

## RESEARCH ARTICLE

# Silibinin-Induces Apoptosis in Breast Cancer Cells by Modulating p53, p21, Bak and Bcl-xl Pathways

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### Abstract

Nowadays herbal-derived medicines are attracting attention as new sources of drugs with few side effects. Silibinin is a flavonoid compound with chemotherapeutic effects on different cancers such as examples in the prostate, lung, colon and breast. In the present study, the cytotoxic effects of silibinin on MCF7 breast cancer cells were investigated. Apoptosis was determined by flow cytometry and the impact of silibinin on the expression of pivotal genes including Bak, P53, P21, BRCA1, BCL-X1 and ATM was analyzed. Treatment for 24h had a significant dose-dependent inhibitory effect on cell growth ( $p < 0.05$ ) with dose- and time- dependent induction of apoptosis ( $p < 0.05$ ). In addition, there were significant increases in BRCA1, ATM, Bak and Bcl-XL gene expression at the mRNA level with different concentrations of silibinin for 24 or 48 h ( $p < 0.05$ ). Taken together, the results suggest that silibinin inhibits the proliferation and induces apoptosis of MCF-7 cells by down-regulating Bak, P53, P21, BRCA1, BCL-X1 and thus may be considered as an effective adjuvant drug to produce a better chemopreventive response for the cancer therapy.

**Keywords:** Silibinin - MCF-7 - cell-cycle arrest - apoptosis

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### Introduction

Breast cancer is the second leading cause of cancer death among women and also is the most common malignancy in women accounting for 1.2 million new cases annually (Sabzichi et al., 2014). First line treatment options include surgery, then radiation, hormonal therapy, and chemotherapy (Sharifi et al., 2014). Moreover non-selective cytotoxicity of chemotherapeutic agents causes adverse effects even irrevocable damages to normal tissues and healthy organisms. On the other hand, drug resistance makes a weak prognosis in the treatment process. Therefore, searching for effective anti-cancer agents to prevent and treat becomes more and more important for therapeutic protocol. Traditional chemotherapy is one of the main strategies that are applied to control the development and progression of breast cancer (Ramos, 2008). So, investigation on novel compounds from plant sources with higher efficiency and fewer side effects appears absolutely necessary. Moreover, flavonoids are strongly involved in the control and regulation of gene expression and inhibition of effective enzymes in carcinogenesis. By the way, flavonoids have been recognized as strong antioxidants which protect cells against oxygen-derived free radicals (Alhazmi et al., 2014). Silibinin is a compound which has been categorized

as a subgroup of flavonolignans that constitutes an active portion of the plant extract from milk thistle (scientific name: *Silybum marianum*) and its anticancer effects have been particularly indicated in hepatocellular carcinoma (Ghasemi et al., 2013). Silibinin is active in enhancing the function of heart, liver detoxification and enhancing its performance, and bronchitis and gall bladder protection (Cecen et al., 2010). The anti-cancer effects of silibinin in the inhibition of cell growth and proliferation as well as apoptotic death, anti-angiogenesis and cell migration have been mainly determined (Wang et al., 2014).

But the molecular mechanisms associated with the anti-cancer effects of silibinin have not been clearly elucidated. In this study we analyzed cytotoxic and apoptotic properties of silibinin in MCF7 cells and determined p53, p21, Bak, Bcl-XL, ATM and BRCA1 gene expression at mRNA levels.

### Materials and Methods

#### Materials

Silibinin was purchased from sigma (Chemical Co. St. Louis, MO, USA), dissolved in DMSO and stored at -20. The human MCF-7 breast cancer cells were purchased from National Cell Bank of Iran (NCBI, Tehran, Iran). Cells were cultured in RPMI 1640 medium (Gibco, Life

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Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS Gibco, USA), 100 U/ml penicillin and 100 µg/ml streptomycin under a humid atmosphere (37°C, 5% CO<sub>2</sub>, 95% air).

#### Cell culture and MTT assay

Methyl thiazolyltetrazolium bromide (MTT) colorimetric assay was performed to determine the inhibitory effect of silibinin on growth and proliferation of the MCF-7 cells. Briefly, cells in the logarithmic growth phase were harvested and seeded into wells of 96-well plates and 10,000 cells/well were cultivated in 96-well culture plate. After 24 h incubation, cells were treated with different concentrations of silibinin (0, 25, 50, 100, 200, 400, 800 µM) for 24, 48 and 72 h in quadruplicate. Then, the medium of all wells was removed carefully and 50 µM MTT (4 mg/ml) was added to each well and incubated for 4 h, followed by the addition of 200 µM DMSO. Thereafter, 25 µM Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl, pH=10.5) was added and absorbance of each well was read at 570 nm in 15-30 min. Finally, a graph was plotted using SPSS software version 16.00 and IC<sub>50</sub> of silibinin on MCF-7 was determined. A group containing 20% DMSO without silibinin served as vehicle control.

#### Apoptosis assay

Apoptotic rate were determined by flow cytometry using the annexin V-FITC apoptosis kit (Ebioscience, USA), with propidium iodide (PI). The percentage of apoptotic cells was determined by green fluorescence emitted by annexin V-FITC bound to phosphatidyl serine exposed to the outer leaflet of the apoptotic cells membrane. After extensive washing in phosphate-buffered saline (PBS), 1×10<sup>6</sup> cells were rinsed with HEPES buffer, re-suspended in the same buffer, and incubated at room temperature for 5-15 min in the dark after the addition of annexin V-FITC (5 µl). To detect necrotic cells and/or late apoptotic cells, PI was then added to the cell suspension before the analysis performed using a FACS Calibur (BD Biosciences, San Jose, CA). The results of FCM were determined by the analysis of variance (ANOVA).

#### Cell cycle analysis

After reaching 80% confluency MCF7 cells were serum-starved for overnight to synchronize them in the same phase of the cell cycle, and then were treated with silibinin in a humidified atmosphere of 5% CO<sub>2</sub> for 24 h. The cells were collected, washed twice with cold PBS, and centrifuged. The pellet was fixed in 70% (v/v) ethanol at 4°C overnight. The cells were washed once with PBS (with RNaseA) for 30 min at 37°C. Eventually, the cells were resuspended in the dark in a cold PI solution (50 µg/ml) for 30 min at 4°C. Analysis of Samples was performed by Flowcytometry using multicycle software.

#### Total RNA extraction and cDNA synthesis

Total RNA was extracted by the use of the RNxPlus (Cinnagen, Iran) Reagent according to the manufacturer's instructions. The concentration of extracted RNA

was measured using a Nano Drop spectrophotometer (Termoscientific, Wilmington, DE, USA). After RNA extraction, cDNA was synthesized using the First-Strand Synthesis kit (Vivantis, USA) according to the manufacturer's instructions. The synthesized cDNA was immediately used in PCR or stored at -70°C for next use.

#### Real-time PCR assay

The real-time PCR method was used for the analysis of mRNA expression levels in the control and treated MCF-7 cells. The GAPDH gene expression was used as a house keeping gene. The real-time PCR reaction was done in triplicate using the Power master mix 2x (ABI reagent in the Biosystems Applied, USA) according to the manufacturer's instructions. The amplification conditions were as follows: (5 min at 95°C and a two-step cycle of 95°C for 15 s and 64°C for 40 s for 40 cycles), Sequences of primers are shown in Table 1. Changes in genes expression levels between the control and treated MCF-7 cells were calculated by the 2-ΔΔCT method (Livak and Schmittgen, 2001).

#### Statistical analysis

Data were expressed as mean±SD from three independent experiments. Statistical analysis was performed using ANOVA test and the differences were considered significant at p<0.05.

## Results

#### *Silibinin inhibited the proliferation and cell growth of MCF-7 cells*

The effect of silibinin on the cell growth was assessed at different concentrations on the MCF-7 cell line. At a concentration of 200 µmole after 24 h, the treatment with silibinin represented a remarkable inhibitory effect on the cell growth compared to untreated control cells (p<0.01). The inhibitory effect of silibinin on the cell growth and its least cytotoxicity were observed after 48 h of treatment at the concentration of 100 µmole. The statistical difference was observed for inhibitory effect of silibinin on the cell growth at a concentration of 50 µmole after 72-h treatment compared to other concentrations (p<0.05). At 50 µmole concentration, the inhibitory effect of silibinin on the cell growth at 72 h after treatment was comparable with 24 h. (Figure 1)

The concentration-dependent inhibitory effect of silibinin on the cell growth was assessed at three time points based on its IC<sub>50</sub> (the results could be observed briefly in Table 1). The level of IC<sub>50</sub> in inhibition of cell growth at 24 h after treatment was estimated to be about 200 µmole (Figure 2). IC<sub>50</sub> level decreased to 160 µmole when we incubated the cells for 48 h. Incubation of the cells for 72 h showed no significant difference (140 µmole).

The trend of inhibitory effect of silibinin on the cell growth or cell division at 50-200 µmole concentrations at 48 and 72 h was almost similar compared to control cells, and this effect was more than that at 24 h. On the whole, our results showed that the inhibitory effect of silibinin on the cell growth was time- and concentration-dependent.

*Effect of silibinin on the apoptosis incidence*

The effects of different concentrations of silibinin on the incidence of apoptosis and necrosis were measured at 48 h after treatment. The results are shown in Figure 4-4. Incidence rate of apoptosis at 25-200  $\mu$ mole concentrations was similar; that is, more than 30% apoptosis was observed. Incubation of the cells with 150  $\mu$ mole for 48h showed a maximum 40% apoptosis.

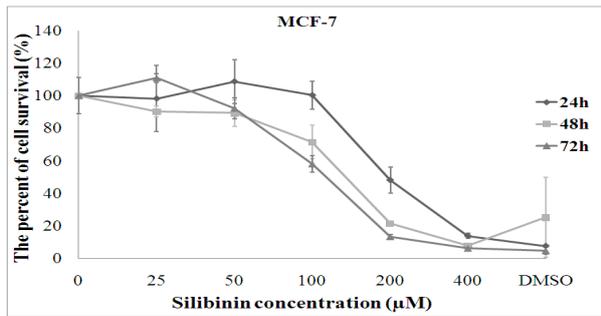
The relative frequencies of apoptotic and necrotic cells in the culture medium in the presence of different concentrations of silibinin using flowcytometry with Annexin-V staining showed that the apoptosis was the main type of cell death involved in the inhibition of cell survival compared to necrosis (Figure 3).

*Silibinin caused MCF7 cells cycle arrest at G0/G1 phase*

After MCF7 cells were treated with control, 25, 50, 100, 150 and 200 $\mu$ M of silibinin for 48h, the portion of cells at the G0/G1 phase improved from 52.69 $\pm$ 2.16% ( $p < 0.05$ ), 66.90 $\pm$ 2.32% ( $p < 0.01$ ), 70.58 $\pm$ 0.98% ( $p < 0.05$ ), 71.58 $\pm$ 0.77% ( $p < 0.01$ ) and to 75.12 $\pm$ 1.63% ( $p < 0.01$ ), compared with control (46.27 $\pm$ 1.36%) respectively. There was a significant difference of the proportion of cells cycle arrest disruption at the G0/G1 phase compared with G2/M and S phases ( $p < 0.05$ ). It shows that silibinin successfully arrests MCF7 cells in the G0/G1 phase of cell cycle (Figure 4).

*Modulation of apoptotic - correlated gene after treated with silibinin*

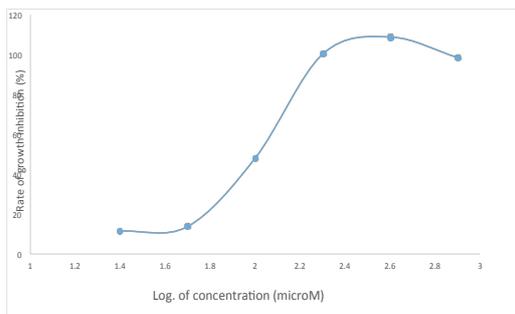
Standard curves drawn with increasing concentrations of silibinin using primers represented the same slope of curve with the PCR efficiency of 101% for GADPH, 100% for P53, 102% for P21, 101% for ATM, 101% for BRCA1, 101% for Bcl-xl, and 102% for Bak. The melting curve indicated the specific amplification of control house-keeping gene with the specific primer used in the study (Table 2). To analyze the relative changes in gene expression, the fold change was calculated by the formula



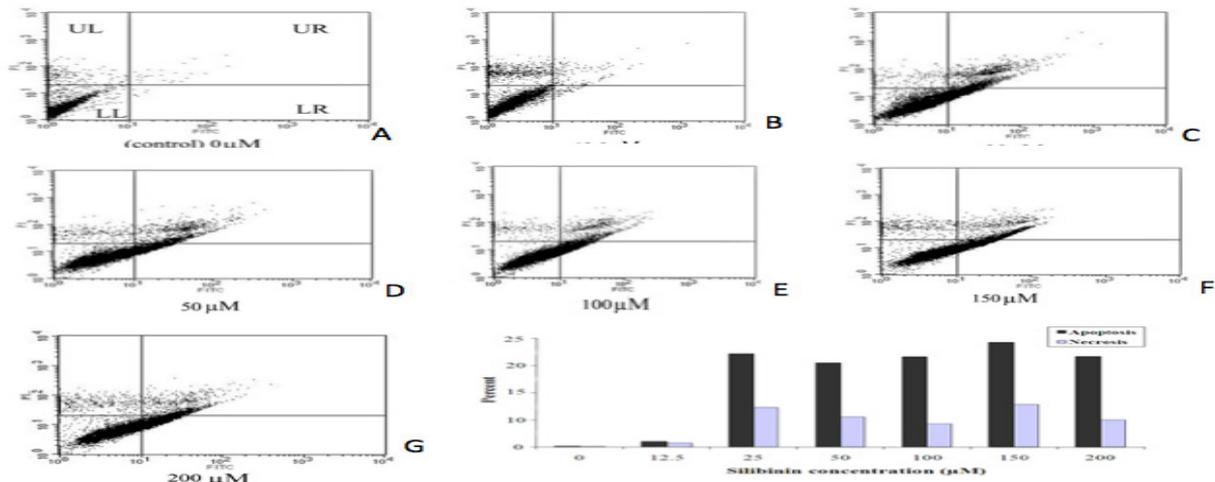
**Figure 1. Average Percentage and 95% CI of Inhibition by Treatment of MCF-7 Cells with Silibinin at Concentrations of 0, 25, 50, 100, and 200 to Reduce Cell Proliferation (MTT assay for 24 hours)**

**Table 1. Time-dependent Inhibitory Affect of Silibinin on Cell Survival and Related IC50 value**

Time	Silibinin concentration( $\mu$ M)					DMSO	IC50 ( $\mu$ M)
	Control	50	100	200	400		
24 hrs	0	0	0	52	86.2	92.5	199
48 hrs	0	10.7	28.5	78.5	92.2	92.5	158
72 hrs	0	7.7	42	86.8	94	95.5	141



**Figure 2. A Typical Curve to Estimate IC50 value and Mean Percentage of Inhibition by Silibinin Treatment at Concentrations of 0, 25, 50, 100, 200, 400 and 800  $\mu$ M to Reduce Cell Proliferation in MCF-7 Cells (MTT Assay for 24 hours)**

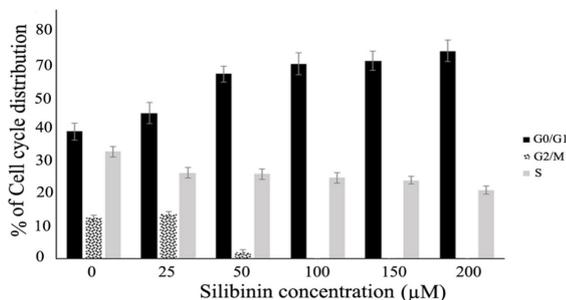


**Figure 3. The Effects of Different Silibinin Concentrations ( $\mu$ M) on the Rates of Apoptosis and Necrosis of MCF-7 Cells were Depicted in Bar Diagram.** The values of necrosis and apoptosis were shown in percentage (%) from the most appropriate individual test. Flowcytometric measurement was performed by staining with annexin-V and the corresponding scatter plots were depicted for different treated concentrations of silibinin as control (0 $\mu$ M; a), 12.5(b), 25(c), 50 (d), 100 (e), 150 (f), and 200  $\mu$ M (g). Apoptosis, the percentage of necrosis and combination of these effects were also underlined in each scatter plot

2- $\Delta\Delta CT$ ). Briefly, the expression of genes P53 and P21 at different concentrations of silibinin showed significant difference compared to untreated control cells. The expression level of genes BRCA1 and ATM significantly increased compared to untreated control cells, which could be associated with the effect of silibinin on DNA repair

in chromosome damages (Figure 5).

The expression of Bak, an important pro-apoptotic gene, also showed a significant increase compared to untreated control cells, while the expression of anti-apoptotic gene, Bcl-xl, showed a significant decrease ( $p < 0.05$ ). Comparing the results with those of P53 expression showed that cells were led to P53-dependent apoptosis.



**Figure 4. Effects of Silibinin on Cell Cycle in MCF-7 Cells.** The MCF-7 cells were treated with either control or different concentration of silibinin (25, 50, 100, 150 and 200 μM). After 48h cell cycle arrest was investigated by flow cytometry analyze. The data are mean ± SD of triplicate for each treatment respectively

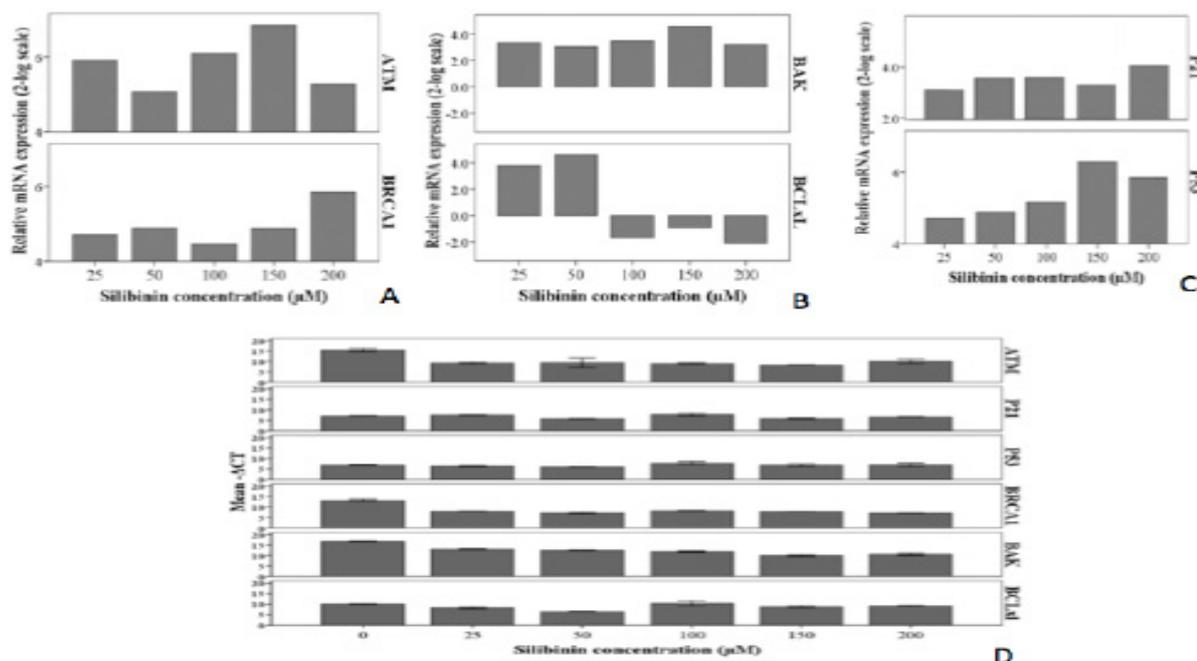
### Discussion

Recently herbal-derived medicines have been investigated as a novel source of anti-cancer drugs (Nasiri et al., 2013). Silibinin has introduced with more properties for anticancer application, chemopreventive efficacy and nontoxic roles in humans (Kauntz et al., 2012). General in vitro and in vivo studies demonstrate that the chemoprotective role of silibinin accompanied by cell proliferation, angiogenesis and metastasis in several cancers (Ghasemi et al., 2013). Aim of this study is was to detect that silibinin had a potential inhibitory effect on MCF-7 cells in reducing cell growth and increasing apoptosis.

In the present study, at concentrations greater than 25

**Table 2. Primer Sequences Used in Quantitative Real-time PCR Assay**

Gene	Primer Sequence (5'-3')	
BRCA1	F: GCG TCC CAC AAA TAA AT	R: CTT GAC CAT TCT GCT CCG TT
ATM	F: ATC TCA GCT TCT ACC CCA ACA	R: GTG AGC TTT CTA GGT TTG ACC TC
P21	F: GCT CAG GGG AGC AGG CTG AAG	R: CGG CGT TTG GAG TGG TAG AAA TCT GT
P53	F: TCA ACA AGA TGT TTT GCC AAC TG	R: ATG TGC TGT GAC TGC TTG TAG ATG
Bcl-xl	F: ATG GCA GCA GTA AAG CAA GCG C	R: TTC TCC TGG TGG CAA TGG CG
Bak	F: TCC AGA TGC CGG GAA TGC ACT GAC G	R: TGG TGG GAA TGC GCT CTC ACA AGG
GapDH	F: AAG CTC ATT TCC TGG TAT GAC AAC G	R: TCT TCC TCT TGT GCT CTT GCT GG



**Figure 5. The Effect of Silibinin on Transcriptional Levels of an Array of Apoptosis and DNA Repair Markers in Breast Carcinoma.** The relative mRNA expression in the absence or presence of treatment with various concentrations of silibinin (for 48 h) were shown distinctively in MCF-7 cell lines. A: DNA repair related genes; B: Apoptotic related genes; C: Cell cycle arrest related genes; D: The mean  $-\Delta\Delta CT$  for all assessed genes. The results are representative of 3 independent experiments. The values are expressed as mean ± SD

$\mu$ mole, silibinin inhibited the cell growth and increased the incidence of apoptosis in MCF-7 cell line. Several studies showed that silibinin induced apoptosis via increased Bcl-2 family levels, and reduced antiapoptotic molecules in different carcinoma cells models (Kauntz et al., 2011; Hagelgans et al., 2014). How silibinin induced cell death and associated signal pathway of apoptosis is not clear. Ghasemiet al showed that silibinin had a strong cytotoxic effect on liver cell line (HepG2 and Hep3B) and caused the cells undergo apoptosis (Ghasemi et al., 2013). In a similar study, silibinin had an inhibitory effect on the growth of MCF-7 cells against UV radiation (Noh et al., 2011). Flowcytometry results showed silibinin could induce cell apoptosis successfully in a time- and dose-dependent manner. Meanwhile, in response to the silibinin behavior, cell cycle was arrested in the G0/G1 phase.

Another study with similar results showed that silibinin exerts time-dependent effects on colorectal cancer cells (Akhtar et al., 2014). Consistent with this explanation, the data of our study show evidently that silibinin caused G0/G1 arrest was associated with a noticeable increase in p53, ATM mRNA level. Studies have shown that silibinin has apoptotic and anti-growth effects on different cell lines (Chhabra et al., 2013). Wang et al demonstrated the anti-inflammatory and apoptosis-inducing effects of silibinin in brain cells in association with increased expression of Bcl-2 gene (anti-apoptotic) and decreased expression of Bak gene (apoptotic) after stroke (Wang et al., 2013).

Tyagi et al investigated the effect of silibinin on the testicular cancer cells and proposed that the expression of P53 was increased as a result of treatment with silibinin. They showed that caspase 2 was activated and expression of Bid gene was increased (Tyagi et al., 2006). In another study, it was found that silibinin protects dermal and epidermal cells against UV radiation through increasing the expression of P53 gene (Wang et al., 2013). In a parallel study with similar results, Roy et al in their study on prostate cancer cells reported the expression of P21 after treatment with silibinin increased (Roy et al., 2012). Mateen et al observed that the treatment of small and non-small lung cancer cells with silibinin inhibited the HDACi (histone deacetylase inhibitors) in NSCLC lung cancer cell line and increased in general the acetylation of histones H3 and H4. They further treated the cells with silibinin and HDACi and observed the expression of P21 (Mateen et al., 2012). Roy et al observed that silibinin increased the expression of P53, and activated the P21 as well. As a result, the progression of cell cycle was stopped, DNA repair and proof-reading was performed (Roy et al., 2009). Our results indicated high levels of ATM not only induced DNA repair but also caused promotion apoptotic pathway. Our data confirm that over expression of ATM is parallel with BRCA1 and Bak.

In 2013, Liu et al scrutinized the effect of silibinin in neutralizing the effect of UV radiation in L929 dermal cells of mouse and observed that silibinin stimulated ATM gene expression, ATM protein synthesis and P53 gene expression. Caspase 3 protein synthesis was also evaluated as a result of activation of apoptosis induction pathway in cells (Liu et al., 2013). Furthermore, in another research it was found that silibinin inhibits the

carcinogenic effect of UV radiation through apoptosis induction or DNA repair of damaged cells via activating P53- and ATM/ATR-dependant protein kinase in mouse JB6 cells (Dhanalakshmi et al., 2005). Fan et al understood that silibinin stimulated activation of ROS-JNK-P53 cycle towards the cell death. They also indicated that silibinin increased P53 and PUMA expression level and in turn Bcl-2 and Bax. It also decreased the MMP (mitochondrial membrane potential) (Fan et al., 2012).

In the study of Roy et al on prostate cancer cells, silibinin stimulated P21/Cip1 gene expression, while it reduced CDKs and cyclins kinase activity. It also activated Kip1/P27 genes following increasing the level of binding to CDK2 and as a result induction of G1-phase cell cycle arrest (Roy et al., 2007). In another study by Guet et al, silibinin was recognized as a leading factor in increasing the P53 and Cip1/p21 expression levels and controlling the chromosomal damage caused by UV radiation (Gu et al., 2005). Further in another study, the treatment of LNCaP prostate cancer cells with silibinin represented the ATM gene expression (Farooqi et al., 2010).

In conclusion, our results, as shown in Figure 5, obviously confirmed that silibinin induces apoptosis through increasing of p53, p21, Bak, ATM in mRNA levels. This study addressed that silibinin inhibited proliferation, induced apoptosis and caused cell cycle arrest at G0/G1 phase in human breast cancer MCF7 Cells, the molecular events identified that silibinin efficacy was associated with the up-regulation of Bak, P53, P21 and down-regulation of Bcl-xl.

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