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Inhibition of galectin-3 mediated cellular interactions by pectic polysaccharides from dietary sources

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Abstract Pectic polysaccharides from dietary sources such as *Decalepis hamiltonii*—swallow root (SRPP), *Hemidesmus indicus* (HPP), *Nigella sativa*—black cumin (BCPP), *Andrographis serpyllifolia*—(APP), *Zingiber officinale*—ginger (GRPP) and, citrus pectin (CPP) were examined for galectin inhibitory activity. Inhibition of (a) galectin-3 of MDA-MB-231 cells induced hemagglutination of red blood cells; (b) galectin-3 mediated interaction between normal/metastatic human buccal cells (NBC)/(MBC) and; (c) invasion of MDA-MB-231 and MBC in the invasive chamber was assessed. Results indicated that SRPP inhibited hemagglutination at Minimum Inhibitory Concentration (MIC) of 1.86 μg ml^{-1} equivalent of carbohydrate as apposed to those of BCPP (130 μg ml^{-1}), APP (40 μg ml^{-1}), HPP (40 μg ml^{-1}) and CPP (25 μg ml^{-1}). GRPP even at concentration >1–6 mg ml^{-1} did not inhibit agglutination. Also SRPP showed ~15 and 2 fold potent anti hemagglutination activity relative to that of galectin-3 specific sugars—galactose (MIC-27.1 μg ml^{-1}) and lactose (MIC-4.16 μg ml^{-1}) respectively. Further, SRPP at 10 μg ml^{-1} inhibited agglutination of NBC by galectin-3 of MDA-MB-231 cells. Modified swallow root pectic polysaccharide (MSRPP) of 50 kDa retained anti hemagglutination activity (MIC of 1.03 μg ml^{-1}) and inhibited MDA-MB-231 and MBC invasion by 73 and 50% with an IC_{50} of 136 and 200 μg ml^{-1} respectively. Both SRPP and MSRPP induced apoptosis up to 80% at 100 μg ml^{-1} concentration by activating ~2 and 8 folds of Caspase-3 activity. Sugar composition analysis and its correlation with the galectin inhibitory property indicated that pectic polysaccharides with higher arabinose and galactose content—arabinogalactan inhibited hemagglutination significantly.

Keywords Galectin-3 · Pectic polysaccharide · Hemagglutination · Anti metastatic activity · Arabinogalactan · Modified pectic polysaccharide

Abbreviations

SRPP	swallow root pectic polysaccharide
HPP	*Hemidesmus* pectic polysaccharide
BCPP	black cumin pectic polysaccharide
APP	*Andrographis* pectic polysaccharide
GRPP	ginger pectic polysaccharide
CPP	citrus pectic polysaccharide
NBC	normal human buccal cells
MBC	metastatic human buccal cells
MIC	Minimum Inhibitory Concentration
MSRPP	modified swallow root pectic polysaccharide

Introduction

Plant and plant products are being used as a source of diet and medicine since long. Diet invariably contains carbohydrates of various size, concentrations and chemical sequences starting from simple monosaccharide to complex polysaccharides. Among them pectic polysaccharides in particular have been shown to play critical therapeutic roles against cancer [25], immuno-modulation [40], ulcer [14] etc. Recently pectic polysaccharides have gained importance due to their role played in controlling cancer metastasis through the blockade of galectin present on the metastatic cancer cells [12].

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Galectins are a group of proteins, which are evolutionarily conserved family of mammalian lectins with multifunctional properties [30]. Galectins are a family of carbohydrate binding proteins that recognize structural variations among β-galactoside containing glycoconjugates. They are widely distributed in the animal kingdom from lower invertebrates to mammals. Sixteen galectin proteins representing a high degree of sequence identity in their carbohydrate recognition domains (CRD) have been described [7, 8, 24, 35]. Galectin-3, an approximately 31–42 kDa protein is one of the β-galactoside binding proteins that bind to the carbohydrate portion of cell surface glycoconjugates. Recent studies have indicated that galectin-3 expression is correlated with metastatic potential in certain malignancies [3]. Results of several investigations have revealed the possibility of galectin-3 as a diagnostic marker in certain cancers and also one of the target proteins for cancer treatment [16]. Further, higher levels of galectin-3 have been shown to correlate with the advancement of the cancer disease and it is believed that galectin-3 of cancer cells binds to normal cells and transforms them to cancer cells and hence establishes secondary tumors. Galectin-3 hence has been implicated in tumor spread and metastasis [36]. Studies have also indicated that oral administration of modified citrus pectin reduced the rate of cancer cell spread and inhibited metastasis in animal models [27]. However, despite a promising role of dietary pectins against metastasis, only few reports are available on identification of such components from dietary sources, which can potentially be explored for the effective management of metastasis. We designate such components—pectic polysaccharides that block the binding of galectin-3 to β-D-galactoside residues present on the extracellular matrix components of normal cell and basement membranes, as galectin inhibitors. In the present study we report a. quantitative levels of galectin-3 in various normal and metastatic cells and; b. effective inhibition of galectin-3 mediated agglutination, cell invasion and cancer cell-normal cell interaction by dietary galectin inhibitors.

Materials and methods

Materials

Minimum essential medium (MEM), Dulbecco's modified eagle's medium (DMEM), glutamine, sodium bicarbonate, penicillin, kanamycin, F12 mixture, FBS (fetal bovine serum), MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl 2-H-tetrazolium bromide), HEPES -(4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid), triton X 100, sucrose, tween 20, EDTA (Ethylenediaminetetraacetic acid), skimmed milk powder, PNPP (paranitrophenyl phosphate), diethanolamine, Alsever's medium, trypsin, hematoxylin, eosin, acridine orange, carbohydrate standards such as rhamnose, arabinose, xylose, mannose, galactose and glucose, protease, termamylase, glucoamylase etc., were purchased from Sigma Chemical Co, St. Louis, MO, USA. Alkaline phosphatase conjugated-rabbit anti mouse IgG, Peroxidase conjugated and fluorescein isothiocyanate conjugated-goat anti mouse IgG secondary antibodies were procured from GENEI, Bangalore, India. The other chemicals such as hexane, ammonium oxalate, iodine solution, sodium phosphate buffer, perchloric acid, acetic acid, sodium acetate, glutaraldehyde, glycine, sodium chloride, amberlite IR 120 H^+ resin, sulphuric acid and solvents used were of the analytical grade purchased from a local chemical company, Sisco Research Laboratories, Mumbai, India

Preparation of galectin-inhibitory/pectic polysaccharide from dietary sources

Fresh *D. hamiltonii* (root), *N. sativa* (seeds), *A. serpyllifolia* (leaves), *Z. officinale* (rhizome) and *H. indicus* (root) were purchased from a local market (Devaraja Market, Mysore, Karnataka, India), while citrus pectin and Larchwood arabinogalactan were procured from Sigma Chemical Co, USA. Raw samples were chopped into small pieces and air-dried in the dark in a ventilated hood. The air dried samples were ground and defatted in a soxhlet apparatus using hexane in the ratio of 200 mL g^{-1} (w/v). The defatted powder was air dried and preserved in dry condition until further extraction of pectic polysaccharides.

Pectic polysaccharides were isolated following the ammonium oxalate extraction of 10 g of defatted samples after removing proteins, amylose and amylopectins by specific enzymatic (protease, termamylase and glucoamylase) digestions at their optimum reaction conditions as per the protocol described by Phatak et al., [26]. The residue thus obtained was extracted with 200 mL of 0.25% (w/v), ammonium oxalate solution pH-3.5, boiled for 1 h at 70°C with occasional shaking. The contents were filtered and the supernatant was precipitated with four volumes of absolute ethanol at 4°C and the precipitate was collected after 1 h by centrifugation at 5,000 g for 20 min. The pellet was washed twice with 50 ml of ethanol, resuspended in 10 ml of water and lyophilized to get pectic polysaccharide. Total sugar content was estimated by Phenol-sulphuric acid method [29]. For cell uptake studies, small molecular weight forms of the selected pectic polysaccharide were prepared by acid precipitation method [20]. Briefly pectic polysaccharide was solubilized as a 1.5% solution in distilled water, and its pH was increased to 10.0 with 3N NaOH for 1 h at 50–60°C. The solution was then cooled to room temperature while its pH was adjusted to 3.0 with 3N HCl and stored overnight. Samples were precipitated the next day with 95% ethanol

and incubated at −20°C for 2 h, filtered, washed with acetone, and dried on Whatman No.1 filter paper. Polysaccharides obtained were designated as modified pectic polysaccharide (MPP). MPP from swallow root is designated as modified swallow root pectic polysaccharide (MSRPP).

Screening of cell lines for galectin-3

Growth and maintenance

Normal cell lines such as mouse embryonic fibroblast (NIH-3T3), monkey kidney fibroblast (VERO) and cancerous cell lines namely human embryonic kidney (HEK 293), human epidermoid carcinoma (A431), lung carcinoma (A549), mouse melanoma (B16F10), human breast adenocarcinoma (MCF-7), human cervix carcinoma (HeLa) and human breast carcinoma (MDA-MB-231) were cultured and screened for galectin expression. Buccal cells were isolated by scraping buccal mucosa of normal individual (NBC) as well as from metastatic cancer patients (MBC). Cells were grown in MEM—high glucose (4.5 g/l) with 2 mM glutamine (VERO, A549 and HeLa cells) or DMEM—high glucose (4.5 g/l) with 4 mM glutamine (A431, B16 F10, HEK293, MCF-7, MDA-MB-231, NIH 3T3, NBC and MBC cells), 1.5 g l^{-1} sodium bicarbonate, penicillin (100 units ml^{-1}) and kanamycin (0.1 mg ml^{-1}) with 10% fetal calf serum at 37°C in a humidified chamber with 95% air and 5% CO_2. For MCF-7 and NIH 3T3 cells, additional F12 mixture in 1:1 (v/v) ratio with the prescribed media was added.

Cultured cell growth and survival was monitored by MTT assay [11]. Cells (2.8×10^4 cells ml^{-1}) were cultured with the specified medium in a 96 well microplate and after 72 h, 25 µl of MTT solution (5 mg ml^{-1}) was added, incubated at 37°C for 4 h. 100 µl of lysis buffer was added and cells were continued to incubate at 37°C overnight (about 16 h) to dissolve the dark blue crystals and absorption of formazan solution was measured at 570 nm in a microplate reader (Spectra Max-340, Molecular Devices, Germany).

Preparation of cell extract

The flask (25 cm^2) containing confluent cells were placed on an ice tray for 10 min. and the medium was removed with gentle washing in PBS. Added 1 ml of 20 mM ice cold phosphate buffer saline (PBS) of pH 7.4 and the cells were scraped using a cell scraper and extracted as per the protocol of Dharmesh et al. [5]. Cell suspensions were centrifuged at 4°C for 3 min at 1,500 g. Supernatant was discarded and the cell pellet was washed thrice with PBS. To the washed cell pellet was added, 1.0 ml of 25 mM HEPES buffer, pH 7.4 containing 250 mM sucrose, 1 mM EDTA and 1% triton X 100, mixed gently and the cell suspension was passed through 18 followed by 20, 22 and 26 gauge needles (five times each) and stored at −20°C. The extract was referred as "Cell Extract" for further experiments.

Microscopy

During the experiments microscopic observations were made between treated and control cells using the inverted microscope with described magnification (Leica DMLS model, Germany) to follow the morphological features. Also cells subcultured, fixed onto the glass slides were stained with filtered solution of hematoxylin and eosin reagents. Stained cells were observed under the microscope to follow the changes if any noticeable in either the nucleus or cytoplasm (hematoxylin stains nucleus violet and eosin stains the cytoplasm, pink), after treatment with indicated concentrations of galectin Inhibitors and compared with those of untreated controls. Since some of pectic polysaccharides treated cells indicated morphological features typical of apoptosis, apoptosis assays were also performed and images were captured under 40× magnification.

Determination of galectin-3 expression in cell extracts and media by immunoassays

ELISA, Immunostaining, Dot blot, Western blot and Immuno fluorescence analysis were performed for the detection of galectin levels in cells/media and to understand its distribution in various cells employing the protocol standardized previously [31]. For all these assays monoclonal anti human galectin-3 antibody (Becton Dickinson Co., USA) was employed at dilutions 1: 1,000 and 1:100 for ELISA and immunoblot/immunoflourescence analysis respectively as primary antibody. Alkaline phosphatase conjugated rabbit anti mouse IgG (GENEI, Bangalore, India) at 1:5,000 dilution followed by paranitrophenylphosphate (PNPP) were used as secondary antibody and substrate respectively for ELISA. A calibration curve of galectin was established by ELISA using 0.02 μg to 0.1 µg 100 μl^{-1} concentration of pure galectin obtained from Sigma Chemical Co., USA, and levels of galectin expressed in each cell line was measured as absorbance in the microplate ELISA reader (Molecular Devices, Spectramax 340, Germany) and quantitated against the calibration curve. 1:1,000/500 diluted goat anti-mouse IgG peroxidase/fluorescein isothiocyanate conjugates were employed as secondary antibodies for immunoblot and immunofluorescence analysis respectively. BCIP (5-bromo-4chloro-3-indolyl phosphate) was used to detect peroxidase binding in western and dot blot analysis. Fluorescence was captured by blue filter in fluorescent microscope (Leica DMLS model, Germany). Results were compared between normal and cancer cells.

Evaluation of galectin inhibitory properties

Agglutination inhibition assay

Microplate agglutination assay was performed for the evaluation of potential dietary galectin inhibitors employing the protocol of Nowak et al. [22]. Briefly, human erythrocytes were prepared from 10 ml of fresh blood (collected in Alsever's medium), washed four times with five volumes of 0.15 M NaCl. A 4% erythrocyte suspension in 0.02 M PBS, pH 7.4 containing 1 mg ml^{-1} trypsin was incubated for 1 h at 37°C. The trypsin treated cells were washed with five volumes of 0.15 M NaCl and fixed in five volumes of 0.02 M PBS pH 7.4 containing 1% glutaraldehyde for 1 h at room temperature. Glutaraldehyde fixation was terminated by the addition of five volumes of 0.1 M glycine in PBS, pH 7.4 at 4°C and the fixed erythrocytes were employed for the hemagglutination assay. Hemagglutination assays were done in microtitre agglutination assay plate using MDA-MB-231 media in presence of serially diluted pectic polysaccharide in 0.15 M NaCl. The reaction mixture contained 150 μl of 4% erythrocyte suspension with or without serially diluted pectic polysaccharide from various dietary sources (Table 1). Minimum Inhibitory Concentration (MIC) of the substances was determined to compare the bioefficacy of the dietary sources. For buccal cells, slide agglutination assay was performed in the presence or absence of 10 μg ml^{-1} of MSRPP followed by microscopic observation with and without hematoxylin and eosin staining.

Inhibition of cell invasion

Matrigel invasion chamber with pore size (0.8 μm, BD Biosciences, USA) was used to measure cell invasion *in vitro* [1]. SRPP/MSRPP at 50 & 100 μg ml^{-1} were added to the cell (MDA-MB-231 and Buccal cells) suspension (in 0.5 ml medium without serum). The control and SRPP/MSRPP treated cell suspensions (0.5 ml of 3×10^4 cells) were added to each Matrigel insert, the bottom chamber contained growth medium with 5% FBS. After 24 h chambers were removed, cells that remained in the upper chamber were counted under the inverted microscope. Percent of cells invaded (% cell invasion) were calculated as 1. (No. of cells invaded into the bottom chamber) (BC)/(Total No. of cells)×100, and 2. (No. of cells in upper chamber (UC) at 0 h)-(No. of cells in UC at 24 h)/(Total No. of cells)×100.

Effect of SRPP/MSRPP on metastatic MDA-MB-231 cells

Since MDA-MB-231 cells expressed higher levels of galectin-3, they were selected to study the effect of one of the best inhibitory polysaccharides—SRPP. MDA-MB-231 was seeded at density 2×10^4 cells/well in a six well plate. After 24 h, the cells were washed four times with PBS and incubated with SRPP (50 & 100 μg ml^{-1} concentrations) in serum free DMEM medium for 24 h at 37°C. After incubation, collecting the media and cells; cells were washed three times with PBS, trypsinized, washed and suspended in PBS. Immunostaining was performed on both treated and untreated cells. Cells were observed microscopically with hematoxylin and eosin stain and indication of apoptosis if any was recorded.

Table 1 Agglutination inhibitory activity of various dietary polysaccharides and SRPP fractions (Purified on DEAE-cellulose column chromatography with 0.05 M-2 M ammonium carbonate elution) against MDA-MB-231-galectin-3 induced hemagglutination of rabbit erythrocytes

Samples	Agglutination inhibition MIC in μg eq. carbohydrate/mL
Galactose *	27.10
Lactose *	4.16
SRPP	1.85
Swallow root pectic polysaccharide	
HPP	40
H. indicum pectic polysaccharide	
BCPP	130
Black cumin pectic polysaccharides	
APP	
A. serpylifolia pectic polysaccharide	40
GRPP	>6 mg no inhibition
Ginger pectic polysaccharide	
CPP*	25
Citrus pectic polysaccharides	
MSRPP	1.03
Modified Swallow root pectic polysaccharide	
MCPP	14
Modified Citrus pectic polysaccharides	
Arabinogalactan *	200
SRPP fractions (% Yield)	
Fractions	
Neutral (16.7)	–
0.05 M (4.26)	0.25
0.10 M (14.89)	–
0.15 M (62.31)	0.04
0.20 M (1.84)	–

Values are expressed as mean ± SEM (n=3)

Apoptosis assay

Apoptosis assay was performed using ethidium bromide and acridine orange dye method [28] as well as observing characteristic features of cells by microscopy. Briefly MDA-MB-231 and Buccal (1×10^4 cells/well) cells were treated with SRPP/MSRPP at 50 and 100 μg ml^{-1} for 72 h and 1 h respectively. Twenty five microliters of cell suspension of both treated and untreated cells were mixed with 1 μl of dye mix containing 100 μg ml^{-1} each acridine orange and ethidium bromide and observed under the microscope at 40×. Viable cell nuclei stained green with acridine orange and apoptotic cell nuclei stained red with ethidium bromide were counted. Percent apoptosis was measured and compared. In order to understand the probable route of apoptosis, levels of Caspase activity was measured [39] using caspase-3 specific peptide- substrate *N*-acetyl-ASP-Glu-Val-ASP-P-nitroanilide (Ac-DEVD-pNA). The release of P-nitroaniline moiety from the substrate was measured at 405 nm in microplate reader (SpectraMAX plus, Molecular Devices).

Sugar composition analysis of pectic polysaccharides and fractions of SRPP

Ten milligrams pectic polysaccharide was subjected to acid hydrolysis by refluxing the sample for 6–8 h with 10% sulfuric acid. Hydrolyzed samples were neutralized with barium carbonate, deionised using amberlite IR 120 H^+ resin, alditol acetates prepared as described [33] and subjected for sugar composition analysis in a Shimadzu GLC (Kyoto, Japan) fitted with OV 225 column with a column temperature of 200°C and injector temperature of 250°C at 40 ml min^{-1} gas flow rate. Since highly active SRPP and CPP showed higher levels of arabinose and galactose, SRPP was further subjected to fractionation on DEAE cellulose Ion exchange column chromatography [37] and resolved fractions were analyzed for sugar composition analysis as described above. Correlation between the activity in the fractions and their sugar compositions were determined.

Statistical analysis

All the experiments were carried out in triplicates and the results are expressed as mean ± standard deviation (SD). Correlation between the activity and sugar composition was calculated as coefficient of determination -R^2 using linear regression model to understand the strong, moderate or weak linear trend employing the statistical programme SPSS for Windows; Version 10.0. P value was calculated by the Mann-whitney test. Duncan's Multiple Range Test (DMRT) was performed to understand the degree of significance between controls and treated samples.

Results

Isolation of pectic polysaccharides from dietary sources

Pectic polysaccharide from dietary sources resulted in 0.6–6.2% of yield upon ammonium oxalate extraction. Swallow root had the highest yield of pectic polysaccharide (6.2%), as it was a fleshy tuberous root, while it was very low in case of black cumin (0.8%) which had high amount of oils stored in it. The pectin yields of ginger and A. *Serpyllifolia* were 0.6 and 4% respectively. Further, the molecular weight of SRPP being higher, it was subjected to controlled hydrolysis using acid precipitation procedure, and recovered 80% of the polysaccharide as MSRPP with low molecular weight ~50 kDa.

Determination of galectin-3 expression using ELISA, dot blot and western blot analysis

Optimal cell growth conditions were established for each cell line and ensured their normal growth. Various cell lines used for the study showed characteristic morphology and their proliferation rate was monitored through MTT assay. The characteristic morphology of normal and cancer cells were evidenced by hematoxylin and eosin staining, where normal cells showed smaller nucleus and an enlarged cytoplasm while the reverse- enlarged nucleus and a narrow cytoplasm was found in cancer cells (Fig. 3b).

Galectin-3 was found in both cells as well as in the media (Fig. 1). MDA-MB-231 and HeLa cells exhibited ~15 to 16 fold (0.217–0.267 μg/10^6 cells ml^{-1}) higher levels than Normal NIH-3T3 and VERO cells (0.014 μg & 0.016 μg/10^6 cells ml^{-1}). A 431, HEK-293, B16F10 and MCF-7 cells showed 0.024, 0.045, 0.066 and 0.070 μg/10^6 cells ml^{-1}, respectively (Fig. 1a). Figure 1a and b reveals the comparative levels of galectin-3 in cells and their respective medium. Normal cells NIH-3T3 and VERO-exhibited basal levels substantiating the literature result [18] that galectin-3 is specifically expressed in cancer cells, particularly increased levels in the metastatic cells (Fig. 1a). Immuno-histological analysis of MDA-MB-231 showed that it expresses galectin-3 inside the cell in a granular-grain like/particle like manner (Fig. 2a), distributed both in the cytoplasm and the nucleus [4], which is evident when compared to staining of cells with H and E (Fig. 2b). Galectin-3 appears also to be extracellular [41] since it is secreted into the media (Fig. 1b). Dot blot (Fig. 2e) and western blot (Fig. 2f) analysis further confirms that galectin-3 is found only in metastatic MDA-MB-231 cells and not in Normal NIH-3T3 cells. A 42-kDa protein band was observed in MDA-MB-231 cells extracts upon western blotting and also on SDS-PAGE analysis. Figure 2c substantiates the higher-level expression of galectin-3

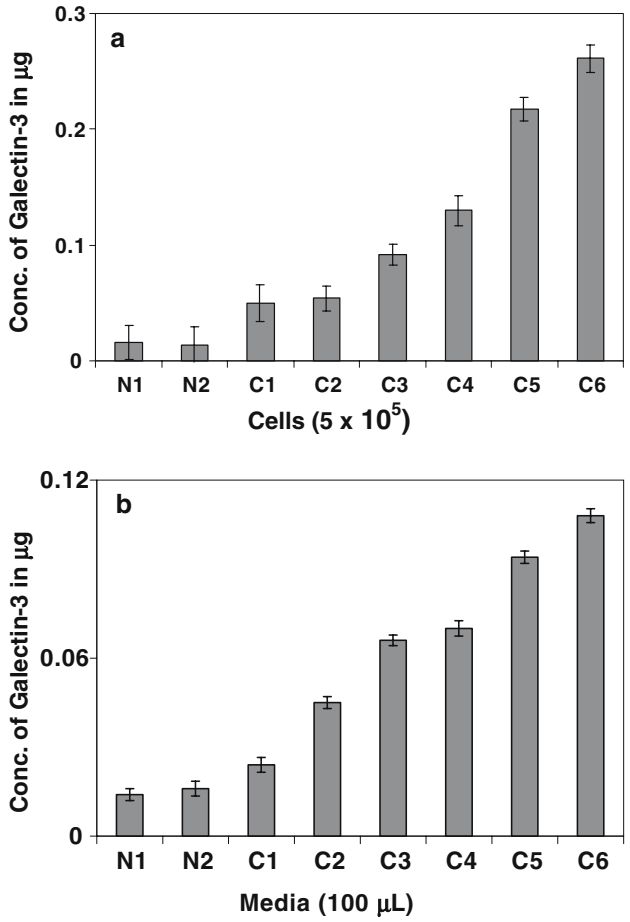

Fig. 1 Galectin-3 levels in normal and cancer cells: Normal cells-N1-NIH 3T3, N2-VERO; Cancer cells-C1-A-431, C2-HEK-293, C3-B16F10, C4-MCF-7, C5-HeLa, C6-MDA-MB-231. 100 µL each of above mentioned cell's extract corresponding to 5×10^6 cells was loaded on ELISA plate and ELISA was performed employing alkaline phosphatase conjugated Rabbit- anti mouse IgG. Absorbance at 410 nm was measured in a microtitre plate reader after incubating with 100 µl of 1 mg ml^{-1} paranitrophenyl phosphate in 1% diethanolamine buffer of pH 9.8 at 37°C for 30 min. Galectin content in µg was determined by the generated calibration curve with R value of >0.99. Figure 1 provides comparative levels of galectin-3 in various cell lines (**a**) and in the media (**b**). Data has been consolidated from six experiments. Values are expressed as mean ± SD

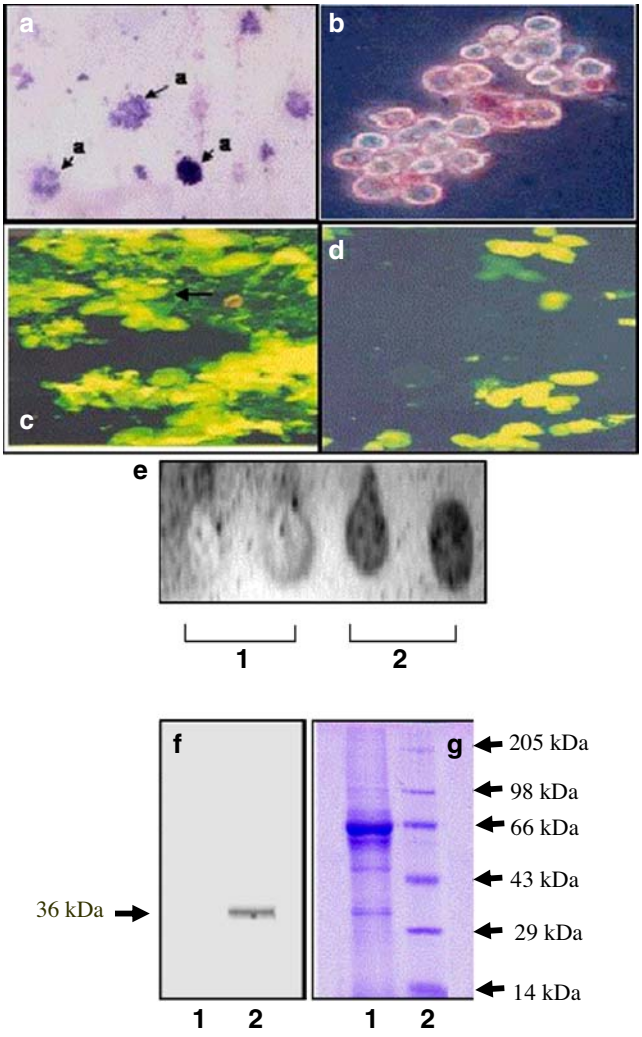

Fig. 2 Confirmation of presence of Galectin-3 in metastatic cells by immunotechniques—**a** Immunostaining of MDA-MB-231; **b** H and E staining of MDA-MB-231 cells with enlarged nucleus; **c** Immunoflourescence of metastatic buccal mucosal cells in comparison with normal buccal cells (Fig. 1d); **e** Dot blot analysis of 1-NIH 3T3 and 2-MDA-MB-231 cell lysate; **f** Western blot analysis resulting no Galectin-3 band in NIH-3T3 cell extract (*lane 1*) and presence of ~36 kDa galectin-3 in MDA-MB-231 cell extract (*lane 2*); **g** SDS-PAGE profile of MDA-MB-231 medium upon coomassie staining (*lane 1*). *Lane 2* shows the profile of molecular weight markers. *Arrow* indicates the molecular size

(Green fluorescence, indicated by arrows) by Immunoflourescence technique only in the metastatic buccal mucosal cells in comparison with negligible fluorescence in normal buccal cells (Fig. 2d).

Determination of potential galectin inhibitory activity of dietary pectic polysaccharides

Hemagglutination assay

Pectic polysaccharides isolated from different dietary sources were evaluated for their galectin inhibitory activity based on hemagglutination assay. Minimum Inhibitory Concentration of the polysaccharide (MIC) in inhibiting the galectin mediated agglutination of red blood cells was determined and results were compared with standard galectin specific sugars—galactose and lactose. Results presented in Table 1 revealed that SRPP showed a potent agglutination inhibition with MIC of 1.86 μg ml^{-1}. MIC of 27.1 and 4.16 μg ml^{-1} was observed for galactose and lactose that are specific for galectin-3 respectively. Practically no inhibition was observed in pectic polysaccharide of Ginger—GRPP even at concentrations >6 mg ml^{-1}. Citrus pectin had higher inhibition with MIC of 25 μg ml^{-1} than,

HPP (40 μg ml^{-1}), APP (40 μg ml^{-1}) and, BCPP (130 μg ml^{-1}). Among the standards, lactose showed a good inhibitory activity with MIC of 4.16 μg ml^{-1} than galactose (MIC-27 μg ml^{-1}). Data thus suggested that at least 15 and 2 fold increase in the activity was exhibited by SRPP over galactose and lactose respectively. Also, SRPP gave ~14 fold increased activity when compared to that of another reported source—Citrus pectin which showed an MIC of 25 μg ml^{-1}. Modification of SRPP to MSRPP also retained galectin inhibitory activity (MIC of 1.03 μg ml^{-1}) despite decrease in the size. Normal buccal cells (Fig. 3a) upon treatment with galectin isolated from cancer buccal cells, resulted in agglutination (Fig 3b) and; this was inhibited by MSRPP at 10 μg level (Fig. 3c). Observation of these cells upon H&E staining at higher magnification (40×) reveals visible changes in cells such as uniformly flattened regular morphology (Fig. 3d) got disrupted with disintegrated nucleus (Fig. 3e) and, such cells were protected by prior treatment with polysaccharides (Fig. 3f). SRPP/MSRPP/CPP/MCPP treated samples showed significant reduction in agglutination with P value<0.001. MSRPP was more potent than SRPP, similar to that of MCPP.

Cell invasion assay

Filtration of metastatic MDA-MB-231 cells treated with and without SRPP/MSRPP/CPP through matrigel coated invasive chamber indicated more number of cells equivalent to the absorbance of ~0.8 as per MTT assay in treated cells in the upper chamber, while an absorbance of only 0.296 was observed in untreated controls. The cells invading through matrigel were also counted. Data suggested concentration dependence and ~73% inhibition of cell invasion at 100 μg mL^{-1} of SRPP/MSRPP/CPP (Fig. 4a). Similar results were observed with the penetration of cancer buccal cells (Fig. 4b). ~63% of metastatic cancer buccal cells were invaded and metastatic cancer buccal cells treated with MSRPP at 100 μg ml^{-1} resulted in the significant inhibition of ~43% of penetrating cells as evidenced by accumulation of these cells in the upper chamber. Results thus suggest that the isolated MSRPP has anti-invasive property similar to that of CPP [20].

Apoptotic effect of SRPP/MSRPP on MDA-MB-231/ buccal cells

In the control cells (Fig. 5a), predominant granular pattern was observed upon immunostaining. Galectin was found both in the nucleus and the cytoplasm. Upon treatment of control cells (Fig. 5a) with SRPP (Fig. 5b) and MSRPP (Fig. 5c), cells showed distinct morphological changes as evident from nuclear/chromatin structures, oozing out of cytoplasmic contents and formation of apoptotic bodies (Fig. 5b), increase in cell volume and membrane disruption (Fig. 5c), cell membrane blebbing and intracellular bridges (Fig. 5d) typical of apoptosis was observed. These observations were substantiated by the loss of 75% and 90% of cell viability at 50 and 100 μg ml^{-1} respectively. A typical necrotic type apoptosis in SRPP (Fig. 5b) and cell lysis resulting in ghost cells by MSRPP treated cells (Fig. 5c), lytic apoptosis was evident. Results were substantiated by the appearance of 40–60% of

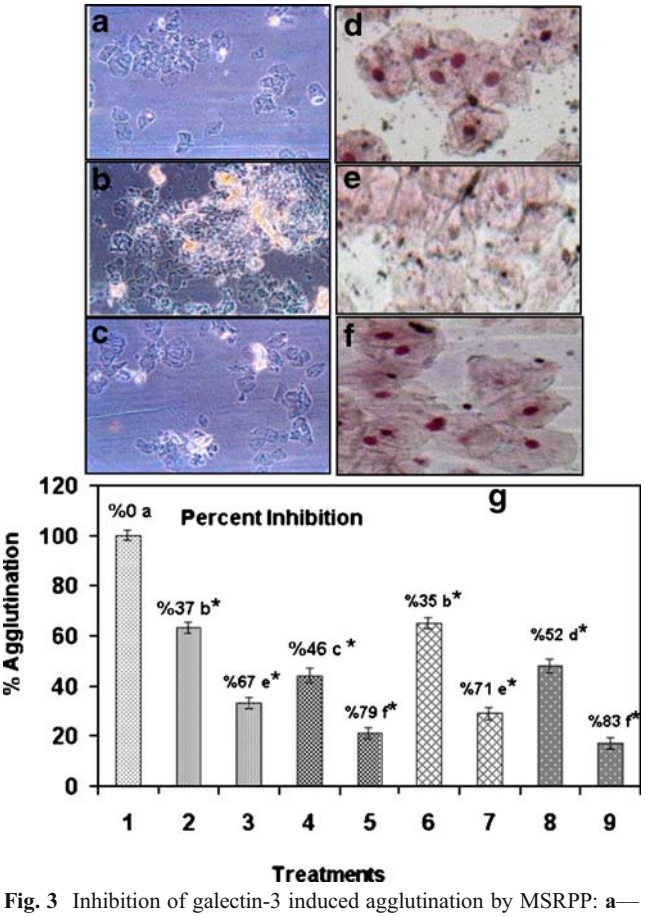

Fig. 3 Inhibition of galectin-3 induced agglutination by MSRPP: **a**—Normal buccal cells (NBC)-free, regular and flattened; **b**—Treatment of **a** with galectin-3 isolated from MDA-MB-231 cells revealing irregular shape and size of cells with clumping; **c**—Same as **b**, but pretreated with MSRPP showing protection against galectin-3 induced changes. Fig. **d**–**f** are similar to **a**–**c**, but cells were stained with hematoxylin and eosin, photographed at 40× and magnified to 400×. Figure 4**a**–c represent the photography at 40×. Figure 4**g** represent the graphical representation of % agglutination in control and SRPP/MSRPP/CPP/MCPP treated cells. % agglutination was determined by counting the (number of cells remaining free/total number of cells) ×100. % agglutination inhibition is indicated on the bar graph. In *bars* 1–9 the dose dependent inhibition of agglutination is evident: 1. Normal Buccal cells treated with Galectin-3 at 10 µg ml^{-1}, 2. and 3 same as 1, but pretreated with SRPP at 50 and 100 µg ml^{-1}, 4. & 5 same as 1, but pretreated with MSRPP at 50 and 100 µg ml^{-1}, 6. & 7 same as 1, but pretreated with CPP at 50 and 100 µg m^{-1}, 8. and 9 same as 1, but pretreated with MCPP at 50 and 100 µg ml^{-1}. All treatments indicated significant inhibition at P value<0.001. Comparative efficacy was calculated by DMRT and represented as **a**–**f** on top of the each bar along with % inhibition

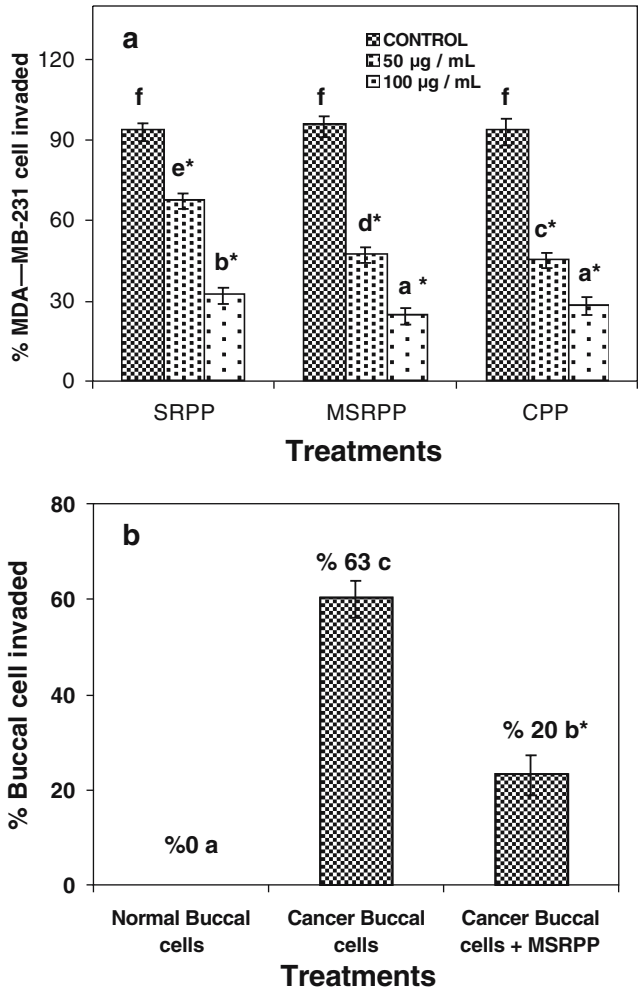

Fig. 4 Inhibition of MDA-MB-231 cell invasion *in vitro* by SRPP/MSRPP/CPP **a**—polycarbonate filters were coated with matrigel and placed in Boyden chamber. MDA-MB-231 cells suspensions (3×10^4) in 0.5 ml of DMEM medium without fetal bovine serum were loaded on to the upper chamber of the Boyden chamber in presence and absence of 50 and 100 µg ml^{-1} of SRPP/MSRPP/CPP. After incubation for 72 h at 37°C in 5% CO_2, cells invaded through the matrigel were counted; % cell invasion was calculated. % Cells remaining in the Upper chamber in NBC is taken as 100 and relative % invasion is represented; **b**. Fresh metastatic buccal cells were subjected to invasive chamber assay. Number of metastatic cells remained in the upper chamber in presence and absence of MSRPP (100 µg ml^{-1}) were counted. Results on % invasion were compared with normal buccal cells (NBC) by DMRT analysis, where no penetration into the lower chamber was observed indicating the non-invasive nature of NBC. Values from three different experiments in triplicates were significant with p value<0.05

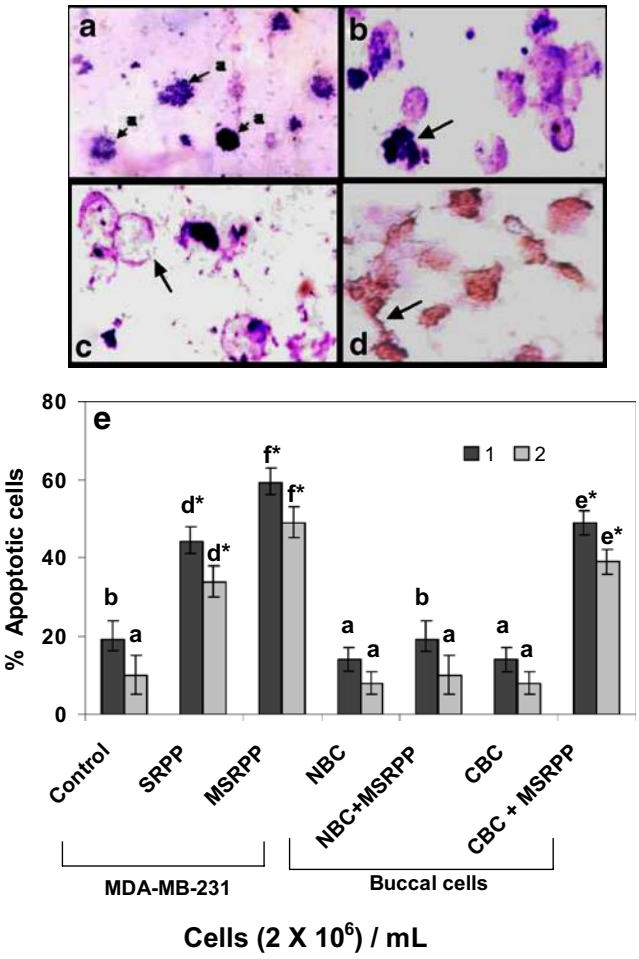

Fig. 5 Measurement of apoptosis in metastatic-MDA-MB-231 and non-metastatic cells: cells were treated with SRPP and MSRPP at 100 µg ml^{-1} for 24 h and subjected to immunostaining using anti-galectin-3 antibody. Control MDA-MB-231 cells showing granular structure (**a**) was disrupted by SRPP treatment. Cell shrinkage and oozing of cellular contents are evident (**b**). Increase in the cell volume and disruption of the membrane (**c**), membrane blebbing, cell–cell bridge formation (**d**). Figure 5f shows bar graph with percent apoptotic cells during the treatment with galectin inhibitory polysaccharide. Number of cells lysed as per microscopic observation (1) /ethidium bromide stained cells (2) were counted and percent apoptosis was calculated. P value was calculated to be <0.05. DMRT analysis reveals significant activity with MSRPP treatment

apoptotic cells as evaluated by acridine orange and ethidium bromide staining methods (Fig. 5e) both in MDA-MB-231 and metastatic cancer buccal cells. Interestingly, there was no cell death of normal buccal cells indicating the differential effect of MSRPP (Fig. 5e), which is a desirable character for anticancer drugs. Further, since Caspase-3 is generally involved in induction of apoptotic death of cancer cells [9, 17], level was measured in SRPP and MSRPP treated cells. Results indicated ~3 (A-0.123) and 8 (A-0.324) fold increase in the absorbance in SRPP and MSRPP treated cells respectively, when compared to that of the control (A-0.043). Dose dependent increase in the activity in 50 and 100 μg ml^{-1} concentration of SRPP and MSRPP was also observed.

Sugar composition analysis of pectic polysaccharides and fractions of SRPP

The sugar composition analysis of galectin-3 inhibitory polysaccharide from the dietary sources (Table 2) showed

the presence of rhamnose, arabinose, xylose, mannose, galactose and glucose in varying proportions. Only traces of xylose and mannose were present in all the samples. Galactose was present to a moderate extent in SRPP (32%), CPP (19%) and APP (26%) while absent in GRPP. In addition, higher levels of uronic acid content of ~113–657 mg g^{-1} (Table 2) was also observed except in GRPP and HPP where 22-24 mg g^{-1} was detected. Rhamnose and arabinose were present in all the samples. The relative percentages of rhamnose/arabinose in SRPP/CPP/APP/BCPP/GRPP and HPP were 16, 50, 05, 30, 08, 23, 49, 42, 06, 24 and 4/14, respectively.

In order to understand the relation between type of sugars in dietary pectic polysaccharide and galectin inhibitory activity, correlation coefficient-R^2 was determined. Results indicated a coefficient-R^2 of 0.06851, 0.6341, −0.1678, −0.2217, 0.695 and −0.3925 for rhamnose, arabinose, xylose, mannose, galactose and glucose respectively. A moderate association was found between arabinose and galactose; while no correlation existed between the activity and other sugars. Results were substantiated by sugar composition and galectin inhibitory activity in purified fractions of SRPP. SRPP $_{0.05}$ M and SRPP $_{0.15}$ M fractions which exhibited galectin inhibitory activity with an MIC of 0.025 μg ml^{-1} and 0.004 μg ml^{-1} did not contain rhamnose, mannose and glucose indicating that these sugars are not involved in the activity. Also the potent fraction of SRPP–SRPP $_{0.15\ M}$ fraction although was more active than 0.05 M fraction, did not contain xylose indicating that xylose also may not be involved in the activity. Arabinose was absent in 0.05 M SRPP fraction. Galactose thus appears to be important for galectin inhibitory activity. It should be noted here that a ~6.25 fold higher active SRPP $_{0.15}$ M fraction contained 2.8 fold lesser galactose than that found in 0.05 M fraction, but contained additionally 66% of arabinose indicating that both arabinose and galactose may be important for the activity. Further, to confirm the fact that arabinose and galactose are important for the galectin—inhibitory property, purified larch wood arabinogalactan that contained 98% (w/w) of arabinogalactan was examined for galectin inhibitory property. Galectin inhibitory activity with an MIC of ~200 μg ml^{-1} was obtained, indicating that not only the presence of arabinose and galactose may be essential for the activity, while a precise arabinose-galactose structure may be involved. Our future studies are directed towards the same and are being explored currently in our laboratory.

Discussion

Cancer is a complex disease involving multiple steps such as tumor initiation, promotion, and metastasis. Dietary compounds containing flavonoids, antioxidants, glycosides etc., are known to reduce the risk of cancers [6] particularly by inhibiting reactive oxygen species induced oxidative stress. Several synthetic and natural antioxidants may exert anticancer properties at the very early stage of cancer disease such as initiation step. However, the advanced step of the disease, metastasis; essentially is the ability to spread to other tissues and organs makes cancer a potentially life-threatening disease [15, 32]. So there is a great concern in understanding what makes metastasis possible for a cancerous tumor and to block the invasion by safer molecules to control the disease. A need for newer and safer compounds particularly to block galectin-3 that mediate metastasis i.e., galectin inhibitors existed when galectin-3 plays a key role in crucial events of metastasis.

Table 2 Relative percent sugar composition analysis and uronic acid content of pectic polysaccharides isolated from various dietary sources and SRPP fractions. SRPP fractionated on DEAE cellulose column chromatography using 0.05 M-2 M Ammonium carbonate were also analysed for sugar composition to correlate with agglutination inhibitory activity

Sugars	Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid (mg/g)
SRPP	16	50	02	00	32	00	141
CPP	05	30	03	00	19	43	295
APP	08	23	03	10	26	30	110
BCPP	49	42	02	00	04	03	30
GRPP	06	24	08	04	00	58	24
HPP	04	14	02	03	06	71	22
MSRP	14	35	03	–	48	–	162
MCPP	17	04	18	–	54	07	657
SRPP-DEAE-column fractions							
0.05 M	–	–	24	–	76	–	3.0
0.10 M	17	83	–	–	–	–	21
0.15 M	7	66	–	–	27	–	113
0.20 M	–	100	–	–	–	–	1.0

Values are expressed as mean ± SEM (n=3)

These galectin inhibitors are believed to be safer since they exert their effects by binding to galectin-3 found only in cancer cells and not in normal cells. A study by Nangia-Makker et al. [19] had demonstrated the antimetastatic property of pectin hydrolyzates by inhibiting angiogenesis, reduction in the mean tumor volume by 7 weeks following the oral intake of modified citrus pectin. In the current study we aimed at blocking the galectin-3 molecule, which is found to be involved in metastasis progression of various types of cancers [34], through dietary galectin inhibitors. Various normal cell lines such as NIH-3T3, VERO and cancer cells-HEK 293, A431, B16F10, MCF-7, HeLa and MDA-MB-231 were screened for the expression of galectin-3. The presence of at least 5–15 fold higher levels of galectin-3 in metastatic MDA-MB-231 and HeLa cells as well as in their media compared to only basal levels in normal cell lines as per quantitative monoclonal anti-galectin-3 based ELISA suggested the correlation between galectin-3 levels with metastatic potential of cells similar to the earlier report of Takenaka et al. [36]. Data also indicated that galectin-3 may be an universal molecule involved in metastasis since the level of expression correlated with the metastatic stage of the cells irrespective of their tissue origin. This data is further supported by our studies on thorough screening of galectin-3 in biopsy tissues, serum and urine samples of different types of cancer patients (unpublished data). Among the dietary sources tested, swallow root pectic polysaccharide yielded 14 fold higher galectin-3 inhibitory activity than other dietary pectic polysaccharides [10] as well as standard galectin-3 binding sugars such as galactose and lactose (2 fold higher).

It was reported that polysaccharides containing arabino-galactan residues inhibits metastasis [13, 38]. Modified citrus pectin (MCP) containing galactose residues [21] has also been mentioned as a possible anti metastatic agent. Presence of both arabinose and galactose as major sugars in SRPP and CPP correlated with their higher galectin-3 inhibitory activity compared to the lower activity of other dietary polysaccharides together with lower levels of galactose. Lack of galectin inhibitor activity in GRPP may be attributed to lack of galactose. Results thus suggest that galactose is important for galectin-inhibitory property. However, studies, particularly with CPP (MIC 25 μg ml^{-1} equivalent of carbohydrate) exhibiting −1.6 fold better activity than APP (MIC ~40 μg ml^{-1} equivalent of carbohydrate) although latter contained 26% galactose when compared to 19% galactose of CPP indicated that just percentage of galactose alone may not be important for the activity; many other components may also contribute to the activity. Studies with purified fractions of SRPP and larch wood arabinogalactans further confirms the fact that rather than galactose alone some arrangement with arabinose and galactose as arabinogalactans may be important for the activity. Despite higher levels −76% of galactose in 0.5 M fraction; it showed poorer activity than SRPP 0.15 M fraction, which could be due to the absence of arabinose; similarly 0.1 M and 2 M fractions did not show any activity since they are devoid of galactose. Larch wood arabinogalactan with 98% of homogeneous arabinogalactan exhibited ~100 fold lesser galectin inhibitory activity than SRPP indicating that not only the presence of precise sugar residues such as arabinose and galactose are important for the galectin inhibitory activity but also the arrangement of these sugar residues are crucial for the activity. Detailed structure—function relationship studies are therefore underway.

SRPP showing galectin inhibitory activity, inhibited other galectin-3 mediated events in metastasis such as invasion, apoptosis etc. Evidently as per Figs. 4 and 5, inhibition of invasion and induction of apoptosis were observed. Interestingly SRPP also exhibited differential effects such as induction of apoptosis in only metastatic cells- MDA-MB-231 and not in normal cells. Apoptosis was mediated by activated caspase-3, a protease that is rapidly activated when cells are exposed to apoptotic conditions that cleaves poly (ADP ribose)-polymerase. No SRPP induced toxicity was also observed. SRPP therefore may be considered as a potent-galectin blocker which can be employed in the arrest of metastasis. Marked reduction in cell invasion (Fig. 4), inhibition of agglutination of buccal cells (Fig. 3) may further emphasize the importance of dietary carbohydrate as potential cancer-preventive and therapeutic agents as already highlighted in case of citrus pectin [2]. The complex nature of carbohydrate may enunciate the development of new antagonists for galectin-3. The identified polysaccharide has additional advantage of being non-toxic and inexpensive. It should be highlighted here that galectin inhibitors may also have a greater impact in inhibiting matrix metalloproteinase activity. Matrix metalloproteinases of metastatic cells help invading into normal cell via acting on galectin-3 itself on extracellular matrix, since galectin-3 is the substrate for matrix metalloproteinases [23]. The binding of pectic polysaccharide to galectin-3 of cancer cell may also abolish the invasive action of metalloproteinases. These observations taken together suggest that SRPP can reduce tumor cell invasiveness by suppressing galectin-3 mediated cell adhesion to extracellular matrix proteins in the basement membrane of normal cells and hence may subject such cells to apoptosis and hence may become a potential cancer therapeutic agent.

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