Iranian Journal of Fisheries Sciences
http:doi.org/ 10.22092/ijfs.2025.132839 2025, 24(2) 259-275

*Research Article*

# Effect of a long-time exposure of green and chemical synthesized silver nanoparticles on antioxidant defense system and immune response in Asian seabass (*Lates calcarifer*)

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**Keywords**

Nanoparticles,
AgNPs,
Barramundi,
Antioxidant system,
Proinflammatory activity

**Article info**

Received: December 2023
Accepted: December 2024
Published: March 2025

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**Abstract**

The unique behavior of silver nanoparticles (AgNPs) led to their increasing use in a wide range of products and the high discharge of these particles in the aquatic ecosystem. Regarding little information about the toxicity of AgNPs in the aquatic environment, the prediction of possible environmental hazards is the hot topic of current research studies. This study aimed to investigate the effects of long-time exposure of Asian seabass (*Lates calcarifer*) to green (using brown macroalga extract) and chemically synthesized AgNPs on oxidant/antioxidant and immunological responses. The $LC_{50}$ of both synthesized AgNPs was determined on fish (mean weight: 15±2.2 g) after 96 h ($LC_{50}$ for green synthesized AgNPs= 18.27 mg/l and for chemical synthesized AgNPs= 1.32 mg/L). Fish were exposed to green and chemical synthesized AgNPs concentrations of 0 (control), 5, 10, and 25% $LC_{50}$ for 60 days. For each treatment, 90 juveniles (22±1.5 g) were distributed in fiberglass tanks (300 L) and fed for 60 days. Fish were sampled at 1, 30, and 60 days after exposure. The liver tissue of fish samples was isolated and homogenized and then the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH), and malondialdehyde (MDA) was determined. The complement pathway activity (ACP), total immunoglobulin (Ig), total protein, albumin (ALB) concentration, and globulin (GLO) were measured in fish blood samples. The results showed that green synthesized AgNPs first increased the activity of the antioxidant system and then significantly reduced its activity. However, after exposure to chemically synthesized AgNPs, the activity of the antioxidant system showed a significant decrease. Both green and chemically synthesized AgNP exposure caused an increase in proinflammatory activities and immune proteins, and these activities were more pronounced after exposure to green-synthesized AgNP.

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## Introduction

Nowadays, silver nanoparticles (AgNPs) have attracted great attention due to the wide range of their applications in health electronics, biotechnology, cosmetics, toy production, and clothing (Beyene *et al.*, 2017; Radwan *et al.*, 2021). It has been estimated that 63 tons of AgNPs per year globally enter water bodies (Keller *et al.*, 2013). A modeling study predicted that environmental concentration for AgNPs in aquatic ecosystems ranges between 0.03 and 0.08 mg $L^{-1}$ (Mueller and Nowack, 2008). Therefore, the continued widespread use of AgNPs has led to the contamination of aquatic ecosystems (Blaser *et al.*, 2008). In the aquaculture industry, the AgNPs has wide applications in various sectors such as aquatic nutrition, water treatment, and disease control (Alishahi *et al.*, 2011; Johari *et al.*, 2016; Swain *et al.*, 2014), leading to direct toxic effect of ionic silver on fish.

In fish, AgNPs can enter the body through the skin or food consumption (Rastgar *et al.*, 2022). These particles can be deposited in the cell nucleus of vital organs and can be accumulated in fish gills and liver tissues. Therefore, the ability of fish respiration to hypoxic (low oxygen) conditions would be affected and caused respiratory stress and oxidative stress (Asharani *et al.*, 2008; Forouhar Vajargah *et al.*, 2018; Gobi *et al.*, 2018). The AgNPs inside the animal body can release Ag+ ions "Trojan horse mechanism" that caused severe toxic effects (Kettler *et al.*, 2016). In addition, the formation of AgNPs corona protein "proteins aggregated on the surface of nanomaterials" after entering these particles into the blood and immune cells of fish can initiate oxidative stress and inflammation (Khan *et al.*, 2018; Rastgar *et al.*, 2022). There is a close interrelationship between oxidative stress and inflammation after exposure to AgNPs and this linking can contribute to the pathogenesis of many diseases (Johnston *et al.*, 2018). It seems that reactive oxygen species (ROS) generation after exposure to AgNPs can increase the production of ROS, and depletion of antioxidants leads to adverse effects on cell macromolecules such as proteins, lipids, and DNA (Huang *et al.*, 2010). To quench excess ROS, the enzymatic and non-enzymatic antioxidant systems are activated. In parallel, ROS can induce the redox-sensitive mitogen-activated protein kinase (MAPK) and the nuclear factor kappa-light-chain enhancer of activated B cells (NF-KB) cascades that can activate pro-inflammatory response (Xia *et al.*, 2006).

There are numerous techniques for synthesising AgNPs, including physical, chemical, and green synthesis (Akter *et al.*, 2018). The most commonly used compound for synthesising AgNPs is silver nitrate. A plethora of inorganic and organic reducing agents can be employed in the reduction process, including sodium citrate, ascorbate, sodium borohydride, Tollen's reagent, elemental hydrogen, ethylene glycol, and dimethylformamide (Iravani *et al.*, 2014). Recently, environmentally friendly processes of AgNPs synthesis that do not utilise toxic chemicals have been developed. One of the organisms that plays a pivotal role in the bioremediation of toxic and precious metals and their bioconversion to different nontoxic forms is algae (Mehta and Gaur, 2005). The algae not only accumulate metals by chelation

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and chemical transformation, but also they can produce bio-mineral structures and metal nanoparticles (Mahdieh *et al.*, 2012).

The Asian seabass or barramundi (*Lates calcarifer*) has a high market price due to its delicately-flavored white meat. This fish is an important euryhaline carnivorous fish that has a fast growth rate and is a good selection for aquaculture (Singh, 2000). Therefore, contamination of water sources with different chemicals, including engineered nanomaterials can have many adverse effects on the body of this fish. This study aimed to evaluate and compare the effects of sub-lethal levels of chemical synthetic and biosynthetic of AgNPs on inflammatory and enzymatic antioxidant systems and some biochemical performance changes.

## Materials and methods

*Chemical synthetic of silver nanoparticles*

Chemically synthesized silver nanoparticles that were produced by photochemical reduction of silver nitrate solution in the presence of hydrazine and alkyl benzene sulfonate were purchased from Nanomaterials, Inc. Bought (US). These particles have a purity of 99.99%, an average size of 20 nm, and a concentration of 4000 mg/L (Mukherji *et al.*, 2019), for further details on this batch of material). The growth experiment of the fish was conducted in Faculty of Veterinary Medicine, Department of Aquatic Health, Shahid Chamran University of Ahvaz, Iran (2019/December). This project was approved by the Shahid Chamran University's institutional ethics committee in Ahvaz, Iran, under approval number EE 98.24.3.71483. The National Academy of Sciences' guide for the care and use of laboratory animals was followed in all animal operations for this work (NIH publications No. 8023, revised 1978).

*Green synthesis of silver nanoparticles*

*Screening and selection of macroalgae*

Fresh brown macroalga, *Sargassum ilicifolium*, was collected from the shores of the Chabahar coastal region in the Oman sea, Southeast of Iran. Samples were transferred to the laboratory in polythene bags and cleaned thoroughly with fresh water to remove adhering debris and associated biota. Then samples were rinsed with sterile distilled water. After cleaning, the algae were dried in shade for a week at room temperature and pulverized using a shredder (Govindaraju *et al.*, 2009).

*Preparation of aqueous extract*

The dried macroalgae powder (15 g) was heated with 300 mL distilled water at 60°C for 10 min. The crude extract was passed through Whatman No.1 filter paper and kept at 4°C for further use.

*Synthesis of silver nanoparticles*

Green synthesis of AgNPs was performed according to the method described by Bita *et al*. In brief, 17 mg of silver nitrate was dissolved in 100 mL of distilled water (1 mM). Then, 10 mL of algae extract was added to 90 mL of 1 mM silver nitrate solution (to reduce Ag+ions). The bioremediation of Ag+to Ag0 ions was investigated by observing the color change of the resulting solution. A particle sizer was used to determine the properties and ensure the production and quality of silver

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nanoparticles synthesized using the macroalgae extract (Bita *et al.,* 2015).

*Experimental fish and conditions*

Two hundred and fifty Asian seabass (*Lates calcarifer*) fingerlings with an initial weight of 15.63±2.2 g were obtained from a private fish farm, Ramoz sea bass fish breeding company in Bushehr city and transferred to the research laboratory of the Aquatic Health Department of the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz (Iran). The fish were acclimatized in 100 L glass aquaria for two weeks with a 12 h light/dark photoperiod with the initial stocking density of five fish per aquarium in triplicates per treatment. The water quality was daily assessed (temperature 29.5±1°C, pH 7.9±0.1, salinity 15ppt , and DO 5.1±0.5 mg/L). Fish were fed twice a day with a commercial pellet diet at a feeding rate of 2% of body weight. During the adaptation period, 40% of the reservoir water was replaced every other day and replaced with new water to prevent the accumulation of ammonia and other metabolites. All experiments were conducted according to the Ethics Committee of the Shahid Chamran University of Ahvaz.

*Determination of 96-h $LC_{50}$ value for chemical and green synthesis of silver nanoparticles*

To determine the appropriate $LC_{50}$ of each synthesized AgNPs, a pilot study with three concentrations for each synthesized AgNPs was performed. Then, based on the pilot study, the required concentrations in the first phase of the research were determined. The concentration of silver ions in the suspension of synthesized silver nanoparticles was determined to be 23 mg/ mL silver ions. The lethal range was determined based on the lowest concentration at which 96 casualties were observed and the first concentration at 100% mortality. To do this, five fish in each aquarium with a volume of 30 L, equipped with an aeration system, were transferred and exposed to increasing concentrations of chemically synthesized AgNPs (0.1, 2, 4, 8 ppm) and green synthesized AgNPs (0, 10, 20, 40, 50, 60 ppm). During both pilot experiments and $LC_{50}$ determination, fish movements and behaviors including gill cap movements, loss of balance, surface breathing, body color, rotational swimming, jumping movements, and floor rest were evaluated. Mortality was monitored continuously, and fish were considered dead when they did not show gill cap movements and response to mechanical stimuli (Bilberg *et al.,* 2012). After a preliminary test and determination of the lethal range, the $LC_{50}$ determination test was performed according to the standard O.E.C.D method in 1998 in a stationary manner (for 96 h, in 6 treatments with different concentrations). After recording the losses, $LC_{10}$, $LC_{50}$, and $LC_{90}$ were determined at 24, 48, 72, and 96 h with 95% confidence by using Finney's probit analysis.

*Experimental design and sampling*

The fish in 300-liter tanks were divided into three groups with three replications for each dose. The food pellets use in this study have an average composition of 47% crude protein, 18% crude fat, 4200 kcal/kg digestible energy, 2% crude fibre, 14% ash,

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1.1% phosphorus and a maximum moisture content of 12%. The first group was exposed to 5% $LC_{50}$ (0.066 mg/L), 10% $LC_{50}$ (0.132 mg/L) and 25% $LC_{50}$ (0.33 mg/L) of chemically synthesized AgNPs. The second group was exposed to 5% $LC_{50}$ (0.91 mg/L), 10% $LC_{50}$ (1.82 mg/L) and 25% $LC_{50}$ (4.56 mg/L) of green synthesized AgNPs and the control group remained unchanged for 60 days. To maintain water quality, food particles remained as wastage was removed to avoid interaction between nanoparticles and organic matter, and 30% of tanks water was daily exchanged (Joo *et al.,* 2013). After that, AgNPs in each experiment were re-dosed based on the respective treatment. To maintain AgNPs solubility, water was well mixed continuously by aeration (Wang *et al.*, 2011). Three fish were taken from each tank om 1, 30, and 60 days after exposure. Then, the fish were anesthetized using clove powder (200 mg/l, 20 min) and the blood samples were drawn from the caudal vein of the anesthetized fish with a sterile heparinized syringe and were transferred to tubes. Then the blood was centrifuged (200 ×g, 10 min., 4°C) to obtain plasma and stored at -80°C until further assays. The fish was sacrificed by decapitation and the livers were dissected out and immediately frozen in liquid nitrogen and stored at -80°C until further assays.

*Antioxidant parameters*

All antioxidant activities were assayed by spectrophotometry at 25°C. First, liver samples were homogenized in 0.1 M sodium phosphate buffer (pH 7.5) with a ratio of 1:10 w/v. Then homogenized samples were centrifuged (12000 ×g, 15 min., 4°C) for collection of supernatant and assaying antioxidant parameters. The malondialdehyde content (MDA) was measured by a commercial chemical colorimetrical assay kit based on the manufacturer's protocol (MDA assay kit; ZellBio GmbH) (Cao *et al.,* 1995). An indirect inhibition assay of nitroblue tetrazolium (NBT) reduction method was used to measure Superoxide dismutase (SOD) activity by commercially available kits (ZellBio GmbH, Germany) (Crouch *et al.,* 1981). Catalase activity (CAT) and reduced glutathione (GSH) were examined according to the method of Aebi (1974) and Davies *et al* (Davies *et al.,* 1984), respectively.

*Immunological parameters*

*Complement pathway activity*

Determination of complement pathway activity (ACP) was assayed according to the previous methods (Yano *et al.,* 1988; Sunyer and Tort, 1995; Hastuti *et al.,* 2020). In brief, 50 µL of activated and inactivated serums at different times was poured separately into sterile microtubes, followed by 350 µL of PBS containing $Ca^{2+}$ and $Mg^{2+}$. Finally, 100 microliters of 5% washed rabbit red blood cells were added to each microtube. The microtubes were incubated at 37°C for 45 min. The microtubes were centrifuged at 200 g for 5 min, and 100 microliters of the supernatant of each microtube were collected and poured into a plate of 96 microplate chambers in an active serum column. At a certain time and in the side column, the inactive serum was poured into the pits at the same time and was read at 450 nm.

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*Total immunoglobulin concentration (Ig)*

For determination of total immunoglobulin (Ig) concentration, the zinc sulfate precipitation method was applied. Briefly, 0.7 mM zinc sulfate buffer was prepared (pH: 8.5). Then, 12.5 µL of serum (from time=0 and time=60s) were poured separately into sterile microtubes, and 850 µL of zinc sulfate was added to each microtube and mixed well. After 2 h at room temperature, 100 microliters of the solution were poured into 96-well ELISA plates and read at 590 nm. Based on reliable sources and considering the age of the fish in this experiment, the amount of immunoglobulin in the standard prototype was considered to be 15 mg/ mL (Siwicki and Anderson, 1993).

*Total protein, albumin (ALB), and globulin (GLO)*

Measuring total protein and albumin (ALB) concentrations was done by applying a commercial kit (Zist Shimi kits) according to the manufacturer's instructions. Then, subtracting the ALB values from the total serum protein was used for calculating Globulin (GLO).

*Statistical analysis*

The normality of data was determined using the Kolmogorov-Smirnov test, and quantitative data were presented as mean±standard deviation. Two-way ANOVA with Multiple Comparisons Test was performed to compare the different groups, followed by Tukey's test ($p$<0.05). $LC_{50}$ calculations were carried out using standard probit analysis techniques. All analyses were performed using SPSS Version 24.

## Results

The results of AgNPs synthesized by the chemical and green method are given in Table 1.

*Lethal concentration results*

There were no moralities during the acclimation period and in the control group. $LC_{50}$ for fish after exposure to chemical and green synthesized AgNPs were calculated as 1.32 mg/L and 18.27 mg/L, respectively (Table 2).

**Table 1: Characteristic of AgNPs synthesized by chemical and green methods.**

| AgNPs synthesize method | mean±Span[a] | d(10)[b] | d(50)[c] | d(90)[d] |
|---|---|---|---|---|
| Green synthesize | 2.90±1.99 | 4.49 | 7.49 | 8.77 |
| Chemical synthesize | 7.1±1.49 | 3.66 | 7.88 | 11.92 |

[a] Span shows the particle size propagation and is the result of d90-d10/d50 and has the role of STED standard deviation in statistical calculations. [b] Average diameter of the first decile of particles (10% of particles with the smallest diameter). [c] Average diameter of half particles (50% particles with a smaller diameter). [d] Average diameter of the first nine deciles of particles (90% of particles).

**Table 2: Different lethal concentrations ($LC_5$-$LC_{90}$ at 96 h) of synthesized AgNPs by chemical and green method in Asian seabass (Lates calcarifer) (mg/L).**

| | $LC_5$ | $LC_{15}$ | $LC_{25}$ | $LC_{50}$ | $LC_{90}$ |
|---|---|---|---|---|---|
| Green synthesize AgNPs | 4.39 | 9.13 | 13.11 | 18.27 | 42.5 |
| Chemical synthesize AgNPs | 0.299 | 0.764 | 1.56 | 1.32 | 3.58 |

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*Subletal AgNPs exposure and antioxidant parameters*

The results showed that long-time exposure to sub-lethal concentrations of green synthesized AgNPs caused a significant increase in MDA levels in fish livers compared to the control group. Long time exposure to sub-lethal concentrations of chemical synthesized AgNPs caused a significant increase in MDA levels at 5 and 10% $LC_{50}$ compared to the control group whereas the level of fish liver MDA significantly decreased after 60 days of exposure to 25% $LC_{50}$. In both exposures to sub-lethal concentrations (5% $LC_{50}$) of green and chemical synthesized AgNPs, no significant difference in fish liver SOD activity was observed. Fish liver SOD activity increased after exposure to sub-lethal concentrations (10 and 25% $LC_{50}$) of chemical synthesized AgNPs in a time-dependent manner. The same results were observed only after exposure to 25% $LC_{50}$ of green synthesized AgNPs, while exposure to green synthesized AgNPs with 10% $LC_{50}$ caused a significant increase in fish liver SOD activity. The pattern of changes in liver CAT activity after long-time exposure to green and chemical synthesized AgNPs was quite similar to the pattern of changes in liver SOD activity. After exposure of fish to a 5% $LC_{50}$ of green and chemical synthesized AgNPs, there was no significant change in liver GSH. The sub-lethal concentration of green and chemical synthesized AgNPs (25% $LC_{50}$) caused a significant decrease in fish liver GSH content in a time-dependent manner ($p$<0.05; Fig. 1).

*Sublethal AgNPs exposure and immunological parameters*

Analysis of complement pathway activity (ACP) in fish serum after long-time exposure to sub-lethal concentrations of green synthesized AgNPs showed a significant increase in ACP in a time and concentration-dependent manner compared to the control group. On the other hand, increases in ACP in fish serum after long-time exposure to sub-lethal concentrations of chemical synthesized AgNPs were observed only after exposure to 10 and 25% $LC_{50}$. Long-time exposure to green and chemical synthesized AgNPs caused a significant increase in total immunoglobulin concentration of fish serum compared to the control group in a time and concentration-dependent manner ($P$<0.05; Fig. 2).

The total protein in fish serum significantly decreased after long-time exposure to sub-lethal concentrations of green synthesized AgNPs in a time and concentration-dependent manner compared to the control group. After long-time exposure to sub-lethal concentrations of chemical synthesized AgNPs (10 and 25% LC50), decreasing in total protein in fish serum was observed . Exposure (5, 10, and 25% $LC_{50}$) to both green and chemical synthesized AgNPs caused a significant decrease in globulin (GLO) and albumin levels in a time-dependent manner compared to the control group ($P$<0.05; Fig. 3).

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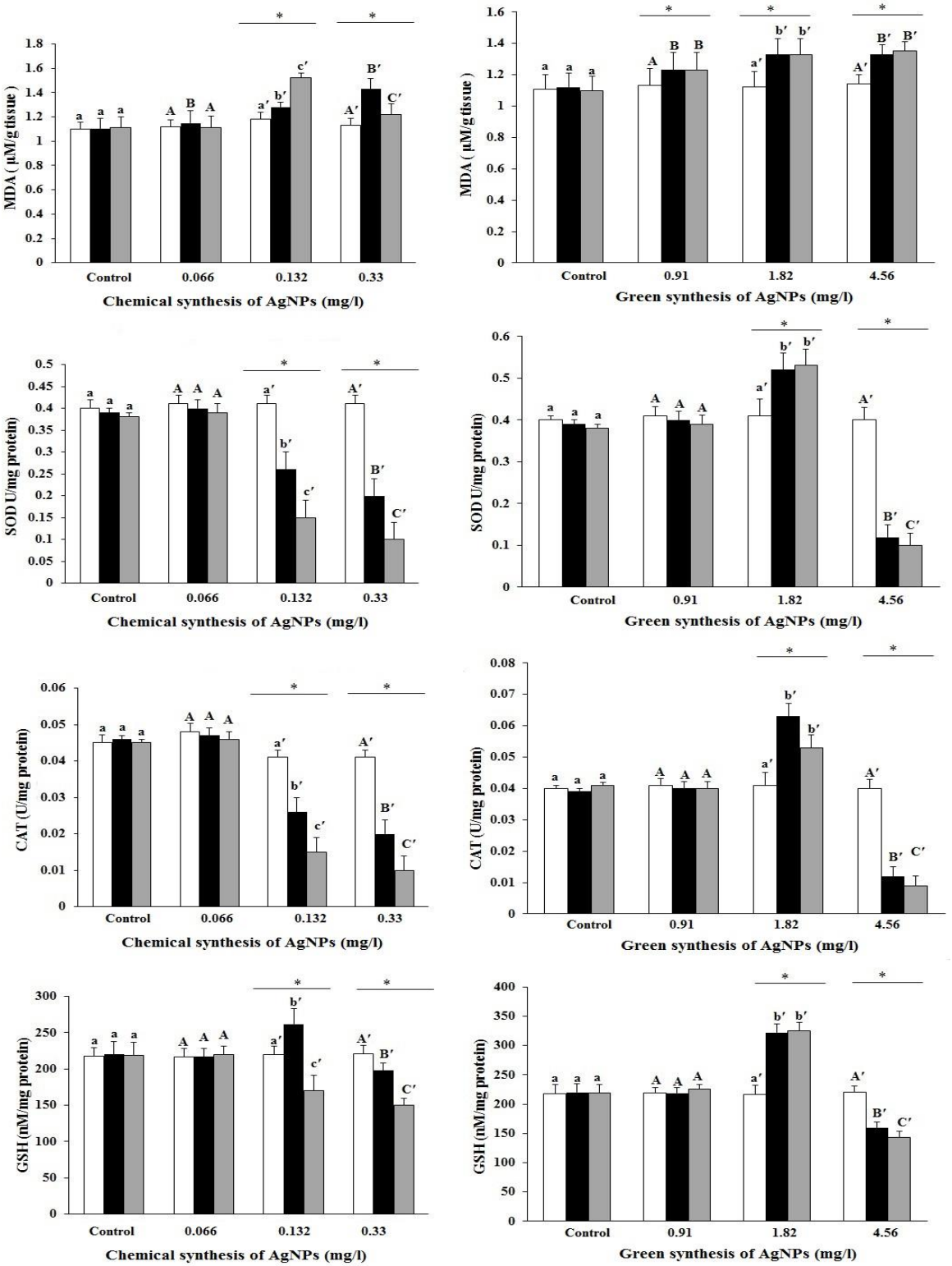


**Figure 1: The changes of the antioxidant parameters in Asian seabass (*Lates calcarifer*) liver exposed to sub-lethal levels of green and chemical synthesized silver nanoparticles after 60 days of exposure. White bar:1 day, black bar: 30 days, and gray bar: 60 days after exposure. Different letters indicate a significant difference between groups and * indicates a significant difference compared to the control group ($p$<0.05; Mean±DS).**

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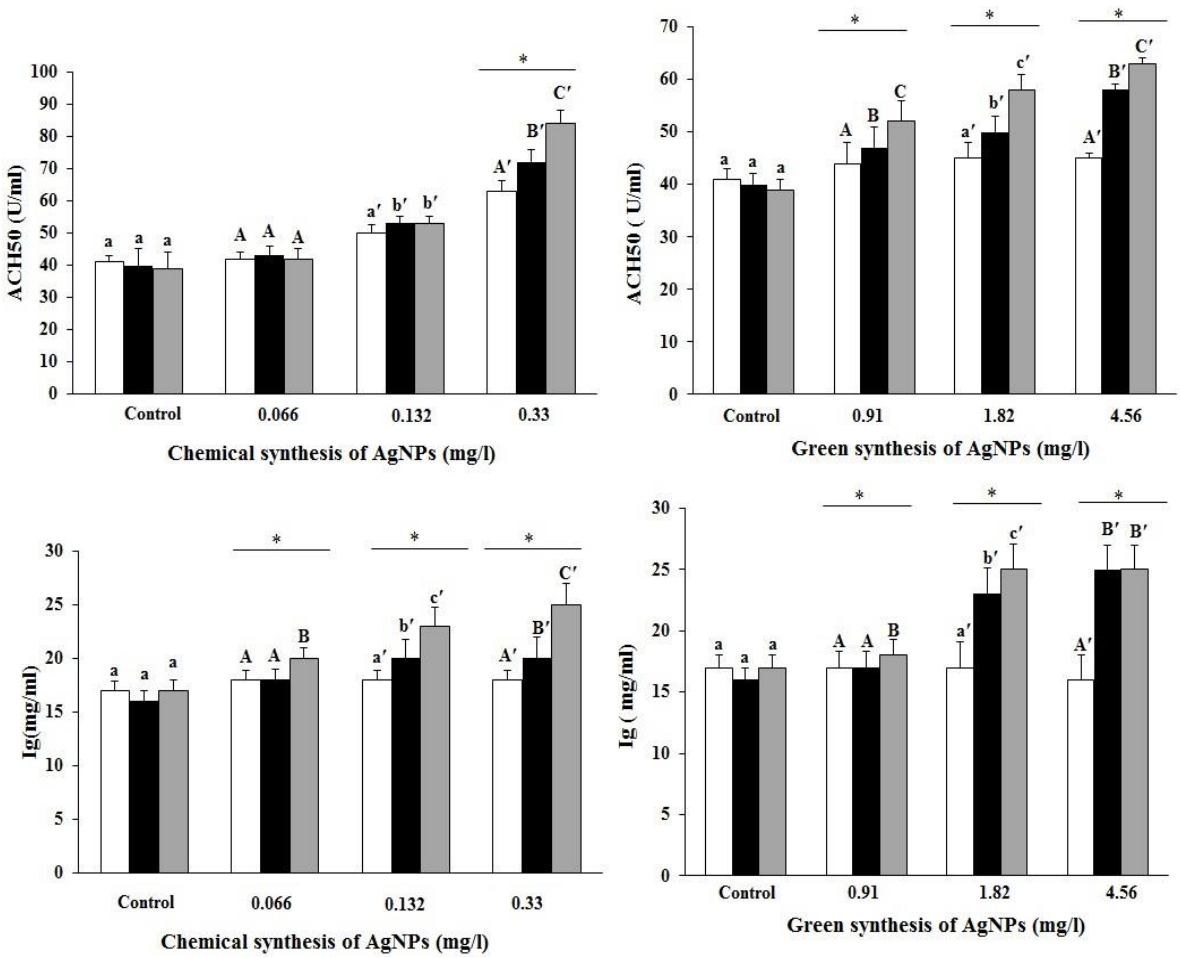


**Figure 2: The changes in complement pathway activity and total immunoglobulin concentration in Asian seabass (*Lates calcarifer*) serum exposed to sub-lethal levels of green and chemical synthesized silver nanoparticles after 60 days of exposure. White bar:1 day, black bar: 30 days, and gray bar: 60 days after exposure. Different letters indicate a significant difference between groups and * indicates a significant difference compared to the control group ($p$<0.05) (Mean±DS).**

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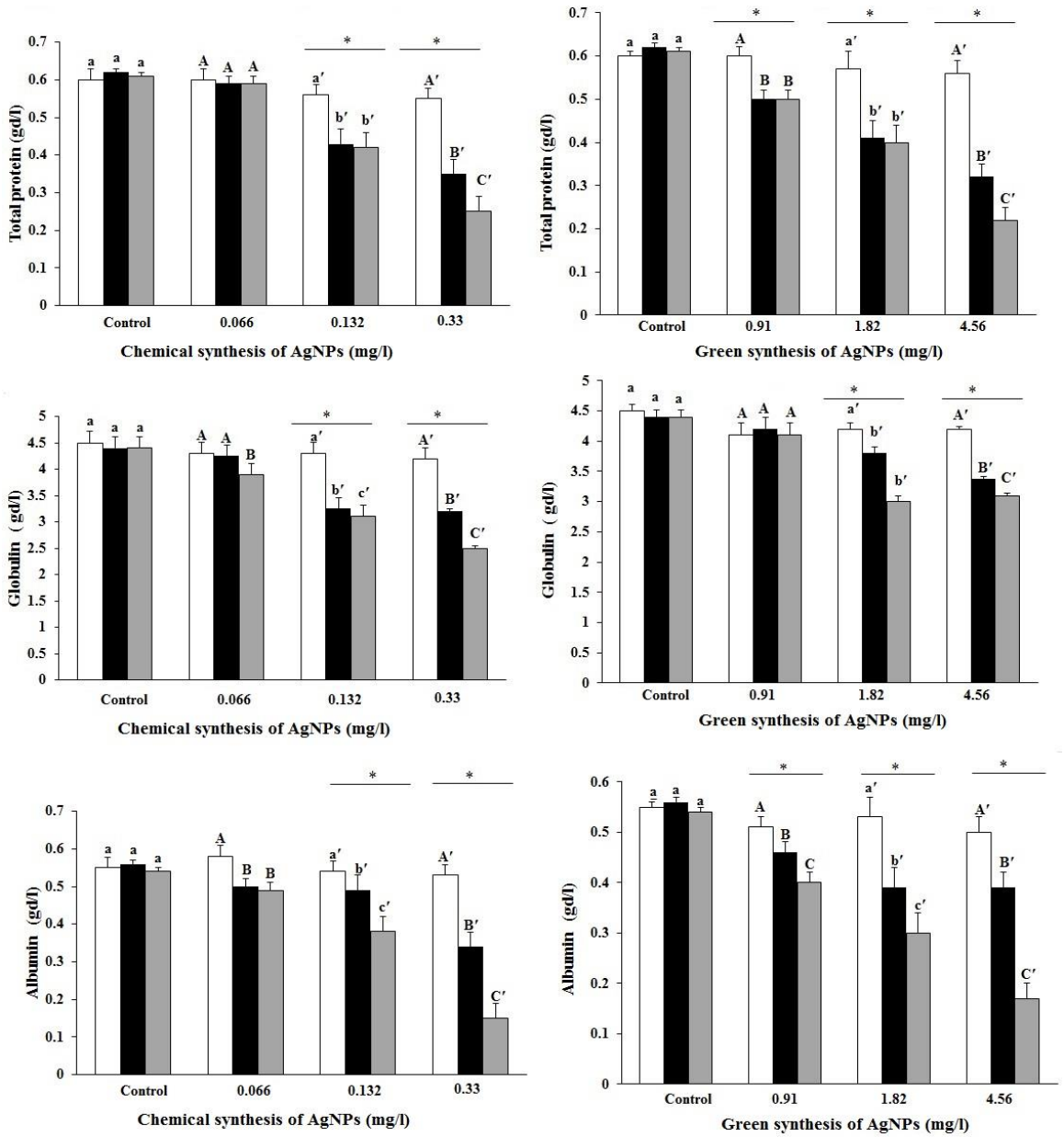


**Figure 3: The changes in the level of total protein, globulin, and albumin in Asian seabass (*Lates calcarifer*) blood exposed to sub-lethal levels of green and chemical synthesized silver nanoparticles after 60 days of exposure. White bar:1 day, black bar: 30 days, and gray bar: 60 days after exposure. Different letters indicate a significant difference between groups and * indicates a significant difference compared to the control group ($p$<0.05)(Mean±DS).**

## Discussion

The increasing use of engineered nanometal base particles can lead to an environmental impact that is gaining a lot of attention, associated with its hazardous effects on living organisms and also its novel beneficial properties (Akter *et al.*, 2018; Rastgar *et al.*, 2022). To measure acute toxicity in toxicological studies, the use of $LC_{50}$ as an important parameter and an initial procedure to screen the toxicity of the nanoparticles has been recommended. In the present study, the 96-h $LC_{50}$ value of green synthesized AgNPs and chemical synthesized AgNPs were 18.27 mg/L and 1.32 mg/L, respectively, suggesting that

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these compounds are highly and moderately toxic to Asian seabass. Previous studies demonstrated that AgNPs toxicity depends on the shape, size, dose, synthesized methods, duration of exposure, coating materials, and species (Khan *et al.*, 2017). In a related experiment,(Bita *et al.*, 2015) found that the 96-h $LC_{50}$ values of green synthesized AgNPs and chemical synthesized AgNPs were 9.669 and 1.569 mg/L, respectively. It seems that the differences in the $LC_{50}$ values among the same fish species are related to differences in body weight, sex, age, ambient conditions, water temperature, and feeding conditions of experimental fish (Hedayati *et al.*, 2012; Shaluei *et al.*, 2013).

The present study revealed that the levels of MDA significantly increased after long-time exposure to green synthesized AgNPs and chemical synthesized AgNPs. On the other hand, the GSH levels significantly decreased compared to the control group in a time and concentration-dependent manner. Changes in liver antioxidant enzyme levels (SOD and CAT) were different in the two groups of synthesized AgNPs. Antioxidant enzymes of the liver after exposure to green synthesized AgNPs increased and then decreased in a time-dependent manner. These enzymes after exposure to chemical synthesized AgNPs (10 and 25% $LC_{50}$) decreased in a time-dependent manner. The MDA is a lipid peroxidation product and can effectively indicate the degree of hepatic oxidative damage (Hamed and Abdel-Tawwab, 2021). The depletion of the liver antioxidant system (GSH,SOD and CAT) in both green and chemical synthesized AgNPs exposure at the highest concentration may be a logical explanation for the increase in MDA in several ways: the induction of reactive oxygen species (ROS) synthesis after AgNPs exposure can increase demand and use of the tripeptide for lipid hydroperoxide metabolism (Afifi *et al.*, 2016; Shaban *et al.*, 2013). These reactions lead to declines in GSH levels (Shaban *et al.*, 2013). The inhibitory effect of AgNPs exposure on SOD-CAT system in the fish liver as the first defense against oxygen radicals toxicity may be attributed to ROS overproduction (Hamed and Abdel-Tawwab, 2021). In other hands, production of ionic Ag from AgNPs in fish liver may over oxidate polyunsaturated fatty acids that resulted in cell and mitochondrial membrane destruction and MDA production (Ale *et al.*, 2018). Increasing ROS production after exposure of hepatoma cell line to Ag NPs in the previous study can also confirm increasing MDA levels in our findings (Bermejo-Nogales *et al.*, 2016).

After exposure to 10% $LC_{50}$, comparing the changes in the antioxidant system between the green synthesized AgNPs and the chemical synthesized AgNPs showed that the free radicals in the liver of fish exposed to the green synthesized AgNPs were effectively eliminated or the oxidative chain reaction was successful. In a similar study, Rajkumar *et al* reported that exposure to AgNPs in *Labo rohita* caused a significant decrease in liver CAT and SOD activity at a high sub-lethal concentration (Rajkumar *et al.*, 2016). Another study reported that common carp (*Cyprinus carpio*) exposed to low levels of Ag-NPs (12.5% of $LC_{50}$) can increase antioxidant enzymes activity, however, increasing

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exposure levels to 25% and 50% of $LC_{50}$ diminished these enzyme's activity compared to the control (Vali *et al.,* 2020).

In this study, proinflammatory reactions such as increased ACP and Ig and albumin levels and decreased total protein and globulin levels after exposure to green and chemical synthesized AgNPs were observed over time and depending on the concentration. The intensity of these reactions after exposure to green synthesized AgNPs was more than the chemical synthesized AgNPs. These results were similar to previous studies in which a significant decrease in total proteins and globulin contents in *Clarias gariepinus* were observed after exposure to AgNPs (Naguib *et al.,* 2020). In another study, Mansour *et al*. (2021) reported a decrease in total protein and globulin in Nile tilapia (*Oreochromis niloticus*) after 28-day exposure to AgNPs (Mansour *et al.,* 2021). Previous studies demonstrated that nano metal base particles such as AgNPs can influence the immune system via direct and indirect pathways (Fadeel, 2012; Rastgar *et al.,* 2022). These particles can apply direct cytotoxic actions on the immune cells and changes in the immune system reactions such as the production of immune proteins, or these nanoparticles can directly interact with immune proteins such as the complement factors (Rastgar *et al.,* 2022). Sulfur-containing macromolecules such as proteins have a strong affinity for both ionic and nano-form of silver (Linhua *et al.,* 2009). Therefore, the formation of AgNPs corona protein as a metabolic damage implication can be a reason for the decrease in immune proteins level (Ellis and Lynch, 2020). During indirect immune reactions, the AgNPs may cause tissue damage and DAMP release that results in triggering of inflammatory response such as activation of Ig and complement systems (Fadeel, 2012).

The long-time exposure of Asian seabass (*Lates calcarifer*) to graded series of green synthesized AgNPs at concentrations over 5% $LC_{50}$ induced a linear toxicity response. These concentrations increased the activity of the antioxidant system and then significantly reduced its activity by increasing the production of free radicals. However, after exposure to chemical synthesized AgNPs, the activity of the antioxidant system showed a significant decrease. Although both green and chemical synthesized AgNP exposure caused an increase in proinflammatory activities and immune proteins, these activities were more pronounced after exposure to green synthesized AgNP. Therefore, to validate the regulations of AgNPs use and discharge in the natural water bodies, it is necessary that the safety levels of these particles, even the green synthesized ones, are evaluated for different aquatic organisms.

**Conflicts of interest**:

The authors declare no conflict of interest.

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