Chemical and biological insights on aronia stems extracts obtained by different extraction techniques: From wastes to functional products

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Abstract

The present study aimed to evaluate chemical and biological potential of aronia stems for providing new raw material for food and pharmaceutical industries. Aqueous extracts of aronia stems were prepared by three different techniques (microwave-assisted, ultrasound-assisted and subcritical water extraction). For biological activities, antioxidant, antimicrobial and cytotoxic activities were evaluated. For chemical characterisation, phenolic compounds and essential elements were analysed as well as total phenolic contents. Subcritical water extracts exhibited the strongest antioxidant activity with the highest content of phenolics. Minimum inhibitory concentrations for analysed extracts were in the range from 9.76 to 156.25 μg/mL in antimicrobial assays. The extracts exerted prominent cytotoxicity against different cell lines. Rutin was detected as the major compound in the studied extracts and these extracts were also rich in essential elements. Our study suggested that subcritical water extract of aronia stem could be considered as a new raw material in developing novel functional/industrial products.

Keywords

Subcritical water extraction; Plant waste; Aronia stems; Natural bioactive agents

1.Introduction

Aronia (Aronia melanocarpa), commonly known as black chokeberry, represents one of the richest sources of dietary phytochemicals [1]. This plant has long traditional use, and different health benefts have been reported for it. Inter alia, it has been reported that fresh aronia berries demonstrate significantly stronger antioxidant properties compared to other berries and fruits [2]. Scientific evidence showed that both aronia and its extracts may be utilized in the prevention of diabetes and diabetes-associated complications [3], cardiovascular diseases [4] and colon cancer [3]. Other studies demonstrated its antiinflammatory, antimutagenic, hepatoprotective, cardioprotective, anticancer and lipid-lowering effects [2], [5], [6], [7].

Besides its traditional use, aronia berries are widely used for industrial production of fruit syrups, juices, jams, marmalades, jellies, alcoholic and nonalcoholic drinks [2]. Moreover, this plant and its extracts are also often used as food preservatives since their polyphenolic constituents inhibit lipid peroxidation [8], [9]. In recent years it has been highlighted as natural food colorant [2]. Current technology of aronia processing does not anticipate the use of stems after their separation from berries. To our knowledge there are no reported scientific evidence on the bioactivity and possible valorization of these aronia wastes.

Plant wastes are growing rapidly providing new raw materials both in scientific and industrial areas. Plant waste can be a significant source of valuable compounds such as bioactive molecules useful in the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceuticals and cosmetic products.

Isolation of bioactive compounds from plant waste is usually performed by using organic solvents. This approach has numerous drawbacks which are primarily related to the toxicity of organic solvents and negative impact on the environment. In last decades, the efforts have been directed to the development of environmentally-benign technologies and thus many studies focuses on the development and application of green technologies. Unconventional extraction techniques contain different approaches such as ultrasonic or microwave extraction.

Subcritical water extraction (SCW) represents an innovative green technology of exceptional potential. In this technique, water remains in its liquid state at temperature above boiling point under high pressure. The intermolecular hydrogen bonds begin to

weaken with increase of temperature, thus reducing water polarity. Consequently, the polarity of water at these conditions becomes equivalent to that of common organic solvents. More precisely, the dielectric constant of water at ambient conditions is approximately 80, whereas the dielectric constant of methanol is 33, and that of ethanol 24. With the increase in temperature and pressure the dielectric constant of water decreases. At 250 °C and 50 bar the dielectric constant of water is 27 Because of this wide range of possible polarity changes, subcritical water has the ability to efficiently solubilize numerous compounds. In contrast, the solubility of compounds which are well soluble in ambient water doesn't change so dramatically with water heating and pressurisation [10]. Another important advantage of subcritical water has not any negative environmental impact. Within these perspectives, SCW has gained great interest in both scientific ad industrial areas.

Within this article, new insights on aronia stems, which is a waste in aronia processing technologies, were investigated. To the best of our knowledge, there are no literature data about this waste applications and its biological potential. Thus, this study is the first report on the biological and chemical profiles of aronia stems. The recovery of bioactive molecules was compared with three different extraction techniques (ultrasound-assisted, microwave-assisted and subcritical water extraction) were compared. The extracts were also characterized with respect to phenol, flavonoid and mineral contents as well as to their antioxidant, antimicrobial and anticancer properties.

2 Materials and methods

2.1 Chemicals and reagents

Cirsimarin, resazurin, amricin, nystatin, sabourand dextrose, Tween 20 and 80 and cis-diamminedichloroplatinum (cis-DDP) were purchased from Tedia Company (USA). Folin-Ciocalteu reagent, trichloroacetic acid, 1,1-diphenyl-2-picryl-hydrazyl-hydrate (DPPH), galic acid, linoleic acid, thiobarbituric acid (TBA), 2-deoxyribose, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, sodium phosphate, ammonium molybdate, ammonium thiocyanate, Butylated hydroxytoluene (BHT), α-tocopherol, sulfuric acid, iron(II) chloride, nystatin, β-carotene and rutin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Iron(II) sulfate was obtained from Zorka (Šabac, Serbia). Amracin was purchased from Galenika a.d. (Belgrade, Serbia). All chemicals and reagents were of analytical reagent grade.

2.2 Plant material

Aronia (Aronia melanocarpa) was collected in Southeast region of Serbia in August 2014. Separated stems were stacked in a crate with perforated bottom, in order to ensure air flow. Drying was performed naturally in the draft and dark until moisture content of 10%. Dry stems were packed in glass jar and stored in the dark until use.

2.3. Sample preparation

2.3.1. Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction (UAE) was performed in ultrasonic water bath (Branson, USA) during 30 min. In volumetric flask 4 g of stems were mixed with 100 mL of deionised water and sonificated. Obtained extracts were filtrated.

2.3.2. Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) was performed in an open system by using a modified domestic microwave oven. Stems were mixed with the water in the ratio as in the case of UAE. The extraction procedure was performed at 580 W during 30 min.

2.3.3. Subcritical water extraction (SCW)

Subcritical water extraction (SCW) was performed in a homemade subcritical water extractor/reactor previously described by Cvetanović et al. [11]. Pressurization of the vessel was performed with 99.99% nitrogen in order to prevent oxidation at high temperatures. The operating pressure was 40 bar while the process temperature was 140 °C. Stirring was at the frequency range of 3 Hz. Extraction duration was 30 min. Obtained extracts were filtrated and stored in the refrigerator until analysis.

2.4. Determination of total extraction yield, total phenolic content (TPC)

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. Based on the mass difference extraction yield was calculated as expressed in percentage (%).

The total phenolic content (TPC) in obtained aronia stem extracts was determined by Folin–Ciocalteu procedure [12], [13] using chlorogenic acid as a standard. Absorbance was measured at 750 nm. Content of phenolic compounds was expressed as mg of chlorogenic acid equivalent (CAE) per g of dry extract (mg CAE/g). All experiments were performed in triplicate.

2.5. Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) was determined using aluminum chloride colorimetric assay [14]. Results were expressed as mg of rutin equivalent (RE) per g of dry extract (mg RE/g). All experiments were performed in triplicate.

2.6. Determination of antioxidative capacity

2.6.1. Determination of lipid peroxidation

Determination of inhibitory activity against lipid peroxidation was carried out according to the thiocyanate method [15]. Dried extracts were dissolved in water to the final concentration of 1 mg/mL of dried extract. This solution was further used for preparation of serial dilutions, and 0.5 mL of each diluted solution 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 h. After that, 0.1 mL of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL of FeCl2 (20 mM), and 0.1 mL of ammonium thiocyanate (30%). The mixture was stirred for 3 min 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 IC50 values (the concentration of the test solution for inhibiting 50% of linoleic acid oxidation).

2.6.2. Measurement of ferrous ion chelating ability

The ferrous ion chelating ability was measured by the decrease in absorbance at 562 nm of the iron(II)-ferrozine complex [16]. Reaction mixture was prepared by mixing sample solutions (1 mL) with 0.125 mM FeSO4 (1 mL). Immediately after, 1 mL of ferrozine (0.3125 mM) was added. The prepared mixture was allowed to equilibrate for 10 min before absorbance measurement. The chelating activity was calculated in the following way (Eq. (1)):

(1)I (%) = (A0 − A1)/A0 × 100

where A0 is the absorbance of the control, A1 is the absorbance of the extract/standard. The results were expressed as inhibitory concentration at 50% (IC50), which is the concentration of the test solution for achieving 50% of the chelating ability.

2.6.3. Determination of hydroxyl radical scavenging activity

The ability to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out according to the method described in the literature [17]. Extracts of aronia stems were mixed with 500 µL of 5.6 mM 2-deoxy-d-ribose in KH2PO4–NaOH buffer (50 mM, pH 7.4), 200 µL of premixed 100 µM FeCl3 and 104 mM EDTA (1:1 v/v) solution, 100 µL of 1.0 mM H2O2, and 100 µL of 1.0 mM aqueous ascorbic acid. Reaction mixture was incubated at 50 °C for 30 min. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube. The samples were vortexed and heated in a water bath at 50 °C for 30 min. The degree of 2-deoxyribose oxidation was estimated from the absorbance of the solution at 532 nm. The scavenging activity was calculated according to Eq. (1). The values were presented as the means of triplicate analyses.

2.6.4. ABTS+ radical scavenging assay

ABTS+ radical scavenging activity was determined following the procedure previously described [18]. Briefly, ABTS+ was generated in the reaction of 7 mM ABTS+ stock solution with 2.45 mM potassium persulfate. The mixture was left to stand in the dark at room temperature for 12–16 h before use. The ABTS+ solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance at 730 nm of 0.70 ± 0.02. Upon addition of 10 µL of the sample to 4 mL of diluted ABTS+ solution, the absorbance was measured after 30 min. Gallic acid, ascorbic acid and butylated hydroxytoluene (BHT) were used as reference antioxidants. The scavenging activity was calculated according to Eq. (1). All samples were analysed in triplicate.

2.6.5. Determination of total antioxidant capacity

The total antioxidant activity of samples was evaluated by the phosphomolybdenum method previously described [19]. Extracts (0.3 mL) were mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The mixtures were incubated at 95 °C for 90 min. After cooling to room temperature, absorbance was measured at 695 nm. The blank was prepared by using methanol instead of an extract. All tests were performed in triplicate. Ascorbic acid was used as the standard, and total antioxidant capacity was expressed as micrograms of ascorbic acid per gram of dry extract (µg AA/g).

2.7. Determination of antibacterial and antiungal activity

The antibacterial activity was tested against the Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Klebsiella pneumonia ATCC 13883, Proteus vulgaris ATCC 13315, Proteus mirabilis ATCC 14153 and Bacillus subtilis ATCC 6633. The antifungal activity was tested against the Aspergillus niger ATCC 16404 and Candida albicans ATCC 10231. Antibacterial and antiungal activities were estimated by measuring their minimum inhibitory concentrations (MIC). MICs of the extracts and cirsimarin against the test bacteria were determined by microdilution method in 96 multi-well microtiter plates [20]. All tests were performed in Muller-Hinton broth (MHB) with the exception of the yeast, in which case Sabouraud dextrose broth was used. A volume of 100 µL stock solutions of extracts (in methanol, 200 µL/mL) and cirsimarin (in 10% DMSO, 2 mg/mL) were pipetted into the first row of the plate. Fifty microliters of Mueller Hinton or Sabouraud dextrose broth (supplemented with Tween 80 to a final concentration of 0.5% (v/v)) were added to other wells. A volume of 50 µL from the first test wells was pipetted into the second well of each microtiter line, and then 50 micro liters of scalar dilution was transferred from the second to the twelfth well. Ten microliters of resazurin indicator solution (prepared by dissolving 270-mg tablet in 40 mL of sterile distilled water) and 30 µL of nutrient broth were added to each well. Finally, 10 µL of bacterial suspension (106 CFU/mL) and yeast spore suspension (3 × 104 CFU/mL) were added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Standard antibiotic amracin was used to control the sensitivity of the tested bacteria, whereas nystatin was used as a control against the tested

yeast. Plates were wrapped loosely with cling film to prevent dehydration and prepared in triplicate. The plates were placed in an incubator at 37 °C for 24 h for the bacteria and at 28 °C for 48 h for the yeast. Subsequently, color change was assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated, and the obtained value was taken as the MIC for the tested sample and a standard drug.

2.8. Measurement of cytotoxic activity by MTT assay

The influence of extracts on the growth of malignantly transformed cell lines was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The following cell lines were used: RD (cell line derived from human rhabdomyosarcoma), Hep2c (cell line derived from human cervix carcinoma − HeLa derivative) and L2OB (cell line derived from murine fibroblast).

Cells were seeded (2 × 105 cell/mL; 100 µL/well) in 96-well cell culture plates in nutrient medium (Minimum Essential Medium (MEM) Eagle supplemented with 5% of Hep2c, RD and L2OB) and grown at 37 °C in humidified atmosphere for 24 hThen, corresponding extract (stock solution: 5 mg of extract dissolved in 1 mL of absolute ethanol) and control (absolute ethanol) diluted with nutrient medium to desired concentrations were added (100 µL/well) and cells were incubated at 37 °C in humidified atmosphere for 48 h. Pure nutrient medium (100 µL) represented positive control for each cell line. After incubation period, supernatants were discarded and MTT (dissolved in D-MEM (Dulbecco's modification of Eagle's medium) in concentration of 500 µg/mL) was added in each well (100 µL/well). Immediately after, all wells were incubated at 37 °C in humidified atmosphere for 4 h. Reactions were halted by adding 100 µL of sodium dodecyl sulfate- SDS (10% in 10 mM HCl). After overnight incubation at 37 °C, absorbance was measured at 580 nm using a spectrophotometer (Ascent 6-384 [Suomi], MTX Lab Systems Inc., Vienna, VA 22182, USA). The number of viable cells per well (NVC) was calculated from a standard curve plotted as cell numbers against A580. Corresponding cells (grown in flasks), after cell count by haemocytometer, were used as standards. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min and then treated with MTT/D-MEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells (ut supra). The

number of viable cells in each well was proportional to the intensity of the absorbed light, which was then read in an ELISA plate reader at 580 nm. Absorbance (A) at 580 nm was measured 24 h later. Cell survival (%) was calculated by dividing the absorbance of a sample with cells grown in the presence of various concentrations of the investigated extracts with control optical density (the A of control cells grown only in nutrient medium), and multiplying by 100. The blank absorbance was always subtracted from the absorbance of the corresponding sample with target cells. IC50 concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. The results of the measurements were expressed as the percentage of positive control growth taking the cis-diamminedichloroplatinum (Cis-DDP) determined in positive control wells as the 100% growth [21], [22], [23]. All experiments were done in triplicate.

2.9. Elemental analysis

Elemental analysis of aronia water extracts was performed by inductively-coupled plasma mass spectrometry (ICP-MS) after microwave-assisted digestion. The samples (3 mL) were transferred into Teflon vessels and 5 mL of nitric acid (p.a. SIGMA) and 1.5 mL of hydrogen peroxide (30%, p.a., MERCK) were added. The microwave heating consisted of three steps as follows: 5 min from room temperature to 180 °C, 10 min hold at 180 °C and 20 min vent. After cooling to room temperature, the digested samples were quantitatively transferred into disposable flasks and diluted to 100 mL with deionized water (ELGA). Measurements were performed using the instrument “iCap Q” (Thermo Scientific, Bremen, Germany), equipped with collision cell and operating in kinetic energy discrimination (KED) mode. The following isotopes were measured: 39K, 23Na, 24Mg, 44Ca, 55Mn, 57Fe, 63Cu, 66Zn, 77Se. Torch position, ion optics and detector settings were adjusted daily using tuning solution (Thermo Scientific Tune B), in order to optimize measurements and minimize possible interferences. For quantitative analysis, five-point calibration curve was constructed for each isotope in the concentration range of 0.1 − 2.0 mg/L. Additional line of the peristaltic pump was used for on-line introduction of multi-element internal standard (6Li, 45Sc − 10 ng/mL; 71Ga, 89Y, 209Bi − 2 ng/mL) covering wide mass range. Concentration of each measured isotope was corrected for response factors of both higher and lower mass internal standard using interpolation method [24], [25]. The quality of the analytical process was controlled by the analysis of the standard reference material (NIST SRM 1577c).

2.10. HPLC-DAD analysis

The HPLC analyses of phenolic components were performed using the Agilent-1200 series with a diode array (DAD) for multi wavelength detection. The column was thermostated at 25 °C. After injecting 5 μL of sample, the separation was performed in an Agilent-Eclipse XDB C-18 4.6·150 mm column. Two solvents were used for the gradient elution: eluent A was water with 2% HCOOH and eluent B 80% ACN plus water with 2% of acetic acid. The elution program used was as follows: from 0 to 10 min 0% B, from 10 to 28 min, 25% B, from 28 to 30 min 25% B, from 30 to 35 min 50% B, from 35 to 40 min 80% B, and finally for the last 5 min gradually decreases 80-0% B. Phenolic compounds in the samples were identified by comparing their retention times and spectra with retention time and spectrum of standards for each component. Quantitative data were calculated from the calibration curves.

3 Results and Discussion

3.1 Extraction yield, total phenolic content and phenolic profile

Extraction yield is very important, especially for economical aspects of large-scale industrial applications. Thus the first step in this research was comparing extraction methods for achieving the highest extraction yield. The results showed that there was a significant difference between the SCW extracts and all other extracts. Namely, total extracted compounds from aronia stems by using this extraction technique was 37.58% which is more than twice higher yield than in case of UAE (15.32%), and 1.6-fold higher than in case of MAE (23%). Those results were in correlation with data from the literature [26].

Total phenolic and flavonoid contents of stems extracts were determined and the results are presented in Fig. 1. Significant differences between the contents in the samples obtained by different extraction techniques were noticed. Subcritical water (SCW) extracts were remarkably richer in total phenols in comparison to other extracts. Appareantly, SCW extracts (48.62 mg CAE/g) were almost three times richer in comparison to extracts obtained by microwave-assisted extraction − MAE (16.65 mg CAE/g) and even nine times in the case of extracts prepared by ultrasound-assisted extraction − UAE (5.22 mg CAE/g). The same ratio between extraction efficiencies was observed also in terms of total flavonoid

contents. The total flavonoid contents of SCW, MAE and UAE were 39.19, 12.12 and 3.94 mg RE/g respectively (Fig. 1).

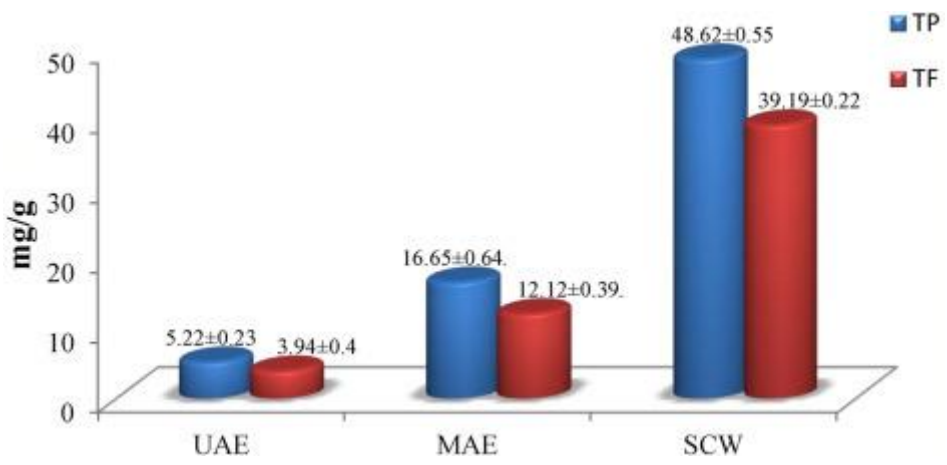

Fig. 1. Total phenolic content (TPC) and total Flavonoid content (TFC) of aronia stems extracts obtained by different extraction techniques. Results are mean values ± SD from three replicates.

Fig. 1. From these results, SWE is the best effective technique for extracting more phenolics from aronia stems. This fact could be explained with important change of water polarity for recovering phenolics, which encompasses different components with varying polarities. In addition, subcritical water potentiates dissociation of analytes bound to matrix constituents. Phenolic compounds are easily oxidized at high temperature so it is very important to demonstrate that they do not degrade under proposed SWE conditions [27]. In this regard, pressurisation with nitrogen in SCW extraction was of great importance. When comparing different techniques obtained results were in arrangement with literature data. Cvetanović et al. [26] demonstrated advantages of SCW in comparison to UAE and MAE in the extraction of phenolic compounds from chamomile. In the same study it was reported that MAE extraction approach was more successful than UAE.

Polyphenolic composition of aronia berries is well investigated. A series of factors, such as habitat/location, harvest date, cultivar, fertilization, and maturation of the berries can affect presence of such compounds and their quantity [28], [29], [30]. Literature data suggest that aronia berries contain high levels of procyanidins, anthocyanins and phenolic acids. Procyanidins have been identified as the major class of polyphenolic compounds in this plant [31], but also, aronia constitute a rich source of phenolic acids. Among others,

chlorogenic and neochlorogenic acids are the two dominating phenolic acids identified in the fruits [32]. On the other hand, there is no data in terms of aronia stem composition. In order to identify compounds present in aronia stem extracts, HPLC-DAD analysis was performed. Identification of compounds was done based on retention times and spectra of used standards. In HPLC analysis eighteen standards were used, of which ten belonged to phenolic acids, and the rest of them to flavon, flavanon and flavonol classes. In analysed samples up to fourteen compounds were identified (Table 1). Among identified compounds rutin was the most abundant in the aronia stem extracts. The level of rutin was reached to 7.335 mg/g extract in SCW, followed by MAE (4.552 mg/g extract) and UAE (0.738 mg/g extract). In regards to phenolic acids, synapic acid (0.617 mg/g extract) was the most abundant acid in the aronia stem extracts, while caffeic acid (0.001 mg/g extract) was present at lowest concentration. Protocatehuic and vanillic acids were not detected in these extracts.

Table 1. Phenolic components in aronia stem extracts obtained by different extraction techniques.[a]

No	Components	MAE	UAE	SCW
1	Protocatehuic	nd	nd	nd
2	p-Hydroxybenzoic acid	nd	nd	0.392
3	Caffeic acid	nd	0.001	0.012
4	Vanillic acid	nd	nd	nd
5	Chlorogenic	nd	0.007	0.066
6	Syringic acid	nd	0.047	0.223
7	p-coumaric	0.079	0.016	0.147
8	Ferulic acid	0.351	0.029	0.200
9	Syndpic acid	0.560	0.127	0.617
10	Rutin	4.552	0.738	7.335
11	Luteolin-gly	nd	nd	0.254
12	Apigenin-gly	0.433	nd	nd
13	Rosmarinic	0.430	nd	nd
14	Quercetin	1.962	0.021	0.789
15	Luteolin	0.164	0.046	0.368
16	Ndringenin	0.292	0.013	0.168
17	Kaempferol	0.276	0.051	0.286
18	Apigenin	0.278	0.064	0.358

a mg per g of dry extracts. nd:not detected.

Conducted analysis showed that SCW extracts were the most aboundant with polyphenols. Apart of rutin, the concentration of apigenin, kaempferol, luteolin, synapic acid, p-coumaric acid, syringic acid and chlorogenic acid were the highest in this extract. Moreover, the

presence of p-hydroxybenzoic acid as well as luteolin-glygoside was noticed only in this sample. Likewise, MAE was also pointed as sample with significant amount of polyphenolic compounds. Information on the quantitative composition showed the highest concentration of ferulic acid, querectin and naringenin in this sample, while apigenin-glucoside and rosmaric acid were only presented here.

To the best of our knowledge, this is the first comprehensive analysis of phenolic compounds in stems of aronia. Thus, it could be a valuable basis for its further utilization.

3.2. Antioxidant activity

The antioxidant activity of aronia steam extracts was assessed by different methods. It is recommendable to evaluate the antioxidant potential by different methods that involve different mechanisms. In the present study, the antioxidant capacity was evaluated by five different methods.

Thiocyanate assay was performed to evaluate the inhibition of lipid peroxidation. The method measures the amount of peroxide produced during the initial stages of lipid oxidation. Inhibitory activity of the aronia extracts against lipid peroxidation was calculated and the results are given in Table 2. The obtained results showed that all tested extracts have remarkable potential for inhibiting lipid peroxidation. As it can be seen from Table 2, the activity can be ranked as SCW > MAE > UAE. SCW extracts had the lowest IC50 value of 30.10 μg/mL. MAE and UAE also showed similar activity, with IC50 values of 33.73 μg/mL and 34.57 μg/mL, respectively. Lipid peroxidation is linked to physiological process in biological damages thus, aronia stem could have great potential for protecting these damages.

Table 2. Antioxidant capacity of extracts obtained by different extraction techniques and comparison with standard antioxidant compounds.

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Samples	IC_{50} (μg/mL)			
	Inhibitory activity against lipid peroxidation	**Metal chelating activity**	**Hydroxyl radical scavenging activity**	**ABTS radical scavenging assay**
UAE	34.57 ± 0.93[a]	39.06 ± 0.43	41.56 ± 0.68	10.09 ± 0.11
MAE	33.73 ± 0.22	36.45 ± 0.14	40.83 ± 0.39	8.44 ± 0.29
SCW	30.10 ± 0.19	33.12 ± 0.33	38.61 ± 0.41	6.79 ± 0.84
Gallic acid	255.43 ± 11.68	–	59.14 ± 1.10	1.96 ± 0.41
Ascorbic acid	>1000	–	160.55 ± 2.31	10.98 ± 0.95
BHT	1.00 ± 0.23	–	33.92 ± 0.79	7.23 ± 0.87
α-Tocopherol	0.48 ± 0.05	–	–	–

[a] Results are mean values ± SD from three replicates.

Antioxidant capacity of the samples was also characterized by measuring ferrous ion chelating ability. It is well known that during the Fenton reactions iron may participate in HO generation and may cause oxidative damage in biological systems [33]. Inhibition of ferrous ion activity contribues to protect against oxidative damage by reducing the production of reactive spacies and molecular damage. Therefore it is important to measure the ability of the extracts to act as ferrous ion chelators. The results are presented in Table 2. Among the examined extracts, SCW extracts showed the strongest ferrous ion chelating capacity with the lowest IC50 value (33.12 μg/mL) followed by MAE extracts (36.45 μg/mL). UAE extracts had the lowest ferrous ion chelating capacity (39.06 μg/mL). Hydroxyl radical is considered as the most reactive. In biological systems, this radical species is generated in the Fenton reaction in the presence of reduced transition metals (such as Fe2+) and H2O2. In this study inhibition activity of the extracts against hydroxyl radicals was determined by measuring the level of oxidation of 2-deoxy-d-ribose by ˙OH with subsequent measurement

of the products by their reaction with thiobarbituric acid (TBA). The results are given in Table 2.

The examined extracts showed significant hydroxyl radical scavenging activity, with IC50 values in the range from 38.61 to 41.56 μg/mL, close to that of BHT. Galic acid and ascorbic acid showed lower capacity in comparison to the examined extracts. Similar to other antioxidant assays, the highest activity was obtained by using subcritical water. The activity for MAE (IC50 = 40.83 μg/mL) and UAE (IC50 = 41.56 μg/mL) extracts were close.

Anti-radical activity was tested by ABTS assay as well.The tested samples were capable to scavenge this radical cation (Table 2). Again, SCW extracts showed the greatest radical scavening potential (IC50: 6.79 μg/mL). The result revealed very good antiradical activity, even better than two of three tested commercially purchased standards. Namely, with their IC50 values of 7.23 and 10.98 μg/mL, respectively, BHT and ascorbic acid showed lower activity than SCW extracts. MAE was more efficient than UAE. Nevertheless, with IC50 value of 10.09 μg/mL UAE extracts of aronia stems were still quite good antioxidants.

Total antioxidant power of the extracts was detected by phosphomolybdenum method. The results are given in Fig. 2. Extracts obtained by SCW extraction technique showed the highest total antioxidant capacity, with 147.2 μg AA/g dry extract. MAE extraction technique was stronger (139.14 μg AA/g) in comparison to UAE (125.49 μg AA/g).

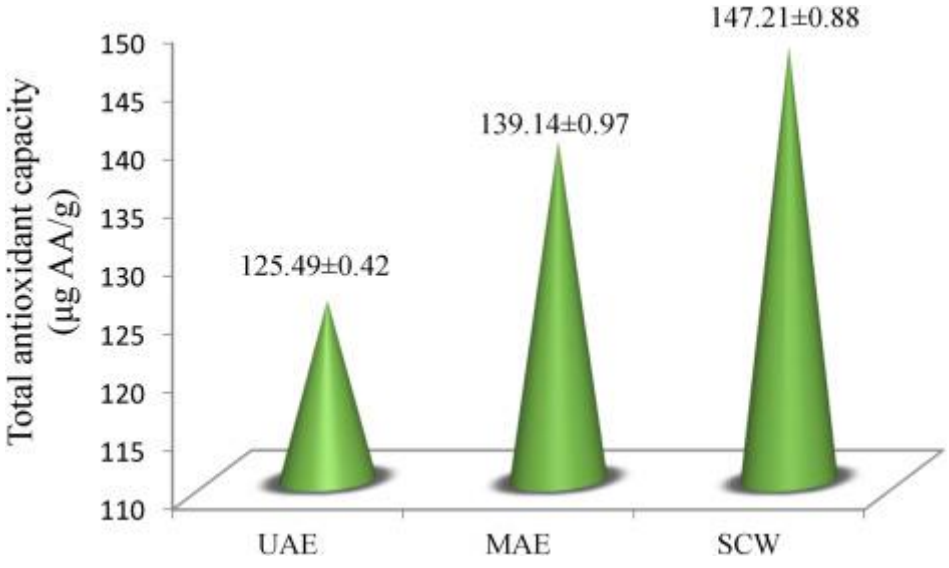

Fig. 2. Total antioxidant capacity of aronia stems extracts obtained by different techniques. Results are mean values ± SD from three replicates.

Taken together, SCW extracts exhibited the best antioxidant ability in the test system with higher levels of phenolics. At this point, SCW extracts from aronia stems could be considered as a valuable raw material for designing natural antioxidants.

3.3. Antibacterial activity and antifungal activity

Antibacterial activity of extracts was determined against selected non-pathogenic or facultative pathogenic bacteria. Both Gram positive and Gram negative bacteria were used. Measured inhibitory concentrations for each extracts are presented in Fig. 3.

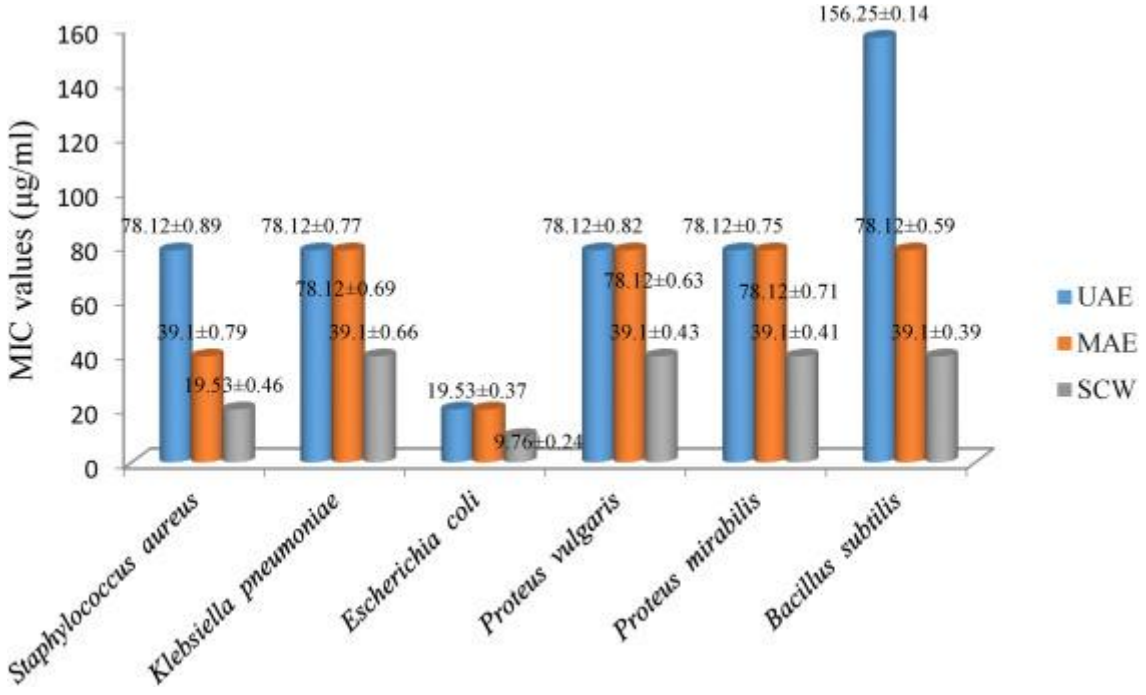

Fig. 3. Antibacterial activity of aronia stems extracts.

Minimum inhibitory concentrations were in the range from 9.76 to 156.25 µg/mL, which are considered to be a very good antibacterial activities [34]. Escherichia coli expressed the highest sensitivity to aronia stem extracts, while the most resistant was Klebsiella pneumonia. Significant difference between extracts obtained by different extraction techniques was observed. SCW extracts had noticeably higher antibacterial efficiency. In the case of Bacillus subtilis, SCW extracts had stronger antibacterial effect with 39.10

μg/mL than UAE (156.25 g/mL) and MAE (78.12 g/mL) extracts. For Escherichia coli MIC value was as low as 9.76 μg/mL indicating exceptionally high antibacterial activity. Likewise, all other bacterial strains were much more sensitive to SCW extracts. Extracts obtained by MAE the highest antibacterial activities were noticed against Escherichia coli (19.53 μg/mL) and Staphylococcus aureus (39.10 μg/mL). In the case of other bacterias MIC values were 78.12 μg/mL. Although samples obtained by UAE technique expressed the lowest antibacterial activity, they still indicated quite high antimicrobial activity.

The high concentrations of phenols and flavonoids in aronia extracts could be related to high antibacterial capacity. In this context, plant phenolics possess antibacterial activity and can be successfully applied to control invasion and growth of pathogens [35]. Also, some researchers suggested that the B ring of flavonoids may play a role in intercalation or hydrogen bonding in stacking of nucleic acid bases and that this mechanism may explain the inhibitory action on DNA and RNA synthesis in bacteria [36]. Flavonoids can also act via inhibition of energy metabolism or inhibition of cytoplasmic membrane function [37]. In general, antimicrobial activity may be related to multiple mechanisms and phytochemicals [35].

The antifungal ability of aronia stem extracts was determined aganist Candida albicans and Aspergillus niger. The results are presented in Table 3. Nystatin was used as a reference standard.

Table 3. Antifungal activity of aronia stem extracts.

Table 3. Antifungal activity of aronia stem extracts.

Fungus	MIC values (µg/mL)			
	SCW	MAE	UAE	Nystatin
Candida albicans ATCC 10231	19.53 ± 0.33[a]	19.53 ± 0.38	19.53 ± 0.29	1.95 ± 0.05
Aspergillus niger ATCC 16404	9.76 ± 0.11	19.53 ± 0.41	39.10 ± 0.21	0.97 ± 0.04

[a] Results are mean values ± SD from three replicates.

As can be seen, the studied extracts exhibited significant antifungal activity. The extracts have the same activity against Candida albicans. The differences in the extracts noted against Aspergillus niger. SCW extracts (9.76 µg/mL) had the highest antifungal activity followed by MAE (MIC = 19.53 µg/mL) and UAE (MIC = 39.1 µg/mL). According to these results, the antifungal activity might be explained by phenols and flavonoids present in extracts. It is well known that owing to the ability of plant phenols to inhibit spore germination, they have been proposed against fungal pathogens in humans [38]. Besides, previous researchs indicated that antifungal activity is correlated with rutin [39] which is identified as dominant polypohenols in observed extracts. Its concentration in MAE, and especially in SCW extracts is quite higher than in UAE which can be linked with higher antifungal activity of MAE and SCW against Aspergillus niger.

3.4. Cytotoxic activity

The cell growth inhibitory activity of aronia stem extracts was evaluated in vitro by MTT assay. cis-diamminedichloroplatinum was applied as standard compound and obtained results are presented in Table 4.

Table 4. Cytotoxic activity of aronia stem extracts.

Cell lines	IC_{50}values (μg/mL)			
	SCW	MAE	UAE	*cis*-DDP[e]
Hep2c cells[a]	12.01 ± 0.94[d]	21.84 ± 0.28	13.27 ± 0.71	0.94 ± 0.55
RD cells[b]	20.97 ± 0.40	19.96 ± 0.61	11.12 ± 0.18	1.4 ± 0.97
L2OBcells[c]	2.98 ± 0.37	5.91 ± 0.37	3.19 ± 0.76	0.72 ± 0.14

a

Cell line derived from human cervix carcinoma.

b

Cell line derived from human rhabdomyosarcoma.

c

Cell line derived from murine fibroblast.

d

Mean value ± 2SD.

e

Cis-diamminedichloroplatinum.

According to the present results, it can be noticed that different extracts expressed different degree of effectiveness. UAE extracts showed the highest cytotoxic activity against RD cell line. On the other hand SCW demonstrated the greatest activity against Hep2C and L2OB cell lines, while MAE had the lowest cytotoxic activity. However, obtained results indicate exceptionally high cytotoxic activity of all examined extracts. Low IC50 values (in the range from 2.98 to 21.84 μg/mL) could be related or at least partly explained by high content of polyphenols in the samples since number of studies have suggested that antioxidant and antitumor potentials are closely associated with this group of secondary metabolites. According to the previous disscussed results, SCW extracts possess the highest concentration of total phenols as well as total flavonoids which can be linked with highest biological activity of this sample. Further, the highest concentration of particular compounds were quatified in SCW extracts. More precisely, SCW extracts had the highest content of kaempferol, luteolin, rutin, synapic and p-coumaric acid which are well known as potent bio-

active molecules. In addition to this, SCW extracts were opulent with apigenin, which is declared as one of the most promising molecules in terms to cytotoxic activity [11].

Although the fact that there is no evidence in terms of biological activity of aronia stems, there is numerous studies which dealing with biological activity of other parts of aronia as well as of its constituents. Rugină et al. [40] dealt with cytotoxic activity of anthocyanin fraction of aronia. It was showed that such fraction in concentration of 200 μg/mL inhibited the survival of HeLa cells for 40%. Furthermore, antileukemic effects of aronia leaf extracts were determined against promyelocytic HL60 cell line in study conducted by Skupień et al. [41]. The effects of aronia juice on the mouse embryonal carcinoma stem cell line P19 were investigated in the study by Sharif et al. [42] where was demonstrated that aronia juce inhibited cell proliferation, induced cell cycle arrest in S phase and triggered apoptosis. The cytotoxic effects of the infusion and decoction prepared from dried chokeberry against malignant HeLa, Fem-X and LS174 cells were tested by Šavikin et al. [43]. In that study, it was showed that berry teas prepared as a decoction showed significantly stronger cytotoxic activity in comparison to the activity of the infusion. Depending on the cell lines measured IC50 values for decoction were in the range from 11.2 to 21.4 μg/mL. Obtained values were comparable with results obtained in our study, however different cell lines were used. Thus, the results could be compared with the results obtained for activity of some other plants. Mašković et al. [44] reported that ethanolic extracts of Onosma aucheriana obtained by using ultrasound extraction were able to inhibit growth of these cell lines but obtained IC50 values (IC50(Hep2C) = 40.34, IC50(RD) = 50.57, IC50(L2OB) = 25.54 μg/mL) were much higher than that one obtained in this study. Further, in the study reported by Cvetanovic et al. [26] extracts of chamomile prepared by different extraction techniques were less effective against observed tumor cells. Moreover, it was showed that subcritical water extracts of chamomile were also inferior to aronia stems extracts indicating that this kind of plant material could be good sources of biologically active molecules. Also in comparison to results obtained for water extracts of ginger, achieved results were better [45].

3.5. Elemental analysis

In aronia stem extracts twelve elements were analyzed. With its concentration range between 1176.03 and 1464.49 μg/mL (Table 5) iron was the most abundant element in the examined samples. This could be of great significance since this mineral plays a role in

oxygen and electron transfer. Moreover, it is essential element for hemoglobin, myoglobin, cytochromes and some enzymes [46].

Table 5. The contents of macro and micro elements in aronia stem extracts obtained by different extraction techniques.

Elements	Samples		
	SCW	MAE	UAE
	Microelements (μg/mL)		
Cr	1.102 ± 0.002[a]	1.733[a] ± 0.001	7.226[a] ± 0.001
Co	2.880 ± 0.02	4.820 ± 0.002	6.020 ± 0.02
Cu	149.770 ± 0.97	174.090 ± 0.015	153.350 ± 0.48
Fe	1176.030 ± 0.75	1210.380 ± 0.02	1464.490 ± 1.26
Mn	126.056 ± 0.935	116.311 ± 0.91	97.967 ± 0.28
Ni	429.600 ± 0.51	450.410 ± 0.48	512.440 ± 0.36
Se	1.160 ± 0.02	2.730 ± 0.003	1.220 ± 0.09
Zn	281.547 ± 0.980	327.413 ± 0.67	255.856 ± 1.166
	Macroelements (μg/mL)		
Ca	112.100 ± 1.4	110.900 ± 0.85	98.490 ± 0.53
Mg	22.187 ± 0.969	23.797 ± 1.229	23.439 ± 1.321
Na	14.456 ± 0.467	10.478 ± 0.698	12.557 ± 0.571
K	55.141 ± 1.115	52.357 ± 0.655	48.092 ± 1.003

[a] Results are mean values ± SD from three replicated.

Besides iron, significant concentrations were determined for other microelements: Mn, Zn and Ni. Macroelements had higher concentrations in SCW extracts. UAE extracts had also the highest concentrations of Cr, Co, Fe, Ni, whereas the concentrations of Cu and Zn were the highest in MAE samples. The concentration of Mn was significant in SCW extracts.

On oppose to the content of microelements whose concentrations varied depending on the extraction techniques applied, there were no significant differences in the content of microelements. The study showed that the dominant macroelement in the extracts was Ca. Its highest content was found in SCW extracts (112.10 μg/mL). Dietary Reference Intake (DRI) for this mineral established by the Institute of Medicine, USA, is 1000 μg per day for adults [47]. Therefore, only 9 mL of the SCW extracts satisfies the daily requirements. A second most present macronutrient in the extracts of aronia stems was potassium, which was detected the highest concentration in the SCW extracts (55.14 μg/mL). Sodium was also detected in the remarkable concentration in SCW extracts (14.45 μg/mL). The maximum concentration of magnesium was in MAE extracts (23.79 μg/mL). This macroelement plays a vital role in a wide range of biochemical and physiological processes, particularly those involving energy metabolism and consumption.

4. Conclusion

Aronia stem extracts were tested for their biological activity and chemical profile. Generally, SCW was noted as the most effective technique with the highest concentration of bioactive compounds. Plant wastes can be considered as valuable raw materials for developing novel-functional products. In this respect, aronia stems may have a potential for application in food and pharmacological fields.

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