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Disease resistance efficacy of macroalgae mediated silver nanoparticles and its prophylactic activity against *Pseudomonas aeruginosa* infection in *Oreochromis mossambicus*

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Abstract: Bacterial pathogens are major threat to the aquaculture industry and small scale aqua farming. The emergence of various virulence pathogens during the culturing period leads to mass mortality and influence the loss of aquaculture production. As a result it causes economic imbalance. This relies on the very importance position to serve the protein rich sources for human consumption. In order to improve the sustainable aquaculture production the biologically synthesized silver nanoparticles have been used as an alternative method for commercial antibiotics to control such pathogenic infection. Marine microalgae Caulerpa racemosa was used as a silver reducing agent in this study. This controls Pseudomonas aeruginosa infection in tilapia. The bioactive compounds present in the extracts showed potential antioxidant and antibacterial activities against P. aeruginosa infection. The formation of AgNPs was observed by the visualization of the colour change and further confirmed by the UV spectroscopy. It was characterised by Dynamic light scattering measurements (DLS). The antibacterial activity was tested against the fish pathogen. It was found to be effective.

Keywords: Algal extract, Nanoparticles, Biosynthesis, Antibacterial activity, Tilapia fish, Fish pathogen.

1. Introduction

Aquaculture is the fastest growing sector. The production system has enlarged enormously because of the worldwide importance of the protein resources from the aquaculture and

fisheries producing sectors. Due to the over exploitations of the production and requirement this system attained greater importance [1, 2]. Due to the increase in the human population, the requirement of proteins is largely produced by the fisheries sectors. The overall importance and need of large production depends on the physiological and environmental factors such as poor hygiene, nutrient depletion and overcrowding which causes the negative impact on the fish production. The emergence of opportunistic pathogens during the climatic changes affects the fish production effectively. In order to ensure aquatic animal health management during all seasons, commercially available remedy has been followed to overcome such situations [3]. The continuous use of commercial drugs, veterinary medicines and antibiotics cause resistance to the fishes against the particular pathogens. The use of such inorganic chemicals and its residuals cause side effects to the fish consumers. The use of such antimicrobial drugs in the aquatic environment causes a serious problem in the environment because of the rapid spread of antibiotics through water. Therefore, the present study investigates the use of biologically synthesized silver nanoparticles as an alternative to the antimicrobial drugs. The silver nanoparticle was synthesized using marine algae. This has been developed to increase the aquaculture production. The use of metallic silver as an antibacterial agent against various pathogenic organisms has been well-studied from olden ages [4, 5, 6].

2. Materials and methods

2.1.Collection and maintenance of fishes

Healthy fingerlings of tilapia (*Oreochromis mossambicus*) were used for the study. Fishes were collected from a local fish farm in Walajapet, Tamil Nadu. The fishes were maintained in the laboratory with continuous aeration.

2.2.Collection of seaweeds and processing

Seaweed *C. racemosa* was collected from the coastal regions of Mandapam. Impurities and debris present in the sea weeds were removed by washing in water. Then the seaweed was powdered and extracted by maceration method. Studies were further carried out to determine the potential antioxidant and radical scavenging activities according to the method of [7]

2.3.Biosynthesis of silver nanoparticles

Dried powder extract of *C. racemosa* algal extracts was used for the biosynthesis of AgNps. The algal broth was prepared by varying the biological and chemical parameters for the successful formation of silver nanoparticles. 5 grams of the extract was added to 100 ml of aqueous medium and kept under stirring condition for 30 mins at 50^{o}C. 1mM of silver nitrate solution was also added to the reaction mixture.

2.4.Characterization of AgNPs

The reductions of Ag+ ions were observed biometrically through UV visible spectroscopy. The optical density of the reaction mixture containing silver nanoparticles was determined by the absorption peak. The absorption peak was recorded at the wavelength between 300-700nm (UV-1800, Shimadzu, Singapore). Dynamic light scattering technique was used for the particle size anlaysis using the colloidal suspension. The density or size of the nanoparticles was measured on Brookhaven Instrument (model 90 Plus) particle size analyzer. This was used to determine the particle size distribution. Scanning Electron

Microscope (SEM) analysis was employed to determine the external morphology and approximate size of the synthesized nanoparticles using (Hitachi S-4500) instrument.

2.5.In vitro Antibacterial activity

Antibacterial activity of AgNPs was determined by the standard antimicrobial assay methods. Well diffusion and disc diffusion was performed according to the modified method of [8]. 25 µl of the syntheised AgNPs was loaded onto the well. Miliqwater was kept as a control. In disc diffusion Ciprofloxacin disc was used a standard. Nanoparticles loaded disc was used as test to determine the zone of inhibition [9].

2.6.Bacterial culture

The gram negative bacterial pathogen of *Pseudomonas aeruginosa* was purchased from Microbial type culture collection (MTCC) was used in the present study and the stain was further confirmed by the biochemical analysis described by Bergy's Manual of Systemic bacteriology.

2.7.Pathogenecity experiment

Pathogenicity of *P.aeruginosa in* tilapiawas carried out by bath exposure for the experimental pathogenicity. The detailed methodology was already reported in our earlier study [10].

2.8.Confirmation of pathogenicity

Confirmation of the pathogenecity was carried out by re-isolating the specific bacterial disease causing pathogen from the moribund fishes to satisfy Koch's postulates. The infected parts of fishes were cut and homogenized. The samples were inoculated on to nutrient agar medium by spread plate technique for the confirmation of the bacterial pathogen.

2.9.In vivo Treatment of bacterial disease using AgNPs

In vivo treatment study of the synthesized silver nanoparticles by biological method was tested against the pathogenic bacteria *P. aeruginosa* to study the potential antibacterial activity against the disease causing pathogen. Immersion route was followed to treat the bacterial infection. The method of [10, 11, 7] was followed. Fishes of 10 per tank/ dosage were arranged in the glass tanks. Fresh water was used throughout the experiment with the proper biochemical and physiochemical parameters. The bacterial culture was diluted serially with different dilution ranging from $10^{-1}$to $10^{-7}$and the bacterial culture was added to the tank containing 1lit of water. 30µl of the synthesised particle was added to the experimental tanks to check the efficacy of the nanoparticles against the pathogenic microorganism.

3.0. Results and discussion

The seaweeds collected from the coastal region were tested for its antioxidant capacity to fight against the free radicals. Free radical formations were initiated in the cells of human and animal system during the extensive stress or cell damages caused by internal or external factors. The radical scavenging properties of *C. recemosa* were tested. The assays such as Total polyphenol, Total flavonoids, Total antioxidant assay and free radical scavenging assays such as hydroxyl radical scavenging assay and DPPH assays were performed. Total

polyphenol, Total flavonoids, Total antioxidant, hydroxyl radical scavenging assay and DPPH was found to be 12.32 mg/g, 18.44mg/g, 38.12mg/g equivalence, 82.2%, 49.05% respectively. The results are shown in the (figure 1).

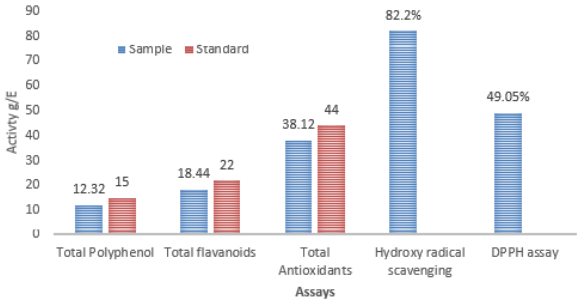

Figure 1. Antioxidant activity

These results were supported by the report of [12] who reported the antioxidant potential antioxidant activity of *Caulerpa scalpelliformis* showed the maximum of 21.34±0.05 mg/ml significantly higher in the methanolic extract of *Cheatomopha antennina.*
Biosynthesis of silver nanoparticles was prepared and confirmed by the color change from brownish yellow to dark brown. The intensity of this brown color is well developed during incubation period and is responsible for the excitation of the surface plasmon resonance (SPR). The reduction of Ag^{+} ion was characterized by the presence of reducing agents in the seaweed extract. The solution mixture showed the characteristic absorbance peak of AgNPs at 420 to 430 nm.

This supports the published data of [13-15] who reported the absorbance of silver nanoparticle at 420nm. The size characterization of biosynthesized silver nanoparticles was achieved primarily with the DLS using the scattering technique, which exhibited the size distribution of 148nm. The results are shown in figure 2. This indicates the incidence of light on the particles exhibited in the Brownian motion. This method partially confirms the particle size range at nanoscale. This result supports the report of [16] who determined the structure and size of the nanoparticle synthesis by the sophoro lipids by varying the effects of synthesis and structure

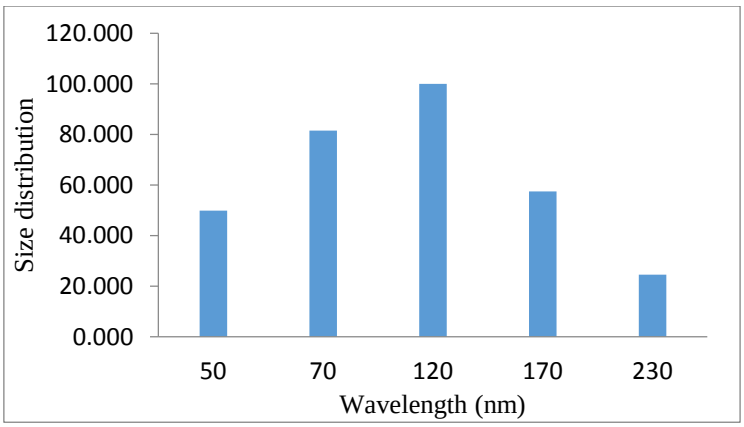

Figure 2. Size distribution by DLS

Further the SEM analysis of the nanoparticle was studied to determine the structure and accurate size of the nanoparticle. This technique illustrated the particles present in the

colloidal suspension of the silver nanoparticle which exhibited spherical shape. The mean diameter of the particle size was found to be 125nm. These results supports the result of [16]. The detailed reports of morphology and size of the nanoparticles are essential to the various biomedical applications like targeted drug delivery and nano drug formulations. Our results also agreed with the published report of [17] who studied the synthesis of silver nanoparticles by *Aeromonas paniculata*. The results revealed an average particle size of 55nm with a spherical shape.

Antibacterial activity of the nanoparticle was studied against the fish pathogen *P. aeruginosa*. The activity of nanoparticles in both well diffusion and disc diffusion was found to be effective against the tested pathogen. The zone of inhibition for the synthesized silver nanoparticle was found to be 15mm. The commercial antibiotic ciprofloxacin showed comparatively 18mm zone of inhibition. Disease controlling efficacy of this nanoparticle was further confirmed through the in vivo activity. The results of the present study showed that there was 70% of survival in the fish which was treated with synthesized silver nanoparticle. The inhibitory effect of the silver nanoparticles against fish pathogen was reported in the studies carried out by [17] using *Aeromonas hydrophilia.* This corroborates with the present study. Use of such novel antimicrobial agents promotes the health and disease management in the aquaculture production.

Controlling bacterial infection in fishes using the silver nanoparticles with different concentrations of formulated nanoparticles were reported for optimum exposure in in vivo treatment of bacterial infection of fishes. Prepared nanoparticles were dispersed and challenged through immersion of nanoparticles in water containing disease-causing pathogen and experimental fishes. Disease controlling efficacy of the silver nanoparticles synthesized from the seaweed was optimized for the effective challenge against the fish pathogen. However the use of antimicrobial drug and chemical used in aquaculture are banned due to the developing resistance against the pathogens. In order to overcome such situation this kind of biologically synthesized antimicrobial formulation has been preferred. Our study reported that the percentage of survival during the successive treatment was found to be nearly 70%. These results corroborated with the report of [18]. Similarly the use of such nanoparticles based treatment studies against fish pathogens have been discussed in the study of [18] who studied the disease controlling efficacy of biologically synthesized nanoparticles to control the pathogenicity of *Vibio harveyii in Feneropenaeus indicus.* The protective efficacy of AgNps was found to be effective in controlling bacterial infection during the in vivo treatment with the survival rate of 71% and mortality of 29%.

4.0. Conclusion

In conclusion the use of such biologically synthesized nanoparticle delivery systems needs to be optimized for use in aquaculture disease management system. The results of the present study suggest an alternative treatment method for treating the bacterial infection in fishes. Further studies needs to be carried out on how to deliver this biosynthsised nanoparticle in the aquatic environment. The use of such novel nano antimicrobial drugs with natural bioprotectant efficacy was found to be one of the effective alternative methods of treatment to commercial chemical drugs.

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