3. INTRODUCTION

Human diseases caused by the aberrant behaviour of a single cell type are very difficult to treat by chemotherapy; the obvious examples are cancer, aging and autoimmune diseases. Successful therapy should ideally eliminate the abnormal cells while leaving all normal cells functionally undisturbed. In this context chemotherapy suffers from its limitations, as most of chemotherapeutic drugs act both on abnormal as well as normal cells. The most successful anticancer drugs have been designed to target neoplastic cells while causing minimal damage to normal cells. Even in the best situations, undesirable side effects are inevitable. It seems unlikely that new reagents will emerge in which a single compound combines selectivity towards target cells with potent cytotoxicity (Lord, 1987; Pastan, 1986). Hence there is a need for search of new molecules which can selectively target cancer cells to exert its effect.

Breast cancer is one of the most prevalent cancers worldwide and the leading cause of cancer death in females, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 (Jemal, 2011). The mammary cell proliferation induced by estrogen is considered the first step of breast cancer development. However, hormone-independent growth is observed in later stages (Brody, 2003). Typically, the treatment of breast cancer involves hormonal therapy with tamoxifen or other selective estrogen receptor modulators (Fisher, 1998; Cuzick, 2002; Martino, 2004). Though many tumors initially respond to
chemotherapy, however, breast cancer cells can subsequently gain resistance to the treatment and survive (Campbell, 2001). Considering this limitation, identification of novel agents that are relatively safe but can suppress growth of human breast cancer is highly desirable. Accordingly, much effort has been made to develop various approaches to target cancer cells by targeting the cancer associated antigens rather than focusing only on receptors.

Glycosylation changes that occur in cancer often lead to the expression of tumour-associated carbohydrate antigens; these antigens are usually associated with a poor prognosis and a reduced overall survival. Cellular models have shown the implication of these antigens in cell adhesion, migration, proliferation and tumour growth. Among these, tumor-associated carbohydrate antigens like T/TF and Tn are the attractive candidates for the development of anticancer immune stimulation (Heimburg-Molinaro, 2009) and drug targeting strategies. George Springer was the pioneer of the study of TF antigen in breast cancer. TF antigen is frequently over expressed in breast cancer and is co-related with the tumour development. There is a debate regarding the expression of TF in breast cancer in relation to prognosis or metastatic behavior (Cazet, 2010). However, TF antigen is shown to play a leading role in docking breast cancer cells onto endothelium by specifically interacting with galectin-3 expressed on endothelial cells. Importantly, TF antigen-bearing glycoproteins are also capable of mobilizing circulating galectin-3 to the surface of endothelial cells. Multivalent galectin-3 anchored to endothelial cells then assist in harbouring circulating metastatic cancer
cells for development of breast cancer (Glinsky, 2001). Considering the importance of this antigen in breast cancer growth and progression, the anti TF-Ab’s and TF specific lectins are gaining clinical implications as they can be tools to differentiate malignant tumors from benign and the degree of glycosylation associated with metastasis (Mody et al, 1995). Apart from recognition these molecules are also known to inhibit the cancer cell proliferation and metastasis following their interaction with TF in TF expressing cells (Almogren, 2012).

Glycan array analysis of Sclerotium rolfsii lectin (SRL) has shown its specificity towards TF antigen and its derivatives. SRL binds with high specificity to “α-anomers” but not the “β-anomers” of the TF antigens and its derivatives (Chachadi, 2011). The present study investigated the interaction of SRL with human cancerous and metastatic breast tissues and its effect on the proliferation of human breast cancer (MCF7 and ZR-75) and normal mammary (HMEC and MCF-10A) epithelial cells.

3.1 Materials and Methods

Bovine serum albumin (BSA), Bovine sub-maxillary mucin (BSM), N-hydroxy succinimido biotin, ethylenediaminetetra-acetic acid (EDTA), 2-mercaptoethanol, Triton X-100, trypan blue, DAPI, formaldehyde, Propidium Iodide, RNase A, DTT, sodium azide, bromophenol blue, formaldehyde, CM-cellulose, and glycine were obtained from Sigma Chemical Co. (St. Louis, USA). Isopropyl alcohol, methanol, ethanol were from Himedia, India. Acrylamide, bis-acrylamide, Tris, sodium dodecyl
sulphate (SDS), hybond poly vinylene difluordine (PVDF) membrane were obtained from GE Life Sciences (USA). Protease inhibitor cocktail was from Roche (Germany). Sepharose-4B was from Pharmacia. Fetal calf serum (FCS) was from Gibco Invitrogen (Paisley, UK). 3-3’ diaminobenzidine chromogen/H2O2 substrate in buffered solution (DAB kit) was obtained from Bangalore Genei, India. Calcein AM fluorescent dye was from Invitrogen Eugene, Oregon. Caspase Glo3/7 Assay kit was from Promega, Madison, USA and caspase inhibitors, caspase-3 [z-VAD(OMe)], caspase-8 (z-IETD), caspase-9 (Z-LEHD), were from Calbiochem, Nottingham (UK). Polyclonal mouse antibodies to FasL, FADD, caspase-8, -9, β-actin and BID were procured from Santa Cruz Biotechnology, CA, USA. Rabbit polyclonal anti active, Species specific HRP labeled secondary antibodies were procured from BioRad, Hercules, USA. Annexin-V detection kit was procured from Biosvision (USA) and tissue culture grade plastic-ware were procured from corning. All other chemical used were of analytical grade.

3.1.1 Purification of SRL

_Sclerotium rolfsii_ lectin (SRL) was purified from mature sclerotial bodies of the fungus _S. rolfsii_ as described in chapter-II.

3.1.2 Activation of Sepharose-4B and coupling of SRL

SRL coupled Sepharose-4B required for growth inhibitory studies was prepared by activating the Sepharose-4B and coupling with the SRL as described earlier in chapter II.
3.1.3 Conjugation of SRL with FITC

FITC conjugated SRL used for confocal microscopy was prepared as described earlier in chapter II and the labeled SRL was stored at 4 °C till further use.

3.1.4 Biotinylation of SRL

Biotinylation of SRL required for histochemistry was prepared as described in chapter II.

3.1.5 Desialylation of bovine submaxillary mucin and glycophorin A

Asialo Bovine submaxillary mucin (aBSM) and asialo glycophorin A required for the inhibition studies were prepared by acid hydrolysis as described by Spiro RG, 1964. aBSM was prepared as described earlier and asialo glycophorin A was prepared as follows, asialo glycophorin A was dissolved in 0.05 M H₂SO₄ containing 0.15M NaCl. The mixture was incubated at 80 °C for 1 h. The incubated sample was then centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant obtained was dialysed against distilled water extensively and lyophilized.

3.1.6 Lectin histochemistry

Human breast tissue samples (normal, primary and metastatic cancer tissues) were procured from S. L. Raheja Hospital, Mumbai, India, with the approval of the ethical committee (IRB No.08/2009). Tissues were obtained during surgery, fixed in buffered formalin and embedded in paraffin for routine pathological examination.
Additional 5 μm sections were prepared for lectin histochemistry after the pathological diagnosis was confirmed. Lectin histochemistry of biotinylated SRL was carried out as mentioned earlier in chapter II. SRL binding was then evaluated through optical analysis by measuring the mean area of stained cells scored arbitrarily as intense (++++), moderate (++), weak (+) and no binding (−).

3.1.7 Cell culture

MCF-7 and ZR-75 are human epithelial metastatic breast cancer cell lines, derived from breast adenocarcinoma. These cells retain characteristics of differentiated mammary epithelium and are reported to express both the wild type and variant forms of oestrogen, progesterone and other steroid hormones receptor. MCF-7 and ZR-75 cells were obtained from the European Cell Culture Collection via the Public Health Laboratory Service (Porton Down, Wiltshire, UK). MCF7 and ZR-75 cells were cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin and100 μg/ml streptomycin (complete DMEM) at 37°C in 5% CO2.

Human Mammary Epithelial Cells (HMEC) are primary breast cells derived from reduction mammoplasty tissue and ideally suited model for normal cells. These cells grow for a finite life span and eventually senesce. HMECs were purchased from Lonza (Walkersville, MD, USA) and were cultured in Mammary epithelial basal media (MEBM) containing necessary supplements of Bovine pituitary extract (BPE), Human epidermal growth
factor (hEGF), Human insulin, Hydrocortisone, Gentamicin (30 mg/ml) and Amphotericin (15 mg/ml).

MCF-10A cells are immortalized, non-transformed epithelial cell line derived from human fibrocystic mammary tissue. These cells are defined as "normal" breast epithelial cells as they have a near diploid karyotype and are dependent on exogenous growth factors for proliferation. They also lack the ability to form tumors in nude mice and the ability to grow in anchorage independent assays. MCF-10A cells are an excellent model system for understanding epithelial cell biology. Non tumorigenic MCF-10A cells were a kind gift from Dr. Milind Viadya (ACTREC) and were cultured in DMEM-F12 (1:1) complete media containing necessary supplements of EGF (100 mg/ml), Hydrocortisone (1mg/ml), CholeraToxin (1mg/ml), Insulin (10mg/ml), Penicillin (100 units/ml) and Streptomycin (100 μg/ml) and maintained at 37 ºC in 5% CO₂.

### 3.1.9 Assessment of cell surface binding of SRL to Human mammary epithelial and breast cancer cells

**a. Flow Cytometry**

Surface binding for SRL to MCF-7, MCF10A and HME cells was analysed by flow cytometry using FITC labeled SRL. MCF-7, MCF10A and HME cells in the exponential growth phase were harvested by gentle trypsinization at 37 ºC. Cells were washed using 25 mM phosphate buffered saline (pH 7.2 PBS) and centrifuged at 1000 rpm for 4 min at room
temperature (RT). Cells (0.5 ×10^6) were incubated with 3% BSA to block the non-specific binding sites. After blocking cells were stained with FITC-labeled SRL for 1 h at 4 °C. Carbohydrate mediated binding was analysed by pre-incubating the FITC-SRL with 100 µg/ml of TF expressing glycoprotein for 1 h at 37 °C before staining with the cells. Cells were washed to remove excess lectin and resuspended in PBS. Data was acquired for 10,000 events on BD FACS Calibur cytometer (Becton Dickinson, San Jose, CA) and analyzed using cell quest-pro software. The results were presented as overlay frequency histograms with relative fluorescence on X-axis and number of events on Y-axis. Unstained cells processed similarly were used as negative control.

b. Confocal Microscopy

Cell surface binding of SRL to MCF-7 and HME cells was also analysed by the confocal microscopy. MCF-7 and HME cells were grown on cover slips were processed as mentioned earlier.

3.1.9 Assessment of cell proliferation

The effect of SRL on proliferation of human breast cancer cell lines MCF-7 and ZR-75 was monitored by Calcien AM assay. Subconfluent MCF-7 or ZR-75 cells were seeded at 4 × 10^4 cells/ml in 96-well plates in DMEM complete medium for 48 h. The medium was replaced with incomplete media and the cells were incubated with various concentrations of SRL for different time points.
In other experiment SRL (20µg/ml) was pre-incubated with 100µg/ml of TF-expressing glycoproteins before treating with MCF-7 or ZR-75 cells and incubated for 72h. MCF-7 cells were preincubated with Pan caspase-inhibitor [Z-VAD(OMe), 50µM] before treating with SRL (20µg/ml) and incubated for 48h. MCF-7 cells were treated with Sepharose-conjugated SRL and SRL having same haemeagglutination units, and incubated at 37°C for 72h. The cells were then processed by Calcein AM method and proliferation was measured as described earlier.

The effect of SRL on proliferation of human Mammary epithelial cell lines (MCF-10A and HMEC’s) was monitored by Calcein AM and MTT assay respectively. MTT assay measures the viable and metabolically active cells. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells in part by the action of dehydrogenases to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan crystals are then solubilised and quantified by spectrophotometry.

Sub confluent, HMEC and MCF-10A cells were seeded at 4x10^4 cells/ml in 96 well plates in MEGM (Mammary Epithelial Cell Growth Medium) or DMEM-F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) media respectively with appropriate supplements for 48h. The medium was replaced with serum-free DMEM-F12 for MCF-10A with supplements containing 0.5% BSA (w/v) and both the cells were incubated
with various concentrations of SRL for different time intervals (24, 48 and 72h). The cells were labeled with Calcein AM and the fluorescent intensity was measured as mentioned above. HMEC cell viability was measured by MTT assay. Briefly, after each time point 10µl of MTT (5 mg/ml) was added to each well followed by lysis in 100 µl of 10% SDS in 0.01 N HCl. Finally, absorbance was measured at 570 nm with reference wavelength of 640 nm, using ELISA plate reader. Percentage viable cell number was calculated, with respect to controls considered as 100%.

3.1.10 Assessment of cellular apoptosis

3.1.10.1 Detection of apoptosis by fluorescence microscopy

DNA fragmentation is a characteristic feature of apoptosis and can be easily detected with DNA-specific dyes. Diamidino-2-phenylindole (DAPI) is a DNA-specific dye that displays a blue fluorescence. This dye can pass through intact, living cell membranes but apoptosis increases cell membrane permeability and uptake of DAPI, leaving a stronger blue stain. For these reasons, the intensity of the fluorescence dyes can be useful in identifying cells undergoing apoptosis. MCF-7 cells were grown on cover slips and treated with or without SRL (20µg/ml) for 48h. The cells were fixed with 2% para-formaldehyde for 10 min, permeabilized with 0.1% Triton-X-100 for 10 minutes and counter stained with 1mg/ml DAPI for 5 min. After washing with PBS, cells were observed using fluorescent microscopy (Olympus, Japan) 40X magnification.
3.1.10.2 Detection of apoptosis by Annexin-V/PI staining

SRL induced apoptosis was determined by using FITC-Annexin-V labeling kit and cell were processed as per the manufacturer’s instructions. Briefly, MCF-7 cells were grown in 6 well plate for 24h before treating with SRL. Cells were treated with SRL (20 µg/ml) for 24, 36 and 48h in DMEM with 0.5 % BSA in CO₂ incubator. After each time point the cells were collected by gentle trypsinization and resuspended in binding buffer at a concentration of 0.5x10⁶ cells/ml. Cell suspension was incubated with 5 µl of FITC Annexin-V and 5 µl propidium iodide for 15 min at room temperature in dark. After incubation 400 µl of binding buffer was added to each tube and analyzed by flow cytometry. The following controls are used to set up compensation and quadrants unstained cells, cells stained with FITC-Annexin V (no PI) and cells stained with PI (no FITC-Annexin-V). The percentages of cells positive for Annexin-V, PI alone and both Annexin-V and PI were calculated by dot blot analysis using Cell Quest Pro software (BD).

3.1.10.3 Measurement of cellular apoptosis by cell cycle analysis

Induction of apoptosis and distribution of cells in different phases of cell cycle following SRL treatment was monitored by propidium iodide (PI) staining. PI is a membrane impermeable DNA stain which can be used to discriminate between live and dead cells; it can penetrate cells after plasma membrane integrity is lost. PI is a DNA staining dye that intercalates between
the strands of nucleic acids i.e. DNA and RNA. Depending on the phase of cell cycles like G0/G1, S and G2/M, the total DNA content of cells changes from 2N to 4N and this DNA content can be detected by the amount of PI taken up by the cell. RNase A is used to remove all the RNA that might give false staining as PI does not distinguish between the double or single stranded nucleic acids. Analysis of PI stained cells on flow cytometer on FL2-A separates the cells in different phases of cell cycles giving a typical twin peak profile representing G0/G1 and G2/M phases. The cells in the S phase form a plateau between the two peaks. Apoptosis leads to condensation and fragmentation of chromosomal DNA in cell nucleus. Fixation of cells with precipitating fixatives (such as ethanol) causes the leakage of these cleaved low molecular weight DNA fragments from the nucleus and as a consequence, apoptotic cells can be identified as a hypodiploid peak, while the healthy cells generate a typical cell cycle histograms.

MCF-7 cells were seeded at 5x10^5 cells/ml in 6 well plate (2 ml/well) for 24 h before the treatment of SRL. Cells were incubated with or without SRL (20 µg/ml) for 24, 36 and 48h in a CO₂ incubator. After each time point cells were harvested by gentle trypsinization and washed twice in cold PBS. Cells were fixed in 70 % chilled ethanol for 30 min at 4 °C (at this stage cells can be stored for 6-7 days at 4 °C) and washed by centrifuging at 1500 rpm for 8 min. Cells were rehydrated in PBS and treated with 50 µl RNase A (5 mg/ml in PBS, DNase free) for 10 min at RT to avoid RNA contamination. Cells were stained with 450 µl Propidium Iodide (50 µg/ml in PBS) for 2 h in dark
at RT, the DNA content was analyzed on the FL-2A channel of Flow cytometer (FACS Calibur, BD Bioscience, USA) equipped with a 488 nm argon laser at linear scale for cell cycle analysis. The data was analyzed by Cell Quest Pro software (BD) for the distribution of cells in different phases of cell cycle. Results were presented as frequency histograms with fluorescence intensity in linear scale on the X-axis and the events or cell counts on the Y-axis. Markers were applied to differentiate the cells which are in different phases of cell cycle (hypodiploid, G0/G1, S, and G2/M) and compared between control and treated cells.

3.1.10.4 Role of caspases in SRL induced cell death

The Caspase-Glo 3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities and is used to measure caspase activities in SRL treated cells. Briefly, MCF-7 cells were seeded in 96 well plate at 4×10^4 cells/ml and grown for 48h. Cells were pre-incubated with the specific inhibitors for caspase-8 (z-IETD-FMK), caspase-9 (z-LEHD-FMK) or pan-caspase (z-VAD-FMK) inhibitor followed by treatment of SRL (20 µg/ml) for 48h. After 48 h of treatment 100µl of Caspase-Glo 3/7 reagent was added to each well of a 96-well plate and incubated at room temperature for 30 minutes and then all the contents were transferred to 96-well white-walled plate and the luminescence of each well was measured in a plate-reading luminometer as described earlier.

In another experiment MCF-10A cells were cultured at 4×10^4 cells/ml in DMEM-F12 complete media for 48h before treatment with SRL (20µg/ml) for 48h. Cell apoptosis was then determined by measuring caspase-3/7
activity using Caspase Glo3/7 assay according to manufacturer’s instructions.

3.1.10.5 Western blotting

The expression of different proteins upon treatment with SRL in MCF7 cells was determined by using Western blotting studies. MCF7 cells were grown in 6 well plates for 24 h before treating with the SRL. MCF7 cells were treated with SRL (20µg/ml) for different time intervals 0, 12, 24, 36 and 48h. At specific time intervals the cells were harvested by using cell scraper and washed using ice cold PBS. Cells were lysed using RIPA lysis buffer (120 mM NaCl, 1.0% Triton X-100, 20 mM Tris–HCl, pH 7.5, 100% glycerol, 2 mM EDTA and protease inhibitor cocktail, Roche, Germany). Total protein of each sample was analyzed by modified Lowry method using DC protein assay kit (BioRad). Equal amount protein was resolved on SDS-PAGE and blotted on to PVDF membrane (Millipore, Bedford, MA, USA). Blots were incubated with 5 % BSA to block the non specific binding. The blots were probed with primary antibodies for FasL, FADD, caspase-8, -9, active caspase-3, and PARP for 4h at room temperature. Blots were washed thrice and incubated with species specific secondary antibodies conjugated with horse radish peroxidase for 1h. The blots were washed thrice and the bands were visualized by chemiluminescence using Super Signal West Femto Maximum Sensitivity Substrate (Pierce, USA) as per the manufacturer’s instructions. The membranes were exposed to X-ray films, fixed and developed. Blots were reprobed for actin upon stripping the blots using stripping buffer (50 mM Tris-HCl pH 6.7, 2 % SDS, 100 mM mercaptoethanol) for 30 min at 60 °C and followed by incubating with actin
antibody and processed as mentioned above.

3.1.10.6 Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

MitoTracker Red CMXRos is a lipophilic, positively charged dye. This can penetrate intact live cells and enter the negatively charged mitochondria where it accumulates in an inner-membrane potential-dependent manner. When the $\Delta\Psi_m$ collapses in apoptotic cells, the dye no longer accumulates inside the mitochondria and becomes more evenly distributed throughout the cytosol.

MCF-7 cells were grown on cover slips were treated with or without SRL for 24 and 36h and stained with 50nM MitoTracker Red CMXRos for 15 min in dark. Cells were washed with PBS and captured live by confocal laser scanning microscope (Zeiss LSM 510, Germany) equipped with 579 nm Argon lasers.

3.1.11 Statistical Analysis

Each experiment was performed at least three times, each time in triplicate. Results were analyzed by one-way ANOVA followed by ‘Newman-Keuls’ multiple comparisons using Stat direct software and data considered significant when $P < 0.05$.

3.2 RESULTS

3.2.1 SRL shows preferential binding to cancer tissues

SRL binding patterns were evaluated by measuring the mean area of stained cells and optical analysis was carried out by considering the intensity of staining. Results of these studies showed distinct pattern of SRL staining
between breast cancer and normal epithelium. SRL showed strong binding to cancerous and metastatic tissues and very weak binding (-/++) to the normal tissues of human breast (Fig. 1). The binding was more intense in metastatic adenocarcinoma than the primary or non metastatic adenocarcinoma which were scored as ++ +/++ and + respectively.

Binding of SRL to breast cancer tissue was seen in secretory gland epithelia, mainly to the apical surface of adenocarcinoma cells in all the patients. The preferential binding of SRL to cancer and metastatic tissues further supports the glycan specificity of SRL to TF and its derivatives, which are known to be over-expressed in cancerous especially in metastatic tissues.

![Fig. 1. SRL binding to normal and cancerous human breast tissues.](image)

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3.2.2 Binding of SRL to human breast cancer and normal mammary epithelial cells

The expression of TF antigen is reported in more than 90% of human carcinomas, including breast cancer. Since SRL recognizes TF and its derivatives, studies were conducted to determine the binding of SRL to human breast cancer and normal mammary epithelial cells MCF-7, HMEC’s and MCF10-A respectively. SRL binding was determined by staining MCF-7, MCF10-A and HMEC’s cells with FITC-SRL (2μg/ml) followed by flow cytometry analysis. Flow cytometry histograms of FITC-SRL in comparison with unstained cells are presented in Fig. 2. It is evident from the data that more than 90% of MCF-7 and HMEC’s cells are positive for SRL binding with MFI of 854 and 473 compared to the unstained cells that had MFI of 13.2 and 13.54 respectively. The carbohydrate mediated binding of SRL was determined by using competing glycoconjugate or TF-expressing glycoprotein like asialo mucin, which resulted in significant decrease in the binding of lectin to the MCF-7 cells. The MFI decreased to 35.5 for asialo mucin by pre incubation with SRL followed by staining with MCF-7 cells, which suggests SRL binding is mediated by carbohydrate binding sites. The results of SRL binding studies to human breast cancer and normal mammary epithelial cells MCF-7 and HMEC’s respectively suggest that, though SRL binds to both human breast cancer and normal epithelial cells, the intensity of binding is seen more in cancer as compared to normal cells, Which clearly suggest its specificity towards cancer associated glyans like TF, which are known to be over-expressed in cancer especially during metastasis.
Fig. 2. Cell surface binding of FITC-SRL to human breast cancer cells (MCF-7) and human mammary epithelial cells (HMEC). SRL shows intense binding to MCF-7 (A) and light binding to HMEC (B) and MCF-10A (C) cells when assessed with FITC-SRL and analyzed by flow cytometry (open diagram, SRL-treated cells; filled diagram: untreated control cells).

3.2.3 Cell surface binding of SRL to human breast cancer and normal mammary epithelial cells

Cell surface binding of SRL was also visualized by confocal microscopy after staining the cells with FITC labelled SRL at 63X magnification. FITC-SRL is shown to bind MCF-7 and HMEC’s cell surface...
as observed by the confocal microscope and photographed (Fig. 3). Uniform and intense fluorescence on the cell surface suggested the high expression of SRL recognizing receptors on the surface of MCF-7 cells. However similar cell surface binding was also seen in HMEC’s but the intensity of staining is less, suggesting less number of receptors being recognized by SRL.

![Fig.3. Surface binding of FITC-SRL to MCF-7 (A) and HME cells (B) by confocal microscopy. MCF-7 and HME cells grown on cover slips were stained with FITC-SRL (2 µg/ml) and DAPI and visualized by Confocal microscope. Images were captured at 63X magnification which shows the intense surface binding of intense surface binding of SRL.](image)

**3.2.4 Effect of SRL on the proliferation of human breast cancer and normal cells**

SRL showed strong binding to breast cancer cells and hence the biological response following SRL binding was studied. To investigate the effect of SRL on proliferation of MCF-7 and ZR-75 cells were treated with increasing concentrations (0–40 µg/ml) of SRL for 24, 48 and 72h respectively. MCF-7 cells treated with SRL at different concentration showed dose- and time-dependent inhibition of the cell growth. At 20 and 40µg/ml SRL caused 47.7±5.6% ($P < 0.0001$) and 58.2±2.7% ($P < 0.0001$) growth inhibition in MCF7 cells respectively after 48h and 76.2±1.5% ($P < 0.0001$)
and 77.9±2.7% (P <0.0001) inhibition respectively after 72h. Whereas SRL treated ZR-75 cells showed 66±3.8% (P < 0.0001) and 70.4± 7.4% (P< 0.0001) inhibition at 20 and 40μg after 72h (Fig. 4A and B). These results suggest that SRL exhibits strong growth inhibitory effect towards MCF-7 and ZR-75 cells in time and dose dependent manner.

Carbohydrate mediated binding growth inhibitory effect of lectin was confirmed by using TF expressing glycoproteins. SRL (20 μg/ml) was preincubated with the asialo bovine mucin (aBSM) 100 μg/ml, before treating with the MCF-7 and ZR-75 cells. Pre incubation of SRL with aBSM resulted in significant inhibition of its growth inhibitory effect and the cell viability increased to 66.28 ±0.25% (P<0.0001) and 62.33±2.0% (P<0.0001) when compared to SRL treated cells 23.8± 1.5 (Fig.4A). Similiarly, SRL induced growth inhibition was also prevented by asialo glycophorin A by 58.3 % at 48h (Fig. 4C), suggesting that the growth inhibitory effect of SRL is mediated by its carbohydrate binding sites.
Fig. 4. SRL inhibits human breast cancer cell growth. (A) SRL causes dose-dependent inhibition of human breast cancer MCF-7 and ZR-75 cell growth. The cells were incubated without or with different concentrations of SRL for 72 h. SRL-mediated cell growth inhibition is prevented by the treatment of TF-expressing glycoprotein. MCF-7 and ZR-75 cells were incubated without or with 20 μg/ml SRL in the presence of 100 μg/ml of bovine asialo mucin (aBSM) for 72 h. SRL causes time dependent inhibition of MCF-7 cell growth (B). The MCF-7 cells were incubated without or with different concentrations of SRL for 24, 48 and 72h before assessment of cell proliferation. (C) SRL-mediated cell growth inhibition is prevented by the treatment of TF-expressing glycoprotein but not by sugars. MCF-7 cells were incubated without or with 20 μg/ml SRL in the presence of 100 μg/ml of asialo glycophorin A and 50nM of sugars like GluNAc and GalNAc for 48 h before cell proliferation was assessed by Calcein AM method. Data represent Mean ±SD of triplicate determinations from three different assessments. *p<0.05; ***p<0.001.
MCF-10A are nontumorigenic cells derived from human fibrocystic mammary tissue, and have been used extensively as a representative normal mammary epithelial cell line. Primary breast cells HMECs are derived from reduction mammoplasty tissue and ideally suited model for normal cells. HMECs and MCF-10A cells were incubated with increasing concentrations (0–40µg/ml) of SRL in their respective media and their growth was then monitored for 24, 48 and 72h. SRL inhibits MCF10-A cell growth by 20±3.1% (P<0.0001), 31.5±1.0% (P<0.0001) and 36.7±4.2% (P<0.0001) at 40µg/ml for 24, 48 and 72h respectively (Fig. 4A). SRL has similar effect on HMECs and inhibits growth by 40.0±2.2% (P<0.0001) at 40µg/ml for 48h (Fig.4B). The results demonstrate that SRL at afore mentioned concentration has relatively marginal effect on HMEC and MCF-10A cells compared with its effect on breast cancer cells.

Fig. 5. Effect of SRL on normal human breast epithelial cells. MCF-10A cells (A) and HMECs (B) were incubated with different concentrations of SRL in serum free media for different time intervals. The cell proliferation was measured by Calcein AM and MTT method for MCF-10A and HMEC respectively. The experiments were carried out in triplicates, and the data were expressed as Mean ± SD percentage of control. *p<0.05; ***p<0.001
3.2.5 SRL-mediated growth inhibition is a direct consequence of cell surface receptor binding

SRL showed strong binding to MCF-7 cells as studied by flow cytometry. Cell surface binding of SRL was also observed by confocal microscopy indicating the high expression of SRL recognizing receptors on the MCF-7 cell surface. To determine whether SRL binding to the surface receptors is sufficient to induce the growth inhibitory effect, Sepharose-conjugated SRL was used for testing the inhibitory effect. Sepharose-conjugated SRL, hence non-internalizable SRL showed similar growth inhibitory effect on MCF-7 cells as that of native SRL (Fig. 5). This indicates that SRL cell surface binding is sufficient to trigger its growth inhibitory effect and lectin internalization is not required.

Fig.5. Sepharose-immobilized, hence non-internalizable SRL retained the growth inhibitory effect in MCF-7 cells. The cells were incubated without or with SRL or Sepharose-SRL (c-SRL), at concentrations equivalent to SRL that produces 128 and 256 hemagglutination units (HAU), for 72h before the cell proliferation was assessed by Calcein AM. Data represent Mean ± SD of triplicate determinations from three different assessments. ***P < 0.0001 when compared to control.
3.2.6 SRL mediated cell growth inhibition is associated with induction of cell apoptosis

3.2.6.1 SRL induced morphological changes in nuclei of MCF7 cells

The nuclear morphology of normal cells is round, clear-edged and uniformly stained. Apoptotic cells show irregular edges around the nucleus, chromosome condensation, heavier colouring with dyes and with nuclear pyknosis, an increased number of nuclear body fragments. During apoptosis chromosomal DNA is first broken into large fragments (50 - 300 kilobases) before they are cleaved into smaller nucleosomal fragments (approximately 200 base pairs). Under the fluorescence microscope, MCF-7 cells treated with SRL presented the morphological features of early apoptotic cells, such as nuclear condensation and increased pyknosis, as identified by DAPI staining in Fig. 6.

![Untreated Cells vs SRL treated Cells](image)

**Fig.6: SRL induced morphological changes in MCF-7 cells.** MCF-7 cells grown on cover slips were treated with SRL for 48h and stained with DAPI and visualized by fluorescence microscope. Images were captured at 40X magnification. Arrow indicated condensed and fragmented nuclei.
3.2.6.2 Detection and quantification of apoptosis by Annexin-V/PI staining

The apoptotic potential of SRL was determined and quantified by Annexin-V/PI staining, to distinguish viable, early, late apoptotic and necrotic cells. MCF-7 cells treated with SRL showed significant increase in the early apoptotic cells (only Annexin V positive) to 50.8% and 81% after 36 and 48h respectively, when compared to respective controls 4% at respective time points (Fig.7A). At 48h only small percentage (15%) of cells were viable (Annexin-V/PI negative) and a total of 85% were undergoing apoptosis or dead. These results further confirmed quantitatively the apoptosis induction by SRL. Comparison of the cells in different phase’s early/late apoptotic and necrosis cell population are presented in Fig.7B.

Fig.7. SRL induces apoptosis in MCF-7 cells. (A) Annexin-V cell surface binding in MCF-7 cells after incubation with SRL (20μg/ml) for 24, 36 or 48h and stained for Annexin-V/PI. The percentage of cells in each quadrant was shown as inserts. (B)The graph indicates percentage of cell population in different phases.
3.2.6.3 SRL induced apoptosis determined by cell cycle analysis

Induction of the apoptosis can be determined by the observation of increase in the hypodiploid populations by cell cycle analysis. MCF-7 cells treated with SRL (20 μg/ml) at different time points were subjected to cell cycle analysis after propidium iodide (PI) staining to observe percentage of cells undergoing apoptosis and the distribution of cells in the different phases of cell cycle by flow cytometry. The results of the cell cycle analysis of MCF-7 cells after SRL treatment are presented in Fig.8.A. The DNA content of MCF-7 cells measured following 24h of SRL treatment showed 20.8% cells in hypodiploid (Sub G1), 67.3% in G1/G0 phase, 16.79% in S phase, and 15.8 % in the G2/M phase compared to untreated cells with 0.4% hypo diploid, 41.6 % in G1/G0 phase, 27.3% in the S phase, and 30.9% in the G2/M phase. Gradual increase in the hypodiploid population which is an indication of apoptosis was observed in SRL treated cells at 24h (20.8%), 36h (25.9 %) and at 48h (41%) cells compared to control (0.4%) confirming that SRL induces apoptosis in MCF-7 cells. Comparison of the distribution of cell populations in different phases between SRL treated and untreated are presented in Fig.8.B

**Fig.8.** (A) MCF-7 cells were exposed to SRL (20μg/ml) for 24, 36 and 48h and were stained with propidium iodide and DNA content was measured by flow cytometry followed by assessment using Mod Fit LT mac2.0. (B)The graph indicates percentage of cells in subG1, G1, S, and G2–M phases of the cell cycle.
3.2.6.4 SRL mediated growth inhibition is linked to induction of apoptosis assessed by Calcein AM assay

To deduce, whether SRL mediated cell growth inhibition is due to induction of apoptosis. MCF-7 cells were pre incubated with Pan caspase-inhibitor [Z-VAD(OMe)] to block the activation of executioner caspases, which inturn will inhibit the SRL induced apoptosis. The presence of pan-caspase inhibitor largely diminished the growth inhibitory effect of SRL on MCF-7 cells (Fig.9) indicating that induction of cell apoptosis is directly linked with SRL-mediated cell growth inhibition.

Fig.9. SRL-mediated cell growth inhibition is associated with induction of apoptosis. MCF-7 cells were incubated without or with SRL (20μg/ml) in the presence or absence 50 μM pan-caspase inhibitor z-VAD-(OMe) for 48 h before the viable cells were assessed by Calcein AM method. *P < 0.05, **P < 0.01, ***P < 0.001.
3.2.6.5 Involvement of different caspases in SRL induced apoptosis by caspase 3/7 Glo assay

To deduce role of different caspases in SRL induced activation of caspases-3/7. The activity of caspases-3/7 was measured by caspases-3/7 Glo assay in presence or absence of specific pharmacological inhibitors for different caspases. Pre incubation of -8, -9, and a pan caspase inhibitors with MCF-7 cells before treating with SRL for 48h and subsequently measuring caspases-3/7 activity showed an increase in caspase-3/7 activity by 2.02 fold (P < 0.001) when compared with the untreated cells (Fig.10). On the other hand, treatment with the same concentration of SRL (20 μg/ml) for 48h showed no significant effect on caspases-3/7 activity of non-tumorigenic MCF-10A cells (Fig. 6D). The presence of inhibitors to caspase-8, caspase-9 or pan-caspase could all largely prevent SRL-induced caspase 3/7 activation (Fig.10), indicating the possible involvements of both the caspase-8 mediated (extrinsic) and caspase-9 mediated (intrinsic) apoptosis signalling pathways.

Fig.10. SRL induced cell apoptosis involves different caspases. (A) MCF-7 cells were incubated without or with SRL (20 μg/ml) in the presence or absence of inhibitors (50μM) to caspase-8 (z-IETD), caspase- 9 (z-LEHD) or pan-caspases (z-VAD-(OMe) for 48h before the caspases-3/7 activity was assessed by Caspase-3/7 assay. ***P < 0.001. (B) MCF-10A and MCF-7 cells were incubated without or with SRL (20 μg/ml) for 48 h before the caspase 3/7 activity was assessed ***P < 0.001.
3.2.6.6 Western blotting studies to demonstrate that SRL-induced apoptosis potentially involves both extrinsic and intrinsic pathways

The involvement of caspase-8,-9, -3 and -7 has been shown by using caspases-3/7 Glo assay and specific pharmacological inhibitors. To delineate the mechanism(s) of SRL-induced apoptosis in MCF-7 cells, the effect of SRL on activation of initiator caspases-8 and -9 and effector caspase-3 and cleavage of PARP was measured by western blotting studies using specific antibodies. Expression of different proteins in SRL treated MCF7 cells were also observed at different time points (0, 12, 24, 36 and 48h) and presented in Fig.11. Cells treated with SRL showed a time-dependent increase of active caspase-8 and -9 and decrease of their inactive forms. Activation of caspase-3 was observed at 36h and reached maximum at 48h.

Detection of cleaved fragments of Poly ADP-ribose polymerase (PARP) is a hallmark of the apoptosis. SRL induced cleavage of PARP was observed in a time dependent manner. Native PARP band (116 kDa) decreased as the time increased and the cleaved fragment (89 kDa) was observed at 36 and 48h, suggesting its inactivation. (Fig.11). SRL treated MCF-7 cells showed to induce a time-dependent increase in the expressions of FasL and FADD, which leads to activation of caspase-8.

Activated caspase-8 triggers downstream events either directly by activating caspase-3 or by cleaving the Bid factor. Increased expression of t-Bid was also observed in time dependent manner in MCF-7 cells suggesting its cleavage following SRL treatment, thus implicating its role in lectin-induced apoptosis. Fig.11
Fig. 1. SRL induced apoptosis in MCF-7 cells involves both extrinsic and intrinsic pathways. MCF-7 cells were incubated with SRL (20 μg/ml) for different time intervals. At specific time intervals whole cell protein was obtained, separated by electrophoresis, blotted on to Immobilon polyvinylidene difluoride membrane and probed with antibodies against FasL, FADD, pro-caspase-8, tBID pro-caspase-9, or active caspase, -8, -9, and PARP. The bands were visualized using chemiluminescence kit. The blots were stripped and reprobed for the β-actin which was used as loading control.
3.2.6.7 SRL-induced apoptosis potentially involves intrinsic pathways as assessed by loss of MMP in SRL treated MCF-7 cells

The signals received within the cells such as DNA damage, excessive ROS, changes in the calcium levels attributes to the formation of large pores across inner and outer mitochondrial membrane and leads to loss of mitochondrial membrane potential (MMP). Pre incubation of MCF-7 cells with caspase-9 inhibitor has shown the protection of SRL induced caspases-3/7 activation. Hence it was essential to analyze whether SRL induced activation of caspases-3/7 is due to the loss of mitochondrial membrane potential (MMP). Hence the cells following SRL treatment were visualized by confocal microscopy using a mitochondrial specific fluorescent dye MitoTracker Red CMXRos. MCF-7 cells treated with SRL for 24 and 36h showed a time dependent decrease in the uptake of TMRE as observed by confocal microscopy. Intact mitochondria were stained red in the control cells, whereas SRL treated cells showed diffused mitochondria in the cytosol and also weak fluorescent signal as the time increased, suggesting the mitochondrial damage and loss of membrane potential leading to activation of caspase-9 involved in intrinsic apoptotic pathway (Fig.12).
Fig. 12. SRL induces loss of mitochondrial membrane potential. MCF-7 cells grown on cover slips were treated with SRL (20 µg/ml) for 24, 36 or 48h and stained with MitoTracker Red CMXRos and visualized by confocal microscope. Images were captured at 63X magnification which shows the intense staining of mitochondria in untreated cells and gradual decrease in intensity of SRL-treated cells.
3.3 DISCUSSION

The present study shows that *Sclerotium rolfsii* lectin, SRL has exquisite binding specificity towards the TF and related O-glycans and shows strong binding to cancer/metastatic tissues but weak binding to normal tissue in human breast. SRL inhibits growth of human breast cancer cells *in vitro* as a result of its cell surface binding and subsequent induction of cellular apoptosis, but relatively has marginal effect on proliferation of normal epithelial MCF-10A and HMEC cells.

Although advances are made in diagnosis and treatment, breast cancer mortality rates are still high, demanding the need for novel and more advanced therapies. The development of novel agents for the prevention and treatment of human breast cancer is therefore highly essential. In the search for novel diagnostic and therapeutic cancer agents, lectins have received increasing attention in recent years (Liu, 2010; Kinghorn, 1999). Lectins serve as novel therapeutic agents; as they exhibit effective antitumor activities with a wide range of mechanisms (Fu, 2011). Amongst this group of proteins are TF-Ag binding lectins, which recognize the core 1 structure of O-linked mucin type glycans (T/TF antigen). To date only a few studies of TF antigen binding lectins from fungi have been reported (Almogren, 2012; Milton, 2005), also the exact mechanisms by which these lectins induce cell growth inhibition are merely understood. Hence there is a need to explore new sources for these TF antigen binding lectins that are effective antitumor agents as they are gaining wide range of pharmacological applications.
Histochemical studies of SRL show intense staining to cancerous and metastatic tissues but very light to normal human breast tissues. The presence of low and high levels of TF antigen in normal epithelium and metaplastic lesions of the breast respectively has been demonstrated using monoclonal antibodies for TF-Ag [Cazet, A; 2010]. The observed differential binding of SRL to normal, primary and metastatic tissues can be explained based on differential expression of TF-Ag. However, binding of lectin with normal and breast cancer cells when tested by flow cytometry showed almost equal binding but the mean fluorescent intensities were noticeably different.

The differential binding of lectins to normal and cancerous tissues can be of clinical significance particularly as diagnostic agents. The noted examples include Roman snail lectin, also known as Helix pomatia agglutinin (HPA) has been used to detect axillary lymph node metastasis and its progression in breast cancer (Leathem, 1985; Taylor, 1991). Another example is Cratylia mollis lectin (Cra Iso-1) that is used to characterize normal and transformed breast cells (Beltrao, 1998). Considering the differential binding of SRL to normal and cancerous tissue it can be further explored for its possible diagnostic application.

SRL strongly inhibits human breast cancer cell growth in dose and time dependent manner. In contrast, SRL has less or marginal effect on normal breast epithelial cells. The preferential toxicities of lectins to malignant cells when compared to normal cells has been reported
(Fang, 2012; Bryce, 2001) and various mechanisms have been proposed to explain this. This may be caused by the numerical difference of molecules recognizable by lectins on the cell surface such as an increased density of lectin binding sites after transformation or altered distributions of these receptors on the surfaces of malignant cells and the differential cellular uptake efficiency of these molecules by the cancer and normal cells. For example *Triticum vulgaris* agglutinin (WGA), *Phaseolus vulgaris* agglutinin also known as phytohemagglutinin-L (PHA-L), *Momordica Charantia* lectin (MCL) and Con A were reported to preferentially bind and inhibit the growth of cancer cells rather than normal (Wang, 2003; Lochner et al., 2003; Bryce et al., 2001; Khurrum et al., 2002; Fang et al., 2012). The growth inhibitory effect of SRL was blocked by the presence of TF-expressing glycoproteins, suggesting that the growth inhibitory effect of SRL is mediated by its carbohydrate binding. The observed growth inhibitory effect of SRL is executed by binding to the cell surface receptors. It may not be necessary that molecules such as lectins must bind strongly to exposed cell surface components in order for internalization and executing their effects. In fact, this is observed with SRL and as well reported in case of PNA, where the stimulatory effect of PNA does not require internalization of lectin (Irazoqui, 2001; Petrossian, 2007).

The inhibition of cell cycle progression and increase in hypo diploid population is one of the molecular events associated with the anti-proliferative efficacy of SRL in MCF-7 cells as evident by cell analysis.
Cancer cells are marked by a proliferation disorder and an apoptosis obstacle. The inhibition of proliferation and the induction of apoptosis are regulated by a network of signaling pathways and transcription factors, which are possible targets for a rational cancer therapy (Thompson, 1995; Kinloch, 1999). Many anticancer agents reported to date are known to cause cell death by apoptosis. Cell apoptosis is an organized mode of cell death that is characterized by a variety of morphological features, including changes in the plasma membrane such as loss of membrane asymmetry which is an early event in apoptosis (Manon van Engeland, 1998). Our results showed that following SRL treatment noticeable morphological changes as well nuclear fragmentations were observed indicating possible induction of apoptosis. This was further supported by the time dependent increase in early apoptotic population, indicating SRL induced apoptosis in MCF-7 cells.

Apoptosis is executed by caspases and caspase-3 is a key protease associated with DNA fragmentation and apoptosis (Ashe, 2003). The increased activity of caspase -3/7 was observed in SRL treated cells, which was blocked in presence of inhibitors to caspase-8, caspase-9 or pan-caspase, indicating the possible involvements of caspase-8,-9,-3 and 7, which further supports SRL mediated growth inhibition is associated with induction of apoptosis possibly involving both extrinsic and intrinsic pathways.

Two main pathways have been involved in activation of caspase-3, including the caspase-8–regulated plasma membrane extrinsic pathway and
the caspase-9–regulated cell damage intrinsic pathway (Pop, 2009; Boatright, 2003). Effector caspases are common to both the extrinsic and intrinsic death pathways (Green, 1995). PARP is involved in the repair of DNA damage, during apoptosis caspase-7 and caspase-3 cleave PARP into two fragments, p89 and p24, thus suppressing PARP activity (Gambi, 2008). MCF-7 cells following SRL treatment showed increased levels of FasL and FADD proteins and resulted in caspase-8 activation, Truncation of BID leading to mitochondrial trans-membrane potential collapse is as evidenced by results of confocal microscopy and activations of caspase-9 and caspase-3 was demonstrated by western blotting. The present investigation showed that the molecular mechanisms involved in SRL induced apoptosis of MCF-7 cells seemed to proceed via both the extrinsic and intrinsic pathway as shown by activation of caspase-8, caspase-9, caspase-3/7, and cleavage of PARP.

In conclusion, this study shows that SRL suppresses the growth of human breast cancer cells by induction of cell apoptosis whilst it produces only marginal effect on normal epithelial cells. As a naturally-occurring lectin that binds specifically to a cancer-associated glycans, SRL has the potential to be further investigated for its clinical application in breast cancer therapeutics.