Apocynum venetum L. (A. venetum) has long been used in oriental folk medicine for the treatment of some liver diseases; however, the underlying mechanisms remain to be fully elucidated. Acetaminophen (APAP) is a widely used analgesic drug that can cause acute liver injury in overdose situations. In this study, we investigated the potential protective effect of A. venetum leaf extract (ALE) against APAP-induced hepatotoxicity. Mice were intragastrically administered with ALE once daily for 3 consecutive days prior to receiving a single intraperitoneal injection of APAP. The APAP group showed severe liver injury characterized by the noticeable fluctuations in the following parameters: serum amino-transferases; hepatic malondialdehyde (MDA), 3-nitrotyrosine (3-NT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione (GSH). These liver damages induced by APAP were significantly attenuated by ALE pretreatments. A collective analysis of histopathological examination, DNA laddering and western blot for caspase-3 and cytochrome c indicated that the ALE is also capable of preventing APAP-induced hepatocyte death. Hyperoside, isoquercitrin and their derivatives have been identified as the major components of ALE using HPLC-MS/MS. Taken together, the A. venetum possesses hepatoprotective effects partially due to its anti-oxidant action.

Keywords: Apocynum venetum; Flavonoids; Acetaminophen; Hepatotoxicity; Oxidative Stress; HPLC-MS/MS.

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Drug-induced liver injury has become the leading cause of acute liver failure (ALF), of which acetaminophen (APAP) is the most causative agent (de Achaval and Suarez-Almazor, 2011). APAP (chemically N-Acetyl-p-Aminophenol) is a widely used analgesic and antipyretic drug that can cause ALF in humans when consumed in overdoses (Larson et al., 2005). Approximately, 10% of APAP is metabolized in liver by cytochrome P450 (CYP450) and converted into a highly reactive electrophilic molecule called N-acetyl-para-benzoquinone-imine (NAPQI) (Bunchorntavakul and Reddy, 2013). This toxic metabolite is responsible for the generation of oxidative stress associated with APAP-induced liver injury. Excessive NAPQI directly depletes the cellular storage of glutathione (GSH), subsequently adducting proteins and initiating lipid peroxidation, thus could lead to massive hepatocyte death in the centrilobular zone (James et al., 2003). This pathological process is accompanied by various biological changes including reactive species (RS) accumulation, mitochondrial dysfunction, caspase-3 activation, DNA fragmentation and necrotic/apoptotic cell death (Cover et al., 2005; McGill et al., 2012a; Xie et al., 2014). APAP overdose in rodents also served as a standard model for liver injury, which is frequently used to evaluate the hepatoprotective potential of new chemical drugs and herbal therapeutics (McGill et al., 2012b). Many compounds have been tested for their potential to reduce APAP intoxication, and those possessing anti-oxidant properties are of particular interest, (Ojo et al., 2006) including natural compounds (Wang et al., 2010; Yousef et al., 2010; Ji et al., 2013; Lu et al., 2013; Noh et al., 2013) and medicinal plants (Mamat et al., 2013; Yuan et al., 2010).

The dried leaves of Apocynum venetum L. (A. venetum, Apocynaceae family) are commonly known as Luobuma in China. It is one of the popular herbs of traditional Chinese medicine and Uygur medicine that is used for calming liver, soothing nerves, dissipating heat and promoting the diuresis. Currently, the Luobuma is not only used as an effective medicine but also as a popular dietary supplement both in East Asia and North America (Xie et al., 2012). Published studies show that, A. venetum possess a wide variety of health benefits, including the antihypertensive (Kim et al., 2000a; Tagawa et al., 2004; Kwan et al., 2005), antihyperlipidemic (Yokozawa and Nakagawa, 2004), free radical scavenging (Kim et al., 2000b; Yokozawa et al., 2002; Cao et al., 2003; Liang et al., 2010), antidepressant (Butterweck et al., 2001, 2003; Shirai et al., 2005; Zheng et al., 2011; Kuo et al., 2011; Zheng et al., 2012) and anxiolytic (Grundmann et al., 2007, 2009) effects. A. venetum leaf extract (ALE) has also been proven for its protective effect against chemicals-induced toxicity including carbon tetrachloride, D-galactosamine/lipopolysaccharide and D-galactosamine/TNF-α-induced liver injury (Xiong et al., 2000). Yang et al. (2009) demonstrated that ALE has protective effect against fatty liver disease of rats with metabolic syndrome. The potent anti-oxidant and radical scavenging activities of flavonoids present in ALE were considered to play a major role in preventing liver disease (Xiong et al., 2000; Yang et al., 2009), however, active constituents of ALE and the underlying mechanisms remain to be fully established. Hence, the present study was aimed to investigate the effect of ALE on liver injury induced by APAP overdose and to explore its possible mechanism.
Materials and Methods

Preparation of ALE

The leaves of *A. venetum* were collected in Lop Nur, Xinjiang, China in June 2011 and were authenticated by the Botanist Dr. Jianjun Hu, Xinjiang Production & Construction Crops Key Laboratory of Protection and Utilization of Biological Resources, Xinjiang, China. A total of 50 g of dried leaves were extracted twice with 300 mL of 70% ethanol in a refluxed condenser for 1 h each. The obtained extracts were combined and evaporated to dryness (12.5 g). Dried extract was dissolved in 20 mL hot water and then filtered. The filtrate was chromatographed on a macroporous resin (D101, Cangzhou BonChem, China) column and eluted sequentially with water and 70% ethanol. The aqueous ethanol fraction was collected, frozen, lyophilized to dryness (2.5 g) and stored at 4°C for further studies.

HPLC-ESI-MS/MS Analysis

The extract was reconstituted in a mixture of 0.1% formic acid and acetonitrile containing 0.1% formic acid at a ratio of 50:50 (v/v) to a final concentration of 10 mg/mL. The 10 μL aliquot of the extract was separated on a Waters Xterra® MS C18 column (4.6 × 150 mm, 3.5 μm) at 30°C using an Agilent 1200 series HPLC system (Agilent Technologies, USA) with a gradient mobile phase composed of 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). A gradient was optimized as follows: 0–5 min, 0%–10% B; 5–14 min, 10%–15% B; 14–19 min, 15%–20% B; 19–35 min, 20% B. The flow rate was fixed at 0.8 mL/min and detection wavelength was set at 254 nm. Mass spectrometry was performed on an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, USA) run in the positive ion mode throughout the m/z 50–1,000 range under following conditions: gas temperature, 350°C; drying gas flow rate, 10 L/min; nebulizer pressure, 40 psi; and the capillary voltage, 4,000 V.

Animals

All animal experimental protocols were reviewed and approved by the Ethics Committee of Northwest A&F University for the use of Laboratory Animals. Six weeks old male Kunming mice (20–25 g) were purchased from the Experimental Animal Center of the Fourth Military Medical University (Xi’an, China). All animals were acclimatized to the laboratory environment for 1 week before the experiment. Mice were allowed to freely access to drinking water and food under constant room temperature (22 ± 2°C) and humidity (50% ± 10%) conditions with an automatic 12 h light and 12 h dark cycle.

Treatment of Animals

Mice were intragastrically administered ALE (50 or 100 mg/kg) once daily for 3 consecutive days. For mortality experiment, mice were intraperitoneally injected with 500 mg/kg
of APAP and the survival rate was observed for 7 days \((n = 8/\text{group})\). In order to demonstrate the protective effects of ALE, the mice were injected intraperitoneally with 300 mg/kg of APAP after 1 h from the last administration of ALE \((n = 8/\text{group})\). Twenty-four hours after the APAP challenge, the experimental animals were weighted and sacrificed to collect blood and liver tissue samples. The serum was separated from the blood samples by centrifugation at 3,000 rpm for 10 min at 4°C and stored at −20°C until further analysis. The right lobe of liver was fixed in 10% formalin for morphological analysis. The remaining liver tissues were thoroughly washed in cold PBS and divided into small portions, snap frozen in liquid nitrogen and stored at −80°C for biochemical analysis.

**Serum ALT and AST Assays**

Enzymatic activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated spectrophotometrically using commercial diagnostic kits (Nanjing Jiancheng Institute of Biotechnology, China).

**Hepatic MDA, SOD, GSH, GPx and GR Determinations**

Frozen liver tissues were thawed and homogenized in ice-cold PBS. The homogenate was centrifuged at 3,000 rpm for 10 min at 4°C and the supernatants were assayed for malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and GSH levels using the commercially available assay kits as per manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, China). The protein concentrations in tissue homogenates were measured by Bradford protein assay using bovine serum albumin as the standard (Tiangen Biotech, China).

**Histology and Immunostaining**

Liver tissues were fixed in 10% formalin and embedded in paraffin for histological assessment. Samples were subsequently sectioned \((5 \mu m)\), stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus, Japan). The detection of nitrotyrosine (NT) protein adducts was performed using ultrasensitive immunohistochemistry kit (Fuzhou Maxim Biotech, China) with anti-3-NT antibody \((1:400)\) (Abcam, UK), cleaved caspase-3 \((1:100)\) and cleaved caspase-8 \((1:100)\) (Boster, China), respectively.

**Western Blot Analysis**

Liver tissues were homogenized in ice-cold lysis buffer (Beyotime Institute of Biotechnology, China) containing 1 mM PMSF and then centrifuged at 12,000 \(g\) for 10 min at 4°C. The protein concentration was determined using the BCA assay kit (Beyotime Institute of Biotechnology, China). Proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore Corporation, USA). After blocking in TBST containing 5%
skimmed milk powder, the membranes were incubated overnight at 4°C with primary antibodies against cleaved caspase-3 (1:200), cytochrome c (1:200) (Boster, China) and glyceraldehyde phosphate dehydrogenase (GAPDH, 1:1,000) (Tianjin Sungene Biotech, China). Bolts were then incubated with a 1:3,000 dilution of horseradish peroxidase-conjugated secondary antibodies (Tianjin Sungene Biotech, China) for 2 h at room temperature. Protein bands were visualized by ECL reaction (Genshare Biological, China). The protein levels were quantified using Gel-Pro Analyzer software (Media Cybernetics, USA) and normalized to GAPDH.

**DNA Fragmentation Analysis**

DNA was extracted using a DNA ladder extraction kit with spin column (Beyotime Institute of Biotechnology, China). Ten microliters of the DNA sample was separated on a 1.0% agarose gel containing 0.5 μg/mL ethidium bromide and the DNA band pattern was visualized by the UV transilluminator (UVP, UK).

**Statistical Analysis**

All the experimental data are expressed as mean ± SD. The significant difference from the respective control in all experiments was assessed by one-way analysis of variance (ANOVA) using SPSS (IBM Corporation, USA). Values of $p < 0.05$ were considered as statistically significant.

**Results**

**Identification of Compounds in the ALE**

As shown in HPLC chromatogram and total ion chromatogram of ALE (Fig. 1), a total of 17 constituents were separated, in which 16 constituents were identified by comparing the retention times, UV and mass spectra with the data available in previously published literature (Kamata et al., 2008; Liu et al., 2009; Lin et al., 2012; An et al., 2013; Zhao et al., 2014). Peaks 1–10 and 12–17 were identified as quercetin-3-O-β-D-glucosyl-β-D-glucopyranoside, gallicatechin or epigallocatechin, chlorogenic acid, catechin, myricetin, rutin, hyperoside, isoquercitrin, malonated hyperoside, trifolin, acetylated hyperoside, malonated isoquercitrin, astragalin, acetylated isoquercitrin, quercetin and kaempferol, respectively (Table 1). Based on the peak areas of the HPLC chromatogram, hyperoside, isoquercitrin, acetylated hyperoside and malonated isoquercitrin were identified as the major constituents within ALE. Peak 11 need to be further investigated.

**Effects of ALE on APAP-Induced Mortality**

Mice were observed for 7 days to determine the effects of ALE on survival rate of mice following APAP overdose. In the group intoxicated with 500 mg/kg of APAP (lethal dose)
without any pre-treatment, 67% of mice were died within a day (24 h) and the remaining mice all died by day 2 (48 h) (Fig. 2). Pretreatment with ALE drastically extended the survival rate after the lethal APAP dose. The survival rate increased to 75% in the ALE50 + APAP group, while over 80% of the mice survived in the ALE100 + APAP group.

Figure 1. HPLC-MS/MS analysis of A. venetum extract. HPLC chromatogram (A) and total ion chromatogram in positive mode (B) of ALE constituents. Peak numbers refer to Table 1.
Table 1. Characterization of Compounds Separated from the ALE

<table>
<thead>
<tr>
<th>Peak</th>
<th>RT (min)</th>
<th>[M + H]+ (m/z)</th>
<th>Major Fragment Ion (m/z)</th>
<th>Compound Identified</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.10</td>
<td>627.1</td>
<td>303.0, 465.1</td>
<td>Quercetin-3-O-β-D-glucosyl-β-D-glucopyranoside</td>
<td>Zhang et al., 2010; An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>2</td>
<td>9.45</td>
<td>307.0</td>
<td>139.0</td>
<td>Gallocatechin or epigallocatechin</td>
<td>Kamata et al., 2008; Zhao et al., 2014</td>
</tr>
<tr>
<td>3</td>
<td>10.25</td>
<td>355.1</td>
<td>193.0</td>
<td>Chlorogenic acid</td>
<td>Zhang et al., 2010; Zhao et al., 2014</td>
</tr>
<tr>
<td>4</td>
<td>13.91</td>
<td>291.1</td>
<td>123.0, 139.0, 164.8</td>
<td>Catechin</td>
<td>Kamata et al., 2008; Liu et al., 2009; Zhao et al., 2014</td>
</tr>
<tr>
<td>5</td>
<td>15.87</td>
<td>319</td>
<td>180.8, 152.8</td>
<td>Myricetin</td>
<td>Lin et al., 2012; Zhao et al., 2014</td>
</tr>
<tr>
<td>6</td>
<td>16.65</td>
<td>611</td>
<td>303.0, 465.1</td>
<td>Rutin</td>
<td>An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>7</td>
<td>18.04</td>
<td>465.1</td>
<td>303.0</td>
<td>Hyperoside</td>
<td>Zhang et al., 2010; An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>8</td>
<td>18.37</td>
<td>465.1</td>
<td>303.0</td>
<td>Isoquercitrin</td>
<td>Zhang et al., 2010; An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>9</td>
<td>20.1</td>
<td>551.1</td>
<td>507.1, 303.0, 465.0</td>
<td>Malonated Hyperoside</td>
<td>Kamata et al., 2008; An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>10</td>
<td>20.51</td>
<td>449.1</td>
<td>287.0</td>
<td>Trifolin</td>
<td>Zhang et al., 2010; An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>11</td>
<td>21.41</td>
<td>611.0</td>
<td>303.0, 465.2</td>
<td>Not identified</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>22.07</td>
<td>507.1</td>
<td>303.0, 465.0</td>
<td>Acetylated Hyperoside</td>
<td>Zhang et al., 2010; An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>13</td>
<td>23.87</td>
<td>551.1</td>
<td>303.0</td>
<td>Malonated Isoquercitrin</td>
<td>An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>14</td>
<td>25.17</td>
<td>449.0</td>
<td>287.0</td>
<td>Astragalin</td>
<td>Zhang et al., 2010; An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>15</td>
<td>26.02</td>
<td>507.1</td>
<td>303.0</td>
<td>Acetylated Isoquercitrin</td>
<td>Zhang et al., 2010; An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>16</td>
<td>27.29</td>
<td>303.0</td>
<td>151.0, 273.0, 257.0</td>
<td>Quercetin</td>
<td>Zhang et al., 2010; An et al., 2013; Zhao et al., 2014</td>
</tr>
<tr>
<td>17</td>
<td>29.46</td>
<td>287.0</td>
<td>229.0, 257.0</td>
<td>Kaempferol</td>
<td>An et al., 2013; Zhao et al., 2014</td>
</tr>
</tbody>
</table>

Figure 2. Effects of ALE on the survival rate following APAP overdose. Mice were administered with ALE (50 and 100 mg/kg, i.g.) for 3 days prior to a single injection of APAP (500 mg/kg, i.p.), and the survival was monitored for 7 days (n = 8).
group (Fig. 2). Death was not observed in the group treated with ALE alone during the experimental period (not shown).

**ALE Protects Against APAP-Induced Hepatic Dysfunction**

Plasma AST and ALT activities were significantly increased in the APAP group, confirming the hepatotoxicity of APAP overdose (Fig. 3). In the group that received APAP after pretreatment with ALE (50 and 100 mg/kg), the ALT and AST activities were significantly reduced compared to those of the APAP group, indicating that ALE prevented APAP-induced liver toxicity. The ALE100 group did not show any hepatotoxic effect (Fig. 3).

### Table 2. Effects of ALE on the Oxidative Damage Markers in Liver Homogenate after APAP Overdose

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/mg prot)</th>
<th>SOD (U/mg prot)</th>
<th>GSH (nmol/mg prot)</th>
<th>GPx (U/mg prot)</th>
<th>GR (U/mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.59 ± 0.14</td>
<td>229.60 ± 14.27</td>
<td>38.97 ± 6.00</td>
<td>387.75 ± 10.75</td>
<td>30.02 ± 4.87</td>
</tr>
<tr>
<td>ALE100</td>
<td>1.66 ± 0.20</td>
<td>218.81 ± 7.82</td>
<td>40.56 ± 5.22</td>
<td>380.76 ± 16.35</td>
<td>33.62 ± 4.23</td>
</tr>
<tr>
<td>APAP</td>
<td>3.23 ± 0.31***</td>
<td>96.08 ± 12.65***</td>
<td>14.90 ± 3.66***</td>
<td>250.03 ± 29.00***</td>
<td>15.50 ± 1.79***</td>
</tr>
<tr>
<td>ALE50 + APAP</td>
<td>1.92 ± 0.30***</td>
<td>164.37 ± 21.21***</td>
<td>27.82 ± 2.78***</td>
<td>341.78 ± 16.02***</td>
<td>25.45 ± 4.68***</td>
</tr>
<tr>
<td>ALE100 + APAP</td>
<td>1.73 ± 0.33***</td>
<td>193.48 ± 7.32***</td>
<td>34.75 ± 5.70***</td>
<td>363.09 ± 19.96***</td>
<td>29.39 ± 4.83***</td>
</tr>
</tbody>
</table>

*Notes: Mice were administered with ALE (50 and 100 mg/kg, i.g.) for 3 days prior to a single injection of APAP (300 mg/kg, i.p.), and were sacrificed at 24 h after APAP treatment. Data are expressed as mean ± SD, n = 8. **p < 0.01 and ***p < 0.001 compared to the control group; *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the APAP group.*
ALE Inhibited APAP-Induced Oxidative Stress in Liver

APAP administration has resulted in a significant increase in MDA level, while SOD activity was markedly decreased as compared with the control group (Table 2). Treatment with ALE normalized the activity levels of MDA and SOD in liver homogenate after 24 h. In addition, APAP decreased the liver homogenate GSH level as well as GPx and GR activities when compared to the control. ALE administration prevented the depletion of the GSH level and the decline of GPx and GR activities.

Figure 4. Effects of ALE on histological alterations in the liver after APAP overdose. Mice were administered with ALE (50 and 100 mg/kg, i.g.) for 3 days prior to a single injection of APAP (300 mg/kg, i.p.). Livers were processed for histological examination 24 h after APAP injection. Typical images were chosen from each experimental group. (Original magnification: 40 × 10) (A) Control, (B) APAP, (C) ALE50 + APAP, (D) ALE100 + APAP, (E) Quantitative analysis of necrotic area. Data are presented as mean ± SD, n = 8. ***p < 0.001 compared to the control group, ###p < 0.001 compared to the APAP group.
ALE Alleviated APAP-Induced Histopathology Changes in Liver

Histology of liver tissue from the control mice showed normal hepatic architecture. Hepatocytes were polyhydral in shape with central rounded vesicular nuclei while few cells were binucleated. The cytoplasm was acidophilic. Hepatocytes were arranged in plates around a central vein and separated by blood sinusoids lined by endothelial cells and Kupffer cells (Fig. 4A). Groups treated with ALE alone showed the same normal histology of the control (not shown).

Liver sections of mice that received APAP alone revealed widespread hepatocellular damage. Almost half of the centrilocular hepatocytes were swollen with marked cytoplasmic

![Figure 5](image)

Figure 5. Effects of ALE on NT formation after APAP overdose. Mice were administered with ALE (50 and 100 mg/kg, i.g.) for 3 days prior to a single injection of APAP (300 mg/kg, i.p.). Livers were processed for immunostaining 24 h after APAP injection. Typical images were chosen from each experimental group. (Original magnification: 40 × 10) (A) Control, (B) APAP, (C) ALE50 + APAP, (D) ALE100 + APAP, (E) Quantitative analysis of 3-NT expression. Data are presented as mean ± SD, n = 8, *p < 0.05, **p < 0.001 compared to the control group, and ***p < 0.001 compared to the APAP group.
Figure 6. Western blot analysis of effects of ALE on activation of caspase-3 and cytochrome c after APAP overdose. Mice were administered with ALE (50 and 100 mg/kg, i.g.) for 3 days prior to a single injection of APAP (300 mg/kg, i.p.). Livers were obtained after 24 h intoxication. The expression of cytochrome c and cleaved caspase-3 were detected by western blot analysis. GAPDH was used as an internal control for normalization. The western blot (A) represents one of three independent experiments with similar results. Relative amount for cytochrome c (B) and cleaved caspase-3 (C) was quantified by densitometric analysis with Gel-Pro Analyzer software and normalized to GAPDH expression. The control is set as 1.0 value. Each value represents the mean ± SD, n = 3. ***p < 0.001 compared to the control group, ##p < 0.01, and ###p < 0.001 compared to the APAP group.
Figure 7. Immunochemistry of effects of ALE on caspase-8 and caspase-3 cleavage after APAP overdose. Mice were administered with ALE (50 and 100 mg/kg, i.g.) for 3 days prior to a single injection of APAP (300 mg/kg, i.p.). Livers were processed for immunostaining 24 h after APAP injection. Typical images were chosen from each experimental group. (Original magnification: 40 × 10) (A–D) activation of caspase-8 in (A) Control, (B) APAP, (C) ALE50 + APAP, and (D) ALE100 + APAP groups; (E) quantitative analysis of cleaved caspase-8. (F–I) activation of caspase-3 in (F) Control, (G) APAP, (H) ALE50 + APAP, and (I) ALE100 + APAP groups; (J) quantitative analysis of cleaved caspase-3. Data are presented as mean ± SD, n = 8. *p < 0.05 and ***p < 0.001 compared to the control group; ****p < 0.001 compared to the APAP group.
vacuolation and condensed nuclei. The intervening hepatic sinusoids were congested. In addition, inflammatory cell infiltration was observed (Fig. 4B).

Pretreatment with ALE markedly attenuated the APAP-induced necrotic lesions. Only a small number (about 10%) of hepatocytes exhibited cytoplasmic vacuolation and dark nuclei in the ALE50 + APAP group (Fig. 4C). The hepatic architecture of the ALE100 + APAP group appeared almost like the control hepatocytes, however the swelling of liver cells were observed in the centrilobular zone (Fig. 4D).

**ALE Inhibited APAP-Induced 3-Nitrotyrosine (3-NT) Adduction**

APAP significantly increased 3-NT protein adduction that was dominantly expressed in the hepatocytes of the centrilobular region (Fig. 5B), whereas, no such expression was observed in control group (Fig. 5A) and ALE alone group (not shown). Pre-administration of ALE at the doses of 50 and 100 mg/kg markedly decreased the amount of 3-NT adduction by 79% and 92% when compared with the APAP group (Figs. 5C and 5D).

**ALE Reduced APAP-Induced Caspase-8, Caspase-3 Activation and Cytochrome C Release**

As shown in Figs. 6 and 7, cleaved caspase-8, cleaved caspase-3 and cytochrome c levels were significantly elevated in the APAP-treated mouse liver. ALE was able to inhibit
APAP-induced cleaved activation of caspase-8, caspase-3 and also prevented release of cytochrome c into the cytosol when compared to the APAP group.

**ALE Inhibited APAP-Induced Nuclear DNA Damage**

Genomic DNA fragmentation was assessed in the liver tissues as shown in Fig. 8. Loss of large genomic DNA with concomitant appearance of a ladder-like fragmentation pattern typical of apoptosis was observed following the APAP treatment. High molecular weight DNA was clearly remained in the ALE group compared to APAP group.

**Discussion**

*A. venetum* has long been used in oriental folk medicine for the treatment of some liver diseases. Previous studies demonstrated that *A. venetum* could prevent xenobiotic compounds induced liver injury as well as fatty liver disease. It has been considered that the anti-oxidant properties of *A. venetum* were responsible for its hepatoprotective effects (Xiong et al., 2000; Yang et al., 2009).

In our present study, pretreatment with ALE significantly improved the survival rates of mice that were intoxicated with a lethal dose of APAP (500 mg/mL), as well as attenuated APAP overdose (300 mg/mL) induced the increase of serum aminotransferase and hepatic histopathological lesions. Thus, suggesting that ALE possess the ability to prevent APAP-induced hepatotoxicity.

Reactive oxygen species (ROS) generation preceded by NAPQI-induced GSH depletion, plays a critical role in APAP toxicity (Hinson et al., 1995; Brown et al., 2012). A key protective action against APAP-induced liver injury is through anti-oxidant enzymes, including GPx, GR and SOD. APAP overdose has previously been demonstrated to reduce anti-oxidant enzyme activities and a few treatments have been tested to recover enzymatic function such as NAC (Wang et al., 2010; Carvalho et al., 2013) and SAMe (Brown et al., 2012). Our results confirmed that APAP depleted the liver homogenate GSH level, increased MDA level and also depressed the GPx, GR, and SOD activities, suggesting the impairment in the liver homogenate redox homeostasis, accumulation of ROS and formation of lipid peroxidation. The mice treated with ALE were able to normalize the levels of GSH and MDA, and returned the activities of GPx, GR and SOD in the liver homogenate. These results suggested that ALE protects against APAP toxicity by maintaining the markers of oxidative damage at control levels, which may be due to the powerful anti-oxidant and free radical scavenging activities of various flavonoids (Xiong et al., 2000; Yang et al., 2009).

In addition to generating ROS, APAP also causes the production of reactive nitrogen species (RNS), leading to NT adduction of proteins (Hinson et al., 2000). Numerous proteins associated with anti-oxidant defense, energy supply or fatty acid metabolism are nitrated in the APAP exposed liver (Abdelmegeed et al., 2013). Immunochemistry staining with anti-3-NT antibody confirmed that APAP-induced massive NT adduction that was localized to the centrilobular region where APAP toxicity was most prevalent. ALE
pretreatment significantly decreased 3-NT adducts, inhibiting the APAP mediated nitration of tyrosine residues in proteins.

APAP hepatotoxicity is a necrotic event associated with caspase activation involved apoptosis (Kon et al., 2004). Both apoptotic and necrotic cell death are preceded by a rapid release of cytochrome c from mitochondrial to cytoplasm (Jemmerson et al., 2002), followed by the activation of caspases in apoptotic cell death but not in necrotic cell death (Li et al., 1999). Cytochrome c release is also used to assess mitochondrial function associated with APAP toxicity. Caspase-8 can initiate apoptosis directly by cleaving and thereby activating executioner caspases (McIlwain et al., 2013). Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly(ADP-ribose)polymerase (PARP) (D’Amours et al., 1998) and DNA fragmentation factor-45/inhibitor of caspase-activated DNase (Wolf et al., 1999), culminating with DNA damage and cell death. Our results showed that APAP greatly enhanced cytochrome c leakage, as well as activated caspase-8 and caspase-3. Simultaneously, APAP produced massive hepatocellular genomic DNA fragmentation and cytoplasmic vacuolation. Consistent with the protection observed by ALE for the anti-oxidant enzyme activities, ALE treatment decreased the expression levels of cytochrome c, suppressed caspase-8 and caspase-3 cleavage, as well as inhibited nuclear DNA damage in the liver of APAP mice, suggesting that ALE protects against APAP-induced hepatocyte necrosis and apoptosis.

HPLC-MS/MS results confirmed that ALE is rich in flavonoids with hyperoside, isoquercitrin, acetylated hyperoside and malonated isoquercitrin as major constituents. It has been shown that hyperoside and isoquercitrin possess potent anti-oxidative and free radical scavenging activities (Kim et al., 2000b; Sukito and Tachibana, 2014). Hyperoside has protective effects against CCl4-induced mice liver injury when administrated intraperitoneally (100 mg/kg) 30 min before and 2 h after CCl4 treatment through the enhancement of the anti-oxidative defense system (Choi et al., 2011). Administration of isoquercitrin (230 mg/kg, i.p., 30 min after Cd2+ treatment) effectively restored liver and kidney function following Cd2+ intoxication, which attributed to the scavenging abilities of isoquercitrin for ROS and RNS (Li et al., 2011). Acetylated hyperoside and malonated isoquercitrin are the derivatives of hyperoside and isoquercitrin, respectively. The biological activities of these flavonoids’ derivatives have been rarely investigated. It is speculated that they were biotransformed to their corresponding glycosides hyperoside and isoquercitrin by metabolic enzymes or intestinal microflora after oral administration (Valentová et al., 2014). However, further investigations are indispensable to support this hypothesis. Since theoretically calculated amounts of hyperoside and isoquercitrin received by mice were approximately equal to 15.7 mg/kg and 28.9 mg/kg respectively, which were less than the mentioned doses in previous reports (Choi et al., 2011; Li et al., 2011). We speculate that these two compounds might be partly involved in the hepatoprotective function of A. venetum. Further investigations to elucidate the participation of individual component(s) from ALE in this hepatoprotective effect are currently in progress.

In addition to the direct anti-oxidant properties, attention has also been paid to the influence of flavonoids on CYP450 enzymes (Breinholt et al., 2002; Melo-Filho et al.,
2014). Published literature has shown the protective effects of oleanolic acid and puerarin against CCl₄-induced hepatotoxicity through the inhibition of CYP2E1, thus possibly reducing the level of ROS generated by CCl₄ bioactivation (Jeong, 1999; Hwang et al., 2007). Studies have been conducted regarding the expression of CYP2E1 in mice treated with APAP (Zhang et al., 2010), revealing that inhibition of CYP2E1 activity and expression can alleviate APAP-induced hepatotoxicity (Ganetsky et al., 2013; Gum and Cho, 2013). It had been confirmed that flavonoids such as kaempferol, quercetin and myricetin were present in ALE; they were able to inhibit the activity of CYP2E1 in vitro and in vivo (Oliveira et al., 2002; Shih et al., 2013; Tang et al., 2013). Hyperoside and isoquercitrin were also reported to alter the expressions of some CYP 450 enzymes, (Song et al., 2013; Vrba et al., 2012) although their activity on CYP2E1 is still unclear. In the present study, 3 consecutive days administration of ALE prior to APAP might have suppressed the expression of CYP2E1 and thereby reduced oxidative stress generated by APAP metabolization.

Conclusions

The present study provides evidence that ALE exerted beneficial effects against APAP hepatic toxicity. The hepatoprotection mediated by ALE was in part due to its anti-oxidant abilities for scavenging free radicals, maintenance of cellular anti-oxidants levels and anti-oxidant enzymes activities. Besides, ALE inhibited APAP-induced hepatocyte apoptosis by suppressing cytochrome c release, caspase activation and DNA fragmentation. In conclusion, ALE is a potent candidate in liver APAP toxicity protection.

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