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Ecotoxicological Assessment of Citrate-Stabilized Silver Nanoparticles in Fish Model (*Poecilia reticulata*) with Implications for Aquatic Environmental Health

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Abstract: The increasing use of silver nanoparticles (AgNPs) in commercial and biomedical applications has raised concerns regarding their uncontrolled release into aquatic environments and potential risks to freshwater organisms. This study aimed to synthesize and characterize citrate-stabilized AgNPs and evaluate their ecotoxicological effects in fish model *Poecilia reticulata*. The nanoparticles were characterized using UV-Vis spectroscopy, FTIR, and TEM, confirming spherical morphology with sizes ranging from 12–45 nm and a surface plasmon resonance peak at 420 nm. Acute toxicity was assessed through a 96-hour semi-static assay, yielding an LC_{50} value of 1.12 mg/L. Sublethal exposure (0.36 mg/L for 21 days) resulted in significant behavioral alterations, including erratic swimming and reduced activity. Histopathological analysis revealed dose-dependent damage in gill and liver tissues, including lamellar fusion and hepatocellular necrosis. Bioaccumulation studies confirmed the uptake of silver in muscle tissues. Overall, these findings demonstrate that citrate-coated AgNPs induce multi-organ toxicity in freshwater fish and highlight the need for stricter environmental monitoring and regulation of nanoparticle discharge.

Keywords: *Silver Nanoparticles; Citrate-Coated; Ecotoxicity; Poecilia Reticulata; Freshwater Fish; Histopathology.*

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I. INTRODUCTION

Engineered silver nanoparticles (AgNPs) are extensively used in a wide range of consumer and industrial products, including medical devices, textiles, cosmetics, food-contact materials, and water-treatment systems due to their strong broad-spectrum antimicrobial activity [1], [2]. The rapid increase in the production and application of AgNP-containing materials has raised concerns regarding their continuous release into wastewater streams and subsequent entry into freshwater ecosystems [3], [4], [5]. Once in aquatic environments, AgNPs undergo various physicochemical transformations such as aggregation, surface coating exchange, sulfidation, and partial dissolution to ionic silver (Ag^+) [6],[4] which significantly influence their stability, bioavailability, and toxicity [2], [3].

Fish are considered reliable bioindicators for evaluating nanoparticle toxicity [7]because their gills, skin, and digestive tract are directly exposed to contaminants present in the surrounding water [8], [9]. The gill epithelium, in particular, provides a large and highly vascularized surface area that facilitates rapid interaction between aquatic pollutants and biological tissues. Several studies have reported that exposure to AgNPs can induce oxidative stress [10], [11] behavioral alterations, and histopathological damage in fish, with the gills and liver being vulnerable target organs [12], [13]. Furthermore, the relative contribution of particulate silver and dissolved Ag^+ ions to overall toxicity may vary depending on several factors, including nanoparticle size, surface coating, concentration, and water chemistry [2], [11].

Among freshwater fish species, *Poecilia reticulata* (red guppy) is a widely used model species in ecotoxicological research due to its small size, ease of laboratory maintenance, rapid life cycle, and sensitivity to a wide range of environmental pollutants [14], [15]. These characteristics

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make guppies suitable organisms for assessing the biological effects of emerging contaminants such as engineered nanoparticles [7], [12].

Citrate-stabilized silver nanoparticles represent one of the most commonly studied nanoparticle formulations in laboratory and commercial applications. Sodium citrate functions both as a reducing agent and as a stabilizing ligand that provides electrostatic repulsion between nanoparticles, thereby preventing aggregation in aqueous environments. The presence of citrate on the nanoparticle surface can influence dissolution kinetics, particle stability, and interactions with biological membranes, ultimately affecting toxicity responses in aquatic organisms [11], [16].

Understanding the toxic effects of nanoparticles requires both physicochemical characterization and biological testing. Characterizing nanoparticles by measuring properties such as particle size, shape, and surface plasmon resonance helps confirm successful synthesis and stability. At the same time, biological assessments such as acute toxicity testing, behavioral observations, bioaccumulation analysis, and histopathological examination help reveal how these nanoparticles affect living organisms and contribute to toxicity mechanisms [8], [10].

Therefore, the present study aimed to synthesize and characterize citrate-stabilized silver nanoparticles and evaluate their toxicological effects in *Poecilia reticulata*. The investigation integrated nanoparticle characterization with acute toxicity testing (96-h LC_{50}), behavioral observations, tissue bioaccumulation, and histopathological analysis in order to provide a comprehensive assessment of the ecotoxicological impact of AgNP exposure in freshwater fish.

II. MATERIALS AND METHODOLOGY

➢ *Synthesis of Silver Nanoparticles*

Silver nanoparticles were chemically synthesized using sodium citrate as the reducing and stabilizing agent. A 0.8 mM solution of silver nitrate was prepared, to which 15 ml of 10 mM sodium citrate solution was added. The mixture was heated at 80 °C for 20 minutes with continuous stirring until a reddish-orange color appeared, indicating nanoparticle formation. The colloidal solution was then cooled to room temperature and stored at 4 °C for further analysis.

➢ *Characterization of Silver Nanoparticles*

Silver nanoparticles were characterized using UV-Visible Spectroscopy (UV-Vis), Fourier Transform Infrared Spectroscopy (FTIR), and Transmission Electron Microscopy (TEM). UV-Vis analysis confirmed the surface plasmon resonance (SPR) peak at approximately 420 nm, indicating successful nanoparticle synthesis. FTIR analysis identified functional groups involved in nanoparticle stabilization. TEM analysis determined the size, morphology, and crystalline structure of the nanoparticles, revealing spherical particles with sizes ranging from 12–45 nm.

➢ *Experimental Fish and Acclimatization*

Healthy red male guppies (*Poecilia reticulata*) around 3 months old (juvenile stage) were collected from *Aquatic Biosystems, Vamanjoor, Mangaluru.* After collection, fish were carefully transported to the laboratory and kept under observation to ensure they were active and free from any visible signs of disease or stress. For acclimatization, the fish was maintained in fiber-reinforced plastic tanks with a total capacity of 50 liters, filled up to 40 liters of dechlorinated tap water. The water was maintained at 26 ± 1 °C with a pH of 7.2-7.6 and dissolved oxygen levels of 5-7 mg/L. The fish were fed daily and the water was renewed regularly to maintain optimal conditions.

The experimental protocols involving the use of *Poecilia reticulata* in this study were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of St Aloysius (Deemed to be University), Mangaluru. All experiments were conducted in accordance with relevant guidelines and regulations for the care and use of laboratory animals.

➢ *Acute Toxicity Testing (96-hr LC_{50})*

The acute toxicity assay followed a semi-static protocol over a 96-hour exposure period. Fish were distributed in 2 L glass tanks containing 1.5 L dechlorinated tap water and ten healthy, three-month juvenile male *Poecilia reticulata* (red guppy) were introduced into each tank. Test groups were exposed to various AgNP concentrations (0.5-10 mg/L), while a control group was monitored and maintained without exposure to AgNP (temperature: 26 ± 1 °C, pH 7.2-7.6, dissolved oxygen 5-6 mg/L).

Mortality was recorded at 24-hour intervals, and dead fish were removed immediately to avoid contamination and alteration of results. The LC_{50} value was determined by Finney's probit analysis method (1971), performed using Microsoft Excel by plotting mortality percentages converted to probit values against the logarithmic concentrations of AgNPs. This statistical approach enabled an accurate estimation of the AgNP concentration responsible for 50% mortality in *Poecilia reticulata*.

➢ *Sub-Lethal Exposure Study*

Based on the 96-hour LC_{50} value (1.12 mg/L), a sub-lethal concentration corresponding to one-third of the LC_{50} (0.36 mg/L) was selected for chronic exposure studies. Healthy *Poecilia reticulata* were exposed to this concentration for durations of 14 and 21 days under controlled laboratory conditions. A control group was maintained simultaneously under identical conditions without nanoparticle exposure. At the end of each exposure period (14 and 21 days), fish were sacrificed for subsequent bioaccumulation and histopathological analyses.

➢ *Histopathological Analysis*

After the 21-day exposure period, tissues from gills, liver, kidney and intestine were dissected from both control and AgNP-exposed groups after they were humanely euthanized using clove oil. Tissues were fixed in 10% neutral buffered formalin for 24-48 hours, dehydrated through a

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graded alcohol series, embedded in paraffin, sectioned at 4µm using a rotary microtome, and stained with Hematoxylin and Eosin (H&E). Sections were examined under a light microscope at 10X and 40X. Observed features were compared against control tissues to identify lesions, degenerative changes, and tissue damage induced by AgNPs.

➢ *Bioaccumulation Study*

For the bioaccumulation study post-exposure, muscle tissues were harvested from n=3 randomly selected fish from both the control and AgNP-exposed (0.36 mg/L) groups at the end of the 21-day exposure period. Tissues from each group were pooled into three independent replicates to ensure statistical representativeness and to provide sufficient biomass for ICP-MS detection. The tissues from both control and treated fish were then excised and cleaned thoroughly with deionized water to eliminate surface-bound nanoparticles. Samples were preserved in 0.25 M sucrose solution at -14 °C for analysis, muscle tissues were digested using concentrated nitric acid (HNO3) and analyzed for silver content using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) at the National Institute of Technology Karnataka (NITK), Surathkal.

III. RESULTS AND DISCUSSION

➢ *Characterization of Silver Nanoparticles*

Citrate-coated silver nanoparticles (AgNPs) were successfully synthesized and confirmed through UV-Vis, FTIR, and TEM analyses. A distinct surface plasmon resonance peak at 420 nm as shown in Fig. 1 in the UV-Vis spectrum indicated the formation of stable, spherical nanoparticles [1], [17].

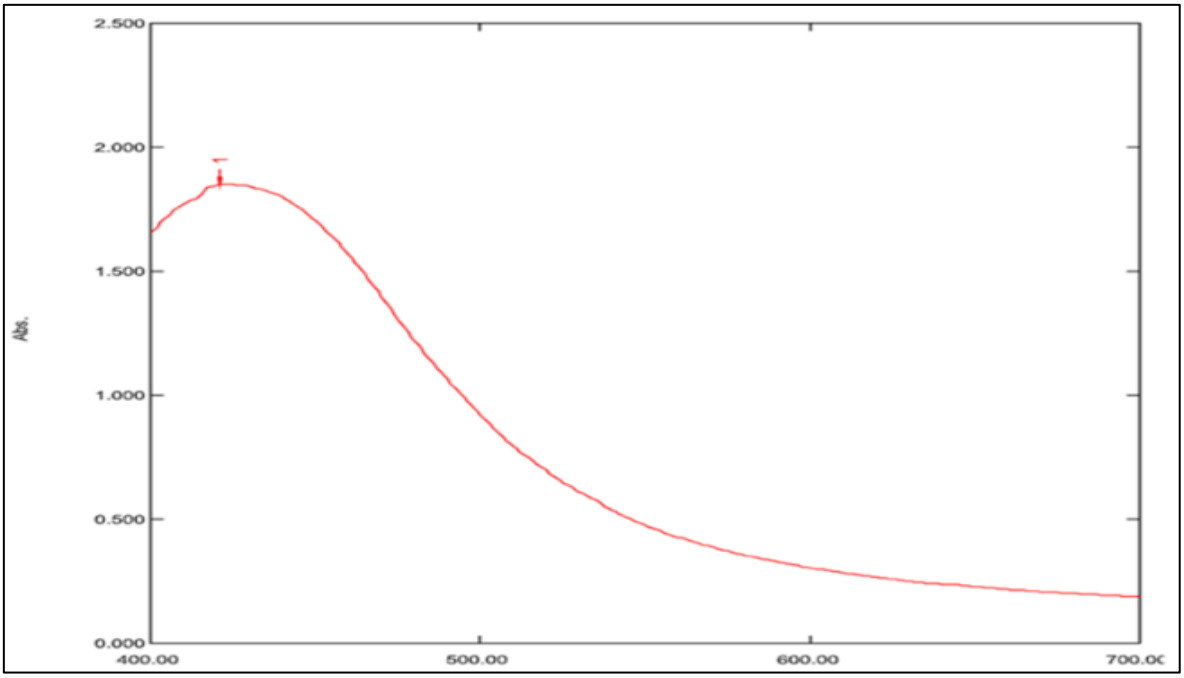

Fig 1 UV-Visible Absorption Spectrum of Synthesized AgNPs Showing a Characteristic SPR Peak at 420 nm.

Fig. 2 shows FTIR spectra with characteristic peaks for hydroxyl (O–H), carbonyl (C=O), and ether (C–O) groups, confirming the presence of citrate capping on the nanoparticle surface [18].

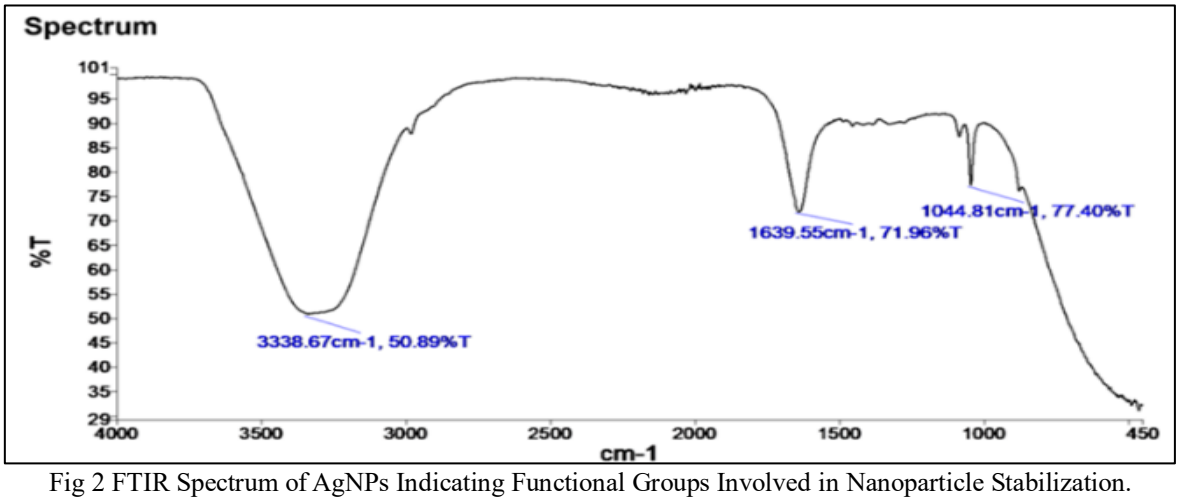

Fig 2 FTIR Spectrum of AgNPs Indicating Functional Groups Involved in Nanoparticle Stabilization.

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TEM images revealed well-dispersed, spherical nanoparticles ranging from 12 to 45 nm in size. The particles showed clear lattice fringes as shown in Fig. 3, suggesting their crystalline nature [19]. Minimal agglomeration indicated effective stabilization by sodium citrate. Overall, these results confirmed that the chemical synthesis method produced uniform, stable AgNPs suitable for toxicity studies [8].

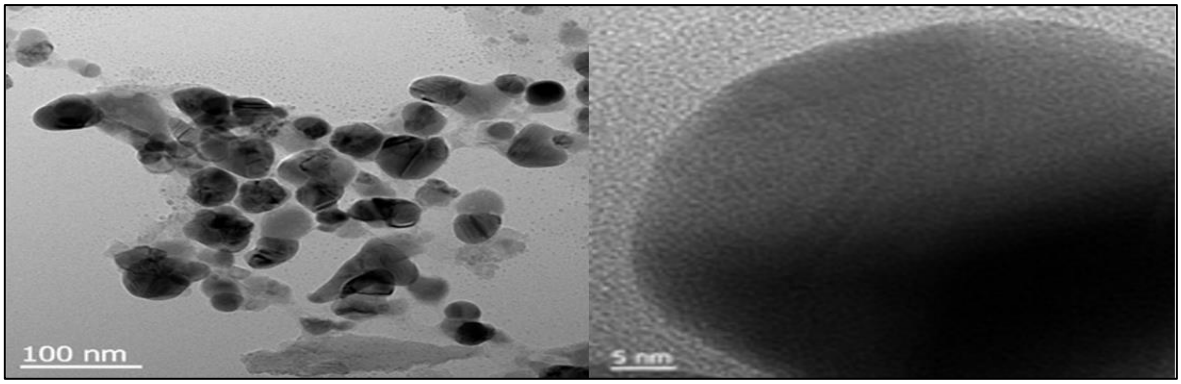

Fig 3 TEM images of chemically synthesized silver nanoparticles (AgNPs) showing spherical morphology and particle size distribution. (a) Well-dispersed spherical nanoparticles with size variation, suggesting effective use of capping agents in minimizing aggregation. (b) High-resolution TEM image showing distinct lattice fringes, confirming the crystalline nature and face-centered cubic (FCC) structure of silver nanoparticles.

Recent literature increasingly supports the view that AgNP toxicity arises from a combination of nanoparticle-specific interactions and the release of Ag^+ ions, rather than from either mechanism alone [2], [3], [4], [7]. Surface coating plays a key role in influencing these processes, as citrate-stabilized nanoparticles generally dissolve more readily than polymer-coated particles such as polyvinylpyrrolidone (PVP), leading to differences in biological responses [10], [11], [16]. The toxicity observed in the present study is therefore consistent with coating-dependent effects reported previously in aquatic organisms.

The surface coating of nanoparticles plays a critical role in determining their environmental behavior and biological interactions. In the present study, citrate was used as a stabilizing agent, which imparts a negative surface charge and enhances nanoparticle dispersion in aqueous environments. However, citrate-coated AgNPs are also known to exhibit relatively higher dissolution rates compared to polymer-coated nanoparticles, leading to increased release of biologically active silver ions (Ag^+) [11], [16]. This enhanced dissolution can significantly contribute to toxicity, as Ag^+ ions readily interact with cellular components, disrupt membrane integrity, and interfere with enzymatic processes [2], [10]. Therefore, the observed toxicity in *Poecilia reticulata* may result from a combined effect of nanoparticle-specific interactions and ionic silver release, emphasizing the importance of surface chemistry in modulating nanoparticle toxicity.

➢ *Acute Toxicity Assessment of AgNPs*

The acute toxicity of citrate-coated AgNPs was evaluated in *Poecilia reticulata* through a 96-hour semi-static exposure test. Fish were exposed to increasing concentrations of AgNPs ranging from 0.5 to 10 mg/L. Mortality was recorded at 24-hour intervals for 96 hours. No mortality occurred in the control group, while in treated groups, mortality increased progressively with rising AgNP concentrations, indicating a clear dose-dependent toxic effect as shown in Fig. 4. Probit analysis of mortality data revealed a 96-hour LC_{50} value of 1.12 mg/L, indicating significant toxicity at low concentrations.

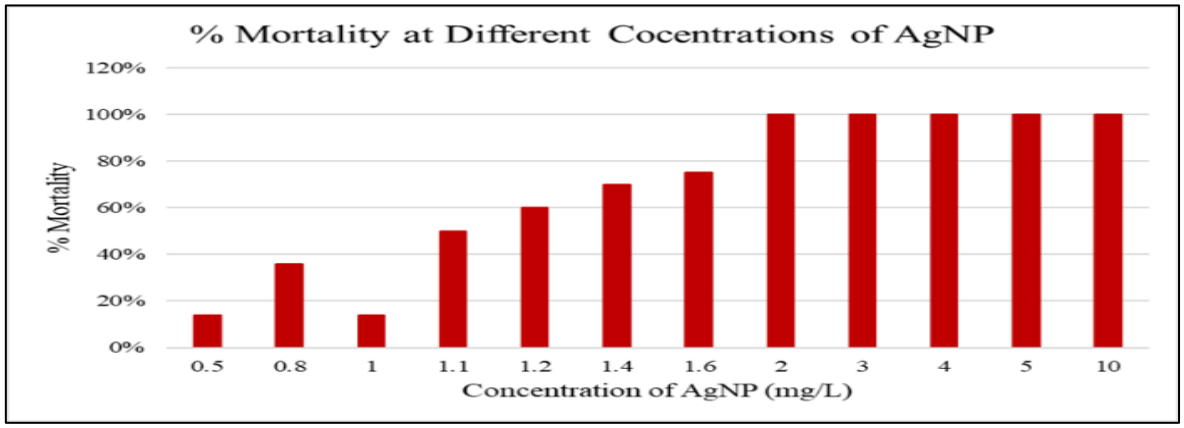

Fig 4 Percentage Mortality of Poecilia Reticulata at Different Concentrations of Citrate-Coated Silver Nanoparticles (AgNPs) after 96 Hours of Exposure. A concentration-Dependent Increase in Mortality was Observed, with Complete Mortality at Concentrations ≥2 mg/L.

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The 96-hour LC_{50} value of 1.12 mg/L obtained in this study indicates a high level of acute toxicity of citrate-coated AgNPs to *Poecilia reticulata*. This value is comparable to previously reported LC_{50} ranges for silver nanoparticles in freshwater fish species, which typically fall within the sub-milligram to low milligram per liter range. For instance, studies in *Danio rerio* and other freshwater fish have reported LC_{50} values ranging from approximately 0.5 to 2 mg/L, depending on nanoparticle size, coating, and exposure conditions [12], [20]. Variations in toxicity across studies may be attributed to differences in physicochemical properties of nanoparticles, water chemistry, and species-specific sensitivity [2], [3]. The relatively low LC_{50} observed in the present study further confirms the potent toxic nature of AgNPs and highlights their ecological risk even at low environmental concentrations.

➢ *Behavioral and Morphological Changes*

During the 21-day exposure to a sub-lethal concentration of citrate-coated AgNPs (0.36 mg/L; one-third of LC_{50}), *Poecilia reticulata* displayed several abnormal behaviors compared to the control group. Treated fish showed erratic swimming, reduced feeding, surface breathing, loss of balance, isolation, and increased mucus secretion. Morphological changes included body darkening, and discoloration. These effects became more pronounced over time. In contrast, the control group remained active, responsive, and displayed normal appearance and behavior throughout the study period.

These behavioral alterations are commonly associated with physiological stress and toxicity in fish. Changes in swimming and respiratory activity suggest possible neurotoxic effects or gill damage due to nanoparticle interaction with epithelial surfaces [21]. Increased mucus production is considered a defensive mechanism in fish to reduce surface contact with harmful agents [22]. Morphological darkening and reduced feeding are typical signs of chronic stress and metabolic disruption, often reported during heavy metal and nanoparticle exposure [23].

Overall, the behavioral and physical changes observed in AgNP-exposed guppies indicate sub-lethal toxicity, potentially linked to nanoparticle interference with the nervous, respiratory, and endocrine systems.

➢ *Bioaccumulation Study*

After 21 days of exposure to citrate-coated silver nanoparticles (AgNPs) at 0.36 mg/L, *Poecilia reticulata* muscle tissues showed a silver concentration of 0.03 ppm, as determined by ICP-MS using Ag-107 in KED mode (RSD = 1.4%). While silver levels in the control group remained below the detection limit, the treated group exhibited a mean concentration of 0.03 ± 0.005 ppm as Fig. 5 shows. This indicates that even at relatively low exposure concentrations, AgNPs are capable of crossing biological barriers and accumulating within edible muscle tissues.

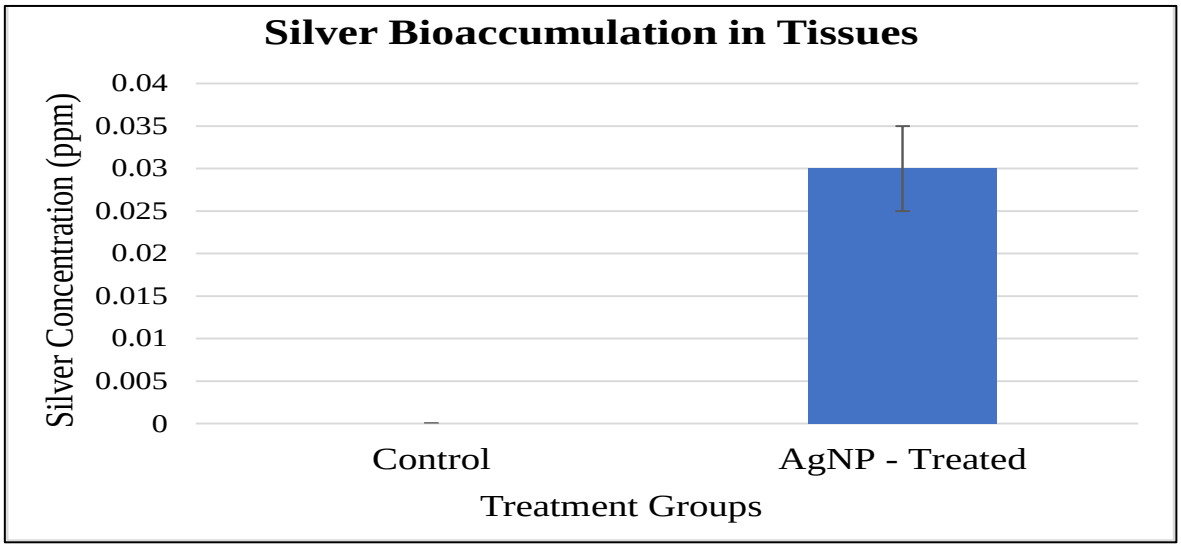

Fig 5 Bioaccumulation of Silver in Muscle Tissues of Poecilia Reticulata after 21 Days of Exposure to Citrate-Stabilized AgNPs (0.36 mg/L). No Detectable Silver was Observed in the Control Group (ND: Not Detected), while the Treated Group Showed a Mean Concentration of 0.03±0.005 ppm. Error Bars Represent Standard Deviation.

Although the concentration appears low, it is toxicologically relevant and aligns with previous studies showing bioaccumulation of silver in fish tissues at similar levels [9], [24]. The accumulation likely includes both nanoparticulate and ionic silver, as AgNPs can dissolve in water and release Ag^+ ions, which are highly bioavailable and toxic [2]. These results confirm that AgNPs or their ionic products can rapidly accumulate in fish tissues, even at sub-lethal concentrations.

➢ *Histopathological Studies*

The histopathological changes were observed in gill and liver tissues of *Poecilia reticulata* due to toxicity caused by silver nanoparticles.

Control fish showed normal hepatic architecture with intact hepatocytes and clear sinusoids. In contrast, treated fish exhibited cytoplasmic vacuolization and mild sinusoidal congestion by day 14. By day 21, more severe alterations

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were evident, including nuclear pyknosis, necrosis, and inflammatory infiltration as shown in Fig. 6A-H

Control gill tissues displayed well-organized primary and secondary lamellae. However, AgNP-exposed fish showed progressive structural damage. At day 14, gill tissues exhibited epithelial lifting and lamellar disorganization. By day 21, there was severe hyperplasia, lamellar fusion, and hemorrhage as shown in Fig. 7A-H.

The observed toxic effects of citrate-stabilized silver nanoparticles (AgNPs) in *Poecilia reticulata* may be strongly associated with oxidative stress mechanisms. Silver nanoparticles are known to induce the generation of reactive oxygen species (ROS), which can disrupt cellular redox balance and lead to oxidative damage of lipids, proteins, and nucleic acids [10], [13]. Elevated ROS levels can impair antioxidant defense systems, including key enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), resulting in cellular dysfunction and tissue injury[10]. Although oxidative stress biomarkers were not directly quantified in the present study, the histopathological alterations observed in liver and gill tissues—such as cellular degeneration, necrosis, and epithelial damage—are consistent with ROS-mediated toxicity reported in previous nanoparticle studies [23], [25]. These findings suggest that oxidative stress may play a central role in mediating AgNP-induced toxicity in freshwater fish.

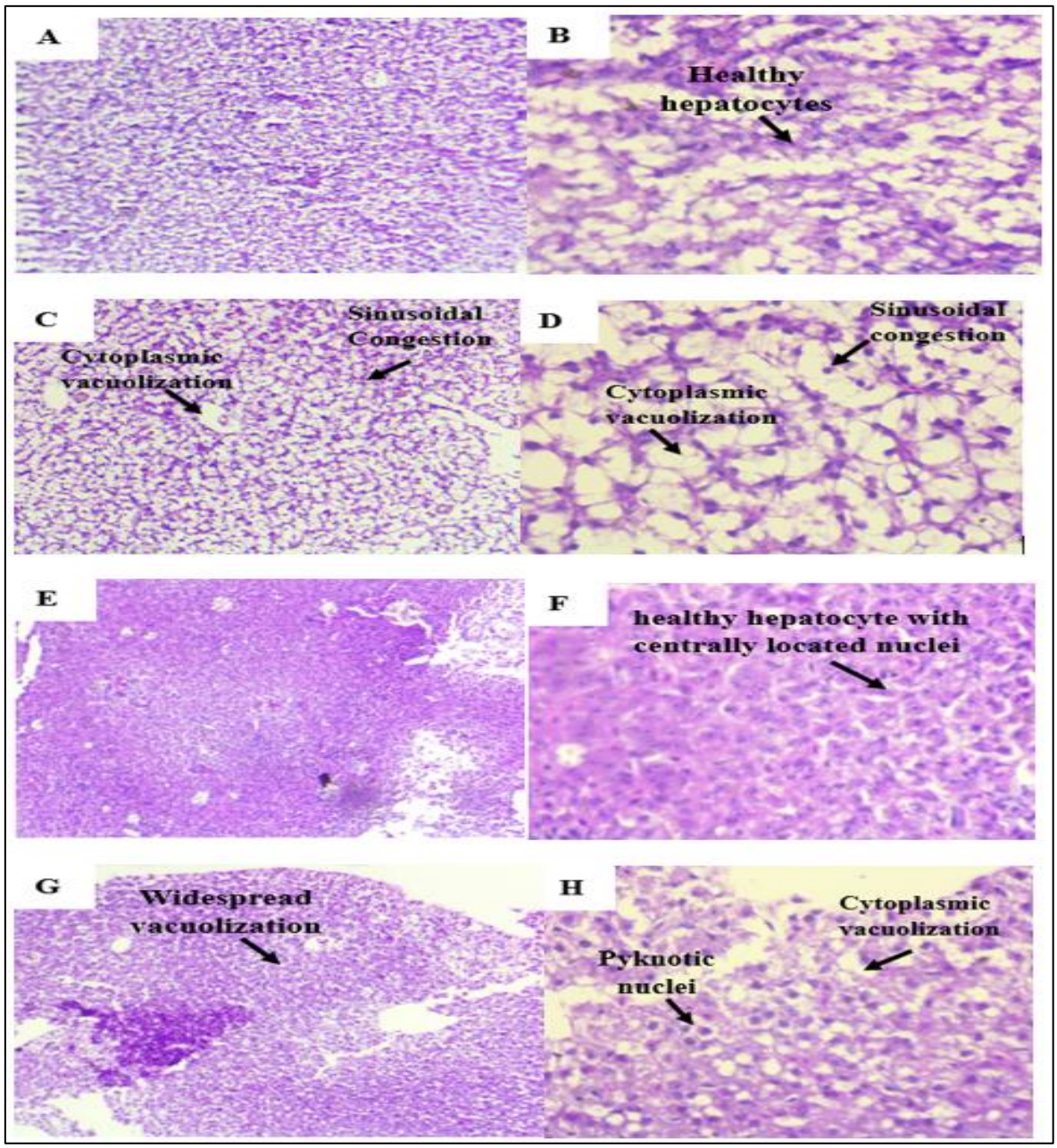

Fig 6 Histological sections of liver tissue of Poecilia reticulata. Control group after 14 days showing normal hepatocytes (a, b). AgNP-treated fish after 14 days showing cytoplasmic vacuolization and sinusoidal congestion (c, d). Control group after 21 days showing healthy hepatocytes with centrally located nuclei (e, f). AgNP-treated fish after 21 days showing pyknotic nuclei and extensive cytoplasmic vacuolization (g, h). Scale bars: 200 µm (a, c, e, g) and 50 µm (b, d, f, h).

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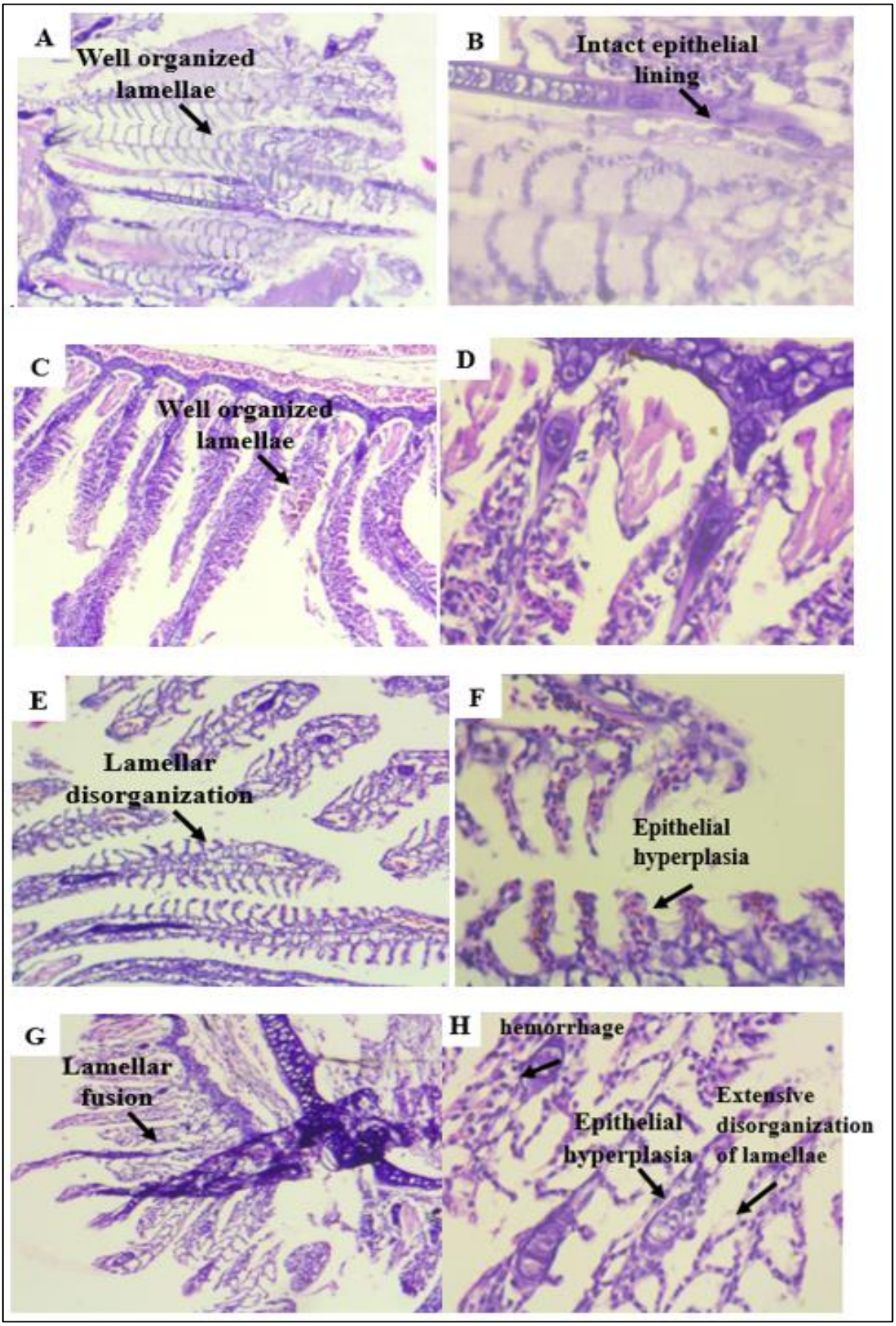

Fig 7 Histological sections of gill tissue of Poecilia reticulata. Control group after 14 days showing well-organized lamellae with intact epithelial lining (a, b). Control group after 21 days showing normal lamellar architecture (c, d). AgNP-treated fish after 14 days showing lamellar disorganization and epithelial hyperplasia (e, f). AgNP-treated fish after 21 days showing lamellar fusion (g, h). Scale bars: 200 µm (a, c, e, g) and 50 µm (b, d, f, h)

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IV. CONCLUSION

This study demonstrated that citrate-coated silver nanoparticles (AgNPs) pose significant toxicological risks to freshwater fish *Poecilia reticulata*. The 96-hour LC_{50} value of 1.12 mg/L confirmed the acute toxicity of AgNPs, while prolonged sub-lethal exposure (0.36 mg/L for 21 days) led to pronounced behavioral changes, tissue bioaccumulation, and histopathological damage in vital organs such as the liver and gills. Together, these results provide clear evidence of AgNP-induced physiological and cellular disturbances, emphasizing the environmental risks associated with their release into aquatic ecosystems [5], [26]. A key strength of this work lies in the integration of nanoparticle physicochemical characterization with organism-level, behavioral, and histopathological endpoints.

To build on these findings, future studies would benefit from targeted mechanistic assays, including quantification of reactive oxygen species [10], antioxidant enzyme activities (superoxide dismutase, catalase, glutathione peroxidase), metallothionein induction, and gene expression profiling, to better disentangle particulate versus ionic contributions to toxicity [10], [13]. Additionally, incorporating environmentally realistic exposure scenarios—such as chronic low-concentration exposures and complex water chemistries representative of wastewater effluents—will further enhance ecological relevance and risk assessment utility.

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