TLC profiling, nutritional and pharmacological properties of Siberian ginseng *(Eleutherococcus senticosus*) cultivated in Poland

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Abstract: The chemical composition and pharmacological activity of *E. senticosus* cultivated in Poland were investigated. Studies included the assay of TPC and TFC, 2D-TLC identification of phenolic acids, HPTLC-detection of antioxidants, and antioxidative, antileukemic, anti-MMPs properties of *E. senticosus*. The ethanolic extracts from the roots, spring leaves, fruits, and the chloroform extract from the roots were tested. The richest in polyphenols are the fresh fruits (57.5 mg/g), while in flavonoids the spring leaves (27.4 mg/g). The antioxidant ability both in extracts and single phenolic constituents were checked out by the measurement of the DPPH radical scavenging activity, iron (II) chelating and lipid peroxidation inhibitory activity. Using HPTLC-DB test eleutherosides B and E1 have been found as the phenolic antioxidants. Thirty six percent of apoptotic cells have been observed in Jurkatt 45 line by the treatment with the chloroform extract from the roots. Only the chloroform extract from the roots and the ethanolic one from the dried fruits have shown the inhibitory activities against MMPs. It is noteworthy, that our studies have been done for the first time, and the plant material has come from another geographical zone (Poland) than native (Asia).

Keywords: Eleutherosides, 2D-HPTLC, HPTLC-DB, Jurkatt 45, MMPs.

INTRODUCTION

Eleutherococcus senticosus Rupr. et Maxim. (Araliaceae) called as Siberian ginseng, occures in Northern Russia, China, Korea, and Japan. *E. senticosus* similarly to *Panax ginseng* [C.A. Meyer. (Araliaceae)], *Schisandra chinensis* [Turcz. Baill (Schisandraceae)] or *Aralia mandshurica* [Rupr. et Maxim. (Araliaceae)] is considered a plant adaptogen. Its properties are compared to *Panax ginseng*, and it is thought to be one of the most important plants in Traditional Chinese Medicine. For these reasons, it has increased interest in public opinion (Panossian *et al*., 1999). The roots have been used in folk medicine in the treatment of many diseases including diabetes, hypertension and cancer. The fruits have been used for a long time as a food and as an ingredient of the fermented wine. The leaves are used as a tonic, as a functional beverage commercially marketed for reducing liver damage and accelerating alcohol detoxification (Park & Rim, 2006). The fruits and leaves of *E. senticosus* are consumed in China, Korea or Northern Russia. In the Olympic Games, the players of the Old Soviet Union have increased records after administering *E. senticosus* (Park & Rim, 2006; Dae Sung Choi, 2007).

The pharmacological activity of natural products is very often believed to be the result of the combined actions of several of its constituents. The main compounds of *E. senticosus* are polyphenols. These compounds include eleutherosides, phenolic acids and flavonoids. Among polyphenols deserve special attention eleutherosides, especially eleutherosides B, E, E1. Eleutherosides B and E constitute 80% of all *E. senticosus* glycosides (Rice-Evans & Packer, 2003; Załuski *et al*., 2011). As additional constituents, the following compounds are present: glycosides of triterpenic acids (eleutheroside I, K, L, M), glycosides of sterols (eleutheroside A), isofraxidin, eleutherans (A, B, C, D, E, F, G), ciwujianosides, essential oil, acanthopanaxosides. It has been believed that for pharmacological and nutraceutical properties of *E. senticosus* most important are polyphenols including eleutherosides (Hikino *et al*., 1986; You *et al*., 2006; Richter *et al*., 2007). They clearly play a role both in the interactions between the plant and its biotic or abiotic environment and nutritional qualities of fruits and vegetables. The attempts to determine phenolic acids, especially the combined forms, have been significantly improved during the last two decades because of their importance as food constituents (Rice-Evans & Packer, 2003; Stalikas, 2007). Within polyphenols, the major active nutraceutical and pharmacological ingredients in plants are flavonoids. Some flavonoids act as antioxidants and metal chelators. They also have long been recognized to possess anti-inflammatory, antiallergic, hepato-protective, antithrombotic, antiviral, and anticarcino-genesis activities (Tapas *et al*., 2008).

Keeping in mind its rich biological activity and long-term use by the Asian, we have decided to evaluate the quality of *E. senticosus* cultivated in Polish climate conditions as a raw herbal material. This is the only one species of *Eleutherococcus* genus, which monograph can be found in Polish Pharmacopeia IX. So far the constituents with

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antioxidative, antileukemic and anti-MMPs activity of the plant remain unknown. Therefore it is necessary to check out the pharmacological activity of crude extracts and single compounds present in different parts. It is vital to check the chemical composition and biological activity of species cultivated in Poland. The chemical compounds and biological activity of plants depend on the geographical zone of the growth. This species is cultivated at the botanical garden in Rogów, which lies in the Central Polish Lowlands region with geographic data such as 51° 49′N and 19° 53′E. The average, long-term temperature is -20.1°C, what classified the garden to the 6bth sub-climate (according to USDA Frost Hardiness Zones) and to the second zone according to the Kórnik's category. These plants are grown on the acidic, luvic, and sandy soils (Tumiłowicz & Banaszczak, 2007).

Polish climate conditions can have an influence on the composition and the pharmacological effect of *E. senticosus*. In this case the content of phenolic compounds, the nutritional and pharmacological properties have been examined. In the world literature, the most information is available on the roots. While the chemical and biological activity of the fruits and leaves remains poorly investigated.

MATERIAL AND METHODS

Reagents

Folin-Ciocalteau reagent, matrix metalloproteinases, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), linoleic acid, EDTA, BHA, ascorbic acid, RPMI 1640 medium, bovine serum, DMSO were obtained from Sigma-Aldrich, and Annexin-V from BD Pharmingen^{TM} The standards of eleutherosides (B, E, E1), isofraxidin, phenolic acids were obtained from ChromaDex (Santa Ana, CA). All others reagents were of analytical grade.

Plant material

The roots, leaves and fruits of *E. senticosus* were collected at the arboretum in Rogów (Poland). Spring leaves were collected in June 2012, while roots and fruits in October 2012. All plant samples were deposited at the Department of Pharmaceutical Botany, Medical University of Lublin, Poland, Cat. Nr. 333.

Methods

Dried material extraction with 75% ethanol

The air-dried and powdered roots, leaves and fruits (15 g each) from *E. senticosus* were soaked in 150 mL 75% ethanol for 24 h. Next, the samples were subjected to triple USAE type extraction (ultrasonic bath -Polsonic, Warsaw, Poland) using 150, 2 x 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 lyophilisation.

Dried material extraction with chloroform

The roots were extracted the same way as extraction with 75% ethanol using chloroform.

Fresh material extraction with ethanol

The fresh fruits (10g) were deluged with 200 ml 99.9% ethanol and were soaked for 24 h. Next, the extraction was performed as shown above (dried material extraction with 75% ethanol).

Total phenolic content (TPC)

To determined the total phenolic content of extracts, the method of Singleton and Rossi (1965) was used. TPC was expressed as gallic acid equivalents (GAE/g dry sample). Every assay was performed in triplicate.

Total flavonoid content (TFC)

The TFC in investigated samples was determined using the colorimetric method (Shohael *et al*., 2006). TFC were expressed as mg of quercetin equivalent (QEs/g/dry sample) and mean±SD was calculated for the triplicate extracts.

Isolation and two-dimensional TLC (2D-TLC) identification of free and released after hydrolysis phenolic acids

Isolation and 2D-TLC were performed according to the method described by Smolarz *et al*. (1993). The extraction was performed as described in the part *Dried material extraction with 75% ethanol*, using 100 g of each sample (the roots -S_R, spring leaves -S_L and fresh fruits -S_O). Alkaline and acid hydrolysis were performed to release esterified acids. Free phenolic acids were marked as A and esterified acids as B.

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 chloroform extracts from the roots, and the ethanol extracts from the roots, spring leaves, fresh and dried fruits at the following concentration 0.1; 0.5; 1.0; 2.0mg/mL. Moreover, the DPPH scavenging was measured for phenolic acids fractions at concentration 0.125; 0.25; 0.5; 1.25mg/mL. All measurements were done after 5 min incubation of DPPH with the sample. Besides, the eleutherosides B, E, E1, isofraxidin were used as the naturally present components in *E. senticosus* (0.1; 0.5; 1.0; 2.0mg/mL). As a standard ascorbic acid, tocopherol, BHA, EDTA were used. Next, EC_{50} value was assayed. Every assay was performed in triplicate.

DPPH assay

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

Inhibition of linoleic acid peroxidation

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

Metal chelating activity

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

TLC screening for antioxidants-direct bioautography technique (DB)

TLC was done according to the method of Załuski *et al.* (2012). Briefly, TLC was performed on 10 cm x 10 cm glass Si60 $HPTLC_{F254}$ plates using P1 (chloroform: methanol: water, 70:30:4; v/v) and P2 (chloroform: methanol: toluene: ammonium, 9:6:3:2; v/v) mobile phases. The plate was developed "face down" in the same direction, using the P1 and after that P2 phases to the distance of 90 mm from the position of application. The plate was dried at room temperature for 20 min. After this time the plate was immersed into 0.5% DPPH solution for 5sec. Active compounds appeared as yellow-white spots against a purple background. White spots were visualized under day light after 1 min, and then after 1, 10, and 20 h. Ethanol solutions of standards (eleutheroside B, E, E1, isofraxidin) were used.

Cytotoxicity assay, apoptosis and necrosis staining

In cytotoxicity assay, *iiin vitro* model according to the method of Bogucka-Kocka *et al.* (2008) was employed. The trypan blue assay and annexin-V-Fluos assay were used to determine the cytotoxicity and influence on the induction of apoptosis. The Jurkat 45 cells were stimulated with ethanol extracts from roots, leaves and fruits and chloroformic extract from roots dissolved in DMSO at concentration between 1 and 600µg/mL.

The amount of apoptotic cells per sample was determined as the percentage of annexin V positive cells per sample. Data were processed according to the Multi Scan software.

Determination of metalloproteinase inhibitory activity with azocoll

It was done according to method described by Grzywnowicz *et al*. (2010). The extract concentration was 0.5 mg/mL and the substrate (azocoll) of 0.1%. As a positive control TIMP-1 (tissue inhibitor of MMP) and *o*-phenantroline were used. Every assay was performed in triplicate.

STATISTICAL ANALYSIS

All assays were performed in triplicate. To statistical analysis the Statistica 7.0. (StatSoft, Cracow) program was applied. All statistical tests were carried out at significance level of $\alpha = 0.05$.

RESULTS

Total phenolic and total flavonoids content

The first step of work was to assay the total content of polyphenols and flavonoids in the ethanolic extracts (table 1). The obtained results have shown that all samples have high content of polyphenols ranging between 11.4 and 57.5 mg/g. The fresh fruits had the highest content of these constituents in contrast to the roots, which had the lowest ones. In the case of flavonoids, the spring leaves were the richest.

Table 1: TPC and TFC in ethanolic extracts from *E. senticosus* (mg GAE/g and QEs/g dry sample*).

	Roots	Spring leaves	Fresh fruits	Dried fruits
TPC	11.4±0.55	27.4±0.84	57.5±0.05	26.3±0.991
TFC	8.7±0.12	23.9±0.29	1.7±0.008	7.4±0.261

* Results in terms of mean ± standard deviation

2D-TLC of phenolic acid

Free phenolic acids and released by hydrolysis were detected and identified by comparison of spots of the examined compounds and of standard substances (table 2).

Table 2: Phenolic acids in *E. senticosus* (A-free acids, B-released after hydrolysis, SR-the roots, SL-the leaves, SO-the fresh fruits).

Phenolic acids	SR_A	SL_A	SO_A	SR_B	SL_B	SO_B
Homoprotocatechuic acid	-	+	+	-	-	-
Protocatechuic acid	+	+	+	+	+	+
Caffeic acid	+	+	+	+	+	+
p-Coumaric acid	+	+	+	+	+	+
Syringic acid	+	+	-	+	+	+
Vanillinic acid	+	+	+	+	+	+
Ferulic acid	+	+	+	+	+	+
Ellagic acid	-	+	+	+	+	+
Gallic acid	+	-	-	+	+	+
Sinapic acid	-	+	+	+	+	+

Free phenolic acids present in all samples were protocatechuic, caffeic, *p*-coumaric, vanillinic and ferulic acid. Sinapic, ellagic and homoprotocatechuic acids were identified in the leaves (SL_A) and the fresh fruits (SO_A). Free gallic acid was only in the roots (SR_A), syringic acid was both in the roots and in the leaves. We did not detect homoprotocatechuic acid in the extracts after hydrolysis. The other acids were detected in all samples.

Antioxidant activity

As it is shown in table 3, all extract have antioxidative properties. In the DPPH test, the ethanol extract of the spring leaves proved the most active (1.1 mg/mL). It has been found that the dried fruits have the strongest antiperoxidation activity (2.9 mg/mL). Interesting enough, all extracts have shown high metal chelating activity. Generally, the ethanol extracts from the roots and spring leaves have shown the highest and simultaneously similar activity to all three methods.

Table 3: The antioxidant properties of ethanolic extracts from the roots, spring leaves, fresh and dried fruits and chloroformic extracts from the roots of *E. senticosus* (1-DPPH radical scavenging activity, 2-Ability to lipid antiperoxidation, 3-The iron (II) chelating activity).

Samples and standards	1*	2*	3*
	(EC_{50} mg/mL)		
Roots -ethanol	1.5±0.07	3.1±0.01	0.5±0.05
Roots - chloroform	143.6±0.55	4.1±0.01	0.7±0.02
Spring leaves - ethanol	1.1±0.26	3.3±0.01	0.5±0.04
Fresh fruits - ethanol	5.2±0.25	3.8±0.01	0.5±0.02
Dried fruits - ethanol	14.0±0.1	2.9±0.03	0.5±0.05
Eleutheroside E	-	1.6±0.02	-
Eleutheroside E1	2.1±0.05	-	-
Eleutheroside B	-	1.5±0.03	-
Isofraxidin	4.0±0.32	-	-
Ascorbic acid	0.04±0.05	-	-
α - tocopherol	0.0051±0.1	-	-
BHA	-	0.1±0.001	
EDTA	-	-	0.01±0.1

* Results in terms of mean ± standard deviation

The results for the extracts were compared with the results for eleutherosides B, E, E1 and isofraxidin as naturally occurring compounds in *E. senticosus*. The DPPH scavenging activity was shown only by eleutheroside E1 and isofraxidin, while eleutherosides B and E inhibited oxidation of linoleic acid.

HPTLC- DB of antioxidants

The results obtained for the screened extracts indicated several compounds with DPPH reduction properties. As standards we used eleutherosides B, E and E1. We observed the plates after 1 min, 1, 2, 5, 10 and 24 h from the time of immersion of the plate in 0.5% DPPH* solution. After 1 min, the ethanol extracts from the roots and the fruits showed areas of activity on the high R_f 0.73 (corresponding to eleutheroside E1). Additional yellow-colored compound was observed to migrate at the R_f 0.57. No changes in decolorization of the chromatogram after 1 and 5 h were noticed, while new spots have been detected after 10 h. The R_f was 0.67 and it was the same as eleutheroside B. Another compound with antioxidant activity was at the R_f 0.76. Ones of the active compounds were eleutherosides E1 and B, while eleutheroside E did not show the same properties (fig. 1). Taking into account the speed of DPPH* decolorization by eleutheroside E1, it can indicate on its strong antiradical activity, what has been confirmed by spectrophotometric test.

Antioxidative activity of phenolic acid fraction

Antioxidant capacity is often associated with the presence of phenolic acids. The DPPH radical-scavenging activity of phenolic acids was significantly high and ranged between 0.13 and 3.5 mg/mL (table 4). The most active was the fraction of free acids from the fruits (0.13 mg/mL), while the released fraction had the lower activity (3.5 mg/mL). Generally free phenolic acids fractions have shown higher activity than ester linked ones, of which special attention in further studies should be paid to this from the fresh fruits.

Cytotoxic and apoptotic activity

In next step of the work we have evaluated a cytotoxic and apoptotic activity of *E. senticosus* against Jurkatt 45 cell line. The concentration of extracts varied from 1 to 600μg/mL. All tested extracts have shown cytotoxic activity, among of which the strongest have the chloroform one from the roots (IC_{50} 2.85μg/mL), next the ethanol extracts from the roots (134.77μg/mL), the spring leaves (199.72μg/mL), the fresh fruits (331.22μg/mL) and the dried fruits (274.4μg/mL).

The Annexin V test was used to determine the apoptotic and necrotic changes in cells. In this aim, cells were treated with the lowest IC_{50} value. The Jurkatt 45 cells were stimulated for 24 h at the extract concentration 2.85 μg/mL. It showed that the examined extract caused the death of cells via apoptosis. Apoptotic cells constituted 36% of the total, while in control group was less than 5%.

Inhibition of metalloproteinases

Our results show for the first time that *E. senticosus* is able to inhibit MMPs. The activity is dependent on the type of the extracts and MMPs. All used MMPs were inhibited by the chloroform extract from the roots in the range of 0.02-18.2%. The highest inhibitory level was measured in the case of MMP-2 (18.2%), next for MMP-3 (16.69%), MMP-9 (11.85%) and MMP-1 (0.02%). The highest inhibition was shown by the ethanol dried fruits against MMP-2 (20%) while the lowest inhibition was shown by the ethanol extract from the roots, only 2.3% against MMP-1, none against remaining MMPs.

TIMP-1 and *o*-phenantroline were used as control inhibitors. TIMP-1 is a naturally occurring inhibitor in human cells and its inhibitory level was 61.5%, at the concentration 0.1µg/mL. While, *o*-phenantroline at the concentration 5 mM has inhibited the MMP activity about 99%.

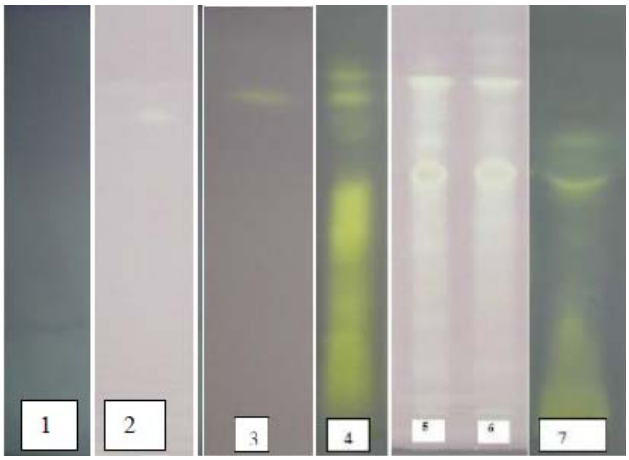

Fig. 1: Bioautographs showing the DPPH scavenging activity after 10 h for standards: 1-eleutheroside E, 2-eleutheroside B, 3-eleutheroside E1; ethanolic extracts from: 4-the roots, 5-the fresh fruits, 6-the dried fruits, 7-the leaves of *E. senticosus*.

DISCUSSION

Plants extracts and secondary metabolites have a large contribution in health prevention, therapy of many illnesses and convalescence. One of more valuable source of secondary metabolites with long-term use in TCM is *E. senticosus*. As far, not all pharmacological activities of its have been confirmed by research. With this in mind, we studied the phytochemical composition and pharmacological activity of extracts from *E. senticosus*. It is worth noting that we investigated all parts of the plant (roots, leaves and fruits).

Total phenolic content, based on dry extract, of *E. senticosus* measured in this study was higher than other reports from Korea. Lee *et al*. (2011) has reported that TPC is dependent on the ethanol concentration. The greatest amount of polyphenols has been detected in the extract from the stem bark, with 60% ethanol concentration (790 mg/L of GAE). The obtained results in our work indicate that *E. senticosus* is rich in the phenols.

One of the polyphenolic groups is phenolic acids. 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. It is very important that *E. senticosus* contains syringic and vanillic acids, whose distribution appears to be very limited. Other acids were determined for the first time in *E. senticosus* growing in Poland.

For many years an increase in leukemia incidents has been observed, especially in young people. Taking into account the cytotoxic activity of *E. senticosus*, no information was found on cytotoxicity on Jurkatt 45 cell line. Similar studies have been performed using other cell lines, such as MOLT-4F (IC_{50}; 14.29µg/mL), PC-3, HCT-15, SW-620, ACHN and A549. The obtained IC_{50} value in all cases were on the level nearly 30µg/mL (Yu *et al*., 2003). Chon *et al*. (2009) have shown cytotoxic activity of seedlings of *E. sessiliflorus* towards Calu-6 and SMU-601 cell lines. The IC_{50} values were 25µg/mL and 196.7 µg/mL, respectively.

Table 4: Antiradical activity of phenolic acids in *E. senticosus* (A-free acids, B-released after hydrolysis; SR-the roots, SL-the leave, SO-the fresh fruits), (EC_{50} mg/mL).

Extract	EC_{50} mg/mL*
SR_A	0.85±0.03
SL_A	0.41±0.02
SO_A	0.13±0.006
SR_B	2.10±0.005
SL_B	2.0±0.004
SO_B	3.5±0.05

* Results in terms of mean ± standard deviation

One of the negative functions of MMPs is to contribute to the tumorigenesis. According to our research, some constituents of the investigated species have inhibitory activity towards MMPs. Because MMPs are the zinc dependent enzymes, for this reason, we suggest that mechanism of azocoll's proteolysis by MMPs is connected with the chelation of ions metal at the active site of the enzyme.

Comparing our own results with that of others, it shows, that the chloroform extract from the roots has a significantly high cytotoxic activity. We can suggest, that *E. senticosus* might be a new source of chemical compounds with nutritional and pharmacological properties. Keeping in mind its rich biological properties and long-term use by the Asians, it should become more popular in Europe's countries. In light of this, consumption, especially of the fresh fruits or the roots extract products can act protectively against free radical.

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