Introduction

With the increase of global exchange and diversification of dietary habits, ethnic medicine and ethnic foods are becoming more popular and more widely consumed (Maynard 2015; Apekey et al. 2019). Meanwhile, assessment and study of safety in ethnic medicine or ethnic foods are gradually becoming very important. Roots and stems of Kadsura coccinea (Lem.) A. C. Smith. [Schisandraceae] is a tropical or subtropical plant. The roots and stems of K. coccinea have been used as a decongestant, tonic and digestive agent in Vietnamese or Chinese traditional medicine. The roots and stems of K. coccinea are also used as traditional foods of the Li nationality. Additionally, the Li folk people use this plant to stew pork and beef because of the sedative effect and strong aroma (Huang et al. 2019). Moreover, the fruits of K. coccinea offer high nutritional value and a new taste (Sun et al. 2009; Huang et al. 2019). The roots and stems of K. coccinea are reported to have a variety of activities, including antifungal effects (Hu et al. 2016), anti-HIV action (Pu et al. 2008; Liang et al. 2013), anti-proliferation (Hu et al. 2016), acetylcholinesterase inhibitory activities (Woo et al. 2020) and antitumor (Liu et al. 2014, 2018). However, to the best of our knowledge, there are few studies on the toxicity of the roots and stems of K. coccinea.

Zebrafish (Danio rerio) is an important vertebrate model for toxicity and efficacy studies (Ye et al. 2017; Forsatkar et al. 2018). It is reported that about 70% of the homology of the human disease genome can be found in the zebrafish genome (Postlethwait et al. 1998; Langheinrich et al. 2002). Moreover, there are many advantages in working with zebrafish, including rapid reproduction, quick development, easy observation, embryonic transparency and convenient high-throughput screening (Mushtaq et al. 2013; Kanungo et al. 2014; Peng et al. 2019). Zebrafish has emerged as a highly promising model for research on the toxicology of food and drugs (Tian et al. 2019; Tran et al. 2019).

In the present study, hydroethanol extract (KCH) and water extract (KCW) of Kadsura coccinea root and stem were used to evaluate the developmental toxicity in zebrafish embryos and larvae. The expression of hepatotoxicity maker genes, oxidative stress and apoptosis levels in zebrafish larvae was measured after treatment to reveal the underlying toxicity mechanisms. This...
study can help us better understand the developmental toxicity and the underlying toxicity mechanisms of *K. coccinea*.

**Materials and methods**

**Preparation of Kadsura coccinea extract**

*Kadsura coccinea* was purchased from Guangxi Wuzhou Pharmaceutical (Group) Co. Ltd. (Wuzhou, China). It was identified by Professor Songji Wei of Guangxi University of Chinese Medicine according to the Quality Standards for Yao Medicinal Materials in Guangxi (Guangxi Food and Drug Administration 2014). A voucher specimen of *K. coccinea* (ZHUANG No. 201908, Jiagang Deng) was deposited in the Guangxi Key Laboratory of Pharmaceutodynamics Research of Chinese Medicine, Guangxi University of Chinese Medicine (Nanning, China). Dried roots and stems of *K. coccinea* were ground, then extracted by continuous reflux extraction using ethanol 95% (v/v) or water in proportion 1:10 (v/v) for 1 h. This extraction process was repeated twice. The filtered extract was dried to give ethanol extract (KCH) and water extract (KCW). Next, KCH stock solutions of 50 mg/mL were prepared with ultrapure water containing 0.5% dimethyl sulphoxide (DMSO). The stock solution was diluted to give a serial solution with culture water at different concentrations. Similarly, 50 mg/mL KCW stock solutions were prepared with ultrapure water. The stock solution was diluted to give a serial solution with culture water at different concentrations.

**The main compounds of KCH and KCW**

Chromatographic analysis of KCH and KCW was performed using an Agilent ultra-high-performance liquid chromatography system LC-1290 (Agilent, Santa Clara, CA). The gradient system, consisting of acetonitrile (solvent A) and water (solvent B), was as follows: 0–1 min, 10% B; 1–15 min, 10–100.0% B; 15–8 min, 100.0–10% B. The volume of sample was 3 μL for each injection. The column temperature and flow rate were 45°C and 0.3 mL/min, respectively. The main compounds of KCH and KCW were detected by a Q-TOF mass spectrometer (Agilent, Santa Clara, CA), with an electrospray ionization interface in positive ionization mode. The full scan range was from 100 to 1000 m/z.

**Zebrafish maintenance**

The AB strain of adult zebrafish and Tg (fabp10a: dsRed; ela3l: EGFP) transgenic line were purchased from the China Zebrafish Resource Center (Wuhan, China). Zebrafish were maintained with standardized conditions at 28°C in a 14 h light/10 h dark cycle. In order to obtain eggs, male and female adult zebrafish (1:1) were placed into a mating tank. The next morning, the normal fertilized eggs were collected and maintained in an incubator at 28°C according to standard methods (Kimmel et al. 1995). All of the experiments followed standard ethical guidelines under control of the Guangxi University of Chinese Medicine (approval number DW20190525-69).

**KCH and KCW toxicity in Zebrafish embryos**

According to a previously published procedure (Xia et al. 2018), normal developing embryos of 6 h post-fertilization (hpf) and normal developing larvae of 72 hpf were, respectively, selected by a stereomicroscope (Leica, Wetzlar, Germany). The embryos were randomly transferred into 24-well plates with 10 embryos in each well and the same procedure was applied for larvae. Then, a total volume of 2 mL of serial concentrations of KCH and KCW was added into each well. The exposure solutions were replaced, and dead embryos or larvae were recorded and removed every 24 h. The lethal curves of KCH and KCW in embryos or larvae were drawn to measure the mean lethal concentration (LC50).

**KCH on morphological analyses of the embryos**

KCH was selected for further study because LC25 value of KCH in embryos was much lower, than that of KCW. Normal developing embryos at 6 hpf were exposed to KCH at concentrations of 0.5% DMSO, LC10 (15 μg/mL), 1/2 LC10 (7.5 μg/mL) and 1/4 LC10 (3.75 μg/mL) as previously described (Xia et al. 2018). These embryos were photographed to observe morphological changes at 24, 48 and 72 hpf. The spontaneous movement of embryos was measured at 24 hpf. Hatching rates of KCH were calculated at 48 and 72 hpf. Ten larvae selected randomly from each group were photographed to assess the body length and eye area at 72 hpf.

**Assessment of KCH on the hepatotoxicity**

KCH was selected for study of hepatotoxicity because KCH (the LC50 value of 45 μg/mL) in larvae was significantly more toxic than KCW (the LC50 value of 2011 μg/mL). Zebrafish of the Tg (fabp10a: dsRed; ela3l: EGFP) transgenic line exhibited the DsRed RFP in the liver. The normal developing larvae of this zebrafish were selected with a stereomicroscope at 72 hpf and exposed to KCH at concentrations of 0.5% DMSO, LC10 (30 μg/mL), 1/2 LC10 (15 μg/mL) and 1/4 LC10 (7.5 μg/mL) as previously procedure (Jia et al. 2019). The exposure solutions were replaced every 24 h. At 72 h post exposure (hpe), lateral photographs of 10 larvae selected randomly from each group were obtained with a fluorescence stereomicroscope. The area and fluorescence intensity of liver in zebrafish larvae were determined with ImageJ software.

Moreover, liver function of larvae treated with KCH was investigated at 72 hpe by assessing the hepatic alanine transaminase (ALT) and aspartate transaminase (AST) activity with commercial kits. The zebrafish larvae treated with KCH were tested for ALT or AST activities according to the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**Measurement of reactive oxygen species generation**

Generation of reactive oxygen species (ROS) in larvae was measured by using a ROS assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as previously described (Jia et al. 2019). After the KCH treatment, 10 larvae selected randomly from each group were incubated with 10 μM DCFH-DA for 1 h in the dark and rinsed three times with culture water. These larvae were anaesthetized with 0.1% tricaine, and lateral images were acquired via a fluorescence microscope. The fluorescence intensity was quantified via ImageJ software (Bethesda, MD).
Detection of antioxidative enzyme and lipid peroxidation activities

Total superoxide dismutase (T-SOD) activity and malondialdehyde (MDA) level were measured in larvae as previously described (Jia et al. 2019). The quality of RNA was evaluated by using TRIzol reagent (Takara, Dalian, China) as previously described (Kim et al. 2019). Ten larvae selected randomly from each group were incubated with 5 μg/mL acridine orange staining solution in the dark for 30 min at 28°C. The larvae were subsequently washed with PBS for three times. The larvae were anaesthetized with 0.10% tricaine, and lateral images were acquired via a fluorescence microscope. The fluorescence intensity of larvae treated with KCH were markedly inhibited, compared to the 0.5% DMSO group (Figure 2(d)). Compared with the 0.5% DMSO group, the hatching rates at 72 hpf in the 7.5 and 15 μg/mL KCH treatment group were significantly inhibited, compared to the 0.5% DMSO group at 72 hpf (Figure 2(f)). The eye area of larvae treated with KCH or KCW were acquired by using a UPLC-Q-TOF. Totally, 7 and 10 main compounds were identified in KCH and KCW according to the retention time, major fragment ions, molecular ions, online database (TCMSP: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform) and previously published papers (Ru et al. 2014; Woo et al. 2020), respectively. Four identical compounds in KCH and KCW were found. The details of compounds in KCH and KCW are listed in Tables 2 and 3.

Effects of KCH and KCW toxicity in embryos

As shown in Figure 2(a,b), the mortality rates of embryos treated with KCH or KCW were measured. At 72 hpf, the LC_{50} and LC_{10} values of KCH in embryos were 24 and 15 μg/mL. The LC_{50} and LC_{10} values of KCW in embryos at 72 hpf were 1447 and 810 μg/mL.

Effects of KCH on morphological analyses of embryos and larvae

As shown in Figure 2(c), the morphological changes of KCH were observed on embryos and larvae at 24, 48 and 72 hpf. The spontaneous movements of embryos at 24 hpf treated with 3.75 and 7.5 μg/mL KCH were markedly inhibited, compared to the 0.5% DMSO group (Figure 2(d)). Compared with the 0.5% DMSO group, the hatching rates at 72 hpf in the 7.5 and 15 μg/mL KCH treatment group were significantly inhibited (Figure 2(e)). The body length of larvae in the 15 μg/mL KCH treatment group was significantly inhibited, compared to the 0.5% DMSO group at 72 hpf (Figure 2(f)). The eye area of larvae treated with KCH was inhibited at 72 hpf (Figure 2(g)).

Effects of KCH and KCW toxicity in larvae

The legality rates of KCH or KCW in zebrafish larvae were observed (Figure 3(a,b)). At 72 hpe, the values of LC_{50} and LC_{10} of KCH in larvae were 45 and 30 μg/mL, respectively. The values of LC_{50} and LC_{10} of KCW in embryos at 72 hpe were 2011 and 1765 μg/mL, respectively.

Effects of KCH on hepatotoxicity

Morphological changes of larvae liver were observed in KCH treatment groups at 72 hpe (Figure 4(a)). The liver areas and fluorescence intensity of larvae treated with KCH were markedly decreased, compared to the 0.5% DMSO group (Figure 4(b,c)). Compared with the 0.5% DMSO group, increased ALT and AST levels in larvae in the 15 and 30 μg/mL KCH treatment groups were observed at 72 hpe (Figure 4(d,e)).

Table 1. The primer sequences of quantitative real-time PCR.

| Gene   | Primer Sequence                  | Reference   |
|--------|----------------------------------|-------------|
| fabp10a | Forward CACGAGCAGAAATCAGGCA     | Liu et al. (2018) |
| Reverse GTCATGACACAGGCTTC       |             |
| gclc   | Forward AAAGTCCGGAAGCTG           | Liu et al. (2018) |
| Reverse AACGTCCATTTGCTG         |             |
| gsr    | Forward CACCTGGAAGGGGCTACG       | Liu et al. (2018) |
| Reverse AAACTGATGCTGGACACATC    |             |
| nqo1   | Forward CTCAGGGATTGTCCCTG        | Liu et al. (2018) |
| Reverse CAGGACACCTACTGGTAA      |             |
| kep1   | Forward ACATGGAAGTCAGTCACC       | Xia et al. (2018) |
| Reverse GGCATATGCTTACGACTG      |             |
| nrf2   | Forward CACCCAACATGACATCACG      | Xia et al. (2018) |
| Reverse ATTCGCCACCTGATGTAAT     |             |
| Cu/Zn-Sod | Forward GGTGCAACAGGAAAAGTC   | Xia et al. (2018) |
| Reverse ATCCATCCACGACGCCAGA     |             |
| bax    | Forward GCATTTCACCGGTTCTC        | Zhang et al. (2019) |
| Reverse TGCGATACCAACATGCTG      |             |
| PS3    | Forward ACCATGGGGACCAAACGCTAG   | Zhang et al. (2019) |
| Reverse CAGAGTGTCTCTCTCTCTG     |             |
| casp9  | Forward CTGACGCGAAGCCATAATCG    | Zhang et al. (2019) |
| Reverse AGAGGACATGGGAAATGCTG    |             |
| casp3  | Forward CCCCTGCCCATCACTA         | Zhang et al. (2019) |
| Reverse ATCTCCTACGACATCT        |             |
| JI-actin | Forward CACACTTTAATGCGCTAAGCA | Zhao et al. (2018) |
| Reverse CATTGTGAGGGCGGAAAGT      |             |

Statistical analysis

Each group was performed in three biological replicates and three technological replicates, and the results are expressed as mean ± standard deviation (SD). Differences were compared by using one-way ANOVA, followed by Tukey’s multiple comparison tests to compare differences among multiple groups. p Values <0.05 were considered to be significant.

Results

The main chemical component analysis of KCH and KCW

As shown in Figure 1(a,b), total ion current chromatograms of KCH and KCW were acquired by using a UPLC-Q-TOF. Totally, 7 and 10 main compounds were identified in KCH and KCW according to retention time, major fragment ions, molecular ions, online database (TCMSP: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform) and previously published papers (Ru et al. 2014; Woo et al. 2020), respectively. Four identical compounds in KCH and KCW were found. The details of compounds in KCH and KCW are listed in Tables 2 and 3.

Real-time quantitative PCR assay

To further illustrate hepatic toxicity induced by KCH at the molecular level, the mRNA expression levels of some essential hepatic biomarker genes were detected and compared. To clarify the underlying mechanism of toxicity, the mRNA expression levels of oxidative stress and apoptotic genes were detected. The total RNA of 30 larvae treated with KCH at 72 hpe was extracted by using TRizol reagent (Takara, Dalian, China) as previously described (Jia et al. 2019). The quality of RNA was evaluated by using the OD260/OD280 ratio. The cDNA of these RNAs was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany), and quantitative real-time polymerase chain reaction (RT-PCR) was performed by using FastStart Essential DNA Green Master (Roche, Mannheim, Germany). The primer sequences of these genes are shown in Table 1.
Effects of ROS generation

As shown in Figure 5(a,b), compared with the 0.5% DMSO group, the ROS levels in the 15 and 30 l g/mL KCH groups were considerably higher, illustrating that ROS levels increased in zebrafish larvae treated with KCH.

Effects of T-SOD activities and MDA contents

As shown in Figure 5(e), at 72 hpe, the T-SOD activities of larvae in the 30 l g/mL KCH group were markedly decreased than that in the 0.5% DMSO group. Compared with the 0.5% DMSO group, the increased MDA contents of larvae treated with 30 l g/mL KCH were obviously observed at 72 hpe (Figure 5(f)).

Effects of apoptotic cells

As shown in Figure 5(c,d), compared with the 0.5% DMSO group, the fluorescence intensity of apoptotic cells in the 15 and 30 l g/mL KCH groups was significantly increased at 72 hpe.

Effects of the mRNA expression levels of hepatotoxicity markers gene

The mRNA expression levels of hepatotoxicity marker genes in larvae were examined by using real-time quantitative PCR to further study the liver toxicity. At 72 hpe, the mRNA expression levels of gclc (glutamate-cysteine ligase,
Figure 2. Effects of KCH and KCW on legality curves and morphological analyses of the embryos. (a) The legality curves of KCH on zebrafish embryos at 24, 48 and 72 hpf. (b) The legality curves of KCW on zebrafish embryos at 24, 48 and 72 hpf. (c) Effects of KCH on morphological analyses of the embryos at 24, 48 and 72 hpf. (d) The embryo spontaneous movement in the KCH group at 24 hpf. (e) The hatching rates of KCH-treated larvae at 72 hpf. (f) The body length of KCH-treated larvae at 72 hpf. (g) The eye area of KCH-treated larvae at 72 hpf. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Figure 3. Effects of KCH and KCW on larval toxicity at 24, 48 and 72 hpe. (a) Mortality concentration of KCH. (b) Mortality concentration of KCW.

Figure 4. Effects of KCH on the hepatotoxicity in larvae at 72 hpe. (a) Liver morphology of KCH-treated larvae. (b) Liver areas of KCH-treated larvae. (c) Liver fluorescence intensity of KCH-treated larvae. (d) AST levels of KCH-treated larvae. (e) ALT levels of KCH-treated larvae. *p < 0.05, **p < 0.01 and ***p < 0.001.
Figure 5. Effects of ROS generation, apoptotic cells, T-SOD activities and MDA contents in KCH treatment groups at 72 hpe. (a) ROS fluorescence staining in larvae treated with KCH. (b) ROS fluorescence levels in larvae treated with KCH. (c) Apoptosis of the larvae treated with KCH. Red arrow represents apoptotic cells. (d) The fluorescence intensity of apoptosis in the larvae treated with KCH. (e) The T-SOD activities in larvae treated with KCH. (f) The MDA levels in larvae treated with KCH. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Figure 6. The mRNA expression levels of genes in larvae treated with KCH at 72 hpe. The mRNA expression levels of hepatotoxicity marker genes gclc (a), nqo1 (b), gsr (c) and fabp10a (d). The mRNA expression levels of oxidative stress-related genes keap1 (e), nrf2 (f) and Cu/Zn-Sod (g). The mRNA expression levels of apoptosis-related genes Bax (h), P53 (i), Casp9 (j) and Casp3 (k) (d). *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.
catalytic subunit) and \textit{nqo1} [NAD(P)H dehydrogenase, quinone 1] decreased in the 30 \mu g/mL KCH treatment group (Figure 6(a,b)]. Moreover, the mRNA expression levels of \textit{gsc} (glutathione reductase) and \textit{fabp10a} (fatty acid-binding protein 10a, liver basic) decreased in the 15 and 30 \mu g/mL KCH groups (Figure 6(c,d)).

**Effects of the mRNA expression levels of oxidative stress and apoptotic gene**

Since oxidative stress of zebrafish embryos exposure with KCH was significantly elevated at a cellular level, the transcriptions of oxidative stress-related genes in larvae were examined. The transcriptions of \textit{keap1} (Kelch-like ECH-associated protein 1), \textit{nrf2} (nuclear factor erythroid 2-related factor 2) and \textit{Cu/Zn-Sod} (copper, zinc superoxide dismutase) were significantly up-regulated in the 15 and 30 \mu g/mL KCH groups at 72 hpe. The mRNA expression levels of apoptosis-related genes in larvae were detected. Compared with those in the 0.5\% DMSO group, \textit{Bax} (BCL2 associated X), \textit{P53} (tumour protein p53), \textit{Casp9} (caspase-9) and \textit{Casp3} (caspase-3) transcription levels were obviously up-regulated in the 15 and 30 \mu g/mL KCH group at 72 hpe.

**Discussion**

In the present study, we found that the LC50 value of KCH (24 or 45 \mu g/mL) was lower than that of KCW (1447 or 2011 \mu g/mL) in embryos or larvae, so KCH was chosen for the careful examination of the decided tests in zebrafish larvae. Hatching of zebrafish embryos is as a key point of developmental toxicology, and failure or delay of hatching because of various reasons such as inhibition of chorionase, hatching enzyme or the weakening of spontaneous muscle movement (Zhao et al. 2018). Our results obviously showed that the hatching rate and spontaneous movement of larvae exposed with KCH were inhibited. Concomitantly, the significant shortened body length was also observed. In the organ developmental and functional study of zebrafish, measurement of liver fluorescence intensity, liver size and ALT and AST levels are important endpoints for assessing liver toxicity (He et al. 2013). ALT and AST play a role in gluconeogenesis to catalyse the transfer of amino groups from aspartic acid or alanine to ketoglutaric acid to produce oxaloacetic acid and pyruvic acid. AST and ALT enzyme mainly are found in liver and has been used in clinical studies as to indicate hepatocellular injury (Kwo et al. 2017). In our study, the decreased liver area and fluorescence intensity, as well as the increased ALT and AST levels in larvae treated with KCH were remarkably observed. This demonstrated hepatocyte damage and hepatic dysfunction in zebrafish larvae after KCH treatment.

Furthermore, the expression levels of marker genes in liver development were measured after KCH treatment and the results were consistent with morphology observation. The genes \textit{gclc} (Liu et al. 2016; Jia et al. 2019), \textit{nqo1} (Qu et al. 2014; Liu et al. 2018), \textit{gsc} and \textit{fabp10a} (Liu et al. 2013; Verstraeten et al. 2016) are often used as marker genes of liver developmental toxicity. In this study, the results of qRT-PCR revealed that KCH remarkably decreased the expression levels of \textit{gclc}, \textit{nqo1}, \textit{gsc} and \textit{fabp10a}, indicating that KCH could induce the developmental hepatotoxicity.

Oxidative stress is often used as a toxic mechanism of drugs or food. The excessive increase in ROS generation can induce oxidative stress and lead to physiological lesions and various biochemicals in the body (Li et al. 2010; Huang et al. 2011; Landete 2013). SOD is a key antioxidant enzyme that prevents the body from oxidative damage (Dobashi et al. 2000; Zhang et al. 2009). MDA is the main oxidation product and reflects oxidative damage in the body (Draper and Hadley 1990; Yang et al. 2018). The increased generation of ROS and MDA levels, and reduced T-SOD activity, were found. Meanwhile, the observed increased of \textit{Keap1}, \textit{Nrf2} or \textit{Cu/Zn-Sod} transcriptions indicated that the antioxidant defence system was activated to eliminate ROS and reduce oxidative stress induced by KCH. This illustrates that oxidative stress occurred in the larvae after KCH exposure. The generation of ROS can result in apoptosis (Zou et al. 2016). The number of apoptotic cells of the larva in the KCH treatment group was significantly increased. Additionally, this study shows that the apoptosis factors, \textit{Bax}, \textit{P53}, \textit{caspase-3} and \textit{caspase-9}, were obviously increased in the 15 and 30 \mu g/mL KCH groups, compared with the 0.5\% DMSO group. These results show that oxidative stress and apoptosis induced the developmental toxicity of KCH on zebrafish larvae.

**Conclusions**

This study offered a better understanding of the toxicity of roots and stems of \textit{Kadsura coccinea} on zebrafish embryo and larvae \textit{in vivo}. The embryo was treated with KCH and KCW from 6 hpf to 72 hpf, and the LC50 value of KCH (24 \mu g/mL) in embryos was obviously lower, relative to the value of KCW (1447 \mu g/mL). The inhibition of spontaneous movement of embryos and hatching were observed in zebrafish after KCH treatment, as well as short body length and small eye size. In addition, the LC50 value of KCH (45 \mu g/mL) in larvae was significantly lower than the value of KCW (11 \mu g/mL), and KCH exhibited developmental toxicity on the liver of zebrafish larvae. The genes related to classical hepatotoxicity, oxidative stress and apoptosis in zebrafish larvae were activated after KCH treatment. These results illustrated that KCW extract was safer than the KCH extract in zebrafish embryos and larvae. The KCH extract was the main toxic extract of roots and stems of \textit{Kadsura coccinea}. Moreover, oxidative stress and apoptosis were the developmental toxic mechanisms of the KCH extract. These results might provide theoretical basis of developmental toxicity for the roots and stems of \textit{Kadsura coccinea} in clinical and dietary applications.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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