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# Toxicity of activated-charcoal purified *Centella asiatica* (L.) Urb. methanolic extract on zebrafish embryos

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## ABSTRACT

**Background:** Purification of *Centella asiatica* methanolic extract using activated charcoal (ACP-CME) to isolate its non-aromatic compounds is a novel method that requires rigorous foundational safety data for the next validation stage.
**Objectives:** The study aimed to determine the $LC_{50}$ and identify toxicity signs of activated charcoal-purified *Centella asiatica* methanolic extract (ACP-CME) on zebrafish embryos.
**Methods:** Fish Embryo Acute Toxicity (FET) was conducted following the 2013 standardized guidelines from OECD number 236. The zebrafish embryos were divided into eight groups (n=20/group), with an additional internal control in each group (n=4), namely 5 ACP-CME groups (ACP-CME at concentrations of 15.625, 31.25, 62.5, 125, and 250 ppm), a negative control group (distilled water), a positive control group (3,4-dichloroaniline), and a solvent control group (DMSO 0.2%). The observation was performed every 24 hours for 96 hours on four lethality parameters (coagulation, somite formation, tail-bud detachment, and visible heartbeat). The lethal concentration 50 ($LC_{50}$) was calculated using Microsoft Excel 2019 to determine the signs and levels of toxicity of the extract based on the observational data.
**Results:** ACP-CME exhibited concentration-dependent lethality caused by coagulation and absence of heartbeat, with 100% mortality at 250 ppm. Some abnormalities caused by ACP-CME exposure were spotted at concentrations of 31.25 and 125 ppm, with the occurrence of 15% and 10%, respectively.
**Conclusion:** The $LC_{50}$ of ACP-CME was 39.56 ppm. Malformation effects included lordosis, kyphosis, delayed tail-bud detachment, abnormal fin, somite formation failure, hatching failure, and yolk sac edema.

## INTRODUCTION

Purification through adsorption is a well-recognized attribute of activated charcoal. Although its use in purifying plant-based extracts is uncommon, activated charcoal has been shown to adsorb non-aromatic contaminants in extracts through π–π effective dispersion and donor-acceptor processes.[1] Structural dispersion, or π–π stacking, is a non-covalent interaction between π (pi) electron systems of aromatic compounds.[2] Activated charcoal selectively interacts with molecules containing aromatic groups, allowing non-aromatic substances such as triterpenoids to pass through the carbon layer.[3] The interaction of activated charcoal and non-aromatic compounds was expected to result in the low toxicity level of *Centella asiatica*, as most aromatic compounds are separated from the purified extract. Aromatic compounds such as flavonoids can have toxic effects at specific concentrations. Hence, it was anticipated that the extract would have a low level of toxicity, as the majority of the aromatic components had been isolated from the pure extract. This phenomenon is elucidated by aromatic compounds, including flavonoids, which can display hazardous properties in particular quantities.[4] Although activated

charcoal is commonly used as an oral decontaminant in an emergency department for patients suffering from acute oral medication poisoning, the use of activated charcoal in patients has also shown no significant impact on death rates.[5] But, the safety and efficacy of using the novel method of activated charcoal as a purifying agent for herbal extract, as in *Centella asiatica* methanolic extract, has not been established. The optimization of *Centella asiatica* methanolic extract using activated charcoal (ACP-CME) has the potential to alter the structure and therapeutic properties as the concentration of non-aromatic compounds in *Centella asiatica* becomes purer. Therefore, further studies are essential to determine the safety of this purification method.[6]

*Centella asiatica* is a plant known for its various beneficial properties. Recent studies have shown its potential for wound healing and neuroprotection.[7] *Centella asiatica*, locally known as *Pegagan*, is commonly used in Indonesia for its herbal medicinal properties and skincare benefits, which are attributed to its diverse pharmacological activities. The wide application of *Centella asiatica* prompts a more focused purification method to get the necessary compounds from this plant. The effectiveness of a secondary purification process that utilizes activated charcoal remains unclear. The initial testing phase is crucial to the next stage of safety testing, as it holds significant importance to the overall process by laying the groundwork for the safety data that will be developed and enhanced. The Zebrafish Embryo Acute Toxicity Test is one of the in vivo methods for toxicity testing due to its similarity to human genetics. There is also a strong link between the teratogenic effects of embryo abnormalities and those in humans.[8,9] The transparency of zebrafish embryos allows for precise observation of developmental processes and potential toxic effects. Zebrafish Embryo Toxicity test integrates acute toxicity testing, encompassing lethality metrics and teratogenic data related to abnormalities. This method has been globally standardized, particularly in accordance with OECD guidelines No. 236 in 2013. Since this test is the initial phase, it is crucial to identify any adverse effects to ensure that only safe and effective substances progress to the more complex and costly testing phases. The results of this study enable further experimentation in rat models, allowing for the comprehensive collection of toxicity data that can be utilized to systematically and ethically advance new extracts and their applications.

Purification using activated charcoal is expected to enhance the efficacy of *Centella asiatica* as a foundational element in herbal medicine applications within Indonesia. This purification is crucial for removing impurities and potentially harmful substances, thereby enhancing the efficacy and safety of the extract. This study adds to the existing literature by providing empirical evidence regarding the safety of purified *Centella asiatica* extracts. This information is crucial for advancing standardized herbal supplements, enhancing consumer protection, and increasing confidence in the use of traditional therapeutic agents. Furthermore, it facilitates the incorporation of conventional medicine practices into Indonesia's healthcare framework by generating scientific evidence on commonly used botanical components. The results provide significant insight about the safety profile of *Centella asiatica* and validate its safe application in both traditional and contemporary Indonesian medical practices. This highlights the necessity of thorough scientific research on herbal medicines and contributes to the health and medical sciences. Investigation into the mechanisms underlying the reported toxic effects, to develop innovative therapeutic applications or improve safety protocols for herbal formulations. This study aimed to determine the $LC_{50}$ and investigate the toxicity indicators of activated charcoal-purified *Centella asiatica* methanolic extract (ACP-CME) on zebrafish embryos.

## METHODS

### Study design

This experimental study aimed to assess the acute toxicity of ACP-CME on zebrafish embryos using the Fish Embryo Acute Toxicity (FET) test, following the OECD Test Guideline 236.

### Population and sample

The ACP-CME was acquired from the Bachelor of Pharmacy Program at the University of August 17, 1945, Jakarta, and purified using activated charcoal (Sigma-Aldrich C9157-500G).

Phytochemical screening was conducted following previously established methods.[3]

**Embryos Preparation**

The breeding stage exhibited a male-to-female ratio of 2:1, respectively. The zebrafish parents were maintained in aquarium water at pH 7.3, a temperature of 28˚C, and a lighting cycle of 14 hours of light and 10 hours of darkness. During the light cycle, zebrafish parents were segregated into separate aquariums based on sex, with males and females housed apart and brought together just before the onset of the dark cycle. The next day, fertilization was initiated in the male fish by commencing the light period, which encouraged the fertilization of eggs from the female zebrafish. Fertilized fish eggs were collected and placed in a petri dish, followed by embryo selection.[10] Embryos used were fertilized and less than 6 hours, with a standard age of 3 hours to ensure compound penetration through the embryo membrane. Blastula development began approximately 15 minutes after fertilization at 26˚C, progressing through cell divisions into 4, 8, 16, and 32 blastomeres.

**Experimental intervention**

Embryos that met the inclusion criteria were separated into three control groups and five groups treated with varying concentrations of ACP-CME (n=20). Five treatment groups received ACP-CME at concentrations of 15.625, 31.25, 62.5, 125, and 250 parts per million (ppm). Control groups included a positive control group using 3,4-dichloroaniline (3,4-DCA), a negative control (distilled water), and a solvent control (0.2% dimethyl sulfoxide, DMSO). All group assignments followed OECD standard guidelines. Each group consisted of twenty-four embryos in a 24-well plate, with an additional four embryos as an internal control.

**Observation**

Embryos were observed under a Zeiss Stereo microscope every 24 hours for 96 hours post-exposure to ACP-CME. Four lethality parameters (embryo coagulation, absence of somite formation, lack of heartbeat, and failure of tail detachment) were included in the observation to calculate the $LC_{50}$ as quantitative data. Developmental abnormalities were included as qualitative data.[10] The test results were validated through the following criteria:

a) Fertilization rate per batch was ≥ 70%
b) Water temperature remained at 26 ± 1 °C throughout the test
c) Survival in the negative control was ≥ 90% at 96 hours
d) Mortality in the positive control was ≥ 30% at 96 hours
e) Hatching rate in the negative control was ≥ 80% at 96 hours
f) Dissolved oxygen concentration was ≥ 80% in both the negative control and the highest test concentration at 96 hours

**Data analysis**

The percentage of each parameter observed was calculated with the formulas below:[11]

1. % mortality
   = (number of dead embryos)/(total embryos) ×100%
2. % coagulation
   = (number of coagulated embryos)/(total embryos) × 100%
3. % somite formation
   = (number of embryos with somite formation)/(total viable embryos) ×100%
4. % tailbud detachment
   = (number of embryos with tailbud detachment)/(total viable embryos) ×100%
5. % visible heartbeat
   = (number of embryos with visible heartbeat)/(total viable embryos) ×100%
6. % abnormalities

= (number of embryos with abnormalities)/(total embryos) × 100%

Data analysis was conducted using Microsoft Excel 2019 to calculate the percentage and determine the $LC_{50}$ through a probit analysis.

**Ethical statement**

The study received ethical approval from the Medical and Health Research Ethics Committee of the Faculty of Medicine at Universitas Islam Indonesia (Approval number: 5/Ka.Kom.Et/70/KE/X/2023).

## RESULTS

### $LC_{50}$

Zebrafish embryos included in this study were collected from egg batches with a fertilization rate of ≥ 70% and were in normal conditions when selected. Water temperature was maintained at 26 ± 1°C throughout the study. FET test results were verified according to the standard guidelines of OECD 236, where negative and solvent control groups showed survival rates of at least 90% and hatching rates of at least 80%, while the positive control had a mortality rate of 80% (Figure 1). The four measured mortality parameters were determined according to the OECD 236 standard guidelines: coagulation, tail-bud separation, somite development, and visible heartbeat. The findings indicate that ACP-CME significantly influences the duration of tail-bud detachment, heartbeat, and coagulation, but has little effect on somite development (Table 1). Embryo mortality increased in a dose-dependent manner with ACP-CME, with the highest concentration of 250 ppm causing 100% mortality (Figure 1). The mortality data were used to calculate the LC50 of ACP-CME using a probit analysis, revealing an LC50 of 39.56 ppm.

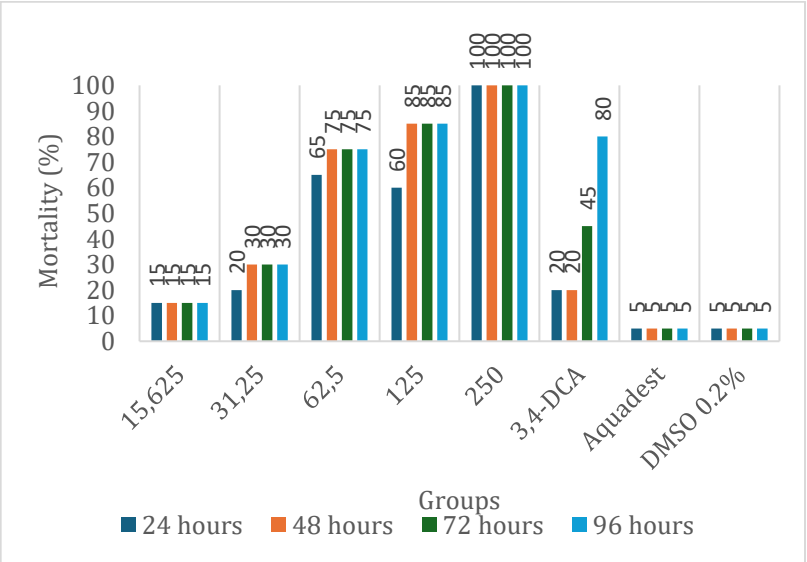


Figure 1: Graph of the mortality rate of ACP-CME in different concentrations (ppm) compared to control groups (%)

Table 1: The percentage (%) of lethality parameters

| Group | | Coagulation (%) | | Somite formation (%) | | Tail bud detachment (%) | | Visible heartbeat (%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | 24 h | 96 h | 24 h | 96 h | 24 h | 96 h | 24 h | 96 h |
| Positive control | | 25 | 30 | 100 | 100 | 100 | 100 | 0 | 28.6 |
| Negative control | | 5 | 5 | 100 | 100 | 100 | 100 | 0 | 100 |
| Solvent control | | 5 | 5 | 100 | 100 | 100 | 100 | 0 | 100 |
| ACP-CME | 250 ppm | 85 | 100 | 100 | 100 | 50 | 50 | 0 | 0 |
| | 125 ppm | 55 | 65 | 100 | 100 | 77.8 | 88.9 | 0 | 37.5 |
| | 62.5 ppm | 65 | 65 | 100 | 100 | 85.7 | 85.7 | 0 | 71.4 |
| | 31.25 ppm | 20 | 25 | 93.8 | 93.8 | 87.5 | 87.5 | 0 | 93.3 |
| | 15.625 ppm | 15 | 15 | 100 | 100 | 100 | 100 | 0 | 100 |

Note: Control (+) = 3,4-dichloroaniline (DCA); control (-)= dilution water; solvent control=DMSO 0.2%.

## Abnormalities

Among the control groups, the positive control group exhibited an abnormality rate of 70%, with 15% of embryos demonstrating normal heartbeats and surviving up to 96 hours of exposure. Morphological anomalies were observed at dosages of 31.25 and 125 ppm, with proportions of 10% and 15%, respectively (Table 2). The morphological abnormalities induced by ACP-CME included kyphosis, lordosis, delayed tail-bud detachment, tail abnormalities, somite failure to develop, hatching failure, and yolk sac edema (Figure 2). The abnormal tail, characterized by lordosis and kyphosis, accounted for 10% of all tails abnormalities. Additionally, other morphological defects listed in Table 2 included yolk sac edema, inability to hatch despite a heartbeat being present after 96 hours post-fertilization, and a sand-serrated caudal fin, as illustrated in Figure 2.

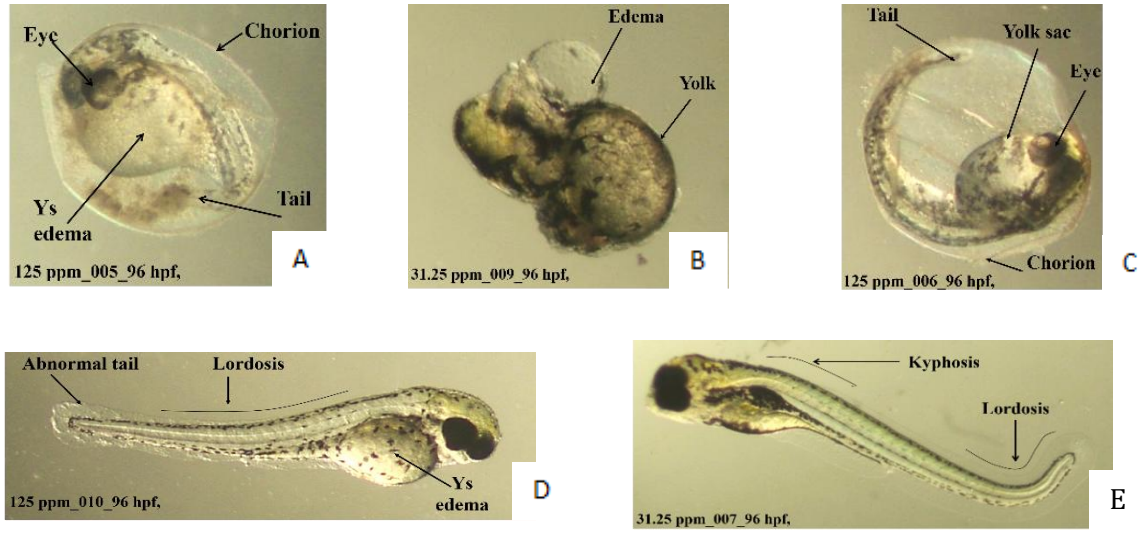


Figure 2: The abnormalities after 96 hours of ACP-CME exposure. (A) edema in the yolk sac in the concentration of 125 ppm, (B) undeveloped somite in the concentration of 31.25 ppm, (C) hatching failure in the concentration of 125 ppm, (D) lordosis, yolk sac edema, and abnormal tail tip in the concentration of 125 ppm, (E) kyphosis and lordosis in the concentration of 31.25 ppm.

Table 2: The percentage (%) of zebrafish embryo abnormalities after 96 hours

| Groups | Variation of treatment | Abnormal embryo | | Coagulation | Normal embryo |
|---|---|---|---|---|---|
| | | Death | Alive | | |
| Control groups | Positive control | 55% | 15% | 30% | 5% |
| | *Cardio edema* | 50% | 0% | - | - |
| | *Somite malformation* | 25% | 0% | - | - |
| | *Abnormal tail* | 35% | 10% | - | - |
| | *Others* | 35% | 15% | - | - |
| | Negative control | 0% | 0% | 5% | 95% |
| | Solvent control | 0% | 0% | 5% | 95% |
| ACP-CME | 250 ppm | 0% | 0% | 100% | 0% |
| | 125 ppm | 15% | 10% | 65% | 5% |
| | *Cardio edema* | 0% | 0% | - | - |
| | *Somite malformation* | 0% | 0% | - | - |
| | *Abnormal tail* | 10% | 10% | - | - |
| | *Others* | 15% | 5% | - | - |
| | 62.5 ppm | 0% | 0% | 65% | 35% |
| | 31.25 ppm | 5% | 5% | 25% | 65% |
| | *Cardio edema* | 5% | 0% | - | - |
| | *Somite malformation* | 0% | 5% | - | - |
| | *Abnormal tail* | 0% | 0% | - | - |
| | *Others* | 0% | 5% | - | - |
| | 12.5 ppm | 0% | 0% | 10% | 90% |

Control (+) = 3,4-dichloroaniline (DCA); control (-)= dilution water; control (solvent)=DMSO 0.2%.

## DISCUSSION

Activated charcoal is a type of charcoal that has been treated with oxygen, resulting in an irregular structure with small pores and a large surface area. It eliminates impurities or undesirable substances from *Pegagan* extract via physical or chemical interactions, including Van der Waals forces, hydrogen bonds, and covalent bonds. Additionally, activated charcoal has an excellent pore structure and thermostability, making it easy to maintain and adjust for diverse applications.[12] Activated charcoal is a standard material used in water purification, pharmaceuticals, and other chemical processes that deal with micro pollutants since it can quickly adsorb impurities.[13] Its fundamental uses in environmental and industrial contexts include bleaching, removing, separating, and modifying compounds in liquid and gas phases.[14] In medicine, activated charcoal is well-established, particularly for treating oral drug poisoning by absorbing the substance.[5,15] Furthermore, activated charcoal is cost-effective and readily available, supporting its use as an environmentally friendly method for purifying herbal medicine.[16]

The assessment of acute toxicity in zebrafish embryos is a widely recognized method standardized by OECD guideline 236 in 2013. Our study employed acute toxicity tests focused on lethality parameters to determine the $LC_{50}$ and evaluated teratogenic abnormalities. Quantitative data encompassed four lethality parameters and the $LC_{50}$ value, while the qualitative data detailed the specific anatomical abnormalities. Exposure to ACP-CME at 250 ppm resulted in a 100% mortality rate. The mortality rate of ACP-CME decreased in a dose-dependent manner, achieving 15% at the lowest dosage of 15.625 ppm.

The $LC_{50}$ of ACP-CME in zebrafish embryos was determined to be 39.56 ppm, classified as Category 2: toxic to aquatic life according to the Globally Harmonized System (GHS) classification

for acute toxicity.[17] Although classified as toxic, ACP-CME exhibited reduced toxicity compared to the ethyl acetate fraction of *Centella asiatica*, which had an $LC_{50}$ of 26.61 ppm as reported by Hayati.[11] The findings suggest that purification with activated charcoal may more effectively reduce toxicity than ethyl acetate fractionation. The lower toxicity of ACP-CME is hypothesized to result from the absence of aromatic alkaloid compounds, which are known for their bitter taste and defensive roles in plants, serving as a deterrent against diseases and predators.[18] Supporting this, another study on *Pegagan* methanol extract showed an $LC_{50}$ of 25.47 ppm in brine shrimp larvae, reinforcing the idea that more selective purification via activated charcoal yields a less toxic extract.

Lethality evaluation was conducted through four key parameters: tail-bud detachment from yolk, somite development, visible heartbeat, and the absence of coagulation.[10] Failure to meet any of the established parameters leads to embryo death. Table 1 indicates a negative correlation between the levels of ACP-CME and the percentage of embryos displaying visible heartbeats. A recent study using ethyl acetate fractionation on *Centella asiatica* ($LC_{50}$ = 26.61 ppm) revealed that the highest concentration leading to a total absence of heartbeat in embryos was 100 ppm, with the most significant heartbeat response (95%) at 6.25 ppm.[11] These data suggest that ACP-CME adversely affects the cardiac function of zebrafish embryos at higher concentrations compared to the ethyl acetate-fractionated *Centella asiatica*. The lower cardiac effects observed in ACP-CME are believed to result from the absence of aromatic components, such as flavonoids, present in the ethyl acetate fraction of *Centella asiatica*. Flavonoids in *Centella asiatica* are thought to inhibit sympathetic nerve activity and enhance parasympathetic activity, causing a depressant effect on heartbeat.[19] Additionally, terpenes in the extract may interact with ion channels and mitochondrial pathways in cardiac tissues.[20,21] Thus, the absence or reduced levels of these compounds in ACP-CME could partially explain the observed differences in heartbeat suppression.

ACP-CME has a concentration-dependent effect on coagulation in zebrafish embryos, as seen in Table 1. Coagulation mainly occurs within the first 24 hours post-fertilization and is rarely observed beyond early development stages. It is closely linked with gastrulation arrest, reflecting complex interactions during early embryogenesis.[22] Exposure to ACP-CME can disrupt cellular processes in a developing zebrafish embryo, leading to developmental abnormalities and mortality. Coagulation is recorded to be influenced by gene expression, protein activity, and cellular signaling pathways.[23]

Table 1 illustrates that ACP-CME has a notable impact on the duration of tail-bud detachment but has minimal effect on somite development. The unusual shortened tail length delays the detachment of the tail bud from the yolk, potentially due to disturbances in notochord progenitors, neural tube, or alterations in the signaling pathway.[24–26] Hatching failure after 96 hours post-fertilization could be attributed to the application of 0.2% DMSO. The solvent control group demonstrated a failure-to-hatch rate of 5%, similar to the exclusion of the ACP-CME group. Zebrafish embryos typically hatch between 48 and 72 hours post-fertilization, and an extended incubation period beyond this period correlated with a decreased likelihood of hatching. It is likely due to the use of DMSO as the solvent, which elevated DMSO concentration more than 0.1% can impair the chorion's barrier function, increasing permeablity to substances in the perivitelline space and causing developmental delays, including hatching failure.[24]However, studies have shown that DMSO at doses below 0.5% does not affect survival rates or embryo morphology, thus in line with previous findings.[25]

Genetic, muscular, or idiopathic factors can alter the standard curve of the spine, which develops during early embryogenesis. Lordosis is defined as when the vertebral bodies in the haemal region bend, which makes the spine point in the opposite direction.[26] This condition contrasts with kyphosis, which involves excessive thoracic curvature, and scoliosis, characterized by lateral spinal deviations.[27] One recognized factor contributing to these malformations is the disruption of signaling pathways associated with urotensin II-related peptides (URPs), specifically Urp1 and Urp2. Studies conducted by Bearc[28] using zebrafish models have demonstrated that cerebrospinal fluid (CSF) flow, propelled by ciliary motility, is crucial for

preserving normal spine morphology. Neurons lining the spinal canal produce URPs, and while zebrafish lacking Urp1 maintain normal spinal structure, those deficient in Urp2 develop mild deformities. The simultaneous absence of both peptides resulted in pronounced caudal curvature, akin to human lordosis. Notably, skeletal deformities such as lordosis in juvenile zebrafish can recover one week after cessation of exposure to the lordosis-inducing agent, coinciding with upregulation of musculoskeletal recovery-related genes.[26]

Another abnormality that occurred due to ACP-CME is edema. Fluid accumulation around the heart or yolk sac in zebrafish embryos, known as edema, may occur due to a failure in the water permeability barrier or heightened vascular permeability.[29] This condition presents as yolk sac edema, characterized by fluid accumulation in the yolk syncytial layer, or yolk edema, which involves fluid pockets within the yolk sac. Potential causes include kidney dysfunction, circulatory failure, or ionic imbalance.[29] Some studies suggest that edema may be a temporary stress response rather than a pathological condition.[8,22] Despite frequent documentation by brightfield microscopy, the underlying mechanisms remain unclear.[30]

During development, zebrafish regulate water homeostasis through permeability barriers surrounding the embryonic body and yolk sac, with some species-specific variations.[29] Disruptions of these barriers thus leads to edema.[29] The yolk sac epithelium is also involved in ion regulation; however, the vulnerability to environmental toxins is not fully elucidated.[31,32] As a metabolically active site that can bioaccumulate hydrophobic compounds, the yolk sac may significantly influence embryonic development by mediating toxicant exposure. [31,33]

Aligned with the Sustainable Development Goals (SDGs), which promote sustainable resource use, environmental protection, and human health, this study presents a novel and environmentally friendly alternative for purifying herbal medicines, *Centella asiatica* methanol extract purification by activated charcoal. This method aimed to improve health and well-being by reducing toxicity and enabling the creation of safer herbal medicines for human use. It also supports the provision of clean water and sanitation by reducing the risk of water contamination caused by harmful solvents since the purification method uses environmentally friendly materials. Promoting the use of more environmentally friendly and recyclable materials aligns with SDGs 12 (Responsible Consumption and Production). Furthermore, reducing environmental impacts, including emissions and chemical residue, aligns with SDGs 13 (Climate Action). Ultimately, this method has the potential to protect both aquatic and terrestrial ecosystems from pollution, thereby contributing positively to the goals of SDG 14 (Life Below Water) and SDG 15 (Life on Land). This study demonstrates that adopting greener processing methods for herbal extracts can advance multiple SDGs simultaneously.

The study has several limitations. A 95% confidence interval was not calculated for the $LC_{50}$, and the observations were limited to 96 hours post-fertilization (hpf). Long-term ACP-CME effects on zebrafish embryos were not evaluated. The critical assessment of cardiac effect and quantitative heart rate observation was absent. Extending the observation period would allow for accurate heart rate tracking. Further research should focus on developing sophisticated cardiac evaluation methods to demonstrate the benefits of ACP-CME more effectively.

## CONCLUSION

The $LC_{50}$ of ACP-CME was established at 39.56 ppm, with the observed abnormalities manifested in the form of lordosis, kyphosis, delayed tail-bud separation, aberrant fin, failure of somite development, failure of hatching, and yolk sac edema. Further investigation is essential to comprehensively understand the impact of ACP-CME on the heart rate and cardiac function of zebrafish embryos.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

**ACKNOWLEDGMENTS**

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**DATA AVAILABILITY**

The data underlying the findings of this study is available from authors upon reasonable request. The data includes the results of toxicity testing on zebrafish embryos and has been structured in a shareable format in accordance with privacy policies and research ethics.

**SUPPLEMENTAL DATA**

All relevant data has been presented in this manuscript, and there is no additional data provided separately.

**AUTHOR CONTRIBUTIONS**

Each author contributed to this manuscript. The first author contributed to the data collection, results and statistical analysis, and manuscript preparation. The second author contributed to the experimental design of the Fish Embryo Toxicity Test, result analysis, editing, review, and submission of the manuscript. The third author contributed to the concept and idea, experimental methods, especially in extraction methods, and review of the manuscript.

**DECLARATION OF USING AI IN THE WRITING PROCESS**

The authors declare the use of AI to assist in citation searching.

**LIST OF ABBREVIATIONS**

ACP-CME: activated-charcoal *Centella asiatica* methanolic extract; DMSO: dimethyl sulfoxide; DCA: Dichloroaniline; hpf: hours post fertilization; Ys: yolk sac; tb: tail bud.

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