

Investigating the cytotoxic effect of chamomile aqueous extract on 4T1 and 47D cells and level of caspase3 protein in breast cancer cells T-47D

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Abstract

Introduction: Breast cancer is one of the most common cancers of women in the world. Many methods have been devised to treat breast cancer, such as radiation therapy, surgery and chemotherapy. Clinical effectiveness of chemotherapy has been limited due to side effects, toxicity and drug resistance. The studies have shown that some medicinal herbs have anti-cancer effects which do not cause side effects of chemotherapy. One of these herbs is Chamomile, the anti-cancer effect of which is being studied by researchers. The effect of Chamomile aqueous extract on cellular survival of breast cancer cells T-47D, 4T1 and protein level of caspase3 in breast cancer cells T-47D has been investigated in this study.

Methodology: Breast cancer cells 4T1 and T-47D were treated by blue extract of Chamomile with various concentrations, for 24 hours and then using MTT method, the rate of cytotoxic effect of Chamomile was checked on the above mentioned cells and following that the apoptotic effect of Chamomile extract on treated cells was investigated using ELISA method for determining the amount of caspase3 protein.

Results: This study showed that Chamomile extract significantly reduced breast cancer cells survival, concentration-dependent, compared to the control group. Dependent on concentration and time, the Chamomile extract also increased caspase3 protein, indicating induction of apoptosis in the mentioned cells.

Conclusion: Chamomile extract, dependent on concentration and time, was able to kill tumour cells that can arise from increasing the level of caspase3 protein. These results can be used in future studies on the anti-cancer effect of Chamomile extract.

Key words: breast cancer, Chamomile extract, caspase3, cell lines of 4T1 and T-47D

Introduction

Cancer is nowadays considered as one of the most important health problems around the world. According to the statistics of World Health Organization, after cardiovascular diseases, cancer is the second highest cause of death in the world. According to the report of the Iran Cancer Statistics Center, more than 51,000 new cases of cancer, are annually identified and 35,000 deaths occur each year because of cancer in the country (1).

Among female cancers, breast cancer is the most important health issue. Breast cancer is the most common cancer in women and the second highest cause of death in women after lung cancer (2-6). The prevalence of breast cancer is different in various countries. The highest prevalence is in the USA and North Europe and the least prevalence has been reported in Asia (3). Breast cancer is rare in women younger than 20 years old and uncommon in women younger than 30 years old. The incidence rapidly increases up to 50 years old. Average age of cancer incidence is 62 years old. 94% of breast cancers occur in women older than 40 and only 6% has been reported in women younger than 40 (5). The most important symptom of breast cancer is a breast lump. If a lump is diagnosed in examination or mammography, breast cancer diagnosis should be proven or rejected through biopsy (6). In spite of affluent developments in controlling and treating cancer, much research has been conducted to recognize the mechanisms, involved in its growth and production of effective drugs. Moreover, a wide range of these studies have been allocated to medicinal herbs.

Using various medicinal herbs, such as garlic, Echinium, *Althaea officinalis*, mulberry tree leaves and *Rosmarinus officinalis* is common in traditional medicine of Iran for different reasons (7). The relation of Terpenoids in herbs with strong anti-inflammatory effects has been proven (8). Chamomile has active compounds such as Terpenoids and Flavonoids. Among these five herbs, Chamomile, in addition to evident antioxidant properties, contains the most anti-inflammatory effects. Because of its anti-inflammatory property, this herb is widely used for treating skin abnormalities as well as different types of cosmetic products (9). Apoptosis is cellular physiologic death which has an effective role on controlling body pathologic conditions. The diseases with infinite power of proliferation, such as cancer, are created as the result of a changing genome. The studies show that several pathologic changes lead to the growth of malignant cells. These changes include self-sufficiency in cell proliferation, resistance to cell inhibitory signals, Angiogenesis, escaping from apoptosis, lack of limitation in proliferation of cell and attack on other tissues. One of these changes is escaping from apoptosis so that resistance to apoptosis is one of the potential symptoms of cancer. Reducing the sensitivity to apoptosis leads to increase of treatment threshold for classical cases such as radiotherapy and chemotherapy. Therefore, one of goals in treating cancer is activation of apoptosis paths (caspases) in cancer cells. In this study, two cell lines of breast cancer including 4T1 and T-47D were used. 4T1 is

a mouse breast carcinoma cell which has tumorous and offensive properties and can metastasize from the primary lump to other points of the body such as lymph, blood, liver, lung and brain. The mentioned property makes it a proper experimental model for breast cancer. T-47D cells are also extracted from breast duct carcinoma of a 54-year-old woman. This line of cell contains epithelium morphology and steroid receptors such as estrogen and progesterone. T-47D lacks epidermal Growth Factor Type 2 (her2). A few studies have investigated the role of Chamomile aqueous extract in preventing meiosis as well as apoptosis induction dependently on dosage and time (10). Hence in the current study, the effect of treatment with Chamomile aqueous on properties of cytotoxic, apoptotic and also effectiveness on caspase3 protein in cell lines of breast cancer are investigated.

Materials and Methods

1- Extraction manner

In the current experimental study, the flowering head branches of *Matricaria chamomila* herb were extracted in flowering season (early March). The herb was collected in Khuzestan Province (Dezful) in an area with geographical coordinates of 48 degrees and 24 minutes north and 32 degrees and 22 minutes east. Flowering head branches were dried in shade and at room temperature. To provide aqueous extract, chamomile dry flower was turned to powder by mill and mixed and boiled with distilled water. Then the contents inside the container were filtered using Whatman filter paper and the obtained extract was concentrated using a rotary machine. The concentrated solution was kept in a freezer at -80°C until testing.

Different concentrations of chamomile aqueous extract were treated in culture environments including 2600-1300-650-325 micrograms per milliliter concentrations. Selection of concentrations was conducted based on previous studies (11).

2- Cell culture

Cell lines 4T1 and T-47D were prepared from Tehran Institute Pasteur cell bank and cultured in culture environment of RPMI 1640, enriched with Fat Bovine Serum (FBS) of 10% and penicillin antibiotic 5%. After reaching proper coating level in 96-cell plates, 10,000 cells were cultured in each well and were incubated for 24 hours in 37°C and 5% carbon dioxide and 95% of incubator moisture. The different concentrations of extract including 2600-1300-650 and 325 micrograms per milliliter were added to the cell and they were treated for 24 hours.

3- Testing the effect of chamomile aqueous on the rate of cell survival

The rate of cells survival was determined using MTT test, as it was explained before (12-13). Based on the activity of succinate dehydrogenase enzyme in mitochondria of living cells, this method turns MTT yellow solution to insoluble purple formazan crystals that can be evaluated by ELISA Reader machine after dissolving in dimethyl sulfoxide. The rate of 10,000 cells were transferred to a 96-cell plate.

They were incubated at 37°C for 24 hours. Then they were treated by different concentration of chamomile extract (2600-1300-650 and 325 micrograms per milliliter) and finally, the volume of wells reached to 100 microliters.

The plates, containing cell and extract, were incubated in the equal conditions for 24 hours. After the considered time, 96-cell plates were taken out of the incubator and 10 microliters of MTT solution was added to each well and they were put in incubator for 3 hours. Then 100 microliters of DMSO was replaced with incubated environment with MTT and they were kept in the dark for half an hour.

After being dissolved in DMSO, Formazan crystals create a purple solution the rate of which indicates the biological potential of treated cells. Optical absorption of plates was evaluated in wavelength of 570 nanometers (14).

To investigate the toxicity effect of chamomile aqueous extract on treated cancer cells, the following formula was used.

1-ODexp/ODcont×100= the rate of cells mortality

4- Investigating caspase3 activity through ELISA method

To evaluate caspase3 activity, CASP3 (human) Cell-Based ELISA Kit was used and implemented according to the following protocol:

1. 200 microliters of culture area containing 20,000 T-47D cells were cultured for a night at 37°C and with 5% of carbon dioxide.
2. The chamomile aqueous was added to the cells with different concentrations (1300, 2600 micrograms).
3. 100 microliters of fixing solution was added and incubated in room temperature for 20 minutes.
4. 100 microliters of quenching buffer was added and incubated in room temperature for 20 minutes.
5. 200 microliters of blocking buffer was added and incubated in room temperature for 1 hour.
6. 50 microliters of initial anti-body (anti-caspase3 or anti-GAPDH) was added and incubated one night at 4°C.
7. The secondary antibody (HRP-conjugated) was added and incubated at room temperature for 1 and a half hours.
8. 50 microliters of reaction substrate was added and incubated at room temperature for half an hour.
9. 50 microliters of stop solution was added and samples absorption was read at a wavelength of 450 nanometers.

Statistical analysis

On average experiments were calculated three times. The collected data were analyzed using SPSS16 software and two-way ANOVA test and the diagrams were drawn using EXCEL software.

Findings

The effect of chamomile aqueous extract on stopping cell proliferation:

The effect of different concentrations of chamomile aqueous extract was evaluated on the rate of cell mortality in cell lines. As shown in Diagrams 1 and 2, in both cell

lines, 2,600 micrograms per milliliter concentration of extract in 24 hours after treatment prevented cell proliferation significantly in comparison with the control group ($P<0.05$). Chamomile extract lacked significant effect in cell cytotoxicity at concentrations of 162 and 325 micrograms per milliliters so these concentrations were eliminated as follows.

The effect of chamomile aqueous extract on caspase3 activity

The Caspase enzyme family is of main apoptosis intermediates and their activity increases within apoptosis procedure. The results of investigating caspase3 activity showed that the value of caspase3 protein at 1 and 3 hours, affected by 2600 and 1300 micrograms per milliliters of chamomile aqueous extract, significantly increased in comparison with control group (Diagram 4). Therefore, chamomile aqueous extract can be said to lead to cell mortality in breast cancer cells through caspase3 activity compared to control group. GAPDH has been also shown as an interior standard in the diagram ($P<0.05$).

Discussion and Conclusion

In spite of important developments in diagnosing and treating cancer, breast cancer is still one of the common problems of women. One of the goals in treating cancer is returning the apoptosis mechanism in cancer cells. New medicines have always been required and researchers all around the world have made a wide range of efforts to identify new, natural or synthesized compounds with anti-cancer properties. The studies have shown that herbal medicines are more important in preventing cancer due to their lesser side effects (15). The chamomile aqueous extract through MTT method in breast cancer cells was investigated in this study and the obtained results of cytotoxicity indicated that the extract significantly and concentration-dependently increases breast cancer cells mortality in 4T1 and T-47D cell lines.

Dependent on concentration and time, the Chamomile extract also increased caspase3 protein, indicating induction of apoptosis in mentioned cells.

In this field, Janmejai, K. et al conducted a study, the results of which showed a significant decrease in cancer cells liveability after Chamomile treatment. The apoptotic anti-proliferative effects of chamomile extract on cancer cells was also observed in this study (16). As mentioned before, chamomile has strong anti-cancer effects. In a study by Asgari et al, it was observed that the rate of antioxidant markers activity in liver cells of rats, faced with oxidant composition after chamomile extract, significantly increased (17).

Sedighara concluded in a study that chamomile has antioxidant and anti-inflammatory effects. In this investigation, the rate of herbs' antioxidant power and copper ion survival and their anti-inflammatory power were tested through tests for preventing serum protein denaturation (18). Given that apoptosis is an important

Diagram 1- The comparison of cytotoxicity percentage of T-47D cells, treated with concentrations of 2600, 650 and 1300 of chamomile aqueous extract with MTT test

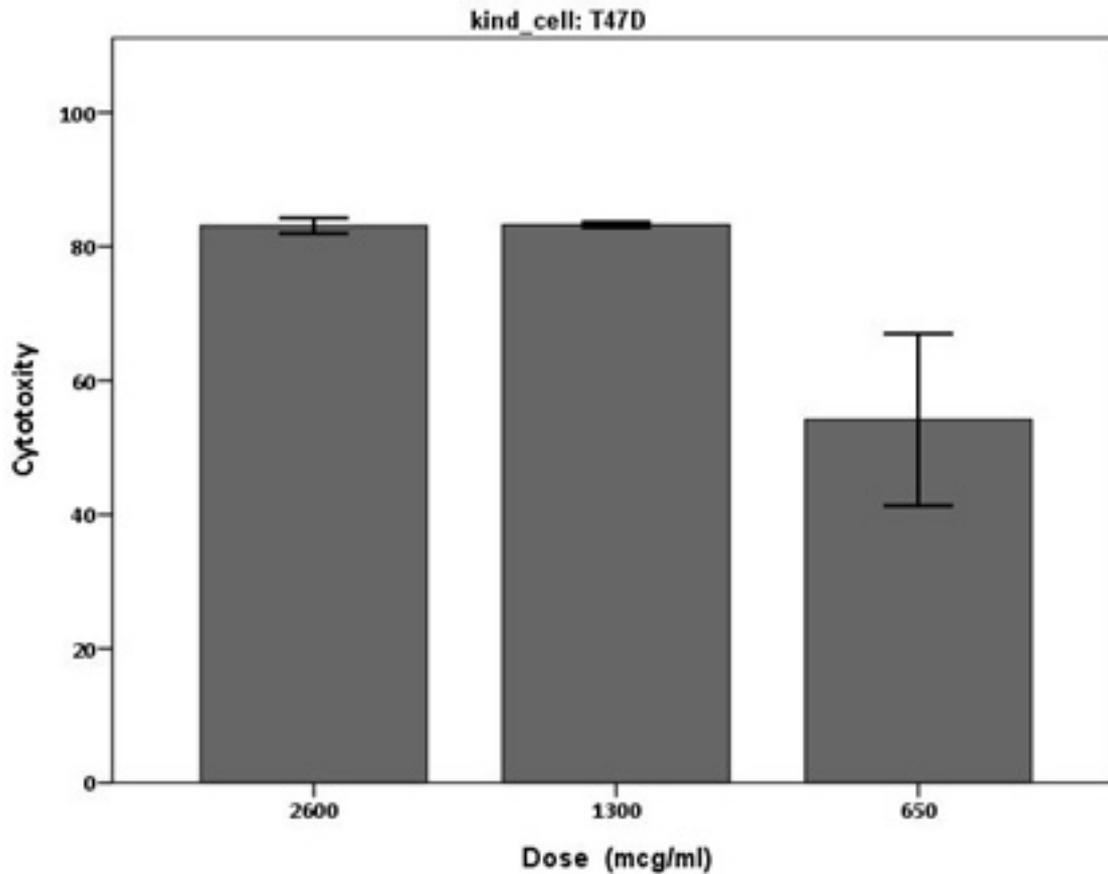


Diagram 2- The comparison of cytotoxicity percentage of 4T1 cells, treated with concentrations of 2600, 650 and 1300 of chamomile aqueous extract with MTT test

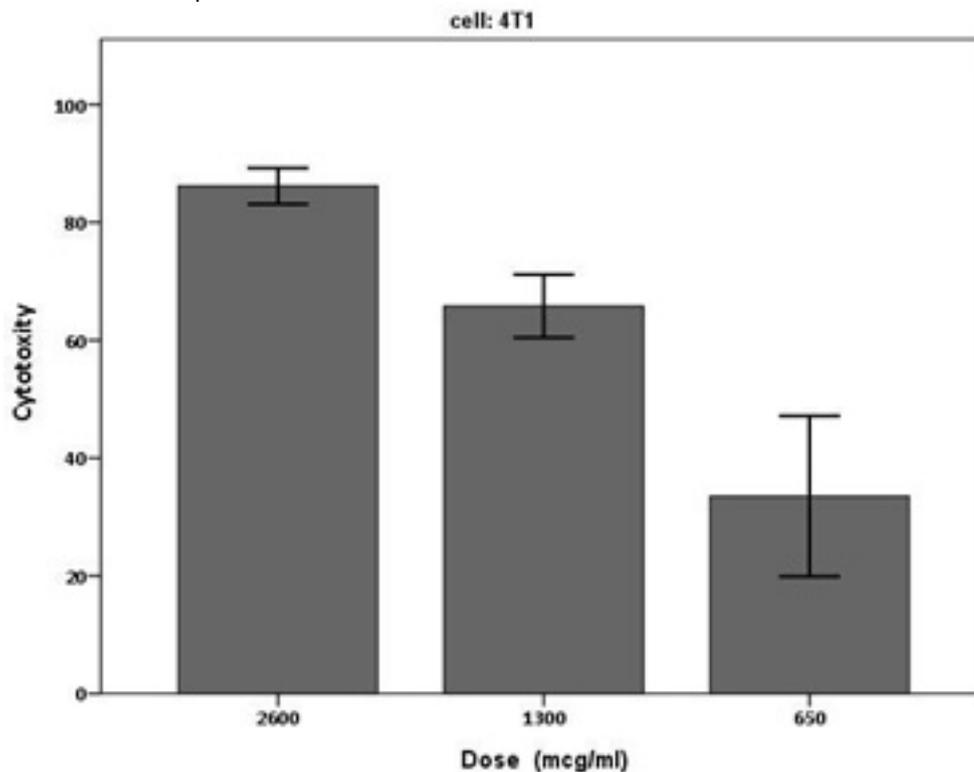


Diagram 3- The changes of caspase3 gene, affected by concentrations of 2600, 650 micrograms per milliliters of chamomile aqueous extract three hours after treatment

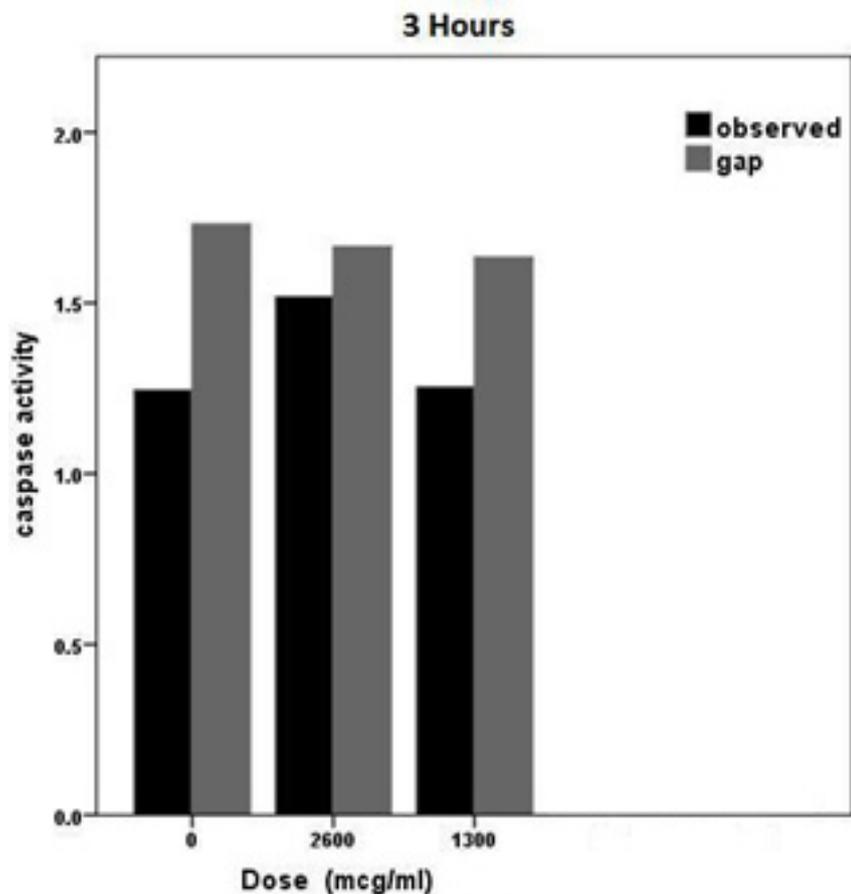
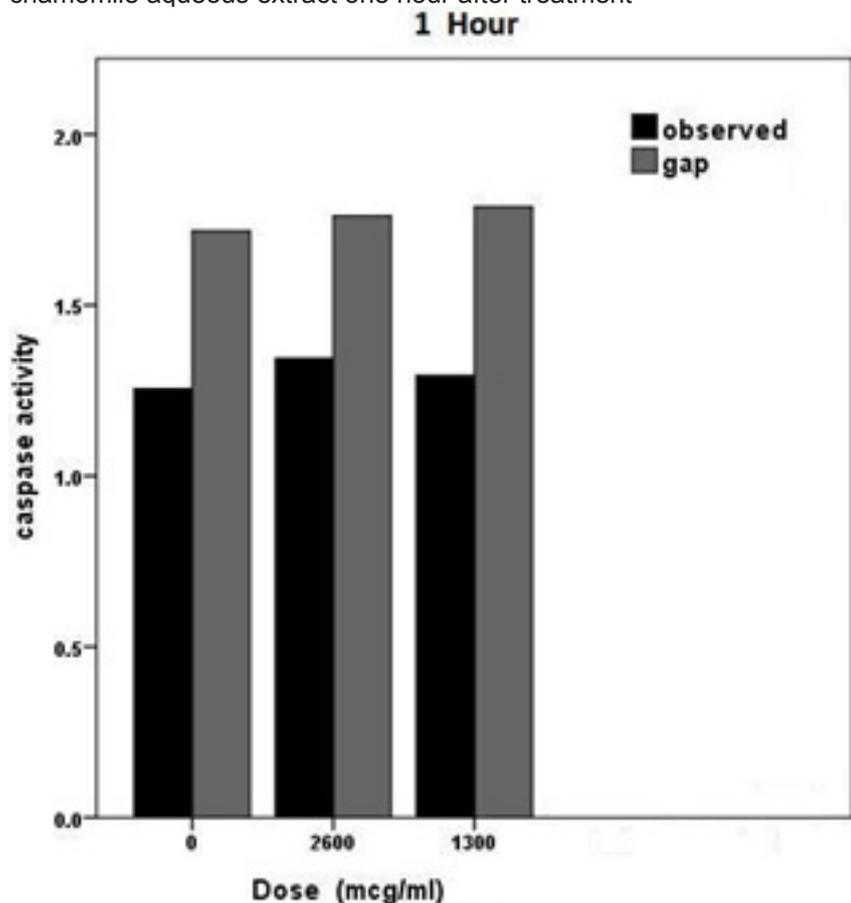


Diagram 4- The changes of caspase3 gene, affected by concentrations of 2600, 650 micrograms per milliliters of chamomile aqueous extract one hour after treatment



process to control cells proliferation and remove cancer cells (19), as the next step, the capability of apoptosis creation in breast cancer cells was investigated. Caspases are of pivotal intermediates in apoptosis mechanism. During cell death process, caspase3 activity first begins being influenced by apoptosis stimulus. By proteolytic failures, this caspase then causes caspase 8 and 9 activation which are of great importance in the process of beginning apoptosis. It was shown in this study that treating T-47D cells with chamomile extract increases caspase3 activity. The apoptotic effects of chamomile through flow cytometry method was investigated in the past studies but its accurate mechanism is unknown.

In this study, the obtained results of morphologic investigation and cells survival test through MTT method showed that inhibitory effect of chamomile aqueous extract began from the low concentration (325 micrograms per milliliter) to 2600 micrograms per milliliter from the initial hours and after 24 hours of treatment, inhibitory effect increased on cancer cells. The obtained results show that in different times, by increasing the concentration, the rate of cancer cells survival decreases, that is probably because of extract effect on apoptosis path. Cytotoxic effect of chamomile aqueous extract can be applied through increasing the rate of caspase3 gene expression. The results of this study generally showed that time and concentration-dependent chamomile aqueous increased proliferation inhibition in breast cancer cells (caspase-dependent path).

In addition, to confirm previous findings, these data have proposed some evidence based on the effect of chamomile as an effective Chemo-preventive factor with apoptotic effect. The commentary studies in cell levels are recommended to find the mechanism of chamomile (Flavonoids) in molecular dimensions and also clinical studies for confirming the effectiveness of this compound on treating cancer.

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