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Immunostimulatory plant polysaccharides impede cancer progression and metastasis by avoiding off-target effects

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ABSTRACT

An unexploited homo-polysaccharide (PSM001) isolated from the seed kernel of Kottukonam variety of *Mangifera indica*, demonstrated selective cytotoxicity against cancer cells both *in vitro* and in murine models while maintaining the immunostimulatory potential. Galactoxyloglucan (PST001) isolated from the seeds of *Tamarindus indica*, was previously established to be an effective anticancer and immunomodulatory agent. Cancer metastasis, with key features including invasion, migration, increased angiogenesis and colony formation is only likely to accentuate in the coming decades, considering the ground realities of the modern lifestyle and environmental factors and hence both the polysaccharides were tested towards the management of malignancy. It was a startling observation with both the biopolymers in inhibiting various processes involved in the metastatic cascade. A quick perusal of the issue at hand would throw up the promising ability of both PSM001 and PST001 to inhibit lung metastatic nodules of C57BL/6 mice wherein the combinatorial treatment of these polysaccharides with vincristine delivered superior therapeutic output. Later, vascular endothelial growth factor and multiple matrix metalloproteinases were found to be the lead players in the polysaccharide mediated metastatic inhibition. Having considered the complexities associated with the chemotherapy in metastatic cancer in terms of palpable immunosuppression, the aftermaths with the co-administration of an immunostimulatory agent which itself possess unique anticancer and anti-metastatic potentials with a potent chemotherapeutic agent will be enormously consequential.

1. Introduction

Cancer is a medical condition highlighted with the uncontrollable proliferation of cells which ultimately disturbs the homeostasis of the body. Several cell-signalling pathways and numerous molecular targets have been recognized to play profound roles in carcinogenesis, which may be either biochemically facilitated, genetically predisposed or epigenetically organized [1]. Discrimination of cancer cells from the normal counterparts is the bottleneck in the early diagnosis and subsequent treatment of this life threatening disease which can invade almost all organ parts of the body. Since the current treatment regimen fails to halt cancer towards metastasis and the fact that almost 90% of cancer deaths arise from metastasis [2], the need for an anti-metastatic drug is very high. The underlying fact that conventional chemotherapeutic drugs are not effective in managing metastasis creates urgency for an effective combination approach which could potentially abstain the progression of cancer cells into metastases.

There is a large repository of natural products currently being employed to treat solid tumors, as well as disseminated cancers. A hunt for the biologically active molecules with tremendous structural diversity like polysaccharides represents most attractive group owing to their many unique structural and physiochemical features. Polysaccharides, which comprises of multiple chains of monosaccharide units joined together by glycosidic bonds are omnipresent in the nature holding major functions of storage and providing structural integrity. Several polysaccharides isolated from algae, mushrooms and higher plants exhibited potent antitumor and antimetastatic activities with minimal or virtually no side effects. One among the striking feature of

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polysaccharides among other biological macromolecules is their ability to enhance the host immune system. As conventional chemotherapeutic drugs are cytotoxic to both normal and cancerous cells and weaken the immune system, the use of polysaccharides in cancer therapy would be highly beneficial [3].

Asian countries such as Bangladesh, India, Philippines and Pakistan are gifted with the wide distribution of *Mangifera indica* and *Tamarindus indica* two flowering plants belonging to the family Anacardiaceae and Fabaceae respectively. Various studies demonstrated the numerous medicinal properties including antioxidant, antiviral, antihelminthic, antimicrobial, immunomodulatory and hepatoprotective abilities with the different compounds of these plants. Polysaccharides isolated from the mature raw pulp of *Mi* established strong antioxidant effects with reno protective and hepatoprotective ability in rats against cyclophosphamide induced toxicity [4,5]. Structural analysis confirmed poly galacturonic acid as the main pectic polysaccharide which is responsible behind the antioxidant abilities [6]. A study on the chemical composition of polysaccharides extracted from raw and ripe mango fruit pulp revealed a drop in the molecular weight of arabinogalactan and rhamnogalacturonan during the ripening process [7]. However, lack of valid scientific reports about the polysaccharides from *Mangifera indica* L attracted our psyche towards exploring the bioactivities of this polymer. A galactoxyloglucan (PST001) from the seed kernel of *Tamarindus indica* was exploited as a potent antitumor and immunomodulatory agent by our group which executes cancer-cell-specific cytotoxicity through the TRAIL-DR4/DR5 apoptotic pathways [8,9]. This galactoxyloglucan was the spine behind the biogenic synthesis of gold (PST-GNPs) and silver (SNP@ PST) nanoparticles which also demonstrated cancer-selective cytotoxicity with excellent biocompatibility [10,11]. However the efficiency of PST001 against metastasis was yet to be explored. Since metastatic progression is the backbone of cancer mortality, and considering the immense therapeutic value of these polysaccharides, the current study aims to evaluate the anti-metastatic capability of both PSM001 and PST001 in both human and murine cancer cells both *in vitro* and in appropriate murine models (Scheme 1). As the antitumor properties of PSM001 were not established, present study furthermore evaluated the mechanism of antitumor activity of PSM001. Although potential studies and multidisciplinary approaches are necessary for the effective management of metastatic cancer, the current exploration will aid fuel for further investigations with these non-toxic polysaccharides.

2. Materials and methods

2.1. Isolation and purification of polysaccharides

Fresh ripe fruits of *Mangifera indica* (Kottukonam variety) and seed kernels of *Tamarindus indica* were used as the raw material for the isolation of polysaccharide PSM001from *Mi* and PST001 from *Ti* respectively using standard procedures [8] reported before. Briefly, the material was powdered and mixed with petroleum ether at room temperature for 72 h, followed by extraction with methanol. The solution was subjected to several cycles of ethanol precipitation and dialysed against distilled water. The contents of the dialysis bags were shaken with chloroform (50 mL) in a separating funnel to remove the denatured protein. Purification was done by gel filtration chromatography using Sephadex G-200 column (Pharmacia Fine Chemicals), Ultrogel AcA-44 chromatography resins (LKB) and 0.001 M phosphate buffered saline (PBS) as the eluent buffer. About 500 mg of the crude polysaccharide was suspended in buffer and run through Sephadex G-200(3 cm × 75 cm) column which was equilibrated with the buffer. Phenol-sulphuric acid solution was mixed and the fractions were monitored at 280 nm and 490 nm. The obtained fractions under the peak were collected, lyophilised (CHRIST ALPHA 2-4 LD PLUS, Germany) and maintained at 4 °C. Detailed procedures adopted for the structural elucidation of PSM001 including molecular weight estimation and identification of sugar components is described in the supporting information file (SI Sections 1.1–1.4).

2.2. Culture and maintenance of cell lines

The human cancer cell lines A549 (adenocarcinoma alveolar basal epithelial cells), A375 (melanoma cells), HCT116 (colorectal carcinoma cells), MCF-7(breast carcinoma cells) and murine melanoma cell line B16F10 (murine melanoma cells) were obtained from National Centre for Cell Sciences, Pune, India. All cell lines were maintained in DMEM media with 10% fetal bovine serum, 1% penicillin G and streptomycin, at a temperature of 37 °C in a humidified incubator (Heraeus BB 15) with 5% CO_2 atmosphere. The murine transplantable lymphoma cell line, Ehrlich ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) were maintained in the peritoneal cavity of mice by intraperitoneal (*ip*) transplantation of 1×10^6cells per mouse. All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) and were in adherence to the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.3. Animal models

Male C57BL/6 mice and female BALB/c mice (6–8 weeks old) were housed respectively at Rajiv Gandhi Centre for Biotechnology and Regional Cancer Centre, Trivandrum, Kerala, India. The animals were fed with mice chow and water *ad libitum*. They were placed in cages with six mice per cage with 50–60% humidity in a room with specifications of temperature at 21–23 °C, 50–60% humidity and 12 h light cycle.

2.4. In vitro cytotoxicity assay

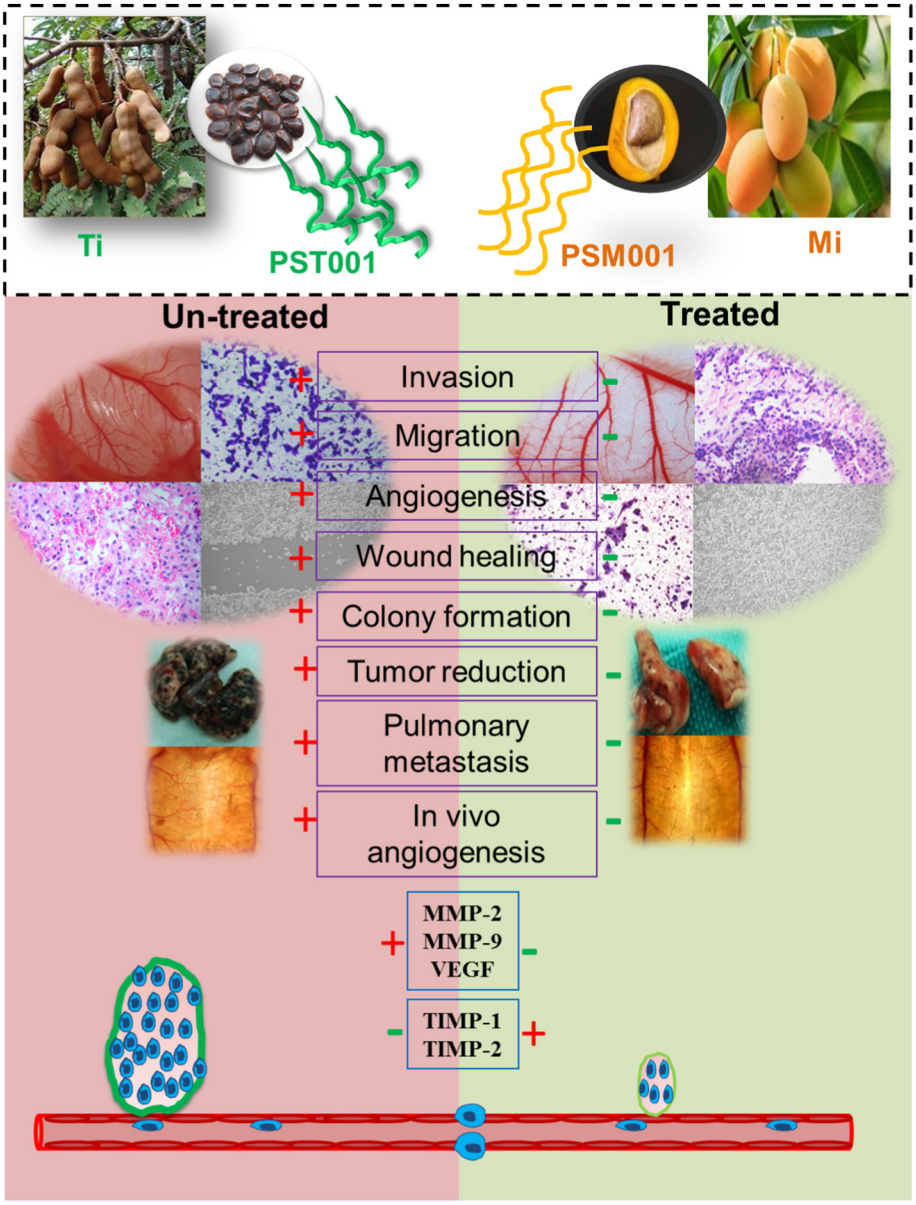
The cytotoxic effect of PSM001 on cancer cells was evaluated by MTT assay as previously described [8] for a wide concentration range (0.001–1000 μg/mL) with doxorubicin (Dox) as a positive control with 24,48 and 72 h duration. The absorbance was measured at 570 nm using a microplate spectrophotometer (BioTek Power Wave XS).

2.5. Apoptosis evaluation

Estimation of apoptosis mediated cell death by morphological assays was conducted in cancer cell lines treated with PSM001 (1000 μg/mL) for 72 h. Initially, cells were observed for any visible gross morphological changes under phase-contrast objective (Olympus 1 × 51, Singapore) to view the apoptotic or non-apoptotic cells. Live-dead assay using acridine orange - ethidium bromide was performed as previously described [10] and the cells were observed under a FITC filter (Olympus 1 × 51, Singapore). Observation for any apoptosis-related changes with Hoechst 33342staining was performed as previously described [10] using a 4′, 6-diamidino-2-phenylindole filter (Olympus 1 × 51, Singapore).

2.6. Toxicity evaluation in BALB/c mice

Acute toxicity studies with PSM001 were performed on 5–6 week-old male BALB/c mice as previously described [12,13]. PSM001 was dissolved in phosphate buffered saline (PBS) and administered (*ip*) in increasing dosage up to 2000 mg/kg body weight to several groups of experimental animals. All animals were observed continuously for 4 h and were housed overnight and mortality, if any, was recorded. Any behavioural changes were tabulated, and the lethal dose causing 50% mortality (LD_{50}) was assessed. Sub-acute toxicity studies were done on 1/5th, 1/10th and 1/20th doses of the LD_{50}. Three groups of 6–8 week old BALB/c mice (6 mice/group) received PSM001 as a single dose per day administered *ip* for a total of 14 days. Body weight and signs of



Scheme 1. PST001 isolated from *Tamarindus indica* and PSM001 isolated from *Mangifera indica* inhibits cancer proliferation, angiogenesis, invasion and metastasis *in vitro* and *in vivo* through the suppression of MMPs and up regulation of TIMPs.

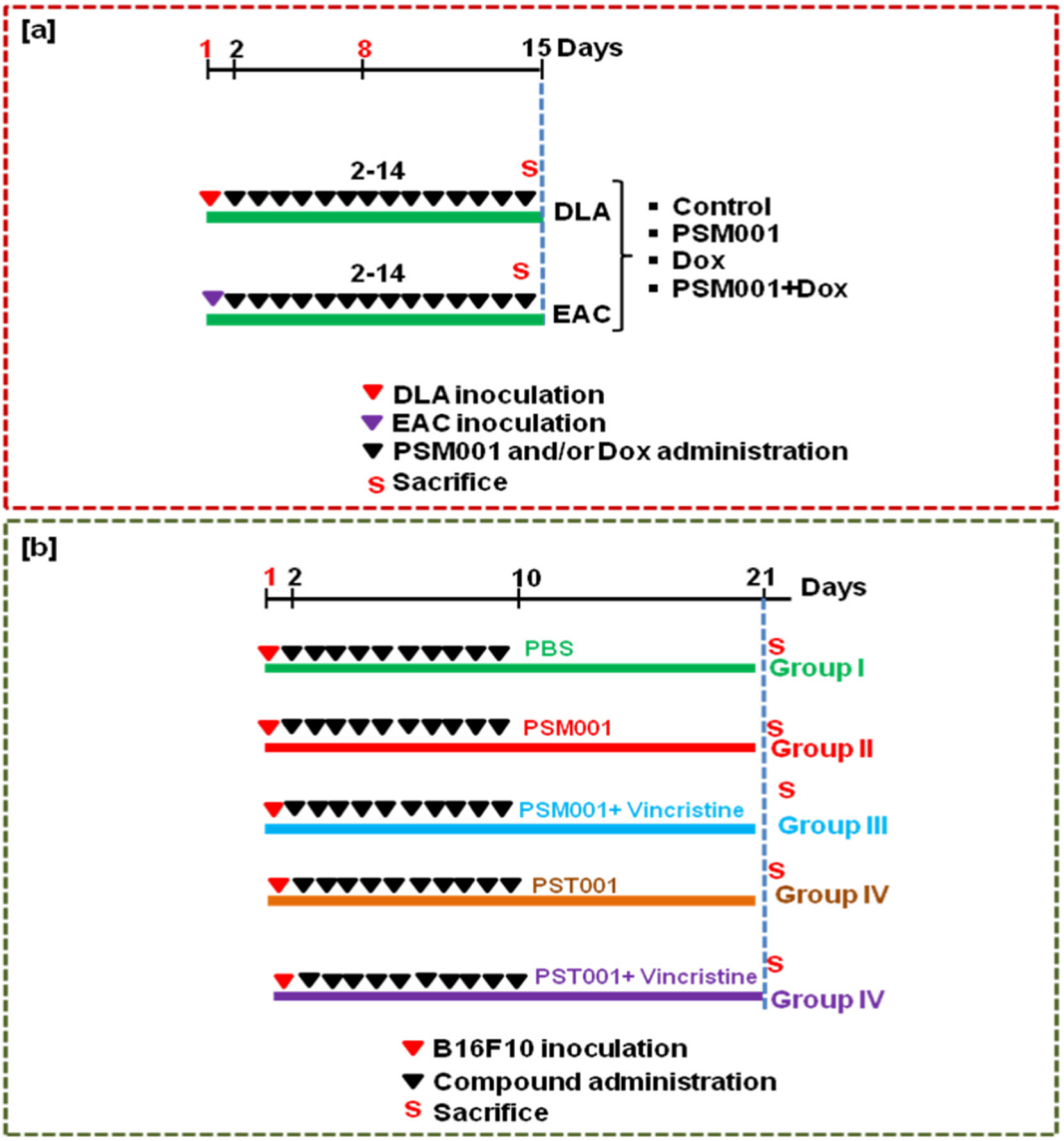
toxicity were measured daily. The mice were sacrificed on the 15th day and vital organs like kidney, liver and spleen were subjected to histopathological examination and blood collected *via* cardiac puncture was analysed for biochemical and hematological parameters.

2.7. In vivo tumor reduction studies

Female BALB/c mice (6–8 weeks) were separated into different groups and were maintained as per the recommended procedures for the tumor reduction studies for which, DLA or EAC cells (1×10^6cells) were given as *ip* injection. The experimental design comprises of a vehicle control group which received PBS, doxorubicin (Dox) at dose 2.25 mg/kg which served as the positive control, PSM001 at a dosage of 200 mg/kg body weight and the combination group of PSM001 and Dox as summarised in Scheme 2a. In group I, DLA cancer cells were injected on day1 and for group II, EAC cancer cells were injected on day 1. Treatments were given consecutively from day 2–14 for both the groups and mice were sacrificed on 15thday. Briefly, the mice were euthanized and then the abdomen of each mouse was incised over a clean vessel and the volume of the ascites fluid obtained (V1) was calculated. The peritoneal cavity was washed thoroughly with isotonic saline until the return was clear, and the volume of the added saline (V2) was then noted. The volume of ascites tumor (V3) was calculated using the formula: V3 = (V1 + V2) − V2. The mean survival time (MST) and percentage of increase in life span (%ILS) was calculated as previously reported [12,13]. MST = (A + B) / 2, where A is the day of the first death and B is the day of last death. The percentage of increase in life span was calculated using the following formula: %ILS = (T − C) / C × 100, where T and C are the MST of treated and control animals, respectively.

2.8. Chorioallantoic membrane (CAM) assay

Fertilized eggs obtained from White Leghorn were purchased from the poultry farm (Kudappanakunnu, Kerala, India).Viable eggs were maintained in a humidified incubator at 37 °C. A small incision of 10 mm diameter was made on the third day, after carefully removing a part of the egg shell. The cavity was re-sealed with cellophane tape and



Scheme 2. [a] Schematic for DLA and EAC tumor reduction experiments. Female BALB/c mice were divided in to two groups, each group subdivided in to 4 treatment options (n = 6/treatment group). Tumor cells were inoculated and compounds were administered as shown. Mice were sacrificed on day 15 and parameters were assessed. [b] Schematic for B16F10 murine melanoma cells induced pulmonary metastasis assay. Female C57BL/6 mice were divided in to five groups, each group were administered with different treatment options as shown. Mice were sacrificed on day 21 and parameters were assessed.

returned back to the incubator. On the 8th day, the seal was opened and PSM001 (500 and 1000 μg/mL) and PST001 (100 and 200 μg/mL) were added onto the CAM of respective eggs, space resealed and replaced back to the incubator. On the 11th day, the CAM was observed for the effect of polysaccharides on neovascularization guided by a stereomicroscope and photographed using a camera (Nikon COOLPIX L310) [14].

2.9. Invasion and migration assay

In order to evaluate the effect of polysaccharides in the invasive and migratory potential of cancer cells, assay was done on 24 well cell culture plate transwell inserts (353097, Corning Falcon, USA). For the invasion assay, matrigel (354234, Corning Falcon, USA) was coated on the inner side of 24 well transwell chambers (8 μm pore size polycarbonate filters) and kept at 37 °C in 5% CO_2 incubator. The upper compartment of the transwell chambers were seeded with cancer cells (1×10^6 cells/ml) in serum free medium. PSM001and PST001 was added along with the cancer cells. DMEM medium enriched with 10% serum served as the chemo attractant and was added onto the24 well cell culture plates. The plates were incubated for 24 h at 37 °C in 5% CO_2 incubator. The cells present in the upper chamber of the transwell inserts were removed using cotton swab and 1% crystal violet were applied to stain the filters. The invaded cells located at the lower compartment of the inserts were photographed and counted using an optical microscope (Olympus 1 × 51) at 10× magnification. The same procedure was opted for the migration assay by using transwell inserts without matrigel coating [15].

2.10. Wound healing assay

For the wound healing assay, cancer cells (1×10^6 cells/mL) were seeded onto 6 well cell culture plates and upon confluency, a sterile 100 μL micropipette tip was used to create a wound on the cell monolayer. PSM001 and PST001was added to the cells at respective concentrations and kept at 37 °C in 5% CO_2 incubator. The cells were viewed in a phase contrast microscope (Olympus 1 × 51) at 10× objective and photographed at various time points (0, 12 and 24 h) until

the wounded area was completely closed in the control wells [16]. The wound width was measured using Image J software and migration rate was calculated as follows: Migration rate (%) = (wound width of drug treated cells / wound width of untreated cells) × 100.

2.11. Colony formation assay

Colony formation assay was performed with cancer cells (1000 cells/mL), seeded on a 6 well cell culture plate which were kept overnight at optimal conditions. Vehicle control (DMEM medium), PSM001 (500 and 1000 μg/mL) and PST001 (100 and 200 μg/mL) were added to the respective wells and incubated for 72 h. The medium was replaced with fresh DMEM enriched with 10% FBS and kept for 9 days. Later, the medium was removed, cells washed with PBS, fixed in 70% ethanol and stained with 1% crystal violet in ethanol. The plate was kept for air drying and the colonies were observed, counted and photographed in an IX51 Olympus phase contrast inverted microscope [17].

2.12. Western blotting

Proteins were extracted from cancer cells treated with PSM001 and PST001 using RIPA buffer (Thermo Scientific, Rockford, USA).The total protein concentration was determined by Coomassie Plus protein assay reagent and bovine serum albumin (BSA) standards (Pierce, Rockford, USA). Equal quantity of proteins (~50 mg) was fractionated by 10% SDS-PAGE and electro blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). After the membranes were blocked with 5% BSA, they were incubated overnight with the specific primary polyclonal antibodies: anti MMP-2, anti MMP-9, anti TIMP-1, anti TIMP-2, anti VEGF and anti β actin raised in rabbit (Bioss, USA).The membranes were washed with TBST buffer three times and incubated with horseradish peroxidase conjugated goat anti rabbit secondary antibody (Jackson, Immuno Research Laboratories, USA) for 1 h at room temperature. After removing the unbound antibody with TBST, the proteins were visualised by enhanced chemiluminescence method in Flourichem analyser (FlourChem M, Protein Simple, USA). Quantitation of the immunoreactive bands was done with the aid of Image J software (version 1.48, NIH, USA) and normalised with the respective β-actin band, which was used as an internal control [18].

2.13. Peritoneal angiogenesis assay in BALB/c mice

To evaluate the anti-angiogenic capacity of the polysaccharides in murine models, EAC cells (5×10^6 cells/mice) were injected into the peritoneal cavity of six-eight week old female BALB/c mice (6 mice/group). While the vehicle control group received PBS, the treatment groups received PSM001 and PST001 respectively at 200 mg/kg from 6th day of tumor cell administration to 12th day. On the 13th day, the mice were sacrificed and the peritoneum was photographed using a Nikon COOLPIX L310 camera to evaluate the extent of angiogenesis. The paraffin embedded 5 μm sections of peritoneum fixed in buffered formalin was prepared and immunohistochemical analysis was done using primary rat anti-mouse CD31antibody and secondary horse radish peroxidase conjugated goat anti-rat antibody (Southern Biotech, Birmingham, USA). The sections were observed on an Olympus IX51 microscope with 10× objective [19].

2.14. Pulmonary metastasis assay in C57BL/6 mice

The anti-metastatic potential was evaluated with B16F10 murine melanoma cells (1×10^6cells/mice) injected to male C57BL/6 mice (6–8 weeks old) *via* tail vein administration. The experiment involved five groups with each group consisting of six animals as summarised in Scheme 2b. Group I served as vehicle control which received PBS. Group II, III, IV and V received PSM001 (200 mg/kg), PSM001 (200 mg/kg) and vincristine (0.065 mg/kg), PST001 (200 mg/kg), PST001 (200 mg/kg) and vincristine (0.065 mg/kg) i.p for 10 days respectively. Half of the animals from each group were sacrificed on day 21. The lungs were evaluated for the extent of metastases, characterised by blackish metastatic colonies. After the numbers of colonies in the lungs were counted under a dissecting microscope, histopathological analyses of lungs were carried out [19]. Tumor formation and survival was checked for > 2 months. The mortality of each animal was monitored and the percentage increase in life span (% ILS) was evaluated.

2.15. Statistical analysis

Data is represented as mean ± standard deviation (SD) of three replicates which was analysed using GraphPad PRISM v 5.0 (GraphPad Software Inc., San Diego, CA). Statistically significant differences were considered if $P < 0.05$, as determined using one-way analysis of variance (ANOVA). The images were quantified using Image J software version 1.5 I (NIH, USA). IC_{50} values were calculated using the Easy Plot software (Spiral Software, MD).

3. Results

3.1. Isolation and characterisation of polysaccharides

The polysaccharide (PST001) isolated from the seed kernels of *Ti* was found to be pH neutral, and the total sugar content was 98% as determined by the phenol-sulphuric acid method. The detailed structural characterisation of PST001 was performed previously and the major monosaccharides glucose, xylose, and galactose units, are present in the ratio of 2.8/2.25/1.0 [8,20]. The molecular weight lies within the range of $2.5 \times 10^5$to 6.5×10^5 Da. However, the detailed structural elucidation of PSM001 isolated from *Mi* is a new insight for glycobiology. Molecular weights were determined by gel filtration chromatography using sephadex G-200 with a series of dextrans as standards. The molecular weight obtained was 30 kDa, PSM001 was found to be pH neutral, and the total sugar content was 94% with a yield around 1%. The homogeneity of the purified polysaccharide was tested using sodium dodecyl sulphate polyacrylamide gel electrophoresis and PSM001 was verified to be homogeneous in nature with excellent solubility in water. Complete hydrolysis and thin layer chromatographic analysis and co-chromatography using standard sugars revealed that PSM001 is a homopolymer of glucose units. Partial hydrolysis, periodate oxidation, methylation, hydrolysis, acetylation and trimethylsilylation were also carried out and analysed by various spectroscopic procedures such as ^{1}HNMR, FABMS and GC–MS. The native polysaccharides also were analysed by ^{1}HNMR spectroscopy and FABMS (SI Sections 2–2.3).

3.2. Selective cytotoxicity of PSM001 on cancer cells through apoptosis

The behavior of PSM001 on cancer cells was evaluated using the MTT assay. PSM001 exhibited an increased cytotoxicity in a dose dependent manner in all the cancer cell lines tested and was found to be an effective cytotoxic agent especially against human cancer cell lines A375, HCT116, A549, MCF-7 and murine cancer cells B16F10, DLA and EAC (Fig. S1a–g). PSM001 demonstrates a maximal cytotoxic effect on EAC cells after 72 h. The IC_{50} values obtained for PSM001 in EAC, A375 and DLA cells are 552 μg/mL, 650 μg/mL and 820 μg/mL respectively. It also displayed a growth inhibition of 47.2%, 44.5%, 42.11% and 39.5% in B16F10, A549, HCT116 and MCF-7 cells respectively. Later, the effect of the polysaccharide on isolated human peripheral lymphocytes (Fig. S1h) was tested which revealed that PSM001 not only proved to be non-toxic towards lymphocytes but also displayed excellent proliferation with a proliferation index of 1.3 after 72 h as compared to the control.

In order to determine the mechanism of cell death induced by

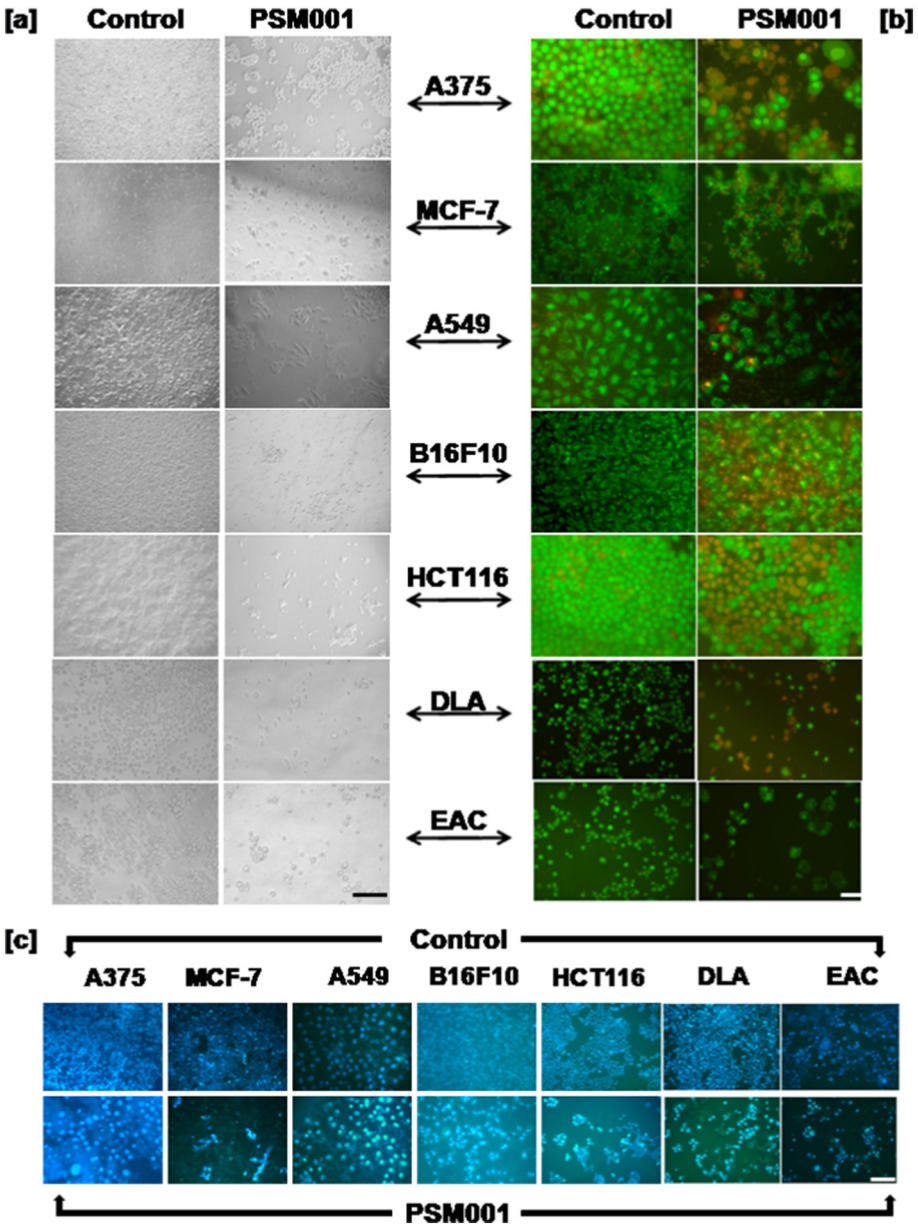


Fig. 1. Apoptotic evaluation of PSM001 treated cancer cells for morphological evaluation with [a] phase contrast microscopy [b] acridine orange- ethidium bromide staining and [c] Hoechst staining after 72 h incubation. The scale bar represents 100 μm.

PSM001, apoptotic assays were performed. The phase contrast microscopic images of PSM001 treated cancer cells revealed characteristic changes in the cellular morphology such as distorted shape, membrane blebbing, and the presence of apoptotic bodies compared to the control group(Figs. 1a; S2a). Acridine orange-ethidium bromide staining showed a change in color from green to yellow/red with associated apoptotic features (Figs. 1b; S2b). PSM001 treated cancer cells demonstrated increased number of early apoptotic cells and also late apoptotic cells visualised as bright green spots in the nuclei and orange cells depicting the condensed chromatin and fragmented nucleus. The most distinct changes of apoptotic cells are DNA fragmentation and nuclear condensation, which could be clearly spotted by Hoechst 33342 nuclear staining (Figs. 1c; S2c). There was an evident chromatin condensation and nuclear cleavage both in murine cancer cells (DLA, EAC and B16F10) and human cancer cells after the application of PSM001.Thus the mode of cytotoxicity induced by PSM001 could be confirmed through the execution of programmed cell death pathways.

3.3. Comprehensive toxicity profiling of PSM001 in BALB/c mice

Evaluation of the effect of the administered agent on the normal health of experimental animals could be well addressed through the detailed toxicological analysis. Hence, we assessed the toxicity of PSM001 administration in male BALB/c mice up to 2000 mg/kg. As all the animals in the experimental groups survived with no observed levels of toxicity, the LD_{50} dose was taken as 2000 mg/kg. Hence for the long term toxicity evaluation, 1/5th (400 mg/kg), 1/10th (200 mg/kg) and 1/20th (100 mg/kg) doses of LD_{50} were selected. Administration (ip) of these doses for 14 consecutive days rule out the possibility of any significant toxicity in the vital organs like kidney, liver and spleen as evidenced from the histopathological evaluation with hematoxylin and eosin staining of the organs. Almost all the evaluated parameters remained unaltered in the kidney upon PSM001 treatment (Fig. 2a–d, Table S1). Even though enlargement of glomerulus and mild degenerative changes was observed with 400 mg/kg, the lower dosages largely unaffected the kidney structure and function. There was no

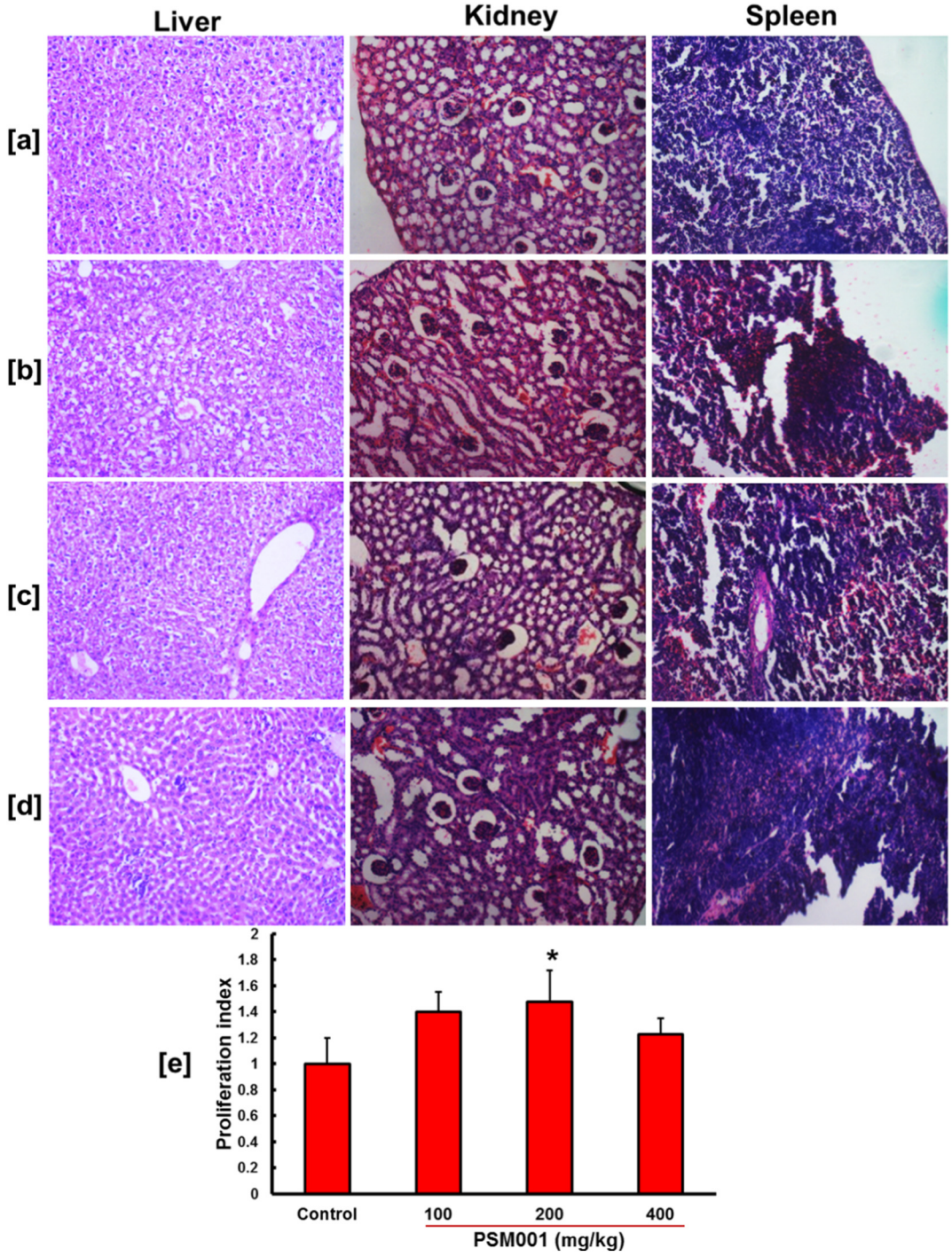


Fig. 2. Hematoxylin & eosin staining with various organs of BALB/c mice after 14 days administration of [a] normal saline, and various concentrations [b] 100 mg/kg, [c] 200 mg/kg and [d] 400 mg/kg PSM001. [e] Proliferation index of lymphocytes isolated from the bone marrow cells after 14 days administration of PSM001 evaluated using MTT Assay. Statistical significance are at $^{*}p < 0.05$ with respect to control.

indication of any major abnormality in the liver with 100 and 200 mg/kg groups, but 400 mg/kg displayed moderate changes with Kupffer cell proliferation, lymphocyte infiltration and central vein congestion (Fig. 2a–d, Table S2). Spleen was found to be largely devoid of any noticeable changes except for the presence of lymphocytes and macrophages (Fig. 2a–d; Table S3). Detailed sub-acute toxicity evaluation exposed the absence of any major biochemical (Table S4) and hematological (Table S5) abnormality emphasising the absence of any major dose-dependent toxicity upon PSM001 treatment. There was a significant increase in the lymphocyte proliferation index of the lymphocytes isolated from the bone marrow with the group administered with 200 mg/kg (Fig. 2e) of PSM001 indicating the immunostimulatory potential of this polysaccharide.

3.4. Effect of PSM001 in ascites tumor bearing mice

Murine ascites tumor cells DLA and EAC bearing mice were evaluated on the 15th day of PSM001 administration for the effects on body weight, tumor volume, tumor cell count, percentage of viable cells, and %ILS. PSM001 significantly reduced the tumor volume and number of

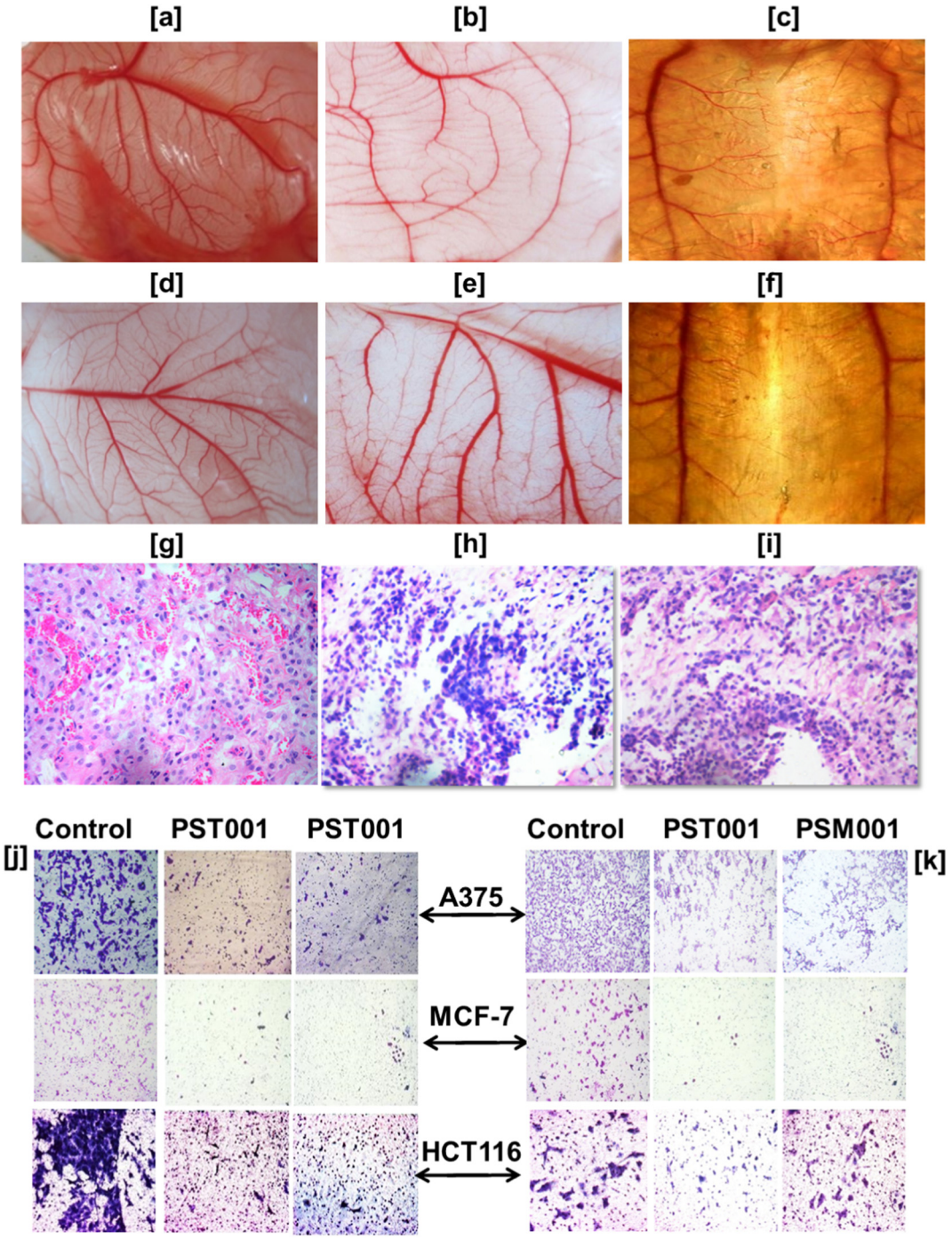


Fig. 3. Inhibition of neovascularization of Chorioallantoic membrane (CAM) by plant polysaccharides PST001 and PSM001. Chick allantoic membrane devoid of polysaccharide treatment [a, d] and treated with PST001 [b] and PSM001 [c]. *In vivo* angiogenesis assay with EAC cells on BALB/c mice with PST001 [c] and PSM001 [d] as viewed under a stereomicroscope. Hematoxylin & eosin staining of the peritoneum sections of vehicle control [g], PST001 [h] and PSM001 [i] treated mice. Effect of plant polysaccharides on the invasion [j] and migration [k] of cancer cells on transwell inserts.

viable cell counts when compared to the vehicle control (Table S6). PSM001 exhibited an increased efficacy in both DLA and EAC model with better results in EAC group (Fig. S3a–f). Dox was also effective in diminishing the tumor volume and number of live tumor cells and the combination group of PSM001 and Dox, which was most effective to alleviate the tumor burden and increase the life span. Although Dox alone could bring about significant reduction in the tumor volume, the survival percentage could not be improved marginally.

3.5. Anti-metastatic potential of plant polysaccharides

The chick chorioallantoic membrane assay was utilised as a means of evaluating the anti-angiogenic potential of both PSM001 and PST001. Both the polysaccharides PST001 (Fig. 3a, b) and PSM001 (Fig. 3d, e) decreased angiogenesis to a greater extent up on comparison with the un-treated membranes. The angiogenesis assay was further extended to an ascitic tumor bearing BALB/c mice which also demonstrated a decrease in the neovascularisation with both PST001 (Fig. 3c) and PSM001 (Fig. 3f) treatment. Also, hematoxylin & eosin staining of

the peritoneum sections showed marked decrease in the number of endothelial cells upon comparison with the control mice (Fig. 3g–i). Next, the effects of PSM001 and PST001 on the metastatic progression were evaluated using invasion and migration assay. Human and murine cancer cell lines exhibited a significant decrease in the rate of invasion (Figs. 3j; S4a) and migration (Figs. 3k; S4b) subjected to the treatment of these polysaccharides. Both PST001 and PSM001 treated cancer cells showed marked decrease in the number of invaded (Table S7) and migrated cells (Table S8) with high inhibition rates. Further, the extent of anti-metastatic potential was illustrated with wound healing assay. The 'wound healing' phenomenon of cancer cells were delayed to a greater extent on A375 (Fig. S5), A549 (Fig. S6), B16F10 (Fig. S7), MCF-7 (Fig. S8) and HCT116 (Fig. S9) cells by *in vitro* wound healing assays up on comparison with the un-treated cells for a period up to 24 h. These results clearly point out the efficacy of PSM001 and PST001in restricting cancer cell progression towards metastasis (Fig. S10). Cancer cells are imprinted with the potential for colony formation which greatly helps them to establish metastatic colonies. The effect of polysaccharides on the clonogenic potential was also evaluated on the cancer cell lines. PSM001 and PST001 reduced the clonogenic potential in different cancer cells (Fig. 4). The most potent effect was seen in A375 cells followed by HCT116 cells. Though PSM001 reduced colony formation in most of the tested cell lines, PST001 was weakly effective in A549 and MCF-7 cells.

3.6. Molecular mechanism underlying the anti-metastatic effects of the polysaccharides

The gross observation of the superior anti-angiogenic and anti-metastatic potential of the polysaccharides prompted us to investigate the underlying molecular mechanism of these processes. Hence we evaluated the protein expression of key players in the above mentioned physiological processes. Since both the polysaccharides produced most favourable effects in A375 cells, it was chosen for the mechanistic studies. Both the polysaccharides actively suppressed MMP-9 and MMP-2 expression in A375 cells (Fig. S11). PSM001 markedly reduced the MMP-2 expression but PST001 displayed potent effect with MMP-9. An up-regulation in the TIMP-1 and TIMP-2 expression was noticed with both PSM001 and PST001treated cancer cells suggesting the involvement of key players in the anti-angiogenic and anti-metastatic effects.

3.7. Polysaccharides inhibit pulmonary metastasis in C57BL/6 murine models

The pulmonary metastasis model with B16F10 melanoma cells in C57BL/6 mice could be used to evaluate the anti-metastatic property of compounds. Administration of polysaccharides alone and in combination with commonly used chemotherapeutic drug vincristine produced auspicious observations. Lung metastatic nodules were effectively reduced up on treatment with PSM001 and PST001 when compared with the control (Fig. 5a upper panel). The histopathological sections of the dissected lung tissues revealed reduced signs of metastasis in all the treatment groups (Fig. 5a lower panel). The effectiveness of these polysaccharides was increased in the combination treatment group with vincristine (Fig. 5b). The combinatorial treatment of PSM001 and vincristine was the most efficient one among all the tested groups. There was no significant change in the body weight after treatment proving their safety and efficacy against cancer.

4. Discussion

PST001 was previously reported to be a galactoxyloglucan with a (1 → 4)-β-D-glucan backbone substituted with side chains of D-xylopyranose and β-D-galactopyranosyl (1 → 2)-α-D-xylopyranose linked (1 → 6) to glucose residues [8,20]. However, the structural elucidation of PSM001 was not reported previously. The major monosaccharide unit of the polysaccharide form *Mangifera indica* is glucose. The higher yield of PST001 than PSM001 makes the isolation of the former an easy task. The anticancer potential of PST001 and its various nano-formulations were much explored but the cytotoxic evaluation of PSM001remained largely untapped. Many of the natural polysaccharide demonstrated cancer targeted cytotoxicity and hence we evaluated the cytotoxic potential of PSM001 on a panel of cancer cell lines. The cytotoxic behavior of PSM001 was limited to higher concentrations and longer incubation periods suggesting that it is not as effective as PST001 as cancer chemotherapeutic agent. Later the mode of cytotoxic behaviour of PSM001 was elucidated to be through the execution of programmed cell death. Many polysaccharides [8,12,13,21] and polysaccharide derived nanoparticles [10,22,23] are reported to execute apoptotic cell death. Most of the clinically used chemotherapeutic agents suppress the host immune system which will further mount an array of off-target toxicity effects. The enhanced lymphocyte proliferation suggested the extensive application of PSM001 as an immunomodulator. In this juncture, the detailed toxicological profiling of PSM001 was explored in BALB/c mice which rules out the possibility of any lethal effects with the absence of LD_{50}. Detailed evaluation of hematological, biochemical and histopathological parameters after the long term toxicity profiling helps to establish PSM001 as a safer agent for *in vivo* administration. Most plant-derived polysaccharides are non-toxic in nature and exhibited significant immune stimulatory effects. Our experience with the polysaccharides PST001 and PSP001 also reported to be non-toxic in nature [9,12,13]. After having established as a non-toxic polymer with moderate cytotoxic potential, we thought of evaluating the anticancer potential of PSM001 in murine ascitic models. The significant reduction in the tumor burden was more pronounced in the group co-administered with doxorubicin, suggesting the potential of PSM001 as a combination agent with known cancer chemotherapeutics. PSM001 significantly reduced the tumor volume and increased the life span of tumor bearing mice. Although PSM001 did not achieve maximal tumor reduction, the increase in life span was the highest, which correlates with the immunomodulatory effects of the compound. Most chemotherapeutic agents have severe side effects and hence co-administration with an immunomodulator like PSM001 will limit their off-target effects and improve the clinical application.

The observation of a significant improvement in the reduction of side effects and enhanced tumor reduction with the combination therapy of PSM001 elated our mode of approach towards the polysaccharides. We have previously established the excellent anti-tumor and immunomodulatory effects of PST001 and its various nano-formulations [9,13,24,25]. Both the polysaccharide can significantly reduce the side effects of the commonly used chemotherapeutic agents to generate synergistic effects. Pectic polysaccharides from Korean citrus hallabong, polysaccharides isolated from mushroom *Cordyceps sinensis* and *Inonotus obliquus* and brown algae *Ascophyllum nodosum* demonstrated similar pattern of moderate cytotoxicity in cancer cells *in vitro* with prominent anti-metastatic effects *in vivo* [15,26,27]. This information along with our own previous experience with PSP001as an excellent anti-metastatic agent [19] stimulated the need for evaluating the potency of PST001 and PSM001 in preventing cancer metastasis. Tumor metastasis accounts for about 90% of all cancer related deaths and hence effective strategies needed to be devised to address this issue. Tumor metastasis is a multistep process involving a typical metastatic cascade of events such as uncontrolled cell proliferation, tissue remodelling, angiogenesis, invasion and migration [28]. Many biologically active molecules and polysaccharides such as fucoidan have been demonstrated to act as a chemo-preventative agent as well as inhibiting lung carcinoma metastasis through down-regulating various key players involved in the metastatic cascade [29].

Angiogenesis is the process of the formation of new blood vessels from existing ones. This process is an essential prerequisite of a tumor to establish with in a new niche and hence many approaches using targeted monoclonal antibodies are in clinics to prevent angiogenesis to

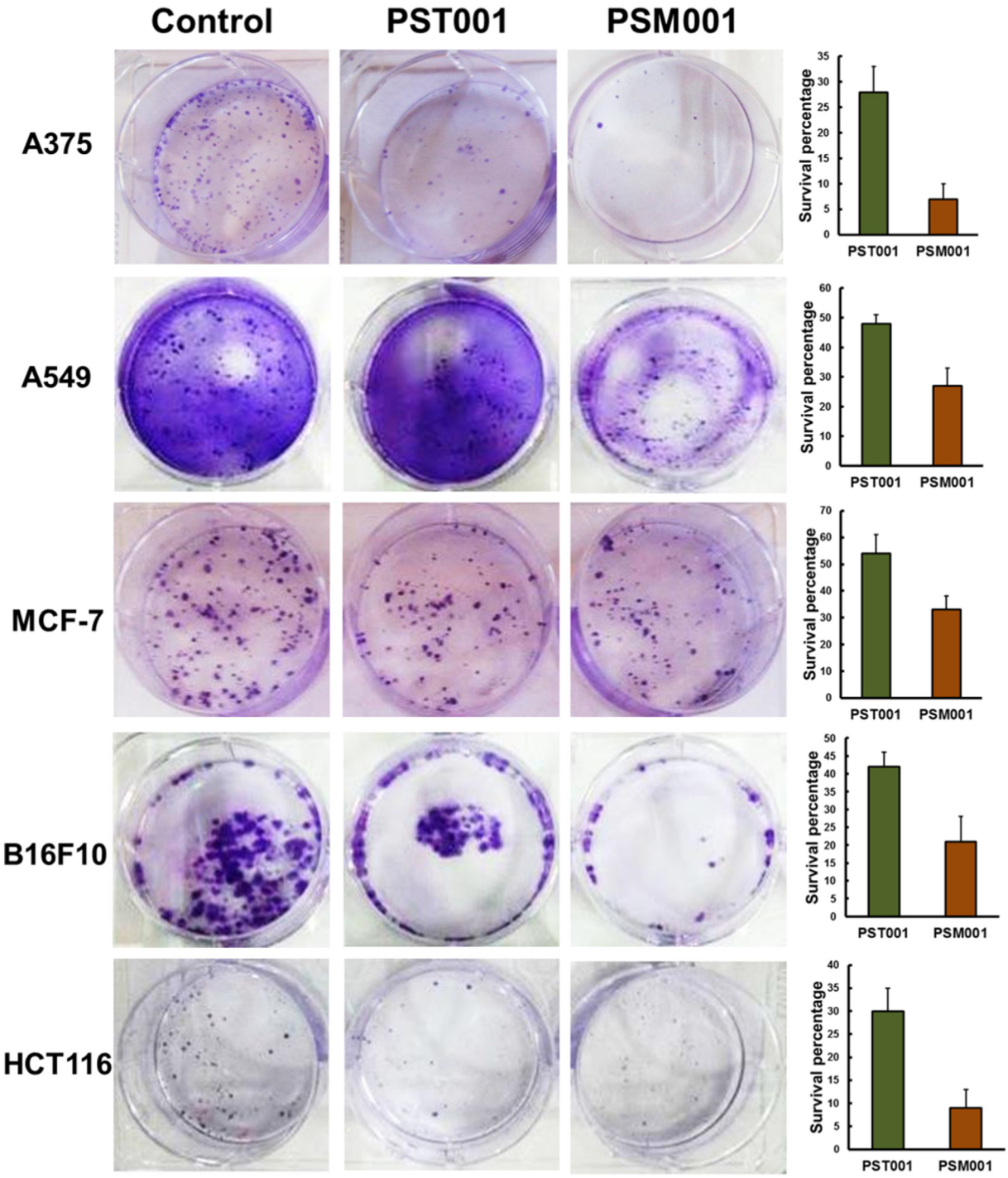


Fig. 4. Inhibition of colony formation ability of human and murine cancer cells treated with polysaccharides. The number of colonies formed was counted and survival percentage with respect to control was calculated. Survival percentage after polysaccharide treatment was calculated with respect to control which was normalised to zero. Data represented as Mean ± SD.

check cancer metastasis. Most of the anti-metastatic agents retard the process of angiogenesis [30,31] and hence the polysaccharides were initially screened for the effects on neovascularization with the *in vitro* CAM assay and *in vivo* peritoneal angiogenesis assay. Both PST001and PSM001 produced moderate effects on CAM and did not harm the viability of the embryos unlike other anti-angiogenic phytochemicals, suggesting non toxicity and detrimental side effects to normal tissues. Invasion and migration of cancer cells through the circulatory system towards distant organs by degrading the extracellular matrix is one among the crucial steps of metastasis. The wound healing assay established the inhibitory role of PST001 and PSM001 in preventing cell invasion and migration. Both the polysaccharides impede the invasion and migration of cancer cells effectively through the suppression of matrix metalloproteinase. The colony formation ability of cancer cells accounts for their metastatic capability and clonogenic assay is still being widely used as a standard experimental procedure to check the effect of cytotoxic agents on the inhibition of colony formation capability of cancer cells [32]. The cancer cells will undergo rapid proliferation and become aggressive to successfully establish a favourable niche through metastasis. A significant reduction in the colony

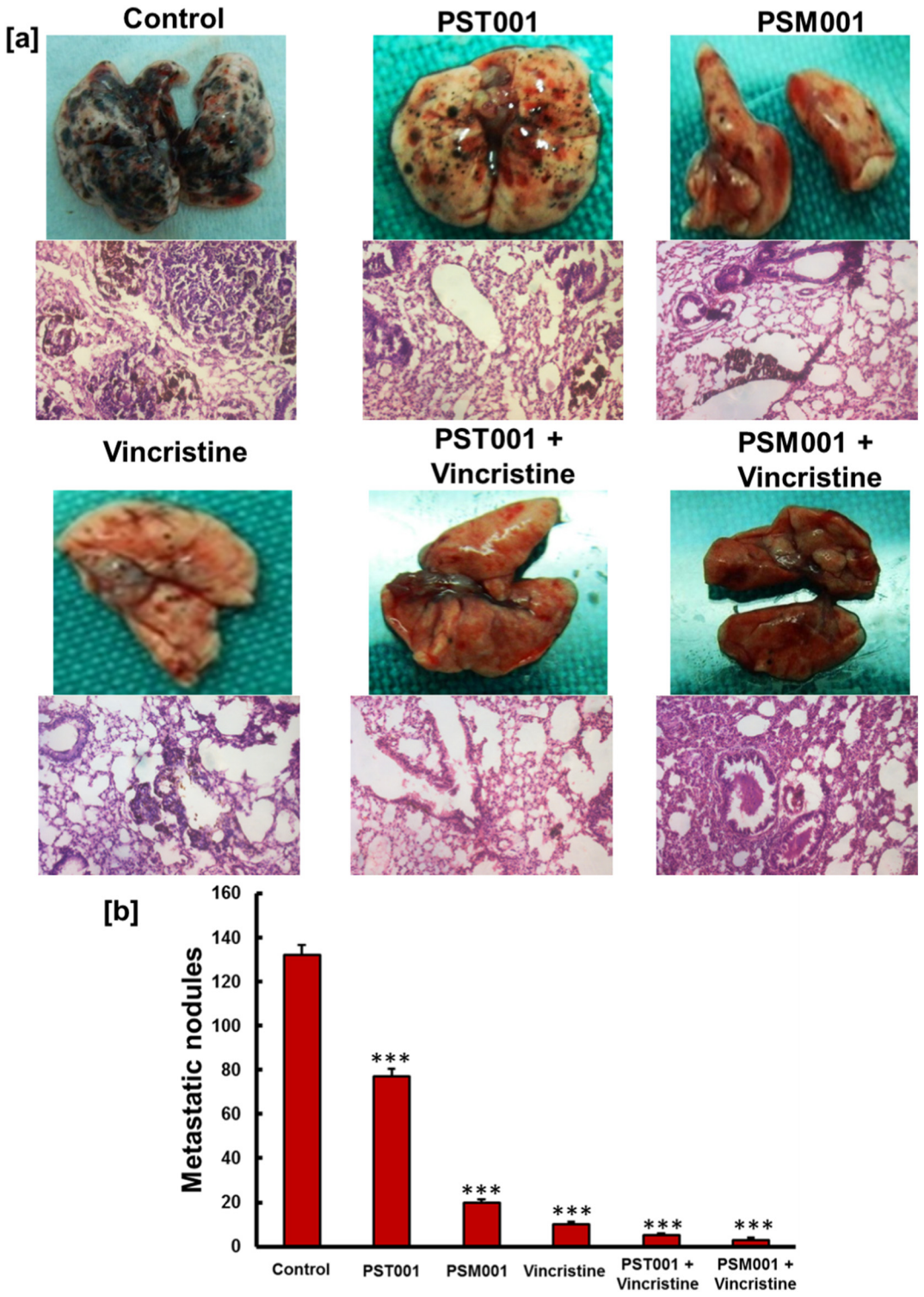


Fig. 5. Effects of polysaccharides, Vincristine and combination therapy on pulmonary metastasis induced by B16F10 cells in C57BL/6 mice. [a] Upper panel shows photographic images of lung colonisation of melanoma cells in C57BL/6 mice on 21st day and the lower panel shows its histopathological features. [b] Inhibition rate of lung colonisation by PST001, PSM001, vincristine and its combination. Statistical significance are at $***p < 0.001$ with respect to control.

formation ability of aggressive cancer cells like A375 and HCT116 cells by both the polysaccharides indicates its role in augmenting the efficiency of host defense system to retard its metastatic potential. The inhibitory capacity towards the colony formation was less pronounced with PST001 up on comparison with the excellent inhibition demonstrated by PSM001 suggesting the outstanding potential of the latter in preventing cancer metastasis. Although PST001 was observed to be a better candidate in terms of cytotoxic potential than PSM001, the latter exhibited many fold improvement in the anti-metastatic capacity.

The greater anti-angiogenic and anti-metastatic potential exhibited by the polysaccharides on cancer cells encouraged us to assess the protein expression of key players in the above mentioned physiological processes. Many members of the metalloproteinase family like MMP-2 and MMP-9 play an active role in the invasion process towards

metastasis [33,34]. MMPs facilitate metastasis by cleavage of extracellular matrix components like laminin 5 and collagen Type IV and exposes hidden sites that favors migration [35,36]. MMPs have also been implicated in the inhibition of activated T cells in tumor resulting in immune suppression. Inhibition of MMP-2 and MMP-9 by PSM001 and PST001 potentiates its role in preventing the complex process of metastasis. The increased lymphocyte proliferation mediated by PSM001 and PST001 strengthens the immune system to suppress the tumor growth and metastasis thereby improving the overall survival. Tissue inhibitors of metalloproteinases (TIMPs) are known to inhibit angiogenesis, tumor growth and also metastasis. Their mechanism of action is through MMP inhibition, preventing the multiplication of cancer and endothelial cells. [37–40]. PSM001 and PST001 up-regulated TIMP-1 and 2 expressions which could be the underlying factor behind their anti-angiogenic and anti-metastatic effects.

After having observed promising observations with the metastatic inhibition of the polysaccharide with the *in vitro* assays, we used pulmonary metastasis model using B16F10 cells in C57BL/6 mice as an *in vivo* system. This experimental metastasis model has addressed only the late events of metastatic cascade which includes the invasion and growth of melanoma cells at the secondary site. A more reasonable approach would be a spontaneous metastasis model of human melanoma xenografts. The PSM001 and PST001 treated C57BL/6 mice showed significant decrease in the number of metastatic lung nodules indicating the anti-metastatic potential of these polysaccharides. Furthermore, the combinatorial approach of these polysaccharides with the chemotherapeutic drug vincristine potentiates their adjuvant capability and augments the efficacy in the suppression of metastases. Corn pectic polysaccharide (COPP) isolated from *Zea mays* L. [41] and pectic polysaccharide purified from *Angelica gigas* [42] inhibited pulmonary metastasis induced by B16F10 melanoma cells through the down-regulation of MMP-9, MMP-2 and VEGF, levels. Circulating tumor cells (CTCs) plays a crucial role in the progression of metastasis. These disseminated tumor cells are faced with an unfavorable atmosphere in a strong host immune response in the peripheral blood [43]. A dysfunctional immune system on the contrary leads to increase in CTCs and poor prognosis of cancer patients [44]. The immunomodulatory effect of the non-toxic polysaccharides PSM001 and PST001 strengthens the immune system and hence can be exploited as a means to suppress the CTCs and thereby bringing out an effective therapeutic strategy against cancer metastasis.

5. Conclusion

Increasingly laid out for all to see and fast becoming a fixture of much discussion is an issue that pertains to the relation between cancer and immune system. In the present study, a largely un-tapped polysaccharide was isolated and characterised from the seed kernel of *Mangifera indica* and its potency as an anticancer agent was evaluated. PSM001 not only demonstrates direct cytotoxic effects in several cancer cell lines through the induction of apoptosis but also significantly improved the survival of ascetic tumor bearing mice. Further, a galactoxyloglucan isolated form *Tamarindus indica* which was previously established to be a potent anticancer agent was investigated for its anti-metastatic properties along with PSM001 using the *in vitro* and *in vivo* models. The outstanding anti-metastatic effect of both the polysaccharides inhibiting many key players of the multifactorial metastatic cascade was deeply elucidated. The anti-angiogenic and anti-metastatic activities exhibited by both PSM001 and PST001 were effectively translated in murine models which specifically and scientifically established our hypothesis. The current investigation provides evidence that the non-toxic polysaccharides could be used as an adjuvant or as single agent for the treatment of metastatic cancer. Moreover the attempts of using these polymers for the fabrication of nanoparticles could yield more light in the quest for targeted medicine with limited off-target effects.

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Appendix A. Supplementary data

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