Boletus aereus protects against acute alcohol-induced liver damage in the C57BL/6 mouse via regulating the oxidative stress-mediated NF-κB pathway

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**ABSTRACT**

**Context:** Alcoholic liver disease, caused by abuse and consumption of alcohol, exhibits high morbidity and mortality. *Boletus aereus* Bull. (Boletaceae) (BA) shows antioxidant, anti-inflammatory and antimicrobial effects. **Objectives:** To investigate the hepatoprotective effects of BA using an acute alcohol-induced hepatotoxicity mice model. **Materials and methods:** The composition of BA fruit body was first systematically analyzed. Subsequently, a C57BL/6 mice model of acute alcohol-induced liver injury was established by intragastrically administration of alcohol, which was intragastrically received with BA powder at 200 mg/kg and 800 mg/kg for 2 weeks, 60 mg/kg silybin treatment was used as positive control group. By employing the pathological examination, ELISA, RT-PCR and western blot, the regulation of BA on oxidative stress signals was investigated. **Results:** The LD\textsubscript{50} of BA was much higher than 4 g/kg/p.o. In acute alcohol-damaged mice, BA reduced the levels of alanine aminotransferase (18.3%) and aspartate aminotransferase (27.6%) in liver, increased the activity of liver alcohol dehydrogenase (35.0%) and serum acetaldehyde dehydrogenase (18.9%). BA increased the activity of superoxide dismutase (13.4%), glutathione peroxidase (11.0%) and 800 mg/kg BA strongly reduced chemokine (C-X-C motif) ligand 13 (14.9%) and chitinase-3 like-1 protein (13.4%) in serum. BA reversed mRNA over-expression (>70%) and phosphor-stimulated expression (>45.0%) of an inhibitor of nuclear factor κ-B (NF-κB), an inhibitor of nuclear factor κ-B α and nuclear factor κ-B in the liver. **Conclusions:** BA is effective in ameliorating alcohol-induced liver injury through regulating oxidative stress-mediated NF-κB signalling, which provides a scientific basis for further research on its clinical applications.

**Introduction**

Alcoholic liver disease (ALD), caused by the abuse and consumption of alcohol, exhibits extremely high morbidity and mortality (Ren et al. 2018). Continued heavy alcohol consumption will exacerbate alcoholic fatty liver disease (AFLD), which develops into fatty hepatitis, liver fibrosis and even hepatic carcinoma (Gao and Bataller 2011). About 90% of alcohol is metabolized in the liver to acetaldehyde by alcohol dehydrogenase (ADH), and then decomposes into acetic acid under the metabolism of acetaldehyde dehydrogenase (ALDH), before finally being converted into non-toxic and harmless substances (Jelski et al. 2008). However, heavy alcohol consumption damages the liver cells, resulting in a decrease in the activity of the above enzymes, thus causing the accumulation of acetaldehyde, which is responsible for alcoholism (Setschedi et al. 2010).

Among the cellular components most vulnerable to acetaldehyde are the mitochondria, where in mitochondrial damage

induces the over-accumulation of reactive oxygen species (ROS) and the suppression of antioxidant activities (Farfan Labonne et al. 2009). Under physiological conditions, ROS are efficiently eliminated by antioxidant defense systems including superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) (Dai et al. 2016). However, hyper-levels of ROS, acting as a secondary messenger, enhance the signal transduction of nuclear factor kappa B (NF-κB), a reduction/oxidation (redox)-sensitive factor, from the cytoplasm into the nucleus (Bubici et al. 2006). Normally, NF-κB binds to the inhibitor of nuclear factor kappa-B (IκB) protein to form a complex that blocks the nuclear localization signal of NF-κB. However, when the cells are stimulated by alcohol, IκB protein is phosphorylated by the activated IκB kinase and then the nuclear localization signal of NF-κB is exposed and NF-κB is transported into the nucleus (Wu et al. 2014). The inhibition of NF-κB activation and cytokine synthesis is a potential mechanism for the treatment of alcohol-induced liver injury (Abhilash et al. 2014).
Presently, raw materials for liver cell metabolism, agents for alcohol metabolism and opioid receptor antagonists are among the commonly used ALD treatment clinically (Ozkol et al. 2017). However, after chronic administration of these agents, various adverse effects including drug dependence, vomiting, dermatitis, dizziness and leukopenia are noted in ALD patients (Triantafyllou et al. 2010). Natural products, especially fungi, show considerable biological effects in humans.

In our group, _Antrodia camphorata_ (Fomitopsidaceae) has been confirmed to display hepatoprotective effects in acute alcohol-induced mice via regulating oxidative stress signalling (Liu et al. 2017). _Boletus aereus_ Bull. (Boletaceae) (BA), rich in carbohydrates, amino acids and fatty acids and widespread in Europe (Beugelsdijk et al. 2008), shows a variety of biological functions such as antioxidant (Zheng et al. 2014), anti-inflammatory (Wu et al. 2016), antitumor (Lemieszek et al. 2017) and antimicrobial (Kosani et al. 2017) properties. BA polysaccharides can increase the activities of antioxidant enzymes and reduce the lipid peroxidation in mice administered with alcohol (Guo et al. 2015). However, the effects of BA on acute alcohol-induced liver injury and its underlying mechanisms have not been systematically reported.

In this study, we first systematically analysed the components of BA fruiting bodies. Subsequently, BA’s hepatoprotective effects and the underlying mechanisms related to oxidative stress-mediated NF-kB signalling, were explored systematically in mouse models with acute alcohol-induced hepatotoxicity. Our data provide valuable evidence to support the use of BA as a functional food to treat ALD.

### Materials and methods

**B. aereus compounds analysis**

_B. aereus_ fruiting bodies, purchased from TengHui Agriculture (Kunming, Yunnan, China), were verified by professor Li Yu from Jilin Agricultural University and dried at 60 ± 2°C, crushed and passed through a 60-mesh sieve.

### Main components detection

The quantities of the main components including total sugar, reducing sugar, total protein, total ash, crude fat, total flavonoids, total triterpenes, mannitol, nucleotides, vitamins, sterols and polyphenols were measured using the phenol-sulfuric acid method (Jain et al. 2017), 5-dinitrosalicylic acid colorimetric estimation (Zhang et al. 2014), Kjeldahl method (Chromy et al. 2015), ash content analysis, Soxhlet extraction (Smith and Tschinkel 2009), aluminium chloride colorimetry (Manurung et al. 2017), vanilin-glacial acetic acid-perchloric acid colorimetry (Li et al. 2014), iodometry (Rivera-Jacinto et al. 2009), high performance liquid chromatography (HPLC) (Mattila et al. 2001; Krpan et al. 2009), UV spectrophotometric assay (Araújo et al. 2013) and Folin-Ciocalteu method (Musci and Yao 2017), respectively.

**Amino acids detection**

The appropriate amounts of BA samples dissolved in 6 mol/L hydrochloric acid were hydrolyzed at 110 ± 1°C for 22 h, then dried under vacuum. Buffer (1 mL, 19.6 g sodium citrate and 16.5 mL hydrochloric dissolved in 1 L of deionized water, pH 2.2) was added to prepare test samples. The amino acids content was quantified by an automatic amino acid analyzer (L-8900, Hitachi, Tokyo, Japan).

### Mineral content detection

The levels of mercury (Hg), lead (Pb), selenium (Se), arsenic (As), cadmium (Cd), zinc (Zn), iron (Fe), manganese (Mn), chromium (Cr), calcium (Ca), copper (Cu), sodium (Na) and potassium (K) were detected by inductively coupled plasma optical emission spectrometry (Hurel et al. 2017).

### Fatty acid detection

BA powder was extracted with a solution mixture of ether: petroleum ether (1:1) for 24 h. The crude fatty acid was dehydrated with anhydrous sodium sulphate and then methyl-esterified with 5% potassium hydroxide-methanol solution and the fatty acid content determined by gas chromatography-mass spectrometry (QDP2010, Shimadzu, Tokyo, Japan).

### Animal experiments

Healthy male C57BL/6 mice (8–10 weeks; 18–22 g) (SCXK (LIAO) 2015-0001) were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). The mice were kept in a controlled environment at a temperature of 23°C ± 1°C with 50% ± 10% humidity and a 12-h light-dark cycle and free access to water and food.

### Acute oral toxicity study

The experimental protocol was carried out under the Ethical Committee of Animal Research of Jilin University (2017SY010). The male C57BL/6 mice (n = 8) were intragastrically administrated with 4 g/kg of BA (the maximum intragastric dose), and then observed continuously for the first 4 h for any mortality changes and abnormal behaviours, intermittently for the next 24 h, and occasionally thereafter for 14 days for any delayed effects.

### Animal model development and drug treatment process

The animal experiment was carried out in accordance with the Guiding Principles of Jilin University Animal Ethics Committee (2017SY0106). As a clinically used liver protectant, silybin (Sil) can reduce oxidative stress, and prevent the increase of alanine aminotransferase (ALT), tumour necrosis factor-α (TNF-α) and lipid peroxidation. Based on its clinically used doses, 60 mg/kg of Sil was chosen for the present experiments. The mice were fed for 7 days to acclimatize to the environment, and then randomly divided into five groups (n = 10). The control mice were intragastrically administrated with saline at 0.1 mL/10 g twice per day at 9:00 am and 4:00 pm for 14 days. All other mice were intragastrically administrated with 13 g/kg of 56% ethanol (Ergouotou wine, Beijing Shunxin Agricultural Co. Ltd, China) at 9:00 am once a day. After 7 h, the mice were intragastrically administrated with saline at 0.1 mL/10 g body weight (model group; n = 10), 60 mg/kg of Sil (Tianjin Tasly Sants Pharmaceutical Co. Ltd, China) (positive control group; n = 10) and BA at doses of 200 mg/kg and 800 mg/kg (BA-treated groups; n = 10) at 4:00 pm (Supplementary Figure IS). The administration lasted for 14 days,
and the body weight of each mouse was monitored daily. After the last treatment, the mice were fasted overnight, and then blood was sampled from the caudal vein of each mouse. After the mice were sacrificed under euthanasia, organs including liver and kidney were quickly collected, and portions of the organs were fixed in 10% formalin buffer, while the remaining parts were stored at −80°C. The organ index was calculated by the following formula:

\[
\text{Organ index (mg/10g)} = \frac{\text{organ weight (mg)}}{\text{body weight (10g)}}
\]

**Biochemical indicators detection**

A portion of the liver tissues were homogenized with physiological saline on ice and centrifuged at 3500 rpm for 10 min. The levels of aspartate aminotransferase (AST; CK-E90386M), ALT (CK-E90314M), ADH (CK-E92648M), ALDH (CK-E92649M), chemokine (C-X-C motif) ligand 13 (CXCL13; CK-E95658M), chitinase-3-like 1 protein (YKL-40; CK-E95772M), thrombopoietin (TPO; CK-E93965M), interleukin-7 (IL-7; CK-E20125M), plasminogen activator inhibitor type 1 (PAI-1; CK-E93562M), retinol-binding protein 4 (RBP4; CK-E20170M), nitric oxide (NO; CK-E20293M), ROS (CK-E91516M), catalase (CAT; CK-E92636M), GSH-Px (CK-E92669M) and malondialdehyde (MDA; CK-E20347M) in the serum and liver and the levels of high-density lipoprotein (HDL; CK-E92669M) and vitamin B2 and 0.42% was vitamin B3; however, adenosine and vitamin A, B1, B2, B6, C, D2, D3 and E were not detected (Table 1). Among the 13 detected minerals, K, Fe and Na were the most abundant (Table 1). Thirty-five fatty acids were measured, among which the contents of linoleic acid, oleic acid and hexadecanoic acid were significantly higher than those of the other fatty acids; however, capric acid, undecanoic acid, tridecanoic acid, myristoleic acid, cis-10-pentadecenoic acid, elaidic acid, trans-linoleic acid, α-linolenic acid, γ-linolenic acid, dihomo-γ-linolenic acid, eicosapentaenoic acid, cis-13,16-docosadienoic acid methyl ester, docosahexaenoic acid and octanoic acid were not detected (Table 1).

**Histopathological examination**

Portions of the liver and kidneys were excised and fixed in 10% neutral formalin buffer. Afterwards, the fixed tissues were dehydrated with gradient ethanol (70, 80, 90, 95 and 100%), and then cleared twice in xylene, and embedded in paraffin. Histopathological examination of each protein band was performed using Image J analysis software.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

RT-PCR was performed according to a method described previously with some modification (Zhang et al. 2017). Briefly, the RNA was isolated from the liver tissues using Trizol (Invitrogen, USA), and then synthesized by QuantScript RT Kit (Tiangen Biotech (Beijing) Co. Ltd., China). β-Actin primers were used as an internal control. The conditions of PCR amplification were as follows: denaturation at 95°C for 5 min, followed by 36 cycles at 95°C for 45 s, 57°C for 45 s and 72°C for 45 s. The primer sequences are listed in Supplementary Table 1S.

**Western blot analysis**

A portion of the liver tissues was homogenized with radioimmunoprecipitation assay (RIPA, Sigma-Aldrich) lysate containing 2% phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, MO) and 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The total protein concentration was determined using the BCA protein assay kit (Merck Millipore, Burlington, MA). Proteins (40μg) were boiled at 100°C for 5 min with loading buffer (Solarbio Biotechnology Co., Ltd, Beijing, China), separated by 12% polyacrylamide gel and transferred onto 0.45 μm polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Burlington, MA). The membranes were blocked using 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and solubilized with Tris-buffered saline (TBS) at 4°C for 4 h. The blocked membranes were incubated with primary antibody against phosphor (P)-inhibitor of nuclear factor kappa-B kinase α/β (IKKα/β) (ab195907), total (T)-IKKα/β (ab178870), P-1kBα (ab12135), T-1kBα (ab32518), P-NF-κB p65 (ab86299), T-NF-κB p65 (ab7970) (Abcam, Cambridge Science Park, UK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ABS16; Merck Millipore, Burlington, MA) at 4°C overnight. The membranes were washed five times with TBS containing Tween 20, and then incubated in horseradish peroxidase-conjugated goat anti-rabbit (IH-0011; Dingguo, Beijing, China) and goat anti-mouse secondary antibody (IH-0031; Dingguo, Beijing, China) diluted 2000-fold with 2% BSA for 4 h at 4°C. The blots were visualized using Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500; Merck Millipore, Burlington, MA) and Gel Imaging System (Tanon Technology Co., Ltd., Shanghai, China). Quantitative gray-scale analysis of each protein band was performed using Image J analysis software.

**Statistical analysis**

All statistical analyses were performed using SPSS 23.0 software (IBM Corporation, USA) and the data were presented as means ± standard errors of the mean (S.E.M.). Differences were tested by one-way analysis of variance (ANOVA). p values of < 0.05 were considered statistically significant.

**Results**

**Composition of BA**

For the main components of BA, 30.60% was total sugar, 4.80% was reducing sugar, 1.44% was triterpenes, 0.23% was flavonoids, 17.90% was mannitol, 12.20% was crude fat, 24.30% was total protein, 2.03% was polyphenols, 1.40% was sterols, 0.02% was vitamin A, B1, B2, B6, C, D2, D3 and E were not detected (Table 1). A total of 17 amino acids were determined, among which the contents of methionine (0.60%), glutamic acid (0.39%) and aspartic acid (0.32%) were higher than those of the others (Table 1). Among the 13 detected minerals, K, Fe and Na were the most abundant (Table 1). Thirty-five fatty acids were measured, among which the contents of linoleic acid, oleic acid and hexadecanoic acid were significantly higher than those of the other fatty acids; however, capric acid, undecanoic acid, tridecanoic acid, myristoleic acid, cis-10-pentadecenoic acid, elaidic acid, trans-linoleic acid, α-linolenic acid, γ-linolenic acid, dihomo-γ-linolenic acid, eicosapentaenoic acid, cis-13,16-docosadienoic acid methyl ester, docosahexaenoic acid and octanoic acid were not detected (Table 1).
Main components (%) | Total sugar | 30.60 | Mannitol | 17.90 |
|----------------------|-----------|-------|---------|-------|
| Total ash            | 5.60      | Protein | 1.40    |
| Total triterpenes    | 1.44      | Total flavonoids | 0.23 |
| Reducing sugar       | 4.80      | Nucleotide | ND    |
| Crude fat            | 12.20     | Sterols | ND      |
| Polyphenols          | 2.03      | Vitamin A | ND    |
| Vitamin B1           | ND        | Vitamin B6 | 1.71 |
| Vitamin B2 (× 10^{-2}) | 4.16   | Vitamin B2 | ND    |
| Vitamin C            | ND        | Vitamin D2 | ND    |
| Vitamin D3           | ND        | Vitamin E | ND    |
| Amino acid (%)       |           |         |         |
| Aspartic acid (Asp)  | 0.32      | Isoleucine (Iso) | 0.18 |
| l-Threonine (Thr)    | 0.21      | Leucine (Leu) | 0.30 |
| Serine (Ser)         | 0.19      | Tyrosine (Tyr) | 0.16 |
| Glutamic acid (Glu)  | 0.39      | Phenylalanine (Phe) | 0.18 |
| Glycine (Gly)        | 0.30      | Lysine (Lys) | 0.16 |
| Alanine (Ala)        | 0.26      | Histidine (His) | 0.16 |
| Cystine (Cys)        | 0.03      | Arginine (Arg) | 0.17 |
| Valine (Val)         | 0.15      | Proline (Pro) | 0.15 |
| Methionine (Met)     | 0.60      |         |         |
| Minerals (%)         |           |         |         |
| Mercury (Hg) (× 10^{-5}) | 8.76 | Manganese (Mn) (× 10^{-5}) | 2.38 |
| Lead (Pb) (× 10^{-5}) | 2.32 | Calcium (Ca) (× 10^{-6}) | 1.83 |
| Selenium (Se) (× 10^{-6}) | 6.81 | Copper (Cu) (× 10^{-5}) | 2.70 |
| Arsenic (As) (× 10^{-6}) | 6.58 | Sodium (Na) (× 10^{-7}) | 2.56 |
| Zinc (Zn) (× 10^{-3}) | 1.02 | Potassium (K) | 1.74 |
| Fatty acid (%)       |           |         |         |
| Capric acid (C10:0)  | ND        | γ-Linolenic acid (C18:3n6) | ND    |
| Undecanoic acid (C11:0) | ND | Arachidic acid (C20:0) (× 10^{-3}) | 5.44 |
| Lauric acid (C12:0) (× 10^{-4}) | 3.60 | Palmitic acid (C20:1) (× 10^{-3}) | 7.34 |
| Myristic acid (C14:0) (× 10^{-3}) | 6.63 | Eicosa-8,11,14-trienoic acid (C20:3n3) (× 10^{-5}) | 9.78 |
| Myristic acid (C14:1n5) | ND | Dihomo-gamma-linolenic acid (C20:3n6) | ND    |
| Pentadecanoic acid (C15:0) (× 10^{-2}) | 1.73 | Arachidonic acid (C20:4n6) (× 10^{-3}) | 2.71 |
| Hexadecenoic acid (C15:1n5) | ND | Eicosapentaenoic acid (C20:5n3) | ND    |
| Palmitoleic acid (C16:1n7) | 0.03 | Heptadecanoic acid (C17:0) | 1.53 |
| Heptadecanoic acid (C17:0) | 0.01 | Erucic acid (C22:1n9) (× 10^{-3}) | 1.87 |
| Heptadecenoic acid (C17:1n7) | 0.01 | Cis,13,16-Docosadienoic acid Methyl ester (C22:2) | ND    |
| Stearic acid (C18:0) | 0.18 | Docosahexaenoic acid (C22:6n3) | ND    |
| Oleic acid (C18:1n9) | 1.23 | Tricosanoic acid (C23:0) (× 10^{-2}) | 1.72 |
| Linoleic acid (C18:2n6) | 2.66 | Tetraicosanoic acid (C24:0) (× 10^{-2}) | 1.85 |
| Translinoleic acid (C18:2n6t) | ND | Nervonic acid (C24:1n9) (× 10^{-3}) | ND    |
| α-Linolenic acid (C18:3n3) | ND | Octanoic acid (C8:0) | ND    |
| **ND:** not detected.**

### Table 2. The effects of BA and Sil on the levels of ALT, AST, ADH and ALDH in serum and liver of alcohol-treated mice.

| Alcohol (13 g/kg) | Serum | Liver |
|-------------------|-------|-------|
|                   | CTRL  | Model | Sil (60 mg/kg) | BA (200 mg/kg) | BA (800 mg/kg) |
| AST (U/L)         | 127.8 ± 1.6 | 147.8 ± 2.6<sup>a</sup> | 134.2 ± 2.7 | 137.9 ± 3.0 | 133.4 ± 6.5 |
| ALT (U/L)         | 47.4 ± 0.6 | 53.5 ± 0.4<sup>a</sup> | 48.4 ± 0.7 | 51.7 ± 1.6 | 50.0 ± 0.9 |
| ALDH (μmol/L)     | 8.6 ± 0.2 | 7.4 ± 0.2<sup>a</sup> | 8.6 ± 0.1<sup>**</sup> | 10.0 ± 0.3<sup>***</sup> | 8.8 ± 0.3<sup>**</sup> |
| Liver             |       |       |               |               |               |
| AST (U/g)         | 55.0 ± 0.6 | 65.3 ± 1.8<sup>a</sup> | 57.3 ± 1.0<sup>a</sup> | 43.2 ± 2.4<sup>**</sup> | 47.3 ± 2.3<sup>**</sup> |
| ALT (U/g)         | 17.1 ± 0.3 | 20.2 ± 0.5<sup>a</sup> | 18.2 ± 0.4 | 16.5 ± 1.1<sup>a</sup> | 14.0 ± 0.7<sup>**</sup> |
| ADH (μmol/g)      | 4.8 ± 0.1 | 3.3 ± 0.1<sup>***</sup> | 5.0 ± 0.1<sup>***</sup> | 3.2 ± 0.2 | 3.3 ± 0.1 |
| ADH (mg/g)        | 2.6 ± 0.1 | 2.0 ± 0.2<sup>**</sup> | 2.8 ± 0.1<sup>***</sup> | 2.7 ± 0.2<sup>**</sup> | 3.2 ± 0.2<sup>***</sup> |

All data are presented as mean ± S.E.M. (n = 10).<sup>**</sup>p < 0.05; <sup>***</sup>p < 0.01 compared with control group; <sup>***</sup>p < 0.001 and <sup>****</sup>p < 0.0001 compared with alcohol group.

**Acute oral toxicity analysis**

Compared to the control group, there was no obvious behaviour abnormality in 4 g/kg of BA-treated mice. In this study, due to the 4 g/kg of BA is the maximum intragastric dose, its LD<sub>50</sub> must be much higher than 4 g/kg.

**Hepatoprotective effect of BA**

Compared with normal mice, the bodyweight of alcohol-only treated mice lost 18.1% significantly at 7th day (p < 0.05; Supplementary Table 2S). During the 2-week treatment period, relative to the alcohol-only treated mice, BA and Sil failed to influence the bodyweight of the mice (p > 0.05; Supplementary Table 2S).
AST and ALT serve as biomarkers for liver function, directly reflecting the degree of liver injury. ADH and ALDH are the main enzymes responsible for alcohol metabolism (Kaviarasan and Anuradha 2007). Compared with the healthy mice, extremely high levels of AST (>15.6%) and ALT (>12.9%), and low levels of ADH (23.1%) and ALDH (>14.0%) in the liver and/or serum, were noted in the alcohol-only treated mice (p < 0.05; Table 2). Compared with the alcohol-only treated mice, similar to Sil, BA only strongly enhanced the levels of ALDH (>18.9%) in the serum (p < 0.05; Table 2). In the liver, BA treatment remarkably reduced the levels of AST (>27.6%) and ALT (>18.3%) (p < 0.05; Table 2), and enhanced the levels of ADH (>35.0%) (p < 0.01; Table 2). Sil regulated the levels of AST, ADH and ALDH (p < 0.05; Table 2), but not ALT (p > 0.05; Table 2).

Heavy drinking can attenuate the oxidation of fatty acids and the deposition of fat in the blood and liver cells, resulting in the accumulation of TC and a substantial reduction in HDL (Tang et al. 2017). Compared with the non-alcohol-treated healthy mice, elevated TC levels and suppressed HDL levels were observed in the liver of the alcohol-only treated mice (p < 0.05; Figure 1), which were regulated back to their normal levels by BA (p < 0.01; Figure 1). BA at 800 mg/kg failed to enhance the hepatic levels of HDL (Figure 1(b)). Sil only enhanced the low hepatic level of HDL in the alcohol-damaged mice (p < 0.001; Figure 1(b)).

Pathological section image analysis revealed that the hepatic lobule structure was damaged in the alcohol-only treated mice, manifested by the disordered arrangement of hepatic cells, shrivelled nucleuses, swollen hepatocytes, diffuse fat vacuoles and infiltration of inflammatory cells (Figure 2(a)), which were all alleviated by BA administration (Figure 2(a)). Glomerular hyperaemia, tubular epithelial cell edema, narrow stenosis and interstitial infiltration of inflammatory cells were noted in the kidneys of the alcohol-only treated mice (Figure 2(b)). Comparatively, the alcohol-induced renal injury was significantly reduced in the BA-treated mice, and only mild edema and inflammatory cell infiltration were observed (Figure 2(b)).

**Effects of BA on inflammatory cytokines**

Alcohol causes the accumulation of inflammatory factors in the liver of mice, leading to the occurrence of hepatitis (Fung and
Table 3. The Effects of BA and Sil on anti-inflammatory activity of serum, liver in alcohol-treated mice.

|                   | CTRL                | Model               | Sil (60 mg/kg)   | BA (200 mg/kg)   | BA (800 mg/kg)   |
|-------------------|---------------------|---------------------|-----------------|-----------------|-----------------|
| **Serum**         |                     |                     |                 |                 |                 |
| CXCL13 (pg/mL)    | 499.8 ± 6.3         | 564.8 ± 10.1 *     | 504.5 ± 9.0 *   | 599.5 ± 44.7    | 480.4 ± 17.8 *  |
| YKL-40 (ng/mL)    | 52.8 ± 1.3          | 62.1 ± 2.8 *       | 52.9 ± 2.3     | 64.6 ± 4.0      | 53.8 ± 1.7 **  |
| TPO (pg/mL)       | 97.2 ± 2.3          | 86.0 ± 1.5 *       | 96.0 ± 3.1 *   | 101.4 ± 2.7 *   | 97.4 ± 4.2 *    |
| IL-7 (pg/mL)      | 87.5 ± 2.1          | 102.5 ± 2.8 *      | 95.2 ± 3.0    | 109.4 ± 2.4     | 93.7 ± 1.2     |
| PAI-1 (pg/mL)     | 924.8 ± 14.5        | 980.7 ± 13.9       | 915.0 ± 25.7   | 731.3 ± 6.6 **  | 837.6 ± 12.7 **|
| RBP4 (ng/mL)      | 38.0 ± 0.3          | 33.7 ± 0.4 *       | 36.8 ± 0.8     | 36.1 ± 0.3      | 34.3 ± 1.2     |

All data are presented as mean ± S.E.M. (n = 10).
* p < 0.05, ** p < 0.01 compared with control group; *** p < 0.05 and **** p < 0.01 compared with alcohol group.

Table 4. The effects of BA and Sil on antioxidant status of serum and liver in alcohol-treated mice.

|                   | CTRL                | Model               | Sil (60 mg/kg)   | BA (200 mg/kg)   | BA (800 mg/kg)   |
|-------------------|---------------------|---------------------|-----------------|-----------------|-----------------|
| **Serum**         |                     |                     |                 |                 |                 |
| ROS (U/mL)        | 342.2 ± 5.7         | 356.7 ± 3.6         | 329.0 ± 3.2     | 349.8 ± 15.1    | 338.6 ± 3.3     |
| MDA (nmol/mL)     | 18.4 ± 0.3          | 20.6 ± 0.5 *        | 18.8 ± 0.4      | 17.5 ± 0.4 *    | 18.4 ± 0.7 *    |
| NO (μmol/L)       | 28.7 ± 0.8          | 33.7 ± 1.1 *        | 30.5 ± 1.2      | 35.0 ± 1.6      | 32.4 ± 1.9      |
| SOD (U/mL)        | 214.7 ± 7.4         | 161.8 ± 4.3 *       | 199.2 ± 4.2 **  | 196.1 ± 5.4 **  | 183.5 ± 6.3 *   |
| GSH-Px (U/mL)     | 269.1 ± 5.9         | 233.6 ± 2.8 *       | 261.4 ± 6.0 *   | 287.8 ± 7.1 **  | 259.4 ± 6.0 *   |
| CAT (U/mL)        | 43.6 ± 0.8          | 37.2 ± 1.0 *        | 44.2 ± 0.5 *    | 46.1 ± 1.1 **   | 39.0 ± 0.7      |

The antioxidant status was measured in mice serum and liver. All data are presented as mean ± S.E.M. (n = 10).
* p < 0.05, ** p < 0.01 compared with control group; *** p < 0.05 and **** p < 0.01 compared with alcohol group.

Antioxidative effect of BA

Excessive ROS and MDA can induce lipid peroxidation and trigger degradation processes, thus affecting the cell membrane (Wen et al. 2017). NO can induce the production of cytokines and mediate the inflammatory response in the late stage of oxidative stress (Zhao et al. 2017). Extremely high levels of ROS (27.4%), MDA (16.3%), NO (13.4%) and low activities of SOD (23.5%), GSH-Px (17.9%) and CAT (21.2%) were noted in the liver of the mice with alcohol-induced hepatotoxicity compared with healthy mice (p < 0.05; Table 4), which were all restored to healthy levels by 14-day BA administration (p < 0.05; Table 4). In the acute alcohol-exposed mice, similar changes of oxidative factors were noted in the serum as in the liver samples (p < 0.05; Table 4), except for the ROS levels, which were not changed significantly. The 14-day administration of BA strongly reduced the hyper-level of MDA (>10.7%), and enhanced the hypo-levels of SOD (>13.4%), GSH-Px (>11.0%), CAT (23.9% in dose of 200 mg/kg) in the serum of the mice with alcohol-induced hepatotoxicity (p < 0.05; Table 4). Sil significantly regulated the chosen oxidative factors in both serum and liver (p < 0.05; Table 4), except for ROS, MDA and NO in the serum, and MDA and NO in the liver (Table 4).

Effects of BA on the activation of NF-κB p65

In the process of alcohol metabolism, ROS can activate the NF-κB signalling, which is responsible for accelerating the synthesis of inflammatory mediators (Zhou et al. 2018). Compared with Pyrsopoulos 2017). Here, acute alcohol exposure-induced dramatic increases in the levels of CXCL13 (17.8%), YKL-40 (28.7%), IL-7 (21.0%) and PAI-1 (18.6%), and reductions in the levels of TPO (18.2%) and RBP4 (21.8%) in the liver. These changes were strongly suppressed by BA administration, except for the TPO (p < 0.05; Table 3). Enhanced levels of CXCL13 (13.0%), YKL-40 (17.6%) and IL-7 (17.1%), and reduced levels of TPO (11.5%) and RBP4 (11.3%) in the serum, were noted in the acute alcohol-exposed mice, compared with the healthy mice (p < 0.05; Table 3). Comparatively, BA administration resulted in the reduction of serum levels of CXCL13 (14.9% in dose of 800 mg/kg), YKL-40 (13.4% in dose of 800 mg/kg), PAI-1 (>14.6%) and the enhancement of serum level of TPO (>13.3%) (p < 0.05; Table 3). The non-dose-dependent manner of BA was noted during its regulation on the levels of inflammatory cytokines. Furthermore, BA failed to influence the serum levels of IL-7 and RBP4 in the acute alcohol-exposed mice (Table 3). Sil administration significantly regulated the levels of all six chosen factors in the liver, but showed beneficial effects only on CXCL13, YKL-40 and TPO in the serum of the acute alcohol-exposed mice (p < 0.05; Table 3).
healthy mice, acute alcohol administration caused the enhancement of the mRNA levels of IKK, IκBα and NF-κB (>70.0%) in liver, which were strongly suppressed by BA and Sil (p < 0.001; Figure 3(a)). Furthermore, the enhanced phosphorylated expressions of IKKα/β, IκBα, and NF-κB p65 (>45.0%) in the liver of the mice induced by acute alcohol exposure were all successfully suppressed after 14-day BA and Sil administration (p < 0.01; Figure 3(b)).

Discussion

In this study, we confirmed the hepatoprotective effect of BA in mice with acute alcohol-induced hepatotoxicity, and clarified the mechanisms related to oxidative stress-mediated NF-κB signalling. BA, an edible fungus, contains 21 fatty acids, 17 amino acids and 13 minerals, indicating its extremely high nutritive value. The detection of zero or low levels of heavy metals suggests that BA is safe for consumption. Potential antioxidants such as vitamins, total polyphenols, and total sterols were detected in BA, which regulate oxidative stress in the body and reduce ROS production, thereby inhibiting the activation of the NF-κB signalling pathway. The total flavonoids in Polygoni Perfoliati Herba previously showed anti-lipid peroxidation and anti-inflammation effects in mice with alcohol-induced liver injury (Ya et al. 2016). A triterpene from the bark of Betula platyphylla Suk. (Betulaceae) alleviates alcoholic liver injury, possibly through blocking fatty acid synthesis and activating the adenosine monophosphate-activated protein kinase (AMPK) signalling pathway (Bai et al. 2016). BA is rich in polysaccharides, total flavonoids, vitamins, polyphenols and triterpenoids, which provide a strong nutritional foundation for its hepatoprotective effect. Additionally, the detection of multiple active compounds in BA allows us to explain the non-dose-dependence manner during its hepatic protection, which is in fact a common feature of pharmaceutically active natural products (Ma et al. 2015).

Alcohol is first metabolized to acetaldehyde by ADH, after which acetaldehyde is metabolized to acetic acid by ALDH (Jelski et al. 2008). Heavy drinking results in a decrease of the...
activities of the ADH and ALDH enzymes, which eventually leads to over-accumulation of acetaldehyde in the liver. This triggers the immune system, resulting in inflammation and severe liver damage, highlighted by large increases in the levels of AST and ALT activity, pro-oxidation enzymes and inflammatory cytokines (Karpypak et al. 2017). Our data confirmed the protective effects of BA against acute alcohol hepatotoxicity. A large number of adipocytes were noted in the liver of the mice with acute alcohol exposure. As the synthesis and degradation of cholesterol are mainly carried out in the liver, TC levels directly reflect the liver’s reserve function (Komorov’sk’yi 1998). HDL is also mainly synthesized by the liver, and is considered as an important indicator of coronary artery disease in clinics (Ayhan et al. 2017). BA significantly enhanced the HDL levels and reduced the TC content in the liver, indicating its regulation of the abnormal alternations of lipid metabolism caused by acute alcohol exposure.

It has been confirmed that BA has the regulation effect on oxidative stress (Zhang et al. 2018), which is recognized as a crucial causal factor of acute alcohol-induced liver injury. This is especially the case when the liver has lower levels of antioxidant protection to cope with the generation of ROS (Diaz-Aguirre et al. 2016). ROS and MDA cause oxidative damage to biological macromolecules including nucleic acids and proteins (Sun et al. 2018). As another important factor of oxidative stress, NO is responsible for the formation of hydroxyl radicals, which contribute to alcohol-induced liver damage (Yamasaki et al. 2001). Encouragingly, BA not only reduced the hyper-levels of oxidative factors, but also increased the activities of antioxidative factors in the serum and liver of mice with acute alcohol injury. The nitrosoimide adenosine dinucleotide phosphate (NADPH) oxidase subunits, but also increased the activities of antioxidative factors in the serum and liver of mice with acute alcohol injury. The nitrosoimide adenosine dinucleotide phosphate (NADPH) oxidase complex converts O$_2$ to superoxide (O$_2^-\:\:\:\:\:\:\:\\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\\:\:\:\:\:\:\:\:\:\:\:\:\:\:\\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\:\\\..
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