International Journal of Pharmacy and Pharmaceutical Science

**ISSN Print:** 2664-7222
**ISSN Online:** 2664-7230
IJPPS 2024; 6(2): 01-07
www.pharmacyjournal.org
Received: 03-04-2024
Accepted: 11-05-2024

**Bhagyasree S**
Department of Pharmacy,
JJTU University,
Vidyanagari, Churu
Jhunjhunu Road, Chudela,
Jhunjhunu, Rajasthan, India

**Sunbee Prakash**
Department of Pharmacy,
JJTU University,
Vidyanagari, Churu
Jhunjhunu Road, Chudela,
Jhunjhunu, Rajasthan, India

**Corresponding Author:**
**Bhagyasree S**
Department of Pharmacy,
JJTU University,
Vidyanagari, Churu
Jhunjhunu Road, Chudela,
Jhunjhunu, Rajasthan, India

# Green synthesis of marine sponge silver nanoparticles and its antioxidant, cytotoxic activity

**Bhagyasree S and Sunbee Prakash**

**DOI:** https://doi.org/10.33545/26647222.2024.v6.i2a.116

**Abstract**
Marine sponges have emerged as a rich source of bioactive compounds with potent anti-cancer activities. This study explores the anti-cancer potential of marine sponge-assisted silver nanoparticles (AgNPs) through comprehensive collection, identification, synthesis, and evaluation processes. Marine sponges were collected from the Rameswaram district in Tamil Nadu, India, and identified using genomic sequencing techniques. Ethanolic extracts from these sponges facilitated the green synthesis of AgNPs, which were characterized by particle size and zeta potential analysis. The synthesized AgNPs demonstrated significant antioxidant activity in DPPH radical scavenging assays, indicating their ability to neutralize free radicals effectively. Cytotoxicity assays revealed a dose-dependent inhibition of L6 cell proliferation, with high concentrations showing substantial biological activity. Further, acridine orange/ethidium bromide staining confirmed the induction of apoptosis in L6 cells treated with marine sponge-assisted AgNPs. These findings suggest that marine sponge-derived AgNPs possess promising therapeutic potential, particularly in developing targeted anti-cancer therapies and antioxidant treatments. The study underscores the need for further research into the molecular mechanisms of these nanoparticles and their optimization for specific biomedical applications.

**Keywords:** Marine sponge, extract, $AgNO_3$, nanoparticles, MTT assay

## Introduction
Marine sponges exhibit potent anti-cancer activities. These substances have been studied for their ability to inhibit the growth of cancer cells, induce apoptosis, and interfere with the formation of blood vessels that nourish tumors [1]. The deep blue expanse of the ocean hides a treasure trove of secrets, and among its many enigmatic inhabitants, marine sponges emerge as unlikely warriors against one of humanity's greatest adversaries: cancer. In the silent depths of the underwater world, these seemingly unassuming organisms reveal a remarkable arsenal of bioactive compounds that exhibit potent anti-cancer activities, holding the promise of groundbreaking advancements in the fight against this devastating disease [2]. Marine sponges, belonging to the phylum Porifera, are ancient and diverse organisms that have thrived in the ocean for millions of years. While their ecological roles as filter-feeders and contributors to marine ecosystems have long been recognized, it is their newfound role in the realm of medicine that has captured the attention of scientists and researchers worldwide. At the heart of this medical intrigue are the bioactive compounds produced by marine sponges. These compounds, often referred to as secondary metabolites, have demonstrated a remarkable ability to combat cancer cells with unprecedented specificity and efficacy. The quest for novel anti-cancer drugs has driven researchers to explore the vast biodiversity of marine sponges, uncovering a treasure trove of compounds with immense therapeutic potential [3]. One of the notable classes of compounds found in marine sponges with anti-cancer properties is the group of compounds known as alkaloids. These nitrogen-containing organic molecules have exhibited cytotoxic effects against a variety of cancer cell lines. Researchers have isolated alkaloids from different sponge species, revealing their potential as lead compounds for the development of anti-cancer drugs. In addition to alkaloids, marine sponges produce polyketides, terpenes, and peptides, each with its unique chemical structure and anti-cancer activities. These compounds target various cellular pathways involved in cancer development and progression, making them valuable candidates for the development of targeted therapies.

The significance of marine sponge-derived compounds in the realm of cancer research lies in their ability to address some of the key challenges faced in cancer treatment [4]. Many conventional cancer treatments, such as chemotherapy, often lack specificity and can cause severe side effects due to their impact on healthy cells. In contrast, the compounds from marine sponges have shown a remarkable degree of selectivity, targeted cancer cells while sparing normal, healthy cells. Furthermore, the complexity and diversity of marine sponge metabolites provide a vast library of potential drug candidates. The unique environmental conditions in which marine sponges thrive, including deep-sea trenches and coral reefs, contribute to the production of compounds with distinctive chemical structures and biological activities. These compounds may hold the key to developing novel therapies that can overcome the challenges posed by drug resistance and heterogeneity in cancer cells. The journey from the depths of the ocean to the laboratory bench involves the painstaking process of isolating, characterizing, and testing these marine-derived compounds. Researchers must navigate the complexities of both the marine environment and the intricate biochemistry of these compounds to unlock their full potential. As the field of marine sponge-derived anti-cancer compounds advances, researchers are optimistic about the prospect of translating these discoveries into tangible clinical applications. The road to developing new anti-cancer drugs is long and arduous, but the promise of more effective, targeted, and less toxic treatments is a beacon of hope for patients and clinicians alike. The anti-cancer activities exhibited by marine sponges represent a remarkable intersection of marine biology and medical science [5]. These ancient organisms, hidden beneath the waves, may hold the key to revolutionizing cancer treatment and providing new avenues of hope for those facing this formidable disease. The exploration of marine sponge-derived compounds marks a frontier in drug discovery, where the secrets of the ocean offer a lifeline in the pursuit of a cancer-free future.

## Material and Methods

### Marine sponge collection and identification

Marine sponges were collected from the Rameswaram district, Tamil Nadu, India, using a grab sample collection method. The coordinates of the location are approximately 9°17'18.60″ " N 79°18'45.76"″ E, and it is known for its coastal ecosystem. This collection method involves the use of a specialised grab sampler, an instrument designed to collect samples from marine water. The sampler is lowered into the water, and when it reaches the desired depth, it is triggered to close, thus capturing a "grab" of the sponge [6].

### Marine sponge identification

The collected marine sponge was cleaned with sterile water to remove the sediment, bacteria, and algae. The cleaned marine sponge was sent to the genomic sequencing centre Biokart India Pvt. Ltd. (Bangalore, India). The resulting sequences were compared with other published sequences in the NCBI GenBank database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify sponges. Alignment of the DNA sequence of the isolate with phylogenetically related genera was performed using MEGA software (Tamura *et al* 2021). Alignment and subsequent phylogenetic analysis provided information regarding the evolutionary relationship of the isolate with reference to other related sponge species [7].

### Extraction by Soxhlet

Using distilled water, the marine sponge was washed, dried after collection, and examined to confirm that it was disease-free. The overlay was cleaned with 0.1% mercury chloride for 20 s. The marine sponge was eventually dried in the sun after being cleaned thrice with distilled water. To separate the bioactive compounds, marine sponge was crushed and 500 mg of the powder is placed in the "thimble" composed of robust filter paper and put in chamber E of the Soxhlet apparatus. To remove the ethanolic extract from the solution, heat was added to Flask-A and the vapour was condensed in condenser D. The unprocessed substance is sprayed into the needle of the unprepared medicine and withdraws itself when it interacts with the material. When the liquid level in chamber E reached the top of the syphon tube in chamber C, the liquid in chamber E became a soufflé and was placed in flask A. The procedure continued until one drop of the solvent from the syphon tube was completely evaporated. Crude methanolic extract was collected and preserved for phytochemical analysis [8].

**Fig 1:** Ethanolic extract of marine sponge

### Green synthesis of silver nanoparticles using methanolic extracts

### Preparation of 1mM silver nitrate aqueous solution

Silver nitrate (0.017 g) was dissolved in distilled water (100 ml of distilled water. This concentration (1 mM) indicates the number of moles of silver nitrate per litre of the solution. Silver nitrate solution, and an amber-coloured bottle. Silver nitrate is light sensitive, and exposure to light can cause decomposition.

### Green synthesis of marine sponge assisted silver nanoparticles

1 mM solution of silver nitrate in double-distilled water provided a controlled source of silver ions for the experiment. The choice of concentration indicated the precision of the approach. A unique aspect of this experiment involved combining the silver nitrate solution with a marine sponge ethanol extract at a specific ratio of 1: 5. The marine sponge extract likely contributed distinct properties to the synthesis process. During the experiment, a magnetic mixer set at 500 revolutions per minute was used

for mixing. Controlled cooling below the boiling point suggests a deliberate effort to maintain the optimal conditions for the reaction. Recognising the light sensitivity of silver nitrate, the reaction occurs in a dimly lit environment. This precaution was aimed at preventing unwanted reactions and at maintaining the stability of the reaction mixture. Following this, the mixture was centrifuged at 10,000 rpm. This step effectively separated the green nanoparticle-containing silver pellet from the rest of the reaction mixture, visually indicating the presence of the nanoparticles. To ensure the purity of the nanoparticle-containing silver pellet, it was washed multiple times with deionised water. This process aims to remove any remaining silver ions and residues from the marine sponge extract, thereby enhancing the quality of the final product. After purification, the nanoparticle-containing silver pellet was moved to a cold, dry, and shadowy location for further analysis. This careful choice of environment suggests a meticulous approach for analysing the synthesised nanoparticles.

**Characterization of marine sponge assisted silver nanoparticles**

**Particle size analysis-marine sponge assisted silver nanoparticles:** Analysing the particle size of marine sponge-assisted silver nanoparticles involves dispersing dried and powdered nanoparticles in water to achieve a suitable scattering intensity. The analysis was conducted using a Malvern Zeta Size Analyzer.

**Zeta potential analysis-marine sponge assisted silver nanoparticles:** The zeta potential of the marine sponge-assisted silver nanoparticles was determined using a Malvern Instruments Zeta-Sizer. The sample was prepared in water and the instrument comprised a zeta cell and polycarbonate cells with gold electroplated electrodes. The zeta potential, measured by this method, provides insights into the surface potential of silver nanoparticles and is crucial for assessing their stability.

**MTT assay of silver nanoparticles-marine sponge assisted silver nanoparticles:** To assess the cytotoxicity of marine sponge-assisted AgNPs on L6 cells derived from rat skeletal muscle, Mosmann's original MTT assay (1983) was employed, with some modifications. Cells in regular DMEM were counted using a haemocytometer and diluted until the cell density reached below 1.104 cells/mL. Subsequently, the cells were added to each well of a 96-well plate and allowed to adhere for one day. Controlled amounts of marine sponge AgNPs, ranging from 250 to 7.8125 g/mL L6 cell treatment, were then applied to each well. Following a 24-hour incubation at 37 °C in an incubator with 95% air and 5% $CO^2$, the L-6 cells were further incubated for an additional 4 h at 37 °C after dilution with fresh cultivation fluid and addition of the colourant. Proliferation inhibition (%) was calculated using the following formula: (optical density of control - optical density of test) × 100.

**Acridine orange/ethidium bromide (AO/EB) staining technique to measure apoptotic induction of marine sponge assisted silver nanoparticles**

The acridine orange/ethidium bromide (AO/EB) staining technique was employed to assess apoptotic induction by marine sponge-assisted AgNPs. Acridine orange (100 µL) and ethidium bromide (100 µL) were dissolved in PBS to create a 200-µliter dye mixture. L6 cells, isolated on a platform with six channels at a density of 5 x 10^4 cells per channel, were incubated for one day. After turning on the cells, rinsing with PBS-cold water, and treating them with nanoparticles for 24 h, they were subjected to a mixture of AO (100 g/mL) and EB (100 g/mL) at room temperature for five minutes. The examination of coloured cells was conducted using a 40x fluorescent microscope, revealing that the majority of cells in the study area underwent apoptosis. The number of cells exhibiting apoptosis-like characteristics was calculated as the ratio of the total number of cells in the field.

## Results and Discussion

**Marine sponge collection and identification**

Marine sponges were collected from the Rameswaram district of Tamil Nadu, India, using a grab sample collection method. The coordinates of the location are approximately 9°17'18.60″ " N, 79°18'45.76"″ E, and it is known for its coastal ecosystem.

**Fig 2:** Marine sponge collections site

**Genotypic identification of marine sponge**

The cleaned marine sponge was sent to the genomic sequencing centre Biokart India Pvt. Ltd. (Bangalore, India).

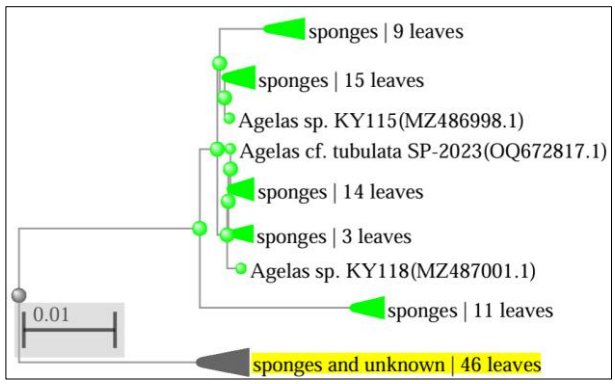


**Fig 3:** Genotypic identification of marine sponge by COX 1 gene analysis

**Extraction by Soxhlet**

In the exploration of marine sponges, the extractive values of ethanolic solvents were investigated, accompanied by the documentation of critical physical and chemical characteristics. In this study, ethanol served as the solvent for extraction, yielding an extract with a semi-solid consistency and a distinctive bark black color. The recorded yield percentage for the extract was 7.67%. These findings

provide valuable insights into the extraction properties of ethanol concerning marine sponges, offering information on both the physical attributes and the quantitative aspects of the obtained extract. The semi-solid consistency implies a certain viscosity, while the brown coloration suggests the presence of specific compounds or pigments within the extracted material. The documented yield percentage quantifies the efficiency of the extraction process, offering essential data for researchers and practitioners interested in harnessing the properties of marine sponges for various applications, such as pharmaceuticals, biotechnology, or other industrial uses [7].

**Characterisation of marine sponge assisted silver nanoparticles**

Zeta potential is a crucial parameter in the characterization of nanoparticles, providing information about their surface charge and stability in colloidal suspensions. The importance of zeta potential in nanoparticle detection and various applications lies in its influence on particle behavior, interactions, and potential applications. Zeta potential represents the electric potential at the shear plane around a charged nanoparticle in a colloidal suspension.

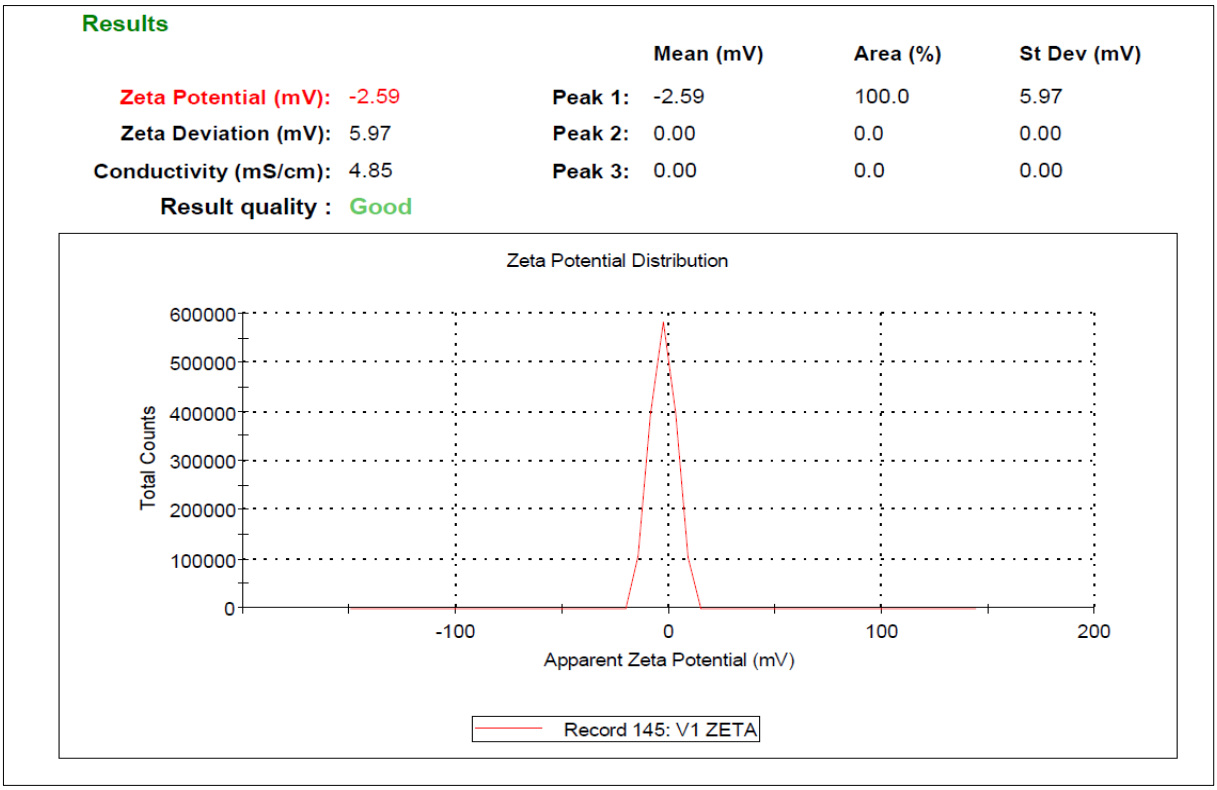


**Fig 3:** Zeta potential of marine sponge assisted silver nanoparticles

**MTT assay of silver nanoparticles-marine sponge assisted silver nanoparticles**

In this study, the MTT assay was employed to assess the inhibitory activity of marine sponge-assisted silver nanoparticles across a range of concentrations in L6 cell lines. The obtained results reveal a dose-dependent response, showcasing the impact of varying concentrations on the inhibition percentages. At the highest concentration of 125, the inhibitory effect is strikingly high, recording at 78.01%. This suggests a robust and potent biological activity of the nanoparticles, potentially influencing critical cellular processes assessed by the MTT assay. As the concentration decreases, a gradual reduction in inhibition percentages is observed, indicating a diminishing returns pattern. For instance, at a concentration of 62.5, there is a notable decrease in inhibition compared to the highest concentration, emphasizing the concentration-dependent nature of the observed effects. The concentration of 31.25 demonstrates a further reduction in inhibition, hinting at the presence of a potential threshold concentration below which the inhibitory effect becomes less pronounced. The practical implications of these findings lie in the optimization of marine sponge-assisted silver nanoparticles for specific applications. Understanding the concentration at which optimal inhibition occurs is crucial for tailoring these nanoparticles to achieve desired outcomes, particularly in fields such as biomedicine or environmental remediation. While the MTT assay provides a quantitative measure of metabolic activity, further investigations are warranted to unveil the biological significance of the observed inhibitory effects. Questions about the specific cellular processes influenced by these nanoparticles and their underlying mechanisms prompt avenues for future research. The reproducibility and reliability of the results are paramount considerations. Ensuring the experiments were conducted under controlled conditions and demonstrating consistency upon repetition are essential for drawing meaningful conclusions. Additionally, safety considerations are raised, especially at higher concentrations where the inhibitory effects are pronounced, necessitating a thorough assessment of the toxicity profile of marine sponge-assisted silver nanoparticles. In conclusion, this study contributes valuable insights into the concentration-dependent inhibitory activity of these nanoparticles, paving the way for further exploration and application in diverse fields [8].

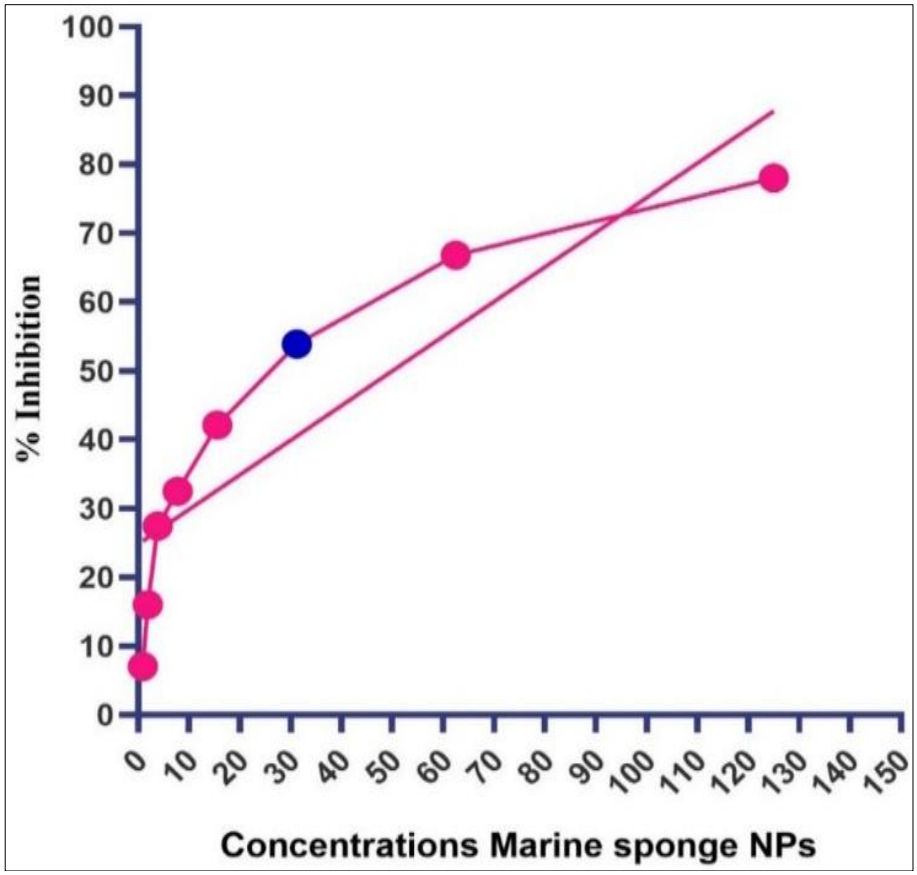


**Fig 4:** MTT assay activity of marine sponge assisted silver nanoparticles

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay holds paramount importance in biomedical research and drug development. This colorimetric assay is widely utilized for assessing cell viability and proliferation by measuring the metabolic activity of cells. The assay relies on the reduction of the yellow MTT dye by mitochondrial dehydrogenases in living cells to form purple formazan crystals [9]. The intensity of the resulting color is directly proportional to the number of viable cells, providing a quantitative measure of cell viability. One of the key strengths of the MTT assay lies in its versatility, enabling researchers to evaluate the effects of various compounds, nanoparticles, or environmental factors on cellular health. It plays a pivotal role in drug discovery, allowing for the screening of potential therapeutic agents and the determination of their cytotoxicity or efficacy [10]. Moreover, the MTT assay is crucial in elucidating the mechanisms of action of drugs and nanoparticles, offering insights into their impact on cellular metabolism. MTT assay is applied in fields such as toxicology, environmental science, and cancer research. It serves as a valuable tool for assessing the cytotoxic effects of environmental pollutants, studying the responses of cells to different treatments, and understanding the intricacies of cancer cell behavior. In summary, the MTT assay's significance lies in its ability to provide rapid, quantitative, and reproducible assessments of cell viability, making it an indispensable tool in diverse areas of biological and medical research.

**Acridine orange/ethidium bromide (AO/EB) staining technique to measure apoptotic induction of marine sponge assisted silver nanoparticles**

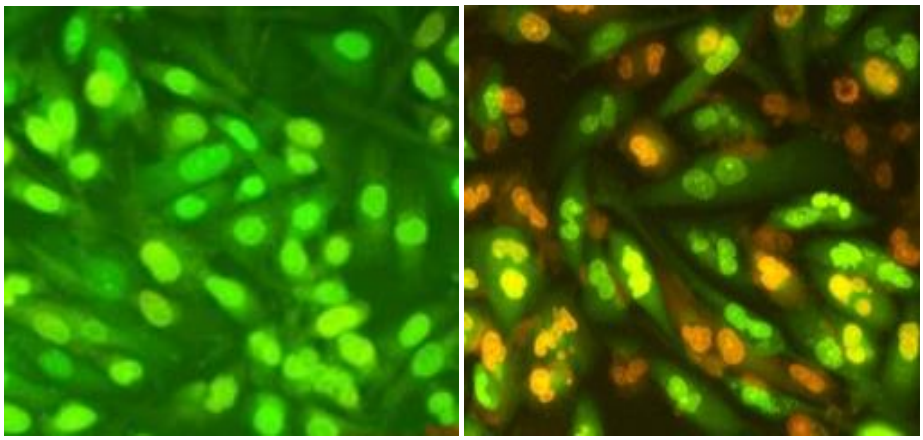

**Fig 5:** A: L6 control cells, B: Marine sponge assisted silver nanoparticles treated L6 cells (24 hr).

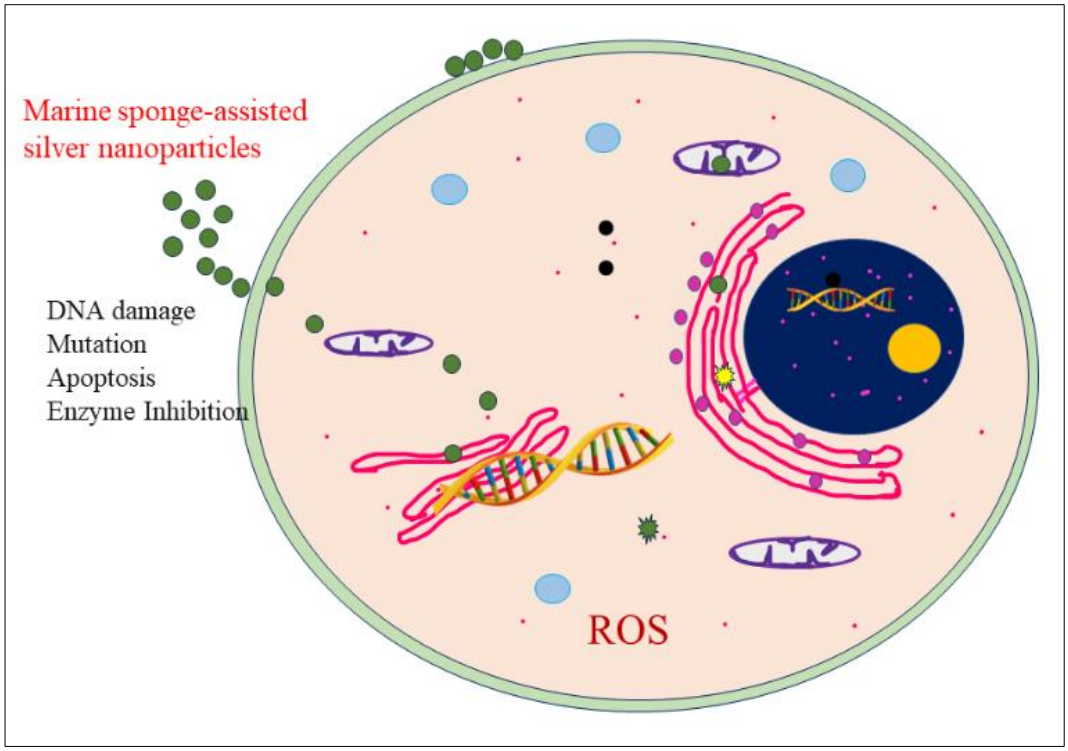


**Fig 6:** Marine sponge assisted silver nanoparticles and its cytotoxic activity

The investigation into the cytotoxic effects of marine sponge-assisted silver nanoparticles on L6 cells has unveiled compelling findings, as depicted in the microscopic images. In Figure A, the control L6 cells exhibit the typical morphology and characteristics expected of healthy cells. However, in Figure B, where L6 cells were subjected to marine sponge-assisted silver nanoparticles for a 24-hour period, discernible alterations in cellular morphology suggest the induction of apoptosis. Apoptosis, or programmed cell death, is a fundamental biological process crucial for maintaining tissue homeostasis and eliminating damaged or unnecessary cells. The observed changes in L6 cells treated with marine sponge-assisted silver nanoparticles strongly indicate the activation of apoptotic pathways. These alterations include cell shrinkage, membrane blebbing, and the formation of apoptotic bodies, all indicative of the controlled and orchestrated process of apoptosis. The implications of these findings are noteworthy, especially in the context of potential biomedical applications of marine sponge-assisted silver nanoparticles. The ability to induce apoptosis in L6 cells suggests a potential avenue for exploiting these nanoparticles in targeted therapies, particularly in the context of cancer treatment where apoptosis plays a pivotal role in eliminating cancerous cells. It is essential to delve deeper into the molecular mechanisms underlying the observed apoptotic effects. Assessing key markers associated with apoptosis, such as caspases and DNA fragmentation, would provide a more comprehensive understanding of the signaling pathways involved. Furthermore, the concentration and duration of nanoparticle exposure must be carefully considered to delineate the optimal conditions for inducing apoptosis while minimizing potential adverse effects on normal cells [11]. This information is critical for the development of safe and effective nanoparticle-based therapies. The microscopic evidence of apoptosis in L6 cells following treatment with marine sponge-assisted silver nanoparticles marks a significant stride in understanding the cytotoxicity profile of these nanoparticles. Further investigations into the molecular pathways, dose-response relationships, and potential therapeutic applications will undoubtedly contribute to the growing body of knowledge in the field of nano-medicine and cellular biology.

## Conclusion

The study presents a comprehensive exploration of marine sponge-assisted silver nanoparticles (AgNPs), encompassing their collection, identification, synthesis, and evaluation of their bioactivities. Marine sponges were collected from the Rameswaram district in Tamil Nadu, India, and identified through genomic sequencing. The ethanolic extracts from these sponges were utilized to synthesize AgNPs via a green synthesis method. The synthesized nanoparticles were characterized by particle size and zeta potential analysis. Their antioxidant activity was confirmed through DPPH radical scavenging assays, demonstrating substantial free radical neutralization capabilities. The MTT assay revealed dose-dependent cytotoxicity of the nanoparticles on L6 cells, indicating significant biological activity. Additionally, the acridine orange/ethidium bromide staining technique showed that marine sponge-assisted AgNPs could induce apoptosis in L6 cells, suggesting their potential for applications in targeted therapies, particularly in cancer treatment. These findings underscore the promising therapeutic potential of marine sponge-assisted AgNPs in biomedicine, especially in developing antioxidant therapies and cancer treatments. Further research into their molecular mechanisms and optimization for specific applications is warranted.

## Consent for Publication

Not applicable.

## Funding

Not applicable.

**Conflict of Interest**
Nil.

**Acknowledgement**
The authors wish to acknowledge the Centre for Biotechnology and Phyto Pharmacognosy Research provide facilities to carry out the necessary work.

**Reference**
1. Sathiyanarayanan G, Saibaba G, Kiran GS, Yang YH, Selvin J. Marine sponge-associated bacteria as a potential source for polyhydroxyalkanoates. Crit. Rev. Microbiol. 2017;43(3):294-312. DOI:10.1080/1040841X.2016.1218389.
2. Kiran GS, Sekar S, Ramasamy P, *et al*. Marine sponge microbial association: Towards disclosing unique symbiotic interactions. Mar Environ Res. 2018;140:169-179. DOI:10.1016/j.marenvres.2018.06.011.
3. Bai X, Liu Y, Wang H, Zhang H. Natural Products from the Marine Sponge Subgenus Reniera. Molecules. 2021;26(4):1097. DOI:10.3390/molecules26041097.
4. Messaoudi O, Benamar I, Azizi A, *et al*. Characterization of Silver Carbonate Nanoparticles Biosynthesized Using Marine Actinobacteria and Exploring of Their Antimicrobial and Antibiofilm Activity. Mar Drugs. 2023;21(10):536. DOI:10.3390/md21100536.
5. Hamed AA, Kabary H, Khedr M, Emam AN. Antibiofilm, antimicrobial and cytotoxic activity of extracellular green-synthesized silver nanoparticles by two marine-derived actinomycete. RSC Adv. 2020;10(17):10361-10367. DOI:10.1039/C9RA10443G.
6. Shkryl YN, Veremeichik GN, Kamenev DG, *et al*. Green synthesis of silver nanoparticles using transgenic Nicotiana tabacum callus culture expressing silicatein gene from marine sponge Latrunculia oparinae. Artif Cells Nanomed Biotechnol. 2018;46(8):1646-1658. DOI:10.1080/21691401.2017.1388248.
7. Alkhilaiwi FA, Fadil SA, Aljoud FA, *et al*. Evaluation of Cytotoxicity of the Methanolic Extract of Red Sea Marine Sponge Xestospongia Testudinaria and Its Related Compounds Against MCF-7 Human Breast Cancer Cells. Breast Cancer (Dove Med Press). 2023;15:879-890. DOI:10.2147/BCTT.S326518.
8. Bechmann N, Ehrlich H, Eisenhofer G, *et al*. Anti-Tumorigenic and Anti-Metastatic Activity of the Sponge-Derived Marine Drugs Aeroplysinin-1 and Isofistularin-3 against Pheochromocytoma *In vitro*. Drugs. 2018 Mar;16(5):172. DOI:10.3390/md16050172.
9. Muthiyan R, Nambikkairaj B, Mahanta N, *et al*. Antiproliferative and Proapoptotic Activities of Marine Sponge Hyrtios erectus Extract on Breast Carcinoma Cell Line (MCF-7). Pharmacogn Mag. 2017;13(Suppl 1). DOI:10.4103/pm.pm_346_16.
10. Liu Z, Frank M, Yu X, *et al*. Secondary Metabolites from Marine-Derived Fungi from China. Prog Chem Org Nat Prod. 2020;111:81-153. DOI:10.1007/978-3-030-53275-6_3.
11. Santos JD, Vitorino I, De la Cruz M, *et al*. Bioactivities and Extract Dereplication of Actinomycetales Isolated from Marine Sponges. Front Microbiol. 2019;10:727. DOI:10.3389/fmicb.2019.00727.