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TLC Screening for Eleutherosides B, E, and E1, and Isofraxidin in the Roots of Six *Eleutherococcus* Species Cultivated in Poland

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Summary. Because chemical and pharmacological studies of *Eleutherococcus* species growing in Asia showed their biological activity depends on glycosides known as eleutherosides, we used TLC to search for eleutherosides in ethanol extracts of the roots of *E. senticosus*, *E. setchuensis*, *E. sesiliflorus*, *E. henryi*, *E. gracilistylus*, and *E. divaricatus* cultivated in Poland. Eleutherosides B, E, and E_1 were identified in all the roots investigated and isofraxidin was identified in the roots of *E. senticosus*. Satisfactory separation of the eleutherosides from other components of the extracts was achieved by use of two mobile phases in two-step elution. The identity of the resolved compounds was confirmed by comparing their R_F values and UV spectra with those of standards.

Key Words: TLC, *Eleutherococcus* spp., eleutherosides, solid phase extraction

Introduction

The *Eleutherococcus* genus, which belongs to the *Araliaceae* family, includes approximately 40 species native to Eastern and South Asia. The root and stem bark of these plants have been used for a long time as tonics and prophylactics in oriental herbal medicine [1–4].

Eleutherococcus senticosus (Siberian ginseng, *Acanthopanax senticosus*) is the only species for which monographs can be found in the European or American herbal pharmacopoeias [5]. The active ingredients of this plant are typically concentrated in the roots and mainly consist of chemically distinct glycosides called eleutherosides (eleutherosides A–M). Other components of the roots include ciwujianosides, eleutherans, isofraxidin, β-sitosterol, essential oil, phenylopropanes, and polysaccharides. Eleutherosides B and E constitute about 80% of all glycosides in *E. senticosus* [6–8].

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According to the European Pharmacopoeia monograph, the minimum combined amounts of eleutherosides B and E in the roots of *E. senticosus* should be 0.08% [5].

The chemical compounds (mainly eleuthrosides) isolated from this species have been shown to have wide biological activity. These compounds have anti-stress, anticholesteremic, anti-inflammatory, antioxidant, antibacterial, and immunostimulatory effects [9, 10]. In addition to *Eleutherococcus senticosus*, *E. gracilistylus* and *E. sessiliflorus* have also been used, also in traditional medicine [11, 12]. These species have antitumor, antioxidant, and diuretic properties [13, 14].

Because the therapeutic effect of *Eleutherococc*us depends on the composition of eleutherosides as active ingredients, the objective of this study was identification of eleutherosides in ethanol extracts of the roots of six species of *Eleutherococcus* cultivated in Poland.

Thin-layer chromatography (TLC) is an effective and easy method for detection of compounds in natural mixtures. This method was used as a pilot technique for separation and identification of important plant components. Eleutherosides, besides TLC, have been analyzed by HPLC, ^{1}H NMR, ^{13}C NMR, and MS [15].

Most often it is not easy to separate multicomponent mixtures of an extract. The World Health Organisation cites several methods based on high-performance liquid chromatography for quantitative analysis of the main eleutherosides (B and E) in radix eleutherococci (the dried roots and rhizomes of *E. senticosus*) [16–19]. Chromatographic separation of eleutherosides from the roots of *E. senticosus* by thin-layer chromatography is described in the European Pharmacopoeia. In our work, the mobile phase proposed by Wagner [20] in Ph. Eur. was only partially satisfactory for the species cultivated in Poland. Our methods, which use a modification of Wagner's mobile phase for detection of isofraxidin and two new mobile phases for the detection of eleutherosides B, E, and E_1, are presented below.

Experimental

Reagents and Materials

Eleutherosides B, E, and E_1 and isofraxidin were obtained from ChromaDex (Santa Ana, CA, USA). Ethanol was purchased from Stanlab (Lublin, Poland), and the other solvents used were from Polish Reagents (POCh, Gliwice, Poland). All were of analytical-grade.

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The autumn roots of *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim., *Eleutherococcus setchuensis* (Harms) Nakai, *Eleutherococcus sessiliflorus* (Rupr. & Maxim.) S. Y. Hu, *Eleutherococcus gracilistylus* (W. W. Smith) S. Y. Hu, *Eleutherococcus henryi* Oliv. and *Eleutherococcus divaricatus* (Siebold & Zucc.) S. Y. Hu were collected from the arboretum in Rogów (Poland) in October 2008. Voucher specimens were deposited at the Department of Pharmaceutical Botany, Medical University of Lublin, Poland.

Extraction Procedure and Sample Preparation

Air-dried and powdered roots (15 g) from *Eleutherococcus spp.* were soaked in 150 mL 75% ethanol in a round-bottomed flask for 24 h. The samples were then sonicated in an ultrasonic bath (Polsonic, Warsaw, Poland) at room temperature for 15 min. The extracts were carefully filtered and the plant material was re-extracted for 15 min with 100 mL of the same solvent. The filters through which the samples were filtered were then sonicated with 100 mL 75% ethanol, so 350 mL of each extract was obtained.

The extracts were purified by SPE. Each extract (12 mL) was applied to a Bakerbond octadecyl (C_{18}) SPE microcolumn (500 mg, 3 mL; J.T. Baker, Phillipsburg, 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 to 0.6 mL in a nitrogen stream. Samples prepared in this underwent chromatographic analysis.

Hydrolysis of extracts was performed by the method described by Yuan et al. [21]. Sulphuric acid (15%, 1.9 mL) was added to 20 mL of each extract. The extracts were heated at 100°C for 2 h under reflux. The hydrolysates were extracted three times with 5 mL ethyl acetate and the solvent was evaporated to furnish 1-mL extracts.

Thin-Layer Chromatography

Chromatography was performed on 10 cm × 10 cm glass HPTLC plates coated with 0.25 mm layers of silica gel Si 60 with fluorescence indicator F_{254} (Merck, Darmstadt, Germany). Ethanolic solutions of all standards (1 mg mL^{-1}, 1 µL) and plant extracts (3 µL) were applied to the plates as bands 8 mm long, 30 mm from the left edge, and 8 mm from the bottom, by means of a Camag automatic TLC sampler (Camag, Muttenz, Switzerland). Chromatograms were developed 'face down', at ambient temperature (20 ± 1°C), in the same direction, in horizontal DS chambers (Chromdes, Lublin, Poland) previously saturated with mobile phase vapour for 20 min.

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Chloroform–methanol–water 70:30:4 (*v*/*v*) (P1), chloroform–methanol–toluene–aqueous ammonia 9:6:3:2 (*v*/*v*) (P2), and chloroform–methanol–water 75:25:4 (*v*/*v*) (P3) were used as mobile phases. The extracts were developed twice, first with P1, then with P2. The hydrolysates were developed once with P3. Chromatograms were developed to a distance of 90 mm from the origin. The plates were then dried at room temperature for 20 min.

Spots were visualized under UV light at 254 and 365 nm before and after derivatisation with Liebermann–Burchard reagent. To prepare the reagent, 5 mL sulphuric acid was carefully added to 5 mL acetic anhydride, and this mixture was then mixed with 50 mL 95% ethanol. All the chemicals, used to prepare the regent were ice-cold. The plates were immersed in the reagent for 1 s, then heated at 110°C for 10 min.

Chromatograms were recorded with a TLC 2010 Diode Array Scanner (J & M, Aalen, Germany) working at the wavelengths 200–600 nm (optical resolution was less than 2 nm).

Results and Discussion

Plants of the *Eleutherococcus* genus contain many active compounds, of which eleutherosides are predominant. Ethanolic extracts of six *Eleutherococcus* roots were prepared for investigation of eleutheroside B (syringin 4-β-D-glucoside), eleutheroside E ((−)-siringaresinol 4,4″-*O*-β-D-diglucoside), and eleutheroside E_1 ((+)-siringaresinol 4-*O*-β-D-glucoside) content. Isofraxidin (the aglycone of eleutheroside B_1, isofraxidin 7-*O*-glucoside) was studied in the ethyl acetate fraction obtained after acid hydrolysis. The crude extracts obtained were purified by solid-phase extraction (SPE). This procedure was used to remove ballast substance from the extracts. To confirm complete elution of the eleutherosides from microcolumns, columns were also eluted with 5 mL 75% ethanol. The eluate was evaporated, and the residue dissolved in 0.3 mL 75% ethanol and applied to an HPTLC plate, and the plate was developed with chloroform–methanol–water 70:30:4, then chloroform–methanol–toluene–aqueous ammonia 9:6:3:2. Eleutherosides B, E, and E_1 were eluted specifically and completely from the SPE column with 4 mL 75% ethanol.

In this work thin-layer chromatography was used as a rapid method appropriate for qualitative analysis of eleutherosides. In the first stage, the mobile phase used by Wagner (chloroform–methanol–water 70:30:4) for separation of standards and compounds in plant extracts was used. The chromatogram obtained for these standards was satisfactory. The retardation factors (R_F values) obtained for the eleutherosides and isofraxidin are listed in *Table I*.

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Table I. R_F values of isofraxidin and the eleutherosides

Compound	R_F values		
	Wagner	Modified Wagner (P3)	P1 + P2
Isofraxidin	0.87	0.57	–
Eleutheroside E	0.30	–	0.31
Eleutheroside B	0.37	–	0.44
Eleutheroside E_1	0.52	–	0.57

Wagner's system was modified because of the high R_F of isofraxidin. The modified mobile phase was chloroform–methanol–water 75:25:4 (*v*/*v*) (P3). Isofraxidin was visible as an intense blue zone in UV light at 365 nm.

The eleutherosides, which were not seen in daylight and were poorly visible under ultraviolet illumination (only eleutheroside B is seen in ultraviolet light at 254 nm) were visualized by use of Liebermann–Burchard reagent, giving coloured forms both in daylight and under UV illumination. Use of the same chromatographic conditions for separation of the components of real plant samples was only partially satisfactory. The problem was with chromatography of eleutheroside B, as shown on the chromatogram presented in *Fig. 1*. Polish climatic conditions might have affected, to some

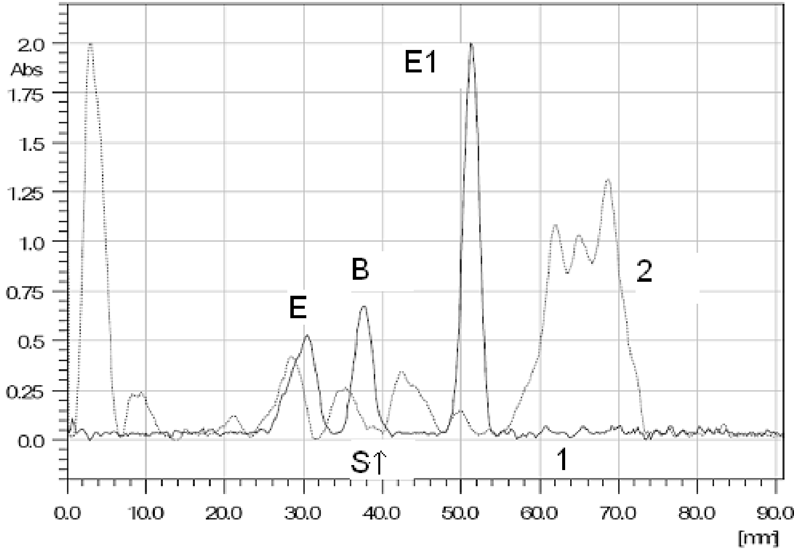

Fig. 1. UV absorption scans of standards and the extract obtained from the roots of *E. senticosus* developed with Wagner's mobile phases (241 nm). 1. Standards (E, eleutheroside E; B, eleutheroside B; E_1, eleutheroside E1); 2. Extract of the root of *E. senticosus* (S, co-eluting substances)

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extent, the chemical components of these Asian species. Probably for this reason, the co-eluting substances marked on the chromatogram as 'S' were present in the extract obtained from *E. senticosus*. A co-eluting substance seen in the chromatogram as an intense blue fluorescent band does not correspond to eleutheroside B (UV, 366 nm). A blue band is directly above eleutheroside B. The R_F value of this band is 0.39.

Two new mobile phases were used for separation of eleutherosides in plant extracts. The plates were developed first with chloroform–methanol–water 70:30:4 (*v*/*v*), then with chloroform–methanol–toluene–aqueous ammonia 9:6:3:2 (*v*/*v*), with drying after each run. Two-step elution with these mobile phases enabled optimum separation of eleutheroside B from other substances. The presence of eleutherosides B, E, and E_1 in the extracts was confirmed by the analysis of UV spectra *(Table II)*. As is apparent from *Fig. 2*, combination of these two mobile phases enabled complete separation of the eleutheroside zones from the mixture. From these chromatographic variables it is apparent that eleutherosides B, E, and E_1 are components of all the species of *Eleutherococcus* investigated (*E. senticosus*, *E. setchuensis*, *E. sesiliflorus*, *E. henryi*, *E. gracilistylus*, and *E. divaricatus*).

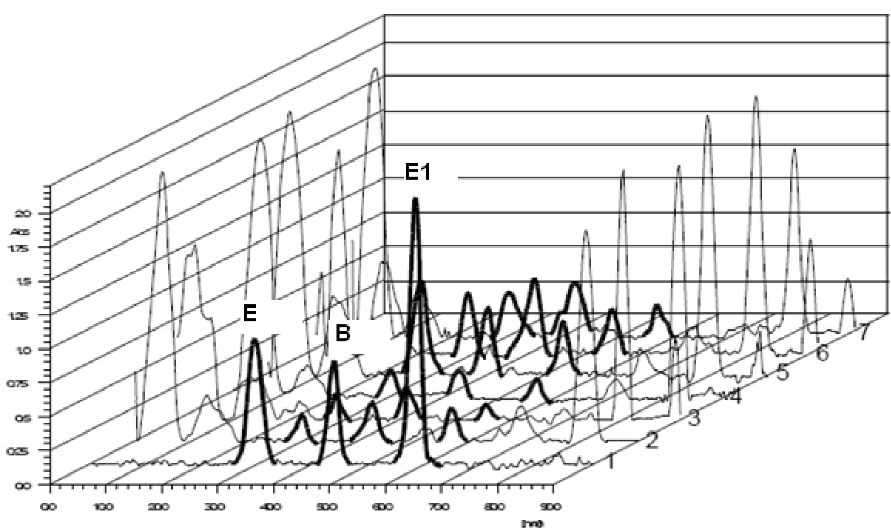

Fig. 2. UV absorption (241 nm) scans of the eleutherosides developed with P1, then P2. 1. Standards (E, eleutheroside E; B, eleutheroside B; E1, eleutheroside E_1. 2–7, extracts from the roots of *E. senticosus*, *E. setchuensis*, *E. henryi*, *E. gracilistylus*, *E. divaricatus*, and *E. sessiliflorus*, respectively

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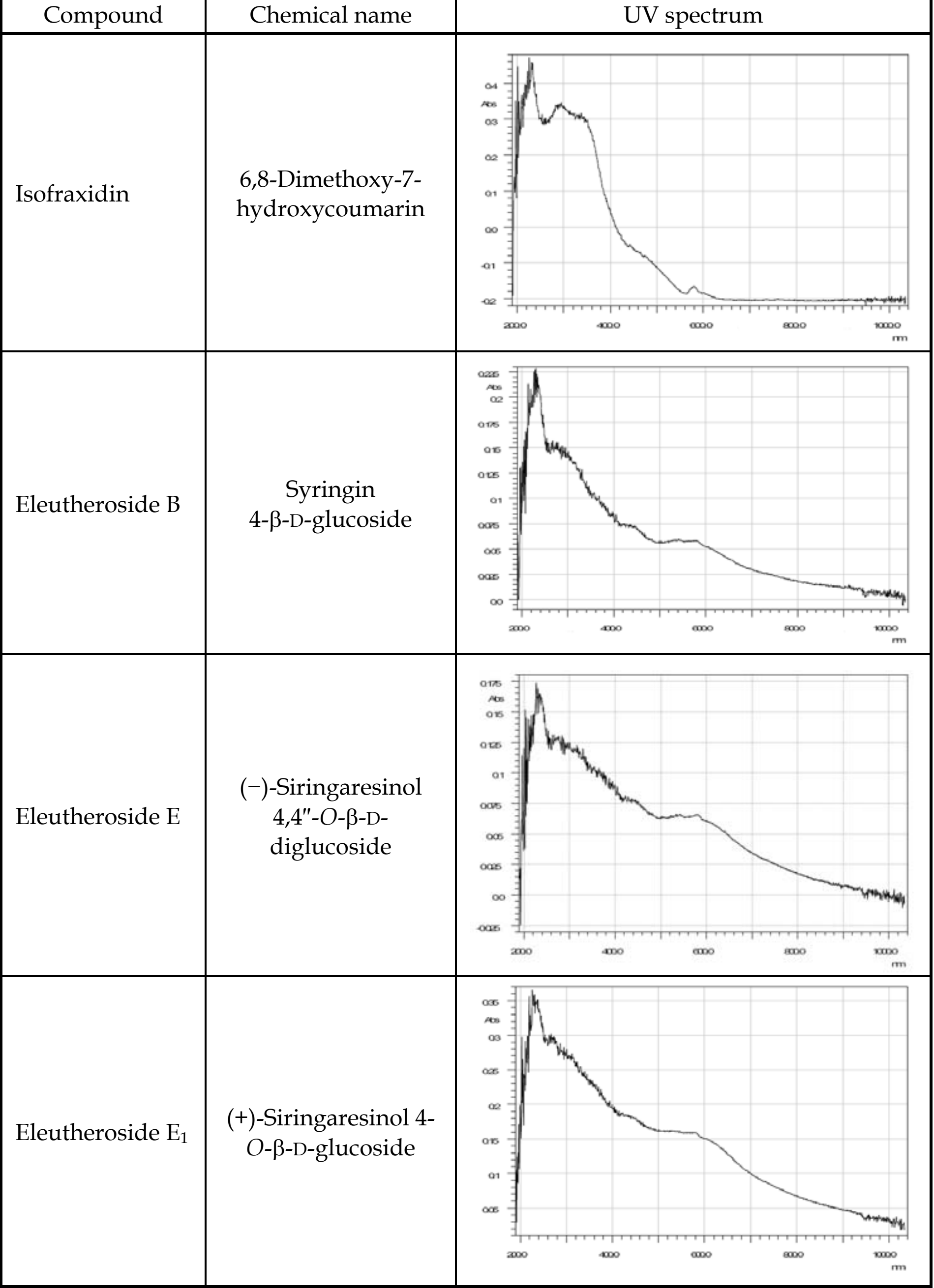
Table II. UV spectra of isofraxidin and eleutherosides B, E, and E1 (the spectra of the eleutherosides were acquired from zones after derivatisation with Liebermann–Burchard reagent)



Compound	Chemical name	UV spectrum
Isofraxidin	6,8-Dimethoxy-7-hydroxycoumarin	
Eleutheroside B	Syringin 4-β-D-glucoside	
Eleutheroside E	(−)-Siringaresinol 4,4″-*O*-β-D-diglucoside	
Eleutheroside E_1	(+)-Siringaresinol 4-*O*-β-D-glucoside	

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Isofraxidin was detected only in the roots of *E. senticosus (Fig. 3)*. This compound has previously been isolated from this plant by Ovodov [22].

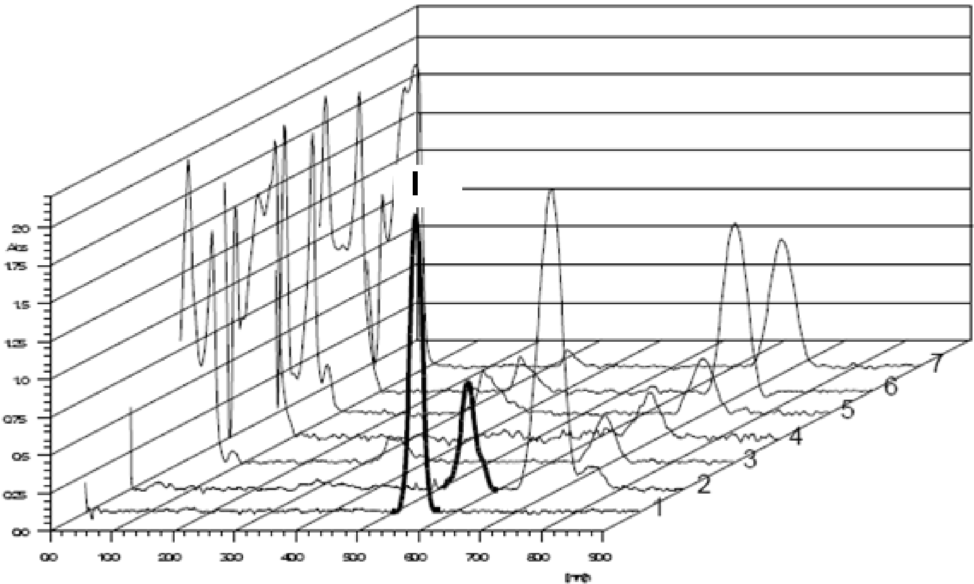

Fig. 3. UV absorption (339 nm) scans of isofraxidin developed with P3. 1. Isofraxidin standard. 2–7, extracts from the roots of *E. senticosus*, *E. setchuensis*, *E. henryi*, *E. gracilistylus*, *E. divaricatus*, and *E. sessiliflorus*, respectively

The literature indicates that eleutheroside E has been found in all these samples, irrespective of geographical origin. Eleutheroside E_1 has been detected in *E. sessiliflorus* only, whereas eleutheroside B is present in all plants except those grown in the Democratic People's Republic of Korea [16–18].

These chromatographic systems are suitable for analysis of eleutherosides B, E, and E_1 in plants of the *Eleutherococcus* genus growing in Poland and might also be helpful for monitoring the quality of the crude drug.

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