A Polyphenol Extract of *Hibiscus sabdariffa* L. Ameliorates Acetaminophen-Induced Hepatic Steatosis by Attenuating the Mitochondrial Dysfunction in Vivo and in Vitro

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Oxidative stress is the major contributor to acetaminophen (AAP)-caused liver damage. It promotes mitochondrial oxidative stress and collapses the mitochondrial membrane potential to cause cell death. We have previously shown that a polyphenol extract of *Hibiscus sabdariffa* L. (HPE) potentiated the antioxidative effect. We further examined in this study the possible mechanism of HPE against AAP-caused liver damage. BABL/c mice were orally fed with HPE (100, 200 or 300 mg/kg) for two weeks prior to an i.p. injection of 1000 mg/kg of AAP. The mice were decapitated 6 h after the AAP injection to collect the blood and liver for further determination. The results show that pretreating with HPE increased the level of glutathione (GSH), decreased the level of lipid peroxidation, and increased catalase activity in the liver. A histopathological evaluation shows that HPE could decrease AAP-induced liver steatosis accompanied by a decreased expression of AIF, Bax, Bid, and p-JNK in the liver. An *in vitro* assay revealed that HPE could reduce AAP-induced death of BABL/c normal liver cells (BNLs), reverse the lost mitochondrial potency and improve the antioxidative status, similarly to the results of the *in vivo* assay. We show in this study that HPE possessed the ability to protect the liver from AAP-caused injury. The protective mechanism might be regulated by decreasing oxidative stress and attenuating the mitochondrial dysfunction.

Key words: acetaminophen; glutathione; polyphenol; *Hibiscus sabdariffa*; apoptosis inducing factor

Acetaminophen (AAP) is widely used as an analgesic and antipyretic agent. AAP at a normal dose is conjugated by glucuronic acid and eliminated in bile to be metabolized. AAP is toxic to liver at 7.0 grams in one day for adults or 150 mg/kg for children. An overdose of AAP is metabolized by cytochrome P-450 to form the reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI). It is conjugated with glutathione, and this reaction causes glutathione depletion to induce the generation of reactive oxygen species (ROS). Such AAP mechanisms as causing a mitochondrial dysfunction and inducing ROS generation are therefore involved in hepatotoxicity.1,2 These events may initiate direct damage to hepatocytes and cause cell apoptosis or necrosis.

Previous studies have shown that the mitochondrial pathway, mitochondrial permeability transition (MPT), can regulate AAP-induced necrotic and apoptotic death.3 AAP can also induce the pro-apoptotic Bcl-2 family members, Bax and the truncated form of Bid, to translocate to mitochondria and lead to the onset of cell apoptosis.4 Recent studies have shown oxidative stress to be responsible for activating c-Jun N-terminal kinase (JNK); activated JNK (pJNK) would be translocated to the mitochondrial membrane and further enhance the oxidative stress to rupture the inner and outer mitochondrial membrane, and lose the cell mitochondrial membrane potential.5–7 Such mitochondrial membrane proteins as the apoptosis inducing factor (AIF) can be released to the cytosol and translocated to the nucleus to cause large-scale DNA fragmentation and cell death.5,8,9 These factors indicate that decreasing oxidative stress to retain the mitochondrial function might be a promising strategy in AAP-induced liver injury.

A wide variety of phenolic substances present in dietary and medicinal plants possess striking antioxidative and anti-inflammatory properties. The flower of *Hibiscus sabdariffa* Linnaeus calyx (the Malvaceae family and local name Karkaday) is commonly used in cold and hot beverages and as a supplement due to its perceived potential of health benefits. Our previous

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Abbreviations: AAP, acetaminophen; NAPQI, N-acetyl-p-benzoquinone imine; HPE, polyphenol extracts of *Hibiscus sabdariffa* L.; ROS, reactive oxygen species; MDA, malondialdehyde; BNL, BABL/c normal liver cell; MPT, mitochondrial permeability transition; JNK, c-jun N-terminal kinase; AIF, apoptosis inducing factor; GSH, glutathione
studies have shown a *Hibiscus sabdariffa* L. extract to contain various polyphenols, and to have the antioxidative potential to inhibit the development of atherosclerosis in cholesterol-fed rabbits by reducing LDL oxidation and to decelerate diabetic nephropathy by improving the antioxidative status.\(^\text{10,11}\) Additionally, Fernández-Arroyo *et al.* have shown in vitro the polyphenolic fraction from aqueous *Hibiscus sabdariffa* L. to possess high reducing capacity in a ferric reducing antioxidant power (FRAP) assay and significant capability to scavenge peroxyl radicals in an oxygen radical absorbance capacity (ORAC) assay.\(^\text{12}\) We investigated in this present study the protective effect of a *Hibiscus sabdariffa* L. polyphenol extract (HPE) on acetaminophen induced liver injury in BABL/c mice. We also examined whether the protecting mechanism involved decreasing the oxidative stress and improving the mitochondrial function.

**Materials and Methods**

**Preparation and assay of HPE.** *Hibiscus sabdariffa* L. obtained from Taitung Hsien Farmers’ Association in Taiwan was identified by Associate Professor Yi-Ching Li of Chung Shan Medical University. A voucher specimen has been kept for future reference at the Department of Pharmacology of Chung Shan Medical University. The extraction of HPE was carried out as previously described.\(^\text{15}\) Briefly, dried flowers were extracted with methanol, and then the samples were filtered and concentrated in a vacuum rotary evaporator. The resulting residue was treated with hexane to remove the pigments and further extracted with ethyl acetate. The extracted portion was re-dissolved in water and lyophilized to obtain HPE. All these procedures were performed at room temperature. The components of HPE, including protocatechuic acid (8.83%), catechin (9.97%), epigallocatechin (EGC, 10.23%), epigallocatechin gallate (EGCG, 20.20%), and caffeic acid (18.10%), were determined by an HPLC analysis.

**Animals and experimental design.** The animal experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC of CSMC) at Taichung in Taiwan. Male BABL/c mice (27 ± 2.5 g) used in the studies was purchased from National Laboratory Animal. The experiments were housed under laboratory conditions (18–23°C, 55–60% humidity, 12 h light/dark cycle) for at least 1 week before the study. The mice were provided with a standard diet (Lab Chow, Purina Mills, USA) and water ad libitum, and assigned to six groups of ten mice per group. Group 1: normal, BABL/c mice fed with the standard diet, without any treatment. Group 2: DMSO control, i.p. injected with DMSO:H\(_2\)O (5:5), fed with the standard diet. Group 3: AAP 1000 mg/kg, fed with 100 mg/kg of AAP and i.p. injected with 1000 mg/kg of AAP. Group 5: HPE 200 mg/kg, fed with 200 mg/kg of HPE and i.p. injected with 1000 mg/kg of AAP. Group 6: HPE 300 mg/kg, fed with 300 mg/kg of HPE and i.p. injected with 1000 mg/kg of AAP. The BABL/c mice were killed 6h after the AAP injection. And the blood and liver tissues were collected for analysis.

**Determination of ALT, AST, ALP, albumin, creatinine, and blood urea nitrogen (BUN).** The level of liver damage was examined by detecting plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphate (ALP) by enzymatic colorimetric methods with an AU 2700 automatic analyzer (Olympus Co., Tokyo, Japan). Albumin, creatinine, and BUN were measured by enzymatic colorimetric methods with the AU 2700 analyzer to evaluate the renal function.

**Pathological histology of the liver.** After removing from each animal, the liver was immediately fixed in 10% buffered formaldehyde, and stained with hematoxylin and eosin (H&E) for a histological examination by the conventional method. The severity of liver damage was evaluated by examining the specimen sections under five randomly selected high-power fields (×100). Image Pro Plus 4.0 was used to quantify the percentage area in each liver specimen.

**Thiobarbituric acid-reacting substances (TBARS).** Lipid peroxidation was determined by measuring TBARS. A 0.5-g liver specimen was homogenized with 5 mL of a 50 mm phosphate buffer at pH 7.4, and centrifuged (1000 × g) for 30 min to obtain the supernatant. The protein content of the supernatant was determined with a Bio-Rad protein assay reagent, using bovine serum albumin as the standard. The 0.3 mL of the supernatant was added 0.3 mL of TBA (1% thiobarbituric acid in 0.3% NaOH) to react for 40 min at 95°C in the dark. After the reaction, the sample was analyzed by a Hitachi F2000 spectrophotofluorimeter with excitation at 532 nm and emission at 600 nm. The concentration of TBARS is expressed as the equivalent of MDA as mmol/mg of protein.

**Catalase assay.** The catalase activity in the liver homogenate was assayed according to a previous method. Briefly, the concentration of protein was determined with Bio-Rad protein assay reagent and adjusted to 50 mg/mL, before 20 μL of the homogenate was added to 980 μL of an H\(_2\)O solution containing 30 μL of ddH\(_2\)O, 50 μL of 1 M Tris-HCl and 5 mM EDTA at pH 8.0. After 10 s at room temperature, the optical density of the H\(_2\)O solution was recorded at 405 nm for 1 min. One unit of catalase activity is defined as H\(_2\)O consumed units/mg of protein.

**Determination of the GSH content.** We determined the liver GSH content according to the method of Hissin and Hilf. A stock solution of the o-phthalaldehyde (OPT) fluorescent probe was freshly prepared in methanol (1 mg/mL). Ten μL of the homogenate was mixed with 100 μL of OPT to incubate for 15 min in the dark. We then monitored the fluorescence intensity with excitation at 350 nm and emission at 420 nm. The result is expressed as ng of GSH/mg of protein.\(^\text{13}\)

**Cell culture.** BALB/c normal liver cells (BNL) were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in a humidified atmosphere of 95% air:5% carbon dioxide at 37°C and with DMEM containing 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin.

**Flow cytometry.** A 1 × 10⁶ amount of cells was cultured in 100-mm Petri dish; after being treated with the indicated concentrations of HPE for 1 h, 5 m of AHP was added and the mixture incubated for 48 h. The cells were harvested, washed twice with PBS, 0.5 μM propidium iodide was added, and the mixture was incubated in the dark for 20 min. The fluorescence emitted from the propidium iodide-DNA complex was quantified by FACScan cytometry (Becton Dickinson, San Jose, CA, USA) as dead cells after excitation by the fluorescent dye.

**Measurement of the mitochondrial membrane potential.** The induction of mitochondrial permeability transition was determined as the reduction of rhodamine 123 in AAP alone and in AAP with the HPE-treated cells. Briefly, 1 mL of the cell suspension (10⁴ cells/mL) was stained with rhodamine 123 to a final concentration of 1 μM in an incubator for 1 h at 37°C. The cells were then centrifuged for 3 min at 300 g and resuspended in fresh PBS. The green fluorescence caused by rhodamine 123 was detected by FACScan cytometry with a 525 BP filter.\(^\text{14}\)

**Western blotting.** After treating with the desired concentration of HPE, the medium was removed, and the remainder rinsed with PBS at room temperature. A 0.5-mL amount of a cold RIPA buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, and 150 mM NaCl at pH 7.5) was added with the fresh protease inhibitor. The cells were scraped off and the lysate was centrifuged at 10000 × g for 10 min. The cell lysate (50 μg/mL) was mixed with an equal volume of the electrophoresis sample buffer, and the mixture boiled for 10 min, before analyzing by SDS–PAGE and transferring the protein from the gel to a nitrocellulose membrane (Millipore, Bedford, MA, USA) by using electrobloating apparatus. The proteins were added with the ECL
Statistical differences were analyzed by one-way ANOVA and treated groups (Transduction Laboratories, Lexington, KY, USA). Results (Santa Cruz Biotech, CA, USA), and the anti-C11 antibodies used in this study were JNK, P-JNK, Bax, Bid and AIF analyzed by the LAS-3000 imaging system (Fuji, Tokyo, Japan). The western blotting detection reagents (Amersham Biosciences, USA) and analyzed by the LAS-3000 imaging system (Fuji, Tokyo, Japan). The antibodies used in this study were JNK, P-JNK, Bax, Bid and AIF (Santa Cruz Biotech, CA, USA), and the anti-α-tubulin antibody (Transduction Laboratories, Lexington, KY, USA).

Statistical analysis. Each result is reported as the mean ± SD. n = 10.

<table>
<thead>
<tr>
<th>Parametera,b</th>
<th>Normal</th>
<th>DMSO</th>
<th>AAP 1000 mg kg⁻¹</th>
<th>AAP +100 mg kg⁻¹</th>
<th>AAP +200 mg kg⁻¹</th>
<th>AAP +300 mg kg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>35.80 ± 4.00</td>
<td>35.80 ± 3.90</td>
<td>36.80 ± 2.70f</td>
<td>29.00 ± 1.90</td>
<td>31.80 ± 1.60d</td>
<td>37.30 ± 9.00e</td>
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<tr>
<td>Liver weight, LW, g</td>
<td>2.41 ± 0.22</td>
<td>2.06 ± 0.45</td>
<td>2.87 ± 0.62e</td>
<td>1.97 ± 0.37f</td>
<td>2.29 ± 0.43f</td>
<td>2.11 ± 0.44f</td>
</tr>
<tr>
<td>LW/BW, %</td>
<td>6.73 ± 1.44</td>
<td>5.75 ± 1.75</td>
<td>7.80 ± 1.73c</td>
<td>6.79 ± 1.95f</td>
<td>7.20 ± 1.52d</td>
<td>5.66 ± 4.63f</td>
</tr>
<tr>
<td>ALT, U dl⁻¹</td>
<td>53.25 ± 24.15</td>
<td>71.25 ± 13.62</td>
<td>1962.40 ± 324.35c</td>
<td>1505.25 ± 135.28d</td>
<td>1273.24 ± 132.14d</td>
<td>1193.25 ± 51.24d</td>
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<tr>
<td>AST, U dl⁻¹</td>
<td>89.25 ± 25.47</td>
<td>172.20 ± 33.25</td>
<td>1575.00 ± 268.51c</td>
<td>1363.32 ± 259.47d</td>
<td>1202.13 ± 76.54d</td>
<td>372.53 ± 57.81f</td>
</tr>
<tr>
<td>ALP, U dl⁻¹</td>
<td>107.25 ± 14.36</td>
<td>145.75 ± 23.54</td>
<td>182.00 ± 45.26c</td>
<td>138.25 ± 23.56d</td>
<td>142.75 ± 21.63</td>
<td>146.30 ± 28.37d</td>
</tr>
<tr>
<td>Albumin, g dl⁻¹</td>
<td>3.10 ± 0.40</td>
<td>2.90 ± 0.28</td>
<td>2.06 ± 0.31f</td>
<td>2.60 ± 0.24</td>
<td>2.88 ± 0.26f</td>
<td>3.10 ± 0.41f</td>
</tr>
<tr>
<td>BUN, mg dl⁻¹</td>
<td>22.00 ± 2.13</td>
<td>21.00 ± 1.95</td>
<td>58.50 ± 3.24d</td>
<td>46.50 ± 5.20d</td>
<td>42.50 ± 10.55d</td>
<td>42.30 ± 13.32d</td>
</tr>
<tr>
<td>Creatinine, mg dl⁻¹</td>
<td>0.40 ± 0.05</td>
<td>0.40 ± 0.06</td>
<td>0.81 ± 0.16d</td>
<td>0.71 ± 0.16d</td>
<td>0.52 ± 0.22f</td>
<td>0.41 ± 0.30f</td>
</tr>
</tbody>
</table>

*Data presented as mean ± SD, n = 10.  
*AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; BUN, blood urea nitrogen. Statistical significance analyzed by Student’s t-test. *p < 0.05, compared to the normal group; *p < 0.05, *p < 0.005, and †p < 0.0005, compared to the AAP-treated group.

Results

**Effect of HPE on the histopathological change to liver steatosis in the BALB/c mice**

The histopathological evaluation of the liver showed that HPE could decrease AAP-induced liver injury. Liver sections from normal BALB/c mice demonstrated the typical structure, the AAP-treated liver showing lipid droplets that appeared in the hepatocytes, this being a pattern for steatosis but not for severe fatty liver. Pretreating with HPE (100, 200 or 300 mg/kg) markedly reduced the steatosis in a dose-dependent manner (Fig. 1).

**Effect of HPE on the liver and kidney functions of AAP-treated BALB/c mice**

A significant elevation of ALT and AST was found in the AAP-treated mice, while treating with HPE significantly decreased the levels of ALT and AST in a dose-dependent manner. The increase in serum BUN and creatinine induced by AAP was significant, whereas treating with various concentrations of HPE reduced the level of BUN and creatinine. A significant decrease of serum albumin was found in the AAP-treated animals (p < 0.05) and a significant improvement in the HPE-treated groups (p < 0.005) (Table 1).

**Effect of HPE on the MDA content, catalase activity, and GSH of the AAP-treated BALB/c mice**

The MDA content was significantly higher in the group with AAP-induced liver injury, while the activities of catalase and GSH were significantly lower. Under the same conditions, pretreating with HPE (100, 200 or 300 mg/kg) reduced about 59%, 66%, 81% of lipid peroxidation. Catalase increased about 28%, 31%, 18% of the activities in the group of pretreatment of HPE (100, 200, 300 mg/kg). The level of glutathione increased 76%, 32%, 3% of the same ones (Table 2).
The AAP-treated liver samples showed overexpressed P-JNK, AIF, Bid, and Bax. Pretreating with HPE markedly reduced the protein overexpression when compared with non treatment (Fig. 2).

**Effect of HPE on protein expression in BABL/c mice with AAP-induced liver damage**

The AAP-treated liver samples showed overexpressed P-JNK, AIF, Bid, and Bax. Pretreating with HPE markedly reduced the protein overexpression when compared with non treatment (Fig. 2).

**Effect of HPE on the viability of BNL cells treated with AAP**

BNL cells were treated with AAP (5 mM) with or without HPE (0.05, 0.1, 0.5 or 1 mg/mL) for 48 h, and the viability of the cells was determined by flow cytometry after PI staining. Figure 3 shows that treating with HPE (0.05, 0.1, 0.5 or 1 mg/mL) significantly increased the cell viability. The same treatment rescued the AAP-caused loss of mitochondrial potential as shown in Fig. 4.

**Table 2. Oxidative Parameters in Liver Specimens of the Mice**

<table>
<thead>
<tr>
<th>Parameter&lt;sup&gt;ab&lt;/sup&gt;</th>
<th>Normal</th>
<th>DMS</th>
<th>1000 mg kg&lt;sup&gt;-1&lt;/sup&gt; AAP HPE</th>
<th>AAP +100 mg kg&lt;sup&gt;-1&lt;/sup&gt; HPE</th>
<th>AAP +200 mg kg&lt;sup&gt;-1&lt;/sup&gt; HPE</th>
<th>AAP +300 mg kg&lt;sup&gt;-1&lt;/sup&gt; HPE</th>
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<tr>
<td>MDA, μmol/g tissue&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.97 ± 0.38</td>
<td>0.80 ± 0.19</td>
<td>2.24 ± 0.87&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.92 ± 0.82&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.75 ± 0.27&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.42 ± 0.22&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT, units/g protein&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.73 ± 0.40</td>
<td>1.95 ± 0.29</td>
<td>0.85 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.09 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.12 ± 0.30&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.01 ± 0.28&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSH, μmol/g protein&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.47 ± 0.26</td>
<td>1.39 ± 0.30</td>
<td>0.56 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.99 ± 0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.74 ± 0.25&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.78 ± 0.28&lt;sup&gt;e&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup>Data presented as mean ± SD, n = 10.
<sup>b</sup>MDA, malondialdehyde content; CAT, catalase activity; GSH, glutathione.
<sup>c</sup>Statistical significance analyzed by Student’s t-test. *p < 0.001, compared to the normal group; **p < 0.005, and *p < 0.0005, compared to the AAP-treated group.
HPE also showed markedly reduced protein expression of tBid and Bax as found with the in vivo examination.

**Effect of HPE on the MDA content, catalase activity, and GSH level in AAP-treated BNL cells**

The MDA content was significantly higher in the AAP-treated BNL cells, while the catalase activity and the level of GSH were significantly lower. Pretreating with HPE improved the oxidative status in a dose-dependent manner as shown in Table 3 as well as the parameters in the in vivo assay.

**Discussion**

Acetaminophen (AAP) is an analgesic and antipyretic drug but which can precipitate liver injury at high doses. Bajt et al. have shown that oxidative stress preceded cell injury in mice hepatocytes exposed to AAP, while Oz et al. have reported that green-tea polyphenols could improve the antioxidative status to
reduce AAP-induced liver toxicity.\textsuperscript{15} Hsu \textit{et al.} have also found that \textit{Ganoderma amboinense} reduced AAP-caused hepatotoxicity by reducing GSH depletion, and MDA and ROS increases.\textsuperscript{16} \textit{Hibiscus sabdariffa} L. is a common medicinal herb and has been used for treating liver disease in the south-west of Nigeria.\textsuperscript{17} Our previous investigation has also indicated that a water extract of \textit{Hibiscus sabdariffa} L. could protect individuals from acetaminophen-induced liver injury by reducing oxidative stress.\textsuperscript{18} We have shown in this study the functional components in \textit{Hibiscus sabdariffa} L., and propose that HPE might be the major contributor to improving the oxidative status in AAP-induced liver damage. We have also previously demonstrated that HPE could inhibit lipopolysaccharide-induced inflammation by improving the antioxidative conditions and decelerating the inflammatory responses.\textsuperscript{19} It can be concluded that HPE possessed good hepato-protective ability resulting in its antioxidative characteristics.

Mitochondria play a critical role in causing cell death during AAP-induced liver injury. Bajt \textit{et al.} have demonstrated that such mitochondrial intermembrane proteins as cytochrome c, the second mitochondrial activator of caspases (Smac), endonuclease G and AIF were released after AAP exposure.\textsuperscript{20} These released proteins could further induce mitochondrial dysfunction and cell death. Our present results show that HPE had the ability to eliminate the release of intermembrane proteins, decrease the loss of mitochondrial membrane potential, and reduce cell death. Green tea polyphenols have also been proved to reduce cell death by regulating Bcl-2, another mitochondrial membrane protein.\textsuperscript{21}

Adams \textit{et al.} have shown the disappearance of Bax in the cytosol of animals with AAP. AAP-treated bcl-2 (−/−) mice presented microvesicular steatosis without progressing to vast necrosis.\textsuperscript{22} These data suggest that Bax may play the role of AAP-induced hepatotoxicity and that the overexpression of Bcl-2 would unexpectedly increase AAP hepatotoxicity. We have demonstrated in this study that HPE could reduce the expression of Bax and tBid to protect the liver from AAP-induced injury.

These results indicate that the protecting mechanism of HPE in AAP-caused liver damage might involve inhibiting oxidative stress and restoring the mitochondrial function. Our current findings in Table 1 also show that HPE possessed the ability to decelerate AAP-caused kidney damage, although we need to examine the detailed mechanism, the protective effect of HPE in AAP-induced liver damage can be further applied.

\textbf{Acknowledgments}

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\textbf{References}