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Anti-Oxidant Activities of *Acanthopanax senticosus* Stems and Their Lignan Components

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The antioxidant activities of *Acanthopanax senticosus* stems were evaluated in CCl_4-intoxicated rats. The *n*-butanol fraction from the water extract of the stems, when pretreated orally at 200 mg/kg/day for 7 consecutive days in rats, was demonstrated to exhibit significant increases in antioxidant enzyme activities such as hepatic cytosolic superoxide dismutase, catalase and glutathione peroxidase by 30.31, 19.82 and 155%, respectively. The *n*-butanol fraction whereas showed a significant inhibition of serum GPT activity (65.79% inhibition) elevated with hepatic damage induced by CCl_4-intoxication. Eleutheroside B, a lignan component, isolated from the *n*-butanol fraction was found to cause a moderate free radical scavenging effect on DPPH, its scavenging potency as indicated in IC_{50} value, being 58.5 μM. These results suggested that the stems of *A. senticosus* possess not only antioxidant but also hepatoprotective activities.

Key words: *Acanthopanax senticosus*, Araliaceae, s-Transaminases CCl_4-intoxication, Antioxidant enzymes, Eleutheroside B, Eleutheroside E, DPPH

INTRODUCTION

Reactive oxygen species (ROS) are generated as byproducts of biological reactions or from exogenous factors (Cerutti, 1991). The involvement of ROS in the pathogenesis of a large number of diseases is well documented (Cross, 1987). It is suggested that free radical damage to cells leads to the pathological changes associated with aging (Beckman and Ames, 1998). Free radicals may also be a contributory factor in a progressive decline in the function of the immune system (Pike and Chandra, 1995). Cooperative defense systems that protect the body from free radical damage include the anti-oxidant nutrients and enzymes. The anti-oxidant enzymes include superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-px) and indirectly glutathione reductase. Their roles as protective enzymes are well known and have been investigated extensively both *in vivo* and *in vitro* model systems. The first three enzymes directly catalyze the transformation of peroxides and superoxides to nontoxic species. Glutathione reductase reduces oxidized glutathione to glutathione, a substrate for glutathione peroxidase. The consequences of oxidative stress are serious, and in many cases are manifested by increased activities of enzymes involved in oxygen detoxification. Identification of new anti-oxidants remains a highly active research area because anti-oxidants may reduce the risk of various chronic diseases caused by free radicals.

Approximately fifteen species of the genus *Acanthopanax* are known to be self-grown in the Korean peninsula. *A. senticosus*, which is distributed in northern Asia, has traditionally been used as a tonic and a sedative, as well as in the treatment of rheumatism and diabetes (Perry, 1980; Yook, 1990). It had been studied extensively and shown to exhibit a variety of activities such as anti-bacterial, anti-cancer, anti-inflammatory, anti-gout, anti-hepatitis, anti-hyperglycemic, anti-leishmanicidic, anti-oxidant, anti-pyretic, anti-xanthine oxidase, choleretic, hemostatic, immunostimulatory, hypocholesterolemic and radioprotectant effects (Davydov and Krikorian, 2000). Investigations

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on the compounds from *A. senticosus* have revealed the presence of phenolic compounds such as isofraxidin, eleutherosides B and E from the stem barks (Nishibe *et al.*, 1990), eleutheroside E_2 and isomaltol 3-*O*-α-D-glucopyranoside from the roots (Li *et al.*, 2001), and chiisanoside, chiisanogenin and hyperin from the leaves (Lee *et al.*, 2003), *etc.* Recently, we reported the effects of the water extract from the stem bark of *A. senticosus* on hyperlipidemia in rats (Lee *et al.*, 2001).

This paper deals with hepatoprotective as well as anti-oxidant activities from *A. senticosus* stems and estimation of the activities of lignans.

MATERIALS AND METHODS

Instruments and reagents

Sodium azide, ethylenediamine tetraacetic acid (EDTA), β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), cumene hydroperoxide, glutathione reductase, carbon tetrachloride (CCl_4), xanthine, potassium cyanide (KCN), sodium dodecylsulfate, cytochrome C, pyridine and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chem. Co. (St. Louis, MO). All other chemicals and reagents were analytical grade. ^{1}H- and ^{13}C-NMR spectra were recorded with BRUKER AVANCE 500 NMR spectrometer in DMSO using TMS as internal standard. MS spectra were measured with JEOL JMS-AX505WA mass spectrometer.

Plant materials

The stems of *Acanthopanax senticosus* (Rupr. & Maxim.) Harms were collected at Jilin Province, China in Oct. 2002, and verified by Prof. S. H. Cho, Kongju National University of Education, Korea. A voucher specimen of this plant was deposited at the R & D Center for Functional Foods, Institute of Food and Culture, Pulmuone Co. Ltd., Korea.

Extraction and isolation

The air-dried powdered stems of *A. senticosus* were extracted with H_2O under reflux. The resultant extract was combined and lyophilized to afford the residue. The H_2O extract was suspended in H_2O and then extracted successively with equal volumes of $CHCl_3$, EtOAc, and *n*-BuOH. Each fraction was evaporated *in vacuo* to obtain $CHCl_3$, EtOAc, *n*-BuOH, and H_2O fractions.

A portion of the *n*-BuOH fraction (20 g) was chromatographed on silica gel column (7×60 cm) eluting with a gradient of $CHCl_3$-MeOH to afford compounds **1** (326 mg, 95:5), and **2** (697 mg, 90:10).

Compound **1**; FAB-MS *m/z*: 373 $[M+H]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H (ppm): 6.73 (2H, s, H-2,6), 6.46 (1H, d, *J* = 15.9 Hz, H-7), 6.33 (1H, dt, *J* = 15.9, 5.1 Hz, H-8), 4.84 (1H, d, *J* = 7.5 Hz, glycosyl H-1'), 4.11 (1H, dd, *J* = 5.1, 1.4 Hz, H-9a), 4.09 (1H, dd, *J* = 5.1, 1.4 Hz, H-9b), 3.77 (6H, s, 2×OMe); ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C (ppm): 152.7 (C-3,5), 133.0 (C-4), 131.0 (C-7), 129.0 (C-8), 128.1 (C-1), 104.5 (C-2,6), 103.1 (Glc C-1'), 77.4 (Glc C-5'), 76.5 (Glc C-3'), 74.9 (Glc C-2'), 71.0 (Glc C-4'), 62.0 (C-9), 60.5 (Glc C-6'), 56.3 (OMe).

Compound **2**; FAB-MS *m/z*: 743 $[M + H]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6) δ_H (ppm): 6.67 (4H, s, H-2',6'), 4.88 (2H, d, *J* = 7.3 Hz, glycosyl H-1"), 4.67 (2H, d, *J* = 3.6 Hz, H-2), 4.28 (2H, dd, *J* = 8.5, 6.6 Hz, H-4_{eq}), 4.20 (2H, dd, *J* = 8.5, 3.0 Hz, H-4_{ax}), 3.76 (12H, s, 4×OMe), 3.19 (2H, m, H-1); ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C (ppm): 153.2 (C-3',5'), 138.1 (C-4'), 134.1 (C-1'), 104.6 (C-2', 6'), 103.3 (Glc C-1"), 85.7 (C-2), 77.5 (Glc C-5"), 76.7 (Glc C-3"), 74.5 (Glc C-2"), 72.1 (C-4), 70.2 (Glc C-4"), 61.2 (Glc C-6"), 57.0 (OMe), 54.2 (C-1).

Anti-oxidant assay *in vivo*

Male Sprague-Dawley rats weighing 200-250 g were used in all experiments. Animals were maintained on 12 h light/dark cycle at approximately 22°C and allowed food and water *ad libitum.* Eight animals were used in each group. All treatments were conducted between 9:00 and 10:00 o'clock to minimize variations in animal response due to circadian rhythm. Rats were injected i.p. with a mixture of CCl_4 in olive oil (1:1) at a dose of 0.6 mL/kg to induce hepatotoxicity. Control animals were given the vehicle alone. Rats were pretreated with silymarin and the fractions given p.o. at a dose of 200 mg/kg/day for seven consecutive days prior to the administration of CCl_4. Animals were sacrified 24 h after CCl_4 dosing and blood was collected by decapitation for the determination of serum transaminases.

Hepatic tissues were carefully excised and homogenized in cold 1.15% KCl-10 mM phosphate buffer with EDTA (pH 7.4) and centrifuged at 10,000 rpm for 10 min. The supernatant was further centrifuged at 40,000 rpm for 60 min to obtain cytosolic extract for the measurement of liver cytosolic SOD, catalase, and GSH-px activities. The protein content was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Hepatocellular damage was estimated by measuring sGOT and sGPT activities (Reitman and Frankel, 1957). Blood was collected from the abdominal aorta of each rat. The blood was centrifuged at 3,000 rpm at 4°C for 10 min to separate the serum. sGOT and sGPT activities were expressed as Karmen unit.

SOD was assayed by the method of McCord and Fridovich (1969). The reaction mixture containing 0.5 mM xanthine as substrate (300 μL), 0.05 mM KCN (100 μL), 1% sodium deoxycholate (100 μL), xanthine oxidase (20 μL), cytosolic extract (20 μL) and 0.1 mM cytochrome C

(300 μL) was placed in a 1 cm cuvette and the rate of increase in absorbance at 550 nm was recorded for 5 min. SOD activity was expressed as unit/mg protein. Catalase was assayed by the method of Rigo and Rotilio (1977). The cytosolic extract of liver (40 μL) diluted 10 times was added with 0.13 mM phosphate buffer (pH 7.0, 500 μL), distilled water (660 μL) and 15 mM H_2O_2 (1800 μL), and thoroughly mixed. The rate of changes in the absorbance at 240 nm for 5 min was recorded. Catalase activity was expressed as unit/mg protein. GSH-px was assayed by the method of Burk *et al.* (1978). The reaction mixture containing 0.3 mM phosphate buffer with 4.0 mM EDTA (pH 7.2, 1000 μL), 26.56 mM sodium azide (500 μL), 294.37 mM GSH (60 μL), 8.4 mM NADPH (110 μL), 1 mM cumene hydroperoxide (320 μL), glutathione reductase (5 μL) and cytosolic solution (30 mL) was placed in 1 cm cuvette and the rate of changes in absorbance was recorded at 340 nm for 5 min. GSH-px activity was expressed as unit/mg protein.

DPPH assay *in vitro*

The hydrogen-donating ability of lyophilized extracts was examined on the basis of the method of Blois (1958) in the presence of DPPH stable radical. Used as reagent, DPPH evidently offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic anti-oxidants (Cao *et al.*, 1997). The samples and positive control, L-ascorbic acid, were diluted with methanol to prepare sample solution equivalent to 400, 80, 40, 8 and 4 μM of dried sample/mL solutions. 40 μL of DPPH (1.5×10^{-4} M) solution was pipetted into each well of 96 well plate followed by 160 μL of the sample solutions. The mixture was incubated at 37°C for 30 min. Absorbance of the methanolic DPPH-tincture was measured at 540 nm. The inhibition percentage (%) of radical scavenging activity was calculated using the following equation.

$$\text{Inhibition (\%)} = \frac{(A_o - A_s)}{A_o} \times 100$$

Where A_o is absorbance of the control and A_s is absorbance of the samples at 540 nm.

Statistical analysis

Multiple comparison test was applied for detecting the significance of difference between different groups.

RESULTS AND DISCUSSION

The water-soluble fractions of *A. senticosus* stems were tested for their free radical scavenging effects, and the effects on hepatocellular damage in CCl_4-intoxicated rats were evaluated.

The effects of the fractions of *A. senticosus* stems on the serum transaminases in CCl_4-intoxicated rats were estimated and the results are shown in Fig. 1. In the CCl_4-intoxicated control group, the sGOT and sGPT activities increased significantly when compared with the normal group. In contrast, the group treated with the *n*-BuOH fraction decreased significantly these elevated transaminase activities. The *n*-BuOH fraction inhibited the sGOT and sGPT activities by 8.09 and 65.79%, respectively. Silymarin inhibited the sGOT and sGPT activities by 18.85 and 46.36%, respectively.

The *n*-BuOH fraction causes a significant elevation of free radical scavenging-enzyme activities such as SOD, catalase and GSH-px. As shown in Fig. 2, silymarin caused significant elevation of SOD, catalase and GSH-px activities by 70.48, 42.32 and 200%, respectively. The *n*-BuOH fraction increased the SOD, catalase and GSH-px activities by 30.31, 19.82 and 155%, respectively. Increase in the catalase activity with respect to CCl_4 treatment indicates that the *n*-BuOH fraction can play an important role in scavenging hydrogen peroxide. Restoration

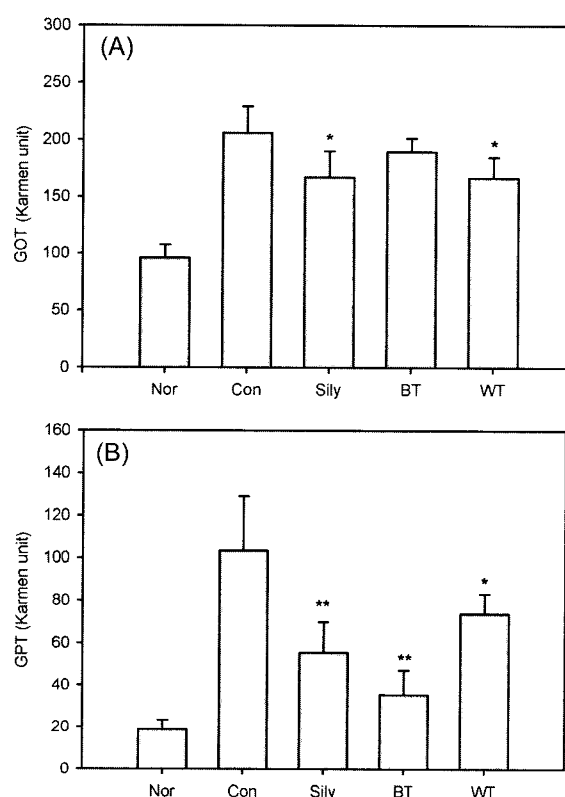

Fig. 1. Effects of the fractions from *A. senticosus* stems on the sGOT (A) and sGPT (B). Rats were pretreated with silymarin and each fraction given orally at a dose of 200 mg/kg, for 7 days, prior to the administration of CCl_4. Rats were sacrified 24 h after CCl_4. Heparinized blood sample was collected. Hepatocellular damage was estimated by measuring sGOT and sGPT activities. Significantly different from the control; *$p<0.05$, **$p<0.01$ (Nor, Normal; Con, Control; Sily, Silymarin; BT, the *n*-BuOH fraction; WT, the H_2O fraction).

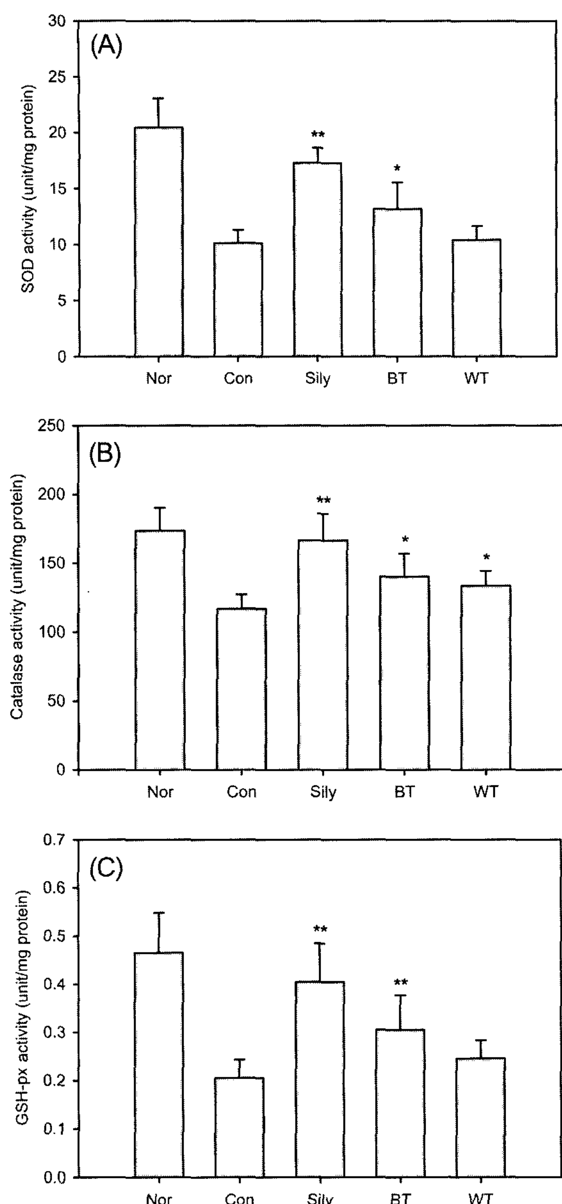

Fig. 2. Effects of the fractions from *A. senticosus* stems on the liver cytosolic SOD (**A**), catalase (**B**) and GSH-px (**C**) activities. Rats were pretreated with silymarin and each fraction was given p.o. at a dose of 200 mg/kg/day for seven consecutive days, prior to the administration of CCl_4. Hepatic tissues were carefully excised, homogenized, and centrifuged to obtain cytosolic fractions for the measurement of liver cytosolic SOD, catalase, GSH-px activities. Significantly different from the control; *$p<0.05$, **$p<0.01$ (Nor, Normal; Con, Control; Sily, Silymarin; BT, the *n*-BuOH fraction; WT, the H_2O fraction).

of SOD activity indicates that the *n*-BuOH fraction can help in cellular defense mechanisms by preventing cell membrane oxidation. Similarly, an increase in glutathione peroxidase activity indicates that the *n*-BuOH fraction also helps in the restoration of vital molecules such as NAD, cytochrome, and glutathione.

In conclusion, the *n*-BuOH fraction of *A. senticosus* stems showed the recovery of liver function by radical-scavenging activities such as SOD, catalase and GSH-px in CCl_4-intoxicated rats. The present study demonstrated that *A. senticosus* possess not only the anti-oxidant, but also the hepatoprotective activities in rats.

A chromatographic separation of this active *n*-BuOH fraction from *A. senticosus* stems led to the isolation of compounds **1** and **2**. Compounds **1** and **2** were obtained as white crystals from MeOH. The structures of **1** and **2** were elucidated as lignans (Fig. 3) such as eleutherosides B (= syringin) and E (= acanthoside D), respectively, with authentic samples as described in the literatures (Sutarjadi *et al.*, 1978; Kinjo *et al.*, 1991).

Free radical scavenging effects of eleutherosides B (**1**) and E (**2**) isolated from *A. senticosus* on DPPH were tested and the results are indicated in Table I. DPPH was reduced with the addition of eleutheroside B (**1**) in a concentration-dependent manner. When compared by IC_{50}, the free radical scavenging potency of eleutherosides B (**1**) was 58.5 µM, which was weaker than that of L-ascorbic acid as a positive reference drug, indicating potential anti-oxidant properties in the *n*-butanol fraction. But there is no free radical scavenging effect of eleutheroside E (**2**) on DPPH. Accordingly eleutherosides B (**1**) isolated from the active *n*-BuOH fraction of *A. senticosus* stems may be the main compound of the anti-oxidant and the hepato-

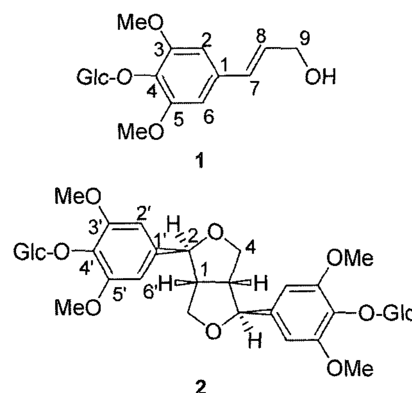

Fig. 3. Structures of eleutherosides B (**1**) and E (**2**) from *A. senticosus* stems

Table I. Free radical scavenging effects of eleutherosides B (**1**) and E (**2**) on DPPH

Treatments	Concentration (µM)					IC_{50} (µM)
	4	8	40	80	400	
L-Ascorbic acid[a]	36.9[b]	85.6	98.9	99.2	99.8	4.0
1	3.4	8.9	32.9	53.4	95.2	58.5
2	-	-	-	3.4	17.1	-

[a] A reference compound
[b] Percent inhibition

protective activities in rats. This compound is the major compound in the crude drug responsible for the pharmacological activities of *A. senticosus*.

These results suggest that the stems of *A. senticosus* possess not only the hepatoprotective, but also the antioxidant activities in rats.

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