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## Anti Proliferative Effect of Fungal Polysaccharide on HeLa and MCF-7 Cell Lines

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### ABSTRACT

Chitosan (N-acetyl D- glucosamine) is a natural polysaccharide present in crustacean shells, some fungi and yeast. It is a versatile polysaccharide irrespective of its sources. In the present research work, fungal chitosan is characterized for its anti proliferative effect on HeLa and MCF-7 tumour cell lines by MTT assay. The results showed that GI<sub>50</sub> of fungal chitosan was found to be 75µg/ml and 80µg/ml on HeLa and MCF-7 cells respectively.

**Keywords:** Antiproliferative, Fungal chitosan, HeLa cell lines, MCF-7 cell lines, MTT assay

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## INTRODUCTION

Cancer is due to the breakdown of the mechanism that controls the growth and proliferation of cells and is one among the principal causes of morbidity and death worldwide. According to a study conducted by WHO, in India, nearly 1 million patients are tormented by this disease and each year nearly 700,000 people die of cancer<sup>1</sup>. Among women, breast and cervical cancers are the foremost cause of mortality<sup>2</sup>. In recent era, the research for more efficient choice in the treatment of cancer has mobilized researchers from host of diverse areas. Sensible results have obtained from the use of substances formed by microbes, including fungi<sup>3</sup>.

Chitosan, a natural polysaccharide that exists in crustacean shells and also a cell wall component of some fungi is well-known for its multi functional biological activities<sup>4</sup>. The anti-tumour or anti proliferative activity of crustacean chitosan has been illustrious since 1986<sup>5</sup>, and several other mechanisms are projected. These embody the regulation of immunity<sup>6</sup>, the direct killing of tumor cells, or inflicting tumor cell death and inhibit tumor angiogenesis<sup>7</sup>.

In the present research work, fungal chitosan was screened for its antiproliferative effect on human epithelial cervical cancer (HeLa) and human breast adenocarcinoma (MCF-7) cell lines.

## MATERIALS AND METHOD

### **Microbial culture and its maintenance:**

The microorganism used in this study was *Mucor rouxii* MTCC 386 obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh, India. The fungus was maintained at 4°C on PDF slant and transferred on a monthly basis.

### **Cultivation conditions:**

The standard inoculum was prepared by the spores of *Mucor rouxii* MTCC 386, harvested from 7 day petriplate culture on PDA medium at 28°C. The spore suspension was prepared and adjusted to 10<sup>8</sup> spores/mL using a hemacytometer for counting<sup>8</sup>.

The fermentation broth consisted (g/L) of glucose (20), peptone (10), yeast extract (1), ammonium sulphate (5), dipotassium hydrogen orthophosphate (1), sodium chloride (1), magnesium sulphate 7-hydrate (5), and calcium chloride 2-hydrate (0.1). The pH of the YPD broth was adjusted to 4.5 using dilute HCl and autoclaved at 121°C for 15 min<sup>9</sup>.

For submerged cultivation 10% v/v of the standard inocula was inoculated to 300 mL of fermentation broth in the seven 500 mL capacity Erlenmeyer flasks and incubated at 28°C for 7 days in a rotary shaker at 200 rpm. The experiment was carried out in triplicate and the results are expressed as mean ± S.D.

**Chitosan isolation:**

After cultivation of culture in fermentation broth, the fungal mycelia was harvested for every 24 hours by filtration (No.1; Whatman) washed with distilled water until a clear filtrate was obtained and then dried at 60°C to a constant weight.

The chitosan isolation was carried out by suspending the fungal mycelia with 1M NaOH solution (1:30 w/v) and autoclaved at 121°C for 15 min. The alkali insoluble fractions were collected after centrifugation at 12,000x g for 15 min. The residues were further extracted using 10% acetic acid (1:40% w/v) at 60°C for 6 hours. The separation of acid soluble chitosan from acid insoluble chitin was done by filtration by no.1 Whatman filter paper. The chitosan was precipitated from the extract by adjusting the pH to 10 with 4MNaOH. The chitosan was finally washed with distilled water, 95%ethanol, acetone and dried at 60°C.

**Chitosan characterization:**

The IR spectra of the KBr discs containing chitosan and a commercial chitosan (Cochin Fisheries, Cochin, Kerala, India) were measured from 4000cm<sup>-1</sup> to 400cm<sup>-1</sup> with a JASCO FT-IR spectrophotometer.

**Cell Lines and Culture Conditions:**

HeLa cell line and MCF-7cell line were obtained from National Centre for Cell Sciences, Pune (NCCS) and were maintained as stocks in RPMI-1640 media( Hi media, Mumbai, India) supplemented with 10% foetal bovine serum (FBS-Gibco's, USA),100U/ml of penicillin-G sodium,50µg/ml of streptomycin and 2µg/ml of amphotericin-B (Himedia, Mumbai,India) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C and the culture medium was changed twice a week<sup>10,11</sup>.

**Trypsinization:**

The media was aspirated from each flask, being sure to change pipettes between cell lines to prevent cross contamination. Monolayer was rinsed with 5-10 ml PBS to remove traces of serum and the rinsing solution was aspirated. 1 ml of 0.25 % trypsin-EDTA in Hank's Balanced Salt Solution (HBSS)was added to each flask and spread evenly over cell monolayer and depending on cell type, the flask was either placed in hood or in incubator for 2-5 mins. The flask was gently 'tapped' for dislodging the cells. Then the cells were resuspended in 8 ml of the medium containing serum to stop the action of the trypsin. Gentle pipetting was carried out up and down for breaking up the clumps. Cell suspension was transferred to a properly labelled 15 ml centrifuge tube. The tubes were centrifuged at 1000 rpm for 5 mins. The pellet was resuspended in 5-10 ml of medium depending upon the size of the pellet or cell number.

**MTT assay:**

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) measures the metabolic activity of the viable cells. MTT measures the metabolic activity of the viable cells. The assay is non-radioactive and can be performed entirely in a microtiterplate (MTP). It is suitable for measuring cell proliferation, cell viability or cytotoxicity. The reaction between MTT and 'mitochondrial dehydrogenase' produces water-insoluble formazan salt. This method involves culturing the cells in a 96-well microtiterplate, and then incubating them with MTT solution for approximately 2 hours. During incubation period, viable cells convert MTT to a water-insoluble formazan dye. The formazan dye in the MTP is solubilised and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number. This is applicable for adherent cells cultured in MTP.<sup>12</sup>

0.1 ml of the cell suspension (containing  $5 \times 10^5$  cells /100  $\mu$ l) and 0.1 ml of the test solution (6.125 -100  $\mu$ g/ml) in DMSO such that the final concentration of DMSO in media is less than 1 %) were added to the 96 well plates and kept in carbon dioxide incubator with 5 % CO<sub>2</sub>, at 37°C for 72 hours. Blank contains only cell suspension and control wells contain 1 % DMSO and cell suspension. After 72 hours, 20  $\mu$ l of MTT was added and kept in carbon dioxide incubator for 2 hours followed by 80  $\mu$ l of lysis buffer (15 % SLS in 1:1 DMF and water). The plate was covered with aluminium foil to protect it from light. Then the 96 well plates are kept in rotary shaker for 8 hours. After 8 hours, the 96 well plates were processed on ELISA reader for absorption at 570 nm. The readings were averaged and viability of the test samples was compared with DMSO control. The percentage growth inhibition was calculated using the following formula

% Growth Inhibition =  $100 - (\text{Mean OD of Individual Test Group} / \text{Mean OD of Control Group} \times 100)$

**RESULTS AND DISCUSSION**

The results for cell growth inhibition by fungal chitosan, a polysaccharide from *Mucor rouxii* MTCC 386 against HeLa and MCF-7 cell lines for various concentrations (25-400 $\mu$ g/ml) are shown in Table 1. As the concentration increases there is an increase in the cell growth inhibition. The results obtained showed that fungal chitosan had a very prominent anticancer activity.

**Table 1 Anti proliferative effect of fungal chitosan on HeLa and MCF-7 cell lines**

Fungal Chitosan	Conc (µg/ml)	O.D at 570nm	%Growth Inhibition		GI <sub>50</sub> (µg/ml)	
			HeLa	MCF-7	HeLa	MCF-7
	25	0.36±0.03	20	14	75	80
	50	0.27±0.03	40	36		
	100	0.18±0.04	58	56		
	200	0.10±0.04	74	78		
	400	0.05±0.03	92	90		

n=3, Mean±S.D

## CONCLUSION

According to the results obtained, fungal chitosan, a polysaccharide may decrease the viability of HeLa and MCF-7 tumor cells. This may be due to activation of macrophages, by the production of cytokines according to Yasouori Maeda and Yoshiyuki Kimura, 2004<sup>13</sup>. Nevertheless, further in vivo experiments are needed to determine tumor-specific effects and optimum doses of fungal chitosan, which might provide deeper insights to evaluate long-term effects of fungal chitosan. Taken into account its antiproliferative effects, there is possibility that this fungal polysaccharide might serve as a promising material for a safer therapeutic option in the treatment of cervical and breast cancer.

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