EFFECT OF EMODIN ON DOXORUBICIN-INDUCED CYTOTOXICITY AND APOPTOSIS IN MCF-7 AND MCF-10A CELLS

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EFFECT OF EMODIN ON DOXORUBICIN-INDUCED CYTOTOXICITY AND APOPTOSIS IN MCF-7 AND MCF-10A CELLS

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ABSTRACT

EFFECT OF EMODIN ON DOXORUBICIN-INDUCED CYTOTOXICITY AND APOPTOSIS ON MCF-7 AND MCF-10A CELLS

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Emodin (3-Methyl-1,6,8-trihydroxyanthraquinone), is a phytoestrogenic component of *Rheum and Polygonum* plant extracts which has been used to treat several diseases since ancient times. It has been shown to have anti-microbial, anti-oxidant and anti-cancer effects in nature. The anti-tumor drug doxorubicin, a widely used chemotherapeutic agent, is used for the treatment of many cancer types including lung, gastric, ovarian and breast cancer. In this study, the effects of pre-, co- and alone treatment of doxorubicin and emodin in MCF-7 and MCF-10A cell lines were investigated.

MCF-7 and MCF-10A cells were cultured in the presence of various concentrations of emodin and doxorubicin at 6, 24 and 72 hours. The effect of emodin varies according to the presence of doxorubicin (pre-treatment, co-treatment, post-treatment) on both cell lines. Emodin pre-treatment (0.4 and 4 μM) for 24-hour prior to doxorubicin treatment (0.1, 0.83, 2.5 μM) caused to increase in cell viability of MCF-10A cells, comparing to doxorubicin alone treatment. Whereas no effect was observed in MCF-7 cells. Emodin post- and co-treatment with doxorubicin for 72-hour inhibited the survival of MCF-7 and MCF-10A cells in a concentration dependent manner, shown by trypan blue and XTT.

Apoptotic effects of doxorubicin and emodin were investigated by flow cytometry. While emodin (0.4 μM) did not induce apoptosis in both cell lines, doxorubicin alone, pre- and co-treatment (0.83 μM) with emodin (0.4 μM) induced late apoptosis/necrosis in MCF-7 and MCF-10A cells. Mitochondrial membrane potential loss was not observed after doxorubicin and emodin treatment in both cell lines.

Keywords: Emodin, Doxorubicin, MCF-7, MCF-10A, cytotoxicity, apoptosis

ÖZ

DOXORUBICIN İLE İNDÜKLENEN MCF-7 VE MCF-10A HÜCRELERİNDE, EMODİNİN SİTOTOKSİSİTE VE APOPTOZ ÜZERİNE ETKİSİ

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Emodin (3-Metil-1,6,8-trihidroxilantrakuinon), eski zamanlardan beri çeşitli hastalıkların tedavisinde kullanılan, *Rheum* ve *Polygonum* bitkilerinin bir bileşeni olup, anti-mikrobiyal, anti-kanser ve anti-oksidan etki gösteren bir fitoestrojendir. Anti-tümör ilacı olarak bilinen doksorubisin, akciğer, mide, yumurtalık ve göğüs kanseri gibi bir çok kanser tipinin tedavisinde kullanılan kemoterapötik bir ajandır. Bu çalışmada, MCF-7 ve MCF-10A hücre hatlarına uygulanan doksorubisinin, öncesinde, sonrasında ve doksorubisinle birlikte verilen fitoestrojen emodinin etkileri araştırılmıştır.

MCF-7 ve MCF-10A hücreleri çeşitli emodin ve doksorubisin konsantrasyonları varlığında 6, 24 ve 72 saat boyunca kültüre edilmiştir. Emodinin her iki hücre hattı üzerindeki etkisi, doksorubisin öncesinde, sonrasında ve beraberinde uygulanmasıyla değişiklik göstermiştir. Doksorubisin (0.1, 0.83, 2.5 μM) öncesinde 24 saat süreyle uygulanan emodin (0.4 ve 4 μM), MCF-10A hücrelerinde, hücre sayısında artışa neden olurken, MCF-7 hücrelerinde herhangi bir etki göstermemiştir. Doksorubisin beraberinde ve sonrasında 72 saat süre ile alınan emodin her iki hücre hattında da konsantrasyona bağlı olarak hücre büyümesini engellemiştir.

Doksorubisin ve emodinin MCF-7 ve MCF-10A hücrelerindeki apoptotik etkileri akım sitometrisi ile çalışılmıştır. 24 ve 72 saat süreyle uygulanan emodinin (0.4 μM), her iki hücre hattında da apoptotik bir etki göstermediği saptanmıştır. Doksorubisinin (0.83 μM) ise 72 saat boyunca tek başına, emodin öncesi ve emodinle birlikte uygulanması her iki hücre hattının normalden erken ya da geç apopotoza geçmesiyle sonuçlanmıştır. Çalışılan konsantrasyonlarda hücrelere doksorubisin ve emodin uygulanması mitokondriyal membran potansiyel değişimine yol açmamıştır.

Anahtar Kelimeler: Emodin, Doksorubisin, MCF-7, MCF-10A, sitotoksisite, apoptoz

To My Family...

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LIST OF SYMBOLS AND ABBREVIATIONS

ABCB 1 : ATP binding cassette, subfamily B

Akt : Protein Kinase B

Apaf-1 : Apoptotic Protease-Activating Factor 1

ATP : Adenosine triphosphate

Bcl-2 : B-cell lymphoma 2

Bid : Bax like BD-3 Protein

BRCA 1 : Breast Cancer 1

Caspase : Cysteine-dependent aspartate-directed proteases

CAT : Catalase

DAPI : 4′,6-Diamidino-2-phenylindole dihydrochloride

DMSO : Dimethyl sulfoxide

EGFR : Epidermal growth factor receptor

ER : Estrogen receptor

FasR : Tumor necrosis factor receptor superfamily member 6 (TNFRSF6)

GSH : Reduced glutathione

GST : Glutathione S-transferase

GPx : Glutathione peroxidase

GR : Glutathione reductase

HER2 : Human epidermal growth factor receptor 2

HSP 90 : Heat shock protein 90

MAPK : Mitogen-activated protein kinases

MCF-7 : Michigan Cancer Foundation - 7

MCF-10A : Michigan Cancer Foundation - 10A

MRP-1 : Multi drug resistance protein 1

MTT : (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

mTOR : Mammalian target of rapamycin

NADH : Nicotinamide adenine dinucleotide hydrogen

NADPH : Nicotinamide adenine dinucleotide phosphate hydrogen

NER: Nucleotide excision repair

NFkB : Nuclear Factor-kappa B

PARP : Poly ADP ribose polymerase

PgR : Progesterone receptor

PgP : Phosphoglycolate phosphatase

PI3K : Phosphatidylinositide 3-kinases

p53 : Protein 53

ROS : Reactive oxygen species

SOD : Superoxide dismutase

Src : Sarcoma proto-oncogene

TNBC : Triple negative breast cancer

TNF : Tumor necrosis factor

Trx : Trithorax

VEGF : Vascular endothelial growth factor

XTT:(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) inner salt

CHAPTER 1

INTRODUCTION

1.1 Estrogens

Estrogens are steroid hormones, mainly produced by the ovaries of premenopausal women (V. Okoh et al., 2011). They are known as the primary female sex hormones and their function in women is the formation of secondary sex characteristics. Estrogens are also responsible for the maturation of sperms in male species (R.A Hess, D. Bunick, J. Bahr, 2001). They are also produced by fat cells and the adrenal glands. They are involved in bone formation, promote fat deposition, increase cortisol levels and improve lung functions in the body. There are three major groups of estrogens in human body; Estrone (E1), 17β-Estradiol (E2) and Estriol (E3). Among them, 17β-Estradiol (E2) is the most potent estrogen circulating in the body (Figure 1.1, http://alaskadigitalvisions.com/femalehormones/).

Cells accept 17β-Estradiol (Estradiol, E2) freely and it binds to the estrogen receptors, ERα and ERβ. The estrogen receptors are the ligand-inducible transcription factors those turn hormone signals to the actions in different organs. They are encoded by two different genes (ESR1-ESR2). The ERs have DNA binding domain (DBD) that is responsible for DNA binding whereas -COOH terminal is responsible for ligand binding domain (LBD). There are four molecular pathways which ERs display regulatory actions in the cell (Figure 1.2).

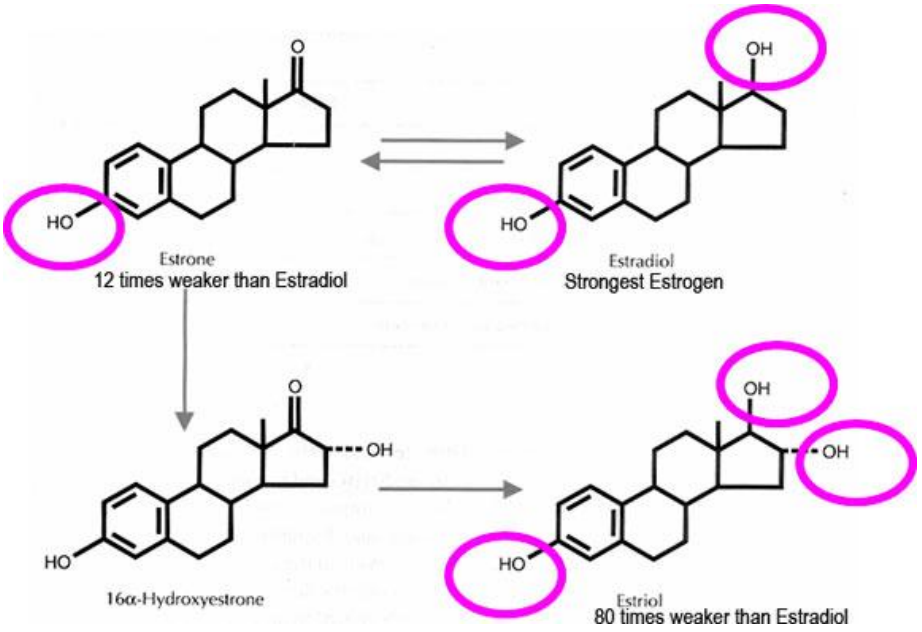

Figure 1.1 Major estrogen isoforms circulating in the body.

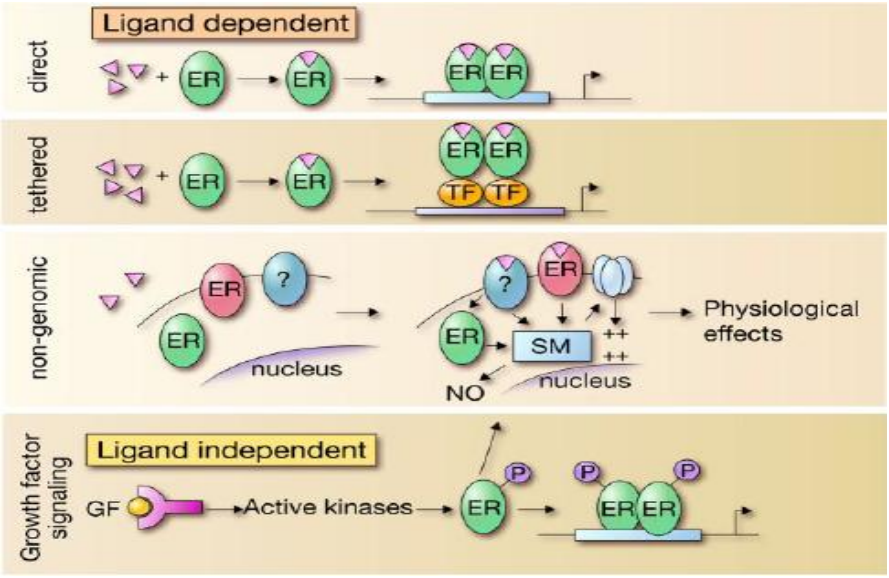

Figure 1.2 Schematic representation of ERs involved molecular pathway. The main pathway includes ligand binding and direct DNA activation. The tethered pathway is based on protein-protein interaction with transcription factors. The non-genomic pathway covers ligand activation of ERs in the cytoplasm and the ligand independent pathway implicates some other signalling routes like growth factor signalling (Heldring et al., 2007).

ERα and ERβ are localized and concentrated differently in human body. They are mainly expressed in the breast, ovary and uterus (J.-M. Renoir et al., 2003). Because of cell proliferation effects of estrogens, ERα-blocking anti-estrogens are widely used for breast cancer treatment (Clarke M., 2006). There are two main hormone therapies; those using anti-estrogens and others using aromotase inhibitors. Selective estrogen receptor modulators' are ER ligands and they bind to estrogen receptors to inhibit ER mediated transcriptional effects (Lonard DM., 2000). Clinical studies demonstrate that using selective estrogen receptor modulators for treatment of breast cancer leads to resistance in cancer therapy. The resistance occurs via increased tyrosine kinase signalling or increased acivity of transcriptional factors; that is why 50 % patients with breast cancers do not respond to hormone therapies those using selective estrogen receptor modulators like tamoxifen, toremifene, raloxifene (Ali S., Coombes RC., 2002). The second way is to use aromatase inhibitors in endocrine therapies. They reduce estrogen production in tumor cells. In premenapousal women, inhibition of estrogen production is an option to treat ER (+) tumors. In post menapousal women who can produce estrogen only in fat tissue responsed more succesfully to cancer therapy. But in endocrine therapies, after certain time resistance to treatment may occur.

However, as mentioned above estrogen is involved in many metabolic processes. Especially after menapouse in women, when estrogen levels decrease, there are some side effects like hot flashes, irregular heartbeat, high levels of LDL etc. Hormone replacement therapy (HRT) has been available to overcome these side effects and increase estrogen levels. Observational evidence suggests that HRT (for longer than 10 yeras exposure) increase the risk of breast cancer (Marsden J., 2002).

In the environment, there are some natural substances which show estrogenic activity; those produced by plants are known as phytoestrogens (Fang H, Tong W, Shi LM., 2001). Epidemiological studies indicate that phytoestrogens in the diet may decrease hormone-induced cancers (Witorsch RJ., 2002).

1.1.2 Phytoestrogens

Phythoestrogens are estrogen-mimicking phenolic compunds which are derived from plants. The main classes of phythoestrogens are the isoflavonoids, the lignans, the coumestans and the anthraquinones (Sirtori et al., 2005). They are found in the legume family, high fiber foods, beans (soybeans, cereal beans, split beas etc.). The anthraquinones are found in vegetables like cabbage and lettuce.

The structures of phytoestrogens share common features with the mammalian estrogen 17β-estrodiol (Figure 1.3). A phenolic ring and hydroxyl groups enable them to bind to the estrogen receptors, ERα and ERβ (H.-S. Seo et al., 2011). Due to the activation of the estrogen receptors, phytoestrogens distrupt estrogenic signaling (Mueller et.al. 2004). Some phytoestrogens show higher affinity for ERβ than ERα.

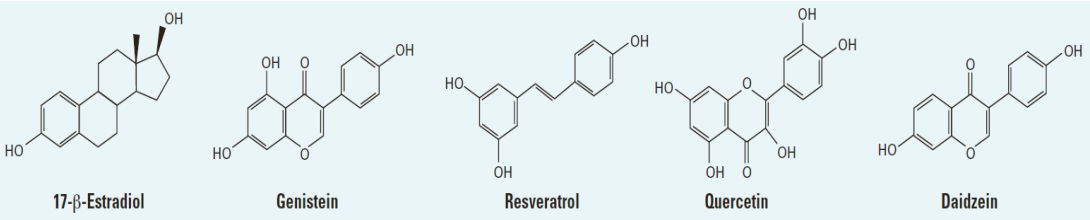

Figure 1.3 Structures of 17β-estrodiol and the phytoestrogens (Mense et al., 2008).

Phytoestrogens may inhibit but also trigger estrogen dependent tumor growth depending on concentrations and time periods (Allred et al. 2001; Bhat et al. 2001; Cotroneo et al., 2002). It was found that, at low concentrations, phytoestrogens exert stimulation activity; whereas at high concentrations, they limit growth (Lemos 2001; Schmitt et al., 2002). Phytoestrogens show their effects with many mechanims; they are involved in cell cycle regulation, protein kinase inhibition, cell proliferation, apoptosis, angiogenesis and aromatase inhibiton (Basly et al., 2000; Cappelletti et al., 2000) (Figure 1.4). Their antioxidant action occur through hydroxyl gorups. The hydrogen donating abilities of phytoestrogens indicate their role in cellular systems. By this way, they may protect cell and inhibit the growing of cancer (Mitchell et al., 1998).

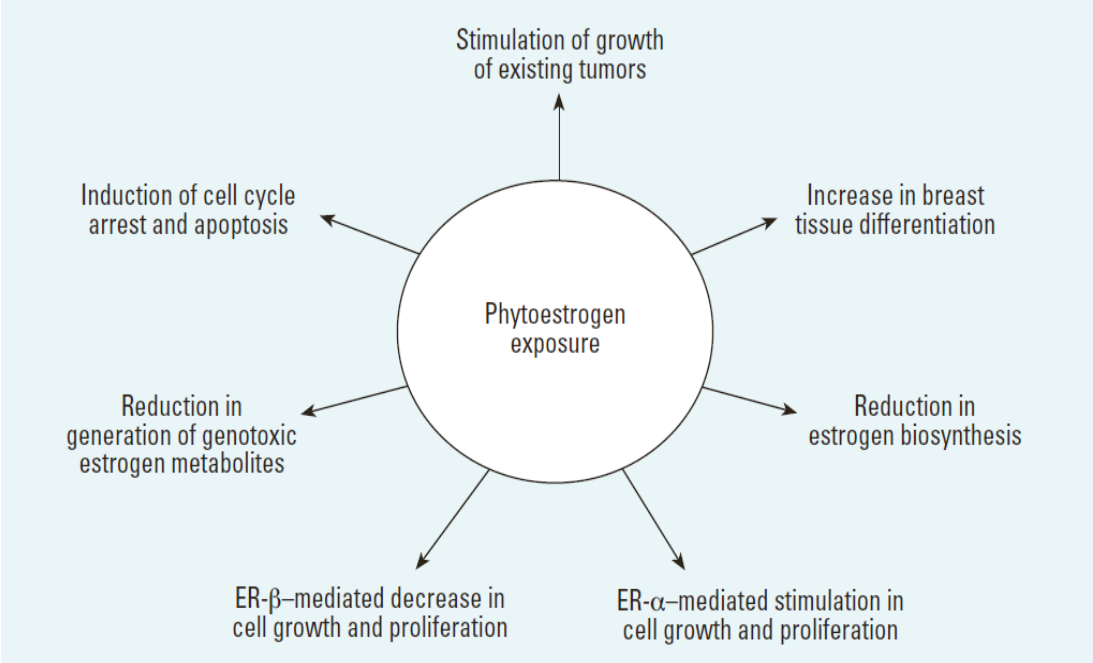

Figure 1.4 Actions of phytoestrogen in the cell (Mense et al., 2008).

It is known that diet and nutrition has an important role in the development of cancer. In Asia, where women consume high amount of soybean products, fewer post menopausal symptoms were reported and fewer breast cancer cases were seen compared to women in Western countries (Usui, 2006). Due to the dietary habits, breast cancer occurence can differ in the worldwide (Adlercruitz, 2002).

Pharmacologic properties of phytoestrogens in breast cancer have been studied by several researchers. Though some of the studies showed the protective effects of phytoestrogens, others observed no connection between phytoestrogens and breast cancer (Hirohata et al., 1985; Key et al., 1999; Nomura et al., 1978, 1985;
Trock et al., 2006; Wu et al., 1998; Yuan et al., 1995). On the other hand, synthetic antiestrogens like tamoxifen has been applied for the treatment of some types of breast cancer. Thus, antiestrogenic and antioxidant effects of phytoestrogens might be used for the cancer treatment. In an animal study, potential interactions of phytoestrogen consumption with hormonal breast cancer treatment was conducted. The study showed that, low doses of phytoestrogen genistein, reduced the anti-

proliferative action of tamoxifen whereas at high doses, anti-proliferating activity is enhanced (Liu B, Edgerton S, Yang X, et al., 2005). This and other studies have shown that the concentration is very imporant for the treatment. Chinese medicine may develop future therapies for cancer but should be examined by more human studies.

1.1.2.1 Emodin

Emodin (6-Methyl-1,3,8-trihydroxyanthraquinone) is an anthraquinone derivative, isolated from the barks and roots of plants and used to treat several diseases in China. It is found in Chinese herbs, *Rheum and Polygonum* (Matsuda et al., 2001). Emodin which is found in *Rumex patientia L.* also used as a purgative agent in Turkish medicine (Baytop T., 1984). Recent studies demonstrated that, emodin has antimicrobial, purgative, anti-inflammatory, anti-angiogenic, hepatoprotective and antiproliferative effects in nature (Shi YQ, Fukai T., 2001). The dose dependent additional effect of emodin with chemotherapeutic drugs makes it a targeted compound in the tratment of cancer.

Emodin was to be shown mutagenic in prokaryotic systems. It is converted to hydroxyemodin by cytochorome P450 enzymes. This metabolic product of emodin produces oxygen and breaks DNA strands. Therefore, this structural conversion can explain the mutagenic activity of emodin (Kodama M, Kamioka Y., 1987). Another possible mechanism could be topoisomerase II inhibiton, because of the non-covalent binding of emodin to DNA (Mueller SO, Stopper H., 1999). However, no mutagenic and genotoxic effects of emodin was observed in mammalian cells (Bruggeman IM, van der Hoeven JC., 1984).

Emodin exerts antibacterial properties against several microorganisms, such as *Escherichia coli* K12, *Staphylococcus aureus*. In the respiratory chain, inhibition of electron flow and the generation of superoxide radicals may explain the antibacterial effects of emodin (Anderson DO, Weber ND., 1991).

Emodin also found to be an antiangiogenic compound (Kumar A, Dhawan S., 1998). Though emodin has so much diverse effects, the mechanism is still unclear. It might be due to the prevention of transcription factor NF-kB which is related to prosurvival signalling and also in angiogenesis (Li HL, Chen HL., 2005). It was also found that emodin can inhibit the disruption of I-kB which is an inhibitor subunit of NF-kB in the tumor cells (Kumar A, Dhawan S., 1998).

Recent studies showed that, emodin supresses the basic fibroblast growth factor (bFGF)-induced proliferation and hence the invasion of human vascular endothelial cells (HUVECs) (Kwak HJ, Park MJ., 2006). It also leads to inhibiton of protein kinase CK2 which influences signalling molecules like p53, PTEN, p38, MAPK and phosphatidylinositol 3-kinase (PI3 kinase)-Akt pathway which is activated by angiogenic agents. Future studies are required to show the effect of emodin on angiogenesis.

Emodin exerts its antioxidant capacity via different mechanisms. It protects cell constituents as well as enhances antioxidant defences in the cell. Furthermore it inhibits lipid peroxidation and radical formation (Huang SS, Yeh SF., 1995). Studies demonstrated that, in the presence of an oxidant, treatment with emodin protects of cell constituents (Sato M, Maulik G., 2000). Chang LC., Sheu HM. showed that, in human fibroblast WI38 cells, emodin restores cisplatin-induced DNA damage and increases nucleotide excision repair (NER) capacities (1999). It was also reported that inhibit 1-nitropyrene-induced DNA damage and its mutagenicity is inhibited in the presence of emodin (Su HY, Cherng SH., 1995).

Glutathione S-transferase and glutathione peroxidase enzymes are responsible for the adjustment of cell status and redox-dependent signaling (Kalinina EV, Chernoc NN., 2010). These enzymes protect organisms from oxidative degradation by their reduction capabilities of hydropeoxides (Bhabak KP, Mugesh G., 2010). Emodin cooperates with peroxide metabolizing enzymes to maintain cell status (Singh KB, Trigun SK., 2011). However, it behaves like a prooxidant in tumor cells.

The role of emodin in controlling apoptosis actualizes through reactive oxygen species (ROS) generation in the cells. Because of emodin's quinone structure, it is an electron acceptor. In this way, it interacts with molecular oxygen and generates superoxide anion in the cell (Rahimipour S, Bilkis I., 2001). ROS disrupt proteins as well as nucleic acids and block transcription factors in the cancer cells and prevent cell survival (Yi J, Yang J., 2004).

The cytotoxic effect of emodin occurs through different molecular mechanisms, whereas these mechanisms are not well discovered yet (Figure 1.5). Recent studies demonstrate that emodin has inhibitory effects on cell survival in the CH27 cells (lung squamous carcinoma cells) and triggers generation of ROS in HL-60 cells (human promyeloleukemic cells) (Lee HZ, 2001, Chen YC, Shen SC., 2002). Emodin-induced apoptosis occurs via p53-dependent pathway in several cancer cell lines (HePG2/C3A human hepatocellular carcinoma cells, PLC/PRF/5 human liver hepatoma cells, SK-HEP-1 human liver adenocarcinoma cells) (Shieh DE, Chen YY., 2004). Emodin also triggers apoptosis in myeloma cells and blocks the interleukin-6-induced JAK2/STAT3 pathway (Muto A, Hori M., 2007). In MDA-MB-453 breast cancer cell lines, emodin derivative, azide methyl anthraquinone, supresses HER2/neu-overexpressing and induces apoptosis (Yan YY, Zheng LS., 2011).

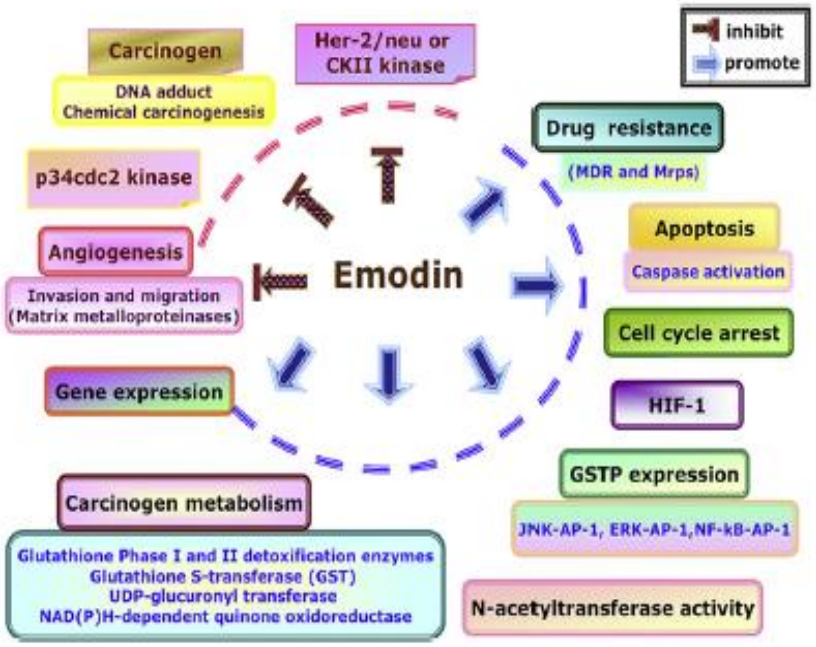

Figure 1.5 The molecular mechanism of emodin (Shu-Chun Hsu, Jing-Gung Chung, 2012)

As mentioned before, emodin generates reactive oxygen species (ROS) and it occurs through ATM-p53-Bax-dependent pathway in adenocarcinoma cells (Lai JM, Chang JT., 2009). Another study reported that, emodin derivative pyrozole induces apoptosis via the caspase activation (caspase-3 and -9 dependent pathway) (Yu CX, Zhang ZQ., 2008).

It has been indicated that combined treatment with emodin poses supplementary effect in chemotherapy by prevention of tyrosine kinase activities (Zhang L, Lau YK., 1999). Moreover, emodin/celecoxib combination via inhibition of antiapoptotic kinase Akt enhances cell death and apoptosis in WB-F344 rat-liver epithelial stem (WBneu) cells. (Lai GH, Zhang Z., 2003). Another study was about how emodin combined treatment with arsenic-trioxide (As_2-O_3) triggers apoptosis compared to As_2-O_3 alone treatment in HeLa (human cervix adenocarcinoma) cells (X. Wang, J. Yang., 2005). Emodin additional effect with other chemotherapeutic drugs as

cisplatin, doxorubicin has been shown in lung cancer cells via overexpression of HER2/neu tyrosine kinase (Zhang L. et al., 1996).

Multi drug resistance (MDR) is an important obstacle which occurs in cancer therapies. When chemotherapeutic agents combine with radiation therapy, it leads to resistance in tumor cells against to therapy. Emodin has been found to induce ROS-mediated suppression of MDR by downregulation MDR related protein-1 (MRP1) in gallbladder cancer cells (Huang XZ, Wang J., 2008). Furthermore, in tumor bearing mice, combined treatment with emodin and cisplatin inhibits tumor growth by down regulation of MRP1 expression (Wang W, Sun YP., 2010).

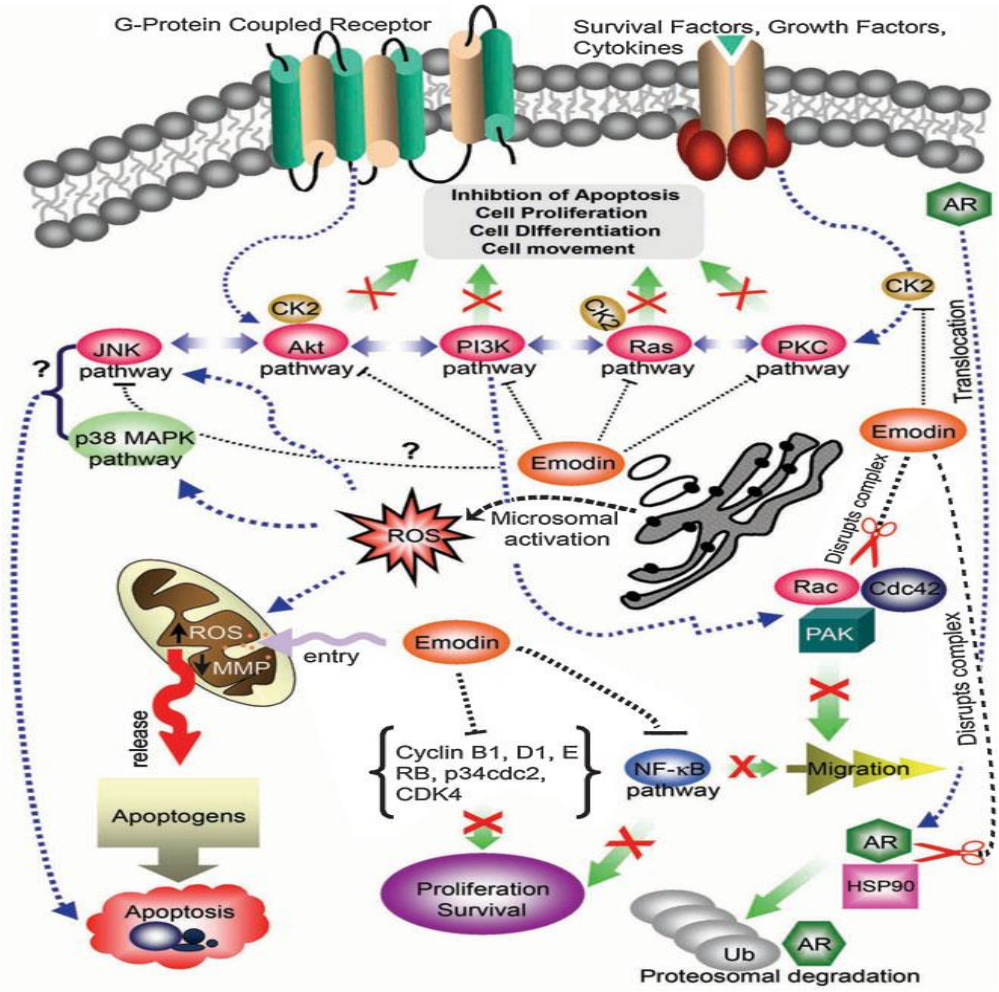

Figure 1.6 The general diagram of emodin action in apoptosis (Srinivas et al., 2007).

1.2 Breast Cancer

Breast cancer is a widespread disease in women that there are one million new cases in a year in the Western world. Breast cancer usually begins in the milk producing gland cells, ducts as well as stromal tissues of the breast. The scientists are not sure what exactly leads to breast cancer, but there are some risk factors which may impact of developing breast cancer. Breast cancer is known to triggered by the genetic abnormalities. However, only 5-10 % of cases occur due to the inherited abnormalities from the parents. Women, who carry BRCA1, BRCA2 and TP 53 genes, are at high risk group for developing breast cancer. These genes can be inherited from the family. Instead, 90 % of cases occur due to the aging process, diet, environmental conditions, exposure to estrogen and radiation etc. In this way, the majority of breast cancer is not hereditary.

In recent years, there has been wide range of treatment choices against to this type of cancer. Surgery, radiation, hormonal (anti-estrogen) therapy, chemotherapy, biological (targeted drug) therapy are the possible treatments in a patient who has breast cancer. There are several factors to make decision on the best treatment in breast cancer including; the type, stage and grade of breast cancer cells, whether or not the cells are sensitive to hormone, the age of patient and the overall health of patient (http://www.medicalnewstoday.com/articles/37136.php).

1.2.1 Therapeutic Approaches to Breast Cancer

The first target of breast cancer therapies are the estrogen receptors (ERs). Because the estrogen receptors are mainly expressed by breast cancer cells which make them sensitive to the growth promotion hormone, estrogen. Therefore, most of breast cancers are estimated to be hormone-responsive and avoidable by using anti-estrogenic compounds. Moreover, aromatase inhibitors have been reported to be a new estrogen therapy for breast cancer (Forbe J., 2008). Despite targeted and endocrine agents are developed in the recent studies, resistance to therapy is a

considerable handicap for the treatment of disease. As the molecular pathways of breast cancer are discovered, it is known that there will be more effective treatment solutions for the breast cancer patients (Eleri D., 2010).

Steroid estrogen receptor modulators (SERMs) are great opportunities to apply ER+ breast cancer cells. Tamoxifen is a widely used SERM to affect ER+ breast disesase. It is a competitive inhibitor of estrogen receptors and inhibits ER transcriptional activity. However, in other tissues, tamoxifen may lead to endometrial cancer (Cohen I., 2004) and tamoxifen treatment is limited to five years; the reason may be drug resistance or occuring agonist effect of tamoxifen (Fisher B, Dignam C., 2001). Aromatase inhibitors also show their activity via inhibiton of estrogens. These compunds exert more efficient activity compared to tamoxifen which used for the extented therapy after tamoxifen (L. Orlando et al., 2010).

Epidermal growth factor receptors (EGFRs) are highly expressed in breast cancer cells and they exert resistant profiles in their phenotypes. They are known to sustain the stimulation of cancer cells during tamoxifen treatment and it is suggested to take EGFR small molecule inhibitors with tamoxifen in the treatment process.

Farnesyl transferase inhibitors are used to prevent farnesylation in different type of tumors. RAS proteins are important factors in signal transduction as well as cellular transformation. They interact with downstream effectors of RAF and PI3K pathways which are responsible for cell growth. The activity of PI3K/Akt pathway increases in breast cancer and it promotes cell-cycle regulatory genes. This pathway is regulated by mammalian target of rapamycin (mTOR) kinases. Thus, prevention of mTOR function is a beneficial therapeutic approach in the treatment (E. Davies, S.Hiscox, 2011). mTOR targeted therapies lead to 50 % reduction in the risk of recurrence. (Valabrega G, Montemurro F., 2007).

Human epidermal growth factor receptor-2 (HER2) is over expressed in breast cancer cell lines. HER2 activation leads to activate cell survival and angiogenesis.

Targeted therapies for HER2+ breast cancer cells are currently implemented to breast cancer patients. Furthermore, the heat shock protein (HSP90) inhibitors also supress HER2 expression. HSP 90 is a molecular agent that arranges the role of oncogenic signalling proteins. The clinical studies demonstrate that the suppression of HSP90 leads to block many transduction signalling pathways in breast cancer cell lines in vitro and in vivo (Di Cosimo S, Baselga J., 2008). There are also antibody based therapies in breast cancer treatment which target HER2 by binding extracellular domain of HER2 (Brockhoff G, Heckel B., 2007).

Simultaneously 15 % of breast cancers in the world are the basal like subtype of breast cancer. They are generally called as "triple negative" phenotype (ER, PgR, HER2 negative). Triple negative breast cancer (TNBC) cells are more offensive and metastatic cells (Rouzier R, Perou CM., 2005). The therapy of TNBC does not have an efficient targeted therapy. Because of this, the recent studies are focused on a number of therapeutic targets; such as poly (ADP-ribose) polymerase (PARP) inhibitors, EGFR inhibitors, vascular endothelial growth factors (VEGF) inhibitors, mTOR inhibitors and Src inhibitors. TNBCs are deficient of DNA repair mechanism like BRCA1-associated breast cancers and these type of cancers are easily affected by poly ADP ribose polymerase (PARP) inhibitors, so that PARP inhibitor agents may be used for the efficient therapy of TNBCs in the future (Hastak K, Alli E., 2010). Data to date demonstrates that, it is not clear if EGFR and VEGF inhibitors have a major effect in TNBCs (Berrada N, Delaloge S., 2010), but VEGF targeting monoclonal antibody, bevacizumab has much influence with given chemotherapy. In addition, mTOR inhibitors with cisplatin (chemotherapeutic agent) show synergistic effect together in patients with TNBC (Wong SW, Tiong KH., 2011).

1.2.2 Doxorubicin and Side Effects

The anti tumor drug doxorubicin (DOX), a widely used chemotherapeutic compound, is used for the treatment of many cancer types including non-Hodgkin's lymphoma, multiple myeloma, lung, gastric, sarcoma, ovarian and breast. Doxorubicin is an anthracycline agent that isolated from *Streptomyces peucetius* and it had shown as an anitumor agent in the 1960s (Weiss RB., 1992). DOX is called as Class I anthracycline which means it supresses DNA and RNA synthesis. Doxorubicin shows its cell death mechanism by three main pathways, (i)intercalation (Figure 1.7, Wikipedia File:Doxorubicin), (ii) poisoning of topoisomerase II enzyme and (iii) ROS generation.

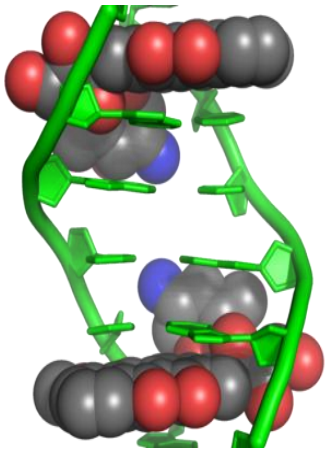

Figure 1.7 Intercalation of DNA strand by doxorubicin molecules.

Topoisomerase II enzyme which involved in DNA synthesis is inhibited by doxorubicin and this inhibition leads to cell death (Perego P., Corna E., 2001). In this process, TP53 gene which has an important role in apoptosis and DNA-damage, has reported to involve in DOX-apoptosis pathway. Moreover, several studies has indicated that TP53 up-regulation is increased by anthracycline treatment (Penault-LIorca, Cayre Anne, 2003). DOX also interacts with nucleotides along DNA forming and distrupts DNA transcription.

DOX undergoes electron reduction to form DOX-semiquinone structure by several enzymes. These oxidoreductases like mitochondrial NADH dehydrogenases, cytosolic enzymes NAD(P)H dehydrogenase, xanthine oxidase involve in this process. Re-oxidation of DOX-semiquinone structure leads to reactive oxygen species (ROS) formation. Oxygen free radicals that are generated by DOX inhibit DNA synthesis and lead to cell membrane damage as shown in Figure 1.8 (Medical Economics : Thomson Healthcare, 2002).

Some researchers investigated that DOX induced H_2O_2 and p53 have distinct roles in endothelial and tumor cells apoptosis. While p53 plays a crucial role in tumor cell apoptosis, H_2O_2 mediated mechanism is responsible for DOX-induced apoptosis and largely independent of p53 activation in endothelial cells. These results showed that DOX-induced apoptosis occurs via distinct signal transduction pathways in normal and tumor cells (Suwei Wang, 2004). However, little is known about p53 involves DOX-induced apoptosis in endothelial cells.

While DOX is a widely used anti-tumor agent in the clinic, resistance is a big problem and it reduces the efficacy of treatment. ABCB1 (MDR1, Pgp) and ABCC1 (MRP1) transporters are thought to cover in this resistance mechanism. ABCB1 shows its resistance through drug efflux pumps. Several studies demonstrated that inhibiton of these transporters supress resistance occuring against DOX activity (Nagata Junko, Kijima Hiroshi, 2002).

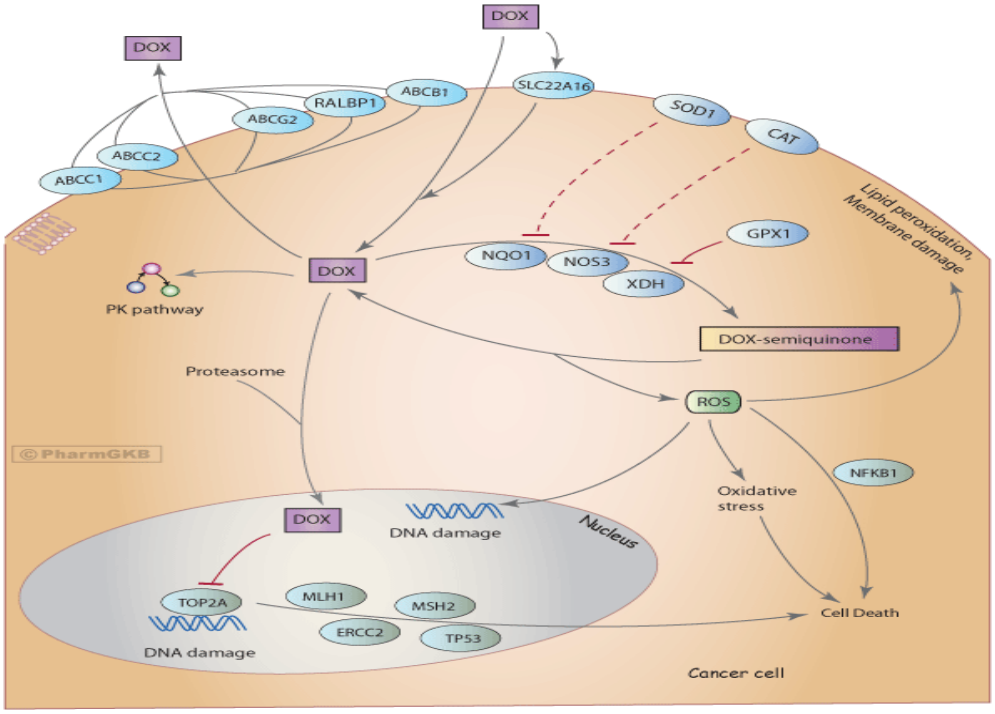

Figure 1.8 Mechanism of doxorubicin action in tumor cells.
(http://www.pharmgkb.org/pathway/PA165292163)

The most dangerous adverse effect of DOX is cardiotoxicity. When the cumulative dose of DOX increase, it might lead to cardiomyopathy and death. DOX is thought to affect of cardiac cells (myocytes) by generation of reactive oxygen species but also might be inhibiting of topoisomerase II enzyme. Inhibition of mitochondrial respiration and peroxidation of lipid membranes occurs through generation of oxygen radicals. These radicals also alter calcium release from ryanodine receptor and induce calcium release. Myocardial tissue can not break down oxygen radicals because the enzyme which destroys free radicals is absent in cardiac tissue (Medical Economics:Thomson Healthcare, 2002). Therefore, cardiac cells are more sensitive to DOX-induced toxicity than other tissues in the body. Another common side effects of DOX are typhlitis, an inflammation of cecum and it's linked to infection (Kaczmarek A, Brinkman BM., 2012), neutrepenia; a decrease in white blood cells, alopecia; hair loss, skin erruptions, dyspigmentation, reactivation of Hepatitis B, nausea and vomiting.

DOX has also been shown to induce apoptosis in endothelial cells via reactive oxygen species and hydrogen peroxide formation (Wang Suwei, Kotamraju S., 2002). Because of the red color of DOX and heavy adverse effects, it is also called as "red devil" and "red death" (Bloch Richard, Bloch Annette 2007; Groopman J., 2007).

1.3 Breast Endothelial Cells

1.3.1 Tumorigenic, MCF-7 Cells

MCF-7 breast cancer cell line was separated from a 69 years old Caucasian woman in 1970. Prior to MCF-7 cells, there was no possibility to provide mammary cell line that living more than months (Glodek Cass, 1990). 70 % of breast cancer cells are ER(+) like MCF-7 breast adenocarcinoma cells. Therefore, these cells are used as a in vitro model to understand estrogen role in breast cancer cells. In MCF-7 cells, estrogen signal transduction mechanism stimulates cell growth through G1 to S phase (Doisneau-Sixou et al., 2003). In addition to their estrogen sensitivity, MCF-7 cells are also sensitive to cytokeratin. Their growth can be suppressed as using tumor necrosis factor alpha (TNF-α) (http://www.mcf7.com/).

In MCF-7 cells, the mechanism of chemotherapeutic agents which induce apoptosis has been investigated in several studies. Activation of p53 transcriptional activity which in turn stimulates apoptosis, caspase-3 activation, oxygen free radicals formation, estrogen receptor inhibition pathways have been shown to suppress tumor growth by numerous chemotherapeutic agents (Wang S, Konorev A., 2004).

1.3.2 Non-Tumorigenic, MCF-10A Cells

MCF-10 breast endothelial cells were isolated from a 36-year-old woman who has a fibrocystic disease and the immortalized MCF-10A cells came out spontaneously in the cell culture. Considering this, MCF-10A cell line is known as immortalized, non-transformed human mammary epithelial cell line. These cells share common features of normal breast epithelial cells (Soule HD, Maloney TM., 1990).

MCF-10A cells are negative for estrogen receptors (ER-). However, cytokeratins and the p53 family member, ΔN63α are expressed by MCF-10A cells. There are several studies that have developed clones from MCF-10A cell line which express ERα and stimulated growth in the presence of 17-β-estradiol (E2) without being epidermal growth factor (EGF) in the culture (Abde M Abukhdeir, Brian G. Blair, 2006).

1.4 Combination Effects of Natural Compounds and ROS-Producing Anti-Cancer Agents in Cancer Therapy

ROS production is a technique used by medicinal approaches for cancer, including chemotherapy due to their implications in stimulating cell death. Accordingly, ROS are applied to destroy cancer cells in medicine (Ozben T., 2007). Physiologically generated ROS are eliminated by enzymatic anti-oxidizing agents like catalase, superoxide dismutase (SOD), peroxidases, glutathione (GSH), thioredoxin (Trx) in healthy cells. At very high concentrations, ROS can not be detoxified by these enzymatic agents. Therefore, ROS damage the cell components and may lead many human diseases including cancer (D'Autreaux B, Toledano MB, 2007; Fruehauf JP, Meykens FL Jr., 2007).

Due to the crucial role of ROS in triggering formation of tumor cells, treating cancer with antioxidants has accepted as a therapeutic approach. Nevertheless, consuming of antioxidant as a supplementation is getting important. Because the effect of antioxidants in dietary remains unclear during cancer therapy. Some researchers

suggest that antioxidants may reduce toxic effects of chemotherapy, whereas others indicate that they trigger ROS production to induce cytotoxicity during radiotherapy or chemotherapy (Seifried HE, McDonald SS., 2003; Conklin KA., 2000).

ROS-producing agents using with chemotherapy accelerate the therapeutic effect of the treatment in cancer cells. Jie Wang showed that emodin through enhance of ROS, can trigger toxic effects of ROS-dependent anti-cancer agents including cisplatin, ATO and taxol in different types of cancer cells. The anti-cancer effect may applied through triggering growth prevention, apoptosis, or restoring anoikis. A natural compound at non-toxic concentrations may induce effect of chemotherapeutic drugs. It is called as "ROS + ROS concept". This effect shows fewer effect on healthy cells in vitro and leads no toxic impact in vivo in mice (Jie Wang, 2008). Moreover, some researchers have suggested an another theory that when both cancer and healthy cells are subjected to same concentrations of ROS producing or stimulating agents, the ROS level would be higher in tumor cells to trigger death, because of the higher basal ROS level in tumor cells than normal cells (Figure 1.9).

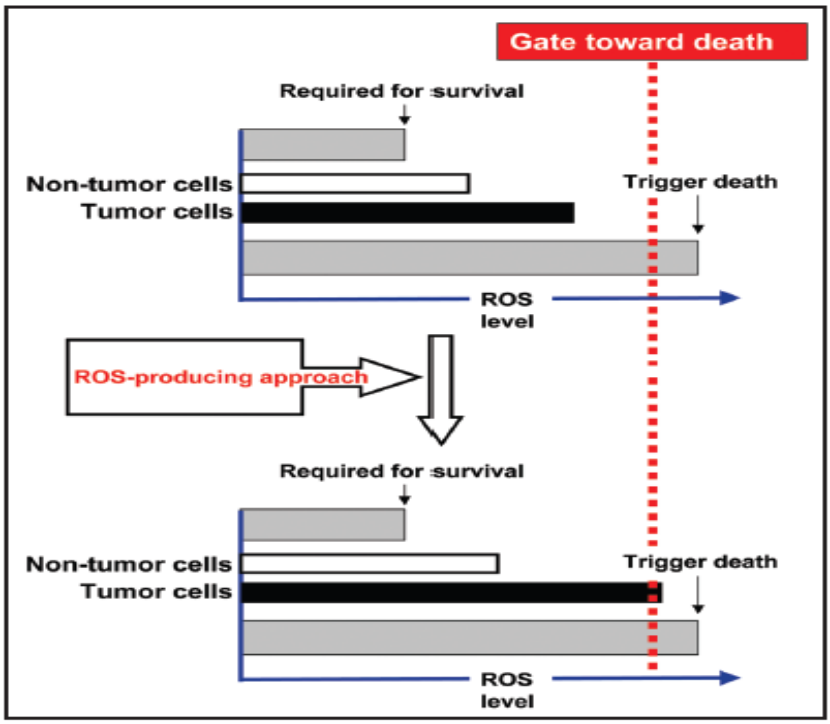

Figure 1.9 ROS-threshold theory (Jie Wang, Jing Y., 2008).

In addition, inhibition of cellular antioxidant systems may also be concluded as excess ROS levels in cancer cells. Among these, superoxide dismutase (SOD), the the glutathione (GSH) system are important components for anticancer drug inventions. Antioxidant system in animals is described briefly below and on the following page :

The GSH system contains NADPH, glutathione reductase (GR) and glutaredoxin. GSH system is arranged by GSH-related enzymes including, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione transferase (GST). GPx, catalyzes the reduction of hydroperoxides while using GSH, in this way GPx protects cell components against oxidative damage. Actually, glutathione metabolism is one of the most effective mechanisms (Esterbauer H, Gebicki J., 1992).

$$ROOH + 2GSH \xrightarrow{GPX} ROH + GSSG + H_2O$$

In animals, hydrogen peroxide is detoxicated by catalase (CAT) enzyme. Catalase intereacts with hydrogen peroxide to generate water and molecular oxygen with peroxidase activity (Turrens JF., 1984).

$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$

$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$

Superoxide dismutase (SOD), is the enzyme which catalyzes the reactive superoxide anion to molecular oxygen and to the less reactive species hydrogen peroxide (Fridovich I., 1995).

$O_2^{\cdot -} + O_2^{\cdot -} + 2H \xrightarrow{SOD} H_2O_2 + O_2$

1.5 Cytotoxicity Tests

Cytotoxicity assays are used for assessing cell viability as a function of redox potential. The use of tetrazolium salts, including XTT (Tetrazolium Hydroxide) to assay cell viability, cell proliferation is a wide-spread application. The XTT assay process prevents radioactivity and is enable for fast assignation and precision results. The assay principles rely on the activity of mitochondrial enzymes that are inactivated following cell death. Cleavage of tetrazolium salt to colored formazan occurs through succinate-tetrazolium reductase system by the mitochondrial enzymes of the active cells. XTT, a yellow tetrazolium salt is reduced to a soluable orange formazan dye, that can be evaluated by absorbance at 450 nm in a spectrophotometer (Figure 1.10). MTT (Diphenyltetrazolium Bromide) is being used since 1950s. MTT is also a colorimetric method as XTT; however Scudiero in 1988 who represented XTT method also showed lots of advantages of XTT upon MTT. Unlike MTT, XTT does not require solubilization before quantitation. Because MTT generates a non soluable formazan and it is necessary to disperse the dye in order to get results while XTT assay give immediate results after incubation.

Moreover, the sensitivity of the XTT reduction is reported to be better than MTT assay. The formazan color is greatly comparative to the activity of cell.

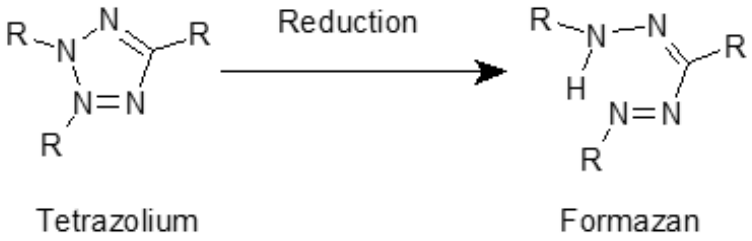

Figure 1.10 Tetrazolium reduction

1.6 Apoptosis and Necrosis

Cell death can be classified into two classes, apoptosis and necrosis. Apoptosis is known as "programmed cell death" and it is arranged by a several of cellular signaling pathways. For cell death to be called as apoptosis, cellular fragmentation, separation of chromosomal DNA, internucleosomal DNA degradation, packaging of the death cell into apoptotic forms without plasma membrane degradation must be observed. Apoptotic bodies are cleared by macrophages, thus there is no inflammation around the dying cell. Unlike apoptotis, necrosis is known as a passive form of cell decease. Necrosis is the consequent of ATP depletion that cells can not find the energy to live up and it is initiated mainly by cellular events such as physical damage. The morphological differences of necrotic cells are breakdown of the plasma membrane, vacuolation of the cytoplasm and initiation of inflammation around the dying cell. Necrotic cells display changes in nuclear morphology but do not demonstrate the condensation of chromatin and fragmentation of DNA into 200 bp fragments which is characteristic for apoptosis. Apoptosis and necrosis are completely different in many respects. Sometimes the degree of stimuli is important that it decides if cell undergoes to apoptosis or necrosis. Low concentrations of

stimulative agents like hypoxia can trigger apoptosis however same stimulative agents can lead to necrosis at high doses (Elmore, 2007).

Apoptosis is needed for cell survival and development of the organism. Excess amount of the cells are produced throughout the development and later on undergo apoptosis to generate tissues (Meier, 2000). Apoptosis also consists during the nomal aging process and works as a defending operation when cells are damaged by several agents or diseases such as cancer (Norbury and Hickson, 2001). Irradiation, toxic agents or drugs used for chemotherapy during cancer treatment lead to DNA damage which may result in apoptotic cell death via a p53 pathway. Fas or TNF receptors are expressed by some cells that may cause apoptosis through ligand binding and protein cross linking (Hirsch, 1997; Zeiss, 2003). Apoptosis is an organized and energy-dependent system that caspases are involved in this process from initiating stimuli to cell decease. There are two main apoptotic pathways, called as intrinsic and extrinsic pathways:

The intrinsic apoptotic pathway is controlled by the Bcl-2 proteins that dominate the extrication of cytochrome c from the mitochondria (Cory and Adams, 2002; Kuwana et al., 2002). Bcl-2 is an oncogene that blocks cell killing in place of cell proliferation (Vaux, Cory and Adams, 1988). Bcl-2 is placed on the outer membrane of mitochondria and its' functions are inhibition of cytochrome c release, prevention of ROS generation, stabilization of mitochondrial transmembrane potential (Green DR., Reed JC., 1998). The Bcl-2 family covers anti-apoptotic and pro-apoptotic proteins and is divided into three classes; pro-survival proteins such as Bcl-X_L; pro-apoptotic proteins, Bax and Bak and pro-apoptotic "BH3-only" proteins (Bouillet and Strasser, 2002). Bax and other apoptotic proteins of Bcl-2 family induce cytochrome c release (Borner, 2003). Tumor supressor, p53, induces expression of Bax, Noxa, PUMA, Bid, Fas, p21. Bax was shown as a first member to be stimulated by p53. In reply to stress conditions, cytochrome c from the mitochondria is released by the Bax which leads to caspase-9 activation (Adams and Cory, 1998). Caspases activates

DNA fragmentation factor (DFF) that results in DNA fragmentation and phagocytosis occurs as a final event (Earnshow et al., 1999).

The extrinsic signalling pathways that trigger apoptosis include death receptors which are known as the tumor necrosis factor (TNF) receptors (Locksley et al., 2001). TNF receptor family have similar domains, a cytoplasmic domain and a "death domain" (Wang H. et al., 2001). The death domain is responsible for transmitting deceased signals. The best known ligands and corresponding to the death receptors are FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR5 (Chicheportiche et al., 1997; Ashkenazi et al., 1998; Li, B. et al., 1998; Suliman et al., 2001; Rubio-Moscardo et al., 2005). The best defined extrinsic stage of apoptosis is the FasL/FasR and TNF-α/TNFR1 patterns. In this phase, the receptors binds with the homologues trimeric ligands. After ligand binding, cytoplasmic adapter proteins that display corresponding death domains bind to the receptors. Activated receptor complex leads to caspase-8 activation. Then, activation of caspase-8 trigger to caspase-3 activation (Scaffidi et al., 1998). Another way to activate caspase-3 that caspase-8 seperates Bid protein and its carboxy terminal invase to mitochondria and leading to release of cytochrome-c. Binding of cytochrome c to the Apaf 1 protein activates caspase-9 and -3 (Luo et al., 1998). Apoptotic pathway was shown in Figure 1.11.

Mitochondria has a very important act in apoptosis. The mitochondrial apoptotic pathway is induced by both external and internal signals. Death receptors can be given as an example to the external signals and reactive oxygen species, DNA damage can be given as internal signals. Extrication of cytochrome c from the mitochondria activates the effector caspases. Following activation, apoptosis initiated by caspase-9 and additional caspases. Death receptors also trigger apoptosis in mitochondria through activation of initiator caspases.

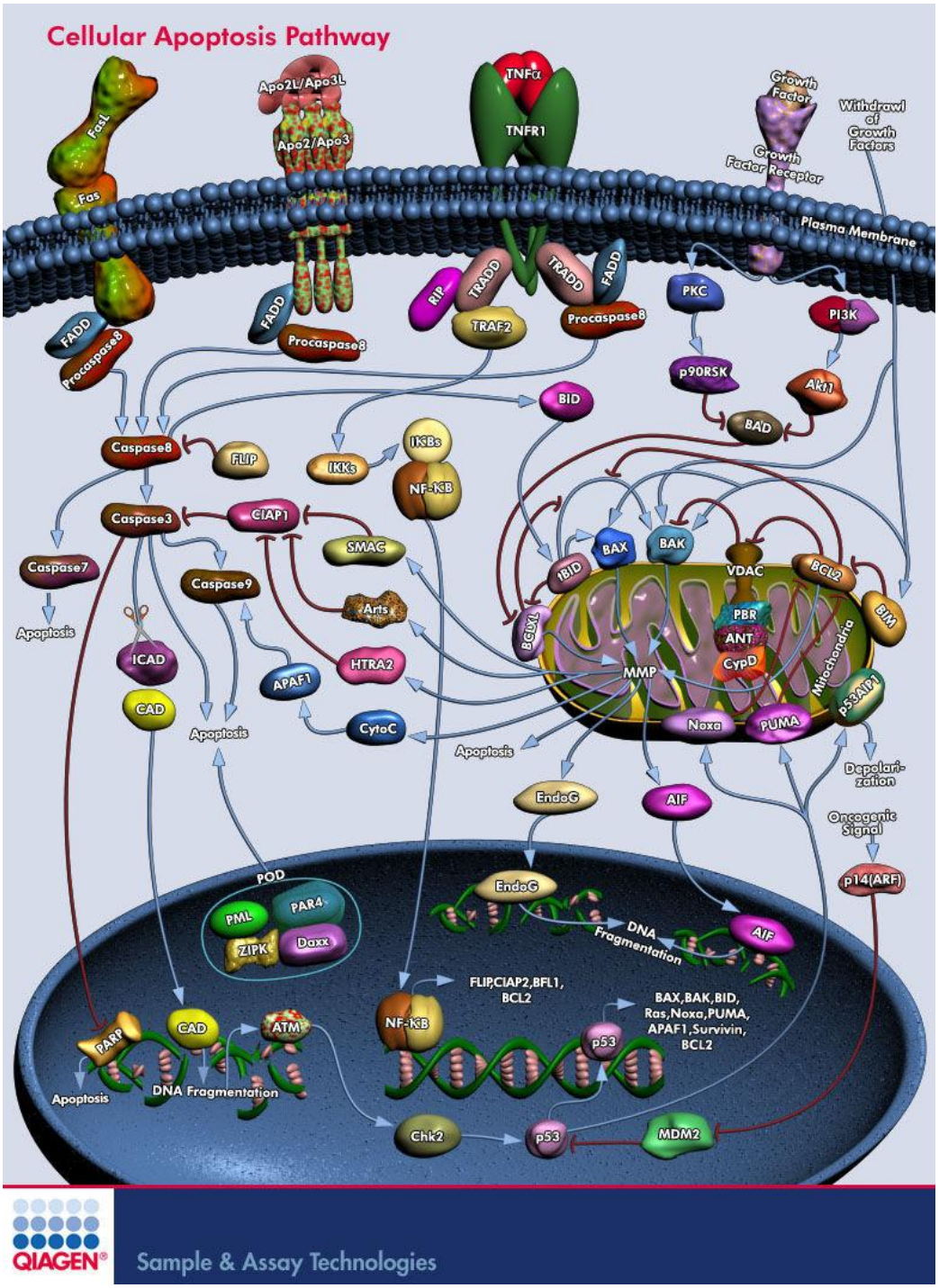

Figure 1.11 Cellular apoptosis pathway (Qiagen Sample and Assay Technologies).

1.7 Scope of the Study

Natural products in the dietary have very important roles in the treatment of cancer. Their consumption with the chemotherapeutic drugs changes the way of treatment. Drug-pyhtochemical interactions screening indicates possibilities for improved therapeutic strategies and contribute to the development of new combined cancer therapies.

Emodin (3-Methyl-1,6,8-trihydroxyanthraquinone) is a compound of several Chinese herbs. As a phytoestrogen, emodin has been used as a purgative agents since ancient times (Srinivas et. al., 2007). It has also immunosuppressive, anti-bacterial, hepatoprotective, purgative, anti-cancer and antioxidant effects. By this way, emodin may interact with the chemopreventive drugs and affect their anti-cancer mechanisms in cancer patients.

The aim of the study was to investigate the interaction effect of emodin with chemotherapeutic drug doxorubucin. This study was planned to evaluate proliferative, cytotoxic and apoptotic effects of emodin when administrated with chemopreventive anti-cancer drug, doxorubicin in both breast cancer cell line, MCF-7 and breast endothelial cell line, MCF-10A.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 MCF-7 and MCF-10A Cell Lines

MCF-7 (human adenocarcinoma cell line, ATCC® HTB-22™) and MCF-10A cell lines (human mammary epithelial cell line, ATCC® CRL-10317™) were donated by Assoc. Prof. Dr. A. Elif Erson Bensan (METU, Ankara, Turkey).

2.1.2 Chemical and Other Materials

Dimethyl sulphoxide (DMSO) was purchased from AppliChem GmbH (Darmstadt, Germany). 0.5 % (w/v) trypan blue, gentamicine, XTT Cell Proliferation Kit were purchased from Biological Industries (Haemek, Israel). 25-cm^2 (T25) and 75-cm^2 (T75) tissue culture flasks and 96 well microplates, cryovials (sterile, DNase and RNase free, non pyrogenic) were obtained from Greiner Bio-One (Frickenhausen, Germany). Fetal bovine serum, horse serum, penicilin-streptomycin (pen-strep), Dulbecco's Phosphate Buffered Saline (PBS), Trypsin/EDTA, Roswell Park Memeorial Institute Medium (RPMI) 1640, Dulbecco's Modified Eagle medium/Ham's F-12 (DMEM/Ham's F12) were purchased from Biochrom Ltd. (Cambridge UK). Epidermal Growth Factor (EGF) was from Peprotech (New Jersey, USA). Emodin, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), hydrocortisone, insulin, paraformaldehyde and sodiumhydroxide were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Doxorubicin (Sigma-Aldrich)

was a generous gift from Assist. Prof. Dr. Meltem Demirel Kars (Selcuk University, Konya, Turkey). BD™ MitoScreen (JC-1) and Annexin V:PE Apoptosis Detection Kit I were purchased from BD Biosciences (New Jersey, USA). For cell culture studies, all the reagents and chemicals were cell culture grade.

2.2 Methods

2.2.1 Preparation of Emodin Solution

Emodin dissolves in DMSO and in basic environment. The precipitation of emodin occurs in the acidic environment. Emodin was dissolved in 2 % DMSO and in basic distilled water pH (11,89). After preparation of emodin solution, it was diluted 20 fold (1:19) into culture medium and ready for cell treatment. Hence, the concentration of 2 % DMSO was decreased 0.1 % following the addition of culture medium. Emodin concentrations used in the experiments were 0.4, 4 and 10 μM.

2.2.2 Cell Lines and Culture Conditions

MCF-7 cells were grown in complete growth media that containing RPMI-1640 culture medium with phenol red containing 10 % fetal bovine serum and 0.2 % gentamicin and incubated at 37 ^{o}C in a 95 % air and 5 % CO_2. The medium was changed every 2 days.

MCF-10A cells were grown in complete medium containing DMEM/Ham's F:12 cell culture medium with phenol red containing 5 % horse serum, 100 μl EGF from 100 μg/ml stock, 250 μl hydrocortisone from 1 mg/ml stock, 500 μl insulin from 10 μg/ml stock, 5ml pen/strep and incubated at 37 ^{o}C in a 95 % air and 5 % CO_2. The medium was changed every 2 days.

2.2.3 Cell Thawing

The cryovials in which MCF-7 and MCF-10A cells found frozen were taken out from the nitrogen tank for thawing the cells and immediately were placed into the water bath (37°C) for dissolution of the frozen cells. After the freezing medium was completely aspirated, the cells were put T25 flasks those containing 8 ml culture medium and left in CO_2 incubator for 24 hours. Following day, the cells were observed under the light microscope and if no cell destruction was detected, centrifuging (100 g RT, 5 min) was done to remove culture medium and the cells were placed into the T75 flasks including 20 ml complete growth medium in order to remove DMSO.

2.2.4 Cell Passaging

Every 2−3 days the media was changed until the cells achieved at 80 % confluency and covered the flask layer. After aspirating the cultue medium, attached cells on the layer were washed with 2 ml Hank's Balanced Salt Solution (BSS) or Dulbecco's Phosphate Buffered Saline (PBS) to elude medium. After washing twice with PBS or BSS, 2 ml Trypsin/EDTA was added to distinguish the cells from the flask layer. After trypsinization step, the flask placed into the incubator at 37 °C, 4-5 minutes for MCF-7 cells and 20-25 minutes for MCF-10A cells. The trypsin activation was blocked by adding culture medium. Trypan blue (Biological Industries) stain was used while the cells were counting under the light microscope.

2.2.5 Cell Counting with Trypan Blue

Trypan blue dye is used for the differentiation of viable cells from non-viable cells. The trypan blue method is based on staining the cells which membranes' are damaged. For this reason, trypan blue only colors death cells while viable cells keep out dye.

In a microcentrifuge tube, 30 µl of 0.5 % (w/v) trypan blue was added on the same volume of cell suspension. Then, the pipetting was done until get a single suspension. 10 µl of the mixture was loaded into the hemacytometer. The coverslip was placed on the counting surface of hemacytometer to evenly spread the liquid. The chamber is then located on the microscope.

The main divisions of hemacytometer seperate into 9 large squares. The surface area of these squares are 1 mm^2 and the depth of chamber is 1 mm. Each square of the hemacytometer indicates a total volume of 1 $mm^3 = 10^{-4}$ cm^3, since 1 cm^3 is equal to 1 ml, the determination can be done using the equations as shown in example:

Cell number per ml = The average cell count per square × DF × 10^4

DF = Dilution Factor (was done with trypan blue dye)

10^4 = Calculated according to the sizes of hemacytometer

Total cell number = cells per ml × the total volume of cell suspension

Example : 113 (average cell count) × 2 (dilution factor) × 10^4 = 2.26×10^6 cells/ml

2.26×10^6 (cells per ml) × 7 ml (original volume) = 15.05×10^6 (total cells)

2.2.6 Cell Freezing

All cell cultures are susceptible to the microbial contamination. As soon as the cells become available from culturing, they should be frozen as a stock. It is important that the cells can be stocked for a long-term and when needed working stocks can be prepared from the frozen cells. The best method for cryopreservation of the cells is stocking them in liquid nitrogen (-190°C) in the existence of dimethylsulfoxide

(DMSO). Freezing medium, containing 10 % DMSO and 90 % FBS, was utilized for MCF-7 nad MCF-10A cells cryopreservation.

Following trypsinization, the cells were counted and 1 ml freezing medium was used for cryopreservation of every 3-4x10^6 cells. After counting, cell suspension was spinned with centrifuge to precipitate cells and the medium was aspirated. The cells with freezing medium were placed into the cryovials. It is a significant consideration that cells should be avoided to subject freezing medium for a long time before freezing. The cryovials were located in the isopropyl alcohol box (Mr. Frosty) and stored at - 80°C for 24 hours. After 24 hours the cryovials were carried into the liquid nitrogen tank which is at -190 °C.

2.2.7 Cell Treatment with Emodin (EMO) and Doxorubicin (DOX)

MCF-7 and MCF-10A cells were seeded at a density of 5 × 10^4 cells/ml into 96 well plates for cytotoxicity studies and incubated at 37 °C. After overnight incubation, cells were treated with EMO (0.4, 4, 10 μM) and DOX (0.1, 0.83, 2.5 and 3.25 μM) for 6, 24 and 72 hours. EMO was first dissolved in DMSO 2 % and basic distilled water (pH:11,89), then diluted with complete RPMI 1640 medium for MCF-7 cells and DMEM Ham's F:12 medium for MCF-10A cells. DOX was dissolved directly in distilled water and then diluted with complete medium. DMSO was added to the wells to make the cell membrane more conductive to EMO and DOX. It was kept at a final concentration 0.1 % in each well. Cells set as controls which grown in 0.1 % DMSO containing medium.

During 96 hours incubation time, the effects of different EMO and DOX concentrations at different time periods (6, 24, 72 h) on the cell viability were evaluated by XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5-carboxanilide) Assay.

2.2.8 Measuring Viability of MCF-7 and MCF-10A Cells using XTT Assay

The effects of EMO and DOX on the proliferation of MCF-7 and MCF-10A cells were measured by XTT cell proliferation kit (Biological Industries, Israel) in 96 well microtiter plates.

MCF-7 and MCF-10A cells (5000 cells/well) were seeded into the 96-well microtiter plates and left for overnight incubation at 37 ^{o}C in 5% CO_2 in order to let them attach and grow. After overnight incubation, old medium was aspirated. For the pre-treated or alone treated group 50 µl medium and 50 µl of EMO or DOX solutions were added in order to reach final concentrations; 0.4, 4, 10 µM for EMO and 0.1, 0.83, 2.5, 3.25 µM for DOX.

For the co-treated group, EMO and DOX concentrations were added in quadruplicate to the wells which already containing of seeded cells (25 µl EMO+25 µl DOX+50 µl medium). No cells were seeded into the blank wells (50 µl of complete medium which containing 0.1 % DMSO and 50 µl EMO or DOX solutions were added). The aim of blank wells was to determinate if any background of EMO or/and DOX absorbance and interaction with the XTT solution. The 96 well microtiter plate presentment of XTT assay was shown in Figure 2.1.

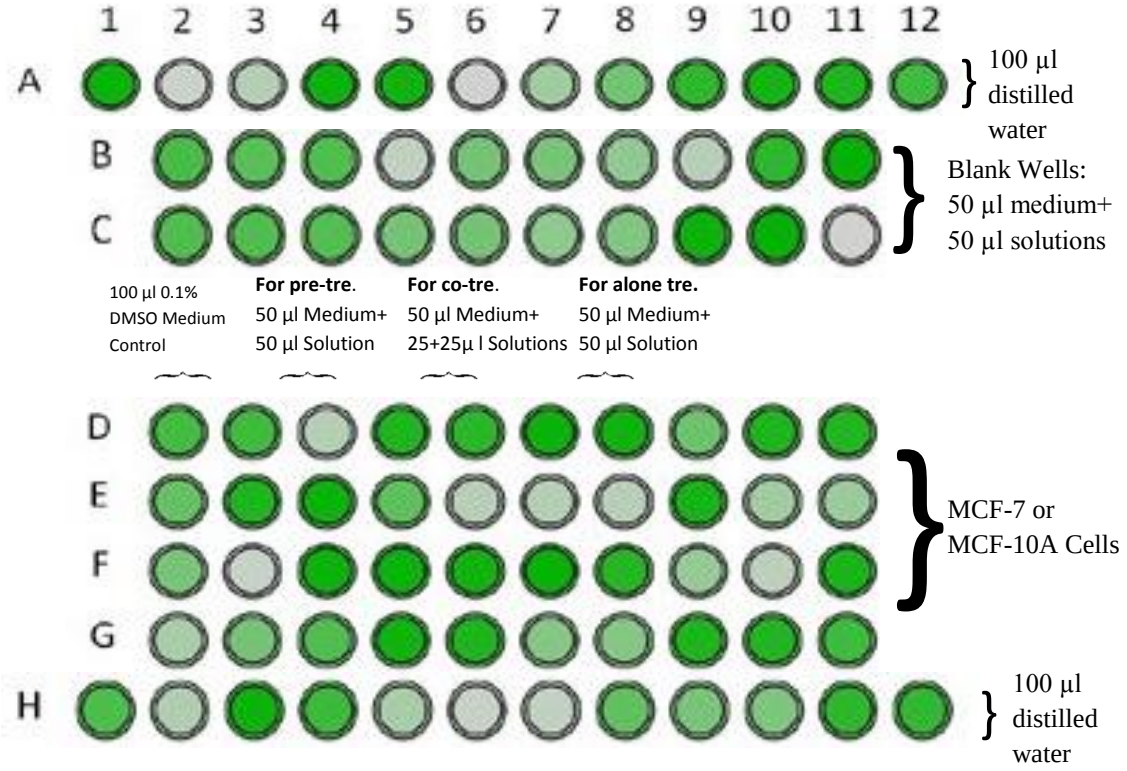

Figure 2.1 The general represantation of XTT assay.

The distilled water (100 µl) was added to the wells from A1 to A12, A1 to H1, A12 to H12 and H1 to H12 in order to prevent evaporation of solutions in the CO_2 incubator. The cells were seeded to D, E, F, G wells. B and C were the blank wells. The complete medium which containing 0.1% DMSO (100 µl) was added to B2 to G2 control wells.

For alone and pre-treatment (Pre-tre) of EMO (0.4, 4, 10 µM) and DOX (0.1, 0.83, 2.5, 3.25 µM), solutions were prepared as two times more concentrated as 0.8, 8, 20 µM for EMO and 0.2, 1.66, 5, 6.5 µM for DOX. (50 µl 0.1 % DMSO containing medium + 50 µl DOX or EMO solutions in order to reach final concentrations). For combined treatment (Co-tre) of EMO and DOX, solutions were prepared as four times more concentrated as 0.16, 16, 40 µM for EMO and 0.4, 3.32, 10, 13 µM for DOX. (50 µl 0.1 % DMSO containing medium + 25 µl DOX solution + 25 µl EMO solution, in order to reach final concentrations).

In the pre-treated group, following EMO treatment for 6 or 24 hours, EMO solutions were aspirated and various concentrations of DOX solution were added into the wells and cells were left in the incubator for 72 hours. The same mechanism was valid for DOX pre-treated group. In the co-treated group, cells were exposed to various concentrations of DOX and EMO solutions at the same time and left in the incubator for 72 hours. In the alone treated group for 24 hours, the concentrations were aspirated after 24 hours, then complete medium was added into the wells and cells were left to incubation for 72 h.

At the end of the treatment time, 100 µl of phenazine metho-sulfate is mixed with 5 mL of XTT reagent and 50 µl of this solution was added to each well. After 5 hours, the orange colored formazan crystals that were generated by mitochondrial enzymes of viable cells were measured at 460 nm with ELISA reader (Biotek, Epoch). The results were given as percentage of viable cells, calculated using equation 2.1.

Equation 2.1

$$\%\ Cell\ Viability = \frac{Abs_S\ (with\ cell) - Abs_S\ (cell\ free)}{Abs_C(with\ cell) - Abs_C(cell\ free)}\ x\ 100$$

Where,

$Abs_{S\ (with\ cell)}$ = Average absorbance of cells treated with EMO or DOX

$Abs_{S\ (cell\ free)}$ = Average absorbance of EMO or DOX in cell free medium

$Abs_{C\ (with\ cell)}$ = Average absorbance of control cells

$Abs_{C\ (cell\ free)}$ = Average absorbance of cell free medium

Abs = Absorbance at 450 nm

2.2.9 Viable Cell Counting with Trypan Blue

MCF-7 and MCF-10A cell lines (1×10^5 cells/ml; 1 ml/well) were seeded in 6-well plates. Following overnight incubation, complete medium was changed with 500 µl fresh medium and cells were treated with 500 µl of 2.5 µM DOX or/and 0.4 µM EMO solutions and they incubated for 72 hours. After incubation time, cells were washed with 500 µl PBS and detached with 300 µl of trypsin. Cells were collected after adding 700 µl of medium. Cell counting was carried out with hemocytometer (Neubauer). Cells those were incubated in medium containing 0.1 % DMSO were considered as controls set as 100 % cell viability.

2.2.10 Flow Cytometry

Cell death is typically debated as either apoptosis or accidental cell death (necrosis). The mechanisms of apoptosis and necrosis are different from eachother. Apoptosis is an active, programmed cell death mechanism that avoids inflammation while necrosis has been defined as passive, resulting from environmental confusions (Susan L., Brad T., 2005). In this section, characterization and differentiation of apoptosis and necrosis were evaluated with flow cytometry. All measurements were done on a FACSAria III system (BD Biosciences, USA). Analysis was examined using FACSDIVA version 6.1.3 software.

2.2.10.1 Detection of Apoptosis by Annexin V-APC

Apoptosis detection with Annexin V after EMO and DOX treatment was carried out using APC- labeled recombinant human Annexin V and 7AAD antibody. Annexin V-Apc (λ ex: 633, λ em: 660 nm) is detected in FL4 on dual laser instruments and 7AAD (λ ex: 488, λ em: 647 nm) is detected in FL3 channel on most instruments.

MCF-7 and MCF-10A cells (1 x 10^5 cells/ml; 1 ml; 6-well plate) were either left untreated (control) or treated with EMO and/or DOX for 24 h and 72 h. Then cells were aggregated by centrifugation (400xg, 5 min, 25 ^oC). After centrifugation, cells were washed with PBS twice and diluted with 1X binding buffer. 100 µl cell suspansion was added to the 5 ml polypropylene tube and incubated with 5 µl Annexin V and 5 µl 7 AAD solution for 15 min at room temperature in the dark. Finally, 400 µl binding buffer was added to the tubes. The samples were examined by flow cytometry.

The following controls were used to determine compensation and quadrants on flow cytometer; (i) unstained cells, (ii) EMO and DOX treated cells stained with Annexin V-APC alone and (iii) EMO and DOX treated cells stained with 7-AAD alone.

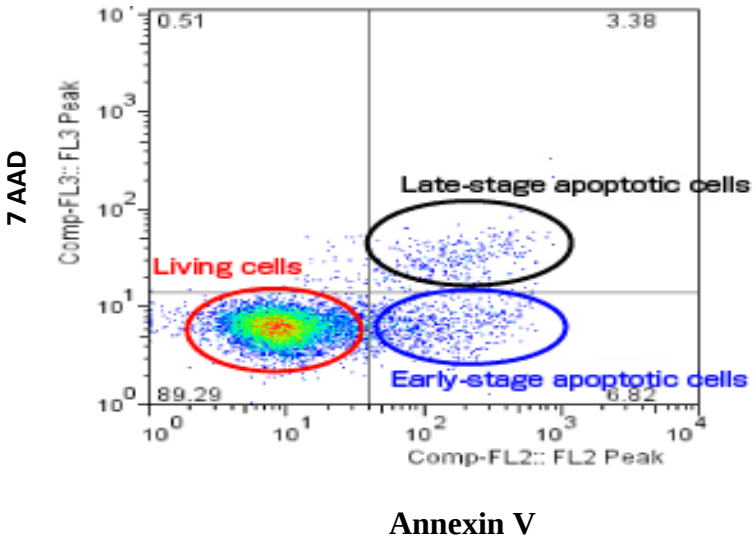

Figure 2.2 Determination of living cells and apoptotic cell populatios (http://www.furukawa.co.jp/bio/english/appli.htm)

The aim of flow cytometric analysis is to separate and prominent the populations. In these populations, apoptotic cells are stained positive for Annexin V and unstained for 7-AAD; necrotic or death cells are stained positive for both Annexin V and 7-AAD. Cells which unstained for Annexin V and 7-AAD were not dead or undergoing apoptosis (Figure 2.2).

2.2.10.2 Detection of Mitochondrial Membrane Potential ($\Delta\psi_m$)

During apoptosis, loss of mitochondrial transmembrane potential ($\Delta\psi_m$) occurs in mitochondria. Therefore, $\Delta\psi_m$ is an important factor to detect mitochondrial function. A cytofluorimetric lipophilic cationic dye 5,5',6,6'- tetrachloro-1,1',3,3'-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1), gets in mitochondria and expand color from red to green during membrane potential loss (Figure 2.3). In viable cells which have high mitochondrial $\Delta\psi_m$, JC-1 forms complexes known as J-aggregates with intense red fluorescence, but in apoptotic cells which have low $\Delta\psi_m$, JC-1 remains in the monomeric form which displays only green fluorescence. The percentage of green to red fluorescence is linked to membrane potential. Mitochondrial size, shape and density do not influence fluorescence signals.

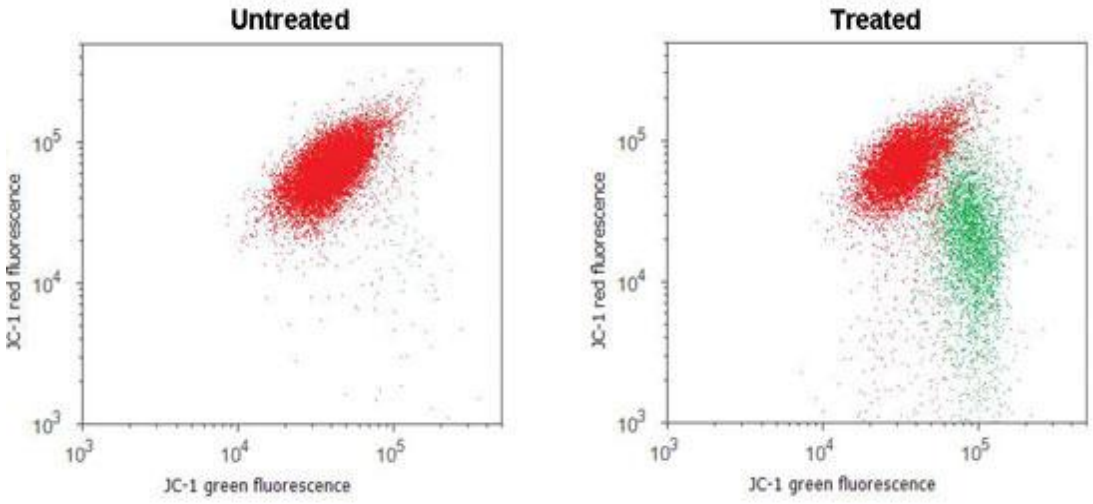

Figure 2.3 Shift of fluorescence emission demonstrating mitochondrial membrane potential in healthy and apoptotic cells (http://bitesizebio.com/13697/flow-cytometric-apoptosis-assays/).

2.2.11 Microscopic Analysis of EMO and DOX Treated Cells

2.2.11.1 Morphology of Treated Cells

MCF-7 and MCF-10A cells (5 x 10^4 cells per ml; 1 ml well; 24 well-plate) were either left untreated (control) or treated with 0.4 μM emodin and/or 2.5 μM doxorubicin for 24 and 72 hours. Cells were viewed at a 200-fold magnification with an inverted microscope (DM IL LED, Leica Microsystems, Wertzlar, Germany).

2.2.11.2 DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) Staining

4',6-diamidino-2-phenylindole (DAPI) is a fluorescent stain that emits blue fluorescence and binds to A-T regions of DNA. Nuclear staining was applied using DAPI. Briefly, MCF-7 and MCF-10A cells were treated with 0.4 μM EMO and 2.5 μM DOX for 24 and 72 hours. After 96 h total treatment time, culture medium was aspirated and cells were bathed with PBS thrice. After washing, cells were fixed using 3.7 % methanol free para-formaldehyde for 10 min at room temperature then washed with PBS twice. Finally, cells were mounted with 1 μg/ml DAPI (Sigma Aldrich) for 25 min and washed with PBS twice to visualize. The images were obtained by a confocal laser microscope system at 403 nm (A1R+/A1+, Nikon Instruments, Inc. Tokyo, Japan).

2.3 Statistical Analysis

Results are denoted as mean ± standard deviation (± SD). All analyses and graphs were carried out with GraphPad Prism version 5 (GraphPad Software, CA, USA). Two-way ANOVA test was applied to determine the statistical significance between control cells and DOX-EMO treated cells. A statistically significant difference was considered to be at $P < 0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

Many of the chemotherapic agents used to beat cancer in medicine were improved from natural products. Phytoestrogens has been used for treating several diseases in China and today they are still present in the dietary as lignans, stilbenes etc. Pre-clinical and clinical studies have indicated that daily consumption of phytoestrogens has beneficial effects to inhibit carcinogenesis by detoxification with anti-oxidant enzymes (S. Vadodkar, 2012). The mechanism of cytotoxic or protective effect of phytoestrogens with chemotherapeutic drugs is still not clear. In this study, emodin (EMO), a phytoestrogen and doxorubicin (DOX), a chemotherapeutic drug were used together on both healthy and cancer breast cell models. Alone treatment, pre-treatment (pre-tre) and co-treatment (co-tre) with different concentrations of EMO and DOX were determined by looking at their protective, cytotoxic and apoptotic properties on both MCF-7 and MCF-10A cells.

3.1 Cytotoxicity of Emodin and Doxorubicin in MCF-7 and MCF-10A Cells

3.1.1 XTT Cell Cytotoxicity Assay

Metabolically active MCF-7 and MCF-10A cells were identified using XTT (*2,3-bis-(2-methoxy -4 -nitro-5 sulfophenyl)-2H-tetrazolium-5-carboxanilide)* cell toxicity assay upon treatment with EMO or DOX for 6, 24 and 72 h, EMO pre-tre for 24 h, DOX pre-tre for 24 h and EMO-DOX co-tre for 72 h. In the XTT assay, tetrazolium salt was reduced by mitochondrial enzymes of the metabolically active cells to form orange coloured formazan and it was evaluated by ELISA plate reader at 460 nm. Cell viability results acquired from XTT assay were transformed to % cell viability

and the control cells set as 100 %. Averages of measurements, treatment with 0.4, 4, 10 μM EMO and 0.1, 0.83, 2.5, 3.25 μM DOX concentrations for 6, 24 and 72 hours were displayed as percentage of the control measurements.

96-well plate was used fot the XTT assay after overnight incubation time at 37 ^{o}C. % cell viabilities were measured for MCF-7 and MCF-10A cells and the results were displayed in Table 3.1-8 and Figure 3.1-10.

Natural substances' extracts and some chemicals may reduce tetrazolium salt. If there were higher metabolically active cells, additional reduction might be formed in the results of XTT assay (Shoemaker et al., 2004). Therefore, it is preferable to reckon cells with trypan staining when examining these materials like EMO and DOX.

Table 3.1 Effects of EMO (0.4, 4, 10 μM) pre-tre (Pre) for 6 h and DOX (0.1, 0.83, 2.5 μM) alone (A), co-tre (Co) for 72 h on the growth of MCF-7 and MCF-10A cells.

Cell Viability % ± SD*						
DOX (μM)		**0**	**0,1**	**0,83**	**2,5**	**EMO (μM)**
MCF-7	A	100	85± 2.82	76±4.24	61± 7.07	**0**
MCF-10A	A	100	83 ± 3.53	76 ± 0.70	50 ± 7.07	
	Pre		64± 5.54	53±11.44	51±4.95	**0,4**
MCF-7	Co	95 ± 2.82	43 ± 17.67	37 ± 9.89	25 ± 7.07	
	Pre		78± 4.95	72± 5.54	51± 2.12	
MCF-10A	Co	105 ± 3.53	74 ± 0.70	66 ± 1.41	47 ± 4.24	
	Pre		76± 4.24	63±3.53	NA	**4**
MCF-7	Co	96 ± 4.24	55 ± 1.41	41 ± 1.41	21 ± 3.53	
	Pre		75±9.03	63± 11.44	NA	
MCF-10A	Co	65 ± 8.48	61 ± 0.70	55 ± 2.12	44 ± 1.41	
	Pre		74±1.41	NA	NA	**10**
MCF-7	Co	92 ± 7.07	47 ± 2.12	33 ± 2.12	23 ± 4.24	
	Pre		60±4.24	52±1.41	NA	
MCF-10A	Co	59 ± 7.07	43 ± 7.78	41 ± 4.95	34 ± 1.41	

NA: Not applicable. $P<0.05$, analyzed by two way ANOVA

*SD was derived from two independent experiments..

MCF-7 and MCF-10A cells were incubated for overnight then treated with 0.4, 4, 10 μM EMO for 6 h, respectively. Following 6 h EMO pre-tre, cells were treated with 0.1, 0.83, 2.5 μM DOX solution for 72 h. Cells also were incubated with EMO and DOX co-tre for 72 h, in applied concentrations. After total cell treatment, the cell proliferation was obtained with XTT Assay. The absorbance values were measured at 460 nm in ELISA plate reader. % Cell viability of control cells was shown as 100 %.

Cell viability was 83% after 0.1 μM DOX alone treatment for 72 h in MCF-10A cells. In pre-treatment schedule of EMO (0.4, 4, 10 μM) given for 6 h before 0.1 μM DOX exposure for 72 h, cell viabilities were measured as 78, 75 and 60%.

Afer 0.83 μM DOX-alone exposure for 72 h, cell viability was 76% in MCF-10A cells. In pre-treatment schedule of EMO (0.4, 4, 10 μM) given for 6 h before 0.83 μM DOX exposure for 72 h, cell viabilities were measured as 72, 63 and 52%.

After 2.5 μM DOX-alone treatment, cell viability decreased to 50% and in pre-treatment schedule of EMO 0.4 μM given for 6 h before 2.5 μM DOX exposure for 72 h, cell viability was measured as 50% in MCF-10A cells.

Consequently, pre-tre of EMO for 6 h did not show any substantial effects against DOX-cytotoxicity in MCF-10A cells, for this reason we decided to extend the time of pre-treatment from 6 hours to 24 hours to demonstrate the difference in a time dependent manner.

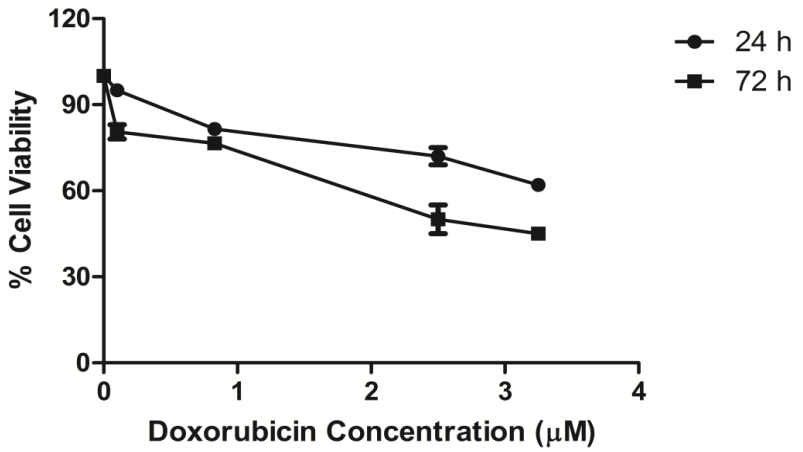

Figure 3.1 Effects of 0.1, 0.83, 2.5, 3.25 μM DOX for 24 h and 72 h treatment on the growth of MCF-7 cells.

In applied DOX concentrations, MCF-7 cell viability was affected in both dose and time dependent manner.

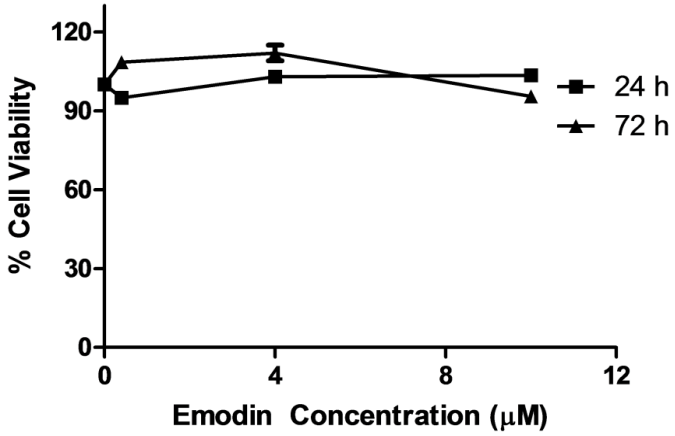

Figure 3.2 Effects of 0.4, 4, 10 μM EMO for 24 h and 72 h treatment on the growth of MCF-7 cells.

In applied EMO concentrations, MCF-7 cell viability decreased in a time dependent manner but not in a dose dependent manner.

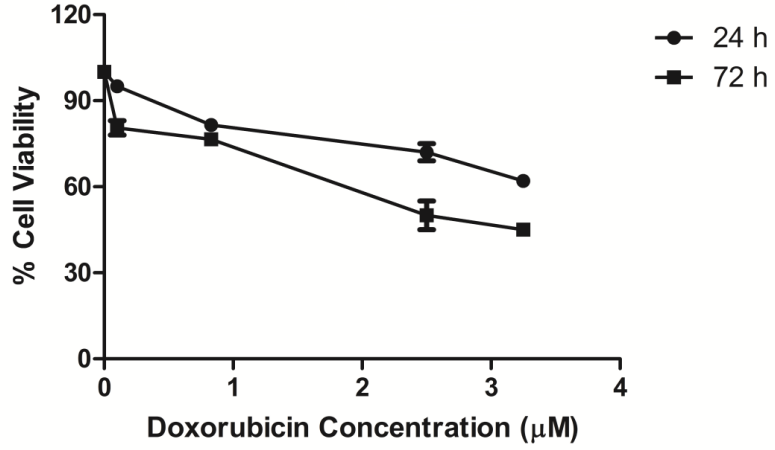

Figure 3.3 Effects of 0.1, 0.83, 2.5, 3.25 μM DOX for 24 h and 72 h treatment on the growth of MCF-10A cells.

In applied DOX concentrations, MCF-10A cell viability was affected in both dose and time dependent manner.

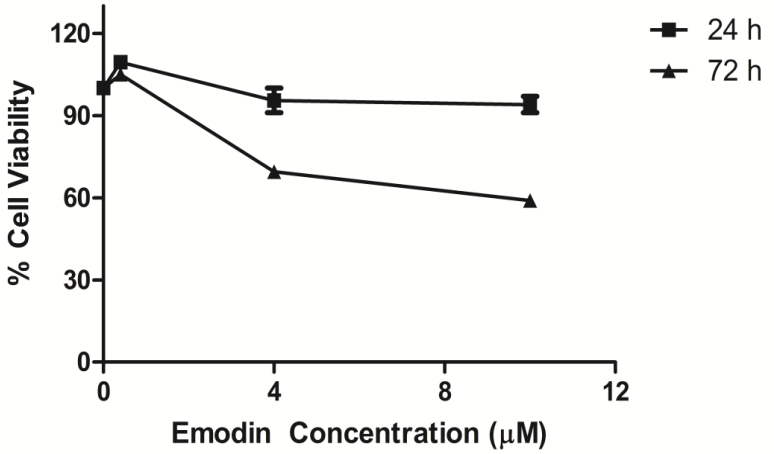

Figure 3.4 Effects of 0.4, 4, 10 μM EMO for 24 h and 72 h treatment on the growth of MCF-10A cells.

In applied EMO concentrations, MCF-10A cell viability decreased in a time dependent manner but not in a dose dependent manner.

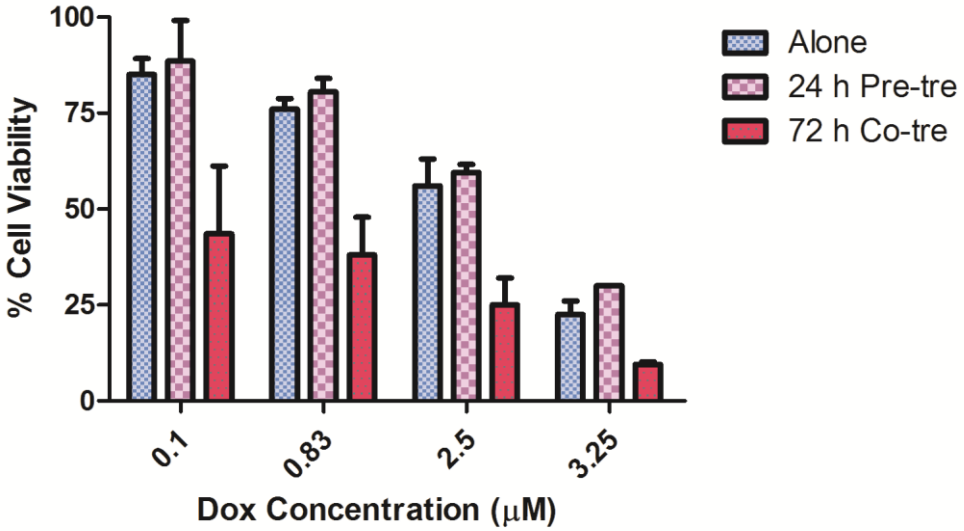

Figure 3.5 Effects of DOX alone (0.1, 0.83, 2.5, 3.25 μM) for 72 h, 0.4 μM EMO pre-tre for 24 h and EMO-DOX co-tre for 72 h, in applied concentrations, on the cell survival of MCF-7 cells. Cytotoxicity was measured by XTT Assay.

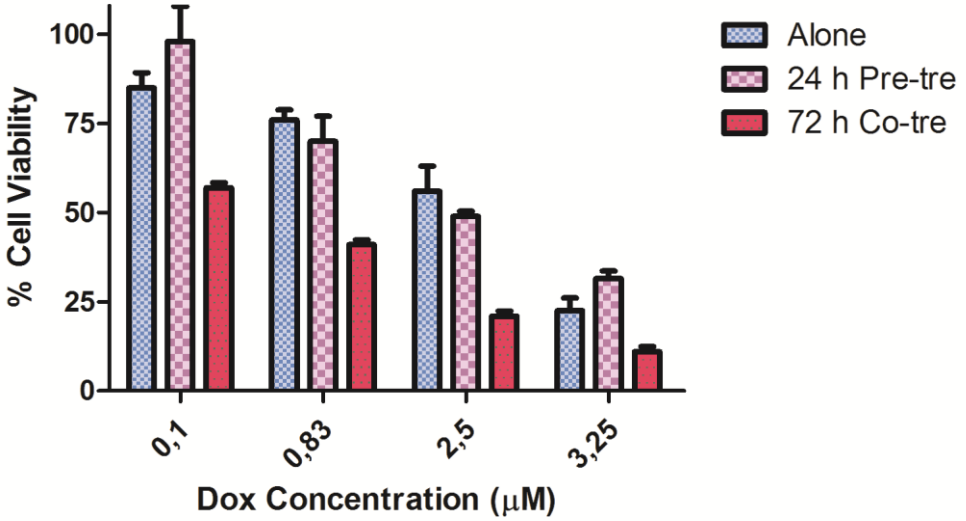

Figure 3.6 Effects of DOX alone (0.1, 0.83, 2.5, 3.25 μM) for 72 h, 4 μM EMO pre-tre for 24 h and EMO-DOX co-tre, in applied concentrations, for 72 h on the cell survival of MCF-7 cells. Cytotoxicity was measured by XTT Assay.

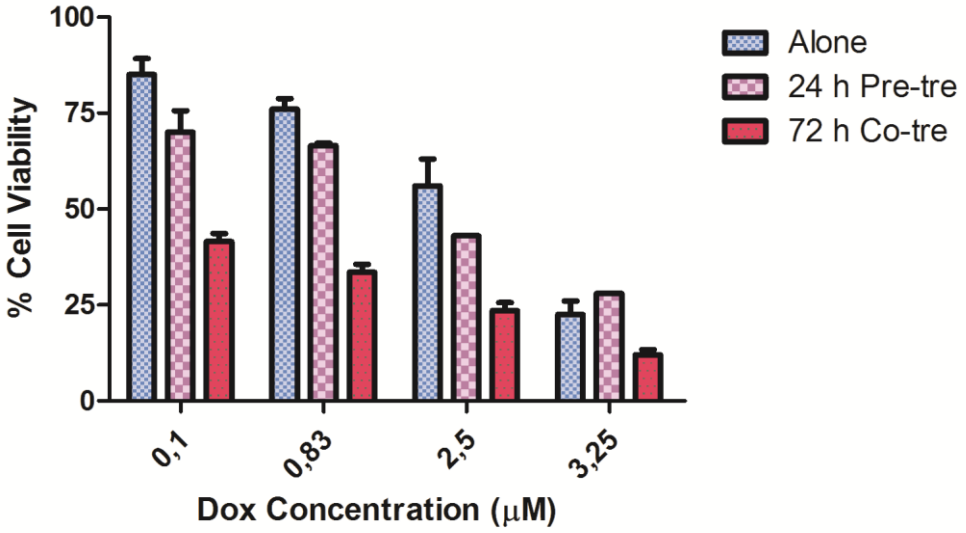

Figure 3.7 Effects of DOX alone (0.1, 0.83, 2.5, 3.25 μM) for 72 h, 10 μM EMO pre-tre for 24 h and EMO-DOX co-tre for 72 h, in applied concentrations on the cell survival of MCF-7 cells. Cytotoxicity was measured by XTT Assay.

Table 3.2 % Viability in MCF-7 cells obtained with XTT Assay after pre-tre of EMO (0.4, 4, 10 μM) for 24 h and DOX (0.1, 0.83, 2.5, 3.25 μM) for 72 h.

Parameter (Conc., μM)	**Cell Viability (%) ± SD***				
Dox 72h / Emo 24h	**0**	**0.1**	**0.83**	**2.5**	**3.25**
0	100	85 ± 2.82	76 ± 4.24	56 ± 7.07	26 ± 3.53
0.4	92 ± 3.53	88 ±10.6	80 ± 3.53	59 ± 2.12	30 ± 2.12
4	94 ± 2.12	91 ± 9.89	72 ± 7.07	48 ± 1.41	30 ± 2.12
10	94 ± 1.41	70 ± 5.65	67 ± 0.70	43 ± 2.82	28 ± 4.24

*SD was derived from two independent experiments. $P<0.05$, analyzed by two way ANOVA

MCF-7 cells were incubated for overnight then treated with 0.4, 4, 10 μM EMO for 24 h, respectively. Following EMO pre-tre for 24 h, EMO solution was aspirated and then the cells were treated with 0.1, 0.83, 2.5 and 3.25 μM DOX solution for 72 h. After 96 h total cell treatment, the cell proliferation was obtained with XTT Assay. The absorbance values were measured at 460 nm in ELISA plate reader. The percentage of cell viability in the control group was shown as 100%.

Emodin did not show any substantial effects alone in applied concentrations for 24 h (viability, >90%). However, DOX (3.25 μM) alone treatment for 72 h inhibited metabolic activity of MCF-7 more than 50%. Pre-tre of EMO (0.4, 4, 10 μM) for 24 h, followed by the addition of DOX solution (3.25 μM) for 72 h was the most effective treatment to inhibit cell proliferation for MCF-7, in applied concentrations. (% cell viability 26, 30, 30, 28). We also demonstrated that pre-tre of EMO (10 μM) for 24 h, followed by the addition of 0.1, 0.83, 2.5 μM DOX concentrations induced anti-proliferative effect of DOX in MCF-7 cells (% cell viability; 70, 67, 43).

0.1, 0.83, 2.5 μM DOX treatment for 72 h did not show an inhibitory effect higher than 50% in cell viability of MCF-7 cells. Moreover, none of the doses (0.4, 4, 10 μM) of EMO for 24 h was found to be cytotoxic to MCF-7 cells (92%, 94%, 94%).

From the cell viability results, it was concluded that, in applied concentrations, pre-treatment schedule of DOX given 72 h after EMO exposure for 24 h was found to induce cytotoxic effect in MCF-7 cells except the lowest concentration of EMO, that is 0.4 μM. Also, pre-tre of EMO for 24 h, in applied concentrations, was found to be ineffective before DOX (3.25 μM) exposure for 72 h.

Table 3.3 % Viability in MCF-7 cells obtained with XTT Assay after co-tre of EMO (0.4, 4, 10 μM) and DOX (0.1, 0.83, 2.5, 3.25 μM) for 72 h.

Parameter (Conc., μM)	**Cell Viability (%) ± SD***				
Dox 72h / **Emo 72h**	**0**	**0.1**	**0.83**	**2.5**	**3.25**
0	100	85 ± 2.82	76 ± 4.24	61 ± 7.07	26 ± 3.53
0.4	95 ± 2.82	43 ± 17.67	37 ± 9.89	25 ± 7.07	9 ± 0.70
4	96 ± 4.24	55 ± 1.41	41 ± 1.41	21 ± 3.53	10 ± 4.24
10	92 ± 7.07	47 ± 2.12	33 ± 2.12	23 ± 4.24	12 ± 5.05

*SD was derived from two independent experiments. $P<0.05$, analyzed by two way ANOVA

MCF-7 cells were incubated for overnight then treated with 0.4, 4, 10 μM EMO and 0.1, 0.83, 2.5, 3.25 μM DOX co-treatment for 72 h. After 72 h total cell treatment, the cells were obtained with XTT Assay. The absorbance values were measured at 460 nm in ELISA plate reader. The percentage of cell viability in the control group was shown as 100%.

As shown in Table 3.3, co-treatment group, in all applied concentrations of EMO and DOX for 72 h triggered growth inhibition in MCF-7 cells. Combined-treatment of these two compounds is more effective to reduce cell proliferation than 24 h pre-treatment of EMO concentrations in breast cancer cell model.

Table 3.4 The comparison effects of DOX (0.1, 0.83 μM) pre-tre for 24 h and EMO (0.4, 10 μM) pre-tre for 24 h on the growth of MCF-7 cells.

Parameter (Conc., μM)	**Cell Viability % ± SD***			
EMO	**0**	**0,4**	**10**	**DOX**
DOX pre 24 h	100	NA	NA	**0**
EMO pre 24 h	100	92 ± 3.53	94 ± 1.41	
DOX pre 24 h	86 ± 4.24	NA	**45 ± 5.05**	**0,1**
EMO pre 24 h	85 ± 2.82	88.5 ± 10.6	**70 ± 5.65**	
DOX pre 24 h	74 ± 5.05	**40 ± 2.12**	NA	**0,83**
EMO pre 24 h	61 ± 7.07	**80 ± 3.53**	67 ± 0,70	

NA: Not applicable,. $P<0.05$, analyzed by two way ANOVA

*SD was derived from two independent experiments.

MCF-7 cells were incubated for overnight then treated with 0.1, 0.83 μM DOX for 24 h. Following 24 h DOX pre-tre, DOX solutions were aspirated and then the cells were treated with 0.4 and 10 μM EMO for 72 h. After 96 h total cell treatment, MCF-7 cell viabilities were obtained with XTT Assay.

DOX pre-tre for 24 h results were compared to the results of EMO pre-tre for 24 h as shown in Table 3.4. Cell viability was decreased by 45% at 0.1 μM DOX pre-tre for 24 h. However, it remained 70% at 10 μM EMO pre-tre for 24 h.

After pre-tre of 0.4 µM EMO for 24 h, the percentage of cell viability was 80 % but; it was decreased by 40 % at 0.83 µM DOX pre-tre for 24 h.

In case of DOX (0.1, 0.83 µM) pre-tre for 24 h, the detrimental effects were increased further by 45% and 40% than the response received in cells exposed to EMO (0.4, 10 µM) pre-tre for 24 h (70%, 80%).

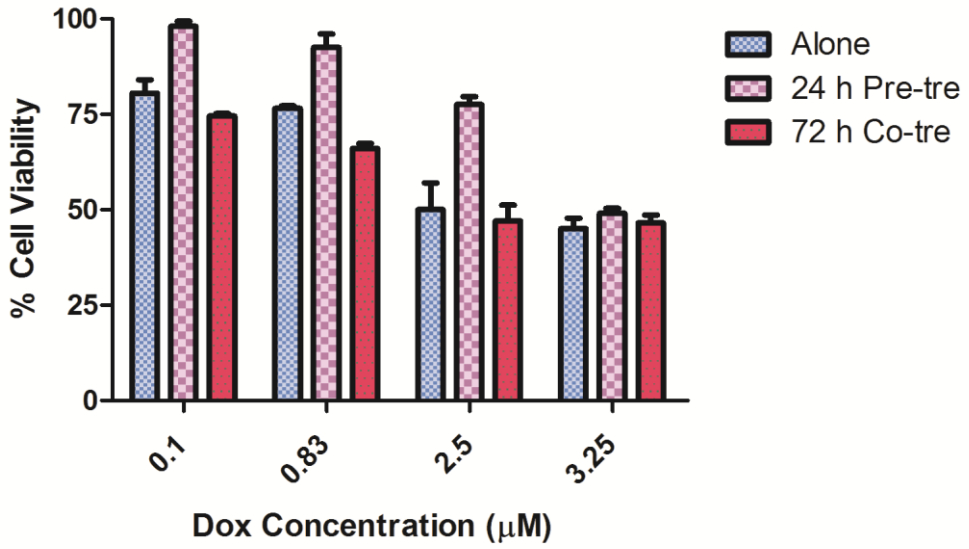

Figure 3.8 Effects of DOX alone (0.1, 0.83, 2.5, 3.25 μM) for 72 h, 0.4 µM EMO pre-tre for 24 h and EMO-DOX for 72 h, in applied concentrations on the cell survival of MCF-10A cells. Cytotoxicity was measured by XTT Assay.

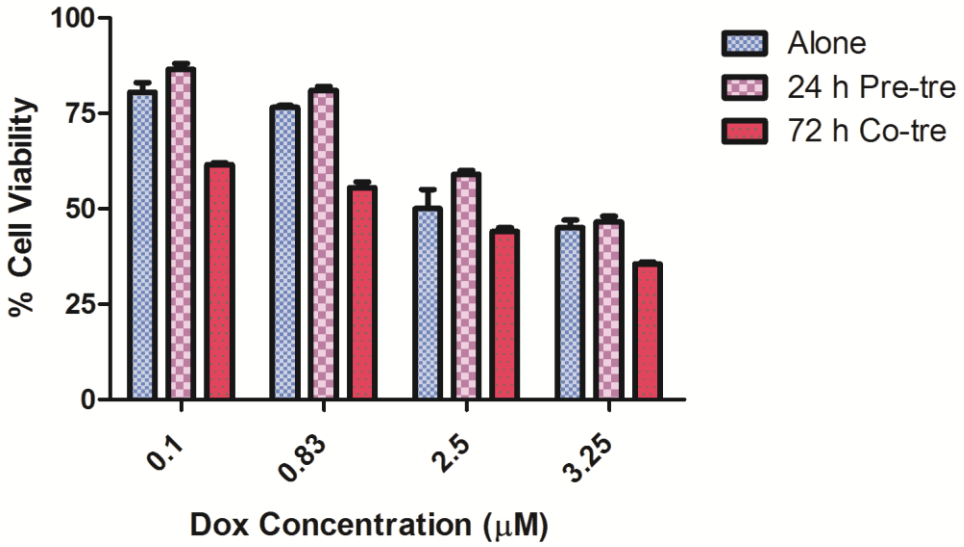

Figure 3.9 Effects of DOX alone (0.1, 0.83, 2.5, 3.25 μM) for 72 h, 4 μM EMO pre-tre for 24 h and EMO-DOX co-tre for 72 h, in applied concentrations on the cell survival of MCF-10A cells. Cytotoxicity was measured by XTT Assay.

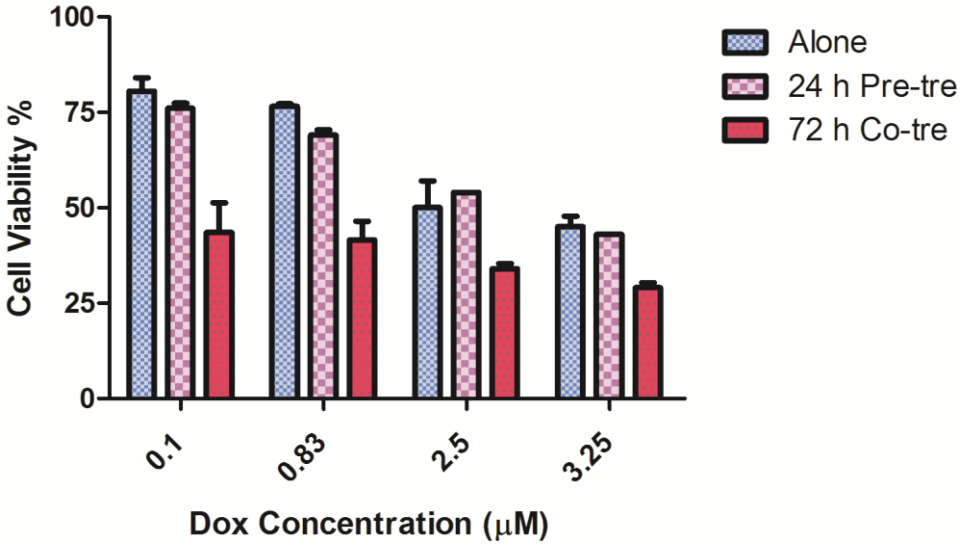

Figure 3.10 Effects of DOX alone (0.1, 0.83, 2.5, 3.25 μM) for 72 h, 10 μM EMO pre-tre for 24 h and EMO-DOX for 72 h, in applied concentrations on the cell survival of MCF-10A cells. Cytotoxicity was measured by XTT Assay.

Table 3.5 % Viability in MCF-10A cells obtained with XTT Assay after pre-tre of EMO (0.4, 4, 10 μM) for 24 h and DOX (0.1, 0.83, 2.5, 3.25 μM) for 72 h.

Parameter (Conc., μM)	**Cell Viability (%) ± SD***				
Dox 72h / Emo 24h	**0**	**0.1**	**0.83**	**2.5**	**3.25**
0	100	81 ± 3.53	76 ± 0.70	49 ± 7.07	44 ± 2.82
0.4	109 ± 4.24	97 ± 1.41	92 ± 3.53	76 ± 2.12	48 ± 4.24
4	100 ± 8.48	85 ± 2.12	82 ± 1.41	58 ± 1.41	46 ± 7.07
10	91 ± 3.53	76 ± 7.78	69 ± 1.41	54 ± 1.41	43 ± 3.53

*SD was derived from two independent experiments. $P<0.05$, analyzed by two way ANOVA

MCF-10A cells were incubated for overnight then treated with 0.4, 4, 10 μM EMO for 24 hours, respectively. Following EMO pre-tre for 24 h, EMO solution was aspirated and then the cells were treated with 0.1, 0.83, 2.5 and 3.25 μM DOX solution for 72 h. After 96 h total cell treatment, the cell proliferation was obtained with XTT Assay. The absorbance values were measured at 460 nm in ELISA plate reader. The percentage of cell viability in the control group was shown as 100%.

Emodin did not show any substantial effects alone in applied concentrations for 24 h (viability, >90%). However, DOX (2.5 and 3.25 μM) alone treatment for 72 h inhibited metabolic activity of MCF-10A cells more than 50%. Pre-tre of EMO (0.4 μM) for 24 h, followed by the addition of DOX concentrations (0.1, 0.83, 2.5, 3.25 μM) for 72 h was the most effective treatment to protect cell proliferation against DOX cell toxicity (% cell viability 109, 97, 92, 76, 48), in applied concentrations for MCF-10A.

Additionally, pre-tre of 4 μM EMO for 24 h enhanced cell proliferation but not as much as 0.4 μM EMO pre-tre for 24 h (% cell viability 100, 85, 82, 58, 46).

We also demonstrated that pre-tre of EMO (10 μM) for 24 h followed by addition all the doses of DOX for 72 h was found to be ineffective against DOX cytotoxicity in MCF-10A cells (% cell viability 76, 69, 54, 43 %).

Table 3.6 % Viability in MCF-10A cells obtained with XTT Assay after co-tre of EMO (0.4, 4, 10 μM) and DOX (0.1, 0.83, 2.5, 3.25 μM) for 72 h.

Parameter (Conc., μM)	**Cell Viability (%) ± SD***				
Dox 72h / Emo 72h	**0**	**0.1**	**0.83**	**2.5**	**3.25**
0	100	83 ± 3.53	76 ± 0.70	50 ± 7.07	44 ± 2.82
0.4	105 ± 3.53	74 ± 0.70	66 ± 1.41	47 ± 4.24	46 ± 2.12
4	65 ± 8.48	61 ± 0.70	55 ± 2.12	44 ± 1.41	35 ± 0.70
10	59 ± 7.07	43 ± 7.78	41 ± 4.95	34 ± 1.41	29 ± 3.53

*SD was derived from two independent experiments. $P<0.05$, analyzed by two way ANOVA

MCF-10A cells were incubated for overnight then treated with EMO (0.4, 4, 10 μM) and DOX (0.1, 0.83, 2.5 and 3.25 μM) co-tre for 72 h. After 72 h treatment, cells were obtained with XTT Assay. The absorbance values were measured at 460 nm in ELISA plate reader. The percentage of cell viability in the control group was shown as 100%.

As shown in Table 3.6, in case of combined treatment of EMO and DOX, in applied concentrations, the detrimental effects of DOX were significantly increased in MCF-10A cells.

Table 3.7 The comparison effects of DOX (0.1, 0.83 μM) pre-tre for 24 h and EMO (0.4, 10 μM) pre-tre for 24 h on the growth of MCF-10A cells.

Parameter (Conc., μM)	**Cell Viability % ± SD***			
EMO	**0**	**0,4**	**10**	**DOX**
DOX pre 24 h	100	105 ± 3.53	59 ± 7,07	**0**
EMO pre 24 h	100	109 ± 3,53	91 ± 3,53	
DOX pre 24 h	97 ± 7.78	NA	**50 ± 5.05**	**0,1**
EMO pre 24 h	83 ± 3.53	97 ± 1.41	**76 ± 7.78**	
DOX pre 24 h	84 ± 7.78	**66 ± 1.41**	NA	**0,83**
EMO pre 24 h	76 ± 0.70	**92 ± 3.53**	69 ± 1.41	

NA: Not applicable. $P<0.05$, analyzed by two way ANOVA

*SD was derived from two independent experiments.

MCF-10A cells were incubated for overnight then treated with 0.1 and 0.83 μM DOX for 24 h. Following DOX pre-tre for 24 h, DOX concentrations were aspirated and then cells were treated with 0.4 and 10 μM EMO for 72 h. After 96 hours total cell treatment, MCF-10A cells were obtained with XTT Assay.

DOX pre-tre results were compared to the results of EMO pre-tre for 24 h as shown in Table 3.7 in MCF-10A cells. In pre-treatment schedule of 0.1 μM DOX given 24 h before 10 μM EMO exposure for 72 h, cell viability was recorded as 50% in MCF-10A cells. However, it remained close to 80% at 10 μM EMO pre-tre for 24 h, before 0.1 μM DOX exposure for 72 h.

In pre-tre schedule of 0.4 μM EMO given 24 h before 0.83 μM DOX exposure for 72 h, cell viability was 92 % in MCF-10A cells, but it was decreased by 66% at 0.83 μM DOX pre-tre for 24 h.

Of important note is that, in case of DOX pre-tre for 24 h, in applied concentrations, was found to show induce cell toxicity in MCF-10A cells.

Table 3.8 Overall data of % Cell Viability in MCF-7 and MCF-10A cells obtained with XTT Assay after alone (A) DOX and EMO treatment for 72 h, pre-tre (Pre) of EMO (0.4, 4, 10 μM) for 24 h and co-tre (Co) of EMO (0.4, 4, 10 μM) and DOX (0.1, 0.83, 2.5, 3.25 μM) for 72 h.

Pre-tre 24 h Co-tre 72 h		**Cell Viability % ± SD***					
DOX		**0**	**0,1**	**0,83**	**2,5**	**3,25**	**EMO**
MCF-7	A	100	85 ± 2.82	76 ± 4.24	61 ± 7.07	26 ± 3.53	**0**
MCF-10A	A	100	83 ± 3.53	76 ± 0.70	49 ± 7.07	44 ± 2.82	
MCF-7	Pre		88 ±10.6	80 ± 3.53	59 ± 2.12	30 ± 2.12	
	Co	95 ± 2.82	43 ± 17.67	37 ± 9.89	25 ± 7.07	9 ± 0.70	
MCF-10A	Pre		97 ± 1.41	92 ± 3.53	76 ± 2.12	48 ± 4.24	**0,4**
	Co	105 ±3.53	74 ± 0.70	66 ± 1.41	47 ± 4.24	46 ± 2.12	
MCF-7	Pre		91 ± 9.89	72 ± 7.07	48 ± 1.41	30 ± 2.12	
	Co	96 ± 4.24	55 ± 1.41	41 ± 1.41	21 ± 3.53	10 ± 4.24	
MCF-10A	Pre		85 ± 2.12	82 ± 1.41	58 ± 1.41	46 ± 7.07	**4**
	Co	65 ± 8.48	61 ± 0.70	55 ± 2.12	44 ± 1.41	35 ± 0.70	
MCF-7	Pre		70 ± 5.65	67 ± 0.70	43 ± 2.82	28 ± 4.24	
	Co	92 ± 7.07	47 ± 2.12	33 ± 2.12	23 ± 4.24	12 ± 5.05	**10**
MCF-10A	Pre		76 ± 7.78	69 ± 1.41	54 ± 1.41	43 ± 3.53	
	Co	59 ± 7.07	43 ± 7.78	41 ± 4.95	34 ± 1.41	29 ± 3.53	

*SD was derived from two independent experiments. $P<0.05$, analyzed by two way ANOVA

Obviously, the overall data indicates that when phytoestrogen EMO applied with chemotherapeutic drug DOX for 72 h, in applied concentrations, a dose dependent increase in the intensity of the damage was observed. The cytotoxicity in MCF-7 and MCF-10A cells from DOX and EMO co-tre (at a concentration range of 0.4-10 μM for EMO, 0.1-3.25 μM for DOX) was higher than that from DOX and EMO alone for 72 h and EMO pre-tre for 24 h for both cell lines.

Xin-zhi Huang proved that co-treating DU-145 (human prostate carcinoma cells), with DOX (1,66 μM) and EMO (50 μM) caused an enhancement of viability repression induced by DOX at these concentrations. Also, at high EMO concentrations, ROS measurements showed that co-treatment of CDDP 10 μg/ml (chemotherapeutic agent) with EMO (50 μM) caused to increase of ROS generation in prostate carcinoma cells (Xin-zhi Huang, 2008). Another study demonstrated that using EMO (40, 80 μM) as an adjuvant therapy with anti cancer drugs may trigger the achievement of the treatment and chemotherapeutic drug gemcitabine can be given as an example to this type of therapy. It was showed that the co-treatment of EMO and gemcitabine inhibited the cell proliferation in Panc-1, pancreatic cancer cells, compared to gemcitabine treatment (72.4% and 54.7%) (Sheng Zang Lin, 2012).

As shown in Tables 3.4-7, the decrease in percent cell viability of DOX pre-treated group for 24 h was observed compared to the EMO-pre treated group for 24 h and co-treated group for 72 h in both cell lines. The increase in percent cell viability of 0.4 μM EMO pre-treated group was observed in MCF-10A cells. I.Wally showed that 1 μM cisplatin (CCDP, chemotherapeutic drug) in the presence of 0.5 μM EMO induced cell proliferation in human embriyonic kidney cells (HEK-293) as compared to cisplatin treated cells (I. Wally, 2012).

We also showed that a dose dependent increase of EMO caused to decrease cell viability in the both pre-treated and co-treated group in MCF-7 and MCF-10A cells. In other studies, EMO (40, 120 μM) has been demonstrated to inhibit cell viability in human kidney (HK-2) cells and has anti-proliferative actions on many cell lines (Wang et. al., 2007). It has also facilitated apoptosis in HeLa cells induced by arsenic trioxide (As_2O_3) (Yi J, 2004).

According to our XTT results, we decided to choose 2.5 μM DOX and 0.4 μM EMO concentrations for our further experiments. Because 0.4 μM EMO, at this concentration, was not toxic to both MCF-7 and MCF-10A cells and 2.5 μM DOX for 72 h reduced the cell viability by 61% in MCF-7 cells and 50% in MCF-10A.

3.1.2 Viable Cell Countig with Trypan Blue (TBE)

In order to elude absorbance mixing of DOX and EMO solutions on XTT assay, trypan blue staining was applied. MCF-7 and MCF-10A cells (1×10^5 cells/ml; 1 ml/well ; 6 well-plate) were either left untreated (control) or treated with EMO (0.4 μM) and DOX (2.5 μM) for 24 h pre-tre and 72 h (alone, co-tre) as described in 2.2.8 XTT assay.

The effects of EMO (0.4 μM) and DOX (2.5 μM) alone for 24 h, pre-tre and co-tre for 72 h on the cell growth and cell viabilities of MCF-7 and MCF-10A were detected by trypan blue counting. Averages of viable cell counts were shown as a vertical column bar graphs (Figure 3.11-12).

After trypan blue staining, cell viabilities of control cells were arranged as 100 %. Control cells were grown in 0.1% DMSO containing complete medium.

The columns indicate the average of dublicate measurements from two biological replicates. Graphs were drawn using Graph Pad Prism program.

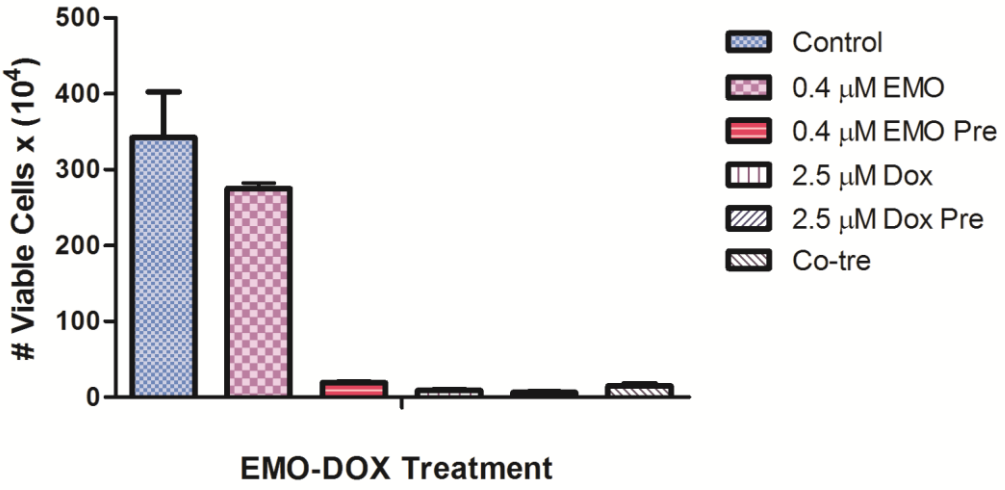

Figure 3.11 Effects of alone tre, pre-tre and co-tre of DOX (2.5 μM) and EMO (0.4 μM) on cell survival in MCF-7 cells acquired by the TBE Assay. MCF-7 cells were incubated for overnight and then treated with DOX and/or EMO for 24 h and 72 h.

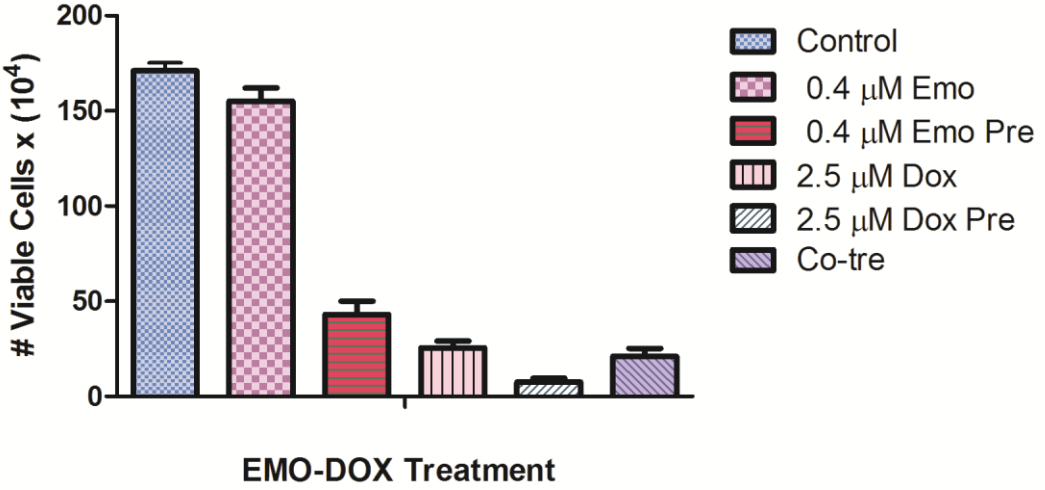

Figure 3.12 Effects of alone tre, pre-tre and co-tre of DOX (2.5 μM) and EMO (0.4 μM) on cell survival in MCF-10A cells acquired by the TBE Assay. MCF-7 cells were incubated for overnight and then treated with DOX and/or EMO for 24 h and 72 h.

The effects of DOX and EMO on viabilities of MCF-7 and MCF-10A cells were also shown in Table 3.9

Table 3.9 Effects of alone, pre-tre, and co-tre of DOX (2.5 μM) and EMO (0.4 μM) on viabilities of MCF-7 and MCF-10A cells for 24 h and 72 h. Viable cell counts were recorded after trypan blue staining.

% Cell Viability ± SD*				
	MCF-7		MCF-10A	
Paremeter (μM)	2.5 μM DOX	0.4 μM EMO	2.5μM DOX	0.4 μM EMO
Control	100	100	100	100
Alone	3.38 ± 2.82	90.50 ± 7.07	14.91 ± 3.53	90.64 ± 7.07
Pre-tre	2.37± 4.24	4.7 ± 5.65	4.38 ± 2.12	27.14 ± 7.07
Co-tre	6.10 ±2.82		12.28 ± 5.65	

*SD was derived from two independent experiments. $P<0.05$, analyzed by two way ANOVA

These results showed that EMO did not show any substantial effect alone in applied concentration for 72 h (viability, >90 %) in both MCF-7 and MCF-10A cells. DOX (2.5 μM) alone treatment for 72 h inhibited metabolic activity of MCF-7 and MCF-10A cells more than 50 % (3.38 %, 14.9 1% respectively). Moreover, 24 h pre-tre of DOX induced cytotoxicity compared to DOX-alone treatment in both cell lines (2.37 %, 4.38 %). However, cytotoxicity of DOX was lowered more than one half by EMO-pre tre in MCF-10A cells which indicating protective effect, but there was not a significant change in MCF-7 cell viability with 24 h EMO pre tre (27.14 %, 4.7 %, respectively). Co-tre of EMO and DOX for 72 h also induced cell viabilities of both MCF-7 and MCF-10A cells (6.10 %, 12.28 %, respectively).

Considering these results, in applied concentrations, EMO exposure for 24 h before DOX treatment reduce cytotoxic effects of chemotherapeutic agent DOX in healthy breast cell model, MCF-10A while it is not effective in breast cancer cell model, MCF-7. Waly et. al. demonstrated that EMO treatment at concentration as low as 0.5 μM did not induce cell death for HEK 293 (Human Embriyonic Kidney 293) cells. EMO also showed protective effect against chemotherapeutic agent cisplatin induced cytotoxicity in HEK 293 (Human Embriyonic Kidney 293) cells (Waly et. al., 2013).

However; EMO exposure after DOX treatment did not show any protective effects of in both cell lines; and also EMO and DOX exposure together caused to induce cell toxicity in both breast cell models. EMO was also proved to show anti-cancer activities and inhibit cell growth at higher concentrations in MCF-7 cells (Sakalli E., M.Sc. Thesis, METU 2011). Recent studies demonstrated that the effect of higher concentrations of EMO (10, 30, 50, 80, 100 μg/ml) inhibited the cell proliferation in a dose dependent manner in IL-6 (Interleukin-6) kidney fibroblasts (Wang et. al., 2007).

3.1.3 Light Microscopic Analysis of Cell Morphology

The morphological changes and viabilities of MCF-7 and MCF-10A cells treated with EMO and/or DOX were observed by inverted microscope as displayed in Figure 3.13 and 3.14. Both cell lines (1×10^5 cells/ml; 1 ml/well, 6 well-plate) were either left untreated (control) or treated with 0.4 μM EMO and/or 2.5 μM DOX for 24 h and 72 h.

After incubation with DOX alone, EMO pre-tre, DOX pre-tre and DOX-EMO co-tre, the morphological changes and growth inhibition in MCF-7 cells were illustrated (Figure 3.13, b, d, e, f). Compared to control cells, shape elongation and decreased cell growth was observed clearly. Control and EMO (0.4 μM) alone treated MCF-7 cells for 72 h were round in normal shape (Figure 3.13, a, c).

Chen et. al. demonstrated that DOX (0.1 μM) treated MCF-7 cells for 48 h, showed a significant cell aggregation and formed short processes. Continuous exposure to different concentrations of DOX induced morphological alterations (Chen et. al., 2002).

DOX induced morphological changes and caused cytotoxicity in MCF-10A cells (Figure 3.14, d, e). However, EMO pre-tre for 24 h in MCF-10A cells caused a significant increase in cell viability and form stable cell shape (Figure 3.14, b), compared to DOX alone, DOX pre-tre and EMO-DOX co-tre (Figure 3.14, d, e, f).

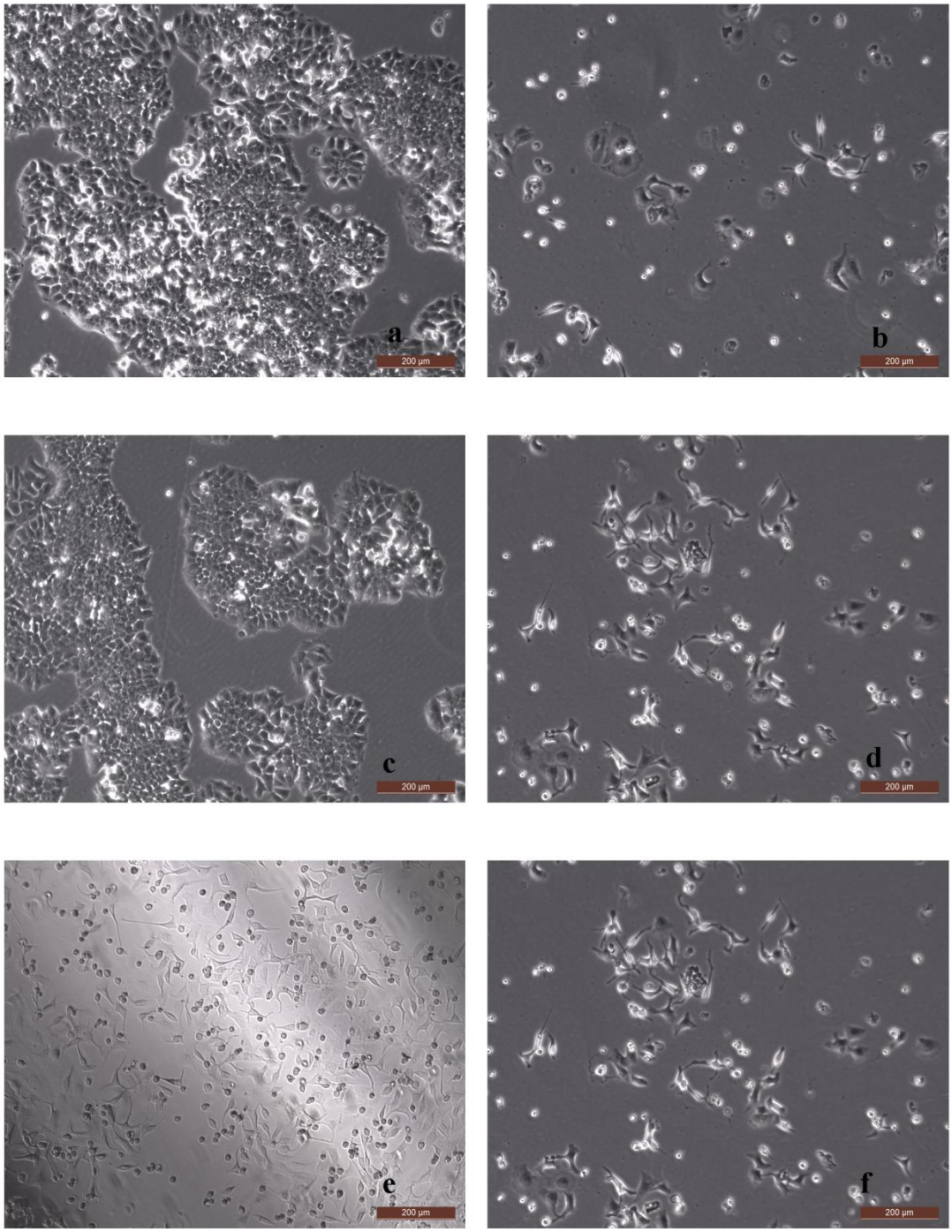

Figure 3.13 Light microscopic analysis of EMO and DOX treated or untreated MCF-7 cells on morphological and cell growth changes for 24h and 72h. a) Control b) 0.4 µM EMO pre-tre for 24 h c) 0.4 µM EMO for 72 h d) 2.5 µM DOX pre-tre for 24 h e) 2.5 µM DOX for 72 h f) 2.5 µM DOX+0.4 µM EMO co-tre for 72 h. The cells were photographed under inverted microscope (DM IL LED, Leica Microsystems, magnification 100X, bar: 200 µm).

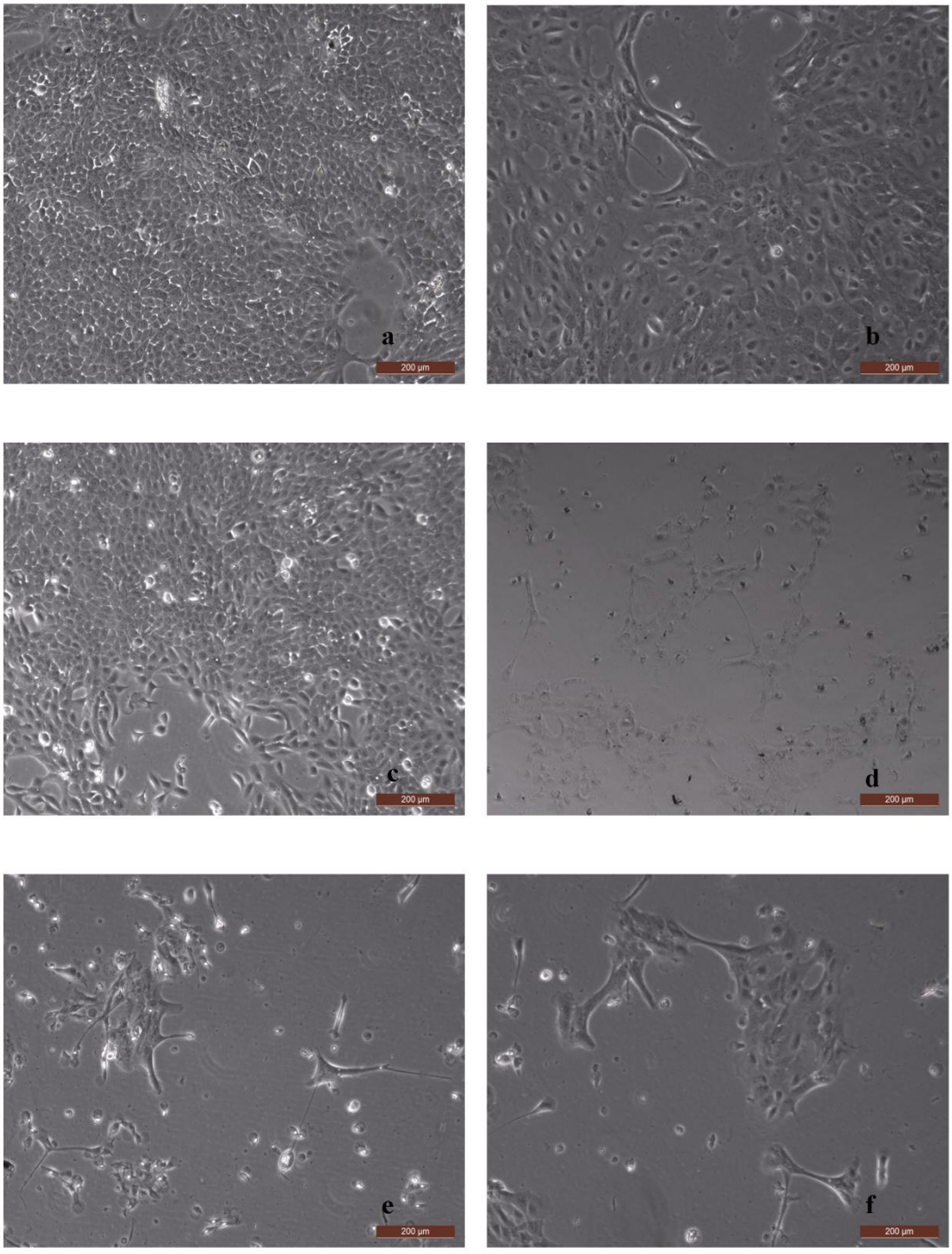

Figure 3.14 Light microscopic analysis of EMO and DOX treated or untreated MCF-10A cells on morphological and cell growth changes for 24h and 72h. a) Control b) 0.4 µM EMO pre-tre for 24 h c) 0.4 µM EMO for 72 h d) 2.5 µM DOX pre-tre for 24 h e) 2.5 µM DOX for 72 h f) 2.5 µM DOX+0.4 µM EMO co-tre for 72 h. The cells were photographed under inverted microscope (DM IL LED, Leica Microsystems, magnification 100X, bar: 200 µm).

3.1.4 DAPI (4',6-diamidino-2-phenylindole) Staining Assay

The fluorescent stain DAPI visualizes nuclei in living cells. Following light microscopic analysis, DAPI staining was used to determine the changes in the number of nuclei and to define cell morphology after EMO and/or DOX treatment of MCF-7 and MCF-10A cells (Figure 3.15-16).

The results of DAPI staining were correlated with light microscopic analysis. Cell growth inhibition in MCF-7 was observed when cells were treated with 0.4 μM EMO pre-tre for 24 h, 2.5 μM DOX alone for 72 h, 2.5 μM DOX pre-tre for 24 h and 2.5 μM DOX-0.4 μM EMO co-tre for 72 h (Figure 3.15, b, d, e, f). DOX-treated cells' nuclei were observed as elongated in MCF-7 cells (Figure 3.15, d, e).

2.5 μM DOX alone treatment for 72 h also induced cytotoxicity in MCF-10A cells (Figure 3.16, e) but, 0.4 μM EMO treatment for 72 h in MCF-10A cells did not cause any decrease in cell viability, compared to 2.5 μM DOX alone treatment (Figure 3.16, c, e). The lowest cell viability was observed at 2.5 μM DOX pre-tre for 24 h and 2.5 μM DOX-0.4 μM EMO co-tre for 72 h in MCF-10A cells (Figure 3.16, d, f).

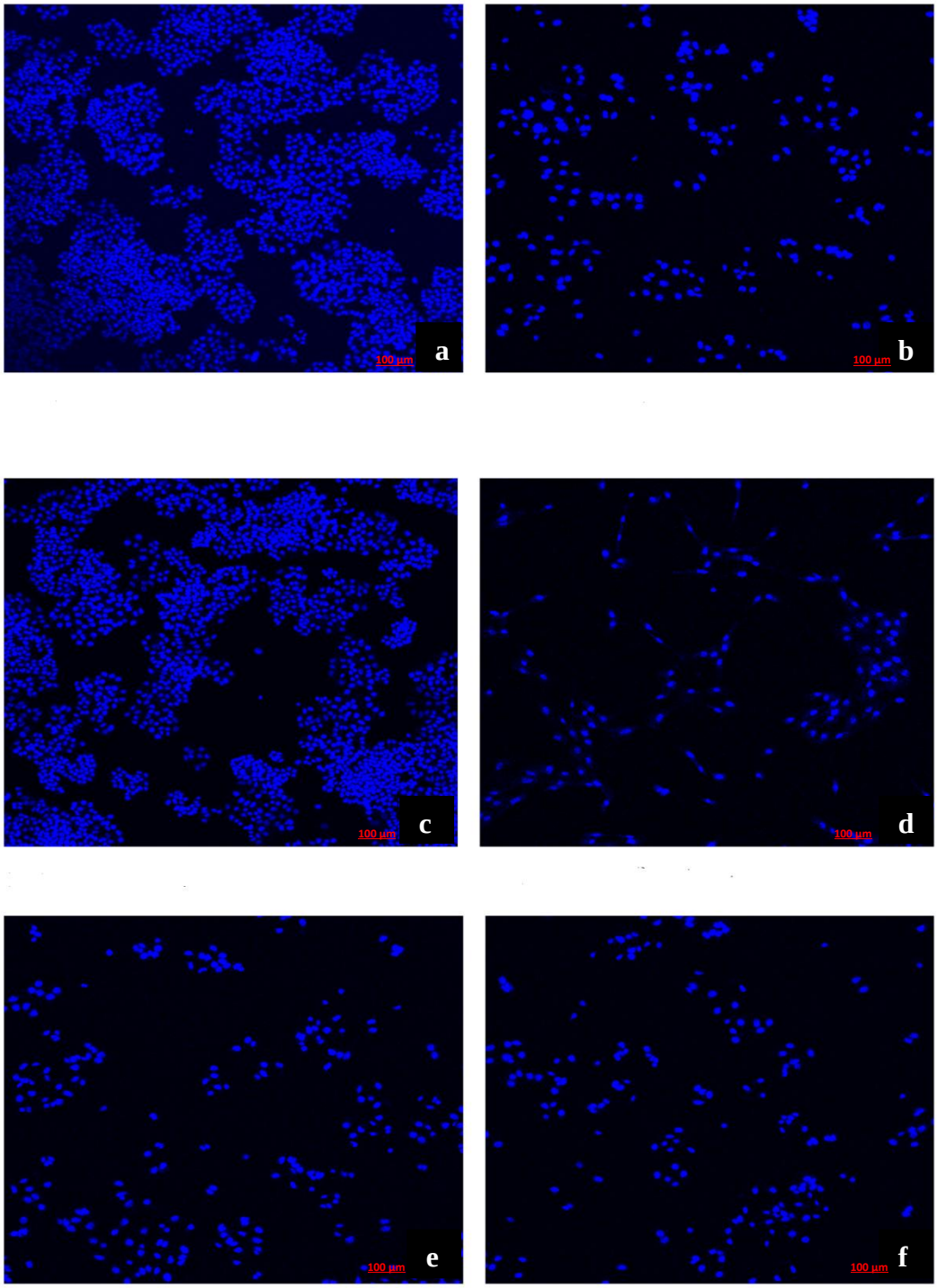

Figure 3.15 Fluorescence microscope cell nucleus analysis of EMO and DOX treated MCF-7 cells for 24 h and 72 h. a) Control b) 0.4 µM EMO pre-tre for 24 h c) 0.4 µM EMO for 72 h d) 2.5 µM DOX pre-tre for 24 h e) 2.5 µM DOX for 72 h f) 2.5 µM DOX+0.4 µM EMO co-tre for 72 h. The cells were photographed under confocal microscope (A1R+/A1+, Nikon Instruments, Inc. magnification 100X, bar: 100 µm).

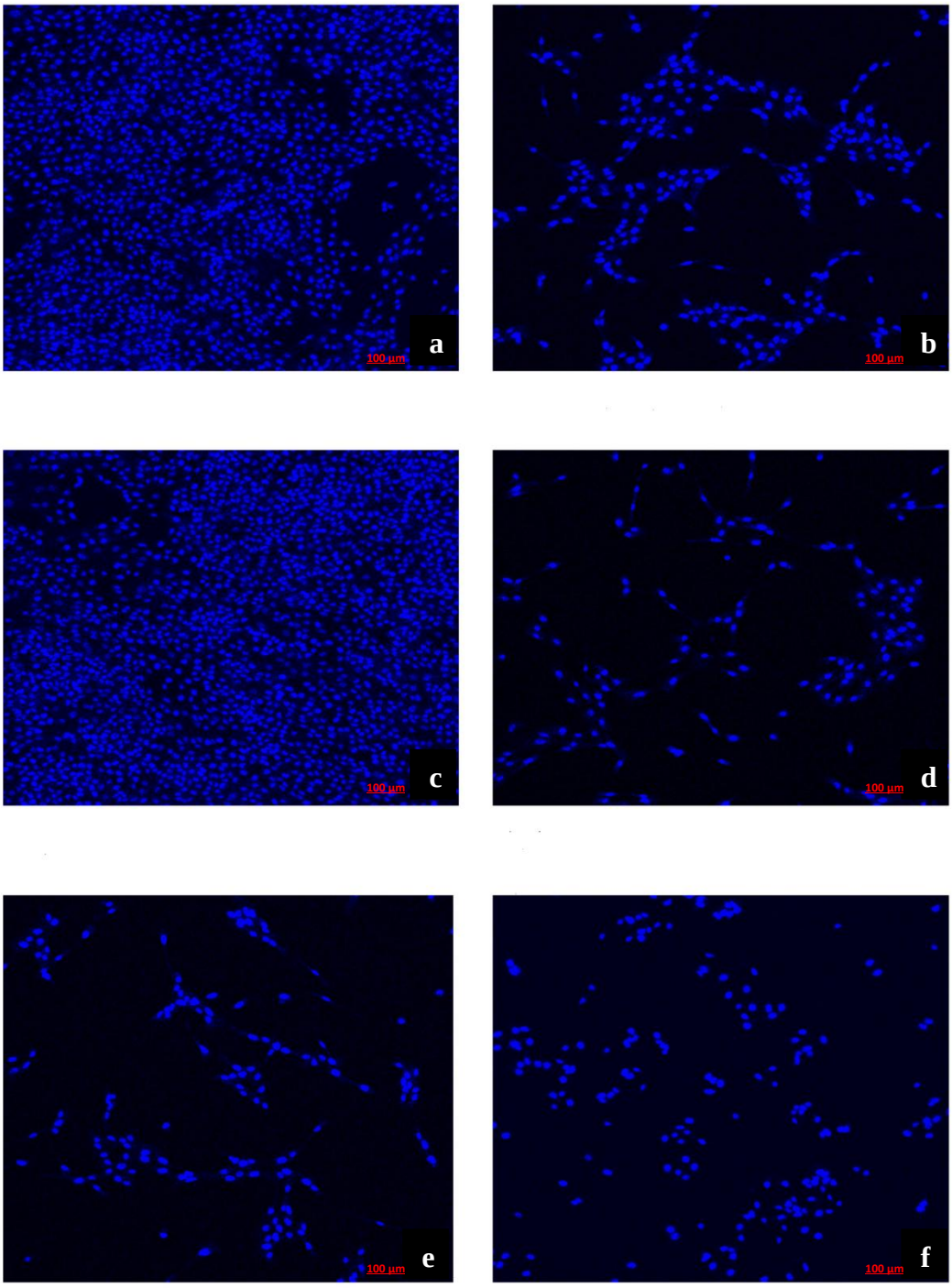

Figure 3.16 Fluorescence microscope cell nucleus analysis of EMO and DOX treated MCF-7 cells for 24 h and 72 h. a) Control b) 0.4 µM EMO pre-tre for 24 h c) 0.4 µM EMO for 72 h d) 2.5 µM DOX pre-tre for 24 h e) 2.5 µM DOX for 72 h f) 2.5 µM DOX+0.4 µM EMO co-tre for 72 h. The cells were photographed under confocal microscope (A1R+/A1+, Nikon Instruments, Inc. magnification 100X, bar: 100 µm).

3.2 Apoptotic Cell Death Analysis

3.2.1 *Dose and Time Kinetics for Emodin and Doxorubicin induction of Apoptosis*

Apoptotic cell death analysis was performed to search the induction of apoptosis by EMO and DOX in dose and time dependent manner. The cells were dual-stained with Annexin-V and 7-AAD. Co-staining with 7-AAD provide to display necrotic cells.

In this study, MCF-7 and MCF-10A cells were treated by 0.4 μM EMO and/or 2.5 μM DOX (pre-tre for 24 h, co-tre for 72 h and alone for 72 h). After treatment, cells were stained with annexin V-apc and 7-AAD. We detected live cells, non-apoptotic cells (annexin V and 7AAD negative), early apoptotic cells (annexin V positive) and necrotic or death cells (annexin V and 7-AAD positive) by flow cytometry.

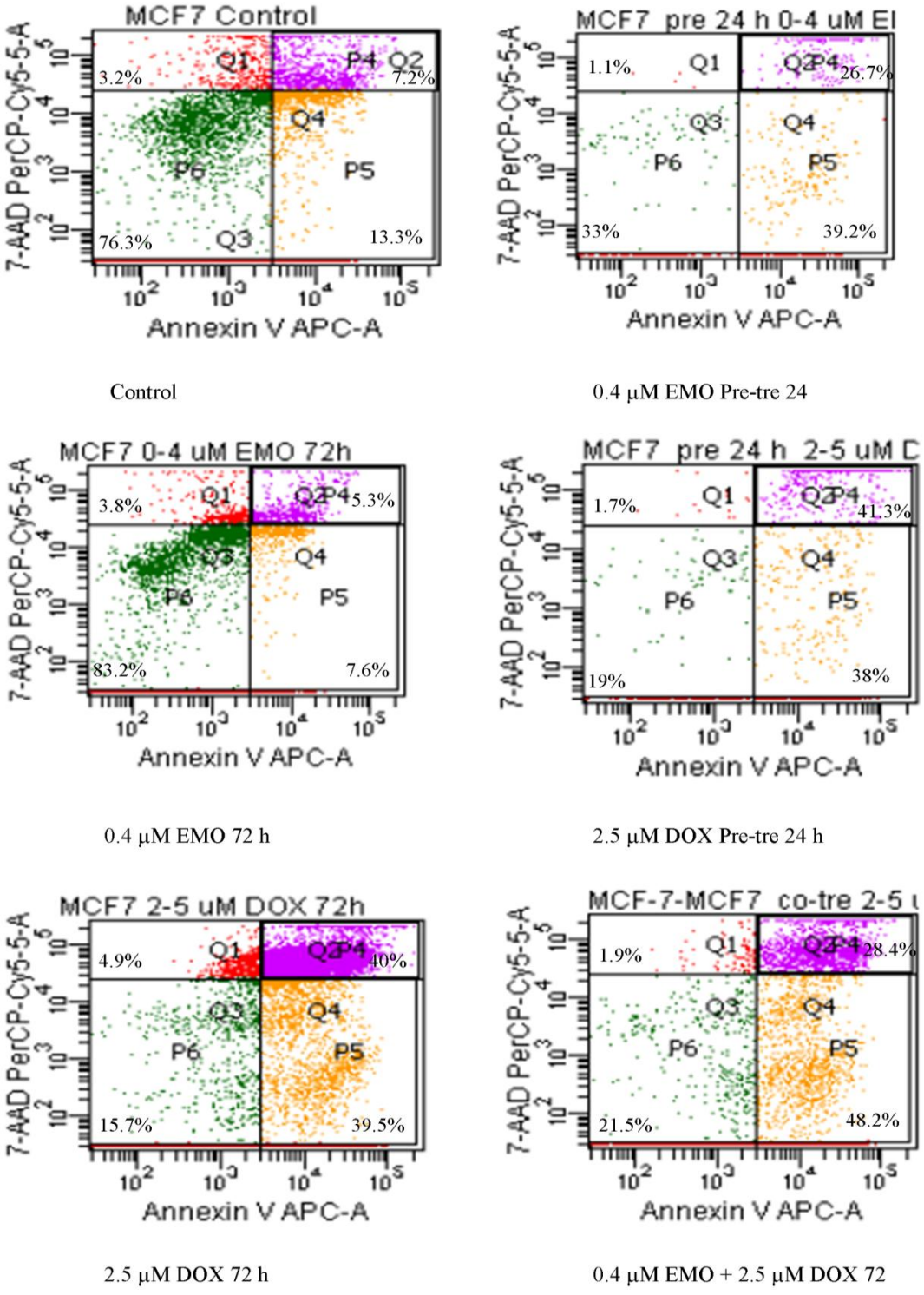

Figure 3.17 0.4 µM EMO and/or 2.5 µM DOX induced apoptosis in MCF-7 cells. FACS analysis of either untreated (control) or treated with EMO (0.4 µM) and/or DOX (2.5 µM) for 24 h, 72 h (alone, pre-tre, co-tre)

After dyeing with Annexin V-apc and 7-AAD, cells visible in the lower right quadrant display positive Annexin V-apc staining that define living cells, no DNA staining with 7-AAD showing early apoptotic cells. Necrotic or late apoptotic cells show up in the upper right section.

As shown in Figure 3.17, after exposure of MCF-7 cells to 0.4 μM EMO alone for 72 h and 2.5 μM DOX alone for 72 h, viabilities were observed as 83.2 % and 15.7 %, respectively in the cell population of live cells stage.

In the pre-treated group, after exposure of MCF-7 cells to 0.4 μM EMO for 24 h prior to 2.5 μM DOX treatment for 72 h and exposure of 2.5 μM DOX for 24 h prior to 0.4 μM EMO for 72 h (96 h total treatment for both group), viabilities were observed as 33 % and 19 %, respectively in the cell population of live cells stage.

In the co-treated group, after exposure of MCF-7 cells to 0.4 μM EMO and 2.5 μM DOX co-tre for 72 h, cell viability was 21.5 % in the cell population of live cells stage.

The maximal apoptosis was observed in 2.5 μM DOX-pre treated for 24 h and DOX alone treated MCF-7 cells for 72 h (% viability, 19 and 15.7 respectively).

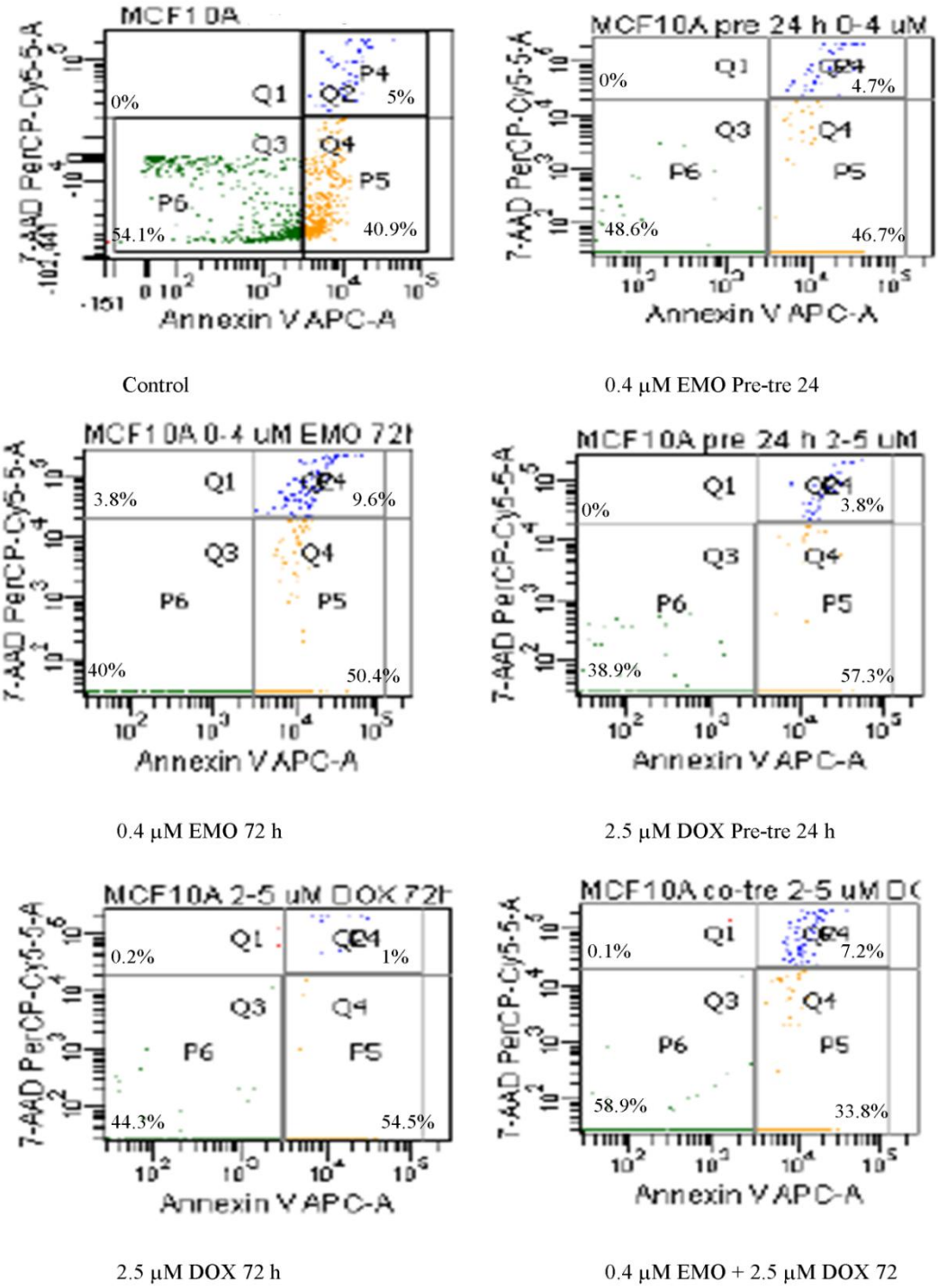

Figure 3.18 0.4 µM EMO and/or 2.5 µM DOX induced apoptosis in MCF-10A cells. FACS analysis of either untreated (control) or treated with EMO (0.4 µM) and/or DOX (2.5 µM) for 24 h, 72 h (alone, pre-tre, co-tre).

As shown in Figure 3.18, after exposure of MCF-10A cells to 0.4 μM EMO alone for 72 h and 2.5 μM DOX alone for 72 h, cell viabilities were observed as 40 % and 44.3 %, respectively in the cell population of live cells stage.

In the pre-treated group, after exposure of MCF-10A cells to 0.4 μM EMO for 24 h prior to 2.5 μM DOX treatment for 72 h and exposure of 2.5 μM DOX for 24 h prior to 0.4 μM EMO for 72 h (96 h total treatment for both group), viabilities were observed as 48.6 % and 38.9 %, respectively, in the cell population of live cells stage.

In the co-treated group, after exposure of MCF-10A cells to 0.4 μM EMO and 2.5 μM DOX co-tre for 72 h, cell viability was 58.9 %, respectively in the cell population of live cells stage.

The maximal apoptosis was observed in 2.5 μM DOX-pre treated for 24 h and DOX alone treated MCF-10A cells for 72 h (% viability 38.9 and 44.3 respectively).

3.2.2 *Detection of Mitochondrial Membrane Potential ($\Delta\psi_m$) Changes in MCF-7 and MCF-10A Cells*

In this study, MCF-7 and MCF-10A cells were treated by 0.4 μM EMO and/or 2.5 μM DOX (pre-tre, co-tre and alone treatment) for 24 and 72 hours, then cells were stained with JC-1 to identify mitochondrial membrane potential by flow cytometry. After staining with JC-1 dye, cells appearing in the upper section indicates normal mitochondrial membrane potential while cells in the lower section indicates loss of mitochondrial membrane potential.

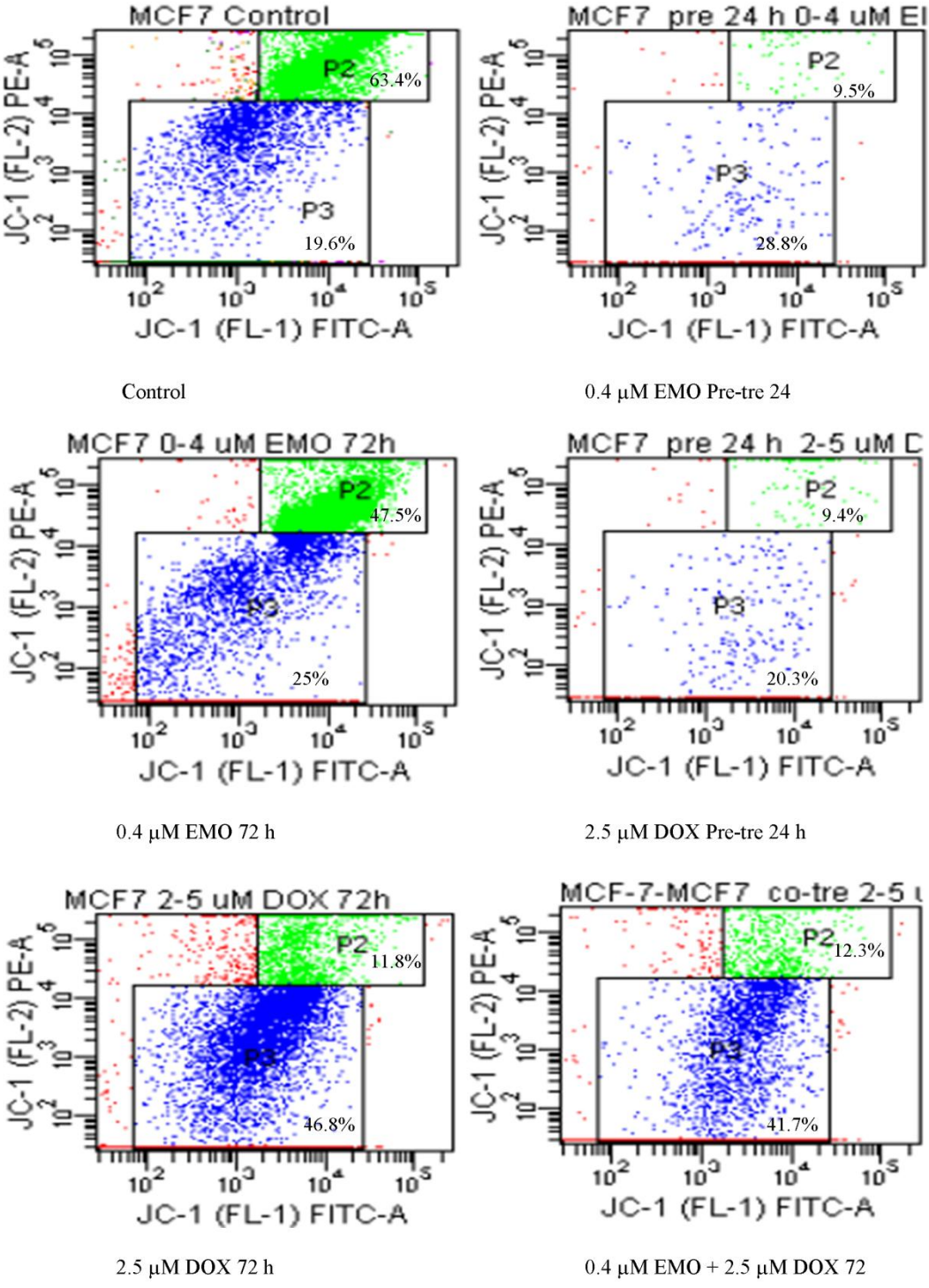

Figure 3.19 EMO and/or DOX induced alterations in mitochondrial membrane potential ($\Delta\psi_m$) in MCF-7 cells. FACS analysis of either untreated or treated with EMO (0.4 µM) and/or DOX (2.5 µM) for 24 h, 72 h (alone treatment, pre-tre, co-tre).

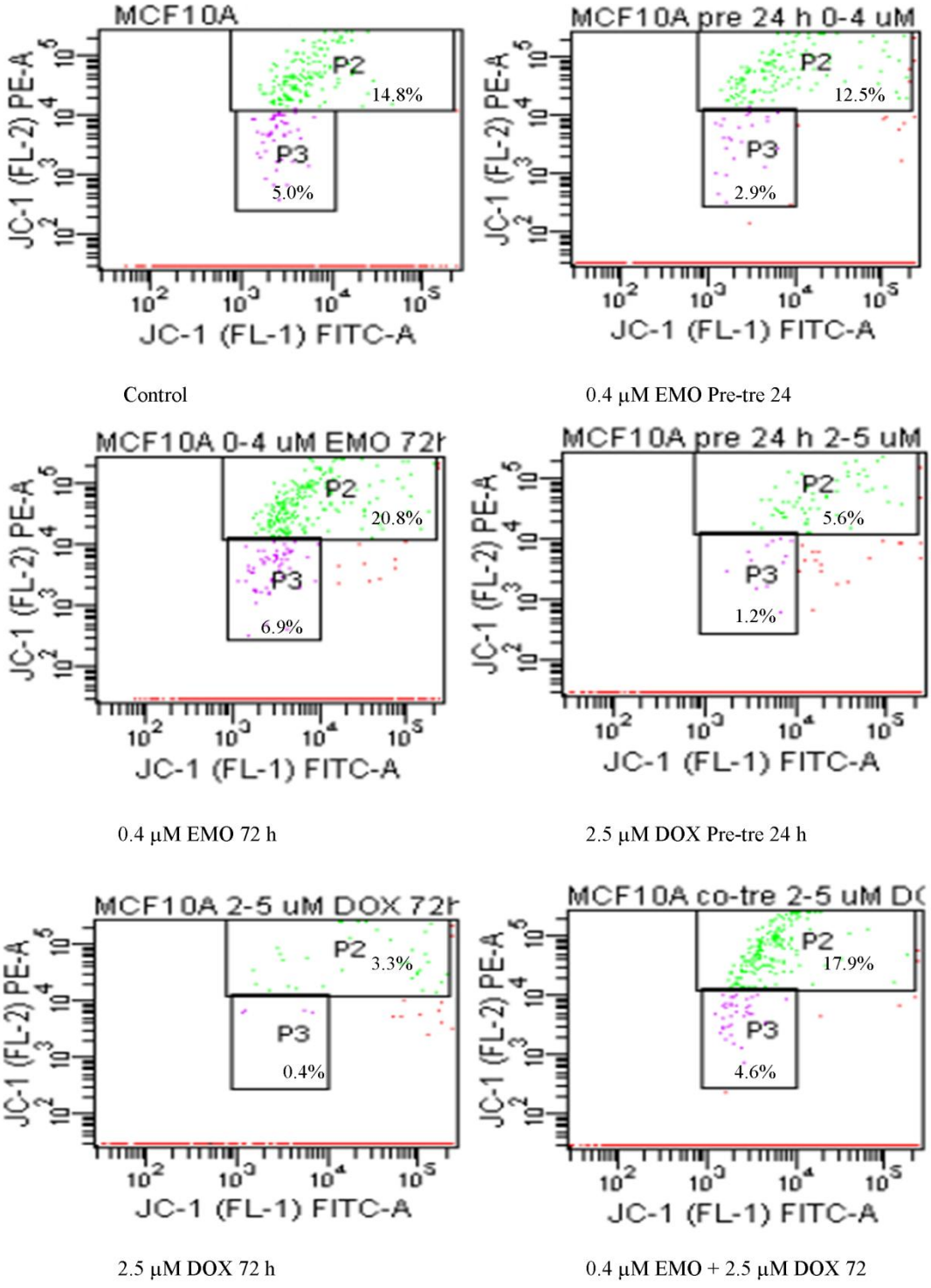

Figure 3.20 EMO and/or DOX induced alterations in mitochondrial membrane potential ($\Delta\psi_m$) in MCF-10A cells. FACS analysis of either untreated or treated with EMO (0.4 µM) and/or DOX (2.5 µM) for 24 h, 72 h (alone treatment, pre-tre, co-tre).

As shown in Figures 3.19 and 3.20, cell viabilities of DOX and/or EMO treated MCF-7 and MCF-10A cells were too low to detect $\Delta\psi_m$. As a result, we were unable to draw a conclusion about the effect of DOX and EMO, in applied concentrations, on mitochondrial membrane potential at this time. Because of the low cell viability, we decided to study with lower concentration of DOX (0.83 μM) for our further studies and repeated microscopic analysis and flow cytometry experiments with 0.83 μM DOX and 0.4 μM EMO concentrations.

As displayed in Figure 3.21, after exposure of MCF-7 cells to 0.4 μM EMO alone for 24 h-72 h and 0.83 μM DOX alone for 24 h-72 h, viabilities were observed as 85.1 %, 90.4 %, 35.4%, 16.2%, respectively in the cell population of live cells stage.

In the pre-treated group, after exposure of MCF-7 cells to 0.4 μM EMO for 24 h prior to 0.83 μM DOX treatment for 72 h and exposure of 2.5 μM DOX for 24 h prior to 0.4 μM EMO for 72 h (96 h total treatment for both group), viabilities were observed as 38.7 % and 19 %, respectively in the cell population of live cells stage.

In the co-treated group, after exposure of MCF-7 cells to 0.4 μM EMO and 0.83 μM DOX co-tre for 72 h, cell viability was observed as 37.3 %, respectively in the cell population of live cells stage.

We showed that the maximal early apoptotic and late apoptotic MCF-7 cells were detected in applied concentrations of DOX alone treated cells for 72 h, DOX pre-treated cells for 24 h and EMO-DOX co-treated cells for 72 h (% viability 66.8, 66.2, 51.4, respectively).

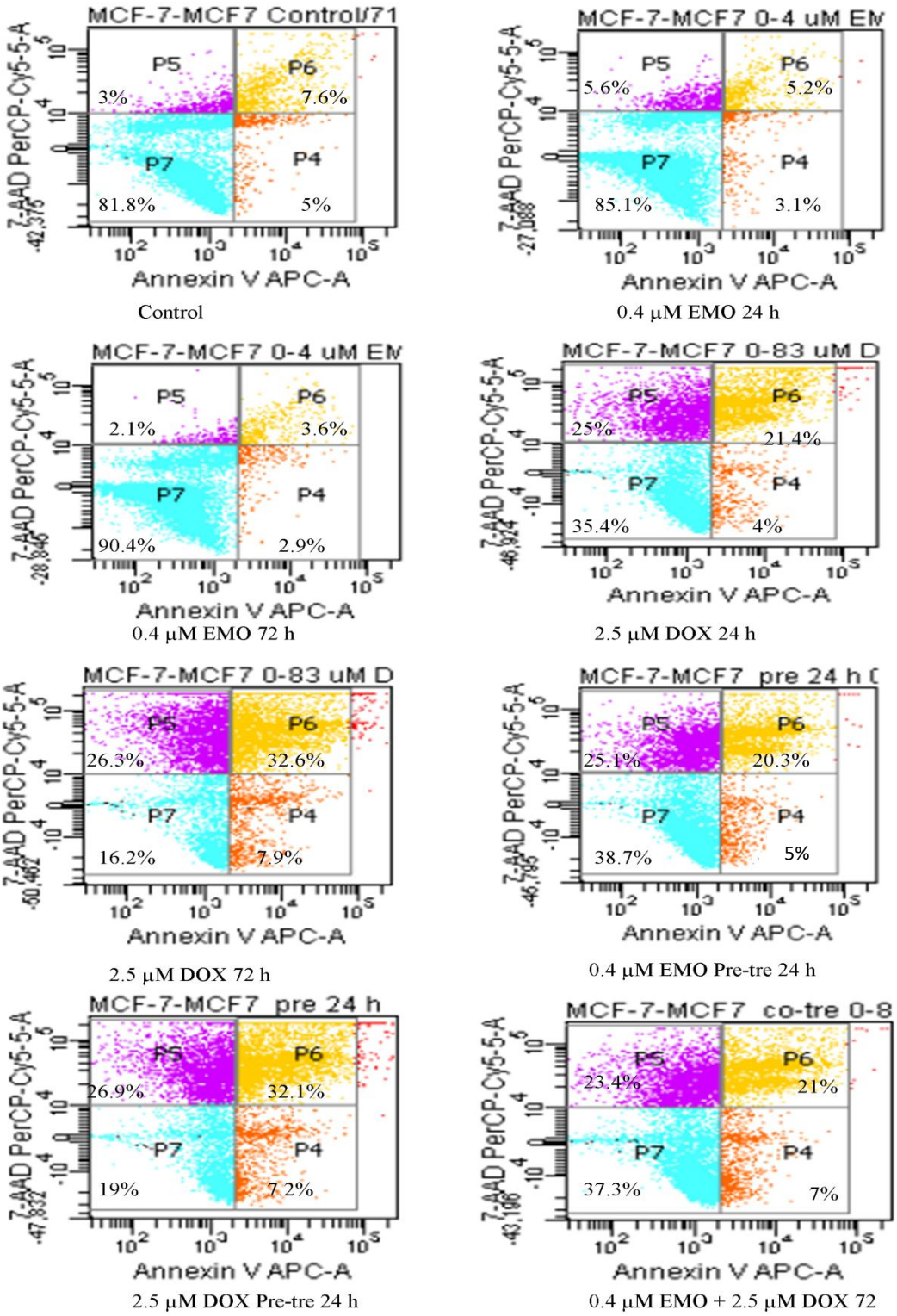

Figure 3.21 EMO and/or DOX induced apoptosis in MCF-7 cells. FACS analysis of either untreated ot treated with EMO (0.4 µM) and DOX (0.83 µM) for 24 h, 72 h (alone treatment, pre-tre, co-tre).

As displayed in Figure 3.22, after exposure of MCF-10A cells to 0.4 μM EMO alone for 24 h-72 h and 0.83 μM DOX alone for 24 h-72 h, cell viabilities were observed as 96.9 %, 95.8 %, 76.5 %, 36.5 %, respectively in the cell population of live cells stage.

In the pre-treated group, after exposure of MCF-10A cells to 0.4 μM EMO for 24 h prior to 0.83 μM DOX treatment for 72 h and exposure of 2.5 μM DOX for 24 h prior to 0.4 μM EMO for 72 h (96 h total treatment for both group), cell viabilities were observed as 72 % and 33.6 %, respectively in the cell population of live cells stage.

In the co-treated group, exposure of MCF-10A cells to 0.4 μM EMO and 0.83 μM DOX co-tre for 72 h, cell viability was 74.6 % in the cell population of live cells stage.

We demonstrated that the maximal early apoptotic and late apoptotic MCF-7 cells and MCF-10A cells were detected in applied concentrations of DOX-treated cells for 72 h and DOX pre-treated cells for 24 h (% viability 16.2 and 19 for MCF-7 cells, and 36.5, 33.6 for MCF-10A cells, respectively).

The important note is that while cell viability was 72 % at 0.4 μM EMO pre-tre for 24 h in MCF-10A cells, it decreased to 38.7 % in MCF-7 cells.

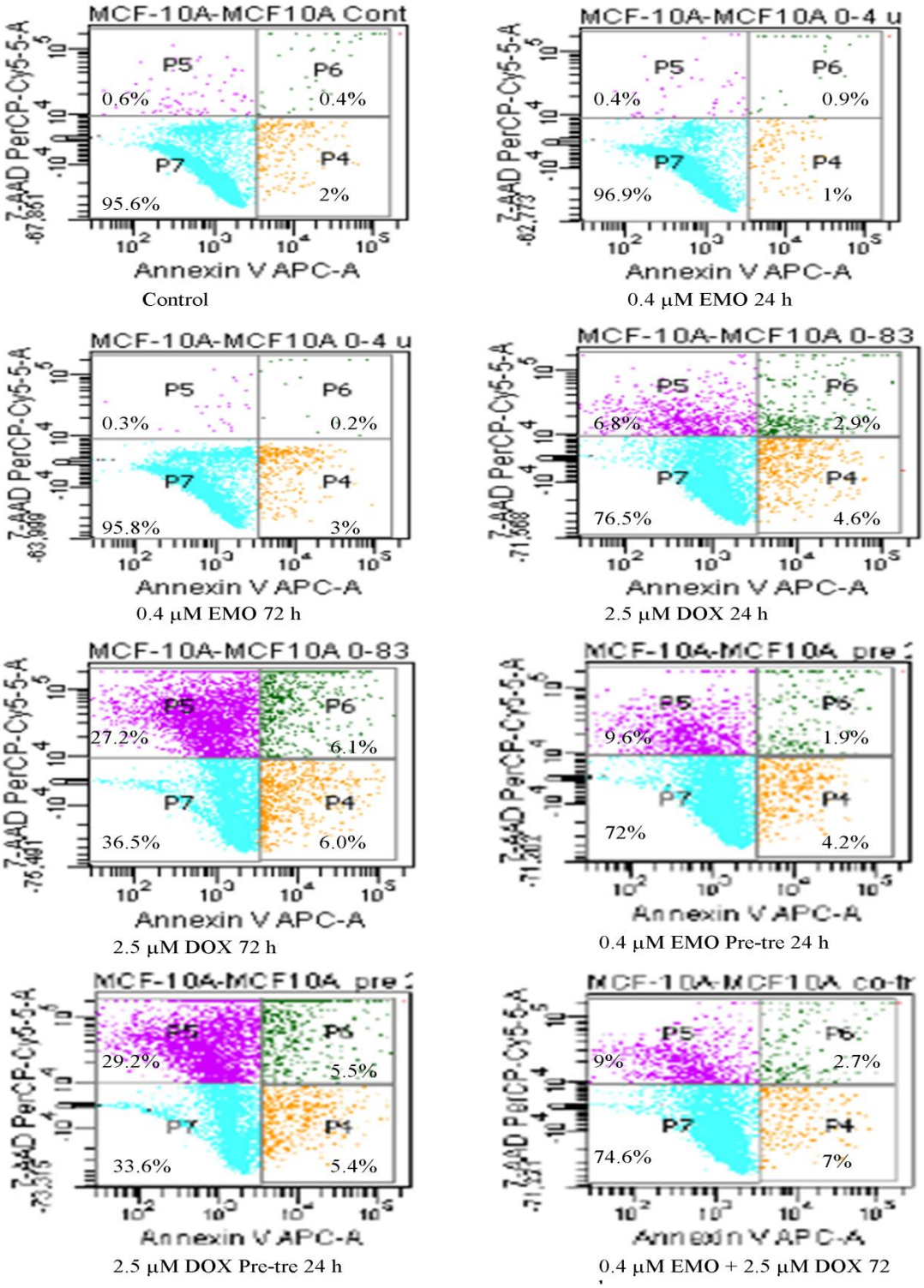

Figure 3.22 EMO and DOX induced apoptosis in MCF-10A cells. FACS analysis of either untreated ot treated with EMO (0.4 µM) and DOX (0.83 µM) for 24 h, 72 h (alone, pre-tre, co-tre).

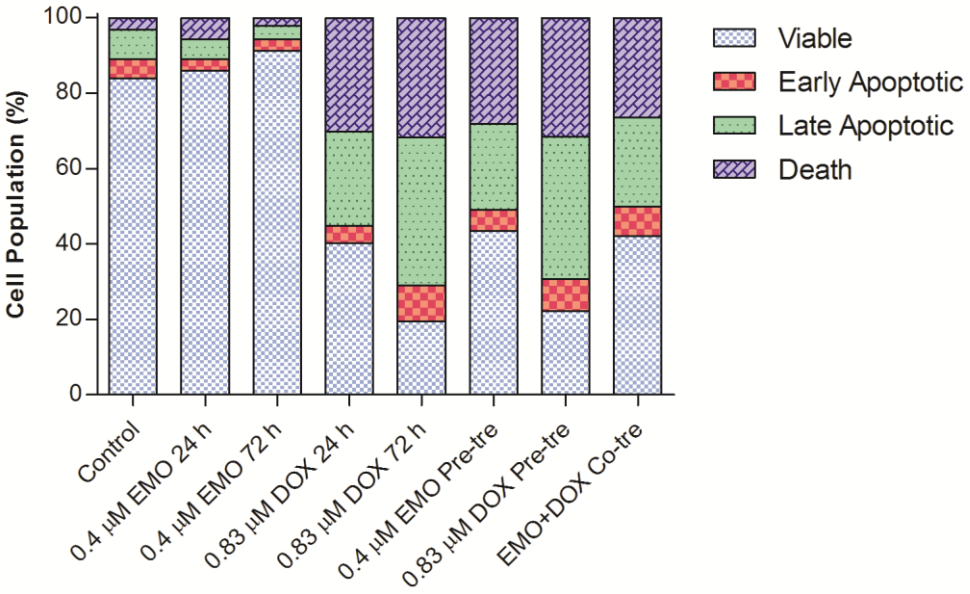

Figure 3.23 Percents of cell population of MCF-7 cells treated with EMO and/or DOX for 24 h and 72 h, analyzed by flow cytometry.

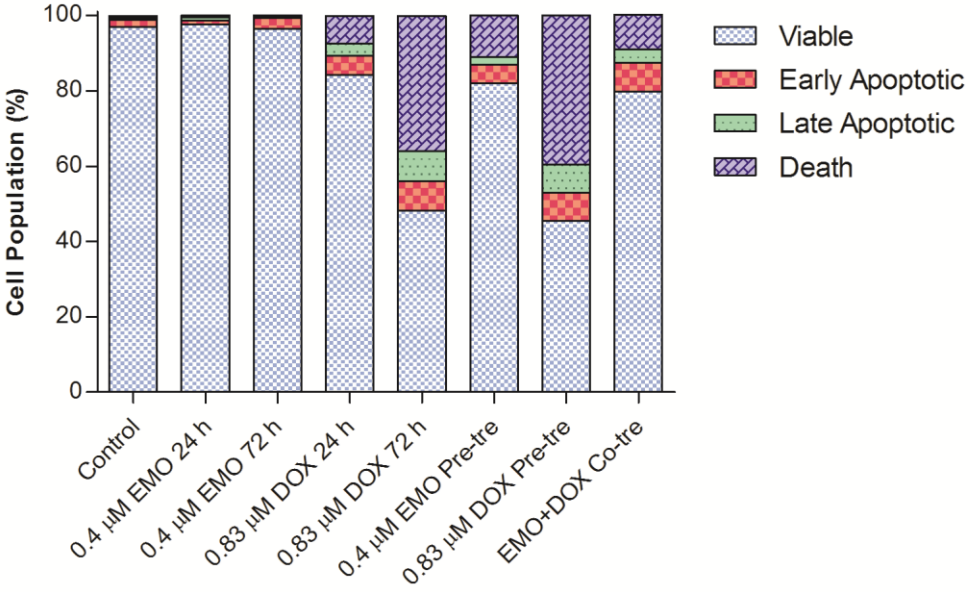

Figure 3.24 Percents of cell population of MCF-10A cells treated with EMO and/or DOX for 24 h and 72 h, analyzed by flow cytometry.

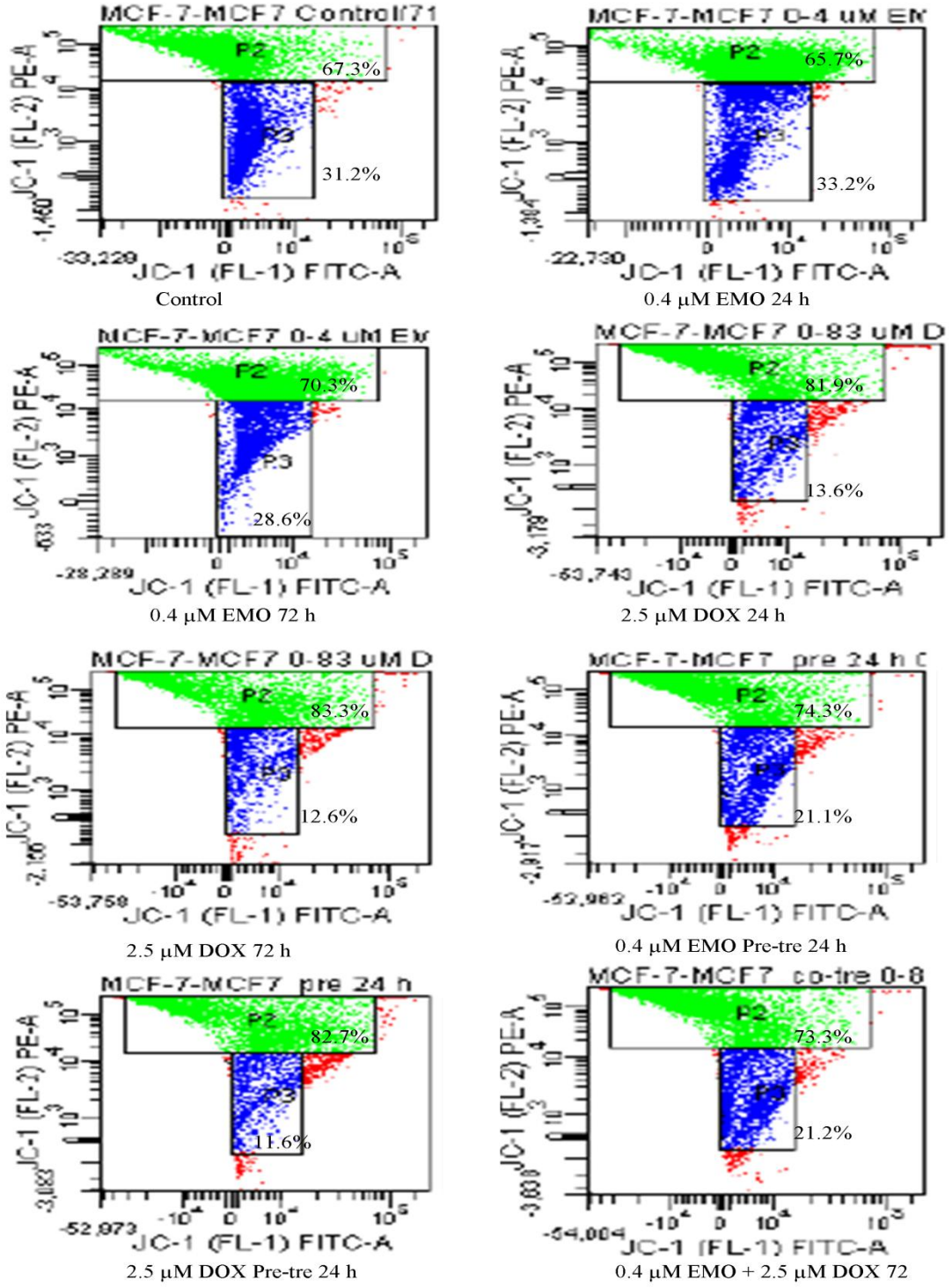

Figure 3.25 EMO and/or DOX caused alterations in mitochondrial membrane potential ($\Delta\psi_m$) in MCF-7 cells. FACS analysis of either untreated ot treated with EMO (0.4 µM) and/or DOX (0.83 µM) for 24 h and72 h (alone, pre-tre, co-tre).

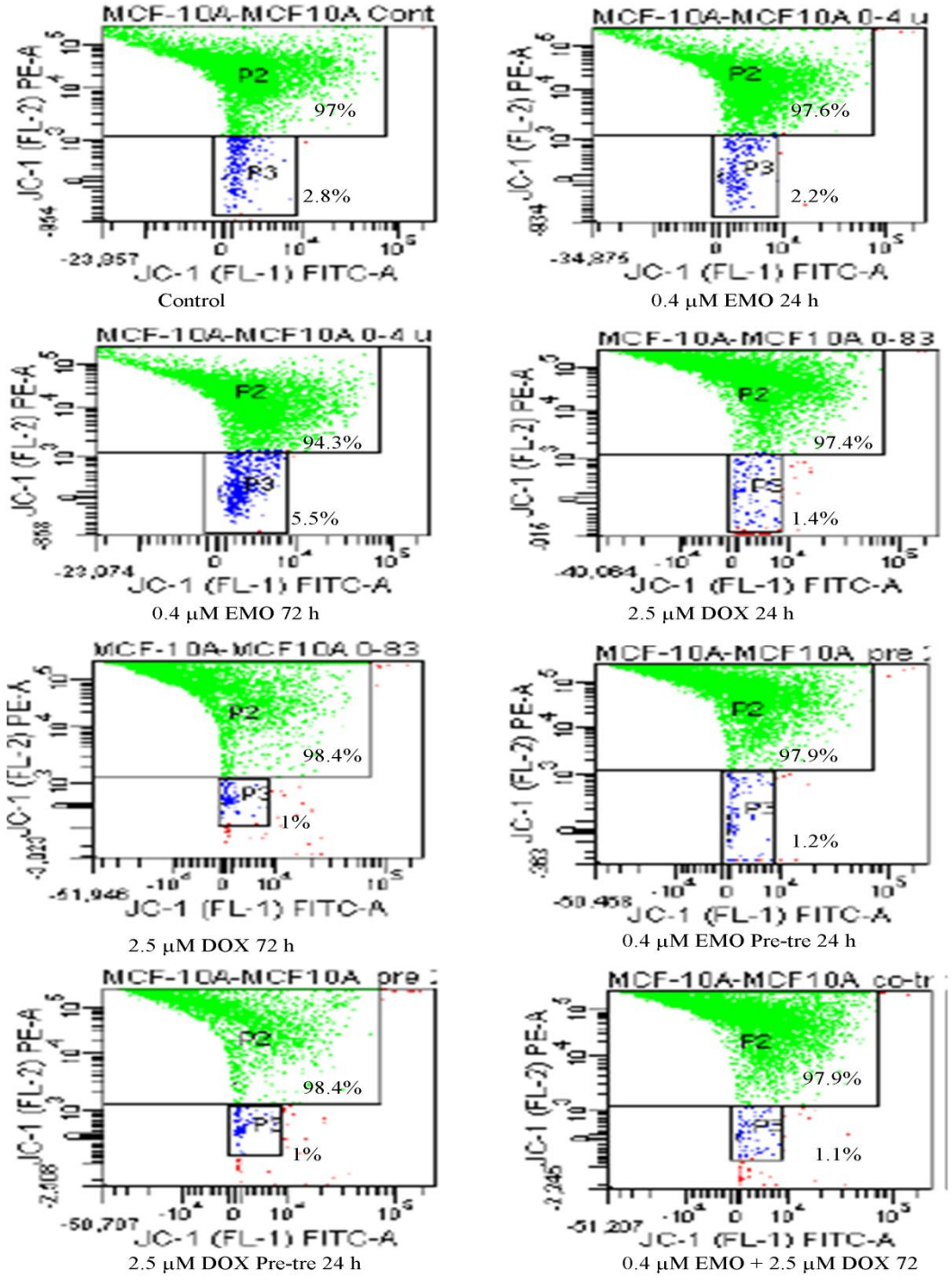

Figure 3.26 EMO and/or DOX caused alterations in mitochondrial membrane potential ($\Delta\psi_m$) in MCF-10A cells. FACS analysis of either untreated ot treated with EMO (0.4 µM) and/or DOX (0.83 µM) for 24 h and 72 h (alone, pre-tre, co-tre).

According to JC-1 staining results in both MCF-7 and MCF-10A cells (Figure 3.25-Figure 3.26, respectively), 0.83 μM DOX and 0.4 μM EMO treatments (alone treatment for 24 h and 72 h, pre-tre for 24 h, co-tre for 72) did not affect mitochondrial membrane potential (mitochondrial membrane potential loss in treated MCF-10A cells, between 1.1 %-5.5 % and in MCF-7 cells between 11.6 % - 33.2 %).

3.*2.3 Microscopic Analysis of EMO and DOX Treated Cells*

The results of DAPI staining was correlated with flow cytometry analysis. The inhibition of proliferation in MCF-7 cells was observed when the cells were treated with 0.83 μM DOX alone for 24 h and 72 h, 0.4 μM EMO pre-tre for 24 h, 0.83 μM DOX pre-tre for 24 h and 0.83 μM DOX - 0.4 μM EMO co-tre for 72 h (Figure 3.27, B, C, D, G, H). Furthermore, the morphological changes were detected in applied concentrations of DOX pre-treated, DOX-alone treated and DOX-EMO co-treated in MCF-7 cells (Figure 3.27, C, D, G, H,).

In MCF-10A cells, 0.83 μM DOX alone-tre for 24 h and 72 h, pre-tre for 24 h and co-tre with 0.4 μM EMO for 72 h also induced morphological changes (Figure 3.28, C, D, G, H). However, 0.4 μM EMO alone-tre for 24 h-72 h and pre-tre for 24 h in MCF-10A cells did not cause morphological alterations in MCF-10A cells (Figure 3.28, B, E, F).

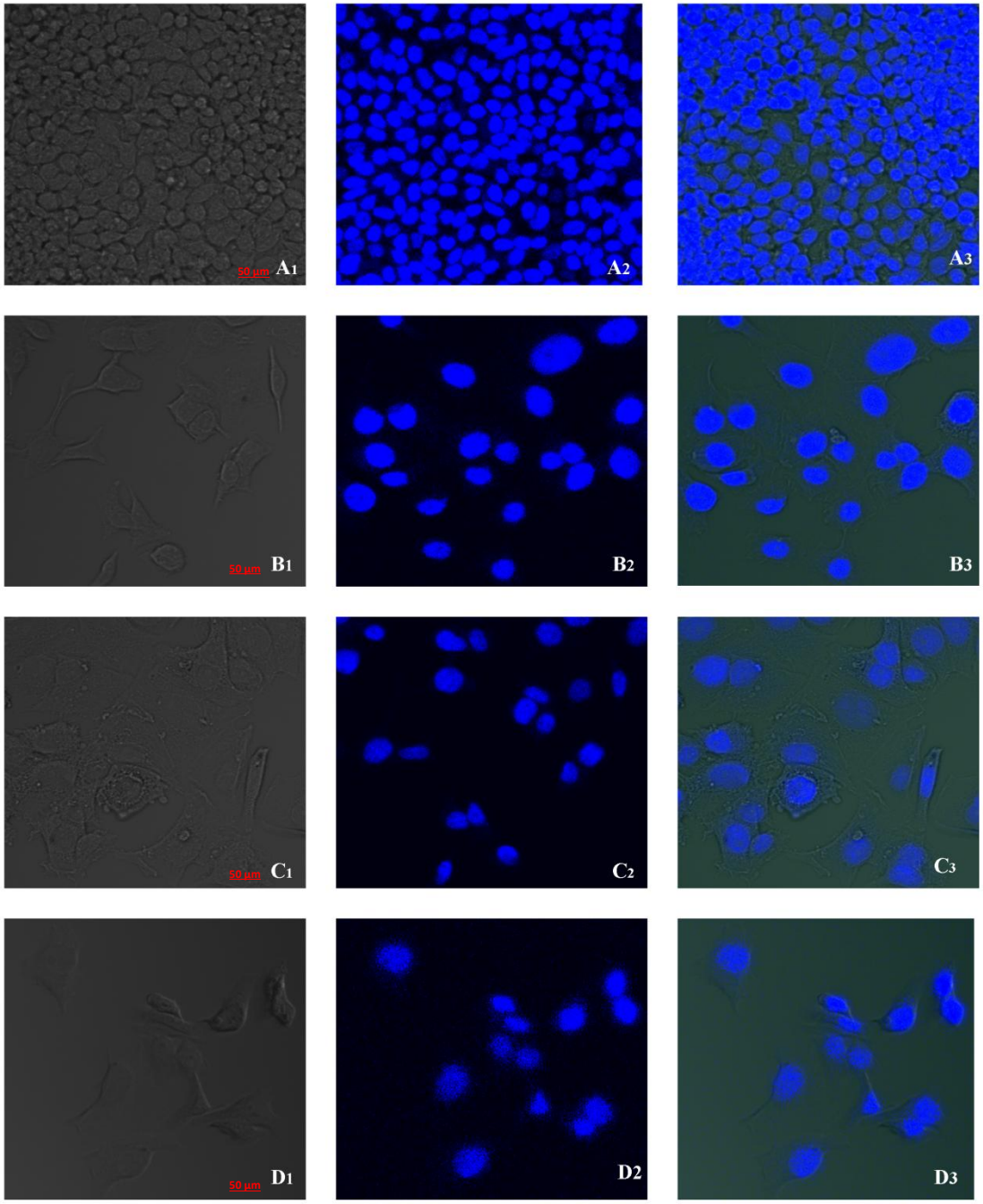

Figure 3.27 Confocal fluorescence images of EMO and DOX treated or untreated (control) MCF-7 cells with ($_1$) Transmission bright field, ($_2$) DAPI staining of the cell nucleus, ($_3$) overlay of bright field and DAPI images. A) Control, B) 0.4 µM EMO pre-tre for 24 h prior to 0.83 µM DOX treatment for 72 h, C) 0.83 µM DOX pre-tre for 24 h prior to 0.4 µM EMO treatment for 72 h, D) 0.4 µM EMO+0.83 µM DOX co-tre for 72 h. The cells were photographed under confocal microscope (A1R+/A1+, Nikon Instruments, Inc. magnification 100X, bar: 50 µm).

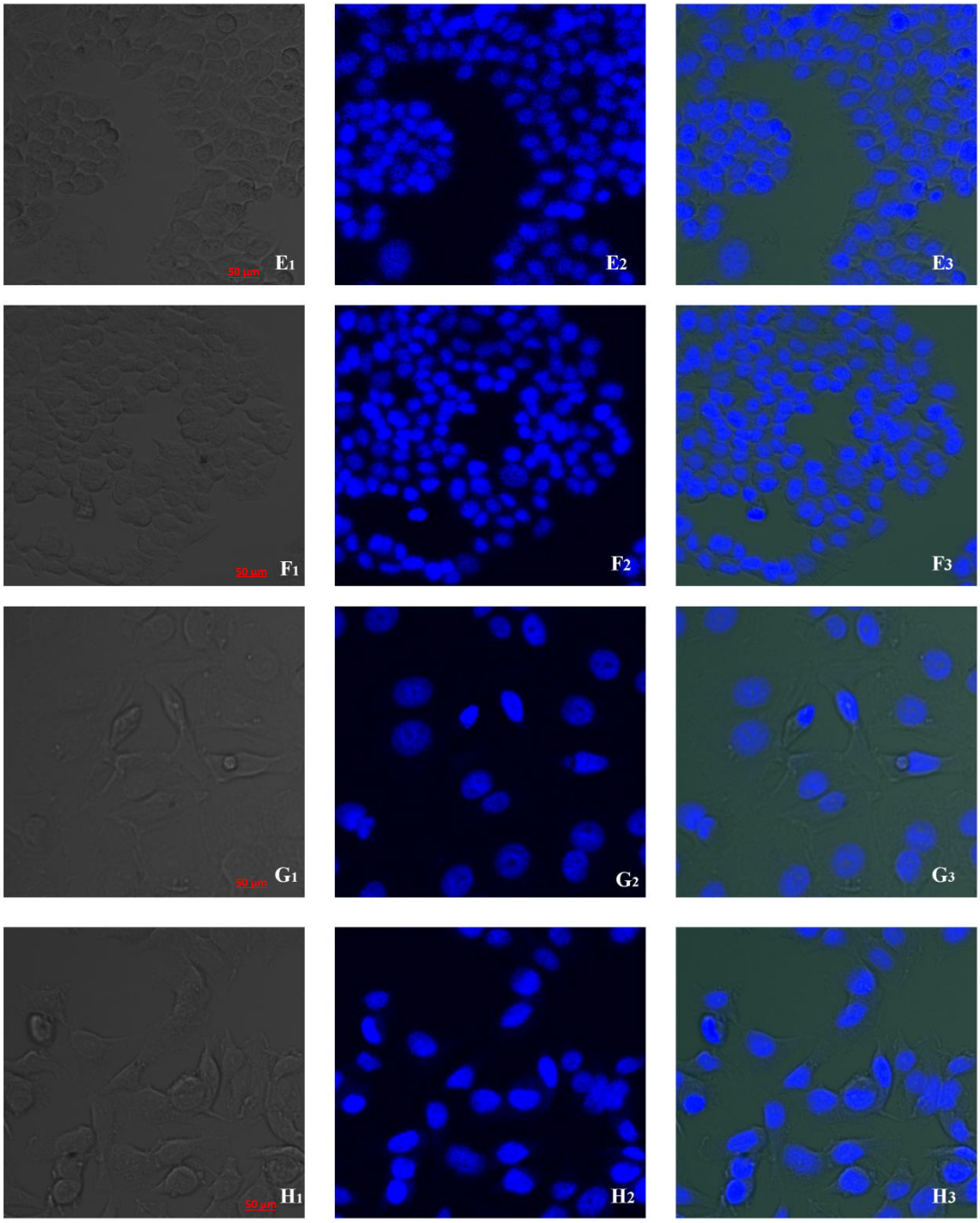

Figure 3.27 (cont'd) E) 0.4 µM EMO alone treatment for 24 h, F) 0.4 µM EMO alone treatment for 72 h, G) 0.83 µM DOX alone treatment for 24 h, H) 0.83 µM DOX alone treatment for 72 h.

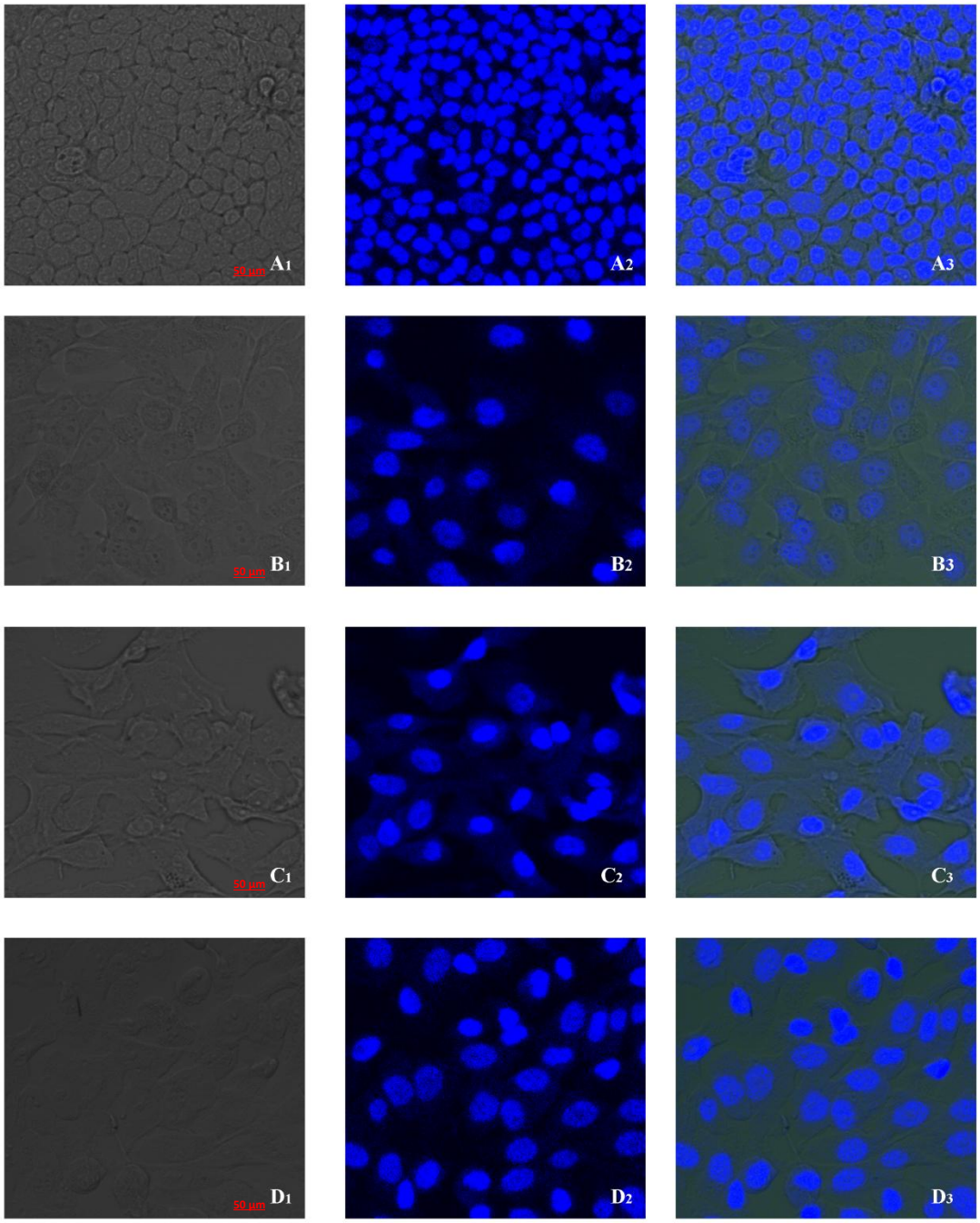

Figure 3.28 Confocal fluorescence images of EMO and DOX treated or untreated (contol) MCF-10A cells. A) Control, B) 0.4 µM EMO pre-tre for 24 h prior to 0.83 µM DOX treatment for 72 h, C) 0.83 µM DOX pre-tre for 24 h prior to 0.4 µM EMO treatment for 72 h, D) 0.4 µM EMO - 0.83 µM DOX co-tre for 72 h. (The cells were photographed under the same conditions with Figure 3.27).

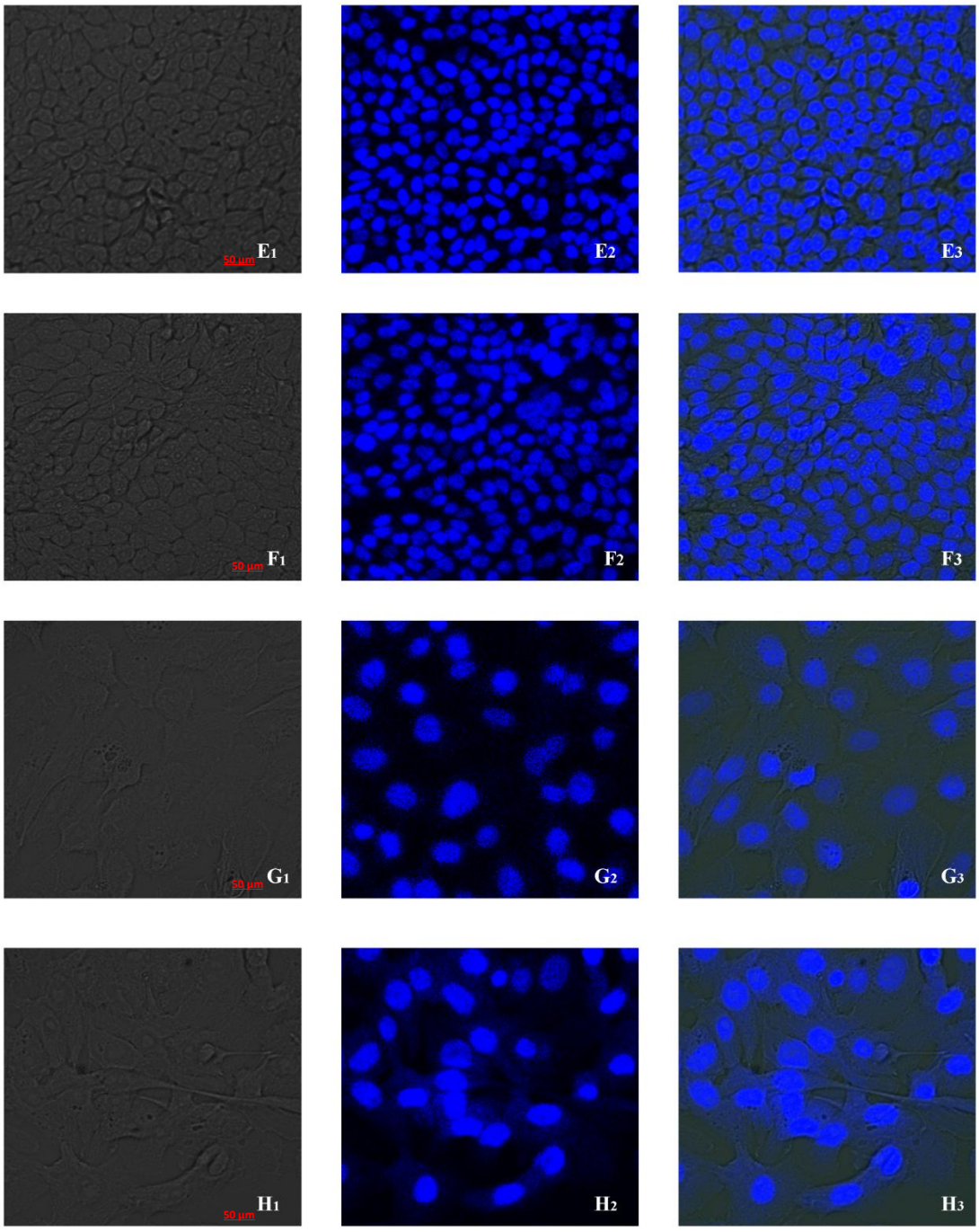

Figure 3.28 (cont'd) E) 0.4 µM EMO alone treatment for 24 h, F) 0.4 µM EMO alone treatment for 72 h, G) 0.83 µM DOX alone treatment for 24 h, H) 0.83 µM DOX alone treatment for 72 h.

CHAPTER 4

CONCLUSION

In this study, the effect of phytoestrogen emodin and the chemotherapeutic agent doxorubicin on MCF-7 and MCF-10A cell lines were examined by comparing their alone treatment, pre-treatment and co-treatment effects on both cell proliferation and apoptosis. The major outcomes are as follows;

- The results of both trypan blue and XTT cell viability assay showed that, exposure to phytoestrogen EMO (0.4 μM) before 24 h from chemotherapy reduce cytotoxic effects of chemotherapeutic agent DOX (0.83 and 2.5 μM), upon treatment for 72 h, in healthy breast cell model while it is not effective in breast cancer cell model. 0.4 μM EMO treatment after 24 h from the chemotherapy induce cytotoxic effects of chemotherapeutic agent DOX (0.83 and 2.5 μM) in both cell lines. Also, their combined exposure (0.4 μM EMO and 0.83 μM DOX) upon treatment for 72 h, triggers inhibition of proliferation and growth in both cell lines. We can conclude that, phytoestrogens which contain EMO could be used in order to reduce the toxic side effects of chemotherapy in healthy breast cells.

- Flow cytometric analysis to detect apoptosis of DOX and EMO treatments indicated that EMO (0.4 μM) treatment for 24 and 72 h did not cause apoptosis in both cell lines. Whereas, DOX (0.83 μM) treatment for 72 h, DOX pre-treatment for 24 h prior to EMO (0.4 μM) treatment for 72 h and co-treatment of EMO (0.4 μM) and DOX (0.83 μM) for 72 h, induced late stage apoptosis/necrosis in both cell lines. On the other hand, EMO pre-tre (0.4 μM) for 24 h prior to DOX (0.83 μM) treatment for 72 h, induced

apoptosis/necrosis only in MCF-7 cells. These results were in accordance with XTT assay and microscopic analysis results.

- The effects of EMO and DOX treatments on mitochondrial membrane potential was carried out by JC-1 staining and it was observed that neither DOX nor EMO treatments, in applied concentrations, caused loss of mitochondrial membrane potential in both cell lines. The results showed that EMO and DOX treatment did not affect cell death by mitochondrial pathway in MCF-7 and MCF-10A cells.

- Considering all the results obtained in the present study, EMO, as a dietary phytoestrogen could be consuming at low doses before chemotherapy to reduce the cytotoxic effect of chemotherapeutic agents in healthy cells and it should not be consumed during/after chemotherapy, especially at high doses, because of the additive effect on chemotherapy in healthy cells.

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