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RESEARCH ARTICLE

Embryonic toxicology evaluation of citrus fruit (*Citrus reticulata* and *Citrus limonum*) peel mediated hydroxyapatite nanocomposite

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Abstract

Nanotechnology is a rapidly evolving scientific field due to advantages such as improved bioavailability, enhanced bioactivity and targeted drug delivery. However, concerns exist regarding its potential toxicity, as nanoparticles can enter the biological systems and induce inflammatory responses, oxidative stress, neurotoxicity and genotoxicity. These risks necessitate comprehensive toxicological evaluations to ensure their safe biomedical use. Green synthesis, particularly using citrus fruit peels, presents an eco-friendly alternative for nanoparticle production by leveraging bioactive compounds to reduce toxicity. This study aimed to assess the embryonic toxicity of citrus fruit (*Citrus reticulata* and *C. limonum*) peel-mediated hydroxyapatite nanocomposites, providing insights into their safety and guiding future research toward sustainable nanotechnology applications. Green synthesis of *C. reticulata* and *C. limonum*-mediated hydroxy apatite nanocomposites was successfully carried out. The toxicity of developed nanocomposite was assessed using embryonic toxicology evaluation in zebrafish. Stereo microscopic analysis was done for the assessment of morphologic features, developmental stages and potential malformation of zebrafish embryos. At a 24 hr interval, hatching rate and embryonic viability rates were measured. Sublethal toxicity was observed in the zebrafish embryonic toxicology evaluation, with a dose-dependent hatching rate and embryonic viability. Overall, *C. reticulata* and *C. limonum* -mediated hydroxyapatite nanocomposites exhibited very low toxicity based on zebrafish embryonic toxicology evaluations.

Keywords: embryonic toxicology; green chemistry; hydroxy apatite; nanocomposite; nanoparticle; reactive oxygen species

Introduction

Nanotechnology is an emerging and rapidly advancing field due to its promising advantages, notably targeted medication, ease of application, improved bioavailability, enhanced bioactivity, anti-microbial properties, osteoconductivity and osteoinductivity (1) Nanoparticles are available in different forms based on their physical and chemical characteristics, including metal nanoparticles, ceramic nanoparticles, carbon-based nanoparticles, lipid-based nanoparticles, semiconductor nanoparticles and polymeric nanoparticles. They also exist in different structural configurations such as nanorods, nanotubes, nanocrystals, nanoclusters, nanofibers, nanofilms and nanocomposites. The properties exhibited by these nanoparticles and nanocomposites are extensive and highly diverse. Their large surface area, elevated particle number per unit mass and strong mechanical and durability provide a wide range of applications, including increased absorbability (2). Their sensitive optical characteristics also makes them suitable for chemical sensing (3). Nanoparticles additionally possess higher thermal conductivity and distinct mechanical properties compared to their micro- and macro-sized counterparts, enabling them to exhibit superior functional performance. These unique features, either individually or synergistically, make them ideal for biomedical applications, including targeted drug delivery, bioimaging, biosensors, hyperthermia management and photoablation treatment (4).

However, a number of concerns stills persist regarding the safety of nanomaterials for the human body and the environment. Nanoparticles and nanocomposites may be released into the environment during production as well as during disposal. This may lead to uncontrolled infiltration of these particles into the biological systems of plants, animals and humans. Routes of entry include ingestion, inhalation and dermal absorption. Once inside the body, nanoparticles can be distributed to various organs, including the blood cells, lungs, heart, brain, liver, kidney, spleen, thymus and immune cells. Toxic manifestations of nanoparticles are generally observed in the form of inflammatory changes, macrophage malfunction and genotoxicity. The immunological response against nanoparticles often involves the production of Reactive Oxygen Species (ROS) and alternation in cytokine levels. An inverse relationship has been observed in which decreasing nanoparticles size leads to increased cytotoxicity and ROS production.

Additionally, nanoparticles have been shown to stimulate peripheral blood mononuclear cells to produce higher levels of interferon (IFN)-γ, interleukin (IL)-12 and tumour necrosis factor (TNF)-α (5).

Blood cells internalize nanoparticles, resulting in haemolysis, which can be fatal if prolonged. Plasma protein adsorption was decreased following nanoparticle functionalization. Procoagulant effects have also been observed when nanoparticles interact with platelets (6). Endothelial damage has been reported in several studies (7). Together, these effects may lead to activation of the coagulation cascade, potentially causing significant harm to biological systems.

Nanoparticles have been reported to demonstrate neurotoxic effects, such as neuroinflammation, impaired neurotransmitters, altered synaptic plasticity and abnormal signalling pathways. These neurotoxic outcomes are due to widespread generation of ROS, which induces oxidative stress leading to cell death, autophagy and impaired function of Blood Brain Barrier (BBB)(8). When inhaled, nanoparticles activate resident alveolar macrophages in the lungs, resulting in lung fibrosis. Pulmonary injury is directly associated with vascular dysfunction, thrombosis and myocardial ischaemia (9).

Genotoxicity and DNA damage have also been associated with exposure to nanoparticles. These particles interact with DNA-associated proteins and disrupt repair mechanisms, resulting in DNA damage. Direct interaction of nanoparticles with the genome can lead to structural damage and secondarily genotoxicity may rise from ROS or Reactive Nitrogen Species (RNS) generated within the cell. Nanoparticles have also been reported to cause locus-specific and global DNA hypomethylation (10). Aluminium oxide, titanium oxide and zinc oxide nanoparticles have been shown to induce DNA damage, with dose-dependent genotoxicity observed for aluminium oxide and titanium oxide nanoparticles. Furthermore, fullerenes have been implicated in DNA strand breakage and chromosomal abnormalities (11).

Hence, thorough toxicity evaluations are required to establish the safety of nanoparticles for biomedical applications and to understand their interaction with living organisms. Multiple assays are available to evaluate nanoparticle toxicity, including cellular interaction assays, cytotoxicity assays, genotoxicity assays, viability assays, apoptosis assays, oxidative stress and inflammation assays, endotoxin assays and teratogenicity assays (12). Embryonic toxicology evaluation using zebrafish (*Danio rerio*) is an established toxicity model, capable of providing more comprehensive information than cell line studies alone while remaining cost-effective. It is also considered as an accepted alternative to animal studies until embryos reach five days post-fertilization, the stage at which they become protected animals (13).

Exposure to lethal substances, especially during the vulnerable embryonic stage of development, can lead to long-term complications such as growth retardation, congenital abnormalities or even developmental death (14). Hence, embryonic toxicology evaluation can be effective method to find toxicity of the emerging nanoparticles and nanocomposites. The interactions between nanoparticles and biological systems are complicated and their toxicity profiles are greatly influenced by factors such as particle size, shape, surface charges and chemical composition (15). The potential impact of nanoparticles and nanocomposites on embryonic development is of greater significance, since early exposure can interfere with normal developmental processes, leading to permanent health problems.

The use of green synthesis or green chemistry to generate nanoparticles from naturally occurring, non-toxic materials is an intriguing approach for minimizing environmental and biological toxicity. Citrus fruits are an excellent source for the green synthesis of nanoparticles, as they contain a wide range of bioactive compounds, including flavonoids, polyphenols, coumarins and tetranortriterpenoids (limonoids and carotenoids). They also contain ascorbic acid, tocopherols, tocotrienols and elements such as zinc, copper, selenium, iron and manganese(16). These components contribute to the antibacterial, anti-inflammatory and antioxidant activities. Various fruit parts, including peels, seeds and seed oils can be utilized. Citrus peels, in particular, are not only rich in bioactive compounds but also represent an abundant agricultural waste product, making them an ecofriendly and sustainable alternative for nanoparticle and nanocomposite development.

Currently only a small quantity of nanoparticles is used in practical use, while many nanomaterials remain under investigation regarding their safety, mechanism of interaction, pharmacokinetics and biological effects(17). Hence, this study seeks to provide a comprehensive evaluation of the embryonic toxicology associated with citrus fruit peel mediated hydroxy apatite (nHAP) nanocomposites. By examining the effects of these nanomaterials on developing embryos, we aimed to gain deeper understanding of their potential risks and to establish a framework for assessing their safety. Through this investigation, we seek to balance the remarkable benefits of green nanomaterials with the need for stringent toxicological assessments, ultimately guiding future research toward safer and more sustainable applications in both the biomedical and scientific sectors.

Materials and methods

Sample preparation

Preparation of citrus fruit (*C. reticulata* and *C. limonum*) peel mediated HAP nanoparticles

Samples were freshly harvested, fully mature *C. reticulata* and *C. limonum* fruit peels collected in January (post-winter season). Green synthesis of citrus fruit peel-mediated HAP nanoparticles was carried following the standard protocol (18).

Preparation of chitosan solution

Chitosan solution was prepared by mixing 500 mg of medium molecular weight chitosan and 1 mL glacial acetic acid. It is then diluted with 49 mL of distilled water. This mixture was kept in a magnetic stirrer for 24 hr at 650 rpm.

Preparation of citrus fruit (*C. reticulata* and *C. limonum*) mediated HAP nanocomposites

The prepared green synthesized HAP nanoparticles was mixed with the chitosan solution and kept in a magnetic stirrer at 450 rpm for 3 hr to obtain the nanocomposite solution.

Embryonic toxicology assessment using zebrafish (*Danio rerio*)

Local procurement of wild-strain zebrafish variety (*D. rerio*) was carried out. These aquatic animals were kept in separate tanks with a regulated pH of 6.8–8.5, a 14:10 hr light/dark cycle and temperature of 28.0 ± 20 °C. The fish were fed twice daily with either optimized feed or commercially procured dry bloodworms. Three male and one female zebrafish were placed in each breeding tank to obtain embryos. Viable eggs were retrieved and rinsed thrice with freshly prepared E3 medium without methylene blue. Fertilized eggs (20

embryos per 2 mL solution per well) were transferred into 6-, 12- and 24-wells plates for toxicological evaluation. Three replications were performed for both test and control groups.

Freshly prepared *C. reticulata* and *C. limonum* mediated HAP nanocomposites at five concentrations (5, 10, 20, 40 and 80 µg/mL) were introduced directly into the E3 medium to begin the study. To ensure even distribution of the nanocomposite, the solution was subjected to ultrasound vibrations for 15 min, while maintaining the pH between 7.2 and 7.3. From 24 to 96 hr post-fertilization, embryos were exposed to the different nanocomposte concentrations. The E3 medium used for incubation was supplemented with the respective *C. reticulata* and *C. limonum* HAP nanocomposites. To maintain a dark experimental environment, the well plates were wrapped in aluminium foil and kept at 28 °C. Control groups were included in the study. Dead embryos were removed every 12 hr.

Using a stereomicroscope, the developmental stages of zebrafish embryos were examined throughout the exposure period after fertilization. At 24 hr intervals for three consecutive days, the hatching rate and embryonic viability were recorded. The study objectives include assessment of hatching rates, embryo viability and identification and documentation of any malformations in embryos and larvae of both test and control groups. A COSLAB-Model HL-10A microscope was used to capture images of the deformed embryos. The percentage of abnormal embryos was also recorded every 24 hr.

Results

The hatching rate of zebrafish embryos was observed for different concentrations of control and test groups. The control group expressed 100 % hatching rate, which served as the baseline for comparison. For *C. reticulata*-mediated HAP nanocomposites, the hatching rate was 100 % at 5 µg/mL, similar to control group. However, as the concentration of the nanocomposite increased, the hatching rate began to decline. At 10 µg/mL and 20 µg/mL, the hatching rate decreased to 80 % and it further decreased to 60 % at 40 µg/mL and 80 µg/mL (Fig. 1).

For *C. limonum*-mediated HAP nanocomposites, the hatching rate was 100 % at 5 µg/mL and 10 µg/mL, mirroring the control. The hatching rate decreased to 80 % at 20 µg/mL and 40 µg/mL, with further reduction to 60 % at 80 µg/mL (Fig. 2). These results indicate that increasing the concentration of nanocomposite led to a progressive decline in hatching rate.

Regular monitoring of zebrafish embryo viability was performed to assess health and survival following exposure to different nanocomposites concentrations. The control group showed 100 % viability rate. A dose-dependent decline in viability was observed in the test groups. For *C. reticulata* mediated HAP nanocomposites, viability remain 100 % at 5 µg/mL and 10 µg/mL, but 80 % at 20 µg/mL, 40 µg/mL and 80 µg/mL (Fig. 3). Similarly, *C. limonum*-mediated HAP nanocomposites, viability was 100 % at 5 µg/mL, 10 µg/mL and 20 µg/mL and declined to 80 % at 40 µg/mL and 80 µg/mL (Fig. 4).

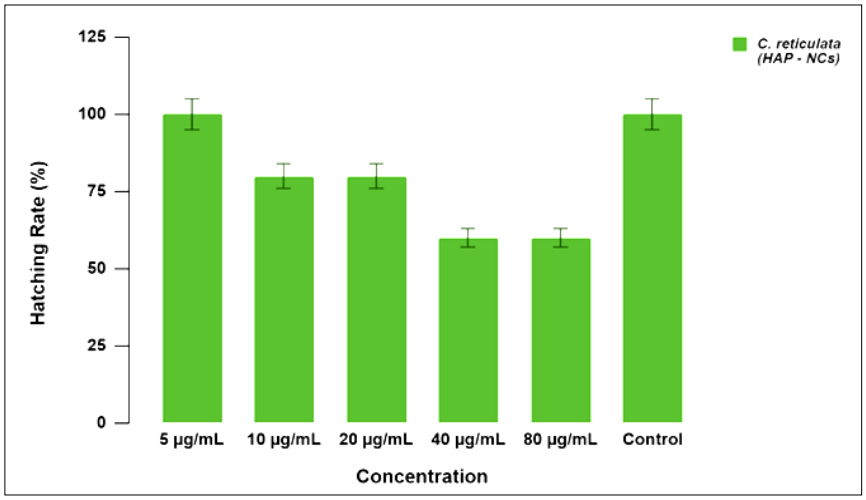

Fig. 1. Hatching rate of *C. reticulata* mediated HAP nanocomposites.

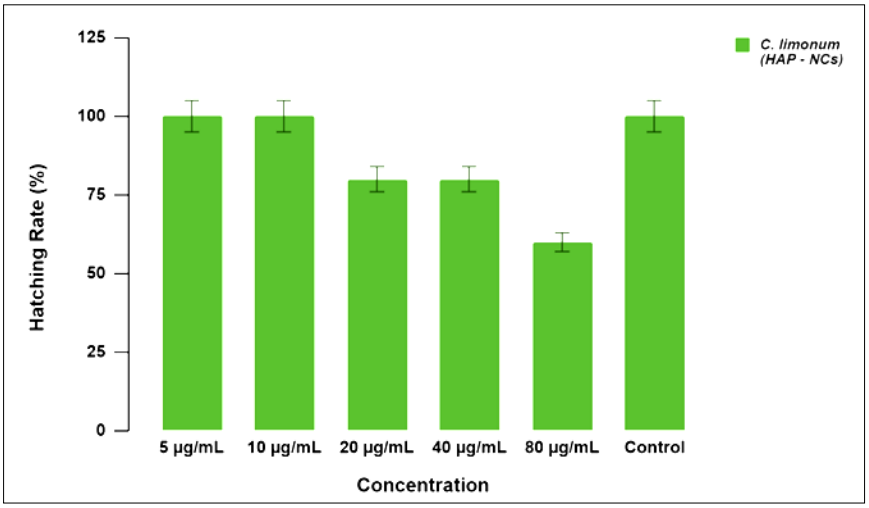

Fig. 2. Hatching rate of *C. limonum* mediated HAP nanocomposites.

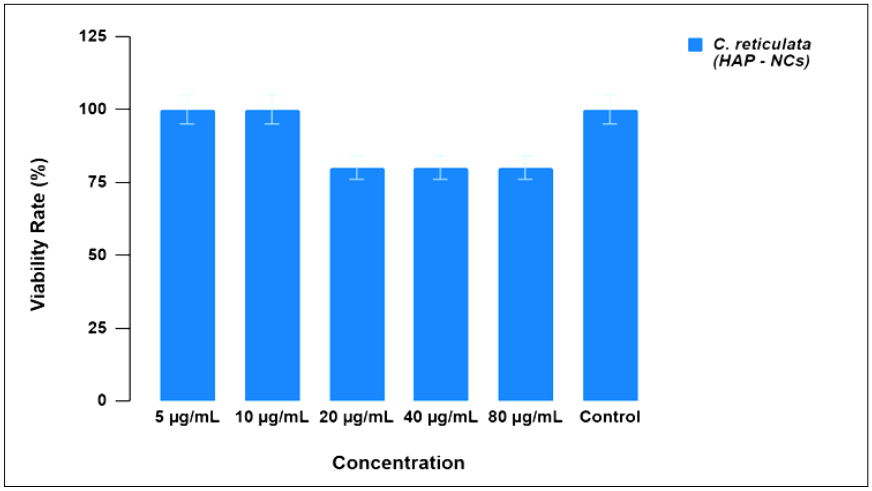

Fig. 3. Viability rate of *C. reticulata* mediated HAP nanocomposites.

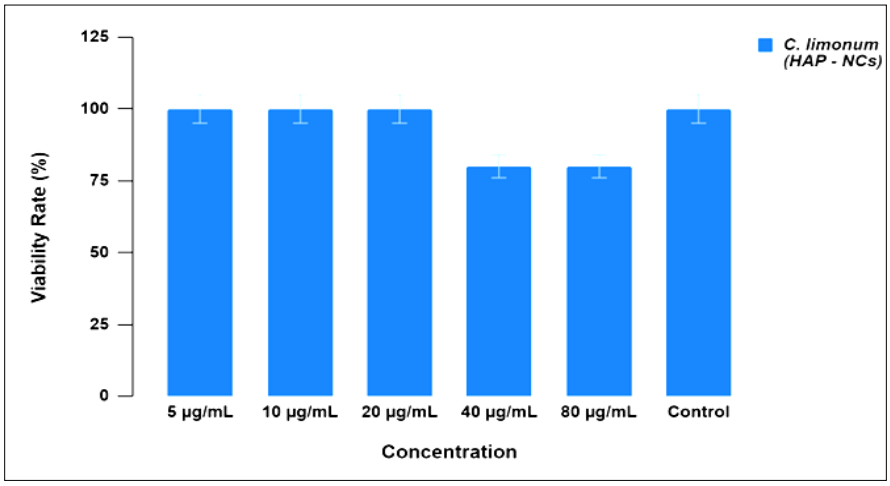

Fig. 4. Viability rate of *C. limonum* mediated HAP nanocomposites.

No morphological abnormalities were observed throughout the 24 hr (day 1), 48 hr (day 2) and 72 hr (day 3) observation periods. Additionally, no malformations were detected in either the control or test groups, indicating that the changes in hatching and viability were not associated with visible developmental defects (Fig. 5, 6).

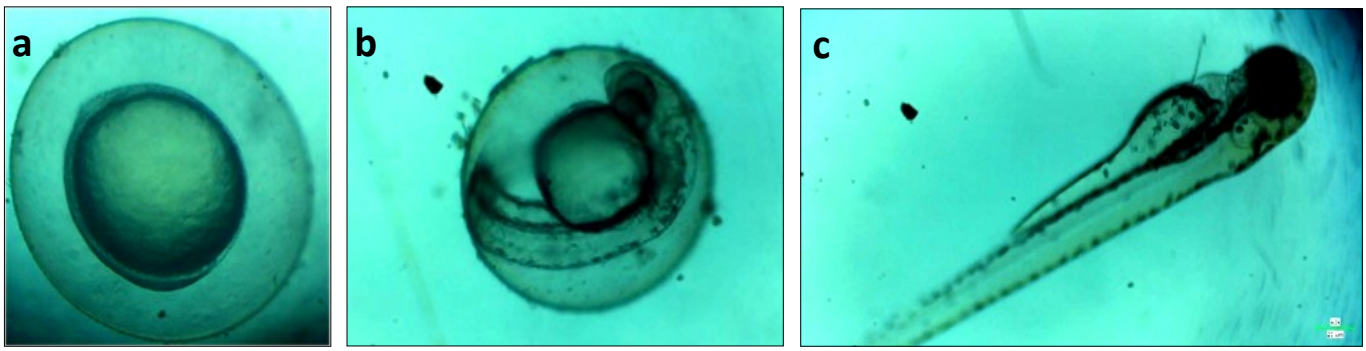

Fig. 5. Morphology of zebrafish embryo exposed to different concentration of *C. reticulata* mediated HAP nanocomposites. a) Day 1; b) Day 2; c) Day 3

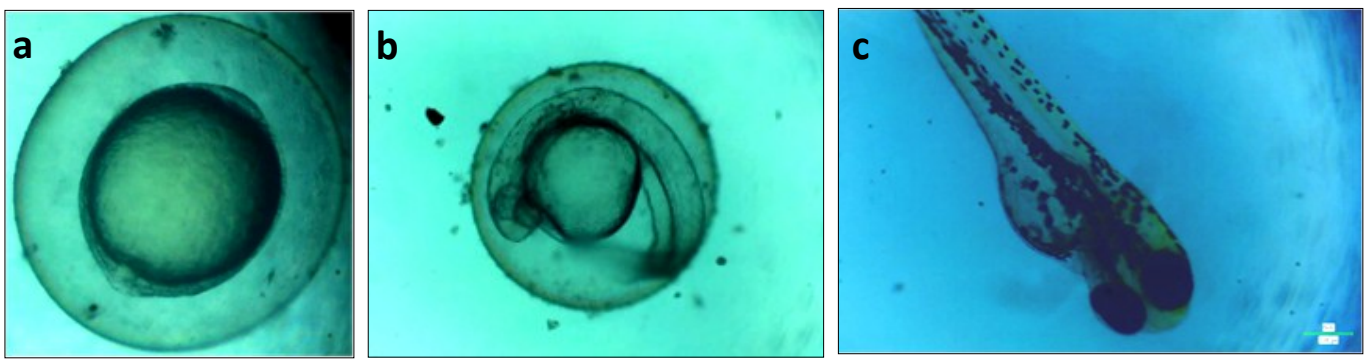

Fig. 6. Morphology of zebrafish embryo exposed to different concentration of *C. limonum* mediated HAP nanocomposites. a) Day 1; b) Day 2; c) Day 3

Discussions

This study evaluated the embryonic toxicity of citrus fruit peel-mediated HAP nanocomposites synthesized using *C. reticulata* and *C. limonum* in zebrafish *(D. rerio)* embryos, focusing on hatching rate, viability and morphological integrity. Zebrafish embryo toxicity evaluation is a widely accepted *in vivo* model for evaluating the biocompatibility and toxicity of nanomaterials due to their transparency, rapid development and genetic similarity to humans. No significant morphological changes or malformations were observed during exposure period. A dose dependant hatching and embryonic viability rates were observed.

Under experimental set up the control group exhibited 100 % hatching and embryonic viability rates validating the typical developmental process. In contrast, the test group evidently demonstrated decrease in hatching rate with increase in concentration. Hatching rates of *C. reticulata*-mediated HAP nanocomposites decreased from 100 % at 5 µg/mL, the lowest concentration to 80 % at 10 µg/mL and 20 µg/mL and further reducing to 60 % at 40 µg/mL and 80 µg/mL concentrations. Although hatching rates were comparatively higher at lower concentrations, a similar pattern was seen in *C. limonum*-mediated HAP nanocomposites with a slightly broader safety margin indicating possible variations in nanoparticle interaction brought on by bioactive chemicals originating from plants. This correlates with the findings of a previous study- that phytochemical profile of plant extracts significantly influences the biological properties of green-synthesized nanoparticles (19). Also, the findings align with the previous study result indicating nanoparticles can impair embryonic development by interacting with chorion permeability, thereby delaying hatching of the embryos (20,21).

Similarly embryonic viability rates followed a dose dependant decline. While lower concentrations such as 5 µg/mL and 10 µg/mL did not affect viability, exposure to 20 µg/mL and above led to an 80 % viability rate for both *C. reticulata* and *C. limonum*. This decrease raises the possibility of low levels of cytotoxicity at elevated nanocomposite concentration which could be connected to both oxidative stress and nanocomposite accumulation. However, in contrast to other nanoparticles like metal oxide nanoparticles which have demonstrated to generate increased embryonic lethality, the lack of full viability at any tested dosage (22-26). The test results of the current study shows that the viability values were not anywhere near the lethality concentration LD_{50} suggests that citrus fruits (*C. reticulata* and *C. limonum)*-mediated HAP nanocomposites have a mild toxic effect and its potential use. Interestingly, while prior studies often report malformations such as pericardial edema, spinal curvature or yolk sac deformities at high nanoparticle doses, our study did not observe such anomalies (27).

Zebrafish embryos in this study maintained normal morphology at the observation periods in either control or tested groups. Any kind of malformations were also ruled out, mirrors findings, in contrast to toxic nanoparticles resulting in severe developmental defects such as pericardial oedema, spinal curvature and craniofacial anomalies (28-30). This implies that, under the tested conditions *C. reticulata* and *C. limonum* mediated HAP nanocomposites do not have teratogenic consequences. According to earlier research on green- synthesized nanoparticles, the presence of bioactive components like phytochemicals, polyphenols and flavonoids may help reduce toxicity through their antimicrobial and antioxidant qualities (31).

Despite the absence of morphological abnormalities, the decrease in hatching and viability rates of this study suggests possible sub-lethal poisoning similar to the study (32). Aggregation of nanocomposites on the chorion which obstruct gas exchange and nutrition absorption, may be the cause of decrease in hatching rate of the embryo. Furthermore, oxidative stress brought on by nanocomposite may change developmental signals and postpone hatching. A similar concentration-dependent toxicity profile for silver nanoparticles, with reduced hatching rates and survival observed at higher exposure levels, even in the absence of visible morphological abnormalities (33). According to studies, nanoparticles can induce the production of ROS, which leads to cellular damage and cell death (34). Although this study did not directly quantify ROS, the observed reduction in embryo viability at higher concentrations of nanocomposite raises the possibility that oxidative stress is a contributing factor.

The study results emphasize the importance of evaluating the potential toxicity and biocompatibility of green synthesized nanoparticles and nanocomposites prior to its use in biomedical application. The dose-dependent effects on hatching and viability rates highlights the necessity for dosage adjustment in future biomedical applications, even though *C. reticulata* and *C. limonum*-mediated HAP nanocomposites demonstrated very low toxicity. Furthermore, emphasis should be given for the environmental effects of their release into different ecosystems, owing to its extended exposure to nanocomposites impact on biological systems of species at various stage of development.

Even though the study results offer insightful information about the embryonic toxicity of citrus fruit peel-mediated HAP nanocomposites, it is important to recognize the limitations. Molecular level analysis of the embryos including gene expression and oxidative stress markers was not done. The role antioxidant components of the green synthesized nanocomposites in reducing nanoparticle induced stress must be further assessed. To evaluate chronic toxicity, it is also necessary to investigate the consequences of long-term exposure and possible bioaccumulation of nanocomposites and nanoparticles in zebrafish.

Conclusion

The embryonic toxicology assessment of C. *reticulata* and *C. limonum*-mediated HAP nanocomposites reports dose-dependent sublethal toxicity. Hence, dosage adjustment is necessary when using these nanocomposites for biological applications. The absence of morphological defects, however, indicates a very mild toxic effect, which may be mitigated by the bioactive compounds present in the citrus fruit peel extracts. These findings provide foundation for further research aimed at optimizing the toxicity profile and enhancing the biocompatibility of green-synthesized nanoparticles for biomedical and environmental applications.

Authors' contributions

LAS carried out the study. DG, RS, JA and DSS participated in the design and coordination of study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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