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Antiproliferative effect of chitosan extracted from crab shells on human lung adenocarcinoma cell line (A549)

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Abstract

The Present study was made to evaluate the antiproliferative effect of chitosan extracted from crab shells against human lung adenocarcinoma cell line (A549). Chitosan was extracted from crab shells which includes deproteinization, demineralization, deacetylation. MTT method to find out the toxicity and cell viability of chitosan in both normal and cancer cells (A549). The Propidium iodide staining and DNA fragmentation is to analyze the apoptotic bodies in A549 cell line. Chitosan appeared creamy white in colour and the total carbohydrate content was estimated as 0.07 mg/ml. The antiproliferative effect of chitosan against A549 cells clearly emphasizes, that there is a decrease in the cell viability. The 50 % inhibition (IC50) of the cell growth was found at 20 µg/ml.The cyto-localization of nuclear morphology and DNA fragmentation assay revealed the induction of apoptotic cell death in A549 at 24 hours. Chitosan exhibits the inhibitory effect by inducing loss of cell viability, morphology change and DNA fragmentation in A549 cells due to the presence of free protonated amino groups on the polymer chain. Our preliminary studies support that chitosan could be an efficient therapeutic agent for cancer.

Keywords: Chitosan, DNA fragmentation, Propidium Iodide.

Introduction

Most of the naturally occurring polysaccharides viz, cellulose, dextrin, pectin, alginic acid, agar, agarose and carrageenan are naturally acidic in nature, whereas chitin and chitosan are examples of highly basic polysaccharides [1]. Chitin is mainly extracted from shrimp and crab shells. Chitin, calcium carbonate and proteins accounts about 90 % of the dry shell weight [2]. The major shell components are chitin, proteins, lipids, pigments and trace elements. Chitin is a biopolymer and a major component of the supporting tissues of organisms such as crustaceans, fungi and insects. It is the most important organic constituent of 'Invertebrates' exoskeleton. Chitin is the second most abundant natural biopolymer after cellulose [1]. Chitosan solubility is affected by the presence of free amine groups present in the chitosan chain, which allows it to dissolve in diluted aqueous acidic solvents [3]. Crab is a decapod crustacean, there are about 4500 species that come in different size and colors. About 30-40 % of crustacean shell waste consists of protein, 30-50 % calcium carbonate and 20-30 % chitin. These proportions vary with species and season [4]. Extracted chitin from crab shells can be used to produce chitin-derived products, such as chitosan for bioplastic and nano structured film. The waste of these natural polymers is a major source of surface pollution of chitosan in coastal areas. The production of chitosan from crustacean shell obtained as a food industry waste is economically feasible, especially if it includes the recovery of carotenoids. These shell contain considerable quantities of astaxanthin, a carotenoid that has so far not been synthesiszed and which is marketed as a fish food additive in aquaculture, especially for salmon [5]. Cancer is the second largest non-communicable disease and it has a sizable contribution in the total number of deaths. Cancer is one of the leading causes of death across the globe. It has been estimated that about 13 million people were diagnosed with cancer and eight million men and women died of cancer in the year 2010. A total of 1,596,670 new cancer cases and 571,950 deaths are occurred in United States in 2011. There have been significant improvements in diagnosis and treatment of several cancers, particularly an increased survival rate for cancer patients who are diagnosed at early stages. Regardless, in most cancers diagnostic, surgical and therapeutic procedures have not yet evolved, cancer elimination and prevention are still a major challenge. For many decades, cancer drug development strategies led to several promising drugs, some of which have proven to be successful in cancer prevention and treatment. Despite the advances in the drug development, clinical intervention options are still limited for many types of human cancers [6]. The disposal of this waste creates severe problems for human life so that the present work is aimed at investigating utilization of waste crab shells in synthesis of chitosan with following objectives.

- Extraction of chitosan from crab shell waste
- To estimate the total carbohydrate content of extracted Chitosan
- To determine the toxicity effect of chitosan on vero cell line
- To study the cytotoxicity of chitosan on Human Lung Adenocarcinoma cell line (A549)
- To observe the morphological variation of cancer cells treated with Chitosan
- To determine the apoptosis by Propidium iodide staining and DNA fragmentation

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Figure 1: Crab shell

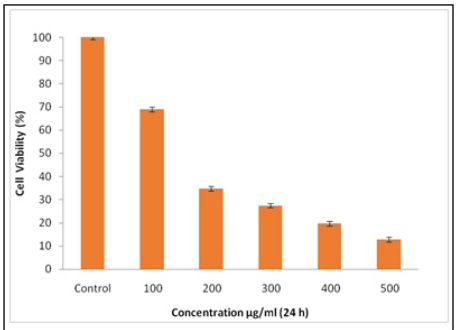

Figure 2: Toxicity assessment of chitosan treated with Vero cells

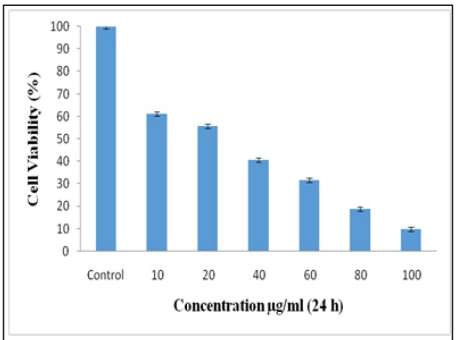

Figure 4: Effect of different concentration of chitosan on cell viability of A549 cells

Material and Mathod

2.1. Collection of crab shell

The crab shells were collected from light house fish market, Chennai, Tamilnadu, India. The collected crab shells were washed with tap water and air dried for 48 hours. The dried samples were made into powder using mortar and pestle.

2.2. Extraction of Chitosan

Extraction of chitin and chitosan involves washing of crushed exoskeletons. Crushed crab exoskeletons were placed in 200 ml beaker and soaked in boiling sodium hydroxide 8 % (w/v) for three hours in order to dissolve the proteins and sugars thus isolating the crude chitin. The crab shell samples are removed from the hot plate, and allowed to cool for 30 minutes at room temperature [7].

2.3. Quantification of total carbohydrate

Five different concentrations (0.2 – 1 ml) of working standard solution in test tubes and add water to bring the volume to 1ml in each test tube. Then add 4 ml of anthrone reagent and mix the contents and place it in water bath for 10 min and then cool the test tube to room temperature and measure the optical density in a photoelectric colorimeter at 620nm or by using a red filter. Simultaneously prepare a blank with 1ml of double distilled water and 4 ml of anthrone reagent. Construct a calibration curve on a graph paper, by plotting the glucose concentration (10 – 100 mg) on X axis and absorbance at 620 nm on Y axis. Compute the concentration of the sugar in the sample from the calibration curve. While calculating the sugar concentration the unknown sample, the dilution factor has to be taken into account [8].

$$\text{Concentration of unknown} = \frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \text{Conc. of standard}$$

2.2 Cell Culture

Lung Adenocarcinoma cancer cell line (A549) and African green monkey kidney cell line (Vero) was obtained from National Centre for Cell Science (NCCS), Pune, India. Dulbecco's modified eagle medium (DMEM), Trypsin-EDTA, Fetal Bovine Serum (FBS), 3-(4, 5- dimethylthiazol-2yl)-2, 5- diphenyltetrazolium bromide (MTT), sodium bicarbonate, Dimethyl sulphoxide (DMSO) and antibiotic solution were purchased from Hi Media Laboratories, Mumbai, India. Cell culture materials were purchased from Tarsons Products Pvt, Kolkata, India.

Toxicity assessment

Vero cells (5×103 cells/ml) were plated in 96 well plates with DMEM medium containing 10% FBS. The cells were incubated for 24 hours under 5 % CO2, 95 % O2 at 37°C. The medium was removed and washed with PBS and then fresh medium was added and kept for 1 hour in the incubator. Then, these medium was removed and the control wells received again fresh medium and treatment plates received 100, 200, 300, 400 and 500 µg/ml of the crude chitosan. Then the cultures plates were again incubated as above. After 24 hours10 µl of MTT solution (5 mg/ml) was added to each well and the cultures were further incubated for 3 hours and then 100 µl DMSO was added and the formed crystals were dissolved gently by pipetting 2 to 3 times. A micro plate reader was used to measure absorbance at 570 nm [9]. Growth inhibition rate was calculated as follows:

$$\text{Percentage of Growth inhibition} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100$$

Cell viability assessment

A549 cell line (5×103 cells/ml) were plated in 96 well plates with DMEM medium containing 10% FBS. The cells were incubated for 24 hours under 5 % CO2 at 37°C. The medium was removed and washed with PBS. The fresh medium was added and kept for 1 hour in the incubator. Then, these medium was removed and the control wells received again fresh medium and treated plates received 10, 20, 40, 60, 80 and 100 µg/ml of the crude chitosan. Then the cultures plates were again incubated as above. After 24 hours10 µl of MTT solution (5 mg/ml) was added to each well and the cultures were further incubated for 3 hours and then 100 µl DMSO was added and the formed crystals were dissolved gently by pipetting 2 to 3 times. A micro plate reader was used to measure absorbance at 570nm [9]. Growth inhibition rate was calculated as follows:

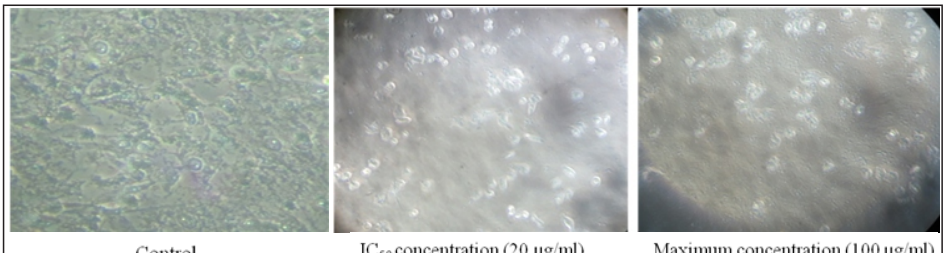

Fig. 4: Photomicrographs showing the morphological observation of control and A549 cells treated with chitosan (40 X maginification)

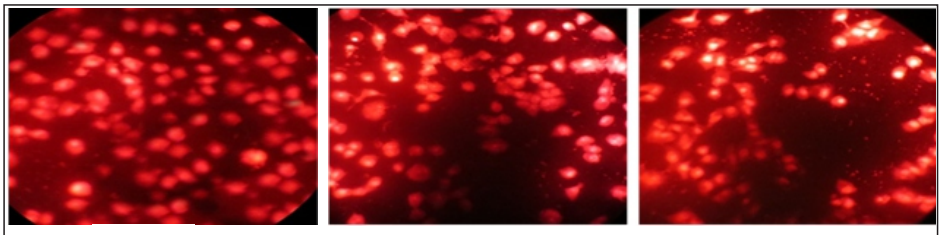

Fig. 5: Photomicrographs showing effect of chitosan on Propidium Iodide stained with A549 cells (40 X maginification)

$$\text{Percentage of Growth inhibition} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100$$

Cell morphological study

The general cell morphological changes was observed when crude chitosan is treated with A549 cancer cell lines and assessed using inverted light microscopic visualization. For that cancer cells (5×104 cells/ml) were plated in 6 well plates with DMEM medium containing 10% FBS. The cells were incubated for 8 hours under 5 % CO2 at 37˚C. Then the medium was removed and the control wells received again fresh medium and the treatment wells received 20 µg/ml (IC50) and 100 µg/ml (Max.Conc) of crude chitosan. Then the culture plates were incubated as above. After the incubation time the cells were visualized and photographed under inverted light microscope (Radical) at 20X magnification [10].

Propidium iodide staining method

A549 cell line (5×104 cells/ml) were seeded in 6 well plates with DMEM medium containing 10 % FBS. The cells were incubated for 8 hours under 5 % CO2 at 37˚C. Then the medium was removed and the control well received again fresh medium and the treated plates received 20 & 100 µg/ml of chitosan. Then the culture plates were incubated as above. After completion of incubation time the cells were washed with PBS and fixed in methanol:acetic acid (3:1 v/v) for 10 minutes and stained with 50 µg/ml of Propidium iodide for 20 minutes. After staining the cells were visualized immediately under fluorescence microscope (Radical) at 20X magnification [11].

DNA-fragmentation analysis by Agarose Gel Electrophoresis

A549 cell line (3×105 cells/ml) were plated in 6 well plates with DMEM medium containing 10% FBS. The cells were incubated for 8 hours under 5 % CO2 at 37˚C. The medium was removed and washed with PBS and then fresh medium was added and kept for 1 hour in the incubator. Then, these medium was removed and the control wells received again medium and treatment plates received appropriate concentrations of chitosan. Then the cultures plates were again incubated as above. After completion of incubation time, the DNA was extracted from the cell lysate as follows. The cells were washed with PBS and then added 0.5 ml of lysis buffer, transferred to a microfuge tube and incubate for one hour at 37˚C. To this 4µl of proteinase K was added and the tubes were incubated at 50˚C for 3 hours. To each tube, 0.5 ml of phenol : chloroform : isoamyl alcohol (25:24:1) was added, mixed and centrifuged at 10,000 rpm for 10 minutes to separate the DNA containing upper aqueous phase. Phenol-chloroform extraction was repeated twice, followed by chloroform extraction alone. To the resulting aqueous phase, 2 volumes of ice-cold absolute ethanol and 1/10th volume of 3 M sodium acetate were added and incubated for 30 minutes on ice to precipitate DNA. DNA was pelleted by centrifuging at 15,000 rpm for 10 minutes at 4°C, the supernatant was aspirated and the pellet was washed with 1 ml of 70 % ethanol. After repeating the above centrifugation step remove the last traces of the supernatant fraction. The pellet was allowed to dry at room temperature for approximately 30 minutes and resuspended in 50 µl of TE buffer. The DNA was quantified by UV-visible spectroscopy and 10 µg of DNA was electrophoresed in a 1% agarose gel containing Ethidium Bromide in a mini gel tank containing TBE buffer for 1 hour under 90 V. Then the gel was examined under UV transilluminator (Biorad) and photographed [12].

Statistical analysis

The data obtained in the present study was subjected to standard statistical analysis by using Microsoft excel data to find out the standard deviations.

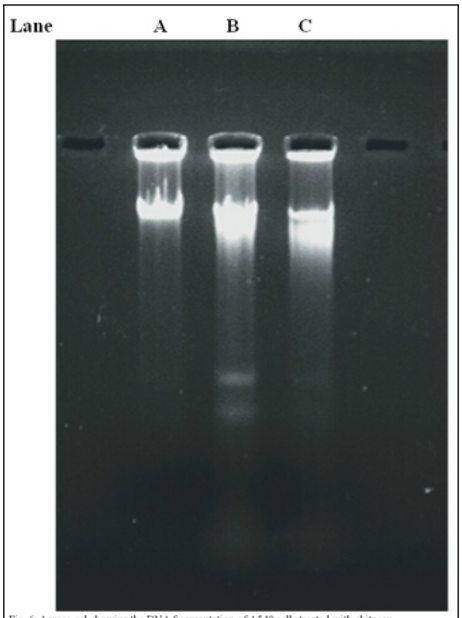

Fig. 6: Agrose gel showing the DNA fragmentation of A549 cells treated with chitosan
Lane A: Control; B: Maximum Concentration (100 µg/ml); Lane C: IC50 Concentration (20 µg/ml)

Result

The crab shells were collected from light house fish market, Chennai, Tamilnadu (Fig 1). Chitosan was extracted by deproteinization, demineralization and deacetylation methods. The dry weight of the chitosan (after lyophilization) was found to be 1.92 g in 20 gm of raw crab shell. The quantification of chitosan was done by Anthrone method, with glucose as standard. The carbohydrate content was estimated to be 0.07 mg/ml.

Toxicity assessment of chitosan on vero cell line

The toxic effect of chitosan was tested by MTT method using African green monkey kidney cancer cell line (Vero). The treatment of chitosan on Vero cell line was investigated over a range of concentration (100 – 500 µg/ml).The different concentration of chitosan inhibits the growth of Vero cell line in time-dependent manner (Fig.2). In the present study the cell viability got decreased once the concentration is increased. So, the percentage of cell viability is decreased from 30 % to 80 % gradually.

Cell viability assessment of chitosan on A549 cell line

The toxic effect of chitosan was tested by

MTT method using lung cancer cell line (A549). The treatment of chitosan on lung adenocarcinoma A549 cancer cell line was investigated over a range of concentration (10 – 100 µg/ml).The different concentrations of chitosan inhibits the growth of cancer cell line in time-dependent manner (Fig.3). In the present study inhibition concentration (IC50) value was observed at 20 µg/ml of chitosan in 24 hrs incubation.

Morphology observation of A549 cell line

Morphological observation of A549) cell line were observed in both control and treated cells (Fig.4). Microscopic observations were monitored using Radical light inverted microscope where in treated cells showed distinct cellular morphological changes indicating unhealthy cells, whereas the control appeared normal. Control cells were irregular confluent aggregates with rounded and polygonal cells. The cells treated with chitosan appeared to shrink became spherical in shape and cell spreading patterns were restricted when compared to control.

Propidium Iodide Staining

To confirm whether the toxic effect induced by chitosan involves nuclear changes and the nuclear condensation was studied by the Propidium Iodide staining method. In case of control cells a negligible number of positive cells were present whereas in case of A549 cells, treated with 20 µg/ml (IC50) of chitosan with exposure time of 24 hours showed progressive increase in the number of propidium iodide positive cells. In maximum concentration 100 µg/ml apoptotic bodies is higher when compare to control (Fig.5).

DNA fragmentation

DNA fragment analysis is a typical assay to find out the DNA damage or fragments appeared in DNA from which the chitosan extracted from crab shells. The DNA fragmentation of A549 cells were detected on 0.8 % agarose gel electrophoresis after exposing with
20 & 100 µg/ml of chitosan. In the present investigation, A549 cells were treated with chitosan for 5 hrs at 20 µg/ml and 100 µg/ml concentration it clearly shows fragmented DNA whereas control cells did not provide ladder (Fig.6).

Disucssion

Chitin is one of the most abundant biopolymers in nature and a major component in the supporting tissues of organisms such as crustacean, fungi and insects. It is a linear polysaccharide composed of α-(1,4)-linked -2 acetomido-2-deoxy D-glucose units which can be de-N-acetylated by boiling in 50% sodium hydroxide [3]. Chitosan is an amino polysaccharide prepared by processing crustaceans waste [13]. Many biochemists have found chitosan as biocompatible, biodegradable and non-toxic which made wide pharmaceutical applications such as wound dressings, blood coagulants, cartilage and bone tissue engineering scaffolds, drug delivery systems [14]. Carbohydrate based vehicles with a capability at reducing dosing frequency, improving drug pharmacological activity and delivering drugs at the specified site appear to be promising as pharmaceutical drug carriers [14]. The antitumor activity of chitosan was reported in early 1970's. This activity suggested mainly due to its cationic property by amino groups and later it was accepted that the molecular weight also plays a major role in antitumor activity [16]. Chitosan possesses good mucoadheshive properties resulting from the cationic behavior and the presence of free hydroxyl and amino groups allowing the polymer to interact with mucin by hydrogen and electrostastic bonding. Hence, it is regulated as a suitable excipient to buccal, nasal, ocular and vaginal forms [14]. The toxicity effect of different concentrations of chitosan was observed in vero cells upto 100 µg/ml concentration 80% of cells are viable. So, it is non-toxic and it can be used as a drug for cancer cells. Cytotoxicity analysis was performed for the MCF-7 and VERO cell lines, which shows the effectiveness of the sample towards the destruction of MCF-7 and its non-toxicity towards VERO. 50% cell viability for the MCF-7 cells was obtained at a sample concentration of 31.2 µg ml−1 [15]. A wide variety of medical applications for chitin and chitin derivatives (chitosan) have been reported over the last three decades [5]. Antiprolierative effect of prawn shells chitosan on lung cancer cell line (A549) has already been reported [17] and also antiproliferative effect of chitosan on urinary bladder cell line [6]. So based on the above reports the study on the cytotoxic effect of chitosan on A549 cell line was conducted. Cancer cell lines have been widely used for research purposes and proved to be a useful tool in the genetic approach and its characterization shows that they are, in fact, an excellent model for the study of biological mechanisms involved in cancer [18]. The stronger cytotoxic effect of chitosan on A549 cells might be related to the high positively charged amino groups in the chitosan molecule being attracted to the cancer cell membrane which has a greater negative charge than that of normal cells [19]. In this study, the efficacy of chitosan extracted from crab shells on A549 cell line was assessed. MTT assay was performed to check the viability of cells at different concentrations ranging from (10 - 100 µg/ml). Viable cells with active metabolism convert MTT into a purple coloured formazan product, with an absorbance maximum near 570 nm absorbance values in spectrophotometer. The readings were then calculated to yield the viability percentage, using the standard formula. The lysed cells lose the ability to convert MTT into formazan. Thus, MTT assay serves as a tool to check the active metabolism and viability of cells. IC50 value was observed at 20 µg/ml which indicates that chitosan is concentration dependent and has anti-prolifertive effect on A549 cells. This result is similar to the reports of Leena et al., (2014) who reported the anti-proliferative effect of prawn shells on lung cancer cell line. The toxic effect of CSO-INPs on A549 and HeLa cells were moderate as compared to bare INPs treatment. Higher toxicity of iron oxide nanoparticles could be attributed to high release of iron ions in intracellular space and in situ degradation [20]. This toxicity of nanoparticles was found to be time and dose dependent. Results clearly indicate that the cell viability decreased with increase in dose and time. The A549 cell morphology was observed and compared between the treated and untreated cells for any visible change in cell/organelle morphology that can give evidence or the cytotoxicity of the IC50 concentration. The morphological observation before treatment showed the cells to be healthy and with process protruding out from the cell body. The nucleus membrane is compact, distinct and it was observed through inverted microscope at 40 X magnification. The cells treated with IC50 and maximum

concentration showed variations in their morphology such as shrinkage, widespread detachment of cells from the culture plate substratum after 24 hours of incubation. Control cells were irregular confluent aggregates with rounded and polygonal cells. Biosynthesized AgNPs treated cells appeared to shrink, became spherical in shape and cell spreading patterns were restricted when compared to control. Similar results were observed by other groups in dermal fibroblast cells treated with citrate-coated gold nanoparticles [21] and U251 cells treated with AgNPs [22]. Propidium iodide is a small fluorescent molecule that binds to DNA but cannot passively traverse into cells that possesses an intact plasma membrane. PI uptake versus exclusion can be used to discriminate dead cells, in which plasma membrane become permeable regardless of the mechanism of death, from live cells with intact membranes [25]. In later stage of apoptosis, cells split to form apoptotic bodies [23] that each apoptotic body contains only part of the original cell's DNA content when stained with PI, this population is known as the sub G1 population and characterized by DNA content of less than 2n chromosomes. In addition, apoptotic cells demonstrate specific morphological changes such as chromatin condensation and plasma membrane blebbing. Use of flurochrome, such as PI, that is capable of binding and labelling DNA makes it possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis, and subsequent identification of hyplodiploid cells [24]. In our study, the cellular degradation was clearly seen in treated A549 cells alters cytotoxic ability was confirmed using PI staining. As expected the control cells showed very few number of PI positive cells with red fluorescence, whereas the cells treated with chitosan showed a greater number of PI positive cells (IC50 and maximum concentration), which supports the inhibitory effect of chitosan which was extracted from crab shells. Similar experiments with PI staining was previously carried out as synthesis of alkaloid loaded chitosan nanoparticles for enhancing anticancer activity in A549 lung cancer cell line [26]. DNA fragmentation provides a method for separation of fragmented and intact DNA fractions was analyzed by agarose gel electrophoresis. In apoptotic cells, specific DNA cleavage becomes evident in electrophoresis analysis as a typical ladder pattern due to multiple DNA fragments. This protocol generally capable of providing good results and is a qualitative method of analysis. Similar kind of DNA patterns have been reported on urinary bladder cell line T24 [6]. Thereby it is possible that chitosan causes apoptosis in A549 cells which clearly indicated that the crab shell induced the apoptotic cell death in A549 cells. Our results shows that the control cells didn't show any ladder form whereas the treated A549 cells shows fragmentation in both IC50 (20 µg/ml) and maximum concentration (100 µg/ml). In conclusion, chitosan exhibits the inhibitory effect by inducing loss of cell viability, morphology change and DNA fragmentation in A549 cells due to the presence of free protonated amino groups on the polymer chain.

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