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Evaluation of chitosan and chitosan silver nanocomposites against bacterial pathogens of tropical tasar silkworm, *Antheraea mylitta Drurry*

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Abstract

Aim: The present research work was carried out to evaluate the effect of commercially available shrimp chitosan and chitosan silver nanocomposites (CSN) on the inhibition of bacterial pathogens of tropical tasar silkworm.

Methodology: Synthesis of chitosan silver nanocomposite was by carried by using chemical precipitation method. The synthesized nanocomposites were characterized using different techniques. Further, antimicrobial potential of the chitosan and chitosan silver nanocomposites was evaluated by *in vitro* inhibition assay. The minimum inhibitory concentration of both chitosan and chitosan nanocomposites was estimated against bacterial pathogens of tasar silkworm.

Results: Chitosan at different concentrations showed antibacterial activity against the bacterial pathogens (*Bacillus* spp., *Micrococcus* spp., and *Serratia* spp.) of tropical tasar silkworm under *in vitro* conditions. The 0.2% concentration of chitosan and CSN was identified as a minimum inhibitory concentration (MIC) against bacterial pathogens.

Interpretation: Based on the findings of this study, the 0.2% concentration of CSNs may be considered as minimum inhibitory concentration against bacterial pathogens infecting tropical tasar silkworms.

Key words: Bacterial pathogens, Chitosan silver nanocomposites, Tropical tasar silkworm

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Introduction

Tasar silkworm is infected by microsporidia, bacteria, fungi, and viruses which causes yield loss of up to 40% (Sahay *et al.,* 2000). Different types of symptoms were observed in Tropical tasar silkworms *viz.,* sealing of anal lips, rectal protrusion, and chain type excreta. The development of control measures against bacterial diseases is of prime importance in the existing scenario. The antimicrobial property of silver has been thoroughly investigated in comparison with any other inorganic antibacterial agent (Russell and Hugo, 1994). Silver damages different biological processes in microorganism by altring the cell membrane structure and functions and expression of proteins associated with ATP production (Yamanaka *et al.,* 2005), although its specific antimicrobial mechanisms are still not completely understood. Silver nanoparticles exhibit interesting properties such as tunable size, shape, ease of surface modifications and ability of silver ions release which makes them suitable for obtaining materials with designed and required properties. Silver, gold and copper nanoparticles are considered as agents of high antibacterial activity against many dangerous and resistant bacterial strains. Silver nanoparticles constitute a promising alternative in fighting down chronic bacterial infections, which already exist in antibacterial commercial products based on AgNPs (Takai *et al.,* 2002).

Chitosan an abundant natural polymer consists of β-1,4-linked N-acetyl-D-glucosamine and D-glucosamine units. Depending on the conditions of deacetylation process (deacetylation of chitin provides chitosan), different forms of chitosan are available. Those forms vary in the deacetylation degree (indicating the number of free amino groups) and the average molecular weight of the polymer (Dash *et al.,* 2010). Applications of chitosan are a consequence of its biodegradability, biocompatibility, and non-toxicity. Moreover, chitosan exhibits antibacterial activity against both, Gram-positive and Gram-negative bacterial strains (Raafat and Sahi, 2009). Its activity has been demonstrated in many bacteria, fungi, and yeasts (Fang *et al.,* 1994). The exact mechanism of the antibacterial action of chitosan has not been elucidated yet. There is a hypothesis stating that the source of chitosan antimicrobial activity is its polycationic nature. The interactions between protonated amino groups and negatively charged microorganisms' cell wall components causes leakage of the intracellular components. Higher the density of positive charge (higher deacetylation degree), the stronger is antimicrobial action (Takahashi *et al.,* 2008).

The ability of chitosan metal ions complexation deprives the bacterial cells of essential components (Verma *et al.,* 2020). Polymeric nanocomposites are advanced, functional materials based on the nanoparticle's finely dispersed polymeric matrix or/and are covered with polymer (core-shell structures). Most of the practical applications of AgNPs demand polymeric or other outer layers for nanoparticles. Polymers are usually the most suitable matrix due to specific surface morphology, chain structure enabling nanoparticle incorporation, and their homogenous dispersion. Functional groups of polymers are responsible for nanoparticle synthesis (chemical reduction) (Dallas *et al.,* 2010). Nanoparticle aggregation or modifications after connection with physiological fluids is prevented due to permanent connection between AgNPs and polymeric matrix (Kumar, 2000). Chitosan stabilization provides long-term stability of materials. Due to low toxicity, chitosan seems to be an ideal choice as a reducing and stabilizing agent, and the main component of nanocomposites.

Another important advantage of chitosan usage as a matrix is its bacteriostatic activity. Biocide activity of AgNPs in connection with bacteriostatic chitosan increase the chance of success in fighting bacterial infections. The synergic antibacterial action of chitosan-silver nanocomposites has been proved to fully exploit (Potara *et al.,* 2011). According to application, chitosan-silver nanocomposites occur in different forms: colloids, powders or membranes (Thomas *et al.,* 2009). The present research work was carried out to evaluate the effect of chitosan silver nanocomposites for the inhibition of bacterial pathogens of tropical tasar silkworm.

Materials and Methods

Subculture of pathogens: The bacterial isolates *Bacillus* spp., *Micrococcus* spp. and *Serratia* spp., which are pathogenic to tropical tasar silkworms, were pre-maintained in the Silkworm Pathology Section of Central Tasar Research and Training Institute, Piska Nagri, Jharkhand (INDIA). They were sub-cultured on the Nutrient Agar (NA) media following the standard inoculation method (Murali *et al*., 2010; Badway *et al*., 2019).

Synthesis of chitosan silver nanocomposite: Chitosan silver nanocomposite (CSN) were synthesized by adding chitosan (200mg) to beakers containing a 200ml aqueous silver nitrate solution (1 mM) in a magnetic stirrer at 90±1°C with constant stirring. Freshly prepared 0.3 M NaOH (0.4 ml) was added to the reaction mixture. The formation of AgNPs was observed by a gradual change in color of the reaction to yellow. The reaction was continued for 25 min, and the solution was cooled to room temperature. The nanocomposite powder at the bottom of the beaker was separated through Whatman No 1 filter paper and washed thrice with Milli-Q water. The powder was air-dried and stored for further studies.

Characterization of nanoparticles: The synthesized nanocomposite was subjected to Ultraviolet-visible (UV–vis) absorption spectra at room temperature using Motras Scientific Instruments UV Plus UV/VIS Spectrometer (India) at 300–700 nm. The Zeta potential of the synthesized nanoparticles was measured Malvern Zetasizer instrument at 25°C. The size and morphology of the NPs were studied by Field Emission Scanning Electron Microscope (FESEM). To prepare the samples for SEM, a drop of the aqueous solution of synthesized AgNPs was drop-cast on an aluminum stub with double-sided copper or carbon

tape, then it was dried under ambient temperature for three hours. The samples were dried for two days. To prevent the sample from being charged during the analysis, gold sputtering was done on the sample's surface. The samples were observed using a FESEM (Zeiss-Ultra 55 model; Carl Zeiss; Germany) equipped with a dispersive energy X-ray (EDX) spectrometer.

Preparation of chitosan and CSN solutions: The stock solutions of chitosan (2%) and chitosan silver nanocomposite (CSN) were dissolved in 0.5% (v/v) aqueous acetic acid and diluted to obtain the required stock. The pH of the chitosan and chitosan-silver nanocomposites solutions was adjusted to 5.5–6.0 with 1 M NaOH.

***In vitro* inhibition study:** Pathogenic bacterial suspension of *Bacillus* spp., *Micrococcus* spp. and *Serratia* spp. were prepared in sterilized distilled water and the initial concentration of bacteria was adjusted to approximately 10^8 colony-forming units (CFU) ml^{-1}. The suspension of microbial cultures was inoculated on the entire surface of the Nutrient agar media in a Petri plate using sterile swab sticks. The sterile discs of diameter 6 mm were impregnated with chitosan and CSN solutions (0.1, 0.2, 0.5 and 1.0% with and without binder) along with ampilicin which served as positive control and placed onto the cultured Nutrient agar plates and incubated at 37°C for 24 hrs. The zone of inhibition around each disc was measured (mm). The experiment was conducted in triplicate. The promising concentration of composite was used for further studies. The promising concentration of the chitosan-silver composite was screened against all pathogenic bacteria species by gel diffusion method to confirm the minimum inhibition concentration (MIC).

Data analyses: For data analyses, the statistical computer application package SPSS 23.0 was employed. The data generated were an average of three independent experiments on antimicrobial activity. Data were subjected to Analysis of variance (ANOVA) and the means were compared for significance using Duncan's Multiple Range Test (DMRT; P = 0.05).

Results and Discussion

The tasar silkworm, *Antheraea mylitta* Drurry. is an economically important insect. Diseases are the major threats to successful crops in the tasar industry. Among the diseases, bacterial diseases are causing considerable yield loss to the tasar silk farmers (Sahay *et al.,* 2000). The nanoparticles can be designed for specific application which possesses unique physico-chemical, optical, and biological properties (Feynman, 1991). Inorganic nanoparticles have gained significant importance due to their ability to withstand adverse processing conditions (Whitesides, 2003). The surface area of nanoparticles influences the antimicrobial activity. Higher antimicrobial activity was noticed in small particles due to high particle penetration in comparison with big particles (Badway *et al*., 2019).

Studies have reported that nanosized silver particles exhibit antimicrobial properties (Rai *et al.,* 2009). The results of the present study showed that nanocomponents present in the chitosan-silver nanocomposites resulted in antimicrobial activity against bacterial pathogens of tropical tasar silkworm. Different mechanisms have been explained to study the inhibitory effect of silver nanoparticles on bacteria *viz.,* disruption of cell membrane affecting bacterial cell viability, DNA replication inactivation, and enzyme functions inhibition (Holt and Bard, 2005; El-Maadawy *et al*., 2022). The silver nanoparticle (less than 20 nm diameters) gets adhered to sulfur-containing proteins of cell membranes of

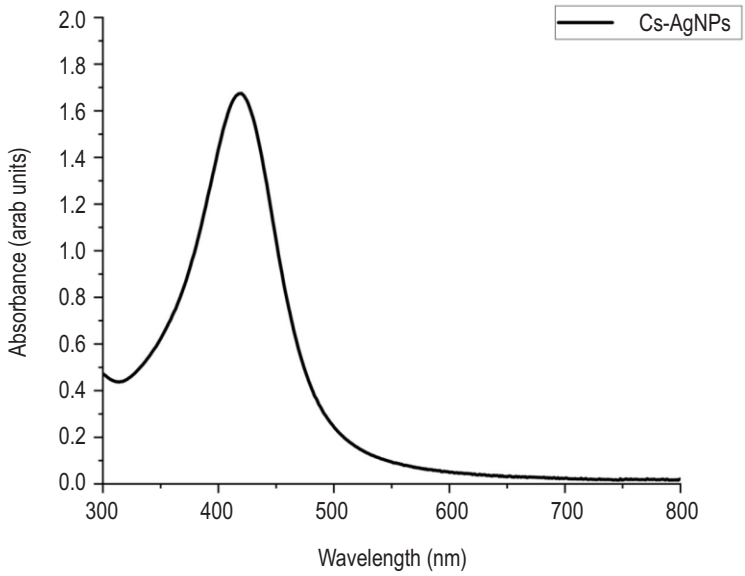


Fig. 1: UV-Vis absorbance spectra of CSN dispersed in 0.1% acetic acid in water.

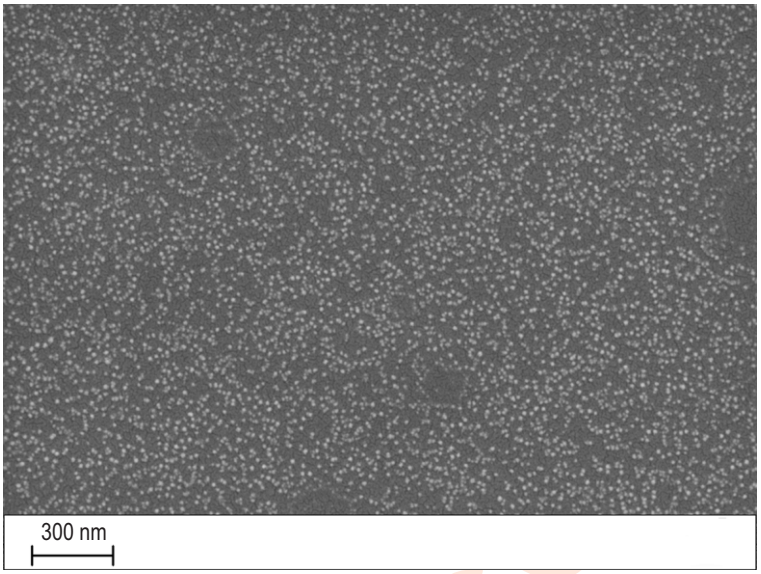


Fig. 2: SEM micrograph of CSN at 300 K X magnification.

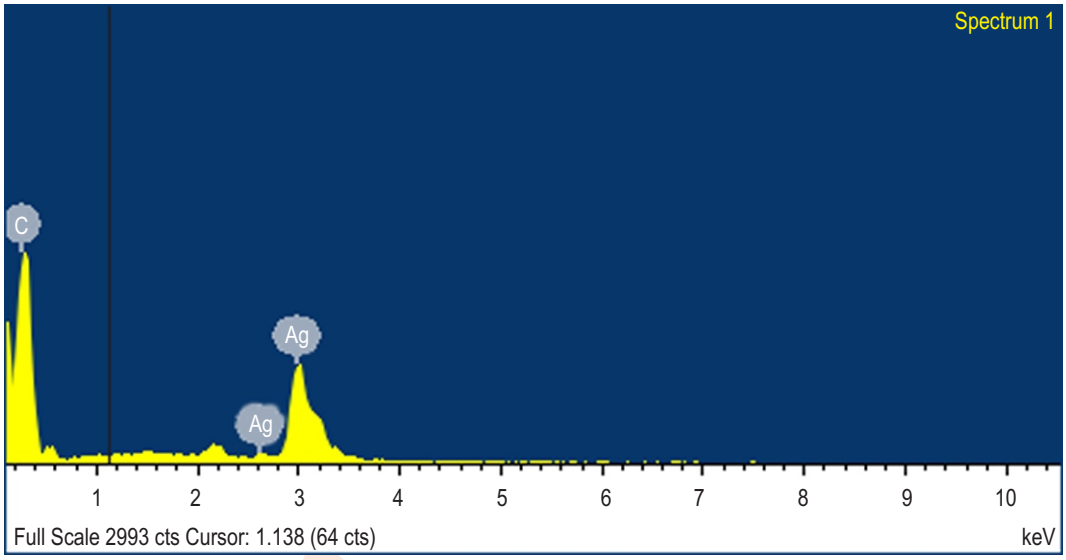


Fig. 3: EDS spectra for CSN.

bacteria that increase permeability of the membrane, which leads to death of the bacteria (Morones *et al.,* 2005).

The CS-AgNps were insoluble in water, so it was dispersed in 0.2% acetic acid solution, and UV-Visible spectroscopic analysis was conducted. The spectra displayed a strong peak at 420 nm (Fig. 1), the characteristic surface plasmon resonance (SPR) band of AgNPs, indicating the formation of silver nanoparticles. The zeta potential of synthesized nanoparticles was + 57.78 mV. The synthesized nanoparticles were stable at ambient conditions for six months without any sign of aggregation. The FESEM analysis showed the production of small spherical AgNPs (Fig. 2). The EDX analysis of nanocomposite showed a prominent silver peak, indicating the formation of AgNPs (Fig. 3). The XRD pattern also confirms the presence of two peaks corresponding to chitosan and silver (Fig. 4). Due to the presence of functional groups (amino and hydroxyl), chitosan shows many interesting properties. Application of chitosan are a consequence of its biodegradability, biocompatibility and non-toxicity. Moreover, chitosan exhibits

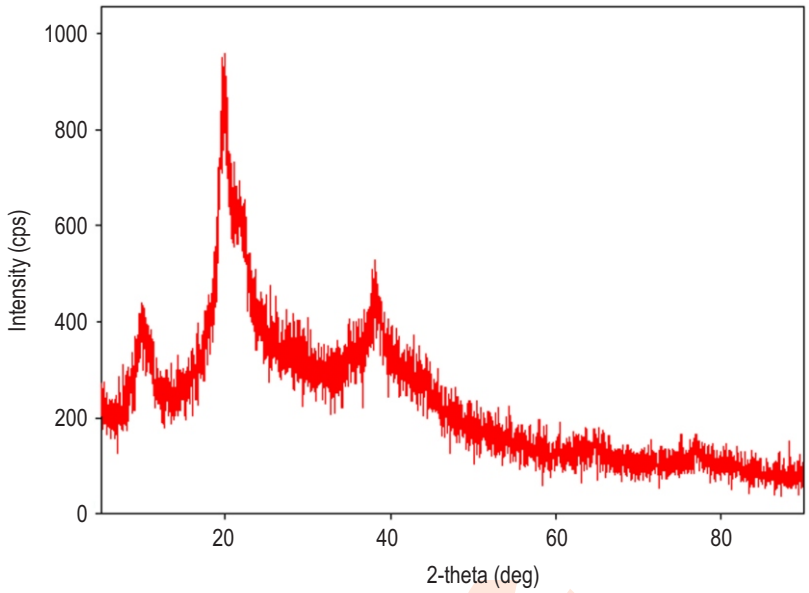
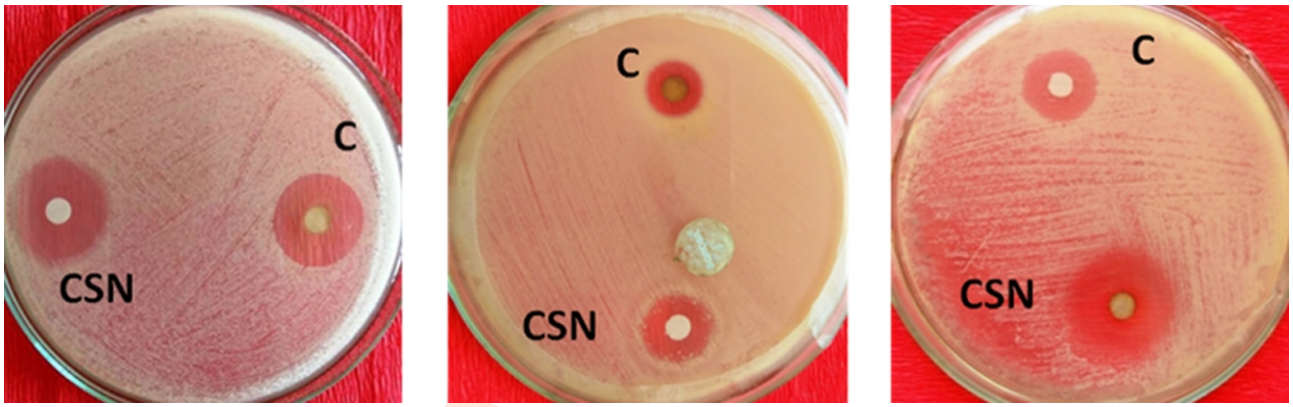


Fig. 4: XRD pattern of CSN



Bacillus spp. (CTE) *Micrococcus* spp. (RP) *Serratia* spp. (SAL)

Fig. 5: Representative photographs showing the zone of inhibition against bacterial pathogens infecting the tropical tasar silkworm by using chitosan-silver nanocomposites.

antibacterial activity against both, Gram-positive and Gram-negative bacterial strains (Kumar, 2000). In the present study, the antibacterial activity of chitosan was evaluated against bacterial pathogens. The results revealed that the chitosan showed antibacterial activity against pathogenic bacterial species tested in the present study (Table 2).

All the concentrations of chitosan showed the inhibition of pathogens on par with the standard (Ampicilin 0.1%). Among the different concentration used in the experiment, 0.2% chitosan showed maximum inhibition of pathogens in comparison with other treatments (Table 1). Different concentrations of chitosan-silver nanocomposites showed more inhibition of all pathogenic bacterial species in comparison with only chitosan treatment (Table 1,2). All the concentrations of composites showed the inhibition of pathogens on par with the standard (Ampicillin 0.1%). Among the different concentrations used in the experiment, 0.2% concentration of chitosan showed maximum inhibition of pathogens in comparison with different treatments used in this experiment (Table 1; Fig. 5). Chitosan-silver nanocomposites showed more zone of inhibition in comparison with chitosan and positive control (Table 3). Based on the observation made in this study, the 0.2% concentration of chitosan-silver nanocomposites can be considered as Minimum Inhibitory concentration against

Table 1: Inhibition of different pathogenic bacteria by chitosan

Concentrations	Zone of inhibition (mm)		
	***Bacillus* spp. (CTE)**	***Micrococcus* spp. (RP)**	***Serratia* spp. (SAL)**
0.1	1.21±0.35^{d}	1.53±0.23^{d}	1.29±0.35^{c}
0.2	1.74±0.45^{a}	1.91±0.27^{a}	1.59±0.25^{b}
0.5	1.22±0.25^{d}	1.72±0.37^{c}	1.33±0.27^{c}
1.0	1.53±0.55^{c}	1.84±0.45^{b}	1.32±0.37^{c}
Standard (Ampicilin)	1.69±0.75^{b}	1.74±0.25^{c}	1.72±0.35^{a}

Values represent mean of three replications ±S.E.; Values with different small alphabets are significantly different according to Duncan's Multiple Range Test (DMRT, (P<0.05) (SAL= Sealing of Anal lips, CTE= Chain type excreta, RP= Rectal protrusion).

Table 2: Inhibition of different pathogenic bacteria by chitosan-silver nanocomposites

Concentrations	Zone of inhibition (mm)		
	***Bacillus* spp. (CTE)**	***Micrococcus* spp. (RP)**	***Serratia* spp. (SAL)**
0.1	1.27±0.57^{c}	1.47±0.27^{d}	1.22±0.57^{c}
0.2	1.89±0.33^{a}	1.93±0.37^{a}	1.69±0.75^{b}
0.5	1.26±0.27^{c}	1.66±0.24^{c}	1.30±0.53^{c}
1.0	1.57±0.27^{b}	1.79±0.19^{b}	1.31±0.33^{c}
Standard (Ampicilin)	1.59±0.53^{b}	1.65±0.25^{c}	1.72±0.27^{a}

Values represent mean of three replications ±S.E.; Values with different small alphabets are significantly different according to Duncan's Multiple Range Test (P<0.05) (SAL= Sealing of Anal lips, CTE= Chain type excreta, RP= Rectal protrusion).

Table 3: Comparative study on inhibition of different pathogenic bacteria between chitosan and chitosan-silver nanocomposites

Treatments	Zone of inhibition (mm)		
	***Bacillus* spp. (CTE)**	***Micrococcus* spp. (RP)**	***Serratia* spp. (SAL)**
Chitosan (0.2%)	1.49±0.33^{c}	1.37±0.37^{c}	1.42±0.75^{c}
Chitosan-Silver Nanocomposites (0.2%)	1.98±0.75^{a}	1.94±0.75^{a}	1.93±0.75^{a}
Standard (Ampicilin)	1.59±0.66^{b}	1.65±0.25^{b}	1.72±0.25^{b}

Values represent mean of three replications with standard error, values with different small alphabets are significantly different according to Duncan's Multiple Range Test (P<0.05) (SAL= Sealing of Anal lips, CTE= Chain type excreta, RP= Rectal protrusion).

test bacterial pathogens. Polymeric nanocomposites are advanced, functional materials based on the nanoparticles finely dispersed polymeric matrix or/and covered with polymer (core-shell structures). Most of the practical applications of AgNPs demand polymeric or other outer layers for nanoparticles (Zhao *et al*., 2022).

Polymers are usually the most suitable matrix due to specific surface morphology, chain structure enabling nanoparticle incorporation, and their homogenous dispersion. In some cases, functional groups of polymer may be responsible for nanoparticle synthesis (chemical reduction). Designing hybrid nanomaterials, which involve different antibacterial activity mechanisms, is ingenious but not effortless. `Due to the permanent connection between AgNPs and polymeric matrix it is possible to omit a problem of nanoparticles aggregation or undesired modifications after contact with physiological fluids. Chitosan stabilization provides long-term stability of materials. Due to low toxicity, chitosan seems to be an ideal choice as a reducing and stabilizing agent and major component of nanocomposites. Another important advantage of chitosan usage as a matrix is its bacteriostatic activity (Dallas *et al.,* 2011). The synergic antibacterial action of chitosan-silver nanocomposites has been proved. The chitosan and its derivates neutralizes the negative charge of the microbes which causes the distruption of cells which alters membrane permeability and damages the

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genetic material of the microbes. In the present study, the efficacy of chitosan-silver nanocomposites was evaluated against the bacterial pathogens of tasar silkworm. The results revealed that the chitosan-silver nanocomposites showed more zone of inhibition against all three pathogens tested in the present study (Table 3).

Based on the result of this study, the 0.2% of Chitosan-silver nanocomposites may be considered as Minimum Inhibitory concentration against bacterial pathogens infecting tropical tasar silkworms. Formulation of chitosan silver nanocomposites can be evaluated under outdoor conditions.

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Authors' contribution: K.N. Madhusudhan: Characterization of nanocomposites and antimicrobial activity; **Shivakumar:** Synthesis and characterization of nanocomposites; **V.P. Gupta:** Analysis of results of antibacterial activity; **A.H. Naqvi:** Analysis of results; **K.P. Kirankumar:** Analysis of results; **Babulal:** Analysis of results.

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Data availability: Not applicable.

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