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Original Article

Evaluation of *in vitro* Anti-Cancer Activity of Vinorelbine Ditartrate Loaded PLGA Chitosan Nanoparticles Using A549 Human Lung Cancer Cell Line

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ABSTRACT

Background: The purpose of this research was to determine if vinorelbine ditartrate Loaded PLGA Chitosan Nanoparticles have any anticancer effects when tested *in vitro*. Many different types of cancer may be effectively treated with vinorelbine. However, they have limited medical use due to undesirable side effects. To circumvent these unwanted impacts, a customized drug delivery method was developed, where we have synthesized and characterized vinorelbine ditartrate Loaded PLGA Chitosan Nanoparticles by Box-Bhenken design method, in our previous study. **Materials and Methods:** *In vitro* anti-cancer activity of nanoparticles was evaluated by Sulphorodamine B (SRB), MMP Estimation, ROS Estimation and Cellular Apoptosis. **Results:** The nanoparticles showed IC_{50} value on A549 is 434.7 at 1000 µg/mL and more cell viability on SRB assay. Nanoparticles slightly increased the MMP in the A549 cells in dose-dependent manner. ROS result indicate that formulation showed anticancer activity due to enhanced production of ROS. The result of DCFDA positive cells events shows 83.58% for unstained, 85.74% for control and 76.73% for vinorelbine ditartrate treated cells which is very close to control and the MFI-positive cells shows 488.40 for unstained, 507.43 for control and 520.66 for treated cells which is comparable to control. Vinorelbine ditartrate significantly raised number of late and early apoptotic cells while decreasing number of viable cells, according to a cell apoptosis experiment conducted on cells after 48 hr of treatment. **Conclusion:** Vinorelbine ditartrate caused A549 cell death and these data showed that apoptosis was the mechanism by which this occurred. Therefore, NCI-A549 cells may be treated with these nanoparticles.

Keywords: Cell apoptosis, Matrix Metalloproteinase, Reactive oxygen species, Sulphorodamine B, Vinorelbine Ditartrate.

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INTRODUCTION

Non-Small Cell Lung Cancer (NSCLC) represents predominant form of lung malignancy, accounting for nearly 85% of overall lung cancer cases. 5-year surviving rate for individuals suffering from NSCLC is a mere 17.3%. This aggressive neoplasm is a major reason of lung cancer-related fatalities in USA, surpassing combined mortality of colon, breast, pancreas, and prostate cancers.[1] The primary subtypes of NSCLC include adenocarcinoma (approximately 40%), squamous cell carcinoma (around 25-30%), and large cell carcinoma (approximately 10-15%).[2] Treatment modalities such as chemotherapy, radiotherapy, and operation is utilized for NSCLC management; however, these interventions are associated with notable adverse effects. Vinorelbine ditartrate is semi-synthetic derivative derived from vinca alkaloid vinblastine, which originates from *Catharanthus roseus* G. Don. Vinorelbine ditartrate was found in 1980s by Pierre Potier and his colleagues at French Centre National de la RechercheScientifique (CNRS). French government authorized use of vinorelbine ditartrate, marketed as Navelbine® IV, for treatment of bronchial carcinoma in 1989. Afterwards, in 1991, it was approved in treating non-small cell lung cancer.[3] Leukemias, lymphomas, advanced testicular cancer, Kaposi's carcinoma and other malignancies are often treated with vinorelbine ditartrate in conjunction with other chemotherapy medicines.[4,5] It shows remarkable effectiveness in patients with metastatic Non-Small Cell Lung Cancer (NSCLC) and Advanced Breast Cancer (ABC).[6] Metaphase cell blockage results from the suppression of mitotic microtubule production, which is the mechanism of action.[7] Nevertheless, the formulation of Navelbine® IV (vinorelbine ditartrate), the product that is sold to the public, presents difficulties as its made at a low pH (~3.5), which leads to venous irritation and phlebitis when administered directly into the vein like an aqueous solution.[8] This problem is probably caused by changes in pH and rapid formation of

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vinorelbine ditartrate salt at injection site. Further, medication causes dose-limiting hematologic adverse impacts like phlebitis and granulocytopenia when given in free form and has extensive dispersion. Consequently, there is a pressing need for a new strategy in the formulation of aqueous injections for vinorelbine ditartrate. Over the past few years, researchers have experimented with anticancer drugs encapsulated in both natural and synthetic polymers to combat cancer cell lines. This approach aims to overcome drug resistance primarily caused by p-glycoprotein receptors.

Due to its bioavailability, mucoadhesivity, biocompatibility, non-toxicity, and low immunogenicity, cationic polysaccharide chitosan has drawn focus in pharmaceutical and biomedical settings.[9-11] According to (9), chitosan is made from deacetylated chitin, a naturally occurring polymer in crustaceans such as shrimp and crabs and it is composed of (1→4) connected D-glucosamine and N-acetyl-D-glucosamine units. Lysozyme and bacterial enzymes in colon are thought to degrade it in vertebrates.[12] For these reasons, chitosan is a promising medication carrier for cancer treatments. It is possible to target certain malignancies using chitosan nanoparticles by adding ligands. No change is made to the physicochemical and biological properties of chitosan by adding targeted ligands.[12,13] This research used PLGA because it is a cost-effective option compared to other targeting polymers, it is straightforward to conjugate to drug delivery carriers and it is stable in processing, storage, and applications.

In our previous study, we have already developed vinorelbine ditartrate Loaded PLGA Chitosan Nanoparticles and its characterization were performed. This research study involved the assessment of *in vitro* anticancer activity against NCI-A549 non-small cell lung cancer cell line.

MATERIALS AND METHODS

Materials

Tri chloro acetic acid was purchased from Fisher Scientific-28444. Acetic acid was purchased from SRL chem. EDTA, DMEM and FBS were purchased from HIMEDIA. SRB solution was purchased from Ottohemi and A549 cell line was procured from National Centre for Cell Science (NCCS), Pune, India.

Table 1: Inhibitory concentration of Vinorelbine Ditartrate Loaded PLGA Chitosan Nanoparticles.

Sample code	IC_{50} value (µg/mL)
Vinorelbine ditartrate	434.7

Methods

Anticancer activity of Vinorelbine Ditartrate Loaded PLGA Chitosan Nanoparticles

Sulphorodamine B SRB Assay

Cytotoxicity of samples was assessed using SRB Assay on A549 cell line. The cells were grown in a 96-well plate for 24 hr at 37°C with 5% CO_2 in 5% FBS (HIMEDIA-RM 10432-500 M) and DMEM (HIMEDIA-AT149-1L) medium supplemented with 1% antibiotic solution. The next day, cells were exposed to various doses ranging from 1 to 1000 µg/mL. (incomplete medium was used to create various concentrations). Following a 24 hr incubation period, 100 µL of Tri Chloro Acetic Acid (TCA, 10%-Fisher Scientific-28444) was introduced to every well. Following this, the plates were rinsed with DM water and allowed to air dry at room temperature. The SRB Solution (Ottokemi-3520-42-1) was added to every well and allowed to sit for 1 hr at a final concentration of 0.04%. The plates were rinsed with 1% (v/v) acetic acid (SRL Chem-641-9-7) to remove any unbound dye after 1 hr of incubation and then left to air-dry at room temperature. To dissolve protein-bound dye, a Tris base solution (pH=10.5) was added to well and agitated on an orbital shaker for 10 min. Plate reader, an Elisa (iMark, Biorad, USA) set at 510 nm, was then used for reading.[14]

MMP Estimation with Flow Cytometry

A549 cells were grown and exposed to the material at an IC_{50} level for duration of 24 hr. After incubation period, medium was drawn and cells were collected in a 1.5 mL tube using trypsin EDTA. They were then washed once with 500 µL of cold PBS. Lastly, 400 µL JC1 staining solution with 2 µM JC-1 dye was added to cell pellet, and samples were collected using a flow cytometer within a hour.[15]

Reactive Oxygen Species (ROS) Estimation with Flow Cytometry

At a density of 50,000 to 100,000 cells/well, A549 cells were seeded in 6-well plates using 1 mL of DMEM media augmented with 10% FBS and 1% antibiotic solution. The plates were then incubated for 24 hr at 37°C with 5% CO_2. It was necessary to remove the old medium after incubation and replace it with new culture media before to treatment. After that, cells were cultured for another 24 hr after being treated with sample at IC_{50} level. The cells were collected in 1.5 mL tube after incubation, and 500 µL

Table 2: Relative MMP (Low) wrt control and treated and Relative MMP (high) wrt control and treated.

Sample code	Relative MMP (Low) wrt Control and treated	Relative MMP (high) wrt Control and treated
Control	100	100
Treated	101.9874038	95.0262697

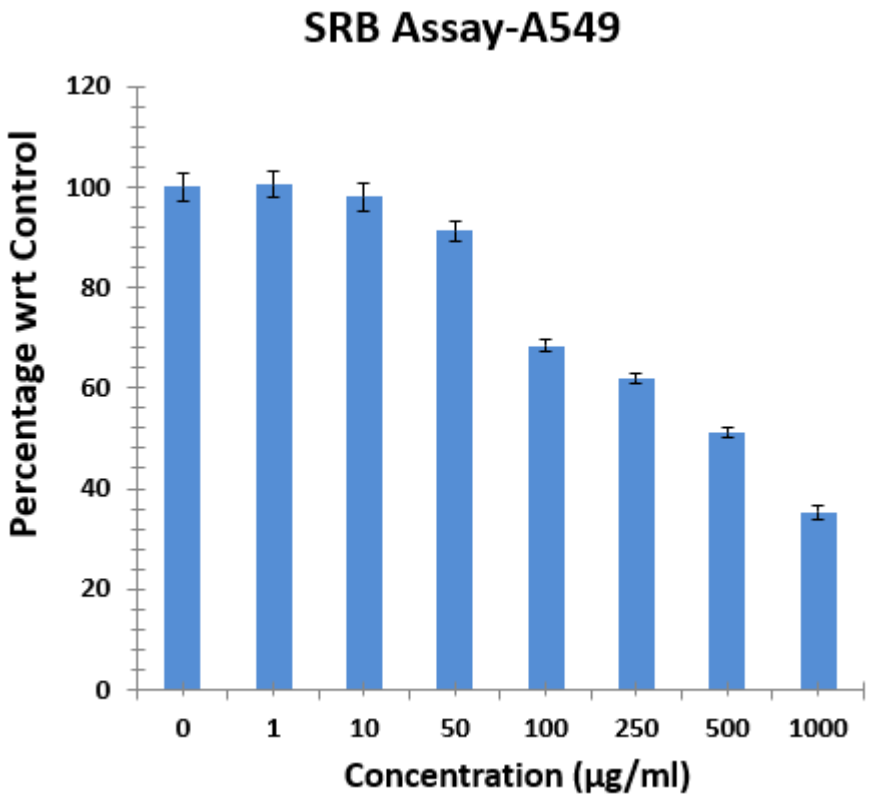


Figure 1: SRB Assay of vinorelbine ditartrate loaded PLGA chitosan nanoparticle at A549 cell line.

Table 3: ROS levels in cells and ROS Positive Population Percentage.

Sample code	All	DCFDA +ve Cells: Events	% DCFDA Stained Cells	MFI-Positive Cells
UNSTAINED	10000	8358	83.58	488.4090691553
CONTROL	10000	8574	85.74	507.430370888733
TREATED	10000	7673	76.73	520.669229766714

of cooled PBS was used to wash them once more. The media was then withdrawn and the cells were harvested using trypsin EDTA. Flow cytometry samples were collected within 1 hr after cell pellet was dissolved in 100 µL of PBS containing 2 µM DCFDA.[12]

Cellular Apoptosis with Flow Cytometry

We cultivated A549 cell lines (obtained from NCCS Pune) and exposed them to compound's IC_{50} dosage. A total of six groups of cells were created: Unstained cells, Control group, PI alone, Annexin only and Treatment. Following treatment, cells were washed twice using cold PBS and resuspended in 1X binding buffer at concentration of $1x10^6$ cells/mL. Correspondingly labelled tubes were then treated with Annexin V FITC and PI. Every tube was incubated at room temperature for 15 min after vortexing and then 1X binding buffer was added. Within 1 hr, the tubes were examined using BD FACS Canto II flow cytometer.[1]

RESULTS

There was zero microbial or fungal contamination in the cell lines obtained from NCCS, Pune. Vinorelbine ditartrate was subject of cytotoxicity investigation. In order to find IC_{50} (50% growth inhibition) using SRB assay, vinorelbine ditartrate was tested for cytotoxicity against A549 cell line at various doses.

Sulphorodamine B (SRB) Assay

Table 1 and Figure 1 display findings of Sulphorodamine B (SRB) test, while Figure 2 provides a visual representation of same. Increasing concentrations of the test substance were shown to increase percentage growth inhibition, as indicated in (Figure 2). At concentrations up to 1000 µg/mL, vinorelbine ditartrate had an impact on the A549 cell line, with an IC_{50} value of 434.7 (Table 1). Thus, the vinorelbine ditartrate shows less cellular viability which means that it increases the cytotoxicity of formulation towards cells. Vinorelbine ditartrate shows significant effect against A549

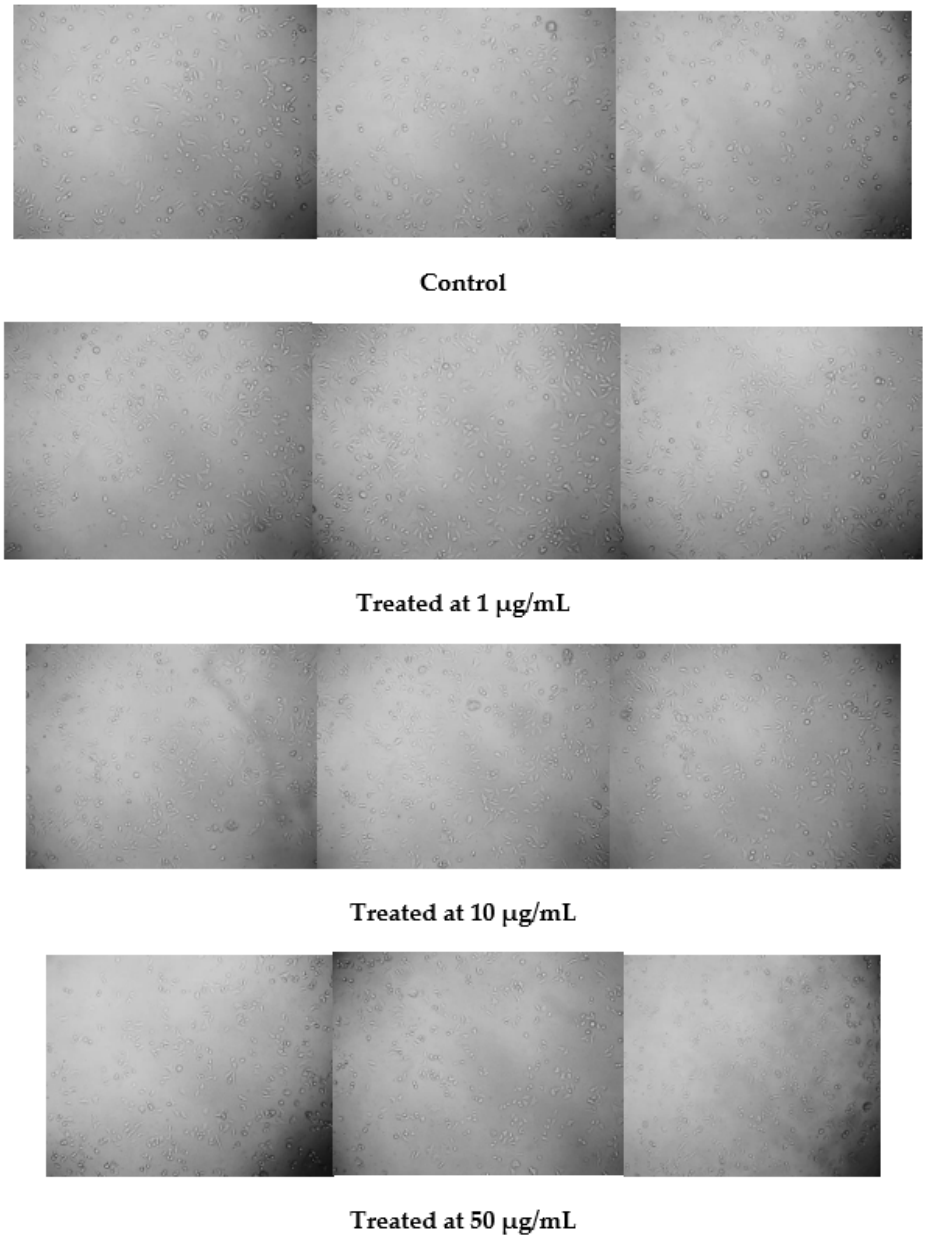


Figure 2: Cell Viability of vinorelbine ditartrate loaded PLGA chitosan nanoparticle on A549 cell line (A) Control (B) Treated at 1 µg/mL (C)Treated at 10 µg/mL (D) Treated at 50 µg/mL (E) Treated at 100 µg/mL (F) Treated at 250 µg/mL (G) Treated at 500 µg/mL (H) Treated at 1000 µg/mL.

Table 4: Cell Apopyosis and Necrosis.

Sample code	Live	Early Apoptosis	Late Apoptosis	Necrosis	Total
Control	6044	312	634	505	7495
Sample	3714	2896	1312	44	7966
Unstained	6340	99	25	0	6464
Sample code	Live %	Early Apoptosis %	Late Apoptosis %	Necrosis %	Percentage
Control	80.6404	4.162775183	8.458972648	6.73782522	99.9333
Sample	49.553	38.63909273	17.50500334	0.58705804	106.21333
Unstained	84.5897	1.320880587	0.333555704	0	86.18666

lung cancer cell line having higher cytotoxicity and minimal cell viability.

Matrix Metalloproteinase (MMP) Assay

To assess impact of vinorelbine ditartrate upon A549 cell line, mitochondrial membrane potential was measured utilising JC-1 dye. Absorption of JC-1 dye is a measure of mitochondrial membrane potential. So, it's a way to monitor cell death directly. Result of MMP in Table 2 showed that mitochondrial membrane potential in A549 cells was modestly elevated in dose-dependent manner by vinorelbine ditartrate (Figures 3 and 4).

Reactive Oxygen Species (ROS)

Apoptosis and Reactive Oxygen Species (ROS) have been the subject of numerous investigations. We also looked at ROS production, which is a major contributor to cell death. Oxidative stress occurs when ROS levels are significantly elevated as a result of exposure to stressful environmental conditions. With that in mind, we looked into the possibility that vinorelbine ditartrate elevated ROS levels in A549 cells. In response to vinorelbine ditartrate, A549 cells released reactive oxygen species. The result of DCFDA positive cells events shows 83.58% for unstained, 85.74% for control and 76.73% for vinorelbine ditartrate treated cells which is very close to control and the MFI-positive cells shows 488.40 for unstained, 507.43 for control and 520.66 for treated cells which is comparable to control (Table 3). From the mentioned result in Table 3, it can be concluded that formulation showed anticancer activity due to enhanced production of ROS.

Apoptosis Assay

Utilizing phase-contrast microscopy, we were able to identify alterations in cell shape and cell death because of dose-dependent

Table 5: All events and cell apoptosis time.

All Events: Events			
Left Bottom:Events	**Right Bottom:Events**	**Left Top:Events**	**Right Top:Events**
Live	Dead	Early	Late

Table 6: Effects of nanoparticles on viability and apoptosis in A549 (A) Unstained, Cells were stained with (B) Annexin V-FITC (C) PI and (D) Sample.

Sample code	All events: Events	Left Bottom: Events	Right Bottom: Events	Left Top: Events	Right Top: Events
Unstained	6,464	6,340	0	99	25
Sample 7	7,966	3,714	44	2,896	1,312
PI	8,143	6,726	1,238	61	118
Control	7,495	6,044	505	312	634
Annexin	7,511	2,933	0	4,535	43

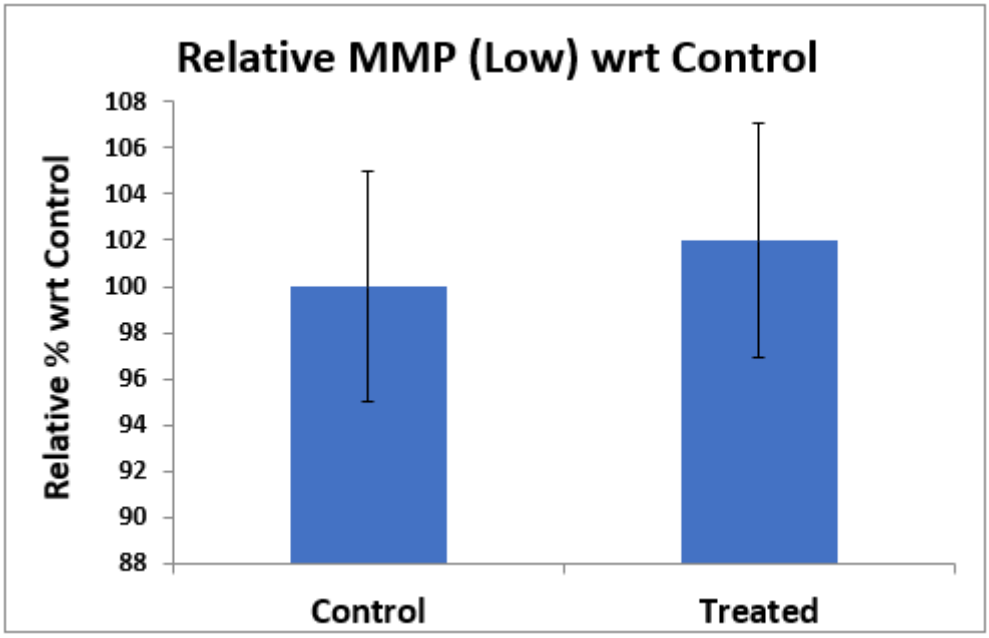


Figure 3: Relative MMP (Low) wrt Control and Treated.

reduction in viabilities of A549 cells treated with vinorelbine ditartrate over 48 hr. Treatment with high doses of vinorelbine ditartrate resulted in more rounded cell morphologies and less interaction with neighbouring cells in A549 (Figure 5) cells compared to untreated A549 cells (Table 4). Vinorelbine ditartrate was shown to have the ability to change cell shape and cause cell death. Staining vinorelbine ditartrate-treated A549 cells with Annexin V and PI provided further proof of drug's effects. During cell death, lipid Phosphatidylserine (PS) is moved from intracellular to the extracellular space, a process known as a "flip-flop," which enables the staining of PS with Annexin V. Also, during necrosis or late apoptosis, holes form in cell membranes that allow PI to attach to DNA. Cells treated with vinorelbine ditartrate exhibited apoptosis, as seen by Annexin V-FITC/PI staining (Figure 6). Vinorelbine ditartrate significantly enhanced number of early and late apoptotic cells while decreasing number of viable cells whenever treated with cells for 48 hr. Vinorelbine ditartrate caused A549 cell death and these data showed that apoptosis was the mechanism by which this occurred (Tables 5 and 6).

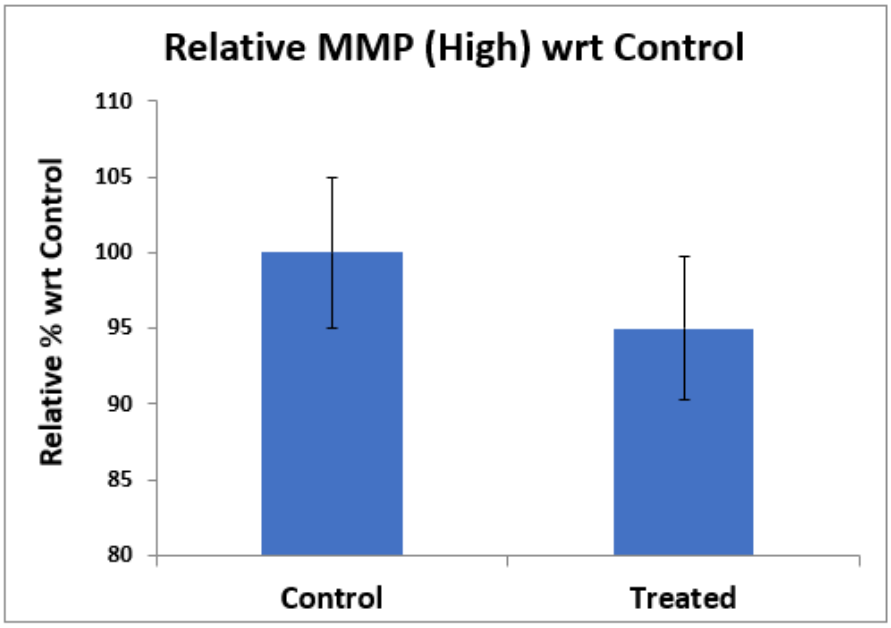
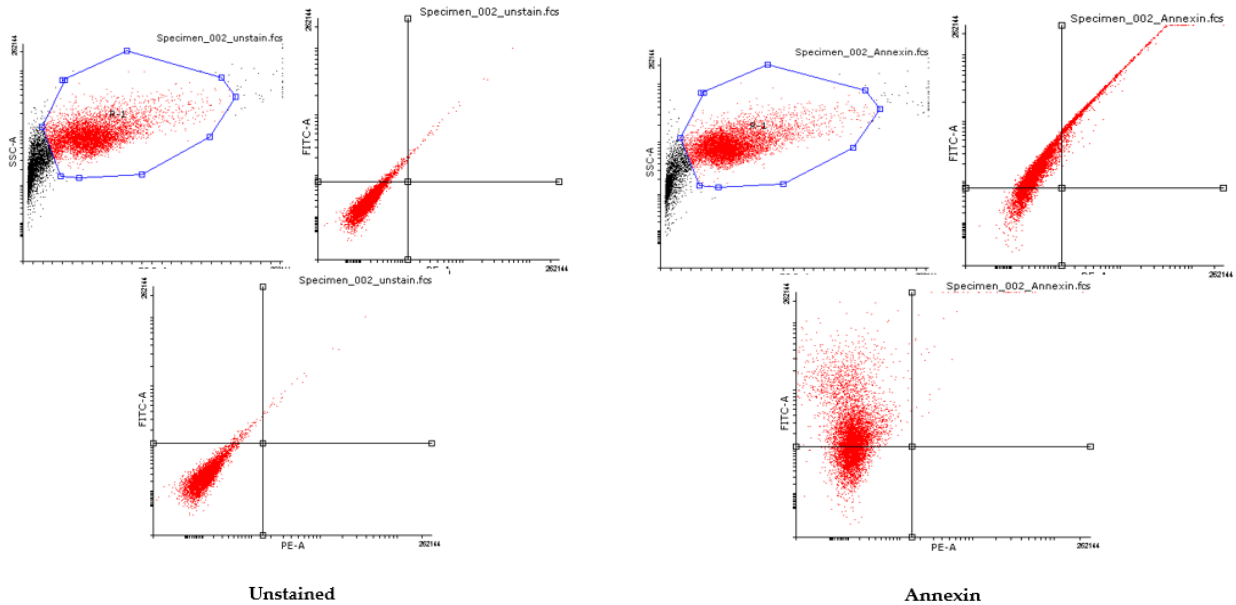


Figure 4: Relative MMP (High) wrt Control and Treated.



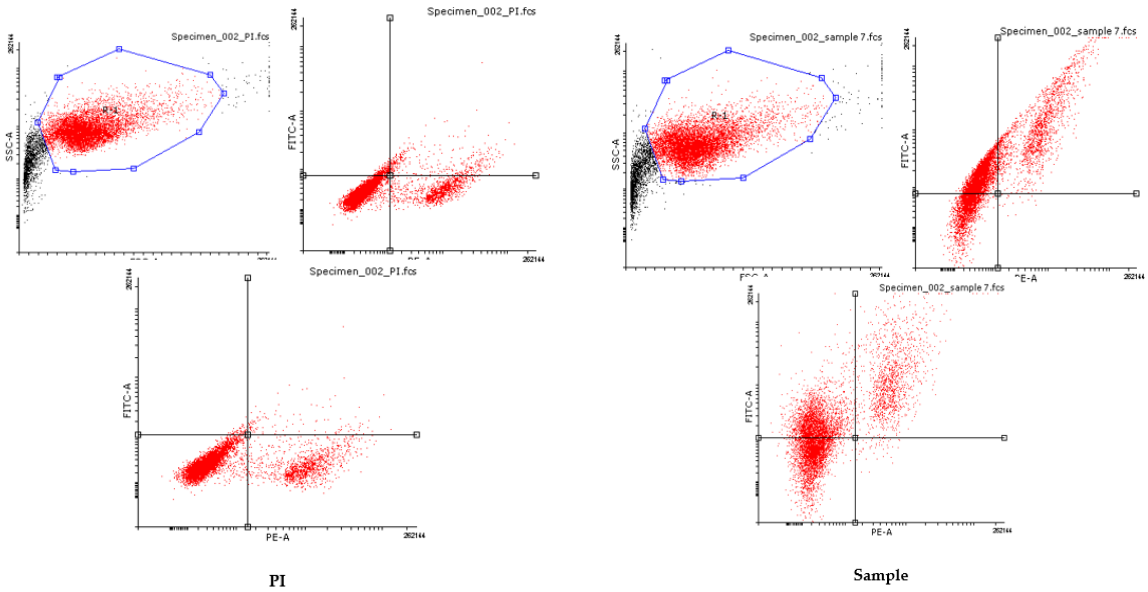


Figure 5: Effects of nanoparticles on viability and apoptosis in A549 (A) Unstained, Cells were stained with (B) Annexin V-FITC (C) PI and (D) Sample.

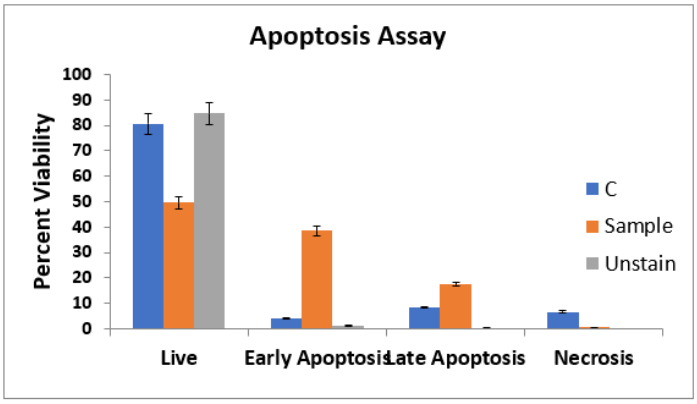


Figure 6: % Percentage viability of live, early apoptosis, late apoptosis and necrosis.

DISCUSSION

The research findings elucidate the cytotoxic effects of Vinorelbine ditartrate on A549 lung cancer cells, revealing its potential as an anticancer agent through various assays. Initial assessments ensured the reliability of cell lines obtained from NCCS, Pune, by confirming their absence of microbial or fungal contamination. Subsequent SRB assays demonstrated dose-dependent growth inhibition, with an IC_{50} value indicating significant impact on cell viability, suggesting Vinorelbine ditartrate's cytotoxicity against A549 cells. Further investigations revealed alterations in mitochondrial membrane potential and increased production of reactive oxygen species, indicative of cellular distress and potential induction of apoptosis. Annexin V/PI staining confirmed apoptosis as the primary mechanism of cell death, supported by morphological changes such as rounding and reduced cell-cell interaction. These findings underscore Vinorelbine ditartrate's potential as an anticancer agent by elucidating its ability to induce apoptosis and modulate cellular functions, warranting further *in vivo* and clinical studies for therapeutic validation and molecular pathway exploration to advance targeted cancer therapies.

CONCLUSION

The NSC-A549 cell line was used to assess the anticancer efficacy of Vinorelbine Ditartrate Loaded PLGA Chitosan Nanoparticles. Vinorelbine ditartrate loaded PLGA chitosan nanoparticles were more cytotoxic than the blank in the SRB experiment. In a dose-dependent manner, nanoparticles marginally raised MMP in A549 cells. The efficiency of vinorelbine ditartrate Loaded PLGA Chitosan Nanoparticles in treating A549 cells was shown by enhanced ROS production and apoptotic morphological alterations associated with NCI-A549 cells. Consequently, A549 lung cancer cells may be treated with vinorelbine ditartrate Loaded PLGA Chitosan Nanoparticles.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ROS: Reactive Oxygen Species; **MMP:** Matrix Metalloproteinase; **SRB:** Sulforhodamine B; **NSCLC:** Non-Small Cell Lung Cancer; **PLGA:** Poly (lactic-co-glycolic acid); **IC_{50}:** Half-Maximal Inhibitory Concentration.

SUMMARY

In this work Sulphorodamine B (SRB), Matrix Metalloproteinase (MMP) estimation, Reactive Oxygen Species (ROS) estimation and cell death were used to assess the nanoparticles' anti-cancer efficacy *in vitro*. Vinorelbine ditartrate loaded PLGA chitosan nanoparticles were more cytotoxic than the blank in the SRB experiment. In a dose-dependent manner, nanoparticles marginally raised MMP in A549 cells. The efficiency of vinorelbine ditartrate Loaded PLGA Chitosan Nanoparticles in treating A549 cells was shown by enhanced ROS production and apoptotic morphological alterations associated with NCI-A549 cells.

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