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CHARACTERIZATION AND ANTICANCER ASSESSMENT OF SILVER NANOPARTICLES SYNTHESIZED FROM SEAGRASS *Enhalus acoroides*

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ABSTRACT

Seagrass possess various pharmacological active compounds which have been utilized for the biosynthesis of silver nanoparticles and act as biocompatible, stable and reliable alternative instead of the current chemical and physical approaches. *Enhalus acoroides* possesses various pharmacological properties and the present study was aimed at assessing the chemical characteristics and anticancer effects of synthesised silver nanoparticles (AgNPs) with the extracts of *E. acoroides* against HeLa cell lines. The nanoparticle was synthesised from the aqueous extract and characterisation of nanoparticles were carried out using UV-Vis, FTIR, SEM, TEM and EDX analysis. Further these nanoparticles were subjected to cytotoxicity study using HeLa cell line. The UV-Vis spectrum at 200-1100 nm and FTIR analysis revealed various functional groups which corresponds to numerous phytochemicals like alkaloid, flavonoids and polyphenols. The crystalline structure of the nanoparticle synthesised was confirmed using XRD, which was exhibited by silver nanoparticles by EDX analysis. The spherical shape and average size of the synthesised nanoparticles was visualised. The morphological characteristics was confirmed using SEM and TEM. The IC_{50} value was found to be 72.89 µg mL^{-1} demonstrating the potency of silver nanoparticles against the HeLa cell line. The cytotoxicity assay using MTT revealed that the synthesised nanoparticle exhibited a dose-and time-dependent cytotoxicity against HeLa cell lines. Thus, the study concluded that *E. acoroides* silver nanoparticle is an effective strategy for cervical cancer treatment.

Keywords: cervical cancer, cytotoxicity, electron microscopy, marine drugs, nanoparticle, therapeutics.

INTRODUCTION

Nanotechnology has garnered significance in biomedical field owing to the limitations posed by ineffective drug choices for curing many infections. It has nowadays been employed in diverse fields like drug engineering, biopharmaceuticals, wastewater treatment, magnetic devices, sensors, drug delivery systems (Saber *et al.*, 2018). Nanotechnology utilises the distinctive qualities of nanoparticles to revolutionise and improve applications in the fields of medicine, electronics, and environmental science. Nanoparticles due to their unique properties including their size and shape have witnessed a surge in attention across multiple fields, owing to their and the utility of their small sized particles (Kumar *et al.,* 2018). Among the various nanoparticles, AgNPs have emerged intriguing due to their minute size and large surface area-to-volume-ratio. These unique properties makes them more prominently used nanoparticle with wide range of pharmaceutical applications (Dadashpour *et al.,* 2018). The cost effectiveness and the size make nanoparticles advantageous in the field of biomedicine compared to other methods. These nanoparticles are more precise compared to the crude extract. Nanoparticles synthesized using natural plant products offer eco-friendly solutions, leveraging bioactive compounds for enhanced biomedical and environmental applications.

For the past five decades, scientists worldwide have been drawn to the study of marine natural products. these organism represent an extraordinary reservoir of novel bioactive compounds, exhibiting a diverse array of structural and chemical characteristics (Blunt *et al.,* 2013). Seagrasses are an important component of marine ecosystems. It varies greatly within different regions (Boutahar *et al.,* 2020). Genera such as *Halodule, Thalassia, Cymodoceae* and *Enhalus* are widely distributed in the tropical coasts (Short F *et al.,* 2007). *Enhalus acoroides* is largest seagrass that belongs to the family *Hydrocharitaceae*, which is known for its unique characteristics and ecological significance within coastal ecosystems. The characteristics of *E. acoroides* include large, round fruits measuring 4-6 cm in diameter and long strap-like rhizomes with a substantial diameter of 1.5 cm. The seagrass is found to exhibit various pharmacological properties such as antioxidant, antibacterial, cytotoxic, haemolytic, antidiabetic and antitumor activity (Menajang *et al.,* 2020). These pharmacological properties as they are found to possess various bioactive classes such as tannins, alkaloids, polyphenols, flavonoids, saponins, and steroids (Amudha *et al.,* 2017). Phytocompounds such as n-tetracosanol-1, 1-nonadecene and triacontane are some major metabolites found in *Enhalus acoroides* (Amudha *et al.*, 2018; Gono *et al.,* 2022). Though many studies have been conducted to study the anticancer activity of *E. acoroides* this is the first study that was aimed to investigate the potential anticancer effects of silver nanoparticles from *E. acoroides* extract on HeLa cervical cancer cell line, utilizing MTT assays and morphological examination.

MATERIALS AND METHODS

Preparation of seagrass extract

The *Enhalus acoroides* samples were thoroughly rinsed with running water to eliminate any debris. The cleaned samples were blended with electric blender with 75% hydroalcoholic extract and the solvent was evaporated using rotary evaporator (Shaffai *et al.,* 2023).

Synthesis of silver nanoparticle

For the study, AgNPs were synthesized from the hydroalcoholic extract of *E. acoroides.* The AgNPs synthesis was carried out by Moteriya and Chanda's protocol (Moteriya & Chanda *et al.,* 2018). To 0.1 mL of hydroalcoholic extract 0.9 mL of 1 mM silver nitrate solution was added and incubated at room temperature for 1 hour and color change was monitored (pale yellow to yellow; clear green to mixed green color). This solution was air dried and washed with distilled water. The powdered samples were kept in -20°C until further use (Hemlata *et al.,*2020).

Characterisation of nanoparticle

The functional group characterisation was carried out using FT-IR (Shimadzu IRTracer 100) and UV-Vis spectrum (Shimadzu UV 5600 Plus) was used to understand the absorbance of nanoparticle which is proportional to its wavelength (Kumar *et al.,* 2019).

Electron microscopy (SEM and TEM) and EDX analysis of nanoparticles

The ZEEISS-SEM equipment was used to evaluate both the dimensions and the form of the nanomaterials. The desiccated state of silver nanoparticles was subjected to ultrasonic treatment using distilled water. A minute droplet of silver nanoparticles was deposited onto a glass slide and allowed to undergo evaporation. The ZEEISS-SEM instrument operated under a total vacuum pressure of approximately 10-5 torr. The voltage is 10 kilovolts. The transmission electron microscopy (TEM) analysis was conducted by CM30–Philips at an operational voltage of 80 kilovolts (kV). The sample underwent compositional analysis using energy dispersive X-ray spectroscopy (EDXS) in conjunction with the scanning electron microscope (SEM). The Ag sample was analysed using the SEM equipment for EDX analysis (Mohamed *et al.,* 2022).

Cell lines and culture medium

The human cervical cancer (HeLa) cell lines were obtained from the National Centre for Cell Science, Pune and cultured in Eagle's Minimum Essential Medium that contained fetal bovine serum (10%) and the cells were kept at 37°C with 5% carbon dioxide, 100% humidity and 95% air. The cells were passaged once in a week by changing the medium twice a week (Thavamani, *et al.,* 2013).

Cytotoxicity activity of silver nanoparticle synthesised from E. acoroides

The monolayer cell culture was treated with trypsin to detach the cells, resulting in a cell density of 1.0 x 10^5 cells mL^{-1}. The media used for this process contained 10% FBS. 100 microliters of the diluted cell suspension (1x105 cells/well) have been added into each well of a 96-well plate. Following a 24-hour incubation period, a partially formed monolayer was established. The excess supernatant was then removed, and the monolayer was rinsed with cell culture media. Subsequently, 100 microliters of various concentrations of test samples were introduced to the partial monolayer on a 96-well plate. The cells were cultured at 37°C for 24 h in an incubator? containing 5% carbon dioxide. Following incubation, the test solution was disposed away and 20 microliters of MTT were introduced into each well. The plate was placed in a controlled environment with 5% carbon dioxide at 37°C for 4 h. The liquid above the sediment was obtained and 100 microliters of dimethyl sulfoxide (DMSO) was added. The plate was agitated to dissolve the formazan that had been produced. The measurement of absorbance was conducted at a wavelength of 570 nm using a UV Vis spectrophotometer (Shimadzu UV 5600 Plus) (Dadashpour *et al.,* 2018). The percent viability was calculated using the following formula: % viability = sample absorbance/control absorbance x 100.

Statistical analysis

The experiments have been carried out three times, with each trial being repeated three times. The software programme GraphPad InStat (version 3), which runs on MS-Windows, was adopted to compute the IC_{50}, which is the quantity of samples essential to reduce the concentrations by fifty percent. This was executed graphically employing a linear regression strategy. The results of the experiment were reported as the mean value plus or minus the standard deviation (Narayanan *et al.,*2024).

RESULTS

Characterization of silver nanoparticle from Enhalus acoroides

FTIR and UV-Vis spectrum analysis: The FTIR study revealed distinct bond types at various peaks 3691.28 (hydroxyl group/-OH) stretch, 3448.63 (hydroxyl group/-OH) stretch, 2921.99 (methylene group/CH_2) stretch, 2852.06 (methyl group/CH_3) stretch, 1643.77 (Carbonyl group/C=O) stretch, 1558.63 (amide group/C=O) stretch, 1449.99 (methyl group/CH3) stretch, 1384.74 (methyl/methylene group CH_3/CH_2) stretch, 1339.95 ((methyl group/CH3) stretch 1190.05 (carbon-oxygen group/C-O) stretch, 1064.91 (carbon-nitrogen group/ C-N) stretch, 1105.52 (carbon-oxygen group/C-O) stretch, 848.30 (Carbon-hydrogen group/ C-H) stretch, 878.07 (Carbon-hydrogen group/ C-H) stretch, 780.59 (Carbon-hydrogen group/ C-H) stretch and 619.37 (Carbon-bromine/ C-Br) stretch (**Fig. 1**). The FTIR analysis revealed that hydroxyl group is the most prominent one compared to other groups with spectrum at various stretches. Similarly in a study with *Capparis zeylanica* extract silver nanoparticle biosynthesised was characterized using FTIR analysis where the most prominent functional groups were carbonyl, hydroxyl, amino and carboxylic groups with bonds at 3455.17 cm^{-1}, 2426.90 cm^{-1} and 1120.099 cm^{-1} (Nilavukkarasi *et al.,* 2020).

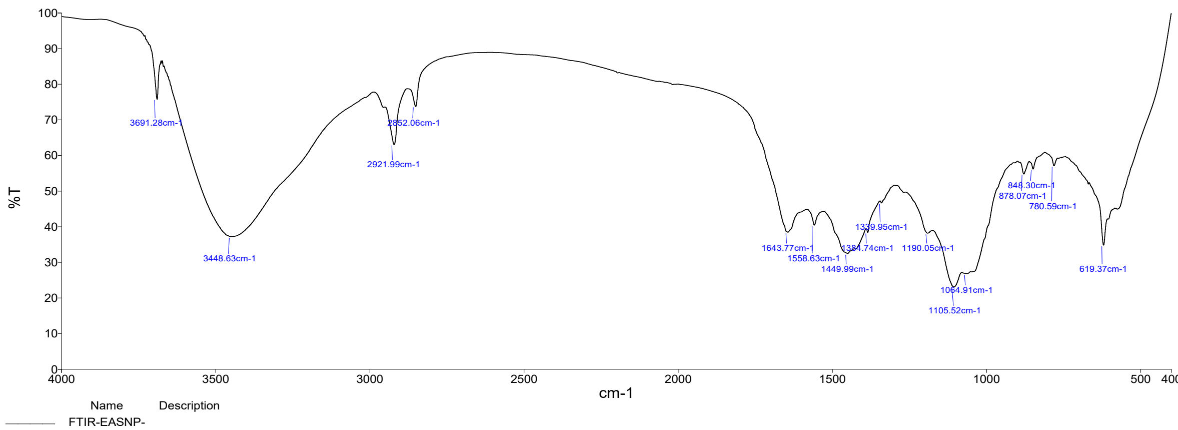

Fig. 1: Fourier-transform infrared (FTIR) spectrum analysis was conducted to characterize the functional groups present on the surface of silver nanoparticles synthesized using *Enhalus acoroides* extract.

The synthesis of the AgNPs in hydroethanolic extract of seagrass was visualized using the absorption spectra at a wavelength range of 200-1100 nm **(Fig. 2).** It was noted that upon addition of leaf extracts to the silver nitrate solution dark brown coloration was observed. Previous studies suggested that a peak at 200-370 nm has been observed for silver nanoparticles that attribute to spherical shape of the synthesized NPs. UV-Vis spectrum analysis is the most widely used technique for the characterization of the synthesized nanoparticle (Mahadevan *et al.,* 2017) where the peak occurred between 200-1100 nm, which is indicative of silver nanoparticle. In a study with *C. zeylanica,* the analysis revealed that a broad spectrum was observed at 310 to 395 nm for a silver nanoparticle synthesis (Nilavukkarasi *et al.,* 2020).

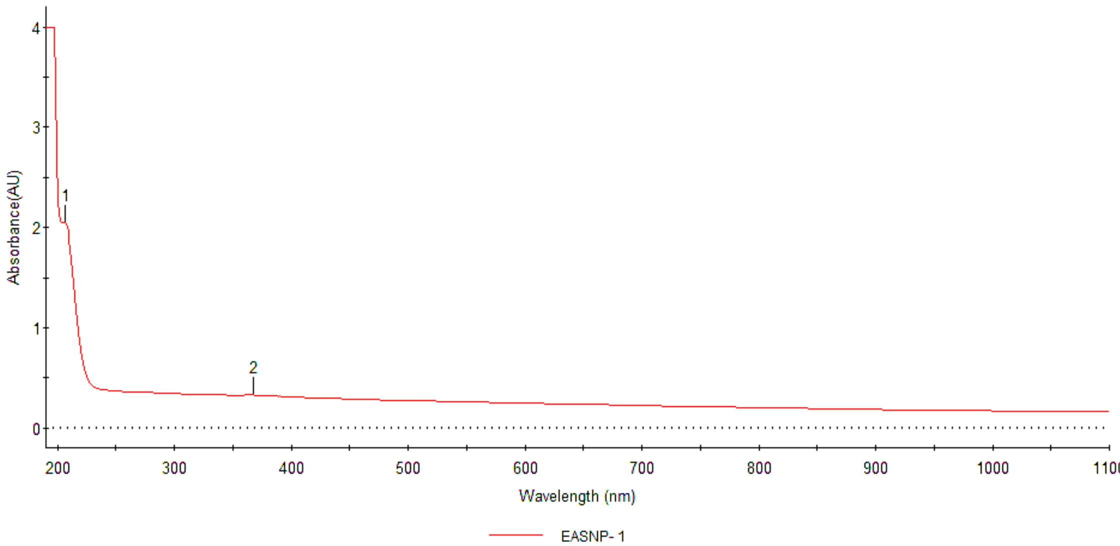

Fig. 2: Ultraviolet-visible (UV-Vis) spectrum analysis was employed to examine the optical properties and stability of silver nanoparticles synthesized using *Enhalus acoroides* extract.

The SEM analysis was conducted to examine the morphology and dimensions of the nanoparticles (NPs). The results revealed the production of polydisperse spherical NPs with varying diameters ranging from 33 to 71 nm. Additionally, the nanoparticles exhibited a cubic structure. Many of the nanoparticles aggregated whereas only a small portion of them exhibited dispersion, as revealed using scanning electron microscopy (SEM) (**Fig. 3a**). To determine the size of the nanoparticles, a histogram-based dispersion assessment was conducted employing the ImageJ 1.53 version programme (**Fig. 3b**). The data was analysed by plotting a graphical representation of the data to examine the variation in the sizes of the particles. The results showed that the nanoparticle diameters ranged from 22.01 to 75.38 nm, with an average size of 44.39 ± 16.19 nm. The nanoparticle exhibited a wide distribution of particle sizes, ranging from 20 to 50 nm. This indicates that the nanoparticles were synthesised to be less than 100 nm. Scanning electron microscopy (SEM) was conducted to examine the morphology and dimensions of the nanoparticles (NPs). The research indicated that the size of the nanoparticles varied within a certain range 22.01-75.38 nanometre with an average size of 44.39 ± 16.19 nanometre. The nanoparticle exhibited a wide distribution of particle sizes, ranging from 20 to 50 nm, indicating that the synthesised nanoparticles were less than 100 nm. In a similar study the morphology of the synthesized silver nanoparticle was observed and the particle size was noted at 28 nm, which suggested that the nanoparticle is spherical in shape (Othman *et al.,* 2019).

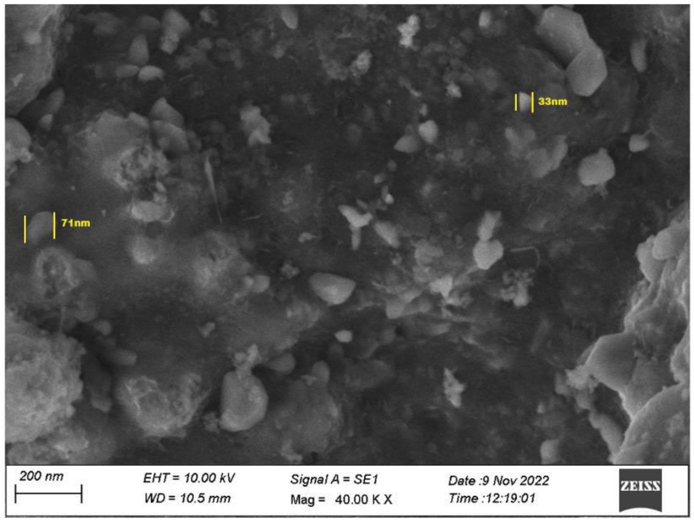

Fig. 3a: Scanning electron microscopy (SEM) analysis was used to observe the surface morphology and particle size of silver nanoparticles synthesized using *Enhalus acoroides* extract (33 to 71nm).

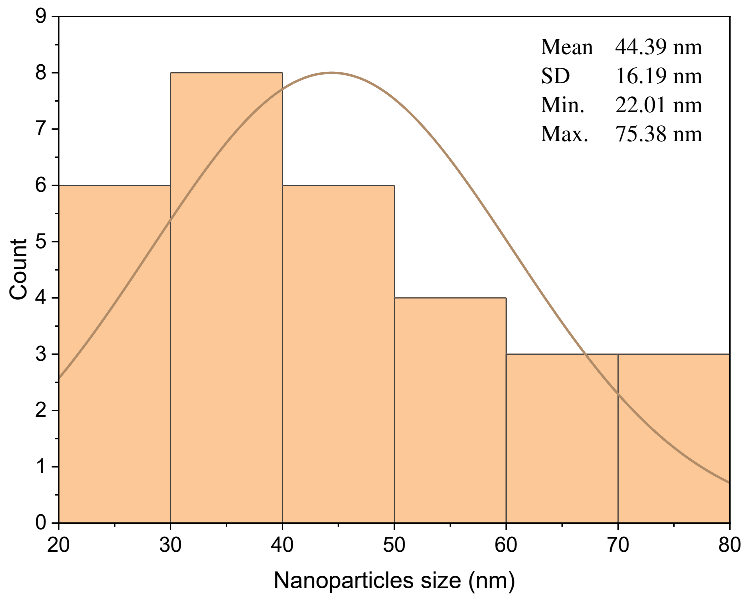

Fig. 3b: The histogram illustrates the particle size distribution of silver nanoparticles synthesized using *Enhalus acoroides* extract. This graphical representation is essential for understanding the range and frequency of particle sizes within the sample.

Transmission Electron Microscopical (TEM) analysis: The transmission electron microscopy (TEM) study demonstrated that the particles have spherical shape and are evenly distributed, with a wide range of sizes ranging from 23.15 to 63.78 nm (**Fig. 4**).

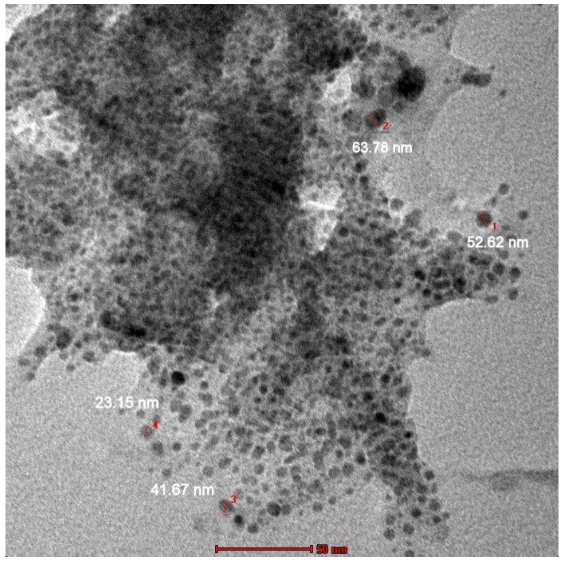

Fig. 4: Transmission electron microscopy (TEM) analysis was conducted to determine the internal structure, morphology, and precise size of silver nanoparticles synthesized using *Enhalus acoroides* extract. TEM provides high-resolution images that reveal detailed information about the nanoparticles at the atomic or molecular level.

Energy Dispersive X-Ray Spectroscopic Analysis (EDXS) analysis: The investigation employing energy dispersive X-ray analysis (EDXS) was conducted to validate the synthesis of silver nanoparticles. The existence of silver as the constituent element and the production and purity of silver nanoparticles synthesised from extract are shown by the EDX peaks corresponding to the element silver. The EDX elemental analysis of the synthesised silver nanoparticles revealed that silver accounted for the largest fraction (68.05%), subsequently followed by oxygen (28.58%), sodium (2.60%), and silicon (0.77%) (**Fig. 5** & **Table 1**). In the present study, The EDX elemental analysis of the synthesised silver nanoparticles revealed a predominant concentration of silver (68.05%), oxygen (28.58%), sodium (2.60%) and silicon (00.77%). Similarly in a study the Edx analysis of silver nanoparticles was found to reveal the presence of silver, chloride, carbon, aluminium, oxygen and silicon at 71.37, 17.89, 2.93, 1.29, 3.69 and 2.83% respectively. This study was concordant with green synthesis of silver nanoparticles mediated by *Benincasa hispida* peel (Devi *et al.,* 2016) and leaf of *Momordica charantia* leaf extracts (Devi *et al.,*2016). In the current study, Transmission electron microscopy analysis revealed the spherical structure of the AgNPs with size distribution of 23.15 to 63.78 nm. In a study conducted by Mariadoss et al., documented that AgNPs synthesized using the extract of *Malus domestica exhibited* spherical morphology with polydispersity ranging from 40 to 100 nm (Mariadoss et al., 2019)and in a similar study the silver nanoparticle synthesised from *Origanum majorana* revealed the polydispersity with a size range of 10 to 60 nm (Yassin *et al.,*2022).

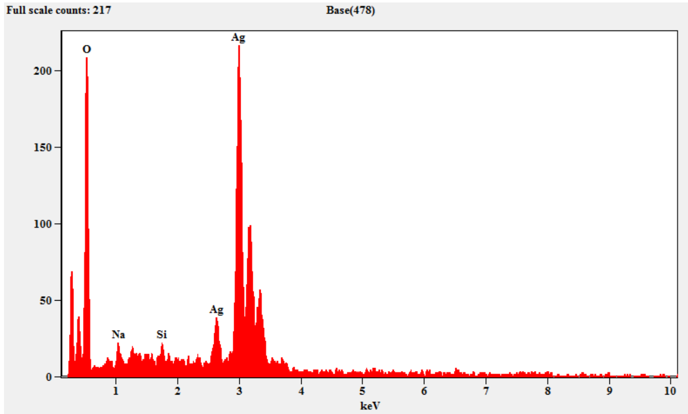

Fig. 5: Energy-dispersive X-ray (EDX) spectrum analysis was performed to determine the elemental composition of silver nanoparticles synthesized using *Enhalus acoroides* extract. This technique is crucial for confirming the presence and purity of silver within the nanoparticles.

Table 1: Elemental composition of nanoparticles

S. No.	Elements/ series	Weight (%)	Atomic weight (%)
1.	Ag L	68.05±2.48	83.55
2.	Na K	2.60±0.50	3.15
3.	Si K	0.77±0.19	0.76
	O K	28.58±1.15	12.54
Total		100.00	100.00

Cytotoxicity activity of AgNPs from Enhalus acoroides

The present study has been undertaken to investigate the anticancer activity of silver nanoparticle on HeLa cell line. The result of anticancer evaluation of silver nanoparticles were examined by MTT assay using HeLa cell line and the different concentrations used for the study is represented in the **Table 2**, where at a concentration of 50 µg mL^{-1} highest cytotoxicity was observed indicating higher potential of AgNPs from *Enhalus acoroides* extract. As the concentration increases there is an increase in the cell viability. The IC_{50} value of AgNPs from *Enhalus acoroides* extract was found to be 72.89 µg/ml. The suppression of cell division may occur due to the activation of programmed cell death and the suppression of cellular expansion. The quantity of formazan crystals generated by MTT is directly correlated with the quantity of live cells. Figure 6 illustrates the cytotoxic effects of AgNPs on HeLa cell line. The cell lines were exposed to different concentrations of silver nanoparticle and cell viability was assessed using the MTT assay. As the concentration of AgNPs increases a significant decrease in cell viability is observed indicating a dose-dependent cytotoxic effect. The IC_{50} value which is the concentration of AgNPs required to inhibit 50% of the cell population is 72.89 µg mL^{-1} demonstrating the potency of silver nanoparticles against the HeLa cell line.

The cytotoxicity evaluation of the AgNPs of *Enhalus acoroides* revealed that the IC_{50} value was 72.89 µg/ml in another study with *Solanum tuberosum* AgNPs, the cytotoxicity was evaluated on four different cell lines such as Skov-3, HDF, U118 and Caco-2, where the IC_{50} value was found to be 4.31±0.15µg/ml, 43.39 ±0.28 µg/ml, 23.37 ±0.23 µg/ml and 6.43 ±0.1 µg/ml respectively (Xu et al., 2023). In a study, anticancer activity of the silver nanoparticle synthesized from *Delonix regia* was found that they were as efficient as compared to the standard Doxorubicin against hepatocarcinoma, colon carcinoma and breast cancer cell line (Abu-Dief *et al.,* 2020; Morais *et al.,* 2020).

Table 2: Cell viability of Ag-NPs treated on HeLa cell line assessed by MTT assay

S. No	**Concentration (µg mL^{-1})**	**Cell viability (%)**
1	6.25	94.6
2	12.5	84.9
3	25	62.07
4	50	52.7
5	100	49.7
	IC_{50}	**72.89 µg mL^{-1}**

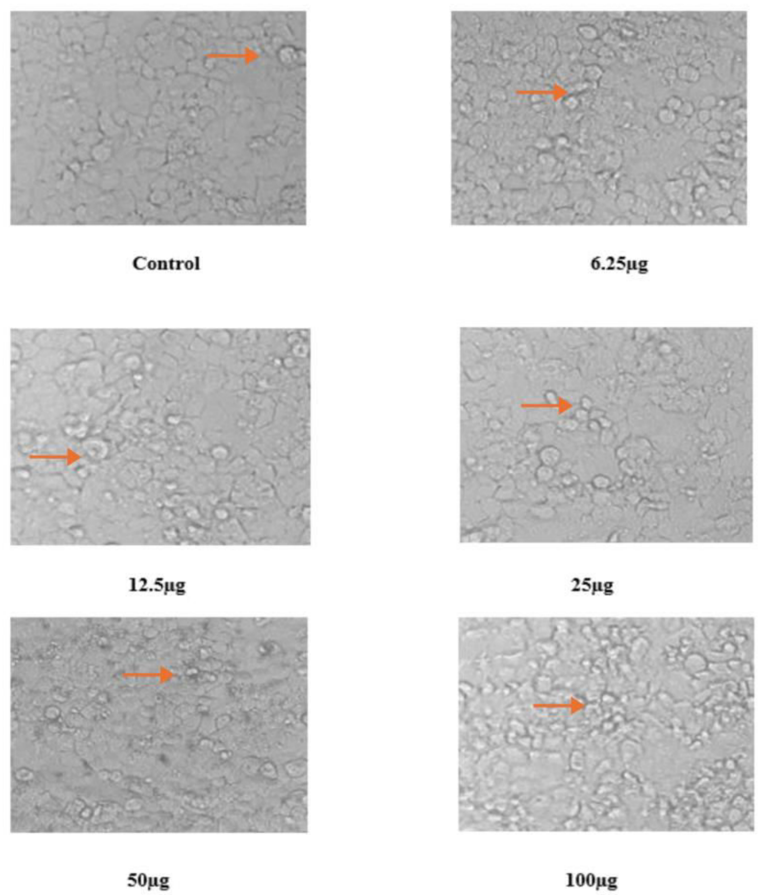

Fig. 6: Light micrographs of AgNPs extract cytotoxicity of HeLa (cervical cancer) cell line. AgNPs treatment caused morphological changes like detachment from the substratum, with rounding of cells and cell shrinkage. The above result affirms that the cytotoxicity of AgNPs from *E. acoroides* extract substantially increased with increase in concentration**.**

CONCLUSION: In conclusion, this study successfully synthesized silver nanoparticles (AgNPs) using *Enhalus acoroides* extract and comprehensively characterized them through various analytical techniques, including FTIR, EDX, SEM, TEM and UV-Vis spectrum analysis. The FTIR analysis revealed functional groups present in the AgNPs, while EDX provided insight into their elemental composition. SEM and TEM imaging showed the morphology size and distribution of the nanoparticles, confirming their nano-scale dimensions. UV-Vis spectrum analysis demonstrated the surface plasmon resonance characteristics of the silver nanoparticles. Furthermore, the efficacy of the synthesized nanoparticles was evaluated on the HeLa cell line, demonstrating promising results. The silver nanoparticles exhibited notable cytotoxic effects on HeLa cells, indicating their potential applications as an anti-cancer agent. These findings underscore the biocompatibility and therapeutic potential of AgNPs derived from *Enhalus acoroides*, highlighting their biomedical applications.

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ETHICAL APPROVAL: This study does not involve experiments with animals or human subjects.

CONSENT FOR PUBLICATION: Not applicable.

AVAILABILITY OF DATA AND MATERIALS: Data and materials used/analyzed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS: The authors declare that there are no conflicts of interest.

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AUTHOR'S CONTRIBUTIONS: Taslima Nasreen & B. N. Poojitha- Methodology, writing – Original Draft Preparation; P. Amudha-Conceptualization, Supervision, writing-review and editing; R.Vidya-Project administration; M. Jayalakshmi-Resources and data curation. All authors have contributed equally to the article.

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