

## Original Article

# Tartary buckwheat extract alleviates alcohol-induced acute and chronic liver injuries through the inhibition of oxidative stress and mitochondrial cell death pathway

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**Abstract:** Alcohol use disorder (AUD) is an enormous public health problem that poses significant social, medical, and economic burdens. Under AUD, the liver is one of the most adversely affected organs. As current therapies and protective drugs for AUD-mediated liver injury are very limited, the prevention and therapy of alcoholic liver disease are urgently needed. The present study aims to investigate the beneficial effects of tartary buckwheat extract (TBE), the important component of Maopu tartary buckwheat liquor, on both alcoholic-induced acute and chronic liver injuries. We show that the TBE administration, similar to curcumin, significantly reduces the elevated serum aspartate aminotransferase and alanine aminotransferase levels, improves liver index, alleviates the elevated contents of hepatic malondialdehyde, and restores the decreased contents of hepatic glutathione both in acute and chronic liver injuries in alcohol-exposed rats. Furthermore, histopathological analyses show that a medium dose of TBE (16.70 ml/kg body weight) alleviates hepatocyte morphology changes in both acute and chronic alcohol exposure models. We also show the protective effects of TBE on the cell death rates of alcohol-exposed primary cultured hepatocytes, HepG2 hepatoma, and Huh 7 hepatoma cells. Furthermore, we demonstrate that TBE exerts hepatoprotection partly through inhibiting the mitochondrial cell death pathway by reducing cytochrome c release, caspase-9 and -3 activities, and the number of TUNEL-positive cells. These effects of TBE were accompanied by enhanced levels of Bcl-2 and Bcl-xL and autophagic cell death pathway by reducing Beclin-1 expression, as well as through promoting its anti-oxidant capacity by suppressing reactive oxygen species production. This study demonstrates, for the first time, the protective effect of TBE against alcohol-induced acute and chronic liver injury *in vivo* and *in vitro*. Given the dietary nature of tartary buckwheat, pueraria, lycium barbarum, and hawthorn, the oral intake of TBE or liquor contained TBE, e.g., Maopu Tartary buckwheat liquor, compared with pure liquor consumption alone, may have the potential to alleviate alcoholic-induced liver injuries.

**Keywords:** Tartary buckwheat extract (TBE), alcoholic liver injuries, oxidative stress, mitochondrial cell death

## Introduction

Consumption of excessive amounts of alcohol has steadily increased and become a norm in both developed and developing countries [1]. In the United States, the prevalence of alcohol use disorder (AUD) as measured by general-population surveys has raised social and eco-

nomic concerns about increased alcohol-related morbidity and mortality over the past 10 to 15 years [2]. AUD is also a worldwide health problem and the liver is one of the most adversely affected organs. The liver is a vital organ that is extremely important in maintaining energy balance and homeostasis but vulnerable to various pathologies including alco-

hol, western-style high-fat high fructose diet, hepatitis viruses, toxic drugs/compounds, bile duct impairment, etc [3, 4]. Abusive alcohol consumption leads to serious acute and chronic health problems including hepatic steatosis, hepatitis, fibrosis, cirrhosis, and eventually, in some cases, to hepatocellular carcinoma and hepatic failure [5].

AUD is a multifactorial disorder that requires a multidisciplinary treatment approach, depending on the organs affected. Clinical research on the use of psychosocial and behavioral treatments for AUD in patients with liver disease is limited [6]. AUD currently is difficult to manage with present therapeutic treatments. Effective and safe AUD treatment, also called alcoholism treatment, which would improve treatment outcomes through working on the molecular mechanisms of alcohol-induced liver injury, is urgently needed.

Among the treatment strategies, the dietary supplements including Chinese herbal medicine have been widely applied. Herb-derived medicines play an important role in the management of various liver diseases. Indeed, many studies have demonstrated the effectiveness of herbal medicines for preventing or treating hepatic fibrosis, chronic hepatitis B and viral hepatitis [7].

Buckwheat is an herbaceous plant, which belongs to the polygonaceae family. Tartary buckwheat (*Fagopyrum tataricum*) and common buckwheat (*Fagopyrum esculentum*) are the two types of buckwheat that are used as both food and medicinal plants [8]. Tartary buckwheat grain is known as a dietary source of protein with favorable amino acid composition, fibers, vitamins (B1, B2, and B6), starch, and essential minerals [9, 10]. Tartary buckwheat seeds contain more flavonoids such as more rutin than common buckwheat seeds and traces of quercitrin and quercetin, which are not found in common buckwheat seeds [9]. Maopu tartary buckwheat liquor is made principally from high-quality fragrant, strong flavor Chinese and sauce flavor Chinese spirits, blending Chinese herbal medicine extracts from tartary buckwheat (plus pueraria, lycium barbarum, and hawthorn). It has been confirmed that the content of total flavonoids, including tartary buckwheat flavonoids in Maopu tartary buckwheat liquor is > 50 mg/L.

As a medicinal herb, ethanol extracts of leaf and flower of buckwheat are used as medicinal preparations [10]. The effects of dietary TBE on antioxidant activity and growth performance in ewe lambs and the obesity-induced inflammation in obese rats have been reported [11]. Repeated administrations of buckwheat flower and leaf extracts (at the dose of 10 ml/kg of body weight for 21 days) have restored the enzymatic activities of superoxide dismutase and catalase in the liver and brain of mice [10]. The effects of an ethanol extract of germinated buckwheat on suppressing fatty liver in C57BL/6 mice fed a western-style high-fat diet have been observed [12]. In addition, buckwheat has pharmaceutical activities including strong antioxidant activity [13, 14] and hypercholesterolemia, obesity, and gallstone formation effects [15]. Interestingly, the anti-hypertensive effects of tartary buckwheat flavonoids in hypertensive rats [16] and anti-hypoglycemic and anti-hypolipidemic effects of flavonoids from tartary buckwheat in type 2 diabetic mice have been observed [17]. Additionally, hepatoprotective and hypoglycemic effects of TBE in high fructose-fed mice have been demonstrated [18]. However, the effects of TBE/Maopu tartary buckwheat liquor on alleviating alcohol-induced liver damage in *in vivo* rat model and *in vitro* cellular model have not been systematically studied. Thus, this present study was designed to investigate the ameliorating effects of Maopu tartary buckwheat liquor (tartary buckwheat extract concentrate or tartary buckwheat alcohol extract) on alcohol-induced acute and chronic liver injury models of Sprague Dawley (SD) rats and look into the underlying mechanisms of action. Curcumin, a root of the plant *Curcuma Longa*, has been widely investigated in experimental animal models for its significant protective effects on acute and/or chronic alcoholic-induced [19], carbon tetrachloride-induced [20, 21], ischemia-reperfusion-induced [22] liver injuries, and brain damage [23, 24], as well as thioacetamide-induced liver cirrhosis [25]. Consequently, Curcumin has been used as a positive control in our study.

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are among the most sensitive biochemical diagnostic markers of liver function. Abnormal upregulation of these enzymes in the serum or plasma reflects

injury and necrotic damage of hepatic cells [26], and alcohol consumption is associated with increased serum levels of ALT and AST [27]. In fact, oral administrations of alcohol to rats could induce acute hepatic injury with elevated levels of serum ALT and AST [28]. Thus, we determined the serum levels of ALT and AST to assess the protective effects of TBE on ameliorating the alcohol-induced acute and chronic liver injuries along with hepatocyte histomorphology evaluation with H&E staining and liver index calculation. Oxidative stress is a key contributing factor in the pathogenesis of alcohol-induced liver injury. Malondialdehyde (MDA) is a product of lipid peroxidation and its level is often increased in an alcohol-damaged liver. Alcohol treatment is associated with increased levels of MDA, glutathione (GSH) and reactive oxygen species (ROS) [27]. Our current results clearly demonstrate that the protective effects of TBE against alcohol-induced acute and chronic liver injuries partly result from its antioxidant capacity and anti-apoptotic effect on mitochondrial-dependent apoptosis with elevated autophagic cell death pathway.

## Materials and methods

### Materials

Ethanol (200 proofs) and curcumin were purchased from Sigma (St Louis, MO, USA). Tartary buckwheat extract (TBE, Chinese herbal medicine ethanol concentrated extracts from tartary buckwheat plus pueraria, lycium barbarum and hawthorn) were provided by Jing Brand Research Institute (Daye, Hubei, China). Briefly, Maopu tartary buckwheat liquor was concentrated under reduced pressure at 60-70°C to an alcohol-free concentrated solution, and then the alcohol-free concentrated solution was justified with Maopu liquor and water to prepare the 10-fold Maopu Tartary Buckwheat Extract (TBE). The major contents or ingredients of TBE are total flavonoids (including buckwheat flavonoids), puerarin, and total polysaccharides (including lycium barbarum polysaccharides). The buckwheat experimental groups include three doses of buckwheat extract: low (8.35 ml/kg bw), medium (16.70 ml/kg bw), and high (41.75 ml/kg bw). The three doses of working solutions of TBE were prepared by adjusting the TBE concentration.

### Animals

Young male and female SD rats were purchased from Taconic Biosciences (New York, USA). The animals were housed in the Harvard Medical School animal facility under specific pathogen-free conditions and received humane care. All the animal experiments were approved by the Harvard Medical School Institutional Animal Care and Use Committee.

### Acute alcoholic liver injuries and treatments in rats

It has been reported that 4.0 g/kg body weight (bw) (oral gavage 50% alcohol by volume fraction) induced significant acute liver injury in SD rats [29]. Consequently, in this present study, acute alcoholic liver injury was stimulated by intragastric administration of 4.0 g/kg bw ethanol into SD rats. Rats, at 6-8 weeks old of age, were randomly divided into five different groups. Each group contained about 6-9 animals with equal or near-equal numbers of male and female rats. Pretreatment of TBE was designed to determine the role of TBE in a “preventive” manner against alcohol-induced liver damage. Rats were intragastrically administered saline (control group), low (8.35 ml/kg bw), medium (16.70 ml/kg bw), or high (41.75 ml/kg bw) doses of buckwheat ethanol extracts, or 75 mg/kg curcumin (positive control group) once daily in the morning for 4 consecutive days. This was based on the stomach capacity 10 ml/kg bw. From the 5<sup>th</sup> day, rats were intragastrically administered 4.0 g/kg bw of ethanol (acute liver injury model group, also called the ethanol model group) or saline (control group), 4 hours after daily treatments in the morning with saline, low, medium or high doses of TBE, or curcumin. The same treatments were conducted daily for 7 consecutive days. The body weight of each rat was measured every day.

### Chronic alcoholic liver Injuries and treatments in rats

Chronic alcoholic liver injury was produced by daily intragastric administrations of 3.0 g/kg/day bw ethanol (37.5% volume fraction) into SD rats. Rats, at 6-8 weeks old of age, are randomly divided into five different groups. Each group contained about 6-9 animals with an equal or near-equal number of male and fe-

male rats. Rats were weighed twice per week to adjust the doses of treatments. Rats were intragastrically administered once daily in the morning for 7-10 consecutive days with saline (control group), low, medium, or high doses of TBE, or 75 mg/kg curcumin (positive control group). After 4 hours, the control group of rats was intragastrically administered with saline while all other groups of rats were intragastrically administered with 3.0 g/kg bw ethanol. The same treatments were conducted daily for about 8~9 consecutive weeks.

### *Measurement of serum aspartate aminotransferase*

After the last administration, rats were fasted for 16-18 hours and the rat blood was freshly collected by the abdominal aortic method, while their livers were also harvested. The whole blood was kept for 30 min at room temperature, and then centrifuged for 15 min at 3000 rpm. The serums from indicated treatment groups were submitted to the measurements of serum AST levels by using BioAssay Systems AST activity assay kits per the manufacturer's instructions. This assay is based on the quantification of oxaloacetate produced by AST, which Oxaloacetate and NADH are converted to malate and NAD<sup>+</sup> by the enzyme malate dehydrogenase. The decrease in NADH absorbance at 340 nm is proportionate to the AST activity.

### *Measurement of serum alanine aminotransferase*

The serum samples of rats were obtained as described above in AST measurements for the indicated groups. Serum ALT levels were determined by using Cayman's Alanine Transaminase Colorimetric Assay Kit per the manufacturer's instructions. Measurement of the ALT activity is carried out by monitoring the rate of NADH oxidation in a coupled reaction system employing lactate dehydrogenase. The oxidation of NADH to NAD<sup>+</sup> is accompanied by a decrease in absorbance at 340 nm.

### *Measurements of hepatic malondialdehyde contents*

The liver tissue harvested in the same area of rats with the indicated treatments from acute and chronic alcoholic liver injuries was weighed,

homogenized in PBS, and centrifuged at 14,000 rpm and then 4 ml supernatants of 5% liver homogenates were collected for the MDA analysis [30]. The measurements were obtained spectrophotometrically at 535 nm with Bioassay MDA ELISA kit by following the manufacturer's instructions. MDA levels were calculated in nmol/mg protein.

### *Measurements of hepatic glutathione contents*

The liver samples of rats were obtained as described above in MDA assay with the indicated treatment. GSH contents in different liver samples were determined by using the Glutathione (GSH) colorimetric detection kit (Arbor Assays), according to the manufacturer's instructions. GSH levels were expressed as  $\mu\text{mol/g}$ .

### *Determination of serum ROS production*

According to the manufacturer's instructions (MyBioSource, Inc) and as previously described [31], ROS antibody was pre-coated on an ELISA plate. The serum samples were added into ELISA plate wells and incubated for 90 min at 37°C. Detecting biotin labeling polyclonal antibody was then added into ELISA plate and incubated for 60 min. Next, the avidin-peroxidase conjugates were added into ELISA plate and incubated for 30 min. Finally, TMB substrates for coloring were added into ELISA plate for 30 min. Absorbance of serum samples at 450 nm was measured using a plate reader and serum ROS (IU/ml) was obtained.

### *Hepatocyte morphology and hematoxylin and eosin (H&E) staining*

The whole liver was weighed and cut into a cross-section from the middle of the left hepatic lobe of each rat and fixed in 4% paraformaldehyde, followed by dehydration with different concentrations of ethanol and xylene, and then replaced xylene with hot paraffin in a tissue processor. Finally, the tissue was embedded in paraffin solution. The waxed tissue was cut into sections (5  $\mu\text{m}$  thick) under a microtome. The sections were passed through graded ethanol solutions, xylene, water and then stained with H&E dye (all reagents from Fisher Scientific, Santa Clara, CA). The histopathological changes of the liver cells were observed under



a light microscope and photo images were taken.

## *Terminal dUTP nick end labeling (TUNEL) assay*

The DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) is designed for the specific detection and quantitation of apoptotic cells. The extent of hepatocyte apoptosis was evaluated as previously described [32]. Briefly, cells grown on slides were fixed in 4% formaldehyde for 15 min, washed twice in PBS, and treated with proteinase K<sup>+</sup> solution (20 µg/mL) for 10 min at room temperature. The slides were then washed and fixed again in 4% formaldehyde for 5 min, washed and added 100 µl Equilibration Buffer for 10 min at room temperature. Then, the sections were incubated with TUNEL reaction mixture for 2 h at 37°C in a humidified chamber. The reaction was stopped in 2 × SSC buffer for 15 min, and the slides were washed 3 times and stained using Vectashield Mounting Medium with DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories, Inc, Burlingame, CA, USA). The stained sections were observed under the fluorescence microscope (Leica DMI8, Germany). The resulting images were processed and analyzed using imaging software Fiji ImageJ.

## *Cultures of hepatocyte cells and alcohol-induced cytotoxicity*

Huh 7 cells and HepG2 hepatoma cells were cultured in a humidified atmosphere of 95% air plus 5% CO<sub>2</sub> in a 37°C incubator in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 µM streptomycin, and 100 U/mL penicillin as previously described [30]. Primary hepatocytes were prepared by aseptically isolating the liver tissues from pups of Swiss mouse (postnatal day 3-5), removing the blood vessels, carefully translocating into Petri dishes before cutting with scissors (0.1 cm × 0.1 cm) and washing. Cells were cocultured with digestion medium (trypsin + EDTA) at 37°C for 20 min, and stopped digestion (by adding 10% FBS), filtered with cell strainer, and centrifuged at 2,000 rpm for 10 min to be collected (~2 × 10<sup>7</sup>). Hepatocyte cytotoxicity was induced by exposure to ethanol (100 mM) with or without low (1:2500 dilution from the low dose for animal experiment mentioned above), medium (1:2500 dilution), and high dose (1:2500 dilu-

tion) of TBE or curcumin (1:2500 dilution) for 24 hours. The HepG2 or Huh 7 hepatoma cells were then incubated with Hoechst 33342 (1:500) for 5 min, and changes to cell nuclei were observed by a Nikon fluorescence microscope. The morphologies of HepG2 or Huh 7 cell nuclei were observed and nuclear damages were analyzed with Hoechst 33342 staining.

## *MTS assay*

Huh 7 cells and HepG2 hepatoma cells were randomly assigned to six groups: the untreated control group, ethanol group, ethanol (100 mM) treated groups with low, medium, and high concentrations of TBE, or curcumin for 18-24 hours. The cytotoxicity/viability of HepG2 hepatoma cells in the different groups was measured by MTS assay, a measure of mitochondrial function and cell survival. The MTS assay was performed according to the manufacturer's instructions (Promega) as previously described [33, 34].

## *LDH assay*

Mouse primary hepatocytes were cultured and randomly assigned to six groups: the untreated control group, ethanol group, ethanol (100 mM) treated groups with low, medium, or high concentrations of TBE, or curcumin for 18-24 hours. The rates of cell death were determined quantitatively by the lactate dehydrogenase (LDH) assay according to the manufacturer's instructions (Roche Applied Science) as previously described [33, 34]. Briefly, the supernatant of cultured cells was collected and then centrifuged at 1,000 or 700 rpm for 10 min. The reaction mixture was then prepared by mixing 250 µl of solution 1 with 11.25 ml of solution 2 for 100 well tests. The above supernatant and reaction mixture (1:1) were added and incubated in the 96 well-plate (100 µl/per well) for 30 min at RT. The absorbance value (A) at 492 nm was measured with an automatic multi-well spectrophotometer.

## *Determination of mitochondrial membrane potential in hepatocytes*

HepG2 hepatoma cells were randomly assigned to six groups: the untreated group and alcohol treated groups with low, medium, or high concentrations of TBE for 18 hours. Rh 123 staining (Invitrogen) (20 µM for 5-10 min) was

performed to detect mitochondrial membrane potential ( $\Delta\Psi_m$ ) in living cells. The images were taken with a Nikon fluorescence microscope [33, 34].

#### Western blot analysis

Western blot analysis was performed to determine the levels of interested proteins, using our standard methods as described previously [35]. In brief, each sample (50  $\mu$ g of protein) was loaded onto a 10% or 12% SDS-PAGE gel using a constant current and then transferred to PVDF membranes on a semi-dry electro-transfer unit (Bio-Rad). Cytosolic fractionations and whole-cell lysates were performed as described previously [32, 35]. Cytosolic fractionation lysates were incubated with primary antibodies to anti-cyto. c (1:500, PharMingen), and whole-cell lysates were incubated with anti-Bcl-2 (1:1000, abcam), anti-Bcl-xL (1:500, Cell Signaling), anti-Beclin-1 (1:1000, Cell Signaling), anti-caspase-3 (1:500, Cell Signaling), anti-caspase-9 (1:500, Cell Signaling) or anti- $\beta$ -actin (1:10,000, Sigma). Released cyto. c or changes of Bcl-2, Bcl-xL, Beclin-1, caspase-3, and caspase-9 were analyzed by Western blot. The level of  $\beta$ -actin in each sample was used as a loading control. Then, the PVDFs were incubated with the respective HRP-conjugated secondary antibody for 2 h at room temperature. Images were detected with the enhanced chemiluminescence system (ECL, Pierce) and were captured on autoradiographic films. Films were scanned and densitometric quantification analysis of the bands was performed with NIH Image J software.

#### Statistical analysis

Statistical significance of histopathological, biochemical tests, and cytotoxicity data was evaluated by Student's t-test or ANOVA: \* $p$  values < 0.05, # $p$  values < 0.05, \*\* $p$  values < 0.01, ## $p$  values < 0.01, \*\*\* $p$  values < 0.001, and ### $p$  values < 0.001 were considered significant. Two-tailed  $p$ -values were always used.

### Results

#### *Tartary buckwheat extract reduces serum ALT and AST activities elevated in alcohol-induced acute and chronic liver injury in vivo*

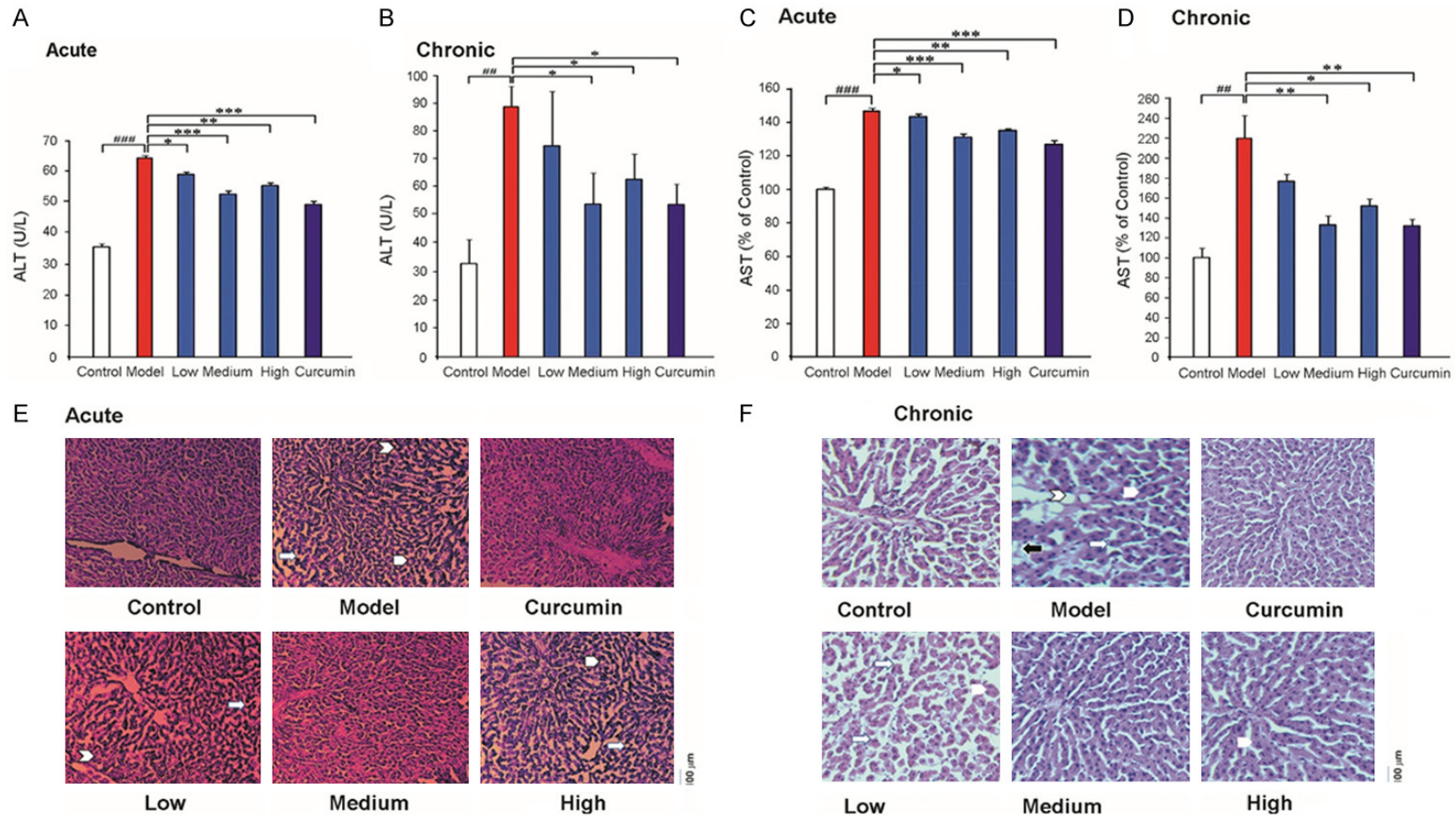
Serum ALT level is commonly measured clinically as a diagnostic biomarker for liver health

and has been most commonly used as a reliable indicator of liver injury [36]. Alcohol consumption increases the levels of serum ALT [27]. Consistent with the previous report that 4.0 g/kg bw alcohol-induced a significant ALT elevation in SD rats [29], we show a significant elevation of serum ALT activity in an acute rat model of liver injury. Specifically, oral gavage administration of ethanol (4.0 g/kg bw) induced a significant 1.9-fold increase in serum ALT enzyme level in the model group (i.e., liver injury group) compared with the control group (saline) (**Figure 1A**). Interestingly, the alleviating effects of TBE resulted in the reduction of ALT activities by 8%, 18%, and 15% by the low dose, medium dose, and high dose groups, respectively. We also used positive control curcumin, which has been widely reported for its protective effects on acute and chronic alcohol-induced liver injury [19]. Serum ALT activity assays revealed a 24% decrease of ALT activity in the positive control curcumin group compared to the acute injury model group, which is similar to the 18% reduction in serum ALT activity by a medium dose of TBE (**Figure 1A**).

Next, we investigated whether TBE has hepatoprotective effects on a rat model of chronic alcoholic liver injury (long-term). Chronic alcoholic liver injury was induced by intragastric administration of 3.0 g ethanol/kg bw/day. In rats, this dose is approximately equivalent to 2-3 drinks in humans (30 g of ethanol; 0.5 g/kg) [37]. Ethanol administration of 3.0 g/kg bw/day for 8-9 weeks was sufficient to produce liver injury with a significant 2.7-fold increase in ALT levels compared with the control saline group (**Figure 1B**). Thus, our results demonstrate that chronic ethanol administration induced more severe liver injury (2.7-fold) in rats than acute ethanol administration (1.9-fold). Interestingly, 16%, 39%, and 29% decreases in the ALT activity were found in the buckwheat low, medium, and high dose groups, respectively. Among the three doses of TBE, the 39% reduction in serum ALT activity in the medium dose group showed similar protective effects as the positive control curcumin group (40% reduction in ALT activity compared to the model group).

Serum AST level is part of diagnostic tests for liver function although the AST is less liver specific than the ALT level. Elevated serum AST

## Tartary buckwheat extract alleviates alcohol-induced liver injury



**Figure 1.** Tartary buckwheat extract reduces aspartate aminotransferase and alanine aminotransferase activities and attenuates alcoholic-induced acute and chronic liver injury with hepatocyte histomorphology changes. The SD rats were randomly divided into six groups, including negative control (white), positive ethanol injury model (red), 4.0 g/kg bw alcohol (A, C, acute) and 3.0 g/kg alcohol administration in (B, D, chronic), TBE groups (light blue) with low, medium, and high doses, or curcumin (blue) 75 mg/kg bw. Rats were treated with chronic ethanol oral gavages (8~9 weeks) and administration of TBE or curcumin (9~10 weeks). The serum activities of ALT and AST are shown. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Positive ethanol injury model vs. negative control ## $P < 0.01$ , ### $P < 0.001$ .  $N = 6\sim9$ /group. The liver sections from alcoholic-induced acute (E) and chronic (F) liver injury models with indicated treatments were stained with H&E, and dehydrated in graded ethanol solutions, cleared with xylene, examined under light microscope. The pathological changes were observed under a phase-contrast microscopy.  $\Rightarrow$  indicates broken hepatic architectures.  $\Rightarrow$  indicates swollen hepatocytes.  $\Rightarrow$  indicates ballooning degeneration.  $\leftarrow$  indicates mild hyperplasia of fibrous. Bar scale: 100  $\mu$ m.



**Table 1.** The effects of tartary buckwheat extract on liver index in acute alcohol-induced rats

Liver index × 100	Control	Model	Low	Medium	High	Curcumin
Liver weight/body weight × 100 ± SEM	3.291 ± 0.066	3.917 ± 0.209	4.086 ± 0.36	3.693 ± 0.071	3.75 ± 0.122	3.916 ± 0.155
	$P = 0.018^*$		0.709	0.335	0.502	0.996

The effects of buckwheat extract on liver index in acute alcohol-injury rats. Body and liver weights were weighed, and liver indexes analyzed for the acute liver injury rats in the absence or presence of treatment with TBE or curcumin, as shown in **Figure 1A** and **1C**. N = 6~8/group. \* $P < 0.05$ , positive ethanol injury model vs. negative control group.

**Table 2.** The effects of tartary buckwheat extract on liver index in chronic alcohol-induced rats

Liver index × 100	Control	Model	Low	Medium	High	Curcumin
Liver weight/body weight × 100 ± SEM	3.057 ± 0.11	3.57 ± 0.10	3.09 ± 0.25	3.093 ± 0.071	3.00 ± 0.07	3.00 ± 0.11
	< 0.01 <sup>##</sup>		> 0.05	< 0.01 <sup>**</sup>	< 0.00 <sup>***</sup>	< 0.01 <sup>**</sup>

The effects of buckwheat extract on liver index in chronic alcohol-injury rats. Body and liver weights were weighed, and liver indexes analyzed for the chronic liver injury rats in the absence or presence of treatment with TBE or curcumin, as shown in **Figure 1B** and **1D**. <sup>##</sup> $P < 0.01$ , positive ethanol injury model vs. negative control group.

<sup>\*\*</sup> $P < 0.01$  and <sup>\*\*\*</sup> $P < 0.001$ , TBE or curcumin group compared with the positive ethanol model group. N = 6~8/group.

levels may also be seen in disorders of the skeletal muscle, heart, and kidney. Alcohol consumption increases the levels of serum AST [27]. Consistent with the previous reports on elevated AST after 4.0 g/kg bw/day ethanol administration [29], the acute administration of 4.0 g/kg bw ethanol induced a significant 1.5-fold elevation of AST activities in the model group compared to the control (**Figure 1C**). It is logical to find that acute ethanol administration induced less AST elevation (1.5-fold) than ALT elevation (1.9-fold) since elevated ALT is more specific to liver injury. The low, medium, and high doses of TBE reduced of the elevated serum AST activities by 3%, 10%, and 8%, respectively. Our result revealed a 13% decrease of serum AST activity in the positive control curcumin group compared to the acute injury model group. This value is similar to the 10% reduction in serum AST by a medium dose of TBE (**Figure 1C**).

We then evaluated whether similar results could be obtained in a rat model of chronic alcoholic liver injury. Consistently, rats with chronic alcoholic liver injury model showed a significant 2.2-fold elevation of serum AST activity (**Figure 1D**). Our AST results also confirmed that the chronic ethanol administration induced more severe liver injury (2.2-fold) than the acute administration (1.5-fold increment). Interestingly, chronic ethanol administration also induced less elevation in serum AST (2.2-fold) than the ALT level (2.7-fold) since ALT is more specific to liver injury. Furthermore, the administration of low, medium, and high doses of TBE significantly reduced ALT levels by 20%, 39%, and 30%, respectively. As the posi-

tive control, the curcumin group decreased the serum AST levels by 39% compared to the chronic liver injury group. This value has the same alleviating effects to the effects on the 39% reduction of AST activities by the medium dose of TBE (**Figure 1D**).

The greater elevation of serum ALT and AST demonstrates that chronic ethanol administration (3.0 g/kg bw) in rats for consecutive 8-9 weeks causes more severe liver injury than the acute liver injury (4.0 g/kg/day bw ethanol administration for 7 consecutive days). Our results, for the first time, show that a medium dose of TBE exhibited similar or same effects as curcumin on reducing the elevated serum ALT and AST levels in both acute and chronic ethanol-induced injury models.

#### *Tartary buckwheat extract inhibits liver index elevated in alcohol-induced chronic liver injury in vivo*

Liver index (the ratio of liver weight/body weight) depends on the metabolic demands of the organism. Increased liver weights are commonly observed and may reflect generalized accumulations of fat, glycogen, and water [38]. Ethanol causes an increased liver index in experimental animal models including rats [39] and mice [40]. We analyzed the liver index by calculating the ratios of liver weight/body weight in rat. Compared with their respective control groups liver index was significantly elevated in both alcoholic-induced acute (**Table 1**, <sup>#</sup> $P = 0.018$ ) and chronic injury model groups (**Table 2**, <sup>##</sup> $P < 0.01$ ) following alcohol exposure.



Although the low, medium, and high doses of TBE did not significantly reduce the liver index elevated in acute alcohol-injury model, a decreasing trend in liver index was observed in the medium dose TBE group ( $3.693 \pm 0.071$ ) compared with the acute liver injury group ( $3.917 \pm 0.209$ ) (**Table 1**). Moreover, similar to the positive control, the administrations of both medium and high doses of TBE significantly reduced the alcohol-induced liver index elevated in the chronic alcohol injury model. In addition, the low dose of TBE showed a decreasing trend in the liver index ( $3.09 \pm 0.25$ ), compared with the chronic injury model group ( $3.57 \pm 0.10$ ) (**Table 2**).

*Tartary buckwheat extract attenuates acute and chronic ethanol-induced hepatocyte histomorphology changes in rats*

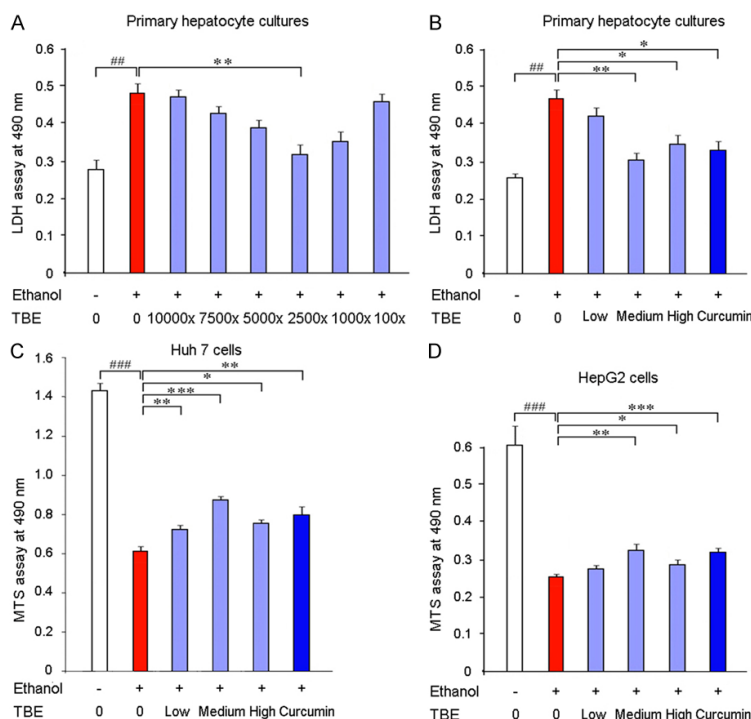
We assessed the protective effect of TBE on hepatic morphology change after acute ethanol exposure. H&E staining showed hepatocyte degeneration in the ethanol-exposed group as characterized by disorganized/broken hepatic architectures, unclear lobular boundaries, abnormal liver plate arrangements with the swollen and irregular size of diffuse hepatocytes. In addition, some of the hepatocytes are balloon-like and visibly scattered with infiltration of inflammatory cells. Interestingly, administration of the medium dose of TBE significantly restored the histopathological changes of acute alcoholic liver injury and the protective effect of TBE was similar to that of the positive control curcumin. However, the histopathological changes in the low and high doses of TBE showed only a trend of improvement (**Figure 1E**).

The H&E staining showed unclear liver lobular contours, broken hepatocyte cords, and irregular liver plate arrangements in the chronic alcohol injury model compared with the untreated negative control group. In the chronic injury models, some hepatocytes were obviously swollen, and the sizes of diffused hepatocytes were not uniform. The number of low, round vacuoles increased, and some low vacuoles merged into high vacuoles that pushed the nucleus to one side within hepatocytes. The histopathology analysis showed ballooning degeneration (**Figure 1F**) and visibly scattered necrotic hepatocytes with occasionally Mallory corpuscles in the chronic alcohol group. In ad-

dition, mild hyperplasia of fibrous tissue was detected around the portal vein area and visible infiltration of lymphocyte-based inflammatory cells (**Figure 1F**). Compared with the chronic alcohol group, the medium and high dose of TBE groups as well as the positive control curcumin group, but not the low dose group, showed the clear outlines of the hepatic lobules and the regular arrangements of the liver plates although the hepatic cords were broken. In addition, many hepatocytes were slightly swollen and consistently just a low number of vacuoles were observed, while hepatocyte necrosis was not clearly observed. Furthermore, TBE treatment decreased the level of fibrous tissues around the portal area with less infiltration of inflammatory cells. However, the histopathological changes in the low dose group showed only a trend of improvement (**Figure 1F**). Overall, the pathological changes in the medium TBE group were most similar to that of the positive drug curcumin group, indicating the protective effects of the medium and high doses of TBE on alcohol-induced chronic liver injury.

*Tartary buckwheat extract prevents ethanol-induced death of primary hepatocytes, HepG2 and Huh 7 cells in vitro*

To further evaluate the cytoprotective effect of TBE on alcohol-induced primary cultured hepatocytes and investigate the mechanisms underlying alcohol-induced cellular damage, primary cultured hepatocytes were randomly assigned to eight groups: the untreated control, ethanol and treated groups with concentrations of medium dose of TBE ranging from  $100 \times$  to  $10,000 \times$  dilution for 18-24 hours and then the cytotoxicity of primary cultured hepatocytes was determined by the LDH activity measurement. We tested a serial of doses of ethanol from 1 mM to 200 mM to induce HepG2 cell death (data not shown) and found that 100 mM ethanol was the optimum dose since this dose could induce about 40-50% of HepG2 cell death. Our data were consistent with the previous studies reporting that 100 mM ethanol could induce oxidative stress [41, 42], cell apoptosis [42], or lipid accumulation [43] on HepG2 cells. Ethanol exposure (100 mM) significantly reduced cell viability, compared with the untreated negative control group and medium dose TBE with  $2500 \times$  dilution significantly



**Figure 2.** Tartary buckwheat extract inhibits ethanol-induced death of primary hepatocytes, HepG2 cell, and Huh 7 cells. (A) Primary cultured hepatocytes were randomly assigned into eight groups: the untreated control (white), ethanol (100 mM, red) and treated groups with different concentrations of TBE (light blue) from 100 to 10,000 × dilution for 18-24 hours. The cytotoxicity of primary cultured hepatocytes was measured by LDH assay. N = 6~8/group. (B-D) Primary cultured hepatocytes (B), HepG2 cells (C), and Huh 7 cells (D) were randomly assigned into six groups: the untreated control (white), ethanol (100 mM, red) and ethanol-treated groups with low, medium, and high concentrations of TBE (light blue), or curcumin (blue) for 18-24 hours. The cytotoxicity/viability of cultured hepatocyte cells was measured by LDH assay (B) or MTS assay (C, D). N = 6~8/group.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ ,  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ , and  $^{***}P < 0.001$ .

inhibited ethanol-induced cell death (**Figure 2A**). LDH activity assay revealed that medium and high doses of TBE significantly inhibited ethanol-induced cell death, in a similar manner to the positive control curcumin group (**Figure 2B**).

The human hepatoma cell line HepG2, originally derived from a hepatocellular carcinoma biopsy, has been widely used as a cellular model by us and other researchers to investigate the underlying mechanisms of oxidative stress-mediated injury of hepatocytes elicited by  $H_2O_2$  [30, 45], or alcohol [46]. In addition, Huh 7, a well-differentiated hepatocyte-derived carcinoma cell line originally taken from a liver tumor in humans, is used extensively in liver research. The cytotoxicity/viability of cultured HepG2 and Huh 7 cells was also measured by

MTS assay. Consistently, medium and high doses of TBE and curcumin significantly inhibited ethanol-induced death of HepG2 (**Figure 2C**) and Huh 7 (**Figure 2D**) cells, demonstrating again the potent hepatoprotective effects of TBE against the death of cultured hepatocytes.

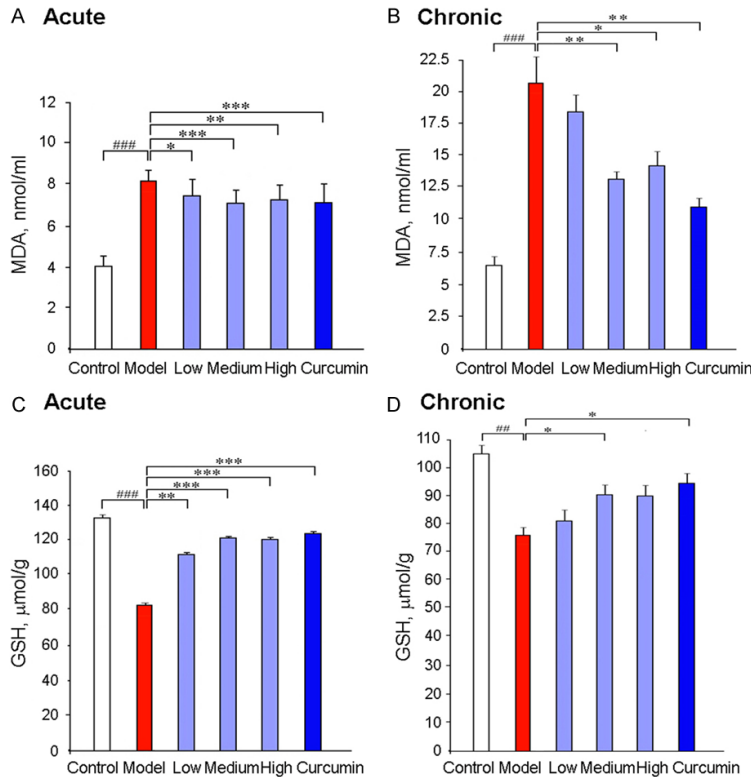
Taken together, our results show that buckwheat extract not only attenuates acute and chronic ethanol-induced hepatocyte morphology changes in rats *in vivo*, but also inhibits ethanol-induced death of cultured cells of hepatic origin *in vitro*.

*Tartary buckwheat extract reverses the altered levels of malondialdehyde and glutathione in alcohol-induced acute and chronic liver injury in rats*

Oxidative stress is a major contributing factor in the development of alcoholic liver diseases, including inflammation, fibrosis, cirrhosis, and cancer. The liver redox balance is subsequently altered through the oxidative alcohol metabolism [47, 48]. The presence of po-

tent antioxidant molecules in buckwheat helps the re-establishment or proper control of the physiological levels of free radicals [48]. MDA is known as an end product of lipid peroxidation and is a significant marker of the increased oxidative stress. GSH is a major non-protein thiol antioxidant, which plays a central role in coordinating antioxidant defense in the living organisms. It is involved in the maintenance of normal cell structure and function, probably through its redox and detoxification reactions [49].

We hypothesized that decreased oxidative stress is responsible for the buckwheat extract-mediated protection against acute and chronic ethanol-induced liver injury. To determine whether the protective effect of TBE supports the cellular antioxidant defense mechanisms,



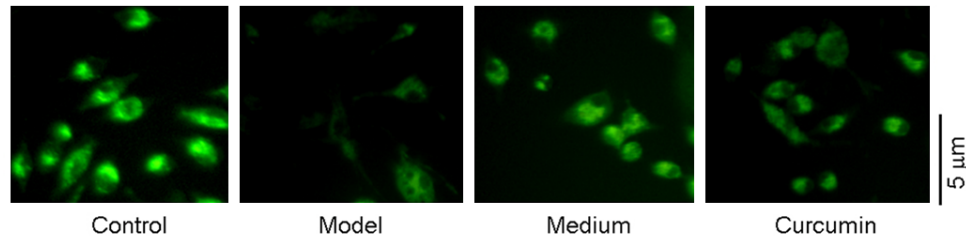
**Figure 3.** Tartary buckwheat extract reverses the levels of oxidative stress markers malondialdehyde and glutathione in alcohol-induced acute and chronic liver injury rats. The alcohol-induced acute and chronic liver injury rat models were established as in **Figure 1**. The liver tissues from differently treated rats were collected. The levels of hepatic MDA (A, B) and GSH (C, D) in the indicated treatment rats were determined. Positive ethanol injury model vs. TBE or curcumin \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Positive ethanol injury model vs. negative Control ## $P < 0.01$ , ### $P < 0.001$ .  $N = 6\sim 9$ /group.

the hepatic levels of two oxidative stress markers MDA and GSH were determined. As demonstrated in **Figure 3A**, oral gavage administration of 4.0 g/kg bw ethanol significantly increased the MDA level by 2-fold in the acute injury group compared with the negative control group. Interestingly, the administration of low, medium, and high doses of TBE significantly reduced the elevated MDA levels by 9%, 13%, and 11%, respectively. There was a 13% decrease in the positive control curcumin group, relative to the ethanol-exposed group. In addition, the hepatic MDA level was significantly elevated by 3.2-fold in the chronic ethanol-exposed group (**Figure 3B**). The greater elevation of MDA demonstrates that chronic ethanol administration (3.2-fold) caused more severe oxidative stress than the acute liver injury following alcohol exposure (2.0-fold). The MDA lev-

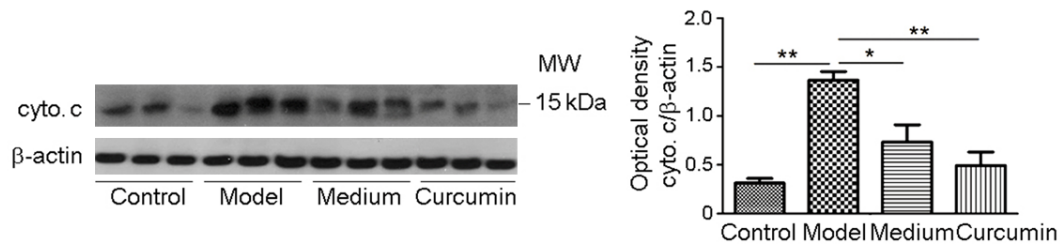
els were 11%, 37%, and 31% decreased by low, medium, and high doses of TBE, respectively. There was a 47% decrease in the positive control curcumin group, relative to the ethanol-exposed control. Among the three doses of buckwheat extract, the 13% reduction of MDA levels in acute alcoholic liver injury model in the medium TBE dose group shows the same protective effect as the positive control curcumin group compared to the ethanol-model group and the 37% reduction of MDA in chronic alcoholic liver injury model in the medium dose group shows similar protective effect to the positive control curcumin group (47% reduction) compared to the model group. The GSH levels were significantly decreased in alcoholic-induced acute liver injury model (**Figure 3C**). Oral gavage administration of 4.0 g/kg bw ethanol decreased the GSH level by 63% in the ethanol-exposed group compared with the untreated control group. Interestingly, the GSH contents were increased by 34%, 46%,

and 45% after treatment with low, medium and high doses of TBE, respectively, while curcumin increased the GSH level by 49%. We next determined whether TBE also increased the GSH levels in the chronic alcoholic liver injury model. Consistent to the acute liver injury model, chronic alcohol exposure for 8~9 weeks decreased the GSH level by 72% (**Figure 3D**). However, the GSH levels were increased by 7%, 19%, and 19% over the ethanol positive control group after treatment with low, medium, and high doses of TBE, respectively, while curcumin increased it by 24%. Among the three doses of buckwheat extract, the 46% and 49% elevated GSH levels by the medium TBE dose in acute and chronic alcoholic liver injury models, respectively, were similar to the protective effect by the curcumin group (19% and 24% enhancements).

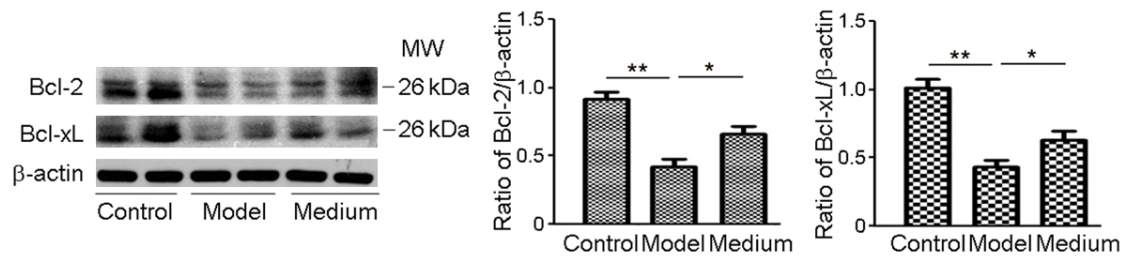
# A Rh123 staining



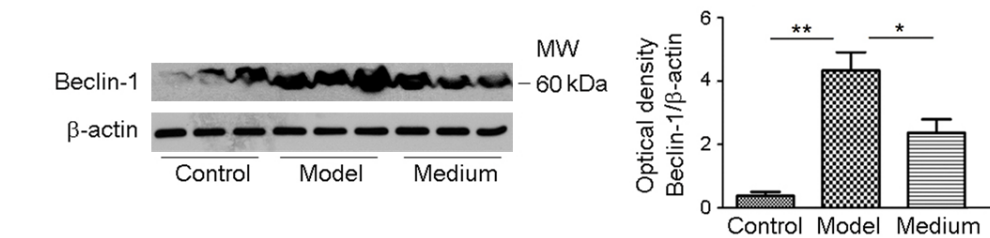
# B



# C



# D



**Figure 4.** Tartary buckwheat extract inhibits alcohol-induced mitochondrial apoptotic and autophagic cell death pathways. (A)  $\Delta\Psi_m$  observed with Rh 123 staining in Huh 7 cells under fluorescence microscopy. Bar scale 5  $\mu m$ . (B-D) The liver tissues were collected in indicated chronic alcohol-treated or non-treated rats. Cytosolic fraction lysates were prepared and analyzed by Western blotting using specific antibodies to cyto. c (B) and re-probed with anti- $\beta$ -actin as a loading control. Whole-cell lysates were extracted and analyzed by Western blotting using specific antibodies to Bcl-2 (C), Bcl-xL (C), or Beclin-1 (D) and then re-probed with anti- $\beta$ -actin as a loading control. Densitometric scans of these gels quantified the intensity of the bands (each lane contained 50  $\mu g$  proteins, representing a different rat). The bar graphs showed the quantitative densitometry analysis of cyto. c, Bcl-2, Bcl-xL, or Beclin-1. \* $P < 0.05$  and \*\* $P < 0.01$ .

## Tartary buckwheat extract suppresses alcohol-induced mitochondrial and autophagic cell death pathways

Despite its high incidence, death rate and severity, alcohol and the link between alcohol exposure and regulated forms of cell death are poorly understood. Measurement of mitochondrial membrane potential ( $\Delta\Psi_m$ ), a sensitive indicator of mitochondrial function, is used as a more specific test for early mitochondrial injury. Given that the loss of the  $\Delta\Psi_m$  is an impor-

tant event associated with the progression of mitochondrial dysfunction or even cell death [50], the depolarization of  $\Delta\Psi_m$  of HepG2 cells was determined by Rhodamine 123 (Rh 123) staining. The green fluorescence resulting from the accumulation of Rh 123 in untreated healthy control cells displayed a punctuate staining pattern (Figure 4A, left panel). Rh 123 staining became diffuse after alcohol insult for 18-24 hours (Figure 3A, middle panel), presumably due to the loss of  $\Delta\Psi_m$  mitochondrial depolarization. TBE treatment significantly pre-



served alcohol-induced dissipation of  $\Delta\Psi_m$  in HepG2 cells (**Figure 4A**, right panel), proving that buckwheat alleviates mitochondrial stress due to alcohol exposure.

It is well-established that alcohol causes mitochondrial dysfunction [47]. Therefore, we next investigated whether TBE inhibits alcohol-induced mitochondrial dysfunction/mitochondrial cell death pathways in a chronic rat model of liver injury. The decline of  $\Delta\Psi_m$  results in the release of cytochrome c (cyto. c) from mitochondria to the cytoplasm followed by activation of a cascade of caspases including caspase-9 and -3 that eventually execute hepatocytes cell death [51]. We hypothesized that TBE inhibits the release of cyto. c. Western blot of the cytosolic fraction lysates showed elevated levels of cyto. c in alcohol-exposed rats, but TBE blocked the release of cyto. c. into the cytoplasm (**Figure 4B**). The mitochondrial apoptosis process is regulated by various proteins, including Bcl-2 and Bcl-xL, two anti-apoptotic molecules in the mitochondria. Western blot analysis demonstrated that the expression of Bcl-2 and Bcl-xL was decreased in chronic ethanol-treated rats, and medium dose of TBE effectively inhibited the decreased levels of Bcl-2 and Bcl-xL (**Figure 4C**). Additionally, the levels of Beclin-1, an autophagy marker, were greatly increased in ethanol-exposed mice, whereas the administration of TBE significantly reduced the elevated levels of Beclin-1 (**Figure 4D**). Thus, TBE also inhibits the alcohol-induced autophagic cell death pathway.

The release of apoptogenic factors, such as cyto. c from mitochondria into cytosol, triggers the activation of downstream caspases. The cleaved caspase-9 can target and activate caspase-3, which is the “executioner” caspase, catalyzing the final steps of the apoptotic signaling cascade [52]. Western blot analysis showed that both caspase-9 (**Figure 5A**) and caspase-3 (**Figure 5B**) were activated in the liver after chronic alcohol exposure. Interestingly, medium dose TBE effectively inhibited the alcohol-mediated activation of both caspase-9 and caspase-3. In parallel, positive control curcumin significantly inhibited caspase-3 activation in chronic alcohol-treated rats (**Figure 5B**).

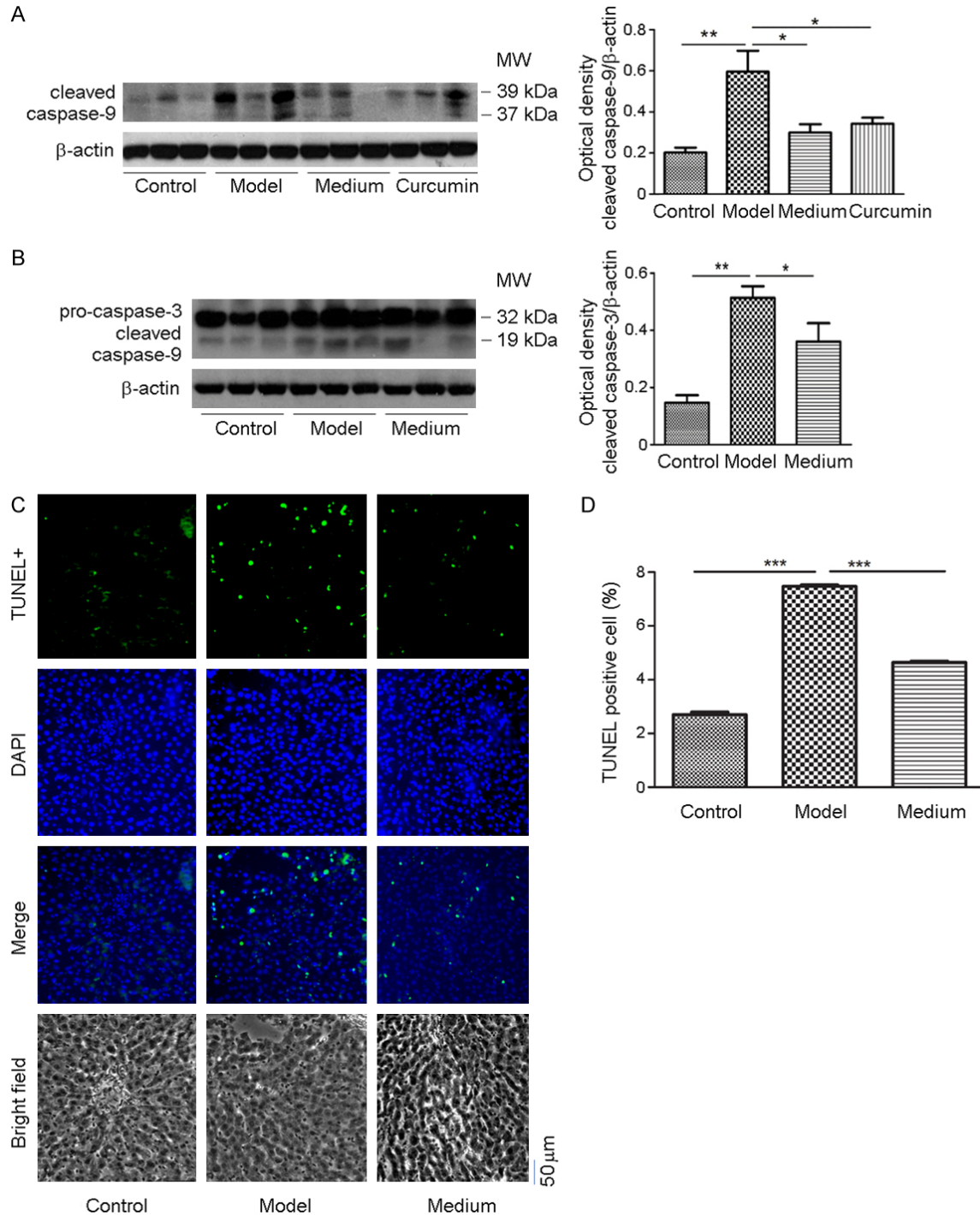
The TUNEL assay is a method of demonstrating apoptotic cell death. To investigate the mecha-

nisms underlying the antiapoptotic effects of buckwheat, the number of TUNEL positive cells (green) in different groups were compared. Compared to the untreated negative control group, more TUNEL-positive cells were observed in liver sections of the chronic alcohol-treated group (**Figure 5C**). Furthermore, our quantitative data showed that TUNEL-positive cells in chronic alcohol-treated rats was reduced significantly by 37.80% after treatment with medium dose TBE (**Figure 5D**). Therefore, by employing the measurements of  $\Delta\Psi_m$ , the release of cyto. c into the cytosol, the expression of Bcl-2 and Bcl-xL, the cleavage (activation) of caspase-9 and caspase-3, and number of TUNEL-positive cells as apoptotic markers, we demonstrate that the mechanisms for the protective effects of buckwheat consist of, at least partly, the inhibition of alcohol-induced mitochondrial dysfunction and autophagic cell death pathway.

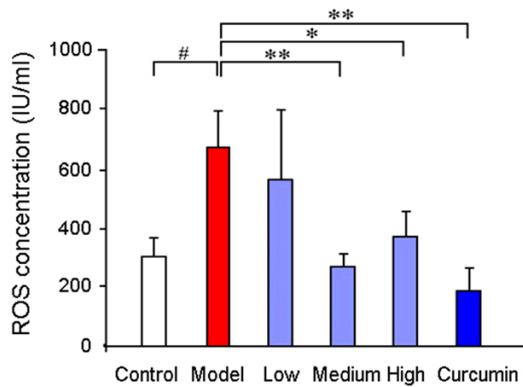
#### *Tartary buckwheat extract reverses the altered levels of ROS in alcohol-induced liver injury*

There is a connection between ROS generation and cell autophagy and/or apoptosis. ROS production is a key factor in discriminating between cell death and survival [30]. As a vital marker of oxidative stress, ROS production plays an important role in the pathogenesis of various liver disorders including alcoholic liver disease [30].

To test the effects of TBE on oxidative stress induced by alcohol, the production of ROS was further measured. As shown in **Figure 6**, oral gavage administration of ethanol significantly increased serum ROS production by 2.2-fold in the chronic injury group compared with the negative control group. The ROS production was 60% decreased by medium dose of TBE and there was a 72% decrease in the positive control curcumin group, relative to the ethanol-exposed control. The ROS content was decreased 45% by high dose of TBE compared to the ethanol-exposed control. Although the low and high doses of TBE did not significantly reduce the ROS level, a decreasing trend (8%) was observed, relative to the ethanol-exposed control (**Figure 6**). Furthermore, our results of the increased ROS generation by TBE on alcohol-induced liver injury model are consistent with the previous reports that buckwheat pos-



**Figure 5.** Tartary buckwheat extract inhibits alcohol-induced caspase-9 and -3 activation and reduces TUNEL-positive apoptotic cells. (A, B) The liver tissues were collected from the indicated chronic alcohol-treated or non-treated rats and whole-cell lysates were extracted and analyzed by Western blotting using antibodies to caspase-9 (A) or caspase-3 (B) and then re-probed with anti-β-actin as a loading control. Densitometric scans of these gels quantified the intensity of the bands (each lane contained 50 μg proteins, representing a different rat). The bar graphs showed the quantitative densitometry analysis of active-caspase-9 and active-caspase-3. (C) The representative apoptotic TUNEL-positive cells (green) observed under fluorescence microscopy are shown. Bar scale 50 μm. (D) TUNEL-positive cells (%) is shown to compare the extent of hepatocyte apoptosis in different treated rat liver tissues. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .



**Figure 6.** Tartary buckwheat extract reverses the production of ROS in alcohol-induced chronic liver injury rats. The alcohol-induced chronic liver injury rat model was established as in **Figure 1**. The serum from different treated rats was collected. The content of serum ROS in the indicated treatment rats was determined. Positive ethanol injury model vs. TBE or curcumin \* $P < 0.05$ , \*\* $P < 0.01$ . Positive ethanol injury model vs. negative control # $P < 0.05$ .  $N = 6\sim 9/\text{group}$ .

sesses a strong antioxidant property as functional foods [13, 14].

## Discussion

*Tartary buckwheat extract alleviates alcohol-induced acute and chronic liver injuries, at least partly, through inhibition of alcohol-induced oxidative stress and mitochondrial cell death pathways*

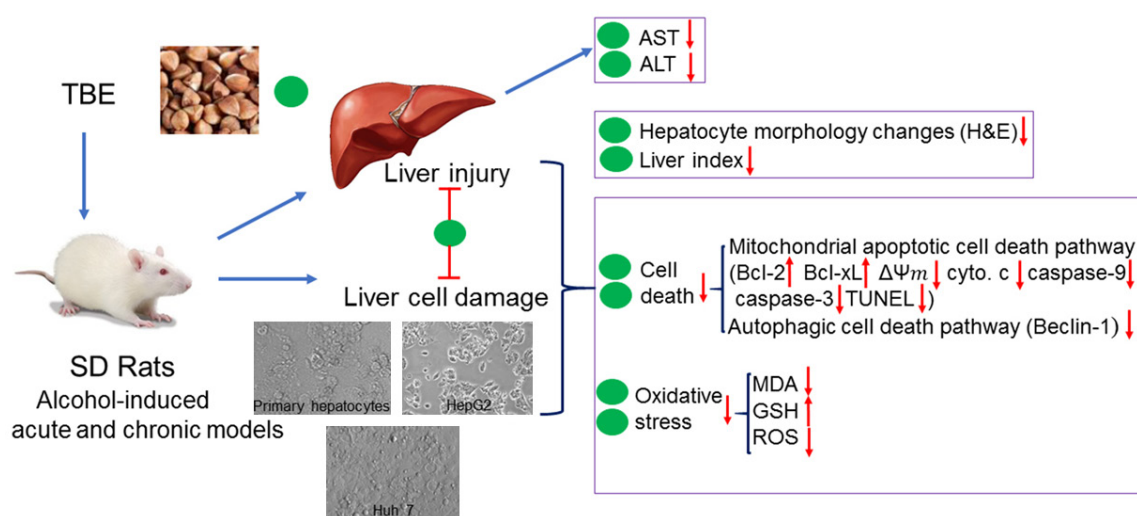
Liver injury is commonly evaluated by serum ALT and/or AST activities, liver index, and histological analyses. In the present study, we successfully established both acute (short-term) and chronic (long-term) alcoholic liver injury models in rats. As a summary diagram (**Figure 7**), our data showed for the first time that TBE not only reduced the elevated serum AST and ALT activities (**Figure 1**) and liver index (**Tables 1** and **2**) but also alleviated pathological dysmorphology of hepatocytes, such as ballooning degeneration and irregular hepatic architectures (**Figure 1**), as similar to the preventive effects of positive control curcumin. These results indicate the protective effects of TBE against alcohol-induced acute and chronic liver damage. Although TBE showed potentially beneficial effects on alcohol-induced liver injuries, it was not able to completely reverse or totally protect from alcohol-induced damages.

TBE contains very high antioxidant properties that can neutralize ROS and inhibit lipid peroxi-

dation [8, 53]. In our study, TBE reversed the elevated MDA and ROS contents and the decreased GSH in alcohol-induced acute and/or chronic liver injury rats, as similar to the preventive effects of positive control curcumin (**Figures 3** and **6**), indicating TBE could inhibit oxidative stress in the liver caused by short-term and long-term alcohol administration.

There is an association between ROS and cell death. Excessive oxidative stress triggers lipid peroxidation and leads to the destruction of cellular components and cell death [54]. Our study shows that ethanol exposure not only caused acute and chronic liver injury in rats *in vivo*, but also damaged the cultured cells of hepatic origin including primary hepatocytes, HepG2 hepatoma, and Huh 7 cells *in vitro* (**Figure 2**). It appears that the effects of TBE did not always exhibit dose-dependence, rather the medium dose had the most beneficial effects compared to the low and high doses. Such potential adverse effects of TBE itself at high doses may explain the dose-independence of the protective effects of TBE on alcohol-induced liver injuries and cell toxicity. It has been reported that the overproduction of ROS results in lipid peroxidation including MDA, damage to the mitochondrial membrane ( $\Delta\Psi_m$ ), release of mitochondrial apoptogenic factors including cyto. c into the cytoplasm, followed by caspase activation such as caspase-9 and 3 and finally cellular apoptosis in hepatocytes during malaria [55]. Additionally, several hepatocyte apoptosis-related proteins including cyto. c, Bcl-2, Bcl-xL, caspase-9 and caspase-3 can affect mitochondrial DNA damage and cell death through mitochondrial antioxidant and redox states [56]. Indeed, in the present study, we demonstrate that TBE, not only reversed the elevated MDA and ROS contents and the decreased GSH level in alcohol-exposed liver injury rats, but also preserved  $\Delta\Psi_m$ , prevented the alcohol-induced release of cyto. c and decreased Bcl-2 and Bcl-xL levels (**Figure 4**), and subsequently suppressed alcohol-induced activation of caspase-9 and caspase-3 as well as reduced the number of TUNEL-positive apoptotic cells in alcohol-exposed liver sections (**Figure 5**). In addition, autophagy influences mitochondrial recycle and can thus modulate hepatic apoptosis via mitochondrial death pathway [57]. Our result also show that TBE inhibited autophagic cell death pathway by reducing Beclin-1 expression (**Figure 4**).

## Tartary buckwheat extract alleviates alcohol-induced liver injury



**Figure 7.** A summary diagram to show the protective effects of tartary buckwheat extract against alcohol-induced acute and chronic liver injury. TBE (●) inhibits (→) hepatocyte morphology changes, cell death, and oxidative stress as well as improves liver index and oxidative stress, possibly by anti-mitochondrial apoptosis, antioxidant and anti-autophagy activities. The symbol ↓ means decrease and ↑ means increase.

Taken together, our data, for the first time, demonstrated the underlying mechanisms by which TBE protects from the alcohol-induced liver injury through inhibition of oxidative stress and the mitochondrial and autophagic cell death pathways (**Figure 7**). Since the “preventive” treatment of TBE in the present study shows the protective role of TBE in improving alcohol-induced liver damage, to mimic clinical situation, “therapeutically” post-treatment after alcohol treatment can be considered in the future research.

### *Tartary buckwheat extract may be used for the treatment of alcohol-induced liver injury*

AUD is a leading cause of preventable morbidity and mortality, which is an enormous public health problem and socioeconomic burden worldwide. Depending on the organs affected, alcoholism is a multifactorial disorder that requires a multidisciplinary treatment approach. So far, conventional treatment strategies have used a combination of reduction of ethanol-mediated organ damage by controlling drinking frequency and amounts with increased local and systemic protective mechanisms of the body, through the use of antioxidant supplementation [58]. However, these approaches are insufficient to address AUD-associated problems. The identification of safe and effective treatments/therapies capable of ameliorating the alcohol-induced organ damage, including liver injury, are urgently needed.

To date, despite great efforts to identify protective agents, specific and highly effective treatments remain limited for alcoholic-induced liver injury, which turns out to be deadly [59]. However, these approaches are insufficient to address AUD-associated problems. The identification of safe and effective treatments/therapies capable of ameliorating the alcohol-induced liver injuries is urgently needed. Treatment of affected patients is aimed at reducing the complications and exacerbation of liver damage. Among the strategies, the Chinese herbal medicine has been widely applied to complement western medicine [60]. However, virtually no studies have been reported to show the beneficial effects of tartary buckwheat extract on alleviating alcohol-induced liver injury. Buckwheat is a plant that is being used as an effective herb and as a safe functional food. Tartary buckwheat is a healthy and nutritionally important dietary supplement [61].

Given that it is very cheap, safe with no toxicity, and can be eaten orally, it may be a valuable herb for combating AUD-associated pathologies. Our results about the preventive effects of TBE against alcohol-induced acute and chronic liver injuries in rats and in cultured hepatocytes *in vitro* and mechanistic studies are novel and may contribute to the develop-



ment of TBE as a potential treatment for AUD-associated organ damage, including alcohol-induced liver injury, while our studies on the molecular mechanisms of action of TBE may contribute to the understanding of the pathogenesis of AUD. Consequently, Maopu tartary buckwheat extract could significantly increase the overall health of alcoholic individuals by reducing alcohol-induced liver damage.

## *The possible effects of biologically active contents in tartary buckwheat extract*

Previous studies have shown the neuroprotective and hepatoprotective effects of individual components of buckwheat [10]. Tartary buckwheat is the healthy and nutritionally important food. The important constituents responsible for the hepatoprotective effects of tartary buckwheat or ethanol extracts of tartary buckwheat include flavonoids and quercetin [61, 62]. The biological active components aid the physiological functions of the liver, while reducing the oxidative liver damage [52, 53]. The high content of flavonoids rutin and orientin are normally responsible for the important antioxidant properties of TBE [63]. Tartary buckwheat contains about 100-fold greater amount of rutin in its seeds than that of common buckwheat. Flavonoids prevent liver damage by reducing harmful oxidative stress, resulting from oxidative alcohol metabolism. Recent studies proved that rutin has also hepatoprotective effects in the case of type 2 diabetes [64]. The content of total flavonoids, including tartary buckwheat flavonoids in Maopu tartary buckwheat liquor is > 50 mg/L. Consequently, Maopu tartary buckwheat extract may have the potential to alleviate alcoholic-induced liver injuries and thus could increase the overall health of alcoholics by reducing liver damage.

Currently, traditional Chinese medicine is being applied to complement western medicine [60]. Typically, in most traditional Chinese medicines, the formulation consists of a concoction of multiple herbs. It has been suggested that the combination of herbs with the various natural ingredients may produce additive or synergistic effects and neutralize potential side effects of an individual herb constituent, hence augmenting the effectiveness of the treatment. The hepatoprotective effects of several Chinese medicinal herbs and composite

formulae, which have been commonly used for preventing and treating hepatic diseases, including *Andrographis Herba*, *Glycyrrhizae Radix et Rhizoma*, *Ginseng Radix et Rhizoma*, *Lycii Fructus*, *Coptidis Rhizoma*, curcumin, *XiaoCha-Hu-Tang* and *Shi-Quan-Da-Bu-Tang*, were reviewed [60]. Specifically, the concoction consisting of the 10 Chinese medicinal herbs, named HPE-XA-08, was reported to show potential hepatoprotective and curative effects against damaged liver [65].

In summary, TBE is a Chinese herbal medicine extract from Tartary buckwheat plus *pueraria*, *lycium barbarum*, and hawthorn, which its major ingredients are total flavonoids including buckwheat flavonoids, puerarin, and total polysaccharides including *lycium barbarum* polysaccharides; for the first time, we demonstrate the protective effect of TBE against alcohol-induced acute and chronic liver injury *in vivo* and *in vitro*. Given the dietary nature of buckwheat, *pueraria*, *lycium barbarum*, and hawthorn, the dietary intake of TBE or liquor contained TBE, e.g., Maopu Tartary buckwheat liquor, compared with pure liquor consumption alone, may have the potential to alleviate alcoholic-induced liver injuries.

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## Disclosure of conflict of interest

None.

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