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Intraspecific relationship analysis by DNA markers and in vitro cytotoxic and antioxidant activity in *Eleutherococcus senticosus*

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Abstract

To analyse genetic relationships and intraspecific variation within *Eleutherococcus senticosus*, the polymerase chain reaction (PCR) was performed on total genomic DNAs of 10 *Eleutherococcus* collections. Ten primers were used for amplification, yielding 106 bands, of which 87 were polymorphic. The genetic diversity and genetic distance among 10 collections of *Eleutherococcus* species were used to describe the dendrogram showing the phylogenic relationship. The 10 collections were classified into two groups (groups I and II) at a similarity coefficient of 0.50. Group I included *E. senticosus* from Bukhaedo (Japan), *E. sessliliflorus* from Youngwal (Korea), *E. seoulense* and *E. chiisanesis*, while group II included several internal and Russian collections. The range of polymorphism was from 66.7 to 90.9% in the 87 amplified polymorphic DNA fragments. The similarity value of all collections ranged from 0.41 to 0.92, and the average genetic distance was 0.61. Thus, RAPD analysis was useful in determining genetic relatedness among collections and in identifying different genotypes of *E. senticosus* and other *Eleutherococcus* species. Also, the biological activity on DPPH radical scavenging, antilipid peroxidation in rat liver microsomes and cytotoxic sulforhodamine B (SRB) assay was evaluated using root extracts of *E. senticosus*, Odaesan, Korea. Ethyl acetate and *n*-butanol fractionation revealed strong antioxidant against scavenging on DPPH free radical and also ethyl acetate fractionation exhibited high antilipid peroxidative activities. In the cytotoxic effects were evaluated on seven human cancer cell lines, the values of 50% growth inhibition (GI_{50}) were mostly below 30 μg/ml for crude extracts to be considered as significantly active.

Keywords: *Eleutherococcus senticosus*; Antioxidant activity; Cytotoxicity; Genetic similarity; Polymerase chain reaction (PCR); RAPD

1. Introduction

Eleutherococcus senticosus (*Acanthopanax senticosus* Harms) is a woody shrub found only in north-eastern Asia (Lee, 1979). The distribution of this plant includes the drainage basin of woosuri in Russia, the Hukryong River in China, a region of Bukhaedo in Japan, and the mountains of Baekdu, Seolak Odae, Dukyu and Taegi in Korea (Hahn et al., 1985).

In Korea, *E. senticosus* either fails to set seed or has poor seed setting. A taxonomic study of *E. senticosus* was conducted by Kim et al. (1996b). Kim et al. (1996b) classified *E. senticosus* on the basis of leaf shape, filament length and flower style, and reported that there was a short filament type and a long filament type. When accessions were crossed artificially, they either failed to set seed or set few seeds, demonstrating intraspecific and individual variation. In Korea, these plants are either rare, or in some locations have become endangered because of over-harvesting. Consequently, they are now classified as rare, protected plants by the Environmental Ministry in Korea.

E. senticosus is a useful medicinal plant, and its shoots and roots have been used to promote robustness and as a remedy for many ailments including anti-blood-sugar diabetes, neuralgia, palsy, sex- and learning-behaviour difficulties, cancer and, because of its antioxidant properties, it has been used to treat several stresses (Hahn et al., 1985; Yook et al., 1996). Wagner et al. (1982) analysed the characteristics of lignin and phenylpropan derivatives from root extracts from accessions of *E.*

Abbreviations: DPPH, 1,1-diphenyl-2-picryhydrazyl; FCS, foetal calf serum; OD, optical density; PCR, polymerase chain reaction; TBA, thiobarbituric acid.

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senticosus from Korea, China and Russia by TLC and HPLC. The amount of eleutheroside in *E. senticosus* accessions from Korea was much higher than that in accessions from other countries. The biological activities of water, ethanol and 50% ethanol extracts from *Acanthopanax* root bark were compared in a study by Kim et al. (2000). Cell growth was reduced by 94% in Hep3B (liver cancer) and by 90% in A549 (lung cancer), by adding ethanol extracts from *E. senticosus* root bark. Furthermore, cell growth and the viability of Jurkat (T-cell) was increased by up to 275% by the addition of 50% ethanol extracts from *E. senticosus* root bark. The biological activities of extracts from *E. senticosus* were better than those of other *Eleutherococcus* species.

There are many *Eleutherococcus* species, and about 15 accessions of this species, in Korea. Therefore, it is important to characterize and quantify genetic similarity and diversity both within and between populations. Information concerning the genetic diversity within a plant species is essential for the rational use of genetic resources. It is particularly useful in characterizing individual accessions and cultivars, in detecting duplications of genetic material in collections, and as a general guide in choosing parents for breeding hybrids. Recently, RAPD (Random Amplified Polymorphic DNA), generated by individual short oligonucleotide primers of arbitrary sequence in the polymerase chain reaction (PCR), have provided a rapid means for plant breeders to develop improved cultivars through indirect selection (Phillip et al., 1996). RAPDs are new molecular markers for comparative analyses that are quick and easy to use, independent of many environmental influences, and are practically unlimited in number. The successful application of RAPDs to taxonomic and evolutionary studies has been documented in a variety of plants, including hop (Pillay and Kenny, 1996), *Epimedium* spp. (Yoo et al., 1997; Lim et al., 2000), *Pisum* (Hoey et al., 1996) and common bean (Phillip et al., 1996). The RAPD technique is considered to have many advantages over RFLPs and provides a fast, efficient method for scoring large numbers of genetic markers. It has a wide range of applications, including genetic mapping, phylogenic analysis, the construction of linkage maps, evaluating gene flow between species, identifying individuals, cultivars or species by genomic fingerprinting, and the identification of parents in pedigree analysis (Pillay and Kenny, 1996).

Little information is available on the analysis of the genetic diversity among the accessions of *Eleutherococcus*, antioxidant and cytotoxic activity on *E. senticosus*.

The objectives of these studies were to identify polymorphic RAPD markers to distinguish 10 accessions of *Eleutherococcus*, and to estimate genetic relatedness among the accessions. The biological activities of antioxidant and anticancer activity using root extracts of *E. senticosus*, Odaesan accession, Korea were also evaluated.

2. Material and methods

2.1. Intraspecific relationship analysis by RAPD

2.1.1. Plant materials

Three accessions of *E. senticosus* from three locations: Bukhaedo (Japan), Siberia (Russia), and Yunbyun (China), and four accessions from Korea: Taegisan, Chunchon, Jamgok, Odaesan, were compared with *E. sessliliflorus* from Yeongwal from Korea, *E. seoulense* from Korea and *E. chiisanesis* from Korea. Table 1 shows the *E. senticosus* characteristics collected in this study (Table 1). There are no morphological differences in leaf shape, stem and prickles at node among seven accessions of *E. senticosus* from Japan, Russia, China and Korea, while there are morphological differences compared with *E. sessliliflorus, E. seoulense* and *E. chiisanesis*. *E. senticosus* have small, about 5 mm dense prickles on the surface of the stem while *E. sessliliflorus, E. seoulense* and *E. chiisanesis* have large less dense prickles on the surface of the stem. *E. senticosus* accessions have trichome of leaf surface and *E. sessliliflorus, E. seoulese*, and *E. chiisanesis* have no trichomes of leaf surface. There are differences in flower morphology and fertility among *E. senticosus* accessions: *E. senticosus* accessions from Russia and Japan flowered and produced normal seeds while *E. senticosus* accessions from Korea produced flowers which either aborted or had no seedless berries.

E. sessliliflorus, E. seoulese, and *E. chiisanesis* have normal flower and many seeds.

2.1.2. Total DNA extraction

Total DNA was isolated from young leaves using the CTAB (hexadecyltrimethylammonium bromide) procedure described by Doyle and Doyle (1990). DNA concentration was estimated by subjecting samples to 0.85% agarose gel electrophoresis and staining with ethidium bromide. Staining intensities of the total DNA were compared visually with a DNA molecular weight marker. DNA yields of 1–20 mg/g leaf tissues were obtained, and total DNA was diluted with sterile distilled water to give a final concentration of 10 ng/μl.

2.1.3. PCR procedure

PCR reactions were performed using random primers (OPERON Technology, Alameda, CA, USA). We selected 10 primers that amplified clear bands in preliminary experiments (OPA-01: CAGGCCCTTC; OPA-02: TGCCGAGCTG; OPA-11: CAATCGCCGT; OPA-12: TCGGCGATAG; OPA-19: CAAACGTCGG; OPA-20: GTTGCGATCC; OPB-01: GTTTCGCTCC; OPB-05: TGCGCCCTTC; OPB-09: TGGGGGACTC; OPB-10: CTGCTGGGAC). We repeated experiments only when bands were not clear. PCRs were performed in a 25-μl reaction mixture consisting of 20 mM Tris-HCl, 50 mM KCl, 200 μM each of dATP, dCTP, dTTP and

Table 1
Morphological characteristics of *Eleutherococcus* spp.

Taxa	*Eleutherococcus senticosus*							*Eleutherococcus sessliliflorus*	*Eleutherococcus seoulense*	*Eleutherococcus chiisanesis*
	Chunchon	Taegisan	Jamgok	Odaesan	China	Japan	Russia			
Stem	0	0	0	0	0	0	0	0	1	0
Leaf margin	0	0	0	0	0	0	0	0	0	0
Tufts of hairs	0	0	0	0	0	0	0	0	1	0
Trichome of leaf surface	1	1	1	1	1	1	1	1	0	1
Prickles	2	2	2	2	2	2	2	1	1	0
Elasticity of prickles	1	1	1	1	1	1	1	0	0	0
Prickles at node	2	2	2	2	2	2	2	2	1	1
Shape of prickles	0	0	0	0	0	1	1	0	2	1

Stem; 0: erect, 1: procumbent, leaf margin; 0: simple serrate, 1: double serrate, tufts of hairs; 0: absent, 1: present, Trichome of leaf surface; 0: absent, 1: present, prickles; 0: absent, 1: present, 2: densely present, elasticity of prickles; 0: absent, 1: present, prickles at node; 0: absent, 1: one, 2: two or more, shape of prickles; 0: bristle like, 1: needle, 2: deltoid.

dGTP, 0.5 μM primer, 1 unit Taq DNA Polymerase (Boehringer, Mannheim, Germany), and 10 ng template DNA. Amplifications were performed in a Touchdown (Hybrid) thermal cycler for an initial 5 min denaturation at 95 °C followed by 45 cycles of 1 min at 94 °C, 1 min at 45 °C and 2 min at 72 °C. All PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels in 1×TAE buffer (Sambrook et al., 1989), stained with ethidium bromide, viewed under UV light, and photographed using Polaroid film.

2.2. Statistical analysis

PCR reactions and electrophoresis were repeated at least twice to ascertain the reproducibility of the bands. Only reproducible bands were scored as present (1) or absent (0) in this study. The RAPD data, generated with 10 primers, were used to compile a binary matrix for cluster analysis using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) version 1.80 package (Rohlf, 1993). Genetic similarity among collections was calculated according to Jaccard's similarity coefficient (Jaccard, 1988) using the SIMQUAL (Similarity for Qualitative Data) routine. Jaccard's coefficient is defined as $a/(a+b+c)$, where a is the number of positive matches (i.e. bands common to two collections), and b and c refer to the number of bands present only in collections 1 and 2, respectively. The similarity coefficients were then used to construct a dendrogram using the UPGMA (unweighted pair-group method with arithmetical averages) through the SHAN (sequential, hierarchical, agglomerative and nested clustering) routine of the NTSYS-pc package.

2.3. Test of biological activities on Eleutherococcus senticosus

2.3.1. Sample preparations for biological activities

The dried 5 g ground roots of *E. senticosus* Odaesan, Korea were extracted with 100 ml of MeOH, hexane, EtOAc, *n*-BuOH, aqueous stirred for 24 h at room temperature, and filtered through Whatman No. 4 filter paper. The filtrate was dried in a rotary vacuum evaporator at below 40 °C, then freeze-dried. The obtained crude samples were used to measure biological activity and were kept in a cool chamber at below −35 °C until extracted.

2.4. DPPH radical scavenging activity

The 1,1-diphenyl-2-picryhydrazyl (DPPH) free radical scavenging activity was measured according to Xiong et al. (1996). One ml of 0.15 mM DPPH in ethanol was added to a sample solution containing 4 ml methanol and allowed to react for 30 min at room temperature, after which time the optical density was measured at 517 nm. For the blank, ethanol was used instead of DPPH solution and, for control, methanol was used instead of the sample represented the concentration of tested compound and the ordinate the average percentage reduction of DPPH radical from three separate tests.

2.5. Anti-lipid peroxidation activity in rat liver microsomes

Inhibition of lipid peroxidation in rat liver microsomes was measured by the thiobarbituric acid (TBA) method (Kim et al., 1996a). Rat liver microsomes were prepared according to the method of Ohkawa et al. (1979) with some modifications, and were suspended in 100 mM Tris-HCl buffer (pH 7.4). The reaction was initiated by the addition of 100 μM $FeSO_4 \cdot H_2O$. After 30 min at 37 °C under reciprocal agitation, the reaction was stopped by the addition of 3 M trichloroacetic acid in 2.5 N HCl. Lipid peroxidation was assessed by measuring TBA reactive products. Percentage inhibition was calculated as follows: $\{1-(T-B)/(C-B)\}\times 100$ (%), in which T, C, and B are absorbance values at 530 nm of the compound treatment, the control (peroxidation

without compound) and the 0 time control (no peroxidation), respectively.

2.6. Cytotoxic sulforhodamine B (SRB) assay

Six kinds of human cancer cell lines were used and cultured with RPMI 1640 containing 10% foetal calf serum (FCS). For the SRB assay, cells were cultured as described previously (Kim et al., 1996a,c). A cell suspension (3–40,000 cells/ml) was made in the culture medium and inoculated into each well of a 96-well microtiter plate. One day after plating, a time zero control plate was made, compounds 1–4 were directly treated, and cells were incubated for a further 48 h in a CO_2 incubator. Cells were fixed with 50 μl of a 50% trichloroacetic acid solution for 1 h at 4 °C and plates were washed five times with tap water and air-dried. 100 μl of SRB solution (0.4% in 1% acetic acid) was added and staining was conducted at room temperature for 30 min. Residual dye was washed out with 1% acetic acid and air-dried. Tris solution (10 mM, pH 10.5) was then added to each well. Optical density (OD) was measured with a microtiter plate reader at 540 nm. Growth inhibition was calculated as described previously. Briefly, the OD of the treated well was subtracted from the OD of the time-zero (Tz) plate, and divided by the calculated value of the untreated control. Growth inhibition of 50% (GI_{50}) was calculated by the probit method (Wu et al., 1992).

3. Results and discussion

The 10 primers used produced an average of 10.6 scorable bands per primer, with 8–13 bands per primer in genetic polymorphism and RAPD patterns. The total number of scorable bands amplified in this study was 106, ranging in size from 126 to 2645 bp (Table 2). Of the 106 bands, 87 (82.1%) were polymorphic and 19 bands (17.9%) were monomorphic among the *E. senticosus* collections. Most collections studied possessed unique combinations of bands, thereby enabling their identification. The optimized PCR protocol resulted in highly reproducible banding patterns. The RAPD profiles resulting from the 10 primers are shown in Plate 1. All primers of G+C content represented 60% above, the reported study (Fitsch and Riesenberg, 1996) confirmed DNA amplification was affected the level of G+C content. Thus, RAPD analysis was a useful tool to determine genetic relationship among collections of *E. senticosus*, and in the identification of different types of *E. senticosus*.

The use of Jaccard's similarity coefficient (Jaccard, 1988) to estimate genetic relationship among collections gave similarity values ranging from 0.42 to 0.92, and the average genetic distance appeared to be 0.61 (Table 3). In general, the dendrogram separated the collections into two main branches (Group I, Group II) at a similarity coefficient value of 0.50. Group I included *E. senticosus* from Bukhaedo (Japan), *E. sessliliflorus* from Yeongwal (Korea), *E. seoulense* and *E. chiisanesis*, while Group II included several *E. senticosus* collections from Korea, China, and a Russia collection. Group I was subdivided further into three clusters. The *E. senticosus* collections from Bukhaedo (Japan), which yielded similarity values of 0.52 between group members, could be distinguished from each other collection of *E. senticosus* of Group II. Group II was subdivided further into two clusters, and the genetic similarity between two major clusters of Group II ranged from 0.583. One cluster comprised only one collection from a location in Korea named Chunchon. The genetic similarity between *E. sessliliflorus* Youngwal accession (10) and *E. senticosus* Bukhaedo accession (1) was about 52.1%, whereas the genetic similarity between *E. sessliliflorus* Youngwal accession (10) and *E. senticosus* Chunchon accession (3) was only about 45.8%. Most accessions of *E. senticosus* from Korea, China, and Russia except for *E. senticosus* from Japan were classified as Group II while *E. sessliliflorus*, *E. seoulense*, and *E. chiisanesis* were classified as

Table 2
Nucleotide sequence and G+C content of selected primers that generated polymorphisms and reproducible band profiles and the number of detectable polymorphic bands

Primer no.	Sequence (5′ to 3′)	G+C content (%)	No. of bands (no. of polymorphic bands)	Polymorphism (%)
OPA-1	CAGGCCCTTC	70	8 (7)	87.5
OPA-2	TGCCGAGCTG	70	11 (10)	90.9
OPA-11	CAATCGCCTG	60	9 (6)	66.7
OPA-12	TCGGCGATAG	60	12 (8)	66.7
OPA-19	CAAACGTCGG	60	11 (9)	81.8
OPA-20	GTTGCGATCC	60	11 (10)	90.9
OPB-1	GTTTCGCTCC	60	10 (9)	90.0
OPB-5	TGCGCCCTTC	70	13 (10)	76.92
OPB-9	TGGGGGACTC	70	13 (12)	92.3
OPB-10	CTGCTGGGAC	70	8 (6)	75.0
Total			106 (87)	82.1

Table 3
Similarity coefficient matrix of 10 accessions of *Eleutherococcus* spp.

	P1	p2	p3	p4	p5	p6	p7	p8	p9	p10
P1	1.0000 000									
P2	0.5000 000	1.0000 000								
P3	0.5000 000	0.5625 000	1.0000 000							
P4	0.5833 333	0.5625 000	0.4375 000	1.0000 000						
P5	0.6041 667	0.5000 000	0.4166 667	0.9166 667	1.0000 000					
P6	0.5208 333	0.7500 000	0.7083 333	0.5000 000	0.4791 667	1.0000 000				
P7	0.5416 667	0.7291 667	0.6458 333	0.5208 333	0.5000 000	0.9166 667	1.0000 000			
P8	0.5833 333	0.7291 667	0.6041 667	0.5208 333	0.4583 333	0.7500 000	0.7708 333	1.0000 000		
P9	0.6041 667	0.5833 333	0.5833 333	0.5833 333	0.5625 000	0.6458 333	0.7083 333	0.7500 000	1.0000 000	
p10	0.5208 333	0.5833 333	0.4583 333	0.7916 667	0.8125 000	0.5625 000	0.5833 333	0.5416 667	0.5208 333	1.0000 000

P1: *E. senticosus* Bukhado, Japan; P2: *E. senticosus* Siberia, Russia; P3: *E. senticosus* Chunchon, Korea; P4: *E. seoulense*; P5: *E. chiisanesis*; P6: *E. senticosus* Taegisan, Korea; P7: *E. senticosus* Jamgok, Korea; P8: *E. senticosus* Odaesan, Korea; P9: *E. senticosus* Yunbyun, China; p10:. *E. sessliliflorus* Yeongwal, Korea.

Group I which means that there were genetic relationships within and among species. Although there were morphological differences between *E. senticosus* and other *Eleutherococus* spp. including *E. sesslilifloru*s, *E. seoulense*, and *E. chiisanesis*, a major morphological difference was that *E. senticosus* had small and dense prickles on the surface of the stem. Flower morphology and fertility also differed: *E. senticosus* accessions from Russia and Japan flowered and produced normal seeds while *E. senticosus* accessions from Korea produced flowers which either aborted or had no seedless berries. *E. senticosus* from Japan had similar morphological characteristics to *E. senticosus* accessions from Korea, China, and Russia but classified as Group I. The genetic relationship between Japan accession and other accessions of Korea, China, and Russia should be more studied in the future.

A dendrogram based on the similarity values was generated using UPGMA clustering analysis (Fig. 1).

3.1. Test of biological activities on Eleutherococcus senticosus

A free radical, DPPH, was used to analyse the antioxidant activity of extracts from the roots of *E. senticosus*. EtOAc and butanol fractionation revealed strong antioxidant activities, and methanol and water extracts of *E. senticosus* had high antioxidant activities (Table 4). The antioxidant activities in the EtOAc and butanol fractions were higher than or similar to that in α-tocopherol. Extracts from the leaf and stem of *E. senticosus* also had strong antioxidant activities, with the antioxidant activity of root extracts being higher than those from the leaf and stem (data not shown). The effect of the hexane layer to restrain the oxidative process is eight times higher than the effect of α-tocopherol. Generally, the scavenging effects of root extracts of *E. senticosus* against DPPH free radical are almost same only in EtOAc layer, but the antioxidative effects of the extracts were greater than α-tocopherol in all the other fractions. The DPPH assay measures hydrogen atom-donating activity and free radical-quenching antioxidant capacity. DPPH, a purple-coloured stable free radical, is reduced to the yellow-coloured diphenylpicryl hydrazine by antioxidants or reducing agents (Punchard and Kelly, 1996). The antioxidant activity can be measured using the simple DPPH method, which has broad applicability. In this study, the antioxidative activity values determined by the DPPH method varied greatly

Table 4
Scavenging effects of root extracts of *E. senticosus* Odaesan, Korea against DPPH free radicals

Fraction	Antioxidant activity RC_{50} (μg/ml) DPPH removal activity
MeOH extract	20
Hexane layer	>100
EtOAc layer	10
n-BuOH layer	16
Aqueous layer	38
α-Tocopherol	12

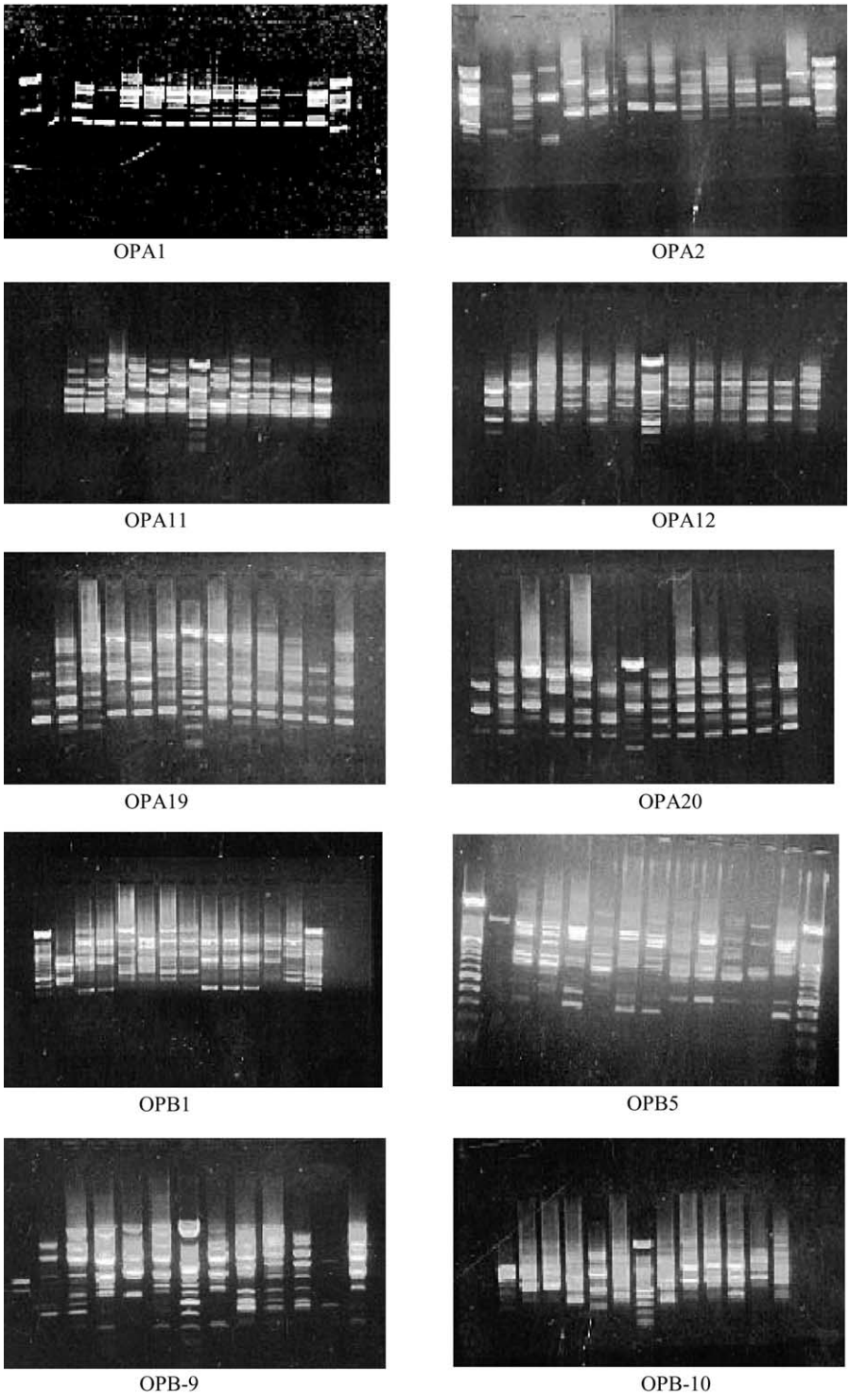

Plate 1. Randomly and specifically amplified polymorphic DNAs from accessions of *Eleutherococcus*. The sequences of each primer in OPA (#1, 2, 11, 12, 19, 20) and OPB (#1, 5, 9, 10) are shown in Table 2.

due to the crude extracts used as substrates. Therefore, accurate and detailed isolation, identification and extraction of antioxidative substances are important prerequisites for obtaining reliable results.

EtOAc fraction shows strong antilipid peroxidative activity and butanol fractions also have good inhibitory activity on lipid peroxidation in rat liver microsomes (Table 5). Ethyl acetate fractionation showed strong inhibitory activity on lipid peroxidation in rat liver microsomes when compared with other fraction layers. But methanol, hexane and the aqueous fraction layer have lower inhibitory activity on lipid peroxidation in

Table 5
Inhibition of lipid peroxidation in rat liver microsomes by root extracts of *E. senticosus* Odaesan, Korea

Fraction	Antilipid peroxidative activity RC_{50} (μg/ml)
MeOH extract	100
Hexane layer	>100
EtOAc layer	18
n-BuOH layer	56
Aqueous layer	100
α-Tocopherol	2.8

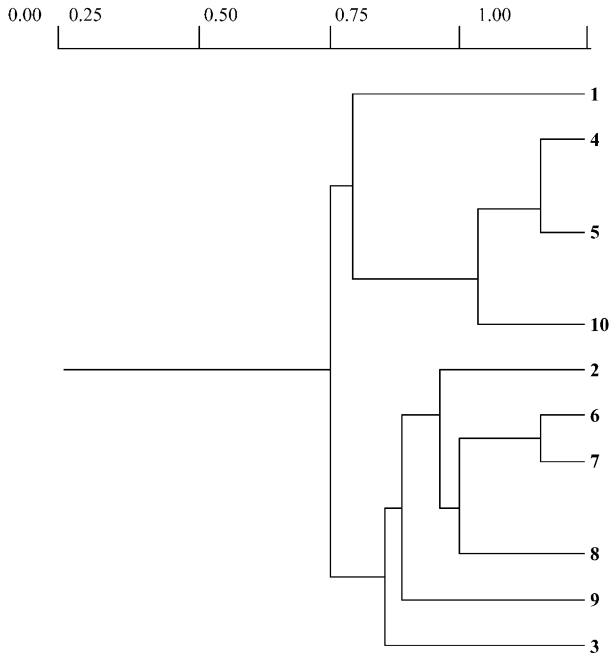

Fig. 1. A dendrogram of 10 accessions of *Eleutherococcus senticosus* Max. based on DNA polymorphism by PCR analysis. 1. *E. senticosus* Bukhado, Japan; 2. *E. senticosus* Siberia, Russia; 3. *E. senticosus* Chunchon, Korea; 4. *E. seoulense*; 5. *E. chiisanesis*; 6. *E. senticosus* Taegisan, Korea; 7. *E. senticosus* Jamgok, Korea; 8. *E. senticosus* Odaesan, Korea; 9. *E. senticosus* Yunbyun, China; 10. *E. sessliliflorus* Yeongwal, Korea.

rat liver microsomes, compared with α-tocopherol and ethyl acetate.

The cytotoxic effects of root extracts of *E. senticosus* were evaluated on six kinds of human cancer cell lines (Table 6). The values of 50% growth inhibition (GI_{50}) were mostly below 30 μg/ml for crude extracts to be considered as significantly active. The values of 50% growth inhibition in the hexane layer were much lower than those in the EtOAc layer, the BuOH layer, the aqueous layer, and in the MeOH extraction. Some cell lines, such as prostate (PC-3), renal (ACHN) and leukaemia (MOLT-4F), showed relatively low GI50 values. Kim et al. (2000) reported that the 50% ethanol extracts of *E. senticosus* inhibited the cell growth of Hep3B and A549. In screening immunomodulating activities, the cell growth and viability of Jurkat (T-cell) were increased and activated up to 275% by adding ethanol extracts.

Previous studies have reported that extracts of *E. senticosus* were effective on gastric ulcer (Fujikawa et al., 1996), immunomodulating (Xie, 1989; Li, 1991; Shen et al., 1991), serum-lipid levels (Shi et al., 1990) and oxidative inhibition (Kim et al., 2000). Therefore, *E. senticosus* is a useful medicinal plant for antioxidant medicine. The immunostimulating activity on T-cells and the direct cytotoxicity activity of extracts of *E. senticosus* might help inhibit tumour growth in a synergistic manner.

In the future research, it is necessary to investigate whether the effect of the extracts on antilipoperoxidant activity could partly explain the inhibition of lipid peroxidation, through decrease of reactive oxygen metabolites by inducing antioxidative genes, such as glutathione peroxidase, ascorbate peroxidase and superoxide dismutase. Also there is possible involvement of mechanisms related to free radical scavenging effects by the extracts of *E. senticosus*. It also is necessary to measure the antiperoxidative activity in addition to decrease in active oxygen formation by the extracts of *E. senticosus*. The mechanisms behind this anticarcinogenic effect from the extracts of *E. senticosus* are not known, although it has been shown that the extracts of *E. senticosus* are able to prevent the metabolic activation of carcinogens, inhibit

Table 6
Cytotoxic activities of root extracts from *E. senticosus* Odaesan, Korea

Fraction	GI_{50} (μg/ml)						
	Prostate (PC-3)	Colon (HCT-15)	Renal (ACHN)	Colon (SW-620)	Lung (A549)	Leukaemia (MOLT-4F)	GI_{50}
MeOH extraction	>30	>30	>30	>30	>30	>30	>30
Hexane layer	20.51	>30	20.22	24.79	>30	14.29	23.30
EtOAC layer	>30	>30	>30	>30	>30	>30	>30
n-BuOH layer	>30	>30	>30	>30	>30	>30	>30
Aqueous layer	>30	>30	>30	>30	>30	>30	>30
Adriamycin	0.13	0.16	0.13	<0.03	<0.03	0.05	0.09

tumor promotion and cell proliferation, and act as agents that induce antioxidant or oxidative-related enzymes. Further investigations will give us clearer answers on the role of the extracts of *E. senticosus*.

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References

Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12, 13–15.

Fritsch, P., Rieseberg, L.H., 1996. The use of random amplified polymorphic DNA (RAPD) in conservation genetics. In: Smith, T.B., Wayne, R.K. (Eds.), Molecular Genetics Approach in Conservation. Oxford University Press, London, pp. 54–73.

Fujikawa, T., Yamaguchi, A., Morita, I., Takeda, H., Nishibe, S., 1996. Protective effects of *Acanthopanax senticosus* Harms from Hokkai do and its components on gastric ulcer in restrained cold water stressed rats. Biological Pharmacy Bulletin 19, 1227–1230.

Hahn, D.R., Kim, C.J., Kim, J.H., 1985. A study on chemical constituents of *Acanthopanax koreanum* Nakai and its pharmaco-biological activities. Yakhak Hoeji 29, 357–361.

Hoey, B.K., Crowe, K.R., Jones, V.M., 1996. A phylogenetic analysis of *Pisum* based on morphological characters, and allozyme and RAPD markers. Theoretical and Applied Genetics 92, 92–100.

Jaccard, A., 1988. Nouvelles recherches sur la distribution florale. Bulletin Society Vaudise Science Nature 44, 223–270.

Kim, H.M., Han, S.B., Kim, M.S., Kang, J.S., Oh, G.T., Hong, D.H., 1996a. Efficient fixation procedure of human leukemia cells in sulforhodamine B cytotoxicity assay. Journal of Pharmacology Toxicology Methods 36, 163–169.

Kim, K.Y., Hun, D.Y., Kim, S., Park, H.K., 1996b. Studies on gathering seeds of *Eleutherococcus senticosus* Max. Korean Journal of Breeding 28, 120–121.

Kim, W.G., Kim, J.P., Kim, C.J., Lee, K.H., Yoo, I.D., 1996c. Benzastatins A, B, C, and D: new free radical scavengers from *Streptomyces nitrosporeus* 30643. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. Journal of Antibiotics 49, 20–25.

Kim, S.K., Kim, Y.G., Lee, M.K., Han, J.S., 2000. Comparison of biological activity according to extracting solvents of four *Acanthopanax* root bark. Korean Journal of Medical Crop Science 8, 21–28.

Lee, W.T., 1979. Distribution of *Acanthopanax* plants in Korea. Korean Journal of Pharmacology 10, 103–107.

Li, X.Y., 1991. Immunomodulating Chinese herbal medicines. Mem. Inst. Oswaldo Cruz. 86 (Suppl. 2), 159–164.

Lim, J.D., Seong, E.S., Choi, K.J., Kim, S.K., Chung, I.M., Heo, K., Yu, C.Y., 2000. Morphological characteristics and RAPD analysis of *Epimedium* spp. Korean Journal of Medical Crop Science 8, 102–108.

Ohkawa, H., Ohishi, N., Ygi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95, 351–358.

Phillip, N.M., Afanador, L., Kelly, J.D., 1996. Recombination-facilitated RAPD marker-assisted selection for disease resistance in common bean. Crop Science 36, 86–90.

Pillay, M., Kenny, S.T., 1996. Random amplified polymorphic DNA (RAPD) markers in hop, *Humulus lupulus*: level of genetic variability and segregation in F_1 progeny. Theoretical and Applied Genetics 92, 334–339.

Punchard, N.A., Kelly, F.J., 1996. Introduction. In: Punchard, N.A., Kelly, F.J. (Eds.), Free Radicals. A Practical Approach Series. IRL Press, pp. 1–8.

Rohlf, F.J., 1993. NTSYS-PC Numerical Taxonomy and Multivalent Analysis System, version 1. 50. Exeter Publishers, New York.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour. Cold Spring Harbour Laboratory Press, New York.

Shen, M.L., Zhai, S.K., Chen, H.L., Luo, Y.D., Tu, G.R., Ou, D.W., 1991. Immuno-pharmacological effects of polysaccharides from *Acanthopanax senticosus* on experimental animals. International Journal of Immunopharmacology 13, 549–554.

Shi, Z., Liu, C., Li, R., 1990. Effect of a mixture of *Acanhopanax senticosus* and *Elsholtzia splendens* on serum-lipids in patients with hyper lipemia. Chung Hsi I Chieh Ho Tsa Chih. 10, 155–156.

Wagner, H., Heur, Y.H., Obermeier, A., Tittel, G., Bladt, S., 1982. Die DC- and HPLC-analysis der *Eleutherococcus* Droge, Dc-and HPLC-analysis of *Eleutherococcus*. Planta Medica 44, 193–198.

Wu, L., Smythe, A.M., Stinson, S.F., Mullendore, L.A., Monks, A., Scudiero, D.A., Paull, K.D., Koutsoukos, A.D., Rubinstein, L.V., Boyd, M.R., Shoemaker, R.H., 1992. Multidrug-resistant phenotype of disease-oriented panels of human tumour cell lines used for anticancer drug screening. Cancer Research 52, 3029–3034.

Xie, S.S., 1989. Immunoregulatory effect of polysaccharide of *Acanthopanax senticosus* (PAS). I. Immunological mechanism of PAS against cancer. Chung Hua Chung Liu Tsa Chih 11, 338–340.

Xiong, Q., Kadota, S., Tadata, T., Namba, T., 1996. Antioxidative effects of phenylethanoids from *Cistanche deserticola*. Biological Pharmacy Bulletin 19, 1580–1585.

Yoo, K.O., Ahn, S.D., Yu, C.Y., Park, K.Y., Lim, H.T., 1997. Intraspecific variations of the *Epimedium koreanum* by randomly and specifically amplified polymorphic DNA markers. Journal of Korean Horticulture Science 38, 183–187.

Yook, C.S., Rho, Y.S., Sed, S.H., Leem, J.Y., Han, S.H., 1996. Chemical components of *Acanthopanax divaricatus* and anticancer effect in leaves. Yakhak Hoeji 40, 251–261.