



Original Article

## Toxicity Effects of Lipid Nanoparticles Containing *Artemisia Absinthium L* Essential Oil on BRC-03 Breast Cancer Cell Line

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### Abstract

**Background & Objective:** According to research, breast cancer is the second most common cancer among women. Nanomaterials of various materials significantly increase the solubility, stability and effective drug delivery, and in recent years research on the effectiveness of essential oils and plant extracts in inhibiting the growth of cancer cells is expanding. *Artemisia* belongs to the Asteraceae family. In many studies, the effectiveness of the essential oil and extract of this plant in inhibiting the growth of cancer cells has been reported.

**Materials & Methods:** In this experiment, nanoparticles containing *Artemisia absinthium L* essential oil (thistle) were first synthesized by homogenizer & sonication method and then their physicochemical properties were determined, such as particle size, particle size distribution, zeta potential, percentage of loading efficiency. Then, the anti-apoptotic effect of lipid nanoparticles containing *Artemisia* essential oil in breast cancer cells (BRC-03) was evaluated.

**Results:** The results of cellular effect of lipid nanoparticles containing essential oil of *Artemisia absinthium L* (thistle) on breast cancer cells showed that increasing the concentration of lipid nanoparticles containing essential oil of this plant reduced the survival rate of cancer cells. Breast (BRC-03) was purified compared to essential oil ( $p < 0.05$ ).

**Conclusion:** Nano-essential oil of this plant is effective in reducing the IC<sub>50</sub> of the drug and in increasing the cytotoxicity of the essential oil of the plant. Lipid nanoparticles containing essential oil of *Artemisia absinthium L* (thistle) activated the apoptotic pathway in breast cancer cells.

**Keywords:** Breast cancer, *Artemisia absinthium L*, Anti-apoptotic, nanoparticles

### Introduction

Breast cancer has become one of the leading causes of death among women in the world and the incidence is increasing significantly compared to other cancers. Most cytotoxic drugs used in the clinic have a large volume of distribution, which leads to more accumulation

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at the site of healthy tissue and causes toxicity. Therefore, there is a lot of research to find new anti-cancer compounds. The use of herbal medicines and new compounds with toxicity, cost and side effects is less considered by medical research in the treatment of cancer (1, 2). Plant essential oils are rich in natural and essential biologically active substances that have antibacterial, antifungal, antiviral, insecticidal, antioxidant and anti-cancer properties. Evidence from the World Health Organization



(WHO) shows that about 70% of the world's population prefers to use traditional and herbal remedies to treat their illnesses. In Iran, the use of alternative supplements has increased significantly in the last decade (3). Approximately, 60% of anticancer agents are obtained from medicinal plants and other natural sources (4). However, many plants still have anti-cancer potential that have not yet been studied. Therefore, the alternative solution to the harmful effects of synthetic drugs is to use complementary alternative drugs. One of these valuable medicinal plants is *Artemisia*, which belongs to the Asteraceae family (5). Many species of this family have shown therapeutic properties due to the presence of metabolites such as flavonoids, abscitin, coumarins, steroids, polyethylene, mono and sesquiterpenes, and succitropen location (6). *Artemisia absinthium* has opened, softening, diluting and appetizing properties and its nature is astringent and bitter. Its collecting power is high due to its bitter taste. It is a laxative of bile and bile secretions and other soda mixtures that accumulate in the stomach and is diuretic. Its amount is appetizing and tonic and its large amount are effective for opening menstruation (7). One of its important effects is cleansing the arteries of the chest and lungs, and due to the presence of Nestonin, it repels intestinal worms and strengthens the stomach and digestion. Another important property is a strong disinfectant. The wormwood plant is used to treat depression, insomnia, anemia and dizziness. This herbal medicine also has anti-intestinal worm properties. Sweet wormwood contains a compound called *artemisinin*, which causes cancer cells to die. *Artemisinin* kills everything from parasites to bacteria, fungi and cancer cells, when it has nothing to do with healthy cells. There is also a trick to killing cancer cells with the help of *artemisinin* (8). *Artemisinin* can kill cancer cells alone (a study showed that 28% of breast cancer cells were destroyed), but its real anti-cancer power is released when combined with iron as it is activated to search for and destroy cancer cells.

That is why the use of sweet rye is useful in treating malaria. The malaria parasite contains iron and cancer cells are usually high in iron. They need iron to multiply and steal it from other cells, so they can continue to multiply rapidly. Other studies published this year show that *Artemisinin* has the potential to kill cancer cells in the head and neck, bladder, blood, lung, prostate, colon and bone (9). Encapsulation of plant essential oils is one of the most effective methods to increase the solubility and stability of essential oils in adverse environmental conditions and control of its active compounds. The development of nano-sized formulations of bioactive compounds due to its small size has more advantages compared to other encapsulation systems. These systems have a higher intracellular absorption due to their small size and are targeted. Since plant essential oils are fatty in nature, solid lipid nanoparticles (SLN) can be used as a very useful option for transporting plant essential oils due to their biocompatibility, low toxicity and good degradability along with the ability to encapsulate hydrophobic biological compounds (10-12). Therefore, considering the importance of breast cancer and the antioxidant potential of *Artemisia Absinthium L*, This study was performed to evaluate the cytotoxic effects of lipid nanoparticles containing *Artemisia Absinthium L* essential oil on BRC-03 breast cancer cell line by MTT method.

## Materials & Methods

### *Essential oil (aerial branches) Artemisia Absinthium L*

This research has been done granting to the ethical protocols of research centers (IR. IAU.PS.REC.1396.165). This plant with herbarium number was prepared in the Faculty of Pharmacy of Kerman University of Medical Sciences (PMP-1538) in Kerman province in the middle of summer 2021 and was well ground. Then, 500 g of dry plant powder was ground and essential oil was extracted by Clevenger. The amount of essential oil obtained was about 2.5 grams (equivalent to 5% w/w).



The ingredients in *Artemisia Albsinthim L* were identified by GC/MS (Agilent Technologies, USA). Then, uptake in the range of 200-400 nm it was taken by UV-Vis (SHIMADZU, Japan).

### Preparation of solid lipid nanoparticles containing *Artemisia Albsinthim L* essential oil

The uniformity method at high temperature and under ultrasonic waves was used to fabricate nanoparticles. Lecithin surfactant and solid fat (myristic acid) were selected for this study. For this purpose, 0.25g of myristic acid and 0.25g of the prepared essential oil were heated on 5 to 10 degrees above the melting point of myristic acid (53.9), ie 60°C, on a heater stater. In a separate bowl, 50cc of distilled water and 1 g of lecithin were mixed at the same temperature of 60°C. The mixture of myristic acid and extract was kept on the stirring heater for 60 hours at 60°C for further dissolution. The mixture of water and lecithin was then gradually added to the mixture of myristic acid and essential oil. After finishing the work, the sample was cooled to ambient temperature to form nanoparticles. Placebo (nanoparticles without plant essential oil) was also prepared according to the above-mentioned method.

### Determination of particle size and zeta potential

The parameters were measured at room temperature at a 90° angle using a zetasizer (Maloren UK Zen 3600). To investigate the parameters, the device was calibrated based on the aqueous phase (RI = 1.3) and then passed through a needle filter with a size of 0.22 µm and the particle size, zeta potential and particle size distribution were analyzed in 3 replications.

### Determining the percentage of essential oil loaded

In this part 3 ml of a solution of nanoparticles containing essential oil was poured into a dialysis bag and distilled water was poured on the other side. After 4 hours, the remaining liquid inside the dialysis bag and the initial solution of the nanoparticles were spectroscoped using a spectrophotometer. The difference between these two solutions is the amount of very small particles of nanoparticles in which the essential oil is not loaded.

### Investigation of nanoparticle morphology by SEM

The best nanoparticles were diluted with deionized water according to the amount of essential oil loading and particle size in a ratio of 1 to 10. The sample was then placed on a copper grid large enough to form a thin layer. Then, one to two drops of 2% phosphotungstic acid were added. After one minute, the excess material was removed from the copper grid with a paper filter to create a thin layer for imaging. The sample was then allowed to dry at room temperature. Then, the morphology of nanoparticles was observed by transmission electron microscope at different magnifications.

The morphology of the SLN was observed using a scanning electron microscope of the Quanta 200 model of FEI Company at an accelerator voltage of 20 kV. A drop of nanoparticle suspension was placed on a graphite surface. After drying at room temperature, the sample was coated with gold ion.

### Infrared spectroscopy with Fourier transform

Infrared spectroscopy with Fourier transform was used to identify and confirm the presence of various materials. The FT-IR spectrum of SLNs was recorded in the range of 500 to 4000 nm using the AVATAR model manufactured by Thermo USA. Essential nanoparticles and non-essential nanoparticles were tested on the machine.

### Cytotoxicity test

#### Cell culture

For this test, human breast carcinoma cells (BRC-03) prepared from Pasteur Institute of Iran were used. Cells of this cell line were cultured in one-time flask of cell culture in the DMEM culture medium of 10% FBS at 37 °C with a pressure of 5% CO<sub>2</sub> and 95% vapored.

### Studied groups

In order to evaluate the anti-cancer effects of essential oil loaded on lipid nanoparticles, cells were cultured for 96 hours in 96-well plates in the following groups with specific concentrations.



**Group 1:** *Artemisia Absinthium L* essential oil 24-h treatment with concentrations of 10, 50, 100, 500, 1000  $\mu$ / mL

**Group 2:** Nanoparticles containing *Artemisia Absinthium L* 24-h treatment with concentrations of 10, 50, 100, 500, 1000  $\mu$ g / mL

**Positive control:** Cis-platinum 24-h treatment with concentrations of 1, 5, 10, 20, 50  $\mu$ / mL.

**Negative control:** DMSO 5% treatment 24 (DMSO was used in positive control because it was used as a solvent in the extract).

### Determination of toxicity and cell viability

Cell viability percentage was determined by MTT assay. Toxicity measurements were cultured separately *Artemisia Absinthium L* cells with a concentration of  $10^4$  per well in a 96 plate for 24 h. Then, the cells were treated with the same volume of fresh culture medium. Different concentrations of extract and SLN containing extract (10, 50, 100, 500, 1000  $\mu$ g /mL) were injected in 3 replicates in wells. After 24 h, 20  $\mu$ L of MTT solution was added to each well with a concentration of 5 mg /ml and incubated for 4 h. After that, the fluid was removed and 150  $\mu$ L DMSO was added to each well. At each step

to remove the fluid, centrifuge was performed. Adsorption was recorded at 570 nanometers wavelength using Raider microprocess (ELISA Reader) and finally calculated according to the sub-percentage of survival and the percentage of cells in the cell.

### Statistical analysis

For statistical analysis of the results, SPSS software and Two-way ANOVA method were used and significant results were measured at  $P < 0.05$ .

### Results

The results of the compounds in *Artemisia Absinthium L* essential oil by examining the spectra of chromatography gas (GC/MS), the calculation of inhibition indexes and comparison of mass specters of compounds with standard combinations, 40 different compounds in *Artemisia* essential oils were identified. Essential oil efficiency was estimated at 1.5cc. The main combinations are:  $\beta$ -Pinene (21.6),  $\beta$ -Thujone (19.87%), sabinene (9.8%), trans-Verbenol (7.0) Linalool (5.1%) and Germacrene D (5.1%) (Table 1).

**Table 1.** Identify the percentage of compounds in *Artemisia Absinthium L* essential oil by GC /MS.

<sup>a</sup> RI: Retention indices on DB-5 column

| Compounds           | RI <sup>a</sup> | GC area (%) |
|---------------------|-----------------|-------------|
| $\alpha$ -Pinene    | 932             | 3.9         |
| Camphene            | 946             | 2.53        |
| Thuja-2,4(10)-diene | 953             | 0.52        |
| Sabinene            | 969             | 9.8         |
| $\beta$ -Pinene     | 974             | 21.6        |
| sabinene            | 975             | 7.8         |
| Linalool            | 998             | 5.1         |
| $\alpha$ -Terpinene | 1014            | 0.11        |
| <i>p</i> -Cymene    | 1020            | 1.32        |
| 1,8-Cineole         | 1026            | 4.12        |



|                                   |       |       |
|-----------------------------------|-------|-------|
| Santolina alcohol                 | 1034  | 0.41  |
| cis-Arbusculone                   | 1046  | 0.19  |
| $\gamma$ -Terpinene               | 1054  | 0.23  |
| $\beta$ -Thujone                  | 1112  | 19.78 |
| <i>trans</i> -p-Menth-2-en-1-ol   | 1136  | 0.35  |
| <i>trans</i> -Verbenol            | 1142  | 7.00  |
| Sabina ketone                     | 1154  | 0.32  |
| Pinocarvone                       | 1160  | 0.11  |
| <i>cis</i> -Chrysanthenol         | 1162  | 2.21  |
| Santolinyl acetate                | 1171  | 4.06  |
| Terpinen-4-ol                     | 1174  | 1.58  |
| Thujenal                          | 1181  | 0.12  |
| <i>p</i> -Cymen-8-ol              | 1182  | 0.81  |
| $\alpha$ -Terpineol               | 1186  | 0.14  |
| Myrtenal                          | 1195  | 0.61  |
| Myrtenol                          | 1196  | 0.55  |
| Verbenone                         | 1204  | 1.00  |
| <i>trans</i> -Carveol             | 1215  | 0.18  |
| Cumin aldehyde                    | 1238  | 0.44  |
| Carvone                           | 1239  | 0.43  |
| Carvotanacetone                   | 1244  | 0.17  |
| Piperitone                        | 1249  | 0.27  |
| <i>cis</i> -Chrysanthenyl acetate | 1261  | 1.89  |
| n-Decanol                         | 1266  | 0.18  |
| Bornyl acetate                    | 1284  | 0.59  |
| <i>trans</i> -Sabinyl acetate     | 1289  | 0.35  |
| Carvacrol                         | 1298  | 0.23  |
| Myrtenyl acetate                  | 1324  | 0.11  |
| Z-Jasmone                         | 1392  | 0.16  |
| Germacrene D                      | 1422  | 5.1   |
| Total                             | 96.51 |       |

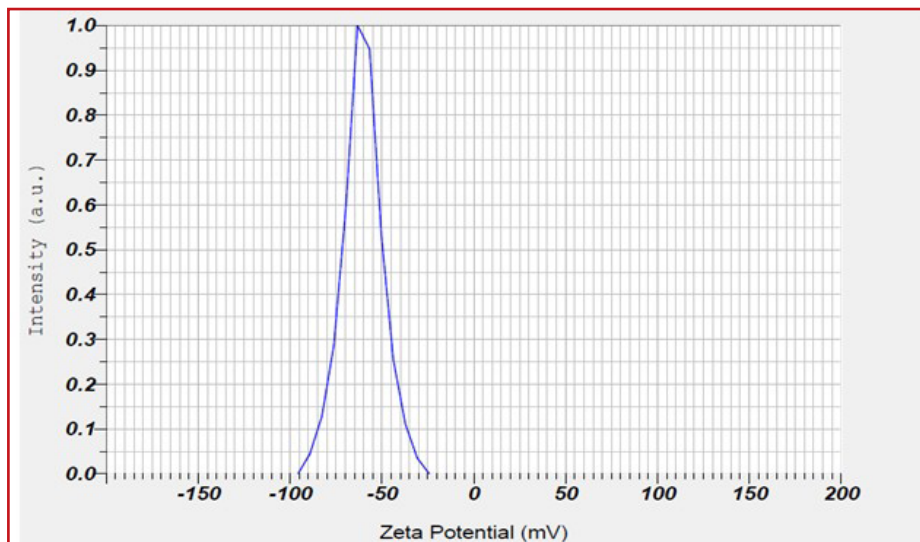
### The results of the particle size and zeta potential in the loaded extract

The average particle size and zeta potential of the extract formulated by the SLN method are listed in Table 2. The potential of the nanoparticles containing essential oil in this study was 62.5, which shows the proper negative charge and have acceptable stability particles. The nanoparticles made without extract had 88.5 zeta potential. The size of the nanoparticles containing extract and extract was 14.5 and 86.6 nm, respectively. Figure 1, 2 and Zeta potential

and the particle size of nanoparticles contain essential oils. In the case of loaded extract percentage, the results of spectrophotometer absorption were measured in the range of 190 to 1100 nm for the solution before and after passing the dialysis bag. In the case of the main solution (before passing the dialysis bag, the average of 0.411 and the solution after passing the dialysis bag was calculated. Figure 3 Shows Spectrophotometric diagram of nanoparticles containing essential oils before and after passing dialysis bag.

**Table 2.** The particle size and potential of nanoparticles

| Investigated factors   | Zeta Potential Average (MV) | Average particle size (nm) |
|--|-----------------------------|----------------------------|
| The values obtained for nanoparticles containing <i>Artemisia Absinthium</i> essential oil | -5.62                       | 130.5                      |
| The values obtained for nanoparticles without essential <i>Artemisia Absinthium</i>        | -5.88                       | 86.5                       |



**Figure 1.** The potential of the nanoparticle nanoparticles containing essential Oils

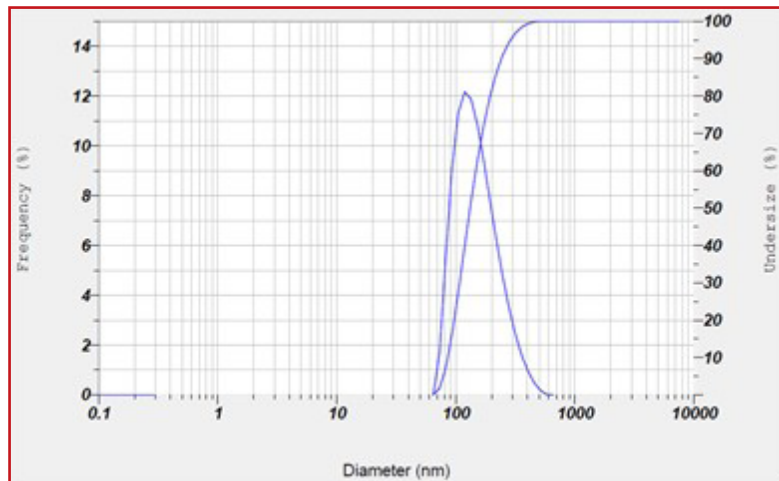


Figure 2. particle size of nanoparticles containing essential oils

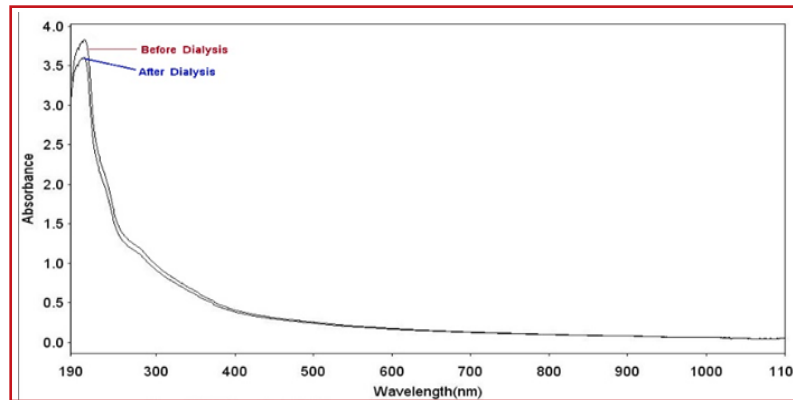


Figure 3. Spectrophotometric diagram of nanoparticles containing essential oils before and after passing dialysis bag

**The results of the morphology of lipid nanoparticles containing *Artemisia Absinthium L* essential oil**

The images of SEM microscopes are shown in figure 4. They showed the shape of spherical nanoparticles.

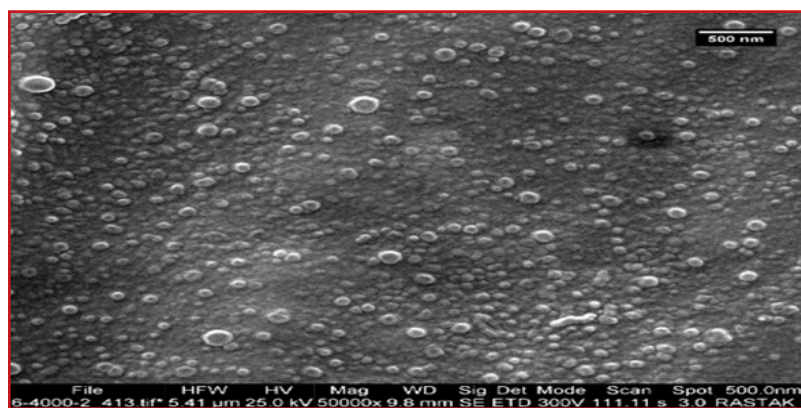


Figure 4. Image of the SEM microscope from nanoparticles containing Artemisia Absinthium L essential oils

### Results of infrared spectroscopy

The results of infrared spectroscopy are displayed in figure 5. In the case of nanoparticles containing alcohol, the essential oil of alcohol

was similar to the structural similarity with the microstrate acid (the acid used in the formulation of nanoparticles) similar similar to the graphic peak with 83.04%.

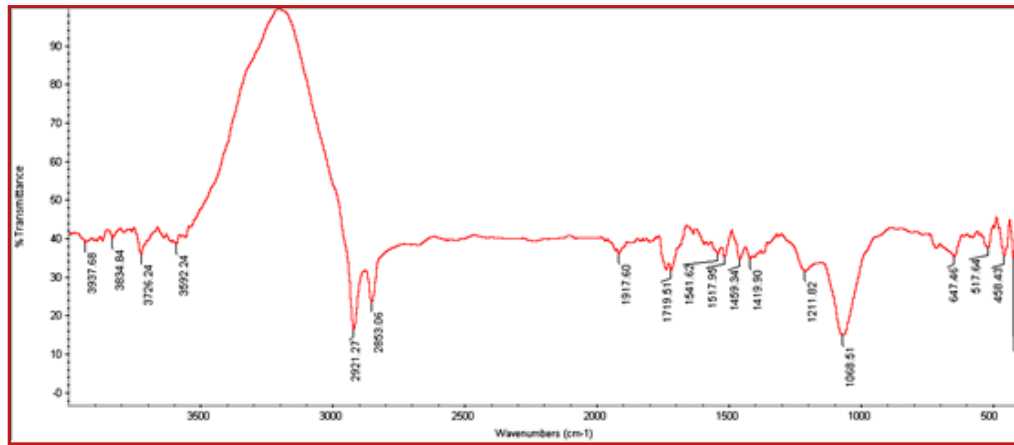


Figure 5. Absorption of FT-IR nanoparticles containing essential oils

### Results from MTT test

Figure 6 shows the results of the MTT test on different groups of testing, and table 3 shows the results of the IC50 levels of different study groups. After comparing the results of different MTT groups using ANOVA statistical techniques, the following conclusions were obtained:

at concentrations of 500 and 1000  $\mu\text{g} / \text{mL}$ , there was a significant difference between the percentage of survival in the treatment group with essential oils with nanoparticles without essential nanoparticles. (Respectively  $P < 0.001$  and  $P < 0.01$ ). This result indicates the effectiveness of essential oil and formulation compared to non-essential lipid nanoparticles.

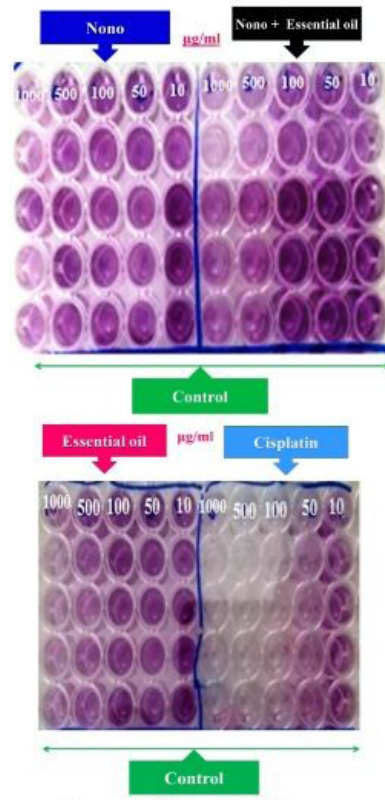


Figure 6. MTT test in 24-hour treatment



**Table 3.** IC50 levels of different study groups

| 24-hour treatment |               |   |                                 |           |
|-------------------|---------------|---|---------------------------------|-----------|
| Study groups      | Essential oil | Nanoparticles containing essential oils | Nanoparticles without essential | Cisplatin |
| IC50 µg/ml        | 1195          | 557.1                                   | 3556                            | 8.193     |

### Discussion

In this study, the antifungal effects of solid lipid nanoparticles essential oil of *Artemisia Absinthium L* on BRC-03 cells were investigated. Our results are in agreement with the findings of Gharehmatrossian et al. (2012), which in their study on the evaluation of the effect of methanolic essential oil of *Artemisia* showed that this essential oil had antioxidant properties and cell lesions and has the potential to use cancer (13). They showed that increasing the concentration of *Artemisia* essential oil reduces the survival of cancer cells and, in other words, have a dose-dependent effect. Similarly, Sharif et al. (2015) showed that methanolic essential oil has the ability to inhibit cell growth in concentration-dependent concentration (14). Other findings from the study of the molecular effect of lipid nanoparticles containing *Artemisia* plant essential oil on cervical cancer cells showed that lipid nanoparticles containing *Artemisia* plant essential oil increased the expression of Bax gene and 1.7 equality of Bcl-2 gene expression (15) Since Bax and Bcl-2 account for important genes in apoptosis, it seems that *Artemisia* essential oil contained in lipid nanoparticles activates the apoptotic pathway in cervical cancer cells. Our results are in agreement with the findings of Imam et al. (2009) in their study on the toxicity of *Artemisia annua* on gastric cancer cells that methanolic essential oil inhibit the apoptosis process significantly (16). Ahmadi et al. (2015) showed that the effect of *A. Aucheri* essential oil on neuroblastoma cells showed that the essential oil of this plant has a high anti-tumor impact on

neuroblastoma cells. Their results showed that this effect was carried out by influencing *Bax* and *Bcl2* genes (17). Based on the results of our study and other studies, the mechanisms of anti-apoptotic activity are that after treating *Artemisia* cytochrome c, the free mitochondria and the activity of Caspase 3 also increased and the activity of this apoptotic protease leads to a polymeric polymeras. Apoptotic induces and DNA failure and ultimately inhibits the activity of NF-kB anti-apoptosis factor. In this way, anti-apoptotic factor activity is suppressed and the death of the cell is stimulated and induced (18,19). As a result of the interactions of special proteins from the *BCL-2* family, cytochrome c entered cytosol from the space between the two mitochondrial membranes, and with the formation of a complex called apoptosome, the cascade pathway activates the cascaza and the cell in the path of dying. Directed. The *Bcl-2* family is a type of tragic proteins that have once traced the membrane width and placed in the membrane of the membrane organelles inside the cell and interact with each other. The pre-apoptotic members of this family are *Bak* and *Bax*, which increase the expression of these two cytochrome C to cytosol and the onset of apoptosis (20, 21). With *BAD* dephosphorylation and its release from cytosolic protein attached to serine 3-3-14 This *BAD* protein is attached to *Bcl-2* and prevents its interaction with *Bax* protein and increases the permeability of the mitochondrial membrane permeability. Cytochrome C is free from the space between



the two membranes and entered cytosol. Then, by connecting to the apoptozomed complex, the apoptosomal and caspase protein (Caspase 9), and this complex also activates the Caspase of the performer (Caspase 3), which is driven by the activity of this cell caspase to death. Therefore, according to the results and studies on cancer cells, lipid nanoparticles containing *Artemisia Absinthium L* essential oils are probably prevented by inducing apoptosis from the continuation of cell division (22-24).

### Conclusion

Finally, the evidence of the cytotoxicity of lipid nanoparticles containing the essential oil of *Artemisia absinthium L* showed that the use of lipid nanoparticles containing the essential oil of the plant can play the role of apoptosis activity in breast cancer.

### Acknowledgments

Department of biology, Roudehen branch, Islamic Azad University, Roudehen, Iran. This research has been done granting to the ethical protocols of research centers (IR.IAU. PS.REC.1396.165).

### Conflicts of Interest

The authors declare no conflict of interest.

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