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TERATOGENICITY AND BACTERICIDAL ACTIVITY OF NANOSYNTHESIZED *JUSTICIA ADHATODA* IN THE EMBRYOS OF *DANIO RERIO*

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Keywords:

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ABSTRACT: In the present study, *Justicia adhatoda* was used for synthesizing AgNPs and characterized using UV-visible, FTIR, XRD, and SEM analysis and checked for its teratogenic effect and bactericidal activity. UV–Vis spectra analysis revealed that the synthesized AgNPs had a peak around 434 nm, while XRD and SEM analysis showed that AgNPs have spherical shapes with sizes ranging from 22 to 29 nm. Fourier transmission infrared (FTIR) analysis authorized the presence of bio interactive hydroxyl, amide, and carbonyl groups playing an important reduction role in the process of nano-synthesis. The acute developmental toxicity of *Justicia adhatoda* leaf extracts with nanosynthesised AgNPs on the embryos of the zebrafish, *Danio rerio*, revealed that the mortality, hatchability, heartbeat rate, teratogenicity, and embryogenicity were all concentration-dependent. The lethal effects were prominent in biosynthesized AgNPs, whereas sub-lethal and teratogenic effects like lordosis, scoliosis, pericardial edema, yolk sac edema, head malformation, tail malformation, and growth retardation were expressed in 79% of the embryos. Pericardial edema and Tail malformation were the most prevalent in higher concentrations signifying the possible alteration of WNT genes. The biosynthesized AgNPs showed strong bactericidal activity. This study opens up newer dimensions in the advancement of the studies on the toxicity of nanosynthesised plant extracts.

INTRODUCTION: Nanotechnology is a field that is creating step by step, influencing all circles of human life, what's more, making a developing feeling of fervor in the existing sciences, especially biomedical gadgets and biotechnology. Nano-materials can be well-defined as a material with sizes ranging between 1 and 100 nm, which influences the frontiers of nanomedicine, starting from biosensors, microfluidics, drug delivery, and microarray tests to tissue engineering [1-2]. The applications of nanoparticles and nanomaterials are proliferating on various fronts due to their enhanced properties based on the size, bio-distribution, and morphology [3].

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Synthesizing nanoparticles using biological methods has the advantages of non-toxicity, reproducibility, and well-defined morphology [4]. Among different nanoparticles, silver (Ag) nanoparticles are known to exhibit strong biocidal effects on different bacterial species [5]. It is generally accepted that free silver ions, present or released from their nano-materials, can bind to cell membrane structures, destabilizing the membrane potential and causing proton leakage [6].

The study aimed at biosynthesizing silver nanoparticles using *Justicia adhatoda* a highly valuable Ayurvedic medicinal plant with high abortifacient, antimicrobial, antitussive, leukoderma, jaundice, tumors, mouth troubles, eyesores, cardio-vascular protection, anticholinesterase, and anti-inflammatory [7] properties and eventually assessing its bactericidal activity. Nowadays, the zebrafish embryo is a promising alternative model for screening the developmental toxicity of compounds, and its small size, transparency, rapid *in-vitro* develop-

ment, high fecundity, and economic husbandry requirements, make it suitable for studies of developmental toxicity, drug screening, and in evaluating teratogenic effects of the compounds, and most importantly its similarity with humans at the molecular and physiological levels [8-10].

The goal of the study was to assess the inherent embryotoxicity and teratogenic effects of biosynthesis of AgNPs using *Justicia adhatoda* leaves on embryos of zebrafish. Especially analysis determination of the (1) antibacterial effectiveness, (2) toxicity and LC_{50} of the synthesis AgNPs, and (3) Morphological aberrations of zebrafish embryos triggered by the synthesized AgNPs.

MATERIALS AND METHODS:

Materials: Silver nitrate ($AgNO_3$) was obtained from Sigma- Aldrich. All other reagents were of analytical grade. Muller Hinton agar (MHA) was acquired from Hi-media laboratories, Mumbai, India. Healthy adult Zebrafishes, *Danio rerio*, were collected from the Centre for Marine Science and Technology, Manonmaniam Sundaranar University. *Staphylococcus aureus* - MTCC 916 and *Klebsiella pneumoniae* - MTCC 503 were obtained from Microbial Type Culture Collection (MTTC), Chandigarh.

Plant Collection and Preparation of the plant extract: The fresh leaves of *Justicia adhatoda* were collected, washed under running tap water to remove dust, and shade dried for 20 days. 10gm of the dried leaf sample was weighed, added to 100ml of distilled water, and boiled at 80 °C for 60 min. The extract was collected by simple filtration using Whatman no. 1 filter paper, and the extract was stored in a refrigerator at 4 °C.

Biosynthesis of Silver Nanoparticles and Characterization: Silver nanoparticles were biosynthesized by adding 10ml of leaf extract into 90 ml of an aqueous solution of 1mM Silver Nitrate ($AgNO_3$) for the reduction of silver nitrate into Ag+ ions and kept in the dark room at 37 °C for 24 hours. The biosynthesized AgNPs were detected by a UV-Vis spectrophotometer within the range of 300-500 nm [11]. The functional groups of AgNPs were identified by the Fourier-transform infrared (FTIR) spectrometer (Perkin-Elmer, Germany) with the scanning spectrum of 500–4000 cm^{-1} [12]. Phase distribution, crystallinity, and purity of the bio-synthesized silver nanoparticles were determined by XRD [13-14]. The surface morphology, size, and shape of the silver nanoparticles were examined by Scanning Electron Microscope (SEM) [15].

Antibacterial Activity: *Staphylococcus aureus* (Gram-positive) and *Klebsiella pneumoniae* (Gram-negative) were used for examining the bactericidal activity of biosynthesized AgNPs. The inoculum was prepared by aseptically adding the fresh culture into 2 ml of sterile 0.145 mol/L saline tube, and the cell density was adjusted to 0.5 McFarland turbidity standard to yield a bacterial suspension of 1.5×10^8 cfu/ml. Nutrient agar was served as a medium for bacterial growth. Biosynthesized AgNPs at concentrations of 25µl, 50µl, 75µl, 100µl was tested against bacterial strains and compared with Streptomycin as a positive control. The plates were incubated at 37 °C for 24 h, and the zone of incubation around the well was observed.

Zebrafish Husbandry and Selection of Embryos: Zebrafish embryo-toxicity assays were followed by standard guidelines of OECD [16]. Wild-type (AB strain) of male and female 2:1 ratio was maintained at 28 ± 1 °C in the dark (12 h) and light (12 h) period. Hatching was achieved successfully within 30 min after exposing them to the morning light. After fertilization at 3–5 h, the embryos were carefully harvested cleanly. Healthy embryos were taken for further studies 1 mL of embryo medium was dispersed in well, and the collected viable embryos were placed in 6 well plates [17].

Toxicity Assays: Experiment solutions were prepared by diluting the stocks of the biosynthesized AgNPs. Thirty viable eggs were transferred into 6 well plates, with five different concentrations such as 0.1%, 0.5%, 1%, 5%, and 10% of leaf extract and biosynthesized AgNPs by suspending them in 3ml of embryo water. Zebrafish embryo development, toxicity, and teratogenicity assays were performed for 72 hours under the compound microscope [18]. Mortality, hatchability, heartbeat, and teratogenicity of embryos were recorded at 12, 24, 48, and 72 h, and LC_{50} values were calculated. Malformed embryos were recorded and the picture of embryos in each well was taken.

Teratogenicity Assays: A teratogenicity assay was performed to determine the teratogenic effect of the compound on the embryos [19]. Thirty embryos transferred to 6 well plates containing different concentrations of biosynthesized AgNPs (0.1%, 0.5%, 1%, 5%, and 10%) were examined under a compound microscope for abnormalities every 24 hours. Zebrafish embryos were tested for retarded growth, tail malfunction, head malformation, limited movement, scoliosis, edema, and so on.

Statistical Analysis: All the experiments were done in triplicates. Data were recorded as the mean with standard deviation (SD). For the embryo/larval bioassays, One-way analysis of variance (ANOVA) was used to detect significant differences between the control and treated groups. $p>0.05$ was considered statistically significant.

RESULTS AND DISCUSSION: A visible color change of the extract from pale yellow to reddish-brown on the addition of 1Mm $AgNO_3$ was observed within 24 h, indicating the formation of AgNPs.

UV-Visible Spectroscopy Analysis: UV spectroscopy analysis confirmed the formation and stability of metal nanoparticles, shown in **Fig. 1**. The reduction of $AgNO_3$ to AgNPs using *Justicia adhatoda* plant extract was confirmed by the change in color of the suspension from yellow to grayish brown. UV-vis spectrometric analysis showed a peak centered at 434 nm, showing a surface plasmon resonance effect resulting from the excitation of surface plasmon reverberation in silver nanoparticles [20].

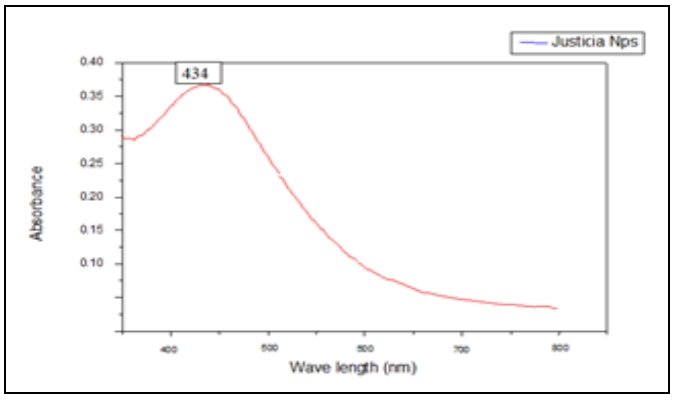

FIG. 1: UV-VISIBLE SPECTRUM OF *JUSTICIA ADHATODA* AgNPs

Fourier Transmission Infrared Spectroscopy: The biosynthesized AgNPs showed different FTIR peaks at 3453 cm^{-1}, 2368 cm^{-1}, 2064 cm^{-1}, 1643 cm^{-1}, and 1499 cm^{-1} **Fig. 2**. The strong and broadband at 3453 cm^{-1} at O-H stretching indicates vibration of the hydroxyl group, H-bonded alcohols, phenols, or N-H stretching of I and II amines and amides. The peak at 2368 cm^{-1} corresponds to C-H stretch alkanes and O-H carboxylic acids [21]. The peak at 2064cm^{-1} contributes to C=0 stretch α, β-unsaturated aldehydes, and ketones, the peak at 1643 cm^{-1} assigned to O-H bend indicates carboxylate. The band at 1499 cm^{-1} indicates the presence of C-N stretch aliphatic amines. Hence, the observations discuss a strong interaction between Ag and O-H group of flavonoids and amino acid groups in protein molecules in *J. adhathoda* leaf extract [22]. The functional biomolecules are hydroxyl, phenols, and amine groups involved in the reduction of silver ions, as confirmed by the FTIR spectrum.

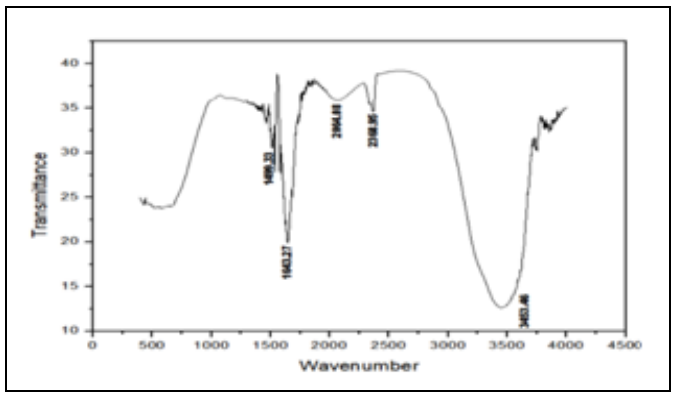

FIG. 2: FTIR SPECTRUM OF BIOLOGICALLY SYNTHESIZED AgNPs

X-ray Diffraction: XRD spectra showed strong diffraction peaks at 38.31°, 46.37°, 64.53°, and 77.40° degrees of 2θ which corresponds to 111, 200, 220, and 311 crystal planes. The high peaks in the XRD analysis indicated the active silver composition with indexing **Fig. 3**. The same result was reported by [23] and the XRD pattern exhibits sharp Bragg peaks corresponding to the (111) plane with high intensity which indicates good face-centered, cubic, and crystalline nature (correlated to JCPDS card: number 04-0783). The average particle size of AgNPs synthesized by the present green method can be calculated using the Debye–Scherrer equation [24].

$$D = 0.9\lambda/\beta \cos\theta$$

Where d is the mean diameter of nanoparticles, λ is the wavelength of the X-ray radiation source, and β

is the angular FWHM of the XRD peak at the diffraction angle θ [25]. Using, Debye-Scherrer equation, the average grain size of the AgNPs was calculated and found to be 22 nm **Fig**. **3**. Similar results were also reported by researcher [26].

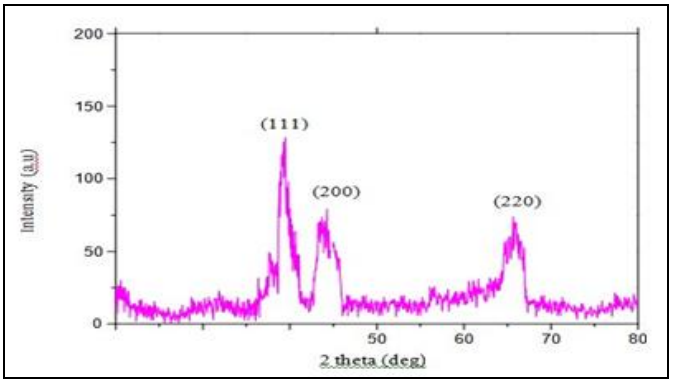

FIG. 3: XRD PATTERNS OF AgNPs SYNTHESIZED USING *JUSTICIA ADHATODA* EXTRACT

Scanning Electron Microscope analysis of Silver Nanoparticles: The SEM image of the bio-synthesized silver nanoparticles was predominantly spherical **Fig. 4**. The presence of biomolecules in the leaf extract has resulted in the synthesis of spherical silver nanoparticles and the aggregation may be due to the presence of secondary metabolites in the leaf extract [27]. The size of the biologically synthesized silver nanoparticles was noticed, ranging from 22 to 29 nm.

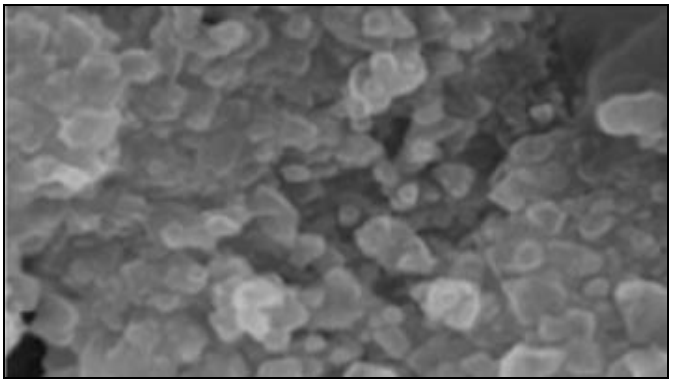

FIG. 4: SEM IMAGE OF AgNPs BIO-NANO-SYNTHESISED USING *JUSTICIA ADHATODA*

Antibacterial Assay: By disc diffusion method, biologically synthesized AgNPs were tested against *Staphylococcus aureus* and *Klebsiella pneumoniae* using varying concentrations of 25µl, 50 µl, 75µl, and 100 µl, and the formation of inhibition zone was observed **Fig. 5**. 100 µl concentration showed the highest antibacterial activity in *K. pneumoniae* (15 mm) and *S. aureus* (14 mm). The minimum inhibition zone was found at 25 µl in *S. aureus* (10mm). In *Klebsiella*, AgNPs at the concentration of 100µl showed more activity compared to Streptomycin, while in *Staphylococcus*, streptomycin was found to be efficient than AgNPs. Compared to *S. aureus*, *K. pneumonia* was more affected by biologically synthesized AgNPs even in low concentrations. Cell wall composition as Gram-positive bacteria is said to possess a cell wall consisting of a thick layer of polysaccharide which is hard to be penetrated by NPs, while the opposite is true with Gram-negative bacteria [28]. The accumulation of AgNPs on the cell membrane is believed to alter the membrane causing it to lose permeability which leads to cell death [29]. These outcomes were normal dependent on the size and morphology of AgNPs since circular morphology and low size are firmly identified with their natural activity.

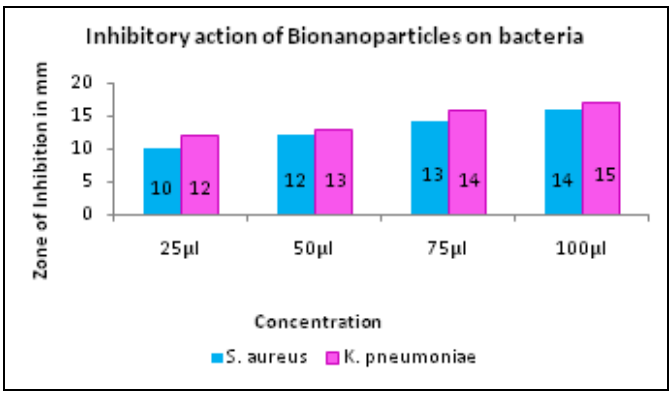

FIG. 5: GRAPH SHOWING ANTIBACTERIAL ACTIVITY OF *JUSTICIA ADHATODA* COATED AgNPs ON BACTERIA

Toxicity Assays on the Embryos of *Danio rerio:* Biologically synthesized AgNPs showed toxicity in zebrafish embryos, and LC_{50} values were calculated **Fig. 6**. The mortality, hatchability, heart-beat, teratogenicity, and malformations were observed at different stages and compared with control embryos **Table 1A, 1B**. As shown in **Fig. 7A**, the mortality was recorded at 12 h, 24 h, 48, 72 h, and the mortality rate was found to be increased with increasing concentration. At the lowest concentration of biosynthesized AgNPs, (0.1%), the mortality rates are detected as 30%. The lethal concentration (LC_{50}) of biosynthesized AgNPs was found to be 0.5% (0.49) **Fig. 6**. On the other hand, leaf extract showed no mortality from 0.1% to 1%, but at higher concentrations (10%) the mortality rates were increased by up to 44%. Hatching indicates the successful embryonic processes in the development of zebrafish.

Usually, it takes place between 48-72 h pf; depending on the thickness of the chorion and enzymes (chorionase) it releases [30]. The percentage of hatchability of embryos treated with fresh and nanosynthesized plant extracts is shown in **Fig. 7B**. The hatching rate is decreased in higher concentrations of 5% and 10% in leaf extract, while biosynthesized AgNPs showed hatchability only in 0.1% and 0.5% i.e. 76% and 55%. The results clearly showed that the hatchability was affected by the amount of treatment concentration.

In zebrafish, the normal embryonic heartbeat rate is 120–180 beats per minute [31]. While comparing the results, the heartbeat rate is decreased when the leaf extract and bio-nanosynthesized AgNPs concentration increases in the exposed embryos. The heartbeat rate of the bio-nanosynthesized AgNPs was 101 beats/min at 0.5% concentration. The comparative analysis of the heartbeat rate on embryos was mentioned in **Fig. 7C**.

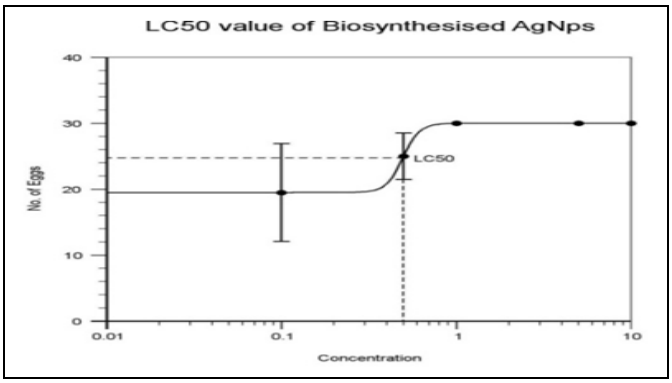

FIG. 6: LC_{50} VALUE OF BIOSYNTHESIZED AgNPs ON ZEBRAFISH EMBRYOS

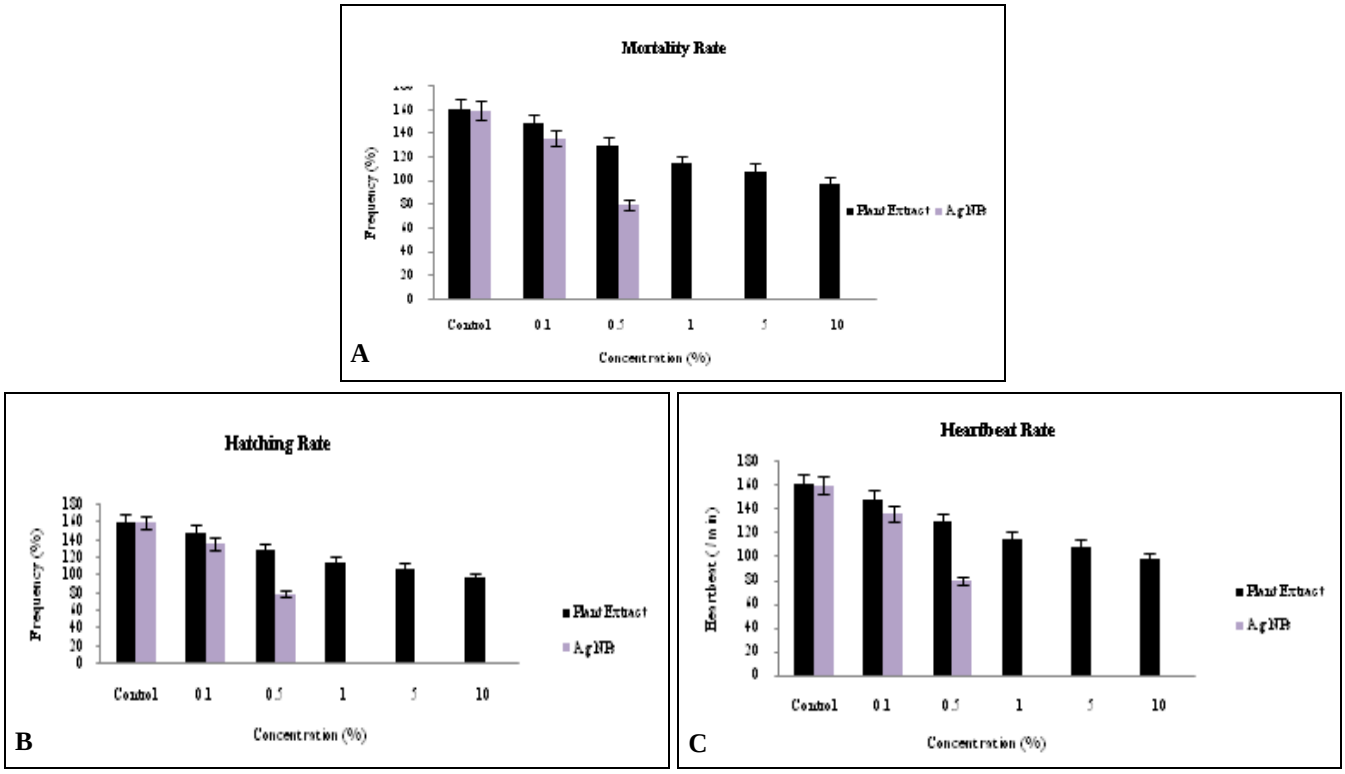

FIG 7: GRAPH REPRESENTING THE TOXICITY OF *JUSTICIA ADHATODA* AND BIOSYNTHESIZED AGNPS IN TERMS OF (A) MORTALITY, (B) HATCHABILITY AND (C) HEART RATE IN ZEBRAFISH EMBRYOS. DATA ARE REPRESENTED AS MEAN ± STANDARD OF THREE INDEPENDENT EXPERIMENTS

Teratogenicity of Zebrafish Embryos: The zebrafish embryos showed several teratogenic effects in biosynthesized silver nanoparticles and leaf extract. The biosynthesized silver nanoparticles caused zebrafish embryos to increase malformation in a dose-dependent manner with developmental abnormalities such as yolk sac edema, malformation of head, scoliosis/flexure, tail malformation, pericardial edema, and mostly limited movement were observed **Fig. 8**. Tail malformation and pericardial edema were the most marked teratogenic effects of the biosynthesized AgNPs and leaf extract. The malformations of the tail were observed as tail tip broadening, bend tail, and a very short tail **Fig. 8 G, H, I-L** [32]. The mortality was more evident with increasing concentrations of the biosynthesized silver nano-particles.

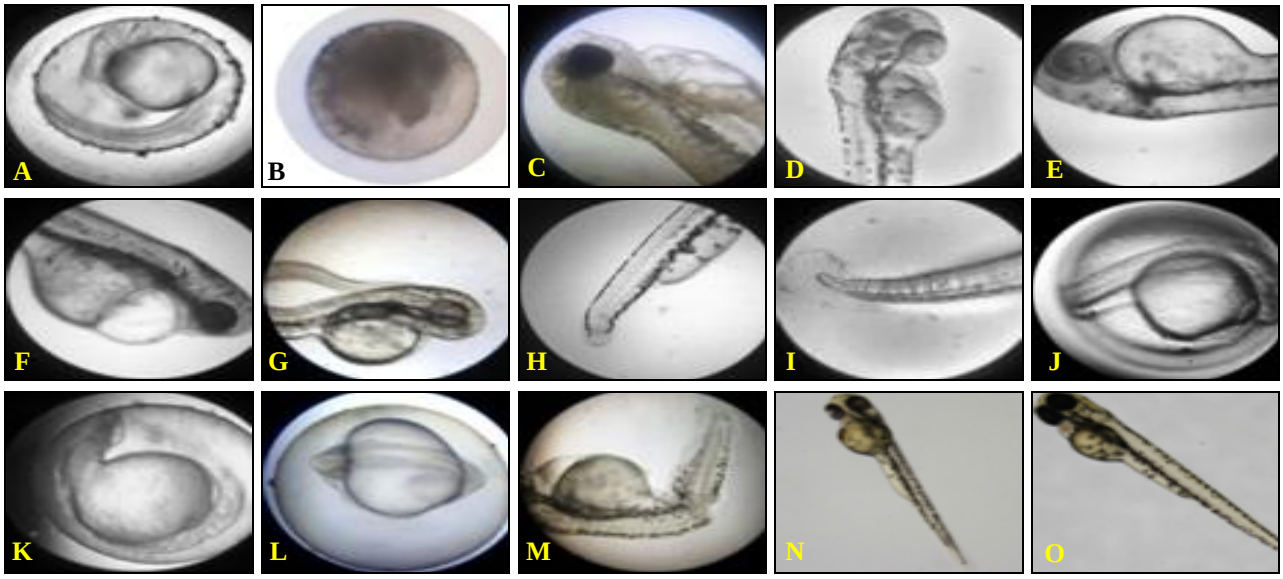

FIG. 8: MICROSCOPIC IMAGES OF NORMAL CONTROL EMBRYO AT (A) 24 HPF (B) EGG SHOWING COAGULATION (C) SCOLIOSIS (D) HEAD MALFORMED EMBRYO AT 60 h (E) YOLK SAC EDEMA (F) PERICARDIAL EDEMA AT 60 h (G) CURVED TAIL(H) EMBRYO WITH DEFORMED TAIL(I-L) TAIL MALFORMATION (M) TAIL NECROSIS (N-O) NORMAL EMBRYOS AT 48 h AND 72 h

TABLE 1A: TERATOGENICITY CHART OF *D.RERIO* EMBRYOS

Type of toxicity	Control	*J. adhatoda* leaf extract	Biosynthesized AgNPs
Lethal	-	+	+++
Coagulation	-	+	++
Lack of heartbeat	-	+	++
Lack of hatching			
Sublethal			
Pericardial edema	-	+	+++
Yolk sac edema	-	+	++
Tail deformation	-	+	++
Reduced heart rate	-	++	++
Limited movement	-	++	++
Teratogenic			
Tail malformation	-	+	+++
Head malformation	-	+	++
Scoliosis	-	-	+
Tail length	-	+	+

TABLE 1B: TOXICOLOGICAL ENDPOINTS AT DIFFERENT STAGES OF DEVELOPMENT OF ZEBRAFISH EMBRYOS (HPF)

Types of toxicity	Exposure time							
	J. adhatoda leaf extract				Biosynthesized AgNPs			
	12h	24h	12h	24h	12h	24h	12h	24h
Lethal endpoints								
Coagulation	*				*	*		
No heartbeat		*	*	*			*	*
Lack of hatching			*				*	*
Sublethal developmental endpoints								
Formation of somites								
Spontaneous movements	*			*	*		*	*
Heartbeat frequency				*			*	*
Edema			*	*			*	*
Endpoints of Teratogenicity								
Malformation of the head			*	*			*	*
Malformation of the tail			*	*		*	*	*
Tail length							*	*
Scoliosis				*			*	*

Statistical Analysis of Teratogenicity: Teratogenic data were given as the mean ± Standard deviation. Statistical analysis was done using Excel software. ANOVA tests agree with a calculated p-value of 0.04, p-value of less than 0.05 ($p < 0.05$). Hence, it was accepted as statistically significant between control and experimental groups.

CONCLUSION: The leaf extract of the medicinal plant *Justicia adhatoda* and biologically synthesized AgNPs caused concentration-dependent toxicity in zebrafish embryos. The exposure caused mortality and some altered physiological and morphological functions, namely decreased movement, hatching rate and heartbeat rate, and teratogenic effects in treated embryos. This proved that nanosynthesised extracts showed more lethality than leaf extract. Our results revealed that tail malformation was prevalent in higher concentrations signifying the possible alteration of WNT genes. This could be due to Wnt signaling, which is a crucial pathway, playing an important role in the early stages of vertebrate embryo development. Wnt gene roles in cell fate determination, differentiation, proliferation, and apoptosis during embryonic development [33].

β-catenin, a key regulator of this classical signaling pathway, is associated with developmental wnt3a and wnt8a are involved in the regulation of the classical Wnt pathway, and inhibition of wnt3a can cause deformities of the zebrafish body joints and the posterior structure of the body. Inhibition of wnt8a results in the absence of tissue near the anterior plate and axis of the spinal cord [34-35]. Down-regulation of Wnt signaling may interfere with cardiomyogenic differentiation and disrupt cardiac formation [35].

The results showed that the expression levels of wnt3a and wnt8a could be significantly down-regulated to induce abnormal body patterning of *Danio rerio*. Thus this probes newer dimensions of research that need to be aimed at the influence of Wnt genes in the occurrence of tail abnormalities in *Danio rerio* exposed to bio-nanosynthesised leaf extracts.

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CONFLICTS OF INTEREST: The author declares that they have no conflicts of interest with the contents of this article.

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