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## ORIGINAL ARTICLE

# LC-ESI-MS/MS profiling of phenolics from *Eleutherococcus* spp. inflorescences, structure-activity relationship as antioxidants, inhibitors of hyaluronidase and acetylcholinesterase

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## KEYWORDS

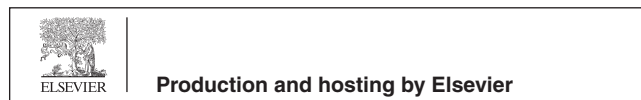
*Eleutherococcus* spp.;  
LC-ESI-MS/MS;  
Phenolic acids;  
Antioxidants;  
AChE;  
Hyal;  
SAR

**Abstract** Nature is a source of many plant-based molecules used as pro- or drugs. *Eleutherococcus* species are native to Asia and the North Russia, and are traditionally used to treat various diseases. In turn, neither secondary metabolites of the species cultivated in the West Europe nor the bioactivity is known. No differences in the phenols and flavonoids content in the inflorescences were found. The richest in polyphenols was *E. giraldii* (5.18 mg/g), while in flavonoids it was *E. gracilistylus* (1.80 mg/g). Using LC-ESI-MS/MS, protocatechuic and *trans*-caffeic acids have been identified as the most abundant compounds in *E. gracilistylus*, *E. giraldii*, *E. senticosus* (833.4; 855.6; 614.7 and 280.8; 156.0; 167.6 μg/g DE). It was observed that all species were able to chelate Fe<sup>2+</sup> with the EC<sub>50</sub> value of 0.2, 0.6, 0.3 mg/mL for *E. gracilistylus*, *E. giraldii*, *E. senticosus*, respectively. *E. gracilistylus* exhibited the strongest antiperoxidation and anti-DPPH<sup>\*</sup> activity (EC<sub>50</sub> 3.2 and 0.48 mg/mL). The weak inhibitory potential has been observed in case of AChE inhibition at the level of 16.17 and 12.2% for *E. gracilistylus*, *E. giraldii*. We report for the first time that the extracts inhibited Hyal activity in the range from 16.4 to 60.7%. To our best knowledge, no information was

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available on this activity of the inflorescences and this provides a background to study inflorescences in more detail. Considering the SAR, an antioxidant activity may be correlated with a high amount of protocatechuic and *trans*-caffeic acids and their chemical structure.

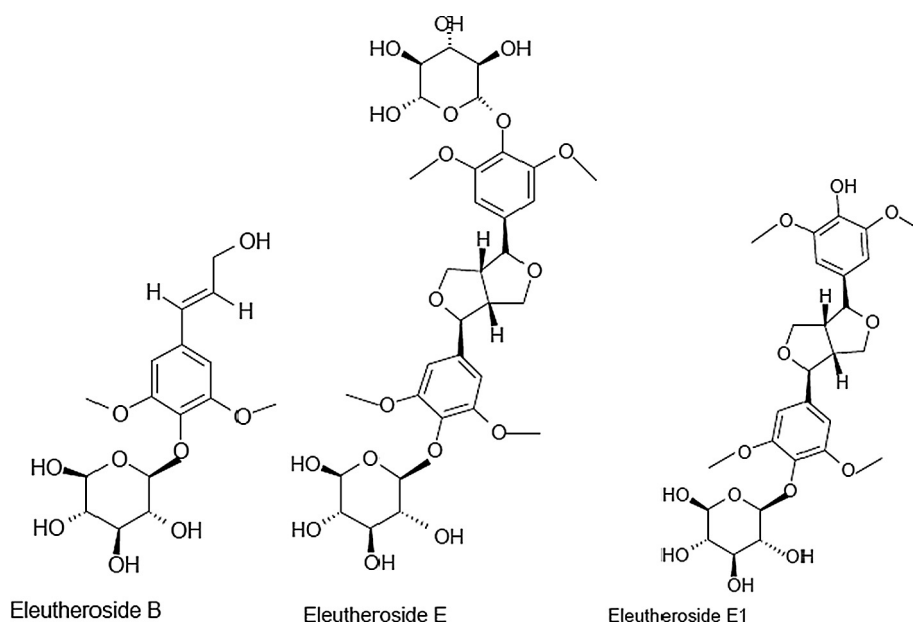
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## 1. Introduction

Plants are a major source of phytochemicals for drug discovery and for laboratory synthesis of drugs. About 80% of the world population is using medicinal plants as their major source for medication in primary health care, and about 120 plant derived compounds are used in western medicine. Phytochemicals can be used as small-molecule drug precursors, which can be converted into drugs by chemical modification, ex. 10-deacetylbaicatin, isolated from *Taxus baccata*, is used in the semisynthetic method to produce paclitaxel. Many synthetic analogues have been made such as analgesics based on morphine, and local anesthetics based on cocaine. The pharmaceutical industry is searching for new, renewable sources of drugs, very often plant-based drugs, because the use of herbs or a combination of herbs and synthetic drugs can reduce toxicities and maximize therapeutic outcomes (Verpoorte, 2000; Salim et al., 2008; Dhanani et al., in press). A promising source of plant-based biologically active compounds is the *Eleutherococcus* Maxim. genus, found in eastern Asia and far western Russia. The major secondary metabolites present in *Eleutherococcus* are phenols, such as eleutherosides (derivatives of lignans, coumarins, phenylpropanoids), flavonoids, phenolic acids, and anthocyanins (Fig. 1). The *E. senticosus* products attract global attention as a novel medicinal plant and since a few years, have become popular as dietary supplement in the United States and European countries. Imported products of this plant have become available in North America, with a market share of 3.1% of the \$12 billion medicinal herbal industry (Załuski et al., 2010; Watson, 2003). The

1994 DSHEA (Dietary Supplement Health and Education Act) regulation allows a direct commercialization of *E. senticosus* as a supplement for consumption in the United States without the regulation of the FDA (Food and Drug Administration) (Arouca and Grassi-Kassisse, 2013). Preparations of the roots of *E. senticosus* are given in cases of asthenia with weakness and fatigue, e.g., in convalescence. This indication has been officially accepted by the Community Herbal Monograph on *Eleutherococcus senticosus* (Rupr et Maxim) Maxim Radix (EMA/HMPC/244569/2006), published by the European Medicines Agency. The fruits have been used for a long time as an ingredient of the fermented wine, the leaves as a tonic, as a functional beverage commercially marketed for reducing liver damage and accelerating alcohol detoxification.

The *E. senticosus* products, which are available in the herbal drugs market, are imported from China. Because of a lack of the assessment of plant material there have been many cases of the poor quality of plants supplied by Chinese traders, leading to financial losses for some pharmaceutical companies. This is particularly important in the pharmaceutical drug development process and to avoid that, the establishment of the new source of important medicinal plants in Europe is required. Moreover, new approaches of extraction and analytical tools are also needed. Keeping in mind their long-term use by the Asian, we have decided to evaluate the quality of some *Eleutherococcus* species cultivated in Polish climate conditions as a raw herbal material. To our knowledge, there are no such phytochemical reports concerning Polish cultivars. As part of a program to search for bioactive constituents from *Eleuthero-*



**Figure 1** Chemical structure of eleutheroside B, E and E1.

*coccus* species, this study was focused on the establishment of TPC, TFC, phenolic acids, antioxidant and anti-AChE, and anti-Hyal activities. The aim of this work was also to discuss the antioxidant and anti-enzymatic mechanism, as well as to search for the structure-activity relationship.

## 2. Materials and methods

### 2.1. Reagents

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), linoleic acid, EDTA, BHA, ascorbic acid,  $\alpha$ -tocopherol, DMSO, physostigmine, hyaluronidase from bovine testes type I-S, *Streptococcus equi* hyaluronic acid, DTNB (5,5'-Dithiobis(2-nitrobenzoic acid), ACTI (acetylthiocholine iodide), sodium phosphate buffer pH 7.0 were obtained from Sigma-Aldrich. The standards of phenolic acids were obtained from ChromaDex (Santa Ana, CA). LC grade methanol (MeOH) was purchased from J.T. Baker (Phillipsburg, USA). Ultrapure water was prepared using a Millipore Direct-Q3 purification system (Bedford, MA, USA). All others reagents were of analytical grade.

### 2.2. Plant material

The inflorescences of *E. senticosus* (Rupr. & Maxim.) Maxim., *E. giraldii* (Harms) Nakai, *E. gracilistylus* (W.W Smith) S.Y. Hu were collected at the arboretum in Rogów (Poland) in June 2015. All plant samples were deposited at the Department of Pharmacognosy, Collegium Medicum, Bydgoszcz, Poland, Cat. Nr. ES 01.; ES 02.; ES 03. The inflorescences were air-dried and stored in a dark place, at room temperature before an extraction.

### 2.3. Dried material extraction with 75% ethanol

The air-dried and powdered inflorescences (15 g each) were soaked in 150 mL 75% ethanol for 24 h. Next, the samples were subjected to triple UAE type extraction (ultrasonic bath -Polsonic, Warsaw, Poland) using 150, 2  $\times$  100 mL of 75% ethanol. The extraction was performed at room temperature for 15 min for each cycle. Finally, 350 mL of each extract was obtained. The solvents were dried with an evaporator under vacuum conditions at 45 °C and subjected to lyophilization. The extraction yield was calculated based on the dry weight of the extract [%].

### 2.4. Total phenolic content (TPC)

The total phenolic content of extracts was determined using the method of Singleton and Rossi (Singleton and Rossi, 1965). TPC was expressed as gallic acid equivalents (20–100  $\mu$ g/mL;  $y = 0.0026x + 0.044$ ;  $r^2 = 0.999$ ; g GAE/100 g dry extract). Every assay was done in triplicate.

### 2.5. Total flavonoid content (TFC)

The TFC was determined using aluminum chloride and 2,4-dinitrophenylhydrazine colorimetric methods (Chang et al., 2002). TFC was expressed as means ( $\pm$ S.E.) g of quercetin

equivalent (QEs/100 g dry extract for FeCl<sub>3</sub> method, 20–100  $\mu$ g/mL;  $y = 0.0041x + 0.236$ ;  $r^2 = 0.999$ ) and as means ( $\pm$ S.E.) g of hesperetin equivalent (HEs/100 g dry sample for 2,4-DNPH method, 250–1000  $\mu$ g/mL;  $y = 6.374x - 0.098$ ;  $r^2 = 0.988$ ). Every assay was done in triplicate.

### 2.6. LC-ESI-MS/MS conditions of analysis of phenolic acids

The samples were analyzed according to modified method of Nowacka et al. (2014). For this purpose an Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a binary gradient solvent pump, a degasser, an autosampler and column oven connected to a 3200 QTRAP Mass spectrometer (AB Sciex, USA) equipped with an electrospray ionization source (ESI) and a triple quadrupole-ion trap mass analyzer was used. The separation of the analytes was carried out on a Zorbax SB-C18 column (2.1  $\times$  50 mm, 1.8- $\mu$ m particle size; Agilent Technologies, USA) maintained at 25 °C, using 3  $\mu$ l injections. The solvents used were as follows: water containing 0.1% HCOOH (solvent A) and methanol containing 0.1% HCOOH (solvent B). The following gradient elution program at a flow rate of 370  $\mu$ L min<sup>-1</sup> was applied: 0–1 min – 5% B; 2–4 min – 20% B; 8–9.5 min – 70 % B; 11.5–15.5 min – 5% B. Mass spectrometer was controlled by the Analyst 1.5 software. ESI worked in the negative-ion mode with the curtain, nebulizer and turbo-gas (all nitrogen) set at 30, 60 and 60 psi, respectively. The ion spray needle voltage was –4500 V and capillary temperature 400 °C. For each compound the optimum conditions of Multiple Reaction Mode (MRM) were determined in the direct infusion mode. Triplicate injections were made for each standard solution and sample. The analytes were identified by comparing retention time and  $m/z$  values obtained by MS and MS<sup>2</sup> with the mass spectra from corresponding standards tested under the same conditions. The calibration curves obtained in MRM mode were used for quantification of all analytes. The identified phenolic acids were quantified on the basis of their peak areas and comparison with a calibration curve obtained with the corresponding standards. Linearity ranges for calibration curves were specified.

The limits of detection (LOD) and quantification (LOQ) for phenolic compounds were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

### 2.7. Antioxidant activity

The antioxidant activities were evaluated using three methods: DPPH radical scavenging assay, determination of inhibition of linoleic acid autooxidation and metal chelating activity. We used the ethanol extracts at the following concentrations of 0.1, 0.5, 1.0, and 2.0 mg/mL. As a standard ascorbic acid, tocopherol, BHA, and EDTA were used. Absorbance was measured on a multidetection BIOTEK spectrophotometer; next, EC<sub>50</sub> value was assayed. Every assay was done in triplicate.

#### 2.7.1. DPPH assay

The anti-radical activity of the extracts was determined by the method of Brand-Williams et al. (1995).

### 2.7.2. Inhibition of linoleic acid peroxidation

The lipid anti-peroxidation activity was determined according to Kuo et al. (1999).

### 2.7.3. Metal chelating activity

The ability to metal ions chelating was determined by the method of Guo et al. (2001).

### 2.7.4. TLC-DPPH dot-blot test

The TLC-DPPH dot-blot test was used, 1  $\mu$ L of extracts (10  $\mu$ g/ $\mu$ L) was applied on silica gel plates, and the plate was immersed for 5 s in freshly prepared 0.2 mmol methanolic DPPH\* solution. After removing DPPH\* excess, the decolorization of DPPH\* was observed after 1, 5, 10, and 30 min.

### 2.8. Anti-enzymatic studies: anti-hyaluronidase and anti-acetylcholinesterase activities

The ability of the extracts to inhibit Hyal was determined by the spectrophotometric method of Yus et al. (Yus and Mashitah, 2012). The extract concentration was 1.0 mg/mL in 10% water ethanol solution. The final concentration in the reaction's mixture was 22  $\mu$ g/0.161 mL. Aescin was used as the positive control at the following concentrations: 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 mg/0.161 mL. The ability of the extracts to inhibit AChE was determined by the spectrophotometric method of Ellman et al. (1961). The extract concentration was 1.0 mg/mL in 10% water ethanol solution. The final concentration in the reaction's mixture was 22  $\mu$ g/0.2 mL. Physostigmine was used as the positive control at the following concentrations: 2, 3, 4, 15, 30, and 40  $\mu$ g/0.195 mL. Every assay was done in triplicate.

### 2.9. Statistical analysis

Determinations were performed by triplicate. The obtained data were subjected to statistical analysis using Statistica 7.0. (StatSoft, Cracow). The evaluations were analyzed for one-factor variance analysis. Statistical differences between the treatment groups were estimated by Spearman's (*R*) and Pearson's (*r*) test. All statistical tests were carried out at significance level of  $\alpha = 0.05$ .

## 3. Result and discussion

### 3.1. UAE type extraction yield

Investigation of the extraction yield for the *Eleutherococcus* inflorescences has not been performed earlier. An analysis of

the extraction yield is important in terms of the quality of medicinal plants and their usage in the treatment as well as obtaining the substrates for drug synthesis. Therefore, it is necessary to determine the yield of extraction with no changes in the structure of extracted compounds. In present study, the green extraction (i.e. UAE) has been used; as it is considered to be environmentally friendly with additional benefits of low energy and solvent consumption, furthermore the time of extraction is also reduced. The results are presented in Table 1. The highest yield was obtained for *E. gracilistylus* (26.60%), and next for *E. senticosus* (20%) and *E. giraldii* (15.24%). Comparing the results of freshly dried fruits of *E. senticosus* and *E. henryi* (44.02 and 27.40%, respectively) it is seen that the fruits contain more soluble compounds in 75% ethanol (Załuski et al., 2015). It may result from the presence of polysaccharides, which are also soluble in the aqueous alcohols, influencing on the yield.

### 3.2. TPC and TFC in the inflorescences

TPC was estimated using the Folin-Ciocalteu method and expressed as gallic acid equivalent (g/100 g dry extract). In order to evaluate TFC, two methods were used. One of them is a spectrophotometric assay, based on the formation of a complex between aluminum ion and the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Besides, aluminum chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. The second procedure is also a spectrophotometric assay, based on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with flavanones, forming 2,4-dinitrophenylhydrazones. The results obtained with the use of these two methods were added up to evaluate the total content of flavonoids. As it is shown in Table 1, there are no differences in polyphenols and flavonoids content. The results varied from 4.15 to 5.18 g/100 g. The highest content was recorded in the extract from *E. giraldii*, whereas the lowest one in the extract from *E. gracilistylus*. The summed amount of flavonoids was between 1.34 and 1.80 g/100 g. We found that flavonones/dihydroflavonol content obtained by DNPH reaction was similar to that obtained by FeCl<sub>3</sub> reaction. The contents of phenolic compounds were within the range of those previously reported for the various raw materials of *Eleutherococcus* spp., cultivated in Poland. The TPC content found in the 75% ethanolic extracts from the spring leaves ranged from 20.3 to 37.2 mg/g, followed by the fresh fruits (6.1–19.7 mg/g) and the roots (6.9–10.6 mg/g) (Załuski et al., 2012). Other studies revealed that the content of TPC and TFC in the fruits is not changed during storage and quantified between 4.11 and 4.35 g/100 g for the freshly dried fruits from *E. senticosus* and *E. henryi*. After 1-year stor-

**Table 1** Yield, TPC and TFC in extracts from the inflorescences of *E. gracilistylus*, *E. giraldii* and *E. senticosus* (g GAE/100 g and QEs/100 g dry extract).<sup>a</sup>

Sample	Yield (%)	TPC	Flavonoid content		TFC
			FeCl <sub>3</sub>	DNPH	
<i>E. gracilistylus</i>	26.64	4.15 ± 1.1	9.23 ± 0.3	7.67 ± 0.9	1.80 ± 0.5
<i>E. giraldii</i>	15.24	5.18 ± 0.5	5.9 ± 0.1	5.74 ± 0.04	1.34 ± 0.02
<i>E. senticosus</i>	20	4.84 ± 0.7	7.52 ± 0.1	5.87 ± 0.5	1.44 ± 0.5

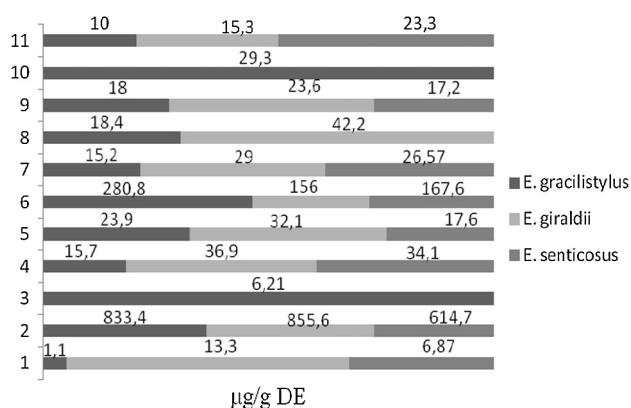
<sup>a</sup> Results are means ± standard deviation of triplicates.

age the amount did not change significantly, and was between 3.85 and 4.13 for the freshly dried fruits from *E. senticosus* and *E. henryi* (Załuski and Janeczko, 2015). Heo et al. (2011) studied the ethanol, methanol and water extract of the *E. senticosus* fruits growing in Korea, but reported a lower concentration of phenols than that was now estimated (0.3, 0.6 and 0.6%, respectively). It was found that the ethanol, methanol and water extract of *E. senticosus* fruits growing in Korea possess the following content of flavonoids: 0.20, 0.23 and 0.3% (Heo et al., 2011). These results reveal that the inflorescences may become a new source of phenolics and flavonoids which can be the chemopreventive compounds of human diet.

### 3.3. LC-ESI-MS/MS phenolic acids profile

Among seventeen phenolic acids (gallic, protocatechuic, gentisic, 4-OH-benzoic, 3-OH-benzoic, vanilic, *trans*-caffeic, *cis*-caffeic, syringic, *trans-p*-coumaric, *cis-p*-coumaric, *trans*-ferulic, veratric, salicylic, 3-OH-cinnamic, *trans*-sinapic, *cis*-sinapic) just eleven were qualitatively and quantitatively determined in the inflorescences. The concentrations of individual compounds, which were quantified by comparison of peak areas with the calibration curves obtained for the corresponding standards, are reported in Fig. 2. Results of the optimization of conditions of LC-ESI-MS/MS analysis and an example chromatogram of analyzed samples are given in Fig. 3, Figs. S1 and S2, Tables S1, S2. Protocatechuic and *trans*-caffeic acids occur in the highest amount (614.7–855.6; 156.0–280.8 µg/g DE). We did not detect 3-OH-benzoic, *cis*-caffeic, syringic, *cis*-ferulic, 3-OH-cinnamic, *trans*-sinapic, and *cis*-sinapic.

Phenolic acids have not yet been studied in the inflorescences of species native to Asia and Russia. Only a few studies have focused on the assessment of phenolic acids present in roots, leaves and fruits. Data in the literature indicated that Kurkin et al. (1991) identified free phenolic acids (syringic, *p*-coumaric, vanillic, *p*-hydroxybenzoic, caffeic and ferulic acids) and depside (chlorogenic acid) in the roots of *E. senticosus* growing in Russia. In turn, Li et al. (2006) identified protocatechuic, chlorogenic and caffeic acids in the roots of Chinese sample. Bączek identified rosmarinic, chlorogenic, fer-



**Figure 2** Concentrations of phenolic acids in the inflorescences (µg/1 g DE). 1. Gallic acid, 2. Protocatechuic acid, 3. Gentisic acid, 4. 4-OH-benzoic acid, 5. Vanilic acid, 6. *trans*-caffeic acid, 7. *trans-p*-coumaric acid, 8. *cis-p*-coumaric acid, 9. *trans*-ferulic acid, 10. Veratric acid, 11. Salicylic acid.

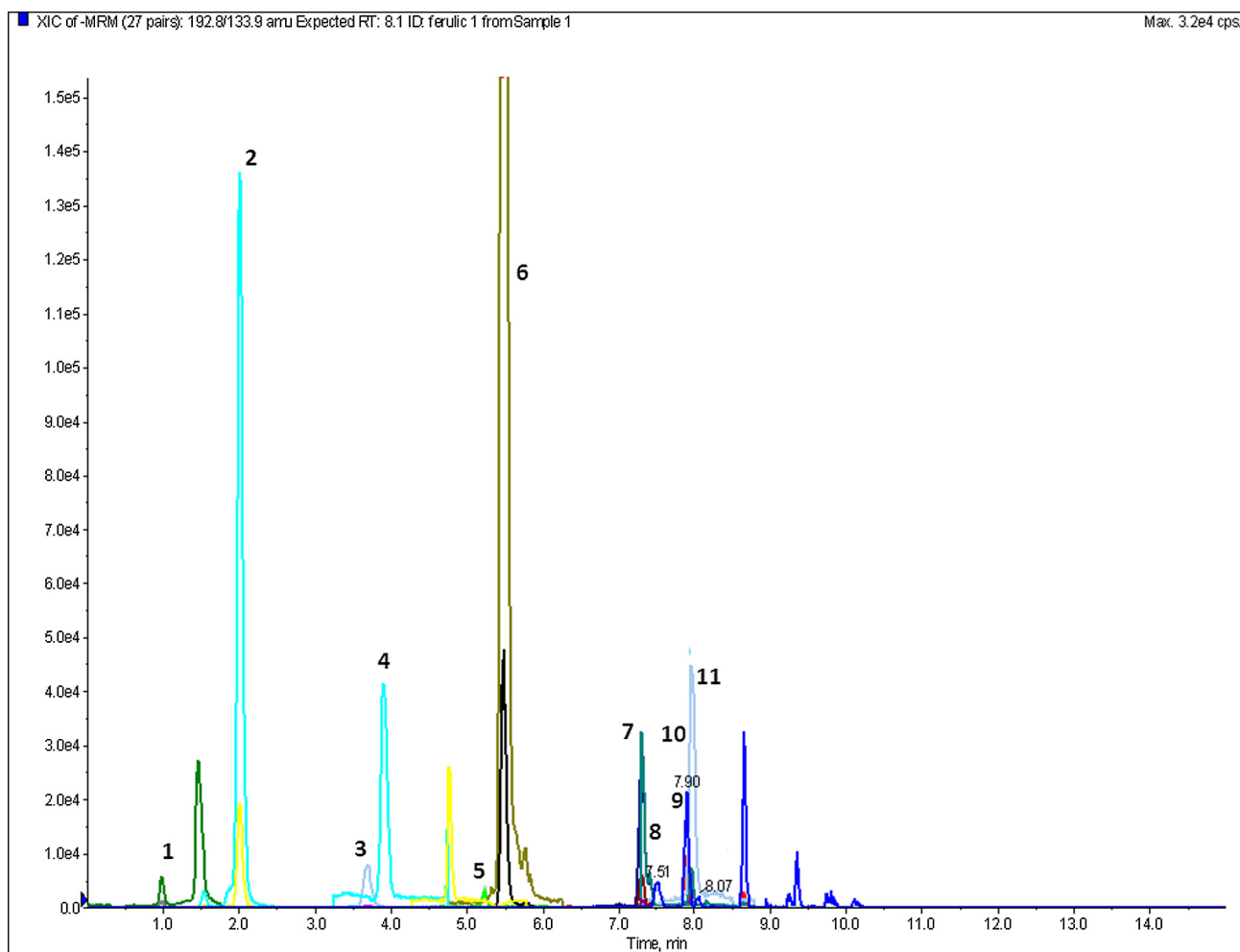
ulic and caffeic acids in the roots, fruits and stem barks of six species (Bączek, 2012, 2009). It is worth noting that *E. gracilistylus* contains gentisic and veratric acids, the compounds whose occurrence in nature is limited. Protocatechuic acid has been identified in *Hibiscus sabdariffa* L. (2.8 and 11.9 mg/g aqueous and ethanol extracts from roselle calyx) and *Euterpe oleracea* Mart. (630 mg/L of oil) (Chao and Yin, 2008; Pacheco-Palencia et al., 2008). In turn, a content of protocatechuic acid in *Allium cepa* L. depended on a type of raw material. The highest content was determined in a dried material (76.3 µg/g) contrary to a fresh material (5.8 µg/g) (Drozd et al., 2011). Comparing the results obtained in this work with the cited ones, it is concluded that *Eleutherococcus* spp. contain more protocatechuic acid, in some cases, a few fold more than those. This acid also occurs in various fruits such as berries (raspberry, blueberry, mulberry, cranberry, and gooseberry), wine, honey, and soybean. Protocatechuic acid has been found to have various activities such as antibacterial, antioxidant, antidiabetic, antiulcer, and anti-inflammatory, cardiac, and for this reason, it would be valuable to use the inflorescences as a dietary ingredient.

### 3.4. Antioxidant activity and the structure-activity relationship

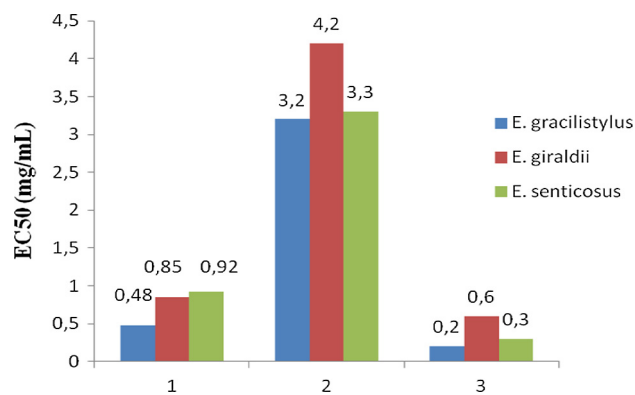
Free radicals are constantly generating in human body, which causes damages at the biochemical and molecular levels. To avoid that, antioxidants from plant sources are provided to our body in order to protect it. Antioxidant capacities of the extracts were expressed in terms of EC<sub>50</sub> value. It was found that all extracts were able to effectively reduce free radicals, and among the three species tested, the extract from *E. gracilistylus* showed the strongest scavenging effect compared with the others (Fig. 4). The EC<sub>50</sub> value in the DPPH test was 0.48 mg/mL: ascorbic acid and α-tocopherol 40 and 5.0 µg/mL. Recently in our previous study we reported that the leaves and fruits of *Eleutherococcus* species have a high antioxidant capability.

Free radical scavenging activity has been confirmed by means of TLC-DPPH\* dot-blot test using silica gel as the stationary phase (Fig. 5). Regions of the TLC plate which contain DPPH\* inhibitors show up as yellow spots against a purple background. We observed the plates after 1, 5, 10, and 30 min from the time of immersion of the plate in 0.2 mmol DPPH\* solution. After 1 min, the extracts showed areas of activity at concentration 10 µg/spot. It is noteworthy that in the spectrophotometric assay, all extracts showed strong anti-radical properties, and this was confirmed in the autography test. It suggests, that the extracts contain compounds which are able to donate hydrogen or electron and may be considered as antioxidants.

On the basis of the obtained results, a relationship between the structure of protocatechuic and *trans*-caffeic acids, as predominant compounds in the extracts, and their antioxidative activity were established (SAR). Some authors have reported only a correlation between DPPH\* radical scavenging activity and total phenols in the *Eleutherococcus* species, while the antioxidative mechanism of action of their individual compounds remains undescribed. Studies about the antioxidant potential of phenolic compounds in plant material have stated that it is impossible to predict the antioxidant power of a given product by studying just one type of phenolic compounds,



**Figure 3** An exemplary chromatogram of phenolic acids in the inflorescences ( $\mu\text{g}/1 \text{ g DE}$ ). 1. Gallic acid, 2. Protocatechuic acid, 3. Gentisic acid, 4. 4-OH-benzoic acid, 5. Vanilic acid, 6. *trans*-caffeic acid, 7. *trans-p*-coumaric acid, 8. *cis-p*-coumaric acid, 9. *trans*-ferulic acid, 10. Veratric acid, 11. Salicylic acid.



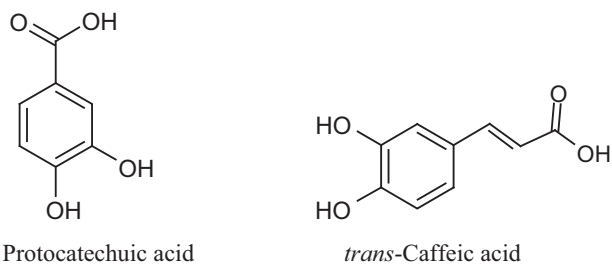
**Figure 4** Biological activity of extracts (1-DPPH radical scavenging activity; 2-Ability to lipid antiperoxidation; 3-The iron (II) chelating activity).

because there is the possible existence of synergistic or antagonistic effects between the various antioxidants present in plants. The antioxidant effectiveness of extracts is attributed to the active compounds present in them, such as phenolic acids. It was determined that the predominant compounds

are protocatechuic and *trans*-caffeic acids, which are known as natural antioxidants, occurring in plant food. Despite the fact that many studies on the radical scavenging activity of protocatechuic and *trans*-caffeic acids toward DPPH\* were reported, some biological actions of phenolic antioxidants are still poorly clarified. Some studies have confirmed that the radical scavenging activity is influenced by the number of hydroxyl groups on the aromatic ring; however, it is not a general rule. It should be mentioned that the antiradical activity may be changed by other compounds present in extracts, such as non-phenolic ones, or complexes/conjugates (Załuski et al., 2015). It is stated that the higher the number of hydroxyl groups, the higher the radical scavenging activity. Taking a closer look at the structure of these acids, it is seen that they possess the two free OH groups, which can enhance the free radical scavenging activity of phenolic acids (Fig. 6). In addition to this, the hydroxyl groups in both acids are in *para* position with respect to the COOH group. These group can be a donor of hydrogen reducing the DPPH\* radical. According to Li et al., the possible mechanism of the DPPH\* reduction may be correlated with the available hydroxyl groups in protocatechuic and *trans*-caffeic acids (Figs. 7 and 8) (Li et al., 2011, 2012).



**Figure 5** A “dot-blot” TLC-DPPH\* test performed on silica gel plate, 1 min after immersion in DPPH\* methanolic solution.



**Figure 6** Structure of protocatechuic and *trans*-caffeic acids.

#### 3.4.1. Metal ions chelating activity

The results obtained in this test indicated that extracts can bind  $\text{Fe}^{2+}$  with  $\text{EC}_{50}$  values from 0.20 to 0.60 mg/mL, which are in agreement with results for other *Eleutherococcus* species (Zaluski et al., 2012). The  $\text{EC}_{50}$  value for EDTA was 10  $\mu\text{g/mL}$ . According to Li et al. the mechanism by which, the main phenolic acids present in the extracts, can act as chelators is related to the structure of them (Figs. 9 and 10). Compounds containing two or more of the  $-\text{OH}$ ,  $-\text{SH}$ ,  $-\text{COOH}$ ,  $-\text{S}-$ ,  $-\text{O}-$ ,  $\text{C}=\text{O}$ ,  $-\text{NR}_2$ ,  $-\text{PO}_3\text{H}_2$  have chelating activity. Some authors suggest that PCA is a better antioxidant than the positive control Trolox ( $\text{EC}_{50}$  566 and 1520  $\mu\text{g/mL}$ ) (Li et al., 2011).

#### 3.4.2. Anti-lipid peroxidation activity

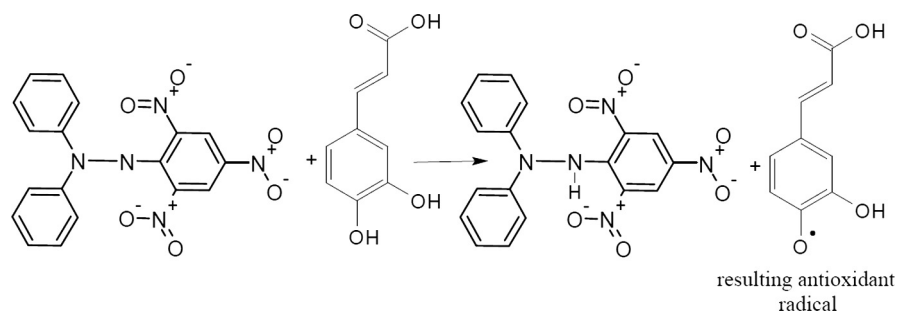
Lipids are important constituents of cell membranes and are very often damaged by ROS, generating lipid peroxidation. That results in the cell structure destabilization. The extracts exhibited a level of antiperoxidation between 3.2 and 4.2 mg/mL, BHA 100  $\mu\text{g/mL}$ . The obtained results are similar to those for the roots, leaves and fruits of *Eleutherococcus* species (Zaluski et al., 2012). The polarity of phenolic components is a key factor that confers their solubility and

ability to access lipid phase and breaking chain reactions. In natural systems both lipophilic and hydrophilic antioxidants are needed. The hydrophilic antioxidants are localized in the aqueous compartments of cells. It was confirmed that the rate of scavenging of radicals within the membrane becomes slower as the radical goes deeper into the interior of the membranes from the surface (Niki et al., 2005).

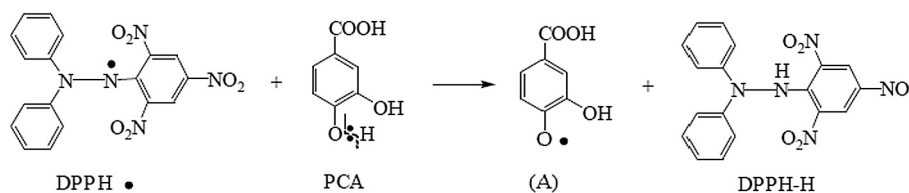
#### 3.5. Anti-Hyal and anti-AChE activity

Overactivity of Hyals has an influence on a faster degradation of ECM by hydrolyzation of the  $\beta 1 \rightarrow 4$  glycosidic bond of hyaluronic acid (HA). In turn, overactivity of AChE influences on a degradation of acetylcholine changes the neurotransmission. Therefore, in current phytochemical studies the main attention is paid to searching for plants Hyal and AChE inhibitors. As far, the inhibitors of AChE have been identified in alkaloids, e.g. physostigmine or galantamine. Identification and characterization of new inhibitors would be valuable for developing new, safer drugs (Zaluski et al., 2015). The results obtained in this work, presented in the Table 2, indicate that all extracts act as inhibitors of Hyal in the range of 16.6 and 60.7 %, and inhibitors of AChE (2.9–16.7 %). The mechanism by which the extract compounds may act as Hyal inhibitors can be based on the chelation of  $\text{Ca}^{2+}$  which plays as the activating ions in the reaction mixture. In order to compare the anti-enzymatic activity of the extracts analysed, physostigmine and aescin were used as the standard compounds because of their well recognized activity (Fig. 11). Physostigmine inhibited AChE in the dose-dependent way and 100% of inhibition was observed at concentration 30  $\mu\text{g}/195 \mu\text{L}$  of the reaction mixture. In turn, aescin inhibited Hyal at the level of 100% at concentration 800  $\mu\text{g}/161 \mu\text{L}$  of the reaction mixture.

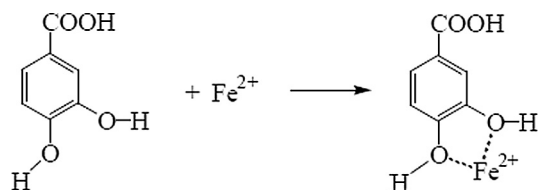
Inhibitors of Hyal have been mainly found in flavonoids and triterpenes. Among flavonoids 7-O-butyl naringenin had



**Figure 7** The proposed reaction mechanism between DPPH\* and caffeic acid (Li et al., 2012).



**Figure 8** The proposed reaction mechanism between DPPH<sup>•</sup> and protocatechuic acid (Li et al., 2011).



**Figure 9** The proposed reaction of protocatechuic acid binding for Fe<sup>2+</sup> (Li et al., 2011).

a high value with 44.84% inhibition at 200  $\mu$ M concentration. Taking into account the chemical structure of flavonoids, their inhibitory activity toward hyaluronidase increases with the number of hydroxyl groups, especially in 2,3' position (quercetin) and 5' (myricetin). Some authors state, that the inhibition is dependent on the number of free, available hydroxyl groups and extension of side-chains. It was noticed that the inhibitory activity was decreased after glycosylation or substitution of hydroxyl groups. In turn, another compound, chlorogenic acid inhibited Hyal with IC<sub>50</sub> 2.25 mM. A promising source of Hyal inhibitors may be some compounds present in essential oil, such as *B*-caryophellene (IC<sub>50</sub> 4.16  $\mu$ g/mL) and 1.8-cineole (IC<sub>50</sub> 1.17 mg/mL). The mechanism of action may be related to the formation of complexes of polyphenols present in the extracts with ions present in the reaction medium (Załuski et al., 2015; Moon et al., 2009).

Inhibitors of AChE have been mainly found in alkaloids which are most widely used in medicine. Most known are galanthamine (IC<sub>50</sub> 3.2  $\mu$ M), voacangine (IC<sub>50</sub> 4.4  $\mu$ M), coronaridine (IC<sub>50</sub> 8.6  $\mu$ M), and piperidinium (IC<sub>50</sub> 0.42  $\mu$ M). Other inhibitors have been found in the terpenic groups, of which most active appeared (+)-2-carene and (+)- $\alpha$ -pinene (IC<sub>50</sub> 0.2 and 0.4  $\mu$ M). The inhibition of AChE of these compounds is dependent on their structure and influenced by hydrocarbon chain, the presence of allylic methyl, isopropenyl groups, and the position of C-C double bonds (Załuski et al., 2015).

### 3.6. Correlation between phenolic compounds and biological activity

The correlation coefficients (*R* values) in Table 3 indicated that, TPC and protocatechuic acid were of significant positive

**Table 2** Anti-hyaluronidase and anti-acetylcholinesterase activities of extracts.

Sample	Anti-Hyal <sup>a</sup> (% inhibition)	Anti-AChE <sup>a</sup>
<i>E. gracilistylus</i>	16.4 $\pm$ 0.05	16.7 $\pm$ 0.01
<i>E. giraldii</i>	60.7 $\pm$ 0.01	12.2 $\pm$ 0.01
<i>E. senticosus</i>	57.5 $\pm$ 0.05	2.9 $\pm$ 0.02

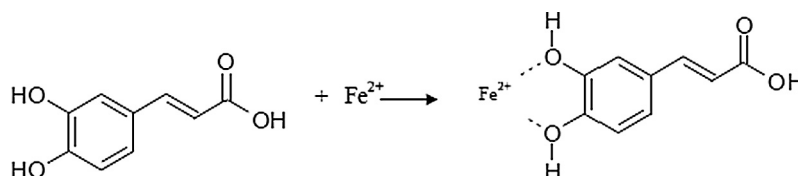
<sup>a</sup> Results are means  $\pm$  standard deviation of triplicates.

correlations (*R* 0.61–0.71; 0.63–0.72) with antioxidant levels. Just TPC, among four analyzed chemical parameters, could influence the anti-Hyal and a high correlation was observed (*R* 0.96). No correlation between *trans*-caffeic acid and antiradical and anti-Hyal activities was found. A significant correlation was observed between TFC, protocatechuic acid, *trans*-caffeic acid and anti-AChE activity.

Despite the fact, that many studies have revealed a high correlation between TFC and antiradical activity, in our studies, in some cases, such correlations were surprisingly low. It was expected for flavonoid to be more active as antioxidants; however, no correlation was found. According to Zieliński et al., the chemical interactions (synergism, antagonism, and additional effects) among various phenolic compounds may take place in extracts, and affect the correlation (Zieliński et al., 2014). The latest studies show that, non-phenolic compounds may also significantly influence the antiradical activity of extracts.

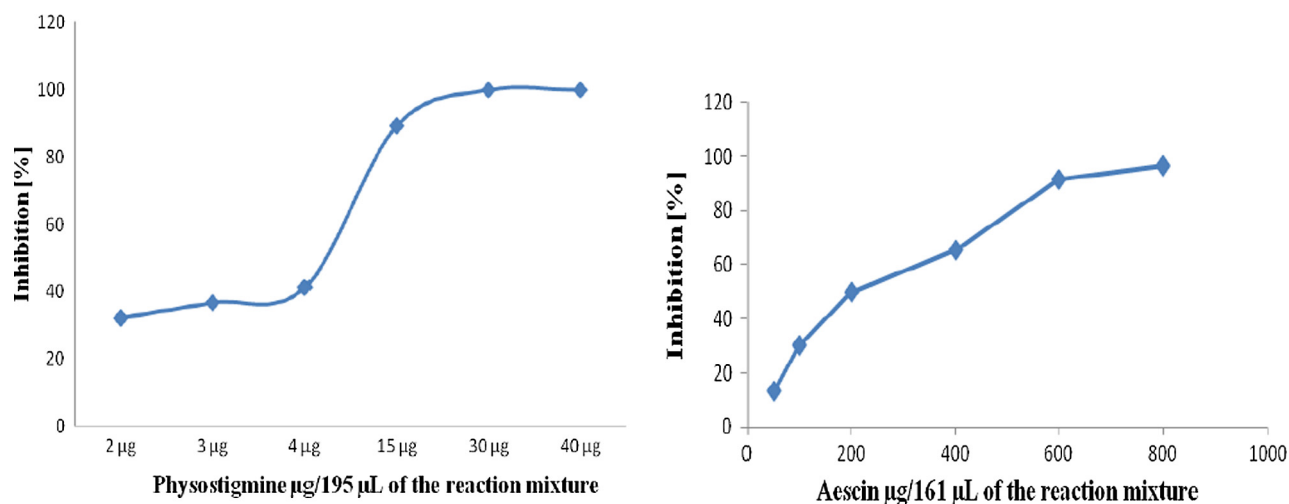
## 4. Conclusions

A high antioxidative capability of extracts may result from the presence of protocatechuic and *trans*-caffeic acids as the predominant compounds. Their radical scavenging activities may have potential use for promoting human health, especially in the developing countries. Thus, the raw material can be used as a commercially available ingredient of beverages, tea products and dietary supplements. It is important that a change of geographical zone has not had an influence on the phytochemical and biological profile of species.



**Figure 10** The proposed reaction of *trans*-caffeic acid binding for Fe<sup>2+</sup> (Li et al., 2012).





**Figure 11** Inhibition of AChE by physostigmine and Hyal by aescin as the standard compounds (%).

**Table 3** The *R* values between concentrations of phytochemicals and activity.

	TPC	TFC	Protocatechuic acid	<i>trans</i> -Caffeic acid
DPPH*	0.69	-0.60	0.63	-0.49
Anti-LPO	0.71	-0.62	0.62	-0.51
Fe <sup>2+</sup>	0.61	-0.50	0.72	0.15
Anti-Hyal	0.96	-0.98	-0.36	-0.99
Anti-AChE	-0.49	0.59	0.91	0.69

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jsps.2016.11.002>.

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