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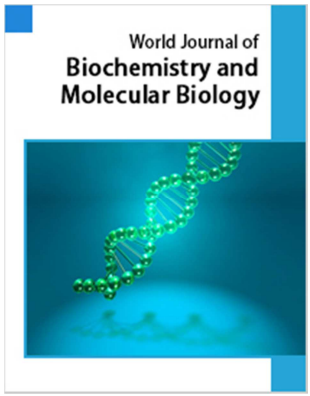

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Silibinin Is an Inhibitor of miR-24-3p Gene Expression in T47D Breast Cancer Cell Line

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Abstract
MiR-24-3p has been considered as an attractive molecular target for breast cancer therapy. The main objective of the present study was to assess the inhibitory effects of silibinin, a herbal component, on the proliferation, apoptotic induction and the expression of miR-24-3p gene in T47D human breast cancer cell line. Human breast cancer cell line of T47D was treated with various concentrations of silibinin. Cellular viability and the apoptotic rate were assessed by MTT assay flow cytometric analysis, respectively. Expression of human miR-24-3p gene was measured with real-time PCR. The present study has demonstrated that the silibinin inhibited the cellular growth in a dose- and time-dependent manner, and effectively induced a considerable amount of apoptosis in of T47D cells. In addition, silibinin caused a decrease in miR-24-3p expression. The present findings suggest that silibinin could successfully reduce the cell viability and miR-24-3p gene-expression in breast cancer cells. These antineoplastic effects of silibinin were strengthened by its inhibiting effects on the cell proliferation, and apoptosis-induced down-regulation of miR-24-3p expression in T47D cells.

1. Introduction

Phytochemicals in are gaining considerable attention as the anticancer agents. They are capable of regulating multiple signaling pathways, associated with aggressive phenotypes. Because of their pharmacological safety, these phytochemicals can be applied in monotherapy or in association with chemotherapeutic drugs [1].

Silibinin, a major bioactive component of silymarin, is isolated from the milk thistle (*Silybum marianum*) plant. It has been reported that silibinin exhibits anticancer activities against various solid tumors-bearing cancers, including breast cancer in both in vitro and in vivo models [2-4].

Breast cancer, the most frequent neoplasm in women worldwide, is a common and highly heterogeneous malignancy. Breast cancer is one of the main gynecological cancers in the world [5, 6]. The research evidences indicate that breast cancer is not a single disease, but instead is a collection of diseases with diverse clinically relevant biological and phenotypical features [7, 8]. Recent technological advances in molecular profiling have led to the identification of an increasing number of molecular subtypes in breast cancer and their underlying genetic drivers that may be affected by numerous biological factors, including miRNAs [8].

MicroRNAs (miRNAs) are a highly abundant type of endogenous small non-coding RNAs (18–25 nucleotides in length) that regulate gene expression at post-transcriptional level by inhibiting the protein translation or by degrading the mRNA of the target gene [9]. About half of the miRNA upstream genes, located in tumor-associated region on chromosome, and the miRNAs themselves are abnormally expressed in a variety of tumors, suggesting that miRNAs may function as tumor suppressor factors or the oncogenes [6, 10]. A large number of studies have shown that miRNAs play major role in a wide range of developmental processes, including the cell proliferation, cell cycle, cell differentiation, metabolism, apoptosis, developmental timing, fate and expression of neuron genes, brain morphogenesis, muscle differentiation and stem cell division [8, 11-13]. Aberrant expression levels of miRNAs have been observed in many solid tumors, including breast cancer [14]. Recent studies have demonstrated that miR-24-3p is found to be deregulated in a variety of cancers and the over-expression of this gene could promote the cell proliferation [15].

The major goal of our study was to assess the anti-proliferative and apoptotic effects of silibinin on the human breast cancer cell line of T47D and its potential to inhibit the expression of miR-24-3p gene.

2. Materials and Methods

2.1. Reagents

Silibinin (Catalogue No. S0417), 3-(4,5-Dimetrylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DAPI (4', 6-diamidino- 2-phenylidole) were purchased from Sigma (St. Louis, Missouri, USA). Fetal bovine serum (FBS), RPMI 1640 and Penicillin/streptomycin were provided from GIBCO (Invitrogen, Carlsbad, CA). DMSO and Bradford solution were acquired from Merck (Darmstadt, Germany). Annexin V-FITC/PI Kit and 2-steps RT-PCR kit were obtained from Invitrogen (Life Technologies, Carlsbad, CA, USA). RNA Isolation Kit was purchased from Qiagen (Venlo, Limburg, Netherlands) and SYBR® Premix Ex TaqTMII was provided by Sinagen (Tehran, Iran).

2.2. Cell Culture and Silibinin Administration

T47D cell line was obtained from Pasteur Institute (Tehran, Iran). and cultured in $RPMI_{1640}$ medium containing 10% FBS and antibiotics under a humid atmosphere (37°C, 5% CO_2, 95% air). For silibinin treatment, appropriate amounts of the pre-prepared silibinin stock solution were added into the RPMI 1640 medium to achieve the indicated concentrations and then administered to the cancer cells for the period of 24, 48 and 72 h, respectively. Whereas, the DMSO solution without silibinin was used as the control treatment.

2.3. Measurement of Cell Viability

After the treatment with silibinin, cell viability was evaluated using the MTT assay. Cell samples were incubated with 100 µl of MTT reagent for 3h at 37°C. Then, the culture medium was removed and insoluble formazan crystals, formed in living cells by the activity of mitochondrial dehydrogenenases, were revealed by adding 100 µL of DMSO to each well, followed by gentle stirring for 10 min. at room temperature. A 0.1 ml aliquot of each sample was then transferred to 96-well plates and the absorbance of each well was measured at 570 nm with ELISA Reader (Awarnesse, USA).

Growth inhibition was measured by dividing the mean absorbance of treated wells by the mean absorbance of control wells (drug-free wells), and expressed as a percentage value. The concentration of silibinin that could inhibit the growth of 50% population of T47D cells (IC50) was defined as the drug concentration, at which the cell growth was inhibited by 50% as compared with the drug-free controls.

2.4. Apoptotic Analysis by Flow Cytometry

T47D cells were plated in 6-well plate under the standard culture conditions. After 48 h of incubation, the cells were fed with fresh medium and treated with DMSO alone or administered with different doses of Silibinin (100 and 150 µM). After 48 h of treatment, the medium was aspirated and the cells were washed twice with ice-cold PBS, trypsinized, and the cell pellets were collected. For the apoptosis analysis, a population of 5×10^5 cells were washed with 1 ml PBS (pH 7.4) and then resuspended in binding buffer according to the manufacturer's protocols. Cell aliquots were then incubated with Annexin-V- FITC and PI, and incubated for 15 min at 4°C in the dark. The levels of apoptotic induction were determined by FACScan cytometer and Cell Quest Software (FACS Calibur; Becton-Dickinson, San Jose, CA, USA). All the experiments were performed in triplicate.

2.5. RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cell cultures, which were about 70% confluent using the Trizol Reagent. About 1 mL of the Trizol solution was added to cell pellet and vortexed. The reaction mix was then incubated at room temperature for 5 min. then, a volume of 200µL chloroform was added to the solution and mixed well. Then, the mixture was incubated at room temperature for 5 minutes, followed by centrifugation at 12,000 g for 15 minutes at 4°C. The aqueous phase was collected into a separate 1.5 mL micro-centrifuge tube, 500µL isopropyl alcohol was added to it and mixed well. Then, the reaction mixture was incubated at room temperature for 10 minutes, followed by

centrifugation at 12,000 rpm for 10 minutes at 4°C. RNA was then pelleted with 75% absolute alcohol and stored in RNase-free water at 80°C for further use. The RNA concentrations were determined by UV-Visible Spectrophotometer and the purity of RNAs were estimated at the wavelength range of 260-280 nm. The integrity of RNA was confirmed by electrophoresis of the individual samples on a 2% agarose gel.

After RNA preparation, complementary DNA (cDNA) was reverse-transcribed using the 2-steps RT-PCR kit according to the manufacturer's instructions. The synthesized cDNA was immediately used in a real-time PCR or stored at -80°C for further experiments.

2.6. Real-Time PCR Amplification

The expression of miR-24-3p and rRNA-5s mRNAs was determined using real-time PCR. Each cDNA sample was amplified using SYBR Green on the ABI 7500 Fast Real-time PCR System (Applied Biosystem, CA). The reaction solutions consisted of 2 µl of cDNA and 0.5 µl primers in a final volume of 20 µl of supermix. PCR reaction parameters were as follows: denaturation at 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and the extension process at 72°C for 30 seconds. The experiment was performed in the form of three independent experiments with triplicate

For each sample, the ΔCt values were determined by subtracting the average of duplicate Ct values of target gene from the average of duplicate Ct values of the reference gene. The relative gene expression level was also normalized relative to a positive calibrator, consisting of one of the samples from the calibration curve. The relative gene expression level of the calibrator (ΔCt calibrator) was also determined by subtracting the average value of the duplicate Ct values of target gene from the average of duplicate Ct values of the reference gene. The results were expressed as 'N-target' and determined as follows:

$$\text{N-target} = 2^{(\Delta Ct\ sample - \Delta Ct\ calibrator)}$$

2.7. Statistical Analyses

The data were analyzed using SPSS for Windows (Version 16.0) software. Statistical significance of the difference in growth inhibition and expression levels of miR-24-3p and rRNA-5s, between the control and treated groups was assessed using Student's t-test. A statistically significant difference was considered to be present at $P < 0.05$ value.

3. Results

3.1. Silibinin Inhibits the T47D Cell Viability

Human ductal breast epithelial T47D cells were exposed to silibinin at the concentrations of 50-4000 µM for 24, 48 and 72 h and the amount of induced cytotoxicity was determined using MTT assay. The MTT results have shown that as the concentration of silibinin increased to 50, 75, 100, 150, 200, 250, 300 and 350 µM, the cytotoxicity was also induced in a dose-dependent manner. In MTT assay, the maximum growth inhibitory effect was observed at 25 µM dose of silibinin for 24 h (Figure 1).

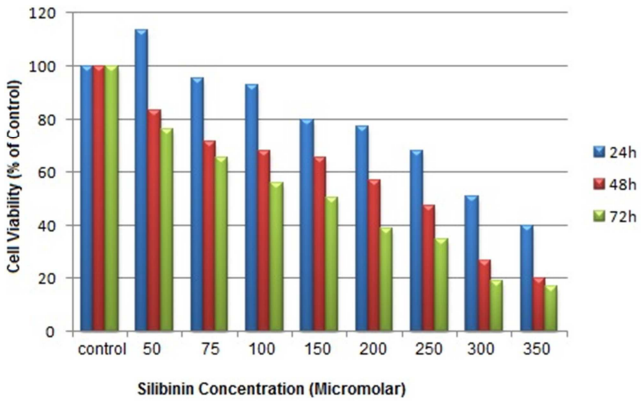

Figure 1. *Effect of silibinin on T47D cell viability.*

T47D cells were cultured as described in "Materials and Methods" and treated with either DMSO or 25–350 µM of silibinin for 24, 48 and 72 h, and the cell number was determined at the end of the exposure period.

Data analysis of cytotoxicity assay showed that cytotoxic effect of silibinin on T47D breast cancer cell line was 373/42, 176.98, and 126.72 µM after the 24, 48, and 72 hours of exposure, respectively ($P<0.05$); indicating a dose- and time-dependent response (Figure 2).

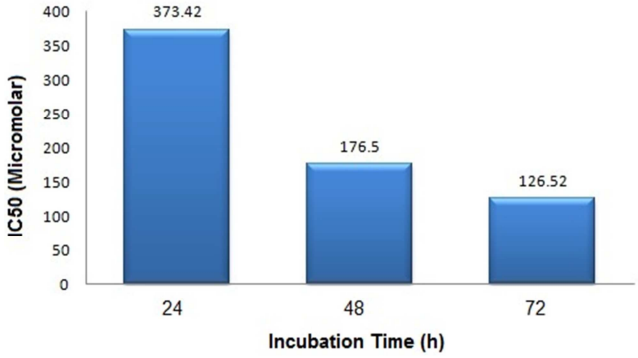

Figure 2. *Silibinin activity against the breast cancer cell line T47D following 24, 48 and 72 hour incubation, as assessed using the MTT assay. Data are reported as IC50 versus time.*

3.2. Silibinin Induces Apoptosis in T47D Cells

Many chemical compounds can inhibit the growth of tumor cells, but not all of them can trigger a good amount of apoptosis. To determine the apoptotic-induction capability of silibinin, we performed flow cytometric analysis with Annexin V-FITC conjugated to PI. As shown in Figure 3, the population of apoptotic cells increased from 1.47% in control to 44.35% ($p < 0.05$) after 48 h treatment with 100 µM silibinin.

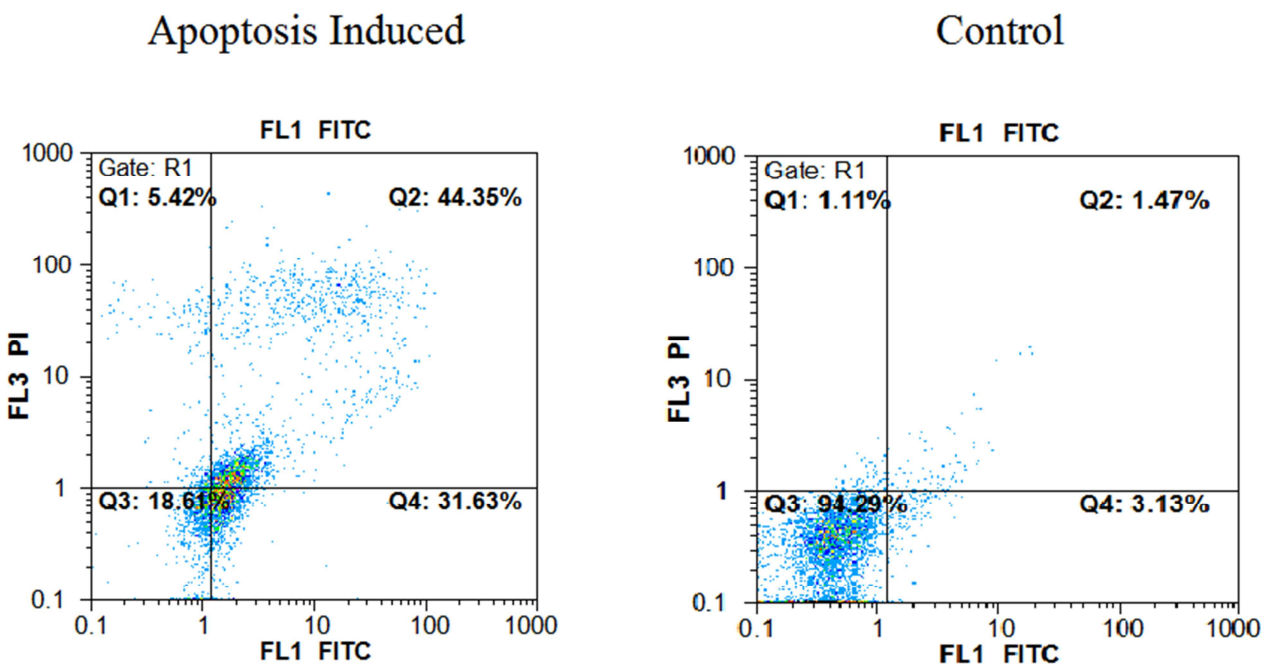

Figure 3. *Silibinin induces apoptosis in T47D cells.*

The T47D cells treated with 100 µM of Silibinin for 48 h were assessed for apoptosis by staining with Annexin V-FITC and PI. Data are reported as the mean ± SD of 3 determinations ($P<0.05$ for 100 µM silibinin treatment vs control).

3.3. Silibinin Inhibits the miR-24-3p Expression in T47D Cells

The expression of miR-24-3p transcript was determined by Real time PCR using ABI PRISM 5700 sequence detection system (Applied Biosystems). Continuous measurement of the PCR product was enabled by the incorporation of SYBR-Green fluorescent dye into the double stranded PCR products. The level of miR-24-3p transcript was normalized to the transcript level of rRNA 5s and ultimately, the ΔΔCt value was calculated for each sample for the purpose of statistical analysis according to Yuan et al. (2006) [16] Finally, the ΔΔCt values were transformed to absolute values using the formula: $2^{-\Delta\Delta Ct}$.

The results of qRT-PCR analysis revealed that silibinin treatment caused a significant increase in the expression of miR-24-3p in T47D cells, as compared to untreated control cells ($p<0.05$).

4. Discussion

Several studies have shown that silibinin, a non-toxic bioactive component of milk thistle, has promising anticancer and chemopreventive efficacy against various epithelial cancers including prostate, skin, colon, breast, lung and ovarian cancer etc [17-22]. It has been shown to modulate the expression of large numbers of miRNAs, small 22-25 nucleotide non-coding RNAs in cancer cells that lead to a reduction in tumor growth [1, 23, 24]. miRNAs also play an important role in the modulation of chemosensitivity in tumor cells [25-27]. miR-24-3p is found to be deregulated in a variety of cancers [15]. Several studies have reported that miR-24-3p might function differently in cell proliferation in different kinds of cells. For example, miR-24-3p was able to inhibit cell proliferation in HeLa cells, in contrast, it promotes cell proliferation of transforming growth factor (TGF) β-treated hepatocellular carcinoma cells (HuH7), as well as A549 lung carcinoma cells [15].

The aim of this study was to assess the potential of silibinin to inhibit the expression of miR-24-3p gene. In this study, the cytotoxic effects of the silibinin (50 – 350 µM) on T47D breast cancer cells was investigated by MTT assay after 24, 48 and 72 h of treatment. After the administration of cancer cells with different concentrations of silibinin, the level of miR-24-3p gene expression was measured by reverse-transcription real-time PCR.

This study demonstrates that silibinin caused 6.75%, 55.75% and 68.25% ($P<0.05$) inhibition in cell growth in terms of a reduction in cell number after 24, 48 and 72 h of treatment (Figure 2). We observed that silibinin could exhibit a dose-dependent inhibitory effect on the viability of T47D cell line and reduces miR-24-3p promoter activity, resulting in a decreased total miR-24-3p RNA.

5. Conclusion

The present results convincingly demonstrate the major biological effect of silibinin in T47D cells on the growth inhibition, and that the cell death is the prime factor in the reduction of cell number. Silibinin inhibits the expression of miR-24-3p and could probably be used as drug candidate for breast cancer therapy through miR-24-3p targeting in the future.

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