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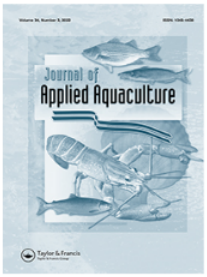

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In vivo and in vitro assays using biosynthesized silver nanoparticles on *Aeromonas hydrophila*-infected *Clarias gariepinus*

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ABSTRACT

The biosynthesis of nanoparticles using silver (Ag^{+}) and plant extracts such as *Myrmecodia* sp. has high potential as an antibacterial agent. Present research aimed to evaluate in vitro and in vivo antibacterial assays of silver nanoparticles (AgNPs) against *Aeromonas hydrophila*. The results showed that 40% AgNPs provided the optimum inhibition index (7.7 ± 0.45 g mm). Meanwhile, *Aeromonas hydrophila* infected-fish without an AgNPs injection showed significantly reduced of erythrocytes (20.89 ± 0.03 × 10^{6} μL^{-1}), hemoglobin (1.20 ± 0.10 g dL^{-1}), and neutrophils, while the levels of leukocytes (11.73 ± 0.09 × 10^{3} μL^{-1}), platelets (29.67 ± 2.91 × 10^{3} μL^{-1}) and lymphocytes were found to be significantly increased. Additionally, the infected-fish that were injected with either AgNPs or *Myrmecodia* sp. extract showed an improved survival rate compared to the control group. The biogenerated-AgNPs demonstrated potent antimicrobial activity, either in vitro or in vivo, and beneficial for use in fish cultivation.

KEYWORDS

Nanoparticle; fish; blood profiles; survival; green synthesize; *Aeromonas hydrophila*

Introduction

The bacterial species *Aeromonas hydrophila* has been recognized as a fish pathogen and has caused mass mortalities in fish cultures (Hardi et al. 2018a, 2018b; Hayatgheib et al. 2020, Li et al. 2020; Nugroho et al. 2017). Many attempts to prevent and cure Motile Aeromonas Septicemia (MAS),

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which is mostly caused by *A. hydrophila*, have been attempted using antibiotics (Odeyemi, Asmat, and Usup 2021; Pauzi et al. 2020) and several plant extracts (Abd El-Gawad et al. 2020; Mohammadi et al. 2020a, 2020b; Yousefi et al. 2021). However, the use of antibiotics to cure MAS has led to resistance (Ansari, Rahimi, and Raissy 2011; Odeyemi, Asmat, and Usup 2021; Radu et al. 2003) and damage to the environment due to the accumulation of antibiotic residues (Holmström et al. 2003; Lulijwa, Rupia, and Alfaro 2020; Yousefi et al. 2021). Thus, the use of plant extracts as an alternative medicinal agent to cure and prevent MAS has gained momentum in the research community.

Recent developments in science and technology, particularly in the field of nanotechnology, have resulted in a novel concept for the synthesis of nanoparticles of any desired size and shape using various elements of plant extracts (Ghotekar et al. 2020; Hasnain et al. 2019; Khatua et al. 2020). Nanotechnology has led to the new concept of synthesizing nanosized particles using biological methods (El-Adawy et al. 2021; Kumar et al. 2019; Rajasekar et al. 2020). The synthesis of nanoparticles using biological processes, also known as green synthesis or biosynthesis, has numerous advantages including lower expenses, environmental friendliness, and superiority over chemical and physical approaches (Khodadadi, Bordbar, and Nasrollahzadeh 2017; Kumar et al. 2017; Ovais et al. 2018; Rafique et al. 2017). Additionally, for the synthesis process, most chemical methods involve the use of harmful chemicals, additives or capping agents, and nonpolar solvents and, thus, are not appropriate for use in the biomedical field. Thus, the requirement to establish a safe, efficient, biocompatible, benign, and environmentally friendly process for the biosynthesis of nanoparticles has compelled many researchers to develop the green synthesis of nanoparticles (Bandeira et al. 2020; Rana, Yadav, and Jagadevan 2020; Salem and Fouda 2021).

Nanoparticle biosynthesis using plant extracts is an expanding topic of study that offers advantages over microbial synthesis due to the lack of complexity and the potential for large-scale production (Ali et al. 2020; Das et al. 2013). Plant extract-based nanoparticle techniques can be utilized as an alternative to the chemical methods that are extensively employed because they are straightforward, fast, inexpensive, and can accommodate nanoparticles of a large scale. There are several naturally occurring phytomolecules in plant extracts, including flavonoids, alkaloids, and other phenolic compounds called polyphenols that are water-soluble (Ghildiyal et al. 2020; Jovanović et al. 2017; Tepal 2016). These polyphenol-based phytomolecules have good reducing properties and a high affinity for adsorption on the surface of nanoparticles (Rice-evans et al. 1995). The phenolic phytomolecules have mostly antioxidant properties because of their ability to be reducers and individual oxygen quenchers. To date, significant research has been conducted on the potential sources of the phenolic content in many plant categories and plant sources, such as leaves,

berries, bulbs, fruits, oil seeds, spices, tissues and roots, and extracts from whole plants (Li et al. 2013). Phytomolecules such as flavonoids, phenolic acids, tannins, stilbenes, and lignin differ greatly between sections of the same plant; however, these elements are particularly common in the leaves, flowering fleshy tissues, and woody forms including the stems, bark, and bulbs (Azzini et al. 2014; Larson 1988; Sarker, Oba, and Daramy 2020).

In addition to serving as reducing agents, the physiologically active plant extracts result in functional silver nanoparticles (AgNPs) (Nugroho et al. 2021; Sarker, Oba, and Daramy 2020). Moreover, the physiological conditions of temperature and pressure allow for the effortless phytomolecules-based synthesis and functionality of the AgNPs (Huang et al. 2014). Due to the dual presence of phytomolecules, plant-based extracts are ideal for use during the production of AgNPs for diverse biological applications, since the nanoparticles may be used directly without any post-treatment (Mie et al. 2014). Furthermore, due to the distinct morphology, stability, and regulated geometry of AgNPs, attention has recently been focused on many other types of nanoparticles. Metallic nanoparticles (NPs) have shown promise in a variety of applications, including synthetic biology, cellular transport, health care, and aquaculture. AgNPs in particular have shown improved antibacterial efficacy against a wide range of pathogens known to cause infectious illnesses.

Previous studies have revealed that nanoparticle-mediated plant extracts can be used as antibacterial agents for fish bacterial pathogens (El-Adawy et al. 2021; Kumar et al. 2019; Velmurugan et al. 2014). AgNPs that were biosynthesized using *Psidium guajava* leaf extract were shown to suppress the growth of *Pseudomonas aeruginosa* MTCC 741 (Bose and Chatterjee (2016)). Mangrove plant (*Rhizophora mucronata* [Lamk, 1804])-mediated AgNPs synthesis also exhibited antibacterial (*Proteus* spp., *Pseudomonas fluorescens*, and *Flavobacterium* spp.) activity against *Dascyllus trimaculatus* (Umashankari et al. 2012).

Several studies have been performed to implement AgNPs as an antibacterial agent in the field of aquaculture; however, limited studies have involved the biosynthesis of AgNPs mediated using an ethanolic extract of *Myrmecodia* sp. bulbs to determine antibacterial activity, either in vitro or in vivo, against *A. hydrophila*. Thus, the present study aimed to produce AgNPs using the ethanolic extract of *Myrmecodia* sp. bulbs, characterize the resulting AgNPs, and evaluate antibacterial activity in vitro against *A. hydrophila* and in vivo in *A. hydrophila*-infected fish.

Materials and methods

Myrmecodia sp. bulb extract

A bulb of *Myrmecodia* sp. was washed with deionized water and dried for 12 hours at 50°C. A mill was used to grind the bulb to obtain a powder. The powder was soaked with ethanol, stirred, and left out for 48 hours. After the sample was filtered using Whatman paper (Whatman Grade 1 filter papers), the filtrate was evaporated using a rotary evaporator, yielding a crude *Myrmecodia* sp. bulb extract. The crude extract was kept at 4°C until it was utilized for AgNP biosynthesis.

Green synthesis of AgNPs

The AgNPs were synthesized by combining 0.5 M $AgNO_3$ solution and the *Myrmecodia* sp. bulb extract at a specific ratio (1:4). The solution was shaken and incubated for 24 hours at 60°C. The biosynthesis reaction of the AgNPs was identified by the color change that occurred when the *Myrmecodia* sp. ethanolic bulb extract was added to the $AgNO_3$ solution.

Characterization of AgNPs

UV-visible spectroscopy is a method that is widely used to determine the structure and characteristics of AgNPs. During the present study, the formation of AgNPs was analyzed under the guidance of a UV-visible spectroscope. The reaction mixture was exposed to wavelengths that ranged from 300 nm to 700 nm, and the peaks were analyzed to determine the wavelengths at which the AgNPs were synthesized. Fourier-transform infrared (FTIR) spectroscopy is critical for studying the surface absorbents found on nanoparticles. Because of the large surface area of AgNPs, adsorbents can influence the production of new characteristics and therefore extra peaks, compared to naked nanoparticles. An FTIR spectrophotometer was used to examine the powdered AgNPs sample (Perklin Elmer Spectrum 100). An x-ray diffractor analyzer was used to examine the transparent structure of the AgNPs. This characterization provided a stable determination of the size, shape, and crystalline structure of the AgNPs. Additionally, to determine the crystalline nature of the AgNPs, a powdered sample was subjected to x-ray diffraction (XRD) using an x-ray diffractometer Bruker AXS D8 Advance diffractometer (x-rays of wavelength $\lambda = 1.54056$ Å, 40 kV and 35 mA). The size range of the AGNPs obtained was determined using Scherrer's equation, and the width was determined using Bragg reflection. Furthermore, scanning electron microscope (SEM) analysis of the AgNPs was performed using an Evo MA 10 Carl Zeiss Scanning Electron Microscope, while transmission electron microscopy (TEM) (model JEM-1400) was conducted to structurally characterize the AgNPs.

In vitro antibacterial activity of the AgNPs

The antibacterial activity of the AgNPs against the fish pathogenic bacteria *A. hydrophila* was determined using an in vitro assay. The *A. hydrophila* were cultured in brain heart infusion broth, while the in vitro assay was conducted using a paper disk diffusion method. The paper disk was soaked with each treatment, namely 10%, 20%, 30%, and 40%, of ethanolic *Myrmecodia* sp. bulb extract or AgNPs. As a negative and positive control, sterile water and 0.1% Chloramphenicol antibiotic were employed, respectively. The disk paper was placed on nutrient agar and incubated for 24 hours at 37°C. The resultant clean zone was measured using a caliper by gauging the vertical and horizontal diameters. The average diameter and inhibition index were calculated according to the method and equation described by Valgas et al. (2007) and Naseer et al. (2020):

$$\text{Inhibition index} = \frac{d1+d2}{2} - dc$$

Where d1: horizontal diameter (mm); d2: vertical diameter (mm); dc: paper disc diameter (5 mm)

In vivo antibacterial assay of AgNPs

In total, 480 fish (initial weight 42 g; total length 19 cm) were used and randomly distributed into six groups of four replicates (20 fish per plastic box). The six groups were named as follows: C = Control group, P1 = fish injected with *A. hydrophila* and *Myrmecodia* sp. bulb extract, P2 = fish injected with *A. hydrophila*, P3 = fish injected with *A. hydrophila* and AgNPs, P4 = fish injected with *A. hydrophila* and Gentamicin. The density of *A. hydrophila* was 10^8 CFU mL^{-1} (volume per injection was 0.2 mL). A total of 0.2 mL each of *Myrmecodia* sp. ethanolic bulb extract, AgNPs, and 0.1% Gentamicin was injected intraperitoneally into the fish. At 0 hours, 24 hours, and 48 hours post-injection, all the fish were tested for erythrocytes, leukocytes, hemoglobin (Hb), platelets (PLT), and the quantity and percentage of lymphocytes, monocytes, and granulocytes. The survival rate was assessed every 24 hours after the injection time up to 96 hours. During the in vivo antibacterial assay, water quality parameters such as temperature, pH, and dissolved oxygen (DO) were monitored.

Data analysis

The data on the biosynthesis and characterization of nanoparticles are provided descriptively, whereas the inhibition index, erythrocytes, leukocytes, Hb, and PLT are presented as the mean standard error and were evaluated using the t-test for a comparison of two groups and ANOVA for a comparison of more than two groups. The lymphocytes, neutrophils, and survival rate are presented

in graph form. The statistical data were processed using SPSS 26 software. The Kruskal–Wallis test was performed, followed by the Mann–Whitney test to identify significant differences. The significance was set at $P < .05$.

Results and discussion

Biosynthesis of AgNPs

Myrmecodia sp. is a well-known medicinal plant, and its bulb is frequently used as a herbal medication (Moatar et al. 2020; Widyawati et al. 2020). The bulb of *Myrmecodia* sp. is used to treat wounds, uric acid disorder, stroke, and diarrhea (Sanjaya et al. 2014). The bulb of *Myrmecodia* sp. has also shown potentially for use as a reducing agent for the biosynthesis of nanoparticles due to active compounds. The findings of the present study revealed that the addition of *Myrmecodia* sp. ethanolic bulb extract to $AgNO_3$ resulted in a change in the color of the solution, from light yellow to reddish yellowish-brown (Figure 1). This indicated that Ag+ were being decreased and AgNPs were being biosynthesized. Previous research verified that the reduction of Ag+ to AgNPs using *Psidium guajava* leaf extract was accompanied by a visible shift in the color of the reaction mixture from colorless to yellow, then to reddish-brown (Bose and Chatterjee 2016). The UV–VIS spectra confirmed the color development of the AgNPs (Figure 2). According to Zuas, Hamim, and Sampora (2014), flavonoids found in *Myrmecodia* sp. bulb extract are responsible for decreasing Ag^+ and capping AgNPs. Several flavonoids present in the bulb extract were discovered to function as reducing and/or capping agents during the production of AgNPs in the study.

Furthermore, the mechanism by which the flavonoids decrease Ag^+ and cap AgNPs is unknown. Makarov et al. (2014) highlighted that several types of -OH groups present in flavonoids play an important role in reducing the Ag^+ to silver metal and capping the metal via chelation due to the proximity of carbonyl and hydroxyl groups as well as the

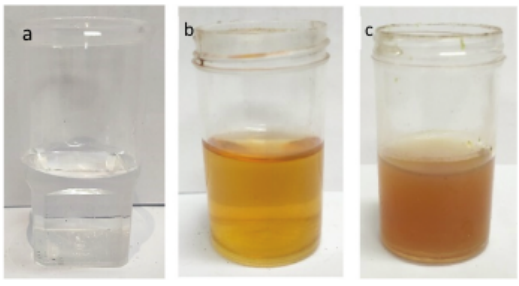

Figure 1. Biosynthesis of AgNPs using *Myrmecodia* sp. ethanolic bulb extract. A: $AgNO_3$ solution; B: initial mixture reaction; C: reaction mixture after 1 hour.

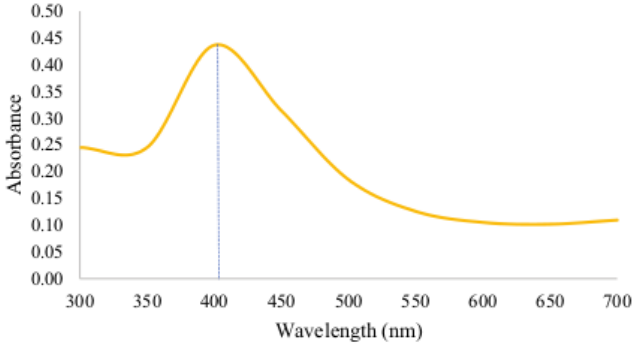

Figure 2. UV-VIS spectroscopy of the AgNPs biosynthesized using the ethanolic bulb extract of *Myrmecodia* sp.

catechol moiety of the flavonoids. It has also been suggested by Ahmed et al. (2016) that by tautomerically converting flavonoids from enol to keto form, a reactive hydrogen atom that reduces metal ions can be released to create nanoparticles.

characterization of biosynthesized agnps

The UV-VIS spectroscopy and XRD analysis supported the biosynthesis of AgNPs using *Myrmecodia* sp. ethanolic bulb extract. Figure 2 shows the UV-VIS spectrum recorded from a UV-VIS spectrometer reaction medium. The silver plasm resonance range occurred at 400 nm. The findings of the present study are similar to those of previous studies and revealed that the UV spectrum of the AgNPs has a broad band form in the visible area of 350 nm to 550 nm. This area of the wavelength is a typical AgNPs surface plasmon resonance absorption band (Zuas, Hamim, and Sampora 2014).

To confirm the crystalline existence of the particles, XRD was performed. The XRD pattern revealed several Braggs reflections that could be indexed based on the face-centered cubic structure of AgNPs. The XRD pattern of the AgNPs biosynthesized from ethanolic extract of *Myrmecodia* sp. bulbs is shown in Figure 3. The XRD spectrum indicated that the nanocrystals, as shown by the peaks at 20 values, were 27.77, 29.32, 32.18, 46.22, 47.93, 54.77, 57.48, 76.75, which corresponded to (111), (111), (200), (220), (311), (222), (400), and (420) sets of planes and may be indexed as the band for face-centered cubic structures of silver. The XRD data showed that the AgNPs produced through the reduction of Ag^{+} by *Myrmecodia* sp. ethanolic bulb extract were crystalline in nature. The average size of the AgNPs was predicted using the Debye-Scherrer equation. The average crystal size of the AgNPs was estimated to be 43.59 nm, which demonstrated that the AgNPs synthesized using the biosynthesis method were nanocrystalline in nature.

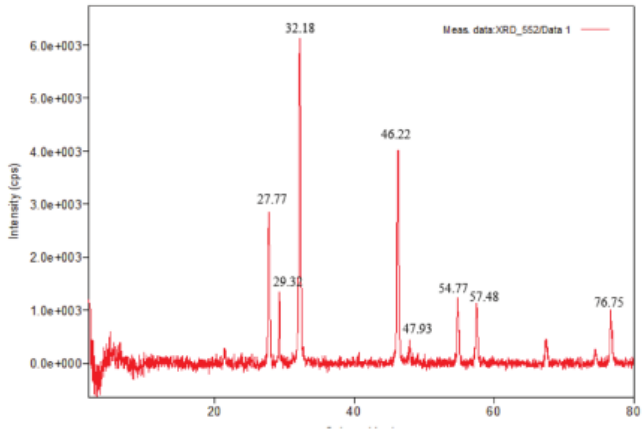

Figure 3. XRD pattern of the AgNPs biosynthesized using the ethanolic extract of *Myrmecodia* sp. bulbs. The Ag peaks are marked with the values provided.

Furthermore, FTIR was used to evaluate the potential biomolecule responsible for the reduction of Ag^{+} and the capping of the bioreduced silver nanoparticles that were biosynthesized using *Myrmecodia* sp. ethanolic bulb extract. Peaks in the FTIR range were observed at 3873 cm^{-1}, 2924 cm^{-1}, 2167 cm^{-1}, 1612 cm^{-1}, 1527 cm^{-1}, 1442 cm^{-1}, 1288 cm^{-1}, 1211 cm^{-1}, and 817 cm^{-1}. The curve of the AgNPs biosynthesized using the *Myrmecodia* sp. ethanolic bulb extract (Figure 4) revealed a broad band at 3410 cm^{-1}, which corresponded to O-H stretching of high concentrations of alcohols or phenols; multiple broad band at 2924 cm^{-1}, which corresponded to the C-H stretching

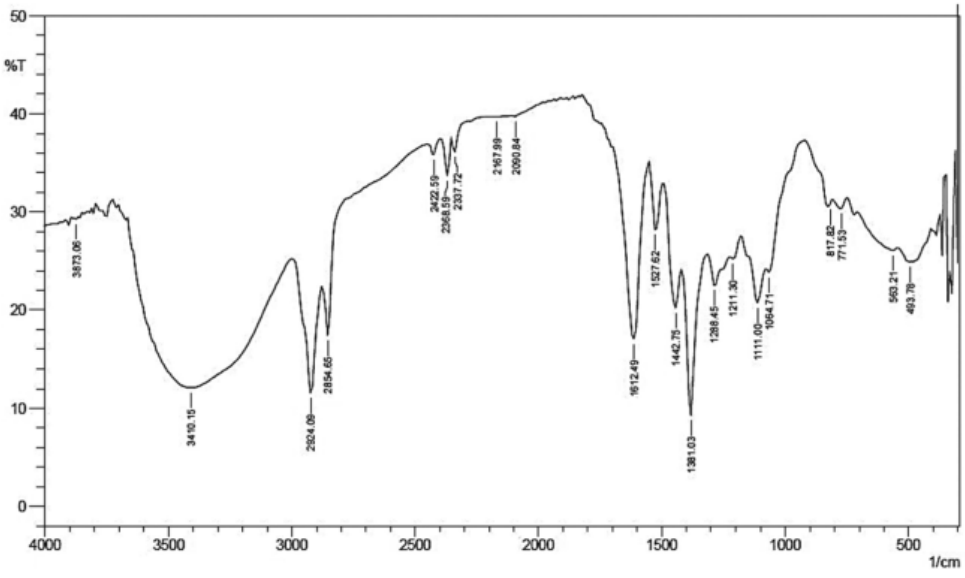

Figure 4. FTIR spectral of the synthesis of AgNPs using an ethanolic extract of *Myrmecodia* sp. bulbs.

Table 1. The FTIR of AgNPs biosynthesized using Myrmecodia sp. ethanolic bulb extract.

Absorption peak (cm^{-1})	Bond	Functional group	Intensity
493.70	Fingerprint		-
563.21			
771.53	C-H	Alkane	Strong
817.82			
1064.71	C-O	Alcohol, Ethers, Carboxylic acid, Ester	Strong
1111			
1211.3			
1288.45	C-N	Amines, Amide	Strong
1381.03	C-H	Alkane	Strong
1442.75			
1527.62	C=C	Aromatic compound	Weak to medium
1612.49	C=C	Alkene	Weak to medium
2090.84	Triple bonds		-
2167.99	C≡C	Alkyne	Weak to medium
2337.72	Triple bonds		-
2368.59			
2422.59			
2854.65	C-H	Alkane	Strong
2924.09			
3410.15	O-H	Alcohol Hydrogen bond, Phenol	Weak to medium
	N-H	Amina, Amide	Medium
3873.06	Single bond stretch		-

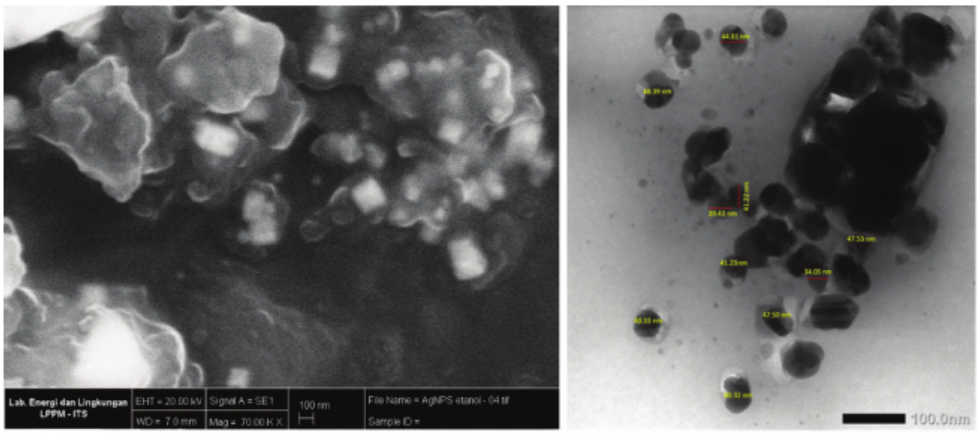

Figure 5. (a) SEM and (b) TEM analysis of AgNPs biosynthesized using an ethanolic extract of *Myrmecodia* sp. bulbs.

of alkane; a medium band at 1288 cm^{-1}, which corresponded to the stretching of C–N, and amide; a weak to strong band at 1527 cm^{-1}, which corresponded to aromatic C = C C-C stretching; and a medium band at 1211 cm^{-1}, which corresponded to C-O stretching in any carboxylic acid (Table 1).

SEM images were used to examine the morphology of the AgNPs (Figure 5a). Most of the particles had a cubic form and were less than 100 nm in size. The SEM images also displayed the biomolecule coating of the biosynthesized AgNPs. This layer confirmed the function of the *Myrmecodia* sp. ethanolic bulb extract metabolites in the synthesis and stabilization of the biosynthesized

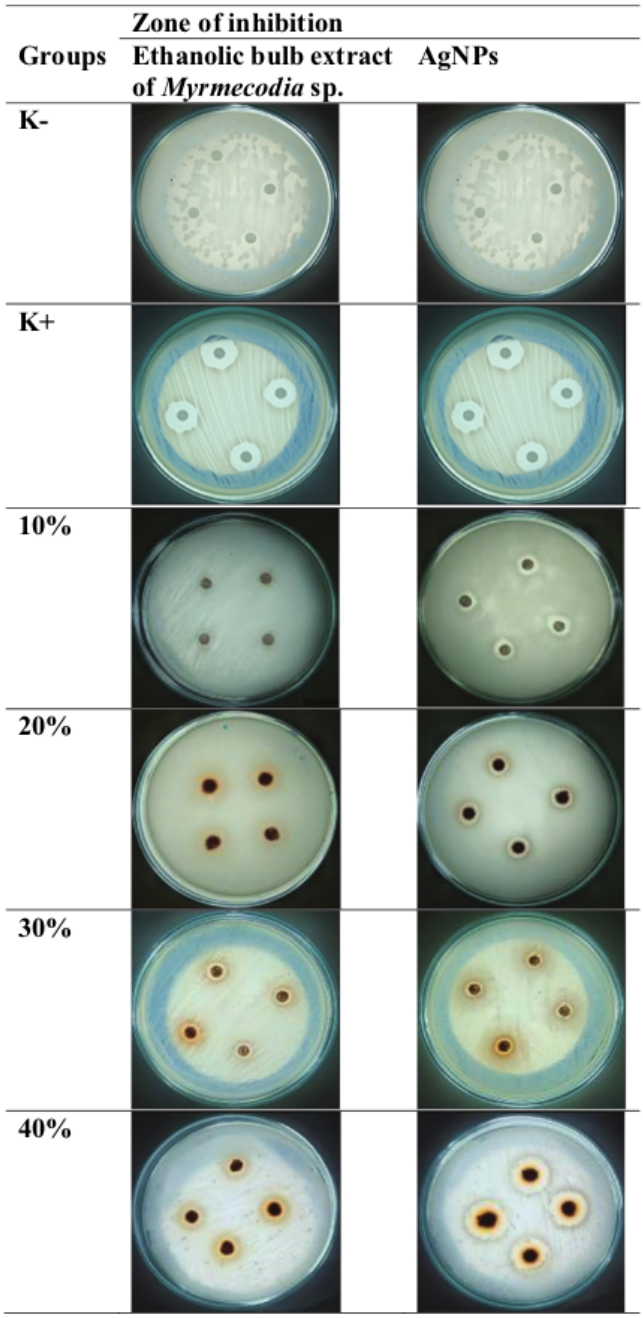

Figure 6. Zone of inhibition results of the antibacterial activity test of the biosynthesized AgNPs against *Aeromonas hydrophila*. Note: Control (K-) = Sterile water, Control (K+) = Chloramphenicol 0.1% antibiotic.

AgNPs. These findings are consistent with those obtained in a previous study performed by (Kanagasubbulakshmi and Kadirvelu 2017) which revealed that biosynthesized Fe_3O_4 nanoparticles were stable, had a cubic shape, and ranged from 30 nm to 100 nm in size. Furthermore, TEM micrographs were used to

document the size and shape of the biosynthesized AgNPs (Figure 5b). The results revealed that the AgNPs particles were cubic shape and ranged in size from 34.05 nm to 48.39 nm. The results of biosynthesized AgNPs in this present study were similar in size to those previously reported by Sur et al. (2018), Reetha (*Sapindus mukorossi*) and Shikakai (*Acacia concinna*), 20–40 nm; Sana and Dogiparthi (2018), leaf extract of *Micrargeria wightii*, 30 to 70 nm; and (Agarwal, Kumar, and Rajeshkumar 2021), leaves of lemongrass (*Cymbopogon* sp), size range of 50 nm.

In vitro antibacterial activity of AgNPs

The antibacterial activity of the biosynthesized AgNPs against *A. hydrophila* was confirmed via the formation of a circular inhibition zone around a well impregnated with AgNPs. The antimicrobial effect was strongest at a concentration of 40% AgNPs (7.7 ± 0.45 mm) (Figure 6). When treated with 0.1% Chloramphenicol antibiotic as a positive control, the inhibition index was 10.11 ± 0.23 mm. Additionally, an inhibition index of 2.2 ± 0.6 mm was observed for the *Myrmecodia* sp. ethanolic bulb extract (Table 2). These findings are comparable to those obtained in previous studies on the antibacterial action of AgNPs on fish diseases and other microorganisms (El-Adawy et al. 2021; Gupta et al. 2014; Kumar et al. 2019).

Several antibacterial action pathways for AgNPs have been postulated. Evidence suggests that AgNPs can interfere with sulfur- and phosphorus-containing proteins or enzymes, as well as the phosphorous moiety of DNA in the bacterial system, resulting in the suppression of an organism's enzyme system (Sulaiman et al. 2013). Le et al. (2012) revealed that AgNPs bind to the cell surface of bacteria, enter the cell, dissolve the cytoplasm, and kill the bacteria. Furthermore, AgNPs increase cell permeability and interfere with normal transport across the plasma membrane. As a result, the control of transport across the membrane is disrupted, resulting in bacterial cell death.

Table 2. Inhibition index (mm) comparison between the ethanolic extract of *Myrmecodia* sp. and AgNPs against *A. hydrophila*.

	Inhibition index (mm)	
Groups	Ethanolic bulb extract of *Myrmecodia* sp.	AgNPs
K-	0	0
K+	$_{1}10.11 \pm 0.23^{a}$	$_{1}10.11 \pm 0.23^{a}$
10%	$_{1}1.03 \pm 0.48^{b}$	$_{1}0.95 \pm 0.16^{b}$
20%	$_{1}2.02 \pm 0.27^{c}$	$_{2}4.02 \pm 0.7^{c}$
30%	$_{1}2.97 \pm 0.1^{c}$	$_{2}3.6 \pm 0.93^{cd}$
40%	$_{1}2.2 \pm 0.6b^{c}$	$_{2}7.7 \pm 0.45^{e}$

Control (K-) = Sterile water, Control (K+) = Gentamicin antibiotic. The Mean ± Standard error followed by various superscripts (a,b,c,d,e) in the same column indicates a significant difference among the groups at $P < 0.05$ (ANOVA). The numerical subscripts (1,2) before the Mean ± Standard error in the same row indicate a significant difference at $P < 0.05$ (t-test).

Furthermore, the antibacterial activity of AgNPs within bacterial cells will result in a close relationship with the bacterial biological components. When nanoparticles penetrate a cell, they disrupt the bacterial growth signaling system by altering the tyrosine phosphorylation of putative peptide substrates that are essential for cell survival and division. Moreover, nanoparticles have the potential to cause DNA to lose its ability to replicate, potentially leading to cell death. The higher the number of negative charges on gram-negative bacteria, the stronger the contact between such nanoparticles and the bacterial cell wall, which is consistent with the finding that gram-negative bacteria were more deeply impacted by AgNPs than gram-positive bacteria (Durán et al. 2016; Hwang et al. 2012; Li et al. 2019).

In vivo antibacterial activity of AgNPs

In the present study, the potential for AgNPs as a common antimicrobial substance against *A. hydrophila* was analyzed through an in vivo assay using infected *C. gariepinus*. Fish can potentially be infected with *A. hydrophila* can have a strong antibiotic resistance (Chandrarathna et al. 2018). *Aeromonas hydrophila* is known to be a ubiquitous and opportunistic fish pathogen (Yu et al. 2004) that causes MAS, a common disease in fish (Zhang et al. 2016). Fish infected with *A. hydrophila* have shown significantly higher blood profiles such as erythrocyte, hemoglobin, and hematocrit levels (Harikrishnan and Balasundaram 2008). Erythrocytes, hemoglobin, leukocytes, and lymphocytes have been recognized as critical indicators of blood conditions in fish regarding innate immune system defense and function (Ballarin et al. 2004).

The results of the present study demonstrated that during a 96 h in vivo assay, *C. gariepinus* fish infected with *A. hydrophila* showed significantly reduced erythrocytes, hemoglobin, and neutrophils, and increased leukocytes, PLT, and lymphocytes (Table 3 and Figure 7). The lowest erythrocyte ($20.89 \pm 0.03 \times 10^6$ μL^{-1}) and hemoglobin (1.20 ± 0.10 g dL^{-1}) values were recorded in the P3 fish group at 96 hours post-injection (Table 1). This result is similar to that obtained in a previous study which revealed that fish infected with bacteria may experience a reduction in erythrocytes and hemoglobin. Moreover, platelet levels may increase significantly due to a wound at the injection location and bacterial infection.

The neutrophil levels of the infected fish without an AgNPs injection were also significantly reduced ($P < .05$) at 96 hours post-infection. The leukocyte, platelet, and lymphocyte levels in the P3 group of infected fish without the AgNPs injection showed a significant increase during the 96 h in vivo assay. This finding is similar to that obtained in a previous study which revealed that an injection of 1×10^6 CFU mL^{-1} of *Enterococcus* in Nile Tilapia resulted in a substantial improvement in the white blood cell and lymphocyte numbers

Table 3. Blood profiles of Clarias gariepinus during a 96-hour in vivo assay using AgNPs that were biosynthesized using Myrmecodia sp. bulb extract.

		Groups					
Parameters	Hours	K	P1	P2	P3	P4	P5
Erythrocytes	0	$_{1}1.11 \pm 0.01^{a}$	$_{1}1.10 \pm 0.03^{a}$	$_{1}1.10 \pm 0.04^{a}$	$_{1}1.87 \pm 0.02^{a}$	$_{1}1.11 \pm 0.10^{a}$	$_{1}1.06 \pm 0.05^{a}$
(10^{6} μL^{-1})	48	$_{1}1.06 \pm 0.04^{a}$	$_{1}1.02 \pm 0.03^{a}$	$_{1}1.97 \pm 0.01^{a}$	$_{1}1.02 \pm 0.08^{a}$	$_{1}1.02 \pm 0.04^{a}$	$_{1}1.08 \pm 0.03^{a}$
	96	$_{1}1.13 \pm 0.09^{a}$	$_{1}1.93 \pm 0.03^{a}$	$_{1}1.20 \pm 0.04^{a}$	$_{2}0.89 \pm 0.03^{b}$	$_{1}1.97 \pm 0.02^{a}$	$_{1}1.81 \pm 0.06^{a}$
Hemoglobin	0	$_{1}3.17 \pm 0.22^{a}$	$_{1}3.40 \pm 0.20^{a}$	$_{1}3.57 \pm 0.18^{a}$	$_{1}2.67 \pm 0.18^{a}$	$_{1}3.07 \pm 0.22^{a}$	$_{1}3.37 \pm 0.19^{a}$
(g dL^{-1})	48	$_{1}3.07 \pm 0.03^{a}$	$_{1}3.07 \pm 0.09^{a}$	$_{1}3.20 \pm 0.10^{a}$	$_{1}2.93 \pm 0.12^{a}$	$_{1}3.23 \pm 0.03^{a}$	$_{1}3.63 \pm 0.18^{a}$
	96	$_{1}3.70 \pm 0.26^{a}$	$_{1}3.07 \pm 0.09^{a}$	$_{1}3.17 \pm 0.19^{a}$	$_{2}1.20 \pm 0.10^{b}$	$_{1}3.23 \pm 0.09^{a}$	$_{2}3.33 \pm 0.27^{a}$
Leukocytes	0	$_{1}9.27 \pm 0.92^{a}$	$_{1}9.73 \pm 1.77^{a}$	$_{1}9.67 \pm 0.84^{a}$	$_{1}9.70 \pm 1.89^{a}$	$_{1}9.73 \pm 1.72^{a}$	$_{1}9.30 \pm 1.23^{a}$
(10^{3} μL^{-1})	48	$_{1}9.63 \pm 0.64^{a}$	$_{1}9.10 \pm 1.79^{a}$	$_{1}9.60 \pm 0.90^{a}$	$_{1}9.87 \pm 0.47^{a}$	$_{1}9.37 \pm 0.64^{a}$	$_{1}9.03 \pm 0.43^{a}$
	96	$_{1}9.63 \pm 2.24^{a}$	$_{1}9.23 \pm 0.72^{a}$	$_{1}9.03 \pm 1.63^{a}$	$_{2}11.73 \pm 0.09^{b}$	$_{1}9.87 \pm 0.72^{a}$	$_{1}9.40 \pm 1.52^{a}$
Platelets	0	$_{1}23.67 \pm 3.28^{a}$	$_{1}25.67 \pm 2.91^{a}$	$_{1}24.67 \pm 2.28^{a}$	$_{1}23.88 \pm 5.00^{a}$	$_{1}24.00 \pm 5.29^{a}$	$_{1}26.33 \pm 4.33^{a}$
(10^{3}/	48	$_{1}23.33 \pm 2.40^{a}$	$_{1}25.33 \pm 2.03^{a}$	$_{1}25.33 \pm 2.60^{a}$	$_{1}24.00 \pm 3.54^{a}$	$_{1}25.33 \pm 2.91^{a}$	$_{1}25.33 \pm 0.67^{a}$
μL^{-1})	96	$_{1}23.67 \pm 2.03^{a}$	$_{2}30.67 \pm 6.39^{b}$	$_{2}30.00 \pm 2.31^{b}$	$_{2}29.67 \pm 2.91^{b}$	$_{2}29.33 \pm 1.33^{b}$	$_{2}31.33 \pm 12.86^{b}$

The superscript letters (a, b, c) indicate significantly different means for the various treatments at $P < 0.05$. The subscript numbers (1, 2, 3) indicate significantly different means at different times at $P < 0.05$. K = Control (K), injected with placebo (P1), injected-fish with AgNPs (P2), infected fish with *A. hydrophila* (P3), Infected-fish with *A. hydrophila* and injected with AgNPs (P4), infected fish with *A. hydrophila* and injected with Gentamicin (P5). The density of *A. hydrophila* was 10^{8} CFU mL^{-1} (volume per injection was 0.2 mL). The 0.2 mL of *Myrmecodia* sp. ethanolic bulb extract, AgNPs, and Gentamicin was injected at a concentration of0.1%.

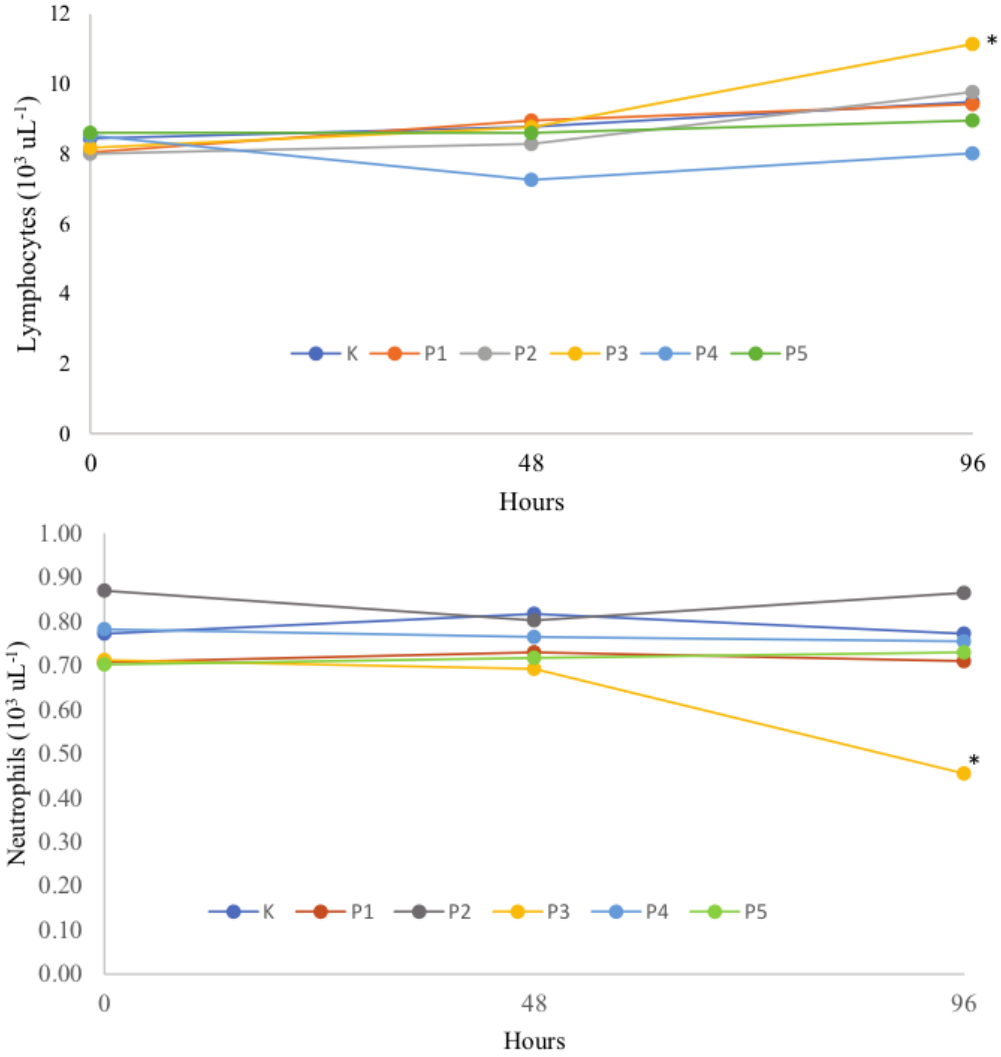

Figure 7. Percentage of lymphocytes and neutrophils of *C. gariepinus* fish infected with *A. hydrophila*. Note: (K) = control (K), injected with placebo (P1), injected-fish with AgNPs (P2), infected fish with *A. hydrophila* (P3), Infected-fish with *A. hydrophila* and injected with AgNPs (P4), infected fish with *A. hydrophila* and injected with Gentamicin (P5). The density of *A. hydrophila* was 10^8 CFU mL^{-1} (volume per injection was 0.2 mL). The 0.2 mL of *Myrmecodia* sp. ethanolic bulb extract, AgNPs, and Gentamicin was injected at a concentration of 0.1%.

compared to the non-injected control. An injection of 1×10^6 CFU mL^{-1} of *Enterococcus* also showed a substantial increase in neutrophils and a decrease in monocytes in saline-injected Nile Tilapia (Martins et al. 2008). In contrast, groups of fish infected with *A. hydrophila* and injected with AgNPs showed stable blood parameters during an in vivo assay.

The present research also indicated that the application of an AgNPs injection in infected fish, which contained an active phytochemical ingredient, could maintain the blood status of the fish, even in the challenge test with *A. hydrophila*. Similarly, no change in the value of the blood parameters of *A. hydrophila*-infected *Betta* sp. immersed with a *Terminalia catappa* leave extract was reported by (Nugroho et al. 2017). Plant-based biosynthesized nanoparticles are mostly nontoxic, environmentally friendly (Borase et al. 2014), suitable for humans (Gomathi et al. 2020), and inexpensive (Gan et al. 2012) compared to antibiotic or synthetic compounds. Several bioactive ingredients in the AgNPs biosynthesized using ethanolic bulb extract, namely, saponins, triterpenoids, quinones, phenolics, tannins, and flavonoids may stimulate the immune system of fish, particularly in infection conditions. The bioactive compounds identified in the AgNPs may boost the innate immune system or survival and have the potential to be widely employed in fish culturing. However, the mechanism underlying how the AgNPs boost the immune system and survival is unclear.

Lyu and Park (2005) stated that biocompounds, such as flavonoids, from plant extracts, may enhance cellular immunity by increasing Th1 cytokines, namely Interleukin 2 and Interferon gamma. Besides being an antioxidant that may preserve the heme iron in its ferrous state and improve erythropoiesis (Shatoor 2011; Uboh, Okon, and Ekong 2010), flavonoids, as a biocatalyst may be involved in the production of leukocytes, the promotion of leukocytes as nonspecific cellular immunity, and the reduction of erythrocyte hemolysis by triggering erythropoiesis (Nugroho et al. 2016). Thus, on the biological membranes of erythrocytes, they provide protection from free radicals which cause oxidative damage and enhance the antioxidant activity (Kitagawa, Fujisawa, and Sakurai 1992; Varghese et al. 2010). Additionally, saponins may be involved in the production of interleukin and interferon, resulting in improved macrophage and T-cell activities in fish (Helal and Melzig 2011; Xiong et al. 2012).

Previous studies have largely focused on the survival of fish after treatment with plant extracts (Hardi et al. 2019, 2018a; Nugroho et al. 2019). However, few studies have focused on the effects of the injection of AgNPs in comparison with antibiotics in infected fish. The results of the present study demonstrated the survival rate of *C. gariepinus* fish infected with *A. hydrophila* and injected with AgNPs during a 96-h challenge test (Figure 8). Furthermore,

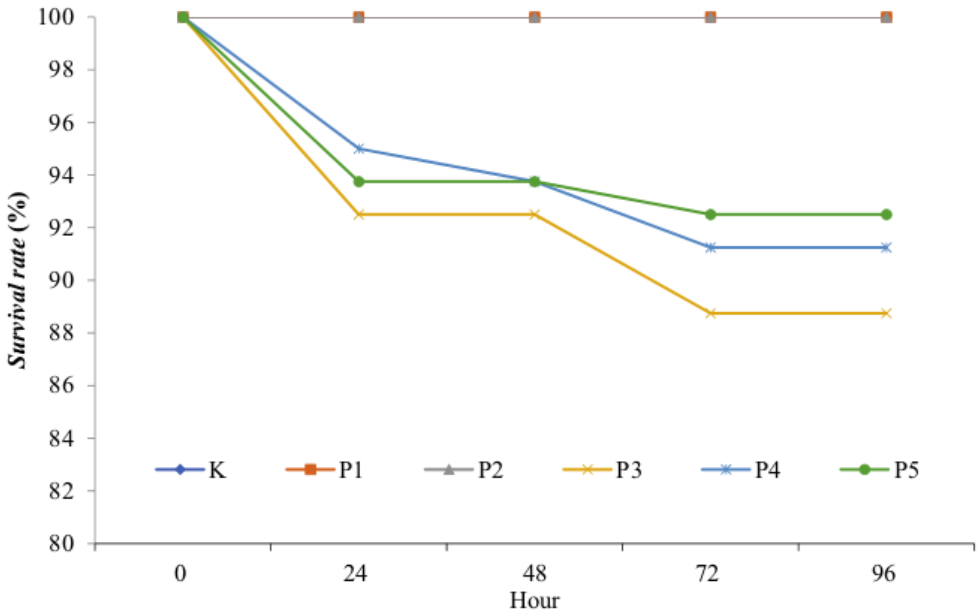

Figure 8. Survival rate of *C. gariepinus* fish during the AgNPs in vivo assay that were challenged with *A. hydrophila*. Note: Control (K), injected with placebo (P1), injected-fish with AgNPs (P2), infected fish with *A. hydrophila* (P3), Infected-fish with *A. hydrophila* and injected with AgNPs (P4), infected fish with *A. hydrophila* and injected with Gentamicin (P5). The density of *A. hydrophila* was 10^8 CFU mL^{-1} (volume per injection was 0.2 mL). The 0.2 mL of *Myrmecodia* sp. ethanolic bulb extract, AgNPs, and Gentamicin was injected at a concentration of 0.1%.

during a 96 h in vivo test, the water quality parameters such as DO, pH, and temperature were recorded at an average of 8.28 ± 2.30 mg L^{-1}, 7.08 ± 0.17, and 27.49 ± 0.49C, respectively, which were appropriate for the culturing of *C. gariepinus*.

Conclusion

The present study demonstrated that the biosynthesis of AgNPs using an ethanolic extract of *Myrmecodia* sp. bulbs effectively played a role as a lowering and balancing agent in the biological development of NPs. The resulting AgNPs can potentially be used as an antibacterial nanobiological agent in the field of aquaculture. The SEM, TEM, and XRD analyses showed that the AgNPs ranged in size from 34.05 to 48.39 nm. The FTIR analysis revealed that the AgNPs that were biogenerated contained several active biocompounds and demonstrated potent antimicrobial activity either in vitro or in vivo. Additional research is needed to assess the toxicological effects of AgNPs in fish, whether acute or chronic, and to investigate the toxicity mechanisms and histopathological, biochemical, and physiological impacts on various fish organs such as the

muscle, gonad, and liver. As the requirement for green synthetic pathways increases, the application of AgNPs as antibacterial agents in fish culturing has a promising future.

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Disclosure statement

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