The Role of Milk Thistle Extract in Breast Carcinoma Cell Line (MCF-7) Apoptosis with Doxorubicin

Hussein Rastegar[1], Hamidreza Ahmadi Ashtiani[2,3,4], Soghra Anjarani[5], Saeed Bokaee[6], Arash Khaki[7], and Leila Javadi[8]

[1] Food and Drug Research Center, Ministry of Health and Medical Education, Tehran, Iran

[2] Department of Biochemistry and Nutrition, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran

[3] Department of Clinical Biochemistry, School of Medicine, Tarbiat Modares University and Institue of Medicinal Plants, Tehran, Iran

[4] Department of Nutritional Hygiene, Faculty of Veterinary, Tehran University of Medical Sciences, Tehran, Iran

[5] Reference Research Center Lab, Ministry of Health and Medical Education, Tehran, Iran

[6] Faculty of Veterinary Medicine, Tehran University of Medical Sciences, Tehran, Iran

[7] Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[8] Department of Nutrition Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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Abstract- Breast cancer is the most commonly diagnosed invasive malignancy and first leading cause of cancer-related deaths in Iranian women. Based on silymarin's unique characteristics, its application in chemotherapy combined with doxorubicin can be effective to enhance the efficacy together with a reduced toxicity on normal tissues. The present study focus on evaluate the efficacy of silymarin in combination with doxorubicin, on viability and apoptosis of estrogen-dependent breast carcinoma cell line (MCF-7). After being cultured, MCF-7 cells were divided into 8 groups and treated as follows: 1st group received 75 µg silymarin, groups 2, 3, and 4 were treated with 10, 25, and 50 nM doxorubicin, respectively, and groups 5, 6, and 7 respectively received 10, 25, and 50 nM doxorubicin as well as 75 µg silymarin. Viability percentage and apoptosis of the cells were assessed with Trypan Blue staining after 16, 24, and 48 hours. Silymarin has a synergistic effect on the therapeutic potential of doxorubicin. Use of silymarin in combination with doxorubicin can be more effective on the therapeutic potential of doxorubicin and decreases its dose-limiting side effects.

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Introduction

Breast cancer is the most common type of cancer and first leading cause of cancer-related deaths in women worldwide (1). It is responsible for death of approximately 400,000 women per year all around the world (2). According to latest statistics reported by Iranian Ministry of health and medical education, breast cancer is the most prevalent cancer among Iranian women and in comparison with other types of cancer, is the first cause of death of women with age of 20-59 years old (3). Currently, chemotherapeutic drugs with systemic toxicity as doxorubicin are used for treatment of this prevalent malignancy; nevertheless, their treatment efficacy is not only limited, but also their use is accompanied by numerous side effects. Doxorubicin belongs to a large family of cytotoxic factors called anthracyclines and has a wide anti-tumoral performance; however, its use is limited due to its systemic toxicity, its inhibitory effects on the immune system, and its heart toxicity. Furthermore, the main problem related to chemotherapeutic methods currently used against breast cancer is that, concentration of drug cannot reach effective dose in cancerous tissues unless its toxic effects threaten other normal tissues (4). Hence, decreasing dose-limiting side-effects and maximizing the efficacy of the desired drug are among the most significant challenges in chemotherapy (5-7).

One of the relatively new and effective methods against cancer is use of combination chemotherapy, i.e.

Corresponding Author: Leila Javadi
Department of Nutrition Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
Tel: +98 914 3040306, Fax: +98 411 3331632, E-mail: javadi.biochem@gmail.com

using nontoxic or very low toxic phytochemical elements along with common chemotherapeutic factors, in order to reduce the toxicity of these factors on normal tissues and increasing their anti-tumoral effects through synergic process (8). Hence, different phytochemical factors have been studied due to their anti-tumoral effects as potential candidates of combination chemotherapy (4). Recent studies have indicated the positive effects of flavonoid compounds on different types of cancer. In addition to increasing apoptosis in cancerous cells (9), these compounds have synergic effect in some cases on the treatment process of common drugs against cancer (4). Many studies have referred to antioxidant effects (10,11), anti-cancer effect (12,13) and apoptosis stimulatory effect of silymarin (Milk Thistle extract) (8, 14, 15). These properties as well as its nontoxicity for normal tissues, its lack of side effects even in high doses (16-19), and its being inexpensive and available have made silymarin an appropriate candidate for combined use with doxorubicin in order to reducing side effects and increasing treatment effects of this drug. Therefore, we have studied the effect of silymarin on apoptosis caused by doxorubicin in breast carcinoma cell line (MCF-7) along with the changes in doxorubicin dose.

Materials and Methods

For this study, breast carcinoma cell line (MCF-7) obtained from cell bank of Pasteur Institute, Iran, was cultured in 50ml flasks in RPMI 1640 nutrient medium supplemented by 10% FBS (Fetal Bovine Serum), 100 units/ml penicillin, and 100 µg/ml streptomycin (*p*H = 7-7.2) at 37 ^{o}C, with 10% CO_2 and 90% air (12,20,21). Afterward, the cells were developed after two passages by trypsinisation (Sigma). Subsequent to be counted, 5×10^4 cells were transferred into each well in 6-well plates. Fresh culture medium was again provided for cells, and we let them 24h to bind to the plate (21). Then, cells were divided into 8 groups and cultured in four 6-well plates. Two wells were assigned to each group, and rows of the plate were alternately empty.

Gradually 1g of silymarin powder was dissolved in 5 ml ethanol (Sigma,code:S0292) at room temperature without light; so, a silymarin solution with concentration of 0.2 g/ml was prepare and subsequently 1.87 µl of it was added to cellular plates such that ethanol's concentration in culture medium would not exceed 0.1% (ethanol of medium equaled 0.03%) (22,23). Doxorubicin (Sigma) was directly dissolved in some amount of culture medium and prepared as 10X stock. Through the addition of proper amounts of this stock, the desired concentrations of doxorubicin in medium were prepared and transferred to cell-containing wells after evacuation of the previous medium. Finally, the groups were treated as follows: group 1 received 75 µg silymarin per 1 ml of culture medium; groups 2, 3, and 4 were treated with concentrations of 10, 25, and 50 nM doxorubicin, respectively; groups 5, 6, and 7 received concentrations of 10, 25, and 50 nM doxorubicin, respectively, in addition to 75 µg silymarin per 1 ml of culture medium; cells in group 8 as a negative control received neither silymarin nor doxorubicin, and only ethanol was added to them equal to that of other groups. In time intervals 16h, 24h, and 48h during incubation, viability of cells from each group was studied by two methods, including light microscopy and Trypan Blue staining (24-26).

In Trypan Blue staining for determination of cell viability, the cells were initially trypsinised and completely separated from the plate. Afterwards, 100 µl of the cellular suspension from each well of the plate (including all study groups as well as the control group) was mixed with 100 µl of Trypan Blue solution (Sigma) and immediately the number of stained, and non-stained cells was determined by a hemocytometer (Neobar lam). In this type of staining, the stained cells are died while the non-stained cells are alive (26,27).

Apoptosis cells in each sample were randomly counted in 10 different zone of the microscope and considering the initial number of cells in medium, number of live cells was calculated as a percentage of the total number of cells. At last, one-way analysis of variance (ANOVA) and Tukey test were used to analyze the results.

Results

The results as well as comparison of data obtained from Trypan Blue staining are provided in Tables 1-3.

In each column, letters denote significant difference between groups. Furthermore, except the difference between groups 4 and 7 at observation time=24h which was significant (P=0.031), other differences were very significant (P<0.01).

Study of cellular plates under light microscope confirmed the results obtained by Trypan Blue staining. Died cells could be easily observed in cellular plates owing to the creation of dark granules and bubble-like shapes in the cells, as well as separation of cells from cellular layer formed at bottom of plates and formation of suspended cellular masses (28).

Table 1. Statistical results of one-way ANOVA test for viability percentage of MCF-7 cells treated by silymarin and different doses of doxorubicin in Trypan Blue staining.

	16 h	24 h	48 h
	Mean± SD	Mean± SD	Mean± SD
G1	34.40±2.319 c	33.30±2.214 d	30.90± 2.183 d
G2	34.20±2.440 c	32.50±1.650 d	30.60±2.119 d
G3	31.50±2.121 bc	28.60±1.506 c	23.40±2.633 c
G4	26.20±1.751 a	23.70±2.263 b	17.10±1.524 b
G5	30.00±1.826 b	28.00±1.826 c	23.20±2.616 c
G6	24.10±2.183 a	19.40±1.430 a	14.50±2.550 ab
G7	24.90±1.912 a	21.00±1.944 a	12.30±1.889 a
G8	38.20±2.150 d	34.80±1.619 d	36.56±2.186 e

Table 2. Statistical results of one-way ANOVA test for the number of MCF-7 live cells treated by silymarin and different doses of doxorubicin in Trypan Blue staining.

Live	Mean± SD
G1	12.70±1.567 b
G2	14.80±2.201 b
G3	23.60±2.413 c
G4	28.40±1.506 d
G5	25.40±1.647 c
G6	30.20±1.619 d
G7	29.20±1.932 d
G8	8.60±1.506 a

Table 3. Statistical results of one-way ANOVA test for comparison of viability percentage after 16, 24, 48 hours among eight groups.

	Mean± SD	*P*-value
16 h	30.44±5.180 c	<0.001
24 h	27.66±5.732 b	
48 h	23.41±8.334 a	

Discussion

Combination chemotherapy has been proposed as an appropriate method for maintaining effective therapeutic characteristics of the major drug while removing the limitations of previous methods with the purpose of modifying chemotherapeutic regimes (30). In this regard, numerous studies have been performed on chemical compounds from herbal origin which possess the inhibitory effect on oncogenic factors or trend of cancer. Use of herbal therapeutic compounds with strong anti-cancerous effects and lower toxicity or at least the same toxicity at a longer period of time has been recommended as the potentially suitable alternative; flavonoids have a specific position among such compounds (31,32). Considering the significance of breast cancer in Iran and worldwide (1-3,34), the ability of silymarin as one of these flavonoid compounds acquired from *Silybum marianum* plant (33) in simultaneous administration with doxorubicin was investigated in the present study with the aim of decreasing doxorubicin's dose and accordingly reducing its side effects. To study such mutuality, estrogen-dependent breast carcinoma cell line was selected in this research. Also, doxorubicin is a drug with a wide spectrum of anti-cancerous properties, which is used in the treatment of different types of tumors, including breast cancer, lung cancer, and prostate cancer (6,35). It has nonetheless some side effects, including its toxicity to the immune system (6) and heart (6,36). Accumulation of drug dose in body remarkably causes congestive heart failure (CHF) (6,37). Also, a little increase in its dose leads to an exponential increase in the rate of CHF, such that these problems reach from 5% in the dose of 400 mg/m^2 to 16% in the dose of 500 mg/m^2, 26% in the dose of 550 mg/m^2, and 48% in the dose of 700 mg/m^2, and the effect of doxorubicin dose accumulation is not faded by increasing the interval between administration times (38,39). On the other hand, besides possessing characteristics such as being antioxidant, anti-cancerous, apoptotic, inhibitor of growth and angiogenesis, as well as being inexpensive and available which suggest it as a candidate for being combined with a chemotherapeutic drug, silymarin has properties for reducing or treating the negative side effects of doxorubicin (10,12,13,40).

Negative effects of doxorubicin on the heart are to some extent due to causing oxidative damage and induction of apoptosis. Moreover, it maintains skeleton structure of cells by inhibition of lipid peroxidation in blood red globules (41,42). Hypertension and

considerable hepatic disorders are among consequences of using anthracyclines, especially in old women (43,44). Silymarin's mechanisms of effect are various and different. The most significant effects of this herbal extract include its antioxidant effects, inhibition of lipid peroxidation, and renovation of inter-cellular glutathione reservoirs (45-48), as an important enzyme in the process of detoxification by hepatic cells which plays the inter-cellular antioxidant role. Silymarin enhances protein synthesis and production in hepatic cells and hence increases the liver's renovation rate (49,50-54).

Besides these useful effects which can be expected in combining silymarin and doxorubicin, silymarin has an inhibitory effect on the resistance caused by frequent use of doxorubicin. Zhang *et al.* reported that silymarin has an inhibitory effect on all drug resistances by glycoprotein P (P-gp) intermediate and exerts this effect through direct reaction with this membrane protein (12,21). In relation to silymarin's anti-cancerous effects on breast carcinoma cells, Agrawal *et al.* published results of their study in 1998 which indicated growth stop of these cells in G1 cell cycle after their treatment with silymarin and they showed that silymarin can exert a strong anti-cancerous effect on breast carcinoma cells. These researchers attributed such effect to induction of Cip/p21 by silymarin, by which the kinase activities of CDKs and their related cyclines are inhibited, and the cell stops at G1 phase (13). Anti-cancerous effects of silymarin are not limited to cell apoptosis; rather, Jiang *et al.* have provided evidence indicating anti-angiogenesis effects of silymarin in tumors. According to their study, silymarin has an inhibitory effect on secretion of some major cytokines in angiogenesis process. 5-to-6-hour treatment of prostate and breast carcinoma cells with silymarin led to decrease in vascular endothelial growth factor (VEGF) dose secreted in cells' medium without causing any observable change in morphology of cells (6). Besides, anti-metastatic effect of silymarin on MCF-7 cells has been studied by Lee and colleagues (9). According to evidence presented by these researchers, silibinin inhibits invasive malignancy of cancer cells through inhibiting the expression of MMP-9 gene dependent on AP-1. This study demonstrated that silibinin can inhibit the invasion of cancer cells induced in PMA pathway which results in metastasis inhibition in an *in vitro* model (55,56).

In the present research, when studying the groups treated with a combination of silymarin and doxorubicin at observation time of 16h, the lowest viability was determined in group 6, whose difference with group 5 was very significant ($P<0.01$) while it had no significant difference with group 7. After 24h, lowest number of live cells was observed in group 6 whereas the highest number of them was recorded in group 5, such that the comparison of the results between these two groups was very significant ($P<0.01$); however, the difference between results of groups 6 and 7 was not evaluated to be significant. Also, after 48h the lowest viability was seen in group 7 with a significant difference compared to group 5 ($P<0.01$), but its difference with group 6 is not significant. Hence, at observation times of 16h and 24h the best response was obtained from group 6 (silymarin combined with 25nM doxorubicin). Though the lowest viability after 48h was observed in group 7, considering the absence of significant difference between groups 6 and 7, it can be stated that half of doxorubicin dose combined with silymarin (group 6) has had the same effects son cellular viability and apoptosis compared to group 7; in addition, reduction of drug dose to half of its initial amount has a remarkable importance in decreasing undesirable side effects. In view of the mentioned points, combination of silymarin and 25nM doxorubicin is the best selection for this therapeutic regimen.

During the current research, silymarin dose was chosen to be 75 µg/ml (4). To decrease the number of variables, this dose was considered constant, and viability of cells was measured in the presence of this silymarin dose by the addition of three different doses of doxorubicin. Comparison of the groups treated with a combination of silymarin and doxorubicin (groups 5, 6, and 7) with groups treated with only doxorubicin (groups 2, 3, and 4) at equivalent doses of doxorubicin showed that, when comparing groups 2 and 5 the number of live cells after 16h, 24h, and 48h is lower in group 5 and this difference is very significant ($P<0.01$). Also, comparing the groups 3 and 6 the treatments containing silymarin exhibited lower number of live cells at all three observation times ($P<0.01$). Comparing the groups 4 and 7 the number of live cells at all three observation times is lower in the treatments containing silymarin; this difference was not significant at 16h, but it was significant at 24h ($P= 0.031$) and 48h ($P<0.01$). So the groups containing silymarin compared to those which received only doxorubicin have a lower number of live cells in all cases. Therefore, it seems that the presence of silymarin along with doxorubicin leads to more decrease in cell viability and increase in doxorubicin's effect in induction of cell death. Also, comparing the groups 4 and 6 the cell viability in group

4 which received 50nM doxorubicin is at the three observation times lower in comparison with group 6 treated with half of doxorubicin dose as well as constant amount of silymarin. It thus seems that the presence of silymarin can result in the positive effect in decreasing undesirable side effects and possibility of drug resistance due to reducing the doxorubicin dose to half while maintaining the anti-cancerous effect of doxorubicin. Comparing the group treated with only silymarin (group 1) and control group (group 8), it is obvious that cell viability after 48h and 16h in group 8 is higher compared to group 1 (P<0.01). At observation time of 24h, cell viability was lower in group 1, but the difference was not statistically significant. In order to evaluate the efficacy of doxorubicin, groups 2, 3, and 4 were separately compared with group 8. Comparing groups 2 and 8 at 16h and 48h, number of live cells in group 2 was significantly lower (P<0.01), while the difference at 24h was not significant. Comparing the groups 3 and 8, number of live cells at all three observation times is much lower in group 3 (P<0.01). Also, comparing the groups 4 and 8, a significant difference is observed at all three observation times (P<0.01). As was expected, these results demonstrate that doxorubicin causes cell death and reduces live cells. In all the three groups 2, 3, and 4 the decrease in cell viability is directly related to doxorubicin dose administered, and the dose increased at equal periods of time leads to lower cell viability. Also at a constant dose in all three groups, more cell death and less viability was observed as time passed, which is entirely consistent with anti-cancerous and anti-apoptotic properties of doxorubicin. Study of cells by light microscope as a qualitative and rapid method in all cases confirmed the results of Trypan Blue staining method. In the current study, we made use of Trypan Blue staining which is a biological dye (negatively-charged chromophore) which selectively stains died cells or tissues. As cells selectively pass compounds through their membrane, live cells with intact membrane do not pass dye, whereas damage to membrane easily causes the cell to become blue (58). Advantage of this method is its being inexpensive, simple, and available, in which cells with intact membrane do not absorb the dye while only those cells whose membrane permeability has been changed are capable of dye absorption. Furthermore, this method cannot induce cell death mechanism, so it can be used for recognition of cells which are in necrosis or apoptosis status (59). In other words, Trypan Blue staining provides an estimation of the number of apoptotic died cells (59). To identify the cells at initial stages of apoptosis, TUNEL technique can be used (60). In a research by Zi *et al.*, anti-cancerous effects of silymarin on breast carcinoma cell line MAD-MB was studied, and inhibitory effect of silymarin on cell growth cycle was investigated. Also, Trypan Blue staining was used for measurement of cell growth by which the live cells were counted (13). In another study by Zhaoa *et al.*, silymarin and silibinin were compared regarding growth inhibition of human carcinoma cells, including breast, uterine, and prostate carcinoma cells. They also used Trypan Blue staining for evaluation of viability of treated cells (8). Another study has shown that silymarin has anti-cancerous effects on several epithelial cancers such as endothelial cell of blood vessels, as well as prostate and breast cancer. The same method of staining was utilized for counting apoptotic cells (9). In studying the antioxidant and anti-cancerous effects of a compound called Antrodia camphorate (a traditional Chinese drug in Taiwan) breast carcinoma cell line MCF-7 was used, and Trypan Blue staining was applied to evaluation of cell growth and viability of cells (61,62).

It can be concluded that silymarin's has a synergistic effect on apoptosis induced by doxorubicin in human breast carcinoma cell line (MCF-7). Optimum dose of doxorubicin with a constant dose of silymarin (75 µg/ml) was 25 nM, by which cell viability was evaluated to be equal with the group treated only with 50 nM doxorubicin. Regarding the obtained results and considering the point that currently the main problem of chemotherapy methods in breast cancer is to reach the dose of these factors to effective concentration in tissue without causing unacceptable toxicity in neighbor normal tissues and avoiding to cause primary or secondary resistance in cancer cells (63), it seems that this study has been successful to provide an optimized chemotherapeutic regime by the combination of doxorubicin and silymarin *in vitro* for the first time in Iran. The fact that silymarin is native to Iran as well as high death statistics caused by breast cancer justify further studies in this field.

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