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ORIGINAL ARTICLE

Effect of Trans Chalcone on Mammary Induced Cancer in Balb/c mice

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ABSTRACT
Cancer is uncontrollable growth of cells. Breast cancer is one of the most prevalent types of cancer mainly treated through chemotherapy that produces significant side effects. Nowadays, natural components like Chalcone are widely used in cancer treatments. This study is going to investigate effects of inducing apoptosis by trans-chalcone in 4t1 breast cancer cells on Balb/c mice. Drug toxicity is measured by MTT assay during 24 and 48 hour time period at concentration of 20, 30, 40, and 50 μM. Haematoxylin-Eosin examination is conducted after treating tumor induced mice. Then, significance of results is assessed through SPSS and ANOVA ($p < 0.05$). The drug toxicity has been measured as to be 50% based on MTT assay during 24 hour time period for all concentration. Apoptosis has been also observed in histological study.Trans chalcone medicines can be used as an effective therapy in treating breast cancer.
Keywords: trans chalcone, breast cancer, Balb/

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INTRODUCTION

Breast cancer is one of the most common cancers has been diagnosed in women across the world. There are more than two million women suffer from breast cancer across the world at present. Breast cancer is the second major cause of death in women. Studies show that the breast cancer is the most prevailing type of cancer between North America and Eastern Europe's women. It has been estimated that every 1 out of 8 American women suffer from breast cancer that is on a rise in industrial countries. Breast tumor in most women develops into metastatic cancer that leads to their death. In Iran, the breast cancer is also recognized as one of the most prevalent types of cancers. Based on studies, the breast cancer occurs in women aged between 15 and 84 while those between 40 and 49 are more vulnerable to it. Recently, the rate of breast cancer has been reported as to be about more than 5000 cases a year that will have increased to more than 15000 cases a year by 2030. The breast cancer is mostly treated by chemotherapy, radiotherapy, and operation. Despite advances made in the chemotherapy and radiotherapy in the last decade, there are still far from producing desirable effect in treating the breast cancer and the metastatic breast cancer is still incurable and remains as a significant clinical challenge. Inherent or acquired resistance of cancer cells to chemotherapy is the major problem leading to death of patients suffering from the breast cancer [1-2].

In the Western countries, the breast cancer mainly incur among women above 50 year old while, according to studies conducted in Iran, the number of patients with the breast cancer aged between 40 and 49 is more than other age ranges. In addition, the young population of patients with breast cancer in Iran is more than that of in the Western countries [3].

Given the importance of breast cancer in women and tsunami of breast cancer in Iran and important role of women in Iranian society as mother and wife, this paper chose the $4t_1$ breast cancer cells in mice as the case of study and conducted an in vivo study on this sort of cancer.

Generally, many chemical medicines used in chemotherapy bring about changes in cell division process leading to a halt in proliferation and differentiation of cancer cells. It is accomplished through a wide variety of methods such as induction of apoptosis, alteration in DNA structure, Inhibition of

topoisomerase, tyrosine kinases, Inhibition of mitosis, inhibition of transcription, inhibition of replication and so forth [4-5].

In addition to importance of cytotoxicity effects of the given medicines on the cancer cells, the lowest side effects of them on healthy cells is also of clinical importance in synthesizing the mentioned drugs [6]. Morphologic properties of process of cellular apoptosis include cell size reduction, peripheral heterochromatin condensation, shrinkage of the cytoplasm, and phagocytosis of fragmented spherical cytoplasmic bodies by macrophage or Phagocytic cells. Apoptosis provides solutions to anti-cancer treatment. Many components have been reported so far that can produce anti-cancer effects through induction of apoptosis. Accordingly, today, drug intervention receives attention as one of interesting strategies in the chemotherapy to facilitate death of malignant cells through induction of apoptosis [7-9].

In a study conducted to examine effect of strawberry extract on the breast cancer in T47D cell and tumor-implanted mouse, it was revealed that the death caused by the toxification of strawberry extract is of apoptosis type. The strawberry extract contains antioxidant and flavonoid components. The study showed that the strawberry extract could activate P27 when TP53 was mutated that led to active apoptotic cascades [10].

According to results of studies on effect of trans chalcone and nitro chalcone produced by staining haematoxylin-eosin, it would be concluded that cell size reduction, peripheral heterochromatin condensation, shrinkage of the cytoplasm, and phagocytosis of fragmented spherical cytoplasmic bodies are caused by apoptosis death caused, which is the result of treating cancer with chalcones.

To cause apoptosis death, genes relevant to this pathway must be activated. However, determining type of internal and external pathways of apoptosis and type of activated genes require performing further studies.

Chalcone

Chalones are of flavonoids or isoflavonoids nature widely found in dietary plants. Chemically, flavonoid has open chain consisting of two aromatic rings connected to each other by 3 unsaturated carbons. Chalone is a type of flavonoid with massive biological activities that is characterized by anti-inflammatory, anti-invasive, antitumor, and antibacterial properties [11].

Chalcones can make apoptosis induction and mitochondrial respiration. The effect of diverse types of chalones against cytotoxicity of various human cell lines, including breast adenocarcinoma (MCF-7), lung adenocarcinoma (A549), prostate cancer (PC3), colon adenocarcinoma (HT-29), and liver normal cells (WRL-68), has been investigated in previous studies. Most compounds were a cytotoxic agent. Of the chalcones, 1,5,23, 25 components were able to produce apoptosis. Performing cytotoxicity assessment and 3,7,8,9 caspase activities (enzymes involved in apoptosis) assay, it was revealed that level of ROS (reactive oxygen species) increases.

Accordingly, it is concluded that ROS increases in this compounds and, consequently, directs apoptosis in MCF-7 cells [12].

Trans chalcone

Trans chalcone is one type of chalcones. Trans chalcones provide protection against stresses caused by hydrogen peroxide H_2O_2 (oxidative stress). Free radicals and, above all, ROS derivatives are most important factors contributing to oxidative stress reaction. Today, radical damage is viewed as an effective factor in many diseases, namely Alzheimer, Atherosclerosis, and cancer [13].

Anti-proliferative property has been found in a great number of Chalcone compounds like xanthahumol and their effects have been proven in vitro situations. Xanthahumol is an anti-cancer medicine working through a wide range of biological activities. Phenolic compounds could produce highly beneficial effects on health and have anti-inflammatory, anti-proliferative, and anti-viral properties. Therefore, flavonoids are main part of human diet. Structure of these antioxidants consists of more than 4000 thousands functional structures (moiety). Accordingly, they seem to be of great use in treating cancer [14].

MATERIAL AND METHODS

Epithelial cells of mouse breast issue were used in this study. Table (1) shows the properties of this cell type.

Tab. 1 identification of cell type: $4T_1$ [http://www.atcc.org]

Organism	Mus musculus, mouse
Strain	BALB/cfC3H
Tissue	Mammary gland
Disease	This tumor is an animal stage IV human breast cancer
Morphology	Epithelial
Growth properties	adherent

10 to 20 percent of Fetal Bovine Serum (FBS) has been given to the epithelial cells in RPMI1640 medium. The given cells produce acid metabolites while growing that, in turn, causes a gradual change in color of cell culture medium from purple to yellow. That is why the cell culture medium is usually changed every 24-48 hours.

There are various methods indirectly measure survival and proliferation rate of cells. Most of these methods measure activity rate of a cell enzyme inside a living cell. MTT assay is one of most recognizable methods to do so. MTT assay is a quantitative test used to measure proliferation rate of cells in face of different factors and to determine toxicity level of this factors on the cells. This assay is based on ability of mitochondrial reductase enzyme of living cells to resuscitate and transform MTT yellow tetrazolium rings to insoluble purple crystals of Formazan, which cannot pass through cell membrane. These crystals, then, become soluble by a detergent [9].

A reaction is made in the living cells during the MTT assay that is illustrated in figure (1).

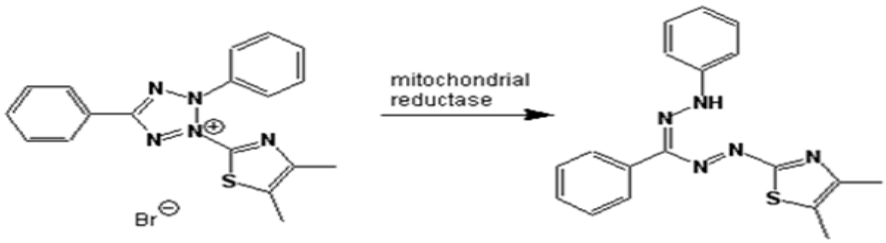


Fig. 1 the reaction made in living cells during the MTT assay

Optical density of resultant solution can be measured by spectrophotometer at 570 nm wavelength.

The resuscitation reaction, mentioned above, only happens in the living cells. Obtained numerical value is directly proportionate to number of the living cells. This measurement compare amount of Fromazan produced by treated cells with that of produced by control cells, which received no treatment [12].

Preparing concentrations of trans chalcone medicine

Molecular weight of trans chalcone equals to 208.26 micromole. That is, each mole of trans chalcone equals to 208.26gr of this substance. To reduce the error while preparing the medicine concentration, we first prepared a solution with higher concentration, e.g. 200$\mu\ mol\ /\ lit$.

$1\ mol$ $\quad$ $208.26\ gr$

$200\ \mu mol/lit$ $\quad$?

$208.26\ \mu mol/lir \times\ 200gr$=$41652\ \mu gr$ ⟹ $0.041652\ gr/lit$

0.041652 gr of this substance should be dissolved in 10 ml of DMSO according to the abovementioned calculation.

Results have been analyzed through SPSS19 and ANOVA and the difference in probability level less than 0.05 has been considered to be significant.

RESULTS

Having prepared cell suspension, the cells were treated by trans chalcone with 20,30,40,50 µM concentration after 24 hour since they had been planted in a 96 well plate. Next, the MTT assay has been conducted in 24 and 48 hour time periods.

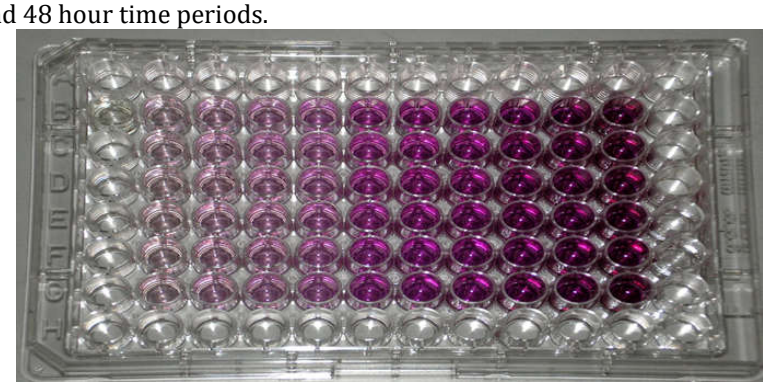
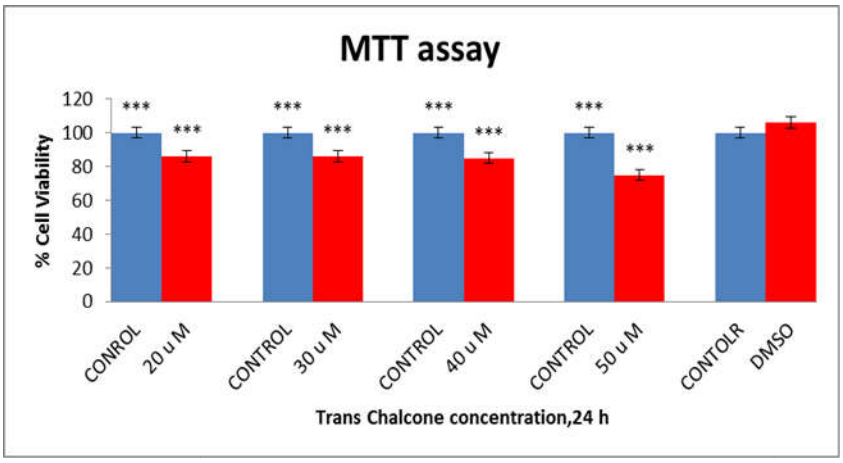


Fig. 2 The MTT assay for breast cancer cells treated by nitro chalcone and trans chalcone in 96 well plate

The control group, which did not receive the treatment, and treatment group, which received the trans chalcone, were studied at various concentration. The assay was repeated 3 times for each concentration of trans chalcone. Furthermore, o.5% DMSO was applied as pharmaceutical solvent in the assay.

Results obtained from conducting MTT assay on trans chalcone in 24 hour

Lack of 50% toxicity was observed 24 hour after treating the cells by different trans chalcone concentration. However, the highest toxicity belonged to 50 µM dose of medicine. Statistically, data was normally distributed and showed a significant result.

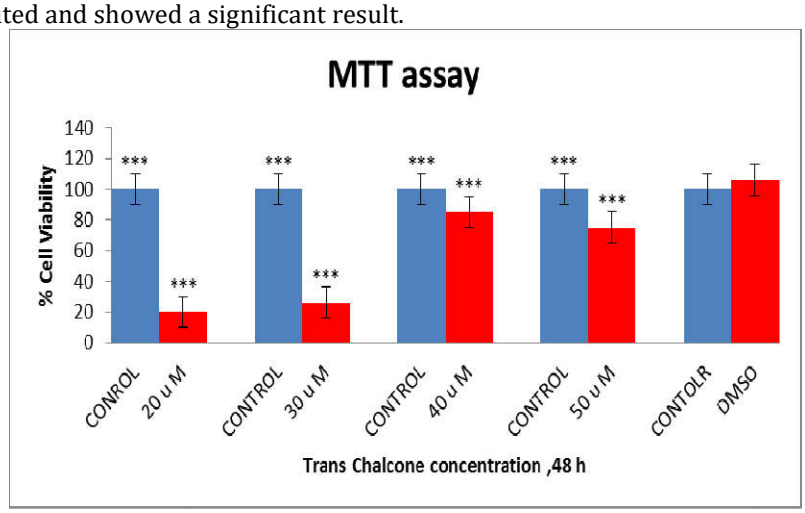


Diag.1 : The MTT results concerning treating $4T_1$ cells for 24 hour based on various concentration of trans chalcone (horizontal axis) and survival percentage of breast cancer cell treated by trans chalcone (vertical axis).

This assay was repeated 3 times on the treatment group with various concentration of trans chalcone for the control group, which did not receive the treatment. 0.5% DMSO, as the pharmaceutical solvent, and 20,30,40,50 µM of the medicine were used in the assay. Additionally, the level of significance was determined to be $p < 0.05$ through SPSS and ANOVA, which is shown by *.

Results obtained from conducting MTT assay on trans chalcone in 48 hour

50% toxicity was observed 48 hour after treating the cells by 20 and 30 µM concentration of trans chalcone. However, the highest toxicity belonged to 20 µM dose of medicine. Statistically, data was normally distributed and showed a significant result.



Diag. 2 : the MTT results concerning treating $4T_1$ cells for 48 hour based on various concentration of trans chalcone (horizontal axis) and survival percentage of breast cancer cell treated by trans chalcone (vertical axis).

This assay was repeated 3 times on the treatment group with various concentration of trans chalcone for the control group, which did not receive the treatment. 0.5% DMSO, as the pharmaceutical solvent, and

20,30,40,50 μM of the medicine were applied in the assay. Additionally, the level of significance was determined to be $p < 0.05$ through SPSS and ANOVA, which is shown by *.

Results of staining haematoxylin-eosin without pharmaceutical treatment

Picture below belongs to the group of mice received no treatment. Apoptosis did not happen in this group.

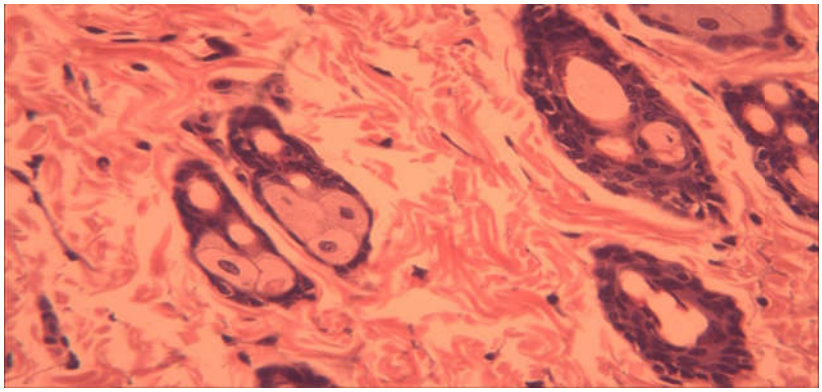


Fig. 3 Immunohistochemistry analysis of mice received no treatment (x40 zoom)

Haematoxylin-eosin staining has been used in this analysis while no apoptosis death cell happened.

Results of staining haematoxylin-eosin after treating with trans chalcone

Finally apoptosis observed in the cancer cells after mice received 12 mg/kg trans chalcone.

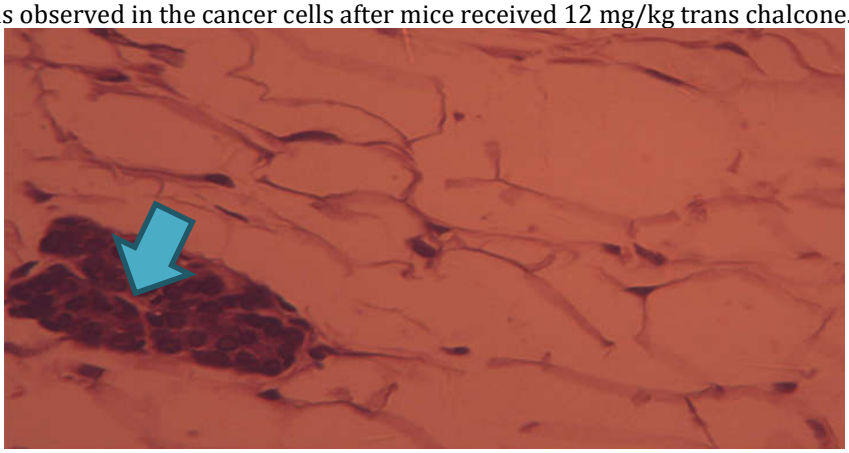


Fig. 4 Immunohistochemistry analysis of mice received treatment (x40 zoom)

Haematoxylin-eosin staining has been used in this analysis and the arrow shows the apoptosis death cell. The mice received 12 mg medicine in this phase.

DISCUSSION

This paper investigates the effect of chalcones (trans chalcone) due to their proven anticancer effects. Chalcones are natural compounds found in the plants; accordingly, their usage in chemotherapy will produce no side effects. Since, they have no or little toxic effects on the healthy cells as opposed to synthetic drugs.

Chalcone derivatives with anti-inflammatory properties are a good choice for cancer treatment considering main challenges lie in treating cancer, i.e. activation of immune system and inflammation.

According to a study conducted in 2014, flavonoid compounds include Furan rings producing anti-cancer effect on breast and colon adenocarcinoma in vitro situation [3].

In another study carried out on trans chalcone drugs, favorable level of toxicity of this drug (IC50) on $4T_1$ cell type is confirmed by results of MTT assay that proves trans chalcone capacity to induce death signals in vitro situation. Additionally, the said study reveals that toxicity level of IC50 is less than 50% in 24 hour treatment with trans chalcone. Trans chalcone drug have less toxic effect in 48 hour treatment.

In a study performed on Hepatocellular carcinoma (Hep G2) after trans chalcone treatment, it is observed that trans chalcone decreases H_2O_2 in doses higher than 20 μM and, then, protect the cells against oxidative stresses [2].

The results of given study also shows suitable level of toxicity for trans chalcone. However the level of toxicity rose by an increase in dosage of the medicine during 24 hour. The decrease in cell numbers is

likely to be relative to cells protection against oxidative stresses and, consequently, a decrease in their proliferation.

In an study conducted using 20 mg/kg trans chalcone in vivo and vitro situations the toxicity of the drug is observed to be suitable and the apoptosis cells death is also confirmed according to results of Immunohistochemistry and Western blot analysis [6].

The present study is performed in vivo and in vitro situations using 12 mg/kg trans chalcone and suitable level of toxicity is observed that confirms results of Immunohistochemistry and apoptosis. At the end, it could be asserted that the lower dosage of trans chalcone, used in this study, is more suitable that that of used in previous studies.

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