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# Inhibitory activity of ginger oil against breast cancer cells

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# INHIBITORY ACTIVITY OF GINGER OIL AGAINST BREAST CANCER CELLS

A Thesis  
Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

In

The Department of Food Science

By  
Namrata Karki  
B. Tech., Kathmandu University, 2008  
August, 2011

**Dedicated to my parents;**

Naresh Karki and Nanita Karki

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## ABSTRACT

Breast cancer is the leading cause of cancer related mortality in women. The estimated new cases and death for 2010 in the US for females were 207,090 and 39,840 and for men were 1,970 and 390 respectively. Breast cancer cells contain cancer stem cells (CSCs), which represent less than 5% of the total cancer cell population, but accounts for continuous proliferation and growth. Breast cancer is highly metastatic and can spread to various parts of the body. Signaling through cell surface markers, including CD44/CD24/ALDH1 in breast cancer, pro-survival factors, including Hsp90 and other client proteins, angiogenic biomarkers and telomerase activity are essential for cancer cell renewal and could represent putative targets for inhibiting breast cancer cell development. The aim of this research was to determine the *in vitro* anti-carcinogenic property of ginger oil against breast cancer cells (MDA-MB-231). We investigated the inhibitory activity of ginger oil against the biomarkers that are essential for breast cancer cell viability or proliferation. Cells were incubated with vehicle (DMSO), or ginger oil at concentrations, 0.05% to 0.2% of the volume of the media for 72 h at 37 °C and 5% CO<sub>2</sub>. Ginger oil dose-dependently inhibited the viability and proliferation of breast cancer cells *in vitro*. The mechanism of breast cancer cell inhibition included induction of caspase-mediated apoptosis, cell cycle arrest, inhibition of Hsp90 and some of its client proteins, down-regulation of cell surface biomarkers, CD44/ALDH1 in breast cancer cells, inhibition of the biomarkers of angiogenesis, and down-regulation of the activity of telomerase.

## CHAPTER 1: INTRODUCTION

Interest in dietary bioactive compounds to prevent chronic degenerative diseases and enhance health arises from evidence supporting a role of these compounds in human health. Chronic degenerative diseases are of interest because there are no known cure for most of these diseases and currently available treatments usually only provides a temporary reduction of symptoms. Breast cancer as a chronic disease is a commonly identified cancer in female in both economically developed and developing country (Jemal and others 2011). There are various treatments approaches that have been aimed towards the treatment of the disease but the success rate of the chemotherapeutic drugs are reported to be low with high rate of recurrence and various side effects.

Natural compounds or dietary agents have been used as an important tool to treat cancer because of its low cytotoxicity and less adverse effects. Ginger because of its aroma and taste, has been used for culinary purposes from ages. It has also been reported in use for medicinal purpose for more than 2500 years. Ginger is found to be rich of bioactive compounds and various researches have been carried out to explore the beneficial property of ginger and its extracts (Grzanna and others 2005; Ali and others 2008; Sharma and others 1994). Essential oils of ginger are of interest because of its richness in various functional compounds, mostly terpenes; monoterpenes and sesquiterpenes, which gives the oil its biological activity(Daferera and others 2002). These bioactive compounds can either remain unmodified or undergo metabolic transformation into active or inactive derivatives and to fully understand the potential of ginger oil in human health requires an understanding of the effect of bioactive compounds in different regulatory mechanism from molecular and cellular level.

Our aim was to evaluate the effect of oil from ginger against breast cancer. The main objectives of this study were to:

- 1) Determine the effect of ginger oil on the viability of breast cancer cells
- 2) Determine the effect of ginger oil on induction of apoptosis in breast cancer cell
- 3) Determine the anti-angiogenic activity of ginger oil *in vitro* using human coronary artery endothelial cells and hormone-independent breast cancer cells.
- 4) Determine the effect of ginger oil on the replicative potential of breast cancer cells
- 5) Determine the effect of ginger oil on expression of various signal transduction molecules in breast cancer cells

## CHAPTER 2: LITERATURE REVIEW

### 2.1 Breast Cancer

According to National Cancer Institute at the National Institute of Health, breast cancer is defined as, “Cancer that forms in tissues of the breast, usually the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk). It occurs in both men and women, although male breast cancer is rare.” Breast cancer is the leading cause of cancer related mortality in women. The estimated new cases and death for women in 2010 in the US were 207,090 and 39,840 and for men were 1,970 and 390 respectively.

The rate of breast cancer in female in developed countries like Northern Europe, Australia/New Zealand and North America, has been reported to be 2-5 times higher than in developing countries Asia and sub-Saharan Africa, as shown in figure 2.1 (Jemal et al. 2011). Some of the major causes of higher incidence of cancer are due to the adopted life-style of “westernized diets”, lack of physical activity, alcohol, smoking and many others.

Different types of cancer originate from and maintained by a small population of cells within, which are known as cancer stem cells. They represent less than 5% of the total cancer cell population and these cells are also the key to breast cancer aggressiveness and lethality. . Cancer stem cells possess the ability of self-renewal and differentiation. This small population of cancer stem cells forms a bulk tumor by using several self-renewal and differentiation pathways and are resistant to chemo- and radiation therapy. Therefore efforts have been made to target these pathways in order to abolish the recurrence and resistant behavior of cancer stem cells (Reya and others 2001). Breast cancer stem cells are characterized as  $CD44^+/CD24^-/AlDh1^+$  cells (Charafe-Jauffret and others 2010).

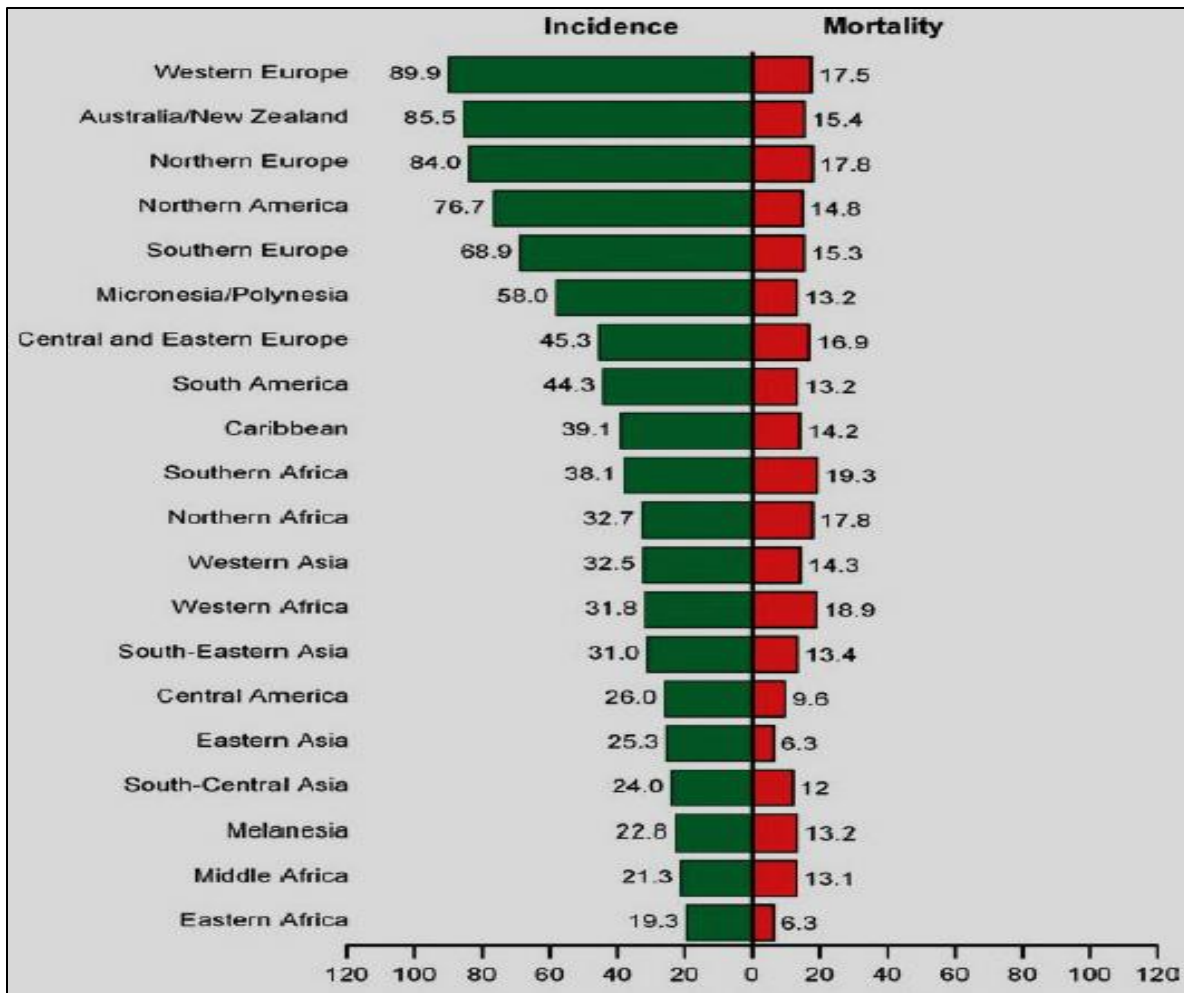


Figure 2.1: Incidence and Mortality Rate of Breast Cancer (GLOBOCAN 2008)

Breast cancer can metastasize to lymph nodes and distant organs from the primary site, which is usually the cause of death. As shown in figure 2.2, the data presented by Lee et al. suggests that the rate of metastasis of breast cancer cells to organs like lung, liver and bone are higher than to other different organs (Lee 1983). Various treatment strategies for patients with breast cancer have been developed like, chemotherapy, radiation therapy, hormonal therapy or adjuvant therapy that target specific properties of cancer cells. These therapies increases the rate of survival along with increased long term side effects (Weigelt and others 2005). To progress in

search for cure of cancer requires understanding of the properties of cancer, which are briefly explained in section 2.2.

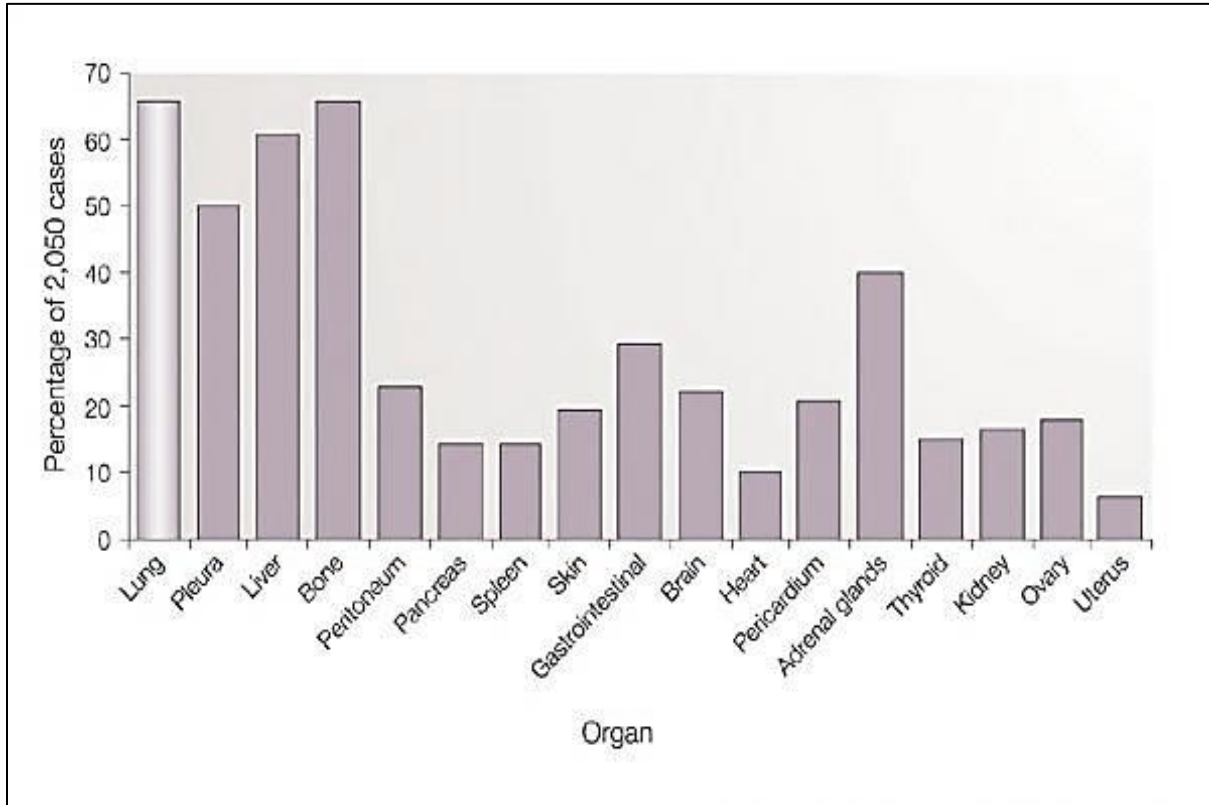


Figure 2.2: Primary breast cancer cells metastasis to various distant organs (Lee 1983).

## 2.2 Features of Cancer Cells

Most cancer cells have similar characteristics present in a normal cell except that some of the cells signaling processes are highly up regulated while some of them are heavily down regulated. Causes of cancer can be inherited through genes or can be adapted due to several activities like exposure to carcinogens. As biomarkers are defined as molecular, cellular, functional measurable parameters indicative of a particular genetic, epigenetic or functional status of a biological system (Ludwig and Weinstein 2005), cancer cell biomarkers, which are the property of tumor cells can be used for diagnosis, prognosis, staging and treatment.

As described by Hanahan and Weinberg, the characteristics of cancer cells can be listed in six different categories as described below and also shown in figure 2.2:

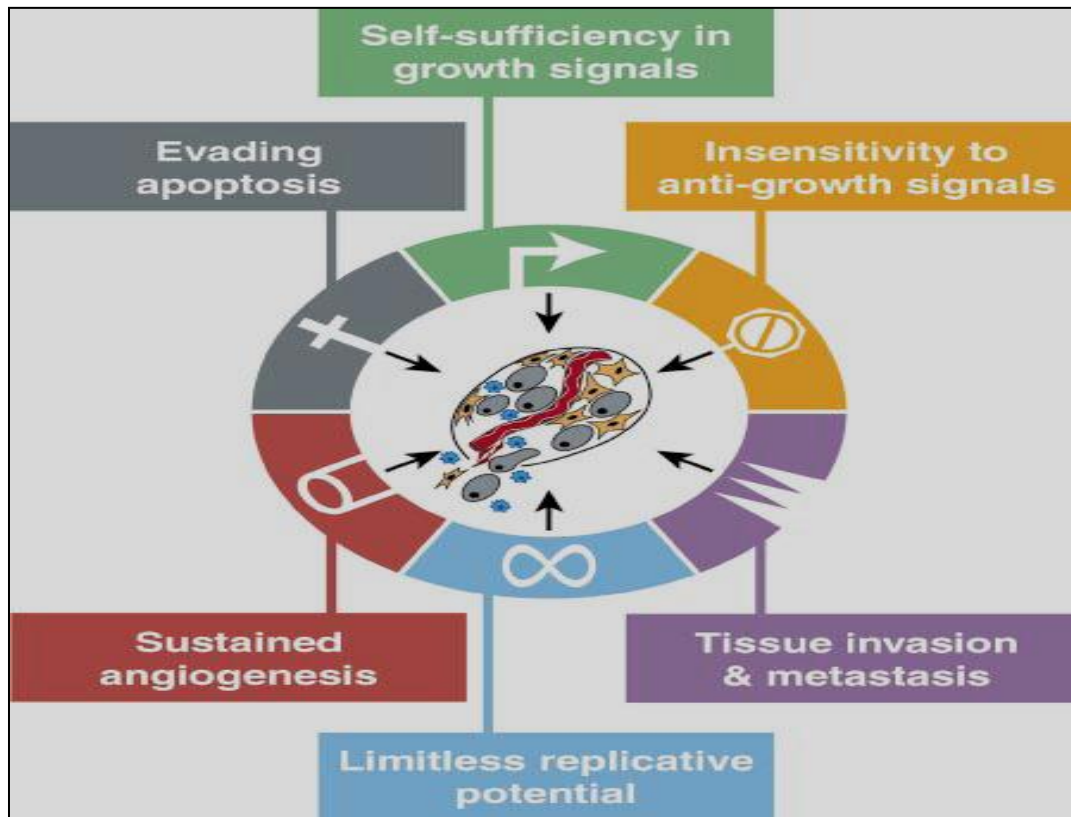


Figure 2.3: Six hallmarks of Cancer (Hanahan and Weinberg 2000).

### 2.2.1 Growth Signals and Cancer

There are various soluble growth factors that are present in a cell, based on which cell function normally. Normal cells usually recognize different kinds of growth signals and respond accordingly, but cancer cells do not require those exogenous growth signals and can grow independent of the signal from the tissue environment. Cancer cells are able to synthesize most soluble mitogenic growth factors and positively respond to the signals. Some of the growth factors produced by cancer cells are platelet derived growth factor (PDGF) and tumor growth factor (TGF). Cancer cells being self-sufficient in growth signal is also explained by higher



expression of the receptors. Due to the high level of receptors present in cancer cell, even very low level of growth factors are detected, which usually would not be recognized in normal cells. For example in breast cancer cells, epidermal growth factor receptor (EGF-R) and Her2/*neu* receptor are overexpressed (Slamon and others 1987).

Normal cells have the property of obeying anti-growth signal whereas cancer cells resist anti-growth signals. Normally when cells receive anti-growth signal, they get into quiescent stage or permanently lose their ability to proliferate, but cancer cells deregulate this phenomena by disrupting pRb proteins and mutating various proteins like p15 and p21 because of which the CDK-cyclin complexes become unresponsive and disregard the anti-growth signals (Zuo and others 1996).

### **2.2.2 Apoptosis and Cancer**

Apoptosis is also called programmed cell death. It is a normal process that occurs during development of cell. When stimuli for death are received, cells undergo various morphological changes and die in a regular and controlled manner. When signals are received by cells, there are various pathways that are active in order to cause apoptosis. During early phase of apoptosis, there are families of proteins called caspases that are activated. These groups of proteins create a cascade that leads to cleavage or change in many structural proteins, nuclear proteins or enzymes eventually causing cell death.

But in case of cancer, the process of apoptosis is uncontrolled. Cancer cell gain the ability of escaping apoptosis by mutating tumor suppressor genes like p53, overexpressing apoptotic-oncogenes like bcl-2 and c-myc, and inhibiting pro-apoptotic proteins like Bax and cytochrome-C.

### **2.2.3 Limitless Replicative Potential**

Cancer cells acquire the property of dividing several times that results in uncontrolled cell growth. The property of uncontrolled cell division and duplication can be defined by two different processes;

#### **2.2.3.1 Cell Cycle**

Cell cycle maintains cell division and growth processes so as to maintain a balance between cell division and cell death. In a eukaryotic cell, cell cycle is divided into four different phases: Gap1 (G1) phase or phase for preparation of DNA synthesis, Synthesis (S) phase or the DNA replication phase, Gap2 (G2) or the preparation for mitosis phase and Mitosis (M) phase or the dividing phase. The phase other than the M phase is also called the interphase.

DNA of cell can undergo many physical or chemical attacks that can cause its damage. The source for DNA damage can be extrinsic or intrinsic. Damaged DNA causes cancer. In a normal cell cycle, cell containing damaged DNA goes through a process called DNA repair, apoptosis or can halt the progression of cell cycle. For the assurance of this process, various checkpoints are present in a cell cycle. But in case of cancer, there is deregulation of the cell cycle process, cell with damaged DNA skip the checkpoints of cell cycle and keep dividing and replicating (Paulovich and Hartwell 1995).

#### **2.2.3.2 Activity of Telomerase**

Telomerase is a reverse transcriptase enzyme responsible for protecting and lengthening the telomeric ends of DNA. It is normally active only in few types of normal cells like the stem cells, germ cells and proliferating cells, but its activity has also been recorded in cases of cancer.

Telomeres are the TTAGGG sequences of DNA that are present at the ends of the chromosomes. The function of telomere is to maintain the stability of the DNA. With each cell division, the length of telomeres shortens, and eventually it reaches a limit where the cell is directed to arrest or senescence. The natural process of telomere shortening is evaded by activation and up regulation of telomerase, which is found considerably in cancer cells.

Telomerase is found to be very active in 90% of breast cancers and not in normal cells and therefore is a potential target for both prevention and treatment of breast cancer. Breast cancer cells have very short telomeres and inhibitors of telomerase can have a significant effect on further shortening the cells causing growth arrest and cancer (Herbert and others 2001).

#### **2.2.4 Tissue Invasion and Metastasis**

Cancer cells are can migrate to distant organs through blood vessels, seed there and grow. This property of cancer cell is because of their ability of activating various extracellular proteases like Matrix Metalloproteinase (MMPs) and inhibiting Tissue Inhibitors of Metalloproteinase (TIMPs), both are very important for tissue remodeling. In a healthy condition, there is always a balance between MMPs and TIMPs, which prevents the degradation of extracellular matrix (ECM) proteins. Over expression of MMPs is associated with degradation of tissues in many chronic inflammatory diseases like cancer. Process of metastasis and tumor growth is influenced by increase in MMPs activity (Tang and others 2005).

In case of breast cancer cells only MMP-2 and MMP-9 are over expressed, which belongs to gelatinase group, Gelatinase A and Gelatinase B respectively and are responsible for tissue invasion, metastasis, and angiogenesis (Joyce and Pollard 2009). Cancer cells are also capable of altering the binding property of cadherins and cell adhesion molecules. In the

process of migrating through the blood vessels they also evade the immune system by release of immunosuppressive compound. Therefore tissue invading and metastasizing property of cancer cells can be controlled by targeting the tissue degrading enzymes, inhibiting tumor angiogenesis, and making tumor cells immune sensitive (Grothey 2005).

### **2.2.5 Tumor Angiogenesis**

Angiogenesis is the process of formation of blood vessels from pre-existing vessels. This process happens throughout the life starting from the embryo. Angiogenesis helps to recover different physiological defects in our body, like wound healing, but it can be dangerous too. There are two types of angiogenesis; Physiological and Pathological.

Cases of pathological angiogenesis can arise due to enhanced or excessive angiogenesis in the system or due to impaired or decreased rate of angiogenesis. Reduced angiogenesis causes many non-healing wounds cases like gastric or oral ulcers, diabetic ulcers, impaired bone fracture healing and cardiovascular diseases like atherosclerosis, diabetes, and restenosis, and also dented nervous system causing Alzheimer disease, amyotrophic lateral sclerosis, and diabetic nephropathy. Some of the other diseases characterized by abnormal angiogenesis are Crohn disease, osteoporosis, hair loss, kin pupura, telangiectasia, nephropathy, neonatal respiratory distress, pulmonary fibriosis, and emphysema. On the other hand, high level of angiogenesis creates many diseases like hemangiomas, cancer, psoriasis, obesity, diabetic retinopathy, atherosclerosis, asthma, nasal polyps, rheumatoid arthritis, inflammatory bowel disease, and endometriosis (Dulak 2005).

Basically a tumor cannot grow in size beyond 1-2 mm until it is supplied with nutrients and oxygen. For a tumor to gain its size and cancerous properties, angiogenesis plays a very

critical role. In order to supply itself with nutrients and oxygen, the tumor turns the angiogenic switch and begins to express growth factors such as vascular endothelial growth factors (VEGF) promoting formation of blood vessels and directional growth. Therefore, targeting angiogenic mechanism has been one of the important anti-cancer approaches.

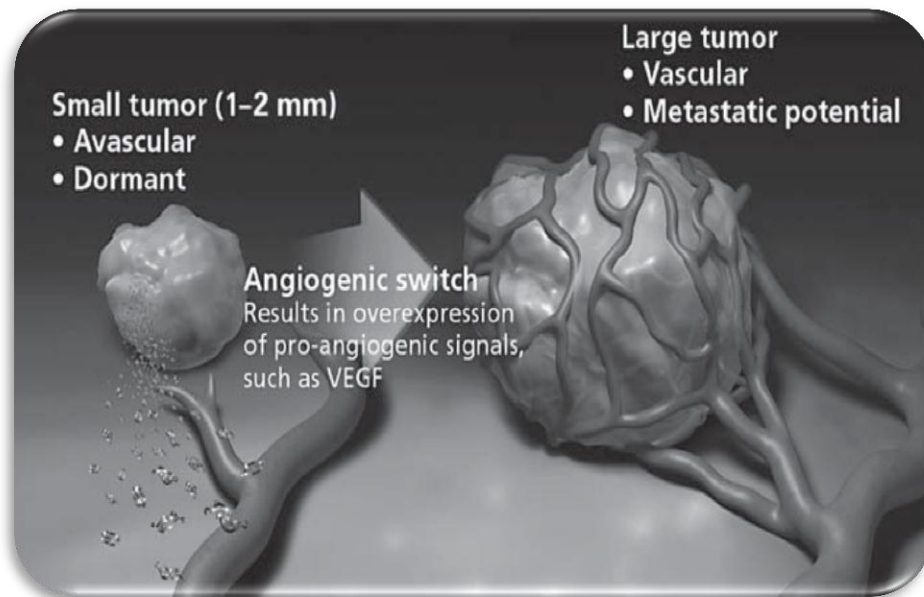


Figure 2.4: Angiogenic switch

### 2.3 Current Approaches against Breast Cancer Cells and Associated Risks

Breast cancer is a frequently occurring chronic disease that is diagnosed in females. According to the statistics provided in 2008, breast cancer has been found to cause 23% of the total cancer worldwide and 14% of the cases has been reported to cause death (Jemal et al. 2011). Various treatment approaches have been designed to eradicate breast cancer, which are listed below;

- Surgery
- Medicine

- Monoclonal antibodies
- Chemotherapeutic drugs
- Hormonal Therapy
- Radiation
- Preventive chemotherapy
- Dietary approaches

### 2.3.1 Drugs against Breast Cancer

There are various drugs that have been introduced to treat breast cancer. The problem related with the drugs provided is, their side effects to non-tumoral tissues. Some of them are listed below (Pegram and others 2004; Cuzick and others 2011):

- |                              |               |
|------------------------------|---------------|
| ✓ 4-hydroxycyclo phosphamide | ✓ Gemcitabine |
| ✓ Carboplatin                | ✓ Anastrozole |
| ✓ Docetaxel                  | ✓ Letrozole   |
| ✓ Paclitaxel                 | ✓ Tamoxifen   |
| ✓ Vinorelbine                | ✓ Raloxifen   |
| ✓ Epirubicin                 | ✓ Trastuzumab |
| ✓ Doxorubicin                |               |

Table 2.1: Some of the side effects associated with mode of treatment

<b>Radiation therapy</b>	<b>Chemotherapy</b>	<b>Hormonal therapy</b>
Lymphodema	Hair loss	Stroke
Fractures of breast bone	Nausea, vomiting	Blood clots
Pain in heart muscles	Anemia	Uterine cancer

---

(Table 2.1 continued)

Radiation pneumonitis	Joint pain	Menstrual problems
Effect on heart	Change of taste	
Risk of cancer	Fatigues	
Swelling of breast	Weight gain	
Fatigue	Cognitive dysfunction	
Cardiac weakness	Peripheral neuropathy	
	Heart failure	
	Leukemia	

---

## 2.4 Functional Foods and Cancer

Functional foods are foods containing physiologically-active components, which provide health benefits beside nutrition, when consumed on a regular basis (Hasler 1996). The Institute of Medicine's Food and Nutrition Board (IOM/FNB, 1994) has defined functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" Dietary agents contain many bioactive compounds in them and had been effectively used as a source of traditional medicine. Profound interest in health-benefiting foods have initiated various research to recognize activity of functional foods against different chronic degenerative diseases like, cancer, heart disease, osteoporosis, arthritis, age related diseases and many other. This correlates with what Hippocrates said 2500 years ago as, "Let food be *thy* medicine and medicine be *thy* food". Epidemiological studies focusing on the mechanism of functional food against cancer have demonstrated that consumption of fruits and vegetables causes reduced risk of various cancers (Rafter 2002).

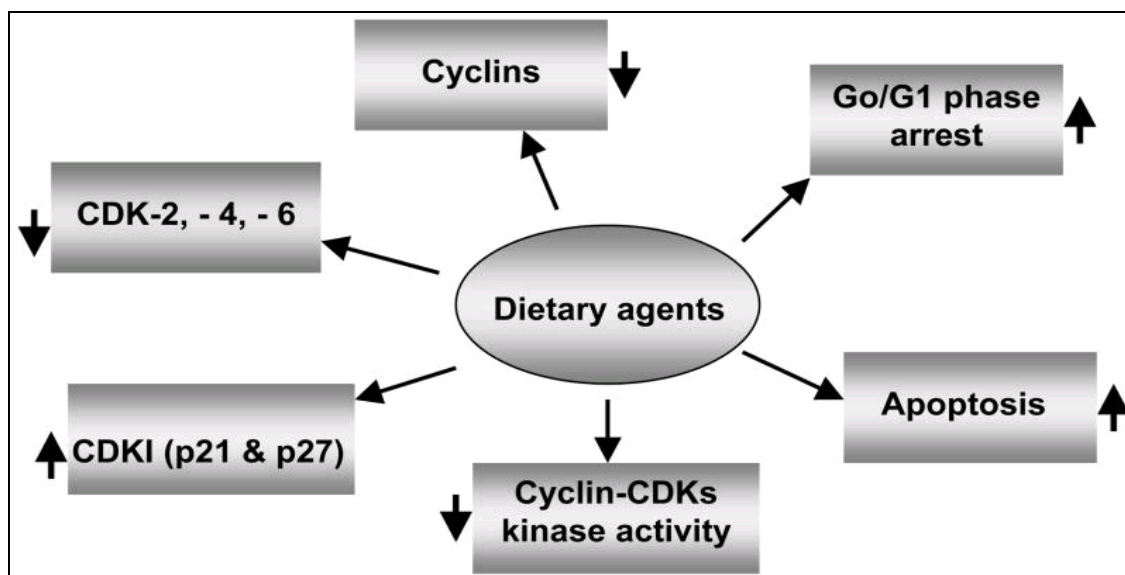


Figure 2.5: Molecular targets of cell cycle regulation in cancer cells *in vitro* and *in vivo* by dietary agents. Upward arrows (↑) indicate enhancement, and downward arrows (↓) indicate a reduction in the levels, or inhibition of the activity of the target molecules (Meeran and Katiyar 2008).

Table 2.2: Dietary Agents against Cancer Stem Cells (Li and others )

Natural dietary compound	Food origin	CSC	Elements of self-renewal pathways
<b>Curcumin</b>	Turmeric	Breast CSCs	$\beta$ -catenin, TCF-4, Frizzled-1; Notch-1
<b>Sulforaphane</b>	Cruciferous vegetables	Pancreatic CSCs, breast CSCs	$\beta$ -catenin, GSK3 $\beta$ (?), Wnt-9a
<b>Soy isoflavone (especially genistein)</b>	Soy		GSK3 $\beta$ , $\beta$ -catenin, Wnt-5a, Sfrp-2; Notch-2
<b>Epigallocatechin-3-gallate</b>	Green tea		HBP1, $\beta$ -catenin
<b>Resveratrol</b>	Grapes, berries, plums, and peanuts		$\beta$ -catenin, GSK3 $\beta$ ; Notch-1
<b>Lycopene</b>	Tomatoes, watermelon, papaya, pink grapefruit		$\beta$ -catenin
<b>Piperine</b>	Black and long pepper	Breast CSCs	Wnt/ $\beta$ -catenin
<b>Vitamin D<sub>3</sub></b>	Fish, egg yolk, beef liver		TCF-4, E-cadherin



## 2.5 Ginger

*Zingiber officinale* belongs to the family Zingiberaceae (Park and Pezzuto 2002). It originated in South-East Asia and now is well known around the world. Ginger plant is an herbaceous, rhizomatous, perennial plant, which is usually 2-3 feet tall and is found naturally in tropical and sub-tropical region. The rhizome part of the plant or the ginger is an aromatic, pale yellowish, thick lobed structure and possesses a pungent smell. Ginger is commonly used as a flavoring agent in food. It is usually used as a fresh paste, or in dried form or in slices. It has also been used in cosmetic and medicinal purposes and used as a herbal medicine in Ayurvedic and Chinese tradition (Ali et al. 2008). It is reported to be rich in anti-oxidant content and has been used as a medicine from ancient time as back as 2500 years (Grant and Lutz 2000).

Table: 2.3 Molecular Target of Ginger in Cancer (Aggarwal and Shishodia 2006)

Name	Active compound	Molecular Target
<b>Ginger (<i>Zingiber officinale</i>)</b>	Gingerol	↓TNF, ↓NFK-B, ↓AP-1,
	Paradol	↓COX-2, ↓ODC, ↓iNOS, ↓p38MAPK, ↓HIF, ↓VEGF, ↑caspase-3, ↓Bcl2

Ginger possesses its health-enhancing property because of the presence of the pungent compounds and the volatile compounds. The odor of the ginger is mainly due to the essential oil present (Ali et al. 2008). Gingerols, shogaols and paradols are some of the pungent compounds and terpenes and sesquiterpenes are the volatile ones. Ginger has found to be effective against

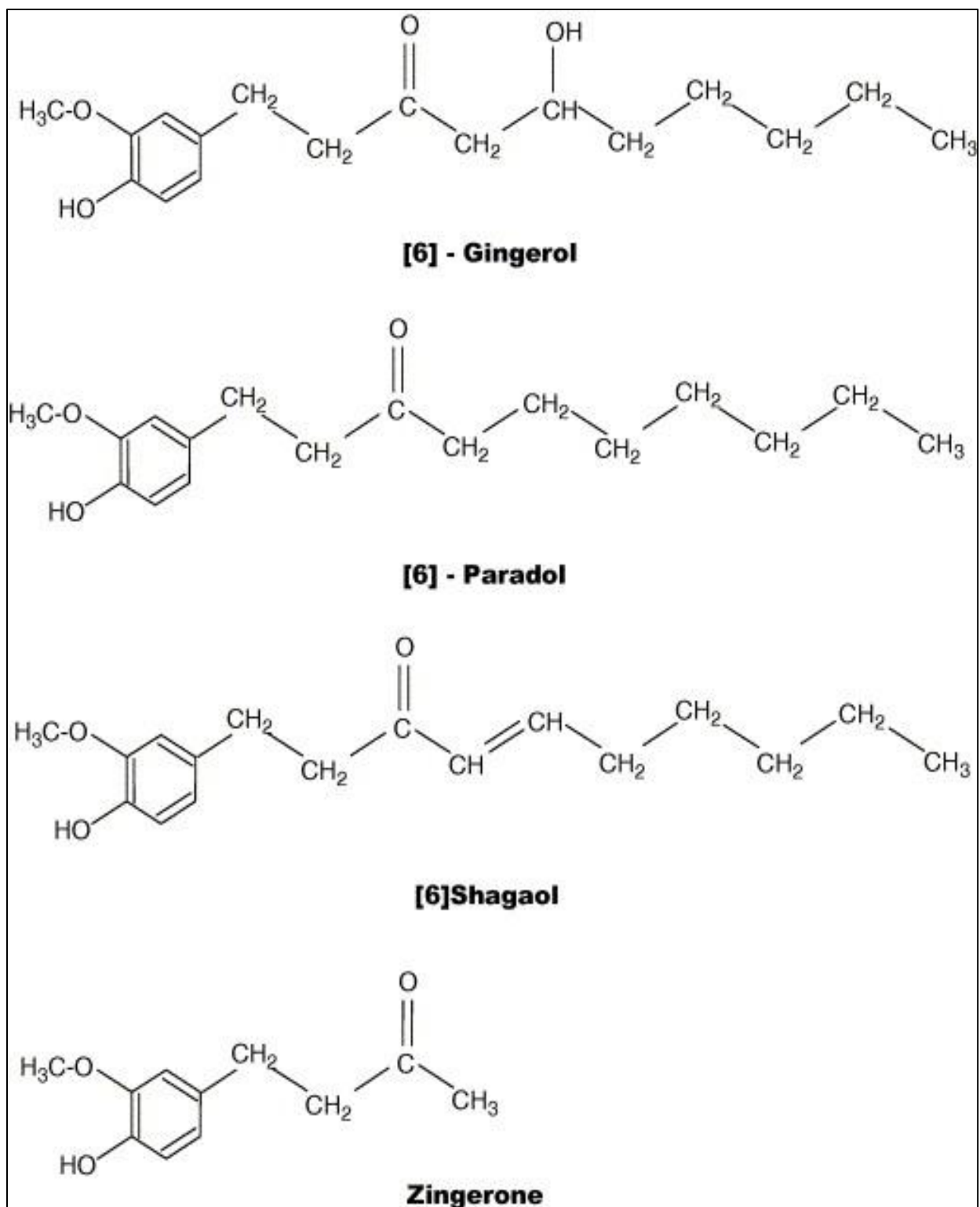


Figure 2.6: Non-Volatile Pungent Components of Ginger (Shukla and Singh 2007)

many diseases like arthritis, urinary infections, gastrointestinal disorders and pain. It is also an effective antiviral and antimicrobial agent and has potential to reduce diabetes mellitus, cardiovascular and gastric disorders (Shukla and Singh 2007).

Hsu et al. have also demonstrated 6-dehydrogingerdione (DGE) as a major component of ginger that has its effect against breast cancer. It was found that DGE activated caspase, up regulated p21 level and down regulated cyclin B1, cyclin A, Cdc2, Cdc25C and thus arrested cells at G2/M phase and caused apoptosis (Hsu and others 2010). 6-gingerol, another component of ginger has been found to have negative effect on NF- $\kappa$ B activation, cell proliferation, and angiogenesis (Kim and others 2005) and also arrests cell cycle at G1 phase by downregulating cyclin D1 (Ippoushi and others 2003).

## **2.6 Ginger Oil**

Ginger contains 9% of lipids or glycolipids and 5-8% of oleoresin (Chrubasik and others 2005). The percentage of essential oil in ginger is 3%. Essential oils are also called volatile oil or ethereal oil and are the aromatic oily part that is usually obtained from various parts of plants like flowers, buds, seeds, leaves, stem, bark, herbs, twigs, fruits or roots. Oils from plant have been used as a traditional medicine from ages. According to Food and Drug Administration, ginger essential oil has been regarded as a GRAS (Generally Recognized as Safe) product under the Code of Federal Regulation (CFR) parts; 182.10 and 182.20.

Aromatic plants contain terpenes; mostly monoterpenes and sesquiterpenes, which provide them their biological property. Terpenes have very good property of penetration because of which they have been used as drug penetration enhancers (Aqil and others 2007). The factors

that contribute to permeation capability of terpenes are its lipophilicity, size and chirality, degree of unsaturation, boiling point, and energy of vaporization.

Sesquiterpenes are the abundant one in the volatile oil of ginger, and consists of zingiberene, curcumene, farnesene, b-sesquiphenalbrene, and bisabolene. The flavor and taste of ginger is derived from the volatile components in it (Shukla and Singh 2007).

Studies have been carried out to evaluate the composition of ginger oil. Lawrence et al. found 114 compounds in the volatile oil of ginger (Lawrence 1984). Nogueira de Melo et al. have found curcumene, beta-mycrene, 1,8-cineol, citral and zingiberene as the major component of ginger oil (Nogueira de Melo and others 2011).

Anti-inflammatory, antinociceptive, and immunomodulatory properties of ginger oil has been demonstrated by Nogueira de Melo (Nogueira de Melo et al. 2011). The experiment carried out by Sharma et al on arthritic rats has suggested that ginger oil contains anti-rheumatic and anti-inflammatory properties (Sharma et al. 1994).

Table 2.4: Classification of Terpenes on Basis of Isoprene Units

<b>Number of isoprene units (C<sub>5</sub>H<sub>8</sub>)<sub>n</sub></b>	<b>Types of terpenes</b>
C <sub>10</sub> H <sub>16</sub>	Monoterpenes
C <sub>15</sub> H <sub>24</sub>	Sesquiterpenes
C <sub>20</sub> H <sub>32</sub>	Diterpenes
C <sub>25</sub> H <sub>40</sub>	Sesterterpenes
C <sub>30</sub> H <sub>48</sub>	Triterpenes
C <sub>40</sub> H <sub>64</sub>	Tetraterpenes

Table 2.5: Classification of Terpenes on Basis of Their Chemical Composition

<b>Class</b>	<b>Example</b>
Alcohols	Linalool, geraniol
Esters and alcohols	Linalool, linalyl acetate, menthol, menthyl acetate
Aldehydes	Cinnamaic aldehyde, cinnamic aldehyde
Ketones	Carvone, thujone
Phenols	Eugenol, thymol
Ethers	Anethole, cineole
Peroxides	Ascaridole

## CHAPTER 3: MATERIALS AND METHODS

### 3.1 Ginger Oil (GO)

To carry out this research, commercially available ginger oil was purchased from Sigma Aldrich. According to the data provided, the oil was all natural, kosher, halal grade and obtained by steam distillation. *Zingiber officinale roscoe* of China origin was the source of the oil and it could be tasted in concentration as low as 30 ppm. The boiling point of the oil was listed as 254°C and density as 0.871g/ml at 25°C.

### 3.2 Cell Cultures

In this study, hormone independent MDA-MB-231(HTB-26) human breast cancer cells, which are ER<sup>-</sup>, p53 mutated and a model of more advanced stage breast cancer (Hsu et al. 2010) were used to model the molecular mechanism for the inhibitory effect of ginger oil against cancer.

The cell line gets its name from M.D. Anderson-Metastatic Breast- 231. The cell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD). MDA-MB-231 is an epithelial adenocarcinoma cells isolated from the breast of *Homo sapiens* and have adherent growth properties. They were sub-cultured as a mono-layer according to the instructions provided by ATCC.

The MDA-MB-231 human breast cancer cell line was maintained in Dubecco's Modified Eagle's Medium (DMEM) containing 10% of fetal bovine serum (FBS), 15mM HEPES, and non-essential amino acids. Cell propagation media was purchased from Invitrogen (Carlsbad, CA).

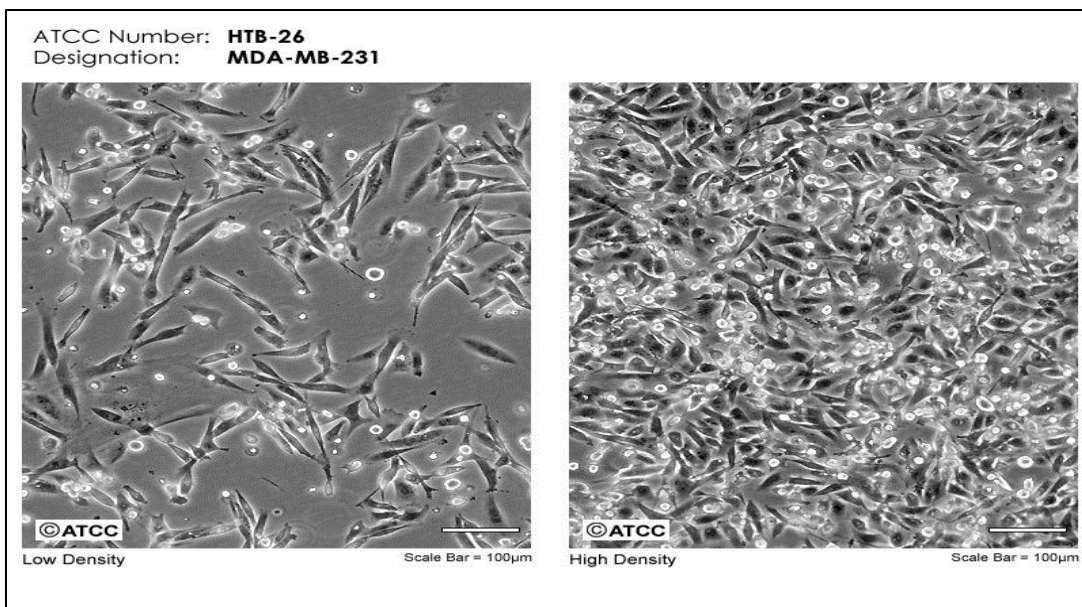


Figure 3.1: Morphology of MDA-MB-231 Cells

### 3.3 Gas Chromatography-Mass Spectrometric (GC-MS) Analysis of Ginger Oil

Gas Chromatographic analysis was performed with a Leco TOF GC/MS system equipped with an Agilent 6890 GC, under the following conditions: DB-5 capillar column (30 m X 0.25mm, 0.25 µm) (J &W Scientific, Albany, NY) was used with a flow rate of 1.0 mL/min. Ginger oil was diluted 1:10 with dichloromethane. One microliter injections were made in splitless mode at an injection temperature of 300 °C. The GC oven was initially held at 50 °C for one minute then increased at 3 °C/min to 80 °C and then at a rate of 10 °C/min to 340 °C. Data was collected from m/z 35 to m/z 1000 at 20 spectra per second.

### 3.4 Cell Viability Assay

#### 3.4.1 MTS Cell Proliferation Assay

Breast cancer cells ( $5 \times 10^3$  per well) were seeded on 96-well plates. The cells were grown for 24 h prior to treatment. Ginger Oil was mixed with dimethylsulfoxide in a ratio of 3:1 and

ginger oil at concentration of 0.05%, 0.1%, 0.15% or 0.2% of the total volume of the media used were added to the cells. The cells were incubated for 24, 48 and 72 hours at 37°C in an incubator with 5% CO<sub>2</sub>. After incubation, cells were treated with the MTS reagent according to the protocol provided by the supplier, CellTiter 96 AQueous One solution (Promega, Madison, WI). The number of viable cells was directly proportional to the absorbance at 490 nm due to the reduction of formazan product from MTS. Absorbance was read in a BioRad Model 680 micro plate reader (Hercules, CA). Cell viability was presented in terms of percentage of control. The experiment was performed in triplicates.

### **3.4.2 Caspase Assay for Apoptosis**

After performing cell viability assay, caspase assay was performed in order to determine the activity of caspase in the cells treated with ginger oil. Breast cancer cells MDA-MB-231, 5X10<sup>3</sup> cells were seeded in a 96 well plate and after 24 h cells were treated with vehicle (DMSO), ginger oil at concentration of 0.05% or 0.1% of the total volume of the media used. The plate was incubated for 72 hours and caspase-9 assay was performed using the Caspase-Glo 9<sup>RM</sup> (Promega, Madison, Wisconsin). After incubation, luminescence was measured using, The Reporter<sup>TM</sup> Microplate Luminometer. Results were presented in terms of percentage of control. Each experiment was performed in triplicate.

### **3.5 Cell Cycle Analysis**

Breast cancer, MDA-MB-231, 0.5x10<sup>6</sup> cells were grown on T-25 flasks and were treated with vehicle and ginger oil at concentration of 0.05%, 0.1%, 0.15% or 0.2% of the total volume of the media used. After 72 hours of incubation, cells were trypsinized and suspended in PBS and counted using trypan blue. Cells (2x10<sup>5</sup>) were selected and centrifuged at 1000 rpm (200g)



for ten minute at room temperature. The supernatant was discarded and the cells were re-suspended in 100ul of PBS and 900ul of 95% cold methanol. Methanol was added drop by drop to the pellet; the cells were vortexed and incubated for 30 min at 4°C.

After incubation cells were centrifuged at 2000 rpm for 5 min at room temperature. Then methanol was removed making sure not to lose cells. After that cells were suspended in 450µl Phosphate Buffer Saline (PBS) (Santa Cruz Biotechnology, Santa Cruz, CA), 25µl RNase (Roche Diagnostics, Indianapolis, IN) (500 µg/ml) and 25µl Propidium Iodide (Sigma-Aldrich, St. Louis, MO) (1mg/ml). The samples were incubated in dark for 24 hours and flow cytometric analysis was performed using Beckman-Coulter Epics flow cytometer using CXP software.

### **3.6 *In Vitro* Angiogenesis**

The assay was performed using the *in vitro* angiogenesis assay kit from Millipore<sup>TM</sup>. Following the preparation of ECMatrix<sup>TM</sup> using the manufacturer's instructions,  $7.5 \times 10^3$  human coronary artery endothelial cells (HCAECs) were seeded on the surface along with the 150ul supernatant from the untreated MDA-MB-231 cells and cells treated with 0.05% and 0.1% ginger oil. The cells were then incubated at 37°C for 6 hours and tube formation was analyzed and photographed using a Leitz phase-contrast inverted microscope at 10X magnification.

### **3.7 Telomerase Activity**

Telomerase is the enzyme responsible for adding the telomeric repeats at the end of DNA sequence. Its activity has been reported in immortal cells, cancer cells and germ cells.

Telomerase activity in the samples was measured using a PCR based assay (TRAPEZE<sup>®</sup> XL Telomerase Detection Kit, Millipore<sup>TM</sup>, Billerica, MA). MDA-MB-231 cells were first treated with ginger oil at concentration of 0.05% and 0.1% of the total volume of media used. After 72 h

of incubation, cells were lysed with CHAPS lysis buffer provided in the kit to extract the protein. Protein concentration determination of the lysate was done by Bradford method using BSA as standard. The concentration of the protein of the samples used for detection of telomerase was made to 750ng/μl. And then the protein samples were then combined with TRAPeze reagents to perform PCR. Manufacturer's instruction was used for making the standards provided in the kit.

As telomerase is a heat sensitive enzyme, negative telomerase samples were also prepared by heat treating them at 85°C for ten minutes. The primer provided in the kit is Ampifluor® primers that only glow after incorporation. After running the PCR, to quantitate telomerase activity, fluorescein and sulforhodamine emission detection was done using Cytofluor® Multi-well Plate Reader (PerSeptive Biosystems, Framingham, MA). The excitation and emission filters for fluorescein detection was at 485nm and 535nm respectively. While for sulforhodamine detection, the excitation and emission filter was at 585 nm and 620 nm respectively. The reading was taken using fluorometer. The results were expressed in terms of percentage of control. The experiment was done in triplicates.

### **3.8 Western Blot Analysis of Biomarkers**

MDA-MB-231 cells were incubated with varying concentration of ginger oil, 0.05% and 0.1% of the volume of media for 72 hours. Cells were then washed with Phosphate Buffered Saline (PBS), lysed in radioimmunoprecipitation assay buffer (RIPA) supplemented with protease inhibitor, sodium orthovanadate and phenylmethanesulfonylfluoride (PMSF). After 30 min incubation on ice with the lysis buffer, cells were centrifuged at 14,000 rpm for 10 min, and the supernatant was saved; protein concentration was determined by the Bicinchoninic acid (BCA) method using BCA protein Assay Reagents (Thermo Scientific, Rockford, IL). Equal

amounts (1000  $\mu$ g) of total cell extract protein from control and GO treated cells were mixed with LDS sample buffer (Invitrogen, Carlsbad, CA), brought to desired volume with water, boiled for 3 minutes centrifuged at high speed for 30 sec, and loaded onto SDS polyacrylamide gels; 4-12% Bis-Tris gels or 3-8% Tris-acetate gels (Invitrogen, Carlsbad, CA) depending on the size of the protein.

Proteins of interest were then separated and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). Membranes were blocked with 5% Bovine Serum Albumin (BSA) in Tris Buffer Saline (TBS) containing 0.05% Tween 20 (TBS-Tween) for 1h. The PVDF membranes were incubated with primary antibody in TBS-Tween containing 5% BSA overnight at 4 °C with gentle agitation following the manufacturer's instructions. Visualization of the bound primary antibody was done by probing with alkaline phosphatase-conjugated secondary antibodies and exposure to chromogenic detection reagent BCIP (Invitrogen, Carlsbad, CA).

Primary antibodies like NF- $\kappa$ B, Cadherin E, Bax, Bcl2, CD44, ESA, Aldh1, VEGF, cathepsin D and the alkaline phosphatase- conjugated secondary antibody was purchased from Santa Cruz Botechnology<sup>®</sup> (Santa Cruz, CA). Other primary antibodies like Hsp90, PDGFR- $\beta$ , p-IGF-1R, Her2, pERK, Histone, phospho-p38 was purchased from Cell Signaling Technology<sup>®</sup> (Danver, MA).

### **3.9 Interleukin-8 (IL-8) and Tumor Necrosis Factor- $\alpha$ (TNF- $\alpha$ ) Levels in MDA-MB-231 Cell Supernatants**

To determine the effect of inhibition by GO on IL-6, IL-8 and TNF- $\alpha$  expression, we measured their levels in the supernatants of vehicle, 0.05% and 0.1% GO treated cells by ELISA

using commercial kits from Prepotech (Rocky Hill, NJ). Manufacturer instructions were used and the test was done in triplicate and results were expressed in percentage of control.

### **3.10 Statistical Analysis**

Results are expressed as means  $\pm$  SD of experiments each conducted in triplicate. Statistical analysis was conducted using the Statistical Analysis Software (SAS) (version 9.2). Analysis of variance (ANOVA) was conducted to examine the differences between treatments followed by Tukey analysis. A P-value of  $< 0.05$  was considered to be statistically significant.

## CHAPTER 4: RESULTS AND DISCUSSION

### 4.1 GC-MS Analysis of Ginger Oil

To determine the components of the ginger oil GC-MS analysis of the oil was done. Trace level of compound present were detected and all of those compounds are presented in Table 4.1. Among those many compounds present in ginger oil, the major compounds were eucalyptol (14.2%), cuparene (12.3%), bicycloheptane (11.4%),  $\gamma$ -muurolene (10%),  $\alpha$ -farnesene (8%),  $\alpha$ -pinene (4%), and  $\beta$ -myrcene (2.2%). However the level of zingiberene, curcumene and citral were found to be as low as 0.7%, 0.2% and 0.4%, which contrasts with the findings of Nogueira de Melo et al., who had reported ar-curcumene, beta-myrcene, 1,8-cineol, citral and zingiberene as the major component (Nogueira de Melo et al. 2011).

The effect of sesquiterpenes has been studied in many tumor cell lines. Cadinol and  $\gamma$ -muurolene exhibit anti-cancer effect on colon cancer and breast cancer cells (Chang and others 2000). And the results from GC-MS analysis shows that ginger oil contains both cadinol,  $\gamma$ -muurolene at concentrations of 0.1% and 10.9% respectively. Elmene, which is also one of the components found in ginger oil, also has been studied for its anti-tumor activity in leukemia cells. The results found from the study by Yang suggest that elmene is effective in inducing apoptosis, which gives property of anti-tumor activity to it (Yang and others 1996)

Essential oils from berries, *Schinus molle L.* and *Schinus terebinthifolius* have been shown to have anti-cancer properties against breast cancer cells (Bendaoud and others 2010), and some of the compounds of the oil were similar to the one found in ginger oil like, germacrene D, terpinoid, phellandrene, pinene, cymene, terpineol, and  $\alpha$ -himachalene. This suggests that properties of ginger oil against cancer are due to the presence of all these bioactive molecules.

Table 4.1: GC-MS Analysis of Ginger Oil

Compound	Percentage %	Compound	Percentage %
1,7,7-Trimethyltricyclo[2.2.1.0(2,6)]heptane	0.2	6-Octen-1-ol, 3,7-dimethyl-, acetate	0.2
$\alpha$ -Pinene	4.0	Cyclohexene	1.2
Bicyclo[4.1.0]heptane, 7-(1-methylethylidene)-	11.4	$\alpha$ -Copaene	0.9
$\beta$ -Pinene	0.4	$\beta$ -Elemene	1.9
$\beta$ -Myrcene	2.2	Zingiberene	0.7
1-Phellandrene	0.9	Germacrene D	0.6
Eucalyptol	14.2	Germacrene B	2.1
$\alpha$ -terpinolene	0.4	$\beta$ -Sesquiphellandrene	2.0
3-Methyl-2-(2-methyl-2-butenyl)-furan	0.5	neoalloocimene	0.6
Linalool	0.8	(R)-Cuparene	12.3
m/z 168 Me Borneol Ether	0.2	$\alpha$ -Farnesene	8.0
m/z 136 & 154 Terpenoid	0.1	alpha-Himachalene	4.8
Camphor	0.1	$\gamma$ -Muurolene	10.9
exo-methyl-camphenilol	0.1	$\alpha$ -Farnesene	2.5
Rose furan epoxide	0.5	Camphogen	0.2
1-borneol	1.7	Sesquisabinene hydrate	0.8
4-Terpineol	0.2	$\alpha$ -curcumene	0.2
$\alpha$ -Terpineol	1.1	Zingiberenol	1.1
n-Decanal	0.3	$\alpha$ -Gurjunene	1.0
$\beta$ -Citronellol	0.3	Bicyclogermacrene	0.6
Z-Citral	0.2	$\tau$ -Cadinol	0.1
GERANIOL	0.5	$\beta$ -Eudesmol	1.9
E-Citral	0.4	$\alpha$ -cedrane	0.9
1-Bornyl acetate	0.3	Mint sulfide	0.3
2-Undecanone	0.4	Nerol	0.3
$\alpha$ -Elemene	0.2		

## 4.2 Effect of Ginger Oil on Cell Viability

To determine the number of viable cells after the treatment with ginger oil, cell proliferation assay was performed using CellTiter96® Aqueous from Promega (Madison, Wisconsin). The assay is based on the colorimetric principle to determine the number of viable cells. After adding MTT reagent into the cells, formazan product is formed from the tetrazolium salt containing MTT reagent. The formazan is an aqueous soluble, colored product formed from the mitochondrial activity of the viable cells and is read at 492 nm. The absorbance at 492 nm is directly proportional to the number of viable cell present (Malich and others 1997). This assay has been used to quantitate cell proliferation and survival of living mammalian cells (Mosmann 1983).

To investigate the effect of ginger oil on the growth of MDA-MB-231 breast cancer cells, cells were incubated for 24h, 48h and 72h with 0.05%, 0.1%, 0.15%, and 0.2% of ginger oil with respect to the volume of media used for cell growth. Cell proliferation was then measured at 490nm using MTS assay. As shown in figure 4.1, cell growth was reduced only by 1%, 5%, 9%, and 26% in the cell treated with 0.05%, 0.1%, 0.15%, and 0.2% of ginger oil for 24 h. But after 48 h, cell growth was reduced by 20%, 26%, 40%, 60% in terms of 100% of control with 0.05%, 0.1%, 0.15% of ginger oil and 72 h of incubation caused reduction of 52%, 61%, 66%, and 73%

Literatures suggest that [6]-gingerol, one of the major compounds of ginger is able to inhibit growth and proliferation of colon cancer cells at a concentration of 100µM/L (Jeong and others 2009). Other group of study has shown that after 72 h incubation of [6]-gingerol in colorectal cancer cell, the rate of cell growth was reduced by 22% and 28% with 150µM and

200 $\mu$ M [6]-gingerol respectively. The growth was also reduced in CaCo2 and HT-29 cells by 8 and 28% respectively (Lee and others 2008).

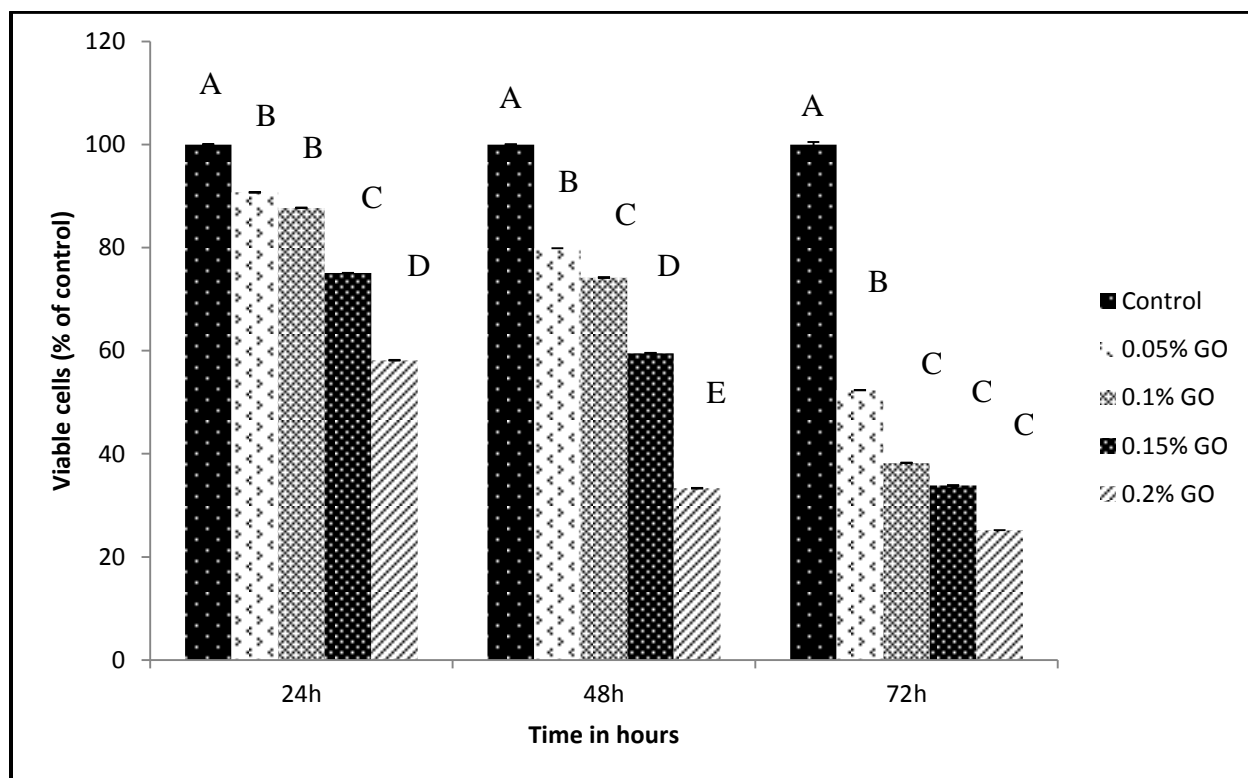


Figure 4.1: Effect of Ginger oil on the viability of MDA-MB-231 cells with respect to time and concentration. Values (means  $\pm$  SD, n=3) not sharing common letters, ABCDE are significantly different ( $\alpha=0.05$ ).

#### 4.3 Effect of Ginger Oil on the Activity of Caspase for Apoptosis

To further evaluate the cause of apoptosis, caspase assay was performed by determining the caspase activity on the cells with vehicle and ginger oil treated cells, Caspase-Glo®-9 Assay from Promega was used. Caspases are group of proteases that plays a very important role in the process of apoptosis in mammalian cells. This caspase assay is a luminogenic assay that measures the caspase activity. The reagent provided includes substrates for caspase activity, luciferase activity, and cell lysis. The cell lysis causes the caspase to release and then break the



substrate; this cleavage causes the release of glow, which is measured as a luminescent signal. The amount of luminescence is directly proportional to the activity of caspase in the system.

As shown in figure 4.3, our results demonstrated that the activity of caspase in the cells treated with ginger oil was higher than in control. In case of the cells treated with 0.1% ginger oil the level of caspase activity was significantly increased as high as 136% more than the control.

Activated caspases can cleave various intracellular proteins, protein kinases, molecules for DNA repair and many components of nucleus and cytoplasm. This process leads to interference of the survival pathways and creates many morphological and biological changes, which causes apoptotic cell death (Earnshaw and others 1999).

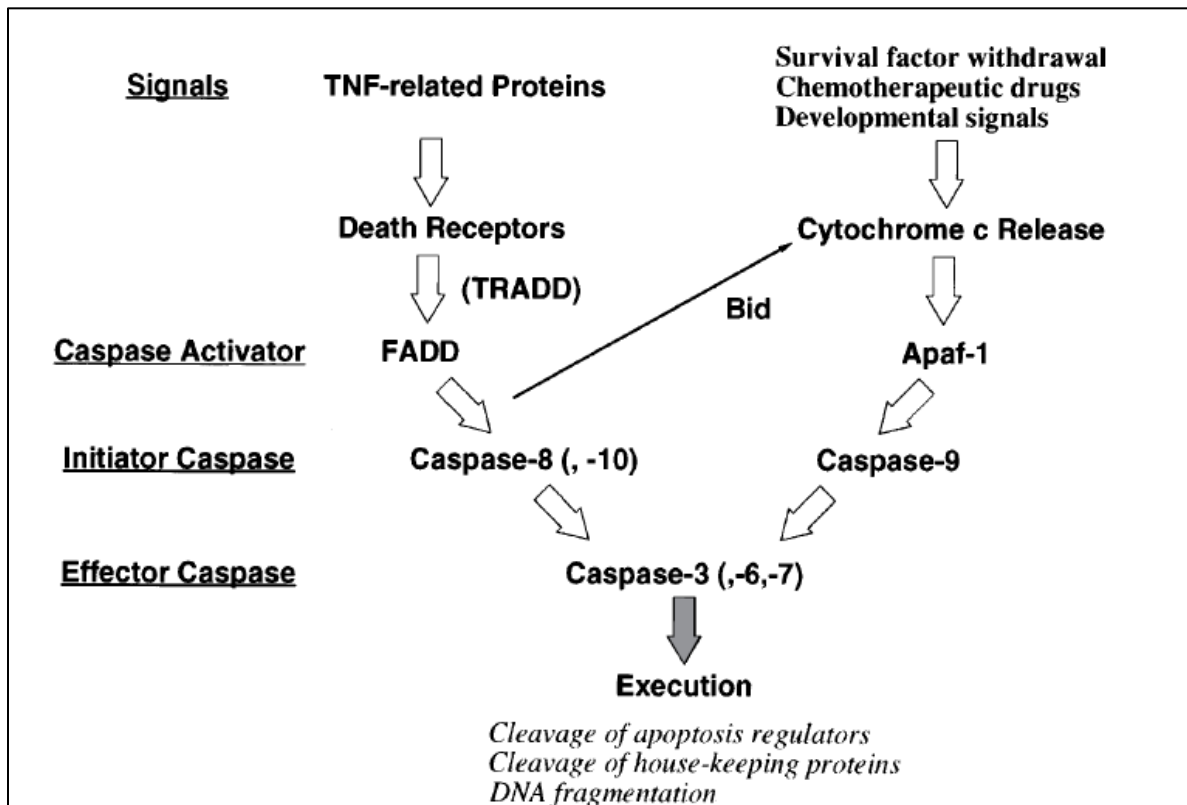


Figure 4.2: Pathway of Caspase Activation.(Nunez and others 1998)

Among various caspases, caspase8, caspase9, and caspase10 act as an initiator caspase that can trigger the activation of different other caspases. Caspase 9 is a type of caspase that is activated by mitochondrial initiated pathway. This creates a series of caspase cascade that ultimately destroys cell (Chen and Wang 2002). This caspase is responsible for a chain of caspase cascades. It activates downstream caspases like caspase -3, -6 and -7 (Budihardjo and others 1999).

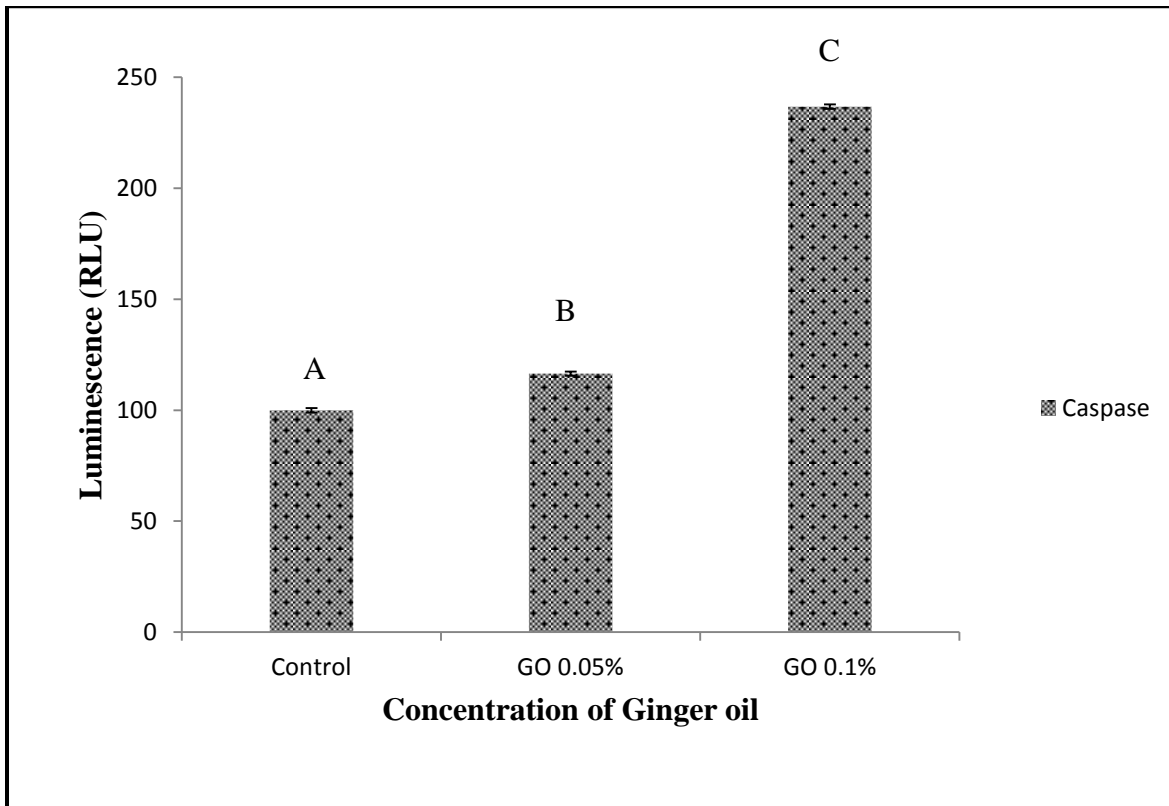


Figure 4.3: Dose Dependent Effect of Ginger Oil on the Activity of Caspase9. Values (means  $\pm$  SD, n=3) not sharing common letters, ABC are significantly different ( $\alpha=0.05$ ).

#### 4.4 Ginger Oil Modifies Breast Cancer Cell Cycle Progression and Cell Cycle Regulatory Protein Expression

To determine the effect of ginger oil on the cell cycle and to confirm apoptosis, cell cycle analysis of the cells treated with vehicle and 0.05% or 0.1% of ginger oil was done by flow cytometry. As shown in figure 4.4, the data demonstrated that ginger oil induced apoptosis and

sub-G1 phase formation in MDA-MB-231 breast cancer cells. The results from cell cycle analysis showed that ginger oil treatment resulted in the increase of sub-G1 phase. The percentage of the cells in sub G1 phase of the vehicle was 1.83% and in those treated with 0.05% or 0.1% of ginger oil was 14.80% and 69.19% respectively.

During apoptosis, the DNA of the cell undergoes degradation due to many enzymes. To analyze this phenomenon, DNA analysis can be done. The sub G1 phase of the cell cycle provides the percentage of those cells with damaged DNA. When the cell loses the DNA, the cell stains less and then appears at the left side of the G1 phase, that is why it is called the sub-G1 phase (Kajstura and others 2007).

Cell cycle analysis also demonstrated that ginger oil treatment resulted in an increase of the G2 phase and S phase. G2 phase of the cycle showed that the control had 15.98% cells, 0.05% GO treated cells had 20.86% cells, and 0.1% GO treated cells had 17.68% cells. The S phase and decrease of the G1 phase of the cell cycle in MDA-MB-231 cells. Ginger oil increased the cell cycle arrest at G2 and S phase.

To determine the level of cell cycle regulatory proteins, western blot analysis of some of those proteins was done. Cyclin dependent kinase and cyclin proteins; Cyclin D1, CDK-4, and CDK-2 proteins were analyzed. Cyclin dependent kinase and cyclin are responsible for the transition from G1 to S phase. As shown in figure 4.4, ginger oil effectively decreased the expression of cyclin D1, CDK4 and CDK2 required for G1 progression.

Cyclin dependent kinase (CDK) and cyclin proteins are the one responsible for the transition from G1 to S phase. Cell cycle is regulated by the phenomena of activation and deactivation of these CDK and cyclin complexes. There are eleven different kinds of CDKs

identified in cells of mammals, which are CDK1-CDK11, but only CDK2, CDK3, CDK4, and CDK6 make a complex with G1 cyclins and are responsible for the transition from G1 phase to the S phase. Checkpoint proteins checks for DNA damage, causes cell cycle arrest, and sends signals to the other cascade mechanisms like cell cycle repair or apoptosis. Dysregulation of the checkpoint process in a cell may result in cardiovascular diseases, neurodegenerative disorders, cancer, and many others.

CDK4 and CDK6 are activated during mid G1 phase and complexes with cyclin D while, CDK2 complexes with cyclin E and cyclin A during late G1 phase (Morgan 1997). CDK2 has also been reported as a potential target for treatment of breast cancer at early stage. Cyclin Ds in the G1 phase are activated as soon as cells enter the cell cycle, and the complex passes signals for mitogenic pathways. Cyclin D1 independent of CDKs also interacts with histone acetylase and other transcription factors (Diehl and others 1998). Cyclin E controls the beginning of DNA replication and the CDK2/cyclinE complex also prompts the duplication of centrosomes. Cyclin E is found to be overexpressed in cases of lungs cancer and breast cancer. Cyclin A assists in assembling the components for DNA synthesis and hence activates the S phase and at the same time it also makes sure that no other new complexes are assembled so it assures G1 phase has ended before the beginning of the S phase (Coverley and others 2002).

Besides triggering the mechanism for repairing DNA damage, CDK-cyclin complexes are also supposed to maintain or regulate the length of telomeres and structure of chromatin, controlling transcription of DNA repairing genes, arranging cytoskeleton and staffing repairing proteins to the site. If the checkpoints fail to detect the damage, it may lead to various abnormalities like mutation, aneuploidy and chromosome instability, which eventually leads or causes tumorigenesis (Hartwell and Kastan 1994).

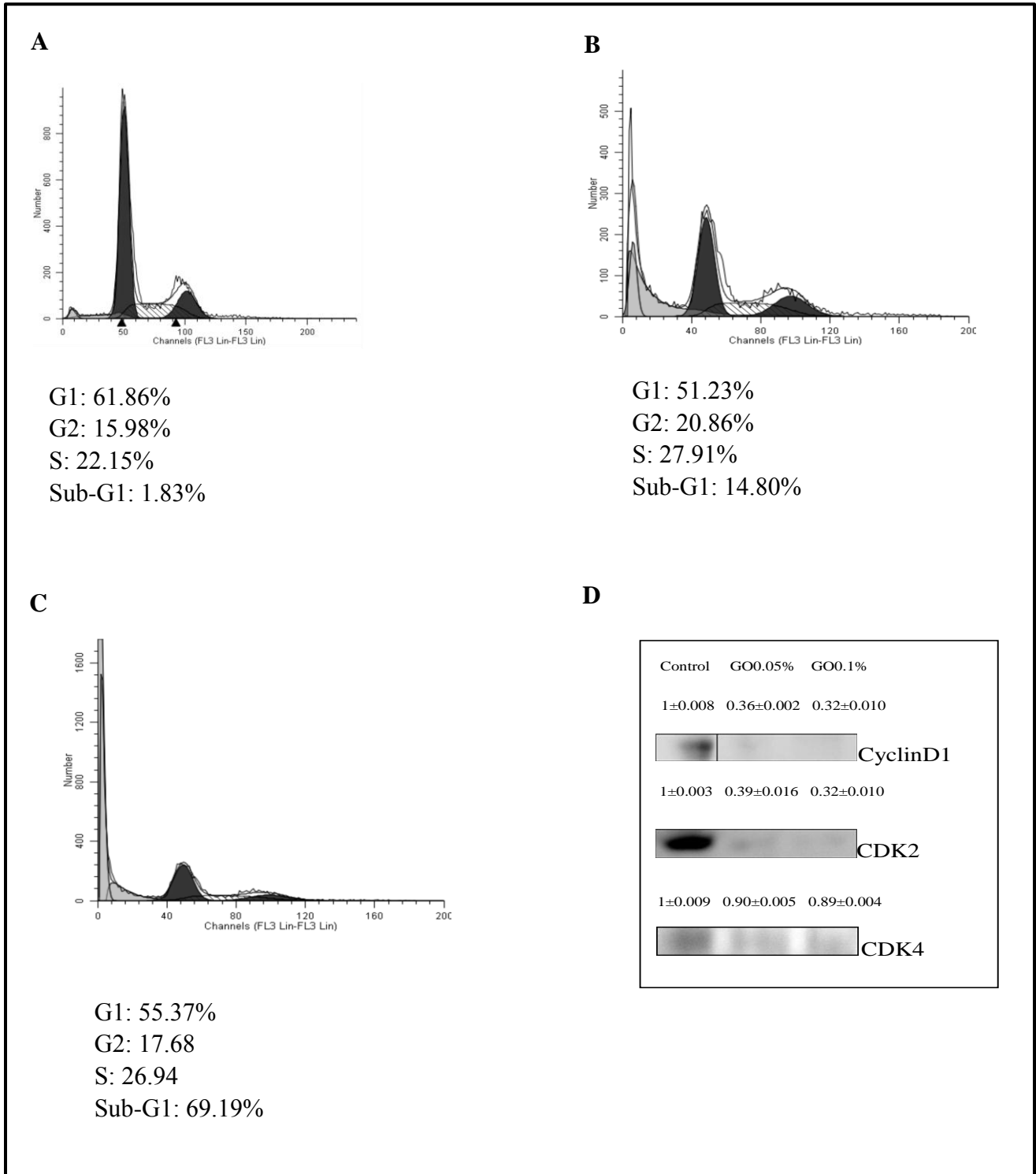


Figure 4.4: Cell Cycle Analysis of A: Control cells, B: Cells Treated with 0.05% GO, C: Cells Treated with 0.1% GO, D: Western Blot Analysis of Cell Cycle Proteins.

#### 4.5 Effect of Ginger Oil on Tumor Necrosis Factor- $\alpha$ (TNF- $\alpha$ ) Levels

To determine the level of TNF- $\alpha$  expression in the treated cells ELISA was done. TNF- $\alpha$ , a pro-inflammatory cytokine is a peptide hormone that effects death of tumor cells, inflammation and immune response. Our in vitro experiments show that ginger oil can inhibit the proliferation of MDA-MB-231 breast carcinoma cells. As shown in figure 4.5, ginger oil can significantly inhibit the expression of TNF- $\alpha$  at concentrations of 0.05% and 0.1%.

TNF- $\alpha$  was primarily discovered as a molecule responsible for antitumor activity, but recent studies have proved that this molecule facilitates tumor initiation, promotion, and metastasis. Increase in TNF- $\alpha$  level increase the property of tissue invasion and metastasis through the up regulation of NF- $\kappa$ B pathway (Hagemann and others 2005). TNF-  $\alpha$  secreted by tumors induce the secretion of matrix metalloproteases (MMPs) that help in the process of invasion and metastasis, and also increases the production of VEGF (Szlosarek and Balkwill 2003). Inflammation is one of the major properties required for promotion of tumor. Among all the cytokines responsible for cell-cell communication, TNF-  $\alpha$  is a major one. The proinflammatory effect of TNF-  $\alpha$  is primarily because of its capability to activate NF- $\kappa$ B, which further causes expression of many inflammatory genes.

Hanahan and Weinberg have classified cancer with six different major hallmarks and they have also reported that chronic inflammation is caused by the dysregulation of TNF-  $\alpha$  production. TNF- $\alpha$  promotes carcinogenesis by influencing all six hallmarks of cancer. It does so by activating reactive oxygen intermediates (ROIs) that in turn activates telomerase giving it limitless replicative potential, by up regulating inflammatory enzyme like; nitric oxide synthase, cyclo-oxygenase, by triggering the production of matrix metalloproteases responsible for

invasion and metastasis, and by activating many other cytokines and cell adhesion molecules (Hanahan and Weinberg 2000; Szlosarek and others 2006).

TNF-  $\alpha$  is found to be a growth factor for most of the tumors including breast cancer. As TNF can mediate so many pathways for carcinogenesis, it has been a potential target for therapy against various chronic diseases. Various dietary phytochemical agents have been reported to decrease the level of TNF-  $\alpha$  expression; curcumin, green tea polyphenols, resveratrol, kaempferol, apigenin, and gingerol (Yang and others 1998; Surh and others 1999; Kowalski and others 2005).

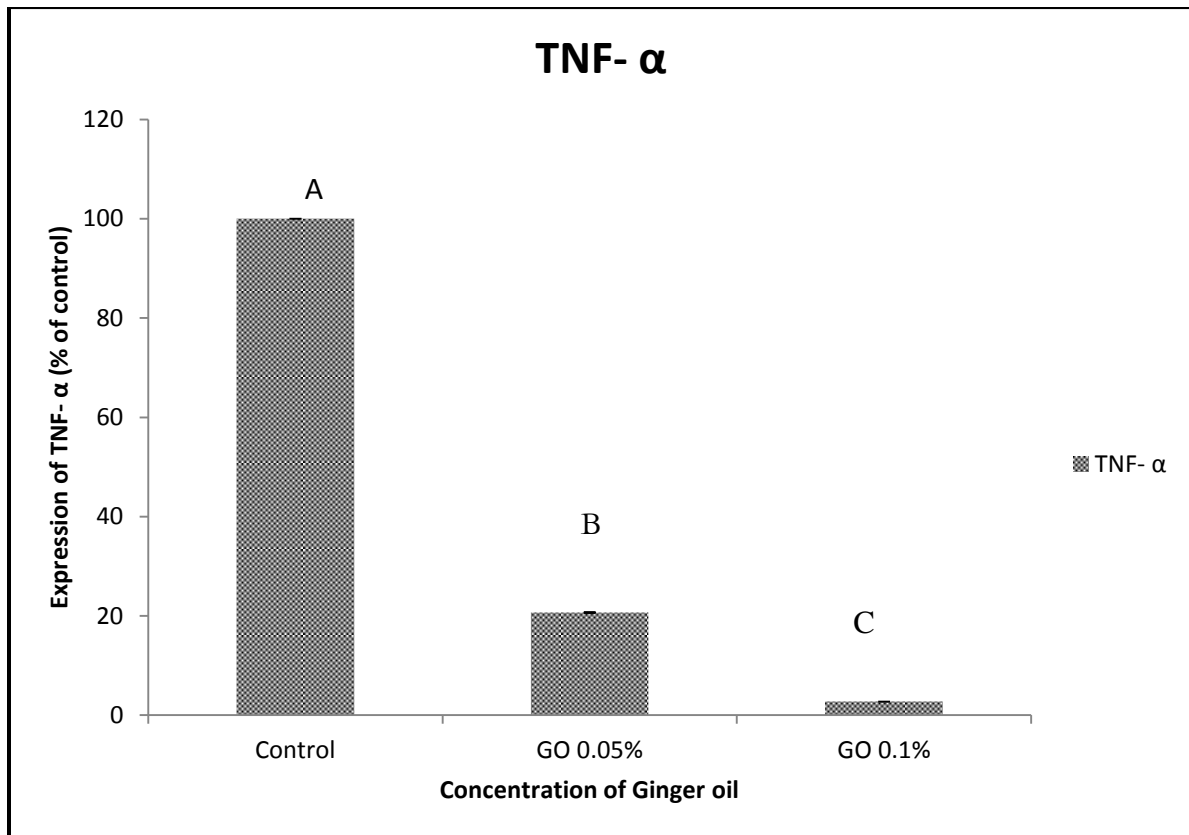


Figure 4.5: Effect of Ginger Oil on the Expression of TNF- $\alpha$ . Values (means  $\pm$  SD, n=3) not sharing common letters, ABC are significantly different ( $\alpha=0.05$ ).

#### **4.6 Inhibition of Telomerase Activity**

Telomerase are found to be active in cancer cells and dormant in normal cells. Telomerase gives cancer cells its property of immortality. Also high telomerase activity correlates with high malignancy and aggressive nature of cancer. Therefore telomerase has been a potential target against cancer. As shown in figure 4.6, we have demonstrated that ginger oil at concentrations of 0.05% and 0.1% could significantly inhibit telomerase expression by 21 % and 49% respectively in compare with the control.

Researches have shown that ethyl acetate extraction of ginger down regulates the expression of human telomerase reverse transcriptase (hTERT) gene eventually decreasing the level of telomerase in lung cancer cells (Tuntiwechapikul and others 2010). Some of the other sources of dietary agents that have been able to inhibit telomerase are polyunsaturated fatty acids like eicosapentanoic acid and docosahexaenoic acid(Eitsuka and others 2005), epigallocatechin gallate from green tea (Naasani and others 2003), sulfoquiniivosyldiacylglycerol from plants and seaweed (Eitsuka and others 2004), sulforaphane from broccoli (Moon and others 2010).

#### **4.7 Effect of Ginger oil on Expression of Various Signal Transduction Molecules**

To determine the effect of ginger oil on different signal transduction pathways, expression of proteins that are over expressed in case of breast cancer were investigated. There are various signaling proteins that are activated for the proliferation and anti-apoptotic property of cancer cells. Therefore molecules that are involved in different processes of cell regulation were investigated in this study using western blot and ELISA, and the obtained results are presented below.



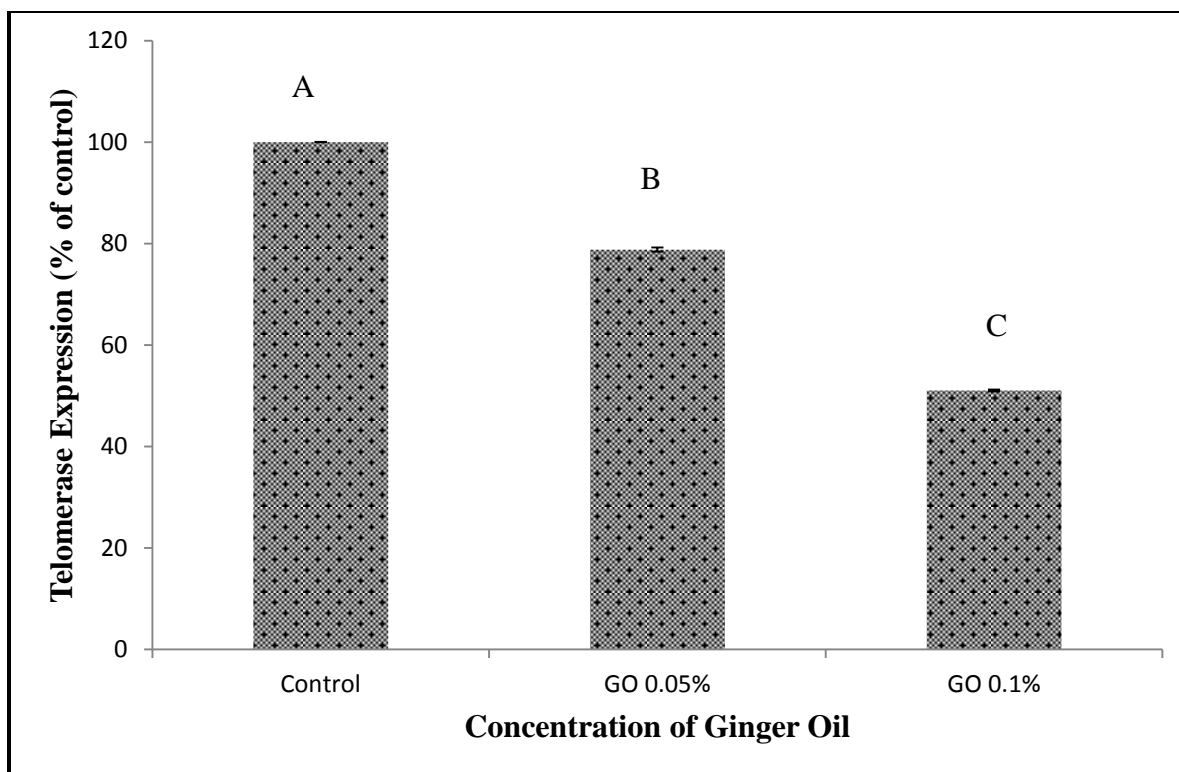


Figure 4.6: Effect of Ginger Oil on the Activity of Telomerase. Values (means  $\pm$  SD, n=3) not sharing a common letters, ABC are significantly different ( $\alpha=0.05$ ).

#### 4.7.1 Effect of Ginger Oil on Expression of Oncogenic Proteins

##### 4.7.1.1 Nuclear Factor-KappaB (NF- $\kappa$ B)

Expression of NF- $\kappa$ B supports the cell to proliferate and survive however down regulation of this protein molecule directs cell towards apoptosis (Aggarwal and others 2004). As shown in figure 4.7, the results from the western blots show that there was down regulation of NF- $\kappa$ B by ginger oil at concentrations, 0.05% and 0.1%. This can also be supported by the findings of Aggarwal that gingerol from ginger down regulates NF- $\kappa$ B pathway (Aggarwal and Shishodia 2006). Some of the genes that regulate NF- $\kappa$ B are bcl-2, survivin, TRAF 1 and TRAF 2 and they act by blocking the apoptotic process so these genes and proteins are highly expressed in cancer (Aggarwal and Shishodia 2004). The treatment of cancer cells with ginger oil also showed to decrease the expression of apoptosis suppressor proteins like Bcl-2.

High activity of NF- $\kappa$ B activity is also associated with high level of VEGF mRNA expression, which means that inhibition of NF- $\kappa$ B, should lead to inhibition of VEGF production (Shibata and others 2002). Our results (Figure 4.12) also demonstrate that cells treated with ginger oil shows significant decrease in VEGF levels.

#### **4.7.1.2 Heat Shock Protein 90 (Hsp90)**

Heat shock response is a very important property that cancer cells possess and Hsp90 is one of the very important molecular chaperones involved in this response. Hsp90 is a master regulator that gives activity and stability to many oncoproteins like Her2, many kinases and transcription factors. It is overexpressed in cancer cells and in case of breast cancer; it is associated with decreased survival (Pick and others 2007). Oncoproteins undergo several mutations and this phenomenon makes them resistant to inhibitors and for cases like this, targeting Hsp90 might be a useful solution as many oncoproteins depend on Hsp90 to maintain their oncogenic property and mutations (Porter and others 2010).

As shown in figure 4.7, we could demonstrate that Hsp90 expression level was reduced in cells that were treated with ginger oil at concentrations of 0.05% and 0.1%. The reduction was from 15% to 18% compared to the control.

#### **4.7.1.3 E Cadherin**

Cadherins are the calcium ions dependent transmembrane glycoproteins responsible for cell adhesion. Breast E Cadherin is expressed in normal epithelial cells of breast and the reduced expression of E cadherin has been found to induce the property of invasion and metastasis in breast cancer (Oka and others 1993; Siitonen and others 1996). As shown in figure 4.7, E cadherin was expressed in the cells treated with ginger oil.

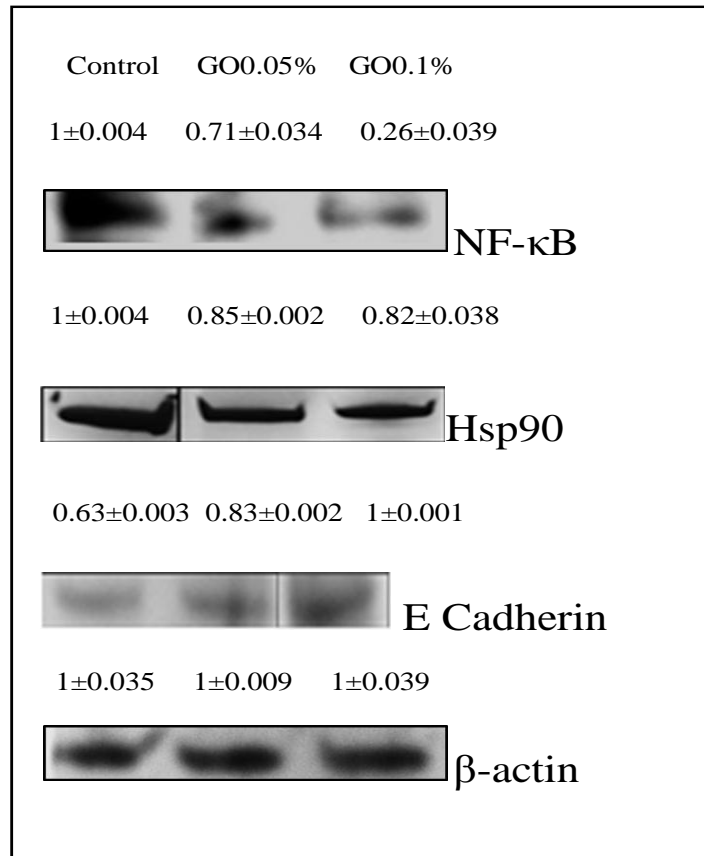


Figure 4.7: Expression of Oncogenic Proteins

## 4.7.2 Effect of Ginger Oil on Expression of Growth Factor Receptors

In case of pathological conditions like cancer, growth factors are highly expressed. This makes cancer cell very sensitive even to very a lesser amount of growth signals. As shown in figure 4.8, the expression of PDGFR-β, p-IGF-1R or Her2 was highly down-regulated in cells treated with ginger oil at both concentration of 0.05% or 0.1%.

### 4.7.2.1 PDGFR-β

Platelet derived growth factors (PDGF) are secreted by endothelial cells for the formation of blood vessels in mammals (Hoch and Soriano 2003). These growth factors further activate receptor tyrosine kinase, PDGFR-β (Hellstrom and others 1999). PDGFR signaling pathway has

important functions in process of embryogenesis but its over-expression has been found in many pathological diseases like cancer (Carvalho and others 2005).

#### **4.7.2.2 p-IGF-1R**

Insulin-like growth factor 1 receptor is found in phosphorylated condition in all breast cancer and accounts for poor survival. IGF-1R is a receptor tyrosine kinase that is considered as oncogene responsible for proliferation, survival, transformation, cell to cell interactions, and differentiation. Expression of this receptor is fundamental for the process of metastasis and cell survival (Zhang and others 2011).

#### **4.7.2.3 Human Epidermal Growth Factor Receptor 2 (Her-2)**

As shown in figure 4.8, ginger oil dose dependently at concentrations of 0.05% and 0.1% inhibited the expression of Her-2, which is overexpressed in 25-30% of breast cancers. Its level is highly increased in malignant cell; it gives aggressive property to tumor. Her2 overexpression increases the aggressive form of the disease therefore there is less chance of survival of patients.

#### **4.7.3 Effect of Ginger Oil on Expression of Pro-Apoptotic, Anti-Apoptotic and Cell Survival Proteins**

As shown in figure 4.9, ginger oil at concentrations of 0.05% and 0.1% of volume of media used, could increase the level of pro-apoptotic protein like Bax and at the same time ginger oil could drop the expression of anti-apoptotic protein like Bcl-2. Literature suggest that constituents of ginger, galanals A and B can successfully inhibit Bcl-2 and enhance Bax in case of leukemia (Miyoshi and others 2003).

P38/MAPK pathway regulates variety of cellular responses to stress and inflammation. Although p38 is considered as inducer of apoptosis and inhibitor of cell proliferation, very high expression of phosphorylated p38 is found to be associated with cell survival (Esteva and others

2004; An and others 2011). The level of phosphorylated p38 is considered to be very high in breast cancer (Mao and others 2010).

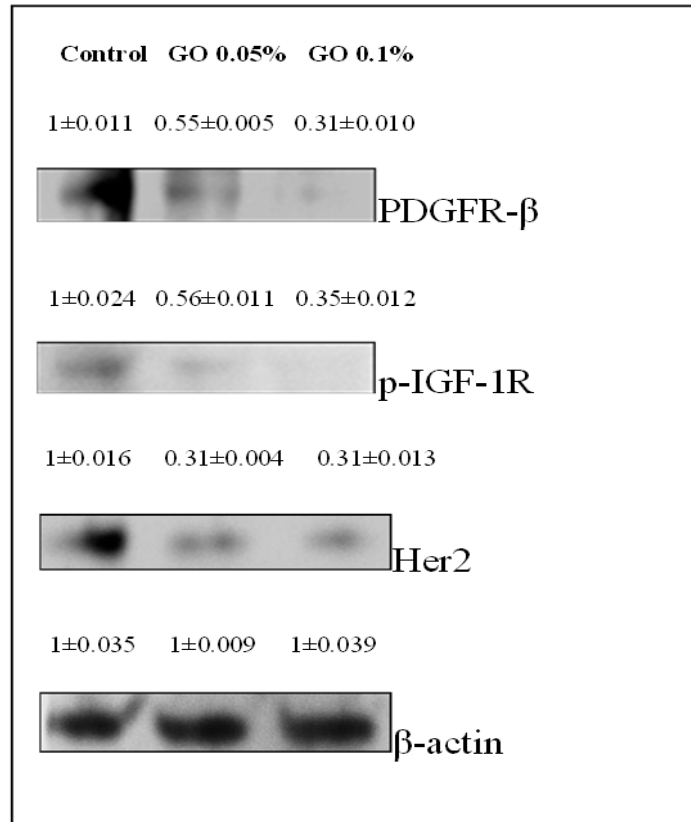


Figure 4.8: Expression of Growth Factor Receptors

As shown in figure 4.9, we have found out that ginger oil down regulates the expression of phosphorylated p38. Our outcomes are consistent with the observations made by others, where p38 MAPK activity modulated the regulation of several cytokines that included IL-8 and TNF- $\alpha$ . Researchers have shown that inhibition of p38 activation leads to strong inhibition of Tumor Necrosis Factor (TNF- $\alpha$ ) and Interleukin-8 (IL-8) (Westra and others 2004), which strongly correlates with our findings presented in figure 4.5 and figure 4.13 respectively. Our results suggest that ginger oil can significantly inhibit the expression of this oncogene, phosphor-p38 at concentrations as low as 0.05% and 0.1% of volume of the media.

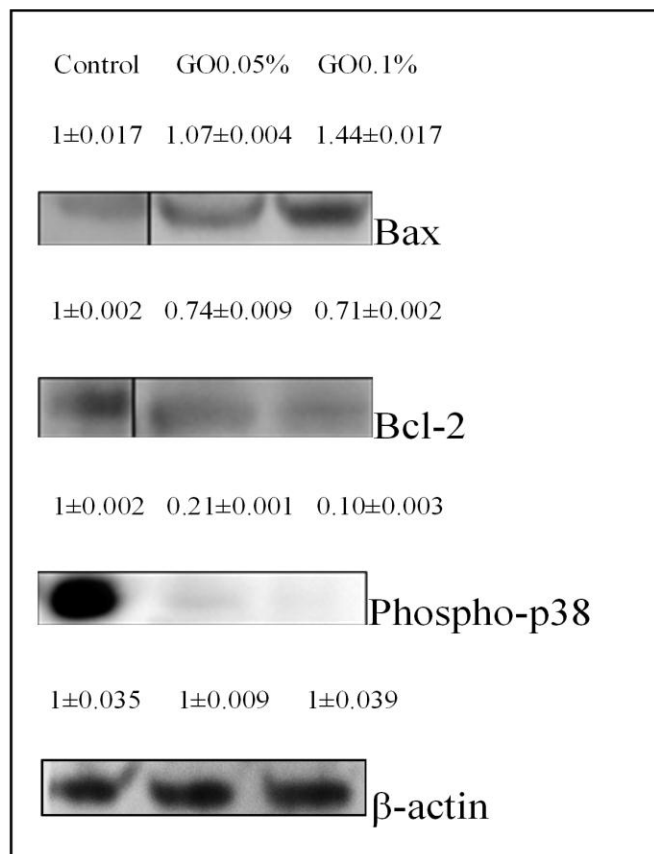


Figure 4.9: Expression of Proteins Involved in Apoptosis and Survival

#### 4.7.4 Effect of Ginger Oil on Expression of Cell Surface Biomarkers

Our results as shown in figure 4.10, demonstrate that ginger oil was effective against inhibiting breast cancer stem cell surface biomarkers like Aldh1/CD44/ESA. These data emphasizes the anti-carcinogenic activity of ginger oil against breast cancer stem cells. Aldh1 is a marker for normal and malignant human mammary stem cells. Curcumin and piperine have been reported to inhibit the expression of Aldh1, breast stem cell marker (Kakarala and others 2010). Dietary agents like sulforophane in combination with drug has also been reported to inhibit Aldh1 (Kallifatidis and others 2011). CD44<sup>+</sup> stem cells are found to be resistant against chemo- or radiation- therapy induced cell death (Takaishi and others 2009). Epithelial –specific antigen (ESA) is also expressed in breast cancer stem cells and all these biomarkers containing

breast cancer cells have very good self-renewal potential and are very aggressive with poor prognosis (Manson and others 2007).

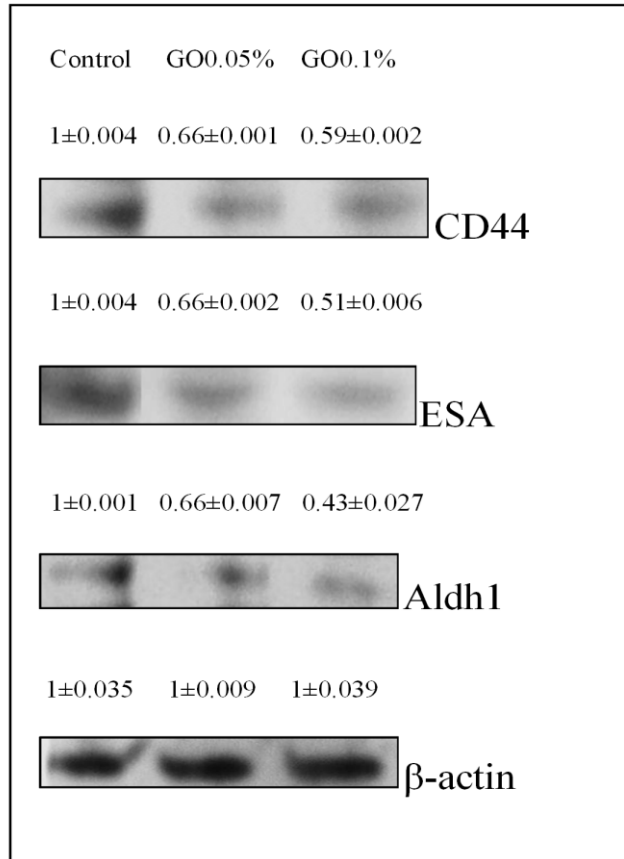


Figure 4.10: Expression of Cell Surface Markers

## 4.8 Effect of Ginger Oil in Process of Angiogenesis

### 4.8.1 Effect of Ginger Oil on Endothelial Cell Tube Formation

To determine the effect of ginger oil in the process of angiogenic tube formation, *in vitro* angiogenesis kit from Millipore was used. The process of angiogenesis is a multi-step process and this assay gives the basis for studying the tube formation. The matrix provided in the assay resembles the basement membrane, and it contains the basement proteins that are required to form tube. Endothelial cells when placed in the matrix, align in a way to form a hollow tube. To

test the inhibitory effect of ginger oil on tube formation, media of the cells untreated and treated with ginger oil was introduced to the seeded human coronary artery endothelial cells and the effect was observed after 6 hours.

The results of this experiment showed that in presence of ginger oil at concentrations as low as 0.05% and 0.1%, endothelial cells alignment was disrupted, which is presented in figure 4.11. While the controls; one with only endothelial cells and the other with endothelial cell with the media from the untreated MDA-MB-231 cells were able to form highly organized, extensively aligned capillary-like structure. Researchers have been able to show that [6]-gingerol, a pungent compound from ginger is able to inhibit VEGF induced endothelial cell proliferation and reduced the size of the tube *in vitro* and *in vivo* at a concentration of 10  $\mu$ M (Kim et al. 2005).

## **4.8.2 Effect of Ginger Oil on Expression of Angiogenic Biomarkers**

### **4.8.2.1 Vascular Endothelial Growth Factor**

To support these results of *in vitro* angiogenesis, we also analyzed the expression of vascular endothelial growth factor (VEGF) by western blot. Our results (Figure 4.12) showed that VEGF expression was significantly reduced in the cells treated with ginger oil at concentrations of 0.05% and 0.1%. VEGF is a dimeric glycoprotein, which is the most important growth factor for inducing angiogenesis. High level of VEGF is associated with high malignancy and poor prognosis in cases of breast cancer (Shibata et al. 2002; Heffelfinger and others 1999). Angiogenesis is regulated by many molecules and VEGF being one of the very important one. It is a glycoprotein with a molecular weight of less than 45 kDa. VEGF is expressed highly in



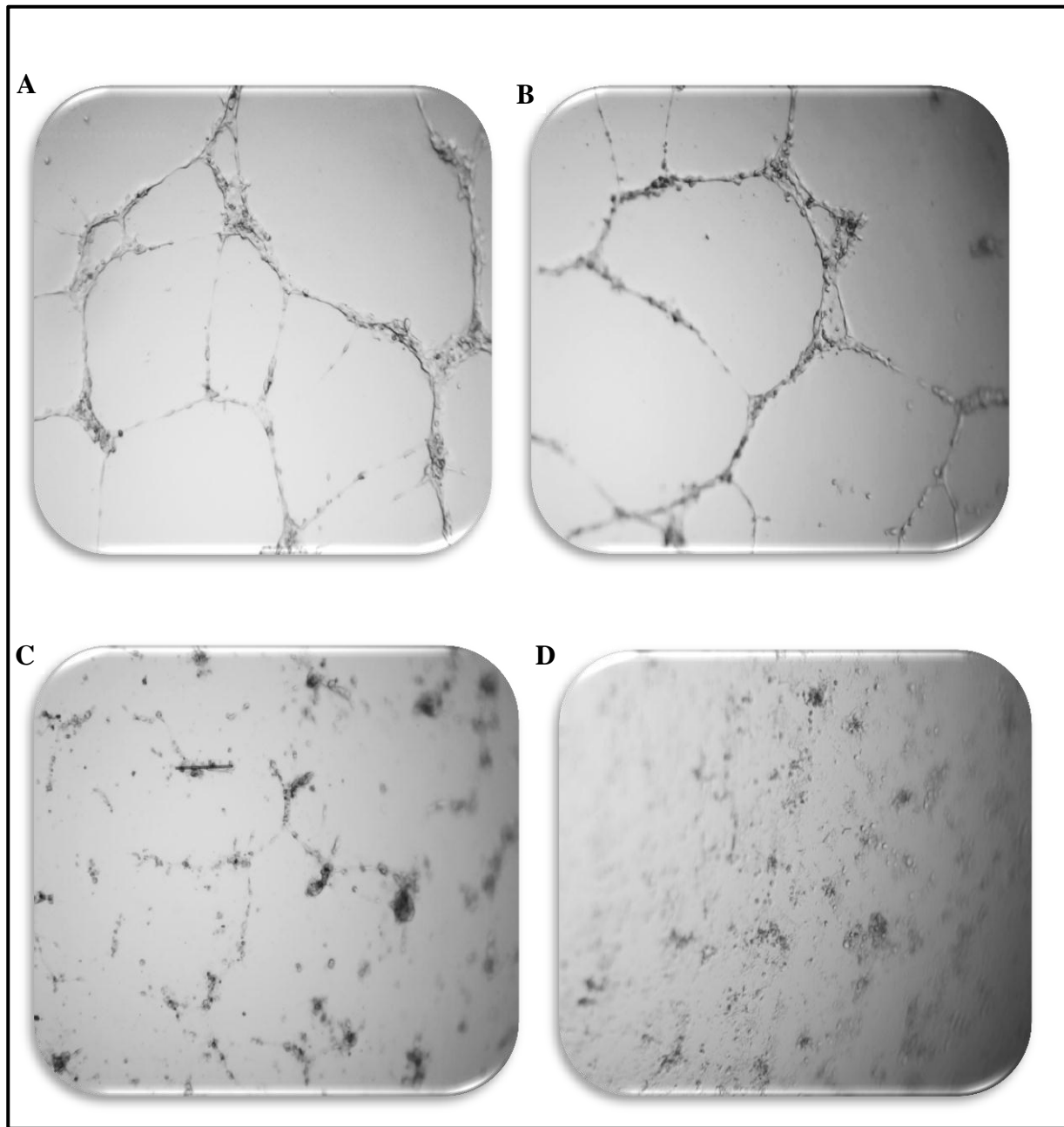


Figure 4.11: Inhibitory Effect of Ginger Oil on *in-vitro* Angiogenesis Tube Formation. A: Tubes Formed by Human Coronary Artery Endothelial Cells (HCAECs). B: Aligned HCAECs for Tube Formation in Presence of Media from Untreated Cells. C: Disrupted HCAECs in Presence of the Media from 0.05% GO. D: Disrupted HCAECs in Presence of the Media from 0.05% GO.

physiological cases like wound healing and embryonic development and in pathological cases like cancer. In case of cancer, when a tumor cannot grow beyond a certain size, it starts secreting VEGF molecules. VEGF receptors are present in endothelial cells, once VEGF molecule binds itself with these receptors, the physiological response of endothelial cell changes, directing the growth of blood vessels towards the tumor, eventually supplying nutrients and oxygen.

#### **4.8.2.2 Phospho-erk**

Phospho-erk is one of the major biomarker of signalling events for monitoring angiogenesis (Sessa and others 2008) and is the down streaming molecule in MAPK pathway . The intracellular signaling cascade involving MAPK /ERK leads to anti-apoptotic pathway and stimulates growth signals (Stuart and others 2006). As shown in figure 4.12, the results of the treatment of MDA-MB-231 cells with ginger oil showed inhibition of phosphorylated ERK when compared with the control.

#### **4.8.2.3 Cathepsin D**

Our results show that MDA-MB-231 cells treated with ginger oil had reduced level of cathepsin D expression. The result is shown in figure 4.12. Cathepsin D is an acidic lysosomal aspartyl endoprotease and is considered one of the biomarkers of breast cancer. It is reported to be overexpressed in pathological condition and is responsible for malignancy. Evidences suggest that the expression of cathepsin D is increased by 2 to 50 times in breast cancer cells as compared to normal mammary cells or other cells (Capony and others 1989). It plays role in cell proliferation, apoptosis, extra cellular matrix degradation, activation of growth factors, and angiogenesis (Leto and others 2004). Literatures suggest that cathepsin D in breast cancer cells, boosts anchorage-independent cell proliferation leading to tumorigenesis and metastasis (Glondou

and others 2002). Cathepsin is supposed to be up regulated and heavily secreted in ER negative MDA-MB-231 cells (Garcia and others 1996). Literatures suggest that MDA-MB-231 cells are model of breast cancer cells with high cathepsin D level with more metastatic properties, which makes cathepsin D a potential target for breast cancer therapy (Rocheftort and others 2000).

#### 4.8.2.4 Histone Deacetylases (HDACs)

As shown in figure 4.12, HDACs are down regulated with the treatment of ginger oil. HDACs are the enzymes involved in epigenetics, most commonly in DNA methylation and acetylation. It controls most of the genes responsible for angiogenesis, cell cycle, and apoptosis (Shankar and Srivastava 2008). Also histone deacetylase have been reported to induce apoptosis by up regulating the pro-apoptotic proteins and inhibiting the anti- apoptotic proteins. Histone inhibitors have also been reported to reduce the level of VEGF expression in tumor cells of mice (Shankar and others 2005).

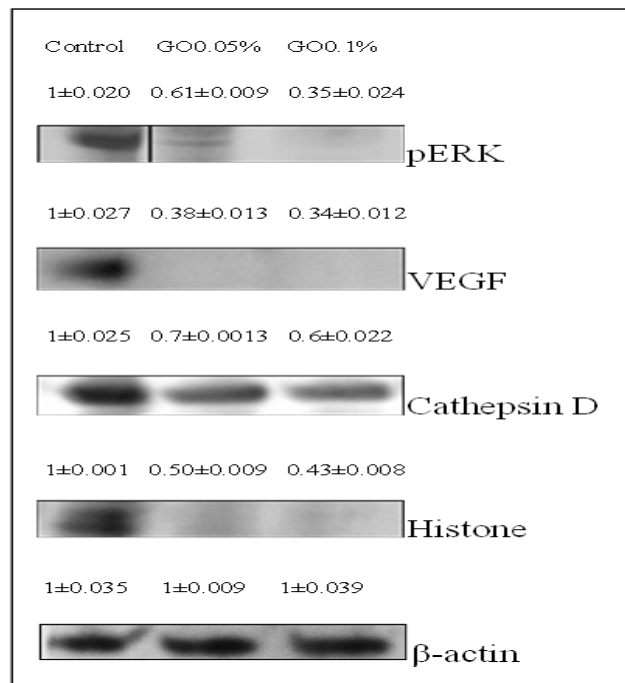


Figure 4.12: Expression of angiogenic biomarkers

### 4.8.3 Effect of Ginger Oil on Interleukin-8 (IL-8) Levels

To determine the level of expression of IL-8, ELISA was performed. IL-8 is produced mostly by monocytes, macrophages, T cells and vascular endothelial cells. Its secretion is regulated by NFκB. IL-8 is reported to function in formation of new micro vessels, tumor neovascularization, help in tumor growth and metastasis, and activate the ERK2/1 pathway (Heidemann and others 2003). It has also been found out that the level of IL-8 expression increases with decrease in the level of estrogen receptor. IL-8 is one of the major molecules found to be responsible for the properties like invasion and angiogenesis in breast cancer (Lin and others 2004). Dietary agents such as curcumin, quercetin, resveratrol, genistein, green tea polyphenols, theaflavin, and capsaicin has been reported to down regulate the expression of IL-8 (Hidaka and others 2002; Shen and others 2003; Porath and others 2005). Literatures show that ginger is able to inhibit the expression of IL-8 in ovarian cancer cells (Rhode and others 2007). Another group of researchers have shown that ginger extract at a concentration of 100µg/ml was able to inhibit the expression of chemokines in human synoviocytes (Phan and others 2005). Researches have shown that ginger can effectively modulate the biochemical pathway in chronic inflammation with changing expression of various chemokines and cytokines genes including cyclooxygenase-2 (Grzanna et al. 2005) .

In our study we have used MDA-MB-231 cell line, which is ER<sup>-</sup> and according to the findings of Lin et al. as mentioned above the level of IL-8 expression is high in ER<sup>-</sup> cells. And this level of IL-8 was significantly dose dependently decreased in the cells treated with 0.05% and 0.1% of ginger oil. Obtained results are presented in figure 4.13.

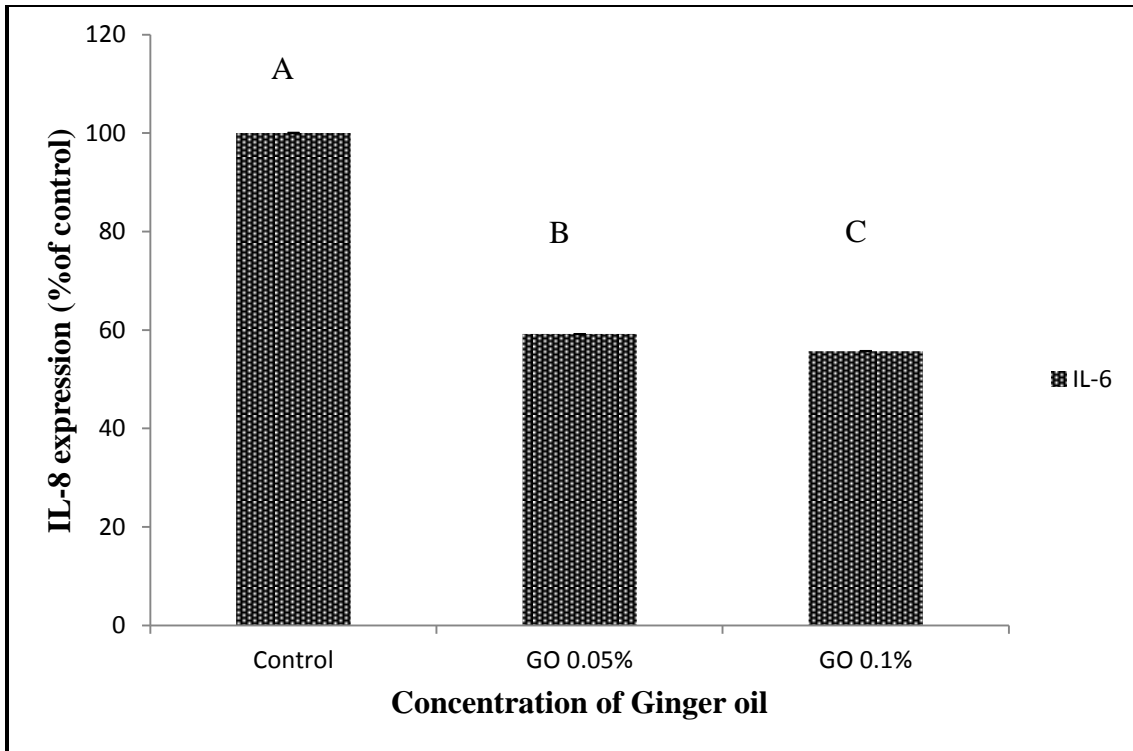


Figure 4.13: Dose Dependent Inhibition of Ginger Oil on the Expression of Interleukin-8 (IL-8). Values (means  $\pm$  SD, n=3) not sharing a common letters, ABC are significantly different ( $\alpha=0.05$ ).

## CHAPTER 5: SUMMARY AND CONCLUSIONS

Data accumulated from this research propose that dietary phytochemicals, like ginger oil have the potential to manage deregulated signaling pathways or restore checkpoint pathways and apoptosis in cancer. Biological activities of ginger oil show promising future in treatment of cancer. However, this requires further investigation to figure out mode of action of ginger oil and additional research to determine the amount of regular intake.

In summary, the major finding of this study is that ginger oil inhibits the viability of breast cancer cells through caspase-mediated pathway, as well as down regulates Hsp90 and its related client proteins. Ginger Oil induced apoptosis with the increase in caspase activity, cell cycle arrest in the S-phase and increase in the level of sub-G1 phase. Ginger oil also decreased protein levels of cyclin D1, cyclin dependent kinase (Cdk)-2, Cdk-4 and Bcl-2 level while it increased the level of apoptotic proteins such as Bax. In addition, ginger oil inhibited *in vitro* angiogenesis and biomarkers associated with angiogenesis. Ginger oil also significantly reduced the expression of various oncoproteins and repressed the activity of telomerase that are expressed in breast cancer cells. Hence, ginger oil is an essential oil with various anti-cancer properties. So far we have demonstrated that ginger oil inhibits hallmarks of breast cancer cells, however, it remains to demonstrate that ginger oil is also as effective against cancer cells *in vivo*.

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