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Original Research Article

Fish Mucus (*Cyprinus carpio*) Mediated Green Synthesis of Silver Nanoparticles and Vitro Investigations on their Biochemical, Biological and Characterization

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Abstract

In recent years, biogenic approaches to crafting silver nanocomposites have garnered considerable attention outstanding to their potential in developing semi-healthcare and para-pharmaceutical consumer products. This study presents a novel, environmentally benign method for synthesizing silver nanoparticles operating the previously unexplored mucus derived from the Common carp (*Cyprinus carpio*). Thorough characterization of the resultant materials using UV–Visible Spectroscopy and FTIR Spectroscopy techniques confirms the successful formation of silver nanoparticles within the common carp mucus matrix. Subsequent testing against a diverse selection of bacterial strains, including Gram-positive (*Escherichia coli*) and Gram-negative (*Bacillus subtilis*), as well as a fungal strain (*Terbinafine*), using the well diffusion method, reveals potent antibacterial and antifungal properties exhibited by the silver nanoparticles embedded in the mucus matrix. Further experiments were conducted to ascertain the inhibitory concentration against both bacterial strains. Cytotoxicity assessments conducted via in vitro analysis using blood intriguingly heightened cytotoxic activity of the biogenically synthesized silver nanoparticles within the biocompatible mucus, suggesting potential applications in anticancer therapies. Moreover, evaluation of antioxidant properties (DPPH, TPC, TFC) and enzymatic activities (SOD, POD, CAT, TSP) of the mucus-based nanoparticles demonstrates promising outcomes, indicative of their potential utility in formulating antimicrobial.

Keywords: Fish mucus; Nanoparticles; Silver: characterization: Biologicals activities: Biochemical activities.

INTRODUCTION

The common carp (*Cyprinus carpio)* is a tremendous fish with a deep body. The color of the carp varies from silver to look like green, with brass and grey on the sides and back. The belly is yellow colored the lower fins orange red. It has a spine, partly scaled cheeks and gill covers. Barbells are whisker-like appendages located around the corners of its lips. Any kind of base and water that varies in clarity from clear to muddy can be used. Because of their extensive range and detrimental effects on wetlands and small lakes, carp are among the most destructive aquatic invasive species. (Joseph R & Tomelleri., 2018).

Mucus, the epithelium surface, is coated in a slick, slimy film. Water, proteins, and enzymes are all present in this layer. Because water is a suitable substrate for bacteria and parasitic microorganisms, the skin is always vulnerable to pathogen assaults (Wang *et al.*, 2011). Mucus functions as a biological and mechanical obstacle. It has lectins and proteins that bind carbohydrates, which may help protect against infections that aren't enzymes or antibodies. Numerous resistance components, including immunoglobulin, reactive protein, complement, lysozyme and hemolysin are found in fish outer body mucus. (Tateno *et al.*, 2011).

Nanoparticles, ranging in size from 1 to 100 nm, are found within the interfacial coat. This layer is an essential component that influences all its properties. The interfacial layer contains organic and inorganic molecules as well as ions. Inorganic molecules that are coated by organic molecules are referred to as stabilizers passive agents surface ligands or passive ligands. Nanoparticles often possess optical properties due to their small size which allows them to confine their

electrons and exhibit quantum effects. In living things, they can pass across cell membranes. The link between bulk materials and atomic structure is provided by nanoparticles. Diffusion is accelerated by their high surface area to volume ratio (Batista *et al.,* 2015). As far as the authors are aware not much has been studied in relation to mucus and nanoparticles. Thus, the purpose of this work is to examine how common carp (*Cyprinus carpio*) mucus binds to silver nanoparticles. Furthermore, the biological activities of the nanoparticles and mucus including their antibacterial, antioxidant, antibiofilm, and cytotoxic qualities will be investigated.

MATERIAL METHOD

Sample Collection:

The University of Agriculture Faisalabad's fish farm, which is located to the Zoology Department, provided samples of common carp (*Cyprinus carpio)* fish mucus. To gather the mucus different amounts of carp fish were taken out from the outer body surface.

Collection of Mucus:

The fish mucus was together with the help of spatula exactly from the outer most side of the fish mucus was not amassed from the lower side of the fish body to prevent from the contagion of urine and sperms. Fish mucus samples were meticulously collected into Eppendorf tubes, with consideration given to the weight for the next process. Subsequently, these mucus samples were promptly placed within containers filled with crushed ice, maintaining a temperature of -20°C, to ensure preservation. Following this preservation step, the samples were transported to the Medicinal Biochemistry Laboratory within the Department of Biochemistry at the University of Agriculture, Faisalabad, to undergo further comprehensive analyses and investigations.

Preparation of crude fish mucus sample:

The crude mucus extract was obtained from previously preserved fish mucus. The skin mucus harvested from the fish was utilized to prepare the crude mucus extract, followed by centrifugation at 1500 rpm for 15 minutes. The resulting supernatant was collected for quantitative and qualitative assays aimed at evaluating the biochemical components. (Tur & Kumara, 2016).

Preparation of silver solution:

To obtain silver oxide, 0.069 g of Ag NPs salt was measured on an analytical balance and mixed with distilled water to a total volume of 100 ml.

Synthesis of Mucus based Silver Nanoparticles:

Silver nanoparticle solution and common carp (*Cyprinus carpio*) crude mucus were used to generate mucus-based Ag nanoparticles. Every solution was made with distilled water. Next, a uniform mucus solution containing 1 mM silver nitrate and 0.5% (w/v) was prepared. Mucilage-based Ag nanoparticles were produced by autoclaving the mixture at 120 °C for 15 minutes. (Munir *et al.,* 2016).

Biological activities of crude fish mucus and silver nanoparticles-based mucus.

Crude mucus and nanoparticles-based mucus are used to check the antibacterial activity and antifungal activity with diffusion method.

Antibacterial activity:

Bacillus subtilus, a gram-positive strain, and *Escherichia coli*, a gram-negative strain, are employed in antibacterial activities using the diffusion method technique. (Cavalieri *et al.,* 2005).

Antifungal Activities:

Crude mucus and nanoparticles-based mucus were tasted against the two different antifungal strains activity to use the well diffusion method (Phillott & Parmenter, 2012).

Minimum Inhibitory Concentration (MIC):

The lowermost attentiveness of an antimicrobial (such as an antibiotic, bacteriostatic, or antifungal medication) that prevents a bacterium from growing appreciably during and for the night incubation is known as the minimum inhibitory absorption (Wiegand *et al.,* 2008).

Antioxidant activity:

Several antioxidant techniques were used to assess the antioxidant activity of crude mucus and AgNps-FM.

Total phenolic content (TPC):

The total phenolic content of crude mucus and Ag nanoparticles-based mucus was determined using the method of Mamelona *et al.,* (2007). Total phenolic compounds (TPC) were determined in gallic acid equivalents (GAE) using the following formula.

T-C x V/M

Total flavonoid content (TFC):

Using the technique, the total flavonoid content of Ag nanoparticles-based mucus and crude fish mucus was determined. (Chang *et al.,* 2006).

2,2-Diphenyl-1-Pierylhydrazyl (DPPH)

To determine DPPH, we used the method described by Garcia *et al.,* (2014) criminal proceedings were initiated. The standard utilized was ascorbic acid. To calculate DPPH activity, use this formula:

DPPH inhibitory activity (%) = Ablank-Asample / Ablank *100

Inhibition of microbial biofilm:

Inhibition of microbial biofilm was determined against gram positive and gram-negative bacteria was determined to use the method of Shahid *et al.,* (2015).

To calculate the amount of microbial biofilm inhibition, use this formula:

$$\textbf{inhibition}\ \% = 100 - \frac{\text{Optical density of sample}}{\text{Optical dnsity of negative control}} \times 100$$

Cytotoxic activity:

A hemolytic method was used to determine the cytotoxicity.

Hemolytic activity:

The hemolytic activity of the crude fish mucus will be assessed using animal blood as a substrate. Dilutions of fish mucus will be prepared in phosphate buffered saline (PBS). Subsequently, the plates containing the dilutions will be incubated at room temperature. Powell *et al.,* (2000).

Biochemical analysis:

The biochemical analysis of fish mucus encompassed catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) assays, as well as protein estimation. Catalase activity (CAT) was assessed following the method outlined by Mohebbi-Fani *et al.*, (2012). Superoxide dismutase (SOD) activity was evaluated with slight modifications according to Kumari and Khare (2014). The activity of peroxidase (POD) as a hydrogen donor was determined using guaiacol, as described by Jayaseelan *et al.*, (2014). Protein estimation was conducted following the method outlined by Hiwarale *et al.,* (2016).

Characterization:

UV visible spectroscopy

Field UV absorbance measurements were conducted following the procedures outlined by Zamtsov *et al.,* (2006).

Fourier transformed infrared spectroscopy (FTIR):

For the spectroscopic analysis of the solid component of fish mucus and nanoparticle-based fish mucus, Fourier-transform infrared spectroscopy (FTIR) is employed, as described by Bragadeeswaran *et al.*, (2011).

Statistical Analysis:

Statistical analysis involved the calculation of simple means and standard deviations. Typically, bar graphs were employed to visually represent the data, facilitating the examination of the study hypothesis regarding the characteristics of interest.

RESULTS

Biological Activity.

Antioxidant Activity of crude Fish Mucus and nanoparticles-based mucus.

Using a variety of tests, the antioxidant activity of fish mucus was ascertained.

Total Phenolic Contents (TPC):

Crude fish mucus contains the minimum number of phenolic contents as compared to nanoparticles-based mucus. crude fish mucus contains lower phenolic content and very low level of antioxidant activity. Total phenolic contents of crude fish mucus and fish mucus-based nanoparticles are determined by Folin-Ciocalten colorimetric procedure (Turkoglu *et al*., 2010).

Total Flavonoids Content (TFC):

The evaluation of mutual sample of crude mucus and nanoparticle based after comparison we find that crude fish mucus contains lower number of total flavonoids as compared to nanoparticles-based mucus. (Turkoglu *et al*., 2010).

Reducing Power:

Because of the reducing capacity of a combination, the reducing power test is frequently used to evaluate an antioxidant's ability to donate an electron. It is the main indicator of antioxidant activity. (Duan *et al*., 2007). The capacity to reduce iron (III) reducing power test is used to determine the sample extract. The assay's reducing power fluctuates based on each sample's concentration. An increase in the sample's or mixture's reducing power indicates an increase in the reaction mixture's absorbance. The capacity of the crude fish mucus sample to contribute electrons and reduce the Fe3+/ferric cyanide complex into the ferrous form, which forms the blue hue, is shown by its weak reducing power. Depending on the sample's lowering power, the test sample's color shifts from yellow to either green or blue. At 700 nm, absorbance was measured.

Free Radical Scavenging Activity (DPPH):

A well-known free radical that contributes significantly to the absorption band at 517 nm is DPPH. The deep violet color of the DPPH solution is neutralized by an antioxidant molecule, which causes the color to vanish and turn yellow. The DPPH scavenging test was used to measure mucus extract's capacity to scavenge free radicals. Every sample's capacity to scavenge was dilution dependent. The reaction mixture's lower absorbance suggested increased DPPH radical scavenging activity. (Gulcin *et al*., 2006).

Total phenolic content, DPPH, Total flavonoid content, and reducing power. The triplicate data ± SD is shown below.

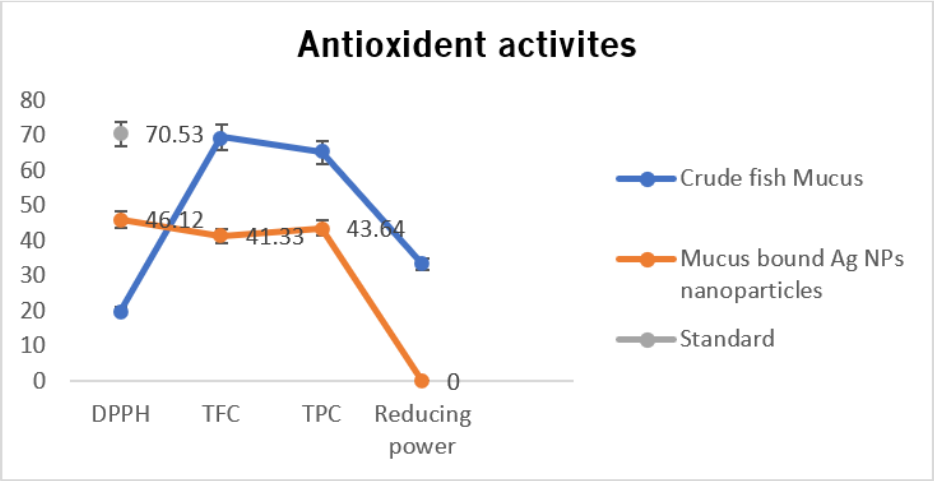


Table 1: Total phenolic content, Total flavonoid content, and reducing power. The triplicate data ± SD is shown below

Sample	DPPH (%)	TFC (ug/ml)	TPC (mg)	Reducing power
Crude fish Mucus	20.13 ± 0.06	69.62 ± 1.27	65.43 ± 0.32	33.28 ± 0.97
Mucus bound Ag NPs nanoparticles	46.12 ± 0.14	41.33 ± 1.19	43.64 ± 1.72	0.87 ± 0.13
Standard	70.53 ± 73			

Biofilm Inhibition:

A biofilm is a thin gum-like coating that adheres to a solid appear. It is made up of a collection of microorganisms with surface-assigned cells. An extensive extracellular matrix composed of extracellular polymeric elements such proteins, polysaccharides, and DNA surrounds this cell. (Flemming and Wingender, 2010).

Microorganisms are pathogens having more resistant to certain antimicrobial treatments when they are in the biofilm state. In addition to withstanding difficult circumstances, bacteria in a biofilm are immune system resistant. Fish pure mucus and mucus-based nanoparticles might prevent the formation of biofilms was the aim of this experiment. Initially, the antibiofilm activity of both samples was assessed; the findings are shown in the table.

The presence of certain polysaccharides and enzymes that start molecule inhibition or receptor inhibition in the quorum pathway which is required for the creation of biofilm is the cause of biofilm resistance. Lectins are crucial for bacterial infection and settling, and they have a significant function in the creation of biofilm, which polysaccharides have inhibited. (Rendueles *et al*., 2013).

Table 2: *Bacillus subtilis* and *Escherichia coli* biofilm inhibition by mucus-based nanoparticles and extracts.

Sample	*E. coli* mean±SD	*B.subtilis* mean±SD
Crude fish Mucus	20.45 ± 0.09	29.23 ± 0.54
Nanoparticles based sample	25.85 ± 1.07	31.79 ± 1.73

Antibacterial activity:

Crude fish mucus and nanoparticles was tested against the antimicrobial activities. For this activity two bacterial strains were used, one was gram positive and other one was gram negative. *E. coli*, *B. subtilis*,

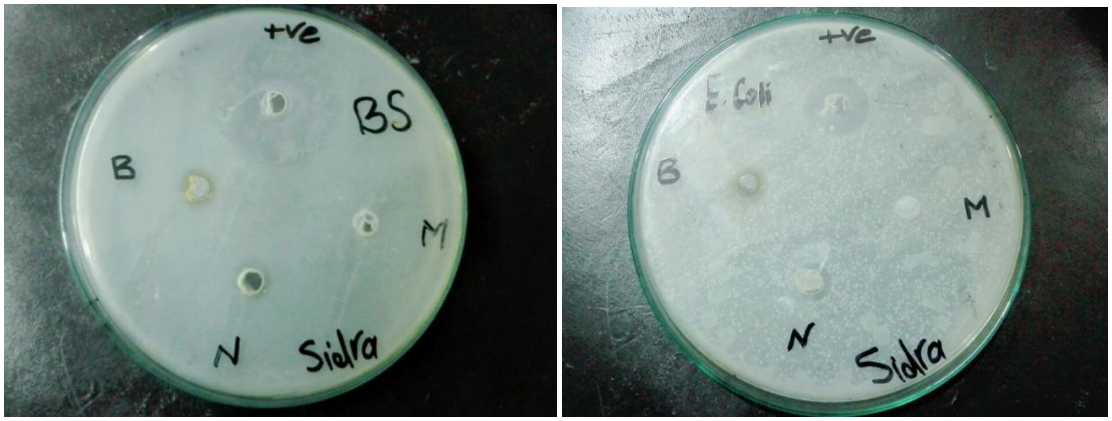


Fig 1: (a) Zone of inhibition between B. subtilis and crude fish mucus based on nanoparticles. Both positive and negative control have an inhibition zone. (b) Zone of inhibition between *E. Coli* and mucus based on nanoparticles and crude mucus. Both positive and negative control have an inhibition zone

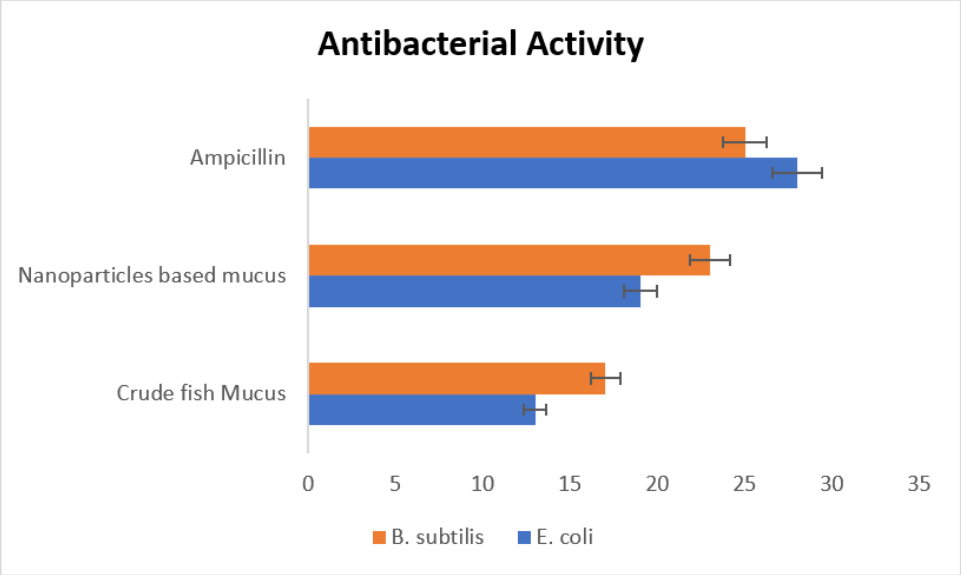


Table 3: Ampicillin as a control antibiotic is used and mucus-based nanoparticles, and crude fish mucus are used in various fish microorganisms are all targets of the antibacterial action of these materials

Sample	***E. coli* (mm)**	***B. subtilis* (mm)**
Crude fish Mucus	13	17
Nanoparticles based mucus	19	23
Ampicillin	28	25

Native fish species, such common carp, exhibit low antibacterial activity when compared to invasive fish species such as AgNps-FM. (Balasubramanian *et al*., 2012). The fish release antibacterial proteins that enable them to permeate the target cell membrane and serve as a barrier for protection. Fish mucus contains antibacterial glycoproteins that could kill bacteria by creating large holes in the target cell membranes. These glycoproteins are what give fish mucus its antibacterial action. (Kuppulakshmi *et al*., 2008).

Antifungal Activities:

The antifungal properties were evaluated against Terbinafine, crude fish mucus and mucus-based nanoparticles Ag nanoparticles attached to fish mucus were not as effective against fungal growth as fish mucus in its pure form. Terbinafine had the strongest antifungal activity overall. The outcomes are listed below.

Table 4: Terbinafine, mucus-based nanoparticles, and crude fish mucus (Common carp) microorganisms were found to have antifungal activity

Sample	*A.niger* (mm)	*S. cerevisiae* (mm)
Crude fish Mucus	13	12
Nanoparticles based mucus	14	17
Terbinafine	25	20

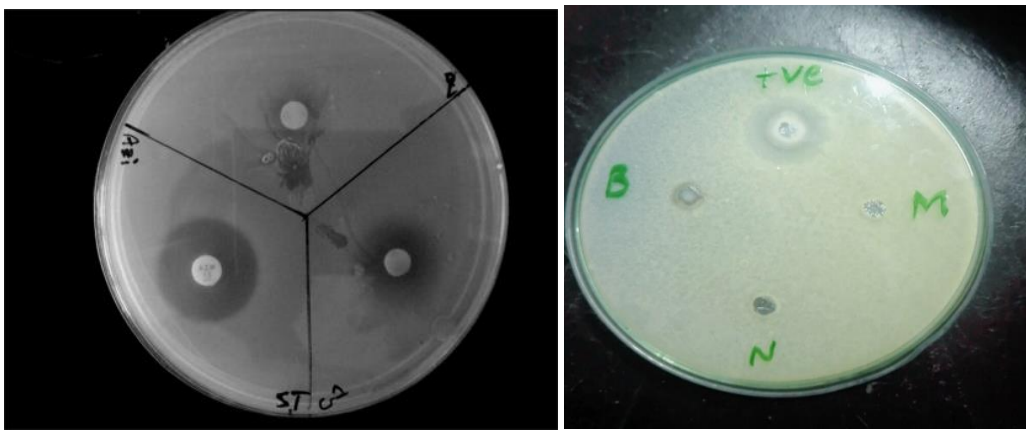


Fig 2 (a) Various samples' antifungal activity against A. niger (b) several samples' antifungal properties against S. cerevisiae

Minimum inhibitory concentration (MIC):

The lowest dose of an antimicrobial (such as an antibiotic, bacteriostatic, or antifungal) that would stop a bacterium from developing noticeably after an overnight incubation is known as minimum inhibitory absorption, or MIC in microbiology. (Wiegand and others, 2008). The MIC of Bacillus subtilis and E. Coli, two bacteria, was measured. Every sample had its MIC data recorded. MIC studies, the silver nanoparticles extract most frequently inhibited *E. Coli*, and *B.subtilis*, with at least ten of the extracts under investigation showing inhibition against any of these two bacteria.

Table 5: MIC of *B. subtilis, E. Coli*, crude fish mucus, and mucus-bound and free Ag nanoparticles

Sample	% inhibition of *B.subtilis*	% inhibition of *E.Coli*
Crude fish Mucus	33.69	30.75
Mucus based Ag nanoparticles	40.48	57.42

Cytotoxic Activity

Hemolytic activity:

Animal blood is used to check the hemolytic activity, EDTA solution was used to be hemolytic, which thwarted the blood from clotting. The table below exhibits the ratio hemolysis of crude mucus extract. Crude mucus has a weaker hemolytic activity than mucus based on nanoparticles. Triton-X was utilized as a positive control, and its hemolysis percentage was 90%.

Table 6: Hemolytic test of Ag nanoparticles and crude fish mucus. The provided data represents the three replicates' average ± standard deviation

Sample	Hemolytic activity
Crude Mucus	0.97±0.33
Nanoparticles based mucus	0.03±0.47
Triton-X	89

Biochemical analysis

Both samples are used to check biochemically analyzed using protein estimation, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) tests.

Catalase (CAT)

In this instance, H_2O_2 was employed as a substrate, and a UV-vis spectrophotometer was utilized to watch how the catalase enzyme broke down H_2O_2. the absorption at 240 nm. Mucus, Ag nanoparticles based, and crude fish mucus were tested for their catalase activity. The findings indicate that crude fish mucus has a low level of catalase activity.

Peroxidase activity (POD):

Guaiacol was used as a hydrogen donor in the POD activity experiment, and absorbance was measured at 470nm. crude Mucus, nanoparticles-based mucus was tested for peroxidase activity. The findings showed that crude fish mucus contains minimum peroxidase activity.

According to the table below nanoparticles-based mucus showed a high level of activity.

Protein estimation:

Samples were diluted for the protein estimation test to acquire protein. Standard albumin bovine serum was used. To set up the standards, a standard consisting of 200–2000 micrograms of protein were generated using bovine serum albumin (2 mg/ml in 1000 ul volumes). The spectrophotometer was used to measure the absorbance at 595 nm. The amount of total soluble protein in crude mucus and nanoparticles mucus-based Ag was determined.

Superoxide Dismutase (SOD):

Phosphate buffer (pH 7.5), riboflavin, Triton-X, Nitro Blue Terazolium, and methionine were the reagents utilized in the SOD test. After 15 minutes of UV light exposure, riboflavin was added at the conclusion. At 560 nm, the absorbance was measured. The activity of superoxide dismutase was assessed in three different forms of mucus-based nanoparticles and crude fish mucus. The findings showed that nanoparticles-based mucus had the highest superoxide dismutase activity. Superoxide dismutase activity was also demonstrated by crude fish mucus, demonstrated lower activity in comparison.

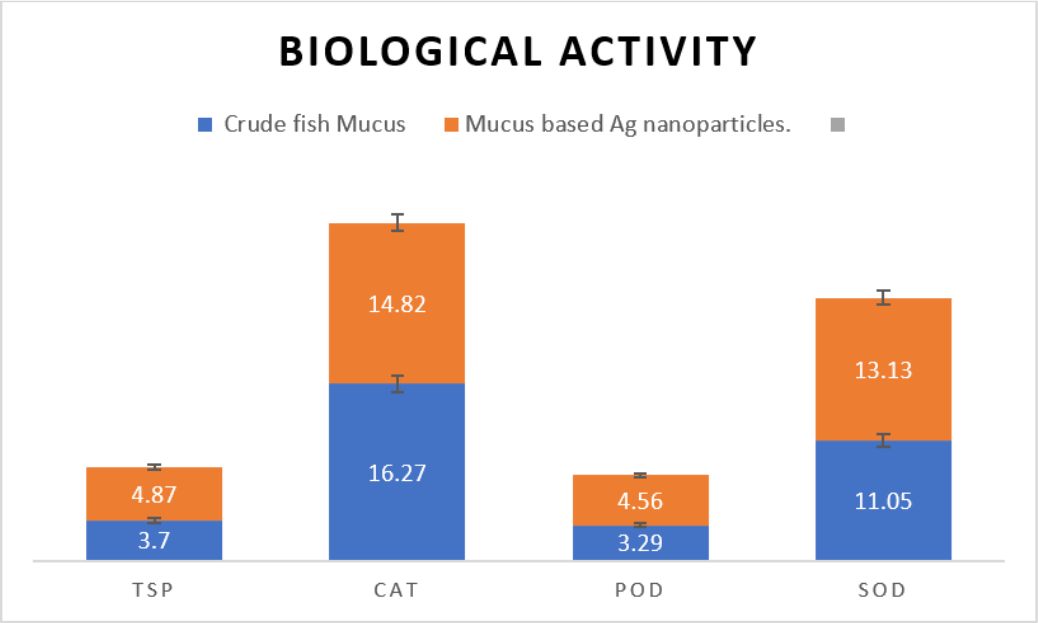


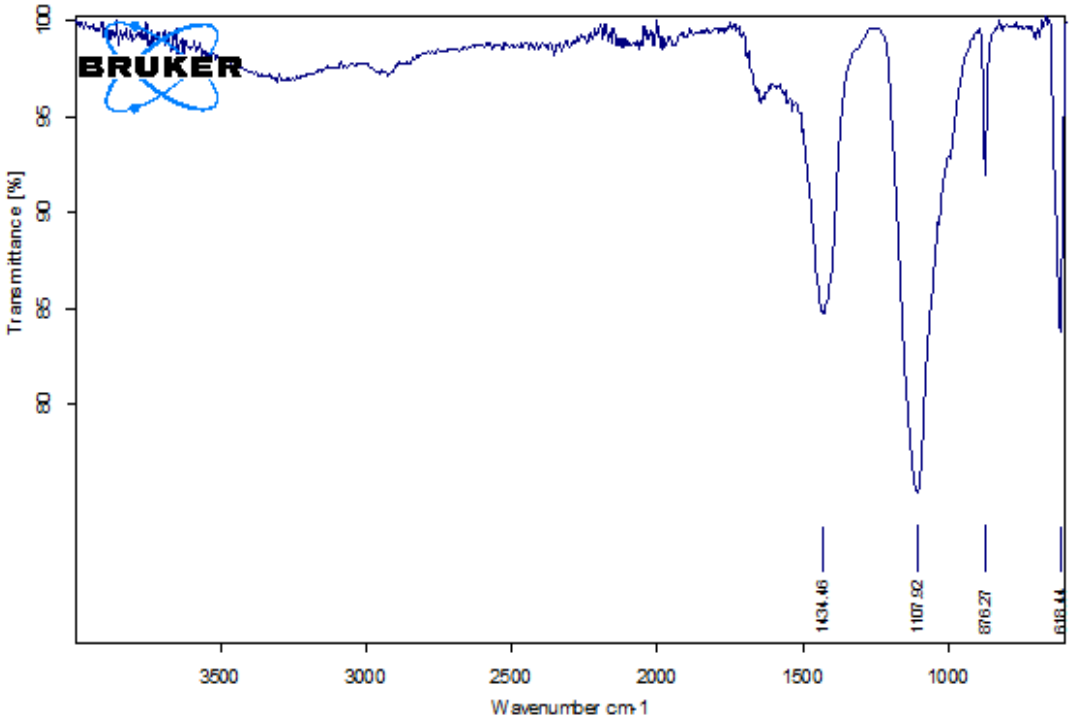
Table 7: POD, TSP, CAT, and SOD values of free, mucus-based Ag nanoparticles, pure fish mucus, and mucus-based nanoparticles. The value of the triplicates ± SD is shown

Sample	TSP	CAT	POD	SOD
Crude fish Mucus	3.70 ± 0.23	16.27 ± 0.71	3.29 ± 0.14	11.05 ± 0.14
Mucus based Ag nanoparticles.	4.87±0.17	14.82 ± 0.26	4.56± 0.83	13.13 ± 0.12

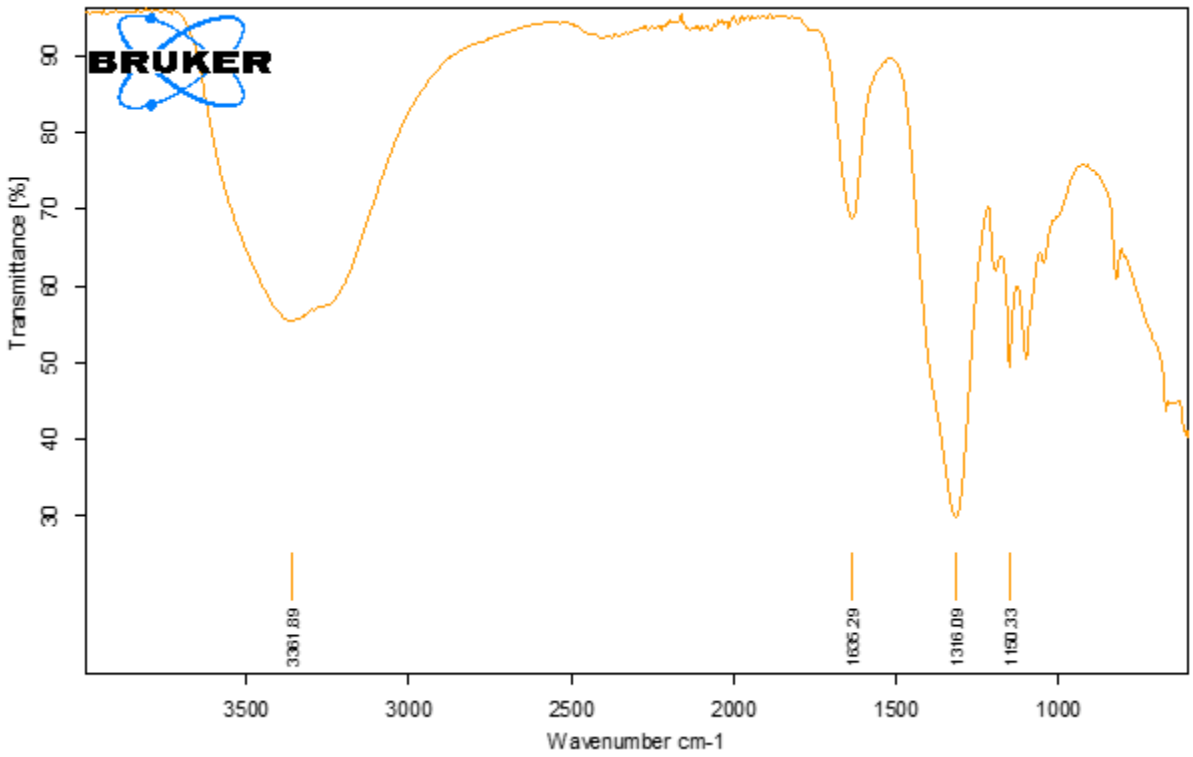
Characterization:

(a) Fourier infrared spectroscopy:

Different peaks representing mucus, binding material, and nanoparticles-based mucus were seen in the FT-IR spectra. The sample's spectrum revealed distinct peaks in each case. At different wavelengths, distinct functional groups can be found in the peaks detected in dry mucus. The functional groups alkane, alkyl amine, and alkyl halides are visible in the FTIR spectrum at wavelengths of 1434.16, 1107.92, 876.27, and 618.44, in that order. (b) Ag nanoparticles based on mucus exhibit four peaks at various wavelengths in their FTIR spectra. Alkanes, alkyl ketones, alkyl amines, and secondary amines (N–H) are the functional groups with wavelengths of 3361.89, 1635.29, 1316.09, and 1150.33, respectively.



(A)

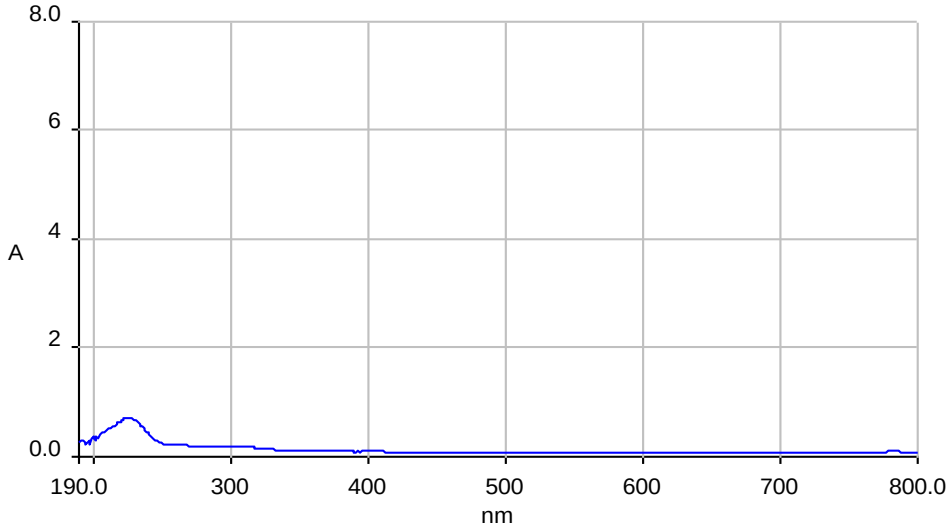


(B)

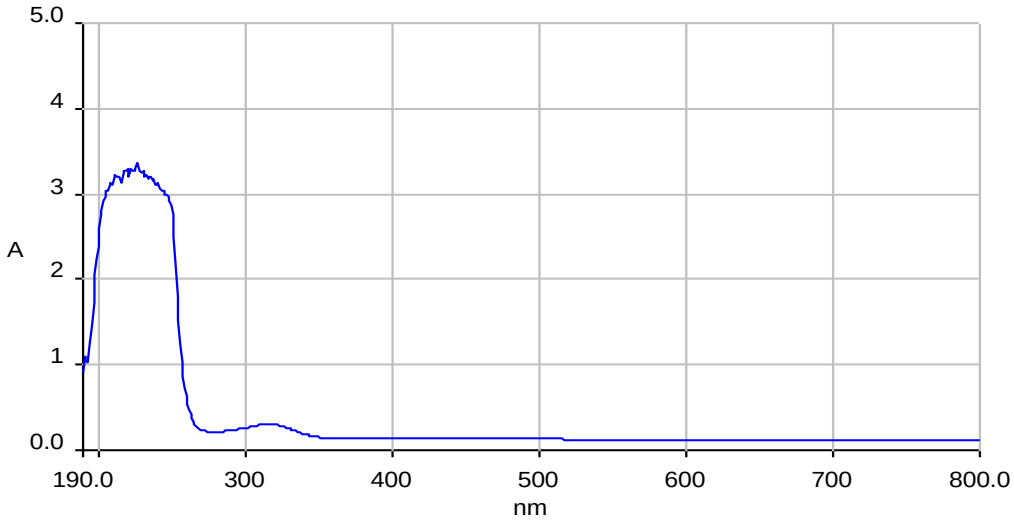
Fig 3: (a) An illustration of the mucus FTIR spectra (b) FTIR spectra of mucus-based Ag nanoparticles shown graphically.

UV spectra

The typical range of UV-vis spectra employed is 200 nm to 1100 nm, where peaks belonging to various functional groups can be seen. The highest peak in this spectrum is located at 250 nm, while the lowest peak is located at 1100 nm (0.5 nm). The measured spectrum peak peaks about 200–300 nm, while the peaks start to diminish after 300 nm. The maximum absorbance is 250 nm.



(A)



(B)

Fig 2: (a) UV spectrum represented graphically. crude carp mucus (b) UV spectra represented graphically for mucus-based nanoparticles

Both pure fish mucus and mucus-based nanoparticles peaked between 190 and 300 nm in diameter. The duration of the peak accounts for the disparity. Ag nanoparticles based on mucus exhibited a peak at a length of 3–4 whereas pure mucus showed a peak at a length of 1-2.

CONCLUSIONS

We have presented an environmentally sustainable method for the biogenic synthesis of silver nanoparticles utilizing a unique biomaterial, namely the naturally secreted mucus of the common carp, Cyprinus carpio. In addition to its role in the bio-reduction and bio-stabilization steps during the synthesis of AgNPs, we have explored the potential of utilizing silver nanoparticles to combat antimicrobial resistance and other pathogenic organisms, capitalizing on their demonstrated efficacy. To this end, we have evaluated the antimicrobial performance of the resulting AgNPs-FM composite samples against various pathogens. Notably, we have observed significant antimicrobial activity against the deadly pathogens E. coli and B. subtilis, which often exhibit resistance to multiple antibiotics in clinical settings. Building upon our initial investigations, we propose the potential for future immunotherapeutic applications based on AGNPs-FM,

suggesting the development of topical treatments utilizing fish mucus for effective and scar-free wound healing.

Conflict of interest; The authors have no conflict of interest.

Data availability: The data of the manuscript will be available on request.

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