Original Article

Silibinin: An Inhibitor of *Mir-181a* Gene Expression in Sk-Br-3 Breast Cancer Cell Line

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ABSTRACT

Background: *Mir-181a* has been considered as an attractive molecular target for breast cancer therapy. The main objective of this work is to assess the inhibitory effects of silibinin, a herbal substance, on proliferation, apoptosis and *Mir-181a gene* expression in human breast cancer cell line SK-BR-3. **Materials and Methods:** Human breast cancer cell line SK-BR-3 was treated with various concentration of silibinin. Cell viability was assessed by MTT assay and apoptosis by flow cytometry. Expression of human *mir-181a gene* was measured with real-time PCR. **Results:** Silibinin inhibits growth of SK-BR-3 cells in a dose- and time-dependent manner and effectively induces apoptosis of SK-BR-3 cells. In addition, silibinin caused a decrease in *Mir-181a* level. **Conclusion:** These findings suggest that cell viability along with *Mir-181a gene* expression in breast cancer cells could be reduced by silibinin. These results suggest that silibinin inhibits the proliferation of SK-BR-3 cells, and it induces apoptosis by down-regulating *Mir-181a* expression in breast cancer cells.

Key words: Silibinin, Cell Viability, Apoptosis, Sk-Br-3 Cell Line, *Mir-181a.*

INTRODUCTION

Breast cancer is a leading cause of death in women worldwide.[1] There has long been standing interest in the identification of natural products for the treatment of various diseases for thousands of years. Natural products possess immense pharmacological significance in the development of drugs including cancer, and were discovered through plant bioprospecting. The majority of drug candidates, such as paclitaxel, etoposide, camptothecin, Vinca alkaloids, indole alkaloids, podophyllotoxin derivatives, etoposide and teniposide, currently used in clinical cancer chemotherapy, were originally derived from plants.[2] The efficacy of chemotherapy, radiotherapy, hormonal therapy, or surgery, which are mainly used for the treatment of cancer, are well known for side effects; hence, the identification of novel natural products that possess better effectiveness against cancer, but less harmful effects have become desirable, and therefore, natural products are continuously being explored worldwide.[3-5]

Silibinin is a polyphenolic flavonoid compound, isolated from milk thistle (*Silybum marianum*).[6] It has been demonstrated that silibinin is an efficacious chemopreventive and chemotherapeutic agent[7,8] without any toxic or adverse effects.[9] The anticancer efficacy of silibinin is clearly evident from the published reports against various cancers including prostate, skin, lung, colon, breast, hepatic, ovarian, cervical, kidney and gastric carcinomas.[10] Therefore, this compound can have potential clinical applications in combination chemotherapeutic approaches. *MicroRNAs* (*miRNAs*) are a highly abundant *type* of endogenous small non-coding RNAs (*18–25 nucleotides* in length) that regulate gene

Submission Date: 06-01-2017;
Revision Date: 15-02-2017;
Accepted Date: 13-07-2017

DOI: 10.5530/ijper.51.4.89

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expression at post-transcriptional level by inhibiting the protein translation or by degrading the mRNA of the target gene.[11] 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.[12,13] 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.[14-17] Aberrant expression levels of miRNAs have been observed in many solid tumors, including breast cancer.[18] Recent studies have demonstrated that *miR-181a* expression was dramatically and selectively upregulated in metastatic breast tumors, particularly triple negative breast cancers, and was highly predictive for decreased overall survival in human breast cancer patients.[19]

The major goal of our study was to assess the antiproliferative and apoptotic effects of silibinin on the human breast cancer cell line of SK-BR-3 and its potential to inhibit the *expression* of *miR-181a gene*.

Materials and methods

Cell culture and silibinin administration

SK-BR-3 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.

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 3 h 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 SK-BR-3 cells (IC_{50}) was defined as the drug concentration, at which the cell growth was inhibited by 50% as compared with the drug free controls.

Apoptotic analysis by flow cytometry

SK-BR-3 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 (274 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.

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 200 µL chloroform was added to the solution and mixed well. Then, the mixture was incubated at room temperature for 5 min, followed by centrifugation at 12,000 g for 15 min at 4°C. The aqueous phase was collected into a separate 1.5 ml microcentrifuge tube, 500 µL isopropyl alcohol was added to it and mixed well. Then, the reaction mixture was incubated at room temperature for 10 min, followed by centrifugation at 12,000 rpm for 10 min 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 concentration was determined by UV-Visible Spectrophotometer and the purity of RNA was estimated at the wavelength range of 260-280 nm. The integrity of RNA was confirmed by electrophoresis on a 2% agarose gel.

The miR-Amp kit (Parsgenome, Tehran, Iran) was used for cDNA synthesis. First, poly-(A) tail was added to miRNAs with polyA polymerase at 37°C. RNA polyA tail was mixed with RT-enzyme, reaction buffer, and miR

specific primers for cDNA synthesis, then incubated at 45 °C for 60 min and inactivated at 85 °C for 1 minutes.

Quantitative real time polymerase chain reaction

The expression of *miR-181a* 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.

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:

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

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-181a* 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.

RESULTS

Silibinin induced loss of SK-BR-3 cell viability

Human breast adenocarcinoma SK-BR-3 cells were exposed to silibinin at the concentrations of 75-1000 µM for 24, 48 and 72 h and cytotoxicity was determined using MTT assays. MTT results have shown that as the concentration of silibinin increased to 50, 75, 100, 150, 200, 250, 300 and 350 µM, cytotoxicity was observed in dose-dependent fashion (Figure 1).

Data analysis of cytotoxicity assay showed that cytotoxic effect of silibinin on SK-BR-3 breast cancer cell line was 274 and 374 µM upon 48 and 72 hours of exposure, respectively (P<0.05), indicating a dose- and time-dependent response relationship.

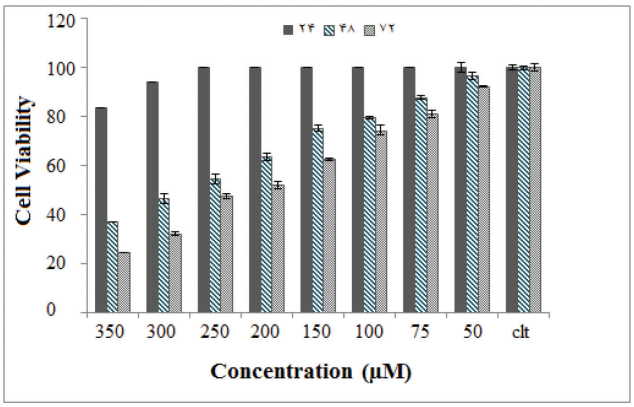

Figure1: Effect of silibinin on SK-BR-3 cell viability. SK-BR-3 cells were cultured as described in "Materials and Methods" and treated with either DMSO or 50–350 µM of silibinin for 24, 48 and 72 h, and cell number was determined at the end of the exposure period.

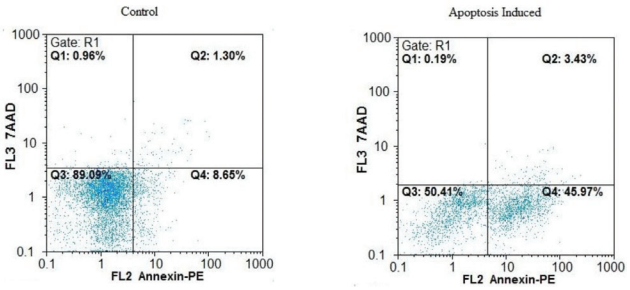

Figure 2: Silibinin induces apoptosis in SK-BR-3 cells. SK-BR-3 cells were treated with 274 µM silibinin for 48 h. Phosphatidylserine (PS) externalization was determined by the combined annexin V/propidium iodide (PI) assay. Cells that stained positive for annexin V represented cells with intact membranes and externalized PS (percentages are indicated in the lower right panel).v Cells that stained positive for annexin V/PI represent cells that had lost membrane integrity (percentages are indicated in the upper right panel). Data are reported as means±SD of 3 determinations. P< 0.05 for 274 µM silibinin treatment *vs* control.

Silibinin induced apoptosis of SK-BR-3 cells

Many chemical compounds can inhibit the growth of tumor cells, but not all of them can trigger apoptosis. To determine whether apoptosis was induced by the compounds mentioned above, we performed flow cytometric analysis with Annexin V-FITC conjugated to Propidium Iodide (PI). As shown in Figure 2, apoptotic cell population increased from 8.65% in control to 45.97% (p all <0.05) after 48 h treatment with 274 µM silibinin.

Silibinin inhibited the *mir-181a* expression in SK-BR-3 cells:

The expression of *mir-181a* transcript was determined by real-time PCR using ABI 7500 Fast Real-time PCR

system (Applied Biosystems). Continuous measurement of the PCR product was enabled by incorporation of SYBR Green fluorescent dye into the double stranded PCR products. The transcript level of *mir-181a* 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*.[20] Finally, the ΔΔCt values were transformed for absolute values using the formula $2^{-\Delta\Delta Ct}$.

The qPCR analysis revealed that silibinin treatment caused a significant decrease in the expression of *miR-181a* in SK-BR-3 cells, as compared to untreated control cells ($p<0.05$).

DISCUSSION

Several studies have shown that silibinin as a natural flavonoid, induces various cell functions including growth inhibition, cell cycle arrest, antiproliferative effect and apoptotic induction which could be applied as anticancer agent.[21-25] It has been shown to modulate the expression of large numbers of miRNAs, small [22-25] nucleotides long non-coding RNAs, in cancer cells that lead to reduction of tumor growth.[26-28] Also, miRNAs play an important role in the modulation of chemosensitivity of tumor cells.[29-31] Deregulation of *miR-181a* has been demonstrated in a variety of cancer.[32] Several studies have reported that *miR-181a* might function differently in cell proliferation in different cells. For example, overexpression of *miR-181a* in colon cancer cells can promote cell proliferation through the enhanced glycolysis .[33] Bhattacharya *et al* reported that *miR-181a* regulates OPN-dependent metastatic function in hepatocellular cancer cell lines.[34] Another study showed that *miR-181a* functioned as a tumor suppressor by inducing apoptosis, triggering growth inhibition and inhibiting invasion in glioma cells.[35] The aim of this study was to assess potential of silibinin to inhibit of expression of the *miR-181a gene*. In this study, cytotoxic effects of the silibinin (50 – 350 μM) on SK-BR-3 breast cancer cells was investigated by MTT assay after 24, 48 and 72 h treatment. With different concentrations the level of *miR-181a gene* expression was measured by reverse transcription real-time PCR.

In this study, we observed that silibinin has dose-dependent inhibitory effect on the viability of SK-BR-3 cell line and reduces *miR-181a* promoter activity, resulting in decreased total *miR-181a* RNA.These results convincingly show that the major biological effect of silibinin in SK-BR-3 cells is growth inhibition, and that cell death is the prime reason for the reduction in cell number. Silibinin inhibits the expression of *miR-181a* and it could probably be used as drug candidates for breast cancer therapy through *miR-181a* targeting in the future.

CONCLUSION

From the results obtained by this study, it may be concluded that silibinin significantly reduces the growth of Sk-Br-3 cells. The therapeutic effect of silibinin on the treatment of breast cancer may be mediated by inhibition of miR-181a gene expression. For verifying this hypothesis and the possible therapeutic implication of silibinin on breast cancer, further studies in this direction are necessary.

ACKNOWLEDGEMENT

All authors read and approved the final manuscript. We would like thank Rozhan Basati and Zahra Maharat for their suggestions on the study.

CONFLICT OF INTEREST

The authors have no conflict of interest in this article.

ABBREVIATION USED

OPN: Osteopontin; **ΔCt:** Normalized threshold cycle.

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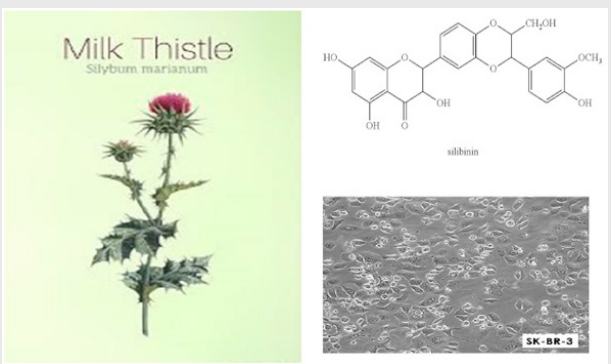
PICTORIAL ABSTRACT

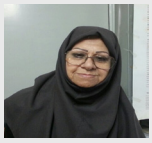


SUMMARY

- The effect of silibinin was investigated in Sk-Br-3 breast cancer cells.
- *Mir-181a* gene expression was analysed after treatment with IC_{50} concentration of silibinin.
- Silibinin inhibited cell proliferation and promoted apoptosis in human SK-BR-3 cells. In addition, silibinin caused a decrease in *mir-181a* level.

About Authors

Parvaneh Shahinfar: She obtained her M.Sc degree in 2016 from Kharazmi University (Tehran, Iran). Her research interests include design, synthesis, and investigation of molecules that have the potential to be useful chemosensors and drug delivery agents.



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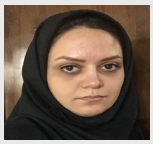

Elnaz Birjandian: Elnaz Birjandian is pursuing her Ph.D at Tehran University. Her research interests include screening different plant extracts and their isolates for their potential pharmacological activity.

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Cite this article: Shahinfar P, Motamed N, Birjandian E and Shabanpour O. Silibinin: an Inhibitor of Mir-181a Gene Expression in Sk-Br-3 Breast Cancer Cell Line. Indian J of Pharmaceutical Education and Research. 2017;51(4):597-602.