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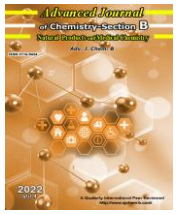

Original Research Article

A New Therapeutic Approach Based on Silymarin in the Treatment of Breast Cancer

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ABSTRACT

One of the goals of nanotechnology is to mount molecules and drugs on carrier materials, and then send and release them into the cell. Since so far, the researches carried out on the loading of the active substance of silybum marianum, silybin in in lipid nanocarriers have been very few. Therefore, in this research, the effect of silybin lipid nano-system on BT-474 cancer cell line has been investigated.

Methodology: By preparing a stock solution of silybin in in isopropyl and PBS solvents, the wavelength at which silybin has the most absorption in the range of 200 to 700 was prepared by spectrophotometric method. To make nanoliposomes containing silybin in, the thin layer hydration method was used. The MTT method was used to check the toxicity of constructed lipid system and to determine the percentage of cell viability.

Results: The results of drug-free nanoliposome toxicity and its comparison with the control show that drug-free nanoliposome is not toxic on BT-474 cells and does not cause side effects on body cells.

Conclusion: The data obtained from investigating the effect of silybin in and liposomal nanoparticles carrying silybin in on BT-474 breast cancer cell line by MTT method, in addition to confirm the anticancer properties of silybin showed the anticancer properties of silybin loaded in nanoparticles. The lipid system is more than its free state.

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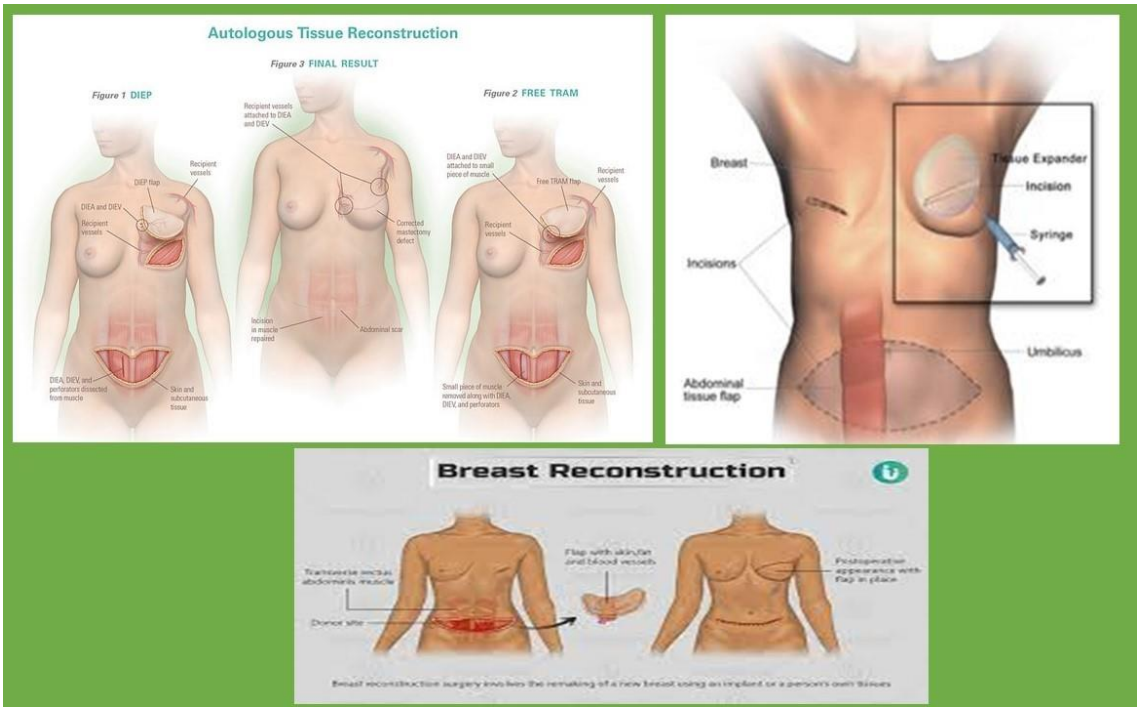
GRAPHICAL ABSTRACT



Introduction

Currently, one of the most important causes of death in the world is cancer. It is estimated that in 2020, about 15 million new cases of cancer and about 10 million cancer deaths will be reported. Breast cancer is the most common cancer among women [1-3]. According to the statistics of World Health Organization (WHO), one out of every 8 to 10 women will get breast cancer [4-6]. According to the Iranian statistics, there is a possibility that one woman out of every 10 to 15 women will get breast cancer, but the age of breast cancer in Iranian women is at least a decade lower than that of women in developed countries [7]. The average age of breast cancer diagnosis in western countries is 56 years and in Iran it is 45 years. Breast cancer is the second most common cause of cancer death [8-10]. Due to the mechanism complexity and the presence of multiple factors in causing cancer, no definitive treatment has been found for this disease, but today, various treatment methods are used to treat breast cancer. Therefore, new targeted cancer treatments that have led to the specificity of tumor treatment and reduced toxicity have been investigated. In modern pharmacy, many efforts are made to optimize the pharmacological performance and reduce the side effects of the drug, and due to the low absorption of the drug in the body, drug nanocarriers are used to control the drug release [11-13]. The types of carriers used are micelles, liposomes, and niosomes. Liposomes are known as bi-layered lipid sacs that are ideal models of biological cell membranes that function by minimizing harmful effects on the health of cells and tissues. Silybum marianum is also known by the name of martial and known names in the world such as English milk thistle or Scottish thistle. Silybum marianum seeds contain approximately 4-6% silymarin and the extract of this plant contains 65-80% silymarin and 20-35% fatty acid. Silybinin, which is also known as silybin, is the main compound found in the alcoholic extract of silybum marianum, and it

contains approximately 50-70% of this extract [14]. Silybinin is a flavonoid of the natural flavonolignan type, which, according to studies, has the ability to inhibit cancer growth by inducing apoptosis in all types of cancer cells and endothelial cells, which indicates its anti-angiogenic effects, but its molecular mechanism is not well-defined [15-17]. This compound has shown therapeutic effects in many cancers cell lines, including prostate, colon, breast, and lung cancer cells. Although the effect of this substance in cancer treatment is not clearly known, the effect of silybin in the treatment of cancer is due to its anti-free radical and anti-angiogenic function. So far, in none of the studies conducted on this compound, its effect on breast cancer cell line has not been evaluated (Figure 1) [18-20].

New technologies are used to reduce side effects and increase the effectiveness of chemotherapy agents [21-23]. Among these new methods is the use of nanotechnology in the field of medicine. One of the goals of nanotechnology is to mount molecules and drugs on carrier materials, and then send and release them into the cell [24]. Since the researches carried out so far on the loading of the active substance of silybum marianum plant, silybin, in lipid nanocarriers are very few. Therefore, in this research, the effect of silybin lipid nano-system on BT-474 cancer cell line has been investigated [25-27].

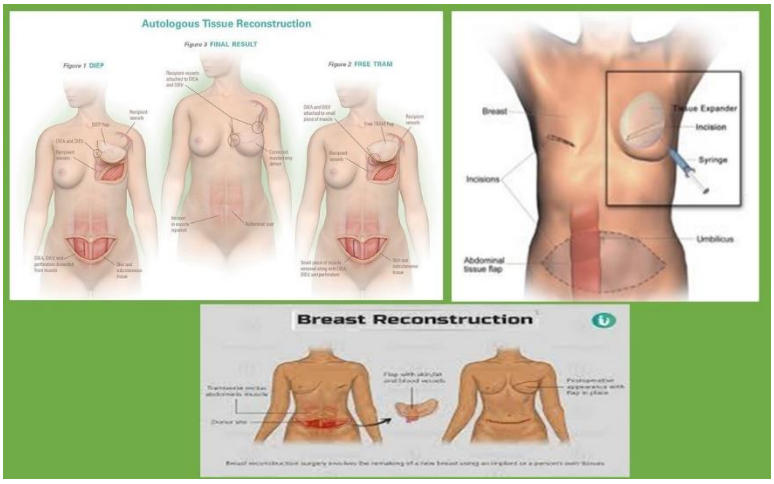


Figure 1. New technologies are used to reduce side effects and increase the effectiveness of chemotherapy agents

Materials and Methods

Silybin in drug was prepared and purchased in the form of a special powder of silybin in from Sigma (USA), casterol, and soy phosphatidylcholine were purchased from Merck (Germany) [28-30].

Determining the maximum wavelength and drawing the standard filicinin chart

Using the preparation of stock solution of silybinine in isopropyl and PBS solvents, the wavelength at which silybinine with the most absorption in the range of 200 to 700 was prepared by spectrophotometric method [31-33]. To draw the calibration chart of silybinine, the specific dilutions of silybinine with isopropanol and PBS solvents were prepared using the standard series method and the absorbance of the sample was read using a spectrophotometer at the maximum wavelength of silybinine, and then the calibration chart of silybinine in isopropanol and PBS were drawn and the linear equation and its regression coefficient were determined. Likewise, the experiments were repeated 3 times [34-36].

Table 1. Proportions and molar concentrations used in the manufacture of liposomes

SPC (mol %)	Cholesterol (mol %)	Concentration of filicinin (mg/mL)	Lipid to silybin ratio
70	30	Ec=0.5	L/D=20

Synthesis of liposomes containing silybinin

To make nanoliposomes containing silybin in, the thin layer hydration method was used, the summary of which is as follows. At first, cholesterol and soybean phosphatidylcholine (SPC) were dissolved in chloroform solvent at 40 °C on a rotary table and thin film was made under vacuum conditions [37-39]. Hydration was done by adding a specific volume of PBS buffer at 50 °C for 1 hour. To reduce the size of the particles, a sonicate probe with an ultrasonic power of 100 watts and a frequency of 28±5% KHz was used for 1 hour. During the process, temperature conditions were controlled to prevent damage to the nano system [40]. To remove the existing impurities and separate the particles with undesirable size from the suspension containing the sample, the filtration method was used. Dialysis bag was used to separate drugs that are not loaded in liposome and are in free solution [41-43].

Isopropyl solvent was used to measure the amount of silybinine incorporated. Different volumetric dilutions of isopropyl-liposome containing silybin in were prepared in triplicate and the absorbance of each was determined using a spectrophotometer and the data reproducibility was also evaluated [44-46]. Next, the amount of filicinin accumulation was calculated using the linear equation obtained from the calibration chart and using the following relationship [47].

The amount of penetration (%) = × 100

To investigate the release process under Bronton conditions, PBS buffer with a neutral pH of 4.7 was used [48-50]. Therefore, 1 ml of the sample suspension was transferred into the dialysis bag and the dialysis bag containing the sample was placed in the falcon containing the PBS buffer and stirred at a temperature of 37 °C at different time intervals compared with the environment sampling [51-53]. Dialysis was carried out around the bag and the same temperature and fresh buffer was replaced in the same ratio. Then, the absorbance of the collected samples was determined using a spectrophotometer, and finally using the line equation obtained from the calibration graph of silybin in in PBS buffer, the silybin concentration was measured at different times and its graph was drawn [54-56]. Morphology, size, and zeta potential of nanoparticles zeta sizer device was used at room temperature and at an angle of 90 degrees to measure the size of particles and zeta potential of nanoparticles [57]. JPK0 instruments atomic force microscope (AFM) was used to investigate the surface morphology of nanoparticles (shape, smoothness, and agglomeration) because currently the use of microscopic methods is one of the most important methods for examining the nanoparticles morphology [58-60].

Determination of cell viability

The cell line in this study was BT-474 breast cancer cell line, which was obtained from Pasteur Institute of Iran. Cells in 75 mL flasks, 1 serving of cell culture containing RPMI 1640 culture medium enriched with L-glutamine with 10% bovine serum (FBS), 1% penicillin-streptomycin in an incubator at 37 °C and 5 CO_2% and 95% humidity were cultivated. The MTT method was used to check the toxicity of the constructed lipid system and to determine the percentage of cell viability. To measure toxicity, breast cancer BT-474 cells were transferred to 96-well plates and after 24 hours of incubation of cells with

liposome containing silybin in and without silybin in as well as free silybin in in 4 repetitions for they were treated for 24 hours. Next, 20 ml of MTT solution with a concentration of 5 mg/mL prepared with PBS buffer was added and incubation was established again for 3 hours to metabolize MTT. Next, after removing the supernatant, 180 µl of DMSO solution is added to the wells to dissolve the purple crystals. At the end, light absorption at the wavelength of 570 nm and the reference wavelength of 630 nm was measured using an ELISA reader and the percentage of cell viability was obtained using the following equation [61-63].

Cell viability (%) = × 100

The results of the absorption spectrum of silybinine were prepared and drawn using a spectrophotometer in the range of 200-700 nm (Chart 1). Examining this spectrum shows that silybinin has the highest absorption at the wavelength of 380 nm.

Chart 1. Silibinin absorption chart at different wavelengths. The wavelength of 380 nm was considered as the maximum wavelength

Hairy liposome calibration containing silybinine in PBS and isopropyl buffer

The standard diagram for silybinine in PBS solvent is a straight line with the equation Y = 0.0088x - 0.0053, which has a coefficient of determination (R^2) of 0.9989 (Chart 2). This equation is a first-order equation and confirms the linear relationship between absorption and concentration. The standard graph of silybinine in isopropyl buffer is further a straight line with the first-degree equation Y=0.0227x-0.0083 and the coefficient of determination 0.9991 (Chart 3).

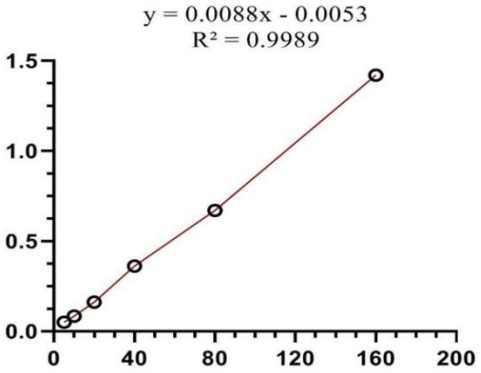


Chart 2. Silibinin calibration chart in PBS buffer.

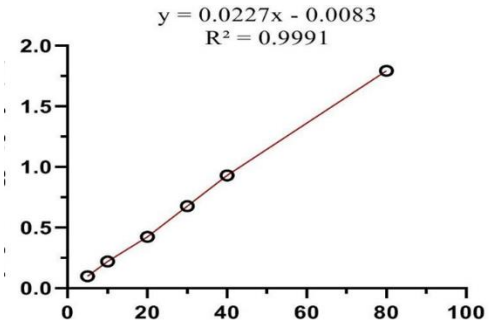


Chart 3. Silybinine chiration diagram in isopropyl buffer.

Investigating the loading percentage of silybinin in the liposomal nano system

In this step, to measure the amount of loaded silybinin, the linear equation obtained from calibration graph in isopropanol solvent was used, and by placing the data in the equation, the amount of loading was measured as 2.14 ± 64%. Qualitative and quantitative analysis of the release pattern was drawn using the standard diagram of silybinin in PBS at a temperature of 37 °C. Examining the release pattern indicates that liposomal nano-systems containing silybinin have a slow and continuous release process. In addition, there is a strong release of silynine in the first 10 hours, which seems normal considering the concentration gradient created between the dialysis bag and the PBS buffer around it. Then, the release process continues

with a more or less gentle and constant slope, and in 48 hours, the release rate reaches its maximum value, and from this time onward, we are facing a slow slope of the release process. As can be deduced from the graph, the maximum amount of release after 48 hours is equal to 58.42%.

Investigation of liposomal nanocarriers carrying silybinine and without silybinine

The results of drug-free nanoliposome toxicity and its comparison with the control show that drug-free nanoliposome is not toxic on BT-474 cells and does not cause side effects on body cells. The results of the MTT test after 48 hours of incubation of BT-474 cell line with different gels and liposomal form of silybinin show that the effect of free silybinin and nanoliposome silybinin in 48 hours from the concentration of 25 and 5.12 µg/ml, respectively, and more than that became significant compared with the control group. These findings show that free silybinin inhibits the growth of cancer cells in 48 hours with a higher dose, while the nanoliposome form of silybinin with a lower dose can cause more toxicity in cancer cells compared with the control and thus inhibit cell growth [64].

The findings show that in all different concentrations of silybinin, the death rate of cancer cells treated with nanoliposome silybinin significantly increased compared with when free silybinin is used. Furthermore, the IC50 value of free silybin in is 6.220±5.8 µg/ml and nanoliposome silybin in is 7.38±1.2 µg/ml in the effect on BT-474 breast cancer cells, which demonstrates that silybinin in the nanoliposome form with a lower drug concentration and dosage produces higher toxicity in cancer cells than the free form, and it can be concluded that free silybinin requires 7.5 times more concentration of the drug than liposome silybinin to achieve the IC50 value of encapsulated silybinin [65].

Discussion

The data obtained from investigating the effect of silybinin and liposomal nanoparticles carrying silybinin on BT-474 breast cancer cell line by MTT method, in addition to confirming the anticancer properties of silybinin, showed that the anticancer property of silybinin in the state loaded in the lipid nano-system compared with its free state is more, which can be related to the property of slow release of the drug and the stability of the liposomal nano-system carrying silybinine. Cancer is the second cause of death after cardiovascular diseases in many societies including Iran. Due to the high side effects of chemotherapy drugs, efforts to find a new compound with complete plant origin and low risk are much needed. Silibinin exerts inhibitory effects against the tumor and thus stops the cell cycle in cancer cells. This drug can prevent the onset and progression of colon cancer. Silybinin can also inhibit angiogenesis, which prevents hypoxia and the formation of endothelial tubes, development, and proliferation of cancer cells. In 2001, researchers used silybinin on MCF-7 breast cancer cells and reported a decrease in survival and an increase in apoptosis in these cells. A study showed the effect of silybinin in inhibiting growth and increasing cell death in T47D breast cancer cell line. Consistent with the above studies, our study also confirmed the effect of silybinin on BT-474 breast cancer cells and its anticancer properties. The use of drug carriers such as liposome and nisome improves the drug performance in cancer treatment. Many studies have been done on lipid nanocarriers that show their importance in drug delivery, reducing drug concentration and improving its performance. The researchers proved that liposomes toxicity containing doxorubicin is higher than the free form of drug on breast cancer cells, and the liposome form works better on these cells. Several other researchers also investigated the toxicity of free curcumin and curcumin-containing liposomes on bone cancer cells and concluded that liposome curcumin has more toxicity on bone cancer cells compared with its free form. In another study, researchers showed

that liposomes containing the anticancer drug hydroxyurea have a more toxic effect than the free form of drug on breast cancer cells. It has been reported that silybinin in the form of niosomes had an effect on human breast cancer cells and it was determined that encapsulated silybinin has more toxicity than the free form of drug on cancer cells. Along with the above studies, our study also proved that the toxicity effect of liposomes containing silybinin is greater than the free form of silybinin on BT-474 breast cancer cells. The researchers synthesized nanoliposomes containing bergamot extract with a size of 186 nm and a zeta potential of 6 and showed that nanoliposomes, in addition to increase the extract solubility, it can improve its anticancer indicators. Reducing the size of particles was one of the advantages of our study compared with the above study. Some other researchers also prepared lipid systems containing silybinine with 24% drug loading in the system and while confirming the slow release of the system, they showed that the maximum drug release in 75 hours is close to 25%.

Among the advantages of this study compared with the Ochi Ardebili study is the higher drug loading percentage. In a study, researchers synthesized lipid nano-systems containing beta-carotene with a size between 80 and 90 nm and a loading rate of 68.83% and a dispersion index of 3.0%. The results of their study were very similar to the results of our research in terms of drug loading. In another study, they synthesized liposomes containing zenian essential oil with 186 nm, electric charge of -1 to -6.7 mV, and 36% coverage with the drug, which showed that the liposome system improved the drug performance.

Conclusion

In this study, we succeeded in loading the drug silybinin in the nano-liposomal system and also its characterization, and evaluated its effect on BT-474 breast cancer cells compared with free form of the drug. In this study, a formulation of nanoliposomes containing silybinin was synthesized, which finally led to the creation of an optimal formulation of nanoliposomes containing silybinin with suitable physicochemical properties, slow release, greater toxicity, and better performance than the free form of silybinin on cancer cells. The results of our studies revealed that it is possible to use optimally synthesized nanoliposomes as a carrier to deliver silybinine as an anticancer drug to cancer cells, including breast cancer, to deal with this type of cancer. Therefore, it is suggested to carry out a series of supplementary researches, such as investigating the clinical course of nanoliposomes containing silybinin with the aim of treatment or the treatment progress in animal models, as well as investigating the simultaneous effect of encapsulated silybinin on breast and ovarian cancer.

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