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Research Article

Analysis of Antimicrobial Potential of Silver Nanoparticles Synthesized by Fucoidan Isolated from *Turbinaria conoides*

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ABSTRACT

The present study investigates the antibacterial potential of fucoidan synthesized silver nanoparticles (FAgNps) against the chosen human and fish pathogens. The fucoidan used for the synthesis of FAgNps was isolated from a brown sea weed *Turbinaria conoides*. Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) method were performed to analyze the antimicrobial efficacy. ATR-FTIR analysis revealed the presence of polar functional groups in the fucoidan molecule capable of synthesizing and stabilizing silver nanoparticles. The fucoidan synthesized nanoparticles were mostly spherical, ranging in size from 5 to 20 nm and with antibacterial activity against gram negative being more distinct and pronounced than gram positive. This study highlighted the prospect of using fucoidan for the synthesis of antimicrobial silver nanoparticles against human and fish microbial pathogens, making them relevant in various medicinal units and antimicrobial control system. Further study is required to get the better understanding of mode of action of fucoidan synthesized silver nanoparticles (FAgNps) against the pathogens

Keywords: Antimicrobial activity, *Turbinaria conoides*, fucoidan, Silver Nanoparticles

INTRODUCTION

Resistance developed by various pathogen strains against the action of antimicrobial medicines[1] is a major concern and highlight the need for searching novel and safe antibacterial agents. As numerous studies have revealed the potential of chemical moites isolated from marine and other natural sources to suppress pathogenic activity can be effectively improved by their integration with nanoparticles. As they usually have superior or special traits than the bulk composition, has intended this investigation to improve the potential of fucoidan to suppress the infections caused by pathogens[2,3]. Among the various nanoparticles used in medicinal applications, silver nanoparticles due to their distinctive physiochemical properties, were found to exhibit superior potential to inhibit pathogenic activity through unique mode of action. As silver nanoparticles are relatively non toxic to human body at low concentration and because of their broad range of antibacterial potential[4], they are used in the development of antimicrobial combinations. Marine environment is unique source for safe biologically active molecules[5,6]. In this regard, even though manifold studies on the biological activities of fucoidan have been reported, effect of silver nanoparticles integration on the pathogenic activity is poorly known. Hence, fucoidan a sulfated polysaccharide abundant in brown seaweed, *Turbinaria conoides*, which is plentifully found in mandapam coast[7], has been used to prepare fucoidan-silver nanoparticles (FAgNPs) combination to enhance their bioactivity against representative pathogens of public concern[8].

MATERIALS AND METHODS

Sampling and preparation of algal sample

Turbinaria conoides, a brown seaweed, belonging to sargassacea family was collected from Mundapam coast, Gulf of mannar, south east coast of India, 9°45' N latitude and 79°E, during pre-monsoon season was used for the isolation of fucoidan.

Extraction, purification and characterization of fucoidan from Turbinaria conoides

Prior to extraction of fucoidan, 20g of powdered seaweed samples was treated with ethanol and shaked in a mechanical shaker for about 12 h at room temperature in order to remove lipids and pigments. Method developed by Yang *and co-workers* in 2008 was used with modification to extract fucoidan[9]. The algal powder was boiled in 1N HCl solution, at 75 °C for 5 h using a reflux condenser under nitrogen atmosphere. The hot extract solution was filtered with a glass membrane filter paper. In order to remove alginic acid from the extract, filtrate was uniformly mixed with 1% $CaCl_2$ and the solution was kept overnight at 5 °C. The solution was then centrifuged at 20000×g for 5 minutes and the supernatant was collected. Prior to fucoidan precipitation, supernatant was mixed with ethanol (99 %) to prepare a solution containing 30% ethanol concentration and was placed at 5 °C for 6 hours. After which they were centrifuged at 20000×g for 10 minutes and the supernatant was

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collected and ethanol concentration in the supernatant was made to 70% using ethanol (99%). This supernatant was placed at 5°C for overnight to precipitate fucoidan and intact fucoidan was obtained through filtration of the solution with a nylon membrane filter paper (0.45µm). The crude fucoidan extract were dissolved in distilled water (1:25 w/v) at 60 °C and centrifuged at 5,000 rpm. The supernatant was dialyzed using dialysis membrane (6-8,000 dt molecular weight cut off) against two volumes of double distilled water for 24 h at room temperature and then freeze-dried. The crude fucoidans obtained were purified using DEAE cellulose 52 (SRL, India) ion-exchange chromatography[10]. The qualitative characterization of the purified fucoidan was carried out using ATR-FTIR spectroscopy (Spectrum 400 FT-IR, PerkinElmer) described by Kemp[11]. The spectra were recorded between 4000 and 600 cm^{-1} wave number and evaluated.

Synthesis and characterization of FAgNps

Stock solutions of fucoidan and silver nitrate were prepared. A 1 mL silver nitrate solution (0.001- 0.01 mol/L) was transferred to separate boiling tubes containing 10 mL aliquots of 10 ppm solutions of fucoidan (prepared by suitable dilution of fucoidan stock solution using Milli-Q water). The reaction times and conditions were optimized after conducting several sets of trial experiments. The boiling tubes containing the reactant mixtures were heated in an air oven at 75 °C while maintaining a pH of about 11.5. A whitish gray precipitate formed instantly upon the addition of silver nitrate solution at first turned colorless and subsequently to pale yellow, indicating the formation of the silver nanoparticles. The time taken for synthesis of F-AgNps was 15 min and solution was stable. The resultant solution of FAgNps was further analyzed using HRTEM (JOEL3010) to understand the distribution and shape of these nano-molecular units. The samples HRTEM were prepared by drop casting method. Drops of aqueous solution of FAgNPs were added to carbon coated copper grids which were dried overnight at room temperature and thereafter used for further analysis.

Antimicrobial activity

Antimicrobial activity were assessed against clinical and fish pathogens. The clinical pathogens such as *Escherichia coli, Klebsiella pneumoniae, Vibrio cholera, Streptococcus spp., Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Shigella sonnaie* were assessed and fish pathogens used in this study were *Vibrio alginolyticus* and *Vibrio parahemolyticus.*

Antibacterial assay

Pathogen collection were reconstituted in Muller-Hinton's broth, cultures were resuscitated under aerobic conditions at 37 °C and 200 rpm to reach exponential growth. The concentration (107 colony-forming units (CFU)/mL) was routinely estimated by spectrophotometric turbidity measurement at 600 nm on a spectrophotometer and by CFU counts on tryptic soy agar (TSA). The microorganism suspension was smeared thoroughly on the test media before assaying the activity of the fucoidan and FAgNPs.

The minimum inhibitory concentration (MIC) was tested in pathogenic strains. Equal volumes of each bacterial strain culture, were applied to 5 mL of Muller-Hinton's broth with different concentration of FAgNPs, in the test tubes ranging from 2.0, 5.0, 8.0, 10.0, 12.0, 15.0, 20.0, 25.0, 30 and 50 µg/mL, respectively. Whereas, the control used for the study was prepared without FAgNPs. These cultures were then incubated at 37°C for 24 h. After the incubation period, turbidity was measured. MIC was defined as the lowest concentration of F-AgNPs that completely inhibited the visible growth of the test microorganisms and the minimum bactericidal concentration (MBC) corresponding to the lowest concentration at which no colony is visualized (lethality higher than 99.9%). After appropriate dilution, aliquots from tubes (100 µL) that seems to have little or no cell growth were plated on agar plates to differentiate between the bacteriostatic or the bactericidal effects.

RESULT AND DISCUSSION

Presence of polar and reducing functional groups in the fucoidan, which facilitated the reduction of alkaline silver nitrate to FAgNPs[12], was confirmed by IR results (Fig.1). Broad band at 3332 cm^{-1} corresponds to the presence of hydrogen bonded OH stretching, COO^{-} stretching bands in the fucoidan molecule isolated from *T. conoides* was confirmed from the presence of 1615 cm^{-1} [13]. Presence of ester sulphate linkage in the molecule was inferred from the peak at 1226 cm^{-1} and 1181 cm^{-1}. Normally in sulphated polysaccharide absorbance band at 1250 cm^{-1} indicate the presence of sulphate ester[14-19]. Presence of 3,6 anhydrogalactose in the molecule) and characteristics of agaro colloids is inferred from absorbance band at 1315, cm^{-1} 976, 962 and 872 cm^{-1} [15,19,20]. On the other hand 1025 cm^{-1} corresponded to the glycosidic linkage stretch vibration of C-O-H [21]. Further, absorption bands detected at 826 cm^{-1}, showing that 2-sulfate galactose, galactose-6- sulfate and sulfate on C-2 of 3, 6-anhydrogalactose were present in this fucoidan molecule[22].

HRTEM analysis revealed the presence of truncated spherical F-AgNPs (Fig.2). Distribution of FAgNPs ranged between 5-20 nm.

The antibacterial potential of FAgNPs was examined using the MIC and MBC tests. Different concentrations of the FAgNPs samples were incubated with Gram-negative bacteria and Gram positive bacteria) in Muller-Hinton's broth. Growth of pathogens was studied by visual inspection as indicated by turbidity. Clear solution may correspond to either bacteriostatic effect or a bactericidal effect. Suppose FAgNPs did not kill but only withdrawn the growth of bacteria (bacteriostatic), upon plating the colonies will be present. And if FAgNPs is bactericidal, bacterial colony would be absent. Bacteriostatic test results of FAgNPs against gram positive and gram negative bacteria are shown in Table 1. It is apparent that the MICs against gram positive are higher than gram negative relatively, and this can be

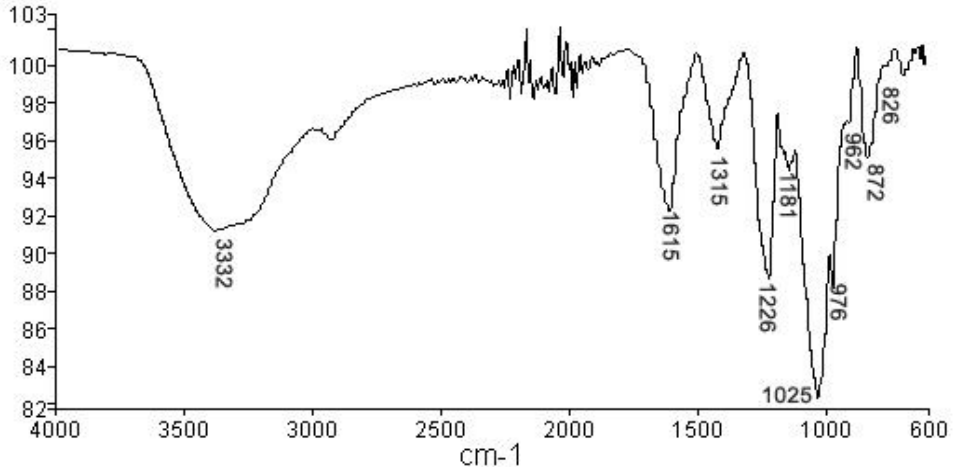

Figure 1: ATR-FTIR image of fucoidan isolated from *T. conoides.*

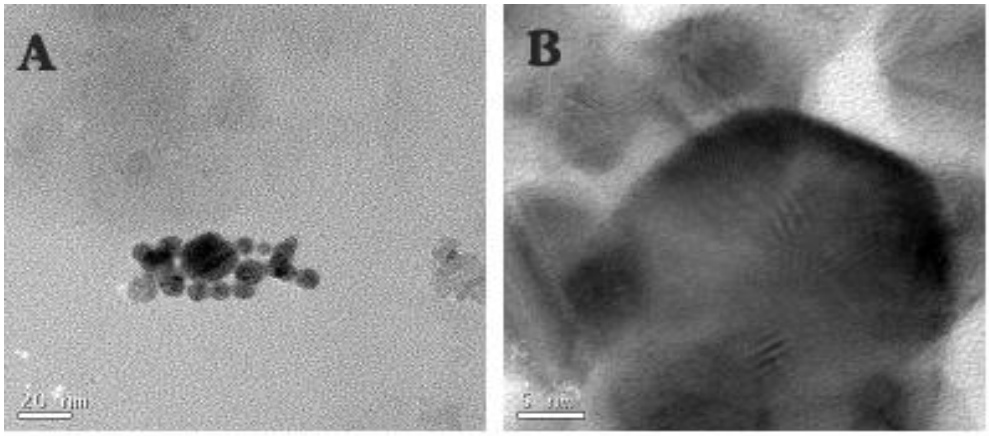

Figure 2: HRTEM images of fucoidan synthesized truncated spherical silver nanoparticles (FAgNPs).

endorsed to the nature and depth of the membrane and cell wall of gram positive microbes. The bactericidal test of the FAgNPs towards these pathogens was carried out, and the MBC is read by the presence of live bacteria on the agar plate.

The MIC and MBC results of all samples of FAgNPs against various gram positive and gram negative pathogens are summarized in Table 1 and because the MIC and MBC values of FAgNPs toward microbes were unchanged, the results of MBC were not given in the table. As per the MIC results, antibacterial potential of FAgNPs observed against gram negative and gram positive could be ascribed to truncated spherical shape and surface coverage of fucoidan. These results can become substantiation on the theory put forward by pal and coworkers relating the antibacterial activity of silver nanoparticles to their shape[23]. In the case of gram negative bacteria, it is assumed that the FAgNPs activity could persist for longer time intervals when compared to their effect on gram positive. As it is observed that biopolymers with cationic nature could effectively bind to negatively charged surface of bacteria (bacteria-adsorbing effect), it is presumed that antibacterial activity of the polymer will be reduced considerably by the adsorbed cell membrane remnants/inactive microbes. In contrast, the FAgNPs molecular units may prolong the inhibition of microbial activity even after the active sites are completely masked by adsorbed materials, thus showcasing persisting activity[24]. This mode of operation has been previously proved for silver-incorporated chitosan film[25] and in gelatin synthesized silver nanoparticles[26].

Even though, the mechanism by which the nanoparticles are able to go through the bacteria is not clearly understood, numerous investigations have been performed in the case of silver nanoparticles. These reports point up that depending on the shape and size, silver nanoparticles can change the membrane morphology of pathogens and alter the membrane permeability and influence the transport and cell signaling prevailing in the plasma membrane[27] .Various investigations has highlighted the presence of silver nanoparticles adsorbed to the cell membrane and inside the cell is an adequate evidence for the understanding of the biocidal action of FAgNPs[28,29]. As per the theory of hard and soft acids and bases, silver ions on the surface of FAgNPs will tend to have an enhanced affinity to react with phosphorus and thio compounds. Hence, sulfur-containing proteins on membrane surface of the pathogens might be favored sites for the binding of silver nanoparticles. On the other hand, silver nanoparticles entering the cells will have direct influence on proper

Table 1: The antibacterial activity MIC values of FAgNPs (µg/mL) against gram negative and gram positive pathogens

FAgNPs	Gram Negative								Gram Positive	
	V. parahaemolyticus	*E. coli*	*V. alginolyticus*	*V. cholera*	*P. aeruginosa*	*K. pneumoniae*	*S. sonnei*	*S. typhi*	*S. aureus*	*Streptococcus spp.*
0.001 mol/L	15	15	20	25	25	25	20	30	30	50
0.002 mol/L	12	12	15	15	20	20	15	30	25	30
0.003 mol/L	10	10	12	10	15	15	15	25	25	25
0.004 mol/L	8	8	8	10	8	10	12	20	20	15
0.005 mol/L	2	5	5	8	5	8	8	10	12	12

functioning of sulfur-containing proteins and enzymes and phosphorus containing genetic materials[30]. To conclude, the changes in the cell membrane permeability and consequent impact on the cell signaling and biochemistry might be directly influencing the multiplication of pathogens and, finally causing their death.

CONCLUSION

Summing up, we have developed a facile and coast effective approach to prepare fucoidan capped spherical silver nanoparticles, which are stable in the aqueous solutions. It is predicted that this green synthesis procedure can be easily extended to other similar polysaccharides. In general, *Turbinaria conoides* represent a source of renewable biomass that can be mass cultured and harvested for sustainable processes and products using fucoidan. More research is necessary to recover fucoidan in relation to scale-up for large scale production. Our results supports the hypothesis that silver nanoparticle synthesized from fucoidan can be used for formulation of novel bactericidal products.

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