



Biological Assessment of Chitosan Nanoparticle Extracted from Carapace of *Sartoriana spinigera* Crab as a Potent Anti-cancer Agent through Apoptotic and Necrotic Pathway

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Authors' contributions

This work was carried out in collaboration between both authors. Author SECK designed the study, wrote the protocol, performed statistical analysis and prepared the original draft of the manuscript. Author NS managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Natural products have acquired interest in the pharmaceutical industry for their various properties of being non toxic and biocompatible, and also for having the efficiency to cure various diseases. One such serious disease is Breast cancer which is known to be the most common cancer among females worldwide and for the cure of which, various research work is being done. Chitosan is a

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natural biopolymer obtained from deacetylation of chitin found abundantly in exoskeleton of Arthropoda including crustaceans. Because of its deacetylated form and cationic nature, it has proved to have various health benefits. In the present study, chitosan nanoparticles have been prepared from carapace of *Sartoriana spinigera* which is a crab found abundantly in the freshwater bodies of Jharkhand, India. Study on enhancing Caspase 3/7 activity of MCF-7 cells has been done by treatment with chitosan nanoparticle which showed that after treatment chitosan nanoparticle was able to show increased necrosis of MCF-7 cells which was significantly higher than that of control group at 5 % significance level. Analysis of Annexin V revealed that chitosan nanoparticle increased the phenomenon of early apoptosis as compared to control group at 1 % significance level, confirming chitosan nanoparticle as a potent anti cancer agent against MCF-7 cell line.

Keywords: Annexin V; breast cancer; Caspase 3; chitosan nanoparticle; MCF-7; *Sartoriana spinigera*.

1. INTRODUCTION

According to reports of IARC- WHO, Breast cancer accounts for more than 6.5 lacs deaths worldwide, and that breast cancer cases and deaths are likely to rise globally (Kim *et al.*, 2025). Although pharmaceutical industry is progressing in terms of new anti-cancer drugs, the society is in demand of natural products that are equally effective as commercial drugs, but with lesser side effects and lower cost consumption. One such natural product is Chitosan, which is a deacetylated form of chitin, an abundant polymer found in the exoskeleton of crustaceans, insects, mollusks and cell wall of fungi (Tang *et al.*, 2023). Chitosan is made up of about 20 % (1,4)-2 acetamido-D-glucopyranose and 80 % of (1,4)-2-amino-D- glucopyranose (Zitouni *et al.*, 2013). Chitosan has been known to possess wound healing capacity (Rajinikanth B *et al.*, 2024), antioxidative efficacy (Ivanova & Yaneva, 2020), antimicrobial activity (Guarnieri *et al.*, 2022) and more. Various research work on chitosan have proved it to possess anti cancer property through angiogenic mechanism. Furthermore, chitosan enhances immune system which reduces the chance of cancer occurrence (Shakil *et al.*, 2021).

Commercially used synthetic anti cancer drugs include Doxorubicin, Cisplatin, which have severe side effects on cancer patients (Živanović *et al.*, 2023). In such terms, chitosan being a natural product is encouraged in clinical use. Nanoparticle of any such molecule further increases its efficiency of performing a function due to its increased surface area and increased potency of target delivery.

Caspase 3 and 7 are the most important enzymes that play main role in apoptotic pathway. Caspase 3 is responsible for fragmentation of DNA and morphological

changes in a cell to undergo apoptosis, whereas, Caspase 7 is important for decreasing cell viability (Lakhani *et al.*, 2006). Any molecule that has the potency to act as anti cancer agent must have the capacity to increase the activity of Caspase 3 and 7. Annexin V is a protein (Vascular anticoagulant α), whose binding to apoptotic cells is proportional to other apoptotic activities such as changes in nucleus structure and DNA fragmentation (Koopman *et al.*, 1994).

Most of the extraction process of chitosan has taken place from exoskeletons of marine fauna. Work based on freshwater source is very less. In the present study, chitosan was extracted from carapace of a freshwater crab *Sartoriana spinigera* which is abundantly found in the fresh water bodies of Jharkhand, Odisha (Pati *et al.*, 2012) and north eastern states of India, during the rainy season. *Sartoriana spinigera* belongs to Least Concern category as per IUCN (International union for conservation of nature) red list (IUCN, 2018), and is not cultured commercially due to lack of scientific knowledge regarding its medicinal value although it is consumed by the tribals to cure various ailments and is of ethnobiological significance.

Aim of the present study is to extract chitosan from carapace of *Sartoriana spinigera*, convert it into nanoparticle form and study its anti cancer efficacy against MCF-7 (Breast cancer) cell line by studying Caspase 3/7 activity and Annexin V activity.

2. METHODOLOGY

2.1 Synthesis and Characterization of Chitosan Nanoparticle (ChNP)

Specimen of *Sartoriana spinigera* were collected from markets of Ranchi after identification of their morphology. Specimen of crabs were sent to ZSI

(Zoological survey of India) for proper identification of species. Chitosan was prepared from carapace powder of *Sartoriana spinigera* by undergoing 3 processes. Demineralization of carapace powder was done by treatment in 2N HCl for 24 hours and then bringing the pH to neutral. Demineralized powder was then deproteinized in 4% NaOH at 110 °C to obtain chitin. Deacetylation of chitin in 40% NaOH at 80 °C was done to obtain chitosan (Takiguchi, 1991).

Crude chitosan was converted to ChNP by sTPP (Sodium tripolyphosphate) method following Anand *et al.*, (2018). 2 gm of crude chitosan obtained from *Sartoriana spinigera* was treated with 1% acetic acid and put in magnetic stirrer at 37°C. The yellow coloured solution turns translucent white. Sodium tripolyphosphate is gradually added drop by drop in the solution until net like nanoparticles start emerging.

Efficiency of chitosan to perform any function is based on characteristics such as Degree of Deacetylation (DD%), Particle size, crystallinity and surface morphology.

Degree of deacetylation (DD%) was calculated following Brugnerrotto *et al.* (2001). Crystallinity of chitosan nanoparticle was studied by X-ray diffraction at 2 θ . Particle size was estimated by Zeta sizer. Scanning electron microscope was used to study the morphology of surface of ChNP at different magnifications (Kachhap & Saxena, 2024).

2.2 Analysis of Caspase 3/7 Using Flow Cytometry

Caspase 3/7 activity of MCF-7 cells after treatment with ChNPs was done following Nan *et al.*, (2019). MCF-7 cells were plated at density of 2.5×10^5 cells per well in a 96 well plate and incubated for 24 hours at 37°C. The cells were then treated with different concentrations of ChNP for 24 hours. ChNP of concentration 131 $\mu\text{g/mL}$ and 262 $\mu\text{g/mL}$ were used. After treatment, trypsin was added to each well to detach cells from the well and washed with PBS and then centrifuged at 2000 RPM for resuspension. Caspase 3/7 green detection reagent was added to 1 mL cell suspension and mixed. The cells were incubated in dark for 30 minutes and then stained with 1 μL of SYTOX AADvanced for 5 minutes. The 96 well plate was

then read using FACS (Fluorescence -activated cell sorting) Aria.

2.3 Analysis of Annexin V Using Flow Cytometry

Apoptosis detection by Annexin V assay was done following Moghaddam *et al.*, (2017). MCF-7 cells were plated in 6 well plates at a density of 2×10^5 cell/well and incubated overnight. The cells were then treated with 131 $\mu\text{g/mL}$ concentration of ChNP and incubated for 24 hours. They were then trypsinized and washed with PBS and resuspended with binding buffer and 5 μL of Annexin V- FITC and Propidium iodide and incubated for 15 minutes in dark. After staining, binding buffer was added and cells were filtered and analysed using flow cytometer.

3. RESULTS AND DISCUSSION

3.1 Characterization of Chitosan Nanoparticle

Degree of deacetylation (DD%) of ChNP (Chitosan nanoparticles) was calculated and found to be 92.25%. Crystallinity of ChNP by X-ray diffraction showed most prominent and highest peak at 20° and other peaks at 12°, 28° and 34° at 2 θ . Peaks at higher degree indicate high degree of deacetylation. Particle size of ChNP was found to be 297 ± 5 nm determining its nanoparticle nature. Surface morphology was studied by SEM which showed oval shaped molecules with smooth surface (Fig. 1).

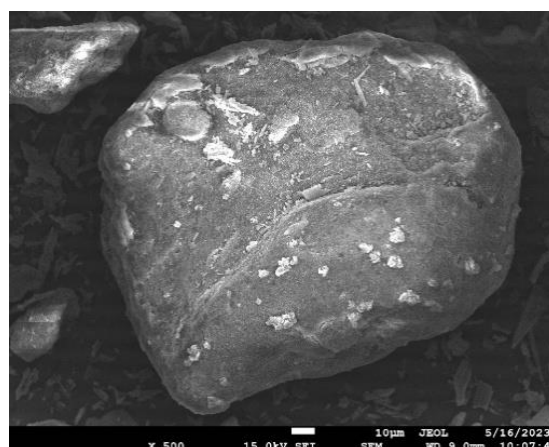


Fig. 1. SEM image of oval and smooth surface of chitosan nanoparticle extracted from carapace of *Sartoriana spinigera*

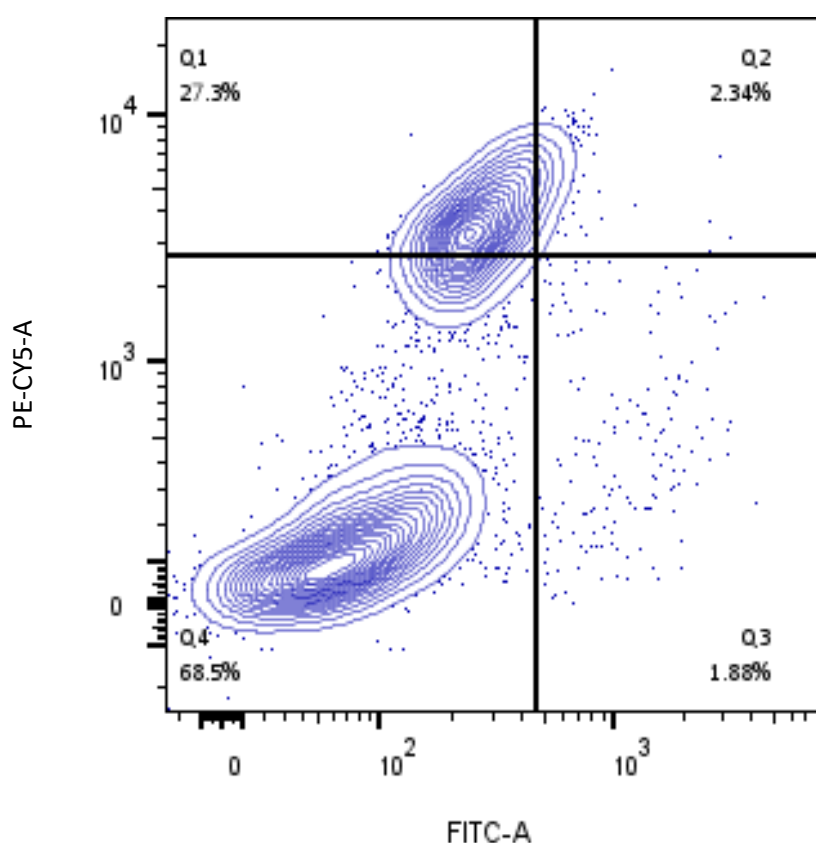


Fig. 2. Graph showing mode of MCF-7 using Caspase 3/7 detection and flow cytometry after treatment with 262 µg/mL of ChNP. Q1= necrosis, Q2=early apoptosis, Q3= late apoptosis, Q4= live cells

3.2 Enhancement of Caspase 3/7 Activity in MCF-7 Cells after Treatment with Chitosan Nanoparticle

Analysis of Caspase 3/7 activity revealed that ChNP extracted from carapace of *Sartoriana spinigera* was able to show decreasing cell viability with increasing concentrations. At 131 µg/mL, ChNP showed cell viability of 72.3% while at 262 µg/mL, it was found to be 68.5% (Fig. 2 and 3). Statistical analysis by Student's t test showed that both the concentrations of ChNP were able to cause decrease in cell viability as compared to the control group at 0.1% significance level. At 131 µg/mL, ChNP showed early apoptosis of 2.34%, and late apoptosis of 1.88%. Whereas at 262 µg/mL, early apoptosis of 7.78% and late apoptosis of 4.02% was observed. However, statistical analysis by Student's t test showed no significant difference between the result of caspase 3/7 enhancing capacity at different concentrations. It was also

observed that at 131 µg/mL, ChNP showed cell necrosis of 24.5% and at 262 µg/mL cell necrosis of 27.3% was noted.

3.3 Enhancement of Annexin V Concentration to Initiate Apoptosis in MCF-7 Cells After Treatment with Chitosan Nanoparticles

Analysis of Annexin V revealed that ChNP at concentration 131 µg/mL was able to show increased early apoptosis of 0.35% of MCF-7 cells (Fig 4), which was significantly higher than that of control group at 1% significance level, when analysed by Student's t test. It was also observed that ChNP showed high necrosis at 15.59 % which is significantly higher than that of control group at 5% significance level.

Both results confirm the presence of apoptosis and necrosis in MCF-7 cells treated with ChNP.

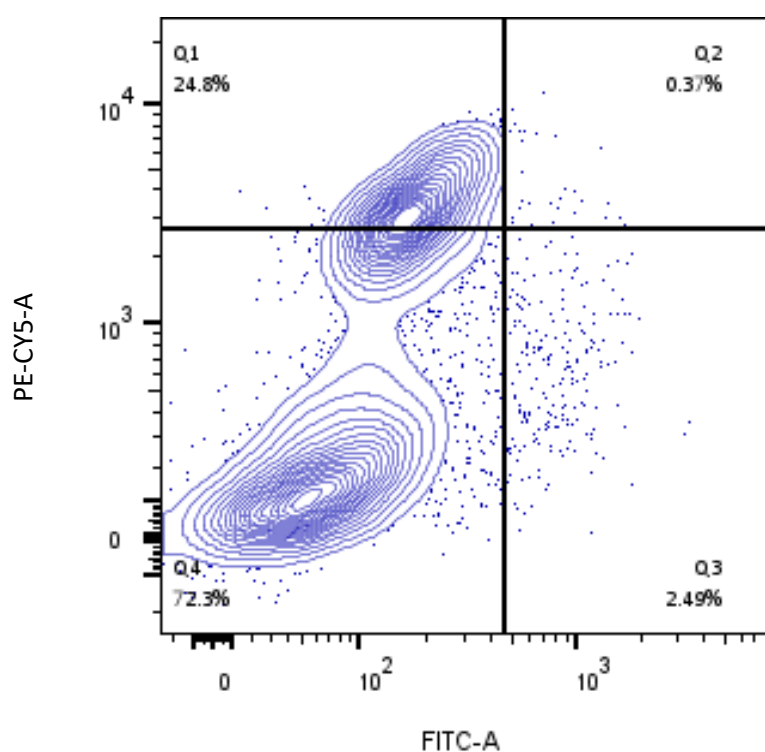


Fig. 3. Graph showing mode of MCF-7 using Caspase 3/7 detection and flow cytometry after treatment with 131 µg/mL of ChNP. Q1= necrosis, Q2=early apoptosis, Q3= late apoptosis, Q4= live cells

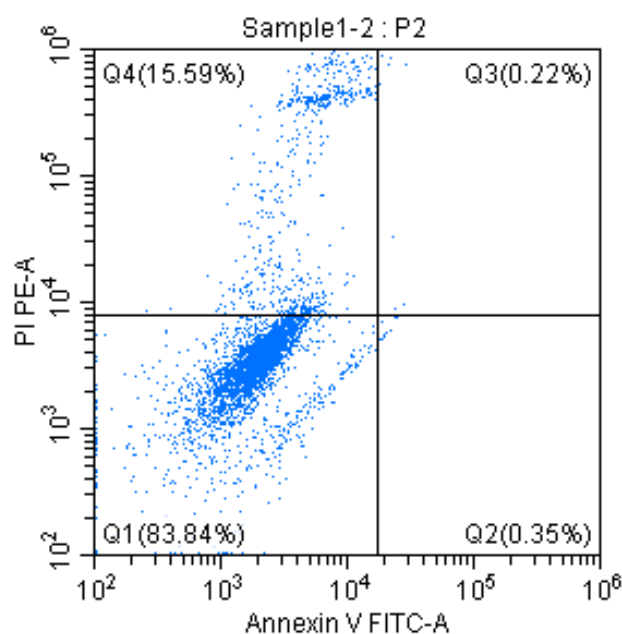


Fig. 4. Graph showing mode of MCF-7 using Annexin V detection and flow cytometry after treatment with 131 µg/mL of ChNP. Q1= live cells, Q2=early apoptosis, Q3= late apoptosis, Q4= necrosis

Similar results were obtained by Abedian *et al.*, (2019), in which it was found that at high concentration of 2 mg/mL, and treatment for 48

hours, ChNP was able to show increased apoptosis by 7.75% in MCF-7 cells. Necrosis was found to be increased as 15.84% in MCF-7

cells. The result of present study also corroborates with the study conducted by Jiang (2011), according to which sulphated chitosan was able to show significant apoptosis of MCF-7 cells when treated for 24 hours. Analysis of caspase activity was done by Wimardhani *et al.*, (2014), on Ca9-22 cells. Their study confirmed that apoptosis might not be the mechanism of inducing cell death by low molecular weight chitosan. It has also been reported by Zhang *et al.*, (2010) that cancer cells are more negatively charged than normal cells, as a result of which cationic chitosan has high affinity towards cancer cells. Thus, chitosan can disrupt the tumour cell membrane by either attaching to a specific receptor or by the process of endocytosis.

4. CONCLUSION

In the present study, chitosan nanoparticle extracted from carapace of freshwater crab *Sartoriana spinigera* was able to show characters of high degree of deacetylation and satisfactory particle size. When treated with ChNP, MCF-7 cells showed decreased cell viability with increasing concentration by the process of significant necrosis, indicating that chitosan nanoparticle has anti cancer efficacy by following necrotic pathway against cancer cells. ChNP was also able to induce early apoptosis in MCF-7 cells which was significantly higher than that of control group. Carapace of *Sartoriana spinigera* is a source of chitin from which chitosan can be prepared and can be utilized for synthesis of ChNP which is a potent anti cancer agent.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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