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Anti-tumor activity of ginger extracts in MCF-7 breast cancer cells

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A B S T R A C T

Breast cancer is the most common form of cancer in women, and the second leading causes of death in the world. Clinical efficacy of chemotherapy is limited due to side effects, toxicity, and drug resistance. Plant-derived anticancer drugs are new promising compounds, which show their anticancer activity through activation of apoptotic pathways. Ginger is a flowering plant with active phenolic compounds that exhibit anticancer activity. Here we studied the effect of ginger acetone, ethanol and methanol extracts on the MCF-7 breast cancer using MTT assay, Real time-PCR and normal inverted microscope. MCF-7 breast cancer cells were incubated with 2.5, 5 and 10 µg/mL of ginger extracts for 48 h. The survival of incubated MCF-7 cells with ginger extracts, indicated significantly decreased. The most dramatic effect was noted in cells incubated with acetone extracts, 10 µg/mL for 48 h compared to the control. Moreover, by increasing the concentration of ethanolic ginger extract, the cell vitality decreased significantly. Real-time PCR results showed that the expression of Bcl-2 was significantly decreased in cells incubated with ginger extracts. Decrease of Bcl-2 expression can cause increase apoptotic pathways and reduction of cancer cell survival. Thus, extract of ginger destroys breast cancer cells and other cancers cells and improves health.

Keywords: Cell culture; Ethanol extract; Apoptosis; MTT

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1. Introduction

Cancer is one of the most serious health problems in the world, which affects peoples of different generations, ages and breeds (Ayob & Ramasamy, 2018). Cancer is a group of diseases, which are diagnosed by uncontrolled cell growth, and cancer cells could spread to other parts of the body (metastasize). Cancer metastasis is the main cause of death related to cancer (El Kichaoui et al., 2016). In recent years, cancer is recognized as a leading cause of death (12%) worldwide (Siegel et al., 2016). After cardiovascular disease, cancer is the second leading cause of death in developed countries and the third leading cause of death in developing countries. If prevention and control strategies were not implemented, more than 85 million people will die in the world due to cancer over the next 10 years (Siegel et al., 2016). Cancer incidence and mortality are increasing exponentially, with 19.3 million new cases and approximately 10 million deaths in 2020 (Talib et al., 2022).

Breast cancer is the most common malignant disease observed in women, and it is the leading cause of death in developed and developing countries (Anderson et al., 2014). Despite the massive efforts put into cancer therapy, relatively little success has been achieved in some strategies. These efforts are limited due to experienced surgeons, lack of specificity, high costs, and side

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effects (El Kichaoui et al., 2016). Several types of therapy including mastectomy, chemotherapy, radiotherapy, hormonal therapy and targeted therapy are used to treat breast cancer (DeSantis et al., 2014).

Breast cancer is an abnormal growth in the epithelial cells of the breast tissue. This abnormal growth occurs in the lobules or in the ducts that connect the lobules to the nipple (Allred, 2010). Based on statistics analyses, breast cancer is the most common diagnosed cancer in women worldwide with 2.26 million new cases in 2020 (Łukasiewicz et al., 2021).

In spite of the therapeutic methodologies such as surgery, chemotherapy, and radiotherapy, the mortality rate still remains high for patients with cancer, which suggest that these strategies are not ideal (Chabner & Friedman, 1992). About 5-10% of all breast cancers are caused by inherited genetic factors. Early reports indicate that more breast cancers are associated with mutations in the BRCA1 and BRCA2 genes (Larsen et al., 2014).

The human genome consists of 3 Ras genes. The H-Ras gene is on the short arm of chromosome 11 at position 5 and 6.5 Kbp in the genome and has 6 exons. Accumulating evidence show that mutations in Exons 1 and 2 of the H-Ras may play significant roles in a variety of human cancers. The K-Ras gene on the short arm of chromosome 12 is located at position 1.12 and consists of 6 exons. The mutation in this gene is commonly seen in the cancers.

The mutation is common in exon 1 of K-Ras. The N-Ras gene is located on chromosome 1 at position 2.13 (Rajasekharan & Raman, 2013). Proto-oncogene RAS is a protein bound to the guanine nucleotide. It becomes activated in response to the binding of extracellular signals to the TCRs, RTKs, and PMAs.

RAS proteins are responsible for controlling cell signal pathways for growth, migration, adhesion, cellular structure, survival and differentiation (Rajalingam et al., 2007).

The Bcl-2 gene is located at position of 21.33q18 (Hwang et al., 2012). For the first time, the Bcl-2 oncogene was detected in a transposition between chromosomes 18 and 14 in B cell leukemia (Marzo & Naval, 2008). In the MCF-7 cells, the tumor burden and metastasis increases with the increased expression of Bcl-2. The Bcl-2 gene detected by immunochemistry has contributed to the prognosis of breast cancer, which is an independent factor (Redondo, 2013). In human, Bcl-2 oncogene is located on chromosome 18, and codes for a 24-kDa protein that appears in the core membrane, the endoplasmic network, and mitochondrial membrane (Hockenbery et al., 1990). Many oncogenes act by stimulating growth, but Bcl-2 prolongs the life of the cell without stimulating cell proliferation (Baer, 1994).

Traditional medicine still accounts for treatment of around 75-80% of the world's population, especially for developing countries, to treat, diagnose or prevent illness. Because it is believed herbal medicines are cheap and affordable without side effects. An ideal anticancer drug should be able to kill cancer cells without harming normal cells. This can be achieved through inducing apoptosis of cancer cells (Pal & Shukla, 2003). Plant-derived compounds are new promising anticancer drugs that show their anticancer properties by activating the apoptotic pathways (Pfeffer & Singh, 2018). According to recent studies, natural compounds in combination with chemotherapy have shown an ability to increase anticancer activity and overcome drug resistance (Dasari et al., 2022). Natural products have created promising avenues for cancer treatment (Hashem et al., 2022).

Zingiber officinalis belongs to the family of *Zingiberaceae*, which is distributed in India, China, Southeast Asia, Mexico, and other parts of the world (Banerjee et al., 2011). The anticancer properties of ginger (*Zingiber officinalis)* have been attributed to Vanilloid including 6-gingerol and 6-paradol, as well as other components such as shogoal and zingerone (Shukla & Singh, 2007).

Ginger has been used as a medicine in India and China for a long time (Kizhakkayil & Sasikumar, 2012). In India, ginger is daily used in cooking. About 50% of the medicinal remedy in Europe and USA originality used plant extracts or their constituents (Zeydi, 2016). In addition, plants have significant anti-cancer properties, and it has been estimated that more than 60% of currently used anti-cancer chemotherapeutic drugs are derived from natural products (Stankovic et al., 2011).

Due to its strong anti-inflammatory activities, ginger is considered as a medicine for osteoarthritis and rheumatoid arthritis (Shukla et al., 2007; Zick et al., 2009). Regarding the anticancer activity, it has been shown that ginger and its compounds prevent the proliferation and induction of apoptosis of different types of cancer cells in vitro (Hu et al., 2012). Also, the use of ginger to prevent colorectal cancer has attracted attention (Zick et al., 2015). Some works have shown that 6-gingerol, which is the major gingerbread phenolic compound of ginger can inhibit NF-KB activation, and increased expression of Caspase 3 and 9 gene in liver cancer (Abdullah et al., 2010). According to a previous study, ginger extracts significantly inhibited the growth of ovarian cancer cells by increasing the expression of P53 and inducing apoptosis (Pashaei-Asl et al., 2017). Also, ginger induces apoptosis in HL-60 leukemia promyelocytes through reduced Bcl-2 expression (Wang et al., 2003). Ginger also inhibited the growth and proliferation of skin carcinoma cells by inducing apoptosis through the disturbances in the BAX/Bcl-2 ratio (Nigam et al., 2009). However, the anticancer activity of ginger extract and its constituents against breast cancer has been poorly investigated

In this study, we examined the anticancer activity of ginger extract against breast cancer cells in vitro and investigated the anticancer properties of ethanolic, acetone and methanolic extracts of ginger on MCF-7 breast cancer cells. The effects of extracts on the expression of K-Ras and Bcl-2 genes and line cell morphology were also evaluated.

2. Material and Methods

2.1. *Preparation of herbal extracts*

The fresh ginger rhizomes were first grated, dried in an oven at 40°C for three days, and then powdered. Ginger powder (10 g) was added to 100 ml of ethanol, acetone, or methanol solvents. The mixtures were subjected to ultrasound waves for 10 min and then shaken for 24 h at room temperature. The mixture was passed through a filter paper and the solvent was removed from the filtrate by a rotary evaporator. The extracts were stored in a refrigerator at 4°C until needed. To prepare a stock solution from the extracts, 50 mg of dried powder was dissolved in 100 µL of DMSO using a vortexer. The mixture was filtered through a 0.2 micron filter and different concentrations (2.5, 5 and 10 µg/mL) were prepared from the stock.

2.2. *Cell culture*

A human breast cancer cell line (MCF-7) was purchased from the Iranian National Cell Bank (Pasteur Institute, Tehran, Iran). Cells were cultured in RPMI-1640 medium containing 10% fetal

bovine serum (FBS), penicillin (100U/ml) and streptomycin (100 µg/ml) (Sigma, Germany). The cells were grown at 37ºC in a humidified atmosphere containing 5% CO_2.

2.3. Cytotoxicity assay

The cytotoxic activity of ginger extracts on cultured cells was measured using the MTT assay (Behbahani, 2014). Briefly, cancer cells were cultured in 96 well plates and incubated with various concentrations of the extract (2.5, 5 and 10 µg/mL). Cisplatin was used as a positive control. Untreated cells were also used as a control group. After incubations for 48 h, MTT solution with a concentration of 5 mg/ml was added to the each well. After incubation at 37ºC for 2 h, 100 µL of DMSO was added to each well. Thirty minute after incubation at room temperature, absorbance at 570 nm was measured using a plate reader (Awareness Technology, USA).

2.4. Real time-PCR (RT-PCR)

The expression of Bcl-2 and K-Ras was determined using RT-PCR as described previously (Bong et al., 2006). Total RNA was prepared from the untreated and treated MCF-7 cells using an RNX PLUS kit (Cinnagen, Iran) according to the manufacturer instructions. The RNA was then transcribed to cDNA using a reverse transcriptase kit (Promega, USA). RT-PCR was performed using the Corbett Rotor-Gene 6000 system (Corbett Life Science, Australia). The primers are indicated in Table 1. Cycling conditions were as follows: initial denaturation step at 95°C for 3 min followed by 45 cycles at 95°C for 10 seconds, 58°C for 30 seconds and 72°C for 20 seconds. A negative control was included in each run to access specificity of primers and possible contamination.

Table 1. Primers Used for Real time-PCR.

Genes	Sequence of Primer (5´ to 3´)	GC %	TM
K-Ras	F TTCCTCAGGGCTCAAGAGAA	51/8	50
	R ATTGGGCAGCAAAGAGATGT	49/7	45
Bcl-2	F GTCTGGGAATCGATCTGGAA	51/8	50
	R CATAAGGCAACGATCCCATC	51/8	50
HPRT	F TATGGCGACCCGCAGCCCT	57/6	68
	R CATCTCGAGCAAGACGTTCAG	54/4	52

2.5. Morphological studies

Morphological studies of MCF-7 cell lines were performed using conventional inverted microscope. Untreated cells served as negative control. Morphological changes of cells were observed under normal inverted microscope after 48 hours after incubations with the ginger ethanolic extracts for 48 h.

2.6. Statistical analysis

All data were expressed as mean ± SD. Data were analyzed by SPSS statistical software (version 21 SPSS Inc.). One-way analysis of variance (ANOVA) followed by a Tukey post hoc test was performed to evaluate significance differences among different groups. Significant difference was set at $p < 0.05$.

3. Results

3.1. Effects of ginger extracts on cell viability

Cytotoxic effects of ethanolic, acetone and methanolic ginger extracts on cell viability were assessed using the MTT assay. As shown in Fig. 1, the acetonic extracts of ginger were more cytotoxic compared to the ginger ethanolic and methanolic extracts after 48 hours ($p < 0.05$).

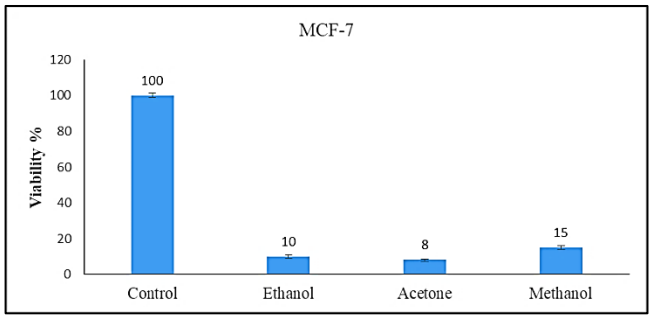

Fig. 1. The survival rate of MCF-7 breast cancer cells under the influence of 10 µL concentration of ethanol, acetone and methanol extracts of ginger after 48 h in MTT test.

3.2. Effects of different concentrations of ginger ethanolic extract on cell viability

Different concentrations of ginger ethanolic extract (2.5, 5 and 10 µg/mL) were tested for cytotoxicity against MCF-7 cells. The ginger ethanolic extract showed a dose-dependent cytotoxic activity in MCF-7 cells. There was a significant difference in cell viability between ginger ethanolic extracts-treated cells and untreated cells. The viability decreased with increasing concentration of ethanol extracts, which was significant among different concentrations in MTT test (Fig. 2; $p < 0.05$).

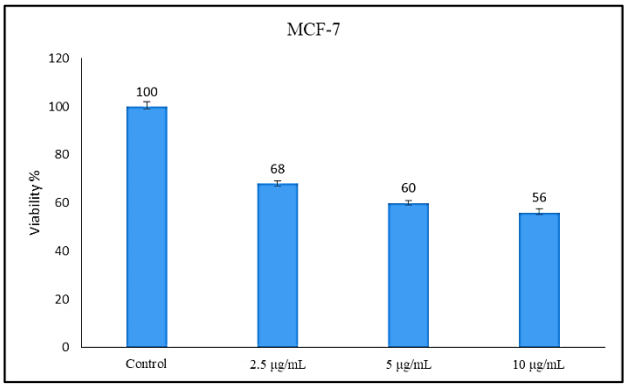

Fig. 2. The survival rate of MCF-7 breast cancer cells under treatment with 2.5, 5, and 10 µg/ml ethanolic extract of ginger after 48 h in MTT test.

3.3. *Effects of ginger ethanolic extract on expression of Bcl-2 and K-Ras*

The expression of Bcl-2 and K-Ras in MCF-7 cells incubated with ginger ethanolic extract (10 µg/mL) were determined by real time PCR analysis. Fig. 3 shows that the expression of Bcl-2 was decreased, while that of K-Ras was increased in cells incubated with ginger ethanolic extract (10 µg/mL) for 48 h compared with untreated cells ($p < 0.05$).

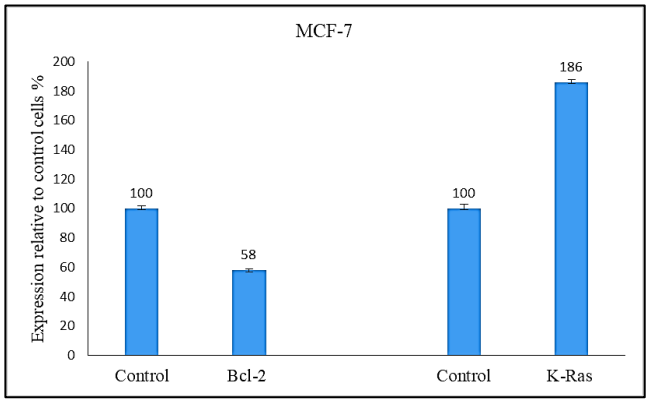

Fig. 3. Effects of ginger ethanolic extracts (10 µg/mL) on expression levels of Bcl-2 and K-Ras genes in MCF-7 cells.

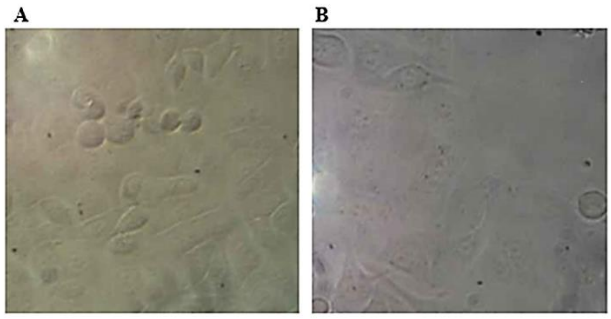

Fig. 4. Cell morphology of MCF-7 cell line with ginger ethanolic extract treatment for 48 h (B) and without treatment (A).

3.4. *Effects of ginger extract treatment on morphology of MCF-7 cells*

The results of morphological analysis of MCF-7 cells incubated with ethanolic ginger extract for 48 h are shown in Fig. 4.

4. Discussion

Despite of advances in developing surgery, chemotherapeutic drugs and radiotherapy, there is serious concern about cancer-related mortality. Also, the side effects of these therapies are of concern to patients. Nature is an enriched and reliable source of treatments. Ginger possesses potent anti-oxidant and anti-inflammatory activity (Shukla & Singh, 2007). Recent studies have shown the anti-cancer effect of ginger on various human cancer cells such as breast cancer (MCF-7), prostate adenocarcinoma (PC-3), cervical cancer (Hela), lung cancer (A549), and colon cancer (Pashaei-Asl et al., 2017).

Our work identified ginger as a medicinal plant reducing the viability of MCF-7 breast cancer cells. Furthermore, these results indicated that such treatment decreased the expression of Bcl-2 reducing cell viability.

Based on the cell viability results (Fig. 1), the ethanol, acetone, and methanol extracts of ginger affected on survival of MCF-7 cells. Also, the effect of acetone ginger extract on cancer cell survival was more substantial than ethanol and methanol extracts. According to the survival rate results (Fig. 2), increasing ginger ethanolic extract concentration further reduced the survival of cancer cells, which perhaps affects the apoptotic pathways. According to previous study, ginger extract can cause induce apoptotic in many cell lines such as head and neck squamous cell carcinoma (Kotowski et al., 2018), Human gastric cancer cell (Mansingh et al., 2018), Ovarian cancer cell (Lechner et al., 2019) and colorectal cancer cell (Saeedifar et al., 2020).

To elucidate the underlying mechanisms involved in the apoptotic properties of ginger extracts, the expression of apoptosis-related genes were evaluated. The data obtained from statistical analysis showed Bcl-2 expression was significantly decreased in cells incubated with ginger extracts compared with control samples, while K-Ras expression was increased compared with the control (Fig. 3).

B-cell lymphoma 2 (Bcl-2) protein is an anti-apoptotic protein, which can inhibit cell death. Its expression is also a molecular marker for risk of breast cancer recurrence (Merino et al., 2016). In this study, we found that the expression of Bcl-2 was decreased in cells incubated with ginger ethanolic extract (10 µg/mL) for 48 h compared with untreated cells. The effect of ginger ethanolic extract on reduction of expression of Bcl-2 gene has been also reported as a molecular mechanism in a recent study investigating the effects of Teucrium polium extract on human umbilical vein endothelial cells (HUVECs) viability (Sheikhbahaei et al., 2018). Study of the effects of apoptosis and anti-transplantation of jujube on MCF-7 breast cancer cells showed enhanced apoptosis associated with increased expression of BAX and decreased expression of the Bcl-2 (Abedini et al., 2016). Also according to another study, ginger extract can caused induced apoptosis due to Bcl-2 reduced expression (Zadorozhna et al., 2021; Chatupheeraphat et al., 2021). According to morphological results (Finger 4), observationally, there was a sharp difference between MCF-7 cells treated and untreated with ginger at the morphological level. This was agreement with MTT assay results.

In summary, our results indicate that increasing of ginger extract concentration reduces the survival of breast cancer cells, perhaps by affecting the apoptosis pathways. Bcl-2 expression was significantly decreased in cells incubated with ginger extracts compared with control samples. The increase in cell death can be attributed to the reduction in Bcl-2 expression. However, the K-Ras expression was increased in cells incubated with ginger extract compared with the control. Morphological analysis indicated that ethanolic ginger extracts caused significant morphological changes in MCF-7 cells compared to untreated cells.

5. Conclusion

Ginger is an herbal medicine, which has anti-oxidant, anti-inflammatory and anti-cancer activity. Our results established that ginger extracts can cause reducing of MCF-7 breast cancer cells viability. Based on these results, acetonic extracts were more

efficacious in decreasing the viability of MCF-7 cells compared to ethanolic and methanolic extracts. The increase in the ethanol extract of ginger concentration further decreases cancer cell survival, which may affect apoptotic pathways. Increase apoptotic pathways can be attributed to decrease Bcl-2 expression. Thus, ginger destroys breast cancer cells and other cancers cells and improves health.

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Conflict of interest

The authors have no conflict of interest to declare.

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