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Original article

Synthesis of biogenic silver nanoparticles using butter fruit pulp extract and evaluation of their antibacterial activity against *Providencia vermicola* in Rohu

Ravi Mani [a,*], Parameswaran Vijayakumar [a], T. Stalin Dhas [a], Karthick Velu [a], D. Inbakandan [a], C. Thamaraiselvi [b,*], Babett Greff [c], Murugesan Chandrasekaran [d], Saeedah Musaed Almutairi [e], Faris S Alharbi [e], Dina S. Hussein [f], Maisari Utami [g,*]

[a] Centre for Ocean Research (DST-FIST Sponsored Centre) (MoES-ESTC Cell), Sathyabama Institute of Science and Technology, Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai 600119, India
[b] Department of Biotechnology, Mother Teresa Women's University, Kodaikanal, Tamil Nadu, India
[c] Department of Food Science, Faculty of Agricultural and Food Sciences, Széchenyi István University, 15-17 Lucsony Street, Mosonmagyaróvár 9200, Hungary
[d] Department of Food Science and Biotechnology, Sejong University, Gwangjin-gu, Seoul 05006, Republic of Korea
[e] Department of Botany and Microbiology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia
[f] Department of Chemistry, College of Sciences and Health, Cleveland State University, Cleveland 44115, USA
[g] Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Islam Indonesia, Yogyakarta, Indonesia

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ABSTRACT

Objective: Development of antimicrobial materials using nano approach and several industries like aquaculture greatly depend on novel biogenic materials. Biogenic techniques to develop nanomaterials with potent antimicrobial activity have been explored recently. The present study demonstrates the green synthesis of AgNPs using butter fruit (*Persea americana*) pulp extract and its antibacterial efficacy against the fish pathogen *Providencia vermicola* using Rohu fish.

Results: The AgNPs were prepared and characterized using various spectroscopic and microscopic techniques. The infrared spectroscopic analysis identified that the fruit biological molecules were involved in the stabilization of AgNPs. Transmission electron microscopic analysis revealed that particles size ranged from 20 to 50 nm. Further, the nanoparticles (5 μg) encapsulated fish feed were given to *P. vermicola* infected Rohu fish. The survival rate observed was 72 % in experimental group as compared to the control group. Total plate count and histopathological results indicated that the AgNPs treated groups showed significant reduction of bacterial population and restore the tissues in the normal range.

Conclusion: The results suggest that the green synthesized (AgNPs) using butter fruit pulp have good efficiency in reducing the infection caused by *P. vermicola* in Rohu fish.

1. Introduction

Nanoscale materials have been used in variety industry such as agriculture, aquaculture, food, pharmaceutical etc. because of their unique chemical and physical properties. Among various nanoscale materials, AgNPs nanoparticles have been used in a wide range of applications over the past few decades (Al-Dhabi et al., 2018, 2019; Gurusamy et al., 2019; Roopan et al., 2019; Smetana et al., 2008; Valsalam et al., 2019). Smetana et al. demonstrated the biocidal properties of highly reactive metal oxide nanoparticles against Gram positive and Gram negative bacteria (*Escherichia coli and Staphylococcus aureus*) (Smetana et al., 2008). Limited studies have been done on the role of biogenic nanoparticles in the aquaculture industry as a functional feed. Aquaculture industries being one of the fastest growing industries across the world producesing high-grade animal proteins and increasing income as well as employment (Subasinghe, 2003). Extensive study has been conducted on Indian Major Carps (IMC) that includes *Labeo rohita*, *Cirrhinus mrigala*, and *Catla catla*. The fish species cultured under farming con-

* Corresponding authors.
E-mail addresses: ravimicro2018@gmail.com (R. Mani), thamaraibiotechnology@gmail.com (C. Thamaraiselvi), maisariutami@uii.ac.id (M. Utami).

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dition were highly susceptible to various diseases, including bacterial, fungal, parasitic, and viral infections (Capkin et al., 2010; Giri et al., 2015; Kole et al., 2017). due to environmental stressors (Pieterse et al., 2005; Arasu et al., 2019; Al-Dhabi et al., 2019; Valsalam et al., 2019; Roopan et al., 2019; Gurusamy et al., 2019). The family Enterobacteriaceae contain wide range of pathogens such as *Providencia vermicola, Alcaligenes faecalis, Salmonella, Shigella* sp. and *Yersinia enterocolitica* (Bansemir et al., 2006; Bejerano et al., 1979; Castro et al., 2008; Ramkumar et al., 2014; Talpur, 2014; Tobback et al., 2007; Zhang et al., 2009). *Providencia* species are identified from varieties of animals including fish, sheep, cattle, dogs, flies, birds, cats, and guinea pigs. Meanwhile, *Providencia rettgeri* and *P. vermicola*s species were isolated from fish in India (Bejerano et al., 1979; Ramkumar et al., 2014). Although, control with antibiotics play key role of drug resistant pathogens, food safety problems, and environmental hazards fish (Austin and Austin, 1989). *P. vermicola* is an important pathogen of *L. rohita*, which cause a systemic infection leading to disease and death (Ramkumar et al., 2014). (Talpur, 2014). In Avocado (*P. americana*) commonly called as butter fruit belonging to family Lauraceae. *P. americana* are a commercially valuable fruit and are cultivated in tropical climates throughout the world. *P. americana* has numerous health benefits and contains a variety of vitamins, minerals, and phytochemicals such as lutein, phenolic antioxidants, and phytosterols (Alvizouri-Muñoz et al., 1992; Carranza et al., 1995; Carranza-Madrigal et al., 1997; Colquhoun et al., 1992; Grant, 1960; López Ledesma et al., 1996; Pieterse et al., 2005; Kumar et al., 2018). In continuation with the previous efforts, the present study focused on biogenic nanoscale AgNPs preparation using butter fruit pulp and to assess its antibacterial activity against fish pathogen *Providencia vermicola* in Rohu fish (*Labeo rohita*).

2. Materials and methods

2.1. Materials

Silver nitrate ($AgNO_3$) was purchased from HiMedia, Mumbai, India. Pathogenic strain of *P. vermicola* was obtained from Aquaculture Biotechnology Laboratory, Vellore Institute Technology, India which was isolated from natural diseased fishes. Further, the bacterial species were cultured in TSA medium and used for experimentation.

2.2. Preparation of pulp extract for GC–MS analysis

Fresh butter fruit was collected from local market, washed thoroughly, and stored at ambient temperature. Fruit pulp of *P. americana* was washed carefully in running tap water, homogenized, frozen and freeze-dried. Further, 50 mL of methanol and 4 mL of isopropanol were mixed with 50 g of the freeze-dried fruit pulp sample. The mixture was agitated for 30 min by using a vortex mixer and the supernatant were taken and centrifuged at 5000 rpm for 5 min. The extract was evaporated to dryness and re-dissolved in 5 mL of methanol and stored at 5 °C in a sealed bottle for GC–MS.

2.3. Synthesis of silver nanoparticles

Synthesis of AgNPs was done by preparing 5 mM of aqueous solution of silver nitrate ($AgNO_3$). 5 mL of extract of *P. americana* fruit pulp was added to 45 mL of 5 mM $AgNO_3$ solution for bioreduction process at room temperature in dark condition and allowed. Incubation for 24 h. Change in colour from yellow to brown markedly shows the reduction of AgNPs thus indicating the formation of AgNPs. Centrifugation is done using Remi CM-12 PLUS cooling micro centrifuge at 4,500 rpm for 20 min to purify the produced AgNPs. Supernatant was disposed and the obtained pellets was washed thoroughly with double distilled water to wash away the unreacted $AgNO_3$ and the extract. Then air-dried refined pellets were collected and analyzed by further characterization (Fig. 1).

2.4. Characterization

The formation of AgNPs was monitored using UV–vis absorption spectroscopy (UV-1800, Shimadzu, Japan), at wavelength of 300 to –800 nm. Fourier transform infrared spectroscopic (FTIR) analysis was carried out to identify the role of fruit biomolecules in the stabilization of AgNPs using IRAffinity-1s, Shimadzu, Japan. X-ray diffraction (Philip PW 1830) examination of *P. americana* with reduced AgNPs was carried out using films of the AgNPs solution coated on glass substrate at 40 kV and 30 mA with CuKα radiation. TEM, from FEI-Tecnai G2 20 Twin characterised at 200 kV. Chromatography interfaced to a mass spectrometer (GC–MS) was analysed by SHIMADZU QP 2010T equipment with the following conditions: capillary column – 624 ms (30 m × 0.32 mm × 1.8 m) operating in an electronic mode at 70 eV; with a constant flow of 1.491 mL/min helium (99.99 %) was utilized as carrier gas with injection volume of 1.0 mL keeping injector temperature at 140 °C, and ion source temperature of 200 °C. The oven temperature is set as 45 °C. FTIR (Shimadzu, 84000S) was analysed in the sequence of functional groups responsible for biogenic AgNPs.

2.5. In vitro antibacterial test

By the disc diffusion method, antibacterial activity was tested fruit pulp extract alone (Test 1) and fruit pulp extract mediated AgNPs (Test 2). *P. vermicola*, approximately 1×10^5 CFU/mL were used in the present experimentation.

2.6. Preparation of feed containing silver nanoparticles

Biogenic AgNPs encapsulated fish feed (5 μg/L per gm feed) was prepared following the protocol of Lotha et al., 2018a, 2018b. Briefly, the commercial fish feed was ground using mortar and pestle and then mixed with biogenic AgNPs. The mixture was made thinly in size of 1.5 mm pellets using 5 mL syringe. The prepared feed was dried at 35 °C for 45 min and stored at room temperature in a airtight container for further investigation.

2.7. Collection and maintenance of fish

Adult rohu fish were collected from fish farm, Walaja, Vellore district, Tamil Nadu, India and carefully transport to aquaculture research facility, Sathyabama Institute of Science and Technology, Chennai. Before the start of experiment, fishes were made to acclimatize for 20 days. The fish were kept in FRP tanks (500 L) and were daily fed twice with commercial pellets feed.

2.8. Inhibitory study of AgNPs on P. vermicola

Antibacterial efficiency of orally administrated AgNPs in rohu fish was determined by oral challenge in fish with *P. vermicola*. The experimental fish were separated into four groups, each group contain 10 fish.

Group I: Fish fed with feed containing Butter fruit extract
Group II: Fish fed with feed containing AgNPs (5 μg/gm)
Group III: commercial pellet feed (Negative control)
Group IV: Fish fed with Fish fed commercial pellet feed with 1×10^5 CFU of *P. vermicola* (Positive control)

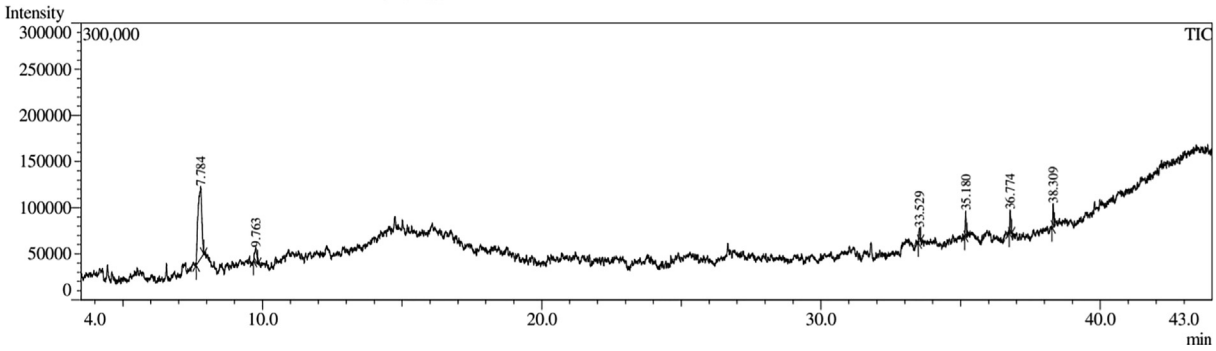

Fig. 1. GC-MS chromatogram of *Persea americana* pulp extract.

The experiments were conducted for 10 days at the end of the experiments, the muscle & gill tissues were dissected out and analysed for the disease infectivity.

2.9. Histopathological analysis

Tissues of muscle and gills were fixed with Bouin's solution. Tissues were then dehydrated with ethanol and embedded in paraffin wax. After deparaffinization using xylene solution for few hours, sections were made using microtome, stained using hematoxylin-eosin and observed using light microscope.

2.10. Statistical analysis

Data are given as mean ± Standard deviation (STD). A Statistical analysis was run by one-way ANOVA for antibacterial activity, $p < 0.05$ was taken to indicate statistical significance.

3. Results and discussion

Fig. 1 shows the gas chromatograms of the extract indicates the respective compounds with molecular weight. Compounds were determined by correlating their mass fragmentation patterns with similar compounds in WILEY library. The major active compounds present are 2,3,6-Trimethyl-7-Octen-3-OL (14.36), (R)-(−)-14-Met hyl-8-hexadecyn-1-ol, E-11-Methyl-12-tetradecenyl propanoate (2.16), Methyl 11-oxo-9-undecenote and 9-octadecenoic acid (z)-,2,3-dihydroxypropyl ester. The bioactive compounds present in the fruit were responsible for its ability to combat disease and its medicinal properties (Fig. 2).

Fig. 3a shows a sharp peak at 420 nm which indicate the formation of AgNPs. The peak represents the surface plasmon resonance of AgNPs which is characteristic function of these nanoparticles (Fatima et al., 2020; Iniyan et al., 2017; Lotha et al., 2018a,b). Furthermore, FTIR analysis was conducted to characterize the biomolecules involved in the capping and efficient stabilization of AgNPs (Fig. 4). The peak at 3500 cm^{-1} indicated the presence of –OH or –COOH groups. Likewise, the peak located at 2900 cm^{-1} showed that a $–CH_2$ group was involved in the formation of AgNPs. The peak at 1650 cm^{-1} indicated the role of Amide I group in the functionalization of AgNPs.

The XRD pattern of the biogenic AgNPs is shown in Fig. 3b. Multiple peaks were obtained at **2θ** values of 38.29°, 44.42°, 64.64°, and 77.60°, corresponding to (100), (200), (220), and (311) planes of the face centered cubic (fcc) structure of crystalline silver, respectively. The peak corresponding to the (100) plane was more intense than the other planes suggesting it as the predominant orientation (Bankar et al., 2010). Fig. 3c and Fig. 3d show the transmission electron micrographs of the synthesized AgNPs. The average sizes of the particles ranged from 20 nm to 50 nm and they seemed to be polydisperse in nature.

(Anjugam et al., 2018; Iniyan et al., 2017; Lotha et al., 2018a,b). For instance, AgNPs synthesized from red algae inhibited the growth of certain fish pathogens (*Vibrio harveyii, V. parahaemolyticus, V. alginolyticus*, and *V. anguillarum*) (Fatima et al., 2020). In the present study, the antibacterial activity of *P. americana* aqueous extract and biosynthesized AgNPs was evaluated against *P. vermicola* in a freshwater fish, *L. rohita.* According to Ramkumar et al. (2014) this bacteria can cause serious ulcerative lesions on the abdomen of rohu. Therefore, agar well diffusion test was carried out to determine the *in vitro* antibacterial activity of biologically synthesized AgNPs at different concentrations (5, 25, 50, and 100 µg). The clear zone around the well (zone of inhibition) indicated the presence of bioactive compounds. AgNPs at a concentration of 100 µg excerted strong antibacterial activity against *P. vermicola* (Table 1). The population of rohu has been mortality caused by several factors including the disease caused by bacterial pathogens. Among these, motile *P. vermicola* caused serious ulcerative lesion in the abdomen correlated with work of Ramkumar et al, 2014. The biologically synthesized nanomaterials such as

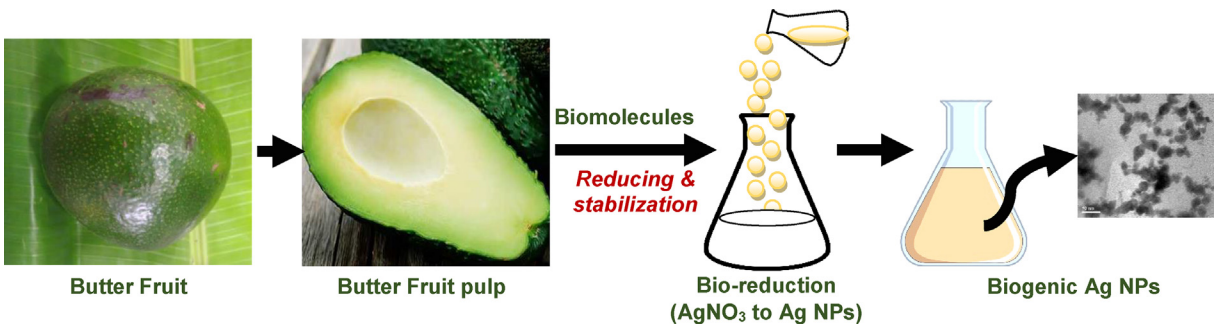

Fig. 2. Schematic diagram of the biosynthesis of silver nanoparticles using *Persea americana* pulp extract.

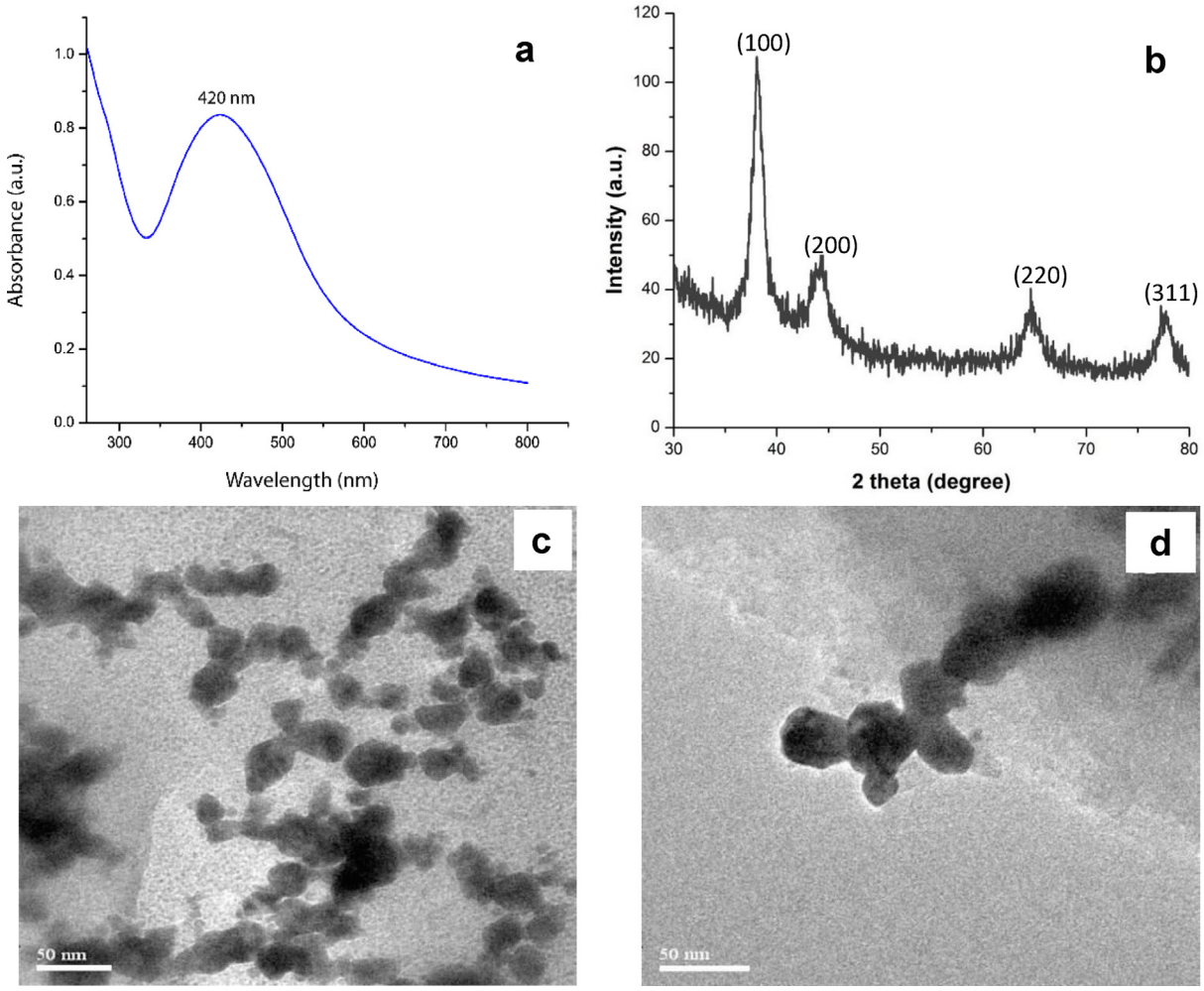

Fig. 3. (a) UV–Visible absorption spectra of AgNPs synthetized using the extract of *Persea Americana* fruit (b) XRD pattern of AgNPs synthetized using the extract of *Persea Americana* fruit (c and d) TEM micrograph of AgNPs synthetized using the extract of *Persea americana* fruit (scale bar 50 nm).

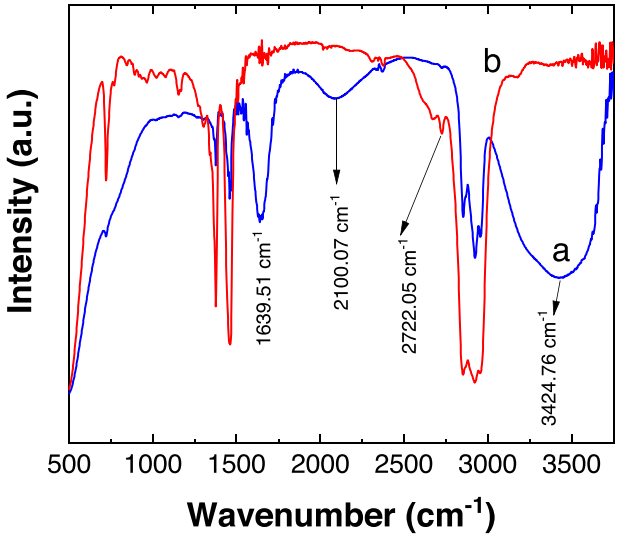

Fig. 4. FTIR spectrum (a) AgNPs synthetized using the extract of *Persea americana* fruit (b) Extract of *P. americana* fruit.

Table 1
The antibacterial activity of *P. americana* fruit extract and silver nanoparticles against *P. vermicola*, determined by disc diffusion method.

S.No.	Disc impregnated with	Inhibition Zone (mm) *P. vermicola A. caviae A. hydrophila*
1	AgNPs	24 ± 3.8 17 ± 2.6 18 ± 3.8
2	*P. americana* extract	< 1 < 1 < 1
3	Control	0 0 0

Values are means ± SD of three replicates.

Ppβ-GBP-AgNPs, silver chloride, plant nutraceuticals capped AgNPs, AgNPs capped with capsaicinoids were studied antibacterial and antibiofilm activity against Gram positive and Gram-negative bacteria (Iniyan et al., 2017; Lotha et al., 2018a, 2018b; Anjugam et al., 2018). Biosynthesis of AgNPs using red algae and it elicits superior antibacterial activity against fish pathogens (*Vibrio harveyii, Vibrio parahaemolyticus, Vibrio alginolyticus and Vibrio anguillarum*) (Fatima et al., 2020).

The results of total plate count carried out on randomly selected fish that survived showed that they were free of *P. vermicola* (Fig. 5). In the present study, the efficacy of AgNPs with *P. americana* against *P. vermicola* was also determined. Four test groups

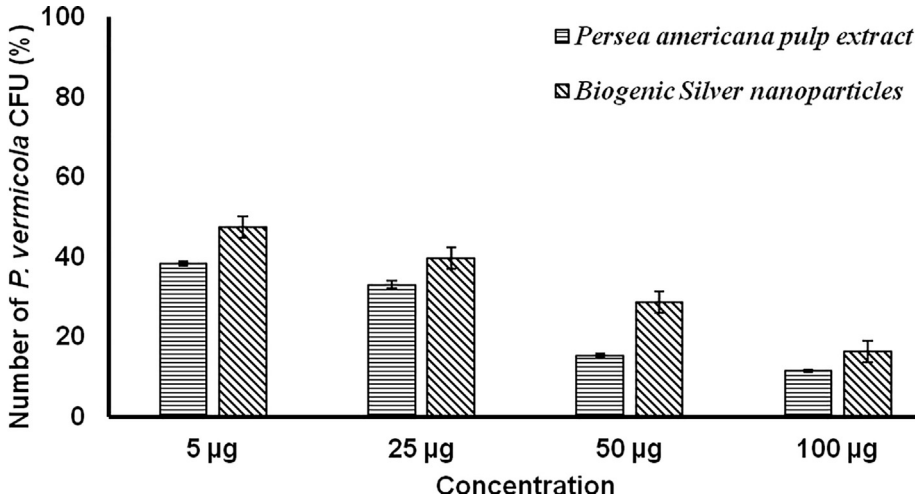

Fig. 5. The total viable counts of *Providencia vermicola* as a function of the concentration of silver nanoparticles in Luria–Bertani agar plates expressed as a percentage of the number of colony forming units (CFU) grown on silver-free control plates. Test I – Extract of *Persea americana* fruit; Test II – Silver nanoparticles.

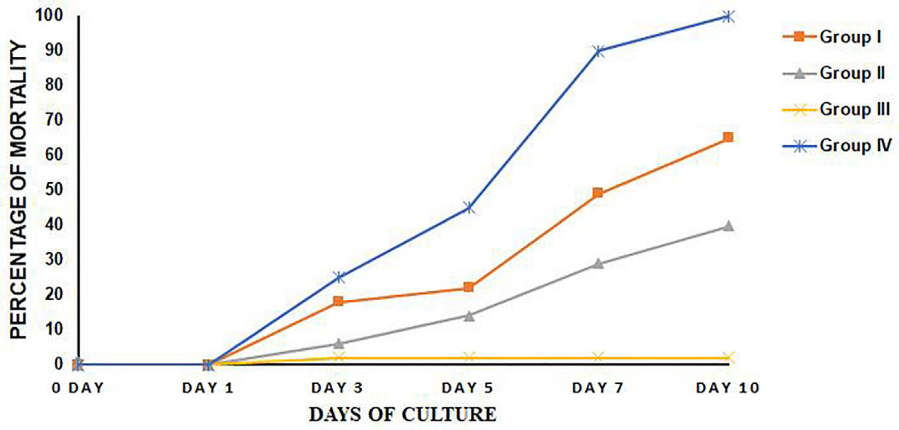

Fig. 6. Mortality (%) of the fish (*Labeo rohita*) in the experimental groups. Group I – Butter fruit; Group II – AgNPs; Group III – Negative control Group IV – Positive control (Infected with *P. vermicola*).

were prepared including the positive and negative controls. Each group of the fish were fed with different feeds and their survival rate was tested after 10 days (Fig. 6). In group I (fish fed with feed containing AgNPs) the mortality percentage was 34.74% at the end of experimental period (10 days of post infection;d.p.i). In group II, infected fish were administered with pellets coated with butter fruit extract. In this group, the mortality rate was 43% after 10 d. p.i. Group III (positive control) showed 100 % mortality at the end of the experiment due to the infection caused by *P. vermicola*. Commercially available feed was used in group IV (negative control) resulting in the highest survival rate (98%).

The biogenic AgNPs would be a lead for less toxic and eco-friendly standard green approach to develop AgNPs inhibiting pathogenic microbes in aquaculture environment. Biogenic AgNPs were highly stable during throughpot the feeding condition and have significant antibacterial effects of *P. vermicola* in fish. Green synthesis of AgNPs is more cost-effective and it's suitable for large-scale production of nanoparticles in control condition to their stability, shape and size (Arasu et al., 2019; Li et al., 2007; Song and Kim, 2009; Valsalam et al., 2019) Fig. 5.

Aeromonas hydrophila injected intraperitoneally into *Clarias batrachus* fingerlings was pathogenic and causing 93% mortality in fish infected with bacteria containing 10 colony forming unit/ml with peak mortalities occurring on days 14 and 15. At lower dosages however mortalities were significantly lower.

Histopathological examination of gill tissue of uninfected control and treated group fish revealed no observable changes in primary and secondary lamellae with pillar cells and histology of its cellular constituents whereas realterations like oedema, desquamation, necrosis, hypertrophy, and fusion of lamellae could be seen in gills infected with *P. vermicola* (Fig. 7a-f). In control fish and treated groups, there were no observable changes rather their primary lamellae and secondary lamellae, central venous sinus, chloride cells were seen. But in case of infected fish, rapid multiplication of bronchial chloride cells that resulted in fusion of lamellae and thereby forming Aneurism. The localization of aneurism occurs and bulging of blood vessel looks alike blood-filled balloon that increases the risk of rupturing, resulting in severe haemorrhage, other complications, or death. When experimented with freshwater fish Aphanius dispar exposed to deltamethrin similar results were observed, like vacuolization, uplift of the lamellar epithelium and blending of secondary lamellae (Al-Ghanbousi et al., 2012). The histological changes in the gills of fish were mostly associated with circulatory interruption and regressive and gradual changes (van Dyk et al., 2009). The treated *L. rohita* showed muscle fibre inflammations.

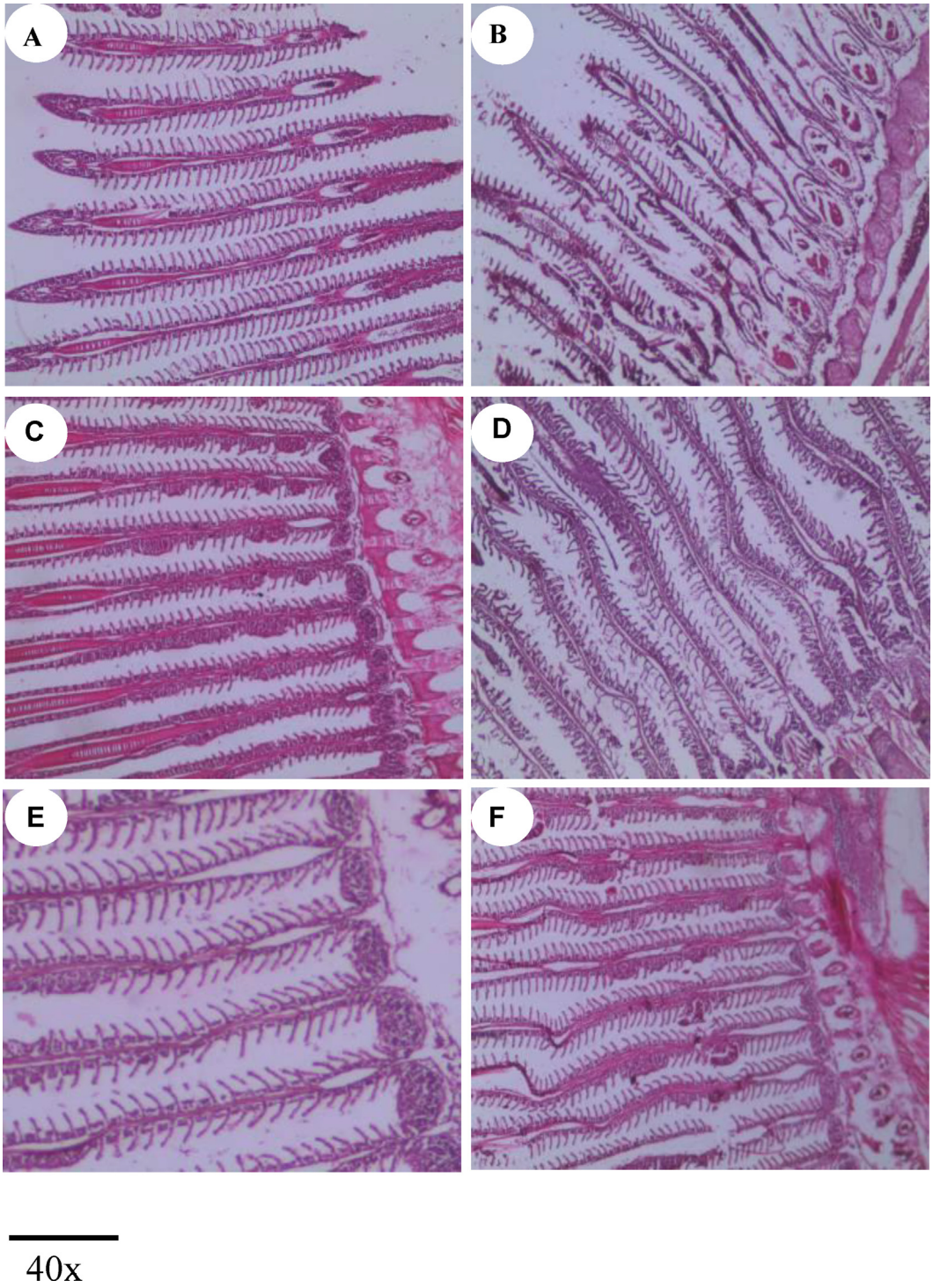

Fig. 7. Histological appearance of the gill tissue in control (A, C and E) and infected group, B – Intramuscular exposed; D – Oral and F – immersion gills after 48 h. A; Uninfected control fish gills tissues occurrence of healthy primary lamellae, secondary lamellae and normal cellular like chloride cells and pillar cells; (B, D and F) – Gill of exposed fish showing lamellae fusion, formation of aneurism and epithelial lifting.

4. Conclusion

The present study shows the efficient green way of synthesizing AgNPs using Avogadro fruit pulp and its antibacterial activity against *P. vermicola*. Well diffusion method was employed to demonstrate the efficacy of AgNPs shows in Table 1 found very effective. The experimentally infected rohu fish was treated with AgNPs encapsulated feed (5 mg/g) shows strong protection against *P. vermicola* based on the mortality, total bacterial count, and histopathology analysis (Capkin et al., 2010). Eco-friendly, convenient green synthesized method will be used for various applications, due to the pharmaceutical importance avocado fruit it provides additional benefits.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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