The Hepatoprotective Activity of Kinsenoside from Anoectochilus formosanus

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Carbon tetrachloride (CCl4) causes chronic hepatitis, featuring an increase in hepatic hydroxyproline, spleen weight and serum GPT levels and a decrease in plasma albumin levels. Crude extracts of fresh whole plants of Anoectochilus formosanus showed inhibition of chronic hepatitis induced by CCl4 in mice. Bioactivity-guided fractionation and spectroscopic analysis revealed that kinsenoside was the most active compound. In an in vitro study, the LD50 values for H2O2-induced cytotoxicity in BALB/c normal liver cells were significantly higher after kinsenoside pretreatment than after vehicle alone, further confirming that kinsenoside shows significant antihapatotoxic activity. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: Anoectochilus formosanus; liver disease; kinsenoside.

INTRODUCTION

Anoectochilus formosanus Hayata (Orchidaceae) is an important ethnomedicinal plant of Taiwan, used as a folk medicine for liver fibrosis, diabetes and for the treatment of cardiovascular diseases (Kan, 1986). Since this herb is very precious in the Taiwanese herb market, unlimited collection has seriously depleted its levels in natural habitats. In addition, colonial propagation by conventional methods is slow. However, vegetative propagation of A. formosanus using tissue culture has been achieved in Taiwan (Shiau et al., 2002).

Lin et al. (1993; 2000) showed that A. formosanus possesses hepatoprotective effects on carbon tetrachloride (CCl4)- and acetaminophen-induced acute hepatitis. Du et al. (2003) showed that aqueous extracts of A. formosanus inhibited cell damage induced by CCl4 in primary cultured rat hepatocytes. Recently, it was also confirmed that an aqueous extract of A. formosanus attenuated hepatic fibrosis induced by both CCl4 and dimethylnitrosamine in rats (Shih et al., 2004; 2005). However, the hepatoprotective compounds in A. formosanus are still unknown. The purpose of this research was to find the most active compounds from A. formosanus in both in vivo and in vitro experiments.

MATERIALS AND METHODS

Plant material. Anoectochilus formosanus plants were purchased from Yu-Jung Farm (Pu-Li, Taiwan) where they are cultivated. The plants were identified by the Institute of Chinese Pharmaceutical Sciences, China Medical University, where a plant specimen has been deposited.

Reagents. Hydroxyproline, p-dimethylaminobenzaldehyde, polyvinylpyrrolidone and hydrogen peroxide were provided by Sigma Chemical Co. (St Louis, MO, USA). Carbon tetrachloride was obtained from Shimakuyu Pure Chemicals (Osaka, Japan). Dulbecco’s modified Eagle’s medium was purchased from Hyclone (Logan, Utah, USA). Penicillin, streptomycin, amphotericin B and fetal bovine serum were purchased from Gibco Laboratorie (Grand Island, NY, USA).

Extraction and isolation. Fresh whole plants of A. formosanus (10 kg) were extracted with water and the filtrate was partitioned successively with ethyl acetate. Both the ethyl acetate fraction (AFEE) and water soluble portion (AFEW) were evaporated under reduced pressure, yielding 47.4 g of a greenish oily residue and 218.4 g of a red residue, respectively. The AFEW (210 g) was subjected to a Diaion HP-20 column (Nippon Ressui Co., Japan) and eluted with H2O, 10%, 20% and 50% methanol in water, and 100% methanol to give five fractions (AFEW-1–AFEW-5). The dry weight of fractions AFEW-1–AFEW-5 was 141.38 g, 22.06 g, 8.16 g, 9.21 g and 3.78 g, respectively.

The AFEW-2 fraction (10 g) was purified further on silica gel (Si 60 F245; Merck, Germany) with chloroform/ethanol (15:8) as the mobile phase to give four fractions. Fraction 4 (4.5 g) was applied to preparative high performance liquid chromatography (HPLC) to...
yield a pure compound (4.1 g). The conditions used for preparative HPLC were as follows: pump, Shimadzu LC-10ATvp; refractive index detector, Shimadzu RID-10A; column, Mightysil ODS RP-18 GP Aqua column (i.d. 4.6 mm, 250 mm long; 5 µm particle size); guard column, Mightysil 4.6 mm × 6 mm. The solvent system used was Milli-Q water at a flow rate of 0.5 mL/min. The kinsenoside content in AFEW, AFEW-2 and AFEW-3 fractions was approximately 18%, 82% and 35%, respectively. No kinsenoside was detected in fraction AFEW-1. Kinsenoside could be detected in fractions AFEW-4 and AFEW-5, but the content was very low.

CCl₄-induced chronic hepatitis in mice. All animals received humane care and the study protocols were in compliance with our institution’s guidelines for the use of laboratory animals. Male ICR mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and were housed in an air-conditioned room at 21°–24 °C with 12 h of light. Mice were allowed free access to a pellet diet and water throughout the study, and were used for experiments when they reached 24–26 g.

Chronic hepatitis was induced in mice by oral administration of 0.1 mL of CCl₄ (diluted 1:19 in olive oil) per 10 g body weight twice a week for 3 or 8 weeks. On the days of CCl₄ treatment, the time interval between CCl₄ and drug administrations was 5 h to avoid absorption interference. After the blood was drawn at the end of the experimental period, all mice were killed at the same time and the liver and spleen were quickly removed and weighed after washing with cold normal saline and blotting dry. The largest lobe of liver was weighed and then completely dried at 100 °C for determination of the hydroxyproline content according to the method designed by Neuman and Logan (1950). Dried liver tissue (approx. 60 mg) was hydrolysed, then oxidized by H₂O₂ and reacted with p-dimethylaminobenzaldehyde; the absorbance of the colored product was determined at 540 nm. The amount of hydroxyproline is expressed in µg/g wet tissue. The levels of plasma glutamate-pyruvate transaminase (GPT) and albumin were assayed spectrophotometrically (Cobas Mira Plus Chemistry Analyzer, Switzerland) using clinical test kits (Roche Diagnostics, Mannheim, Germany).

Experiment I (3 week CCl₄ treatment): Chronic hepatitis was induced in mice by CCl₄ administration for 3 weeks. Since AFEW did not dissolve in water, it was suspended in polyvinylpyrrolidone (PVP). Therefore, the experiments were divided into two parts. For part I, mice were divided into three groups, which received CCl₄ with H₂O, CCl₄ with aqueous A. formosanus extract (AFE; 2000 mg/kg) or AFEW (1500 mg/kg) p.o. daily beginning on day 1 to the end of week 3. The control group received olive oil vehicle with H₂O. In part II, the mice were divided into two groups which received CCl₄ with PVP or CCl₄ with AFEW (500 mg/kg) p.o. daily beginning on day 1 to the end of week 3. The control group received olive oil vehicle with PVP. After 1 week of CCl₄ treatment, blood was collected via the retro-orbital sinus of mice for serum GPT assays.

Experiment II (8 week CCl₄ treatment): Chronic hepatitis was induced in mice by CCl₄ administration for 8 weeks. Since AFEW-4 did not dissolve in water, it was suspended in PVP. Therefore, the experiment was also divided into two parts. In part I, mice were divided into six groups, which received CCl₄ with H₂O, CCl₄ with AFEW (1500 mg/kg), AFEW-1 (1055 mg/kg), AFEW-2 (130 mg/kg), AFEW-3 (50 mg/kg) or AFEW 5 (190 mg/kg) p.o. daily beginning on day 1 to the end of week 8. The control group received olive oil vehicle with H₂O. In part II, mice were divided into two groups, which received CCl₄ with PVP or CCl₄ with AFEW-4 (75 mg/kg) p.o. daily beginning on the day 1 to the end of week 8. The control group received olive oil vehicle with PVP.

Cell culture and hydrogen peroxide cytotoxicity assay. BALB/c normal liver (BNL) cells were purchased from Bioresources Collection and Research Center in Taiwan. Cell lines were grown in Dulbecco’s modified Eagle’s medium with 50 IU/mL penicillin, 50 µg/mL streptomycin, 50 IU/mL amphotericin B and 10% fetal bovine serum. For cytotoxicity assays, cells (5 × 10⁴ cells/well) were plated into 96-well culture plates and allowed to attach overnight. The medium was replaced with fresh medium (as control) or with various concen-

trations of AFEW, AFEW-1 and kinsenoside for 16 h. The cells were then washed with PBS and further treated with various doses of hydrogen peroxide (0.125–4.00 µM) for 2 h. After H2O2 cytotoxic treatment, the percentage of viable cells was determined using a CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA). Reagent solution (20 µL) was added to each well and incubated at 37 °C in humidified 5% CO2 for 1 h. Then the absorbance was measured at 490 nm using a 96-well plate reader (Multiskan Spectrum, Labsystem, Thermo Electron Corporation, Finland).

Statistical analyses. Data from in vivo experiments were treated by one-way analysis of variance and Dunnett’s test was applied. The significance level was set at p < 0.05. The LD50 values and 95% confidence limits for H2O2-induced BNL cell death were calculated according to the method of Litchfield and Wilcoxon (1949). The significance of differences between the LD50 values was also tested using this method.

RESULTS AND DISCUSSION

Experiment I

CCl4 treatment caused hepatocellular damage in mice, as indicated by an increase in plasma GPT activity after 1 and 3 weeks of CCl4 administration (Table 2). Mice treated with AFE (2000 mg/kg, daily) or AFEW (1500 mg/kg, daily) showed protection against CCl4-induced hepatitis, with the level of GPT being reduced. In contrast, AFE (500 mg/kg, daily) enhanced the increase in plasma GPT levels caused by CCl4 administration for 1 and 3 weeks. These results clearly show that AFEW had hepatoprotective action. Therefore, AFEW was further studied.

Experiment II

The AFEW was fractionated and five (AFEW-1 AFEW-5) fractions were collected. An animal model of chronic hepatitis induced in mice by oral administration of CCl4 twice a week for 8 weeks was used to investigate the hepatoprotective effect of AFEW-1–AFEW-5. In the CCl4-treated group, they were marked increases in GPT activity compared with the control group in week 8. Mice treated with AFEW (1500 mg/kg, daily), AFEW-1 (1050 mg/kg, daily) AFEW-2 (130 mg/kg, daily) or AFEW-5 (190 mg/kg, daily) showed reduced GPT levels (Table 3), thus these fractions showed actions in reducing the liver damage induced by CCl4.

Liver fibrosis is a common end stage of most forms of chronic hepatitis, involving the hyperaccumulation of collagen (Neubauer et al., 2001). The amount of collagen can be reflected by the hydroxyproline content, which is a characteristic component of collagen.

Table 2. Effect of AFE, AFEE and AFEW on the activities of serum GPT in CCl4-treated mice after 1 and 3 weeks

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose (mg/kg)</th>
<th>Week 1</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive oil + H2O</td>
<td>–</td>
<td>40.3 ± 3.5</td>
<td>37.9 ± 2.0</td>
</tr>
<tr>
<td>CCl4 + H2O</td>
<td>–</td>
<td>299.3 ± 15.9d</td>
<td>1266.5 ± 162.0d</td>
</tr>
<tr>
<td>CCl4 + AFEW</td>
<td>2000</td>
<td>160.5 ± 47.5e</td>
<td>600.0 ± 142.7e</td>
</tr>
<tr>
<td>CCl4 + AFEW</td>
<td>1500</td>
<td>195.5 ± 29.7e</td>
<td>306.0 ± 22.6e</td>
</tr>
<tr>
<td>Olive oil + PVP</td>
<td>–</td>
<td>39.1 ± 2.9</td>
<td>37.5 ± 1.8</td>
</tr>
<tr>
<td>CCl4 + PVP</td>
<td>–</td>
<td>170.7 ± 17.4d</td>
<td>740.0 ± 120.2d</td>
</tr>
<tr>
<td>CCl4 + AFEE</td>
<td>500</td>
<td>492.6 ± 103.6e</td>
<td>1398.0 ± 268.6e</td>
</tr>
</tbody>
</table>

All values are mean ± SE (n = 8). a p < 0.001 compared with olive oil + H2O group. b p < 0.05, c p < 0.01 compared with CCl4 + H2O group. d p < 0.001 compared with olive oil + PVP group. e p < 0.05 compared with CCl4 + PVP group.

Table 3. Effect of AFEW and AFEW-1 to AFEW-5 on the levels of plasma GPT and albumin, spleen weight and contents of hepatic hydroxyproline in CCl4-treated mice after 8 weeks

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose (mg/kg/day)</th>
<th>Plasma GPT (U/L)</th>
<th>Plasma albumin (g/dL)</th>
<th>Spleen weight (g)</th>
<th>Liver hydroxyproline (µg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive oil + H2O</td>
<td>–</td>
<td>36.4 ± 4.0</td>
<td>3.5 ± 0.1</td>
<td>0.18 ± 0.01</td>
<td>504.0 ± 26.8</td>
</tr>
<tr>
<td>CCl4 + H2O</td>
<td>1500</td>
<td>436.9 ± 62.5e</td>
<td>3.4 ± 0.1e</td>
<td>0.21 ± 0.03e</td>
<td>1062.0 ± 189.5e</td>
</tr>
<tr>
<td>CCl4 + AFEW</td>
<td>1500</td>
<td>343.0 ± 19.0e</td>
<td>3.4 ± 0.1e</td>
<td>0.15 ± 0.01e</td>
<td>688.0 ± 51.3e</td>
</tr>
<tr>
<td>CCl4 + AFEW-1</td>
<td>1500</td>
<td>177.8 ± 20.0e</td>
<td>3.1 ± 0.1</td>
<td>0.18 ± 0.01</td>
<td>660.1 ± 61.7e</td>
</tr>
<tr>
<td>CCl4 + AFEW-2</td>
<td>130</td>
<td>246.9 ± 32.8e</td>
<td>3.2 ± 0.2</td>
<td>0.16 ± 0.01</td>
<td>845.2 ± 110.7</td>
</tr>
<tr>
<td>CCl4 + AFEW-3</td>
<td>50</td>
<td>353.8 ± 20.4</td>
<td>3.3 ± 0.3</td>
<td>0.20 ± 0.02</td>
<td>858.5 ± 54.0</td>
</tr>
<tr>
<td>CCl4 + AFEW-5</td>
<td>190</td>
<td>258.6 ± 23.8e</td>
<td>3.2 ± 0.2</td>
<td>0.18 ± 0.02</td>
<td>499.6 ± 27.6</td>
</tr>
<tr>
<td>Olive oil + PVP</td>
<td>–</td>
<td>38.3 ± 3.3</td>
<td>3.4 ± 0.2</td>
<td>0.22 ± 0.02</td>
<td>1029.0 ± 170.6e</td>
</tr>
<tr>
<td>CCl4 + PVP</td>
<td>–</td>
<td>388.7 ± 59.0e</td>
<td>2.8 ± 0.1e</td>
<td>0.17 ± 0.03</td>
<td>898.0 ± 98.6</td>
</tr>
</tbody>
</table>

All values are mean ± SE (n = 10). a p < 0.01, b p < 0.001 compared with olive oil + H2O group. c p < 0.05, d p < 0.01 compared with CCl4 + H2O group. e p < 0.001 compared with olive oil + PVP.
(Hanauske-Abel, 1996). In the present study, AFEW-1 and AFEW-2 treatment significantly reduced the increase in hepatic hydroxyproline caused by CCl₄ (Table 3). Liver fibrosis is often accompanied by hypoalbuminemia and splenomegaly (Gill and Kirkbain, 1997). As shown in Table 3, both AFEW and AFEW-2 diminished the hypoalbuminemia and splenomegaly caused by CCl₄ in mice. According to these results, AFEW-2 was the most hepatoprotective fraction.

**In vitro experiment**

In the in vitro experiment, the major compound of AFEW-2, kinsenoside, was used to replace AFEW-2 in cell experiments. The hepatoprotective effect of AFEW (100 and 200 µg/mL), AFEW-1 (75 and 150 µg/mL) and kinsenoside (20 and 40 µg/mL) on injury induced by H₂O₂ in BNL was studied. As shown in Table 4, the LD₅₀ concentrations of H₂O₂ were markedly increased by pretreatment with AFEW and kinsenoside, but not by AFEW-1, indicating that the hepatoprotective effect of AFEW is mostly due to kinsenoside.

AFEW-1 decreased plasma GPT levels and hepatic hydroxyproline content in CCl₄-treated mice, suggesting that other active constituents are also present in AFEW-1. It is well known that besides hepatocytes, hepatic stellate cells (HSC) and Kupffer cells also play important roles in the pathogenesis of liver disease (Bataller and Brenner, 2005; Xidakis et al., 2005).

## REFERENCES


Shih CC, Wu YW, Hsieh CC, Lin WC. 2004. Effect of *Anoec-


Further Studies on the Hepatoprotective Effects of Anoectochilus formosanus

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The purpose of this study was to investigate the hepatoprotective effects of Anoectochilus formosanus effective fraction (AFEF) on chronic liver damage induced by carbon tetrachloride (CCL4) in mice. CCL4 (5%; 0.1 ml/10 g body weight) was given twice a week for 9 weeks, and mice received AFEF throughout the whole experimental period. Plasma GPT, hepatic levels of hydroxyproline and malondialdehyde were significantly lower in mice treated with AFEF compared with those treated with CCL4 only. Liver pathology in the AFEF-treated mice was also improved. RT-PCR analysis showed that AFEF treatment increased the expression of methionine adenosyltransferase 1A and decreased the expression of collagen(α1)(I) and transforming growth factor-β1. These results clearly demonstrated that AFEF reduced the hepatic damage induced by CCL4 in mice. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: Anoectochilus formosanus; carbon tetrachloride; hepatoprotective action.

INTRODUCTION

Anoectochilus formosanus Hayata (Orchidaceae) is an important ethnomedicinal plant of Taiwan. This herbal plant is also called ‘King Medicine’ because of its diverse pharmacological effects, such as anithyperglycemia (Shih et al., 2002), antiosteoporosis (Shih et al., 2001), anti-hyperlipolysis (Du et al., 2003), antifatigue (Ikeuchi et al., 2005) and hepatoprotection (Du et al., 2003). Recently, health food products of A. formosanus have been developed for the Taiwanese market.

Since this herb is very precious in the Taiwanese herb market, unlimited collection has seriously depleted its levels in natural habitats. In addition, clonal propagation by conventional methods is slow. In Taiwan, the vegetative propagation of A. formosanus by tissue culture has been achieved, and this technology has been used in commercial application by agricultural applications. The current market price of the fresh herb collected from tissue culture is approximately US $220/kg.

In previous studies, it was demonstrated that aqueous extracts of A. formosanus can inhibit liver fibrosis induced by both carbon tetrachloride (CCL4) and dimethyl nitrosamine in rats (Shih et al., 2004, 2005). In a preliminary study of bioguided fractionation, it was found that one fraction from the water-soluble portion has hepatoprotective action, and identified kinsenoside (3-(R)-β-D-glucopyranosylxybutanolide) as the major component of this fraction (Du et al., 2001). It was shown that kinsenoside has a hepatoprotective effect on liver damage induced by CCL4 in mice (Wu et al., 2007).

Lin et al. (2000) found that the methanol extract of A. formosanus could prevent acetaminophen-induced liver injury in small doses, but it might cause liver injury in large dose. In our previous work, it was found that the ethyl acetate fraction from the aqueous extract of A. formosanus enhanced the liver injury induced by CCL4 (Wu et al., 2007). These results indicated that A. formosanus contained some hepatotoxins. In the present study, an A. formosanus effective fraction (AFEF) was prepared that contained kinsenoside, and excluded the hepatotoxic fraction, to examine the hepatoprotective effect.

MATERIALS AND METHODS

Preparation of AFEF. A. formosanus plants were purchased from Yu-Jung Farm (Pu-Li, Taiwan) where they are cultivated. The plants were identified by the Institute of Chinese Pharmaceutical Sciences, China Medical University, where a plant specimen (Number: CMCP 1253) has been deposited.

Fresh whole plants of cultured A. formosanus were extracted with water and the filtrate was partitioned with ethyl acetate successively. The aqueous fraction was further filtered and evaporated under reduced pressure yielding a purpuric residue (AFEF). The AFEF yield was approximately 2%. The content of kinsenoside in AFEF was approximately 180 mg/g measured by HPLC according to the method reported by Wu et al. (2007).

Animals. ICR male mice were obtained from the National Laboratory Animal Breeding and Research Center, National Science Council. The experimental animals were housed in an air-conditioned room at 21–24 °C with 12 h of light. The mice were allowed free
access to a pellet diet and water throughout the study. When they reached 26–30 g, the mice were used for experiments. Mice were divided randomly into four groups (control group and three CCl₄-treated groups) 1 day before administration of the test substance. All animals received humane care and the study protocols were in compliance with our institution’s guidelines for the use of laboratory animals.

**CCL₄-induced chronic hepatitis in mice.** Chronic hepatitis was induced in three groups of 10 mice by oral administration of 0.1 mL/10 g body weight of CCl₄ diluted 5:95 (v/v) in olive oil twice a week for 9 weeks. The animals received only CCl₄, CCl₄ with AFEF (0.5 and 1.0 g/kg, p.o., daily). The drugs were given when the chronic injury model started, and total drug treatment duration was 9 weeks. On the days of CCl₄ treatment, the time interval between CCl₄ and AFEF administration was 5 h to avoid absorption interference. After 3 and 6 weeks of CCl₄ treatment, blood was collected from the mice via the retro-orbital sinus. At the end of the experiment, the mice were killed under ether anesthesia and blood was withdrawn from the abdominal vein. The liver was quickly removed and weighed after washing with cold normal saline and blotting dry. The livers were divided into four parts, which were: (i) submerged in 10% neutral formalin for the preparation of pathological sections; (ii) after weighing, completely dried at 100 °C for the determination of collagen content; (iii) kept in liquid nitrogen for RTPCR analysis; (iv) stored at −80 °C in reserve.

**Assay of liver functions.** A 50 μL sample of blood was added to an Eppendorf tube containing 150 μL of 5% sodium citrate solution; the plasma was separated by centrifugation (1700 × g at 4 °C for 10 min). The level of plasma glutamate-pyruvate transaminase (GPT) was assayed using clinical test kits (Roche Diagnostics, Mannheim, Germany) spectrophotometrically (Cobas Mira; Roche, Rotkreuz, Switzerland).

**Assay of hydroxyproline.** Hydroxyproline determination followed a method designed by Neuman and Logan (1950). Dried liver tissue after hydrolysis was oxidized by H₂O₂ and reacted with p-dimethylaminobenzaldehyde; the absorbance of the colored product was determined at 540 nm (U-2001; Hitachi, Tokyo, Japan). The amount of hydroxyproline is expressed in μg/g wet tissue.

**Assays of lipid peroxidation and protein.** Livers were homogenized in nine volumes of ice-cold 0.15 M KCl/1.9 mm ethylenediaminetetraacetic acid. The homogenate was used for the determinations of lipid peroxidation and protein. Lipid peroxidation was measured by the method of Ohkawa et al. (1979) using 2-thiobarbituric acid. Protein was measured by the Lowry method using bovine serum albumin as the standard (Lowry et al., 1951). Lipid peroxidation is expressed as the amount of malondialdehyde/mg protein.

**RNA extraction and RT-PCR analysis.** Total RNA was isolated from the livers of the mice using the acid guanidinium thiocyanate–phenol–chloroform extraction methods as described by Chomczynski and Sacchi (1987). A sample of 5 μg of total RNA from each liver sample was subjected to reverse transcription (RT) by MMLV reverse transcriptase in a 50 μL reaction volume. Aliquots of the RT mix were used for amplification by polymerase chain reaction (PCR) of fragments specific to methionine adenosyltransferase IA (MATIA), collagen (α₁(I)) and transforming growth factor-β1 (TGF-β1) using the primer pairs listed in Table 1. The levels of expression of all the transcripts were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue sample. The primer pairs were designed using the Primer select program (Primer 3). The identities of the PCR products were confirmed by sequence analysis. The PCR products were run on a 2% agarose gel and recorded on Polaroid film; bands were quantitated using a densitometer.

**Pathological examinations.** After formalin fixation, tissue samples were sliced, embedded in a standard manner and stained with hematoxylin/eosin and Sirius red. The incidence and degree of cell necrosis was scored as normal (0), very slight (1), slight (2), moderate (3) or severe (4). Fibrosis was graded according to the method of Ruwart et al. (1989). To avoid sampling errors, all biopsies were obtained from the same lobe and these semi-quantitative grades were assigned without knowledge of the sample treatment.

**Statistical analysis.** Data were presented as mean ± SD. All other experimental data, except the pathological findings, were analysed by one-way analysis of variance using Dunnett’s test. Liver pathological examination data were analysed by the Kruskall-Wallis non-parametric test, followed by a Mann-Whitney U-test. A value of p < 0.05 was considered statistically significant.

### RESULTS

**Plasma GPT activity.** As shown in Table 2, the levels of GPT of mice at 3, 6 and 9 weeks after CCl₄ treatment

<table>
<thead>
<tr>
<th>Table 1. Primer sequences for PCR amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>Collagen (α₁(I))</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>MAT1A</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Table 2. Effect of AFEF on the activities of plasma GPT in CCl₄-treated mice at 3, 6 and 9 weeks

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (g/kg)</th>
<th>Week 3</th>
<th>Week 6</th>
<th>Week 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>35.9 ± 3.8</td>
<td>34.0 ± 7.3</td>
<td>33.4 ± 10.7</td>
</tr>
<tr>
<td>CCl₄ + H₂O</td>
<td>–</td>
<td>574.3 ± 56.7</td>
<td>759.0 ± 322.6</td>
<td>650.6 ± 170.9</td>
</tr>
<tr>
<td>CCl₄ + AFEF</td>
<td>0.5</td>
<td>420.4 ± 159.2</td>
<td>543.8 ± 166.2</td>
<td>440.9 ± 221.2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>310.3 ± 109.3</td>
<td>331.0 ± 108.3</td>
<td>464.6 ± 194.3</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 10). * p < 0.01 compared with control group.

Table 3. Effect of AFEF on the hepatic hydroxyproline and malondialdehyde contents in CCl₄-treated mice

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (g/kg)</th>
<th>Hydroxyproline (μg/g tissue)</th>
<th>Malondialdehyde (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>490.5 ± 76.1</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>CCl₄ + H₂O</td>
<td>–</td>
<td>691.0 ± 167.1</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>CCl₄ + AFEF</td>
<td>0.5</td>
<td>622.9 ± 51.8</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>552.2 ± 79.0</td>
<td>3.9 ± 0.3</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 10). * p < 0.01 compared with control group.

We were all clearly higher than the levels in the control group. AFEF treatment (0.5 and 1.0 g/kg) lowered the CCl₄-induced increase in GPT in mice at 3, 6 and 9 weeks.

Hepatic hydroxyproline lipid peroxidation content.

As shown in Table 3, CCl₄ induced the liver damage in mice, indicating increases of approximately 141% and 131% in hepatic hydroxyproline and malondialdehyde levels, respectively. The administration of AFEF (1.0 g/kg) significantly decreased the hepatic levels of hydroxyproline and malondialdehyde to 80% and 83%, respectively.

RT-PCR analysis of liver tissue.

Fragments specific to MAT1A, collagen (α1)(I) and TGF-β1 were amplified by RT-PCR (Fig. 1A). The values from densitometric analysis were normalized to the corresponding GAPDH transcript and are expressed as the MAT1A/GAPDH, collagen(α1)(I)/GAPDH and TGF-β1/GAPDH ratios (Fig. 1B). MAT1A presents low mRNA levels in CCl₄-damaged liver. Administration of AFEF (1 g/kg) significantly increased the level of MAT1A in CCl₄-treated mice. The levels of collagen I and TGF-β1 mRNA in liver were significantly increased by CCl₄ treatment. Administration of AFEF (1 g/kg) significantly decreased the levels of collagen I and TGF-β1 mRNA expression.

Pathological changes.

The livers of CCl₄-treated mice showed gross necrosis, broad infiltration of lymphocytes around the central vein and fibrosis (Fig. 2B, 2E). Histological examination of the livers in the mice treated with AFEF (1.0 g/kg) showed a normal lobular pattern.
with a mild degree of necrosis and lymphocyte infiltration (Fig. 2C, 2F). The average scores of necrosis and fibrosis in AFEF (1.0 g/kg)-treated rats were significantly reduced compared with the control group (2.3 vs 3.2, \( p < 0.05 \) for necrosis; 2.5 vs 3.4 for fibrosis, \( p < 0.05 \)).

**DISCUSSION**

The present study revealed a beneficial effect of AFEF in preventing liver fibrosis induced by CCl4 treatment. The improvement brought about by AFEF was also seen in the plasma biochemical parameters.

Plasma GPT activity is the most commonly used biochemical marker of liver damage (Friedman et al., 2003). CCl4 treatment caused hepatocellular damage in mice, as indicated by a drastic increase in plasma GPT activity after 3, 6 and 9 weeks of CCl4 administration. Mice treated with AFEF showed protection against CCl4-induced damage, with the levels of plasma GPT being reduced. This phenomenon was also confirmed by histological examination.

Methionine adenosyltransferase (MAT) catalyses the formation of S-adenosylmethionine, the principal biological methyl and propylamino donor, and is essential for normal cell function (Fontecave et al., 2004). In mammalian tissue, three different forms of MAT (MAT I/III and MAT II) have been identified, which are the products of two different genes (MAT1A and MAT2A) (Mato et al., 2002). The expression of MAT1A is primarily restricted to adult liver (Kotb et al., 1997). Liver damage caused by hepatotoxins initiates a cellular response involving the generation of reactive oxygen substances (Mato et al., 2002). This leads to the inactivation of MAT1A and a concomitant reduction in adenosylmethionine content, which in turn reduces MAT1A expression (Lin and Lin, 2006; Mato et al., 2002). Thus, in response to liver injury, MAT1A expression is decreased. Consistent with this, it was found that the expression of MAT1A was reduced in the livers of mice with chronic CCl4 injury. In this study, the decrease in MAT1A expression in chronic CCl4-injured mice was also diminished by AFEF treatment. These results provide further evidence that AFEF possesses a hepatoprotective effect.

Hepatic fibrosis is a consequence of chronic hepatitis and involves the abnormal accumulation of extracellular matrix proteins, particularly collagen (Schuppan and Porov, 2002). Hydroxyproline is the characteristic
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The amount of collagen can be reflected by determining the content of hydroxyproline and can be used to express the extent of fibrosis (Hanauske-Abel, 2003). When CCl4 was applied in this experiment to induce liver fibrosis, the content of hydroxyproline in liver obviously increased; AFEF can reduce the content of hydroxyproline. This was also confirmed by the histopathological examinations.

Many studies have shown that levels of collagen I increase during liver fibrosis (Lin *et al.*, 2006). Therefore, the effect of AFEF on the mRNA expression of collagen I was investigated. Treatment with AFEF was effective in reducing the amount of collagen I mRNA expression. This result further confirms that AFEF could abrogate the actions of hepatic fibrosis.

TGF-β1 is a profibrogenic cytokine, because it directly stimulates extracellular matrix production by both Kupffer cells and stellate cells (Nakamura *et al.*, 2005; Breitkopf *et al.*, 2005). Increased levels of TGF-β1 mRNA expression have been found in patients with liver fibrosis as well as in experimental models of liver fibrosis (Chen *et al.*, 2002; Lin *et al.*, 2006). In this experiment, CCl4 treatment increased, while AFEF significantly reduced TGF-β1 mRNA expression. This result suggests that AFEF ameliorated liver fibrosis by reducing TGF-β1 secretion.

Increased free radical production and lipid peroxidation have been proposed as a major cellular mechanism involved in CCl4 hepatotoxicity (Basu, 2003). Furthermore, a close relationship has been reported between lipid peroxidation and fibrogenesis in rats in which fibrosis was induced by CCl4 administration (Comporti *et al.*, 2005). The results confirm the findings that hepatic lipid peroxidation is increased during hepatic fibrogenesis. It was also found that AFEF inhibited CCl4-induced hepatic lipid peroxidation. These results indicated that AFEF might inhibit lipid peroxidation, and consequently attenuate the development of liver fibrosis.

There have been reports on the hepatoprotective action of *A. formosanus* since 1993. These reports include experimental acute hepatitis in rats induced by CCl4 or acetaminophen (Lin *et al.*, 1993; 2000), chronic hepatitis in rats caused by CCl4 or dimethylnitrosamine (Shih *et al.*, 2004; 2005), and CCl4 or H2O2 induced cytotoxicity in cultured hepatocytes (Du *et al.*, 2003; Wu *et al.*, 2007). Numerous studies have indicated that *A. formosanus* extracts are good free radical scavengers (Wang *et al.*, 2002; Shih *et al.*, 2003; Wang *et al.*, 2005), suggesting that the ameliorative effects of *A. formosanus* on liver injury induced by hepatotoxins are due, at least in part, to its free-radical scavenging ability. In the present study, RT-PCR was used to analyse the mRNA expressions of *MAT1A*, collagen (*α1*/*β1*) and TGF-β1. The results also confirmed the hepatoprotective actions of AFEF.

Acknowledgements

This study was supported by grants from the National Science Council of the Republic of China (NSC 91-2317-B-039-11)

REFERENCES


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DOI: 10.1002/ptr


A Standardized Aqueous Extract of *Anoectochilus formosanus* Ameliorated Thioacetamide-Induced Liver Fibrosis in Mice: The Role of Kupffer Cells

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Received November 9, 2009; Accepted January 9, 2010; Online Publication, April 7, 2010
[doi:10.1271/bbb.90824]

*Anoectochilus formosanus* is used in traditional folk medicine as an hepatoprotective agent. The purpose of this study was to investigate the effects of a standardized aqueous extract of *A. formosanus* (SAEAF) on thioacetamide (TAA)-induced liver fibrosis. An *in vitro* study showed that the inhibitory effect of kinsenoside, a major component of SAEAF, on tumor necrosis factor α (TNF-α) secretion from Kupffer cells might be derived at least partly from downregulation of LPS-receptor Toll-like receptor 4 (TLR4) signaling. Hepatic fibrosis was produced by TAA (200 mg/kg, i.p.) 3 times per week for 12 weeks. Mice in the three TAA groups were treated daily with distilled water and SAEAF (1.0, 0.2 g/kg) via gavage throughout the experimental period. The mice that received the SAEAF treatment had significantly reduced plasma alanine aminotransferase activity, relative liver weights, and hepatic hydroxyproline contents. A histological examination also confirmed that SAEAF reduced the degree of fibrosis caused by TAA treatment. RT-PCR analysis showed that SAEAF treatment reduced mRNA expression of collagen (α1(I)), lipopolysaccharide-binding protein, CD14, TLR4, and TNF receptor 1. An immunohistochemical examination also indicated that SAEAF reduced the number of CD68-positive cells (macrophages). In conclusion, oral administration of SAEAF significantly reduced TAA-induced hepatic fibrosis in mice, probably through inhibition of hepatic Kupffer cell activation.

Key words: Anoectochilus formosanus; kinsenoside; thioacetamide; Kupffer cells; liver fibrosis

*Anoectochilus formosanus* Hayata (Orchidaceae) is an indigenous, valuable Taiwanese medicinal plant, very popular as an herbal tea in Taiwan and other Asian countries. It is also called King Medicine because of its diverse pharmacological uses, e.g., anti-osteoarthritis, anti-hyperglycemia, anti-hyperlipidemia, and for treatment of cardiovascular disease.1–4 In recent and earlier experimental studies, aqueous extracts of *A. formosanus* revealed hepatic protection and inhibitory action on liver fibrosis in rats and mice.5–8) Recently, we found that kinsenoside (Fig. 1), a high-yielding component of *A. formosanus*,9 prevented CCl4-induced liver fibrosis in mice.9) Except for kinsenoside, the various fractions showed hepatoprotective action.8) Thus, kinsenoside can be used as a marker compound in the quality control of *A. formosanus* preparations, but the mechanisms underlying the antifibrotic action of *A. formosanus* are still unclear.

Bioactivity-guided fractional studies showed that the ethyl acetate fraction enhanced CCl4-induced hepatitis in mice.8) In this study, a standardized aqueous extract of *A. formosanus* (SAEAF) was prepared, excluding the ethyl acetate fraction. Then phytochemical analysis by high-pressure liquid chromatography (HPLC) was done to quantify the content of kinsenoside.8)

Kupffer cells, hepatic macrophages, activated with the release of fibrogenic mediators are known to become active before hepatic stellate cell activation. Transformation of stellate cells with deposition of collagen and other extracellular matrix proteins is considered a key factor in the pathogenesis of liver fibrosis.9) Thus, modulation of Kupffer cells can disrupt the sequence of events leading to organ injury by damping down the fibrogenic stimulus.9) Evidence of the role of Kupffer cells has been confirmed by attenuating liver cirrhosis in animals selectively depleted of Kupffer cells.10,11)

It has been reported that regenerative nodules and liver fibrosis occur more prominently in rats injected with thioacetamide (TAA) than in those injected with CCl4. The histology closely resembles that of human cirrhosis.12) It has been found that Kupffer cells are involved in the progression of TAA-induced liver fibrosis.10) In addition, several previous studies have demonstrated that elevated levels of circulating tumor necrosis factor α (TNF-α) occurred in patients with liver cirrhosis.13) In general, Kupffer cells are the major cellular source of TNF-α production in the liver.14) Toll-like receptor (TLR)-4, one of the most important lipopolysaccharide (LPS) receptors, plays an important role in the activation of macrophages.15) TNF-α signals

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Abbreviations: ALT, alanine aminotransferase; LPS, lipopolysaccharide; SAEAF, standardized aqueous extract of *Anoectochilus formosanus*; TAA, thioacetamide; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor α; TNFR1, TNF receptor 1

through activation of TLR-4 in Kupffer cells, which play an essential role in the development of liver fibrosis. In screening tests, we observed an ability of kinsenoside to inhibit LPS-induced TNF-α production in Kupffer cells. Hence, in order to elucidate the role of Kupffer cells in the anti-fibrotic effect of SAEAF. We examined the molecular changes of Kupffer cells in TAA-induced liver fibrosis in mice.

**Materials and Methods**

Preparation of SAEAF and measurement of kinsenoside. A. formosanus plants were purchased from Yu-Jung Farm (Pu-Li, Taiwan). The plants were identified by the Institute of Chinese Pharmaceutical Sciences, China Medical University (Taiwan), where a plant specimen (no. CMCP 1253) has been deposited. Fresh whole plants of cultured A. formosanus were extracted with water, and the filtrate was partitioned with ethyl acetate. The aqueous fraction was further filtered and evaporated under reduced pressure to yield a purpuric residue, SAEAF. The SAEAF yield was approximately 18%.

Isolation and treatment of Kupffer cells. Kupffer cells were isolated from male Wistar rat (350–450 g in body weight) liver according to a published procedure, with slight modifications. Briefly, the liver was perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with perfused with 200 ml of 0.02% type IV collagenase in Hanks’ balanced salt solution (HBSS, 20 ml/min) and removed for smashing with

Liver fibrosis was induced in three groups of 10 mice each by intraperitoneal injection of 200 mg/kg in body weight. TAA dissolved in saline was administered 3 times per week, for 12 weeks. The animals received TAA with distilled water (0.1 ml/kg in body weight) or SAEAF (0.2 and 1.0 g/kg, po, daily). The control group was given an intraperitoneal injection of saline (0.1 ml/kg body weight) 3 times per week, and distilled water (0.1 ml/kg body weight, po, daily) for 12 weeks. The drugs were administered when the chronic injury model was induced. The drug treatment duration was 12 weeks in total. At the end of the experimental period, the mice were sacrificed under CO₂ anesthesia and blood was withdrawn from the abdominal vein. Liver samples were also collected for histological and biochemical examination.

**Assay of liver functions.** Blood was centrifuged at 4,700 rpm at 4 °C for 15 min to separate out the plasma. The plasma alanine aminotransferase (ALT) level was measured using clinical kits (Roche Diagnostics, Mannheim, Germany) and a spectrophotometric system (Cobas Mira; Roche, Rotkreuz, Switzerland).

<table>
<thead>
<tr>
<th>mRNAs</th>
<th>Size</th>
<th>Oligomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>375 bp</td>
<td>Sense5'-CTTAGCTGGAATTCATATTCCGGAGCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense5'-AATGGAGAAGGCTCGGGAACCTTG-3'</td>
</tr>
<tr>
<td>Collagen(a1(I))</td>
<td>174 bp</td>
<td>Sense5'-GGTCTTCAAGGAGGGTCTGATGG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense5'-GACACGCTCACAACGAGTCT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>76 bp</td>
<td>Sense5'-AGGCTTCTCCGATCTGAGATCG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense5'-CCTGCTTCCACACCTTCTTGA-3'</td>
</tr>
<tr>
<td>LBP</td>
<td>127 bp</td>
<td>Sense5'-ACCCCTTGAACCTGGACTGAC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense5'-ACAGTTGCCCAGCCTTAAAGT-3'</td>
</tr>
<tr>
<td>TLR4</td>
<td>85 bp</td>
<td>Sense5'-TGGAGACCATGACGCTCCTGA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense5'-TCATACCCCTCTGGAAGGAGG-3'</td>
</tr>
<tr>
<td>TNFR1</td>
<td>228 bp</td>
<td>Sense5'-TACCCCAAGGGGAGTACGGCA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense5'-GCTCTGCGCCGGCGAAGC-3'</td>
</tr>
</tbody>
</table>

Fig. 1. Structure of Kinsenoside.
Assay of hydroxyproline. Hydroxyproline determination was performed by the method designed by Neuman and Logan.18 After hydrolysis with 6 N HCl, the dried liver tissue was oxidized with hydrogen peroxide and reacted with p-dimethylaminobenzaldehyde and the absorbance of the colored product was determined at 540 nm (U-2001; Hitachi, Tokyo). The amount of hydroxyproline was expressed as μg/g in wet tissue.

RT-PCR analysis. Total RNA was isolated from the mouse livers and from Kupffer cells cultured by the acid guanidinium thiocyanate-phenol-chloroform extraction method, as described by Chomczynski and Sacchi.19 A 5-μg sample of total RNA from each liver sample was subjected to reverse transcription (RT) by moloney murine leukemia virus reverse transcriptase in a 50-μl reaction volume. Aliquots of the RT mix were used in amplification by polymerase chain reaction of fragments of collagen (α1(1)), LPS binding protein (LBP), CD14, TLR4, and TNF receptor 1 (TNFR1) with the mice primer pairs listed in Table 1. The expression levels of all the transcripts were normalized to that of GAPDH mRNA in the same tissue sample. PCR product identities were confirmed by sequence analysis. The PCR products were separated on a 2% agarose gel and recorded on Polaroid film; the bands were quantified with a densitometer. The mean ratio of each group was calculated as the average for 10 animals. The fragments shown in Fig. 3 reflect the pooled data for 10 samples.

Pathological and immunohistochemical examinations. After formalin fixation, tissue samples were sliced, embedded in standard manner, and stained with Sirius red to detect collagen fibrils. Fibrosis was quantified with a computerized image analysis system (Image-Pro Plus version 5.1; Media Cybernetics, Bethesda, MD). Three or four samples per slide were examined in 10 animals per experimental group. In each sample, 10 non-overlapping areas were evaluated, and observed through the objective lens with a magnification of 40×. Data for fibrosis were expressed as the mean percentage of fibrotic area to the whole area of the liver using 10 fields per sample.

Expression and localization of CD68 in the liver were detected by immunohistochemical staining, as previously described.20 For single staining of CD68, deparaffinized tissue sections were incubated with a monoclonal anti-CD68 antibody (Abbiotec, San Diego, CA). Specific staining was visualized using an immunodetection kit (SuperSensitive link-label IHC detection system; BioGenex, San Ramon, CA) and 3,3′-diaminobenidine. With an automated software analysis program, the percent immunostained/field areas of the digital photomicrographs were quantified.

Statistical analysis. Results were expressed as mean ± SD. All experimental data were analyzed by one-way analysis of variance following the Dunnet test. A p value of <0.05 was considered statistically significant.

Results

Effects of kinsenoside on TNF-α production and mRNA expression of TLR4 and TNF-α in LPS-stimulated Kupffer cells

As shown in Table 2, culture treatment with 0.5 μg/ml of LPS for 24 h caused a dramatic increase in TNF-α production. Kinsenoside inhibited TNF-α production in a concentration-dependent manner. The Kupffer cells did not undergo any change in viability after exposure to LPS + kinsenoside (Table 2).

Table 2. Effects of Kinsenoside on the Viability and Production of TNF-α in Kupffer Cells after LPS Stimulation

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (μM)</th>
<th>Cell viability (optical density)</th>
<th>TNF-α (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>1.08 ± 0.05</td>
<td>62.2 ± 21.9</td>
</tr>
<tr>
<td>LPS + Vehicle</td>
<td>—</td>
<td>1.18 ± 0.07</td>
<td>228.0 ± 31.5*</td>
</tr>
<tr>
<td>+ SAFAF</td>
<td>100</td>
<td>1.15 ± 0.02</td>
<td>77.2 ± 75.4**</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.28 ± 0.04</td>
<td>91.3 ± 13.2**</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.29 ± 0.12</td>
<td>189.4 ± 57.5</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 3). *p < 0.01 as compared with the control group. **p < 0.01 as compared with the LPS + vehicle group.

Fig. 2. Effects of Kinsenoside on TLR4 and TNF-α mRNA expression in LPS-Stimulated Kupffer Cells

A. Fragments were amplified by RT-PCR. The expression levels of TLR4 (B), and TNF-α (C) mRNA were measured and quantified densitometrically. Values were normalized to GAPDH mRNA expression. *p < 0.01, **p < 0.001 as compared with the control group. ***p < 0.01 as compared with the TAA + vehicle group.

The LPS-activated Kupffer cells treated with kinsenoside showed suppression of TLR4 and TNF-α mRNA expression in a concentration-dependent manner (Fig. 2B and C).

Body weight and relative liver weight

An earlier study demonstrated that the variation and level of critical hepatic damage in response to TAA can be monitored easily by changes in body weight.12 In this study, marked reductions in body weight were found under TAA treatment. The final mean body weight in the
The reduction in body weight using TAA treatment was significantly less with SAEAF treatment (0.2 and 1.0 g/kg). Treatment with SAEAF of 0.2 and 1.0 g/kg led to 9% and 11% reductions in relative liver weight respectively.

**Plasma ALT activity**

Plasma ALT activity is a routine liver function test. As shown in Table 4, when treated with TAA, there was marked increase in plasma ALT activity. Plasma ALT activity increased 2.8-fold in the TAA-treated mice as compared with the control mice (Table 4). Administration of SAEAF (1.0 g/kg) significantly reduced the TAA-induced increase in ALT activity. Treatment with SAEAF (1.0 g/kg) led to a 33% reduction in plasma ALT activity.

**Hepatic hydroxyproline**

Hepatic hydroxyproline was used to evaluate hepatic fibrosis. TAA treatment increased the hepatic hydroxyproline level by approximately 165% (Table 4). Administration of SAEAF (1.0 g/kg) significantly reduced the hepatic content of hydroxyproline, to 78% that of the TAA + H2O group.

**Table 3. Effects of SAEAF on Body and Liver Weight in TAA-Treated Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (g/kg)</th>
<th>Body weight (g)</th>
<th>Relative liver weight (g/100 g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>27.8 ± 1.6</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>TAA + H2O</td>
<td>—</td>
<td>16.8 ± 0.8**</td>
<td>7.0 ± 0.2***</td>
</tr>
<tr>
<td>+ SAEAF</td>
<td>1.0</td>
<td>22.9 ± 3.0***</td>
<td>6.2 ± 0.3**</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>17.7 ± 2.6</td>
<td>6.4 ± 0.2**</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 10). **p < 0.01, ***p < 0.001 as compared with the control group. **p < 0.05, ***p < 0.01 as compared with the TAA + H2O group.

**Table 4. Effects of SAEAF on Plasma ALT Activities and Hepatic Hydroxyproline Contents in TAA-Treated Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (g/kg)</th>
<th>Plasma ALT (U/L)</th>
<th>Hepatic hydroxyproline (µg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>36.1 ± 3.2</td>
<td>441.1 ± 59.8</td>
</tr>
<tr>
<td>TAA + H2O</td>
<td>—</td>
<td>99.5 ± 33.3***</td>
<td>726.1 ± 71.4**</td>
</tr>
<tr>
<td>+ SAEAF</td>
<td>1.0</td>
<td>66.7 ± 16.7**</td>
<td>565.5 ± 45.7**</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>78.5 ± 22.1</td>
<td>621.6 ± 89.6</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 10). ***p < 0.001 as compared with the control group. **p < 0.01 as compared with the TAA + H2O group.

TAA-treated group was about 60% that of the control group (Table 3). The reduction in body weight using TAA treatment was significantly reduced with SAEAF treatment (0.2 and 1.0 g/kg). Treatment with SAEAF of 0.2 and 1.0 g/kg led to 9% and 11% reductions in relative liver weight respectively.
Pathological changes
A primary consideration in assessing efficacy in hepatic fibrosis is its effect on liver histology. Liver sections of the control mice showed low ratios of fibrotic area (Fig. 4A and E). When treated with TAA, there were marked increases in the collagen surrounding the liver lobules, which resulted in large fibrous septa (Fig. 4B and E), and the fibrosis area was 20-fold greater in the TAA-treated mice than in the control mice. Treatment with SAEAF (1.0 g/kg) led to only very low levels of collagen detected (Fig. 4C and E). Treatment with SAEAF (1.0 g/kg) led to a 34.4% reduction in the fibrosis area ratio.

To determine whether SAEAF reduced Kupffer cell numbers in the TAA-treated mice, hepatic CD68 expression was quantified. In the control livers, when stained with CD68 antibodies, Kupffer cells were barely detected (Fig. 5A). CD68 immunoreactivity increased 10-fold in the TAA-treated mice as compared with the control mice (Fig. 5B and E). In the SAEAF (1.0 g/kg)-treated mice livers, in contrast, CD68 immunoreactivity was reduced (Fig. 5C and E). Treatment with SAEAF (1.0 g/kg) led to a 45.5% reduction in the CD68 area ratio.

Discussion
In this study, our data indicate that SAEAF inhibits the development of TAA-induced liver fibrosis in mice. They also indicate that inhibition of Kupffer cell activation plays an important role in bringing about the ameliorative effects of SAEAF.

Kupffer cells reside in the lumen of the sinusoids and are constantly exposed to gut-derived bacteria, microbial debris, and bacterial LPS. On activation, Kupffer cells release various cytokines and play an important role in the pathogenesis of various liver diseases. It is well known that LPS stimulates Kupffer cells to secrete TNF-α by triggering TLR4 signaling. Downregulation of TLR4 expression significantly attenuated LPS-induced cytokine secretion.21,22 In the present study, kinsenoside, a major component of SAEAF, decreased LPS-induced TNF-α production in isolated rat Kupffer cells. In addition, LPS-stimulated mRNA expression of TLR4 and TNF-α in Kupffer cells and kinsenoside pretreatment efficiently decreased the expression of proinflammatory factors TLR4 and TNF-α. These results indicate that the inhibitory effect of kinsenoside on TNF-α secretion from Kupffer cells under LPS stimulation might be derived at least partly from downregulation of LPS-receptor TLR4 signaling.

Liver fibrosis is a consequence of chronic hepatitis, and it involves abnormal accumulation of extracellular...
matrix proteins, particularly collagen I. Hydroxyproline is the main component of collagen. Many studies indicate that collagen I levels increase during liver fibrosis. In the present study, administration of SAEAF reduced the hydroxyproline hepatic content and mRNA expression level of collagen (α1(I)), which had been increased with TAA treatment in mice. Histological examination using Sirius red also showed that SAEAF reduced TAA-induced liver fibrosis.

As an acute-phase protein, hepatic LBP expression is increased in many models of acute-phase response and liver injury. TAA is also used to induce fulminant hepatic failure. Some studies reveal that the level of serum endotoxin in rats increases in TAA-induced fulminant hepatic failure. In the circulatory system, endotoxin promotes the hepatic synthesis of LBP. As anticipated, we found that hepatic LBP expression was upregulated in TAA-induced hepatitis in mice.

In this study, SAEAF treatment significantly inhibited the hepatic mRNA expression of LBP. The major portion of pathological LPS enters the host through the gastrointestinal tract in both the normal and the pathological state. This suggests that SAEAF limited the entry of endotoxin into the liver through gastrointestinal protection.

Kupffer cells play an important role in liver fibrogenesis. Evidence shows that depletion of Kupffer cell function attenuates TAA-induced hepatoxity. CD14/TLR4 is a component of the LPS receptor and has a key intermediary role in endotoxin-induced activation of Kupffer cells. LBP enhances the binding of LPS to the CD14/TLR4 complex receptor on the Kupffer receptor, causing TNF-α production. TNF-α is known to be one of the earliest events in hepatic inflammation, triggering a wound-healing response that includes fibrogenesis. This proinflammatory activity resembles one of the cascades initiated by the binding of TNF-α to its receptors TNFR1. Since the level of TNF-α mRNA in the liver is changeable, TNFR1 mRNA expression was monitored instead in this study.

Our data showed that TAA significantly increased the hepatic CD14 level and TLR4 and TNFR1 mRNA expression, indicating activation of Kupffer cells. This might be explained in two ways. First, TAA increases the hepatic LBP expression level. Second, it is possible that parenchyma cells (due to TAA metabolism in those cells) participate in the activation of Kupffer cells. SAEAF treatment of mice significantly inhibited the TAA-induced hepatic mRNA expression of CD14, TLR4, and TNFR1. This also confirmed an in vitro experiment that showed the ability of kinsenoside to inhibit the activation of Kupffer cells. Perhaps SAEAF inhibited the activation of Kupffer cells by reducing LBP production.

After liver injury, the number of Kupffer cells increases in the damaged liver, and they are located principally around the regions of damage and fibrosis. In this study, hepatic CD68 expression, a macrophage marker, was quantitated. CD68-positive cells were markedly increased in the TAA-treated mice, indicating either the proliferation of resident Kupffer cells or the recruitment of circulating macrophages to the liver. Low levels of CD68 positive cells in the liver were detected in the SAEAF-treated mice. Thus SAEAF treatment was also associated with a decrease in number of Kupffer cells.

Plasma ALT activity is the most commonly used biochemical marker of hepatocyte injury. In the present study, plasma ALT activity was found to increase in cases of TAA-induced liver injury. SAEAF reduced plasma ALT activity, which increased under TAA administration. When the liver is damaged, it can initiate regenerative actions, that, in turn, increase the liver weight. When the liver is severely damaged, liver cirrhosis can develop, resulting in atrophy. In this study, TAA-induced hepatic fibrosis in mice was accompanied by hepatomegaly. TAA-induced hepatomegaly in mice was reduced with SAEAF treatment. These results indicate that SAEAF can protect against TAA-induced liver damage.

In conclusion, our data indicate that the beneficial effect of SAEAF against TAA-induced liver fibrosis in mice may occur partly through inhibition of Kupffer cell activation.

Acknowledgments

This study was supported by grants from the National Science Council of the Republic of China (NSC94-2317-B-039-001).

References