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Article

Akebia quinata Extract Combined with Korean Traditional Juice Activates Antioxidant Enzymes and Reduces Apoptosis and Inflammation

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Abstract: In this study, we investigated the effects of *Akebia quinata* ethanol extract (AE), *Akebia quinata* sikhye (AS), and *Akebia quinata* water extract (AW) on alcohol-induced liver injury in rats. The hepatoprotective, anti-inflammatory, anti-apoptotic, and antioxidant effects of AE, AS, and AW were examined. Experimental animals were randomly divided into five groups: normal, ethanol, AE (10 mL/kg), AS (10 mL/kg), and AW (10 mL/kg). Each group was administered the respective treatment orally once per day for 21 days. CYP2E1 mRNA expression was significantly lower in the AE, AS, and AW groups than that in the ethanol group. Pro-inflammatory cytokines including cyclooxygenase-2, IL-6, and TNF-α increased significantly in the ethanol group but these increases were ameliorated with AE, AS, and AW treatment. Moreover, the expression of the apoptosis-associated proteins Bax, p53, procaspase-3, and PARP decreased after treatment with AE, AS, and AW. The expression of antioxidant enzymes including BCL-2, SOD, and GST slightly decreased in the ethanol group, but AE, AS, and AW treatment augmented their activities. AQ extracts and AS attenuated ethanol-induced increases in the levels of phosphorylated *p*-AKT, *p*-ERK, and *p*-JNK. These results demonstrate that AQ is a competence indicator for inhibiting alcoholic liver injury.

Keywords: *Akebia quinata* extracts; alcohol; liver disease; CYP2E1; COX-2

1. Introduction

Alcohol consumption is customary in most cultures but, unfortunately, can often lead to alcohol abuse and alcoholic liver disease. Worldwide, people aged 15 years or older consumed 6.1 litres of alcohol on average in 2013 (WHO, 2014). Excessive alcohol consumption can cause many diseases, including cirrhosis of the liver, unintentional injuries, and cancer [1]. The progression of alcoholic liver disease (ALD) features three histological stages, starting with simple steatosis, which progresses to inflammation and necrosis, and finally to fibrosis and cirrhosis. While the early stages of ALD are usually reversible with abstinence, chronic alcoholic hepatitis, which causes the later stages, is difficult to be controlled due to inflammation-induced hepatic damage, oxidative damage, and apoptosis of hepatocytes [2]. The ingestion of alcohol induces the accumulation of reactive oxygen species (ROS) that cause oxidation and lipid peroxidation of cellular components, resulting in injury to hepatocytes [3]. This oxidative stress, mediated by CYP2E1, appears to play a major role in ethanol-induced liver injury. One specific ethanol-inducible form of cytochrome P450 (CYP) catalyses ethanol oxidation more rapidly rates than other CYP enzymes. Under physiological conditions, only a small amount of ethanol is oxidized to acetaldehyde by CYP2E1, but chronic alcohol abuse leads to induction of the microsomal system, and a

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consequent increase in CYP2E1 expression and oxidative damage [4]. In addition, pro-inflammatory cytokines that trigger inflammatory responses and apoptosis have been observed in the livers of animal models of ALD as well as in chronic alcoholics. Targeting enhanced levels of inflammatory cytokines, including IL-1β and TNF-α, has been investigated as a potential therapeutic approach to modifying ALD progression [5]. Generally, ALD-related chemical drugs have serious side effects and cannot be tolerated by everyone. Therefore, it is necessary to investigate non-pharmaceutical substance and natural food components including antioxidant enzymes for use in ALD therapy.

Akebia quinata (AQ), commonly referred to as chocolate vine, grows naturally and is widely distributed throughout East Asia, including in China, Japan, and Korea [6]. AQ is consumed as the entire fruit, including the pulp and seed. The dry ripe fruit of AQ is used as an analgesic, antiphlogistic, and antidiuretic in traditional Chinese medicine [7]. Although previous in vivo and in vitro studies have shown anti-tumour effects of the water extract of the whole fruit of *Akebia trifoliata*, to date, the molecular mechanism underlying the anti-tumour effects of the ethanolic extract of AQ seed or the individual ingredients remains to be clarified [8]. It has been reported that many saponins such as the glycosides of oleanolic acid and hederagenin have been isolated from the stem of AQ. In addition akequintoside D, roseoside II, 3-o-caffeoylquinic acid, methyl-3-o-caffeoylquinate, 3,4,5-trimethoxyphenyl-bD-glucopyranoside, cuneataside D, 3,4-dimethoxyphenyl-6-O-(a-L-rhamnopyranosyl)-b-D-glucopyranoside have all been isolated from the stem of AQ [1]. It has been reported that an anti-inflammatory agent derived from a saponin of oleanolic acid and hederagenin was taken from a stem bark of AQ [9]. The AQ fruit possesses antioxidant activity and while the water extract has free radical scavenging and antibacterial activity [10]. The saponins obtained from AQ induced cytotoxicity and apoptosis of HepG2 liver cancer cells [11]. Triterpene glycosides, from the stems of AQ, have also been reported to have antioxidant and protective effects and induced DNA damage in human lymphocytes [12]. In a previous study, we reported on the antioxidant, antimicrobial, and anti-tumour activities of the fruit and leaf of AQ. We reported that hederagenin alleviates the pro-inflammatory and apoptosis response by ethanol in both in vitro and in vivo models. However, the molecular mechanism of the effect of AQ extracts on alcoholic liver disease remains widely unclear.

Therefore, we examined whether AQ extracts and AQ sikhye, a traditional fermented juice, have antioxidant, anti-inflammatory, or anti-apoptosis effects against alcohol-induced liver damage in rat. The mechanism we investigated for the mediation of these effects was the phosphorylated *p*-AKT, *p*-ERK, and *p*-JNK signalling pathway. The present study objected to investigate the possible improvement and therapeutic effects of AQ ethanol extract (AE), AQ sikhye (AS) and AQ water extract (AW) on alcohol-induced liver disease in rat and clinical study.

2. Materials and methods

2.1 Preparation of AQ sikhye and a beverage containing AQ water extract

The fruit of AQ was obtained from the Jiri mountain. AE and AW were prepared as follows: The air-dried and milled fruits of AQ (1 kg) were subjected to extraction with methanol (40 L) for 24 h at 27℃. The filtered extract was concentrated under a vacuum concentrator (Ecopspin 314, PS1E1AF01, Biotron Inc., Korea). A sample having a concentration of 50 mg/kg was then dissolved in 1 mL of ethanol to make the AE. After the ethanol extraction of AQ, 100 g of residue was dried and dissolved in distilled water for 24 h at 25℃ in a shaking incubator to prepare the AW. AS was prepared by mixing and fermenting steamed rice (250 g), malt (125 g), and water (1 L) at 37°C for 12 h. After cooling, 1% AQ and 4L sterilized water were added to the mixture and heated at 100°C for 1 h. The samples were stored at 4°C until needed.

2.2 Animal experiments

All experiments were performed with approval from the Institutional Animal Care and Use Committee at Konkuk University (IACUC approval number KU 15057), Seoul, Republic of Korea. Male

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Wistar rats weighing 200 g and aged 5–6 weeks (Orient bio. Korea) were used in this study. The animals were housed in a temperature-controlled (21–22°C) and light-controlled (12 h light, 12 h dark cycle) environment with 70% humidity and were given free access to water and food. The experimental animals were randomly divided into five groups: (1) a normal (sham) group (n=8), (2) a group fed 25% alcohol (n=8), (3) a group fed 25% ethanol + AE (50 mg/kg) (n=8), (4) a group fed 25% ethanol + AS (n=8), and (5) a group fed 25% ethanol + AW (1 g/L) (n=8). During 21 days, the rats in the ethanol-treated group were orally administered 1 mL of 25% ethanol each day and were given free access to water. Rats in the AE group received 1 mL of 25% ethanol orally and 1 mL of AE by oral injection. Rats in the AS and AW groups received of 1 mL of 25% ethanol orally, and then 100 mL AS or AW as a drink. The amount of water consumed was measured daily, and weight gain was measured weekly. After 21 days, the rats were killed by decapitation following a 16-h fast. Blood samples were collected from the heart. Plasma was separated from the collected blood centrifugation for 30 min at 1610 g. The liver, kidney, and spleen were excised and the organs were weighed, and then were stored -70°C in the deep freezer.

2.3 Biochemical assays

Hepatotoxicity was assessed by quantifying the activities of serum aspartate alanine aminotransferase (ALT) and alanine aminotransferase (AST) using the methods described by Reitman and Frankel [13]. The levels of total cholesterol (TC) in the serum were detected using commercial kits (Sigma, St. Louis, MO) by modifying the cholesterol oxidase method described by Alain et al [14]. The serum triglyceride (TG) concentrations were measured enzymatically using a kit from Sigma, using a modified lipase glycerol phosphate oxidase method [15].

2.4 Histological Analysis

Liver and kidney specimens from the rats were fixed overnight in 10% formaldehyde, embedded in paraffin, and cut into 5-μm-thick sections. The slices were stained with haematoxylin and eosin (HE) for routine histopathological examination, and were examined and imaged using a light microscope at × 100 magnification to determine the degree of hepatic steatosis.

2.5 Reverse Transcription-polymerase chain reaction (RT-PCR)

Total RNA was separated from the liver tissue by using Trizol solution (Trizol, Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using a first strand cDNA synthesis kit (18080-051, Invitrogen, Carlsbad, CA, USA). PCR was performed using the KAPA Taq Extra PCR kit (KR0355, Kapa Biosystems, Wilmington, DE, USA). Primer sequences were as follows: GAPDH: 5′-ATCCCATCACCATCTTCCAG-3′, 5′-CCTGCTTCACCACCTTCTTG-3′, ALDH2: 5′-GCTGTCAGCAAGAAAACATTCCCC-3′, 5′-CTTGTCAGCCCAGCCAGCATAATA-3′, ADH: 5′-ACCATCGAGGACATAGAA-3′, 5′-GTGGAGCCTGGGGTCAC-3′, TNF-α: 5′-GTAGCCCACGTCGTAGCAAA-3′, 5′-CCCTTCTCCAGCTGGAAGAC-3′, COX-2: 5′-CTGCATGTGGCTGATGTCATC-3′, 5′-AGGACCCGTCATCTCCAGGGTAATC-3′, IL-6: 5′-CAAGAGACTTCCAGCCAGTTC-3′, 5′-GAAACGGAACTCCAGAAGACC-3′. PCR was initiated at 95 °C for 3 min followed by 30 cycles at 95 °C for 30 s and 50–60 °C for 30 s. The number of cycles and annealing temperature for each primer pair were optimized. A final extension of 72 °C for 10 min was included. The amplified PCR products were subjected to electrophoresis at 100 V through 1.2% agarose gels for 40 min. A 100 bp DNA ladder was used as a molecular marker. The bands were visualized with ethidium bromide and analysed using BandScan (C-digit, Licor, Lincoln, Nebraska, USA).

2.6 Western blotting analysis

The rat livers were lysed in RIPA lysis buffer containing protease inhibitor (Roche, Mannheim, Germany) and centrifuged at 10 000 g for 30 min at 4°C. The total protein levels were determined using a Bio-Rad protein kit (Bio-Rad, Richmond, CA, USA). The proteins were seperated to 10–15% sodium

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dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto Immobilon-P transfer membranes (Millipore Co., Bedford, MA, USA), which were blocked with 5% bovine serum albumin. After blocking with 5% bovine serum albumin (Sigma-Aldrich), membranes were probed with primary antibodies as follows: β-actin (Cell Signaling Technology, Beverly, MA, USA), Bcl-2 (Abcam, Cambridge, UK), glutathione S-transferase (GST) (Cell Signaling Technology), SOD-1 (Santa Cruz Biotechnology, Dallas, Texas, USA), Bax (Cell Signaling Technology), pro-caspase-3 (Abcam), Cytochrome C (Cell Signaling Technology), p53 (Abcam,), PARP-1 (Abcam), *p*-AKT (Cell Signaling Technology), *p*-ERK (Cell Signaling Technology), and *p*-JNK (Cell Signaling Technology) by overnight incubation at 4 °C and then memebranes were incubated with a secondary antibody as follows : goat anti-rabbit IgG (H+L) horseradish peroxidase (HRP)-conjugated secondary antibody (Zymax, San Francisco, CA, USA). Immunodetection was performed using a chemiluminescence method (Thermofisher, Waltham, MA, USA) and relative densities of bands were quantified using an NIH image J program (http://rsb.info.nih.gov/ij/). All data were normalized to the actin content of the same sample.

2.7 Measurement of alcohol concentration in human breath

All study protocols were in accordance with the ethical and legal guidelines program at Konkuk University. Ten men and 10 women participated in the measurement of alcohol concentration in breath after the consumption of alcohol. All studies used an AS, controlled two-group (alcohol vs. AS) within subjects design in which participants drank water + alcohol on one night and AS + alcohol on the other night (counter balanced order) with order randomization stratified by gender. The subjects (n=20) consumed water or AS (100 mL) 30 min before consuming 18.7% ethanol (380 mL). AS was administered in an amount equivalent to 1% AQ or an equivalent amount of water. The alcoholic beverages used for Study 1 (n = 20) were high level of alcohol (18.7%) and water and those for Study 2 were high level of alcohol (18.7%) and AS (n = 20). After consumption of the alcoholic beverages, the levels of ethanol in the breath of humans were measured using breath analysers (AL6000LITE; Sentech, Korea).

2.8 Sensory testing of a hangover in humans

Ten women and 10 men participated in the sensory testing of a hangover after the consumption of alcohol. Sweat, drunkenness, and dizziness, were examined and then scored on a 7-point scale [16].

2.9 Statistical analysis

The statistical program SPSS version 18.0 was used to calculate the means and standard deviations for each test interval, and the significance of each zone was verified using the Duncan test method at a significance level of 5% ($p<0.05$).

3. Results

3.1 Effects of AQ on body weight gain and organ weight

The average liver weights of the AS and AW groups were significantly different than that of the normal group. However, the result of ratio liver/weight gain also showed no significant change (Table 1). The kidney weights were significantly different between the groups (Table 1). In addition, the spleen weight was slightly lower in the ethanol group that that in the normal group (Table 1).

3.2 Effects of AQ on biochemical markers of liver injury

In order to investigate the protective effects of AE, AS, and AW treatment on liver injury, we measured the expressions of AST, ALT, TC, and TG in the serum (Figure 1). AST levels in the ethanol-treated group were significantly greater than in the normal group. Assuming that the AST level of the ethanol-treated group was 100%, AST of the AE, AS and AW groups were 84.6%, 63%, and 74.5%, respectively ($p < 0.05$) (Figure 1A). The AST of the ethanol-treated group increased 52.1% compared to the normal group. These results suggest that AQ has a protective effect against alcoholic liver damage.

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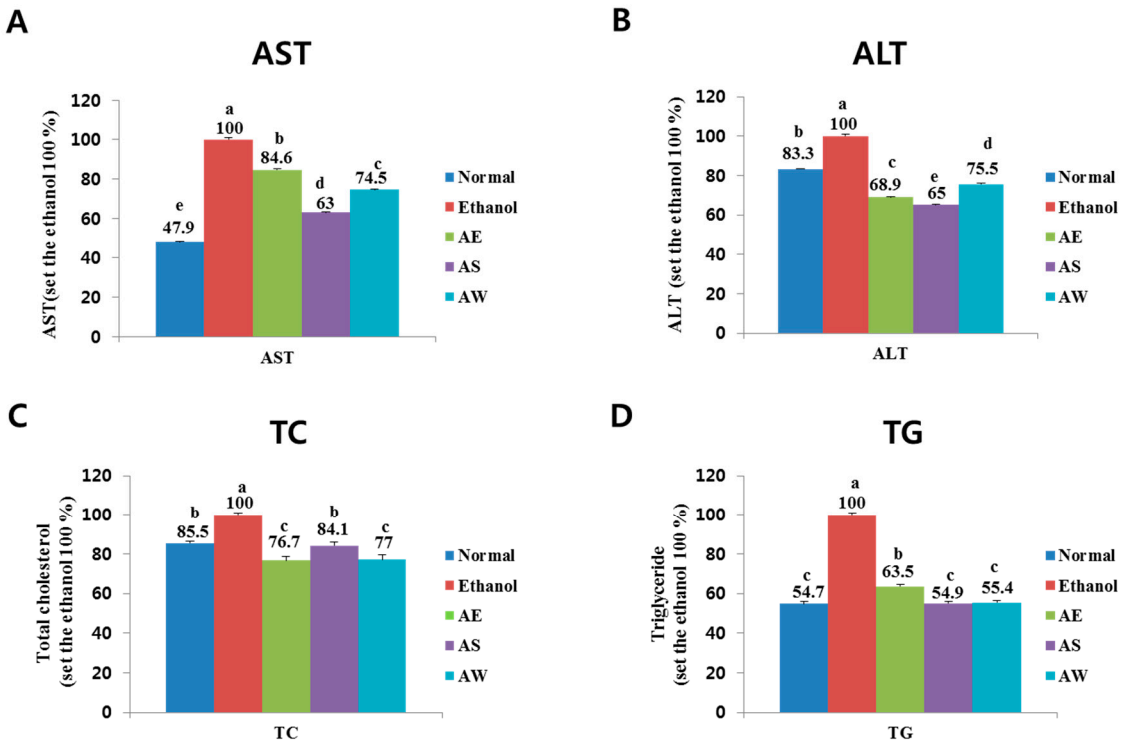

Figure 1. Effects of *Akebia quinata* extract and juice on serum AST, ALT, TC, and TG levels in ethanol-treated rats. The values of AST (A), ALT (B), TC (C) and TG (D) levels when ethanol group was set to 100%. Results are expressed as the mean ± standard deviation (SD). Values not sharing a common superscript (a, b, c, d and e) differed significantly (Duncan's multiple range test) ($p < 0.05$). AST, aspartate aminotransferase; ALT, alanine aminotransferase; TC, total cholesterol; TG, triglyceride; AE, *Akebia quinata* ethanol extraction group; AS, *Akebia quinata* sikhye group; AW, *Akebia quinata* water extraction group.

Assuming that ALT levels in the ethanol-treated group were 100%, ALT levels in the AE, AS and AW groups decreased 31.1%, 35%, and 24.5%, respectively (Figure 1B).

The TC levels in the ethanol-treated group were slightly higher than in the normal group. Assuming that TC levels in the ethanol-treated group were 100%, The TC levels in the AE, AS, and AW groups decreased 23.3%, 15.9%, and 23%, respectively (Figure 1C).

Finally, TG levels increased 45.3 in the ethanol treated group than in the normal group ($p<0.05$) (Figure 1D). Assuming that the TG levels in the ethanol-treated group were 100%, the TG levels in the AE, AS, and AW groups were 63.5%, 54.9%, and 55.4%, respectively, ($p < 0.05$) (Figure 1D). Therefore, AE, AS, and AW were beneficial in lowering blood TG levels. Our results show that AQ decreased the risk of ethanol-induced hepatotoxicity.

3.3 Histopathological analysis of liver and kidney tissues

Histological examination after H&E staining observed normal liver lobular architecture in the normal rats. However, the livers from ethanol-treated rats showed micro- and macro-vesicular steatosis and accumulative inflammatory cell infiltration. Those pathological changes were alleviated by AE, AS, and AW treatment (Figure 2A). The livers of AE, AS, AW treated rats showed a similar pattern to the normal group. These histopathological images showed that the AS group recovered more rapidly than the others groups.

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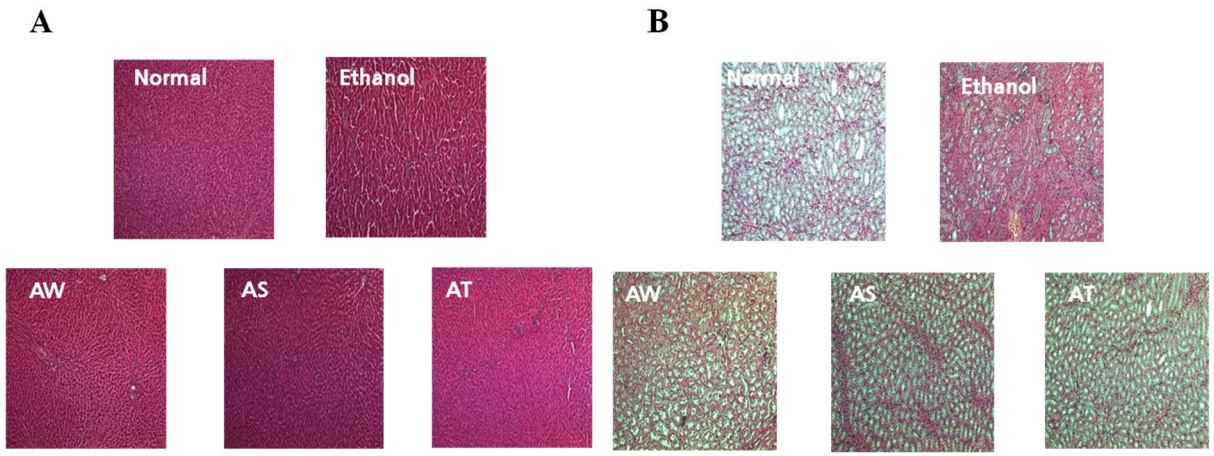

Figure 2. Representative photomicrographs (magnification ×100) of haematoxylin and eosin-stained sections of the liver (A) and kidney (B). AE, *Akebia quinata* ethanol extraction group; AS, *Akebia quinata* sikhye group; AW, *Akebia quinata* water extraction group.

Hepatological image of the kidney showed no significant difference between the normal, AE, AS, and AW groups in the glomerular and renal corpuscle tissues. Ethanol induced kidney damage including congestion, haematuria, and the infiltration of inflammatory cells adjacent to the glomerulus. The kidney lesions improved in the AE, AS, and AW groups compared to that in the ethanol-treated group. The results of the ALT, AST, TG, and TC serum levels and the histopathological findings may provide strong evidence of the hepatocyte protective effect of AE, AS, and AW.

3.4 Effects of AQ on hepatic ADH, ALDH2, and CYP2E1 mRNA expression

We investigated changes in the mRNA expression of ADH, ALDH2, and CYP2E1 using RT-PCR (Figure 3). Hepatic ADH mRNA expression levels were higher in the ethanol-treated group than in the normal group. However, ADH mRNA expression was considerably lower in the AE, AS, and AW groups than in the ethanol-treated group. The level of ALDH2 mRNA expression was slightly lower in the ethanol group than that in the control group; however, this effect was partially attenuated in the AE, AS, and AW groups, where the ALDH2 mRNA expression was higher than that in the ethanol-treated group. Thus, treatment with AQ increased the ethanol-induced inhibition of ALDH2 mRNA expression.

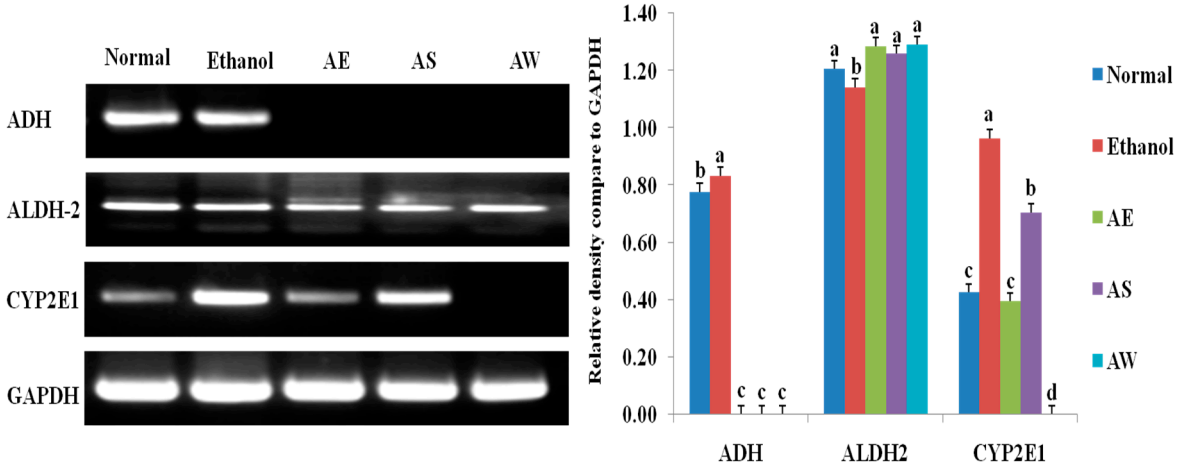

Figure 3. Effects of AQ extract and *Akebia quinata* sikhye on the mRNA expression of ADH, ALDH2, and CYP2E1 analysed using reverse transcription-polymerase chain reaction (RT-PCR). Results are expressed as the means ± standard deviation. Significant differences ($p < 0.05$) are represented using different letters (a, b, c and d). AQ, *Akebia quinata;* ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase;

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CYP2E1, Cytochrome P450 2E1 AE; *Akebia quinata* ethanol extraction group; AS, *Akebia quinata* sikhye group; AW, *Akebia quinata* water extraction group.

Ethanol-induced oxidative stress shows to play an important role in ethanol-induced liver injury. Therefore, we examined whether AQ extracts and AS could have a preventive effect against ethanol-induced liver injury by regulating the CYP2E1 enzyme in an in vivo model. CYP2D1 mRNA expression increased 2.3-fold in the ethanol-treated group over the normal group. However, the AW treated group showed significantly decreased levels over the other groups. In total, the levels of CYP2E1 mRNA expression in the AE, AS, and AW treatment groups were reduced 2.5-fold, 1.4-fold, and 10-fold compared to the ethanol-treated group.

3.5 AQ inhibits the inflammatory response induced by ethanol.

The level of TNF-α mRNA expression in the ethanol-treated group was significantly higher (10-fold) than in the normal group ($p<0.05$); however, TNF-α mRNA expressions in the AE, AS, and AW groups were dramatically reduced (10-fold) than in the ethanol-treated group (Figure 4). Similarly, IL-6 mRNA expression levels significantly increased in the ethanol-treated group ($p<0.05$), but were reduced in the AE, AS, and AW groups (Figure 4).

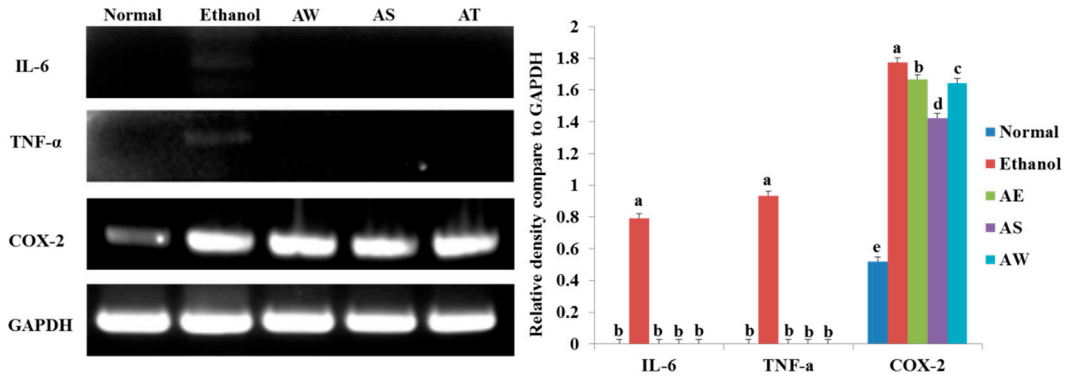

Figure 4. Effects of AQ extracts and AS on inflammation-related gene expression in the livers of ethanol-exposed rats and on mRNA expression of COX-2, TNF-α and IL-6 expression in the liver, assessed using reverse transcription polymerase chain reaction (RT-PCR). Results are expressed as the mean ± standard deviation. Significant differences ($p < 0.05$) are represented using different letters (a, b, c, d and e). TNF, tumour necrosis factor; IL, interleukin; COX, cyclooxygenase; AQ, *Akebia quinata;* AE, *Akebia quinata* ethanol extraction group; AS, *Akebia quinata* sikhye group; AW, *Akebia quinata* water extraction group.

COX-2 mRNA expression was detectable in normal liver tissue and significant upregulation of COX-2 gene expression ($p < 0.05$) was observed in the ethanol-treated group. In addition, COX-2 mRNA expressions in the AE, AS, and AW groups were slightly lower than that in the ethanol-treated group. Thus, the AQ extracts and AS suppressed ethanol-induced increases in COX-2, TNF-α, and IL-6 mRNA expression.

3.6 Effects of AQ extracts and AS on apoptosis

Apoptosis plays a vital role in liver injury, such that understanding the molecular mechanism and regulating factors of how hepatocyte apoptosis proceeds is crucial for the treatment of liver diseases. Therefore, we examined the impact of AQ on apoptosis-associated protein expression in ethanol-induced liver injury via western blot analysis. As shown in Figure 5, p53 expression increased in the ethanol-treated group and reduced 1.33-fold in AE group, 1.37-fold in AS group, and 1.72-fold in AW group, respectively.

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As shown in Figure 5, expression of the pro-apoptotic protein Bax significantly increased in the ethanol-treated group. In contrast, the expression of Bax in the AS and AW groups reduced 2.11-fold and 1.63-fold, respectively. The expression level of the anti-apoptotic protein Bcl-2 attenuated in the ethanol-induced liver. Further, the Bax/Bcl-2 ratio was increased in the ethanol group. In contrast, treatment with AS and AW reversed the expression levels of Bcl-2 and Bax and decreased the Bax/Bcl-2 ratio.

The expression of cytochrome C showed a slight reduced in the ethanol-treated group. However, in the AE group, cytochrome C expression dramatically decreased by 2.71-fold (Figure. 5). In addition, cytochrome C levels in the AS and AW groups were enhanced 1.86-, 1.84-fold compared to those in the ethanol-treated group, respectively

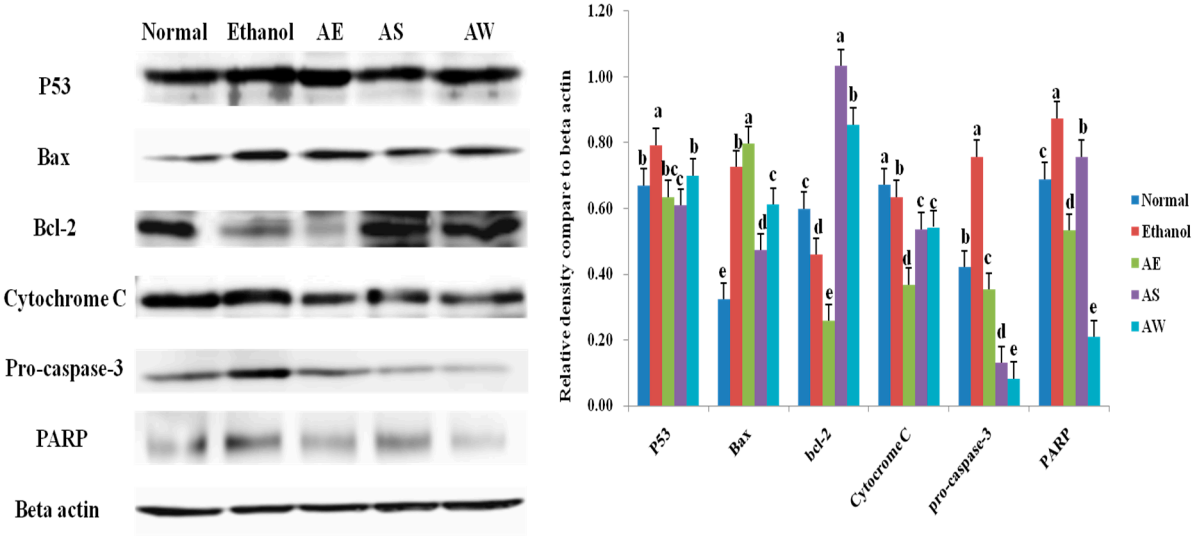

Figure 5. Effects of AQ extracts and AS on apoptotic signalling cascades in ethanol-treated hepatocytes. Extracts of liver proteins from the different groups were subjected to western blotting. Expression levels of p53, Bax, cytoplasmic Bcl-2, cytochrome C cytosol, pro-caspase-3, and PARP are shown. Results are expressed as the mean ± standard deviation. Significant differences ($p < 0.05$) are represented using different letters (a, b, c, d, and e). AQ, *Akebia quinata*; AE, *Akebia quinata* ethanol extraction group; AS, *Akebia quinata* sikhye group; AW, *Akebia quinata* water extraction group.

The protein expressions of pro-caspase-3 and PARP-1 in the AE, AS, and AE groups were higher than in the normal group. As shown in Figure 5, the expressions of pro-caspase-3 and PARP-1 dramatically increased in the AE, AS, and AW groups compared to that in the ethanol-treated group. This may suggest that AQ extracts and AS ameliorates apoptosis in alcoholic liver damage.

3.7 Treatment with AQ extracts and AS restores the expression of anti-oxidative proteins including SOD and GST.

An important protective strategy against oxidative damage is the induction of antioxidant enzymes. Therefore, the activities of antioxidant enzymes, including SOD and GST, were determined to evaluate the protective effect of AE, AS, and AW treatment. The expression of SOD decreased in the ethanol-treated group compared with that in the normal group, whereas it was restored to normal levels with AE, AS, and AW treatment (Figure 6).

The protein expression of GST slightly decreased in the ethanol-treated group (Figure 6). Similarly, the upregulation of activated GST was observed in ethanol-treated rats, an effect that was increased by the addition of AS. These results suggested that, in vivo, AE, AS, and AW treatment promoted antioxidant activity.

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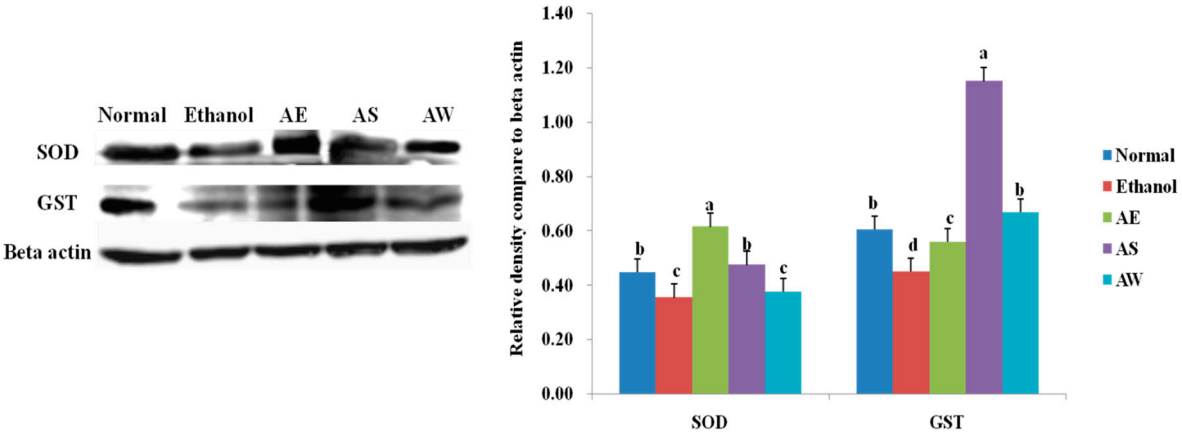

Figure 6. Effects of AQ extracts and AS on antioxidant-related protein expression in the livers of ethanol-exposed rats and expression levels of liver SOD and GST, assessed via western blotting. Results are expressed as the mean ± standard deviation. Significant differences ($p < 0.05$) are represented using different letters (a, b, c, and d). SOD, Superoxide dismutase; GST, glutathione S-transferases; AQ, *Akebia quinata*; AE, *Akebia quinata* ethanol extraction group; AS, *Akebia quinata* sikhye group; AW, *Akebia quinata* water extraction group.

3.8 Effects of AQ extract and AS on phosphorylation of AKT, ERK, and JNK

Several studies have indicated that the AKT, ERK, and JNK pathway is important in the regulation of cell survival and proliferation. Therefore, we examined the effects of AQ on the activation of the phospho-AKT (*p*-AKT), phospho-ERK (*p*-ERK), and phospho-JNK (*p*-JNK) pathways in ethanol-induced liver injury. As shown in Figure 7, the downregulation of *p*-AKT, *p*-ERK, and *p*-JNK was observed in the ethanol-treated group, while their levels increased with the addition of AE. Thus, these results suggested that AQ extracts and AS supplement activated the *p*-AKT, *p*-ERK, and *p*-JNK pathway.

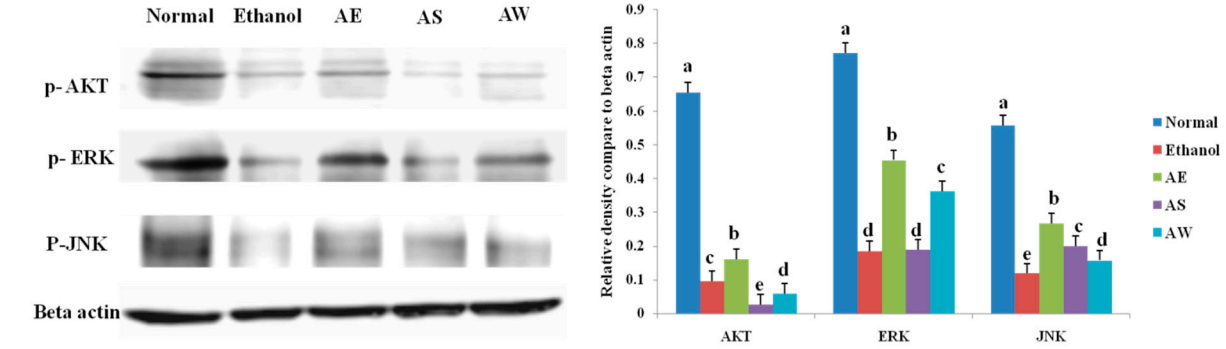

Figure 7. Effects of AQ extracts and AS on the expression levels of *p*-AKT, *p*-ERK, and *p*-JNK in the liver of ethanol-exposed rats. Results are expressed as the mean ± standard deviation. Significant differences ($p < 0.05$) are represented using different letters (a, b, c, d, and e). AQ, *Akebia quinata*; AE, *Akebia quinata* ethanol extraction group; AS, *Akebia quinata* sikhye group; AW, *Akebia quinata* water extraction group.

3.9 Levels of ethanol in the breath

AQ extracts and beverage have been used as protective agents for alcohol-induced liver injury in animals. However, the effects of AS have not been investigated in humans thus far. Therefore, we performed a randomized single-blind crossover trial with 20 healthy young men and women to examine effects of AS on alcohol hangovers. The characteristics of the subjects who participated in the trial were as follows: average age, 20 years; profession, Konkuk University students; average height, 169.2 ± 10.2 cm and average weight, 63.5± 11.3 kg. We measured the levels of ethanol in the breath using a breath analyser (Table 2). The level of alcohol in the control group, those who consumed water before drinking

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alcohol, showed the highest alcohol levels at 0 min, and these levels decreased with time. However, the alcohol concentration in the group that consumed AS before drinking alcohol was lower than that in the control groups. At 30 min, the alcohol level in the AS-treated groups (0.11) was significantly lower than that in the normal group (0.17). The alcohol levels in the breath of humans showed similar patterns to those observed in a model of ethanol-treated wistar rats. Thus, our results show that AS has protective effects against alcohol-induced liver damage. (Table 3) In particular, the levels of alcohol in the women in the AS group were significantly lower than those in the men (Figure 3). Our results show that AS may alleviate alcohol-induced hangovers in humans.

3.10 Hangover sensory test

The results of the survey on seven characteristic traits of hangovers are shown in Figure 8. Headache, fever, and nausea were marginally alleviated by treatment with AS. The nausea and headach were observed significant difference in AS group than control group. But, drunkenness, thirst, and dizziness showed no difference between the control and AS groups. Practically, this study has several limitations, including a small sample size and the and some results that were barely significant. Thus, our results showed that consumption of AS after drinking reduced the symptoms of hangover. To the best of our knowledge, this is the first study to examine the hepatic protective effect of AS in the hangover on humans. Therefore, we believe that this study can be useful as an exploratory study show the need for larger human studies to elucidate the effects of AQ on alcohol hangovers. Our results suggest that AS alleviates alcohol hangover and that AQ fruit may have protective effects in alcohol-induced liver injury.

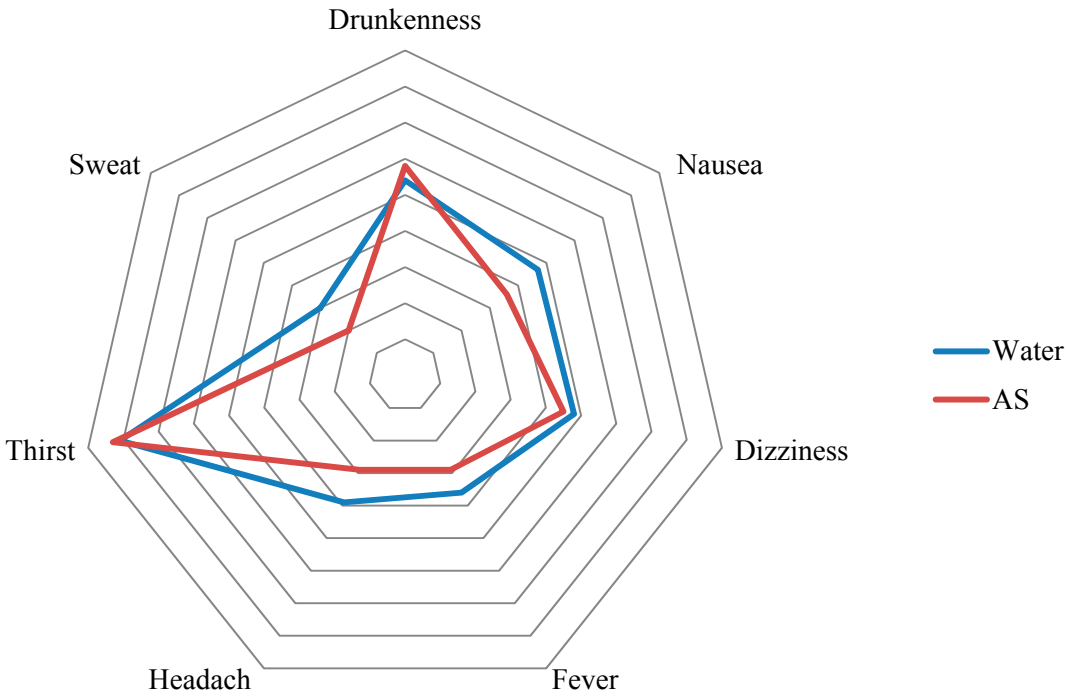

Figure 8. Spider-web diagram of sikhye prepared with AQ by sensory test. Except for drunkenness and thirst, AQ showed a slight improvement in all other variables. AQ, *Akebia quinata*; AS, *Akebia quinata* sikhye group.

4. Discussion

Our results showed that supplementation of AE, AS, or AW for three weeks suppressed the progression of alcoholic-induced oxidative stress injury in liver rats. AE, AS, or AW treatment reduced alcohol-induced liver injury and inflammation, as illuminated by attenuated serum AST, ALT levels, as

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well as decreased serum TC and TG levels. In addition, AE, AS, and AW decreased the inflammatory response to ethanol, as evidenced by significantly alleviated levels of hepatic COX-2, IL-6 and TNF-α mRNA expression. Furthermore, AE, AS, and AW supplementation inhibited the effects of alcohol on ADH, ALDH2, and CYP2E1 levels, displaying a protective mechanism against alcohol-induced liver injury. AE, AS, and AW also reduced apoptosis in the liver of rats exposed to alcohol, as shown by the suppressed ratio of Bax/Bcl-2, and decreased levels of p53, pro-caspase-3, and PARP. AE, AS, and AW supplementation increased antioxidant activity as demonstrated by the levels of SOD and GST expression. Finally, our study found that alcohol exposure reduced the expression of *p*-JNK, *p*-AKT and *p*-ERK. These results suggest that this is the first evidence that AE, AS and AW reduces liver injury through antioxidant, anti-inflammatory, and anti-apoptotic mechanisms in rats exposed to ethanol.

The ethanol metabolic pathway plays a significant role in the pathogenesis of ALD [17]. Up-regulation of ADH increases susceptibility to ethanol toxicity in hepatic cells while increased ALDH has a protective effect on alcohol-induced liver injury [18]. Moreover, CYP2E1-dependent microsomal alcohol oxidative stress system, was an important pathway of alcohol metabolism in the liver [19]. We found that AE, AS, and AW decrease ethanol-induced ADH expression, while ALDH2 expression increased with AE, AS, and AW treatment compared to the ethanol group. AE, AS, and AW supplementation also inhibited ethanol-induced hepatic CYP2E1 mRNA expression. Our results indicate that the protective effects exerted by AE, AS, and AW may be attributable to protection of the alcohol metabolic pathway via prevention of acetaldehyde accumulation.

Pro-inflammatory cytokines appear to be pivotal mediators of hepatic inflammation related with excessive ethanol intake**.** Especially, the release of inflammatory cytokines, such as COX-2, TNF-α and IL-6 can induced to hepatocyte apoptosis [18]. Decreases in COX-2, TNF-α and IL-6 levels have been used as important regulators of an inflammatory response to potentially toxic agents [18]. In our study, COX-2 levels in the livers of ethanol-treated rats were significantly increased. However, AE, AS, and AW treatment significantly ameliorated the ethanol-induced increased of liver COX-2 levels. The expressions of COX-2, IL-6, and and hepatic TNF-α mRNA were elevated after alcohol treatment. These increases were dramatically decreased by AQ extracts and AS.

Apoptosis has been associated in the pathogenesis of liver diseases. The activation of p53 in hepatocytes increased apoptosis and induced liver fibrosis. Bcl-2 also plays an important role in cell apoptosis in that the Bcl-2 family regulates apoptosis, with the Bax/Bcl-2 ratio serving as a rheostat to decide cell susceptibility to apoptosis [20]. In the present study, a sharp decline in Bax and p53 protein expression was observed in the ethanol-treated group. The protein expression of Bcl-2, however, was upregulated in the ethanol-treated group. Therefore, the ratio of Bax to Bcl-2 that had changed in the ethanol-treated tissue was eventually reversed in the presence of AQ extracts and AS that inhibited apoptosis. The generation of free radicals in rat livers after ethanol treatment and consequent oxidatively damaged biomolecules are responsible for apoptotic cell death [21]. PARP is one of the potential target molecules of effector caspases; PARP cleavage has been regarded as an evidence of caspase activation and has been widely used as an indicator of cell apoptosis [22]. In the present study, we found loss of full length PARP in liver cells exposed to ethanol. In our study, after treatment with AE, AS, and AW, the level of the native form of PARP was restored suggesting the anti-apoptotic activity of these compounds.

The pathogenesis of alcohol-induced liver disease accompanies the side effects of ethanol metabolites and oxidative tissue injury [23]. Alcohol exposure impairs enzymatic and non-enzymatic mechanisms that protect cells against reactive oxygen species, such as glutathione S-transferase (GST) and superoxide dismutase (SOD) [23]. Our results confirmed the involvement of oxidative stress in alcohol-induced liver injury, and the levels of enzymatic antioxidants, including SOD and GST, were restored by treatment with AE, AS, and AW.

AKT is major role in regulating cell signals that are pivotal for cell survival and death. Activation of the AKT pathway stimulates cell survival and is involved in the overexpression of Bcl-2 [24]. Our study demonstrated that the AS-treated group decreased AKT in the alcoholic liver. In cultured hippocampal neurons and endothelial cells, enhancement in activated ERK have been shown to orchestrated the inhibition of apoptosis, preventing the loss of activated ERK, which may be a mechanism by which AQ

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extracts inhibit ethanol-induced apoptosis [25]. We found that the ethanol-treated group inhibited the phosphorylation of ERK. However, AE reversed the change in the phosphorylation of ERK that had been induced by ethanol.

In conclusion, our study presents for the first time that AQ extracts and AS have various protective effects against alcoholic-induced oxidative stress injury. The protective activities of AE, AS, and AW against ethanol toxicity in the liver include the suppression of acetaldehyde through the activation of ALDH2. Further, our study shows that AE, AS, and AW can protect against alcohol-induced liver injury by reducing inflammatory regulators such as COX-2, TNF-α and IL-6. Moreover, AE, AS, and AW decreased mediators of apoptosis by inhibiting the p-AKT, ERK, and JNK signalling pathways. Our findings demonstrated that AQ extracts and AS are competence indicator for the prevention and treatment of alcohol-induced liver injury.

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Table. 1 Effects of *Akebia quinata* frutis on body weights and liver weights.

Treatment	Normal	Ethanol	AE[a]	AS[b]	AW[c]
Weight gain(g/day)	5.6 ± 1.81 [a]	6.7 ± 1.2 [a]	5.9 ± 0.34[a]	5.3 ± 0.35[a]	5.3 ± 0.48[a]
Beverage intake (mL/day)	-	-	-	138 ± 9.50	142.3 ± 10.30
Liver (g)	10.9 ± 1.43[a]	9.6 ± 0.92[b]	9.6 ± 1.21[b]	8.8 ± 0.64[c]	8.3 ± 1.24[c]
Kidney (g)	2.6 ± 0.10[a]	2.4 ± 0.17[a]	2.4 ± 0.29[a]	2.2 ± 0.08[b]	2.3 ± 0.25[b]
Spleen (g)	0.9 ± 0.09[a]	0.8 ± 0.15[b]	0.8 ± 0.12[b]	0.7 ± 0.09[b]	0.7 ± 0.17[b]
L.W/B.W	0.05 ± 0.03[a]	0.04 ± 0.03[a]	0.04 ± 0.09 [a]	0.04 ± 0.05[a]	0.03 ± 0.10[a]

[a] AE : *Akebia quinata* ethanol extraction group.

[b] AS : *Akebia quinata* sikhye group

[c] AW : *Akebia quinata* water extraction group

Different superscript in a row are significant differences ($p < 0.05$).

Table. 2 Effects of AS on the levels of ethanol in the breath of humans.

	0	30 min	60 min	90 min
Water	0.19 ± 0.06[a]	0.17 ± 0.04 [a]	0.14 ± 0.04 [a]	0.13 ± 0.04 [a]
AS[a]	0.12 ± 0.1 [b]	0.11 ± 0.06 [b]	0.12 ± 0.05 [a]	0.11 ± 0.05 [a]

Results are presented as mean ± standard deviation and different superscript in column are significant differences ($p < 0.05$). Water group decreased constantly. And there was good effect between 30 min to 60min. In AS group, the effects maintained constantly.

[a] AS : *Akebia quinata* sikhye group

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