

Research Article

Safety Evaluation of Hot Water and Ethanol Extracts of *Crescentia cujete* L. Bark in Zebrafish Embryo Model for Potential Functional Ingredient Applications

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ABSTRACT

The exploration of natural plant sources for functional ingredients has gained significant attention in food science, with emphasis on bioactive compounds that can enhance human health. *Crescentia cujete* L. (calabash tree) is traditionally valued in folk medicine, yet the safety of its bark extracts for functional food applications remains underexplored. This study evaluated the safety performance of hot water and ethanol extracts of *C. cujete* bark using the zebrafish embryo toxicity (ZFET) model, an established in vivo tool for early toxicological screening. Fertilised zebrafish embryos were exposed to graded concentrations of each extract from 0 to 120 hr post-fertilisation (hpf), and endpoints including survival, hatching, heart rate, and morphological development were monitored. The hot water extract demonstrated a significantly higher median lethal concentration (LC₅₀) of 9.69 mg/mL, while the ethanol extract exhibited a markedly lower LC₅₀ value of 0.42 mg/mL ($p < 0.001$), suggesting higher relative safety of the aqueous preparation. Dose-dependent reductions in survival and hatching rates were observed, with ethanol extracts producing more severe developmental delays and cardiac abnormalities. Heart rate disturbances, pericardial edema, and axial malformations were most prominent at concentrations near the ethanol LC₅₀ value, whereas the hot water extract induced only mild effects at higher concentrations. No significant changes in heart rate were observed with the hot-water extract, whereas significant reductions were observed at higher concentrations of the 50% ethanol extract ($p < 0.05$). These findings indicate that the extraction solvent significantly influences the safety profile of *C. cujete* bark. The hot water extract demonstrated lower developmental toxicity in the zebrafish embryo model and warrants further investigation through phytochemical characterisation and mammalian toxicity studies. By establishing the first zebrafish-based safety evaluation of *C. cujete* bark, this study highlights the importance of integrating toxicological validation in the development of novel plant-derived functional ingredients.

Key words: Bark, calabash, functional food, toxicity, zebrafish

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INTRODUCTION

The development of functional ingredients has become a central focus in food science and technology, as modern consumers increasingly seek natural dietary components that provide health benefits beyond basic nutrition. Functional ingredients are typically bioactive compounds derived from plants, animals, or microorganisms that deliver physiological benefits, such as enhancing immunity, reducing inflammation, and protecting against chronic diseases (Kalogerakou *et al.*, 2024; Mekinić & Šimat, 2025). Among these, plant-derived phytochemicals—including flavonoids, phenolic acids, terpenoids, alkaloids, and saponins—are of particular interest, owing to their broad biological activities and long-standing role in traditional medicine (Vignesh *et al.*, 2024; Frumuzachi, 2025). The incorporation of such bioactivities into functional foods and nutraceuticals offers significant potential, provided that both

efficacy and safety are scientifically validated (Faienza *et al.*, 2024; Hajzer *et al.*, 2025).

Crescentia cujete L. (commonly known as the calabash tree) is a tropical species traditionally used in folk medicine across Asia, Africa, and Latin America. Different parts of the plant, including the fruit, leaves, and bark, have been reported to contain diverse secondary metabolites with therapeutic potential. Ethnobotanical reports suggest its applications for treating respiratory illnesses, digestive problems, skin infections, and inflammatory disorders (Olaniyi *et al.*, 2022; Gonzales *et al.*, 2023). Phytochemical studies indicate that *C. cujete* contains flavonoids, phenolics, tannins, terpenoids, and saponins, compounds known for antioxidant, antimicrobial, and anti-inflammatory effects (Gutierrez, 2022; Gonzales *et al.*, 2023). While most research has centred on its fruit extracts, the bark has received less scientific attention despite evidence that

bark tissues are often rich in condensed tannins and other potent phytochemicals relevant to food applications.

Safety validation is a critical prerequisite in the development of functional food ingredients. It was therefore hypothesised that the extraction solvent determines the developmental toxicity of *C. cujete* bark extracts, and specifically that the semi-polar 50% ethanol extract would yield a significantly lower LC₅₀ than the aqueous hot water extract in the zebrafish embryo toxicity assay. This hypothesis was tested by comparing the concentration–response relationships of the two extracts for survival, hatching, heart rate and morphological development. In a previous preliminary study, Sagrin *et al.* (2019) evaluated *C. cujete* fruit and bark extracts using the brine shrimp lethality assay (BSLA) and reported low toxicity, suggesting potential for safe use. On the other hand, a recent report by Liman *et al.* (2023) demonstrated that *C. cujete* leaf extract exhibited moderate toxicity, as determined by BSLA, with an LC₅₀ value of 383.27 µg/mL. However, the brine shrimp assay is a general cytotoxicity test and does not provide detailed insights into developmental, physiological, or organ-specific toxicity. Thus, more advanced bioassays are required to establish comprehensive safety profiles.

The zebrafish embryo toxicity (ZFET) model has emerged as a reliable and ethically favourable *in vivo* system for early-stage toxicological assessments. Zebrafish (*Danio rerio*) share high genetic homology with mammals, display transparent embryonic development, and enable real-time observation of endpoints such as survival, hatching, morphological malformations, and cardiac activity (Kimmel *et al.*, 2023; Lee & Kim, 2024). This model is recognised by regulatory guidelines and has been increasingly applied to evaluate natural products, medicinal plants, and food-derived bioactive compounds (OECD, 2013; Shen *et al.*, 2024).

By providing LC₅₀ values and dose–response outcomes, the ZFET assay serves as a bridge between preliminary *in vitro* screening and mammalian studies.

Although *C. cujete* has shown promise as a medicinal and nutritional resource, scientific validation of the safety of its bark extracts for functional food use is scarce. Previous cytotoxicity studies, such as Sagrin *et al.* (2019), provide limited evidence, and no systematic evaluation of its developmental and physiological safety has been reported. Moreover, extraction methods significantly influence phytochemical composition and biological effects, yet the comparative safety outcomes of aqueous versus ethanol bark extracts remain unexplored. Without such data, the translation of *C. cujete* bark into functional food applications remains uncertain.

This study aimed to evaluate the safety performance of hot water and ethanol extracts of *C. cujete* bark using the zebrafish embryo toxicity (ZFET) model. Specifically, the work sought to determine the LC₅₀ values and to assess survival rate, hatching rate, heart rate, and morphological development of zebrafish embryos exposed to the extracts. By comparing aqueous and ethanol extracts, this study provides new insights into the influence of extraction solvents on safety profiles, thereby supporting evidence-based selection of preparation methods for potential functional food and nutraceutical applications.

MATERIALS AND METHODS

Sample collection

C. cujete bark was collected from six reproductively mature trees in a private orchard in Puchong and Universiti Putra Malaysia (UPM) campus, Selangor, Malaysia. Although the exact ages of the trees were unknown, only healthy trees showing no visible signs of disease or mechanical damage were selected. Sampling was conducted between May and June 2020, during the relatively dry mid-year

period. Bark was collected from third-order branches measuring 1.0–1.5 cm in diameter and located approximately 1.3 m above ground level. Approximately 500 g of bark was collected from each tree. The samples from all six trees were pooled in approximately equal proportions and homogenised to produce a single composite sample. Three independent extractions were prepared from the composite sample, and each extract was analysed separately. The species was authenticated by Dr Mohd Firdaus Ismail, and the corresponding voucher specimen (SK 3136/17) is preserved at the Herbarium, Institute of Bioscience, Universiti Putra Malaysia. Bark samples were manually removed from the trunk using sterilised stainless-steel tools, sealed separately in black polyethylene bags, transported in a cooler to the UPM Food Analysis Laboratory, and refrigerated at 4°C until processing.

Sample preparation

Bark samples were washed with distilled water, chopped into 1 cm² fragments, and stored at –20 ± 1°C for 24 hr. Samples were freeze-dried (FreeZone 12 L, Labconco Corporation, Kansas City, USA) for 3–4 days, pulverised, and stored at –20°C before extraction (≤4 weeks).

Extraction procedures

Hot water extraction (HW)

Approximately 20 g bark powder was refluxed with 500 mL distilled water at 98 ± 2°C for 1 hr (Lin *et al.*, 2016). The extract was cooled, filtered, frozen (–18°C, 24 hr), and lyophilised for 1 week.

Ultrasound-assisted ethanol extraction (ET)

Approximately 5 g bark powder was mixed with 100 mL of 50% ethanol (v/v, analytical grade), sonicated at 40 kHz, 40°C for 2 hr (Branson® 3510, Branson Ultrasonics Corporation, Danbury, USA), filtered, and concentrated under reduced pressure (Rotavapor R-210, Büchi Labortechnik AG, Flawil, Switzerland) (Sagrin *et al.*, 2019). Extracts were frozen, lyophilised, and were stored at –18°C (≤4 weeks) for further analyses.

Zebrafish maintenance and breeding

The experimental procedures involving zebrafish were conducted in accordance with protocols approved by the IACUC at the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. The Zebrafish Embryo Toxicity (ZFET) assay was carried out following the established guidelines from the Organisation for Economic Co-operation and Development (OECD) (OECD, 2013). Adult zebrafish were maintained at the Faculty of Biotechnology, UPM. Breeding was induced overnight, and fertilised embryos were collected, rinsed in Danio-SprintM solution, and incubated for 2 hr. Only viable embryos were used. Each exposure group, including the untreated control, was tested with 24 embryos (one embryo per well in 96-well plates), and the assay was performed in two independent biological replicates using embryos from separate breeding events, giving a total of 48 embryos per exposure group. Before exposure, embryos were examined under a stereomicroscope and staged according to the criteria of OECD Test Guideline: only fertilised embryos showing symmetrical cleavage, an intact transparent chorion and no sign of coagulation at the 4- to 64-cell stage (approximately 1–2 hpf) were retained. Unfertilised, coagulated, irregularly cleaving or mechanically damaged embryos were discarded and replaced before exposure began.

Zebrafish embryo toxicity assay

Stock solutions were prepared at 10 mg/mL (HW) and 5 mg/mL (ET). Dilutions (96-well plate) produced test concentrations of 4–10 mg/mL (HW) and 0.08–1.25 mg/mL (ET). One embryo per well was exposed to 200 µL of solution. Controls used Danio-SprintM medium only. Embryos were monitored every 24 hr up to 120 hpf for survival, hatching, heart rate, and morphological malformations. Images were captured using an inverted microscope with a digital camera (Thunder Imager 3D Live Cell & 3D Assay, Leica Microsystems GmbH, Wetzlar, Germany). Heartbeats were counted manually for three embryos per concentration (1 min).

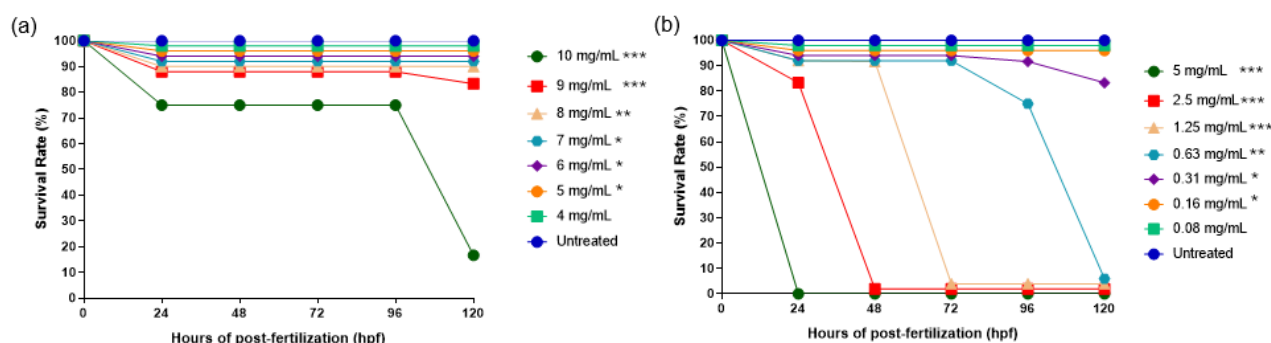


Fig. 1. Survival rate of zebrafish embryos at 0 to 120 hr after exposure to *Crescentia cujete* (a) barks extracted in hot water at concentrations of 4-10 mg/mL, (b) barks extracted in 50% ethanol at concentrations of 0.08-5.0 mg/mL. No embryos survived at concentrations tested >0.63 mg/mL after 120 hr post fertilisation (hpf). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ are considered significant as compared to untreated zebrafish embryos.

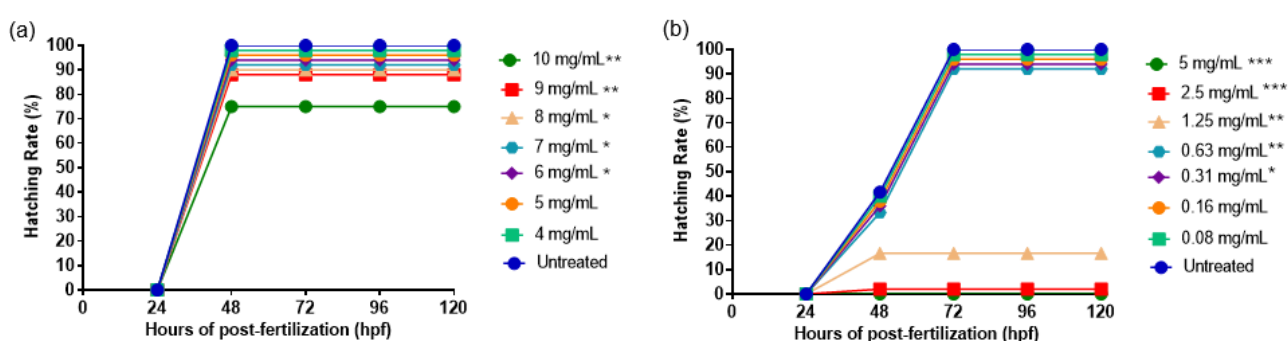


Fig. 2. Hatching rate of zebrafish embryos at 0 to 120 hr after exposure to *Crescentia cujete* (a) bark at concentrations of 4-10 mg/mL. A high hatching rate (>70%) was observed at all concentrations. (b) barks extracted in 50% ethanol at concentrations of 0.08-5.0 mg/mL. A low hatching rate (<20%) was observed at concentrations >1.25 mg/mL due to a high mortality rate. A high hatching rate was observed at concentrations <0.63 mg/mL (>90%). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ are considered significant as compared to untreated zebrafish embryos.

Determination of lethal concentration (LC₅₀) and statistical analysis

The LC₅₀ values and their 95% confidence intervals (CIs) were estimated using four-parameter logistic regression with the IC₅₀ Calculator (AAT Bioquest). The regression models showed good fit, with R² values ranging from 0.955 to 0.997. GraphPad Prism 8.0 (GraphPad Software, Inc.) was used for data analysis. Results were expressed as mean $\pm$ SEM. One-way ANOVA with Dunnett's test was applied ($p < 0.05$, * $p < 0.01$, ** $p < 0.001$).

RESULTS AND DISCUSSION

The zebrafish embryo toxicity (ZFET) assay provided comprehensive insights into the safety profile of *C. cujete* bark extracts prepared by hot water (HW) and ethanol (ET) methods. Outcomes were assessed across four key endpoints: survival, hatching, mortality, heart rate, and morphological characteristics over a 120-hr exposure period.

Survival rate

The survival rate of zebrafish embryos to *C. cujete* bark extracts revealed clear differences between the two solvents (Figure 1). The hot water extract had high survival rates across concentrations up to 8 mg/mL, with only slight mortality observed at the maximum tested dose of 10 mg/mL (Figure 1a). By contrast, embryos exposed to the 50% ethanol extract displayed significantly reduced survival beginning at 0.31 mg/mL, with survival dropping sharply above 0.63 mg/mL (Figure 1b).

The higher toxicity of the ethanolic extract may be associated with ethanol-extractable compounds, although further phytochemical characterisation is required to confirm this hypothesis. Ethanol and other semi-polar solvents extract alkaloids, terpenoids, and certain

phenolics that are more bioavailable and toxic than the more polar constituents of aqueous extracts (Chahardehi *et al.* 2020). These results are consistent with the findings of Zahid *et al.* (2025) and Limanan *et al.* (2023), who reported that the use of ethanol as an extraction solvent resulted in a higher yield of plant secondary metabolites, especially flavonoids, which in turn contributed to toxic effects on zebrafish embryo development. From a functional ingredient perspective, the concentration-dependent toxicity highlights the importance of determining safe intake thresholds. Low-dose bark extracts may be safely incorporated into food matrices, but higher doses could present risks. Therefore, survival data serve as a foundation for establishing safe functional ingredient dosages.

Hatching rate

In embryos treated with the hot water bark extract (Figure 2a), hatching rates remained high (>90%) at concentrations ≤ 8 mg/mL, with minor delay observed only at the highest concentration (10 mg/mL), where hatching at 48 hpf dropped (70%). In contrast, for ethanol bark extract (Figure 2b), hatching rates dropped sharply even at concentrations >0.63 mg/mL. At 1.25 mg/mL, the hatching rate was very low (<20%), likely due to high mortality rates.

Delayed hatching is often associated with impaired enzymatic degradation of the chorion, which is essential for embryo release (Zhang *et al.* 2018). Plant-derived phenolics and flavonoids can bind proteins or alter enzyme activity, which may explain the delayed hatching observed. Similar outcomes were reported with *Moringa oleifera* seed and leaf extracts, where phenolic compounds disrupted normal hatching processes or delayed hatching in zebrafish embryos (Mohamad Shariff *et al.*, 2020; Ganesan *et al.*, 2020). This also accords with earlier observations by Hosseini *et al.* (2025), who

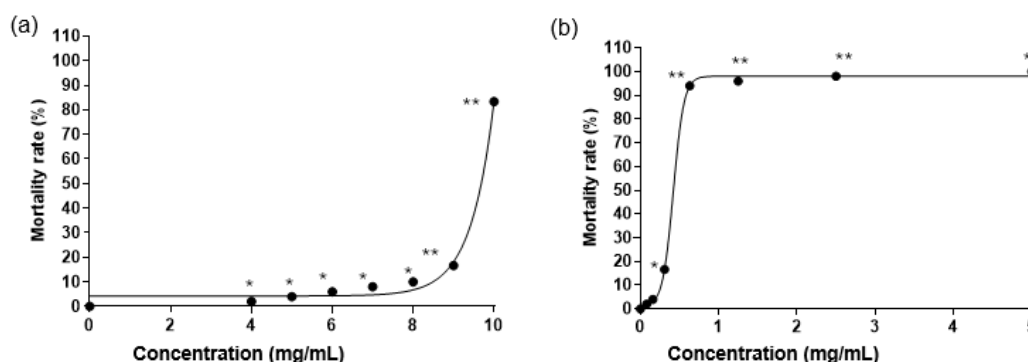


Fig. 3. Mortality rate of zebrafish embryos at 120 hr post-fertilisation (hpf) after exposure to *Crescentia cujete* bark (a) extracted in hot water at concentrations of 4-10 mg/mL. The LC_{50} value towards zebrafish embryos was 9.69 mg/mL. (b) Extracted in 50% ethanol at concentrations of 0.08-5.0 mg/mL on zebrafish embryos; mortality rate after 120 hr post-fertilisation (hpf). The LC_{50} value towards zebrafish embryos was 0.42 mg/mL. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ are considered significant as compared to untreated zebrafish embryos.

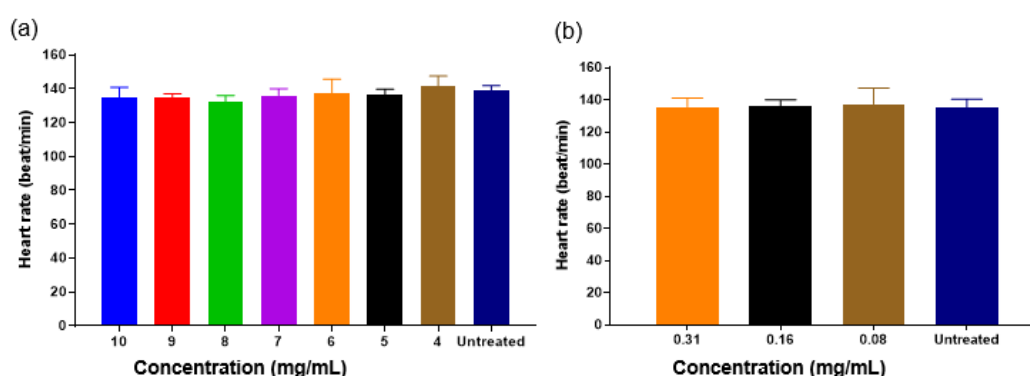


Fig. 4. Heart rate of zebrafish embryos at 96 hr of post fertilization (hpf) after exposed to *Crescentia cujete* barks (a) extracted in hot water at concentrations of 4-10 mg/mL (b) extracted using 50% ethanol at concentrations range 0.08-0.31 mg/mL on heart rate of zebrafish embryos at 96 hpf. Results are presented as mean $\pm$ SEM for $n = 24$. No data at concentrations > 0.31 mg/mL due to death. No significant differences were observed between any treatment and the untreated control ($p > 0.05$).

showed that excess concentrations of polyphenols interfere with the oxidative balance and cellular metabolism in an animal model by chelating metal cofactors such as Fe^{2+} , Zn^{2+} , and Cu^{2+} thereby reducing enzyme efficiency. In the context of functional foods, delayed hatching suggests that excessive exposure to ethanol extracts could interfere with normal development. However, at lower concentrations where no hatching delays occurred, the extracts remain promising as functional additives, which reflects the importance of precise concentration or accurate dosage control to ensure future safe applications in humans.

Mortality rate

The LC_{50} values were calculated at 9.69 mg/mL for hot water extracts and 0.42 mg/mL for ethanol extracts, confirming the relatively higher toxicity of the latter (Figure 3a, b). The lower LC_{50} value of ethanol extracts suggests a more potent concentration of active compounds.

These findings are in agreement with Sagrin *et al.* (2019), who reported higher cytotoxicity in ethanol extracts of *C. cujete* using brine shrimp assays. The consistency across different extraction models strengthens the evidence that solvent type influences extract potency. Similar LC_{50} ranges have been reported for other functional botanicals such as *Curcuma longa* and *Allium sativum*, where aqueous fractions were generally safer than ethanol fractions (Ton *et al.* 2006; Park *et al.* 2015). According to the US Environmental Protection Agency classification, LC_{50} values exceeding 100 mg/L are categorised as practically nontoxic to aquatic animals (USEPA, 2008). Accordingly, the hot-water and 50% ethanol extracts, with

LC_{50} values of 9,690 and 420 mg/L, respectively, fall within this category, although the ethanol extract showed greater relative toxicity. For functional ingredient development, the LC_{50} values indicate that bark extracts are safe within a wide concentration range. However, ethanol extracts require stricter dosing limits due to their stronger bioactivity. These values provide essential toxicological benchmarks for future formulation and regulatory considerations.

Heart rate

Heart rate assessment showed no significant difference from control in embryos exposed to hot water extract (Figure 4a), with average beats per min (bpm) remaining between 137–140 bpm at concentrations up to 10 mg/mL. Conversely, 50% ethanol extract (Figure 4b) caused measurable reductions in heart rate (bradycardia) at concentrations ≥ 0.31 mg/mL, along with pericardial edema that prevented reliable measurements at higher doses.

Cardiotoxicity in zebrafish, expressed as bradycardia and pericardial edema, is a common response to toxic phytochemicals and xenobiotics (Jayasinghe *et al.* 2019). Neither extract caused a statistically significant change in embryonic heart rate under the tested conditions. However, the pericardial oedema observed following exposure to the ethanol extract warrants further investigation.

Morphological characteristics

Morphological abnormalities, including pericardial edema, yolk sac edema, and spinal curvature, were observed in embryos treated with higher ethanol extract concentrations (≥ 200 μ g/mL). Hot water extracts produced fewer malformations, with only mild pericardial edema observed at the highest dose. Figure 5a and Figure 5b illus-

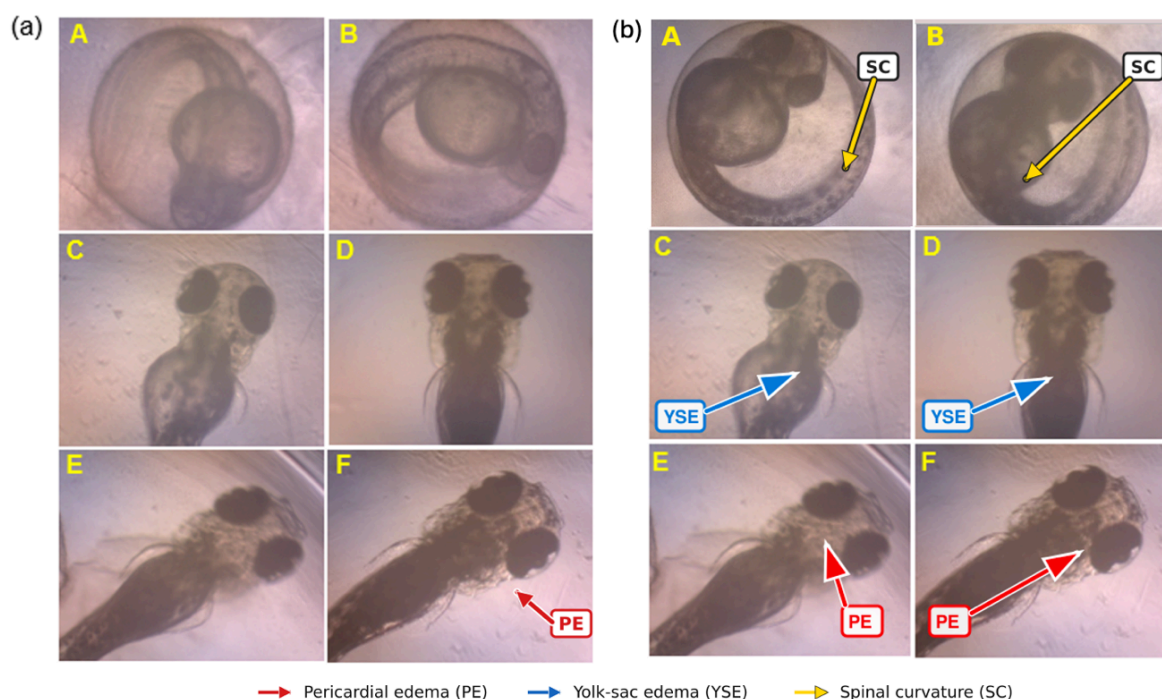


Fig. 5. Zebrafish embryo and larval development after treatment with *Crescentia cujete* bark extract in (a) hot water at a concentration of 8.0 mg/mL, (b) 50% ethanol extract at a concentration of 0.31 mg/mL. Images were captured using an inverted microscope at 100X, at 0 hpf (A) and 24 hpf (B), whereas images were captured at 40X magnification at 48 hpf (C), 72 hpf (D), 96 hpf (E), and 120 hpf (F). hpf, hr post fertilisation. Yellow arrows indicate spinal curvature (SC), blue arrows indicate yolk-sac oedema (YSE), and red arrows indicate pericardial oedema (PE).

trate representative images of zebrafish embryos with morphological changes after exposure. Such teratogenic effects are commonly associated with compounds capable of interfering with embryonic signalling pathways (Nguyen *et al.*, 2024). Similar solvent-dependent differences have been reported in other bark extracts. For example, *Cassia fistula* stem bark extracts exhibited an aqueous LC₅₀ of 213.6 ppm, while the ethanol extract showed an LC₅₀ of 63.5 ppm in zebrafish (Udaya *et al.*, 2020).

In brine shrimp lethality assays conducted by Sagrin *et al.* (2019), the ethanol extracts of *C. cujete* demonstrated higher toxicity compared to aqueous extracts. In other medicinal plants such as *Azadirachta indica* and *Ocimum sanctum*, ethanol fractions were reported to be more bioactive yet exhibited higher embryotoxicity than water-based extracts (Sathishkumar *et al.*, 2022; Choudhury *et al.*, 2023). Similarly, *Allium sativum* extracts demonstrated dose-dependent morphological effects in zebrafish embryos, including delayed hatching and axial malformations, highlighting the importance of dosage optimisation in functional ingredient applications (Islam *et al.*, 2023).

A cumulative body of evidence has shown the potential use of different *C. cujete* parts as functional ingredients. A recent study by Isiaq (2025) demonstrated modulation of inflammatory pathways in a mouse paw edema model after being fed ethanolic extract of *C. cujete* fruit for 12 hr. Furthermore, treatment with a cream-based formula incorporated with 2% *C. cujete* fruit in Sprague Dawley rats for seven days promoted the generation of new cells and collagen by improving fibroblast growth factor and preventing inflammation (Wijayanti *et al.*, 2024). In vivo, administration of *C. cujete* extract in hypoxic rat models reduced malondialdehyde levels and elevated catalase activity, suggesting protection against oxidative stress in cardiac tissues (Limanan *et al.*, 2023). Despite the limited in vivo studies on *C. cujete* bark, the dichloromethane fraction exhibited potent antioxidant activity (IC₅₀ = 95.83 ± 19.64 µg/mL), while the water fraction showed mild antibacterial activity

against *Escherichia coli*, indicating the plant's potential as a source of bioactive compounds for functional ingredient development (Nitami *et al.*, 2018). Therefore, the present zebrafish embryo toxicity data could serve as important insights for assessing the potential health risks associated with *C. cujete* extracts. Nevertheless, further investigations are warranted to determine the LC₅₀ values of different solvent fractions, particularly the dichloromethane and aqueous extracts, to guide dose selection for pre-clinical studies before investigating their efficacy in humans. Such evaluations are essential for functional food or nutraceutical applications from *C. cujete* extracts. Moreover, this study supports the feasibility of using the zebrafish model as a reliable tool for early-stage safety screening of *C. cujete*-derived bioactive compounds.

CONCLUSION

This study evaluated the safety of hot water and 50% ethanol extracts of *C. cujete* bark using the zebrafish embryo model. Both extracts demonstrated concentration-dependent effects on survival, hatching, mortality, heart rate, and morphology, with ethanol extracts exhibiting higher toxicity. LC₅₀ values confirmed this difference, at 9.69 mg/mL for hot water extracts and 0.42 mg/mL for ethanol extracts. The safety evaluation of *C. cujete* bark extracts provides critical insights for their potential use as functional food ingredients. Functional ingredients are defined not only by their health-promoting bioactivity but also by their safety in human consumption. The present study demonstrates that hot water extracts of *C. cujete* bark exhibit a wider safety margin, making them more promising for incorporation into nutraceuticals and functional foods. Ethanol extracts, richer in phytochemicals, present higher toxicity risks and would require precise dosage regulation. Overall, the study supports the potential of *C. cujete* bark as a source of safe functional ingredients, provided that extraction methods and dosage are carefully optimised.

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ETHICAL STATEMENT

This study was conducted in accordance with the ethical standards of the institutional and/or national research committee.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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