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Aqueous extract from *Chrysophyllum cainito* bark exhibits embryonic toxicity in *Danio rerio* and negligible acute toxicity in adult Wistar rats

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Abstract: *Chrysophyllum cainito* has been used as a traditional medicine to treat a wide range of diseases, but the toxicity profile of this plant remains unknown. This study aimed to evaluate the acute toxicity of the aqueous extract of *C. cainito* (CE) bark based on OECD guidelines in two different *in vivo* experimental models: acute single-dose oral toxicity in adult Wistar rats and the zebrafish embryo acute toxicity test. All concentrations of CE (500-4000 mg/kg) tested during a 14-day period in both male and female rats showed no effect on behavior, body weight, organ weights, biochemical and hematological parameters. In contrast, CE significantly delayed zebrafish embryo hatching and decreased embryo survival rates in a dose-dependent manner. Hatched larvae were notably sensitive to CE-induced toxicity compared to unhatched fish embryos. Acridine orange staining showed that CE induced apoptosis in the yolk sac region that is responsible for supplying nutrients to support larval growth and development. According to OECD guidelines, CE was identified as GHS category 5, a substance with low to no acute toxicity. However, as embryotoxicity was observed in zebrafish, CE use during pregnancy should be exercised with caution until further examination of its safety.

Keywords: *Chrysophyllum cainito*; embryotoxicity; acute toxicity; zebrafish; Wistar rat

INTRODUCTION

Traditional herbal medicines offer an alternative for the treatment of common ailments such as diabetes, bacterial infections, fever. In developing countries, patients with chronic diseases are the major consumers of herbal medicines due to traditional beliefs, local accessibility and the low cost of these remedies, which is typically less than that of conventional modern medication [1,2]. The widespread use of traditional herbal medicine is also attributed to exaggerated claims regarding their broad preventative and therapeutic effects [3]. Unfortunately, the safety information regarding most folk herbal medicine is based on non-scientific sources or misleading scientific evidence. Furthermore, medicinal plants, while widely used, can also exert toxic adverse effects if misused, particularly in vulnerable populations, including pregnant women and children [4]. Concerns regarding herbal medication are increasing as more reports evaluate and highlight the toxicity of herbal medicinal plants [5,6]. It has been reported that changes in herbal extraction methods of the same medicinal plant can lead to increased toxic effects in brine shrimp [5]. The risk of herbal medicine misuse may lead to potential adverse effects, toxicity, mutagenicity and carcinogenicity [7].

The pharmacovigilance scheme employs a scientific approach to detect, estimate, understand and limit the adverse effect of drugs. According to the pharmacovigilance system introduced by the World Health Organization [8], safety is deemed fundamental for all therapeutic products, including herb-derived medicinal and healthcare products. Recent pharmacovigilance concerns describe the use and misuse of herbals, folk and complementary medicines. Unlike modern medicines, efficacy is not a legal issue for folk remedies. Although most herbal folk remedies are generally believed to be safe, their potential adverse effects remain to be elucidated [9].

Chrysophyllum cainito is a tropical fruit tree considered to exert multiple therapeutic effects, including

hypoglycemic activity, antioxidant, antihypertensive, antiinflammatory and antibacterial properties [10]. The bark of *C. cainito* has been traditionally used in Côte d'Ivoire and the southwest of Vietnam as a folk treatment for diabetes [10,11]. Despite the widespread use of the *C. cainito* extract, there is a lack of evidence regarding its safety and toxicity profile.

In vivo pharmacological evidence supporting the use of *C.cainito* in diabetes in our previous work showed that the aqueous extract of *C. cainito* bark was indeed effective in reducing blood glucose levels in the mouse model [12]. However, to safely recommend the integration of *C. cainito* as part of the therapeutic remedy for diabetes, its toxicity should be considered in parallel with its benefits [13,14]. Therefore, this study aimed to evaluate the safety of the *C. cainito* bark extract (CE) using internationally accepted guidelines provided by the Organization for Economic Co-operation and Development (OECD). The safety profile of CE was assessed by a single-dose acute toxicity test performed in a Wistar rat model, and to examine whether CE is safe for use in pregnancy, fish embryo acute toxicity tests were conducted.

MATERIALS AND METHODS

Ethics statement

All experimental procedures were approved and conducted with strict adherence to the guidelines of the Institutional Animal Care and Use Committee, Suranaree University of Technology, Thailand (Approval No: 1/2559 and 10/2559). According to the current regulations of the European Union and the Institutional Animal Care and Use Committee (IACUC), zebrafish were subjected to ethical regulation after 5 days (120 h) postfertilization [15,16], as evidence suggests that zebrafish would be capable of nociception and distress at 5 days postfertilization [15,17-19]. No zebrafish embryos older than 72 h postfertilization were used in this study.

Chemicals

All chemicals were purchased from Sigma Aldrich (Missouri, USA) unless stated otherwise.

Plant collection and *C. cainito* bark extraction

C. cainito bark was collected from Mo Cay Nam district, Ben Tre Province, Vietnam. Plant verification was performed as indicated in our previous study [12]. Vouchered specimens of leaf, fruit, flowers, and stem were stored at the Suranaree University of Technology Botanical Garden (collection ID: H. DOAN-1).

C. cainito bark aqueous extraction was performed as described [12]. Briefly, shade-dried *C. cainito* bark was chopped and ground with a blender before extraction. Fifty grams of the finely ground bark were mixed with 200 mL of deionized water. Four cycles of aqueous extraction were performed for 2 h per extraction cycle. All extraction procedures were performed on a shaker at room temperature. The combined supernatant was filtered with cotton gauze then centrifuged in a Sorvall Biofuge Stratos Centrifuge (Thermo Fisher Scientific, USA) at 8000 *x g* for 15 min, at room temperature to remove any remaining bark residue. The extract was concentrated by rotary evaporation and freeze-dried for 48 h using a lyophilizer. The dried *C. cainito* extract was stored at -20°C until further use.

Acute toxicity study in rat model

Animal husbandry

Male and female Wistar rats were obtained from the Laboratory Animal Facility, Suranaree University of Technology. The animals were housed in stainless steel cages lined with wood shavings at Laboratory Animal Facility, Suranaree University of Technology, under standard conditions of 25±2°C, 45-50% relative humidity and a 12-h light/dark cycle. Rats had *ad libitum* access to standard pellet food and water. All animals were acclimatized for seven days before an acute toxicity test was performed.

Acute toxicity in rats

Based on OECD Guideline no. 423 [20], healthy male (120-150 g) and female Wistar rats (100-140 g) were selected for the acute toxicity test. After seven days of acclimatization, rats were fasted overnight. The animals were divided into five treatment groups (3 animals of each sex per group; N=6 per group) as

follows: control group (that received deionized water) and four treatment groups that were administered a single dose of increasing concentrations of CE at 500, 1000, 2000 and 4000 mg/kg of rat body weight. Minor dose deviations from the OECD guidelines were chosen. The animal sample sizes were calculated based on the "resource equation" method [21].

Prior to treatment, CE was freshly prepared by dissolving the lyophilized CE in sterile distilled water. All treatments were administered by oral gavage. Post-treatment, the rats were monitored for signs of toxicity at 0.25, 0.5, 1, 4 and 24 h, then daily for 14 days. According to OECD guidelines, signs of toxicity indicative of animal suffering were considered as experimental endpoints, and any affected animals were immediately euthanized [20]. Rats were weighed on days 0, 7 and 14. At the end of the experiment, all rats fasted overnight and were killed by CO_2 inhalation. Necropsy was performed, and the internal organs were examined. Rat organs (liver, heart, kidney, lung, spleen, testis and ovary) were isolated and weighed. Blood samples were collected for hematological analysis (Mindray BC-6800 Auto Hematology Analyzer) and biochemical analysis (Mindray BS-800 Automatic Chemistry Analyzer). The following liver and kidney function parameters were monitored: alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), blood urea nitrogen, total protein and albumin.

Developmental toxicity in zebrafish embryos

Zebrafish housing and breeding

Adult zebrafish (*Danio rerio*) were sourced from a local fish shop that routinely supplies zebrafish for breeding purposes to Suranaree University of Technology. Adult zebrafish were housed in glass tanks, reared in reversed osmosis water, exposed to room temperature of 26±2°C and a 12-h light/dark cycle. Male and female fish were raised in separate tanks. Zebrafish were routinely fed three times per day with commercial micropellet (morning and evening at 7.00 and 17.00 h) and frozen artemia (noon, 12.00 h). Zebrafish were monitored daily for signs of disease, and routine feces removal was performed to maintain water quality. To stimulate breeding and egg fertilization, active male and female fish (ratio 2:1) were placed in the same spawning tank fitted with a transparent barrier and a spawn trap 1-2 h before the dark cycle. At the beginning of the light cycle, the transparent barrier was removed to allow male and female fish to mate. After 45-50 min, adult zebrafish were removed from the spawning tank and the fish eggs were collected and rinsed with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$).

Fish embryo acute toxicity test (FET)

Acute toxicity of CE on zebrafish embryos was performed according to OECD guideline 236 [22] with minor amendments [23]. Healthy fertilized eggs were chosen under a stereomicroscope and maintained in E3 medium. Selected fertilized eggs were placed into a 24-well plate, one egg/well. Fifteen to twenty eggs were used per treatment and five independent experiments were performed. At about 3-4 h postfertilization (hpf), eggs were exposed to different concentrations of CE (0, 15.63, 31.25, 62.5, 125, 250 and 500 μg/mL) for 72 h. The developmental morphologies of embryos were monitored at 24, 48, and 72 h post-treatment administration (hpta). Parameters that were monitored included: hatching rate, death and noticeable abnormal embryonic development. Fish embryo images were captured using AM423X/AM4023CT Dino-Eye C Mount digital cameras (AnMo Electronics Corp., Taiwan) fitted on Olympus SZX7/SZ61 stereomicroscopes (Olympus, Japan). Images were acquired by DinoCapture 2.0 software (AnMo Electronics Corp., Taiwan).

Fish larvae acute apoptosis test

From the LC_{50} calculated from the FET test, an acute apoptosis screening test was performed to determine whether CE induces selective or non-specific toxicity. Healthy fertilized eggs were maintained in E3 medium until hatching at 72 hpf. Newly hatched larvae were placed into 24-well plates containing the designated treatment conditions, including 0, 31.25 and 62.5 μg CE/mL, and were exposed to the treatment for either 0.5 or 1 h. At least two replicates were performed per treatment condition.

After the designated treatment, zebrafish larvae were stained with 3.33 μg/mL of acridine orange (cat#

A8097, Sigma-Aldrich, Missouri, USA) at 37°C for 15 min. The stained larvae were then washed with E3 medium for 5 min. Washing was repeated three times. The stained larvae were then immobilized in an ice-water bath for at least 30 min prior to image capture, after which all larvae were euthanized in an ice-water bath overnight (>12 h) [24]. Fluorescent zebrafish larvae imaging was carried out using an Olympus DP72 fluorescent microscope (4x objective lens, FITC channel, 400 ms exposure) fitted with a Nikon Eclipse 80i camera. Images were taken using Olympus CellD software (Olympus, Japan) and were processed by Fiji ImageJ software [25].

Table 1. Body weight, mortality, and symptoms of toxicity of rats treated with CE.

Treatment (mg/kg)	Body weight (g)			Mortality	Symptoms of toxicity
	Day 1	Day 7	Day 14		
Male rats					
Control	183.33±6.67	233.33±12.02	263.33±6.67	None	None
CE 500	176.67±3.33	226.67±6.67	263.33±6.67	None	None
CE 1000	160.00±11.55	203.33±17.63	243.33±16.67	None	None
CE 2000	176.67±8.82	220.00±10.00	263.33±12.02	None	None
CE 4000	166.67±3.33	213.33±6.67	253.33±3.33	None	None
Female rats					
Control	156.67±3.33	183.33±8.82	200.00±5.77	None	None
CE 500	146.67±8.82	170.00±11.55	186.67±13.33	None	None
CE 1000	150.00±0.00	176.76±3.33	190.00±5.77	None	None
CE 2000	153.33±3.33	176.67±6.67	193.33±3.33	None	None
CE 4000	143.33±8.82	163.33±8.82	180.00±5.77	None	None

Statistical analysis

All quantitative results are expressed as the mean±SEM. Statistical analysis was performed using Graphpad Prism version 9.2.0 (GraphPad Software, USA). Normality tests were performed to determine whether the data sets used for statistical analysis were parametric or nonparametric. For multiple comparisons of parametric data, results were analyzed by one-way ANOVA followed by Tukey's test. Kruskal-Wallis one-way ANOVA and Dunn's post-hoc test were performed for multiple comparisons of nonparametric data. For multifactor comparisons, two-way ANOVA and Tukey's post-hoc test were performed. $P<0.05$ was considered as a statistically significant difference.

RESULTS

C. cainito bark extract exhibited negligible acute oral toxicity in Wistar rat

As safety data regarding oral route administration of *C. cainito* bark are currently unavailable, it is important to determine the safety category of CE.

Table 2. The acute toxicity test of CE on serum biochemical parameters in rats.

Parameters		Treatment (mg/ kg BW)			
	Control	500	1000	2000	4000
Male					
AST (U/L)	204.67±33.82	364.67±59.74	224.67±36.86	252.00±63.79	241.33±30.07
ALT (U/L)	39.33±2.33	43.33±8.35	40.00±3.46	41.00±5.57	45.33±6.77
ALP (U/L)	107.67±10.40	94.33±5.36	125.00±18.61	99.67±14.15	77.33±15.06
BUN (mg/dL)	20.80±2.15	23.77±1.19	21.43±2.54	23.90±3.46	22.43±2.87
Total Protein (g/dL)	6.27±0.21	6.00±0.20	6.30±0.00	6.07±0.12	6.03±0.31
Albumin (g/dL)	3.73±0.09	3.57±0.03	3.77±0.03	3.90±0.17	3.93±0.12
Female					
AST (U/L)	274.67±14.08	316.33±21.18	251.00±40.28	392.33±58.67	268.00±23.46
ALT (U/L)	48.67±6.89	37.33±2.85	43.33±5.04	53.00±8.08	39.33±0.88
ALP (U/L)	52.67±6.98	48.00±8.89	79.00±8.54	57.33±10.71	74.33±12.72
BUN (mg/dL)	26.20±0.20	28.07±1.62	24.33±3.64	32.57±2.55	23.87±1.74
Total Protein (g/dL)	6.47±0.09	6.00±0.20	6.27±0.19	5.73±0.07	6.13±0.07
Albumin (g/dL)	3.97±0.03	3.77±0.13	3.83±0.07	3.67±0.12	3.67±0.03

Values are expressed as the mean±SEM (n=3). There are no significant differences in any parameters between groups ($P>0.05$; one-way ANOVA).
Abbreviation: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; BW, body weight

Table 3. The acute toxicity test of CE on hematological biochemical parameters in rats

Parameters		Treatment (mg/ kg BW)			
	Control	500	1000	2000	4000
Male					
RBC (× 10^6 /μL)	7.80 ±0.25	7.76±0.27	8.91±0.43	8.09±0.06	8.03±0.14
HGB (g/dL)	14.87±0.66	13.93±1.48	17.33±0.83	15.85±0.12	16.23±0.38
HCT (%)	52.00±0.58	52.67±1.45	61.67±3.18	55.50±0.58	56.67±1.45
WBC (× 10^3/μL)	4.82±2.29	9.97±2.25	9.69±2.38	8.53±0.50	9.92±2.57
LYM (× 10^3/μL)	84.73±0.64	81.23±3.01	86.30±1.44	86.90±2.16	84.63±1.42
MON (× 10^3/μL)	2.33±0.17	2.77±0.38	1.93±0.19	2.33± 0.20	2.77±0.13
NEU (%)	11.53±0.60	12.10±1.31	10.40±0.95	8.97±1.48	10.87±1.52
PLT (× 10^4/μL)	49.10±13.92	77.50±14.07	79.70±11.11	86.33±4.88	80.03±4.18
MCV (fL)	67.00±1.16	67.67±0.67	69.00±0.58	68.33±0.33	70.33±0.67
MCH (pg)	19.07±0.35	17.87±1.49	19.47±0.12	19.43±0.19	20.20±0.15
MCHC (g/dL)	28.60±1.00	26.50±2.4	28.23±0.34	28.53±0.45	28.80±0.12
RDW-CV (%)	14.33±0.33	14.57±0.29	15.00±0.38	14.63±0.48	14.73±0.27
Female					
RBC (× 10^6 /μL)	8.61±0.11	9.01±0.50	8.30±0.27	8.40±0.44	8.87±0.13
HGB (g/dl)	17.00±0.06	19.20±0.98	16.83±0.55	15.77±1.47	17.43±0.41
HCT (%)	57.33±0.88	58.67±4.18	54.67±2.03	53.33±3.33	57.00±1.53
WBC (× 10^3/μL)	6.52±1.92	7.45±1.27	5.03±1.67	4.08±0.80	5.29±1.63
LYM (× 10^3/μL)	76.36±5.66	88.50±1.85	85.80±0.57	80.37±0.90	83.67±1.53
MON (× 10^3/μL)	3.80±0.76	1.83±0.48	4.10±0.53	3.77±0.92	2.93±0.69
NEU (%)	13.80±3.10	6.93±1.56	7.80±0.57	11.13±0.90	12.13±0.96
PLT (× 10^4/μL)	103.73±2.89	57.40±34.22	52.33±27.23	43.80±15.55	84.73±9.48
MCV (fL)	66.33±0.33	65.00±1.00	66.00±0.58	63.67±0.88	64.00±1.00
MCH (pg)	19.70±0.23	19.83±1.36	20.30±0.20	18.67±1.13	19.67±0.18
MCHC (g/dL)	29.77±0.46	30.67±2.59	30.63±0.17	29.33±1.45	30.57±0.28
RDW-CV (%)	14.03±0.23	14.13±0.54	12.93±0.07	13.27±0.33	13.33±0.13

Values are expressed as mean±SEM (n=3). There are no significant differences in any parameters between groups (P>0.05; one-way ANOVA). Abbreviation: BW, body weight; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; WBC, white blood cell; LYM, lymphocyte; MON, monocyte; NEU, neutrophil; PLT, platelet; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW-CV, red cell distribution width - coefficient of variation.

Preliminary toxicity studies are typically performed using *in vivo* rodent models. To ensure that CE toxicity could be classified based on the widely accepted Globally Harmonized System (GHS) of classification, our study compliantly assessed the safety of CE extract using the OECD acute oral toxicity test guidelines. A single dose of CE or vehicle control was orally administered to healthy Wistar rats. Then symptoms of toxicity (tremors, convulsions, salivation, diarrhea, lethargy and coma) were checked every day for 14 days. Compared to the untreated control, doses of CE at 500 mg/kg up to 4000 mg/kg did not produce any noticeable adverse effects or toxicity (Table 1). No mortality was observed in any of the treated groups for the duration of the study. There were no significant changes in body weight (Table 1), serum biochemical parameters (Table 2) and hematological parameters (Table 3). Necropsy of key internal organs (liver, kidneys, lungs, heart, or digestive and reproductive organs) showed no noticeable abnormalities compared to the control animals. There were no changes in the relative organ weights of liver, kidneys, lungs, heart, spleen, testis/ovaries (Table 4) when compared to the control group (P>0.05). CE did not induce mortality and signs of toxicities at 4000 mg/kg.

The embryotoxic effect of CE on zebrafish

As CE may be beneficial for the management of diseases during pregnancy, it is crucial to establish whether CE induces embryotoxicity. CE treatments at 72 hpta induced noticeable embryotoxicity in

Table 4. Relative organ weights of rats treated with CE.

Organs	Treatment (mg/ kg BW)				
	Control	500	1000	2000	4000
Male					
Liver	3.86±0.12	3.91±0.04	3.45±0.16	3.75±0.21	3.55±0.09
Heart	0.43±0.03	0.39±0.02	0.43±0.04	0.42±0.02	0.44±0.02
Kidneys	0.77±0.01	0.81±0.02	0.75±0.02	0.79±0.01	0.74±0.02
Lungs	0.57±0.03	0.58±0.09	0.60±0.05	0.54±0.00	0.66±0.07
Spleen	0.26±0.01	0.30±0.03	0.29±0.01	0.26±0.01	0.29±0.03
Testis	1.23±0.01	1.25±0.03	1.26±0.04	1.27±0.04	1.27±0.06
Female					
Liver	3.73±0.35	3.68±0.04	3.42±0.10	3.52±0.11	3.64±0.05
Heart	0.49±0.04	0.41± 0.01	0.46±0.02	0.45±0.01	0.47±0.02
Kidneys	0.79±0.05	0.75±0.01	0.77±0.02	0.69±0.05	0.73±0.04
Lungs	0.67±0.09	0.73±0.09	0.81±0.09	0.79±0.12	0.74±0.05
Spleen	0.34±0.04	0.31±0.05	0.26±0.00	0.29±0.03	0.27±0.02
Ovaries	0.08±0.01	0.06±0.01	0.08±0.00	0.08±0.01	0.09±0.00

Values are expressed as mean±SEM (n=3). There are no significant differences in any parameters between groups (P>0.05; one-way ANOVA). Abbreviation: BW, body weight.

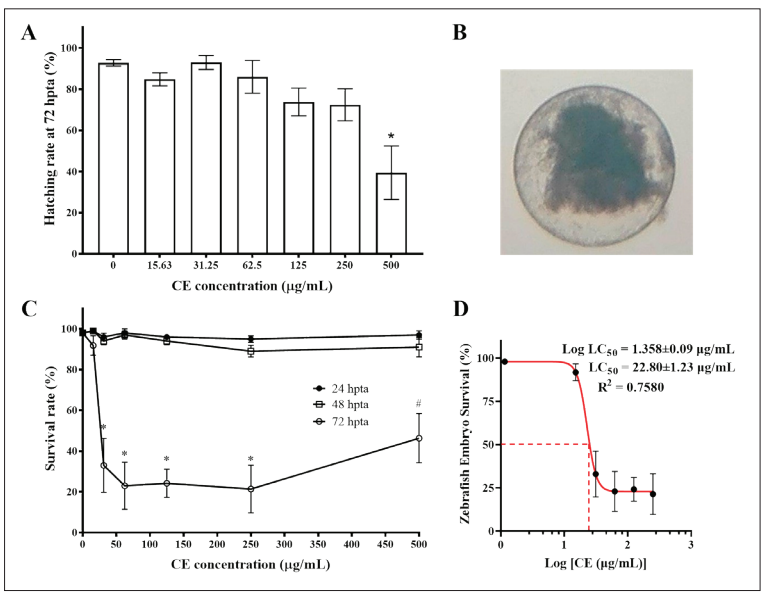

Fig. 1. Acute toxicity of CE on zebrafish embryos. **A** – Effect of CE on zebrafish hatching rate. The cumulative hatching rate is expressed as the percentage of hatched embryos at 72 h post-administration (hpta) of CE normalized to the initial unhatched embryos at 0 hpta, regardless of viability. Statistical analysis was performed using Kruskal-Wallis one-way analysis of variance with Dunn's post-hoc test. * P<0.05 in comparison to the untreated control. **B** – Representative image of CE-induced embryo coagulation. **C** – Cumulative effect of CE on zebrafish embryo survival rate at 24, 48, and 72 h. Embryos exhibiting signs of coagulation, lack of heartbeat at late developmental stages or apparent decomposition were considered dead. Repeated-measure two-way ANOVA with Tukey's post-hoc test was performed. * P<0.05 in comparison to 24 and 48 hpta at the same CE concentration. # P<0.05 in comparison to 24 hpta at 500 µg/mL CE. **D** – Median lethal concentration (LC_{50}) of CE at 72 hpta. The survival data of embryos exposed to log [CE (µg/mL)] at 72 hpta were fitted to a sigmoidal non-linear regression model (red line). All data shown are expressed as mean±SEM (n=5).

zebrafish embryos. Although the overall hatching rate of zebrafish embryos was not significantly affected by CE treatments at 15.63-250 µg/mL, a trend of reduced hatching rate in a concentration-dependent manner was observed. Exposure to CE at 500 µg/mL significantly decreased the hatching rate compared to the untreated control (P=0.0051; Fig. 1A).

Survival rates of embryos, including egg and larvae, were observed at 24, 48 and 72 hpta. Before hatching, between 24-48 hpta, the major lethal effect observed was coagulation (Fig. 1B). Our results showed insignificant changes in mortality between 24 and 48 hpta (Fig. 1C). However, treatments of CE at 31.25-500 µg/mL significantly decreased the survival rate at 72 hpta as compared to the untreated control and similar doses at 24-48 hpta (Fig. 1C). The median lethal concentration that caused 50% embryonic death (LC_{50}), calculated at 72 hpta, was 22.80±1.23 µg/mL (Fig. 1D). Representative images of zebrafish embryos treated with CE were monitored at 24, 48 and 72 hpta (Fig. 2). No apparent abnormalities in the morphology of the embryos were observed in any of the treatment conditions. Overall,

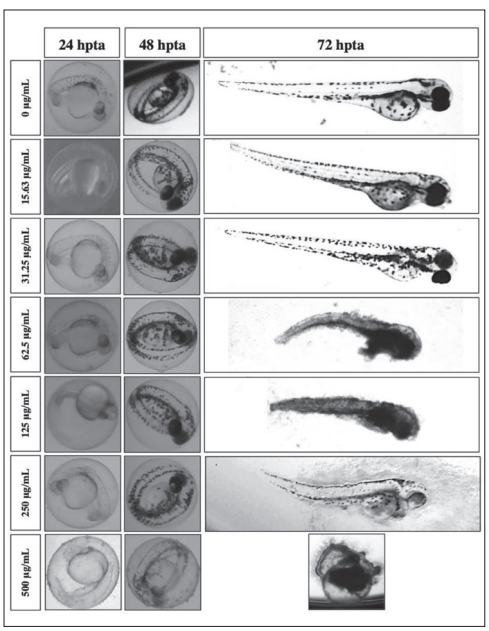

Fig. 2. Effect of CE on zebrafish embryo morphology. Representative brightfield images of zebrafish embryos treated with designated concentrations of CE at 24, 48 and 72 hpta.

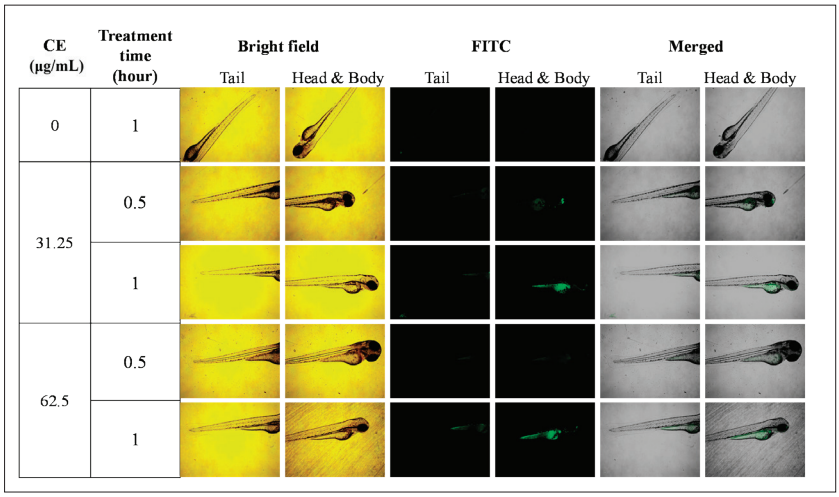

Fig. 3. Apoptotic effects of CE in zebrafish larvae**.** Healthy larvae (72 hpf) were exposed to designated concentrations of CE for 0.5 or 1 h. Acridine orange dye was used to visualize cell apoptosis in live larvae**.** Apoptosis signals (green) were detected on the FITC channel using a fluorescence microscope at 40× magnification**.** Apoptotic signals were overlaid onto brightfield images from the same field of view and are presented as merged images. Representative images of CE-induced apoptosis in yolk-sac regions of hatched larvae are shown (n=2).

although CE potentially lacked teratogenic effects, the extract demonstrated notable embryotoxicity when exposed to hatched larvae.

Apoptosis induced by CE

It was noted that hatched larvae rapidly died within a few hours after exposure to CE. To broadly assess how rapidly CE induced mortality in these larvae, healthy larvae were exposed to CE concentrations above the LC_{50} (30.25 and 60.5 μg/mL) for either 0.5 or 1 h. Acridine orange staining was performed to detect CE-induced apoptosis in larvae. Compared to the untreated control, signs of apoptosis became manifest in the yolk sac and yolk sac extension regions of the larvae in a dose and time-dependent manner (Fig. 3).

DISCUSSION

C. cainito bark aqueous extract has been reported to contain multiple bioactive compounds such as phenols, tannins, terpenoids, glycosides, saponin, phenolics and antioxidants [12]. We demonstrated that CE exerted antidiabetic effects in a mouse model and exhibited anticancer effects in the HepG2 hepatocellular carcinoma cell line [10]. Despite the potential benefits of CE, there is a lack of evidence regarding the safety of the extract. Due to the misconception that herbal remedies are always safe and beneficial for prophylaxis and multiple diseases, herbal remedy-associated toxicities are unclear and remain an understated concern. Safety reports regarding most herbal remedies, including *C. cainito,* are currently unavailable.

Toxicological and safety evaluation of herbal preparations are commonly performed in mouse and/or rat models [26-28]. The first recommended step to determine the systemic safety and toxicity of unknown compounds often involves identifying the lowest observed adverse effect level (LOEAL) and LC_{50} using a "single-dose" acute toxicity test [29-31]. Subsequently, further subacute and chronic repeated dose toxicity studies may then be performed to provide an accurate picture of the typical use of *C. cainito* for the treatment of subchronic (inflammation and viral infections) and chronic diseases (diabetes) [10]. The current study attempted to evaluate the safety and classified the acute toxicity class of CE according to the GHS classification. In compliance with international standards, this study used the OECD "single-dose" acute oral toxicity and FET guidelines as the means to assess the preliminary toxicities of CE.

We performed a single-dose acute oral toxicity test in a Wistar rat model to establish the LC_{50} range of CE, and to identify potential signs of toxicity necessary for toxicant classification. We showed that none of the tested concentrations of CE (500, 1000, 2000 and 4000 mg/kg) induced mortality or any apparent signs of toxicity (distress behavior, appearance, weight loss) during the 14-day duration of the experiment, suggesting that the LD_{50} of CE was higher than 4000 mg/kg, and indicating that the extract was well tolerated even at the OECD's highest dose range (2000-5000 mg/kg).

Despite the lack of apparent external signs of toxicity, it was critical to assess the effect of CE on the

morphology of key internal organs as any manifested abnormalities could indicate morbid signs of toxicity. The liver and kidneys are functionally crucial for drug/toxicant metabolism and elimination, respectively [32,33]. Therefore, altered serum levels of liver and kidney biomarker enzymes potentially indicate hepatotoxicity and renal toxicity. Altered blood biochemistry and hematologic parameters are also considered as signs of toxicity as the blood is deemed one of the most sensitive systems often affected by toxicants [26]. Our data revealed that none of the doses of CE significantly affected the morphology of any key organs, liver and kidney functional biomarkers, the hematological profile or the blood chemistry of Wistar rats. Cumulatively, these data indicated that single oral exposure to CE had no acute toxic effects in adult Wistar rats. Based on the GHS, the acute toxicity data suggested that the aqueous extract of *C. cainito* bark could be classified as category 5, exhibiting low to no acute toxicity [20,34].

As the CE demonstrated cytotoxicity in a highly proliferative hepatocellular carcinoma cell line but not in normal fibroblast cells [35], we speculated that CE might affect proliferative cells during embryonic development. Furthermore, *C. cainito* may potentially be used for treatment of gestational diabetes – the condition when diabetes is present during pregnancy. Hence, we believed that it was imperative to assess the effect of CE on embryotoxicity. Zebrafish embryos are commonly used for developmental and embryotoxicity assessment. The transparency and rapid development of zebrafish embryos enable mutagenesis and teratogenicity screening [36]. The early stage of zebrafish embryonic development is also susceptible to toxicant-induced toxicities [37]. Using the FET guideline, our results showed that CE exerted noticeable embryotoxicity at 72 hpta in a relatively concentration-dependent manner.

A delayed hatching rate is considered a sign of embryotoxicity [38]. We showed that the highest dose of CE (500 μg/mL) significantly inhibited the hatching rate at 72 hpta. Although the current study did not further investigate the mechanism of CE-induced delayed hatching, multiple plausible mechanisms associated with toxicant-induced delayed hatching were proposed. Zebrafish hatching is likely dependent on multiple enzymes, including zebrafish hatching enzymes (ZHE) 1 and 2 [39] and CD63, a proteolytic enzyme belonging to the tetraspanin family [40]. Previous studies suggested that these proteolytic enzymes are required for chorion-softening necessary for zebrafish hatching. We hypothesized that CE potentially modulated the activities of the hatching enzymes, resulting in hatching delay. However, further investigation is required to decipher the exact mechanism of CE on zebrafish hatching.

Unlike the adult Wistar rat model, we demonstrated that zebrafish embryos were sensitive to CE-induced toxicity with an LC_{50} of 22.80±1.23 μg/mL. Clear signs of observed embryotoxicity were embryo death in the form of egg coagulation and larvae death. None of the tested concentrations of CE induced any significant zebrafish embryo death at 24-48 htpa as compared to the control. At 72 hpta, except for the 500 μg/mL treatment that induced delayed hatching, CE caused significant zebrafish embryo death in a dose-dependent manner. However, it should be noted that the control zebrafish embryos were completely unhatched at 24 hpta, partially hatched at 48 hpta and completely hatched at 72 hpta. Therefore, the highest CE concentration (500 μg/mL) induced less toxicity as the embryo:larvae ratio was altered due to the delayed hatching. We speculated that the unhatched embryos were protected by the egg chorion, whereas the hatched larvae were directly exposed to CE, leading to enhanced sensitivity to CE-induced toxicity. The chorion acts as a protective barrier that prevents and reduces direct embryo exposure to toxicants [41]. We noted that newly hatched larvae often died within a few hours after direct CE exposure at 31.25-250 μg/mL. Thus, we postulated that the sensitivity of zebrafish to CE differed at different developmental stages where the hatched larvae were most sensitive to CE-induced toxicity.

In highly proliferative hepatocellular carcinoma cells, CE-induced caspase-3 cleavage subsequently resulted in apoptotic cell death [35]. Since CE caused relatively rapid zebrafish larvae death, we hypothesize that CE induced apoptotic cell death in these larvae. Hence, low concentrations of CE were chosen to assess whether CE induced organ-specific or non-specific toxicity in healthy zebrafish hatchling (72 hpf). The acridine orange staining results showed that CE exhibited a time- and concentration-dependent apoptotic effect. Interestingly, CE only induced selective apoptotic cell

death within the yolk sac and yolk sac extension regions. In zebrafish larvae, it is well recognized that the yolk and yolk sac are metabolically active. Zebrafish embryos exhibit similar anatomical traits to the early stages of the human embryo before blastocyte implantation and placenta formation. In terms of function, both the human and zebrafish embryonic yolks serve as the sole nutrient supply vital for the growth and development of embryos [42]. The yolk sac epithelium contains functionally active receptors involved in transporting nutrients, potentially playing key roles in toxicokinetics, notably the accumulation and distribution of toxicants. Toxicant accumulation in the yolk can induce toxicity by either damaging the yolk or altering the rate of nutrient usage, leading to yolk utilization impairment and embryo starvation [43]. Furthermore, maternal exposure may lead to toxicant deposition in the embryonic yolk of zebrafish [44,45]. We therefore speculated that CE was rapidly absorbed and selectively accumulated in the yolk and yolk sac extension of the larvae, resulting in cytotoxicity and apoptotic cell death. Unfortunately, to the best of our knowledge, the ability of the active ingredients of CE to cross the placenta has not been investigated. Human teratogenicity of the extract remains to be elucidated.

Collectively, although no acute toxicities were observed in the adult Wistar rat model, the current study demonstrated that CE exerted acute embryotoxicity in zebrafish embryos. Our data shows that CE markedly induced selective toxicity in the embryonic yolk region, consequently affecting the survival rate of zebrafish embryos. Therefore, caution should be exercised when using CE preparations in female patients planning for pregnancy or during pregnancy. Further investigation of CE is required to understand the mechanism of toxicity and assess the safety of extract use during pregnancy. As the extracts of this plant are often used for long-term management of diabetes, further thorough investigations should be performed to assess the safety of CE for subchronic and chronic applications.

CONCLUSION

The current study is the first to evaluate the safety of *C. cainito* bark extract, commonly used as a folk remedy for multiple ailments. Based on the OECD single-dose acute oral toxicity guidelines, our study in the adult Wistar rat model suggests that the safety of *C. cainito* bark extract could be classified as GHS category 5, i.e. low to no toxicity. In contrast, the extract interfered with normal embryonic hatching in the zebrafish model and reduced the survival rate of embryos. Hatched zebrafish larvae were more susceptible to CE than unhatched embryos likely due to the protection of the chorion. CE induced apoptosis and demonstrated selective toxicity in the zebrafish larvae yolk and yolk sac extension regions. Further study is necessary to decipher the mechanism of CE-derived embryonic toxicity and to determine the repeated subchronic and chronic safety of CE.

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Data availability: All data generated or analyzed during this study are included in this published article and its supplementary information file which can be accessed via the link: http://www.serbiosoc.org.rs/NewUploads/Uploads/Doan%20et%20al_7224_Supplementary%20Material.pdf.

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Supplementary Material

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Doan%20et%20al_7224_Supplementary%20Material.pdf