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Synthesis of chitosan nanoparticles from Tasar silkworm, *Antheraea mylitta* pupae and its antioxidant efficacy and antibacterial activity against fish pathogens

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Abstract

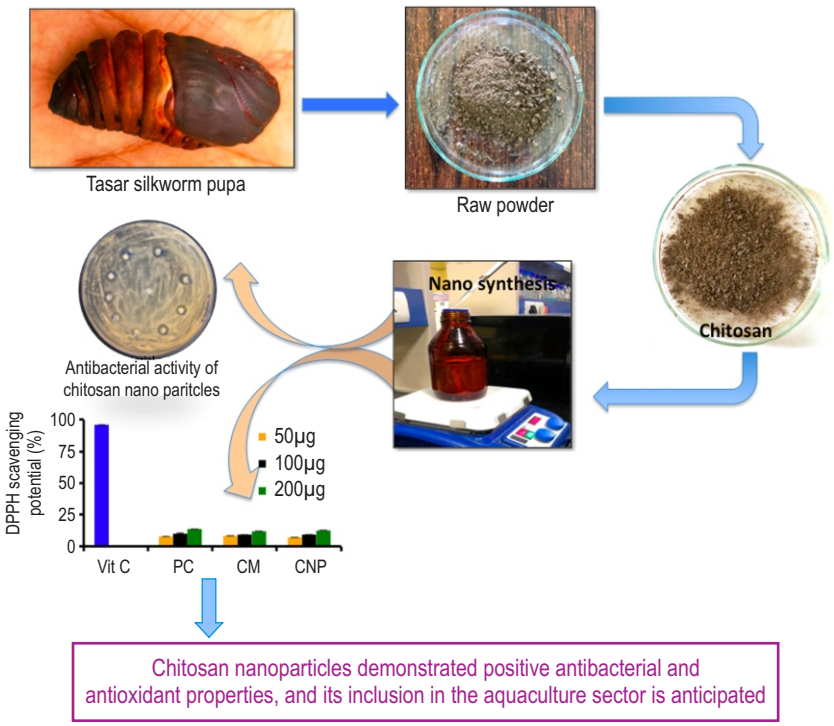
Aim: To synthesize and characterize chitosan nanoparticles (CNPs) from the pupae of tasar silkworm, *Antheraea mylitta*. The antioxidant and antibacterial activities of CNPs against different strains of selected bacterial fish pathogens were also evaluated.

Methodology: CNPs were prepared using sodium tripolyphosphate anions. Characterization of nanoparticles was done by FTIR, Zeta potential analysis and Particle size distribution analysis. The antibacterial activity of chitosan and CNPs against *Aeromonas hydrophila* (ATCC-7966), *Escherichia coli* (ATCC-25922), and *Staphylococcus aureus* (ATCC-25923) was evaluated by disc diffusion method.

Results: CNPs inhibited the growth of selected bacteria and the maximum zone of inhibition (14 mm) was observed against *E. coli* and *A. hydrophila*. Chitosan and CNPs had free radical scavenging effects on hydrogen peroxide (H_2O_2) and 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Interpretation: CNPs obtained from silkworm pupae have potential antibacterial as well as antioxidant activities and can be incorporated in aquafeed to prevent fish diseases.

Key words: *Aeromonas hydrophila, Antheraea mylitta*, Antibacterial activity, Antioxidant activity, Chitosan nanoparticles



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Introduction

Fish pathogens cause disease outbreaks and the excessive use of antibiotics is regarded crucial for the fishery sector, generally due to the emergence of certain antibiotic-resistant bacterial pathogens. So it is economically and ecologically essential to maintain healthy and disease-free farmed fish (Cabello *et al.,* 2013; Pelgrift and Friedman, 2013). Nanotechnology offers a cutting-edge method for using nanoparticles in brand-new applications for the diagnosis and treatment of fish diseases (Shaalan *et al.,* 2018; 2020). Chitosan alterations that have been reported over the past few years, such as synthesis of chitosan nanoparticles, have drawn a lot of interest because of their prospective applications, especially in terms of pharmaceutical industry (Ahmed and Aljaeid, 2016). Due to their accessibility, biocompatibility, biodegradability, high permeability, cost-efficiency, excellent gel-forming ability, lack of toxicity, antimicrobial, antioxidant, and even anti-tumor effects, chitosan nanoparticles are regarded as a high potential candidate for use as biomaterial applications. The well-founded polymeric and cationic characteristics of chitosan nanoparticles, as well as their small size and large surface-to-weight ratio and other favorable factors, all contribute to the interest in using them in the biomedical area.

Chitosan [poly-(b-1/4)-2-amino-2-deoxy-D-gluco-pyranose] is a biodegradable naturally occurring polysaccharide made up of β-(1-4) D-glucosamine and N-acetyl-D-glucosamine chains that are sporadically arranged. It can be synthesized by alkaline deacetylation of chitin (Du *et al.,* 2008). Numerous researchers have studied various methods for producing chitosan nanoparticles, including ionic gelation method, self-assembling (Liu *et al.,* 2005), ultrafine milling (Zhang *et al.,* 2012), reverse micelles (Banerjee *et al.,* 2002), and spray drying. The emergence of bacteria that are resistant to antibiotics has received a lot of attention lately. According to studies, microbial resistance has affected every single antimicrobial agent. Researchers have been motivated to create novel antimicrobial substances that affect harmful bacteria, which are growing increasingly resistant to antibiotics. However in this regard, there are no reports on the emergence of bacteria resistance to chitosan. Chitosan's polycationicity in acidic media, or the ability of its amino group to be protonated to NH^{3+} in an acidic environment and produce antifungal or antimicrobial activities, influences its antimicrobial properties in addition to some physico-chemical properties (Yilmaz and Hüsnügül, 2019). Additionally, chitosan is more effective at low pH and has antibacterial properties in an acidic medium (Qi *et al.,* 2004).

Use of antimicrobial treatments, including antibiotics, is being reduced by the use of nanomaterials with great antibacterial activity, which is regarded as the most sophisticated and promising reagents (Shaalan *et al.,* 2018). When compared with chitosan, CNPs demonstrates greater antibacterial activity against various strains of fungi, Gram-positive and Gram-negative bacterias (de Campos *et al.,* 2005; Qi *et al.,* 2004; Divya *et al.,* 2017; Xing *et al.,* 2021). Considerable emphasis has been paid on the antioxidant activity, particularly on the utilization of naturally occurring antioxidants to reduce oxidative damage, among other biological applications. Generally, the antioxidant activity of chitosan and its derivatives has attracted great attention (Rajalakshmi *et al.,* 2013). Several researchers have examined the antioxidant qualities of commercial chitosan made from sources other than silkworm pupae (Song *et al*., 2013; Wu *et al.*, 2019). However, it is difficult to obtain data on the antioxidant properties of pupal bioproducts. Most insects, including beetles, grasshoppers and silkworms are significant sources of chitosan. One of the main by-products of the reeling sector is silkworm pupae.

On a dry weight basis, it is estimated that 40,000 metric tonnes of pupae are generated yearly and are regarded as waste (Mahesh *et al.,* 2015). In the silk reeling sectors, discarding silkworm pupae is a serious problem where the silk reelers dump dead pupae after reeling out the silk, posing pollution and environmental risk. According to Suresh *et al.* (2012), silkworm pupae are a potential source of chitin who's deacetylated derivative, chitosan is more frequently used for commercial applications than chitin. Over the past few decades, the mulberry silkworm has been widely studied and used for its biomedical purposes. However, to produce biomaterials, the non-mulberry silkworm, *A. mylitta* is rarely reported, which is widely distributed throughout the nation in varied geoclimatic environments. This work focus on the potential antibacterial and antioxidant activities of chitosan and chitosan nanoparticles derived from *A. mylitta*, as a source of natural biomaterials that can efficiently work against the potential fish pathogens.

Materials and Methods

Extraction of chitosan: The silkworm pupal powder was obtained from collected pupae for extraction of chitin and chitosan. Three basic steps for extraction of chitin and chitosan are Deproteinization, Demineralization, and Deacetylation. Before deproteinization, the raw powder was treated with hexane for 48 hr in batches in 1:3 (w/v) ratio. The defatted sample was treated with 10% NaOH in 1:10 ratio (w/v) at 80° C for 24 hr with constant stirring (REMI, 1-MLH) at 250 rpm. The materials were then washed 4-5 times in deionized water to remove excess alkali. Hydrochloric acid (7%) in 1:10 (w/v) ratio was used for demineralization of silkworm pupae at ambient temperature for 24 hr. The material was washed properly and allowed to dry in a hot air oven for 12 hr at 50°C and finally chitin was obtained. Deacetylation was carried out by treating chitin with 55% NaOH solution for 9 hr at 90°C with constant stirring at 250 rpm. After washing and proper drying, the final product obtained was chitosan. Due to the presence of pigment, one additional step was conducted, Thereafter, the chitosan was properly dried in a hot air oven at 50° C for 10 hr for further studies.

Preparation of chitosan nanoparticles (CNPs): In the present work, CNPs were synthesized from the chitosan extracted from pupae of tasar silkworm using sodium tripolyphosphate as a

cross linking agent by ionotropic gelation method.It's a simplistic approach to chemical reaction that demands minimal organic solvents, can be carried out at room temperature and enables the non-toxic and effective encapsulation of fragile and delicate molecules. Chitosan powder with 1% acetic acid (5 mg ml^{-1}) was mixed properly using a magnetic stirrer for about 20 min. The pH was maintained in the range of 3.0-6.5. STPP solution was added drop wise to the above-prepared chitosan solution with continuous stirring and stored in a deep freezer until further processing.

Particle size and zeta potential: The size, charge, and distribution of silkworm CNPs were determined using the dynamic light scattering (DLS) technique (LENOVO, Litesizer 500). This characterization analysis was run in triplicate on diluted CNPs suspensions in deionized distilled water. At 25°C under 90° scattering angle, the DLS value was measured (Du *et al.,* 2008).

Fourier transform infrared spectroscopy(FTIR): The FTIR spectra were conducted in Central Instrumentation Facility, OUAT, Bhubaneswar. The CNPs were synthesized and characterized by (FTIR-spectro, PerkinElmer, Spectrum Two). The mid IR region of 400-4000 cm^{-1} was used for reading the FTIR spectrum. The sample was directly placed in the KBr crystal and the spectrum was measured in the transmittance mode.

Antibacterial activity test using single disc diffusion method: Antibacterial sensitive tests of chitosan (PC), chitosan nanoparticles (CNPs) from tasar silkworm pupae, and commercial chitosan (CM) of different concentrations were done using a single disc diffusion method (Chabbert, 1963). Selected fish pathogenic bacteria were grown in Nutrient broth and plated using a sterile swab, onto petridishes containing Muller Hinton Agar (Hi-media). Each sample, PC (100 µg $1ml^{-1}$), PC (500 µg 1 ml^{-1}), CM (100 µg $1ml^{-1}$), CM (500 µg $1ml^{-1}$), and CNPs (5 mg $1ml^{-1}$) was poured on a sterile paper discs measuring 6 mm diameter (Hi-Media). Discs with acetic acid were taken as control. Antibacterial activity of chitosan, as well as CNPs, was determined after 24 hr of incubation at 37°C by measuring the zone of inhibition (mm).

Antioxidant activity: The antioxidant activity of CNPs was estimated by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) method (Blois, 1958). The H_2O_2 scavenging power of chitosan and chitosan nanoparticles was determined following the method of Mukhopadhyay *et al.* (2016).

Statistical analyses: Results are presented as mean ± SD and the difference among the mean doses was tested for significance by Students t-test at probability level P≤ 0.05.

Results and Discussion

The chitosan nanoparticles from silkworm pupae were synthesized based on an ionic gelation interaction between negatively charged tripolyphosphate and positively charged chitosan at room temperature (de Campos *et al.,* 2001; Xu and Yumin, 2003). The hydrodynamic diameter of the particles was 27.46 nm and particle size 32.40 nm, with a narrow size distribution ranging from 70 to 140 nm (polydispersity index of 0.6). Hence, the small particle diameter range allows the nanomaterial to reach homogeneous equilibrium with respect to diffusion in a very short time as a result of high mobility in the solution under the influence of applied field and Brownian motion (Alnarabijiand Husein, 2020). The stability of mean zeta potential of CNP was shown at 25.0°C with a conductivity of 1.914 mS cm^{-1}, preventing the electrode polarization and degradation. The zeta potential values typically ranged from +100mV to -100mV, which is an important tool for understanding the state of nanoparticle surface and predicting the long-term stability of the nanoparticles. The results show that all these effects lead to a narrow range of sample concentrations that will yield a satisfactory quality result.

Characterization of chitosan and chitosan nanoparticles was carried out by FTIR studies and is shown in Fig.1. After deacetylation, FTIR spectra of chitosan and CNPs were performed at different vibrations. The FTIR spectra of CNPs sample showed that the functional groups and wavenumbers were changed due to reduction and stabilization. The combined peaks of NH_2 and OH group stretching vibration in the chitosan sample were observed at a band of 3298 cm^{-1}. The band at 1635 cm^{-1} is attributed to the $CONH_2$ group. The 1586 cm^{-1} peak of γ (NH_2) bending vibration was sharper than the peak at 1635 cm^{-1}, which shows high degree of deacetylation of chitosan. The peak was sharper in the chitosan nanoparticles and a shift from 3298 to 3326 cm^{-1} was observed, which indicated enhanced hydrogen

Table 1: Antibacterial activity of chitosan and chitosan nanoparticle from tasar silkworm pupae against selected fish pathogens

Chitosan and Chitosan nano particles	*E. coli* (ATCC-25922)	*S. aureus* (ATCC-25923)	*A. hydrophila* (ATCC-7966)
		Zone of inhibition (mm)	
PC (0.1 mg ml^{-1})	15	-	12
PC (0.5 mg ml^{-1})	14	-	12
CM (0.1 mg ml^{-1})	13	-	14
CM (0.5 mg ml^{-1})	12	-	13
CNPs (5 mg ml^{-1})	14	12	14

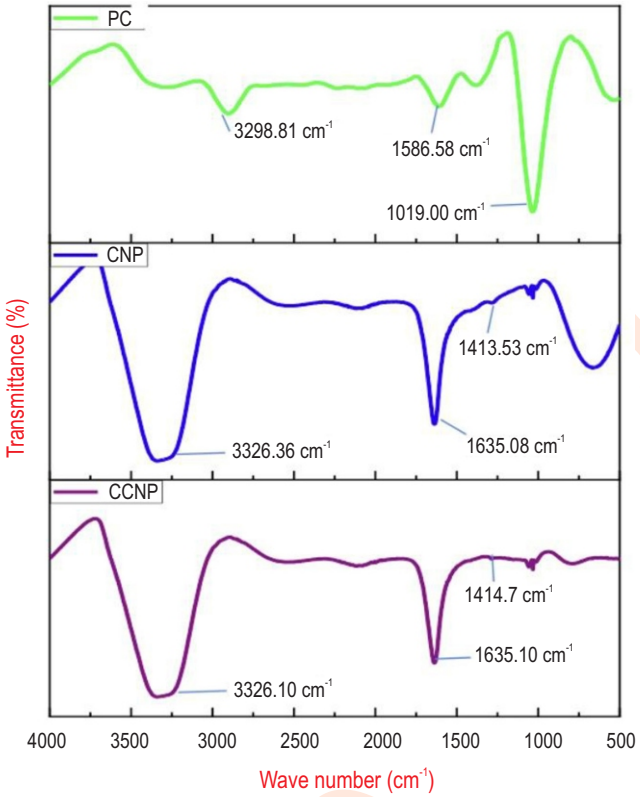

Fig. 1: FTIR comparison of PC (Pupae chitosan) and CNP (Pupae chitosan nanoparticles) and CCNP (commercial chitosan nanoparticles).

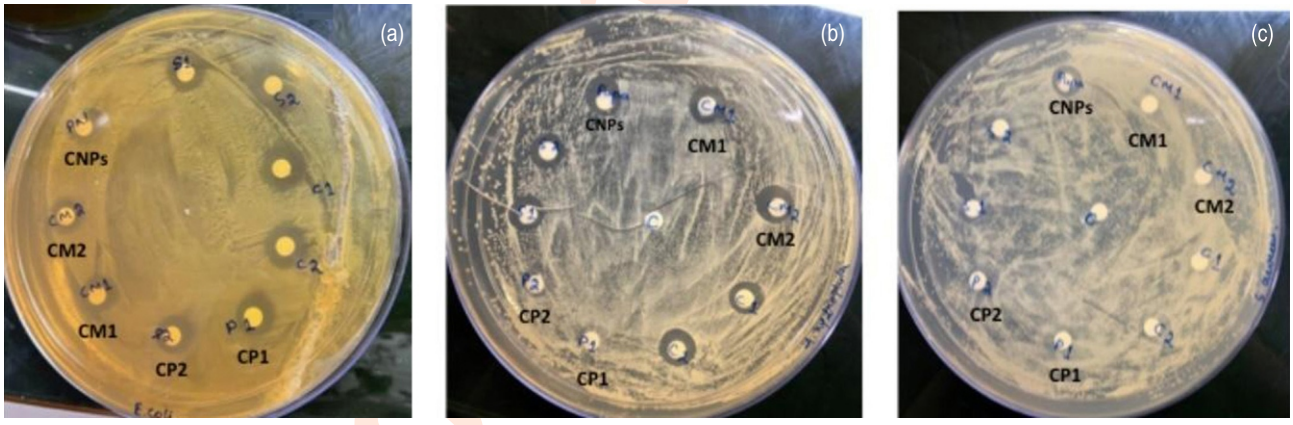

Fig. 2: Antibacterial activity of chitosan and CNPs against (a) *E. coli*, (b) A. hydrophila and (c) *S. aureus*.

bonding. Two new absorption bands appeared at 1635 and 1413.53 cm^{-1}, which indicates the cross-linking of ammonium groups with tripolyphosphate molecules (Yongmei and Yumin, 2003). Thus, the interaction of the ammonium groups of chitosan with polyphosphoric groups of sodium polyphosphate provides enhancement of both inter-and intramolecular interaction in CNPs. It is interesting to note that previous studies have also reported similar findings regarding the inhibitory effects of chitosan nanoparticle and copper-loaded nanoparticles on the growth of various bacterial species such as *E. coli*, *S. choleraesuis*, *S. typhimurium*, and *S. aureus*. The study by Qi *et al.* (2004) provides additional evidence to support the potential use of these nanoparticles as antimicrobial agents. The antibacterial activity of both chitosan and CNPs of different

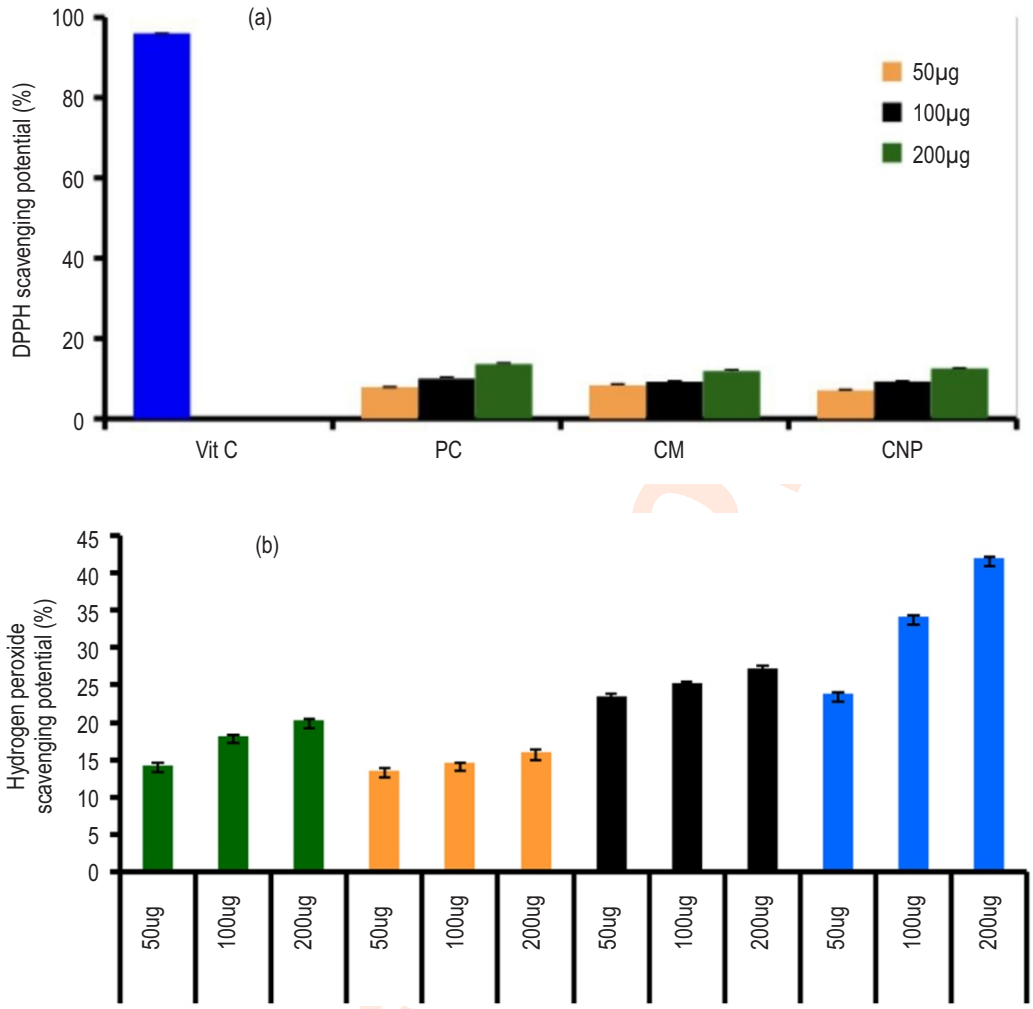

Fig. 3: Scavenging potential (%) of chitosan and CNPs of tasar silkworm pupae (a) DPPH and (b) Hydrogen peroxide.

concentrations was studied against selected fish pathogens. The results are represented in Table 1 and Fig. 2. Both commercial chitosan (CM) and chitosan of pupae (PC) of different concentrations showed zones of inhibition against *E. coli* (ATCC-25922) and *A. hydrophila* (ATCC-7966), whereas The maximum zone of inhibition (14, 12, and 14 mm) was noticed in chitosan nanoparticle (CNPs) against *E. coli* (ATCC-25922), *S. aureus* (ATCC-25923), and *A. hydrophila* (ATCC-7966), respectively. Different concentrations of extracted chitosan samples exhibited no antibacterial activity against Gram-positive bacteria, *S. aureus* (ATCC-25923). Several factors influence the antibacterial activity of chitosan, where the polycationic structure of chitosan is a necessity. The most common mechanism of antibacterial activity of chitosan is disruption of cell membrane, due to binding of negatively charged bacterial cell surface (Nagy *et al.,* 2011; Yilmaz and Hüsnügül, 2019). Another possible mechanism is that chitosan acts as a chelating agent and produces toxins that ultimately inhibit microbial growth (Divya *et al.,* 2017) whereas the CNPs exhibit superior activities for their small size and quantum size effect. Qi *et al.* (2004) evaluated the antibacterial activity of chitosan nanoparticles and copper-loaded nanoparticles against *S. typhimurium*, *S. choleraesuis, S. aureus*, and *E. coli*. A similar study reported the antibacterial potency of chitosan nanoparticles against *S. aureus, E. coli* and *Pseudomonas aeruginosa* (Bangun *et al.,* 2018). Generation of free radicals occur either during the normal metabolic process or from exogenous factors, and membrane lipids peroxidation initiates easily, leading to the accumulation of lipid peroxides (Halliwell and Gutteridge,1990) Hydrogen free radical of DPPH shows a characteristic absorption at 517 nm (DiMambro *et al.,* 2003; Lin and Chou *et al.,* 2004).

The decrease in its absorbance at 517 nm determines the reduction capability of DPPH, which is induced by antioxidants. In this study, DPPH was used to determine the free radical scavenging activity of commercial chitosan (CM), pupae chitosan (PC), and chitosan nanoparticles (CNPs). The total DPPH

scavenging capability of chitosan and CNPs at different concentrations (50 µg, 100 µg, and 200 µg) is shown in Fig. 3(a).The declining colour intensity was directly proportional to the DPPH inhibition. The present study reveals that the increasing concentration of chitosan and CNPs suppresses the DPPH activity. The maximum inhibition was observed at 200 µg. Both chitosan and CNPs were found to be equally satisfactory in the DPPH radical-scavenging activities. The scavenging activity of hydrogen peroxide, at various concentrations, shows the oxidative damage induced by Fe^{3+}/ H_2O_2 on deoxyribose, which is plotted in Fig. 3(b).The commercial chitosan (CM) shows nearly 20% inhibition at the highest concentration (200 µg), and nearly 16% was noticed in pupae chitosan. Whereas chitosan nanoparticles exhibited 27% inhibition at maximum concentration, and 42% was observed in standard Ascorbic acid. The reducing power of chitosan and chitosan nanoparticles increased with the increasing concentration (Zhang *et al*., 2008; Hosseini *et al*., 2018). The present study manifests the H_2O_2 scavenging potential, while ascorbic acid showed more effectiveness to scavenge H_2O_2 due to electron donor, which accelerates the conversion of H_2O_2 to H_2O .

Conclusion: Chitosan extracted from tasar pupae are effective antimicrobial and antioxidant agents. The attention has been focused on chitosan nanoparticles due to its non-toxicity, biocompatibility, biodegradability, accessibility, and high permeability. In the current study, the chitosan and the chitosan nanoparticle was extracted from silkworm pupae, which was synthesized and characterized. Aquaculture industry can utilize this biopolymer since it has been shown to have antioxidant and antibacterial properties along with physico-chemical and functional properties against potential fish pathogens. Further investigation of chitosan nanoparticles in an *in vivo* model is required. The focus has been on the capability of chitosan nanoparticles to develop novel and more potent antimicrobial compounds.

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Authors' contribution: B. Baisakhi: Performed experiment, investigation, formal analysis of the data and drafting of manuscript; **D. Mohanty:** Performed experiment and investigation; **B.K. Das:** Conceptualization and investigation; **S. Ananta:** Performed experiment and investigation; **K. Jena:** Conceptualization, investigation and supply of samples; **J. Pradhan:** Conceptualization, investigation, formal analysis of data and drafting of manuscript.

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