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PRELIMINARY STUDIES ON THE CHEMICAL CHARACTERIZATION AND ANTIOXIDANT CAPACITY OF POLYPHENOLS FROM *SAMBUCUS SP.*

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The aim of this study was focused on the chemical characterization of phytochemical compounds and their antioxidant capacity of *Sambucus nigra* L. (black elder) and *Sambucus ebulus* (dwarf elder) berries. The primary sources of antioxidant capacities in the evaluated elderberry extracts were phenolic acids and flavonoids (flavonols and anthocyanins) determined by liquid chromatography-mass spectrometry (LC-PDA-MS). The most abundant anthocyanin in elderberry fruits was cyanidin-3-*O*-sambubioside, which accounted for more than half of all anthocyanins identified in the berries. Quercetin-3-*O*-rutinoside was the major flavonoid found in elderberry extracts, in higher quantity in *Sambucus nigra*. The antioxidant capacity data obtained for crude-extracts proved that the *Sambucus ebulus* had the highest antioxidant activity, being the richest one in anthocyanins.

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1. Introduction

Sambucus nigra L. (black elder) and *Sambucus ebulus* L. (dwarf elder) belong to *Adoxaceae* family, being two widespread species which grow on sunlight-exposed locations in most parts of Europe. *Sambucus nigra* L. cultivars are planted for ornamental purposes and the elderflower extracts are used as beverage or food flavorings [1]. Recently, much attention has been focused on natural compounds that may be beneficial in reducing oxidative stress-induced diseases. Elderberries contain organic acids and flavonols glycosides [2] and anthocyanins [3, 4]. Nevertheless, the anthocyanins present in elderberries are important for the beneficial health effects associated with their antioxidant properties and their ability to protect against colon cancer [5], influenza A and B virus [6], respective *Helicobacter pylori* [7] infections has been demonstrated.

The antioxidant activity of flavonols and anthocyanins is due to the presence of *ortho* 3',4'-dihydroxy moiety in the B ring and *meta* 5,7-dihydroxy arrangements in the A ring [8, 9]. Literature data on the overall chemical composition of elderberry fruits are scarce, only few studies revealed anthocyanin and polyphenolic content levels of various *Sambucus nigra* L. cultivars [2-4, 10]. Even if the content of phenolics in berries is affected by genetic differences,

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environmental conditions, degree of maturity at harvest it is very important for pharmaceutical and alimentary industry to know the chemical composition and the antioxidant capacity of different species [10]. Therefore, the objective of our study was to characterize the phenolic acid, flavonol and anthocyanin content from *Sambucus nigra* L. and *Sambucus ebulus* L. berry extracts and to evaluate their antioxidant capacity.

2. Material and methods

2.1. Reagents

The standard compounds, including cyanidin-3-*O*-glucoside, cyanidin, gallic acid (GAE), quercetin, and 2,2'-Diphenyl-1- picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, MO). Reagents such as butylhydroxytoluene (BHT), acetonitrile, formic acid, ethanol and methanol were purchased from Merck (Darmstadt, Germany). Cyanidin-3-sambubioside, cyanidin-3,5-diglucoside standard compounds were purchased from Extrasynthese (Lyon, France). Water used for experiments was treated in a Milli-Q water purification system.

2.2. Extraction

The fruits of black elder and dwarf elder were harvested at the end of August, 2011 at their optimum fruit maturity (Hunedoara, Romania). Vouchers No. 530 and 531 were deposited in the Herbarium of the Department of Pharmaceutical Botany of the Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. For obtaining extracts 5g of fruits were extracted by grinding the sample at 20.000 rpm by ultraturax with acidified methanol (0.3% HCl, v/v). The extracts were collected after a centrifugation at 3500 rpm and the residues were re-extracted until the extraction solvents became colorless. The crude extracts were filtrated by multiple layers of cotton and then were concentrated by rotary evaporation at 35°C. Prior to HPLC analysis were redisolved in acidified methanol, centrifuged at 5000 rpm and filtered through 0.45 μm nylon filter.

2.3. LC-MS analysis

Samples were analysed on an Agilent Technologies 1100 HPLC system (Chelmsford, MA) equipped with G1322A degasser, G13311A binary gradient pump, G1313A autosampler and G1316A UV detector. Volumes of 5 μl were injected on a Zorbax SB C-18 column (3.5 μm, 100 x 3.0 mm) at temperature of 48°C. Data were collected at 330 nm until 17.5 min then at 370 nm. Mobile phase was consisted in methanol (solvent A) and acetic acid 0.1% (v/v) (solvent B). The solvent elution started with a linear gradient with 5% A and continued for 35 min up to 42% A. The flow rate was 1 ml/min min. The HPLC system was coupled with an Agilent mass spectrometer (LC/MSD Ion Trap VL). Identification of flavanols was performed via LC-MS in negative mode ionisation on an Agilent 1100 Technologies mass spectrometer equipped with an electrospray ionisation (ESI) interface. Nitrogen was used as desolvation and collision gas. Desolvation gas (nitrogen) was heated at 360 ºC and nebulizer pressure was set to 65 psi [11,12,13].

2.4. HPLC-PDA analysis

In order to obtain the anthocyanin-rich fraction the elderberry crude-extracts were semipurified following the procedure described previously by He et al. (2011) [14]. The aqueous extract (50 μL) was filtered through a C-18 Sep-Pak cartridge (sorbent mass = 50 mg) (Waters Corp., Milford, MA), previously activated with methanol followed by equilibration with 0.01% aqueous HCl. Anthocyanins and other polyphenolics were adsorbed onto the Sep-Pak column while sugars, acids, and other water-soluble compounds were removed by washing the minicolumn with 2 volumes of 0.01% aqueous HCl. The second fraction, containing polyphenols

(other than anthocyanins), was subsequently eluted with 2 volumes of ethyl acetate. Anthocyanins were eluted with 4 volumes of methanol containing 0.01% HCl. The acidified methanol fraction was concentrated by using a rotaevaporator at 35˚C until the methanol was completely removed. The anthocyanin-rich fraction obtained was then dissolved in acidified deionized and sterile water. For the acid hydrolysis of anthocyanin-rich fraction (2 ml) was mixed with 2M HCl (2 ml) in a screw-cap test tube. The mix was capped and hydrolyzed for 1 h at 90˚C, then cooled in an ice bath.

2.5. Radical scavenging activity (DPPH)

The DPPH scavenging activity assay was done according to a method reported by Brand-Williams et al. [15]. A volume of 1ml of freshly prepared DPPH solution (0.1 g/l) solution was allowed to react with 1ml sample. The ability of the polyphenolic compounds to act as free radical scavengers against DPPH radical was tested by measuring the disappearance of the absorbance after 30 min at 40 °C from the moment when antioxidant solution was applied. The antioxidant activity was calculated as follows:

$$\% \text{ DPPH scavenging activity} = (1-[A_{sample}/A_{control\ t=o}])\ 100$$

The absorbance was read at 517 nm, against a reagent blank (JASCO V-630, Japan). Standard curve was prepared using different concentrations of butylhydroxytoluene and the results were expressed as percent of control.

2.6. Statistical analysis

The data were expressed as mean ± standard deviation (SD) from three replicates for each sample. Significance of difference was defined at the 5% level ($p<0.05$).

3. Results and discussion

3.1. Polyphenol compounds composition

The profile of phytochemicals of the investigated elderberry cultivars by HPLC-MS analysis revealed the presence of four polyphenolic compounds in both *Sambucus sp.* fruit extracts (Fig. 1-2). One phenolic acid - p-coumaric acid, two flavonoid glycosides quercetin-3-*O*-glucoside (also known as isoquercetrin) and quercetin-3-*O*-rutinoside (also known as rutin) and the aglicon quercetin we identified in the black elderberry crude-extract. Quercetin-3-*O*-rutinoside was the major phenolic compound found in the fruits of *Sambucus nigra* (29.02 mg/100 g FW) and the results are in agreement with those obtained by other authors. Quercetin-3-*O*-glucoside (8.69 mg/100 g FW) was the second major polyphenol identified in our samples, according to data reported previously [16]. The aglicon quercetin in our study was approximately 1/50 of the amount of quercetin-3-*O*-rutinoside comparing to 1/10 of the amount reported by Veberic et al.,(2009) [2]. In the *Sambucus ebulus* berry extract hyperoside (1.60 mg/100 g FW) and quercetin-3-*O*-rutinoside (1.20 mg/100 g FW) were the major polyphenolic compounds identified. After the acidic hydrolysis of the extracts the identified compounds were kaempferol and quercetin (Table 1, Fig.1b, Fig.2b). Kaempferol was found in fewer amounts (0.23 mg/100 g FW) in *Sambucus nigra* crude-extract comparing to *Sambucus ebulus* (0.57 mg/100 g FW) sample.

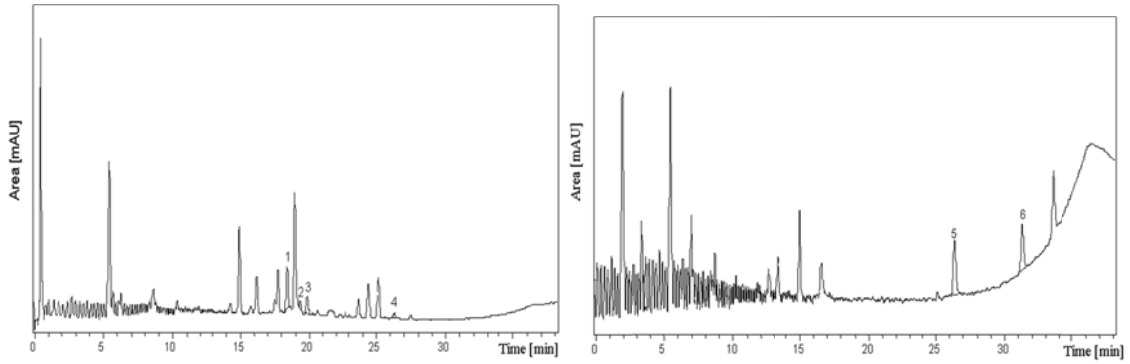

*Fig.1. Individual polyphenols of Sambucus ebulus crude-extract (a) and hydrolyzed extract (b). Peak identification: **1**- hyperoside; **2**- quercetin-3-O-glucoside, **3**- quercetin-3-O-rutinoside; **4**- quercetin; **5**-quercetin, **6**-kaempherol.*

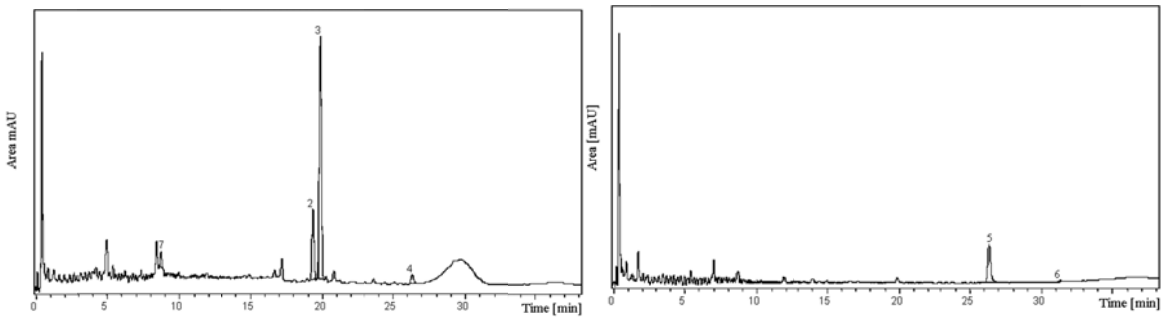

*Fig.2. Individual polyphenols of Sambucus nigra crude-extract (a) and hydrolyzed extract (b). Peak identification: **2**- quercetin-3-O-glucoside, **3**- quercetin-3-O-rutinoside; **4**- quercetin; **5**-quercetin, **6**-kaempherol, **7**- p-coumaric acid.*

3.2. Anthocyanins composition

The anthocyanins from *Sambucus sp.* berries have been separated and identified by HPLC-DAD analysis. Elderberries were found to contain five major anthocyanins (cyanidin-3-*O*-sambubioside-5-glucoside, cyanidin-3,5-*O*-diglucoside, cyanidin-3-*O*-sambubioside, cyanidin-3-*O*-glucoside) these consisted of three aglycons (cyanidin, pelargonidin) glycosylated with glucose and sambubioside. The anthocyanin quantification was performed using cyanidin 3-*O*-sambubioside pure standard. The data regarding anthocyanin identification and quantification are shown in Table 2 and Fig. 3, 4. Cyanidin-3-*O*-sambubioside and cyanidin-3-*O*-glucoside were the major anthocyanins identified based on their retention times, UV-VIS spectra comparing with standards and published data. Cyanidin 3-sambubioside and cyanidin-3- *O* -glucoside contents were higher in *Sambucus ebulus* (244.79 mg/100 g FW, respectively 84.53 mg /100 g FW) comparing to those detected in *Sambucus nigra* (134.94 mg/100 g FW, respectively 44.83 mg /100 g FW). The unidentified compound (peak 3) could be a cyanidin-3-*O* -rutinoside according to literature data and it UV maximum absorbance at 516 nm. Consistent with our results, other authors reported the major anthocyanins in *Sambucus nigra* L. as cyanidin-3-*O* -sambubioside and cyanidin-3-*O*-glucoside [2, 4]. Similar amounts for cyanidin-3-*O*-sambubioside from elderberries, varied between 270 and 630 mg/ 100 g FW were reported previously [2]. Regarding the total anthocyanin content (TAC) obtained this was higher for *Sambucus ebulus* (502.73 mg/100 g FW) then for *Sambucus nigra* (272.87 mg/100 g FW), data in agreement with the results obtained by others authors [2, 16]. The extracts were subjected to acid hydrolysis in order to release anthocyanidins from the glycosilated forms and then analyzed by HPLC-PDA analysis. The presence of cyanidin-3-*O*-glucoside in hydrolyzed extract may be explained by an incomplete hydrolization procedure. Anthocyanidins such as cyanidin and pelargonidin were found in *Sambucus nigra* and *Sambucus ebulus* extracts (Table 1). Pelargonidin-3-*O* -glucoside and

pelargonidin-3-*O*-sambubioside were detected for the first time in elderberry extracts by Wu et al., (2004) [4].

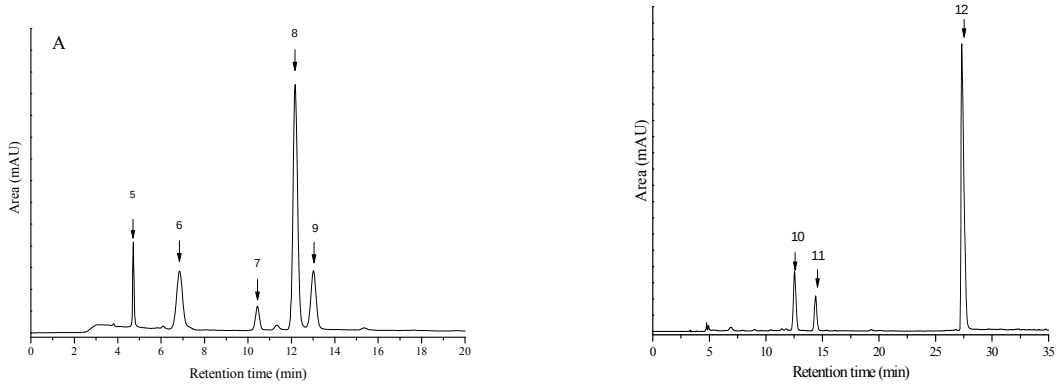

Fig.3. Individual anthocyanins and anthocyanidins of Sambucus ebulus anthocyanin rich-fraction (A) and hydrolyzed extract (B). Peak identification ***5****- Cyanidin-3-O-sambubioside-5-glucoside;* ***6****- Cyanidin-3,5-diglucoside,* ***7****- Unknown;* ***8****- Cyanidin-3-O-sambubioside;* ***9****- Cyanidin-3-O-glucoside,* ***10****- Cyanidin-3-O -glucoside;* ***11****- Pelargonidin;* ***12****- Cyanidin.*

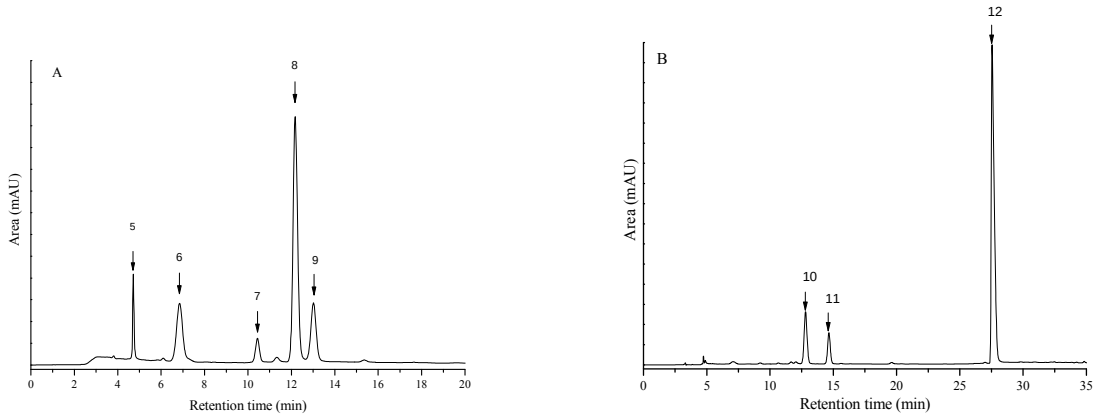

Fig.4. Individual anthocyanins and anthocyanidins of Sambucus nigra anthocyanin rich-fraction (A) and hydrolyzed extract (B) extracts. Peak identification ***5****- Cyanidin-3-O-sambubioside-5-glucoside;* ***6****- Cyanidin-3,5-diglucoside,* ***7****- Unknown;* ***8****- Cyanidin-3-O-sambubioside;* ***9****- Cyanidin-3-O-glucoside,* ***10****- Cyanidin-3-O-glucoside;* ***11****- Pelargonidin;* ***12****- Cyanidin.*

Table 1. The aglycons from the Sambucus sp. berries extracts after acid hydrolysis (mg /100 g FW)

Identified compound	λmax	R_t	Peak	*Sambucus nigra*	*Sambucus ebulus*
				(mg/100 g FW)	
Total flavonol aglycons				2.60 ± 0.16	1.06 ± 0.06
Quercetin	280, 365	23.0	5	2.37 ± 0.13	0.49 ± 0.08
Kaempferol	270, 370	31.5	6	0.23 ±0.18	0.57 ± 0.05
Total anthocyanindins				121.87 ± 2.05	175.03 ± 2.39
Cyanidin-3-*O*-glucoside	278, 516	12.7	10	19.56 ± 1.24	32.22 ± 2.21
Pelargonidin	274, 520	14.6	11	11.89 ± 1.52	19.72 ± 1.98
Cyanidin	275, 523	27.5	12	109.98 ± 3.41	155.31 ± 2.98

Table 2. Retention times and the individual polyphenols concentration in Sambucus nigra and Sambucus ebulus berry extracts

Compound	λmax (nm)	Peak	R_t	Concentration (mg/100 g FW)	
				Sambucus nigra	*Sambucus ebulus*
Phenolic acid				0.55 ± 0.01	-
p-coumaric acid	284	7	8.72	0.55 ± 0.01	-
Total flavonoids				38.26 ± 1.43	3.44 ± 0.045
Hyperoside	366	1	18.70	-	1.60 ± 0.04
Quercetin-3-*O*-glucoside	366	2	19.70	8.69 ± 1.23	0.44 ± 0.02
Quercetin-3-*O*-rutinoside	367	3	20.20	29.02 ± 2.1	1.20 ± 0.07
Quercetin	370	4	23.10	0.55 ± 0.98	0.20 ± 0.05
Total anthocyanins				272.87 ± 1.11	502.73 ± 1.83
Cyanidin-3-*O*-sambubioside-5-glucoside	275, 525	5	4.7	23.34 ± 0.68	42.92 ± 1.56
Cyanidin-3,5 -diglucoside	275, 516	6	6.8	47.1 ± 0.78	91.63 ± 3.21
Unknown	271, 518	7	10.4	22.66 ± 0.21	38.86 ± 0.87
Cyanidin-3-*O*-sambubioside	279, 518	8	12.2	134.94 ± 2.65	244.79 ± 3.25
Cyanidin-3-*O*-glucoside	275, 517	9	13	44.83 ± 1.23	84.53 ± 0.26

nd- not detected

3.3. Antioxidant activity evaluation by DPPH assay

Various phytochemical components, such as phenolic acids, flavonols and anthocyanins are known to be responsible for the antioxidant capacity of fruits. The antioxidant activity of elderberry crude extracts was evaluated by DPPH assay and the data obtained are presented as percentage values in Table 3. The method used is based on the reduction of the radical DPPH in the presence of hydrogen donating antioxidant. The strongest radical scavenging activity showed *Sambucus ebulus*, while the activity of *Sambucus nigra* berry crude extract was considerably lower. The butylated hydroxytoluene (BHT), a synthetic scavenger showed a reduced antioxidant activity than those exerted by polyphenolic compounds from *Sambucus ebulus* extract. The antioxidant activity evaluated by oxygen radical absorbance capacity was higher for elderberries than for gooseberries, black and red currants [4]. In agreement with literature data our results showed that the antioxidant activity of elderberries is positively correlated to their anthocyanin content. Similar results were obtained for chokeberries, blackberries, red raspberries, black raspberries and strawberries extracts [17-19].

Table 3. Radical scavenging activity determined by DPPH assay

Elderberry crude-extracts	DPPH radical scavenging activity (%)
Sambucus nigra	63.26 ± 1.05
Sambucus ebulus	83.17 ± 1.21
BHT	81.39 ± 0.85

4. Conclusions

Elderberries are predominately used as processed food or dietary supplements, therefore it is useful to know their content on phenolic acids, anthocyanins and flavonoids. *Sambucus nigra* and *Sambucus ebulus* both contained especially anthocyanins as cyanidin 3-*O*- sambubioside and cyanidin 3-glucoside. The species with the highest amounts of total analyzed anthocyanins and flavonoids was *Sambucus ebulus,* which also showed the higher potential to scavenge the radical DPPH.

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