Synthesis of Green Zinc Oxide Nanoparticles Mediated by *Syzygium cumini* Induced Developmental Deformation in Embryo Toxicity of (*Daniorerio*) Zebrafish

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ABSTRACT: *This study investigated the synthesis of Zinc oxide (ZnO) nanoparticles using Syzygium cumini fruit (Indian blackberry) seed extract. The seed extract of Syzygium cumini fruit has properties of anti-diabetic, anti-inflammatory, and anti-bacterial, and traditionally it has long been used in Indian folklore medicine. Zebrafish embryos and larvae were treated with 5 different concentrations (0, 25, 50, 75, 100, 150 µg/mL) of Zinc oxide (ZnO) nanoparticles from 4 hours post fertilization (hpf). The results showed that exposure to 50-150 µg/mL zinc oxide nanoparticles (ZnO NPs) induced developmental toxicity in these embryos, causing mortality, hatching delay, and malformation. Exposure of 50-150 µg/mL Zinc oxide (ZnO) nanoparticles to zebrafish embryo caused coagulated unhatched phenotype, spinal curvature, axis bent, tail malformation, yolk sac, and pericardial edema, at 72-96 hpf. These results will assist in elucidating the mechanisms of the developmental toxicity of green synthesized Zinc oxide nanoparticles during the embryonic development of zebrafish.*

KEYWORDS: *Green synthesis; ZnO NPs; Zebrafish embryo; Invivo toxicity; Developmental deformities.*

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INTRODUCTION

Nanotechnology has sparked tremendous interest in several sectors over the last few decades, including medical diagnostics, gene delivery, medication administration and imaging, and cell tracking, among others, with numerous potential uses [1, 2]. Semiconducting metal oxides, such as TiO_2, CeO_2 and ZnO, have been found to have the potential in physical, chemical and biological application [3, 4]. Because of their physicochemical properties, nanomaterials are now widely used in a variety of applications. In this regard, metal oxide nanoparticles are of enormous importance due to their various fascinating properties such as high melting points, temperature holding capacity, resistance to acids and alkalis, and anti-microbial properties [5]. Furthermore, green-engineered nanomaterials have demonstrated a promising function in aquatic ecological conservation and environmental studies [6-8]. In general, the ecotoxicity of nanomaterials has gotten a lot of attention across the world [9, 10]. ZnO nanoparticles have been well-known in recent years for their contributions to nanotechnology research [11-13] and a wide range of applications such as paint, UV detectors, cosmetics, gas sensors and antibacterial agents, and microelectronics [14, 15]. The toxicity of ZnO nanoparticles has been studied in a variety of species, including algae [16], plants [17], bacteria [18] and embryonic and adult zebrafish [19-21]. In general, nanoparticle solubility is critical to the toxicity of these nanoparticles and their effects on ecosystems. As a result, it is claimed that nanoparticles with high stability may be able to enter, aggregate, and remain within organisms. ZnO nanoparticles are quickly bio-accumulated by aquatic species and have hazardous consequences [22, 23] Zebrafish are commonly employed as model animals in high tide acute toxicity research, and their immense potential in nanomaterial toxicity assessment is being investigated. Furthermore, the zebra fish has a combination of compensatory characteristics that make it appealing, such as great homology to the human genome, small size, low cost, excellent repeatability, transparency, and quick embryonic development. Several investigations have found that ZnO nanoparticles are harmful to zebrafish [24] exhibit that ZnO nanoparticles induce a concentration-dependent decrease in hatching rates. Previous research has looked at the oxidative stress caused by zinc oxide nanoparticles [25-27]. Green biosynthesis of nanoparticles offers an advantage over chemical or physical approaches, and it has a wide range of applications in healthcare sectors. Pharmacological research has revealed that *Syzygium cumini* has antihyperglycemic, anti-inflammatory, antibacterial, cardioprotective, and antioxidant properties [28]. The primary goal of the research is to investigate the developmental toxicity of green-produced ZnO nanoparticles using UV-Vis spectroscopy, X-Ray Diffraction (XRD), Fourier Transforms InfraRed(FT-IR), Spectroscopy Atomic Force Microscopy (AFM), and Scanning Electron Microscopy (SEM). The current study found that green-produced ZnO nanoparticles caused death, hatching delays, and developmental abnormalities in zebrafish eggs, and the findings will help to understand the causes of toxicity of green-generated ZnO nanoparticles.

EXPERIMENTAL SECTION

All chemicals used in this study were of analytical grade and purchased from Merck, India, and used as obtained without additional purification. Zinc Oxide was purchased from Sigma–Aldrich, Acetic acid, hydrochloric acid, ethanol absolute were used as the starting materials (Sigma–Aldrich). Distilled water was used in all preparation procedures. All chemicals and reagents were of analytical grade and used without any further modification.

Plant materials

The fresh *Syzygium cumini* fruit (Jammun) seed was collected from the garden of Sri Paramakalyani Centre for Environmental Science campus, Manonmaniam Sundaranar University, Alwarkurichi.

Preparation of seed extract

The dried and finely cut seeds (10 g) were boiled in a 250 mL Erlenmeyer ask with 100 mL of double distilled water for fifteen minutes. Then the extract was filtered through ordinary filter paper or Whatman No. 1 filter paper. The filtrate was collected and it was kept in a refrigerator at 4°C for further experiments.

Synthesis of Zinc oxide nanoparticles

Typical synthesis process of ZnO nanoparticles 10mL of pure mixed with aqueous solution of 90 mL of 1mM Zinc

Oxide (ZnO) solution and kept at room temperature for constant stirring at 120 rpm. Changes in the color solution (300 to 700 nm) noted by visual inspection and the absorbance calculated by UV-Vis spectroscopy at different times and wavelengths confirmed the synthesis of ZnO nanoparticles.

Purification and characterization of Zinc oxide nanoparticles

The bioremediation of Zinc oxide ions in an aqueous solution using seed extract was observed by a double-beam UV-vis spectrophotometer at different wavelengths from 300 to 700 nm (Perkin Elmer Singapore). The synthesized ZnO nanoparticles are purified by distilled water by repeated centrifugation at 8000 rpm 15 min. The crystalline nature of ZnO nanoparticles was analyzed by XRD (Panalytical 'X' Part Pro X-ray Diffractometer) and the particle morphologic structure was characterized by Scanning Electron Microscope (SEM). The functional groups present in the plant seed extract responsible for ZnO nanoparticles formation were characterized by FT-IR (Perkin Elmer Singapore). The dried ZnO nanoparticles were measured at the wavelength range from 400 to 4000 cm^{-1}.

Fish maintenance and nanoparticles exposure

Local sellers in India provided wild type zebrafish (Danio rerio). The fish were adapted in a zebrafish aquarium at the Sri Paramakalyani Centre for Environmental Science campus, Manonmaniam Sundaranar University, Alwarkurichi, India, for one month in a glass aquarium of 50 L capacity and maintained in continuously well aerated water containing 2 mg/L instant ocean salt at approximately 28°C under a 14:10 hr light-dark cycle and pH of 7.2. (6.8-8.5). Fish were fed twice daily with a commercially available dry flakes meal (Basic Flake, China) and once daily with live Artemia nauplii (Inve Aquaculture Nutrition, Thailand). Zebrafish embryos were obtained by mating one female and two males in each breeding tank. To summarise, men and females were physically separated by a transparent block during the night, which was removed during the next day's morning light cycle to enable for reproduction to eliminate residues on the egg surface, eggs were washed numerous times with E3 medium. The fertilised eggs were immediately put on culture plates with 6, 12, 24, and 48 wells (20 embryos in 2 mL solution/well). There were three repetitions of each experimental treatment and control group. For 24hpf to 96hpf, healthy fertilised embryos were treated with Zinc oxide nanoparticles at concentrations of (0, 25, 50, 75, 100, and 150 µg/mL). Dead embryos were removed from plates every 24 hours in groups exposed to zinc oxide nanoparticles. All of the experimental plates were covered in foil to keep light out and kept at 28°C. The Institutional Animal Ethics Committee of Sri Lanka accepted all protocols. All protocols were approved by the Institutional Animal Ethics Committee of Sri Paramakalyani Centre for Environmental Science campus at Manonmaniam Sundaranar University, Alwarkurichi, following International guidelines for animal care.

Embryo toxicity test

The embryonic developmental stage of zebrafish embryo was studied under a stereomicroscope during the whole exposure period following fertilisation. For 24–96 hpf, the embryos were treated to Zinc oxide nanoparticle doses of 0, 25, 75, 100, and 150 µg/mL . Every 24 hours, the embryonic death and hatching rate were assessed. The hatching rate is the proportion of hatching embryos to the total number of live embryos in each well. Malformations were documented and photographed in both the control and treatment groups of embryos and larvae. Every 24 hours, photos of deformed embryos were obtained using a stereomicroscope (Optica, Italy Model; T3 15 A) and the proportion of aberrant embryos was counted.

RESULTS AND DISCUSSION

The color shift indicates the creation of ZnO nanoparticles. The purpose of this work is to look into the manufacture of zinc oxide nanoparticles utilizing the fruit seed extract of the *Syzygium cumini* plant. One of the procedures used to validate the production of ZnO nanoparticles in an aqueous solution is the identification of color changes from a colorless solution to a dark brown tint (Fig. 1). When the plant seed extract was treated with a ZnO nanoparticles solution, the manufacturing of nanoparticles began within minutes, confirming the color change. At the beginning of the process, after adding plant seed extract to the aqueous ZnO nanoparticles solution, the hue changed to dark brown. The intensity of the color was proportional to the incubation period. The color formation is due to the excitation of the surface Plasmon resonance effect of ZnO nanoparticles. The typical absorbance peak was observed at 361 nm. The lack of any additional peak in the spectrum demonstrates that the synthesized products are solely ZnO nanoparticles [29]. (See Fig. 1). It has been

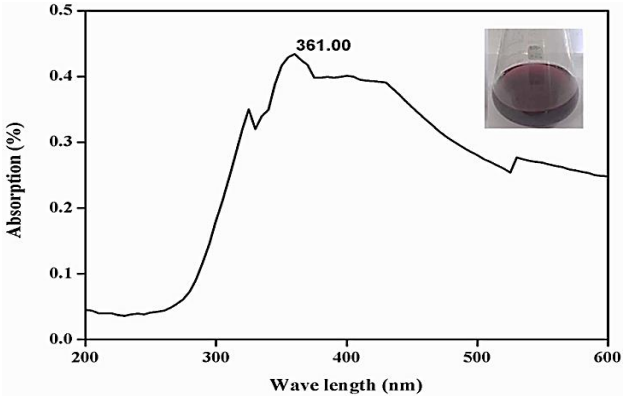

Fig. 1: UV spectrum of green synthesized Zinc oxide Nanoparticles.

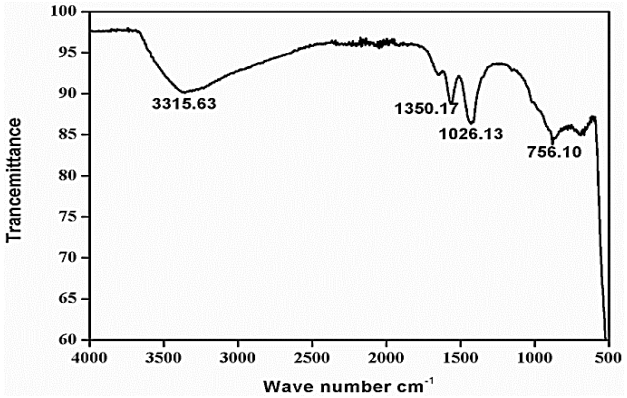

Fig. 2: FT-IR spectrum of green synthesized Zinc oxide Nanoparticles.

observed that the strength of the absorption peak in the UV–Visible spectrum is proportional to the particle size of nanoparticles. As particle size reduces, the absorption peak changes to a lower wavelength, resulting in a blue shift. Similarly, the other samples exhibit a minor blue shift as particle size decreases, implying that the strength of the absorbance peak exhibits a slight blue shift as particle size decreases. The absorption peak is affected by the type of polyols employed, the temperature, and the reaction duration [30, 31].

Fourier transform – infrared spectrophotometer studies

Fourier Transform Infra-Red Spectroscopy is an analytical technique used to quantify the intensity of light in the infrared vs the wavelength (wave number). The FT-IR spectrum is used to investigate the biomolecules in plant seed extracts. The FT-IR spectrum of *Syzygium cumini* plant seed extract revealed a significant band at 3315.63, 1350.17, 1026.13 and 756.10 cm^{-1}. The FT-IR spectra of ZnO nanoparticles generated in plant seed extract of *Syzygium cumini* in Supplementary data Fig. 2 displayed a distinctive peak at 3315.63 cm^{-1}, which was attributable to stretching vibrations of the hydroxyl group [32,33] and the peaks at 1350.17 cm^{-1} were attributed to –CH stretching, demonstrating the existence of CH_2, CH_3 groups [34]. Metal oxides usually display absorption bands in the fingerprint area below 1026.13 cm^{-1} due to interatomic vibrations. [35] Peaks in the infrared range at roughly 756.10 $^{-1}$ relate to ZnO and demonstrate the stretching vibration of Zn-O [36]. This finding suggests that fruit seed extract of *Syzygium cumini* plant seed extract is adsorbed on produced ZnO nanoparticles [34]. According to the literature [37], changes in particle sizes might result in varying wavenumbers and frequencies.

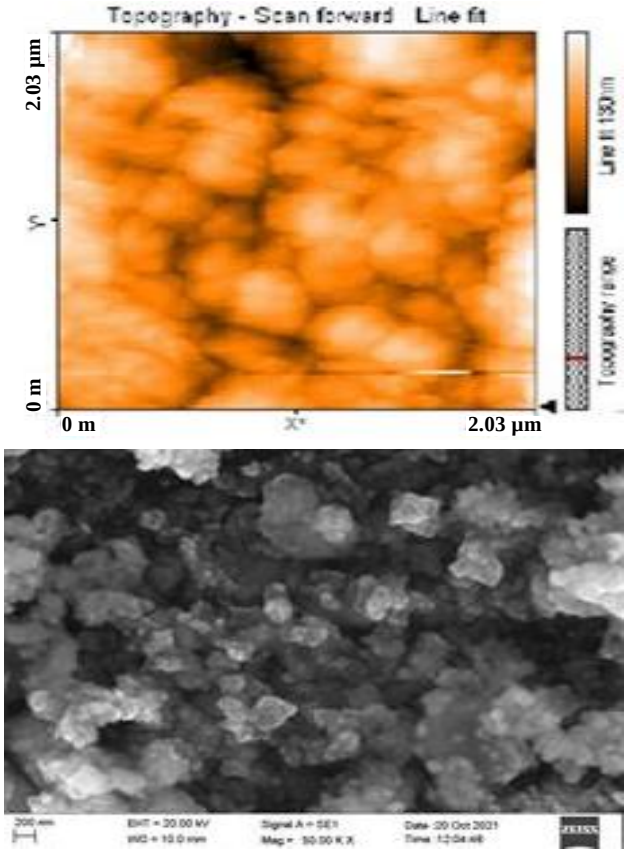

Fig. 3: Morphological Structure of green synthesized Zinc oxide nanoparticles

Analysis of Zinc oxide nanoparticles using SEM and AFM

SEM and AFM were used to determine the surface shape and dispersion of the produced ZnO nanoparticles (Fig. 3). The ZnO nanoparticles generated are generally spherical and rod-shaped, with some particles aggregating into bigger particles with no well-defined forms and being polydispersed in nature. The particle size ranges from 50

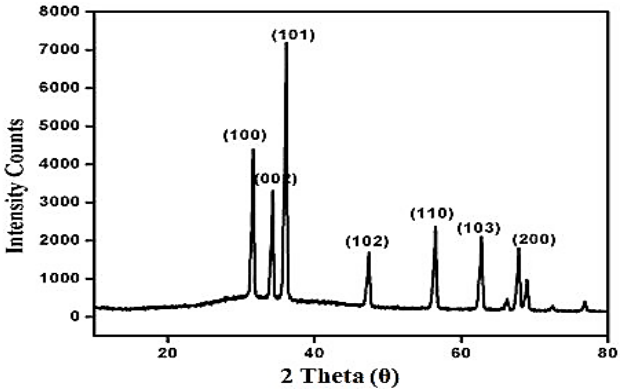

Fig. 4: XRD Patterns of Biologically synthesized ZnO Nanoparticles.

to 200 nm. The surface shape of the ZnO nanoparticles is further confirmed by the AFM photos.

Finally, the pictures from the SEM and AFM demonstrate that the produced ZnO nanoparticles are predominantly spherical and rod-shaped, with aggregates [38] Atomic Force Microscopy (AFM) analysis is a widely used technique for determining the size of nanoparticles. AFM provides information regarding the roughness of ZnO NPs [39]. The XRD data also validated the size of the NPs [40]. The size of ZnO NPs assessed by AFM 3D image was 30–82 nm.

X-ray diffraction analysis

XRD revealed the presence of bio-reduced metal nanoparticles (Fig. 4). The purpose of this research was to determine the crystallization, size, and structure of zinc oxide nanoparticles (Fig. 4). Peaks in the XRD diffraction pattern of ZnO nanoparticles are detected at two values of 31°, 34°, 36°, 47°, 56°, 62°, and 66°, which correspond to the set of lattice planes (100), (002), (101), (102), (110), (103). (200).

The ZnO nanoparticles were compared to a standard pure zinc oxide provided by the Joint Committee on Powder Diffraction Set (JCPDS File No; 76-0704). So, all diffraction peaks fit well with hexagonal wurtzite structure of ZnO nanoparticles, which proves that ZnO nanoparticles was successfully synthesized by green synthesis method [41]. Through Debye Sherrer's formula, the average particle size of ZnO nanoparticles was 37 nm at (101). There was no XRD pattern related to impurity. Thus, the XRD data demonstrates the purity of the produced ZnO nanoparticles. There was agreement between our experimentally observed and computed spacing data.

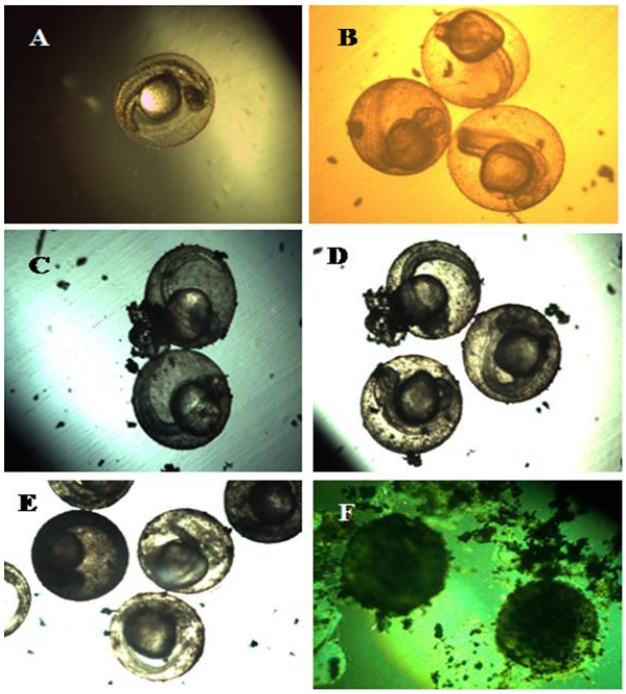

Fig. 5: Aggregations of ZnO NPs on Zebrafish Chorion Representative Images of embryos exposed to green synthesized ZnO Nanoparticles at 72-96 hpf: A- control, B- 25 µg/mL, C- 50 µg/mL, D- 75 µg/mL hpf: E- 100 µg/mL, F- 150 µg/mL.

Aggregation of ZnO NPs on Zebrafish Chorion

The spherical and rod-shaped nanoparticle aggregates stick into the surface of the chorion of an unhatched embryo after 24 hours of exposure to ZnO nanoparticles, and the aggregation of the nanoparticles at the surface of the chorion increased with the concentrations of the nanoparticles (Fig. 5).

These findings are consistent with those of [21], who investigated the effects of different concentrations of ZnO nanoparticles suspensions on embryonic chorion.

Effect of green synthesized Zinc oxide nanoparticles on zebrafish embryo

Because of their immense potential as an in vivo animal model, zebrafish embryos have been employed as model animals for nanomaterials toxicity studies. More crucially, because of their similarity and relevance to the physiological reactions of mammals, toxicological studies acquired from the Zebrafish model may be extended to human biology [42]. Zebrafish embryos were exposed to ZnO nanoparticles 50-200 nm at various doses (0, 25, 50, 75,100, and 150 µg/mL) to assess their toxicity. Largely sized ZnO nanoparticles accumulated on the outer surface of the embryonic chorion

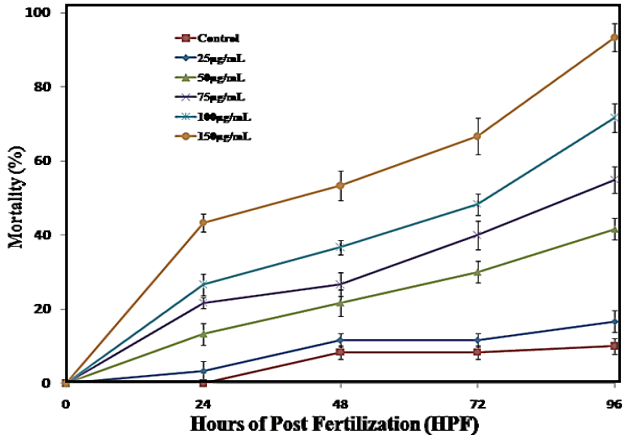

Fig. 6: Effects of Green produced ZnO NPs on Zebrafish Embryo Mortality from 24-96 hpf. The results demonstrated that embryos exposed to ZnO NPs observed increased in mortality rate.

in a concentration-dependent manner, as seen in Fig. 5. A similar finding was made using ZnO nanoparticles, graphene oxide (GO), and selenium NPs [43].

At certain time periods, the effect of zebrafish embryos and larvae exposed to varying concentrations of green-produced ZnO nanoparticles was studied (Fig. 6). During the 24-96 h exposure interval, 25µg/mL of ZnO nanoparticles had no effect on embryonic mortalities as compared to the control group (Fig. 6). The 25g/mL ZnO nanoparticles treated group, on the other hand, demonstrated a substantial increase in mortality in a dosage and time-dependent way. These findings are consistent with those of, those who discovered an increase in mortality after exposing zebrafish embryos to ZnO nanoparticles.

Zinc oxide nanoparticles induced developmental deformities in embryo

At 96 hpf, zebrafish embryos exposed to 25-150 µg/mL ZnO nanoparticles had a number of developmental defects, which were seen and reported. At 96 hpf, zebrafish embryos exposed to 50-150g/mL ZnO nanoparticles showed a dose-dependent increase in deformity.

At 96 hpf, embryos treated with 50 µg/mL ZnO nanoparticles had a characteristic morphological abnormality, including an unhatched coagulated egg, Spinal Curvature (SC), Axis Bending (AB), Tail Malformation (TM), Pericardial Edema (PCE), and Yolk Sac Edema (YSE). Embryos exposed to ZnO nanoparticles were unable to hatch and eventually perished in certain categories (Fig. 9 B & Fig. 5 E, F). At 96 hpf, a greater proportion of fish had enlarged yolk sacs and pericardial edoema (Fig. 9 F).

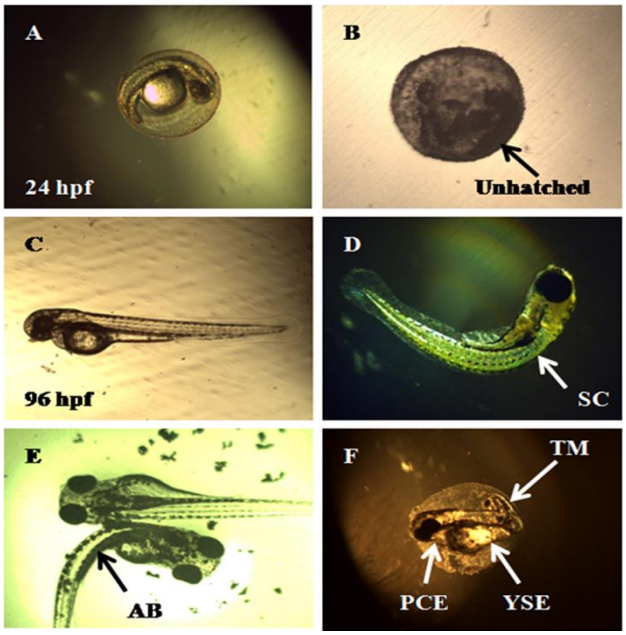

Fig. 7: Malformations of zebrafish embryos caused by exposure of green synthesized ZnO nanoparticles. The control group shows the normal appearance at 24hpf (A), Unhatched coagulated embryo (B). Control 96 hpf embryo (C), Spinal curvature (D), Axis bent (E), Embryo shows the Pericardial Edema (PCE), Yolk Sac Edema (YSE), and Tail malformation Axis abnormalities and Spinal cord curvature (C). Pericardial Edema (PE) and Yolk Sac Edema (YSE) (F).

In addition to our observations, numerous more have been made several other reports on ZnO nanoparticles showed similar developmental deformities in zebrafish embryos [44, 45].

Effect of Zinc oxide nanoparticles on hatching of zebrafish embryo

Zebrafish embryos have a hatching time of 48-72 hours under natural conditions, which is required for optimal embryonic development. The hatching rates of zebrafish embryos exposed to various concentrations of ZnO nanoparticles aggregates at early embryonic stages are depicted in (Fig. 7). In the control group, all of the embryos hatched successfully. When compared to the control exposure group, hatching rates with 25 and 50 µg/mL ZnO nanoparticles were delayed. However, those treated with 50µg/mL ZnO nanoparticles had significant embryonic hatching delays and toxicity. On embryonic zebrafish, ZnO nanoparticles aggregates caused a similar hatching impairment [46]. Our findings demonstrated that exposure to green-produced ZnO nanoparticles resulted in a dose-dependent influence on embryonic toxicity.

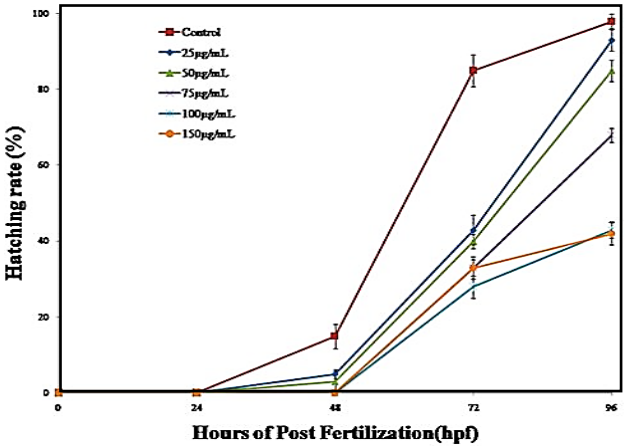

Fig. 8: Effects of Green synthesized ZnO NPs on hatching rates of zebrafish embryos from 24-96 hpf. The results showed a strong inhibition of the hatching rate after embryos were exposed to ZnO NPs.

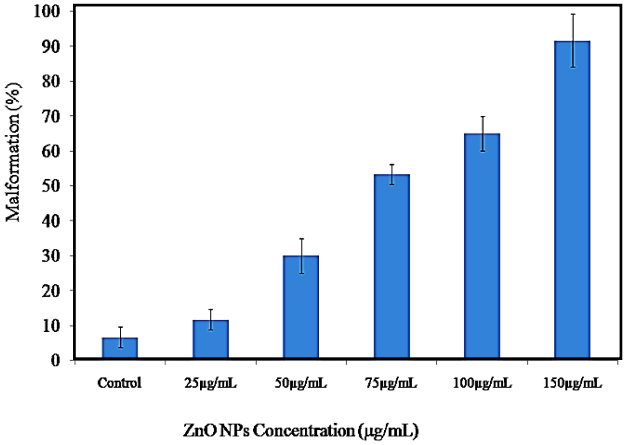

Fig. 9: Malformation of zebrafish embryos exposed to green synthesized ZnO nanoparticles. Malformed (%) rate of zebrafish embryos exposed to green synthesized ZnO nanoparticles at 96hpf. In each concentration total number of malformed embryos i.e Unhatched edema, coagulated, Spinal cord and axis curvature, Yolk sac and Pericardial edema was calculated. N= 20 embryos.

The normal embryonic development is hampered as a result of the hatching delay. ZnO nanoparticles were freshly produced from the seed extract of *Syzygium cumini* Jammun fruit (Indian blackberry), a traditional medicinal tropical plant from India, in our work. Our findings reveal that exposing zebrafish embryos to green-manufactured ZnO nanoparticles causes developmental toxicity, hatching delay, and deformity, which may give more meaningful experimental evidence for the safety and toxicity investigations of green-created ZnO nanoparticles The embryonic chorion was linked to the hatching delay caused by green produced ZnO nanoparticles. The zebrafish chorion is made up of minute pore canals that are around 0.5–0.7 mm in diameter and play an important role in the transfer of $02/Co_2$ nutrients and excretory materials enter and exit the embryo Furthermore, the zebrafish embryo is enveloped by a cellular envelope composed of three intercrossing layers, allowing materials to be transported by passive diffusion [47, 48]. Our findings indicate that exposure to green produced large size (50-200nm) ZnO nanoparticles aggregates may cause chorion pore canal blockage, resulting in hatching delay, which may directly interfere with embryonic development and toxicity.

Hatching delay was found using ZnO nanoparticles, which was consistent from our observations. However, the determination of toxicity levels is affected by differences in nanoparticle size, shape, technique of manufacture, solubility, and mode of exposure. Furthermore, expertise in Nanotoxicology regarding different antioxidant plant-mediated green produced nanoparticles to zebrafish embryonic developmental toxicity research is still inadequate. As a result, more specific experimental results are required to address the impact of embryonic chorion toxicity studies using various green designed nanoparticles.

CONCLUSIONS

The current work shows that green-produced ZnO nanoparticles are harmful to developing embryos. We generated ZnO nanoparticles using a unique *Syzygium cumini* Jammun fruit (Black plum, Indian blackberry) seed extract, which has a wide range of therapeutic applications in biopharmaceuticals, and ZnO nanoparticles are presently employed as biomedicine. As a result of our findings, we believe that exposure to ZnO nanoparticles might be a potentially dangerous factor to the environment and human health. However, additional research into the relationship between distinct green-designed ZnO nanoparticles exposure, adverse effects, and biological pathways is required for the safety assessment of ZnO nanoparticles.

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