

**Phytochemicals from *Graviola* fruit selectively inhibit breast cancer cells growth
involving regulating EGFR Signaling pathway**

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ABSTRACT

There is a growing interest in using naturally-occurring compounds as cancer chemopreventive or chemotherapeutic agents. This study investigated the anticancer potential of the graviola fruit extract (GFE) on specific human breast cancer (BC) cells. GFE was found in our preliminary screening to selectively inhibit the growth of certain human BC cells (MDA-MB-468) but did not affect non-transformed breast epithelial MCF-10A cells. GFE treatment was very effective against the growth of MDA-MB-468 BC cells with an IC₅₀ of 4.8 µg/ml. *In vitro*, effects of GFE treatment on MDA-MB-468 BC cells were further examined for apoptosis and cell proliferation. Apoptosis, determined qualitatively and quantitatively, was enhanced and accompanied by caspase-3 activation. GFE treatment also induced cell cycle arrest at the G1 cell cycle phase and significantly reduced the percentage of MDA-MB-468 cells in S-phase following 24h of exposure. Moreover, the results from analysis of the mRNA expression of epidermal growth factor receptor (EGFR), which plays an important role in regulating cell development and death, by qRT-PCR, suggested that GFE-induced selective growth inhibition of MDA-MB-468 BC cells is associated with a significant inhibition of EGFR gene expression in the cells. *In vivo*, dietary treatment with GFE significantly inhibited MDA-MB-468 tumor growth implanted in mice by reducing tumor wet weight and significantly reduced EGFR and p-ERK protein expression in tumors. Overall, GFE attenuated cell proliferation, induced apoptosis, modulated cell cycle regulation and downregulated EGFR gene expression both *in vitro* and *in vivo*. These discoveries support the further studies to fully elucidate the antitumor potential of GFE and its components as a dietary agent for BC.

DEDICATION

I would like to dedicate this thesis to my parents Mingliang Dai and Huiling Fan, my brother Wei Zhang for their love and support.

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LIST OF ABBREVIATIONS AND TERMS

ANOVA	Analysis of variance
BC	Breast cancer
cDNA	Complimentary DNA
DAPI	4',6'-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
p-ERK	Phosphorylated extracellular regulated protein kinases
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde phosphate dehydrogenase
GFE	Graviola fruit extract
HEPES	Hydroxyethyl piperazine ethanesulfonic acid
HRP	Horseradish peroxidase
MTT	Methyl thiazolyl tetrazolium bromide
PI	Propidium iodide
PR	Progesterone receptor
qRT-PCR	Quantitative real time-polymerase chain reaction
RIPA	Preparation of modified radioimmunoprecipitation
TBST	Tris buffered saline with Tween
TGF- α	Transforming growth factor-alpha
THF	Tetrahydrofuran

CHAPTER 1: INTRODUCTION

Breast cancer remains one of the most common cancers among women (Bray, McCarron & Parkin, 2004). In 2009, a prediction estimated 193,370 new cases and 40,170 death from BC (Cancer.org, 2009). Annual health care costs total in the billions (Smigal et al., 2006).

Breast cancer is extremely difficult to treat due to several distinct classes of tumors that exhibit different treatment responses (Lopez-Otin & Diamandis, 1998). Thus, treatment of breast cancer patients varies and must be individualized. For example, women diagnosed with estrogen receptor negative (ER-) breast cancers tend to have more aggressive tumors, fewer treatment options, and a higher mortality rate (Dunnwald, Rossing & Li, 2007). A great number of drugs used to treat breast cancer have been introduced onto the market, including Taxol, Doxorubicin, Tamoxifen, etc. However, most of these drugs confront several barriers when being used. For example, Taxol is required to be dissolved in a toxic solvent prior to administration, while Tamoxifen has been reported to be effective in only one-third of the breast cancer patients (Forbes, 1997). Considering the heterogeneity of BC and the limitations of current therapies such as severe side effects and drug resistance, there is an urgent need to explore alternative strategies to prevent and treat BC.

Development of novel mechanism-based nutritional agents that can selectively target BC may offer an intriguing strategy for controlling BC. Accumulating evidence suggests a strong effect of the diet or its components on BC development and progression, either through effects on hormonal status or via direct tumor-promoting or anticarcinogenic effects (Rock & Demark-Wahnefried, 2002a). Epidemiologic studies have consistently indicated that increased consumption of fruits and vegetables is associated with reduced risk of developing cancer (Hong & Sporn, 1997). This beneficial effect is due in part to

the fact that fruits and vegetables contain fiber, antioxidants, vitamins, minerals, and other potentially anti-neoplastic compounds (Terry, Terry & Wolk, 2001). Specific food bioactive components, notably sulfur-containing glucosinolates and green tea polyphenols, were associated with reduced risk of BC (Duffy & Cyr, 2003; Mukhtar & Ahmad, 2000). Therefore, exploring natural food sources for bioactive compounds with anti-tumorigenic, and other systemic protective effects is reasonable and logical.

Recent screening identified a specific extract of *graviola* fruit (GFE) that significantly inhibited the growth of human MDA-MB-468 BC cells at a low dose ($IC_{50} = 4.8 \mu\text{g/mL}$) but did not affect non-transformed breast cells (MCF-10A). Graviola (*Annonaceous muricata L.*) is an Amazon fruit tree that grows in the tropics of North and South America, and is also known as soursop and guanabana. Leaves and stems of graviola have been traditionally used as a herbal remedy for a variety of purported health promoting effects, including supporting healthy cell growth and immune function (Adewole & Caxton-Martins, 2006). Graviola fruits have been widely consumed by indigenous people in fresh or processed forms for centuries. However, research on graviola fruit is extremely limited despite its regular consumption and there is no published study investigating the effect of graviola fruit on breast and other cancer.

It is known that MDA-MB-468 cells contain an amplified EGFR gene and show very high expression levels of EGFR (Filmus, Trent, Pollak & Buick, 1987). Interestingly, preliminary data showed that GFE treatment significantly down-regulated EGFR expression in MDA-MB-468 BC cells. The epidermal growth factor receptor (EGFR) has been identified as a promising target for BC therapies. Moreover, molecular inhibition of EGFR signaling by anti-EGFR monoclonal antibody or specific small molecule inhibitors has shown anti-tumor effects in clinical trials (Goss et al., 2005). Therefore, the study of Graviola phytochemicals' mechanisms of action may strengthen its effects on breast cancer

cell growth and, in turn, could have a significant impact on treatment.

Specific Aims:

1. Identification of anticancer capacity of GFE *in vitro*.

MTT assay will be employed to detect the cell viability of Human BC MDA-MB-468, MDA-MB-231, and MCF-7 cells and non-transformed breast epithelial MCF-10A cells after being treated by GFE. Then, cell apoptosis, cell cycle and EGFR mRNA expression assays will be carried out on the cell line which shows the lowest cell viability.

Null hypothesis: There is no difference in anticancerous capacities among GFE treated cells and non-treated cells.

Alternative hypothesis: There is a difference in anticancerous capacities among GFE treated cells and non-treated cells.

2. Identification of anticancerous capacity of GFE *in vivo*.

MDA-MB-468 Breast cancer cells will be implanted into athymic nude mice and generate the xenografts. The tumor size and body weight of the mice will be monitored. After extraction of proteins from the xenografts, EGFR and p-ERK protein expression and cytokines level will be measured.

Null hypothesis: There is no difference in EGFR and p-ERK protein expression and cytokines level among GFE treated cells and non-treated cells.

Alternative hypothesis: There is a difference in EGFR and p-ERK protein expression and cytokines level among GFE treated cells and non-treated cells.

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CHAPTER 2: REVIEW OF LITERATURE

2.1 Breast cancer and its traditional treatment

2.1.1 Breast cancer

Breast cancer refers to cancers originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Cancers originating from ducts are known as ductal carcinomas; those originating from lobules are known as lobular carcinomas. There are many different types of breast cancer, with different stages (spread), aggressiveness, and genetic makeup; survival varies greatly depending on those factors.

According to the American Cancer Society, besides skin cancer, breast cancer is the number one form of cancer diagnosed in women with the highest rate of occurrence in North America (Smigal et al., 2006). Since 1940, incidence rates of invasive breast cancer show four distinct phases: between 1940 and 1980, a steady increase of about 1% per year occurred, possibly due to a gradual increase in underlying risk factors, such as delayed childbearing and having fewer children; between 1980 and 1987, incidence rate increased by about 4% per year, mainly due to a greater capability of detecting smaller tumors during mammography screening. In this time frame, the number of small tumors <2.0cm more than doubled and incidence of tumors >3.0cm decreased by 27% (Garfinkel, Boring & Heath, 1994). Between 1987 and 1998, incidence rates of breast cancer have increased by 0.5% per year with the decline in incidence rate possibly attributed to increased detection and earlier diagnosis from mammography screening (Howe et al., 2001). However, breast cancer incidence rates decreased by about 2% per year from 1999 to 2006. This decrease may be due, at least in part, to lower use of hormone replacement therapy (HRT) after the results of the Women's Health Initiative were published in 2002. The American Cancer Society's most recent estimates for breast cancer in the United States for 2009 indicated

that about 192,370 new cases of invasive breast cancer will be diagnosed in women, and about 62,280 new cases of carcinoma in situ will be diagnosed, while about 40,170 women will die from breast cancer.

Breast cancer is the second leading cause of cancer death in women, exceeded only by lung cancer. The chance that breast cancer will be responsible for a woman's death is about 1 in 35 (about 3%). However, breast cancer mortality rates demonstrate a declining tendency in the past few decades. It was relatively stable between 1950 and the late 1980s, but there has been a decrease from then on. For all races, combined, between 1989 and 1995, mortality rates from breast cancer decreased by 1.6% annually and continued to decrease by 3.4% annually between 1995 and 1998 (Howe et al., 2001). Death rates from breast cancer continue to decline since 2000, with larger decreases in women younger than 50. These decreases are believed to be the result of earlier detection through screening and increased awareness, as well as improved treatment.

The survival rate from breast cancer is influenced by several factors. The time from diagnosis is considered where survival is 86% within the first five years after diagnosis, 76% after 10 years, 58% after 15 years and 53% after 20 years (Ries LAG, 2008). Other factors include age at the time of diagnosis, where it is speculated that younger women have lower survival rates because their tumors may be more aggressive and less responsive to hormonal treatment (Marcus, Watson, Page & Lynch, 1994), stage of the cancer at diagnosis, ethnicity (Ries LAG, 2008) and socioeconomic factors (Eley et al., 1994).

There are different kinds of risk factors. Some factors, such as a person's age or race, can't be changed. Others are linked to cancer-causing factors in the environment. Still others are related personal behaviors, such as smoking, drinking, and diet. Well established risk factors account for 47% of cases while 5% are attributable to hereditary syndromes

(Madigan, Ziegler, Benichou, Byrne & Hoover, 1995). In particular, carriers of the breast cancer susceptibility genes, BRCA1 and BRCA2, are at a 30-40% increased risk for breast and ovarian cancer, depending on in which portion of the protein the mutation occurs (Venkitaraman, 2002). Breast cancer incidence increases with age and is the leading overall cause of cancer death in women between the ages of 45 and 55 within the United States. About 1 out of 8 invasive breast cancers are found in women younger than 45, while about 2 out of 3 invasive breast cancers are found in women age 55 or older, according to the statistics from American Cancer society. Besides age, risk increases if a woman has a family history of breast cancer (Morabia & Costanza, 1998) or drinks too much alcohol (Boffetta, Hashibe, La Vecchia, Zatonski & Rehm, 2006). Breast cancer risk is, in part, associated with higher serum levels of endogenous hormones, in particular that of 17 β -estradiol, whose metabolite--16 alpha-hydroxyestrone may act to enhance cancer development (Suto et al., 1999).

2.1.2 Breast cancer cell lines

There are more than 50 types of cell lines that have been discovered from breast cancer tissue. As shown in **Table 2.1**, these different kinds of cell lines varied based on genomic features, transcription profiles, genomic deregulation of gene expression, etc. (Neve et al., 2006). Although the genes of all cell lines are clustered either luminal, Basal A or Basal B, they still show different biological differences, such as ER, PR positivity, HER2 overexpression, TP53 protein levels or mutational status. In this study, MCF-7, MDA-MB-231, MDA-MB-468 and MCF-10A were used for the research, since these four different cell lines could be representative for each type. MCF-7 is the first hormone-responsive breast cancer cell line, expresses a high level of estrogen receptor (ER) and responds to the mitogenic and antimitogenic stimuli of estrogens and antiestrogens, respectively. Thus, MCF-7 provides an excellent model for the study of the hormonal regulation of ER in breast cancer (Levenson & Jordan, 1997). The MDA-MB-468 cell line represents an aggressive ER negative phenotype, since proliferation of these cells is

also inhibited by 17 β -estradiol, and it is comparable to effects observed in other cell lines stably transfected with the ER (Wang, Smith, Burghardt & Safe, 1997). The MDA-MB-231 cell line is also a representative of an ER negative phenotype breast cancer cell line. However, compared to MDA-MB-468 and MCF-7, it has invasive characteristics. It is reported that the invasive capacity of highly metastatic MDA-MB-231 breast cancer cells is much higher than that of poorly metastatic MCF-7 breast cancer cells (Zuo, Shields & Chakraborty, 2006). Highly invasive cancer cells are capable of passing through matrix membranes and may affect the body using different signaling pathways. MCF-10A cells, which are often used to represent normal breast epithelial cells, mainly express myoepithelial marker, and appear to have arisen from basal cells of the mammary epithelium (Spink, Cole, Katz, Gierthy, Bradley & Spink, 2006). Therefore, using MCF-10A cells as control cells, it is easier to discover the drug action during the chemotherapies.

2.1.3 Traditional treatments of breast cancer

Surgery, and then drugs, radiation, or both are the main methods to treat breast cancer. Treatments are given with increasing aggressiveness according to the prognosis and risk of recurrence. Early cancers with good prognosis (T1, N0) may be treated with lumpectomy plus radiation alone or hormone therapy alone. Later cancers with poorer prognosis and greater risk of recurrence may be treated with more aggressive chemotherapy with uncomfortable and life-threatening side effects, in order to increase the likelihood of cure and lower the risk of recurrence.

Surgery could be divided into two kinds-- lumpectomy and mastectomy. Lumpectomy removes only the breast lump and a surrounding margin of normal tissue but how much is removed depends on the size and location of the tumor and other factors. However, side effects of these operations can include pain, temporary swelling, tenderness, and hard scar tissue that form at the surgical site. As with all operations, bleeding and infection at the

surgery site are also possible. Mastectomy involves removing all of the breast tissue, sometimes along with other nearby tissues. However, aside from post-surgical pain and the obvious change in the shape of the breast, possible side effects of mastectomy include wound infection, hematoma (buildup of blood in the wound), and seroma (buildup of clear fluid in the wound) (Munoz, Shamash, Friedman, Teicher & Wise, 1986).

Radiotherapy is given after surgery to the region of the tumor bed, to destroy microscopic tumors that may have escaped surgery. Radiation therapy can be delivered as external beam radiotherapy or as brachytherapy (internal radiotherapy). Radiation can reduce the risk of recurrence by 50-66% when delivered in the correct dose. The main short-term side effects of external beam radiation therapy are swelling and heaviness in the breast, sunburn-like skin changes in the treated area, and fatigue.

Drugs, in addition to surgery, are called adjuvant therapy. Hormone therapy is one class of adjuvant therapy. Some breast cancers require estrogen to continue growing, much like the MCF-7, BT474, T47D breast cancer cell lines. They can be identified by the presence of estrogen receptors (ER+) and progesterone receptors (PR+) on their surface. These ER positive cancers can be treated with drugs that block the production of estrogen or block the receptors, such as tamoxifen or an aromatase inhibitor.

Tamoxifen (**Figure 2.1A**) is an antagonist of the estrogen receptor in breast tissue. It has been the standard endocrine therapy for hormone-positive early breast cancer and used for many years to reduce the risk of recurrence in localized breast cancer. Results from the Breast Cancer Prevention Trial (BCPT) have shown that women at increased risk for breast cancer are less likely to develop the disease if they take tamoxifen. Women in the study took either tamoxifen or a placebo for 5 years. After 7 years of follow-up, women taking tamoxifen had 49% fewer breast cancers than women who took the placebo, although there

was no difference in the risk of dying from breast cancer (Fisher et al., 1998). Tamoxifen is approved for reducing breast cancer risk in women at high risk. However, tamoxifen can also cause severe side effects, including increased risks of endometrial cancer and blood clotting (Zikmund-Fisher et al., 2008), has affects on the central nervous system (Legault et al., 2009), and may reduce breast cancer risk in women with BRCA2 gene mutations, but the same may not be true for those with BRCA1 mutations (King et al., 2001). Therefore, the patients should consider the possible benefits and risks of tamoxifen before deciding if it is right for them.

Aromatase inhibitors, such as letrozole (Figure 2.1B), anastrozole, and exemestane are also being studied as breast cancer chemopreventive agents in post-menopausal women (Mouridsen et al., 2009). Aromatase is an enzyme that synthesizes estrogen and aromatase inhibitors block the synthesis of estrogen. This lowers the estrogen level, and slows the growth of estrogen-sensitive cancers. These drugs are already being used to help prevent breast cancer recurrences. They work by blocking the production of small amounts of estrogen that post-menopausal women normally make. But they can also have side effects such as causing joint pain and stiffness and bone loss (Harper-Wynne et al., 2002), leading to a higher risk of osteoporosis. None of these drugs is approved for reducing the risk of developing breast cancer at this time.

Chemotherapy is given for more advanced stages of disease and can also be used as the main treatment for women whose cancer has already spread outside the breast and underarm area at the time it is diagnosed, or if it spreads after initial treatments. The length of treatment depends on whether the cancer shrinks, how much it shrinks, and how a woman tolerates treatment. In most cases, chemotherapy is most effective when combinations of more than one drug are used. One of the most common treatments is cyclophosphamide (Figure 2.1C) plus doxorubicin (Adriamycin) (Figure 2.1D), known as

CA; Sometimes a taxane drug, such as docetaxel, is added, and the regime is then known as CAT. Taxane compounds, like taxol (**Figure 2.1E**), attack the microtubules in cancer cells. Other treatments, which produce equivalent results, are cyclophosphamide, methotrexate, and fluorouracil (**Figure 2.1F**) (CMF), cyclophosphamide, epirubicin, and 5-fluorouracil (CEF), cyclophosphamide, doxorubicin (Adriamycin), and 5-fluorouracil (CAF), etc. Chemotherapy drugs work by attacking cells that are dividing quickly, which is why they work against cancer cells. But other cells in the body, such as those in the bone marrow, the lining of the mouth and intestines, and the hair follicles, also divide quickly. So these cells are also likely affected by chemotherapy, which can lead to side effects: hair loss, mouth sores, loss of appetite, nausea and vomiting, increased chance of infections, easy bruising or bleeding. Moreover, some longer-term effects may occur, for example, damage to the heart muscle is the most dangerous complication of doxorubicin use (Osman, Nemnem, Abou-Bakr, Nassier & Khayyal, 2009). Several drugs used to treat breast cancer, including the taxanes (docetaxel and paclitaxel), platinum agents (carboplatin, cisplatin), and ixabepilone, can damage nerves outside of the brain and spinal cord. This can sometimes lead to symptoms (mainly in the hands and feet) such as numbness, pain, burning or tingling sensations, sensitivity to cold or heat, or weakness. (Argyriou, Koltzenburg, Polychronopoulos, Papapetropoulos & Kalofonos, 2008). Other native effects also include increase risk of leukemia (Renella et al., 2006) and chemobrain, a cognitive dysfunction (memory, concentration) that results from chemotherapy (Weiss, 2008).

Although traditional methods of treatment, especially drugs, elicit positive effects of decreasing mortality of breast cancer patients, negative effects which make the patients frustrated and are sometimes lethal, are also apparent. Because drug resistance occurs with long term use of specific drugs, and various kinds of breast cancer types result from mutation, identifying alternative ways to regulate growth of breast cancers, especially the

ways that has high selectivity to control specific types, is imperative.

2.2 Nature-derived nutrients and phytochemicals from fruits and vegetables may be alternate cancer killers

In order to prevent the drug resistance and inevitable severe side effects, in recent years, it has been suggested that dietary modification is an alternative and useful strategy to lower the risk of cancer. It has even been suggested that one-third of all cancer deaths could be avoided this way (Willett, 1995). Epidemiological studies have consistently shown that increased consumption of fruits and vegetables is linked with declining risk of developing cancer (Block, Patterson & Subar, 1992). Among all the nutrients in the fruits and vegetables, it is suggested that phytochemicals are the major contributor to these health benefits in the cancer prevention (Liu, 2003). Focused on breast cancer, Birt and coworkers had found that the higher the isoflavone or flavone intake, the less estrogenic or antiestrogenic activity the body shows, thus the lower probability the breast cancer might occur (Birt, 2001). An earlier study by Freudenheim and coworkers have discovered that there was a reducing risk of breast cancer associated with the high intake of several nutrients present in vegetables, among which, beta-carotene and lutein contribute the most (Freudenheim et al., 1996). In a review focusing on the survival of breast cancer patients and their intake of fruit and vegetables, five of the eight cohort studies showed an inverse relationship between vegetables and fruit intake and survival of breast cancer patient, with a 20-90% reduction in death risk (Rock & Demark-Wahnefried, 2002b). These studies convincingly show that dietary modification may be an effective way to prevent and perhaps even treat breast cancer.

2.2.1 Vitamins may inhibit breast cancer *in vitro* and *in vivo*

Several nutrients have been reported to alter the growth of breast cancer cells *in vitro* and *in vivo*. Among them, Vitamins A, C, E, D and the B group play important roles in

combating different types of breast cancer cells.

Vitamin A was the first vitamin to be studied with respect to carcinogenesis. In the late 1950s and throughout the 1960s, a number of reports demonstrated that vitamin A deficiency increased the number of spontaneous and chemically induced tumors in experimental animals (Niles, 2000). Moreover, retinoids, which are naturally occurring and hormonally active vitamin A metabolites, have been shown to have antitumor activities both *in vitro* and *in vivo* (Pettersson, Couture, Hanna & Miller, 2004). In addition, many researchers have examined the ability of the retinoids all-*trans*-retinoic acid, 9-*cis*-retinoic acid and *N*-(4-hydroxyphenyl) retinamide (fenretinide) to protect against breast cancer and have reported that these compounds prevent breast cancer cell proliferation and promote the apoptosis of these cells by binding to the estrogen receptor (Rosenauer, Nervi, Davison, Lamph, Mader & Miller, 1998; Simeone & Tari, 2004). So it seems that Vitamin A may be a useful agent to control breast cancer of ER positive type. Moreover, Paik and coworkers discovered that, in breast cancer cells under the treatment of Vitamin A, the overexpression of *cis*-retinol dehydrogenase which converts 9-*cis*-retinol to 9-*cis*-retinoic acid, increased production of 9-*cis*-RA, which in turn suppressed cell proliferation (Paik, Blaner & Swisshelm, 2005).

Vitamin C is an essential nutrient involved in many biochemical functions. The biochemical roles of Vitamin C are related to its ability to act as an electron donor or reducing agent. In its role as a free radical scavenger, Vitamin C is believed to protect cellular biopolymers, including genetic material, and could protect against the initiation and progression of carcinogenesis. However, Vitamin C supplementation showed no effects on directly prevention of cancer in several studies (Salonen et al., 2000), but it is believed that carcinogenesis is due to oxidative damage of mitochondrial DNA by reactive oxygen species (ROS) arising from catechol estrogen redox cycling, which is a process in

estrogen metabolism (Cai et al., 2004; Roy & Liehr, 1999). Thus, Vitamin C could prevent the initiation of breast carcinogenesis by neutralizing free radicals before they can damage DNA and initiate tumor growth. In this way, Vitamin C indirectly prevents breast cancer, instead of treating the cancer.

Vitamin E is important not only for its cellular antioxidant and lipid-lowering properties, but also as an antiproliferating agent. It has also been shown to contribute to immunoregulation, antibody production, and resistance to implanted tumors. Vitamin E is a generic term that refers to an entire class of compounds that are further divided into two subgroups called tocopherols and tocotrienols. Just as there are several forms of tocopherol (α , β , γ , and δ), there are also α -, β -, γ -, and δ -tocotrienols. Tocopherols are commonly found in high concentrations in vegetable oils, animal fats, grains, vegetables, and fruits, whereas tocotrienols are relatively rare and found in appreciable levels only in a few specific vegetable fats such as palm oil and rice bran oil. Previous research has shown that tocotrienols can exert direct inhibitory effects on cell growth in human breast cancer cell lines *in vitro* (Nesaretnam, Dorasamy & Darbre, 2000; Nesaretnam, Guthrie, Chambers & Carroll, 1995) and that inhibitory effects occurred regardless of estrogen receptor status of the cells (Nesaretnam, Stephen, Dils & Darbre, 1998). Later, Nesaretnam reported that, after 72 hour treatment of tocotrienols, c-myc binding protein MM-1, a 23-kDa highly basic protein, and interferon-inducible protein 9-27 (IFITM-1), involved in the cell cycle, could exert inhibitory effects on cell growth and differentiation of the tumor cell lines. These data suggest that tocotrienols are able to affect cell homeostasis, possibly independent of their antioxidant activities (Nesaretnam, Ambra, Selvaduray, Radhakrishnan, Canali & Virgili, 2004).

Besides Vitamin A, C, E, it has been found that Vitamin D may inhibit the proliferation of MCF-7 breast cancer cells by blocking the Vitamin D receptor (Brenner et al., 1995), and

deficiency of folic acid (Vitamin B11) in experimental studies causes DNA damage that resembles the DNA damage seen in cancer cells (Jennings, 1995). As an important group of nutrients, vitamins mostly derived from fruits and vegetables appear to be a large anti-cancer source in the human diet and therefore dietary manipulation to reduce risk of cancer should focus on increasing vitamin intake.

2.2.2 Plant-derived phenolic compounds and other phytochemicals play an important role in treating breast cancer

Another type of nutrients that may have a promising role in the prevention and/or treatment of breast cancer are plant-derived phenolic compounds with antioxidant properties. For example, many natural food sources contain bioactive compounds with antioxidant activities (as flavonoids and other phytochemicals) that may have benefit as primary or adjunct therapy for oxidative stress-associated disease states such as cancer. Recent research indicates that many beneficial health effects result from the consumption of fruits due to their phenolic compounds (Arts, Hollman, Mesquita, Feskens & Kromhout, 2001; Aviram, 2003; Aviram & Dornfeld, 2001; NamDeuk et al., 2002; Schubert, Lansky & Neeman, 1999). Many phyto-phenolic extracts have been documented to possess a high antioxidant capacity. For example, pomegranate juice is reported to exhibit three times the antioxidant capacity compared to that of red wine, and is comparable to green tea (Gil, Tomas-Barberan, Hess-Pierce, Holcroft & Kader, 2000; Plumb, de Pascual-Teresa, Santos-Buelga, Rivas-Gonzalo & Williamson, 2002). Additional research is accumulating indicating that phyto-compound nutrients may be important modulators of human mammary cancer cell growth, and further study of their mechanism of action may result in new treatments for mammary cancer cell growth.

Flavonoids are a group of naturally-occurring, low molecular weight compounds and constitutes of large class of polyphenolic compounds that are abundant in vegetables, fruits and plant-derived beverages. Over 6500 flavonoids have been described, and the average

intake of total flavonoids from the Western diet was estimated to be 200 mg to 1 g per day (Harborne & Williams, 2000). Flavonoids compounds can be divided into different structural groups, such as flavones, isoflavones, flavonols and flavanones. A number of different chemical compounds including quercetin, genistein and catechin (Figure 2.2A) have shown great inhibition to the growth of different types of breast cancer cells, like MCF-7, MDA-MB-231.

Quercetin (Figure 2.2B), a flavonol, is widely distributed in all kinds of green vegetables. According to the research of Rodgers, 100 μ M of quercetin inhibited cell proliferation of MCF-7 by 78% compared to control cells as detected by MTT assay. Moreover, at 25 μ M, quercetin decreased intracellular protein contents to $57\pm 6\%$ of control cell values; at 50 μ M it caused a decrease to $53\pm 2\%$, and at 100 μ M to $41\pm 6\%$. It also inhibited protein, DNA and RNA synthesis (Rodgers & Grant, 1998). Obermeier et al. suggest that one of the mechanisms by which quercetin might exert their anticancer effects is through interaction with the cytochrome P450 mixed function oxidase (MFO) system either by inhibition or induction of the isoforms of this enzyme. This may lead to decreased metabolic activation of potential carcinogens (Obermeier, White & Yang, 1995). Moreover, the effects of flavones and flavonols, including quercetin, on several different enzyme systems including cytochrome P450 isoenzymes, protein tyrosine kinase, RNA and DNA polymerases and epoxide hydrolase have been reviewed by Middleton and Kandaswami. Quercetin has been shown to inhibit human P-form sulphotransferases (Walle, Eaton & Walle, 1995), and induce glutathione S-transferase (Prochaska & Talalay, 1988).

Genistein (Figure 2.2C), the major isoflavonone contained in soybeans, has various activities, including estrogenic, growth-promoting, and anti-carcinogenic effects (Fotsis, Pepper, Adlercreutz, Hase, Montesano & Schweigerer, 1995; Zhou, Mukherjee, Gugger, Tanaka, Blackburn & Clinton, 1998). Epidemiological studies, as well as work performed

with animal models, suggest that it has a chemopreventive effect for breast, colon, and skin tumors (Shao, Wu, Shen & Barsky, 1998). Genistein kills cancer cells in culture by causing apoptosis. In breast cancer cells, the effects of genistein are dependent on the estrogen receptor (ER) status. Although genistein at low concentrations (<10 μ M) is growth stimulatory in ER-positive breast cancer cells (Fioravanti, Cappelletti, Miodini, Ronchi, Brivio & Di Fronzo, 1998), it is growth suppressive at all concentrations in ER-negative cell lines (Monti & Sinha, 1994). Different mechanisms may be involved in the observed chemopreventive and antiproliferative action of genistein. Some early studies, which focused on the antiestrogenic activity of genistein, showed its ability to bind *in vitro* sheep uterine estrogen receptors and human breast cancer ER (Martin, Horwitz, Ryan & McGuire, 1978) and suggested that its chemopreventive activity was the result of interference at the ER level in the tumor-promoting effect of estrogens, since its structure is similar to 17 β -estradiol (**Figure 2.2D**). Others analyzed the ability of genistein to inhibit protein tyrosine kinases (PTK), particularly epidermal growth factor receptor (EGF-R) autophosphorylation, by competing with ATP rather than with the protein substrate (Akiyama et al., 1987) and indicated the genistein antiproliferative effect as a result of interference in the tyrosine kinase cascade activated by mitogens. A direct effect of genistein on angiogenesis through inhibition of endothelial cell proliferation (Fotsis et al., 1993) may also account for the delay in tumor appearance and especially for the antimetastatic activity (Weidner, Semple, Welch & Folkman, 1991). The antiproliferative effect of genistein has also been ascribed to direct inhibition of topoisomerase-II by a non-intercalating irreversible binding to DNA (Markovits et al., 1989) and to induction of apoptosis. Li and coworkers discovered that genistein, in a concentration-dependent manner, suppresses the protein levels of MEK5, total ERK5, and phospho-ERK5, effects that are consistent with inhibition of cell growth and induction of apoptosis, thus inhibition of the MEK5/ERK5/NF- κ B pathway may be an important mechanism by which genistein suppresses cell growth and induces apoptosis (Li et al., 2008).

Besides flavonoids, many other phytochemicals, which are derived from fruits and vegetables, have been uncovered to show potent anti-breast cancer activities in recent research, including β -carotene and related carotenoids, resveratrol (**Figure 2.2E**) and sulforaphane (**Figure 2.2F**). Resveratrol from red wine, also highly abundant in skins of red grapes, could interfere with cell cycle control and induce apoptosis in a concentration- and cell-specific manner exposed in MCF-7 or MDA-MB-231 breast cancer cells (Poza-Guisado, Alvarez-Barrientos, Mulero-Navarro, Santiago-Josefat & Fernandez-Salguero, 2002) by inducing mitochondrial intrinsic apoptotic pathway as well as Ca^{2+} -calpain-dependent cell death (Sareen, Darjatmoko, Albert & Polans, 2007). Sulforaphane, a prominent isothiocyanate present in cruciferous vegetables, at the concentration of 15 μM , could inhibit proliferation and mitosis of MCF7-green fluorescent protein- α -tubulin breast tumor cells by 50%, and it significantly modified microtubule organization in arrested spindles without modulating the spindle microtubule mass, in a manner similar to that of much more powerful antimetabolic drugs, taxol and vincristine (Azarenko, Okouneva, Singletary, Jordan & Wilson, 2008).

2.2.3 Vegetable and fruits extracts display synergistic effects of multiple bioactive compounds

Recent research suggests that the health benefits of fruits and vegetables are due to the additive and synergistic effects of multiple nutrients, rather than to a single compound (Liu, 2003). Interestingly, Kim and coworkers found that Vitamins A, C and A+C reduced MCF-7 cell proliferation by 20.7%, 23.3% and 75.7% relative to the untreated cell proliferation, respectively. The synergistic ratio of Vitamin A and Vitamin C was 1.72 (Kim, Pie, Park, Park, Kim & Kim, 2006). Therefore, there is a growing interest in looking for anticancer resources from the extracts of the fruits and vegetables first, before isolating the bioactive compounds, since the synergistic effects of multiple nutrients may lead to an unexpected effect, such as minimizing the side effects, increasing the

acceptability to the patients.

Several fruit and vegetable extracts had been investigated for their anti- breast cancer activity. Green tea, which contains abundant gallocatechins, procyanidins and phenolic acids, is a good example. A case-control study on breast cancer patients revealed that high daily consumption of green tea was associated with a lower recurrence rate among Stages I and II patients (Nakachi, Suemasu, Suga, Takeo, Imai & Higashi, 1998). Similarly, cranberry phytochemical extracts significantly inhibited human breast cancer MCF-7 cell proliferation, induced apoptosis and G1/S blockage in cell phase (Sun & Hai Liu, 2006). Litchi fruit pericarp extracts, which contain significant amounts of polyphenolic compounds, inhibit MCF-7 and MDA-MB-231 cells proliferation in vitro, and lead to a 40.7% tumor mass volume reduction when fed to nude mice for 10 weeks (Wang et al., 2006).

2.2.4 Graviola and acetogenins

On this big background, in our lab, the screening of a variety of fruit extracts for anti-proliferation of breast cancer cells was conducted by selecting aggressive ER negative MDA-MB-468 breast cancer cells as the target and non-transformed normal breast cell MCF-10A as the control. As shown in **Fig. 2.3**, cell viability were determined by the MTT assay, and we discovered that among more than 200 hundred fruit extracts, like Tusi, Sylvester leaf, Fenugreek seed, red raspberry, graviola, and juniper berry, guava, strawberry, broccoli, wolfberry, blueberry, and tomato, after 48 hours only 25µg/ml graviola fruit extracts showed the 74% inhibition of MDA-MB-468 breast cancer cells while having no effect on MCF-10A non-transformed normal breast cells. Therefore, in this study, graviola fruit extracts were used for further research.

Graviola (*Annonaceous muricata* L.) is a small, upright evergreen tree, 5–6 m high, with large, glossy, dark green leaves. Graviola is indigenous to most of the warm tropical areas

in South and North America, including the Amazon. The fruit is sold in local markets in the tropics, where it is called guanábana in Spanish-speaking countries and graviola in Brazil. The fruit pulp is excellent for making drinks and sherbets and, though slightly sour-acid, can be eaten out of hand.

In a long history, all parts of the graviola tree, including the bark, leaves, roots, fruit, and fruit seeds, have been traditionally used as a herbal remedy for a variety of purported health promoting effects, including supporting healthy cell growth and immune function (Adewole et al., 2006). Generally, the fruit and fruit juice are taken for worms and parasites, to cool fevers, as a lactagogue (to increase mother's milk after childbirth), and as an astringent for diarrhea and dysentery. The crushed seeds are used as a vermifuge and anthelmintic against internal and external parasites, head lice, and worms. The bark, leaves, and roots are considered sedative, antispasmodic, hypotensive, and nervine, and a tea is made for various disorders toward those effects. Several studies over the years have demonstrated that leaf, bark, root, stem, and seed extracts of graviola had anticancer (Jaramillo, Arango, Gonzalez, Robledo & Velez, 2000), antibacterial, antifungal (Heinrich et al., 1992), anti-parasitic (Bories et al., 1991), anti-malarial, insecticidal and anti-depressive (Hasrat, De Bruyne, De Backer, Vauquelin & Vlietinck, 1997) properties.

Among all the bioactive properties, the anticancer property seems to be the most important one. In a 1976 plant screening program by the National Cancer Institute, graviola leaves and stem showed active cytotoxicity against cancer cells and researchers have been following up on these findings since that time. Many bioactive compounds and phytochemicals have been found in graviola, such as acetogenins, flavonoids, alkaloids, phenolic acids, etc. However, annonaceous acetogenins have been the focus of most research over the past 30 years. Graviola produces these natural compounds in its leaf, stem, bark, and seeds. Three separate research groups have isolated these acetogenin

compounds in graviola which have demonstrated significant antitumor and anticancer properties, and selective toxicity against various types of cancer cells (Rieser, Fang, Rupprecht, Hui, Smith & McLaughlin, 1993; Wu et al., 1995; Zeng, Wu, Oberlies, McLaughlin & Sastrodihadjo, 1996). Many of the acetogenins have demonstrated selective toxicity to tumor cells at very low dosages - as little as 1 part per million, with several cancer cell lines, including breast cancer (Rieser, Gu, Fang, Zeng, Wood & McLaughlin, 1996).

According to the structure, all of the annonaceous acetogenins compounds (**Figure 2.4A**) consist of one or two tetrahydrofuran (THF) rings in the middle and a γ -lactone on the tail, which is connected by an inter-hydrocarbon chain, while another hydrocarbon chain is connected to the THF rings with several oxygenated moieties. They can be classified into four different types, bis-adjacent (THF) acetogenins, bis-non-adjacent THF acetogenins, mono- THF acetogenins and non-THF acetogenins. Most of them had already been reported to have strong anti-breast cancer activity. Oberlies discovered that multidrug resistant MCF-7 breast cancer cells was invisible after exposure to 1.0 $\mu\text{g/ml}$ of bullatacin (**Figure 2.4B**), as a representative of bis-adjacent-THF acetogenins, for 48 hours (Oberlies, Croy, Harrison & McLaughlin, 1997). Annonacin (**Figure 2.4C**), a type of mono- THF acetogenin, induced apoptotic cell death when treated to MCF-7 breast cancers with the IC_{50} of 0.433 $\mu\text{g/ml}$ (Yuan et al., 2003). Squamocin (**Figure 2.4D**) inhibited proliferation of both parental and multidrug resistant MCF7 breast cancer cell lines identically by blocking the cell cycle in the G1-phase (Raynaud et al., 1999). These three typical acetogenins had been isolated from the leaf and seed of graviola. However, there is little research on anticancer activity of graviola fruit.

Research had been conducted to elucidate the mechanism to cytotoxicity of acetogenins, and it is widely believed that, after acetogenins enter the cell, the THF rings will be fixed

on the hydrophilic part of the inner-membrane of mitochondria, which allows the γ -lactone in the tail of the chain to interact with ND1 subunit of NADH- ubiquinone oxidoreductase (Zafra-Polo, Gonzalez, Estornell, Sahpaz & Cortes, 1996), which is Complex I of the respiratory chain. After cross-linking of the Val144 and Glu192, Complex I is blocked, and the proton from NADH cannot be pumped out, the whole respiratory chain was blocked, thus ATP can not generated, leading to energy exhaustion and cell apoptosis (Sekiguchi, Murai & Miyoshi, 2009). However, the molecular mechanism is still not clear.

2.3 Cell apoptosis induced by phytochemicals and its molecular mechanism

2.3.1 Cell apoptosis and development of its research

Cell death is a regular phenomenon in biology; some cell death is due to physiological changes such as aging, metabolism, while others may be as a result of pathological abnormality or environment changes. Up till now, there are mainly two kinds of cell death that had been discovered and studied by scientists: that is, necrosis and apoptosis. Necrosis is the premature death of cells and living tissue and is always caused by external factors, such as infection, toxins or trauma, while apoptosis is the process of programmed cell death that involves a series of biochemical events leading to a characteristic cell morphology change, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation, etc.

Cell death is a completely normal process in living organisms and was first discovered by scientists over 100 years ago. In 1842, German scientist Carl Vogt discovered a different cell death other than necrosis when focused on tadpoles' development. Later, in 1885, anatomist Walther Flemming delivered a more precise description of the process of

programmed cell death after studying follicular cells. However, it was not until 1965 that the topic was resurrected. While studying liver tissues using electron microscopy, John Foxton Ross Kerr was able to distinguish apoptosis from necrosis by discovering that some of the liver cells shrink gradually without accompanying inflammation and finally disappear (Kerr, 1965). After that, the word “apoptosis”, which is from Greek meaning “falling of petals from flowers” was brought into the biological kingdom (Kerr, Wyllie & Currie, 1972).

Apoptosis occurs when a cell is damaged beyond repair, infected with a virus, or undergoes stressful conditions such as starvation. Damage to DNA from ionizing radiation or toxic chemicals can also induce apoptosis via the actions of the tumor-suppressing gene *p53*. The "decision" for apoptosis can come from the cell itself, from the surrounding tissue, or from a cell that is part of the immune system. In these cases, apoptosis functions to remove the damaged cell, preventing it from sapping further nutrients from the organism, or halting further spread of viral infection (Evan & Littlewood, 1998). Thus, the importance of cell apoptosis is same as cell growth, development and proliferation in the human body.

The research interest in cell apoptosis grew rapidly after 1972. Four major stages of research are apparent (Farber, 1994). Stage one (1885-1971): description and concept formation of cell apoptosis. During this period, the research on cell apoptosis mainly focused on observation and description, such as the physiological difference between necrosis and apoptosis and the selection of experimental material. Brenner indicated that *Caenorhabditis elegans* seems to be the best material for apoptosis research (Brenner, 1974). Stage two (1972-1987): focused on morphology and biochemistry research. During this period, advanced biological technology was introduced into the research. Through electron microscopy, the difference between necrosis and apoptosis were

discovered morphologically (Kerr et al., 1972); DNA fragmentation was detected by electrophoresis (Wyllie, Beattie & Hargreaves, 1981); the increase of calcium concentration was one of the reason that apoptosis occurred, etc. Stage three (1988-current): molecular biology method used in research. With the development of biotechnology, cell apoptosis was continued to be researched at the molecular level, searching for the related genes and their regulation, the various signaling pathways and the interaction between the related molecules are the three main fields that scientists focused on. Stage four (1992-current): clinical applications. The aim for this apoptosis research is to help to elucidate the mechanism of some specific diseases. Since it has discovered that many diseases had involvement of cell apoptosis, especially cancers, induction of cancer cell's apoptosis maybe a potential means to treat the cancer. An international meeting on cell apoptosis (Shanghai, China, 2002) took clinical research on chronic disease by inducing cell apoptosis as one of the main topics. Several molecular mechanisms of cell apoptosis have been elucidated, however due to the complexity of signaling pathways and the variety of tumors, cell apoptosis research is still ongoing.

2.3.2 Morphological and biochemical changes during cell apoptosis

As shown in **Fig. 2.5**, apoptotic cells shrink while retaining intact plasma membranes. Their cellular contents are packaged into apoptotic bodies that subsequently will be phagocytosed by neighboring cells or by professional phagocytes. Usually, the apoptotic cell death pathway does not lead to inflammation. In contrast, necrosis is characterized by cell swelling, plasma membrane damage, and loss of cytoplasmic contents into the extracellular space, which leads to uncontrolled presentation of antigens to the immune system and may cause inflammatory reactions (Fehsel, Kolb-Bachofen & Kroncke, 2003).

During apoptosis, morphological changes experience three stages (Cummings, Winterford & Walker, 1997). Firstly, apoptosis initiation: in this stage, some specific structure changes on the surface of the cell membrane, such as the disappearance of microvillous.

However, cell membrane still maintains its integrity and selectivity of penetration. In the cytoplasm, mitochondria mostly complete, but the ribosome gradually detached from reticulum; Chromatin begins to condense and migrate towards the nuclear membrane periphery. Secondly, the appearance of apoptotic bodies: Nuclear chromatins are fragmented into small pieces and gather with some cell organs, like mitochondria, wrapped with shrunked cell membranes. From appearance, some bubble-like convex morphological changes occur on the surface of the cell. Later, each small aggregation separates from each other and forms a single apoptotic body. Finally, each apoptotic body is combined into the adjacent cells by endocytosis.

In addition to morphological changes, at the biochemical level, several important proteins and enzymes showed significant changes, too. When cell experience apoptosis, DNA is fragmented into small pieces, which is about 180-200bp, and electrophoresis would show a DNA ladder, however, compared to necrosis, DNA will be fragmented randomly, so that electrophoresis band will be continuous and murky. Since Mg^{2+}/Ca^{2+} dependent-endonuclease should be responsible for the chromatin condensation while DNA ladder nuclease, the enzyme that cuts genomic DNA between nucleosomes generating DNA fragments, is responsible for the characteristic DNA ladder pattern observed after DNA electrophoresis, their activities during the apoptosis should be relatively higher than in normal cells (Barisic, Petrik & Rumora, 2003; Khodarev & Ashwell, 1996). Moreover, in the apoptotic cells, tissue transglutaminase, which functions to catalyse a Ca^{2+} -dependent acyl-transfer reaction between the γ -carboxamide groups of a peptide bound glutamine residue and the ϵ -amine group of peptide-bound lysine, resulting in the production of highly cross linked protein aggregates, is found at high levels (el Alaoui, Mian, Lawry, Quash & Griffin, 1992).

Cell apoptosis is similar to cell proliferation, which is the basic life phenomenon and

contributes to the balance of cell numbers in the human body, thus it is also regulated by gene and related proteins. In cell apoptosis regulation, the caspase family is the typical proteins.

Caspase, is the acronym of aspartate-specific cysteine protease. Overexpression or activation of caspase can both induce cell apoptosis. The mammalian caspase family contains 14 members, of which 11 human enzymes are known (**Fig. 2.6**). Phylogenetic analysis indicates that this gene family is composed of two major subfamilies that are related to either ICE (caspase-1; inflammation group) or to the mammalian counterparts of ced-3 (apoptosis group) (Zimmermann, Bonzon & Green, 2001a). Caspases share similarities in amino acid sequence, structure, and substrate specificity (Nicholson & Thornberry, 1997). They are expressed as proenzymes that contain three domains: an N-terminal prodomain, a large subunit containing the active site cysteine within a conserved QACXG motif, and a C-terminal small subunit. Caspases are among the most specific proteases, with an unusual and absolute requirement for cleavage after aspartic acid residues (Stennicke & Salvesen, 1998). An aspartate cleavage site separates the prodomain from the large subunit, and an interdomain linker containing one or two aspartate cleavage sites separates the large and small subunits. The presence of aspartate at the cleavage site is consistent with the ability of caspases to auto-activate or to be activated by other caspases as part of an amplification cascade.

The caspase in apoptosis group can be divided into two groups: the apoptotic initiators (caspase-2, -8, -9, and -10) and apoptotic executioners (caspase-3, -6, and -7). The most prevalent caspase in the cell is caspase-3. It is the one ultimately responsible for the majority of the apoptotic effects, although it is supported by two others, caspase-6 and -7. Together, these three executioner caspases presumably cause the apoptotic phenotype by cleavage or degradation of several important substrates. For example, caspase-3 cleaves

the inhibitor of CAD/DFF40 complex and allowed the nuclease to cut the chromatin, resulting in high- and low- molecular weight DNA fragmentation and the cell's break up (Enari, Sakahira, Yokoyama, Okawa, Iwamatsu & Nagata, 1998).

2.3.3 Detection of cell apoptosis

Detection of cell apoptosis mostly is based on the unique morphological and biochemistry characteristics of the apoptotic cells. As the research on cell apoptosis has progressed, a number of methods for detection of apoptosis have been established, including fluorescent microscopy, flow cytometry, immunological staining, etc.

2.3.3.1 Observation of morphological changes

Regular microscopy: the apoptotic process involves a sequence of cell shrinkage, increased cytoplasmic density, chromatin condensation, and segregation into sharply circumscribed masses that abut the nuclear membrane and can form blister-like protrusions ("budding"). The latter then separate to produce membrane-bound apoptotic bodies. Initially, the mitochondria and the Golgi apparatus show no signs of swelling and the nuclear membrane remains intact for an extended period of time (Sgonc & Gruber, 1998). After Giemsa's or Wright's staining, the chromatin's condensation can be easily recognized. In addition, combined with using electron microscopy, some micro-structural changes can also be recognized.

Fluorescent microscopy: using fluorescent dye, which would insert into DNA of the cells and stain them, is convenient for distinguishing the apoptotic cells from necrotic cells by differences in fluorescence. Regular fluorescent dyes for apoptotic cells include Hoechst 33342, Hoechst 33258, DAPI (4',6'-diamidino-2-phenylindole) and Annexin-V while propidium iodide (PI) and ethidium bromide (EB) are preferred for necrotic cells. Hoechst 33342 (Schmid, Uittenbogaart & Jamieson, 2007) and DAPI (Florent, Godard, Ballet, Gauduchon & Sola, 1999) could enter the complete cell and form strong and non-covalent

linkage with adenine- and thymine-rich regions in the minor groove of DNA, after exciting by UV light, the chromatin would show a blue fluorescence. Since the apoptotic cells have a relatively high permeability, the bright blue would indicate the condensed chromatin (**Fig. 2.7A**), even the apoptotic bodies. However, PI and EB could not enter the intact cells, only necrotic cells with incomplete cell membrane could be stained by PI and show a red fluorescence. Thus, combined with these two dyes, the apoptotic cells can be recognized from others.

2.3.3.2 Analysis by Flow Cytometry

Flow cytometric analyses based on the detection of morphological changes, DNA fragmentation, DNA loss, and membrane changes are increasingly used for quantitative investigations of apoptosis. Thus, the light scattering properties of cells can be used to analyse the changes in size and granularity. Apoptosis is accompanied by water loss, cell shrinkage, and nuclear fragmentation, whereas necrosis is not. Thus, apoptotic and necrotic cells differ in light scatter patterns ([Carbonari et al., 1994](#)); apoptotic cells give lower forward scatter and higher side scatter values than viable cells, reflecting the smaller size and different nucleus/cytoplasm consistency. In principle, flow cytometric analysis of morphological parameters is a very rapid and objective way of enumerating apoptotic cells.

Mainly two kinds of experiments would be used by flow cytometer. The first one is the detection of DNA content and cell cycle distribution by PI staining. Flow cytometry showed the DNA content in different phases of the cell cycle according to the fluorescence intensity from PI, since PI will insert into DNA of the cells. Once the cells experience apoptosis, apoptotic bodies which contain less DNA will be detected by the flow cytometer, and form a different apoptotic peak before the normal G₀/G₁, S/ G₂ or M peak (**Fig. 2.7B**). However, we can only estimate the percentage of the cells that may undergo apoptosis, but

cannot distinguish early apoptosis, late apoptosis or necrosis.

The other experiment that can be done using a flow cytometer is the quantitative analysis of cell apoptosis. The translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane during apoptosis can be detected by annexin V, which binds preferentially to phosphatidylserine in the presence of Ca^{2+} (Koopman, Reutelingsperger, Kuijten, Keehnen, Pals & van Oers, 1994). To discriminate between apoptotic and necrotic cells, the annexin V-FITC binding assay should be combined with PI staining. Since the normal cells with the intact cell membrane will not be stained by neither annexin V nor PI, apoptotic cells will show annexin V positive but PI negative, while both annexin V and PI could attach to the necrotic cells and late apoptotic cells, three different types of cells could be distinguished (Fig. 2.7C). Combining the fluorescent intensity reading from flow cytometer, the percentage of different cells can be obtained. However, some cell apoptosis occurs without the translocation of phosphatidylserine, and this method could be only used in early apoptosis.

2.3.3.3 Detection by methods of molecular biology and histological chemistry

(1) Analysis of DNA degradation

The most striking biochemical event in apoptosis is the DNA cleavage between nucleosomes that produces fragments in multiples of approximately 180-200 bp (Wyllie, 1980). This phenomenon is most often analyzed by agarose gel electrophoresis, which measures DNA fragmentation in nuclear extracts showing the typical “DNA-ladder” configuration (Compton & Cidlowski, 1986) (Fig. 2.7D). An improved method for the detection of DNA fragmentation visualizes the DNA after Southern blotting with a radiolabeled total cellular DNA probe instead of ethidium bromide staining, increasing sensitivity (Facchinetti, Tessarollo, Mazzocchi, Kingston, Collavo & Biasi, 1991)

(2) Analysis of endonucleases

Because DNA fragmentation is a primary event in apoptosis and may be the actual cause of cell death, the identification of the endonuclease(s) which is (are) responsible for this cleavage is of great interest in the study of apoptosis. Nuclease assays have been described that detected DNA-degrading activity in SDS-polyacrylamide gels in which a nonradioactive or ³²P-labeled nucleic acid substrate was incorporated into the gel matrix, and nuclease activity was ascertained by loss of the substrate from the gel (Caron-Leslie & Cidlowski, 1991).

(3) Histological chemistry method

Determination of the specific gene expression and related protein contents is also a regular way to detect cell apoptosis. The expression of pro-apoptotic genes, such as ced-3, ced-4, c-Myb, c-myc, p53, p21, ICE, bax, and anti-apoptotic genes, like ced-9, Bcl-2, Bcl-xL, MCL1, are usually determined. Proteins caspase-3, caspase-9, EGFR, Her-2 can be detected by western blot (Batsi et al., 2009; Borralho et al., 2007; Sharifi, Eslami, Larijani & Davoodi, 2009).

2.3.4 Cell apoptosis signaling pathways

2.3.4.1 Traditional pathways

Through years of research on cell apoptosis signaling pathways, 2 general types of signaling pathways leading from a triggering event to the activation of an initiator caspase and from there to apoptotic death have been identified. The first involves death receptors such as the TNF receptor- 1 and the Fas molecule, and the second one depends on the participation of mitochondria (Fig. 2.8).

The death receptors of the tumor necrosis factor receptor (TNFR) family include TNFR1, Fas (CD95), DR3/WSL, and the TRAIL/Apo-2L receptors (TRAILR1/ DR4, TRAIL-R2/DR5). When these death receptors are bound by their ligands (TNF or

lymphotoxin, Fasligand), membrane-bound signaling complexes are formed. These complexes recruit procaspase-8 molecules via adapter molecules, such as the Fas-associated protein with death domain (FADD) and/or the tumour necrosis factor receptor associated protein with death domain (TRADD). High local concentration of procaspase-8 results in its autoactivation. Under such conditions, the low intrinsic protease activity of procaspase-8 is sufficient to allow the proenzyme molecules to cleave and activate each other and other downstream caspases, like caspase3, 6, 7, leading to the induction of the apoptotic process (Thornberry & Lazebnik, 1998).

The mitochondrial pathway of apoptosis is used extensively in response to diverse forms of cellular stress (DNA damage, growth factor withdrawal, cell-cycle perturbation, exposure to cytotoxic drugs). These stressors promote release of cytochrome c from mitochondria and it seems that pro-apoptotic members of the Bcl-2 family, including Bax, Bad, Bim, and Bid, might have a role in the perturbation of mitochondrial membrane integrity. Once released into the cytosol, cytochrome c associates with the apoptotic protease-activating factor 1 (Apaf 1) and procaspase-9 to form apoptosome. Both Apaf 1 and cytochrome c are required for caspase-9 activation. It seems that Apaf 1 is not only a transient activator of caspase-9; it is rather an essential regulatory subunit of caspase-9 holoenzyme (Srinivasula et al., 2001). Therefore, Apaf 1/caspase-9 complex is nowadays thought to represent the true active form of caspase 9 (Rodriguez & Lazebnik, 1999). Finally, activated caspase-9 continued to active caspase-3, leading to apoptosis.

The death-receptor and mitochondrial pathways converge at the level of caspase-3 activation. The evidence for the cross-link and integration of these two pathways is provided by Bid, a pro-apoptotic member of the Bcl-2 family. In some cell types, death receptor-associated caspase-8 activation is insufficient to activate downstream caspases. In these cells, caspase-8 can propagate the death signal by engaging the mitochondrial

pathway through proteolytic processing of the Bid. Truncated Bid translocates to mitochondria where it causes cytochrome c release (Zhao et al., 2009).

2.3.4.2 EGFR pathway

Besides this two main signaling pathway, some other important pathways have also been well researched in recent years, and EGFR signaling pathway is one of them.

The epidermal growth factor receptor (EGFR; ErbB-1; HER1 in humans) is the cell-surface receptor for members of the epidermal growth factor family (EGF-family) of extracellular protein ligands (Herbst, 2004). The epidermal growth factor receptor is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4).

EGFR exists on the cell surface and is activated by binding of its specific ligands, including epidermal growth factor and transforming growth factor α (TGF α), amphiregulin, betacellulin, epigen, epiregulin, etc. (Linggi & Carpenter, 2006). Upon activation by its growth factor ligands, EGFR undergoes a transition from an inactive monomeric form to an active homodimer. In addition to forming homodimers after ligand binding, EGFR may pair with another member of the ErbB receptor family, such as ErbB2/Her2/neu, to create an activated heterodimer. EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity. As a result, autophosphorylation of several tyrosine residues (Y992, Y1045, Y1068, Y1148 and Y1173) in the C-terminal domain of EGFR occurs (Downward, Parker & Waterfield, 1984). This autophosphorylation elicits downstream activation and signaling by several other proteins that associate with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains. These downstream signaling proteins initiate several signal transduction cascades, principally the MAPK, Akt and JNK pathways (Fig. 2.9), leading to DNA synthesis and

cell proliferation (Oda, Matsuoka, Funahashi & Kitano, 2005).

However, EGFR is frequently dysregulated in human epithelial tumors, via autocrine stimulation, overexpression, or mutation; dysregulation is often associated with an adverse prognosis in an array of tumor types, including central nervous system, head and neck, GI, and breast cancer (Arteaga, 2001). It is well established that EGFR overexpression correlates inversely with estrogen receptor (ER) status in patients with breast cancer (Lichtner, 2003) and is associated with an advanced tumor stage (Salomon, Brandt, Ciardiello & Normanno, 1995), poor prognosis (Bange, Zwick & Ullrich, 2001), and resistance to chemotherapy, hormone therapy, and radiation (Brabender et al., 2001). Thus, EGFR is thought to serve as a target for cancer, including breast cancer therapy.

Phytochemicals from natural products have already been used for treating cancer, and several convincing results have been reported that the mechanism is related to the EGFR signaling pathway. Hogan and coworkers indicated that silibinin, the primary active flavonoid component isolated from the milk thistle plant [*Silybum marianum* (L.) Gaertneri] seed extract, inhibits proliferation and promotes cell cycle arrest of HCT-116 human colon cancer by inhibiting the of TGF- α ligand-binding to EGFR (Hogan, Krishnegowda, Mikhailova & Kahlenberg, 2007). Sah and coworker discovered that epigallocatechin-3-gallate, a bioactive green tea polyphenol, inhibited EGFR expression in cervical tumor cells (HeLa, Caski, and SiHa), resulting in a very selective decrease in ERK 1/2 phosphorylation state and activity of these kinases which are downstream in the signaling pathway (Sah, Balasubramanian, Eckert & Rorke, 2004). For breast cancer, genistein, an isoflavone isolated from soybean, could delay the growth of breast tumor via decreased estrogen levels and activity, and down-regulation of EGFR expression synergistically with tamoxifen (Mai, Blackburn & Zhou, 2007). Moreover, resveratrol, from red grape skin, inhibit MDA-MB-231 breast cancer cell invasion by altering the

cytoskeleton through modulating EGFR signaling, while offsetting the stimulating effect of estradiol (Azios & Dharmawardhane, 2005).

Since there is no report concerning the molecular mechanism of anti- breast cancer properties of acetogenins compounds in Graviola, and the EFGR signaling pathway is one of the important pathways that regulate the breast cancer's proliferation, in this study we examined graviola fruit extracts' possible anticancer mechanism through the EFGR signaling pathway.

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2.5 Figures and Tables

Table 2.1 Source, clinical, and pathological feature of tumors are used to derive breast cancer cells. (With permission, Copyright © 2006 Elsevier)

Table 1. Source, clinical, and pathological features of tumors used to derive breast cancer cell lines used in this study

Cell line	Gene cluster	ER	PR	HER2	TP53	Source	Tumor type	Age (years)	Ethnicity	Culture media	Culture conditions	
1	600MPE	Lu	+	[-]	-		IDC			DMEM, 10% FBS	37°C, 5% CO ₂	
2	AU565 ^d	Lu	-	[-]	+	++ ^{WT}	PE	AC	43	W	RPMI, 10% FBS	37°C, 5% CO ₂
3	BT20	BaA	-	[-]	+	++ ^{WT}	P.Br	IDC	74	W	DMEM, 10% FBS	37°C, 5% CO ₂
4	BT474	Lu	+	[+]	+		P.Br	IDC	60	W	RPMI, 10% FBS	37°C, 5% CO ₂
5	BT483	Lu	+	[+]	-		P.Br	IDC, pap	23	W	RPMI, 10% FBS	37°C, 5% CO ₂
6	BT549	BaB	-	[-]	+	++ ^M	P.Br	IDC, pap	72	W	RPMI, 10% FBS	37°C, 5% CO ₂
7	CAMA1	Lu	+	[-]	+		PE	AC	51	W	DMEM, 10% FBS	37°C, 5% CO ₂
8	HBLL100	BaB	-	[-]	+	++	P.Br	N	27		DMEM, 10% FBS	37°C, 5% CO ₂
9	HCC1007 ^d	Lu	+	[-]	+	[+/-]	P.Br	Duc.Ca	67	B	RPMI, 10% FBS	37°C, 5% CO ₂
10	HCC1143 ^d	BaA	-	[-]	+	++ ^M	P.Br	Duc.Ca	52	W	RPMI, 10% FBS	37°C, 5% CO ₂
11	HCC1187 ^d	BaA	-	[-]	+	++ ^M	P.Br	Duc.Ca	41	W	RPMI, 10% FBS	37°C, 5% CO ₂
12	HCC1428 ^d	Lu	+	[+]	+	[+]	PE	AC	49	W	RPMI, 10% FBS	37°C, 5% CO ₂
13	HCC1500 ^d	BaB	-	[-]	-		P.Br	Duc.Ca	32	B	RPMI, 10% FBS	37°C, 5% CO ₂
14	HCC1569 ^d	BaA	-	[-]	+	- ^M	P.Br	MC	70	B	RPMI, 10% FBS	37°C, 5% CO ₂
15	HCC1937 ^d	BaA	-	[-]	+	[-]	P.Br	Duc.Ca	24	W	RPMI, 10% FBS	37°C, 5% CO ₂
16	HCC1954 ^d	BaA	-	[-]	+	[+/-]	P.Br	Duc.Ca	61	EI	RPMI, 10% FBS	37°C, 5% CO ₂
17	HCC202 ^d	Lu	-	[-]	+	[-]	P.Br	Duc.Ca	82	W	RPMI, 10% FBS	37°C, 5% CO ₂
18	HCC2157 ^d	BaA	-	[-]	+	[+]	P.Br	Duc.Ca	48	B	RPMI, 10% FBS	37°C, 5% CO ₂
19	HCC2185 ^d	Lu	-	[-]	+	[+]	PE	MLCa	49	WH	RPMI, 10% FBS	37°C, 5% CO ₂
20	HCC3153 ^d	BaA	-	[-]	+	[-]				RPMI, 10% FBS	37°C, 5% CO ₂	
21	HCC38 ^d	BaB	-	[-]	+	++ ^M	P.Br	Duc.Ca	50	W	RPMI, 10% FBS	37°C, 5% CO ₂
22	HCC70 ^d	BaA	-	[-]	+	++ ^M	P.Br	Duc.Ca	49	B	RPMI, 10% FBS	37°C, 5% CO ₂
23	HS578T	BaB	-	[-]	+	++ ^M	P.Br	IDC	74	W	DMEM, 10% FBS	37°C, 5% CO ₂
24	LY2	Lu	+	[+]	+	+	PE	IDC	69	W	DMEM, 10% FBS	37°C, 5% CO ₂
25	MCF10A ^b	BaB	-	[-]	+	+/- ^{WT}	P.Br	F	36	W	DMEM/F12*	37°C, 5% CO ₂
26	MCF12A ^b	BaB	-	[-]	+	+	P.Br	F	60	W	DMEM/F12*	37°C, 5% CO ₂
27	MCF7	Lu	+	[+]	+	+/- ^{WT}	PE	IDC	69	W	DMEM, 10% FBS	37°C, 5% CO ₂
28	MDAMB134V1	Lu	+	[-]	+	+/- ^{WT}	PE	IDC	47	W	DMEM, 10% FBS	37°C, 5% CO ₂
29	MDAMB157	BaB	-	[-]	-		PE	MC	44	B	DMEM, 10% FBS	37°C, 5% CO ₂
30	MDAMB175VII	Lu	+	[-]	+	+/- ^{WT}	PE	IDC	56	B	DMEM, 10% FBS	37°C, 5% CO ₂
31	MDAMB231	BaB	-	[-]	+	++ ^M	PE	AC	51	W	DMEM, 10% FBS	37°C, 5% CO ₂
32	MDAMB361	Lu	+	[-]	+	- ^{WT}	P.Br	AC	40	W	DMEM, 10% FBS	37°C, 5% CO ₂
33	MDAMB415	Lu	+	[-]	+	+	PE	AC	38	W	DMEM, 10% FBS	37°C, 5% CO ₂
34	MDAMB435	BaB	-	[-]	+	++ ^M	PE	IDC	31	W	DMEM, 10% FBS	37°C, 5% CO ₂
35	MDAMB436	BaB	[-]	[-]	+	[-]	PE	IDC	43	W	L15, 10% FBS	37°C, no CO ₂
36	MDAMB453	Lu	-	[-]	+	- ^{WT}	PF	AC	48	W	DMEM, 10% FBS	37°C, 5% CO ₂
37	MDAMB468	BaA	[-]	[-]	+	[+]	PE	AC	51	B	L15, 10% FBS	37°C, no CO ₂
38	SKBR3 ^c	Lu	-	[-]	+	+	PE	AC	43	W	McCoy's 5A, 10% FBS	37°C, 5% CO ₂
39	SUM1315MO2 ^e	BaB	-	[-]	+	[+]	Sk	IDC			Ham's F12, 5% I-E	37°C, 5% CO ₂
40	SUM149PT ^e	BaB	[-]	[-]	+	[+]	P.Br	Inf Duc.Ca			Ham's F12, 5% I-H	37°C, 5% CO ₂
41	SUM159PT ^e	BaB	[-]	[-]	+	[+]	P.Br	AnCar			Ham's F12, 5% I-H	37°C, 5% CO ₂
42	SUM185PE ^e	Lu	[-]	[-]	+	[-]	PE	Duc.Ca			Ham's F12, 5% I-H	37°C, 5% CO ₂
43	SUM190PT ^e	BaA	-	[-]	+	[+/-]	P.Br	Inf			Ham's F12, 5% I-H**	37°C, 5% CO ₂
44	SUM225CWN ^e	BaA	-	[-]	+	++	CWN	IDC			Ham's F12, 5% I-H	37°C, 5% CO ₂
45	SUM44PE ^e	Lu	[+]	[-]	+	[-]	PE	Ca			Ham's F12, 5% I-H**	37°C, 5% CO ₂
46	SUM52PE ^e	Lu	[+]	[-]	+	[-]	PE	Ca			Ham's F12, 5% I-H***	37°C, 5% CO ₂
47	T47D	Lu	+	[+]	+	++ ^M	PE	IDC	54		RPMI, 10% FBS	37°C, 5% CO ₂
48	UACC812	Lu	+	[-]	+	- ^{WT}	P.Br	IDC	43		DMEM, 10% FBS	37°C, 5% CO ₂
49	ZR751	Lu	+	[-]	+	-	AF	IDC	63	W	RPMI, 10% FBS	37°C, 5% CO ₂
50	ZR7530	Lu	+	[-]	+	- ^{WT}	AF	IDC	47	B	RPMI, 10% FBS	37°C, 5% CO ₂
51	ZR75B	Lu	+	[-]	+	+/-				RPMI, 10% FBS	37°C, 5% CO ₂	

AC, adenocarcinoma; AF, ascites fluid; AnCa, anaplastic carcinoma; ASC, acantholytic squamous carcinoma; BaA, Basal A; BaB, Basal B; Ca, carcinoma; CWN, chest wall nodule; Duc.Ca, ductal carcinoma; F, fibrocystic disease; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; Inf, inflammatory; LN, lymph node; Lu, luminal; MC, metaplastic carcinoma; MLCa, metastatic lobular carcinoma; N, normal; Pap, papillary; ND, not done; P.Br, primary breast; PE, pleural effusion; Sk, skin; W, White; B, Black; H, Hispanic; E, East Indian.

ER/PR/HER2/TP53 status: ER/PR positivity, HER2 overexpression, and TP53 protein levels and mutational status (obtained from the Sanger web site;¹⁶ mutant protein; ^{WT}, wild-type protein) are indicated. Expression data are derived from mRNA and protein levels presented in this article (Table S2 and Figure S4). Square brackets indicate that levels are inferred from mRNA levels alone where protein data is not available.

Media conditions: FBS, fetal bovine serum; I, Insulin (0.01 mg/ml); H, hydrocortisone (500 ng/ml); E, EGF (20 ng/ml); DMEM, Dulbecco's modified Eagle's medium, GIBCO #11965-092; RPMI, RPMI medium 1640, GIBCO #27016-021; Ham's F12, F-12 nutrient mixture (Ham), GIBCO #11039-021; DMEM/F12, Dulbecco's modified Eagle's medium; Nutrient mix F-12 [D-MEM/F-12], GIBCO #11039-021; L15, Leibovitz's L-15 medium, GIBCO #11415-064. *For MCF10A and MCF12A, supplement DMEM/F12 media with 5% horse serum, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, and 500 ng/ml hydrocortisone. **For serum-free (SF) medium, supplement Ham's F12 with 0.5 µg/ml bovine serum albumin, 5 mM ethanolamine, 10 mM HEPES, 5 µg/ml transferrin, 10 µM T3, 50 µM Se, 5 µg/ml insulin, 1 µg/ml hydrocortisone. ***For serum-containing F12 media, supplement media with 5% FBS, 5 µg/ml insulin, and 1 µg/ml hydrocortisone or 10 ng/ml EGF.

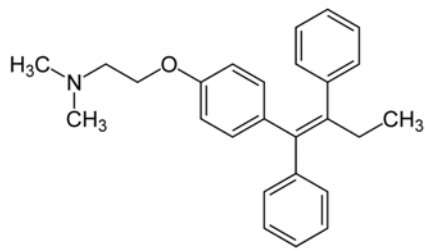
^dAU565 and SKBR3 were derived from the same patient.

^eDerived from a reduction mammoplasty.

^bProvided by Steve Ethier (http://www.cancer.med.umich.edu/breast_cell/Production/index.html).

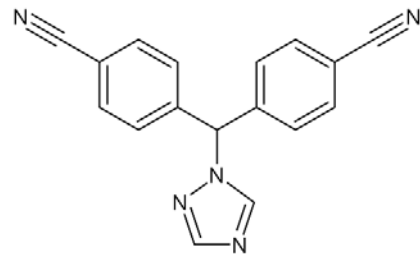
^cProvided by Adi Gazdar, now available through ATCC [Gazdar et al., 1998].

A



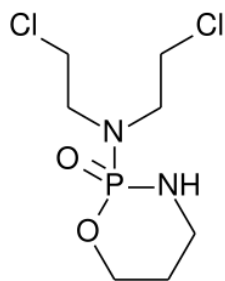
Tamoxifen

B



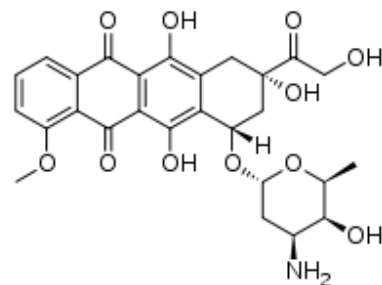
Letrozole

C



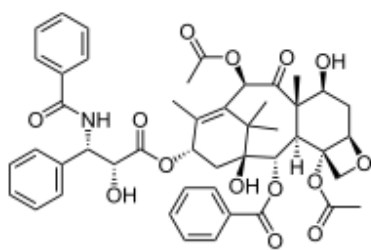
Cyclophosphamide

D



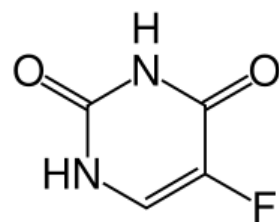
Doxorubicin

E



Taxol

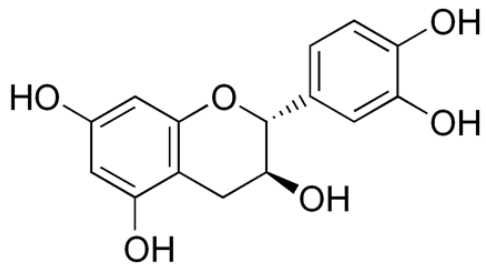
F



5- Fluorouracil

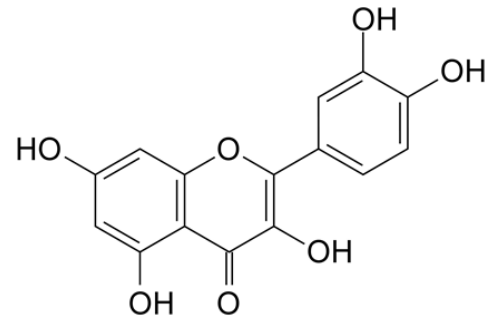
Figure 2.1 Different kinds of drugs which used to treat breast cancer

A



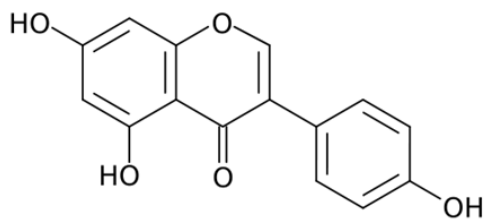
Catechin

B



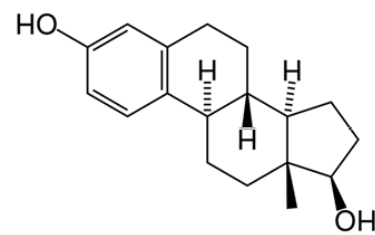
Quercetin

C



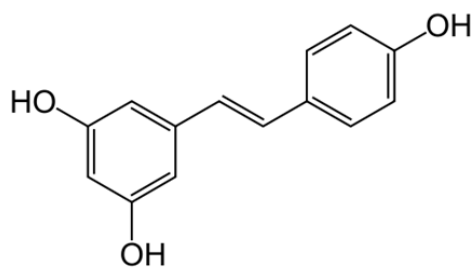
Genistein

D



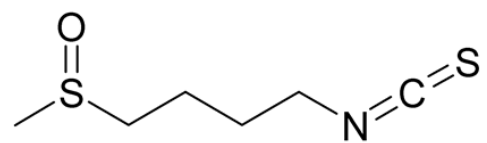
Estradiol

E



Resveratrol

F



Sulforaphane

Figure 2.2 Different kinds of anticancer natural products from fruits and vegetables

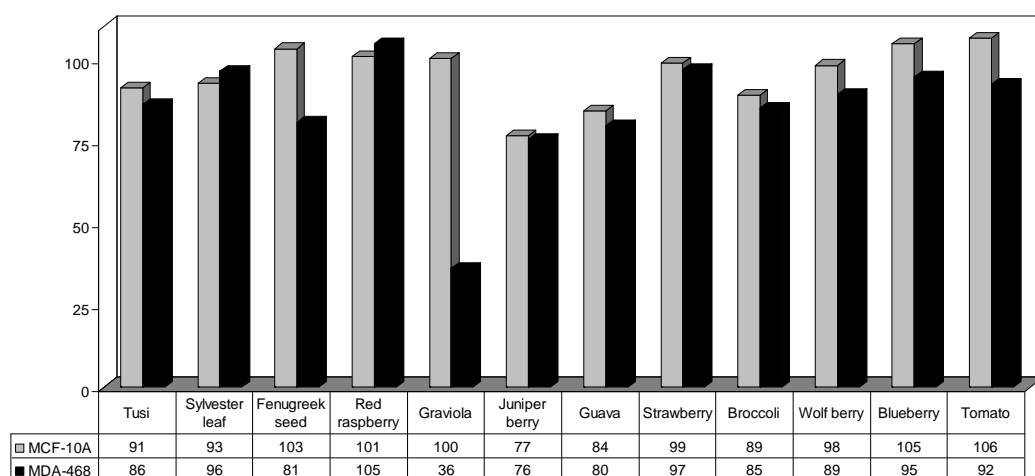
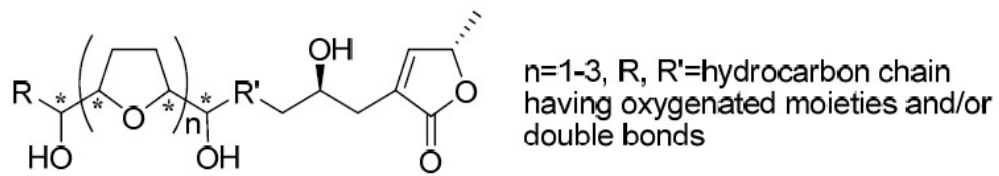


Figure 2.3 Screening of a variety of fruit extracts for anti-proliferation of normal and aggressive human breast cancer cell lines

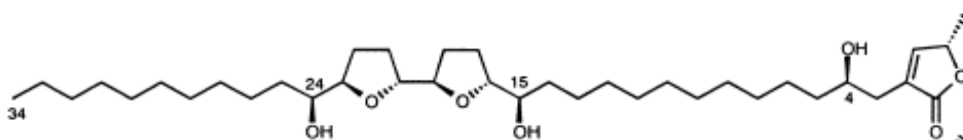
Cells (MCF-10A and MDA-468) were grown in experimental medium containing 25 μ g/mL (Tusi, Sylvester leaf, Fenugreek seed, red raspberry, graviola, and juniper berry) or 200 μ g/mL (guava, strawberry, broccoli, wolfberry, blueberry, and tomato) for 48 hours. MTT cell proliferation assays were performed in quadruplicate and results were analyzed with SPSS software using ANOVA and Tukey's Highest Significant Difference test for multiple comparisons between groups. Significance was set at p-values < 0.05. Mean percentages \pm SD are presented. Treatments significantly inhibited cell proliferation compared to untreated controls (p<0.0001). Significant dose and time responses were noted (p<0.0001).

A



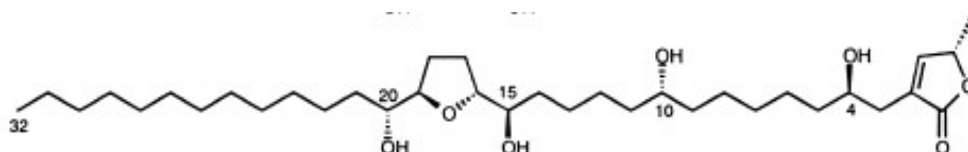
Acetogenins

B



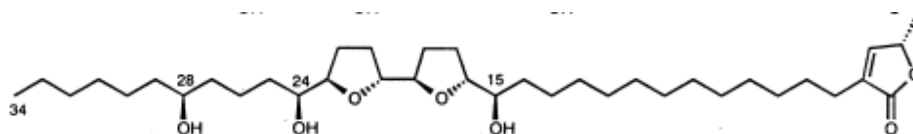
Bullatacin

C



Annonacin

D



Squamocin

Figure 2.4 Different kinds of acetogenins from Graviola

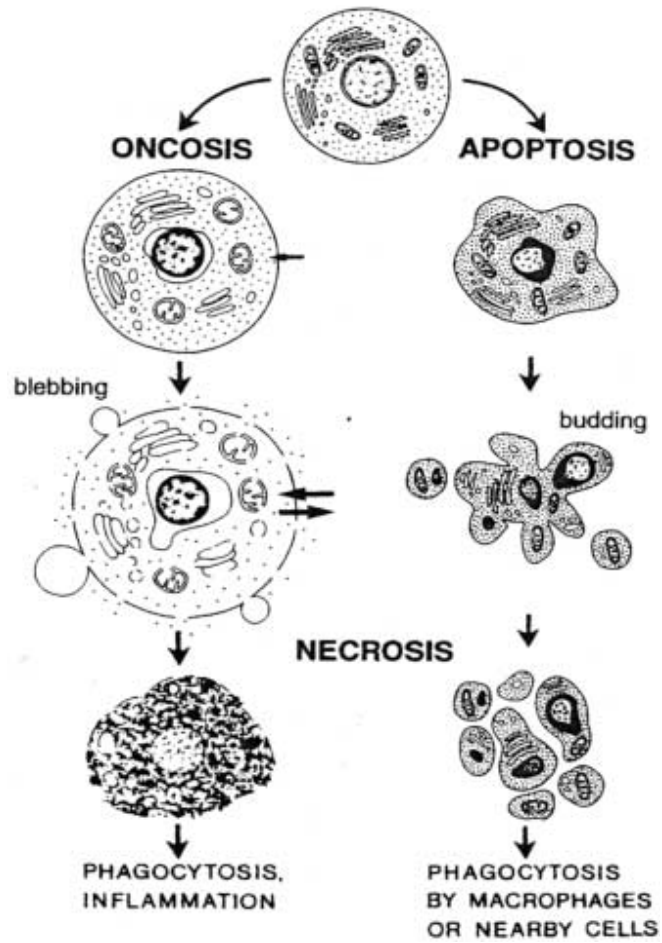


Figure 2.5 The Difference between Apoptosis and Necrosis (Van Cruchten & Van Den Broeck, 2002)

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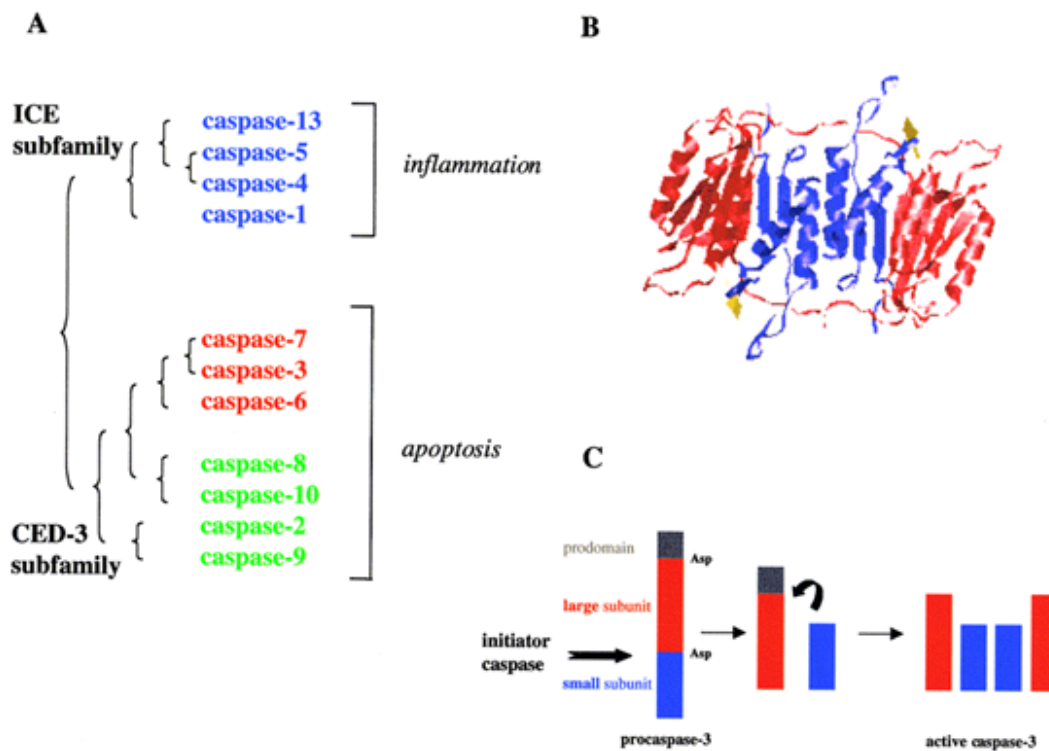


Figure 2.6 The caspase family

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A: Caspases segregate into two major phylogenetic subfamilies (ICE, CED-3). Based on their proteolytic specificities, caspases further divide into three groups: inflammatory caspases (blue) that mediate cytokine maturation, whereas the apoptotic caspases are either effectors of cell death (red) or upstream activators (green). **B:** Crystal structure of mature caspase-3 in complex with a tetrapeptide aldehyde inhibitor (yellow). The active caspase is a tetramer of a large (red) and a small (blue) subunit, each of which contributes amino acids to the active site. **C:** Activation of procaspase-3 by cleavage. (Zimmermann et al., 2001a)

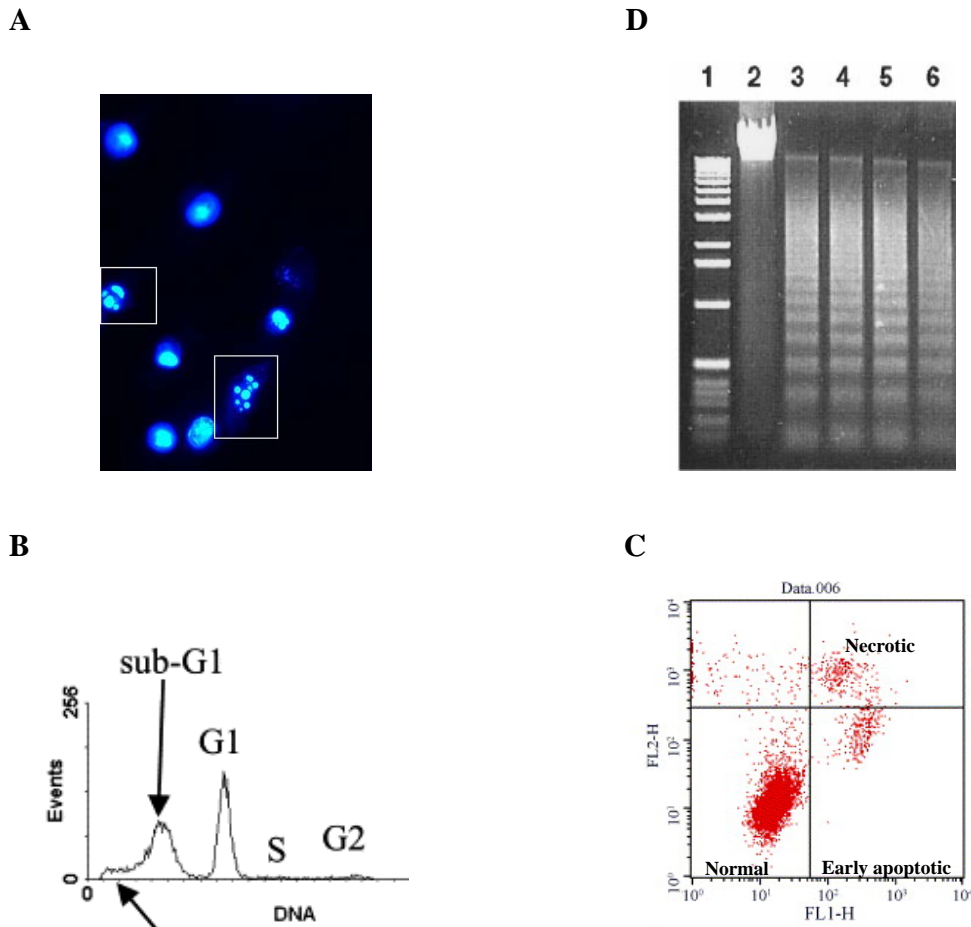


Figure 2.7 Characteristics of apoptotic cells

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A: Apoptotic Chinese Hamster Ovary cells that stained with Hoechst 33258. **B:** Marine haemopoietic cell line BAF3 experience apoptosis after IL-3 removal for 16 h and induced G1 arrest in cell cycle (Ormerod, 2002). **C:** Flow cytometric analysis of statin-induced apoptosis in IM-9 using avidin–biotin complex. Cells were treated with pravastatin (3 mM) for 16 h, followed by staining with biotin-annexin V, avidin-FITC and PI (Yasuda, Matzno, Iwano, Nishikata & Matsuyama, 2005). **D:** Total DNA from replicate cultures was prepared using the adsorption protocol, and fragmentation was assessed by agarose gel electrophoresis and ethidium bromide staining. H9 T cells: lane 1, 1-kb ladder; lane 2, DNA from nonapoptotic cells; lanes 3 to 6, replicates of CD95 triggered H9 cells. (Daniel et al., 1999)

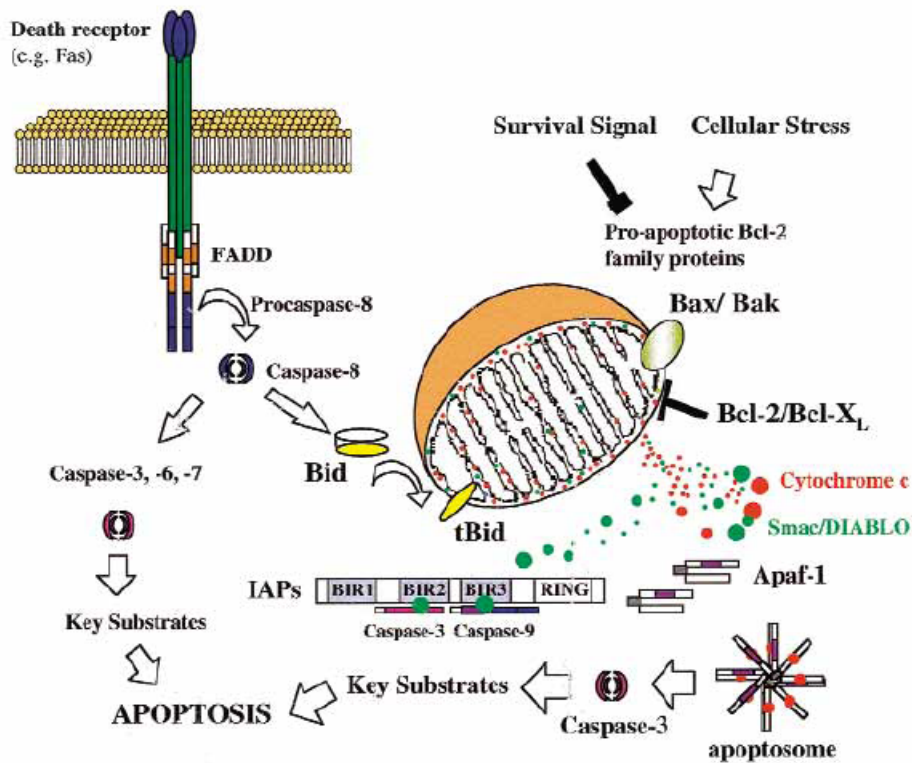


Figure 2.8 Apoptotic signaling pathways

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Cellular stress induces proapoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome *c*. Cytochrome *c* catalyzes the oligomerization of Apaf-1, which recruits and promotes the activation of procaspase-9. This, in turn, activates procaspase-3, leading to apoptosis. Alternatively, the activation of caspase-8 by ligation of the death receptor Fas is illustrated. Ligated Fas recruits FADD to the intracellular region, which in turn recruits procaspase-8. The procaspase-8 transactivates, and the mature caspase now can cleave and activate procaspase-3, leading to apoptosis. Signaling from the Fas receptor to mitochondria involves cleavage of the BH3-only protein, Bid, by caspase-8. Bid subsequently induces cytochrome *c* release and downstream apoptotic events (Zimmermann & Green, 2001b).

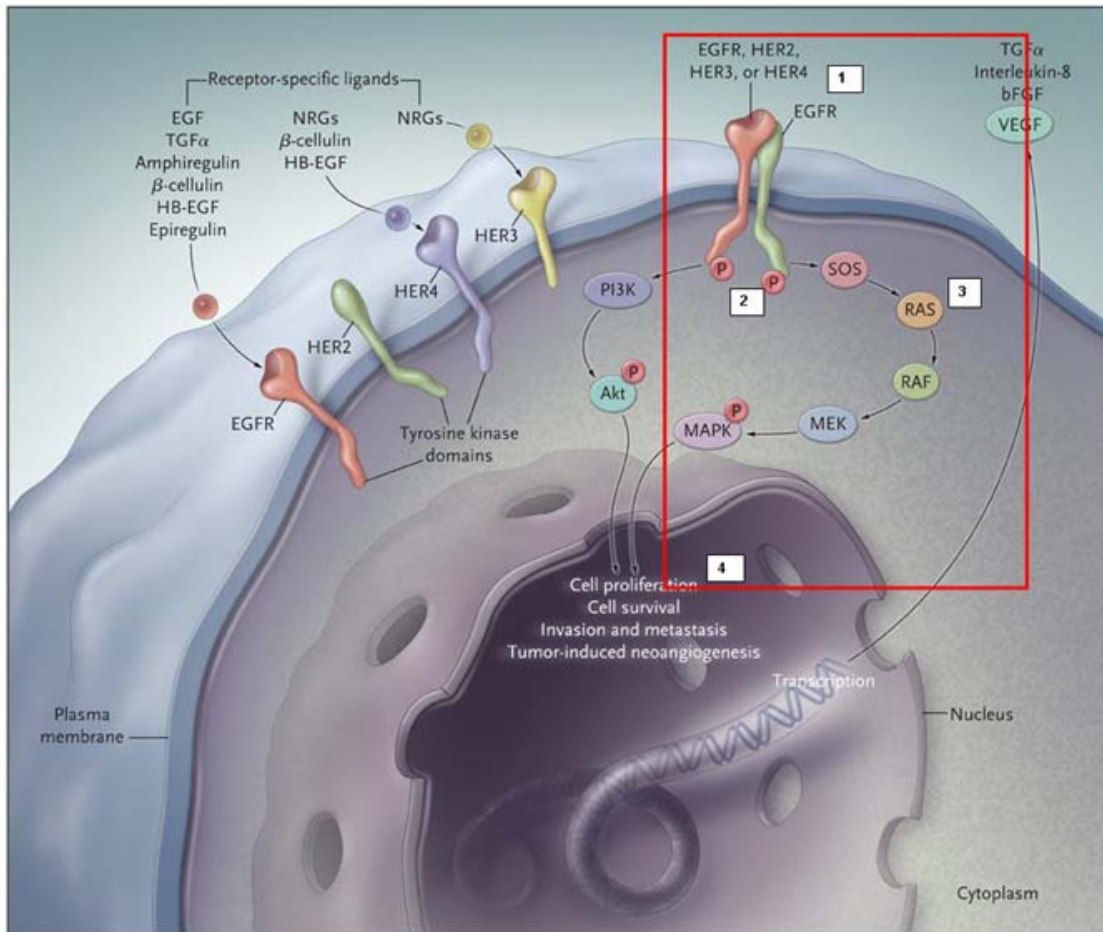


Figure 2.9 EGFR signaling pathways

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<https://www.cobas-roche.co.uk/site/WhatsNew.aspx?articleid=82>

CHAPTER 3: GRAVIOLA FRUIT PHYTOCHEMICALS SELECTIVELY INHIBIT THE GROWTH OF HUMAN BREAST CANCER CELLS IN VITRO AND IN VIVO BY DOWN REGULATION OF EGFR GENE EXPRESSION.

Abstract:

Overexpression of EGFR in breast cancer is associated with poor prognosis and resistance to chemotherapy and hormone therapy. In this study, we identified a specific extract of graviola fruit (GFE) that significantly down-regulated EGFR expression in MDA-MB-468 human breast cancer cells both *in vitro* and *in vivo*. GFE treatment selectively inhibited the growth of MDA-MB-468 cells ($IC_{50}=4.8 \mu\text{g/ml}$) without adversely affecting non-transformed breast epithelial cells (MCF-10A). GFE treatment significantly down-regulated the mRNA expression of EGFR, arrested cell cycle in the G0/G1 phase, and induced apoptosis in MDA-MB-468 cells. We further assessed the potential anti-tumor effect of dietary GFE at a dosage relevant to human intake using ovariectomized mice implanted with MDA-MB-468 cells. Mice were fed diets containing GFE (200 mg/kg in the diet) for 5 weeks. Dietary treatment of GFE significantly inhibited MDA-MB-468 tumor growth by reducing tumor wet weight by 32% compared with the control. GFE treatment also significantly reduced EGFR and p-ERK protein expression in tumors (by 56% and 54%, respectively), and lowered pro-inflammatory serum cytokine levels. These findings suggest that GFE may modulate EGFR and its signaling pathway delaying breast tumor development and that GFE may have a protective effect for women with EGFR (+) breast cancer.

Keywords: graviola (*Annona Muricata* L); breast cancer; MDA-MB-468 cell; epidermal growth factor receptor.

3.1 Introduction

Breast cancer (BC) remains one of the most common cancer among women in the United States (Bray et al., 2004). It is estimated by The American Cancer Society that 192,370 women are diagnosed with BC and of those 40,170 women may die of BC in 2008 (Ries LAG, 2008). Considering the heterogeneity of this disease and the limitations of current therapies such as severe side effects and drug resistance, there is an urgent need to explore alternative strategies to prevent and treat BC. Development of novel mechanism-based nutritional agents that selectively target cancer cells may offer an intriguing dietary strategy for controlling BC. Accumulating evidence suggests a strong effect of the diet or its components on the development and progression of BC, either through effects on hormonal status or via direct tumor-promoting or anticarcinogenic effects (Rock et al., 2002a). Data from epidemiologic studies have supported a significant association between greater fruit and vegetable intake and a lower risk of cancer (Prentice et al., 2006; Steinmetz & Potter, 1996). The health-promoting effect of fruits and vegetables is due in part to the fact that they are comprised of fiber, antioxidants, vitamins, minerals, and other potentially antineoplastic compounds (Terry et al., 2001). Specific food bioactive components, notably sulfur-containing glucosinolates and green tea gallic catechins, were associated with reduced risk of BC in epidemiologic and animal data (Duffy et al., 2003; Mukhtar et al., 2000) and are currently under clinical investigation.

The epidermal growth factor receptor (EGFR) has been identified as a promising target for cancer therapies. EGFR is a type I tyrosine kinase receptor belonging to a family of epidermal growth factor receptors that also includes HER2, HER3, and HER4. Binding of a ligand such as the EGF or transforming growth factor α (TGF α) results in a signaling cascade that produces diverse effects including cell migration, maturation, differentiation, metastasis, angiogenesis, and inhibition of apoptosis (Woodburn, 1999). High expression

of EGFR is commonly considered as the main mechanism by which EGFR signaling is increased in cancer cells (Ciardiello & Tortora, 2003). It is well established that the EGFR or its family members are highly expressed in a variety of human tumors including BC (Salomon et al., 1995). Its overexpression correlates inversely with estrogen receptor (ER) status in patients with BC (Lichtner, 2003) and is associated with an advanced tumor stage (Salomon et al., 1995), poor prognosis (Bange et al., 2001), and resistance to chemotherapy, hormone therapy, and radiation (Brabender et al., 2001). Molecular inhibition of EGFR signaling by anti-EGFR monoclonal antibody or specific small molecule inhibitors has shown anti-tumor effects in preclinical and clinical trails (Goss et al., 2005; Moulder, Yakes, Muthuswamy, Bianco, Simpson & Arteaga, 2001). Therefore, EGFR is a rational target for new BC therapies.

We have recently identified that a specific extract of graviola fruit (GFE) significantly inhibited the growth of human MDA-MB-468 BC cells but not affect non-transformed breast (MCF-10A) cells. We further found that GFE treatment significantly down-regulated EGFR mRNA expression in MDA-MB-468 BC cells. Graviola (*Annonaceous muricata L.*) is an Amazon fruit tree that grows in the tropics of North and South America, and is also known as soursop and guanábana. Leaves and stems of graviola have been traditionally used as a herbal remedy for a variety of purported health promoting effects, including supporting healthy cell growth and immune function (Adewole et al., 2006). Graviola fruits have been widely consumed by indigenous people in fresh or processed forms for centuries. However, research on graviola fruit is extremely limited despite its regular consumption and there is no published study investigating the effect of graviola fruit on breast or other cancers. This study was designed to determine the *in vitro* and *in vivo* effect of GFE treatment on the growth of human MDA-MB-468 BC cells through a mechanism involving down regulation of EFGR expression.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Laboratory animal diet and dietary components were purchased from Dyets (Bethlehem, PA). Reagents for RNA extraction and purification were purchased from Qiagen (Valencia, CA). Reagents for cDNA synthesis and qRT-PCR were purchased from Bio-Rad (Hercules, CA). Primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). MTT (methyl thiazolyl tetrazolium bromide), propidium iodide, Triton X-100, DAPI (4',6'-diamidino-2-phenylindole) were purchased from Sigma Chemical Company (St. Louis, MO). Annexin V-FITC cell apoptosis kit was obtained from Zymed Laboratories Inc. (San Francisco, CA)

3.2.2 Graviola Fruit Extraction and Purification

The dried graviola fruit (*Annona muricata* L.) was acquired in powder form from Earthfruits (South Jordan, UT). The fruit extract was prepared with 50% aqueous acetone extraction which was subsequently filtered and lyophilized. We were able to obtain approximately 100-120mg GFE from 10g dried graviola fruit powder which was equivalent to about 100g fresh fruit. All of the acquired fractions were lyophilized and reconstituted in DMSO for investigation.

3.2.3 Cell Culture

Human BC MDA-MB-468, MDA-MB-231, and MCF-7 cells and non-transformed breast epithelial MCF-10A cells were obtained from American Type Culture Collection (Manassas, VA). BC cells were maintained in DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS). In addition, MCF-10A cells were supplemented with 100 ng/mL cholera enterotoxin, 10 µg/mL insulin, 0.5 µg/mL hydrocortisol, 20 ng/mL epidermal growth factor, 5% horse serum, 10 mM HEPES (RPMI) and 2.2g/L sodium bicarbonate, while MCF-7 cells were supplemented with 0.5nmol/L

estradiol and 5 $\mu\text{mol/L}$ insulin. All cultures were maintained in media in the presence of 100 units/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin in 5% CO_2 at 37°C in a humidified atmosphere of 5% CO_2 in air as a monolayer culture in plastic culture plates. Media were changed every 48 h, and cells were passed when they reached 85-95% confluence, as observed by a light microscope.

3.2.4 Cell Viability Assay

The cell viability was determined by measuring the ability of cells to transform MTT (methyl thiazolyl tetrazolium bromide) to a purple formazan dye. BC and non-transformed breast epithelial cells were seeded in 96-well tissue culture plates (8.0×10^3 cells/well). After 24 h, cells were treated with GFE or its fractions for 48 – 96 hr. After incubation, the medium was removed and cells were washed with HBSS, and incubated for 4 h with MTT reagent solution. The MTT solution was aspirated and the formazan crystals were dissolved in DMSO for 20 minutes on a plate rocker protected from UV light. The color intensity of the formazan solution, which reflects the number of cells under the specific growth conditions, was measured at 570 nm using a Victor³ multilabel plate reader (PerkinElmer, USA). The cell viability of treatment groups was expressed as percentage of the control.

3.2.5 Caspase-3 activity

After GFE treatment, the cells were harvested and suspended in the cell lysis buffer and incubated on ice for 1 h. After centrifugation at 11000 x *g* for 30 min, the supernatants were collected and immediately measured for protein concentration and caspase-3 activity with a caspase-3 activation detection kit following manufacturers protocol (TruPoint™ Caspase-3 Assay Kit, PerkinElmer Life and Analytical Sciences, Norton, OH). Briefly, cell lysates were placed in 96-well plates, and a caspase-3 specific substrate (EU-CDKVDK-QSY 7) was added. Plates were incubated at 37 °C for 1 h, and caspase activity was determined by measuring the fluorescence intensity with the excitation and

emission wavelengths set at 340 and 615 nm, respectively.

3.2.6 DAPI analysis

To detect morphological evidence of apoptosis, cells were examined for nuclear condensation using the fluorescent dye 4',6'-diamidino-2-phenylindole (DAPI) to stain the nuclei. Cell nuclei were visualized following DNA staining with DAPI. MDA-MB-468 cells were seeded into 24-well plates (5×10^4 cells/well), allowed to attach in CM for 24h onto sterile glass cover slips, and then maintained for another 24h in EM supplemented with GFE (0, 5, 25, or 100 $\mu\text{g/ml}$) in triplicate. At the end of incubation, the morphology of cells was monitored under an inverted light microscope. Cells were fixed with 1% paraformaldehyde for 20 min and washed with PBS, and then incubated with DAPI (1 $\mu\text{g/ml}$) for 10min. After washed with PBS, cells were observed using fluorescent microscope (Nikon, Japan) with a peak excitation wave length of 340 nm.

3.2.7 Annexin V-FITC/PI staining analysis

MDA-MB-468 BC cells were plated in 6-well plates with 2.5 mL of medium with the final concentration of 5×10^5 cells/well. After attaching for 24h, the cells were treated by 5, 25, 50, 100 $\mu\text{g/ml}$ crude graviola extracts for 48 h respectively. The cells were then washed twice with PBS, harvested by trypsinization and suspended in the 190ul binding buffer. Subsequently, 10 μl of 1 $\mu\text{g/ml}$ Annexin V-FITC and 10 μl of 20 $\mu\text{g/ml}$ PI were added separately before incubation for 10 minutes at the room temperature in the dark. Finally, cells were analysed using a FACS Aria flow cytometer (BD Bioscience, San Jose, California).

3.2.8 Cell cycle analysis

MDA-MB-468 BC cells at 80-90% confluence were treated with GFE at different concentrations: 5, 25, 50, and 100 $\mu\text{g/ml}$ for 48 h. After trypsinization, floating and formerly attached cells were collected, washed and suspended at a density of 1×10^6

cells/ml in 0.5ml PBS, then 4.5ml of 70% ethanol was pipetted slowly into the suspension while vortexing at top speed. Cells were stored in ethanol at 4°C for 2 h, followed by centrifugation, washed with PBS and resuspended in 1ml PI/Triton X-100 staining solution with DNase-free RNase A at 37°C for 30 minutes before analysis by using a FACS Aria flow cytometer, gated on forward light scatter, using a dichroic, a band-pass filter and 488 nm argon laser .

3.2.9 Analysis of EGFR mRNA expression using relative quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

After collecting 1.5×10^6 of MDA-MB-468 cells which were treated by 5 µg/ml, 25µg/ml and 100 µg/ml GFE for 48 h, total RNA was isolated using the RNeasy Mini Kit (Qiagen Company), and quantified by UV absorbance. We selected one low-dose (5 µg/ml) and one high-dose GFE (100 µg/ml) treatment for PCR analysis. cDNA was generated using 10 ng of RNA and iScript Reverse Transcription Reagents (Bio-Rad, Hercules, CA). Primers were designed using Beacon Designer 5 (Premier Biosoft International). The oligonucleotide primers specific for EGFR and GAPDH (EGFR: forward primer, 5'-CCGTCGCTATCAAGG AATTAAG-3'; reserve primer, 5'-GTGGAGGTGAGGC AGATGG-3'; GAPDH: forward primer, 5'-TTGGTATCGTGGAAGGACTC-3'; reverse primer, 5'-TAGAGGCAGGGATGATGTTC-3') were used. PCR and analysis of PCR products were performed using the iCycler (Bio-Rad) and the SYBR-green detection system were used. The reverse transcription of RNA was performed in a final volume of 20 µl containing 4 µl 5× iScript Reaction Mix, 1 µl iScript Reverse Transcriptase, 15 µl nuclease-free water and RNA template (total RNA content is 200ng). After incubation at 25°C for 5 minutes, 42°C for another 30 minutes, the reaction was terminated by raising the temperature to 85°C for 5 minutes. For PCR, supermix containing 20nM of primers was added to 0.5 µl of the newly synthesized DNA to bring the final volume to 25 µl. The reaction mixture was first heated at 95°C for 3 minutes and amplification was carried out in 40 cycles at 95°C for 10s, 55°C for 45s, followed by a final incubation at 55°C for 1

minute, with a iCycler iQ multicolor Real-time PCR Detection System (Bio-rad). PCR products were detected by fluorescence scanning along with the iCycler iQ, and the content of EGFR expression were calculated and normalized to the house keeping gene GAPDH. Data were analyzed using a comparative threshold cycle (Ct) method. Each sample was run in triplicate in separate tubes to permit quantification of target genes normalized to controls, GAPDH.

3.2.10 Athymic nude mice

Female athymic BALB/c (nude) mice (5-wk of age) were purchased from Charles River Laboratories (Wilmington, MA) and acclimated for a week. During the study, the mice were maintained under standard light/dark cycle (12 h light, 12 h dark).

3.2.11 Animal treatment and analysis of tumor growth

After one week of acclimation, MDA-MB-468 cells (1×10^5 cells per 40 μ l of Matrigel® [Collaborative Biomedical Products, Bedford, MA]) were injected at 40 μ l per site into each of the four spots of back of the athymic mice. Mice were divided into two groups: MDA-MB-468 control (n=4) and 200 μ g/g GFE (n=6) and dietary treatment began. American Institute of Nutrition 93 growth diet (AIN93G) semi-purified diet (Dyets, Bethlehem, PA) was selected as a base diet for control mice as it has been established as meeting all of the nutritional requirements of mice (Reeves, 1997). Treatment animals were fed AIN93G diet plus GFE (200 μ g/g diet) and dietary treatment lasted for 5 weeks when the average tumor surface area of the control group reached 90.8 mm². Tumor surface area and body weight were measured weekly and surface area was determined using the formula [$\text{length}/2 \times \text{width}/2 \times \pi$] (Gottardis, Wagner, Borden & Jordan, 1989). Food intake was measured throughout the study. At the end of the study, uterine wet weight was measured. Tumors and blood samples were collected for further analysis.

3.2.12 Western blot analysis

We examined dietary GFE-induced changes in protein expression of EGFR, phosphorylated-ERK (p-ERK), and p-Akt. Blots were stripped and re-probed with antibody that recognizes total ERK or Akt. Frozen tumors (4 tumors/control and 6 tumors/GFE) were pulverized with mortar and pestle in liquid N₂ and lysed and homogenized in RIPA buffer. Homogenates were centrifuged at 10,000g for 10 minutes at 4°C and supernatant collected for analysis. Protein from tumors (10-15 µg) was loaded into a 7.5% gradient gel, electrophoresed at 100V for 1.5 hrs, and transferred to nitrocellulose membrane at 100V for 1 hour. The membrane was blocked for 1 hour in 5% milk-TBST at RT, washed 3x with TBST, incubated overnight at 4° in primary antibody (EGFR, p-ERK, and p-Akt, Santa Cruz: Santa Cruz, CA) washed 3x with TBST, incubated for 1 hr at RT in secondary antibody (goat anti-rabbit-HRP, bovine anti-mouse-HRP, goat anti-rabbit-HRP, respectively) (Santa Cruz: Santa Cruz, CA), and activated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.: Rockford IL). The membrane was then exposed to film and developed manually. Protein bands were analyzed using Image J (NIH: Bethesda, MD) and the bands from each film were normalized to β-actin (1°Ab Goat, 2°Ab Bovine anti-goat-HRP) control.

3.2.13 Serum cytokine levels

We evaluated the dietary GFE-induced changes in cytokine levels in pooled serum using multiplex Enzyme Linked Immunosorbant Assay (Quansys Biosciences, Logan, UT). Inflammatory mediators (interferon gamma [INF-γ], interleukin-6 [IL-6], IL-1β), monocyte chemo attractant protein 1 (MCP-1), and macrophage inflammatory protein-1α (regulated on activation normal T-cell expressed and presumably secreted, RANTES) levels were measured. MCP-1 and RANTES are chemokines co-related with advanced breast cancer (Luboshits et al., 1999), and might be involved in angiogenesis and vascularization, and metastatic breast cancer (Saji et al., 2001). IL-1β is a

pro-inflammatory cytokine that exerts tumor regression (Elkordy et al., 1997). Values were collected from larger sample sets instead of the individual tests. A value that fell outside the standard curve was extrapolated using linear regression.

3.2.14 Statistical analysis

Values are expressed as the mean \pm SEM. Data from in vitro experiments, tumor wet weight at final week, Western blot analysis, body weight gain, and feed intake were analyzed using one-way or repeated-measures analysis of variance according to the characteristics of the data set using the SAS program (SAS, Cary, NC). If the overall treatment F-ratio was significant ($p < 0.05$), the differences between treatment means were tested with Tukey's multiple comparison test.

3.3 Results

3.3.1 GFE treatment selectively inhibited growth of human BC cells

The inhibitory effect of GFE on the growth of BC cells including MDA-MB-468, MDA-MB-231, and MCF-7 and non-transformed breast epithelial cells MCF-10A were determined by employing a MTT assay. All cells were treated by GFE at the doses of 0.5, 1, 5, 50, 100, and 200 $\mu\text{g/ml}$ for 96 h. As shown in **Figure 3.1A**, GFE treatment selectively inhibited the growth of all three BC cells while the non-transformed MCF-10A cells were not affected with GFE dose up to 200 $\mu\text{g/ml}$ media, suggesting the different susceptibilities of cancer and non-transformed cells to GFE cytotoxicity. Among the BC cells, MDA-MB-468 cells were most sensitive to GFE treatment; the cell growth was inhibited by 29.5% with the GFE dose as low as 0.5 $\mu\text{g/ml}$ and the growth inhibition was dose-dependent. We determined the IC_{50} of GFE for MDA-MB-468 cells was 4.8 $\mu\text{g/ml}$, which is comparable to IC_{50} of 4 $\mu\text{g/ml}$ (10 μM) that was defined by NCI as the minimal requirement for an active antitumor compound (Geran, 1997). In contrast, GFE treatment only resulted in moderate growth inhibition of BC MDA-MB-231 and MCF-7 cells. For

example, GFE treatments at 100 µg/ml only inhibited the cell growth of MDA-MB-231 and MCF-7 by 32.2 and 20.8%, respectively, while the growth of MDA-MB-468 cells was inhibited by 76.3%. These observations suggest that GFE-induced growth inhibition is not only cancer-specific but also high selective against the different cancer cells. Therefore, the further mechanistic studies on antitumor properties of GFE were using BC MDA-MB-468 cells.

3.3.2 Time-responses of GFE on the growth of MDA-MB-468 cells

To further evaluate the time responses of GFE treatments on the growth of MDA-MB-468 cells, we treated the cancer cells with different doses of GFE for 24, 48, 72, and 96 h. As shown in **Figure 3.1B**, for all the GFE treatments, the growth inhibition of the BC cells was more pronounced as the exposure time to GFE increased, suggesting the growth inhibitory activity of GFE can be at least maintained during 96 h. When the MDA-MB-468 cells were treated with the low GFE concentrations (1 and 5 µg/ml), a significant growth inhibition was observed until the incubation time were 48 h or longer. For instance, the cell viability was reduced to 91.53% ($p>0.05$), 69.29% ($p<0.05$), 54.73%, and 50.20% of control levels after 24, 48, 72, or 96 h of GFE treatments at 5 µg/ml, respectively. These data along with the results shown in **Figure 3.1A** demonstrated that GFE treatments inhibited the growth of MDA-MB-468 BC cells in both dose- and time-dependent manner and the significant growth inhibition was shown with GFE treatment as low as 0.5 µg/ml.

3.3.3 GFE treatment induced apoptosis in MDA-MB-468 BC cells

To assess whether the GFE-induced growth inhibition of MDA-MB-468 cells involves apoptosis, we performed nuclear DAPI staining, caspase-3 activation, and PI-Annexin-V-FITC staining measured by flow cytometry to evaluate whether GFE induced cell apoptosis. For qualitative assessment of apoptosis, cells after 48 h GFE treatment at 25 µg/ml were fixed on culture slides and examined under a fluorescent light microscope for identification of presence of nuclear changes associated with apoptosis.

These changes include nuclear condensation, chromatin modulation, and the presence of apoptotic bodies. Fluorescence staining with DAPI revealed a strong the morphologic changes in cell nuclei. After exposure to different doses of GFE, MDA-MB-468 cells showed condensed or fragmented nuclei, which is characteristic of early apoptosis (**Figure 3.2A**). To corroborate the microscopic data, the extent of apoptosis was quantitated by measuring the level of caspase-3 activation following GFE exposure (**Figure 3.2B**). GFE treatment significantly activated the caspase-3 activity in a dose-dependent manner. The enzyme activity was increased by 17.8 and 91.9% over the vehicle-treated controls after 24h incubation with GFE at 12.5 and 200 $\mu\text{g/ml}$ media, respectively ($p < 0.05$). As another index of apoptosis, we measured the apoptotic cell population with flow cytometry using Annexin V-FITC/PI staining (**Figure 3.2C**). The results showed a noticeably increased early stage apoptosis (by 20.8%) after GEF treatment (100 $\mu\text{g/ml}$) for 24 h. Collectively, these results demonstrated that GFE-induced growth inhibition of MDA-MB-468 cells was at least in part due to apoptosis induction.

3.3.4 GFE treatment induced G1 phase arrest in MDA-MB-468 BC cells

To further elucidate the mechanisms of GFE-induced growth inhibition that may accompany the induction of apoptosis and account for the reduced viable cell number as shown in **Figure 3.2D**, we examined the effect of GFE on cell cycle distribution with PI staining measured by flow cytometry. Concomitant with a growth inhibitory effect, GFE treatment induced both G0/G1-phase arrest in a dose-dependent manner. The cell population in the G0/G1, S, and G2/M phases were 41.8, 34.8, and 20.9% in control MDA-MB-468 cells, while after 24 h of incubation with 25, 50, 100 $\mu\text{g/ml}$ GFE, the G0/G1 population was enhanced by 3, 5, and 6%, respectively. This increase in the G0/G1-phase cell population paralleled a concomitant decrease in the S and G2/M phase cell populations. Concurrently, the apoptotic cell population of MDA-MB-468 cells was increased after GFE treatment. The G1/S ratio has been used as an index of G1 arrest ([Sun et al., 2006](#)). After a 24 h exposure to GFE, the G1/S ratio of the treatment group was significantly

higher than that of control group ($p < 0.05$) and a constant increasing pattern of the G1/S ratio was observed in the treatment group as GFE concentration increased. The GFE treatment at 100 $\mu\text{g/ml}$ increased the G1/S ratio of the BC cells by 72.13%, suggesting that GFE induced a significant GI arrest in MDA-MB-468 cells.

3.3.5 GFE treatment down regulated mRNA expression of EGFR in MDA-MB-468 breast cancer cells

The MDA-MB-468 cell line is known to have an amplified EGFR gene and consequently these cells show very high expression levels of EGFR (1.5×10^6 receptors per cell) (Filmus et al., 1987). We were therefore interested whether the exceptional selectivity of GFE against the growth of MDA-MB-468 cells is related to EGFR signaling. To investigate whether GFE-induced growth inhibition involves modulation of EGFR in MDA-MB-468 cells, we examined the effect of GFE on mRNA expression of EGFR by qRT-PCR. As shown in **Figure 3.3**, GFE treatment induced a strong down regulation of EGFR mRNA expression in MDA-MB-468 cells. GFE at 5 and 100 $\mu\text{g/ml}$ significantly down regulated the EGFR gene expression by 30 and 54%, respectively. However, at 25 $\mu\text{g/ml}$, it shows upregulated EGFR gene expression, whose mechanism is not clear.

3.3.6 Effect of dietary GFE on the growth of MDA-MB-468 tumors implanted in athymic mice

After 5 weeks of dietary treatment, average tumor surface areas were $90.82 \pm 9.15 \text{ mm}^2$ for the control group, and $80.97 \pm 5.62 \text{ mm}^2$ for the GFE (200 $\mu\text{g/g}$) group, respectively. There was no statistical difference between the control and the GFE group. However, we observed a significant reduction of tumor wet weight in the GFE group (by 32%) (**Figure 3.4**). Average tumor weight was $253.29 \pm 54.68 \text{ mg}$ for the control group, and $171.15 \pm 23.23 \text{ mg}$ for the GFE group, respectively ($p < 0.05$).

There was no significantly difference in food intake between the control and the GFE

treatment groups (data not shown); however, there was a significant difference in body weight gain between two groups. Body weight gain was calculated using a formula; body weight gain = (body weight at wk 5 – tumor weight) – body weight at wk 1. Average body weight gain was 2.58 ± 0.42 g for the control group, and 0.46 ± 0.44 g for the GFE group, respectively ($p < 0.05$).

3.3.7 Effect of dietary GFE on protein expression in tumors

We examined whether dietary GFE modulates EGFR and its signaling pathway effectors by Western blot analysis. As shown in **Figure 3.5**, dietary treatment of GFE significantly reduced EGFR and p-ERK protein expression in tumors (by 56% and 54%, respectively) compared with the control group ($p < 0.05$). However, no change was detected in p-Akt and NF- κ B expression in tumors (data not shown).

3.3.8 Effect of dietary GFE on serum cytokine levels

We also examined whether dietary GFE-induced tumor growth inhibition involves modulation of circulating cytokine levels by multiple ELISA assays. The results showed that the dietary GFE increased the serum IL-1 β level but lowered IL-6 level in the athymic mice (**Table 3.1**).

3.4 Discussion

Traditional cancer therapeutics has relied heavily upon the ability to inhibit DNA replication or cell division. While this approach has proven effective in some patients, lack of tumor-cell selectivity has limited response rates and complicated treatment with numerous adverse effects. Thus, identification of novel-targeted chemotherapeutics that can selectively inhibit tumors is of major importance in efforts to decrease the burden of BC. One such strategy to control BC growth and metastasis could be its prevention and treatment by phytochemicals present in diets that targets specific BC cells. We have identified GFE as such a dietary agent with anticancer potential through our preliminary

screening of hundreds of food extracts and compounds on growth inhibition of BC cells. GFE showed excellent selectivity against certain BC cells. For instance, the ER- BC MDA-MB-468 cells were exceptional sensitive to GFE treatment with an IC₅₀ of 4.8 µg/ml, while GFE only modestly inhibited the growth of MDA-MB-231 and MCF-7 cells (**Figure 3.1A**). The GFE treatment with a dose as low as 0.5µg/ml can significantly inhibit the growth of MDA-MB-468 cells. More importantly, GFE did not affect the growth of non-transformed breast epithelial MCF-10A cells with the treatment dose up to 200µg/ml (**Figure 3.1A**). Collectively, these observations suggest that GFE may selectively target specific mechanisms in certain BC cells such as MDA-MB-468 cells. This unusual cancer-cell selectivity of GFE offers a significant opportunity for the development of GFE components as novel dietary agents for controlling human BC.

To reveal the potential mechanisms governing the GFE-induced growth inhibition on BC MDA-MB-468 cells, we further examined whether or not GFE induced apoptosis or cell cycle arrest or both. Apoptosis regulates tissue homeostasis and is a critical mechanism for cancer chemoprevention and chemotherapy (Kleinridders et al., 2009). In BC, cells become resistant to apoptosis partially due to disruptions of apoptotic signaling pathways and changes in the expression of proteins and enzymes associated with tumor resistance (Crowe & Chandraratna, 2004). In this study, we found that GFE treatment led to a dose-dependent increase in enzymatic activity of caspases-3 (**Figure 3.2B**) and apoptotic cells death. The GFE-induced apoptosis was also confirmed through morphological changes and nuclear condensation in MDA-MB-468 cells (**Figure 3.2A**). Incubation of the cancer cells with GFE did not cause significant necrotic cell death as shown on (**Figure 3.2C**). In addition, cell cycle deregulation is closely associated with apoptosis and disruption of the cell cycle may eventually lead to apoptotic death, thus, inhibition of deregulated cell cycle progression in cancer cells is another effective strategy to control tumor growth (Noble et al., 2005). Chemotherapeutic agents often induce cell cycle arrest

at the G0/G1 or G2/M phases (Athar, Kim, Ahmad, Mukhtar, Gautier & Bickers, 2000; Sun et al., 2006). Our data clearly showed that the increase of G0/G1 phase cells was accompanied by a significantly decrease of S phase cells and moderate decrease of G2/M phase cells, revealing that GFE treatment brought about a blockage effect at the G1/S transition and induced dose-dependent G0/G1 cell cycle arrest (**Figure 3.2D**). Together, these observations suggest that GFE-induced growth inhibition of MDA-MB-468 cells is in part attributable to its induction on apoptosis and G0/G1 cell cycle arrest in the cancer cells. However, the specific mechanism of the GFE treatment on the cell cycle machinery and expression of several related proteins deserved further investigation.

EGFR is involved in many cellular processes including cell proliferation, motility, adhesion and angiogenesis (Woodburn, 1999). Deregulation and overexpression of EGFR have been implicated in the pathogenesis of breast tumors, leading to transformation of cells *in vitro* and tumor formation in nude mice in an EGF-dependent manner (Chung et al., 2008). MDA-MB-468 cells exhibit EGFR gene amplification. The extreme sensitivity of MDA-MB-468 cells to GFE treatment prompted us to examine whether GFE-induced growth inhibition is mediated through inhibition of EGFR signaling. Therefore, we incubated MDA-MB-468 cells with different doses of GFE for 24h and found that GFE induced a strong dose-dependent down regulation of EGFR mRNA expression (**Figure 3.3**). After GFE treatment (100 µg/ml), EGFR gene expression was down regulated as much as 54% versus the control. Previous research has demonstrated that molecular inhibition of EGFR signaling induced apoptosis and blocked cell cycle progression in the G1 phase in different experimental models such as colorectal cancer cells (Woodburn, 1999). Here we also observed that the GFE treatment caused apoptosis induction and G0/G1 cell cycle arrest in MDA-MB-468 cells (**Figure 3.2D**). The underlying mechanism is unclear, but the induction of apoptosis and G0/G1 cell cycle arrest may be mediated by GFE-induced down regulation of EGFR gene expression in

MDA-468 cells.

Over two decades, targeting EGFRs has been intensively studied in cancer therapy (Zhang et al., 2007). Activation of EGFR occurs frequently in both benign and malignant hyperproliferative BC and triggers a cascade of downstream intracellular signaling pathways. This activation involves at least two major pathways: the highly conserved Ras/mitogen activated protein kinase (MAPK)-dependent pathway ([Ras/Raf/extracellular signal-regulated kinase [MEK]/extracellular signal-regulated kinase [ERK]) and the phosphatidylinositol 3-kinase (PI3-K)-dependent pathway (PI3K/phosphatase and v-akt murine thymoma viral oncogene homolog [Akt]/mammalian target of rapamycin [mTOR]). The activation of Ras/Raf/MEK/ERK pathway results in cell survival, proliferation, migration, angiogenesis, and inhibition of apoptosis (Normanno et al., 2006; Schlessinger, 2000). The activation of the PI3K/Akt/mTOR pathway also regulates proliferation, survival, mortality and morphology (Farhana et al., 2004; Woodburn, 1999; Yarden & Sliwkowski, 2001). Therefore, blocking EGFR and its signaling cascade is a promising targeted anticancer therapy. In this study, we conducted a feeding study of GFE to confirm that results from the *in vitro* studies can be reproduced *in vivo*. Dietary intake of GFE significantly reduced tumor weight (**Figure 3.4**), confirming its anti-cancer efficacy at a dosage relevant to human exposure. We further examined whether the GFE-induced tumor growth inhibition is due to modulation of EGFR and its signaling pathways, through measuring the protein expression of EGFR, p-ERK, and p-Akt in tumors. We found that dietary GFE treatment significantly reduced EGFR expression by 56% and inhibited ERK phosphorylation by 54% (**Figure 3.5**), but did not change p-Akt levels, suggesting that dietary GFE treatment may modulate EGFR and Ras/Raf/MEK/ERK signaling pathway resulting in tumor inhibition. In addition, we observed that dietary GFE treatment altered the serum cytokine levels, causing a significant increase in IL-1 β and a decrease in IL-6 (**Table 3.1**). Besides its health-beneficial role in inflammation, the cytokine IL-6 also acts

as a paracrine/autocrine growth factor of many tumor cells and confers cancer therapy resistance, which correlates with worse prognosis in breast cancer patients (Bertucci et al., 2005; Neve et al., 2006). The tumor-suppressing property of local small amounts of IL-1 β induces anti-tumor immunity, whereas large amounts induce broad inflammation (due to its paracrine effect on the tumor's microenvironment) that contribute to invasiveness and metastasis (Apte & Voronov, 2002; Elkordy et al., 1997). The underlying mechanism of GFE on cytokines is unclear but it is likely that the tumor inhibition is partly mediated by anti-inflammation activity. Overexpression of EGFR and/or erbB2 can lead to MAPK-induced activation of NF- κ B (Van Laere et al., 2006), which contributes to the progression of BC from ER(+) to more aggressive ER(-) breast tumor growth (Nakshatri, Bhat-Nakshatri, Martin, Goulet & Sledge, 1997; Sovak et al., 1997), (most predominantly in ER(-) and ErbB2(+) breast tumors (Biswas et al., 2004)). A transcription factor, NF- κ B, exists in the cytoplasm in the form of a complex with an inhibitor of κ -B (I κ B). Cytokines, chemokines, and intracellular stress lead to the phosphorylation of I κ B by I κ B kinase (IKK), releasing NF- κ B, which can then translocate into the nucleus and modulate the transcription of target genes (Siebenlist, Franzoso & Brown, 1994). However, we did not observe a significant change in NF- κ B expression by GFE treatment.

It is worthy of note that our current study also demonstrated the ability of dietary GFE to reduce body weight and inguinal fat pad mass. Although the mechanism for weight reduction is unclear at this point, one of possible mechanism could be involvement with adipocytokines. The management of normal body weight is regulated by adipocytokines that act on the brain to regulate food intake (Matsuzawa, Funahashi & Nakamura, 1999). The adipocytokines are biologically active polypeptides that are produced by white adipose tissue, preadipocytes, and mature adipocytes and act by endocrine, paracrine, and autocrine mechanisms (Matsuzawa et al., 1999; Rose, Komninou & Stephenson, 2004). Leptin, a product of the obese (*ob*) gene is a neuroendocrine hormone (MacDougald, Hwang, Fan &

Lane, 1995) bound to the leptin receptor in plasma membranes (Houseknecht, Mantzoros, Kuliawat, Hadro, Flier & Kahn, 1996) which belongs to a family of class I cytokine receptors (Tartaglia, 1997). Like other class I cytokine receptors, the leptin signaling is thought to be transmitted mainly by the Janus kinases (JAKs)/signal transducers and activators of transcription (STAT) pathway (Ahima & Osei, 2004; Bahrenberg et al., 2002).

To the best of our knowledge, this is the very first report demonstrating that phytochemicals in the fruits can selectively down regulate EGFR mRNA expression in human BC MDA-MB-468 cells, resulting in cell apoptosis and G1 progression arrest. We showed that GFE selectively inhibits the growth of human BC cells while non-transformed breast epithelial MCF-10A cells remained unaffected, suggesting GFE and its components possess unique antitumor properties and have the potential as novel dietary agents for controlling human BC. We further confirmed the efficacy of GFE *in vivo*. Dietary GFE acted as an anti-EGFR agent in nude mice xenografts and inhibited EGFR-expressing MDA-MB-468 tumor growth by reducing EGFR and interfering EGFR/ERK pathway. In summary, GFE is a potential effective anti-EGFR agent *in vitro* and *in vivo*. However, we understand that advanced human breast tumors overexpress not only EGFR, but also erbB2 and/or erbB3. Therefore, in future study we will further evaluate effect of GFE treatment on the combined expression pattern of EGFR family members and on the dimerization pattern) in order to test the efficacy of GFE as a targeted therapy.

3.5 Literature Cited

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3.6 Figures and Tables

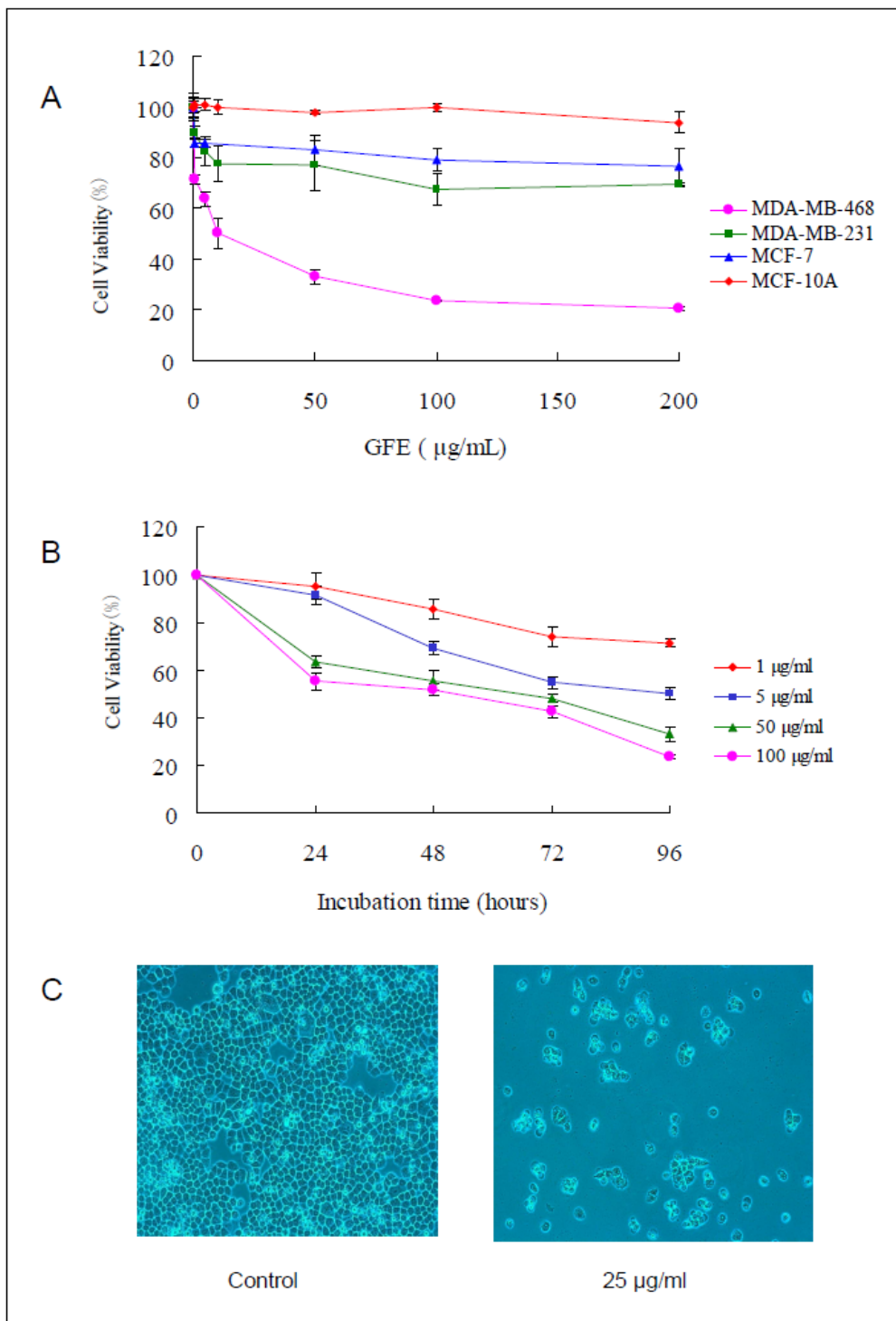


Figure 3.1 GFE treatment selectively inhibited growth of human BC cells.

A, Dose-dependent effects of GFE on ER- human BC cell lines, MDA-MB-231 and MDA-MB-468, and ER+ human BC cell lines, MCF-7 and MCF-10A, respectively. Cells were grown in experimental medium containing 5, 10, 25, 50, 100, or 200 $\mu\text{g}/\text{mL}$ of extract for 96 h. B, Time- and dose- effects of GFE on human BC MDA-MB-468 cells. Cells were grown in experimental medium containing 1, 5, 50, or 100 $\mu\text{g}/\text{mL}$ of GEF for 24, 48, 72 or 96 h treatment. Data are expressed as the percentage of control cells, mean \pm SEM. C, MDA-MB-468 cells treated with 0 and 25 $\mu\text{g}/\text{ml}$ GFE for 48 h. Cells were seeded at 5×10^4 cells/ml on a 48 well plate for 48 h with the GFE treatment of 0 and 25 $\mu\text{g}/\text{ml}$.

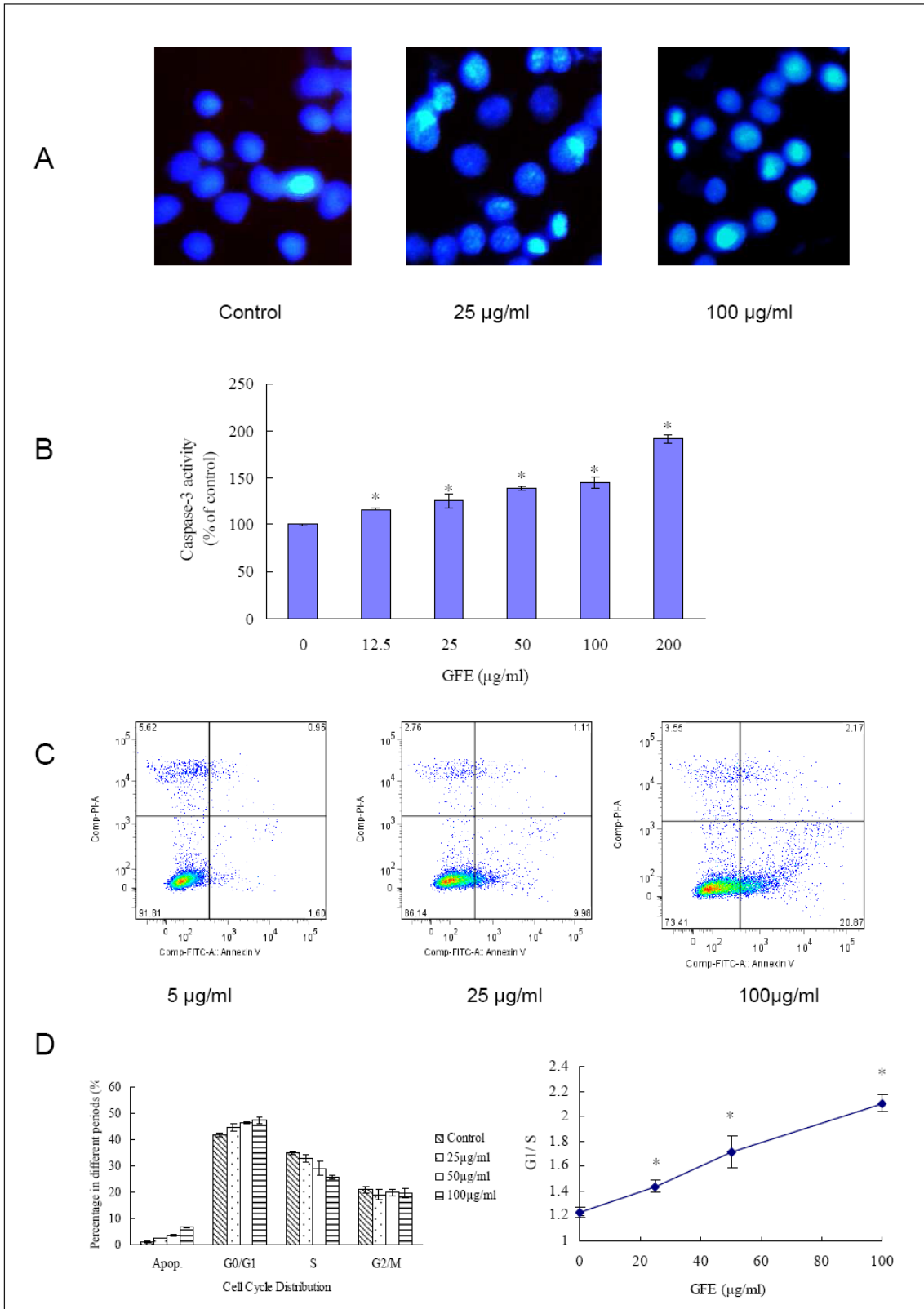


Figure 3.2 GFE treatment induced apoptosis in MDA-MB-468 BC cells

A, DNA-DAPI for apoptotic analysis of GFE influence on human BC MDA-MB 468 cells (seeded at 1×10^5 cells/mL). Cells were grown in the media containing 5, 25, 100 $\mu\text{g/mL}$ of GFE for 24 h. Fluorescence microscopy (60X) was performed in quadruplicate. B, Caspase-3 activity as a function of different concentrations of GFE. Cells were grown in the media containing 0, 12.5, 25, 50, 100, or 200 $\mu\text{g/mL}$ of GFE for 48 h. Data are expressed as a percentage of control cells, mean \pm SEM, and asterisks indicate a statistically significant difference compared to control cells ($P < 0.05$). C, GFE treatment (24h) induced apoptosis in MDA-MB-468 cells as detected by flow cytometry. MDA-MB-468 BC cells were incubated in medium supplemented with 10% FBS containing 0, 25, 100 $\mu\text{g/ml}$ GFE. After 24h, the cells were harvested, stained with Annexin V-FITC and PI, and analyzed by flow cytometry. The lower right quadrants represent the cells in the early stage of apoptosis. The upper right quadrants contain the cells in the late stage of apoptosis or necrosis. D, Cell cycle distribution after exposure to different concentrations of GFE. Cells were grown in experimental media containing 0, 25, 50, or 200 $\mu\text{g/ml}$ of GFE for 24 h. Data are expressed as a percentage of control cells, mean \pm SEM. Asterisks indicated a statistically significant difference compared to control cells ($P < 0.05$).

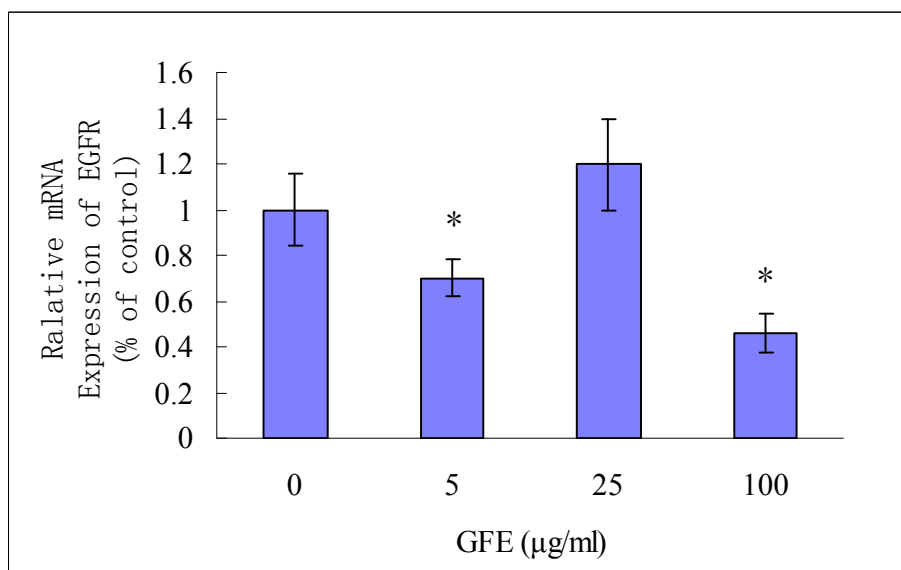


Figure 3.3 EGFR expression analyzed after treatments of GFE on human BC cells

Cells (1×10^6 cell/m) were grown in the media containing 0, 5, 25 or 100 µg/ml of extract for 48 h. EGFR expression was determined from each treatment in triplicate from 3 different cell passage experiments. Results were analyzed with SPSS software using ANOVA and Tukey's Highest Significant Difference test for multiple comparisons between groups. Mean percentages \pm SEM are presented. Treatments significantly inhibited cell proliferation compared to untreated controls ($p < 0.05$).

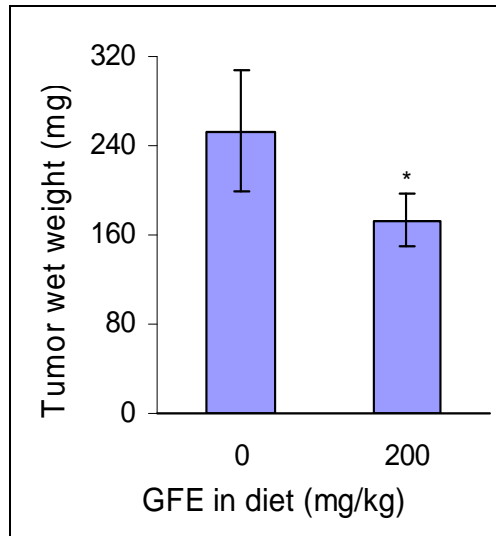


Figure 3.4 Effect of dietary GFE on the growth of MDA-MB-468 tumors implanted in athymic mice.

Female athymic mice (n=10) received MDA-MB-468 cell injection at 6-wk of age, and divided into two treatment groups; control (n=4) and 200 $\mu\text{g/g}$ diet GFE (n=6). After five weeks of dietary treatment, animals were sacrificed and individual tumor wet weight (15 tumors in the control group and 21 tumors in the GFE groups) was measured. Data are expressed as average tumor wet weight ($\text{g} \pm \text{SEM}$) for all tumors in each treatment. Bars with different letter are significantly different, $p < 0.05$.

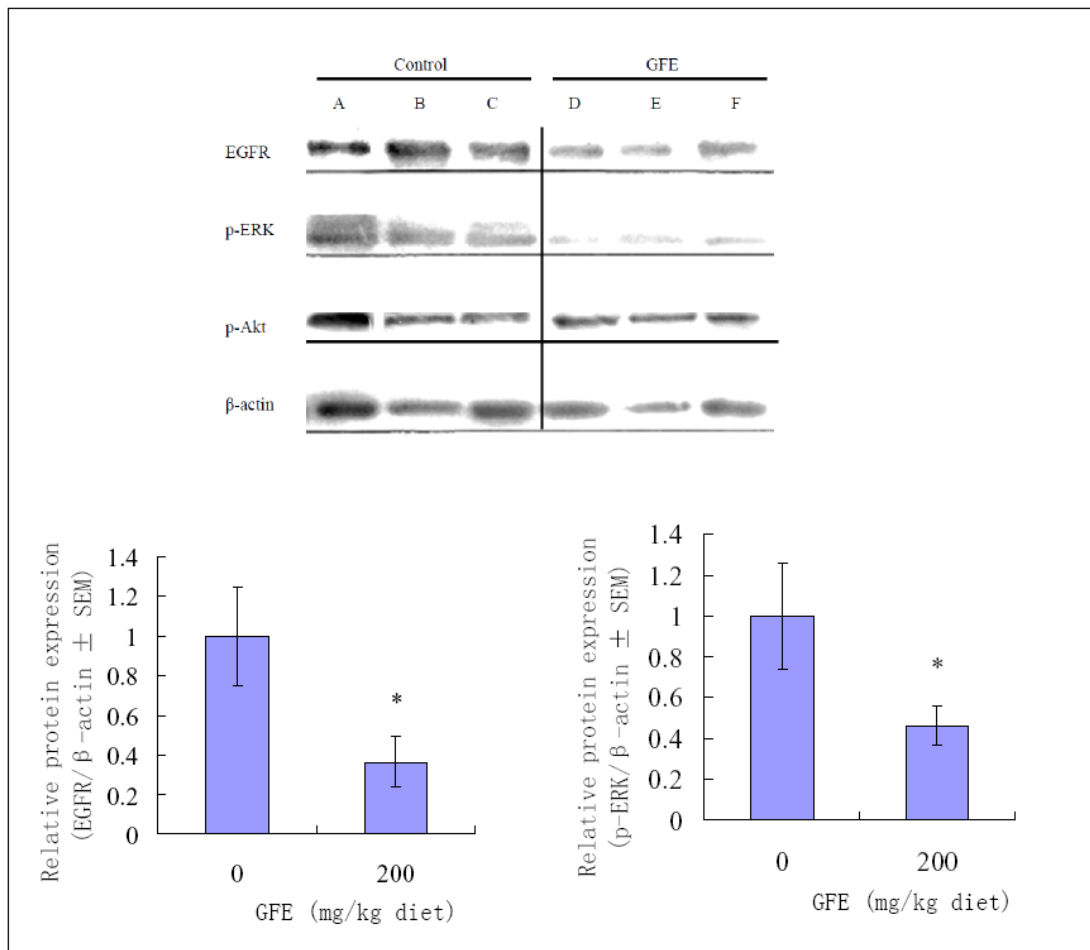


Figure 3.5 Effect of dietary GFE on EGFR and p-ERK protein expression in tumors.

Tumors (4 tumors in the control group (A, B, C) and 5 tumors in the GFE group (D, E, F)) were analyzed using Western blot analysis. Numbers on the Y axis represent the ratio of the target protein to the standard protein expression level; bars \pm SEM. β -actin was used as a standard for the quantity analysis. Bars with different letter are significantly different, $p < 0.05$.

Table 3.1 Analyte Concentration (pg/ml)

Cytokine	Control	GFE	Change%	p-value
IL-1 β	<57.2*	136.7 \pm 19.1	+ 139.0%	< 0.01
IL-6	35.8 \pm 1.7	22.0 \pm 0.9	- 38.5%	< 0.01
MCP-1	39.5 \pm 2.7	33.9 \pm 1.7	- 14.2%	< 0.05
IFN γ	32.7 \pm 2.9	38.1 \pm 2.9	+ 16.5%	< 0.05
RANTES	14.1 \pm 2.4	< 7.7 *	- 45.4%	< 0.05

* under the detection limit