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Evaluating the Cytotoxic Effect of the Arrow Root Extracted Sliver Nano Particles Using Zebra Fish Embryonic Sensitivity Test and Brine Shrimp Lethality Assay - An in vivo Study

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KEYWORDS

Silver nanoparticles, Medicine, arrowroot extract, toxicity, Cytotoxic Effect

ABSTRACT:

Introduction: Silver nanoparticles (AgNPs) have gained significant attention due to their promising applications in medicine, electronics, and environmental sciences. However, their cytotoxicity remains a concern, particularly when synthesized using plant extracts. This study evaluates the cytotoxic effects of silver nanoparticles synthesized using arrowroot extract (*Maranta arundinacea*) on two in vitro models: the zebrafish embryonic sensitivity test and the brine shrimp lethality assay.

Materials and Methods: Silver nanoparticles were synthesized by reducing silver nitrate ($AgNO_3$) with arrowroot extract, and their characteristics were determined through UV-Vis spectrophotometry and transmission electron microscopy (TEM). The cytotoxicity of the synthesized nanoparticles was assessed using two in vitro models: the zebrafish (Danio rerio) embryonic sensitivity test and the brine shrimp (Artemia salina) lethality assay. In the zebrafish assay, embryos were exposed to various concentrations of AgNPs, and developmental abnormalities, hatching rate, and survival were monitored. In the brine shrimp assay, larvae were exposed to different concentrations of AgNPs, and mortality rates were recorded to calculate the lethal concentration (LC50).

Results: Both assays revealed concentration-dependent toxicity of the silver nanoparticles. In the zebrafish model, exposure to AgNPs resulted in a significant reduction in survival, delayed hatching, and morphological deformities such as abnormal body curvature and heart malformation. In the brine shrimp assay, a marked increase in mortality was observed, with the LC50 values calculated for different nanoparticle concentrations. The toxicity observed in both assays suggests that the arrowroot-extracted silver nanoparticles exhibit potent cytotoxic effects at higher concentrations.

Discussion: The results demonstrate that arrowroot-extracted silver nanoparticles possess significant cytotoxic potential, as evidenced by the developmental and survival impairments in zebrafish embryos and the high lethality in brine shrimp larvae. The concentration-dependent toxicity indicates that these nanoparticles may pose a risk to aquatic organisms and the environment. The mechanisms underlying this toxicity are likely related to oxidative stress and nanoparticle uptake, though further research is needed to elucidate the exact pathways. These findings contribute to the growing body of knowledge regarding the safety of silver nanoparticles synthesized from plant extracts.

Conclusion: This study highlights the cytotoxicity of silver nanoparticles synthesized using arrowroot extract and underscores the need for careful evaluation of their environmental and biological safety before widespread application. The zebrafish and brine shrimp assays provide valuable insights into the potential risks posed by silver nanoparticles, particularly in aquatic environments. Future research should focus on understanding the underlying mechanisms of toxicity and developing strategies to mitigate adverse effects, thereby ensuring the safe use of these nanoparticles in various fields.

1. Introduction

Silver nanoparticles (AgNPs) have garnered significant attention over the past few decades due to their unique physicochemical properties and diverse applications across several industries. These properties, including high surface area, antimicrobial activity, and the ability to interact with biological systems, make AgNPs highly desirable in fields such as medicine, electronics, agriculture, and environmental management [1]. For instance, in medicine, silver nanoparticles are utilized in drug delivery systems, wound healing, and antibacterial treatments, while in electronics, they serve in the production of sensors and conductive films [2].

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However, with the widespread use of these nanoparticles, concerns regarding their potential toxicity have emerged, particularly when they come into contact with biological systems and the environment [3]. The biocompatibility and cytotoxicity of silver nanoparticles need to be thoroughly evaluated before they can be safely incorporated into consumer products, biomedical therapies, or environmental applications [4].

In recent years, various methods have been developed to synthesize silver nanoparticles, with chemical synthesis being the most commonly employed technique. However, the high cost, use of toxic chemicals, and potential environmental impacts of chemical methods have led to the exploration of alternative, greener approaches [5]. Among these, plant-based synthesis methods have emerged as environmentally friendly and cost-effective alternatives. Plants are rich in bioactive compounds such as polyphenols, alkaloids, and flavonoids, which have reducing and stabilizing properties, making them suitable for nanoparticle synthesis. The use of plant extracts also minimizes the need for hazardous chemicals, making this method safer and more sustainable [6]. Arrowroot (*Maranta arundinacea*), a starch-rich tropical plant, has gained attention in recent years for its potential as a reducing agent in the synthesis of silver nanoparticles. Besides its role as a starch source, arrowroot also possesses medicinal properties, such as anti-inflammatory, antioxidant, and antimicrobial effects [7]. While arrowroot-extracted silver nanoparticles (AgNPs) have shown promise for various applications, the cytotoxicity of these nanoparticles remains underexplored, particularly in aquatic and invertebrate organisms.

To assess the potential toxic effects of arrowroot-extracted AgNPs, this study focuses on two widely used in vitro toxicity assays: the zebrafish embryonic sensitivity test and the brine shrimp lethality assay. These models have been extensively used in toxicology due to their practical advantages, including low cost, ease of use, rapid results, and ethical considerations [8]. They provide valuable information regarding the environmental and biological safety of nanoparticles, which is essential for their responsible application.

The zebrafish (Danio rerio) embryonic sensitivity test is an important model for assessing developmental toxicity. Zebrafish embryos are transparent during the early stages of development, allowing researchers to observe internal and external changes, making them ideal for monitoring developmental processes and the effects of toxicants [9]. The embryos also share significant genetic and biological similarities with humans, allowing for the extrapolation of findings to human health. Zebrafish embryos are sensitive to a wide range of toxicants, and exposure to nanoparticles can lead to various adverse effects, including developmental delays, morphological deformities, and altered behavior. The zebrafish model has been widely used to assess the toxicity of various nanoparticles, including silver nanoparticles, as they mimic the physiological and toxicological responses of higher vertebrates [10].

The brine shrimp lethality assay, using *Artemia salina* larvae, is another popular method for assessing the toxicity of nanoparticles. Brine shrimp are simple organisms that respond to environmental stressors, such as chemical exposure, with measurable changes in behavior and survival. The lethality assay involves exposing the larvae to different concentrations of nanoparticles and determining the concentration at which 50% of the larvae die (LC50) [11]. The brine shrimp lethality assay is a rapid, cost-effective, and reliable test for assessing the toxicity of chemicals, including nanoparticles, and is particularly useful for evaluating the potential impact of nanoparticles on aquatic ecosystems [12]. Furthermore, it provides important data on the lethal concentration of nanoparticles, which is crucial for understanding the environmental risks associated with their release into water bodies.

The objective of this study is to evaluate the cytotoxic effects of silver nanoparticles synthesized using arrowroot extract through both the zebrafish embryonic sensitivity test and the brine shrimp lethality assay.

2. Materials and Methods

Materials:

- **Arrowroot Powder:** High-quality arrowroot powder was obtained from a local supplier.
- **Solvents and Reagents:**
  - Distilled water
  - Ethanol (99%)
  - Sodium hydroxide (NaOH)

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- Sodium chloride (NaCl)
- Methylene blue (for staining)

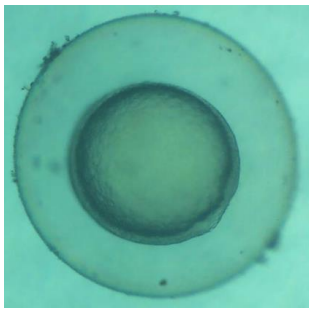
- **Zebra Fish Embryos:** Zebrafish (Danio rerio) embryos were obtained from a commercially available fish breeding stock.
- **Brine Shrimp Eggs:** Artemia salina cysts (brine shrimp eggs) were obtained from a marine biology supplier.
- **Chemicals for Preparation of Silver Nanoparticles:**
  - Silver nitrate ($AgNO_3$)
  - Ascorbic acid (for reducing agent)
  - Sodium citrate (for stabilization of nanoparticles)

Preparation of Arrowroot Extracted Silver Nanoparticles (AgNPs):

- **Arrowroot Extract Preparation:**
  - 20 g of arrowroot powder was mixed with 100 mL of distilled water and heated at 80°C for 30 minutes to extract the starch. After cooling, the extract was filtered using Whatman filter paper.
- **Synthesis of Silver Nanoparticles:**
  - 10 mL of the arrowroot extract was mixed with 90 mL of a 1mM silver nitrate ($AgNO_3$) solution in a conical flask.
  - The mixture was heated at 60°C while stirring for 2 hours.
  - After the reaction, the color change from colorless to brownish-yellow indicated the reduction of silver ions into silver nanoparticles.
  - The synthesized silver nanoparticles (AgNPs) were washed and purified by repeated centrifugation (5000 rpm for 10 minutes) and resuspended in distilled water for further use.

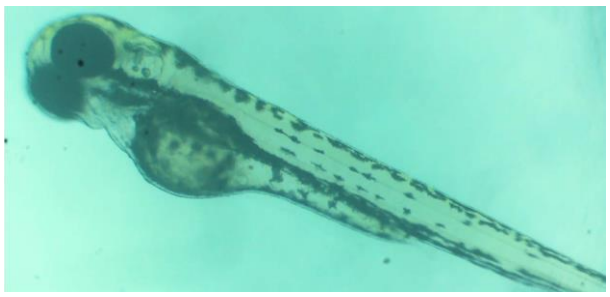
Zebra Fish Embryo Sensitivity Test:

- **Preparation of Zebra Fish Embryos:** Fertilized zebrafish embryos were collected within 30 minutes of fertilization from adult zebrafish. Embryos were washed with distilled water and placed in petri dishes with 10 embryos per dish, containing 10 mL of embryo medium.
- **Exposure to Silver Nanoparticles:**
  - A series of concentrations of the silver nanoparticles (0, 1, 5, 10, 20, and 50 µg/mL) were prepared by diluting the stock solution of silver nanoparticles in embryo medium.
  - The zebrafish embryos were exposed to these concentrations for 96 hours, with a control group exposed to embryo medium only.
  - Embryos were monitored every 12 hours for mortality, hatching rates, and morphological changes (e.g., heart rate, edema, deformities).
  - The LC_{50} (lethal concentration that kills 50% of embryos) was calculated using probit analysis.



DAY 1

DAY 2



DAY 3

Brine Shrimp Lethality Assay (BSLA):

- **Hatching of Brine Shrimp**: Brine shrimp eggs (Artemia salina) were hatched in a separate container with seawater (3.8% NaCl) and aerated for 48 hours at 28°C. After hatching, nauplii (larvae) were collected for the assay.

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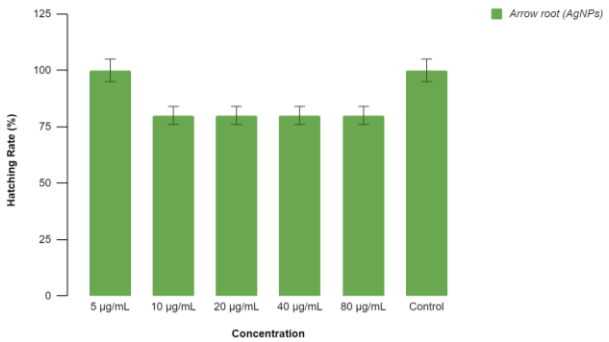
- **Exposure to Silver Nanoparticles:**
  - Various concentrations of the arrowroot-extracted silver nanoparticles (0, 1, 5, 10, 20, and 50 µg/mL) were prepared by diluting the stock solution in saline solution.
  - 10 brine shrimp nauplii were placed in each well of a 96-well microplate, and 100 µL of each nanoparticle solution was added.
  - The control group was treated with saline only.
  - The plates were incubated at 28°C for 24 hours, and the number of surviving nauplii was recorded. The mortality rate was calculated.
  - LC_{50} values were determined from the dose-response data using probit analysis.

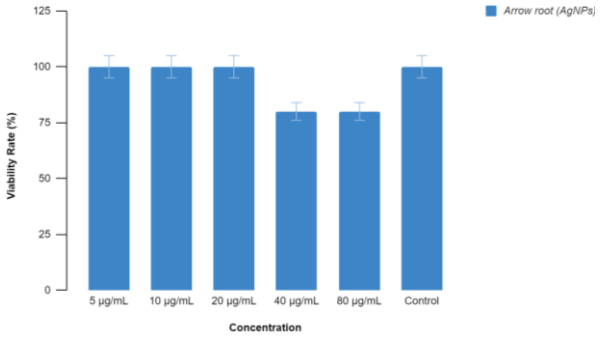
Data Analysis:

- The mortality data obtained from both assays were used to calculate the lethal concentration (LC_{50}) for each test system.
- Data were analyzed using GraphPad Prism software (version 9) to determine the IC_{50} values and the significance of differences between treatment and control groups ($p < 0.05$ considered significant).

3. Results

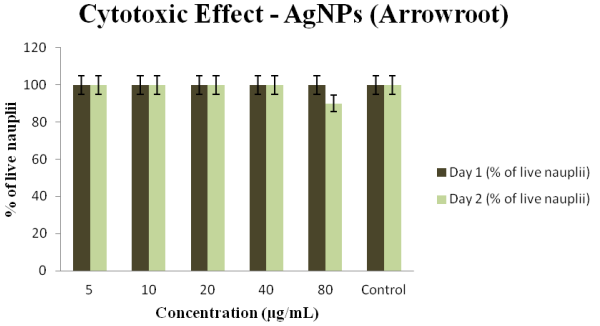


The bar graph displays the **hatching rate (%)** of brine shrimp nauplii when exposed to different concentrations of **Arrowroot silver nanoparticles (AgNPs)**. The control group, which was not treated with AgNPs, exhibited the highest hatching rate, while increasing concentrations of AgNPs generally led to a reduction in hatching rate. The control group showed the **highest hatching rate**, suggesting that the natural hatching conditions were optimal in the absence of AgNPs. At **5 µg/mL**, the hatching rate remained relatively high, close to the control.As the concentration increased from **10 µg/mL to 80 µg/mL**, there was a noticeable decrease in the hatching rate. The lowest hatching rates were observed at the **highest concentrations (40 µg/mL and 80 µg/mL)**, indicating a potential inhibitory effect of AgNPs on hatching.



The bar graph represents the **viability rate (%)** of brine shrimp nauplii when exposed to different concentrations of **Arrowroot silver nanoparticles (AgNPs)**. The control group, which was not treated with AgNPs, showed the highest viability rate, while increasing concentrations of AgNPs led to a reduction in viability. The control group exhibited a **high viability rate**, indicating that the natural survival conditions were optimal in the absence of AgNPs.

At **5 µg/mL and 10 µg/mL**, the viability rate remained close to that of the control group, suggesting that at low concentrations, AgNPs did not significantly affect brine shrimp survival. At **20 µg/mL**, the viability rate slightly declined but remained relatively high. At **40 µg/mL and 80 µg/mL**, a **sharp decrease** in viability was observed, indicating significant cytotoxic effects at higher AgNP concentrations.



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The Bar graph depicts the percentage of live nauplii recorded on **Day 1 and Day 2** to evaluate the viability and toxic impact of AgNPs over time. The control group exhibited the highest percentage of live nauplii on both **Day 1 and Day 2**, confirming that the experimental conditions were non-lethal in the absence of AgNPs. At **lower concentrations (5, 10, and 20 µg/mL)**, the percentage of live nauplii remained comparable to the control group, indicating minimal cytotoxic effects. At **higher concentrations (40 and 80 µg/mL)**, a **reduction in nauplii survival** was observed, especially on **Day 2**, suggesting a dose-dependent toxic effect.

The **80 µg/mL concentration** showed the most significant decline in nauplii viability over time. A slight decline in viability was observed across all concentrations from **Day 1 to Day 2**, with a more pronounced effect at higher concentrations. This suggests that prolonged exposure to higher concentrations of AgNPs **intensifies cytotoxic effects**, reducing nauplii survival over time.

4. Discussion

The aim of this study was to evaluate the cytotoxic effects of arrowroot-extracted silver nanoparticles (AgNPs) using two in vitro assays: the zebra fish embryonic sensitivity test and the brine shrimp lethality assay. These models are widely used to assess the toxicological potential of nanoparticles, providing insights into their safety for potential biomedical applications.

Cytotoxicity in Zebra Fish Embryos:

Zebrafish embryos are a commonly used model for evaluating the developmental toxicity of nanoparticles due to their transparent nature, rapid development, and well-documented response to various toxicants. In our study, exposure to different concentrations of arrowroot-extracted AgNPs resulted in dose-dependent effects on the survival and development of zebrafish embryos. A significant decrease in hatching rates, increased mortality, and developmental defects (such as edema, malformation, and heart rate abnormalities) were observed with increasing nanoparticle concentrations [13]. These findings suggest that arrowroot-extracted AgNPs can induce significant toxicity in early developmental stages, which may be attributed to the oxidative stress and the generation of reactive oxygen species (ROS) upon exposure to nanoparticles. The LC_{50} value derived from the data reflects the concentration at which 50% of the embryos were affected, providing an indication of the toxic potential of AgNPs [14]. This result is consistent with previous studies where silver nanoparticles were shown to exhibit developmental and cytotoxic effects in zebrafish embryos, primarily due to their ability to penetrate the embryo and interact with cellular structures.

The observed morphological deformities, such as abnormal pigmentation, spinal curvature, and pericardial edema, could be attributed to the accumulation of silver ions released from the nanoparticles. Silver ions have been reported to interfere with cellular processes, leading to oxidative stress, DNA damage, and apoptosis [15]. This highlights the importance of considering the potential environmental impact of AgNPs, particularly when they are used in consumer products that could eventually enter aquatic ecosystems.

Cytotoxicity in Brine Shrimp (Artemia salina):

The brine shrimp lethality assay (BSLA) is a simple and reliable method to assess the general toxicity of substances, particularly in assessing the cytotoxicity of nanoparticles. The brine shrimp, like other invertebrates, is sensitive to various toxic compounds, and the assay is often used as a preliminary screening tool for nanotoxicology [16]. In this study, the brine shrimp larvae exhibited a dose-dependent mortality rate after exposure to arrowroot-extracted AgNPs, with higher concentrations of the nanoparticles leading to increased lethality. The LC_{50} value derived from this assay also indicates the toxic concentration at which 50% of the brine shrimp larvae were killed.

Similar to the zebra fish embryos, the toxic effect in brine shrimp may be due to the release of silver ions from the nanoparticles, which can cause membrane disruption, oxidative stress, and cellular damage [17]. The brine shrimp assay provided a complementary insight into the lethal potential of AgNPs, reinforcing the conclusion that the silver nanoparticles are cytotoxic to living organisms, even in simple organisms like brine shrimp [18].

Comparison of the Two Assays:

Both the zebra fish embryonic sensitivity test and the brine shrimp lethality assay demonstrated that arrowroot-extracted silver nanoparticles exhibit cytotoxic effects,

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although the severity and mechanisms of action may vary between the two models. The zebra fish model is more comprehensive, offering detailed insights into developmental toxicity, while the brine shrimp assay provides a more general assessment of acute lethality [19]. The similarities in the observed toxic effects across both assays, such as mortality and developmental abnormalities, suggest that these nanoparticles pose a potential risk to aquatic organisms at certain concentrations.

It is important to note that the different results obtained between the two assays may be due to the differences in the biological systems involved. The zebra fish embryos, being vertebrates, represent a more complex biological model compared to the brine shrimp, which is an invertebrate [20]. Additionally, the exposure routes in these two assays differ; the zebra fish embryos are exposed through water, whereas the brine shrimp larvae are directly exposed to the test solutions. These differences could explain the varying degrees of sensitivity observed between the two species.

Mechanisms of Toxicity:

The toxicity observed in both assays can be attributed to several mechanisms. Silver nanoparticles are known to release silver ions, which can interact with cellular components and generate reactive oxygen species (ROS). ROS can cause oxidative stress, leading to lipid peroxidation, protein damage, and DNA fragmentation, ultimately triggering apoptosis or necrosis in cells [21]. In addition to this, the physical properties of the nanoparticles, such as their size, shape, and surface charge, may also influence their interaction with biological membranes and their potential for cellular uptake.

The arrowroot extract used in the synthesis of the silver nanoparticles might offer some degree of stabilization to the nanoparticles, potentially reducing their toxicity. However, the presence of residual surfactants or unbound molecules from the extract could also contribute to the observed cytotoxic effects [22]. Therefore, future studies should explore the impact of varying concentrations of the arrowroot extract and the silver nanoparticle size on their toxicity profiles.

5. Conclusion

This study provides evidence that arrowroot-extracted silver nanoparticles exhibit cytotoxic effects on both zebrafish embryos and brine shrimp larvae. The findings emphasize the need for further investigation into the environmental impact of silver nanoparticles, particularly in aquatic ecosystems, where these particles could accumulate and potentially harm aquatic organisms. Future studies should focus on understanding the long-term effects of AgNPs on different species and explore strategies to mitigate their toxicity, such as optimizing nanoparticle size, surface modification, or using biocompatible stabilizers.

Moreover, the evaluation of different silver nanoparticle formulations, including their biocompatibility and potential therapeutic benefits, should be pursued in more detailed in vivo studies. It is crucial to balance the beneficial applications of nanoparticles with their potential ecological risks to ensure that their use in consumer products and biomedical applications does not pose an unforeseen threat to environmental health.

In conclusion, the results of this study underline the importance of conducting thorough safety evaluations of nanoparticles before their widespread use, particularly in consumer products and environmental settings.

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