Research Article

Isolation of chitosan from fish scales of *Catla catla* and synthesis, characterization and screening for larvicidal potential of chitosan-based silver nanoparticles

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ABSTRACT

Background and Objectives: Chitosan is the major component present in fish scales and crustacean shell waste. Chitosan was isolated from scales of fish waste of Catla (*Catla catla*) by chemical method involving demineralization, deproteinization, and deacetylation. **Methods:** Chitosan isolated from fish scales was confirmed qualitatively by Richard's method. Further, it was quantitatively analyzed and was found to contain 6 g of chitosan (100 g of dried fish scales). Chitosan had been characterized using Fourier transform infrared spectrometry analysis. A new method of preparing silver nanoparticles combined with chitosan is used here. It was characterized using ultraviolet and scanning electron microscopy analysis. The potential of chitosan-based silver nanoparticles against Culex (III instar) and Anopheles (IV instar) was screened, and biochemical parameters of the larval tissues were also estimated. **Results:** Protein, carbohydrate, lipid, acid phosphatase, alkaline phosphatase, and lactate dehydrogenase content were found to be decreased in the treated larval tissues when compared to the control in both mosquito species. **Conclusions:** Decreased levels of biochemical contents are indicating the high toxic capacity of chitosan silver nanoparticles.

KEY WORDS: *Anopheles, Catla catla*, Chitosan, Chitosan-based silver nanoparticle, *Culex*, Fourier transform infrared, Larvicidal activity, Standard error mean, Ultraviolet

INTRODUCTION

The seafood industries waste possesses environmental hazard due to its easy deterioration, even though the wastes generated are biodegradable, it degrades slowly. About 130 million ton of fish waste is generated each year in the world.[1] Chitosan is a homopolymer of ß-(1→4)-linked N-acetyl-D-glucosamine while chitin is a linear chain of acetylglucosamine groups, and chitosan is obtained by removing enough acetyl groups (CH_3-CO) and this is soluble in most diluted acids.[2] Natural polymers have also been used in the preparation of nanosilver because they are non-toxic and biocompatible. Starch[3] and chitosan[4] have been used as stabilizers for the preparation of metal nanoparticles. Mosquitoes serve as a vector of several diseases, causing serious health problems to human and they transmit diseases, namely yellow fever, human lymphatic filariasis, and malaria. Mosquito problem has become acute in recent years and many programs have been launched to control these vectors.[5] Mosquitoes are the principle vectors of many vector-borne diseases affecting human beings and animals, in addition to being nuisance pests. Vector-borne diseases in India, for example, malaria, dengue, chikungunya, filariasis, Japanese encephalitis, and leishmaniasis, cause thousands of deaths per year.[6] The biological method employing chitosan extract is a simple and viable route of synthesis which is alternative to chemical method. The chitosan has been used to synthesize silver nanoparticles. However, till date, there are no scientific reports available on the synthesis of silver nanoparticles using chitosan. Hence, this necessitated to isolate the chitosan and synthesize chitosan-based silver nanoparticles were characterized (ultraviolet [UV] and scanning electron microscopy [SEM]) and evaluate the larvicidal activity.

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MATERIALS AND METHODS

Sample Preparation

The fish scales waste was collected from local market at Ukkadam, Coimbatore and Tamil Nadu and brought to the laboratory for isolation of chitosan. Then, they were washed thoroughly with water to remove all other wastes. The scales were completely dried under sunlight.

Extraction of Chitosan

The dried scales were demineralized with 1% HCL. Samples were allowed to soak for 36 h to remove the minerals (calcium carbonate and calcium phosphate). Then, the scales are washed in water. The demineralized scales are then deproteinized using 0.5 N NaOH at room temperature for 18 h. This step is to remove proteins from the scales to obtain chitin. The chitin further deacetylated to get the chitosan. The deacetylation process is carried out by adding 50% NaOH and then heated for 2 h at 80°C in water bath. Afterward, the samples are washed continuously with the water and filtered to obtain the solid matter, which is the chitosan. The samples were then left uncovered oven dried. The powder obtained from deacetylation is called chitosan.

Preliminary Study

The obtained chitosan was allowed to dry at 55°C for 2–4 h. After on the dried precipitate, 2–3 drops of iodine/potassium iodide solution were added and mixed, and the mixture was acidified with 2–3 drops of 1% H_2SO_4. The chitosan obtained before and after chemical treatment was noted. The amount of chitosan powder from dried fish scales was calculated.

Characterization of Chitosan

The infrared spectral analysis of the isolated chitosan sample was measured by Fourier transform-infrared spectrometry (FTIR), (Frontier Spectrometer, Perkin Elmer, USA) in the wavelength range of 450–4000/cm at a resolution of 4/cm.

Synthesis of Chitosan-based Silver Nanoparticles

The chitosan powder was dissolved in 2% (v/v) acetic acid at room temperature for 12 h to achieve 1% (w/v) chitosan solution. For the synthesis of the chitosan-AgNPs, the silver nitrate ($AgNO_3$) was used as a source of Ag^+. A stock solution of 52 mM $AgNO_3$ solution 15 ml of 52 mM was added into 30 ml of 1% (w/v) chitosan solution and stirred vigorously for 1 h. Then, the homogeneous solution was heated at 121°C and 15 psi for 15 min in an autoclave. After heat treatment, the solution color, absorption spectrum, AgNP size and morphology, and crystal structure were investigated.

Characterization of Chitosan-silver Nanoparticles

UV-visible Spectroscopy

The characterization was done by UV-visible spectroscopy in the range of 200–800 nm and the plots were shown respectively and confirmed the presence of chitosan-AgNP.

SEM

The morphological features of synthesized silver nanoparticles from chitosan were studied by SEM (JSM 6450 LV).

Collection and Maintenance of Larvae

Culex larval strains in Stage III and *Anopheles* larval strains in Stage IV were collected from National Centre for Disease Control, Mettupalayam, Coimbatore, and were brought to the laboratory safely without disturbance. Larvae were maintained in trays with 200 ml distilled water.

Screening of Larvicidal Activity

Larvicidal activity of chitosan-AgNPs against *Culex* and *Anopheles* larvae was investigated at five different concentrations (2 ppm, 4 ppm, 6 ppm, 8 ppm, and 10 ppm) of nanoparticles. 20 larvae were transferred to conical flasks containing 150 ml distilled water and different concentrations of nanoparticles. Control was set up by transferring 20 larvae to separate flask and normal food was provided to it. Mortality was observed after 24 h of exposure and percentage mortality was calculated.

Biochemical Analysis

Estimation of total proteins

Lowry's method was adopted to estimate protein content in the larvae. Reaction of protein with Folin–Ciocalteu become purple-blue proportional to the amount of proteins and read at 620 nm. Further, protein concentration was calculated with optical density.[7]

Estimation of total carbohydrates

Carbohydrate was estimated as described in the method of Nelson.[8] Proteins were removed from the tissue homogenate and the filtrate containing glucose only as reducing substrate was heated with alkaline copper reagent and subsequently treated with arsenomolybdate reagent. The blue color thus developed was read at 540 nm and protein content was calculated.

Estimation of Lipids

Total lipids present in the larval tissue were estimated following the method of Bragdon.[9] Lipid content was separated from the non-lipid components by chloroform-methanol solution and lipid in the aqueous phase was reduced by sulfuric acid-dichromate

mixture. The resultant green color was measured at 600 nm, and the concentration of lipid was calculated.

Estimation of Acid/Alkaline Phosphatases (ALPs)

Activity of acid/ALPs in larval homogenates was evaluated according to Fiske-Subbarow method. 0.1 ml of filtrate was added to 2 ml of buffer substrate and incubated for 1 h. 0.8 ml of acid molybdate and 0.2 ml of ANSA reagent were added. The final volume was made up to 10 ml with distilled water. The intensity of color developed was measured at 660 nm against reagent blank.

Estimation of Lactate Dehydrogenase (LDH)

The King (1965) method was followed for evaluating LDH activity. 0.2 ml NAD + solution was added to the test tubes containing 1 ml of the buffered substrate. The sample (0.01 ml) was also added to the test tubes. Test tube samples were incubated for exactly 15 min at 37°C and then arrested by adding 1 ml of color reagent (2, 4-dinitrophenyl hydrazine) to each tube. The incubation then continued for an additional 15 min. After the contents were cooled to room temperature, 10 ml of 0.4 N NaOH was added to each tube to make the solutions strongly alkaline. At exactly 60 s, after the addition of alkali to each tube, the intensity of color was measured at 440 nm.

RESULTS AND DISCUSSION

Isolation of Chitosan

The demineralized, deproteinized, and deacetylized scales were obtained as the result of demineralization, deproteinization and deacetylation process, respectively. The chitosan was obtained as reported by Kumari and Rath.[10]

Table 1: Yield of chitosan

Fish scales	% yield
Before treatment	100 g
After treatment	6 g

Confirmation of Chitosan

The results for the confirmation analysis of chitosan obtained. The color of chitosan appeared to be white. The polysaccharide test showed dark brown on the addition of iodine/potassium iodide solution. Further addition of 1% H_2SO_4, the dark brown color turns to dark purple. This indicates the presence of chitosan.[11]

Percentage Yield of Chitosan

The total yield of chitosan from the *Catla catla* fish scales after the chemical treatments was shown in Table 1.

Characterization of Chitosan

FTIR results clearly indicated the presence of functional groups including amino and hydroxyl groups. Adsorption bands of FTIR of chitosan were observed as shown in Figure 1. The peaks appear at 3637.75/cm, 2974.23/cm, and 2384.02/cm corresponds to axial OH group, C-H and C-N asymmetric bands, respectively. The same results obtained by Nidhi Nigam[12] at 3526/cm, 3000/cm, and 2364/cm, respectively.

The peaks 759.95/cm and 825.53/cm corresponds to N-H stretch bonds (primary and secondary amines) for chitosan. The same peaks obtained for chitosan from shrimp and crab at 747/cm and 902/cm.[13] The bands of 1421/cm, 1584/cm 3526/cm indicating – CN (secondary amide), C=O (Carbonyl stretching) respectively and the results were observed by Yateendra.[14] The similar results were obtained for the fish scale chitosan at the peak 1435.04/cm and 1585.49/cm.

Synthesis of Chitosan-based Silver Nanoparticles

The color changes from light yellow to light pink show the formation of silver nanoparticles after heating at 121°C. This color change phenomenon indicated that Ag+ was reduced to Ag°. Then, the silver atoms agglomerated into oligomeric clusters and eventually formed AgNPs.[15]

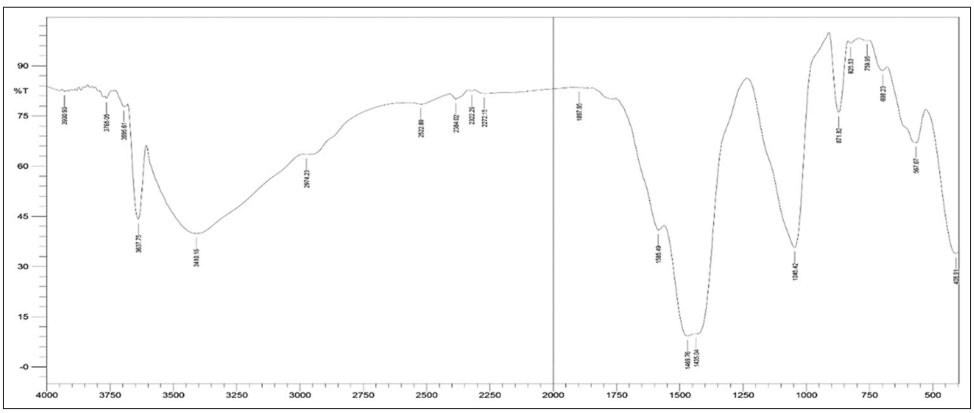

Figure 1: Fourier transform-infrared spectrometry of chitosan

Table 2: Mortality rate of Ch-AgNP-treated larvae

Species and instar	Exposure time	Concentration of Ch-AgNP (ppm)	Mortality %	LC_{50}	LC_{90}
Culex (III) instar)	24 h	2	5.26	50.46	616.9
		4	10.52		
		6	14.73		
		8	17.89		
		10	21.05		
Anophela (IV) Instar)	24 hrs	2	2.12	33.80	181.9
		4	4.25		
		6	9.57		
		8	14.89		
		10	20.21		

LC_{50}: Median lethal concentration; LC_{90}: 90% lethal concentration

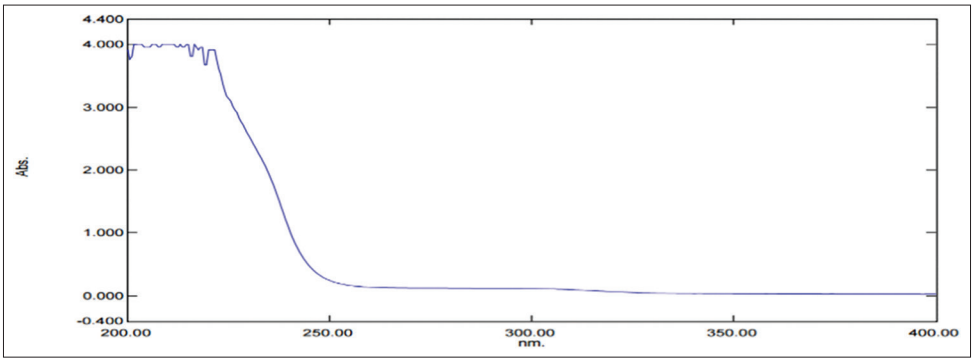

Figure 2: Ultraviolet spectrum of chitosan-based silver nanoparticles

Characterization Studies of Chitosan-based AgNPs

UV-visible spectroscopy

UV-visual spectroscopic analysis is widely used method to identify the formation of metal nanoparticles by analyzing the unique optical properties, which depend on the size and the shape of the nanoparticles. Figure 2 shows the chitosan-silver nanoparticles formation at 209 nm and 221 nm. Chitosan itself is transparent in the UV and visible region. Hence, its optical properties hard was too hard to characterize by spectroscopy methods. The peak was due to the plasma vibrations of silver showing some possible interactions between chitosan and silver nanoparticles.[12]

SEM

The morphology of nanoparticles of chitosan-based silver nanoparticles was characterized using SEM. The SEM shows the morphology of the nanoparticles. The SEM micrograph of 10 µm of the chitosan-silver nanoparticles was shown in Figure 3. The silver nanoparticles synthesized by chitosan-silver nanoparticles were mostly spherical in shape.[16]

Larvicidal activity

Mortality rate

The mortality rate of chitosan-based silver nanoparticles treated with larval tissues of III instar larvae of *Culex* and IV instar larvae of *Anophela* was shown in Table 2 within 24 h of exposure. The LC_{50} value was identified for *Culex* as 50.46 and *Anophela* as 33.80. The LC_{90} values were 616.9 and 181.9 for

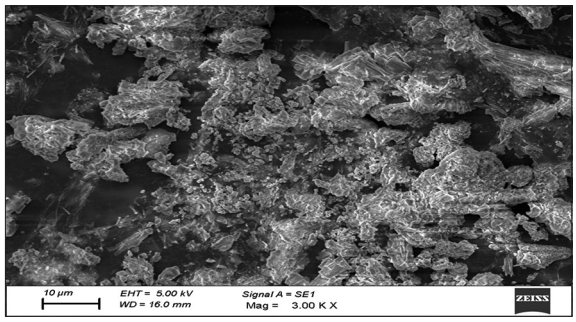

Figure 3: Scanning electron microscopy image of chitosan based silver nanoparticles

Culex and *Anophela,* respectively. There is a need for increasing the nanoparticle concentration to increase the mortality rate against well developed and higher stage larvae.[17]

Content of Protein, Carbohydrate, and Lipid

Protein levels in the larvae treated with Ch-AgNP were found to be decreased when compared to control. *Culex* and *Anophela* after treatment with Ch-AgNP (ppm) and control were shown in Tables 3 and 4. The protein content was reduced from 0.744 µg/g in control to 0.105 µg/g in 10 ppm of *Culex* and from 0.521 µg/g in control to 0.112 µg/g in 10 ppm of *Anophela*. Reduction of protein content in *Pericallia ricini* might be due to insecticidal inference of the extract with the hormones regulating protein synthesis. This view is supported by Ramakoteswara *et al.*[18]

Table 3: Effect of Ch-AgNP on protein, carbohydrate, and cholesterol content of *Culex* (III) instar larvae

Ch-AgNP (ppm)	Protein (µg/g)	Carbohydrate (µg/g)	Cholesterol (µg/g)
C	0.744±0.002	0.850±0.006	0.028±0.003
2	0.567±0.003	0.687±0.002	0.019±0.000
4	0.353±0.003	0.692±0.002	0.015±0.003
6	0.327±0.002	0.757±0.002	0.012±0.002
8	0.292±0.002	0.807±0.002	0.007±0.002
10	0.105±0.004	0.838±0.001	0.004±0.000

Values are mean±SD of three samples in each group. SD: Standard deviation

Table 4: Effect of Ch-AgNP on protein, carbohydrate, and cholesterol content of *Anophela* (IV) instar larvae

Ch-AgNP (ppm)	Protein (µg/g)	Carbohydrate (µg/g)	Cholesterol (µg/g)
C	0.521±0.003	0.86±0.002	0.069±0.003
2	0.319±0.003	0.635±0.004	0.044±0.004
4	0.284±0.004	0.658±0.009	0.041±0.000
6	0.264±0.003	0.679±0.003	0.034±0.004
8	0.256±0.004	0.726±0.004	0.026±0.002
10	0.112±0.002	0.762±0.002	0.015±0.004

Values are mean±SD of three samples in each group. SD: Standard deviation

Table 5: Levels of ACP, ALP, and LDH in the *Culex* (III) instar larvae treated with Ch-AgNP

Ch-AgNP (ppm)	ACP (IU/L)	ALP (IU/L)	LDH (IU/L)
C	1.402±0.002	1.750±0.005	0.163±0.002
2	1.487±0.002	0.669±0.004	0.099±0.004
4	1.460±0.005	0.442±0.002	0.065±0.004
6	2.58±0.004	0.340±0.004	0.054±0.004
8	0.909±0.003	0.231±0.002	0.048±0.003
10	0.596±0.003	0.225±0.005	0.031±0.003

Values are mean±SD of three samples in each group. SD: Standard deviation, ALP: Alkaline phosphatase, ACP: Acid phosphatase, LDH: Lactate dehydrogenase

Table 6: Levels of ACP, ALP, and LDH in the *Anophela* (IV) instar larvae treated with Ch-AgNP

Ch-AgNP (ppm)	ACP (IU/L)	ALP (IU/L)	LDH (IU/L)
C	1.634±0.005	1.265±0.003	0.156±0.04
2	1.432±0.002	1.586±0.004	0.115±0.005
4	0.842±0.002	1.038±0.003	0.129±0.003
6	1.334±0.004	0.745±0.004	0.109±0.012
8	0.924±0.004	0.676±0.004	0.094±0.004
10	0.386±0.004	0.224±0.004	0.075±0.005

Values are mean±SD of three samples in each group. SD: Standard deviation, ACP: Acid phosphatase, LDH: Lactate dehydrogenase

Carbohydrate (glucose) content of Ch-AgNP treated different strains of larval tissues of *Culex* and *Anophela* was tested with different ppm concentrations and the carbohydrate content in *Culex* control was 0.850–0.838 µg/g in 10 ppm and in *Anophela* control was 0.86–0.762 µg/g in 10 ppm was shown in Tables 3 and 4. The larvicidal activity was studied by Preeti *et al.*[17] in *Anopheline* and *Culicine* larvae by the leave extract of *Artemisia annua* and seeds of *Azadirachta indica* resulting in the reduction of glucose content.

A decrease in lipid content from 0.028 µg/g to 0.069 µg/g of control larval tissue was observed in *Culex* and *Anophela,* respectively. When this two larvae species treated with Ch-AgNP, it was decline to 0.004 µg/g in 10 ppm of *Culex* and to 0.015 µg/g in 10 ppm of *Anophela* was shown in Tables 4 and 5. The content of lipid was reduced when the concentration of toxic level increased. Similar type of results was observed in the study of larvicidal activity by Brindha *et al.*[19] in the species of *Aedes aegypti* when treated by TiO_2 nanoparticles.

Activity of Acid Phosphatase (ACP), ALP, and LDH

In the ACP assay, the ACP activity seems to be declined in both *Culex* and *Anophela* on the treatment with nanoparticles. The activity of enzyme level in both control and nanoparticles treated larvae of both the species was shown in Tables 5 and 6. Similar type of study was carried out by Hassan *et al.* (2018) using silver nanoparticles against *Aedes albopictus* larvae and pupae.

The levels of ALP in the larvae treated with Ch-AgNP have been represented in Tables 5 and 6. Activity of this enzyme in the larval tissues was decreased by the treatment of toxic agent. Our results are in accordance with the observation of Brindha *et al.*,[19] who reported the reduction of ALP level in *Aedes aegypti* larvae with the treatment of TiO_2 nanoparticles.

LDH is a biochemical parameter widely used in clinical area to diagnose damages in tissues and organs. LDH is involved in the production of energy and probably causes chemical stress.[20] The assay of total LDH content present in the *Culex* and *Anophela* larval tissues of both control and Ch-AgNP treated was taken. Tables 5 and 6 summarize the activity of LDH. The level of LDH was found to be downregulated at 10 ppm concentration in both species.

CONCLUSION

Chitosan was isolated from fish scales (*C. catla*). Extraction of chitosan was successfully done. From the biochemical test, the purple color confirmed the presence of chitosan. The percentage yield was received as 100 g and 6 g from the scales before and after chemical treatments, respectively. The functional groups of the components were separated based peak ratio through FTIR. The peaks 759.95/cm and 825.53/cm corresponds to N-H stretch bonds (primary and secondary amines) for chitosan. This indicated the presence of chitosan. The color changes from light white to light pink indicated the formation of chitosan-silver nanoparticles. After that, synthesized nanoparticles further characterized. The UV-visible spectrometer analysis showed an absorption band at 221 nm, which confirms the newly synthesized nanoparticles. SEM analysis shows that the chitosan-silver nanoparticles were spherical in shape. The LC_{50} value was obtained as 50.46 and 33.80 for *Culex* and *Anopheles,* respectively. LC_{90} value also calculated for both the species as 616.9 for *Culex* and 181.9 for *Anopheles.* Decreased levels of biochemical contents are indicating the high toxic capacity of chitosan-silver nanoparticles.

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