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Research Article

Ribosomal inactivating plant protein: The regulator of tumor suppressor and oncogenes in breast cancer

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ABSTRACT

Background: Breast cancer, a leading malignancy in females, accounts for a significant proportion of cancer related deaths worldwide (~12%). Novel medicines are required to control disease burden. Plants genera is a big source for naturally occurring anticancer compounds. Riproximin, a purified protein obtained from a plant, has shown substantial anticancer potential against cancer cells. The current study was designed to identify cytotoxic effects and expressional changes in tumor suppressors and oncogenes at transcriptome levels induced by the selected ribosomal inactivating plant protein (riproximin) in breast cancer cells.

Methods: In preliminary phase, toxic effects of the protein were identified against breast cancer cells (MDA-MB-231) by a dye reduction assay. In a separate experiment, the cells were exposed to the protein and expressional modulations in three selective tumor suppressors and oncogenes (REL, FOS, KITLG) identified by using a ready-made PCR methodology.

Results: Riproximin induced anti-proliferative effects in the breast cancer cells. Exposure of the cells with various concentrations of riproximin showed concentration dependent effects of the compound on expressional profile of the selected genes. The three genes were substantially induced in the cells where maximum induction was observed in FOS (~40fold) followed by KITLG (~20fold) and REL (~5fold) gene.

Conclusion: Riproximin bears significant cytotoxic potential and modulates tumor suppressors and oncogenes in breast cancer cells. Targeting these gene families by using riproximin can be instrumental in cancer treatment in future.

Key Words: Plant proteins, Tumor suppressors, Oncogenes, Breast cancer

INTRODUCTION

Breast cancer involves uncontrolled cell division and invasion of the cells from primary sources of origin to other secondary areas including bones, lungs, brain and liver (metastasis). Breast cancer is the most prevalent malignancy in females [1]. Around two million new cases of breast cancer were diagnosed in 2018, making up 23% of all cancer types [2]. Poor prognosis, adverse effects of the existing medication, and the lack of comprehensive treatment choices are the main challenges to control the disease burden.

Breast cancer treatment is challenging mainly due to the different molecular subtypes and heterogeneous characteristics of disease. Chemotherapy, radiations, hormonal therapy and surgery are the mainstream approaches that are being utilized to treat breast cancer [3]. Despite the availability of various drugs, statistics of breast cancer related mortality all over the world are alarming. Chemotherapeutic drugs, the main domain of therapeutic options, are designed to kill tumor cells without causing damage to normal cells. Although, chemotherapeutic drugs successfully kill the cancer cells, but they also have multiple side effects including unintended destruction of normal host cells in the gastrointestinal tract, bone marrow, hair follicles, blood cells and other tissues. In addition to this, resistance is witnessed in patients against chemotherapeutic entities. Circumstances demand to investigate new therapeutic options, preferably of natural origin, to have minimal side-effects and better disease control.

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In this context, the enzymes known as ribosomal inactivating proteins (RIPs) obtained largely from plant species have received the attention of the scientific community. RIPs depurinate the ribosomal RNA (rRNA), causing irreversible damage to ribosomes and preventing cells from synthesizing proteins. Numerous plant species, fungi, and microbes contain these RIPs [5]. RIPs are powerful inducer of apoptosis in cancer cells. Apart from site-specific ribosomal N-glycosidase activity, RIPs can remove adenine residues from nucleic acid molecules and numerous adenine residues from ribosome [6].

In Toxicology and Chemotherapy division of German Cancer and Research Centre (DKFZ), Germany, several protein fractions were obtained from the *Ximenia americana* aqueous extracts. A 60 kDa protein called *riproximin* was discovered to be an active component of the various isolated *X. americana* fractions [7]. Two polypeptide chains (A and B) make up riproximin are joined by a disulfide bridge, forming a heterodimer. The catalytic activity of riproximin is attributed to its active chain (A-chain), which also results in the hydrolysis of a particular adenine from the large ribosomal subunit. When binding to cell surface molecules, the binding chain (B-chain) is implicated [8]. Riproximin has exhibited potent anticancer activity as revealed by toxic effects against various cancer cell lines. An in-depth analysis of riproximin's anti-proliferative effects on cancer cells showed that this protein potently hindered the capacity of the cancer cells to migrate and form colonies by causing cell cycle arrest and nucleic acid fragmentation. Riproximin considerably decreased the proliferation of the breast cancer cell lines (MDA-MB-231 and MCF-7. Apoptosis-induction, nuclear DNA breakage and cell cycle arrest in response to riproximin exposure were also detected. Riproximin has also demonstrated antineoplastic activity against tumor cell lines of colorectal and pancreatic origin [9-15].

Tumor-suppressors and oncogenes are two very important gene families which play a pivotal role while inhibiting or promoting malignant transformation. These gene families have received substantial attention as prognostic indicators and therapeutic targets because of their significant functions. Expression deregulation of tumor suppressors and oncogenes in transformed cells is well-known phenomenon. These modifications are often accompained with poor prognosis and treatment resistance. Understanding the role of these gene families and precise targeting is a vital reseach area to control cancer burden. Considering their importance in tumor biology, it is worthwhile to investigate natural substances as modulators of oncogene and/or of tumour suppressors [16-18]. The purpose of this investigation was to determine whether riproximin may have potential effects on the expression patterns of tumor suppressors and oncogenes in breast cancer cells. The findings are valuable to comprehend the molecular processes being affected by riproximin in target cancer cells for inducing antineoplastic affects.

METHODS

Cancer Cell Lines

The human breast tumor cell line (MDA-MB-231), obtained from ATCC, was cultured and maintained in RPMI-1640, a cell culture medium that contains glutathione (a reducing agent) and vitamins. The media was enriched with 10% fetal calf serum (FBS), essential amino acid (2mM L-glutamine), antibiotics (streptomycin, 100g/ml and penicillin, 100IU/ml). Sustaining exponentially increasing cell populations, standard humidified conditions (100%) along with 5% CO_2 and 37**°**C temperature were provided to incubate the cells.

Source Compound

Purified riproximin (dissolved in PBS) was provided by our collaborator, Prof. Dr. Martin R. Berger, previous head of Toxicology and Chemotherapy unit, German Cancer Research Centre (DKFZ), Heidelberg University, Germany.

Toxicity Assay

The assay was performed to observe cell viability and proliferation following treatment with riproximin. At pre-optimized cell densities, the cells were seeded in 96-well plates (4000 cells/well) and treated for three-time intervals (24, 48, and 72 hours) with riproximin (0.78-50ng/ml). Following treatment intervals, MTT solution (10mg/ml in PBS) was added (10µl/well) and cells were incubated for three hours under normal cell culture conditions. Formazan crystals, formed by the viable cells, were dissolved by adding 100µl of an acidic solvent (0.04 N HCL in 2-propanol). ELISA reader was used to determine the optical density of the solution (Anthos Microsystem, Krefeld Germany). A reference filter for 560 and 690 nm wavelengths was used. Cell survival rates were estimated as percentage of untreated cells. Inhibitory concentrations (IC) values were computed using GraphPad Prism 6 software.

Treatment, RNA Extraction and cDNA

In 6-well culture plates, the human breast tumor cell line (MDA-MB-231) was grown at a density of 150,000 cells/well/2ml medium and exposed to the increasing concentrations (5, 10, 50ng/ml) of riproximin for 48 hours. Cells were trypsinized (0.05% trypsin) after the exposure period, and palettes were recovered by centrifugation at 1500rpm for 5 minutes and stored at -80^{0}C. Likewise, the cell palettes of untreated cells as controls were also collected and stored in similar conditions. By using the GeneJet RNA extraction kit

(Thermo Fisher, K0731), the total RNA was extracted from treated and untreated control cell palettes. Utilizing the Nanodrop ND2000, RNA concentration was measured. Reverse transcriptase was used to create cDNA from 1000ng of isolated RNA per sample (Thermo Fisher, K1622). A PCR based amplification of the reference gene (HPRT1), and gel electrophoresis was performed to verify the prepared cDNA samples.

Primer Design, Optimization and Real Time PCR

Designed primers (Table 1) were optimized by using gradient PCR methodology. For this purpose, a prepared cDNA sample was used and synthesized primers were tested for at least three potential annealing temperatures. The amplified products were analyzed by using gel electrophoresis. Quantitative real time PCR was performed for selected genes (FOS, REL, KITLG) by using SybrGreen florescence dye (Thermo Fisher Scientific, Cat#K0221), prepared cDNA samples treated with various concentrations of the riproximin (5, 10, 50ng/ml/ 48 hours). All the samples were amplified in triplicate to highlight the concentration dependent effects of riproximin.

Data Presentation

After amplification procedures by qRT-PCRs and normalization of data, fold changes were calculated and determined by the Livak $2^{-\Delta\Delta CT}$ method by comparing Cq (quantification cycle) values of experimental (riproximin treated) and untreated control samples, as it is the most commonly and accurate method used for relative gene expression analysis.

RESULTS

Riproximin and Cytotoxicity

A dye reduction assay (MTT) was performed to evaluate cytotoxic effects of riproximin on selected breast cancer cells (MDA-MB-231). The cell line was treated with increasing concentrations of riproximin for 24-72 hours. Riproximin exposure inhibited proliferation of MDA-MB-231 cells. Lighter color of formazan crystals formed after reduction of MTT molecules by mitochondrial enzymes, produced in purple, showed a smaller number of viable cells (Figure 1). The effects were calculated numerically as percentages of untreated controls via GraphPad Prism 6 software (Table 2). The decline in growth curve reflects decreased optical density by ELISA reader, which in turn reflects lighter purple color produced by a smaller number of viable cells. Reduction in proliferation was gradual in the cells when exposed to riproximin as shown in Figure 2. In other words, the effects were time dependent as minimum inhibition of proliferation was observed after 24 hours exposure time (26%) while maximum inhibition was observed after 72 hours (81%), when 50ng/ml riproximin was applied. In addition to this, the inhibitory effects on cellular proliferation were concentration dependent especially for late time intervals (48 and 72 hours) as more toxic effects were observed with increasing concentrations of riproximin. Overall, the protein showed strong toxic effects against the MDA-MB-231 breast cancer cells as observed previously [9].

RNA and cDNA

To identify the impact of riproximin on expressional profiling of tumor suppressor and oncogenes, the cell line was exposed to three various concentrations (5, 10, 50ng/ml) of the test compound (riproximin) for 48 hours. After exposure intervals, the total RNA was extracted from untreated controls and treated cells (MDA-MB-231) followed by RNA quantification before further proceedings. A substantial amount of RNA (≥50ng/µl/sample) was extracted from untreated and treated samples of cell line. Equally important, the extracted RNA was good in terms of quality as reflected by 260/280 ratios (1.9-2.1). Total RNA extracted from controls and treated samples were used to synthesize cDNA and verified via PCR based amplification of reference gene (HPRT1) as described. Agarose gel electrophoresis methodology was used for visualization of the amplified product of samples. Single discrete amplified bands were witnessed during gel electrophoresis, which showed successful synthesis of cDNA from all the samples (Figure 3).

Primer Optimizations

The designed primers for selected genes (FOS, KITLG, REL) were optimized via gradient PCR technique using three different annealing temperatures (56, 59 and 62°C). Amplified products demonstrated specific amplification of the genes at the checked temperature as shown in Figure 4. This in turn shows a successful design of highly specified primers for the selected genes.

Expression Profiles of Genes

The cDNA samples from the cell line (MDA-MB-231) were used for real-time PCR based experiment while using SybrGreen master mix along with gene specific primers. cDNA samples synthesized after exposure with riproximin at different concentrations (5, 10 and 50ng/ml) were used to investigate expressional modulation in selected three genes. qRT-PCR methodology was used to investigate the expressional changes while cDNA samples of untreated cells were used for comparison as controls. HPRT1 was used as reference to normalize the data while all the cDNA samples were amplified in triplicates. Overall, riproximin exposure induced the expression of selected genes. The approach also highlighted the concentration dependent effects of riproximin on selected genes. Expression of REL gene was substantially increased by riproximin exposure in

MDA-MB231 cell line at all concentrations. Maximum induction in MDA-MB-231 cells (5.8fold) was observed at highest concentration of riproximin (50ng/ml). Similar findings were observed for KITLG gene where the induction was moderate at lower concentrations of the riproximin (5, 10ng/ml) and highly effective and intensified with further increasing concentrations (50ng/ml). Regarding FOS gene, expression of the gene was substantially increased after riproximin exposure in MDA-MB-231 cells at all concentrations. Maximum induction in MDA-MB-231 cells (36.4fold) was observed at highest tested concentration of riproximin (50ng/ml) (Figure 5).

Table 1: Primer Sequences

Genes	Product (bp)	Forward Primer	Reverse Primer
FOS	189	GATACACTCCAAGCGGAGAC	CCCAGTCAGATCAAGGGAAG
KITLG	155	AGCAGTAGCAGTAATAGGAAGG	ACTGCCCTTGTAAGACTTGG
REL	136	GTGCCAGGATCACGTTAATTT	AACCCCTGTAGGCATTTCTC

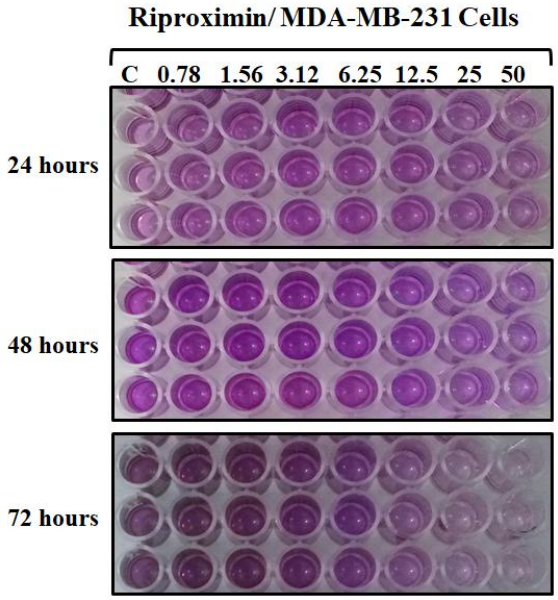

Figure 1: Effects of riproximin on proliferation of MDA-MB-231 cells determined by MTT dye reduction assay.

Table 2: MDA-MB-231 cells survival after riproximin treatment

	Riproximin (ng/ml)							
	0.0	**0.78**	**1.56**	**3.1**	**6.25**	**12.5**	**25**	**50**
24 hours	100	99	108	107	102	92	78	74
48 hours	100	102	104	93	81	66	49	42
72 hours	100	79	80	78	66	36	24	19
	Cell survival (% of untreated controls)							

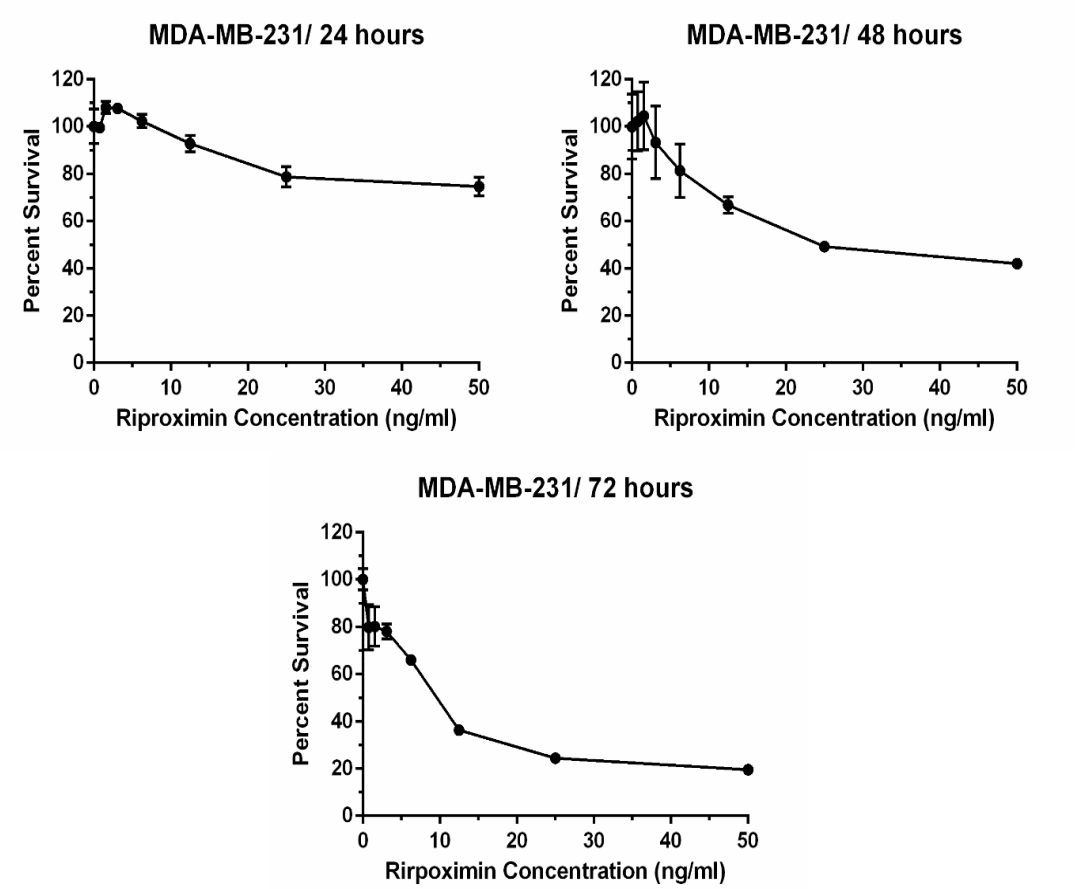

Figure 2: Inhibition of proliferation in MDA-MB-21 cells in response to riproximin exposure. Inhibitory effects were determined by MTT dye reduction assay.

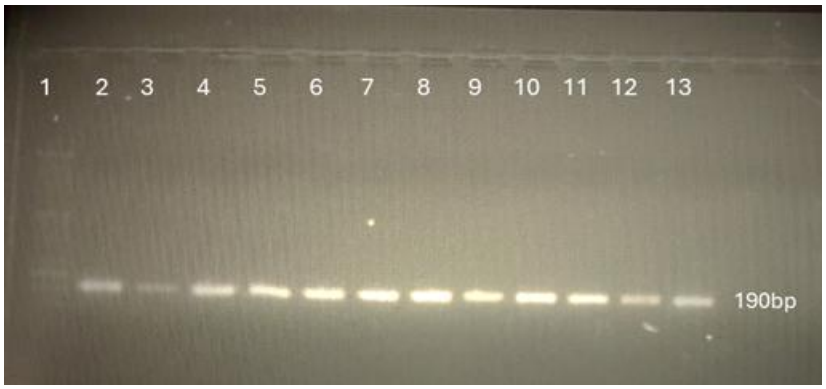

Figure 3: Electrophoresis of amplified cDNA of HPRT gene from MDA-MB-231 cells for cDNA verification. Sample distribution (left to right): M: DNA marker (50bp) 1-3: MDA-MB-231 untreated control, 4-13: MDA-MB-231 treated with riproximin.

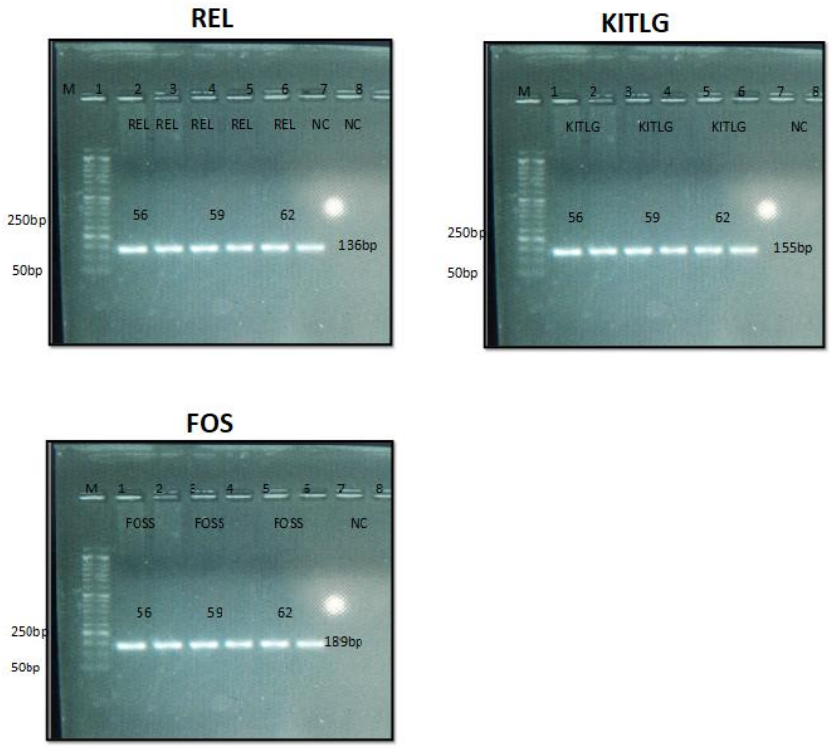

Figure 4: Electrophoresis of amplified products of three genes (REL, KITLG, FOS) via gradient PCR methodology. Discrete specified amplified bands were shown at all three different annealing temperatures.

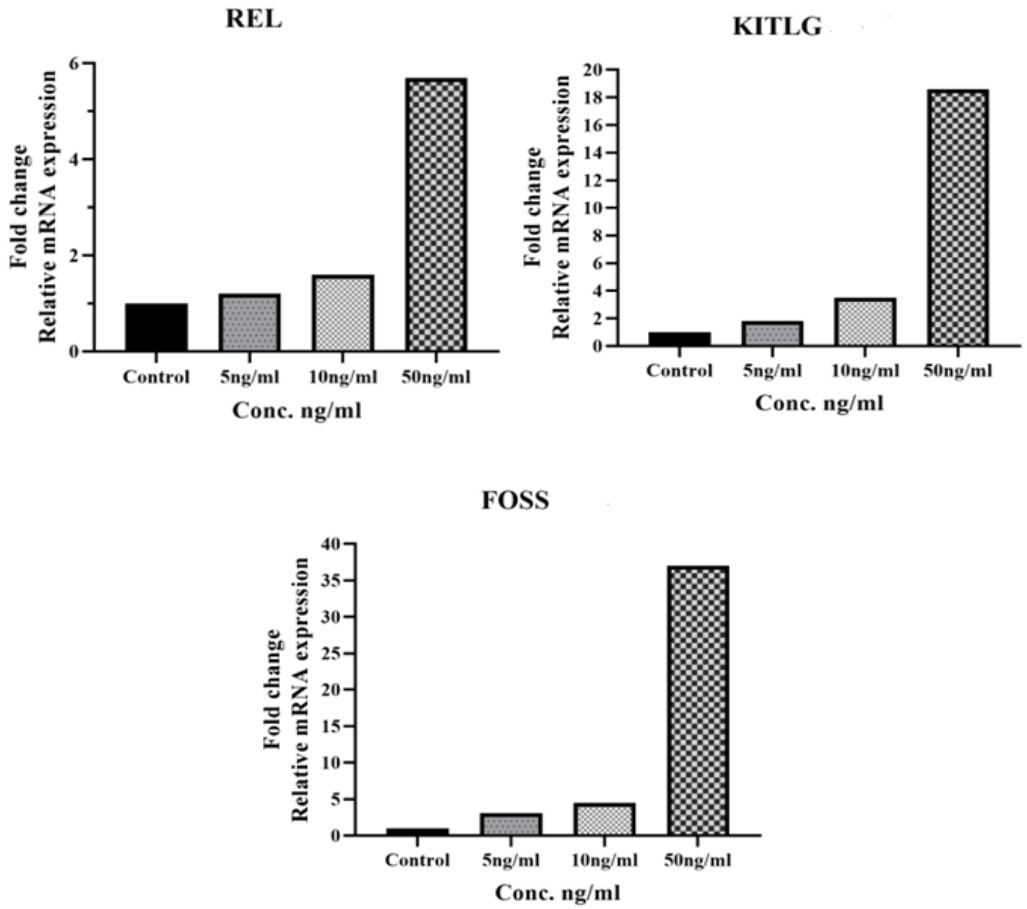

Figure 5: Expressional changes in REL, KITLG and FOS gene in MDA-MB-231 cells. Cells were exposed to three different concentrations of riproximin, and expressional levels were calculated by qRT-PCR.

DISCUSSION

The most frequent type of cancer among women worldwide is breast cancer. It is currently the most prevalent cancer. Breast cancer affects women of all ages after puberty; however, the incidence rises with age [15]. Five most used therapeutic approaches for breast cancer treatment are: surgery, radiation, chemotherapy, targeted therapy and immunotherapy. Treatment of this cancer during advanced stages of the disease is challenge (5years survival<25%). Main hurdles for management of this disease include complexity of the disease, side-effects of the current treatment and limited therapeutic options. Situation demands to evaluate novel compounds for its effective treatment. Anticancer compounds obtained from natural resources often proved to be better therapeutic remedies as they impose less side effects as compared to most of the synthetic treatment modalities.

Considering this, riproximin is a naturally occurring anticancer plant protein obtained from a commonly growing plant (*X. americana*). A number of anti-neoplastic effects of this protein have been proven against cancer cells. Riproximin belongs to a bigger family of proteins known as RIPs. These proteins are extraordinary proteins because of their tremendous anticancer activities. A few RIPs show vigorous toxicity towards cancer cells and low toxicity towards normal cells and suppress cancer development generally through apoptosis [11]. The cellular machinery involved in protein synthesis, which is demonstrated to promote the growth of cancer cells and is significantly impacted by various oncoproteins during cancer transformation. An appealing target for anti-cancer therapy is ribosomal machinery. RIPs are distinguishing catalytic proteins, which permanently suppress protein synthesis in their susceptible cells in eukaryotes through altering the component of the 28S rRNA. As a traditional medication for different types of cancer in African countries, *X. americana* plant gained the special importance. A ~60-kDa dynamic protein part of this fraction with substantial anticancer properties was identified and called as riproximin [11]. The cytotoxic mechanism of action was founded to be depended on cell take-up of riproximin followed by the A-chain induced depurination of the 28S ribosomal RNA and elicitation of unfolded protein response. The distinctiveness of riproximin relied upon the B-chain associated bond to the cell surface glycans, which presence is significant for later incorporation into cells and cytotoxicity [8]. Riproximin has been shown as a potent anticancer compound against various cancers including breast, colorectal, oral and pancreatic. For such broad-spectrum effects, the compound interferes with various signalling pathways and gene families. Among the various gene families, oncogenes and tumor suppressors are capable of driving or inhibiting the progression of cancer. Oncogene activation and tumor suppressor deactivation might be viewed as "brakes" of cell growth and is significant phenomenon in the emergence and spread of cancer. One basic idea is that oncogenes are often activated, and tumor-suppressors are down-regulated during cancer cell transformation. Tumor suppressor genes are crucial to the pathogenesis of cancer. These genes play a variety of activities in healthy cells, but in cancer cells, their mutation and deregulation lead to abnormal molecular processes. The genes are involved in ubiquitination, cellular differentiation, DNA damage repair, and cell cycle regulation in normal cells. Tumor suppressors exhibit abnormal functioning due to genetic alterations [19]. Due to vital roles in cancer pathogenesis, tumor-suppressors and oncogenes are being target for treatment purposes. For this purpose, inhibitors of oncogenes and inducers of tumor-suppressors are being exploited.

At molecular levels, riproximin modulates multiple signaling cascades leading to anti-proliferative, anti-migratory, cytostatic and apoptotic effects in human cancer cells. In proposed study, riproximin mediated effects on expression profiling of tumor suppressor and oncogenes were investigated in breast cancer cells. For this purpose, human breast cancer cell line (MDA-MB-231) was selected and exposed to various concentrations of riproximin. First, cytotoxic effects of riproximin on the cell line were determined by MTT dye reduction assay. Effects of riproximin against MDA-MB-231 cells are already published [9], but needed to be confirmed before proceeding to assure active status of the available compound. For this purpose, the selected cell line (MDA-MB-231) was cultured in 96-well plates and exposed to various concentrations of riproximin (0.78-50ng/ml) followed by MTT dye-based assessment of viable cell fractions at three different time intervals (24, 48 and 72 hours). As shown in the results section, riproximin induced antiproliferative effects in a time and concentration manner. This is important particularly for future clinical utilization, as it shows that with higher concentrations and prolonged exposure, the protein can halt proliferation of cancer cells more effectively. But equally important would be to carefully monitor the potential side-effects including hemolytic, GI tract and bone marrow associated. Furthermore, as a matter of fact, there are various molecular sub-types of breast cancer and the cell line (MDA-MB-231) used in this study was triple negative for ER, PR, HER2. Thus, impact of riproximin on other molecular sub-types of breast cancer cells needed to be explored for broader utilization against this disease. Nevertheless, the anti-proliferative effects of riproximin against MDA-MB-231 cells were comparable with the already published data, which showed active anticancer potential of available in house riproximin fractions.

To investigate the effects of riproximin on tumor suppressors and oncogenes in breast cancer cells was the major objective of this study. For this purpose, following the exposure period (48 hours), cell pellets were collected from the culture plates and good quality of extracted RNA were used to profile the genes by using gene specific primers for REL, KITLG and FOSS. Among these genes, REL gene is known for its dual role as tumour suppressor or oncogene depending upon the physiological conditions and it interferes with vital signalling cascades, immune modulator and regulator of fibrosis [20]. KITLG gene plays important role in cell survival pathways and its prognostic relevance gene has been identified recently in breast cancer [21]. Exact molecular role of FOS gene is not cleared as reports claimed its proto-oncogenic and tumour suppressor roles at various instances [22]. Nevertheless, riproximin showed substantial potential of up-regulating of these genes in breast cancer cells and need due attention to understand the exact mechanisms of regulations.

Overall, riproximin proved to be a significant cytotoxic compound and induced substantial anti-proliferative effects in breast cancer cells. The compound also showed a potential to up-regulate expression of tumor suppressor and oncogenes in the target breast cancer cell line (MDA-MB 231). To understand the gene-level modification that riproximin affects in breast cancer cells, further *in vitro* research studies are necessary.

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