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ORIGINAL ARTICLE

HPTLC-profiling of eleutherosides, mechanism of antioxidative action of eleutheroside E1, the PAMPA test with LC/MS detection and the structure–activity relationship

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SAR

Abstract Human body is constantly generating free radicals, which causes oxidative stress. Despite naturally occurring antioxidant systems in human body, free radicals cause lipid, proteins and DNA oxidation. New antioxidants are still urgent as well as their mechanisms of action should be explained. In this study, we investigated the mechanism by which eleutherosides B, E and E1 may act as antioxidants, identified eleutherosides in *Eleutherococcus lasiogyne* and *Eleutherococcus giraldii*, and explained *in vitro* the absorption of eleutheroside E1 based on passive transport. The DPPH* and DB-HPTLC tests were used to assess the antioxidant activity. Of the three eleutherosides, only eleutheroside E1 exhibited a strong anti-DPPH* activity (EC_{50} 37.03 μg/mL; 63 mMol) compared to the raw extracts (EC_{50} 170 and 180 μg/mL for *E. lasiogyne* and *E. giraldii*). This activity was also confirmed by the DB-HPTLC autography technique. According to Załuski's hypothesis, the antioxidant mechanism of eleutheroside E1 is based on the complexation of DPPH* molecule with its aryl radical. During this reaction, the aryl radical of eleutheroside E1 (E1*) and DPPHH are created. Next, the aryl radical (E1*) is complexed with another DPPH* molecule. Additionally, the aryl radical can be stabilized by the presence of the methoxy groups in the aromatic ring, which increases its antioxidative action. The HPTLC-identification of extracts showed the presence of eleutherosides B, E and E1 in both species. The PAMPA test coupled with LC/MS detection showed a low permeability of eleutheroside E1 across artificial membrane. Because eleutherosides belong to the polyphenols, the TPC and TFC were quantified. The TPC and TFC

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varied from 51.4 to 49.3 mg/g dry extract for TPC, and from 5.73 to 4.91 mg/g dry extract for TFC, for *E. giraldii* and *E. lasiogyne*, respectively. In conclusion, eleutheroside E1 in its pure form could be a chemopreventive ingredient of new pharmacological or dietary products, stimulating the GALT. These findings can explain partially the adaptogenic activity of eleutheroside E1 on the GALT, which has been still unknown.

1. Introduction

Human body is constantly generating free radicals, which causes oxidative stress. Several factors such as drugs, chlorinated compounds, and deficiency of natural antioxidants, tobacco and pollution may increase the ratio of free radicals. Despite naturally occurring antioxidant systems in human body, free radicals cause lipid, proteins and DNA oxidation. These damages at the molecular level may influence the etiology of diseases, such as cancer, atherosclerosis, diabetes, neurodegenerative disorders and aging. Some evidences indicate that the diet rich in antioxidants may be protective against abovementioned diseases (Ahmed et al., 2013; Cooke, 2012; Cieśla et al., 2012). One of the special sources of antioxidants is plants belonging to the *Eleutherococcus* Maxim. genus (native to China, Korea, Japan and Russia). It is believed that the chemical compounds are eleutherosides A, B, B1, E, E1, I, K, L and M, which are the glycosides of coumarins (eleutheroside B1), lignans (eleutheroside B, E, E1), sterols (eleutheroside A), and triterpenic acids (eleutheroside I, K, L, M). Eleutheroside B and E are responsible for the pharmacological effect, for the sum of which the standardization of products with *Eleutherococcus* extracts is carried out (Załuski et al., 2010; Park et al., 2006; Polish Pharmacopoeia VIII, 2008). It is thought, these two compounds are responsible for adaptogenic properties of *Eleutherococcus* spp. The concept of adaptogen was first used by a Soviet scientist, dr Nikolai Lazarev, who was researching substances which produced a "state of nonspecific resistance". Adaptogens act as drugs via increasing non-specific resistance of the body. Other studies showed that extracts from *E. senticosus* added as adjuvants to vaccines for the prevention and/or treatment of microbial, viral infections and/or cancer, may affect the expression of proteins associated with immune response to microbial infection, viral infection and/or cancer. Some authors believe that eleutheroside B and E are responsible for the adaptogenic activity of *Eleutherococcus*, whereas others believe it is the result of the synergistic action of all the constituents.

Pharmacokinetic studies have demonstrated that these eleutherosides are very well absorbed in the human intestines, with a peak plasma concentration occurring at 30 min. The eleutherosides may be absorbed by the cell by means of active glucose and galactose carriers. Some studies have shown that the accumulation time of eleutheroside B is between 2 and 4 h. The experimental results demonstrated that eleutherosides B and E are cumulated in the plasma, heart, kidney and liver. Concentrations of eleutheroside B and E were higher in the liver and kidney, which demonstrates that both eleutherosides B and E are metabolized and excreted primarily by the liver and kidneys (Feng et al., 2006). The adaptogenic activity of eleutheroside E1 and its pharmacokinetic parameters remain still unknown. In addition its distribution in *Eleutherococcus* spp. is poorly investigated.

Previous phytochemical studies on *E. giraldii* have reported the presence of eleutherosides E and B4, isofraxidin, β-sitosterol, hederagenin glycosides, sesquiterpenes, polysaccharides, hyperoside, syringing, syringaresinol and lipid acids. Besides, in the stem bark of *E. giraldii*, chlorogenic, caffeic and protocatechuic acids were identified, as well as thymine, uracil, xanthine, adenine, hypoxanthine, adenosine, liriodendrin and allantoin (Yuan et al., 2009; Wang et al., 2005; Dong-Liang et al., 1994; Li et al., 2009; Zhao and Yuan, 1991; Liu et al., 2007; Wang et al., 2005).

These constituents play key functions in ill-health prevention and in the treatment of many diseases. It has been reported that they have antiinflammatory, immunostimulative, anticancer and hepatopreventive properties; an inhibitory effect on COX-1 and COX-2 was also observed. It is used in the treatment of anemia and rheumatoid arthritis. The tea from *E. giraldii* can resist hyperlipemia, and inhibit absorption of cholesterol and the growth of tumor cells. In addition, the tea has a protecting effect on the liver. The polysaccharides can be used to treat neutropenia, thrombocytopenia and viral infectious diseases (Wang et al., 1992; Deng et al., 1994).

Keeping in mind their rich biological activity and long-term use by the Asian, we have decided to evaluate the quality of *Eleutherococcus* species cultivated in Polish climate conditions as a raw herbal material. The chemical compounds and biological activity of plants depend on the geographical zone of the growth. These species are cultivated at the botanic 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, which classified the garden to the 6bth sub-climate (according to USDA Frost Hardiness Zones) and to the second zone according to the Kórnik category. These plants are grown on the acidic, luvic, and sandy soils (Tumilowicz and Banaszczak, 2007).

Studies on eleutherosides are important for drug discovery, design and clinical application. In this report we aimed at searching new antioxidants in *E. giraldii* and *E. lasiogyne*, as well as we suggested the hypothetical mechanism of the antiradical action of eleutheroside E1 and B. The PAMPA test of eleutheroside E1 with LC/MS detection was also performed. From the pharmacological point of view, an *in vitro* explanation of the antiradical mechanism of chemical compounds is needed because *in vivo* experiments are sometimes unable to clearly explore the mechanism of their action. Of course, the impact of absorption, distribution and metabolism within the body cannot be reflected through *in vitro* testing, but to explain the causes of all changes, *in vitro* tests must also be performed. Because the therapeutic effect of *Eleutherococcus* spp. depends on the presence of the eleutherosides as active ingredients, one

of the objectives of this work was HPTLC-the identification of eleutheroside B, E and E1.

2. Materials and methods

2.1. Materials

The standards of eleutherosides B, E and E1 were obtained from ChromaDex (Santa Ana, CA). 1.1-diphenyl-2-picryl-hydrazyl (DPPH*), ascorbic acid, and L-α-phosphatidylcholine were purchased from Sigma–Aldrich. Methanol, ethanol, 2,4-DNPH and $FeCl_3$ were obtained from POCH (Lublin, Poland). All others reagents were of analytical grade.

2.2. Plant material

The roots of *E. giraldii* (Harms) Nakai and *E. lasiogyne* (Harms) S.Y. Hu were obtained from arboretum in Rogów (Poland). The roots were collected in October 2013. Voucher specimens were deposited at the Department of Pharmacognosy, Jagiellonian University of Cracow, Poland marked as EG-011 and EL-02.

2.3. Accelerated solvent extraction (ASE)

The air-dried and powdered roots (2 g) from *E. giraldii* and *E. lasiogyne* were placed in 5 mL extraction cell with 3 g of neutral silica gel. The ASE cell was placed into ASE for extraction process (Dionex ASE 159). During the extraction process, 75% ethanol was delivered into the extraction cell. Pressure (1000 psi) was applied to maintain the solvent in its liquid state. The extraction process was repeated three times using after 18 mL 75% ethanol. The extraction temperature was 40 °C, the extraction time was 15 min. Following extraction, the extract containing the target analytes was purged from the cell using nitrogen into a collection vial for analysis. After the three extraction cycles 54 mL each of extracts was obtained. The solvents were evaporated under reduced pressure at 45 °C and the residue was subjected to lyophilization (Labconco Freezone 1).

2.4. Solid phase extraction (SPE)

The extracts were purified by SPE according to Załuski et al. (2011). Each extract dissolved in 2 mL 75% ethanol was applied to a Bakerbond SPETM Octadecyl C18 microcolumns (500 mg, 3 ml; J.T. Baker, Phillipspurg, NJ, USA) previously activated with 2 mL 99.8% ethanol, then with 2 mL distilled water, and finally with 2 mL 75% ethanol. After the application of the sample solution the target analytes were eluted with 4 mL 75% ethanol. Collected eluates were concentrated in a nitrogen stream and subjected to lyophilization. Samples prepared in this underwent chromatographic analysis.

2.5. DPPH assay

The ability of the extracts to scavenge DPPH free radicals was determined by the modified method of Brand-Williams et al. (1995). The methanol solvents of the extracts at the following concentrations: 0.1; 0.5; 1.0; 1.5 mg/mL were used. A 40 µL sample of extract was mixed with 160 mL of methanol solution of DPPH* (0.2 mmol/L). Absorbance was measured at the wavelength of 515 nm at 1, 5, 10, 30, 60, 90 min on a multidetection BIOTEK spectrophotometer. Pure methanol was used as a control sample. Antioxidant activity was expressed as a percentage of inhibition calculated according to the following formula:

$$\% \text{ inhibition} = [(A_{C(0)} - A_{A(t)})/A_{C(0)}] \times 100$$

where $A_{C(0)}$ – absorbance of the control sample at 0 time, $A_{A(t)}$ – absorbance of the experimental sample measured after 5 min. Ascorbic acid was used as a positive control (12.5; 25; 50; 75 µg/mL) and ethanolic solutions of eleutheroside B, E, E1 (0.1; 0.5; 1.0; 2.0 mg/mL). Next, EC_{50} value was assayed. Every assay was performed in triplicates.

2.6. PAMPA test with LC/MS detection

The PAMPA test was performed according to the modified method of Załuski et al. (2016). Permeation experiments of eleutheroside E1 and verapamil (as a positive control) were carried out in hydrophobic PVDF 96-well filter plates (Millipore, Molsheim, France). Each well of the donor plate was coated with 1% (v/v) liquid membrane (L-α-phosphatidylcholine) dissolved in *n*-dodecane for 20 min to completely evaporate the solvent. Next, in each well of donor plate, 150 µL 40 µM of eleutheroside E1 in 0.1 M PBS pH 7.4 was transferred. In each well of acceptor plate, 300 µL 0.1 M PBS/4.7% MeOH acceptor solution was transferred and covered by the donor plate, creating a PAMPA sandwich. Each substance was measured in triplicates at iso-pH conditions (the same pH value in donor and receptor compartment). The donor plate was covered to prevent evaporation and the whole system set up to interact with the vibratory mixer (500 rpm). After 4 h, PAMPA sandwich was removed from the mixer and vibration, and the concentration of the investigated substances in the donor and receptor compartments were quantified from an appropriate calibration line using LC–MS. Before LC/MS analysis, the donor solution was diluted four times 0.1 M PBS/4.7% MeOH. A UltiMate 3000RS/DAD Dionex system equipped with a binary gradient solvent pump, a degasser, an autosampler and column oven connected to amaZon SL Bruker Daltonics Mass spectrometer was used. Chromatographic separations were carried out at 25 °C, on an Ascentis Express C18 column (2.1 × 50 mm, 2.7-µm particle size; Sigma–Aldrich) with a mobile phase consisting of water containing 0.1% HCOOH (solvent A) and ACN containing 0.1% HCOOH (solvent B), using 10 µL injections. The flow rate was 1 mL min^{-1} and the gradient was as follows: 0–0.5 min – 5% B; 3–3.3 min – 95% B; 3.4–4.0 min – 5% B.

The MS system was equipped with electrospray ionization source (ESI) operated in the negative-ion mode. ESI worked at the following conditions: capillary temperature 300 °C, curtain gas at 40 psi, and nebulizer gas at 9 L/min. Nitrogen was used as curtain and collision gas. For each compound the optimum conditions of Multiple Reaction Mode (MRM) were determined. Triplicate injections were made for each solution. The analytes were identified by comparing retention time (R_t 1.5 min) and $[M]^+$ at m/z 579.2 values obtained by MS 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 (10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.63 μM, 0.31 μM in 0.1 M PBS/4.7% MeOH). As a positive control verapamil was used (250 μM, 125 μM, 62.5 μM, 31.25 μM, 15.63 μM, 7.81 μM, 3.91 μM in 0.1 M PBS). The following formula was applied to determine Pe:

$$\text{Pe } (10^{-6}\ \text{cm/s}) = C_x - \ln(1 - C_{\text{drug acceptor}}/C_{\text{drug equilibrium}});$$
$$\text{where } C = V_{\text{donor}} \times V_{\text{acceptor}}/(V_{\text{donor}} + V_{\text{acceptor}}) \times \text{Time} \times \text{Peak area}$$

2.7. Total phenolic content (TPC)

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

2.8. Total flavonoid content (TFC)

The TFC in investigated samples was determined using aluminum chloride and 2,4-dinitrophrnylhydrazine colorimetric methods (Chang et al., 2002). TFC were expressed as means (±S.E.) mg of quercetin equivalent (QEs/g/dry sample for $FeCl_3$ method, 20–100 μg/mL; $y = 0.0041x + 0.236$; $r^2 = 0.999$) and as means (±S.E.) mg of hesperetin equivalent (QEs/g/dry sample for 2,4-DNPH method, 250–1000 μg/mL; $y = 6.374x - 0.098$; $r^2 = 0.988$). Every assay was performed in triplicates.

2.9. HPTLC of eleutherosides B, E and E1

Chromatographic analysis was preceded by a clean-up procedure, on C18 microcolumns, to purify the sample. To check whether the eleutherosides were completely eluted from the SPE microcolumns, the cartridges were flushed with 10 mL 75% ethanol. The HPTLC-densitometric examination of the eluate, showed the absence of bands characteristic for the investigated eleutherosides. It confirmed the complete yield of eleutherosides from the microcolumns.

2.9.1. Polar adsorbent

The HPTLC analysis was done according to the chromatographic method described by Załuski et al. (2010). Ethanolic solutions of all standards (1 mg/mL, 1 μL) and plant extracts (10 mg/mL, 3 μL) were used. After development, bands in the extracts were identified by matching their R_f values and UV scan with those obtained for standards.

2.9.2. Nonpolar adsorbent

The HPTLC analysis was done according to the method described by Cieśla et al. (2011). Ethanolic solutions of all standards (1 mg/mL, 1 μL) and plant extracts (10 mg/mL, 3 μL) were used. After development, bands in the extracts were identified by matching their R_f values and UV scan with those obtained for standards.

2.10. HPTLC screening for antioxidants – direct bioautography technique (HPTLC-DB)

The Si60 HPTLC-F254 and Si60 HPTLC RP18-WF254 plates were developed according to the method described in the HPTLC of eleutherosides B, E and E1 parts. After development, the plates were dried at room temperature for 20 min. and were dipped into the 0,5% methanolic solution of DPPH* for 5 s. Active compounds appeared as yellow–white spots against a purple background. White spots were visualized under daily light after 1 min, 1, 10 and 24 h (Camag Visualiser). Ethanolic solutions of standards (eleutheroside B, E and E1; 1 mg/mL, 1 μL) and plant extracts (10 mg/mL, 3 μL) were used.

2.11. Statistical analysis

All determinations were performed in triplicates. 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 Person's (r) test. All statistical tests were carried out at a significance level of $\alpha = 0.05$.

3. Results and discussion

3.1. Antioxidant properties

To our best knowledge, our study is the first to report the antioxidant properties of eleutherosides B, E, E1, and *E. giraldii* and *E. lasiogyne*. The first step of the work was to establish a change of absorbance in time. As is shown in Fig. 1 (Suppl. Mat.), the optimal time is 30 min. which correlates with that of the original method of this analysis. After this time no changes in absorbance were noticed. On the basis of the obtained results, all species were found to reduce the DPPH* radical (Table 1). Both extracts have similar antioxidative activity and the EC_{50} value ranged between 0.17 and 0.18 mg/mL for *E. lasiogyne* and *E. giraldii*, respectively. The results for the extracts were compared with the results for eleutherosides B, E and E1 as naturally occurring compounds in the investigated samples. Of the three eleutherosides, eleutheroside E1 exhibited strong anti-DPPH* activity (EC_{50} 37.03 μg/mL; 63 mMol), which was higher than ascorbic acid (41.0 μg/mL; 232 mMol).

Table 1 DPPH radical scavenging capacity of *E. giraldii*, *E. lasiogyne*, eleutherosides E1, E and ascorbic acid.

Species	EC50 [mg/mL]*[μg/mL]^
E. giraldii	0.18* ± 0.06
E. lasiogyne	0.17* ± 0.07
Eleutheroside E1	37.03^ ± 0.2
Eleutheroside E	5.40* ± 0.9
Ascorbic acid	41.0^ ± 0.05

On the basis of statistical data, the correlation between total phenolic content and radical scavenging activity was revealed. It is in agreement with the general information on polyphenols rich in hydroxyl group in C-3 or C-5 position in the molecule.

3.2. The PAMPA test of eleutheroside E1 with LC/MS detection

Permeability is one of the key parameters determining the pharmacokinetic behavior of orally administered drugs. We used PAMPA to study *in vitro* the absorption of eleutheroside E1 and the nature of the mechanisms underlying passive transport of eleutheroside E1, that still remain unclear. The permeability classification is divided into three groups: low < 5; moderate 5–20; and high $> 20 \times 10^{-6}$ cm/s. The chromatogram and spectra of eleutheroside E1 in the positive- and negative-ion modes are shown in Fig. 2S, Suppl. Mat. The Pe value for eleutheroside E1 was established as 0.5×10^{-6} cm/s, the recovery was 88.95%. The results obtained indicate a low absorption of eleutheroside E1 with a partial binding to the membrane. Verapamil was used as a relatively high permeability compound (Pe 16.9×10^{-6} cm/s, the recovery was 75.86%). It is worth noting that binding to the membrane may influence the prolongation of eleutheroside E1 time action, and its stimulating effect on the GALT. GALT represents almost 70% of the human immune system, which is stimulated by different molecules supplied to the body, not always absorbable (Vighi et al., 2008). Our findings can explain partially the adaptogenic activity of eleutheroside E1. Usually, the researchers are focused on the activity of the crude extracts and their stimulation effect on thymus and medulla, whereas the influence of the extracts or eleutherosides on the GALT activity still remains unexplained. Only one study reports on the influence of the *Eleutherococcus senticosus* seeds extract on the TLRs, *in vitro*. It has been demonstrated that the extract may activate B cells and macrophages via the activation of TLR2 and/or TLR4 (Han et al., 2003). In other studies, extracts from *E. senticosus* were used as adjuvants for vaccines for the prevention and/or treatment of microbial, viral infections and/or cancer. It was suggested that they may affect the expression of proteins associated with immune response to microbial infection, viral infection and/or cancer. In further studies, the PAMPA test should be modified with the use of Caco-2 test to predict better bioavailability based on the active transport. Further investigations are also needed to evaluate its metabolism *in vitro* and clinical significance.

3.3. Antioxidative mechanism of eleutheroside action

On the basis of the obtained results, a relationship between the structure of eleutherosides and their antioxidative activity was established. 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 glycosides has not yet been described. For this reason we have tried to explain the antiradical action of these compounds (eleutherosides B, E, E1) and we have proposed the mechanism by which these eleutherosides may act as antioxidants. This was called "Załuski's eleutheroside hypothesis". The interaction of potential antioxidants with DPPH* depends on their structural conformation. As it is known, antioxidant activity increases with the number of OH groups on a given aromatic ring, but with no more than three. Eleutherosides E and E1 are the glycosides of syringaresinol belonging to diphenols (furofuran lignans), while eleutheroside B is the glycoside of syringing belonging to monophenols (Fig. 1). In our test of eleutherosides E and E1, only eleutheroside E1 showed strong antiradical properties. A close look at the structure of eleutheroside E1 and eleutheroside E demonstrates the presence of one free OH group in the phenyl ring of eleutheroside E1. In the case of eleutheroside E, all of the OH groups are esterified by glucose and do not participate in the reduction reaction. Fig. 2 shows the suggested mechanism of the antiradical action of eleutheroside E1. In the structure of eleutheroside E1 there are four methoxy groups (two in each aromatic ring) and the one free OH group in the phenyl ring (Table 2).

According to the author's hypothesis, the antioxidant mechanism is based on the complexation of the DPPH* molecule with the OH group of eleutheroside E1. During this reaction, the aryl radical of eleutheroside E1 (E1*) and DPPHH are created (Eq. (1)). Next, the aryl radical (E1*) is complexed with another DPPH* molecule (Eq. (2)).

$$\mathrm{DPPH}^* + \mathrm{E1} \leftrightarrow \mathrm{DPPHH} + \mathrm{E1}^* \quad (1)$$

$$\mathrm{DPPH}^* + \mathrm{E1}^* \leftrightarrow \mathrm{DPPH} - \mathrm{E1} \quad (2)$$

Additionally, the aryl radical can be stabilized by the presence of the methoxy groups in the aromatic ring, which increases its antioxidative action. Several authors have indicated that the antioxidative properties depend on the number of hydroxyl groups in the chemical formula, especially on the OH group in the *ortho* and *para* positions. Eleutheroside E1 has the one free OH group in the *para* position, which in eleutheroside E is esterified by glucose. Additionally, the effectiveness of radical scavenging is strengthened by the 3-OH group attached to the 2,3-double bond. According to Pokorny, the efficiency of the *para* diphenols is in part due to the stabilization of the aryloxyl radical by hydrogen bonding or by regeneration of another diphenol. This author has reported that the presence of more than three hydroxyl groups on a given aromatic nucleus did not improve the efficiency (Brand-Williams et al., 1995; Chang et al., 2002; Vighi et al., 2008; Pokorny, 1987). On the basis of the above findings, the hypothesis seems to be true concerning the antioxidant activity of eleutheroside E1. Moreover, the foregoing reasoning confirms that the anti-DPPH* activity of eleutherosides is in relation to their chemical structure and localization of the active groups.

Among other eleutherosides, eleutheroside B was active in the autography test, i.e. its yellow band was observed after 24 h from the immersion time. Taking into account the chemical structure of eleutheroside B, we can see that eleutheroside B has two OH groups. One of them is directly connected with the aromatic ring and is esterified by glucose. The second, free OH group is in the propenyl chain. The position and ability of these groups is important. The lack of a free OH group directly linked to the aromatic ring may be one of the causes for weaker antioxidant properties. The second cause may be the absence of the double C=C bond by the carbon connected with the OH group. Some authors believe that this bond

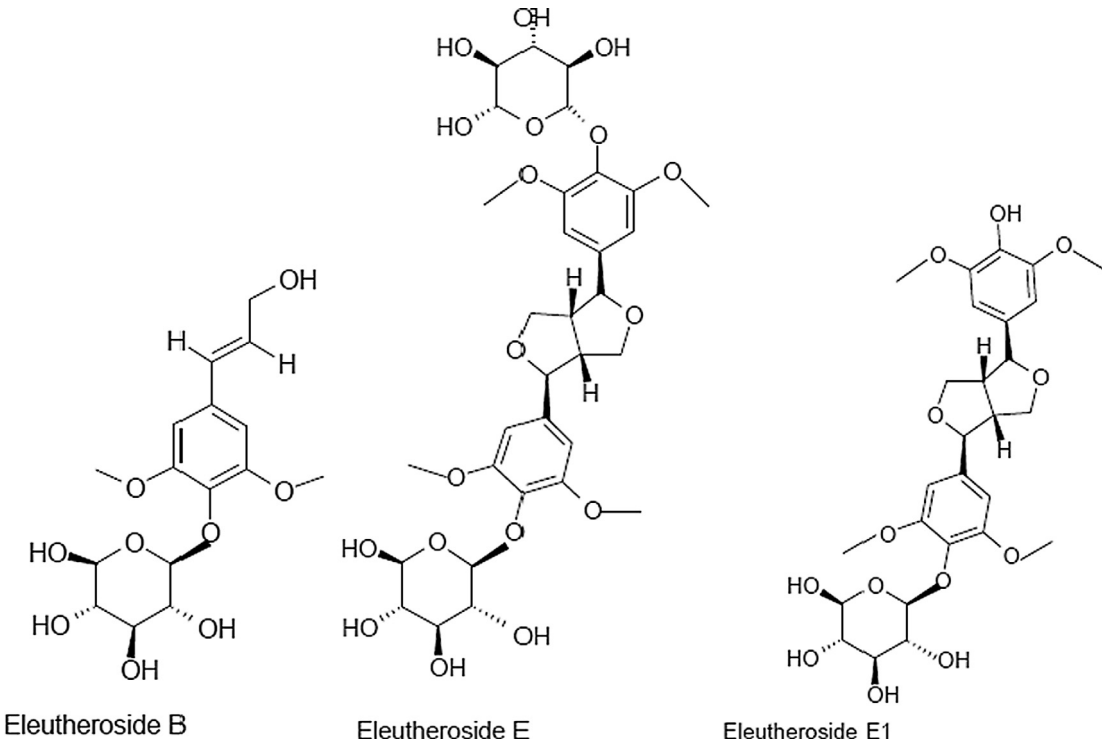

Figure 1 Chemical structure of eleutherosides B, E and E1.

participates in stabilizing the radical by resonance and increases the antiradical efficiency (Cuvelier et al., 1992).

3.4. Total polyphenol and flavonoids content (TPC, TFC)

TPC was estimated using the Folin–Ciocalteu method and expressed as a gallic acid equivalent (mg/g dry extract). As it is shown in Table 3, both species contain the similar amount of TPC (51.4 and 49.3 mg/g dry extract for *E. giraldii* and *E. lasiogyne*, respectively). No data were found in the world literature. One of the most common procedures used to evaluate the TFC 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. The summed amount of flavonoids was 5.7 and 4.9 mg/g dry extract for *E. lasiogyne* and *E. giraldii,* respectively. We found that flavonoid content obtained by aluminum chloride reaction was much higher than those obtained by DNPH reaction (Table 3). It can indicate, that the species contain more compounds with the OH group which are responsible for high antioxidant activity of extracts.

3.5. HPTLC determination of eleutherosides B, E and E1

It is believed that eleutherosides B (syringing 4-β-D-glucoside), E ((−)-syringaresinol-4,4′-O-β-D-diglucoside), and E1 ((+)-syringaresinol-4-O-β-D-glucoside) are the main active compounds in *Eleutherococcus spp*. According to Polish and European Pharmacopoeia, the roots should contain a minimum of 0.08% for the sum of eleutherosides B and E (Polish Pharmacopoeia VIII, 2008; European Pharmacopoeia V, 2005). The chromatography of the eleutherosides was done using the HPTLC technique as previously described by Załuski et al. (Załuski et al., 2010; Cieśla et al., 2011). To ensure the presence of the investigated eleutherosides in the samples, the resolution of eleutherosides was performed using two stationary phases: Si60 HPTLC RP18-WF254 and Si60 HPTLC-F254. After development, bands in the extracts were identified by matching their R_f values and UV scan and spectra with those obtained for the standards. Purity of the eleutheroside peaks in all of the analyzed samples was examined by taking the spectra at the peaks' center and flanks (Fig. 3S, 4S; Suppl. Mat.). As is shown in Fig. 3S, the investigated eleutherosides are present in all samples. The results indicated that there was no difference in the UV scan and spectra of each plant sample and the standards. In the applied chromatographic system, the eleutherosides are characterized by the following R_f values: (nonpolar system): eleutheroside B-0.56, eleutheroside E-0.46, eleutheroside E1-0.32; (polar system): eleutheroside E-0.29; eleutheroside B-0.42, and eleutheroside E1-0.58. Data in the literature indicates that eleutheroside E was detected in *E. giraldii* growing in China (Wang and Sun, 2005). No information on eleutherosides in *E. lasiogyne* was reported.

3.6. HPTLC-DPPH* test

The next step was to identify the free radical scavenging compounds using the autography technique. Autography is a simple and rapid technique for the detection of the antioxidant and radical scavenging properties of many plant samples.

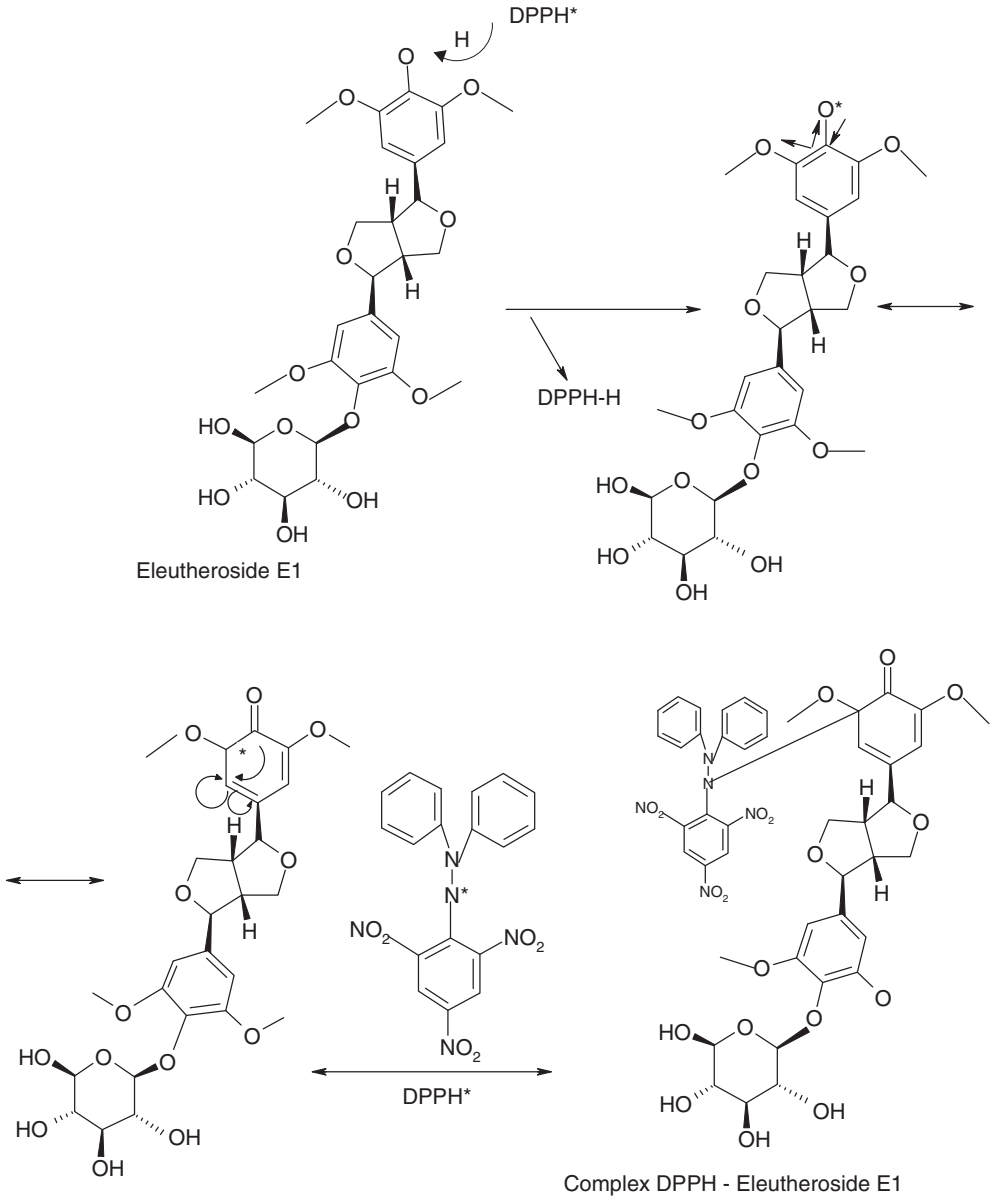

Figure 2 Potential mechanism of the reaction of DPPH* with eleutheroside E1.

Table 2 Localization of active groups in the tested eleutherosides.

Eleutherosides	Number of free OH groups	Substitution position	Number of OCH_3 groups
E1	1	*Para*	2 × 2 (*meta–meta*)
E	0	–	2 × 2 (*meta–meta*)
B	1	Propene chain	2 (*ortho*)

Table 3 Total polyphenol and flavonoid content [mg/g dry extract].

Species	TPC	Flavonoid content		TFC
		$FeCl_3$	DNPH	
E. giraldii	51.4 ± 0.5	4.8 ± 0.11	0.11 ± 0.04	4.91 ± 0.02
E. lasiogyne	49.3 ± 0.5	5.5 ± 0.08	0.17 ± 0.01	5.73 ± 0.03

Regions of the TLC plate which contain DPPH* inhibitors show up as yellow spots against a purple background. In the present study yellow spots were observed after 1 min, 1, 10 and 24 h from the time of immersion of the plate in 0.5% DPPH* solution. Eleutherosides B, E and E1 were used as natural compounds. All of the extracts showed areas of inhibition of DPPH*. Four spots were noticed in the roots of *E. giraldii* and three in the roots of *E. lasiogyne* after 1 min. In each of the tracks on the chromatographic plate one additional spot was detected after 24 h. After 1 min on the Si60 HPTLC-F254 plate, the first yellow spots were observed in extracts from the roots at R_f 0.58 as well as in all of the extracts at R_f 0.37. A yellow-colored inhibitory substance was observed to

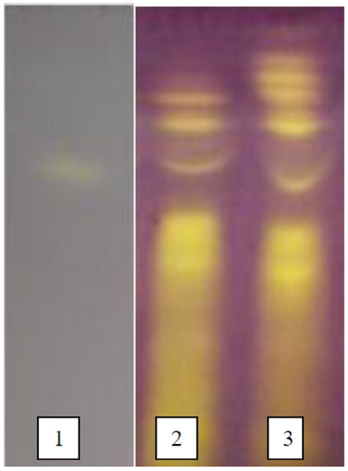

Figure 3 Bioautograph showing the DPPH* scavenging activity after 1 min for standards: 1 – eleutheroside E1; ethanolic extracts from: 2 – the roots *E. giraldii*, 3 – the roots *E. lasiogyne*.

migrate at the same R_f as eleutheroside E1 (R_f 0.58). It is noteworthy that in the spectrophotometric assay, only eleutheroside E1 showed strong antiradical properties, and this was confirmed in the autography test (Fig. 3).

No changes in decolorization of the chromatogram after 1 and 10 h were noticed. Additional yellow bands were observed after 24 h. The R_f value was 0.42 and was the same as for eleutheroside B. Yellow bands were not observed at a high R_f for eleutheroside E. Using the Si60 HPTLC RP18-WF254 plate, the first yellow spot was observed in extracts from the roots at R_f 0.32, and this was the same R_f as for eleutheroside E1. Other yellow bands were observed at a high R_f of 0.15; 0.5 and 0.61. New yellow bands were not determined after 1, 10 and 24 h.

4. Conclusions

One way of evaluating the adaptogenic activity of natural compounds is to determine their ability to reduce DPPH*. On the basis of the obtained results, such properties of the extracts have been observed. In addition, several single compounds of the extract have anti-DPPH* activity, such as eleutheroside E1 and E in the spectrophotometric test and eleutheroside B and E1 in the autography test. *E. giraldii* and *E. lasiogyne* are a promising, natural source of the chemical substances with antioxidant properties, eg. eleutheroside E1. To support the hypothesis, it would be worth to characterize the reaction products using LC/MS in order to determine intermediate antiradical compounds, as well to quantify eleutheroside E1 in those roots.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.sjbs.2016.01.018.

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