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Antiparasitic effect of biogenic iron nanoparticles against the fish ectoparasite *Argulus siamensis*: *In vitro* study

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Abstract

Argulus, an ectoparasite of fish which is ubiquitous in wild and culture ponds, poses a major challenge with severe economic losses to the global aquaculture industry. With the increase in intensification of aquaculture practices, there is a dire need to discover new therapeutic options in the treatment of argulosis owing to the limited effectiveness of existing drugs and chemicals and their significant side effects. The use of metal nanoparticles has shown promising results in the management of several parasitic infections. In this prelude, antiparasitic effect of biosynthesised iron nanoparticles was evaluated against *Argulus siamensis* under *in vitro* condition. Iron nanoparticles (FeNPs) were biosynthesised using fresh *Bauhinia racemosa* leaf extract as a reducing agent and were characterised using UV-VIS spectrophotometry, dynamic light scattering (DLS) technique, zeta potential measurements, transmission electron microscopy (TEM), scanning electron microscopy (SEM), and fourier transform-infrared spectroscopy (FT-IR). For estimating antiparasitic efficacy of FeNPs under *in vitro* test, ten adult and juvenile parasites each were challenged for 6 h separately in 20 ml of five different concentrations of FeNPs test solutions *viz*. 1.00, 1.25, 1.50, 1.75 and 2.00 mg ml^{-1} in triplicate along with control groups for adults whereas, for juveniles, it was 0.75, 1.00, 1.25, 1.50 and 1.75 mg ml^{-1}. Formation of FeNPs was measured in 370-400 nm UV range. DLS showed an average FeNPs particle size of 119.8 nm with a polydispersity index of 0.311. Zeta potential measurements showed negative surface charges (-11.3 mV) whereas, SEM and TEM micrographs revealed synthesised nanoparticles were nearly spherical and size ranged from 60-270 nm. Further, FT-IR spectrum showed the presence of Fe-O, N=O and O-H groups. Argulocidal effectiveness in both cases was found to be concentration-dependent. The highest argulocidal activity of FeNPs was observed at concentration of 1.75 mg ml^{-1} for juveniles and 2.00 mg ml^{-1} for adult argulids which led to 100 and 87% mortality, respectively, in 6 h, however no mortality was recorded in control group up to 16 h. Furthermore, the calculated 6 h-EC50 of biosynthesised FeNPs for juvenile and adult argulid parasites was determined as 0.97 and 1.27 mg ml^{-1}. Results of the present study showed that short term bath treatment with biosynthesised FeNPs is effective against argulid parasites. However, further research is required to evaluate its therapeutic potential under *in vivo* condition.

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Introduction

Globally, argulosis remain a formidable challenge to fish health, posing a serious threat to sustainable fish farming. *Argulus* spp. are obligate ectoparasites of fish and a large group of branchiuran crustaceans (Ananda Raja *et al.,* 2020; Ananda Raja, 2022). They can survive off host for many days which stands on the way to develop any effective therapy (Mikheev *et al.,* 2015; Ananda Raja *et al.,* 2022). They spread rapidly and feed upon mucus, epidermal tissue and blood from the host (Bandilla *et al.,* 2006; Walker *et al.,* 2011), causing ulceration and immunological suppression as well as increasing the risk of subsequent infections with bacteria and fungus (Saurabh *et al.,* 2011; Kar *et al.,* 2017; Ananda Raja *et al.,* 2020; Ananda Raja,

2022). In addition, this parasite may act as a carrier to transmit other fish pathogens to the host body like spring viremia of carp virus (SVCV), nematode larvae (dracunculoid and skrjabillanid), and the fungus *Saprolegnia* (Gresty *et al.,* 1993; Avenant-Oldewage, 2001; Ahne *et al.,* 2002; Hadfield and Smit, 2019).

In recent years, intensification of aquaculture practices with the development of new technologies has resulted in increased occurrence of argulosis. Interestingly, Sahoo *et al.* (2013) reported that argulus affects 48% of water bodies used for carp aquaculture, resulting in an annual loss of ₹300 crores in Indian carp culture, signifying the seriousness of the infestation. Further, between 2014 and 2018, a passive surveillance-based study conducted in five carp dominating Indian states highlighted that 19.51% of cases were attributable to argulosis among diverse parasitic outbreaks (Sahoo *et al.,* 2020). Therefore, it is now critical to focus on *argulosis*, which has a direct impact on the freshwater aquaculture industry.

Owing to huge monetary losses, several efforts were made to control argulosis in various aquaculture systems; nonetheless, the struggle against argulosis still remains as tolerance or resistance development to the existing parasiticides. Although several veterinary drugs and insecticides such as organophosphates, pyrethrin compounds, benzoyl phenyl ureas and avermectins are being extensively used to control fish lice, currently no FDA-approved drug is available to treat/manage Argulus parasites (Treves-Brown, 1999; Toovey and Lyndon, 2000; Piasecki and Avenant-Oldewage, 2008; Moller, 2011; Hemaprasanth *et al.*, 2012; Mayer *et al.,* 2013; Ananda Raja *et al.,* 2020; Ananda Raja, 2022). The ectoparasite has been shown to develop resistance to majority of the chemical treatments used, requiring multiple medication during a single culture operation (Hakalahti-Siren *et al.,* 2008). Alternatively, a variety of non-chemical control approaches have been used to control infestations by reducing *Argulus* populations in aquaculture ponds. These include the use of bamboo poles/mats to collect parasite egg clutches (Gault *et al.,* 2005), light traps for juvenile/adult collection (Kehayias and Tsounis, 2019), argulid parasite predating fish *Puntius gonionotus* (Ni *et al*., 2010) and phytotherapy (Prakash and Rao, 1996; Kumar *et al.,* 2012; Banerjee *et al.,* 2014; Kumari *et al.,* 2019; Pereira *et al.,* 2020). However, the lack of purity of crude extracts alters antiparasitic activities and stability. Further, the requirement of a large treatment dose and poor storage stability is the major bottleneck in the commercialisation of the plant-based products (Kumari *et al.,* 2021). Nowadays, development of vaccines against Argulus has received considerable interest (Kar *et al.,* 2017; Das *et al.,* 2018; Ambuali *et al.,* 2020) but it is in infant stage.

Nanomedicines are an emerging field of study in human and veterinary medicine and many nanoparticles have been extensively studied for their potential pharmacological effects. Recent literature reports highlight the green synthesis of metal nanoparticles using a variety of biological sources, including plants, seaweeds, algae, yeasts and bacteria (Iravani, 2011; Mahdavi *et al.,* 2013; Silva *et al.,* 2016; Mukherjee *et al.,* 2021). Use of plants for synthesis of nanoparticles has gained importance due to presence of variety of phyto-constituents, which are crucial for synthesis reaction and provides various functional groups for the stabilising, oxidising, capping and reducing metal oxide precursors (Wu *et al.,* 2015, Vasantharaj *et al.,* 2019).

Bauhinia racemosa Lam. belonging to the Fabaceae family, commonly known as bidi leaf tree is distributed throughout India (Prabhu *et al.,* 2021). The phyto-constituents of the *B. racemosa* leaves includes alakloids (Sashidhara *et al.,* 2012), flavonoids, phenols (Rahman *et al.,* 2016), terpenoids, propanoids, lipids, steroids (Gawade and Farooqui, 2018) and coumarin (El-Hossary *et al.,* 2000), extensively used in the traditional medicine. Therefore, *B. racemosa* is a promising alternative for reducing metal oxide precursors, enabling the formation, stabilisation and functionalisation of the iron nanoparticles. Metal nanoparticles (NPs) have shown high biocidal activity against various pathogens. Furthermore, metal nanoparticles have also been explored to cure parasitic infections of fish and several groups of parasites including microsporidians Heterosporis saurida (Saleh *et al.,* 2016), protozoan *Ichthyophthirius multifiliis* (Saleh *et al.*, 2017), crustacean copepode *Lernaea cyprinacea* (Abu-Elala *et al.,* 2018) and monogenean *Cichlidogyrus* spp. (Pimentel-Acosta *et al.,* 2019). Iron nanoparticles have also shown to have anti-microbial (Arakha *et al.,* 2015; Gudkov *et al.,* 2021; Kumar *et al.,* 2022), antihelmintic (Dorostkar *et al.,* 2017; Gonzalez-Moragas *et al.*, 2017) and acaricidal effects (Norouzi *et al.,* 2020). Hence, the aim of the present study was to evaluate the argulocidal effects of iron oxide nanoparticles.

Materials and methods

Chemicals, reagents and sample collection

Fresh leaves of *B. racemosa* were collected from the campus of ICAR-Central Institute of Fisheries Education (ICAR-CMFRI), Mumbai, Maharashtra, India. Ferric chloride hexahydrate (97.0%, $FeCl_3{\cdot}6H_2O$) and NaOH were purchased from SRL, India. All the working solutions were freshly prepared using Milli Q water.

Preparation of leaf extract and biosynthesis of Iron oxide nanoparticles

The aqueous extract of fresh *B. racemosa* leaves (1 g finely chopped leaves in 3 ml distilled water) was prepared on water bath at 60°C for 30 min followed by cooling at room temperature and filtration using Whatman™ grade1 qualitative filter paper (GE Healthcare, UK). The leaf extract was added drop-wise to the freshly prepared 0.1 M $FeCl_3{\cdot}6H_2O$ solution in a 1:2 ratio and continuously stirred for 1 h using magnetic stirrer to effectuate the synthesis of iron oxide nanoparticles. Then, 0.1 M NaOH solution was added to adjust the pH of solution to 6.0. Synthesis of nanoparticles was indicated by a colour change from an orange-brown solution to a black precipitate. After an overnight incubation, the solution was centrifuged for 20 min at 8000 rpm, followed by washing of pellet thrice with deionised water. The resulting pellets were dried in a hot air oven at 60ºC till complete drying to obtain powdered nanoparticles.

Characterisation of biogenic FeNPs

Preliminary characterisation of biosynthesised FeNPs was conducted after 24 h of incubation at room temperature using UV-VIS spectrophotometer (Motras Scientific Instrument Pvt. Ltd, India). The biosynthesised NPs was diluted with distilled water at 1:30 ratio and surface plasmon resonances was measured by scanning absorbance spectra between 300-700 nm wavelengths with deionised water as a blank. The particle diameter was

measured by a dynamic light scattering (DLS) analyser (SZ-100, HORIBA Scientific) using dispersed biosynthesised NPs in distilled water at 25°C. The zeta potential was also measured using the same equipment through electrophoretic mobility.

Morphological studies of biosynthesised iron oxide nanoparticles were carried out using FEG-SEM (JEOL, JSM 7600F) at Sophisticated Analytical Instrument Facility, IIT Bombay, India. Further the size and crystallinity of the NPs was carried out using HR-TEM 200 kV (JOEL, JEM 2100F). The lattice fringes and diffraction ring patterns (SAED) were examined in its high-resolution mode. The dried NPs powder was used for SEM studies. For TEM studies, NPs were suspended in distilled water and sonicated to obtain a well dispersed suspension; a drop of this was put on copper grid and kept in an oven for drying before transferring it to the microscope. To determine the particle size using TEM micrograph, a set of 6 images were selected to investigate the size distribution using ImageJ software (Schneider *et al.,* 2012). FTIR spectroscopy of biosynthesised FeNPs were carried out using HYPERION 3000 Microscope with Vertex 80 FTIR System, Bruker, Germany by ATR method in transmittance mode from 450-4000 cm^{-1} with spectral resolution at 0.2 cm^{-1}.

Identification of different life stages of Argulus parasite

The test parasite of fish, *Argulus* sp. was manually collected from *Labeo rohita* sampled in broodstock ponds of Instructional Fish Farm, College of Fisheries, Dholi, Muzaffarpur, Bihar, India and subjected to the laboratory identification as well as evaluation of EC_{50} of biosynthesised iron oxide nanoparticles (FeNPs) under *in vitro* condition. The adult parasites were morphologically identified as *Argulus siamensis* using characteristics described by Sahoo *et al.* (2012). Moreover, adult parasites were placed in a glass aquarium with the host fish to lay their eggs on the glass walls, after laying, eggs were scrapped and transferred to a 1 l beaker with aeration for hatching for 15 days of incubation. The juvenile argulids, which are identified by their underdeveloped sucker, were then used for subsequent tests.

Assessment of *in vitro* antiparasitic activity

Actively swimming parasites were considered for performing the *in vitro* assay to evaluate the antiparasitic efficacy of FeNPs. After initial range-finding test, ten adult parasites were subjected to 20 ml of five different concentrations of FeNPs test solutions *viz*. T_1 (1.00 mg ml^{-1}), T_2 (1.25 mg ml^{-1}), T_3 (1.50 mg ml^{-1}), T_4 (1.75 mg ml^{-1}) and T_5 (2.00 mg ml^{-1}) separately in triplicate for 6 h in definitive test. Similarly, *in vitro* test was performed for juveniles having FeNPs test solution *viz.* T_1 (0.75 mg ml^{-1}), T_2 (1.00 mg ml^{-1}), T_3 (1.25 mg ml^{-1}), T_4 (1.50 mg ml^{-1}) and T_5 (1.75 mg ml^{-1}). In both tests, positive (ICAR-CIFRI Argcure, Glaucus Agrochem Pvt. Ltd., Kolkata, which contains cypermethrin in nano-emulsion) and negative (pond water only) controls were maintained along with treatment groups. Number of parasites surviving in each plate were examined at 0.5, 1, 2, 4 and 6 h post-exposure with test solution. Parasites were considered dead when they failed to respond to a gentle touch or external stimulus and did not exhibit any motion when being transferred to deionised water.

Data analysis

The median effective concentration (EC_{50}) with confidence intervals 95% was determined by probit analysis (Finney, 1971) in SPSS 16.00. The EC_{50} value was calculated through the obtained regression equation (Y= Percentage Mortality; X= Log concentration).

Results and discussion

Preparation of FeNPs

The green approach was used to successfully synthesise FeNPs using *B. racemosa* leaf extract and scheme for biosynthesis is shown in Fig. 1. The instantaneous appearance of the orange-brown solution to a dark black colour served as proof for the formation of nanoparticles. The formation of black precipitates might be attributed to interaction of phyto-constituents with ferric ions, ensuring reduction of metal ion and formation of stabilised FeNP (Singh *et al.,* 2018; Lakshminarayanan *et al.,* 2021).

Characterisation of FeNPs

UV-VIS spectroscopy is an analytical technique involved in measuring the absorption of electromagnetic radiation (Mulvaney, 1996). Scanning of UV-VIS spectrum for biosynthesised NPs was carried at wavelengths from 300-700 nm and the absorption peaks were observed in the range of 370-400 nm due to the surface-excitation plasmon vibrations having λ_{max} at 386 nm (Fig. 2), which is in agreement to the expected values of FeNPs (Jagathesan and Rajiv, 2018; Bibi *et al.*, 2019). DLS technique is widely used to measure the hydrodynamic size of nanoparticles in a liquid phase and provide details on their propensity to aggregate (Frone *et al.,* 2011;

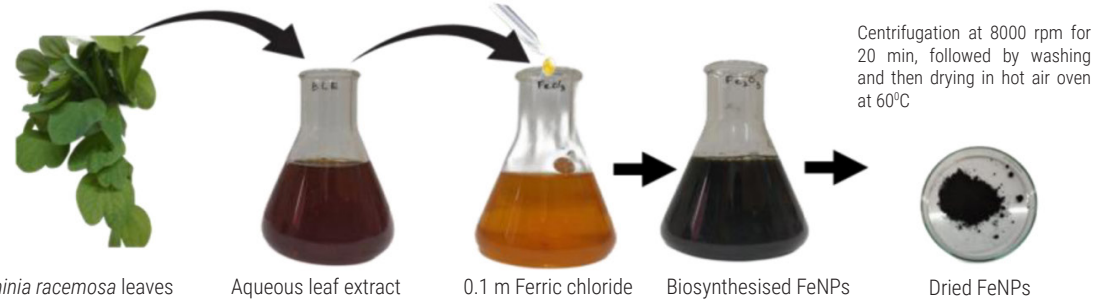

Fig. 1. Illustration of biosynthesis of iron oxide nanoparticles using *B. racemosa* leaf extract

Lim *et al.,* 2013). In addition, the polydispersity index (PDI) is used to provide information about the degree of non-uniformity in the size distribution of the particles (Nobbmann, 2014) and the higher PDI values corresponds to larger size distribution of sample particle (Clayton *et al.,* 2016). The mean particle diameter obtained in DLS is depicted in Fig. 3, the biosynthesised FeNPs were homogenously distributed and their hydrodynamic size (mean±SD) was 158.0±37.6 nm and the PDI was 0.311.

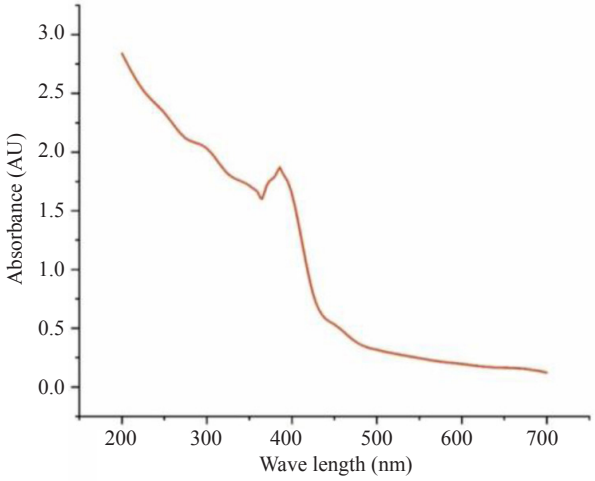

Fig. 2. UV–VIS spectra of biogenic FeNPs synthesised using *B. racemosa* leaf extract

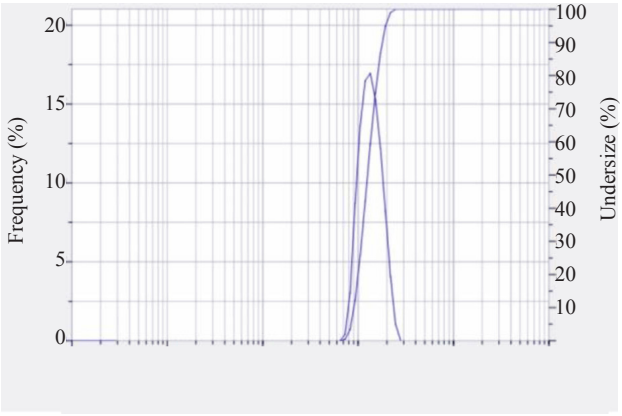

Fig. 3. Particle size distribution of biosynthesised FeNPs using dynamic light scattering (DLS) analyser

Zeta potential measurements are among the characteristics advised for experimental testing of NPs, providing information on the stability of NPs and a negative result indicate that there is less agglomeration of particles in the colloidal phase (Shi *et al*., 2012). The results showed (Fig. 4) that the surface charges of biosynthesised FeNPs were negative (-11.3 mV), which indicates that the NPs are moderately stable. The negative surface charges could be attributed to the polyphenolic compounds present in leaf extract coated on the surface of biosynthesised FeNPs (Harshiny *et al.,* 2015). The FEG-SEM and HR-TEM analyses showed particle size between 60-280 nm with an average of size±SD of 157.60±41.59 nm (Fig. 5a-d). Most NPs are spherical, except for some cubes. These images also reveal that the nanoparticles are agglomerated and consistent with the observations of Rahman *et al.* (2017). FTIR spectroscopy is attributed to the functional groups present in the biosynthesised NPs due to capping and stabilisation by phyto-constituents present in *B. racemosa* leaf extract (Lakshminarayanan *et al.,* 2021). The FT-IR spectrum shows well-defined peaks at 671.56 cm^{-1} extending to Fe-O stretches, 1636.59 cm^{-1} extending to N=O and 3460.98 cm^{-1} extending to O-H of Fe_2O_3 (Lassoued *et al.,* 2017). Moreover, the peaks at 2917.93 cm^{-1}, 2850.20.99 cm^{-1} and 1384 cm^{-1} indicate C-H stretching, C=C stretching and C-O stretching ensuring the presence of alkane, conjugated alkene and secondary alcohol in the plant extract correspondingly (Fig. 6). Therefore, the coating of iron oxide nanoparticles with *B. racemosa* leaf extract can be inferred from these data.

Assessment of *in vitro* parasiticidal activity

FeNPs are physically and chemically stable, biocompatible and environmentally safe (Velusamy *et al.,* 2016). Studies have shown the potential for magnetic nanoparticles to produce microbial toxicity due to a series of interactions such as membrane depolarisation with consequent impairment of cellular homeostasis (Pelgrift *et al.,* 2013), production of reactive oxygen species with lipid peroxidation, DNA damage (Pan *et al.,* 2010) and release of metal ions that affect cell integrity and protein coordination (Saleh *et al.,* 2015). FeNPs are reported to have potent antiparasitic efficacy against mammalian helminthic parasites, roundworm *Toxocara vitulorum* (Dorostkar *et al.,* 2017) and nematodes *Caenorhabditis elegans* (Gonzalez-Moragas *et al.,* 2017; Kumar *et al.,* 2017).

A concentration-dependent effect on both juvenile and adult stages of *A. siamensis* was observed during *in vitro* test for parasiticidal effectiveness of biosynthesised FeNPs. The concentrations at 1.00, 1.25, 1.50, 1.75 and 2.00 mg ml^{-1} led to mortality of 30, 47, 63, 77 and 87%, respectively, in 6 h among the adult parasites (Fig. 7a) whereas, 0.75, 1.00, 1.25, 1.50 and 1.75 mg ml^{-1} test solution resulted in 23, 57, 73, 87 and 100% mortality respectively in 6 h in *Argulus* juveniles (Fig. 7b). The calculated 6 h EC_{50} of FeNPs for adult and juvenile parasites was obtained 1.27 and 0.97 mg ml^{-1}, respectively. In accordance with present findings, Pimentel-Acosta *et al.* (2019) reported a concentration-dependent death

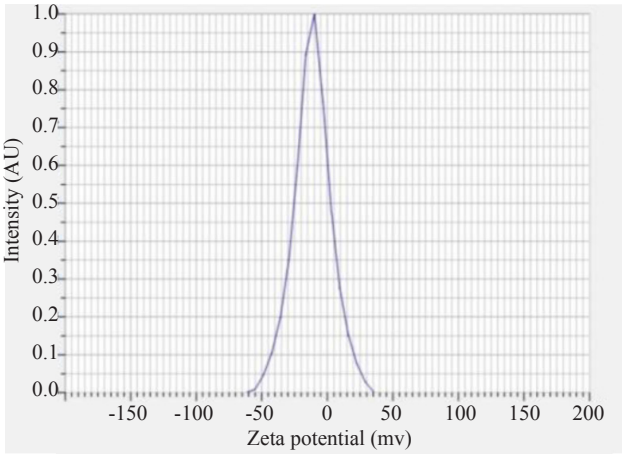

Fig. 4. Zeta potential analysis of biosynthesised FeNPs

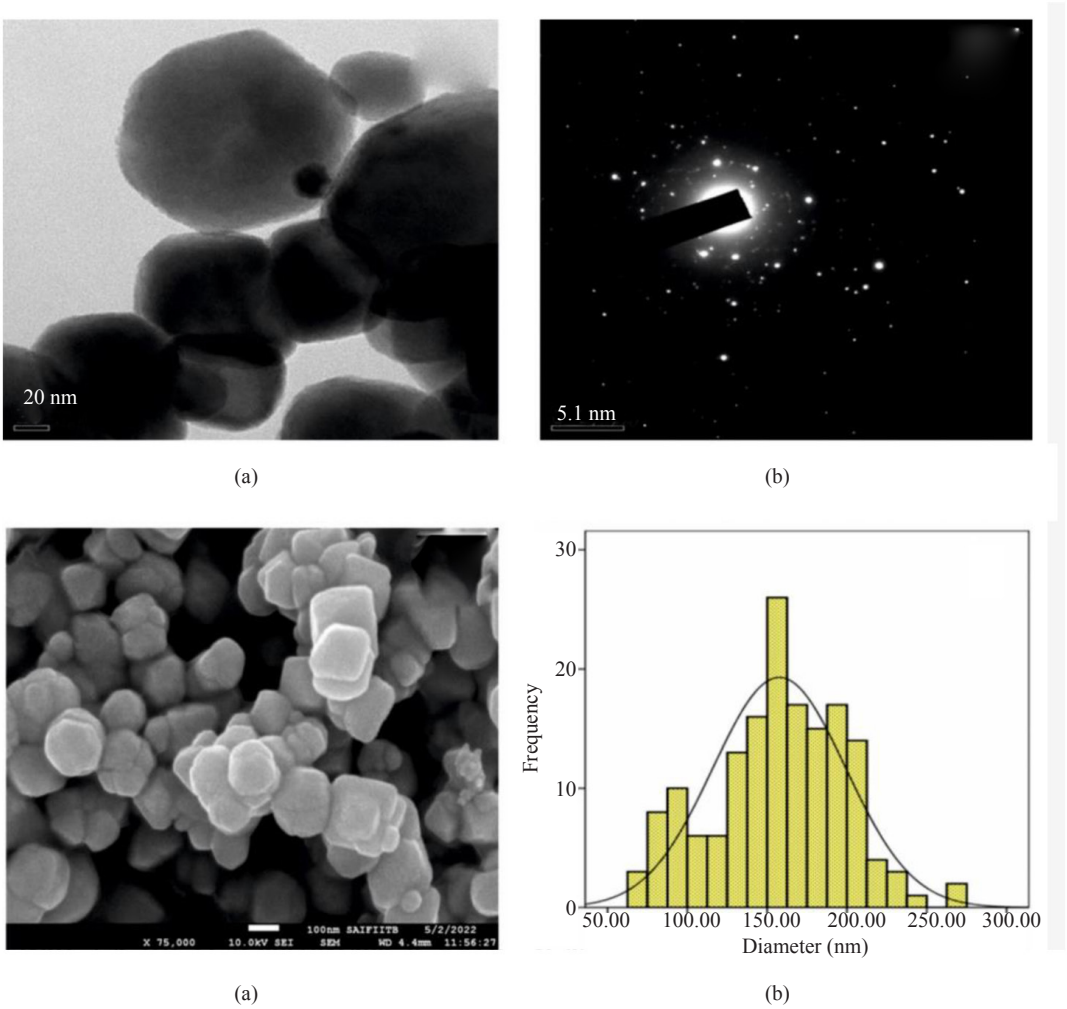

Fig. 5. (a) HR-TEM micrograph, (b) Selective area electron diffraction (SAED) images, (c) FEG-SEM image showing morphology and (d) Histogram showing particle size distribution (measured on SEM images using ImageJ software) of biosynthesised FeNPs

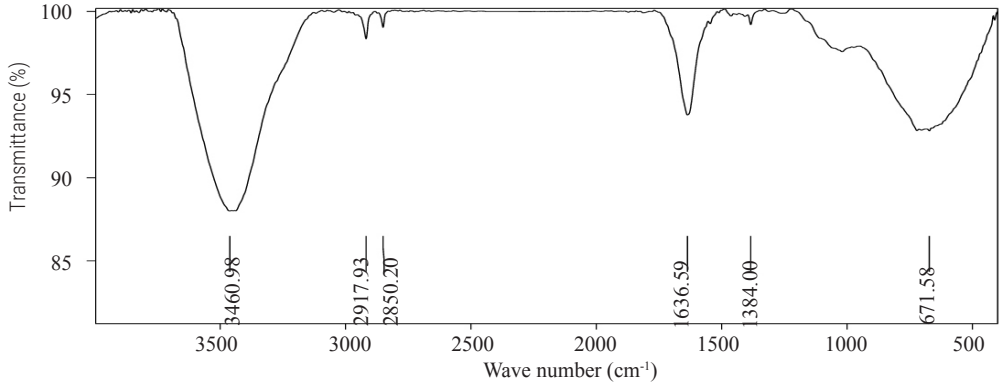

Fig. 6. Fourier transform infrared (FT-IR) spectrum of biosynthesised FeNPs

of monogenean parasite of fish *Cichildogyrus* spp. due to *in vitro* application of silver nanoparticles. Further, gold nanoparticles significantly reduced the microsporidian parasite of fish *Heterosporis saurida*, in a proportional manner to the dosage, with highest dose of 1 µg ml^{-1} NPs inhibited 75% spore, grown in eel kidney cell line EK-1 (Saleh *et al.,* 2016). Later, in a separate study Saleh *et al*. (2017) evaluated *in vitro* effectiveness of gold, silver and zinc oxide nanoparticles against protozoan ectoparasite, *Ichthyophthirius multifiliis*, demonstrating dose- and time-dependent responses that decreases survival time with increasing nanoparticle concentration. After exposure to either 20 ng ml^{-1} gold, 10 ng ml^{-1} silver or 5 ng ml^{-1} zinc oxide nanoparticles, 50% theronts were killed within 30 min and repressed reproduction of tomonts after 2 h exposure.

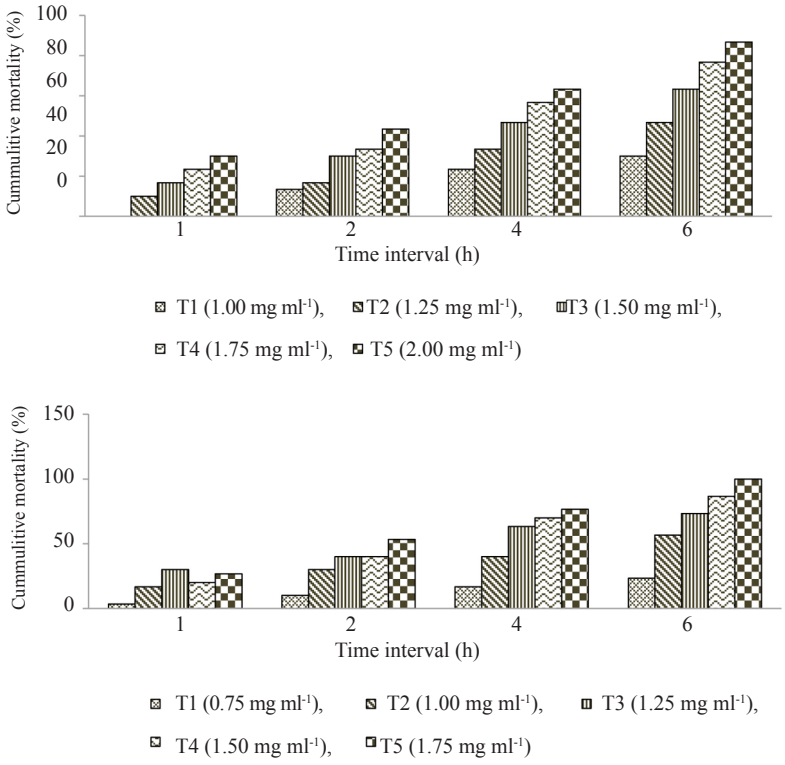

Fig. 7. *In vitro* mortality of *A. siamensis*. (a) Adult parasites and (b) Juveniles treated with test solutions having different concentrations of biogenic FeNPs

The antiparasitic activity of chitosan silver nanocomposite and emamectin benzoate was evaluated on crustacean parasite *Lernaea cyprinacea* (Abu-Elala *et al.,* 2018; Ananda Raja *et al.,* 2023) and severe damage were noticed in the parasite with 100% mortality and considerable increase in host survival. Adsorption of nanoparticles to the whole body of parasite, swelling and fissures in the trunk, and contraction of eggs were observed. A few studies have shown the adsorption of nanoparticles to the exoskeleton of aquatic invertebrates (Dabrunz *et al.,* 2011; Asghari *et al.,* 2012); however, the mechanism of interaction between the nanoparticles and the cuticle is still not clear. Further, Dorostkar *et al.* (2017) recorded the anthelmintic effects of FeNPs against *Toxocara vitulorum* through the induction of oxidative/nitrosative stress. However, Gonzalez-Moragas *et al.* (2017) suggested that IONPs might be endocytosed by a clathrin-mediated process, a putative mechanism of nanotoxicity in the nematode *C. elegans*. Similarly, anthelmintic effects of ZnONPs was evaluated against *Haemonchus contortus,* which induced oxidative/nitrosative damages to biomolecules of the worm (Esmaeilnejad *et al.,* 2018).

Results of the present study shows that short term bath treatment with biosynthesised FeNPs at a concentration of 1.75 mg ml^{-1} can be effective against both juveniles and adults of argulid parasites. Despite the potential of NPs as antiparasiticide in aquaculture, worries about off target effect on host and alteration in efficacy under *in vivo* condition is paving the subsequent investigations. Therefore, additional research is required to assess its therapeutic effectiveness *in vivo* as well as its toxicity to fish and the environment.

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