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Role of Silver Nanoparticles Synthetized by Marine Red Algae, *Acanthophora spicifera*, Against Saprolegniosis in *Oreochromis niloticus*

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Abstract

The anti-fungal activity of *Acanthophora spicifera*-synthetized silver nanoparticles (AS-AgNPs) was tested in vitro against *S. australis*. *In vivo*, 160 *Oreochromis niloticus* (25±0.57 grams) were separated into 4 groups; group 1 and group 2 received 0 mg/L and 0.9 mg/L AS-AgNPs, respectively, and were not exposed to *S. australis*. Group 3 and group 4 were experimentally infected with *S. australis* and imperiled to 0 and 0.9 mg/L AS-AgNPs for 96 hours, respectively. At all levels, survival rates, non-specific immune indices, antioxidant activities, and expression analysis of IL-8 and TNF-α genes in the anterior kidneys were estimated. Moreover, histopathological pictures of the skin and underlying musculature were investigated. Results showed that AS-AgNPs produced an inhibitory zone with a diameter of around 12 millimeters. A significant survival rate (72.50%) accompanied by marked non-specific immune responses and antioxidant activities was recorded in the infected-treated group. The histopathological picture of the skin and underlying musculature of diseased fish (group 3) exhibited degenerative changes in the dermal and epidermal layers of the skin and the myocytes. Intriguingly, infected fish treated with AS-AgNPs (group 4) showed normal histological structures of the skin and underlying musculature. Based on these findings, AS-AgNPs have a curative outcome against the destructive effects of *S. australis* infection and are a potential option for the control of *S. australis* infection in aquaculture.

KEYWORDS
Anti-fungal, *Acanthophora spicifera*, *Saprolegnia australis*.

INTRODUCTION

Aquaculture intensification resulted in a lot of infectious disease outbreaks, which remain a concern and hinder sustainable production. *Saprolegnia* species is critical oomycete freshwater fish fungi, whether they strike under regular circumstances or are fostered under cultured conditions (Meneses *et al*., 2021). They generate cotton-like bulks that might appeared as whitish or greyish on external fish surfaces such as skin, eyes, and gills, resulting in marked mortalities in *Oreochromis niloticus* (*O. niloticus*) (Zahran *et al*., 2017).

The most prevalent infections in fish that impair their health and survival must be treated properly, cheaply, and environmentally friendly for the aquaculture industry to be sustainable. The use of nanotechnology in avoidance of microbial infections has proven to be successful (Luis *et al*., 2019). However, the potential to cause harmful effects may limit the widespread usage of nanoparticles in aquaculture (Mahboub *et al*., 2021). Thus, there has been recent advancement in the green production of nanomaterials. For the manufacture of numerous metallic nanoparticles, it depends on the use of plant materials as bioactive agents, which lessen ecological effluence compared to chemical ones (Kumari *et al*., 2021). Interestingly, AgNPs, are thought to be effectual against viruses, bacteria, and fungi (Sarkheil *et al*., 2017).

Acanthophora spicifera (*A. spicifera*), a particular genus of marine red algae, is a conspicuous group of photosynthetic organisms in marine habitats (Budiyanto *et al*., 2022). These algae are notable for their high amounts of bioactive components such as polysaccharides, phenolic compounds, and colors and are often found in tropical and subtropical habitats (Janarthanan and Senthil Kumar, 2018). These compounds are particularly intriguing for use in pharmaceutical and medical applications since they possess a broad range of biological actions, involving antioxidant, anti-inflammatory, and anticancer characteristics (Kumar *et al*., 2021). *A. spicifera*'s potential use in the production of nanoparticles has recently grasped significant research interest (Ibraheem *et al*., 2016).

The aim of the current study was to inspect the effectiveness of AS-AgNPs in treating *S. australis* infection in *O. niloticus* in an aquaculture setting. Moreover, to determine the safe and effective dosage of AS-AgNPs for treating *S. australis* infection in *O. niloticus*, as well as to evaluate the impact of the treatment on various indicators of fish health, including survival rates, hepatic and renal functions, immune indices, antioxidant activities, immune-gene expression, and histopathological changes.

MATERIALS AND METHODS

Preparation and characterization of AS-AgNPs

AS-AgNPs were prepared according to Ibraheem *et al*. (2016) as follows: Briefly, the *A. spicifera* used in this study was gathered

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from the Red Sea (Hurghada city, Egypt), washed with distilled water (DW), and dried completely before being milled into a fine powder. To prepare the extract, ten grams of the powdered algae were immersed and soaked in 100 mL of 95% ethanol for 24 hours with discontinuous shaking. The resulting mixture was then subjected to centrifugation for 5 minutes at 2000 g at room temperature (RT). Reduction of Silver nitrate was accomplished by adding 10 ml of the algae extract to 900 ml of 0.001M Silver nitrate at RT. Centrifugation at 1000 xg for 10 minutes was used to gather the created nanoparticles. The size and morphology of the obtained silver nanoparticles were analyzed using a zetasizer (Malvern Panalytical, Ltd.) and a transmission electron microscope (TEM).

AS-AgNPs had an average size of 184.6 nm, a negative potential of -12.6 mV, and a conductivity of 0.384 mS/cm, according to the zetasizer measurements (Fig. 1). The round and irregular form of the AS-AgNPs (Fig. 2) and the size range noted in the zetasizer analysis were both validated by the TEM investigation.

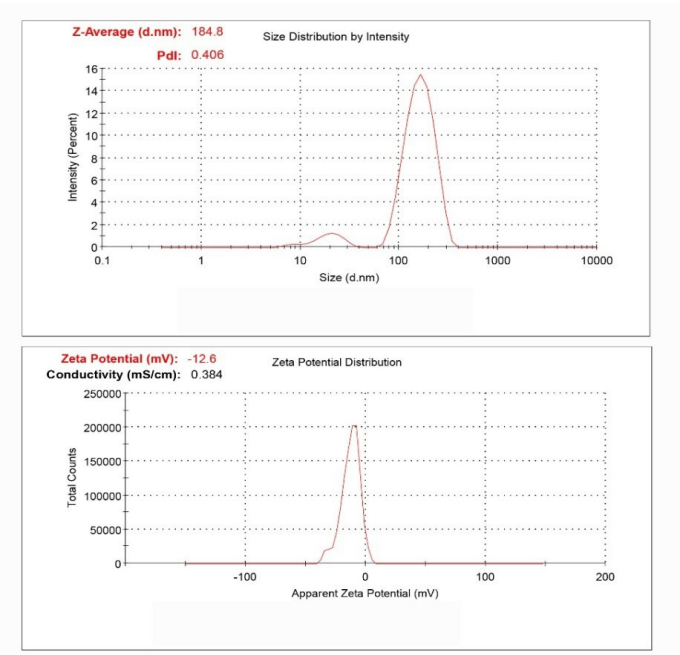

Fig. 1. Zetasizer characterization of *A. spicifera*-produced AS-AgNPs. The average size of AS-AgNPs was 184.8 nm, and they had a negative charge.

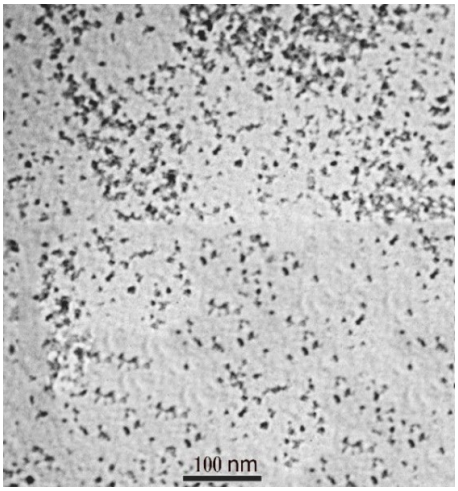

Fig. 2. TEM for biosynthesized AS-AgNPs by *A. spicifera* showed spherical and irregular nanoparticles.

Fungal strains and culture conditions

S. australis was isolated from an outbreak at Abo-Saleh fish hatchery in Beni-Suef province, Egypt, in 2019. It was morphologically and molecularly identified, then submitted to GenBank under accession number MT075633. The selected fungus was preserved on hump seed culture and kept at the Department of Fish Diseases and Management, Beni-Suef University, Egypt. Prior to use, the fungal strain was sub-cultured on sterilized glucose yeast agar (GYA) and incubated at 23°C for 3 days.

In vitro antimicrobial assay

The disc diffusion method, as reported by Prabhu *et al.* (2010), was used to examine the anti-fungal action of AS-AgNPs against *S. australis*, which was grown for three days on a GYA plate. Under aseptic circumstances, sterile discs (5 mm in diameter) were positioned and filled with 150 µl of AS-AgNPs (0.2 mmol) using a micropipette. For three days, plates were incubated at 23°C. A ruler was used to determine the zone of inhibition and to measure it in millimeters.

Ethical statement and fish rearing conditions

Institutional Animal Care and Use Committee (IACUC), approval number 021-221, at Beni-Suef University in Egypt approved all the experimental protocols. At the Department of Fish Diseases and Management, Beni-Suef University, all experimental procedures were completed. The government fish hatchery in Abo-Saleh, Beni-Suef Province, Egypt, provided 280 *O. niloticus*, with an average body weight of 25±0.57 g. These fish were examined for health. They were housed in a 20-liter glass aquarium (70x35x25 cm) filled with tap water that had been de-chlorinated. The fish underwent 14 days of acclimation prior to the experiment. A simple diet was given to the fish at a rate of 2% of their body weight. Throughout the whole experiment, the following water parameters were maintained: 22±1°C water temperature, 7.5±0.4 pH, 6±0.21 mg/L dissolved O2, 0.016±0.003 mg/L NO2, 0.02±0.004 mg/L NH_3, and 12:12 h light / dark).

Preparation of zoospore suspension for experimental infection

According to Mostafa *et al.* (2020), a zoospore suspension from *S. australis* was set up with modifications as follows: The fungal strain was grown on GYA plates for 2 days at 23°C. Some growing hyphae were seized from the brink of mounting plates and dipped in flasks containing water from the aquarium supplemented with 0.1% tween 20 and chloramphenicol (400 mg/L) to avert bacterial impurity and foster zoospore release from fungal hyphae. Flasks were incubated for 24 h at 23°C. After that, zoospores were harvested and diluted. The feasible count of zoospores was estimated by hemocytometer (BT, Wertheim). The experimental infection method in this study was carried out by the Ami-Momi (net-shake) method as illustrated by Hatai and Hoshiai (1993), and the infective dose was adjusted to 2×10^5 zoospores/ml.

Determination of the effective cure concentration of AS-AgNPs

100 experimentally diseased fish with *S. australis* were randomly assigned to five groups of 20 fish each. The fish were given five different doses of AS-AgNPs over the course of 96 h (0, 0.3, 0.6, 0.9, and 1.2 mg/L for groups 1 through 5, respectively). Fish in group 6 did not have AS-AgNPs treatments or experimental infection. The death rates, clinical symptoms, and postmortem abnormalities were monitored and recorded daily in order to find the lowest observable safe and effective concentration.

Treatment trial and sampling

160 fish were divided into four even groups at random, each with 30 fish (30 in triplicate, 10 in each repeat). Group (Gr) 1 served as the control group, whereas Gr 2 and Gr 1 were the non-infected groups subjected to 0 and 0.9 mg/L AS-AgNPs, correspondingly. On the other hand, Gr 3 and Gr 4 were experimentally subjected to 0 and 0.9 mg/L AS-AgNPs and infected with the infective dosage of *S. australis* (2×10^5 spores/L). Daily records were kept of the clinical symptoms, fatalities, and post-mortem lesions. Following the exposure time (96 h), survival rates were determined as described by Ibrahim *et al*. (2022).

After the treatment trial (96 h), experimental fish were subjected to anesthesia using a 40 ppm eugenol solution (Rairat *et al*., 2021). Blood was collected from the caudal vessels of six fish per group deprived of anticoagulant and subjected to centrifugation at 4°C for 15 min for serum parting at 5000 g, then kept at -20°C till biochemical and immune analyses. Tissue samples from the gills and liver were appropriated to detect oxidative and antioxidant activities in branchial and hepatic tissues. Anterior kidneys were incised, immersed in RNA later, and preserved at -80°C aimed at gene expression analyses. Additionally, tissue samples from the skin and underlying musculature were kept in 10% formalin for histopathological examination.

Non-specific immunological characteristics

Serum nitric oxide (NO) level was measured spectrophotometrically (Bryan and Grisham, 2007). While complement-3 quantification was carried out using a commercial kit (C3, Cusabio), involving measurement of the OD following the immune sensitization to the anti-complement-3 antibody at 450 nm, in addition, activity of lysozyme was reckoned in serum, as illustrated in a previous approach described by Ghareghanipoora *et al*. (2014), via the disintegration of *Micrococcus lysodeikticus* (Sigma-Aldrich). Briefly, serum sample was combined with a suspension of *Micrococcus lysodeikticus* (0.22 mg/mL in 0.05 Mol PBS, pH 6.3), and the mixture was then allowed to react at 25°C for 5 min. The OD was then measured using a T80 UV/V spectrometer from PG Instrument Ltd) five times at intervals of 1 min for 5 min. Lyophilized chicken egg-white lysozyme (Sigma-Aldrich) was used to create a calibration curve that was used to calculate lysozyme concentrations.

Evaluation of antioxidant activities

Six tissue samples from each group, including liver and gill tissues, were washed three times with a salt chloride solution (0.65 %). Then thoroughly homogenized in PBS (pH 7.4) after that. The homogenized tissues were then subjected to centrifugation at 5000 g for around 20 min. For the purpose of quantifying oxidative and antioxidant markers, the lightest layer was painstakingly amassed in sterilized tubes (Fernandez-Botran *et al*., 2002). Malondialdehyde (MDA), a byproduct of lipoperoxidation, was measured at the branchial and hepatic levels exploiting the thiobarbituric acid approach (Ohkawa *et al*., 1979). Thiobarbituric acid's active chemicals were estimated at 535 nm in terms of the Malondialdehyde that was generated. According to Aebi, (1984) method, the catalase (CAT) activity in the gill and liver supernatants was assessed. A combination of enzymatic procedures using hydrogen peroxide (H_2O_2), potassium phosphate (pH 7.2), and homogenized supernatant were prepared to measure CAT activity. A spectrophotometer (T80 UV/V spectrometer, PG Instrument Ltd) operating at 240 nm was used to examine the molar attenuation coefficient of hydrogen peroxide. Catalase activity was measured as the rate at which hydrogen peroxide was broken down by the enzyme (1 mol/min/mg of hippocampus tissue protein) (U/mg protein). Additionally, the superoxide dismutase (SOD) activity of the gill and liver was evaluated in accordance with Nishikimi *et al*. (1972). This approach is contingent on the ability of adrenaline oxidation at pH around10 to produce superoxide radicals (O^-_2) and adrenochrome. Depending on how the absorbance at 480 nm changed, the inhibition of SOD activity was examined. According to Benzie and Strain (1996), total antioxidant capacity of serum (TAC) was assessed. The calculation of TAC is based on how well the antioxidants in the serum sample under investigation react with a significant quantity of exogenously given H_2O_2. Based on the enzymatic process that causes the modification of 3, 5, and dichloro-2-hydroxybenzensulfonate to a colored substance, the remaining amount of H_2O_2 is estimated at 505 nm (LTQ Velos Orbitrap spectrometer, Thermo Scientific).

Immune-relevant gene expression

Following the manufacturer's instructions, 60 mg of anterior kidney tissues from two fish per replication (n.= 6 per group) were used to extract total RNA using 1 mL of Quiazol (Qiagen). The amount of the obtained total RNA was evaluated using a NanoDrop One UV-Vis Spectrophotometer (Thermo Scientific) to quantify concentration at 260 and 280 nm. The cDNA was created from the extracted RNA using a high capacity cDNA reverse transcription Kit (catalogue number K1641, Maxima First Strand cDNA Synthesis, Thermo Scientific) according to the manufacturer's instructions. In 20-µl reaction tubes, 2 ng of total RNA from a 4 µl RNA sample, 3 µl of 6X buffer reaction mix, 3 µl of maximal enzyme mixture, and 10 µl of purified water were used to create the cDNA. At -80°C, the reactions were kept safe. Interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α) real-time quantitative PCR (RT-qPCR) estimation were accomplished exploiting the specific primer sets (ShineGene Molecular Biotech) (Table 1). RT-qPCR was carried out using the FastStart Universal SYBR Green Master Kit (ROX; Roche). Each 20 µl qPCR reaction contained 10 µl of highly purified water, 8 µl of FastStart Universal SYBR Green Master (ROX), 1 µl of cDNA, and 0.5 µl of primers. The qPCR reactions were carried out with the use of the 7,500 RealTime PCR System (Applied Biosystems). The thermal cycler was set up to accomplish 40 cycles at 94°C for 10 s, 58°C for 15 s, and 72°C for 10 s for denaturation, annealing, and extension, respectively, over a period of 10 min at 95°C for initial denaturation. According to Karsi *et al*. (2004), the expressions of genes of interests were calculated as a relative fold change to the reference gene (β-actin).

Histopathological examination

Each group's tissue samples (skin and underlying musculature) were taken, and then they were fixed in 10 % formalin. The tissue samples were gradually subjected to dehydration in ethanol (adjusted from 60 % to 100 %), cleaned in xylene, and then embedded in paraffin. The slices of the paraffin blocks were made with a microtome (Leica RM 2155). The slices (5 mm thick) were subjected to staining with hematoxylin and eosin (H&E) (Sultana *et al*., 2016).

Statistical analysis

Data from immunological and biochemical analyses, antioxidant indices, and fold changes of immune-relevant genes (IL-

8 and TNF- α) were examined by one-way analysis of variance (ANOVA) by SPSS 18 (SPSS, Chicago, Illinois, USA). At a significance level P ≤ 0.05, Duncan's multiple range tests were utilized to identify mean differences.

RESULTS

Antimycotic activity of AS-AgNPs against S. australis

The produced nanoparticles showed a strikingly clear zone in the growing plates of the tested fungus. With a zone of inhibition of around 12 mm against *S. australis*, AS-AgNPs demonstrated in vitro antimycotic efficacy (Fig. 3).

Concentration of AS-AgNPs for effective therapy

Table 2 shows that experimentally infected fish groups (Gr 1 and Gr 2) imperiled to 0.00 and 0.30 mg/L AS-AgNPs had erratic swimming, a deficiency in the escape reflex, and external cutaneous lesions with cotton-like lumps on various regions of external body surfaces. For Gr 1 and Gr 2, the recorded mortality rates were 80 % and 70 %, respectively. The *S. australis* diseased fish imperiled to 0.60 mg/L (Gr 3) displayed decreased swimming activity, a subpar response to the escape reflex, and 40 % mortality rate. However, 0.9 mg/L MS-AgNPs (Gr 4) treatment of infected fish reduced the prior clinical indications with the lowest mortality rate (25 %) when compared to other groups.

Treatment trial

The non-infected fish (Gr 1 and Gr 2) imperiled to 0 and 0.9 mg/L AS-AgNPs, subsequently, did not exhibit aberrant clinical symptoms or mortalities at the level of the treatment trial (Table 3 & Fig. 4-D). The *S. australis*-infected fish (Gr 3) that were

Table 1. Oligonucleotide sequences of immune-relevant genes for qPCR amplification.

Gene	Primer sequence	Size of Product (bp)	GenBank number	Reference
β-actin	F: CCACCCAAAGTTCAGCCATG R: ACGATGGAGGGGAAGACAG	121	XM_003443127.5	Rowida *et al*. (2022)
IL-8	F: GCACTGCCGCTGCATTAAG R: GCAGTGGGAGTTGGGAAGAA	180	NM_001279704.1	Ming *et al*. (2013)
TNF-α	F: GAGGTCGGCGTGCCAAGA R: GGTTTCCGTCCACAGCGT	119	NM_001279533.1	Chen *et al*. (2016)

β-actin: beta actin; IL-8: interleukin 8; TNF-α: tumor necrosis factor alpha.

Table 2. Assessment of *O. niloticus*'s observable clinical symptoms and mortality rates of experimental *S. australis* infected fish after treatment with various doses of AS-AgNPs for 96-h.

Group	Concentration (AS-AgNPs mg/L)	Observable clinical findings			Mortality number (N=20)	Mortality %
		Irregular swimming	Escape reflex deficit	Exterior cutaneous lesions		
Gr 1	0	++++	++++	++++	16/20	80
Gr 2	0.3	++	++	++	14/20	70
Gr 3	0.6	+	+	-	20-Aug	40
Gr 4	0.9	-	-	-	20-May	25
Gr 5	1.2	-	-	-	20-Jun	30
Gr 6	Control -ve	-	-	-	-	0

(-) stands for no lesion, (+) for a light injury, (++) for a medium injury, and (++++) for a serious injury. Gr 6 denotes fish that have not been experimentally infected or given AS-AgNPs treatment.

Table 3. Assessment of *O. niloticus*'s observable clinical symptoms and mortality rates of experimental *S. australis* infected fish after treatment with 0.9 mg/L of AS-AgNPs for 96-h.

Group	Concentration (AS-AgNPs mg/L)	Observable clinical findings			Mortality number (N=40)	Survival rates
		Irregular swimming	Escape reflex deficit	Exterior cutaneous lesions		
Gr 1 (Non-infected)	0	-	-	-	-	100
Gr 2 (Non-infected)	0.9	-	-	-	-	100
Gr 3 (Infected)	0	++++	++++	++++	32/40	20
Gr 4 (Infected)	0.9	+	+	-	Nov-40	72.5

(-) stands for no lesion, (+) for a light injury, (++) for a medium injury, and (++++) for a serious injury.

Table 4. Impact of AS-AgNPs on immunological parameters of *O. niloticus* experimentally infected with *S. australis* for 96 h.

Immune -parameters	Fish groups			
	Gr 1	Gr 2	Gr 3	Gr 4
Nitric oxide (NO) (µmol/L)	15.55±1.34^{a}	16.18±1.47^{a}	10.30±1.28^{b}	13.12±0.88^{c}
Complement (C3) (g/dL)	0.35^{a}0.02^{a}	0.34±0.03^{a}	0.27^{a}0.04^{b}	0.30±0.01^{c}
Lysozyme (lyz) (µg /mL)	10.42±1.50^{a}	9.39±0.51^{a}	4.23±0.53^{b}	5.18±0.98^{c}

Gr 1 and Gr 2 are *S. australis*-free fish exposed to 0 mg/L and 0.9 mg/L AS-AgNPs, respectively. Fish infected with *S. australis* and submerged in 0 mg/L and 0.9 mg/L AS-AgNPs are represented by Gr 3 and Gr 4. The mean±standard error (±SE) is used to express values. Values with various superscript letters on the same line differ considerably (P ≤ 0.05) from each other.

Table 5. Influence of AS-AgNPs on the branchial, hepatic, and serum antioxidant activations of experimentally infected *O. niloticus* with *S. australis* for 96 h.

Indicators	Fish groups			
	Gr 1	Gr 2	Gr 3	Gr 4
Branchial antioxidant activity				
MDA, malondialdehyde (nmol/mg tissue)	5.62±0.45 [a]	6.30±0.72 [a]	15.35±1.2 [b]	9.79±1.3 [c]
SOD, superoxide dismutase (U/mg tissue)	22.59±2.87 [a]	23.34±1.1 [a]	12.62±0.86 [b]	15.46±1.78 [c]
CAT, catalase (U/mg tissue)	11.83±0.63 [a]	12.45±0.64 [a]	4.53±0.03 [b]	6.22±0.02 [c]
Hepatic antioxidant activity				
MDA, malondialdehyde (nmol/mg tissue)	14.60±1.35 [a]	16.32±1.31 [a]	30.47±0.62 [b]	26.96±0.
SOD, superoxide dismutase (U/mg tissue)	81.15±1.40 [a]	83.35±1.02 [a]	45.53±0.54 [b]	56.52±1.43 [c]
CAT, catalase (U/mg tissue)	11.80±0.31 [a]	13.67±0.46 [a]	4.96±0.37 [b]	6.25±0.73 [c]
Serum antioxidant activity				
TAC, total antioxidant capacity (ng/mL)	0.66±0.05 [a]	0.59±0.07 [a]	0.24±0.01 [b]	0.32±0.04 [c]

Gr 1 and Gr 2 fish are uninfected with *S. australis* and were exposed to 0 mg/L and 0.9 mg/L AS-AgNPs, respectively. Fish infected with *S. australis* and submerged in 0 mg/L and 0.9 mg/L AS-AgNPs are represented by Gr 3 and Gr 4. The mean±standard error (±SE) is used to express values. Values in the same line that have a different superscript letter are substantially different at P ≤0.05.

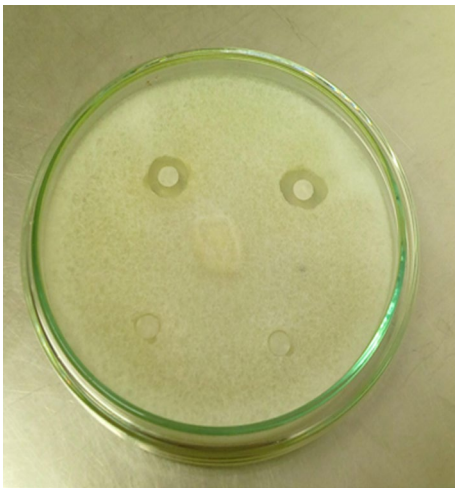

Fig. 3. Anti-fungal activity of AS-Ag-NPs against *S. australis*.

left untreated showed irregular swimming patterns, a diminished escape reflex, exterior cutaneous hemorrhages, eroded skin, opaque eyes with cotton-like masses on various external body surfaces, and a low survival rate (20 %) (Table 3 & Fig. 4- A, B and C). However, treatment of diseased fish with 0.9 mg/L AS-Ag-NPs (Gr 4) minimized the symptoms listed above, except for slow swimming and a marginally diminished escape reaction with the highest survival rate (72.50 %) (Table 3 & Fig. 4- E).

Immunological indices

As shown in Table 4, Gr 2 did not exhibit any appreciable differences from Gr 1 in the immunological parameters of nitric oxide, complement, and lysozyme. In contrast to Gr 1, Gr 3 displayed a significant reduction in these immunological markers. Additionally, when compared to Gr 3, treatment of the diseased fish in Gr 4 with 0.9 mg/L AS-AgNPs changed these variables.

Tissues and serum antioxidant activities

Gr 3 showed significantly higher malondialdehyde (MDA) activity in both livers and gills compared to Gr 4, while Gr 1 and Gr 2 showed no significant change (Table 5). When compared to Gr 1 and Gr 2, the hepatic and branchial superoxide dismutase (SOD) levels of Gr 3 and Gr 4 significantly decreased (P ≤ 0.05). There was no discernible difference between Gr 1 and Gr 2, while Gr 3 and Gr 4 had the lowest levels of branchial and hepatic catalase

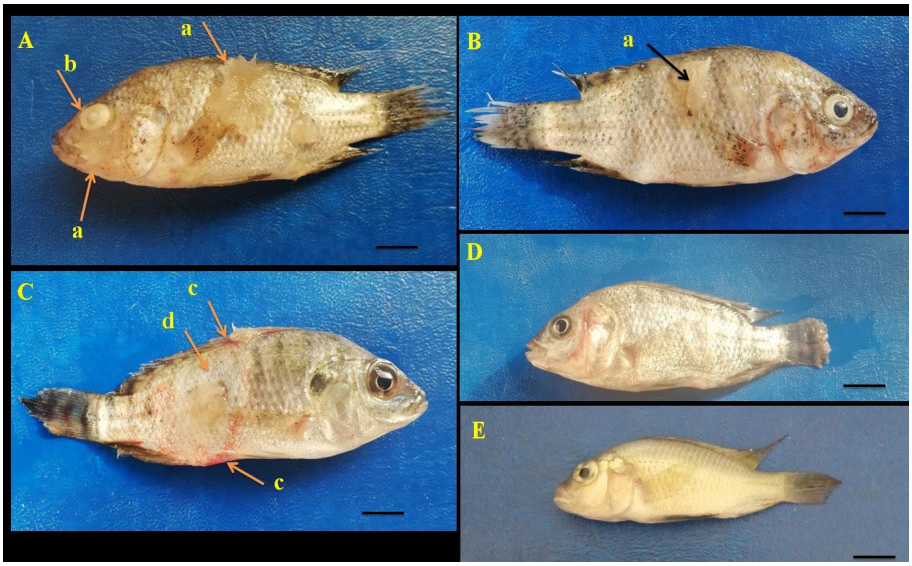

Fig. 4. A, B, and C represent fish infected and treated with 0.00 mg/L AS-AgNPs, which showed a: cotton wool-like growth on skin and fin surfaces; b: eye opacity; c: hemorrhages; and d: eroded skin. D represents fish from the non-infected groups treated with 0.00 mg/L AS-AgNPs and 0.9 mg/L AS-AgNPs that showed normal appearance and swimming behavior. E represents fish infected and treated with 0.9 mg/L AS-AgNPs that showed a normal appearance with sluggish swimming and a mild response to the escape reflex.

(CAT) and serum total antioxidant capacity (TAC).

Expressions of immune genes

Fig. 5- A & B showed that there were significant up-regulations of IL-8 and TNF-α expressions in the anterior kidneys of Gr 2 (2.87 & 3.63-fold, subsequently), in comparison with Gr 1. Moreover, the highest levels of IL-8 and TNF-α up-regulations were recorded in Gr 4 (7.24 and 10.22 fold), followed by G3 (5.62 and 6.28 fold), respectively.

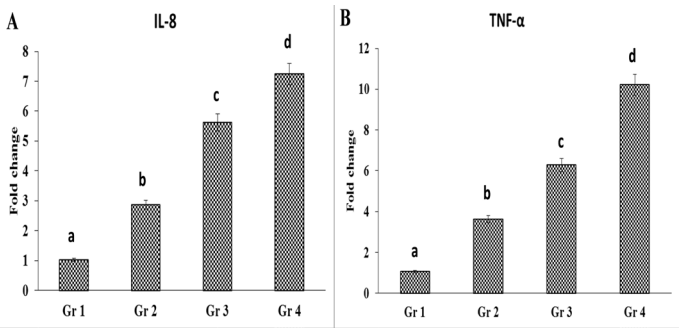

Fig. 5. Impact of AS-AgNPs on IL-8 (A) and TNF- α (B) expression levels in the head kidney of *O. niloticus*. Gr 1 and Gr 2 fish are uninfected with *S. australis* and were exposed to 0 mg/L and 0.9 mg/L AS-AgNPs, respectively. Fish infected with *S. australis* and submerged in 0 mg/L and 0.9 mg/L AS-AgNPs for 96 h are represented by Gr 3 and Gr 4. Bars with the superscripts a, b, c, and d are noticeably different ($P \leq 0.05$).

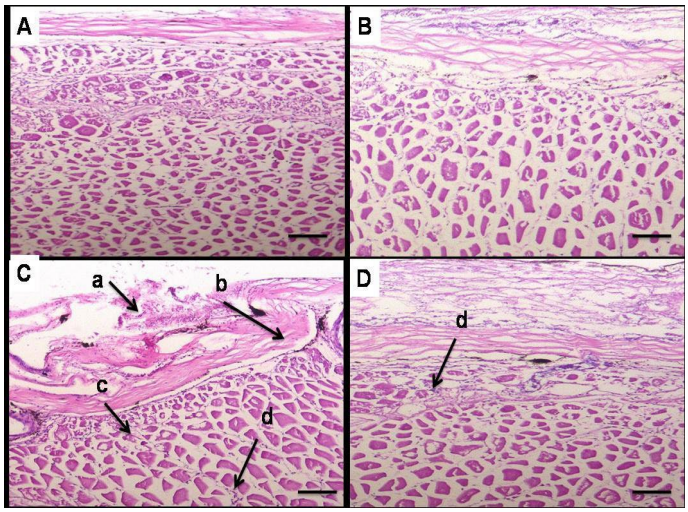

Fig. 6. Photomicrograph (H&E) of the skin and underlying musculature of *O. niloticus* A and B: Gr 1 and 2, showing normal histological structures of the skin and underlying musculature. C: skin and underlying musculature of infected non-treated fish (Gr 3) showing destructive alterations of epidermal and dermal layers of skin (a & b), focal necrotic changes in myocytes (c), and leucocyte cell infiltration among myocytes (d). D: skin and underlying musculature of treated fish (Gr 4) with 0.9 mg/L AS-AgNPs showing normal histological organization of skin and underlying musculature with leucocytes infiltration (d). Scale bar: 100 µm.

DISCUSSION

The aquaculture industry is impacted by a variety of infectious diseases, including mycotic diseases, which cause considerable losses in fish and hatcheries in both wild and cultured habitats (Meneses *et al*., 2021). Due to extensive fish epidermal damage caused by saprolegniosis, which is then followed by secondary bacterial infection and death, fish farms experience notable losses (Zahran and Risha, 2013). Since there aren't many studies on employing green synthesized AS-AgNPs to treat fungus infections, the current study was designed to examine the effect of AS-AgNPs on controlling *S. australis* infection in *O. niloticus*.

A. spicifera is well known for having high levels of bioactive components like uronic acids, polysaccharides, monosaccharides, and phenolic compounds (Janarthanan and Senthil Kumar, 2018). These biological elements might be essential for the creation and stability of metal nanoparticles (Sahayaraj and Rajesh, 2011). Additionally, their proteins may create a cap over the silver nanoparticles to keep them from clumping together and to stabilize them in the aqueous solution (Sahayaraj and Rajesh, 2011). Furthermore, AS-AgNPs had tiny particle size in terms of diameter. This indicated that the particles' uniformity may have contributed to their resistance to aggregation or clustering in the aqueous solution. Our findings showed that AS-AgNPs presented antimycotic action in vitro against *S. australis* with a clear zone of inhibition around 12 mm. (Ibrahim *et al*., 2022) reported a similar outcome and measured an 18 mm inhibitory zone of silver NPs produced by moringa plant against *Saprolegnia* spp. Silver nanoparticles (NPs) have a considerable inhibitory effect on *S. australis*, which can be linked to a number of means that involve the release of free molecules and ions (as silver ions Ag+) from the biosynthesized NPs, both of which have antimycotic merits (Sadjadi *et al*., 2009). Additionally, Ag+ disrupt the microbe's membrane configuration by making it more delicate, hindering its ability to transfer materials, and/or hurting the cellular respiratory system (Dakal *et al*., 2016).

According to the current study, fish that were experimentally infected with *S. australis* displayed cutaneous erosions and hemorrhages, opaque eyes, and cotton-like masses adhered to the fish's skin, eyes, and fins. These symptoms were also accompanied by poorer survivorship. In past research, comparable clinical images were recorded (Mahboub *et al*., 2021). Fish epidermal layer injury, which causes failure in osmoregulation, hemodilution, and secondary infection, followed by mortality, may be to blame for lower survival rates (Zahran and Risha, 2013). A growing survival rate of 75.50 % is observed when *S. australis* is treated with AS-AgNPs, which intriguingly prevents the emergence of prior clinical symptoms in the treated group. This might be interpreted as the antimycotic influence of AS-AgNPs being strengthened by the present study's in vitro antimycotic action (Figure 3). Similar outcomes were seen when *Saprolegnia* spp. infected *O. niloticus* were treated with silver nanoparticles made from moringa, and the treated fish had a 72% survival rate (Ibrahim *et al*., 2022). Also, (Meneses *et al*., 2021) recorded the anti-fungal activity of AgNPs against *Saprolegnia* spp. in angelfish.

Concerning fish innate immunity, (Saeij *et al*., 2000) reported that nitric oxide has broad-spectrum antimicrobial effects on several fish microorganisms. The host's ability to detect potential infections and fight them off depends on the complement system. Furthermore, the foundation for the development of adaptive immunity is the complement activation pathways (Boshra *et al*., 2006). Lysozyme is a crucial mucolytic enzyme as well. It is a component of the fish host's bodily fluids such as plasma, serum, and mucus, and the activity of this component is a key indicator of the fish's innate immune response (Reda *et al*., 2022). In this investigation, *S. australis*-infected fish displayed reduced nitric oxide, complement, and lysozyme immune levels in comparison to non-infected groups. The immune-inhibitory influence might be credited to the use of fish leukocytes that were capable of binding to the wall of the fungal element after detecting the fungal component and activating a potent response of inflammation in the host (Belmonte *et al*., 2014). Related findings revealed changes in the immunological parameters of *Saprolegnia* spp.-infected fish hosts, specifically the lysozyme and complement levels (Ibrahim *et al*., 2022). Treatment with AS-AgNPs for the *S. australis*-infected group in this investigation had the potential to change the immunological parameters (Table 4). This could be viewed as a barrier against infection's progression. The immune-modulatory effects of AgNPs have also been seen in previous studies in *O. niloticus* (El-Houseiny *et al*., 2021; Ibrahim *et al*., 2022).

Fish microorganisms produce more reactive oxygen species (ROS), which inhibits antioxidant enzymes and decreases non-enzymatic antioxidants. These oxidatively damaging effects are a

primary cause of infectious illness pathogenesis (Mozhdeganloo and Heidarpour, 2014). Total serum antioxidant activity (TAC) and tissue antioxidant activities (branchial and hepatic CAT and SOD) decreased in this study, although an elevated MDA level was found in the *S. australis*-infected group. (Baldissera *et al*., 2020; Ibrahim *et al*., 2022) reported similar findings. The *S. australis* infection may result in lipid peroxidation in fish cell membranes, which could increase the MDA enzyme's activity. The antioxidant endeavor of AS-AgNPs might be responsible for changes in the antioxidant indicators of the treated group with AS-AgNPs, which expressed higher levels of SOD and CAT and lower levels of MDA than the infected non-treated fish group. Previous researches have provided evidence of similar results (Mitiku and Yilma, 2017; Ibrahim *et al*., 2022).

In terms of pro-inflammatory cytokines, fish leukocytes release pro-inflammatory cytokines, including IL-8 and TNF-α, whose main function is to start the inflammatory process (Zou and Secombes, 2016). Immune-relevant genes, such as IL-8 and TNF-α, are expressed in fish at the start of infection and are crucial in controlling the inflammatory response (Zou and Secombes, 2016). The up-regulation of the pro-inflammatory genes, IL-8 and TNF-α in the current study was a result of *S. australis* infection (Figure 5). These findings demonstrated that *S. australis* infection impaired *O. niloticus*'s immune response, which was supported by the decreased levels of serum immunological indices involving nitric oxide, complement, and lysozyme (Table 4). The sensitization of fish leukocytes by polysaccharides present in the cell wall of the fungal element, which cause a significant response of inflammatory reaction in fish hosts, may explain the elevations of IL-8 and TNF- α expression levels. Enhancing the production of prostaglandin E2, a metabolic byproduct that has been shown to promote the response of inflammation in fish leukocytes, is another effect of *Saprolegnia* infection (Belmonte *et al*., 2014). Previous reports of related findings were made (Earle and Hintz, 2014; Ibrahim *et al*., 2022). Exposure of the diseased fish group with 0.9 mg/L AS-AgNPs changes the expression levels of immune-relevant genes of concern that might be regarded as having antimycotic action against *S. australis* infectivity (Fig. 5).

According to the results of the current study's histopathological analysis, the non-treated infected fish in this study displayed pathological changes in the skin and underlying musculature that appeared as destructive changes to the epidermal and dermal layers of skin, focal necrotic changes in myocytes, and leucocyte cell infiltration among myocytes (Fig. 6-C). The pro-inflammatory cytokines that started the inflammation and the excessive creation of ROS from the injured tissue brought on by the *S. australis* infection which was accompanied by a high MDA level could be credited with these pathological changes. Figure 6-D showed that AS-AgNPs have a calming impact on the skin and underlying musculature by reducing the negative effects of *S. australis* infection with the exception of certain leukocyte infiltrations. This may be attributable to AS-AgNPs' antioxidant activity, which helps to mitigate all of the negative effects of the infection's ROS production. (El-Houseiny *et al*., 2021; Ibrahim *et al*., 2022) also found similar results.

CONCLUSION

Infection with *Saprolegnia* spp. poses a serious risk to the aquaculture industry. It was necessary to develop an effective therapy free from negative effects due to the dangers and limited effectiveness of chemical drugs. According to the study's results, we highlighted the crucial role AS-AgNPs (0.9 mg/ L) play in lowering the negative effects of *S. australis* infection in *O. niloticus*, including mortality rates, liver dysfunction, immune-antioxidant deficiencies, expressions of pro-inflammatory genes, and histopathological changes. The curing action of AS-AgNPs might work by decreasing the destructive effects of ROS production, inflammation and lipid peroxidation. To reduce the pathogen challenge and achieve sustainable development in the aquaculture industry, more research is required. We propose investigating the anti-fungal, antiviral, and antibacterial effects of AS-AgNPs against additional fish pathogens and in different fish species with different experimental periods.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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