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ChemTech

International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN : 0974-4290
Vol.6, No.9, pp 4102-4107, September 2014

RTBCE 2014[12th August 2014]
Recent Trends in Biotechnology and Chemical Engineering

Antiproliferative effect of Prawn shells Chitosan on Lung cancer (A549) cell line

Leena Regi Saleth[1], Shanthinee Ravichandhren[1], Seethalakshmilllanchezian[2], Ramasamy Sivasamy[1]*

[1]Department of Biotechnology, Rajalakshmi Engineering College, Thandalam, Cheenai, (Tamilnadu) India
[2]Director, Lifeteck Research Centre,Vadapalani, Chennai, (Tamilnadu) India

***Corres.author: sivasamy.r@rajalakshmi.edu.in, Mobile: 9445695957**

Abstract: Chitosan, a deacetylated derivative of chitin is the second most available biopolymer composed of linear polysaccharide of alpha (1-4)-linked 2-deoxy beta-D-glucopyranose. Chitosan is a compound known for its uses, both as biomaterial and for its bioactivity. This current work aims to finding out anticancer potential Chitosan on A549 cells. Acid and salt treatment with subsequent alkali treatment of prawn shells, yielded chitosan which was consequently powdered and dried. It was investigated for its anti proliferative effect on A549- Lung cancer cell line. IC_{50} value of Chitosan on A549 cells was determined by MTT assay and anti proliferative property was further analyzed by using the Flow Cytometry. The results on MTT assay and Flow Cytometry readings assert the significant inhibitory action of Chitosan on A549cells.
Keywords: Chitosan, Anticancer potential, MTT Assay, Flow Cytometry.

Introduction

Cancer is one of the leading causes of death across the globe. The most common four types of cancer worldwide - lung, female breast, bowel (including anus) and prostate - account for more than four in ten (42%) of all new cases., out of which lung cancer ranks first out the most common four types[1.]There has been significant improvements in diagnosis and treatment of several cancers, particularly an increased survival rate for cancer patients who are diagnosed at the early stages. Regardless, in most cancer 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[2-5].

Chitosan is a deacetylated derivative of chitin, which is the second most abundant natural biopolymer, found on earth after cellulose [6]It is the major component of the exoskeletons of crustaceans such as crabs and shrimps. Chitosan is obtained by the deacetylation of chitin and it is a co-polymer of glucosamine and N-acetylglucosamine units linked by 1-4 glucosidic bonds. Chitosan has various biological activities including antimicrobial activity[7], antioxidant properties[8], immuno-enhancing effects [9] and antitumor activity [10]. These activities have attracted more attention, especially the antitumor and antibacterial activity. Chitosan is also applied in several drug delivery systems as it is non toxic[11]. The antitumor activity of Chitosan was reported in

early 1970s[12]. This activity was suggested mainly due to its cationic property exerted by the amino groups, and later it was accepted that the molecular weight also plays a major role for the antitumor activity[13]. This article demonstrates that the anti-proliferative of Chitosan on A549- Lung Cancer cell line using MIT assay and Flow cytometry.

Materials and methods

Cell line and culture

A549 cell line was obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in Minimal Essential Medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 5%CO_2 at 37 ºC.

Reagents

MEM was purchased from Hi Media Laboratories Fetal Bovine Serum (FBS) was purchased from Cistron laboratories Trypsin, methylthiazolyldiphenyl- tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). All of other chemicals and reagents were obtained from Sigma Aldrich Mumbai.

Preparation of Chitosan

The prawn shells obtained from the local market were first suspended in 4 % HCl at room temperature in the ratio of 1:14 (w/v) for 36 h, in order to demineralise the shells. The shells were washed with water to remove any acids and calcium chloride. 5 % NaOH was added to demineralised shells and left undisturbed for 24 h, with a solvent to solid ratio of 12:1 (v/w) (Figure- 1 &2).This step causes deproteinisation of the shells. Succeeding the incubation step, the sample was washed with running tap water thoroughly and was then sun dried. The product obtained was chitin. Deacetylation of chitin produces chitosan [14] for the chitosan preparation, adding 70 % NaOH solution in a solid to solvent ratio of 1:14 (w/v) to the sampleand was incubated at room temperature for 72 h. Uniform and frequent stirring of the incubated sample renders a homogenous mixture. The residue obtained after 72 h was washed with running tap water to neutrality and rinsed with deionized water. The washed product is then filtered, sun dried and finely ground to a powder. The powder was used to subsequent analysis.

Acid Alkali treatment of Chitosan

Figure 1:

Figure 2:

In Vitro assay for Anticancer activity (MTT assay) (Mosmann, 1983)

Before each assay, 90% confluent A549 Cells were dissociated,(1 × 10^5cells/well) plated in 24-well plates and incubated in 37^0C with 5% CO_2 condition.After cells were attached to the plate, the various concentrations of the samples were added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells .The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the

concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100

Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.

Cell cycle distribution

Cellular DNA content was determined by Flow Cytometric analysis of Propidium Iodide (PI) labeled cells. The A549 cells were grown until their morphology was distinct (exponential phase) and seeded at a density of 1ml: 1×10^6 cells per well. These cells were treated with 62.4 µg/ml, 31.2 µg/ml and 15.8 µg/ml concentrations of chitosan sample and incubated for 48hr. Following the treatment, the cells were collected by tripsinisation and fixed in ice cold 70% ethanol at -20ºC overnight. The cells (500µl) were collected and stained with fluorescent stain Propidium Iodide (100µl) [15]. A BD FACSJazz flow cytometer was used for flow cytometric analysis. The DNA content of 10,000 cells per analysis was monitored using BD FACSJazz system and the results were generated using its pertaining software.

Results and Discussion

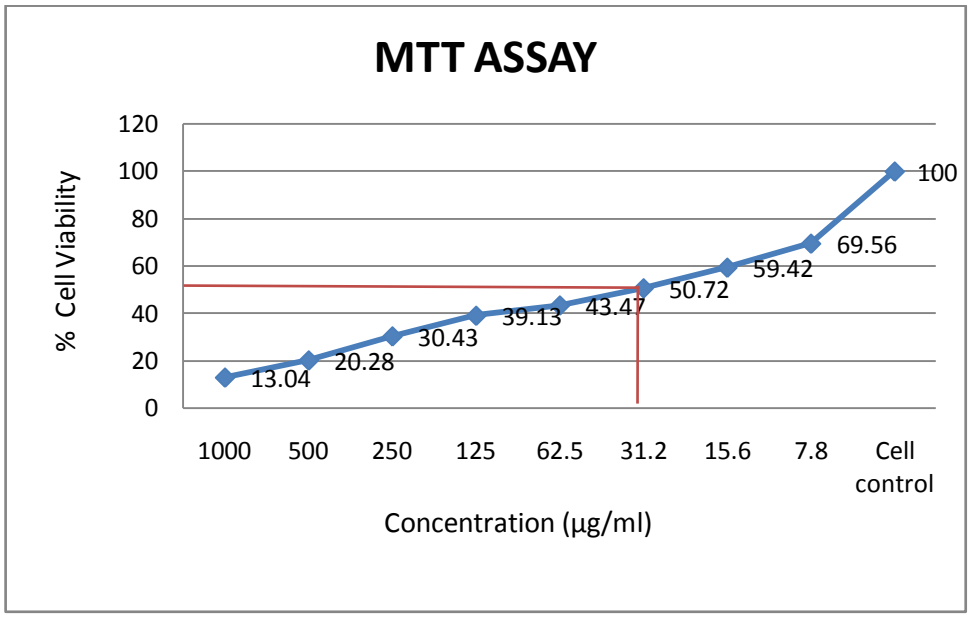
Effect of Chitosan samples on proliferation by MTT Assay

The effect of chitosan on A549 Lung Cancer cell lines was investigated over a range of concentrations (1000µg/ml-7.8µg/ml). Viable cells with active metabolism convert MTT into a purple colored Formazan product, with an absorbance maximum near 570 nm. Using UV Spectrophotometer, absorbance values for all concentrations were obtained. 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 color formation serves as a useful and convenient marker of only the viable cells. With higher incubation periods, the intensity of the purple colour increases. Thus MTT assay serves as a tool to check for active metabolism and viability of cells. At 31.2 µg/ml of chitosan, 50% of cells are viable, which indicates IC_{50} activity of chitosan.

Cell cycle Distribution- Flow Cytometry

To investigate the cellular mechanism of chitosan induced cell growth inhibition, Flow Cytometry was used to detect cell cycle distribution and apoptosis induced cells. The fig: 2 reveal that maximum number of cells have undergone apoptosis, indicated by P1. P3 indicates Cells in their G_0 and G_1 phase. According to the reading, 58% of the cells are in their G_0 and G_1 cell cycle stages on 31.2 µg/ml of chitosan treated A549 cells.

Figure 3: Graphical representation of MTT Assay results. 50% cell viability is obtained at 31.2µg/ml of chitosan dosage. This value corresponds to the IC_{50} concentration of Chitosan.



Anti Proliferative effect of Chitosan *on A549 C*ell line

Figure 4: Normal A549 cell line

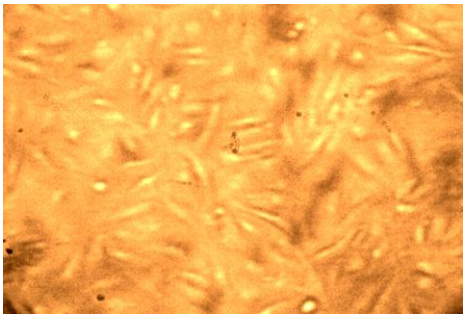


Figure 5: Toxicity-62.5µg/ml

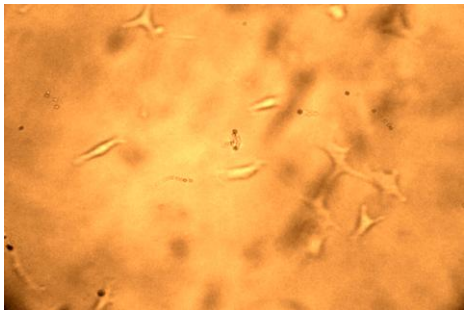


Figure 6: Toxicity-31.2µg/ml

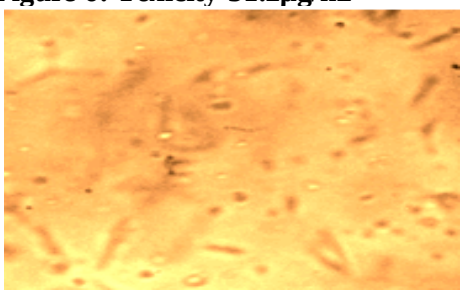
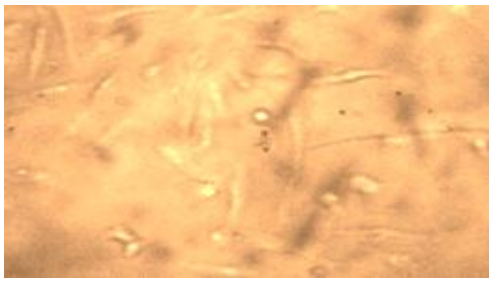


Figure 7: Toxicity-15.6µg/ml

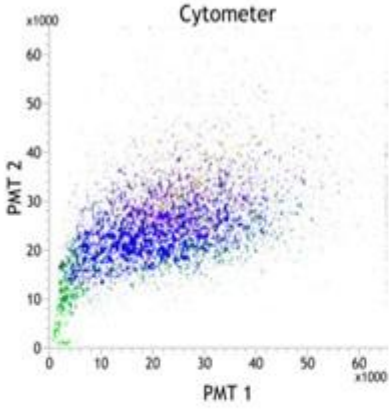


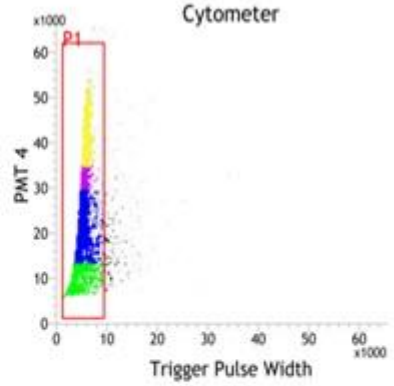
Discussion

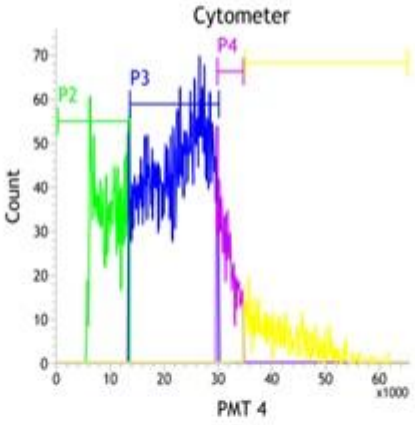
The aim of this work was to evaluate the inhibitory effect of chitosan on A549 cell lines growth and to study its activity as an Antiproliferative agent *in vitro*. MTT Assay was performed to check for viability of cells at different concentrations of chitosan sample. Based on the results IC 50 value was obtained in the concentration of 31.2 µg/ml indicates that chitosan have the concentration dependent Antiproliferative effect on A549 cells (Graph-1; Table 1). The morphology difference of the A549 was also observed based on the concentration ranges from 1000µg/ml-7.8µg/ml (Figure 3-6) A subsequent study, to investigate the Antiproliferative effect of chitosan involves flow cytometric readings. The results reveal varied action of chitosan in different phases of the cell cycle. The phases of cell cycle can be classified into the G_0-G_1, S, G_2 and M phase. Flow Cytometry fluorescent reagents target the DNA of cells. The G_0 and G_1 phase cells have double stranded DNA, S phase is when the DNA begins to replicate,G_2 phase is characterized by two daughter cells and therefore duplicated chromosomes and M phase is characterized by double stranded DNA, each into its new daughter cell. Chitosan induces apoptosis in A549 cancer cell lines indicated by P3 and P4 values. On observing the expression pattern of the BD FACSJazz -flow cytometer, Chitosan has maximum Antiproliferative effect during the P3 region of the graph, which corresponds to G_0-G_1 phase which is discussed in (Figure- 7). Therefore we assuming that it may bind to double stranded DNA and prevent to replication process begins.

Table 1: For serially diluted concentrations of Chitosan (initially 1000 µg/ml to 7.8µg/ml) different OD values were obtained. The corresponding Viability percentage was calculated and tabulated.

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.09	13.04
2	500	1:1	0.14	20.28
3	250	1:2	0.21	30.43
4	125	1:4	0.27	39.13
5	62.5	1:8	0.33	43.47
6	31.2	1:16	0.35	50.72
7	15.6	1:32	0.41	59.42
8	7.8	1:64	0.48	69.56
9	Cell control	-	0.69	100







Statistics: Cytometer

Populations	Events	% Total	% Parent	PMT 4 Mean	Time 1 Mean
All Events	10,000	100.00%	####	22,066	32,415
P1	9,620	96.20%	96.20%	22,100	32,391
P2	2,178	21.78%	22.64%	9,638	32,929
P3	5,845	58.45%	60.76%	22,243	32,324
P4	961	9.61%	9.99%	31,768	32,295
P5	876	8.76%	9.11%	42,011	32,108

Figure 8: BD FACSBazz Flow cytometry readings:

Conclusion

The current study clearly indicates that chitosan has Antiproliferative effect on A549 Lung Cancer cell lines. DNA fragmentation assay could establish the reason for cytotoxicity is due to the induction of apoptosis (programmed cell death) or necrosis. The future study may be extended to other cell lines and to establish mechanism of action.

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