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Synthesis of alkaloid loaded chitosan nanoparticles for enhancing the anticancer activity in A549 lung cancer cell line

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ABSTRACT

The present study was designed with the aim to enhance the in vitro anticancer activity of alkaloids from Sphaeranthusamaranthoides. The alkaloids isolated from this plant have been known to possess anti-cancer properties. They were further coated with Chitosan to form Chitosan nanoparticles so as to enhance this property as well as for better drug delivery. The anti-cancer activity was tested in A 549 lung cancer cell lines. Chitosan have a capability of targeted drug delivery based on the pH.The alkaloids loading and release characteristics was identified by FTIR, the stability of the nanopartilces were confirmed with pH and Temperature variations. Our findings proved that chitosan nanoparticles have pH responsive release characteristics. The effective internalization and anticancer activity of alkaloids were proved by fluorescent microscopy using dual staining. The PI staining and the FACS analysis showed that there is a concentration dependent increase in the accumulation of the cells in the G0-G1 phase. This observation can affordnew directions in the development of target oriented drug delivery systems in response to the PH to overcome the draw backs of the conventional therapies.

Key words: anticancer activity; alkaloids; chitosan; nanoparticles; fluroscent microscopy.

INTRODUCTION

Worldwide lung cancer is leading cause of significant morbidity and mortality leading to death with a dismal 5-year survival rate of only 15 % [1]. Current treatment strategies are strongly dependent on the type of maliganancy and stage at the time of diagnosis but often involve a combination of surgery, chemotherapy, and/or radiation therapy. Naturally available plant products and endophyte extracts are rich source for natural drugs, these will provide unlimited opportunities for new drug findings due to unrelated availability of biochemical compound diversity[2]. According to World health organization (WHO), more than 80% of the population relies on traditional medicine for their primary healthcare needs. India has rich flora and fauna of medicinal plants with a long history in use of herbal medicines which increases the human interactions with the environment. Medicinal plants contain a wide range of bioactive compounds which can be used in the treatment of various chronic as well as infectious diseases[3]. Chemically synthesized drugs leads to develop adverse effects and microbial resistance men switched to ehtnopharmacognosy. They initiate plainly thousands of bioactive compounds from plants with various effective alternatives and safe with fewer side effects. Many beneficial biological activities such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were noted by author for *sphaeranthusamaranthoides*. So, the current study concentrates on the antiproliferative activity of alkaloids from *sphaeranthusamaranthoides*.

Internalization of the drug in chemotherapy is important step; however this can be helped by the carrier molecules. Thenano-sized cytotoxic drug carriers accumulates passively around the solid tumors and targets the tumour tissues, this activates the internalization of the drug intotumourtissues (1-2 polymers). One of the effective carrier molecules is chytosan. Particles with size range between 10 and 1,000nm are defined as particulate dispersions or solid particles. The drugs can be dissolved, entrapped, encapsulated or attached to thenanoparticles matrix[4]. Nano carriers prepared form the biocompatible and biodegradable polymers are being exploited in various targeted drug delivery of the therapeutic agents: such as sustained drug, vaccine and gene delivery [5]. Nanoparticles prepared using Alginate and chitosan (10-100nm) have been employed to deliver drugs to target specific regions, e.g. for colon specific deliver[6]. There are some special features for an ideal targeting system are the following: (1) it has to be biocompatible biodegradable, and possess low antigenicity(2), protection of the drug,(3) maintenance of the integrity till the target is reached, (4) less side effects, (5) easy membrane passage, (6) recognizing the target and then association, (7) association has to favour the controlled drug release, and (8) elimination of the carrier molecule is important upon drug release. While many of these characteristics are widely exploited in literature, vector interactions at cell level are not yet deeply investigated. Among polymers, alginate has several unique properties that have allowed using it as a matrix for the entrapment and/or delivery of a variety of biological agents [7]. Alginate is extracted from some varieties of brown algae and it is a co-polymer, made up of two uronic acids: D-mannuronic acid and L-guluronic acid. The Polyvalent cations present in the alginate are responsible for interchain and intrachain reticulations because they are tied to the polymer when two guluronic acid residuals are close. The reticulation process consists of the simple substitution of sodium ions with calcium ions [8]. The relatively mild gelation process has enabled not only proteins, but also cells [9] and DNA[10] to be incorporated into alginate matrices with retention of full biological activity. Hence in the current study chitosan- alginate nanoparticles exploited and encapsulated with alkaloid and their anticancer activity is assessed. The morphological changes observed for lung cancer cell lines in the current study are basis for the functional changes; in turn, functional changes would affect the morphological structure. The changes in the sub-cellular structures reveal the target delivery of the alkaloids and their effective anti-proliferative nature in A549 lung cancer cell lines.

MATERIALS AND METHODS

2.1 Plant material:

The fresh leaves of *SphaeranthusAmaranthoidesburm.f* was collected from the Thirunalveli district and was identified and authenticated by V.Chelladurai, Research officer-Botany (Retd), Central council for research in Ayurveda and Siddha, Government of India. The leaf was washed and air dried and then shade dried at room temperature. It was subjected mill to make coarse powder and used for extraction with solvent.

Preparation of leaf extract

1kg of the coarse powder of leaves of *sphaeranthusamaranthoidesburm f* was packed in to the thimble of soxhlet apparatus and was subjected to extraction sequentially with ethanol and chloroform.Ethanol and chloroform extracts were subjected to lyophilisation with the help of lyophiliser to a semisolid mass. Preliminary phytochemical screening of the drug was carried out as per the methods and tests described by Dey[11]. Solvent -solvent extraction was done to isolate the alkaloids. The plant extract was partitioned between aqueous tartaric acid solution and ethyl acetate. The plant extract was added to this mixture and left undisturbed for several hours until layers were formed. The presence of alkaloids was identified with the mayer's, hangers and Wagners tests.

1.2. Thin Layer Chromatography

Further the presence of alkaloids was confirmed with TLC. Thin layer chromatography was performed according to the method of Skoog West Holler and Fried[12]. The chloroform dissolved sample was used for test and the spots was detected in the presence of UV light or spray reagent (mayer's reagent).

1.3. Preparation of blank and drug loaded chitosan-Alginate nanoparticles:

The drug loading studies were performed with different concentrations of drug in phosphate buffer.

Blank chitosan nanoparticles are prepared according Paul CatalinBacalaureat [13]with slight modifications. For the synthesis of the drug loaded nanoparticles the drug was incorporated at the rate of 1mg/ml to Chitosan solution. It was then added into the Sodium Alginate solution then the further steps were similar to that of the preparation of blank Chitosan–Alginate nanoparticles[13].

1.4. Characterization of the nanoparticles

The prepared blank and drug loaded nanoparticles are characterised using SEM (SEM-Carl Zeiss SUPRA-55). Nanoparticles isolated from suspension were dried by a lyophilizer. SEM was performed to obtain the information regarding the topography and surface characteristics of the beads.

1.4.1. FTIR

The blank Alginate-Chitosan nanoparticle sample and drug loaded Alginate- chitosan nanoparticle sample was subjected to FT-IR for analyzing the various potential interactions within the nanoparticles. The FT-IR spectrum was obtained using IR Prestige-21 SHIMADZU. The spectral resolution is 4 cm-1 and 20 scans were performed each.

1.5. Drug entrapment efficiency

The amount of drug entrapped was determined by centrifuging the solution and the supernatant obtained was analyzed for drug content spectrophotometrically by measuring the absorbance at 260nm in UV Spectrophotometer. Confirmation for loading of the drug was done by comparing optical absorbance of nanoformulation with and without drug loading at 260nm [14].

Entrapment Efficiency (%)= [(Total amount of drug – Amount of drug left out in the supernatant)/Total amount of drug]*100

1.6. Invitro drug release

For quantitative estimation of the drug loading efficiency, the pellet that was collected during the centrifugation for calculating the Drug Entrapment Efficiency was further dispersed in buffer pH7.2 and was subjected to further centrifugation at 15000rpm for 40minutes. The filtrate obtained was assayed spectrophotometrically for drug content at 260nm.Drug Loading Efficiency%=(Mass of drug in nanoparticle /Mass of nanoparticle recovered)*100 [14].

1.7. Anticancer activity of alkaloids in a549 cell lines

The Alkaloid-rich extract from *Sphaeranthusamaranthoides* has been known to exhibit anti-proliferative effects on cancer cell lines. The extract was further coated with Chitosan-Alginate nanoparticles for better drug delivery and enhancement of the anti-cancer activity on the lung cancer cell lines.

1.8. MTT assay

The proliferation of A549 cells was assessed by MTT assay according to the method of [15]. Cells were plated in 96-well plate at a concentration of 5×10^4 cells/well 24 h after plating. The cells were exposed to chitosan-alginate encapsulated alkaloid nanoparticle in a different concentration ranging from 10-200µg/ml for 24hrs. Spectrophotometrical absorbance of the purple blue formazan dye was measured using an ELISA reader (BIORAD) at 570nm. Optical density of each sample was compared with control optical density and graphs were plotted.

1.9. Ethidium bromide/acridine orange (dual staining)

The ability of the alkaloid fraction in inducing apoptosis was assessed by Ethidium bromide/acridine orange staining was carried out by the method of Gohel [16].according to him the IC50 concentration the concentration of chitosan-alginate nanoparticles selected as 50µg/ml and 100µg/ml. Nearly 300 cells were counted in each sample at two different fields. The percentage of apoptotic cells was determined by [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) ×100].

1.10. Propidiumidodide staining

Nuclear staining was performed using Propidium iodide staining according to the method of Chandramohan[17]. A549 cells were plated at a density of 5×10^4 in 6-well plates. The plates were incubated at 37°C in a humidified CO_2 incubator until they are 70–80% confluent. Then cells were treated with 50µg/ml and 100µg/ml of drug loaded Chitosan-alginate nanoparticles for 24h. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined by fluorescence microscopy and at least 1×10^3 cells were counted for assessing apoptotic cell death.

1.11. Statistical analysis

Data were expressed as mean ± S.E.M and analyzed by Turkey's test to determine the significance of differences between groups. A *p* value lower than 0.05, 0.01 or/and 0.001 was considered to be significant.

RESULTS

3.1. Phytochemical analysis

The prepared plant extract was subjected for the preliminary phytochemical analysis which showed the presence of different bioactive compounds like tannins, saponins, flavonoids, alkaloids, proteins and steroids.

3.2. Solvent – solvent extraction

The alkaloids are isolated using solvent-solvent extraction, the ethyl acetate layer or the aqueous layer (neutralised with ammonia) contained neutral and weakly basic alkaloids. The organic layer contained basic alkaloids .The obtained extract was further subjected to rotary evaporator to get the crude alkaloids.

3.3. Identification and confirmation of alkaloids:

The obtained alkaloid crude extract subjected to confirmatory tests with Mayer's, Wagner's and hager's reagents. All the three qualitative tests indicated the presence of the alkaloids with the respective color changes. The three creamy bands displayed by spraying with mayer's reagent indicates the presence of three different groups of alkaloids in TLC. The bands were also observed under the UV light. The calculated Rfvalue is 5.2/8=0.605.

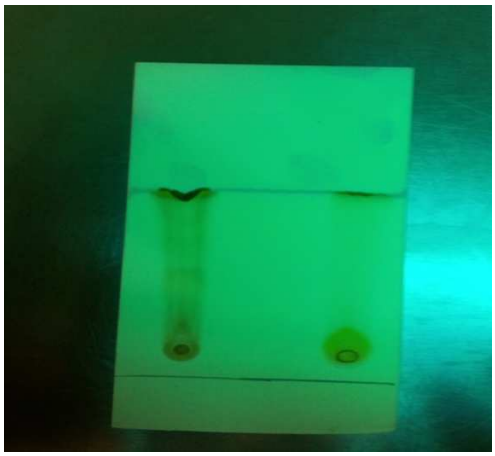


Fig: 1TLCunder UV of Alkaloids

3.4. Alkaloid release from thechitosan nanopartilces

Alkaloid release profile and stability study of chitosan-alginatenanoparticles were analysed by considering the PH and size. The alkaloid release capability was investigated at different PH values (PH 4.2 and 5.0) which is similar to the biological conditions. 750mg/ml of drug was loaded and 625mg was released at12^{th} hr this shows 83.3% of drug release efficacy. The pH value of the medium has a remarkable effect on release of alkaloid from the chitosan-alginate coated nanoparticles. The nanoparticles were around 80-85% stable for both 37^{o}C and 4^{o}C at pH 7.2. The stability of nanopartilces did not significantly change with temperature. The current results indicates that alkaloid loaded chitosan- alginate nanoparticles were stable at both 37^{o}C and 4^{o}C (pH 7.2), so that chitosan-alginatenanopartilces can be stored safely.

3.5. SEM analysis

Fig 2 and Fig 3 shows the SEM images of blank Chitosan-alginate nanoparticles and drug loaded Chitosan-algiantenaoparticles. The particle size of blank chitosan-alginate nanoparticles and drug loaded Chitosan nanoparticles were seen in the range (41nm and 200nm) and spherical in shape.The increase in the size of the drug loaded nanoparticle confirms the successful encapsulation of the drug.

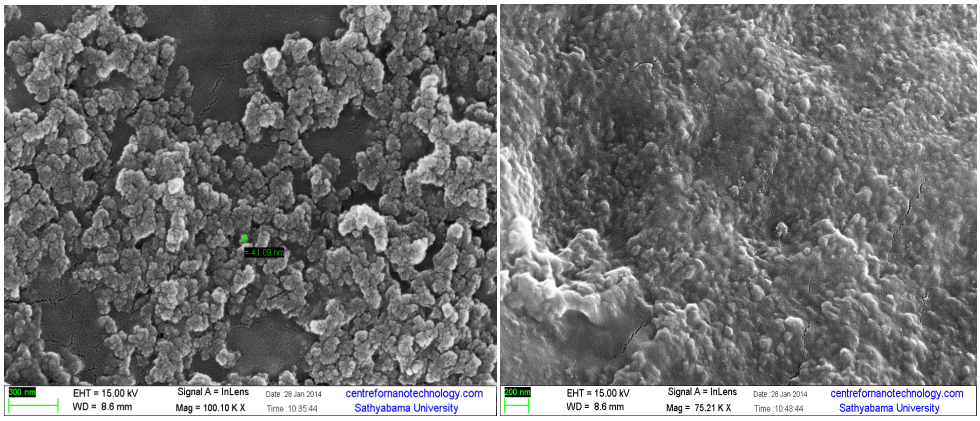


Fig: 2. Blank chitosan nanoparticles

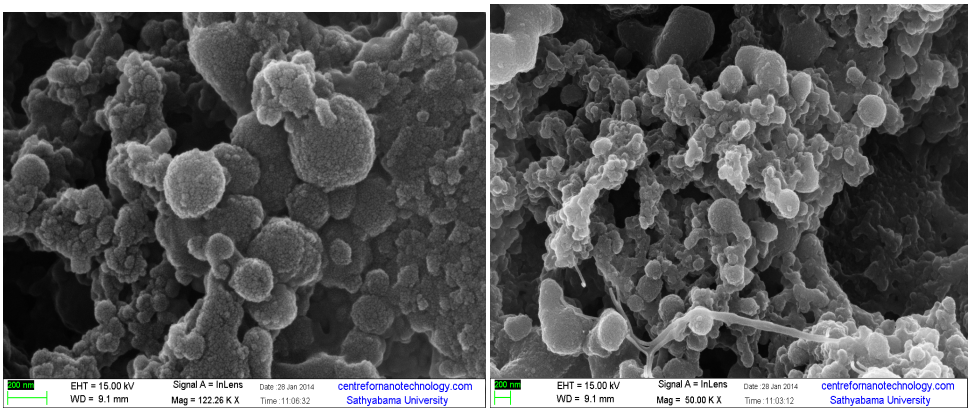
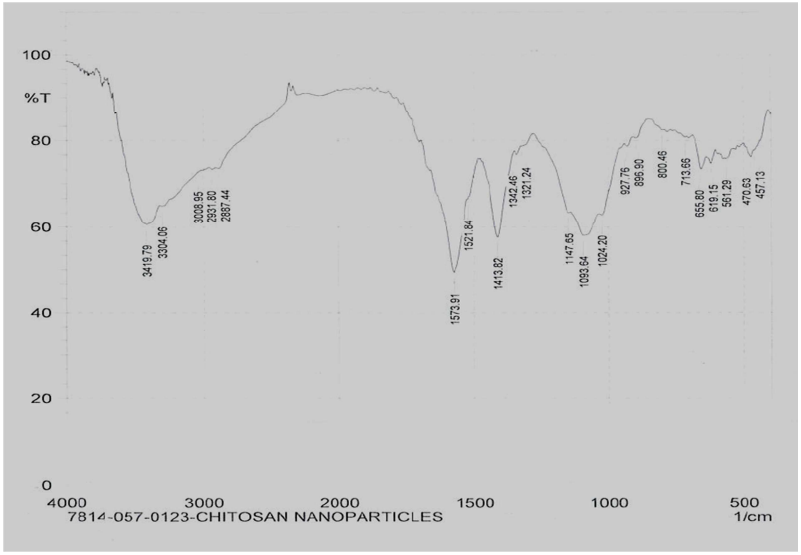


Fig: 3. chitosan Encapsulated nanoparticles

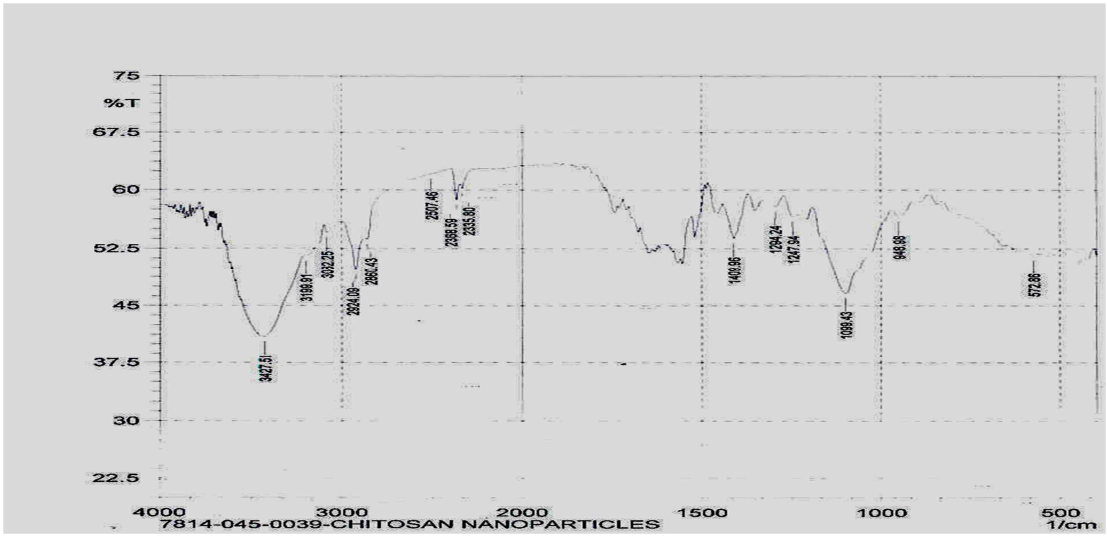
3.6. FT-IR ANALYSIS

FT-IR or Fourier Transform Infrared Spectrophotometry is a technique which is used to obtain an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid,liquid or gas.



Graph:1. FT-IR spectra of blank Chitosan-Alginate nanoparticles

The FTIR spectrum of blank chitosan nanoparticles is shown in Graph.1.The characteristic peaks observed are 457 $cm^{-1}$and 470cm^{-1}(C-I stretching), 561cm^{-1} (C-Br stretching),619 $cm^{-1}$and 655cm^{-1} (C-Cl stretching),713 $cm^{-1}$and 800cm^{-1}(=C-H bending overlapped with C-Cl stretching) , 896$cm^{-1}$and 927cm^{-1}(=C-H bending),1024cm^{-1}(C-F stretching),1093cm^{-1}(C-O stretching overlapped with C-N stretching),1147cm^{-1} (C-O stretching overlapped with C-F stretching),1321cm^{-1} and 1342cm^{-1}(C-F stretching overlapped with C-N stretching),1413 cm^{-1} (-C-H bending overlapped with C=C stretching),1521cm^{-1} and 1573 cm^{-1} (C-C stretching overlapped with N-O stretching),2887cm^{-1} and 2931cm^{-1}(C-H stretching),3008cm^{-1} and 3304cm^{-1}(C-H stretching),3419 cm^{-1} (N-H stretching).



Graph 2: FT-IR spectra of drug loaded Chitosan-Alginate nanoparticles

The FTIR spectrum of drug loaded chitosan nanoparticles is shown in Graph.2.The characteristic peaks observed are 572cm^{-1}(C-Br stretching),948cm^{-1} (=C-H stretching),1099cm^{-1} (O-H stretch overlapped with C-N stretch),1247 cm^{-1} and 1294 cm^{-1} (C-F overlapped with C-N),1409 cm^{-1} (C-Hbending overlapped with (C=C stretching), 2335cm^{-1} and 2368cm^{-1}(C≡C stretching), 2507cm^{-1}(C-N stretching), 2860cm^{-1} and 2924cm^{-1}(C-H stretching),3082 cm^{-1} (C-H stretching),3199 cm^{-1} (O-H stretching),3427cm^{-1}(N-H stretching). The FTIR spectra inferred that the characteristic peak 457cm^{-1} has been shifted to 572cm^{-1}(C-I to C-Br) and 1093cm^{-1} has been shifted to 1099cm^{-1}(C-O stretching overlapped with C-N stretching to O-H stretch overlapped with C-N stretch).This denotes the drug was intact in the formulation and did not react with the polymer.

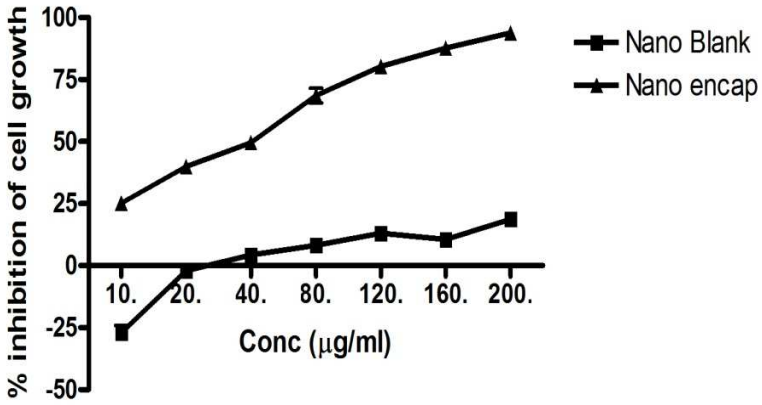
MTT assay

3.7. Cellular internalization and cytotoxicity of nanoparticles.

The internalization of nanoparticles loaded with the alkaloids were investigated by fluresence microscopy on A549 cell lines. The % inhibition of cell growth against A549 lung adenocarcinoma cell lines showed that the Nano encapsulated alkaloid was able to inhibit the cell growth when compared to the Nano Blank. Maximum inhibition was found to be 87% at a concentration of 200µg/ml. The IC_{50} was calculated by linear regression analysis. The IC_{50} of Nano Encap was 52.98µg/ml (Graph :3). Hence further assays were carried out with 50µg/ml and 100µg/ml. The cellular uptake of nanoparticles could be facilitated by the positive surface charges of chitosan –alginate coat.

3.8. Dual Staining assay

To assess the type of cell death induced by the drug in A549 cells, the morphological changes of the cells after double staining with Acridine Orange/Ethidium Bromide (AO/EB) were identified. AO/EB staining uses combination of two different dyes to visualize cells with aberrant chromatin organization (Fig 1&2). AO penetrates into living cells, emitting green fluorescence after intercalation into DNA, but it cannot distinguish viable from non-viable cells. To achieve this, a mixture of Acridine Orange and Ethidium Bromide was used, Ethidium bromide can penetrate in to the non-viable cells and emits red fluorescence in the cells with the altered cell membrane. This differential uptake of these two dyes allows the identification of viable and non-viable cells.



Graph 3: Cytotoxicity of Nano blank and Nano Encap alkaloid against A549 lung adenocarcinoma cells

The Viable cells appears green fluorescence and with intact DNA and nucleus. Early apoptotic cells exhibited green cloured nuclei which had fragmented DNA. Late apoptotic cells are stained orange and red with DNA fragmentation this indicates the necrosis. Besides, some cells exhibited typical characteristics of apoptotic cells like plasma membrane blebbing. Inspite of the inefficiency of the nanoblank in inducing cell death against the tested A549 lung adenocarcinoma cells, further confirmation was done by AO/EB staining along with different concentrations of nanoencapsulated alkaloid. From the data it was clear that only the nanoencapsulated alkaloid was able to induce apoptosis and not the nanoblank which with increasing concentration of drug, the number of viable cells decreased tremendously. The percentage of apoptotic cells after treatment with nanoblank did not show significant difference comparing with control whereas the 50µg/ml and 100µg/ml of nanoencapsulated alkaloid drug showed a drastic increase in apoptotic cells ($p< 0.001$) to 58% and 86% respectively(Graph .4).

Fig 1 AO/EtBr staining of Nanoblank

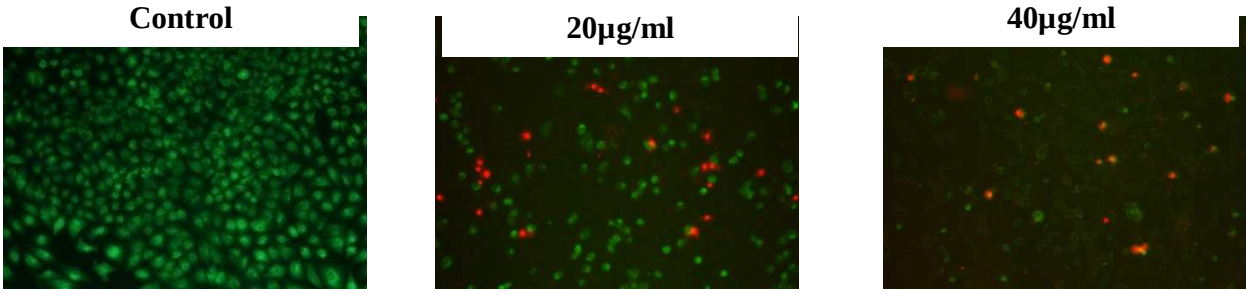
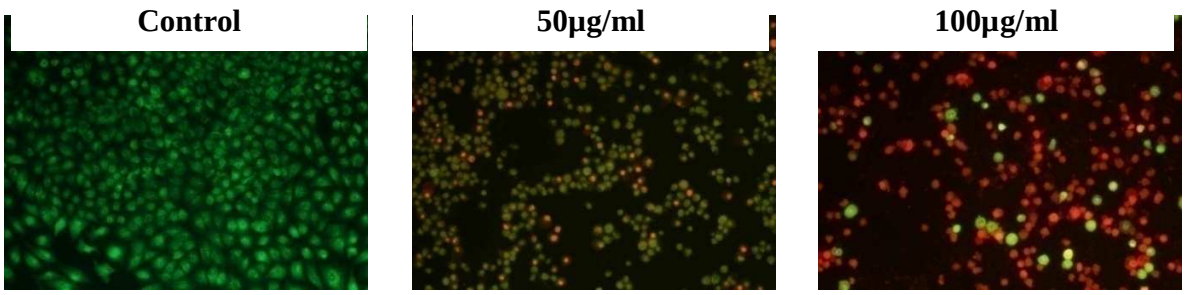
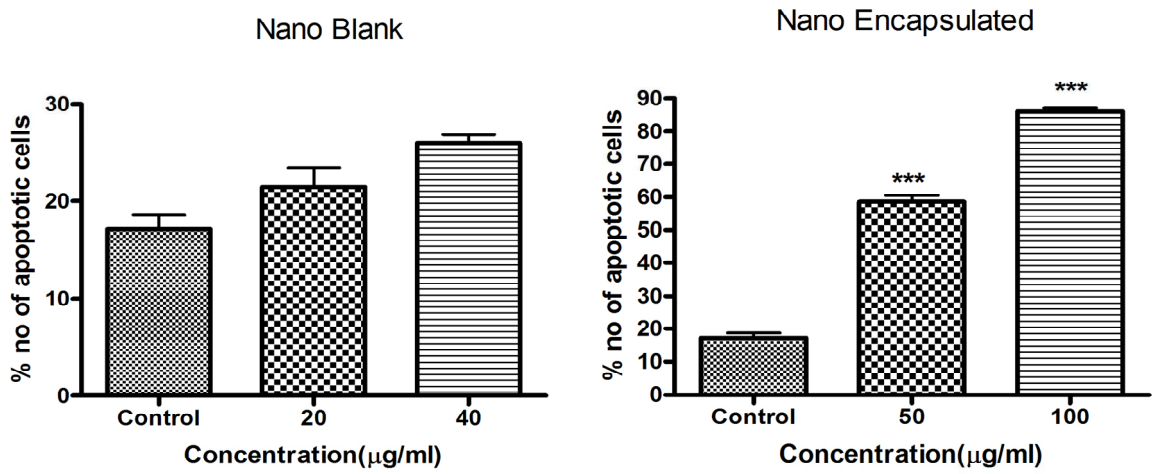


Fig 2 AO/EtBr staining of Nanoencapsulated Alkaloid



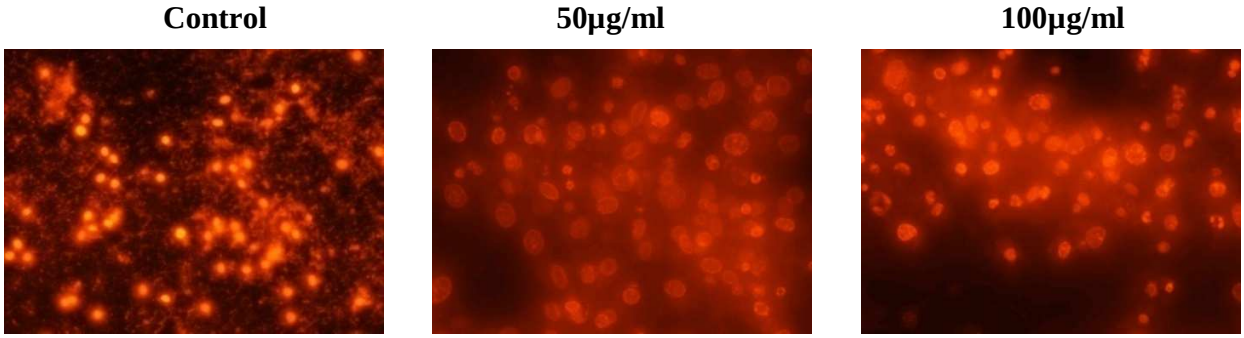
Graph 4: Bar graph showing the percentage number of apoptotic cells in Nanoblank and Nano encapsulated alkaloid



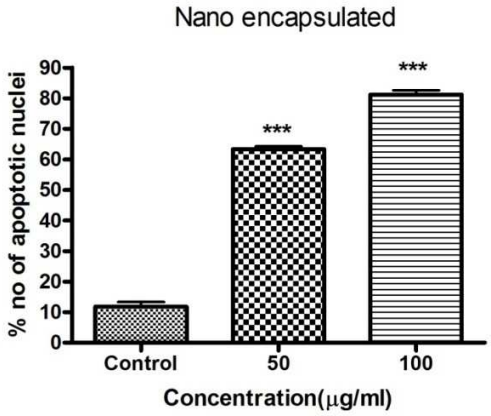
3.9. Propidium Iodide – nuclear fragmentation assay

The pro-Apoptosis stage of the cells was further confirmed by analyzing the nuclear morphology of drug-treated A549 cells. Morphology of nucleus was identified with membrane-permeable PI stain. The cells treated with alkaloids induced characteristic nuclear fragmentation where as the untreated control cells did not show this characteristic change (Fig .3). The apoptotic cells displayed specific features of reduced size, intense fluorescence of condensed nuclear chromatin and formation of membrane blebs. The percentage of apoptotic nuclei after treatment with 50µg/ml and 100µg/ml of the extract increased enormously ($p<0.001$) to 63% and 81%, respectively (Graph .5).

Fig 3 Propidium Iodide staining assay of Nanoencapsulated alkaloid



Graph 5: showing the percentage of apoptotic nuclei

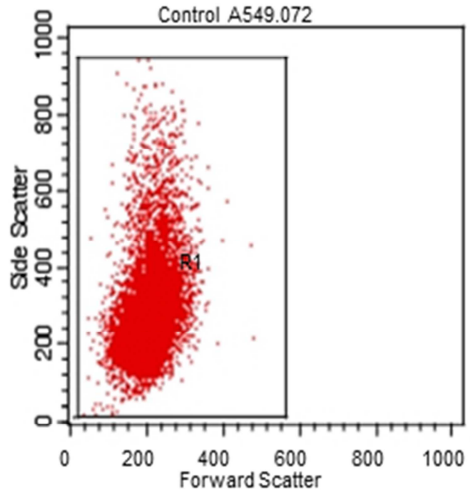


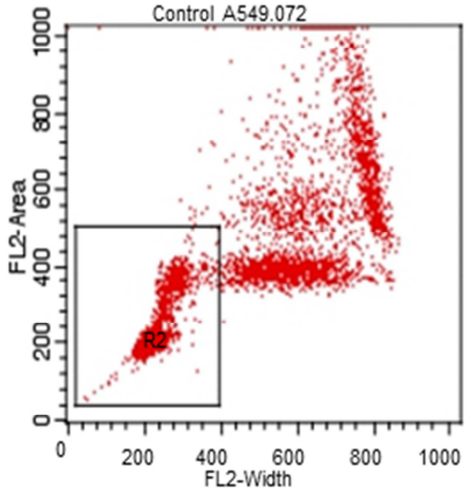
Graph : 5 Percentage of apoptotic nuclei

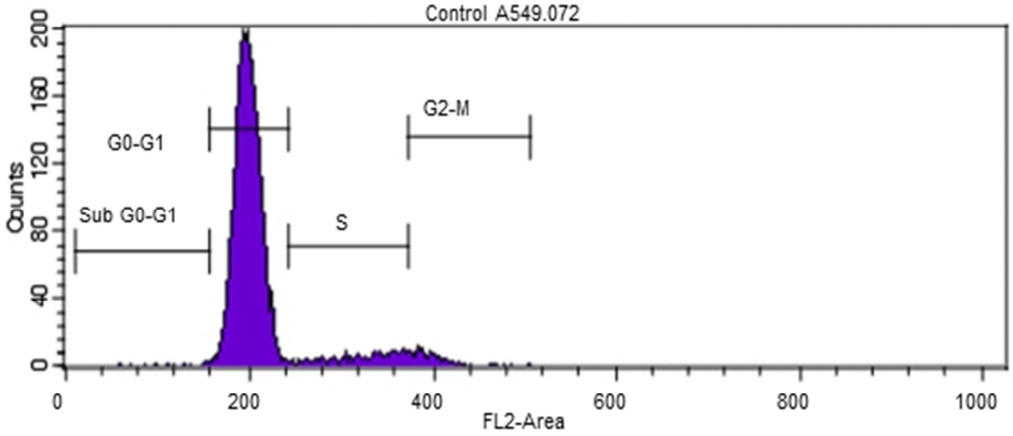
3.10. FACS analysis

The cytotoxicity of chitosan nanoparticles was further evaluated by uptake of the Propidium iodide after treatment with chitosan nanoparticles for 48hrs in different concentration. Normally live cells are impermeable to PI so this specific feature is used to check the uptake of PI to measure the population of cells in which membrane integrity was lost. The no. of dead cell were Quantified by FACS analysis (Nuria*S et al*2004).Surprisingly, chitosan nanoparticles treated A549 cells took up PI in a concentration-dependent manner. With respect to the increase in the concentration the number of cells up taking of PI also increases (Fig. 4). At lower concentration (50µg/ml) nanoencapsulated alkaloid showed arrest at subG0 – G1 with 16% cells accumulated. At higher concentration (100µg/ml), it showed an increased cell population at sub G0 – G1 with 35% with concomitant decrease in the other phases of cell cycle. Sub G0 – G1 phase is the apoptotic phase, hence from the results it is demonstrated that treatment with nanoencapsulated alkaloid induced A549 cell apoptosis maximally.

Fig 4 Cell Cycle arrest induced by different concentrations of Nanoencapsulated alkaloidControl



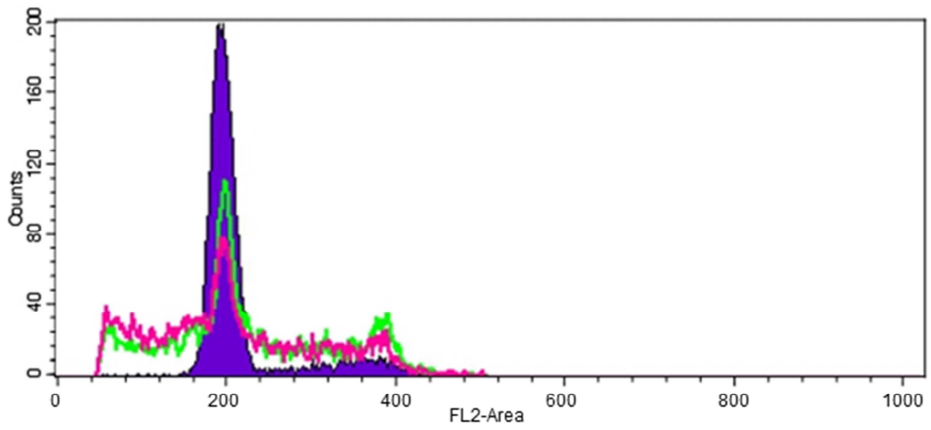


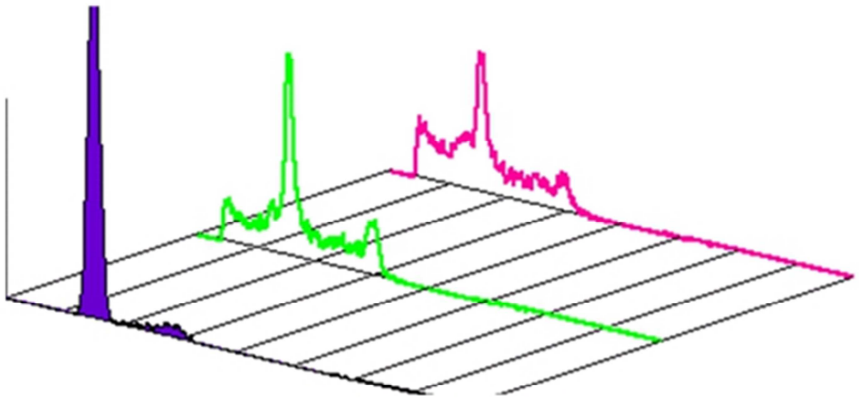


File: Control A549.072 Gate: G2

Gated Events: 6873 Total Events: 10000

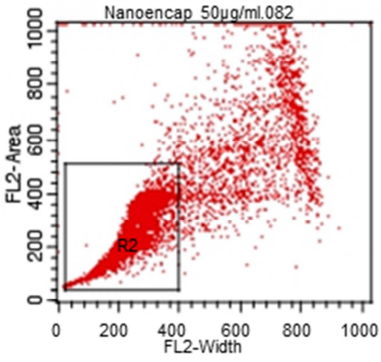
Marker	Events	% Gated	% Total	Mean
All	6873	100.00	68.73	213.40
Sub G0-G1	18	0.26	0.18	117.89
G0-G1	6147	89.44	61.47	198.22
S	479	6.97	4.79	323.06
G2-M	241	3.51	2.41	396.15

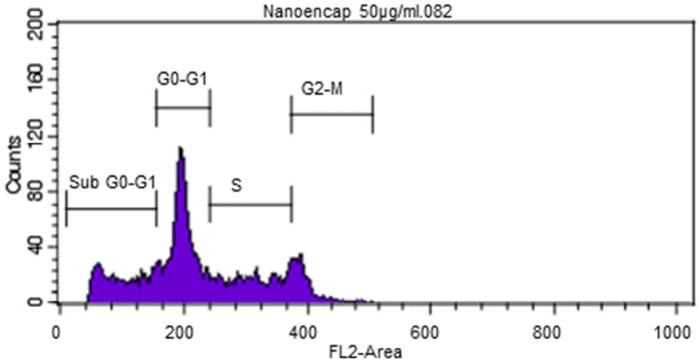




Key	Name	Parameter	Gate
	Control A549.072	FL2-A	G2
	NANOENCAP 50µg/ml.082	FL2-A	G2
	NANOENCAP 100µg/ml.083	FL2-A	G2

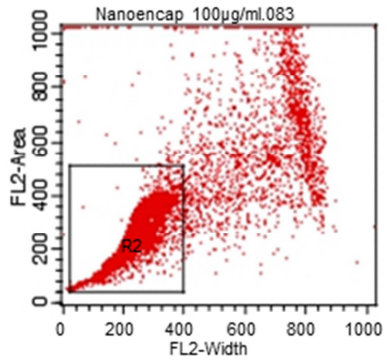
Treatment groups

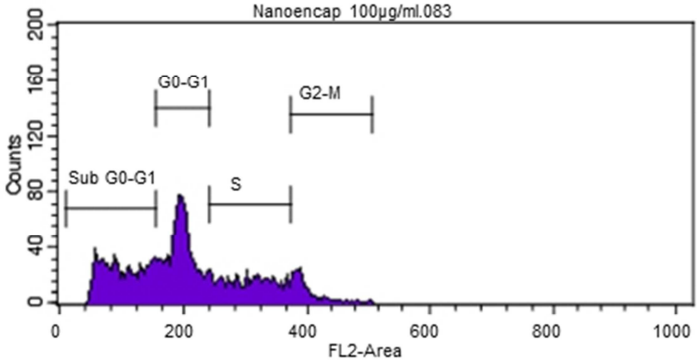




File: Nanoencap 50µg/ml.082 Gate: G2
Gated Events: 7649 Total Events: 10000

Marker	Events	% Gated	% Total	Mean
All	7649	100.00	76.49	226.37
Sub G0-G1	1628	21.28	16.28	102.53
G0-G1	3455	45.17	34.55	199.75
S	1719	22.47	17.19	307.92
G2-M	916	11.98	9.16	395.79





File: Nanoencap 100µg/ml.083 Gate: G2
Gated Events: 7406 Total Events: 10000

Marker	Events	% Gated	% Total	Mean
All	7406	100.00	74.06	209.81
Sub G0-G1	2259	30.50	22.59	103.32
G0-G1	2941	39.71	29.41	197.05
S	1659	22.40	16.59	307.49
G2-M	613	8.28	6.13	400.14

DISCUSSION

Treatment of Cancer is hindered by two major hurdles. One of the obstaclesis notpossessing preferential target cell killing, whereas the second is the reduced internalization through the cell membranes. The combination of the

prodrug and targeting approaches are applied to overcome the first obstacle using antibodies [18][19][20]. the second hindrance can be overcome with most usual method like receptor mediated endocytosis or phagocytosis[21] regardless of serious draw backs such as low internalisation efficiency[22]and need for drug release from end some [23]. Presently there are very less delivery systems are capable of overcoming these two limitations, and none of them provides evidence of macromolecular drug delivery such as proteins or genetic materials. Therefore, an effective system for the delivery of hydrophilic macromolecular drug types, is necessary, and the seek remains. In this regard the alginate- chitosan nanoparticles are most promising strategy as a drug delivery system.

The Alginate- chitosan encapsulated drug systems exhibits decreased clearance and prolonged circulation, thus providing ample time for the release of encapsulated drug at the target site and lowering the undesirable side effects generated by free drug. Moreover the current study proves that the alginate- chitosan-based system delivers high drug payloads, thereby increasing the cytotoxic activity for the same drug concentration. Because the encapsulated nanoparticles displays rapid cell uptake, the findings of this study open up possibilities for the treatment of cancers.

Earlier studies of the anticancer activity of *sphaeranthusamaranthoides* showed inhibition in the growth of lung cancer cell lines. The alkaloids isolated from the leaf extract showed a good targeted delivery and anticancer activity. The present invitrostudy proved that alkaloids isolated from the leaf extract inhibited the cell growth via induction of apoptosis in A549 cell line through concentration and time dependent manner. This was evidenced by the appearance of the membrane disintegration and blebbing of nuclear membrane. Our current results reveals that alkaloids servers as a good apoptotic inducer in invitro.

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