J. Pharm. Pharmacol. 2000, 52: 875–881
Received December 14, 1999
Accepted March 20, 2000

The Ethanol-soluble Part of a Hot-water Extract from *Artemisia iwayomogi* Inhibits Liver Fibrosis Induced by Carbon Tetrachloride in Rats

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Abstract

This study was carried out to investigate the protective effects of the hot-water extract from *Artemisia iwayomogi* (Compositae) on carbon tetrachloride-induced liver fibrosis in rats.

Liver injury was induced by oral administration of carbon tetrachloride ($1\,mL\,kg^{-1}$) twice a week during 4 weeks of *A. iwayomogi* treatment. Extracts from *A. iwayomogi* were prepared and administered to rats orally ($2\,g\,kg^{-1}$ as *A. iwayomogi* for 4 weeks) as follows: group 1, hot-water extract; group 2, ethanol-soluble part of hot-water extract; group 3, ethanol-insoluble part of hot-water extract; and group 4, methanol extract.

In rats treated with the ethanol-soluble part of the hot-water extract, liver hydroxyproline content was reduced to 74% that of carbon tetrachloride control rats ($P < 0{\cdot}05$). Protein expression of alpha smooth muscle cell like actin was also decreased in rats treated with the ethanol-soluble part of the hot-water extract, which indicates inhibition of hepatic stellate cell activation. Liver malondialdehyde levels were significantly lowered in rats treated with the ethanol-soluble part of hot-water extract ($P < 0{\cdot}05$). Serum cholesterol levels in rats treated with hot-water extract, ethanol-soluble or -insoluble parts of hot-water extract or methanol extract were significantly reduced when compared with those of carbon tetrachloride control rats ($P < 0{\cdot}05$).

The ethanol-soluble part of the hot-water extract from *A. iwayomogi* inhibited fibrosis and lipid peroxidation in rats with liver fibrosis induced by carbon tetrachloride. Both hot-water extract (either ethanol-soluble or -insoluble) and methanol extract of *A. iwayomogi* also lowered serum cholesterol levels in fibrotic rats.

Artemisia iwayomogi (*A. messer-schmidtiana* var. *viridis* Besser, Compositae), locally known as Haninjin or Dowijigi, is a perennial herb easily found in Korea. The aerial part of *A. iwayomogi* has traditionally been used for anti-inflammatory, antipyretic, diuretic, liver protective and choleretic purposes in Korea (Yook 1989). With recent renewed interest in the pharmaceutical potential of natural products, many studies were done in the exploration of the biomedical potential of *A. iwayomogi* and it has been evaluated that this medicinal herb has many therapeutic properties. The polysaccharide fraction from *A. iwayomogi* has antitumour (Bae et al 1992) and immunomodulating activity (Koo et al 1994) and a liver protective effect (Lee et al 1998) and the methanol extract has antioxidant activity (Kim et al 1997).

We investigated the protective effect of *A. iwayomogi* on lipid peroxidation and fibrogenic process in rats with liver fibrosis induced by carbon tetrachloride. As traditional Oriental medicine is generally administered to patients as the hot-water extracts called Tang in Korea, hot-water extract as well as a methanol extract of *A. iwayomogi* was used to investigate the liver protective effects in rats with liver fibrosis.

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Materials and Methods

Animals

Male Sprague-Dawley rats (200–220 g) were purchased from Dae Han Laboratory Animal Research

and Co. (Choongbuk, Korea), and had free access to normal standard chow diet (Jae Il Chow, Korea) and tap water. The rats were housed four or five per plastic cages and maintained under $22 \pm 2^{\circ}C$, 50–60% relative humidity and 12-h light–dark cycles throughout the experiment. The rats were maintained in these facilities for at least 1 week before the experiment.

Preparation of Artemisia iwayomogi *extracts*
The aerial part (the whole plant above ground including leaves and stems) of *Artemisia iwayomogi* was obtained from Jin-Ahn Medicinal Plant Experiment Station (Korea) and was authenticated by Prof. Y. C. Kim of the College of Pharmacy, Wonkwang University. The aerial part was extracted with methanol under reflux or extracted with water while heating. The ethanol-insoluble part was obtained by adding two volumes of ethanol to one volume of hot-water extract. The ethanol and hot-water extract mixture was mixed well and left at 4°C for 16 h. The supernatant was considered as the ethanol-soluble part and the precipitate as the ethanol-insoluble part of the hot-water extract. The ethanol-insoluble part was dissolved in water and concentrated under vacuum. All the prepared samples were concentrated under vacuum and freeze-dried. The freeze-dried materials were kept at 4°C during the experiment.

Animal treatment
Rats were dosed with aqueous solutions ($2\ g\ kg^{-1}$ as aerial part of *A. iwayomogi*) orally for 4 weeks as follows: group 1, hot-water extract; group 2, ethanol-soluble part of hot-water extract; group 3, ethanol-insoluble part of hot-water extract; and group 4, methanol extract. During *A. iwayomogi* treatment rats were given carbon tetrachloride orally ($1\ mL\ kg^{-1}$ as carbon tetrachloride, mixed with an equal volume of corn oil) on Mondays and Thursdays for 4 weeks (Bickel et al 1991). Three days after the last carbon tetrachloride treatment, rats were killed under ether anaesthesia and blood and liver samples were collected for further experiment.

Determination of serum biochemical parameters
Blood samples were kept at room temperature for 1 h and centrifuged at $3000\ rev\ min^{-1}$ for 30 min to obtain sera. Sera were kept at −20°C until further assay. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) activities and levels of albumin and total-cholesterol were measured by an Autodry chemistry analyser (SPOTCHEM SP4410, Arkray, Japan).

Determination of hydroxyproline contents in liver
Collagen concentrations in liver were estimated by measuring hydroxyproline by the method of Jamall et al (1981). In brief, specimens of the liver were weighed and completely hydrolyzed in 6 M hydrochloric acid. A portion of the samples was derivatized using chloramine T solution and Erhlich reagent and measured at 558 nm. Standard calibration curve was prepared using trans-4-hydroxy-L-proline (Sigma Chem. Co., USA).

Determination of lipid peroxidation levels in liver
Lipid peroxidation was quantified by the thiobarbituric acid (TBA) method (Plaa & Charbonneau 1994) using the S9 fraction of liver homogenates as an enzyme source; all manipulations were made rapidly on ice to avoid peroxidation. S9 fraction of homogenate (approximately 5 mg protein) was reacted with TBA solution in a boiling water bath for 10 min and then extracted with *n*-butanol for 10 s. The organic phase containing coloured complex was collected for fluorescence measurement. 532 nm and 553 nm were used for excitation and emission wavelengths, respectively. The calibra-tion curve was prepared using 1,1,3,3-tetraethoxypropane (Sigma Chemical Co., USA), a chemical releasing malondialdehyde in acidic conditions. Alterations in lipid peroxidation levels have been used to monitor the extent of carbon tetrachloride-induced liver damage as highly reactive free radicals, metabolites of carbon tetrachloride, react with cellular macromolecules leading to lipid peroxidation and thereby resulting in death of the hepatocytes. The reaction of malondialdehyde, a degradation product of peroxidized lipids, with TBA to produce a TBA-malondialdehyde chromophore has been taken as an index of lipid peroxidation and is the most widely used method for detecting lipid peroxidation.

Histological and immunohistochemical examination of liver
The portion of removed liver was rapidly fixed with 10%-neutralized formalin (pH 7·4) and stained with Masson's Trichrome method for detection of connective deposition. Alpha-smooth muscle cell actin for detection of activated hepatic stellate cells was immunohistologically assessed by the strepavidin–biotin–peroxidase complex method using a LSAB

2 Kit (DAKO Co., USA) and anti-alpha-smooth muscle cell actin monoclonal antibody (Boehringer Mannheim, Germany).

Statistical analysis

The results were evaluated by one-way analysis of variance and Tukey's multiple comparison tests. Values of $P < 0{\cdot}05$ were considered significant.

Ethical considerations

This experiment was carried out under the Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology in 1989.

Results

In liver fibrosis induced by carbon tetrachloride, serum levels of ALT, AST and ALP in carbon tetrachloride-treated control rats were increased to 2743% ($P < 0{\cdot}01$), 1700% ($P < 0{\cdot}01$) and 433% ($P < 0{\cdot}01$), respectively (Table 1). In rats treated with the ethanol-soluble part of the hot-water extract of *A. iwayomogi*, serum levels of ALT and AST were not significantly different from that of carbon-tetrachloride-treated control rats. The serum total-cholesterol concentration in rats treated with carbon tetrachloride was increased to 159% when compared with that of normal control rats ($P < 0{\cdot}01$). The serum total-cholesterol level was decreased to 73–78% ($P < 0{\cdot}05$) that of carbon-tetrachloride-treated control rats in all rats treated with extracts of *A. iwayomogi*. ALT and AST levels in the serum of rats treated with the hot-water or ethanol-insoluble part of the hot-water extract were similar to that of those treated with carbon tetrachloride alone. In rats treated with methanol extract, serum ALT, AST and ALP levels were not significantly different from that of rats treated with carbon tetrachloride alone. Serum biochemical parameters of untreated normal rats were similar to those of normal controls treated with corn oil twice a week for 4 weeks (Table 1).

Liver hydroxyproline contents were increased to 167% in carbon-tetrachloride-treated rats ($P < 0{\cdot}01$) when compared with normal controls. In rats treated with the ethanol-soluble part of the hot-water extract of *A. iwayomogi*, the hydroxyproline content was decreased to 74% that of carbon-tetrachloride-treated controls ($P < 0{\cdot}05$) (Table 2). In rats treated with the hot-water extract, the ethanol-insoluble part of the hot-water extract or the methanol extract, the levels of hydroxyproline in the liver were not significantly different from that of rat liver treated with carbon tetrachloride alone (Table 2). Liver hydroxyproline levels of untreated normal rats were similar to those of normal controls treated with corn oil twice a week for 4 weeks (Table 2).

Malondialdehyde values in the liver of carbon-tetrachloride-treated control rats were increased to 283% ($P < 0{\cdot}01$) that of normal, showing that 4 weeks of carbon tetrachloride treatment increases lipid peroxidation in the liver, significantly. Malondialdehyde values of control rats treated with the extracts of *A. iwayomogi* and corn oil were similar to that of normal controls. The ethanol-soluble part of the hot-water extract from *A. iwayomogi* decreased lipid peroxidation in the liver ($P < 0{\cdot}05$) (Table 2). Malondialdehyde values in the liver of rats treated with the hot-water extract, methanol

Table 1. Serum biochemical values in carbon-tetrachloride-intoxicated rats treated with extracts of *Artemisia iwayomogi* for 4 weeks.

Group	n	AST (int. units L^{-1})	ALT (int. units L^{-1})	ALP (int. units L^{-1})	T-chol (mg dL^{-1})	Alb (g dL^{-1})
Normal	4	92 ± 13	39 ± 5	335 ± 52	91 ± 23	5·3 ± 0·4
Nctrl	4	93 ± 23	42 ± 9	384 ± 75	85 ± 31	5·2 ± 0·4
CCl_4	6	1580 ± 555*	1152 ± 377*	1662 ± 806*	135 ± 19*	5·2 ± 0·4
1	6	1549 ± 489*	1215 ± 331*	1521 ± 335*	101 ± 18†	4·9 ± 0·2
2	6	1039 ± 526*	920 ± 414*	1562 ± 554*	105 ± 9†	5·2 ± 0·2
3	6	1479 ± 372*	1061 ± 206*	2412 ± 664*	101 ± 12†	5·1 ± 0·2
4	6	1905 ± 439*	1435 ± 317*	2324 ± 580*	99 ± 4†	5·0 ± 0·1

Results are shown as the mean ± s.d. Carbon tetrachloride was orally administered 1 mL kg^{-1} as carbon tetrachloride, mixed with an equal volume of corn oil, twice a week for 4 weeks. Each extract of *A. iwayomogi* was administered to rats in each group orally (2 g kg^{-1} as aerial parts of *A. iwayomogi*, daily for 4 weeks). n: Number of rats. Normal: untreated normal rats. Nctrl: normal control rats treated with water daily and corn oil twice a week. CCl_4: rats treated with water daily and carbon tetrachloride twice a week. Group 1: CCl_4-intoxicated rats treated with hot-water extract. Group 2: CCl_4-intoxicated rats treated with the ethanol-soluble part of the hot-water extract. Group 3: CCl_4-intoxicated rats treated with the ethanol-insoluble part of the hot-water extract. Group 4: CCl_4-intoxicated rats treated with the methanol extract. *$P < 0{\cdot}01$, compared with Group Nctrl. †$P < 0{\cdot}05$, compared with Group CCl_4.

Table 2. Hydroxyproline contents and thiobarbituric acid reactive substance values in the liver of carbon tetrachloride-intoxicated rats treated with extracts of *Artemisia iwayomogi* for 4 weeks.

Group	n	Hydroxyproline content (μg (g liver)$^{-1}$)	Thiobarbituric reactive substance (pmol (mg protein)$^{-1}$)
Normal	4	275 ± 23	98 ± 12
Nctrl	4	280 ± 21	109 ± 8
CCl_4	6	466 ± 29**	308 ± 41**
1	6	462 ± 72**	223 ± 40*
2	6	347 ± 57†	203 ± 26†
3	6	462 ± 70**	289 ± 80**
4	6	375 ± 83	234 ± 100*

Results are shown as the mean ± s.d. n: number of rats. Normal: untreated normal rats. Nctrl: normal control rats treated with water daily and corn oil twice a week. CCl_4: rats treated with water daily and carbon tetrachloride twice a week. Group 1: CCl_4-intoxicated rats treated with hot-water extract. Group 2: CCl_4-intoxicated rats treated with the ethanol-soluble part of the hot-water extract. Group 3: CCl_4-intoxicated rats treated with the ethanol-insoluble part of the hot-water extract. Group 4: CCl_4-intoxicated rats treated with the methanol extract. *$P < 0{\cdot}05$, compared with Group Nctrl. **$P < 0{\cdot}001$, compared with Group Nctrl. †$P < 0{\cdot}05$, compared with Group CCl_4.

extract or the ethanol-insoluble part of the hot-water extract were not significantly different from those of carbon tetrachloride control liver. Liver malondialdehyde values of untreated normal rats were similar to those of normal controls treated with corn oil twice a week for 4 weeks (Table 2), showing that the amount and period of corn oil treatment in this experiment had no effect on lipid peroxidation in liver.

The livers of rats treated with carbon tetrachloride for 4 weeks showed inflammation, massive fatty change, gross necrosis, extensive accumulation of connective tissue resulting in destruction of the lobular architecture and formation of septum (Figure 1). Less damage was present in the livers of rats in all groups given the extracts of *A. iwayomogi* (Figure 1). Activated hepatic stellate cells, which express alpha-smooth muscle cell like actin, showed marked proliferation in the livers of rats treated with carbon tetrachloride for 4 weeks. The ethanol-soluble part of the hot-water extract of *A. iwayomogi* markedly reduced the number of alpha-smooth muscle cell like actin-positive cells in liver when compared with rat liver treated with carbon tetrachloride alone (Figure 2).

Discussion

The liver is vulnerable to toxic injury as it intercepts all orally administered agents, noxious substances as well as nutrients (Miyai 1991). Whatever the aetiology may be, chronic liver injury leads to excessive deposition of connective tissues resulting in fibrosis and cirrhosis, which is one of the most common causes of all deaths (Anthony et al 1978). Therefore, prevention or suppression of fibrotic changes in the liver or protection from, and treatment of, liver fibrosis and cirrhosis are important. But therapeutic attempts with liver protective drugs, which prevent liver fibrosis, are still at an experimental stage (Park et al 1997). Both toxicity and therapeutic effects when used in clinical studies have shown problems in developing these drugs.

In recent years, we have screened a number of liver protective agents from natural products, which are traditionally used in Korea for liver diseases. Developing antifibrotics from natural products used in folk medicine may reduce the risk of toxicity and therapeutic effectiveness when the drug is used clinically.

The aerial parts of *Artemisia iwayomogi* (Compositae) have been traditionally used for treating liver diseases in Korea. The constituents of *A. iwayomogi* are reported to be esculetin 6-methylether, esculetin 7-methylether (scopoletin), scopolin, beta-sitosterol, chlorogenic acid, essential oil, fatty acid, sesquiterpene lactones, eudesmanolides and flavonoids (Yook 1989; Valant-Vetschera & Wollenweber 1995). The methanol extract of *A. iwayomogi* is reported to have strong antioxidant activity and this activity was postulated to be due to chlorogenic acid, of which the antioxidant activity was comparable with that of L-ascorbic acid (Kim et al 1997). Polysaccharide extracts of *A. iwayomogi* had a protective effect on liver damage induced by paracetamol, ANIT (1-Naphthyl isothiocyanate), ethanol or bile-duct ligation (Lee et al 1997, 1998). The polysaccharide fraction also suppressed transplanted tumour cell growth, augmented antibody production and increased the population of macrophages and lymphocytes in the spleen both in-vivo and in-vitro, suggesting that the polysaccharide fraction may have immunomodulating and antitumour activity (Bae et al 1992; Koo et al 1994).

Until now, the pharmacological effects of *A. iwayomogi* have been focused on the methanol extract or polysaccharide fraction. But, traditionally, medicinal herbs are generally given to patients as a hot-water extract. So in this study the effects of a hot-water extract of *A. iwayomogi* were studied, as well as those of a methanol extract, in rats with liver injury. Four weeks of carbon tetrachloride treatment induced liver injury with fibrosis, necrosis, inflammation and fatty-acid changes. Serum

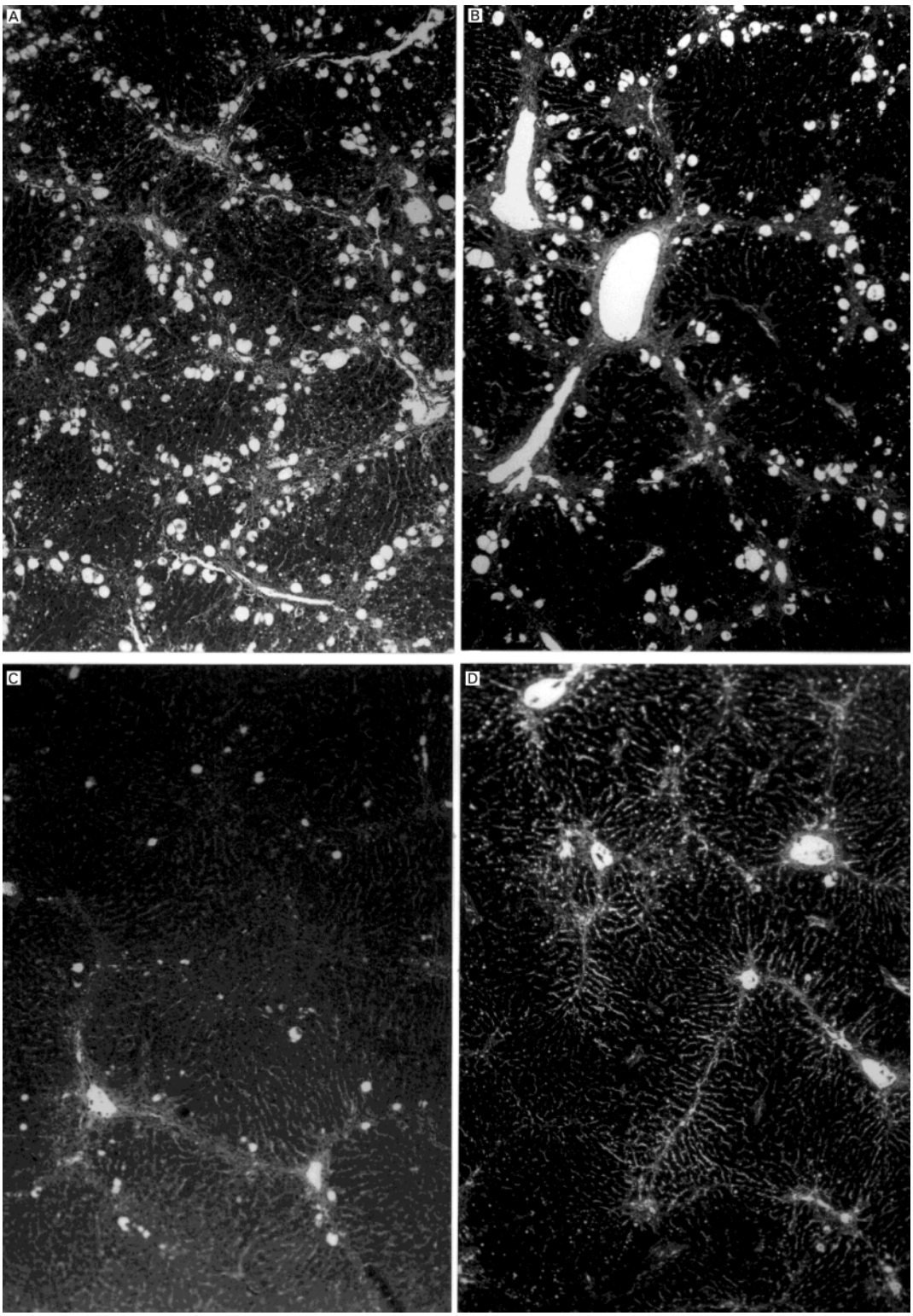

Figure 1. Light microscopical appearance of damaged rat liver treated with extracts of *Artemisia iwayomogi* for 4 weeks (magnification: ×63). Carbon tetrachloride was orally administered (1 mL kg^{-1} as carbon tetrachloride mixed with an equal volume of corn oil) twice a week for 4 weeks. Each extract of *A. iwayomogi* was administered to rats in each group orally (2 g kg^{-1} as aerial parts of *A. iwayomogi*) daily for 4 weeks.

A. Rats treated with water daily and carbon tetrachloride (Haematoxylin & Eosin). B. Rats treated with water daily and carbon tetrachloride (Masson's trichrome staining). C. Carbon tetrachloride intoxicated rats treated with the ethanol-insoluble part of the hot-water extract (Haematoxylin & Eosin). D. Carbon tetrachloride intoxicated rats treated with the ethanol-insoluble part of the hot-water extract (Masson's trichrome staining).

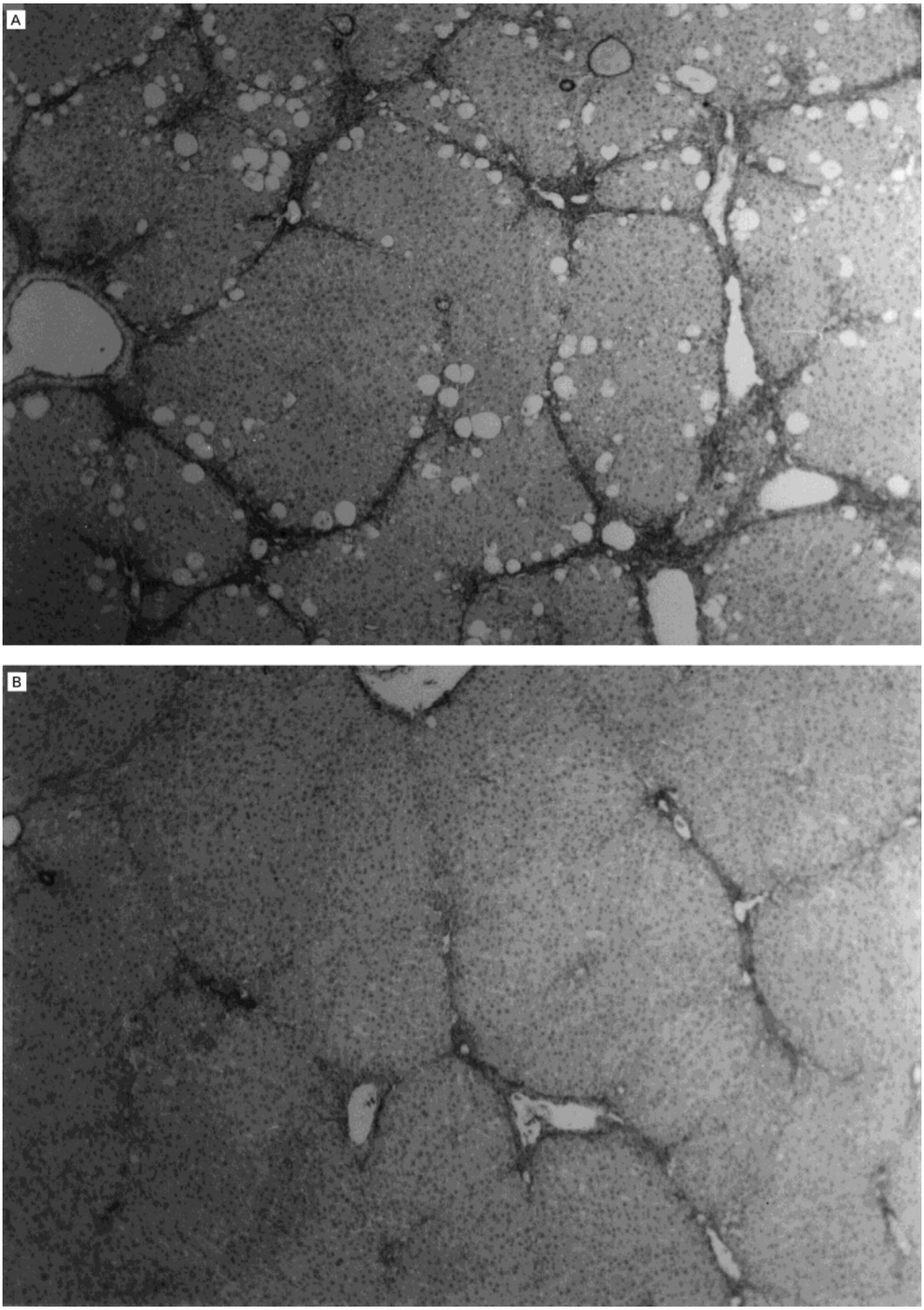

Figure 2. Immunohistochemical appearance of damaged rat liver treated with extracts of *Artemisia iwayomogi* stained against alpha smooth muscle cell like actin (magnification: ×63). A. Rats treated with water daily and carbon tetrachloride (1 mL kg^{-1} as carbon tetrachloride mixed with equal volume of corn oil) twice a week. B. Carbon tetrachloride-intoxicated rats treated with the ethanol-soluble part of the hot water-extract (p.o., 2 g kg^{-1} as aerial parts of *A. iwayomogi*).

total-cholesterol levels were decreased to 73–78% ($P < 0{\cdot}05$) that of carbon-tetrachloride-treated controls in all rats treated with the ethanol-insoluble part of the hot-water extract, the hot-water extract or the methanol extract of *A. iwayomogi*. Treatment with the ethanol-soluble part of the hot-water extract of *A. iwayomogi* for 4 weeks significantly reduced the hydroxyproline content and malondialdehyde values in liver. These results indicate that the ethanol-soluble part of the hot-water extract of *A. iwayomogi* significantly inhibited collagen accumulation and lipid peroxidation in liver damaged by carbon tetrachloride. Treatment with the ethanol-soluble part of the hot-water extract of *A. iwayomogi* for 4 weeks reduced the total-cholesterol level in serum ($P < 0{\cdot}05$) in rats with liver injury induced by carbon tetrachloride. From the immunohistological appearance, the ethanol-soluble part of the hot-water extract reduced the alpha smooth muscle cell like actin-positive area in liver, showing inhibition of hepatic stellate cell activation during the fibrogenic process. The constituents of the ethanol-soluble part of the hot-water extract are thought to be low molecular weight components of *A. iwayomogi*, but further study is needed to identify the active components.

In conclusion, this study demonstrates that the ethanol-soluble part of the hot-water extract of *Artemisia iwayomogi* can effectively improve the liver fibrosis caused by carbon tetrachloride treatment as monitored by reduced levels of connective tissue, lipid peroxidation and hepatic stellate cell activation in the liver.

Acknowledgements

This work was supported by the Medicinal Resources Research Center (98-16-02-01-A-3), sponsored by Korea Science and Engineering, Chollabuk-Do Provincial Government and Wonkwang University and by a Wonkwang University Grant for Dong Hwan Sohn (1998).

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