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Developmental toxicity of *Clerodendrum cyrtophyllum* turcz ethanol extract in zebrafish embryo

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ABSTRACT

Ethnopharmacological relevance: Clerodendrum cyrtophyllum Turcz has been used in traditional medicine for the treatment of various diseases. In spite of its therapeutic applications, research on its toxicity and teratogenicity is still limited.

Aim of the study: The study aimed to investigate the developmental toxicity of the ethanol extract of *C. cyrtophyllum* (EE) in zebrafish embryo model.

Material and methods: Major compounds from crude ethanol extract of *Clerodendron cyrtophyllum* Turcz leaves were determined using HPLC-DAD-Orbitrap-MS analysis**.** The developmental toxicity of EE were investigated using zebrafish embryo model. Zebrafish embryos at 6 h post-fertilization (hpf) were treated with EE at different concentrations. Egg coagulation, mortality, hatching, yolk sac edema, pericardial edema and teratogenicity were recorded each day for during a 5-day exposure. At time point 120 hpf, body length, pericardial area, heartbeat and yolk sac area were assessed. In order to elucidate molecular mechanisms for the developmental toxicity of EE, we further evaluated the effects of the EE on the expression of genes involved on signaling pathways affecting fish embryo's development such as heart development (*gata5, myl7, myh6, has2, hand2*, *nkx 2.5*), oxidative stress (*cat, sod1, gpx4, gstp2*), wnt pathway (β-*catenin, wnt3a, wnt5*, *wnt8a*, *wnt11*), or cell apoptosis (*p53, bax, bcl2, casp3, casp8, casp9, apaf-1, gadd45*bb*)* using qRT-PCR analysis.

Results: Our results demonstrated that three major components including acteoside, cirsilineol and cirsilineol-4'-*O-β*-D-glucopyranoside were identified from EE. EE exposure during 6–96 h post-fertilization (hpf) at doses ranging from 80 to 200 μg/mL increased embryo mortality and reduced hatching rate. EE exposure at 20 and 40 μg/mL until 72–120 hpf induced a series of malformations, including yolk sac edema, pericardial edema, spine deformation, shorter body length. Based on two prediction models using a teratogenic index (TI), a 25% lethality concentration (LD25) and the no observed-adverse-effect level (NOAEL), EE is considered as teratogenic for zebrafish embryos with TI (LC50/EC50) and LD25/NOAEC values at 96 hpf reaching 3.87 and 15.73 respectively. The mRNA expression levels of *p53*, *casp8*, *bax/bcl2*, *gstp2*, *nkx2.5*, *wnt3a*, *wnt11*, *gadd45*bb and *gata5* were significantly upregulated by EE exposure at 20 and 40 μg/mL while the expression of *wnt5*, *hand2* and *bcl2* were downregulated.

Abbreviations: EE, the ethanol extract from *Clerodendrum cyrtophyllum* Turcz leaves; HPTLC, High performance thin layer chromatography; QE, quercetin; GAE, gallic acid equivalent; qRT-PCR, quantitative polymerase chain reaction; dpf, day post fertilization; TI, Teratogenic Index; NOAEL, the no observed-adverse-effect level.

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Conclusions: These results provide evidence for toxicity effects of EE to embryo stages and provide an insight into the potential toxicity mechanisms on embryonic development.

1. Introduction

Congenital abnormalities represent a severe problem, 5–10% of the congenital abnormalities of human newborns are known to be related to environmental factors including therapeutic agents and developmental toxicants (Hong and Jeung, 2013). During pregnancy, the mother can be exposed to a variety of chemicals and medicine. These compounds subsequently can be transported through the placenta and exert adverse effects on reproductive tissues or the embryo. As a result, birth defects and biological dysfunction may be induced (Peters et al., 2008). Toxicological screening is very important for the development of new drugs and, according to international regulatory guidelines, each drug in development for administration to women of childbearing potential must be tested for developmental toxicity (Nishimura et al., 2016).

Clerodendrum cyrtophyllum Turcz (Lamiaceae), a medicinal plant, is widely used in traditional medicine of many Asian countries such as China, India, Japan, Korea, Thailand, and Vietnam for the treatment of colds, high fever, migraines, hypertension, enteritis, dyspepsia, inflammation of the throat, rheumatic arthritis, fever, jaundice, leukorrhea, syphilis, and typhoid (Kar et al., 2014; Zhou et al., 2013). Several therapeutic and pharmacological properties of *Clerodendrum cyrtophyllum* Turcz have recently been reported such as antioxidant, anti-inflammatory (Nguyen et al., 2020a, 2020b), and anticancer (Cheng et al., 2001) activities. Despite their frequent use, studies on its toxicity and its effects on reproductive tissues or embryos are currently unknown.

The zebrafish model is widely used in screening for teratogenity in preclinical developmental toxicity assessment. Compared with traditional animal models (rabbit, rat, mice), the zebrafish has many advantages such as transparency (easy for observation of malformations), small size, requirement of small amounts of test compound, low-cost, easy maintenance, similarity of major organ systems of zebrafish compared to human (Li et al., 2018; Gao et al., 2017). Due to the above advantages, zebrafish is the ideal model for testing drug's preclinical developmental toxicity today.

In this study, we investigated the developmental toxicity induced by the ethanol extract of *Clerodendrum cyrtophyllum* Turcz (EE) in the zebrafish model by focusing on the effects of EE on teratogenicity indicators and on the expression of genes involved in different signaling pathways affecting embryo development such as oxidative stress, apoptosis and the wnt pathway.

2. Materials and methods

Plant collection*:* Leaves of *Clerodendrum cyrtophyllum* Turcz were collected in June 2018 from Vietnam. A voucher specimen (HNU 024106) was kept at the Botanical Museum of Hanoi, University of Science.

Preparation of total extract: The ethanol extract from leaves of *Clerodendrum cyrtophyllum* Turcz was prepared as described in our previous study (Nguyen et al., 2020b). After the extraction, the EE was analysed with various analytical techniques including colorometric methods, **HPLC-DAD- Orbitrap-MS analysis**. The concentration of total phenolic compounds and flavonoid in the extract were 23.3 ± 1.5 GAE mg/g and 2.97 ± 0.01 QE mg/g expressed in dry weight of leaves material (Nguyen et al., 2020b).

2.1. Analysis of samples by HPLC-DAD- Orbitrap-MS analysis

Analyses were performed on an Accela HPLC system (Thermo Fisher Scientific) consisting of a PDA detector connected with a Thermo Fisher Scientific LTQ Orbitrap XL mass spectrometer from the UCLouvain Massmet platform. A Phenomenex® Lichrospher C18, 4.6 × 250 mm column packed with 5 µm particles was applied. Ten µL of samples at 1 mg/mL concentration were injected in the full loop injection mode. The column was eluted at a constant flow rate of 0.8 mL/min using a binary solvent system: solvent A, MilliQ water 0.1% formic acid and solvent B, acetonitrile HPLC grade (0–10 min, 83% A and 17% B; 30 min, 50% A and 50% B; 38 min, 15% A and 85% B; 38 min, 15% A and 85% B). DAD-UV detector was set at 254 nm. HR-MS were measured with APCI source in the negative mode using full-scan MS with a mass range of 100–2000 *m/z*. The following (−) APCI conditions were applied: vaporizer temperature, 400°C; sheath gas (N_2) flow rate, 25 a. u.; auxiliary gas (N_2) flow rate, 25 a. u.; sweep gas (N_2) flow rate, 5 a. u.; capillary temperature, 250°C; capillary voltage, 10 V; tube lens, 125 V.

2.2. Fish and experimental conditions

Adult wild – type AB zebrafish (*Danio rerio*) were maintained at at 28 °C in a recirculating ZebTec housing system. Conductivity was kept at approximately 500 µS/cm, pH at 7.2 with a 12:12 h (light/dark) photoperiod. Fish were fed to apparent satiation twice daily with ZM-400 fry food for zebrafish. The evening before reproduction, males and females were placed together in spawning tanks in a ratio of 2:2. The next morning, eggs were collected within 30 min of spawning. Fertilized eggs were selected and placed in embryo medium at 26 ± 0.5 °C. The use of zebrafish was in accordance with the animal welfare act. Since zebrafish larvae below 120 hpf old are not considered animals (Lackmann et al., 2018), no animal test authorization was requested in accordance with European legislation (EU Directive, 2010/63/EU). All experiments were terminated at 120 hpf.

2.2.1. Preparation of test samples

The ethanol extract of *Clerodendrum cyrtophyllum* Turcz (EE) was dissolved in DMSO for stock solution at 50 mg/mL and then diluted to different concentrations using fresh medium so that the final concentration in each experimental well was 1, 5, 10, 20, 40, 80, 100, 200 µg/mL.

2.2.2. Fish embryo acute toxicity (FET) test on zebrafish

The evaluation of mortality and malformation in zebrafish after exposure to EE was performed according to OECD guideline 236 (OECD, 2013) and literature methods (Huang et al., 2018; Alafiatayo et al., 2019). At 6-h post fertilization (6hpf), fertilized healthy embryos were selected, washed and examined under the microscope. Fertilized embryos were incubated with EE at difference doses (1, 5, 10, 20, 40, 80, 100, 200 µg/mL) in 6-well culture plates. Twenty fertilized embryos were used per treatment per well, and each treatment was repeated three times. The control group was exposed to medium containing only 0.04% DMSO. The plates were then placed in an illuminated incubator at 26 ± 0.5 °C. The exposure solutions were replaced at 24, 48, 72 and 96 hpf, to maintain water quality and the concentrations of EE. We checked for abnormal development and removed dead embryos/larvae when we replaced exposure solutions. Egg coagulation, mortality, hatching, yolk sac edema, pericardial edema, teratogenicity were recorded each day for five days of exposure with the aid of a microscopic image acquisition system (Nikon SMZ 1270). The number of spontaneous movements during 1 min was counted at 24 hpf. Coagulated embryos are milky white and appear dark under the microscope. Sign of death is the absence of heartbeat under the microscope. When larva's

Table 1
Primers pair used in this study.

Gene name	Full name	GenBank Accession No.	Forward and reverse primer sequences (5' -3')
β-actin	*Danio rerio* beta-actin	AF057040	*Fwd:* CCCCATTGAGCACGGTATTG *Rev*: ATACATGGCAGGGGTGTTGA
eef1a	*Danio rerio* elongation factor 1 alpha	L23807.1	*Fwd:* CCAAGGAAGTCAGCGCATAC *Rev*: CCTCCTTGCGCTCAATCTTC
wnt3a	*Danio rerio* Wnt3a	AY613787.1	*Fwd:* GGTACGCAGCCCATAATGTG *Rev:* GGCCAGCTTGTCGTTGATAG
wnt8a	*Danio rerio* wingless-type MMTV integration site family, member 8a	NM_130946.3	*Fwd:* TGTAGACGCGCTGGAAAATG *Rev*: ACTTCCGTGCTTGATCATGC
β-*catenin*	*Danio rerio* b-catenin	U41081.1	*Fwd: ACCTCTGGCACCCTACACAA* *Rev*: *AGGGGAGCCGAGCATATTGA*
wnt5	*Danio rerio* Wnt5	U51268.1	*Fwd: CCGGAAGAATGGCGGTGTAT* *Rev*: *GGCGCTGTCGTATTTCTCCT*
wnt11	*Danio rerio* Wnt11 protein	AF067429.1	*Fwd: TTCGCTACTACGGCCTACAGAT* *Rev*: *ACAGAGCATGAGCCAGAAACG*
sod1	*Danio rerio* superoxide dismutase 1, soluble	NM_131294.1	*Fwd*: ATGGTGAACAAGGCCGTTTG *Rev*: AAAGCATGGACGTGGAAACC
gpx4b	*Danio rerio* glutathione peroxidase 4b	BC095133.1	*Fwd:*TGAGAAGGGTTTACGCATCCTG *Rev*: TGTTGTTCCCCAGTGTTCCT
cat	*Danio rerio* catalase	NM_130912.2	*Fwd:* TCCGGACATGGTTTGGGAT *Rev*: CGATCCGCTTCTTCAACAGG
gstp2	*Danio rerio* glutathione S-transferase pi 2	NM_001020513.1	*Fwd:* GGACTGGATGAAGGGTGACA *Rev*: ACGCTTCTTTACCGGTCTCA
casp 3b	*Danio rerio* caspase 3, apoptosis-related cysteine peptidase b	NM_001048066.2	*Fwd:* TCACAGTAAGTCGGCCATGTTT *Rev*: TCACCTACACCGTCACACTC
casp 9	*Danio rerio* caspase 9, apoptosis-related cysteine peptidase	NM_001007404.2	*Fwd:* TCAGCGGCACAGGTTAAACCTC *Rev*: AGTCTCACGCAGGGAATCAA
apaf 1	*Danio rerio* apoptotic protease activating factor 1	BC116581.1	*Fwd:* AGTTCTTCTGACCACACGCAAT *Rev*: CCTGTTCTGGGAGTTTGTGC
bcl-2	*Danio rerio* Bcl2	AY695820.1	*Fwd:* GGGCGGATCATTGCATTCTT *Rev:* TCTGCTGACCGTACATCTCC
p53	*Danio rerio* tumor suppressor p53	U60804.1	*Fwd:* TACTTGCCGGGATCGTTTGAC *Rev:* TCAGGTCCGGTGAATAAGTGC
bax	*Danio rerio* Bax gene	AF231015.1	*Fwd:* CTGTGTGACCCCAGCCATAAA *Rev*: GATGACAAGGCGACAGGCAA
casp 8	*Danio rerio* caspase 8, apoptosis-related cysteine peptidase	NM_131510.2	*Fwd:* CCTTTTGCCGGATGCAGAAC *Rev*: TCCATCCGACGTCCAAACAC
*gadd45*bb	*Danio rerio* growth arrest and DNA-damage-inducible, beta b	NM_001012386.2	Fwd: CGCTTCAGATCCACTTCACG Rev: TCCCACTTCCTTCAGCTTGA
gata5	*Danio rerio* GATA binding protein 5	NM_131235.2	Fwd: CCACAGACTGGCACCGATAA Rev: GCGTACGGGCTGGAATAAGA
myl7	*Danio rerio* myosin, light chain 7, regulatory	NM_131329.3	Fwd: TGCACAACTAGGGAAGCTGAA Rev: GCAGCAAGGATGGTTTCCTC
myh6	*Danio rerio* myosin, heavy chain 6, cardiac muscle, alpha	NM_198823.1	Fwd: AAGCCACTACCGCCTCTCTA Rev: TGAGGCAAGGTCGTCCAA
has2	*Danio rerio* hyaluronan synthase 2	NM_153650.2	Fwd: CCTGAGCAGCGTGAGGTATT Rev: TGCAGTGGCTTCCCATGAAT
hand2	*Danio rerio* heart and neural crest derivatives expressed 2	NM_131626.3	Fwd: CGCGGATACGAAGCTATCCA Rev: GGCCAACCAGTTCTCCCTTT
nkx2.5	*Danio rerio* homeodomain protein Nkx2.5	U66572.1	Fwd: ACCCGGGTGAAGATCTGAAG Rev: TGGCTAGGTGGTCTCTCTCT

head or tail breaks out of the embryo membrane, hatching is successful. The presence of fluid above or below the yolk sac was considered as yolk sac edema. Pericardial edema was characterised by the inflation of the pericardial cavity to twice its size due to the presence of fluid. Counting formulas of lethality, hatchability and teratogenicity at each time point are given below:

Mortality (%) = death number/total exposed number × 100

Hatching rate (%) = hatched number/total surviving embryos number × 100

Teratogenicity rate (%) = abnormal number/total surviving number × 100

Embryo was considered abnormal if it had spinal curvature, pericardium edema, tail hypoplasia, yolk sac edema, and growth retardation.

Dead embryos or larvae were cleaned at each stage of observation. Embryo medium was changed once a day. At time point 120 hpf, larvae were anesthetized with 0.003% MS-222 (tricaine methane-sulfonate) before observation. Body length, pericardial area, heartbeat, yolk sac area were assessed using a microscope with digital camera and the support tool for measurement (NIS elements imagines software 4.4).

2.2.3. Measurement of body length

For body length, a line starting at the anterior-most point of the head and ending at the tail end was measured. All pictures were taken at the same resolution and magnification with the live fish positioned in a lateral orientation.

2.2.4. Pericardial sac area, yolk sac area

To measure pericardial and yolk sac areas, lateral view images of each embryo were taken at the same magnification, outline of the pericardial sac and yolk sac, respectively, was traced, and the area within each tracing was determined by NIS elements imaging software.

2.2.5. The heart rate

The number of heartbeats for each larva was recorded by counting the beats per 20s under the stereomicroscope when the fish was stationary.

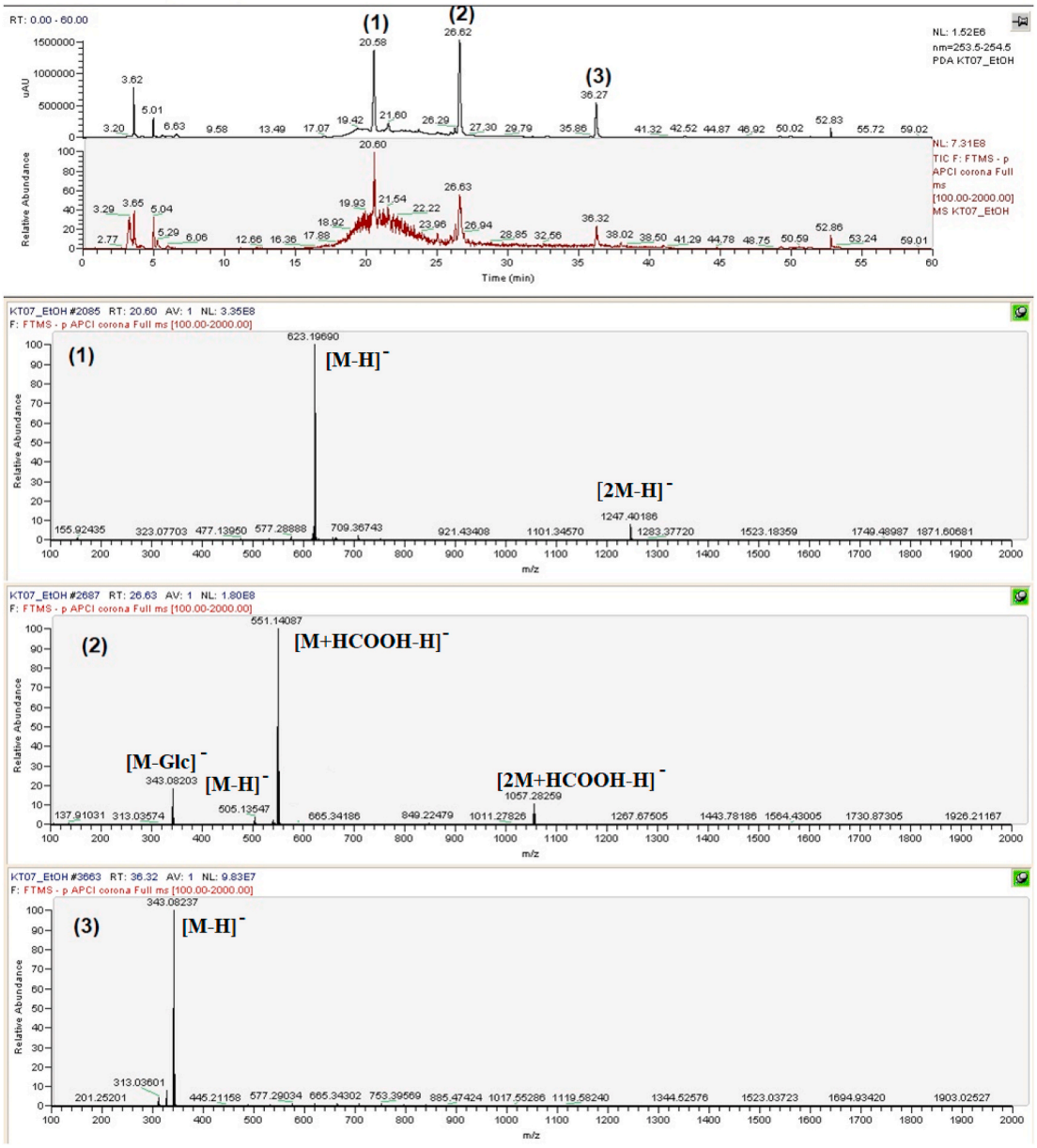


Fig. 1. HPLC-DAD (upper) and HPLC-Orbitrap-MS-TIC (lower) chromatograms of the crude ethanolic extract of *C. cyrtophyllum* in negative ion mode.

Table 2
Identification of major compounds from *C. cyrtophyllum* leaves using HPLC-DAD- Orbitrap-MS method.

N°	Retention time	Λmax (Metwally et al.)	Molecular ion *m/z* [M-H]⁻	Mw	Formula	Error (ppm)	Compound	Reference
1	20.58	243, 230	623.19690	624	$C_{29}H_{36}O_{15}$	−0.235	Acteoside (Verbascoside)	Zhou et al. (2020)
2	26.62	245,275,335	505.13547	506	$C_{24}H_{26}O_{12}$	+2.806	Cirsilineol-4'-*O*-*β*-D-glucopyranoside	(Cong Nhuong et al., 2006), (Zhou et al., 2020)
3	36.45	245,275,343	343.08237	344	$C_{18}H_{16}O_7$	+3.325	Cirsilineol	(Cong Nhuong et al., 2006), (Zhou et al., 2020)

2.2.6. Classification of teratogenicity

2.2.6.1. The ratio LC50/EC50 (teratogenic index- TI) evaluation. A dose-response analysis was performed as previously described by Selderslaghs et al. (2012). Using Graph Pad prism, version 5.0, concentration-response curves for malformed, and mortality for each time point were created. The data were fitted to a sigmoidal equation with variable slope. This dose-response curve was used to determine the $EC50_{48h}$, $EC50_{72h}$, $EC50_{96h}$ (teratogenic effect) and $LC50_{48h}$, $LC50_{72h}$, $LC50_{96h}$ (lethal/embryotoxic effects) values. These were derived from a four parameter equation describing the curve as follows:

Y=Bottom + (Top-Bottom)/(1 + 10^((LogEC50-X)*HillSlope

Where Y is response (percentage of death or malformed individual).
X is log of concentration of EE.
(Bottom ≈ 0, Top ≈ 100).

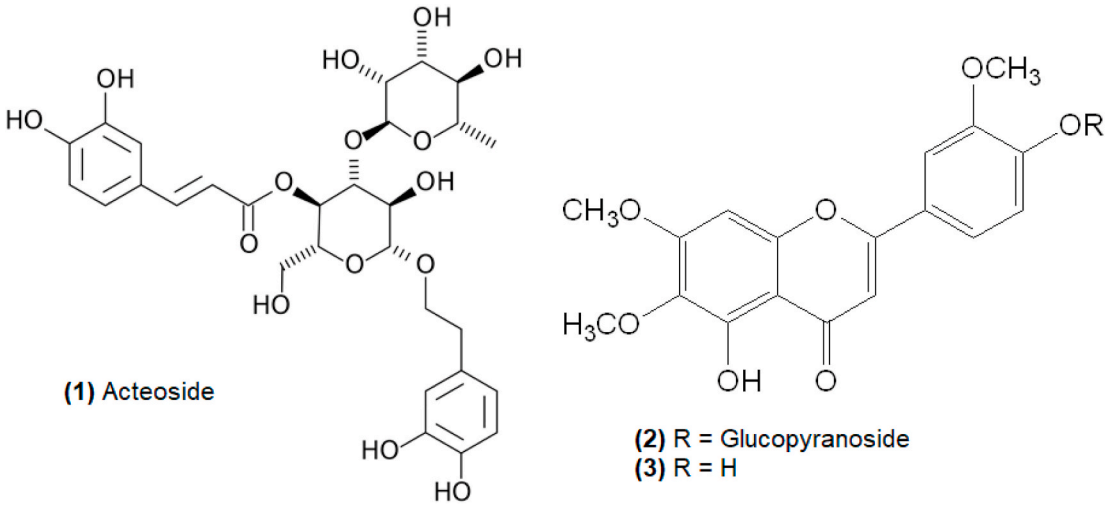


Fig. 2. Structures of major compounds identified from *Clerodendron cyrtophyllum* Turcz leaves.

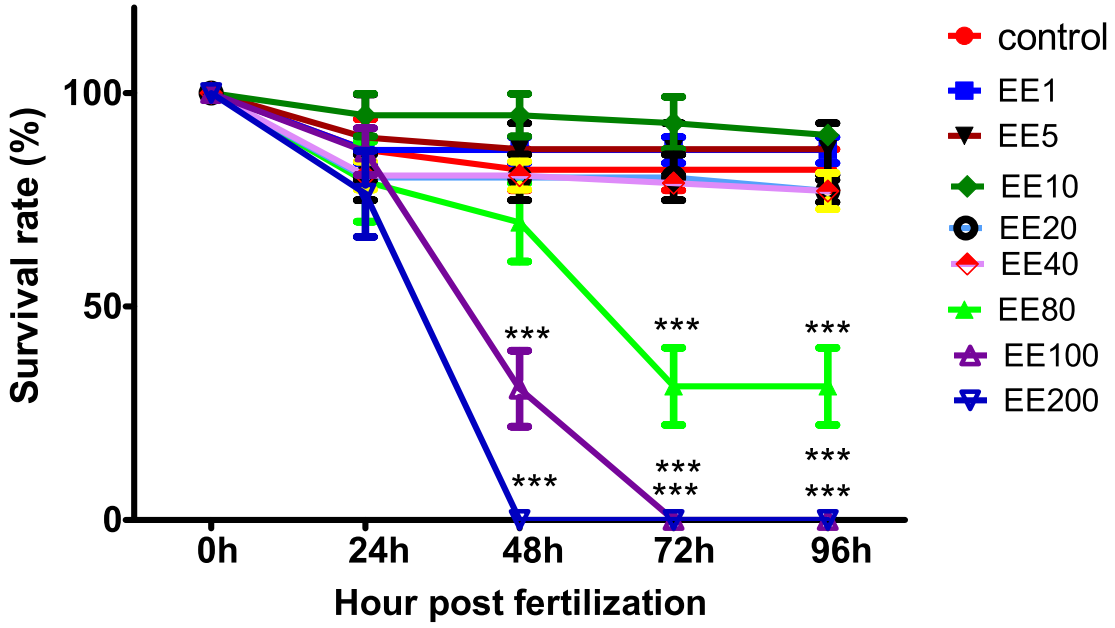


Fig. 3. Survival rate of zebrafish embryo and larvae after treatment with the ethanol extract of *C. cyrtophyllum* (EE) at different concentrations. *The data are presented as mean ± SD. for three different experiments performed in triplicate, ***p < 0.001 compared to the control group.*

Based on values of $LC50_{48h}$, $LC50_{72h}$, $LC50_{96h}$ and $EC50_{48h}$, $EC50_{72h}$, $EC50_{96h}$, TI was calculated as the ratio LC50/EC50 for each time point. TI values allow ranking the compounds according to their teratogenic potency. The higher the TI value the greater the tetratogenic potential of a compound. Cut-off value of TI was 2, TI values higher than 2 indicating that the compound is considered as teratogenic (Selderslaghs et al., 2012).

2.2.6.2. The ratio of the NOAEL to LC25 (teratogenic index). Based on the morphological assessments, the no observed-adverse-effect level (NOAEL) was identified as the highest dose at which there were no observed toxic or adverse effects on the development of zebrafish.

The LC25 of each compound was calculated by curve-fitting of incidence data for dead larvae. The curve-fitting model used was the 4-parameter logistic equation:

Y=Bottom + (Top-Bottom)/(1 + 10^((LogEC25-X)*HillSlope

The ratio of the NOAEL to LC25 (teratogenic index) was calculated as described by Brannen et al. (2010). The highest concordance and predictivity were obtained when ratios greater than or equal to 10 were used to classify a compound as a predicted teratogen, whereas ratios less than 10 resulted in a classification of a predicted non-teratogen.

2.2.7. Analysis of mRNA expression by quantitative polymerase chain reaction (qRT-PCR)

To investigate the possible mechanisms for the developmental toxicity of EE, real-time quantitative polymerase chain reaction (qRT-PCR) was used to evaluate the mRNA levels of marker genes in whole embryos at 2 doses, 20 and 40 μg/mL. These concentrations were found to produce a fully and uniform phenotype in zebrafish embryo. At 6hpf, 20 fertilized eggs were treated with EE at the doses 20 and 40 μg/mL for 48 h. After 48h, zebrafish embryos were collected, frozen and stored at −80 °C for qRT-PCR analysis.

Quantitative PCR

Total RNA extraction, DNase and reverse transcription

Total RNA extraction, DNase and reverse transcription were carried

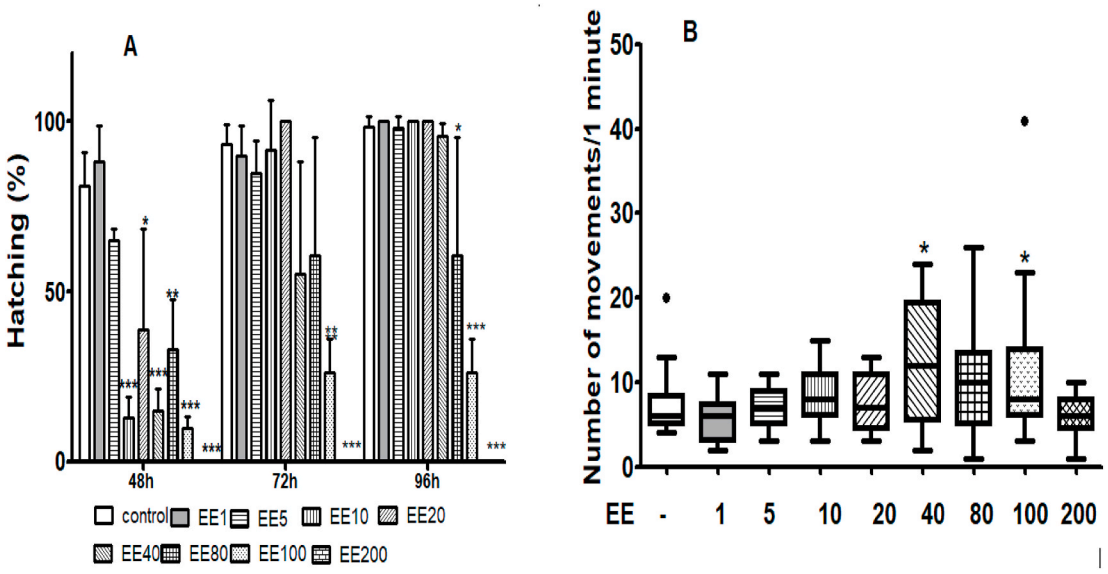


Fig. 4. A. Hatching rate of surviving embryos at 48, 72 and 96 hpf after exposure to EE. *The data are presented as mean ± SD. for three different experiments performed in triplicate. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the control group.* **B.** Number of movements by surviving embryos at 24 hpf after exposure to EE (*Each bar represents median (interquartile range) for 15 different larvae, n = 15, *p < 0.05; **p < 0.01;***p < 0.001 compared to control group).*

out as described in previous our study (Nguyen et al., 2020a) using Trizol Reagent solution (Ambion, Thermofisher Scientific), DNA-*free*™ DNA Removal Kit- Invitrogen and the RevertAid RT kit (Thermo Scientific).

The cDNA sample was diluted and used for real-time qRT-PCR to determine gene expression. β-actin and elongation factor 1α (*eef1a*) were used as housekeeping genes. The expression of genes related to oxidative stress (*cat, sod1, gpx4* and *gstp2*), wnt pathway (β-*catenin, wnt3a, wnt5, wnt8a* and *wnt11*), apoptosis (*p53, bax, bcl2, casp3, casp8* and *casp9, apaf-1, gadd45*bb), heart development (*gata5, myl7, myh6, has2, hand2* and *nkx 2.5*) were determined using specific primers. Primer sequences list are presented in Table 1.

RT-PCR was performed using an ABI Step One Plus Real Time PCR system (Applied Biosystems). The thermal conditions used were 3 min at 95 °C of preincubation, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. For analysis, a standard curve of a pool of the cDNAs of all samples was constructed to calculate the PCR efficiency and the quantity of an unknown sample. The relative gene expressions are presented as the ratio of the quantity of candidate gene/average quantity of housekeeping genes. It was used a pool of 20 larvae per group (n = 3).

2.3. Data presentation and statistical analyses

Data analyses were performed using SPSS 22.0 software (SPSS Inc, Chicago) or GraphPad Prism Software 5.0. Shapiro-wilk test was used for normality check. Data are shown as mean ± SD in the case of data with a normal distribution, a *t*-test and one-way analysis of variance with LSD post hoc test were used to determine differences between the experimental groups. In case the data were not normally distributed, the non-parametric Kruskal-Wallis test was performed, followed by a Mann-Whitney test to determine significant differences between the experimental groups. A p-value less than 0.05 was considered statistically significant. LD50, EC50, LD25 was calculated using GraphPad Prism Software 5.0 (San Diego, CA, USA).

3. Results

3.1. Chemical constituent identification from EE by HPLC-DAD-Orbitrap-MS analysis

Identification of major compounds from the ethanol extract from *C. cyrtophyllum* Turcz leaves was performed by HPLC-DAD-Orbitrap-MS analysis. Major constituents of the ethanol extract were identified based on characteristic properties of the peaks in the chromatograms such as the retention times, UV absorption spectra, experimental mass data, best matching molecular formula provided by the Xcalibur software of the OrbitrapMS fragments and comparison with the reported data of other samples of *C. cyrtophyllum* Turcz leaves.

The results show that three major compounds were present in the crude ethanol extract from *C. cyrtophyllum* (Fig. 1). These compounds could be identified, by comparison of MS molecular formulas as acteoside (Zhou et al., 2020), cirsilineol and cirsilineol-4'-*O*-*β*-D-glucopyranoside (Cong Nhuong et al., 2006; Zhou et al., 2020) which were also previously isolated from other samples of this plant and reported in the literature (Cong Nhuong et al., 2006; Zhou et al., 2020). The HR-MS data of these compounds are summarized in Table 2 and their structures are presented in Fig. 2.

3.2. Embryo mortality and hatchability

The percentage of surviving embryo decreased as the concentration of EE increased (Fig. 3). EE at concentrations below 40 μg/mL had no obvious effects on the survival of embryo and larvae. At 72 hpf, 31.3% of the embryos exposed to EE at 80 μg/mL survived. Meanwhile, no embryo survival was observed at concentrations of 100 and 200 μg/mL at 72 and 96 hpf.

The effects of EE on the embryo hatching are shown in Fig. 4A. Our results show that 81 and 93% of the control zebrafish embryos hatched after 48 hpf and 72 hpf, respectively. However, a significant decrease ($p < 0.05$) in the hatching rate was observed at all concentrations in the range from 10 to 100 μg/mL of EE as compared to control at 48 hpf. By

Table 3
Representative images of teratogenic effects of EE in zebrafish embryos and larvae. PE: pericardial edema, SBL: short body length, SC: Spinal curvature, TM: tail malformation.

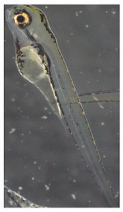
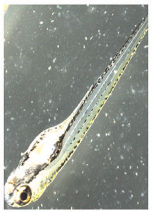
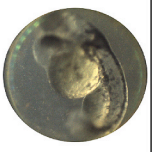
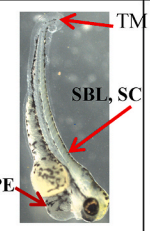
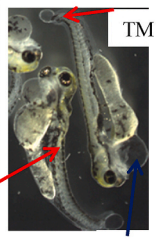
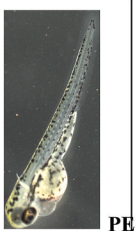
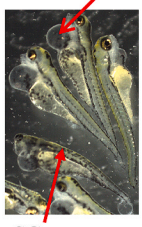
Group	24 hpf	48 hpf	72 hpf	96 hpf	120 hpf
control					
EE 200					
EE 100					
EE 80					
EE 40					
EE 20					

Table 4
LC50, EC50 and TI values for EE.

	LC50 (μg/mL)	EC50 (μg/mL)	TI (LC50/EC50)
48 hpf	93.68	60.31	1.55
72 hpf	79.59	29.23	2.72
96 hpf	79.61	20.57	3.87

Table 5
LC25, NOAEL and ratio LC25/NOAEL values for EE.

	LC25	NOAEL	LC25/NOAEL
48 hpf	81.82	20	4.1
72 hpf	78.65	5	15.73
96 hpf	78.68	5	15.73

96 hpf, at concentrations of EE 80 and 100 μg/mL, hatching rates were 61 and 26% respectively while no hatching was observed at 200 μg/mL. These data indicated a remarkable dose- and time-dependent decrease in the hatching rate in the EE-treated groups compared with those of the control.

The hatching of embryos depends, among other processes, on the embryo movements. To further investigate the mechanism of the low hatchability rate, we measured the effects of EE on the embryo movements at 24 hpf. As shown in Fig. 4B, at 24 hpf, the number of spontaneous movements in normal embryos was 6 movements/min. Exposure to EE had no effects on the spontaneous movement of zebrafish embryo at low concentrations 1, 5, 10 and 20 μg/mL. However, at high concentrations 40 and 100 μg/mL EE significantly increased the number of coiling contractions in the embryos compared with the control group whereas the number of movements was not affected by EE at 200 μg/mL.

3.3. Teratogenic effects of EE in zebrafish embryos and larvae

At 24 hpf, no hatchings of embryos were observed. At 48 hpf, hatching of embryos was observed but no observable effects were noticed in 1, 5, 10 and 20 μg/mL concentrations, while unhatched darkened embryos were observed in EE 100 and 200 μg/mL. At 72, 96 and 120 hpf dead hatched larvae were observed at 80 μg/mL and morphological deformities such as spinal curvature, short body length, yolk retention, pericardial edema, and tail malformation were seen at EE 20 and 40 μg/mL (Table 3).

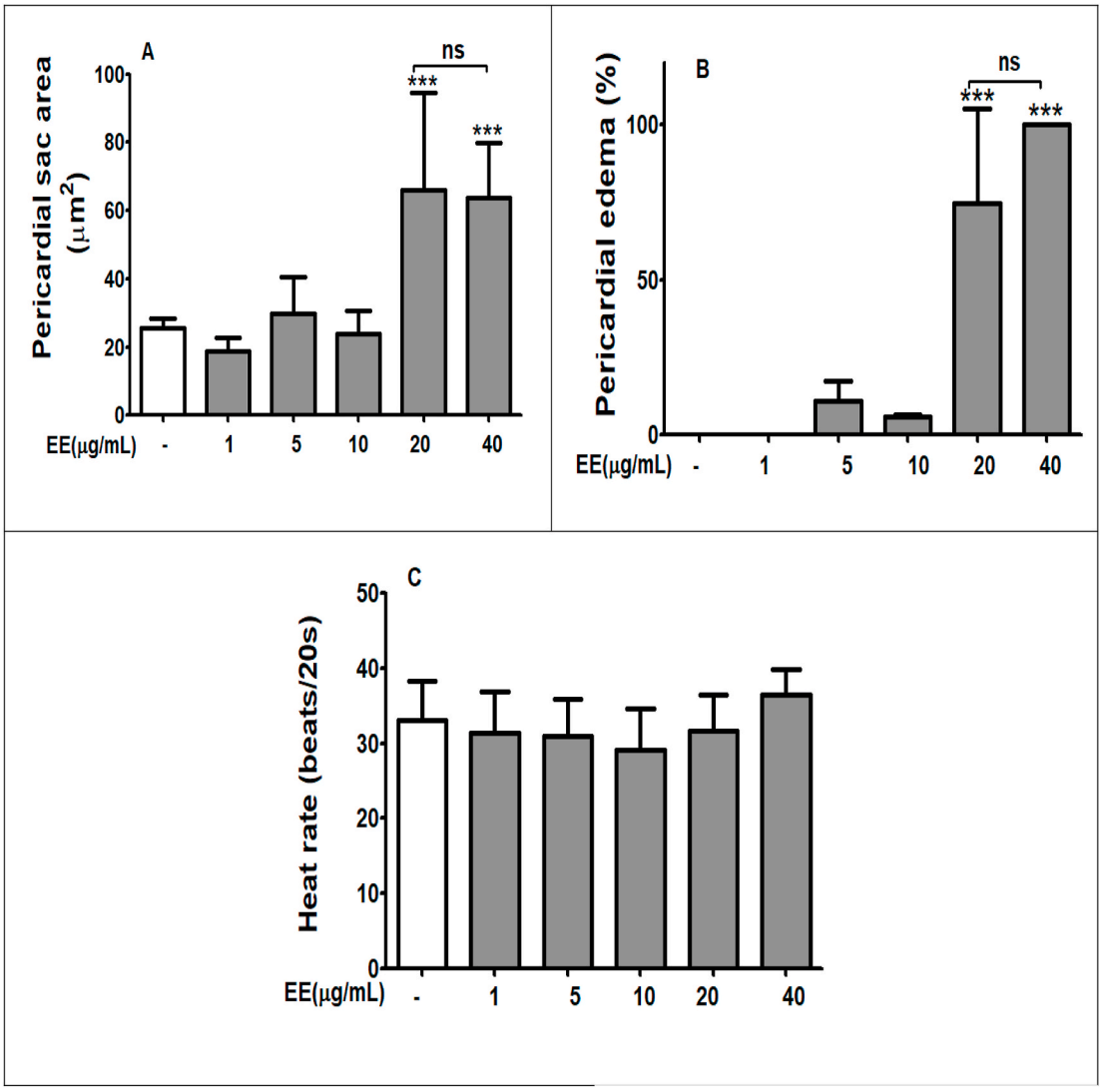


Fig. 5. Effect of ethanol extract from leaves of C. cyrtophyllum (EE) on the cardiovascular system of zebrafish larvae at 120 hpf. Effects of EE on pericardial sac area (**A**) (*each bar represents the mean ± SD for 10 different larvae, n = 10 * p < 0.05; **p < 0.01;***p < 0.001 compared to control group)* and rate of larvae with pericardial edema (**B**) (*each bar represents the mean ± SD. for three different experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control group)*, and heart rate (**C**) (*each bar represents the mean ± SD for 12 different larvae, n = 12, *p < 0.05; **p < 0.01;***p < 0.001 compared to control group)*.

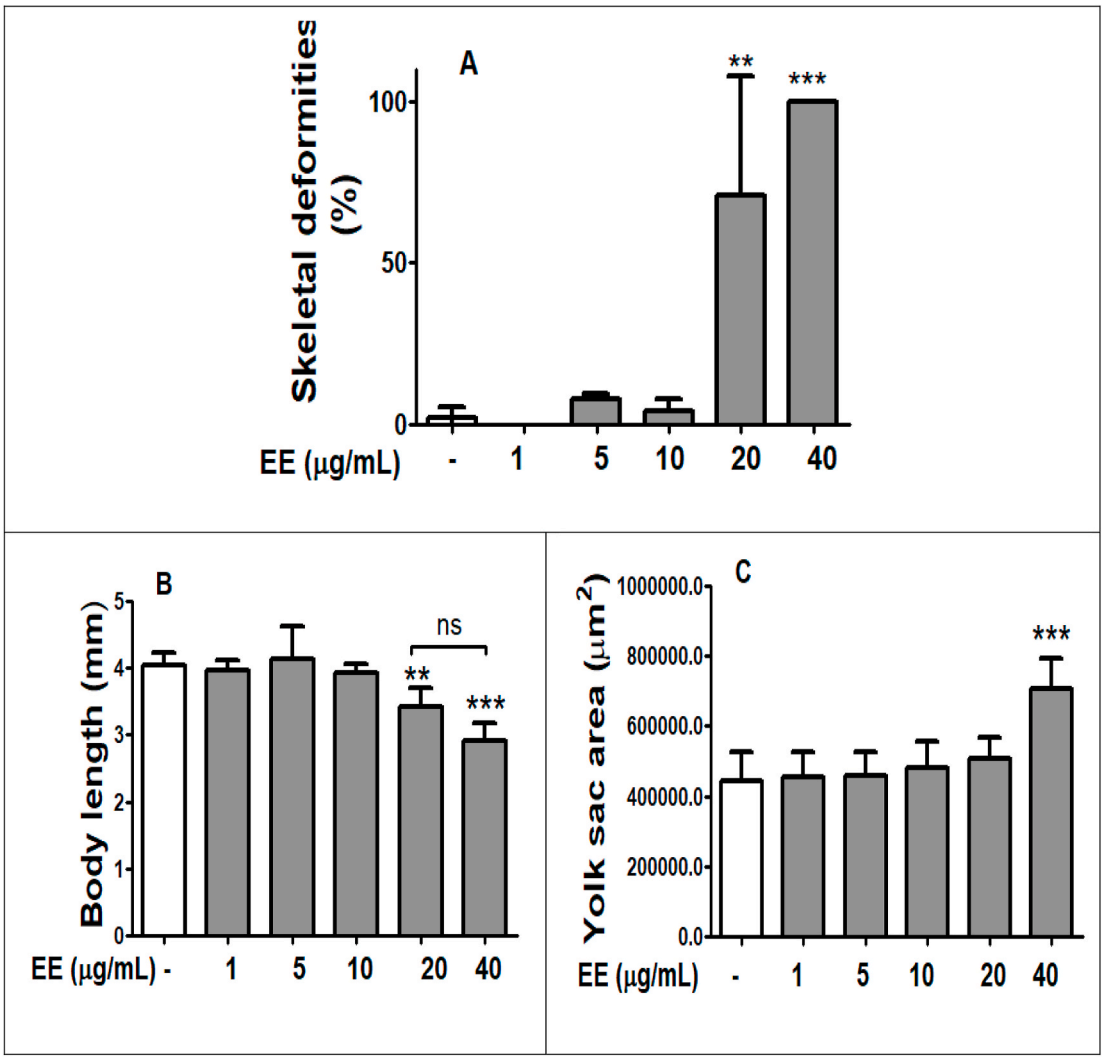


Fig. 6. Effects of EE on rate of skeletal deformities (**A**) (*each bar represents the mean ± SD for three different experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control group)* at 120 hpf; body length (**B**) and yolk sac area (**C**) (*each bar represents the mean ± SD for 12 different larvae, n = 12, *p < 0.05; **p < 0.01;***p < 0.001 compared to control group)*.

3.4. Classification of teratogenicity

In this study, we used 3 criteria for risk classification of EE: LC50, the ratio LC50/EC50 and ratio LC25/NOAEL. The toxicity effect of EE was dose-dependent. The LC50 (for embryotoxic effects/lethality) and EC50 (for particular teratogenic effects) data were obtained from the dose-response curves (Table 4). According to OECD guidelines, toxicity of pollutants against zebrafish is categorized as harmful (10 mg/L < LC50 < 100 mg/L), toxic (1 mg/L < LC50 < 10 mg/L), and highly toxic (LC50 < 1 mg/L). Based on this, EE was found harmful. Based on LC50 and EC50 values, a teratogenic index (TI) was calculated as the ratio LC50/EC50 for each time point. TI values allowed ranking the compounds according to their teratogenic potency. In this study, with TI values of EE higher than 2, the plant extract is considered as a teratogenic compound.

To further confirm the teratogenicity risk related to the use of EE, we also used the ratio LC25/NOAEL for the classification. Ratio LC25/NOAEL greater than or equal to 10 were used to classify a compound as a predicted teratogen. The LC25/NOAEL value of EE was 15.73 at both 72 and 96 hpf (Table 5). Therefore, EE was correctly classified as a teratogen in the zebrafish embryo assay.

3.5. Cardiovascular toxicity

Heart rates, pericardial sac area, pericardial edema rate were recorded to determine the effects of EE on cardiac function. As shown in Fig. 5, at 120 hpf, EE at 20 and 40 µg/mL produced a significant increase in the pericardial sac area and rate of larvae with pericardial edema compared with the control (Fig. 5 A, B). However, no significant differences were observed for heart beat between the different experimental groups (Fig. 5 C).

3.6. Skeletal deformities and yolk sac

As shown in Fig. 6 A, B, compared with the control group, the groups treated with EE 20 and 40 µg/mL increased the skeletal deformity rate and decreased the body length of zebrafish larvae at 120 hpf. At 40 µg/mL, EE even delayed nutrient absorption from yolk sac (Fig. 6C).

3.7. Effect of EE on gene expression

3.7.1. Oxidative stress

To investigate the possible mechanisms of the toxic effects induced by EE, we used qRT-PCR to examine the mRNA expression levels of antioxidant genes in zebrafish embryo after exposure to different EE

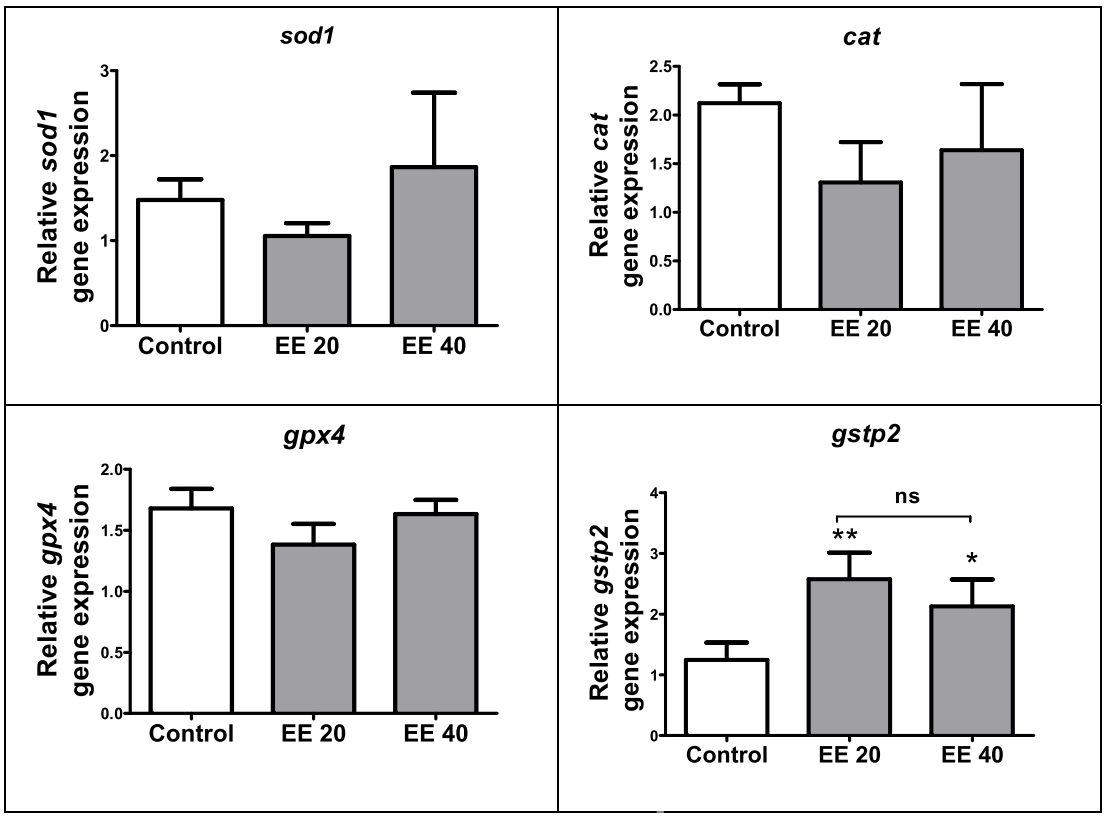


Fig. 7. Effects of EE on antioxidant genes expression.

concentrations from 20 to 40 μg/mL. We observed that the mRNA levels of the oxidative stress-related gene *gstp2* were upregulated upon EE exposure compared with the control group while the level of other antioxidant genes (*sod1, cat* and *gpx4*) was not affected (Fig. 7).

Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group.

3.7.2. Development of body patterning

EE induced skeletal deformities with bending of the body, spinal curvature, and bent tails. To investigate the possible mechanisms of abnormal body patterning induced by EE, we evaluated the effects on the expression of Wnt pathway-related genes. The mRNA level of *wnt3a, wnt11* increased significantly upon EE exposure, while *wnt5* mRNA level decreased. The mRNA levels *β-catenin* and *wnt8a* were not affected by the EE exposure (Fig. 8).

Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group.

3.7.3. Cardiac development

Compared to control, EE at 20 and 40 μg/mL induced a significant increase in the pericardial sac area and in the rate of larvae with pericardial edema. To elucidate molecular mechanism for heart defects, we evaluated the effects of EE on the expression of genes related to heart development, including atrial myosin heavy chain (*myh6*), cardiac myosin light chain (*myl7*), GATA-binding protein 5 (*gata5*), heart and neural crest derivatives expressed transcript 2 (*hand2*), hyaluronan synthase 2 (*has2*) and homeodomain protein nkx2.5 (*nkx2.5*). The mRNA levels of *gata5* and *nkx2.5* increased significantly after exposure to EE at 40 μg/mL. The mRNA levels of *myh6* also increased in the larvae exposed to EE at 20 μg/mL. On the other hand the mRNA levels *hand2* was decreased significantly with EE exposure at 20 and 40 μg/mL. The mRNA levels of *myl7* and *has2* were not affected by EE exposure (Fig. 9).

3.7.4. Cell apoptosis-related genes

To assess whether EE induced apoptosis, the expression levels of apoptosis-related genes *p53, bax, casp9*, *casp3*, *casp8* and *bcl-2*, *apaf-1* were examined. The mRNA expression levels of *casp8, p53* and *bax/bcl-2* significantly increased with increasing EE exposure dose while mRNA levels of *bax*, *bcl-2* decreased (Fig. 10).

3.7.5. Expression of gadd45bb gene

As shown in Fig. 11, the mRNA level of *gadd45*bb was significantly upregulated by the exposure to EE at 40 μg/mL.

4. Discussion

4.1. Identification of compounds from Clerodendron cyrtophyllum turcz leaves

Three main compounds (acteoside, cirsilineol and cirsilineol-4'-*O*-*β*-D-glucopyranoside) were identified from EE in this study. The results are consistent with previous reports for phytochemical investigations from *C. cyrtophyllum* Turcz. Acteoside was found to be a major component (0.803 g, 0.54%) in *Clerodendrum cyrtophyllum* Turcz leaf extracts (Zhou et al., 2020). Cirsilineol and Cirsilineol-4'-*O*-*β*-D-glucoside also were isolated from the ethyl acetate fraction of *Clerodendrum cyrtophyllum* Turcz leaves (Cong Nhuong et al., 2006; Zhou et al., 2020). These compounds are the main active ingredients and maybe responsible for the pharmacological effects but also the toxicological ones of *C. cyrtophyllum*. More research studies are needed to confirm the safety of the combination between cirsilineol or cirsilineol-4'-*O*-*β*-D-glucoside with verbascoside or other compounds in the crude extract, in order to evaluate the possible additive, synergistic or antagonist effects of this mixture.

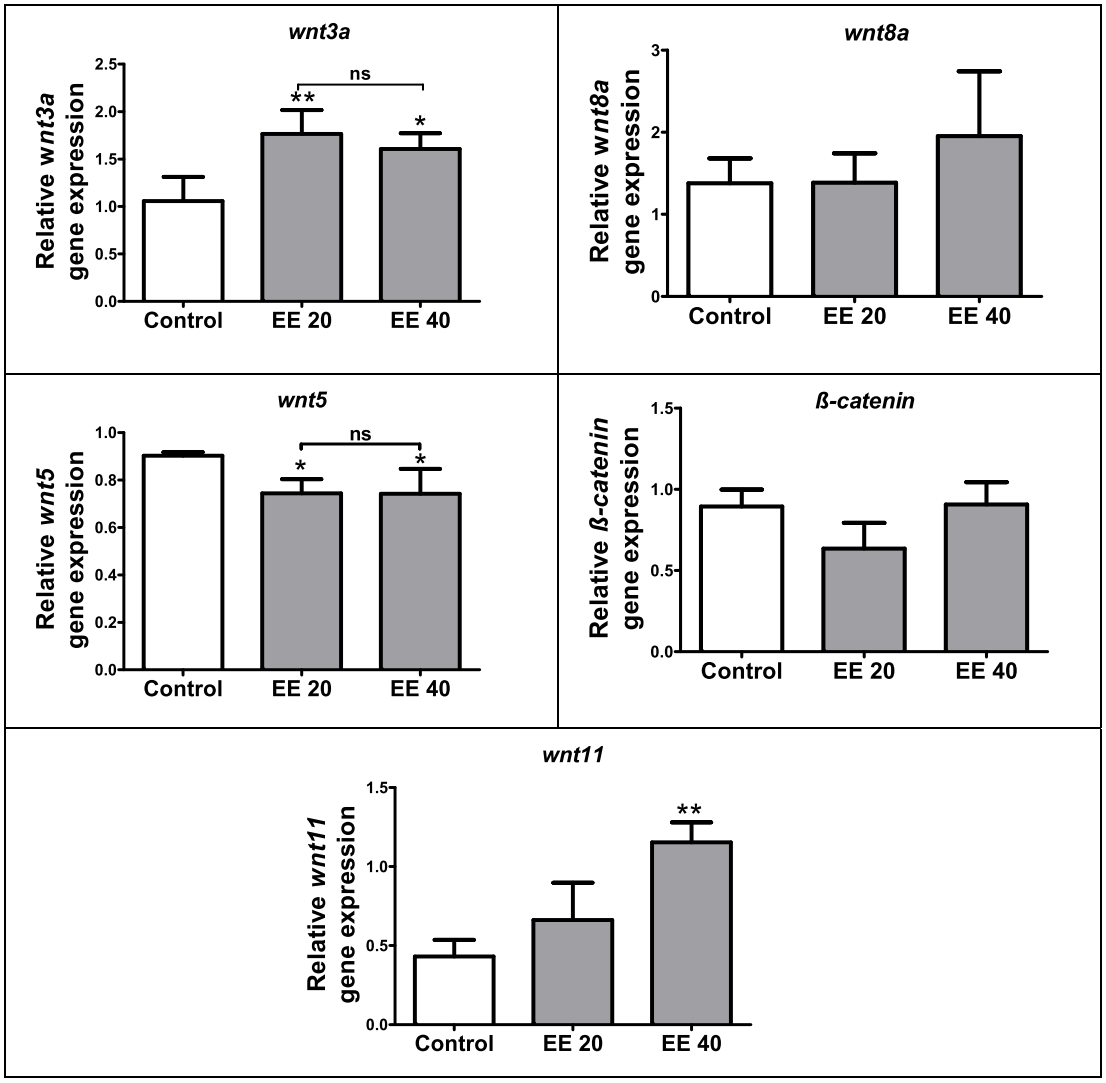


Fig. 8. Effects of EE on the expression of genes related to the development of body patterning.

4.2. *Developmental toxicity of Clerodendrum cyrtophyllum turcz*

In the present study, we reveal that the exposure of zebrafish embryos to ethanol extract of *Clerodendrum cyrtophyllum* impaired their early development by particularly decreasing the survival rate, inducing morphological abnormalities, and delaying the hatching rate. EE treatment at doses of 80–200 μg/mL obviously reduced hatching rate at 72 hpf. Hatching is a key step in the transformation of embryos into larvae and is one of the most important indices to evaluate the developmental toxicity in zebrafish. Delayed hatching may be due to retarded development or the inability of embryos to break the chorion. Hatching of embryos depends on the hatching enzyme activity and embryo movements (Zhang et al., 2015; Qian et al., 2018). In our study, the number of spontaneous movements during embryogenesis in groups exposed to EE from 40 to 100 μg/mL increased at 24 hpf, while the hatching rate decreased at all time points of observation. It can be deduced that hatching enzyme activity might be affected by EE, but this requires further studies.

The exposure of embryos to EE caused several developmental abnormalities, such as yolk sac retention, pericardial edema, spine deformation and bent tails. The deformities were observed to be dose-dependent and increased with the time of exposure. Among the malformations, the most pronounced morphological alterations were pericardial edema and yolk sac edema. Congenital heart disease is the most common birth defect (Zaidi and Martina Brueckner, 2018). Damage to the heart may lead to inhibition of blood transport in embryos and then affect energy transport, further influencing the development of embryos (Qian et al., 2018). The yolk sac plays an important role during the early developmental stage, because it is the only source of nutrition for embryos. The yolk size will decrease along with the embryonic development (Zhang et al., 2015). In this study, exposure to EE at the dose of 40 μg/mL delayed nutrient absorption from yolk sac. Decreased absorption of nutrients can delay or impair growth of larvae. Body length is an important indicator of embryo growth, and the loss of nutrients may induce a shorter body length. In this study, exposure to EE at the doses of 20 and 40 μg/mL exhibited a significant reduction in the body length of larvae. In addition, yolk lipids are the source of triacylglycerol and cholesterol, which are required for the synthesis of cell membranes, bile acids and steroid hormones. Unabsorbed yolk thus can affect the proper development of embryos (Anderson et al., 2011). Other morphological abnormalities observed in EE treated groups were spinal curvature, and bent tails. Spinal deformities are commonly identified as being one of the major pathological traits among fish dwelling in areas polluted with toxic chemicals (Cheng et al., 2000). Spinal curvature, and bent tails can be associated with early defects in somite formation that in turn leads to deformities of the muscle and skeleton. Spinal deformities also might be associated with a reduction in myosin because myotome formation critical for a robust musculoskeletal system formation (Zoupa and

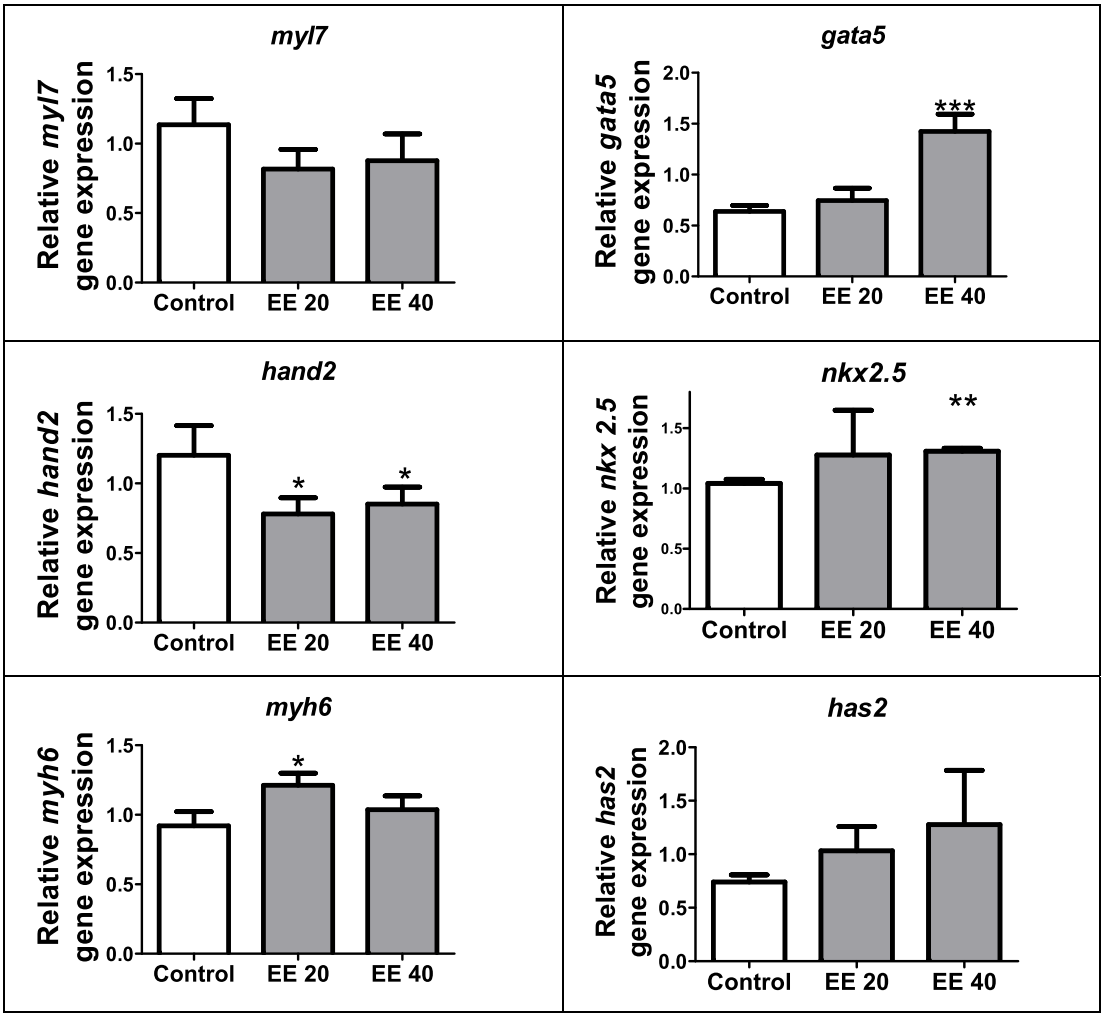


Fig. 9. Effects of EE exposure on the expression of cardiac program related genes.
Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group.

Machera, 2017). Taken together, these results demonstrated that developmental toxicity of zebrafish was induced by EE exposure.

Nkx2.5 expression is the earliest marker of precardiac cells and plays a critical role in differentiation and proliferation of myocardial cell and heart morphogenesis (Han et al., 2015). Knockdown of *nkx2.*5 has been shown to cause a variety of cardiovascular deformities in heart chamber development as well as pericardial edema (Targoff et al., 2008). In this study, the expression of *nkx2.5* was significantly up-regulated with increasing EE concentration, which might reflect a compensatory response to repair or rescue heart development after its impairment. *Gata5* is a transcription factor which is necessary for the development of the heart (Han et al., 2015). Overexpression of *gata5* induces the expression of a few myocardial genes including *nkx2.5* and can produce ectopic foci of beating myocardial tissue (Reiter et al., 1999). In this study, the expression of *gata5* was significantly up-regulated at EE 40 μg/mL, which may enhance the regenerative proliferation of cardiomyocytes in response to the injury. *Hand2* is associated with cardiomyocyte formation, plays pivotal roles in heart morphogenesis and cardiac-specific transcription. Overexpression of *hand2* can enhance cardiomyocyte production, resulting in an enlarged heart with a striking increase in the size of the outflow tract. In contrast, Zebrafish *hand2* mutants can cause a striking cardiac phenotype that features a dramatic deficit of cardiomyocytes (Schindler et al., 2014). In this study, the expression of *hand2* was significantly downregulated at EE 20 and 40 μg/mL. The downregulation of this gene expression provides a potential mechanism for EE-induced cardiotoxicity. These results further confirm cardiotoxicity by EE induction at the gene expression level.

The Wnt signaling pathway, plays an important role in the early stages of vertebrate embryo development. It regulates key events during embryonic patterning, morphogenesis, and skeletogenesis (Li et al., 2018). In this study, treatment with EE up-regulated *wnt3a* and down-regulated *wnt5*. Because Wnt signaling plays an important role in body patterning, the up- or down-regulations of *wnt* genes could consistent with the developmental defects in the EE treated groups. Wnt11 participates in the regulation of cardiac circulation and differentiation (Pandur et al., 2002). In our study, the expression of *wnt11* significantly increased at 40 μg/mL, explaining the heart development defects of zebrafish in the EE treated groups.

In multicellular organisms, normal development depends upon the balance between cell proliferation, differentiation, and death. Apoptosis is the most common mechanism employed during development to remove damaged, misplaced, or otherwise unwanted cells (Eimon and Ashkenazi, 2010). There are two key apoptotic signaling mechanisms in mammals and other vertebrates: the cell-intrinsic pathway and the cell-extrinsic pathway. The intrinsic pathway is regulated by the *bcl-2* gene family and functions through the initiator protease caspase-9 (Eimon and Ashkenazi, 2010; Youle and Strasser, 2008). The extrinsic pathway is triggered by death receptor ligation and functions through a death-inducing signaling complex (DISC) that includes the initiator proteases caspase-8 and -10. The p53 pathway induces apoptosis by

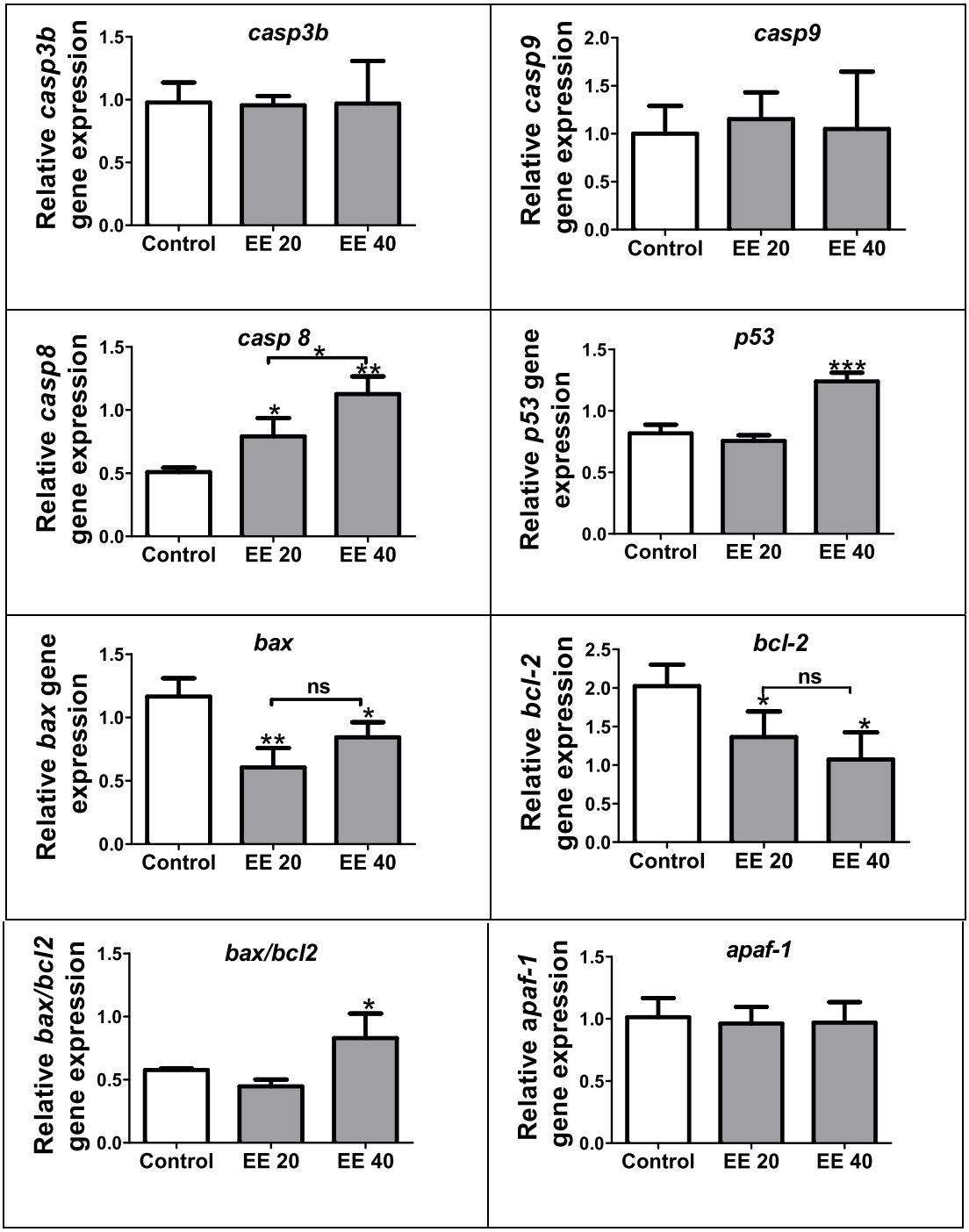


Fig. 10. Effects of EE on the expression of cell apoptosis-related genes.
Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group.

up-regulating the transcription of pro-apoptotic genes, and down-regulating anti-apoptotic genes, including *bcl-2* (Zhang et al., 2015). All pathways converge at the level of the effector caspases (caspase-3, -6 and -7), which carry out the molecular mechanics of programmed cell death (Eimon and Ashkenazi, 2010). We show that exposure of zebrafish to EE significantly decreased the expression levels of *bcl-2, bax* while the expression level of *caspase 8* and *p53* was up-regulated. These results indicate that apoptosis was induced by EE in zebrafish which is consistent with the phenotype of developmental toxicity.

It has been reported that embryonic development is especially sensitive to Reactive Oxygen Species (ROS). ROS can induce cell apoptosis, contributing to abnormal embryonic development (Zhang et al., 2015; Shi and Zhou, 2010). Drugs can induce oxidative stress by the reduction of cellular antioxidant defenses. In our study, the expression levels of *gstp2* were upregulated while the transcription of *sod1*, *cat* and *gpx4* was not affected. The increase of *gstp2* could be explained by a self-protection mechanism to antioxidative stress in fish. These results show that oxidative stress can play an important role in the developmental toxicity of EE.

Gadd 45 participates in cell cycle arrest, cell survival or apoptosis and the regulation of signaling pathways. DNA-damaging agents and cellular stresses can induce transcription of the *gadd45*bb gene. In our study, the mRNA levels of *gadd45*bb were upregulated in the EE treated

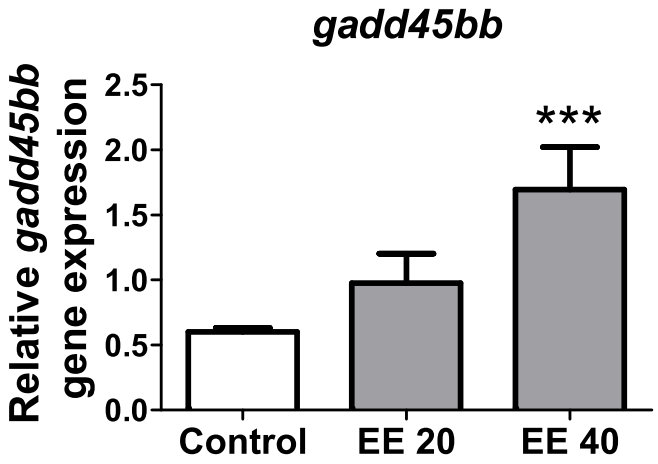


Fig. 11. Effects of EE on the expression of *gadd45*bb.
Each bar represents the mean ± SD. for three different experiments performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group.

group. These results indicate that DNA-damaging and growth arrest were activated in zebrafish embryos exposed to EE and played an important role in its developmental toxicity.

4.3. Classification of teratogenicity

The teratogenic index (TI- LC50/EC50) is a popular approach to classify compounds according to their teratogenic potential. In our study, TI value of EE at 96 hpf was 3.87 (>2) classifying EE as teratogen. Recent studies found that LC25 also was adequate for defining a toxic concentration and the teratogenicity index, which included the LC25, successfully discriminated between teratogenic and non-teratogenic compounds (Brannen et al., 2010). In this study, LC25/NOAEL value of EE at 96 hpf was 15.73 (>10) suggesting that EE is teratogenic. LC25/NOAEL further confirmed the teratogenic potential of EE. The use of EE therefore should be avoided during pregnancy.

4.4. The developmental toxicity of acteoside, cirsilineol-4'-O-ß-D-glucopyranoside and cirsilineol in zebrafish embryo model

In this study, the exposure of embryos to EE caused a decrease of the survival rate, inducing morphological abnormalities, and delaying the hatching rate. Molecular mechanisms for the developmental toxicity of EE were proved to be involved on signaling pathways affecting fish embryo's development such as heart development, oxidative stress, wnt pathway or cell apoptosis. Acteoside, cirsilineol-4'-O-ß-D-glucopyranoside and cirsilineol are major compounds of EE and can contribute to the development toxicity of EE. A previous study showed that acteoside exerted prooxidant short-term effects, increased catalase activity and reactive oxygen species (ROS) signals and reduced blastocyst formation rate with long time exposure (Dell'Aquila et al., 2014). Reactive oxygen species (ROS) production could impair biomolecules like DNA, lipids and proteins, and manifesting unsettled signal transduction that can lead to inappropriate apoptosis and necrosis. Therefore, the effects of EE on the developmental toxicity in zebrafish embryo can be explained by the presence of acteoside. The results on pregnant mice also showed that although acteoside exerted no significant toxicity on the mothers there was still a small rate of fetus with skeletal abnormalities (<2%) including vertebral column deformity (<1%) and limb deformity (<1%) observed in acteoside-treated groups (Etemad et al., 2016).

Cirsilineol-4'-O-ß-D-glucopyranoside and cirsilineol were identified in EE. The toxicity risk analysis showed that cirsilineol possesses a mild risk for skin and ocular irritancy but is non-mutagenic (Pathak et al., 2020). In a previous study, Cirsilineol displayed anti-inflammatory effect through cyclooxygenase inhibitory activity (Kelm et al., 2000). The authors attributed the effect to the inhibition of zebrafish cyclooxygenase enzyme (z-cox/ptgs) which is necessary for the embryo early stage development (Grosser et al., 2002). Two different COX isozymes (COX-1 and COX-2) were widely expressed during development, COX-1 transcripts were detected in all tissues except brain. The most robust COX-2 signals were detected in gills, intestine, and testes, followed by heart, skeletal muscle, and the brain. Knockdown of COX-1 causes growth arrest during early embryogenesis (Grosser et al., 2002). Nonselective inhibition COX of cirsilineol in the embryo stage can explain for the development toxicity of EE.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most prescribed medications for treating inflammatory diseases. NSAIDs work by inhibiting the activity of cyclooxygenase enzymes (COX-1 or COX-2). The recent studies revealed that NSAIDs such as ibuprofen and diclofenac induced the developmental toxicity in zebrafish embryos. At low concentrations, ibuprofen and diclofenac impair the cardiovascular development of zebrafish (Zhang et al., 2020). Exposure to high concentration of ibuprofen and diclofenac significantly decreased the hatching rate, the spontaneous movement and the free swimming distance (Xia et al., 2017). Diclofenac induces short body length, smaller eye, pericardial and body edema, lack of liver, intestine and circulation, muscle degeneration, and abnormal pigmentation in zebrafish larvae. The genes involved in cardiovascular physiology, such as *nppa* and *nkx2.5*, were significantly up-regulated by ibuprofen. The portion of diclofenac transferred into the embryo altered the expression of certain genes, e.g. down-regulation of *Wnt3a* and *Gata 4* and up-regulation of *Wnt8a* (Chen et al., 2014). These results are similar to EE, so the development toxicity of EE may be related to cyclooxygenase inhibitory activity of cirsilineol.

Cirsilineol-4'-O-ß-D-glucopyranoside has not been reported previously for toxicity or pharmacology effect. However, the presence of Cirsilineol-4'-O-ß-D-glucopyranoside can contribute for toxicity of EE, too.

4.5. The relationship between therapeutic use and toxicity

Women are the primary consumers of traditional medicines, and usually continue using them during pregnancy. Pregnant women usually use tradtional medicines as a safe and natural alternative to conventional drugs and often use them to improve their wellbeing or for the treatment of non-life threating conditions (Illamola et al., 2019). However, data on the safety of these herbal treatments during pregnancy are generally insufficient. *Clerodendrum cyrtophyllum* Turcz is widely used in traditional medicine for the treatment of many diseases. Pregnant women also are consumers of *Clerodendrum cyrtophyllum.* The results of this study showed that using EE for pregnant women can induce defects or even mortality for embryo. Due to its teratogenic effects, *Clerodendrum cyrtophyllum* EE should not be recommended during pregnancy.

5. Conclusion

Our results demonstrate that EE caused developmental toxicity to zebrafish embryos/larvae. EE exposure at doses ranging from 80 to 200 µg/mL increased the mortality of embryos and reduced the hatching rate. Exposure at 20 and 40 µg/mL until 72 hpf – 120 hpf induced a series of symptoms of malformation including yolk sac edema, pericardial edema, spine deformation, shorter body length. The mRNA expression levels of *p53, casp8, bax/bcl2*, *gstp2*, *nkx2.5*, *gata5*, *wnt3a, wnt11*, *gadd45*bb were significantly upregulated upon EE exposure at 20 and 40 µg/mL while the expression of *wnt5, hand2* and *bcl2* were downregulated. These results provide evidence of toxicity of EE to embryo stages and provide an insight into the potential toxic mechanisms on embryonic development. As zebrafish embryo development is sensitive to EE, this extract could also have potential risks for the development of human embryos, therefore caution should be taken when

consuming it during pregnancy.

Author contributions

Conceptualization, TH. N and P.K.; Methodology, TH. N, PD. N and D. TLH; Validation, P.K, JQ. L and M.M.; Formal analysis, TH. N, PD. N and HT. P; Investigation, TH. N and D. TLH; Writing— Original Draft Preparation, TH.N.; Writing- Review and editing, P.K., D. TLH, HTP, M. M; Supervision, P.K.; Project Administration, P.K, HT. P and M.M. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

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