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Original Article

The essential oil of *Artemisia capillaris* protects against CCl_4-induced liver injury *in vivo*

Qinghan Gao[a,b,1], Xin Zhao[a,1], Lei Yin[a,1], Yuanbin Zhang[a], Bing Wang[a], Xiuli Wu[a], Xinhui Zhang[a], Xueyan Fu[a,c,*], Weihong Sun[d,*]

[a] School of Pharmacy, Ningxia Medical University, Yinchuan, China
[b] School of Public Health, Ningxia Medical University, Yinchuan, China
[c] Key Laboratory of Hui Ethnic Medicine Modernization, Ministry of Education (Ningxia Medical University), Yinchuan, China
[d] General Hospital of Ningxia Medical University, Yinchuan, China

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ABSTRACT

To study the hepatoprotective effect of the essential oil of *Artemisia capillaris* Thunb., Asteraceae, on CCl_4-induced liver injury in mice, the levels of serum aspartate aminotransferase and alanine aminotransferase, hepatic levels of reduced glutathione, activity of glutathione peroxidase, and the activities of superoxide dismutase and malondialdehyde were assayed. Administration of the essential oil of *A. capillaris* at 100 and 50 mg/kg to mice prior to CCl_4 injection was shown to confer stronger *in vivo* protective effects and could observably antagonize the CCl_4-induced increase in the serum alanine aminotransferase and aspartate aminotransferase activities and malondialdehyde levels as well as prevent CCl_4-induced decrease in the antioxidant superoxide dismutase activity, glutathione level and glutathione peroxidase activity ($p < 0.01$). The oil mainly contained β-citronellol, 1,8-cineole, camphor, linalool, α-pinene, β-pinene, thymol and myrcene. This finding demonstrates that the essential oil of *A. capillaris* can protect hepatic function against CCl_4-induced liver injury in mice.

Introduction

Liver disease, a common disorder caused by viral hepatitis, alcoholism, liver-toxic chemicals, unhealthy dietary habits and environmental pollution, is a global concern (Papay et al., 2009). However, medical treatment for this disease is often hard to administer and has a confined effect. Traditional Chinese herbal medicines, which underlie numerous prescriptions used to treat liver diseases, are still widely used by the Chinese (Zhao et al., 2014). *Artemisia capillaris* Thunb., Asteraceae, according to the Bencao Gangmu, the most famous records of Chinese Traditional Medicine, has been widely used as a medicine to clear heat, promote diuresis and remove jaundice and has also been used as a flavor in beverages, vegetables, and pastries because of its particular fragrance. *A. capillaris* has been regarded as a type of Chinese folk medicine and food by a growing number of people. Therefore, there have been considerable efforts to develop useful herbal medicines, such as *A. capillaris*, for the treatment of liver disease.

In recent years, herbal medicines have gained more attention and popularity for the treatment of liver disease because of their safety and efficacy (Ding et al., 2012). *A. capillaris* has been proven to possess good hepatoprotective activity based on modern pharmacological methods (Han et al., 2006). It is also an important medicinal material in China and is a popular anti-inflammatory (Cha et al., 2009a), choleretic (Yoon and Kim, 2011), and anti-tumor (Feng et al., 2013) herbal remedy.

Phytochemical studies have revealed a number of volatile essential oils, coumarins, and flavonol glycosides as well as a group of unidentified aglycones from *A. capillaris* (Komiya et al., 1976; Yamahara et al., 1989). The essential oil of *A. capillaris* (AEO) is one of the main pharmacological active compounds and confers anti-inflammatory (Cha et al., 2009a) and anti-apoptotic properties (Cha et al., 2009b). However, as AEO is one of the main compounds of *A. capillaris*, the potential hepatoprotective activities of the major constituents from *A. capillaris* should be explored.

In this study, the protective effect of AEO on carbon tetrachloride (CCl_4)-induced hepatotoxicity was evaluated by biochemical methods, such as hepatic reduced glutathione (GSH),

* Corresponding authors.
E-mails: xueyanfu2661@163.com (X. Fu), tungbuo@163.com (W. Sun).
[1] These authors equally contributed to this manuscript; they should be considered first author.

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malondialdehyde (MDA) levels, superoxide dismutase (SOD), and glutathione peroxidase (GSH-P*x*) activity, as well as the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum. The extent of CCl_4-induced liver injury was also analyzed through histopathological observations, accompanied with phytochemical analysis by GC–MS to identify the constituents of AEO.

Materials and methods

Drugs and chemicals

The essential oil of *Artemisia capillaris* Thunb., Asteraceae, was obtained from Kangshen Natural oils Co., Ltd (Jishui, Jiangxi Province, China). CCl_4 was purchased form Damao Chemical Company (Tianjin, China). Bifendate tablets were purchased from Yunpeng, Shanxi Pharmaceutical Co., Ltd. (no. A130602). The diagnostic kits used for the determination of ALT, AST, SOD, MDA, GSH and GSH-P*x* were obtained from the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China).

AEO analysis

AEO was analyzed by gas chromatography–mass spectrometry (GC/MS) using a Shimadzu QP2010 plus GC with Rxi-5Sil MS (30 m × 0.25 mm; 0.25 μm film thickness). The GC/MS was run under the following conditions: a fused-silica capillary column with helium at 1.60 ml/min and an injector at 250 °C. The GC oven temperature was initially held at 40 °C for 5 min. Thereafter, the temperature was raised with a gradient of 3 °C min^{-1} until it reached 230 °C. This temperature was held for 3 min. Finally, the temperature was then raised with a gradient of 1 °C min^{-1} until 260 °C and again held for 10 min. Mass spectra were taken at 70 eV from 33 to 500 Da. Identification of the compounds was based on the comparison of the mass spectral data on a computer matched with NIST (similarity index >85%) and those described in the literature (Sylvestre et al., 2006; Cheng et al., 2008; Ait-Ouazzou et al., 2012; Argyropoulou and Skaltsa, 2012; Ćavar et al., 2012; Ben Mansour et al., 2013; Chen et al., 2013; Gouveia and Castilho, 2013; Murugan and Mallavarapu, 2013; Singh et al., 2013; Bagheri et al., 2014; Da Silva et al., 2014; Desai et al., 2014; Pandey et al., 2014; Qi et al., 2014; Rather et al., 2014; Sadgrove et al., 2014; Sereshti et al., 2014; Tao et al., 2014; Tian et al., 2014). The identification of compounds was performed according to their retention indices relative to C_8–C_{24} *n*-alkanes and MS.

Animals

Male ICR mice at 6–8 weeks of age (18–22 g) were purchased from the Medicine Experiment Animal Center of Ningxia Medicine University. The animals were allowed to acclimatize for two days to animal room conditions and were maintained on a standard pellet diet and water *ad libitum*. The food was withdrawn on the day before the experiment, but the animals were allowed free access to water. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals (1996). The experimental procedures were approved by our institutional animal research ethics committee (approval number: SYXK (NING) 2011-0001).

Rodent model design

The animals were randomly divided into five groups of fifteen mice each. The control group and model group mice were gavaged with sesame oil for 6 days. Positive control group mice were gavaged with bifendate tablets (BT, 10 mg/kg) for 6 days. The experimental groups were treated with 100 mg/kg and 50 mg/kg AEO dissolved in sesame oil for 6 days. On day 6, the control group was treated with sesame oil, and all of the other groups were treated with a single dose of 0.2% CCl_4 in sesame oil (10 ml/kg) by intraperitoneal injection. The mice were then fasted free of water, and blood samples were collected from the retrobulbar vessels; collected blood was centrifuged at 3000 × *g* for 10 min to separate the serum. Cervical dislocation was performed immediately after withdrawal of blood, and liver samples were promptly removed. One part of the liver sample was immediately stored at −20 °C until analysis, and another part was excised and fixed in a 10% formalin solution; the remaining tissues were stored at −80 °C for histopathological analysis (Wang et al., 2008; Hsu et al., 2009; Nie et al., 2015).

Measurement of the biochemical parameters in the serum

Liver injury was assessed by estimating the enzymatic activities of serum ALT and AST using the corresponding commercial kits according to the instructions for the kits (Nanjing, Jiangsu Province, China). The enzymatic activities were expressed as units per liter (U/l).

Measurement of MDA, SOD, GSH and GSH-Px in liver homogenates

Liver tissues were homogenized with cold physiological saline at a 1:9 ratio (w/v, liver:saline). The homogenates were centrifuged (2500 × *g* for 10 min) to collect the supernatants for the subsequent determinations. Liver damage was assessed according to the hepatic measurements of the MDA and GSH levels as well as the SOD and GSH-P*x* activities. All of these were determined following the instructions on the kit (Nanjing, Jiangsu Province, China). The results for MDA and GSH were expressed as nmol per mg protein (nmol/mg prot), and the activities of SOD and GSH-P*x* were expressed as U per mg protein (U/mg prot).

Histopathological analysis

Portions of freshly obtained liver were fixed in a 10% buffered paraformaldehyde phosphate solution. The sample was then embedded in paraffin, sliced into 3–5 μm sections, stained with hematoxylin and eosin (H&E) according to a standard procedure, and finally analyzed by light microscopy (Tian et al., 2012).

Statistical analysis

The results were expressed as the mean ± standard deviation (SD). The results were analyzed using the statistical program SPSS Statistics, version 19.0. The data were subjected to an analysis of variance (ANOVA, $p < 0.05$) followed by Dunnett's test and Dunnett's T_3 test to determine the statistically significant differences between the values of various experimental groups. A significant difference was considered at a level of $p < 0.05$.

Results and discussion

Constituents of AEO

Upon GC/MS analysis, the AEO was found to contain 25 constituents eluted from 10 to 35 min, and 21 constituents accounting for 84% of the essential oil were identified (Table 1). The volatile oil contained monoterpenoids (80.9%), sesquiterpenoids (9.5%), saturated unbranched hydrocarbons (4.86%) and miscellaneous acetylene (4.86%). Compared with other studies (Guo et al., 2004), we found abundant monoterpenoids (80.90%) in the AEO. The results showed that the most abundant constituent of AEO is β-citronellol (16.23%). Other major components of AEO include 1,8-cineole (13.9%), camphor (12.59%), linalool (11.33%), α-pinene

Table 1
Chemical composition of essential oil from *Artemisia capillaris Thun.* (AEO).

Compound	*Rt*[a]	RI^{calc} [b]	RI^{lit} [c]	%[d]
α-Pinene	11.133	928	933	7.21
Camphene	11.340	943	946	1.52
Sabinene	13.269	968	969	1.09
β-Pinene	13.640	975	974	3.99
Myrcene	14.313	987	988	2.02
Iso-cineole	15.612	1012	1012	1.27
p-Cymene	16.025	1020	1020	2.28
Limonene	16.273	1025	1024	2.00
1,8-Cineole	16.424	1028	1026	13.09
Artemisia ketone	17.965	1057	1056	1.44
Terpinolene	19.226	1081	1086	1.30
Linalool	20.112	1098	1095	11.33
trans-Pinocarveol	17.965	1135	1135	1.44
Camphor	22.215	1140	1141	12.59
Terpinen-4-ol	23.998	1176	1174	1.21
α-Terpineol	24.744	1191	1186	1.89
Dodecane	25.173	1199	1200	2.44
β-Citronellol	26.407	1226	1211	16.23
Thymol	29.356	1288	1289	3.22
Longipinene	34.330	1401	1402	2.16
(*E*)-Caryophyllene	34.813	1413	1417	1.73

[a] *Rt* – retention time (min) form a linear temperature program.
[b] RI^{calc} – retention index calculated for each compound.
[c] RI^{lit} – retention index obtain from published literatures.
[d] % – relative abundances from the peak area integration.

(7.21%), β-pinene (3.99%), thymol (3.22%), and myrcene (2.02%). The variation in the chemical composition may be related to the environmental conditions that the plant was exposed to, such as mineral water, sunlight, the stage of development and nutrition.

As previously suggested, few studies have reported the hepatoprotective effects of some of these major compounds. However, there are findings that indicate that major compounds possess an anti-inflammatory effect, such as thymol (Braga et al., 2006), eucalyptol, limonene, and linalool (Ku and Lin, 2013). β-Myrcene and 1,8-cineole were also found to eliminate TCDD-induced oxidative stress in rats (Ciftci et al., 2011).

Measurement of the biochemical parameters in the serum

CCl_4-induced hepatic injury is a commonly used experimental animal model (Johnston and Kroening, 1998). A previous study reported that CCl_4 administration is associated with activation by the cytochrome system (*e.g.*, CYP_2E_1) to form trichloromethyl radicals ($CCl_3^{\bullet}$). It is generally thought that CCl_4 toxicity is due to a reactive intermediate that is generated by its reductive metabolism. This highly reactive intermediate is known to induce lipid peroxidation, oxidative stress, hepatic necrosis and apoptosis (Weber et al., 2003).

In this assay, the results of the hepatoprotective effects of AEO on the enzymatic activities of serum ALT and AST are shown in Fig. 1A and B, respectively. An intraperitoneal injection of CCl_4 induced a notable increase in ALT and AST activities in comparison with untreated control mice ($p < 0.01$); the increased serum activities of ALT and AST enzymes in CCl_4 treated mice confirmed the hepatic damage. Conversely, pretreatment with AEO at doses of 100 mg/kg and 50 mg/kg, as well as bifendate tablets, obviously reversed the toxin-induced increase in the ALT and AST levels compared with toxin model group 2 ($p < 0.01$). When the dose reached 100 mg/kg, the results were as good as BT.

ALT and AST are important metabolic enzymes of the liver. After CCl_4 administration, the serum ALT and AST activities in mice were evaluated. These enzymes normally exist in the cytoplasm, but upon liver injury, they can enter the circulatory system due to toxicity-mediated altered permeability of the cellular membrane (Wills and Asha, 2006). In a previous study, it was reported that administration of CCl_4 in mice caused increased ALT and AST activities.

Our data showed that the liver damage induced by CCl_4 elevates liver marker enzymes. Elevated activities of serum enzymes, ALT and AST are indicative of cellular leakage and the loss of the functional integrity of the cell membrane in the liver, while pretreatment with AEO effectively decreased the amount of AST and ALT leakage.

Measurement of MDA, SOD, GSH and GSH-Px in liver homogenates

The levels of MDA suggest enhanced peroxidation leading to tissue damage and failure of the antioxidant-defense mechanisms to prevent the formation of excessive free radicals (Cheng et al., 2013; Pareek et al., 2013). However, AEO at 50 and 100 mg/kg could markedly prevent the increase in MDA formation (Fig. 1C), which clearly demonstrated the ability of AEO to relieve lipid peroxidation. MDA is well known to be the most abundant individual aldehyde resulting from lipid peroxidation and is commonly used as an indicator of liver tissue damage involving a series of oxidative chain reactions (Huang et al., 2012). In the present research, mice treated with CCl_4 showed a striking increase in MDA levels compared to untreated normal mice ($p < 0.01$). SOD and GSH-P*x* are the major enzymes that play an important role in the elimination of toxic metabolites, which are the major cause of the liver pathology caused by CCl_4 (Cengiz et al., 2013; Xia et al., 2013). Here, administration of CCl_4 to mice sharply decreased the SOD and GSH-P*x* activities in mouse liver tissues, as evidenced by the inhibition of their enzymatic activities. However, the decrease in these enzymatic activities was significantly elevated by pretreatment with AEO, suggesting that they could protect the three antioxidant enzymes in CCl_4-damaged livers. The protective effect of AEO was potent in terms of the SOD and GSH-P*x* activities, which is most likely related to the elimination of toxic metabolites (Fig. 1D and F). SOD is a manganese-containing enzyme in the mitochondria and converts the dismutation of superoxide anions into hydrogen peroxide (H_2O_2) (Reiter et al., 2000). GSH-P*x* is an important enzyme that catalyzes the reduction of H_2O_2 and hydroperoxides and terminates the chain reaction of lipid peroxidation by removing lipid hydroperoxides from the cell membrane (Ai et al., 2013). Similarly, GSH is an important water-phase antioxidant and an essential cofactor for antioxidant enzymes; it protects the mitochondria against endogenous oxygen radicals (Han et al., 2006). The endogenous antioxidant GSH peroxidase inhibits oxidative stress by quickly removing superoxide radicals (Chaudiere et al., 1999) and plays an important role in clearing intracellular hydrogen peroxide and lipid peroxides. Therefore, the level of GSH in the body is considered to be an important indicator of antioxidative capacity (Campo et al., 2001; Iwamoto et al., 2002). Fig. 1E showed that CCl_4 treatment induced a significant decrease in the level of GSH in liver homogenates compared to control livers. The treatment of mice with AEO at 50 and 100 mg/kg significantly increased the hepatic GSH content compared with the CCl_4-treated group. This finding indicated that the free radicals released in the liver were effectively scavenged during AEO treatment. All of these data suggest that the hepatoprotective effects of AEO on CCl_4-induced liver damage in mice are associated with its capacity for free radical scavenging and antioxidation.

Histopathological examination of mouse livers

Furthermore, the histopathological observations substantiated the biochemical analysis, as presented in Fig. 2. The histology of the liver sections from the normal control group showed normal

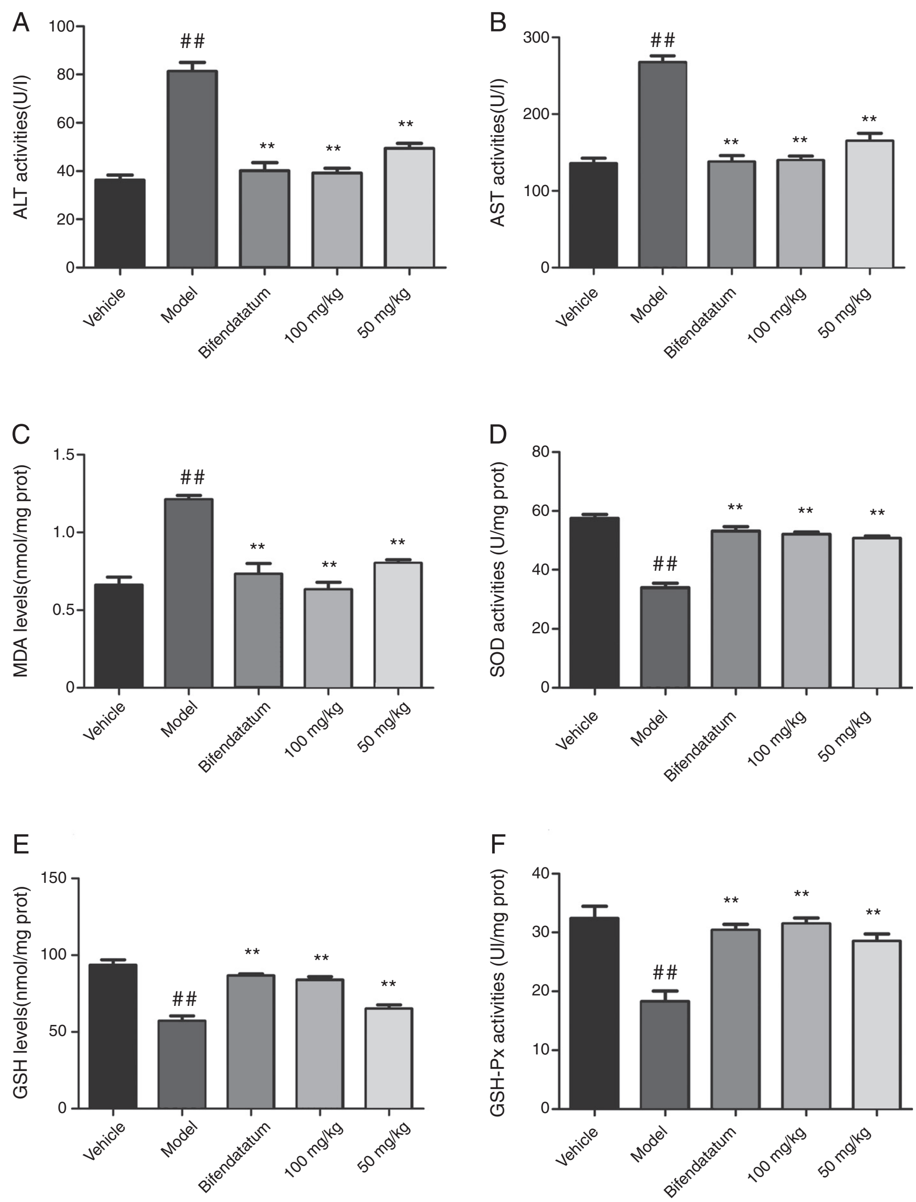

Fig. 1. Effects of oral pretreatment with AEO on CCl_4-induced hepatic injury. The results of the biochemical parameters (ALT, AST) and hepatic levels (MDA, SOD, GSH and GSH-P*x*) are represented as the mean ± SD ($n = 15$). The mice were pretreated with AEO (100, 50 mg/kg) once daily for 6 consecutive days. The control mice were given sesame oil. Differences between the groups were determined by ANOVA followed by Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to the control group.

hepatic cells with a well-preserved cytoplasm and legible nucleus, hepatocytes that were radially arranged around the central vein, and a well-defined sinusoidal line (Fig. 2A). CCl_4 caused damage to the hepatic architecture and produced histological changes, such as severe hepatocellular degeneration and necrosis around the central vein, sinusoidal dilatation, loss of cellular boundaries, inflammatory cell infiltration, and cytoplasmic vacuolation, all of which confirmed the successful establishment of liver injury (Fig. 2B). In contrast, CCl_4-intoxicated mice pretreated with BT showed near normalization of liver tissues with no significant changes in hepatocytes (Fig. 2C). The liver damage was noteworthy and dose-dependently reduced by pretreatment with AEO at different doses, as indicated by the significant reduction in the number of ballooning-degenerated hepatocytes and significantly decreased necrotic area. In the group pretreated with a low dose of AEO (Fig. 2E), the liver sections showed moderate hypertrophy of hepatocytes with a relatively intact central vein, a shrunken sinusoidal area and reduced inflammatory cells. The administration of a high dose of AEO (Fig. 2D) induced a near-normal appearance, suggesting that AEO at a dose of 100 mg/kg was more effective compared to the dose of 50 mg/kg and that AEO could protect the liver from acute CCl_4-induced hepatic damage. This was in good agreement with the results from the serum biochemical markers.

In summary, the results from this study clearly demonstrate that AEO is effective for the prevention of CCl_4-induced hepatic injury in mice. This study indicates the potential for the development of AEO as a potential hepatoprotective agent in cases of CCl_4 induced acute liver injury.

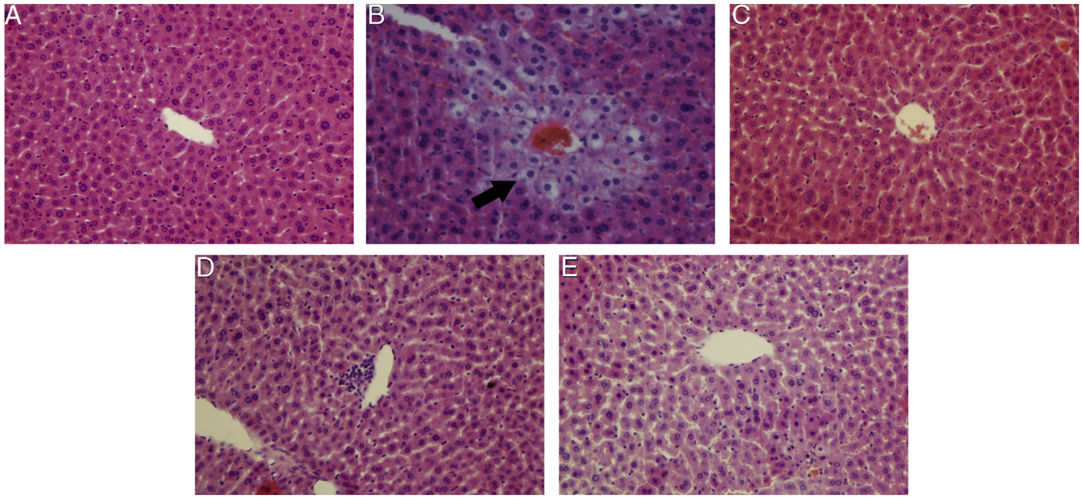

Fig. 2. Effects of AEO on the histological changes of the liver after CCl_4 treatment in mice (original magnification 400×): (A) control group; (B) CCl_4-intoxicated group (model group); (C) bifendatatum (10 mg/kg) + CCl_4; (D) AEO (100 mg/kg) + CCl_4; (E) AEO (50 mg/kg) + CCl_4.

Author contributions

QG, LY and XZ contributed to performing the laboratory work. YZ and XZ contributed to the estimation of the chemical composition. BW contributed to the analysis of the data. LY wrote the manuscript. QG and XW contributed to the critical reading of the manuscript. XF and WS designed the study, supervised the laboratory work and contributed to the critical reading of the manuscript. All of the authors have read the final manuscript and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

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