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Biochemical *In-vitro* Study of Tamoxifen Effect on CYP2D6 Enzyme and Cytotoxicity in Iraqi Premenopausal Women with Breast Cancer

A Thesis

Submitted to the college of science / Al-Nahrain University as partial fulfillment of the requirements for the Degree of Master of Science in chemistry

By

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Mom & Dad

I could never have done this without your faith, and support. Thank you for teaching me to believe in God, in myself, and in my dreams.

Brother & Sister

I am really grateful to both of your

My fiance

My refuge when I anger and gladness…

My Best friends

Donya & Ala'a

Acknowledgement

*Thanking **ALLAH** for giving me the power and health to complete this work.*

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Abstract

Background:

Hormone therapy is the first targeted therapy also called estrogen suppression therapy often used as an adjuvant therapy to reduce the risk of cancer back after surgery or cancer that has spread. Tamoxifen is used to treat early breast cancer in women who have already been treated with surgery, radiation, and/or chemotherapy. It is used to reduce the risk of developing a more serious type of breast cancer in women.

Present study was planned to assess the effect of tamoxifen on cytochrome P4502d6 enzyme activity. As well, study the cytotoxic effect of tamoxifen on MCF-7 breast cancer cell line and survey the apoptosis mechanism for MCF-7.

Subjects and methods:

This study includes (60) pre-menopausal women, their age ranged between (25-48) years. Thirty subjects were apparently healthy, chosen as control group (C group) and sixty patients with breast cancer (P group), were divided into two groups according to type of treatment: thirty five patients under tamoxifen treatment (P1 group), and twenty five patients under fluoxetine and tamoxifen treatment (P2 group). The levels of all parameters (age, weight, height, BMI, waist, hip, WHR) were quantitatively determined in patients and control. The second part of this study include the determination of the cytotoxic effects of tamoxifen against MCF-7 breast cancer cell line and investigation of the mechanism by which the affected living cells toward apoptosis using Caspase -9 activity and High Content Screening (HCS) assay.

Results:

Part I:

- A significant increase ($p<0.01$) in the mean of BMI was observed in the (P1) group and (P2) group in comparison with that of the group (C) (30.3 and 32.1 **vs.** 25.18 Kg/m^2).
- The mean level of serum cytochrome P450 2d6 has significant increase in group (P1) and group (P2) in comparison with that in group (C) (32.2 and 19.43 **vs.** 13.87 U/L) while the result showed a significant decrease in the group (P2) when compared with group (P1)

Part II:

- Cytotoxic effects of tamoxifen against MCF-7 cell line showed half maximal inhibitory concentration IC_{50} value was at 4.506µg/ml which is the most significant cytotoxic toward MCF-7 cell line treated for 24 hours.
- The effect of the tamoxifen on the mechanism of apoptosis reveal a highly increased caspase-9 activity, which observed at 5 µg/ml concentration in MCF-7 cell, IC_{50} was at (4.506 µg/ml)
- The effect of the tamoxifen on the mechanism of apoptosis showed a significant increase with increased concentration (dose dependent) for cell membrane permeability, cytochrome c and nuclear intensity at (100µg/ml of tamoxifen) when compared with (20µg/ml) of doxorubicin as a standard.
- The effect of the tamoxifen on the mechanism of apoptosis showed a significant decrease when the concentration is increased (dose depended) for cell viability and mitochondrial membrane permeability at (100µg/ml) when compared with 20 µg/ml of doxorubicin as a positive control.

Conclusions:

- In patients treated with tamoxifen, the measurement of CYP2D6 activity showed a good indicator of breast cancer
- Tamoxifen possess cytotoxic effect against breast cancer cells (MCF-7), as determined *in-vitro* by MTT assay (4.506 µg/ml).
- A toxic effect of tamoxifen has been demonstrated on MCF-7 cell line by using caspase-9 at IC_{50} concentration (4.506 µg/ml)
- From HCS technique results; tamoxifen showed toxic effect toward MCF7 cell line at (100µg/ml) tamoxifen concentration in a dose-dependent manner with increase in cell membrane permeability, cytochrome C, nuclear intensity, and change in mitochondrial membrane potential and decrease in cell viability level.

Abbreviations

A1	Bcl2-related protein A1
ADME	Absorption, distribution, metabolism, and excretion
ANOVA	Analysis of variance
Bad	BCl2-associated death promoter
Bak	BCl2-homologous antagonist killer
Bax	BCl2-associated x protein
Bcl-xL or Bcl-2	B-cell lymphoma-extra large
Bid	BH3-interacting-domain death agonist
BMI	Body mass index
BRCA1	Breast cancer type 1
BRCA2	Breast cancer type 2
CD95	Cluster of differentiation 95
CT	computed tomography
CYPs	Cytochrome P450s
DD	Death domain
DISC	Death-inducing signaling complex
DR	Death receptors
DR-4	Death receptor 4
DR-5	Death receptor 5
ELISA	Enzyme-linked immunosorbent Assay

EM	Extensive metabolism
ER	Estrogen receptor
Erα or NR3A1	Estrogen receptor alpha or Nuclear receptor subfamily 3, group A, member 1
FADD	Fas-associated protein with death domain
Fas	Apoptosis antigen 1
HCA	High-content analysis
HCS	High content screening
HER2	Human epidermal growth factor receptor 2
IC_{50}	Half maximal inhibitory concentration
MCF7	Breast cancer cell line
MCl-1	Induced myeloid leukemia cell differentiation protein
MRI	Magnetic resonance imaging
MTT	(3-(4, 5 Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
Noxa or PMAIP1	(Latin for damage) Phorbol-12-myristate-13-acetate-induced protein 1
N.S	Non-significant
PCD	Programmed cell death
PM	Poor metabolism

PR	Progesterone receptor
PUMA	P53 up regulated modulator of apoptosis
P-value	Probability value
SSRIs	Selective serotonin reuptake inhibitors
TAM	Tamoxifen
TNM	Tumor, node and metastasis
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor receptor
TNFR-1	Tumor necrosis factor receptor 1
TRAIL	TNF-related apoptosis-inducing ligand
TRADD	TNF-R type 1-associated death domain protein
MCF7	Breast cancer cell line
UEM	Ultra-extensive metabolism
WHR	Waist-to-hip ratio

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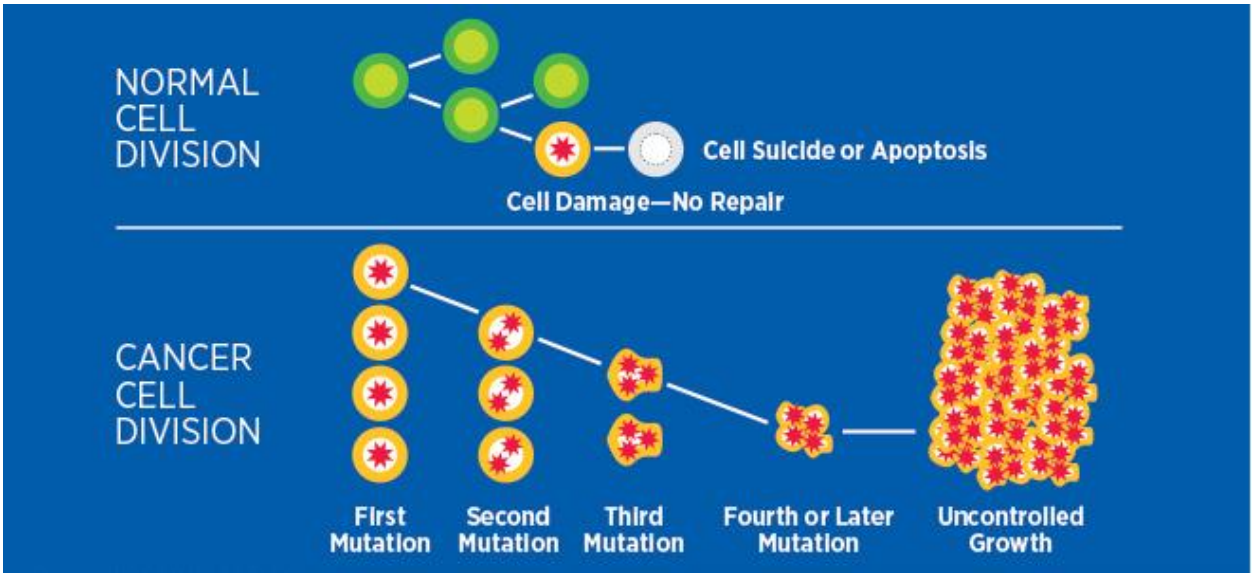
Chapter One

Introduction & Literature Review

1. Introduction

1.1 Cancer Biology

Cancer is a heterogeneous group of diseases described by the uncontrolled development of the cells. This proliferation if permitted to continue and spread be deadly. Around 90% of cancer-related death is because of tumor spread as metastasis [1, 2]. Cancer is a multi –gene and multi- step sickness beginning from the single abnormal cell [3]. The irregularities in malignancy cells usually produced because of mutation in protein coding genes that manage cell division. Aggregation of mutations result in cell increases abnormally and called tumors. A few cells in the tumors submit further series of mutation leading to figuration of malignant cells, which result to metastasis. Since malignant development can happen in virtually all position of the body, so that there are more than 100 different kinds of malignancies [4]. A few properties of malignancy cells are self-sufficiency in development signals, insensitivity to development inhibitory signals, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis, and tissue invasion and metastasis [5]. The rundown has been further amplified, for example, deregulating cellular activity, avoiding an immune reaction, tumor-promoting inflammation, and genome instability and change all together make cancer multiply [6]. Not all tumors are cancerous, but the tumor can be benign and dangerous. Benign tumors are not carcinogenic, they can frequently be removed and as a rule they don't return. Cells in benign tumors don't spread to different parts of the body. While dangerous tumors are malignant, cells in these tumors can attack close-by tissues and spread to different parts of the body and this is called metastasis. A few malignancies don't form tumors for instance; leukemia is a cancer of the blood and bone marrow [7].



Scheme (1-1): comparison between normal and malignant cells [8]

1.1.1. Apoptosis

Apoptosis is a Greek word meaning "falling away from". It is a form of programmed cell death in multicellular organisms [9]. It is one of the main types of programmed cell death (PCD) and involves a series of biochemical events leading to characteristic cell morphology and death [10]. Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called “budding.” Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment; these bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded [11]. There is essentially no inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells because apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis [12]. There is also the issue of distinguishing

apoptosis from necrosis, two processes that can occur independently, sequentially, as well as simultaneously [13]. Apoptosis plays a role in preventing cancer; if a cell is unable to undergo apoptosis due to mutation or biochemical inhibition, it can continue dividing and developed into a tumor [14]. Cells must be replaced when they become diseased or malfunctioning, but proliferation must be compensated by cell death [15].

1.2 Breast Cancer

Breast cancer is one of the most frequent malignant neoplasms happening in women in worldwide, and metastasis is the major reason of cancer-related death in patients [16]. In spite of recent main advances in the understanding of the technique of breast cancer progression and in the development of novel curative modalities, breast cancer remains the main leading reason for mortality among women. Mortality is almost because of metastasis. For instance, somewhere around 25% of patients diagnosed to have breast cancer will finally develop deadly metastasis, frequently decades after the duration of diagnosis and elimination of the primary tumor [17]. Early identification of cancer is a desirable objective as it often permits treatment with lower toxicity and predicts longer survival. In numerous cancers, however, the restricted capabilities of existing diagnostic techniques may contribute to high cancer mortality [18]. The objective of screening for early breast cancer detection is to discover the cancer before it begins to cause symptoms. Finding breast cancer early and getting state-of-the-art cancer treatment are the most important strategies to prevent deaths from breast cancer. Breast cancer that’s found early, when it’s small and has not spread, is easier to treat successfully. Breast

cancer can be diagnosed by accurate physical examination, mammography, ultrasound (U/S), magnetic resonance imaging (MRI) and breast biopsy [19].

1.2.1. Risk Factors

A risk factor is anything that raises a person's chance of getting a disease. Diverse cancers have diverse risk factors. Various comparatively strong risk factors for breast cancer that influence large proportions of the general population have been known for some time. Nonetheless, by far most of breast cancer cases happen in women who have no identifiable danger variables other than their gender [20].

Many factors known to increase the risk of breast cancer are not modifiable such as: Age (is one of the strongest risks); family history of breast cancer increases a woman's own risk, early menarche and late menopause.

Factors that are modifiable involve: Postmenopausal obesity, use of joint estrogen and progestin menopausal hormones, alcohol consumption increases endogenous estrogen levels that may participate to the observed increase in risk among regular drinkers, users of oral contraceptives are probably due to their estrogenic (and probably menses effects), and breastfeeding [21].

Numerous breast cancer risk factors influence lifetime exposure of breast tissue to hormones (early menarche, late menopause, obesity, and hormone use) [22]. Hormones are thought to impact breast cancer risk by increasing cell generation, thereby increasing the likelihood of DNA damage, as well as promoting cancer development. Despite the fact that breast cancer risk accumulates throughout a woman's life [23].

Techniques that may help decrease the danger of breast cancer incorporate avoiding overweight and obesity, engaging in regular physical activity, and minimizing alcohol intake [24].

1.2.2. Stage of breast cancer

The degree of spread at presentation is one of the most critical prognostic variables for patients with breast cancer. Staging is likewise important for frequent breast cancer. Staging and consideration of breast tumor spread outside the breast is commonly divided into regional lymph nodes, particularly axillary nodes, and remote or systemic staging, for locales beyond regional lymph nodes. All patients with invasive breast cancer submit axillary nodal staging as the presence or absence of axillary lymph nodes is a critical thought for further treatment after surgery [25].

The present methods for breast cancer patients staging into prognostic groups, which impacts therapeutic choices, depend on clinicopathological parameters, for example, size and grade of the tumor, absence or presence of lymph node metastases , and distant metastases so that is called Tumor, Nodal and Metastasis (TNM) staging , as showed in table (1-1) [26].

Table (1-1): Clinical staging group of breast cancer, were T: primary tumor present, N: any lymph node detected and M: if the tumor metastasis out from the breast to other organs [27].

Stage	T.N.M staging	Prognosis (5 year overall survival)
Stage 0	Tis, N0, M0	93%
Stage I	T1, N0, M0	88%
Stage IIa	T0 or T1, N1, M0 or T2, N0, M0	
Stage IIb	T2, N1, M0 or T3, N0, M0	74-81%
Stage IIIa	T0-T1, N2, M0 or T3, N1 or N2, M0	
Stage IIIb	T4, N0-N2, M0	41-67%
Stage IIIc	T, N3, M0	
Stage IV	Any T, any N, M1	15%

Tumor, Nodal and Metastasis (TNM) classification from breast cancer according to American Joint Committee on Cancer Staging as presented in table (1-2) as aforesaid [28].

Table (1-2): Tumor, Nodal and Metastasis (TNM) staging of breast cancer [28]

Tumours	**T0/Tis**	**T1**	**T2**	**T3**	**T4**
Tumour Size	**T0**: No primary tumour. **Tis**: Tumour only in breast ducts or lobules.	0-2 cm	2-5 cm	>5 cm	Tumor of any size with extension to chest wall/skin or ulceration ****inflammatory breast cancer** is staged as T4.
Nodes	**N0**	**N1**	**N1mi**	**N2**	**N3**
	No lymph node metastases.	Cancer cells present in 1-3 axillary lymph nodes.	Lymph node tumor > 2 mm.	Cancer cells present in 4-9 axillary lymph nodes.	Cancer cells in infra or supraclavicular lymph nodes, or in >10 axillary lymph nodes.
Metastasis	**M0**	**M1**			
	No evidence of cancer metastasis.	Cancer found in other areas of body.			

1.3 Tumor Grading

Tumor grade is a measure of the degree of differentiation of the tumor. Roughly, grade measures how closely the malignant cells resemble the morphologic and functional characteristics of the tissue of origin [29].

A pathologist also assigns a grade to the cancer, which is based on how closely the biopsy sample looks to normal breast tissue and how rapidly the cancer cells are dividing. The grade can help predict a woman's prognosis. In general, a lower grade number indicats a slower-developing cancer that is less likely to spread, while a higher number indicates a faster-growing cancer that is more likely to spread. The tumor grade is one factor in deciding if further treatment is needed after surgery. For invasive cancers, the histologic tumor grade is sometimes called the BloomRichardson grade, Nottingham grade, Scarff-Bloom-Richardson grade, or Elston-Ellis grade. Sometimes the grade is expressed with words instead of numbers: GX: Grade cannot be assessed (undetermined grade), G1: Well differentiated (low grade), G2: Moderately differentiated (intermediate grade), G3: Poorly differentiated (high grade) and G4: Undifferentiated (high grade) [30].

1.4 Diagnosis

Cancer that’s diagnosed at an early stage, before it’s had the chance to get too big or spread is more likely to be treated successfully. Breast cancers that are found because they can be felt tend to be larger and are more likely to have already spread outside the breast. But screening exams can often find breast cancers when they are small and still confined to the breast. The size of a breast cancer and how far it has spread are some of the most important factors in predicting the outlook (prognosis) of a woman with this disease. Most doctors feel that early detection tests for breast

cancer save thousands of lives each year. Many more lives probably could be saved if even more women and their health care providers took advantage of these tests [31].

Clinical examination, open biopsy and frozen section, imaging tests, biopsy diagnostic mammography, tumor features, ultrasound, estrogen receptor (ER) and progesterone receptor (PR), magnetic resonance imaging (MRI), computed tomography (CT) scan, human epidermal growth factor receptor 2 (HER2). This list describes options for diagnosing this type of cancer, and not all tests listed will be used for every person [32].

1.5 Treatment

The size, stage, grade, if the cancer cells have particular receptors, and other characteristics of the tumor; determine the kinds of treatment which include surgery, radiotherapy, chemotherapy, and hormone therapy [33]. Surgery remains the primary treatment for most women, with breast conservation (plus whole breast radiotherapy) providing similar outcomes to mastectomy. Following mastectomy, breast reconstruction should be considered, although uptake is incomplete [34]. Axillary surgery has moved from clearance via node sampling techniques to sentinel node biopsy as the preferred means for assessment of axillary metastasis in early breast cancer. Neoadjuvant therapy, initially implemented to down-stage inoperable cancers, is increasingly used to assess drug efficacy in individuals and to reduce the extent of surgery required in good responders [35]. Radiotherapy is a treatment for cancer that uses carefully measured and controlled high energy x-rays. In primary breast cancer, it aims to destroy any cancer cells that may be left behind in the breast area after surgery [36]. Chemotherapy means using drugs to destroy

cancer cells. Chemotherapy plays a major role in the treatment of patients with gynecological malignancies [37]. In general, chemotherapy has a smaller therapeutic window compared with drugs of other types; hence, the potential for severe adverse effects associated with chemotherapy has made appropriate patient and drug selection critical [38]. Chemotherapy often has many drawbacks. Many chemotherapeutic agents are toxic to healthy cells, and chemotherapy can cause significant and dangerous side effects, including severe nausea, bone marrow depression, and immunosuppression [39]. Additionally, even with administration of combinations of chemotherapeutic agents, many tumor cells are resistant or develop resistance to the chemotherapeutic agents [40]. Hormonal therapy (also called hormone treatment/therapy or anti-estrogen therapy) is the opposite of the type of women hormones sometimes take to reduce the symptoms of menopause [41]. It lowers estrogen levels instead of increasing them, Hormonal therapy is recommended for most women with breast cancer, and sometimes it is taken by women who have not been diagnosed with breast cancer but are at high risk for it based on their genes or family history [42].

1.6 Cytochrome P450 Enzyme

Cytochrome P450s (CYPs), constituting a superfamily of heme-containing monooxygenases found in all three domains of life, are involved in the metabolism of a diverse array of endogenous (e.g. steroids and lipids) and exogenous (i.e. xenobiotic compounds) [43]. Cytochrome P450 enzymes are essential for the metabolism of many medications. Although this class has more than 50 enzymes, six of them metabolize 90 percent of drugs, with the two most significant enzymes being CYP3A4 and CYP2D6 [44]. Generally they have more flexible active sites to

allow them to act on a wider array of substrates [45]. The name cytochrome P450 derives from the fact that the CO bound heme complex has an absorption band at 450 nm. Their heme active site is linked to the protein via a thiolate linkage of a cysteinate residue covalently bound to the iron centre [46]. CYP450s are hemoproteins and act as the terminal oxidases in the monooxygenase system [47]. The arrangement of P450s in the enzyme gathering is as appeared in Figure (1-1):

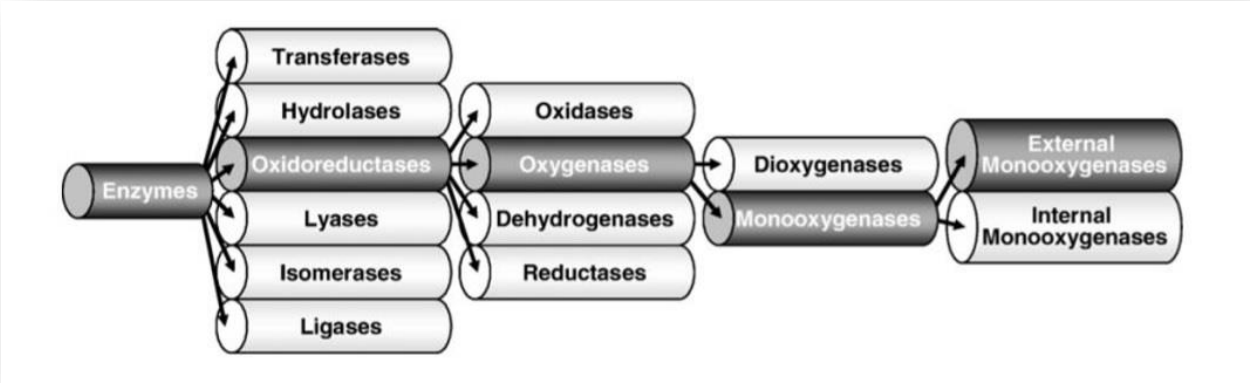


Figure (1-1): Assignment of Cytochrome P450 to enzyme group. P450 are highlighted with dark grey colored sub-groups [48].

Cytochrome P450 primarily catalyzes oxidation reaction by insertion of one atom of molecular oxygen into an organic substrate. Oxidation reactions are manifested as hydroxylation, oxygenation, epoxidation, dealkylation and are usually highly regioselective and stereoselective [49] Figure (1-2). Selective C-H oxyfunctionalization at unactivated carbons, one of the most challenging reactions in synthetic chemistry is performed by P450s especially in water, at room temperature and under atmospheric pressure [50].

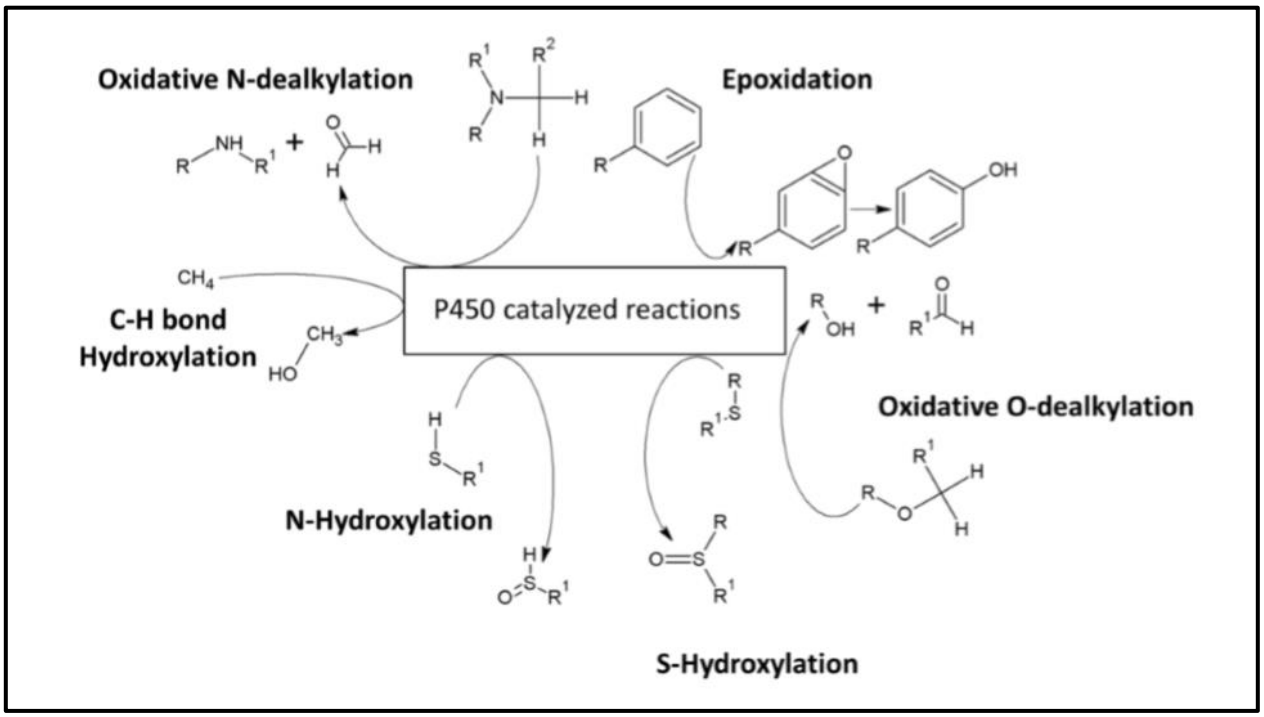


Figure (1-2): Catalyzed reactions by Cytochrome P450 monooxygenases [51]

1.6.1 Catalytic Mechanism

P450s catalyze the incorporation of one atom of molecular oxygen into various substrates. For this reaction P450s obtain their electrons from NADPH or NADP and shuttle it to the catalytic heme center via one or more electron transfer proteins [52].

$$\mathbf{R\text{-}H + NAD(P)H + H^{+} \xrightarrow[O_2]{P_{450}\ \text{monooxygenase}} ROH + NAD(P)^{+} + H_2O}$$

Scheme (1-2): A typical reaction catalyzed by CYP450 monooxygenases during hydroxylation of substrates [53].

The principal characteristic of the consensus mechanism of cytochrome P450 are as summarized in figure (1-3), the oxidation chemistry occur in steps 7 and 8 [54]:

(1) Binding of substrate to the enzyme, at times joined by a twist state change of the iron, to afford an enzyme-substrate adducts 3;

(2) Reduction of the ferric cytochrome P450 by a related reductase with a NADPH-inferred electron to the Fe^{++} cytochrome P450 4;

(3) Binding of molecular oxygen to the ferrous heme to create a ferrous cytochrome P450-dioxygen complex 5 like the case in oxymyoglobin;

(4) A second one-electron reduction and protonation to reach base at the Fe (III)-hydroperoxy complex 6;

(5) Protonation and heterolytic cleavage of the O-O bond in 6 with simultaneous generation of a water molecule to compose a reactive iron-Oxo intermediate7;

And, finally, oxygen-atom transfer from this iron-oxo complex 7 to the bound substrate to form the oxygenated produce complex 8. Product dissociation completes the cycle [55].

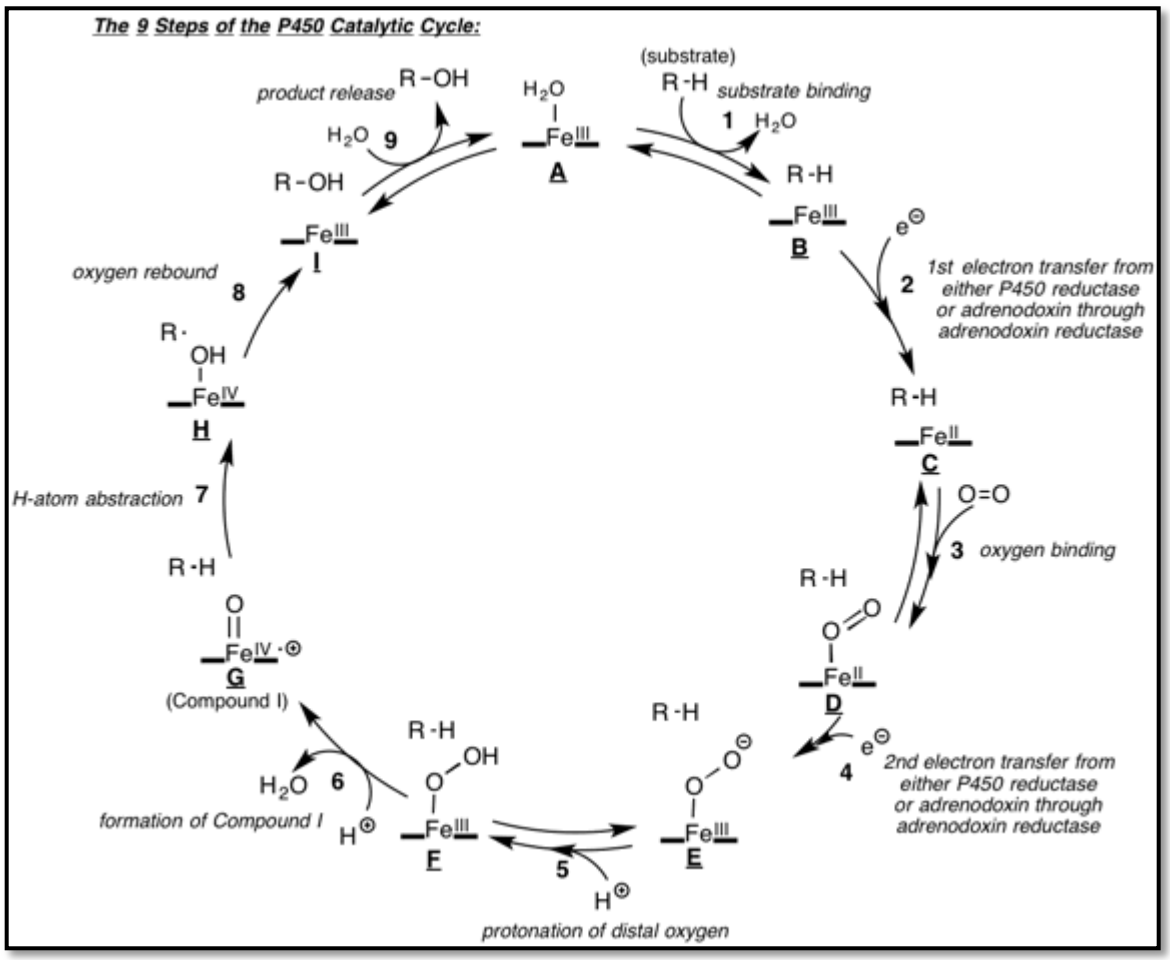


Figure (1-3): Generalized catalytic cycle for CYP450 reactions [56]

1.7 Cytochrome P450 2D6 (CYP2D6)

The human cytochrome P450 (CYP) 2D6 gene is a member of the CYP2D gene subfamily. Even though CYP2D6 only represents (1–5 %) of the CYP liver content, it is responsible for the oxidative metabolism of up to 25% of commonly prescribed drugs such as antidepressants, antipsychotics, opioids, antiarrythmics and tamoxifen, many of which have a narrow therapeutic window . CYP2D6 is encoded by a highly polymorphic gene, with more than 130 genetic variations are described

[57]. The human CYP2D6 enzyme has a high affinity for alkaloids, and it detoxifies alkaloids [58].

1.7.1 Various Aspects of CYP2D6

Cytochrome P450 2D6 (CYP2D6), an individual from the cytochrome P450 mixed-function oxidase system, is one of the most substantial enzymes required in the metabolism of xenobiotic in the body. Whilst CYP2D6 is included in the oxidation of an extensive variety of substrates of all the CYPs, there is significant variability in its expression in the liver. The gene is situated almost two cytochrome P450 pseudo genes on chromosome 22q13.1. Then again, joined transcript variations encoding distinctive isoforms have been found for this gene. CYP2D6 appears the biggest phenol typical variability amongst the CYPs, generally because of genetic polymorphism. The genotype represents the normal, decreased and nonexistent CYP2D6 function in subjects. More than 50 human CYP isoenzymes have been recognized [59]. The genetic base for extensive and poor metabolites variability is the CYP2D6 allele, situated on chromosome 22 [60]. Subjects who have certain allelic variations will appear normal, reduced or no CYP2D6 function be based on the allele. In CYP2D6, genetic polymorphism has been connected to three classes of phenotypes in view of the degree of drug metabolism. Extensive metabolism (EM) of a drug is specific for the ordinary population; poor metabolism (PM) is connected with accumulation of particular drug substrates and is commonly an autosomal recessive characteristic requiring mutation and/or deletion of both alleles for phenotypic expression; and ultra-extensive metabolism (UEM) results in increased drug metabolism and is an autosomal dominant quality arising from gene amplification [61].

1.7.2 Drug Metabolism

Due to the high polymorphic character of CYP2D6, this enzyme is also the site of a number of drug interactions *in-vivo*, which is of clinical significance [62]. The basic purpose of drug metabolism in the body is to make drugs more water soluble and thus more readily excreted in the urine or bile. One common way of metabolizing drugs involves the alteration of functional groups on the parent molecule (e.g., oxidation), the cytochrome P450 enzymes e.g., CYP2D6 drug interactions involving the cytochrome P450 isoforms generally result from one of two processes, enzyme inhibition or enzyme induction. Enzyme inhibition usually involves competition with another drug for the enzyme binding site. This process usually begins with the first dose of the inhibitor, and onset and offset of inhibition correlate with the half-lives of the drugs involved. Enzyme induction occurs when a drug stimulates the synthesis of more enzyme protein, enhancing the enzyme's metabolizing capacity [63, 64].

1.7.3 CYP2D6 phenotype–genotype relationships:

• **Poor metabolizers**: individuals with absent CYP2D6 activity; poor metabolizers are carriers of two nonfunctional alleles of *CYP2D6*, resulting in nonfunctional CYP2D6 enzymes [65].

• **Extensive metabolizers:** individuals with normal activity; most extensive metabolizers carry one or two alleles encoding normally functional CYP2D6 activity [66].

• **Intermediate metabolizers:** individuals with reduced activity; most intermediate metabolizers carry one nonfunctional allele and one intermediate metabolizer allele encoding an enzyme with subnormal CYP2D6 activity [67].

• **Ultrarapid metabolizers:** subjects with increased CYP2D6 activity; genetically determined ultrarapid metabolizers carry at least one duplicated or multiduplicated functional allele [68].

1.8 Tamoxifen (Nolvadex)

Tamoxifen is a hormonal treatment; it stops estrogen from telling breast cancer cells to grow [69]. Chemically known as (z) - 2-[4-(1, 2-Diphenyl – 1 butenyl) phenoxy - N, N – Dimethylethanamine [70]. Tamoxifen (TAM) is an important anticancer drug that is commonly used in the prevention and treatment of breast cancer, and also exhibits antioxidant and cardio protective effects. One mechanism by which TAM inhibits cancer cell growth is competitive blocking of estrogen receptors. However, tamoxifen also inhibits the growth of estrogen-receptor-negative breast cancer cells. This implies the presence of additional mechanisms that are not related to estrogen receptor mediation. Tamoxifen was also found to antagonize protein kinase C without interacting with the enzyme's active site. Other studies have shown that tamoxifen can cause liver toxicity by affecting mitochondria functions. Since tamoxifen is highly lipophilic, modulation of cell membrane structural and mechanical properties may be a reason for its anticancer activity and associated adverse effects [71]. The chemical structure of tamoxifen is shown in figure (1-4):

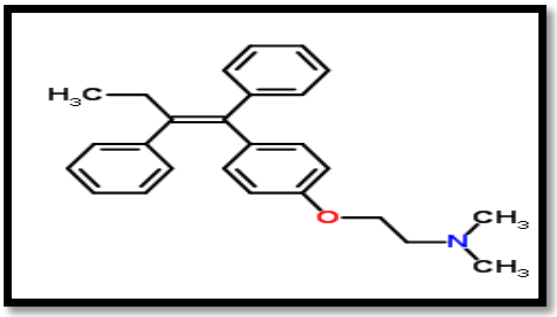
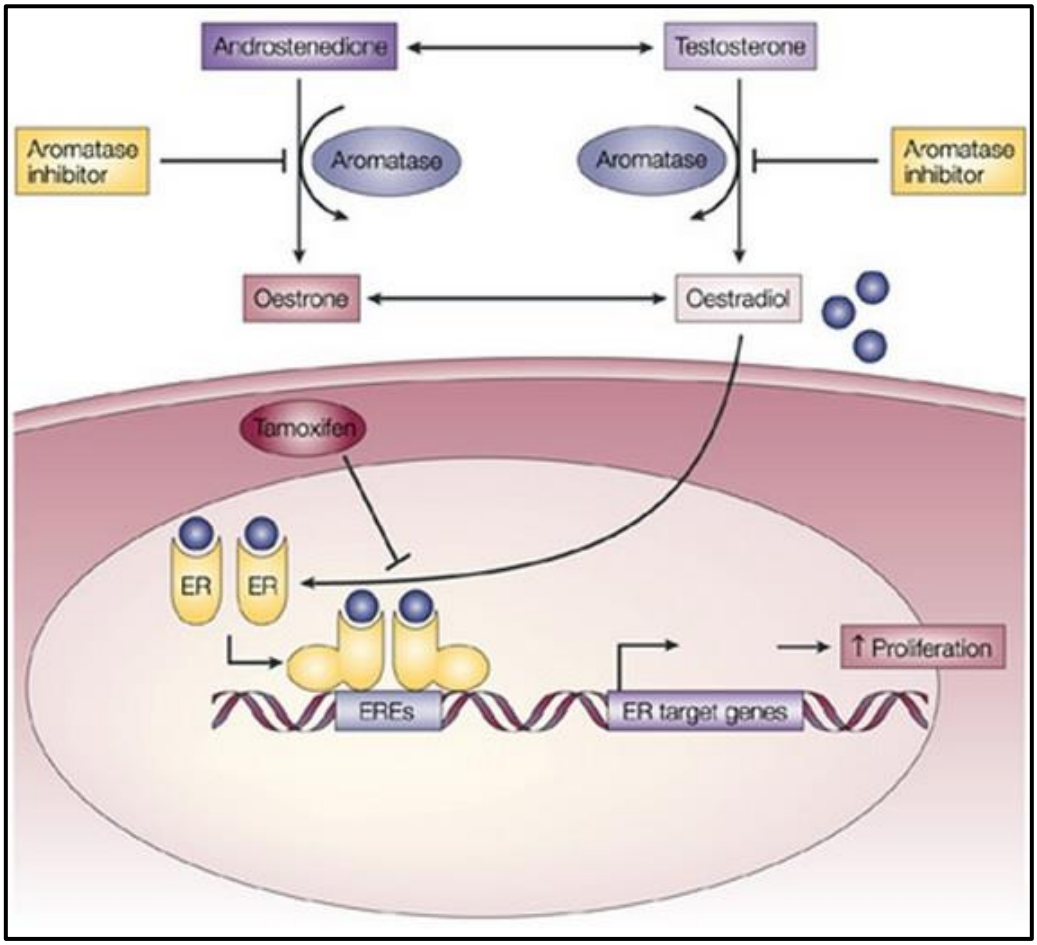


Figure (1-4): Chemical Structure of tamoxifen [72]

1.8.1 Benefits

Tamoxifen was used for palliative therapy of breast cancer in premenopausal women, who are considered poor candidates for surgery, and is also used to treat female infertility [73]. Tamoxifen has been widely used in the endocrine treatment of metastatic breast cancer as adjuvant therapy preoperative treatment, ductal carcinoma in situ and chemoprevention [74]. An adjuvant treatment strategy incorporating an aromatase inhibitor as primary (initial endocrine therapy), sequential (using both tamoxifen and an aromatase inhibiter in either order), or extended (aromatase inhibiter after 5 years of tamoxifen) therapy reduces the risk of breast cancer recurrence compared with 5years of tamoxifen alone [75].

Tamoxifen blocks the action of estrogen at the receptor level, whereas aromatase inhibitors block the synthesis of estrogen in peripheral tissues including the breast [76]. Tamoxifen has been tested as a possible treatment for hepatocellular carcinoma, stomach carcinoma, renal cell carcinoma, melanoma, pancreatic cell carcinoma, cervical carcinoma, ovarian carcinoma and other tumors; however it is not widely used for these treatments [77].



Scheme (1-3): Comparison between aromatase inhibitors and tamoxifen [78]

1.8.2 Metabolism

Tamoxifen is a prodrug, requiring cytochrome P450 enzyme-mediated metabolism to form the active metabolite endoxifen [79]. Tamoxifen is transformed predominantly by the drug- metabolizing enzymes CYP3a4 and CYP2D6 into the therapeutically more efficient drug metabolites 4-hydrox y tamoxifen (4-OH-tamoxifen) and endoxifen [80]. By binding to the tamoxifen and its metabolites regulate, the estrogen-induced translation of estrogen receptor target genes. The metabolites 4-OH-tamoxifen and endoxifen appear up to 100 times higher affinity to the erα than the parental compound thus, the efficacy of tamoxifen firmly

depends on upon its proper bio activation by cytochrome P450 enzymes [81]. CYP2D6 is highly polymorphic and shows a high interindividual variability in its activity. Other enzymes involved in tamoxifen metabolism comprise CYP2C9, CYP2C19 and CYP2B6. These three enzymes are also involved in the formation of 4-OH-tamoxifen and endoxifen, but their contribution may depend on actual tamoxifen concentrations and on CYP2D6 activity [82].

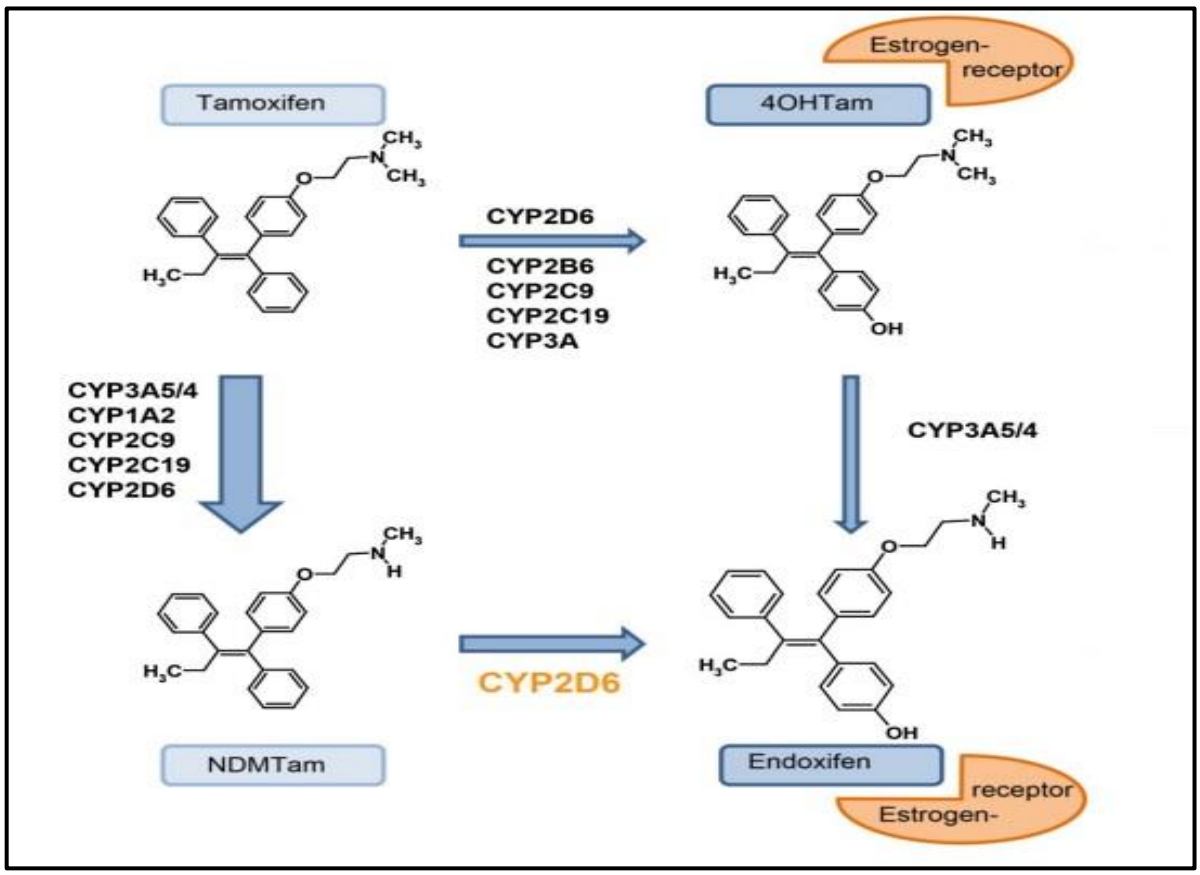


Figure (A)

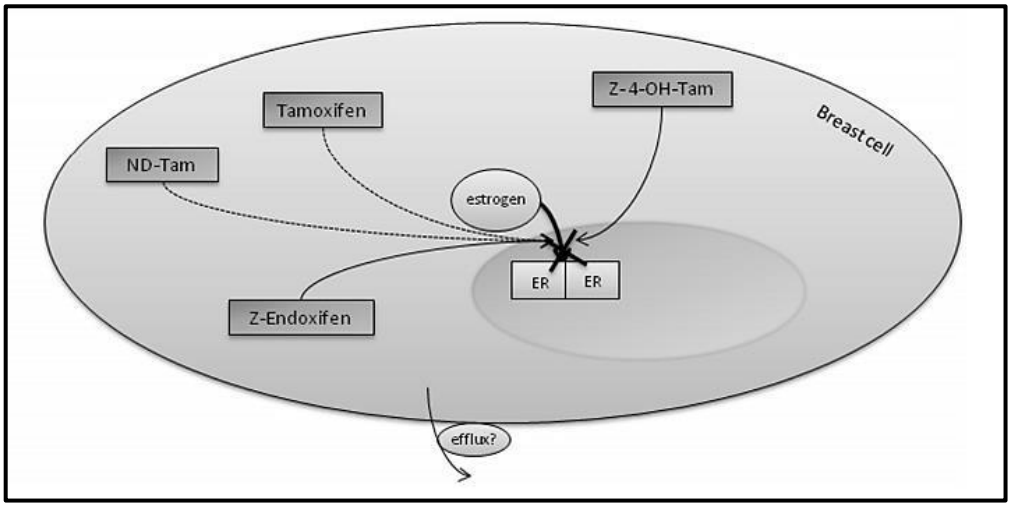


Figure (B)

Figure (1-5): Tamoxifen metabolism (A) in liver (B) in breast [83, 84]

1.9 Cell viability cytotoxicity assays

Cell-based measures are often utilized for screening collections of compounds to limit if the test molecules have impacts on cell proliferation or show direct cytotoxic impacts that in the end lead to cell death. Cell-based tests also are widely used for measuring receptor binding and a variety of sign transduction events that may include the statement of genetic reporters, trafficking of cell parts, or monitoring organelle function. Regardless of the kind of cell-based examine being utilized, it is imperative to know what numbers of suitable cells are staying toward the end of the examination. There are a variety of examine methods that can be utilized to evaluate the quantity of viable eukaryotic cells. MTT and WST methods, which that used for calculation, the number of cells live [85].

1.9.1 MTT cytotoxicity assay

MTT (3-(4, 5 Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) change from yellow to purple formazan in living cells by NADH [86]. MTT assay depends on the formation of dark colored formazan by decrease of the tetrazolium salt MTT by metabolically active cells. After 2-4 hrs. incubation, the water-insoluble formazan color structures crystals, which can be melt in an organic dissolvable and the sum, can be determined semi-automatically using a micro plate reader. Absorbance readings are identified with the number of cells thus providing the possibility to utilize the MTT measure as a proliferation test to evaluate cell development after irradiation. In the present study our adjusted version of the MTT measure is contrasted with the clonogenic examine in order to open the possibility of replacing one by the other [87].

1.10.1 Caspase technique

The caspase, or cysteine-dependent aspartate-directed proteases, belong to a highly protected group of cysteine proteases that have a critical role in the diverse phases of apoptosis, which is a procedure responsible of keeping homeostasis in multicellular organisms [88]. Caspases are endoproteases that break down peptide bonds in a reaction that based on catalytic cysteine residues in the caspase active site and happens simply after certain aspartic acid residues in the substrate. In spite of the fact that caspase-mediated processing can leads to substrate inactivation, it might likewise create active signaling molecules that participate in ordered procedures, for example, apoptosis and inflammation [89]. Caspases included in apoptosis have been sub-classified by their technique of activity and are either initiator caspases (caspase 8 and -9) or executioner caspases (caspase-3,- 6, and - 7)

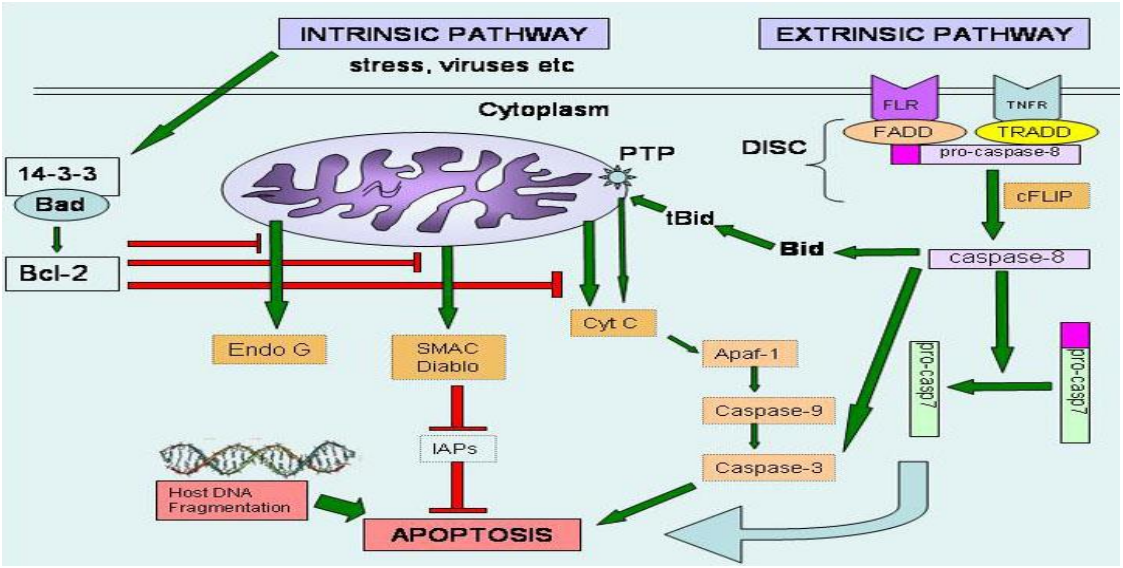
[90]. The initiator caspases group comprises of (caspases -2, -8, -9, and -10). The initiator pro caspases exist as possess long pro domains and monomers, that are activated by dimerization and not by cleavage [91]. Improper activation of the executioner (caspases - 3, - 6, and - 7) is prohibited by their generation as inactive procaspase dimers that must be separated by initiator caspases. This cleavage between the extensive and little subunits permits a conformational change that brings the two active sites of the executioner caspase dimer together and makes a functional full grown protease [92].

1.10.2 Apoptosis

One of the best-characterized systems of programmed cell death is apoptosis, which is intermediate through the activation of individuals from the caspase group of proteases. Initiator caspases stimulate executioner caspases that coordinate their activities to destroy key structural proteins and stimulate other enzymes. The morphological signs of apoptosis result, including DNA fracture and fragmentation blebbing [93]. The apoptotic cascade is separated into two main pathways, extrinsic and intrinsic, which gets triggered by dissolvable molecules that link to plasma-membrane receptors or by different mitochondrial stimuli, respectively [94]. The extrinsic apoptotic pathway is stimulated by death receptors (DR), which are cell-surface receptors that connect specific ligands and transmit apoptotic signals. Such ligands contain dissolvable molecules of the tumor necrosis factor (TNF) family, which are excreted as homotrimers and join to members of the TNF-receptor (TNF-R) family, including TNFR-1, Fas/CD95, and TRAIL receptors DR-4 and DR-5. Ligand restricting causes receptor trimerization and subsequent activation [95, 96]. TNF-Rs have a death domain (DD), which inducts other DD-containing proteins, for example, TNF-R sort 1-associated death domain protein (TRADD) and Fas-

associated protein with death domain (FADD). These proteins connect to initiator caspases-8 and - 10, therefore can homodimerization and subsequent activation of death-inducing signalling complex (DISC) [97]. Taking after the activation of caspases-8 and - 10, the effector caspases-3, - 6, and - 7 are cleaved, prompting cell degradation in the final phase of apoptosis [98].

The intrinsic apoptotic pathway is started by different intracellular stimuli, including oxidative stress, DNA harm, hypoxia, and growth-factor reduction, which stimulate external mitochondrial membrane permeabilization [99]. Mitochondrial integrity can be controlled by different members of the Bcl-2 superfamily, of which there are two subcategories: anti-apoptotic and pro-apoptotic. Bax, Bid, Bak, Bad, Noxa, and PUMA are pro-apoptotic relatives, while Bcl-xL, Bcl-2, Mcl-1, and A1 are anti-apoptotic relatives [100]. Through apoptosis, pro-apoptotic Bax and Bak go on dimerization and insert into the external mitochondrial membrane, setting off the intrinsic apoptotic pathway [101].



Scheme (1-4): Schematic representations of the two main pathways of apoptosis are extrinsic and intrinsic pathway [102].

1.11 High content screening

High- Content Screening (HCS) is a cellular imaging- established approach that played important role in the detection of toxicity and classification of compounds depend on observed patterns of reversible and irreversible cellular damage. HCS supply multi-parametric analysis of compound toxicity at the level of individual cells [103].The Array Scan high-content screening System (Fig. 1-7), is a cytometer depend on a fully automated fluorescence microscope that obtains images from cells seeded in multi-well plates [104]. This instrument proceeds automated measurements, involving the intensity and localization of the fluorescence signals within single cells and within subcellular compartments over a wide cell population. The analysis image is performed in actual time for up to 4 fluorescence channels. The Array Scan has been used to analyze the execution of various cellular events that can be tracked by fluorescence immunocytochemistry, like variations in cellular morphology [105] and intracellular trafficking [106] or to describe NF-kB translocation [107].The major feature of such process resides in the possibility of gaining both intrinsic data for individual cells and population data within a single analysis [108].

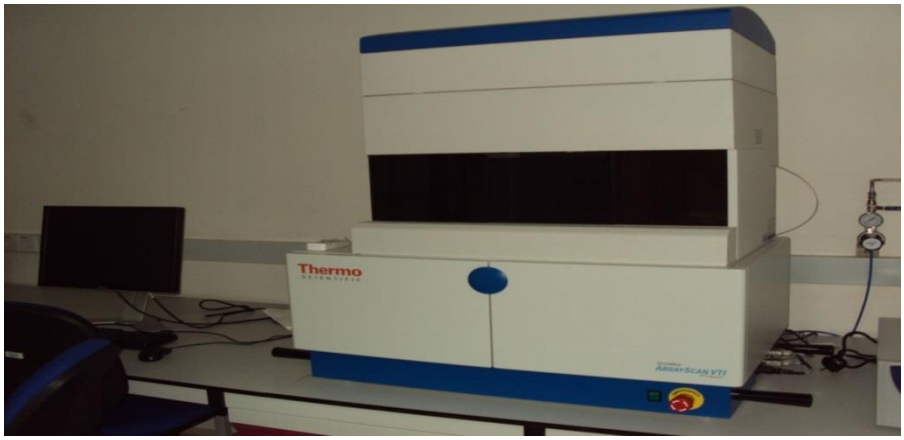


Figure (1-6): Array Scan high-content screening System, Thermo Scientific.

1.12 Aims of study

- Study the influence of tamoxifen on cytochrome P450 2d6 enzyme activity in sera of premenopausal patients with estrogen receptors positive receptors that use tamoxifen as a treatment in comparison with the enzyme activity in patients under tamoxifen and fluoxetine treatment breast cancer and healthy groups.
- Determination of cytotoxic effects of tamoxifen against MCF-7 breast cancer cell line.
- Evaluation of the caspase-9 activity to assess the effect of tamoxifen on CYP2D6 mechanism.
- Inspect the effects of tamoxifen on apoptosis mechanism in MCF7 cell line by HCS technique.

Chapter Two

Experimental Part

2.1 Equipments and apparatuses

Table (2-1): Instruments

Instrument	Suppliers
Micropipettes (8-12 channel)	Gilson, France
Centrifuge	Hittich Universal , Germany
Centrifuge	Becton Dickinson, England
Timer	Junghans, Germany
ELISA	Micro ELISA system(washer and reader) (Thermo, Germany)
Weight scale	Raven equipment limited, England
Length scale	Salter ,England
Incubator	Gallenkamp, United kingdom
Freezer	Haas , Saudi Arabia
CO_2 incubator	Gallenkamp (England)
High Content Screening Array Scan	Thermo Scientific (USA)
Spectrophotometer	Shimadzu(Japan)

Table (2-2): kits

Kit	Suppliers
cytochrome P450 2D6	Shanghai, China
MTT	Sigma, USA
Caspase-Glo ® 9	Promega, USA

HCS	Fisher-Thermoscintific, Japan
Cell line	ATCC, Manassas, VA, USA
Tamoxifen	AstraZenca UK
Doxorubicin	Midindia (India)
DMSO	Sigma (USA)

2.2 Subjects

2.2.1 Breast cancer patients

Sixty premenopausal Iraqi women breast cancer patients were selected according to positive estrogen receptor test, with collected from patients attended to Oncology Teaching Hospital, Medical City, in Baghdad, Iraq during the period from October 2015 to April 2016.

Recorded patients in the present study were subdivided into patients with hormone treatment and patients without any treatment. The study included group (P1) (35) female were treated with tamoxifen (20 mg), group (P2) (25) female were treated with tamoxifen and fluoxetine (20 mg). Their ages ranged from 25-48 years. All clinical screening was carried out by their physician's consultant.

2.2.2 Control subjects

The (30) Iraqi premenopausal control group (group C) with ages ranging from 25 to 48 year, were included in the study matched for age, gender and ethnic background (Iraqi). Control was chosen between subjects that apparently healthy in terms of

non-hypertensive, non-diabetic and with no family history breast cancer or other types of cancer. In addition, they had no history of alcohol consumption or smoking.

2.2.3 Characteristic of patients and control

Breast cancer patients and control were characterized in terms of age, sex, BMI, WHR. Cytochrome P4502D6 (CYP2D6) were measured for patients and control.

2.3 Materials and methods

2.3.1 Blood Sample

Blood samples were drawn using 5 ml syringes by vein puncture. A 5 ml of blood sample was drawn from each patient. The whole blood was directly transferred to a plain tube. These samples were allowed to stand for 10 minutes at room temperature for clotting; the tubes were centrifuged at 3000 (r.p.m) for 10 minutes. The serum was then transferred to other tube using micropipette and stored at -20 $^{\circ}$C until the time of analysis.

2.3.2 Anthropometric indices measurements

2.3.2.1 Body mass index (BMI)

Body Mass Index (BMI) is a simple index of weight over height that is generally used to classify overweight, underweight and obesity in adults. BMI is defined as the weight in kilograms divided by the square of the height in meters (kg/m^2) [109]. Correlation between obesity and BMI has been reported [110].

$$\text{BMI} = \frac{Weight\ Kg}{(Height\ \ m)2}$$

The World Health Organization (WHO) had established guidelines for optimal weight (18.5 - 24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (>30 kg/m^2) adults [111].

2.3.2.2 Waist-to-hip ratio (WHR)

Waist–hip ratio or waist-to-hip ratio (WHR) is the ratio of the circumference of the waist to that of the hips. The WHR has been used as an indicator or measure of the health of a person, and the risk of developing important health conditions Research appears that people with "apple-shaped" bodies (with more weight around the waist) face more health risks than those with "pear-shaped" bodies who carry more weight around the hips (Ratio of ≥0.85 indicate central obesity) [112].

$$WHR = \frac{Waist\ (cm)}{Hip\ (cm)}$$

2.4 Human cytochrome P450 2D6 (CYP2D6) ELISA kit

2.4.1 Principle of the assay

This kit uses enzyme-linked immune sorbent assay (ELISA) based on biotin double antibody sandwich technology to assay Human Cytochrome P450 2D6 (CYP2D6). Cytochrome P450 2D6 (CYP2D6) was added to wells that were pre-coated with Cytochrome P450 2D6 (CYP2D6) monoclonal antibody and then incubated. After incubation, anti CYP2D6 an antibody labeled with biotin was added to unite with streptavidin-HRP, which forms the immune complex. Unbound enzymes were removed after incubation and washing, and then substrate A and B were added. The solution will turn blue and change to yellow with the effect of acid. The shades of the solution and the concentration of Human Cytochrome P4502D6 (CYP2D6) are positively correlated.

2.4.2 Reagent composition

Table (2-3): Reagents and their Quantity

Reagents	Quantity
Standard solution(64U/L)	0.5ml×1
Streptavidin-HRP	6ml×1
Stop Solution	6ml×1
chromogenic reagent A	6ml×1
chromogenic reagent B	6ml×1
Anti CYP2D6 antibodies labeled with biotin	1ml×1
Standard dilution	3ml×1
Washing concentrate	(20ml×30)×1

2.4.3 Assay Procedure

a) The standard solutions were diluted in small tubes following the table below:

32U/L	Standard No.5	120µl Original Standard + 120µl Standard diluents
16U/L	Standard No.4	120µl Standard No.5 + 120µl Standard diluents
8U/L	Standard No.3	120µl Standard No.4 + 120µl Standard diluent
4U/L	Standard No.2	120µl Standard No.3 + 120µl Standard diluent
2U/L	Standard No.1	120µl Standard No.2 + 120µl Standard diluent

b) The number of stripes needed was determined by that of serum to be tested and the standards. It was recommended that each standard solution and each blank well be arranged with multiple wells as much as possible.

c) Sample injection:

1) Blank well: chromogen reagent (A and B) and stop solution were added, each other step operation was the same.

2) Standard solution well: 50µl of standard solution and 50µl Streptavidin-HRP solution were added (biotin antibodies have united in advance in the standard so no biotin antibodies were added).

3) Serum well to be tested: 40µl of the serum was added and 10µl of CYP2D6 antibodies solution and 50µl of the streptavidin-HRP solution. Then was covered with seal plate membrane. Then it was shaken gently and incubated at 37℃ for 1 hour.

d) Preparation of washing solution: The washing concentration (30X) was diluted with distilled water for later use.

e) Washing: The seal plate membrane was removed carefully, and then the liquid was dried and shaken off the remainder. Then, each well was filled with washing

solution, and left to stand for 30 seconds, and then drained. This procedure was repeated five times then bloated on the plate.

f) Color development:

First, 50µl chromogen reagent A was added to each well, and then 50µl of chromogen reagent B solution was added to each well. The solutions were shaken gently. Incubation for 10 minutes at 37℃ away from light was made for color development.

g) Stop: 50µl Stop Solution was added to each well to stop the reaction (color changes from blue to yellow immediately at that moment).

h) The blank well was taken as zero, the absorbance was measured (A) of each well one by one under 450 nm wavelength for 10 minutes after the addition stop solution.

i) According to standards concentrations and corresponding A values, the linear regression equation of the standard curve was calculated. Then according to the A value of serum, the concentration of the corresponding serum was calculated. The statistical software could also be employed.

2.4.4 Calculation

The concentration of cytochrome P450 (U/L) was calculated from the standard curve equation (R^2 =0.998) as shown (Figure 2-1).

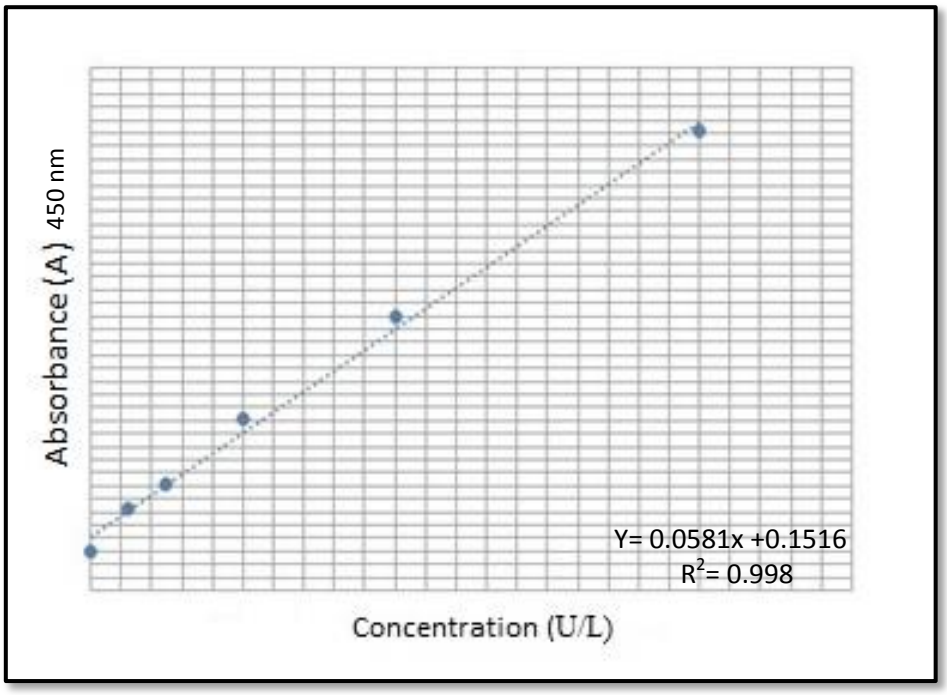


Figure (2-1): The standard curve of cytochrome P450 concentrations

2.5 Biological activity

Caspase-9, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and High content screening were carried out in the Centre of Biotechnology Researches, Al-Nahrain University, Baghdad and Centre for Natural Product Research and Drug Discovery, Department of Pharmacology, Faculty of Medicine, University of Malaya, Kuala Lumpur, respectively. The MCF7 cancer cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

2.5.1 MTT cytotoxicity Assay [113].

The cytotoxic influence of tamoxifen stock solution was performed by using MTT ready to use kit:

2.5.1.1 Kit contents

- MTT solution 1 ml x 10 vials.
- Solubilization solution 50 ml x 2 bottle.

2.5.1.2 Protocol:

- Tumor cell line ($1x10^4$ cells/well) were grown in 96 flat well micro-titer plates, in a final volume of 200 µl complete culture medium per each well.
- The plates were incubated at 37°C, 5% CO_2 for 24 hrs.
- After incubation, the medium was removed and two fold serial dilutions of Tamoxifen (0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 200 µg/ml) were added to the wells.
- Triplicates were used per each concentration as well as the controls (cells treated with serum free medium). Plates were incubated at 37°C, 5% CO_2 for selected exposure time (48 hours).
- After exposure time, 10 µl of the MTT solution was added to each well. Plates were further incubated at 37°C, 5% CO_2 for 2-4 hours.
- The media were carefully removed and 100 µl of DMSO solubilization solution was added per each well for 5 min.
- The absorbance was determined by using an ELISA reader at a wavelength of 570 nm. The data of optical density was subjected to statistical analysis in order to calculate the concentration of compounds required to cause 50% reduction in cell viability for each cell line.

2.5.2 Caspase-Glo® 9 luminescent assay kit

2.5.2.1 Kit Contents

- Purified caspase-9 enzyme
- Stabilizer to dilute purified enzyme (10 mM HEPES buffer (PH 7.4) with 0.1% prionex®)
- Caspase-9 inhibitor Ac-LEHD-CHO
- Caspase-Glo®9 buffer (1 X 10 ml)
- Caspase-Glo®9 substrate (1 bottle lyophilized)
- MG-132 Inhibitor (30 µl)

2.5.2.2 Principle of the assay

Directions are given for performing the assay in a total volume of 200µl using 96-well plates and a luminometer. However, the assay can be easily adapted to different volumes provided the 1:1 ratio of Caspase-Glo® 9 Reagent volume to sample volume is preserved. The protocol includes the option of preparing Caspase-Glo® 9 Reagent to include a proteasome inhibitor (MG-132), which when added to cell-based assays significantly reduces cellular non-specific background activity in cell-based assays, MG-132 Inhibitor can be added to Caspase-Glo® 9 Reagent. Thaw the tube of MG-132 Inhibitor was thawed, mixed, and added to the reagent.

2.5.2.3 Procedure

1. Purified caspase-Glo®9 was diluted in 10 mM HEPES buffer (PH 7.4) with 0.1% Prionex® stabilizer and examine in a total volume of 200µl per well in a 96-well plate.

2. The caspase-Glo®9 buffer and lyophilized caspase-Glo®9 substrate were equilibrated to room temperature before use.

3. The contents of the caspase-Glo®9 buffer bottle were transferred into the amber bottle containing caspase-Glo®9substrat. Mix by swirling the contents until the substrate is thoroughly dissolved to form the caspase-Glo®9.

4. The reagent was allowed to equilibrate to room temperature. Mix thoroughly after adding MG-132 inhibitor.

5. 96-well plates containing cells were removed from the incubator and allowed plates to equilibrate to room temperature

6. 100µl of caspase-Glo®9 reagents was added to each well of a white-walled 96-well plate containing 100µl of blank, treated cells in culture medium.

7. The plate was covered with a plate sealer.

8. Contents of wells were mixed gently using a plate shaker at (300rpm) for 30 seconds.

9. At room temperature was incubated for 30 minutes.

10. The luminescence was measured of each sample in a plate-reading luminometer.

2.5.3 High-Content Screening

2.5.3.1 Cytotoxicity 3 Kit

2.5.3.2 Kit Contents

- Cytochrome c Primary Antibody
- DyLight™ 649 Conjugated Goat Anti-Mouse IgG
- Mitochondrial Membrane Potential Dye
- Permeability Dye
- Hoechst Dye
- Wash Buffer (10X Dulbecco's PBS)
- Permeabilization Buffer (10X Dulbecco's PBS with 1% Triton® X-100)
- Blocking Buffer (10X)
- Thin Plate Seal Assembly

2.5.3.3. Preparation of solutions (per 96-well plate)

1X Wash Buffer	20 ml of 10X Wash Buffer was added to 180 ml ultrapure water. Store buffer at 4°C for up to 7 days.
Fixation Solution	3 ml of 16% paraformaldehyde solution was added to 9 ml of 1X Wash Buffer just before its used.
1X Permeabilization Buffer	1.5 ml of 10X Per meabilization Buffer was added to 13.5 ml of the 1X Wash Buffer. Stored at 4°C for up to 7 days.
1X Blocking Buffer	5 ml of 10X Blocking Buffer was added to 44 ml of 1X Wash Buffer. Stored at 4°C for up to 7 days.
Primary Antibody Solution	15 µl of the Cytochrome c Primary Antibody was added to 6 ml of 1X Blocking Buffer. Solution prepared just before each assay.
Secondary Antibody/ Staining Solution	0.6 µl of Hoechst Dye and 12 µl of the DyLight 649 Goat AntiMouse was added to 6 ml of 1X Blocking Buffer. Solution prepared just before each assay.

Live Cell Staining Solution	117 µl of DMSO was added to the Mitochondrial Membrane Potential Dye to make a 1 mM stock solution. Just before use, 2.1 µl of Permeability Dye and 21 µl of Mitochondrial Membrane Potential Dye were added to 6 ml complete medium pre-warmed to 37°C.

2.5.3.4 Method of HCS

1. Solutions of tamoxifen were diluted at different concentration (100, 50, 25, 12.5, 6.25µg/ml) was prepared and 25 µl was added to the MCF-7 cells. The cells were incubated at 37°C for 24 hours.

2. Fifty µl of live cell staining solution was added to each well.

3. The cells were incubated at 37°C for 30 minutes.

4. The medium and the staining solution and 100 µl/well of fixation solution and was plate incubated for 20 minutes at room temperature.

5. The fixation solution was gently aspirated and 100 µl/well of 1x wash buffer was added.

6. Wash Buffer was removed and 100 µl/well of 1X permeabilization buffer was added and incubated for 10 minutes at room temperature protected from light.

7. Permeabilization buffer was aspirated and washed plate twice with 100 µl/well of 1X wash buffer.

8. Wash buffer was Aspirate and 100 µl of 1X blocking buffer was added and incubated for 15 minutes at room temperature.

9. Blocking Buffer was aspirated and 50 µl/well of primary antibody Solution was added, Incubated for 60 minutes protected from light at room temperature.

10. The primary antibody solution was aspirated and plate washed three times with 100 µl/well 1x wash buffer.

11. Wash Buffer was aspirated and 50 µl/well of Secondary Antibody/Staining Solution was added. Incubated for 60 minutes protected from light at room temperature.

12. secondary antibody/staining solution was aspirated and washed plate three times with 100 µl/well of 1x wash buffer.

13. One hundred µl/well of 1x wash buffer was added.

14. The plate was sealed and evaluated on the array scan HCS reader.

15. Sealed plates were Stored in dark at 4°C. The plates were evaluated within 24 hours after assay completion.

2.6 Statistical analyses

A one-way analysis of variance ANOVA (Duncan) was performed to test whether group variance was significant ($p<0.01$) or not. A t-test was performed to analyze the statistical significance of the difference between group (C) and group (P). One-way analysis of variance (ANOVA) test was used to compare the parameters among groups (C), (P1) and (P2) followed by post hoc test. Data were expressed as a mean ±standard deviation and drowned using Graph Pad Prism version 6.

The Statistical Analysis System- SAS (2012) program was used to study the difference factors in study parameters. Least significant difference –LSD test was used to significant compared between means in this study [114].

Chapter Three

Results & Discussion

Results and Discussion

3.1 Characteristics of the patients and selecting parameters

Sixty Iraqi female breast cancer samples were included in this study. The mean age of the patients (39.72 ± 5.654) years old; ranging from (25-48) years old; ten cases were under (35) years old (16.67%); fourteen cases between (35-39) years old about (23.33%); thirty six cases were between (40-48) years old about (60%). Twenty five cases (41.67%) take inhibition treatment with tamoxifen. While the (mean ±SD) age of control (31.07 ±8.077) ranging from (25-48) years old; two cases were above (45) years old (8%); sixteen cases between (35-39) years old about (64%); seven cases were between (45-48) years old about (28%), shown in the figure and Table (3-1).

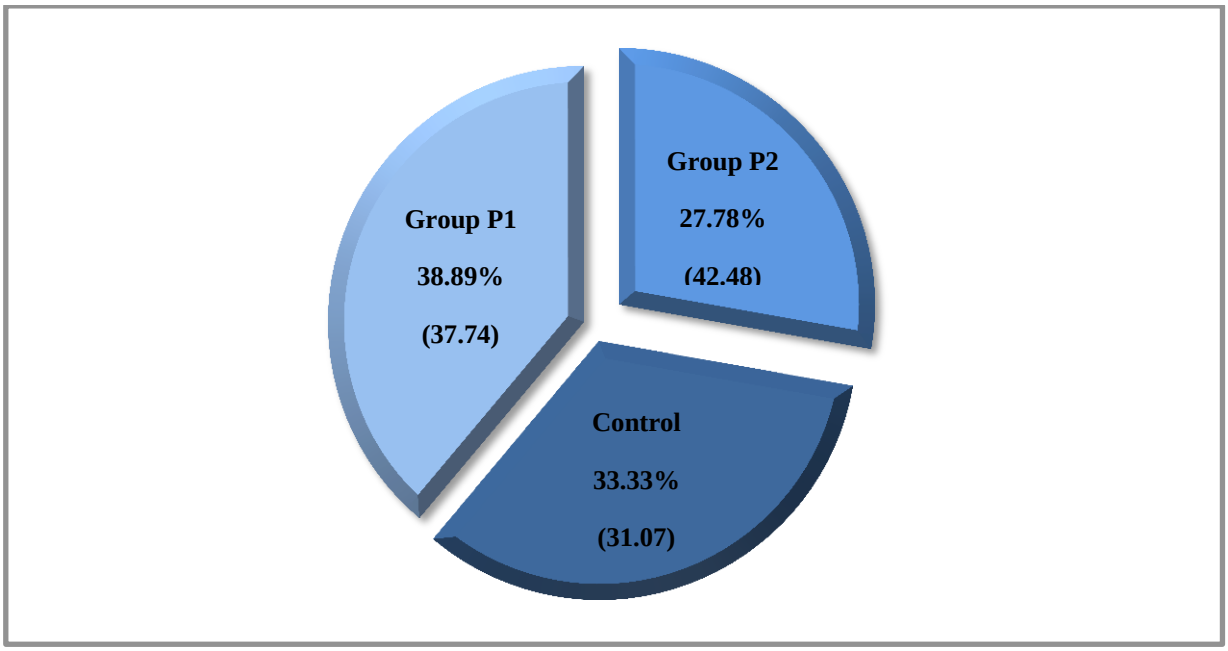


Figure (3-1): Distribution of study groups

3.1.1 Characteristic of patients and control groups

Patient's clinical data has been summarized in Table (3-1) figure (3-2) the mean age (31.07vs. 39.72), weight (67.80 vs. 81.58), BMI (25.18 vs. 31.05) and waist (77.93 vs. 87.92), showed a significant ($P<0.01$) increase of breast cancer patients group (P) ($P=0.00073$), ($P=0.00045$), ($P=0.00094$) and ($P=0.0017$) when compared with healthy control group (C) respectively. In contrast, the mean of WHR was (0.7516 and 0.7529) in the group (P) showed no significant differences ($P<0.01$) respectively, when compared with the group (C).

Obesity is characterized by an excess of the fat body. The most regularly used record being the body mass index (BMI) as weight in kilograms divided by height in meter squared [115]. The results showed a highly statistically significant increase ($p=0.00094$) between breast cancer risks in premenopausal female and body fat as shown in the table (3-1). The study agrees with the result by confirmed a direct association between BMI with the risk of developing the disease [116]. Other study indicated that women who are overweight or obesity before menopause have a 20-40 % higher danger of breast cancer than the individuals who are lean. Despite the fact that being overweight or fat may depress breast tumor hazard before menopause, overweight must be avoided [117].

Other study found that the body shape may also influence breast cancer hazard. Female who put on additional weight around their center sections (apple-shaped), instead of their hips and thighs (pear-shaped), have a little to direct expanded danger of breast cancer [118]. The clinical studies show that after WHR is checked, body shape does not build breast tumor risk [119].

Table (3-1): Characteristics of breast cancer patients

Parameter	Group (c) (n = 30)	Group (p) (n = 60)	P – value
Age (year)	31.07 ± 8.077	39.72 ± 5.654	0.00073
Weight (Kg)	67.80 ± 9.338	81.58 ± 12.74	0.00045
Height (cm)	164.3 ± 6.358	162.1 ± 6.343	N.S
BMI (kg/m^2)	25.18 ± 3.703	31.05 ± 4.470	0.00094
Waist (cm)	77.93 ± 9.563	87.92 ±15.46	0.0017
Hip (cm)	103.4 ± 8.811	104.9 ±12.3	N.S
WHR	0.7529 ± 0.06157	0.7516 ±0.04879	N.S
N.S= Non-significant			

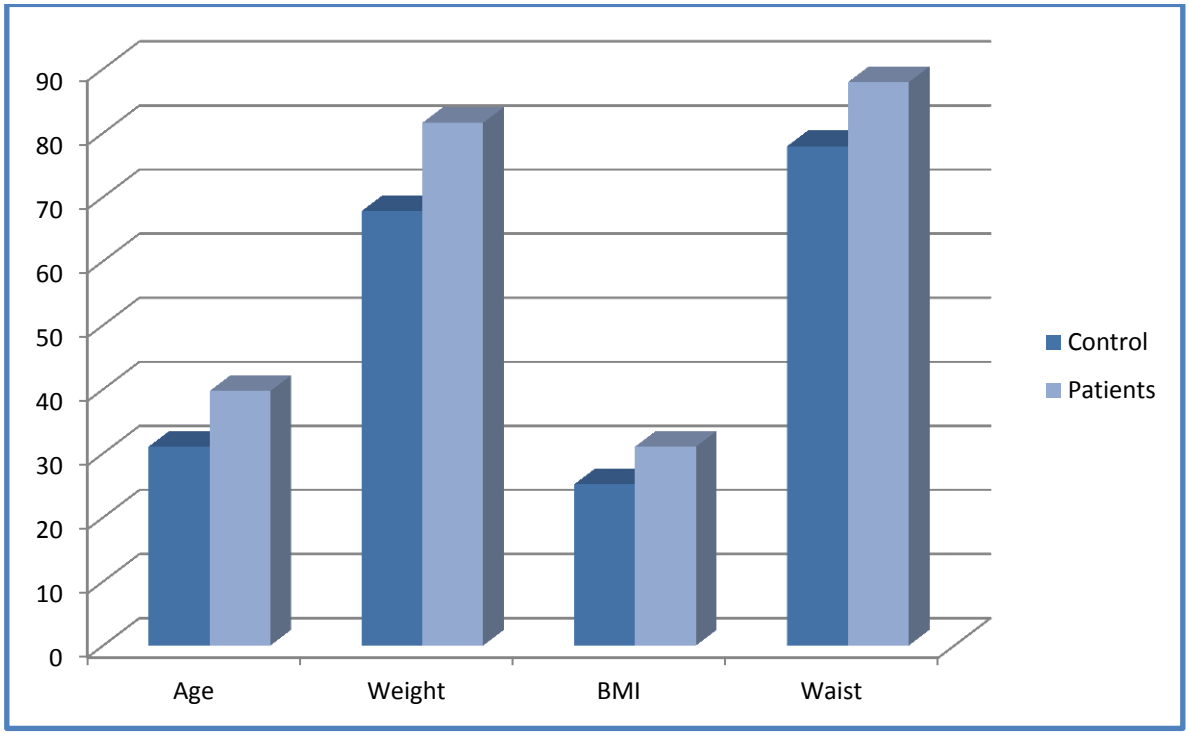


Figure (3-2): Mean distribution of age, weight, BMI and waist in studied patients and control groups

3.1.2 Level of serum cytochrome P450 2d6 (CYP2D6)

Parameter	Group (C) (n=30)	Group (P) (n=60)	P- value
CYP2D6 (U / L)	13.87 ± 2.479	26.88 ± 10.95	<0.0001

Table (3-2): Mean (±SD) level of serum CYP2D6 of control and patients

The result of cytochrome P4502d6 enzyme level in serum samples of breast cancer patients and control groups are shown in the table (3-2) and figure (3-3), revealed the presence of significant differences between groups (C), (P) (P=<0.0001). A significant increase was observed in the mean level of CYP2D6 in the group (P) in comparison with that of the (C) group (26.88 vs. 13.87 U / L) (P<0.0001).

In this experiment the highest level of the CYP2D6 enzyme was noticed after treatment with the tamoxifen, this indicated that the effect of tamoxifen was depending on the CYP2D6 activity. The CYP2D6 is responsible for the metabolic activation of tamoxifen to endoxifen [120]. In breast cells, tamoxifen acts as an antagonist and competes with estradiol to bind to the estrogen receptors (ER) and blocks the reproduction this slows or stops the growth of the tumor by preventing the cancer cells from getting the hormones they need to grow [121].

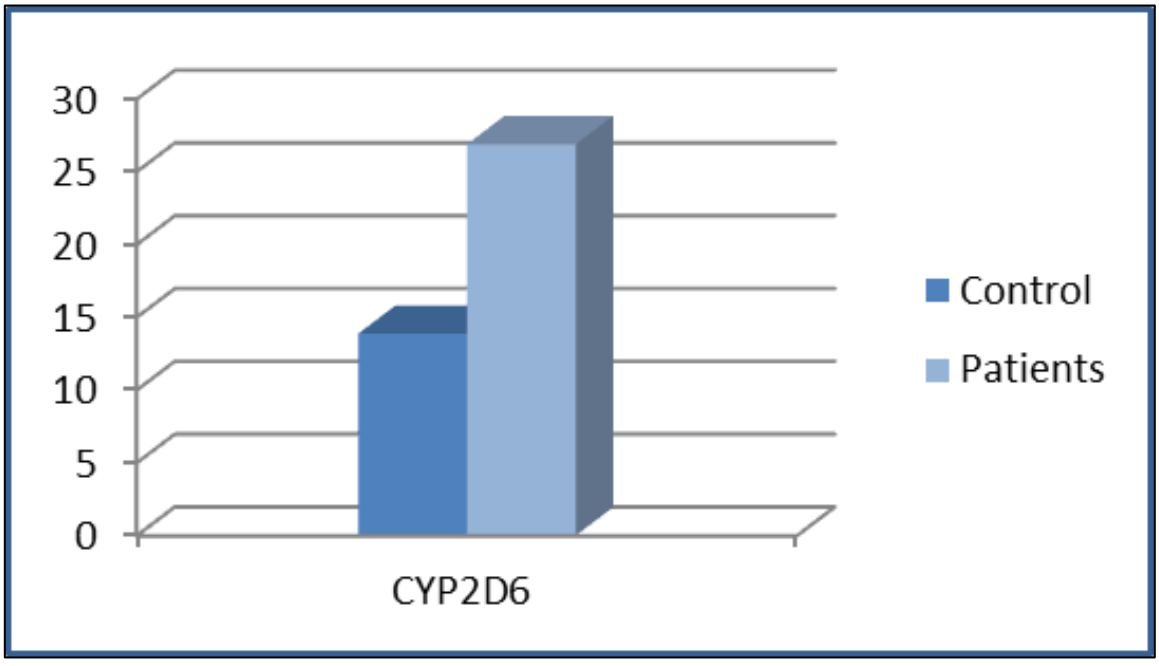


Figure (3-3): Mean distribution of CYP2D6 in studied groups patients and control.

3.2.1 Characteristic of studied groups

The results of this experiment are illustrated in table (3-3) figure (3-4), the result of mean age (37.74 and 42.48 **vs**. 31.07), weight (80.66 and 82.88 **vs.** 67.80) and BMI (30.3 and 32.1 **vs**. 25.18) showed significantly increase in two breast cancer patients female group (P2) and (P1) (P= <0.0001), separately in contrasted with control healthy group (C). BMI showed a significant increment when compared each group with control (P<0.0001).

Before menopause, the ovaries produced estrogens in the body and estrogens mainly come from fat tissue. Fat tissue converts androgens hormones to estrogens [122]. So, the overweight female has higher blood estrogen levels than slim female. Female have higher estrogen levels; have higher BMI are prone to increased risk of breast cancer compared to the female who have lower estrogen levels [123].

Table (3-3): Mean (±SD) level of age, BMI, WHR, in (C), (P1) and (P2) groups.

Parameters	Group (C) (n=30)	Group (P1) (n=35)	Group (P2) (n=25)	P- value
Age (year)	31.07 ±8.077	37.74 ±6.099 a**	42.48 ±3.501 b*** c*	<0.0001
Weight (Kg)	67.80 ± 9.338	80.66 ±10.41 a**	82.88 ±15.57 b**	<0.0001
Height (cm)	164.3 ± 6.358	163.2 ±5.778	160.5 ±6.881	NS
BMI (kg/m^2)	25.18 ±3.703	30.3 ±3.581 a****	32.1 ±5.383 b****	<0.0001
Waist (cm)	77.93 ± 9.563	78.40 ±12.79	80.08 ± 12.69	NS
Hip (cm)	103.4 ±8.811	104.3 ±12.97	105.7 ± 11.51	NS
WHR	0.7529±0.06157	0.7489±0.03959	0.7553 ±0.06005	NS

P***<0.001
a) Indicate significant difference between groups (C) and (P1)
b) Indicate significant difference between groups (C) and (P2)
c) Indicate significant difference between groups (P1) and (P2)

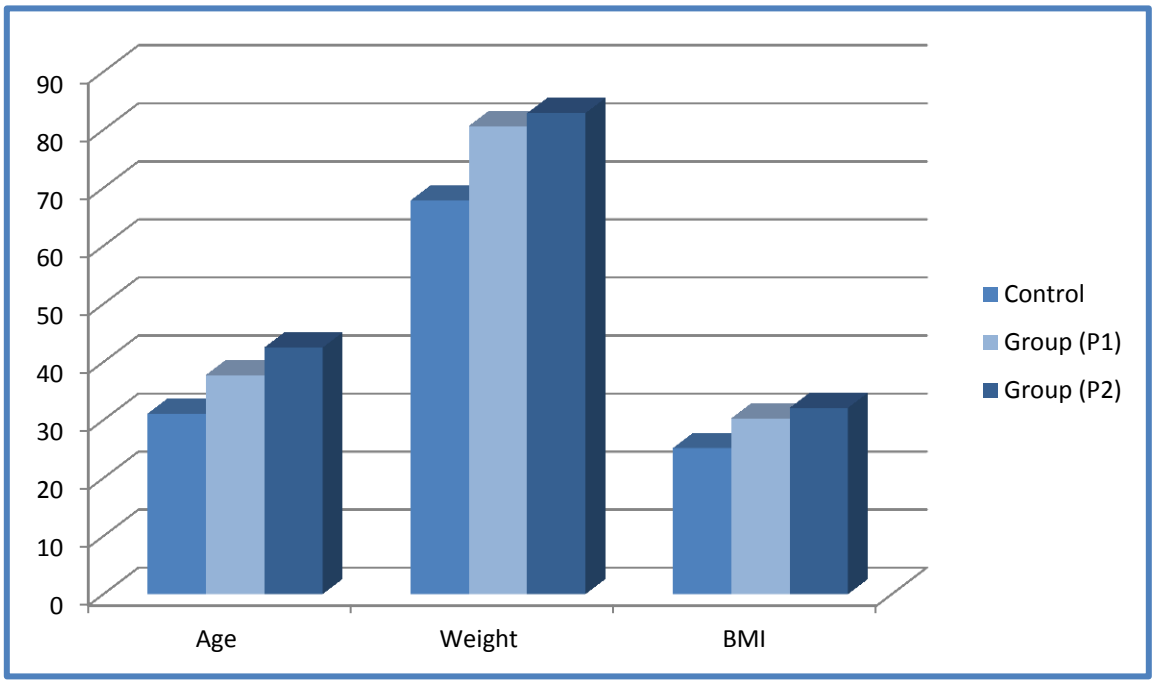


Figure (3-4): Mean distribution of age, weight and BMI in studied groups (P1), (P2) and control

3.2.2 Level of serum cytochrome P450 2d6 (CYP2D6)

The result of CYP2D6 enzyme level in serum samples of two different breast cancer groups and control group are shown in the table (3-4) and figure (3-5). Significant differences between groups (C), (P1) and (P2) (P=<0.0001) were found. A significant increment in the mean level of CYP2D6 was observed in the group (P1) in compared to the control group (32.2 vs. 13.87U/L) (P<0.0001). A significant increase in the mean level of CYP2D6 was also observed in the group (P1) in comparison with a group (P2) (32.2 vs. 19.43 U/L) (P< 0.0221).

This enzyme (CYP2D6) was selected because it is responsible for the metabolism of more than 25% of the available pharmaceuticals. There are treatments can interfere with CYP2D6 action and must be avoided when tamoxifen is taken. For example, some antidepressants, such as fluoxetine (Prozac), can interact with

CYP2D6 and affect how tamoxifen works in the body [124]. This drug falls within selective serotonin reuptake inhibitors (SSRIs) have the capacity to decrease the efficacy of tamoxifen treatment, and can inhibit CYPs [125].

The results of this study show that tamoxifen has potent effect by increasing the CYP2D6 enzyme activity which appears clearly in a group (P1) when compared with control group, which is agree with the results of Zeruesenay Desta study [126]. While group (P2) show the significant decrease in the CYP2D6 enzyme activity when compared with a group (P1), which is compatible with the results of Matthew P. Goetz study [127].

Table (3-4): Mean (±SD) level of serum CYP2D6 of (C), (P1) and (P2) groups.

Parameter	Group (C) (n=30)	Group (P1) (n=35)	Group (P2) (n=25)	P- value
CYP2D6 (U/L)	13.87 ±2.479	32.2 ±11.49 a****	19.43 ±2.962 b* c****	<0.0001
P***<0.001 a) Indicate significant difference between groups (C) and (P1) b) Indicate significant difference between groups (C) and (P2) c) Indicate significant difference between groups (P1) and (P2)				

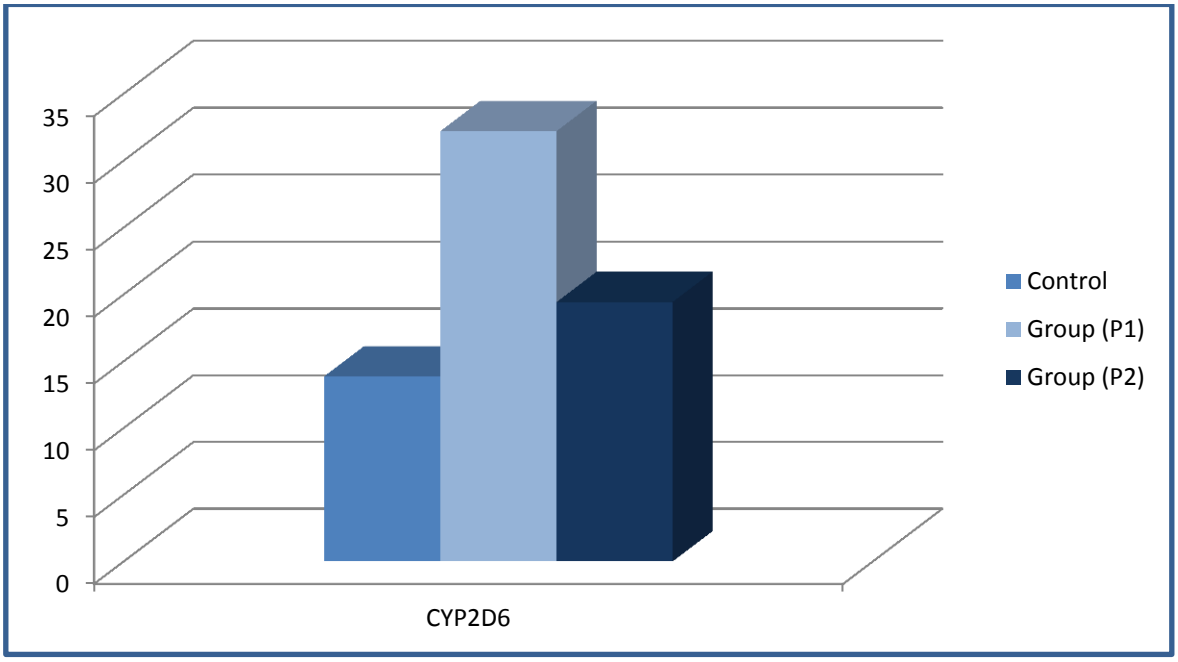


Figure (3-5): Comparison of the mean level of CYP2D6 between the three groups (C), (P1) and (P2).

3.3 Cytotoxic effect of tamoxifen on MCF7 cell line, *in-vitro* study viability assay.

The cytotoxic effect of the tamoxifen was evaluated against breast cancer cells (MCF-7) by using MTT method. The viability rates of MCF-7 cells treated with tamoxifen were shown in figure (3-6).The results indicated that 24 h after the administration of different concentrations of tamoxifen to MCF-7 cells, viability values were 15.28%, 15.60%, 15.29%, 14.58%, 14.42%, 27.65%, 69.65%, and 85.73% respectively, which considered as 100% (Figure 3-6). Nevertheless, the tamoxifen significantly showed the highest cytotoxic activity (15.29% at 50µg/ml) among other concentrations. The tamoxifen significantly showed the highest cytotoxic activity with the IC_{50} value of (4.506µg/ml).

Table (3-5): Cytotoxicity effects of tamoxifen (at different conc.) against MCF-7 tumor cell line viability.

Concentrations µg/ml	Viability % ±SD MCF-7
200	15.28 ±0.3960 [a]
100	15.60 ±0.8365 [a]
50	15.29 ±0.3953 [a]
25	14.58 ±0.06293 [a]
12.5	14.42 ±0.1591 [a]
6.25	27.65 ±3.366 [a]
3.125	69.65 ±8.683 [b]
1.5625	85.73 ±4.617 [c]
0.78125	100.0 ±0.0 [d]
Letters [a,b,c,d] refers to significant result at $p \leq 0.05$	

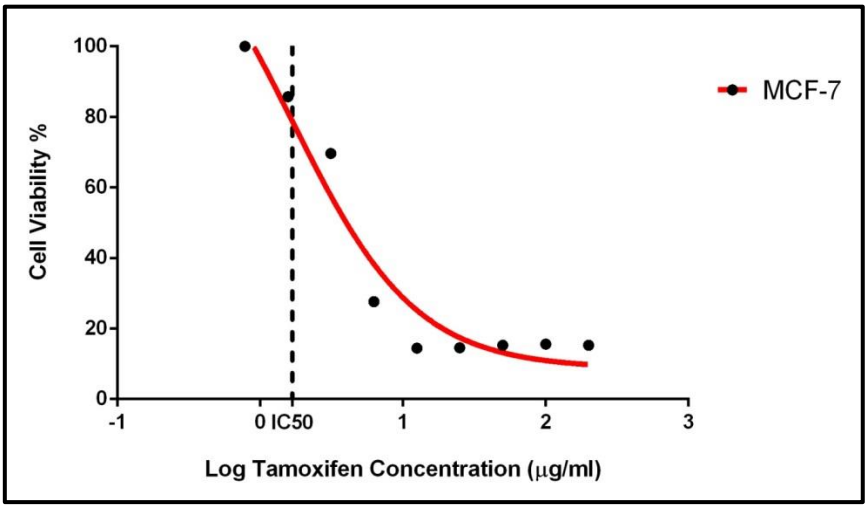


Figure (3-6): Cytotoxicity effect of tamoxifen on MCF-7 cells after incubation for 24 hours at 37ºC.

3.4 Cytotoxic Effect of tamoxifen on MCF7 cell line, *in-vitro* study by using Caspase -9 activity

Caspase is a family of cysteine proteases that is split into executioner caspases such as caspase (-3 or -7), and initiator caspases, such as caspase (-8 and -9). Initiator caspase-8 is activated through extrinsic pathway, while caspase-9 is activated in the case of mitochondrial cytochrome C leakage [128].

The results of caspase-9 activity reveal that there is a significant increase in mean activity which was (15331, 105428) in MCF-7 cells treated with 2.5µg/ml and 5µg/ml of tamoxifen respectively at IC_{50} (4.506 µg/ml) (Figure 3-7) and table (3-6). The caspase-9 activity are directly correlated to the toxic effect of the tamoxifen tested because tamoxifen at pharmacological concentrations has an effect on cell membranes associated with rapid changes in membrane permeability, which leads to decreased viability and death of the cells [129], also it had been shown to rapidly inhibit estrogen-dependent protein kinase C in MCF-7 cells [130]. Tamoxifen induces a rapid mitochondrial death program in estrogen receptors-positive breast cancer cell lines [131], and the significant decrease in mean activity was (27541) treated with 10 µg/ml of tamoxifen. This study revealed that tamoxifen is a potent antagonist of estrogen and induces apoptosis in ER+ cells; however, at concentrations higher than 5µg/ml, it shows an estrogenic behavior and acts as an agonist of estrogen [132]. There is compelling evidence that the majority of cytotoxic drugs initiate apoptosis by triggering the cytochrome C/Apaf-1/Caspase-9-dependent pathway through the mitochondrial (intrinsic pathway) [133].

Table (3-6): Tamoxifen effect on caspase-9 activity in MCF-7 cells after incubation for 24 hours at 37ºC.

Concentrations Tamoxifen	Caspase (Mean ± SD) Luminoscence
2.5 µg/ml	15331 ±5625 [c]
5 µg/ml	105428 ±2462 [a]
10 µg/ml	27541 ±2155 [b]
0 µg/ml DMSO	2365 ±633.1[d]
LSD value	982.073 *
Letters [a,b,c,d] refers to significant result at $p< 0.05$ LSD= Least Significant Difference	

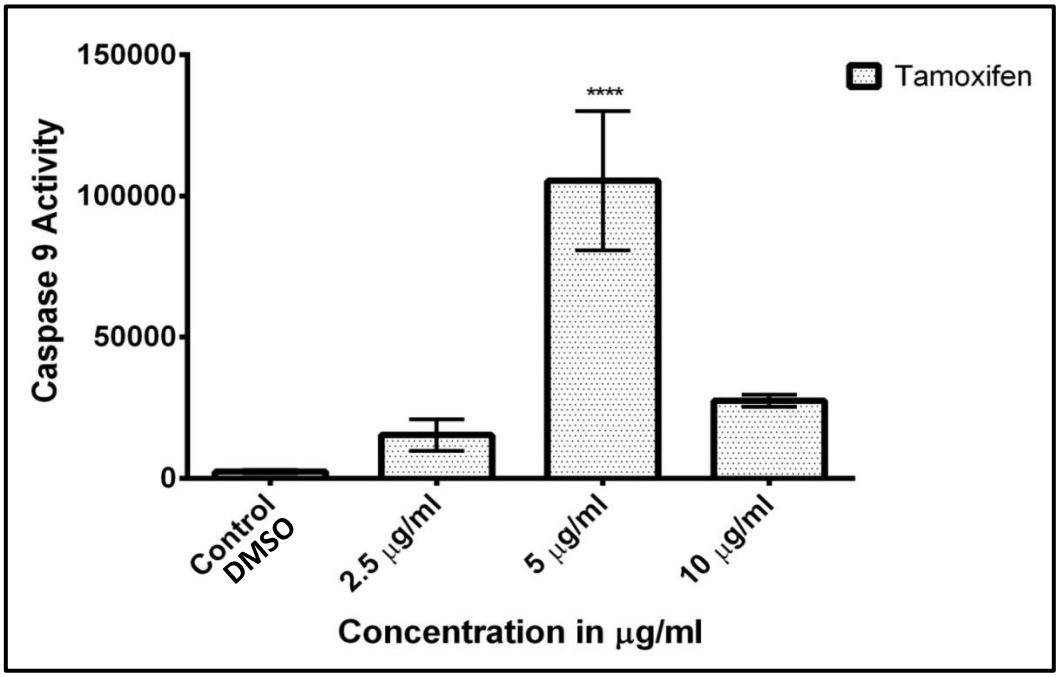


Figure (3-7): The Caspase-Glo luminoscence of MCF-7 treated with tamoxifen.

3.5 Cytotoxic Effect of tamoxifen on MCF7 cell line, *in-vitro* study by using High-Content Screening (HCS).

High-Content Screening (HCS) is a cellular imaging- based approximately, that played a key role in the revelation of toxicity and sorting of compounds depend on observed patterns of reversible and irreversible cellular injury. It provides multipara metric analysis of compound toxicity at the level of individual cells [134].

Table (3-7) appeared that 100 µg/ml has the highest significant effect on the four parameters, which were valid cell count, nuclear intensity, mitochondrial membrane potential and cytochrome C when compared with Doxorubicin 20 µM (Substance act as anti-cancer) which represent the positive control ($p<0.01$). 12.5µg/ml and 6.25 µg/ml appeared results close to those of the untreated cells which act as a negative control with very few significant differences.

The cytotoxic outcomes of MCF-7 cell- tamoxifen interaction were evaluated by High-content Screening Analysis after 24 hours of exposure. Hoechst dye enables monitoring of cell loss, nuclear morphology changes and DNA content which are proportional to the total Hoechst intensity per nucleus. Several cellular parameters (such as cell viability, nuclear morphology, cell membrane permeability, mitochondrial membrane potential changes, and cytochrome C localization and release from mitochondria) were measured via the HCS System.

Table (3-7): Cytotoxicity effect of tamoxifen on multi cellular parameters during 24 h. of incubation at 37° C.

Concentration	HCS Parameter (Mean ±SD)				
	Cell Viability	Nuclear intensity	Cell permeability	MMP*	Cytochrome c
100 µg/ml	854.5 ±181.7 C	661.5 ±67.18 b	112.0 ±12.73 b	174.0 ±24.04 d	421.0 ±55.15 b
50 µg/ml	974.0 ±73.54 b c	504.5 ±7.778 c	86.00 ±4.243 c	299.0 ±11.31 c	283.5 ±23.33 c
25 µg/ml	940.5 ±16.26 b c	407.5 ±10.61 d	61.50 ±21.92 d	418.5 ±16.26 a	191.0 ±36.77 d
12.5 µg/ml	1039 ±86.97 B	422.5 ±3.536 d	70.00 ±18.38 c d	416.0 ±18.38 a	225.0 ±14.14 d
6.25 µg/ml	1073 ±66.47 B	418.5 ±3.536 d	85.50 ±7.778 c	376.5 ±47.38 b	211.5 ±26.16 d
20 µg/ml Doxorubicin	519.5 ±89.80 D	951.5 ±54.45 a	187.5 ±17.68 a	99.00 ±5.657 e	650.0 ±70.71 a
0 µg/ml Untreated cell	1279 ±5.657 A	402.0 ±16.97 d	72.00 ±8.485 c d	384.0 ±41.01 b	212.5 ±31.82 d
LSD value	178.371 **	48.227 **	22.194 **	46.842 **	48.053 **

** =(P<0.01) Similar letters mean the absence of significant differences

LSD = Least Significant Difference

*= mitochondrial membrane potential

3.5.1 Cell Viability

Cell viability is defined as the number of healthy cells contained in a sample. The cell viability measurement plays a fundamental function in all forms of cell culture. Sometimes it is the main purpose of the experiment, for example in toxicity assay [135].

The results of cell viability reveal that there is a significant reduction in cell viability which were 33.19%, 23.85%, 26.46%, 18.76%, 16.11% in MCF-7 cells treated with 100µg/ml, 50µg/ml, 25µg/ml, 12.5 µg/ml and 6.25 µg/ml of tamoxifen respectively (Figure 3-8) and table (3-7) when compared with negative control. The changes in cell viability were directly correlated to the toxic effect of the tamoxifen tested. The differences between standard (+ve control) and all experimental groups were statistically significant ($P<0.01$).

The most significant reduction ($P<0.01$) in cell count was at the 100µg/ml of tamoxifen, were it down to 854.5 cells when compared to 519.5 cell count at 20µg/ml doxorubicin as a standard.

It was found that MCF-7 cell viability was dose dependent after 24 hours of treatment with different concentrations of tamoxifen, which affect the cellular survival of MCF-7 cell and reduced the cell count [136].

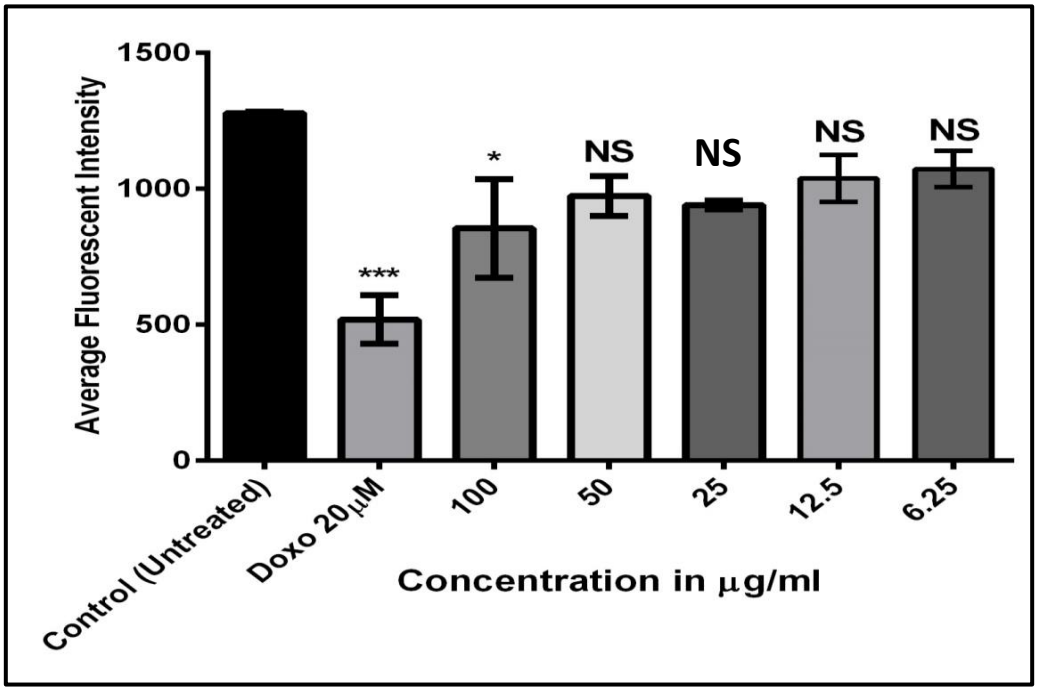


Figure (3-8): Tamoxifen effect on cell viability in MCF7 cell line after 24 hours of incubation at 37º C and evaluated by the Array Scan HCS Reader.

3.5.2 Nuclear Intensity

Nuclear morphology is apoptosis hallmarks in the nucleus [137]. A nuclear morphological change of MCF-7 breast cancer cells was examined by staining the cells with Hoechst 33342 dye.

This study was examined a nuclear morphological changes of MCF7 breast cancer cells that treated with tamoxifen by staining the cells with Hoechst 33342 dye. The results of nuclear intensity as shown in Finger (3-9) showed that MCF-7 nuclear intensity increase significantly when treated with 100, 50, 25, 12.5 and 6.25 μg/ml of tamoxifen and the increasing percentage of nuclear intensity were 64.55%, 25.49%, 1.36%, 5.10% and 4.10% respectively, the influence of tamoxifen was dose dependent.

The nuclear intensity, corresponding to apoptotic changes was significantly increased to 64.55% after tamoxifen treatment in breast cancer cells when compared with 20µg/ml standard, also with other concentrations as shown in Figure (3-9), While at concentration 50, 25, 12.5 and 6.25 µg/ml of tamoxifen wasn't found any significant difference between each other, but shown a significant when compared with 20µg/ml standard [138].

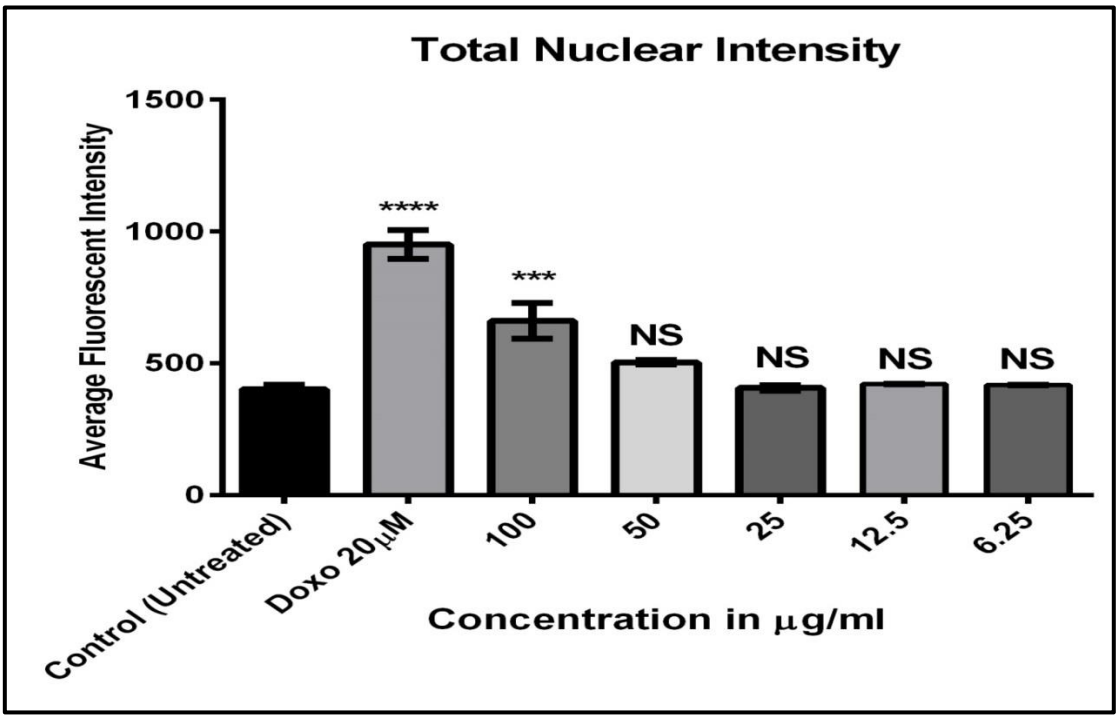


Figure (3-9): Tamoxifen effect on Nuclear Intensity of MCF7 cell line after 24 hours of incubation at 37º C. and evaluated by the Array Scan HCS Reader.

3.5.3 Cell Membrane Permeability

They are employed in particular to give an indication of cell membrane integrity. Because of their charged or polar nature, reporter dye molecules are unable to penetrate intact membranes but able to traverse appropriately damaged ones [139]. This was used in the present study as a key parameter for the evaluation of the cell compound interaction, the high doses of the tamoxifen increased cell membrane blebbing leading to increasing cell membrane permeability. The dose-dependent increase in cell membrane permeability was in best significant at 55.56% compared to standard at 20 μg/mL and with other concentrations when ($p<0.01$), as shown in (Figure 3-10). It has been reported that changes in cell membrane permeability often associated with a toxic or apoptotic response, and the loss of cell membrane integrity is a common phenotypic feature of marked cytotoxicity [140].

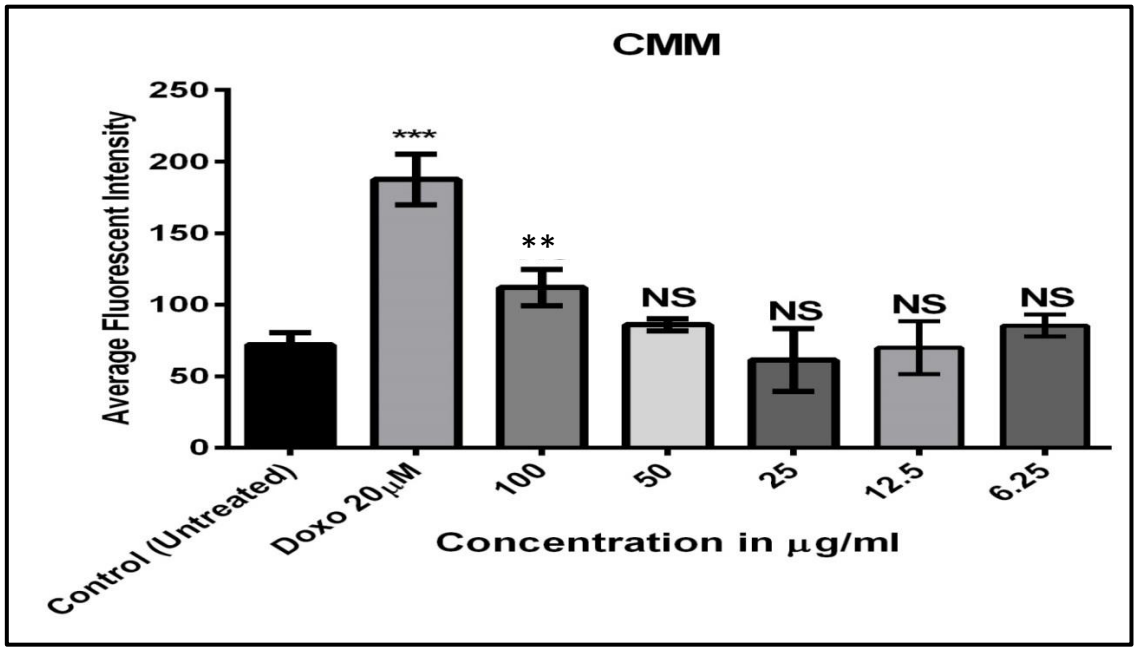


Figure (3-10): Tamoxifen effect on cell membrane permeability in MCF-7cell line after 24 hours of incubation at 37º C. evaluated by the Array Scan HCS Reader.

3.5.4 Mitochondrial membrane potential

Membrane potential is a central characteristic of healthy mitochondria, and membrane depolarization is a perfect indicator of mitochondrial dysfunction, which is increasingly implicated in drug toxicity [141]. To efficient characterization of the cell death signaling events in tamoxifen toxicity, this study investigated the drug effect on changes in mitochondrial membrane permeability.

The results from the figure (3-11) and figure (3-13) revealed that 100, 50, 25, 12.5 and 6.25µg/ml of tamoxifen caused 54.68%, 22.13%, 8.98%, 8.33% and 1.95% decrease in mitochondrial membrane potential intensity respectively, and the effect of tamoxifen was dose dependent. The dose-dependent increased mitochondrial membrane permeability was detected the best significant at 54.68% compared to standard at 20 µg/mL as shown in figure (3-11).

This may be due that cells undergo either apoptosis or necrosis accompanied by changes in mitochondrial function, resulting in loss of mitochondrial membrane potential and release of cytochrome C from mitochondria [142].

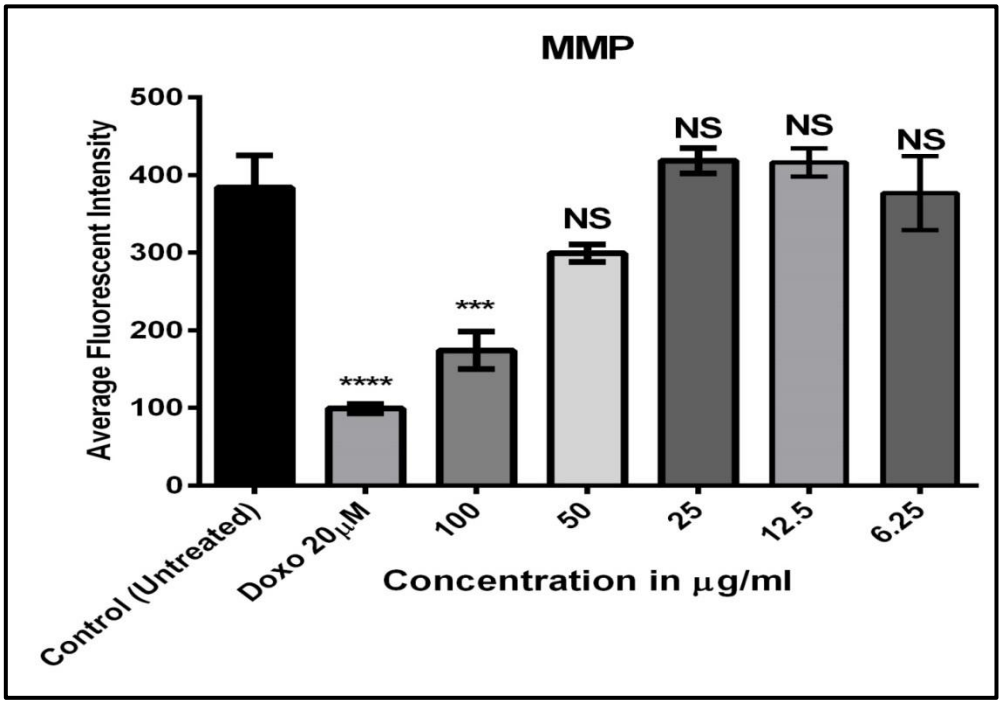


Figure (3-11): Tamoxifen effect on mitochondrial membrane potential in MCF7 cell line after 24 hours of incubation at 37º C. evaluated by the Array Scan HCS Reader.

3.5.5 Cytochrome C Releasing

Cytochrome C (Cyt C) is a major component of the electron transport chain, which is loosely binding on the outer layer of the inner mitochondrial membrane cytochrome C plays an essential role in apoptosis [143]. Results of cytochrome C releasing showed in table (3-7), figure (3-12) and figure (3-13) rise significantly with the increasing of concentration when compared with –ve and the concentrations of increasing tamoxifen concentrations were 98.12%, 33.41%, 10.12%, 5.88% and 0.47% for 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml and 6.25µg/ml respectively ($p<0.01$). The results of this study observed that tamoxifen dose-dependently induced cytochrome C significant release 98.12% compared with

standard at 20 µg/ml as shown in (figure 3-12).These effects may occur by activation of caspase-9 leading to the caspase-3 formation which in turn cleaves target that causes apoptosis and increased phosphorylation of extracellular signal-regulated kinase (ERK) [144].

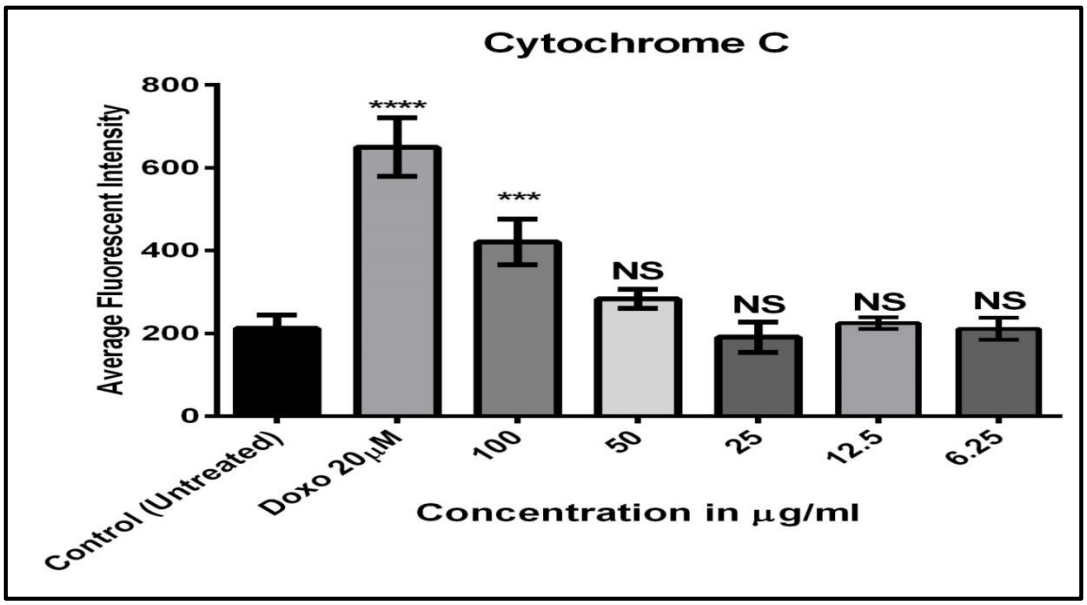


Figure (3-12): Tamoxifen effect on Cytochrome C in MCF7 cell line after 24 hours of incubation at 37º C. evaluated by the Array Scan HCS Reader.

A clear cytotoxic effect was observed at 100 µg/ml of tamoxifen. At this concentration, 33.19% cell loss was observed and 64.55% changes in nuclear morphology occurred. Used these results were as-a key parameters for the evaluation of cell- tamoxifen interaction.

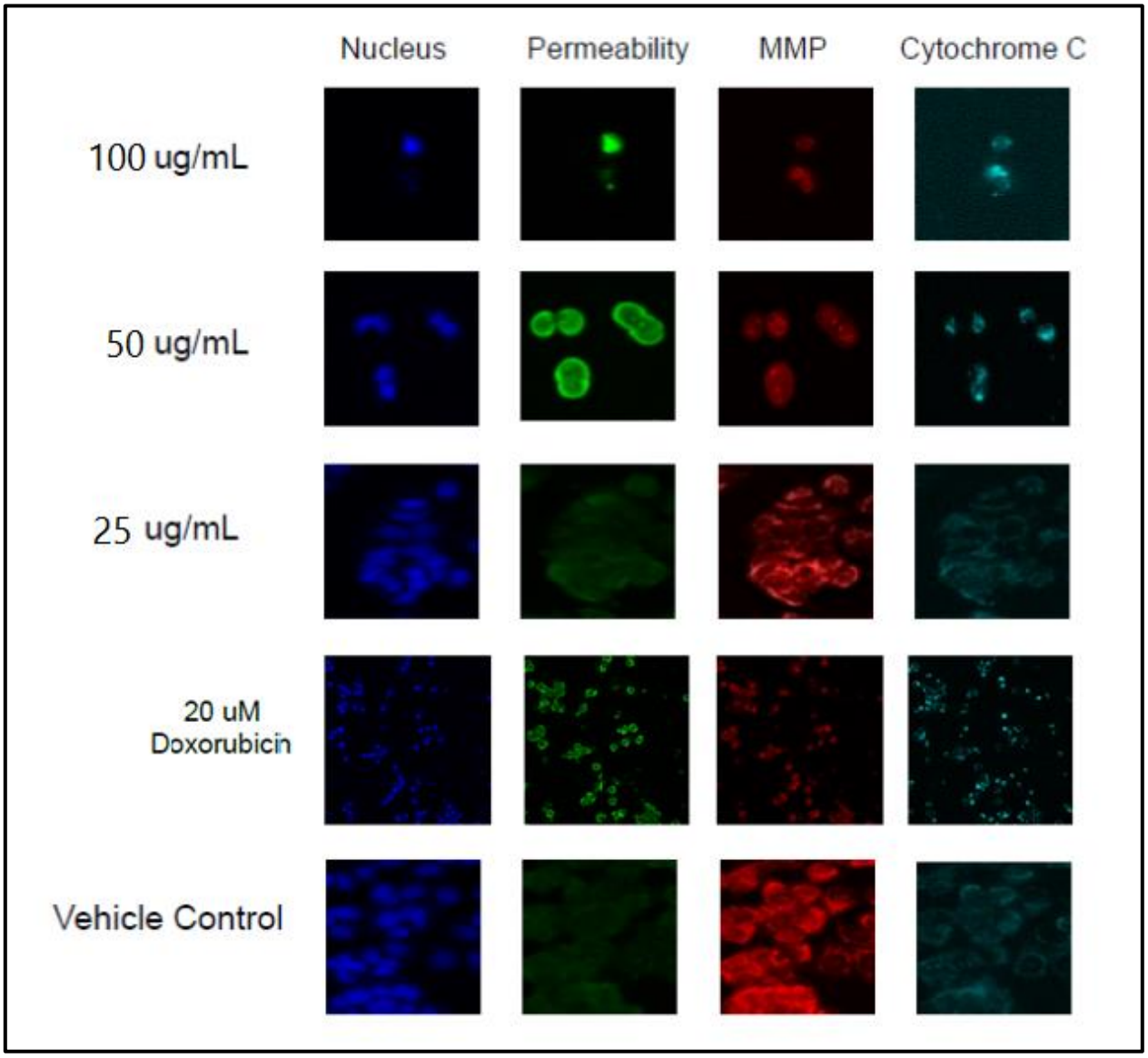


Figure (3-13): Multiparameter cytotoxicity (HCS) analysis of tamoxifen-treated MCF-7cell line after 24 hours of incubation at 37 ° C. Cells were stained with Hoechst 33342 dye which enables monitoring of cell loss, nuclear morphology changes, which is proportional to the total Hoechst intensity per nucleus. Cells were also stained with permeability dye (Excitation 491/Emission 509), which enables monitoring of membrane permeability, and cells were stained with MMP dye (Excitation552/Emission 576) for mitochondrial membrane potential changes, and cytochrome C antibody.

Conclusions & Recommendation

1. Conclusions

a) In patients treated with tamoxifen, the measurement of CYP2D6 activity showed a good indicator of breast cancer

b) Tamoxifen possess cytotoxic effect against breast cancer cells (MCF-7), as determined *in-vitro* by MTT assay (4.506 µg/ml).

c) A toxic effect of tamoxifen has been demonstrated on MCF-7 cell line by using caspase-9 at IC_{50} concentration (4.506 µg/ml)

d) From HCS technique results; tamoxifen showed toxic effect toward MCF7 cell line at (100µg/ml) tamoxifen concentration in a dose-dependent manner with increase in cell membrane permeability, cytochrome C, nuclear intensity, and change in mitochondrial membrane potential and decrease in cell viability level.

e) In tamoxifen treated patients, CYP2D6 is an autonomous predictor of breast cancer result in pre-menopausal women receiving tamoxifen for early breast cancer and it appears that CYP2D6 inhibitors should be avoided in tamoxifen-treated women.

2. Recommendation

a) Role of CYP2D6 polymorphism in response with tamoxifen.

b) Biochemical study of effect of tamoxifen on apoptotic gene product.

c) Study the effect of cytochrome P450 3A4 enzyme activity in breast cancer premenopausal.

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الخلاصة

مقدمة:

يعتبر العلاج الهرموني اول علاج يستهدف السرطان بشكل مباشر ويعرف ايضا بعلاج القمع الاستروجيني، غالبا ما يستخدم بمثابة علاج مساعد للحد من خطر الاصابة بالسرطان مرة أخرى بعد الجراحة أو للحد من السرطان المنتشر. التاموكسفين هو عقار يستخدم في علاج سرطان الثدي المبكر في النساء اللواتي سبق وتم علاجهم بالجراحة، والاشعاع، والعلاج الكيميائي. يستخدم التاموكسفين لتقليل مخاطر الاصابة بسرطان الثدي في النساء.

تهدف هذه الدراسة الى تقييم تاثير علاج التاموكسفين على فعالية أنزيم CYP2D6. وأيضا، لدراسة تأثيره السمي على الخلية بتطبيقه على خط الخلايا السرطانية MCF-7 ودراسة آلية موت الخلايا المبرمج لخلية سرطان الثدي.

العينات، المواد وطرق العمل:

تضمنت هذه الدراسة (٩٠) امرأة قبل سن الياس ، تراوحت أعمارهم من (٢٥-٤٨) سنة. (٣٠) عينة اصحاء تم اختيارهم كمجموعه سيطره (مجموعه C) و (٦٠) عينة لمريضات مصابات بسرطان الثدي ذو مستقبلات استروجينيه موجبة (مجموعه P) تم تقسيمهم الى مجموعتين وفقا لنوع العلاج : (٣٥) مريضا تمت معالجتهم بعقار التاموكسفين مجموعة (P1) و (٢٥)مريضا تمت معالجتهم بعقار الفلوكستين بالاضافة الى التاموكسفين (مجموعة P2). في الجزء الاول من الدراسة تم تحديد مستوى انزيم CYP2D6 كميا للمرضى ومجموعة التحكم بواسطة فحص (ELISA). اما في الجزء الثاني من الدراسة فقد تم تحديد التأثير السمي للتاموكسفين على خلايا سرطان الثدي MCF-7 باتستخدام Cytotoxic Assay والتحقيق في الية موت الخلية المبرمج للخلية الاكثر تحسسا للتاموكسفين باستخدام تقنية High-content screening (HCS) و Caspase-9 activity.

النتائج:

توصلت هذه الدراسة الى النتائج التالية:

الجزء الاول

- زيادة معنوية في معدل BMI لوحظ في مجموعة (P1)و مجموعة (P2)بالمقارنة مع مجموعة (C) (30.3 و 32.1 مقابل 25.18 كغم/مترالمربع)
- معدل مستوى السايتوكروم P450 2D6 المصلي اظهر زيادة معنوية في المجموعة (P1) والمجموعة (P2) بالمقارنة مع مجموعة (C) (32.2 و 19.43مقابل 13.87وحدة/لتر)، بينما اظهرت نقصان معنوي في معدل المجموعة (P2) بالمقارنة مع مجموعة (P1).

الجزء الثاني

- إظهرالتأثير السمي في خط الخلية MCF-7 عند معاملتها بالتاموكسفين ب IC_{50} قيمته (4.506 µg/ml) الاكثر تأثراً بالسمية تجاه خط الخلية السرطانية MCF-7 لمدة 24 ساعة.
- تم الكشف عن تأثير عقار تاموكسيفين على آلية موت الخلايا المبرمج وبتزايد كبير في فعالية كاسباس-٩، الذي لوحظ في تركيز 5 µg/ml في خط الخلية السرطانية MCF-7 و IC_{50} بقيمة (4.506 µg/ml).
- اظهر تأثير التاموكسفين على إلية الموت المبرمج للخلية زيادة معنوية مع الزيادة في تركيز المركب على نفاذية غشاء الخلية، السايتوكروم C و كثافة النووية عند تركيز100 ميكروغرام/مليليتر وذلك بالمقارنة مع 20 ميكروغرام/مليليتر من الدوكسوروبسين كمادة قياسية.
- اظهر تأثير التاموكسفين على إلية الموت المبرمج للخلية نقصان معنوى مع الزيادة في تركيز المركب على بقاء الخلية ونفاذية غشاء المايتوكوندريا عند تركيز 100 ميكروغرام/مليليتر وذلك بالمقارنة مع 20 ميكروغرام/مليليتر من الدوكسوروبسين كمادة قياسية.

الاستنتاج:

- في المرضى اللذين عولجوا بالتاموكسفين، أظهر قياس نشاط انزيم CYP2D6 انه مؤشر جيد لسرطان الثدي
- تم اثبات وجود تاثير سمي للتاموكسفين على خلايا سرطان الثدي MCF-7 باستخدام تقنية MTT (4.506 ميكروغرام/مليليتر).
- تم اثبات وجود تاثير سمي للتاموكسفين على خلايا سرطان الثدي MCF-7 بأستخدام تقنية فعالية كاسباس -٩ ب IC_{50} 4.056 ميكروغرام/مليليتر.
- أظهرت تقنية HCS ان للتاموكسفين تأثير سام باتجاه خط خلية MCF-7 عند 100 ميكروغرام/مليليتر بنمط يعتمد على الجرعة مع الزيادة في نفاذية غشاء الخلية، السايتوكروم C، كثافة النووية، وتغيير في جهد غشاء الميتوكوندريا، وتناقص في مستوى قدرت الخلية على البقاء.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين

كلية العلوم

قسم الكيمياء

دراسة مختبرية كيموحيوية لتأثير التاموكسيفين على أنزيم السايتوكروم بي٤٥٠ ٢د٦ والسمية الخلوية في النساء العراقيات قبل انقطاع الطمث مع سرطان الثدي

رسالة

مقدمة الى كلية العلوم /جامعة النهرين

كجزء من المتطلبات نيل درجة الماجستير في علوم الكيمياء

من قبل

غفران محمد مجيد

بكلوريوس ٢٠١٤

بأشراف

الاستاذ المساعد

د. فراس عبدالله حسن

١٤٣٨ ربيع الاول

ديسمبر ٢٠١٦