Article

The Antifibrotic and Antioxidant Activities of Hot Water Extract of Adventitious Root Culture of *Panax ginseng* (ARCP)

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The anti-fibrotic effects of hot water extract of adventitious root culture of *Panax ginseng* (ARCP) and the possible mechanisms were investigated on CCl_4-induced hepatotoxicity model mice. Fibrosis was induced by a mild treatment of CCl_4. Then silymarin as a positive control drug and ARCP or carrier alone as a negative control were treated. Serum GPT, GOT and ALP activity levels were lowered by 25, 21 and 11% for silymarin treated group and by 48, 39 and 14% for ARCP treated group compared to carrier treated alone. Hepatic collagen for ARCP treatment group was reduced by 18% and MDA contents decreased a little more. Pro-fibrotic gene (TGF-β1, TIMP-1 and α-SMA) expression increased following the CCl_4 treatment, but both the silymarin and the ARCP treatments decreased the expressions of these genes by 20% to 50%. The antioxidant effect of ARCP was studied by DPPH free radical scavenging activity. In addition, a generation of reactive oxygen species (ROS) was also reduced in H_2O_2-treated HepG2 cells upon the ARCP treatment. In summary, ARCP has antioxidant property, and can have some protection against oxidative stress; more importantly, ARCP can efficiently protect mice against CCl_4-induced fibrosis.

Key words: *adventitious root culture, anti-fibrotic effect, antioxidant effect,* Panax ginseng, *tetrachloromethane*

Recently, improvements of the quality of life worldwide has spurred great interests in human health and the longevity of life. Wild ginseng has been considered as a remedy for a broad spectrum of diseases from the past. *Panax ginseng* C.A. Meyer (Araliaceae) is a valuable herb in East Asia, and because of its pharmacological properties, it has also gained popularity in the West [Mahady *et al.*, 2000]. Saponins of wild ginseng have shown to improve immunity function. The immuno-modulating effects of wild and cultured ginseng were compared recently [Mizuno *et al.*, 1994]. There are reports of decrease in cholesterol [Lee *et al.*, 2003], and

whitening effects [Shin, 2001] of wild ginseng roots culture. Especially, ginseng has been found to have liver-protection effect via a cytochrom P450 inhibition to reduce CCl_4-induced hepatic lipid peroxidation [Kim *et al.*, 1997].

Traditionally, *P. ginseng* is categorized as an either cultivated or wild according to the different nurturing methods. Recently, adventitious root culture technique of wild *P. ginseng* has been developed to make mass production possible in a large culture system. However, only a few studies have been reported concerning the pharmacological activities of ARCP [Yoo *et al.*, 2003].

Fibrosis reflects increased the deposition of the physiological components of extracellular matrix, which is the hallmark of aging of various organs, including heart and kidneys. Aging is also associated with variable degrees of fibrosis in the liver [Sun *et al.*, 1998]. OS might represent a direct or an indirect profibrogenic stimulus for HSC, suggested by *in vivo* experimental studies of antioxidants preventing OS, lipid peroxidation and liver fibrosis [Chao *et al.*, 2002]. Among the various cytokines, TGF-β1 plays an important role as a

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Abbreviations: ALP, alkaline phosphatase; α-SMA, α-smooth muscle actin; ALT, alanine transaminase; ARCP, adventitious root culture of *Panax ginseng*; AST, aspartate aminotransferase; HSC, hepatic stellate cells; MDA, malondialdehyde; OS, oxidative stress; ROS, reactive oxygen species; TGF-β1, transforming growth factor β-1; TIMP-1, tissue inhibitors of metalloprotease

profibrogenic factor in chronic liver disease, triggering the expression of procollagen-I and TIMP-1, the key effectors of fibrogenesis [Jeong *et al.*, 2005].

Tetrachloromethane (CCl_4) induced liver damage has been extensively studied and widely used as a model for screening hepato-protectors. CCl_4 is capable of causing liver necrosis, changing the activities of metabolic enzymes in liver [Noguchi *et al.*, 1982], as well as increasing the hepatic lipid peroxidation [Comporti, 1985]. Because the damaging effects of CCl_4 are generated by oxidative stress, many antioxidants [Ilavarasan *et al.*, 2003] have been tried as hepato-protective agents.

In this study, we created a liver damage by pre-treatment of mice with CCl_4 for a month and then treated them with water extracts of ARCP to test the protective or healing effects. This paper reports the mechanism of the anti-fibrotic effect of water extracts of ARCP on CCl_4-induced mouse liver.

Materials and Methods

Chemicals. All the plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA). Silymarin, trichloroacetic acid and 2-thiobarbituric acid were obtained from Sigma (St Louis, MO, USA), and CCl_4 was obtained from Aldrich (Milwaukee, WI, USA). Dulbecco's Modified Eagle's Medium and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals and reagents were of the highest quality available.

Plants materials and extraction procedure. ARCP was supplied by Biovalue Co. (Jeju, Korea). Hot water extract of ARCP was prepared as follow: Five kilograms of wet weight ARCP was placed in the extractor (Cosmos, Inchon, Korea) with 2 L distilled water and extracted for 9 h at 120°C under reduced pressure. The remaining liquid was collected, lyophilized and stored for further use.

Animals. Six-week-old male Balb/c mice about 22 g were obtained from Daehan Lab. (Animal Research Center Co. Ltd, Daejeon, Korea). They were acclimated for 1 week before dosage under controlled environmental conditions at 23°C and 65% relative humidity with a 12-h light/dark cycle. Feed and water were available *ad libitum*.

Experimental design. Mice were divided into five groups with five mice per group (control, CCl_4, silymarin and two different dose ARCP treatment groups). Liver fibrosis was induced in mice by the intraperitoneal injection of CCl_4 (0.15 mL per mouse (diluted 1 : 1 in mineral oil)) once a week for up to 4 weeks. After liver fibrosis was induced, the groups of mice were orally

given silymarin (50 µg/g body weight) or ARCP (100 or 600 µg/g body weight) dissolved in 0.5% sodium carboxymethylcellulose (CMC) every day during a week.

Measurement of AST, ALT and ALP. After treatment, blood was obtained by heart puncture and centrifuged at 2500 × g for 20 min at 10°C to separate the sera. Activities of AST, ALT and ALP in the serum were measured according to manufacturer's manual using Randox TM kit and Screenmaster colorimeter set.

Collagen contents and staining. After development of fibrosis, mice were anaesthetized and laparotomized. The liver was perfused with phosphate-buffered saline and a part of the liver was removed and quickly frozen in liquid nitrogen. The remaining part of the liver was perfusion-fixed with 4% formaldehyde, dehydrated and embedded in Polybed. Sections were cut at a thickness of 5 mm and stained for 1 h in 0.1% (w/v) Sirius red (Direct Red 80, Aldrich, Milwaukee, WI).

A portion of each liver was homogenized in 33 volumes (mL/g) of 0.5 M acetic acid at 4°C using a Polytron PCU-2 homogenizer (Kinematica, Luzern, Switzerland), and the homogenate was disrupted by freeze-thawing and sonication for 2 min for collagen determination [Shimizu *et al.*, 1999]. The results are expressed as micrograms of collagen per milligram of liver tissue.

Polymerase chain reaction for TGF-β1, TIMP-1, and α-SMA. Total RNA was extracted using Trizol (Biostar Biologic technology Co. Ltd. USA.) according to the manufacturer's directions. Then total RNA was reverse-transcribed into cDNA. PCR system contained 2 µL cDNA, 5 µL 10 × buffer, 5 µL 25 mmol/L $MgCl_2$, 1 µL 10 mmol/L dNTP, 1 µL 20 pmol/µL target gene sense and anti-sense primer, 1 µL 20 pmol/µL β-actin primer pair, 3 Unit of Taq DNA polymerase. PCR was carried out as followed: initial denaturation at 95°C for 5 min, 30 amplification cycles (denaturation at 94°C for 45 s, annealing at 55°C for 30 s, extension at 72°C for 1 min) and final extension at 72°C for 7 min. The primers used were listed in Table 1.

Electrophoresis and semi-quantitative analysis. The PCR products were run on 2% agarose gel electrophoresis and visualized with ethidium bromide staining. The expected product sizes were 170 bp of TGF-β1, 170 bp of TIMP-1, 74 bp of α-SMA and 188 bp of β-actin. GS-710 calibrated imaging densitometer system (Bio-RAD) was employed to detect the density of bands of PCR products. The values of TGF-β1, TIMP-1 and α-SMA expression were semi-quantified by scanning densitometry using the ratios of TGF-β1/β-actin, TIMP-1/β-actin and α-SMA to assess the relative level.

Determination of antioxidant activity using DPPH. The free radical scavenging activity of the ARCP extracts

Table 1. Primer pairs used for RT-PCR in this study

	Forward	Reverse
TGF-β1	5'-tgacgtcactggagttgtacgg-3'	5'-gttcatgtcatggatggtgc-3'
TIMP-1	5'-attcaaggctgtgggaaatg-3'	5'-aagaagctgcaggcattgat-3'
α-SMA	5'-cctggcttcgctgtctacct-3'	5'-ttgcggtggacgatgga-3'
β-actin	5'-aggtgacagcattgcttctg-3'	5'-gctgcctcaacacctcaac-3'

was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay described by Blois. ARCP at final concentrations of 2.5, 1, 0.5, and 0.25 mg/mL were tested individually by addition to 150 mM solution of DPPH in ethanol. The mixtures were vigorously mixed and allowed to stand in the dark at RT for 4hrs. The absorbance of the remaining DPPH was measured at 520 nm using a spectrophotometer. These experiments were run in triplicate. The radical scavenging activity was calculated according to following equation:

Radical scavenging activity (%) = [(Ao-A)/Ao]*100%, where Ao is the A_{520} of DPPH without sample (control), A is the A_{520} of DPPH with sample.

Cell culture. The human hepatoma HepG2 (KCLB No.58065) cells were purchased from the Korea Cell Line Bank (Seoul, Korea). They were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Cells were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere. The culture medium was changed twice a week, and the cells were subcultured at 1 : 4 ratio once a week.

Determination of reactive oxygen species. Cellular ROS were quantified by dichlorofluorescein (DCFH) assay, in which the esterified form of 2'',7'-dichoro-fluoresceindiacetate (DCFH-DA) diffuses through the cell membrane and is enzymatically deacetylated by intracellular esterase. The resulting compound, dichloro-fluorescein (DCFH), is reactive with ROS to give an oxidized fluorescent compound, dichlorofluorescein (DCF). HepG2 cells were pretreated with ARCP for 30 min, then washed twice and incubated with 1 mM H_2O_2 for 30 min. DCFH-diacetate was added to the culture plates at a final concentration of 12 mM and incubated for 30 min at 37°C. DCF fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using Genios multiwell fluorescence plate reader (GENios, Tecan, Salzburg, Austria).

Effect of wild ginseng on lipid peroxidation inhibitory activity in *vivo*. Lipid peroxidation was assayed by measuring malondialdehyde (MDA) according to the method of Ohkawa *et al.* In order to evaluate the inhibitory activity of the ARCP on a lipid peroxidation

generated assay system *in vivo*. Liver tissue were sliced and homogenized (13,000 rpm, 3 min) with 25 mM Tris-HCl buffer (pH 7.2) (10% w/v). In a glass test tube, 0.1 mL of the liver homogenate was incubated with shaking for 1 h at 37° C in Tris-HCl buffer (pH 7.2), and then 1.5 mL of 1.0% TBA and 1.5 mL of 20% acetic acid were added and incubated continuously for 1 h at 95°C. The upper lipid solution was then extracted for spectro-photometer analysis at 532 nm absorption.

Statistical analysis. Experimental data were analyzed by Student's t-test and expressed as mean ± SE. A probability value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Serum GPT and serum GOT and alkaline phosphatase activities. After liver fibrosis was induced by the intraperitoneal injection of CCl_4 once a week for 4 weeks, silymarin or ARCP was introduced orally to mice every day for a week. Then, blood was obtained to measure the activities of aspartate aminotransferase (AST/GOT), alanine transaminase (ALT/GPT) and alkaline phosphatase (ALP). Serum GPT, GOT and ALP activities for CCl_4-treated groups were found to be 169%, 160% and 131%, respectively, compared to the non-treated groups (Fig. 1). However, with the addition of silymarin to these CCl_4-treated groups, serum GPT, GOT and ALP activity levels showed 25, 21 and 11%, decrease respectively, compared to the groups with CCl_4 treatment alone. ARCP treated group was lowered by 48, 39 and 14%, respectively. It has been generally reported and accepted that the CCl_4-induced hepatotoxicity results from its hepatotoxic metabolites and trichloromethyl free radical (•CCl_3) [Rechnagel and Glende, 1973]. This free radical can react with sulfhydryl groups, such as glutathione (GSH) and thiol-groups in the protein side chain. Also, it covalently binds with cell proteins, and initiates the lipid peroxidation process in the cellular membrane, which eventually leads to various liver pathological processes [Connor *et al.*, 1990].

Collagen accumulation. Serum levels of GPT, GOT, and ALP indicated fast recovery of hepatocytes. To estimate the progress of fibrosis, we measured collagen
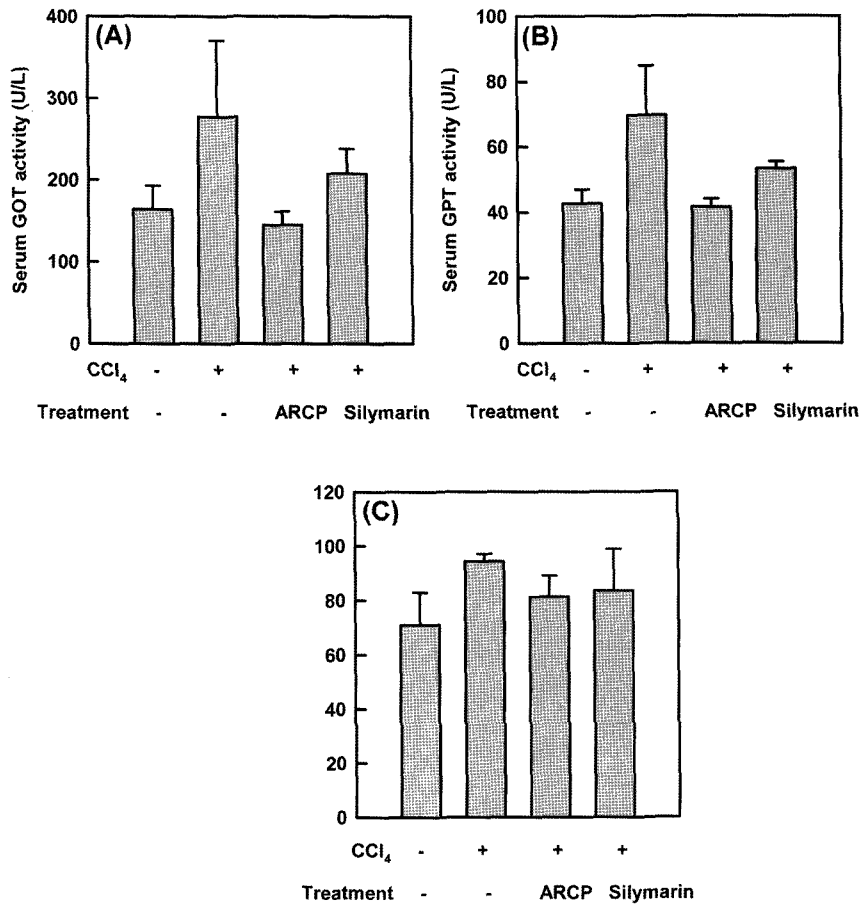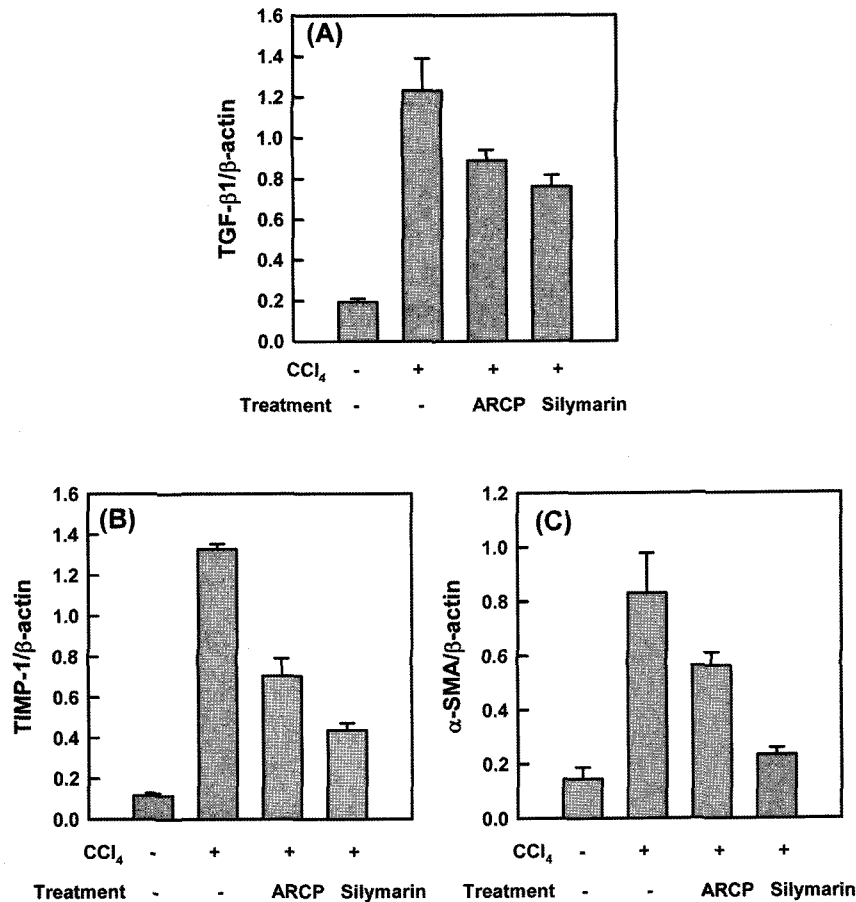
Fig. 1. Effect of ARCP administration on SGOT, SGPT and ALP levels in animals recovering from CCl₄ -induced fibrosis. After liver fibrosis was induced by the intraperitoneal injection of CCl₄ once a week for 4 weeks, silymarin or ARCP was introduced orally to mice every day for a week. Then, blood was obtained to measure the activities of (A) aspartate aminotransferase (AST/GOT), (B) alanine transaminase (ALT/GPT) and (C) alkaline phosphatase (ALP). Enzyme activities as the average ± SE from 5 animals.
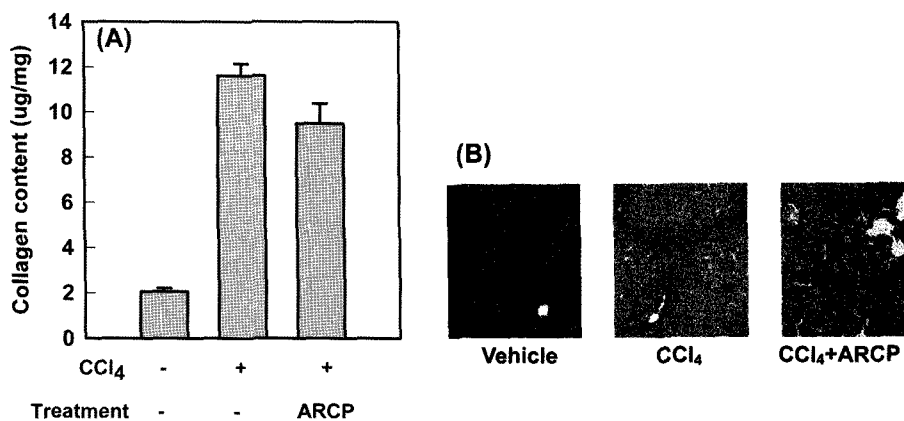
Fig. 2. Effects of ARCP treatment on spontaneous resolution of fibrosis. After liver fibrosis was induced by the intraperitoneal injection of CCl₄ once a week for 4 weeks, vehicle or ARCP was introduced orally to mice every day for a week. Livers were assessed for fibrosis by determination of (A) collagen content and examination of (B) sirus red-stained sections. Collagen contents as the average ± SE from 5 animals.

contents. For CCl₄-treated groups, hepatic collagen contents had 11.6 μg/mg liver weight (Fig. 2). However, for ARCP group, the hepatic collagen contents was 9.48

μg/mg liver weight. These hepatic collagen contents of ARCP-treated group were reduced by 18 % compared to that of CCl₄-treated groups. The state of fibrosis is

(A)

TGF-β1/β-actin

CCl₄   -   +   +   +
Treatment   -   -   ARCP   Silymarin

(B)

TIMP-1/β-actin

CCl₄   -   +   +   +
Treatment   -   -   ARCP   Silymarin

(C)

α-SMA/β-actin

CCl₄   -   +   +   +
Treatment   -   -   ARCP   Silymarin

Fig. 3. Reduced expression of TGF-β1, TIMP-1 and α-SMA transcripts in ARCP treated livers. Semi-quentitative revers-transcription polymerase chain reaction for expression of (A) TGF-β1, (B) TIMP-1 and (C) α-SMA transcripts in livers of mice injured for 4 weeks with CCl₄ followed by recovery for a week of treatment with ARCP and silymarin. Actin was used as an internal control. The relative level of transcription as the average ± SE from the three independent experiments.

controlled by both the rate of development and the reversion of fibrosis. Activation of HSC is the primary event that triggers the process of fibrogenesis and is characterized by the conversion of quiescent vitamin A-storing cells into proliferative, collagen-producing myofibroblastic cells. It is believed that the reversion of fibrosis starts with the apoptosis of activated HSC.

Effect of ARCP on TGF-β1, TIMP-1 and α-SMA expression. Because the fibrosis seems to be delayed based on the collagen contents, the genes related with fibrosis were checked. The major profibrotic cytokine, TGF-β1 expression level was measured by semi-quantitative PCR (Fig. 4-A). The TGF-β1 expression in the CCl₄-treated group revealed 6.4 fold increase compared to CCl₄-non-treated control group. The silymarin-treated group and the ARCP-treated group had increased TGF-β1 expression by 3.9 fold and 4.6 fold, respectively.

Since the expression of tissue inhibitors of metalloprotease (TIMPs) is reported to decrease in fibrotic tissue, the expression of TIMP-1 was semi-quantified by RT-PCR.

Figure 4-B shows that the treatment of ARCP or silymarin after the CCl₄ treatment decreased the expression of TIMP-1 to about 50% and 70%, respectively, compared to the CCl₄ treatment alone.

The elevated expressions of α-SMA in the activated hepatic stellate cells also characterize chronic hepatic fibrosis. In order to conform the effects of ARCP on liver fibrosis, the expressions of α-SMA in the CCl₄ induced fibrosis model were measured by RT-PCR. As expected, expressions of α-SMA increased in the CCl₄ induced fibrosis model animal to about 6 fold. However, silymarin treatment after induction of fibrosis reduced expression of α-SMA to about 70%. The ARCP treatment also reduced the expression of α-SMA to about 30% (Fig. 4-C). Therefore, the results stated above clearly demonstrate that ARCP has anti-fibrosis effects in the CCl₄ induced fibrosis model animal.

Effect of ARCP on lipid peroxidation inhibitory activity. Lipid peroxidation is a freely radical-mediated propagation of oxidative insult to the polyunsaturated
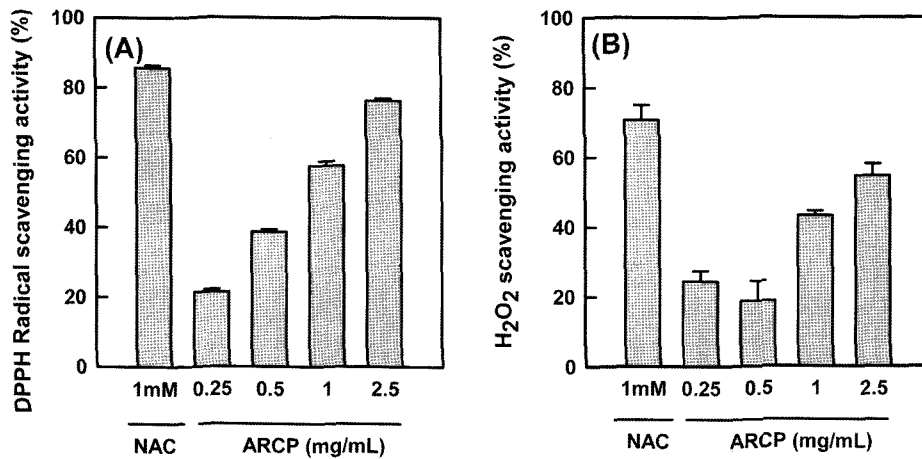
Fig. 4. DPPH radical scavenging activity (A) and H₂O₂ scavenging activity (B) of ARCP and 1 mM NAC. Each experiment was performed at least 4 times and data are expressed as the average percent change from control ± SE.

fatty acids, involving several types of free radicals. Termination occurs through enzymatic means or free radical scavenging activities of antioxidants.

The control's hepatic MDA contents was 11.6 μg/mg liver weight (Table 2). However, for silymarin and ARCP group, the hepatic MDA contents were 0.125 and 0.145 nmol/mg protein, which showed a decrease of 32% and 21% respectively compared to those of the CCl₄ group.

Antioxidant activity. The DPPH radical scavenging activity of ARCP was compared to that of the standard, 1 mM N-acetyl cysteine (NAC), at a concentration of 2.5 mg/mL (Fig. 4A). The radical scavenging activity of ARCP was similar to that of 1 mM NAC, which is equivalent to 0.16 mg/mL.

To investigate ROS scavenging effects of ARCP upon exposure to H₂O₂, the level of intracellular ROS was measured by quantification of DCF fluorescence in HepG2 cells. Cells were treated for 30 min with ARCP then it was removed from the culture medium. 1 mM of H₂O₂ was added to the 30 min of ROS assay. H₂O₂ scavenging activity increased by 24, 19, 43, and 55% with the pretreatment of ARCP at a concentration of 0.25, 0.5, 1 and 2.5 mg/mL, respectively. The results suggest that water extract of wild ginseng is capable of amelioration of hepatocyte oxidation stress caused by H₂O₂.

Anti-oxidant activity of ARCP may relate to loosening of the CCl₄-induced hepatotoxicity because ARCP showed strong radical and H₂O₂ scavenging activities (Fig. 3, 4). A Decrease of MDA contents in ARCP-treated group (Table 2) might be resulted from anti-oxidant activity of ARCP. Wild *P. ginseng* leaves have reported to decrease lipid peroxidation in streptozotocin diabetic rats [Jung *et al.*, 2005]. Hot water extract of *P. notoginseng* was reported to contain protective effects on ethanol-induced hepatotoxicity via the reduction of

Table 2. Inhibitory effect of ARCP on CCl₄ induced lipid peroxidation in the mouse liver homogenate *in vivo*

Group	MDA (nmol/mg protein)	Inhibition rate (%)
Vehicle	0.128 ± 0.0015	-
CCl₄	0.182 ± 0.0014	-
ARCP (600 μg/g)	0.145 ± 0.0013	21%
ARCP (100 μg/g)	0.150 ± 0.0018	17%
Silymarin (50 μg/g)	0.125 ± 0.0016	32%

oxygen-free radical production [Lin *et al.*, 2003]. Hepatocyte apoptotic bodies can be phagocytosed by Kupffer and HSCs, and it significantly increased superoxide production via activation of NADPH oxidase [Canbay *et al.*, 2003]. It is well known that an exposure of HSC to a soluble factor such as a reactive oxygen radical produced from Kupffer cells leads to morphological transition to myofibroblast-like cells [Nieto *et al.*, 2002]. Therefore, it is not an unreasonable conclusion that the radical scavenging activity of ARCP will play anti-fibrotic role by reducing ROS, thus reducing apoptosis.

However, in the experimental design of ours, it opens other possibilities of reducing fibrosis. Because the intraperitoneal injection of CCl₄ at a low dose for four weeks was induced, the treatment of ARCP or the control drug was started without a co-injection of CCl₄. The effects of ARCP on reducing fibrosis may partially be depended on the reversion of fibrosis. Additionally, untreated control mice undergo reversion process upon stopping injection of CCl₄ but the ARCP-or drug-treated mice may reverse fibrosis faster.

In this study, we employed the methods to induce liver fibrosis first by using CCl₄, and then by treating ARCP to

evaluate anti-fibrotic effects. We found the decrease of serum GPT, GOT and ALT after the treatment of ARCP. Collagen contents also decreased significantly in ARCP treated group than the control groups. Other factors related to fibrosis such as expressions of TGF-β1, TIMP-1 and α-SMA also decreased in ARCP treated group. Our future work will focus on the fractionation of active compounds and more detailed study on the mechanism of reducing fibrosis. Especially, we will investigate the possibility of ARCP that induces apoptosis of activated HSC.

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