SWE extraction of polyphenols from aronia stems has already been reported in the literature (Cvetanović et al., 2017a). By comparing with literature data, it was noticed that conditions during SWE process have a great influence on total phenolic content but also on yield of individual components. Namely, in previously reported studies, the yield for the majority of observed compounds was higher probably due to the higher extraction temperature which increases the thermal agitation and hence leads to more efficient extraction. Differences in polyphenolics yield could be also consequence of other parameters, such as pressure and time of extraction. Detailed study on influence of temperature and time, as well as other extraction

parameters in SWE has already been reported (Cvetanović, 2016; Cvetanović et al., 2017b; Cvetanović et al., 2018).

According to the best of our knowledge, the composition of SWE aronia leaves and berries extracts has not been explored in detail. So far, polyphenols from aronia berries were isolated by numerous extraction approaches. The majority of them were conventional techniques. Comparing the results obtained in this study with data from the literature certain differences among SWE and conventional techniques were noticed. Wangensteen et al. (2014) extracted berries of aronia by double reflux extraction for 2 h using ethanol as a solvent. The total phenolics were in the range 98-175 mg GAE/g. However, it should take into account that in mentioned study extraction consumed a lot of time while SWE was performed in 20 min. Furthermore, subcritical water has advantage because of its safety, green character and low price. Thus, the obtained results give enough credits to consider exploitation of aronia potentials by using SWE technique.

3.2. Antioxidant activity

Free radicals play main role in the development of degenerative and chronic diseases such as cancer, atherosclerosis and diabetes mellitus. From this point, antioxidant compounds could considered as vital in preventing and repairing damages caused by free radicals. In the current study, the antioxidant properties of aronia extracts was tested by three assays including: lipid peroxidation, DPPH and ABTS radical scavenging assays. All applied methods are widely used for evaluation of antioxidant abilities of both natural and synthetic molecules. The activity of obtained samples was compared with those of well-known antioxidants (ascorbic acid, BHT

and α-tocopherol). Determined inhibitory concentrations (IC_{50} values) for investigated extracts are presented in Table 4.

In case of DPPH assay, it was noticed that the leaves extract exhibited the strongest scavenging activity, followed by berries and stems, whereby the differences in activity among these extracts were significant ($p < 0.05$). The highest activity of leaves extract may be a consequence of the highest phenolics content. This assumption was supported by several researchers who reported linear correlation between the concentration of total phenols and radical scavenging ability (Chen et al., 2017; Zheng et al., 2017). However, standard compounds were found to be better scavenger of DPPH radicals.

In contrast to DPPH, ability of the extracts to act as scavengers of ABTS radicals can be ranked as follows: berries>leaves>stems. Significant differences among the extracts were noticed at $p < 0.05$. Extract with the lowest activity against ABTS radicals was stems extract, what is in accordance with the results obtained for DPPH test. The differences between the stems and berries extracts ($p = 0.001142$) as well as between stems and leaves extracts ($p = 0.028139$) were significant. In comparison with standards, the extracts expressed lower activity.

An opposite situation was observed in the case of inhibition of lipid peroxidation where maximum antioxidant activity was noticed for the stems extracts. More precisely, the inhibition ability was in the following order: stems>leaves>berries. The differences between the stems and leaves extracts were significant ($p =0.033749$). Significant differences were also noticed between the stems and berries ($p = 0.000231$) as well as between the leaves and berries ($p = 0.000272$) extracts. Ability of the extracts to inhibit lipid peroxidation process was much higher that ability of ascorbic acid. However, activity of BHT and α-tocopherol was higher.

The observed differences in these assays can be explained by complex nature of phytochemicals or their synergetic/antagonist interactions. All of these methods rely on different mechanisms of action, and different compound may participate in scavenging different radicals. Usually, second metabolites, such as polyphenols, are responsible for the activity. However, other compounds might contribute the activity, as well. SWE plant extracts have very complex composition as a consequence of different thermochemical process and reactions in SWE medium and newly formed compounds could be produced by the Maillard reaction or caramelization (Plaza et al., 2010). Furthermore, different compounds may appear in SWE extracts because of their formation during the hydrothermal conversion or rearrangement reactions.

Table 4.

Although several studies indicated the antioxidant effects of aronia berries or its several parts (Jakobek et al., 2007; Bräunlich et al., 2013; Do Thi and Hwang, 2014), there are no data on antioxidant effects of SWE aronia extracts obtained from leaves and berries. In that sense, our results can be considered as a significant contribution. The data could be of the exceptional practical importance, especially from the aspect of food industry. SWE aronia extracts have "green" character, and due to the absence of organic/toxic solvents residue there is no need for their further purification. This lower costs and make them safer for the food industry.

3.3 Enzyme inhibitory activity

Alzheimer's disease (AD) is the main type of dementia and affects about 50 million people worldwide. The expected prevalence is about 131 million by 2050 (ADI**,** 2015)**.** Hence, new therapeutic strategies are needed to manage AD. Among the strategies, cholinergic

hypothesis is one of the most accepted approaches. According to this hypothesis, the inhibition of hydrolysis of acetylcholine in synaptic cleft may alleviate the symptoms of AD. Cholinesterase catalyzes the hydrolysis of acetylcholine, which is a neurotransmitter in synapses. Thus, cholinergic hypothesis is based on inhibition of cholinesterase (Huang and Mucke **,** 2012)**.** For these purposes, several drugs (galatamine, tacrine, donezepil, etc.) are chemically produced as enzyme inhibitors. However, most of them have side effects including gastrointestinal disturbances and toxicity (Silva et al., 2014). From that reason, the investigation on novel enzyme inhibitors from natural resources with minimal side effects has a good perspective for treatment of AD. For this purpose, we tested cholinesterase inhibitory effects of aronia extracts against acetylcholinesterase (AChE) and butyrlcholinesterase (BChE). As can be seen in Table 5, the extract of berries was the most active on AChE with 0.814 mg GALAE/g, followed by leaves and stems. The differences in AChE inhibition activity between berries and stems (p = 0.004899) as well as between stems and leaves (p = 0.045231) were significant, while difference between leaves and berries was insignificant (p = 0.177369). However, only berries extract exhibited inhibitory activity on BChE. These results were not correlated with the total phenolics content. Thus, non-phenolic components (alkaloids, saponins, etc.) could be responsible for the observed cholinesterase inhibitory in the aronia extracts.

Table 5.

For millennia, aging is one of the major problems, being a driving force to great number of pharmaceutical companies in designing new products. A cosmeceutical should be penetrate the skin and provide beneficial physiological effects when compared to an inert cosmetics (Morganti, 2008). Elastase is the main enzyme which causes the breakdown of elastin, which is an important protein in the extracellular matrix. In this regard, elastase is a target enzyme for

controlling aging (Azmi et al., 2014)**.** Therefore, the research for elastase inhibitors may provide new raw materials for developing novel cosmeceuticals. The aronia extracts were tested against elastase and the results are presented in Table 5. All aronia extracts expressed elastase inhibitory potentials. The extract of berries exhibited the greatest inhibitory effect with 3.549 mmol CAE/g, followed by stems (3.118 mmol CAE/g) and leaves (2.345 mmol CAE/g). There are not significant differences among the extracts ($p > 0.05$).

Ability of aronia SWE extracts to inhibit elastase and cholinesetase has not been reported yet. This study offers the first data, which implies that all examined aronias' parts can inhibit the activity of AChE and elastase. Taking into account that berries of aronia are widely used as foods, these findings could improve their value for human health. Two other examined parts (stems and leaves) do not participate in diet, but they could be sources of valuable compounds. In recent decade, the number of aronia processing industrial plants is consequently increasing resulting in great quantities of waste. Current technologies for processing of aronia do not include the use of stems or leaves after their separation from berries. However, this study offers data about potential of this plant waste and their usage in accordance to "green chemistry" principles. The obtained extracts are safe for its utilization as ingredients in numerous functional food products, but also in cosmeceutical and pharmaceutical products.

3.4. Cytotoxic activity

The cytotoxic activity of stems, leaves and berries of aronia extracts malignant cell lines (A-549, LS-174T and HeLa) and normal, lung fibroblasts (MRC5) is presented in Table 6. The obtained results showed that growth of all used malignant cells was inhibited by action of

extracts. Additionally, it was shown that HeLa cells were much more sensitive to the extracts than the other three cell lines.

Table 6.

In the case of examined cells, the extracts made from leaves exhibited the highest activity. Concentrations of leaves extracts which inhibited 50% of the malignant cells growth for A-549, LS-174T and HeLa were 2.01, 1.38 and 0.69 µg/mL, respectively. The differences in cytotoxicity between leaves and berries extracts in case of HeLa and LS-174T cells were significant (p = 0.000291 and 0.000316, respectively). On the other hand, in case of A-549 cells, these extracts expressed insignificantly different activity (p = 0.063233). However, in case of stems, reduction of MTT was notices, which was disturbing further reaction. This can be explained by presence of some non/phenolic compounds which are able to reduce MTT reagent.

In case of normal cell, obtained IC_{50} values were quite similar to these obtained for malignant cells. Namely, IC_{50} values determined for berries and leaves extract were 5.50 and 1.72 µg/mL, respectively. However, both examined extracts showed better selectivity than positive control, chemotherapy medicine, cisplatin (Table 7).

Table 7.

This study showed that constituents of aronia leaves and berries are cytotoxic for all three malignant cell lines. For the first time, our results demonstrated antitumor properties of the SWE aronia extracts. Larger amounts of phenols and flavonoids in leaves extract suggest that this constituents are responsible for better cytotoxicity. As it was mentioned before, different bioactive compounds can be formed in subcritical water medium due to the hydrothermal conversion or because of rearrangement reactions. Furthermore, synergistic effects between

phenolic compounds and other co-extracted and newly formed compounds might contribute the overall cytotoxic activities. Cytotoxic activity of aronia water extracts has been already reported in the literature. Šavkin et al. (2014) reported that aronia berries water extracts prepared in the form of infusion and decoction showed cytotoxic activity agains HeLa cells whereby obtained IC_{50} values were 86.99 and 11.16 µg/mL. In case of LS 174 cells, infusion of aronia expressed IC_{50} value of 73.70 µg/mL while in case of decoctions this value was 21.46 µg/mL. Taking into account that teas are commonly consumed in form of infusion, we compared this literature data with the results from our study. It appeared that SWE extracts are 25 and 13 times more active than infusion in the case of HeLa and LS 174 cells, respectively. In case of decoction, SWE extracts expressed much higher activity, as well.

Such high activity of SWE berries extracts highlighted the advantages of SWE over the traditional techniques. Further, extremely higher activity was noticed in case of leaves SWE extract. Leaves represents non-edible plant part, but obtained results clearly show their abundance in bioactive compounds as well as exceptionally bioactivity. By using SWE technique such potential could be better exploited. In this way, products with added-value could be obtained.

Despite the good results in the clinical application of cisplatin in cancer therapy, a major obstacle to more widespread use of this medicine is the presence of toxic side effects. Nephrotoxicity is the most clinical significant toxicity. Other adverse effects are gastrointestinal, myelosuppression, ototoxicity and neurotoxicity. All side effects are dose-dependent, and affect the application and effectiveness of the cancer therapy. Poor selectivity is important and unsolved problem for patients taking cisplatin (Barabas et al., 2008; Manohar and Leung, 2017).

Both examined extracts exhibited better selectivity than cisplatin with all three malignant cell lines. Selectivity in the anti-cancer action and good aronia extracts cytotoxicity open the way for further anti-cancer potential investigations of these extracts and their active compounds. As we already know one of the cytotoxic mechanisms of cisplatin is generation of superoxide radicals. These radicals are responsible for cisplatin toxicity (Barabas et al., 2008; Manohar and Leung, 2017). Obtained results offers good reasons and good base for further extracts research that would include study of combination therapy cisplatin/SWE aronia efficacy.

3.5. Anti-microbial activity

The antibacterial activity of the SWE extracts of aronia leaves, berries and stems was studied for seven different concentrations (19.5, 39.1, 78.1, 156.2, 312.5, 625 and 1250 μg/mL) against two Gram-positive (*Staphylococcus aureus, Bacillus subtilis*) and four Gram-negative (*Escherichia coli, Klebsiella pneumonia, Proteus vulgaris, Proteus mirabilis*) bacterial strains. Antifungal activity was determined against two fungal species (*Candida albicans, Aspergillus niger*) in the same range of concentrations. The results were determined based on colour changes and expressed as MIC values (Table 8).

Table 8.

According to the obtained results *Klebsiella pneumonia* was showed to be the most resistant toward examined extract. MIC values for this bacteria were 625, 312.5 and 312.5 μg/mL in case of stems, leaves and berries, respectively. The highest antimicrobial activity was noticed in the case of stems extract toward *Proteus mirabilis* (19.53 μg/mL). Strong antimicrobial activity of aronia stems obtained by SWE extraction has been already reported in

the literature (Cvetanović et al., 2017a), and results from this study were comparable with those from the literature. However, there are no published data on extraction of antimicrobial constituents of aronia berries and leaves by SWE.

Confirmed antimicrobial and antioxidant activity makes aronia SWE extracts a good candidate as ingredient for food functionalization. Such food becomes popular due to consumers' concern to avoid food with synthetic additives and to avoid a series of food safety risks. According to the obtained results, we can assume that there is a potential of SWE aronia extracts to improve the shelf life of the food. Additional advantage of such extracts is the fact that SWE represents green and safe technology which is of utmost importance in the process of designing functional food. Moreover, due to the fact that water is still cheap solvent as well as that there is no need for purification of extracts, this technique is cost-effective.

Conclusion

In the present study, biological and chemical fingerprints of subcritical waters extracts of aronia were analysed and discussed. Generally, leaves and berries exhibited considerable biological activity with higher concentration of total phenolics and flavonoids. Also, the extracts had very good cytotoxic potentials against tested cell lines. In comparison to standard chemotherapy in medicine, the extracts exhibited higher selectivity against normal cells. From these results, we could suggest that subcritical water extraction is suitable for preparing novel functional products from aronia. Moreover, the study offers new insights in utilization of plants and plant waste and their application in the productions of functional products. However, more efforts are needed to determine *in vivo* mechanisms or their synergistic/antagonistic actions of the compounds present in the tested extract or theirs full toxicological profile by *in vivo* studies.

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Table 1. Analytical parameters for 18 phenolic compounds used for HPLC-DAD analysis.

Compound	**Calibration curve**	**(R^2)**	**Range (µg/µl)**	**RT* (min)**	**LOD** (µg/µl)**	**LOQ*** (µg/µl)**
Protocatechuic acid	y = 13,307.5x + 0.2	0.9988	0.007–23.000	12.52	0.004	0.013
p-Hydroxybenzoic acid	y = 9934.4x + 0.1	0.9998	0.006–50.000	17.65	0.003	0.010
Caffeic acid	y = 32,241.5x - 0.1	1.0000	0.001–34.000	21.19	0.009	0.030
Vanillic acid	y = 10,781.0x + 0.5	0.9995	0.006–30.000	22.15	0.003	0.010
Chlorogenic acid	y = 10,491.8x - 0.5	0.9998	0.050–26.200	22.25	0.035	0.116
Syringic acid	y = 11,253.6x + 0.5	0.9999	0.020–22.000	23.88	0.011	0.037
p-Coumaric acid	y = 16,239.7x - 0.8	0.9997	0.042–20.000	24.83	0.042	0.140
Ferulic acid	y = 24,685.7x - 0.7	0.9998	0.035–22.000	27.35	0.029	0.097
Sinapic acid	y = 13,332.5x + 1.1	0.9998	0.040–22.200	29.11	0.032	0.106
Rutin	y = 4589.0x - 0.7	0.9999	0.067–17.000	29.30	0.052	0.173
Rosmarinic acid	y = 5385.2x + 0.7	0.9998	0.025–20.000	33.58	0.018	0.060

Compound	Calibration curve	(R^2)	Range (µg/µl)	RT* (min)	LOD** (µg/µl)	LOQ*** (µg/µl)
Quercetin	y = 10,336.2x + 0.3	0.9996	0.045–20.000	36.50	0.033	0.110
Luteolin	y = 15,958.6x - 0.2	0.9998	0.045–7.200	37.31	0.030	0.100
Naringenin	y = 14,797.5x + 1.0	1.0000	0.065–32.000	37.85	0.055	0.183
Kaempferol	y = 13,636.5x + 0.1	0.9997	0.040–27.000	38.29	0.029	0.097
Apigenin	y = 6229.8x + 1.2	0.9996	0.055–16.000	40.10	0.045	0.150

*RT-retention times; **LOD - limit of detection; ***LOQ - limit of quantification.

Table 2. Total phenols and flavonoids of *Aronia* samples (mean±SD)

Sample	Total phenols (mg CAE/g extract)	Total flavonoids (mg RE/g extract)
Stems	49.96±0.15b	25.10±0.38b
Leaves	131.53±0.96a	88.64±0.31a
Berries	13.88±0.02c	10.00±0.25c

CAE - Chlorogenic acid equivalents; RE - Rutin equivalents. Significant differences between the different samples are indicated by different letters ($P < 0.05$) within a column.

Table 3. Phenolics profile of *Aronia* samples

	Compound	berries	leaves	stems
		(mg/g of dry extract)		
1	Protocatehuic acid	nd	nd	0.150
2	*p*-Hydroxybenzoic	0.469	0.128	0.161
3	Caffeic acid	n.d.	n.d.	0.019
4	Vanillic acid	0.238	0.041	n.d.
5	Chlorogenic	0.171	0.029	0.050
6	Syringic acid	0.419	0.055	0.056
7	*p*-coumaric acid	0.175	0.093	0.033
8	Ferulic acid	0.173	0.046	0.051
9	Sinapic acid	1.072	0.547	0.290
10	Rutin	5.544	0.693	1.264
11	Rosmarinic acid	0.236	0.155	0.115
12	Quercetin	1.396	0.105	0.247
13	Luteolin	0.154	0.334	0.364
14	Naringenin	0.172	0.026	0.065
15	Kaempferol	0.271	0.069	0.049

16	Apigenin	0.242	0.210	0.151

n.d. – not detected

Table 4. Antioxidant capacity of *Aronia* samples

	IC_{50} (µg/mL)		
Samples	DPPH scavenging activity	Inhibitory activity against lipid peroxidation	ABTS scavenging activity
Stems	35.43±0.25a	20.41±0.45b	34.57±0.29a
Leaves	30.45±0.94b	21.36±0.20b	32.94±0.54ab
Berries	32.40±0.53b	24.49±0.38b	31.43±0.57a
Ascorbic acid	6.05±0.34	> 1000	2.39±0.93
BHT	15.61±1.26	1.00±0.23	19.32±0.72
α-Tocopherol	-	0.48±0.05	-

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

Table 5. Enzyme inhibitory effects of *Aronia* samples

Samples	AChE Inhibition (mg GALAE/g extract)	BChE Inhibition (mg GALAE/g extract)	Elastase (mmol CAE/g extract)
Berries	0.814±0.016[*] a	0.605±0.066a	3.549±0.113 a
Stems	0.713±0.030b	n.a.	3.118±0.392 ab
Leaves	0.774±0.023a	n.a.	2.345±0.519 b

[*]n = 3, with mean ± standard deviation; GALAE: Galantamine equivalent; CAE: Catechin equivalent; n.a.: not active; Significant differences between the enzyme-inhibitory activities of different samples are indicated by different letters ($P < 0.05$) within a column.

Table 6. Cytotoxic activity of *Aronia* samples

Samples	IC_{50} (µg/mL)			
	A-549	LS-174T	HeLa	MRC-5
Stems	/	/	/	/
Leaves	2.01±1.05b	1.38±0.28b	0.69±0.08b	1.72±0.06b
Berries	4.21±1.06a	5.44±0.24a	3.41±0.01a	5.50±0.07a
Cisplatin	2.83±0.18	7.46±1.02	1.56±0.14	2.48±0.24

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

Table 7. Selectivity index in the antitumor action of extracts and compounds

Samples	**IC_{50} MRC-5/IC_{50}A-549**	**IC_{50} MRC-5/IC_{50} LS-174T**	**IC_{50} MRC-5/IC_{50} HeLa**
Leaves	0.86	1.24	2.49
Berries	1.3	1.01	1.61
Cisplatin	0.88	0.33	1.59

Table 8. Antimicrobial activities (μg/mL) of aronia samples

Microbial strains	**Stems**	***Leaves***	***Berries***	***Amracin***	***Nystatin***
Staphylococcus aureus ATCC 25923	156.25	39.10	78.20	19.53	/
Klebsiella pneumoniae ATCC 13883	625.00	312.50	312.50	39.10	/
Escherichia coli ATCC 25922	19.53	312.50	312.50	19.53	/
Proteus vulgaris ATCC 13315	78.12	39.10	78.20	156.25	/
Proteus mirabilis ATCC 14153	312.50	19.53	78.125	312.50	/
Bacillus subtilis ATCC 6633	625.00	78.12	312.50	78.12	/
Candida albicans ATCC 10231	39.10	39.10	78.20	/	39.1
Aspergillus niger ATCC 16404	19.53	312.5	312.5	/	19.53

Highlights

- Subcritical water extraction as cutting-edge technology for recovery of bioactives
- Examination of subcritical aronia extracts prepared from berries, leaves and stems
- Chemical and biological characterization of obtained extracts
- Possibility of usage safe and health extracts for food functionalization
- Potential of the extracts in food, cosmetic and pharmaceutical industry