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Chemical composition, antioxidative activity and cell viability effects of a Siberian pine (*Pinus sibirica* Du Tour) extract

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ABSTRACT

Siberian pine (*Pinus sibirica* Du Tour) seeds, commonly known as cedar nuts, are ascribed a number of medicinal properties. In this study, we report the qualitative–quantitative composition, antioxidant activity and cell viability-related properties of a defatted aqueous-acetone-soluble *P. sibirica* seed extract. The total phenolic and total tannin contents were estimated at 266 ± 3.9 mg gallic acid/g and 115 ± 7.8 mg tannic acid/g, respectively. Reverse-phase chromatographic analysis of the crude extract indicated the presence of a chromatographic hump indicative of the presence of proanthocyanidins. After acid hydrolysis, the presence of hydroxylated benzoic and cinnamic acids, flavanones and flavan-3-ols was confirmed. After thiolysis, (+)-catechin was identified as more abundant than (−)-epicatechin, suggesting that this molecule was the main terminal unit of the proanthocyanidins within this extract. The extract demonstrated iron(III)-reductive (*AscAE* = 650 ± 5.10 μmol ascorbic acid/g) and iron(II) chelating (EC_{50} = 20.1 ± 2.1) activities and the ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (IC_{50} = 257 ± 2.36 μg/ml) and hydroxyl (IC_{50} = 338 ± 6.49 μg/ml) free radicals. When the effects of *P. sibirica* extract were assessed in a tumourigenic SH-SY5Y neuroblastoma cell line, it was found that the cell viability was diminished in the presence of *P. sibirica* extract (0.2–1.0 mg/ml), as indicated by decreased membrane integrity (LDH assay) and mitochondrial metabolic activity (MTT assay), but the level of p53 protein was not changed (Western blot).

1. Introduction

Pinus sibirica Du Tour (Siberian pine) belongs to the order Pinales, family Pinaceae and subgenus *Strobus*. It is an evergreen tree of 35–40 m height and has a trunk diameter of up to 1.8 m at full maturity. It predominantly grows in Siberia, the Urals and northern parts of Europe. Similar to other members of the *Strobus* subgenus, its needles are clustered inside a deciduous sheath and are approximately 5–10 cm in length. Siberian pine cones are approximately 5–9 cm long, whilst seeds can grow up to 9–12 cm in length. Traditionally known as cedar nuts, the seeds of *P. sibirica* are considered to be a valuable medicinal raw plant material, being ascribed a wide spectrum of traditional properties. For example, a decoction is suggested for rheumatism and arthritis, whilst an infusion is prescribed for neurosis, liver and kidney disorders (Shikov, Makarov, & Ryzhenkov, 2004). The pharmacological actions accredited to *P. sibirica* seeds are related to the presence of both lipophilic and hydrophilic bioactive substances, viz, terpenes, fatty acids and triacylglycerides, vitamins, polyphenols, amino acids and macro/trace elements (Deineka, 2003; Kosman et al., 2001; Makarov, Shikov, Pozharitskaya, Fomicheva, & Fomichev, 2000; Pozharitskaya et al. 2007; Shikov et al., 2004; Skakovskii et al., 2007; von Schantz & Hiltunen, 1983).

Experimental data pertaining to the benefical properties of *Pinus* species in general primarily relate to their anti-inflammatory (Rohdewald, 2002), antioxidant (Guri, Kefalas, & Roussis, 2006; Vuorela et al., 2005), antineoplastic (Li et al., 2007; Potta, Doss, Hescheler, & Sachinidis, 2005) and immuno-modulatory (Li et al., 2007; Rohdewald, 2002) properties, related to their effects upon cyclooxygenase activity (Potta et al., 2005), prostaglandin E_2 production (Karonen et al., 2004; Vuorela et al., 2005), nitric oxide synthesis (Karonen et al., 2004; Shashi, Jaswant, Madhusudana, Kumar, & Nabi, 2006; Virgili, Kobuchi, & Packer, 1998) and regulation of cancer-related proteins (Li et al., 2007) amongst others.

In this study, we have investigated in parallel the chemical composition, antioxidant capacity and effects upon cell viability of a *P. sibirica* seed extract. The purpose of this study was (i) to

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screen an aqueous-acetone extract for antioxidant (ferric reducing, ferrous chelating and free-radical scavenging) properties, (ii) to examine its effects on the cell viability (membrane integrity, metabolic activity and p53 expression) using human SH-SY5Y neuroblastoma cells and (iii) complement this data with a compositional (total phenol and tannin content, in addition to a qualitative-quantitative compositional analysis, facilitated by acid hydrolysis and thiolysis) analysis. The authors believe this information contributes to the limited scientific knowledge relating to *P. sibirica* seeds and may stimulate further scientific study into this rather abundant natural resource.

2. Materials and methods

2.1. Plant material and reagents

Pinus sibirica seeds were obtained from Vigado (Moscow, Russia). A voucher specimen (No. 225) was deposited at the Interregional Center Adaptogen, St. Petersburg, Russia. Chromatographic standards were purchased from Extrasynthese (Genay, France) or Fluka (Buchs, Switzerland). Ultrapure water was used throughout and was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., Billerica, MA). All remaining reagents were of the highest purity available and obtained from the Sigma Chemical Company (St. Louis, MO).

2.2. Extract preparation

Eighty grams of *P. sibirica* seeds were finely ground and defatted with hexane using a Soxhlet apparatus for 8 h. After vacuum filtration, the defatted marc was air-dried then macerated with 300 ml (95:5, v/v) acetone:water at 45 °C for 4 h. Thereafter, the process was repeated but for 20 h at ambient temperature. The resulting extracts were combined, the solvent was removed *in vacuo* (40 °C) and the residue was freeze-dried.

2.3. Acid hydrolysis

In brief, 2 ml of dissolved extract (10 mg/ml) was heated with 180 μl concentrated HCl for 1 h at 95 °C then diluted with 4 ml H_2O and centrifuged at 3000 rpm for 3 min. The supernatant was extracted with 3 × 10 ml of (1:1, v/v) EtOAc/*n*-hexane. The organic layers were pooled and concentrated to 3 ml at 35 °C, then evaporated to dryness under N_2. The residue was dissolved in 1 ml MeOH, filtered (0.45 μm) and analysed by HPLC-PDA.

2.4. Thiolysis

Thiolysis was carried out, as described by Jerez, Pinelo, Sineiro, and Núñez (2006). In brief, the extract was dried over P_2O_5 and dissolved in MeOH to a concentration of 10 mg/ml. A 100 μl aliquot was mixed with 100 μl MeOH, acidified with concentrated HCl, and 200 μl (5%, v/v) toluene-α-thiol in MeOH. The reaction mixture was placed in a sealed test tube, heated at 40 °C for 30 min and then cooled in a water bath. The average degree of polymerisation (DPn) was estimated using

$$\mathrm{DPn} = \left[\frac{\text{Total area of benzylthioethers}}{\text{Total area of catechin and epicatechin}}\right] + 1. \quad (1)$$

2.5. Total phenolic content

The total phenol content of the extract was estimated according to the Folin–Ciocalteu reagent method (Singleton, Orthofer, & Lamuela-Raventos, 1999). In brief, 0.25 ml of sample was transferred to a 25.0 ml volumetric flask containing 6 ml of H_2O, to which was added 1.25 ml Folin–Ciocalteu reagent. After 1 min, 3.75 ml of 20% (w/v) aqueous Na_2CO_3 was added and the volume was made up to 25.0 ml with H_2O. The controls contained all the reagents except the extract. After 2 h at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. The data are presented as mean values ± standard deviation (n = 3).

2.6. Total tannin content

The total tannin content of the extract was estimated according to the USSR State Pharmacopoeia (Anon, 1989). In brief, the extract was dissolved in H_2O and a 25 ml (1 g/125 ml) aliquot was mixed with 500 ml H_2O and 25 ml indigo sulphuric acid. The resulting solution was then titrated using a 0.02 M $KMnO_4$ solution. The data are presented as mean values ± standard deviation (n = 3).

2.7. High performance liquid chromatography analysis

The liquid chromatographic apparatus (Waters 600) consisted of an in-line degasser, pump and controller, coupled to a 2996 photo diode array detector equipped with a Rheodyne injector (20 μl sample loop) interfaced to a PC running Millenium^{32} Chromatography Manager software (Waters Corp., Milford, MA). Separations were performed on a 250 × 4.6 mm i.d., 5 μm particle size, reverse-phase Hypersil BDS-C_{18} analytical column (Agilent Technologies, Santa Clara, CA) operating at ambient temperature (22 °C) with a flow rate of 0.7 ml/min. Detection was carried out with a sensitivity of 0.1 aufs between the wavelengths of 200–550 nm. Elution of the crude extract was effected using a ternary non-linear gradient of the solvent mixtures 0.02% TFA in H_2O (v/v) (solvent **A**) and 0.02% TFA in MeOH (v/v) (solvent **B**). The solvent gradient 1 (analysis of phenol compounds) in volumetric ratios of solvents **A** and **B** was as follows: 0–10 min, 95**A**/5**B**; 10–60 min, 50**A**/50**B**; 60–80 min, 30**A**/70**B**; 80–90 min, 95**A**/5**B**. A 10 min equilibrium time was allowed between injections. The hydrolysed extract was analysed by HPLC. Solvent **A** was H_2O:MeOH (84:16, v/v) adjusted to pH 2.8 with formic acid, and solvent **B** was 100% MeOH. Gradient 2, consisted of a linear gradient from 25% **B** to 50% **B** in 55 min. The flow rate was 0.9 ml/min and the UV detector was set to 280 nm.

Components were identified by comparison of their retention times with those of authentic standards under identical analytical conditions, and UV spectra with an in-house PDA-library. All extracts and standards were dissolved in MeOH at a concentration of 10 and 0.5 mg/ml, respectively. The concentration used for the calibration of reference compounds was 0.01–0.10 mg/ml. All standard and sample solutions were injected in triplicate.

2.8. Iron(III) to iron(II) reductive activity

The iron(III)-reductive ability of the extract was assessed by the method of Oyaizu (1986). In brief, a 1 ml aliquot of dissolved extract was mixed with 2.5 ml (0.2 M, pH 6.6) phosphate buffer and 2.5 ml 1% aqueous (w/v) potassium hexacyanoferrate solution. After 30 min at 50 °C, 2.5 ml 10% aqueous (w/v) trichloroacetic acid (TCA) was added and the mixture was centrifuged for 10 min. A 2.5 ml aliquot of the upper layer was mixed with 2.5 ml H_2O and 0.5 ml 0.1% aqueous $FeCl_3$, and the absorbance was recorded at 700 nm. The mean absorbance values were plotted against concentration, and a linear regression analysis was carried out. The data are presented as mean ascorbic acid equivalent (AscAE) [μmol of ascorbic acid/g (dry weight) of sample] values ± standard deviation (n = 4), calculated from an ascorbic acid standard curve.

2.9. Iron(II)-chelating activity

The iron(II)-chelating ability of the extract was assessed by the method of Carter (1971). In brief, to a 200 μl aliquot of dissolved extract was added to 100 μl (2.0 mM) aqueous $FeCl_2 \cdot 4H_2O$ and 900 μl MeOH. After 5 min, the reaction was initiated by the addition of 400 μl (5.0 mM) ferrozine solution. After 10 min equilibrium, the absorbance at 562 nm was recorded. The controls contained all the reagents except the extract or positive control substance. EDTA was used as a positive control. Percentage inhibition was calculated using Eq. (2), whilst the EC_{50} values were estimated from the % inhibition versus concentration plot using a non-linear regression algorithm. The data are presented as the mean values ± standard deviation ($n = 8$)

$$\%\ \text{Inhibition} = \left(\frac{(\text{Absorbance of control} - \text{absorbance of sample})}{\text{Absorbance of control}}\right) \times 100. \quad (2)$$

2.10. 1,1-Diphenyl-2-picrylhydrazyl free-radical scavenging

The ability of the extract to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH·) free radicals was assessed by the method of Gyamfi, Yonamine, and Aniya (1999). In brief, a 50 μl aliquot of extract dissolved in (50 mM, pH 7.4) Tris–HCl buffer was mixed with 450 μl buffer and 1.0 ml (0.1 mM) methanolic DPPH· solution. After 30 min in darkness at ambient temperature (23 °C), the absorbance was recorded at 517 nm. Controls contained all the reagents except the extract or positive control substance. Percentage inhibition was calculated using Eq. (2), whilst IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data are presented as mean values ± standard deviation ($n = 4$).

2.11. Hydroxyl free-radical-scavenging activity

The ability of the extract to scavenge hydroxyl radicals was assessed by the method of Aruoma et al. (1997). Bovine brain extract (Folch type VII) was mixed with 10 mM phosphate-buffered saline (PBS, pH 7.4) and sonicated in an ice bath until an opalescent suspension was obtained, containing 5 mg/ml phospholipid liposomes. The liposomes (0.2 ml) were combined with 0.5 ml of PBS buffer, 0.1 ml of 1 mM $FeC1_3$, and 0.1 ml extract dissolved in PBS. Peroxidation was initiated by adding 0.1 ml of 1 mM ascorbic acid solution. The mixture was incubated at 37 °C for 60 min, after which 50 μl of 2.0% (w/v) butylated hydroxytoluene solution was added followed by 1 ml 2.8% (w/v) TCA solution and 1 ml 1.0% (w/v) 2-thiobarbituric acid dissolved in 0.05 M NaOH. The samples were vortexed and heated in a water bath at 100 °C for 20 min. The reaction was stopped by a 5-min immersion in an ice bath. Two millilitres *n*-BuOH were added to each tube and the mixture was vigorously vortexed. After centrifugation, the absorbance of the organic layer was recorded at 532 nm. The controls contained all the reagents except the extract or positive control substances. Percentage inhibition was calculated using Eq. (2), whilst IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data are presented as the mean values ± standard deviation ($n = 3$).

2.12. Cell experiments

2.12.1. Cell culture

Human SH-SY5Y neuroblastoma cells were cultured in a Dulbecco's modified Eagle medium (DMEM): nutrient mixture F-12 (1:1) containing 15 mM HEPES buffer and L-glutamine (Gibco; Invitrogen, Carlsbad, CA) and supplemented with 15% heat-inactivated foetal bovine serum (FBS, HyClone, Thermo Fisher Scientific, Waltham, MA), 1% non-essential amino acids (Gibco), penicillin (170 U/ml) and streptomycin (170 μg/ml) (Gibco) at 37 °C in 5% CO_2/humidified air. Cells were grown in 75 cm^2 flasks and detached with 0.5 g/l trypsin-0.53 mM EDTA (pH 7.4). Split ratio was once per week and the medium was changed 1–2 times per week.

2.12.2. MTT assay

The effect of *P. sibirica* extract on cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, essentially according to Mosmann (1983). For cell viability assays 6.0×10^4 cells per well were seeded onto 96-well plates with 200 μl of cell culture medium. The cells were cultured for 18–24 h before treatment with *P. sibirica* extract (0.2–1.0 mg/ml). For viability assays the cell culture medium with 5% heat-inactivated foetal bovine serum was used during the treatment. *P. sibirica* extract was diluted with 15% DMSO in cell culture medium with 5% FBS. The final concentration of DMSO during experiment was below 0.75%. In brief, after the cells had been treated with the *P. sibirica* extract for 24 h, an MTT solution (5 mg/ml) was added to the wells containing cells (final concentration 0.5 mg/ml). MTT was dissolved in Hank's balanced salt solution (HBSS). The cells were incubated for 2.5 h at 37 °C in 5% CO_2/95% humidified air. Thereafter, the medium was removed and added to blue formazan crystals dissolved in 200 μl DMSO; the absorbance was measured at 550 and 655 nm as a background, using a microplate reader. The percentage cell viability was calculated using Eq. (3). Data are presented as mean values ± standard deviation ($n = 3$)

$$\%\ \text{Cell viability} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}}\right) \times 100. \quad (3)$$

2.12.3. LDH assay

The effect of the *P. sibirica* extract upon cell viability was also determined by the measurement of lactate dehydrogenase (LDH) using a commercial kit (CytoTox-ONE Homogenous Membrane Integrity Assay; Promega Corporation, Madison, WI) (Technical bulletin, 2007). In brief, 50 μl of substrate solution was added into the 50 μl of medium from treated cells. The plate was incubated for 30 min, shaking the first 10 min. Thereafter, the fluorescence reaction was stopped using 25 μl of stop solution. The fluorescence was measured using the excitation wavelength of 560 nm and the emission wavelength of 590 nm using a microplate reader. Percent cytotoxicity values were calculated using Eq. (4). Data are presented as mean values ± standard deviation ($n = 3$)

$$\%\ \text{Cytotoxicity} = \frac{(\text{Experimental} - \text{Culture medium background})}{(\text{Maximum LDH release} - \text{Culture medium background})} \times 100. \quad (4)$$

2.12.4. Western-blot analysis of p53 protein

The effect of *P. sibirica* extract on the level of p53 was examined by Western-blot analysis (Maňáková, Puttonen, Raasmaja, & Männistö, 2003). For Western-blot analysis 2.0×10^6 cells were seeded onto 60 mm dishes with 5 ml of cell culture medium. After 18–24 h cells were treated with *P. sibirica* extract (0.05–2.0 mg/ml) for 24 h. The extract was diluted with 15% DMSO in cell culture medium. The final concentration of DMSO was below 1.5%. After treatment, cells were washed twice and scraped from dishes with ice-cold PBS. The cell pellets were separated from PBS by centrifugation

for 5 min and 5000*g* at 4 °C. The cell pellets were stored at −80 °C until the experiments were performed. The total protein was extracted using a cell lysis buffer containing 20 mM HEPES (pH 7.4), 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100 supplemented with Complete Mini protease inhibitor (Roche Diagnostics Corporation, Indianapolis, IN) for 30 min and sonicated 3 × 10 s. The protein concentration was determined using a BCA protein assay kit (Pierce; Thermo Fisher Scientific) and 20 μg of protein were separated by 12% SDS–PAGE. The proteins were transferred onto nitrocellulose membranes and membranes were blocked in TBS containing 0.5% of Tween 20 and 5% of non-fat dry milk for 1.5 h at ambient temperature. The blots were incubated with monoclonal p53 (NCL-p53-DO7, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) and β-actin (A1978, Sigma) antibodies over night at 4 °C. Then, the blots were washed and incubated with HRP-linked anti-mouse IgG secondary antibody (HAF007, R&D Systems, Minneapolis, MN) for 1 h at RT. The protein bands were visualised by chemiluminescent reagent (Super Signal West Pico, Pierce) and GeneSnap with GeneGnome (Syngene). The results were analysed using GeneTools (Syngene, Cambridge, UK) program.

2.13. Statistical analysis

One-way analysis of variance was performed. Significant differences between means were determined by Tukey's pairwise comparison test at a level of $p < 0.05$.

3. Results and discussion

3.1. Extract yield, total phenol and tannin content

The extract yield was 21.5 mg extract/g plant material whilst the total phenol and total tannin content were 266 ± 3.9 mg gallic acid/g (dry weight) extract and 115 ± 7.8 mg tannic acid/g (dry weight) extract, respectively.

3.2. High performance liquid chromatography analysis

The high performance liquid chromatographic analysis of the crude extract is presented in Table 1, whilst the chromatographic profile recorded at 280 nm is presented in Fig. 1. As can be seen from Table 1, the extract contained benzoic acid (protocatechuic, syringic and vanillic acids) and cinnamic acid derivatives (*m*-coumaric and (*E*)-cinnamic acids), flavanones (taxifolin, eriodictyol and naringenin) and flavan-3-ols (catechin, epicatechin and epigallocatechin gallate) (Fig. 2). The most abundant component was eriodictyol (383 ± 1.0 mg/100 g) followed by taxifolin (172 ± 3.1 mg/100 g), whilst the least abundant components were *trans*-cinnamic (12.2 ± 1.2 mg/100 g) and *m*-coumaric (trace) acids. Some components could only be tentatively identified as proanthocyanidins,

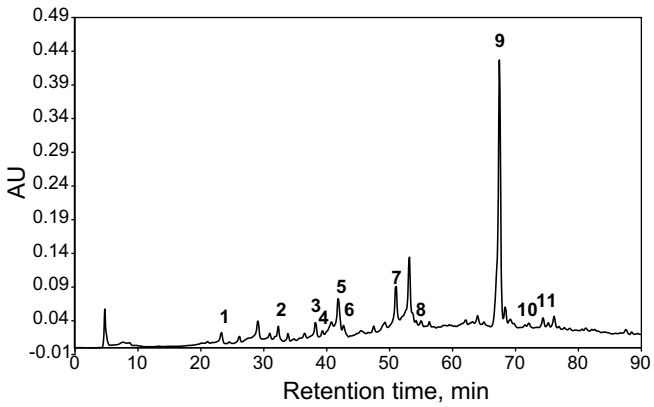

Fig. 1. HPLC–PDA analysis of the aqueous-acetone *P. sibirica* extract with detector response at 280 nm: (**1**) protocatechuic acid; (**2**) (+)-catechin; (**3**) vanillic acid; (**4**) (−)-epigallocatechin gallate; (**5**) syringic acid; (**6**) (−)-epicatechin; (**7**) (+)-taxifolin; (**8**) *m*-coumaric acid; (**9**), (+)-eriodictyol; (**10**) (*E*)-cinnamic acid and (**11**) naringenin.

hydroxylated benzoic and cinnamic acid derivatives, flavan-3-ol and flavonol derivatives.

It can be seen that a "chromatographic hump" (broad signal response) was evident, a phenomenon characteristic of reverse-phase separation of oligomeric proanthocyanidins (Stead, 1998). The "chromatographic hump" observed between 25 and 80 min suggested the presence of co-eluting compounds typical of proanthocyanidins. Reverse-phase HPLC permits a good resolution of compounds with a low DPn value, viz, monomers, dimers and trimers, whilst polymers greater than trimers remain unresolved.

Thiolysis was applied and the resultant chromatogram recorded at 280 nm is presented in Fig. 3. (+)-Catechin (**1**) and (−)-epicatechin (**2**) were detected as the main monomeric phenols. With regard to the benzylthioethers, 4 peaks (**5–8**) were identified after thiolysis. A similar result was obtained when the proanthocyanidin contents of several selected plants were investigated by Jerez et al. (2006). (*E*)-3,4-Benzylthioepicatechin was formed when (−)-epicatechin internal unit-containing proanthocyanidins were subjected to thiolysis. However, the (*Z*)-3,4 isomer was not detected. When the internal units are comprised of (+)-catechin, both (*E*)-3,4 and (*Z*)-3,4-benzylthiocatechin were identified. Furthermore, thiolysis allows distinction between extension and terminal units of proanthocyanidins and can be used to assess the DPn (Souquet, Cheynier, Brossaud, & Moutounet, 1996). Response factors, for both (+)-catechin and catechin-benzylthioether, and (−)-epicatechin and epicatechin-benzylthioether are identical at 280 nm. Therefore, catechin and epicatechin can be used as standards to quantify the benzylthioethers and the DPn value can be assessed using Eq. (1). The DPn value for the *P. sibirica* extract was calculated to be 9.0.

Table 1
HPLC-determined qualitative-quantitative data for the *P. sibirica* extract

Identified components[a]											Σ
PA (23.3)[b]	(+)-Catechin (33.8)	VA (38.2)	(−)-EGCG (39.4)	SyA (41.8)	(−)-EC (42.6)	Taxifolin (51.0)	*m*-CA (56.3)	Eriodictyol (67.4)	(*E*)-CA (72.1)	Naringenin (74.4)	
49.2 ± 0.5	52.5 ± 0.6	85.5 ± 1.0	47.0 ± 1.4	101 ± 0.3	125 ± 3.1	172 ± 3.1	tr.[c]	383 ± 1.0	12.2 ± 1.2	37.0 ± 2.1	1060 ± 5.45

Chromatographic values (mg/100 g (dry weight) extract) are presented as mean values ± standard deviation ($n = 3$).
[a] PA, Protocatechuic acid; VA, vanillic acid; (−)-EGCG, (−)-eipgallocatechin gallate; SyA, syringic acid; (−)-EC, (−)-epicatechin; *m*-CA, *m*-coumaric acid; and (*E*)-CA, (*E*)-cinnamic acid.
[b] Retention time (min).
[c] tr., trace (<0.001 mg/100 g (dry weight) extract).

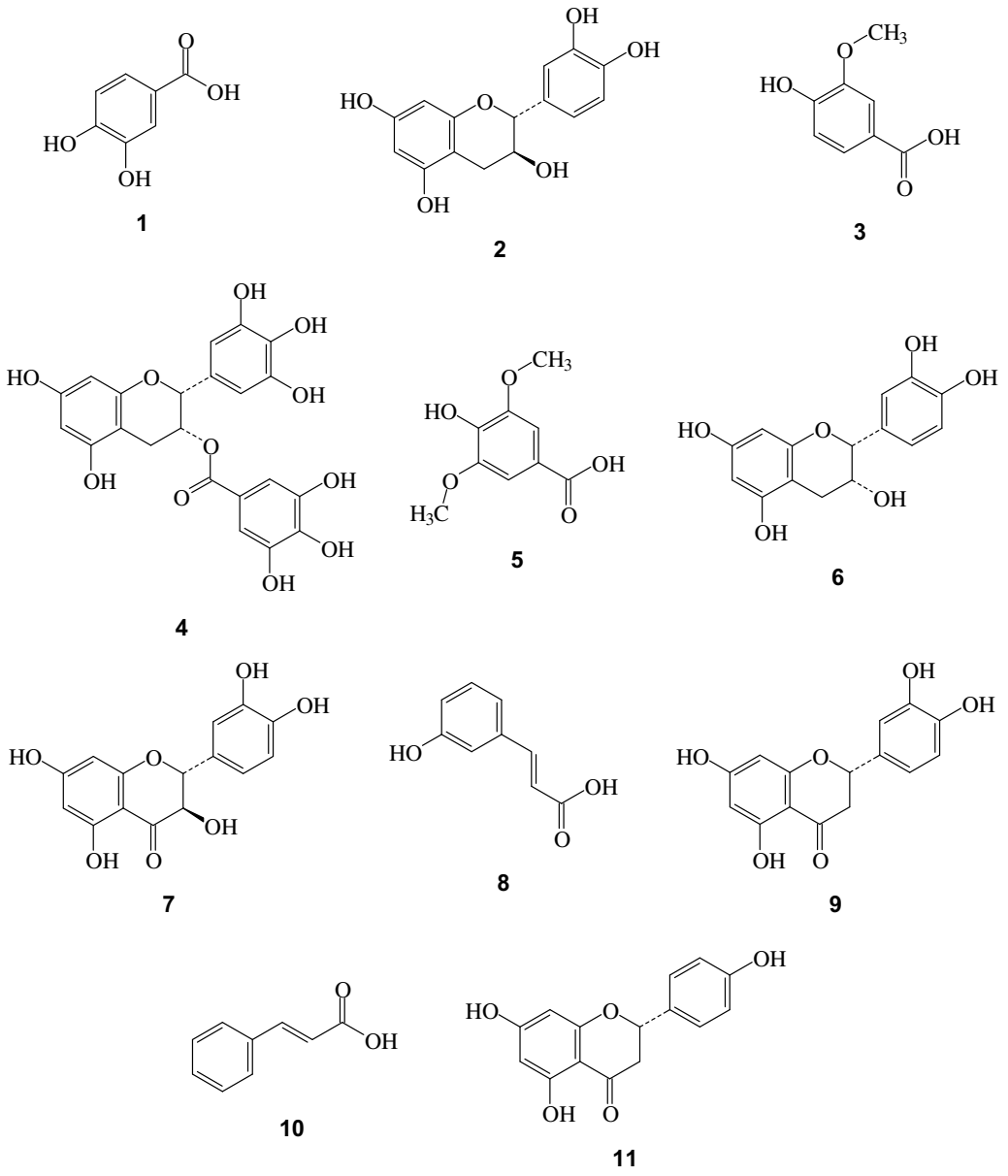

Fig. 2. Structural formulae of the main compounds identified within the crude *P. sibirica* extract: (**1**) protocatechuic acid; (**2**) (+)-catechin; (**3**) vanillic acid; (**4**) (−)-epigallocatechin gallate; (**5**) syringic acid; (**6**) (−)-epicatechin; (**7**) (+)-taxifolin; (**8**) *m*-coumaric acid; (**9**) (+)-eriodictyol; (**10**) (*E*)-cinnamic acid and (**11**) naringenin.

3.3. Iron(III) to iron(II) reductive activity

The presence of reductones is considered to be indicative of antioxidative capacity (Gordon, 1990). Therefore, it was considered important to determine the reductive ability of the extract. The extract reduced iron(III) and did so in a linear fashion across the concentration range used in this study (Fig. 4A). As shown in Table 2, the AscAE value for the extract was 650 ± 5.10 μmol ascorbic acid/g (dry weight) extract. However, when compared to the reference substances, it is clear that the extract was significantly less effective, with ascorbic acid being the most potent reference followed by BHA and BHT.

Despite the poor activity of the *P. sibirica* extract, the data does confirm that the extract does possess electron-donating properties and thus may act as a free-radical chain reaction terminator, transforming free-radical species into stable non-reactive species before they may initiate or propagate detrimental chain reactions.

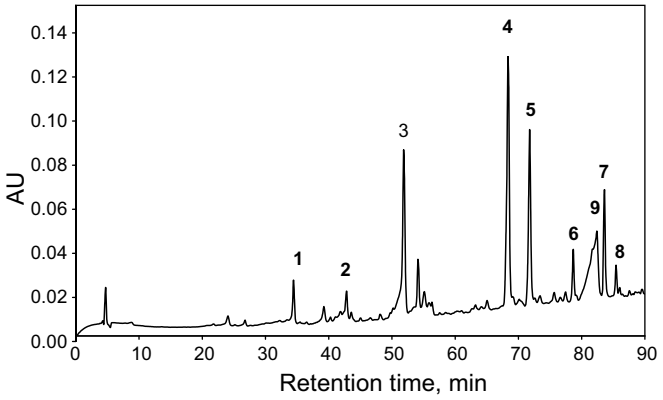

Fig. 3. HPLC-PDA analysis of the aqueous-acetone *P. sibirica* extract after thiolysis with detector response at 280 nm: (**1**) (+)-catechin; (**2**) (−)-epicatechin; (**3**) (+)-taxifolin; (**4**) (+)-eriodictyol; **5**,**6**,**7**,**8** benzylthioethers and **9**, unreacted toluene-α-thiol.

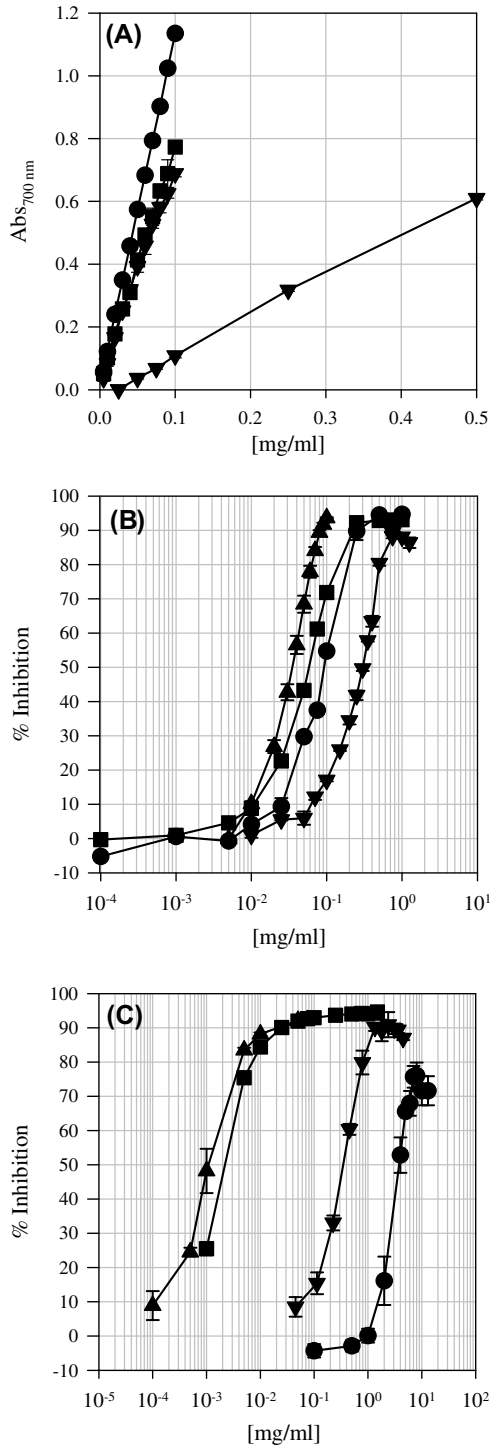

Fig. 4. (A) iron(III) reduction, (B) DPPH· and (C) hydroxyl free radical-scavenging dose–response curves for the *P. sibirica* extract ((—▼—)), ascorbic acid ((—●—)), BHA ((—■—)) and BHT ((—▲—)). Data are presented as mean values ± standard deviation.

3.4. Iron(II)-chelating activity

Transition metals are important because of their ability to catalyse oxidative reactions (Gordon, 1990) and, thus, the ability to chelate these metals is a significant antioxidant-related property (Kehrer, 2000). The extract was able to chelate iron(II) and, as can be seen from Table 2, did so in a concentration-dependent fashion. However, the estimated EC_{50} value, i.e. the concentration of extract required to chelate 50% of the iron(II), was very high (20.1 ± 2.1 mg/ml) especially in comparison to EDTA (0.27 ± 0.02 mg/ml) (Table 2). Polyphenolic compounds should be able to chelate transition metals because of the high charge density of the phenoxide group generated on deprotonation (Hider, Liu, & Khodr, 2001). The extract did chelate iron(II), however, the EC_{50} value was substantial. This suggests that its usefulness in protecting against Fenton chemistry/hydroperoxide decomposition-mediated oxidative damage *in vivo* is unlikely.

Table 2
Iron(III)-reductive capacity, iron(II)-chelating and free radical-scavenging activity data for the *P. sibirica* extract and positive controls

Sample	Iron(III) reduction[b]	Iron(II) chelation[c]	Free Radical scavenging[a]	
			DPPH·	OH·
Ascorbic acid	5680 ± 83.5A	–	85.4 ± 1.58A	2980 ± 358A
BHA	3761 ± 126B	–	53.9 ± 1.52B	1.07 ± 0.12B
BHT	3450 ± 135C	–	38.6 ± 2.94C	1.96 ± 0.09C
EDTA	–	0.27 ± 0.02A	–	–
Extract	650 ± 5.10D	20.1 ± 2.1B	257 ± 2.36D	338 ± 6.49D

Data are presented as mean values ± standard deviation. Different uppercase letters (A–D) within columns indicate statistically significant ($p < 0.05$) difference between values.
[a] Free radical-scavenging expressed as IC_{50} values (μg/ml).
[b] Iron(III) reduction expressed as ascorbic acid equivalents (μmol ascorbic acid/g dry weight extract).
[c] Iron(II) chelation expressed as an EC_{50} value (mg/ml).

3.5. 1,1-Diphenyl-2-picrylhydrazyl free-radical-scavenging activity

A cardinal antioxidant property is the ability to scavenge free-radicals, species believed to participate in the aetiology and pathogenesis of various diseases and premature ageing (Raha & Robinson, 2000; Terranova, 2004). Moreover, free-radical-mediated degradation is considered the principal cause of rancidity (German, 1999). Thus, the free-radical-scavenging activity of the extract was assessed using the model free-radical DPPH·.

The extract scavenged DPPH· free radicals and did so in a concentration-dependent fashion (Fig. 4B). The IC_{50} value, i.e. the concentration of sample required to scavenge 50% of the DPPH·, was estimated at 257 ± 2.36 μg/ml (Table 2). When compared to the IC_{50} values calculated for the reference substances ascorbic acid, BHA and BHT, the extract was the least active.

Despite the antiradical activity of the extract being less than for the references, the extract was able to scavenge DPPH· free radicals, which suggests that it may have a role in preventing free-radical-mediated chain reactions.

3.6. Hydroxyl free-radical-scavenging activity

The DPPH· scavenging model is an useful indicator of antioxidant potential, however, it does not use a biological or food-related substrate, so its value for predicting real activity is limited. Therefore, the extract ability to protect a more relevant matrix (i.e., phospholipid liposomes) against hydroxyl radical damage was assessed. In the ascorbate-iron(III)-liposome assay, hydroxyl free-radicals attack the phospholipid polyunsaturated acyl side chains. An extract capable of inhibiting this can be described as a hydroxyl radical scavenger.

The extract was able to inhibit phospholipid degradation by scavenging hydroxyl free radicals, and did so in a concentration-dependent fashion (Fig. 4C). As shown in Table 2, the extract's IC_{50} value was estimated at 338 ± 6.49 μg/ml. When compared to the references, it can be seen that the extract was significantly less effective than either BHA or BHT, compounds formulated for

maximum antiradical activity. However, it was a significantly better than ascorbic acid (Table 2), a compound possessing both antioxidant and pro-oxidant properties. When used at low concentrations, ascorbic acid demonstrated pro-oxidant activity whilst at high concentrations its antioxidant activity occurred.

The extract inhibited the formation of phospholipid-derived oxidative products, therefore, the extract may be able to protect lipid-rich matrices, e.g. foodstuffs and biological membranes, from oxidative deterioration.

3.7. Cell experiments

The effects of *P. sibirica* extract were studied in the human SH-SY5Y neuroblastoma cell culture, to determine the effects of the extract on the cell viability and for the possible effects on the p53 cell signalling pathway. The cell viability and cell death were assessed using MTT (methylthiazolyldiphenyl-tetrazolium bromide) and LDH (lactate dehydrogenase) assays. Furthermore, the effect of *P. sibirica* extract was also examined on the expression of p53 protein as a marker of apoptosis using Western blot. The SH-SY5Y cells are tumourigenic and have been used for apoptotic studies earlier (Maňáková et al., 2003).

3.7.1. Cell viability assays

When the SH-SY5Y neuroblastoma cells were treated for 24 h with different concentrations of *P. sibirica* extract, the cell viability was estimated using the MTT assay, which measures the metabolic activity of mitochondria. As shown in Fig. 5, all the used concentrations of *P. sibirica* extract decreased the production of formazan compared to the untreated control cells. With higher concentrations (0.6, 0.8 and 1.0 mg/ml) the decrease of cell viability was significant compared to the control cells. Each assay was performed three times in triplicate. The MTT reduction (% of control values) data for the *P. sibirica* extract in SH-SY5Y cells after 24 hours were 84%, 87%, 54%, 57% and 63% MTT reduction at 0.2, 0.4, 0.6, 0.8 and 1 mg/ml doses, respectively.

The cell viability was estimated also by measuring the cell membrane integrity. The effects of *P. sibirica* extract on the membrane integrity in the SH-SY5Y cells are shown in Fig. 6 as measures of LDH enzyme activities. All concentrations increased the release of LDH from the cells into the surrounding culture medium significantly. The most effective concentrations were 0.6 and 0.8 mg/ml increasing the amount of LDH about 320% comparing to the control cells. LDH releases in the cell cultures (% of control) were 199%, 258%, 323%, 326% and 276% at 0.2, 0.4, 0.6, 0.8 and 1 mg/ml concentrations, respectively.

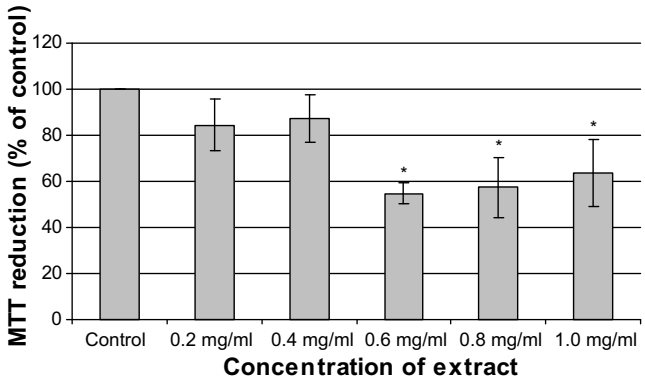

Fig. 5. The reduction of MTT in SH-SY5Y cells measured after 24 h of treatment with different concentrations of *P. sibirica* extract. Data are presented as mean values ± standard deviation from three independent experiments made in triplicate. *Different from the control at a level of $p < 0.05$.

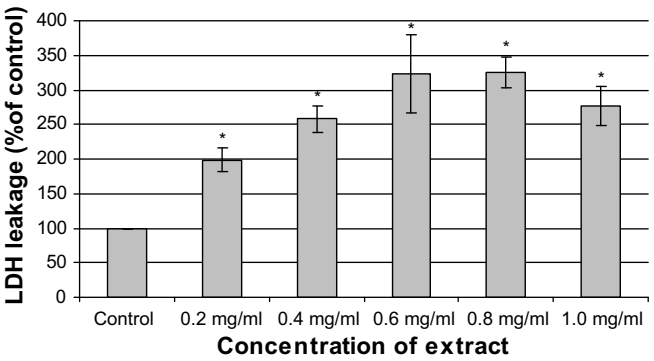

Fig. 6. The leakage of LDH from SH-SY5Y cells after 24 h of treatment with *P. sibirica* extract. Data are presented as mean values ± standard deviation from three independent experiments made in triplicate. *Different from the control at a level of $p < 0.05$.

3.7.2. Western-blot analysis of p53

The amount of p53 protein was measured in the SH-SY5Y neuroblastoma cells treated for 24 h with different extract concentrations. The p53 protein is a tumour suppressor and plays a pivotal role in mediating DNA damage-induced apoptosis (for review, see Roos & Kaina, 2006). Activation of p53 as a transcription factor can lead the cells to apoptosis or to cell cycle arrest. Fig. 7 demonstrates that in the present experiments *P. sibirica* extracts did not have any effects on the p53 amount. However, the highest concentration of 2.0 mg/ml of *P. sibirica* extract seemed to be lethal to the cells, and therefore it was not possible to analyse the p53 amount at this concentration.

To date, the cell viability assessments have been carried out as indicated by MTT reduction, LDH release and p53 measurements. In general, the analysis of cell viability suggests that the *P. sibirica* extract does not activate apoptosis at the concentrations used in the present experiments, since there were no significant changes in the amount of p53 protein. However, there seemed to be some decrease in the cell viability when estimated by LDH and MTT assays. Compounds within the acetone extract of seeds of *P. sibirica* were capable of scavenging DPPH· radicals. In the extract of seeds of *P. sibirica* phenolic compounds were detected by TLC, in minor amounts, but showing high antiradical activity. It is suggested that the decreased cell viability may be caused by necrosis due to a *P. sibirica* extract-mediated disturbance of antioxidant/oxidant balance in the SH-SY5Y cells.

3.8. In summary

P. sibirica seeds are an abundant natural resource which are used for various medicinal purposes, yet pharmacognostic studies are rarely published, especially in English language publications. In this study, we investigated the chemistry of an aqueous-acetone-soluble extract and assessed its performance in a battery of

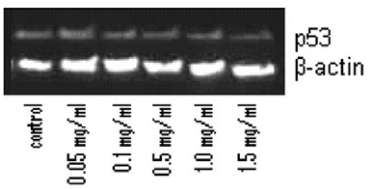

Fig. 7. Representative p53 immunoblots from SH-SY5Y cells after 24 h treatment with different concentrations of *P. sibirica* extract. Four independent experiments were made in duplicate.

antioxidant and cell viability assays. The extract predominantly contained phenolic compounds which would explain the antioxidant activities it demonstrated in antioxidant screening assays. The SH-SY5Y neuroblastoma cell culture data suggests that the extract can decrease the cell viability. However, the extract does not activate apoptosis, based on the analysis of p53 expression, but may have some necrotic effects, as indicated by the LDH and MTT analysis. Therefore, further studies related to the mechanisms of apoptosis, e.g., caspase activation and gene expression, are needed to understand the effects of *P. sibirica* extracts on the cell viability and cell death in detail. This information can be helpful when estimating the beneficial properties of *P. sibirica* extract or other plant extracts as valuable medicinal raw plant materials to be used for drug development.

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