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The Isolation of *Eleutherococcus senticosus* Constituents by Centrifugal Partition Chromatography and their Quantitative Determination by High Performance Liquid Chromatography

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An investigation of *Eleutherococcus senticosus* roots, using centrifugal partition chromatography as a key intermediate separation step, has led to the straightforward isolation of eight constituents. Four of these, sinapaldehyde glucoside, coniferaldehyde glucoside, coniferin and 1,5-di-*O*-caffeoylquinic acid, have been described for the first time in this plant. The quantitative analysis of different *E. senticosus* samples has been performed by high performance liquid chromatography using eleutherosides B and E as external standards.

Keywords: *Eleutherococcus senticosus*; Araliaceae; centrifugal partition chromatography; high performance liquid chromatography; phenolics

INTRODUCTION

Eleutherococcus senticosus Rupr. et Maxim. (syn. *Acanthopanax senticosus* Rupr. et Maxim.) Harms (Araliaceae), known also as "Siberian Ginseng", is a shrub found in Russia, Northern China, Korea and Japan. The roots of the plant are considered to have a similar therapeutic action to ginseng and consequently a great deal of interest has been generated in their alcoholic extracts (Sprecher, 1989). At the same time, *E. senticosus* extracts are more or less devoid of adverse toxic effects (Farnsworth *et al.*, 1985). Most of the work on the pharmacological effects of *E. senticosus* has been performed in the Soviet Union: the roots have stimulant and adaptogenic properties, and they can be used to treat diabetes and to alleviate the side-effects of clinical anticancer drugs (Baranov, 1982). Marked immunostimulant effects have also been observed (Sprecher, 1989). Despite these results, satisfactory explanations for the adaptogenic and other pharmacological actions are still lacking in most cases.

A certain amount of work has been undertaken to characterize the constituents of root extracts and, amongst others, the following compounds have already been isolated: eleutheroside A (daucosterol), eleutheroside B (syringin) (**4**), eleutheroside B_1 (isofraxidin 7-*O*-glucoside) (**2**), eleutheroside B_4 [(−)-sesamin], eleutheroside C (methyl α-D-galactoside), eleutheroside E (liriodendrin, syringaresinol di-*O*-β-D-glucoside) (**6**), chlorogenic acid (5-caffeoylquinic acid) (**7**), isofraxidin, (−)-syringaresinol, caffeic acid, caffeic acid ethyl ester, coniferylaldehyde, sinapyl alcohol, β-sitosterol, oleanolic acid (Wagner *et al.*, 1982; Farnsworth *et al.*, 1985). It is claimed that eleutheroside E has the most pronounced stimulant and anti-stress effects of all the *E. senticosus* glycosides tested (Breckhman and Dardymov, 1969).

Concerning the analysis of *E. senticosus* constituents, some work has appeared on the spectrophotometric or fluorimetric determination of mixtures of the glycosidic components (Lapchik *et al.*, 1969; Solov'eva *et al.*, 1989) or of the individual glycosides after scraping bands off thin layer chromatographic (TLC) plates (Baevskii *et al.*, 1982). Preliminary investigations of TLC and high performance liquid chromatographic (HPLC) conditions for the qualitative and quantitative determination of certain extracts of different origin were carried out (Wagner and Wurmböck, 1977; Wagner *et al.*, 1982; Vanhaelen and Vanhaelen-Fastré, 1984) and very recently a more detailed TLC and HPLC investigation of *E. senticosus* and other *Acanthopanax* species has been reported (Bladt *et al.*, 1990). In this last communication, various commercial extracts and preparations were analysed, as well as dried plant material from China, Korea and Russia.

The present contribution describes the preparative-scale separation of *E. senticosus* root constituents and their use in the quantitative evaluation of *E. senticosus* extracts and pharmaceutical preparations. In view of the variation in content of the constituents with geographical location of the plant, the development of efficient standardization methods is essential for quality control purposes.

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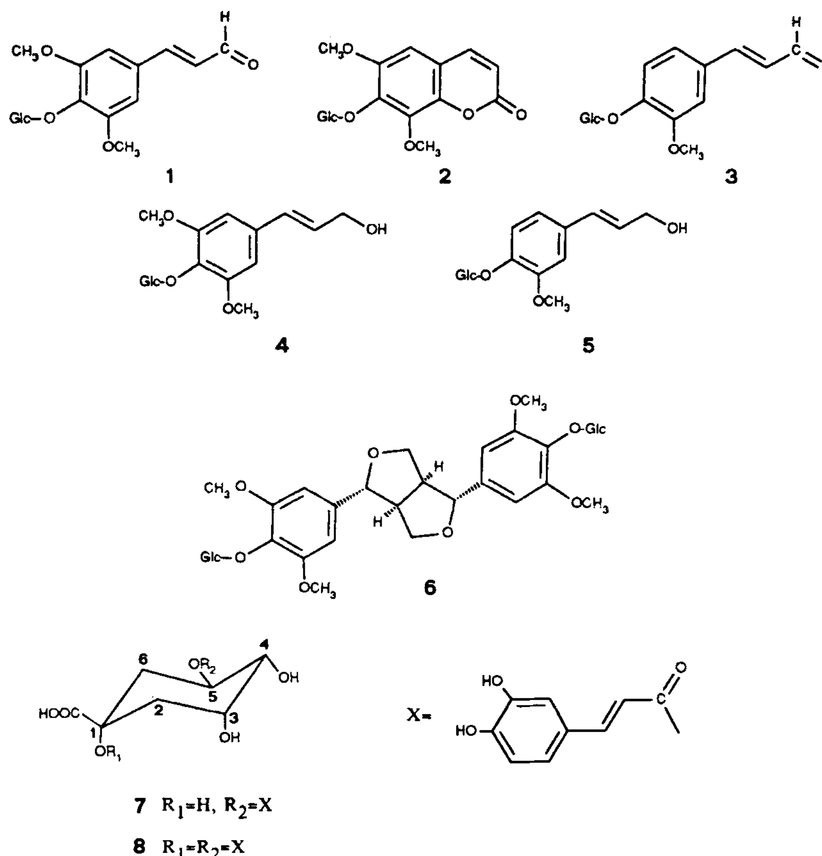

EXPERIMENTAL

Analytical conditions. TLC was carried out on silica gel precoated Al sheets (Merck) with chloroform: methanol: water (70:30:4). Detection was with Godin reagent (Godin, 1954). For normal phase column chromatography (CC), silica gel 60 (63–200 μm) was used. Low pressure liquid chromatography (LPLC) was performed on Lobar size B RP-18 columns (40–63 μm) at 3 mL/min with a Duramat-80 pump. For semipreparative HPLC separations, a μBondpak C_{18} column (10 μm; 10 × 300 mm) was used. The solvent flow-rate was 3 mL/min. Liquid–liquid separations (centrifugal partition chromatography; CPC) were carried out at ca. 20 °C with a multilayer coil separator–extractor (P.C. Inc., Potomac, MD, USA), equipped with a 2.6 mm ID coil (volume 360 mL) and sample loop. The chromatograph was connected to two Waters 6000A pumps, one for each phase of the biphasic solvent system (Slacanin *et al.*, 1989). Rotation was at 700 rpm and detection at 254 nm, with the solvent system chloroform: methanol: water (7:13:8), using the lower phase as the mobile phase at a flow-rate of 3 mL/min.

Qualitative HPLC analyses were performed on a μBondpak 10 μm C_{18} column (300 × 3.9 mm) with a gradient of acetonitrile: water (+0.05% TFA): 10% to 50% acetonitrile over 30 min, at a flow-rate of 1 mL/min. The mobile phase was delivered by a Spectra-Physics SP8750 pump. For detection at 220 nm, a photodiode array detector (HP1040A, Hewlett Packard) coupled with a HP85 personal computer were used.

Quantitative HPLC analyses were carried out on a Spherisorb ODS 5 μm (250 × 4.6 mm) column, using isocratic elution with acetonitrile: water (15:85, v/v; adjusted to pH 2.5 with phosphoric acid). The flow-rate was 1 mL/min. A Varian 2010 pump, connected to a Rheodyne 7125 sample injector (10 μL sample loop), was used for solvent delivery. Detection at 220 nm was with a Varian 2050 variable wavelength detector, peak areas being measured with a Spectra-Physics 4290 integrator.

Spectral data. UV spectra were measured in MeOH. NMR spectra were recorded at 50.1 MHz for ^{13}C and 200 MHz for ^{1}H. Desorption/chemical ionization mass spectra (D/CIMS) were recorded on a Ribermag R 10-1013 quadrupole instrument with NH_3 as reactant gas. Electron impact mass spectra (EIMS) were measured at 70 eV.

Plant material. Dried roots of *E. senticosus* for isolation work (Sample no. 8; see Table 1) were obtained from Heinrich Ambrosius GmbH (Hamburg, Germany) in 1988. The crude drug originated from Siberia. Other commercial samples of dried roots for analytical purposes were purchased from different French and German companies in 1989. The three fluid preparations (extracts) of *Eleutherococcus* were obtained from pharmacies in Switzerland and the Federal Republic of Germany.

Extraction and identification of constituents. Powdered roots (300 g) of *E. senticosus* were treated with dichloromethane and then with methanol. Column chromatography of the methanol extract (15 g) over silica gel (chloroform: methanol: water, 70:30:4) gave five fractions (I–V). CPC of Fraction II (660 mg), followed by LPLC with methanol: water (40:60) gave sinapaldehyde glucoside (**1**) (22 mg). CPC of Fraction III (710 mg) yielded three fractions (IIIA–IIIC). LPLC of IIIA with methanol: water (15:85) gave eleutheroside B_1 (**2**) (35 mg) and impure coniferinaldehyde glucoside (**3**), which was obtained in the pure state (18 mg) after semipreparative HPLC on RP-18 with methanol: water (15:85). Eleutheroside B (**4**) (30 mg) was isolated from

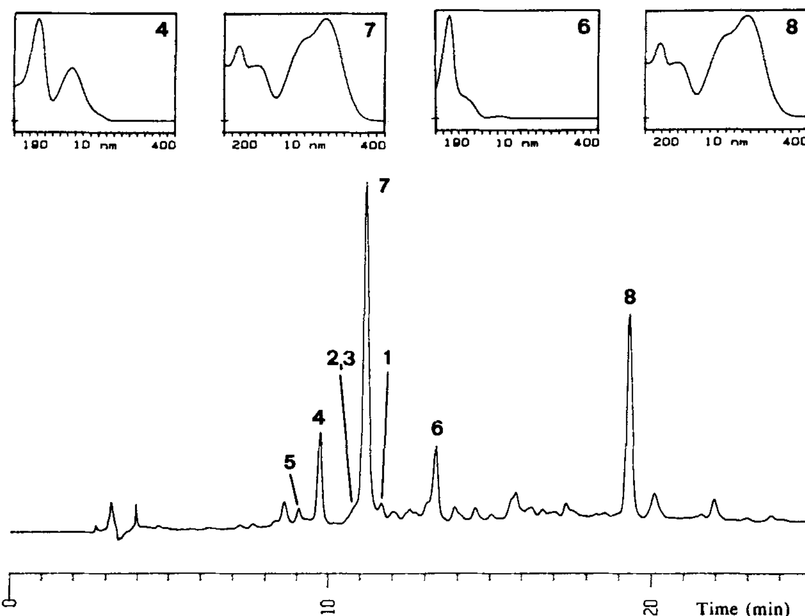

Figure 1. Qualitative HPLC analysis of the methanol extract of *E. senticosus* roots, Sample no. 8 (μBondpak C_{18}; $CH_3CN:H_2O$ (+0.05% TFA) 10:90 to 50:50 over 30 min; 1 mL/min; detection 220 nm). Peak numbers refer to structures mentioned in the text.

Fraction IIIB by LPLC (methanol:water, 20:80) and coniferin (**5**) (25 mg) from fraction IIIC by LPLC (methanol: water, 20:80). CPC of fraction IV (1300 mg) yielded eleutheroside E (**6**) (35 mg).

For the isolation of chlorogenic acid (**7**) and 1,5-di-*O*-caffeoylquinic acid (**8**), methanol extract (6 g) was passed through a column of Sephadex LH-20 (in methanol), to give five fractions (I–V). Chlorogenic acid (**7**) (300 mg) and **8** (8 mg) were obtained from fractions III and V, respectively, by LPLC (methanol:water, 15:85), while saccharose (1.2 g) crystallized from Fraction II.

Eleutheroside B (**4**), coniferin (**5**), eleutheroside E (**6**) and chlorogenic acid (**7**) were identified by comparison (UV, MS, ^1H and ^{13}C NMR) with authentic samples. The diequatorial configuration of the substituents on the lignan moiety of eleutheroside E (**6**) was confirmed by ^1H and ^{13}C NMR spectroscopy (Jolad *et al.*, 1980).

Sinapaldehyde glucoside (**1**). D/CIMS *m/e* (rel. int.): 209 $[(M+H)-162]^+$ (76), 180 (100). UV λ_{MAX} (nm): 205, 238, 315. IR ν_{max} (cm^{-1}): 3350, 1650, 1580, 1120, 1060, 1020, 810. ^1H NMR (CD_3OD): δ 3.20–3.90 (6H, m, H-2′, H-3′, H-4′, H-5′, H-6′a, H-6′b), 3.91 (6H, s, OMe), 4.97 (1H, d,

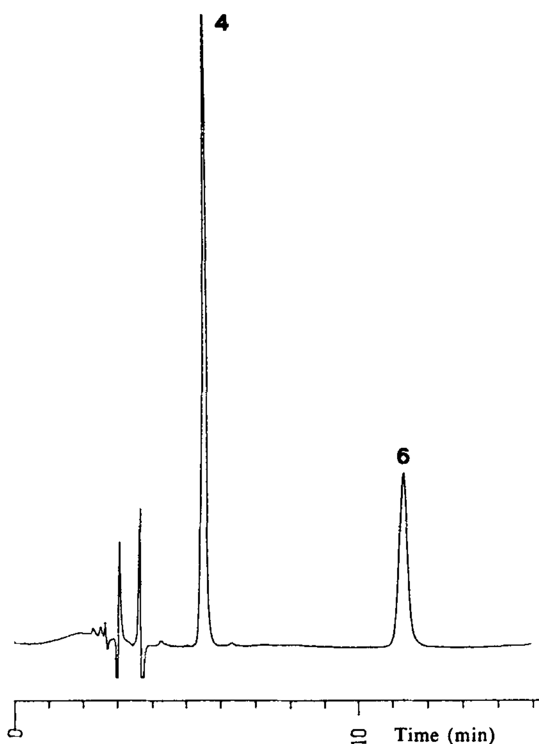

Figure 2. HPLC separation of reference compounds eleutherosides B (**4**) and E (**6**) (Spherisorb ODS; $CH_3CN:H_2O$ 15:85 (+H_3PO_4); 1 mL/min; detection 220 nm). Peak numbers refer to structures mentioned in the text.

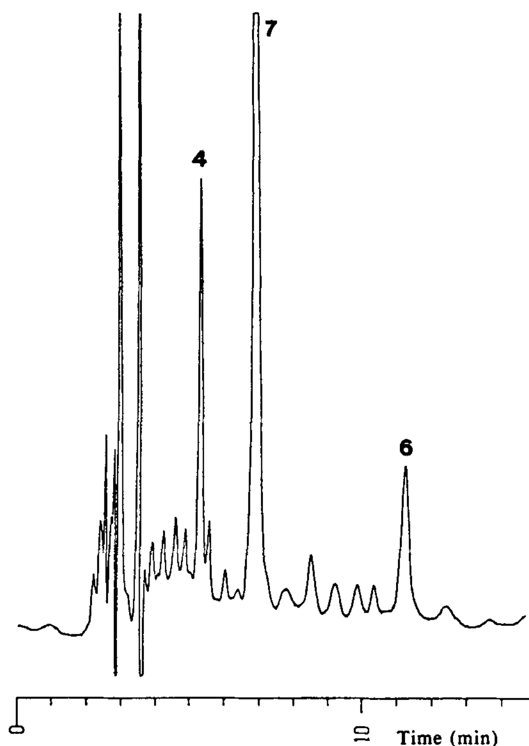

Figure 3. Quantitative HPLC analysis of eleutherosides B (**4**) and E (**6**) from *E. senticosus* root methanol extract, Sample no. 8 (Spherisorb ODS; $CH_3CN:H_2O$ 15:85 (+H_3PO_4); 1 mL/min; detection 220 nm; sample size 10 μL). Peak numbers refer to structures mentioned in the text.

Table 1. Contents of eleutherosides B and E (%) in different commercial samples of *E. senticosus* root

Compound	Samples[a] 1	2	3	4	5	6	7	8	9	10
Eleutheroside B	0.178	0.158	0.160	0.143	0.142	0.107	0.098	0.083	0.055	—
Eleutheroside E	0.113	0.111	0.109	0.109	0.102	0.120	0.110	0.103	0.100	0.123
Total	0.291	0.269	0.269	0.252	0.244	0.227	0.208	0.186	0.155	0.123

[a]Origin of samples: 1–5 (China), 6–9 (Siberia), 10 (Korea).

$J=7.2$ Hz, H-1′), 6.72 (1H, dd, J = 16, 6.6 Hz, H-8), 7.00 (2H, s, H-2, H-6), 7.58 (1H, d, $J=16$ Hz, H-7), 9.64 (1H, d, $J=6.6$ Hz, H-9). ^{13}C NMR (d_6-DMSO): δ 56.49 (OMe), 60.79 (C-6′), 69.87 (C-4′), 74.15 (C-2′), 76.61 (C-3′), 77.37 (C-5′), 102.02 (C-1′), 107.13 (C-2, C-6), 127.92 (C-7), 129.38 (C-1), 136.87 (C-4), 152.76 (C-3, C-5), 153.45 (C-8), 194.26 (C-9).

Eleutheroside B_1 (**2**). D/CIMS *m/e* (rel. int.): 402 $[M+NH_4]^+$ (0.13), 223 $[(M+H)-162]^+$ (100), 180 (11). UV λ_{max} (nm): 220, 241, 306, 329. IR ν_{max} (cm^{-1}): 3400, 1695, 1560, 1125, 1100, 1080, 1020, 925, 870, 820. ^{1}H NMR (d_6-DMSO): δ 3.10–3.79 (6H, m, H-2′, H-3′, H-4′, H-5′, H-6′a, H-6′b), 3.82 (3H, s, OMe), 3.92 (3H, s, OMe), 5.17 (1H, d, $J=7.3$ Hz, H-1′), 6.41 (1H, d, $J=9.6$ Hz, H-3), 7.13 (1H, s, H-5), 7.96 (1H, d, $J=9.6$, Hz, H-4). ^{13}C NMR (d_6-DMSO): δ 56.55 (OMe), 60.76 (C-6′), 61.27 (OMe), 69.85 (C-4′), 74.09 (C-2′), 76.47 (C-3′), 77.48 (C-5′), 102.12 (C-1′), 105.46 (C-5), 114.50 (C-3), 114.71 (C-10), 140.22 (C-8), 141.63 (C-7), 142.36 (C-9), 144.38 (C-4), 149.40 (C-7), 159.79 (C-2).

Coniferaldehyde glucoside (**3**). D/CIMS *m/e* (rel. int.): 358 $[M+NH_4]^+$ (1.04), 341 $[M+H]^+$ (3.49), 179 (100). UV λ_{max} (nm): 209, 229, 292, 344, IR ν_{max} (cm^{-1}): 3400, 1650, 1590, 1265, 1130, 1090, 1070, 800. ^{1}H NMR (d_6-DMSO): δ 3.10–3.80 (6H, m, H-2′, H-3′, H-4′, H-5′, H-6′a, H-6′b), 3.83 (3H, s, OMe), 5.03 (1H, d, $J=7.6$ Hz, H-1′), 6.84 (1H, dd, $J=16$, 7 Hz, H-8), 7.15 (1H, d, $J=8.4$ Hz, H-5), 7.27 (1H, dd, $J=8.4$, 2 Hz, H-6), 7.40 (1H, d, $J=2$ Hz, H-2), 7.66 (1H, d, $J=16$ Hz, H-7), 9.62 (1H, d, $J=7$ Hz, H-9). ^{13}C NMR (d_6-DMSO): δ 55.74 (OMe), 60.57 (C-6′), 69.54 (C-4′), 73.07 (C-2′), 76.80 (C-3′), 77.08 (C-5′), 99.46 (C-1′), 111.42 (C-2), 114.88 (C-5), 123.23 (C-6), 126.94 (C-7), 127.94 (C-1), 149.14 (C-3), 149.18 (C-4), 153.40 (C-8), 194.18 (C-9).

1,5-Di-O-caffeoylquinic acid (**8**). D/CIMS *m/e* (rel. int.): 516 $[M]^+$ (0.86), 354 $[M-162]^+$ (0.82), 192 $[M-324]^+$ (23), 163 (62), 137 (100). UV λ_{max} (nm): 217, 235, 298, 327. IR ν_{max} (cm^{-1}): 3350, 1680, 1590, 1510, 1160, 1115, 820. ^{1}H NMR (d_6-DMSO): δ 1.80–2.60 (4H, m, H-2′a, H-2′b, H-6′a, H-6′b), 3.61 (1H, dd, H-4′), 4.08 (1H, ddd, H-3′), 5.22 (1H, ddd, H-5′), 6.20 (2H, d, H-8), 6.77 (2H, d, H-5), 6.98 (2H, dd, H-6), 7.02 (2H, d, H-2), 7.48 (2H, d, H-7). ^{13}C NMR (d_6-DMSO): δ 36.35, 37.32 (C-2′, C-6′), 68.19, 70.49, 71.00, 73.60 (C-1′, C-3′, C-4′, C-5′), 114.42, 114.88, 115.89, 121.54, 125.72 (C-1, C-2, C-5, C-6, C-7), 145.10 (C-8), 145.66, 148.45 (C-3, C-4), 165.87 (C-9), 175.08 (COOH).

Quantitative HPLC analyses. For sample preparation, 0.500 g pulverized plant material was extracted twice with 80 mL methanol: water (80:20, v/v) at 60 °C for 15 min. After filtration, the filtrate was adjusted to a final volume of 200.0 mL in a volumetric flask. A standard solution containing 0.05 mg/mL of eleutherosides B and E in methanol: water (80:20, v/v) was also prepared and 1 mL of this solution was diluted to 10 mL with the same solvent. A linear relationship between peak area and concentration (1–10 μg/mL) was observed with a correlation coefficient $r=0.9998$ for each glycoside. The relationship between peak areas (y) and concentrations in μg/mL (x) was $y=16861x-1384$ (**4**) and $y=9593x-842$ (**6**). The minimum detection limit was 0.2 ng (**4**) and 1 ng (**6**), which resulted in a signal-to-noise ratio of 3:1. Reproducibility was verified with 10 extracts of identical sample. Relative standard deviations (%) were 5.16% and 5.57%, respectively. Peak purity was checked by examining the UV spectra at different points of the peak with a photodiode array detector. The results were not significantly modified for different extraction solvent mixtures down to methanol: water 20:80 (v/v).

Table 2. Contents of eleutherosides B and E (mg/mL) in commercial *E. senticosus* tinctures

Compound	Samples A	B	C
Eleutheroside B	0.080	0.050	—
Eleutheroside E	0.078	—	0.051

RESULTS AND DISCUSSION

Isolation of pure compounds

Extraction of *E. senticosus* roots (Sample no. 8) was performed first with dichloromethane and then with methanol. A butanol: water partition step was avoided since virtually no eleutheroside E (**6**) is extracted into the butanolic phase. Instead, **6** remains in the aqueous portion, together with some eleutheroside B (**4**), an observation consistent with the results of Jolad *et al.* (1980). The methanol extract was first fractionated by open column chromatography on silica gel and six (**1–6**) pure compounds were isolated by a combination of CPC, LPLC and HPLC. Eleutheroside E (**6**) was obtained by CPC in a single step after preliminary silica gel column chromatography. The two caffeoylquinic acid derivatives **7** and **8** could not be isolated by the same procedure as that used for the other six compounds because they remained adsorbed on the silica gel column. Instead, they were purified from the methanol extract by gel filtration and LPLC.

The dicaffeoyl derivative **8** was easily distinguished from cynarin (1,3-di-*O*-caffeoylquinic acid; IUPAC nomenclature recommendations) (Clifford, 1986) by analysis of the corresponding NMR spectra (Horman *et al.*, 1984). Furthermore, the retention times in HPLC analysis of an authentic sample of cynarin were very different from those of **8**. The ^{1}H NMR spectrum of **8** was compared with those of authentic samples of quinic acid and cynarin (all in d_6-DMSO). While the signal (ddd) for H-3 at 3.88 ppm in quinic acid was shifted downfield to 5.31 ppm in cynarin (cf. Horman *et al.*, 1984; Clifford, 1986), the signal for H-5 (ddd) at 3.76 ppm is shifted downfield to 5.22 ppm in **8**. This results from acylation of the hydroxyl group adjacent to

H-5 (Corse *et al.*, 1965; Clifford, 1986). As both the H-3 and H-4 signals in **8** are only slightly shifted in comparison to the equivalent signals in quinic acid, the second acylation position is at OH-1. The yield of **8** after purification was very low, resulting from the formation of artefacts during the separation. Easy migration of caffeoyl groups under mild conditions from positions 1, 3, 4 and 5 on the quinic acid moiety is well documented (Haslam *et al.*, 1964) and this is presumably the situation encountered with **8**. This dicaffeoylquinic acid has previously been found in the artichoke, *Cynara scolymus* (Asteraceae) (Michaud, 1967).

Sinapaldehyde glucoside (**1**), first isolated from *Fraxinus griffithii* (Oleaceae) (Sutarjadi *et al.*, 1978), is reported here for the first time as a constituent of *E. senticosus* roots. Similarly, there has been no previous mention of the occurrence of coniferaldehyde glucoside (**3**), coniferin (**5**) (Podimuang *et al.*, 1971) or 1,5-dicaffeoylquinic acid (**8**) in the plant. Coniferaldehyde glucoside (**3**) has been synthesized for biosynthetic studies on lignans (Stöckigt and Klischies, 1977).

The exocyclic double bonds of **1**, **3**, **4** and **5** all exhibit *trans* stereochemistry, as deduced from the coupling constants of the H-7 and H-8 protons ($J = 16–17$ Hz) in the ^{1}H NMR spectra.

The ^{1}H-NMR spectrum of eleutheroside B_1 (**2**) gave a doublet at 5.17 ppm for the anomeric proton of the glucose moiety. The magnitude of the coupling constant ($J = 7.3$ Hz) suggested that, contrary to the previously assigned 7-*O*-α-L-glucoside structure (Ovodov *et al.*, 1967), eleutheroside B_1 is in fact isofraxidin 7-*O*-β-D-glucoside (calycanthoside). This result was confirmed by enzymatic hydrolysis with β-D-glucosidase.

Qualitative HPLC analysis of *E. senticosus*

The chromatogram of the methanol extract of *E. senticosus* roots used for extraction of the pure compounds is shown in Fig. 1, together with the UV spectra corresponding to the major constituents. Chlorogenic acid (**7**) and 1,5-di-*O*-caffeoylquinic acid (**8**) dominate the chromatogram, with eleutherosides B (**4**) and E (**6**) assuming secondary importance. Eleutheroside B_1 (**2**) and coniferaldehyde 4-*O*-glucoside (**3**) are masked by the peak due to chlorogenic acid and are only properly visible after passage of the methanolic extract over silica gel, which effectively removes the quinic acids.

Quantitative determination of eleutherosides B and E in root samples of *E. senticosus*.

Reversed phase HPLC has been used to quantitate eleutherosides B and E in commercial samples of *E. senticosus*. For the standardization of raw materials and the various preparations, coevaluation of eleutherosides B and E is recommended for two reasons. First, the two phenolic glycosides represent about 80% of the total glycosidic derivatives eleutherosides A–G (Lapchik *et al.*, 1969). Second, one or the other of eleutherosides B and E may be absent in a particular drug charge.

Quantitative determination was carried out under isocratic conditions using external standards for calibration. The chromatogram of a mixture of the reference compounds eleutheroside B (**4**) and eleutheroside E (**6**) is shown in Fig. 2. A typical chromatogram for the analysis of an extract is shown in Fig. 3. The analysis of different commercial sources of *E. senticosus* showed qualitative and quantitative variations (Table 1). Eleutheroside B was found to be the predominant component in samples originating from China (Samples 1–5). The phenylpropane derivative was, however, present in lower yields than eleutheroside E in the other commercial samples originating from Siberia (Samples 6–9) and could not even be detected in the Korean sample (no. 10).

The contents of eleutherosides B and E in three widely available tinctures are shown in Table 2. While Sample A contained both eleutherosides, Sample B contained syringin, and only syringaresinol di-*O*-β-D-glucoside could be detected in C. The contents of eleutherosides B and E in the two latter samples were lower than in the first, thus indicating that there are some considerable differences among the compositions of commercially available preparations.

CONCLUSIONS

In conclusion, both preparative and analytical aspects of *E. senticosus* root constituents have been presented here. Before suitable analytical procedures can be set up, a source of reference substances is needed for identification and quantitation purposes. This has been achieved by a combination of liquid–liquid and liquid–solid chromatographies. Using the isolated constituents, and particularly eleutherosides B and E, a suitable procedure has been developed for the reproducible quantitation of these substances in *E. senticosus* roots and extracts.

Acknowledgements

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