

# SQUID PENS MEDIATED SILVER NANOPARTICLES SYNTHESIS, ITS CHARACTERISATION, AND BIOLOGICAL ACTIVITIES

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## ABSTRACT

This research scintillates the synthesis of silver nanoparticles from chitosan (C-AgNps) from squid pens as a reducing and stabilizing agent and assesses its antioxidant, antibacterial, and anticancer potentials. The nanoparticles were 240 to 290 nm with excellent stability of +98.2 mV. The C-AgNps possessed an excellent 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity with an IC<sub>50</sub> of 6.65 µg/mL. The C-AgNps showed exceptional antibacterial properties against *S. aureus*, *B. subtilis*, *P. aeruginosa*, and *E. coli* using a good diffusion assay. The C-AgNps also showed outstanding anticancer properties against A375 human skin malignant melanoma. Thus, the C-AgNps could be used as antimicrobial and anticancer agents for novel therapeutics after lead optimization and *in-vivo* clinical trials.

**Keywords:** Chitosan, Silver Nanoparticles, Characterization, Antioxidant, Antibacterial, Anticancer.

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## INTRODUCTION

Marine organisms act as an excellent renewable reducing agent of nanomaterials, which constitutes seashells, pearls, seaweeds, sponges, and fish bones as reducing agents.<sup>1</sup> Recently, attention has been focused on metallic nanoparticles such as silver, copper, gold, titanium, and zinc oxide for versatile applications. Nanomaterials range from 1 to 100 nm with better physical, chemical, optical, electrical, and catalytic properties than bulk materials.<sup>2,3,4</sup> The green synthesis of nanomaterials is rapid, sustainable, and environment-friendly and is often preferred over the other conventional methods. The bioactivities and physicochemical properties of silver nanoparticles highly depend on their shape, size, precursor, agglomeration, capping agents, properties, and reducing agents used, especially for biological activities. The nanoparticles need to be small, non-aggregative and have excellent physicochemical properties.<sup>5</sup> Silver nanoparticles have emerged as a product of broad antimicrobial activity and low toxicity towards mammalian cells and have gained pace.<sup>6,7,8</sup> Chitin, a polymer abundant in marine organisms such as crustaceans, cephalopods, and molluscs, is made of poly-β-(1-4)-N-acetyl-D-glucosamine; in squid pen, it is evidenced as cellulose-containing -OH at position C-2 altered by an acetamido group due to the presence of nitrogen. It has been classified into α-, β- and γ- variants. α-chitin is arranged in antiparallel, which has robust inter- and intramolecular hydrogen bonds and is usually obtained from the exoskeleton of shrimps and crustaceans. β-chitin is arranged in parallel with fragile intramolecular hydrogen bonds extracted from squid pens, and γ-chitin is a combination of parallel and antiparallel strands from microorganisms.<sup>9</sup> Chitin is not soluble in many organic and inorganic solvents, which hinders its extensive utilization. The deacetylated chitosan form of chitin, having contained amino moieties and is soluble in weak solvents like dilute acetic acid. Chitosan finds application in diverse areas, including food technology, nutraceuticals, cosmetics, agriculture, and medicine.<sup>10,11</sup> Standard extraction of chitosan from squid pen consists of demineralization, deproteinization, and deacetylation are the standardized procedure for converting chitin to chitosan.<sup>12</sup> Chitosan shows antibacterial activity, antioxidant, and anticancer activity and acts as a chelator in the biological system.<sup>13</sup> The main objectives of the present studies include the extraction of chitin from squid pens and its deacetylation to chitosan. The chitosan was then used as stabilizing and

reducing agent to synthesize C-AgNps and confirm using physicochemical characterization techniques. The resultant C-AgNps were assessed for antibacterial activities against microbes: *S. aureus*, *B. subtilis*, *P. aeruginosa*, and *E. coli*. Moreover, the nanoparticles were tested for the DPPH antioxidant and anticancer properties against human malignant melanoma cells.

## EXPERIMENTAL

### Materials and Methods

The reagents purchased were of high-purity analytical grade. The microbiological and cell media were purchased from HiMedia Laboratories, Mumbai.

### Collection of Squids

Fresh dead squids were procured from the neighboring fish markets in Chennai, Tamil Nadu. The collected squids were transported to the University laboratory and washed with tap water, then with de-ionized water. Squid pens were isolated from the squids, shade-dried for five days at room temperature, and powdered for pre-treatment.

### Deproteinization and Demineralization of Squid Pens

Deproteinization was performed to dissolve proteins and sugars; this was accomplished by treating them with a hot 1% sodium hydroxide solution for one hour in a water bath (Labman Scientific Pvt. Ltd., India). The final resultant solution was stored at 4°C. Demineralization was performed to remove minerals by soaking squid pens in 100 mL of 1% hydrochloric acid for 24 hours, followed by washing and air drying. The dried powder was stored in air-tight polypropylene tubes in a dark place.

### Deacetylation of Squid Pens to Chitosan

This process was done by adding 100 mL of 50% sodium hydroxide to squid pens and boiling them at 100°C for two hours in a water bath (Equitron, India). The samples were washed thoroughly with 50% sodium hydroxide and filtered. The samples were then uncovered and dried at 120°C for 24 hours in a hot air oven (REMI, India) and kept in a moisture-tight container.

### Synthesis of C-AgNps Using Chitosan as a Reducing Agent

The 0.2 g of chitosan was mixed with 15 mL of 1% glacial acetic acid and stirred for 30 minutes continuously using a magnetic stirrer (REMI, India). 3 mL 0.1 M silver nitrate (Fisher Scientific, USA) and 100 µL of 1 M sodium hydroxide were added to the 3 mL of chitosan solution. The reaction was kept dark for 10 hours of incubation in a shaking incubator (REMI, India). An appearance of yellowish-brown color indicates the formation of C-AgNps. The nanoparticles formed were then centrifuged (REMI, India) at 8000 rpm for 10 minutes, dried at 70°C for 1 day, and maintained in a moisture-tight container and characterized.

### Characterization of C-AgNps

The C-AgNps were characterized using a Shimadzu UV-Visible spectrophotometer (UV-Vis) (Shimadzu, Japan) for  $\lambda_{\text{max}}$  detection and Fourier transform infrared spectroscopy (FTIR) (JASCO, Japan) at  $4 \text{ cm}^{-1}$  to investigate the functional groups. Dynamic light scattering (DLS) and zeta potential (Microtrac Inc., USA) for analysing the particle size and surface electric potential, scanning electron microscope coupled with energy dispersive X-ray spectroscopy (FE-SEM-EDS) (FEI Company of USA (S.E.A) PTE LTD, Singapore) for morphological analysis.

### Antioxidant Activity of C-AgNps

The DPPH free radical scavenging assay was done as reported by Blois, 1958.<sup>14</sup> The test samples viz., L-ascorbic acid, chitosan, and C-AgNps were prepared at diverse concentrations from 20 to 100 µg/mL by mixing with 2 mL of 4 mM DPPH in methanol for thirty minutes. Then, the absorbance was recorded at 517 nm using a UV-Vis spectrophotometer. L-ascorbic acid of 20 to 100 µg/mL was used as a standard. The percentage of the DPPH radical scavenging is calculated using equation (1).

$$\% \text{ free radical scavenging potential} = \left\{ \frac{X-Y}{X} \right\} \times 100 \quad (1)$$

Here X and Y depict the absorbance of the standard (without C-AgNps) and C-AgNps. The IC<sub>50</sub> of C-AgNps was calculated for the DPPH antioxidant property using Microsoft Excel 2019, USA.

### Antibacterial Activity of C-AgNps Using Well Diffusion Assay

Before the assays, all glassware and media were sterilized in an autoclave at 121°C at 15 psi. The antibacterial activity of synthesized C-AgNps studied the antibacterial potentials using the well diffusion method and was performed according to Perez 1990.<sup>15</sup> Four bacterial strains viz., *S. aureus* (MTCC\_96), *B. subtilis* (MTCC\_441), *P. aeruginosa* (MTCC\_1688), and *E. coli* (MTCC\_2939), were subjected to the antibacterial activity of C-AgNps. The Muller Hilton Agar was poured onto Petri plates, 100 µL of overnight bacterial culture was spread using L-rod, and wells were punctured using a cork borer. The centre part of the well consists of antibiotic amoxicillin (10 µg/mL) as control, and the different concentrations of C-AgNps were loaded in four wells. A mixture of 1% acetic acid and chitosan at four different concentrations was the control. Following, the Petri plates were maintained at 37°C for 24 hours to determine the inhibitory growth of the C-AgNps against the clinical pathogens.

### Anticancer Activity of C-AgNps

#### Cell Culture Maintenance

A375 human skin malignant melanoma cell lines were procured from the National Centre for Cell Sciences, Pune, India. The cells were maintained in the log phase in Dulbecco's modified eagle medium supplemented with 10% (v/v) fetal bovine serum, 100 µg/mL streptomycin, and 100 U/mL penicillin. The cells were kept at 37°C with 5% carbon dioxide in a 95% air-humidified incubator (Biobase, Germany).

#### Anticancer Activity of C-AgNps on A375 Cell Lines

The anticancer effect of the C-AgNps was assessed against the A375 cell line by MTT assay.<sup>16</sup> The cells were added to 96-well microplates (1 x 10<sup>6</sup> cells/well) and maintained at 37°C for two days in a 5% carbon dioxide incubator until the 70-80% confluence stage. Then the cells were added with diverse concentrations of 20 to 100 µg/mL of C-AgNps and incubated for 1 day. The untreated and treated cells were visualized under an inverted microscope (Auxilab, Spain) for 1 day to study the structural and morphological changes. The cells were washed with phosphate buffer saline (PBS) of pH 7.4, and 20 µL of MTT solution was aspirated to the wells. Following this, the plates were maintained in the dark at 37°C for two hours. 100 µL DMSO was aspirated to solubilize the formazan and was read spectrometrically at 570 nm.

The cell viability percentage was estimated using equation (2).

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} * 100 \quad (2)$$

## RESULTS AND DISCUSSION

### Extraction of Chitosan from Squid Pens

Chitin was obtained by demineralization followed by deacetylation to obtain chitosan from the squid pen. 1.6 g of crude chitin was obtained, followed by deacetylation, and 2.3 g of chitosan was obtained at the end. Several studies report the predominance of high molecular weight β-chitin in squid pens.<sup>17,18</sup>

### Synthesis of C-AgNps

The C-AgNps were observed visually after incubation, denoted by a change in color from clear to reddish-brown. The chitosan acted as the capping, stabilizing, and reducing agent for reducing silver ions to nanoparticles due to various hydroxyl and carbonyl groups. The nanoparticles synthesized showed a peak λ<sub>max</sub> at 425 nm because of localized surface plasmon resonance.

### Effect of Chitosan Concentration on C-AgNps Synthesis

The C-AgNps was prepared with diverse concentrations of chitosan, namely 0.05%, 0.1%, 0.15%, and 0.2% (w/v). At 0.15% and 0.2% chitosan concentration, the peak absorbance was found between 400-450 nm. As the chitosan concentration increases, the concentration of C-AgNps was also found to increase. The maximum absorbance of C-AgNps was obtained at 0.2% chitosan concentration at λ<sub>max</sub> 425 nm with 2.491 optical density. The chitosan concentration of 0.2% produced the highest yield of nanoparticles, therefore fixing the rest of the research.

## Characterization of Synthesized C-AgNps

### Analyzing the Particle Size and Zeta Potential Using DLS

The average particle size was 240 to 290 nm; however, the dynamic light scattering analysis determines the hydrodynamic particle size surrounding the metalcore; thus, the actual size of the nanoparticles is much less than the hydrodynamic size. The zeta potential measures the surface electric potential of nanoparticles, which was found to be +98.2 mV which concludes that the particles are highly stable and non-agglomerated.

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### Functional Group Determination Using FTIR

FTIR spectrum of chitosan isolated from squid pen showed the bands at 3288, 2887, 1637, 1415, 1381, 1149, 1080, and 1033  $\text{cm}^{-1}$  representing various functional groups (Fig.-1). The peak at 3288  $\text{cm}^{-1}$  showed -NH bond stretching indicative of the primary - $\text{NH}_2$  group. The peak at 2887  $\text{cm}^{-1}$  denoted the -CO stretching bond, which indicates the aldehyde group in chitosan and the band at 1637  $\text{cm}^{-1}$  denoted an amide group. The absorption at 1149  $\text{cm}^{-1}$  showed a stretching C-O-C bond indication of aliphatic ether. The band between 1080 and 1033  $\text{cm}^{-1}$  led to a free amino group, an influential group in the chitosan extract responsible for remarkable biological activities.<sup>20</sup>

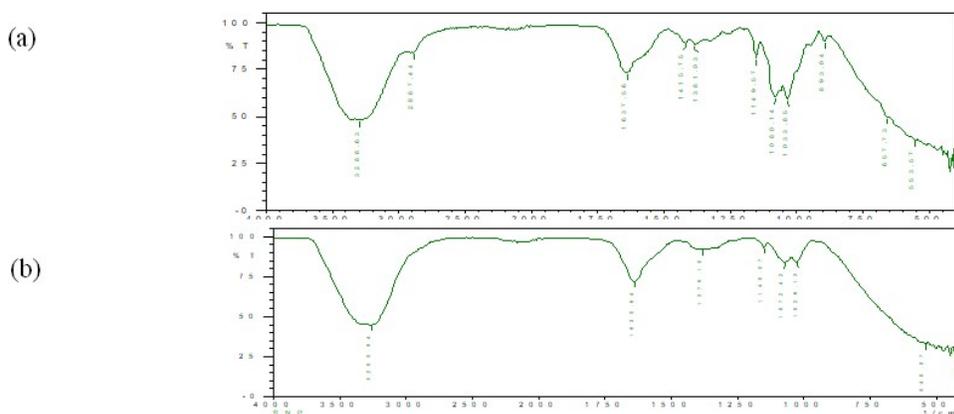


Fig.-1: FTIR Denoting the Functional Groups of a) Chitosan and b) C-AgNps

### Analyzing the Surface Morphology Using FE-SEM-EDS

The extracted chitosan was observed to have clusters and fibril structures. The microscopic structures reveal that the morphology of C-AgNps was spherical and clustered (Fig.-2). The elemental analysis of silver in the nanoparticle suspension was done using EDS at 3 KeV. EDS results showed several peaks that confirmed the presence of silver. Other trace elements like nickel, iron, carbon, oxygen, sodium, aluminum, and molybdenum were also detected.

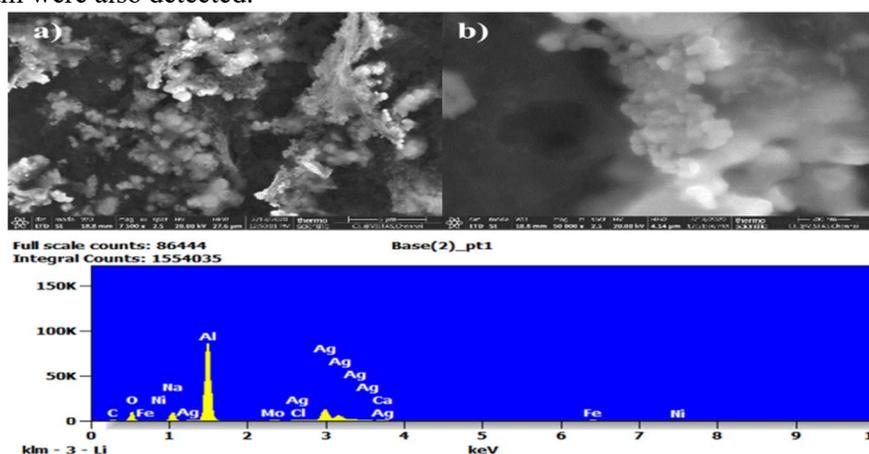


Fig.-2: Surface Morphology and EDAX of C-AgNps Using FE-SEM

## Biological Activity of Chitosan and C-AgNps

### DPPH Antioxidant Activity

The activity of C-AgNps increased serially from 0.975 to 38.46%, from 20 to 100  $\mu\text{g/mL}$ . The best antioxidant activity was attained at a 100  $\mu\text{g/mL}$  concentration of C-AgNps. The experiment used chitosan as a negative control, with only 35.1% as the antioxidant potential at 100  $\mu\text{g/mL}$  concentration. The  $\text{IC}_{50}$  of antioxidant potential was with C-AgNps and was found to be 6.65  $\mu\text{g/mL}$  than only chitosan with an  $\text{IC}_{50}$  of 7.22  $\mu\text{g/mL}$ .<sup>21</sup> The extent of reduction depends on the formation of donor-acceptor interactions playing a vital role in reducing DPPH stable free radicals; this is due to the interaction of the hydroxyl group in chitosan. The control L-ascorbic acid showed an inhibition potential of 75% with an  $\text{IC}_{50}$  of 3.15  $\mu\text{g/mL}$ . Thus, the antioxidant potential of C-AgNps was found to be more reasonable than the control. The  $\text{IC}_{50}$  of C-AgNps synthesized in this research using squid pens is better than many previously reported literatures.<sup>22</sup>

### Antibacterial Activity

The antibacterial activity of C-AgNps showed good results than chitosan against the tested microorganisms. The antibacterial property increased linearly with the concentration of chitosan and C-AgNps. The maximum zone of inhibition against *B. subtilis* was observed for C-AgNps with a zone diameter of 12 mm than chitosan 10 mm and possessed a low  $\text{IC}_{50}$  of 37  $\mu\text{g/mL}$  for C-AgNps (Fig.-3). Amoxicillin control drug possessed a zone diameter of 26 mm against *B. subtilis* at 10  $\mu\text{g/mL}$ . The zone diameters for *S. aureus* for C-AgNps were 10 mm than chitosan 8 mm, respectively, and possessed an  $\text{IC}_{50}$  of 28.5  $\mu\text{g/mL}$  for C-AgNps. It was proved that C-AgNps has growth inhibition of bacterial strain.<sup>23</sup> Amoxicillin control drug possessed a zone diameter of 24 mm against *S. aureus* at 10  $\mu\text{g/mL}$ . The antibacterial potential of C-AgNps and chitosan were outstanding against gram-negative bacteria *E. coli*, with large zones of inhibition comprising 25 mm and 20 mm.<sup>24</sup> The  $\text{IC}_{50}$  of 16.62  $\mu\text{g/mL}$  was obtained for C-AgNps against *E. coli*, denoting that *E. coli* is highly susceptible to C-AgNps. Amoxicillin control drug inhibited *E. coli* with a zone diameter of 23 mm. The synthesized C-AgNps inhibited *E. coli* more effectively than amoxicillin, with a zone diameter of 24 mm. The gram-negative bacteria *P. aeruginosa* was also susceptible to C-AgNps and showed a reasonable zone of inhibition for C-AgNps (9 mm) than chitosan (8 mm) with an  $\text{IC}_{50}$  of 46  $\mu\text{g/mL}$  for C-AgNps. The zone diameter of the control antibiotic against *P. aeruginosa* was 22 mm. Thus, the synthesized C-AgNps showed good antibacterial properties against all four bacteria, particularly effective against *B. subtilis* and *E. coli*. Therefore, the findings were promising and could be taken for further *in-vivo* applications. The antibacterial property of C-AgNps in the present research work was found to be more effective than Garibo *et al.* 2020; Yu *et al.* 2021, thus proving to be an excellent antibacterial agent and could be used for a therapeutic regimen.<sup>25,26</sup>

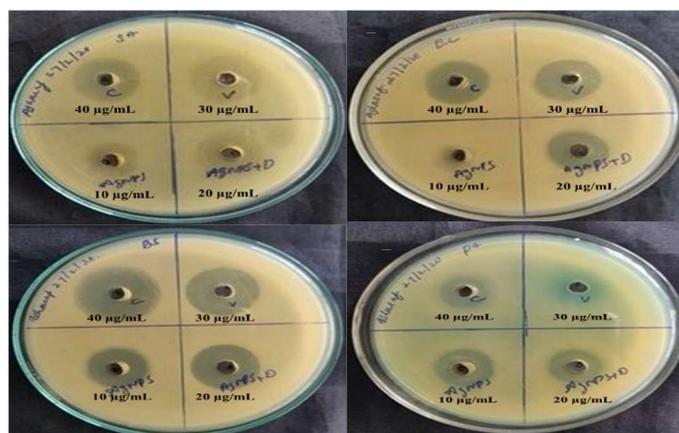


Fig.-3: Antibacterial Activity of C-AgNps against a) *B. subtilis*; b) *S. aureus*; c) *E. coli*; d) *P. aeruginosa*

### Anticancer Activity

An observation from our studies suggested that C-AgNps possessed excellent anticancer activity. The C-AgNps acts at multiple levels to induce the cancer cell (A375 cell line) death, including the destruction of the cell membrane, low negative surface charge, and membrane potential of mitochondria. In the present

study, tumor growth inhibition was significantly induced by C-AgNps (Fig.-4). The anticancer activity of the C-AgNps was tested with concentrations (20 to 100  $\mu\text{g}/\text{mL}$ ). The cell viability significantly reduced from 94.19% to 32.66%, increasing concentration from 20  $\mu\text{g}/\text{mL}$  to 100  $\mu\text{g}/\text{mL}$ . The  $\text{IC}_{50}$  of C-AgNps was 3.72  $\mu\text{g}/\text{mL}$ , which is better than previously reported studies'  $\text{IC}_{50}$  values.<sup>27,28</sup> Thus, the anticancer activity of C-AgNps was dose-dependent with a morbid rate of 67.34% at 100  $\mu\text{g}/\text{mL}$ . The antioxidant capacity of C-AgNps used here is better than the  $\text{IC}_{50}$  of 55  $\mu\text{g}/\text{mL}$  for the DPPH antioxidant activity using silver nanoparticles synthesized using *Brassica oleracea*.<sup>29</sup> Kharat and Mendhulkar, 2016 reported an  $\text{IC}_{50}$  of 126.6  $\mu\text{g}/\text{mL}$  for the DPPH antioxidant activity using *Elephantopus scaber*-mediated silver nanoparticles, whereas the present study reported a better  $\text{IC}_{50}$  of 6.65  $\mu\text{g}/\text{mL}$ .<sup>30</sup> The DPPH antioxidant activity of the present is better than the reported  $\text{IC}_{50}$  of 158.20  $\mu\text{g}/\text{mL}$  from extracts of *Elephantopus scaber* L. and *Biophytum sensitivum*.<sup>31</sup> Behravan *et al.* 2019 reported a reasonable antibacterial activity using silver nanoparticles synthesized using *Berberis vulgaris* against *S. aureus*.<sup>32</sup> The antibacterial activity of C-AgNps in this study against *S. aureus* and *E. coli* with zones of diameters of 10 and 25 mm, is comparable with Sinsinwar *et al.* 2018 with zone diameters of 15 and 13 mm.<sup>33</sup> Francis *et al.* 2018 studied the anticancer activity of biosynthesized leaf essence *Elephantopus scaber* mediated silver nanoparticles against A375 cell lines with an  $\text{IC}_{50}$  of 15.68  $\mu\text{g}/\text{mL}$ . The present study reported an  $\text{IC}_{50}$  of 3.72  $\mu\text{g}/\text{mL}$ , which has a higher inhibition potential.<sup>27</sup> Netchareonsirisuk *et al.* 2016 reported a similar lower  $\text{IC}_{50}$  to the present study with C-AgNps, denoting greater against A375 cell lines killing the cancer cells.<sup>34,35</sup> Parthasarathy *et al.* 2020 studied the mechanism of A375 cancer cell death using ZnO through biochemical assays by measuring SOD, CAT, and GPx antioxidant enzymes, which could be a possible mechanism for the death of these cells.

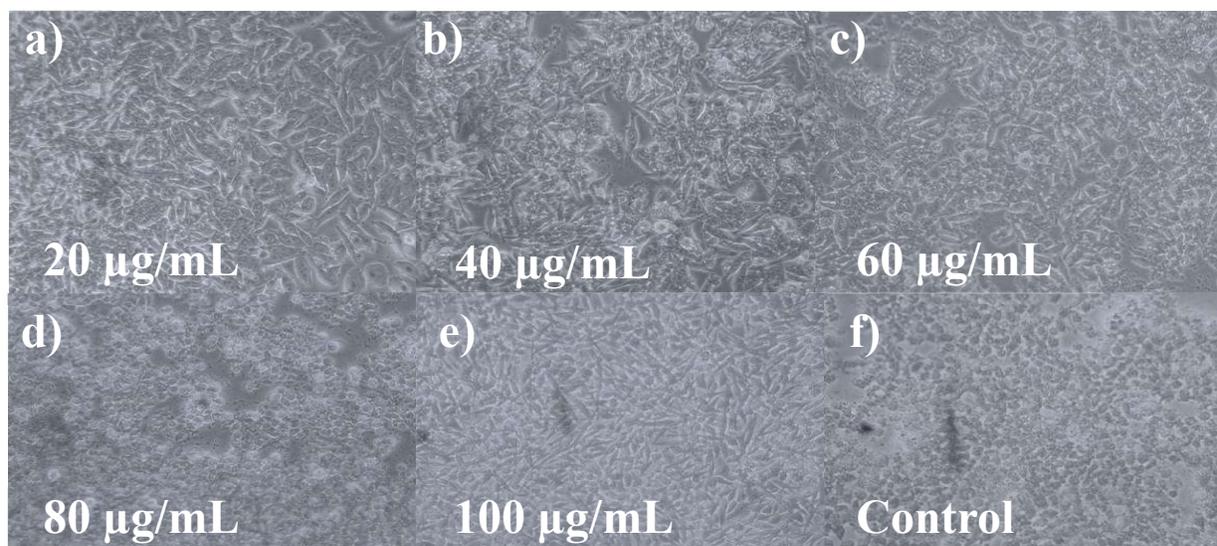


Fig.-4: Anticancer Activity of C-AgNps on A375 Cells

### CONCLUSION

Thus, this research work flashed the limelight on extracting  $\beta$ -chitin from squid pens to produce chitosan. The extracted fraction of chitosan polymer was then used as the stabilizing and reducing agent for chitosan-mediated silver nanoparticles. The hydrodynamic particle of C-AgNps was established using DLS between 240 to 290 nm. The C-AgNps had excellent DPPH antioxidant and antibacterial properties against nosocomial microorganisms with good  $\text{IC}_{50}$  values and excellent inhibition zones. The C-AgNps showed anticancer properties against human skin malignant melanoma with a low  $\text{IC}_{50} = 3.72 \mu\text{g}/\text{mL}$  value. Our future works would focus on scaling the production, molecular docking with virulent bacterial and viral proteins, dynamics simulations, and *in-vivo* studies.

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### CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

### AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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