Investigating the Biochemical Activity of Some Flavonoids, Coumarins and Modified Gold-Nanoparticles as Anti-Breast-Cancer Agents

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INVESTIGATING THE BIOCHEMICAL ACTIVITY OF SOME FLAVONOIDS,
COUMARINS AND MODIFIED GOLD-NANOPARTICLES AS
ANTI-BREAST-CANCER AGENTS

by

Amr Ezzat Mohamed Mahmoud

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Doctor of Philosophy
Department of Chemistry
Advisor: Ekkehard Sinn, Ph.D.

Western Michigan University
Kalamazoo, Michigan
August 2012
WE HEREBY APPROVE THE DISSERTATION SUBMITTED BY

Amr Ezzat Mohamed Mahmoud

ENTITLED Investigating the Biochemical Activity of Some Flavonoids, Coumarins,
and Modified Gold-Nanoparticles as Anti-Breast-Cancer Agents

AS PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF Doctor of Philosophy

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INVESTIGATING THE BIOCHEMICAL ACTIVITY OF SOME FLAVONOIDs, COUMARINS AND MODIFIED GOLD-NANOPARTICLES AS ANTI-BREAST-CANCER AGENTS

Amr Ezzat Mohamed Mahmoud, Ph.D.

Western Michigan University, 2012

Polyphenolic compounds are abundant natural products found in many plants as secondary metabolites. These compounds display many interesting pharmacological activities, which have motivated chemists over the years to explore them, and their synthetic derivatives, as potential drugs, especially anticancer drugs. Breast cancer is the most common cancer in women globally, and in the United States of America the estimated worldwide new breast cancer cases and the number of deaths are 1.38 million and 458 thousand, respectively. Currently in the USA there are 2.6 million women who have been treated for breast cancer, and the estimated number of deaths are 39,520 in 2012. Therefore, this work focuses on investigating the anti-breast cancer activity of three groups of compounds: five naturally occurring flavonoids, five synthetic coumarins, and modified gold nanoparticles. Their pro-apoptotic activities were investigated and their toxicity on normal breast cells tested. The anti-breast cancer activity investigation used four breast carcinoma cell lines MDA-MB-231, MCF-7, BT-474, and SK-BR-3. The primary screening for their ER and HER-2 status indicated that only the MCF-7 and BT-474 cell lines are ER
positive. Both BT-474 and SK-BR-3 express HER-2 protein. The anticancer activity assay MTT indicated that all the tested flavonoid compounds had anti-breast cancer activity and were not toxic on normal breast cells. The five synthetic coumarin compounds displayed variable toxicity from very toxic to moderately toxic. However, only two of them were not toxic to normal breast cells. Out of the third group of compounds, only one modified gold nanoparticle was promising as an anti-breast cancer agent without any toxicity against the normal breast cell. All of the promising anti-breast cancer agents were shown to have pro-apoptotic activities such as formation of apoptotic bodies and fragmentation of DNA.
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<td>Arase</td>
<td>Aromatase</td>
</tr>
<tr>
<td>Cox-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra Acetice Acid</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ES</td>
<td>Estrone Sulfatase</td>
</tr>
<tr>
<td>EStase</td>
<td>Estrone Sulfotranferase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GNPs</td>
<td>Gold nanoparticles</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HDase</td>
<td>17β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Inhibitory concentration for half of maximal activity</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>mg</td>
<td>Milli Gram(s)</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethythiazol-2yl)-2,5-Diphenyl Tetrazolium Bromide</td>
</tr>
<tr>
<td>µl</td>
<td>Micro Liter(s)</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar Concentration</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-Ribose) Polymerase</td>
</tr>
</tbody>
</table>
List of Abbreviations-Continued

PBS ................................................................. Phosphate Buffered Saline
PBST ................................................................ Phosphate Buffer Saline-Tween-20
RT ................................................................. Room Temperature
SDS ................................................................. Sodium Dodecyl Sulfate
SDS-PAGE .................................................. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TBE ................................................................. Tris borate EDTA
WHO .............................................................. World Health Organization
% ................................................................. Percent
CHAPTER 1

INTRODUCTION

1.1. Breast cancer

Cancer is the second leading cause of death in the United States, after heart disease, and one of the biggest health problems all over the world (Jemal, 2009). According to American Cancer Society statistics, in 2012 the estimated total new cancer cases of all types will be 1,638,910, resulting in 577,190 deaths (Siegel, 2012). Breast cancer is the most common cancer in women. The estimated worldwide new breast cancer cases and the number of deaths are 1.38 million and 458 thousand respectively (Jemal, 2011). Currently, there are 2.6 million US women who have been treated for breast cancer (Breast Cancer Facts & Figures, 2011). Furthermore, the estimated new breast cancer cases and the number of deaths in the USA are 229,060 and 39,920 respectively. Michigan has seen an increase in new breast cancer cases over time: 6,480, 7,340 and 7,710 in 2009, 2010 and 2011, respectively (Jemal, 2009; Jemal, 2011; Siegel, 2012).

1.2. Estrogens and breast cancer

Steroid hormones play a major role in the growth and development of normal breast tissues and breast cancer tissues as well. These hormones include
mineralocorticoids, glucocorticoids, progestins, androgens, and estrogens (Sanderson, 2006). Some 20-25% of breast tumor cases over-express Human Epidermal Growth Factor Receptor 2 (HER-2) and 75% of breast cancer growth is controlled by estrogens, including estrone, estriol, and 17β-estradiol (Tan, 1997). The local biosynthesis of estrogens in men occurs primarily in the testes then in bone. On the other hand, in mature and premenopausal women the biosynthesis occurs mainly in the ovaries. But, in postmenopausal women, the mesenchymal cells of the breast are the main source of estrogen biosynthesis and are responsible for breast cancer development (Simpson, 1999).

1.3. Estrogen biosynthesis

The biosynthesis of estrogen hormones starts in the theca cells, where cholesterol is the main precursor of estrogen hormones. This cholesterol (C27) precursor is converted to pregnenolone C21 by cytochrome P-450 through an enzymatic cleavage of the side chain (Simpson, 2000) (see figure 1).

![Figure 1.1. Cholesterol side-chain cleavage reaction.](image-url)
Pregnenolone is then converted to progesterone or 17-hydroxyprogesterone, and finally converted to androstenedione, C<sub>19</sub> steroid (Thomas, 2004). Androstenedione transfers to the granulosa cells, where it is converted to estrogens, C<sub>18</sub> by enzymatic stimulation. First, androstenedione is converted to estrone by aromatase and then estrone is converted to 17β-estradiol by 17β-hydroxysteroid dehydrogenase (Miki, 2007) (see figure 2).
One more important fact is that most estrone molecules are converted to estrone sulfate by estrone sulfotransferase, which is the important storage of estrone. Therefore, this enzyme plays an important role in controlling the estrone level in the body (see figure 3).

![Estrone Conversion](image)

*Figure 1.3. Estrone conversion to estrone sulfate.*

Since cancer cells can develop drug resistance which then applies not only for anticancer drugs but potentially also for the other drugs which have similar structures (Cabara, 1998). Consequently, there is an increasing demand for new anti-breast cancer drugs with high selectivity for the cancer cells only.

1.4. Breast cancer treatment

There are many factors that determine breast cancer treatment including the stage of the cancer, the estrogen receptor status *viz.* ER+ or ER-, the HER-2 status, the size of the breast cancer itself and finally whether it is just a tumor or metastatic breast cancer. Depending on all the previous factors breast cancer treatment would be one or more of the following: surgery, hormonal therapy, immunotherapy, radiation treatment and chemotherapy (Grube, 2001; Wyld, 2007).
1.4.1. Surgery

Surgery is the primary treatment of cancer which depends on the elimination of the cancerous tissues. This treatment may require treatments such as chemotherapy or radiation treatment before or after the surgery to reduce the tumor size or to stop the cancer growth, spread or reoccurrence (Malmstrom, 2003).

1.4.2. Hormone therapy

Hormone therapy is the treatment by which estrogen is blocked from binding to estrogen receptors or by inhibiting the estrogen biosynthesis process itself. This treatment is highly recommended for ER+ breast cancer cases (Puhalla, 2012; Zhan, 2011).

1.4.3. Immunotherapy

It is reported that 20 -25% of breast cancer patients are HER-2 positive which means that their cancer cells over express this protein on their cell surfaces (Tan, 1997). Many studies have indicated the ability of the immune system to fight this specific protein which regulates breast cancer growth. Herceptin (Trastuzumab) is a humanized monoclonal antibody approved by the Food and Drug Administration (FDA) in 1998 as a treatment for HER-2 over expressing breast cancer patients. Its ability to bind to HER-2 receptors can inhibit the breast cancer growth by stopping the growth signal pathway. (Kamal, 2011; Bhargava, 2001).
1.4.4. Radiation treatment

Radiation treatment has been used for decades to fight breast cancer. The high energy radiation kills the cancer cells by damaging the cell’s DNA. Furthermore, it is hard to focus the radiation on just the cancer cells and avoid damage to some normal cells. Radiation treatment is a critical treatment used at early stage of breast cancer growth to reduce the size of the breast tumor. And it is used after other treatments like surgery or chemotherapy to reduce the probability of reoccurrence (Frank, 2004; Reintgen, 2010).

1.4.5. Chemotherapy

The term chemotherapy in general refers to the use of any chemical or drug that treats a specific disease. For breast cancer chemotherapy, it means the use of chemicals as drugs to kill cancer cells selectively. It is the only available treatment for both ER negative and HER-2 negative patients (Bernard-marty, 2004).

1.5. The current approved FDA-breast cancer drugs

According to the National Cancer Institute, there are 47 FDA-approved breast cancer drugs on the USA market. These drugs may be prescribed individually or in combinations of two or more. The 47 US drugs have brand names as follow: Abitrexate , Abraxane, Adriamycin PFS, Adriamycin RDF, Adrucil, Anastrozole, Arimidex, Aromasin, Capecitabine, Clafen, Cyclophosphamide, Cytoxan, Docetaxel, Doxorubicin Hydrochloride, Efudex, Ellence, Epirubicin Hydrochloride, Exemestane, Fareston, Faslodex, Femara, Fluorouracil, Folex, Folex PFS, Fulvestrant, Gemcitabine Hydrochloride, Gemzar, Ixabepilone, Ixempra, Lapatinib Ditosylate, Letrozole,

1.6. Known side effects of the current FDA-approved breast cancer drugs

There are no drugs that are totally selective for cancer cells; healthy cells are affected also. Therefore, currently chemotherapy is a very painful treatment and the treatment can take up to five years. The long treatment period and the significant pain cause many people not to complete their treatment course. Breast cancer treatments side effects are variable and depend on the cumulative dose, the patient age, stage of the cancer itself and presumably on the nature of the drug as well. The common side effects vary from nausea, dry mouth, hot flashes, chest pain, cognitive dysfunction, up to premature ovarian failure because of the loss of estrogen production or release of eggs. The most risky side effects are bone loss and cardiac toxicity (Hurria, 2003; Chargari, 2011; Schimmel, 2004). Even with the most selective anti-breast cancer drugs which target specific a protein, Trastuzumab and Lapatinib are associated with cardiac failure (Schimmel, 2004; Chargari, 2011).
1.7. References


CHAPTER 2

BIOCHEMICAL INVESTIGATION OF FIVE FLAVONOIDs AS ANTI-BREAST CANCER AGENTS

2.1. Introduction

Flavonoids are an extensive class of naturally occurring poly-phenolic compounds found in many plants as secondary metabolites. This group of compounds includes isoflavones, flavanones, flavones, flavonols and flavanols (Fig. 2.1) (Chang 2008; Cermak 2001). Many biochemical activities of this class of compounds have been discovered including anti-inflammatory activity (Zhang 2008; Govindarajan 2007; Garcia-Lafuente 2009; Ramirez-Cisneros 2012), antiviral activity (Wu 2010; Tait 2006; Chiang 2003; Berezin 2002), antibacterial activity (Yao 2011; Rigano 2007), and antifungal activity (Salas 2011; Alcerito 2002). Flavonoids also have the important cancer preventive activity as antioxidants that neutralize the free radicals produced during respiration. Free radicals have the capability to damage many important cellular components, such as DNA, protein and lipids (Hamilton 2001; Peng 2003; Abraham 2008). Finally, the most important biological activity for this study is the activity against different types of cancer (Galati 2004; Li 2007; Rao 2005; Santos 2011). This class of naturally occurring secondary metabolites has been given more attention because of their anti-estrogenic activities, which are critical for the treatment of breast cancers are hormone-dependent (Watanabe 2002). They play an important inhibitory role against
cancer growth regulators such as estrogens, aromatase, estrone sulfatase and cyclooxygenase-2 enzymes.

Figure 2.1. Chemical structure of flavonoids.
2.1.1. Antioxidant activity of flavonoids

The free radicals produced during normal metabolic processes like breathing have been known to be associated with high risk intercellular environment. These radicals can damage important cell’s components like DNA, proteins and lipids. There are many cellular protecting components that can eliminate these radicals such as ascorbic acid, vitamin E, glutathione, glutathione peroxidase, superoxide dismutase, catalase (Cand 1989). The free radicals accumulation during life are known to lead to many diseases such as arthritis, neurodegenerative disorders, atherosclerosis and cancer (Dreher 1996). Flavonoids as natural phenolic constituents extracted from medicinal plants, vegetables and fruits have received great attention as antioxidant. Flavonoids acquired this attention because their important role in scavenging and neutralization free radicals (Pittella 2009). Free radical scavenging is not the only way flavonoids can fight cancer. They have the ability to mimic natural estrogens and even to inhibit aromatase enzymes which have critical role controlling hormone-dependent cancers like prostate and breast cancer (Hodek 2002).

2.1.2. Inhibitory activity of flavonoids against breast cancer

Aromatase enzyme level has been consistently observed to be higher in breast tumor cells and metastatic breast cancer tissues than in normal breast cells (Chetrite 2000; Irahara 2006). Tamoxifen has been used for many years as hormonal therapy against estrogen positive breast cancer. This anti-breast cancer drug used to block the interaction of estrogens and their receptors (Van de Velde 2010). The standard usage of tamoxifen for five years treatment period was the main reason for the occurrence of
patient resistance (Riggins 2007). Because of the critical role of aromatase enzyme in the biosynthesis of estrogen, it was an alternative treatment for breast cancer. Flavonoids have been shown to have anti-aromatase and anti-estrogen activity. The structure similarity between flavonoids and the estrogen skeleton enables flavonoids to bind the estrogen receptors, modulating or inhibiting their activity. More important is the anti-aromatase activity of flavonoids helped to modulate the hormone-dependent cancers, such as prostate and breast cancer (Hodek 2002; Martin 2003).

The anti-breast cancer activity of three flavonoids, quercetin, myricetin and epicatechin had been investigated previously using the MCF-7 cell line. Quercetin decreased cell viability by 50% at 200 μM. But myricetin and epicatechin had very little efficacy. Also, quercetin was found to be more potent in decreasing the level of protein contents of the cells and it inhibited the synthesis of the RNA and DNA of the MCF-7 breast cancer cells (Rodgers 1998).

Apigenin was shown to have anti-proliferation activity against the ER-negative breast cancer cell line SK-BR-3 at higher concentration 100 μM. It induced apoptosis and caused cell cycle arrest at the G2/M transition. Also, at the above concentration apigenin decreased growth regulators such as CDK1, and cyclin A and B, which regulate cells growth at G2 phase. The results indicated that there is no detectable change in CDK2 and CDK4 which regulate cells growth in G1 phase (Choi 2009).

The anti-breast cancer activity of ten flavonoids was investigated using the MDA-MB-231 cell line. Myricetin, catechin, and naringenin were the least cytotoxic with IC_{50} at > 200 μM. Luteolin, and apigenin were the most effective with IC_{50} at < 20 μM.
Genistein, daidzein, genistein, chrysin, kaempferol, and quercetin have cytotoxicity with IC₅₀ ranging from 40 µM to 179 µM (White 2012).

Therefore, the main objective of this work focuses on investigating the anti-breast cancer activity of five naturally occurring flavonoids. Furthermore, to investigate their pro-apoptotic activities such as apoptotic bodies formation and DNA fragmentation and test their toxicity on normal breast cells.

2.2. Materials and methods

2.2.1. Reagents and antibodies

All reagents and solvents were purchased from Sigma-Aldrich Co. (St Louis, MO), unless stated otherwise. Antibodies against β-Actin, HER-2 and the estrogen receptor (ER), were purchased from Cell Signaling Technology (Beverly, MA) for use in Western blot analysis. Western blot and Hoechst 33342 nucleic acid stain were purchased from Invitrogen Life Technologies (Grand Island, NY). Cell proliferation Kit I (MTT) and the apoptotic DNA-ladder Kit were purchased from Roche Applied Science (Indianapolis, IN).

2.2.2. Cell lines and culture conditions

Four breast adenocarcinoma cell lines (MDA-MB-231, MCF-7, BT-474 and SK-BR-3) were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). All cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Grand Island, NY) supplemented with 10% v/v fetal bovine serum, 1% v/v L-glutamine, 1% v/v antibiotic penicillin-streptomycin solution (Sigma-
Aldrich) and 0.37% v/v sodium bicarbonate. All cells were incubated at 37 °C and under 5 % CO₂ in a humidified Fisher Scientific incubator. All cells received fresh media 2 to 3 times/week and were sub-cultured when they reached 70-80% confluence. First, the culture medium was removed and the adherent cells were washed with 1X versene (Invitrogen, 0.2 g/L EDTA.4Na in phosphate-buffered saline). Then the cells were detached using 1X trypsin-EDTA (Invitrogen, 0.25% trypsin, 0.1% EDTA, pH 7.2-8), followed by 2-5 minutes incubation at 37°C and 5% CO₂ in a humidified incubator. Cells were re-suspended in fresh media and counted with a hemocytometer using trypan blue dye (0.47% in phosphate-buffered saline) and seeded in new tissue culture treated flasks or 96 well microplates.

2.2.3. Cellular anti-proliferation activity using the MTT cytotoxicity assay

The effects of five flavonoids, myricetin (compound A), quercetin (compound B), luteolin (compound C), apigenin (compound D), and kaempferol (compound E) on cell proliferation in breast adenocarcinoma cell lines MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 were measured by using the 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay (MTT). The anticancer activity was tested using various concentrations of the flavonoid compounds at 25, 50, 100, 200, 300 μM of each compound. Each treatment concentration was performed in triplicate and the whole experiment was repeated 3 to 5 times, as described previously (Chen, 2009; Xiong, 2010; Chae, 2011; Goel, 2011; Zhou, 2011). The cells were grown in 96-well, flat bottom, tissue culture treated microplates, at the density of 1 x 10⁴ for 2 days at 37°C and under 5% CO₂ in a humidified incubator to promote cellular adhesion. The flavonoid compounds were dissolved in DMSO and
diluted in complete media to a final concentration of 1% to the desire testing concentration. After the incubation period, old media was removed and the adherent cells were first washed by 1X versene then 200 µl new complete media with the compounds were incubated in the same condition. Control wells received only the media and DMSO. MTT salt solution (20 µl) at a final concentration of 0.5 mg/ml was added to each well and incubated for a period of 5 h. After the incubation period, the whole solution in each well was replaced with 200 µl of DMSO to dissolve the formed formazan crystals. The optical density of each well was read at 560 nm and reference wavelength at 650 nm on a Tecan infinity F500 microplate reader (Tecan System Inc., San Jose, CA, USA).

The % of cell viability = \frac{OD \text{ of treated cells}}{OD \text{ of control cells}} \times 100

2.2.4. Morphological examination of apoptotic changes

The morphological apoptotic changes were examined using the chromatin dye staining protocol as described previously (Chen, 2009). Four breast adenocarcinoma cell lines MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 were grown in complete media of DMEM/F12 in a chamber slide, Thermo Scientific (Rochester, New York). All cells were incubated for 2 days at 37 °C and 5 % CO₂. When cells reached 50% confluence, the old media was removed and the adherent cells were first washed with 1X versene. Then 300 µl of new complete media with the compounds (1%) were added and incubated for 2 more days in the same condition. The control chambers received only the media and DMSO (1%). After the incubation period, the culture medium was removed and the adherent cells were washed with PBS (contain: 0.9 mM CaCl and 0.9 mM MgCl₂). And then cells were stained with the chromatin dye Hoechst 33342 (2 µg/ml) at 37 °C for 30
min. The cells were examined using fluorescence microscopy equipped with a blue filter (Leica DM5500 B microscope, Leica Microsystems). Apoptotic cells were identified on the basis of nuclear morphology changes, such as chromatin condensation, cell shrinking, apoptotic bodies formation, and nuclear fragmentation.

2.2.5. Apoptotic DNA analysis

2.2.5.1. DNA isolation

DNA fragmentation analysis was carried out using the apoptotic DNA ladder kit (Roche) using the following protocol: MDA-MB-231, breast carcinoma cell line was grown in complete media of DMEM/F12 in 25 cm² tissue cultural (TC) treated flasks. All cells were incubated for 2 days at 37 °C under 5 % CO₂. When the cells reached 50% confluent, the old media was removed and the adherent cells were first washed by 1X versene. Then new complete media with the compounds (1%) were added and incubated for 2 more days in the same condition. The control flasks received only the media and 1% DMSO. After the incubation period, the culture medium was removed and the adherent cells were washed with 1X versene then the cells were detached using 1X trypsin-EDTA. Afterward, the cells were centrifuged at 5000 RPM at 4 °C for 10 min. After the centrifugation period and the removal of the supernatant, cells were resuspended in 200 µl PBS then mixed with 200 µl of lysis buffer and incubated at RT for 20 min. An equal amount of isopropanol was added and vortexed vigorously for 5 minutes to precipitate the DNA. The precipitated DNA was collected using filter tubes and washed two times with the kit washing buffer. The DNA was eluted using 200 µl pre-warmed (+ 70°C) elution buffer. Then the DNA was stored at -20 °C for later analysis.
2.2.5.2. DNA fragmentation analysis using agarose gel electrophoresis

Apoptotic DNA fragmentation is the hallmark of apoptotic cells. The negatively charged DNA fragments migrate on agarose gel from negative to the positive in an applied electrical field. This DNA electrophoresis was carried out according to the manufacturer’s protocol as follows:

First, 20 µg of DNA sample was mixed with 2 µl of loading buffer (0.1 g SDS, 25 mg bromophenol blue, 7 ml redistilled water and 3 ml glycerol) and 5 µl of DNA marker were loaded in 1% agarose gel (0.4 g agarose in 40 ml Tris borate EDTA and 5 µl of ethidium bromide solution). The gel was allowed to run at 124 volts for 1h in 1x TBE buffer (5.4g Tris, 2.8g boric acid, 2 ml 0.5M EDTA solution and completed to 1000 ml, pH 8.0). The DNA fragmentation was visualized using UV light and Ethidium Bromide dye.

2.2.6. Protein isolation and Western blotting

2.2.6.1. Protein extraction

Cells were seeded in 25 cm² TC treated flasks, and were incubated at 37 °C under 5 % CO₂ in a humidified incubator until 70 to 80% confluent. The culture medium was removed and the adherent cells were washed with 1X versene then the cells were detached using 1X trypsin-EDTA. Afterward, cells were centrifuged at 5000 RPM at 4 °C (centrifuge machine). After the centrifugation period and the removal of the supernatant cells were mixed with lysis buffer then kept in ice for 30 min. Cells were sonicated for 30 seconds then heated to 95-100 °C for 5 min then cooled in ice. The
supernatant that contained protein was then obtained after centrifugation for 5 min and kept in – 80 °C until use.

2.2.6.2. Determination of protein concentration

Protein concentration was determined using the Pierce BCA Protein Assay Kit, Thermo Scientific (Rockford, Illinois). First, the standard protein curve was determined using bovine serum albumin (BSA) (2mg/ml) diluted with distilled water for the final concentration of 2000, 1500, 1000, 750, 500, 250, 125, 25 and 0 μg/ml. Working BCA solution was freshly prepared by mixing 50 parts of BCA reagent A with 1 part of reagent BCA reagent B which produced a green solution. The determination of protein concentration in the standard and protein samples was carried out by mixing 25 μl of each of them separately with 200 μl of the working solution in 96 well plates. After incubation at 37 °C for 30 min, the absorbance was measured at 562 nm on a Tecan infinity F500 microplate reader. Protein concentration was then determined using the standard curve.

2.2.6.3. SDS-PAGE electrophoresis

Equal amounts of protein (20 μg) samples were subjected to electrophoresis using 4-12% Bis-Tris gels (Invitrogen). First, the 4x sample buffer (Invitrogen) was added to each sample and heated at 100 °C for 2 min. Then 20 μl of each protein sample and 10 μl of pre-stained protein marker (Invitrogen) were loaded on the gel using 1x running buffer (Invitrogen) and run at 100v for 50 min.
2.2.6.4. Transfer of proteins to nitrocellulose membrane

The proteins were transferred from the SDS-PAGE gel to the nitrocellulose membrane according to the Invitrogen Western Blotting protocol using a XCell SureLock™ Mini-Cell & Cell II™ Blot Module unit. The trans-blotting sandwich was assembled in the following way: cathode core side (-) 3 Sponge pads, filter paper, gel, nitrocellulose membrane 0.45 μm, filter paper and 3 sponge pads, anode core side (+). All trans-blotting sandwich contents were kept wet all the time and air bubbles were completely and carefully removed from the sandwich before trans-blotting. This sandwich was oriented so that the protein would migrate toward the positive core side. The transfer was run for 90 min at 30 volts using 1x transfer buffer in the buffer core and distilled water in the buffer chamber.

2.2.6.5. Blocking of non-specific sites and blotting the membrane

After transferring, the membrane was first soaked in 0.1% phosphate buffered saline with Tween-20 (PBST; PBS, 0.1 % Tween-20) with shaking for 20 min. Then the membrane was blocked using 5% nonfat dry milk in PBST (Blotto solution) at RT with shaking for 1 h. The membrane was incubated with primary antibodies of HER-2, ER, and β-actin at 1:1000 in Blotto solution at 4 °C overnight. Then the membrane was washed three times with 0.1% PBST, for 20 min each time. The membrane was then incubated with horseradish peroxidase (HRP) secondary antibody at 1:2000 dilution and HRP-conjugated anti-biotin antibody at 1:1000 dilution in Blotto. After a 1 h incubation period with the secondary antibodies, the membrane was washed four times with 0.1% PBST 15 min each.
2.2.6.6. Chemiluminescent detection of protein

The membrane was incubated with the chemiluminescent substrate solution of reagent A and B 5% each in Milli-Q water for 1 min at RT (Invitrogen kit). The membrane was exposed to x-ray film (Kodak BioMax Light Film, Sigma-Aldrich) for 1 min then developed in Dektol developer (Sigma-Aldrich) and fixed using Kodak fixer (Sigma-Aldrich).

2.2.7. Statistical analysis

The results for the cell proliferation assay are expressed as mean ± standard deviation (SD) for three replicates. The statistical difference was determined by using the Enova test. P-values of less than 0.05 were considered statistically significant. IC\textsubscript{50} values were determined using Excel.

2.3. Results

The biochemical activities of five flavonoids (myricetin, quercetin, luteolin, apigenin, and kaempferol) have been investigated as anticancer agents against four breast carcinoma cell lines: MDA-MB-231, MCF-7, BT-474 and SK-BR-3.

2.3.1. Characterization of the breast cancer cell lines using Western blotting

The HER-2 and estrogen receptor (ER) status of the four breast cancer cell lines was determined using Western blot analysis. The BT-474 and SK-BR-3 cell lines show strong bands of similar intensity when probed for the HER-2 protein whereas MDA-MB-231 and MCF-7 cell lines did not show any detectable HER-2 expression (Fig. 2.2). On
the other hand, MCF-7 and BT-474 cell lines showed high ER protein expression, but MDA-MB-231 and SK-BR-3 had scarcely detectable ER protein bands (Fig. 2.3). All the breast cell lines showed similar β-Actin protein expression (Fig. 2.4).

*Figure 2.2. Western blot analysis of HER-2 in the breast cancer cell lines. MDA-MB-231 (A), MCF-7 (B), BT-474 (C) and SK-BR-3 (D) cells. Cells were harvested and proteins were separated using 4-12 % SDS-PAGE, transferred to nitrocellulose membrane and probed with HER-2 antibody as described in section 2.2.7.*
Figure 2.3. Western blot analysis of ER in the breast cancer cell lines. MDA-MB-231 (A), MCF-7 (B), BT-474 (C) and SK-BR-3 (D) cells. Cells were harvested and proteins were separated using 4-12 % SDS-PAGE, transferred to nitrocellulose membrane and probed with ER antibody as described in section 2.2.7.
Figure 2.4. Western blot analysis of β-actin in the breast cancer cell lines. MDA-MB-231 (A), MCF-7 (B), BT-474 (C) and SK-BR-3 (D) cells. Cells were harvested and proteins were separated using 4-12% SDS-PAGE, transferred to nitrocellulose membrane and probed with β-Actin antibody as described in section 2.2.7.
2.3.2. Anti-proliferation activity of the five flavonoids on the four breast cancer cell lines

The first cytotoxicity screening activity of the five flavonoids did not show any activity against the four breast cancer cell lines after a 24 h treatment. The anti-proliferation investigation for these compounds indicated that these flavonoids have a good inhibition efficacy on the breast cancer cells after a 48 h treatment. All these compounds showed time-dependent and dose-dependent suppression of proliferation in all the breast cancer cell lines. Compound A suppressed the growth of the four breast cancer cell lines MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC_{50} at 111, 208, 192 and 139 μM respectively (Fig. 2.6, 8, 10, 12). Compound B inhibited the growth of MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC_{50} at 40, 49.5, 291 and 145 μM respectively (Fig. 2.14, 16, 18, 20). Compound C suppressed the growth of the four breast cancer cell lines MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC_{50} at 50, 81, 90 and 75 μM respectively (Fig. 2.22, 24, 26, 28). Apigenin selectively suppressed the first two cell lines MDA-MB-231 and MCF-7 at the concentration of 100 μM. However, it requires a higher concentration of 200 μM to have offsets on the BT-474 cell line and 300 μM for the SK-BR-3 cell line. Apigenin showed suppression of proliferation in these four breast carcinoma cell lines, MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC_{50} at 57, 38, 61 and 46 μM, respectively (Fig. 2.30, 32, 34, 36). Kaempferol suppressed the growth of the four breast cancer cell lines MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC_{50} at 100, 90, 98 and 141 μM respectively (Fig. 2.38, 40, 42, 44).
Figure 2.5. The effect of compound A on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound A for 24 h.

Figure 2.6. The effect of compound A on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound A for 48 h.
Figure 2.7. The effect of compound A on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound A for 24 h.

Figure 2.8. The effect of compound A on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound A for 48 h.
Figure 2.9. The effect of compound A on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound A for 24 h.

Figure 2.10. The effect of compound A on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound A for 48 h.
Figure 2.11. The effect of compound A on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound A for 24 h.

Figure 2.12. The effect of compound A on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound A for 48 h.
Figure 2.13. The effect of compound B on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound B for 24 h.

Figure 2.14. The effect of compound B on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound B for 48 h.
Figure 2.15. The effect of compound B on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound B for 24 h.

Figure 2.16. The effect of compound B on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound B for 48 h.
Figure 2.17. The effect of compound B on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound B for 24 h.

Figure 2.18. The effect of compound B on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound B for 48 h.
Figure 2.19. The effect of compound B on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound B for 24 h.

Figure 2.20. The effect of compound B on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound B for 48 h.
Figure 2.21. The effect of compound C on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound C for 24 h.

Figure 2.22. The effect of compound C on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound C for 48 h.
Figure 2.23. The effect of compound C on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound C for 24 h.

Figure 2.24. The effect of compound C on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound C for 48 h.
Figure 2.25. The effect of compound C on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 µM of compound C for 24 h.

Figure 2.26. The effect of compound C on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 µM of compound C for 48 h.
Figure 2.27. The effect of compound C on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound C for 24 h.

Figure 2.28. The effect of compound C on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound C for 48 h.
Figure 2.29. The effect of compound D on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound D for 24 h.

Figure 2.30. The effect of compound D on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound D for 48 h.
Figure 2.31. The effect of compound D on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound D for 24 h.

Figure 2.32. The effect of compound D on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound D for 48 h.
Figure 2.33. The effect of compound D on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 µM of compound D for 24 h.

Figure 2.34. The effect of compound D on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 µM of compound D for 48 h.
Figure 2.35. The effect of compound D on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound D for 24 h.

Figure 2.36. The effect of compound D on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound D for 48 h.
Figure 2.37. The effect of compound E on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound E for 24 h.

Figure 2.38. The effect of compound E on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound E for 48 h.
Figure 2.39. The effect of compound E on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound E for 24 h.

Figure 2.40. The effect of compound E on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound E for 48 h.
Figure 2.41. The effect of compound E on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound E for 24 h.

Figure 2.42. The effect of compound E on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound E for 48 h.
Figure 2.43. The effect of compound E on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound E for 24 h.

Figure 2.44. The effect of compound E on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound E for 48 h.
2.3.3. Morphological studies

2.3.3.1. The effect of flavonoids on apoptosis

According to the cell anti-proliferation results, more studies were needed to explore the effect of the tested compounds on the MDA-MB-231 cell line apoptotic pathway. Here apoptosis is a term used to describe the cellular changes of dying cells. Recently, it has been defined as a programmed cell death (Vinatier 1996). These morphological changes include nuclear DNA fragmentation, chromatin condensation, formation of apoptotic bodies and cell shrinkage. (Raffray 1997).

The main morphological features of apoptosis are the formation of apoptotic bodies and nuclear DNA fragmentation. (Chen 2009) These changes can be experimentally observed using specific chromatin dyes such as Hoechst 33342, a stain which enables detection of apoptotic morphological changes using a fluorescence microscope with a blue filter.

Apoptotic morphological changes were determined at the concentration of 100 \( \mu \text{M} \) of each compound. Then the cells were stained in Hoechst for 30 min in dark. The morphological changes observed in this study were similar to the changes produced by 20 \( \mu \text{g/ml} \) crude methanolic extract of the pericarp of *Garcinia mangostana* after a 48h treatment (Moongkarndi *et al.*, 2004). The morphological alterations of the five flavonoids are illustrated in figure 2.45.
Figure 2.45. Morphological and cellular changes induced by the tested flavonoid compounds. The effects of these flavonoids on cell morphology were tested with fluorescent chromatin dye Hoechst 33342 in MDA-MB-231 cells. Cells were treated with 5 flavonoids (myricetin A, querectin B, luteolin C, apigenin D and kaempferol E) for 48 h and then stained with the chromatin dye for 30 min. The two panels are MDA-MB-231 cells, the top panel is a control without any treatment and the lower panel is for the treated cells.
2.3.4. Apoptotic DNA fragmentation analysis

Apoptotic DNA fragmentation is the hallmark of apoptotic cells. This analysis was carried out using the apoptotic DNA ladder kit (Roche). The DNA fragmentation effects of the five flavonoids on the MDA-MB-231 cell line were studied. The cells were treated for 48 h with 100 μM of each compound. Then, 20 μg DNA of each treatment was subject to 1% agarose gel electrophoresis. The DNA electrophoretogram (Fig. 2.46) indicates that neither myricetin nor quercetin have any DNA fragmentation effects (lane 4, 5). However, clear DNA fragments are shown for all of luteolin, apigenin and kaempferol treatments after 48h at the same concentration (lanes 6, 7 and 8).

Figure 2.46. Electrophoretogram demonstrating the apoptotic DNA fragmentation analysis of five flavonoids on MDA-MB-231 Cells. Cells were treated for 48 h with 100 μM of the Compounds. 20 μg DNA of each treatment was subject to 1% agarose gel electrophoresis. DNA Marker (lane 1), positive control (lane 2), untreated cells (lane 3), treated cells with Myricetin (lane 4), treated cells with Quercetin (lane 5), treated cells with Luteolin (lane 6), treated cells with Apigenin (lane 7) and treated cells with Kaempferol (lane 8).
2.4. Discussion

Plant secondary metabolites and their derivatives have played an important role in drug development especially over the last 50 years. Out of the 155 new FDA approved anticancer drugs there were 47% natural products or natural products derivatives (Newman 2007). Phenolic compounds are the most plentiful constituents and broadly distributed in the plant kingdom (Shi 2003). Flavonoids are a large class of polyphenolic compounds found in many plants as secondary metabolites (Chang 2008; Cermak 2001). They have a wide range of pharmaceutical activities including, anti-inflammatory activity (Zhang, 2008; Govindarajan 2007; Garcyia-Lafuente 2009; Ramirez-Cisneros 2012), antiviral activity (Wu 2010; Tait 2006; Chiang 2003; Berezin 2002), antibacterial activity (Yao 2011; Rigano 2007), antifungal (Salas 2011; Alcerito 2002), antioxidant activity (Hamilton 2001; Peng 2003; Abraham 2008) and anticancer activities (Galati 2004; Li 2007; Rao 2005; Santos 2011). The anti-estrogenic activity of flavonoids is very critical for the treatment of breast cancer (Watanabe 2002).

This study is to investigate the anti-breast cancer activity of these five flavonoids myricetin, quercetin, luteolin, apigenin, and Kaempferol on three metastatic breast cancer cell lines MDA-MB-231, MCF-7, and SK-BR-3 and one BT-474 non-metastatic breast cancer cell line in a single experiment. Western blot results indicated that both BT-474 and SK-BR-3 are positively expressing HER-2 protein which means they are depending on this protein on their growth and proliferation (Chen 2009). But, MDA-MB-231 and MCF-7 cell lines do not depend on HER-2 protein for their growth. On the other hand, both of MCF-7 and BT-474 cell lines expressed estrogen receptor protein. This is noteworthy because expression of these two proteins HER-2 and ER is correlated with
the efficacy of the tested flavonoids against each breast cancer cell line. Neither MDA-MB-231 nor SK-BR-3 showed any ER expression. These results are completely similar to the characterization done by Chen (Chen, 2009). The previous oncology studies reported the activity of some of these flavonoids, but they were only on one or two breast cancer cell lines involved in this study. Consequently, it was difficult to compare their results on all the metastatic and non-metastatic breast cancer cell lines. Additionally, some of them are ER positive and others are HER-2 positive, but BT-474 is positive for both ER and HER-2. Accordingly, one experiment studying the effects of all these factors is highly necessary for clarify the anti-breast-cancer activity of these flavonoids.

The goal of this study is to test the cytotoxicity of the five flavonoids on different breast cancer cell lines in one experiment. And, do some apoptotic characterizations studies using these flavonoids and breast cell lines. All the anti-proliferation activity assay results indicated that all the five flavonoids did not show any notable cytotoxicity activity against the breast cancer cells after just 24 h of treatment. The MTT results showed time-dependent and dose-dependent inhibition by the tested compound against the four breast cancer cell lines.

Both MDA-MB-231 and MCF-7 cell lines required lower dosages of the tested compounds compared to the BT-474 and SK-BR-3 cell lines. This may be because flavonoids have a structure similar to that of estrogen. Flavonoids are known to be effective against ER-positive breast cancer cell lines (So, 1997). Additionally, both BT-474 and SK-BR-3 over express HER-2 and depend on this protein for their growth.

The MTT assay results indicated that BT-474 cell line is the most resistant to the tested compounds, requiring higher concentrations to shown cytotoxicity effects.
This may be because it is the only cell line that over-expresses both HER-2 and ER proteins, and therefore may have different pathways maintain its growth.

The cytotoxicity results we present here are similar to those obtained by (Chang 2008) for all of the compounds except luteolin, which required more concentration to show similar activity. However, luteolin results were in agreement with those of (Phromnoi, 2009).

Apigenin showed the lowest IC₅₀ of 38, 61, and 48 µM against MCF-7, BT-474, and SK-BR-3. By comparing the cytotoxicity of all of myricetin, quercetin and kaempferol, and the cytotoxicity of apigenin, it is seen that these have lower activity by 2 to 4 fold that of apigenin. It has been proposed that there are three major structural features that control the cytotoxicity of flavonoids; the C2-C3 double bond, the C3-hydroxyl group and the extent of hydroxylation (Plochmann 2007). All the flavonoids in this study have the same C2-C3 double bond (see figure 2.1). Increasing the number of hydroxyl groups in flavonoids decreases the anticancer activity. Also, the presence of hydroxyl group at C3 position decreases the activity (Plochmann, 2007). According to what was reported in previous literature and our MTT results, these previous two structural features, confirmed that apigenin is more active than the other flavonoids.

More investigations were required to explore the cytotoxicity of these flavonoids on the MDA-MB-231 cell line in particular. Both the studies of apoptotic morphological changes with fluorescent chromatin dye Hoechst 33342 and DNA fragmentation tests showed that flavonoid compounds were cytotoxic to the MDA-MB-231 breast cancer cell line, which clearly showed apoptotic bodies and DNA fragmentation.
2.5. Conclusion

- Flavonoids have anti-breast-cancer activity against both metastatic and non-metastatic cell lines.
- Flavonoids are more active against ER positive breast cancer cell lines.
- Breast cancer cell lines that overexpress both HER-2 and ER are more resistant, require higher concentrations of flavonoids to achieve cytotoxic effects.
- Flavonoids were shown to have pro-apoptotic activity, including formation of apoptotic bodies and fragmentation of DNA.
- Apigenin is the most effective anti-breast-cancer compound. We propose that this is because it has no hydroxyl group at C3 position.
- Myricetin and quercetin are the least active flavonoids. We propose that this is because they have more hydroxyl groups and, in particular have one at the C3 position.
2.6. References


Haza, A. I.; Coto, A. L. and Morales, P. Comparison of the Ability of Myricetin and Quercetin to Modulate the Oxidative DNA Damage Induced by Heterocyclic Amines. *FNS.* 2011, 2, 356-365.


3.1. Introduction

Coumarins are a widely known, large group of natural and synthetic benzopyranic derivatives that have a wide range of pharmacological activities (Thati, 2007; Reddy, 2004). Coumarin derivatives have been reported to have antioxidant activity (Zhang, 2011), anti-inflammatory (Kang, 2009), anti-tumoral activity (Chimichi, 2002), and anticancer activity against many types of cancer, such as pancreatic, colon, lung, breast, and leukemia (Devji, 2011; Saidu, 2012; Sashidhara, 2010; Riveiro, 2009). Also, coumarin compounds have a lot of other biological activities including, anti-microbial, anti-hepatitis C virus, and anti-coagulant activities (Smyth, 2009; Hwu, 2008; Weigt, 2012). Coumarin compounds are also used as early detectors for specific diseases such as Menkes’ and Wilson’s disease, where coumarin compounds are used as fluorescent detectors for the copper II level in cells.

Thati (2007) investigated the anticancer activity of the new synthetic coumarin compound, coumarin-dioxy-acetic acid (cdoaH2), and its copper complex, copper coumarin-dioxyacetic acetate-phenathroline ([Cu(cdoa)(phen)2]). The anti-proliferation results indicated that the coumarin parent compound has anticancer activity with an IC50 of more than 250 μM. But, the metal complex has an IC50 ranging between 2.3 and 14
μM. The copper salt used in the synthesis showed no anticancer activity itself with an IC₅₀ of > 250 μM. (see figure 3.1)

Figure 3.1. Chemical structure of compounds (cdoaH2) and (Cu(cdoa)(phen)2).

Grazul (2009) reported in his review that coumarin metal complexes may be really promising anticancer agents. However, these are still difficult to use in treatment because of potential metal ion accumulations in the body. Extensive research should be carried out using in vitro and in vivo studies to target only the cancer cells not the normal cells.

Mao (2009) synthesized some 5-hydroxycoumarin and pyranocoumarin derivatives, and evaluated their anti-proliferation activity against two breast cancer cell lines, MDA-MB-231 and MCF-7. In general, the results indicated that the MDA-MB-231 cell line required higher dosage to show the same inhibition percentage as the MCF-7 cell line. Modifying the hydroxycoumarin and pyranocoumarin parent compounds with any of benzothiophene, thianthren, and sulfamate moieties could increase the anticancer activity against both the MDA-MB-231 and MCF-7 cell lines.
You (2009) prepared some coumarin analogs from osthole, a traditional Chinese medicine natural product. After synthesizing some compounds, they evaluated their cytotoxicity against MCF-7 and MDA-MB-232 breast cancer cell lines. Only one compound (8e) was shown to have strong anti-breast-cancer activity, with IC$_{50}$ values of 0.24 μM and 0.31 μM against MCF-7 and MDA-MB-231 cells, respectively (see figure 3.2).

![Chemical structure of compound 8e.](image)

*Sashidhara (2010) synthesized 35 coumarin derivatives and tested their cytotoxicity against MCF-7 breast carcinoma cell lines. Out of the 35 compounds only 3 benzocoumarins were markedly more active than the others. Compounds Be- 5, 8 and 9 showed anti-breast cancer activity with IC$_{50}$ values of 3.8, 7.9 and 6.5 μM, respectively (see figure 3.3). However, the anti-breast cancer drug Tamoxifimen which is used to treat early and advanced ER positive breast cancer (Buzdar, 2006) required more dosage, with an IC$_{50}$ of 11.8 μM. These three compounds showed strong activity, but the other ranged between moderate to weak activity with IC$_{50}$ values between 20- 100 μM and > 100 μM respectively.*
Mustafa (2011) evaluated the anticancer activity of 11 synthetic $N1$-(coumarin-7-yl) amidrazones compounds against the MCF-7 breast carcinoma cell line. Only 3 compounds were found to have anti-breast cancer activity: compounds I, M, and N with IC$_{50}$ values of 47.8, 39.7 and 20.2 µM (see figure 3.4.).

Therefore, the main objective of this work focuses on investigating the anti-breast-cancer activity of five synthetic coumarin compounds. Furthermore, to investigate their pro-apoptotic activities such as apoptotic bodies formation and DNA fragmentation and test their toxicity on normal breast cells.
3.2. Materials and methods

3.2.1. Reagents and antibodies

All reagents and solvents were purchased from Sigma-Aldrich Co. (St Louis, MO.), unless stated otherwise. Hoechst 33342 nucleic acid stain was purchased from Invitrogen Life Technologies (Grand Island, NY). Cell proliferation Kit I (MTT) and the apoptotic DNA-ladder Kit were purchased from Roche Applied Science (Indianapolis, IN).

3.2.2. Cell lines and culture conditions

Four breast adenocarcinoma cell lines (MDA-MB-231, MCF-7, BT-474 and Sk-BR-3) were obtained from the American Type Culture Collection (ATCC Manassas, VA). All cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Grand Island, NY) supplemented with 10% v/v fetal bovine serum, 1% v/v L-glutamine, 1% v/v antibiotic penicillin-streptomycin solution (Sigma-Aldrich) and 0.37% v/v sodium bicarbonate. All cells were incubated at 37 °C and 5% CO₂ in humidified Fisher Scientific incubator. All cells received fresh media 2 to 3 times/week and were sub-cultured when they reach 70-80% confluence. First, the culture medium was removed and the adherent cells were washed with 1X versene (Invitrogen, 0.2 g/L EDTA.4Na in phosphate-buffered saline) then the cells were detached using 1X trypsin-EDTA (Invitrogen, 0.25% trypsin, 0.1% EDTA, pH 7.2-8), followed by 2-5 minutes incubation at 37°C and 5% CO₂ in a humidified incubator. Cells were re-suspended in fresh media and counted with a hemocytometer using trypan blue dye (0.47% in
phosphate-buffered saline) and seeded in new tissue culture treated flasks or 96 well microplates.

3.2.3. Cellular anti-proliferation activity using the MTT cytotoxicity assay

The effects of five coumarin compounds (compounds F: J), (see figure 3.5.) on cell proliferation breast carcinoma cell lines MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 were measured by using the 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay (MTT). The anticancer activity was tested using various concentrations of the coumarin compounds at 25, 50, 100, 200, 300 μM of each compound. Each treatment concentration was performed in triplicates and the whole experiment was repeated 3 to 5 times as described previously (Chen, 2009; Xiong, 2010; Chae, 2011; Goel, 2011; Zhou, 2011). The cells were grown in 96-well, flat bottom, tissue culture treated microplates, at the density of 1 x 10⁴ for 2 days at 37°C and 5% CO₂ in humidified incubator to promote cellular adhesion. The tested compounds were dissolved in DMSO and diluted in complete media to a final concentration of 1% to the desire testing concentration. After the incubation period, old media was removed and the adherent cells were first washed by 1X versene. Then 200 μl new complete media with the compounds were incubated in the same condition. Control wells received only the media and DMSO. MTT salt solution (20 μl) at a final concentration of 0.5 mg/ml was added to each well and incubated for a period of 5 h. After the incubation period, the whole solution in each well was replaced with 200 μl of DMSO to dissolve the formed formazan crystals. The optical density of each well was read at 560 nm and reference wavelength at 650 nm on a Tecan infinity F500 microplate reader (Tecan System Inc., San Jose, CA, USA).
The % of cell viability = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100

Figure 3.5. Chemical structure of coumarin compounds.
3.2.4. Examination of morphological apoptotic changes

The morphological apoptotic changes were examined using the chromatin dye staining protocol as described previously (Chen, 2009). Four breast adenocarcinoma cell lines MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 were grown in complete media of DMEM/F12 in a chamber slide, Thermo Scientific (Rochester, New York). All cells were incubated for 2 days at 37 °C and 5 % CO₂. When cells reached 50% confluence, the old media was removed and the adherent cells were first washed with 1X versene. Then 300 μl of new complete media with the compounds (1%) were added and incubated for 2 more days in the same condition. The control chambers received only the media and DMSO (1%). After the incubation period, the culture medium was removed and the adherent cells were washed with PBS (contain: 0.9 mM CaCl and 0.9 mM MgCl₂). And then cells were stained with the chromatin dye Hoechst 33342 (2 μg/ml) at 37 °C for 30 min. The cells were examined using fluorescence microscopy equipped with a blue filter (Leica DM5500 B microscope, Leica Microsystems). Apoptotic cells were identified on the basis of nuclear morphology changes, such as chromatin condensation, cell shrinking, apoptotic bodies formation, and nuclear fragmentation.

3.2.5. Apoptotic DNA analysis

3.2.5.1. DNA isolation

DNA fragmentation analysis was carried out using the apoptotic DNA ladder kit (Roche) using the following protocol: MDA-MB-231, breast carcinoma cell line was grown in complete media of DMEM/F12 in 25 cm² tissue cultural (TC) treated flasks. All cells were incubated for 2 days at 37 °C under 5 % CO₂. When the cells reached 50%
confluent, the old media was removed and the adherent cells were first washed by 1X versene. Then new complete media with the compounds (1%) were added and incubated for 2 more days in the same condition. The control flasks received only the media and 1% DMSO. After the incubation period, the culture medium was removed and the adherent cells were washed with 1X versene then the cells were detached using 1X trypsin-EDTA. Afterward, the cells were centrifuged at 5000 RPM at 4 °C for 10 min. After the centrifugation period and removal of the supernatant, cells were resuspended in 200 µl PBS then mixed with 200 µl of lysis buffer and incubated at RT for 20 min. An equal amount of isopropanol was added and vortexed vigorously for 5 minutes to precipitate the DNA. The precipitated DNA was collected using filter tubes and washed two times with the kit washing buffer. The DNA was eluted using 200 µl pre-warmed (+ 70°C) elution buffer. Then the DNA was stored at -20 °C for later analysis.

3.2.5.2. DNA fragmentation analysis using agarose gel electrophoresis

Apoptotic DNA fragmentation is the hallmark of apoptotic cells. The negatively charged DNA fragments migrate on agarose gel from negative to the positive in an applied electrical field. This DNA electrophoresis was carried out according to the manufacturer's protocol as follows:

First, 20 µg of DNA sample was mixed with 2 µl of loading buffer (0.1 g SDS, 25 mg bromophenol blue, 7 ml redistilled water and 3 ml glycerol) and 5 µl of DNA marker were loaded in 1% agarose gel (0.4 g agarose in 40 ml Tris borate EDTA and 5 µl of ethidium bromide solution). The gel was allowed to run at 124 volts for 1h in 1x TBE buffer (5.4g Tris, 2.8g boric acid, 2 ml 0.5M EDTA solution and completed to 1000 ml,
pH 8.0). The DNA fragmentation was visualized using UV light and Ethedium Bromide dye.

3.2.6. Statistical analysis

The results for the cell proliferation assay are expressed as mean ± standard deviation (SD) for three replicates. Statistical difference was determined by using the Enova test. P-values of less than 0.05 were considered statistically significant. IC\textsubscript{50} values were determined using Excel.

3.3. Results

The anti-breast-cancer activity of five coumarin compounds, compound F to J, has been investigated using four breast carcinoma cell lines, MDA-MB-231, MCF-7, BT-474 and SK-BR-3. According to the Western blot analysis results in chapter 2, section 2.2.1., BT-474 and SK-BR-3 cell lines had comparable, strong expression of the HER-2 protein but, MDA-MB-231 and MCF-7 cell lines did not show any expression. This suggests that BT-474 and SK-BR-3 depend on HER-2 protein for their proliferation. In contrast, the MCF-7 and BT-474 cell lines showed high ER protein expression, but MDA-MB-231 and SK-BR-3 did not show any bands (see figure 3.5). These data suggest that the MDA-MB-231 cell line does not depend on either HER-2 or ER protein for proliferation. The MCF-7 cell line does depend on ER which regulates its growth. Interestingly, BT-474 breast carcinoma cell line does depend on both of HER-2 protein and ER to proliferate. However, the SK-BR-3 breast carcinoma cell line showed to only depends on HER-2 protein for its growth.
Figure 3.5. Western blot analysis of HER-2, ER and β-actin in the breast cancer cell lines. MDA-MB-231 (A), MCF-7 (B), BT-474 (C) and SK-BR-3 (D) cells. Cells were harvested and proteins were separated using 4-12% SDS-PAGE, transferred to nitrocellulose membrane and probed with HER-2, ER and β-Actin antibodies as described in section 2.2.7.

3.3.1. Anti-proliferation activity of the five coumarins on the four breast cancer cell lines

The screening for anticancer activity of the five coumarin compounds was carried out using the well-known 3-(4, 5 dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide assay (MTT). The anti-proliferation tests of these compounds indicated that these coumarin compounds have a significant inhibition efficacy on the breast cancer cells after 48 h treatment. All these compounds showed time-dependent and dose-dependent suppression of proliferation in all of the cell lines. Compound F after 24 h treatment at concentrations of 25, 50, 100, 200, and 300 µM, showed zero to very slight activity against the four breast cancer cell lines, MDA-MB-231 (see figure 3.6), MCF-7 (see figure 3.9), BT-474 (see figure 3.12) and SK-BR-3 (see figure 3.15). However, after 48 h treatment with the same concentrations, it suppressed 99.2% to 99.99% of the growth of the four breast cancer cell lines MDA-MB-231(figure 3.7.), MCF-7 (figure 3.10), BT-474 (figure 3.13), and SK-BR-3 (figure 3.16). Further cytotoxicity investigations have
been done with lower concentrations of 1, 5, 10, 15, 20 μM. The first three concentrations showed little to no activity for all cells. At the concentration of 15 μM, 70% of the MDA-MB-231 cells lost their viability, and at 20 μM all the cells lost their viability (see figure 3.8). Compound F showed no cytotoxicity against the MCF-7 cell line at 1 and 5 μM after 48 h treatment, but this compound suppressed 50% of cell viability at 10 μM and 100% at 15 and 20 μM (see figure 3.11). After 48 h treatment with compound F, 65% of BT-474 cell viability was lost, and 96% of viability lost at 20 μM (see figure 3.14). Only 39% SK-BR-3 cell viability was lost at 15 μM after 48h treatment and 94% at 20 μM (see figure 3.17). All the data collected after 48 h treatment showed significant loss of viability the four breast cancer cell lines MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC₅₀ of 11.3, 10.13, 12.08, and 13.75 μM respectively.

Figure 3.6. The effect of compound F on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound F for 24 h.
Figure 3.7. The effect of compound F on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound F for 48 h.

Figure 3.8. The effect of compound F on MDA-MB-231 cell growth. The cells were treated with 1, 5, 10, 15, 20 μM of compound F for 48 h.
Figure 3.9. The effect of compound F on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound F for 24 h.

Figure 3.10. The effect of compound F on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound F for 48 h.
Figure 3.11. The effect of compound F on MCF-7 cell growth. The cells were treated with 1, 5, 10, 15, 20 μM of compound F for 48 h.

Figure 3.12. The effect of compound F on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound F for 24 h.
Figure 3.13. The effect of compound F on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound F for 48 h (not significant).

Figure 3.14. The effect of compound F on BT-474 cell growth. The cells were treated with 1, 5, 10, 15, 20 μM of compound F for 48 h.
Figure 3.15. The effect of compound F on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound F for 24 h.

Figure 3.16. The effect of compound F on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound F for 48 h.
Figure 3.17. The effect of compound F on SK-BR-3 cell growth. The cells were treated with 1, 5, 10, 15, 20 μM of compound F for 48 h.

The screen for anticancer activity of the coumarin compound G after 24 h treatment at concentrations of 25, 50, 100, 200, and 300 μM, showed little to no activity against the four breast cancer cell lines, MDA-MB-231 (see figure 3.18), MCF-7 (see figure 3.20), BT-474 (see figure 3.22) and SK-BR-3 (see figure 3.24). Further cytotoxicity investigations have been done with the same concentrations for a 48 h treatment period. Compound G showed a dose-dependent effect on viability of the MDA-MB-231 cell line only at higher concentrations. It suppressed 40% of MDA-MB-231 cell viability at 300 μM (see figure 3.19). But, this compound suppressed 50% of the MCF-7 viability at the concentration of 100 μM and 100% cytotoxicity at 200 μM and up (see figure 3.21). Also, compound G had the same efficacy on the viability of the BT-474 cell line. It showed 50% cytotoxicity at 100 μM and 100% anticancer activity at 200 μM and up (see figure 3.23). Compound G showed little activity against SK-BR-3,
where it suppressed up to 70% at the highest used concentration of 300 μM (see figure 3.25). The results indicate that compound G was not effective as an anti-breast-cancer agent after 24 h treatment. It showed efficacy only with MCF-7 and BT-474 cell lines, with IC₅₀ values of 80 and 118 μM, respectively. However, it had lower activity against MDA-MB-231 and SK-BR-3 cell lines with IC₅₀ values of >200 and 141 μM respectively.

![Figure 3.18](image)

**Figure 3.18.** The effect of compound G on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound G for 24 h.
Figure 3.19. The effect of compound G on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound G for 48 h.

Figure 3.20. The effect of compound G on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300μM of compound G for 24 h.
Figure 3.21. The effect of compound G on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound G for 48 h.

Figure 3.22. The effect of compound G on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound G for 24 h.
Figure 3.23. The effect of compound G on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound G for 48 h.

Figure 3.24. The effect of compound G on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound G for 24 h.
Figure 3.25. The effect of compound G on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound G for 48 h.

The anti-breast-cancer activity of the compound H against MDA-MB-231, MCF-7, BT-474, and SK-BR-3 has been investigated. In tests of cytotoxicity against the MDA-MB-231 cell line after 24 h treatment at concentration of 25, 50, 100, 200, and 300 μM, compound H showed 50% activity at only the highest concentration (see figure 3.26), but after a 48 h treatment period it showed up to 67% at 300 μM (see figure 3.27). Compound H showed slight activity against the MCF-7 cell line after 24 h treatment (see figure 3.28). However, it was more effective after 48 h treatment. It suppressed the cells viability with 45 %, 57 % and 99 % at 100, 200, and 300 μM (see figure 3.29). Also, it has the same efficacy on the BT-474 cell line after 24 h treatment period (see figure 3.30). And, it suppressed its viability with 25 %, 51 % and 99.8 % at 100, 200, and 300 μM (see figure 3.31). The SK-BR-3 cell line was more resistant to compound H treatment. Compound H show no to slight activity after 24 h treatment (see figure 3.32).
However, after 48 h treatment period it suppressed the cells viability with 46 % and 59 % at 100 and 200 µM, respectively. Increasing the dosage concentration to 300 µM did not increase the efficacy (see figure 3.33). Accordingly, the results indicated that compound H was not effective as an anti-breast-cancer agent after 24 h treatment. However, at 48 h it was effective against MCF-7 and BT-474 cell lines with IC₅₀ values of 152 and 165 µM, respectively. And, it has lower activity against MDA-MB-231 and SK-BR-3 cell lines with IC₅₀ values of >200 and 187 µM respectively.

Figure 3.26. The effect of compound H on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 µM of compound H for 24 h.
Figure 3.27. The effect of compound H on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 µM of compound H for 48 h.

Figure 3.28. The effect of compound H on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 µM of compound H for 24 h.
Figure 3.29. The effect of compound H on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound H for 48 h.

Figure 3.30. The effect of compound H on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound H for 24 h.
Figure 3.31. The effect of compound H on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound H for 48 h.

Figure 3.32. The effect of compound H on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound H for 24 h.
Figure 3.33. The effect of compound H on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound H for 48 h.

The cytotoxicity of compound I against the MDA-MB-231 cell line after 24 h treatment at concentrations of 25, 50, 100, 200, and 300 μM, showed 20 % activity at only 300 μM (see figure 3.34). But, after a 48 h treatment period it showed anti-breast-cancer activity of 30 % at 100 μM and it suppressed 100% of cell viability at 200 μM and up (see figure 3.35). Compound I showed slight activity against the MCF-7 cell line after 24 h of treatment, the maximum inhibition being 15% at 300 μM (see figure 3.36). However, it was more effective after 48 h treatment. It suppressed the cell viability 40%, 63 % and 99 % at 50, 100, and 200 μM (see figure 3.37). Also, it has the same efficacy on the BT-474 cell line after 24 h treatment period (see figure 3.38). And, compound I suppressed the BT-474 cells viability with 33 % and 99.8 % at 100, and 200 μM (see figure 3.39). Compound I show little to no activity after 24 h treatment against the SK-
BR-3 cell line (see figure 3.40). But, after 48 h treatment period it suppressed the cells viability 36, 44, 51, and 56 % at 50, 100, 200 and 300 μM respectively (see figure 3.41). The results indicate that compound I was not effective as an anti-breast-cancer agent after 24 h treatment. However, it was more effective at 48 h against the MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 cell lines with IC50 values of 129, 88, 134, and 192 μM respectively.

Figure 3.34. The effect of compound I on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound I for 24 h.
Figure 3.35. The effect of compound I on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound I for 48 h.

Figure 3.36. The effect of compound I on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound I for 24 h.
Figure 3.37. The effect of compound I on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound I for 48 h.

Figure 3.38. The effect of compound I on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound D for 24 h.
Figure 3.39. The effect of compound I on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound I for 48 h.

Figure 3.40. The effect of compound I on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound I for 24 h.
The anti-breast cancer activity of compound J against four breast carcinoma cell lines was investigated. The activity of compound J on the MDA-MB-231 cell line after 24 h treatment at concentrations of 25, 50, 100, 200, and 300 μM, showed only 20% activity at the highest concentration (see figure 3.42). However, after a 48 h treatment period it showed anti-breast-cancer activity of 30, 89, and 90 % at 100, 200 and 300 μM (see figure 3.43). Compound J showed slight activity against the MCF-7 cell line after 24 h of treatment, the maximum inhibition was 20 % at 300 μM (see figure 3.44). However, it was more effective after 48 h of treatment. It suppressed the cells viability with 40 %, 62 % and 99 % at 50, 100, and 200 μM (see figure 3.45). It showed simillar efficacy on the BT-474 cell line after 24 h treatment period (see figure 3.46). Compound J suppressed the BT-474 cells viability with 23, 54, 90, and 99 % at 50, 100, 200 and 300 μM, respectively (see figure 3.47). Compound J showed little to no activity after 24 h treatment against the SK-BR-3 cell line, with maximum inhibition of 10% only at 300
μM (see figure 3.48). However, after a 48 h treatment period it suppressed the cells viability with 1, 8, 46, and 48% at 50, 100, 200 and 300 μM, respectively (see figure 3.49). Accordingly, compound J was not effective as an anti-breast-cancer agent after 24 h of treatment. However, it was more effective against the MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 cell lines with IC_{50} values of 141, 91.9, 121.9, and > 200 μM respectively.

Figure 3.42. The effect of compound J on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound J for 24 h.
Figure 3.43. The effect of compound J on MDA-MB-231 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound J for 48 h.

Figure 3.44. The effect of compound J on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound J for 24 h.
Figure 3.45. The effect of compound J on MCF-7 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound J for 48 h.

Figure 3.46. The effect of compound J on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound J for 24 h.
Figure 3.47. The effect of compound J on BT-474 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound J for 48 h.

Figure 3.48. The effect of compound J on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound J for 24 h.
3.3.3. Morphological studies

3.3.3.1. The effect of coumarin compounds on apoptosis

According to the cell anti-proliferation results of the compounds F, G, H, I and J, more studies were needed to explain the effect of the tested compounds on the MDA-MB-231 cell line apoptotic process. Apoptosis as a programmed cell death has many characteristics including nuclear DNA fragmentation, chromatin condensation, formation of apoptotic bodies and cell shrinkage (Vinatier, 1996; Raffray, 1997).

The main morphological features of apoptosis are the formation of apoptotic bodies and nuclear DNA fragmentation. Chen (2009) indicated that these changes can be experimentally observed using a specific chromatin dye like Hoechst 33342, a stain which enables the detection of the apoptotic morphological changes using a fluorescence

Figure 3.49. The effect of compound J on SK-BR-3 cell growth. The cells were treated with 25, 50, 100, 200, 300 μM of compound J for 48 h.
microscope with a blue filter. DNA fragmentation can be detected using DNA electrophoresis.

The apoptotic morphological changes were determined after a 48 h treatment period at the concentration of 100 μM of each compound. Then the cells were stained in Hoechst for 30 min in dark. The morphological changes observed were similar to those produced by 20 μg/ml crude methanolic extract of the pericarp of *Garcinia mangostana* after 48h treatment (Moongkarndi *et al.*, 2004). The morphological alterations of the five coumarins are illustrated (see figure 3.50).

![Figure 3.50](image)

*Figure 3.50.* Morphological and cellular changes induced by the coumarin compounds. The effects of these coumarins on cell morphology were tested with the fluorescent chromatin dye Hoechst 33342 in MDA-MB-231 Cells. Cells were treated with five coumarin compounds (Compounds F: J) for 48 h and then stained with the chromatin dye for 30 min. The two panels are the MDA-MB-231 Cells, the top panel is the untreated control and the lower panel is for the treated cells.
3.3.4. Apoptotic DNA fragmentation analysis

Apoptotic DNA fragmentation is the hallmark of apoptotic cells. This analysis was carried out using the apoptotic DNA ladder kit (Roche) to study the DNA fragmentation effects of the five coumarin compounds on the MDA-MB-231 cell line. The cells were treated for 48 h with 100 μM of each compound. Then, 20 μg DNA of each treatment was subjected to 1% agarose gel electrophoresis. The DNA electrophoretogram (Fig. 3.51) indicates that compound J produced no DNA fragmentation (lane 4). However, clear DNA fragments were observed for all of the other coumarin compounds I, H, G, and F treatments after 48 h at the same concentration (lanes 5, 6, 7, and 8).

Figure 3.51. Electrophoretogram demonstrating the apoptotic DNA fragmentation analysis of five coumarin compounds on MDA-MB-231 cells. Cells were treated for 48 h with 100 μM of the compounds using 1% agarose gel. DNA marker (lane 1), positive control (lane 2), untreated cells (lane 3), treated cells with Com. J (lane 4), treated cells with Com. I (lane 5), treated cells with Com. H (lane 6), treated cells with Com. G (lane 7) and treated cells with Com. F (lane 8).
2.4. Discussion

This study is the first investigation of the anti-breast cancer activity of these five coumarin compounds F, G, H, I and J on three metastatic breast cancer cell lines MDA-MB-231, MCF-7, and SK-BR-3 and one BT-474 non-metastatic breast cancer cell line. The Western blot results described in chapter 2, section 2.3.1. indicate that the MDA-MB-231 breast carcinoma cell line does not express HER-2 or ER, and therefore is not likely to depend on either HER-2 protein or ER for growth. The MCF-7 cell line depends on ER for its growth and proliferation. BT-474 breast carcinoma cell line does depend on both of HER-2 protein and ER for its growth. Finally, SK-BR-3 cell line depends only on HER-2 protein for its growth.

The anti-breast cancer screening results indicate that all of the five coumarin compounds showed no to very slight cytotoxicity against the four breast cancer cell lines after a treatment period of 24 h except for compound F. On increasing the period of treatment to 48 h, these five compounds showed time-dependent and dose-dependent effects.

All four breast carcinoma cell lines completely lost their viability after the treatment period of 2 days at 25, 50, 100, 200 and 300 μM concentrations of compound F. But on lowering the dose concentration to 1, 5, 10, 15 and 20 μM, compound F started to show the anticancer activity at 15 μM for the cells and it killed 100% of the cells at 20 μM. The IC₅₀ against all the cells showed no selectivity for any one of them, which may be because of the presence of the cyano group in the chemical structure of F compound.
Bhattacharya (1997) indicated that cyanide compounds have acute toxicity and caused DNA damage when tested on mammalian cell cultures.

The other four coumarin compounds have no cyano group, and the MTT assay results indicated the all of these compounds are exhibit anti-breast-cancer activity against both the MCF-7 and BT-474 cell lines. According to the Western blot results, these two cell lines over-express ER, in agreement with the results obtained by Chen, (2009). So, these four coumarins may be used to fight breast cancer, especially those that overexpress ER.

The IC_{50} results indicated that the BT-474 cell line always required higher concentrations than MCF-7 to suppress growth. This may be because MCF-7 depends only on the ER protein for its proliferation. But, BT-474 may be more resistant because growth can be supported by either the ER or HER-2 proteins.

In contrast, SK-BR-3 cell line always required the highest dosage of treatment from each compound separately and these four compounds were not effective against it. This may be because this cell line overexpresses HER-2. These last four compounds resemble estrogen molecule and are very selective for ER breast cancer cell lines, and may therefore be able to be used against other ER over-expressing cancer types.

Studies of cellular changes were done to explore the efficacy of these compounds on the MDA-MB-231 cell line. Both the studies of apoptotic morphological changes with the fluorescent chromatin dye Hoechst 33342 and DNA fragmentation tests showed that the five coumarin compounds were cytotoxic on the MDA-MB-231 breast cancer cells, which exhibited apoptotic bodies formation and DNA fragmentation.
3.5. Conclusion

- Five novel coumarin compounds have been evaluated for their anti-breast cancer activities.

- Compound F was a toxic agent for all of the breast carcinoma cell lines used, with an IC$_{50}$ of 15 µM in this experiment.

- Compounds G, H, I and J were more selective for ER over-expressing breast carcinoma cell lines (MCF-7 and BT-474).

- The BT-474 cell line was more resistant than the other ER over-expressing breast carcinoma cell line MCF-7, perhaps because either ER or HER-2 can supported.

- The SK-BR-3 cell line was the most resistant to all the last 4 compounds, which may be because it depends only on the HER-2 protein for its growth, and these compounds are selectively active against only ER positive cells.
3.6. References


4.1. Introduction

The use of nanotechnology has been given much attention especially in the field of cancer treatment. One of these nanotechnology techniques is the use of nano-carriers which have been used as a delivery system for the treatment of cancer and other diseases. Nanoparticles with diameters less than 100 nm were found to be more pharmaceutically effective than small-molecule drugs (Hu, 2012; Joshi, 2012). For any anticancer drug to be effective, it should be selective only for the cancer cells and not damage the normal cells as well. By modifying the nano-carriers surfaces with specific selective moieties, this would yield a highly selective anticancer agent (Byrne, 2008). Currently, there are many types of drug nano-carrier platforms that have been used in the field of cancer research such as: micelles, liposomes, protein nanoparticles, polymeric nanoparticles, carbon nanotubes, dendrimers and metallic nanoparticles (Koo, 2012; Zhang, 2012; Cirstoiu-Hapca, 2010, Rejinold, 2011; Zhou, 2012; Wolinsky, 2008 and El-Sayed, 2006).

Gold nanoparticles have been shown to be more promising than other nanoparticles because they are easy to functionalize and bio-modify with high binding affinity for thiols, amines, and disulfide compounds. In addition they are easy to use as imaging labels, and can be conjugated with multi-functional moieties on the same particle
to enhance the drug delivery system. Most importantly, Au nanoparticles are not toxic to human cells (Jain, 2007).

There are three ways that gold nanoparticles can be internalized into cells: phagocytosis, fluid-phase endocytosis, and receptor-mediated endocytosis. One important technique to overcome the side effects of many anticancer agents in general is using the target therapies (Zhang, 2010). This entails manipulating the properties of receptor-mediated endocytosis in order to target only the cancer cells and not the normal cells (see figure 4.1) (Sudimack, 2000). HER-2 protein and the folate receptor are two proteins found to be over-expressed in many types of cancers, including cancers of the breast, ovaries, endometrium, kidneys, colon, and brain (Zhang, 2010).

Fig. 4.1. A schematic diagram of the folate receptor-mediated endocytosis pathway.

Folic-acid-conjugated nanoparticles were synthesized using glutathione as coupling agent (FA-GSH-GNPs) (see figure 4.2) and evaluated for their binding affinity with human cervical carcinoma cells (HeLa), which is a folate-receptor-positive cancer cell line, and mouse fibroblast (FB) cells which lack folate receptors. The transmission
electron microscopy (TEM) results showed the accumulation of the FA-GSH-GNPs inside HeLa cells but not in the FB cells. The MTT results indicated that FA-GSH-GNPs was not toxic for either of the cell types (Zhang, 2010).

\[
\text{O} \quad \text{C-\(\text{NH-FA}\)}
\]

\[
\text{NH} \quad \text{O=C}\]

\[
\text{CH}_2 \quad \text{HN} \quad \text{C=O}
\]

\[
\text{H}_2\text{N-C-C-\(N-\text{FA}\)}
\]

**Fig. 4.2.** Chemical structure of the FA-GSH-GNP.

11-mercaptoundecanoic acid-modified gold nanoparticles (~7 nm) conjugated with chloroquine were synthesized and their cytotoxicity was evaluated using the MCF-7 breast cancer cell line. The GNPs themselves were found not to be toxic, while the chloroquine compound, at the same concentration of the GNPs induced total cells death of 70%. However, when conjugated with the GNPs it induced 99% cells deaths with an IC\(_{50}\) value of 30 ± 5 µg/ ml (Joshi, 2012).

Therefore, the main objective of this work focuses on investigating the anti-breast-cancer activity of modified gold-nanoparticles. Furthermore, to investigate their
pro-apoptotic activities such as apoptotic bodies formation and DNA fragmentation and test their toxicity on normal breast cells tested.

4.2. Materials and methods

4.2.1. Reagents and antibodies

All reagents and solvents were purchased from Sigma-Aldrich Co. (St Louis, MO.), unless stated. Hoechst 33342 nucleic acid stain was purchased from Invitrogen Life Technologies (Grand Island, NY). Cell proliferation Kit I (MTT) and apoptotic DNA-ladder Kit were purchased from Roche Applied Science (Indianapolis, IN).

4.2.2. Cell lines and culture conditions

Four breast adenocarcinoma cell lines (MDA-MB-231, MCF-7, BT-474 and Sk-BR-3) were obtained from the American Type Culture Collection (ATCC Manassas, VA). All cells were grown in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12) (Gibco, Grand Island, NY) supplemented with 10% v/v fetal bovine serum, 1% v/v L-glutamine, 1% v/v antibiotic, penicillin-streptomycin solution (Sigma-Aldrich) and 0.37% v/v sodium bicarbonate. All cells were incubated at 37 °C and 5 % CO2 in humidified Fisher Scientific incubator. All cells received fresh media 2 to 3 times/ week and were sub-cultured when they reached 70-80% confluence. First, the culture medium was removed and the adherent cells were washed with 1X versene (Invitrogen, 0.2 g/L EDTA.4Na in phosphate-buffered saline) then the cells were detached using 1X trypsin-EDTA (Invitrogen, 0.25% trypsin, 0.1% EDTA, pH 7.2-8), followed by 2-5 minutes incubation at 37°C and under 5% CO2 in a humidified incubator. The cells were re-suspended in fresh media and counted with a hemocytometer using trypan blue dye
(0.47% in phosphate-buffered saline) and seeded in new tissue culture treated flasks or 96 well microplates.

4.2.3. Cellular anti-proliferation activity using the MTT cytotoxicity assay

The effects of five compounds containing two modified gold nanoparticles GNPs (compounds K:O), (see figure 4.3.) on cell proliferation breast carcinoma cell lines MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 were measured using the 3-(4, 5 dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay (MTT). The anticancer activity was tested using various concentrations of these compounds at 1 and 5 mg of each compound. Each treatment concentration was performed in triplicate and the whole experiment was repeated 3 to 5 times, as described previously (Chen, 2009; Xiong, 2010; Chae, 2011; Goel, 2011; Zhou, 2011). The cells were grown in 96-well, flat bottom, tissue culture treated microplates, at the density of $1 \times 10^4$ for 2 days at 37°C and 5% CO$_2$ in a humidified incubator to promote cellular adhesion. The tested compounds were dissolved in DMSO and diluted in complete media to a final concentration of 1% to the desire testing concentration. After the incubation period, old media was removed and the adherent cells were first washed with 1X versene then 200 μl new complete media with the compounds were incubated in the same condition. Control wells received only the media and DMSO. MTT salt solution (20 μl) at a final concentration of 0.5 mg/ml was added to each well and incubated for a period of 5 h. After the incubation period, the whole solution in each well was replaced with 200 μl of DMSO to dissolve the formed formazan crystals. The optical density of each well was read at 560 nm and reference wavelength at 650 nm on a Tecan infinity F500 microplate reader (Tecan System Inc., San Jose, CA, USA).
The % of cell viability = \( \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100 \)

Figure 4.3. Chemical structure of compounds K, L, M, N, and O.
4.2.4. Examination of morphological apoptotic changes

The morphological apoptotic changes were examined using the chromatin dye staining protocol as described previously (Chen, 2009). Four breast adenocarcinoma cell lines MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 were grown in complete media of DMEM/F12 in a chamber slide, Thermo Scientific (Rochester, New York). All cells were incubated for 2 days at 37 °C and 5 % CO₂. When cells reached 50% confluence, the old media was removed and the adherent cells were first washed with 1X versene. Then 300 μl of new complete media with the compounds (1%) were added and incubated for 2 more days in the same condition. The control chambers received only the media and DMSO (1%). After the incubation period, the culture medium was removed and the adherent cells were washed by PBS (contain: 0.9 mM CaCl and 0.9 mM MgCl₂). And then cells were stained with the chromatin dye Hoechst 33342 (2 μg/ml) at 37 °C for 30 min. The cells were examined using fluorescence microscopy equipped with a blue filter (Leica DM5500 B microscope, Leica Microsystems). Apoptotic cells were identified on the basis of nuclear morphology changes, such as chromatin condensation, cell shrinking, apoptotic bodies formation, and nuclear fragmentations.

4.2.5. Apoptotic DNA analysis

4.2.5.1. DNA isolation

DNA fragmentation analysis was done using the apoptotic DNA ladder kit (Roche) using the following protocol: MDA-MB-231, breast carcinoma cell line was grown in complete media of DMEM/F12 in 25 cm² tissue cultural (TC) treated flask. All cells were incubated for 2 days at 37 °C and 5 % CO₂. When cells reached 50%
confluent, the old media was removed and the adherent cells were first washed with 1X versene then new complete media with the compounds (1%) were added and incubated for 2 more days in the same condition, control flasks received only the media and 1% DMSO. After the incubation period, the culture medium was removed and the adherent cells were washed with 1X versene then the cells were detached using 1X trypsin-EDTA. Afterward, the cells were centrifuged at 5000 RPM at 4 °C for 10 min. After the centrifugation period and removal of the supernatant, cells were resuspended in 200 μl PBS then mixed with 200 μl of lysis buffer and incubated at RT for 20 min. An equal amount of isopropanol was added and vortexed vigorously for 5 minutes to precipitate the DNA. The precipitated DNA was collected using filter tubes and washed two times with washing buffer. The DNA was eluted using 200 μl pre-warmed (+ 70°C) elution buffer. Then the DNA was stored at -20 °C for later analysis.

4.2.5.2. DNA fragmentation analysis using agarose gel electrophoresis

Apoptotic DNA fragmentation is the hallmark of apoptotic cells. The negatively charged DNA fragments migrate on agarose gel from negative to the positive in an applied electrical field. This DNA electrophoresis was done according to the manufacturer’s protocol as following:

First, 20 μg of DNA sample was mixed with 2 μl of loading buffer (0.1 g SDS, 25 mg bromophenol blue, 7 ml redistilled water and 3 ml glycerol) and 5 μl of DNA marker were loaded in 1% agarose gel (0.4 g agarose in 40 ml Tris borate EDTA and 5 μl of ethidium bromide solution). The gel was allowed to run at 124 volts for 1h in 1x TBE buffer (5.4g Tris, 2.8g boric acid, 2 ml 0.5M EDTA solution and completed to 1000 ml,
pH 8.0). The DNA fragmentation was visualized using UV light and Ethidium Bromide dye.

4.2.6. Statistical analysis

The results for the cell proliferation assay are expressed as mean ± standard deviation (SD) for three replicates. Statistical difference was determined by using the Enova test. *P*-values of less than 0.05 were considered statistically significant. IC$_{50}$ values were determined using Excel.

4.3. Results

4.3.1. Anti-proliferation activity of the five compounds containing two modified GNPs on four breast carcinoma cell lines

Two gold nanoparticles adducts and their ligands were tested for their anti-breast-cancer activities using four breast carcinoma cell lines. Compound K, showed little to no activity after 24 h treatment period at concentration of 1 and 5 mg against the four breast cancer cell lines, MDA-MB-231 (see figure 4.4), MCF-7 (see figure 4.6), BT-474 (see figure 4.8) and SK-BR-3 (see figure 4.10). Even after 48 h treatment period with the same previous concentrations, it showed very slight activity against the four breast cancer cell lines, MDA-MB-231 (see figure 4.5), MCF-7 (see figure 4.7), BT-474 (see figure 4.9) and SK-BR-3 (see figure 4.11). Similarly, compound L is a one mercapto-coumarin compound showed little to no activity after 24 h treatment period at concentration of 1 and 5 mg against the four breast cancer cell lines, MDA-MB-231 (see figure 4.12), MCF-7 (see figure 4.14), BT-474 (see figure 4.16) and SK-BR-3 (see figure 4.18). Also, after 48 h treatment period with the same concentrations, it showed very little activity against
the four breast cancer cell lines, MDA-MB-231 (see figure 4.13), MCF-7 (see figure 4.15), BT-474 (see figure 4.17) and SK-BR-3 (see figure 4.19).

Figure 4.4. The effect of compound K on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg of compound K for 24 h.

Figure 4.5. The effect of compound K on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg of compound K for 48 h.
Figure 4.6. The effect of compound K on MCF-7 cell growth. The cells were treated with 1 and 5 mg of compound K for 24 h.

Figure 4.7. The effect of compound K on MCF-7 cell growth. The cells were treated with 1 and 5 mg of compound K for 48 h.
Figure 4.8. The effect of compound K on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound K for 24 h.

Figure 4.9. The effect of compound K on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound K for 48 h.
**Figure 4.10.** The effect of compound K on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of compound K for 24 h.

**Figure 4.11.** The effect of compound K on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of compound K for 48 h.
Figure 4.12. The effect of compound L on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg of compound L for 24 h.

Figure 4.13. The effect of compound L on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg of compound L for 48 h.
Figure 4.14. The effect of compound L on MCF-7 cell growth. The cells were treated with 1 and 5 mg of compound L for 24 h.

Figure 4.15. The effect of compound L on MCF-7 cell growth. The cells were treated with 1 and 5 mg of compound L for 48 h.
Figure 4.16. The effect of compound L on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound L for 24 h.

Figure 4.17. The effect of compound L on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound L for 48 h.
Figure 4.18. The effect of compound L on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of compound L for 24 h.

Figure 4.19. The effect of compound L on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of compound L for 48 h.
The first gold nanoparticle derivatives modified with compound L is compound M, and Au-MC nanoparticles were tested for their anti-breast-cancer activities using four breast carcinoma cell lines. Compound M was found to have anti-breast cancer activity after a 24 h treatment period at concentrations of 1 and 5 mg with the four breast cancer cell lines, MDA-MB-231 (see figure 4.20), MCF-7 (see figure 4.22), BT-474 (see figure 4.24) and SK-BR-3 (see figure 4.26). These GNPs showed similar efficacy in all cell lines. So, the results after a 24 h treatment indicated the cytotoxicity activity against the four breast cancer cell lines MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC$_{50}$ values of 1, 4.2, 7.3, and 3.2 mg, respectively. Increasing the treatment period with the same concentrations led to much more anti-breast-cancer activity. Compound M was found to suppress 93 to 94% of MDA-MB-231 cells after 48 h treatment (see figure 4.21), 81 to 89% of MCF-7 cells after 48 h treatment (see figure 4.23), 68 to 77.5% of BT-474 cells after 48 h treatment (see figure 4.25), and 94 to 94.5% of SK-BR-3 cells after 48 h treatment (see figure 4.27).
Figure 4.20. The effect of compound M on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg μM of compound M for 24 h.

Figure 4.21. The effect of compound M on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg of compound M for 48 h.
Figure 4.22. The effect of compound M on MCF-7 cell growth. The cells were treated with 1 and 5 mg of compound M for 24 h.

Figure 4.23. The effect of compound M on MCF-7 cell growth. The cells were treated with 1 and 5 mg µM of compound M for 48 h.
Figure 4.24. The effect of compound M on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound M for 24 h.

Figure 4.25. The effect of compound M on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound M for 48 h.
Figure 4.26. The effect of compound M on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of Compound M for 24 h.

Figure 4.27. The effect of compound M on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of compound M for 48 h.
Compound L is a one mercapto-coumarin compound that was found to have little to no activity after a 24 h treatment period at concentrations of 1 and 5 mg, using the four breast cancer cell lines, MDA-MB-231 (see figure 4.12), MCF-7 (see figure 4.14), BT-474 (see figure 4.16) and SK-BR-3 (see figure 4.18). Also, after 48 h treatment period with the same concentrations, it showed weak activity against the four breast cancer cell lines, MDA-MB-231 (see figure 4.13), MCF-7 (see figure 4.15), BT-474 (see figure 4.17) and SK-BR-3 (see figure 4.19).

Compound O showed weak activity after a 24 h treatment period at concentrations of 1 and 5 mg, using the four breast cancer cell lines, MDA-MB-231 (see figure 4.36), and MCF-7 (see figure 4.38). Compound O had moderate activity against BT-474 cells (see figure 4.40) and no activity against SK-BR-3 cells (see figure 4.42). Even after a 48 h treatment period with the same concentrations, it showed very slight activity against the first two breast cancer cell lines, MDA-MB-231 (see figure 4.37), MCF-7 (see figure 4.39). The activity of compound O was enhanced by up to 40% on BT-474 cells compared to the activity after 24 h treatment (see figure 4.41), no activity against SK-BR-3 cells (see figure 4.43) was observed.
Figure 4.28. The effect of compound N on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg μM of compound N for 24 h.

Figure 4.29. The effect of compound N on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg of compound N for 48 h.
Figure 4.30. The effect of compound N on MCF-7 cell growth. The cells were treated with 1 and 5 mg of compound N for 24 h.

Figure 4.31. The effect of compound N on MCF-7 cell growth. The cells were treated with 1 and 5 mg μM of compound N for 48 h.
Figure 4.32. The effect of compound N on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound N for 24 h.

Figure 4.33. The effect of compound N on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound N for 48 h.
Figure 4.34. The effect of compound N on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of compound N for 24 h.

Figure 4.35. The effect of compound N on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of compound N for 48 h.
Figure 4.36. The effect of compound O on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg μM of compound O for 24 h.

Figure 4.37. The effect of compound O on MDA-MB-231 cell growth. The cells were treated with 1 and 5 mg of compound O for 48 h.
Figure 4.38. The effect of compound O on MCF-7 cell growth. The cells were treated with 1 and 5 mg of compound O for 24 h.

Figure 4.39. The effect of compound O on MCF-7 cell growth. The cells were treated with 1 and 5 mg μM of compound O for 48 h.
Figure 4.40. The effect of compound O on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound O for 24 h.

Figure 4.41. The effect of compound O on BT-474 cell growth. The cells were treated with 1 and 5 mg of compound O for 48 h.
Figure 4.42. The effect of compound O on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of compound O for 24 h.

Figure 4.43. The effect of compound O on SK-BR-3 cell growth. The cells were treated with 1 and 5 mg of compound O for 48 h.
4.3.2. Morphological studies

4.3.2.1. The effect of the GNPs and their ligands on apoptosis

Apoptosis as a programmed cell death has many characterizations including nuclear DNA fragmentation, chromatin condensations, formation of apoptotic bodies and cell shrinking. (Vinatier, 1996; Raffray, 1997). According to the cell anti-proliferation results, compound M was the most effective as anti-breast cancer agent. So, more studies were needed to explain the effect of this GNP and its ligands on MDA-MB-231 cell line apoptotic process.

The main morphological features of apoptosis are the formation of apoptotic bodies and nuclear DNA fragmentation. Chen (2009) indicated that morphological changes can be observed using a specific chromatin dyes like Hoechst 33342, this stain results in the detection of the apoptotic morphological changes using a fluorescence microscope with a blue filter. DNA fragmentation can be detected using DNA electrophoresis.

The apoptotic morphological changes were determined after 48 h treatment period at the concentration of 5 mg of each compound. Then the cells were stained in Hoechst for 30 min in dark. The morphological changes were similar to the changes produced by 20 µg/ml crude methanolic extract of the pericarp of *Garcinia mangostana* after 48h treatment (Moongkarndi *et al.*, 2004). The morphological alterations of compounds K, L, and M are illustrated in figure 4.44.
Figure 4.44. Morphological and cellular changes induced by the tested GNPs and its ligands. The effects of these compounds on cell morphology were tested with the fluorescent chromatin dye Hoechst 33342 in MDA-MB-231 cells. Cells were treated with three compounds (Compounds K: M) for 48 h and then stained with the chromatin dye for 30 min. The two panels are MDA-MB-231 cells, the top panel is a control without any treatment and the lower panel is for the treated cells.
4.3.3. Apoptotic DNA fragmentation analysis

Because DNA fragmentation is the hallmark of apoptotic cells, DNA fragmentation analysis was done using the apoptotic DNA ladder kit (Roche). The DNA fragmentation effects of compounds K, L, and M on the MDA-MB-231 cell line were studied. The cells were treated for 48 h with 5 mg of the each compound. Then, 20 µg DNA sample of each treatment was subject to 1% agarose gel electrophoresis. The DNA electrophoretogram (see figure 4.45) indicates that only the modified gold nanoparticles, compound M, caused fragmentation of the cell’s DNA (lane 6). Neither, Compound K nor compound L showed any DNA fragmentation (lane 4 and 5).

*Figure 4.51.* Electrophoretogram demonstrating the apoptotic DNA fragmentation analysis of K, L, and M compounds on MDA-MB-231 cells. Cells were treated for 48 h with 5 mg of the compounds using 1% agarose gel. DNA marker (lane 1), positive control (lane 2), untreated cells (lane 3), treated cells with Com. K (lane 4), treated cells with Com. L (lane 5), treated cells with Com. M (lane 6).
4.4. Discussion

In this study, two modified gold nanoparticles derivatives and their ligands were tested for their anti-breast-cancer activity against three metastatic breast cancer cell lines (MDA-MB-231, MCF-7, and SK-BR-3) and one BT-474 non-metastatic breast cancer cell line. The MTT assay screening results for the K, L, M, N, and O compounds indicated that all the compounds except compound M, showed little to no cytotoxicity against the four breast cancer cells after treatment periods of 24h and 48h.

The two tested concentrations 1 and 5 mg of the modified gold nanoparticle compound M showed moderate anti-breast-cancer activity after a 24 h treatment period. However, after 48 h treatment compound M showed strong activity against MCF-7 and BT-474 breast cancer cells with IC$_{50}$ values of 4.2 and 7.3 mg, respectively. It also showed very strong activity against MDA-MB-231 and SK-BR-3 cells with IC$_{50}$ values of 1 and 3.2 mg, respectively.

Western blot test results performed in part 2.3.1. indicated that the MDA-MB-231 breast carcinoma cell line not over express the HER-2 or ER protein. The MCF-7 cell line expresses ER for its growth and proliferation. But, BT-474 breast carcinoma cell line expresses both of HER-2 protein and ER for its growth. Finally, the SK-BR-3 cell line expresses only HER-2 protein for its growth. From these results, the modified gold nanoparticle compound M, was found to be a very strong anti-breast-cancer agent against the non-ER over-expressing cell lines. The HER-2 and ER screening results performed in this study were unable to answer the selectivity of the modified gold nanoparticles for compound M. However, it does need further investigations that explore the efficacy of this GNP on the cell cycle and its related regulator proteins.
The pro-apoptotic effects of compound M on the MDA-MB-231 cell line were studied using the fluorescent chromatin dye Hoechst 33342 and DNA fragmentation tests. Compound M was found to be cytotoxic against the MDA-MB-231 breast cancer cell line, including apoptotic bodies formation and DNA fragmentation.

4.5. Conclusion

- Two modified gold nanoparticles and their ligands have been evaluated for their anti-breast-cancer activities.

- Compound M showed cytotoxicity against all of the breast carcinoma cell lines used in this experiment with IC$_{50}$ values ranging between 1 to 7.3 mg.

- Compound M was shown to be more effective against the non-ER over-expressing breast carcinoma cell lines.

- Further investigations are needed to explore the efficacy of this GNP on the cell cycle and its related regulator proteins.
4.6. References


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CHAPTER 5

INVESTIGATING THE TOXICITY OF THE TESTED FLAVONOIDS, COUMARINS AND MODIFIED GOLD-NANOPARTICLES ON NORMAL BREAST CELLS

5.1. Introduction

Although chemotherapy is a very effective way to fight cancer, it is still very unpleasant for the patient, with treatment periods of up to five years, and has a lot of very risky side effects such as bone loss and cardiac toxicity. (Hurria, 2003; Chargari, 2011; Schimmel, 2004). The problems related to chemotherapy have pushed people to not complete their treatment course. A challenge in cancer therapy is to find new anticancer drugs with less side effects and very high specificity toward the cancer cells (Galati, 2004). But even the most selective anti-breast cancer drugs, such as Trastuzumab (a monoclonal antibody) and Lapatinib (a synthetic chemical agent) which target specific protein like the monoclonal antibody, are associated with cardiac failure (Schimmel, 2004; Chargari, 2011). To overcome such problems many researchers have focused on natural products and their derivatives which have been playing an important role in the drug industry, especially over the last 50 years. Out of the 155 new FDA-approved anticancer drugs, 47% of them are natural products or natural product derivatives (Newman 2007). The tested compounds used in this study are: flavonoids, coumarins and coumarins as ligands on modified gold nanoparticles. All of these are phenolic compounds, and phenolic compounds are the most plentiful and broadly distributed fragments in the plant kingdom (Shi 2003). When these compounds are found to have
anti-breast-cancer activity, it becomes important to verify their toxicity on normal breast cells. The objective of this study is to determine the toxicity of all of these tested compounds on normal breast cells.

5.2. Materials and methods

5.2.1. Cell lines and culture conditions

Normal breast cell line MCF-10F line was obtained from the American Type Culture Collection (ATCC Manassas, VA). All cells were grown in Dulbecco’s Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12) (Gibco, Grand Island, NY) supplemented with 10% v/v fetal bovine serum, 1% v/v L-glutamine, 1% v/v antibiotic, penicillin-streptomycin solution (Sigma-Aldrich), 0.37% v/v sodium bicarbonate and mammary epithelial cell growth medium kit (MEGM)( 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone, 95%; chelex-treated horse serum, 5%) (Lonza, Allendale, NJ). The cells were incubated at 37 °C and under 5% CO₂ in a humidified Fisher Scientific incubator. All cells received fresh media 2 to 3 times/week and were sub-cultured when they reach 70-80% confluence. First, the culture medium was removed and the adherent cells were washed with 1X versene (Invitrogen, 0.2 g/L EDTA.4Na in phosphate-buffered saline) then the cells were detached using 1X trypsin-EDTA (Invitrogen, 0.25% trypsin, 0.1% EDTA, pH 7.2-8), followed by 5-15 minutes incubation at 37°C and 5% CO₂ in a humidified incubator. Cells were re-suspended in fresh media and counted with a hemocytometer using trypan blue dye (0.47% in phosphate-buffered saline) and seeded in new tissue culture treated flasks or 96-well microplates.
5.2.2. Cellular anti-proliferation activity using the MTT cytotoxicity assay

The effect of the tested compounds A to O on normal breast cell line MCF-10F cells proliferation was measured by using the 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay (MTT). The experiment was been repeated 2 to 3 times, in triplicate for each concentration of 200, 300 μM or 1 and 5 mg for each compound, as described previously (Chen et al., 2009; Xiong et al., 2010; Chae et al., 2011; Goel and Gude, 2011; Zhou et al., 2011). The cells were grown in 96-well, flat bottom tissue culture treated microplates, at the density of 1 x 10^4 for 2 days at 37°C and under 5 % CO₂ in a humidified incubator to promote cellular adhesion. The compounds were dissolved in DMSO and diluted in complete media to a final concentration of 1% to the desire testing concentration. After the incubation period, old media was removed and the adherent cells were first washed with 1X versene then 200 μl new complete media with the compounds were incubated in the same condition. Control wells received only the media and DMSO. MTT salt solution (20 μl) at a final concentration of 0.5 mg/ml was added to each well and incubated for a period of 5 h. After the incubation period, the whole solution in each well was replaced with 200 μl of DMSO to dissolve the formed formazan crystals. The optical density of each well was read at 560 nm and reference wavelength at 650 nm on a Tecan infinity F500 microplate reader (Tecan System Inc., San Jose, CA, USA).

The % of cell viability = \( \frac{OD \text{ of treated cells}}{OD \text{ of control cells}} \times 100 \)
5.3. Results

The toxicity of three groups of compounds was tested on normal breast cells MCF-10F. The first group consists of five flavonoids compounds, A to E. These compounds were tested for a 48 h period at concentration of 200 and 300 μM. All the flavonoids compounds showed no toxicity against the normal breast cells: see figures 5.1-5.5 for compounds A, B, C, D and E, respectively. The second group of compounds tested consists of five coumarin derivatives. Compound F was found to be very cytotoxic against the normal breast cells at the concentrations of 15 and 20 μM after a 48 h treatment period. Compound F killed 99 to 100% of the normal breast cells at these conditions (see figure 5.6). Compound F was equally toxic to both of the breast cancer cells and the normal cells. Compounds G and H were found to be non toxic against the normal cell when tested for 48 h at concentrations of 200 and 300 μM (see figure 5.7 and 5.8). Compounds I and J were somewhat toxic against the normal breast cell at the same above conditions. The breast cells lost 9 to 17% of their viability when exposed to compound I and J respectively (see figure 5.9 and figure 5.10). The third group of compounds was tested against normal breast cells for 48 h treatment period at concentrations of 1 and 5 mg from each compound. Compounds K, L, M, N, and O were shown to be non toxic under these conditions (see figure 5.11 to 5.15).
Figure 5.1. The effect of compound A on MCF-10F cell growth. The cells were treated with 200 and 300 μM of compound A for 48 h.

Figure 5.2. The effect of compound B on MCF-10F cell growth. The cells were treated with 200 and 300 μM of compound B for 48 h.
Figure 5.3. The effect of compound C on MCF-10F cell growth. The cells were treated with 200 and 300 μM of compound C for 48 h.

Figure 5.4. The effect of compound D on MCF-10F cell growth. The cells were treated with 200 and 300 μM of compound D for 48 h.
Figure 5.5. The effect of compound E on MCF-10F cell growth. The cells were treated with 200 and 300 μM of compound E for 48 h.

Figure 5.6. The effect of compound F on MCF-10F cell growth. The cells were treated with 15 and 20 μM of compound F for 48 h.
Figure 5.7. The effect of compound G on MCF-10F cell growth. The cells were treated with 200 and 300 μM of compound G for 48 h.

Figure 5.8. The effect of compound H on MCF-10F cell growth. The cells were treated with 200 and 300 μM of compound H for 48 h.
Figure 5.9. The effect of compound I on MCF-10F cell growth. The cells were treated with 200 and 300 μM of compound I for 48 h.

Figure 5.10. The effect of compound J on MCF-10F cell growth. The cells were treated with 200 and 300 μM of compound J for 48 h.
**Figure 5.11.** The effect of compound K on MCF-10F cell growth. The cells were treated with 1 and 5 mg of compound K for 48 h.

**Figure 5.12.** The effect of compound L on MCF-10F cell growth. The cells were treated with 1 and 5 mg of compound L for 48 h.
Figure 5.13. The effect of compound M on MCF-10F cell growth. The cells were treated with 1 and 5 mg of compound M for 48 h.

Figure 5.14. The effect of compound N on MCF-10F cell growth. The cells were treated with 1 and 5 mg of compound N for 48 h.
Figure 5.15. The effect of compound O on MCF-10F cell growth. The cells were treated with 1 and 5 mg of compound O for 48 h.
5.4. Discussion

The most important question for anyone who is working with any anticancer agent is, is this agent toxic against the normal cells? In this study, three groups of compounds have been tested against normal breast cells at the same conditions used in tests against breast cancer cells in chapters 2, 3, and 4. The first group of compounds consists of five flavonoids, which were non toxic against the tested breast normal cell line MCF-10F. From the literature it was clear that these flavonoids as a poly-phenolic class of compounds are found in many plants as secondary metabolites (Chang 2008; Cermak 2001). Such phenolic compounds are the most abundant compounds in the plant kingdom and are therefore present in the human daily diet (Shi, 2003; Tapiero, 2002). The estimated daily total flavonoids intake is 189.7 mg/day, and the main source is from tea 157 mg/day (Chun, 2007). The tea extracts and tea polyphenolic compounds were shown to be preventive agents against many types of cancer (Yang, 2008). All the previous literature supported the proposition that flavonoids, even with the high daily consumption, are not toxic and often act as preventive agents.

The second tested group of compounds is coumarin derivatives, compounds F, G, H, I, and J. Compound F was very toxic against the normal cells, which may be due the presence of the cyano group in this compound. Bhattacharya (1997) indicated that cyanide compounds have acute toxicity and caused DNA damage when tested on mammalian cell cultures. On the other hand, the other four coumarin compounds have no cyano group and they have variable toxicity on the normal breast cells. Compounds G and H were nontoxic but compound I and J were found to be somewhat toxic against
normal breast cells. Compound I is less toxic than Compound J. The only difference between them is that the hydroxyl group at carbon atom number 7 in compound I is substituted by an acetate group in compound J. Perhaps the presence of the hydroxyl group helps the compound to act as free radical scavenger compared to the ester, so that it works as a preventive group (Lin, 2008). The toxicity of any compound depends on its structure. Some coumarin compounds are used as antibiotics such as novobiocin and clorobiocin (Laurin, 1999) and other coumarin compounds are toxic (Sashidhara, 2010).

The third group of compounds consists of the modified gold nanoparticles and their ligands. All the compounds, K, L, M, N, and O were found to be nontoxic against the normal breast cell line MCF-10F. According to the anti-breast cancer results for these compounds in chapter IV, it was found that only compound M was toxic and the others were not. A comparison of the toxicity of these compounds against normal cells indicates an advantage in using the gold nanoparticles for cancer treatment, because GNPs are not toxic for human cells (Jain, 2007).
5.5. References


CHAPTER 6
SUMMARY AND FUTURE WORK

6.1. Summary

Breast cancer is the most common cancer in women globally and in The United States of America where the estimated worldwide new breast cancer cases and the number of deaths are 1.38 million and 458 thousand, respectively. Currently, in the USA there are 2.6 million women have been treated for breast cancer and the estimated number of deaths are 39,520 in 2012. The 47 approved-FDA anti-breast cancer drugs in the US markets have a lot of side effects. Furthermore, the most selective anti-breast cancer drugs Trastuzumab and Lapatinib are associated with cardiac failure. This indicates a need for new anti-cancer drugs to overcome the side effect problems and the drug resistance problems.

Polyphenolic compounds are abundant natural products found in many plants as secondary metabolites. These compounds display many interesting pharmacological activities, which have motivated chemists over the years to explore these natural products, and their synthetic derivatives as potential drugs, especially anticancer drugs. To cover many bases in this study, we investigated the anti-breast cancer activity of some flavonoids as a natural products group, synthetic coumarin compounds and even modified gold nanoparticles. This investigation was carried out using both metastatic and non-metastatic breast cancer cell lines to study the difference of the anti-breast cancer activity against them. To understand possible side effects, we also investigated the toxicity of all
the tested compounds on normal breast cells. The first group of compounds consists of
the five flavonoids, myricetin (compound A), quercetin (compound B), luteolin
(compound C), apigenin (compound D), and kaempferol (compound E). Using the
standard anticancer activity test MTT assay, all the five flavonoids did not show any
activity after 24 h of treatment. Furthermore, increasing the treatment period up to 48 h
all the flavonoids showed various anti-breast cancer activities. Compound A suppressed
the growth of the four breast cancer cell lines MDA-MB-231, MCF-7, BT-474 and SK-
BR-3 with IC\textsubscript{50} values of 111, 208, 192 and 139 μM respectively. To compare the
efficacy of compound A on all the cells, the growth inhibition percentage is shown in
figure 6.1. Compound B inhibited the growth of MDA-MB-231, MCF-7, BT-474 and
SK-BR-3 cells with IC\textsubscript{50} values of 40, 49.5, 291 and 145 μM respectively. The growth
inhibition percentage is shown in figure 6.2. Compound C suppressed the growth of the
four breast cancer cell lines MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC\textsubscript{50}
values of 100, 91, 98 and 141 μM respectively. The growth inhibition percentage is
shown in figure 6.3. Compound D showed suppression the four breast carcinoma cell
lines, MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC\textsubscript{50} values of 57, 38, 61 and
46 μM respectively. The growth inhibition percentage is shown in figure 6.4. Compound
E suppressed the growth of the four breast cancer cell lines MDA-MB-231, MCF-7, BT-
474 and SK-BR-3 with IC\textsubscript{50} values of 50, 81, 90 and 75 μM respectively. The growth
inhibition percentage is shown in figure 6.5.
Figure 6.1. The growth inhibition percentage by compound A on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.

Figure 6.2. The growth inhibition percentage by compound B on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.
Figure 6.3. The growth inhibition percentage by compound C on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.

Figure 6.4. The growth inhibition percentage by compound D on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.
Figure 6.5. The growth inhibition percentage by compound E on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.

The most important thing about these results is that the anti-breast cancer activity is reported for these five flavonoids on BT-474 and SK-BR-3 breast carcinoma cell lines. Furthermore, the MTT assay results indicate that compound D is the most powerful anti-breast cancer agent, followed by compound C, E, B, and A. These results are in accordance with those reported by Plochmann (2007), who indicated that there are three structural features controlling the anticancer activity of flavonoids. One is the double bond between carbon atoms 2 and 3, (as indicated in chapter 2) which exist in all the flavonoids compounds. Secondly, increasing the hydroxyl group counts in flavonoids decreases the anticancer activity, especially the one at the carbon atom number 3 (as illustrated in chapter 2). Compound D has no hydroxyl group at carbon 3 and has the lowest hydroxyl groups count as well. On the other hand, both of compound B and A have a hydroxyl group at carbon 3 and have the highest hydroxyl groups count. This may help to interpret the MTT assay results. Compounds C, D, and E especially showed many pro-apoptotic activities such as cell shrinking, formation of apoptotic bodies and
fragmentation of DNA. Additionally, all the tested flavonoids were safe without any toxicity against the normal breast cell line MCF-10F.

The second group of the tested compounds consists of five coumarins, compounds F, G, H, I, and J. These were tested against the previous four breast cancer cell lines and were not toxic after 24 h of treatment. With increasing the treatment period up to 48 h were showed various activity. Compound F was very toxic against all the cells at the concentration of 25, 50, 100, 200, and 300 µM after 48 h of treatment. Further cytotoxicity investigations were done with lower concentrations of 1, 5, 10, 15, 20 µM. Compound F has anticancer activity against the four breast cancer cell lines MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC₅₀ of 11.3, 10.13, 12.08, and 13.75 µM respectively (figure 6.6). Compound G showed selectivity for only the ER +, MCF-7 and BT-474 cell lines with IC₅₀ of 80 and 118 µM, respectively. However, it has lower activity against the MDA-MB-231 and SK-BR-3 cell lines with IC₅₀ of >200 and 141 µM respectively (figure 6.7). Compound H was selective for only MCF-7 and BT-474 cell lines with IC₅₀ of 152 and 165 µM, respectively. It has lower activity against MDA-MB-231 and SK-BR-3 cell lines with IC₅₀ of >200 and 187 µM respectively figure 6.8. Compound I showed anticancer activity against the MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 cell lines with IC₅₀ of 129, 88, 134, and 192 µM respectively figure 6.9. Compound J showed anticancer activity against the MDA-MB-231, MCF-7, BT-474 and Sk-BR-3 cell lines with IC₅₀ of 141, 91.9, 121.9, and > 200 µM respectively figure 6.10.

This group of coumarin compounds showed pro-apoptotic activity such as cell shrinking, formation of apoptotic bodies and fragmentation of DNA. Furthermore, the most important test is the toxicity of these compounds on normal breast cells.
Compounds F, I, and J were toxic against the normal breast cell line MCF-10F. Compounds G and H were safe without any toxicity against the normal breast cell line MCF-10F.

*Figure 6.6.* The growth inhibition percentage by compound F on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.
Figure 6.7. The growth inhibition percentage by compound G on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.

Figure 6.8. The growth inhibition percentage by compound H on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.
Figure 6.9. The growth inhibition percentage by compound I on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.

Figure 6.10. The growth inhibition percentage by compound J on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells.
The third group of compounds tested was the modified gold nanoparticles and their ligands. This group consists of five compounds K, L, M, N, and O. Compounds K, L, N, and O were not toxic against all the four breast carcinoma cell lines after 24 h and 48 h of treatments. Only the modified 2nm gold nanoparticles compound M showed anti-breast cancer activity after both of 24 h and 48 h of treatment at concentrations of 1 and 5 mg. It showed cytotoxicity activity against the four breast cancer cell lines MDA-MB-231, MCF-7, BT-474 and SK-BR-3 with IC$_{50}$ of 1, 4.2, 7.3, and 3.2 mg respectively. The growth inhibition percentage after 24 h and 48 h of treatment is shown on (figures 6.11 and 6.12). Furthermore, the modified gold nanoparticles showed pro-apoptotic activities such as cell shrinking, formation of apoptotic bodies and fragmentation of DNA. Additionally, Compound M was 100% safe on the normal breast cancer cells MCF-10F.

![Graph showing growth inhibition percentage by compound M on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells after 24 h of treatment.](image)

Figure 6.12. The growth inhibition percentage by compound M on MDA-MB-231, MCF-7, BT-474, and SK-BR-3 cells after 48 h of treatment.

In summary, the anti-breast-cancer activity of 15 compounds have been investigated. These 15 compounds consist of natural products, synthetic compounds and modified gold nanoparticles. This investigations which was carried out against four breast cancer cell lines including metastatic and non-metastatic cells, leads to the following conclusion. Out of the 15 compounds, 11 compounds showed anti-breast cancer activities. Not all of these compounds were safe on normal breast cells. Only six compounds showed promising anti-breast-cancer activities without any toxicity on normal breast cells. These included three flavonoids (C, D, and E), two coumarins (G and H), and the 2 nm modified gold nanoparticles M (see table 6.1) and their IC50 values are shown in table 6.2.
Table 6.1

Summary table for all the data after 48 h of treatment

<table>
<thead>
<tr>
<th>Compound</th>
<th>Anti-breast Cancer activity</th>
<th>DNA Fragmentations</th>
<th>Apoptotic bodies formations</th>
<th>Normal breast toxicity</th>
<th>Promising compound</th>
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<tr>
<td>K</td>
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</tr>
<tr>
<td>L</td>
<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>N</td>
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<tr>
<td>O</td>
<td>-</td>
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</table>

*Note.* + indicates positive activities and - indicates negative activities.
Table 6.2
IC₅₀ values of all the tested compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Breast cancer cell lines</th>
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<td>BT-474</td>
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<td>139</td>
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<tr>
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<td>98</td>
<td>141</td>
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<tr>
<td>D</td>
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<td>46</td>
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<tr>
<td>E</td>
<td>50</td>
<td>81</td>
<td>90</td>
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<td>10.13</td>
<td>12.08</td>
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<tr>
<td>G</td>
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<td>80</td>
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<td>141</td>
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<tr>
<td>H</td>
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<tr>
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<td>O</td>
<td>&gt;50</td>
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</table>

Note. All the IC₅₀ values are in μM except for compounds K, L, M, N and O are in mg.
6.2. Future Work

Cancer cells have many growth pathways, which are hard to study in one project. Therefore, there is more work that needs to be done to explore the multiple-pathways cancer cells may follow when another is blocked, such future projects may include

1) Explore the molecular anti-cancer targets such as Cytochrome C, p53 and PARP.

2) Explore the activity of the tested flavonoids, coumarins and modified gold-nanoparticles on some enzymes that are involved in cancer growth pathways, such as COX-1 and 2, topoisomerase, aromatase and sulfotransferase.

3) Try to modify the cyano group in compound F, attaching it to gold nanoparticles and investigate its cytotoxicity.

4) Try to modify the modified gold-nanoparticle with another functional moiety such as folate or estrogen to use the GNP as a delivery system.

5) Investigate possible synergetic activities of the tested compounds when applied together or with synthetic compounds.

6) Investigate the anticancer activity of these compounds using other cancer types that are ER dependent.

7) Test the efficacy of the tested compounds on the cell’s cycle and its related regulator proteins.

8) Perform in vivo studies.