

## Effect of Silibinin on the Induction of Apoptosis and the Inhibition of Cell Growth on the MCF-7 Cell Line

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### Abstract

Given the prevalence of breast cancer, the mortality of patients and the public's convenient access to the herbal extract of milk thistle, we decided to examine the effectiveness of this plant's components on the breast cancer cell line MCF-7. Silibinin is a naturally occurring flavonoid antioxidant found in the milk thistle. Recently, it has demonstrated potent, anti-proliferative effects against various malignant cell lines. In the present study, MCF-7 cells were incubated and treated with various concentrations of silibinin repeatedly. The degree of cell cytotoxicity depended on the dose of the milk thistle's extract and also on the duration of its exposure, imparting an inhibitory effect on the viability of metastatic MCF-7 cells. Finally, the expression of apoptotic genes (BCL-2 and BAX) was assessed by Real time PCR. Cell viability and growth of MCF-7 cell lines were inhibited by silibinin. The results of the present study confirm the efficacy of the herbal supplement against breast cancer. Due to the nature of the product, its low cost and potential accessibility to the public, adding this herbal supplement to the human food diet may be effective in preventing and treating breast cancer.

**Keywords:** Silibinin, MCF-7, Bcl-2, Bax

### Introduction

Breast cancer is a common malignant neoplasm in women and is considered as the main cause of death in women (Fisch et al, 2005; Bray, McCarron, & Parkin, 2004). Chemotherapy is a cancer treatment method or temporary manner of relief via the use of certain medications. Side effects of chemotherapy depend on dosage. It can also lead to tumor recurrence and often lead to drug resistance (Hale et al, 2005; Juzenas, et al, 2008). It is understood that the consumption of some food products can be effective in preventing cancer due to antioxidant properties (Namiki, 1990). Among these products, we can point to silymarin — obtained from milk thistle seeds — which has about 90% of its components allocated to the compound sili, binin (Deep & Agarwal, 2010).

Silibinin is a polyphenolic flavonoid which is commonly isolated from the fruits or seeds of the milk thistle (*Silybum marianum*); silymarin is a flavonolignan complex, composed of silibinin and small amounts of isosilybin A, isosilybin B, silychristin, iso-silychristin, dehydrosilybin, silydianin and taxifolin (Gazak et al, 2007). Silibinin has anticancer properties against many cancers including lung (Chu et al, 2004), skin (Mohan et al, 2004), bladder (Tyagi et al, 2004), liver (Momeny et al, 2008), prostate (Singh and Agarwal, 2004) and colon (Gazak et al, 2007) cancer cells.

Silibinin induces apoptosis by down-regulation, providing for the inhibition of hypoxia-inducible factor-1 alpha in non-small-cell lung cancer cells (Kim et al, 2010). Silibinin up-regulates the expression of cyclin-dependent kinase inhibitors and causes a cell cycle arrest. It also causes a

type of apoptosis which is independent of caspases' activation in the human colon carcinoma HT-29 cells (Agarwal et al, 2004).

The Bcl-2 family of proteins regulates apoptosis, cell cycle and differentiation. Bcl-2 proteins can inhibit the mitochondrial apoptotic pathway via binding to its pro-apoptotic protein Bax, thereby preventing the release of cytochrome c and the activation of the apoptotic cascade (Teijido & Dejean, 2010). Bax promotes apoptosis, induces permeabilization of the outer mitochondrial membrane (OMM), controls transition of cytochrome c through the OMM and maintains Ca<sup>2+</sup> levels of the Endoplasmic Reticulum (Hoppins et al, 2011).

The purpose of this study was to develop an understanding of silibinin's effects on breast cancer cells and also determine its therapeutic value in preventing or treating this disease. Therefore, we examined the anticancer activities of silibinin against the MCF-7 breast cancer cell line.

## Materials and Methods

### *Cell culture and Silibinin treatment*

MCF-7 cells were obtained from the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran. MCF-7 cells were cultured on the RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (100 mg/ml). Apart from the cell lines, the other materials were from Gibco, Scotland. The complex was maintained at 37<sup>o</sup> C in an incubator containing 5% CO<sub>2</sub>. Harvested cells with trypsin (0.25%) (Sigma, USA) were counted by a Neobar slide with trypan blue and were then seeded into 96-well plates (1 \* 10<sup>4</sup> cells/well). Silibinin (Sigma, USA) was dissolved in DMSO (Sigma, USA). The cells were incubated with different concentrations of silibinin (50, 100, 200mg/ ml) at 12, 24 and 48 hours. A group of cells were not treated with silibinin. These were used as a negative control group of cells. All experiments were repeated three times.

### *Measurement of cell viability*

Cell viability was evaluated via a methyl-thiazolyl-tetrazolium-bromide (MTT, Sigma, USA) assay. This method is based on the ability of living cells in converting soluble tetrazolium salt to insoluble formazan. To perform the test, the cells were passaged twice and were then transferred to a 96-well plate, wherein 10 ml of MTT solution (5 mg/ml in PBS) was added to each well containing 100 ml of cultured medium. The dissolved, yellowish MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring via dehydrogenase enzymes during 4 hours of incubation. The produced, insoluble formazan was dissolved in a solution containing 100 ml isopropanol (Merck, Germany) and its optical density (OD) was read against a blank reagent with a multi-well scanning spectrophotometer at a wavelength of 570 nm. Untreated cells were used as control.

The percentage of cytotoxicity was calculated according to the following formulae:

$$\% \text{cytotoxicity} = \frac{1 - \text{mean absorbance of toxicant treated cells}}{\text{mean absorbance of negative control}} \times 100$$

$$\% \text{Viability} = 100 - \% \text{ cytotoxicity}$$

### *RNA Extraction by the TRIzol Reagent*

After incubation, cells were trypsinised and centrifuged for 5 min at 3000 rpm for the cells to pellet. The pellets were washed 2-3 times with sterile PBS for RNA isolation. In detail, 300 µL of the TRIzol solution was added to the cell pellet and was vortexed. The reaction mixture was incubated at room temperature (RT) for 5 minutes and then 80µL of chloroform was added while being thoroughly mixed. Then, the mixture was incubated at RT for 5 minutes, followed by centrifugation at 11,000 g for 15 minutes at 4°C. The aqueous phase was collected into a separate 1.5 mL micro centrifuge tube and 150µL of isopropyl alcohol was added. The reaction mixture was

incubated at RT for 10 minutes followed by centrifugation at 13,000 rpm for 10 minutes at 4°C. RNA was then pelleted with 75% absolute alcohol and centrifuged at 9,000g for 10 minutes at 4°C. The pellets were air-dried and resuspended in 10µL of RNAase-free water for further use.

### REAL-TIME PCR Analysis

The expression of apoptotic genes was analyzed by reverse transcription PCR via a one-step real-time SYBR Green mix. Relevant mRNA levels of two apoptotic genes were tested using the reference gene GAPDH to normalize the gene expression. Quantitative real-time RT-PCR was performed in a reaction volume of 25µL according to the manufacturer's instructions. Briefly, 13µL of master mix, 0.2µL of primer (0.2nM) and 5µL of template RNA (100µg) were added to 0.2mL PCR tubes. After a brief centrifugation, the PCR plate was subjected to 30 cycles using the following conditions for cDNA: 50°C for 10 minutes (i) PCR activation at 95°C for 5 min; (ii) denaturation at 95°C for 5s; and (iii) annealing/extension at 60°C for 10 s. The quantitative RT-PCR data were analyzed using the comparative threshold (Ct) method. GAPDH was used as an internal reference gene to normalize the expression of the apoptotic genes. The Ct cycle was used to determine the expression level in MCF-7 cells treated with silibinin for 48 hours. Sequences of primers that were used are listed in Table 1.

**Table 1: Sequences of primers used**

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
Bax	CGAGAGGTCTTTTCCGAGTG	GTGGGCGTCCCAAAGTAGG	242
Bcl-2	CGGTGGGGTTCATGTGTGTG	CGGTTCAGGTACTCAGTCATCC	90
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226

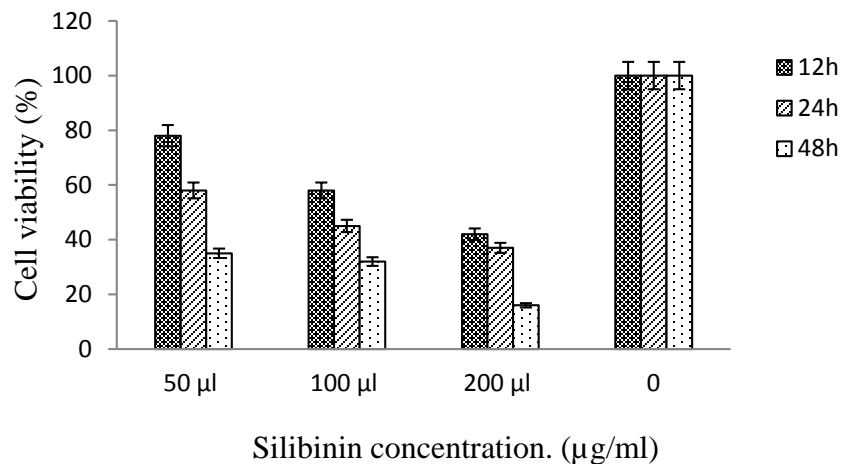
### Statistical analysis

The statistical software (SPSS 19) was used for data analysis. The student t-test and ANOVA were applied to interpret the data pertaining to the different silibinin treatments to evaluate the differences between control groups and treated samples.

### Results

#### *Silibinin Cytotoxicity on MCF-7 cells*

Cell survival was evaluated by the MTT assay which showed that silibinin impedes the growth of MCF-7 cells in a manner that is dependent on dosage and time (Fig. 1).



**Figure 1. Evaluation of silibinin inhibitory effects on cell proliferation by the MTT assay**

In order to check the inhibition activity of silibinin, MCF-7 cells were plated and treated with different concentrations of silibinin (50  $\mu$ l, 100  $\mu$ l and 200  $\mu$ l) (Table 1). The MTT assay was performed after 12, 24 and 48 hours following the silibinin treatment.

Figure 1 shows the relative difference in cell survival, resulting from the 3 separate schedules pertinent to each concentration of silibinin, drawn against the control group.

As results show in figure 1, the inhibitory activity was measured based on cell number. According to the results in Table 2, the cytotoxic effect of silibinin on cancer cells and their growth depends on dose and time.

**Table 2: Comparison between the means of effects of silibinin on cell viability**

Variable time	Silibinin concentration ( $\mu$ l)			
	50	100	200	0
12 hr	78b*	58c	42d	100a
24 hr	58c	65b	37d	100a
48 hr	35b	32b	16c	100a

\*Different letters in each row represent significance by the Duncan test at  $p < 0.05$

According to the diagram, when the cancer cells are treated with silibinin for longer durations, the growth rate declines correspondingly. Higher doses of silibinin prevent cell growth more effectively. Therefore the effect of silibinin concentration could remarkably alter the outcome of cell growth.

The most toxic concentration of silibinin on MFC-7 cells was 200  $\mu$ l after 48 hours of exposure. Statistically, silibinin significantly decreased cell viability in all treatments ( $F_3, 35 = 697.83$   $P < 0.001$ ) and as regards the duration of exposure ( $F_2, 35 = 179.03$   $P < 0.001$ ). However, the magnitude of this decrease in cell viability was different among various combinations of duration  $\times$  concentration ( $F_6, 35 = 29.91$   $P < 0.001$ ). Treating the cancer cells with 200  $\mu$ l of silibinin resulted in the greatest decrease in cell viability, which is when the cells were exposed to silibinin for 48 hours.

#### ***Silibinin's effect on the expression of apoptotic genes***

To investigate the molecular mechanism of silibinin-induced apoptosis in MCF-7 cells, the expression levels of two apoptosis-related genes were examined. To evaluate the effect of silibinin on the expression of the Bax and the BCL 2 genes, the expressions of these two genes were analyzed by real-time PCR.

The Bax protein is known to be a key protein involved in apoptosis induced by various factors, and its role is to act in the internal apoptosis path. To investigate the effect of silibinin on BCL 2 and Bax genes, their expressions of mRNA were analyzed by Real time PCR. Silibinin was observed to increase the level of Bax mRNA expression significantly, while it had no significant effect on the expression of the BCL-2 gene.

#### **Discussion**

Breast cancer is the most common malignant type of cellular proliferation in women worldwide and the incidence of this disease is increasing in a diverse array of countries. Among the causes of cancer, there are numerous factors that are progressing towards dangerous edges such as air pollution, stress, diet and lifestyle. It is known that the consumption of foods, which have antioxidant properties, can prevent cancer or reduce the risk of its occurrence (Ren et al, 2003; . Sylvester and Shah, 2002). Among the many food supplements of interest, in relation to this context, one can point to the extract of *Silybum marianum*. Recent studies on the anticancer effects of this plant extract have been successful, at least to some extent.

The objective of this project was to investigate the effect of silibinin concentration on breast cancer cells, i.e. cell line MCF-7. Results show that these cells are notably sensitive to silibinin,

owing to the changes in apoptotic gene expressions. Silibinin's inhibitory effect was dose-dependent and time-dependent. Our findings showed that the mechanisms of silibinin's potential inhibitory effect on MCF-7 cells are to decrease cell proliferation and increase apoptosis of the cell.

Our results showed that the most toxic concentration of silibinin occurs at 200  $\mu$ l in 48 hours. Also, there is a significant difference between the dose and the duration of exposure when comparing treated cells with control cells ( $p < 0.001$ ).

BCL-2 and Bax are two major proteins that are generally involved in apoptosis. The mRNA levels were performed using a one-step RT-PCR SYBR Green mix quantitative real-time reverse transcription PCR.

The results indicate that silibinin significantly increases the level of Bax mRNA expression, while there was no effect on the expression of BCL-2. These findings confirm previous ones regarding other types of cancer with evidence of silibinin's effectiveness and its impact on apoptotic genes.

In a study conducted by Zhang et al (2012), it was found that silibinin inhibits the growth of gastric cancer SGC-7901 cells by reducing the expression of the protein p34cdc2. Liu et al (2011) studied the inhibitory effects of silymarin on a highly metastatic lung cancer cell line Anip973 and found that silymarin exerts a significant inhibitory effect on the proliferation of Anip973 cells in a temporally and dose-dependent manner. It has also been found that the silymarin can further induce apoptosis. Miscellaneous studies have demonstrated the strong inhibitory effect of silibinin as it displays anti-growth properties and as it exerts apoptotic effects on different cell lines (Singh & Agarwal, 2004). Results show the potent effect of silibinin on apoptotic genes and also on related processes in cells treated in experiments, compared to untreated cells or controls. Further studies shall be needed to determine the precise molecular mechanisms in this regard.

In summary, the results of this study confirm the efficacy of the herbal supplement silibinin, its pro-apoptotic properties against breast cancer cell lines MFC-7. Prospects are in close reach that by using this herbal supplement, the human food diet may become purposefully effective in reducing breast cancer.

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