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Isolation, structure elucidation, antioxidant and hepatoprotective effects of petroleum ether extract of *Artemisia integrifolia* L. against CCl4-induced liver injury in rats

BAO Ha-Si-Er-Dun, WANG Qing-Hu, BA E-Er-Dun-Bu-La-Ga and XU Yan-Hua
Isolation, structure elucidation, antioxidant and hepatoprotective effects of petroleum ether extract of *Artemisia integrifolia* L. against CCl₄-induced liver injury in rats

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The aim of this study was to investigate the chemical constituents of petroleum ether extract from *Artemisia integrifolia* L (AIPEE) and to evaluate its hepatoprotective potential and *in vivo* antioxidant effects. Six compounds, namely eugenol (1), linolenic acid (2), 6,7-epoxy-linolenic acid (3), linoleic acid (4), oleic acid (5) and hexadecanoic acid (6) were isolated from the AIPEE. Oral administration of AIPEE significantly reduced carbon tetrachloride-induced elevations in the levels of plasma markers of hepatic damage and lipid peroxidation. It also rescued histopathologic alterations observed in the liver and levels of oxidative stress markers. AIPEE exhibited antioxidant and hepatoprotective activities *in vivo*, which may be attributable to its chemical constituents such as five fatty acids and eugenol.

**Key words:** Artemisia integrifolia L., fatty acids, eugenol, antioxidant, hepatoprotection.

INTRODUCTION

Liver is the most important organ, which is involved in several vital functions, for example, metabolism, secretion and storage (Sharma et al., 2012; Mistry et al., 2013). It has great capacity to detoxicate toxic substances and synthesize useful principles. Thus, excessive exposure to drugs and environmental pollutants overpower the detoxification mechanisms of the liver and lead to liver injury (Setty et al., 2007).

Oxidative stress, an important factor that induces liver fibrosis, represents a key feature of hepatitis induced by various conditions, including anoxic/reoxygenation injury, autoimmune hepatitis, viral hepatitis, and alcoholic hepatitis (Singh et al., 2008). The CCl₄-induced liver toxicity model is widely used because CCl₄ induces hepatic changes analogous to those observed in chemical hepatitis. In the liver, CCl₄ is converted by the cytochrome P450 system into a trichloromethyl radical. This radical reacts with oxygen to form a trichloromethylperoxyl radical, which reacts with cell macromolecules, inducing lipid peroxidation and provoking hepatocyte membrane breakdown (Halliwell, 2012).

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Liver damage is associated with lipid peroxidation, enzyme leakage, and depletion in the GSH level (Singh et al., 2008). Chronic liver injury may develop into several liver diseases, including hepatic steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma (Srivastava and Shivanandappa, 2010). Conventional or synthetic drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects (Liu et al., 2006). On the other hand, Mengyixue, an indigenous system of medicine in Inner Mongolia, has a long tradition of treating liver disorders with Mongolian medicine (Wang et al., 1998).

Artemisia integrifolia L., belonging to the family Compositae, is distributed throughout Inner Mongolia (Zhang et al., 2008). It is characteristic cuisine of Daur in Hulunbeier of Inner Mongolia, and is considered to be the green vegetables for possessing rich in nutrition and heat-clearing and detoxicating (Tu and Liu, 2007). In Mongolian medicine, A. integrifolia L. is cold in property, with a bitter flavor. It has been used as a folk medicine to treat cardiovascular disease and liver diseases (Zhang et al., 2008). The ethanol extract of A. integrifolia L. possess very good in vitro superoxide, hydroxyl and nitric oxide radical scavenging, and lipid peroxidation inhibiting activities (Liu et al., 2010). The phytochemical screening of the plant extract showed the presence of flavonoids, coumarin, amino acids and proteins (Hu and Feng, 1999). However, up to date, there have been few studies about the hepatoprotective effects of A. integrifolia L.

In the paper, a systematic research was undertaken to investigate the chemical constituents of petroleum ether extract from A. integrifolia L (AIPEE) and to evaluate its hepatoprotective potential and in vivo antioxidant effects.

**MATERIALS AND METHODS**

The UV spectra were recorded on a Shimadzu UV-2001 spectrometer (Shimadzu, Japan). NMR spectra were measured on a Bruker Avance W-500 NMR spectrometer (Bruker, Germany) with tetramethylsilane (TMS) as the internal reference, and chemical shifts are expressed in δ (ppm). Semi-preparative HPLC was performed by using a Japanese liquid chromatograph equipped with a EZ0566 column. Column chromatography was performed by using silica gel (200-300 mesh, Marine Chemical Factory, Qingdao, China).

Commercial assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (TB), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Silymarin was obtained from Ningbo Liwah Pharmaceutical Co. (Ningbo, China). Corn oil was purchased from Martha supermarket (Tongliao, China). High-performance liquid chromatography (HPLC)–grade acetonitrile was purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were analytical grade.

**Plant material**

The aerial part of A. integrifolia L. were collected in Hailaer (126°04’ E and 48°20’ N), Hulunbeier, Inner Mongolia of China, in April 2015, and identified by Prof. Buhebateer (Inner Mongolia University for Nationalities). The aerial part of A. integrifolia L. were placed in the shade to dry. A voucher (NO. 20150410) has been deposited in a warehouse (10-30°C, 30–50% RH, 24 h dark) in the School of Traditional Mongolian Medicine of Inner Mongolia University for Nationalities.

**Extraction and isolation**

The air dried aerial part of A. integrifolia L (2.0 Kg) were powdered and extracted twice under reflux petroleum ether (30 L). Evaporation of the solvent under reduced pressure (0.1 MPa) delivered the petroleum ether extract (965 g). The AIPEE was stored in a refrigerator (0–4°C) for further use.

The AIPEE (30 g) was separated by column chromatography on silica gel and gradiently eluted with petroleum ether-ethyl acetate (80:1 to 20:1) to give 4 fractions (Fractions 1-4). Fraction 2 [210 mg, petroleum ether-ethyl acetate (60:1) elute] was subjected to silica gel column chromatography using petroleum ether-ethyl acetate with increasing polarity (70: 1-50: 1) to give 1 (18 mg) and 2 (43 mg); Fraction 3 [330 mg, petroleum ether-ethyl acetate (40:1) elute] was separated by semipreparative HPLC (The separation was performed on EZ0566 column (250 mm × 4.6 mm, 5 μm) with acetonitrile-water (85:15) as the mobile phase and a detection wavelength at 215 nm (Figure 1). The retention time of compounds 6, 5, 4 and 3 were 10.02, 13.52, 16.21 and 18.02 min, respectively] yielding 6 (17 mg), 5 (22 mg), 4 (24 mg) and 3 (37 mg).

**Animals and experimental design**

Wistar rats (200–300 g) were provided by Changchun Yisheng Laboratory Animal Technology Co., Ltd. (Changchun, China). The rats were maintained under standard animal housing conditions (25 ± 5°C, 40–70% RH, 12 h light/dark cycle) and provided with standard laboratory food (Rat sterile granulated feed, product executive standard: GB14924–2001, license: SCXK–(Jl) 2010–0001) and water ad libitum. All procedures were performed in accordance with National Institute of Health guidelines for the care and use of animals and approved by the Institute Animal Care and Use Committee (IACUC. Approved num: 20120228 ). Rats were randomly assigned to six experimental groups (n = 10), as described later. CCl₄ was administered intraperitoneally (i.p.) at a single dose of 3 mL/kg, diluted in corn oil (vehicle 50%, v/v) to animals in groups 2 to 6: Group 1: Control, received 2.0 mL/kg i.p. of vehicle; Group 2: toxicant (CCl₄, 3.0 mL/kg, i.p) (Milena et al., 2014); Group 3: CCl₄ + AIPEE for 7 d (100 mg/kg, p.o.); Group 4: CCl₄ + AIPEE for 7 d (200 mg/kg, p.o.); Group 5: CCl₄ + AIPEE for 7 d (300 mg/kg, p.o.); Group 6: CCl₄ + Legalon for 7 d (300 mg/kg, p.o.).

AIPEE and Legalon were administered by gastric gavages daily for 7 d starting 24 h after CCl₄-induced liver injury. Legalon was used as a standard drug.

**Collection and processing of tissue sample**

On day 8, animals were anaesthetized (30 mg/kg, i.p. of chloral hydrate), sacrificed by cervical dislocation, and submitted to laparotomy. Blood was collected from abdominal artery into non-heparinized tubes and centrifuged at 4°C, 900 rpm for 10 min to obtain serum for biochemical tests. The livers were quickly excised, washed, and used to determine the levels of the oxidative stress markers (thiobarbituric acid reactive substance [TBARS], SOD, CAT, GPx, and GSH) and for histopathologic studies. All determinations were performed in triplicate.
Preparation of tissue homogenate

Hepatic tissues were homogenized in cold 50 mM potassium phosphate buffer (pH 7.0) using a Potter-Elvehjem homogenizer to give a 10% (w/v) liver homogenate and centrifuged at 600 rpm for 5 min. The filtrate was used for further estimations.

Serum parameters

AST, ALT, and TB levels were analyzed to assess hepatic function using Biosystems kits, according to the manufacturer’s instructions.

Oxidative stress analyses (Das et al., 2000)

The liver homogenate was used to analyze the enzymatic antioxidant activities by SOD, CAT, GPx, and nonenzymatic antioxidants like GSH were evaluated in liver tissue homogenate by respective methods. TBARS levels were determined by a previously described method.

Histopathologic analysis

For histological studies, the liver tissues were fixed with 10% phosphate buffered neutral formalin, dehydrated in graded (50%–100%) alcohol and embedded in paraffin. Thin sections (5 μm) were cut and stained with routine hematoxylin and eosin (H&E) stain for photo microscopic assessment. The initial examination was qualitative, with the purpose of determining histopathological lesions in liver.

Acute toxicity

For the assessment of acute toxicity, wistar rats (200–300 g) were divided into three groups of 10 animals. The AIPEE and legalon were given p.o. at the doses of 1500 mg/kg to the group 2 and group 3, respectively. The control group (group 1) received 2.0 mL/kg i.p. of vehicle. The mortality rate within 72 h period was determined and the LD₅₀ was estimated according to the method described by references (Silva et al., 2005).

Statistical analysis

Data, expressed as the mean ± SD, were analyzed by one-way analysis of variance and Tukey-Kramer posthoc tests using GraphPad Software version 4.0. P < 0.05 was considered significant.

RESULTS

AIPEE was separated by chromatography and assigned six compounds, namely eugenol (1), linolenic acid (2), 6,7-epoxy-linolenic acid (3), linoleic acid (4), oleic acid (5) and hexadecanoic acid (6) (Figure 2). The structures of the compounds were identified by comparing their spectroscopic data with those reported in the literature (Wang et al., 2010; Yang et al., 2008; Deng et al., 2013; Zan et al., 2008).

Eugenol (1): Yellowish liquid; UV (hexane) λmax (nm): 280; IR (KBr) νmax (cm⁻¹): 3351, 1635, 1591, 1487 and 1201 cm⁻¹; 1H-NMR (500MHz, CDCl₃) δH: 7.06 (1H, d, J = 2.0 Hz, H-2), 6.94 (1H, d, J = 8.0 Hz, H-5), 7.10 (1H, dd, J = 8.0, 2.0 Hz, H-6), 2.07 (2H, m, H-7), 5.81 (1H,m, H-8), 5.95 (2H, m, H-9), 3.78 (3H, s, -OCH₃).

13C-NMR (125MHz, CDCl₃) δC: 126.7 (C-1), 109.3 (C-2), 148.5 (C-3), 146.8 (C-4), 114.1 (C-5), 123.5 (C-6), 41.2 (C-7), 145.7 (C-8), 110.2 (C-9), 56.1 (-OCH₃).

Linolenic acid (2): Colorless oily liquid. 1H-NMR (500MHz, CDCl₃) δH: 2.34 (2H, t, J = 7.5 Hz, H-2), 1.62 (2H, m, H-3), 1.31-1.35 (8H, m, H-4-7), 2.05 (2H, m, H-8), 2.80 (4H, t, J = 6.5 Hz, H-11, 14), 5.42-5.35 (6H, m, H-9, 10, 12, 13, 15,16), 2.07 (2H, q, J = 7.5 Hz, H-17, 0.96 (3H, t, J = 7.5 Hz, H-18). 13C-NMR (125MHz, CDCl₃) δC: 80.3 (C-1), 34.1 (C-2), 24.6 (C-3), 29.0 (C-4, 7), 29.1 (C-5), 29.6 (C-6), 27.6 (C-8), 130.2 (C-9), 127.8 (C-10), 25.6 (C-11), 128.2 (C-12), 128.3 (C-13), 25.5 (C-14), 127.1 (C-15), 131.9 (C-16), 20.6 (C-17), 14.3 (C-18), 6.7-epoxy-linolenic acid (3). Colorless oily liquid. 1H-NMR...
Table 1. Effect of AIPEE on serum biochemical parameters in CCl₄-treated rats.

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<tr>
<th>Particulars</th>
<th>Group 1</th>
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<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
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<tr>
<td>Relative weight</td>
<td>3.41 ± 0.22</td>
<td>4.43 ± 0.47a</td>
<td>4.02 ± 0.34b</td>
<td>3.63 ± 0.48#</td>
<td>3.29 ± 0.32###</td>
<td>3.34 ± 0.50###</td>
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<td>ALT (U/L)</td>
<td>48 ± 18</td>
<td>378 ± 49a</td>
<td>303 ± 38##</td>
<td>125 ± 42##b</td>
<td>50 ± 21###b</td>
<td>53 ± 13###b</td>
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<tr>
<td>AST (U/L)</td>
<td>124 ± 29</td>
<td>718 ± 142a</td>
<td>542 ± 81##</td>
<td>358 ± 69##b</td>
<td>109 ± 32###b</td>
<td>127 ± 26###b</td>
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<td>TB (mg/dL)</td>
<td>1.22 ± 0.18</td>
<td>2.71 ± 0.42a</td>
<td>2.07 ± 0.19a</td>
<td>1.89 ± 0.31##b</td>
<td>1.28 ± 0.24###</td>
<td>1.30 ± 0.20###</td>
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Values are expressed as the means ± SD; *P < 0.001, #P < 0.01 and ##P < 0.05 compared with Group 1; a#P < 0.01 and b#P < 0.05 compared with control group.

Oleic acid (5): Colorless oily liquid. ¹H-NMR (500MHz, CDCl₃) δ: 5.40 (1H, d, J = 5.5 Hz, H-9), 5.34 (1H, d, J = 55.5 Hz, H-9), 2.37 (2H, t, J = 7.5 Hz, H-2), 1.65 (2H, m, H-3), 1.31-1.33 (16H, m, H-4~7, 12~15), 2.04 (4H, t, J = 7.5 Hz, H-8, 11), 1.28 (2H, m, H-16), 1.34 (2H, m, H-17), 0.92 (3H, t, J = 6.5 Hz, H-18). ¹³C-NMR (125MHz, CDCl₃) δ: 179.1 (C-1), 33.4 (C-2), 24.6 (C-3), 29.0 (C-4, 15), 29.6 (C-5, 13, 14), 29.2 (C-7, 12), 27.3 (C-8), 128.0 (C-9), 130.3 (C-10), 27.2 (C-11), 31.5 (C-16), 22.5 (C-17), 14.0 (C-18).

Hexadecanoic acid (6): Colorless oily liquid. ¹H-NMR (500MHz, CDCl₃) δ: 2.37 (2H, t, J = 7.5 Hz, H-2), 1.66 (2H, m, H-3), 1.28-1.32 (16H, m, H-4~13), 1.27 (2H, m, H-14), 1.34 (2H, m, H-15), 0.91 (3H, t, J = 6.5 Hz, H-16). ¹³C-NMR (125MHz, CDCl₃) δ: 179.4 (C-1), 33.9 (C-2), 24.7 (C-3), 29.0 (C-4, 13), 29.2 (C-5, 12), 29.6 (C-6, 7, 8, 9, 10, 11), 31.9 (C-14), 22.7 (C-15), 14.2 (C-16).

The levels of AST, ALT, and TB in serum were analyzed and the results are shown in Table 1. The oxidative stress analyses of liver homogenate are shown in Table 2. Figure 3 shows representative photomicrographs of liver obtained from different treatments.

No mortality was observed in groups of rats treated with the AIPEE and legalon. LD₅₀ values for the AIPEE...
Table 2. Effect of CCl₄, AIPEE and Legalon on CAT, SOD, GPx and GSH in rats.

<table>
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<th>Particulars</th>
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<td>CAT (U/mg protein)</td>
<td>51.1 ± 12.2</td>
<td>16.5 ± 1.53²</td>
<td>25.6 ± 2.63ᵃ</td>
<td>39.8 ± 9.81ᵇ</td>
<td>56.8 ± 13.8⁴</td>
<td>46.3 ± 5.31⁴</td>
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<td>SOD (U/mg protein)</td>
<td>53.2 ± 10.1</td>
<td>19.8 ± 4.20ᵃ</td>
<td>20.1 ± 2.03ᵃ</td>
<td>35.8 ± 1.89ᵇ</td>
<td>42.4 ± 9.72⁴</td>
<td>43.8 ± 13.8⁴</td>
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<td>GPx (U/mg protein)</td>
<td>8.96 ± 1.03</td>
<td>2.98 ± 0.82ᵃ</td>
<td>5.21 ± 0.22ᵃ</td>
<td>7.35 ± 0.47ᵇ</td>
<td>8.35 ± 0.68⁴</td>
<td>9.63 ± 1.02⁴</td>
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<td>GSH (mmol/L)</td>
<td>5.02 ± 0.48</td>
<td>2.88 ± 0.37ᵃ</td>
<td>3.96 ± 0.88ᵃ</td>
<td>5.08 ± 0.37ᵇ</td>
<td>5.51 ± 0.72⁴</td>
<td>5.23 ± 0.39⁴</td>
</tr>
<tr>
<td>TBAR (μmol/L)</td>
<td>27.9 ± 6.71</td>
<td>70.8 ± 9.42ᵃ</td>
<td>59.3 ± 7.26ᵃ</td>
<td>33.5 ± 3.34ᵇ</td>
<td>25.3 ± 5.81⁴</td>
<td>32.0 ± 5.02⁴</td>
</tr>
</tbody>
</table>

Values are expressed as the means ± SD; ᵃP < 0.001, ᵇP < 0.01 and ᵇᵇP < 0.05) compared with Group 1; ᵆᵇᵇP < 0.01 and ᵆᵇᵇᵇP < 0.05) compared with Group 2.

Figure 3. Photomicrographs of liver sections obtained from (a) control group, (b) CCl₄ group, (c) CCl₄ + AIPEE (100 mg/kg), (d) CCl₄ + AIPEE (200 mg/kg), (e) CCl₄ + AIPEE (300 mg/kg) and (f) positive control group (Legalon, 300 mg/kg).

and legalon was more than 1500 mg/kg.

DISCUSSION

As is shown in Table 1, the CCl₄-treated rats (Group 2) developed extensive hepatic damage, evidenced by significant increases in the levels of AST, ALT and TB of serum compared with the control group (Group 1). Moreover, AIPEE showed dose-dependent effect on serum hepatic markers. The highest dose at 300 mg/kg caused a significant decrease in AST and ALT activities and TB level (P < 0.001) in CCl₄-intoxicated rats (Group 5). Group 5 and group 6 rats were compared with control group 1 and they showed no significant change.

From Table 2, it is shown that the levels of the hepatic oxidative stress markers CAT, SOD, GPx, and GSH in Group 2 resulted in significant decreases (P < 0.001)
The oxidative damage to cell membranes induced by CCl₄ was also associated with an increase in lipoperoxidation, as evidenced by an increase in TBARS levels and a decrease in the reduced GSH content. The GSH plays an important role in hepatoprotection, decreased GSH content is associated with oxidative stress (Ramachandra Setty et al., 2007). Post-CCl₄ treatment with 200, 300 mg/kg daily of medium and high dose AIPEE over 7 days restored the endogenous antioxidant defense system, and reversed lipid peroxidation in the liver, thereby reducing cell damage. In this matter, AIPEE (300 mg/kg) proved equally efficient to Legalon (Table 2). The hepatoprotective effect observed suggests that the six compounds, namely eugenol (1), linolenic acid (2), 6,7-epoxy-linolenic acid (3), linoleic acid (4), oleic acid (5) and hexadecanoic acid (6), identified from the AIPEE play an important role in plasma membrane stabilization, as well as in repairing liver damage caused by CCl₄. In this regard, our results are in agreement with other studies that have reported hepatoprotective and antioxidant effects of eugenol and fatty acids (Wang et al., 2007; Bao-zhong and Li, 2008; Zhang et al., 2007). Eugenol protects against liver injury because it protects the integrity of cell membrane by removing free radicals in the body, enhancing the activity of SOD, GSH-Px and CAT, and reducing the synthesis of MDA. Linolenic acid can increase the levels of EPA and DHA in platelets and erythrocytes, and reduce the production of arachidonic acid. At the same time, linolenic acid can prevent all kinds of organ damage by scavenging free radicals, preventing protein denaturation and nucleic acid metabolic disorder and protecting the biological membrane. Eugenol has antioxidant activity, which is directly related to the hydroxyl and terminal double bond in its structure. Therefore, the presence of eugenol and fatty acids in AIPEE could contribute to its overall antioxidant and hepatoprotective activity.

Exposure of the liver to CCl₄ triggers a series of events that deregulate cell functions and affect the levels of the endogenous enzymatic antioxidant system, which consists of CAT, SOD, and GPx (Yeum et al., 2009). The significant decrease in the activities of CAT, SOD, and GPx observed upon CCl₄ treatment is also reversed by high dose AIPEE, which restores the enzyme activities to their control values. Furthermore, GSH levels in group 5 were also significantly increased compared with those in groups 1 and 6. These results further support our finding that this extract attenuates hepatic injury in CCl₄-treated rats.

Figure 3 shows representative photomicrographs of livers obtained from different treatments. In the control group, the structure of the hepatic lobule was clear; the cell cord was orderly, and there was no edema, fatty degeneration or visible lesions; the hepatic sinusoid was normal and the nuclear structure was clear (Figure 3a). However, in CCl₄-treated rats (Group 2), the normal structure and most of the hepatic sinusoid had disappeared. Furthermore, hepatocytes exhibited extensive vacuolar degeneration (Figure 3b). Pre-administration of AIPEE and silymarin exerted a protective effect against CCl₄-induced nuclear damage (Figure 3c to f). The overall liver histochitecture in the highest dose at 300 mg/kg was similar to that of the control and the CCl₄/Legalon-treated animals.

As is shown in Figure 3b, the liver damage induced by CCl₄ was developed, which revealed cellular correlates of damage 24 h after CCl₄ administration. This hepatocellular injury led to an increase in liver weight. Rupture of cellular membranes resulted in intracellular content leakage with concomitant elevation of blood hepatic serum markers. Therefore, serum levels of AST, ALT, and TB, which are established indicators of liver injury, were elevated. AIPEE administration induced histologic and biochemical changes reflecting liver recovery toward normality. Analogous results has been reported under similar experimental conditions (Khan et al., 2007; Megahed et al., 2010; Talwar et al., 2013).

CONFLICT OF INTERESTS
The authors have not declared any conflict of interests.

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