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Inhibition of mir-15a Gene Expression by Silibinin in MCF-7 Breast Cancer Cell Line

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KEYWORDS

Silibinin, Breast cancer, MCF-7 cell line, mir-15a, Caspase-3

A B S T R A C T

Recent studies have indicated that microRNA-15a (miR-15a) is dysregulated in breast cancer. The main objective of this work is to assess the inhibitory effects of silibinin, a herbal substance, on proliferation, apoptosis and mir-15a gene expression in human breast cancer cell line MCF-7. Human breast cancer cell line MCF-7 was treated with various concentration of silibinin. Cellular viability was assessed by MTT assay and the *activation* of *caspase-3* was measured using Caspase-Glo® 3/7 assay kit and the level of mir-15a gene-expression was measured by reverse-transcription real-time PCR. Silibinin inhibits growth of MCF-7 cells in a dose- and time-dependent manner and effectively induces apoptosis of MCF-7 cells with a significant activation of caspase 3. In addition, silibinin caused an decrease in mir-15a level. These findings suggest that cell viability along with mir-15a gene expression in breast cancer cells could be reduced by silibinin in MCF-7 cell line. These results suggest that silibinin inhibits the proliferation of MCF-7 cells, and it induces apoptosis by caspase-3 activation in breast cancer cells.

Introduction

Phytochemicals in plant material are gaining increasing attention as 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 (Zhao *et al.*, 2014).

Silibinin, a major bioactive component of silymarin, is isolated from the plant milk thistle (Silybum marianum). It has been reported that silibinin has anticancer activities in various cancers including breast cancer in both in vitro and in vivo models (Ramasamy and Agarwal, 2008; Jeong *et al.*, 2011; Nejati-Koshki *et al.*, 2013).

Breast cancer, which is the most frequent neoplasm in women worldwide, is a common highly heterogeneous malignancy, and is one of the main gynecological cancers in the world (Adams *et al.*, 2008; Li *et al.*, 2014b). The evidence indicates that breast cancer is not a single disease, but is instead a collection of diseases with diverse clinically relevant biological and phenotypical features (Vargo-Gogola and Rosen, 2007). 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 (Riaz *et al.*, 2013).

MicroRNAs (*miRNAs*) are a highly abundant *class* of endogenous small non-coding RNAs (18–25 nucleotides in length) that regulate gene expression at post-transcriptional level through inhibiting protein translation or degrading mRNA of target gene (Khoshnaw *et al.*, 2009). About half of the miRNA upstream genes locate in tumor-associated region on chromosome, and miRNA are abnormally expressed in a variety of tumors, suggesting that miRNAs may function as a tumor suppressor gene or oncogene (Li *et al.*, 2014b; Croce, 2009). A large number of studies have shown that miRNAs play a major role in a wide range of developmental process including cell proliferation, cell cycle, cell differentiation, metabolism, apoptosis, developmental timing, neuronal cell fate, neuronal gene expression, brain morphogenesis, muscle differentiation and stem cell division (Riaz *et al.*, 2013; Ha, 2011; Ardekani and Naeini, 2010; Qian *et al.*, 2011). Aberrant expression levels of miRNAs have been observed in many solid cancers including breast cancer (Allegra *et al.*, 2012).

Recent studies have demonstrated that overexpressed miR-15a in breast cancer cells induced G1 arrest, suppressed cell proliferation, and induced apoptosis (Li *et al.*, 2014a). The major goal of our study was to assess the antiproliferative and apoptotic effecte of silibinin on human breast cancer cell line MCF-7 and its effect on *expression* level of the mir-15a gene.

Materials and Methods

Reagents

Silibinin *(*Catalogue No. *S0417)*, 3-(4,5-Dimetrylthiazol-2-yl)-2,5-diphenyl tetra zolium bromide (MTT) were purchased from *Sigma*. Fetal bovine serum (FBS), RPMI 1640 and Penicillin/streptomycin were provided from GIBCO. DMSO was acquired from Merck. 2-steps RT-PCR kit was obtained from invitrogen. RNA Isolation Kit was purchased from qiagen and SYBR® Premix Ex TaqTMII was provided from sinagen and Caspase-Glo® 3/7 Assay kit was purchased from Promega.

Cell culture and silibinin treatment

MCF-7 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 stock solution of silibinin were added into culture medium to achieve the indicated concentrations (0–300 μg/ml) and then incubated with cells for 24,

48 and 72h whereas DMSO solution without silibinin was used as blank reagent.

Measurement of cell viability

After treatment with silibinin, cell viability was evaluated using the 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. Cell samples were incubated with 100 µl MTT for 3h at 37°C, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 100µl of DMSO. A 0.1 ml aliquot of each sample was then translated to 96-well plates and the absorbance of each well was measured at 570 nm with ELISA Reader.

Growth inhibition was measured by dividing the mean absorbance of treated wells per mean absorbance of control wells (drug-free wells), and is expressed as a percentage. The inhibitory concentrations of 50% of cells (IC50) values were defined as the drug concentrations, at which cell growth was inhibited by 50% compared with drug-free controls.

Media pH assessment

To evaluate the effect of silibinin on acidity of media containing MCF-7 cells, the pH of control and silibinin-treated media were measured using a laboratory pH meter (Metrohm, USA) before and after incubation time (48 h).

Caspase–3 activity assay

MCF-7 cells were treated in duplicat with silibinin (75, 100, 150 and 200 µg/ml) for 48h and cells were then collected and cell lysates prepared in CCLR and the protein concentrations were determined using the Bradford method. Subsequently, the caspase-3 activity assay was conducted with Caspase-Glo® 3/7 assay kit, according to the manufacturer's instructions (Promega, Madison, WI, USA). A 5-10 µg of protein in a 50 µl total volume was mixed with 100 µl of equilibrated Caspase-Glo® 3/7 reagent and incubated for 1h at the room temperature. Afterwards, the luminescence was measured using a TD-20/20 Luminometer.

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. 1 mL of Trizol solution was added to the cell pellet and vortexed. The reaction mix was incubated at room temperature for 5 minutes and followed by 200µL of chloroform was added and mixed well. Then the mix was incubated at room temperature for 5 minutes and followed by centrifugation at 12,000 g for 15 minutes at 4°C. The aqueous phase was collected into a separate 1.5mL micro centrifuge tube and added 500µL of isopropyl alcohol, mixed well.

The reaction mix was incubated at room temperature for 10 minutes followed by centrifugation at 12,000 rpm for 10 minutes at 4°C. RNA was the pelleted with 75% absolute alcohol and pellet and stored in RNase free water at 80°C for further use. Through absorbance measurement, RNA concentrations were determined by UV-vis Spectrophotometer (Eppendorf BioPhotometer) and at 260-280 nm purity of RNAs were estimated. 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 later use.

Real-time PCR amplification

The expression of mir-15a 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 conditions 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 minutes followed by 50 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The experiment was performed by three independent experiments with triplicate

For each sample the ΔCt values were determined by subtracting the average of duplicate Ct values of the 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 of duplicate Ct values of the target gene from the average of duplicate Ct values of the reference gene. Results were expressed as 'N-target' and determined as follows:

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

Statistical analysis

The data were analyzed using SPSS version 16.0 software. Statistical significance of difference in growth inhibition and expression levels of mir-15a and rRNA-5s, between control and treated groups was assessed using Student's t-test. A statistically significant difference was considered to be present at $P < 0.05$.

Results and Discussion

Silibinin induced loss of MCF-7 cell viability

MCF-7 breast cancer cells were exposed to silibinin at the concentrations of 0-300 µg/ml for 24, 48 and 72h 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 and 300 µg/ml, cytotoxicity was observed in dose-dependent fashion. In MTT assay maximum effect was observed at 100 µg/ml dose of silibinin for 48h.

Data analysis of cytotoxicity assay showed that cytotoxic effect of silibinin on MCF-7 breast cancer cell line was 182.7, 118, and 82.26 µg/ml upon 24, 48, and 72 hours of exposure, respectively($P<0.05$); indicating a dose- and time-dependent response relationship (Figure 1).

Silibinin-induced media pH alkalization

Acidification of extracellular pH has roles in driving pro-tease-mediated digestion and remodeling of the extracellular matrix. In this study as display in figure 2, we observed that silibinin significantly increased the pH of the media in dose-dependent manner ($p<0.05$).

Effect of silibinin on caspases activity

The activation of caspase-3 is a hallmark of apoptotic cell death in many cell types (Fischer *et al.*, 2009). In an attempt to identify the mechanism of provocation of strong apoptotic response following the

treatment of cancer cells with the silibinin treatment, we next assessed the caspase-3-like activity in MCF-7 cells *in vitro*. The treatment of MCF-7 cells with silibinin (75, 100, 150 and 200 µg/mL) for 48h showed induced caspase-3-like activity (Figure 3).

Silibinin inhibited the mir-15a expression in MCF-7 cells

The expression of mir-15a 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 incorporation of SYBR-Green fluorescent dye into the double stranded PCR products. The transcript level of mir-15a 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). Finally, the ΔΔCt values were transformed for absolute values using the formula $2^{-\Delta\Delta Ct}$.

As shown in figure 4, silibinin treatment caused a significant decrease in the expression of miR-15a in MCF-7 cells, as compared to untreated control cells ($p<0.05$).

Several studies have shown that silibinin, a non-toxic bioactive component in milk thistle, has promising anticancer and chemopreventive efficacy against various epithelial cancers including prostate, skin, colon, breast, lung, ovarian, etc (Mokhtari *et al.*, 2008; Mateen *et al.*, 2013; Noori-Daloii *et al.*, 2011; Ge *et al.*, 2011; Kim *et al.*, 2009; Singh and Agarwal, 2005). 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 (Zhao *et al.*, 2014; Ahmad *et al.*, 2013; Jia *et al.*, 2013). Also, miRNAs play an important role in the modulation of chemosensitivity of tumor cells (Lei *et al.*, 2014; Ning *et al.*, 2014; Papaconstantinou *et al.*, 2012). MiR-15a regulates the apoptosis and proliferation of cells by functioning in the regulation of multiple intracellular signaling pathways. Luo *et al.* (2013) revealed that overexpression of miR-15a inhibited cellular growth, suppressed migration and arrested cells at the G1 phase, but did not promote cellular apoptosis. Cai *et al.* (2012) found that miR-15a downregulate CCND1 and induce apoptosis and cell cycle arrest in osteosarcoma.

It is well known that chemotherapeutic agents induce apoptosis in most of the cancer cell types via two major ways of death receptor-mediated pathway and mitochondria-mediated pathway (Petak and Houghton, 2001; Debatin *et al.*, 2002). Both pathways converge to a final common pathway, involving the activation of a cascade of proteases called caspases, which can cleave regulatory and structural molecules, and thus induce cell death (Ghobrial *et al.*, 2005). Silibinin could induce cellular apoptosis both via the caspases activation or independent of caspases activation (Tyagi *et al.*, 2006; Agarwal *et al.*, 2003; Tyagi *et al.*, 2004).

In this study, cytotoxic effects of the silibinin (50–300 µg/ml) on MCF-7 breast cancer cells was investigated by MTT assay after 24, 48 and 72h treatment. With 100 µg/ml, the level of miR-15a gene expression was measured by reverse-transcription real-time PCR and then, we investigated the effect of silibinin on the apoptosis and activation of caspase-3 (an effector caspase). The present results have shown that the caspases-related pathway is involved in the silibinin-triggered apoptosis in MCF-7 cells.

Figure.1 Effect of silibinin on MCF-7 cell viability. MCF-7 cells were cultured as described in "Materials and Methods" and treated with either DMSO or 50-300 µg/ml of silibinin for 24, 48 and 72h, and cell number was determined at the end of the exposure period

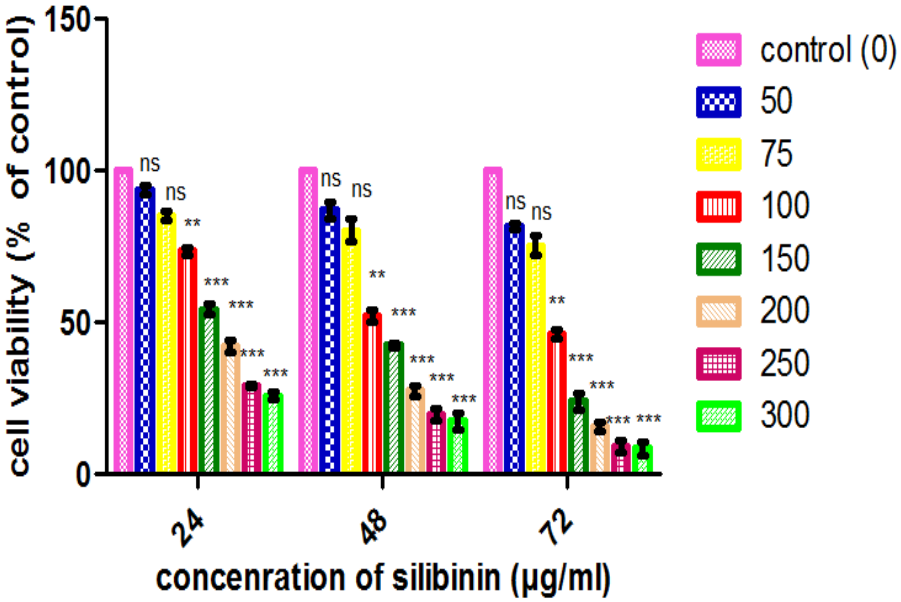


Figure.2 Effect of silibinin on media pH. The media pH was measured immediately after treatment with desired concentration of silibinin and then measured again after 48 h. As shown in figure, silibinin alkalized the extracellular media of MCF-7 cells in the dose dependent manner

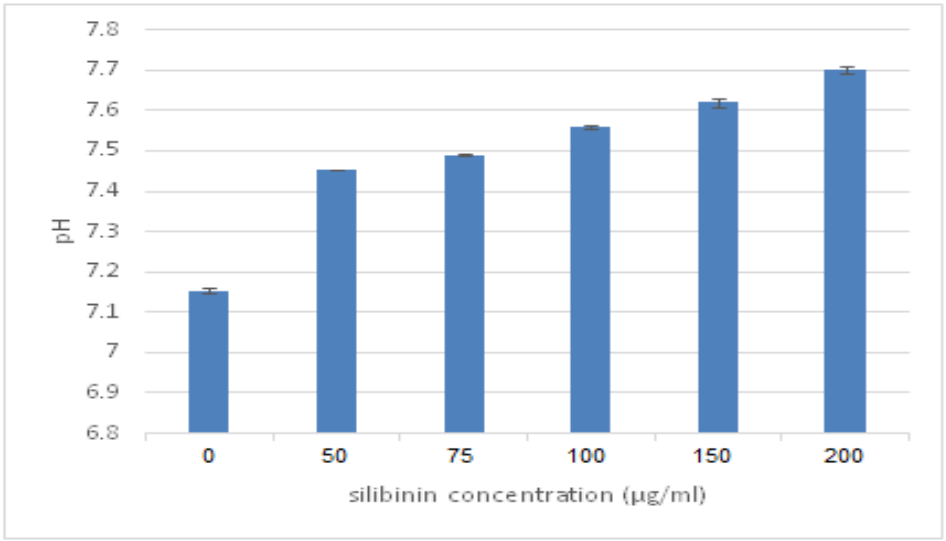


Figure.3 Luminescence is proportional to caspase-3 activity. MCF-7 cells were treated with 75, 100, 150 and 200 µg/ml silibinin for 48 hours to induce apoptosis; an identical population of cells was left untreated. The Caspase-Glo® 3/7 Reagent was added directly to cells in 96-well plates; the final volume was 200µl per well. The assays were incubated at room temperature for various times before recording luminescence with a TD-20/20 Luminometer

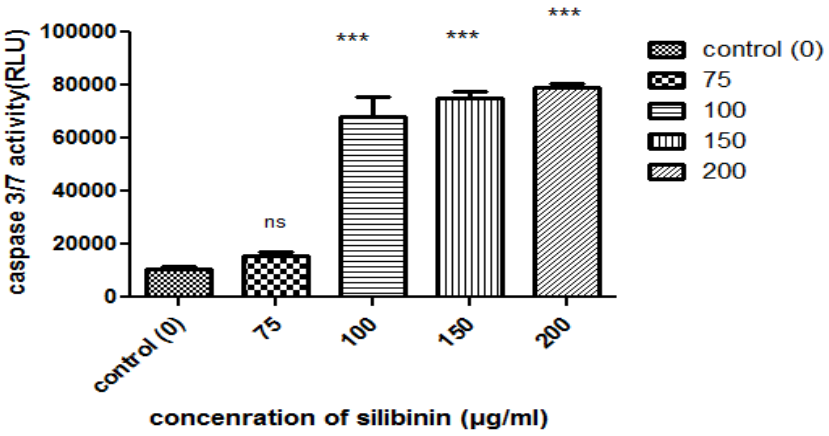
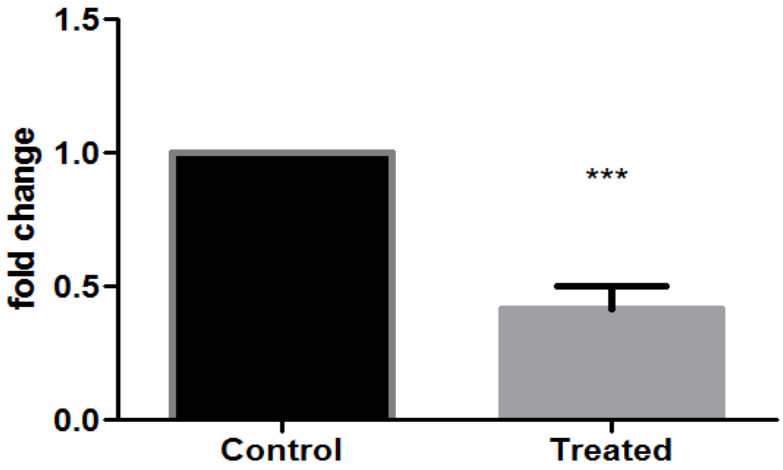


Figure.4 Effects of silibinin on the inhibition of mir-15a expression in MCF-7 breast cancer cells



Unexpectedly, it was found in our study that treatment with silibinin decreased miR-15a gene expression in MCF-7 cell line. This study demonstrates that silibinin has dose-dependent inhibitory effect on the viability of MCF-7 cell line and reduces miR-15a promoter activity, resulting in decreased total miR-15a RNA. These results convincingly show that the major biological effect of silibinin in MCF-7 cells is growth inhibition, and that cell death is the prime

reason for the reduction in cell number activation of caspase-3.

Conclusion

In summary, the findings of present study have demonstrated that silibinin could successfully inhibit the cancer cell proliferation, induce apoptosis by caspase-dependent mechanism and cause the down-regulation of mir-15a while inducing a considerable amount of apoptosis in MCF-7 breast cancer cell line. Further studies are needed to unveil the underlying mechanisms and for the further confirmation of these results.

References

Adams, B.D., Guttilla, I.K., White, B.A. 2008. Involvement of microRNAs in breast cancer. *Semin. Reprod. Med.,* 26(6): 522–36.

Agarwal, C., *et al.* 2003. Silibinin upregulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells. *Oncogene*, 22(51): 8271–82.

Ahmad, J., *et al.* 2013. MicroRNA in carcinogenesis & cancer diagnostics: a new paradigm. *Indian J. Med. Res.*, 137(4): 680–94.

Allegra, A., *et al.* 2012. Circulating microRNAs: new biomarkers in diagnosis, prognosis and treatment of cancer (review). *Int. J. Oncol.,* 41(6): 1897–912.

Ardekani, A.M., Naeini, M.M. 2010. The role of microRNAs in human diseases. *Avicenna J. Med. Biotechnol.,* 2(4): 161–79.

Cai, C.K., *et al.* 2012. miR-15a and miR-16-1 downregulate CCND1 and induce apoptosis and cell cycle arrest in osteosarcoma. *Oncol. Rep.,* 28(5): 1764–70.

Croce, C.M. 2009. Causes and consequences of microRNA dysregulation in cancer. *Nat. Rev. Genet.,* 10(10): 704–14.

Debatin, K.M., Poncet, D., Kroemer, G. 2002. Chemotherapy: targeting the mitochondrial cell death pathway. *Oncogene,* 21(57): 8786–803.

Fischer, U., Janicke, R.U., Schulze-Osthoff, K. 2003. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ.,* 10(1): 76–100.

Ge, Y., *et al.* 2011. Silibinin causes apoptosis and cell cycle arrest in some human pancreatic cancer cells. *Int. J. Mol. Sci.,* 12(8): 4861–71.

Ghobrial, I.M., Witzig, T.E., Adjei, A.A. 2005. Targeting apoptosis pathways in cancer therapy. *CA Cancer J. Clin.,* 55(3): 178–94.

Ha, T.Y. 2011. MicroRNAs in human diseases: from cancer to cardiovascular disease. *Immune Netw.,* 11(3): 135–54.

Jeong, J.C., *et al.* 2011. Silibinin induces apoptosis via calpain-dependent AIF nuclear translocation in U87MG human glioma cell death. *J. Exp. Clin. Cancer Res.*, 30: 44.

Jia, W., Chen, W., Kang, J. 2013. The functions of microRNAs and long non-coding RNAs in embryonic and induced pluripotent stem cells. *Genomics Proteomics Bioinformatics,* 11(5): 275–83.

Khoshnaw, S.M., *et al.,* MicroRNA involvement in the pathogenesis and management of breast cancer. *J. Clin. Pathol.,* 62(5): 422–8.

Kim, S., *et al.* 2009. Silibinin suppresses TNF-alpha-induced MMP-9 expression in gastric cancer cells through inhibition of the MAPK pathway. *Molecules,* 14(11): 4300–11.

Lei, Y., *et al.* 2014. miR-150 modulates cisplatin chemosensitivity and invasiveness of muscle-invasive bladder cancer cells via targeting PDCD4 *in vitro*. *Med. Sci. Monit.,* 20: 1850–7.

Li, P., *et al.* 2014a. MiRNA-15a mediates cell cycle arrest and potentiates apoptosis in breast cancer cells by targeting synuclein-gamma. *Asian Pac. J. Cancer Prev.*, 15(16): 6949–54.

Li, X.Y., *et al.* 2014b. MiRNA-107 inhibits proliferation and migration by targeting CDK8 in breast cancer. *Int. J. Clin. Exp. Med.*, 7(1): 32–40.

Luo, Q., *et al.* 2013. MiR-15a is underexpressed and inhibits the cell cycle by targeting CCNE1 in breast cancer. *Int. J. Oncol.*, 43(4): 1212–8.

Mateen, S., Raina, K., Agarwal, R. 2011. Chemopreventive and anti-cancer efficacy of silibinin against growth and progression of lung cancer. *Nutr. Cancer*, 65(Suppl. 1): 3–11.

Mokhtari, M.J., Motamed, N., Shokrgozar, M.A. 2008. Evaluation of silibinin on the viability, migration and adhesion of the human prostate adenocarcinoma (PC-3) cell line. *Cell Biol. Int.*, 32(8): 888–92.

Nejati-Koshki, K., *et al.* 2013. Inhibition of leptin and leptin receptor gene expression by silibinin-curcumin combination. *Asian Pac. J. Cancer Prev.*, 14(11): 6595–9.

Ning, F.L., *et al.* 2014. MicroRNA-182 modulates chemosensitivity of human non-small cell lung cancer to cisplatin by targeting PDCD4. *Diagn. Pathol.*, 9: 143.

Noori-Daloii, M.R., *et al.* 2011. Multifaceted preventive effects of single agent quercetin on a human prostate adenocarcinoma cell line (PC-3): implications for nutritional transcriptomics and multi-target therapy. *Med. Oncol.*, 28(4): 1395–404.

Papaconstantinou, I.G., *et al.* 2012. A review on the role of microRNA in biology, diagnosis, and treatment of pancreatic adenocarcinoma. *Pancreas*, 41(5): 671–7.

Petak, I., Houghton, J.A. 2001. Shared pathways: death receptors and cytotoxic drugs in cancer therapy. *Pathol. Oncol. Res.*, 7(2): 95–106.

Qian, L., *et al.* 2011. miR-24 inhibits apoptosis and represses Bim in mouse cardiomyocytes. *J. Exp. Med.*, 208(3): 549–60.

Ramasamy, K., Agarwal, R. 2008. Multitargeted therapy of cancer by silymarin. *Cancer Lett.*, 269(2): 352–62.

Riaz, M., *et al.* 2013. miRNA expression profiling of 51 human breast cancer cell lines reveals subtype and driver mutation-specific miRNAs. *Breast Cancer Res.*, 15(2): R33.

Singh, R.P., Agarwal, R. 2005. Mechanisms and preclinical efficacy of silibinin in preventing skin cancer. *Eur. J. Cancer*, 41(13): 1969–79.

Tyagi, A., *et al.* 2004. Silibinin causes cell cycle arrest and apoptosis in human bladder transitional cell carcinoma cells by regulating CDKI-CDK-cyclin cascade, and caspase 3 and PARP cleavages. *Carcinogenesis*, 25(9): 1711–20.

Tyagi, A., *et al.* 2006. Silibinin activates p53-caspase 2 pathway and causes caspase-mediated cleavage of Cip1/p21 in apoptosis induction in bladder transitional-cell papilloma RT4 cells: evidence for a regulatory loop between p53 and caspase 2. *Carcinogenesis*, 27(11): 2269–80.

Vargo-Gogola, T., Rosen, J.M. 2007. Modelling breast cancer: one size does not fit all. *Nat. Rev. Cancer*, 7(9): 659–72.

Yuan, J.S., *et al.* 2006. Statistical analysis of real-time PCR data. *BMC Bioinformatics*, 7: 85.

Zhao, S.F., *et al.* 2014. Induction of microRNA-9 mediates cytotoxicity of curcumin against SKOV3 ovarian cancer cells. *Asian Pac. J. Cancer Prev.*, 15(8): 3363–8.