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GINGER ESSENTIAL OIL IN VITRO INHIBITS CELL GROWTH AND INDUCES APOPTOSIS IN MCF-7 HUMAN BREAST ADENOCARCINOMA CELLS

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ABSTRACT: Ginger plant (*Zingiber officinale*) is known as one of the important medicinal plant and dietary substances that are rich in phytochemicals. It has been used as a food flavoring agent as well as for traditional oriental medicinal purposes for centuries. Therefore, this study aimed to evaluate the inhibition of growth and induction of apoptosis (*in vitro*) in breast cancer cell line (MCF-7) using ginger essential oil (GEO). For this purpose, cells were exposed to 0, 20, 60 and 100 µg/ml of GEO for 72 hr. Cell viability and cell proliferation were performed by using trypan blue and 3-(4,5-dimethylthiazol-2yl)- 2,5-biphenyl tetrazolium bromide (MTT) assays. Cellular morphological changes were examined by phase contrast inverted microscopy. Furthermore, the mitochondrial transmembrane potential assay was evaluated by flow cytometry using tetramethylrhodamine, ethyl ester (TMRE) dye as an indicator of apoptosis. The results revealed that, human breast cancer cell line (MCF-7) was sensitive to the treatment with various GEO concentrations. Treatment of cells with GEO resulted in a reduction in cell viability, growth inhibition, distinct changes in cellular morphology and induction of apoptosis in a concentration dependent manner. The lowest percentage of viable cells was recorded as 20.68% at a concentration of 100 µg/ml of GEO. GEO was found to have cytotoxic activity against MCF-7 cells (*in vitro*) with an IC50 value of 10.12 µg/ml and induces apoptosis by loss of mitochondrial membrane potential ($\Delta\Psi m$). Our findings demonstrate that, GEO inhibited MCF-7 proliferation by inducing apoptosis and may have applications in the field of anticancer drug development.

Key words: Ginger oil, apoptosis, MCF-7 cells, TMRE, breast cancer cells.

INTRODUCTION

In both developed and developing countries breast cancer is the most frequent cancer. It is the most common type of cancer and the second most common cause of cancer death among females in Asia, Western countries and others (Farshori *et al.,* 2013). It is often referred to as one disease, but there are many types of breast cancer. Breast cancers can be divided into 2 major types, noninvasive (in situ carcinomas) and invasive (infiltrating) (Richie and Swanson, 2003). The first type may arise in either ductal or lobular epithelium, but remain confined there, with no invasion of the underlying basement membrane. The second type can start in the ducts or lobules, but then they spread into surrounding breast tissue (ACS, 2014). MCF-7 (human breast adenocarcinoma cell line) is a breast cancer cell line that was first isolated from a 69-year-old Caucasian American woman in 1970. It is the acronym of Michigan Cancer

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Foundation-7, referring to the institute in Detroit, USA where the cell line established in 1973 (Soule *et al.,* 1973). Since then it has become a prominent model system for the study of breast cancer (Simstein *et al.,* 2003; Hamedeyazdan *et al.,* 2012).

From ancient times, it has been known that, some essential oils from medicinal plants possess antibacterial, antifungal and antioxidant properties (Dagli *et al.,* 2015). Ginger is a rhizomatous plant grown in Asia, China, Japan, Austria, Latin America, and Africa (Sasidharan and Nirmala, 2010). Ginger extract contains numerous potent phytochemicals including gingerols, shogaols, zingerols, paradols and others (Jolad *et al.,* 2004). Shogaols and gingerols are the principal active pharmacological components of ginger (Plengsuriyakarn *et al.,* 2012). They have cytotoxic effects against lung, ovarian, and skin cancer cell lines (Kim *et al.,* 2008). In the last several years, many researchers have extensively studied ginger oil as an anticancer agent and they found that, ginger oil exert potential effects against cancer, skin problems, gastrointestinal tract diseases, and central nervous system disorders associated with oxidative and inflammatory stresses (Sang *et al.,* 2009; Dugasani *et al.,* 2010 ; Kubra and Rao, 2012). Furthermore, [6]-shogaol as one of the major active components of ginger has been shown to exhibit anticancer activities against breast cancer (Ling *et al.,* 2010), anti-proliferation activity, (Choudhury *et al.,* 2010) and anti-invasion of human hepatocellular cell (Weng *et al.,* 2010). To our knowledge, in the last few years, much current research has focused its attention to ginger given its anticancer and anti-inflammatory activities (Karimi and Roshan, 2013; Mariadoss *et al.,* 2013; Hakim *et al.,* 2014; Wee *et al.,* 2015 ; Jeena *et al.,* 2016), but unfortunately, there has been little research on cytotoxic activity of ginger essential oil against MCF-7 human breast cancer cells (Yuangang *et al.,* 2010). Our previous study has demonstrated a cytotoxic activity of fungal culture filtrates against MCF-7 cells (Abd El-Rahman *et al.,* 2014). In the recent decades, there has been a growing interest in the use of essential oils in the pharmaceutical industries (Coisin *et al.,* 2012), a systematic study of ginger oil has become very important. Therefore, this study aimed to evaluate the cytotoxic and apoptotic activities of GEO against MCF-7 human breast cancer cells.

MATERIALS AND METHODS

Consumables and Chemicals

Tissue culture plates and serological pipets were purchased from Biofil, Belgium. Commercially ginger oil was purchased from El-Captain Company, Cairo, Egypt. Penicillin streptomycin 10.000 units/ ml, L-glutamine 200 mM, fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) culture medium, were purchased from Gibco, Life Technologies, UK. Trypsin purchased from Serva, Heidelberg, Germany. All other specified reagents and solvents were purchased from Sigma Chemical Company Pvt. Ltd. St. Louis, MO, USA.

Ginger Essential Oil Preparation

Ginger essential oil was purchased from El-Captain company, Cairo, Egypt. Ginger oil (1 ml) dissolved in 1 ml dimethyl sulfoxide (DMSO), the resulting solution was diluted with Dulbecco's Modified Eagle Medium (DMEM) 1: 100 and stored at 4^0C until use.

Cell Line and Culture

Human breast adenocarcinoma cells (MCF-7) were obtained from research and education center pharma 2020, Kazan federal university, Russian federation. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin, streptomycin and 0.5% L-glutamine 200 mM at 37°C in a 5% CO_2 atmosphere with 95% humidity (ESCO, CelCulture, CO_2 incubator). Before beginning the experiments, viability of cells was assessed following the protocol of Siddiqui *et al.* (2008). MCF-7 cells showing more than 95% cell viability.

Experimental Design

Cells were plated in 96-well culture plates (5×10^3 cells/well) followed by incubation at 37°C for 24 hours for attachment and then exposed to various concentrations of GEO (0, 20, 60 and 100 µg/ml) for 24 hours to assess the cell viability assay by trypan blue dye, and cellular morphology by phase contrast inverted

microscope. Cell proliferation and mitochondrial transmembrane potential assays were determined by using MTT and TMRE dyes after 24 and 72 hr., of incubation with GEO respectively.

Trypan Blue-cell Viability Assay

Cell viability of the treated and untreated MCF-7 cells was assessed using the trypan blue cell viability assay. Trypan blue is a widely used assay for staining dead cells. The cell viability assay was performed by adding 50 µl of trypsinized cell suspension to 50 µl of 0.4% trypan blue in a 500µl Eppendorf tube and gently mixed by pipetting up and down. The final solution was allowed to stand at room temperature for 2-3 min and cells were counted using the hemocytometer. The percentage of viable cells was calculated by the following formula:

Viable cells (%) = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100.

MTT-cell Proliferation Assay

MCF-7 cell proliferation was determined by MTT assay (Mosmann, 1983). Cells were plated at a density of 5×10^3 cells per well in 96 well plates and incubated for 24 hr., at 37ºC for attachment and then treated with various concentrations of GEO (0, 20, 60 and 100 µg/ml) followed by incubation at 37ºC in 5% CO_2 incubator for 72 hr. Cells treated with medium only served as a control group. After incubation for 72 hr., the supernatant was discarded and 10µl of MTT dye (5 mg ml^{-1} in PBS) with 90 µl of medium was added to each well and incubated at 37ºC for 4 hr. Then the purple-colored precipitates of formazan were dissolved by gently shaking for 10 min in dimethyl sulfoxide (100 µl). After complete dissolution, the absorbance intensity measured by a microplate reader (TECAN Infinite M200, USA) at 555 nm. The effect of GEO on growth inhibition was assessed as percent growth inhibition of cells and calculated according to Patel *et al.* (2009), using the following formula:

Cell inhibition (%)=100-[(At-Ab)÷(Ac-Ab)]×100

Where:

At = Absorbance value of test compound; Ab = Absorbance value of blank; Ac = Absorbance value of control

Half-maximal inhibitory concentration (IC_{50}) values of test substances were calculated from at least three independent experiments using linear regression of the dose-log response curves by SOFTmaxPro.

Morphological Changes

Morphological changes in human breast adenocarcinoma cells (MCF-7) exposed to various concentrations of GEO were examined using a Zeiss Axio Vert. A1 microscope at 40X magnification. Treated cells were compared with control cells to can detect the morphological changes.

Tmre-mitochondrial Transmembrane Potential Assay

Loss of mitochondrial transmembrane potential was assessed by flow cytometry (Guava easycyte 8ht), using the fluorescent lipophilic cationic dye TMRE. TMRE dye is a red-orange dye, positively-charged, cell permeant that readily accumulates in active mitochondria due to their relative negative charge (Chazotte, 2011). TMRE accumulation in mitochondria is driven by $\Delta\Psi m$; therefore, depolarization of $\Delta\Psi m$ can be represented by the loss of TMRE staining (Akao *et al.,* 2002). After treatment MCF-7 cells with 10.12 µg/ml of GEO (IC50) for 24 hr., cells were stained with 200 nM TMRE for 20 min at 37°C, then washed twice in medium and re-suspended in PBS. Some cells were incubated with 20 mM carbonylcyanide-p-trifluoromethoxy phenylhydrazone (FCCP) for 10 minutes prior to staining with TMRE, served as a positive control for depolarized mitochondria. The TMRE fluorescence density was analyzed using (Guava easycyte 8ht) flow cytometry. The data are presented as percentage of stained cells.

Statistical Analysis

Statistical analysis was conducted using the statistical analysis software SPSS (version 20). Data are expressed as mean ± standard deviation (SD) of experiments each conducted in triplicate. Analysis of variance (ANOVA) was conducted to examine the differences between

treatments followed by LSD multiple comparison test. P-value of < 0.05 was considered to be statistically significant.

RESULTS

Cell viability

After 24 hr., of incubation with various concentrations of GEO (0, 20, 60 and 100 µg/ml), the percentage of MCF-7 viable cells was determined. The percent cell viability in MCF- 7 cells as observed by Trypan blue assay are presented in Fig. 1. Results showed that, GEO induced statistically significant ($p<0.05$) decrease in the percentage of MCF-7 viable cells in a concentration dependent manner. MCF-7 cells exposed to 20 µg/ml and above concentrations of GEO for 24 hr., were found to be cytotoxic, and cell viability was found 95.2, 62.51, 25.12 and 20.68% at 0, 20, 60 and 100 µg/ml of GEO respectively by Trypan blue assay (Fig. 1). Also, as the results showed that, the minimum percent of viable cells was 20.68% at a concentration of 100 µg/ml of GEO (Fig. 1).

Cytotoxicity Screening

Cytotoxicity of GEO was assessed using MTT assay. MCF-7 cells were exposed to GEO with various concentrations (20, 60 and 100 µg/ml) and incubated for 72 hr. After incubation, the percent growth inhibition of cells and half-maximal inhibitory concentration (IC_{50}) of GEO were determined. As shown in Table 1, the percent growth inhibition of MCF-7 cells treated with different concentrations of GEO (20, 60 and 100 µg/ml) for 72 hr., was recorded 85.5, 88.72 and 78.67%, respectively using MTT assay (Table 1). The maximum and minimum percent inhibition of MCF-7 cells were 88.72 and 78.67% at a concentration of 60 and 100 µg/ml of GEO, respectively. Furthermore, the IC_{50} value for MCF-7 cells was 10.12 µg/ml of GEO (Table 1).

Morphological Alterations

Changes in human breast adenocarcinoma cells (MCF-7) morphology were found to be concentration dependent manner. Morphological alterations observed in cells are shown in Fig. 2. Cells exposed to 20 µg/ml and above concentrations of GEO for 24 hr., reduced the normal morphology and cell adhesion capacity as compared to control (Fig. 2). MCF-7 cells exposed to 20, 60 and 100 µg/ml of GEO lost their typical morphology, cells were shrunken and appeared smaller in size (Fig. 2). Furthermore, it was noticed that, cells treated with 60 µg/ml of GEO for 24 hr., appeared abnormal with a rounded shape and spaces between cells were increased compared to those in the control group. Additionally, some cells shrank with a ruptured membrane at a concentration of 100 µg/ml of GEO, suggesting apoptotic cell death. The proportion of abnormal and dead cells increased with increasing GEO concentrations.

Mitochondrial Membrane Potential

Mitochondrial membrane depolarization was detected using a fluorescent dye TMRE in MCF-7 cells treated with GEO. The cytofluorometric analysis of mitochondrial $\Delta\Psi m$ is shown in Fig. 3a. The results presented in Fig. 3a and b had clearly shown that, GEO-induced apoptosis visualized by TMRE staining of MCF-7 cells treated with 10.12 µg/ml GEO (IC_{50}). The percent of MCF-7 stained cells was decreased sharply from 36.20% to 16.49 by 54.45% after treatment with GEO compared with the untreated cells (Fig. 3a) and cells appeared to contain a lower level of TMRE compared to control cells (Fig. 3b). Our findings suggest that, treatment of MCF-7 cells with GEO induces apoptosis because the cell population with relatively low TMRE staining became predominant in the samples exposed to GEO. Loss of mitochondrial membrane potential is indicative of apoptosis. This confirms that, GEO treatment caused more cells to undergo a reduction in mitochondrial membrane potential ($\Delta\Psi m$) and the effect of GEO in inducing apoptosis in MCF-7 cells involves a decrease of mitochondrial membrane potential.

DISCUSSION

Cancer is the cause of more than 6 million deaths worldwide every year (Janakiraman and Johnson, 2016). More than 700,000 new cases of cancer occur in Asian countries and this number is expected to increase each year (Kimman *et al.,* 2012). Breast cancer is the most common malignancy in women with nearly a half

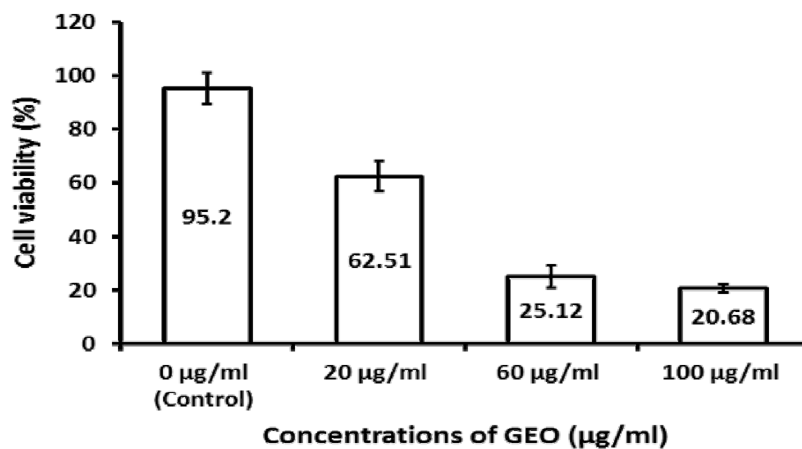

Fig. 1. Cell viability assessments using trypan blue assay following the exposure of various concentrations of ginger essential oil (GEO) for 24 hr., values are mean ± SD of three replicates ($p<0.05$ vs control)

Table 1. Cytotoxicity assessment of ginger essential oil (GEO) against human breast adenocarcinoma cells (MCF-7) as determined by the MTT assay

Concentration of GEO (µg/ml)	MCF-7 (%) inhibition ± SD	IC_{50}
20	85.50 ± 4.44	
60	88.72 ± 2.25	10.12
100	78.67 ± 3.22	

Results represented mean ± SD, n=3

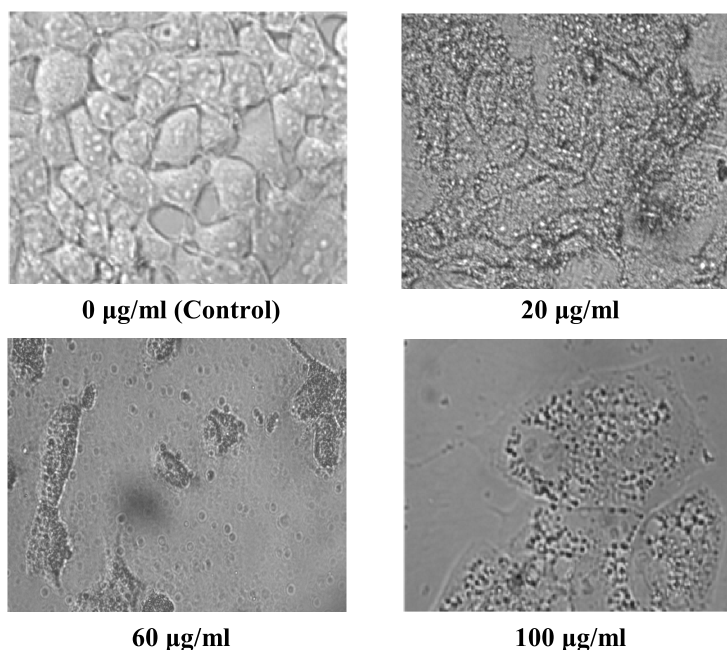

Fig. 2. Morphological alterations in MCF-7 cells exposed to various concentrations of ginger essential oil (GEO) for 24 hr, images were taken using a Zeiss Axio Vert. A1 microscope at 40 X magnification

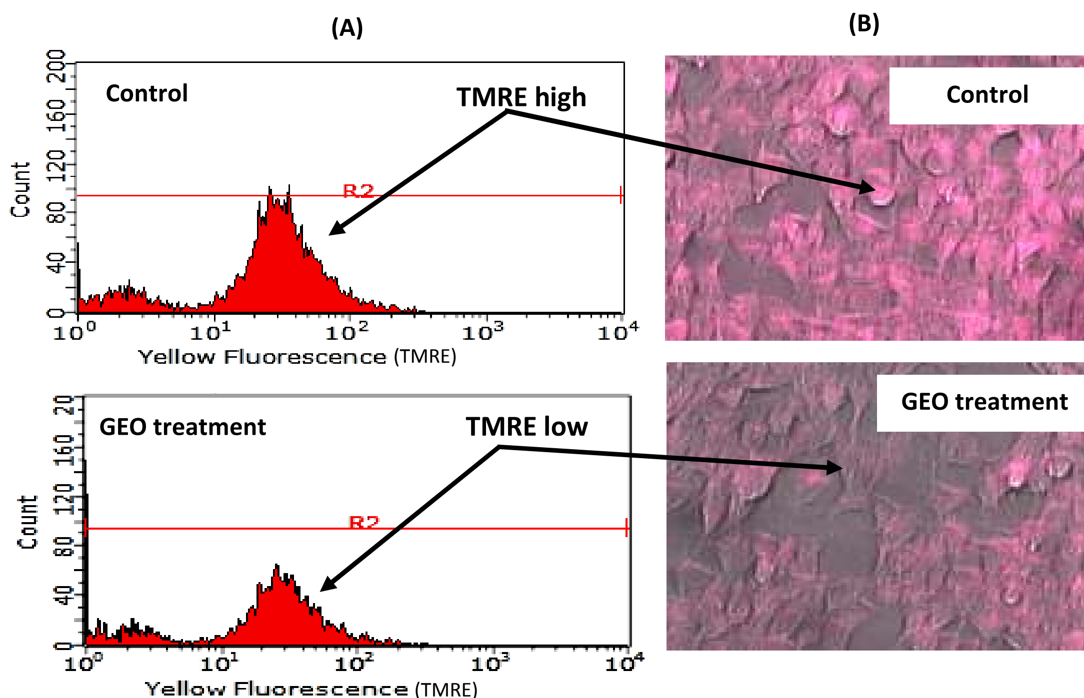

Fig. 3. Analysis of MCF-7 cells exposed to ginger essential oil (GEO) based on TMRE staining. (A): Cytofluorometric analysis of ΔΨm in MCF-7 cells. Upper fluorescence histogram, of MCF-7 control cells, lower fluorescence histogram of MCF-7 cells treated with GEO. (B): Representative images of MCF-7 cells after staining with 200 nM TMRE. Upper panel, TMRE ^{high} in MCF-7 control cells; lower panel, TMRE ^{low} in MCF-7 cells treated with GEO. Scale bar, 20 µm.

million deaths worldwide every year (Slaoui *et al.,* 2014 ; Jahanafrooz *et al.,* 2016). The rates of breast cancer vary worldwide being the highest in Europe, and they are increasing in Asian and Latin- American countries (Jung *et al.,* 2009). In the Arab world, breast cancer incidence rates differ according to region and country. In Egypt, it is the most frequent cancer among females. In Lower, Middle, and Upper Egypt the pattern was dominated by the high frequency of breast cancer (Ibrahim *et al.,* 2014). Surgery, chemotherapy, radiotherapy, and acupuncture point stimulation are used to treat cancer (Janakiraman and Johnson, 2016). These treatments may cause treatment-induced cancer pain and destroy the normal cells along with cancer cells; cancer cells can develop resistance to treatment through mutations (Wiseman and Spencer, 1998). Resistance to chemotherapeutic drugs is the main reason chemotherapy fails (Hutchins-Wolfbrandt and Mistry, 2011). So, there is still an urgent need for more effective cancer treatments. In the potential management of cancer, natural products derived from medicinal plants have gained significant recognition (Desai *et al.,* 2008). Active components derived from plants are important sources of new drugs that are likely to lead to new and better treatments for breast cancer (Cragg and Newman, 2003).

Ginger as a one of medicinal plants has long been used in traditional medicine. (Al-Suhaibani and El-Morshedi, 2015). Much research has focused their attention to ginger as anti-cancer (Shukla and Singh, 2007), anti-oxidant (Jeyakumar *et al.,* 1999) and anti-inflammatory (Park *et al.,* 1998) agent. A number of recent studies have investigated the cytotoxicity of ginger plant extracts against MCF-7 human breast cancer cell line (Rahman *et al.,* 2011; Park *et al.,* 2014 ; Ray *et al.,* 2015).

Ginger essential oil consists of a very complex mixture of various classes of organic compounds which is responsible for its antioxidant activity (El-Baroty *et al.,* 2010). It is a rich source of phenolic compounds such as shogaols, zingerone, gingerols and gengerdiols (Singh *et al.,* 2008). Recently, studies had focused on the anticancer activities of ginger essential oil (Jeena *et al.,* 2013 ; Jeena *et al.,* 2015), but not extensively studied its cytotoxic effects and mode of action in breast cancer cell lines. Currently, the study of the cytotoxic activity of ginger essential oil against human breast adenocarcinoma cells has become more important in the field of anticancer drug development. Hence, the current investigation has evaluated the cytotoxic and apoptotic activities of GEO against MCF-7 human breast cancer cells. To the best of our knowledge, this is the first investigation, that exhibited high cytotoxic activity of GEO against MCF-7 human breast cancer cells and its mechanism of cell death using mitochondrial mechanism.

To determine the cytotoxic effect of GEO against MCF-7 cells, we first studied the effect of GEO on the cell viability. Cell viability assay showed that, GEO induced decrease in the percentage of MCF-7 viable cells in a concentration dependent manner. We further verified this cytotoxic effect of GEO by MTT assay. The MTT assay is based on the reduction of MTT by mitochondrial dehydrogenase by purple formazan product. The GEO were subjected to MTT cell proliferation assay. Increased growth inhibition was observed in MCF 7 cells in a concentration dependent manner by MTT assay. Maximum percentage cell inhibition was observed in MCF 7 cells at a concentration of 60 µg/ml of GEO. As the concentration increased, there was a decreased in cell growth inhibition. It was 78.67% growth inhibition at 100 µg /ml. Furthermore, the IC_{50} value of GEO on MCF-7 cell line was 10.12 µg/ml. Moreover, morphological features in MCF-7 cancer cells after exposure to GEO indicated that, GEO has a substantial ability to alter the morphological and cytological structure of MCF-7 cancer cells.

A similar study conducted by Yuangang *et al.* (2010), conflicts the findings of our study. They found that, ginger essential oil exhibited the lowest cytotoxicity towards MCF-7 cells. The percentage of cell viability was 81.85% in their study. The difference in the results of both studies may be due to the source of oil used in both studies, which could lead to differences in oil constituents. Nevertheless, we can be certain that, the inhibitory effect of GEO on breast cancer cells was recorded for the first time in our study using the mitochondrial observation.

To describe the cell death mechanism induced by GEO, we used TMRE dye as fluorescent probes to monitor the membrane potential of mitochondria. TMRE is a positively-charged dye that use to label active mitochondria (Christensen *et al.,* 2013).

Determination of mitochondrial membrane potential ($\Delta\Psi m$) is widely used for characterization of cellular metabolism, viability, and apoptosis. The loss of mitochondrial membrane potential ($\Delta\Psi m$) is a hallmark of apoptosis (Ahamad *et al.,* 2014). Apoptosis (programmed cell death) is a normal process that occurs during development of cell. The process of apoptosis in case of cancer is uncontrolled. It is reported that, many chemotherapeutic agents exert their antitumor effects by inducing apoptosis in cancer cells (Kamesaki, 1998).

In the present study, we had shown that, GEO was able to induce apoptosis in MCF-7 cells. It has been observed that, there is a reduction in mitochondrial membrane potential ($\Delta\Psi m$) in MCF-7 cells treated with GEO, compared to those in the control group. The effect of GEO in inducing apoptosis in MCF-7 cells involves a decrease of mitochondrial membrane potential.

In conclusion, according to the results from current study it seems that, ginger essential oil may induce apoptosis and reduces cell viability in MCF-7 human breast adenocarcinoma cells. In addition, there is a growing evidence that ginger essential oil had higher anticancer activity against breast cancer. Therefore, essential oil derived from ginger has great potential to be used in the field of anticancer drugs and could be employed in the treatment of cancerous diseases.

Conflict of Interest

The authors declare that they have no conflict of interest.

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زيت الزنجبيل الطيار يثبط النمو ويحدث موت الخلايا المبرمج في خلايا سرطان الثدي MCF-7

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عرف نبات الزنجبيل كواحد من أهم النباتات الطبية وكأحد المواد الغذائية الغنية بالمركبات الكيميائية، ولقد استخدم لعده قرون كماده منكهةبالإضافة إلى استخدامه في العديد من الأغراض الطبية، لذا فقد هدفت هذه الدراسة إلى تقييم تثبيط نمو الخلايا وإداث موت الخلايا المبرمج في خلايا سرطان الثدي MCF-7 باستخدام زيت الزنجبيل الطيار، ولتحقيق هذا الهدف فقد تم تعريض الخلايا الي زيت الزنجبيل الطيار بتركيزات صفر ، ٢٠ ، ٦٠ ، ١٠٠ ميكروجرام زيت/مل معلق خلايا لمده ٧٢ ساعة لقد تم قياس حيوية ونمو الخلايا باستخدام صبغه ازرق التريبيان وصبغه MTT، بالإضافة إلى انه تم فحص التغيرات الخلوية المورفولوجيه باستخدام الميكروسكوب، علاوة على ما سبق فقد تم تقدير جهد نفاذية غشاء ميتوكوندريا الخلايا باستخدام جهاز التدفق الخلوي بواسطة صبغه TMRE كمؤشر لحدوث عمليه موت الخلايا المبرمج، وقد أظهرت النتائج أن خلايا سرطان الثدي MCF-7 كانت حساسة للمعاملة بتركيزات مختلفة من زيت الزنجبيل الطيار، حيث أن معامله الخلايا بزيت الزنجبيل الطيار أدت إلى حدوث نقص في حيوية ونمو الخلايا وحدوث تغيرات خلوية مورفولوجية ملحوظة بالإضافة إلى الحث على حدوث عمليه موت الخلايا المبرمج وذلك ارتباطا بتركيز الزيت الطيار، ولقد سجلت اقل نسبه مئوية لحيوية الخلايا بمقدار ٢٠,٦٨% عند معامله الخلايا بتركيز ١٠٠ ميكروجرام زيت/مل معلق خلايا، ولقد وجد أن زيت الزنجبيل الطيار له نشاط سام ضد خلايا سرطان الثدي MCF-7 بالإضافة إلى أن التركيز المثبط لنمو ٥٠% من الخلايا قد وصل إلى ١٠,١٢ ميكروجرام زيت/ مل معلق خلايا كما انه لديه القدرة على تحفيز عمليه موت الخلايا المبرمج عن طريق فقد الخلايا لجهد نفاذية غشاء الميتوكوندريا، وبالتالي فان النتائج التي توصلنا إليها تثبت أن زيت الزنجبيل الطيار لديه القدرة على تثبيط نمو خلايا سرطان الثدي MCF-7 عن طريق إحداث موت الخلايا المبرمج وبالتالي فمن الممكن ان يكون له تطبيقات في مجال تطوير الأدوية المضادة للسرطان.

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