Chapter 4

Elucidation of Mechanism of action of corn pectic polysaccharide in in vitro and in vivo models
4.1. Hypothesis

Scheme 4.1. Recent research revealed that galectin-3 is associated with several steps of invasion and metastasis, like cell-matrix interaction, adhesion, angiogenesis, and dissemination through blood flow and extravasation. It is hypothesized therefore in this chapter, whether Corn Pectic Polysaccharide (COPP) (a) can bind to galectin-3 on B16F10 (b) and inhibit B16F10 induced lung metastasis (c) in \textit{in vivo} in Swiss albino mice (d) Effect of COPP in galectin-3-mediated functions \textit{in vitro} like cell matrix interaction/adhesion (e), and invasion cells (f) will be addressed.
4.2. Work Plan

**Determination of anti-metastatic activity of corn pectic polysaccharide (COPP)**

**In vitro models**
- Cytotoxicity
- Cell adhesion
- Inhibition of agglutination
- Cell invasion
- Induction of apoptosis

**In vivo models**
- Regression of tumor; Tumor Index; Histopathology
- Melanin/galectin-3 levels / VEGF/MMP/NF-κB
- Uronic acid/Hexosamine/Hydroxyproline/PGI
- Antioxidant Enzymes levels
- Toxicity

**Effect on B16F10 metastatic cells**

**Effect on B16F10 cells induced lung metastasis**

+ Galectin Inhibitors (COPP)

To understand the mechanism of inhibition of metastatic cells and inhibition of induction of lung metastasis *in vivo*
4.3. Introduction

Metastasis is a complex process regulated at many levels and ..., involving multiple rate limiting steps (Roodman, 2004; Tantivejkul, 2004; Glinsky, 2006) and is one of the greatest obstacles in clinical treatment against cancer. The transition from in situ tumor growth to metastatic advanced disease involves the ability of the tumor cells to invade into the local tissue and to cross tissue barriers. To initiate the process, carcinoma cells must penetrate the epithelial basement membrane. The adhesion of tumor cells to extracellular matrix (ECM), such as fibronectin, laminin, matrigel, is crucial for metastasis (Yan and Han, 1997; Sass, 1998; Syrigos et al., 1999; Zou et al., 2005). This is followed by ECM degradation which is believed to include secretion and activation of proteolytic enzymes, such as matrix metalloproteinases (MMPs) (Hejna, et al., 1999; Raffetto and Khalil, 2008) followed by pseudopodial protrusion and locomotion of tumor cells.

Melanoma is a relatively common and one of the most malignant tumors in humans (National Institute of Health report, 1992). The incidence of melanoma has increased significantly since 1930 in both men and women and was found to be attributed to changes in sun exposure and environmental levels of UV light followed by its unique character of exhibiting resistance to chemo/radiation therapy (Shea et al., 1999). It is reported recently that galectin-3, a protein and a member of the group of lectins that binds to β-galactosides (Krześlak and Lipińska, 2004; Vitaly et al., 2010) was found to be expressed in higher levels in melanocytes during tumor progression (Mollenhauer et al., 2003). Increased level of galectin-3 was also reported from our laboratory in B16F10 melanoma cell lines (Sathisha et al., 2007) suggesting that galectin-3 plays a key role in metastatic melanomas similar to what has been observed in other malignant metastatic cancers (Vereecken et al., 2005; 2006; 2009).

The role of dietary carbohydrates in cancer progression and metastasis is an emerging field of clinical importance. Pectin is a natural, complex plant polysaccharide present in all higher plant primary cell walls and, consequently, is a dietary component of all fruits and vegetables. Pectin
accounts for approximately 30% of the primary cell walls of all higher plants except the grass family, where it makes up about 10% of the primary wall. Pectin has multiple roles in plant growth, development, and disease resistance (Ridley et al., 2001), and is used as a gelling and stabilizing agent in the food industry (Thakur et al., 1997). Previous research has shown that pectin can suppress colonic tumor incidence in rats (Heitman et al., 1992) and inhibit cancer cell metastasis in mice and rats (Platt and Raz, 1992; Pienta et al., 1995; Nangia-Makker et al., 2002). Pectin has been shown to bind to B16F10 melanoma cells \textit{in vitro} (Platt and Raz, 1992). Furthermore, when injected intravenously in mice, relatively large sized commercial pectin increased homotypic cell–cell aggregation and metastasis to the lung, while, pH-modified, relatively small sized pectin inhibited lung metastasis (Platt and Raz, 1992), demonstrating a differential response depending upon the type & size of pectin used. Oral administration of a pH-modified citrus pectin significantly reduced metastasis of rat prostate adenocarcinoma to the lung (Pienta et al., 1995). It is noteworthy that those anti-metastatic effects of pectins occurred in the absence of cell toxicity (Inohara and Raz, 1994). From such data, it has been hypothesized that pectins can bind to cancer-cell-surface-galectins (galactose-binding lectins) and interfere with cell–cell or cell–matrix adhesion and may inhibit metastatic lesions (Inohara and Raz, 1994). Modified forms of pectic polysaccharides have also been shown to play critical therapeutic roles against cancers (Nangia-Makker, et al., 2002; Martin et al., 2003) via immunomodulation (Wong et al., 1994).

Initially screening of pectic polysaccharides (Listed in Table 2.1) was performed using a simple and reliable galectin-3 mediated agglutination assay model. Corn pectic polysaccharide, which showed potent galectin-3 inhibition was selected for the determination of anti-metastatic potential in both \textit{in vitro} and \textit{in vivo} models. Effects of COPP on B16F10 metastatic cells such as MDA-MB-231 and \textit{in vivo} melanoma induced lung metastasis models were studied. Results were substantiated by biochemical/tumor markers and histopathological analysis.
4.4. Materials and Methods

4.4.1. Chemicals

Monoclonal anti human galectin-3 antibody and Twenty six and half gauge insulin syringe were purchased from Becton Dickinson Co., USA. B16F10 cell line was procured from National Facility of Animal Tissue and Cell Culture, Pune, India. Dulbecco’s modified eagle’s medium (DMEM), glutamine, sodium bicarbonate, penicillin, kanamycin, FBS (fetal bovine serum), MTT (3-[4,5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2-H-tetrazolium bromide), skimmed milk powder, paranitrophenyl phosphate, hydroxylamine hydrochloride, hydrogen peroxide, glutathione reductase, NADPH, reduced glutathione (GSH), 5, 5'- dithionitrobenzoic acid (DTNB), butylated hydroxy toluene (BHT), 1,1,3,3 tetramethoxypropane (TMP), phospho gluco isomerase (PGI), Tris-HCl, human serum albumin, bovine serum albumin, calf thymus DNA, ethidium bromide, acridine orange, hematoxylin, eosin, diethanolamine, trypsin and monoclonal anti NF-κB (P65 clone NF-12, mouse ascites fluid) antibody were purchased from Sigma Chemical Co. (St. Louis, MO). MMP-2 and MMP-9 monoclonal antibodies were from Santa Cruz Biotechnology, USA. Alkaline phosphatase conjugated-rabbit anti mouse IgG secondary antibodies were procured from GENEI, Bangalore, India. Matrigel invasion chamber with pore size (0.8 μm) was purchased from BD Biosciences, USA. Natural mouse laminin and VEGF Mouse ELISA Kit from Invitrogen, USA. Other chemicals and solvents used were of analytical grade procured from Qualigens, Mumbai, India.
4.4.2. Effect of corn pectic polysaccharides (COPP) on metastatic cells in vitro

4.4.2.1. Growth and maintenance of B16F10

B16F10 melanoma cells obtained from the National Facility of Animal Tissue and Cell Culture, Pune, India; previously shown to depict high metastatic status as evaluated by galectin-3 levels (Sathisha et al., 2007) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) containing sodium pyruvate, l-glutamine and vitamins and supplemented with 10% heat-inactivated fetal calf serum. The cells were grown in flasks in a humidified incubator with 5% CO₂ in air at 37 °C. After the cells were grown to confluence, they were washed several times with phosphate buffered saline (PBS) (pH 7.4) and harvested with brief treatment with 0.25% trypsin. Trypsinization followed by subculturing was done as described in chapter 1. Percent cell viability was assessed by MTT assay.

4.4.2.2. Determination of the cytotoxic activity of COPP

Cytotoxic activity of COPP was monitored by MTT assay (Hansen et al., 1989). Cells were seeded at density 2×10⁴ cells/well in a six well cell culture plate. After 24 h, the cells were washed four times with PBS and incubated with COPP (2-20 μg/mL) in serum free DMEM medium for 48 h at 37 °C. After incubation, 25 μL of MTT solution (5 mg/mL of media) was added, incubated at 37 °C for 4 h. 100 μL of ethyl alcohol and DMSO (1:1, v/v) were added to dissolve the dark blue crystals obtained due to the action of mitochondrial reductase, which converts yellow MTT, a tetrazole to reduced purple formazan in living cells. Absorption of formazan solution was measured at A 570 nm in a microplate reader (Spectra Max – 340, Molecular Devices, Germany). Trypsinization followed by subculturing was done as described in chapter 1. Cell viability was assessed using trypan blue staining method.

4.4.2.3. Effect of galectin-3 blockade by COPP; Inhibition of cell invasion

Galectin-3 has been implicated in cell invasion. Hence the role of COPP on cell invasion was evaluated. Matrigel invasion chamber with pore size 0.8 μm (BD Biosciences, USA) was used to measure cell invasion in vitro.
(Alexander et al., 2008). COPP at 10 & 20 \( \mu g/mL \) was added to B16F10 cells suspension in 0.5 mL medium without serum. The control and COPP treated cell suspensions (0.5 mL of \( 2 \times 10^4 \) cells) were added to each matrigel insert. The bottom chamber contained growth medium with 5% FBS. After 24 h, inserts 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. \((\text{No. of cells invaded into the bottom chamber}) \times 100\) and 2. \((\text{No. of cells in upper chamber at 0 h}) - (\text{No. of cells in UC at 24 h}) \times 100\).

4.4.2.4. Effect of galectin-3 blockade by COPP; Inhibition of cell adhesion

Adhesion assay was carried out according to the method of Hibino, et al., (2004). Microtitre wells were coated with laminin (100 \( \mu g/well \)) overnight at 4 \( ^{\circ} \)C. Wells were washed three times with PBS, blocked with 1% BSA in PBS for 2 h, and washed with PBS. B16F10 melanoma cells pretreated with 10 and 20 \( \mu g \) of COPP were resuspended in DMEM containing 0.1% BSA and added at \( 1 \times 10^4 \) cells/well and incubated for 1 h at 37 \( ^{\circ} \)C. After washing to remove unattached cells, the adherent cells were stained with crystal violet, detached with trypsin – EDTA and relative cell number adhered to the plate were counted under microscope.

4.4.2.5. Effect of COPP on apoptosis

Apoptosis assay was performed using ethidium bromide and acridine orange dye method (Powell et al., 2001) as well as observing characteristic features of cells by microscopy. Briefly, B16F10 (\( 1 \times 10^4 \) cells/well) cells were treated with COPP at 10 and 20 \( \mu g/mL \) for 24 h. respectively. Twenty five microliters of cell suspension of both treated and untreated cells were mixed with 1 \( \mu L \) of dye mix containing 100 \( \mu g/mL \) each acridine orange and ethidium bromide and observed under the microscope at 40 X. Viable cell nuclei stained green with acridine orange and apoptotic cell nuclei stained red with ethidium bromide were counted. The treated and untreated cells were also stained with crystal violet to visualize the cell membrane disruption. Percent
apoptosis was calculated as No of apoptotic cells (stained red)/total no cells x100.

4.4.2.6. Inhibition of galectin-3 induced agglutination of buccal cells by COPP

Buccal cells were obtained from gentle scraping of the inner cheek layer of healthy person, washed 3 times with sterile PBS and suspended in Ham’s F12 media containing 100 U/mL of penicillin and streptomycin. Normal Buccal cells (NBC -1x10^4) were treated with 10 and 20 µg /mL of COPP for 1 h. Galectin isolated from MDA-MB-231 cells (MDA-MB-231 cells were characterized as metastatic in nature with increased galectin-3 levels) were then added to both COPP treated and untreated cells and incubated for 1 h. The cells were then observed and photographed under inverted microscope at 40 X magnifications (Leica DMLS model, Germany). The % agglutination inhibition (free cells) were calculated as: total no. of cells ─ n. of free cells X 100/total no of cells.

4.4.3. Antimetastatic activity of COPP using B16F10 mouse melanoma cells on Swiss albino mice

4.4.3.1. Animals and treatments

6 – 8 weeks old female, Swiss albino mice (body weight of 25–32 g) were used in the study. The animals were maintained in an animal house at room temperature of 25 – 28 °C, water and food pellets were provided ad libitum. Animal experiments were carried out upon the clearance from the Central Food Technological Research Institute Ethics committee, at the animal house facility of CFTRI, Mysore 570 020, India, which has been registered with CPCSEA (Reg. No. 49, 1999), Government of India, New Delhi, India.

The animals were divided into four groups having 6 animals each. COPP was administered by oral intubation at the dose of 200 mg/kg body weight (in 0.2 ml distilled water) everyday for 20 days in group III and IV animals.

For in vivo experimental pulmonary metastasis, B16F10 cells (1 X 10^5 cells) suspended in PBS (20 mM, pH 7.4) were injected through the lateral tail vein.
of Swiss albino mice. Twenty-one days after the inoculation of tumour cells, mice were sacrificed and the lungs were removed and metastatic nodules were counted (Hibino et al., 2004) in various groups of animals. Experimental Groups were as follows;

<table>
<thead>
<tr>
<th>n=6</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Healthy (H): animals were given with only distilled water</td>
</tr>
<tr>
<td>Group 2</td>
<td>Metastatic control (MI): animals were given only B16F10 cells</td>
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<tr>
<td>Group 3</td>
<td>COPP control: animals were given with COPP alone - 200 mg/Kg b.w.</td>
</tr>
<tr>
<td>Group 4</td>
<td>COPP+MI: animals challenged with B16F10 cells following the administration of COPP - 200 mg/Kg b.w.</td>
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Group 3 animals were used to study the toxic effect of COPP also.

At the end of the experiment, animals were sacrificed by cervical dislocation; serum was collected and analyzed for biochemical parameters. Lungs were excised and the metastatic nodules were quantitatively evaluated by two observers. In addition tumors if appeared in tail, the site of injection or elsewhere in the body are noted since it indicates the extent of metastatic spread.

### 4.4.3.2. Preparation of serum, lung and liver homogenates

All the animals after the treatment were anaesthetized with diethyl ether. Serum was collected from blood samples as described in section 3.3.3.4. Liver and lungs were removed and five percent lung/liver homogenates were prepared using cold 0.15 M potassium chloride and centrifuged at 5000 g for 20 min at 4 °C. The supernatants obtained were collected and used for the estimation of galectin-3, melanin content as well as various other biochemical parameters described below.

### 4.4.3.3. Determination of galectin-3

Galectin-3 has been reported earlier as a metastatic marker (Nangia-Makker et al., 2000; Sathisha et al., 2007). ELISA was performed for the detection of galectin-3 levels in the lung tissue homogenate employing the protocol
standardized previously (Rajeshwari et al., 1998). Monoclonal anti human galectin-3 antibody was employed at dilutions 1:1,000 (v:v) as primary antibody. Alkaline phosphatase conjugated rabbit anti mouse IgG at 1:5,000 (v:v) dilution followed by paranitrophenylphosphate were used as secondary antibody and substrate, respectively. The absorbance was measured at 405 nm in a microplate ELISA reader (Molecular Devices, Spectramax 340, Germany).

**4.4.3.4. Estimation of Melanin content**

The melanin content of lung and liver tissue homogenates were measured according to the method of Oka et al., (1996) with slight modification, since tumors were observed both in lung and liver tissues. The liver homogenates were solubilized in boiling with 1 M NaOH for 10 min and the spectrophotometric analysis of melanin content was performed at an absorbance of 400 nm. The entire experiment was performed in triplicates, and results were confirmed by three independent experiments. A spiked tissue homogenate with known amount of melanin to the control lung/liver tissue homogenates was also performed to rule out the interference from homogenates.

**4.4.3.5. Estimation of uronic acid**

The acidic modification of monosaccharides produced by the oxidation of CH₂OH group to COOH group yield uronic acid, where as basic modification yield amino sugars (hexosamines) and these form an integral part of many structural polysaccharides, and glycosaminoglycans found in the ECM.

Uronic acid was estimated (Bitter & Muir, 1962) in 0.5 mL of the sample solution (10 mg/100 mL) in a test tube and kept in ice cold water bath for 10 min. To this was added concentrated sulphuric acid (3 mL) slowly, contents were mixed thoroughly and kept in boiling water bath for 20 min. Contents were cooled to which carbazole solution (0.1 mL, 0.1% prepared by dissolving re-crystallized carbazole in alcohol) was added. The tubes were kept in dark for 2 h and the absorbance was recorded at 530 nm. Uronic acid content was determined against the calibration graph prepared by using D-galacturonic acid (10-50 μg/mL).
For extraction of glycoproteins from the tissues, a known weight of the tissue was homogenized in 7.0 mL of methanol. The contents were decanted and homogenized in 14.0 mL of chloroform. This was again decanted and the residue was successively homogenized in chloroform-methanol (2:1 v/v) and each time the extract was decanted. The residue (defatted tissue) was obtained and the filtrate decanted. A weighed amount of defatted tissue was suspended in 3.0 mL of 2 N HCl and heated at 90 °C for 4 h. The sample was cooled and neutralized with 3.0 mL of 2 N NaOH. Aliquots from this were used for estimation of hexosamine and sialic acid. Hydroxyproline was also estimated, since they represent the component of extracellular matrix - particulary collagen.

### 4.4.3.6. Estimation of hexosamine

Hexosamine was estimated by the method of Wagner (1979). To 1.0 ml of defatted tissue sample was added 2.5 mL of 3 N HCl and kept for 6 h in a boiling water bath and then neutralized with 6 N NaOH. 0.8 ml of the neutralized sample was treated with 1 mL of freshly prepared 2% acetylacetone in 0.5 M Na₂CO₃ in capped tubes and kept in boiling water bath for 15 min. After cooling in tap water, 5 mL of 95% ethanol and 1mL Ehrlich’s reagent were added and mixed thoroughly. The purple red color developed was read after 30 min at 530 nm.

### 4.4.3.7. Estimation of Hydroxyproline

Lung collagen hydroxyproline content is a direct marker of lung fibrosis. During lung fibrosis, extracellular matrix, especially collagen is deposited massively in the alveoli of lungs. Fifteen to thirty percent of collagen is hydroxyproline and it results in defective pulmonary function. Hydroxyproline was measured by the modified alkaline hydrolysis method of Reddy and Enwemeka (1981). Briefly to 100 μL of homogenate sample was added NaOH (2 N final concentration) and the mixture was hydrolyzed by heating in boiling water bath for about 3 - 4 h. Approximately 900 μL of 56 mM chloramine T reagent was added to the hydrolyzed sample and oxidation was allowed to proceed at the room temperature for 25 min. Then 1 mL of 1 M Ehrlich’s reagent (P- dimethylaminobenzaldehyde) was added to the
oxidized sample and the chromophore was developed by incubating the samples at 65 °C for 20 min. The absorbance was read at 550 nm. The hydroxyproline concentration in the samples was calculated using the standard curve of hydroxyproline at 10-50 μg/mL.

4.4.3.8. Estimation of Phosphoglucoisomerase enzyme
Phosphoglucoisomerase (PGI) is an enzyme which isomerizes glucose-6-phosphate to fructose-6-phosphate. The level of PGI has been believed to raise significantly as a metabolic adaptation by cancer cells to differentially favour their own growth (Paschos et al., 2009). Tissue homogenates were dialyzed using 10 kDa cut off dialysis tubing against PBS to remove free sugar that can interfere with the assay. The reaction mixture, consisting of 0.1m L of above homogenates in 1 M Tris buffer (pH 7.4) was preincubated for 10 minutes at 37 °C. Subsequently, the reaction was initiated by the addition of buffered substrate (glucose -6-phosphate) and incubated for 40 min at 37 °C. The reaction was terminated by the addition of TCA (10%) and centrifuged for 10 mins at 1500 g. The supernatant was mixed with 30% HCl and Resorcinol-Thiourea reagent and heated on water bath for 10 minutes at 75 °C. The tubes are immediately cooled in cold running water and the pink-red colour read in the next few minutes at 490 nm.

4.4.3.9. Estimation of VEGF, MMP-2, MMP-9 and NF-kappa B
VEGF, MMP-2, MMP-9 and NF-kappa B was estimated in lung homogenate by ELISA method. Monoclonal antibodies to VEGF, MMP-2, MMP-9 and NF-kappa B were employed as primary antibodies at 1:500 (v/v), 1:100 (v/v), and 1:1000 (v/v) dilutions respectively. Alkaline phosphatase conjugated rabbit anti-mouse IgG (GENEI, Bangalore, India) at 1:5000 (v/v) dilution followed by paranitrophenylphosphate (p-NPP) were used as secondary antibody and substrate respectively for the estimation of MMP-2, MMP-9 and NF-kappa B. Levels of VEGF was estimated in lung homogenate by a VEGF ELISA kit according to the instructions of the manufacturer (Invitrogen, USA). Levels were measured and compared between animals of various group.
4.4.3.10. Biochemical analysis in the serum
4.4.3.10.1. Determination of serum sialic acid
Sialic acid, a family of acetylated derivatives of neuraminic acid, occurs as a terminal component at the non-reducing end of carbohydrate chains of glycoproteins and glycolipids. Neoplasm often have an increased concentration of sialic acid on the tumour cell surface and sialoglycoproteins are shed or secreted by some of these cells which increases their concentration in blood (Khadapkar et al., 1975; Kloppel et al., 1977).

Sialic acid was determined by the method of Warren, 1959. In brief, 0.5 mL of aliquot/plasma was treated with 0.5 mL of water, 0.25 mL of periodic acid and incubated at 37 °C for 30 min. To the reaction mixture added 0.2 mL of sodium meta arsenate and 2 mL of thiobarbituric acid were added and heated for 6 min and added 5 mL of acidified butanol. The absorbance was read at A 540 nm.

4.4.3.10.2. Evaluation of antioxidant and antioxidant enzymes
Serum of all test groups were analyzed for total protein (Lowry et al., 1951), antioxidant enzymes such as SOD (EC 1.15.1.1) CAT (EC 1.11.1.6) and antioxidant GSH employing protocols described earlier (Section 3.3.3.6.1, 3.3.3.6.2 and 3.3.3.7). Enzyme activity for SOD and CAT are expressed as units U/mg protein. To evaluate the toxic effects of COPP, serum of control groups were analyzed for total protein and liver function enzymes like serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) as per the standard protocols (Naik et al., 2007).

4.4.3.11. Histopathology
The lung tissues were fixed in 10% formalin in PBS, and then embedded in paraffin. The paraffin embedded tissue blocks were sectioned using a microtome and the thickness was set between 6 - 8 microns, the tissue sections were fixed on to the microscopic slide and stained with hematoxylin and eosin as per the standard protocol.
4.4.4. Statistical analysis
Values are expressed as mean ± SD (n = 6). The analysis utilized one way analysis of variance (ANOVA) and Turkey-Kramer multiple comparison test for significance at p < 0.05 using graphpad statistical software (Graphpad Instat).
4.5. Results

4.5.1. Determination of antimetastatic activity in the *in vitro* system

4.5.1.1. Cytotoxic activity of COPP towards B16F10 melanoma cells by MTT assay

B16F-10 melanoma cells were incubated with different concentrations (2–20 μg/ml) of COPP. Percentage of cytotoxicity was determined using MTT assay. As shown in Figure 4.1A, treatment of B16F10 cells at 10 and 20 μg/mL showed a dose dependent growth inhibition by reducing the cell number by 28% and 60% respectively. Absorbance observed in the untreated control was considered as 100%. Percent reduction in MTT absorbance reveals toxicity to cells.

4.5.1.2. Inhibition of tumor cell invasion by COPP.

Metastatic B16F10 melanoma cells show highly invasive property through the collagen matrix. Very high number of cells were found in the lower surface of the polycarbonate membrane, but administration of COPP produced significant inhibition in the invasion of the collagen matrix by the tumour cells in a dose dependent manner. At concentrations of 10 and 20 μg/mL, COPP inhibited the invasion by 38% and 63% respectively (Figure 4.1B).

4.5.1.3. Inhibition of cell adhesion by COPP

To investigate whether COPP inhibited tumor metastasis by directly preventing tumor cell adherence, we performed *in vitro* adhesion assays. COPP significantly inhibited B16F10 cell adhesion to laminin matrigel in a dose dependent manner (Figure 4.1C). COPP at 10 and 20 μg/mL reduced cell adhesion by 47% and 65% respectively.

4.5.1.4. Apoptotic effect of COPP on B16F10 cells

Apoptotic effect was observed in B16F10 cell upon treatment with COPP. Cells showed distinct morphological changes as evident from oozing out of cytoplasmic contents, increase in cell volume, membrane disruption and cell lysis resulting in ghost cells (Figure 4.1D). Results were substantiated by the appearance of apoptotic cells as evaluated by acridine orange and
ethidium bromide staining (Figure 4.1E&E2). Nuclear fission and cell disruption were evident in COPP treated cells suggesting cell death. Treatment of B16F10 cells with COPP for 2 h was shown to induce apoptosis of 23% and 48% at 10 and 20 µg /mL of COPP respectively (Figure 4.1F).

4.5.1.5. Inhibition of agglutination
Normal buccal cells were free, regular and flattened (Figure 4.2A1), upon treatment of normal buccal cells with galectin-3 isolated from MDA-MB-231 cells showed irregular shape and size of cells with clumping (Figure 4.2A2). Pretreatment of buccal cells with COPP showed protection against galectin-3 induced changes and also significant reduction in agglutination by 31% and 68% at 10, and 20 µg /mL of COPP respectively. (Figure 4.2A, 3 & 4).

4.5.2. Antimetastatic activity of COPP in vivo in lung metastasis.
B16F10, mouse melanoma cells have been widely used as a well accepted model in vitro and in vivo to understand both the pathogenicity as well as therapeutic aspects of metastasis. Previous studies from our laboratory have shown higher levels of galectin-3 in B16F10 cells, which is implicated in metastasis. Results also have shown the apoptotic effect and hampering of cell invasion, adhesion, agglutination etc., we envisage the in vivo efficacy of Corn Pectic Polysaccharide (COPP) which showed potent inhibition of metastasis probably by inhibiting galectin-3 and galectin-3 mediated metastatic cascade.

4.5.2.1. Effect of COPP on the lung tumor nodule formation
Since the COPP caused a marked reduction of tumor invasion in vitro, this study examined the effect of COPP on in vivo experimental lung metastasis produced by an intravenous injection of B16F10 melanoma cells in swiss albino mice. Inoculation of B16F10 cells in the lateral tail vein resulted in the formation of metastatic nodules in the lungs (Figure 4.3A). In addition, local tumors were observed at the site of injection on the tail (4.3B). There was a significant reduction in the lung tumor nodule formation in mice with COPP treated post B16F10 cell injection compared to mice which were injected with only metastatic B16F10 melanoma cells & left untreated.
Earlier, it has been reported that B16F10 cells mainly form lung tumors (Engbrin et al., 2002). Administration of COPP reduced lung metastasis caused by B16F10 melanoma cells. Quantifiable metastatic nodules in the lungs were measured. Nodular structures with blackish coloration that could be distinguished on the lung surface and which were sufficiently separated from each other to be counted individually, and present in several numbers in metastatic mouse and totally absent in lungs of healthy group of animals also suggested the extent of tumor harboring in the lung for their survival, to seek more oxygen. Metastatic group of animals – group 2 injected with only B16F10 cells developed massive number of tumor nodules between between 201 and 386 randomly distributed over the lung surface with a mean of 290 ± 92.69. There was a significant reduction in the lung tumor nodule formation when the animals were treated with COPP (71.66 ± 26.9) along with tumor cells. It represents a significant reduction of 75 % metastatic nodules compared to that of metastasis induced group (Figure 4.6A) at the concentration of 200 mg/kg b.w.

The localization of metastatic B16F10 colonies varied widely in their size, numbers and distribution between Metastatic induced (MI) and sample treated groups. In MI groups, huge colonies were observed and found to occur across all regions on the lung such as pleural, subpleural and intraparenchymatose region. As observed by other investigators (Martinez et al., 2005) two basic patterns such as linear and solid patterns were observed. Solid tumors were usually larger in size, with bulging appearance and accumulation of numerous melanocytes (Figure. 4.3Ab). Inflammatory infiltrates, actively proliferative hyperchromatic cells indicating increased mitotic potency, disrupted and dysplastic cells, altered glandular architecture of lungs due to invasion process; with typical metastatic characteristics were also observed. The melanin pigment was observed as a blackish-brown colored accumulation. However, such changes were not found in sections of the lung, which were treated with COPP (Figure. 4.3Ac) although few colonies found were only at the peripheral end indicating anti-invasive property.
4.5.2.2. Histopathological analysis of lungs

Histological changes of lung tissues hematoxylin and eosin stained sections of lung tissues (Figure 4.4). Figure 4.4A shows a well organized Alveoli, pleura and alveolar passage lined with healthy ciliated columnar epithelial cells in healthy groups. Intact mucosal layer, well organized, elongated glandular structures in healthy groups. The Lungs of COPP control were exactly similar to normal lung of healthy groups suggesting that COPP does not have any adverse effect on the major organs. (Figure 4.4C). The lungs of control metastatic tumor bearing animals (Figure 4.4B) showed prominent tumor nodules around terminal bronchiole. These tumor nodules are characterized by polygonal tumor cells with prominent nucleolus, intracellular melanin deposition and clear areas of necrosis. This massive infiltration of the neoplastic cells around the main bronchioles, which make alveolar passages indistinguishable, extended to the pleura. This together with fibrosis reduces alveolar space, which in turn leads to reduced vital capacity. Alveoli and pleura were tumor free, alveolar passage lined with healthy ciliated columnar epithelial cells and almost similar to normal lung. Considerable reduction of tumor mass was observed upon COPP administration (Figure 4.4D).

4.5.2.3. Effect of COPP on secondary metastasis

During the experiment later diffusion of tumor cells from lungs were observed in the liver also. As shown in figure 4.5A liver from normal, metastasis induced and COPP treated are provided. Blackish tumor colonies with differences in size were observed. No tumor colonies in liver were observed in COPP treated animals suggesting effective inhibition of secondary metastasis by COPP. Results were substantiated by histopathological analysis (Figure 4.5B).

4.5.2.4. Biomarker analysis in lung homogenate

4.5.2.4a. Galectin-3 levels in lung tissue

Galectin-3 as indicated earlier is a metastatic marker. When levels were determined by galectin-3 specific monoclonal antibody 2.1 fold increase in galectin-3 was observed (Figure 4.6B) in metastasis induced animals when compared to that of healthy lungs. ~ 55% reduction in galectin-3 levels were
observed in COPP treated animals. Results thus support the role of galectin-3 in metastasis.

4.5.2.4b. Melanin content in lungs
Melanin is a chromogenic molecule produced specifically by melanoma cells. In this case it is from B16F10 mouse melanoma cells which were harboured in the metastatic lung, at very high levels. Healthy animal notably did not show melanin content. Measured melanin levels hence indicate the concentration of melanoma cells established in the lung. Basal level of absorbance observed at A 475 nm was taken as blank and absorbance observed in metastatic induced samples over control was recorded and considered as 100% to compare with the treated groups. Figure 4.6C reveals ~59% reduction in group 4 animals treated with COPP. Data substantiates the results observed in histopathology (Figure 4.4B) and reveal that reduced melanin is due to reduced tumor colonies.

4.5.2.4c. Effect of COPP on Phosphoglucoisomerase (PGI) enzyme activity
PGI level was significantly higher in the lungs of metastatic animals (8.43 ± 0.65 nmoles of fructose/min/mg protein) than the healthy controls (3.67 ± 0.48 nmoles of fructose/min/mg protein). PGI levels decreased significantly in COPP treated animals (4.92 ± 1.13 nmoles of fructose/min/mg protein) paralleling improvement of the disease (Table 4.1)

4.5.2.4d. Effect of COPP on VEGF, MMP-2, and MMP-9 activity
There was an increased level of VEGF in the group 2 metastatic induced animals, (12.7±0.96 ng/g tissue) compared to the normal animals (0.68 ±0.15 ng/g tissue). Treatment with COPP reduced the elevated VEGF to 4.58 ± 0.62 ng/g tissue indicating the decreased tumor burden. (Table 4.1). Effect of COPP on the MMP-2 and MMP-9 level of metastatic tumor bearing animals is shown in Table 4.1. The MMP-2 and MMP-9 level was drastically elevated in the metastasis control group (1.31±0.09 and 1.71±0.13 U/mg protein) compared to the normal animals (0.35±0.09 and 0.38±0.03 U/mg protein). COPP treated animals showed a significant reduction of 49 and 60% in MMP-2 (0.67±0.12 U/mg protein) and MMP-9 (0.69±0.16 U/mg protein).
protein) activity. Suggesting that reduction in invasiveness could be due to reduction in metalloproteinase activity.

**4.5.2.5. Effect of COPP on the biochemical parameters of the metastasis bearing animals**

**4.5.2.5a. Effect of COPP on the Uronic acid levels of the lung**

In control metastatic tumour bearing animals, the lung uronic acid level was drastically elevated (298.86 ±13.8 μg/100 mg tissue wet wt), as compared to normal level (45.66 ± 4.21 μg/100 mg tissue wet wt) which was significantly reduced after the simultaneous administration of COPP (123.63±10.42 μg/100 mg tissue wet wt).(Table 4.2).

**4.5.2.5b. Effect of COPP on the lung collagen hydroxyproline content**

Elevated levels of hydroxyproline content in the control tumour bearing animals, (26.96 ±3.0 μg/mg protein) compared to the normal animals (1.73 ± 0.36 μg/mg protein). Treatment with COPP reduced the elevated hydroxyproline content to 8.3 ± 2.2 mg/100 µg/mg protein indicating the decreased tumour burden. (Table 4.2)

**4.5.2.5c. Effect of COPP on the Lung hexosamine content**

There was an increased level of lung hexosamine content in the control tumour bearing animals, (5.46 ± 0.92 mg/100 mg tissue dry wt) compared to the normal animals (1.29 ± 0.28 mg/100 mg tissue dry wt). Treatment with COPP reduced the elevated lung hexosamine content to 2.8 ± 0.61 mg/100 mg tissue dry wt. indicating the decreased tumour burden. (Table 4.2).

**4.5.2.6. Serum analysis for protein, sialic acid, antioxidant and antioxidant enzymes**

The total protein content in group 3 and group 4 (15.26 ± 1.36 and 16.38 ± 1.42 mg/mL) was not significantly different from that of control group (group 1-14.89 ± 1.23 mg/mL). Group 2 showed slightly increase in total protein content of 19.12 ± 1.58 mg/mL, which could be due to increase in tumor burden (Table 4.3)
Effect of COPP on the serum sialic acid level of metastatic tumor bearing animals is shown in Table 4.2. The serum sialic acid level was drastically elevated in the control group (137.89 ± 5.68 μg/mL) compared to the normal animals (31.70 ± 2.2 μg/mL). COPP treated animals showed a significant reduction (52.92 ± 3.76 μg/ml) in the sialic acid content.

Antioxidant enzyme CAT was slightly depleted in metastasis induced groups, however no significant difference observed between healthy, COPP treated group and COPP control animals. SOD was depleted in metastasis induced group (3.6 ± 0.71 U/mg protein) compared to healthy animal group (6.61 ± 0.7 U/mg protein) by ~ 2 fold. Significant recovery of enzyme activity was observed in COPP treated group (~ 75%). Around ~ 3.5 fold reduction in GSH antioxidant level was observed in the metastasis induced group (2.11 ± 0.66 mg/mg protein); while no difference was observed also in group 1 and 3 animals. The GSH level in group 4 was recovered upon COPP treatment (6.49 ± 1.41 mg/mg protein) (Table 4.3).

4.5.2.7. Toxicity of COPP

Toxicity analyses with COPP were arrived out in mice for safety evaluation. The results indicated no lethal effect, when orally fed for 21 days. After the above treatment schedules, animals remained as healthy as control animals with normal food and water intake, body weight gain and behaviour. There were no significant differences in total protein, TBARS levels, SGPT and SGOT levels between normal and COPP treated mice (Table 4.4), indicating no adverse effect on the major organs and further substantiated the non-toxic nature of COPP.
Figure 4.1. (A). Cytotoxicity of COPP towards B16F10 melanoma cells. (B). Inhibition of B16F10 cell invasion through matrigel by COPP. (C). Inhibition of B16F10 cell adhesion by COPP. (D1 & D2). Arrow indicates apoptotic cells. Photograph of crystal violet stained cells suggesting cell disruption and cell death during the treatment with COPP. (E1 & E2). Photograph of apoptotic B16 F10 cells resulting from the treatment with 10 & 20 µg of COPP respectively; viable cell nuclei stained green with acridine orange and apoptotic cell nuclei stained red with ethidium bromide. (F). Measurement of percent apoptotic B16F10 cells during the treatment with COPP.
Figure 4.2. (A). Inhibition of galectin-3 induced agglutination by COPP: **A1**-Normal buccal cells - free, regular and flattened; **A2**-Treatment with galectin-3 isolated from MDA-MB-231 cells revealing irregular shape and size of cells with clumping; **A3&4**-Same as A2, but pretreated with 10 & 20 µg of COPP showing protection against galectin-3 induced changes. (B). Graphical representation of % agglutination in control and COPP treated cells.
**Figure 4.3.** (A). Macroscopic characteristics of the lung metastatic nodules. Aa- Healthy; b- metastatic induced; c- COPP pretreated followed by metastatic induction. (B). Local tumor growth on the tail at the site of B16F10 cells injection.
Figure 4.4. Histopathological photographs of healthy (H), and metastasis induced (MI), COPP treated lungs (Swiss Albino Mice). (A)-Healthy control; (B)-Metastatic induced; (C)- COPP treated; (D)- COPP pretreated followed by metastatis induction.
Figure 4.5. Macroscopic characteristics of the pulmonary metastatic nodules (A). A a- Healthy; b- metastatic induced; c-COPP treated d- COPP pretreated followed by metastatic induction. B. Histopathological photographs of healthy (H), and metastasis induced (MI), COPP treated liver (Swiss Albino Mice). a-Healthy control liver; b-Metastatic induced; c- COPP treated; d- COPP pretreated followed by metastatic induction.
Figure 4.6. (A) Frequency of the lung metastatic nodules of experimental animal groups. (B) galectin-3 levels in serum and lung tissues and (C) melanin levels in lung metastasis and liver tissues. Values are expressed as mean ± SD (n = 6).
Table 4.1. Effect of COPP on VEGF, MMP-2, MMP-9, PGI and NF-κB activity during B16F10 induced lung metastasis. Values are expressed as mean ± SD (n = 6). The analysis utilized one way analysis of variance (ANOVA) and Turkey-Kramer multiple comparison test for significance at p < 0.05 using graphpad statistical software (Graphpad Instat).

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>VEGF (ng/g tissue)</th>
<th>MMP-2 (units/mg protein)</th>
<th>MMP-9 (units/mg protein)</th>
<th>PGI (nmoles of fructose/min/mg protein)</th>
<th>NF-κB (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td>0.68±0.15</td>
<td>0.35±0.09</td>
<td>0.38±0.03</td>
<td>3.67±0.48</td>
<td>0.051±0.01</td>
</tr>
<tr>
<td>Metastatic control</td>
<td>12.7±0.96</td>
<td>1.31±0.09</td>
<td>1.71±0.13</td>
<td>8.43±0.65</td>
<td>2.43±0.21</td>
</tr>
<tr>
<td>COPP Metastasis</td>
<td>4.58±0.62</td>
<td>0.67±0.12</td>
<td>0.69±0.16</td>
<td>4.92±1.13</td>
<td>0.816±0.15</td>
</tr>
</tbody>
</table>

Table 4.2. Effect of COPP administration on the lung biochemical parameters. The lungs were dissected out and assayed different biochemical parameters on the 22nd day after induction of B16F10 melanoma cells through the lateral tail vein. Values are mean±S.D. Values are expressed as mean ± SD (n = 6). The analysis utilized one way analysis of variance (ANOVA) and Turkey-Kramer multiple comparison test for significance at p < 0.05 using graphpad statistical software (Graphpad Instat).

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>Hydroxy proline (μg/mg protein)</th>
<th>Uronic acid (μg/100 mg tissue wet wt)</th>
<th>Hexosamine (mg/100 mg tissue dry wt)</th>
<th>Sialic acid (μg/ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>1.73±0.36</td>
<td>45.66±4.21</td>
<td>1.29±0.28</td>
<td>31.70±2.2</td>
</tr>
<tr>
<td>Metastatic induced</td>
<td>26.96±3.0</td>
<td>298.86±13.81</td>
<td>5.46±0.92</td>
<td>137.89±5.68</td>
</tr>
<tr>
<td>COPP+ Metastasis</td>
<td>8.35±2.2</td>
<td>123.63±10.42</td>
<td>2.8±0.61</td>
<td>52.92±3.76</td>
</tr>
</tbody>
</table>
### Results

#### Table 4.3. Analysis of serum protein, antioxidant and antioxidant enzymes.

Values are mean±S.D. Values are expressed as mean ± SD (n = 6). The analysis utilized one way analysis of variance (ANOVA) and Turkey-Kramer multiple comparison test for significance at $p < 0.05$ using graphpad statistical software (Graphpad Instat).

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Metastasis induced</th>
<th>COPP</th>
<th>COPP+ Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Protein</strong></td>
<td>14.89±1.23</td>
<td>19.12 ± 1.58</td>
<td>15.26±1.36</td>
<td>16.38±1.42</td>
</tr>
<tr>
<td>(mg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SOD</strong></td>
<td>6.61±0.70</td>
<td>3.62±0.71</td>
<td>7.51±0.67</td>
<td>5.21±1.06</td>
</tr>
<tr>
<td>(U/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CAT</strong></td>
<td>28.9±3.06</td>
<td>23.25±2.98</td>
<td>27.96±2.3</td>
<td>25.46±2.02</td>
</tr>
<tr>
<td>(U/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GSH</strong></td>
<td>7.53±0.17</td>
<td>2.11±0.66</td>
<td>7.04±1.83</td>
<td>6.49±1.41</td>
</tr>
<tr>
<td>(mg/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Table 4.4. Toxicity studies of COPP.

SGOT: serum glutamate oxaloacetate transaminase; SGPT: serum glutamate pyruvate transaminase; TBARS: thiobarbituric acid reactive substances. All data are the mean 3 ± SD (n=6). Insignificant differences reveal no toxicity from COPP.

<table>
<thead>
<tr>
<th>Group n=6</th>
<th>SGOT (U/mg protein)</th>
<th>SGPT (U/mg protein)</th>
<th>TBARS (μ moles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>3.78±0.5</td>
<td>4.05±0.63</td>
<td>0.58±0.12</td>
</tr>
<tr>
<td>COPP control</td>
<td>3.53±0.65</td>
<td>3.85±0.35</td>
<td>0.55±0.11</td>
</tr>
</tbody>
</table>
4.6. Discussion
Cancer is a complex disease involving multiple steps such as tumor initiation, promotion, and metastasis. Metastasis is one of the greatest problems in cancer patients. They appear frequently and are the primary cause of mortality in cancer patients (Fidler, 1999). Different mechanisms are involved in the so-called metastatic cascade, including angiogenesis, cellular adhesion, local proteolysis, and tumor cell migration (Kohn, 1993; Fidler, 1999). Development of chemotherapeutic agents which target and intervene in one or more processes in the metastatic cascade should lead to a favourable outcome for a large number of cancer patients.

Dietary compounds containing flavonoids, antioxidants, glycosides etc., are known to reduce the risk of cancers (Doll, 1990) particularly by quenching 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 in the initiation step. However, the advanced step of the disease, metastasis; essentially is the ability to spread to other tissues and organs makes cancer potentially a life-threatening disease (Raz, and Lotan, 1987; Kohn, 1993). 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. The role of dietary components in cancer progression and metastasis is an emerging field of clinical importance. Many stages of cancer progression involve carbohydrate-mediated recognition processes (Nangia-Makker et al., 2002). Modified citrus pectin has been known to reduce the growth of human prostate cancer cells (Pienta et al., 1995). Unmodified form of this pectin appears to inhibit spontaneous metastasis of certain cancer cells. Furthermore, based on in vitro binding experiments, citrus pectin inhibited prostate cancer cell adhesion to endothelial cells by competing for galactose binding lectins – galectins on the cell surface of the cells, essentially acting as a cancer cell “decoy”. Tumor cells that form aggregates appear to have greater metastatic potential. In an in vitro study citrus pectin inhibited tumor cell aggregation (Platt and Raz 1992). Injected citrus pectin was also shown to inhibit...
metastasis of melanoma cells to the lungs of mice (Pienta et al., 1995) and believed to be by competing for galectin binding sites on the tumor cells (Inohara and Raz 1994).

Previously, we have investigated galectin inhibitory activity and inhibition of cell invasion by dietary pectic polysaccharides (Sathisha et al., 2007). This inhibition of tumor nodules correlated with an increase in the life span of the metastatic-tumor-bearing animals 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. Pectin, a natural plant polysaccharide present in all higher plant cell walls, and thus in all fruits and vegetables and in most plant derived foods, is a compound that appears to be able to inhibit cancer metastasis and primary tumor growth in multiple types of cancer in animals. Although pectins were initially recognized as compounds capable of inhibiting metastatic lesions (Heitman et al., 1992; Platt and Raz 1992; Pienta et al., 1995; Nangia-Makker et al., 2002), more recently, pectins have been shown to reduce primary tumor growth, angiogenesis and metastasis in vivo (Nangia-Makker et al., 2002). It has been suggested that the inhibitory effects of pectin on metastatic lesions in the lung are mediated through their binding to galectin-3 (a galactoside-binding lectin), since galectins facilitate cell–cell interactions by binding to galactose-containing molecules on neighboring cancer cells. In human colon, stomach and thyroid cancers, the amount of galectin-3 increased with the progression of cancer. Blocking galectin-3 expression in highly malignant carcinoma cells led to reversion of the transformed phenotype and suppression of tumor growth in nude mice (Honjo et al., 2000, 2001). It has been proposed that the pH-modified citrus pectin blocks binding of galectins, and thus, inhibits tumor cell–cell interactions.

In the current study, the animals treated with COPP showed considerable inhibition of lung metastasis. ~ 72% inhibition of tumor nodules compared to the untreated control group reveals the efficacy of COPP against metastasis. Further, the increased levels of galectin-3 in the positive control
animals and reduction in animals treated with COPP correlated well with the severity of metastasis. These results further strengthen the view of galectin-3 involvement in metastatic process as reported earlier (Inufusa et al., 2001). Tumor nodules are metastatic colonies of B16F10 melanoma cells formed in the lungs and initiate lung fibrosis and collagen deposition. Lung collagen hydroxyproline content is a direct marker of lung fibrosis. Administration of COPP resulted in significant reduction of hydroxyproline content, which in turn causes marked reduction in lung fibrosis. This was well in correlation with histopathological analysis and significant reduction of tumor nodules in COPP treated animals. Hyaluronic acid (HA) and hexosamines form an integral component of tissue matrix and tissue fluid (Delpech et al., 1997; Tammi et al., 2002). Elevated HA concentration in several cancers regardless of the tumor grade (Setala et al., 1999; Hautmann et al., 2001) supports tumor cell migration by interacting with cell surface HA receptors and thereby promoting metastasis (Delpech et al., 1997; Tammi et al., 2002; Turley et al., 2002). Hyaluronidase degrades HA and liberates disaccharide units, which are good promoters of angiogenesis as well as modifiers of proliferation, adhesion and migration of endothelial cells (Rodden et al., 1989; West et al., 1985). Hexosamine has an important role in the synthesis of N-acetyl neuraminic acid (sialic acid), which is a component of glycolipids present on the surface of tumor cells (Voet and Voet, 1995) and sialoglycoproteins are shed or secreted by some of these cells which increases their concentration in blood (Khadapkar et al., 1975; Kloppel et al., 1977). The enhanced level of these monosaccharides and sialic acid in metastatic tumor bearing animals indicates the active growth and proliferation of tumor cells. Reduction in the elevated levels of uronic acid, hexosamine and sialic acid content in the COPP treated animals may be one of the reasons for reduction in tumor growth and secondary metastasis as occurred with group 2 metastatic animals.

One possible explanation for protection is that during dissemination through blood flow, tumor cells cannot adhere to ECM and unadhered cells may die as a result of anoikis. Anoikis is a kind of apoptosis, which is induced by disruption of cell-matrix interaction. Recently it was also shown that over
expression of galectin-3 protects cells from anoikis as well as other apoptotic stimuli (Yoshii et al., 2002). This galectin-3 mediated anoikis resistance may confer advantage during dissemination through blood flow on tumor cells.

Secondly, COPP may also directly prevent cancer cell metastasis by inhibiting galectin-3 mediated cancer cell adhesion to and invasion through the ECM. It is known that metastasis is a highly complicated phenomenon that requires interactions between cancer cells and many other types of cells and ECM. After binding to the ECM, cancer cells invade the ECM, migrate away from primary tumors, to bind and penetrate blood vessel walls, migrate to new sites, and finally form secondary tumors. We found that COPP inhibits cancer cell adhesion to and invasion through the ECM in *in vitro* systems. COPP successfully blocked B16F10 melanoma cells adhesion to the ECM proteins, such as laminin, in a concentration-dependent manner (Figure 4.1C). The binding of pectic polysaccharide to galectin-3 in addition to anti-invasive and anti-adhesion activity may also be responsible for inhibition of secondary tumors (Figure 4.5). These observations together suggest that COPP can reduce tumor cell invasiveness by suppressing galectin-3 mediated cell adhesion to extracellular matrix proteins in the basement membrane and hence may subject such cells to apoptosis. Data on induction of apoptosis of cancer cell and not normal cell (data not shown) further provide evidence for galectin-3 mediated antiapoptosis. Akahani et al., (1997) reported that galectin-3 has an influence on Bcl-2 – an antiapoptotic protein. COPP, by virtue of galectin-inhibiting property therefore may block the effect of galectin-3 in blocking the cancer cell apoptosis.

Matrix metalloproteinases are a family of zinc dependent endoproteinases that are capable of degrading almost all of the components of the extracellular matrix and thereby up regulates invasion and metastasis (Stetler-stevenson et al., 1996; Chambers and Matrisian, 1997). Among the MMPs reported earlier, MMP-2 and MMP-9 are key enzymes for degrading type IV collagen, which is a major component of the basement membrane (Zucker et al., 1993; Bernhard et al., 1994). Several experiments also proved
that MMPs not only break down the physical barrier of extracellular matrix but also modulate the growth factors and cytokines stored in the extracellular matrix, which may promote neoplastic progression (Voet and Voet, 1995). The results indicate that administration of COPP inhibited the activation of matrix metalloproteinases and also inhibited the invasion of B16F10 melanoma cells through the collagen matrix and strongly back up the direct inhibition of activation of matrix metalloproteinases by COPP. It has been well established that the progression of tumors (growth of both primary and metastatic tumors) is critically dependent on angiogenesis (neovascularization) to supply oxygen and nutrition. (Folkman, 2002). Soluble Galectin-3 has also been shown to induce endothelial capillary tube formation \textit{in vitro} and angiogenesis \textit{in vivo}, which suggests that angiogenesis could be mediated by carbohydrate recognition potential of galectin-3 (Nangia-Makker et al. 2000). Therefore, inhibition of VEGF by COPP has been expected to prevent the growth of tumor cells at both primary and metastatic sites, thereby preventing tumor progression.

Phosphoglucoisomerase(PGI)/Autocrine motility factor (AMF) promotes cell motility via binding to a cell surface receptor named AMFR/gp78. This is a seven-transmembrane glycoprotein of 78 kDa (Silletti et al., 1991; Shimizu et al., 1999). When AMF reacts with its receptor, the latter is internalised, stimulates a pertussis toxin-sensitive G protein, activates protein kinase C (PKC) and inositol phosphate is produced; the receptor also undergoes phosphorylation. These molecular alterations are implicated in normal and tumor cell locomotion (Yanagawa et al., 2004; Funasaka and Raz, 2007). Multiple studies have revealed that the expression of AMF and its receptor is associated with increased tumor penetrating ability to normal tissues. AMF are known to promote angiogenesis, and \textit{in vivo} experiments in mice showed that AMF over-expressing tumor cell were able to provoke the development of new capillary blood vessels, which in turn could be prevented by specific AMF inhibitors (Passaniti et al., 1992; Abe et al., 1993). Reduction in the PGI levels in case of COPP treated animals may significantly affect not only the invasion of cancer cells in the primary site, but also the development of distant metastatic lesions, predominantly in the liver.
The decrease in antioxidant (GSH) and antioxidant enzyme (Catalase and superoxide dismutase) levels in metastatic conditions were normalized upon treatment with COPP. This may help in improvising the antioxidant status in animals. Toxicity studies indicated no significant damage to the vital organs such as liver as evidenced by no increase in the liver function enzymes (SGPT and SGOT). Also there was a normal weight gain in COPP treated groups as compared to healthy groups that were fed with standard mice feed suggesting lack of side effects of the selected test components. Additionally since galectin-3 inhibitors show specific affinity to galectin-3, they are targeted only to high galectin-3 expressing B16F10 cells that are harbored in the lung and not normal lung cells per se.

Blockade of galectin-3 mediated lung metastasis hence appears to be due to inhibition of mixed functions that are induced during metastasis. These data stress the importance of dietary carbohydrate compounds as cancer-preventive and/or -therapeutic agents and necessitates further evaluation from multiple angles, since the majority of cancer patients who succumb to their disease; die from metastasis. The role of dietary components in cancer progression and metastasis can be a promising remedial tool as effective therapeutics to explore against complex metastatic melanomas.
4.7. Summary and conclusions

- This chapter addresses *in vivo* efficacy and mechanism action of Corn Pectic Polysaccharide (COPP) on B16F10 induced lung cancer models.

- COPP effectively inhibited the galectin-3 mediated cell adhesion and invasion of B16F10 cells as demonstrated by *in vitro* matrigel assay.

- COPP at the concentration of 200 mg/kg b.w. showed 75% reduction in the B16F10 cells induced metastatic nodules.

- COPP could effectively inhibit lung fibrosis as evidenced by markers like hydroxyproline, sialic acid, uronic acid etc.

- COPP demonstrated its efficacy by inhibiting galectin-3 and also galectin-3 mediated signaling cascades including MMPs, transcription factor (NFkB), caspases, p53 (tumor suppressor gene) etc.

- COPP can therefore be a potent, promising antimetastatic molecule.