Study of the hepatotoxicity induced by *Dioscorea bulbifera* L. rhizome in mice

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**Summary** *Dioscorea bulbifera* L. is a medicinal plant. The present study was undertaken to investigate the hepatotoxicity induced by *D. bulbifera* in mice. Through the acute toxicity of various extracts including the EtOAc fraction (EF) and the non-EtOAc fraction (Non-EF) from ethanol, and the ethanol itself, we found that the EF contains the toxic ingredients of *D. bulbifera* rhizome. On this basis, to study the hepatotoxicity induced by the toxic ingredients, mice were treated with 0.5% sodium carboxymethyl cellulose (CMC-Na) alone or the EF of *D. bulbifera* rhizome at doses of 80, 160, 320, and 480 mg/kg once daily i.g. for fourteen consecutive administrations. Serum samples were collected for determination of the biomarkers for liver injury, such as, alanine transaminase (ALT) and aspartate transaminase (AST). Hepatic tissues were used to assay for the level of lipid peroxide (LPO), amounts of antioxidants such as glutathione, and activities of antioxidant-related enzymes for liver oxidative-antioxidative status in mice. The results showed that ALT and AST were significantly elevated after fourteen consecutive administrations of the EF of *D. bulbifera* rhizome. In addition, the level of LPO increased remarkably, while the glutathione amounts, and the activities of the antioxidant-related and glutathione-related enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPxs), glutathione-S-transferase (GST), glutathione reductase (GR) and glutamate-cysteine ligase (GCL) of hepatic tissues all decreased conspicuously, in livers of mice treated with the EF of *D. bulbifera* rhizome. Taken together, our results indicate that the EF contains the main toxic ingredients of *D. bulbifera* rhizome, and the mechanism of hepatotoxicity induced by it may be due to liver oxidative stress injury in mice.

**Keywords:** *D. bulbifera*, EtOAc fraction, hepatotoxicity, oxidative stress

1. Introduction

The rhizome of *Dioscorea bulbifera* (Dioscoreaceae) is widely distributed in Asia and traditionally used to treat various diseases including thyroid disease, tumors, etc. (1–2). Research results have demonstrated that *D. bulbifera* rhizome could induce hepatotoxicity in mice and rats (3–4). However, both the chemical compounds and mechanism of induced liver toxicity are still not very clear.

It is reported that oxidative stress plays a critical role in liver toxicity induced by alcohol, carbon tetrachloride, acetaminophen, chemotherapeutic agents and so on (5–9). Reactive oxygen species (ROS), produced in the process of oxidative stress, are extremely reactive, which may modify and inactivate lipids, proteins, DNA, and RNA, and thus induce cell injury. To prevent ROS-induced cell injury, the body has developed antioxidant systems including antioxidants and antioxidant enzymes. Among them,
glutathione is one of the common antioxidants (10). Superoxide dismutase (SOD) and catalase (CAT) are two important antioxidant-related enzymes, while glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR), and glutamate-cysteine ligase (GCL) are all glutathione-related antioxidant enzymes. Such antioxidant systems protect the organism from injury induced by ROS. However, with regard to the liver toxicity induced by *D. bulbifera* rhizome, there is no related report about the involvement of ROS in induced liver injury.

The present study was designed to evaluate the toxic ingredients of *D. bulbifera* rhizome through acute toxicity experiments, and then to explore the mechanism that oxidative stress injury has in such hepatotoxicity by measuring the lipid peroxide (LPO) level, glutathione amounts, and activities of antioxidant enzymes including SOD, CAT, GPx, GST, GR, and GCL in mice livers.

2. Materials and Methods

2.1. Experimental animals

ICR male and female mice (20 ± 2 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Certificate No. SCXK 2007-0005, Shanghai, China). The animals were reared in the animal house of Regional Center Animals. They were given rodent laboratory chow and water *ad libitum*, and maintained under controlled conditions with a temperature of 22 ± 1°C, relative humidity 65 ± 10% and a 12/12 h light/dark cycle (lights on at 7:00 am). All the procedures were in strict accordance with the P. R. China legislation on the use and care of laboratory animals using the guidelines established by Institute for Experimental Animals of Shanghai University of Traditional Chinese Medicine and were approved by the university committee for animal experiments.

2.2. Materials

NADPH, reduced glutathione (GSH) and oxidized glutathione (GSSG) were purchased from Roche (Basel, Switzerland). 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), glutathione reductase, 2-thiobarbituric acid (TBA), and other reagents unless indicated were from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Samples and preparation of various extracts

The rhizomes of *D. bulbifera* were collected in Qingyang, Anhui Province and authenticated by Prof. Shou-Jin Liu (Anhui College of Traditional Chinese Medicine, Anhui, China). A voucher specimen was deposited in the herbarium of Institute of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine. Preparation of various extracts is described as follows.

Water or ethanol extract: The powder was soaked in water or 80% ethanol (w/v = 1:10), and incubated at room temperature for 120 min, respectively. The mixtures were extracted three consecutive times, with constant stirring at 100°C once for 60 min for the water extract, and with a rotary evaporator at 85 ± 5°C and once for 180 min for the ethanol extract, respectively. The combined extracts were centrifuged at 4,000 × g for 10 min and the supernatant was decanted to a glass container and concentrated under vacuum using a rotary evaporator under reduced pressure at 45 ± 5°C. Thus, the water (about 53 g) and ethanol (about 78 g) extracts were obtained.

EF and Non-EF from ethanol extract: Half of the above ethanol extract was extracted eight times using EtOAc (v/v = 1:1) at room temperature. After evaporation of solvents, EtOAc (about 16 g) and Non-EtOAc (about 62 g) layers were achieved. The yield of EF from the ethanol extract was about 1.6% of raw medicinal materials.

The above extracts were diluted with 0.5% CMC-Na into different doses for the experiment. All extracts were stored at 4°C before use.

2.4. Acute toxicity

Healthy ICR mice, weighing 20 ± 2 g, were randomly divided into groups of 10 animals (5 males and 5 females). They were fasted from food, but not water 12 h prior to the administration of the test suspension. The control group received water containing 0.5% CMC-Na (vehicle) administered orally *i.g.*. The water extract of *D. bulbifera* rhizome suspended in 0.5% CMC-Na was administered orally at a dose of 14,000 mg/kg, while the ethanol suspension in 0.5% CMC-Na was administered orally at doses of 2,700, 3,200, 3,800, 4,500, and 5,400 mg/kg. The Non-EF ethanol extract of *D. bulbifera* rhizome suspended in 0.5% CMC-Na was administered orally at a dose 13,800 mg/kg, while the EF suspension in 0.5% CMC-Na was administered orally at doses of 320, 480, 640, 761, 905, 1,077, and 1,280 mg/kg. Toxic symptoms such as piloerection, inactiveness, dizziness, hypothermia, loss of righting reflex, etc., and mortality was observed twice a day for fourteen consecutive days after the treatment. The toxicological effect was assessed on the basis of mortality, which was expressed as the median lethal dose (LD₅₀) (II).

2.5. Hepatotoxicity

To determine the hepatotoxicity induced by *D. bulbifera* rhizome, groups of 10 male mice each were administered, once daily 80, 160, 320, or 480 mg/kg, *i.g.*, of the EF (suspended in 0.5% CMC-Na) of the ethanol extract from *D. bulbifera* rhizome for 14 days. Mice in
the control group (Normal) received the vehicle in an identical manner. After the treatment period, mice were sacrificed, blood was collected and livers were removed. Serum samples from blood were used for the assay of biomarkers for liver injury and the liver tissues of partial groups for assay of the mechanism of liver toxicity.

2.6. Serum biomarkers for liver injury

Blood samples were obtained from mice of all groups (10 mice per group) for determination of serum biomarkers for liver injury. Serum alanine transaminase (ALT) and aspartate transaminase (AST) were assayed according to the reported method (12).

2.7. Assay for the mechanism of hepatotoxicity induced by the EF of the D. bulbifera rhizome

LPO level, glutathione amounts, and activities of antioxidant-related enzymes including SOD, CAT, and glutathione-related enzymes such as GST, GPx, GR, and GCL in mice liver tissues of the partial groups were assayed according to the following descriptions.

2.7.1. Assay for liver tissue LPO

Liver tissue was homogenized in cold phosphate-buffered saline (PBS). LPO was determined using the previously reported method (13). Malondialdehyde (MDA) formed as an end product of the LPO and served as an index of the intensity of LPO. MDA reacts with TBA to generate a pink colored product, which has an absorbance at 532 nm. The standard curve of MDA was constructed over the concentration range of 0-40 μM. The LPO level was expressed as micromoles of MDA per milligram of protein based on tissue protein concentration.

2.7.2. Assay for liver tissue glutathione

The quantity of glutathione was measured immediately as described in a previous study (14). The reaction mixture contained: samples, 150 μL of a working solution (100 mM sodium phosphate buffer, pH 7.0, containing 0.53 U/mL of glutathione reductase, 40.7 μg/mL of DTNB, and 1 mM EDTA), and 50 μL of 0.16 mg/mL of NADPH solution. The change in absorbance was determined at 412 nm against the reagent blank after standing at room temperature for 30 min. Glutathione amounts were determined from a standard curve. The glutathione amounts from mouse liver tissue were calculated based on tissue protein concentration.

2.7.3. Assay for liver tissue SOD and CAT activity

Tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 5 min and the supernatant was transferred to new tubes for assay. Liver tissue activity of SOD and CAT was determined using methods from previous studies (15-16) and calculated based on tissue protein concentration.

2.7.4. Assay for liver tissue GPx activity

GPx activity was measured by the utilization of glutathione as reaction substrate according to the previously reported method (17). Mouse liver tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 10 min and the supernatant was transferred to new tubes for GPx assay. Tissue activity of GPx was calculated based on tissue protein concentration.

2.7.5. Assay for liver tissue GST activity

GST activity was measured according to the previously reported method (18) using 1-chloro-2,4-dinitrophenol (CDNB) as substrate. Mouse liver tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 10 min and the supernatant was transferred to new tubes for GST assay. Tissue activity of GST was calculated based on tissue protein concentration.

2.7.6. Assay for liver tissue GR activity

GR activity was assayed spectrophotometrically by following the oxidation of NADPH at 340 nm (19). Briefly, mouse liver tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 10 min and the supernatant was transferred to new tubes for GR assay. The reaction mixture (0.2 mL) contained 0.1 M phosphate buffer (pH 7.0), 1 mM GSSG, and 0.1 mM NADPH and was initiated by addition of supernatant. The activity of GR was calculated based on tissue protein concentration and expressed as mU/mg protein, where 1 unit of GR activity is defined as 1 mmol GSSG catalyzed per minute.

2.7.7. Assay for liver tissue GCL activity

GCL activity was assayed according to the previously reported method (20) with minor modifications. Briefly, mouse liver tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 10 min and the supernatant was transferred to new tubes for GCL assay. Enzyme activity was determined at 37°C in reaction mixtures (final volume, 0.2 mL) containing 0.1 M Tris-HCl buffer, 150 mM KCl, 5 mM ATP, 2 mM phosphoenolpyruvate, 10 mM l-glutamate, 10 mM l-α-amino-butyrate, 20 mM MgCl₂, 2 mM EDTA, 0.2 mM NADPH, 17 μg of pyruvate kinase, and 17 μg of lactate dehydrogenase. The activity of GCL was calculated based on tissue protein concentration and expressed as mU/mg protein, where 1 unit of GCL activity was equal to the oxidation of 1 mM NADPH per min.
2.8. Statistical analysis

All experimental data were expressed as mean ± standard error of mean (SEM). Significant differences between experimental groups were compared by One-Way ANOVA (analysis of variance) followed by Least Significant Difference (LSD) \((p < 0.05)\) using the Statistics Package for Social Science (SPSS) program Version 11.5.

3. Results

3.1. Acute toxicity

Mice were observed for fourteen consecutive days twice a day for toxic symptoms and mortality after oral administration of a single dose of various extracts of \(D.\) bulbifera rhizome, respectively. The results indicated that none of the mice in the group of the water extract (at a dose of 14,000 mg/kg) exhibited any toxic symptoms, while some in the ethanol extract (at a dose of 2,700 mg/kg) showed toxic symptoms. The results suggest that the ethanol extract is the toxic fraction of \(D.\) bulbifera rhizome. Meanwhile, further results indicated that none of the mice in the group of the Non-EF of the ethanol extract (at a dose of 13,800 mg/kg) exhibited any toxic symptoms, while mice in the EF of the ethanol extract (at a dose of 640 mg/kg) showed some toxic symptoms. All these results suggest that the EF is the toxic fraction of \(D.\) bulbifera rhizome.

The toxic symptoms appeared almost 2 h after the EF of the ethanol extract or the ethanol administration itself and slowly progressed to some extent. Most of the animals died on the second or third day after a single administration. The surviving mice could almost come back to the normal state in a week. The LD50 values for a single oral dose of the EF of the ethanol extract and the ethanol itself for ICR mice were 922 and 3,800 mg/kg, respectively (Table 1).

3.2. Hepatotoxicity

3.2.1. Serum biomarkers for liver injury

As for liver injury, serum ALT and AST activities are the generally accepted biomarkers. The obvious elevation of them often reflects liver injury. This study revealed that ALT increased significantly \((p < 0.05)\) in groups of mice at and above the dose of 160 mg/kg after fourteen consecutive administrations of EF of \(D.\) bulbifera rhizome. Meanwhile, AST was found to be elevated in groups of mice after treatment at and above 320 mg/kg for fourteen consecutive days. This suggests that EF of \(D.\) bulbifera rhizome has induced liver injury in mice at and above the dose of 160 mg/kg for fourteen days (Figure 1).

3.2.2. Assay for liver tissue LPO level

As one of the main end products of LPO, MDA amounts reflect the LPO level \((21)\). Figure 2A showed that MDA amounts increased significantly \((p < 0.05)\) in liver tissue of mice. This result demonstrated that EF of \(D.\) bulbifera rhizome could induce liver LPO injury in mice.

3.2.3. Assay for liver tissue glutathione

Glutathione is an antioxidant which helps protect cells against ROS such as free radicals and peroxides \((22)\), and the excessive exhaustion can induce oxidative stress injury. In the present study, glutathione amounts decreased significantly \((p < 0.05)\) in liver tissue of mice after given the EF of \(D.\) bulbifera rhizome at a dose of 480 mg/kg for fourteen consecutive days of administration (Figure 2B). The result suggests that EF of \(D.\) bulbifera rhizome can destroy the balance between cellular oxidants and antioxidants through exhausting cellular glutathione and thus can likely induce liver oxidative stress injury.

3.2.4. Assay for liver tissue SOD and CAT activities

SOD and CAT are both intracellular antioxidant-related enzymes, which participate in the process of oxidative stress. The results in this study showed that the SOD activity decreased significantly (Figure 3A) in livers of mice after treatment with \(D.\) bulbifera rhizome, but the CAT activity did not (Figure 3B). Meanwhile, the

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<th>Table 1. Acute toxicity of various extracts of the (D.) bulbifera rhizome in mice</th>
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Figure 1. Effects of the EF of ethanol extract of \(D.\) bulbifera rhizome on serum ALT and AST activities in mice. Data are presented as mean ± SEM \((n = 10)\). Significant differences with the normal group were designated as * \(p < 0.05\) and ** \(p < 0.01\).
results further confirmed the oxidative stress injury induced by *D. bulbifera* rhizome in mice.

### 3.2.5. **Assay for liver tissue GST, GPx, GR and GCL activity**

GST, GPx, GR, and GCL are all intracellular glutathione-related enzymes, cooperating with glutathione in participating in the course of oxidative stress. Our results showed that GST, GPx, GR, and GCL activity were all significantly \((p < 0.05)\) decreased in liver tissue of mice (Figure 4). Our results further confirmed that *D. bulbifera* rhizome destroyed the balance between cellular oxidants and antioxidants.

### 4. Discussion

The *D. bulbifera* rhizome has many bioactivities including anti-goiter, antitumor and so on (1). However, toxicity, especially hepatotoxicity induced by it greatly affects its use in the clinic (4). The results of the present study demonstrated that the EF of the ethanol extract was the main toxic ingredient of *D. bulbifera* rhizome.

After the liver is injured by hepatotoxins, ALT and AST can leak from the damaged liver into the serum. Therefore, the obvious elevation of serum ALT and AST generally reflects liver injury (12). In the present study, ALT and AST activities both increased significantly in the serum of mice after treatment with the EF of the *D. bulbifera* rhizome for fourteen consecutive days. These results suggest that *D. bulbifera* rhizome can induce mice liver injury.

Many studies have demonstrated that ROS plays an important role in various hepatotoxins-induced liver injury (5,6,23). Hepatic cellular oxidative stress often takes place during the occurrence of the imbalance between oxidants and antioxidants. Moreover, many non-enzymatic antioxidant and glutathione-related enzymes may be changed during this process (24-27). Of them, LPO is a free radical-related process (28). In this study, MDA amounts increased significantly, which indicated potential oxidative stress injury induced by LPO.

There are superoxide anions produced in the process of oxidative stress. As a metalloenzyme, SOD can convert such superoxide anions to hydrogen peroxide (29). The results in Figure 3A showed that SOD activity significantly decreased in livers of mice after given *D. bulbifera* rhizome for fourteen consecutive days, which confirmed that it induced oxidative stress injury in livers. CAT can further detoxify hydrogen peroxide (29). However, the results in Figure 3B indicated that there was no significant difference found in CAT activity in livers of mice treated with *D. bulbifera* rhizome.

Cellular glutathione plays a critical role in protecting hepatocytes against exogenous toxins. Its amounts are related to oxidative damage (30,31). The results showed that *D. bulbifera* rhizome caused excessive exhaustion of liver glutathione amounts and thus likely induced...
damage of liver normal antioxidant oxidant balance and caused oxidative stress injury in mice.

GST, GPx, GCL, and GR are all glutathione-related enzymes. Among them, the cytosolic GSTs exist in almost all aerobic species. It can catalyze the conjugation of electrophilic compounds produced during oxidative stress with glutathione. In the present study, *D. bulbifera* rhizome significantly decreased GST activity in livers of mice and thus contributed to liver oxidative stress injury. GPx catalyzes hydrogen peroxide decomposition to the stable form of hydroxides, specifically using reduced glutathione as the electron provider (32). GCL regulates glutathione as the first and rate-limiting enzyme in GSH de novo synthesis, which protects against free radical damage (20). GR catalyzes the reduction of GSSG to GSH using NADPH resulting from the pentose phosphate pathway (22). The results in the present study indicated that *D. bulbifera* rhizome induced a significant decrease in the activity of GPx, GR, and GCL in mice livers. All of these results confirmed damage on the balance between cellular oxidants and antioxidants induced by *D. bulbifera* rhizome.

In conclusion, the present study shows that the EF of the ethanol extract is the main toxic ingredient of *D. bulbifera* rhizome. Moreover, it also demonstrates that the EF of the *D. bulbifera* rhizome can induce liver toxicity and the mechanism of such hepatotoxicity may be related to liver oxidative stress injury in mice. All of these results remind us to pay attention to liver toxicity induced by *D. bulbifera* rhizome in the clinic. Further studies are in progress in our laboratory to find specific hepatotoxic chemical compounds in *D. bulbifera* rhizome and to explore the molecular mechanisms of its induced hepatotoxicity.

Acknowledgements

This work was financially supported by National Basic Research Program of China (No. 2006CB504704), Shanghai Science and Technology Committee Grants (08DZ1972300), National Natural Science Foundation of China (30701082) and Innovation Program of Shanghai Municipal Education Commission (09ZZ125).

References
