COMPARATIVE EFFECTS OF EMODIN ON BIOLOGICAL ACTIVITIES OF MCF-7 AND MDA-231 CELL LINES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ELİF SAKALLI

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOCHEMISTRY

DECEMBER 2010

Approval of the thesis:

COMPARATIVE EFFECTS OF EMODIN ON BIOLOGICAL ACTIVITIES OF MCF-7 AND MDA-231 CELL LINES

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ABSTRACT

COMPARATIVE EFFECTS OF EMODIN ON BIOLOGICAL ACTIVITIES OF MCF-7 AND MDA-231 CELL LINES

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December 2010, 104 pages

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a phytoestrogenic component of *Rheum* plant extracts which has been used for medical treatment since ancient times. It has been shown to have anti-inflammatory and anti-cancer effects. In our research, we aimed to study the biological effects of emodin on MCF-7 and MDA-231 cell lines.

Cytotoxicity assays showed that emodin treatment for 48hours caused a concentration dependent decrease in viable cell numbers of both cell lines. As determined by cell counting with tryphan blue, IC50 values were 8.40 and 12.17 µg/ml for MCF-7 and MDA-231 cells, respectively.

Apoptotic effects of emodin was investigated by measuring the changes in apoptotic and antiapoptotic gene expressions by qRT-PCR. In MCF-7 cells, Bax expression increased with increasing emodin concentrations, while Bcl-2 expression was downregulated. Bax/Bcl-2 ratio was calculated as 9.2 fold at 10µg emodin/ml treatment for 48 hours, indicating stimulation of apoptosis. However, Bax/Bcl-2 ratio was found 1.6 fold for MDA-231 cells. These results were in accordance with the results obtained from microarray analysis of related gene expressions. MCF-7 cells were more apoptotic in comparison to MDA-231 cells. DNA fragmentation was observed in MCF-7 cells by TUNEL method.

GST enzyme activity that was measured using CDNB as substrate, was increased 100% with respect to control up to 5μg emodin/ml treatment of MCF-7 cells for 48 hours. However, effect of emodin on GST activity in MDA-231 cells was found insignificant. According to microarray analysis results, emodin affected the gene expressions of GST isozymes in MCF-7 cells much more than in MDA-231 cells.

Keywords: Emodin, MCF-7 MDA-231, cytotoxicity, apoptosis, glutathione S-transferase, microarray analysis.

ÖZ

EMODİNİN MCF-7 VE MDA-231 HÜCRE HATLARININ BİYOLOJİK AKTİVİTELERİ ÜZERİNDEKİ KARŞILAŞTIRMALI ETKİLERİ

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Aralık 2010, 104 sayfa

Emodin (1,3,8-trihidroksil-6-metilantrakuinon), eski zamanlardan beri tıpta tedavi için kullanılan bitkisel ilaçların bir bileşeni olup, *Rheum* bitkilerinde bulunan, enflamasyona ve kansere karşı etkiler gösteren bir fitoestrojendir. Biz araştırmamızda, emodinin MCF-7 ve MDA-231 hücre hatlarındaki etkilerini karşılaştırmalı olarak inceledik.

Hücre toksisitesi testleri ve inverted mikroskop görüntüleri, emodinin 48 saat süreyle, artan konsantrasyonlarda, hücre sayısını her iki hücrede de azalttığını gösterdi. Tryphan blue boyasıyla ile hücre sayımıyla IC50 değerleri MCF-7 hücresinde 8.40µg/ml, MDA-231 hücresinde 12.17µg/ml olarak hesaplandı.

Eş zamanlı polimeraz zincir reaksiyonu metoduyla belirlenen gen ekspresyon analizlerine göre, 48 saat süreyle artan konsantrasyonlarda emodin uygulanmasıyla, MCF-7 hücrelerinde Bax gen ifadesi artarken, Bcl-2 gen ifadesi azaldı. Bax/ Bcl-2 oranı, 10 µg emodin/ml uygulamasında, MCF-7 hücresinde 9.2, MDA-231 hücresinde 1.6 olarak belirlendi. Bu sonuç emodinin MCF-7 hücresini apoptoza götürdüğünü gösterdi. Mikrodizin analizinde ilgili gen ifadelerinde benzer değişiklikler saptandı. TUNEL yöntemiyle, konfokal mikroskop kullanılarak, MCF-7 hücresinde DNA kırılmaları gözlendi.

Emodin, 48 saat süreyle, MCF-7 hücrelerinin CDNB substratı kullanılarak ölçülen sitozolik GST enzim aktivitelerini MDA-231 hücrelerine göre önemli oranda daha çok etkiledi. 5µg/ml emodin konsantrasyonuna kadar enzim aktivitesi kontrole göre %100 oranında artarken, yüksek emodin konsantrasyonlarında enzim aktivitesi kontrol seviyesine düştü. MDA-231 hücrelerinde ise emodinin çalışılan konsantrasyonlarında istatistiksel olarak anlamlı değişiklikler yapmadığı belirlendi. Mikroarray analizi sonuçlarına göre, emodin GST izozimlerinin gen ifadelerini MCF-7 hücresinde, MDA-231 hücresine göre çok daha fazla oranda değiştirdi.

Anahtar Kelimeler: Emodin, MCF-7 MDA-231, sitotoksisite, apoptoziz, glutatyon S-transferaz, mikrodizin analizi

To My Family…

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Prof. Dr. Mesude İŞCAN for her valuable guidance, incredible patience, critical discussions and continued advice throughout this study.

I am deeply thankful to Examining Committee Members: Prof. Dr. Tülin Güray, Assoc. Prof. Dr. Nursen Çoruh, Assist. Prof. Dr. Çağdaş D. Son, Assist. Prof. Dr. Belgin İşgör for their valuable comments.

I have special thanks to Pembegül Uyar for her support thoughout the study and also for her friendship, I have learned lots from her. I have also thankful to my labmates, Metin Konuş, Can Yılmaz, Melih Dağdeviren, Elif Güler, Elif Aşık, Emilia Ekenel, Selis Yılmaz and also to Yeşim Kümbet, Şule Şahin, Aslıgül Aksoy, Özge Kaya, Tuğba Çulcu, Deniz Hisarlı, Deniz İrtem, Derya Dilek, Gözde Çıtır, Emrah Sağır, Serdar Karakurt, Rafiq Gulbanov and to Şafak Tecim for their endless support and friendship.

I would like to express my sincere gratitude to my parents Funda and İhsan Sakallı whom also deserve a certificate, and to my sister Fulya Akbuğa whom guided me through all my life, and also her husband Yusuf Akbuğa, their endless love, and support gave me the strength to complete the study.

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LIST OF SYMBOLS AND ABBREVIATIONS

AP-1: Activator protein 1

ARE: Antioxidant Response Element

Bax: Bcl-2–associated X protein

Bcl-2: B-cell lymphoma 2

Caspase: Cysteine-dependent aspartate-directed proteases

CDNB: 1-Chloro-2,4-dinitrobenzene

DMSO: Dimethyl sulfoxide

ER: Estrogen Receptor

ERE: Estrogen Response Element

GSH: Reduced glutathione

GST: Glutathione S-transferases

HER-2: Human epidermal growth factor receptor 2

IC50: Inhibitory Concentration 50

Lck: Lymphocyte-specific protein tyrosine kinase

p53: Protein 53

ROS: Reactive Oxygen Species

RMA: Robust Multiarray Analysis

MMP: Matrix metalloproteinase

MCF-7: Michigan Cancer Foundation - 7

NFkB: Nuclear Factor-kappa B

MDA-MB-231: Monroe Dunaway Anderson - Metastatic Breast

qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction

TUNEL: Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling

XTT:2,3-bis[2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide inner salt

CHAPTER 1

INTRODUCTION

1.1 CANCER

Cancer is the group of diseases forming from the uncontrolled growth and proliferation of cells and it is one of the main causes of death. According to World Health Organization global cancer deaths are expected to increase 45% from 7.9 million to 11.5 million deaths from 2007 to 2030. Cancer is the second cause of all deaths after cardiovascular diseases in developed countries. Lung cancer has the highest incidence. Breast, colon, liver, prostate, cervical, stomach are the other common cancer cases (http://www.who.int/features/qa/15/en/). In Turkey, breast cancer was the most common cancer found in woman in 2002 and 2005 (https://apps.who.int/infobase/report.aspx).

Environmental carcinogens as in the case of the chemicals in the cigarette smoke, hormonal carcinogens e.g. estrogen, pharmacologic carcinogens e.g. antineoplastic drugs, dietary carcinogens e.g. nitrosamines, metal carcinogens e.g. nickel, physical carcinogens e.g. UV irradiation, ionizing radiation, biological carcinogens e.g. viruses and genetic factors are the common risk factors for cancer development. Over production of the oncogenes that promotes the growth, repression of the tumor suppressor genes or failure of DNA repair mechanisms are the reasons for cancer development.

Cancer treatment studies are being continued and lots of progress have been made but the best approach to avoid cancer is the correct life style to prevent it since it

seen that 40% of all types of cancer can be avoided by just practicing the proper life style.

1.1.1 BREAST CANCER

Breast cancer forms in breast tissues like ducts, tubes that carry milk to nipple and lobules, glands that make milk. It is the most common cause of cancer death among women. There are risk factors but still women who have risk factors may not develop the disease and women who have no risk factors may develop it. Risk factors include personal history of breast abnormalities, people having these abnormalities have increased risk of cancer; familial history, if person's mother, sister or daughter had breast cancer the risk increases; age, as all the other cancer types the risk of breast cancer increases with age, because of accumulation of mutations and seen mostly in women older than age 50; age at first live birth, giving birth at younger age decreases the risk; race, Caucasian women have higher risk than African American women; other risks are hormonal therapy, age at menapause, birth control pills, high body mass index, radiation, pollutants from the environment (http://www.cancer.gov/cancertopics/factsheet/estimating-breast-cancer-risk).

In addition to the reasons for general cancer development, it is known that breast cancer is hormon dependent tumor and estrogen plays major role for the formation. Nearly 70% of breast tumors express estrogen receptor (ER) and estrogen maintain the proliferation (Massarweh and Schiff, 2006).

1.2 ESTROGEN

Estrogens are a class of steroid hormones and have important roles in reproductive development and normal sexuality in women. Estrogens are synthesized from cholesterol and secreted mainly from the ovaries and also from testes, adrenal glands, adipose tissue, and placenta. Estrogen has important roles in female reproductive system and necessary for proliferation and differentiation of normal breast epithelium (Lewis and Wambi, 2009). Effects of estrogen can be classified as

follows; in brain delays memory loss; in heart and liver, decreases the formation of plaque in the coronary arteries by regulating the liver's production of cholesterol; in ovaries, stimulates the maturation of the ovaries and start of the menstrual cycle; in vagina, estrogen stimulates the maturation of the vagina; in bones helps to maintain bone density; in uterus, stimulates the maturation of the uterus and prepare it to gain nourishment for the fetus; in breast, estrogen stimulates the development of breasts and milk production (www.healthsystem.virginia.edu).

1.2.1 MECHANISM OF ESTROGEN ACTION

Estrogen exerts its actions through estrogen receptors (ER) which are DNA binding transcription factors and have roles in gene expression which are mostly participated in proliferation. Estrogen receptors may present in the cell membrane, cytoplasm or in the nucleus. There are two different forms of ER, ER α and β, encoded by different genes, on sixth chrmosome *ESR1* and on fourteenth chromosome *ESR2*, respectively. They have different concentrations and localizations in our body. Interactions of the ligand with these receptors determines the action in that tissue. In breast both ER α and β are present. It is seen that ER α and β behave differently at activator protein-1 (AP-1) enhancer element site of the gene upon binding of estrogen, where ER α activates transcription, whereas ER β inhibits transcription but they show similar transcription patterns at ERE (estrogen response element) (Paech et al., 1997). Also it is shown that ER β has lower affinity to estrogen than ER α (Kuiper et al., 1997). Transactivation by estrogen is higher for ER α than ER β (Mosselman et al., 1996). ER β may inhibit proliferation and inhibit growth effects of ER α so ER β may protect against breast cancer (An et al., 2001; Strom et al., 2004).

As it is shown in Figure 1.1, there are two mechanisms of ER for regulating gene expression. First one is direct mechanism named as genomic action through nuclear receptors and estrogen receptor shows its effect directly in the nucleus as a transcription factor (Nemere et al., 2003). Being a steroid hormone, estrogen diffuses across the cell membrane and binds to estrogen receptors in the nucleus,

upon binding, phosphorylation and dimerization occurs, coactivators bind and induce receptor complex to bind promoter regions of target genes and finally increase trancriptional activity. Genes related to division, cell growth, differentiation, survival are expressed. Because of these, if there is estrogen induced over expression of ER, this promotes breast cancer formation and progression (Osborne et al., 2001; Schiff et al., 2005; Lewis and Jordan, 2009).

Second mechanism is nongenomic action of ER. ER found in cell membrane or cytoplasm may initiate cellular signalling by interacting with signalling pathways of some tyrosine kinases, growth factors, G-proteins and finally these cause growth and survival effects of estrogen indirectly (Nemere et al., 2003).

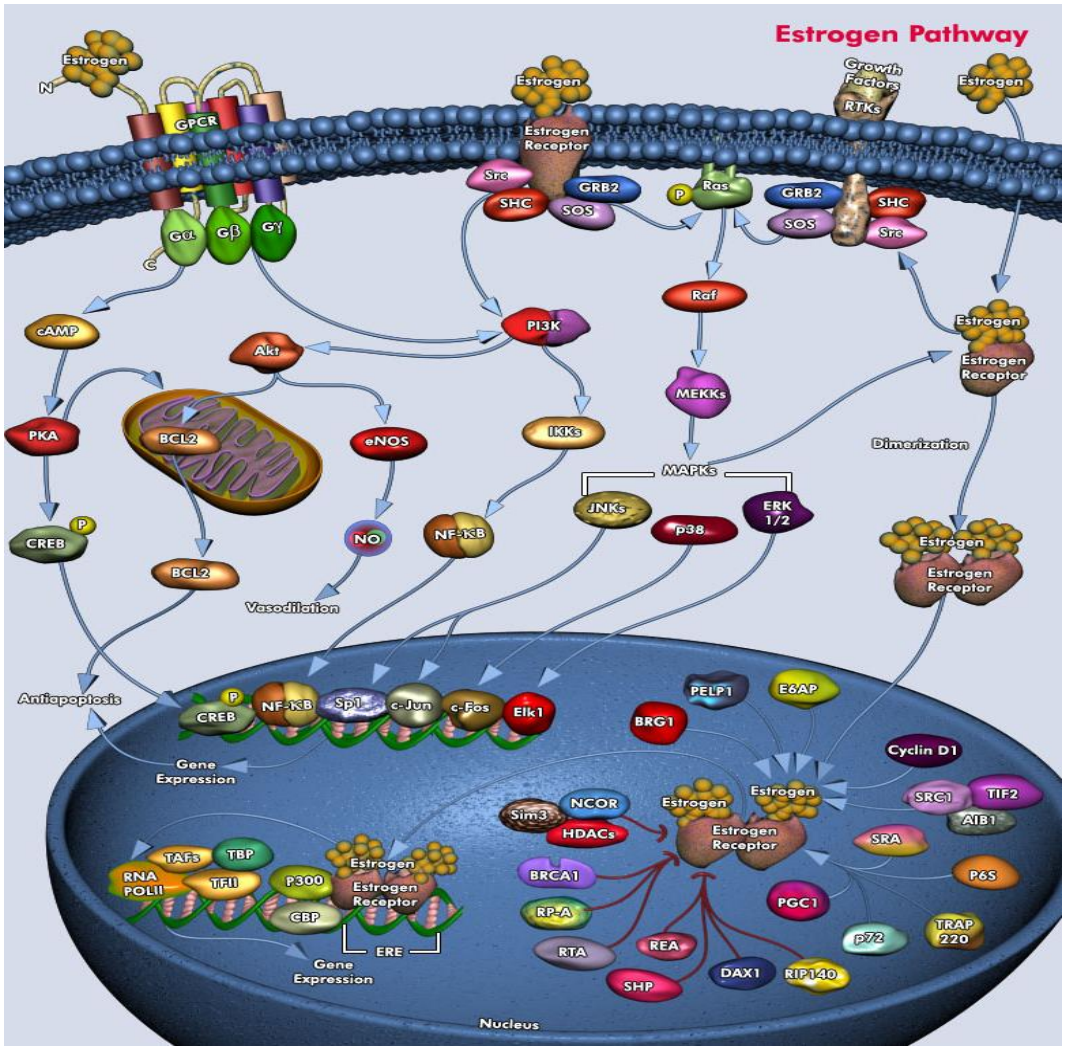

Figure 1.1 Estrogen Pathways (Qiagen Sample and Assay Technologies)

1.2.2 MECHANISM OF ENDOCRINE THERAPY

For treatment of tumors that possess ER, mostly ER antagonists are used, one common example is tamoxifen. Antiestrogen changes the estrogen receptor's molecular conformation and induces binding of corepressors instead of coactivators to ER, this causes inhibition of transcription. There is one important point, binding of coregulatory proteins that are coactivators and corepressors to the receptor is a vigorous process and related to the numbers of coactivators and corepressors in tumor cell. Due to the concentrations of coregulators in specific tissue, tamoxifen can act as agonist or antagonist (Schiff et al., 2003). Tamoxifen ligand inhibits estrogen action in breast but due to its estrogenic activity in bone, it can increase bone density and reduce osteoporosis.

An important obstacle with treatment is the resistance of tumor cell to tamoxifen. This resistance may already be present in tumor cell or may arise during treatment. There is a strong relation with endocrine resistance and growth factor signalling. As it is shown in Figure 1.2 (A), the endocrine sensitive tumors that are estrogen receptor positive and have low levels of growth factor receptors, exerts genomic ER action in the nucleus mainly but nongenomic ER action may also present with ER residing in the cytoplasm or at the membrane. These tumors can be stimulated by estrogen and inhibited by tamoxifen. On the other hand as it is shown in Figure 1.2 (B), endocrine resistant tumors overexpress growth factor receptors and through their pathways both ER activity mechanisms increase, higher cell growth rate and proliferation are achieved (Schiff et al., 2006). To overcome this not only compounds like tamoxifen are enough but also inhibitors of growth factor pathways are necessary. Furthermore, applying tamoxifen alone increases growth factor receptors and causes cells to be more sensitive to growth factor receptors, because of these, growth factor receptor inhibitors should also be used (Nilsson et al., 2001). Also pure antiestrogens like fulvestrant or aromatase inhibitors that block both genomic and nongenomic action of ER might be good alternative for treatment together with growth factor receptor inhibitors (Zhu et al., 2004). As a result additional adjuvant compounds to increase the sensitivity to the current drugs are

needed, these would help to overcome growth factor pathways. It is indicated that ER expression should remain during these treatments (Schiff et al., 2006).

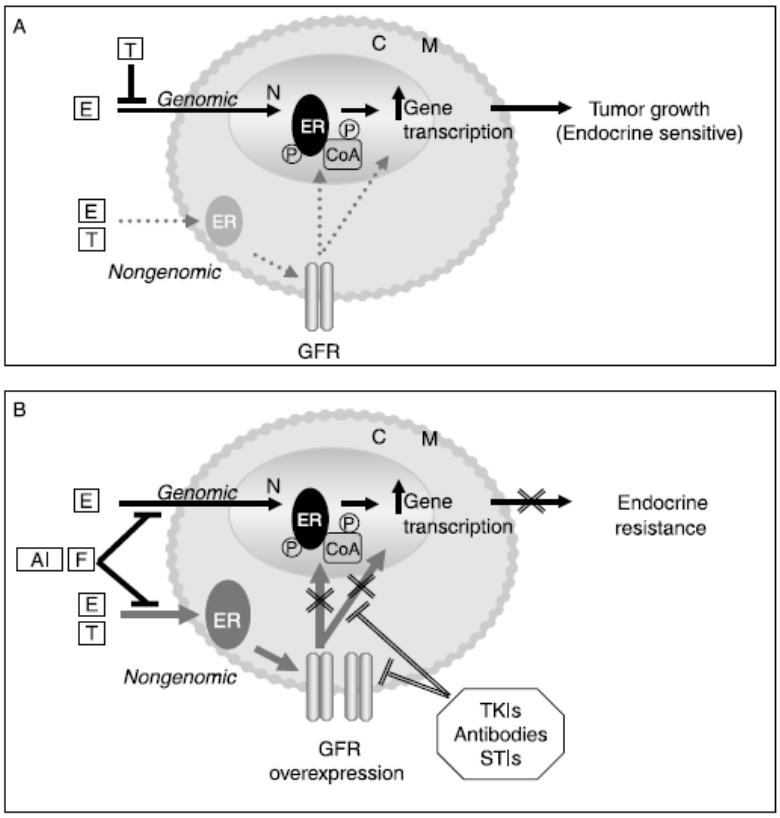

Figure 1.2 ER functions and crosstalk with growth factor receptor pathways in breast cancer cell. (A) Endocrine sensitive tumor (B) Endocrine resistant tumor (Massarweh and Schiff, 2006).

30 % of breast tumors do not express ER (Massarweh and Schiff, 2006). This a big problem for therapy, they do not respond antiestrogen treatment. During the tamoxifen resistance ER continuously expresses but as tumor grows independent of ER through other mitogenic pathways, tumor may lose ER expression. As it is indicated in Figure 1.3, breast cancers that overexpress growth factor receptors like Her-2, EGFR and signalling molecules like EGF, IGF-1, Mek1, Raf1 have more tendency to become ER negative (Schiff et al., 2006) and furthermore, it is also speculated that downregulation of the growth factor receptors with using specific inhibitors make the cell regain the receptor and become endocrine sensitive, if this is

achieved, patients may have the benefit of antiestrogen drugs (Munzone et al., 2006).

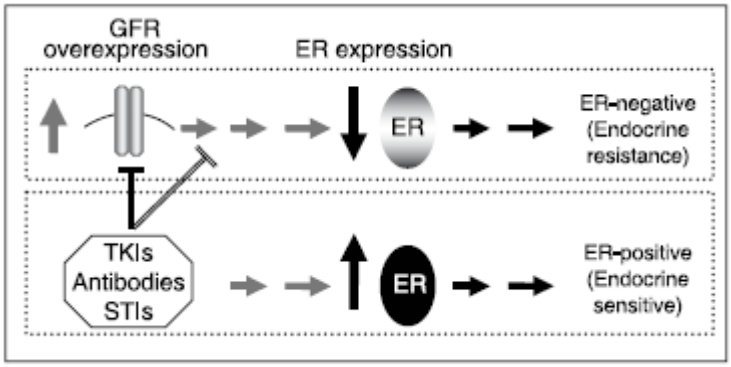

Figure 1.3 The Effect of growth factor receptor signaling upon tumor ER status (Massarweh and Schiff, 2006).

1.2.3 PHYTOESTROGENS

Phytoestrogens are plant derived estrogens. Their structural and functional similarities with estrogen and abilities to bind to estrogen receptor makes them called phytoestrogens. There is a diverse group of compounds known as phytoestrogens. Isoflavonoids, anthocyanins, lignans, resveratrol, antraquinones are some of them (Stoper et al., 2005). Being an antraquinone, emodin is a phytoestrogen. Phytoestrogens occur naturally in plants and have roles in defence mechanisms of plants (Korkina, 2007). Since they have a phenolic ring, they can bind to estrogen receptors. They have the ability to bind both receptors, ERα and ERβ, but have higher affinity to ERβ than ERα. Since phytoestrogens bind to ERβ with a higher affinity than steroidal estrogens, estrogen and phytoestrogens might use different pathways to exert their actions (Setchell, 1998). The binding of phytoestrogens to ERβ is an advantage for breast cancer protection (An et al., 2001; Brzezinski and Debi, 1999; Strom et al., 2004). Furthermore phytoestrogens inhibit aromatase enzyme that is needed to synthase estrogen (Rice et al., 2006). Phytoestrogens exhibit both estrogenic and antiestrogenic activities, which one is dominant depends on the type and amount of the compound, circulating estrogen amounts, type and number of estrogen receptors in that individual (Cassidy et al., 1994). Also their metabolisms may differ individually, because of genetic

polymorphisms or differences in intestinal environment (Webb and McCullough, 2005) or also according to sex, women seems to metabolize them more effectively (Lu and Anderson, 1998).

Phytoestrogens exert stimulatory effects in low estrogenic environments (postmenopausal women), whereas show inhibitory effects in high estrogenic environments (premenopausal women) (Ju et al., 2006; Adlercreutz 2002). Furthermore it is seen that at low concentrations phytoestrogens show estrogenic activity and stimulate growth whereas at high concentrations of phytoestrogens inhibit growth (Lemos, 2001; Schmitt et al., 2002). Phytoestrogens have 10^2 to 10^5 times less estrogenic activity than human estrogen but in the body they are present much more amounts than estrogen (Price and Fenwick, 1985; Adlercreutz, 1993). Phytoestrogens possess anticarcinogenic properties. They show not only estrogenic activities but also nonhormonal actions. They do their anticarcinogenic actions by some suggested mechanisms followed as; ability to induce apoptosis, induce differentiation, suppress angiogenesis and inhibit DNA topoisomerase, they may inhibit enzymes that have roles in cell proliferation, so it is postulated that they can inhibit tumors (Barnes and Peterson, 1995; Messina et al., 1995; Adlercreutz et al., 1995). There is no detailed research on the type, the dose, the duration of use or adverse effects of a phytoestrogen in a cancer patient. Because of this no recommendations can be made. Although there are results showing phytoestrogen can inhibit growth of cancer cell lines, and there is a belief that exposure to phytoestrogens early in life might protect against cancer, however, suggesting them as a part of therapy, will take a long time. Also a decrease in menopausal symptoms is not observed. The results of the studies on patients that recovered from breast cancer do not support using phytoestrogens in preventing its recurrence (Duffy et al., 2007).

1.2.4 EMODIN

Extracts from many plants have been used to heal a number of diseases as natural medicines. Emodin is one of the constituents of plants that are traditionally named as

natural herbal medicines. Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) is found mainly in plants of *Rheum* and *Polygonum* genus (Lee and Tsai, 1991; Matsuda et al., 2001) and from ancient times these plants are being used as purgatives and laxatives.

Emodin, being an anthraquinone, have lots of biological effects including; $p56^{lck}$ protein tyrosine kinase inhibitor (Jayasuriya et al., 1992), HER-2/neu protein tyrosine kinase inhibitor (Zhang et al., 1995), growth inhibitor of human cancer cells, HL-60 human leukemia cells (Yeh et al., 1988), ras transformed human bronchial epithelial cells (Chan et al., 1993), lymphocytic leukemia cells (Kupchan and Karim, 1976). Because of these effects emodin has being investigated as purgative, cell cycle inhibitor, antitumor, antimetastasis and apoptosis inducer agent.

One of the research aspects is its mutagenicity. Emodin was seen to be mutagenic in Salmonella typhimurium some strains with or without metabolic activation (Krivobok et al., 1992). Several reasons of this is speculated; P450 is involved in oxidative metabolism of emodin to form hydroxyemodins, some of them are direct mutagens to the strains investigated, 2-hydroxyemodin can produce reactive oxygen and this can induce DNA strand breaks (Kodama et al., 1987). According to the tests of National Toxicological Program of National Cancer Institute, USA, there is no certain knowledge about the carcinogenic activity of emodin in mice and rats (NTP Toxicology and Carcinogenesis Studies of EMODIN 2001), so these and other studies do not support the genotoxic risk of laxative components to humans when consumed under certain amounts (Brusick and Mengs, 1997). As it is seen, cell system is important for the effects of emodin. Because of its interference of cellular metabolism of microorganisms, emodin can be refered as antimicrobial agent.

In some experimental animal models, emodin has shown anti-inflammatory effects and it is first detected on carrageenan induced edema rats (Goel et al., 1991). For the anti-inflammatory action of emodin, the most accepted mechanism is its inhibition of NF-kB which is an important transcription factor (Kumar et al., 1998; Li et al., 2005).

Emodin has been found to have antioxidant capacity through different mechanisms like radical scavenging, inhibition of radical formation, inhibition of lipid peroxidation, so having antioxidant capacity, emodin protects the cell (Yim et al., 1998; Huang et al., 1995; Jung et al., 2004). Also there are reports indicating that emodin is a prooxidant. Because of its quinone structure, it is an effective electron acceptor interacts with molecular oxygen intracellularly and generates superoxide anion (Rahimipour et al., 2001). There are lots of reports of emodin's ability generating reactive oxygen species (ROS) and by this way induction of apoptosis in tumor cells (Wang et al., 2005; Su et al., 2005; Yi et al., 2004; Jing et al., 2002). The generated ROS may inactivate the transcription factors, proteins and nucleic acids needed for cell proliferation (Yi et al., 2004). Also it is not surprising that in different cell lines emodin has varing effects, from being an antioxidant to ROS generation (Chen et al., 2002).

Emodin induce cytochromes P450 1A1 and 1B1 in human lung adenocarcinoma cell line CL5 and also in MCF-7 breast cancer and NCI-H322 human lung carcinomas. It is because emodin is metabolized to 2-hydroxyemodin and w-hydroxyemodin by P450 enzymes (Wang et al., 2001; Zhang et al., 2003).

Chemical structures of emodin and its metabolites are shown in Figure 1.4.

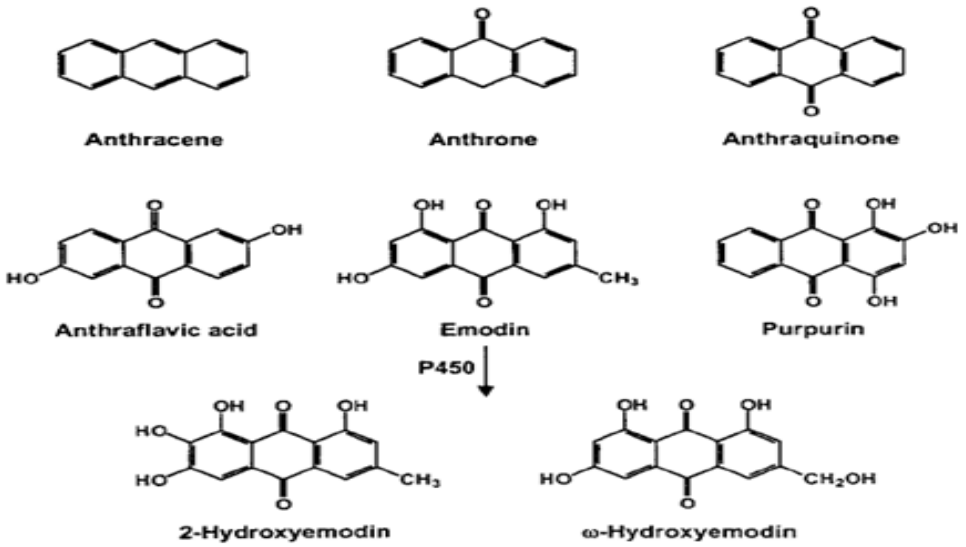

Figure 1.4 Chemical structures of emodin, its metabolites and related compounds (Wang et al., 2001).

The formed metabolites can induce ROS formation. There are lots of reports indicating its role on apoptosis formation. One example is about human tongue cancer SCC-4 cells. Emodin induces ROS formation and this causes apoptosis. ROS formation and distruption of mitochondrial potential is observed, this indicates apoptosis via mitochondrial pathway. Later the observed events are increase of the Bax/Bcl-2 ratio, cytochrome c release and activation of caspase-9 and caspase-3 and finally apoptosis. ROS inhibitor inhibited emodin induced apoptosis and this shows apoptosis took place because of emodin induced ROS formation (Lin et al., 2009). The effects of emodin on SCC-4 cells were shown in Figure 1.5.

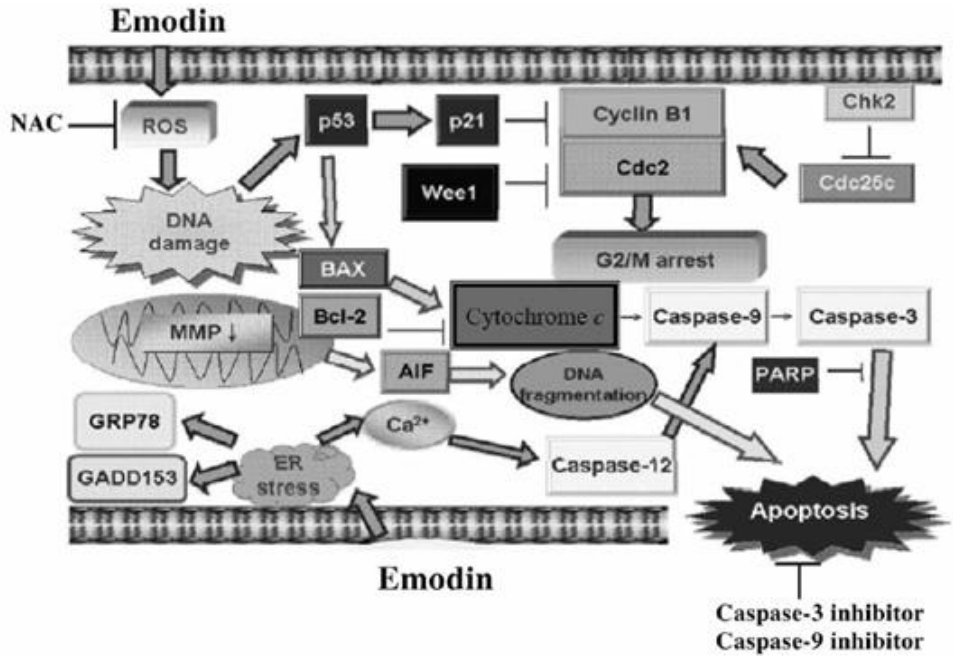

Figure 1.5 Suggested effect of emodin on SCC-4 cancer cells (Lin et al., 2009).

ROS also induces cell motility. As it was indicated in Figure 1.6, in MCF-7 and MDA-231 cell lines ROS induced Lck dependent MEK-1, ERK dependent uPA synthesis and this causes cell motility. Being Lck tyrosine kinase activity inhibitor, emodin inhibited the process (Mahabeleshwar et al., 2004).

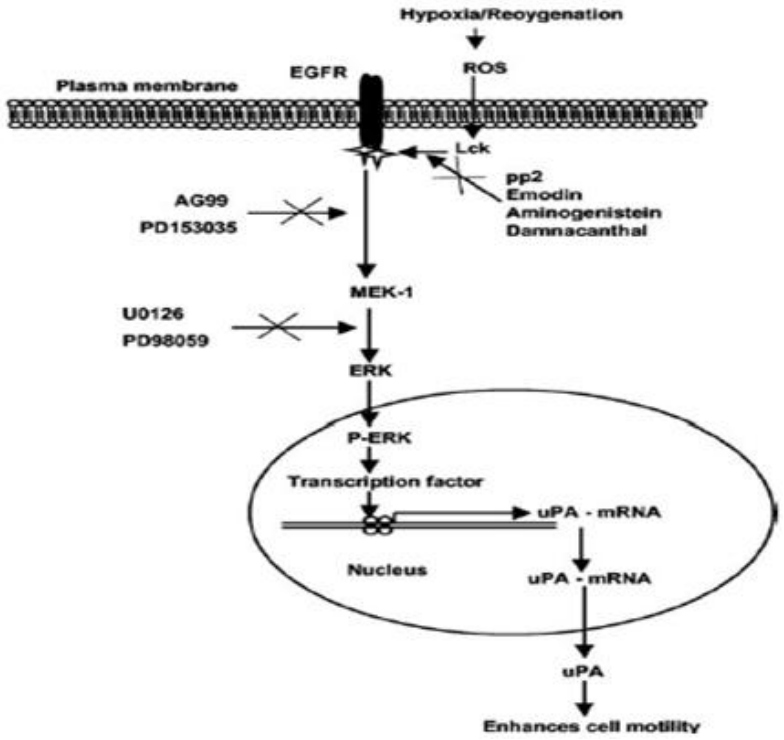

Figure 1.6 The inhibitory effect of emodin on cell motility in MCF-7 and MDA-231 breast cancer cell lines (Mahabeleshwar et al., 2004).

Inhibition of invasion by emodin is also seen in another research using skin squamous and MDA-231 breast cancer cell lines. Emodin caused suppression of MMP (matrix metalloproteinase)-9 expression through inhibiting AP-1 and nuclear factor-kB pathways that has roles in invasion (Huang et al., 2004).

Another important effect of emodin to cancer cells is that it suppresses their growth. Being a protein tyrosine kinase inhibitor, emodin, selectively inhibit the growth of ras-transformed human bronchial epithelial cells (Chan et al., 1993). On HepG2/C3A, PLC/PRF/5 and SK-HEP-1 hepatoma cells, emodin suppressed the growth with the result of sub-G1 and, G2/M arrest by stimulating p53 and p21 expressions (Liu et al., 2003; Shieh et al., 2004). Emodin treatment, suppressed transformation and growth, at the same time induced differentiation of HER-2/neu overexpressing breast cancer cells. Although emodin causes the growth inhibition of MDA-MB453, BT483, AU-565 cells (that overexpress HER-2/neu) at higher levels, MDA-MB231, MCF-7 cells (that express basal levels of HER-2/neu) showed less

inhibition. Also there was no induction of differentiation of these cells by emodin at the treated concentrations. HBL-100 that is immortalized breast cell line did not show proliferation inhibition that worths to record, this shows emodin does not effect normal breast cells (Zhang et al., 1995). It is seen that emodin also sensitizes tumor cells to the effects of chemotherapeutic drugs. It is also shown that emodin inhibited the growth of HER-2/neu overexpressing nonsmall cell lung cancer cells (NCI-H1435 and Her-2/neu-transfected NCI-H460) and sensitized the effect of anti-cancer drugs (etoposide cisplatin, doxorubicin) to NSCLC (Zhang and Hung, 1996).

Emodin is a phytoestrogen as it stated before. These are the molecules produced in plants naturally as indicated before and have effects in the estrogenic actions by directly interacting with receptors or by other ways changing the estrogen levels (Woods, 2002). Although they are less potent than synthetic estrogen, it is speculated that they can be used to cope with the menopausal symptoms and by this way, may decrease the breast cancer occured because of consuming synthetic estrogen (Adlercreutz and Mazur, 1997). The estrogenic activity of the compounds isolated from Rheum palmatum was investigated using a yeast system with a human estrogen receptor (ER) inserted within a plasmid. Emodin was seen to have highest estrogenic activity among the other anthraquinones tested. Also emodin showed high cytotoxicity on MCF-7 and MDA-231 breast cancer cells (Kang et al., 2008). But also there is a report indicating that having a high estrogen receptor binding affinity, emodin induced the proliferation of MCF-7 cells (Matsuda et al., 2001).

1.3 MCF-7

MCF-7 is an ER positive breast adenocarcinoma cell. Having estrogen receptor the cell has the ability to respond to estrogenic chemicals. In MCF-7 cells the mechanism of estrogen in stimulating growth is through G1 to S phase progression. C-myc, cyclins expression increase and retinoblastoma protein is phosphorylated (Doisneau-Sixou et al., 2003; Foster and Wimalasena, 1996). Also estrogen exerts nongenomic action and signal transduction mechanisms related to cell growth are activated (Pietras and Marquez, 2007; Moriarty et al., 2006; Pedram et al., 2006).

1.4 MDA-231

MDA-231 is an ER negative breast adenocarcinoma cell. Breast cancers have differences in their physical and molecular levels. The level of metastasis, the tumor size are important classification parameters and another important one is the presence of estrogen receptor. Nearly 30% breast cancer cases are ER negative. Determination of ER and one of the growth factor receptors Her-2 is important in targeted therapy, patients having these may have the benefit of receiving hormonal therapy and using Her-2 receptor specific drugs. Considering these, patients that are both ER and Her-2 negative have big problems in therapy, they only use classic chemotherapy. Being an ER negative breast cancer MDA-231 is highly aggressive and metastatic. In order to find better treatment, it is important to understand its biology, unique properties with comparing ER positive tumors. (http://grants.nih.gov/grants/guide/rfa-files/RFA-CA-09-026.html)

1.5 CYTOTOXICITY TESTS

Cytotoxicity tests are used to detect viable cell number upon treatment with tested chemicals like cytotoxic agents or growth factors by which cell proliferation is detected. Two largely applied assays use MTT (Diphenyltetrazolium Bromide) and XTT (Tetrazolium Hydroxide) tetrazolium salts for these tests. The assay principles are based on the activity of the mitochondrial enzymes that are inactivated shortly after cell death. The mitochondrial enzymes of the active cells have the ability to reduce tetrazolium salts into colored formazan products that are detected with a spectrophotometer. MTT is being used since 1950s. XTT was first represented by Scudiero in 1988 and shown to have lots of advantages upon MTT. MTT produces a nonsoluble formazan product and it is required to dissolve the dye in order to measure it, whereas XTT results in soluble formazan product which can be measured immediately after addition of the reagent. The activity of cell is greatly proportional to the formazan color.

1.6 APOPTOSIS

Apoptosis is a programmed cell death and is needed for survival and proper development of cells. Many cells are produced excess during development and later undergo apoptosis to form the whole organism (Meier, 2000). The formation of the fingers and toes of the fetus, the loss of frog tail, the neural development of the adult brain (Hutchins, 1998), development of reproductive organs are occured by apoptosis (Meier, 2000). The balance between proliferation and death, regulation of the immune system, removal of the defective and harmful cells represents the importance of the apoptosis. Any defect in its mechanism may result in cancer and several other diseases (Fadeel, 1999a).

Less of survival signals like growth factor or having negative signals like x-ray, ultraviolet light, oxidative stress, chemicals that leads to DNA damage, defects in DNA repair mechanisms, ligation of cell surface receptors are all cause of apoptosis. (http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/A/Apoptosis.html)

There are two main pathways of apoptosis named as intrinsic and extrinsic pathways that are triggered by stimulus from inside and outside respectively.

The intrinsic pathway is triggered by tumor suppressor protein p53 which is activated as a transcription factor upon several factors like oncogene activation, DNA damage and these results in growth arrest or apoptosis. p53 stimulates the expression of apoptotic proteins like p21, Bax, Apaf-1, Puma, Noxa, Fas and DR5 (Vousden et al., 2002) and represses the expression of antiapoptotic proteins such as Bcl-2 and Bcl-XL (Hoffman et al., 2002; Wu et al., 2001). Bcl-2 family members regulates the mitochondrial integrity and control the release of apoptotic proteins to the cytoplasm (Cory and Adams, 2002). Bcl-2 is the first example of an oncogene found to inhibit cell death instead of proliferation (Vaux et al., 1988). Bcl-2 is located on the surface of the outer membrane of the mitochondria and suppresses the generation of ROS, stabilizes mitochondrial membrane potential and inhibits the release of cytochrome c (Reed, 1998). Bax together with other apoptotic members of

the Bcl-2 family causes the cytochrome c release. In response to apoptotic stimulus Bax gains the ability to migrate from cytoplasm to outer membrane of mitochondria causes pore formation, permeability of transition and finally the release of cytochrome c to the cytoplasm (Borner, 2003). Cytochrome c binds to the apoptotic protease activating factor-1 protein, Apaf-1, forming the complex named as apoptosome (Salvesen and Renatus, 2002b). Apoptosome binds procaspase-9 and activates it forming caspase-9 (Denault and Salvesen, 2002). Caspase-9 activates caspase-3. Caspase 3 activates caspase 6 and this activates caspase 7. Apoptosis continues with digestion of structural proteins, DNA condensation, fragmentation. Caspase-3 activates DNA fragmentation factor, DFF, having DNase activity causes internucleosomal DNA fragmentation and final event is the phagocytosis of the cell (Earnshaw et al., 1999).

The extrinsic pathway also named as death receptor pathway is triggered upon binding of specific ligands to cell surface receptors that belongs to the tumor necrosis factor receptor gene superfamily (Ashkenazi, 2002). Activated receptor complex activates caspase-8. There are two pathways for caspase-8 to activate caspase-3. In the first one, caspase-8 directly activates caspase-3 (Scaffidi et al., 1998). In the second pathway, the death signal coming from the activated receptor isn't enough for apoptosis to proceed and needs to be amplified through mitochondrial apoptosis pathways. In this regard, caspase-8 cleaves Bcl-2 interacting protein, Bid, and its carboxy terminal migrates to mitochondria causing the release of cytochrome c. As in the case of intrinsic pathway cytochrome c binds to Apaf-1 and this complex activates caspase-9 and it activates caspase-3 (Luo et al., 1998). Apoptotic pathways were shown in Figure 1.7.

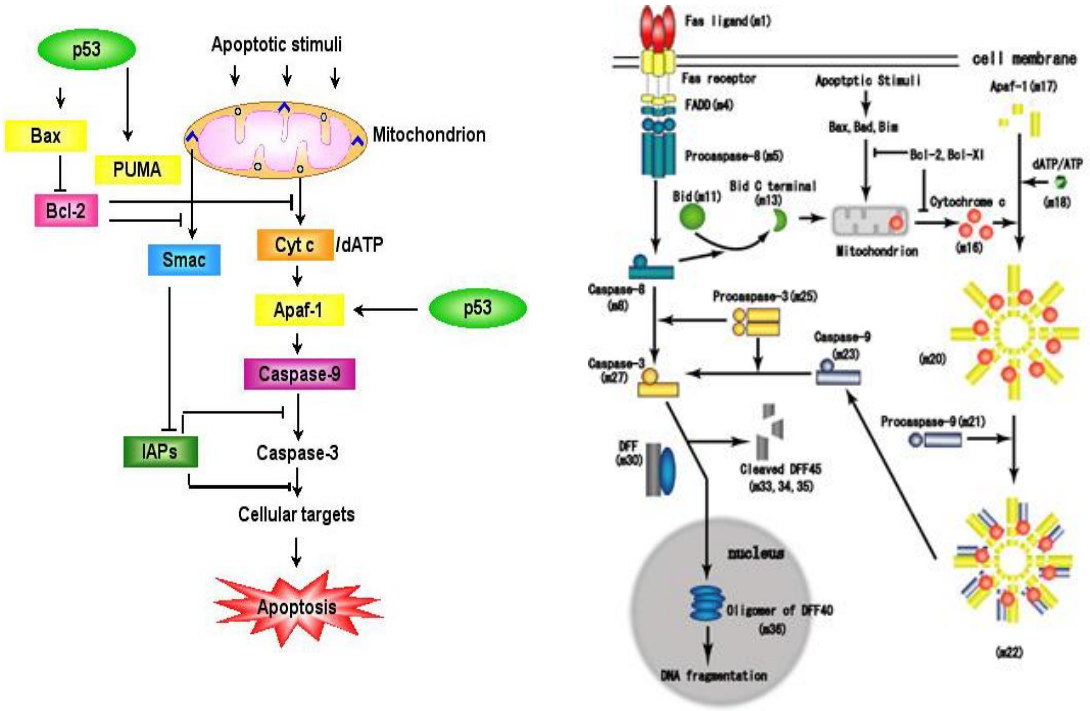

Figure 1.7 Representation of intrinsic and extrinsic apoptosis pathways. (http://www.weizmann.ac.il/home/ligivol/apoptosis_project/apoptotic_pathways.html; http://www.genomicobject.net/member3/GONET/apoptosis.html)

1.7 Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL)

One of the main consequences of apoptosis is DNA fragmentation and this can be visualized by some methods. TUNEL is the most common one. This was first identified by Gavrieli et al. in 1992. DNA strand breaks determined by terminal deoxynucleotidyl transferase which is an enzyme that catalyzes the addition of biotin-dUTP to strand breaks of cleaved DNA (Figure 1.8).

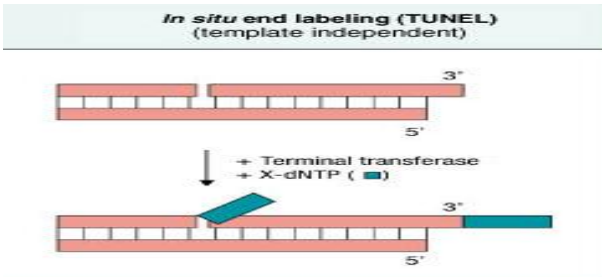

Figure 1.8 Labeling principle of TUNEL (Roche Diagnostics).

1.8 GST ENZYME ACTIVITY

The glutathione S-transferases (GSTs) are primary phase II detoxification enzymes that has also many other important functions in the cell (Wilce and Parker, 1994). Their major activity in the cell is to catalyze the attack of reduced glutathione to compounds that have electrophilic nitrogen, sulphur or carbon atom. They have a wide range of substrate specificities. These include nitrobenzenes, epoxides, heterocyclic amines, quinones, arene oxides, α,β- unsaturated carbonyls (Hayes and McLellan, 1999; Sheehan et al., 2001).

GST enzymes are evolved from thioredoxin enzymes that are antioxidants and are found in many organisms ranging from bacteria to mammals and share structural and sequence similarities with other stress related proteins that are found in a wide range of organisms and it is suggested that a common stress related ancestor that is formed before thioredoxin is responsible for this relationship. (Martin, 1995; Rossjohn et al., 1996). These multiple enzyme families from one ancestor might have been formed from gene amplification, duplication, independently folding domains and inherited in proteins during evolution (Armstrong, 1998; Hansson et al., 1999).

There are three major families of glutathione transferases. Cytosolic and mitochondrial GST are soluble enzymes whereas the third class microsomal GST is membrane associated, named as, membrane associated proteins in eicosanoid and glutathione (MAPEG) metabolism. All three members of GST enzyme family catalyze the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) a reaction involving a nucleophilic attack of a thiolate anion (GS^-) on CDNB's aromatic ring with displacement with Cl and show glutathione peroxidase activity toward cumene hydroperoxide (CuOOH) as it was shown in Figure 1.9. The MAPEG and cytosolic GST enzymes catalyzes isomerization reaction and are involved in the formation of leukotrienes and prostaglandins (Hayes et al., 2005).

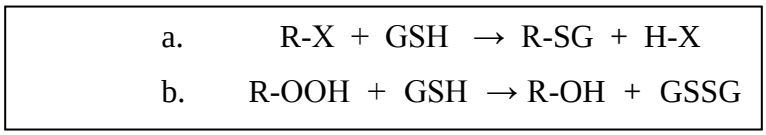

Figure 1.9 General GST reactions. (a) GSH conjugation reaction
(b) GSH peroxidase reaction

The largest family represents cytosolic GST enzymes and have lots of activities as shown in Figure1.10. They have thiolysis, reduction, isomerization activities (Hayes et al., 2005). Beside having these activities, cytosolic GST enzymes can bind covalently and noncovalently to nonsubstrate ligands and have roles in intracellular transport and disposition of xenobiotics. These nonsubstrate hydrophobic ligands are some steroids, bilirubin, heme, lipophilic anticancer drugs and this binding has often roles with the inhibition of the GST enzyme activity (Hayes and Pulford, 1995). Cytosolic GSTs are found in humans in several classes having 60% amino acid homology; Alpha, Mu, Pi, Sigma, Theta, Omega, Zeta. Classification depends on the primary, tertiary structure similarities; immunological, kinetic properties. (Sheehan et al., 2001). The structural differences especially depends on the active site, general structure is similar and it is like the ancestor enzyme thioredoxin. The conserved part of the structure is the N terminal and this includes an important part of the active side; tyrosine, serine or cysteine residue that interact with the thiol group of GSH and lowers the pKa from 9 to 6-7. This is the catalytic activity of the GST enzyme (Atkins et al., 1993).

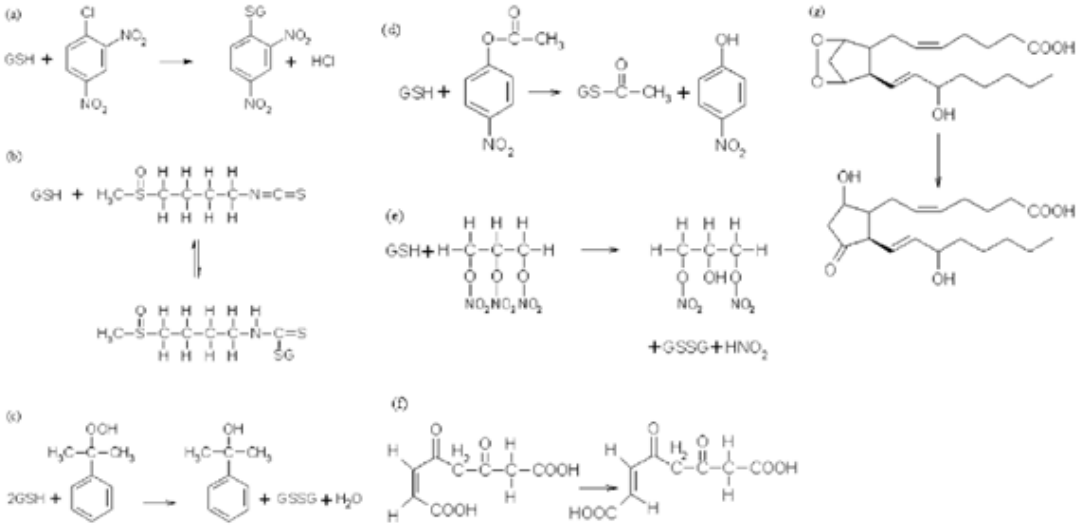

Figure 1.10 Conjugation, reduction, thiolysis and isomerization reactions catalyzed by GST. Substrates are as the following; (a) CDNB (b) sulforaphane (c) CuOOH (d) 4-nitrophenyl acetate (e) trinitroglycerin (f) maleylacetoacetate (g) PGH_2 conversion to PGD_2 (Hayes et al., 2005).

Glutathione transferases have exogenous and endogenous substrates. They metabolize drugs, herbicides, insecticides, chemoterapeutic agents, carcinogens, environmental pollutants. Overexpression of GST enzymes in tumor tissues causes resistance to anticancer drugs and in these cases the goal of the treatment is to lower GSH and GST using some inhibitors. (Lewis et al., 1988). Also GST enzymes may activate xenobiotics for some instances. In this cases, if they transform prodrugs to active forms, the overepression of these enzyme becomes an advantage (Findlay et al., 2004).

Reactive oxygen species; superoxide anion O_2^-, hydrogen peroxide H_2O_2, hydroxyl radical HO• produced during aerobic respiration, are endogenous substrates of GST enzymes. Superoxide dismutase, catalase, GST, glutathione peroxidase, aldehyde dehydrogenase, alcohol dehydrogenase, aldo-keto reductase are enzyme systems that protect the cell against products of oxidative stress. GSH, bilirubin, ascorbic acid, α-tocopherol are the compounds that help this process nonenzymatically (Hayes et al., 2005).

Reactive oxygen species causes the peroxidation of polyunsaturated fatty acids in membranes. This event causes destruction of the membranes. The GST enzymes reduce the end products of lipid peroxidation, as a result, protect the membranes. Base propenals are formed by oxidation of nucleotides and these are detoxified by GST. Oxidation of catecholamines causes the formation of quinone containing compounds, these can produce oxygen radical by redox cycling. GST causes the conjugation of GSH with these compounds and prevents radical formation (Hayes et al., 2005; Dagnino-Subiabre et al., 2000).

GST enzymes have roles in the catabolism of tyrosine and phenylalanine amino acids (Hayes et al., 2005). Also they have roles in the synthesis of steroid hormones; testesteron and progesterone (Johansson and Mannervik, 2001).

GST enzymes catalyze the synthesis and inactivation of eicosanoids, this role has many biological consequences in the cell. GST enzymes has the ability to effect formation and degradation of eicosanoid 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) as shown in Figure1.11. Overexpression of GST effect PPARγ, Nrf-2 and NF-kB dependent gene expressions (Paumi et al., 2004; Itoh et al., 2004; Rossi et al., 2000).

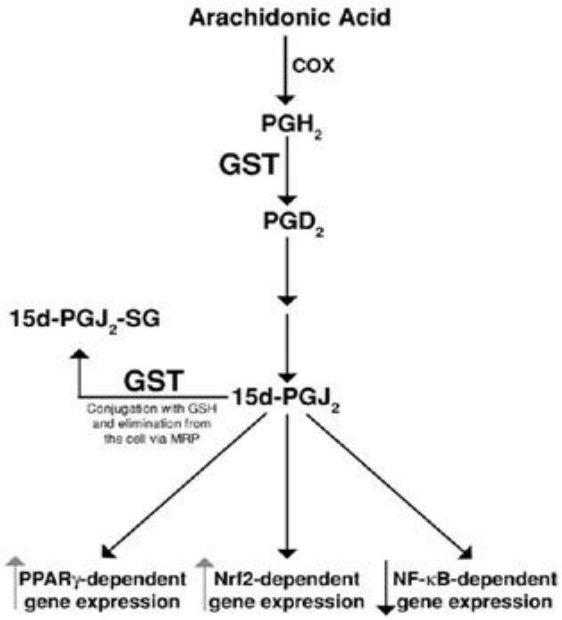

Figure 1.11 The effect of GST enzymes on prostaglandin signalling (Hayes et al., 2005).

Beside GSTs, GSH has also effects on gene expressions, it conjugate with an endogenous lipid peroxidation product, 4-hydroxynonenal (4-HNE). Being an *α,β*-unsaturated carbonyl, 4-HNE is an intracellular signalling molecule. 4-HNE has the ability to activate several cell surface receptors, stimulates expressions of JNK, PKC, p53 and promote apoptosis; also effect several transcription factors like NF-kB, c-Jun, Nrf2. As a result the reaction with GSH has many biological consequences (Echtay et al., 2003; Awasthi et al., 2003; Tjalkens et al., 1999).

GST expression increases during carcinogenesis. Under oxidative stress, Nrf2, transcription factor that is normally located in the cytoplasm, is carried to the nucleus and reaches to ARE (Antioxidant Response Element) sequences, the genes that have ARE sequences are induced as a result. A number of GST enzymes known to have these ARE or related sequences and seem to be regulated and induced by this mechanism. It is seen that ARE sequences are mostly overexpressed during tumorigenesis and the increase of GST enzymes during carsinogenesis might be because of this (Hayes et al., 2005). GST π gene is especially emphasized in this regard. It is seen that in many tumor tissues GST π gene expression is increased and this can be used as a marker (Sato, 1989; Shea et al., 1988; Howie et al., 1990). Also GST μ gene is important in carcinogenesis especially in lung cancer because 50% of human population lacks this gene, so it is highy polymorphic, person who lacks this enzyme is more susceptible to carcinogens (Seidegard et al., 1988; Seidegard et al., 1990).

1.9 MICROARRAY

Microarray is an excellent tool to display thousands of gene expressions meanwhile. In this technology, biotin labeled target is hybridized to probe array and light emitted at 570 nm is proportional to bound target and indicates gene expression level as shown in Figure 1.12 (www.affymetrix.com). By this way differential expressions of genes at different conditions can be monitored.

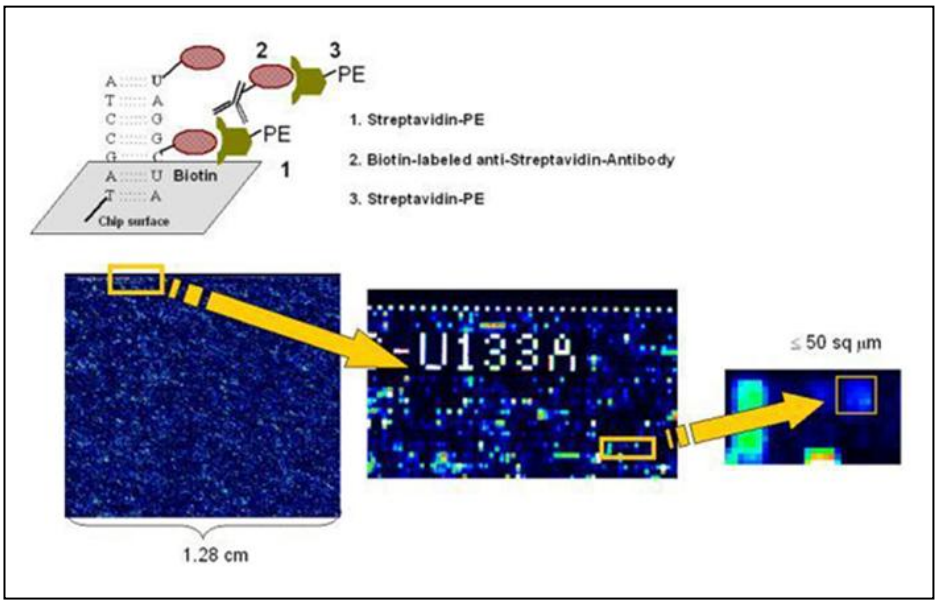

Figure 1.12 Microarray technology (www.affymetrix.com)

1.10 SCOPE OF THE STUDY

Tumors are formed from different cell types. The phenotypic variation among tumor cells results in changes in their responses to chemopreventive treatment. Being an estrogen receptor positive cell line, MCF-7 has the ability to respond estrogen mechanism targeted therapy. MDA-231 cells has no estrogen receptor and it is hard to treat them. Both cells lines are adenocarcinoma of human breast cancer and it is important to investigate the similarities and differences between them to improve the treatment.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a phytoestrogen, exists in plants. Phytoestrogens are known to have anticarcinogenic properties. They can be used with other chemotherapeutic drugs as adjuvant therapy. The exact mechanism of emodin on cell lines is not known. It has estrogen dependent and independent activities having estrogenic or nonestrogenic results. Because of these, it is important to compare the effects of emodin on estrogen receptor positive and estrogen receptor negative cell lines.

In this study, to compare the effects of emodin on biological activities of MCF-7 and MDA-231 cells, the cytotoxicity of emodin, and effects of emodin on apoptotic genes and GST enzyme activities have been investigated. Microarray analysis have been done to obtain large scale analysis of differentially expressed genes and so to investigate the different mechanisms of emodin on investigated cell lines.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Reduced glutathione (Sigma), 1-chloro-2.4-dinitrobenzene (Sigma), ethylenediamine tetraacetic acid (Merck), hydroxymethyl aminomethane (Sigma), boric acid (Sigma), bovine serum albumin (BSA), Folin-Ciaceltau Phenol Reagent, cupper sulfate, sodium-potassium tartrate, sodium hydroxide, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium carbonate, 2-mercaptoethanol (Merck), chloroform (Amresco), isopropyl alcohol (Applichem), ethanol absolute, Triton X-100, Formaldehyde (Sigma), DTT, 20XSSC (Clontech). All chemicals were analytical grade and were obtained from commercial sources having the highest purity available.

For cell culture assays, RPMI-1640 medium with phenol red and without phenol red (Biochrom AG), fetal bovine serum (Biochrom AG), gentamycin (Biological Industries), Hank’s Balanced Salt Solution (BSS) (Biochrom AG), Dulbecco's Phosphate Buffered Saline (PBS) (Biochrom AG), Trypsin/EDTA (Biochrom), Tryphan Blue (Biological Industries), Dimethyl sulfoxide (DMSO) (Applichem). For cell culture studies, all the reagents and chemicals were cell culture grade.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Cell lines and Growth Conditions

MCF-7 and MDA-231 cell lines were from ATCC (American Type Culture Collection). These monolayer cells were grown in complete medium containing RPMI-1640 cell culture medium with phenol red containing 10% fetal bovine serum and 0.2% gentamycin and incubated at 37 ^{o}C in a 95% humidified atmosphere of 5% CO_2.

In order to get rid of the immune factors in the serum and their unwanted effects on cells and assays, previously heat inactivated serum (that was prepared by incubation at exactly 56 ^{o}C for 30 minutes) was purchased and used.

2.2.1.2 Cell Passaging

At about 80% confluency meaning that when 80 % of the flask was covered by monolayer cells, the cells were passaged, otherwise the cells overgrow, detach and die. The medium was discarded using pipettes away from the attached cells without disturbing them. Cells that were still attached to flask that were grown in, were washed with 1-2 ml Hank's Balanced Salt Solution (BSS) or Dulbecco's Phosphate Buffered Saline (PBS) in order to get rid of the medium. 1-2 ml Trypsin/EDTA was added and incubated at 37 ^{o}C for 2-5 minutes to detach the cells. Trypsinization was stopped by addition of complete medium, serum inside the medium stops trypsin. Later detached cells were pipetted up and down to have cell suspension so, to seperate cells from each other and eliminate the clumps. Trypan Blue (Biological Industries) stain was used during counting the viable cells.

2.2.1.3 Viable Cell Counting

Trypan blue staining depends on the metabolic activity of viable cells. Viable cells could dispose the dye out of the cell with their transporters and were seen bright, while the dead ones were observed as blue under microscope. After detaching the cells and obtaining cell suspension, cells could be counted. In a seperate eppendorf, cells were diluted with Trypan Blue. The dilution factor depended on cell number; if the confluency of cells were above 80%, the dilution factor was 3, so 1:2 dilution was done, (20 µl cell suspension and 40 µl tryphan blue), otherwise 1:1 dilution is made. Cell counting was performed using hemocytometer. Following mixing the cell suspension gently, 20 µl of cell suspension in trypan blue was placed to each chamber of the hemocytometer. Coverslip was placed on the hemocytometer to evenly spread the liquid. After counting viable cells on 25 squares at the center of the device each containing 16 small squares, the average of two chambers were taken and the cell number per ml was calculated as follows;

Cell number / ml = The average cell number of the two chambers x DF x 10^4
DF= Dilution factor that was done with Trypan Blue
10^4= Factor calculated from the dimensions of hemocytometer

The dimentions of each chamber on the hemocytometer were 1cm lengh, 1 cm width and 0.1 cm height, having volume of 0.1 cm^3 or 10^{-4} mm^3 (10^{-4} ml).

2.2.1.4 Freezing and Thawing

In order to freeze the cells, freezing medium, that was 10% DMSO containing complete growth medium with serum, was used. After trypsinization, cell suspension was counted because for every 10 million cells, 1 ml of freezing medium should have been used. After counting the cells, cell suspension was centrifuged and cells were precipitated, the supernatant medium was discarded. Cells were resuspended with appropriate volume of freezing medium according to the cell number and placed into the cryovials. The cryovials were stored at -80^oC for 24

hours in a box containing isopropyl alcohol which provided the cells to freeze gradually, and later transferred into tank containing liquid nitrogen having temperature about - 190 ^{o}C. In order to thaw the cells, cryovials were taken from the liquid nitrogen tank and placed into 37 ^{o}C water bath. When the medium completely dissolved, it was placed very slowly into T25 tissue culture flasks containing 10 ml complete medium. The following day, the attached cells were trypsinized and passaged to T75 tissue culture flasks containing 20 ml medium in order to get rid of DMSO.

2.2.2 Cytotoxicity of Emodin

2.2.2.1 Preparation of Emodin Solution for Treatments

Emodin is soluble in DMSO and likes basic environment, in acidic environment it precipitates. Because of its solubility problems, first the stock working emodin solution was prepared containing 2 % DMSO in basic distilled water pH (10,89) and complete RPMI-1640 medium without phenol red. Then working emodin solutions of varying concentrations (from 5 μg/ml to 200μg/ml emodin) were prepared by appropriately diluting the stock working solution in 2 % DMSO containing basic distilled water pH (10,89) and complete RPMI-1640 medium without phenol red. Working solutions were two times more concentrated in DMSO and emodin than that of treatment medium. Hence, DMSO concentration was kept constant at 1 %, and emodin concentrations were varied from 2.5 to 100μg/ml in each well during treatments.

2.2.2.2 XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) Cell Cytotoxicity Assay

The cytotoxic effects of emodin in MCF-7 and MDA231 cells were investigated by using Cell Proliferation XTT Kit of Biological Industries according to manufacturer's instructions.

The tetrazolium salt XTT was reduced to orange colored formazan compounds by the activity of mitochondrial enzymes in metabolic active cells (Figure 2.1). The formed product was water soluble and can readily be quantitated using with ELISA reader at 415 nm.

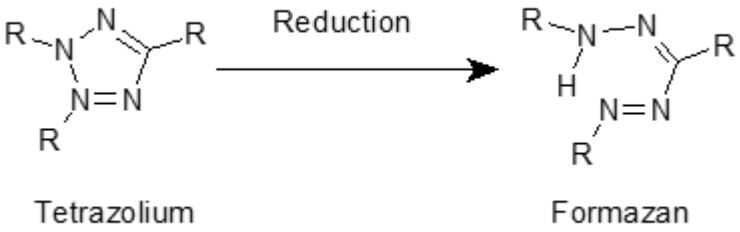

Figure 2.1 XTT Assay Reaction

MCF-7 and MDA-231 cells (10,000 cells/well) were seeded into 96 well plates and left for 24 hours in order to let them attach and grow. After 24 hours, medium was changed, 50 µl fresh complete medium was added and cells were treated with 50 µl of working solutions of emodin in order to reach final emodin concentrations; 0, 10, 20, 40, 80, 100 µg/ml, and DMSO concentration of 1%, and left for 48 hours incubation. One of the control wells in which cells were grown in their growth medium, contain only growth medium (100 µl), the other control well contains 1% DMSO in growth medium which is the solvent of emodin. The 96 well plate representation of XTT assay was shown in Figure 2.2.

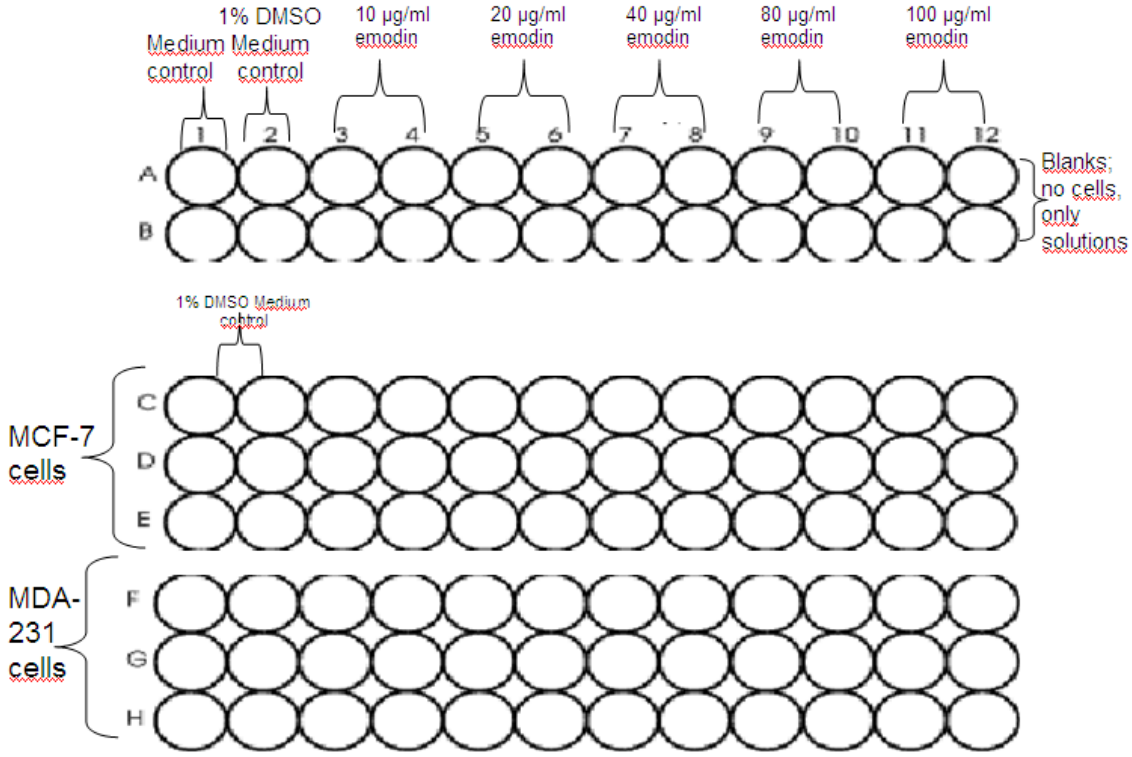

Figure 2.2 96 well plate representation of XTT assay. A1 and B1, no cells, complete medium control, 100 µl; A2 and B2, no cells, 1% DMSO medium control, prepared as 50 µl 2% DMSO medium + 50 µl complete medium, A3 to H3 and A4 to H4, 10 µg/ml emodin, prepared as 50 µl 20 µg/ml emodin + 50 µl complete medium, A5 to H5 and A6 to H6, 20 µg/ml emodin, prepared as 50 µl 40 µg/ml emodin + 50 µl complete medium; A7 to H7 and A8 to H8, 40 µg/ml emodin, prepared as 50 µl 80 µg/ml emodin + 50 µl complete medium; A9 to H9 and A10 to H10, 80 µg/ml emodin, prepared as 50 µl 160 µg/ml emodin + 50 µl complete medium; A11 to H11 and A12 to H12, 100 µg/ml emodin, prepared as 50 µl 200 µg/ml emodin + 50 µl complete medium, C1 to H1 and C2 to H2, 1% DMSO medium control, prepared as 50 µl 2% DMSO medium + 50 µl complete medium. C, D and E wells contain MCF-7; F, G, H wells contain MDA-231 cells.

When the incubation time was completed, 50 µl of reaction mixture that was prepared by adding 0.1 ml activation reagent to 5 ml XTT reagent was added to each well. Following the incubation with XTT reagent mixture for 20 hours in CO_2 incubator at 37 ^{0}C, the absorbance was read at 415 nm with ELISA reader. Emodin

solutions at different concentrations with no cells were used as blanks in order to get rid of the emodin interference and the unwanted effect of serum intensity at that wavelenght. 6 replicates were used. The inhibitory concentration (IC50) values were determined by Graph Pad Prism Program.

2.2.2.3 Viable Cell Counting with Trypan Blue

MCF-7 and MDA-231 cell lines (100.000 cells/well) were placed in 24 well plates. After 24 hours, medium was changed with 500 µl fresh medium and cells were treated with 500 µl of emodin working solutions, that were prepared as described in Section 2.2.2.1, in order to reach final emodin concentrations; 0, 5, 10, 20, 40 µg/ml and left for 48 hours incubation. When the incubation was over, wells were emptied, washed and trypsinized using 100 µl of trypsin and cells were collected by adding 400 µl of medium. 1:1 dilution was done with the trypan blue and cell count was performed with the hemocytometer. Cells that were grown in 1% DMSO containing medium are considered as controls and their counts were set as 100% cell viability. IC50 values were determined by Graph Pad Prism Program.

2.2.3 Preparation of Cell Homogenates

MCF-7 and MDA-231 cell lines (500,000 cells/well) were placed in 6 well plates. After 24 hours, medium was changed with 2 ml fresh complete medium and cells were treated with 2 ml of emodin working solutions, that were prepared as described in Section 2.2.2.1, in order to reach final emodin concentrations; 0, 5, 10, 20 µg/ml and left for 48 hours incubation. When the incubation time was over, the medium was discarded and wells were washed and trypsinized using 200-300 µl of trypsin. Following the detachment of cells, trypsin was inactivated using complete medium. The cells were collected and centrifuged for 5 minutes at 600 X g at 4 ^{o}C. After removing supernatant, the pellet was washed with cold PBS and cells were again centrifuged at 600 X g for 5 minutes at 4 ^{o}C. Isolation was performed according to Cell Fractionation Kit User Manual of Clontech. For homogenization, fractionation buffer mix was prepared by mixing 0.2 µl protease inhibitor cocktail, 0.1 µl DTT to

100 µl fractionation buffer. Following the removal of supernatant, cells were resuspended in 80 µl cold fractionation buffer and left on ice for 10 minutes. Homogenization was done using a sonicator for 3 passes, 1 pass lasting for 5 seconds. In order to be sure if all the cells were burst, a little volume of cells were taken and observed under microscope, if there were not so much shiny rings, the procedure could be continued. Following homogenization, centrifugation was performed at 700 X g for 10 minutes at 4 ^{o}C. Afterwards, supernatant was transfered to a new eppendorf tube and centrifuged at 10,000 X g for 25 minutes at 4 ^{o}C. The supernatant was collected as cytosolic fraction and were kept in aliquots at -80 ^{o}C until enzyme activity measurements. The pellet was resuspended in 10 µl fractionation buffer mix to have mitochondrial fraction.

2.2.4 Protein Concentration Determination

The protein concentrations were determined according to Lowry method. Bovine serum albumine (BSA) protein was used as standart and standard curve was drawn (Fig. 2.3) and using its slope sample protein concentrations were calculated. Cytosolic fractions prepared from MCF-7 and MDA-312 cells were diluted as 1/40, 1/60 in distilled water and 40 µl of them were placed into 96 well plates as triplicates so there were 6 replicates for one sample. 200 µl of Lowry alkaline copper reagent (ACR) was prepared (composed of 2% copper sulfate; 2% sodium potassium tartrate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100 respectively). First copper sulfate and sodium potassium tartrate was mixed and than the remaining component was added and mixed. Following 10 minutes incubation at room temperature, 20 µl Folin Phenol Reagent was added and immediately mixed for 8 seconds. After 45 minutes incubation at room temperature, the intensity of the color was measured by ELISA reader at 660 nm. The intensity of the color is directly proportional to the protein concentration.

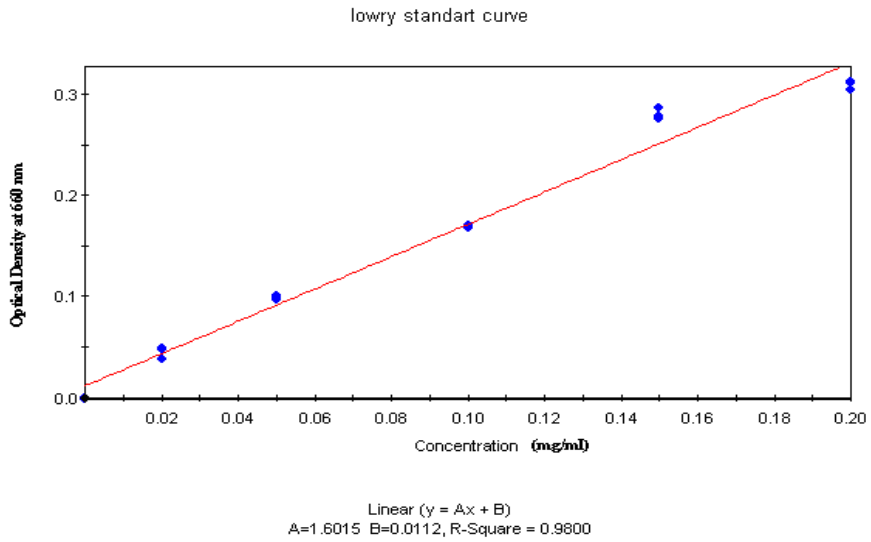

Figure 2.3 Bovine serum albumin standart curve constructed by Lowry method

2.2.5 GST Enzyme Activity Determination

GST enzyme activity was determined using CDNB as substrate and GSH as cofactor. Enzyme activity was measured indirectly by measuring the conjugation of CDNB with GSH using ELISA reader at 340 nm. GST activities expressed as unit/mg where one unit of enzyme was defined as one nmol product formed in one minute.

For MCF-7 cells cytosolic fraction (3 mg protein/ml) and MDA-231 cells cytosolic fraction (3 mg protein/ml) were diluted in 10 mM phosphate buffer, pH 7.4, appropriately, and were used as GST enzyme source in reaction medium. Measurements were done in triplicates. For MCF-7 cells no dilution and two times dilution were used whereas for MDA-231 cells two and four times dilutions were used.

To a typical reaction medium (250 µl) in a single well of a 96 well plate; 162.5 µl dH_2O; 50 µl 500 mM Phosphate Buffer pH 7.4; 10 µl 25 mM GSH; 12.5 µl 20 mM

CDNB and 15 µl enzyme source (45 µg protein) were added. After sample addition solution was mixed and reaction started. For reaction blanks instead of enzyme source, 15 µl water was added into reaction medium. Reactions were measured seperately for each well by recording at every 20 seconds for 10 minutes. Best lines were drawn for each reaction (Fig. 2.4) and the slopes of the best lines were used as the rate of reaction; dA/dt. The slopes of reaction blanks were subtracted from slopes of reactions prior to specific activity calculations according to the following formula.

$$\text{Specific Activity} : \frac{\text{Average Slopes (dA/dt)}}{\varepsilon \text{ CDNB}} \times \frac{250}{15} \times \frac{1}{\text{mg protein}} \times \text{DF}$$

Dilution in the tube is 250/15. εCDNB is 6,29 mM^{-1}/cm^{-1} (modified for ELISA plate reader, www.kamiyabiomedical.com).

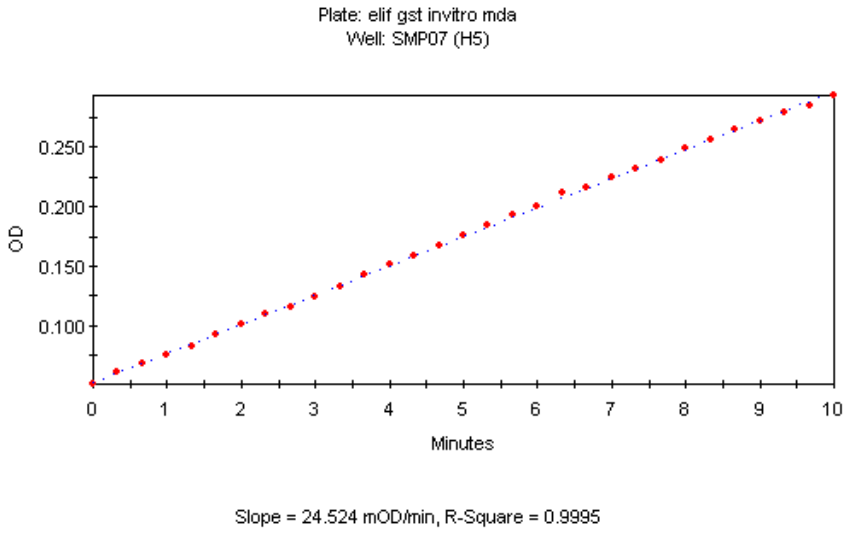

Figure 2.4 Time dependency of GST reaction

2.2.6 RNA Isolation

Isolation of RNA from MCF-7 and MDA-231 cell controls and from varing concentrations of emodin treated MCF-7 and MDA-231 cells was performed using

Qiagen RNeasy Minikit. MCF-7 and MDA-231 cell lines (500.000 cells/well) were placed in 6 well plates. The following day, medium was refreshed and the cells were treated with 0, 5, 10, 20 µg/ml emodin concentrations for 48 hours incubation. At the end of incubation period, the medium was discarded, wells were washed with 1 ml PBS and trypsinized using 200-300 µl of trypsin. After cells were detached, the trypsin was inactivated by adding 5 ml complete medium. Cells were collected into Falcon tubes as for medium control, DMSO control and 5 µg/ml emodin treated cells, 2 wells, for 10 µg/ml and 20 µg/ml emodin treated cells, 3 wells were combined. Tubes were centrifuged at 300 X g for 5 minutes. The supernatant was completely discarded and the cells were lysed by adding 350 µl Buffer RLT. In order to have efficient lysis, the solutions were pipetted 10 times and waited for 5 minutes at room temperature. 70% ethanol was added to the homogenized lysate at the same volume of Buffer RLT and mixed by pipetting. 700 µl of the homogenate was added to RNeasy spin column placed in a collection tube and centrifuged at 12.000 X g for 15 s. Flow-through was completely discarded. For the washing parts, two solutions were used named as Buffer RW1 and Buffer RPE. Following the first centrifugation step, 700 µl of RW1 was added to the RNeasy spin column and centrifuged at 12.000 X g for 15 s. Flow–through was discarded. 500 µl of Buffer RPE was added to the RNeasy spin column and centrifuged at 12.000 X g for 15 s and the flow-through was discarded. 500 µl of Buffer RPE was added to the RNeasy spin column once again and centrifuged at 12.000 X g for 2 minutes. Following washing, the RNeasy spin column was placed to a new collection tube and 25-30 µl RNase- free water was added and centrifuged at 12.000 X g for 1 minutes. The eluate was taken and added to the column and the centrifugation was performed again to have more concentrated RNA. The isolated RNA was kept at -80 ^{o}C until it was used for cDNA synthesis prior to real time PCR. Kit usage brought DNA contamination and in order to get rid of this, Dnase-1 treatment was performed.

RNA purity and concentration was determined by measuring the absorbances at wavelengths of 260 and 280 nm. At 260 nm nucleic acids were measured and at 280 nm proteins gave absorbance. A260/A280 ratio shows the purity of RNA and it

should be between 1.8 and 2.2. One absorbance unit is equal to 40 µg/ml RNA concentration.

Accordingly, the concentration of RNA is calculated as,

A260 X 40 X Dilution factor = µg/ml RNA concentration

2.2.7 Agarose Gel Electrophoresis

Using agarose gel electrophoresis the integrity and purity of the isolated RNA was visualized. 1% agarose gels were prepared using TBE (Tris/Borate EDTA) buffer. Solution was boiled and the agarose was dissolved. Before cooling and solid formation, EtBr (0.5 µg/ml) added to color the gel under UV. High range RNA loading dye was used as marker and the samples were run for 1 hour at 70-90 V.

2.2.8 DNase-1 Treatment

In order to remove DNA contamination, isolated RNAs were treated with DNase-1 enzyme according to Fermantas DNase-1 treatment kit. Reaction was performed according to the Table 2.1.

Table 2.1 DNase I Reaction Mixture

RNA	1 µg
10 X reaction buffer with $Mg2Cl_2$	1 µl
Ribonuclease inhibitor	0.5 µl
DNase-1 enzyme	1 µl
Depc Treated Water	To 10 µl

The reaction was performed at 37 ^{o}C for 30 minutes. Afterwards, 1 µl 25 mM, chelating agent, EDTA was added to the reaction tube in order to prevent RNA hydrolyzation during heating and incubated at 65 ^{o}C for 10 minutes. When the incubation was over, purified RNA was kept at – 80 ^{o}C until used as a template for reverse transcription.

2.2.9 cDNA Synthesis

In order to generate first strand cDNA for use in real time reverse transcription polymerase chain reaction (qRT-PCR), two step protocol was performed. 2 µg Template RNA, 1 µl oligo$(dT)_{18}$ and DEPC-treated water up to 12 µl were added in an eppendorf tube. The solution was briefly mixed and centrifuged and incubated at 65 ^{o}C for 5 minutes. Following incubation, eppendorf tube was placed on ice. For the second step following components were added to tube in the written order (Table 2.2).

Table 2.2 cDNA Synthesis Reaction Mixture

5X Reaction Buffer	4 µl
RiboLock^{TM} RNase Inhibitor	1 µl
10 mM dNTP Mix	2 µl
RevertAid^{TM} M-MulV Reverse Transcriptase	1 µl
Total	20 µl

Following mixing and centrifuging briefly, the tube was incubated for 60 minutes at 42 ^{o}C and the reaction was terminated by heating at 70 ^{o}C for 10 minutes. The syntesized cDNA was kept at -80 ^{o}C until use, if not used immediately.

2.2.10 Quantitative Reverse Transcription PCR (qRT-PCR)

In order to explore the expression levels of interested genes; Bax, Bcl-2, β-actin, qRT-PCR was performed using the instrument of Corbett Research. A standart curve was drawn by amplifying known dilutions of cDNA (Figure 2.5). Four dilutions were enough to draw a curve, these were no dilution, 1/10 dilution, 1/100 dilution, 1/1000 dilution. Every dilution was measured in triplicates. Using this standart curve expression levels of unknown samples were predicted. Each run was

performed two times. Furthermore, negative controls that have no template cDNA was included.

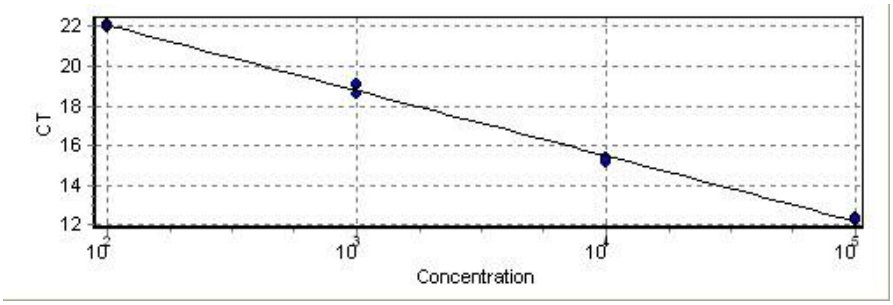

Figure 2.5 qRT-PCR Standart Curve

The Ct value or the threshold cycle meant as the cycle at which a significant increase in fluorescence was first detected. The higher the Ct value, the smaller the copy number, so it takes more reactions to reach the detection level. The slope of the curve was the M value and it should have been between -3.6 and -3.1; the efficiency of the PCR should have been between 90-110%.(Eff = $10^{(-1/slope)}$- 1). Also, R^2 › 0.99 indicated good efficiency.

There was a normalizer gene, in this case, β-actin. This was used as an internal control in order to make better relative gene expression comparisons by normalizing the differences in the amount of RNA added to each reaction.

In order to evaluate the results ΔΔCt method was used.

ΔCt = Ct (target) – Ct (normalizer)

ΔCt is the difference between the C_T values of the target and the normalizer that is β-actin .

ΔΔC= ΔCt (sample) – ΔCt (reference)

Comparative expression level = $2^{-\Delta\Delta Ct}$

DMSO controls for each cell line were reference treatments and other treatment comparisons were done according to these. Subtraction of ΔCt value of DMSO control from ΔCt value of treatments gave the ΔΔCt value and $2^{-\Delta\Delta Ct}$ gave

comparative expression level. So if the ΔCt values of the samples are greater than DMSO control there is a down regulation of gene expression with respect to control, and if the ΔCt values of the samples are smaller than DMSO control there is an up regulation of gene expression with respect to control.

If the result was 1, then there was no change in the expression level. If the result was greater or smaller then 1, that much fold increase or decrease, respectively, in the gene expression level was seen.

For the qRT-PCR reaction, Fast Start Universal SYBR Green Master (ROX) kit of Roche was used. To 5 µl SYBR Green master mix, 2 µl sense and 2 µl antisense primers and 1 µl cDNA were added and the reaction was performed. PCR reaction conditions and programs, and primer sequences are given below for β-actin, Bcl-2 and Bax (Figure 2.6).

β-actin

Initial Denaturation		95 ^{o}C 10 min
Cycling(40repeats)	Denaturation	94 ^{o}C 30 sec
	Annealing	50 ^{o}C 30 sec
	Extension	72 ^{o}C 30 sec

β actin S	CAGAGCAAGAGAGGCATCCT
β actin A	TTGAAGGTCTCAAACATGAT

Bax

Initial Denaturation		95 ^{o}C 5 min
Cycling(40repeats)	Denaturation	94 ^{o}C 30 sec
	Annealing	53 ^{o}C 30 sec
	Extension	72 ^{o}C 30 sec

Bax A	TTGAGGAGTCTCACCCAACC
Bax S	TCTGACGGCAACTTCAACTG

Bcl-2

Initial Denaturation		95 ^{o}C 5 min
Cycling(40repeats)	Denaturation	94 ^{o}C 30 sec
	Annealing	53 ^{o}C 30 sec
	Extension	72 ^{o}C 30 sec

Bcl-2 A	TCTTCAGAGACAGCCAGGAGA
Bcl-2 S	GGATTGTGGCCTTCTTTGAG

Figure 2.6 The Reaction Conditions of qRT-PCR and The Primer Sequences

2.2.11 DNA Fragmentation Assay (Terminal Transferase dUTP Nick End Labeling, TUNEL)

The cells that had undergone to apoptosis were examined by staining the fragmented DNA inside the nucleus of the cell. Assay was performed using Clontech Apoalert DNA Fragmentation Assay Kit. The observation was done using Confocal Laser Scanning Microscope (Hitachi).

MCF-7 and MDA-231 cell lines (100.000 cells/well) were placed in 6 well plates. Each well contained a slide localized at the bottom. The following day, the cells were treated with 0 (DMSO only), 5 μg/ml emodin concentration and left for 12 hours incubation. There were two controls for this assay, one was positive control that had Dnase-1 treatment and the other was negative control that had no dye [Terminal Deoxynucleotidyl Transferase (TdT) minus] because of these, 1 well was used for each of these controls.

When the incubation time was over, the slides were removed from the bottom of well and placed into new 6 well plate to get rid of the medium and unattached cells. All the incubation and washing steps were done in a new plate. Slides were gently washed with PBS and for fixation 4% formaldehyde/PBS was prepared freshly and added to slides for 25 min at 4^{o}C. Following incubation, the slides were washed twice with PBS. The cells were permeabilized by prechilled 0.2% Triton X-100/PBS and incubated for 5 minutes on ice. All the slides were washed with PBS twice for 5 minutes each. At this point in order to prepare a DNase enzyme treated positive control, 100 μl of DNase-1 Buffer was added to slide and incubated at room temperature for 5 minutes. The liquid was removed and 100 μl of DNase-1 Buffer containing 0.5-1 μg/ml DNase was added and incubated for 10 minutes at room temperature. The liquid was removed and the slide was washed with distilled water 3-4 times.

Staining: Having prepared all the samples, microscopy detection part was proceeded. PBS was removed and the cells were covered with 100 μl equilibration

buffer which was a component of the kit and in order to equally spread the liquid, plastic coverslips were put on the slides and equilibration was done at room temperature for 10 minutes.

Following 10 minutes incubation, the coverslips were removed and the liquid was taken. 50 µl of TdT incubation buffer was placed on cells and coverslips were put to equally spread the liquid. The reaction was performed in 5% CO_2 incubator at 37^{o}C for 60 minutes. For TdT minus negative control, instead of TdT enzyme, equal amount of distilled water was added. TdT incubation buffer was prepared for all the samples as follows; 45 µl equilibration buffer, 5 µl nucleotide mix, 1 µl TdT enzyme. and its components must be kept away from light. TdT catalyzed the addition of nucleotides to 3´-OH end of the fragmented DNA, fluorecently labeled dUTPs made the apoptotic cells visible with Confocal Laser Scanning Microscope (Hitachi).

At the end of incubation, coverslips were removed and to terminate the reaction 2X SSC (20XSSC; 3M NaCl, 0.3M NaCitrate, pH 7.0) was added and incubated at room temperature for 15 minutes. Following incubation, slides were washed with PBS twice for 5 minutes each and Confocal Laser Scanning Microscope (Hitachi) used to visualize the fragmented DNAs. If not only the fragmented DNA but also the borders of the cells are also wanted to visualize, the cells can be stained with propidium iodide. As in the case of trypan blue dye, metabolically active cells can export propidium iodide out of the cell, so if the cells are apoptotic, propidium iodide stays in the cell and the cells can be seen. We determined the places of cells by normal visible light and later visualized the fragmented DNAs.

2.2.12 RNA Subunit Detection Using Bioanalyzer

The integrity and the purity of the isolated RNA samples were also analyzed by Bioanalyzer to be sure that the RNA quality was sufficient for microarray analysis. The analysis was performed using RNA nano chip. RNA samples were thawed and diluted 4 times for nanodrop readings and the stock RNAs were immediately removed to refrigerator. The RNA contents were determined using nanodrop.

Having reached to appropriate amounts, the assay was continued. Quantitation range was 25–500 ng/µl for bioanalyzer. For the detection of subunits, Agilent RNA 6000 Nano Assay Protocol was followed and Agilent 2100 Bioanalyzer was used. Firstly, all the kit reagents were waited for 30 minutes to reach to the room temperature. Upon 65µl filtered gel, 1 µl dye was added. 9 µl of prepared gel was loaded to the chip at the bottom of the well marked as 'G' in order to fill all the sample wells and waited for 30 seconds. Again 9 µl of gel was loaded to each of two wells that were placed above the well marked as 'G'. These wells were loaded in order to evenly distribute the voltage. 5 µl of marker was added to each sample wells, in order to be sure the run in that well was allright. After loading markers to each 12 sample wells, 1 µl of samples were loaded to them and later 1 µl of ladder was added to the last remaining well in order to control RNA subunits. Nano chip was vortexed at 1200 rpm for 2 minutes. Before placing the chip to the bioanalyzer, the electrodes of the bioanalyzer were cleaned. 350 µl of RNaseZAP was added to electrode cleaner and placed into bioanalyzer for 1 minute. Later another electrode cleaner was filled with 350µl RNase-free water for 10 seconds. After these cleaning steps, nano chip was placed, the appropriate protocol was selected. For this assay, Eukaryotes Total RNA Nano Series II was selected. The run lasted for 30 minutes.

After marker 18 S and 28 S RNA peaks were should be seen as follows (Figure 2.7)

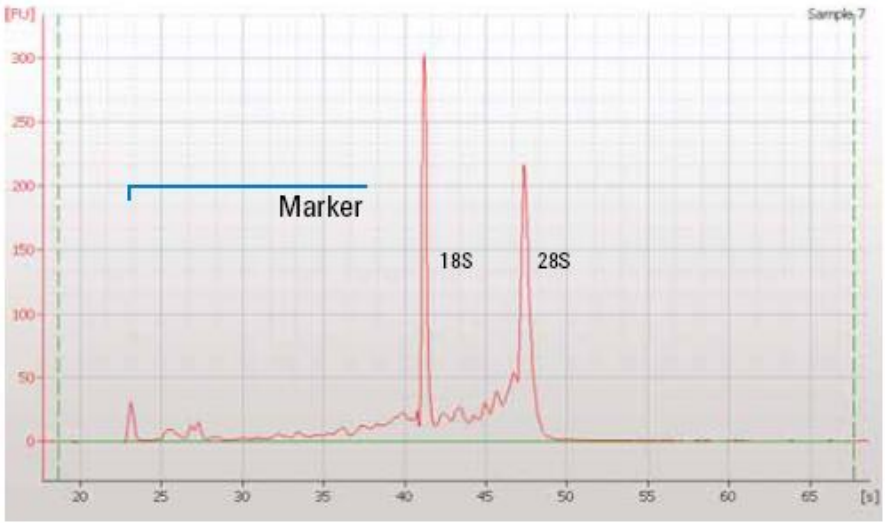

Figure 2.7 RNA peaks of Bioanalyzer given in Agilent RNA 6000 Nano Kit

2.2.13 Microarray

In order to perform microarray, Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array was used and all the kits were supplied from Affymetrix. In the assays; Affymetrix GeneChip® Eukaryotic Poly-A RNA Control Kit, Affymetrix GeneChip® Expression 3'-Amplification Reagents for IVT Labeling, Affymetrix GeneChip® Expression 3'-Amplification Reagents Hybridization Control Kit, Affymetrix GeneChip® Hybridization, Wash, and Stain Kit were used. Applied Biosystems thermal cycler, GeneChip® Scanner 300, Hybridization Oven 640 and Fluidics Station 450 were used in the assays.

2.2.13.1 Total Isolation of RNA

Total RNA isolation from MCF-7 control (1% DMSO treated), 10 μg/ml emodin treated MCF-7, MDA-231 control (1% DMSO treated), 10 μg/ml emodin treated MDA-231 cells and from their biological replicates were performed by Qiagen RNeasy Mini Kit. MCF-7 and MDA-231 cell lines (500.000 cells/well) were placed in 6 well plates. For control cells, 2 columns were used for 12 wells and for treatments, 2 columns were used for 18 wells for each replicate. RNA concentration was measured using nano drop and detection of subunits were checked by agarose gel electrophoresis and bioanalyzer (Agilent 2100 Bioanalyzer).

2.2.13.2 One Cycle cDNA Synthesis

2.2.13.2.1 Preparation of Poly-A RNA Controls for One Cycle cDNA Synthesis

In this part first, poly-A RNA controls were prepared to have exogenous positive controls to observe the labeling process independently from the quality of starting RNA samples, they were amplified and labeled together with the samples. These were genes that were absent in eukaryotic samples (*lys*, *phe*, *thr*, *dap*) and should have given the signal strenght as follows; *lys* < *phe* < *thr* < *dap*. Poly-A RNA controls were supplied in the GeneChip Eukaryotic Poly-A RNA Control Kit. Poly-

A RNA Control Stock and Poly-A Control Dilution Buffer were present in the kit. The appropriate dilutions were made according to total RNA starting amount. For this assay it was 10 µg and the dilutions were as follows (Table 2.3).

Table 2.3 Serial Dilutions of Poly-A RNA Control Stock

Serial Dilutions		
First	Second	Third
1:20	1:50	1:5

2.2.13.2.2 First Strand cDNA Synthesis

For this part, One-Cycle cDNA Synthesis Kit was used.

Table 2.4 RNA/T7-Oligo(dT) Primer Mix Preparation for 10 µg total RNA

Component	Volume
Sample RNA	Variable
Diluted poly-A RNA Controls	2 µl
T7-Oligo(dT) Primer, 50 µM	2 µl
RNase-free water	Variable
Total Volume	11 µl

The components in Table 2.4 were mixed gently, centrifuged briefly 5 seconds and incubated at 70 ^{o}C for 10 minutes. Following incubation the sample was cooled to 4 ^{o}C for 2 minutes followed by centrifugation for 5 seconds.

In a seperate tube First-Strand Master Mix was prepared with components written in Table 2.5.

Table 2.5 First-Strand Master Mix Preparation for One Sample

Component	Volume
5X First- Strand Reaction Mix	4 µl
DTT, 0.1 M	2 µl
dNTP, 10 mM	1 µl
Total Volume	7 µl

After centrifugation for 5 seconds 7 µl of First-Strand Master Mix was taken to each RNA/t7-Oligo(dT) Primer Mix to have the final volume 18 µl. Centrifugation was performed for 5 seconds followed by incubation at 42 ^{o}C for 2 minutes. After this, 2 µl SuperScript II enzyme was added for each RNA sample. After centrifugation for 5 seconds, incubation was done at 42 ^{o}C for 1 hour and sample cooled at 4 ^{o}C for 2 minutes. After incubation at 4^{o}C, centrifugation was done before proceeding to the Second Strand cDNA Synthesis.

2.2.13.2.3 Second Strand cDNA Synthesis

Again, One-Cycle cDNA Synthesis Kit was used. For this part first, Second Strand Master Mix was prepared in a seperate tube with the components in Table 2.6 and it was recommended to prepare the mixture immediately before use.

Table 2.6 Second Strand Master Mix Preparation for One Sample

Component	Volume
RNase-free Water	91 µl
5X Second Strand Reaction Mix	30 µl
dNTP, 10 mM	3 µl
E. coli DNA ligase	1 µl
E. coli DNA Polymerase I	4 µl
RNase H	1 µl
Total Volume	130 µl

After centrifugation for 5 seconds, 130 µl of Second Strand Master Mix was added to the prepared mixture at the end of First Strand cDNA Synthesis part. After centrifugation for 5 seconds, the incubation was started at 16 ^{o}C for 2 hours. After this, 2 µl of T4 DNA Polymerase was added to each sample and incubated at 16 ^{o}C for 5 minutes. At the end of this incubation step, 10 µl of EDTA 0.5M was added and proceeded to the following step.

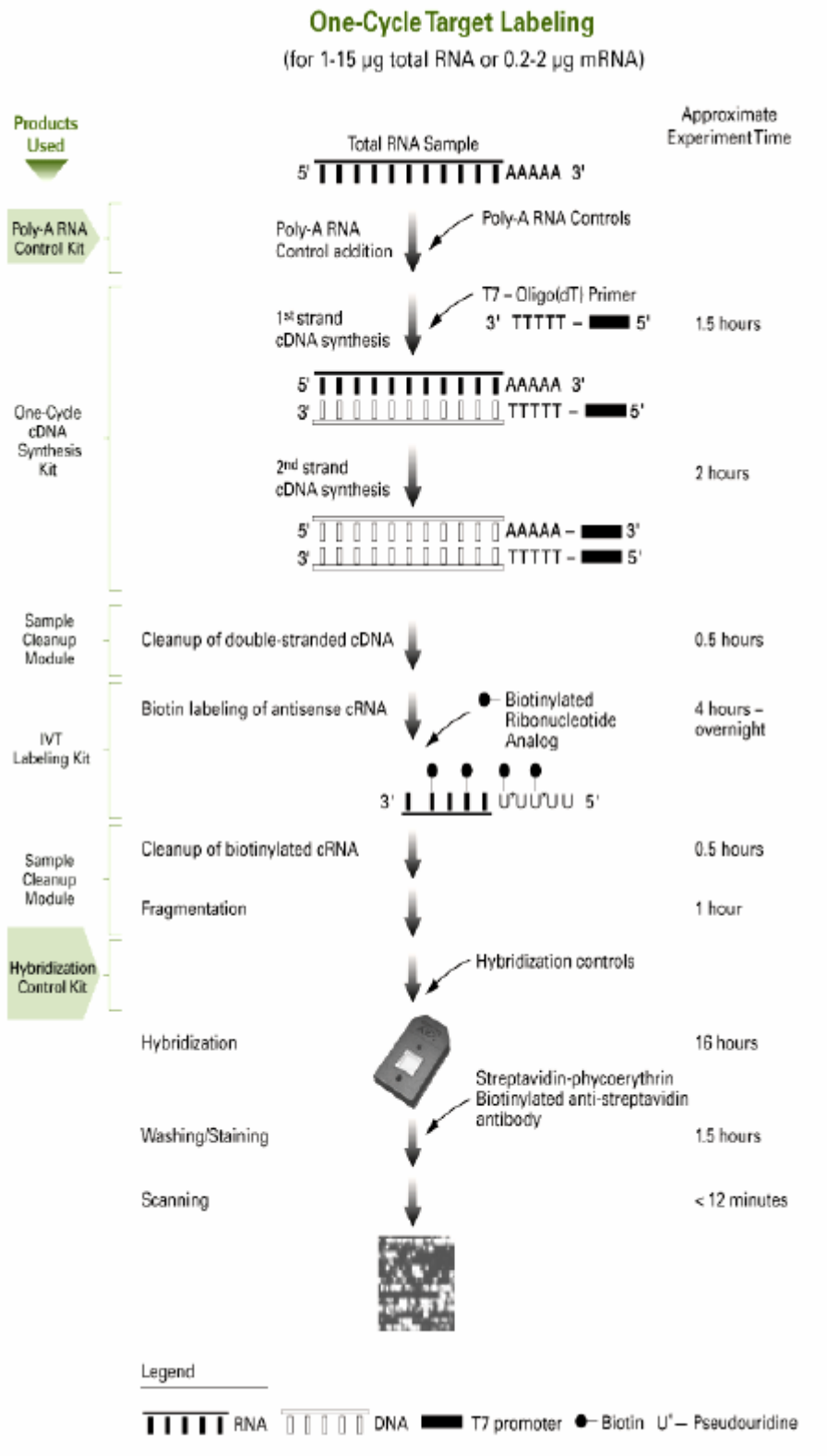

Figure 2.8 GeneChip® Expression Labelling Assays for Expression Analysis (Affymetrix GeneChip® Expression Analysis Technical Manual)

2.2.13.3 Cleanup of Double Stranded cDNA

GeneChip Sample Cleanup Module was used for this part. 600 µl of yellow colored cDNA Binding Buffer was added to the double stranded cDNA synthesized tube and vortexed for 3 seconds. Unchanging of the color told, the assay was going well, this was the first check point. After this 500 µl of the sample was added to the cDNA Cleanup Spin Column that was placed upon 2 ml Collection Tube and centrifuged at 8.000 g for 1 minute and the collection tube was emptied. The same process was applied for the remaining mixture. After this the spin column was placed to a new 2 ml Collection Tube and 750 µl of cDNA wash buffer was added onto it and centrifuged at 8.000 g for 1 minute and the collection tube was emptied (24 ml absolute ethanol should have been added at the first opening of cDNA wash buffer box). After, the caps of the spin column was opened and centrifuged at 25.000 for 5 minutes to let the membrane dry. Later, spin column was placed to a new collection tube and 14 µl cDNA elution buffer was added directly yo spin column membrane, incubated at room temperature for 1 minutes and centrifuged at 25.000 g for 1 minute. The elution volume was 12 µl after centrifugation. cDNA was ready now for the synthesis of biotin labeled cRNA.

2.2.13.4 Synthesis of Biotin Labeled cRNA

GeneChip IVT Labeling Kit was used for this part. The starting material was 10 µg RNA, in this case it was said to use 6 µl of 12 µl eluted cDNA in IVT Labeling Protocol. The remaining cDNA was stored at -20 ^{o}C. The protocol was as in Table 2.7. At this point it was important not to let the reaction mixture place on ice, if this was the case, 10 X IVT Labeling might cause precipitation of the template cDNA.

Table 2.7 IVT Labeling Mixture Preparation

Component	Volume
Template cDNA	6 µl
RNase free water	14 µl
10 X IVT Labeling Buffer	4 µl
IVT Labeling NTP Mix	12 µl
IVT Labeling Enzyme Mix	4 µl
Total Volume	40 µl

The mixture was centrifuged briefly for 5 seconds and incubated at 37 ^{o}C for 17 hours.

2.2.13.5 Cleanup and Quantification of Biotin Labeled cRNA

2.2.13.5.1 Cleanup of Biotin Labeled cRNA

GeneChip Sample Cleanup Module was used for this part. Before cleaning, an aliquot was saved of unpurified IVT product for nano drop readings. Later, 60 µl RNase free water was added to IVT reaction and vortexed for 3 seconds. 350 µl IVT cRNA Binding Buffer was added to mixture and vortexed for 3 seconds. 250 µl absolute ethanol was added and mixed well by pipetting. All the mixture was added to the IVT cRNA Cleanup Spin Column that was placed onto 2 ml Collection Tube, centrifuged at 8.000 g for 15 seconds and the collection tube was emptied. The spin column was transferred to a new collection tube. 500 µl IVT cRNA Wash Buffer (previously 20 ml absolute ethanol was added) was added to the spin column and centrifuged at 8.000 g for 15 seconds, the collection tube was emptied. 500 µl 80 % ethanol was added to spin column and centrifuged at 8.000 g for 15 seconds, the collection tube was emptied. After this in order to dry the membrane, the cap of the spin column was opened and centrifuged at 25.000 g for 5 minutes. The spin column was placed in to a new collection tube and 11 µl RNase free water was added in order to elute the cRNA, centrifuged at 25.000 g for 1 minute. Again 10 µl RNase free water was added directly to the membrane and centrifuged at 25.000 g for 1 minute. The cRNA sample was ready for quantification.

2.2.13.5.2 Quantification of cRNA

Quantification of cRNA at this time of the assay was made to check the purity of the cRNA and to observe the yield if it was enough for further steps. Amount of cRNA after IVT was determined by nanodrop reading. Also, the amount of the aliquot that was taken from unpurified IVT product was measured, and by this way it was checked that if the amount of the purified IVT product was not enough for further steps, it was because of bad handling of the cleanup step. If this was the case the IVT labeling procedure was performed again with the 6 µl of the remaining cDNA that was left to -20 ^{o}C.

In order to calculate the adjusted cRNA yield (labeled cRNA), the following formula was used;

adjusted cRNA yield = RNAm – (total RNAi) (y)

RNAm = amount of cRNA measured after IVT (µg)

total RNAi = starting amount of total RNA (µg) (For this assay 10 µg)

y = fraction of cDNA reaction used in IVT (50%)

2.2.13.6 Fragmenting the cRNA for Target Preparation

The Fragmentation Buffer used in this step was optimized to fragment cRNA to 35 to 200 base fragments by metal induced hydrolysis. Reaction mixture was prepared as in Table 2.8, calculated adjusted cRNA amount was used.

Table 2.8 Fragmentation Reaction Mixture Preparation

Component	49Format (Chip format used in this assay)
cRNA	20 µg (1 to 21 µl)
5X Fragmentation Buffer	8 µl
RNase free water	To 40 µl final volume
Total Volume	40 µl

The mixture was incubated at 95 ^{o}C for 35 minutes and following incubation, put on ice.

2.2.13.7 Eukaryotic Target Hybridization

In this step in order to control the hybridization, GeneChip Expression 3'–Amplification Reagents Hybridization Control Kit and GeneChip Hybridization, Wash and Stain Kit were used. Firstly, Hybridization Cocktail was prepared as in Table 2.9.

Table 2.9 Hybridization Cocktail for Single Probe Array

Component	49 Format (Standart) Array
Fragmented and Labeled cRNA	15 µg
Control Oligonucleotide B2 (3nM)	5 µl
20X Eukaryotic Hybridization Control (*bioB, bioC, bioD, cre*)	15 µl
2X Hybridization Mix	150 µl
DMSO	30 µl
Nuclease free water	to final volume of 300 µl
Total Volume	300 µl

20X GeneChip Eukaryotic Hybridization Controls were heated to 65 ^{o}C for 5 minutes.

At this time it was necessary to take the probe array out of the refrigerator to reach to room temperature in order to prevent cracking that can lead to leaks.

The hybridization cocktail was heated to 99 ^{o}C for 5 minutes.

At the same time the probe array was filled with 200 µl of Pre-Hybridization Mix and incubated at 45 ^{o}C for 10 minutes with rotation at hybridization oven. There were two septa as shown in Figure 2.9. A yellow pipette tip was placed on the upper septa to have air space, and with an angle smaller than 90^{o} the other septa was used

to fill the probe array, as it was filled with Pre-Hybridization Mix, the wetness of the front side was checked.

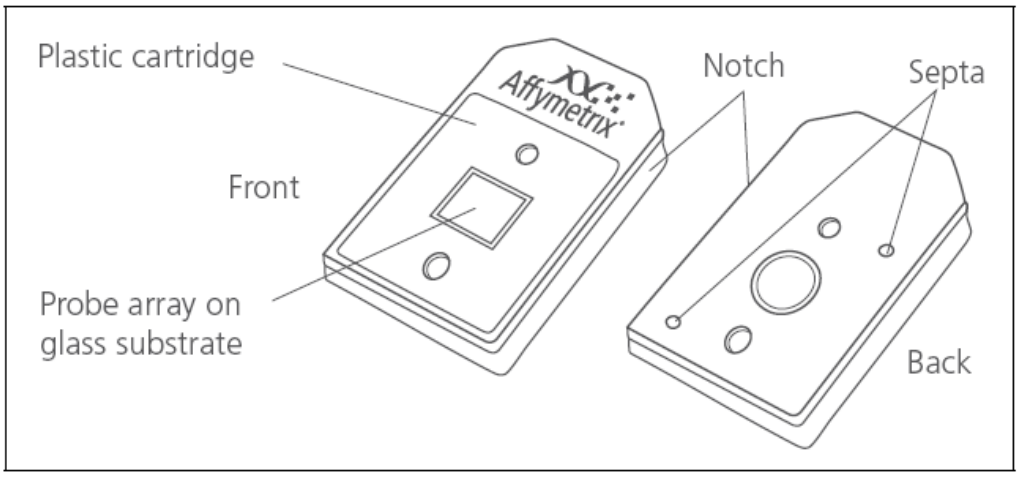

Figure 2.9 GeneChip® Probe Array

Meanwhile, the hybridization cocktail was taken from 99 ^{o}C and placed to 45 ^{o}C again for 5 minutes. Later, the hybridization cocktail was centrifuged at maximum speed for 5 minutes at room temperature to collect all the components bottom of the tube (The maximum speed of the microcentrifuge was 19720 g).

At the end of incubation time, the array was taken from the hybridization oven. Again yellow pipette tip was placed on the upper septa and the Pre-Hybridization Mix was taken from the other septa. The array was refilled with 200 µl Hybridization Cocktail, any bubble was avoided. After filling, the yellow tip was taken and the two septa openings was closed with small sticky papers. Later the array was placed into the hybridization oven in a balanced configuration with rotation at 60 rpm and the temperature was set to 45 ^{o}C. Hybridization lasted for 17 hours.

2.2.13.8 Washing, Staining and Scanning

In this part, GeneChip Hybridization, Wash and Stain Kit was used. Firstly, Fluidics Station 450 was prewashed with distilled water, Wash A (Non-Stringent Wash Buffer) and Wash B (Stringent Wash Buffer) Buffers that were supplied with the kit.

When the hybridization was over, the hybridization coctail was removed and the array filled with Wash A buffer. Three staining steps were performed. First and third were done with SAPE Stain Solution (Table 2.10), while the second was done with biotinylated antibody (Table 2.11). SAPE Solution Mix was prepared fresh before usage.

Table 2.10 SAPE Solution Mixture Preparation

Components	Volume
2X Stain Buffer	600 µl
50 mg/ml BSA	48 µl
1 mg/ml Streptavidin Phycoerythrin (SAPE)	12 µl
Distilled Water	540 µl
Total Volume	1200 µl

1200 µl SAPE solution was divided into two for stainings 1 and 3.

Table 2.11 Antibody Solution Mixture

Components	Volume
2X Stain Buffer	300 µl
50 mg/ml BSA	24 µl
10 mg/ml Goat IgG Stock	6 µl
0.5 mg/ml biotinylated antibody	3.6 µl
Distilled Water	266.4 µl
Total Volume	600 µl

We chosed ‘EukGE-WS2v5_450’ Fluidics Script because our chip format was 49 and feature size was 11 μm. For this script fluidics protocol in Table 2.12 was used.

Table 2.12 Fluidics Protocol

Protocol	EukGE-WS2v5_450
Post Hyb Wash # 1	10 cycles of 2 mixes/cycle with Wash A Buffer at 30 ^{o}C
Post Hyb Wash # 2	6 cycles of 15 mixes/cycle with Wash B buffer at 50 ^{o}C
First Stain	Stain the probe array for 5 minutes in SAPE solution at 35 ^{o}C
Post Stain Wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 30 ^{o}C
Second Stain	Stain the probe array for 5 minutes in antibody solution at 35 ^{o}C
Third Stain	Stain the probe array for 5 minutes in SAPE solution at 35 ^{o}C
Final Wash	15 cycles of 4 mixes/cycle with Wash A Buffer at 35 ^{o}C, holding temp. at 25 ^{o}C

Total procedure lasted for 90 minutes.

After washing and staining, the two septa was closed again and array was scaned using GeneChip Scanner 3000.

2.2.14 Statistical Analysis

For XTT and cell counting assays, GST enzyme analysis and qRT-PCR results, Mann Whitney test; and for microarray analysis one way ANOVA was used as statistical analysis.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 The Absorbance Spectrum of Emodin

Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) being an anthraquinone gives yellow color in visible light. In order to prevent misinterpretation of the results of the experiments that were analyzed by spectrophotometer, the interference of emodin to the optical measurements should be determined.

The typical absorbance spectrum of 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, 100 µg/ml emodin solutions were determined. Figure 3.1 shows the absorbance spectrum of 10 µg/ml emodin solution recorded between wavelengths 250 nm-800 nm.

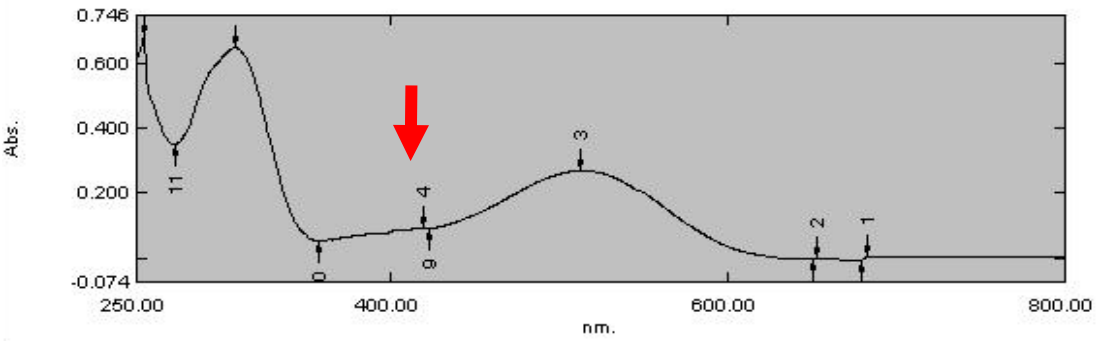

Figure 3.1 The absorbance spectrum of 10 µg/ml emodin solution. Emodin is dissolved in DMSO than diluted to 2% DMSO in basic dH_2O and read against 2 % DMSO in basic dH_2O by Shimadzu double beam UV-Vis spectra

In XTT cytotoxicity assay, measurements are done at 415 nm. As shown in Figure 3.1, emodin absorbs light at 415 nm. The absorbance of emodin solutions prepared

at the concentrations used in XTT assay were determined at wavelength of 415nm in a separate experiment and shown in Table 3.1.

Table 3.1 The absorbances of 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml, 100 μg/ml emodin concentrations at 415 nm.

Emodin Concentrations (μg/ml)	Absorbance at 415 nm
10	0.090
20	0.226
40	0.414
80	0.791
100	1.004

It was clear that the increasing concentrations of emodin in XTT assay would contribute to the absorbance values especially at higher emodin concentrations. Therefore, emodin absorbance values were subtracted from XTT measurements at corresponding concentrations.

3.2 Effects of Emodin on Cell Cytotoxicity

3.2.1 Viable Cell Counting with Trypan Blue

In order to get rid of the absorbance interference of emodin on XTT assay, one of the principle cell culture methods, cell counting with trypan blue was also performed.

Trypan blue staining provided the information about effects of emodin treatment on viable cell number, viable cell counts were converted to percent cell viability by setting control counts as 100% cell viability. Cells that were grown in 1% DMSO containing medium are considered as controls and their counts were set as 100% cell viability.

Averages of counts were expressed as percentage of the control counts and percent cell viability for 48 hour treatment versus 0 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml emodin concentrations were plotted (Figure 3.2).

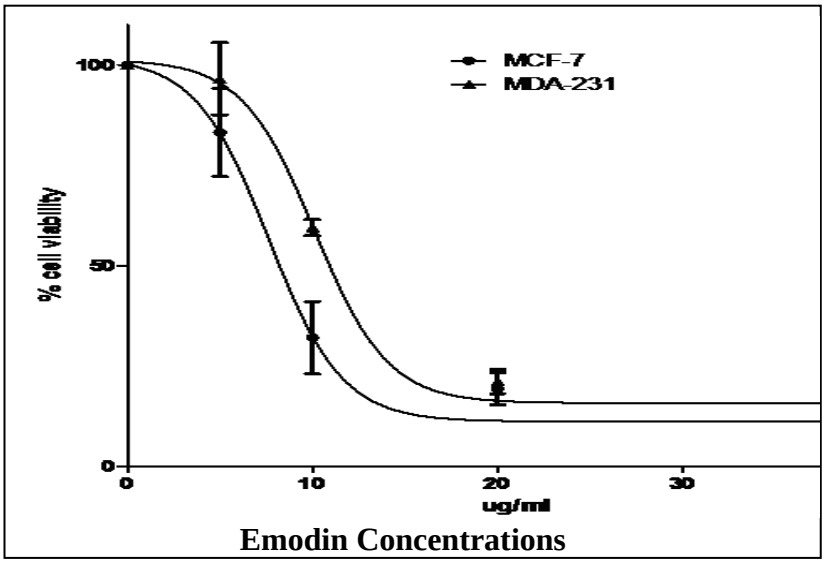

Figure 3.2 Effects of varying emodin concentrations on viability of MCF-7 and MDA-231 cells which were treated with emodin for 48 hours. The viable cells were counted after trypan blue staining. The points indicate the average of duplicate measurements from two biological replicates. Graph was drawn using Graph Pad Prism program.

The effects of emodin on viability of MCF-7 and MDA-231 were also shown in Table 3.2.

Table 3.2 Effects of emodin on viability of MCF-7 and MDA-231 cells which were treated with varying emodin concentrations for 48 hours. Viable cell counts were recorded after trypan blue staining.

Emodin Concentration (µg/ml)	MCF-7 % cell viability ± SD	MDA-231 % cell viability ± SD
0	100	100
5	83.24 ± 11.44	96.60 ± 9.65
10	32.11 ± 9.03	59.51 ± 2.99
20	19.40 ± 4.01	21.20 ± 3.80
40	3.41 ± 0.85	11.21 ± 1.83

IC50 values of two cell lines were shown in Table 3.3.

Table 3.3 IC50 values in MCF-7 and MDA-231 cells obtained by counting with tryphan blue.

IC50 MCF-7 (µg/ml)	IC50 MDA-231 (µg/ml)
8.40	12.17

From the growth inhibition curves (Fig. 3.2), IC50 values that is the emodin concentration at which 50% of cells are viable were calculated as 8.40 µg/ml and 12.17 µg/ml for MCF-7 and MDA-231 cells, respectively. These results showed that although the difference between the two value was not statistically significant, $p>0.08$, the inhibitory effect of emodin was higher in MCF-7 cells (ER+) than MDA-231 (ER-) cells, because the amount of emodin concentration required to kill 50% of cells were lesser in MCF-7 cells. Furthermore, from our results it was also seen that emodin exhibited growth inhibition on both estrogen receptor positive and estrogen receptor negative cells.

Higher inhibitory effect of emodin on MCF-7 cells were also reported in literature. Kang et al (2008) have isolated some known anthraquinones from the roots of *Rheum palmatum* and examined for their estrogenic activity. Among them, emodin was found to have highest estrogenic activity. The fraction that contains emodin caused cytotoxicity in MCF-7 and MDA-231 cell lines with the statistically significant IC50 values of 16.4 and 46.3 µg/ml, respectively, for 48 hours showing that emodin had hormone dependent and also independent effects to cells (Kang et al., 2008).

The inhibitory effects of emodin on MCF-7 and MDA-231 cells were observed on inverted microscope as given in Figures 3.3 and 3.4. respectively.

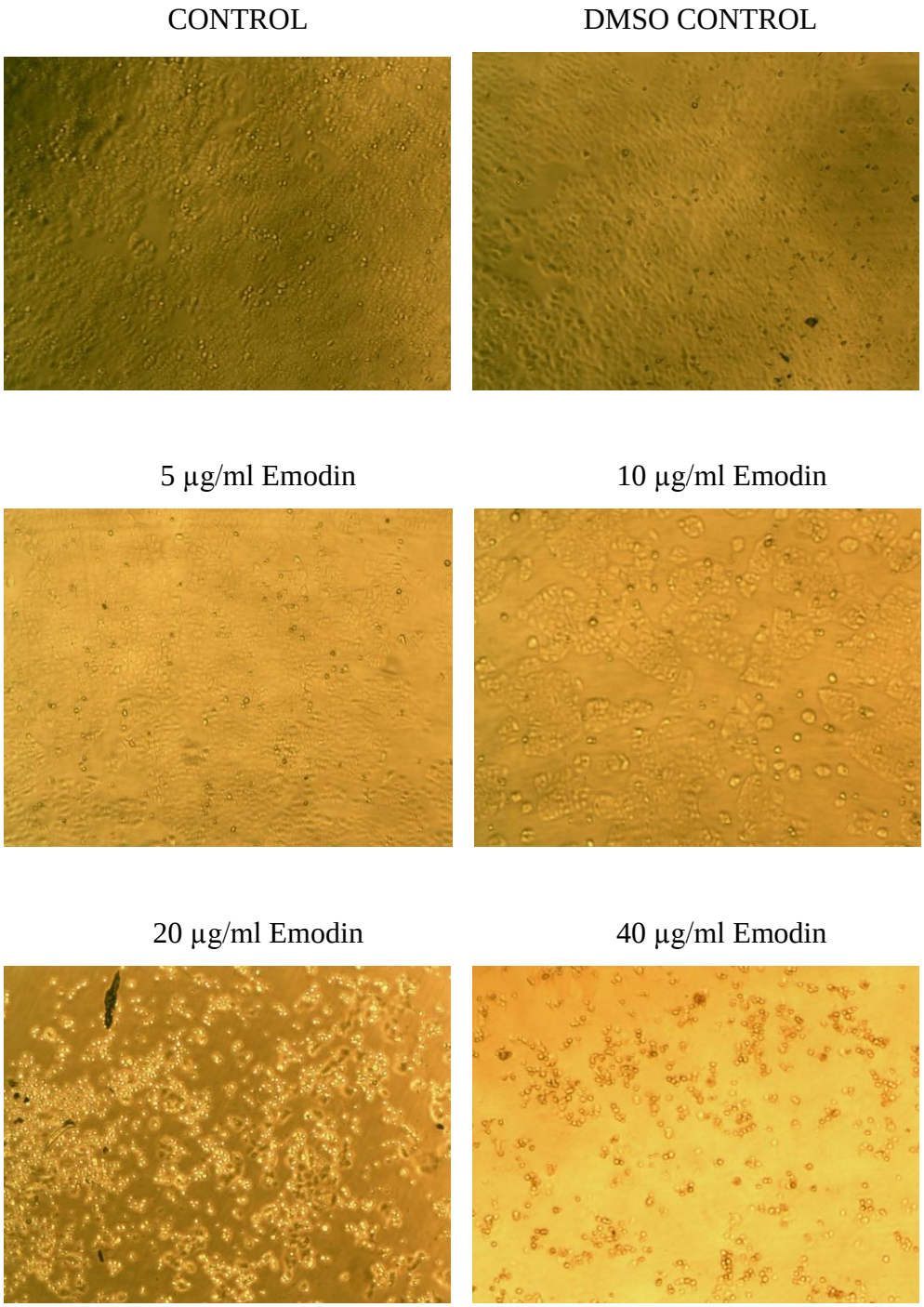

Figure 3.3 Inverted microscope pictures (4X) of MCF-7 cells seeded in 24 well plates treated with 0 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml emodin concentrations for 48 hours.

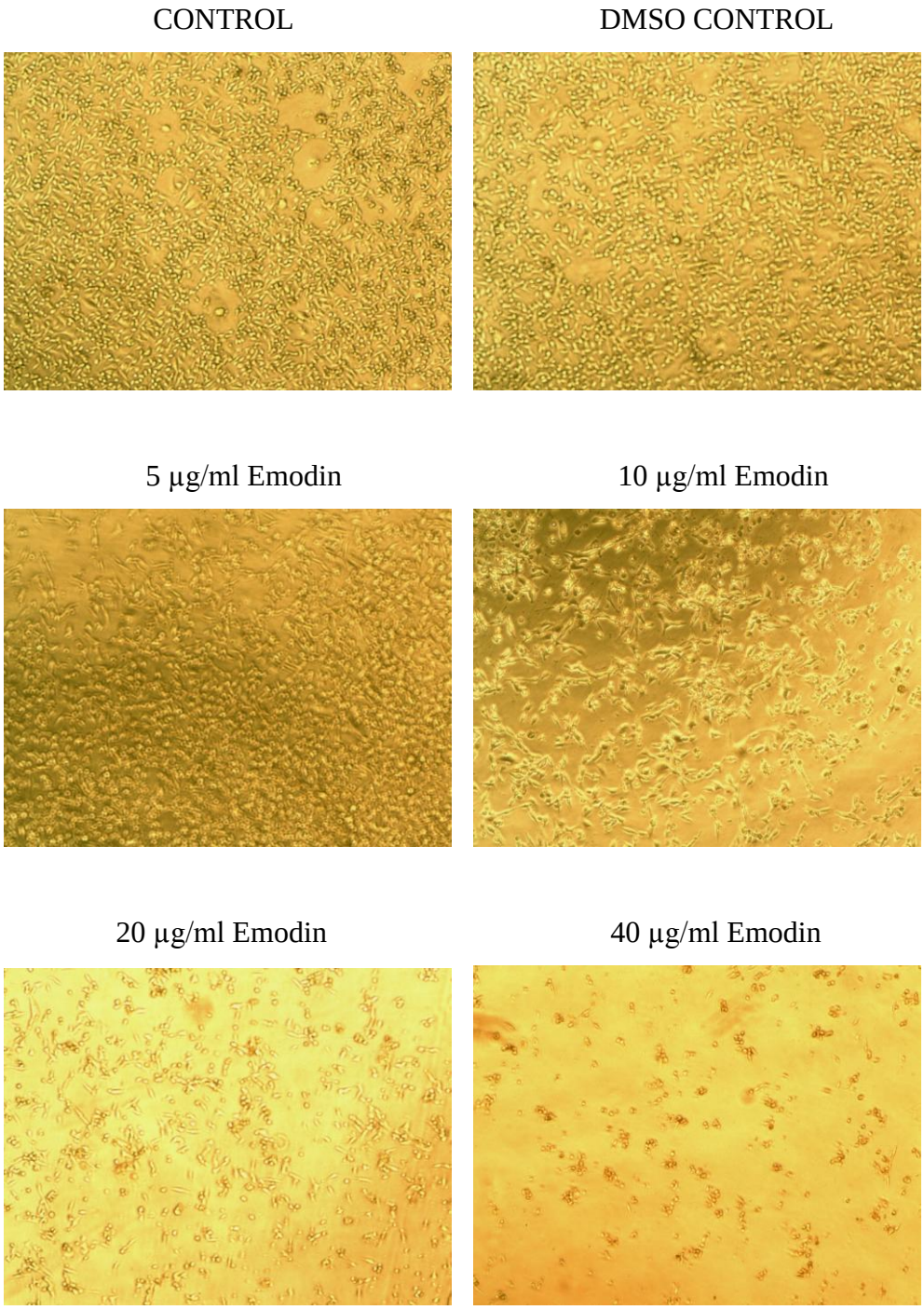

Figure 3.4 Inverted microscope pictures (4X) of MDA-231 cells seeded in 24 well plates treated with 0 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml emodin concentrations for 48 hours

It was observed that the first effect of 0-40 μg/ml emodin to 48 hours treated cells was the detachment of cells. Detached, floating cells were seen in the medium and increased with the increasing emodin concentrations.

In order to examine the effects of potential chemotherapeutic agents to the metastatic cascade of breast cancer cells; their effects on adhesion, invasion and migration are studied as three major metastasis indicators. It was reported by Huang et al. (2004) that emodin significantly inhibited cell adhesion and invasion in MDA-231, HSC5 cells. In 2006 they found that emodin inhibited tumor cell adhesion in MDA-231 HepG2 and HSC5 through disturbing membrane lipid raft clustering by interrupting integrin signalling pathway (Huang et al., 2006). They have shown the inhibitory effect of emodin on adhesion of the 0-40 μM emodin pretreated cells to fibronectin, collagen and laminin that are extracellular matrix components. It was seen that emodin decreases cell adhesion with the increasing concentrations. Furthermore it was also observed that emodin inhibited cell spreading with increasing concentrations. Huang et al. (2005) also showed the inhibitory effect of emodin on the migration and invasion (Huang et al., 2004) in their previous studies.

In 2004 Huang et al. showed that emodin caused suppression of MMP (matrix metalloproteinase)-9 expression through inhibiting AP-1 (activator protein-1) and NF- kB (nuclear factor-kB) and also ERK (extracellular signal regulated kinase) pathways in skin squamous and MDA-231 breast cancer cell lines and inhibited tumor invasion (Huang et al., 2004).

As Huang's results, we also observed detachment of cells upon treatment with emodin and this detachment increased as increasing concentrations.

Another study declaring emodin's inhibitory effects on metastasis belongs to Mahabeleshwar et al.(2004). In MCF-7 and MDA-231 cells, ROS induced Lck dependent MEK-1 and ERK dependent uPA (urokinase-type plasminogen activator) synthesis which caused cell motility were prevented by emodin being a Lck tyrosine

kinase activity inhibitor and the predicted mechanism was given in Figure 1.6 (Mahabeleshwar et al., 2004).

3.2.2 XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) Cell Cytotoxicity Assay

Metabolically active MCF-7 and MDA-231 cells were detected using XTT assay upon treatment with emodin for 48 hours. Mitochondrial enzymes of the metabolically active cells reduced tetrazolium salt, XTT and orange colored formazan product was measured using ELISA plate reader. Cell viability measurements obtained from XTT assay were converted to percent cell viability by setting control results as 100% cell viable. Averages of measurements upon treatment with 0 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml, 100 μg/ml emodin concentrations for 48 hours were expressed as percentage of the 1% DMSO control measurements.

As it was mentioned before, emodin had absorbance values at 415 nm at which XTT measurements were done and absorbance values increased as the emodin concentrations increased as it was given in Table 3.1. In order to prevent interference by emodin, blank wells that were seen in Figure 2.2 were subtracted from sample wells.

Beside emodin's spectral interference, there was another important reason of emodin to cause misinterpretation of the results with XTT assay. Botanical extracts and chemicals in them have the ability to reduce tetrazolium salt MTT, and denser color formed because of this additional reduction might be supposed as if there were higher metabolically active cells (Shoemaker et al., 2004). One of the plant species used in the study was *Rheum palmatum* that contains high amounts of emodin. In order to prevent misinterpretations, it is advisable to use emodin blanks and subtract them from samples or it is better to count cells with trypan blue when studying with colorful, botanical chemicals.

From XTT cell cytotoxicity assay curves IC50 values were calculated as 25.67 μg/ml and 15.10 μg/ml for MCF-7 and MDA-231 cells respectively and these were shown in Table 3.4.

Table 3.4 IC50 values of XTT Cell Cytotoxicity Assay curves of 0 μg/ml, 10 μg/ml, 20 μg/ml, 40 μg/ml 48 hours emodin treated MCF-7 and MDA-231 cells

IC50 MCF-7 (μg/ml)	IC50 MDA-231 (μg/ml)
25.67	15.10

From the IC50 values, it could have been said that emodin had more inhibitory effect on MDA-231 cells than MCF-7 cells but as it was explained above, the XTT results aren't reliable when using botanical chemicals because of this IC50 values of cell counts are preferable to comment. It was concluded that the growth inhibitory effect of emodin was higher in MCF-7 cells than MDA-231 cells. This is understandable because emodin has inhibitory effects on ER of MCF-7 and this might have caused additional inhibitory effects of emodin. Nevertheless, using emodin as an adjuvant therapy with known anticancer drugs might give better results and enhance the achivement of the therapy as in the case of a chemotherapeutic drug, Paclitaxel that is used to treat metastatic breast cancer. It was seen that emodin sensitized HER-2 (proto-oncogene that encodes transmembrane tyrosine growth factor receptor) overexpressing breast cancer cells in athymic mice to the inhibitory effect of Paclitaxel (Zhang et al., 1999).

It was reported by Zhang et al. (1999) that emodin caused much more growth inhibition on HER-2 overexpressing breast cancer cells. MCF-7 and MDA-231 cells that express basal levels of HER-2 were inhibited to a lesser extend by emodin. While emodin inhibited the growth of HER-2 overexpressing breast cancer cell line, MDA-453, as 68%, MCF-7 and MDA-231 cells were inhibited by emodin as 37% and 23% at 40 μM concentration (≈10μg/ml emodin) respectively. Cytotoxicity tests were done using MTT, so the killing percent might be higher than it seemed, as it was explained before and the inhibitory effect of emodin on MCF-7 and MDA-231

cells might be through different pathways and different receptors other than HER-2. Nevertheless in this study, it was shown that the inhibitory effect of emodin on HER-2 overexpressing cell lines were stronger. Beside the effects of emodin on breast cancer cell lines; HBL-100, normal human breast tissue cell line, was insensitive to the inhibitory effects of emodin. Emodin didn't change the protein level of breast cancer cell lines, instead decreased tyrosine phosphorylation activity of the receptor. Also it was seen that emodin preferentially induced differentiation and suppressed transformation of HER-2 overexpressing cell lines (Zhang et al., 1995).

Matsuda et al. (2001) published that emodin caused MCF-7 proliferation at 1 to 10 μM concentrations (≈0.25 to 2.5 μg/ml). They also showed that emodin inhibited binding of estrogen to its receptors (Matsuda et al., 2001). Phytoestrogens have the ability to show estrogenic activity at low concentrations whereas they inhibit growth at high concentrations (Lemos, 2001; Schmitt et al., 2002). The proliferation that was reported by Matsuda et al. (2001) might be because of treatment with low emodin concentrations or because they had misinterpreted the results of MTT assay. They found that emodin inhibited binding of estrogen to ERα at 0,77μM so if this binding was inhibited, estrogenic activity should have been inhibited and cell proliferation should have been decreased.

3.3 Effects of Emodin on Apoptosis

3.3.1 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

qRT-PCR was performed to analyze the expression levels of apoptosis genes Bax and Bcl-2. Increase in the expression of apoptotic gene Bax and decrease in the expression of antiapoptotic gene Bcl-2 indicate the presence of apoptosis. Therefore, the increase of the ratio of Bax expression to Bcl-2 expression is an indicator of apoptosis.

3.3.1.1 Qualification of RNA by Agarose Gel Electrophoresis

In order to visualize the integrity and purity of the isolated RNA, agarose gel electrophoresis was used. It was aimed to see three subunits (28S, 18S, 5S rRNA) of RNA without DNA contamination. It was seen that without Dnase-1 treatment, RNA, that was isolated by kit, contained DNA contamination, so it was necessary to apply Dnase-1 treatment to RNAs that were isolated by kit unless the capacity of the column was filled. As it is seen in Figure 3.5, RNA of MCF-7 control cells without DNase-1 treatment contained DNA (lane 2), after DNase-1 treatment DNA contamination was eliminated but unfortunately, about half of the RNA was also degraded. Also, in order to test the DNA content of the isolated RNA without DNase-1 treatment, qRT-PCR was performed using isolated RNA as sample and amplification of the contaminated DNA was observed.

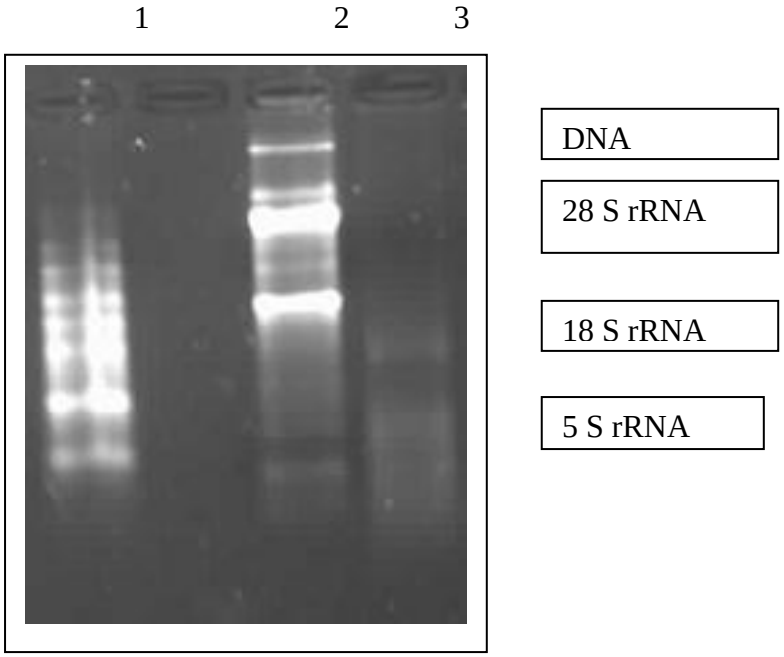

Figure 3.5 Total RNA isolated from control MCF-7 cells using kit (lane2, without DNase-1 treatment and lane3 with DNase-1 treatment) and RNA ladder (lane 1) (1% agarose gel).

3.3.1.2 Qualification of RNA by Bioanalyzer

The integrity and purity of isolated RNA with DNase-1 treatment were also analyzed by Bioanalyzer. The ratio of 28S to 18S is important in this analysis, the more the ratio reaches to 2, the better the integrity, if the ratio is too small, this indicates RNA is fragmented. Figure 3.6 shows the bioanalyzer result of RNA sample after DNase-1 treatment. The ratio of 28S to 18S was 1.5. It was seen from bioanalyzer results that quality of RNA with DNase-1 treatment was better than the quality of RNA without DNase-1 treatment.

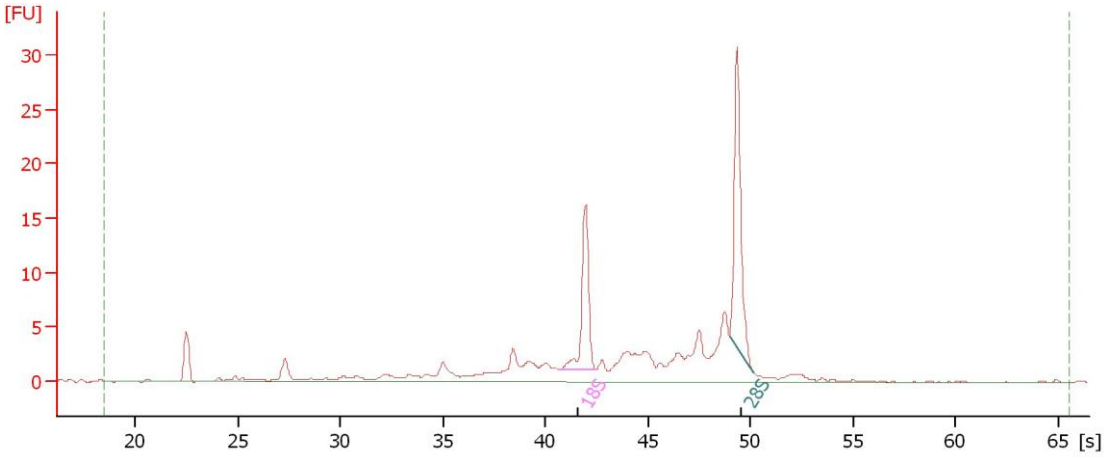

Figure 3.6 Bioanalyzer (Agilent) result of total RNA isolated from control MCF-7 cell line with DNase-1 treatment

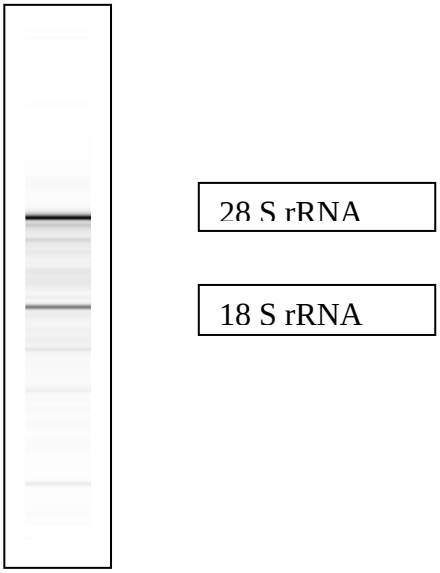

Figure 3.7 Gel image of bioanalyzer (Agilent) result of total RNA isolated from control MCF-7 cell line with DNase-1 treatment

3.3.1.3 Expression Analysis of Bax and Bcl-2 Genes

One important indicator of apoptosis is the change in the expression levels of Bcl-2 family genes. The expression of apoptotic gene Bax increases whereas the expession of antiapoptotic gene Bcl-2 decreases during apoptosis, resulting in increase of Bax/Bcl-2 ratio (Cory and Adams, 2002). The effects of emodin treatment on apoptosis were investigated by qRT-PCR measuring gene expression levels of Bax and Bcl-2 in MCF-7 and MDA-231 cells. The changes in expression levels of corresponding genes in MCF-7 and MDA-231 were given in Figures 3.8 and 3.9 respectively. Fold changes in gene expressions were calculated using $2^{-\Delta\Delta Ct}$ method. Gene expressions in 1% DMSO treated cells were used as controls. Hence, the fold change in 1% DMSO control was considered as 1.

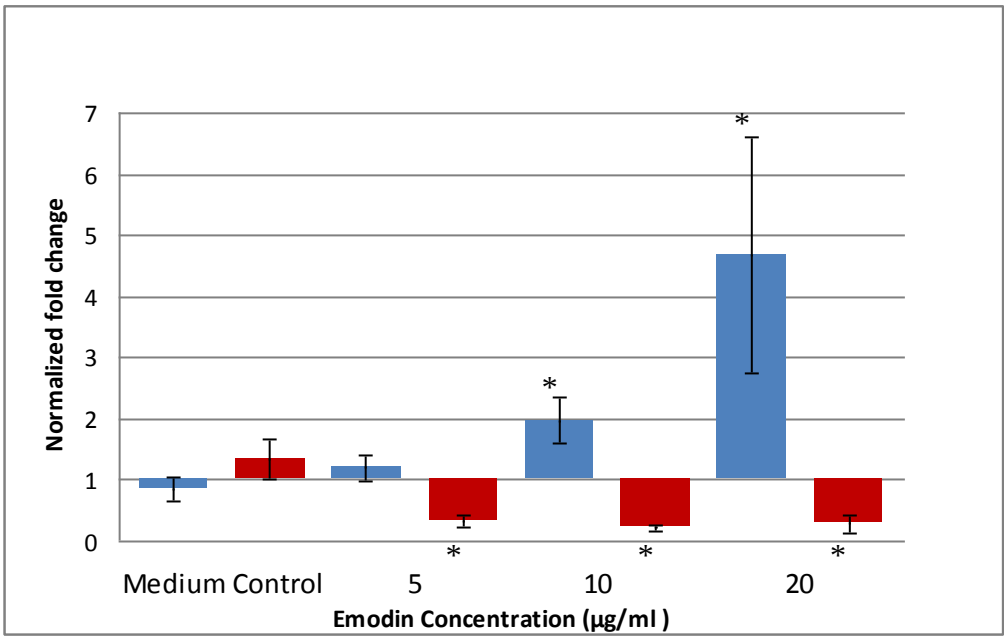

Figure 3.8 Expression of Bax and Bcl-2 genes in MCF-7 cells upon treatment with varying concentrations of emodin for 48 hours. The values are the average of triplicate measurements from two biological replicates. * Statistically significant, $p < 0.005$ (Mann Whitney Test).

As seen in Figure 3.8 Bax expression increased with increasing emodin concentrations. At least 2 fold and 4 fold increases were detected in 10 µg/ml and 20

µg/ml emodin treated MCF-7 cells, respectively. However, Bcl-2 expressions were decreased less than 2 fold in these cells.

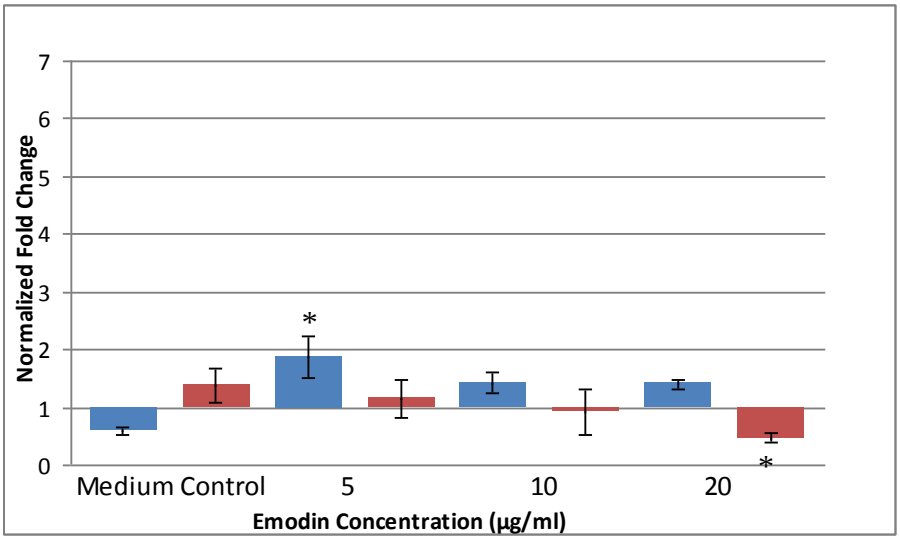

Figure 3.9 Expression of Bax and Bcl-2 genes in MDA-231 cells upon treatment with varying concentrations of emodin for 48 hours. The values are the average of triplicate measurements from two biological replicates. * Statistically significant, $p < 0.005$ (Mann Whitney Test).

In MDA-231 cells, Bax and Bcl-2 expression changes were less than 2 fold (Figure 3.9).

Table 3.5 shows the effects of emodin treatment on Bax/Bcl-2 ratio. Although the ratio increased as the concentration increased in MDA-231 cells, this increase was not pronounced as much as that of MCF-7.

Table 3.5 Effects of emodin treatment on Bax/Bcl-2 ratio in MCF-7 and MDA-231 cell lines treated for 48 hours

Emodin Concentration (µg/ml)	Bax/Bcl-2 ratio	
	MCF-7	MDA-231
Vehicle Control	1	1
5	3.7	1.6
10	9.2	1.6
20	16.1	2.9

Emodin decreased cell viability in both cell lines for 48 hours. Emodin treatment for 48 hours changed the expression levels of Bax and Bcl-2 genes in MCF-7 cells in favor of apoptosis as the emodin concentration increased. In contrast to this effect, emodin did not cause a significant change in the expression of investigated genes in MDA-231 cell line at the treatment concentrations for 48 hours. The increase in the ratio of Bax to Bcl-2 was greater in MCF-7 than MDA-231 cells in all the treatment concentrations, showing MCF-7 cells were more apoptotic. Death in MDA-231 cells might have been due to the different time or concentration necessity for apoptosis in MDA-231 cells. MDA-231 cells might have died through other mechanisms different than apoptosis or these cells might have used different ways to get to apoptosis. In the literature, it was seen that another phytoestrogen, resveratrol treatment for 48 hours at different concentrations up to 200 μM, although decreased cell viability in MCF-7 and MDA-231 cells, caused apoptosis only in MCF-7 but not in MDA-231 cell line (Guisado et al., 2002).

3.3.2 Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL)

Another apoptosis indicator, DNA fragmentation, was examined with TUNEL method. The fragmented DNAs were observed as bright yellow as in Figure 3.10.

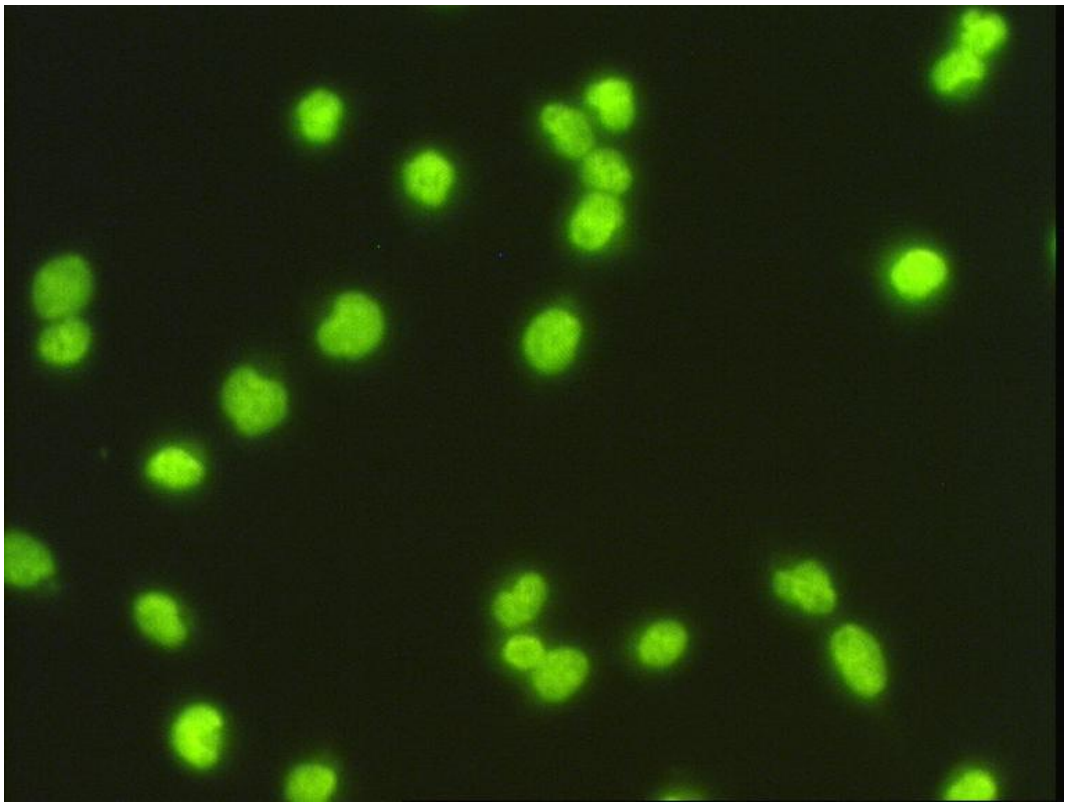

Figure 3.10 TUNEL image of MDA-231 cells treated with DNase-1, positive control indicating DNA fragmented cells (Confocal Laser Scanning Microscope, Hitachi, 100X)

Figure 3.11 and 3.12 are showing MCF-7 cells and MDA-231 cells treated with 5 μg/ml emodin for 12 hours respectively. With these treatment conditions, as it was seen from number of bright yellow dots, emodin caused apoptosis in MCF-7 cells more than MDA-231 cells but the florescence was not as intense as in the positive control. In order not to miss the apoptosis, 12 hours incubation time was selected with the lowest emodin concentration.

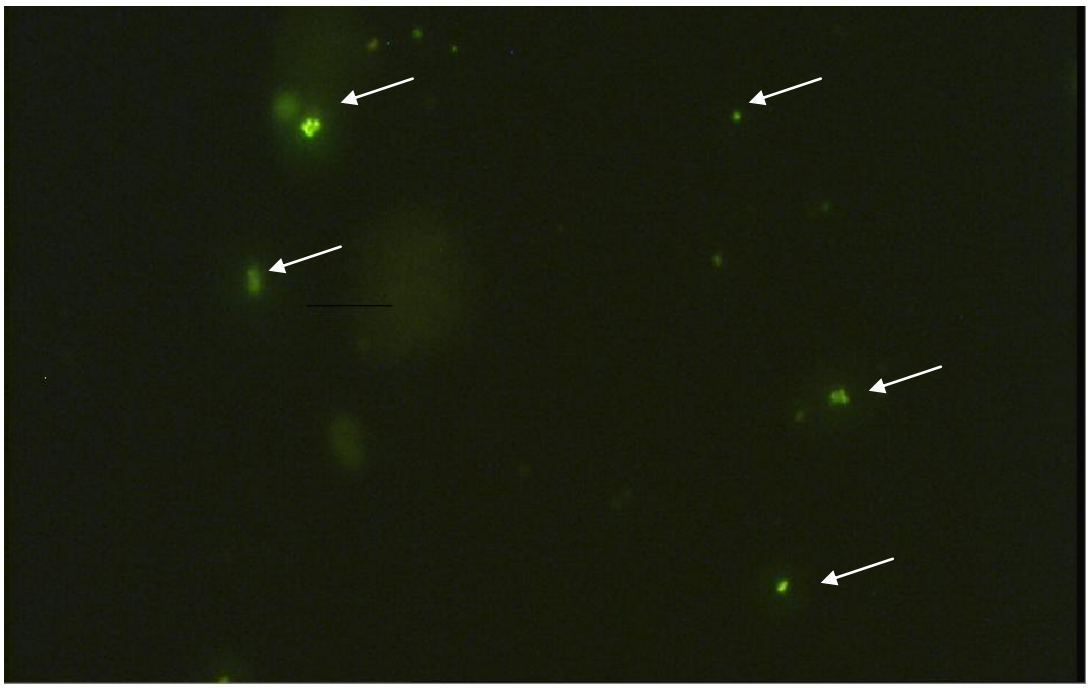

Figure 3.11 TUNEL image of MCF-7 cells treated with 5 μg/ml emodin for 12 hours (Confocal Laser Scanning Microscope, Hitachi, 100X)

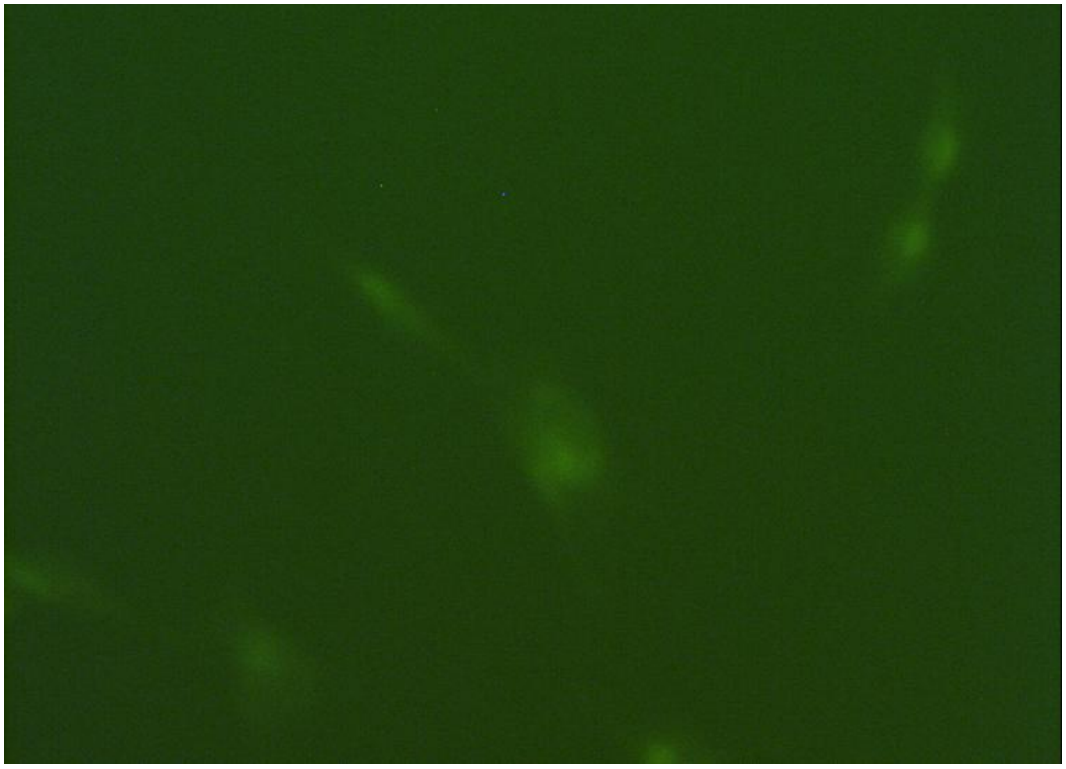

Figure 3.12 TUNEL image of MDA-231 cells treated with 5 μg/ml emodin for 12 hours (Confocal Laser Scanning Microscope, Hitachi, 100X)

Similar to the results of qRT-PCR experiments, the effects of emodin on apoptosis was higher in MCF-7 cells than in MDA-231 cells, so TUNEL results confirmed the expression analysis results.

There are other reports indicating the effects of emodin on apoptosis in several cell lines. In human cervical Bu 25 TK cancer cells and human lung squamous carcinoma CH 27 cells, emodin induced apoptosis through mitochondrial activation of caspase 3 and 9 and with Bax upregulation (Srinivas et al., 2003; Lee, 2001).

It is known that quinones could react with oxygen and produce reactive oxygen species and exert antiproliferative activities by creating ROS. Emodin being an anthraquinone, has this ability. P450 enzymes metabolize emodin to its genotoxic intermediates, 2-hydroxyemodin and w-hydroxyemodin, which increase the ROS formation (Mueller et al., 1998). Su et al. (2005) demostrated that emodin caused apoptosis by generating ROS in human lung adenocarcinoma cells, A549, as a result Bax levels elevated and Bcl-2 levels reduced, mitochondrial membrane potential decreased, cytochrome c was released and subsequently caspase 2, 3, 9 activated, and also survival molecules downregulated. Pretreatment of cells with an antioxidant, ascorbic acid, abolished apoptosis, indicating that apoptosis was generated because of ROS formation (Su et al., 2005). Apoptosis was observed also in human tongue cancer SCC-4 cells (Lin et al., 2009) due to ROS generated by emodin. Furthermore, chemotherapeutic drugs and emodin cotreatment enhanced the therapy. Emodin as a ROS generator, enhanced the sensitivity of HeLa cells to arsenic trioxide (Wang et al., 2005). In our case, ROS generation by emodin might have been the mechanism of apoptosis in MCF-7 and MDA-231 cell lines but it was shown that induction of apoptosis by emodin in HL-60 cells was independent of ROS (Chen et al., 2002), this indicates, that induction of apoptosis by ROS produced by emodin, may be cell line specific.

3.4 Effects of Emodin on GST Enzyme Activity

GSTs are the major Phase II detoxifying enzymes and have many other important functions in the cell as well as their role in apoptosis. Therefore, we studied the effect of emodin on GST enzyme activities of MCF-7 and MDA-231 cells.

GST amount in MCF-7 and MDA-231 cell controls were determined as 3.41 ± 0.63 nmoles/min/mg protein (mean ± SE) and 19.34 ± 6.63 nmoles/min/mg protein (mean ± SE), respectively. It was seen that the GST enzyme activity was higher in MDA-231 cells than in MCF-7 cells. After emodin treatment for 48 hours, in MCF-7 cells, treated up to 5 μg emodin/ml, GST activity was increased statistically significantly about 100% with respect to DMSO control, while it was decreased to control levels at higher emodin concentrations. The increase in GST activity at low emodin concentrations might be the protective response of cells against ROS generation, however, at higher concenrations of emodin, GSH depletion may occur in a short time resulting in drop in GST activity, and the cells began to die. It was observed that emodin did not exhibit statistically significant changes in MDA-231 cells (Figure 3.13).

Figure 3.13 is showing GST enzyme activity of both cell lines. GST activity of DMSO controls were accepted as 100% activity, changes in other samples' activities were calculated with respect to 1% DMSO control.

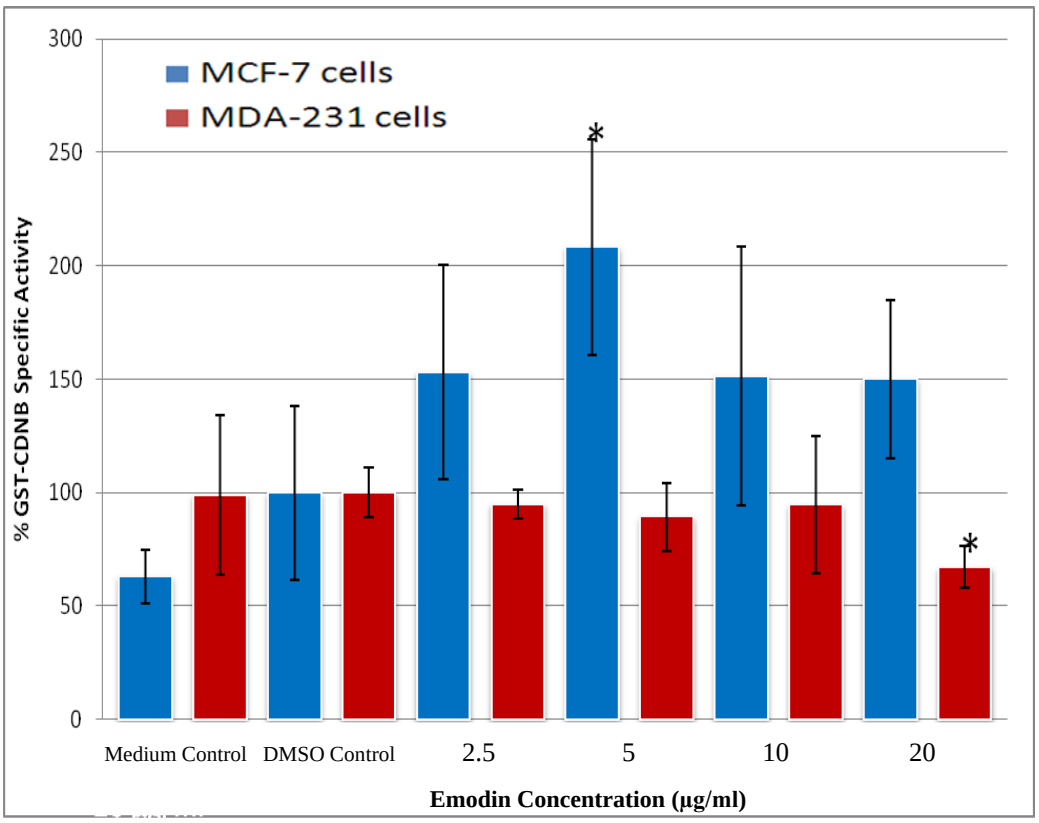

Figure 3.13 Effects of emodin treatment for 48 hours on GST activity against CDNB in MCF-7 and MDA-231 cells. The average of triplicate measurements from three biological replicates. * Statistically significant, $p < 0.05$ (Mann Whitney Test).

The major isozymes contributing to CDNB conjugation in these cell lines were most probably GST Mu, Pi and Omega which are the supressors of apoptosis. Increase of the GST activities results in the decrease of apoptosis. In MCF-7 cells, treated up to 5 µg/ml, GST enzyme activity increased, but at higher concentrations GST enzyme activity decreased. As a result, apoptosis increased significantly after 5 µg/ml emodin treatment when the GST enzyme activity started to decrease. In MDA-231 cells there weren't any significant change both in GST enzyme activity and the expressions of the apoptosis genes.

The differential effects of emodin on GST enzyme activity in MCF-7 and MDA-231 cells is probably because of estrogen receptor content of the cell lines. Emodin being a phytoestrogen, has affinity to estrogen receptor and binding emodin to ER results

in signal transduction effects of emodin in MCF-7 cells which is absent in MDA-231 cells. Therefore emodin binds preferentially to ER rather than other receptors or transporters in MCF-7 cells. MDA-231 cells might have been exposed to the emodin chemical much more than MCF-7 cells, because lacking ER, emodin would transfer inside the cell with other transporters as a result the GST enzymes might have been inhibited in MDA-231 cells.

In order to examine the substrate level effect of emodin, cytosolic proteins of each cell line were isolated and cell free cytosolic GST activity was investigated. It was seen that as the emodin concentration was increased (0-10 µg/ml) in the reaction medium, the enzyme activity decreased to about 50%. By doing so, it became clear that the increase in GST enzyme activity in MCF-7 cells was not because of emodin's chemical effect, rather induction of GST isozymes might have occured.

The effects of emodin treatment on cell free cytosolic GST activity against CDNB was shown in Figure 3.14.

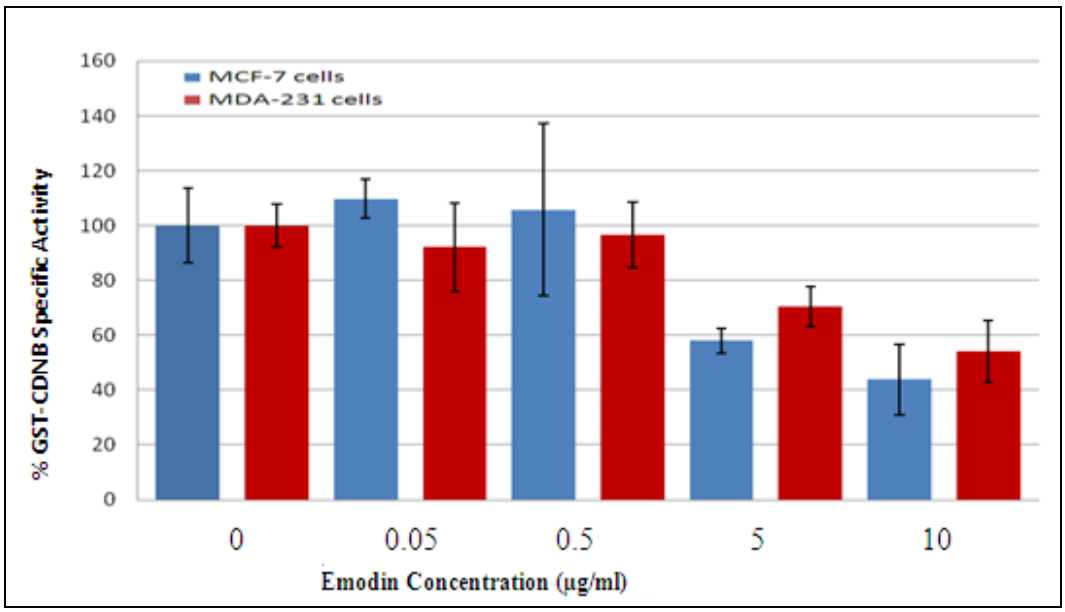

Figure 3.14 Effects of emodin treatment on cell free cytosolic GST activity against CDNB in MCF-7 and MDA-231 cells. The average of triplicate measurements from two biological replicates.

Decrease in cell free GST activity against CDNB as substrate which occured at substrate level, might have been an indication of stimulation of apoptosis. This explanation might have been valid for MCF-7 cells which exhibited apoptosis. MDA-231 cells did not show apoptotic tendency with intrinsic pathway but they might use different ways to get to apoptosis.

3.5 Microarray Analysis

Microarray is an excellent tool to monitor thousands of gene expressions at the same time. GeneChip Human Genome U133 Plus 2.0 Array containing 47,000 transcripts was used for our assays. The array contained 25 base pair long oligonucleotide probes which were the target genes hybridized with. The intensity of the fluorescence that was generated from the hybridization indicates the gene expression, the measurements were done at 570 nm (www.affymetrix.com). In our assay, 48 hours treated, MCF-7 DMSO control, 10 μg/ml emodin treated MCF-7, MDA-231 DMSO control and 10 μg/ml emodin treated MDA-231 cells were analyzed by microarray. There were also biological replicates, therefore totally 8 chips were analyzed.

After hybridization and staining, the array could be visualized. In order to trust the microarray analysis data and to see the assay was done properly, some quality assesments were done. The very first signal for this was the image of the array, there shouldn't be big bubbles, later B2 oligo hybridization should be present at each corner and edges of the array and array name should be seen on upper left of the array as shown in the Figure 3.15. These were the first indicators that hybridization and scaning procedures were applied well.

There were RNA Poly-A controls, *B. subtilis* genes, that were present since the beginning of the assay to control labeling. The signals of them should increase in the order of *lys, phe, thr, dap*. There were hybridization controls, *E.coli* genes *bioB, bioC, bioD* and P1 bacteriophage recombinase gene *cre*. Furthermore housekeeping

genes, β-actin and GAPDH were used to control RNA sample and assay independent of tissue types.

After doing all the quality assessments, the microarray data could be further analyzed.

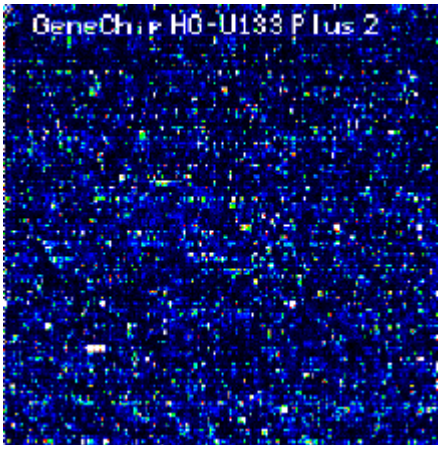

Figure 3.15 Scanned chip image showing the name of the array

In order to analyze the data GeneSpringGX 9.0 (Agilent) Software was used. First all the data normalized by Robust Multiarray Analysis (RMA) to get rid of background and to use only perfect match probes. With this regard, being background, 20% of the intensity was filtered away. After 20 % of intensity value was filtered, 46.089 transcripts out of total 47.000 transcripts were passed for further analyzes free from background intensities.

RMA normalized intensity values were represented as box plots in Figure 3.16. This showed that expression profiles of biological replicates were similiar with each other and also showed that expressions of different cell lines were differed from each other. By means of these normalization, data were comparable with each other.

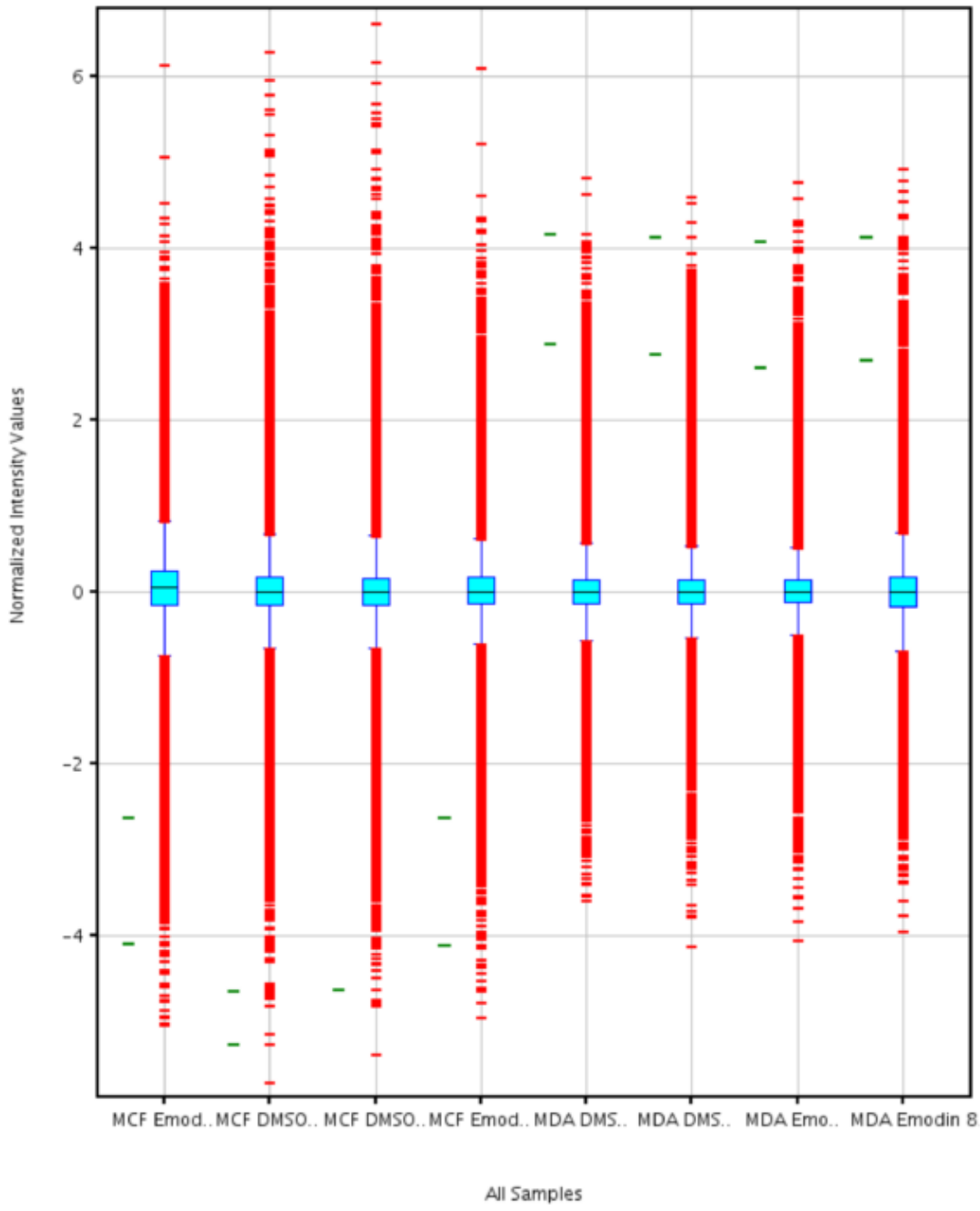

Figure 3.16 RMA normalized intensity values of all the samples, MCF-7 DMSO control, 10 µg/ml emodin treated MCF-7, MDA-231 DMSO control and 10 µg/ml emodin treated MDA-231 with biological replicates

General view of differential expession profiles were also monitored with line graph. Figure 3.17 showed normalized intensity values of all the gene profiles. This representation showed that expression profiles of cells were different from each

other and emodin treatment affected the gene expressions of MCF-7 cells much more than MDA-231 cells. It was seen that there was a change in gene expression in 10 µg/ml emodin treated MCF-7 with respect to MCF-7 DMSO control as it was seen from up or down lines indicating up or down regulation, but the lines were mostly straight between MDA DMSO and 10 µg/ml emodin treated MDA-231 cells, indicating no change in gene expression.

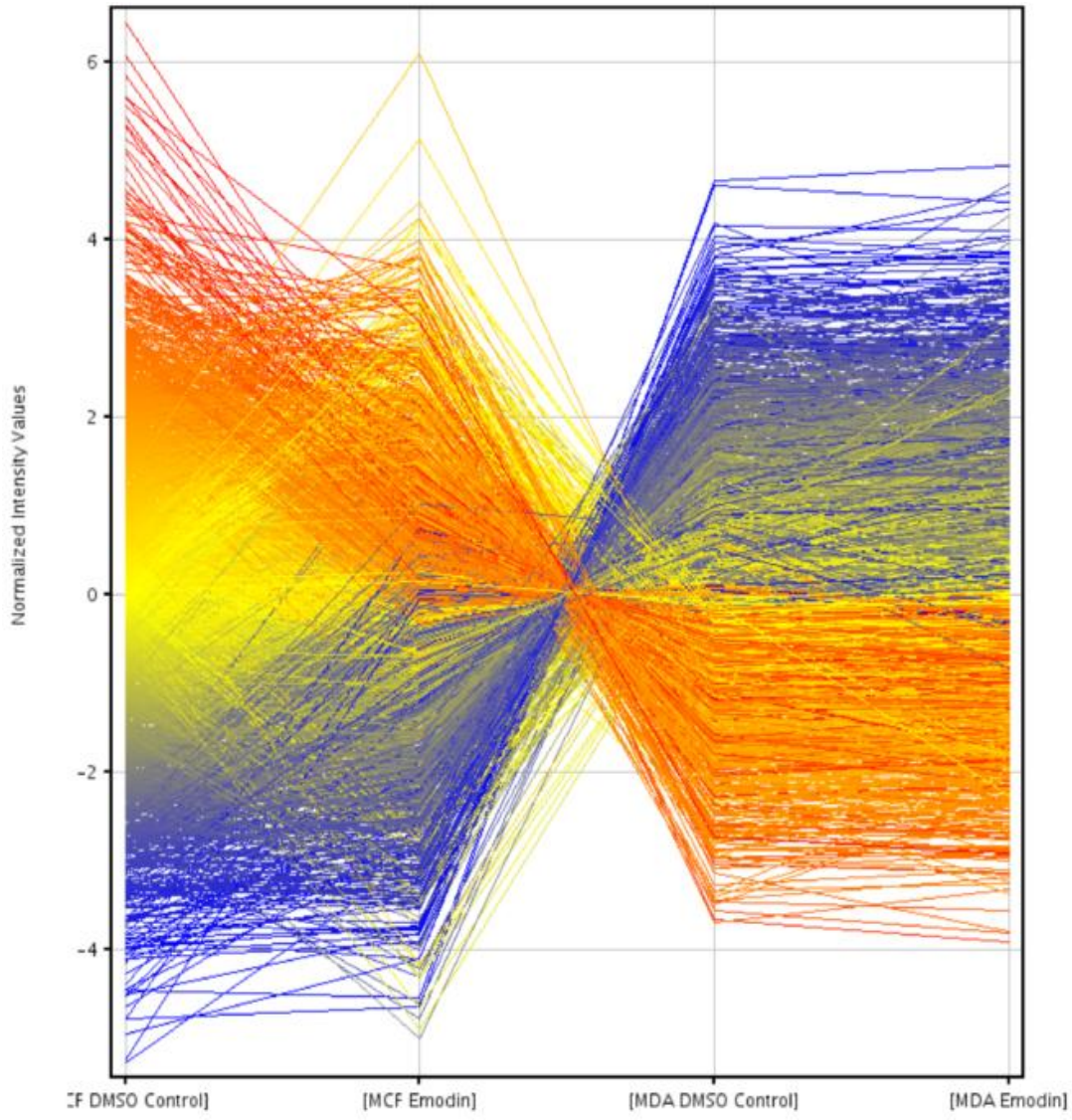

Figure 3.17 Normalized intensity values of all genes represented by line graph

As an indication of significant change in gene expression at least 2 fold change was accepted and using one way ANOVA, $p < 0.05$ was considered as significant

alteration. Filtering on expression (that is showing only change greater than 2 fold) showed that 2978 genes were differentially expressed in MCF-7 cells at least two fold compared to DMSO control under 10 µg/ml emodin treatment. In MDA-231 cells 603 genes were differentially expressed at least two fold compared to DMSO control under 10 µg/ml emodin treatment. After appliying ANOVA test, change in gene expression at least 2 fold together with significant difference meaning $p<0.05$, 2911 genes were differentially expressed in MCF-7 cells at least two fold compared to DMSO control under 10 µg/ml emodin treatment. In MDA-231 cells 586 genes were differentially expressed at least two fold compared to DMSO control under 10 µg/ml emodin treatment. Because of these, it was clear that emodin treatment affected the gene expressions of MCF-7 cells much more than MDA-231 cells as it was indicated our previous assay results. Scatter plots indicated these findings. If we compare the dots between Figure 3.18 and Figure 3.19, it was clear that higher number of genes were differentially altered in MCF-7 cells than in MDA-231 cells.

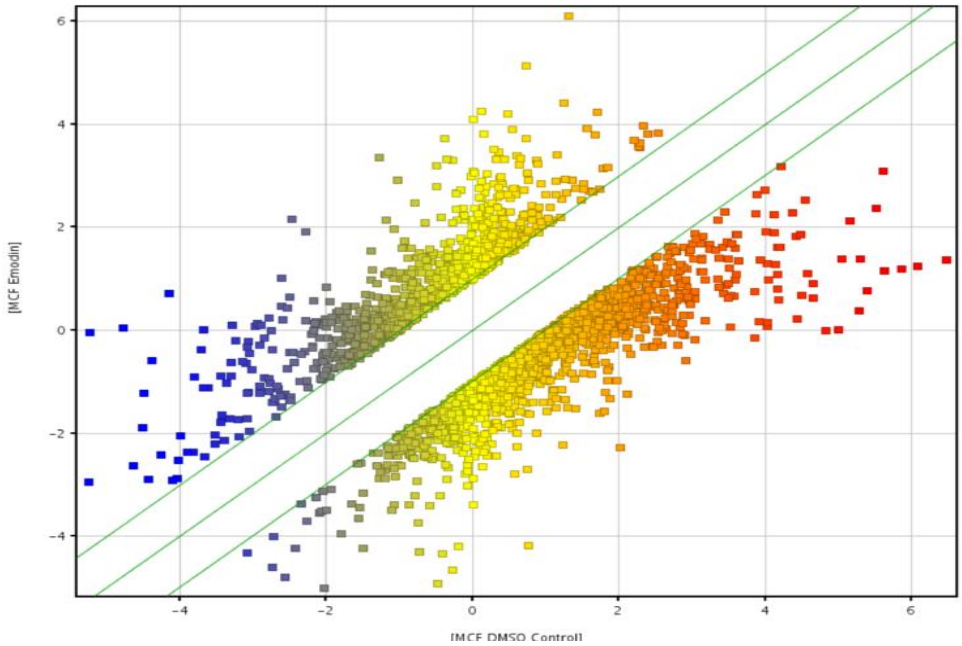

Figure 3.18 Scatter plots of differentially regulated MCF-7 genes under 10 µg/ml emodin treatment with respect to MCF-7 DMSO control. Diagonal lines indicate 2 fold difference lines. Points above the lines indicate up regulated genes and points below the lines indicate down regulated genes.

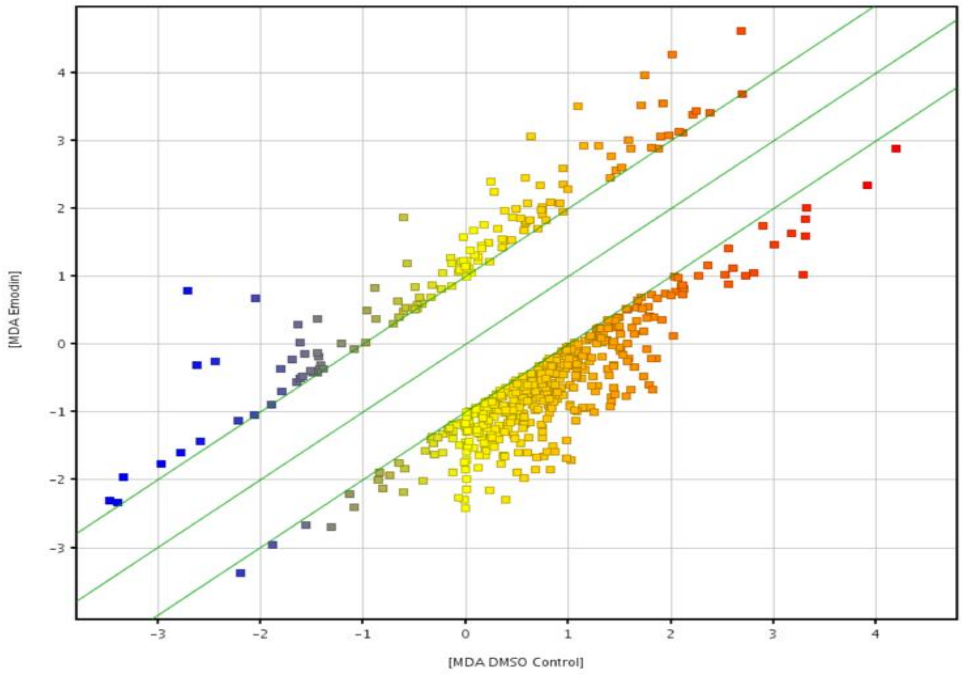

Figure 3.19 Scatter plots of differentially regulated MDA-231 genes under 10 µg/ml emodin treatment with respect to MDA-231 DMSO control. Diagonal lines indicate 2 fold difference lines. Points above the lines indicate up regulated genes and points below the lines indicate down regulated genes.

Venn diagram (Figure 3.20) representation indicated that among the differentially expressed genes that showed at least 2 fold differences of each cell line, 248 genes were common where as 2730 genes were only differentially expressed between MCF-7 DMSO and 10 µg/ml emodin treated MCF-7 and 355 genes were only differentially expressed between MDA-231 DMSO and 10 µg/ml emodin treated MDA-231. This diagram again showed the different effects of emodin on two different cell lines and again an evidence that we used different cell lines.

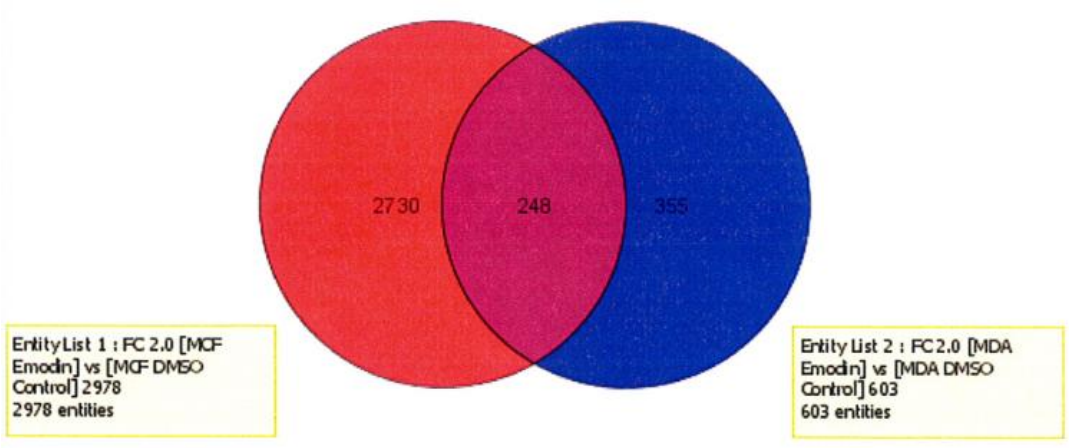

Figure 3.20 Venn diagram; showing numbers of genes altered in MCF-7 and MDA-231 cell lines

3.5.1 Alterations in Apoptosis Genes (Bax and Bcl-2)

Changes in gene expressions were evaluated using normalized data with 1.25 cut off value and accepted as significant with $p < 0.05$. In MCF-7 cells, Bcl-2 was 4.48 fold down regulated in 10 µg/ml emodin treatment with respect to MCF-7 DMSO cells. Bcl-2 gene expression change in MCF-7 cells was also represented in Figure 3.21.

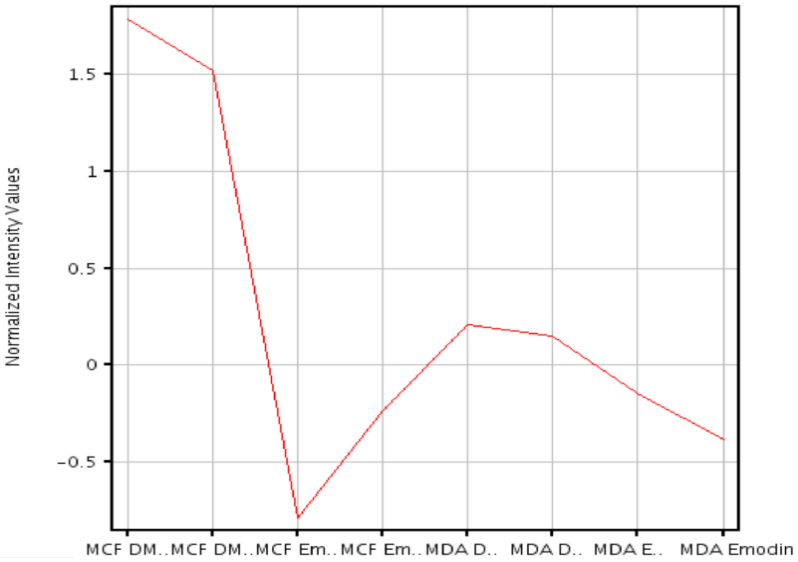

Figure 3.21 Bcl-2 gene expression change in MCF-7 cells

In MDA-231 cells, Bcl-2 was 1.36 fold down regulated with 10 µg/ml emodin treatment with respect to MDA-231 DMSO cells. There were no significant change in Bax expression in both cell lines. Changes in expression of Bax and Bcl-2 genes with emodin treatment in MCF-7 and MDA-231 cells were shown in Table 3.6.

Table 3.6 Changes in expression of Bax and Bcl-2 genes by 10 µg/ml emodin for 48 hr in MCF-7 and MDA-231 cells

Affimetrix probe numbers	Gene Symbol	MCF-7 Emodin vs DMSO	MDA-231 Emodin vs DMSO
203684_s_at	Bcl-2	1.37 ↓	NS
203685_at		4.48↓	1.36↓
207004_at		NS	NS
207005_s_at		NS	NS
208478_s_at	Bax	NS	NS
211833_s_at		NS	NS

As it was seen in Table 3.7, Bax to Bcl-2 ratio was increased to 4.48 fold with respect to 1.0 of control (DMSO), microarray results showed that MCF-7 cells entered the apoptosis. MDA-231 cells however, did not exhibit a significant change. These results were in accordance with qRT-PCR results.

Table 3.7 Effects of emodin treatment on Bax / Bcl-2 ratio in MCF-7 and MDA-231 cell lines treated for 48 hours

Emodin Concentration (µg/ml)	Bax/Bcl-2 ratio	
	MCF-7	MDA-231
10	4.48	1.36

Ontologies of Bax and Bcl-2 genes were shown in Table 3.8.

Table 3.8 Gene ontologies of Bax and Bcl-2 genes

Gene Symbol	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
Bax	Activation of caspase activity by cytochrome c, induction of apoptosis by intracellular and extracellular signals, release of cytochrome c from mitochondria	Mitochondrial outer membrane, mitochondrial permeability transition pore complex	Protein binding, BH3 domain binding
Bcl-2	Oxygen and reactive oxygen species metabolic process, cell proliferation, response to oxidative stress, post embryonic development	Mitochondrial outer memnbrane, endoplasmic reticulum, nuclear membrane	Protein binding, BH3 domain binding, transcription factor binding

3.5.2 Alterations in GST Genes

The effect of emodin on GST isozyme expressions were evaluated by microarray analysis. In enzyme activity assays CDNB was used as substrate to measure total GSTs of MCF-7 and MDA-231 cells. The major isozymes contributing to CDNB conjugation in these cell lines are most probably GST Mu, Pi and Omega. GST Pi, Mu and Omega are the supressors of apoptosis. GST Pi protects tumor cells by detoxifying chemoteraupetic drugs and by blocking apoptosis through MAP kinase pathway.

The expression changes of GST isozymes upon emodin treatment with respect to DMSO control were shown in Table 3.9 and 3.10 for MCF-7 and MDA-231 cells respectively. In MCF-7 cells, all Mu isozymes were supressed, although change in GSTM4 expression was not significant. GSTO1 was also supressed. The supression of gene expression of these isozymes are advantageous for a cancer cell which is expected to enter apoptotic death pathway, because GSTO and GSTMu have antiapoptotic features through MAP kinase signaling pathway. GSTP1 which is highly expressed in cancer cells, exhibited higher expression upon emodin treatment. Since GSTP is also antiapoptotic, its increase is not desirable under these circumstances.

Table 3.9 Changes in gene expression of GST isozymes by emodin in MCF-7

Affimetrix probe numbers	Gene Symbol	MCF-7 Emodin vs MCF-7 Control
202967_at	GSTA4	1.27 ↑
235387_at	GSTCD	1.61 ↓
1554518_at	GSTCD	NS
204550_x_at	GSTM1	1.31 ↓
215333_x_at	GSTM1	1.27 ↓
204418_x_at	GSTM2	1.32 ↓
202554_s_at	GSTM3	NS
204149_s_at	GSTM4	NS
201470_at	GSTO1	1.40 ↓
1557915_s_at	GSTO1	1.43 ↓
200824_at	GSTP1	1.92 ↑
203815_at	GSTT1	1.29 ↑
232193_at	GSTT1	1.60 ↓
209531_at	GSTZ1	1.41 ↓

In MDA-231 cells, GSTM3 and GSTO1 tend to increase in their gene expressions, preventing these cells to enter to the apoptosis. (Table 3.10)

Table 3.10 Changes in gene expression of GST isozymes by emodin in MDA-231

Affimetrix probe numbers	Gene Symbol	MDA-231 Emodin vs MDA-231 Control
215766_at	GSTA1	NS
202967_at	GSTA4	1.50 ↑
235387_at	GSTCD	1.37 ↓
235867_at	GSTM3	1.28 ↑
201470_at	GSTO1	1.33 ↑
1557915_s_at	GSTO1	1.41 ↑
209531_at	GSTZ1	1.28 ↓

Ontologies of GST isozymes were shown in Figure 3.11.

Table 3.11 Gene ontologies of GST isozymes

Gene Symbol	Gene Ontology Biological Process	Gene Ontology Cellular Component	Gene Ontology Molecular Function
GSTA (Alpha) 4	Metabolic process	cytoplasm	glutathione transferase activity
GSTM (Mu) 1	Metabolic process	cytoplasm	glutathione transferase activity
GSTM (Mu) 2	Metabolic process	cytoplasm	glutathione transferase activity
GSTM (Mu) 3	response to estrogen stimulus, establishment of blood-nerve barrier	cytoplasm	glutathione transferase activity, protein binding
GSTM (Mu) 4	Metabolic process	cytoplasm	glutathione transferase activity
GSTP (Pi) 1	anti-apoptosis	cytoplasm	glutathione transferase activity, protein binding
GSTT (Theta) 1	glutathione metabolic process	cytoplasm	glutathione transferase activity
GSTT (Theta) 2	-	Nucleus, cytoplasm	glutathione transferase activity
GSTZ (Zeta) 1	L-phenylalanine catabolic process, tyrosine catabolic process, aromatic amino acid family metabolic process	Nucleus, cytoplasm, mitochondrion	glutathione transferase activity, glutathione peroxidase activity, maleylacetoacetate isomerase activity
GSTO (Omega) 1	L-ascorbic acid biosynthetic process	cytoplasm	glutathione transferase activity, monodehydroascorbate reductase (NADH) activity
GSTO (Omega) 2	Metabolic process	cytoplasm	glutathione transferase activity
GSTCD (C-terminal domain containing)	-	-	protein binding
GSTK (Kappa)	glutathione metabolic process	Mitochondrial inner membrane, mitochondrial matrix, intracellular	glutathione transferase activity, glutathione peroxidase activity, protein binding, protein disulfide oxidoreductase activity

CHAPTER 4

CONCLUSION

In our research, the effects of emodin on MCF-7(estrogen receptor positive) and MDA-231(estrogen receptor negative) cell lines were studied by comparing its effects on proliferation, apoptosis genes and glutathione S- transferase (GST) enzymes. The major findings are as follows;

- To detect the effect of emodin on viable cell number, it was prefered to comment on the counting with tryphan blue. IC50 values of MCF-7 and MDA-231 cells were calculated as 8.40 and 12.17 μg/ml respectively, it was concluded as the inhibitory effect of emodin was higher in MCF-7 cells than MDA-231 cells.

- The expression of apoptosis genes Bax and Bcl-2 that were analyzed by qRTPCR resulted in increase in apoptosis in MCF-7 cell line as the emodin concentration increased (0, 5, 10, 20 μg/ml), upon treatment for 48 hours, whereas no significant change was observed in the expression of apoptosis genes of MDA-231 cell line. An apoptosis indicator, Bax to Bcl-2 ratio of MCF-7 and MDA-231 cell lines were 9.2 and 1.6 respectively at 10μg/ml emodin concentration. It was concluded that the main mechanism of action of phytoestrogens in MDA-231 cell line might not be apoptosis or might be other apoptosis pathway or the onset of apoptosis was earlier and we couldn't catch.

- TUNEL method indicated the presence of apoptosis in both cell line at 5 μg/ml emodin treatment for 12 hours, and as in the case of expression

analysis, emodin caused apoptosis in MCF-7 cells more than MDA-231 cells, nevertheless, this was lesser than control images.

- Microarray analysis showed that after applying ANOVA test, change in gene expression at least 2 fold together with significant difference meaning $P< 0.05$, 2911 genes were differentially expressed in MCF-7 cells at least two fold compared to DMSO control under 10 μg/ml emodin treatment. In MDA-231 cells 586 genes were differentially expressed at least two fold compared to DMSO control under 10 μg/ml emodin treatment. This showed that emodin treatment affected the gene expressions of MCF-7 cells much more than MDA-231 cells. Bax to Bcl-2 ratio is increased to 4.48 value with respect to 1.0 of control (DMSO), microarray results showed that MCF-7 cells enter the apoptosis. MDA-231 cells however, did not exhibit a significant change. These results were in accordance with qRT-PCR results. Furthermore by microarray anaysis it was shown that emodin affected the gene expressions of GST isozymes in MCF-7 much more than MDA-231 cells.

- In order to observe the effect of emodin on GST enzyme activity, cells were treated with 0, 5, 10, 20 μg/ml emodin concentrations for 48 hours. It was seen that in MCF-7 cells GST enzyme activity was increased statistically significantly up to 5 μg/ml with respect to DMSO control and later decreased to control levels, whereas emodin did not exhibit statistically significant changes in MDA-231 cells.

- The chemical effect of emodin on GST enzyme was examined and it was observed that as the emodin concentration increased (0-10 μg/ml), the enzyme activity decreased in both cell lines at about 50%. As a result the increase of GST activity in MCF-7 cell was not because of emodin's chemical effect.

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