

Anti-Cancer Activity of Silymarin on MCF-7 and NCIH-23 Cell Lines

*Pavan Kumar Kalla, Sashikanth Chitti, Seyedeh Tahereh Aghamirzaei,
Radhakrishnan Senthilkumar and Selvam Arjunan*

Indian academy Degree College, Hennur cross, Bangalore 560043, India

Abstract: Silymarin, an active extract milk thistle (*Silybum marianum*) plant, is used for the protection against various liver conditions in both clinical settings and experimental models. Prevailing evidence suggest that the silymarin can prevent the proliferation of cancer cells in both in vivo and in vitro models. It has also been found that silymarin alter the inequality between cell survival and apoptosis by means of interfering with the expressions of cell cycle regulators and proteins involved in apoptosis. Several studies have demonstrated silymarin's anticancer effects by causing cell cycle arrest and inducing apoptosis in different type of cancers. However, there is no report on the comparison of different apoptotic gene expression by the lung and breast cancer cell lines treated with silymarin. The objective of the current study is to find the sensitivity of lung and breast cancer cell lines against silymarin as observed by the apoptotic gene expression and the associated inhibitory activity of silymarin on the proliferation of both the cell lines.

Key words: Silymarin • Mcf-7 • Ncih-23 • Cancer Cells

INTRODUCTION

Over the past decades, numerous medicinal herbs from plants have been considered for their extensive continuum of pharmacological effects. As a result, medicinal plants have been evaluated for their cancer chemopreventive activity as well. It has been observed that a specific concentration of photochemical from the plants possibly produce cancer chemopreventive effects with no significant toxicity. Natural products including fruits and vegetables from plants are assumed to suppress the inflammatory process, which result to neoplastic transformation, hyper proliferation, promotion and progression of carcinogenic process and angiogenesis. It has been found that almost one-third of all cancer deaths in the United States may be prevented by means of an suitable diet intake. Accumulating research evidence suggests that many dietary agents/medicinal [1, 2].

For more than 2000 years, silymarin has been used as a natural medicine for treating hepatitis and cirrhosis and to protect liver from toxic substances. Different actions of silymarin in experimental liver diseases include antioxidative, antilipidperoxidative, antifibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory

and liver regenerating mechanisms [3-5]. Besides, silymarin has been extensively studied, both in vivo and in vitro, for its cancer chemopreventive potential against various cancers [6].

Several studies have demonstrated silymarin's anticancer effects by causing cell cycle arrest and inducing apoptosis in different type of cancers. A study by Li *et al.* [7] have shown that silymarin induces apoptotic cell death in CH11-treated human malignant melanoma A375-S2 cells through an increased expression of Fas-associated proteins with death domain (FADD), which is a downstream molecule of the death receptor pathway, subsequent to the cleavage of procaspase-8 that induces apoptosis. Silibinin also promotes apoptosis of human hematoma HuH7 cells by down-regulating survivin and up-regulating activated caspase-3 and caspase-9 [8]. However, there is no report on the comparison of different apoptotic gene expression by the lung and breast cancer cell lines treated with silymarin. The objective of the current study is to find the sensitivity of lung and breast cancer cell lines against silymarin as observed by the apoptotic gene expression and the associated inhibitory activity of silymarin on the proliferation of both the cell lines.

MATERIALS AND METHODS

Materials: MEM (Minimum Essential Medium), Trypsin EDTA, Phosphate buffer saline (PBS), TRI solutions were purchased from GeNei, Chloroform and Isopropanol were purchased from Qualigens Fine Chemicals Pvt. Ltd. MCF-7 (Breast cancer) and NCI-H23 (Lung cancer) cell lines were obtained from NCCS, Pune.

Methods

Cell Lines: MCF-7 and NCI-H23 cells were cultured and were treated with Silymarin. RNA extraction was done by Trizol reagent. The RNA samples were subjected to cDNA synthesis and real time PCR for apoptotic mRNA expression. Six common apoptotic gene expressions were analyzed in the Silymarin induced MCF-7 and NCI-H23 cells by Real Time PCR.

RNA Extraction by Trizol Reagent: MCF-7 and NCI-H23 cancer cell lines we have procured from NCCS (National Center for Cell Science), Pune, India. Cells were cultured and induced with Silymarin and incubated for 24hrs. After incubation cells were trypsinised and centrifuged for 5min at 3000rpm to pellet the cells. Wash the pellet with 2-3 times with sterile PBS for RNA isolation.

In detail, 300 μ L of Trizol solution was added to the cell pellet and vortexed. The reaction mix was incubated at RT for 5 minutes and followed by 80 μ L of chloroform was added and mixed well. Then the mix was incubated at RT for 5 minutes and followed by centrifugation at 11,000 g for 15 minutes at 4°C. The aqueous phase was collected into a separate 1.5mL micro centrifuge tube and added 150 μ L of isopropyl alcohol, mixed well. The reaction mix was incubated at RT for 10 minutes followed by centrifugation at 13,000 rpm for 10 minutes at 4°C. RNA was the pelleted with 75% absolute alcohol and centrifuged at 9,000g for 10 minutes at 4°C. Air dries the pellet and resuspended in 10 μ L of RNase free water for further use.

Cell Inhibition Activity by MTT Assay: Cells were cultures in a 96 well plate for overnight and add 1mg of plant crude extract dissolve in absolute alcohol and consider it as a stock solution. From this stock solution prepared different dilutions like 12.5 μ g, 25 μ g, 50 μ g, 100 μ g and 200 μ g respectively, added to each well and left one well for non additive, considered it as a control. Cells were cultured for 1-5 days to allow the drug to take effect, at the end of the incubation add 20 μ L of freshly prepared MTT solution (5mg/ml in PBS) and incubated for 1-5 hr to

allow the MTT to be metabolized and followed by remove the media and resuspended formosan MTT metabolic product in 200 μ L DMSO and place a shocker at 150 rpm for 5min. Read optical density of 492nm and substrate background at 620nm.

REAL-TIME PCR Analysis: The expression of apoptotic genes was analyzed by reverse transcription-PCR (RT-PCR; CFX96, Bio-Rad) using a one step real-time SYBR Green mix (Helini Biomolecules). The RNA was prepared Silymarin induced MCF-7 and NCI-H23 cultured cells using TRIZOL reagent and the mRNA levels of six apoptotic genes were tested using reference gene GAPDH to normalize the gene expression. Quantitative real-time RT-PCR was performed in a reaction volume of 25 μ L according to the manufacturer's instructions. Briefly, 13 μ L of master mix, 0.2 μ L of primer (0.2nM) and 5 μ L of template RNA (100 μ g) were added to 0.2mL PCR tube. 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 5 s; 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 and NCI-H23 cells treated with Silymarin for 24hrs.

RESULTS

The MCF-7 and NCI-H23 cells were cultured in MEM complete medium. The medium was supplemented with 10% heat inactivated fetal bovine serum, antibiotics. The cells were maintained at 37° C in a 5% CO₂ incubator and the media were changed frequently. The cell morphology has been analyzed after treatment with Silymarin for 24hrs. As the result, figure 1 showed that, there is an cell death as well inhibition of cell proliferation after drug treatment (Fig 1 B and Fig 1D).

In order to check the inhibition activity of Silymarin on NCIH-23 and MCF-7 cells were plated and treated with different concentration of the drugs like, 12.5 μ g, 25 μ g, 50 μ g, 100 μ g and 200 μ g respectively. At the end of silymarin treatment after 5days, MTT assay was performed. As the result shown in the figure 2, inhibition activity was measured based on the cell number. 58.54 % inhibition activity shown in NCIH-23 cell line in comparison to MCF-7 cell line 48.14% was shown.

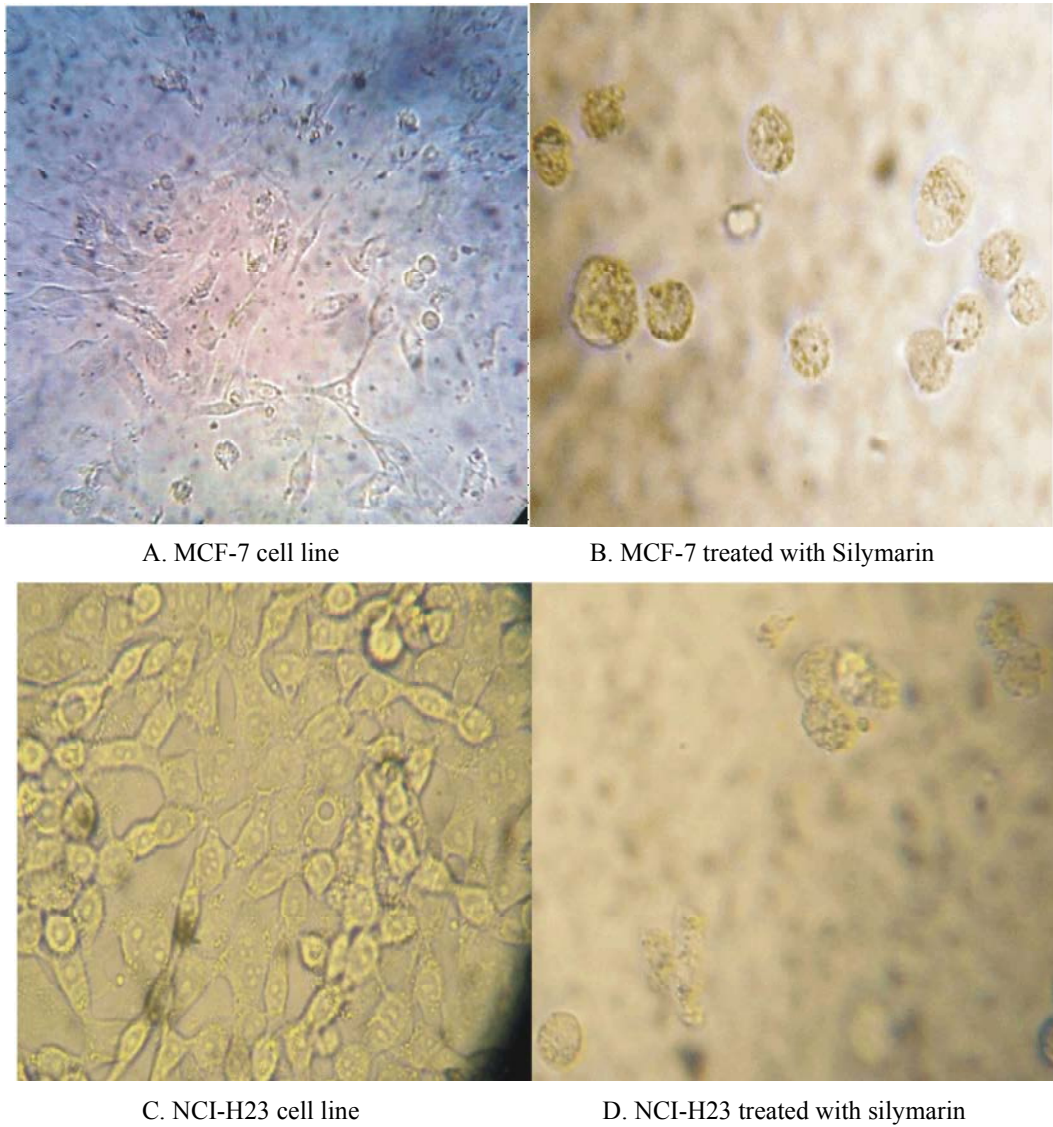


Fig. 1: A and C: MCF-7 cell lines and NCI-H23 cell lines before silymarin treatment; B and D: MCF-7 cell lines and NCI-H23 cell lines after silymarin treatment for 24hrs.

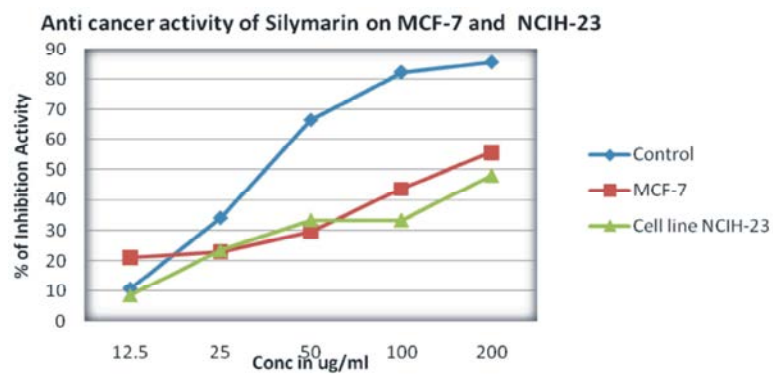


Fig. 2: Anti cancer activity of silymarin on MCF-7 and NCIH-23. Graph showing the percentage proliferative inhibition values of cell lines. All the values are average of triplicates.

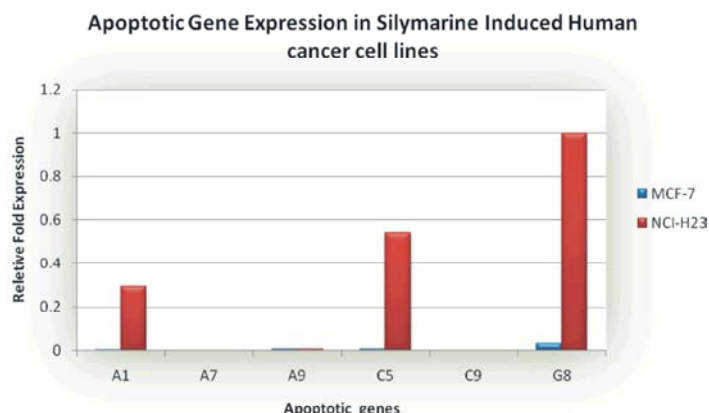


Fig. 3: Graph showing the Quantification of mRNA levels of apoptosis responsive genes

To investigate the molecular mechanism of Silymarin induced apoptosis in MCF-7 and NCI-H23 cells, the expression levels of six apoptosis-related genes were examined. Bcl-2, Bax and p53 are three major proteins generally involved in apoptosis. The relative quantification of apoptotic genes, caspase-3 (0.5407 ± 0.001) APAF1 (0.29284 ± 0.02) and TP53 (1.0000 ± 0.020) were expressed in NCI-H23 (Lung cancer) among six apoptotic genes. mRNA levels were performed using one step RT-PCR SYBR Green mix quantitative real-time reverse transcription PCR using a CF96 Real-Time System. Figures 3 and 4 summarize the gene expression changes of APAF1, caspase-3 and TP53. Silymarin treatment increased the number of transcripts of caspase-3, APAF1 and TP53 by several fold. The expression levels of these genes in MCF-7 cells treated with $49 \mu\text{g/ml}$ ZR extract after for 24hrs.

DISCUSSION

The current study, which was aimed to evaluate silymarin chemotherapeutic effect in human breast cancer MCF7 and lung cancer NCI-H23 cell lines, showed that NCI-H23 cells were found to be more sensitive to silibinin than MCF7. This was observed owing to the change in apoptotic gene expression of apoptotic protease activating factor, caspase 3 and tumor protein 53 which was increasingly expressed by the NCI-H23 cell lines than the MCF7 cell lines. These results may have clinical significance in understanding silibinin treatment to breast and lung cancer. The study also demonstrated that the silymarin had greater inhibitory effects on the proliferation of lung cancer cells than the breast cancer cell line by inducing apoptosis as revealed by the expression of the apoptotic genes [9, 10].

W.D. Liu *et al.*, 2011, [11] studied the inhibitory effects of silymarin to a highly metastatic lung cancer cell line Anip973 and found that silymarin had significant inhibitory effects on the proliferation of Anip973 cells in a temporally and dose-dependent manner. It has also been found that the silymarin can also induce apoptosis [11]. In a study by P. Tiwari *et al.*, [12] the chemotherapeutic effect of silymarin in breast cancer cell MCF7 and T47D was assessed, the T47D cells were found to be more sensitive to silibinin than MCF7 as observed by the inhibitory effect of silymarin and the apoptotic assays [13]. This is consistent with present study results which also showed a less inhibitory effect of the silymarin against the MCF7 cell lines. These results potentially have significance in understanding the molecular mechanism by which the silibinin can induce apoptosis in different cancer cell lines [14, 15].

CONCLUSIONS

It has been concluded that silymarin, which exerted a strong anti-carcinogenic effect against NCI-H23 and MCF-7 cells by inducing the apoptotic gene expression, might be developed as a therapeutic strategy to increase the antitumor. However, further studies are required to evaluate the effects of silymarin on other apoptotic genes and additional thorough investigations relating to its application as a supplementary anticancer agent is essential.

ACKNOWLEDGEMENT

I would like thank to laboratory of the translational Research Institute of Molecular Sciences (TRIMS) for their support and resources to run this research.

REFERENCES

1. Prasain, J.K. and S. Barnes, 2007. Metabolism and Bioavailability of Flavonoids in Chemoprevention: Current Analytical Strategies and Future Prospectus. *Mol. Pharm.*, 4: 846-864.
2. Nishino, H., Y. Satomi, H. Tokuda and M. Masuda, 2007. Cancer control by phytochemicals. *Curr. Pharm. Des.*, 13: 3394-3399.
3. Deep, G.R.P., C. Singh, Agarwal, D.J. Kroll and R. Agarwal, 2006. Silymarin and silibinin cause G1 and G2-M cell cycle arrest via distinct circuitries in human prostate cancer PC3 cells: a comparison of flavanone silibinin with flavanolignan mixture Silymarin, Department of Pharmaceutical Sciences, 25: 1053-1069.
4. Deep, G. Oberlies, N.H. Kroll and R. Agarwal, 2008. Identifying the differential effects of silymarin constituents on cell growth and cell cycle regulatory molecules in human prostate cancer cells. *International Journal of Cancer*, 123: 41-50.
5. Deep, G., R.P. Singh, C. Agarwal, D.J. Kroll and R. Agarwal, 2006. Silymarin and silibinin cause G1 and G2-M cell cycle arrest via distinct circuitries in human prostate cancer PC3 cells: a comparison of flavanone silibinin with flavanolignan mixture silymarin. *Oncogene*, 25: 1053-1069.
6. Post-White, J., E.J. Ladas and K.M. Kelly, 2007. Advances in the use of milk thistle (*Silybum marianum*). *Integr. Cancer Ther.*, 6: 104-109.
7. Li, L.H., L.J. Wu, Y.Y. Jiang, S. Tashiro, S. Onodera, F. Uchiumi and T. Ikejima, 2007. Silymarin enhanced cytotoxic effect of anti-Fas agonistic antibody CH11 on A375-S2 cells. *J. Asian Nat. Prod. Res.*, 9: 593-602.
8. Lah, J.J., W. Cui and K.O. Hu, 2007. Effects and mechanisms of silibinin on human hepatoma cell lines. *World J. Gastroenterol.*, 13: 5299-5305.
9. Fesik, S.W., 2005. Promoting apoptosis as a strategy for cancer drug discovery. *Nat. Rev. Cancer*, 5: 876-885.
10. Agarwal, C., R.P. Singh and S. Dhanalakshmi, 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: 8271-8282.
11. Li, W.D., Mu, L. Song, J. Zhang, J. Liang, C. Wang, N. Liu, F. Tian, X. Li, W. Zhang and X. Wang, 2011. Molecular mechanism of silymarin-induced apoptosis in a highly metastatic lung cancer cell line anip973. *Cancer Biother Radiopharm*, 26(3): 317-24.
12. Tiwari, P., A. Kumar, S. Balakrishnan, H.S. Kushwaha and K.P. Mishra, 2011. Silibinin-induced apoptosis in MCF7 and T47D human breast carcinoma cells involves caspase-8 activation and mitochondrial pathway. *Cancer Invest.*, 29(1): 12-20.
13. Agarwal, C., R.P. Singh and S. Dhanalakshmi, 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: 8271-8282.
14. Dhanalakshmi, S., P. Agarwal, L.M. Glode and R. Agarwal, 2003. Silibinin sensitizes human prostate carcinoma DU145 cells to cisplatin-and carboplatin-induced growth inhibition and apoptotic death. *Int. J. Cancer*, 106: 699-705.
15. Deep, G. and R. Agarwal, 2010. Antimetastatic efficacy of silibinin: Molecular mechanisms and therapeutic potential against cancer, *Cancer and Metastasis Reviews.*, 29(3): 447-463.