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Anticancer Potential of *Vernonia cinerea* Mediated AgNPs Against the Human Pancreatic Cancer PANC

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Abstract: *Vernonia cinerea* (L.) Less is used in folk medicine as a remedy for various diseases. *In vitro* anticancer activity of green synthesized silver nanoparticles from the aqueous extract of *V. cinerea* extract was evaluated in the present study. Physicochemical characterization of the *V. cinerea* synthesized silver nanoparticles (VC-AgNPs) was performed using UV-analysis, FT-IR, SEM, XRD and DLS followed by the evaluation of anticancer activity on human pancreatic Cancer PANC. The nanoparticles were found to be spherical with a 50 nm average particle size and a zeta potential value of −18.6 mV. Silver nanoparticles exhibited significant toxicity to human pancreatic Cancer PANC with an IC_{50} value of 20.4 µg/ml. Following that, morphological alterations related with apoptosis were observed using acridine orange and ethidium bromide staining (AO/EtBr) with various doses of VC-AgNPs. The nanoparticles induced DNA fragmentation and also inhibited cell proliferation in PANC. In the PANC, the cytotoxic efficacy of VC-AgNPs was shown to be in dose dependant manner. Using florescence analysis, the effect of VC-AgNPs on cell cycle arrest was studied. The developed VC-AgNPs exerted potential anticancer activity on human pancreatic Cancer PANC.

Keywords: *Vernonia cinerea* , PANC, Green synthesis, MTT assay, Anticancer activity, Nanoparticles

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Introduction

The burden of cancer is significant worldwide and continues to increase. It was speculated that by the end of 2020, there will be 16 million new cancer diagnoses worldwide, up from the total of over 11 million. In addition, more than 8 million people globally die from cancer each year.

According to estimates it was reported that by 2020, there will be one cancer patient or cancer survivor for every 19 persons. Testicular, prostate, ovarian, bladder, cervix, breast, and head and neck cancers are the most prevalent forms of solid tumours (Amuthan *et al.*, 2021). In terms of cancer-related deaths worldwide, pancreatic cancer (PC) continues to be the deadliest disease, with a 5-year survival rate in the low single digits (1–7%) and an estimated 25,000 deaths per year worldwide. Additionally, pancreatic cancer incidences in developed nations have increased at a 2% annual rate, and the death rate from most cancers has significantly decreased (Raimondi *et al.*, 2009; Jemel *et al.*, 2011). Due to their significant side effects and toxicity on non-cancerous tissues, the current therapeutic drugs utilized to treat pancreatic cancer are both expensive and ineffective. Therefore, research into a novel, affordable, and biocompatible therapeutic strategy to combat pancreatic cancer is necessary.

The most intriguing field for developing new nanoproducts in biotechnology and biocompatible medicine is nanotechnology as an interdisciplinary approach (Sadeghzadeh *et al.*, 2017; Dadashpour *et al.*, 2018). Silver nanoparticles (AgNPs) are among the most significant and well-known nanoproducts used in medicine (El-Sonbaty, 2013). The most promising field for creating novel forms of nanomaterials for use in biomedicine is bionanotechnology (Montazeri *et al.*, 2017). Gold, silver, and copper are among the several noble metal NPs that are often and widely synthesized utilizing physical and chemical techniques. There are many drawbacks to the chemical and physical synthesis of metal NPs, including the high cost, the use of risky and toxic compounds, and potential biological and environmental risks. Plants with a variety of natural compounds can be used as a key source for the biosynthesis of NPs and serve as a stable, dependable, and biocompatible alternative to the current physical and chemical techniques in the search for a unique, cost-effective, and environmentally acceptable method. Additionally, the biological approach has the capacity to better control particle size in comparison to the chemical and physical synthesis methods of metal NPs because control of particle size and morphology are crucial features for application in biomedicine (Dadashpour *et al.*, 2018).

More than 250,000 plants were screened in recent years to find powerful compounds, and some of these chemicals were studied and characterised pharmacologically and chemically. The potential of the tropical rainforest is enormous, undeveloped, and studyable. Therefore, the worldwide scientific collaboration in this field continues with the search for novel cytotoxic substances from microbial, plant, marine, and natural sources. Due to the complexity of the symptoms and signs associated with cancer, very few anticancer compounds have been developed from natural sources (Lalitha *et al.*, 2020).

An annual plant with a wide geographic distribution in India, Bangladesh, Sri Lanka, and the Malay Islands is *Vernonia cinerea* (L.) Less of the Asteraceae family (Harbone, 1998). The herb is widely used in traditional medicine to treat bronchitis, asthma, and colds as well as stomach problems. The plant is advised for the treatment of intermittent fever, filariasis, blisters, boils, and vaginal discharges according to the Indian Ayurveda Pharmacopoeia. The juice of the plant is used to hasten the healing of accidental wounds, filariasis, and toxic viral fevers (Sonibare *et al.*, 2016). The plant's root is traditionally used to treat all sorts of eruptive boils. The seeds are used as a decoction to treat colic and dysuria. Tonsillitis can be treated using the plant's young leaves. Both the leaf juice extract and the leaf extract are used to treat skin conditions and paediatric dysentery, respectively. In addition to these, the herb is used to treat leprosy, arthritis, urinary calculi, fever, cough, and malaria. The plant has qualities that are antibacterial, antimicrobial, antioxidant, analgesic, antipyretic, antiflautulent, anti-spasmodic, and diuretic. Sterols, flavonoids, sesquiterpene lactones, and a terpenoid with the chemical name "leupeol acetate" are a few of the phytochemical substances that have been linked to the plant and have been claimed to have

antihyperglycemic and antiulcer characteristics. *Vernonia cinerea* also has insect-antifeedant properties (Sonibare *et al.*, 2016).

In this present study, Physicochemical characterization of the *V. cinerea* synthesized silver nanoparticles (VC-AgNPs) was performed using UV-analysis, FT-IR, SEM, XRD and DLS and ZETA, followed by the potential *in vitro* cytotoxic and antiproliferative effect of VC-AgNPs in PANC human pancreatic cancer cells. Moreover, our results indicate that VC-AgNPs may prove a source of potential anticancer molecules against human pancreatic cancer PANC.

Materials and Methods

Preparation of V. cinerea leaf extract:

A powdered extract of *V. cinerea* leaves was made from shade-dried leaves. Then, in a temperature-controlled water bath, 5 g of dried leaf powder was heated for 30 min at 90–95°C after being homogenized with 100 mL of double distilled water. Additionally, to remove debris, the final solution was filtered through a cellulose nitrate membrane with pores measuring 0.22 mm in size. In order to create nanoparticles, this extract was chilled (Kovendan *et al.*, 2013).

Synthesis and characterization of VC-AgNPs:

At room temperature, 5 ml of freshly made *V. cinerea* leaf extract and 10 ml of $AgNO_3$ (0.01 M) were thoroughly combined. A visible colour change from yellow to reddish-brown signalled the creation of AgNPs, and spectrophotometric monitoring of the reduction kinetics followed. To continue with characterization using double beam UV-visible spectroscopy (Jasco V-670 UV-visible double beam spectrophotometer), the VC-AgNPs solution was multiplied by five with deionized water. Using deionized water as a blank, the spectra were measured between 200 and 800 nm. Through a zeta sizer (Horiba Scientific SZ-100, UK), the external stability and the average particle size distribution were examined at a 90 degree angle. The XRD grid was coated with dried nanoparticles, and the spectrum scanning range of 10 to 90 (2h value) and AgNPs crystalline structure were all noted. The shape and structure of the purified VC-AgNPs were examined using scanning electron microscopy (SEM). To further examine the functional groups of secondary metabolites, the VC-AgNPs was pelleted with KBr and subjected to Fourier-transform infrared (FTIR) spectroscopy (JASCO FT-IR 4100) at a resolution rate of 4 cm^{-1} (Rajaganesh *et al.*, 2016).

Cell culture maintenance:

The human pancreatic cancer cells (PANC) were purchased from the National Centre for Cell Science (NCCS) in Pune, India. The cells were maintained in Dulbecco modified eagle medium (DMEM), which included 1.5 g/l glucose, 1.5 g/l Na_2CO_3, essential amino acids, and bovine serum. It was also supplemented with balanced salt solution and 2 mM L-glutamine. Concentrations of streptomycin and penicillin (100 IU/100 g) were adjusted to 1 ml/L. The cells were grown in a 5% CO_2 environment at 37°C. Before being treated to 6-OHDA (100 M) for 24 h, the cells were first exposed to different ratios of VC-AgNPs (10, 25, or 50 M) for about 2 h.

Evaluation of cytotoxicity:

To determine the inhibitory concentration (IC_{50}) value, a cell cytotoxicity test (MTT, Hi-Media) was performed. The PANC human pancreatic cancer cells were given 48 h to reach 80% cell confluency while growing in 96 well plates (1x 10^4 cells/well). The culture medium was replaced with fresh medium containing VC-AgNPs at varying concentrations (10, 25, or 50 M), and they were similarly treated with PANC followed by incubation for 48 h. As a result, the culture medium was removed, 100 µl of MTT solution was added, and the wells were then incubated at 37°C for 4 h, along with the removal of the supernatant. Then, 50 µl of DMSO were added to each well and incubated for 15 min to spread the formazan crystals. The optical density (OD) of the PANC at 570 nm was calculated using a Biotek multimode plate reader (USA).

% cell viability = OD of experimental sample/OD of experimental control ×100

Morphological Study:

The PANC pancreatic cancer cells ($1x10^5$) were exposed to different concentrations of VC-AgNPs for 24 h (10 g/ml, 25 g/ml, and 50 g/ml), after which the cells were fixed in acetic acid: ethanol (1:3; v/v) solution. Glass slides were carefully placed on top of the cover slips for the morphologic research. There were three monolayers micrographic in each experimental group. Nikon (Japan) bright field inverted light microscopy was used to investigate the morphological alterations in the cells at 20X magnification.

Apoptotic cell death analysis:

On clean microscopic cover slips, 900 µl of cell suspension ($1x10^5$ cells/ml) was produced as a dye mixture of AO: EtBr, 1:1 (v/v) in deionized water. The PANC cells were pre-treated with 6-hydroxydopamine and then treated with various doses of VC-AgNPs before being collected and PBS-washed. The next step was to stain 10L of AO/EtBr. After 2 min, the cells were given two PBS rinses. Once more, 20X magnification fluorescence microscopy was used to examine the frozen cells. The cells were planted on glass microscope slides and cultured for 24 h in a 24 well plate. The fixed cells were first permeabilise for 10 min at room temperature with 0.2% triton X-100 (50 l), and then they were treated for 3 min with 10 l of DAPI. Glass coverslips assisted in uniformly distributing the dye. A fluorescent microscope was used to see the cells.

Fluorescence microscopic analysis of apoptotic cell death:

On brand-new microscope cover slips, 900 L of cell solution ($1x10^5$ cells/ml) and 1 ml of the dye mixture AO and EtBr each containing 100 mg/ml in distilled water were combined. After being pre-treated with various concentrations of VC-AgNPs, the cancer cells were collected, washed with phosphate buffer solution (pH 7.2), and stained with 10 L of AO/EtBr. After being incubated for 120 sec, the samples were rinsed with PBS and examined using a fluorescence microscope (Nikon Eclipse, Inc., Japan) using a variety of magnifications and a 480 nm excitation filter. Additionally, the cells were given different doses of VC-AgNPs for 24 h after being placed on a cover glass slip in a 6-well plate. Following a 3:1 methanol/acetic acid treatment, the samples were rinsed in PBS. The cells were then viewed using fluorescence microscope (Nikon Eclipse, Inc., Japan) after being stained with 10 µl of DAPI in the dark for 20 min.

Cell cycle analysis:

Pancreatic cancer cells PANC ($1x10^5$) were maintained in a 6-well plate for 48 hours with various doses of VC-AgNPs for the cell cycle investigation. The cells were then separated and treated for 30 min at room temperature in 500 ml of PBS solution containing 25 g of propidium iodide (PI) and 50 g of RNase. The samples were examined using flow cytometry by BD Facsverse USA.

Data Analysis:

The SPSS statistical programme, 16.0 edition, has been used for all the analyses. For data on maximal inhibition and IC_{50} for cell apoptosis, probability analysis was employed (Finney, 1971). Utilising ANOVA, the cell viability data were examined.

Results and Discussion

Characterization of VC-AgNPs:

UV–vis spectra of *VC*-AgNPs showed localized Plasmon resonance of VC-AgNPs (Fig. 1). VC-AgNPs turned dark brown on addition of $AgNO_3$. The overall reaction indicate the bio reduction of Ag^+ to Ag^0. The maximum absorbance peak was found at 436 nm (Poofery *et al.*, 2020). Similar color changes of reddish-brown outcome have been reported in the AgNPs synthesized via leaf extracts of *Ctenolepis garcini* (Narayanan *et al.*, 2021). Also, it is concluded that the phytoconstituents such as phenols, saponins, flavonoids, and triterpenes present in the *C. garcini* aqueous extract might acted as a reducing agent for the green fabrication of AgNPs. The

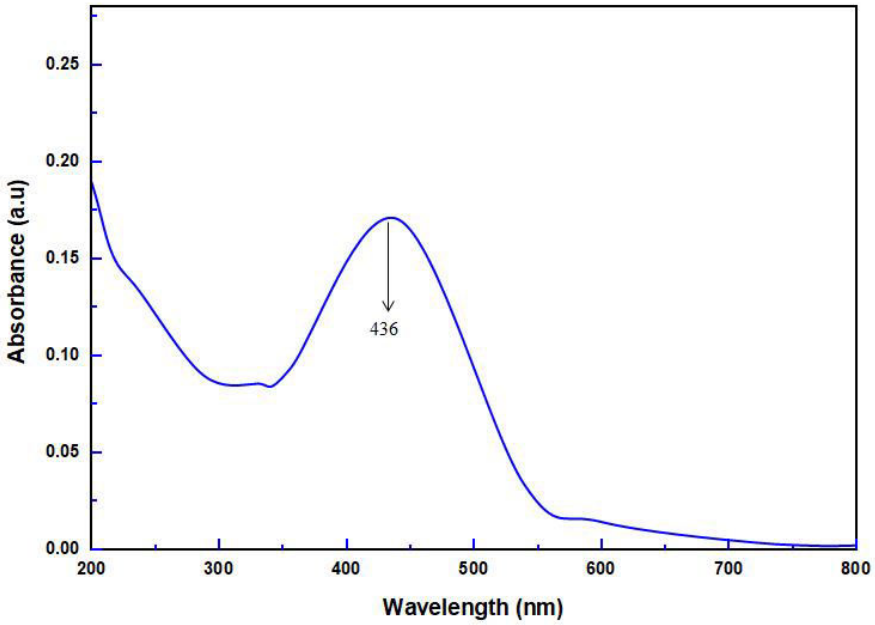

Fig. 1: UV-vis spectroscopy analysis of *V. cinerea* synthesized AgNPs.

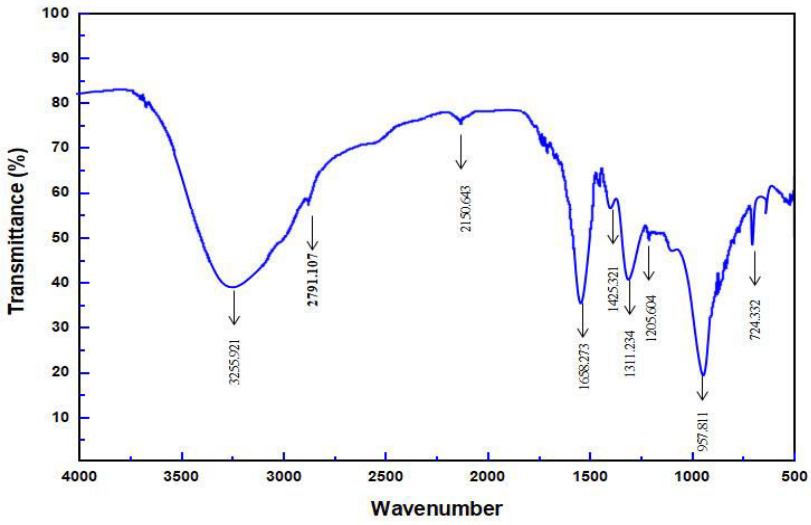

Fig. 2: FTIR analysis of *V. cinerea* synthesized AgNPs.

capping agents might bind to the metallic core via electrostatic or chemical interactions to coat the NPs to prevent or reduce the aggregation phenomena (De Matteis *et al.*, 2020).

FT-IR spectra shows peaks at 3255 cm^{-1} representing a hydroxyl (– OH) group. The peaks at 1658 cm^{-1} represents double bond (C– –C). The peaks 1311 cm^{-1} and 1205 cm^{-1} show amine (C–N) and carbonyl (C– –O) of proteins, (Fig. 2). Our FTIR spectral analysis findings are consistent with Basu *et al.* (2016) and Shankar *et al.* (2004), who identified similar trends in the synthesized AgNPs FTIR spectra from the green material extract. This suggests Ag's binding and capping from the plant extract with biological functional groups such as N H and OH groups. These results are in line with

other experiments in which the spectral peak was found between 2820 and 3590 cm^{-1} and subsequent vibrations of 1 and 2 amines, amides, alcohol, and H-bonded phenols corresponding to N H and O H (Ankamwar *et al.*, 2005). At 1270 to 1650 cm^{-1}, the absorption peak of AgNPs spectra shows C H stretching alkene vibrations (Pacioni *et al.*, 2015), while at 1080–1270 cm^{-1}, the peak reflects the C H in-plane bending of alkenes, alcohols, carboxylic acids, esters, and ethers (Pacioni *et al.*, 2015). Ankamwar *et al.* (Shankar *et al.*, 2004) have also studied the role of bio-molecules in core particle matter.

X-ray diffraction pattern of VC-AgNPs had Bragg's reflections of 111, 200, 220 and 311 planes, which confirms the face-centered cubic (FCC) crystalline structure of VC-AgNPs (Fig. 3), which are corresponding to the data of joint committee of powder diffraction standards (JCPDS) file no-04–0783 (Nguyen *et al.*, 2021). Similar diffraction peaks around 38°, 44°, 64°, and 77° at 2 θ are reported for green synthesized AgNPs by corn silk aqueous extracts (Li *et al.*, 2020) and cactus pear fruit peel infusions (Kamaraj *et al.*, 2020). A high-intensity diffraction peak ~38° implying that the NPs are serene distinctly crystalline. The AgNPs prepared via *C. quinoa* and *T. portulacastrum* leaf extracts are ~19 nm and ~24 nm, respectively (Younas *et al.*, 2021). The reduced size of the resultant material obtained in this study is found to be a noteworthy aspect that has importance in the AgNPs synthesis process.

DLS (photon correlation spectroscopy) is a promising scattering method to analyze the average particle size and size distribution of the nanoparticles. The hydrodynamic diameter of VC-AgNPs was found to be 115.87 nm (Fig. 4). The zeta potential of VC-AgNPs was (-29.8 mV) (Fig. 5). Several earlier studies validate the study. Similarly, the negative zeta charge of PF-AgNPs and St-PFAgNPs observed was due to the capping of the fungal molecules on the surface of PF-AgNPs while the capping of starch did not affect the surface change but increased the high stability as also reported earlier (Madhu *et al.*, 2022). Figure 6 illustrates the SEM studies of VC-AgNPs which confirms the spherical shape of the nanopartcles. Similar observations were reported from AgNPs of *Ajug abracteosa* and *Cinnamomum tamala* (Nahar *et al.*, 2020; Loganathan *et al.*, 2022).

Cytotoxic Assay:

VC-AgNPs was examined for cytotoxicity effects on PANC human pancreatic cancer with MTT bioassay in a 24 h examination (Fig. 7) and the IC_{50} value is shown in Table 1. Figure 7 shows that VC-AgNPs can inhibit the growth of PANCSCs human pancreatic cancer at certain doses, which demonstrated that IC_{50} value was 20.4 µg/ml, whereas 16.5 µg/ml in PANC for doxorubicin (standard). The VC-AgNPs had higher inhibitory activity against cell proliferation as compared to the doxorubicin. Some other studies revealed the potential toxicity of silver NPs in cancer cells (Uygur *et al.*, 2009; Kim *et al.*, 2009). Similarly, the same cytotoxic effect of AgNPs on human lung carcinoma A549 cells has been reported ((Gurunathan *et al.*, 2015; Mittal *et al.*, 2020). Recently, Eswaraiah *et al.* (2020) reported that the marine mangrove plant *Avicennia marina* has anti-cancer properties against various cancer cells. It enhanced the anti-cancer activity than other plant and chemical synthesized AgNPs due to the fluctuated marine environmental conditions such as temperature, salinity, pH, carbon and nitrogen sources (Ramachandran *et al.*, 2018). Very recently, *Allium sativum* synthesized AgNPs showed a significant cytotoxic potential against A549 human lung cancer cells at an IC_{50} of 22 µg/ml (Padmini *et al.*, 2022).

Morphological Analysis:

Figure 8 shows the morphological changes in PANC during 24 h of treatment with the VC-AgNPs IC_{50} concentration. Treatment affects the shape of PANC human pancreatic cancer cells in a dose-dependent manner. Enhanced VC-AgNPs increased cytotoxicity. This extract had incited cell shrinkage, adjusting of the cell and reducing the number of feasible cells. These progressions

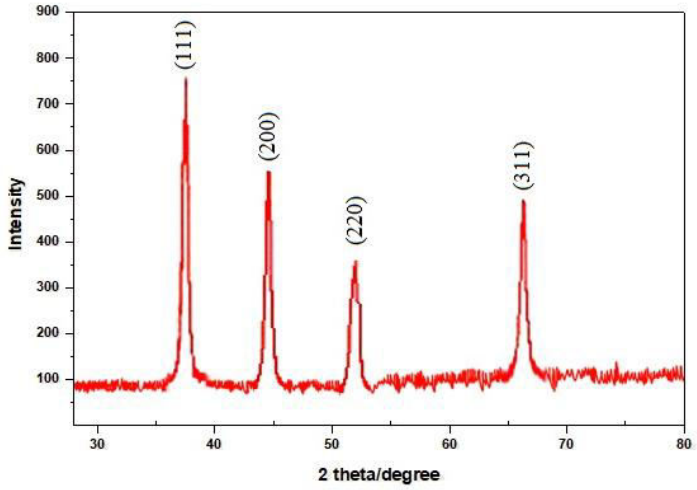

Fig. 3: XRD pattern of *V. cinerea* synthesized AgNPs.

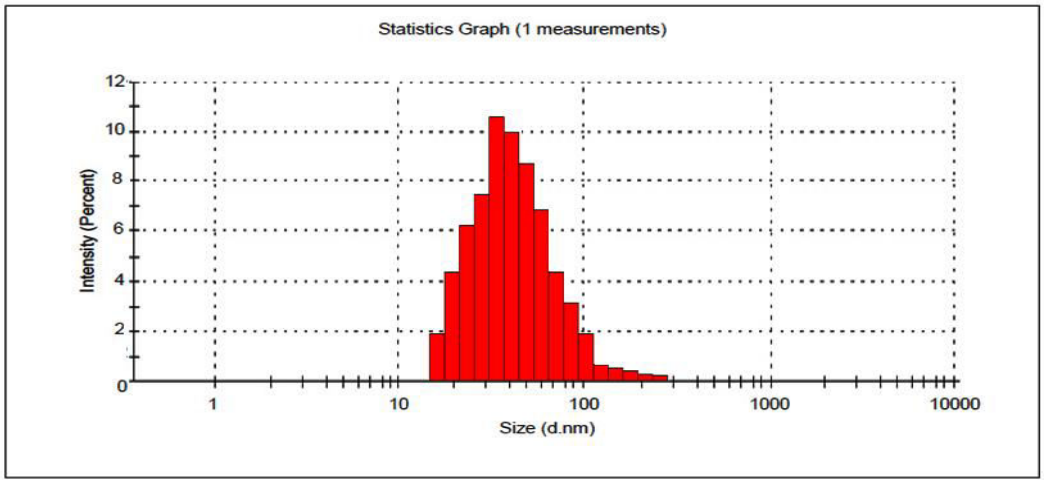

Fig. 4: DLS analysis of *V. cinerea* synthesized AgNPs.

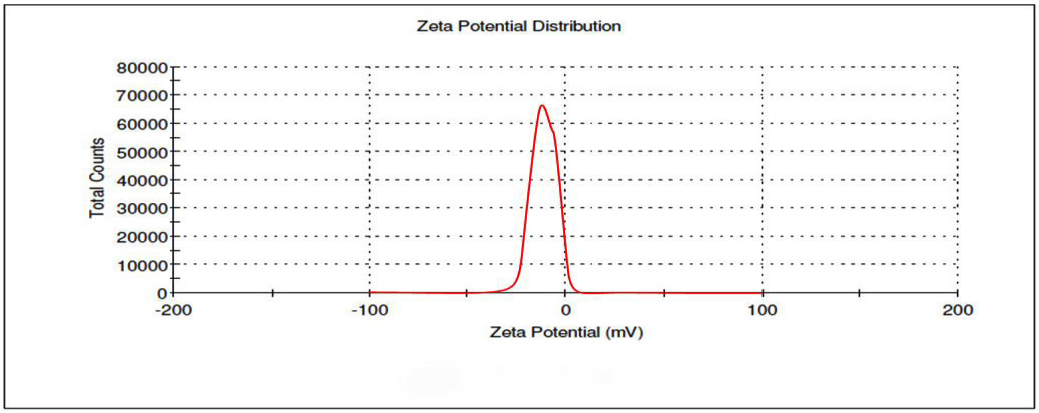

Fig. 5: Zeta potential analysis of *V. cinerea* synthesized AgNPs.

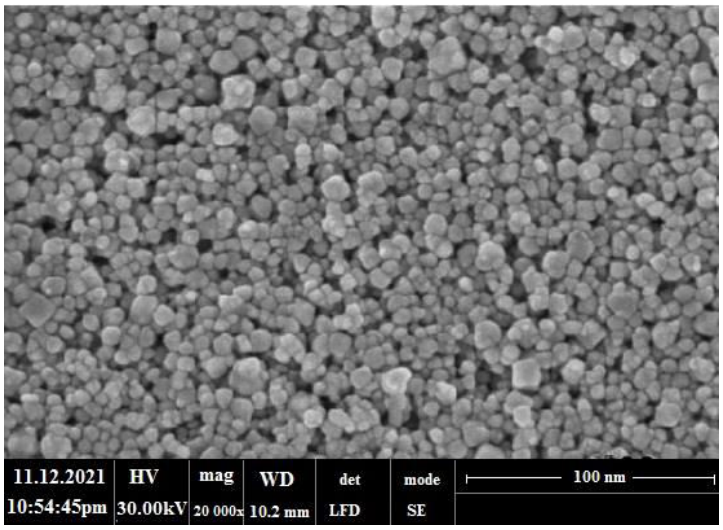

Fig. 6: SEM image of *V. cinerea* synthesized AgNPs.

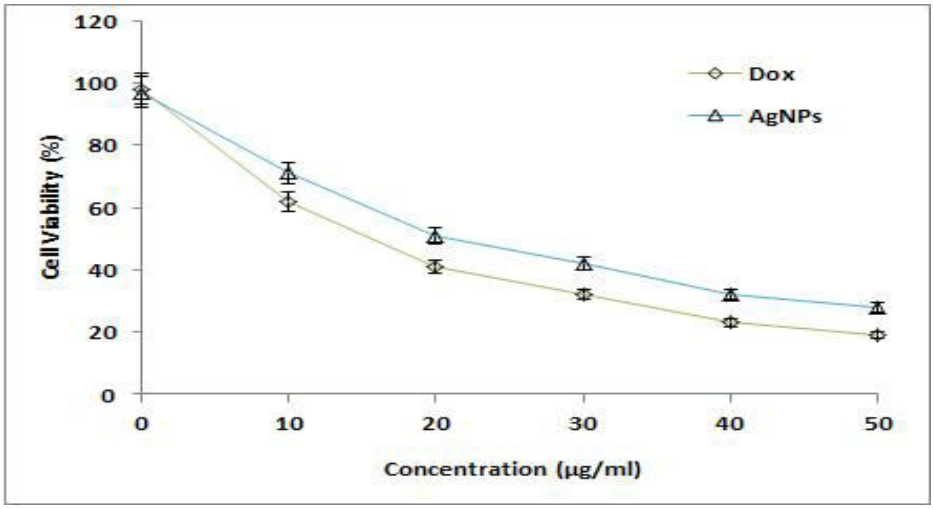

Fig. 7: MTT cytotoxicity analysis: In vitro cell viability of PANC human pancreatic cancer cells after treatment with VC-AgNPs.

demonstrate that VC-AgNPs actuated apoptosis in PANC human pancreatic cancer cells (Figs. 8b, c, d). While the untreated control cells on the other hand, had not shown any significant effect (Fig. 8a). This is in agreement with the reports of Venugopal *et al.* (2017) and Rajivgandhi *et al.* (2018). Also, their AgNPs was synthesized from biological sources and effective role against A549 lung cancer cells at IC_{50} concentration with increased damages (Zhang *et al.*, 2020).

AO/EtBr staining for fluorescence microscopy investigation of nuclear fragmentation:

The effect of the VC-AgNPs apoptogenic activity on cancer cells was studied using fluorescence microscopy. No aggregation of VC-AgNPs was shown during the experiment because most of the VC-AgNPs component was dispersed at all these low concentrations. The induction of apoptosis in PANC human pancreatic cancer cells following treatment with the IC_{50} concentration of VC-AgNPs was assessed using fluorescence microscopy stained with acridine orange/ethidium bromide (AO/EtBr). These findings revealed that live cells fluoresced green, while dead cells fluoresced acridine orange. Untreated control cells showed a high number of live cells (Fig. 9 a). PANC human pancreatic cancer cells treated with VC-

Table 1: Cytotoxic activity of compound (µg/ml)

Compound name	Inhibitory concentration (IC_{50})
AgNPs	20.4 ± 0.1
Doxorubicin	16.5 ± 0.7

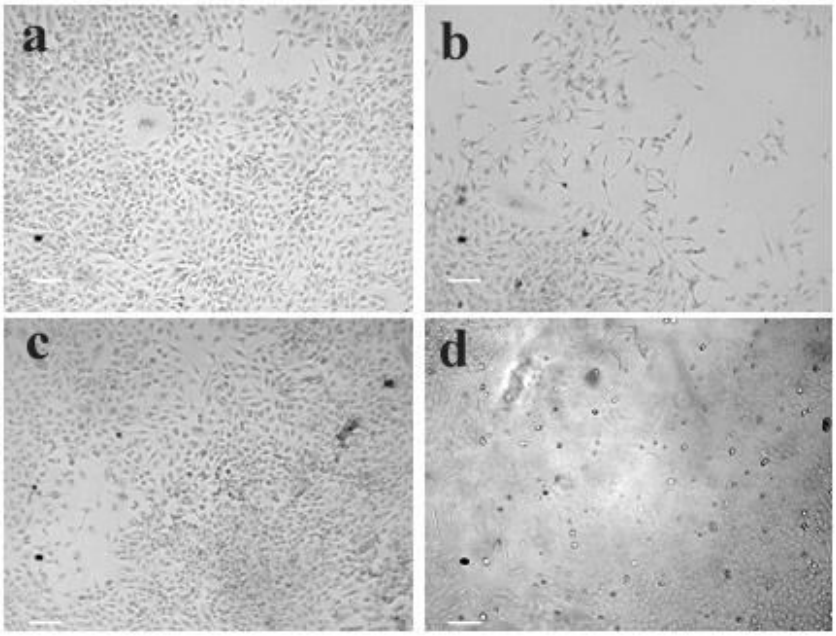

Fig. 8: The morphological changes in PANC human pancreatic cancer cells during 24 h of treatment with the *VC*-AgNPs IC_{50} concentration (a) Control, (b) 10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

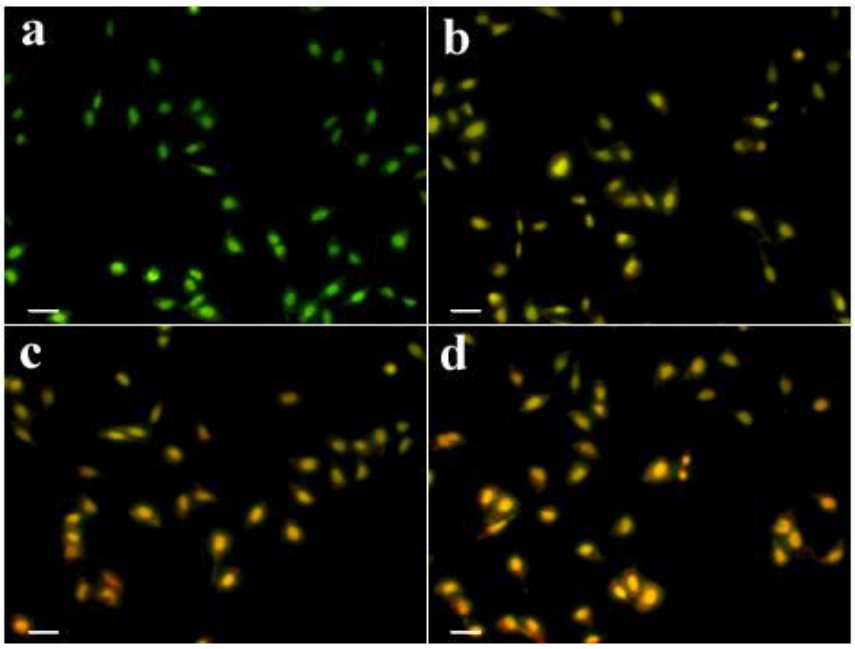

Fig. 9: AO/EtBr staining for fluorescence microscopy investigation of nuclear fragmentation (a) Control, (b) 10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

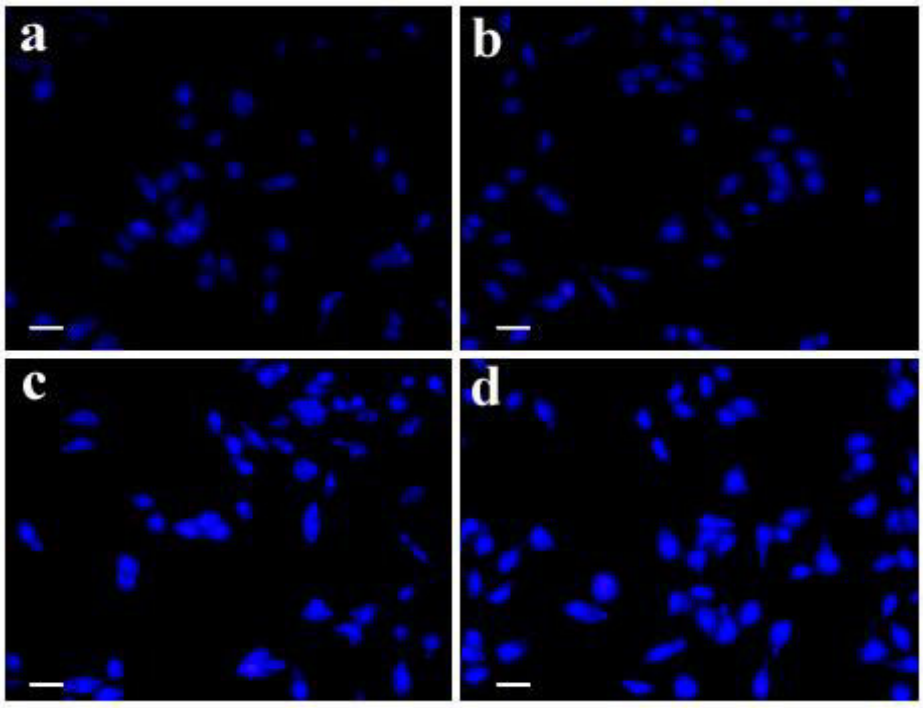

Fig. 10: Fluorescence microscopic analysis of nuclear fragmentation - DAPI Staining, (a) Control, (b) 10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

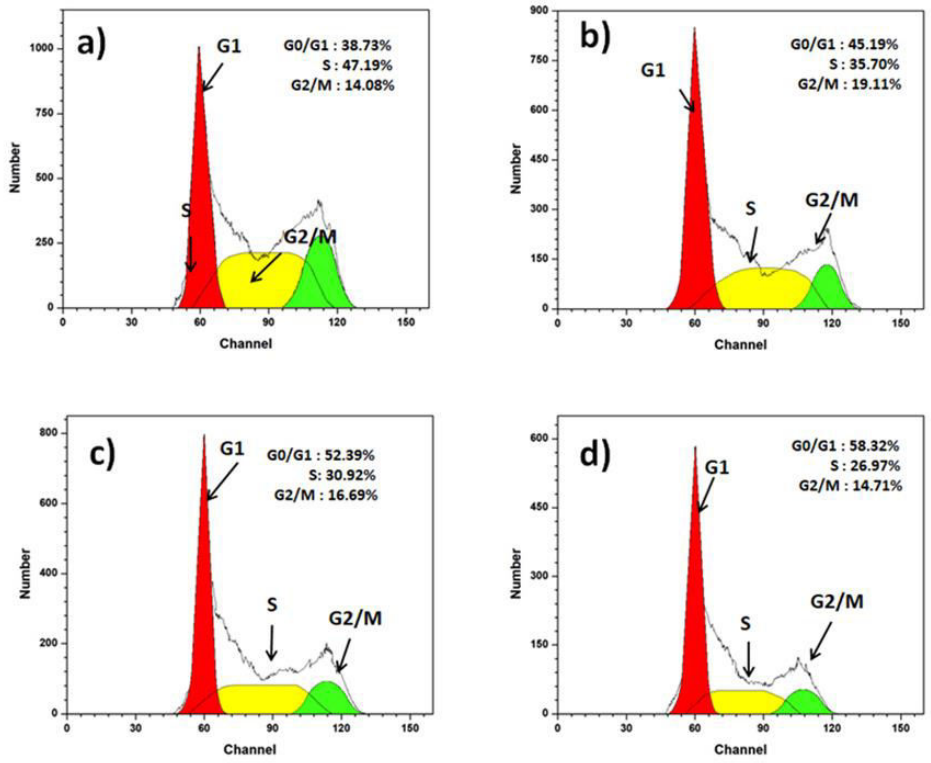

Fig. 11: Cell cycle analysis determined using flow cytometry (a) Control, (b) 10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

AgNPs, on the other hand, exhibited more cellular damage and bodies with nuclear shrinkage, membrane blabbing, and nucleus degradation as orangish bodies (Figs. 9b, c, d). In another study, AgNPs of *Butea monosperma* bark extract shoed the apoptotic cells in KG-1A cell line (Pattanayak *et al.*, 2015). The cytotoxic effect of AgNPs from *Indigofera aspalathoids* using AO/EtBr staining indicated the induction of apoptosis (Krishnasamy *et al.*, 2014). The extent of the anticancer effect was significantly more in the nanobioconjugates than their non-conjugated forms, proving that the anticancer activity of the test materials used can be enhanced markedly by preparing silver nanobioconjugates (Preethi and Padma, 2016).

Fluorescence microscopic analysis of nuclear fragmentation - DAPI Staining:

Furthermore, we evaluated the VC-AgNPs using the DAPI staining method. Figure 4 shows a fluorescence microscopic image of cells labelled with DAPI after 24 h in the presence and absence of VC-AgNPs substance. Figure 10 (a) shows that there were no significant changes in the cells, whereas Bright signals were observed in VC-AgNPs treated cells (Figs. 10 b, c, d), indicating the structure of condensed chromatins and nuclear fragmentations in PANC human pancreatic cancer cells. According to the results of the fluorescence microscopic study, the VC-AgNPs could be used as an efficient cancer therapy agent. Similarly, previous studies that involved AgNPs synthesized through *Cleome viscosa* L fruit extracts (Lakshmanan *et al.*, 2018), *Terminalia chebula* seed extracts (Muthusamy *et al.*, 2021), and *Annona muricata* extracts (Jabir *et al.*, 2021) have also reported the nuclear fragmentation of cancer cells after treatment with AgNPs, indicating the value of DAPI staining to study the photodynamic effect at the nuclear level.

Cell cycle analysis:

The phase of the cell cycle at which the cells are arrested was determined using flow cytometry. The cells in the control group (untreated group) were uniformly dispersed in the G0-G1, S, and G2M phases. VC-AgNPs concentrations of 10, 25, and 50 µg/ml were applied to PANC human pancreatic cancer cells. Cells were arrested in G2M phase for doses of 10 µg/ml and 25µg/ml, whereas cells were arrested at S phase for values of 50 µg/ml. This meant that increasing the concentration prevented PANC human pancreatic cancer cells from synthesizing as they were exposed with VC-AgNPs, as shown in Figure 11.

Conclusion

An aqueous extract of *Vernonia cinerea* was used to prepare mono-dispersed AgNPs in the current research. UV–visible, XRD, SEM, DLS, and FT-IR spectra have characterized the AgNPs. They have a spherical shape with an average of 50 nm in size. *Vernonia cinerea* biological synthesis of AgNPs is a cheap, nonpolluting, and safe technique and is an excellent alternative to chemical and physical methods. Our findings confirmed that *Vernonia cinerea* aqueous extract might be an effective method for AgNPs' biosynthesis and stabilization of silver ions. The cytotoxicity and cell apoptotic of *VC*-AgNPs in PANC human pancreatic cancer cells are studied using MTT assay, AO-EtBr, and DAPI staining assay was found to be increased in dose-dependent aspects. Also, it recommended using VC-AgNPs for potential anticancer applications after thoroughly analyzing their behavior in different systems and anticancer activity in different *in vitro* and *in vivo* models.

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