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RESEARCH ARTICLE

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Cardiotoxicity induced by *Cochinchina momordica* seed extract in zebrafish

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Abstract

Momordica cochinchinensis (Lour.) Spreng is an indigenous South Asian edible fruit, and seeds of *Momordica cochinchinensis* have been used therapeutically in traditional Chinese medicine. Previous studies have shown that *M. cochinchinensis* seed (Momordicae Semen) has various pharmaceutical properties such as antioxidant and anti-ulcer effects as well as contains secondary metabolites with potential anticancer activities such as triterpenoids and saponins. Recent studies reported that water extract and ethanol extract of *M. cochinchinensi* seed were tested on mammals using an acute toxic classic method as OECD guidelines 420. No matter injected intravenously or intramuscularly, animals died within several days. In this study, zebrafish embryos were exposed to various doses of *Cochinchina momordica* seed extract (CMSE) from 2 dpf (days post fertilization, dpf) to 3 dpf. CMSE-induced cardiotoxicity such as pericardial edema, cardiac apoptosis, increased ROS production, cardiac neutrophil infiltration, decreased blood flow velocity, and reduced expression of three marker genes of cardiac functions were found in zebrafish roughly in a dose-dependent manner. These results suggest that CMSE may induce cardiotoxicity through pathways involved in inflammation, oxidative stress, and apoptosis.

KEYWORDS
apoptosis, cardiotoxicity, CMSE, inflammation, oxidative stress, zebrafish

1 | INTRODUCTION

Momordica cochinchinensis (Lour.) Spreng belongs in the Cucurbitaceae family and is geographically restricted to Southeast Asia. All parts of the plant are medicinally important with their use ranging from topical to internal preparations against malnutrition to anticancer effects. *Cochinchina momordica* seed is the dried ripe seed of *Momordica cochinchinensis* (Lour.) Spreng. Chemical analysis has shown that *Cochinchina momordica* seed extract (CMSE) is composed of several compounds, including fatty acids, saponins, proteins, α-spinasterol,

Zheng-Cai Du and Zhong-Shang Xia have equal contribution.

and momordica acid (Bolognesi et al., 1989; Kubota, Sato, Murakami, & Yamagishi, 1971; Murakami, Nagasawa, Itokawa, Tachi, & Tanaka, 1966). Recent studies reported that the ethanol extract of *Cochinchina momordica* seed was able to enhance immune responses and accelerate the healing of gastric ulcers (Jung, Nayoung, Bongcheol, Joo-Hyon, & Bong-Yong, 2010; Sakwiwatkul, Li, Song, & Hu, 2010; Xiao, Bao, & Hu, 2009). They have been used to treat internal and external disorders such as fluxes, liver and spleen disorders, hemorrhoids, wounds, bruises, swelling, and pus (Lim, 2012). Aril is also used to make a tonic for children and lactating or pregnant women and to treat "dry eyes" and night blindness (Burke, Smidt, & Vuong, 2005). In the present study, *Momordica cochinchinensis* seed extracts have been reported to be effective agents against melanoma, lung, breast, and gastric cancer cells. Ethanol extracts of the seeds also showed anticancer activity on lung, breast, esophageal carcinoma, and melanoma cell lines (Liu et al., 2012; Zhao, Han, & Shan, 2010). However, its biological toxicity has not yet been investigated.

Zebrafish, *Danio rerio*, is emerging as a predictive vertebrate animal model for in vivo assessing toxicity, safety, and efficacy of compounds and drugs, and numerous studies have confirmed that mammalian and zebrafish toxicity profiles are strikingly similar (McGrath & Li, 2008). In earlier investigations, we along with others have developed a variety of zebrafish assays and disease models for drug toxicity and metabolism assessment and for cancer drug screening (D'Amico, Li, Glaze, Davis, & Wen, 2012; Li, Luo, Awerman, & McGrath, 2012; McGrath & Li, 2008). As a vertebrate animal, zebrafish and humans have approximately 87% homology in genomes (Milan, Jones, Ellinor, & MacRae, 2006), and their morphological and molecular basis of tissue and organ development is either identical or similar to other vertebrates including humans (Chen & Fishman, 1996; Granato & Nüsslein-Volhard, 1996). The transparency of zebrafish for several days post-fertilization enables in vivo visual observation of internal organs including the cardiovascular system. Larval zebrafish can live for 7 days in a single well of a standard 96-well microtiter plate supported by nutrients stored.

Over the past decades, the zebrafish has become a major model organism for cardiovascular research. First pioneered in developmental biology laboratories (Shin & Fishman, 2002), this role has been consolidated by the acquisition of genetic and genomic resources including the sequencing of the zebrafish genome. Development of the two-chambered zebrafish heart is rapid: contractions begin by 26 h post fertilization (hpf) and looping occurs by 48 hpf and full vascular tree development by 72 hpf. Vascular development begins before 24 hpf with migration of angioblasts initiating formation of the two major axial vessels, the DA and posterior cardinal vein (Gore & Burggren, 2012), which are fully formed and carry blood by 30 hpf.

The present study was aimed to evaluate systematically and comprehensively cardiovascular toxicity and the associated mechanisms produced by CMSE exposure. We found that CMSE was highly toxic to zebrafish. CMSE induced inflammation, oxidative stress, and apoptosis of the zebrafish heart generally in a dose-dependent manner. In addition, the expression of three marker genes for cardiac system functions or development was significantly downregulated following CMSE exposure.

2 | MATERIALS AND METHODS

2.1 | Zebrafish care and maintenance

Three lines of zebrafish were used in this study: wild-type AB line zebrafish and Tg (MPO:GFP) transgenic zebrafish. Zebrafish were housed in a light and temperature controlled aquaculture facility with a standard 14: 10 h light/dark photoperiod and fed with live brine shrimp twice daily and dry flake once a day. Four to five pairs of zebrafish were set up for natural mating every time. On average, 200–300 embryos were generated. Embryos were maintained at 28°C in fish water (0.2% Instant Ocean Salt in deionized water, pH 6.9–7.2, conductivity 480–510 mS cm^{-1} and hardness 53.7–71.6 mg L^{-1} $CaCO_3$). The embryos were washed and staged at 6 and 24 hpf (hours post fertilization). The zebrafish facility at Hunter Biotechnology, Inc., is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International (Zhu, Xu, Guo, & Li, 2015; Zhu, Liu, et al., 2016; Zhu, Xia, et al., 2016).

2.2 | Plant material

Momordica cochinchinensis (Lour.) Spreng seeds used in this study were obtained from Guangxi Wuzhou Pharmaceutical Group Co., Ltd., Wuzhou, Guangxi, China.

2.3 | Extraction and isolation

CMSE was prepared following a procedure reported previously with minor modifications. The frozen sample (9.2 g) was placed in a vessel, protected from sunlight (covered in aluminum foil) and mixed with 92 ml of 70% ethanol under reflux twice for 1.5 h. The mixture was ultra-sonicated for 10 min (Luo, Hu, & Xiao, 2008; Sun et al., 2010). The extract was followed by filtration and then concentrated and evaporated to dryness using a culture dish. The crude extract was weighed, diluted to appropriate working concentrations in the range of 50 mg/ml using milliQ water and mixed using a water-bath sonicator for 10 min.

2.4 | Chromatographic and mass spectrometry conditions

HPLC and LC-MS analysis was performed on a Shimadzu Exion LC system (Kyoto, Japan). The analytes were separated on a Waters BEH C18 column (50 mm, 2.1 mm, and 1.7 μm). The mobile phase consisted of A (water containing 0.1% methanoic acid) and B

(acetonitrile). The linear gradient conditions were optimized as follows: 0–1 min, 5% B; 1–20 min, 5%–90% B; 20–25 min, 90% B; 25–25.1 min, 90%–5% B ; 25.1–30 min, 5% B. The flow rate was 0.3 ml/min. The injection volumes for both sample and standard solutions were 10 μl. LC-MS analysis was performed on AB SCIEX X500 QTOF system in positive and negative ESI mode. Ion source temperature was set to 550°C, and IS voltage was set to 5500 V. Mass calibration was achieved using the integrated calibrant delivery system (CDS) with the TwinSprayer probe (dual ESI needle). High resolution data were acquired using an information dependent acquisition (IDA) method consisting of a TOF-MS survey (100–1000 Da for 200 ms) and up to 10 dependent MS/MS scans (50–1000 Da for 50 ms). Declustering potential (DP) was set to 80 V, and MS/MS fragmentation was achieved using a collision energy (CE) of 35 V with a collision energy spread (CES) of ±15 V. Dynamic background subtraction (DBS) was activated to achieve the most complete MS/MS coverage. No inclusion list was used which allowed non-target identification without the need for a second injection to acquire MS/MS data. All data were acquired and processed using SCIEX OS software version 1.6.1.

2.5 | MNLD and LD_{10} determination

To determine the maximum non-lethal dose (MNLD) and 10% lethal dose (LD10) of the CMSE, zebrafish were treated with CMSE delivered by yolk sac microinjection from 48 to 72 hpf, and mortality was recorded at 24 h post-treatment. Dead zebrafish were defined as the absence of a heartbeat under a dissecting stereomicroscope. In the microinjection assays, six doses (15.6, 39, 80, 125, 250, and 500 ng/fish) with 5 nl of injection volume were used for CMSE in the initial tests. Zebrafish treated with 0.1% DMSO or fish water were used as vehicle controls. If a LD_{10} could not be found from the initial tests, other doses up to 2000 ng/fish and down to 0.1 ng/fish were used. Lethality curves were generated using Origin 8.0 (OriginLab, USA), and MNLD and LD_{10} were estimated from this curve.

2.6 | Cardiovascular toxicity assessment

Drug concentrations used in the cardiovascular toxicity tests are summarized for microinjection assays. Generally, four doses at 1/9 MNLD, 1/3 MNLD, MNLD, and LD_{10} were selected for assessing cardiovascular toxicity. Zebrafish were treated with CMSE from 48 to 72 hpf. At the end of treatment, 10 zebrafish from each group were randomly selected for visual observation and image acquisition under a dissecting stereomicroscope. Heart malformations, pericardial edema, circulation abnormalities, thrombosis, and hemorrhage were used in cardiovascular toxicity assessment and were described as follows.

2.7 | Cardiac apoptosis

Apoptotic cells from larvae in each group were detected by staining with acridine orange. In brief, after CMSE treatment (at 72 hpf), the larvae were incubated with 2.5 mg/ml acridine orange (AO) staining solution in the dark for 30 min and observed for apoptotic cells that would display yellow-green fluorescent spots in the heart under a stereo fluorescence microscope (Nikon AZ100 fluorescence microscope). Nikon NIS-Elements D 3.10 Advanced image processing software was used to capture and analyze the images. The fluorescence signal from apoptotic cells in the heart was measured, and the apoptotic rate was calculated as reported by us (Li et al., 2006). The induction % of heart apoptosis in zebrafish treated with CMSE was calculated based on the following formula: the induction % of apoptosis = $(S_{CMSE}/S_{Vehicle} - 1) \times 100\%$.

2.8 | Hemodynamics analysis

After zebrafish embryos were exposed to different doses of CMSE for 24 h, the blood flow and velocity were detected by ZebraBloodTM (v1.3.2, ViewPoint, Lyon, France) software via recording the motion of erythrocytes with tracking area. Blood flow videos were analyzed to detect changes in pixel density and combined with vessel diameter to generate a flow rate in nl/s for every frame (Parker et al., 2014).

2.9 | Mechanisms of cardiotoxicity

2.9.1 | Inflammation

Thirty Tg (MPO:GFP) transgenic zebrafish were treated with CMSE at doses of 1.5, 4.5, 13.5, and 39 ng/fish from 48 to 72 hpf. After treatment, 10 zebrafish from each group were randomly selected, and images were acquired using a stereo fluorescence microscope. Qualitative image analysis was performed using image-based morphometric analysis. The heart inflammation resolution % in zebrafish treated with CMSE was calculated based on the following formula: The heart inflammation resolution % = $(1 - S_{CMSE}/S_{Vehicle}) \times 100\%$.

2.9.2 | Reactive oxygen species (ROS) assay

Generation of reactive oxygen species (ROS) in control and CMSE-treated zebrafish was detected at 48 hpf using an ROS assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on detection of the fluorescent probe CM-H2DCFDA. The treated zebrafish were incubated with 0.5 mg/ml CM-H2DCFDA for 24 h in dark at 28°C. After rinsing for three times using fish water, zebrafish were transferred into a 96-well microplate (one zebrafish per well), and ROS was measured at 488 nm under a multimode microplate reader (Duan et al., 2015).

2.9.3 | Gene expression analysis

To further investigate the possible cardiovascular toxic mechanism induced by CMSE based on adverse phenotypes and functional

results, calcium channel-related genes (cacna1ab), potassium ion channel-related genes (kcnq3), and the regulatory gene for cardiac troponin C (tnnc1a) were determined by q-PCR. After CMSE-treatment (at 72 hpf), total RNA was extracted from 30 larvae using the TRIzol reagent (Invitrogen, Waltham, USA). The quality of extracted RNA was evaluated on the basis of OD260/OD280 ratio. Reverse-transcription reactions were performed using a reverse transcriptase kit (Takara, Dalian, China). Q-CMSER amplifications were carried out with a real-time CMSER system (Biorad, CA, USA) using the SYBR green system (Takara, Dalian, China) in which there are three technical or biological replicates. The β-actin mRNA was considered as the reference gene; the relative quantification of each gene mRNA level among groups was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Three replicates of RNA extracted from the pools of zebrafish were analyzed. Each reaction contained 5 μl of SYBR Green, 4.5 μl of cDNA, 0.25 μl Forward Primer, and 0.25 μl Reverse Primer. The primers are listed in Table 1.

TABLE 1 Sequences of primer pairs used in the real-time quantitative PCR reaction

Target gene	Primer sequence (5′-3′)	
β-actin	Forward	5′-TCGAGCAGGAGATGGGAACC-3′
	Reverse	5′-CTCGTGGATACCGCAAGATTC-3′
tnnc1a	Forward	5′-GGCAGAGCAACTCACCGAT-3′
	Reverse	5′-GTAGGGTTCTGGCCCAACAT-3′
cacna1ab	Forward	5′-GGAATCGGCAGATGAGGGAG-3′
	Reverse	5′-ATTGCCGACAGAGCCGTAAT-3′
kcnq3	Forward	5′-CATCATCACAACCAGCGACG-3′
	Reverse	5′-TCTGGACCTGATCCCCATGT-3′

Statistical analysis

One-way ANOVA followed by the Dunnett's test was used to compare differences among groups. All statistical analyses were performed using the SPSS 16.0 software (SPSS, USA), and $p < 0.05$ was considered statistically significant. For quantitative analysis, all data were presented as mean ± SE, and results were statistically compared between drug-treated and control zebrafish groups.

3 | RESULTS

3.1 | Identification of phytochemical compounds in Gac fruit parts by LC-MS/MS

Phytochemical compounds obtained from the LC-MS/MS analysis of CMSE are displayed in Table 2. Approximately 25 compounds were

TABLE 2 Phytochemical compounds identified by LC-MS/MS in CMSE

Peak	Calcd *m/z*	Exptl *m/z*	Compound
1	148.06	148.06	Glutamic acid
2	162.11	162.11	L-Carnitine
3	175.11	175.11	L(+)-Arginine
4	130.09	130.09	Pipecolinic acid
5	268.11	268.11	Adenosine
6	132.10	132.10	Leucine
7	284.10	284.10	Guanosine
8	166.09	166.09	Phenylalanine
9	469.13	469.13	Rhein-8-*O*-β-D-glucopyranoside
10	195.07	195.07	Ferulic acid
11	225.08	225.08	Sinapinic acid
12	417.18	417.18	Arenobufagin
13	309.10	309.10	Bisdemethoxycurcumin
14	700.28	700.28	Pinoresinol diglucoside
15	439.16	439.16	Ganoderic acid A
16	465.11	465.11	Hyperin
17	611.16	611.16	Rutin
18	603.17	603.17	Naringin
19	303.05	303.05	Quercetin
20	287.06	287.06	Luteolin
21	148.06	148.06	3-*O*-β-D-glucofuranosidurono-6,3-lactone-gypsogenin
22	162.11	162.11	3-*O*-β-D-glucuronopyranosyl-gypsogenin
23	175.11	175.11	Gypsogenin
24	130.09	130.09	5-Dehydrokarounidiol
25	268.11	268.11	Methyl linoleate

identified by comparing and searching in the standard library information based on the retention time and mass spectra acquired in Figure 1. The present screening highlighted a number of organic acid, polyphenols, and fatty acids distributed among the CMSE.

3.2 | MNLD and LD_{10}

CMSE-induced zebrafish lethality curves were presented in Figure 2. Based on zebrafish lethality curves, MNLD and LD_{10} of drugs were estimated. To assess cardiotoxicity of CMSE, zebrafish were exposed to the testing samples from 48 to 72 hpf. Dose-dependent mortalities were observed for CMSE, and MNLC and LD_{10} were calculated as 13.8 and 39.4 ng/fish, respectively (Figure 2).

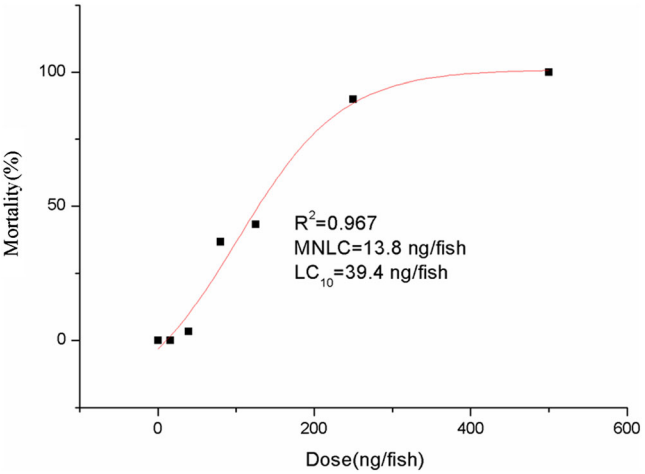

FIGURE 2 The effects of CMSE on zebrafish mortality. Zebrafish were exposed to CMSE at the indicated doses for 24 h

3.3 | Assessment of CMSE-induced cardiovascular toxicity

Based on the morphological assessments of zebrafish embryos, a series of CMSE doses (1.5, 4.6, 13.8, and 39.4 ng/fish) were then performed for the cardiac toxicity experiments. Representative zebrafish malformation of cardiac region was shown in Figure 3A. Pericardial edema was the typical cardiac toxicity phenotype induced

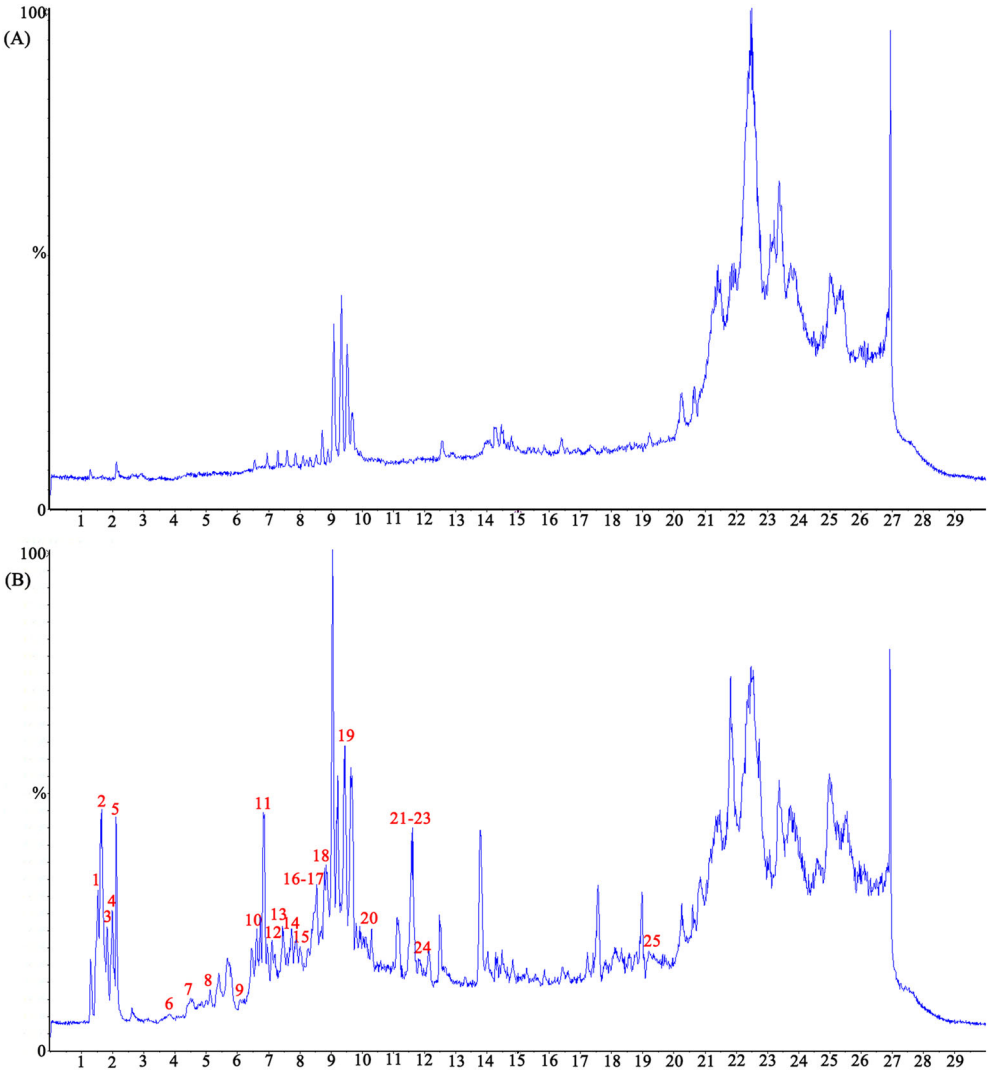

FIGURE 1 LC-MS/MS chromatograms of (A) blank feed sample and (B) samples of CMSE

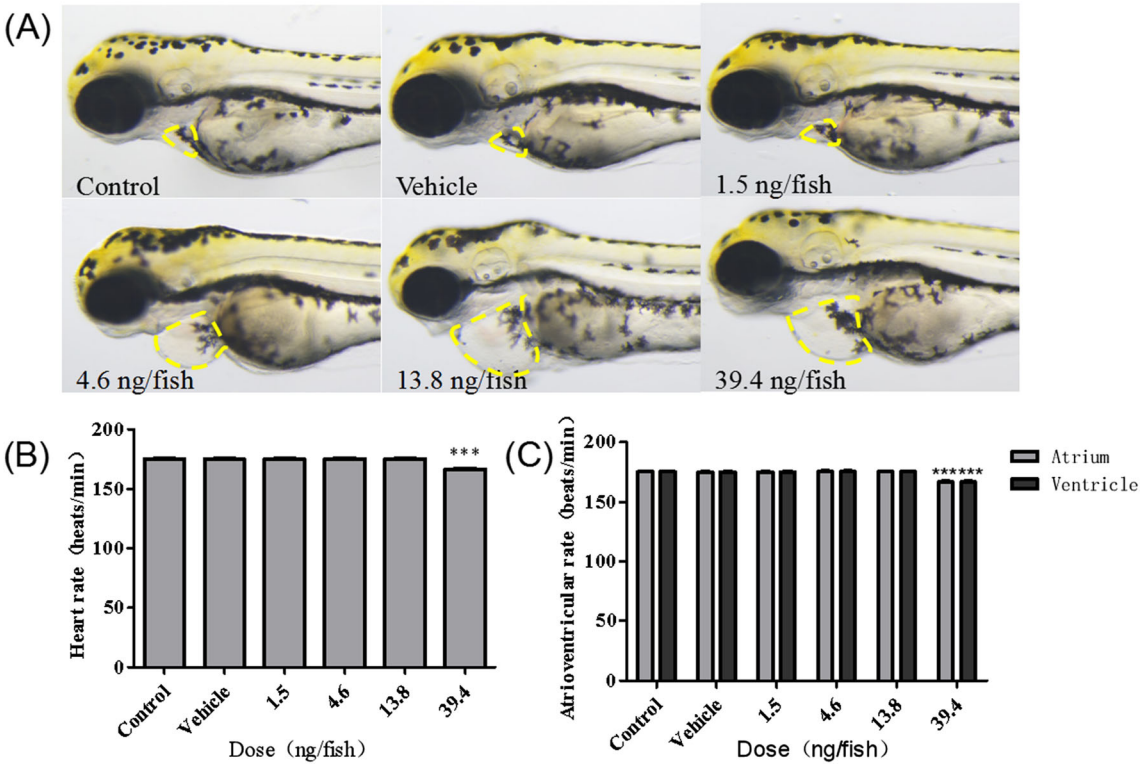

FIGURE 3 Cardiac toxicity phenotype induced by CMSE. (A) Pericardial edema of zebrafish was the typical cardiac malformation. (B) Heart rate and (C) atrium and ventricle ratio of zebrafish exposed to CMSE at 72 hpf. Data were expressed as means ± SE. Compared with vehicle group: ***$p < 0.001$

by CMSE. The heart rate was (175 ± 1.5)/min in vehicle group and had no significant difference as compared with control group. In the zebrafish treated with CMSE at doses of 1.5, 4.6, 13.8, and 39.4 ng/fish, it was (175 ± 2.0)/min, (175 ± 2.1)/min, (175 ± 1.5)/min, (167 ± 2.6)/min, respectively. The heart rate of zebrafish embryos was significantly decreased at the higher concentrations (39.4 ng/fish) as compared with vehicle group (Figure 3B). While the atria/ventricular ratio was 1:1 (Figure 3C), indicating that there was no atrio-ventricular block after zebrafish exposed to CMSE.

3.4 | Cardiac apoptosis

The heart of zebrafish was chosen for the observation of apoptosis induction. There were a little apoptotic cells indicated in the control zebrafish. As shown in Figure 4A, at the highest doses of CMSE (39.4 ng/fish), the fluorescence intensity of apoptotic cells was increased significantly, which was 401% higher of vehicle. The induction percentages of apoptosis were 3.3%, 46.1%, 127.0%, and 401.0% in zebrafish treated with CMSE at 1.5, 4.6, 13.8, and 39.4 ng/fish,

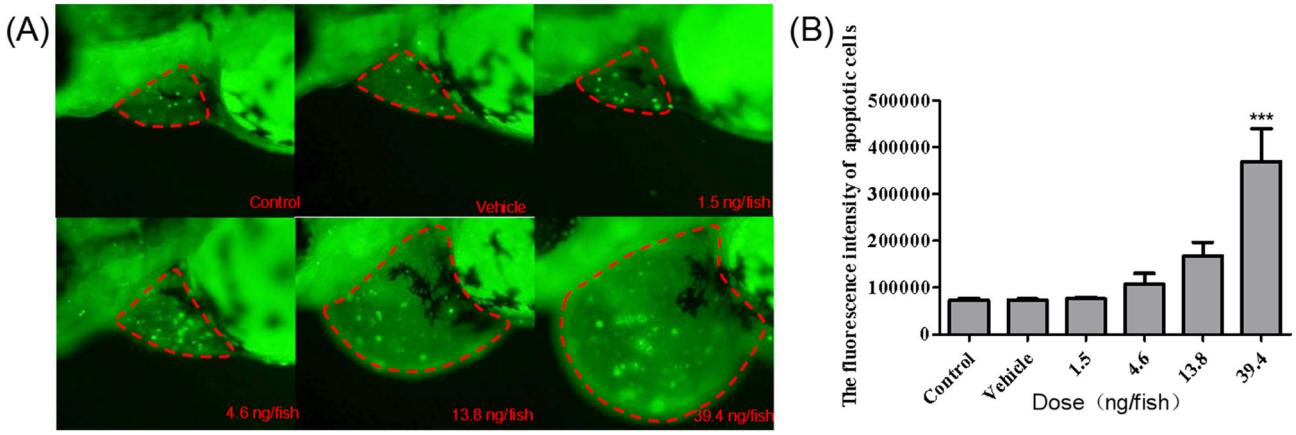

FIGURE 4 Phenotype of CMSE exposure induced cardiac apoptosis: there were no obvious apoptotic cells observed in the control zebrafish, but significant numbers of apoptotic cells were observed in CMSE-treated zebrafish

respectively. Statistically significant inductions of apoptosis were observed as compared with vehicle group in zebrafish treated with CMSE at doses of 1.5 ng/fish ($p > 0.05$), 4.6 ng/fish ($p > 0.05$), 13.9 ng/fish ($p > 0.05$), and 39.4 ng/fish ($p < 0.001$) (Figure 4B).

3.5 | Hemodynamics changes

As shown in Figure 5, the blood flow were gradually decreased in a dose-dependent way and had significant difference as compared to vehicle group. The significantly reduced blood dynamics was found in zebrafish treated at the highest doses (39.4 ng/fish). The blood flow velocity was markedly declined compared with that of vehicle at the higher doses (13.8 and 39.4 ng/fish) (Figure 5B). Both blood flow and blood velocity decreased significantly at the CMSE exposure level higher than MNLC. Our results demonstrated that CMSE could induce the circulation abnormality.

3.6 | Mechanisms of cardiotoxicity

3.6.1 | Inflammation

Since the neutrophils are normally located in the ventral vein region, the recruitment and chemotaxis of neutrophils in Tg (mpo: GFP) zebrafish were observed mainly in the cardiac region after exposed to CMSE (Figure 6A). At the 24 hpi, the higher doses of CMSE (4.6, 13.8, and 39.4 ng/fish) had significant difference compared with control group. The results indicated that CMSE induced cardiac inflammation in zebrafish embryos. The inflammation induction percentages were 35.7%–171.4% (Figure 6B).

3.6.2 | ROS production

To get a closer insight into the mechanisms of CMSE toxicity on zebrafish embryos, we measured the ROS production using the fluorescence intensity of oxidation-sensitive probe CM-H2DCFDA. CMSE treatment resulted in increased ROS production. ROS levels relative to vehicle zebrafish were 36.0%, 73.5%, 96.0%, and 226.0%, respectively, in zebrafish treated with CMSE at concentrations of 1.5, 4.6, 13.8, and 39.4 ng/fish. Statistically significant difference ($p < 0.01$) was found in zebrafish treated with CMSE at 39.4 ng/fish. The results showed that CMSE could induce the production of ROS in whole-embryo in a dose-dependent manner (Figure 7).

3.6.3 | Gene expression

The mRNA levels of genes related to the cardiac functions were determined, including *tnnc1a*, *cacna1ab*, and *kcnq3*, which are known

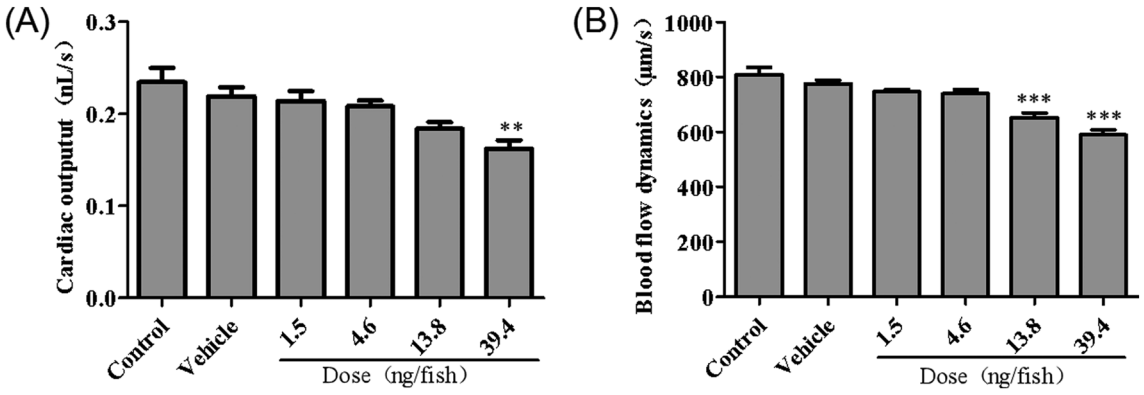

FIGURE 5 Reduced cardiac output and blood flow dynamics in zebrafish treated with CMSE. (A) Cardiac output in zebrafish treated with CMSE; (B) blood flow dynamics in zebrafish treated with CMSE. Data were expressed as means ± SE. Compared with vehicle group: ***$p < 0.001$

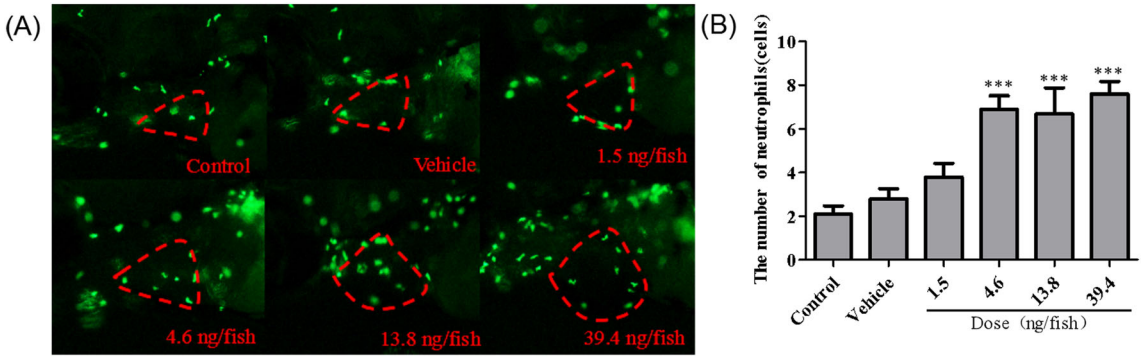

FIGURE 6 Cardiac inflammation in Tg (mpo: GFP) zebrafish induced by CMSE. (A) Neutrophils were recruited and migrated in cardiac region. (B) The number of neutrophils was increased markedly at 24 hpi, respectively. Data were expressed as means ± SE. Compared with vehicle group: ***$p < 0.001$

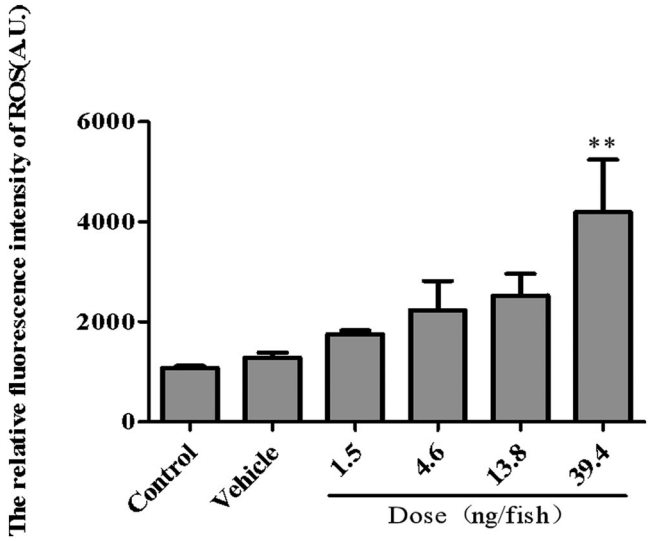

FIGURE 7 Respiratory burst activity of CMSE-treated whole zebrafish, measured by oxidation of H2DCFDA to DCF. Data were expressed as means ± SE. Compared with vehicle group: ***$p < 0.001$

to be expressed in cardiac muscle. As shown in Figure 8, these genes were generally decreased in a concentration-dependent manner in zebrafish treated with CMSE. The relative quantitative expression of *tnnc1a*, *cacna1ab*, and *kcnq3* were 0.56–0.93, 0.45–0.87, and 0.67–0.85, respectively. A statistically significant downregulation of three examined genes was all observed in zebrafish treated with CMSE ($p < 0.05$, 0.01, or 0.001).

4 | DISCUSSION

Cardiovascular toxicity is a major challenge for the pharmaceutical industry. Zebrafish as a new cardiovascular system animal model have been used for assessing drug toxicity, efficacy, and drug screening. *Momordica cochinchinensis* has been demonstrated toxic, especially its seeds (Chen, Zhang, & Shan, 2006), but the toxic target organ has not been identified yet. To the best of our knowledge, this was the first time demonstrating that CMSE induced cardiac toxicity and cardiovascular dysfunction in a whole animal model probably through neutrophil-mediated inflammation, oxidative stress, and apoptosis.

CMSE exposure led to severe abnormalities of the cardiovascular system in zebrafish, including pericardial edema, bradycardia, and cardiac output reduction. These results suggest that CMSE exposure damaged zebrafish cardiovascular system not only morphology but also functions. The reduced heart rate has been validated as one of the most sensitive biomarkers for the cardiovascular toxicity of human drugs in the zebrafish (Liang et al., 2016). Surprisingly, the atria/ventricular ratio was 1:1 in both control and CMSE-treated zebrafish (Figure 3C), hinting that CMSE may not affect on the atrioventricular conduction. Abnormal heart rhythm of human cardiac toxic drugs observed before in zebrafish contains tachycardia, bradycardia, atrioventricular block, and premature contraction or fibrillation (Zhu et al., 2014). These results indicated CMSE had a toxic effect on the heart rhythm, mainly inducing bradycardia but not atrioventricular block.

The zebrafish displayed a remarkable decrease in cardiac output when treated with CMSE for 24 h (Figure 5B). The changes in cardiac output may be resulted from cardiac contraction disturbances (Choi & Park, 2012). The reduction of cardiac output usually caused increase of heart rate in physiologic compensatory reaction of organism, but not in this study. Our results indicated the reduction trend of cardiac output was consistent with the reduced heart rate (Figures 3B and 5A).

Sun et al. observed acute toxicity and pharmacodynamic action of *Cochinchina momordica* seed extract in mice and found that *Cochinchinam momordica* seed extract affected mouse weight, organ index, ear swelling, pain threshold, and so on. They also found that the toxic components in CMSE responsible for its acute toxicity were mainly saponin and cochinchinin (Sun et al., 2010). After injecting chitosan saponins into rats, their blood pressure was temporarily decreased, breathing was excited, and heartbeat was accelerated (Wang, Chen, Huang, Zhu, & Zhang, 2011). The death rate was 50% after intravenous injection (32.35 mg/kg) or intraperitoneal injection (37.34 mg/kg) of saponin in mice (Wang, Zhao, & Zhang, 2010). Saponin had a hemolytic effect on rabbit red blood cells. LD_{50} of cochinchinin in mice with intraperitoneal injection was 16 mg/kg, and the poisoned mouse died quietly (Wang, Zhao, & Zhang, 2010). In this study, we

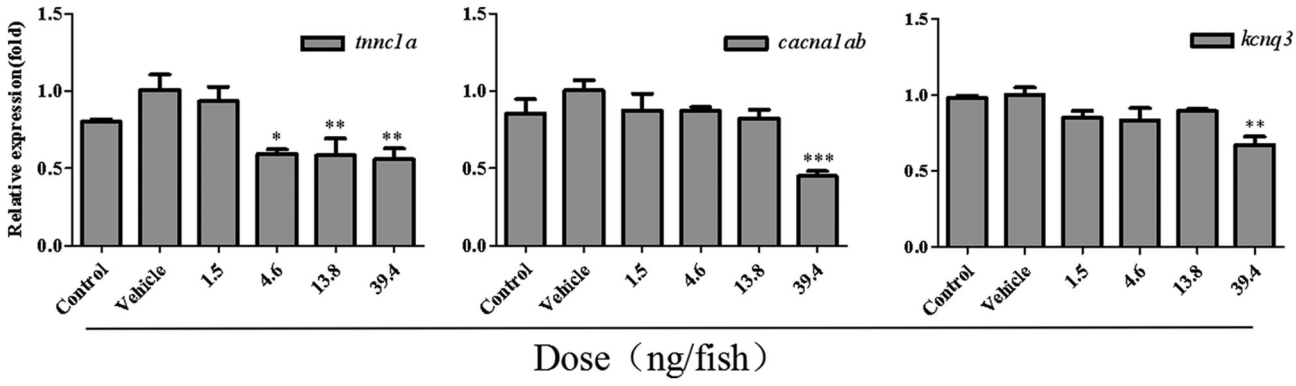

FIGURE 8 Effects of CMSE on calcium signaling pathway and cardiac muscle contraction genes. Q-PCR analysis showed that the genes involved in calcium signaling pathway and cardiac muscle contraction were decreased. Data were expressed as means ± SE. Compared with vehicle group: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

identified approximately 25 compounds from CMSE by comparing and searching in the standard library information based on the retention time and mass spectra acquired (Figure 1). But the exact chemical components that were involved in the CMSE-induced cardiovascular toxicity were not identified yet. Further work to specify the toxic compounds and the preventive agents is in progress in our laboratory.

To explore whether the pericardial edema was probably due to the induction of inflammation and cell death by CMSE, neutrophil analysis and AO staining were performed. AO can bind to the damaged DNA of cells that underwent cellular death (Jin et al., 2018). As expected, cardiac inflammatory cell infiltration and the increased apoptosis were shown in the zebrafish treated with CMSE. These observations imply that the cardiovascular toxicity of CMSE in zebrafish could be caused by the inflammation and the increased apoptosis. The heart tissue is the first organ to form and function in the zebrafish embryos, and the heart rate is an important indicator as assessment of zebrafish cardiac function. It is worthy to mention that the zebrafish with a weak or damaged heart still survive for at least 5 days after fertilization, but impaired heart generally leads to dead in mammals.

A possible explanation for the cardiac toxicity could be the ROS production probably mainly derived from inflammation induced by CMSE. Thus, we monitored the ROS levels using dichlorofluorescein diacetate as an indicator in zebrafish (Zhu et al., 2019). As the doses of CMSE increased, the brightness of the fluorescence gradually enhanced, indicating the ROS generation (Figure 7). Quantitative analysis of ROS showed a 3.3-fold higher than vehicle control at the highest dose (39.4 ng/fish). The generation of intracellular ROS could cause oxidative damage and further deteriorate cardiac dysfunction (Duan et al., 2015).

The decrease in heart rate could be due to abnormal cardiomyocyte contraction or inhibition of different ion channels that generate action potentials in cardiomyocytes (Lin, Hui, & Cheng, 2007). We assessed the genes expression profiles associated with calcium signaling pathway and cardiac muscle contraction in CMSE-exposed zebrafish embryos, which demonstrated significant down-regulation of these genes including calcium channel-related genes (cacna1ab), potassium ion channel-related genes (kcnq3), and the regulatory gene for cardiac troponin C (tnnc1a) (Liu, Chu, Chen, Gui, & Zhu, 2017). These results suggest that CMSE exposure inhibited the calcium signaling pathway and cardiac muscle contraction in zebrafish, leading to cardiac dysfunction.

The discoveries reported in this study, in combination with literature, strongly suggest that cardiovascular system could be the major toxic target organ of CMSE. The data obtained from this investigation could potentially provide valuable information for cardiovascular health risk assessment and management of this clinically extensively used Chinese medicine herb. Further work to identify the toxic compounds responsible for its cardiotoxicity and the potentially preventive agents are in progress in our laboratory.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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