

POLYSACCHARIDE PST001 ISOLATED FROM THE SEED KERNEL OF *TAMARINDUS INDICA* INDUCES APOPTOSIS IN MURINE CANCER CELLS

S.R ARAVIND¹, MANU M JOSEPH¹, SHEEJA VARGHESE¹, PRABHA BALARAM^{1*} AND T.T. SREELEKHA¹

¹Laboratory of Biopharmaceuticals, Division of Cancer Research, Regional Cancer Centre, Trivandrum, Kerala, India

*Present address: Health Campus, INFORMM, Universiti Sains Malaysia

ABSTRACT

The chemotherapeutic agents long used in oncologic treatment produce deleterious side effects. Polysaccharides isolated from plants and mushrooms were widely studied to find an additional agent for cancer therapy. Present study evaluated the in vitro anti-tumor effect of a polysaccharide from seed kernel of *Tamarindus indica* (PST001) on murine cancer cells DLA and EAC. PST001 has antitumor activity and could inhibit the cell proliferation significantly. DLA cells showed an IC₅₀ value of 91.14 µg/ml for PST001 after 48 hours of treatment, whereas EAC cells showed no IC₅₀ value but an inhibition of 46.45% was observed. DLA and EAC cells treated with PST001 showed typical apoptotic morphology when stained with Acridine Orange/ Ethidium bromide. The results of flow cytometry analysis for Caspase-3 activation and Annexin- V staining showed that PST001 could induce apoptosis. Cell cycle analysis also supports apoptosis, where as distribution of cells in the different cell cycle phases are normal in EAC cells and a slight change in DLA cells. DNA isolated from DLA cells treated with 100 µg/ml PST001 showed DNA ladder specific for apoptosis on agarose gel electrophoresis. The above results suggest that PST001 could suppress DLA and EAC cell growth and reduce the cell survival by inducing apoptosis of tumor cells.

Key words: Polysaccharide, Anticancer Activity, *Tamarindus indica*, Apoptosis, Flow cytometry

INTRODUCTION

Chemotherapy is one of the major therapeutic modalities for cancer. Conventional chemotherapeutic agents are highly toxic to normal tissues and are not successful for complete remission of tumors and to prevent metastasis. During the past three decades, many polysaccharides and polysaccharide protein complexes have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants with medicinal properties. The biological activity of the polysaccharides has attracted more attention recently in the biochemical and medical areas because of their

immunomodulatory and antitumor effects. The ability of bioactive polysaccharides and polysaccharide bound proteins to modulate so many important immune functions may be due to the structural diversity and variability of these macromolecules. A successful approach in cancer therapy is to trigger apoptosis but it is often complicated by development of multi drug resistance (MDR) mechanisms. Polysaccharides are able to down regulate P-glyco protein, and reverse MDR (Wei et al. 2008).

Natural products have been the mainstay of cancer chemotherapy for the past 30 years and are likely to provide many of the lead structures, and these will be used as templates for the construction of novel compounds with enhanced biological properties (John Mann. 2002). Tamarind is the most common and commercially important large evergreen tree that is grown abundantly in the dry tracks of Central and South Indian states, and also in other South East Asian countries. Tamarind products are widely used in Asia and some parts of Africa. In Asian countries, especially India, tamarind is mainly cultivated and used as an acidulant, gelling and acidifying agent (Kulkarni G. 2005). Tamarind gum along with xanthan gum hydroxypropyl cellulose is used for nasal muco adhesion studies in powder formulation. Tamarind gum is also used as a bio-adhesive tablet (Nakamura et al. 1996). In our earlier studies we have reported the immunomodulatory and antiproliferative activities of the polysaccharide (PST001) isolated from the seed kernel of Ti (Sreelekha et al. 1993, Aravind et al. 2009). The present study investigated the apoptotic effect of PST001 on murine cancer cells DLA and EAC by assessing cell proliferation, morphological changes by Acridine Orange/ Ethidium bromide staining, DNA laddering, Caspase-3 activation and Annexin V status. The distribution of cells in various cell cycle phases was evaluated by flow cytometry.

1. MATERIALS AND METHODS

1.1 Chemicals

Dulbecco Modified Eagle Medium (DMEM) were purchased from Gibco™ Invitrogen Corporation, USA; antibiotics (Pencillin, Streptomycin and Amphotericin) were obtained from Lonza India PVT Ltd. Mumbai, India; fetal calf serum (FCS) was from Pan Biotech GmbH; MTT from Sigma-Aldrich Co., St. Louis, MO, USA; Caspase-3 and Annexin V were purchased from BD Biosciences, San Jose, CA, USA.

1.2 Isolation and purification of the polysaccharide PST001

Seed kernel of *Tamarindus indica* was collected and shade dried. Hundred grams of powdered seed kernel was extracted with petroleum ether (BP 60⁰–80⁰ C) at room temperature for 72 hours with occasional

stirring in order to remove any fat present in it. The dried material was extracted with distilled water using a soxhlet apparatus. The aqueous extract was centrifuged at 20,000 x g for 15 minutes and polysaccharide was precipitated with ethanol with constant stirring and kept overnight at 4⁰C to complete the precipitation of polysaccharide. The precipitate was pelleted by centrifugation at 20,000 x g for 15 minutes and the residue was dissolved in distilled water. It was treated with equal volume of chloroform in a separating funnel and the denatured protein formed as a gel at the water chloroform interphase was removed and the process was repeated till the interphase became clear. This was followed by dialysis against distilled water for 48 hours at several changes of water. The contents of dialysis bags were collected, concentrated and treated with ethanol to precipitate the polysaccharide. Precipitate was collected by centrifugation at 20,000 x g and the residue was dissolved in distilled water (100ml) and dialyzed against distilled water, concentrated and lyophilized. This was purified by gel filtration chromatography using Sephadex G-200 (Pharmacia Fine Chemicals), 0.001M PBS was used as the eluent, finally lyophilized and stored at -20⁰C.

1.3 Cell lines

Murine cancer cell lines DLA (Daltons Lymphoma Ascites) and EAC (Ehrlich Ascites Carcinoma) obtained under the courtesy of Amala Cancer Research Centre, Thrissur, Kerala, India, and were maintained by weekly intra-peritoneal inoculation of 10⁶ cells/mouse.

1.4 Determination of Cytotoxic Activity of PST001 in culture by MTT Assay

The effect of PST001 on the proliferative capacity of the cancer cells was determined using MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously (Alley et al. 1988). Briefly, cells were seeded (5000 cells/well) in 96-well, flat-bottom titer plates along with different concentrations of PST001 (0.001-1000 µg/mL) and incubated for 24, 48 and 72 hours at 37⁰C in 5% CO₂ atmosphere. After completion of incubation the medium was removed and wells were washed with PBS, 100 µl of the working MTT dye in DMEM media was added and incubated for 2 hours. MTT

lysis buffer (100 µl) was added and incubation continued for 4hrs. The absorbance was measured at 570nm and the proliferation rate (PR) was calculated using the formulae:

$$PR = \frac{\text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

Cytotoxicity of the drug PST001 on the cells was calculated as cell growth inhibition rate (IR).

$$IR = 100 - PR.$$

1.5 Acridine Orange - Ethidium Bromide staining

Acridine orange/ethidium bromide dual staining is a commonly used method to detect apoptosis based on the differential uptake of the two fluorescent DNA binding dyes to determine the viable and non viable cells in a population. Cells were seeded in a 96-well plate and polysaccharide in different concentrations were added and incubated for 24, 48 and 72hr at 37°C in a 5% CO₂ incubator (McGahon et al. 1995). After incubation, plates were taken, media was discarded and 25µl Acridine orange (7.5µg) / Ethidium bromide (25µg) stain was added, mixed well and observed under fluorescent microscope.

1.6 Assessment of apoptosis by DNA laddering

PST001 induced DNA fragmentation was determined using DNA laddering (Ramnath et al. 2007). The DLA and EAC cells incubated with different concentrations of PST001(100 & 200µg/ml) for 24, 48 and 72 hr were washed twice with Tris borate saline (TBS), centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded. The pellet was washed and re-suspended in saline EDTA (SE) buffer and 10 µl of proteinase K (10 mg /ml), 100 µl of 10% SDS and 100 µl of 5M NaCl. Saturated phenol (pH 7.8) was added in equal volume, mixed and centrifuged at 4000 rpm for 15 minutes. The aqueous phase was collected in fresh tubes, a mixture of saturated phenol : chloroform : isoamyl alcohol (25 : 24 : 1) was added in equal volume, mixed and centrifuged at 4000 rpm for 15 minutes. Repeated the above step with an equal volume of chloroform: isoamyl alcohol (24:1), mixed and centrifuged. Precipitated DNA with 1/10th volume of 3M sodium acetate (pH 5.5). Equal volume of isopropanol was added and the precipitated DNA was pooled out on clean micropipette tip, washed with 70% ethanol, air dried and re-suspended in TE buffer (pH 8.0). Purity

and quantification of the DNA was carried out by monitoring the ODs at 260 and 280 nm. Ten microliter (50 ng) of each samples were resolved at 100V for 2 h on a 2% agarose gel containing 0.5 mg/ml ethidium bromide. The resulting DNA fragmentation was visualized under Gel documentation system (Bio Rad).

1.7 Caspase-3 Activity

Caspase-3 activity was measured by using FITC Active Caspase-3 Apoptosis kit, BD Pharmingen. Control and polysaccharide treated cells were washed twice with cold PBS and re-suspended in cytofix or cytoperm solution at a concentration of 1x10⁶ cells / 0.5 ml and incubated for 20 minutes on ice, pelleted, aspirated and discarded cytofix or cytoperm solution. It was then washed twice with perm / wash buffer at a volume of 0.5 ml buffer per 1x10⁶ cells at room temperature. Total amount of experimental samples (tests) was determined and calculated the amount of perm / wash buffer and antibody so that each test will have 100µl perm/ wash buffer and 20µl antibody. Cells were re-suspended in the above calculated perm/ wash buffer with antibody and incubated for 30 minute at room temperature. Each test was washed in 1 ml perm / wash buffer then re-suspended the cells in 0.5 ml perm / wash buffer and analyzed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA).

1.8 Detection of Apoptotic cells with Annexin V assay.

PST001 induced apoptosis on DLA and EAC cells was determined by flow cytometry using Annexin V conjugated FITC apoptotic detection kit (BD Biosciences, San Jose, CA). Briefly, after treatment with PST001 at various time periods (24, 48 and 72 hours) and concentrations (100 & 200µg/ml) cells were washed twice with cold PBS and then re-suspended the cells in 1x binding buffer at a concentration of 1x 10⁶ cells / ml and 1x 10⁵ cells (100µl) were transferred to a 5ml BD Falcon tubes and 5µl of Annexin V FITC and 5µl of Propidium iodide (PI) were added to each tube. Gently vortex the cells and incubated for 15 minutes at room temperature (25°C) in the dark. Four hundred microlitre (400µl) of 1x binding buffer was added to each tube and analyzed by flow cytometry equipped with CellQuest Pro software (FACS Calibur, BD

Biosciences, San Jose, CA). The early apoptotic cells stained with FITC are represented in the lower right quadrant of the FACS histogram, and the late apoptotic cells stained with both FITC and PI are represented in the upper right quadrant of the histogram.

1.9 Cell Cycle Analysis

DLA and EAC cells were seeded in tissue culture flask (2×10^6 cells) and treated with and without PST001 at various concentrations (100 & 200 $\mu\text{g/ml}$) for 24, 48 and 72 hours. Cells were collected by centrifugation at 3000 rpm and washed with PBS. Resuspended and fixed in 70% ice-cold ethanol for 30-60 minutes and treated with 5 μl of RNase A (10mg/ml) at 37°C for 30-50 minutes. Cells were kept on ice for 10 minutes and 10 μl of 1mg/ml PI was added and incubated for dark until analysis. The cell cycle distribution of the cells of each sample was determined using a FACS Calibur instrument (BD Biosciences, San Jose, CA) equipped with Cell Quest Pro software. Modfit LT cell cycle analysis software was used to determine the percentage of cells in the different phases of the cell cycle.

1.10 Statistical analysis

The results were expressed as the mean (SD). The differences between control/PST001 treated groups were evaluated for statistical significance by one way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison tests. $P < 0.05$ was considered statistically significant.

2. RESULTS

2.1 PST001 inhibited proliferation of DLA and EAC cells

The treatment of DLA cells with PST001 (0.001 - 1000 $\mu\text{g/ml}$) resulted in a significant reduction in cell proliferation as assessed by MTT assay ranging from $20.57 \pm 1.01\%$ to $29.29 \pm 1.91\%$ after 24 hours, $21.7 \pm 0.91\%$ to $54.33 \pm 1.6\%$ after 48 hours and $18.04 \pm 1.38\%$ to $45.36 \pm 1.8\%$ after 72 hours of incubation with an IC_{50} value of 91.14 $\mu\text{g/ml}$ (Figure 1a) after 48 hours. Similar dose and time dependent effects were obtained on treatment of EAC cells with PST001 except that no IC_{50} value was obtained for EAC but an inhibition of $46.45 \pm 1\%$ was obtained for 100 $\mu\text{g/ml}$ concentration after 72 hours of treatment (Figure 1 b). These data suggest that PST001 does have cytotoxic effect on murine cancer cells. Cyclophosphamide for DLA and 5-FU for EAC were included as positive controls for comparison (Figures 1c & 1d).

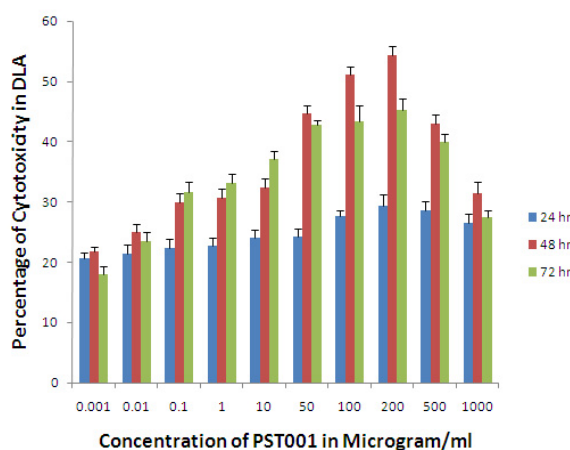


Fig. 1a

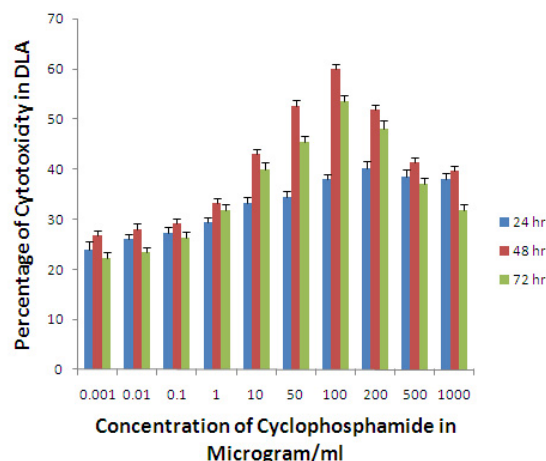


Fig. 1c

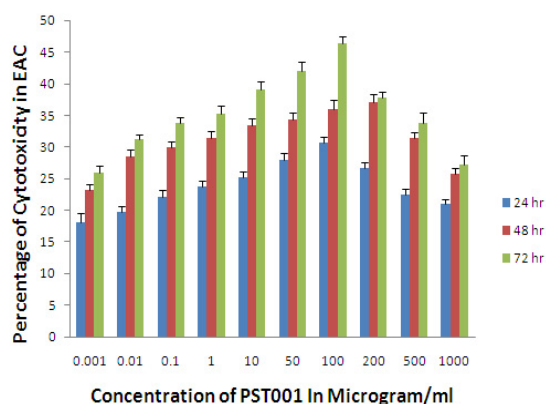


Fig. 1b

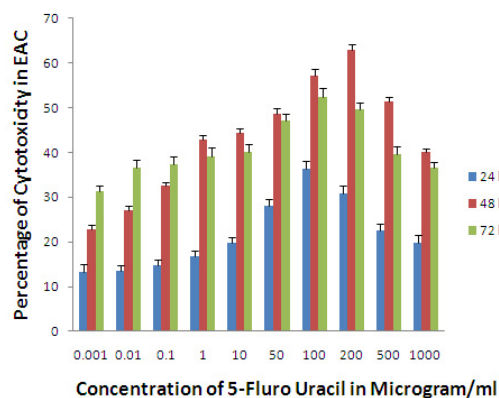


Fig. 1d

Figures 1a & 1b: Cytotoxicity profile of PST001 on DLA and EAC cell lines as measured by MTT assay.
Figures 1c & 1d: Cytotoxicity profile of Cyclophosphamide on DLA and 5-FU on EAC cell lines as measured by MTT assay.

2.2 Effect of PST001 on cell morphology

The morphological examination of DLA and EAC cells were assessed using phase contrast microscope. Significant decrease in the number of DLA and EAC cells treated with PST001 for 48 hours was observed compared to control group. Cells treated with PST001 showed significant morphological changes

(Figures 2 a-f) of apoptosis like blebbing formation, nuclear condensation and apoptotic bodies, which are time and concentration dependent. Nuclear shrinkage and condensation are hallmark of apoptosis, were evident in PST001 treated DLA and EAC cells.

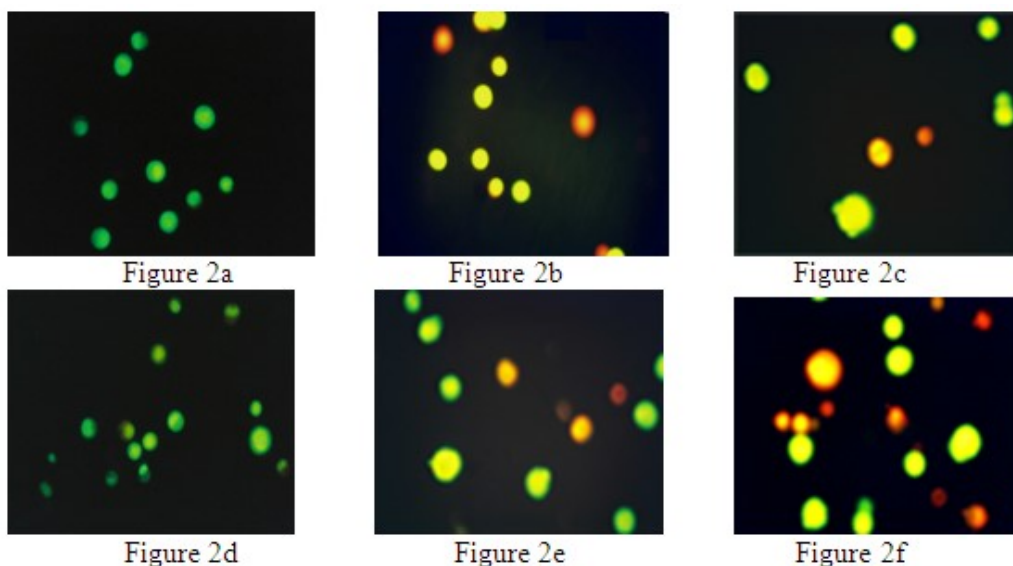


Figure 2: Effects of PST001 on the morphology of DLA and EAC cells were assayed by Acridine Orange/ ethidium bromide staining. After treatment with PST001 for 48 h, DLA and EAC cells were stained with Acridine Orange/ ethidium bromide stain and were observed under a fluorescence microscope. (a) DLA Control; (b) 100µg/ml PST001 on DLA; (c) 200µg/ml PST001 on DLA; (d) EAC control; (e) 100µg/ml PST001 on EAC; (f) 200µg/ml PST001 on EAC.

2.3 Validation of apoptosis by DNA laddering

DNA fragmentation on treatment with PST001 was examined using agarose gel electrophoresis and the results are shown in figure 3. No DNA ladder was observed with PST001 or vehicle treated DLA and

EAC cells except in DLA cells treated with 100 μ g/ml concentration of PST001 after 48 hours. However, it is evident that the exposure of PST001 to DLA cells might cause DNA fragmentation characteristic of apoptosis (figure 3).

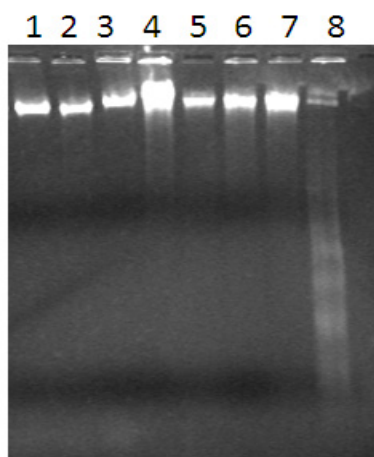


Figure 3: Effects of PST001 on DNA Laddering.

Lane 1- DLA control 24 hr, Lane 2 – PST001 1 μ g/ml 24 hr, Lane 3- PST001 10 μ g/ml 24 hr, Lane 4- PST001 100 μ g/ml 24 hr, Lane 5 - DLA control 48 hr, Lane 6- PST001 1 μ g/ml 48 hr, Lane 7 - PST001 10 μ g/ml 48 hr, Lane 8 - PST001 100 μ g/ml 48 hr.

2.4 Detection of caspase-3 activity by flow cytometry

Caspase-3 enzyme activity stimulated by PST001 was measured using flow cytometry. Caspase-3 activation was time and concentration dependent and varies in DLA and EAC cell, the activity was more in DLA cells than in EAC cells. At a concentration of 100 μ g/ml PST001, Caspase-3 activation was observed to be increased to 7.21 ± 1.65 from 3.68 ± 1 ($P < 0.01$) and to $52.93 \pm 2.47\%$ from $42.78 \pm$

3.43% ($P < 0.001$) in control cells (Figure 4 a) after 48 hours of treatment. However, the activity of caspase-3 was shown to increase even in the DLA control cells following incubation for 48 hours. In EAC, activation of caspase-3 was observed to be $9.47 \pm 0.8\%$ for 100 μ g/ml concentration of PST001 and $5.54 \pm 0.43\%$ ($P < 0.001$) for control cells (Figure 4b) after 48 hours of incubation. At 24 hours of treatment the caspase-3 activation was not significantly increased.

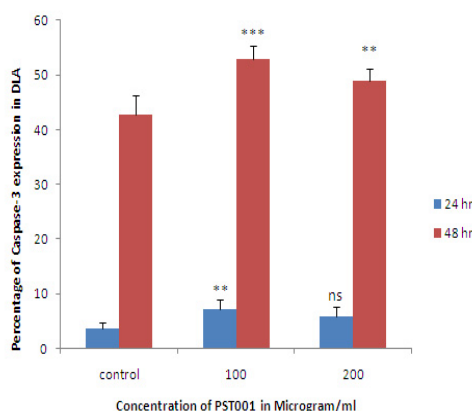


Fig. 4a

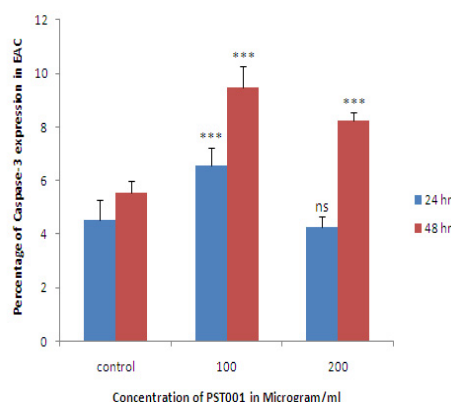


Fig. 4b

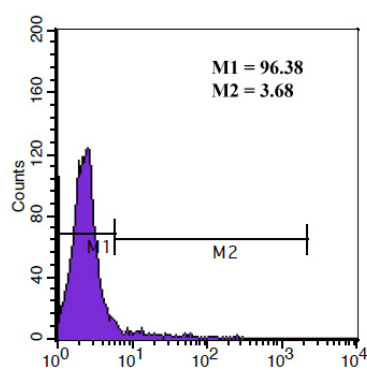


Fig. 4 c

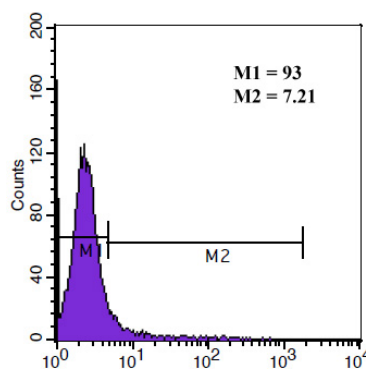


Fig. 4d

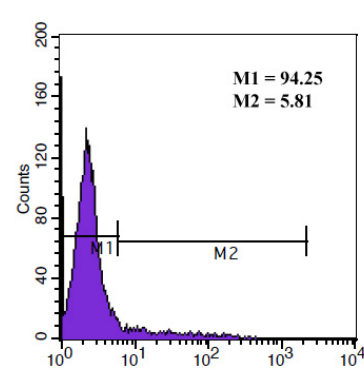


Fig. 4e

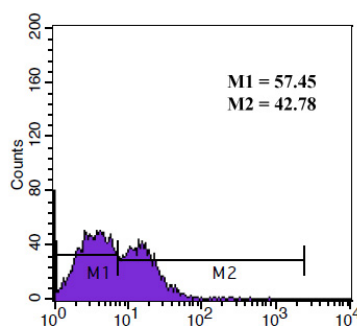


Fig. 4 f

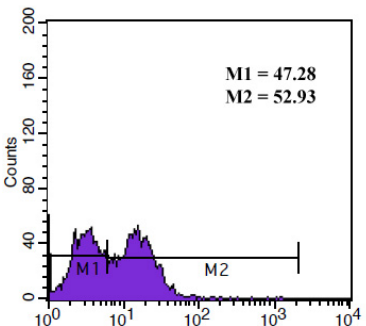


Fig. 4g

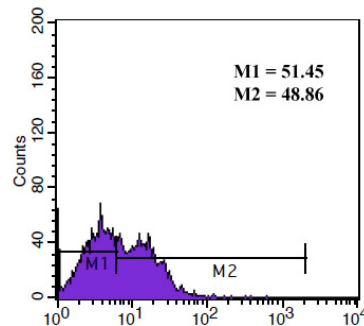


Fig. 4h

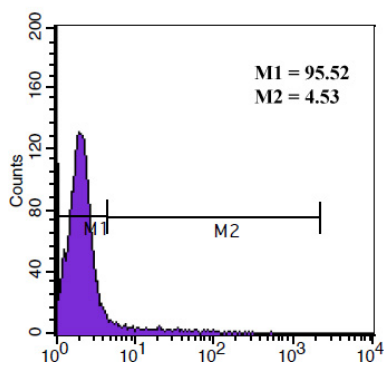


Fig. 4 i

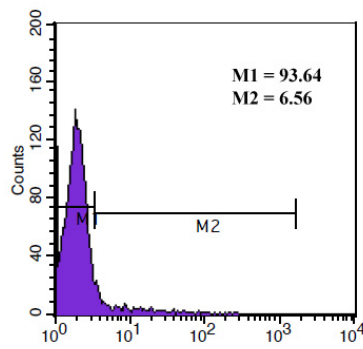


Fig. 4j

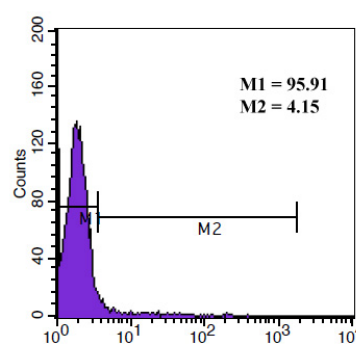


Fig. 4k

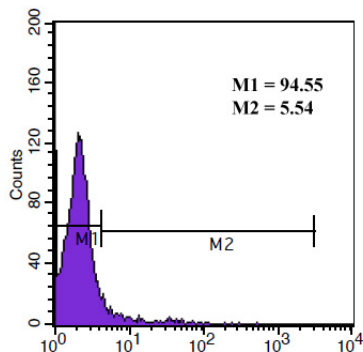


Fig. 4 l

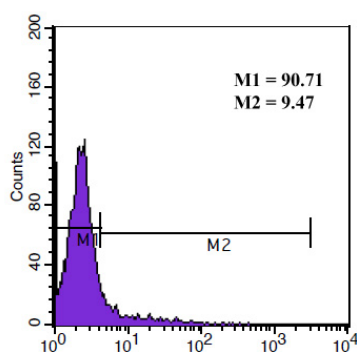


Fig. 4m

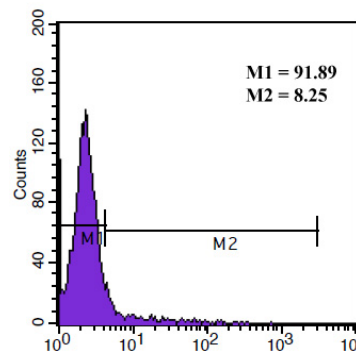


Fig. 4n

Figures 4a & 4b: Histogram for Caspase-3 activity of PST001 in DLA and EAC cells. Figures 4c-n: Images of Caspase-3 on DLA and EAC by flow cytometry. (c) DLA control 24 hr ; (d) 100µg/ml PST001 on DLA 24hr ; (e) 200µg/ml PST001 on DLA 24 hr (f) DLA control 48 hr ; (g) 100µg/ml PST001 on DLA 48hr ; (h) 200µg/ml PST001 on DLA 48 hr; (i) EAC control 24 hr ; (j) 100µg/ml PST001 on EAC 24hr ; (k) 200µg/ml PST001 on EAC 24hr ; (l) EAC control 48 hr ; (m) 100µg/ml PST001 on EAC 48hr ; (n) 200µg/ml PST001 on EAC 48 hr.

2.5 Evaluation of Annexin V staining by flow cytometry

To determine whether the growth inhibition of DLA and EAC cells on treatment with PST001 was associated with apoptosis, these cells were evaluated for Annexin- V staining by flow cytometry. Cells were counted as late or early apoptotic cells, which are shown in the upper and lower right quadrant of the histogram respectively (Figure 5). After 24 hours of treatment, PST001 induced apoptosis on DLA and EAC cells and was significantly increased in DLA cells from $9 \pm 1\%$ in untreated cells to $39.98 \pm 1.8\%$

in $100\mu\text{g/ml}$ ($P < 0.001$) concentration of PST001 treated cells and $8.27 \pm 0.65\%$ in control to $15.24 \pm 0.8\%$ ($P < 0.001$) in PST001 treated EAC cells (Table 1a&b). Annexin V status was also higher for $100\mu\text{g/ml}$ concentration of PST001 for both the cancer cells as in the case of caspase-3. However, the necrotic cells (upper left) stained with PI alone was not appreciably changed after exposure to PST001 (Figure 5). These results suggested that apoptosis not necrosis contributed to the PST001 induced death of DLA and EAC cells.

Table 1a: Annexin V assay of PST001 in DLA cells by flow cytometry.

Concentration in $\mu\text{g/ml}$	24 hr DLA	48hr DLA
Control	9 ± 1	60.04 ± 2.43
100	39.98 ± 1.8 ***	63.42 ± 2.65 ns
200	14.06 ± 1.25 ***	62.29 ± 2.31 ns

Table 1b: Annexin V assay of PST001 in EAC cells by flow cytometry.

Concentration in $\mu\text{g/ml}$	24 hr EAC	48hr EAC
Control	8.27 ± 0.65	24.73 ± 1.43
100	15.24 ± 0.8 ***	34.99 ± 1.65 ***
200	11.35 ± 1 ***	26.19 ± 1.31 ns

Data were expressed as mean \pm SD. Statistically significant differences at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns is the non-significant, as compared with control group.

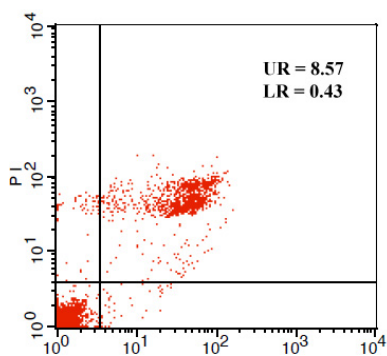


Fig. 5a

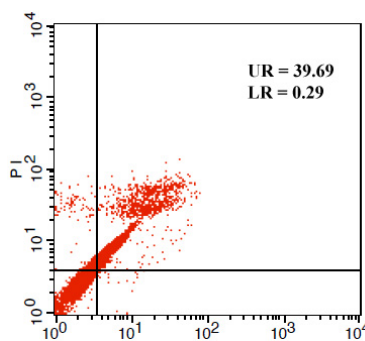


Fig. 5b

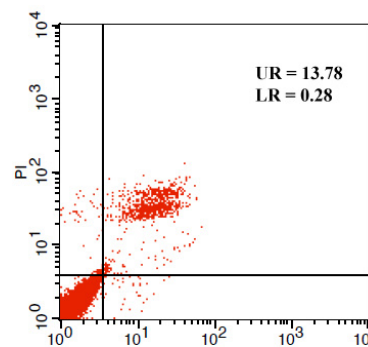


Fig. 5c

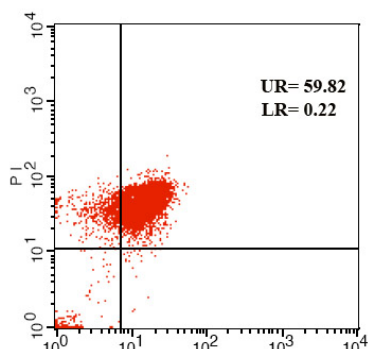


Fig. 5d

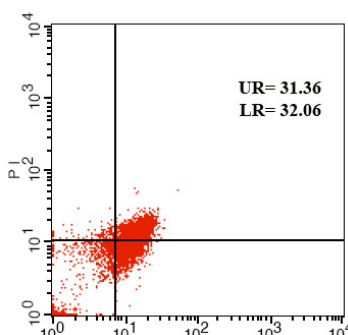


Fig. 5e

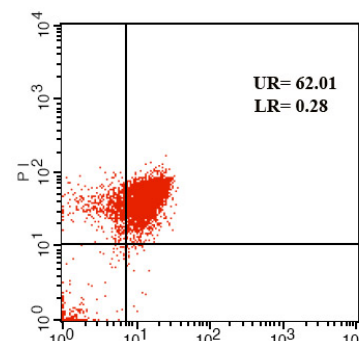


Fig. 5f

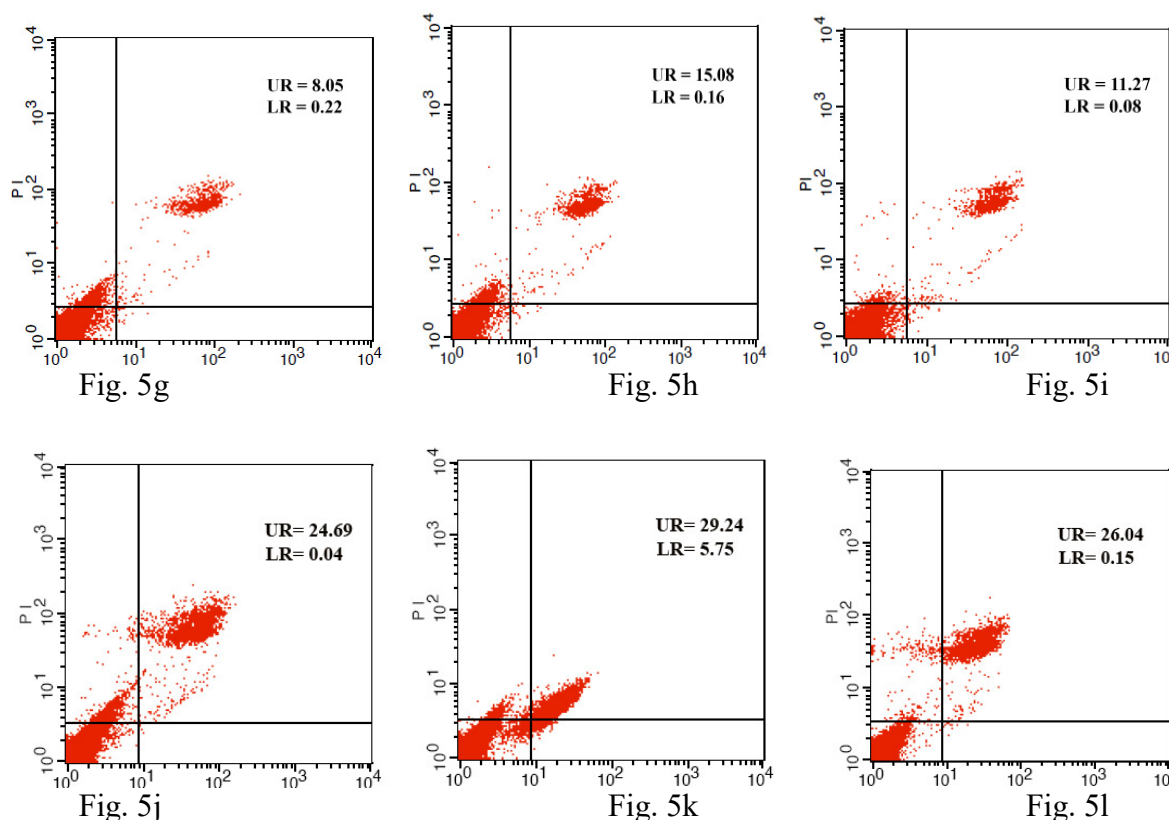


Figure 5a - l: Flow cytometry images of Annexin V on DLA and EAC cells.

(a) DLA control 24 hr ; (b) 100 μ g/ml PST001 on DLA 24hr ; (c) 200 μ g/ml PST001 on DLA 24 hr (d) DLA control 48 hr ; (e) 100 μ g/ml PST001 on DLA 48hr ; (f) 200 μ g/ml PST001 on DLA 48 hr ; (g) EAC control 24 hr ; (h) 100 μ g/ml PST001 on EAC 24hr ; (i) 200 μ g/ml PST001 on EAC 24hr ; (j) EAC control 48 hr ; (k) 100 μ g/ml PST001 on EAC 48hr ; (l) 200 μ g/ml PST001 on EAC 48 hr.

2.6 Effect of PST001 on cell cycle analysis

To determine the effect of PST001 on the cell cycle distribution of cells in different cell cycle phases, control and treated cells were analyzed by flow cytometry and the results are shown in figure 6. We observed that DLA cells accumulated in G0/G1 phase after treatment with PST001 for 48hr. In control cells $23.4 \pm 1.8\%$ G0/G1 cells are present whereas in 100 μ g/ml PST001 treated cells $38.37 \pm 2.75\%$ ($P < 0.001$) and in 200 μ g/ml PST001 treated

cells $50.87 \pm 2.1\%$ ($P < 0.001$) G0/G1 accumulation was observed. After 24 hr incubation, in 200 μ g/ml PST001 treated DLA cells a 20% increase in S phase accumulation was observed (Table 2a). However, EAC showed no significant alteration in cell cycle distribution. For DLA cells the apoptotic cell population was observed to be 13.04% for 100 μ g/ml PST001 concentration after 48hr of treatment and for EAC cells 15.40% apoptosis was evident even at 24hrs of incubation (Table 2b).

Table 2a: Table showing cell cycle analysis of PST001 treated DLA cells

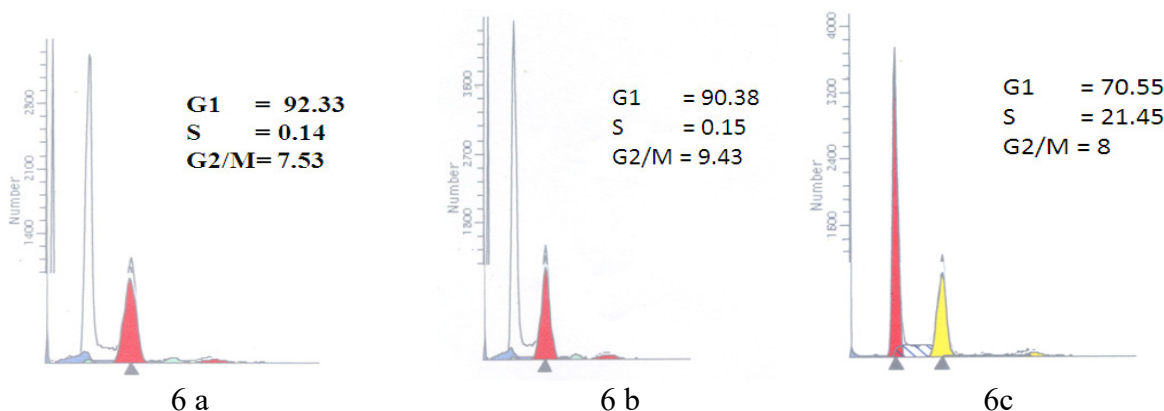
Concentration in $\mu\text{g/ml}$	24 hr DLA			48 hr DLA			Apoptotic Cells
	%G0/G1	%S	%G2/M	%G0/G1	%S	%G2/M	
Control	92.33 \pm 2.84	0.14 \pm 0.25	7.53 \pm 0.89	23.40 \pm 1.80	68.60 \pm 1.72	8 \pm 0.64	3.92
100	90.38 \pm 2.9 ^{ns}	0.15 \pm 0.37 ^{ns}	9.43 \pm 1.92 [*]	38.37 \pm 2.75 ^{***}	53.63 \pm 2.69 ^{***}	8 \pm 0.52 ^{ns}	13.04
200	70.55 \pm 2.2 ^{***}	21.45 \pm 0.45 ^{***}	8 \pm 0.65 [*]	50.87 \pm 2.1 ^{***}	41.13 \pm 1.74 ^{***}	8 \pm 0.35 ^{ns}	10.34

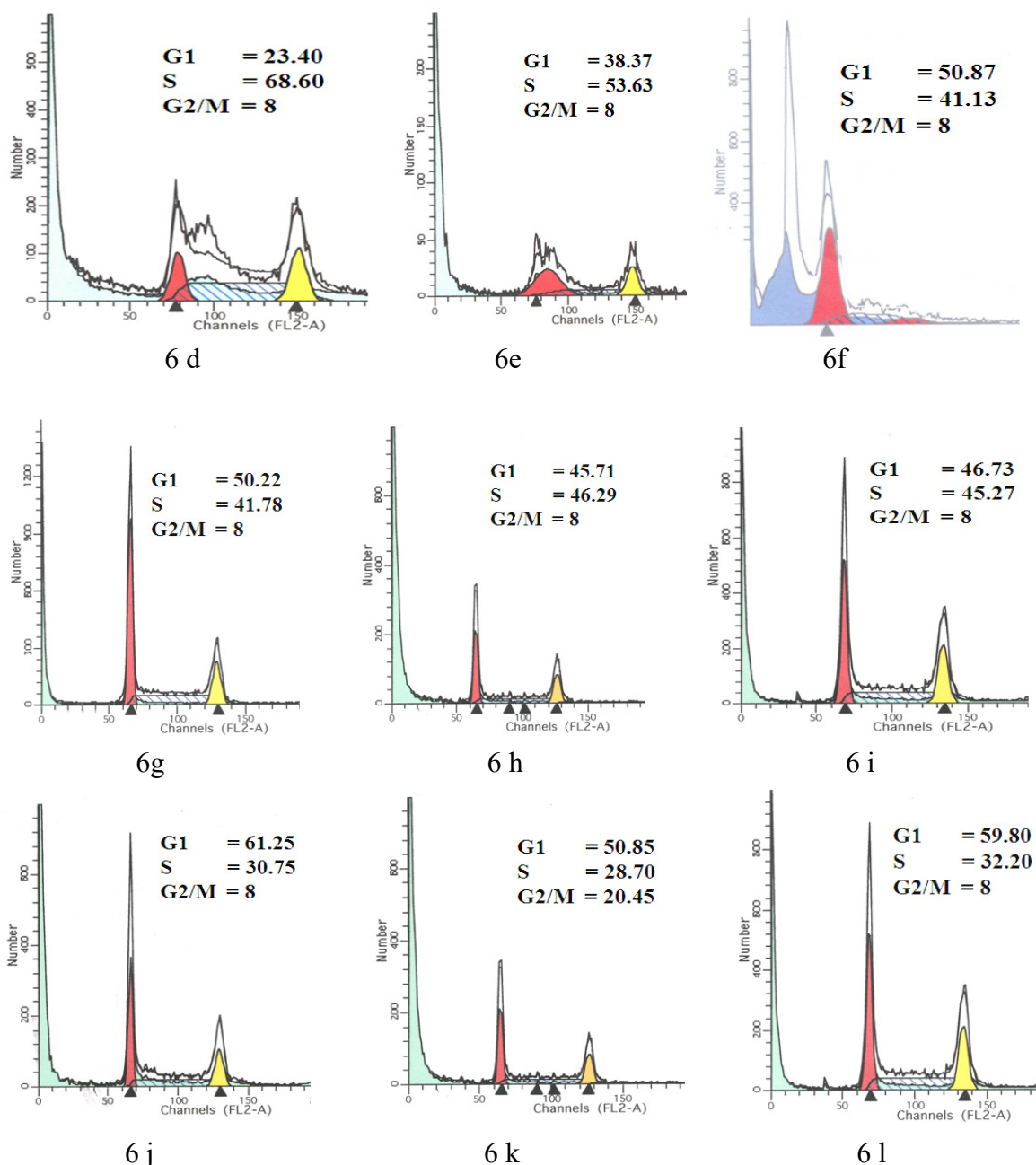
Data were expressed as mean \pm SD. Statistically significant differences at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns is the non-significant, as compared with control group.

Table 2b: Table showing cell cycle analysis of PST001 treated EAC cells

Concentration in $\mu\text{g/ml}$	24 hr EAC				48 hr EAC		
	%G0/G1	%S	%G2/M	Apoptotic Cells	%G0/G1	%S	%G2/M
Control	50.22 \pm 2.89	41.78 \pm 2.72	8 \pm 0.35	0	61.25 \pm 3.84	30.75 \pm 1.7	8 \pm 2.27
100	45.71 \pm 2.95 [*]	46.29 \pm 3.76 [*]	8 \pm 0.39 ^{ns}	3.97	50.85 \pm 2.98 ^{***}	28.70 \pm 2.62 [*]	20.45 \pm 1.31 ^{***}
200	46.73 \pm 2.1 [*]	45.27 \pm 2.8 [*]	8 \pm 0.42 ^{ns}	15.40	59.80 \pm 2.2 ^{ns}	32.20 \pm 2.65 [*]	8 \pm 1.34 ^{ns}

Data were expressed as mean \pm SD. Statistically significant differences at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns is the non-significant, as compared with control group.





Figures 6a - l: Flow cytometry images of cell cycle analysis on DLA and EAC cells.

(a) DLA control 24 hr ; (b) 100 μ g/ml PST001 on DLA 24hr ; (c) 200 μ g/ml PST001 on DLA 24 hr (d) DLA control 48 hr ; (e) 100 μ g/ml PST001 on DLA 48hr ; (f) 200 μ g/ml PST001 on DLA 48 hr ; (g) EAC control 24 hr ; (h) 100 μ g/ml PST001 on EAC 24hr ; (i) 200 μ g/ml PST001 on EAC 24hr ; (j) EAC control 48 hr ; (k) 100 μ g/ml PST001 on EAC 48hr ; (l) 200 μ g/ml PST001 on EAC 48 hr.

DISCUSSION

Apoptosis is a physiological process of cell elimination and the ability to induce apoptosis is an important property of the anticancer agents. During the last decade, a number of plant and mushroom

polysaccharides have been reported for their antiproliferative, apoptotic and immunomodulatory properties. The most potent polysaccharides are Polysaccharide peptide (PSP) isolated from *Coriolus Versicolour* strain-1 in China and Polysaccharide Krestin (PSK), from *Coriolus*

Versicolour in Japan, which are able to fight against cancer and boost the immune system (Dong Y, 1996; Siu-Lung Chan, 2006; Tsang, 2003). Now PSK is one of the most used cancer co-therapeutic substance in Japan.

In the present study, we have showed PST001, a polysaccharide isolated from the seed kernel of *Tamarindus indica* (PST001), was able to inhibit the invitro growth of DLA and EAC cells significantly. Previous reports from our laboratory showed that PST001 has antiproliferative and immunomodulatory activities (Sreelekha et al.1993). Antitumor and antioxidant activities of another polysaccharide PSP001 isolated from *Punica granatum* has also been reported by us (Sreelekha et al. 2008, Joseph et al. 2011). Our findings were in agreement with the reports on tumor growth inhibition by PSK and PSP (Ooi et al. 2000) and many other plant polysaccharides (Yu, 2009; Piero, 2007).

The proliferation inhibition of PST001 was the result of apoptotic induction, as evidenced by the cell morphology analysis showing nuclear shrinkage, chromatin condensation and apoptotic bodies in PST001 treated cells in contrast to control cells. The detection of apoptotic cells by using fluorescent microscopy is based on morphological features including condensation of chromatin and cytoplasm, fragmentation of the cell and apoptotic body formation (Kerr et al. 1972). The budding phenomenon of apoptotic cells lasts only a few minutes, but the formation of apoptotic bodies remains visible histologically for 1 to 2 hours (Barres, 1992; Coles, 1993). DNA fragmentation is very typical of the apoptotic process, with generation of series of multiples of 180bp units. Though, not in all cases, DNA ladder was obtained for DLA cells treated with 100µg/ml PST001 after 48 hours. The fragmentation of chromosomal DNA is a hallmark of apoptosis and may facilitate apoptosis by terminating DNA replication and gene transcription. The degradation of DNA down to oligonucleosomal fragments is a late event of apoptosis (Compton, 1992).

Flow cytometry data of PI single staining and PI – Annexin V dual staining confirmed that PST001 could be able to induce apoptosis on cancer cells. An early indicator of apoptosis is the rapid translocation

and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected by using the binding properties of Annexin V. Caspase-3 was also evaluated by flow cytometry and was supporting the above results. Apoptosis or programmed cell death is an essential physiological process that plays a critical role in development and tissue homeostasis. The progress of apoptosis is regulated in an orderly way by a series of signal cascades under certain circumstances. The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals. Cell cycle analysis showed almost normal cell cycle distribution of cells except in two cases, sub G1 apoptotic population was observed both in DLA and EAC cells. The cell cycle checkpoint maintains the order and fidelity of cell cycle events in eukaryotes (Hatwell, 1994; Nigg, 2001). DNA damage and mutations could be detected by it, and the damaged cells were arrested in specific checkpoint to repair. If the damaged DNA was repaired, the cells could enter into the next phase of cell cycle, or apoptosis would happen (O'Neil, 2006; Brnzei, 2008). It was suggested that DNA damage repair mechanism might be activated by PST001 in DLA and EAC cells which resulted in almost normal distribution of cells in the different cell cycle phases.

This study demonstrated that PST001 is a potent growth inhibitor against DLA and EAC cells. Acute and sub acute toxicity studies were done and no LD50 was observed up to a concentration of 2000mg/kg body weight in mice. Biochemical and histochemical evaluation also showed no abnormalities on treatment with PST001 (Data under publication).

CONCLUSION

The results of this study provide scientific evidence to support the apoptotic inducing activity of PST001 on DLA and EAC cells. Further detailed studies are required to clarify the cellular mechanism by which PST001 induces growth inhibition in DLA and EAC cells.

ACKNOWLEDGEMENTS

The authors thank Kerala State Council for Science, Technology and Environment (KSCSTE) for financial support.

REFERENCES

1. Alley M.C, Scudiero D.A, Monks A. Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. *Cancer Res* 1988; 48: 589–01.
2. Aravind S.R, Vijayan K.K, Prabha Balaram and Sreelekha T.T. Apoptotic Evaluation of Polysaccharide PST001 in Tumor Cells. Proceedings of the 21st Kerala Science Congress. 2009; 553-556.
3. Barres B.A, Hart I.K, Coles H.S, Burne J.F, Voyvodic J.T, Richardson W.E & Raff M.C. Cell death and control of cell survival in the oligodendrocyte lineage. *Cell*.1992;70: 31-46.
4. Branzei D and Foiani M. Regulation of DNA repair throughout the cell cycle. *Nat Rev Mol Cell Biol*. 2008; 9: 297–308.
5. Coles H.S, Burne J.F & Raff M.C. Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. *Development* 1993; 118: 777-784.
6. Compton M.M. A biochemical hallmark of apoptosis: Internucleosomal degradation of the genome. *Cancer Metastasis Rev*. 1992; 11:105–119.
7. Dong Y, Kwan C.Y, Chen Z.N, Yang M.M. Antitumor effects of a refined polysaccharide peptide fraction isolated from *Coriolus versicolor*: in vitro and in vivo studies. *Res Commun Mol Pathol Pharmacol*. 1996; 92(2):140-8.
8. Hartwell L.H and Kastan K.B. Cell cycle control and cancer. *Science*. 1994; 266: 1821–1828.
9. John Mann. Natural products in cancer chemotherapy: past, present and future. *Nature Reviews Cancer*. 2002; 2: 143-148 doi:10.1038/nrc723.
10. Joseph M.M, Aravind S.R, Varghese S, Mini S, Sreelekha T.T Evaluation of antioxidant, antitumor and immunomodulatory properties of polysaccharide isolated from fruit rind of *Punica granatum*. *Molecular Medicine Reports*. 2012, (In press).
11. Kerr J.F, Wyllie A.H, Currie A.R. Apoptosis: A basic biological phenomenon with wide Ranging implications in tissue kinetics. *Br. J. Cancer*. 1972; 26: 239–57.
12. Kulkarni G. Development of controlled release spheroids using natural polysaccharide as release modifier. *Drug Deliv*. 2005; 12:201-6.
13. McGahon A.J, Martin S.J, Bissonnette R.P, Mahboubi A, Shi Y, Mogil R.J, Nishioka W.K, Green D.R. The end of the (cell) line: methods for the study of apoptosis in vitro. *Methods Cell Biol*. 1995; 46: 153-185.
14. Nakamura O.R, Machid Y, Nagai T. Invitro invico nasal mucoadhesion of some water Soluble polymer. *Int j Pharm*. 1996; 134:173-81.
15. Nigg E.A. Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol*. 2001; 2: 21–32.
16. O'Neil N, Rose A. DNA repair. *WormBook*, 2006, 13: 1–12.
17. Ooi V.E and Liu F. Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. *Curr Med Chem*. 2000; 7(7):715-29.
18. Piero Sestili, Chiara Martinelli, Donata Ricci, Daniele Fraternali, Anahi Bucchini, Laura Giamperi, Rosanna Curcio, Giovanni Piccoli, Vilberto Stocchi. Cytoprotective effect of preparations from various parts of *Punica granatum* L. fruits in oxidatively injured mammalian cells in comparison with their antioxidant capacity in cell free systems *Pharmacological Research*. 2007; 56:18–26.
19. Ramnath V, Rekha P. S., Kuttan G. and Kuttan R. Regulation of Caspase-3 and Bcl-2

- Expression in Dalton's Lymphoma Ascites Cells by Abrin Advance Access Publication. 2007; 6(2): 233–238.
20. Siu LC, John H.K. Yeung. Effects of polysaccharide peptide (PSP) from *Coriolus versicolor* on the pharmacokinetics of cyclophosphamide in the rat and cytotoxicity in HepG2 cells Food and Chemical Toxicology.2006; 44:689-694.
21. Sreelekha T.T. Vijayakumar T, Ankathil R, Vijayan K.K, and Nair M.K. Immunomodulatory effects of polysaccharide from *Tamarindus indica*. Anticancer Drugs. 1993; 4(2): 209-12.
22. Sreelekha TT, Vijayan KK, Balaram P. Antimitotic polysaccharide from *Punica granatum*. In Ethnopharmacology - Recent advances. Eds. Pushpangadan P, George V and Janardhanan KK, Daya Publishing House, Delhi, India, 2008. Chapter 19, 231 – 245,
23. Tsang K.W, Lam C.L, Yan C, Mak J.C, Ooi G.C, Ho J.C, Lam B, Man R, Sham J.S, Lam W.K. *Coriolus versicolor* polysaccharide peptide slows progression of advanced non-small cell lung cancer. Respir Med.2003; 97(6):618-24.
24. Wei D.L, Bo D.Z, Wei R, Ji L.L, Zhi L.L. Reversal effect of *Ganoderma lucidum* polysaccharide on multi drug resistance in K562/ADM cell line. Acta Pharmacol Sin 2008; 29(5): 620-627.
25. Yu Cao and Isao Ikeda. Antioxidant activity and antitumor activity (in vitro) of xyloglucan selenious ester and sulfated xyloglucan. International Journal of Biological Macromolecules. 2009; 45:231–235.