Acupuncture and Herbal Medicine

Elucidating the mechanism of hepatotoxicity in Euodia rutaecarpa: insights from QSAR toxicity prediction and metabolomics

Chunqi Yang[1,2], Chengcai Lai[2], Yi Ru[2], Baoying Shen[2], Xiangjun Wu[2,3], Jialu Cui[2], Fangyang Li[2], Cheng Zhang[2], Zhuo Shi[2], Qingyuan Qian[2], Chengrong Xiao[2], Yuguang Wang[2,*], Boli Zhang[2,4,*], Yue Gao[1,2,*]

[1]College of Life Science and Bioengineering, Beijing University of Technology, Beijing, China; [2]Department of Pharmaceutical Sciences, Beijing Institute of Radiation Medicine, Beijing, China; [3]School of Pharmacy, Henan University, Kaifeng, China; [4]State Key Laboratory of Component-based Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China

Abstract

Objective: Euodia rutaecarpa, (Wu Zhu Yu) a Chinese medicine clinically used to treat gastrointestinal disorders, has been widely employed. However, Euodia rutaecarpa is regarded as a small toxic traditional Chinese medicine in the Chinese Pharmacopoeia and other herbal works. Using toxicity predictions combined with *in vitro* and *in vivo* studies, this study aimed to identify the toxic components and toxic target organs of Euodia rutaecarpa, and explore its toxic mechanism from a metabolic perspective.

Methods: The toxic target organs of Euodia rutaecarpa were identified through *in vitro* and *in vivo* studies. *In vitro* toxicity screening was performed by alkaloid enrichment and isolation. The potential toxicity of compounds was predicted by Absorption, Distribution, Metabolism, Excretion, and Toxicity Predictor (ADMET Predictor) based on Quantitative Structure–Activity Relationship (QSAR) construction. In addition, the study integrated the serum metabolomic analysis after the administration of potentially toxic components to clarify the effect of potentially toxic substances on metabolism in mice.

Results: Comparing the acute toxicity in mice of different extraction methods and before and after processing, it was evident that Euodia rutaecarpa alcoholic extract had the highest toxicity, and the target organ of Euodia rutaecarpa toxicity was the liver. The alkaloid fraction of alcoholic extract of Euodia showed strong cytotoxicity. The potential toxicity of Euodia rutaecarpa was calculated and predicted by ADMET Predictor, and alkaloids are suspected to be responsible for the toxicity of Euodia rutaecarpa. Evodiamine significantly reduced the number of cells and increased the mitochondrial membrane potential *in vitro*. Different metabolites were significantly identified by serum metabolomics, of which bile acid metabolism and steroid hormone biosynthesis are the key pathways of hepatotoxicity.

Conclusions: Clarify the scientific significance of clinical use of processed products by comparing the acute toxicity of different extraction methods before and after processing. Combining the toxicity prediction based on QSAR with the toxicity screening *in vitro* and *in vivo*, the potential toxic target organs and toxic components of Euodia rutaecarpa can be identified. Through metabolomics, we preliminarily revealed that the hepatotoxicity of Euodia rutaecarpa may be related to bile acid metabolism and steroid hormone biosynthesis. This study lays the foundation for elucidating the mechanism of Euodia rutaecarpa and evaluating its safety and quality.

Keywords: ADMET predictor, Euodia rutaecarpa, Evodiamine, Hepatotoxicity, LD_{50}, Metabonomic

Introduction

Euodia rutaecarpa (Wu Zhu Yu) is widely distributed in South China and Shanxi, China, and has a long history of clinical use. It is mainly used for pain relief, dehumidification, and the treatment of gastrointestinal diseases, and it has a wide range of pharmacological activities[1]. Euodia rutaecarpa has been listed as a low-toxicity traditional Chinese medicine in the Chinese Pharmacopoeia Commission 2020, and processed products are often used in clinical practice. Evodiamine (EVO), belonging to the quinazolinocarboline alkaloid class, is the main bioactive component isolated from Euodia rutaecarpa. Modern pharmacological studies show that EVO has pharmacological activities such as antibacterial[2], vascular protection[3], central nervous system protection[4], anti-inflammatory[5], and antioxidant properties[6].

Chunqi Yang contributed equally to this work.

**Corresponding author. Yuguang Wang, E-mail: wangyg@bmi.ac.cn; Boli Zhang, E-mail: zhangbolipr@163.com; Yue Gao, E-mail: gaoyue@bmi.ac.cn.*

Received 24 December 2023 / Accepted 27 April 2024

How to cite this article: *Yang CQ, Lai CC, Ru Y, Shen BY, Wu XJ, Cui JL, Li FY, Zhang C, Shi Z, Qian QY, Xiao CR, Wang YG, Zhang BL, Gao Y. Elucidating the mechanism of hepatotoxicity in Euodia rutaecarpa: insights from QSAR toxicity prediction and metabolomics. Acupunct Herb Med 2024;4(2):257–270. doi: 10.1097/HM9.0000000000000108*

In vitro studies have shown that the ethanol extract of Euodia rutaecarpa can significantly inhibit LPS-induced neuroinflammation while exhibiting strong antioxidant effects on neutrophils and microglia[7]. In addition, current research results indicate that Euodia rutaecarpa has significant anti-tumor activity and is a potential therapeutic drug for various cancers, making it a highly promising anti-cancer drug[8]. Panda et al.[9] reviewed the anti-cancer potential and therapeutic effects of EVO alkaloid in combination with various anti-cancer drug and proposed that EVO alkaloid in Euodia rutaecarpa is an exciting anti-cancer drug. According to current research, EVO inhibits cancer growth by activating apoptosis pathways, inhibiting abnormal cell proliferation, and inhibiting oxidative stress[10].

Since ancient times, Euodia rutaecarpa has been known for its low toxicity; adverse reactions also limit its clinical use, such as hepatotoxicity[11], nephrotoxicity[12], cardiotoxicity[13], and gastrointestinal adverse reactions[14]. So far, 165 chemical components have been isolated from Euodia rutaecarpa, mainly including volatile oils, alkaloids, bitterness compounds, etc. Research suggests that volatile oils (eg, evodene)[15] and alkaloids (eg, EVO)[16] are potential toxic components in Euodia rutaecarpa, and their toxicity can be reduced through processing or compatibility in clinical practice. Jing et al.[17] determined the content of volatile oil in Zuojin Tang and Euodia rutaecarpa and conducted a comparative experiment using thin-layer analysis. The results showed that the content of volatile oil in the formula was only 1/10 of that of a single drug. As volatile oil is a toxic component of Euodia rutaecarpa, it can be considered that Huanglian can reduce the toxicity of Euodia rutaecarpa[18]. Xu et al.[19] used high-performance liquid chromatography to investigate the content of main alkaloids in Euodia rutaecarpa before and after the compatibility of Huanglian and Euodia rutaecarpa. The results showed that the content and dissolution rate of Euodia rutaecarpa alkaloids and Euodia rutaecarpa alkaloids in the water decoction after compatibility were significantly reduced. From the fingerprint of the drug pair, Huanglian is beneficial for the dissolution of lipid-soluble alkaloids from Euodia rutaecarpa, showing a proportional relationship. The changes in the content of Euodia rutaecarpa alkaloids after the combination of Euodia rutaecarpa and ginger gradually decrease with the increase in ginger dosage. The above results indicate that the comprehensive toxicity of a single component is lower than that of the entire volatile oil, but the volatile oil is significantly reduced after processing and boiling, while alkaloids and other active ingredients still exert their effects[20]. In long-term toxicity experiments, Euodia rutaecarpa has significant hepatotoxicity and nephrotoxicity, which can significantly reduce the level of manganese superoxide dismutase (MnSOD). It induces oxidative stress in liver mitochondria, leading to mitochondrial vacuolization, adenosine triphosphate (ATP) depletion, and cytochrome C release, thereby inducing cell death[21]. The accumulating evidence indicates that the biological toxicity of Euodia rutaecarpa is characterized by multiple target organs, especially the kidney, liver, and heart. Quantitative structure-activity modeling indicated that EVO may cause cardiotoxicity. Yang et al.[13] showed that EVO causes cardiovascular side effects such as oxidative stress in rat cardiomyocytes and zebrafish. Based on the above research, we believe that using the effective components of Euodia rutaecarpa as the research scope, further exploring the correlation between the various effective components of Euodia rutaecarpa and their toxicity, and identifying the toxic components in Euodia rutaecarpa are crucial. In addition, clarifying the toxic targets and mechanisms of Euodia rutaecarpa provides a theoretical basis for rational clinical use and reduces adverse drug reactions.

Metabolomics is a discipline that deeply understands the normal or diseased state of the organism through quantitative and qualitative analysis of small-molecule metabolites in the organism, reflecting the metabolic changes in the organism. It is the most powerful tool for studying the metabolites and their changes in the organism after diseases and exogenous stimuli, and it can provide more accurate and direct information on the end of the life system and phenotype. At present, metabolomics is widely used in the early detection of liver injury. Through accurate metabolite analysis techniques combined with a comprehensive reference database, it can efficiently and accurately discover biomarkers of liver injury, making it a powerful tool for studying drug-induced liver injury[22]. Based on nuclear magnetic resonance spectroscopy (NMR) and liquid chromatography-gas chromatography-mass spectrometric (LC-GC-MS), it is inferred that traditional Chinese medicine can interfere with energy metabolism, phospholipid metabolism, and other pathways to deeply explore the mechanism of liver injury, gradually becoming a new strategy for comprehensively revealing the toxicity of traditional Chinese medicine through the identification of metabolites and changes in metabolite profiles caused by liver injury caused by traditional Chinese medicine[23].

In this study, the liver toxicity of Euodia rutaecarpa was determined through acute toxicity experiments. In this direction, we predict the hepatotoxicity of the main active ingredient in Euodia rutaecarpa through toxicity prediction and component separation techniques. The study further identified the major components causing hepatotoxicity *in vitro* and *in vivo* and initially explored their *ex vivo* and *in vivo* toxic mechanisms of action.

Reagents and materials

Euodia rutaecarpa was purchased from Tongrentang (Tongrentang Pharmaceutical Co., Ltd., Beijing, China). EVO, rutecarpine, evodine, and synephrine were purchased from the China Institute of Food and Drug Verification (Beijing, China). RPMI 1640 media was obtained from Sigma (St. Louis, MO, USA).

Drug and administration

KM mice (SPF grade) weighing 18 to 22 g were used for the experiment and were purchased from Weitonglihua [Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd., Experimental Animal Production License: SCXK (Jing) 2021-0017]. This study was approved by and in accordance with the Animal Ethics Committee of the Academy of Military Medical Sciences Animal Laboratory of the Laboratory Animal Center

(IACUC-DWZX-2020-60). Experiments were conducted after adoptive rearing.

Mice were randomly divided into a blank control group, an ethanol extract group of Euodia rutaecarpa, an aqueous extract group of Euodia rutaecarpa, and a processed aqueous extract group of Euodia rutaecarpa; with 10 mice in each group (half male and half female). Mice were observed continuously for 14 d, including status, diet, and water intake, after single-dose administration of the drug at 0.2 mL/10 g by oral gavage. In addition, the blank group and the EVO group (L: 1 g/kg, M: 2.5 g/kg, H: 5 g/kg) were treated according to the above method, and continuously administered by gavage for 14 d. Serum was collected and stored at −80°C.

Sample collection

The body weight and survival of mice were recorded to evaluate *in vivo* toxicity for 14 d *p.o. (per os)*. In this experiment, mice were administered pentobarbital, exsanguinated *via* the femoral artery, and organs were removed after execution. Formalin-fixed tissue from the mice was paraffin embedded for pathological analysis. For biochemical testing, blood samples were centrifuged at 3,000 rpm for 10 min, then immediately frozen at −80°C for serum collection.

Histomorphology analysis

Collected tissue was fixed in formalin, paraffin embedded, sectioned, stained with Hematoxylin & eosin (H&E), and viewed under a microscope using normal light.

Drug extraction and separation

These herbs were purchased from Tongrentang (Tongrentang Pharmaceutical Co., Ltd., Beijing, China) and identified by Professor Yang Wenzhi from Tianjin University of Traditional Chinese Medicine. Take the proper number of herbs to 10 times the amount of extract soaked overnight and then decocted to boil, keep a mild boiling condition for 30 min, and then continue to eight times the amount of aqueous decocted twice. Ethanol extract of Euodia rutaecarpa, aqueous extract of Euodia rutaecarpa, and processed aqueous extract of Euodia rutaecarpa was conducted as above. Following extraction, the filtration was collected, concentrated, and freeze-dried to obtain a lyophilized powder.

Previous studies have suggested that the ethanol extract of Euodia rutaecarpa contains the most abundant monomers. Therefore, the study takes the freeze-dried powder of the abovementioned Euodia rutaecarpa ethanol extract and places it in a round bottom flask for ultrasonic extraction, filtration, and concentration. The precipitate is dissolved in methanol and then extracted by ultrasound, filtered, and concentrated to obtain the filtrate. Then the two filtrates were combined and dissolved by adding methylene chloride and methanol (1:1) by sonication. After dissolution, centrifugate the supernatant and elute it with a gel column. During the elution process, use bismuth potassium iodide combined with fluorescent spots under the ultraviolet (UV) lamp to detect and collect the eluate. Concentrate the collected eluate into a total alkaloid sample, and concentrate the remaining eluate into impurities.

The quantitation of alkaloids

Determination of pharmacopeial quality control components and other alkaloids in Euodia rutaecarpa by LC-MS. 100 µL 20% methanol/water solution was added dropwise until the powders were full dissolved, then shake and centrifuge the supernatant for positive and negative ion mode analysis. The profiling was analyzed using Thermo Scientific™Q Exactive™. Positive model: the separation of sample was performed by BEH C_8 column (1.7 µm, 2.1 mm × 100 mm; Waters Corporation, Milford, MA, USA). Mobile phase A was an aqueous solution containing 0.1% formic acid and mobile phase B was acetonitrile containing 0.1% formic acid. The elution procedure was optimized as follows: 0 to 1 minute, 5%(B); 1 to 24 min, 5% to 100% (B); 24.1 to 27.5 min, 100%(B); 27.6 to 30 min, 5%(B). The flow rate was 0.35 mL/min; the injection volume was 5µL and the column temperature was 50°C. Negative model: the separation of sample was performed by HSS T_3 column (1.8 µm, 2.1 mm × 100 mm, Waters). Mobile phase A was an aqueous solution containing 6.5 mM ammonium bicarbonate and mobile phase B was 95% methanol/water containing 6.5 mM ammonium bicarbonate. The elution procedure was optimized as follows: 0 to 1 min, 5%(B); 1 to 18 min, 5% to 100%(B); 18.1 to 22 min, 100%(B); 22.1 to 25 min, 5%(B). The flow rate was 0.35 mL/min; the injection volume was 5 µL and the column temperature was 50°C.

Electrospray ionization (ESI) source was used in both positive and negative ion modes to collect the data. The heated electric spray ion source HESI Positive mode is adopted, and the first level full scan + DDA second level sub ion scan mode is adopted. Spray voltage (kV): +3.8; capillary temperature (°C): 320; Aux gas heater temperature (°C): 350; Sheath gas flow rate (Arb): 35; Aux gas flow rate (Arb): 8; S-lens RF level: 50; Mass range (m/z): 70 to 1,050, full MS resolution: 70,000; MS/MS resolution: 17,500; TopN: 5; NCE/stepped NCE: 20, 40. The heating electric spray ion source HESI Negative mode is adopted, and the first level full scan + DDA second level sub ion scan mode is adopted. Spray voltage (kV): −3.0; capillary temperature (°C): 320; Aux gas heater temperature (°C): 350; Sheath gas flow rate (Arb): 35; Aux gas flow rate (Arb): 8; S-lens RF level: 50; mass range (m/z): 70 to 1,050, full ms resolution: 70,000; MS/MS resolution: 17,500; TopN: 5; NCE/stepped NCE: 20, 40.

High-content screening

ImageXpress Micro 4 Confocal High-Content Analysis System and MetaXpress High-Content Image Acquisition and Analysis Software were used for high-content screening of L02 cells in 96-well black plates. For analysis, three horizontally adjacent images were captured for each well of the experimental and control samples. For 30 min in the dark, the cells were washed and incubated with 10 M Hoechst (350 nm/461 nm), 1 M SYTOX®Green (491 nm/509 nm), and 1 M MitoTrackerTM Red

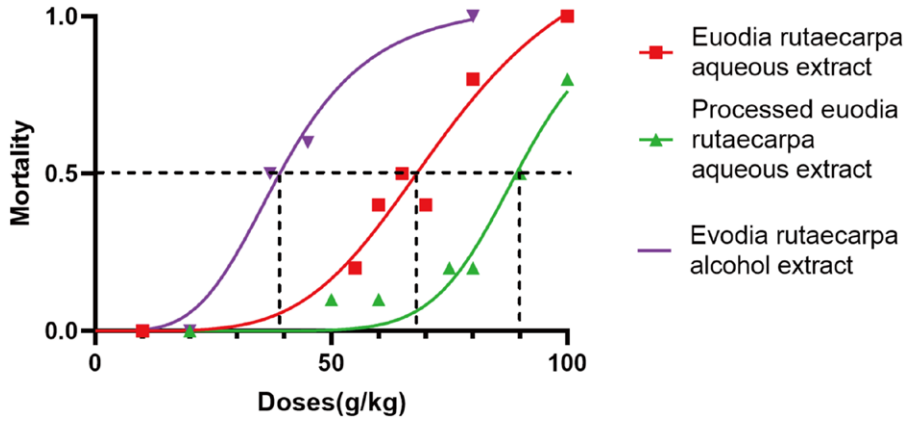


Figure 1. Comparison of acute toxicity of different extraction methods of Euodiae Fructus in mice. (A) The survival rates of single-dose administration of Euodiae Fructus alcohol extract (purple), Euodiae Fructus aqueous extract (red), and processed Euodiae Fructus aqueous extract (green) in mice.

CMXRos (551 nm/576 nm). Using ImageXpressMicro4, cytotoxicity was assessed after removing the dye solution and adding 0.1% Triton X-100.

Toxicological parameter prediction

Absorption, Distribution, Metabolism, Excretion, and Toxicity Predictor 6.5 (ADMET Predictor 6.5) is a toxicity prediction model based on Quantitative Structure–Activity Relationship (QSAR). It is a toxicity prediction model based on QSAR to predict the ADMET properties of compounds and to develop ADMET risk coefficients. The study selected 10 monomers contained in Euodia rutaecarpa, downloaded the three dimensional (3D) molecular structure of each compound in PubChem, and saved it in SDF file format. Import the obtained files into ADMET predictor 6.5 software for toxicity prediction, including: acute toxicity, mutagenicity, chromosomal variability, carcinogenicity, reproductive toxicity, and potential liver toxicity, including the effects of alkaline phosphatase (ALP), glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) indicators. The changes in the levels of five liver drug enzymes are represented by elevated or normal. Other indicators are assigned a value of 0.5 points based on the characteristics and weight of the drug. After software weighting, TOX_Risk is calculated to evaluate the strength of toxicity.

Non-target metabolomic profiling

To detect more metabolites as much as possible, plasma metabolites were measure by untargeted metabolomic. Plasma was isolated form whole blood *via* centrifugation, which was obtained from the above. Then, samples were snap frozen in liquid nitrogen for 5 min and placed in an ultra-low temperature refrigerator at –80°C for subsequent experiments. To detect the metabolite information in the sample as accurately as possible, the study uses high-resolution mass spectrometry (HRMS Technology) to analyze the metabolites quantitatively and qualitatively.

Raw mass spectrometry file obtained by HRMS are imported into Compound Discoverer 3.1 (CD3.1) for spectrum processing and database search to obtain the qualitative and quantitative results of metabolites, then the data are subject to quality control to ensure the accuracy and reliability of the data results. In addition, the multivariate statistical analysis of metabolites, including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), was carried out to reveal the differences of metabolic patterns in different groups. Hierarchical clustering (HCA) and metabolite correlation analysis were used to reveal the relationship between samples and metabolites. Finally, the biological significance of metabolites was explained by functional analysis such as metabolic pathway.

Statistical analysis

We performed the data analysis using mean ± standard error mean (SEM). The data was repeated three times to achieve reproducibility. For comparisons, we used the Student's *t* test. GraphPad Prim 7.0 was used to calculate all the above data.

Results

Discovery of toxicity of Euodia rutaecarpa

Acute toxicity experiments on mice were performed to investigate the toxicity of extracts of Euodia rutaecarpa with different methods. The research results indicate that the median lethal dose (LD_{50}) of different extraction methods for extracting Euodia rutaecarpa is 38.5 g/kg (ethanol extract), 66.4 g/kg (aqueous extract), and 95.8 g/kg (processed product aqueous extract), respectively (Figure 1A, Table 1). Among them, the ethanol extract of Euodia rutaecarpa has the strongest toxicity, and the toxicity after processing is the weakest, indicating the scientific nature of reducing toxicity after traditional Chinese medicine processing. In addition, by recording the weight of mice within 7 d after administration, it was found that there was still a significant difference in weight between the mice and the control group after 7 d of recovery after administration of Euodia rutaecarpa ethanol extract (>40 g/kg), indicating that its toxicity is irreversible in the short term [Supplementary Figure S1, http://links.lww.com/AHM/A113].

Table 1

Survival rate of mice treated with different extraction methods of Fructus Evodiae

Groups	Dose (g/kg)	Total (*n*)	Death (*x*)	Mortality (*x*/*n*, %)	LD_{50} (g/kg)
Alcohol extract group of Fructus	0	10	0	0	35.5
	10	10	0	0	
	20	10	0	0	
	37	10	5	50	
	35	10	6	60	
	80	10	10	100	
Water extract group of Fructus Evodiae	0	10	0	0	66.4
	50	10	2	20	
	55	10	4	40	
	60	10	5	50	
	65	10	5	50	
	70	10	4	40	
	80	10	8	80	
Processed water extract group of Fructus Evodiae	0	10	0	0	95.8
	10	10	0	0	
	20	10	0	0	
	50	10	1	10	
	60	10	1	10	
	75	10	2	20	
	80	10	2	20	
	90	10	5	50	
	100	10	8	80	

LD_{50}: Median lethal dose.

Confirmation of toxic target organs of Euodia rutaecarpa

To further clarify the toxic effects of Euodia of Fructus e on target organs, the study calculated the changes in organ indices after administration of extracts with different extraction methods, and detected the biochemical changes in surviving mice after administration. The results of liver system number calculation show that the ethanol extract of Euodia rutaecarpa (>30 g/kg) can significantly increase the organ index, while the aqueous extract and processed aqueous extract have no significant increase in the organ index of mice. It is important to detect liver disease by measuring AST and ALT levels. The ratio of AST/ALT is usually fixed, but when liver damage persists and becomes severe, mitochondria are also damaged, and AST is released from the cytoplasm of mitochondria, resulting in an increase in the ratio. Our study found that the ethanol extract of Euodia rutaecarpa can significantly increase AST/ALT in mice at low doses (5 g/kg), and the aqueous extract of Euodia rutaecarpa (60 g/kg) also has the same effect, but there is no significant change in the processed Euodia rutaecarpa (Figure 2A–C). The above research results indicate that the ethanol extract of Euodia rutaecarpahas potential toxicity. To clarify the toxic target organs and types of damage caused by Euodia rutaecarpa, pathological examination of mouse tissues after administration of Euodia rutaecarpa ethanol extract was conducted. Pathological examination showed mild punctate or patchy necrosis of liver cells can be observed in the liver, and mixed inflammatory cell infiltration around liver blood vessels gradually becomes severe with increasing dosage (Figure 2D). The results are consistent with biochemical tests.

Isolation and screening of toxic substances from Euodia rutaecarpa

The potential hepatotoxicity of Euodia rutaecarpa was clarified by toxicity screening and relevant literature reports. The toxic monomer components in Euodia rutaecarpa are not known and the study was carried out to analyze the toxicity by extracting the total alkaloids from the ethanol extract of Euodia rutaecarpa [Supplementary Figure S2, http://links.lww.com/AHM/A114]. The method was able to enrich the major alkaloids in Euodia rutaecarpa and isolate other impurities in the extract. The results of mass spectrometry analysis of the extracted total biological samples and the rest of the impurity samples showed that the main alkaloids in Euodia rutaecarpa could be extracted by the above extraction method (Figure 3A). The samples of total alkaloids of Euodia rutaecarpa and other impurity components were further screened for hepatotoxicity by high connotation screening. High-content results showed that total alkaloids were able to significantly reduce the number of L02 cells, as well as lead to nuclear crumpling and

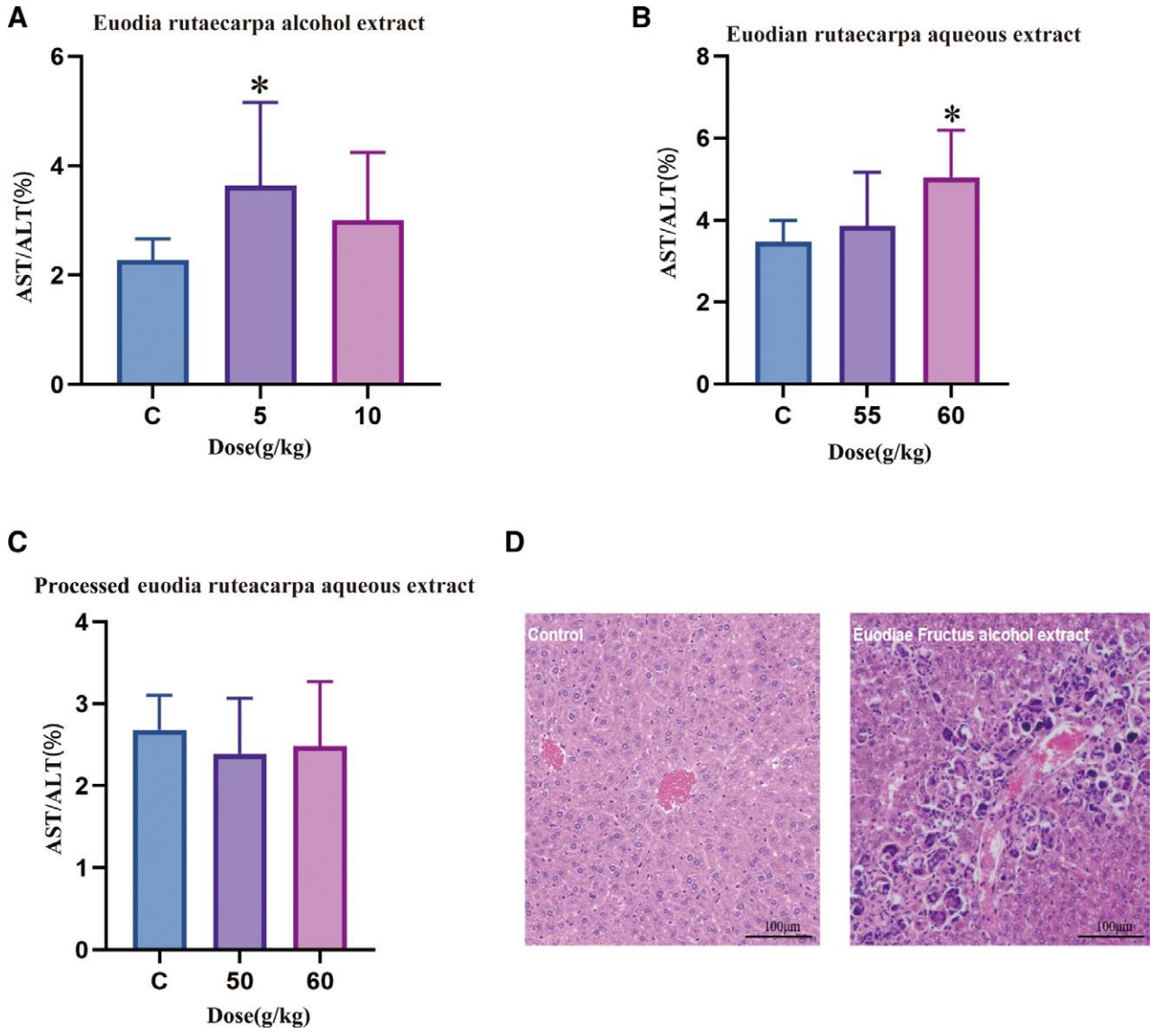


Figure 2. The liver injury effect of Fructus Evodiae under different extraction methods. Effects of Euodiae Fructus alcohol extract (A), Euodiae Fructus aqueous extract (B), and processed Euodiae Fructus aqueous extract (C) on biochemical indicators AST/ALT in mouse liver. (D) Histopathological examination of liver in Euodiae Fructus alcohol extract group (5 g/kg) (H&E staining, ×200). *$P < 0.05$ *vs*. the control group. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; H&E: Hematoxylin & eosin.

cell membrane rupture; whereas no significant changes were observed in the cells after impurity administration. Remarkably, the reduced fluorescence intensity of SYTOX®Green staining in the high-dose group after total alkaloids administration was inconsistent with the cell number, suggesting a decrease in the amount of cell membrane-damaged cells, which may be the result of the cell death itself enzymatically disintegrating into cellular debris. The results of the mitochondrial membrane potential assay showed that the total alkaloids could significantly enhance the mitochondrial membrane potential after administration, and it increased with the increase of the concentration of the administered alkaloids; the impurity group also increased slightly at the same time, but there was no significant difference (Figure 3B, C). The above *in vitro* results indicate that the main toxicity of Euodia rutaecarpa is alkaloids, and its toxicity is related to the increase of mitochondrial membrane potential.

Prediction of toxic substances in Euodia rutaecarpa

To determine the potential liver injury components in Euodia rutaecarpa, a total of 10 monomeric components, including the main alkaloids and potential toxic components reported in literature, were selected for analysis. We calculated the toxicity risk of Euodia rutaecarpa, and the toxicity data are shown in Table 2. The table reveals the parameters of acute toxicity, genotoxicity, liver toxicity, environmental toxicity, and other toxicological effects of individual components in Euodia rutaecarpa, and comprehensively evaluates the toxicity risk coefficient of the compound based on its genotoxicity, liver toxicity, and other factors. The TOX Risk score according to the evaluation rules indicated in column TOX Code which of the scoring rules in TOX_Ris are met.

In terms of predicting cardiac toxicity, EVO and Evodiamide may produce the human Ether-à-go-go Related Gene (hERG) channel toxicity; acute toxicity prediction, Rutaevin and Limonin have lower LD_{50} in

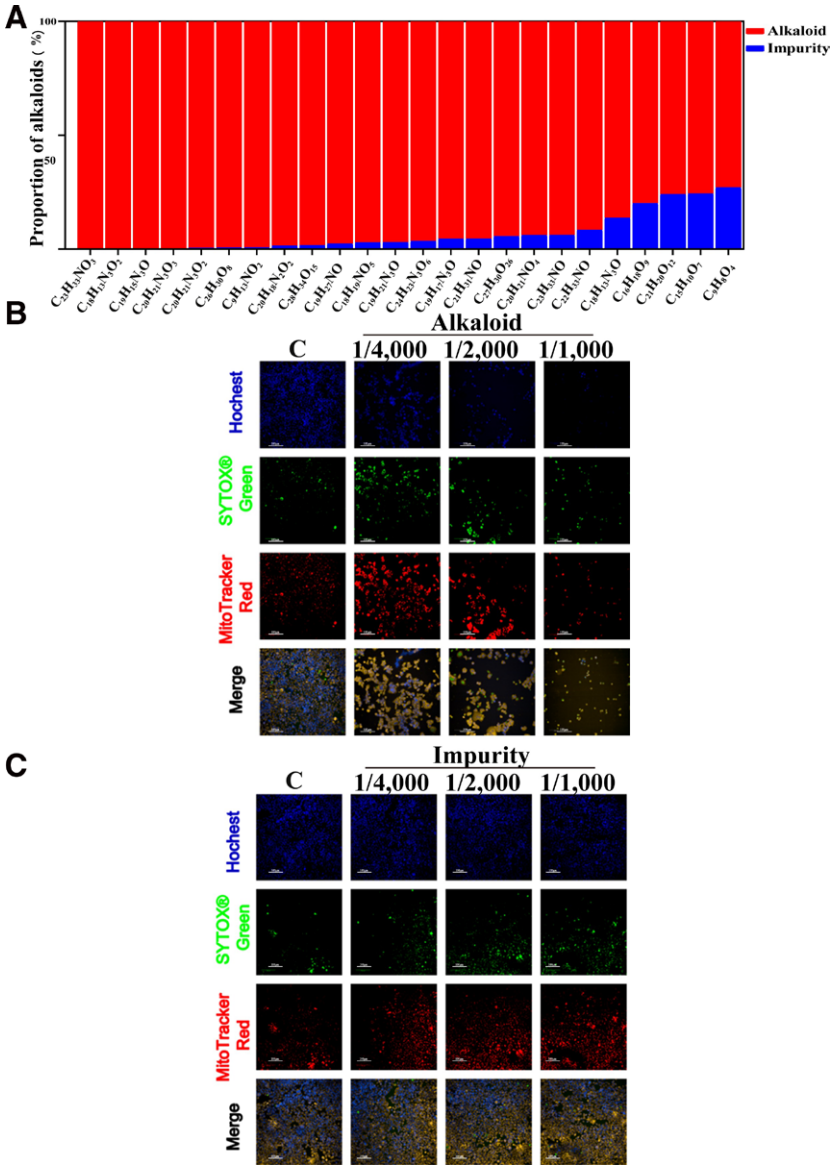


Figure 3. Screening of hepatotoxic components in Euodiae Fructus alcohol extract. (A) Extraction of total alkaloids from the Euodiae Fructus alcohol extract. (B) Hepatotoxic of total alkaloids from the Euodiae Fructus alcohol extract. (C) Hepatotoxic of impurity from the Euodiae Fructus alcohol extract.

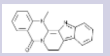
both large and small mice; predicting carcinogenicity, Evodine (rats) and Evolitine (mice) have potential carcinogenicity; predicting genotoxicity, Evolitine, Evodine, and Dehydroevodiamine; Chromosomal variations in mammalian cells cultured with Rutaecarpine exhibit certain genotoxicity; predicting phospholipid disease, the likelihood of various compounds producing phospholipid disease is not high. Predicting reproductive toxicity, dehydroevodiamine, rutaecarpine, and EVO may have the potential to produce reproductive toxicity; predicting liver adverse reactions, except for evocarpine and evodiamide, all compounds can be elevated in the five biochemical indicators of ALP, LDH, AST/ALT/GGT. For the overall evaluation of the 10 compounds, the ADMET risk coefficient is relatively low, but alkaloids such as dehydroevodiamine have higher scores and can significantly improve liver toxicity-related indicators such as GGT, AST, ALT, and LDH, indicating that alkaloids in Euodiae Fructus may have potential liver toxicity consistent with *in vivo* results (Table 1).

Screening for high-content toxicity

To clarify the toxic effects of the single components, the main alkaloids contained in Euodia rutaecarpa and the potentially hepatotoxic components reported in the literature were selected for validation in the study (EVO, Rutecarpine, Limonin, Synephrine, Evocarpine, and Dehydroevodiamine). The change in cell number after administration of each monomer was first determined by HOCHEST staining and the IC_{50} was calculated, with EVO being the most toxic (IC_{50}: 17.24 μM), followed by Evocarpine (IC_{50}: 35.76 μM). Studies of dead cell dyes after administration as a single agent during the period of use revealed a significant increase in fluorescence after the administration of EVO, Rutecarpine, Evocarpine, and Dehydroevodiamine. In conjunction with nuclear

Table 2

Prediction of compound monomer hepatotoxicity in Euodiae Fructus based on ADMET predictor

Structure	Identifier	Name	Formula	MW	TOX_ Risk	TOX_ Code	*Tox*	Rat_ Acute	Rat_ TD50	Mouse_ TD50	Ser_ AlkPhos	Ser_ GGT	Ser_ LDH	Ser_ AST	Ser_ALT	MUTx_ Risk	MUTx_ Code
	7172	Synephrine	C9H13NO2	167.209	0			664.447	47.3	282.859	Normal (92%)	Elevated (57%)	Elevated (49%)	Normal (84%)	Normal (86%)	0	
	196980	Evolitrine	C13H11NO3	229.237	1	MUT		576.283	18.02	53.557	Elevated (54%)	Normal (97%)	Normal (65%)	Elevated (72%)	Elevated (69%)	1.52	S_97; S_98; NIHS
	398789	Evodine	C18H19NO5	329.355	1	MUT		405.149	8.938	62.589	Normal (68%)	Normal (97%)	Normal (83%)	Elevated (68%)	Elevated (41%)	0.61	m_97; NIHS
	9817839	Dehydroevodiamine	C19H15N3O	301.35	2	Xr+; HEPX+; MUT		525.925	4.716	111.139	Elevated (96%)	Elevated	Elevated	Elevated (99%)	Elevated (86%)	1.27	m_97; S_98; m102
	MOL002662	Rutaecarpine	C18H13N3O	287.323	0.5	Xr-		1585.82	6.582	69.429	Elevated (69%)	Normal (97%)	Normal (87%)	Elevated (93%)	Elevated (86%)	1.01	m_97; m_98
	MOL003958	Evodiamine	C19H17N3O	303.366	0.5	Xr+		687.312	3.384	112.466	Normal (66%)	Normal (97%)	Normal (72%)	Normal (78%)	Elevated (53%)	1.01	m_97; m_98
	MOL003959	Limonin	C26H30O8	470.523	1.5	rat; Xr+		15.382	0.828	182.999	Elevated (59%)	Normal (90%)	Normal (94%)	Normal (72%)	Elevated (94%)	0.16	S102
	MOL003974	Evocarpine	C23H33NO	339.524	0			3293.88	69.73	264.079	Normal (92%)	Normal (97%)	Normal (94%)	Normal (84%)	Normal (60%)	0	
	MOL004014	Evodiamide	C19H21N3O	307.398	0			1195.35	5.899	192.837	Normal (83%)	Normal (97%)	Normal (87%)	Normal (91%)	Normal (65%)	0.34	S_97
	MOL004015	Rutaevin	C26H30O9	486.522	1.5	rat; Xr+		10.401	0.345	103.08	Elevated (90%)	Normal (97%)	Normal (94%)	Normal (98%)	Elevated (86%)	0.16	S102

Identifier	Compound identifier. The field used to populate this column is specified when the input file is opened. If an identifier field was not selected, the identifier will be a unique integer ID.
Formula	Molecular formula
TOX_Risk	Risk connected with predicted toxicity: a score in the 0–6 range indicating the number of potential toxicity problems a compound might have. Exceeds 2.0 for 9% of a focused WDI subset
TOX_Code	TOX Risk rule codes: hERG = hERG inhibition, rat = acute rat toxicity, Xr = carcinogenicity in rat, Xm = carcinogenicity in mice, HEPX = hepatotoxicity, MUT =Ames positive
Tox	hERG_pIC50 [4.769, 5.994], log(Rat_Acute) [1.017, 3.518], log(Rat_TD50) [–0.462, 1.843], log(Mouse_TD50) [1.729, 2.452], Ser_AST [0.000, 1.000], Ser_ALT [0.000, 1.000], Ser_LDH [0.000, 1.000], MUT_Risk [0.000, 1.800]
Rat_Acute	LD50 for rat acute toxicity (mg/kg in oral dose that would be lethal to 50% of the rats). RMSE/MAE = 0.60/0.45 (2D) and 0.58/0.44 (3D) in log units
Rat_TD50	TD50 for rat carcinogenicity (mg/kg/day in oral dose) over a standard lifetime. RMSE/MAE = 0.54/0.42 (2D) and 0.52/0.43 (3D) in log units
Mouse_TD50	TD50 for mouse carcinogenicity (mg/kg/day in oral dose) over a standard lifetime. RMSE/MAE = 0.47/0.38 (2D) and 0.46/0.38 (3D) in log units

(*Continued*)

Table 2
Continued

Structure	Identifier	Name	Formula	MW	TOX_ Risk	TOX_ Code	*Tox*	Rat_ Acute	Rat_ TD50	Mouse_ TD50	Ser_ AlkPhos	Ser_ GGT	Ser_ LDH	Ser_ AST	Ser_ALT	MUTx_ Risk	MUTx_ Code
Ser_AlkPhos	Predicts whether or not the compound will cause elevation in the levels of alkaline phosphatase enzyme. Overall accuracy = 91%																
Ser_GGT	Predicts whether or not the compound will cause elevation in the levels of GGT enzyme. Overall accuracy = 94%																
Ser_LDH	Predicts whether or not the compound will cause elevation in the levels of LDH enzyme. Overall accuracy = 95%																
Ser_AST	Predicts whether or not the compound will cause elevation in the levels of SGOT enzyme. Overall accuracy = 84%																
Ser_ALT	Predicts whether or not the compound will cause elevation in the levels of SGPT enzyme. Overall accuracy = 89%																
MUTx_Risk	Enhanced risk of mutagenicity: a score in the 0–4 range that is a weighted sum of the number of "Positive" predictions by a total of eleven individual MUT_* models along with combinations thereof. See the user manual for details. Exceeds 1.0 for 12% of a focused WDI subset																
MUTx_Code	Similar to MUT_Code, but with additional Sx* and mx* codes indicating Yes predictions for combinations of strains. See the user manual for details																

2D: Two dimensional; 3D: Three dimensional; GGT: Glutamyl transpeptidase; LD_{50}: Median lethal dose; LDH: Lactate dehydrogenase; MAE: Mean absolute error; RMSE: Root mean square error; SGOT: Serum glutamic-oxaloacetic transaminase; SGPT: Serum glutamic-pyruvic transaminase; TD_{50}: Median toxic dose; WDI: World drug index.

staining, it was found that Rutecarpine may recrystallize the drug at high concentrations, resulting in false-positive SYTOX® Green staining, whereas Evocarpine and Dehydroevodiamine showed strong cytotoxicity at higher concentrations, whereas EVO showed cytotoxicity at low concentrations. The mitochondrial membrane potential of the monomers was examined and it was found that the mitochondrial membrane potential increased significantly after the administration of EVO and increased with the concentration of the administered drug, which was consistent with the performance of the total alkaloids (Figure 4; Supplementary Figure S3, http://links.lww.com/AHM/A115). Based on these findings, the study concluded that the potential hepatotoxic components in Euodia rutaecarpa are alkaloids, of which EVO are the main toxic components.

Hepatotoxicity induced by Euodia rutaecarpa on metabolism in mice

In the above study, EVO was found to be a potentially toxic component of Euodia rutaecarpa, so to further clarify the key pathway of EVO leading to hepatotoxicity, the study was conducted using EVO administered continuously for 14 d, and then serum was taken from the mice for metabolomics assay. The study used a supervised discriminant analysis statistical method, PLS, to model the relationship between metabolite expression and sample category to achieve prediction of sample category. The PLS-DA model was established for each comparison group, and the model evaluation parameters ($R2$, $Q2$) obtained, if $R2$ and $Q2$ were closer to 1, indicated that the model was more stable and reliable [Supplementary Figure S4B–D, http://links.lww.com/AHM/A116]. As shown in Supplementary Figure S4B–D, http://links.lww.com/AHM/A116, the higher the QC sample correlation (the closer $R2$ is to 1) indicates the better the stability of the whole detection process and the higher the data quality. According to the $Q2$ and $R2$ values after 200 disruptions and modeling, their regression lines can be obtained, and when the $R2$ data is greater than the $Q2$ data and the intercept between the $Q2$ regression line and the Y-axis is less than 0, it can be demonstrated that the model is not "overfitted," and with the increase of the dosage of the administered drugs, a clear separation trend of the dosage groups can be seen when compared with the control group, and the overfitting does not occur. As the dosage increases, a clear separation trend can be seen between the dosing groups and the control group without overfitting, indicating that the PLS-DA model has good explanatory and predictive power. The study set metabolite screening thresholds VIP > 1.0, FC > 1.2, or FC < 0.833 and P value < 0.05, and the differential metabolites are shown in Supplementary Figure S4E, http://links.lww.com/AHM/A116. In the low-dose group, 17 metabolites were up (D-Erythro-sphingosine 1-phosphate; Neodiosmin; Guanosine monophosphate; Isorhapontigenin; Citraconic acid; Epigallocatechin; Glucose 1-phosphate; Prostaglandin A1 ethyl ester; 2-Furoic acid; PC (16:2e/22:2), etc) and 42 metabolites (Guanethidine Monosulfate; Guggulsterone; Isoquinoline; SPK; 4-(anilinomethylidene)-3-methyl-4,5-dihydroisoxazol-5-one; Cer-NS (d18:1/22:0);

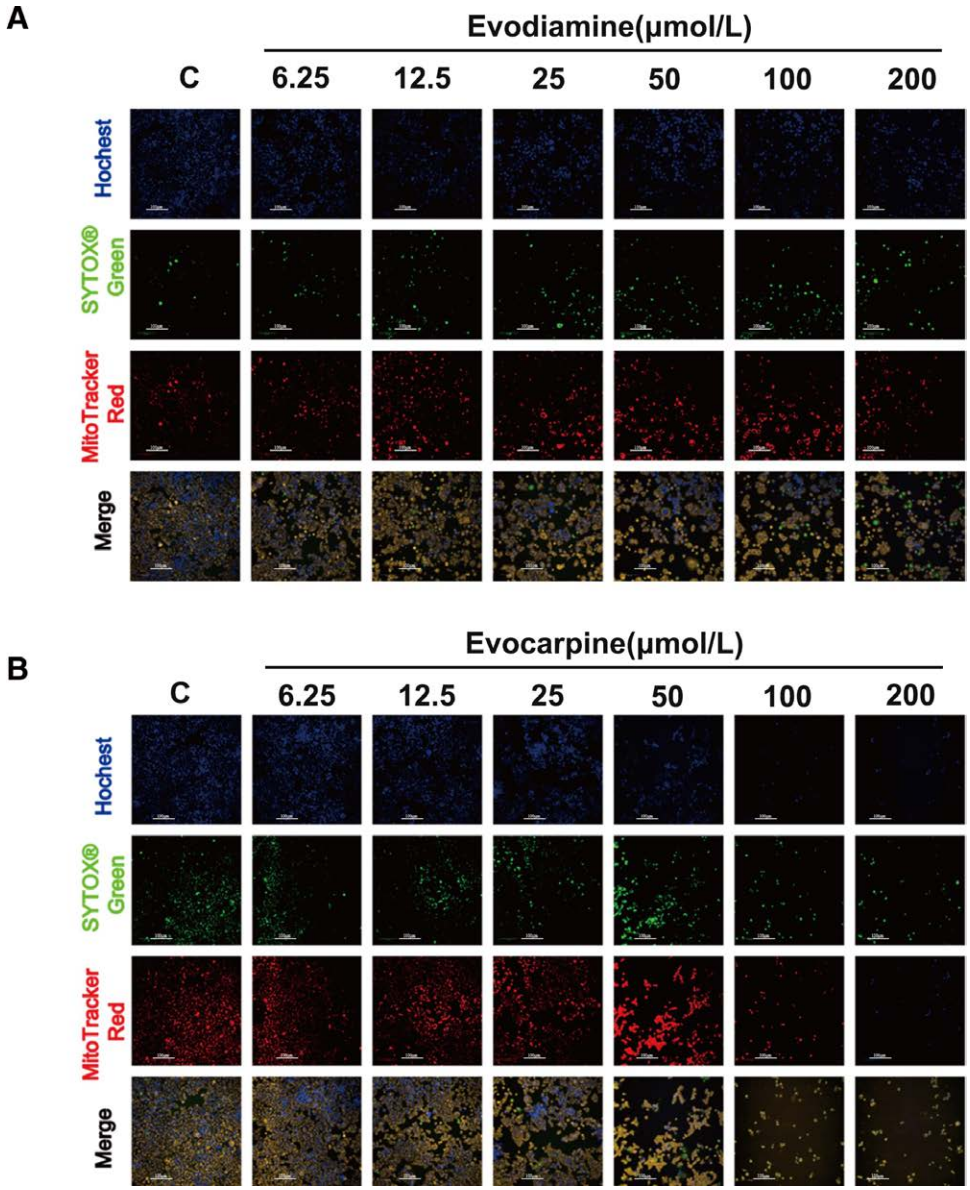


Figure 4. Screening of high-content toxicity of alkaloids in Euodiae Fructus. (A) Toxic effects of different concentrations of evodiamine on L02 cells. (B) Toxic effects of different concentrations of evocarpine on L02 cells.

4-{2-[(2-chlorophenoxy)methyl]-1,3-thiazol-4-yl} benzonitrile; Cortisone; SM (d22:0/14:0); 4-Hydroxyisoleucine, etc) were downregulated compared with the control group (Figure 5A); in the medium-dose group, 32 metabolites were up (PC (19:1/19:1); PC (19:1/19:2); Neodiosmin; Lignoceric Acid; PE (14:0e/22:2); Docosanoic Acid; PC (20:0/22:6); Pentacosanoic acid; Nervonic acid; N,N-Diethyldodecanamide, etc), and 83 metabolites were downregulated ((6E)-7-(2H-1,3-benzodioxol-5-yl)-1-(piperidin-1-yl)hept-6-en-1-one; PC (21:2/20:5); PC (18:0/18:1); PC (20:4e/20:5); 4-{2-[(2-chlorophenoxy)methyl]-1,3-thiazol-4-yl}benzonitrile; 1-Methylhistidine; SPK; 4-Hydroxyisoleucine; PC (14:0e/22:5); PC (17:1/18:1), etc) compared with the control group (Figure 5C); and in the high-dose group, 12 metabolites were up (Norfloxacin Erucic acid; trans-Petroselinic Acid; (+)-EVO; Estrone; PC (19:1/19:2); (±)12(13)-DiHOME; 4-Hydroxyretinoic Acid; LPC 20:2; (±)9-HpODE, etc) and 27 metabolites were downregulated (4-{2-[(2-chlorophenoxy) methyl]-1,3-thiazol-4-yl}benzonitrile; Guanethidine Monosulfate; PC (22:5e/17:2); 1-Methylhistidine; PC (16:0/17:0); PC (17:1/18:1); PC (17:0/17:0); Uracil 1-beta-D-arabinofuranoside; (6E)-7-(2H-1,3-benzodioxol-5-yl)-1-(piperidin-1-yl)hept-6-en-1-one; 6-(3-hydroxybutan-2-yl)-5-(hydroxymethyl)-4-methoxy-2H-pyran-2-one, etc) compared with the control group (Figure 5E). The differential metabolites obtained from different dose groups were analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) as shown in Figure 5B, D, F, and the low-dose, medium-dose, and high-dose groups were jointly enriched for steroid hormone biosynthesis, bile secretion, suggesting that the hepatotoxicity of EVO may be related to steroid hormone synthesis and bile secretion.

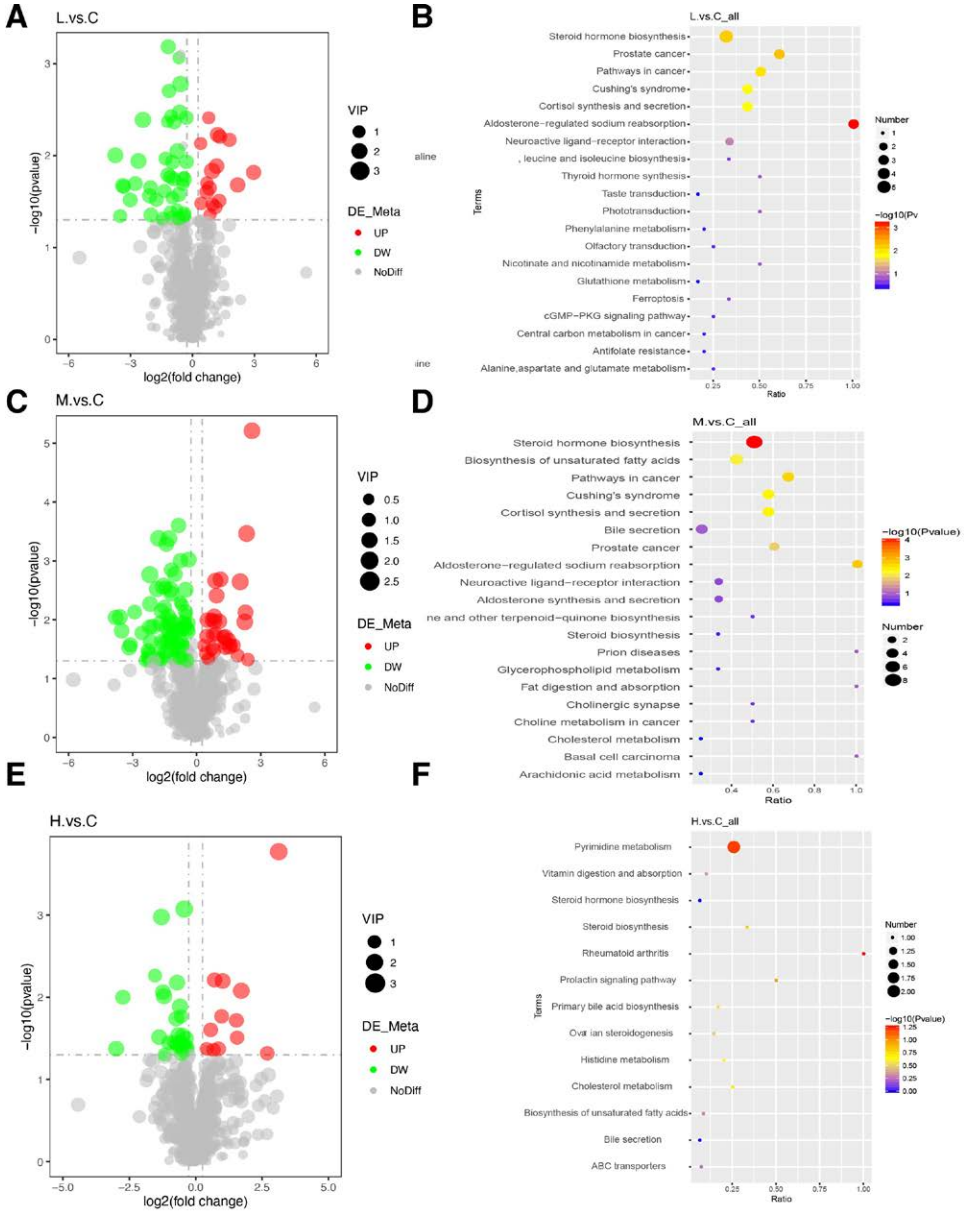


Figure 5. Changes in serum metabolites detected by untargeted metabolomics. (A, B) Volcano plot and KEGG enrichment bubble plot of differential metabolites in mouse serum after continuous administration of evodiamine (1 g/kg) for 14 days. (C, D) Volcano plot and KEGG enrichment bubble plot of differential metabolites in mouse serum after continuous administration of evodiamine (2.5 g/kg) for 14 days. (E, F) Volcano plot and KEGG enrichment bubble plot of differential metabolites in mouse serum after continuous administration of evodiamine (5 g/kg) for 14 days. KEGG: Kyoto Encyclopedia of Genes and Genomes.

Discussion

The Chinese Pharmacopoeia notes that Euodia rutaecarpa is a small toxic traditional Chinese medicine, and there have been reports of adverse reactions caused by improper use of Euodia rutaecarpa in clinical practice[24]. Although previous studies have investigated the chemical composition and pharmacological activity of Euodia rutaecarpa, there are still disagreements regarding its toxic components and target organs. The study conducted acute toxicity experiments in mice to clarify the hepatotoxicity of Euodia rutaecarpa, and research has been conducted using AMET Predictor software to predict the toxicity of active monomers in Euodia rutaecarpa. Using toxic partial enrichment and separation techniques, it was determined that alkaloids are the main toxic component in Euodia rutaecarpa. Moreover, research was conducted on the mechanism of action and *in vivo* metabolic changes of Euodia rutaecarpa to provide a basis for more rational and effective clinical use in the future.

Current pharmacological studies have shown that the pharmacological activity of Euodia rutaecarpa is closely related to the alkaloids it contains, which are fat-soluble and easily soluble in organic reagents such as ethanol, ethyl acetate, and methanol. Previous experiments showed that the solubility of Euodia rutaecarpa in methanol and ethanol was higher than that in ethyl acetate. Related studies also showed that the alkaloid content in the ethanol extract of Euodia rutaecarpa was significantly higher than that in the aqueous extract, so 70% ethanol extraction of Euodia rutaecarpa was used in the

study. Previous studies have shown that the toxicity of Euodia rutaecarpa is closely related to alkaloids[25], while the content of alkaloids in the ethanol extract of Euodia rutaecarpa is high[26]. Related studies have found that the alkaloid dissolution in Euodia rutaecarpa varies under different extraction methods, with the highest alkaloid content in the ethanol extract of Euodia rutaecarpa[27]. This may be related to the toxicity of Euodia rutaecarpa, and the acute toxicity results are consistent[28]. The current research shows that the total alkaloid content in Euodia rutaecarpa after processing is significantly reduced, and with the processing time increasing, the alkaloid content decreases more obviously, while our acute toxicity study in mice shows that the ethanol extract of Euodia rutaecarpa has the highest toxicity, while the alkaloid content after processing is reduced, and the acute toxicity in mice is the lowest. The comparison of the components showed that the reduction of toxicity was related to the reduction of alkaloids such as EVO, rutaecarpine, and evocarpine. Therefore, the technology of alkaloid enrichment and separation was used to enrich and purify the total alkaloids of EVO. The *in vivo* results were also verified by *in vitro* studies. The results of the acute toxicity study in mice showed that the ethanol extract of Euodia rutaecarpa with high alkaloid content was the most toxic, while the processed products of Euodia rutaecarpa with low alkaloid content were less toxic. The characteristic components of Euodia rutaecarpa, such as EVO and rutaecarpine, further clarified the active components of Euodia rutaecarpa toxicity[29].

At present, toxicity prediction of traditional Chinese medicine is an important part of traditional Chinese medicine research, which is mainly divided into toxicity prediction based on QASR, toxicity prediction based on network toxicology, and toxicity prediction based on traditional Chinese medicine properties[30]. The QSAR research method for toxicity prediction of compounds is one of the key technologies in new drug design and research, and is currently an effective means of exploring the relationship between compound structure and toxicity. By establishing structure-activity relationships with a few types of compounds with known structures, it can predict the pharmacological or toxic effect of many compounds with similar structures. This method has been successful in predicting biological activity, structure, and selectivity, pharmacokinetics, and inferring the structure of receptors used by drugs[31]. The traditional toxicological research methods, which cost high experimental period is long, and at the same time, many animals must be used for experiments, while QSAR-based toxicity prediction modeling is an important research tool to screen the toxicological potential of compounds in a high-throughput manner, which is of great significance to reduce the number of animal tests, and to increase the speed of drug toxicity screening and the development of new drugs[32]. Drug toxicity prediction has been widely used in toxicity assessment studies. However, there are still many problems and challenges in using toxicity prediction models for the prediction of Chinese medicine components. However, numerous questions remain. The current QSAR model is mainly used for acute toxicity prediction, while the toxicity of traditional Chinese medicine is generally potential and accumulated for a long time. Therefore, in the toxicity prediction of traditional Chinese medicine, the model still needs further optimization for the toxicity research of long-term and repeated exposure. Since the model is the result of comparison of many compounds, and the current toxicity study of traditional Chinese medicine is not deep enough and the amount of relevant data is small, its use for toxicity prediction is still a gap with the real situation, and there is an over-reliance on and possible bias in the interpretation of the QSAR model. In addition, the toxicity of TCM has the characteristics of multi-components and multi-targets, and the mechanism of toxicity is complex, so the toxicity screening model established by QASR cannot comprehensively investigate the toxicity of TCM, and further research is still needed. Based on the main active ingredients of Euodia rutaecarpa, the present study utilized the toxicity prediction function of ADMET Predictor software to predict the acute toxicity, carcinogenicity, chromosomal variability, mutagenicity, genotoxicity, and bioconcentration index of Euodia rutaecarpa, and to explore the toxicological activity of the drug in the animal body, and to validate the toxicity of Euodia rutaecarpa using the *in vitro* and *in vivo* modeling, to make up for the defects of the single toxicity prediction in the traditional way.

The misuse of traditional Chinese medicine can lead to drug-induced liver damage, which is a common occurrence in clinical practice[33]. However, the mechanisms of action and hazardous targets of traditional Chinese medicine are obscure because of its various targets and complicated composition. Mitochondria are a vital source of energy in the body, taking part in several physiological processes such as energy metabolism. Currently, mitochondria are thought to be major hazardous targets for liver toxicity, as they play an important role in bile acid metabolism and liver homeostasis[34]. According to the research, traditional Chinese medicine might induce mitochondrial structural damage, which can result in aberrant bile acids. Yang et al.[35] showed that emodin inhibits proton transport and activates the mitochondrial apoptosis pathway, which is the primary cause of liver injury. Hasnat et al.[36] uncovered a unique mechanism of liver toxicity in which triptolide causes mitochondrial damage caused by autophagy. Furthermore, Miyazaki et al.'s[37] research suggests that the disruption of bile acid metabolism may be related to the inhibition of BA synthesis and the failure of BA excretion into bile caused by mitochondrial CYP27A1 expression deficiency. Additionally, insufficient taurine supplementation may further trigger various diseases through abnormal BA metabolism[37]. Furthermore, Miyazaki et al.'s[37] research suggests that the disruption of bile acid metabolism may be due to the inhibition of BA synthesis and the failure to excrete BA into bile caused by mitochondrial dysfunction of CYP27A1. Furthermore, insufficient taurine supplementation may cause other disorders due to improper bile acid metabolism[37]. A rate-limiting step in the alternate pathway for the generation of BA is the steroidogenic acute regulatory protein 1 (STARD1), which transports cholesterol to mitochondria. The production of BA is enhanced, and its products promote the pluripotency and self-renewal of liver cells as well as the incidence of inflammation

because of the aberrant expression of STARD1 protein in boosting the acidic pathway of mitochondria[38]. In addition, aberrant bile acids have the potential to harm mitochondria, and a crucial ligand for bile acids is mitochondrial fusion protein (MFN2). Excessive levels of bile acids facilitate the attachment of mitochondria to the endoplasmic reticulum (ER), which causes calcium excess in the mitochondria and initiates pyroptosis and NLRP3 inflammasomes[39]. Conversely, bile acids exhibit a positive feedback regulation of bile acid homeostasis on mitochondrial homeostasis at low physiological concentrations by promoting mitochondrial fusion, which in turn leads to enhanced oxidative phosphorylation and enhanced clearance of bacteria by macrophage mediated-phagocytosis[40]. Interestingly, bile acids can also repair damaged cells. Research has shown that ursodeoxycholic acid can control the harm that bile acids cause to liver cells. The primary advantages of ursodeoxycholic acid therapy include: preventing cytotoxicity because of the higher toxicity of BA; stimulating gallbladder and liver secretion; promoting antioxidant activity, partially because of the increased glutathione levels; and inhibiting apoptosis of liver cells. Because of their protective qualities, other naturally occurring BA or its derivatives, such as butyryl-N-methylglycine or butyrylsalicylic acid[41]. In this study, serum differential metabolites of cortisol, hydrocortisone, and cortisone were all observed in different dose groups of mice after administration of EVO, which was found to affect mainly serum steroid metabolism and bile acid metabolism in mice by analysis of KEGG enrichment of differential metabolites. The above research results indicate that mitochondrial damage caused by EVO may be the cause of bile acid metabolism, and negative feedback regulation of bile acid metabolism exacerbates mitochondrial damage. Furthermore, the liver produces and secretes corticosteroid binding globulin (CBG), which binds to glucocorticoids in the bloodstream. Damage to the liver can cause aberrant synthesis, which can alter phase metabolism and blood glucocorticoid levels[42]. Our research findings suggest that abnormal steroid metabolism may also be related to liver damage caused by EVO; therefore, further research is needed to better understand its mechanism.

In conclusion, we identified and clarified the potential hepatotoxic substances in Euodia rutaecarpa, and the method we used is applicable to the toxicity screening of traditional Chinese medicine compounds due to the complex and diverse characteristics of their compositions. Moreover, through the *in vivo* metabolomics study of EVO, it was preliminary revealed that the hepatotoxicity of EVO may be related to the abnormalities of steroid hormone synthesis and bile secretion caused by mitochondrial damage. The present study presents the active compounds responsible for the hepatotoxicity of EVO, evaluates their safety, and investigates the potential mechanisms of toxicity. This study's methodology is essential for both computational exploration and *in vitro* validation of the toxicological effects and metabolic processes of herbs found in clinical Chinese medicine. These findings contribute to the improvement of the safety of Chinese medicine used in clinical settings.

Conflict of interest statement

Boli Zhang is the Editor-in-Chief of this journal. Yue Gao is editorial board members of this journal. None of the other authors declare any conflicts of interest.

Funding

This study was supported by Major Program of National Natural Science Foundation of China (82192910); and the Innovation Team and Talents Cultivation Program of National Administration of Traditional Chinese Medicine (ZYYCXTD-C-202009 and ZYYCXTD-D-202207).

Author contributions

Chunqi Yang participated in research design, participated in the writing of the paper, and participated in the performance of the research. Chengcai Lai, Yi Ru, Baoying Shen, Xiangjun Wu, Jialu Cui, Fangyang Li, Cheng Zhang, Zhuo Shi, Qingyuan Qian, and Chengrong Xiao participated in the performance of the research and analyzed the data. Yuguang Wang, Boli Zhang, and Yue Gao designed the experiment and corrected the draft, and is the guarantor of this study. All authors contributed to the article and approved the submitted version. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Ethical approval of studies and informed consent

The animal study was reviewed and approved by Animal Ethics Committee of the Academy of Military Medical Sciences Animal Laboratory of the Laboratory Animal Center (IACUC-DWZX-2020-60).

Acknowledgments

None

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

References

[1] Li D, Li Y, Jiang X, et al. Evodiamine: a privileged structure with broad-ranging biological activities. *Mini Rev Med Chem* 2022;22(21):2680–2701.
[2] Yang JY, Kim JB, Lee P, et al. Evodiamine inhibits helicobacter pylori Growth and helicobacter pylori-induced inflammation. *Int J Mol Sci* 2021;22(7).
[3] Jiang XH, Wu QQ, Xiao Y, et al. Evodiamine prevents isoproterenol-induced cardiac fibrosis by regulating endothelial-to-mesenchymal transition. *Planta Med* 2017;83(9):761–769.
[4] Chou CH, Yang CR. Neuroprotective studies of evodiamine in an okadaic acid-induced neurotoxicity. *Int J Mol Sci* 2021;22(10).
[5] Tang Y, Gu W, Cheng L. Evodiamine attenuates oxidative stress and ferroptosis by inhibiting the MAPK signaling to improve bortezomib-induced peripheral neurotoxicity. *Environ Toxicol* 2024;39(3):1556–1566.
[6] Huang G, Kling B, Darras FH, et al. Identification of a neuroprotective and selective butyrylcholinesterase inhibitor derived from the natural alkaloid evodiamine. *Eur J Med Chem* 2014;81:15–21.

[7] Ko HC, Wang YH, Liou KT, et al. Anti-inflammatory effects and mechanisms of the ethanol extract of Evodia rutaecarpa and its bioactive components on neutrophils and microglial cells. *Eur J Pharmacol* 2007;555(2–3):211–217.

[8] Chen PY, Han LT. Study on the molecular mechanism of anti-liver cancer effect of Evodiae fructus by network pharmacology and QSAR model. *Front Chem* 2022;10:1060500.

[9] Panda M, Tripathi SK, Zengin G, et al. Evodiamine as an anticancer agent: a comprehensive review on its therapeutic application, pharmacokinetic, toxicity, and metabolism in various cancers. *Cell Biol Toxicol* 2023;39(1):1–31.

[10] Jiang J, Hu C. Evodiamine: a novel anti-cancer alkaloid from Evodia rutaecarpa. *Molecules* 2009;14(5):1852–1859.

[11] Liang J, Chen Y, Ren G, et al. Screening hepatotoxic components in Euodia rutaecarpa by UHPLC-QTOF/MS based on the spectrum-toxicity relationship. *Molecules (Basel, Switzerland)* 2017;22(8):1264.

[12] Yang CQ, Lian WY, Wang YG, et al. Research progress in pharmacology and toxicology of evodiamine. *Zhongguo Zhong Yao Za Zhi* 2021;46(20):5218–5225.

[13] Yang W, Ma L, Li S, et al. Evaluation of the cardiotoxicity of evodiamine *in vitro* and *in vivo*. *Molecules* 2017;22(6):943.

[14] Yu LL, Liao JF, Chen CF. Anti-diarrheal effect of water extract of Evodiae fructus in mice. *J Ethnopharmacol* 2000;73(1-2):39–45.

[15] Cao JQ, Guo SS, Wang Y, et al. Contact toxicity and repellency of the essential oils of Evodia lenticellata huang and Evodia rutaecarpa (Juss.) Benth. Leaves against three stored product insects. *J Oleo Sci* 2018;67(8):1027–1034.

[16] Sun Q, Xie L, Song J, et al. Evodiamine: a review of its pharmacology, toxicity, pharmacokinetics and preparation researches. *J Ethnopharmacol* 2020;262:113164.

[17] Jing W, Jingjing L, Tao S, et al. Review of Studies on the Compatibility of Huanglian(Coptis Chinensis) and Wuzhuyu(Evodia Rutaecarpa). *Liaoning J Tradit Chin Med* 2015;42(11):2242–2245.

[18] Ma BL, Yao MK, Han XH, et al. Influences of Fructus evodiae pretreatment on the pharmacokinetics of Rhizoma coptidis alkaloids. *J Ethnopharmacol* 2011;137(3):1395–1401.

[19] Xu YC, Wei LX, Zhou YX, et al. Determination of evodiamine and rutaecarpine in Evodia rutaecarpa (Juss.) Benth after compatibility with Coptis chinensis Franch. *Zhongguo Zhong Yao Za Zhi* 2001;26(12):846–847.

[20] Zhao P, Xu H, Xu J, et al. Analysis on changes of chemical compounds in different processed products of Euodiae fructus. *Zhongguo Zhong Yao Za Zhi* 2011;36(5):559–562.

[21] Cai Q, Wei J, Zhao W, et al. Toxicity of Evodiae fructus on rat liver mitochondria: the role of oxidative stress and mitochondrial permeability transition. *Molecules* 2014;19(12):21168–21182.

[22] Matsuyama R, Okada Y, Shimma S. Metabolite alteration analysis of acetaminophen-induced liver injury using a mass microscope. *Anal Bioanal Chem* 2022;414(12):3709–3718.

[23] Guo Q, Zhang QQ, Chen JQ, et al. Liver metabolomics study reveals protective function of Phyllanthus urinaria against CCl(4)-induced liver injury. *Chin J Nat Med* 2017;15(7):525–533.

[24] Zhang W, Ren K, Wu S, et al. Cytotoxicity evaluation and metabolism of hepatotoxicity components of Euodiae fructus in L02 cells. *J Chromatogr B Analyt Technol Biomed Life Sci* 2021;1186:123040.

[25] Huang X, Zhang YB, Yang XW. Indoloquinazoline alkaloids from Euodia rutaecarpa and their cytotoxic activities. *J Asian Nat Prod Res* 2011;13(11):977–983.

[26] Bezek K, Kurincic M, Knauder E, et al. Attenuation of adhesion, biofilm formation and quorum sensing of campylobacter jejuni by Euodia ruticarpa. *Phytother Res* 2016;30(9):1527–1532.

[27] Hainan JI, Na T, Chang LIU, et al. Variations of chemical substances and toxic substances of Evodia rutaecarpa using different extraction methods. . *Chin J Pharmacovigil* 2022;19(12):1309–1314.

[28] Yan WH, Lyu WS, Sun DM, et al. Study on the changes of components in Euodiae Fructus and prepared Euodiae Fructus by UPLC fingerprint combined with multivariate statistical analysis. *Nat Prod Res Dev*. 2022;34(8):1301–1310.

[29] Huang X, Li W, Yang XW. New cytotoxic quinolone alkaloids from fruits of Evodia rutaecarpa. *Fitoterapia* 2012;83(4):709–714.

[30] Xiong G, Wu Z, Yi J, et al. ADMETlab 2.0: an integrated online platform for accurate and comprehensive predictions of ADMET properties. *Nucleic Acids Res* 2021;49(W1):W5–W14.

[31] Nantasenamat C, Isarankura-Na-Ayudhya C, Naenna T, et al. A practical overview of quantitative structure-activity relationship. *EXCLI J* 2009;8:74–88.

[32] Kim J, Kim S, Schaumann GE. Development of QSAR-based two-stage prediction model for estimating mixture toxicity. *SAR QSAR Environ Res* 2013;24(10):841–861.

[33] Shaw D. Toxicological risks of Chinese herbs. *Planta Med* 2010;76(17):2012–2018.

[34] Li HH, Tyburski JB, Wang YW, et al. Modulation of fatty acid and bile acid metabolism by peroxisome proliferator-activated receptor alpha protects against alcoholic liver disease. *Alcohol Clin Exp Res* 2014;38(6):1520–1531.

[35] Yang X, Zhang Y, Liu Y, et al. Emodin induces liver injury by inhibiting the key enzymes of FADH/NADPH transport in rat liver. *Toxicol Res (Camb)* 2018;7(5):888–896.

[36] Hasnat M, Yuan Z, Naveed M, et al. Drp1-associated mitochondrial dysfunction and mitochondrial autophagy: a novel mechanism in triptolide-induced hepatotoxicity. *Cell Biol Toxicol* 2019;35(3):267–280.

[37] Miyazaki T, Sasaki SI, Toyoda A, et al. Impaired bile acid metabolism with defectives of mitochondrial-tRNA taurine modification and bile acid taurine conjugation in the taurine depleted cats. *Sci Rep* 2020;10(1):4915.

[38] Conde de la Rosa L, Garcia-Ruiz C, Vallejo C, et al. STARD1 promotes NASH-driven HCC by sustaining the generation of bile acids through the alternative mitochondrial pathway. *J Hepatol* 2021;74(6):1429–1441.

[39] Henkel AS, LeCuyer B, Olivares S, et al. Endoplasmic reticulum stress regulates hepatic bile acid metabolism in mice. *Cell Mol Gastroenterol Hepatol* 2017;3(2):261–271.

[40] Che Y, Xu W, Ding C, et al. Bile acids target mitofusin 2 to differentially regulate innate immunity in physiological versus cholestatic conditions. *Cell Rep* 2023;42(1):112011.

[41] Perez MJ, Briz O. Bile-acid-induced cell injury and protection. *World J Gastroenterol* 2009;15(14):1677–1689.

[42] Kang HJ, Kang H, Kim B, et al. Evaluation of the diagnostic performance of a decision tree model in suspected acute appendicitis with equivocal preoperative computed tomography findings compared with Alvarado, Eskelinen, and adult appendicitis scores: a STARD compliant article. *Medicine (Baltim)* 2019;98(40):e17368.