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The inhibitory effect of anti- tumor polysaccharide from *Punica granatum* on metastasis

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ABSTRACT

Galactomannan (PSP001) isolated from the fruit rind of *Punica granatum* was demonstrated as an excellent antioxidant, immunomodulatory and anticancer agent both *in vitro* and *in vivo* models. Since the most lethal and debilitating attribute of cancer cells is their ability to evolve to a state of malignancy, with key features like increased angiogenesis, invasion, migration, colony formation, and metastasis, the present study focused on evaluating the effects of the galactomannan on tumor and malignancy. PSP001 effectively reduced the neovascularization in chick embryos highlighting its potential as an angiogenic inhibitor. Furthermore, the invasion, migration and clonogenic capacity of human and murine cancer cells were dramatically inhibited by PSP001. Evaluation of the molecular mechanism of its unique potential revealed the down regulation of key players including VEGF, MMP-2, and MMP-9 with marked elevation of TIMP-1 and TIMP-2. The anti-metastatic potential of PSP001 tested in pulmonary metastasis C57BL/6 mice model deciphered the combinatorial administration with vincristine deliberated better survival rates and decreased metastatic index. The angiogenic inhibition potential of PSP001 was further proved with peritoneal angiogenesis assay in BALB/c mice ascitic tumor model. The outcomes of the current investigation highlight the mode of action of antitumor galactomannan in the reduction of tumor malignancy.

1. Introduction

Cancer is a heterogeneous pathological abnormality and accounts for the second leading cause of mortality globally. It is characterized with the uncontrolled proliferation of cells with resistance towards conventional treatment approaches. The various reasons for developing cancer are lifestyle changes, sedentary lifestyle, consumption of alcohol and tobacco, various viral infections, susceptibility to ionizing radiations and other environmental contaminants. Although the diagnosis of this disease at an earlier stage provides hope for patients with increased survival rates, a complete cure becomes very much distant and difficult once the disease progresses. Although chemotherapy has all the earmarks of being the real treatment methodology it is often accompanied with severe side effects, which could lead to opportunistic infections and even death. One of the major drawbacks of current treatment regime is the elusive nature of cancer cells to evade the defense system of the human body and rapid multiplication and disagreement to undergo apoptosis exhibited by normal cells. Tissue invasion and metastasis is one among the hallmarks of cancer [1]. Tumor metastasis accounts for about 90% of all cancer related deaths [2] and hence effective strategies to tackle this major problem is a major global concern among oncologists and the research community.

Carbohydrates are polyhydroxy aldehydes and ketones which serve diverse roles in the human body. Polysaccharides present an enormous variety of structures and are still under-exploited, thus they should be considered as a novel source of natural compounds for drug discovery. Polysaccharides have drawn greater attention in the medical field owing to their varied health benefits and exhibited structural variability with great capacity to hold biological information [3]. In the present scenario, chemotherapeutic agents that are safe for normal cells with immunostimulatory features while destroying cancer cells is on high demand. Several natural polysaccharides isolated mainly from algae, mushroom, plants and animals possess potent immunomodulatory, antioxidant and antitumor activities with no side effects [4,5]. The anti-metastatic and

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anti-angiogenic nature of polysaccharides further enhances their potential as improved candidates in cancer management [6,7].

Punica granatum (Pomegranate) belongs to the family of Punicaceae, grows mainly in the Middle East, India, China, Spain, Israel, and Latin America. Pomegranate is a fruit-bearing deciduous shrub and the extracts from various parts of this plant has been shown to possess antimicrobial, antidiabetic, cardiovascular protection and is effective against a wide range of diseases like diarrhea, bronchitis, allergies and dental caries. Fruits of pomegranate possess a vast ethno medical history and are considered to be a deep reservoir of medically potent molecules. The phytochemistry and pharmacological activities of all *Punica granatum* parts propose broad clinical uses for the treatment and avoidance of cancer and different other ailments where inflammation is believed to assume a crucial etiological part [8]. PSP001 exhibits excellent antioxidant, immunomodulatory and antitumor effects both *in vitro* and *in vivo* models. Moreover, PSP001 imparts no lethal effects towards normal cells and tissues highlighting its potential role for *in vivo* administration. The detailed mechanistic mode of selective cytotoxicity exhibited by the galactomannan was investigated to be through programmed cell death involving the active participation of caspases [9–11]. Likewise, studies from our laboratory on galactoxyloglucan conjugated doxorubicin nanoparticles exhibited a selective inhibitory effect on cancer cells with minimum toxicity to lymphocytes and RBC cells confirmed the *in silico* analysis at genomic and proteomic levels [12].

Tumor metastasis is a multistage process during which malignant cells spread from the primary tumor to discontiguous organs. Cancer metastasis involves a many-sided multiprocess cascade including cell adhesion, migration, invasion, cell-to-cell and cell-to-extracellular matrix interactions. The major cause of cancer-related deaths is often due to tumor cell adhesion, invasion and migration, which results in the formation of distant metastasis [13]. Circulating tumor cells (CTCs) are cells that are derived from the primary tumor and enter the circulation to reach distant organs and develop metastases. CTCs are a major culprit which triggers the mechanism of metastasis that is responsible for innumerable cancer related deaths [14]. Efforts to control these CTCs could greatly succumb the further extension of tumor towards metastasis. Polysaccharides isolated from the peels of *Citrus sphaerocarpa* was reported to possess anti-angiogenic and anti-migratory effects in HUVEC and MDA-MB231 cells respectively [15]. Even though the immunomodulatory and anticancer potential of PSP001 was investigated in detail, the role of polysaccharide in managing tumor metastasis was not deciphered yet. Since the major dilemma of cancer treatment is its metastatic progression and considering the immense therapeutic value of this polysaccharide, objectives of the current study were to evaluate the antimetastatic capability of PSP001 in human and murine cancer cells both *in vitro* and in appropriate murine models. Further, the effect of PSP001 on clonogenic potential, angiogenesis, invasion and migration was also studied and later in depth investigation into the molecular mechanism of action was also carried out (Scheme 1). Although prospective studies are warranted, toward efficacious treatment approaches in cancer, the current investigation will enable further explorations with this non-toxic polysaccharide in cancer management.

2. Materials and Methods

2.1. Isolation and purification of PSP001 from Punica granatum

PSP001 was isolated from ripened fruit rind of *Punica granatum* using the standard procedure illustrated earlier [11]. Briefly, fruit rind was washed thoroughly and shade dried. Hundred gram of this powdered material was treated with 300 mL of petroleum ether for 72 hours with occasional stirring to completely remove the fat content present. The residue obtained after filtration was dried and subjected to hot water extraction. The filtrate thus obtained was precipitated with four folds of ice cold ethanol and kept overnight at 4 °C to maximize precipitation and centrifuged at 12,000 rpm at 4 °C. The pellet obtained was dissolved in distilled water and added with Sevag's reagent to remove protein. This process was repeated until the interphase became clear. Later the solution was dialyzed across distilled water for duration of 48 hours and was concentrated and the above steps were repeated again for several cycles. 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. Briefly, 500 mg of the lyophilized crude sample was suspended in buffer and chromatographed through Sephadex G-200(3 cm × 75 cm) equilibrated with the buffer. Fractions were monitored at 280 nm and at 490 nm after mixing with phenol-sulfuric acid reagent. For PSP001, a single peak was obtained for both the columns. The fractions under the peak were pooled, lyophilized (CHRIST ALPHA 2-4 LD PLUS, Germany) and stored at 4°C for further analysis.

2.2. Culture and maintenance of cell lines

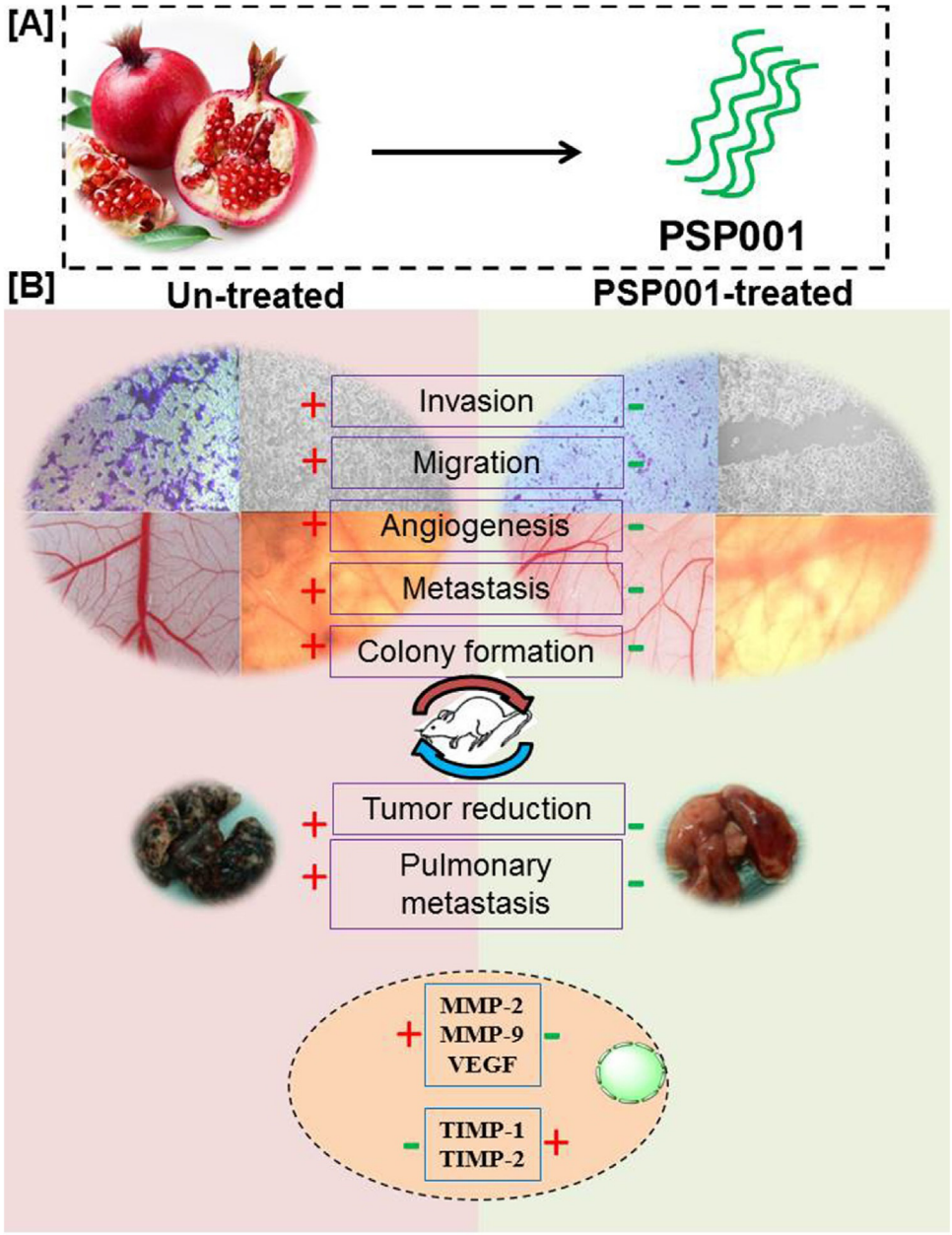
The human cancer cell lines A549 (lung adenocarcinoma), A375 (malignant melanoma) and murine melanoma cell line B16F10 were purchased from NCCS, Pune, India and were maintained in DMEM medium supplemented with 10% bovine fetal calf serum together with antibiotics amphotericin – penicillin mix (Gibco) and kept at 37 °C in 5% CO_2 incubator. The murine transplantable lymphoma cell line, Ehrlich ascites carcinoma (EAC) was maintained in the peritoneal cavity of mice by intraperitoneal (*ip*) transplantation of 1×10^6cells per mouse. All animal experiments were performed in accordance with rules set by the Institutional Animal Ethics Committee (IAEC) and according to the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.3. Chorioallantoic membrane (CAM) assay

Fertilized chicken eggs of White Leghorn were purchased from poultry farm (Kudappanakunnu, Kerala, India). The eggs were candled and observed for viability of embryos, dead embryos were discarded and viable eggs were kept in a humidified incubator at 37 °C. On the fourth day, a small window of 10 mm diameter was created on the egg shell. The window was then closed using cellophane tape and returned back to the incubator. On the 8th day, the window was re-opened and PSP001 at concentrations 50, 100 and 200 μg/mL was added onto the CAM and window was resealed and chick embryos returned back to the incubator. On the 11th day, the CAM was observed for changes in neovascularization using a stereomicroscope and photographed using a camera (Nikon COOLPIX L310) [16].

2.4. Animal models

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



Scheme 1. *PSP001 isolated*from *Punica granatum* inhibits angiogenesis, invasion and metastasis *in vitro* and *in vivo* through the suppression of MMPs and up regulation of TIMPs.

2.5. Invasion and migration assay

The invasive and migration potential of cancer cells in presence of PSP001was carried out using 24 well plate transwell inserts (353097, Corning Falcon, USA). For the invasion assay, the 24 well transwell chambers (8 μm pore size polycarbonate filters) were applied with matrigel (354234, Corning Falcon, USA) on the inner side and kept at 37 °C in 5% CO_2 incubator. Cancer cells were seeded (1×10^6 cells/mL) in serum free medium onto the transwell chambers with or without PSP001 (100 μg/mL). DMEM medium with 10% serum was added onto the 24 well plates which served as the chemo attractant. The plates were kept at 37 °C in 5% CO_2 incubator for 24 hour incubation. The transwell inserts were then taken and cells present in the upper chamber were removed using cotton swab and filters were stained using 1% crystal violet. The invaded cells present at the lower side of the inserts were visualized, counted and photographed using an optical microscope Olympus 1 × 51, Singapore at 10 X magnification. Similarly, the migration assay was carried out using the above protocol except that the transwell inserts were devoid of matrigel coating [17].

2.6. Wound healing assay

Cancer cells (1×10^6 cells/mL) were maintained in DMEM medium on 6 well cell culture plates. After the cells attained confluency, a wound was created using a sterile 100 μL micropipette tip. PSP001 was added to the cells and kept at 37 °C in 5% CO_2 incubator. The cells were viewed at 10 X objective and photographed using a phase contrast microscope (Olympus 1 × 51, Singapore) at various time points (0, 12 and 24 hour) until the monolayer was completely closed [18]. The area of the wound created was quantified using Image J software (version 1.5i). The cell migration rate was calculated using the formula

Cell migration rate = Area of the wound at 0 hour - Area of wound at 24 hour/Area of wound at 0 hour X 100

2.7. Clonogenic assay

Cancer cells (1000 cells/mL) were seeded onto a 6 well cell culture plate and treated with PSP001 for 72 hours. The medium was removed and the cells were kept in fresh medium for 9 days or till the control cells achieved 50 cells per colony. The medium was removed, fixed in 70% ethanol and stained using 1% crystal violet in ethanol. The plate was air dried and the colonies were counted and photographed [19].

2.8. Western blotting

Proteins were extracted from 2×10^6 cells per petridish using RIPA buffer (Thermo Scientific, Rockford, USA). Protein concentrations were determined using Coomassie Plus protein assay reagent and bovine serum albumin (BSA) standards (Pierce, Rockford, USA). Proteins (~50 mg) were separated by 10% SDS-PAGE and transferred to poly vinylidene difluoride (PVDF) membranes (Millipore, MA, USA). Membranes were blocked with 5% BSA and incubated with the specific primary antibodies overnight. The primary antibodies anti MMP-2, anti MMP-9, anti TIMP-1, anti TIMP-2, anti VEGF and antiβ- actin raised in rabbit were purchased from Bioss, USA. The membrane was incubated with horseradish peroxidase conjugated goat anti rabbit secondary antibody (Jackson, Immuno Research Laboratories, USA) for 1 hour at room temperature after washing thrice in TBST. The unbound antibody was washed off with TBST and detected by enhanced chemiluminescence method. The luminescence generated was detected in Flourichem analyzer (FlourChemM, Protein Simple, USA). The resulting bands were then quantitated using Image J software (version 1.48, NIH, USA) and normalized with β-actin [20].

2.9. Peritoneal angiogenesis assay

Ehlrich ascites carcinoma cells (EAC) develops ascites in mice when injected into its peritoneal cavity. Experiments were performed on eight week old female BALB/c mice (6 mice/group).To each group, EAC cells (5×10^6) in 0.5 mL Phosphate buffered saline was injected into the peritoneal cavity. After 5 days of tumor cell administration, PSP001 at 200 mg/kg was given to one group daily while PBS was injected to the vehicle control group. The mice were sacrificed on the 13th day and the peritoneum of control and PSP001 treated mice was removed and photographed using a Nikon COOLPIX L310 camera for evaluation of angiogenesis. The peritoneum was fixed in buffered formalin and processed further to prepare paraffin embedded 5 μm sections. Immuno histochemical analysis using primary rat anti mouse CD31antibody and secondary horse radish peroxidase conjugated goat anti rat antibody (Southern Biotech, Birmingham, USA) and viewed under an Olympus I X 51 microscope with 10 X objective.

2.10. Pulmonary metastasis model

B16F10 melanoma cells (1×10^6cells/mice) in 100 μL PBS were injected via tail vein to male C57BL/6 mice [21]. Three groups were taken for the study with each group containing six animals. Group I was maintained as vehicle control with PBS. In Group II, PSP001 at 200 mg/kg was administered intraperitoneally (*ip*) for 10 days. In Group III, PSP001 at 200 mg/kg and Vincristine at 0.065 mg/kg were administered *ip* simultaneously with the initiation of metastasis and was carried out for 10 days respectively (**SI Scheme**). In Group IV Vincristine at 0.065 mg/kg was administered (*ip*) simultaneously with the induction of metastasis and continued for 10 days. Three animals from each group were sacrificed after 20 days of tumor initiation; dissection of the lungs was carried out. The number of blackish metastatic colonies occupying the entire portion of the lungs was counted under a dissecting microscope.

2.11. In vivo *tumor reduction analysis*

B16F10 melanoma cells were inoculated subcutaneously at 1×10^6 cells/C57BL/6 mice. PSP001 was administered repeatedly for 10 days *ip* at a dosage of 200 mg/kg starting from the 1st day after B16F10 cell administration. Tumor formation and survival was monitored for more than 2 months. A total of 6 animals per group were used for the study. The mortality of each animal was observed and the percentage increase in life span (% ILS) calculated from the formula:

$$\%ILS = [(T - C/C)] \times 100$$

Where T represents the number of survival days of PSP001 treated animals and C represents the number of survival days of control animals.

2.12. Histopathology of lungs

The lung tissues were fixed in 10% formaldehyde solution and dehydrated with graded percentage of ethyl alcohol (50%, 75%, 96% and 99.8%) and later with xylene. The tissues were paraffin embedded and 5 μm sections prepared after freezing on a cold platform were placed on poly lysine coated glass slides. The sections were incubated with xylene and decreasing percentage of aqueous ethyl alcohol (99.8%, 96%, 70%, 50%) and finally rinsed in distilled water. It was stained with Mayer's hematoxylin stain for 12 minutes, rinsed for 15 minutes using tap water and stained in 0.1% eosin solution for 1.5 minutes, dehydrated in graded volumes of aqueous ethyl alcohol (50%, 70%, 96%, 99.8%) and xylene, sealed in DPX and viewed under 10 X objective of Olympus microscope (I × 51).

2.13. 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 and Discussion

3.1. Isolation and Characterization of PSP001

PSP001 isolated from *Punica granatum* is a water soluble polysaccharide, with a neutral pH and sugar content of 97.5% confirmed using phenol- sulfuric acid method with a total yield of 2%. The purification of polysaccharide was done by Gel filtration chromatography with Sephadex G-200 and the molecular weight was determined to be 110 kDa. PSP001 on complete hydrolysis followed by thin layer chromatographic analysis revealed the presence of monosaccharides galactose and mannose. The structure of PSP001 was elucidated as β- 1, 3 galactopyranose backbone along with β-D mannopyranose and α- D mannopyranose side chains [11]. Proposed structure of PSP001 determined after further characterization using techniques like partial hydrolysis followed by periodate oxidation, methylation, acetylation and trimethylsilylation, NMR and FABMS are provided as Supplementary Information.

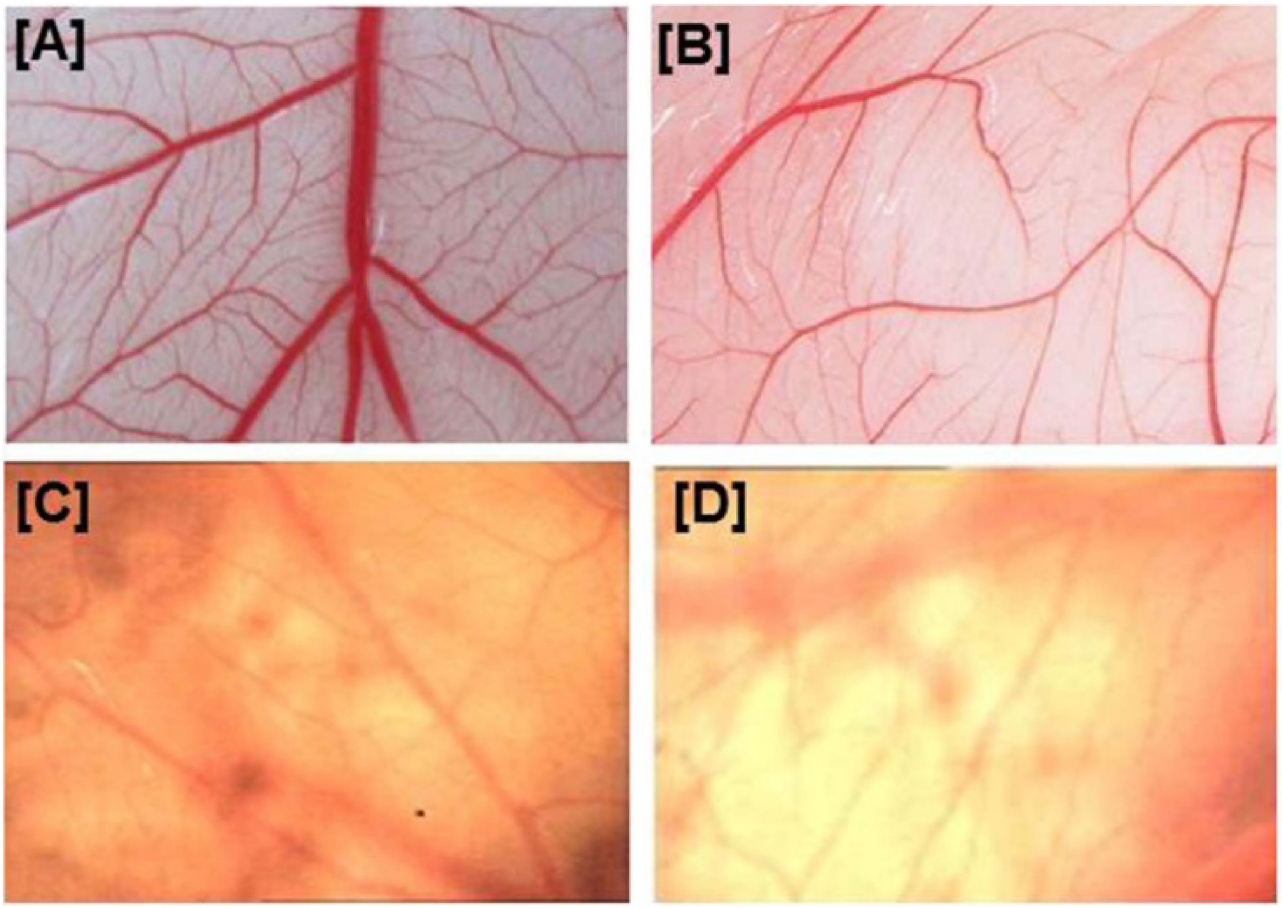


Fig. 1. Inhibition of neovascularization of Chorioallantoic membrane (CAM) by PSP001.CAM was treated with PSP001 at 200 μg/mL on Day 8 and sealed and kept in the incubator. CAM was photographed and analyzed for PSP001 induced changes in neovascularization on the eleventh day. (**A**) Chick chorioallantoic membrane devoid of PSP001 treatment. (**B**) CAM treated with PSP001 at 200 μg/mL. (**C**) Control chorioallantoic membrane as viewed in a stereomicroscope. (**D**) Chorioallantoic membrane treated with PSP001 at 200 μg/mL concentration as viewed in stereomicroscope.

Table 1
Score values for examining the antiangiogenic effect induced on chorioallantoic membrane in chick embryos.

Score value	Observed effect
0	No effect
0.5	Very weak effect, no capillary-free area with reduced capillary density
1	Weak- medium effect, small capillary-free area or area with significantly reduced capillary density
2	Strong effect, wide capillary-free area

3.2. PSP001 retards neovascularization in chick chorioallantoic membrane

Angiogenesis, the formation of new blood vessels from existing ones is an essential prerequisite of a tumor progressing towards metastasis. The spread of the tumor increases vascularization and a manifold increase in the expression of angiogenic factors [22,23]. Many of the anticancer agents execute retarding effects on vascularization and hence PSP001was screened for its anti-angiogenic potential. The negative control Chorioallantoic membrane attained neovascularization as usual. Although PSP001 at concentration of 50 μg/mL decreased angiogenesis (SI. Fig. SS1), a marked inhibition of angiogenesis was noted significantly at 200 μg/mL without affecting the viability of the chick embryos (Fig. 1). A scoring method was adopted as shown in Table 1 [24]. PSP001demonstrated a dosage dependent optimal action on vascularization wherein a dosage of 200 μg/mL received a score of 1. Moreover, the effect of PSP001 on CAM was moderate with a score value of 1 and did not harm the viability of the embryos unlike other anti-angiogenic phytochemicals suggesting non toxicity and detrimental side effects to normal tissues.

3.3. PSP001 inhibits invasion and migration of cancer cell lines

To investigate the effects of PSP001 on invasive and migration ability in cancer cell lines, PSP001 was treated with A549, B16F10 and A375 cells using transwell chamber assay. While the cancer cells freely moved across the matrigel towards the lower chamber of the transwell plate, PSP001 effectively prevented the invasion in all the cancer cells tested significantly (Fig. 2A) with the inhibition rates given in Table 2. PSP001 exhibited inhibition of invasion in MCF-7 (SI. Fig. S2A) and HCT116 cancer cells also (SI. Fig. S2B).The highest inhibition capacity was noted in A549 cells (93.47 ± 2.82) followed by B16F10 cells (92.11 ± 1.93). PSP001 significantly retarded the migration of cancer cells (Fig. 2B) with a maximal effect in A549 cells (91.67 ± 1.65) followed by B16F10 melanoma cells (90.25 ± 1.28) as shown in Table 2.The migration of HCT116 cells was also significantly reduced by PSP001 (SI. Fig.

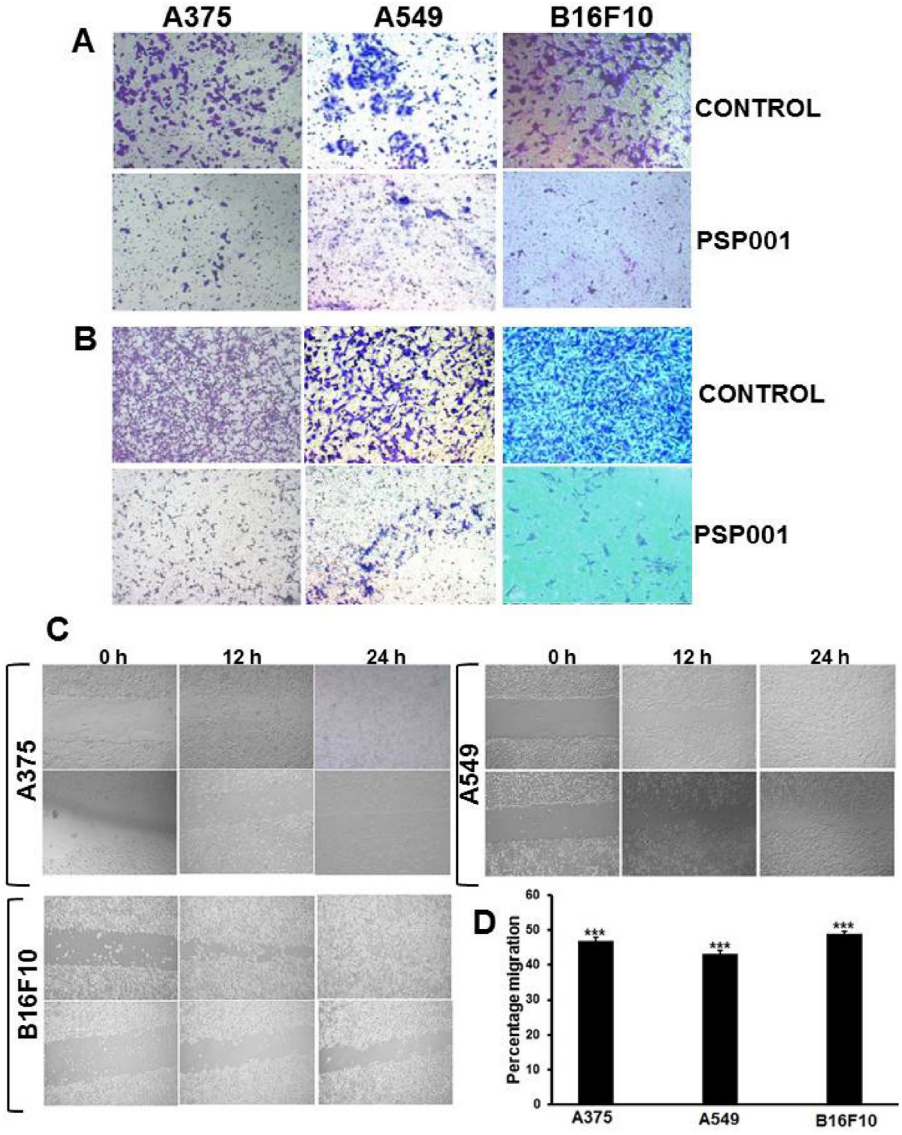


Fig. 2. *Effects* of PSP001 (100 μg/mL) on the invasion and migration of cancer cells*in vitro*. (**A**) Invasion assay and (**B**) migration assay in A375, A549 and B16F10 cells *in vitro*. **(C)** Evaluation of migration rate of PSP001 wherein the upper lane represents the untreated and lower lanes represents cells with PSP001 treatment and **(D)** represents graphical representation of wound healing assay after 24 hour after normalizing with control. Statistical significance are at ***p <0.001 with respect to control.

Table 2
Evaluation of number of cells invaded and migrated with their inhibition rates in control and PSP001 (100 μg/mL) treated cancer cell lines. Values are represented as mean ± SD.Statistical significances are at ***p <0.001 with respect to control.

Cell line	Control		PSP001(100 μg/mL)		Inhibition rate	
	No. of invaded cells	No. of migrated cells	No. of invaded cells	No. of migrated cells	Invasion	Migration
A375	181.6 ± 2.5	504 ± 5.8	34.33 ± 3** *	182.6 ± 5.5***	81.06 ± 1.79	63.95 ± 1.54
A549	101.6 ± 3.5	277.66 ±3.51	6.66 ±3***	23 ± 4.5***	93.47 ± 2.82	91.67 ± 1.65
B16F10	122.3 ± 2.08	480.3 ± 4.04	9.66 ± 2.51***	50 ±3***	92.11 ± 1.93	90.25 ± 1.28

S2C). Wound healing assay clearly demonstrated that the control cells achieved confluency on the "wound" created over a period of 24 hours, while the PSP001 treatment prevents the cancer cells (A375, A549 and B16F10) from wound closure in a dosage dependent manner (Fig. 2C) with significant inhibition in migration rate (Fig. 2D).

The wound healing assay further re-enforces the inhibitory role of PSP001 in preventing cell migration. The prime step of metastasis is the migration of neoplastic cells defined as tumor invasion. Invasion and migration of cancer cells through the circulatory system towards distant organs by degrading the extracellular matrix is one of the crucial steps of metastasis [25]. PSP001 impedes the invasion and migration of cancer cells effectively and inhibits cancer cells ability to cleave the extracellular matrix possibly through the suppression of matrix metalloproteinase like MMP-2 and MMP-9.

3.4. Effect of PSP001 with colony formation ability of cancer cells

As cancer cells acquire the ability of rapid multiplication and tumor formation, PSP001 was tested on cancer cells to check its effects on colony formation. It was noticed that PSP001 markedly reduced the clonogenic ability of cancer cells in a dosage dependent

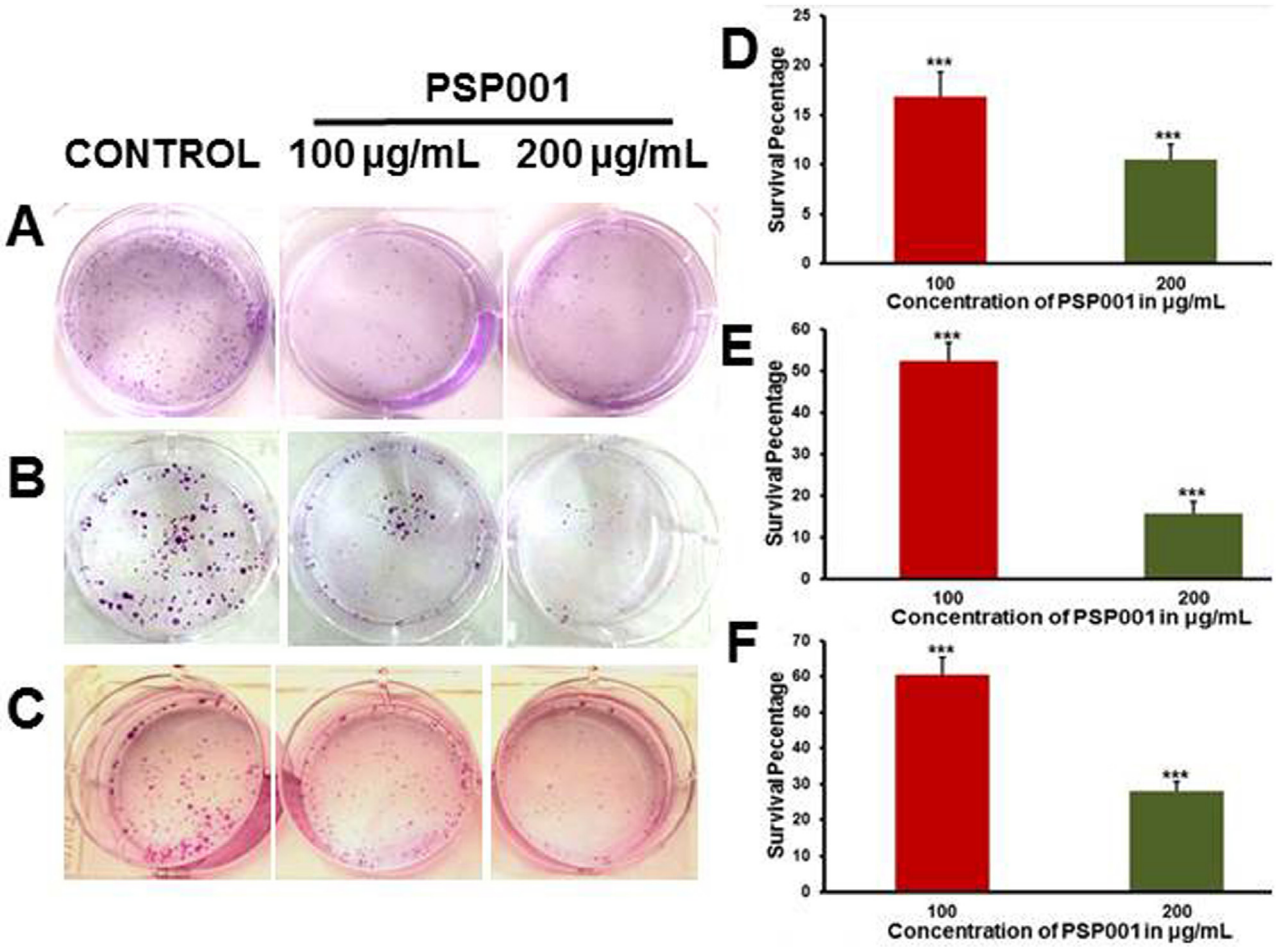


Fig. 3. Inhibition of colony formation ability of cancer cells *in vitro*. Human cancer cells namely A375, A549 and murine melanoma cells B16F10 were treated with PSP001 for 72 hours. The number of colonies formed were counted and survival percentage with respect to control was calculated. Photographs of clonogenic assay on **(A)** A375, **(B)** A549 and **(C)** B16F10 cells. Survival percentage after PSP001 treatment on **(D)** A375, (**E**) A549 and (**F**) on B16F10 cells with respect to control which was normalized to zero. Data represented as Mean ± SD. *p <0.05 with respect to control.

manner as shown in Fig. 3. The highest reduction in the number of colonies was noted for PSP001 at 100 µg/mL with a survival percentage of 16.9± 2.4 in A375 melanoma cells followed by 52.4 ± 4.3 in A549 cells and 60.39 ± 4.8 in B16F10 cells. PSP001 at 200 µg/mL showed active inhibition in colony formation with survival percentage of 10.05 ± 1.5 in A375 cells followed by 15.85± 2.7 in A549 cells and 27.72 ± 2.6 in B16F10 cells. PSP001at 200 µg/mL also efficiently decreased the number of colonies in MCF-7 cells and HCT116 cells with survival percentage of 63 ± 4 and 42.2 ± 2.86 respectively when compared to the control (SI. Fig. S3 A&B). Clonogenic assay is still being widely used as a standard experimental procedure to understand the sensitivity of cytotoxic agents on the colony formation capability of cancer cells [26]. A state of malignancy develops from a complex interaction between the tumor-host environments wherein the neoplastic cells acquire selective mechanisms to evade the host defense mechanisms [27]. These neoplastic cells become immortal, proliferate rapidly and become aggressive to successfully establish a favorable niche through metastasis. They cross the protective proliferative barrier mortality stage 1 regulated mainly by tumor suppressor genes. A significant reduction in the colony formation of aggressive cancer cells like B16F10 and A375 cells by PSP001 indicates its role in augmenting the efficiency of host defense system to retard its metastatic potential.

3.5. PSP001 suppresses the expression of VEGF, MMP-2 and MMP-9 in cancer cells

The superior anti-angiogenic and anti-metastatic potential exhibited by PSP001 on cancer cells prompted us to evaluate the protein expression of key players in the above mentioned physiological processes. Vascular endothelial growth factor (VEGF) and matrix metalloproteinase MMP-2 and 9 in PSP001 treated A375 and A549 cells (Fig. 4), was found to be largely effected. PSP001 down regulates the expression of VEGF in A375 and A549 cells in a dosage responsive manner. Similarly the inhibition of MMP-2 and MMP-9 by PSP001 further expanded its anti-metastatic and anti-angiogenic activities.

Angiogenesis is one of the necessary steps needed for the expansion and growth of tumors. Growth factors like VEGF are released by cancer cells to promote sprouting and branching from existing blood vessels towards the tumor for better supply of nutrients [28]. Matrix metalloproteinases (MMPs) play a significant role in tumor angiogenesis and metastasis. Extra cellular matrix degradation by MMPs facilitates the tumor cell migration, invasion and proliferation. The over expression of MMPs especially MMP-2 and MMP-9 has been universally seen in a wide variety of cancers with direct association to tumor advancement and metastasis [29,30,31]. Galectin- 3 is a substrate of MMPs. The presence of galactose residues in pectic polysaccharides of corn and modified citrus pectin contributes to its anti-metastatic capability mainly

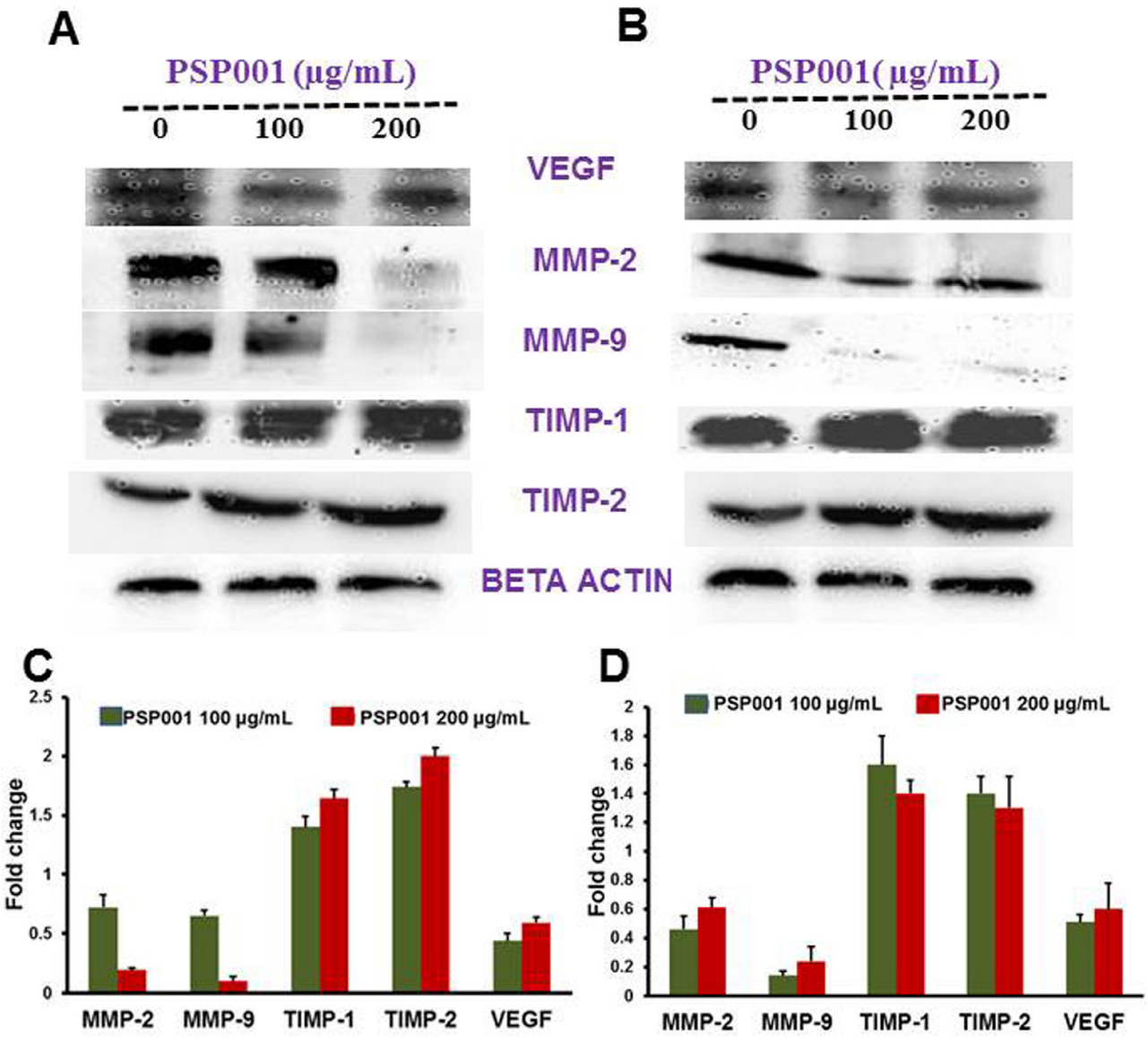


Fig. 4. *Evaluation*of expression levels of proteins involved in angiogenesis and metastasis in vitro. (**A)**Western blot analysis of MMPs, TIMPs and VEGF on PSP001 treated A375 and (**B)** on A549 cells. (**C)** Fold change evaluation of protein involved in metastasis and angiogenesis of PSP001 treated A375 cells with respect to control. (**D)**Fold change evaluation of protein involved in metastasis and angiogenesis of PSP001 treated A549 cells with respect to control. Data are expressed as Mean ±SD.

because galectin- 3 has a galactose binding site which prevents MMPs activation [32,33]. Also, anti-metastatic polysaccharides isolated from *Cordyceps sinensis* and *Colocasia esculenta* also contained monosaccharides galactose and mannose as a main constituent in their structural backbone [34,35]. Hence the anti-metastatic activity exhibited by PSP001 polysaccharide may be attributed in part to its monosaccharide chemical composition and structure.

3.6. Expression of TIMP-1 and TIMP-2 was upregulated by PSP001

Tissue inhibitors of metalloproteinases, TIMPs are inhibitors of active MMPs. TIMP-1 and TIMP-2, the inhibitors of matrix metalloproteinase MMP-9 and MMP-2. Also TIMP-2 inhibits angiogenesis *in vivo* [36]. TIMP-1 and TIMP-2 were up regulated in PSP001 treated A375 and A549 cells with respect to control (Fig. 4). TIMP-2 was noticeably up regulated than TIMP-1 in A375 while TIMP-1 was slightly upregulated than TIMP-2 in A549 cells after PSP001 treatment implicating its role in suppression of metastasis via suppressing angiogenesis. It has been well documented that TIMPs possess anti-angiogenic activity by the suppression of MMPs or through a direct inhibition of endothelial cell proliferation [37,38].

3.7. PSP001 retards peritoneal angiogenesis

As increased angiogenesis is a major feature in tumor malignancy, we checked the effect of PSP001 on tumor cell induced peritoneal angiogenesis *in vivo*. Administration of PSP001 at 200 mg/kg visibly diminished the peritoneal angiogenesis in EAC induced BALB/c mice (SI. Fig. S4A). The immuno-histochemical sections of peritoneum were stained with anti-CD31 antibody which is specific for endothelial cells. The peritoneum of the control BALB/c mice showed CD31 positivity of 36.3± 1.2 whereas only 1.7± 0.5 cells were positive for CD31 in the peritoneum of BALB/c mice treated with PSP001 (SI. Fig. S4B). The H & E sections of the control peritoneum of EAC tumor bearing mice (SI. Fig. S5A) shows focal collection of inflammatory cells along with neoplastic cells and increased angiogenesis while PSP001 treated peritoneum section (SI.Fig. 5B) shows foci of neoplastic cells and

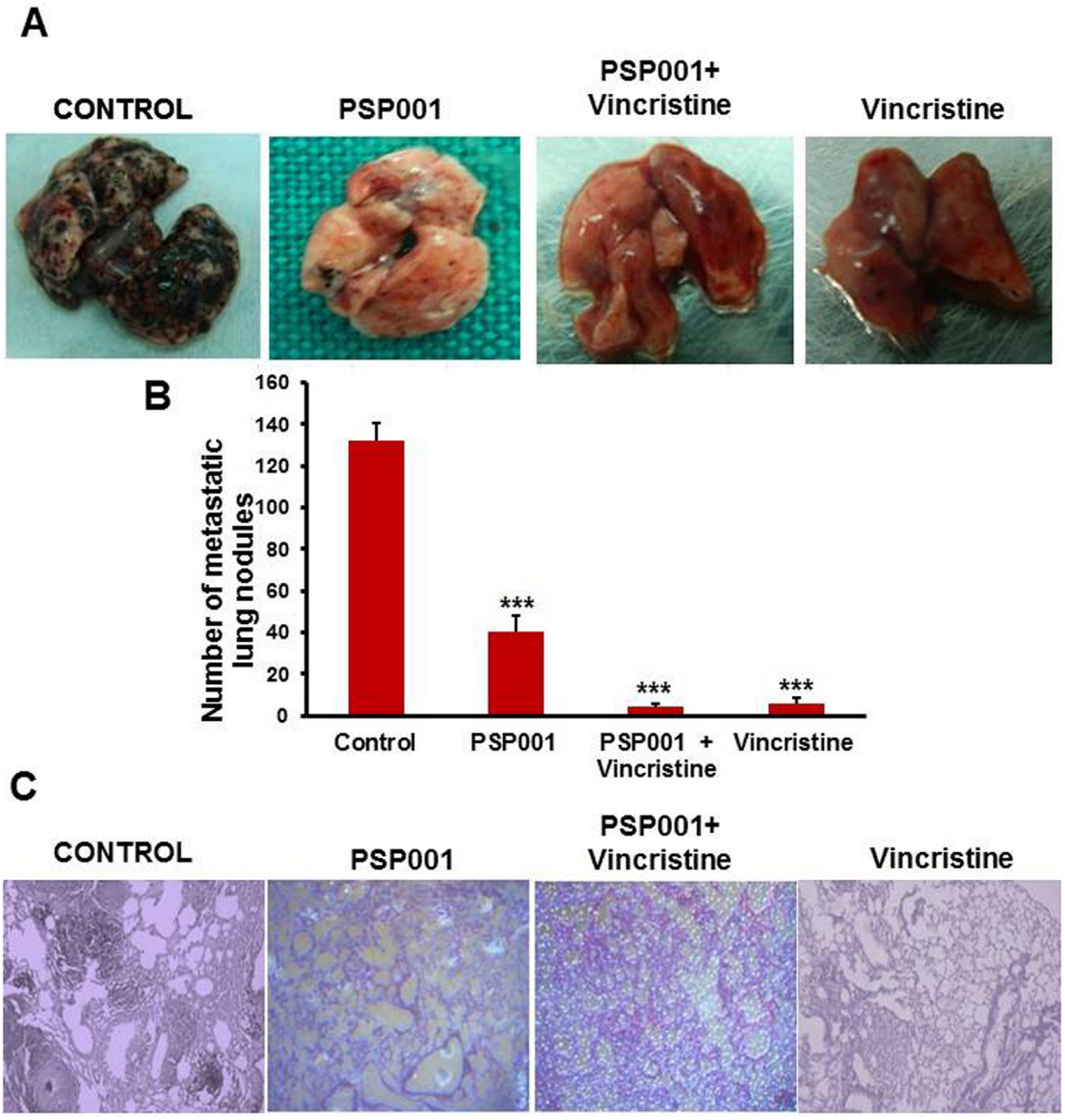


Fig. 5. Effects of PSP001 and vincristine on pulmonary metastasis induced by B16F10 cells in C57BL/6 mice *in vivo*. B16F10 melanoma cells were injected via tail vein on C57BL/6 mice. PSP001, vincristine and its combination were administered to each treatment group (6mice/group) from day 2 for 10 days. The mice were sacrificed on Day 21 and lungs were inspected for metastasis, nodules were counted and subjected to histopathological examination. (**A)** Lung colonization of melanoma cells in C57BL/6 mice on 21st day. (**B)** Inhibition rate of lung colonization by PSP001, vincristine and its combination at doses 200 mg/kg and 0.065 mg/kg respectively. (**C)**Histopathological features of lungs after administration of B16F10 cells *in vivo*. Statistical significance are at *p <0.05 with respect to control.

nearby area with benign cells admixed with angiogenic areas. The control peritoneum sections shows benign region with reactive mesothelial cells and normal vasculature surrounding the tumor cells (SI. Fig. S6A) and also the vasculature in the adjacent normal tissues surrounding the tumor area were not hindered after PSP001 treatment thus unaffecting the normal wound healing process (SI. Fig. S6B). PSP001 hence successfully prevents the migration of endothelial cells during tumor progression without hampering the adjacent normal vasculature (SI. Fig. S7). Endothelial cell migration is an important factor in the process of angiogenesis during tumor growth [39]. The increased angiogenesis at the site of tumor development helps to meet the growing demand of oxygen and nutrient depletion resulting in the tumor dissemination and metastasis. Inhibition of angiogenesis by PSP001 may help to curb the transition of neoplastic cells to metastasis to a great extent.

3.8. PSP001 inhibits pulmonary lung metastasis in C57BL/6 mice model

B16F10 cells were injected via tail vein to create pulmonary lung metastasis in C57BL/6 mice. The cytotoxicity studies of PSP001 in B16F10 cells using MTT assay showed no significant cytotoxicity at 100 and 200 μg/mL at 24 hour incubation (SI. Fig. S8). Hence these cells were used for subsequent anti- metastatic studies of PSP001. Extensive lung metastasis was observed in the vehicle control group characterized by the blackish metastatic nodules, the

significant reduction in the metastatic nodules in the groups treated with PSP001 and combination of PSP001 and Vincristine confirmed the therapeutic potential of PSP001 *in vivo* (Fig. 5A & B). Vincristine, a well- known chemotherapeutic drug minimized the lung nodules but the combined treatment of vincristine and PSP001 markedly reduced the number of lung nodules and improved the survival to a great extent. The histopathological sections of the lung tissue in the vehicle control mice revealed typical features of melanoma cells with melanin pigmentation while there was minimum melanin pigmentation in the PSP001treated sections with prominent effect in the combination treatment of PSP001 and vincristine which was significantly lower than the vincristine treatment (Fig. 5C). Gamma glutamyl transpeptidase is a marker for tumor cell proliferation and metastasis and its level increases manifold during metastasis condition. The elevation of gamma glutamyl transpeptidase and nitric oxide in the control C57BL/6 mice was significantly decreased with PSP001 and combination treatment group of PSP001 and vincristine (SI. Table S1). A significant increase in life span of 61 and 85% was observed in PSP001 and combination treatment groups respectively. The combinatorial treatment approach slightly increased the survival of mice than vincristine alone treated mice (81%). Several pectic plant polysaccharides have been reported to possess anti-metastatic activity *in vivo*. Corn pectic polysaccharide (COPP) isolated from *Zea mays L.* was found to inhibit pulmonary metastasis induced by B16F10 melanoma cells and also down regulated VEGF, MMP-2 and MMP-9 levels *in vitro* [31]. Angelan pectic polysaccharide purified from *Angelica gigas* Nakai inhibited B16F10 induced lung *metastasis in vivo* [40].

One of the crucial steps in the hematogenous metastasis includes the tumor cell extravasation, attachment of circulating tumor cells (CTCs) onto endothelial cells forming a tumor emboli and later transportation through the circulation with colonization at the target secondary organs [41,42]. The primary selection for the secondary sites of metastasis arises at the time of attachment of the CTCs to the endothelial cells at that site [43]. The inhibition of CTCs to colonize at these secondary organs highly helps in controlling the metastatic process. Many reports suggest the presence of tumor infiltrating lymphocytes (TIL) that develop an adaptive antitumor activity in many malignant tumors with increased survival in patients afflicted with melanoma, ovarian, esophageal, colorectal and breast cancers [44–46]. Additionally NK cells along with macrophages have found to possess the ability to block CTCs and prevent metastasis [47,48]. Moreover, the condition of lymphocytopenia has been correlated to metastatic progression due to CTC prevalence [49]. PSP001 acts as a potent immune-enhancer and a powerful free radical scavenger [10,11] both *in vitro* and *in vivo* which will ultimately stimulate the defense mechanism to cope with invading CTCs, preventing metastasis. The antitumor effects of PSP001 *in vitro* and *in vivo* models were also proven in our earlier studies. PSP001, a natural polysaccharide elicits multi faceted approach in inhibiting cancer cells with its antitumor activity, anti-angiogenic activity, decreased colony formation probably by inhibition of binding of tumor cells to the endothelial cells and hence may attribute in the inhibition of tumor progression by arresting CTC expedition towards metastases. The metastasis prevention capacity of PSP001 could be due to the culmination of its antitumor and immunomodulatory properties.

4. Conclusion

The current study showed that the anticancer potential of naturally obtained galactomannan from *P. granatum*, with direct cytotoxic effects in several cancer cell lines and ascitic tumors was through inhibition of tumor metastasis. The anti-angiogenic and anti-metastatic activities exhibited by PSP001 was effectively translated in murine models. PSP001 effectively diminished the crucial steps of metastatic cascade: angiogenesis, invasion, migration and colonization of neoplastic cells. Thus, this study provides evidence of the non-toxic polysaccharide, which could be used as an adjuvant or as single agent for the treatment of metastatic cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2017.05.137

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