Effect of Soyasaponins-Rich Extract from Soybean on Acute Alcohol-Induced Hepatotoxicity in Mice

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ABSTRACT: The protective effects of soyasaponins-rich extract (SRE) from soybean against acute alcohol-induced hepatotoxicity were first investigated in the Institute of Cancer Research mice. Administration of SRE prior to alcohol significantly prevented the increases in serum aspartate transaminase, alanine transaminase, alkaline phosphatase, and lactate dehydrogenase caused by alcohol, as well as hepatic triglyceride, total cholesterol, and malondialdehyde levels. Mice treated with SRE showed a better profile of the antioxidant system with normal superoxide dismutase, glutathione S-transferase, and glutathione peroxidase activities, which were associated with the increase of hepatic glutathione levels relative to the acute alcohol-treated group. Supplement of SRE prevented alcohol-induced hepatic steatosis necrosis, inflammation, and swelling, as indicated by liver histopathological studies. All of these findings demonstrate that SRE has protective effects on acute alcohol-induced liver damage.

KEYWORDS: Soyasaponins, alcohol, hepatotoxicity, mice

■ INTRODUCTION

Alcoholic beverages have been a part of the human diet since antiquity. However, excessive drinking of alcohol will cause serious health problems, such as alcoholic liver disease.[1] After absorption by stomach and intestines, alcohol is carried to the liver via the mesenteric and portal veins. Alcohol is first oxidized to aldehyde by alcohol dehydrogenase, and then, the aldehyde is metabolized to acetate, which is rapidly converted to water and carbon dioxide via the Krebs cycle.[2] Long-term alcohol consumption induces oxidative stress in the liver due to the imbalance between the prooxidant and the antioxidant systems.[3] Oxidative stress is considered as one of the crucial mechanisms responsible for alcoholic liver damage.[4,5] Persistent oxidative stress results in fatty liver, which can lead to inflammation, fibrosis, cirrhosis, and even liver cancer.[6]

Reactive oxygen species (ROS) cause tissue injury through covalent binding and lipid peroxidation.[7] The hepatic tissue damage can be reduced through scavenging the free radicals by antioxidants.[8] Antioxidant systems in the body include the antioxidant nutrients and enzymes. The antioxidant enzymes contain superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).[9] A potential source of ROS in hepatocytes is the microsomal cytochrome P-450 system.[10] The rising of alcohol-induced cytochrome P-450 2E1 activity is regarded as a principal contributor in generating a state of oxidative stress, which results in hepatotoxicity.[11]

Soy saponins are oleanane triterpenes that are found in various glycosides as soy products and other legumes.[12] Soy saponins are mainly divided into three groups based on the structure of the aglycone moiety, A, B, and E (glycosides of soyasapogenols A, B, and E).[13] Soy saponins have been shown to have many biological activities including chemoprotective, hypocholesterolemic, immunostimulatory, antiviral, and anticarcinogenic activities.[14−20] In previous research, soy sapogenol A and soy sapogenol B derivatives showed protective effects on liver injury mediated by

immune response in a concanavalin A-induced hepatitis model.[21,22] As far as we know, the effect of soy saponins on acute alcohol-induced hepatotoxicity has not been studied. Therefore, the present investigation was undertaken with the objective of examing the protective effects of feeding different levels of soy saponins on acute alcohol-induced liver damage in mice.

■ MATERIALS AND METHODS

Chemicals. Commercial antioxidant assay kits for total cholesterol (TC), triglyceride (TG), malondialdehyde (MDA), SOD, glutathione S-transferase (GST), GPx, and glutathione (GSH) were purchased from Nanjing Jiancheng Bioengineering Institute (NJBI) (Nanjing, China). Daidzin, glycitin, and genistin were purchased from Sigma and Aldrich (Sigma-Aldrich Corp., St. Louis, MO). Malonyldaidzin and malonyl-genistin were a gift from Dr. J. M. Sun (Chinese Academy of Agricultural Sciences, China). All other reagents were of analytical or chromatographic grade.

Chemical Characterization of SRE. SRE was purchased from North China Pharmaceutical Co., Ltd. (Hebei Province, China), and the content of total soyasaponins was 80% with spectrophotometer assay according to the National Standards of the People's Republic of China for soybean saponin (GB/T 22464-2008). The main soyasaponins were identified by using an Agilent 1200 LC and 6300 Serries Ion Trap MS (Agilent Technologies, Germany) equipped with a YMC-Pack ODS-A column (250 mm × 4.6 mm, 5 μm, YMC Co., Ltd., Japan). Mobile phase A was 0.5% acetic acid solution, whereas mobile phase B was 0.5% acetic acid in acetonitrile. The gradient elution was carried out as follows: Solvent B increased from 5 to 15% in 15 min, then increased to 30% in 40 min, and finally increased to 60% in 75 min. The column temperature
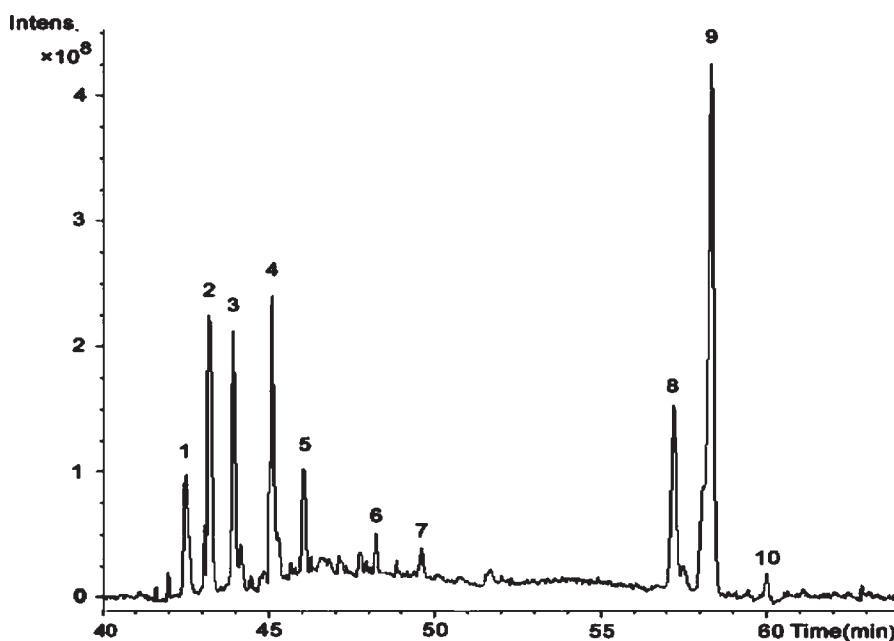
Figure 1. Total ion chromatogram of soyasaponins-rich extract (SRE) by HPLC-MS. Explanation for the peaks: 1, soyasaponin Aa; 2, soyasaponin Ab; 3, soyasaponin Ae; 4, soyasaponin Bb; 5, soyasaponin Bb′; 6, soyasaponin Bd; 7, soyasaponin Be; 8, soyasaponin αg; 9, soyasaponin βg; and 10, soyasaponin βa.

was 30 °C. The injection volume was 5 μL with the flow rate of 0.5 mL/min. Spectra were recorded in negative ion mode between m/z 150 and 1500 u. Major MS parameters were as follows: capillary voltage, 3500 V; nebulizer pressure, 35 psi; dry gas, 9.0 L/min; and dry temperature, 350 °C.

The isoflavones in SRE were analyzed in a Waters Alliance HPLC (Waters Corp., Milford, United States) equipped with an Apollo C18 column [250 mm × 4.6 mm, 5 μm, W. R. Grace & Co. (Alltech), CT]. The column temperature was 40 °C. The mobile phases were composed of (A) 0.1% glacial acetic acid in water and (B) 0.1% glacial acetic acid in acetonitrile. A gradient program at a flow rate of 1.0 mL/min was as follows: Solvent B was increased from 15 to 35% over 50 min and then held at 35% for 10 min. The individual isoflavones were monitored in an UV detector at 260 nm. Identification and quantification of daidzin, glycitin, genistin, malonyldaidzin, and malonylgenistin in SRE were performed by comparing the retention times of authentic standards and areas of external standard.

Animals and Treatments. Male ICR mice, weighing 18−22 g, were obtained from the Department of Laboratory Animal in Peking University (Beijing, China). Animals were kept under the standard conditions of animal house with 12 h light/dark cycle (light 7:00−19:00) at a temperature of 22 ± 2 °C and a humidity of 60 ± 5% and maintained free access to food and water for 1 week prior to use. The ICR mice diet was conducted according to the general quality standard for formula feeds of laboratory animals in China (GB14924.1, 2001). The whole experiment was carried out complying with the European Community guidelines for the use of experimental animals and approved by the Peking University Committee on Animal Care and Use.

Mice were randomly assigned to five experimental groups with 10 mice in each group, according to preliminary experiments and previous literature.[10,23] The experimental design was as follows. Group 1: normal group, mice treated with normal saline (5 g/kg BW); group 2: ethanol group, mice received ethanol (5 g/kg BW); group 3: low dose group, treated mice received ethanol (5 g/kg BW) 30 min after SRE (300 mg/kg BW); group 4: middle dose group, treated mice received ethanol (5 g/kg BW) 30 min after SRE (600 mg/kg BW); group 5: high dose

group, treated mice received ethanol (5 g/kg BW) 30 min after SRE (1200 mg/kg BW). Each group received the appropriate vehicle or ethanol by gastric intubation for 8 days. At the end of the experimental period, animals were sacrificed to collect serum and livers. These were stored at −80 °C until the analysis. Eight of the samples in each group were chosen for the subsequent assay.

Serum Biochemistry. Activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) in serum were measured using an automatic biochemical analyzer (Olympus AU2700, Japan).

Measurement of TC, TG, and MDA. TC, TG, and MDA in liver was determined by kits obtained from NJBI (China). Liver homogenates were prepared in cold Tris-HCl (5 mM containing 2 mM EDTA, pH 7.4) using a homogenizer and centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was used for the assay of hepatic TC, TG, and MDA.

Biochemical Assays. SOD, GST, GPx, and GSH in liver homogenates were determined following the instructions on the NJBI kits.

Histopathology. The livers were removed and preserved in 10% neutral-buffered formaldehyde solution. Fixed tissues were processed for paraffin embedding, cut into sections, and placed on microscope slides (5−6 μm thick). Slides were stained with hematoxylin and eosin (H&E), which mounted in neutral distyrene−dibutylphthalate−xylene medium for microscopic observations and photograph.

Statistical Analysis. Statistical analysis was performed using SPSS software package, version 13.0. The values were analyzed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). All of the results were expressed as means ± standard deviations (SDs) in each group. Differences of $P < 0.05$ were considered statistically significant.

■ RESULTS

Soyasaponins and Isoflavones in SRE. The total ion chromatogram of SRE by high-performance liquid chromatography−mass spectrometry (HPLC-MS) is shown in Figure 1. Soyasaponins were eluted after 40 min and identified by comparing

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Table 1. Structures, Formulas, Observed $[M - H]^-$, and Retention Times of Soyasaponins Detected in SRE

soyasaponins	structure[a]	formula	observed $[M - H]^-$	t_R[b]
Aa	glc(1→2)gal(1→2)glcUA(1→3)A(22←1)ara (3←1)xyl(2,3,4-tri-O-acetyl)	$C_{64}H_{100}O_{31}$	1363	42.5
Ab	glc(1→2)gal(1→2)glcUA(1→3)A(22←1)ara (3←1)glc(2,3,4,6-tetra-O-acetyl)	$C_{67}H_{104}O_{33}$	1435	43.2
Ae	gla(1→2)glcUA(1→3)A(22←1)ara (3←1)xyl(2,3,4-tri-O-acetyl)	$C_{58}H_{90}O_{26}$	1201	43.9
Bb	rha(1→2)gal(1→2)glcUA(1→3)B	$C_{48}H_{78}O_{18}$	941	45.1
Bb′	gal(1→2)glcUA(1→3)B	$C_{42}H_{68}O_{14}$	795	46.0
Bd	glc(1→2)gal(1→2)glcUA(1→3)E	$C_{48}H_{76}O_{19}$	955	48.3
Be	rha(1→2)gal(1→2)glcUA(1→3)E	$C_{48}H_{76}O_{18}$	939	49.6
αg	glc(1→2)gal(1→2)glcUA(1→3)B (22←2′)DDMP	$C_{54}H_{84}O_{22}$	1083	57.2
βg	rha(1→2)gal(1→2)glcUA(1→3)B (22←2′)DDMP	$C_{54}H_{84}O_{21}$	1067	58.3
βa	rha(1→2)ara(1→2)glcUA(1→3) B(22←2′)DDMP	$C_{53}H_{82}O_{20}$	1037	60.0

[a] glc, β-D-glucopyranosyl; gal, β-D-galactopyranosyl; glcUA, β-D-glucoronopyranosyl; ara, α-L-arabinopyranosyl; rha, α-L-rhamnopyranosyl; xyl, β-D-xylopyranosyl; A, soyasapogenol A; B, soyasapogenol B; E, soyasapogenol E; and DDMP, DDMP conjugated to soyasapogenol B. [b] t_R, retention time (min).
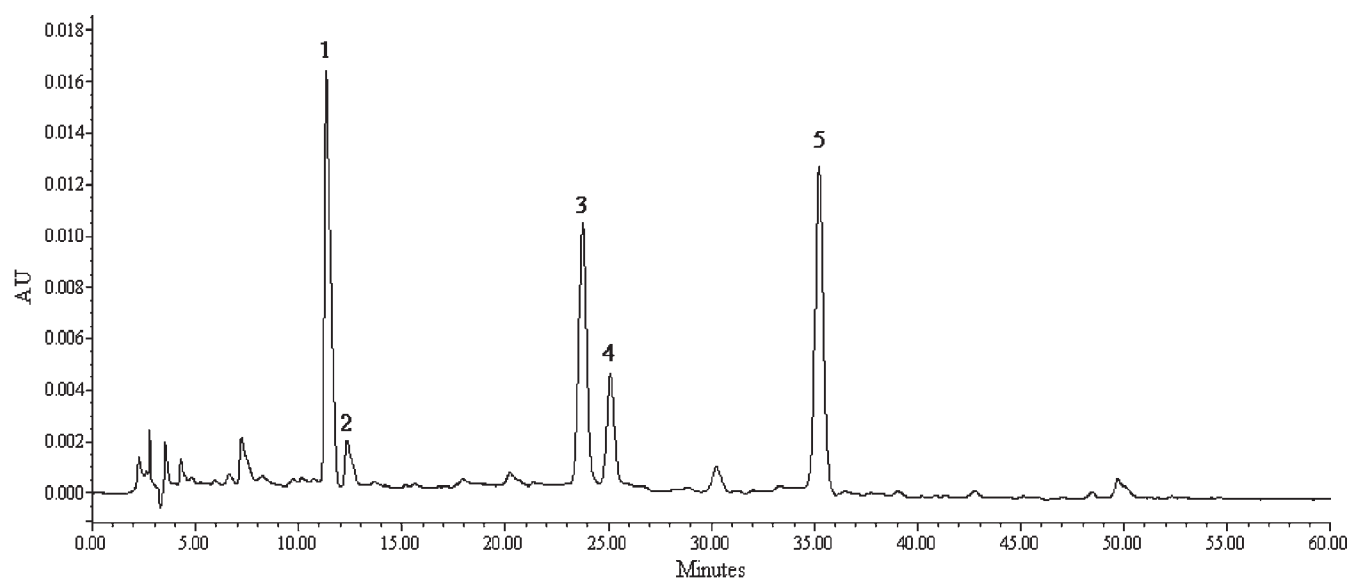

Figure 2. HPLC chromatogram of isoflavones in SRE. Identification of peaks: 1, daidzin; 2, glycitin; 3, genistin; 4, malonyldaidzin; and 5, malonylgenistin.

their retention time and MS data with published data[24,25] in Table 1. Peaks 1–10 were soyasaponin Aa, soyasaponin Ab, soyasaponin Ae, soyasaponin Bb, soyasaponin Bb′, soyasaponin Bd, soyasaponin Be, soyasaponin αg, soyasaponin βg, and soyasaponin βa, respectively. Isoflavones were as follows: daidzin (1.15 mg/g), glycitin (0.09 mg/g), genistin (2.68 mg/g), malonyldaidzin (1.24 mg/g), and malonylgenistin (4.57 mg/g) (Figure 2).

Effects of SRE on Serum Biochemical Markers Levels. Several hepatic enzymes in serum are considered effective biochemical markers for early hepatic damage, such as AST, ALT, ALP, and LDH. Table 2 showed the results of these enzymes in experimental mice. Ethanol administration caused hepatotoxicity in mice, as indicated by the significant increase in serum AST, ALT, ALP, and LDH as compared to the normal group ($P < 0.01$ or $P < 0.05$). Administration of SRE along with alcohol decreased the level of these functional markers relative to the ethanol group. SRE at a dose of 1200 mg/kg ($P < 0.01$) was more effective when compared with two other doses (600 and 300 mg/kg).

Effects of SRE on Hepatic TC, TG, MDA, and GSH Levels. The results of hepatic TC, TG, MDA, and GSH levels are shown in Figure 3. TC, TG, and MDA levels in the ethanol-treated group were significantly ($P < 0.01$ or 0.05) higher than those in the normal group. Those in the SRE-treated groups (group 4 and group 5) were significantly lower than those in the ethanol-treated group ($P < 0.05$ and $P < 0.01$). The GSH level was significantly decreased in the ethanol-treated group, as compared to the normal group. SRE supplement significantly increased the GSH level in group 4 ($P < 0.05$) and group 5 ($P < 0.01$) relative to the alcohol only treatment.

Hepatic Antioxidant Enzyme Activities. SOD, GST, and GPx were considered as an index of antioxidant status of tissues. As presented in Table 3, significant ($P < 0.01$) reduction of hepatic SOD, GST, and GPx activities was observed in the ethanol group as compared with the normal group. There was a significant increase of these antioxidant enzyme activities in SRE treated groups at doses of 600 ($P < 0.05$) and 1200 mg/kg ($P < 0.01$) relative to the ethanol group.

Histopathological Examination of Mice Liver. Histopathological studies of the liver provided supportive evidence for the biochemical analysis. In the normal group, liver slices showed typical hepatic cells with well-preserved cytoplasm, clear nucleus,

Table 2. Effects of SRE on Liver Function Marker Levels in the Serum[a]

group	U/L			
	AST	ALT	ALP	LDH
normal control	101.75 ± 9.22	46.12 ± 3.56	103.54 ± 8.66	1024.22 ± 85.50
ethanol control	127.54 ± 8.35[b]	54.50 ± 4.65[a]	127.32 ± 9.09[b]	1269.34 ± 76.93[b]
ethanol + SRE (300 mg/kg)	121.02 ± 6.06[b]	51.51 ± 3.32	119.25 ± 5.91[a]	1172.58 ± 66.81[a]
ethanol + SRE (600 mg/kg)	114.27 ± 7.93[c]	48.37 ± 6.95	112.64 ± 6.24[c]	1116.04 ± 95.44[c]
ethanol + SRE (1200 mg/kg)	102.72 ± 10.47[d]	44.25 ± 3.20[d]	105.43 ± 9.76[d]	1051.50 ± 74.66[d]

[a] Values of AST, ALT, ALP, and LDH are expressed as means $\pm$ SDs for eight mice. As compared with normal group: [a]$P < 0.05$, and [b]$P < 0.01$. As compared with ethanol group: [c]$P < 0.05$, and [d]$P < 0.01$.
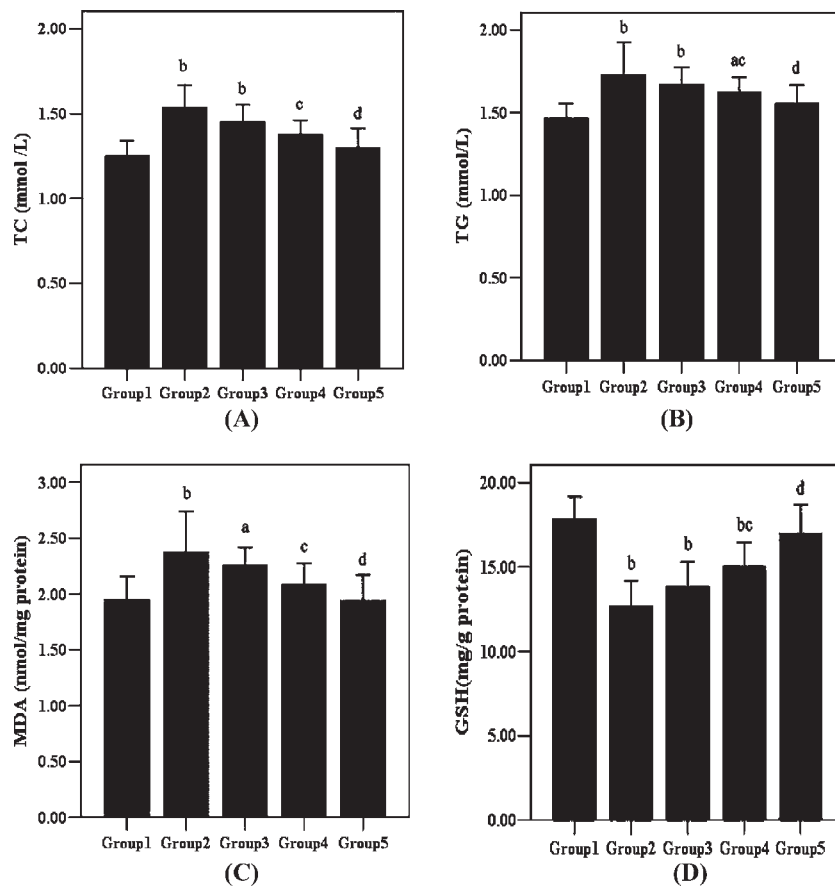

Figure 3. Effects of SRE on the levels of hepatic TC (A), TG (B), MDA (C), and GSH (D). Groups 1–5 are groups of normal, ethanol, ethanol + SRE (300 mg/kg), ethanol + SRE (600 mg/kg), and ethanol + SRE (1200 mg/kg), respectively. Values are expressed as means $\pm$ SDs for eight mice. As compared with normal group: [a]$P < 0.05$, and [b]$P < 0.01$. As compared with ethanol group: [c]$P < 0.05$, and [d]$P < 0.01$.

Table 3. Effects of SRE on Hepatic Antioxidant Enzyme Activities[a]

group	SOD (U/mg protein)	GST (U/mg protein)	GPx (IU/L)
normal control	64.88 ± 5.56	30.59 ± 2.62	479.72 ± 41.39
ethanol control	52.65 ± 7.27[b]	24.39 ± 2.42[b]	284.31 ± 45.41[b]
ethanol + SRE (300 mg/kg)	56.00 ± 6.13[b]	26.51 ± 3.20[a]	312.53 ± 43.90[b]
ethanol + SRE (600 mg/kg)	59.74 ± 4.82[c]	27.65 ± 3.14[c]	338.91 ± 32.92[bc]
ethanol + SRE (1200 mg/kg)	62.30 ± 5.35[d]	29.02 ± 3.54[d]	400.72 ± 49.67[bd]

[a] Values of SOD, GST, and GPx are expressed as means $\pm$ SDs for eight mice. As compared with normal group: [a]$P < 0.05$, and [b]$P < 0.01$. As compared with ethanol group: [c]$P < 0.05$, and [d]$P < 0.01$.

and central vein (Figure 4A). The ethanol group revealed steatosis, macrovesicular necrosis, lobular inflammation, and hepatocellular swelling (Figure 4B). However, these histopathological hepatic damages were reduced in the tissues treated with
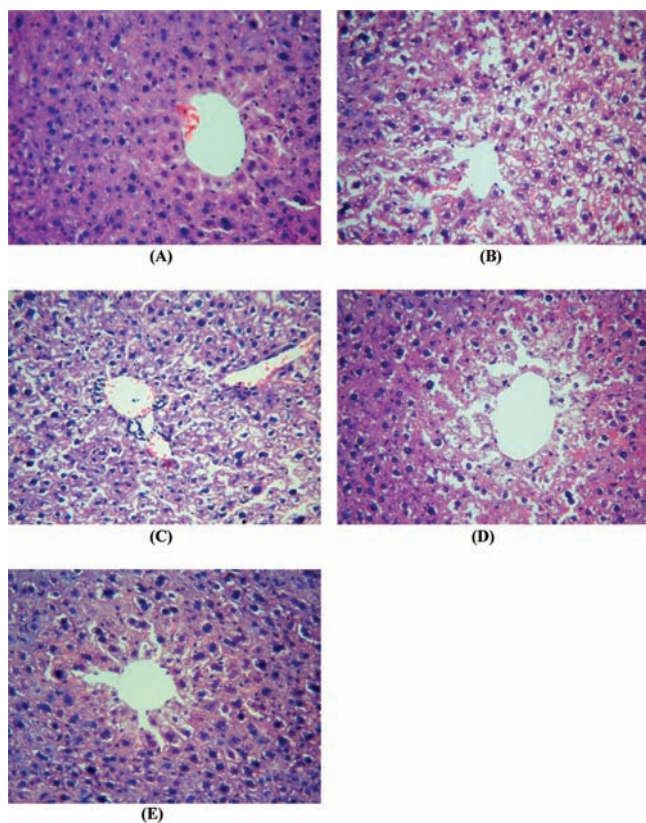
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Figure 4. Histopathological results of liver tissues in mice with H&E under light microscope (magnification, 200×). The normal group (A) shows a central vein surrounded by normal hepatic cells; the ethanol group (B) shows steatosis, necrosis, inflammation, and hepatocellular swelling; the ethanol + SRE (300 mg/kg) and SRE (600 mg/kg) groups (C and D) show a gradual reduction of fatty change and necrosis; and ethanol + SRE(1200 mg/kg) group (E) shows nearly absent histopathological hepatic damage.

SRE (300 mg/kg). As shown in Figure 4C, a decrease of hepatocytes with fatty change and necrosis in the liver was observed. Figure 4D showed a regeneration of hepatocytes, remarkable reduction of hepatocytes with fatty change, and lobular inflammation in the group treated with SRE (600 mg/kg). Histopathological hepatic damage was nearly absent in the group treated with SRE (1200 mg/kg), as presented in Figure 4E. The results of histopathological examination suggested that SRE could protect the liver from acute alcohol-induced hepatic damage.

■ DISCUSSION

Recent reports indicate that saponins from *Platycodon grandiflorum* and from *Panax japonicus* show some protection against ethanol-induced hepatotoxicity in mice.[23,26] Thus, it is reasonable to hypothesize that soyasaponins might demonstrate a similar effect. Soy isoflavones, for example, daidzein (100 mg/kg), was also reported to possess some hepatoprotective activity.[27] Because the isoflavone content in SRE is so low, only 9.73 mg/g, these are not likely the components responsible for the observed response. Other substances with hepatoprotective activity in soybean such as soy protein, soy pinitol, and phosphatidyl choline should not be contained in SRE because SRE was prepared from following procedure. Briefly, the concentrated

extracts from soybean were loaded onto an AB-8 resin, and then, the resin was washed with distilled water. The above substances are of strong polarity, so they must be washed away with water. Taken together, the hepatoprotective activity of SRE should be attributed to the soyasaponins.

Alcohol is mainly metabolized in the liver. Excessive alcohol consumption leads to massive generation of ROS and creates a rapid increase of oxidative stress, which may cause alcoholic fatty liver, alcoholic hepatitis, fibrosis, or cirrhosis.[28] Alcohol-induced hepatic damage is characterized by release of hepatic marker enzymes such as AST, ALT, ALP, and LDH into the circulatory system. The elevation of these enzymes in serum indicates cellular leakage and loss of functional integrity of cell membranes in the liver.[29] Administration of soy saponins (SRE) (1200 mg/kg) significantly decreased the serum levels of these enzymes, which may be a consequence of the stabilization of plasma menbrane as well as repair of hepatic tissue damage caused by alcohol.[9] The results of serum enzymes confirmed the protective effects of SRE on hepatic damage caused by acute alcohol ingestion.

The excessive ROS initiates the lipid peroxidation chain reaction, which produce lipid peroxyl radicals.[3] MDA is a major reactive aldehyde that appears during the final stages of lipid peroxidation of biological membrane polyunsaturated fatty acid.[30] Hepatic MDA activity is commonly used as an indicator of liver tissue damage involving a series of chain reactions.[31] The increase of hepatic MDA concentration suggests enhanced peroxidation leading to tissue damage and failure of the antioxidant defense mechanisms to prevent the formation of excessive free radicals.[32] In our study, acute alcohol administration caused an increase of hepatic MDA contents as compared to the normal group. Administration of SRE decreased the elevation of MDA level, especially at the dose of 1200 mg/kg. These results indicated that the free radicals being released in the liver were effectively scavenged by SRE.

GSH is a nonenzymatic antioxidant found in liver cells that can decrease hydroperoxides and xenobiotic toxicity.[33] Numerous studies have confirmed that acute ethanol exposure can cause a decrease of hepatic GSH.[34] GSH is oxidized to glutathione disulfide by any of the selenium-containing GPx isozymes. The consumption of GSH is due to the overproduction of ROS and subsequent oxidative stress caused by alcohol.[35] The decrease of hepatic GSH leads to the inactivation of methionine adenosyltransferase and the reduction of *S*-adenosylmethionine level, which in turn aggravates the depleted level of intracellular GSH.[36] Our study showed that SRE administration significantly inhibited the alcohol-induced consumption of hepatic GSH.

It is well-known that antioxidant enzymes such as SOD, GST, and GPx provide protection against oxidative stress. SOD is involved in the antioxidant defense mechanism by converting superoxide anions to H_2O_2.[37] GST plays a critical role in cellular protection against ROS. Ethanol or its metabolic products might specifically target GST, and the reduction in enzyme activity may contribute to ethanol hepatoxicity.[38] GPx catalyzes the reduction of hydroperoxides with reduced glutathion to form glutathion disulfide.[39] These antioxidant enzymes are easily inactivated by excessive lipid peroxides or other ROS resulting from acute alcohol-induced liver damage. In the present study, the activities of SOD, GST, and GPx were significantly reduced in ethanol treated group as compared with normal group. SRE supplementation preserved the activities of these enzymes. Administration of SRE at the dose of 600 and 1200 mg/kg could significantly

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normalize the activities as compared with ethanol group. SRE may enhance the innate mechanisms of the antioxidant system or provide its antioxidant capacity against alcohol-induced oxidative stress in mice liver.

The histological observations fundamentally supported the results obtained from serum and hepatic enzyme assays. The hepatic slice of alcohol-intoxicated mice showed fatty changes, necrosis, lobular inflammation, and hepatocellular swelling. Administration of SRE inhibited the alcohol-induced hepatotoxicity, as indicated by a more or less normalization of the liver. The group treated with SRE at a dose of 1200 mg/kg showed a nearly normal liver. These findings also suggested that SRE could protect the liver from hepatotoxicity by repairing and regenerating the hepatocytes.

In conclusion, the present data indicate that SRE supplementation could restrain the hepatic damage caused by acute alcohol exposure. Excessive alcohol consumption will result in oxidative stress via enhanced lipid peroxidation and ROS production. SRE administration antagonized the hepatic damage as evidenced by the activation of hepatic antioxidant system and reversal of lipid peroxidation. SRE not only protect the integrity of plasma membrane but also increase the reparative and regenerative capacity of the liver. Taken together, our present study suggests that SRE may be an effective therapeutic agent in alcohol-induced liver damage.

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