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Toxicity comparison of different active fractions extracted from radix *Sophorae tonkinensis* in zebrafish*

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Abstract: Radix *Sophorae tonkinensis* (RST) is a widely used herb in Traditional Chinese Medicine (TCM) for treating infectious and inflammatory diseases. However, the toxicity data for RST are limited. The aim of this work is to assess and compare the toxicity of the whole RST extract and its five active fractions using the zebrafish model. Five active fractions of RST were prepared using five different types of solvents, which included dealkalized water, ethanol, *n*-butyl ethanol, dichloromethane, and diethyl ether. The chemical profiles of the active fractions were determined by high-performance liquid chromatography (HPLC), and the toxicity observed in the zebrafish model was confirmed using mouse models. In the zebrafish model, cardiovascular toxicity was observed for the fraction extracted using diethyl ether, and hepatotoxicity was observed for the whole RST extract and the fractions extracted using water and ethanol, whereas both cardiovascular and hepatic toxicities were observed for the fractions extracted using *n*-butyl ethanol and dichloromethane. The hepatotoxicity of the fractions extracted using *n*-butyl ethanol and dichloromethane was also observed in mice. Our findings provide the toxicity data for RST and its five active fractions through modeling in a zebrafish, and indicate that the different fractions may each have a different toxicity, which is helpful for the optimal use of RST in clinical practice.

Key words: *Sophorae tonkinensis* Gagnep; Active fraction; Hepatotoxicity; Cardiovascular toxicity; Zebrafish
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1 Introduction

The dried roots and rhizomes of *Sophorae tonkinensis* Gagnep, commonly known as radix *Sophorae tonkinensis* (RST) or "Shandougen" in Chinese, have been used for hundreds of years as a Traditional Chinese Medicine (TCM) for treating acute pharyngolaryngeal infections and sore throats (Tang *et al*., 2013; CPC, 2015). Previous phytochemical studies of this plant have revealed that quinolizidine alkaloids, flavonoids, and triterpenoids are present as its major constituents (Xiao *et al*., 1999; Ding and Chen, 2006; Ding *et al*., 2006; Li *et al*., 2008a; 2008b; He C.M. *et al*., 2013; Pan *et al*., 2015). In the past few years, much more attention has been paid to the analysis of alkaloids in RST, including matrine, oxymatrine, and sophocarpine, due to their various pharmacological activities, which include ameliorating throat inflammation (Li *et al*., 2012; Yoo *et al*., 2014; Lee *et al*., 2015) and suppressing in vitro cancer cell proliferation (Chui *et al*., 2005; Liu *et al*., 2006) as well as hepatoprotective effects (Cho *et al*., 1986; Long *et al*., 2004; Chai *et al*., 2012). Unfortunately, some of the alkaloids extracted from RST have been shown to be highly toxic to humans and livestock

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in ways that damage the nervous system, digestive system, and respiratory system, affecting cardiovascular and liver functions. The frequency of side effects and poisoning from RST including hepatotoxicity and cardiovascular toxicity has increased (Sun *et al*., 2010; Li *et al*., 2011; Zhang and Ding, 2013; CPC, 2015) and those toxicities have limited the application of RST and reduced its medical and economic value.

Most medicinal herbs including RST contain different compounds that buffer, modulate, and modify the effects of any "active principles." The accumulated data suggest that the effects produced by whole plant extracts cannot be mimicked by administering isolated purified constituents of the plant. Herbs generally exert broad actions on a number of whole physiological systems at the same time and these actions are usually oriented in the same general therapeutic direction. The toxicity of Chinese herbs can generally be reduced through traditional processing and the addition of antidote herbs. Recently, the active fractions of Chinese herbs extracted through special solvents have been approved as effective in reducing or removing the toxicity and/or improving the efficacy of the herbs. Matrine and oxymatrine are the major components responsible for the toxicity of RST. There is a certain relationship between the matrine and oxymatrine content and the acute toxicity of the different components of RST, but this relationship is not entirely uniform and consistent (Sun *et al*., 2010).

In the present study, in combination with conventional TCM extraction technologies, RST extract (RSTE) and the five RST active fractions were prepared using five different types of solvents including ethanol, diethyl ether, dichloromethane, *n*-butyl ethanol, and dealkalized water. Zebrafish were exposed to each individual sample and their in vivo toxicity was assessed and compared.

2 Materials and methods

2.1 RST and chemicals

The dried roots of RST (lot#: 1207070) were purchased from Sichuan Neautus Traditional Chinese Medicine Co., Ltd., China. Absolute methanol (lot#: 20130204), dichloromethane (lot#: 20120810), diethyl ether (lot#: 20100419), *n*-butyl ethanol (lot#: T20110518), hydrogen chloride (lot#: T20090312), and anhydrous sodium carbonate (lot#: F20100810) were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); sophocarpidine (lot#: 01-2002) was from the Research Center of Shanghai Chinese Traditional Medicine; standard oxymatrine (lot#: 091110) was from Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China); and trifluoroacetic acid (lot#: FC160) and acetonitrile (lot#: I631730212) were from CNW Technologies GmbH (Düsseldorf, Germany) and Merck & Co. (New Jersey, USA), respectively. All chemicals used in this study were of analytical grade. Ultra-pure water (resistance >18 mΩ) was prepared by Millipore Milli-Q purification system (Bedford, USA).

2.2 Zebrafish and mouse handling

Adult AB strain zebrafish were housed in a Hunter Biotechnology, Inc., zebrafish facility and fed with live brine shrimp and dry flake as recommended by Westerfield (1995). The zebrafish were paired for natural mating and 200–300 embryos on average were generated per pair each time. Embryos were maintained at 28 °C in fish water (0.2% (2 g/L) Instant Ocean salt in deionized water, pH 6.9–7.2, conductivity 480–510 μS/cm, and hardness 53.7–71.6 mg/L $CaCO_3$) and washed and staged at 6 and 24 h post fertilization (Kimmel *et al*., 1995). The Hunter Biotechnology, Inc., zebrafish facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Kunming mice (20–22 g, 4–6 weeks old) were purchased from Shanghai Slac Laboratory Animal Co., Ltd. (certification No.: SCXK (Shanghai) 2012-0002) and housed in large polypropylene cages in a temperature-controlled room ((22±2) °C) and fed with standardized pelleted food (TANUVAS) and clean drinking water ad libitum.

2.3 Preparation of RST extract and active fractions

The RST was weighed and boiled with six times the amount of water for one hour, and the extract was filtered with filter paper to remove suspensions and the extraction was repeated one more time. The filtrates were combined, evaporated under a rotatory evaporator, and lyophilized through quick-freezing to obtain the RSTE.

An amount of RSTE was dissolved in water and separated with 75% ethanol. The precipitate was dried to obtain the ethanol sedimentation fraction from the RST. The supernatant was evaporated under a rotatory evaporator, dissolved in 2% HCl, and extracted four times with a known volume of diethyl ether. The diethyl ether solvent was then removed by evaporation to yield a dried diethyl ether fraction of the RST. The residue was fractionated by a series of solvents, including dichloromethane (dichloromethane fraction), *n*-butyl ethanol (*n*-butyl ethanol fraction), and sodium bicarbonate, and a remainder of dealkalized water fraction. All the active fractions were placed in airtight containers for future use.

2.4 Treatment of RSTE and active fractions

Stock solutions of RSTE and RST active fractions, including dealkalized water extract, ethanol sedimentation extract, *n*-butyl ethanol extract, dichloromethane extract, and diethyl ether extract were prepared in fish water and serial dilutions were made before each experiment. The zebrafish were placed into six-well plates (Nest Biotech, Shanghai, China), 30 zebrafish per well in 3 ml of RSTE solution at designated concentrations for the treatment period (Westerfield, 1995; McGrath and Li, 2008). Fish-water-treated zebrafish served as the control. To protect the RST solutions from light-induced decomposition, all experiments were performed at a constant temperature (28 °C) in the dark. After treatment, the zebrafish were visually observed and photographed with a dissecting stereomicroscope (Olympus Co., Tokyo, Japan) equipped with a high-speed video camera (JVC, Japan). Images were quantified using image-based morphometric analysis (NIS-Elements D 3.1, Japan). At the end of the experiments, all the zebrafish were sacrificed with 0.25 g/L tricaine methanesulfonate, which conforms to the American Veterinary Medical Association (AVMA) requirements for euthanasia by anesthetic (Shen *et al*., 2015).

2.5 Determination of maximum non-lethal concentration and 10% lethal concentration

To determine the maximum non-lethal concentration (MNLC) and 10% lethal concentration (LC_{10}) of RSTE and RST active fractions for toxic target organ identification and cardiovascular toxicity and hepatotoxicity assessment, 30 zebrafish per condition were exposed to RSTE or the RST active fractions for 3 d from 2 to 5 d post fertilization (dpf) (for toxic target organ identification), for 1 d from 2 to 3 dpf (for cardiovascular toxicity assessment), and for 2 d from 3 to 5 dpf (for hepatotoxicity assessment), respectively, following the standard procedures we reported earlier (He J.H. *et al*., 2013; Zhu *et al*., 2014; Shen *et al*., 2015). In the initial experiments, five testing concentrations ranging from 0.1, 1, 10, 100, and 1000 μg/ml were used for each sample and mortality was recorded daily. Zebrafish that lacked an observable heartbeat under a dissecting stereomicroscope were counted as dead zebrafish. If MNLC and LC_{10} were not estimated from the initial tests, additional testing concentrations up to 10000 μg/ml and down to 0.01 μg/ml were tested. Zebrafish mortality curves were generated using Origin 8.0 (OriginLab, USA) and MNLC and LC_{10} were calculated from this curve (Shen *et al*., 2015).

2.6 Target organ identification

To identify the target organs of toxicity induced by RSTE and RST active fractions, 30 zebrafish per condition were treated with each testing sample from 2 to 5 dpf at the nominal concentrations of 1/10 MNLC, 1/3 MNLC, MNLC, and LC_{10} as summarized in Table 1. After treatment, 15 zebrafish were randomly selected from each group for visual observation and photographs. The major organs and tissues of the zebrafish were visually assessed and toxic target organs were identified based on morphological changes and morphometric analyses.

2.7 Cardiovascular toxicity assessment

To confirm cardiovascular toxicity found in the target organ toxicity identification study, 30 zebrafish per condition were treated with *n*-butyl ethanol extract, diethyl ether extract, or dichloromethane extract from 2 to 3 dpf at the nominal concentrations of 1/10 MNLC, 1/3 MNLC, MNLC, and LC_{10} as summarized in Table 1. At the end of the experiment, 15 zebrafish were randomly selected from each group for visual observation and image acquisition. Six specific phenotypic endpoints were validated and used in the cardiovascular toxicity analyses: heart rate, heart rhythm, pericardial edema, circulation, hemorrhage, and thrombosis as described in our earlier report (Zhu *et al*., 2014).

Table 1 Tested concentrations used for RST toxicity studies (*n*=10)

Toxicity study	RST component	Concentration (μg/ml)			
		1/10 MNLC	1/3 MNLC	MNLC	LC_{10}
Target toxicity	RSTE	300.0	1000.0	3000.0	4323.4
	Dealkalized water extract	71.0	236.7	710.2	820.9
	Ethanol sedimentation extract	72.3	240.8	722.5	907.0
	n-Butyl ethanol extract	35.8	119.2	357.5	380.5
	Dichloromethane extract	14.0	46.7	140.0	150.0
	Diethyl ether extract	6.8	22.5	67.5	68.3
Cardiovascular toxicity	*n*-Butyl ethanol extract	47.9	159.6	478.9	538.3
	Diethyl ether extract	8.3	27.6	82.9	93.6
	Dichloromethane extract	45.0	150.0	450.0	
Hepatotoxicity	RSTE	543.2	1810.7	5432.0	7510.8
	Dealkalized water extract	96.0	319.9	959.7	1009.1
	Ethanol sedimentation extract	340.9	1136.2	3408.5	4367.6
	n-Butyl ethanol extract	70.0	262.2	700.0	
	Dichloromethane extract	340.9	1136.2	3408.5	4367.6

To confirm the cardiovascular toxicity induced by dichloromethane extract, a zebrafish histopathology was performed. After treatment, the zebrafish were fixed and dehydrated before paraffin embedding. The embedded zebrafish were longitudinally sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Histological images were obtained using a histological microscope (Leica, Germany) and a pathological diagnosis was performed by a certified pathologist (Zhu *et al*., 2016).

2.8 Hepatotoxicity assessment

The hepatotoxicity observed in the target organ toxicity identification study was further assessed in zebrafish treated with RSTE, dealkalized water extract, ethanol sedimentation extract, dichloromethane extract, or *n*-butyl ethanol extract. Thirty zebrafish per condition were treated with a testing concentration from 3 to 5 dpf at the nominal concentrations of 1/10 MNLC, 1/3 MNLC, MNLC, and LC_{10} as summarized in Table 1. After treatment, 15 zebrafish from each group were randomly selected for visual observation and quantitative image acquisition performed using image-based morphometric analysis (NIS-Elements D 3.1; Japan). Three specific phenotypic endpoints (liver size, liver degeneration, and yolk sac retention) were used for assessing hepatotoxicity as reported by our group (He J.H. *et al*., 2013; Shen *et al*., 2015). Liver size, liver degeneration, and yolk sac retention were calculated based on the formulas below: liver relative size (% of control)=liver area (sample)/liver area (control)×100%; liver degeneration (%)=[1−liver brightness (sample)/liver brightness (control)]×100%; yolk sac retention (% of control)=yolk sac area (sample)/yolk sac area (control)×100%.

2.9 Mouse study

The mice were divided into three groups of 12 mice each (half male and female). Dichloromethane extract and diethyl ether extract of RST (8 g/kg body weight) were administered to the mice via oral administration. Group I served as the control group, and received deionized water only; Group II and Group III received dichloromethane extract and diethyl ether extract once a day, respectively, for seven consecutive days. All the mice were sacrificed at the end of the eighth day after drug administration, and blood was drawn from the eyeball of the mouse and the serum was separated for liver function assays. Total bile acid (TBA), total bilirubin (TBil), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in the blood serum were quantified by an autoanalyzer (Shimadzu CL-7080, Shimadzu, Japan). The mouse livers were immediately removed, sliced, and washed in saline. Liver pieces were fixed in 10% formalin, embedded in paraffin wax, sectioned, and stained with H&E for histopathological analysis.

2.10 Fingerprint analysis of the dichloromethane fraction and the diethyl ether fractions

To establish the fingerprints, 10 mg of dichloromethane fraction or diethyl ether extract was weighed into a 10-ml polypropylene centrifugal tube, and 2 ml of water was added for sonication, filtered with a 0.22-μm Millipore filter, and then a total volume of 10 μl was loaded into the high-performance liquid chromatography (HPLC) column. The analyses were performed on a Waters Alliance e2695 HPLC (Waters Corporation, Milford, USA) coupled with vacuum degasser, quaternary pump, column compartment, and diode-array detector. The instrument was fitted with a Waters XBridge C18 Column (250 mm×4.6 mm, 5 μm), operated at 30 °C. HPLC analysis was performed using a gradient method. Gradient analysis was performed using a mobile phase A (acetonitrile) and B (aqueous solution of 0.1% (1 g/L) trifluoroacetic acid). The gradient profile was as follows: 5% A and 95% B were held for 20 min, followed by a decrease of 70% B over 15 min and 65% B for 15 min, and then 5% A and 95% B were held for a further 10 min. The flow rate used was 1.0 ml/min and chromatograms were collected at 210 nm. The similarity evaluation of the different fractions was performed using the Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A) software, published by the National Pharmacopoeia Committee of China.

2.11 Statistical analysis

Sigmoidal regression for concentration-response curves was used for estimating MNLC and LC_{10} (Origin 8.0) and one-way analysis of variance (ANOVA) followed by Dunnett's test was conducted for comparing differences between the groups. All statistical analyses were performed on SPSS Version 16.0 (SPSS Inc., Chicago, USA) and $P<0.05$ was considered statistically significant. Quantitative data were expressed as mean±standard error (SE) and all experiments were repeated at least three times to confirm the reproducibility of all data (Shen *et al*., 2015).

2.12 Quality control standard

According to our laboratory quality control standard, successful experiments must meet these milestones: (1) zebrafish natural death in untreated (control) groups was ≤10%; and (2) intraplate and interplate coefficients of variation (CV) were ≤25% (Zhou *et al*., 2014).

3 Results

3.1 Toxic target organs

We assessed toxicity and identified toxic target organs for RSTE and RST active fractions in zebrafish, concentration-dependent mortality was demonstrated, and the MNLC and LC_{10} are summarized in Table 1. Pericardial edema and/or reduced heart rates were observable in the zebrafish treated with dichloromethane extract, *n*-butyl ethanol extract, and diethyl ether extract in a dose-dependent manner, but not in zebrafish treated with RSTE, dealkalized water extract, or ethanol sedimentation extract. Liver degeneration was found in zebrafish treated with RSTE, dealkalized water extract, ethanol sedimentation extract, dichloromethane extract, and *n*-butyl ethanol extract, but not in zebrafish treated with diethyl ether extract. These findings suggest that RST diethyl ether extract induced cardiovascular toxicity, RSTE, RST dealkalized water extract and ethanol sedimentation extract induced hepatotoxicity, whereas RST *n*-butyl ethanol extract and dichloromethane extract induced both cardiovascular and liver injuries.

3.2 Cardiovascular toxicity

To confirm and characterize cardiovascular toxicity induced by RST *n*-butyl ethanol extract, diethyl ether extract, and dichloromethane extract, these RST active fractions were exposed to zebrafish at 2 dpf and the atrial and ventricular rates were counted at 4 h after treatment and the systematic cardiovascular toxicity was assessed after 24 h exposure. As summarized in Table 2, *n*-butyl ethanol extract, dichloromethane extract, and diethyl ether extract induced pericardial edema (Fig. 1a) and slowed the blood circulation. As indicated in Fig. 1b, *n*-butyl ethanol extract did not affect heart rate, whereas diethyl ether extract and dichloromethane extract treatment resulted in slower heartbeats in a dose-dependent manner. Statistically significant bradycardia was found in zebrafish treated with diethyl ether extract or dichloromethane extract at 1/3 MNLC and the above concentrations ($P<0.05$ or $P<0.001$). In addition, dichloromethane extract caused zebrafish

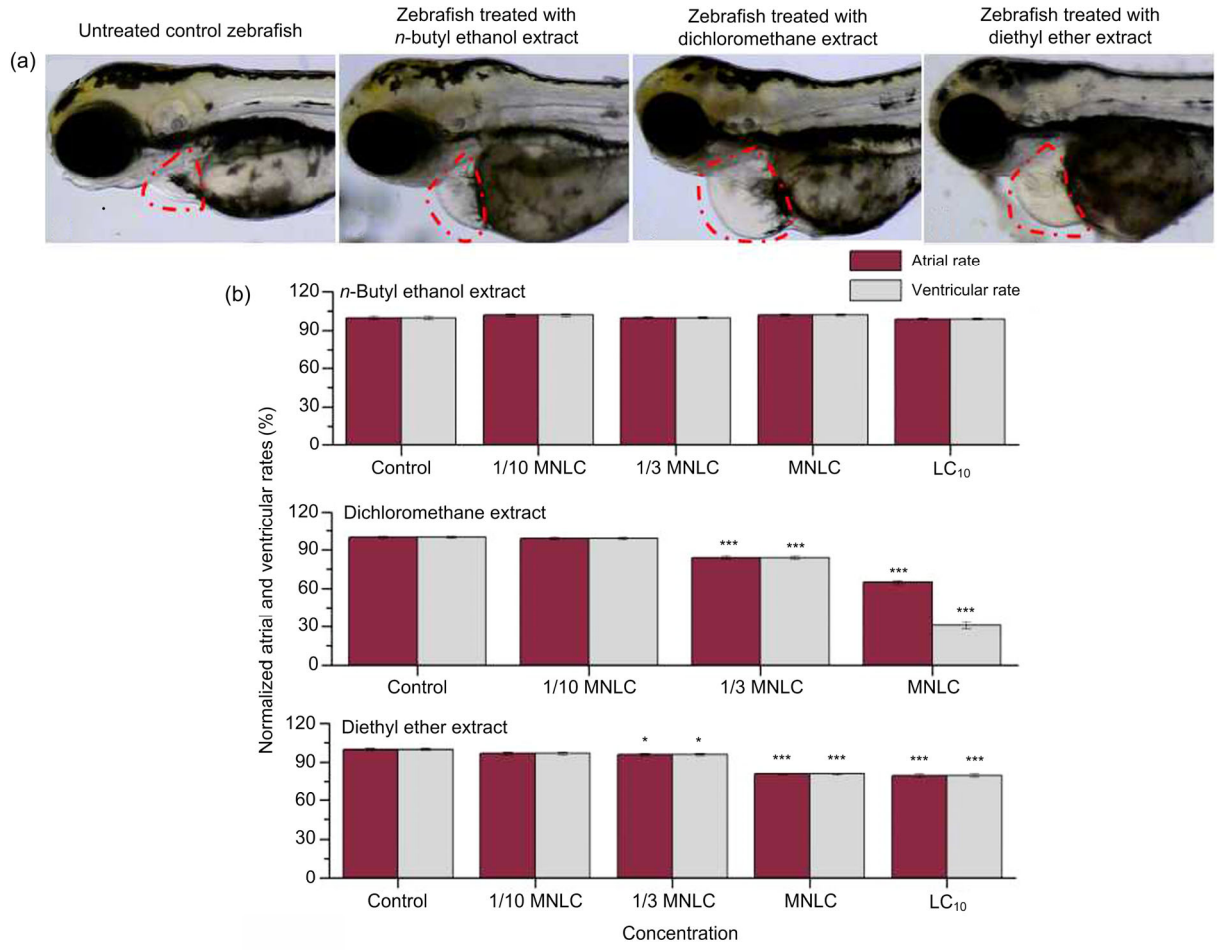

Fig. 1 Cardiovascular toxicity induced by RST active fractions in zebrafish

(a) Pericardial edema: circled area is a zebrafish heart or pericardial edema of untreated control zebrafish and zebrafish treated with *n*-butyl ethanol extract, dichloromethane extract, and diethyl ether extract. (b) Zebrafish atrial and ventricle heart rate changes as compared with the untreated control zebrafish. All the values represent the means±SE (n=10). $^{*}P<0.05$ and $^{***}P<0.001$ vs. control

Table 2 RST active fractions-induced cardiovascular toxicity (n=10)

RST active fraction	Pericardial edema (% of control)	Slow circulation (% of control)
n-Butyl ethanol extract	10% at MNLC & LC_{10}	40% at MNLC
Diethyl ether extract	80% at MNLC; 90% at LC_{10}	100% at MNLC & LC_{10}
Dichloromethane extract	30% at MNLC	100% at MNLC

atrioventricular conduction block at MNLC. No hemorrhage or thrombosis was found from any treatment. Cardiovascular toxicity was confirmed by histopathological examination in zebrafish treated with dichloromethane extract. When compared with a normal zebrafish heart (Fig. 2a), pericardial edema, a misshaped atrium and ventricle as well as reduced number of endothelial cells and cardiomyocytes were seen in the zebrafish treated with dichloromethane extract (Fig. 2b). The overall ranking of cardiovascular toxicity of RST active fractions was dichloromethane extract>diethyl ether extract>*n*-butyl ethanol extract.

3.3 Hepatotoxicity

In the acute toxicity and target organ toxicity study above, we found that RSTE and four of the RST

active fractions (dealkalized water extract, ethanol sedimentation extract, dichloromethane extract, and *n*-butyl ethanol extract) induced zebrafish hepatotoxicity. Here we systematically assessed and characterized the liver toxicity of these RSTEs. Untreated zebrafish exhibited clear liver tissue and were perfused with circulating blood cells. After treatment with RSTE or one of these RST active fractions, the zebrafish liver lost transparency and became dark or brown and liver blood flow was no longer observable. All tested RST active fractions except the ethanol sedimentation extract also induced zebrafish yolk retention and showed an obvious dosage–toxicity relationship (Fig. 3).

We quantified the liver size, liver degeneration, and yolk sac retention in zebrafish treated with RSTE and RST active fractions using an image-based morphometric analysis. As indicated in Table 3 and Fig. 4, the relative liver areas of zebrafish treated with RSTE, dealkalized water extract, ethanol sedimentation extract, dichloromethane extract, and *n*-butyl ethanol extract were 95.8%–96.8%, 98.1%–103.9%, 97.7%–104.8%, 97.1%–100.8%, and 89.9%–102.4% of the untreated control zebrafish, respectively, and a statistically significant reduced liver size ($P<0.01$) was observed only in the zebrafish treated with RSTE at a tested concentration of LC_{10}. The relative liver brightness of zebrafish treated with RSTE, dealkalized

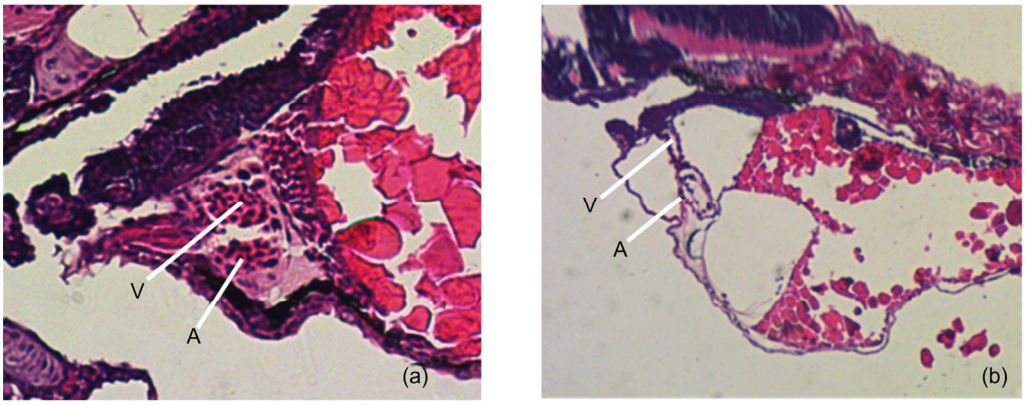

Fig. 2 Histopathological analysis of zebrafish hearts

(a) Untreated larval zebrafish exhibited a normal heart morphology and histology. (b) Larval zebrafish treated with dichloromethane extract showed pericardial edema, misshaped atrium and ventricle as well as reduced number of endothelial cells and cardiomyocytes. A: atrium; V: ventricle

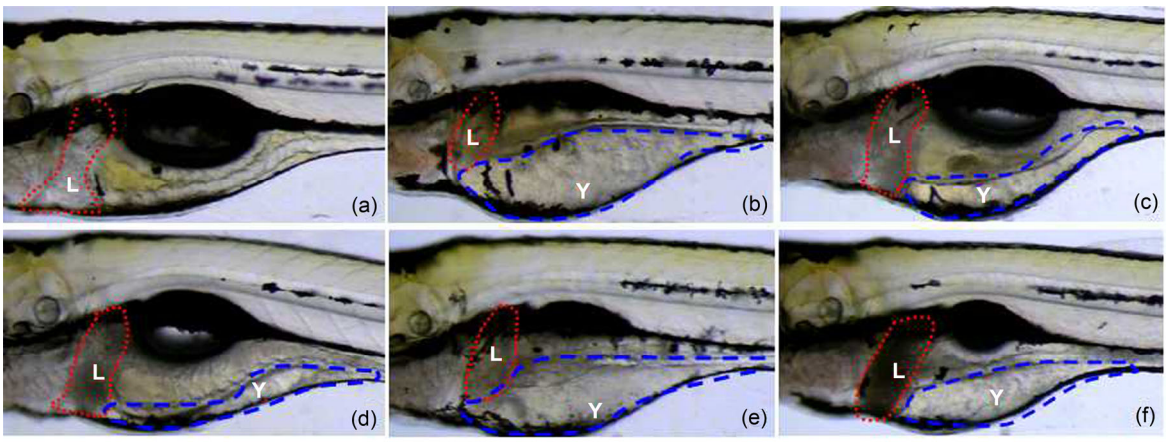

Fig. 3 Visual phenotype of hepatotoxicity in zebrafish at 5 dpf after exposure to RSTE or RST active fractions for 2 d

Untreated larval zebrafish exhibited a clear, healthy liver (a). Larval zebrafish treated with RSTE (b), RST dealkalized water extract (c), ethanol sedimentation extract (d), *n*-butyl ethanol extract (e), and dichloromethane extract (f) exhibited liver tissue degeneration, hepatatrophia, and yolk sac retention. L: liver; Y: yolk sac

Table 3 RSTE and RST active fractions-induced liver toxicity (n=10)

RST active fraction	Liver size change (% of control)	Liver degeneration (% of control)	Yolk sac retention (% of control)
RSTE	88.6% at LC_{10}	9.4% at 1/3 MNLC 24.3% at MNLC 30.1% at LC_{10}	516% at MNLC 538% at LC_{10}
Dealkalized water extract	No	9.5% at MNLC 17.8% at LC_{10}	210% at MNLC 345% at LC_{10}
Ethanol sedimentation extract	No	8.0% at 1/3 MNLC 11.2% at MNLC 16.9% at LC_{10}	No
Dichloromethane extract	No	23.8% at MNLC 25.2% at LC_{10}	573% at MNLC 834% at LC_{10}
n-Butyl ethanol extract	No	11.7% at 1/3 MNLC 20.8% at MNLC	689% at MNLC

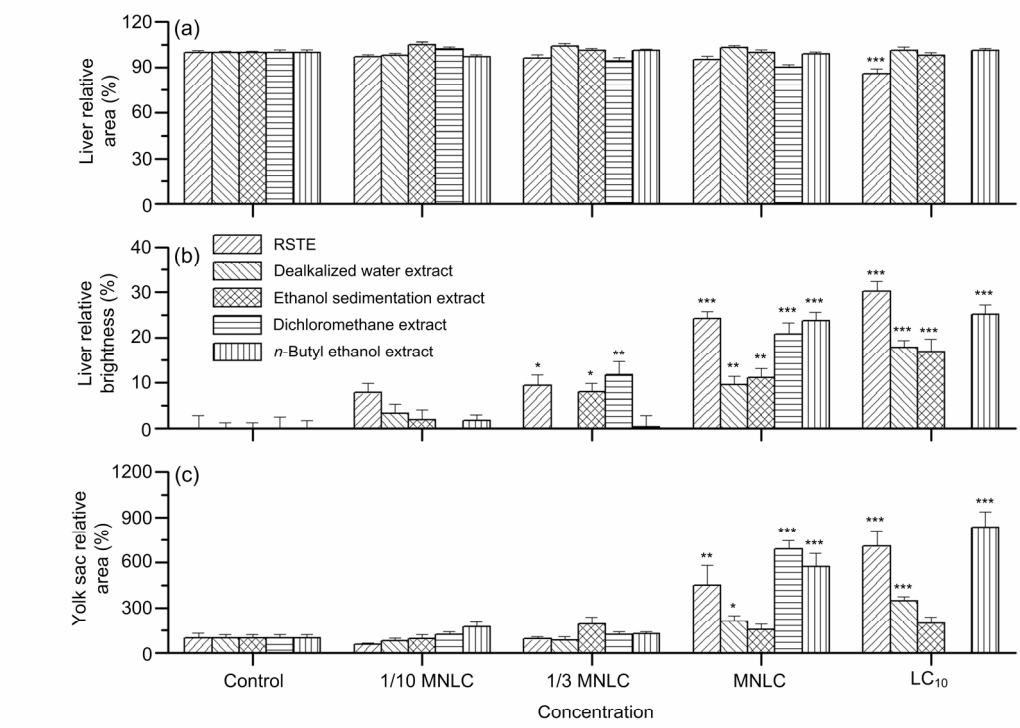

Fig. 4 Zebrafish liver size change, liver degeneration, and yolk sac retention after exposure to RSTE and RST active fractions

(a) Liver size was significantly reduced in zebrafish treated with RSTE ($^{***}P$<0.001 vs. control). (b, c) Liver degeneration and delayed yolk sac absorption were found in zebrafish treated with RSTE and four RST active fractions ($^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 vs. control). All the values are presented as mean±SE (n=10)

water extract, ethanol sedimentation extract, dichloromethane extract, and n-butyl ethanol extract was 7.8%–30.1%, 3.4%–17.8%, 2.0%–16.9%, 1.7%–25.2%, and −1.2%–20.8% of the untreated control zebrafish, respectively; liver degeneration was demonstrated in zebrafish treated with RSTE, ethanol sedimentation extract, and n-butyl ethanol extract at 1/3 MNLC and higher concentrations, and with dealkalized water extract and dichloromethane extract at MNLC and LC_{10} concentrations (P<0.05, P<0.01, or P<0.001). The relative yolk sac areas were 44%–538%, 80%–345%, 96%–196%, 127%–834%, and 124%–689% of the untreated control zebrafish, respectively, in zebrafish treated with RSTE, dealkalized water extract, ethanol sedimentation extract, dichloromethane extract, and n-butyl ethanol extract. Statistically significant delayed yolk sac absorption was found in zebrafish treated with n-butyl ethanol extract

at MNLC (P<0.001), and with RSTE, dealkalized water extract, and dichloromethane extract at MNLC and LC_{10} (Fig. 4). Based on the liver degeneration at MNLC, the hepatotoxic severity was RSTE> dichloromethane extract>*n*-butyl ethanol extract>ethanol sedimentation extract>dealkalized water extract.

To confirm the hepatotoxicity in zebrafish induced by RST dichloromethane extract but not liver damage in zebrafish treated with RST diethyl ether extract, we performed further experiments in mice by analyzing liver functions and liver pathology. Various serum biochemical biomarkers for liver functions in control mice and mice administered either dichloromethane extract or diethyl ether extract are represented in Table 4. Compared with the untreated control mice, ALT activity was significantly increased (P<0.01), whereas TBiL level was statistically decreased in mice administered dichloromethane extract. No liver function changes were found in mice administered diethyl ether extract (P>0.05). The histological observations consistently supported the results obtained from serum enzyme assays. Gross necrosis, massive fatty degeneration, broad lymphocyte, and Kupffer cell infiltration around the central vein, and cellular boundary loss were observed in mice administered dichloromethane extract. No abnormal histopathology was found in the diethyl ether extract group (Fig. 5).

3.4 Fingerprint analyses of RST dichloromethane extract and diethyl ether extract

The fingerprint of the chemical constituents from active fractions of dichloromethane and diethyl ether was determined by HPLC. Under optimum chromatographic conditions, the major components of each active fraction were identified and a well-resolved baseline separation was obtained. The main peaks existing in all of the sample profiles of the same fraction were selected as the common peaks. Quantitative HPLC fingerprint was calculated based on the relative retention time and relative peak area with the reference peak of these common peaks. The similarities among samples were analyzed using the Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A). The results are shown in Fig. 6, where there are four representative chromatograms for each fraction, of which S1, S2, and S3 represent the profiles of three batches of active fractions, respectively, and R shows the profile of the reference. Our results demonstrated a more than 0.96 similarity among these three batches of samples, which means the fingerprint derived from the HPLC assay was a reproducible and reliable method and could be used as a quality control for preparation of active fractions. The main characteristics of the chromatograms obtained from the diethyl ether fraction

Table 4 Liver functions of mice administered the RST active fractions (n=12)

Group	ALT (IU/L)	AST (IU/L)	TBiL (μmol/L)	ALP (IU/L)	TBA (μmol/L)
Control	22.5±5.27	102.8±21.78	1.4±0.47	529.1±203.10	2.1±1.23
Diethyl ether extract	27.1±11.28	102.1±24.46	1.3±0.39	525.3±141.25	1.7±1.04
Dichloromethane extract	42.8±45.66*	105.8±23.85	1.0±0.32*	505.6±143.04	1.7±0.91

All values are expressed as mean±SE (n=12). * P<0.01, compared with untreated control mice

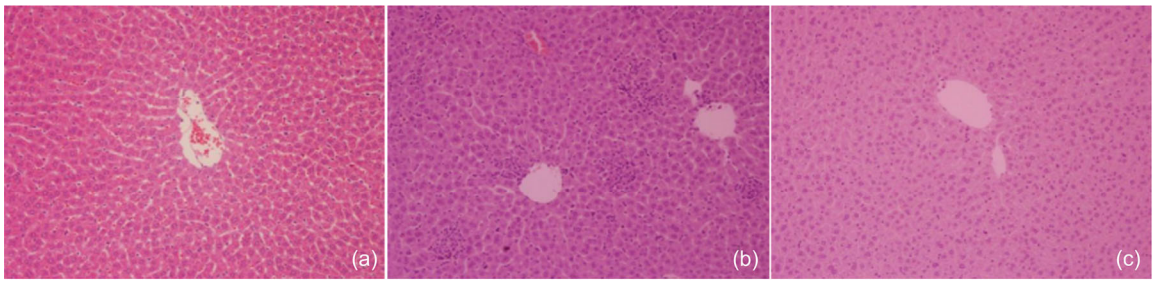

Fig. 5 Histopathology of liver tissue section

(a) The central vein surrounded by a hepatic cord of cells from the untreated control mice; (b) Patches of liver cell necrosis with inflammatory cell infiltration around the central vein and focal necrosis with sinusoidal dilatation in mice treated with the dichloromethane extract group; (c) A normal liver histology from mice treated with diethyl ether extract

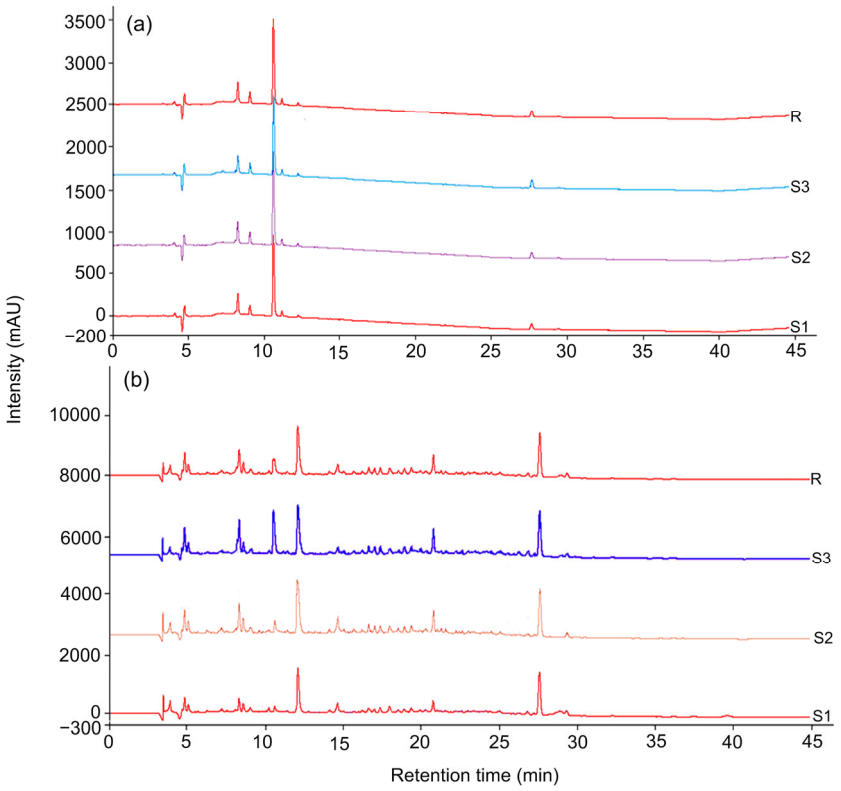

Fig. 6 HPLC fingerprint chromatograms of dichloromethane extract (a) and diethyl ether extract (b) recorded at a wavelength of 210 nm

A Kromasil C18 Column, a linear gradient of acetonitrile (5%–35%) in water containing 0.1% trifluoroacetic acid, and a flow rate of 1.0 ml/min were used for quantitative determination

and dichloromethane fraction were different, implying that the chromatograms of the dichloromethane fraction and the diethyl ether fraction were different.

4 Discussion

In this study, we prepared and obtained RSTE and five RST active fractions by extracting RST using dealkalized water, ethanol, *n*-butyl ethanol, dichloromethane, and diethyl ether. The toxicity of the various active fractions extracted by different solvents from RST was assessed and compared in a zebrafish model. We found that diethyl ether extract induced cardiovascular toxicity, and RSTE, dealkalized water extract and ethanol sedimentation extract induced hepatotoxicity, *n*-butyl ethanol extract and dichloromethane extract induced both cardiovascular and liver injuries. Overall ranking of cardiovascular toxicity of RST active fractions was dichloromethane extract> diethyl ether extract>*n*-butyl ethanol extract. Severity of hepatotoxicity was RSTE>dichloromethane extract> *n*-butyl ethanol extract>ethanol sedimentation extract> dealkalized water extract. Hepatotoxicity in zebrafish induced by dichloromethane extract as well as the absence of liver damage in zebrafish treated with diethyl ether extract was further confirmed in a mouse study. Our findings in this study demonstrated that the toxicity from RST active fractions can be different from the toxicity from the whole RSTE and active fraction technology could be a strategy, or part of a strategy, for reducing or even eliminating RST toxicity.

It appears that dealkalized water extract and ethanol sedimentation extract could not remove whole RSTE-induced hepatotoxicity; and *n*-butyl ethanol extract and dichloromethane extract actually induced additional organ/cardiovascular toxicity in addition to liver toxicity. Hepatotoxicity was not found in diethyl ether extract, but unfortunately, cardiovascular toxicity occurred. We postulate that due

to higher concentrations/amounts or the toxic ability of some chemical components in extracts, compared with RSTE, some active fractions could induce an additional cardiovascular toxicity.

We analyzed the fingerprint chromatograms of the chemical constituents of dichloromethane extract and diethyl ether extract taken as the representatives. Based on the toxicity study findings and HPLC fingerprint profiles, we consider that there was a correlation between the different active fractions and toxic responses. Toxic target organs and toxic levels induced by different active fractions of RST were probably related to the various substances extracted by different solvents. The literature has indicated that the major chemical components are polysaccharide in ethanol extract, flavonoid in *n*-butyl ethanol extract, alkaloid in dichloromethane extract, and organic acid in diethyl ether extract (Wang *et al*., 2004; Qian *et al*., 2015; Zhou *et al*., 2015). Further study to investigate the chemical components that are responsible for toxicity in RST active fractions is in progress.

Zebrafish share physiological, morphological, and histological similarities with mammals and have been recognized as an inexpensive and rapid alternative to rodents for evaluating drug toxicity and safety liabilities (Zhang *et al*., 2003; Hill *et al*., 2005; 2012; Zon and Peterson, 2005; Jones *et al*., 2008; McGrath and Li, 2008; Rekha *et al*., 2008; Yang *et al*., 2010; Hill, 2011; Selderslaghs *et al*., 2012; Zhang and Ding, 2013; Qian *et al*., 2015). As a whole organism, zebrafish are apparently able to capture toxicity associated with toxic metabolites, which are unlikely to be found in vitro (Hill *et al*., 2008). Very recently, several studies regarding the use of zebrafish for hepatotoxicity and cardiovascular assessment were published from our group (He J.H. *et al*., 2013; Zhu *et al*., 2014; Shen *et al*., 2015). These and other studies strongly support the use of zebrafish for toxicity studies, including assessing and comparing the toxicity of Chinese herbs and their active fractions.

5 Conclusions

In summary, this study assessed and compared the toxicity of RSTE and five RST active fractions in a larval zebrafish model. We found that diethyl ether extract induced cardiovascular toxicity, and RSTE, dealkalized water extract, and ethanol sedimentation extract induced hepatotoxicity, whereas *n*-butyl ethanol extract and dichloromethane extract induced both cardiovascular and liver injuries. The effects of dichloromethane extract and diethyl ether extract on the zebrafish liver were confirmed in mice. Our findings suggest that the toxicity from RST active fractions could be different from the whole RSTE and active fraction technology could be a strategy for reducing or eliminating RST toxicity.

Compliance with ethics guidelines

Hong-cui LIU, Xiao-yu ZHU, Jiang-hua CHEN, Sheng-ya GUO, Chun-qi LI, and Zhong-ping DENG declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：使用斑马鱼模型比较山豆根不同有效部位的毒性

目　的：利用斑马鱼模型评价和比较山豆根不同提取方法提取的有效部位的体内毒性。

创新点：首次在斑马鱼模型中证明山豆根提取方法不同，有效部位的毒性有明显差异。研究结果有助于指导山豆根的新药开发与临床应用。

方　法：用五种不同的溶剂（去碱水、乙醇、正丁基乙醇、二氯甲烷和乙醚）提取山豆根，然后通过高效液相色谱法（HPLC）检测有效部位，将 AB 品系斑马鱼分为对照组（养鱼水处理）和实验组（山豆根提取物）。实验组根据采用的提取溶剂不同，分为以下六组：去碱水提取组、乙醇沉提取组、正丁基乙醇提取组、二氯甲烷提取组和乙醚提取组以及山豆根总组分组（对照），观察各种山豆根提取物对斑马鱼的急性毒性与毒性靶器官。

结　论：山豆根乙醚提取组诱发斑马鱼心血管毒性（图 1）；山豆根去碱水提取组、乙醇沉提取组以及山豆根总组分组诱发斑马鱼肝脏毒性（图 3 和图 4）；而山豆根正丁基乙醇提取组和二氯甲烷提取组诱发斑马鱼心血管毒性（图 1 和图 2）和肝脏毒性（图 3 和图 4）。

关键词：山豆根；有效部位；肝脏毒性；心血管毒性；斑马鱼