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DETERMINATION OF EFFICACY AND MECHANISM OF ACTION OF ANTIMETASTATIC COMPONENTS FROM BLACK CUMMINS (*NIGELLA SATIVA*) AND SWALLOW ROOT (*DECALEPIS HAMILTONII*) USING *IN VITRO* BIOCHEMICAL AND CELL CULTURE ASSAY MODEL SYSTEMS

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ABSTRACT
Cell division or cell proliferation is a physiological process that occurs in almost all tissues and under many circumstances. Normally the balance between proliferation and programmed cell death is tightly regulated to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer, disrupt these orderly processes. The uncontrolled and often rapid proliferation of cells can lead to either a benign tumor or a malignant tumor (cancer). Studies have indicated that galectin-3 expression is correlated with metastatic potential in certain malignancies. 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. This study was carried out to determine the efficacy and mechanism of action of antimetastatic components from black cummins (Nigella sativa) and swallow root Decalepis hamiltonii) using in vitro biochemical and cell culture assay model systems. SRPP which showed galectin-3 inhibitory activity as evaluated by inhibition of agglutination of red blood cells, also inhibited invasion and induced apoptosis in vitro. 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.

INTRODUCTION
Cancer development is a long-term process that appears to proceed by step-by-step carcinogenesis events that ultimately spread from one area of the body to other parts of the body during the late metastasis stage. Current clinical therapies of cancer, which include surgery, radiation and chemotherapy, are limited, particularly during the terminal metastasis phase. However, there is increasing evidence from cancer epidemiological and pathological studies suggesting that many human cancers could be prevented or their progression slowed down (Weinstein, 1991). Therefore, there might be opportunities for interference and prevention of cancer progression in the early stages of carcinogenesis by dietary phytochemicals.

Pectic polysaccharide also called pectin is a linear chain of α-(1-4)-linked D-galacturonic acid that forms the pectin-backbone, a homogalacturonan. Into this backbone, there are regions where galacturonic acid is replaced by (1-2)-linked L-rhamnose. From rhamnose, sidechains of various neutral sugars branch off. This type of pectin is called rhamnogalacturonan I. Over all, up to every 25th galacturonic acid; in the main chain is exchanged with rhamnose. Some stretches consisting of alternating galacturonic acid and rhamnose – "hairy regions", others with lower density of rhamnose – "smooth regions". The neutral sugars are mainly D-galactose, L-arabinose and D-xylose; the types and proportions of neutral sugars vary with the origin of pectin.

Recently the role of pectic polysaccharides has gained importance due to their role played in controlling cancer metastasis through the blockade of galectin present on the metastatic cancer cells (Inohara & Raz, 1994) as galectin-3 expression is correlated with metastatic potential in certain malignancies (Bresalier *et al*., 1997). 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 (Konstantinov *et al.*, 1996). 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 bind to normal cells and establishes secondary tumors. Galectin-3 hence has been implicated in tumor spread and metastasis (Takenaka *et al*., 2004). Studies have also indicated that oral administration of modified citrus pectin reduced the rate of cancer cell spread and inhibited metastasis in animal models (Pienta *et al.*, 1995).

Antimetastatic component isolated from swallow root has shown galectin-3 inhibitory activity *in vitro* which reflects inhibition of interaction between galectin-3 and red blood cells. The objective of this study is to investigate the effect of binding of these components on to the cell surface and the changes induced by this interaction, it is important to understand the biopotency of these molecules. Cell-cell interaction and cell invasion being the role played by galectin-3 of cancer cell via β-galactoside containing normal cell, alteration in interaction by pectic polysaccharide resulting in inhibition of cell-cell interaction and cell invasion.

MATERIALS AND METHODS

Effect of galectin-3 blockade by SRPP; Inhibition of cell invasion

Matrigel invasion chamber with pore size (0.8 µm, BD Biosciences, USA) was used to measure cell invasion in *vitro* (Zoltan-Jones *et al.*, 2003). SRPP at 50 & 100 µg/mL were added to the metastatic MDA-MB-231 cell suspension in 0.5 ml medium without serum. The control and SRPP treated cell suspensions (0.5 ml of 3×104 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 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.

Apoptosis assay

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, MDA-MB-231 and Buccal (1×104 cells/well) cells were treated with SRPP at 50 and 100 µg/mL 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 each acridine orange and ethidium bromide and observed under the microscope at 40X. 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 (Chanana *et al*., 2007) using caspase-3 specific peptide-substrate N-acetyl-ASP-Glu-Val-ASP-P-nitroanilide (Ac-DEVDpNA). The release of P-nitroaniline moiety from the substrate was measured at 405 nm in microplate reader (SpectraMAX plus, Molecular Devices).

RESULTS

Antimetastatic components-cell interaction studies

Cell invasion assay

Filtration of metastatic MDA-MB-231 cells treated with and without SRPP/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 of SRPP/CPP (Figure 1).

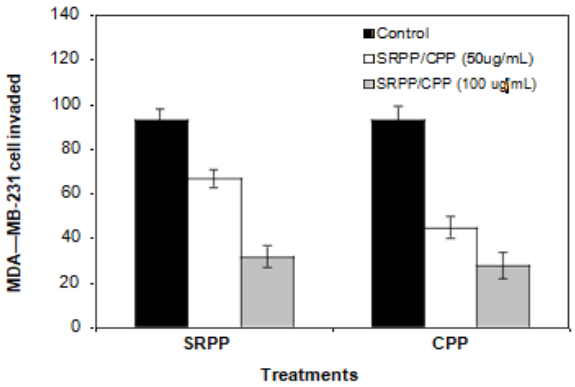

Figure 1: Inhibition of MDA-MB-231 cell invasion *in vitro* by SRPP/CPP. Values are expressed as mean ± SD (n=3).

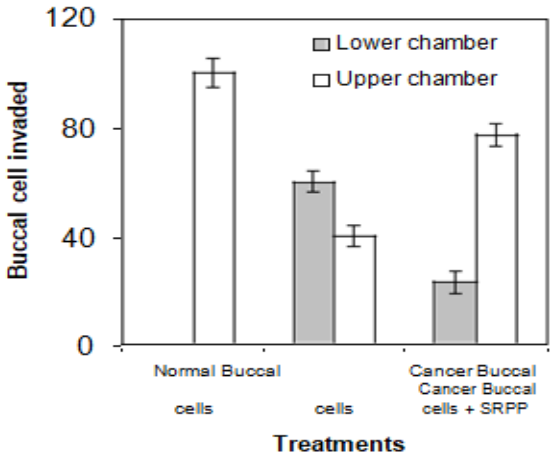

Figure 2: Inhibition of buccal cell invasion *in vitro* by SRPP/CPP. Results on % invasion were compared with normal buccal cells (NBC). Values are expressed as mean ± SD (n=3).

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

In the control cells (Figure 3a), predominant granular pattern was observed upon immunostaining. Galectin was found both in the nucleus and the cytoplasm. Upon

treatment of control cells with SRPP, cells showed distinct morphological changes as evident from nuclear/chromatin structures, oozing out of cytoplasmic contents and formation of apoptotic bodies (Figure 3b), increase in cell volume and membrane disruption, cell membrane blebbing and intracellular bridges typical of apoptosis was observed. Results were substantiated by the appearance of 40–60% of apoptotic cells as evaluated by acridine orange and ethidium bromide staining methods (Figure 3c) in metastatic cancer buccal cells.

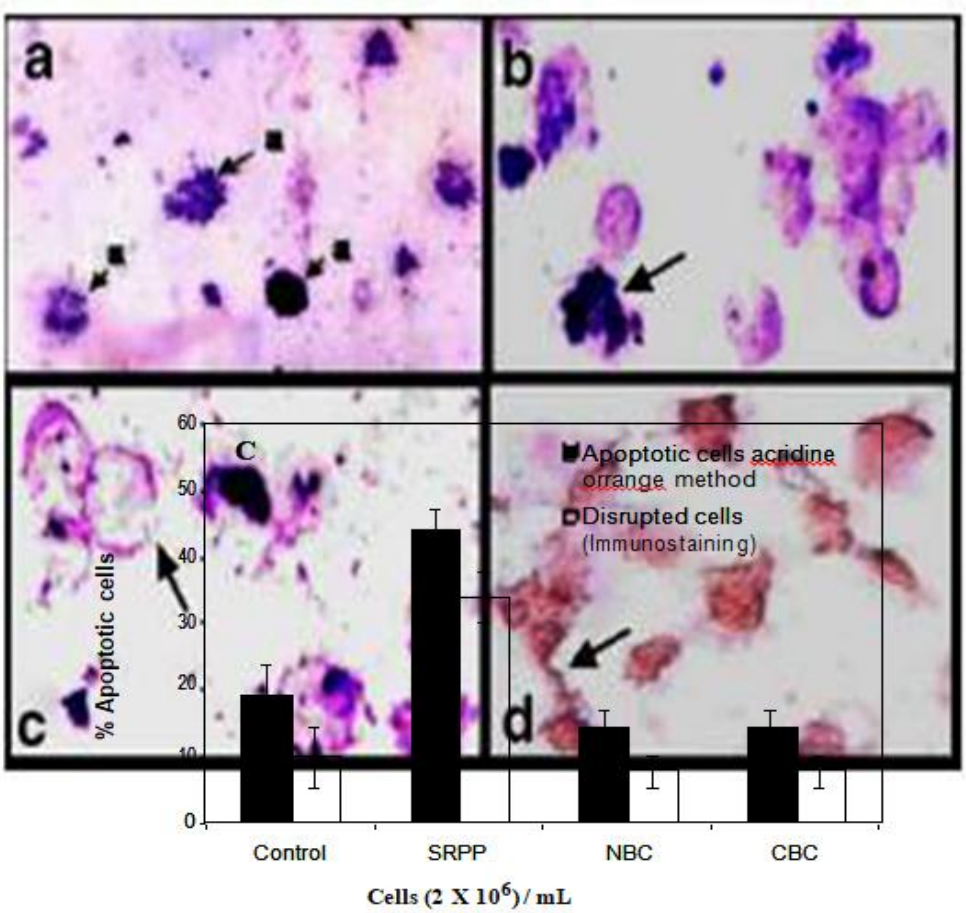

Figure 3. Measurement of apoptosis in metastatic-MDA-MB-231 and non-metastatic cells: cells were treated with SRPP at 100 µg/mL for 24 h and subjected to immunostaining using anti-galectin-3 antibody. Control MDA-MB-231 cells showing granular structure (a) was disrupted by SRPP treatment. Cell shrinkage and oozing of cellular contents are evident (b). Figure C shows bar graph with percent apoptotic cells during the treatment with galectin inhibitory polysaccharide. Number of cells lysed as per acridine method/immunostaining method were counted and percent apoptosis was calculated.

DISCUSSION

Antimetastatic components-cell interaction studies

SRPP which showed galectin-3 inhibitory activity as evaluated by inhibition of agglutination of red blood cells, also inhibited invasion and induced apoptosis in vitro. Data that inhibition of galectin-3 mediated interaction inhibits invasion and induced apoptosis suggest that galectin-3 is a key molecule for successful metastasis. Unpublished data from our laboratory provided evidence for the effect of galectin-3 inhibitory polysaccharide in inhibiting the subsequent signaling cascade of metastasis. For the first time we showed that SRPP can block significantly the activation of matrix metalloproteinase which are involved in favoring invasion of cancer cells and establishment.

From the above results, 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. 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, may further emphasize the importance of dietary carbohydrate as potential cancer-preventive and therapeutic agents as already highlighted in case of citrus pectin (Beuth *et al*., 1987). 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 (Ochieng *et al.*, 1994). 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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