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Swallow root (*Decalepis hamiltonii*) pectic oligosaccharide (SRO1) induces cancer cell death via modulation of galectin-3 and survivin

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ABSTRACT

Swallow root pectic oligosaccharide fraction (SRO1) from swallow root pectic polysaccharide (SRPP) possessed a molecular size of 831 Da. Structural analysis revealed that it is a rhamnogalacturonan I type, bearing arabinogalactan side chain with β-D-(1→4) galactose along with α-L-Araf (1→5)-α-L-Araf (1→3) structure on α-D-GalA-*OAc*-(1→2)-α-L-*Rha*-(1→4)- linear backbone. β-D (1→4) linked galactose being the specific sugar for galectin-3, SRO1 had potentials in inhibiting galectin-3 mediated cancer progression. SRO1 inhibited galectin-3 mediated agglutination, *in vitro*, effectively with MIC of 1.08 μg/mL and down regulated mRNA levels of galectin-3 (~92%) along with its downstream key protein that inhibits apoptosis – survivin (~78%) suggesting the capability of SRO1 in inhibiting galectin-3 mediated cancer promoting pathway. This is the first report, which highlights the inhibition of interplay of galectin-3 and survivin by a dietary pectic oligosaccharide.

1. Introduction

Cancer remains as one of the major causes of mortality worldwide (Chen, Sun, Zhang, Liao, & Liao, 2017; Jin et al., 2013). Galectin-3, a carbohydrate binding protein (32 kDa) has been shown extensively both from our group and others, that it plays a significant role in accelerating the proliferation and metastasis along with inhibition of apoptosis, leading to "difficult-to-treat" condition in the affected patients (Balasubramanian et al., 2009; Takenaka, Fukumori & Raz, 2004). In addition to metastasis, chemoresistant nature of cancer cell is another important bull's eye that needs to be targeted for effective cancer management. Available literature suggests that survivin, a 16 kDa anti-apoptotic protein belonging to "Inhibitor of Apoptosis" (IAP) family, is a key molecule responsible for chemoresistance and is reported to be over expressed in tumour cells (Virrey, Guan, Li, Schönthal, Chen, & Hofman, 2008). Survivin functioning through TRAIL receptor (Tumour necrosis factor − related Apoptosis Inducing Ligand) is known to promote metastasis and angiogenesis; therefore, believed to encompass entire tumorigenesis process including proliferation, migration, invasion and thus collectively, facilitating metastasis (Garg, Suri, Gupta, Talwar & Dubey, 2016).

One interesting observation by Cheong, Shin & Chun, (2010) revealed that the silencing of galectin-3, also silenced the activity of survivin. In fact it appear to be viable to target both galectin-3 and survivin as the signal transduction pathway leading to cancer with metastatic spread encompasses galectin-3 as a key initiator molecule, while survivin is in the downstream of the pathway (Cheong et al., 2010). In this perspective, we are addressing, in this paper, whether an inhibitor of galectin-3 can inhibit survivin and, if so, does it augment apoptosis. Our question is pertinent since several reports suggested that down regulation of galectin-3, increases cancel cell sensitivity to chemotherapeutic molecules suggesting that galectin-3 indeed can be a key target to overcome chemoresistance (Cheong et al., 2010; Lin et al., 2009; Niture & Refai, 2013).

In this scenario, we utilized the previously identified galectin inhibitor from swallow root (*Decalepis hamiltonii)*, a pectic polysaccharide (SRPP) which was the best, effective and safer with an MIC of 1.86 μg/mL (Sathisha, Jayaram, Nayaka & Dharmesh, 2007) as opposed to standard galactose which had a MIC of 25 μg/mL, for elucidation of its activity against survivin. Further, over a decades of research, we also established that low molecular weight oligosaccharides from intact pectic polysaccharides were more potent than their large molecular weight polysaccharides, probably due to the better bioavailability and also accessibility to galectin-3 molecule (Kapoor & Dharmesh, 2017).

In the current study therefore, we envisage the 1. Isolation of a low molecular weight oligosaccharide (SRO1) from Swallow Root Pectic Polysaccharide (SRPP); 2 Comparative evaluation of galectin-3 inhibitory property of SRPP and swallow root oligosaccharide (SRO1); 3. Determination of sugar composition and structural backbone present in SRO1 to understand its structure-function relationship; 4. Effect of galectin-3 inhibitor on apoptosis and proliferation of cancer cells and; 5. Substantiation of mRNA levels as well as protein levels of galectin-3 and

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survivin in presence and absence of the active fraction of SRO – SRO1. Results of the study highlights the modulation of both galectin-3 and survivin in cells by galectin inhibitor and appear to be due to the structural motif present in SRO1. Study thus impinges on the signal transducing effect of galectin inhibitor on the key initiator molecule – galectin-3 and the downstream molecule survivin. Other studies in our laboratory have shown the effect of galectin- 3 inhibitor on apoptosis activating molecule such as caspases also (Jayaram, Kapoor & Dharmesh, 2015; Venkateshaiah, Eswaraiah, Annaiah & Dharmesh, 2017).

2. Material and methods

2.1. Chemicals

Dulbecco's modified eagle's medium (DMEM) (AL007A), antibiotic solution (A018), FBS (fetal bovine serum) (RM9955), skimmed milk powder (GRM-1254), MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl 2-H-tetrazolium bromide) (TC-191), were procured from Himedia, India (Cell Culture grade). 6-diaminidino-2-phenylindole (DAPI) (D9542), mercaptoethanol, protein molecular weight marker (D1992), trypsin, HPLC grade 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. HRP conjugated-goat anti-mouse IgG secondary antibody (ab97023), anti-survivin mouse monoclonal (ab93274) and anti-galectin-3 monoclonal (ab2785) antibodies were procured from Abcam, UK. The other chemicals such as Triton X 100, SDS, Tween 20, TEMED, EDTA (ethylene diamine tetra acetic acid), hexane, ammonium oxalate, iodine solution, sodium phosphate buffer, glutaraldehyde, glycine, sodium chloride, amberlite IR 120H$^+$ resin, sulphuric acid and solvents used were of the analytical grade purchased from Sisco Research Laboratories, Mumbai, India.

2.2. Isolation and physicochemical characterization of SRO

2.2.1. Isolation of swallow root oligosaccharide (SRO) from swallow root polysaccharide (SRPP)

SRPP was isolated according to Phatak, Chang & Brown (1988) using ammonium oxalate extraction method. Defatted samples (100 g) were subjected to protease, termamylase and glucoamylase treatment to degrade proteins, amylose and amylopectin, respectively by providing optimum reaction conditions for enzymes. The deproteinzed and destarched residue was subjected to extraction with 200 mL of 0.5% (w/v), ammonium oxalate solution, pH 3.5; boiled for 3 h at 70°C with occasional agitation. The content was filtered and the supernatant was subjected to precipitation with four volumes (v/v) of absolute ethanol at 4°C. After 3 h, the pellet was separated by centrifugation at 5000 g at room temperature for 20 min and was washed twice with 50 mL of ethanol. The pellet was resuspended in 10 mL of water and lyophilised to obtain Swallow Root Pectic Polysaccharide (SRPP). Oligosaccharides from SRPP- SRO was obtained using the protocol described previously from our laboratory (Kapoor & Dharmesh, 2016). Briefly, to 100 mg of SRPP, 10 mL of 2 M acetic acid was added and kept in a boiling water bath to hydrolyze the polysaccharide. Three volumes of ethanol (v/v) were added after 10 h of hydrolysis and stirred for 30 min at 4°C. The content was centrifuged at 4000*g* for 15 min and excess acid in the supernatant was removed by co-distilling with water. The content was dried completely; the residue obtained was resuspended in water and lyophilised to get Swallow Root pectic Oligosaccharides (SRO).

2.2.2. HPLC analysis; determination of purity and molecular weight

The purity of SRO was analysed using HPLC. 25 µL of 1 mg/ mL (in deionised water) was injected to ultra-hydrogel E-linear column (Waters 2695). Sample was eluted using deionised water at a flow rate of 1 mL/ min and elution was monitored using ELSD 2424 detector system. SRO was resolved into 3 components – SRO1, SRO2 and SRO3. Active fraction SRO1 was re-chormatrographed on the same column under similar conditions and the fraction was found to be 99.5% pure. Molecular weight of SRO1 was estimated according to Kapoor & Dharmesh (2017) using ESI–MS (Alliance, Waters 2695) in a positive mode (100 V). All our further studies were performed with SRO1.

2.2.3. Estimation of total sugar and uronic acid

Total sugar content of SRO1 was estimated by phenol-sulphuric acid method (Rao & Pattabiraman, 1989). To various aliquot of test molecules, 0.3 mL of 5% phenol and 2 mL of concentrated sulfuric acid was added. Absorbance was measured against a blank at 490 nm. Glucose was used as a reference standard. Uronic acid estimation was carried as described previously (Bitter & Muir, 1962). To different aliquot of test molecules, 3 mL of concentrated sulphuric acid was added. The mixture was boiled for 20 min and brought down to 25°C. Carbazol (0.1%) was added and the mixture was incubated in dark for 2 h. The absorbance was measured at 530 nm. The concentration of uronic acid in samples was estimated using galactouronic acid as reference standard.

2.2.4. Sugar composition analysis by gas liquid chromatography (GLC)

GLC was performed to analyse neutral sugar composition of SRO1 according to Thejaswini et al. (2013) and Sathisha et al. (2007). To 10 mg of sample, 10% sulphuric acid was added and subjected to acid hydrolysis by refluxing for 8–12 h. Neutralization and deionization of the hydrolysed sample was achieved by treating with solid barium carbonate and amberlite IR 120H$^+$ resin, respectively. Alditol acetates were prepared and subjected for sugar composition analysis on RTX-1 column using a Shimadzu GLC (Kyoto, Japan) The flow rate was 40 mL/ min; the column temperature was maintained at 200°C and the injector temperature was 250°C (Sathisha et al., 2007).

2.2.5. FTIR and NMR analysis

SRO1 was subjected to IR spectral study using a Perkin-Elmer spectrum 2000 spectrometer. Spectra was followed from 4000 to 400 cm^{-1} in an absorbance mode at a resolution of 4 cm^{-1}. 10 mg of SRO1 dissolved in 600 µL deuterated water was subjected to ^{1}H and ^{13}C NMR analysis for its characterisation. The spectrum was recorded using Bruker AQS 400 MHz NMR spectrophotometer. ^{1}H NMR spectrum was recorded at 500 MHz of 10,330 Hz spectral width containing water presaturation pulse program zgpr. ^{13}C spectrum was recorded at 125 MHz with a spectral width of 28,985 Hz. Based on the signals of FTIR, NMR and sugar composition, tentative structure of SRO1 has been proposed. Molecular weight was calculated using Expasy glycan mass analysis software and compared with the mass obtained by ESI–MS.

2.3. Evaluation of galectin-3 inhibitory, antiproliferative and apoptotic activity of SRO1 and SRPP

2.3.1. Galectin-3 inhibition assay

The test molecules were evaluated for its galectin-3 inhibitory activity based on hemagglutination assay, according to the protocol described by Sathisha et al. (2007). Briefly, human erythrocytes were prepared from heparinised blood and were 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 trypsin was incubated for 1 h at 37°C. The trypsinized 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. Termination of glutaraldehyde fixation was achieved by the addition of five volumes of 0.1 M glycine in PBS, pH 7.4 at 4°C. The fixed erythrocytes were employed for the hemagglutination assay. The assay was performed in a microtitre agglutination assay plate. The reaction mixture contained 150 µL of 4% erythrocyte suspension with or without serially diluted SRO1 and SRPP (50 µg). Minimum Inhibitory Concentration (MIC) of the test molecules required to inhibit the galectin mediated agglutination of RBCs was