Experimental Study on Hepatotoxicity of *Hemsleya sphaerocarpa* Fractions in Normal Mice

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Abstract [**Objectives**] To investigate the effects of various extract fractions of *Hemsleya sphaerocarpa* and their mechanisms of action on hepatotoxicity in normal experimental mice and to provide a scientific foundation for the clinical application of *H. sphaerocarpa*. [**Methods**] The extracts were separated by vacuum rotary evaporation into aqueous, n-butanol, petroleum ether, and ethyl acetate fractions. The resulting extracts from various fractions were subsequently formulated into 10% drug solutions using a normal saline solution. These solutions were administered intragastrically to mice at a dosage of 0.1 mL/10 g once daily. After 14 d of intragastric administration, various indices were assessed, and serum samples were collected from the ocular region of the mice for analysis. [**Results**] 15 g of the aqueous fraction, 20 g of the n-butanol fraction, 30 g of the petroleum ether fraction, and 20 g of the ethyl acetate fraction were obtained through the vacuum rotary evaporation method. After 14 d of intragastric administration, the serum biochemical indices of the mice were assessed. It was observed that alanine aminotransferase (ALT) levels significantly increased in the mice of experimental group. This finding suggests that the ethyl acetate, petroleum ether, and n-butanol extracts of *H. sphaerocarpa* may contribute to liver injury in the subjects. After 14 d of drug withdrawal, the ALT level in the ethyl acetate group exhibited a significant decrease, but remained elevated compared to those in the normal saline group. In contrast, the ALT levels in the n-butanol and petroleum ether groups also demonstrated a significant reduction and were marginally lower than those observed in the normal saline group. Furthermore, the body weights of the mice in both the petroleum ether and n-butanol groups did not show any significant changes throughout the duration of drug administration. [**Conclusions**] The liver injury in mice induced by the ethyl acetate extract of *H. sphaerocarpa* is characterized as the most severe and challenging to self-heal.

Key words *Hemsleya sphaerocarpa*, Extract, Vacuum rotary evaporation, Hepatotoxicity

1 Introduction

Hemsleya sphaerocarpa is a type of Chinese herbal medicine that is prevalent in the folk of the Qianxinan region. It belongs to the genus *Hemsleya* within the family Menispermaceae, and the medicinal component is derived from the tuberous root of the plant[1]. The rhizome of *H. sphaerocarpa* contains natural saponins and panaxadiol, which are two active compounds known for their significant pharmacological effects. These compounds can directly influence the human nervous system, thereby affecting its functionality[2]. It is documented in the *New Selection of Practical Chinese Herbal Medicines of Guangxi* that the plant possesses astringent and mildly bitter properties, is neutral in nature, and is considered non-toxic. Its therapeutic functions include detoxification, astringency, promotion of muscle growth, treatment of poisonous snake bites, management of injuries caused by hot liquids and fire, as well as the alleviation of dysentery and diarrhea. The *Checklist of Medicinal Plants of Guangxi* records that this plant exhibits several therapeutic effects, including the ability to clear heat, provide astringency, eliminate accumulations, and tonify blood following childbirth. Additionally, these plants are utilized in the treatment of gastrointestinal dampness and heat, red dysentery, aphthous stomatitis, abscesses, hemorrhoids, and fire-related injuries. Furthermore, *Hemsleya* species are noted for their content of cucurbitacin, which is associated with hepatoprotective and anti-cancer properties[3]. In the Eastern Han Dynasty, the *Benjing* (*Herbal Classic*) made a clear distinction between 'poisonous' and 'non-toxic' herbs. Within the seven treatises of the *Neijing* (*Internal Canon of Medicine*), medicinal substances were categorized based on their level of toxicity into four classifications: highly toxic, commomly toxic, slightly toxic, and non-toxic. Certain toxic constituents of medicinal plants may coexist with their active ingredients, and the therapeutic and toxic doses of certain medications can be closely aligned, such that improper usage may result in poisoning or fatality. Additionally, a prevalent adverse effect associated with traditional Chinese medicine is the impairment of liver and kidney function, a concern that has persisted for an extended period among both healthcare professionals and patients[4].

The liver serves as the primary site for drug metabolism within the human body. Certain constituents of traditional Chinese medicines may initially be non-toxic; however, during the metabolic process, specific functional groups can be catalyzed by vari-

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ous enzymes or radicals, resulting in the formation of potentially toxic compounds. Numerous relevant studies have indicated that the proportion of liver damage attributable to traditional Chinese medicines within the total cases of clinical drug-induced liver injury varies between 4.8% and 32.6%[5]. At both the toxicological and metabolic levels, the primary compounds responsible for hepatotoxicity are glycosides, alkaloids, and phenylpropanoid compounds, with certain alkaloids demonstrating particularly significant hepatotoxic effects[6].

Liver injury associated with the use of traditional Chinese medicines is a significant challenge to their modernization and international acceptance. Consequently, a primary objective in the contemporary development of these medicines is to promote their appropriate use and to mitigate potential harm. This study investigates the effects of four extracts, including water, n-butanol, petroleum ether, and ethyl acetate, on the liver of mice through subacute toxicity experiments. Additionally, the hepatotoxicity mechanism of *H. sphaerocarpa* was examined by measuring the levels of alanine aminotransferase (ALT) in the serum of mice, aiming to provide a scientific basis and data support for the clinical application of *H. sphaerocarpa*.

2 Materials and methods

2.1 Materials and reagents Dried *H. sphaerocarpa*: purchased from a licensed herbal supplier (Guangxi origin), ground into coarse particles using a pulverizer; 95% ethanol: used to prepare 70% ethanol (Nanning Songyuan Instrument Chemical Glass Co., Ltd.); ALT assay kit (microplate method, Nanjing Jiancheng Bioengineering Institute). Petroleum ether, ethyl acetate, and n-butanol were of analytical reagent (AR) grade.

Standard ICR mice, with a body weight ranging from 25 to 35 g, were obtained from Changsha Tianqin Biotechnology Co., Ltd., with production license No.: SCXK(Xiang) 2024-0021.

2.2 Instruments The following equipment was utilized in the study: the SHB-III circulating water multi-purpose vacuum pump (Zhengzhou Greatwall Scientific Industry & Trade Co., Ltd.), the TWCL-T 5000 magnetic stirrer (Shanghai Biaohe Instrument Co., Ltd.), the FA1204B electronic balance (Techcomp Jingke Scientific Instruments (Shanghai) Co., Ltd.), the RE-3000A rotary evaporator (Shanghai Yarong Biochemistry Instrument Factory), the Mthras LB 943 multifunctional microplate reader (Berthold, Germany), and the HH-ZK intelligent constant temperature water bath (Gongyi Yingyu High Tech Instrument Factory).

2.3 Preparation of solutions

2.3.1 Preparation of 70% ethanol. 95% ethanol and distilled water were mixed in a ratio of 1∶0.356. To achieve a final concentration of 70% ethanol, it is necessary to add 178 mL of distilled water to a 500 mL bottle of 95% ethanol.

2.3.2 Preparation of various extracts. 10 g of extract was accurately measured using an electronic balance and transferred to a small beaker. Subsequently, 100 mL of saline was added to ensure complete dissolution of the extract. The solution was then autoclaved and stored in a refrigerator at 4 ℃ for subsequent use.

2.3.3 Preparation of 0.4 mol/L sodium hydroxide. On the day of the experiment, a 4 mol/L sodium hydroxide solution was diluted with distilled water at a ratio of 1∶9, based on the volume utilized, and subsequently stored at room temperature.

2.4 Drug extraction 1 kg of *H. sphaerocarpa* was subjected to crushing to obtain coarse particles. The material was then extracted using 10 volumes of 70% ethanol through two reflux processes, each lasting 1 h. The resulting extracts were combined and concentrated under reduced pressure until the alcohol odor was no longer detectable. The concentrated *H. sphaerocarpa* extract was subsequently dissolved in hot water and sequentially extracted with petroleum ether, ethyl acetate, and n-butanol. Following the concentration of each extract, the samples were subjected to vacuum drying at 50 ℃ to yield extracts from various fractions of *H. sphaerocarpa*: 15 g of the aqueous fraction, 20 g of the n-butanol fraction, 30 g of the petroleum ether fraction, and 20 g of the ethyl acetate fraction. The extracts were subsequently prepared as a 10% solution in physiological saline, autoclaved, and stored in a refrigerator at 4 ℃ for subsequent use.

2.5 Rearing of mice A total of 40 ICR mice, half male and half female, weighing 25 – 35 g, were acclimatized and maintained in a standard environment for one week. The mice were weighed daily throughout the duration of the experiment. The mice were assigned to five groups based on a 5% picric acid label, utilizing the random number table method: the water fraction group, n-butanol fraction group, petroleum ether fraction group, ethyl acetate fraction group, and a blank control group. Each group consisted of 8 mice, with 4 males and 4 females. The drug was administered once daily through a 24 h dietary water feeding regimen, with all mice receiving a dosage of 0.1 mL/10 g. The mass concentration of the drug solution was established to be 0.1 g/mL.

The mice in the water fraction group received intragastric administration of n-butanol extract. Similarly, the mice in the n-butanol fraction group were administered n-butanol extract via the intragastric route. The mice in the petroleum ether fraction group were given petroleum ether extract intragastrically, while those in the ethyl acetate fraction group received ethyl acetate extract through the same method. The mice in the blank control group were administered normal saline intragastrically.

Each group received the designated drug via intragastric administration for 14 d. Following the final intragastric administration, the mice in each group were randomly divided into two batches, each consisting of four mice, half male and half female. After a 24 h fasting period, a batch of mice from each group was selected for blood sampling via ocular puncture and liver sampling. Whole blood was subjected to centrifugation at 3 000 r/min for 10 min, after which serum was extracted. The isolated mouse serum was subsequently utilized for the determination of ALT levels. Following the collection of blood from the ocular region, the mice were euthanized using the neck dislocation method. The liver tissue was subsequently excised, rinsed with ice-cold saline until

free of blood, and dried on filter paper. The mass of the liver was then measured using a balance, and the liver index was calculated accordingly.

The other batch ceased intragastric administration 14 d post-treatment and resumed normal feeding. On day 28, blood and liver samples were collected using the same method. Serum was subsequently separated, and the ALT levels in the mice were measured utilizing the same indicator kit method, with the data being systematically recorded.

2.6 Determination of ALT levels ALT serves as a significant biomarker in liver function assessments, with fluctuations in its enzymatic activity typically correlating with the extent of hepatocellular damage. At a temperature of 37 ℃ and a pH of 7.4, ALT facilitates the transamination reaction between alanine and α-ketoglutarate, resulting in the production of pyruvate and glutamate. Following this reaction, pyruvate reacts with 2,4-dinitrophenylhydrazine (DNPH) to yield a reddish-brown compound known as acetone phenylhydrazone. The enzymatic activity of ALT can be indirectly quantified by measuring the variation in absorbance at a wavelength of 510 nm.

The experimental procedure was conducted as follows: 20 μL of matrix solution was added to each assay and control well of a 96-well plate. Subsequently, 5 μL of mouse serum was introduced into the assay wells, thoroughly mixed, and incubated at 37 ℃ for 30 min. Following this, 20 μL of DNPH solution was added to each of the two wells, and an additional 5 μL of serum was added to the control wells, which were also mixed thoroughly and incubated at 37 ℃ for 20 min. After incubation, 200 μL of 0.4 mol/L sodium hydroxide solution was added, mixed well, and allowed to stand at room temperature for 15 min. The absorbance at 510 nm was measured using a microplate reader, and the optical density (*OD*) difference between the assay wells and the control wells was calculated to obtain the absolute *OD* value. The activity of ALT was subsequently derived from the standard curve.

3 Results and analysis

3.1 ALT activities in mice Based on the data presented in Table 1, the regression equation was determined to be $y = 553x - 39.181$, $r = 0.945$. According to the analysis conducted using IBM SPSS Statistics 25, the n-butanol and petroleum ether groups exhibited a statistically significant increase in ALT activity compared to the normal saline group on day 14 of administration ($P < 0.05$). In contrast, the other groups did not demonstrate any significant differences at that time point. After 14 d of drug withdrawal, a significant reduction in ALT activity was observed in the n-butanol and petroleum ether groups when compared to the ethyl acetate group ($P < 0.05$). The other groups did not exhibit any significant differences at this time. Furthermore, a comparison among the n-butanol, ethyl acetate, and petroleum ether groups revealed a significant decrease in ALT activity ($P < 0.05$) when assessed after 14 d of drug withdrawal in contrast to the same groups following 14 d of drug administration.

Table 1 Comparison of ALT activity across different groups

Group	14 d of drug administration	14 d of drug withdrawal (28^{th} day)
Ethyl acetate	153.395 ± 79.413 *	50.249 ± 17.165^{bc} *
n-Butanol	228.507 ± 145.135^{d} *	22.493 ± 7.657^{a} *
Petroleum ether	240.638 ± 104.961^{d} *	22.413 ± 14.42^{a} *
Normal saline	23.258 ± 4.478^{bc}	30.969 ± 10.795

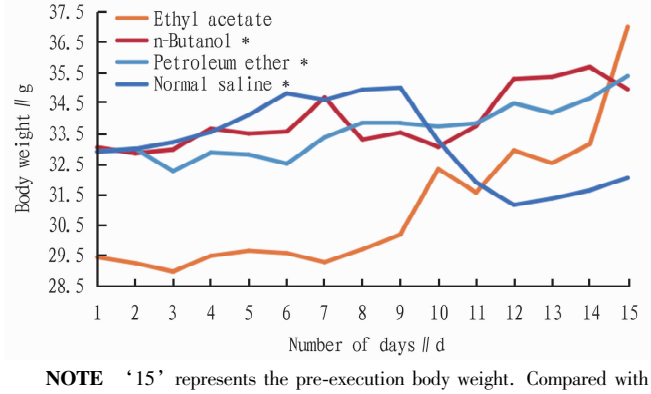
NOTE The results indicate significant differences when comparing the ethyl acetate group (a, $P < 0.05$), the n-butanol group (b, $P < 0.05$), the petroleum ether group (c, $P < 0.05$), and the normal saline group (d, $P < 0.05$). Additionally, comparisons within the same group at different time points are also statistically significant (*, $P < 0.05$).

3.2 Liver index of mice The analysis conducted using IBM SPSS Statistics 25 indicated that there was no statistically significant difference in the liver index among the groups, as presented in Table 2.

Table 2 Comparison of liver index across different groups

Group	14 d of drug administration	14 d of drug withdrawal (28^{th} day)
Ethyl acetate	0.050 ± 0.003	0.045 ± 0.010
n-Butanol	0.047 ± 0.003	0.046 ± 0.003
Petroleum ether	0.047 ± 0.006	0.043 ± 0.006
Normal saline	0.053 ± 0.005	0.044 ± 0.007

3.3 Changes in body weight of mice As illustrated in Fig. 1, the analysis conducted using IBM SPSS Statistics 25 indicated that the body weight of each extraction fraction group exhibited varying degrees of increase when compared to the control group (normal saline group). Notably, the ethyl acetate group demonstrated the most pronounced effect on body weight, with all ethyl acetate extraction fractions showing statistically significant differences from the control group ($P < 0.05$).



NOTE '15' represents the pre-execution body weight. Compared with the ethyl acetate group, * indicates a statistically significant difference ($P < 0.05$).

Fig. 1 Changes in body weight of mice after 14 d of drug administration

4 Discussion

4.1 Definition and importance of drug-induced hepatotoxicity Drug-induced hepatotoxicity is defined as the direct or indi-

rect damage to hepatocytes caused by pharmaceutical agents and their metabolites, leading to alterations in liver function or structural integrity. Given that the liver serves as the primary organ for drug metabolism, its toxic responses are of significant concern in both drug development and clinical practice. Mouse models have emerged as a valuable tool for investigating drug-induced hepatotoxicity, owing to their genetic, physiological, and biochemical similarities to the human liver.

4.2 Possible mechanisms of drug-induced hepatotoxicity

4.2.1 Oxidative stress. ALT levels can serve as an indicator of the extent of hepatic tissue damage. Additionally, ALT is associated with the production of reactive oxygen species (ROS) and free radicals that are generated during drug metabolism, which can lead to lipid peroxidation of hepatocyte cell membranes, thereby damaging cellular structure and function. Research has demonstrated that a reduction in the activity of antioxidant enzymes, coupled with an increase in oxidation products, significantly contributes to hepatotoxicity in mice models[7].

4.2.2 Mitochondrial damage. The incidence of hepatocyte apoptosis and necrosis is intricately associated with the impairment of mitochondrial function. During the initial phase of tissue damage, a significant release of inflammatory mediators occurs, resulting in mitochondrial dysfunction triggers apoptosis via cytochrome crelease and the subsequent release of substantial quantities of ROS, cytochromes, and other factors, which further exacerbate hepatocyte injury[8].

ALT is predominantly localized in the cytoplasm of hepatocytes and serves as a sensitive biomarker for hepatocyte damage. In contrast, AST is primarily found in the mitochondria of hepatocytes[9]. The concentration of these enzymes in the liver exceeds that in the serum, with ALT being the first enzyme to be released into the bloodstream during the initial stages of hepatocyte injury. If the damage progress and involve mitochondrial injury, AST will subsequently enter the circulation. Elevated levels of AST observed in liver function tests indicate more severe hepatocellular injury and may suggest involvement of the mitochondria within hepatocytes.

4.2.3 Inflammation response. Damage to hepatocytes initiates an inflammatory response that results in hypohepatia. This condition not only compromises the liver's capacity to metabolize and excrete endogenous metabolites *in vivo* but also diminishes its efficacy in inhibiting various functional enzymes. Consequently, damaged hepatocytes release elevated levels of ALT into the bloodstream, leading to an increase in serum ALT concentrations[10].

After 14 d of drug administration, ALT levels were significantly elevated in the ethyl acetate, n-butanol, and petroleum ether treatment groups when compared to the normal saline group. Following 14 d of drug withdrawal, ALT levels in the petroleum ether and n-butanol groups exhibited a significant decrease. The infiltration of inflammatory cells and the release of inflammatory mediators are critical characteristics of hepatotoxic reactions[11]. However, this conclusion requires further validation.

4.2.4 Lipid metabolism. The liver serves as a critical metabolic organ within the organism, and pertinent studies have indicated that elevated levels of ALT and AST are closely associated with disorders in lipid metabolism. Hepatic lipid metabolism plays a vital role in regulating the body's lipid homeostasis and in the pathogenesis of metabolic syndrome[12]. Following 14 d of drug administration, a significant increase in weight was observed in the ethyl acetate group, while the ethyl acetate, n-butanol, and petroleum ether groups exhibited an upward trend throughout the duration of the experiment. It is hypothesized that the ethyl acetate, n-butanol, and petroleum ether groups of *H. sphaerocarpa* may induce damage to the mouse liver by influencing fat metabolism. The elevation of ALT and AST levels in mice suggests an impact on hepatic lipid metabolism; however, the experiment did not include the measurement of AST, which necessitates further investigation.

5 Conclusions

The investigation of the hepatotoxicity associated with various fractions of *H. sphaerocarpa* extracts in mice models not only contributes essential insights into the mechanisms underlying drug-induced hepatotoxicity but also paves the way for enhancing the safety of current pharmaceuticals and informs the design and development of novel therapeutic agents. After 14 d of drug administration, the serum biochemical marker ALT in mice exhibited a significant elevation, suggesting that the extracted fractions of ethyl acetate, petroleum ether, and n-butanol from *H. sphaerocarpa* may induce liver injury in these subjects. Following 14 d of drug withdrawal, the serum ALT levels in the ethyl acetate group showed a significant decrease, but remained elevated compared to the normal saline group. In contrast, the ALT levels in both the n-butanol and petroleum ether groups also demonstrated a significant reduction, with values slightly lower than those observed in the normal saline group. Notably, there was no significant difference in the body weight of mice within the petroleum ether and n-butanol groups throughout the drug administration period, which may indicate a potential for spontaneous liver recovery in the mice. In conclusion, it is hypothesized that the ethyl acetate fraction of *H. sphaerocarpa* exerts the most pronounced liver injury in mice and is associated with a reduced capacity for self-repair.

The experiment speculates that *H. sphaerocarpa* induces liver injury associated with abnormal lipid metabolism, oxidative stress, and inflammatory responses in mice models, resulting in elevated serum ALT levels. However, due to the absence of relevant factor assessments, the findings of this study do not fully represent the actual conditions and require further investigation in subsequent research. Future studies should concentrate on the development of prevention and treatment strategies for drug-induced hepatotoxicity to enhance the safety and efficacy of pharmacological interventions.

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4 Conclusion

This study employed a TGF-β1-induced LX-2 cell activation model to investigate the anti-fibrotic mechanism of KA via the TGF/Smad signaling pathway using RT-qPCR and Western blot. The results demonstrate that KA alleviates hepatic fibrosis by significantly suppressing both gene expression levels (TGF-β1, Smad2, Smad3, Smad4) and protein expression levels (TGF-β1, p-Smad2/3/Smad2/3, Smad4), highlighting its therapeutic potential through modulation of the TGF/Smad pathway.

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