Toxicity evaluation of zinc oxide nanoparticles green synthesized using papaya extract in zebrafish

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Abstract. In green synthesis of zinc oxide nanoparticles (ZnO NPs), the use of papaya extract as a capping and reducing agent shows promise for potential applications of these particles in biomedicine. However, toxicity evaluation is necessary to ensure the safety of humans and the environment. The zebrafish model is used to assess toxicity with embryo developmental observation as it is a rapid, simple method for screening of toxicity. The objective of the present study was to assess the toxicological characteristics of ZnO NPs produced from papaya extract using a zebrafish model. The preparation of plant extracts from papaya using two solvents (water and methanol) and characterization of bioactive compounds in the extracts were reported. ZnO NPs were synthesized from both plant extracts and characterized with scanning electron microscopy, X-ray diffraction and Fourier transform infrared spectroscopy. Toxicity evaluation was conducted on zebrafish embryos for 96 h. ZnO NPs synthesized from aqueous and methanol extracts had mean crystallite diameters of 13 and 12 nm, respectively. Mortality, hatching rate and malformation of zebrafish embryos were assessed at different concentrations of ZnO NPs. Both NPs showed high mortality rates at high concentrations, with 100 (aqueous) and 20 mg/l (methanol extract) being lethal for all embryos. Concentrations <10 mg/l for both synthesized ZnO NPs had similar results to the negative control, indicating a safe dosage for embryos. The hatching rate and malformation were also affected, with higher concentrations of NPs causing a delayed hatching rate and malformation in pericardial and yolk sac edema. Whole embryo mRNA expression of immune-associated genes, including *IL-1* and *-10* and *TNF-α,* was upregulated following lethal concentration 50 (LC50) ZnO NP exposure. ZnO NPs synthesized from papaya extract (both in aqueous and methanol environments) had a dose- and time-dependent embryonic toxicity effect. Hence, the present study demonstrated initial toxicity screening of ZnO NPs synthesized from plant extract.

Introduction

Zinc oxide nanoparticles (ZnO NPs) are widely used in various industries, including biomedicine, due to their versatility in optical and chemical properties and rating as generally recognized as safe metal oxide by the US Food and Drug Administration (1). Altering the properties and morphology of ZnO NPs is simple and it can be used to enhance their photocatalytic and photo-oxidizing potential (1). Traditional chemical (such as chemical vapor deposition and the use of toxic chemical reduction reagents such as sodium borohydride) and physical (e.g. sol-gel process and chemical co-precipitation) methods for NP synthesis are known to increase the toxicity and subsequently decrease their biocompatibility (2). To overcome this, green synthesis uses eco-friendly sources and methods. In green synthesis of NPs, substituting toxic reagents with plant-based natural capping agents decreases toxicity and increases biocompatibility. In this regard, papaya is used as a reducing agent to produce ZnO NPs (3). Papaya is widely available and easily accessible throughout the world (4). The fruit is rich in phytochemicals (phenolic and oleic acids and tannins) and biomolecular compounds. In addition to being a good source of nutrition, it is known for anti-inflammatory, antioxidant, antibacterial, antiviral and anti-parasitic properties, which are beneficial to health (5,6).

Despite the promising features of ZnO NPs and green synthesis methods, NP toxicity assessment is crucial. Comprehensive evaluation of the toxicology and hazardous properties of nanomaterials is key to ensuring human and environmental safety (6). The zebrafish (*Danio rerio*) is a widely used model for assessing diverse biological and

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toxicological responses (6-12). The small size of zebrafish and rapid embryonic development allows for cost-effective replication (8,9). Furthermore, notable homologous genes and physiological response similarities (such as immune response) between humans and zebrafish make this model useful for understanding toxicity mechanisms (8). Additionally, the zebrafish model enables real-time and non-invasive tracking of NP biodistribution (13).

Several studies regarding the toxicity of zebrafish in metal oxide NP, specifically in ZnO NP, have been conducted (9-12). However, only a few studies assessed the toxicology evaluation of ZnO NP synthesized via green synthesis methods (3,10,14). The present study prepared ZnO NPs using papaya extract from two solvents, aqueous and methanol, for toxicity evaluation using a zebrafish model. Characterization of ZnO NPs obtained through green synthesis was performed by scanning electron microscopy (SEM), Ultraviolet Visible (UV-Vis) spectrophotometer, X-ray diffraction (XRD) and Fourier transform infrared (FTIR) measurement. Assessment of toxicology was performed in zebrafish by identifying embryonic mortality, hatching rate and malformations. Subsequently, the immune response were assessed by gene expression analysis using quantitative (q)PCR targeting *TNF-α* and *IL-1* as pro-inflammatory gene, *IL-10* as an anti-inflammatory gene and the *elongation factor 1 α promotor* (*EF1α*) used as reference gene. Therefore, the aim of the present study is to investigate the toxicity of green synthesized ZnO NPs using papaya fruit extract on zebrafish embryonic development and immune response.

Materials and methods

Preparation of plant extract. Plant extracts were prepared with two solvents, distilled water and methanol ACS grade (cat. no. 6501-04, CAS no. 67-56-1; Anhui Fulltime Specialized Solvent & Reagent Co., Ltd.). Whole papayas with medium ripeness were used. All fruits were washed with distilled water, cut into small pieces, and dried until excess water was evaporated. A total of 75 g fruits were boiled with distilled water (1:2) for 30 min at 80˚C. The mixture was cooled and filtered with a Buchner funnel using Whatmann filter paper no. 1 three times to remove solid residue. The papaya extract was kept in a fridge at 4˚C until further experiments. For the methanol extraction, the fruits were separated and dried at 70˚C overnight. The dried fruit was ground to a soft powder using a blending machine. A total of 10 g fruits was weighed into a sterile Erlenmeyer flask, then 100 ml 70% methanol was added and left for 72 h at room temperature on the shaker. Filtered extract was evaporated at 40˚C using a rotary evaporator. The crude extract was stored at 4˚C for further processing (15). Bioactive compound analysis was conducted on both extracts using qualitative phytochemical tests, including phenolic, tannin, flavonoid, saponin, triterpenoid, steroid and alkaloid tests described by Ehiowemwenguan *et al* (16).

Green synthesis of ZnO NPs from plant extract. NP synthesis was conducted as described by Bayrami *et al* (17) and Dmochowska *et al* (18) with modifications. A total of ~20 ml papaya extract was diluted in 80 ml distilled water. Then, 6.42 mg zinc nitrate [$Zn(NO_3)2.6H_2O$, Sigma Aldrich; Merck KGaA; cat. no. 228737-100G] was added and stirred using a magnetic stirrer for 10 min. A total of 5 M NaOH was added until the pH reached 12. The solution was oven-dried at 60˚C for 1 h or until white precipitate was formed. The precipitate was rinsed with distilled water and ethanol (3:1) after supernatant was decanted. The pellet was centrifuged at 4,025 x g for 20 min at room temperature and incubated in an oven at 60˚C for 24 h. Then, using a crucible cup, the pellet was furnaced at 400˚C for 2 h, producing a white powder of NPs. The product was stored in a hermetic tube for testing and characterization.

Characterization of ZnO NPs. The structure of synthesized ZnO NPs was determined by SEM analysis (JSM 6510 LA, JEOL Ltd.) using gold (99.9%) coating with sputtering for 90 sec at room temperature, and the size of particles was analyzed using ImageJ software version 1.53t (ImageJ.org). The optical absorption spectra of ZnO NPs were recorded using a UV-visible spectrophotometer (Shimadzu Corporation; cat. no. UV-1900) at a range of 200-600 nm. Diffraction patterns were determined by XRD at 1˚/min with two angles from 20 to 80˚ (Bruker D8 Advance). Cu-K radiation (1.54060 Å) was operated at 40 kV and 40 mA. The results of XRD were analyzed using OriginLab version 2023b (originlab.com) to identify the type, morphology and crystal size of the measured particles. The diffraction peak maximum was observed at the 101 plane and the crystallite size was determined using Scherrer's physical formula as follows: $D=0.94\ \lambda/\beta\cos\theta$ where D is crystallite size, is the X-ray wavelength, and is the full width at half the maximum of the peak (19). Functional groups and compound classes of papaya extract and synthesized ZnO NPs were identified using FTIR (Shimadzu Corporation; Prestige 21) at room temperature with frequencies of 400-4,000 cm^{-1} (14).

Toxicity evaluation in zebrafish. Toxicity evaluations were conducted to identify the lethality of extract and ZnO NPs in fish embryos for 96 h. The mortality, abnormality, and hatching rate were assessed according to standard procedure by OECD Fish Embryo Acute Toxicity Test (FET) no. 236 (20).

Adult wild-type zebrafish (n=30, 4-5 months, 0.4-0.6 g) purchased from a local breeder from Bogor, Indonesia were maintained under standard laboratory conditions. Zebrafish were maintained in a temperature-controlled room at 28˚C with a 14 h light/10 h dark cycle in 12 L tanks with aerator. The zebrafish were fed three times/day with commercial pellets. The eggs of zebrafish were obtained 4-5 h post-fertilization (hpf) from breeding adult fish in a 1:2 female: male ratio and analyzed under stereo microscope (magnification 1.575x) to separate the viable eggs. A total of ~20 viable embryos were transferred to each well of a 24-well plate with 2 ml zebrafish culture medium (5 mM NaCl; 0,17 mM KCl; 0,33 mM CaCl2; 0,33 mM MgSO4, Sigma Aldrich). ZnO NPs synthesized from distilled water [papaya aqueous extract (PAE); 0.01, 0.10, 1.00, 10.00 and 100.00 mg/l] and methanol extract [papaya methanolic extract (PME); 1.25, 2.50, 5.00, 10.00 and 20.00 mg/l] were dispersed in distilled water before being added to the wells. Negative controls (zebrafish culture medium) were used to compare with positive controls (3,4-dichloroaniline) and treated groups, while internal plate controls (also in zebrafish culture medium) were used for checking the quality of the embryosaccording to standard procedure (20). The embryos

Table I. Primer sequences for gene expression analysis.

Primer	Sequence, 5'→3'	Accession no. (NCBI)
EF1α forward	CTGGAGGCCAGCTCAAACAT	AI330352
EF1α reverse	ATCAAGAAGAGTAGTAGTACC	
IL-10 forward	AGCACTCCACAACCCCAATC	AY887900
IL-10 reverse	GACCCCCTTTTCCTTCATCT	
TNF-a forward	CGTCTGCTTCACGCTCCAT	BC124141
TNF-a reverse	CTGGTCCTGGTCATCTCTCC	
IL-1 forward	CGCAGCACAAAATGAAGCAG	NM 212844.2
IL-1 reverse	TGTAAGACGGCACTGAATCC	

EF1α, elongation factor 1α; NCBI, National Center for Biotechnology Information.

were incubated for 96 h at 27±1˚C and observed every 24 h for toxicity evaluation. Toxicity evaluation comprised mortality, malformation, and hatching rate. The mortality and hatching rate were expressed as the number of dead embryos or eggs hatched compared with the control group. Abnormalities were analyzed by observing coagulation of embryos, lack of somite formation, pericardial/cardial edema and non-detachment of the tail. The probit analysis were used to calculate the LC50 dosage which is a method to analyze the relationship between the test compound/treatment and the response (mortality) in a binominal manner (21), The zebrafish embryos used for toxicity evaluation were euthanized using excess clove oil >100 ppm (22). All research procedures were approved by Research Ethics Commission, Padjadjaran University (approval no. 1026/UN6.KEP/EC/2022).

RNA isolation and cDNA synthesis. A total of ~40 zebrafish larvae 96 hpf from each treatment (lethal concentration 50 (LC50) and untreated control group were used for total RNA isolation using Quick-RNA™ MiniPrep Plus (Zymo Research Corp.), according to the manufacturer's instructions. The RNA was quantified using NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies Inc.). cDNA strand was then synthesized from total RNA templates using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The synthesized cDNA was stored at -20˚C for further experiments.

Gene expression analysis. cDNA amplification was performed using SYBR Green Mastermix (GoTaq® qPCR Master Mix; cat. no. A6001; Promega Corporation). Targeted genes associated with the immune response in zebrafish were *IL-10, IL-1,* and *TNF-α*. The housekeeping gene EF1α was used to normalize results. Primer sequences are listed in Table I. DNA amplification was performed according to the manufacturer's instructions using 5 μl 2X PCR premix, 1 μl primer mix, 2 μl nuclease-free water and 2 μl DNA template (nuclease-free water was used as negative control). Thermocycling conditions were as follows: Initial denaturation at 95˚C for 1 min, followed by 40 cycles (30 sec denaturation at 95˚C, 30 sec annealing at 58˚C and 45 sec extension at 72˚C) and 1 min final extension at 60˚C. Amplification and quantification were performed with CFX96 Biorad system (Biorad) and Quantstudio™ 1 RT-PCR, for analysis using QuantStudio Design and Analysis Software version 1.5.2 (Applied Biosystem, Thermo Fisher Scientific). Expression was calculated by normalizing Cq values of the target gene to the Cq value of the housekeeping gene (ΔCq) and normalized to untreated control (ΔCq untreated-ΔCq treated) (23).

Statistical analysis. Data are presented as the mean ± standard error of the mean and statistical significance of differences between groups was analyzed by performing one-way ANOVA followed by Tukey's post hoc test. For assessing mortality and hatching rate, a two-way ANOVA followed by Tukey's post hoc test with dosage was utilized. Data were obtained from three independent experiments. All the statistical analysis was performed using IBM Corp. SPSS Statistics 29.1 for Windows. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Synthesis and characterization of ZnO NPs. PAE and PME underwent preliminary phytochemical screening test to identify the bioactive compounds involved in synthesis of nanomaterials. Key phytochemical components of PME included phenolic compounds, tannins, saponins and triterpenoids, whereas PAE only contained triterpenoid (Table II).

ZnO NPs were synthesized using both PAE and PME, as corroborated by absorption bands at 387.4 and 364.0 nm using UV-Vis spectroscopy, respectively (Fig. 1A). Both bands are characteristic of ZnO NP (1,2). Furthermore, particles were also characterized using XRD, revealing typical hexagonal wurtzite structure of ZnO NPs based on the diffraction angles at 31.641, 34.291, 36.271, 47.481, 56.471, 62.801, 66.351, 67.711, 69.101 and 76.831, corresponding to the reflection planes of 110, 002, 101, 102, 110, 103, 200, 112, 201 and 202, respectively (Fig. 1B). The sharp and narrow diffraction peaks were in accordance with Joint Committee on Powder Diffraction Standards card no. 36-1451, which is a compiled database of diffraction patterns of various high quality powder, confirming the pure crystallite form of the ZnO hexagonal phase (wurtzite structure) (24). The diffraction peak maximum was observed at the 101 plane and. Mean

Table II. Preliminary qualitative screening analysis.

Phytochemical	Papaya aqueous extract	Papaya methanolic extract
Phenol	-	+
Tanin	-	+
Flavonoid (HCl + Mg)	-	-
Flavonoid (H_2SO_4)	-	-
Flavonoid (NaOH 10%)	-	-
Saponin	-	++
Triterpenoid	+	+
Steroid	-	-
Alkaloid	-	-

(-), no secondary metabolites detected; (+), secondary metabolites detected (few); (++), secondary metabolites detected (moderate).

crystallite size of ZnO NP PAE and PME was 13 and 12 nm, respectively. To visualize the structure of ZnO NPs, particles were also subjected to SEM imaging. ZnO NP synthesized had mean particle sizes of 198 and 152 nm for PAE and PME, respectively (Fig. 1C). The particles exhibited nanoflower morphology and surface structure, with only slight variations in thickness.

To identify the chemistry of the compounds from PAE and PME involved in the formation of ZnO NPs, analyses based on FTIR spectra were conducted (Fig. 1D). For PAE, the vibration bands were observed at 586.36, 777.31 and 817.82 (C-H), 1056.99 (C-O stretch of alcohols), 1409.96 (OH bend of phenol), 1622.13 (C=C stretching alkene), 2933.73 (C-H stretching of methylene) and 3423.65 cm^{-1} (O-H stretching of alcohols). For PME, the bands were recorded at 514.99, 628.79, 777.31, 819.75 and 866.04 (C-H vibrations), 700.16 (C-C vibrations), 921.97 (-$CH=CH_2$ vinyl terminal), 1,076.28 and 1,265.30 (C-O stretch of alcohols and phenol), 1,436.97 (C-H bend of methylene), 1,635.64 (C=C stretching alkene), 2,854.65 and 2,924.09 (C-H stretching of methylene) and 3,410.15 cm^{-1} (O-H stretching of alcohols). The functional groups identified by FTIR analysis were consistent with the phytochemical screening which indicated the presence of phenolic compounds, tannins, saponins and triterpenoids.

FTIR analysis of ZnO-PME revealed vibration bands at 422.41, 902.69, 1,382.96 and 1,620.21 (C=C stretching alkene), 2,372.44 (N-H component), 2,931.8 (C-H stretching of methylene) and 3,404.36 cm^{-1} (O-H stretching of alcohols). For ZnO-PAE, bands were observed at 414.7, 896.9, 1,016.49, 1,382.96 and 1,622.13 (C=C stretching alkene), 2,368.59 (N-H component), 2,931.8 (C-H stretching of methylene) and 3,425.58 cm^{-1} (O-H stretching of alcohols). The vibration bands between 400 and 600 cm^{-1} were attributed to the Zn-O group due to vibration of Zn and O atoms in ZnO (25,26).

Toxicity evaluation. Healthy zebrafish embryos (6 hpf) were used to assess the toxicity in terms of mortality, hatching rate and malformation. Mortality is defined as the number of zebrafish embryos that died during observation, while hatching rate is defined as the number of zebrafish embryos that hatched

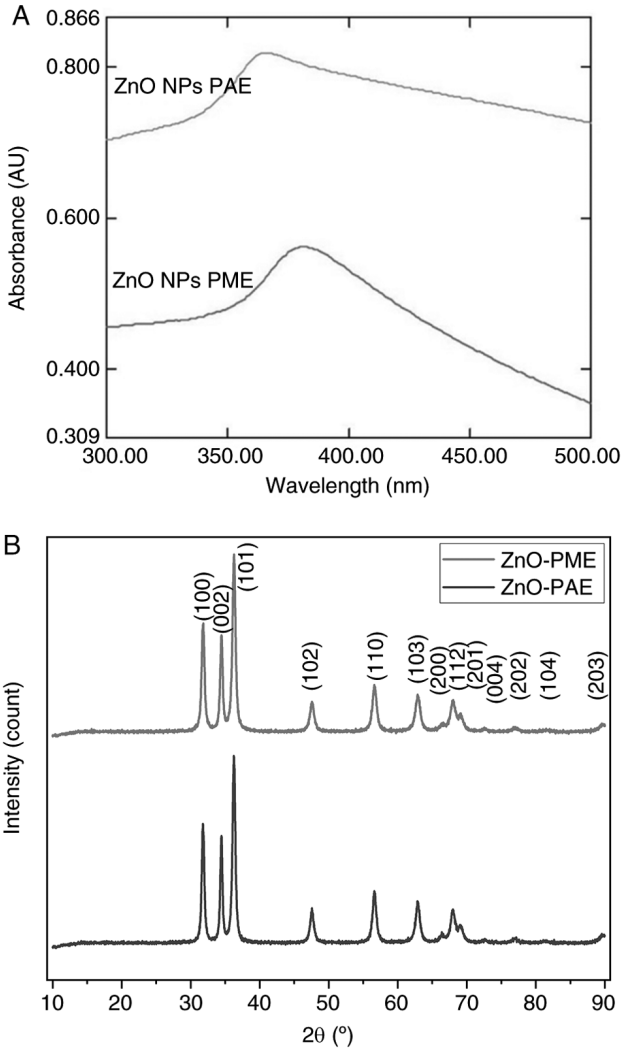

Figure 1. Continued.

from their chorion. Finally, malformation was considered to be a common abnormality that occurs in pericardial edema and yolk sac edema.

Mortality rate. The mortality rate of zebrafish exposed to ZnO NPs synthesized from PAE and PME was observed for 96 h (Fig. 2). Both types of ZnO NP showed a tendency for higher concentrations to cause significant mortality in zebrafish, with similar results for the positive control (3,4-dicholoroaniline) after 96 hpf. The highest concentration of ZnO NP PAE, 100 mg/l (Fig. 2A), led to the death of all zebrafish embryos at 96 h of observation. ZnO NP PME (Fig. 2B) at 20 mg/l showed mortality after 48 hpf. The concentration of 10 mg/l in both ZnO NPs showed a fairly high mortality, in which half of zebrafish embryos died. On the other hand, lower concentrations displayed similar results to the control group. For ZnO NP PAE, concentrations of 0.1 and 0.01 mg/l showed similar results at all time points. Similarly, the results obtained from the lowest concentration of ZnO NP PME which is 1.25 mg/l indicated similar results with that of the control group. The 96-h LC50 values estimated by probit analysis for ZnO NP PAE and PME were 8.246 and 6.568 mg/l, respectively.

Hatching rate. The hatching rate of zebrafish embryos was also investigated for 96 h. At 24 h, no embryos had

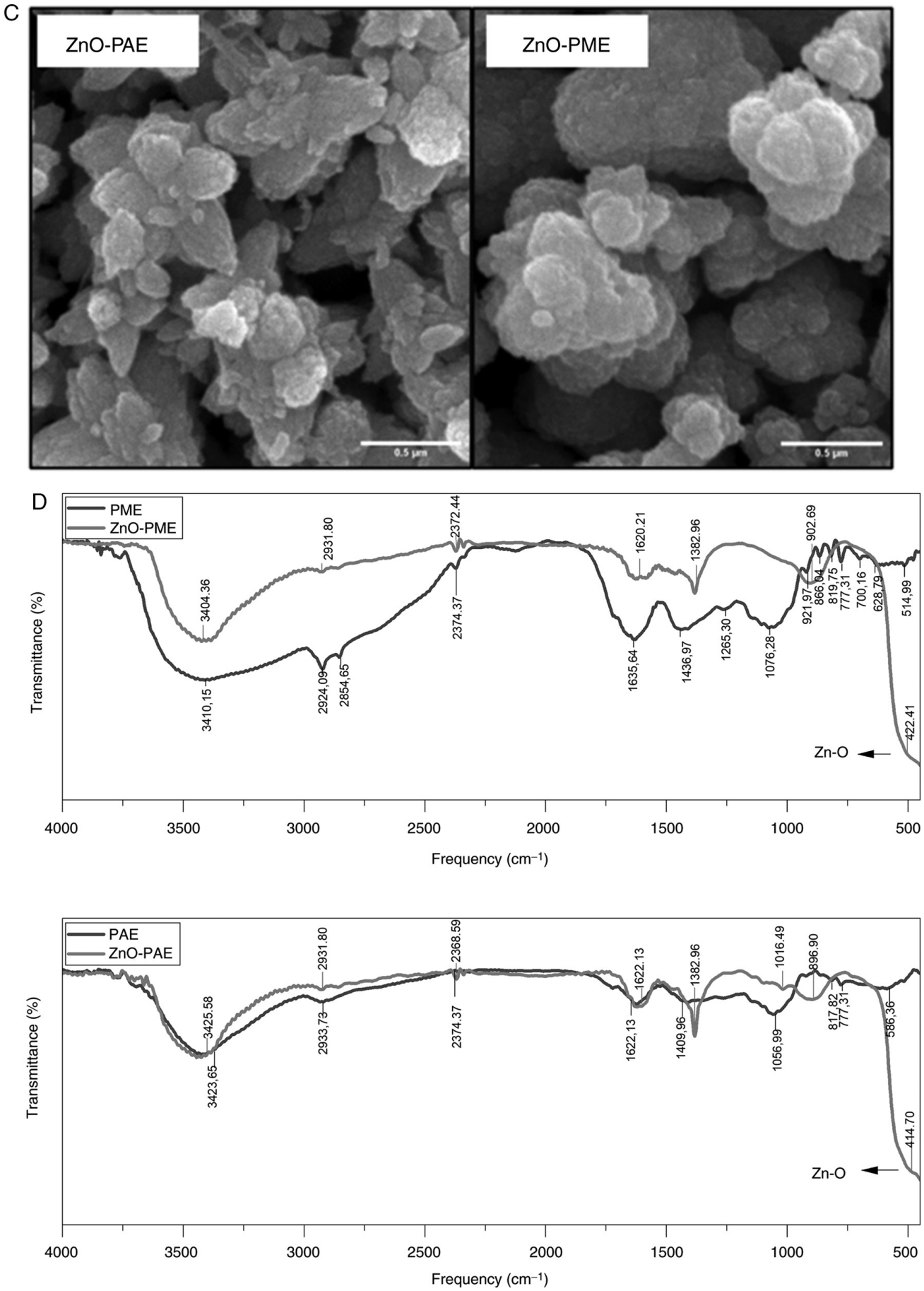

Figure 1. Characterization of ZnO NPs synthesized from PAE and PME. (A) UV-Vis spectrum, (B) XRD pattern, (C) morphology under scanning electron microscopy (magnification, x40,000) and (D) FTIR spectrum analysis of papaya extract. ZnO NP, zinc oxide nanoparticle; PAE, papaya aqueous extract; PME, papaya methanolic extract; UV-Vis, Ultraviolet Visible Spectroscopy; XRD, X-ray Diffraction; FTIR, Fourier Transform Infrared.

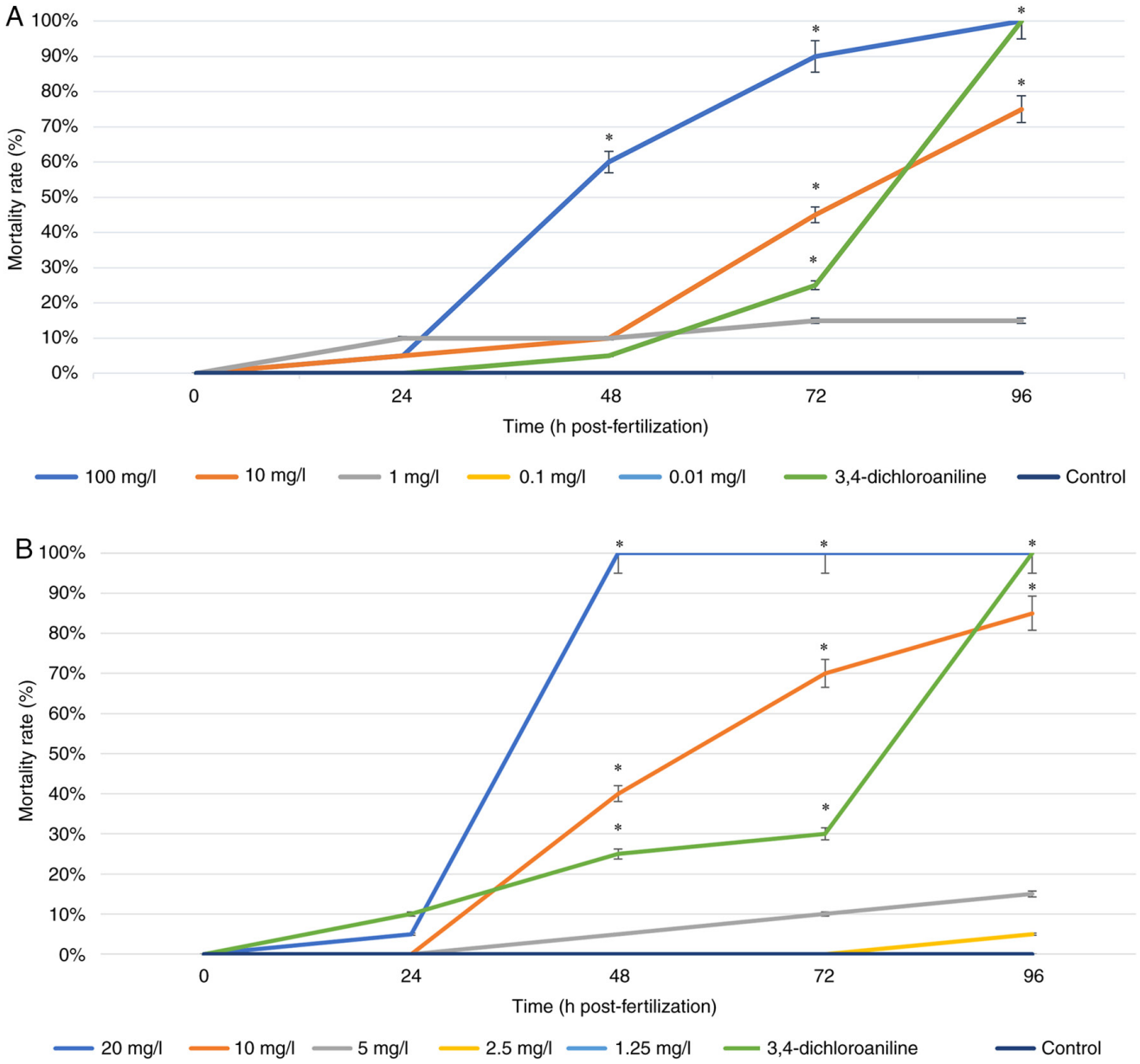

Figure 2. Mortality rate induced by ZnO NPs. (A) Mortality rate of zebrafish embryo exposed to ZnO NPs synthesized from papaya (A) aqueous and (B) methanolic extract. Data are presented as mean ± SEM (20 embryos/group and performed in triplicate). Two-way ANOVA followed by Tukey's test was performed. *P<0.05 vs. control. ZnO NP, zinc oxide nanoparticle.

hatched and the hatching rate significantly increased at 48 h (Fig. 3). In the control group, normal embryos hatched at 48-72 h. The hatching rate in all treatments using both ZnO NPs at low concentrations had the same results as the control group at 96 h of observation. (ZnO NP PAE, 0.10 and 0.01; PME: 1.25 and 2.50 mg/l; Fig. 3A and B, respectivey). The higher concentrations showed a significantly decreased hatching rate, with 100 PAE and 20 mg/l PME preventing all hatching. The concentration at 10 mg/l for both types of ZnO NP yielded a lower hatching rate compared with the positive control, showing that exposure to ZnO NP >10 mg/l led to inhibition of the development of the zebrafish embryo. ZnO NP exerted embryonic toxicity in a dose- and time-dependent manner.

Malformation. After exposure to ZnO NP PAE and PME, larvae showed several abnormalities or morphological alterations typical of metal oxide NP-induced toxicity, such as coagulation of the embryo and pericardial edema (20). Fish embryo acute toxicity tests have four core endpoints: i) Coagulation of fertilized eggs; ii) lack of somite formation; iii) non-detachment of the tail bud and iv) lack of heartbeat (20). Zebrafish embryos exposed to 20 mg/l ZnO NP PAE and PME showed coagulation after 48 hpf (Fig. 4A). Coagulation occurred at 24 hpf (Fig. 4A), indicating early death, and in later developmental stages, where the general development was delayed and the body typically started coagulating from the tail and the yolk sac.

In addition to the endpoints, other observations were recorded as lethal or sublethal endpoints (27). Malformations were identified (arrows) at the spine and sac yolk compared with the control group (Fig. 4B, C and F) following treatment with 5 mg/l ZnO NP PME and 10 mg/l ZnO NP PME and PAE,

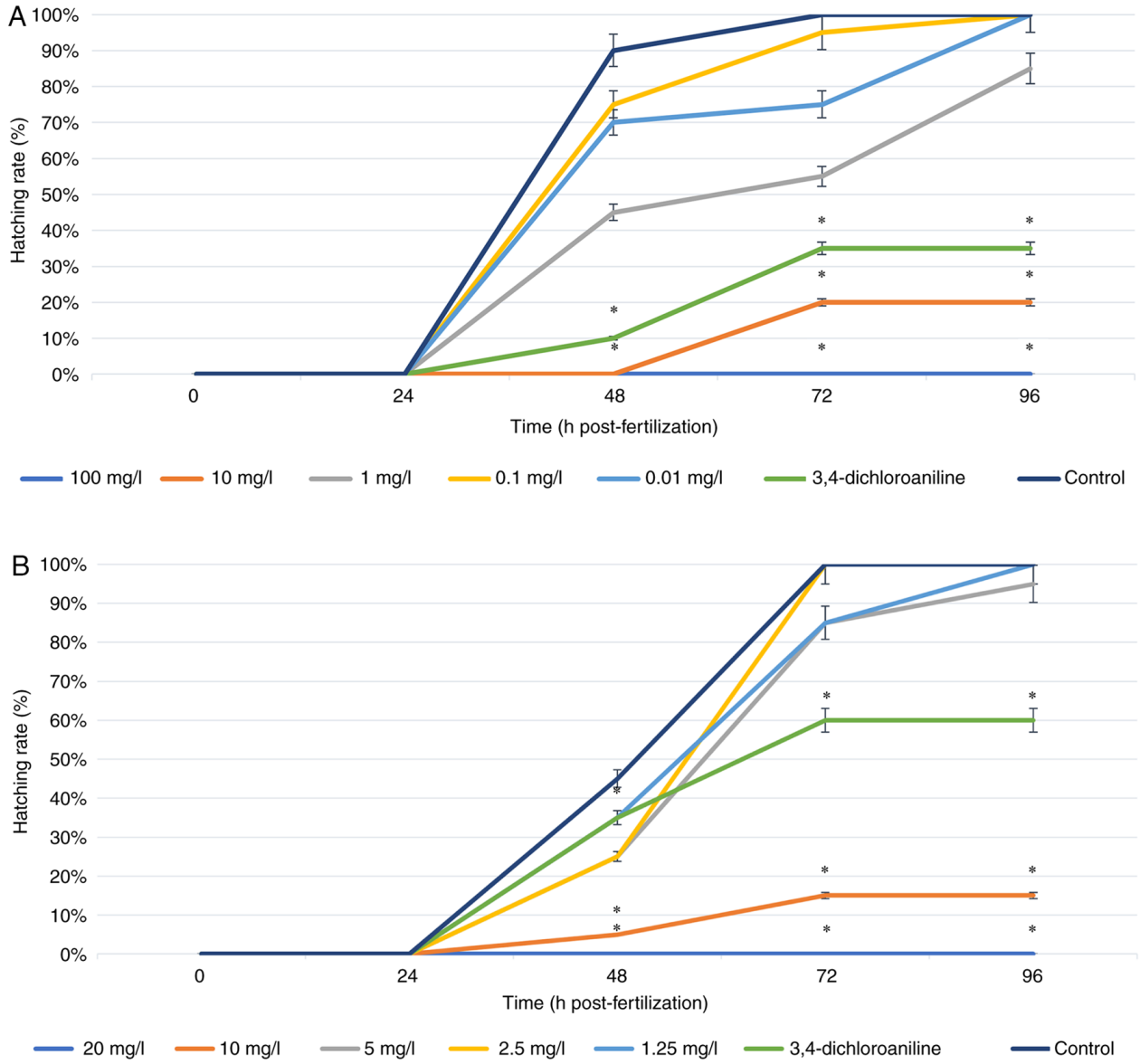

Figure 3. Hatching rate induced by ZnO NP. (A) Hatching rate of zebrafish embryos exposed to ZnO NP synthesized from papaya (A) aqueous and (B) methanolic extract. Data are presented as mean ± SEM (20 embryos/group and performed in triplicate). Two-way ANOVA followed by Tukey's test was performed. *P<0.05 vs. control. ZnO NP, zinc oxide nanoparticle.

indicating a toxic effect on embryonic development. Zebrafish embryos exhibited decreased eye size and the formation of pericardial edema at 72 hpf following exposure to 10 mg/l ZnO NP PME (Fig. 4D). However, lower concentrations of ZnO NP PAE and 1.25 and 2.5 mg/l ZnO NP PME showed no significant difference compared with the control (Fig. 4E).

Expression levels of inflammatory genes. LC50 of synthesized ZnO NPs upregulated transcripts of *IL-1* and *-10* and *TNF-α* (Fig. 5). *TNF-α* and *IL-1* and *-10* mRNA expression levels in whole zebrafish larvae were stable in controls but changed following exposure to 8.246 mg/l ZnO NP PAE and 6.568 mg/l PME for 96 h. The proinflammatory cytokine *TNF-α* mRNA expression levels following ZnO NP PME exposure were higher than following ZnO NP PAE exposure, while the highest *IL-1* mRNA expression was observed following exposure to ZnO-PAE. *IL-10* mRNA expression, an anti-inflammatory cytokine, was observed to be upregulated after exposure to both ZnO NP PAE and PME. This may indicate an immunomodulatory response after exposure to ZnO NPs (28).

Discussion

ZnO NP have unique features associated with biomedical applications, such as relatively high catalytic reactivity and also have a good non-linear optical performance and biochemical stability (2). Synthesis of ZnO NP yields different size, morphology and structure depending on methodology. Green synthesis is one of the most common metal oxide NP synthesis methods using biological substances (3). In the present study, ZnO NP was synthesized from papaya extract. The present study synthesized ZnO NP from papaya extract. Papaya contains abundant phytochemicals (i.e. phenolic, terpenoid, tannin, and alkaloid compounds) and has been shown to be beneficial in the treatment of several diseases,

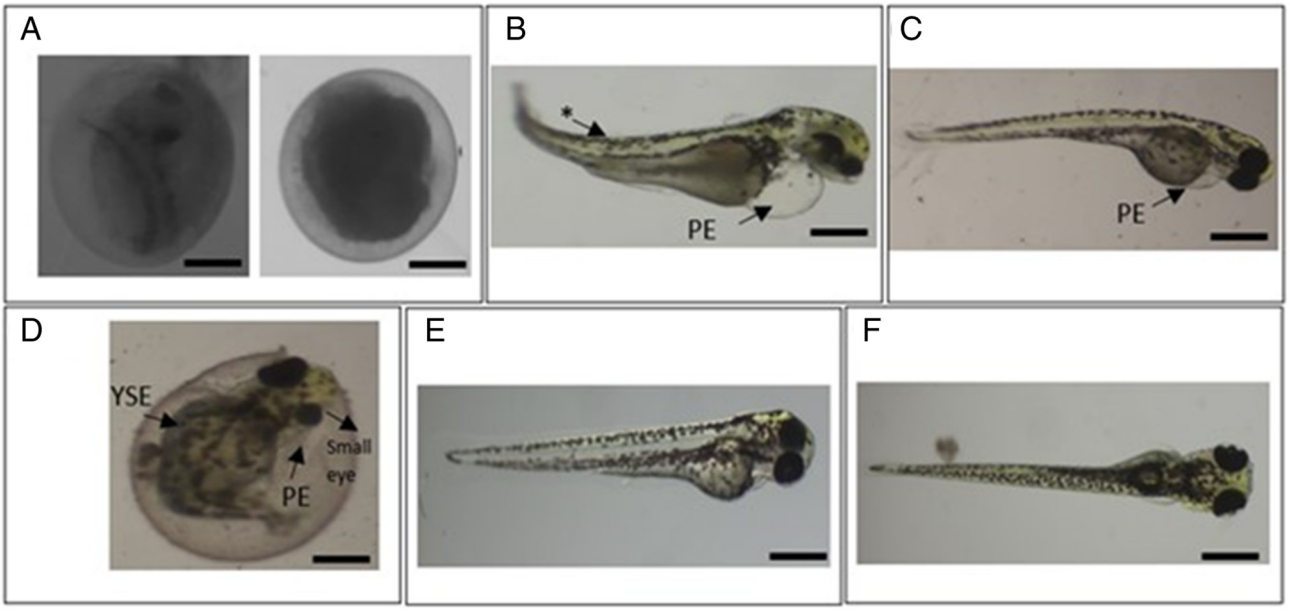

Figure 4. Malformation of zebrafish embryos exposed to ZnO NPs. Representative images of zebrafish embryos exposed to ZnO NP PAE and PME. Observations were made using a stereo microscope; magnification, x2.5. (A) Coagulation of zebrafish embryo exposed to 20 mg/l ZnO NP PAE and PME after 48 hpf. (B) Bent spine and yolk sac edema of zebrafish embryo exposed to 5 mg/l ZnO NP PME at the end of observation. (C) Yolk sac edema of zebrafish embryo exposed to 10 mg/l ZnO NP at the end of observation. (D) Reduced eye size and pericardial edema of zebrafish exposed to 10 mg/l ZnO NP PME after 72 hpf. (E) No visible malformation observed in larva zebrafish after exposure at lower dose of ZnO NP. (F) Normal development of the control zebrafish embryo. ZnO NP, zinc oxide nanoparticle; PAE, papaya aqueous extract; PME, papaya methanolic extract; PE, Pericardial edema.

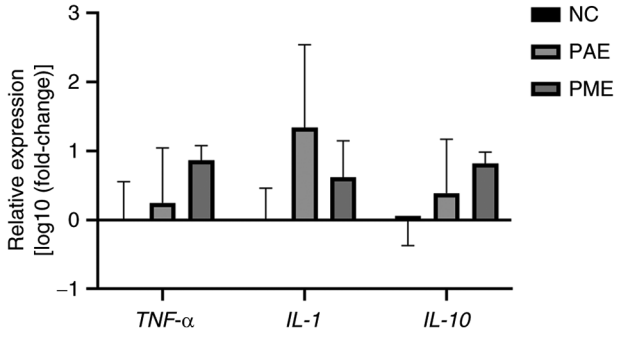

Figure 5. Relative expression of immune response-associated genes. Zebrafish were exposed to zinc oxide nanoparticles synthesized from PAE (8.246 mg/l) and PME (6.568 mg/l) at 4-96 h post-fertilization. The relative expression was normalized to *elongation factor* 1α. All data are presented as mean ± SEM (40 embryos/group and performed in triplicate). One-way ANOVA followed by Tukey's test was performed. PAE, papaya aqueous extract; PME, papaya methanolic extract; NC, negative control.

such as inflammation, hyperglycemia, and hypertension. It also possesses anticarcinogenic, antiparasitic and antimicrobial activities (29). Here, papaya extract was obtained using distilled water and methanol to explore the effects of ZnO NP.

The phytochemical screening was carried out for both extracts to determine the bioactive compound involved in ZnO NP synthesis. Phytochemicals such as terpenoids, alkaloids, phenolics, tannins, amino acids and saponins extracted from plants are potential substitutes for stabilizing and reducing agents (5,30,31). PME produced more phytochemicals such as phenolic compounds, tannins, saponins and triterpenoids compared with PAE, which only produced triterpenoid. These results may be due to differences in the polarity of distilled water and methanol, which dissolve different bioactive compounds during extraction. Papaya might contain high levels of biocompounds that are soluble in water (29). Phenolic and other phytochemical compounds in the papaya fruit extract serve as capping agents for NPs. Phenol, triterpenoid, and saponin are also known for their antioxidant, anticarcinogenic and anti-inflammatory activities (14,30). Coordination with -OH and COOH groups stabilizes and caps synthesized ZnO NPs (31). Terpenoid and phenolic group molecules are responsible for the reduction process (18).

Based on XRD and SEM analyses, there was a slight difference in size between ZnO NP PAE and PME. ZnO NP PAE showed a larger size (SEM, 207 nm; crystallite size from XRD, 13 nm) compared with the ZnO NP PME (SEM, 188 nm; crystallite size from XRD, 12 nm). The difference in size of ZnO NP might be due to the number of phytochemicals involved in the reduction of ZnO NP as PME contained more biocompounds extracted than PAE (29,30). Despite that, surface morphology and crystallite structure analyzed showed a similar nanoflower shape with wurtzite structure, which is typical morphology for ZnO NP (5,26,32,33). Furthermore, the FTIR analysis indicated that PME showed more vibration bands than PAE, indicating that more phytochemicals were contained in PME. Peaks observed from ZnO NP PAE and PME showed that both synthesized ZnO NPs have organic functional groups from phytochemical components of papaya fruit extract, which are strongly attached to the surface of Zn precursor and act as both capping and reducing agents (5,31). As functional groups were similar for ZnO-PME and ZnO-PAE, similar compounds may have been involved in the reductive synthesis using both extracts and remained as the capping agents surrounding NPs, such as phenol and triterpenoid (26). Biomolecules are bifunctional in the formation and stabilization of ZnO NPs in aqueous medium;

the phenolic group prevents agglomeration, allowing metal NPs to form and stabilize (34).

Numerous *in vivo* toxicity assessments of ZnO NP have been conducted in various animal models to determine the effect of ZnO NP in organisms (3,9-11,28,35,36), Toxicity assessment has been performed in several systems (such as pulmonary, renal and reproductive) in mammalian models (28,36). However, assessment in mammalian models is time-consuming, expensive and laborious due to invasive distribution of ZnO NP. For initial screening of nanomaterial toxicity, zebrafish models provide a quick and easy assessment (6). The zebrafish model is typically used in developmental toxicology as embryos are optically transparent and developmental observation is simple using microscopy. Moreover, zebrafish share close homology with the human genome and similar physiological responses to mammalian models (8,29).

Earlier developmental embryos are more susceptible to external substances than adult or larval zebrafish (8,29). Hence, administration of ZnO NPs synthesized from PAE and PME to examine their potential toxicity was performed during the embryonic period (4-96 hpf). The present study indicated that ZnO NPs synthesized from PAE and PME led to a rise in mortality and a decline in hatching rate in a concentration- and time-dependent manner. Similar outcomes have been reported in previous studies of nanomaterials, where the survival and hatching rates of zebrafish embryos decreased (9-11,36-38). Furthermore, the reduced hatching rate results from delays in the development of the embryos and some of the embryos developed malformation in some organs (37,38). Common examples observed in malformed embryos were decreased heartbeat or blood flow, lack pigmentation, delayed or altered development, modified movement, distortion of the spine and formation of various types of oedemata (6,7). Oedemata in zebrafish appears to be of little mechanistic value and is categorized as an unspecific side effect of both acute and sublethal toxicity as changes typical of cardiotoxicity have also been described following exposure to nanomaterials (18,29). The toxic effect of ZnO NPs may be due to the dissolved ZnO NPs in Zn^{2+} ions, which are free in intracellular cells. The disruption of cellular Zn homeostasis in the cell is associated with mitochondrial dysfunction and oxidative stress (27). Zn is known as an element of many transcription factors, like zinc finger protein transcription factors and enzymes (35,39).

The primary molecular mechanism underlying the toxicity of NPs is the formation of reactive oxygen species (ROS), which leads to the induction of inflammation (40). Inflammatory cytokines, such as ILs and TNF are proteins secreted mainly by activated immune cells such as macrophages and neutrophils (41). These key proinflammatory cytokines produced by activated immune cells in zebrafish and the first cytokines produced in the initial stages of inflammation (41,42). *TNF-α* and *IL-1* mediate the innate immune response, which is an important activator of inflammation (43,44). *IL-10* is a potent anti-inflammatory cytokine that suppress the transcription of proinflammatory cytokines (45).

The present study showed that expression of genes associated with the immune response might be stimulated by ZnO NP exposure. Similarly, in whole zebrafish embryos, copper (Cu) NPs (25 nm; 1 mg/l), soluble Cu, and polystyrene NPs (25 nm; 10 mg/l) were able to upregulate more mRNA expression of innate immune genes [*IL-1β* and *immunoresponsive gene 1* (*irg1l*)] on the skin cells (epithelium) than in the intestine. Exposure to CuNPs induces neutrophil migration in the tail as a result of altered transcriptional changes in pro-inflammatory genes, indicating NP-specific inflammatory response (45). ZnO NP exposure induces immunotoxicity in BALB/c mice in an age-dependent manner, as aged mice exhibited altered CD4 and CD8 cells, *IL-6, TNF-α* and *IFN-γ* (28).

Exposure to ZnO NPs promotes production of ROS, which leads to oxidative and endoplasmic reticulum stress, then excessive uptake and formation of fatty acids, which are deposited in the liver and cause non-alcoholic fatty liver disease (46). The cytotoxic activity of ZnO NPs has been shown to be associated with the physicochemical properties of their surface, which may be exert an indirect (influencing kinetics and release of zinc ions, which are the main factor causing toxicity) or direct (interacting with cell membranes) influence (47-49). Toxicity of ZnO NP in the aqueous environment depends on the hydrodynamic diameter and concentration, with larger hydrodynamic diameters and high concentrations associated with higher toxicity (36). Nevertheless, the quantitative association (amount of Zn^{2+} release from ZnO NPs that leads ROS production in cells) of the dose-response relationship for both ZnO NP synthesized should be determined (48). In addition, expression of genes associated with antioxidant, antibacterial, antiviral and anti-parasitic properties need to be assessed in ZnO NPs from PAE and PME.

Here, ZnO NPs synthesized from PME and PAE gave similar outcomes in toxicity, with the mean safe dosage of ZnO NPs <10 mg/l. This is also consistent with previous studies with ZnO NPs synthesized from *Amaranthus caudatus* causing deformities and a lower survival rate at ≥10 mg/ml (50). Although the present study did not conduct experiments with different concentrations of papaya extract to synthesize ZnO NPs or change the shape of ZnO NPs (spheres), to the best of our knowledge, the present study is the first to screen toxicity of ZnO NPs synthesized from plant extracts. One way to reduce the toxicity of ZnO NPs is surface modification with silica coating to prevent dissolution of ZnO NPs (51).

To summarize, the present study indicated that exposure to ZnO NPs synthesized from plant extract leads to developmental toxicity in embryos based on mortality, hatching rate and malformation. ZnO NPs may pose a potential environmental hazard, highlighting the need for further investigation into the association between ZnO NP exposure, adverse effects and the underlying biological mechanisms to evaluate safety of ZnO NPs.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

IW, RMP, NGZ and APS contributed to the conceptualization, study design, formal analysis, and data interpretation. NGZ and APS performed all experiments and wrote the manuscript. RKP performed toxicological experiments and data analysis. NGZ and APS confirm the authenticity of all the raw data. IW and RMP contributed to supervision and writing of original draft. SD contributed to formal analysis, validation, and editing the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran, Bandung, West Java, Indonesia (approval no. 1026/UN6. KEP/EC/2022).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Yadi M, Mostafavi E, Saleh B, Davaran S, Aliyeva I, Khalilov R, Nikzamir M, Nikzamir N, Akbarzadeh A, Panahi Y and Milani M: Current developments in green synthesis of metallic nanoparticles using plant extracts: A review. Artif Cells Nanomed Biotechnol 46 (Suppl 3): S336-S343, 2018.
2. Sadhasivam S, Shanmugam M, Umamaheswaran PD, Venkattappan A and Shanmugam A: Zinc oxide nanoparticles: Green synthesis and biomedical applications. J Clust Sci 32: 1441-1455, 2021.
3. Kalpana VN and Devi Rajeswari V: A review on green synthesis, biomedical applications, and toxicity studies of ZnO NPs. Bioinorg Chem Appl 2018: 3569758, 2018.
4. Asghar N, Naqvi SAR, Hussain Z, Rasool N, Khan ZA, Shahzad SA, Sherazi TA, Janjua MR, Nagra SA, Zia-Ul-Haq M and Jaafar HZ: Compositional difference in antioxidant and antibacterial activity of all parts of the Carica papaya using different solvents. Chem Cent J 10: 5, 2016.
5. Rathnasamy R, Thangasamy P, Thangamuthu R, Sampath S and Alagan V: Green synthesis of ZnO nanoparticles using Carica papaya leaf extracts for photocatalytic and photovoltaic applications. J Mater Sci Mater Electron 28: 10374-10381, 2017.
6. Jia HR, Zhu YX, Duan QY, Chen Z and Wu FG: Nanomaterials meet zebrafish: Toxicity evaluation and drug delivery applications. J Control Release 311-312: 301-318, 2019.
7. Chahardehi AM, Arsad H and Lim V: Zebrafish as a successful animal model for screening toxicity of medicinal plants. Plants (Basel) 9: 1345, 2020.
8. Lee KY, Jang GH, Byun CH, Jeun M, Searson PC and Lee KH: Zebrafish models for functional and toxicological screening of nanoscale drug delivery systems: Promoting preclinical applications. Biosci Rep 37: BSR20170199, 2017.
9. Bai W, Zhang Z, Tian W, He X, Ma Y, Zhao Y and Chai Z: Toxicity of zinc oxide nanoparticles to zebrafish embryo: A physicochemical study of toxicity mechanism. J Nanopart Res 12: 1645-1654, 2010.
10. Machado S, González-Ballesteros N, Gonçalves A, Magalhães L, Sárria Pereira de Passos M, Rodríguez-Argüelles MC and Castro Gomes A: Toxicity in vitro and in zebrafish embryonic development of gold nanoparticles biosynthesized using cystoseira macroalgae extracts. Int J Nanomedicine16: 5017-5036, 2021.
11. Lacave JM, Retuerto A, Vicario-Parés U, Gilliland D, Oron M, Cajaraville MP and Orbea A: Effects of metal-bearing nanoparticles (Ag, Au, CdS, ZnO, SiO2) on developing zebrafish embryos. Nanotechnology 27: 325102, 2016.
12. Zakaria ZZ, Mahmoud NN, Benslimane FM, Yalcin HC, Al Moustafa AE and Al-Asmakh M: Developmental toxicity of surface-modified gold nanorods in the zebrafish model. ACS Omega 7: 29598-29611, 2022.
13. Johnston HJ, Verdon R, Gillies S, Brown DM, Fernandes TF, Henry TB, Rossi AG, Tran L, Tucker C, Tyler CR and Stone V: Adoption of in vitro systems and zebrafish embryos as alternative models for reducing rodent use in assessments of immunological and oxidative stress responses to nanomaterials. Crit Rev Toxicol 48: 252-271, 2018.
14. Singh C, Kumar J, Kumar P, Chauhan, BS, Nath G and Singh J: Green synthesis of silver nanoparticles using aqueous leaf extract of Premna integrifolia (L.) rich in polyphenols and evaluation of their antioxidant, antibacterial and cytotoxic activity. Biotechnol Biotechnol Equip 33: 359-371, 2019.
15. Bere AW, Mulati O, Kimotho J and Ng'ong'a F: Carica papaya leaf extract silver synthesized nanoparticles inhibit dengue type 2 viral replication in vitro. Pharmaceuticals (Basel) 14: 718. 2021.
16. Ehiowemwenguan G, Emoghene AO and Inetianbor JE: Antibacterial and phytochemical analysis of Banana fruit peel. IOSR J Pharm 4: 18-25, 2014.
17. Bayrami A, Parvinroo S, Habibi-Yangjeh A and Rahim Pouran S: Bio-extract-mediated ZnO nanoparticles: Microwave-assisted synthesis, characterization and antidiabetic activity evaluation. Artif Cells Nanomed Biotechnol 46: 730-739, 2018.
18. Dmochowska A, Czajkowska J, Jędrzejewski R, Stawiński W, Migdał P and Fiedot-Toboła M: Pectin based banana peel extract as a stabilizing agent in zinc oxide nanoparticles synthesis. Int J Biol Macromol 165: 1581-1592, 2020.
19. Sharma D, Sabela MI, Kanchi S, Bisetty K, Skelton AA and Honarparvar B: Green synthesis, characterization and electrochemical sensing of silymarin by ZnO nanoparticles: Experimental and DFT studies. J Electroanal Chem 808: 160-172, 2018.
20. Organisation for Economic Co-operation and Development: Test No. 236: Fish embryo acute toxicity (FET) test. Guidelines for the Testing of Chemicals. Paris, France, 2013.
21. Halmi MIE, Rahim MBHA and Othman AR: Estimation of LC50 and its confidence interval for the effect of ferrous sulphate on Catla catla. J Environ Microbiol Toxicol 6: 21-23, 2018.
22. Fernandes IM, Bastos YF, Barreto DS, Lourenço LS and Penha JM: The efficacy of clove oil as an anaesthetic and in euthanasia procedure for small-sized tropical fishes. Braz J Biol 77: 444-450, 2017.
23. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
24. Etape EP, Foba-Tendo J, Ngolui LJ, Namondo BV, Yollande FC and Nguimezong MBN: Structural characterization and magnetic properties of undoped and Ti-Doped ZnO nanoparticles prepared by modified oxalate route. J Nanomater 2018: 9072325, 2018.
25. Awasthi A, Bansal S, Jangir LK, Awasthi G, Awasthi KK and Awasthi K: Effect of ZnO nanoparticles on germination of Triticum aestivum seeds. Macromol Symp 376: 1700043, 2017.
26. Awwad AM, Albiss B and Ahmad AL: Green synthesis, characterization and optical properties of zinc oxide nanosheets using Olea europea leaf extract. Adv Mater Lett 5: 520-524, 2014.
27. von Hellfeld R, Brotzmann K, Baumann L, Strecker R and Braunbeck T: Adverse effects in the fish embryo acute toxicity (FET) test: A catalogue of unspecific morphological changes versus more specific effects in zebrafish (*Danio rerio*) embryos. Environ Sci Eur 32: 122, 2020.
28. Senapati VA, Gupta GS, Pandey AK, Shanker R, Dhawan A and Kumar A: Zinc oxide nanoparticle induced age dependent immunotoxicity in BALB/c mice. Toxicol Res (Camb) 6: 342-352, 2017.
29. Dotto JM and Abihudi SA: Nutraceutical value of Carica papaya: A review. Sci Afr 13: e00933, 2021.
30. Truong DH, Nguyen DH, Ta NTA, Bui AV, Do TH and Nguyen HC: Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of Severinia buxifolia. J Food Qual 2019: 8178294, 2019.

31. Senthilkumar N, Nandhakumar E, Priya P, Soni D, Vimalan M and Vetha Potheher I: Synthesis of ZnO nanoparticles using leaf extract of Tectona grandis (L.) and their anti-bacterial, anti-arthritic, anti-oxidant and in vitro cytotoxicity activities. New J Chem 41: 10347-10356, 2017.
32. Dulta K, Koşarsoy Ağçeli G, Chauhan P, Jasrotia R and Chauhan PK: Ecofriendly synthesis of zinc oxide nanoparticles by Carica papaya leaf extract and their applications. J Clust Sci 33: 603-617, 2022.
33. López-Miranda JL, España Sánchez BL, Esparza R and Estévez M: Self-assembly of ZnO nanoflowers synthesized by a green approach with enhanced catalytic, and antibacterial properties. Mater Chem Phys 289: 126453, 2022.
34. Hu YL, Qi W, Han F, Shao JZ and Gao JQ: Toxicity evaluation of biodegradable chitosan nanoparticles using a zebrafish embryo model. Int J Nanomedicine 6: 3351-3359, 2011.
35. Vandebriel RJ and De Jong WH: A review of mammalian toxicity of ZnO nanoparticles. Nanotechnol Sci Appl 5: 61-71, 2012.
36. Amin N, Zulkifli SZ, Azmai MNA and Ismail A: Toxicity of zinc oxide nanoparticles on the embryo of javanese medaka (Oryzias javanicus Bleeker, 1854): A comparative study. Animals (Basel) 11: 2170, 2021.
37. Duan J, Yu Y, Shi H, Tian L, Guo C, Huang P, Zhou X, Peng S and Sun Z: Toxic effects of silica nanoparticles on zebrafish embryos and larvae. PLoS One 8: e74606, 2013.
38. Zhu X, Zhu L, Duan Z, Qi R, Li Y and Lang Y: Comparative toxicity of several metal oxide nanoparticle aqueous suspensions to zebrafish (*Danio rerio*) early developmental stage. J Environ Sci Health A Tox Hazard Subst Environ Eng 43: 278-284, 2008.
39. Haque E and Ward AC: Zebrafish as a model to evaluate nanoparticle toxicity. Nanomaterials (Basel) 8: 561, 2018.
40. Pandey A and Mishra AK: Immunomodulation, toxicity, and therapeutic potential of nanoparticles. BioTech (Basel) 11: 42, 2022.
41. Rosowski EE: Determining macrophage versus neutrophil contributions to innate immunity using larval zebrafish. Dis Model Mech 13: dmm041889, 2020.
42. Ott LW, Resing KA, Sizemore AW, Heyen JW, Cocklin RR, Pedrick NM, Woods HC, Chen JY, Goebl MG, Witzmann FA and Harrington MA: Tumor necrosis factor-alpha- and interleukin-1-induced cellular responses: Coupling proteomic and genomic information. J Proteome Res 6: 2176-2185, 2007.
43. Kaneko N, Kurata M, Yamamoto T, Morikawa S and Masumoto J: The role of interleukin-1 in general pathology. Inflamm Regen 39: 12, 2019.
44. Iyer SS and Cheng G: Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol 32: 23-63, 2012.
45. Brun NR, Koch BEV, Varela M, Peijnenburg WJGM, Spainkc HP and Vijver MG: Nanoparticles induce dermal and intestinal innate immune system responses in zebrafish embryos†. Environ Sci Nano 5: 904-916, 2018.
46. He M, Li X, Yu L, Deng S, Gu N, Li L, Jia J and Li B: Double-sided nano-ZnO: Superior antibacterial properties and induced hepatotoxicity in zebrafish embryos. Toxics 10: 144, 2022.
47. Czyżowska A and Barbasz A: A review: Zinc oxide nanoparticlesi-friends or enemies? Int J Environ Health Res 32: 885-901, 2022.
48. Choi JS, Kim RO, Yoon S and Kim WK: Developmental toxicity of zinc oxide nanoparticles to zebrafish (*Danio rerio*): A transcriptomic analysis. PLoS One 11: e0160763, 2016.
49. Wu F, Harper BJ and Harper SL: Comparative dissolution, uptake, and toxicity of zinc oxide particles in individual aquatic species and mixed populations. Environ Toxicol Chem 38: 591-602, 2019.
50. Jeyabharathi S, Kalishwaralal K, Sundar K and Muthukumaran A: Synthesis of zinc oxide nanoparticles (ZnONPs) by aqueous extract of *Amaranthus caudatus* and evaluation of their toxicity and antimicrobial activity. Mater Lett 209: 295-298, 2017.
51. Chia SL and Leong DT: Reducing ZnO nanoparticles toxicity through silica coating. Heliyon 2: e00177, 2016.