# THE EFFECT OF COMBINATION OF DRUGS UPON K562 CELL LINE TREATMENT

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
ATILIM UNIVERSITY

AKHLAS SALEM MOHMED ALARBI

A MASTER OF SCIENCE
THESIS
IN
THE DEPARTMENT OF CHEMICAL ENGINEERING AND APPLIED CHEMISTRY

JUNE 2019

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A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

OF

ATILIM UNIVERSITY

BY

AKHLAS SALEM MOHMED ALARBI

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR

THE DEGREE OF MASTER OF SCIENCE

IN

THE DEPARTMENT OF CHEMICAL ENGINEERING AND APPLIED CHEMISTRY

JUNE 2019

Approval of the Graduate School of Natural and Applied Sciences, Atilim University.

_____

Prof. Dr. Ali Kara
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science in Chemical Engineering and Applied Chemistry, Atilim University.

_____

Prof. Dr. Şeniz Özalp Yaman
Head of Department

This is to certify that we have read the thesis " THE EFFECT OF COMBINATION OF DRUGS UPON K562 CELL LINE TREATMENT " submitted by " AKHLAS SALEM MOHMED ALARBI " and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

| | |
|---|---|
| _____ | _____ |
| Assoc. Prof. Dr. Yasemin G. İşgör<br>Co-Supervisor | Assoc. Prof. Dr. S. Belgin İşgör<br>Supervisor |

**Examining Committee Members:**

Assoc. Prof. Dr. Nesrin E. Machin _____
Chemical Eng. and App. Chem. Dept., Atilim University

Assoc. Prof. Dr. S. Belgin İşgör _____
Chemical Eng. and App. Chem. Dept., Atilim University

Asst. Prof. Dr. Pembegül Uyar Arpacı _____
Biotechnology. Department, Selcuk University

Date: June 28, 2019

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Name, Last Name: Akhlas Salem Mohmed Alarbi

Signature:

## ABSTRACT

### THE EFFECT OF COMBINATION OF DRUGS UPON K562 CELL LINE TREATMENT

Akhlas Salem Mohmed Alarbi

M.Sc., Department of Chemical Engineering, and Applied Chemistry

Supervisor: Assoc. Prof. Dr. S. Belgin İşgör

Assoc. Prof. Dr. Yasemin G. İşgör

June 2019, 42 Pages

Nowadays, there is a very strong need for new approaches for cancer treatment. Unfortunately, treating cancer is very difficult for many reasons. The first reason is drug resistance. It is tried to approach new ways and techniques in the laboratory in order to find a permanent solution to drug resistance consequently increased the drug effectiveness. Chronic myelogenous leukemia Cell lines (K562) were used in this study to realize the effect of chemotherapeutic drugs, Doxazosin, Genistein, SU6656 and Adriamycin alone or in their different combinations on Glutathione-S-transferase (GST), Superoxide Dismutase (SOD) and Protein Tyrosine Kinase (PTK) activity. The result showed that, by administered different concentration of Genistein on K562 cells, the GST activity is directly proportional to the concentration of the drug used. While, after the K562 cells treated with different concentrations of Genistein and 7.5µM Doxazosin mesylate in combination, the GST activity was decreased gradually compared with control. On the other hand, when the cells treated with different concentration of SU6656 and 7.5 µM of Doxazosin, the highest GST activity was at the highest concentration of SU6656. By administered 7.5µM Doxazosin on K562 cells, the GST activity was decreased when it was compared with the control, however, the GST activity increased at 0.5 µM of Doxazosin. According to the experimental result, it is found that After K562 cells treated with different concentration of Genistein and 7.5µM doxazosin separately. The SOD activity increased at all concentration relative to the control. While when they administrated together doxazosin 7.5µM with

variable concentration of genistein, the result showed that combined drugs increase the formazan formation 10 times much higher than the result in which each drug used alone. While after the cells treated with 7.5 µM doxazosin in combination with variable concentration of Adriamycin, the SOD activity was increased. According to protein tyrosine kinase assay, the result showed that the activity of PTK enzyme on K562 cells decreased after treated with 7.5µM Doxazosin or by using variable concentration Genistein either alone or in their combination. while the PTK activity on K562 cells increased by using Adriamycin. PTK activity was decreased when 7.5µM Doxazosin and variable concentration of SU6656 were administered in combination and the PTK activity was decreased when K562 cells treated with 7.5µM Doxazosin and variable concentration of Genistein in combination. As a conclusion, the results showed that Doxazosin mesylate is less toxic than the Genistein and Su6656. The activity of PTK decreases after using Doxazosin. However, Genistein can be considered as a potent inhibitor for GST enzyme activity which is an advantage to reduce drug resistance. On the other hand, according to the result, Adriamycin has the strongest toxic effect on the K562 cells. On the other hand, Doxazosin decreases the toxicity of Genistein and SU6656 and Adriamycin when they used with them in combination.

**Keywords:** Cell lines, K562, Chemotherapy, drug resistance, Genistein. SU6656, Adriamycin, Doxazocin mesylates, Antioxidant Enzymes, Glutathione-S-Transferase (GST), Superoxide Dismutase (SOD), Protein tyrosine kinase (PTK).

# ÖZ

## K562 HÜCRE HATTI TEDAVİSİNDEKİ İLAÇLARIN BİRLEŞTİRİLMESİ ETKİSİ

Akhlas Salem Mohmed Alarbi

Yüksek Lisans, Kimya Mühendisliği ve Uygulamalı Kimya Anabilim Dalı
Danışman: Doç. Dr. S. Belgin Isgör

Eş -Danışman Doç. Yasamin G. İsgör

Haziran 2019, 42 sayfa

Günümüzde kanser tedavisi için yeni yaklaşımlara ihtiyaç duyulmaktadır. Ne yazık ki, kanser tedavisi birçok nedenden dolayı çok zordur. Bunlardan İlki ilaç direncidir. İlaç direncine kalıcı bir çözüm bulmak için günümüzde yeni yöntem ve tekniklere yaklaşılmaya çalışılıp buna bağlı olarak ilaç etkinliği arttırdı. Bu çalışmada kronik Miyeloid lösemi Hücre hatları (K562) kullanılarak, sadece Doxazosin, Genistein, SU6656 ve Adriamycin' ilaçlarının tek başına ya da kombinasyon şeklinde verilerek hücrenin Glutatyon-S-transferaz (GST), Superoksit dismutaz (SOD) ve protein tirozin kinaz (PTK) aktivitesi üzerindeki etkileri çalışılmıştır. Sonuçlar, K562 hücrelerine uygulanmış farklı Genistein konsantrasyonlarının GST aktivitesi üzerine etkisinin, kullanılan ilacın konsantrasyonuyla doğrudan orantılı olduğunu göstermiştir. K562 hücrelerinin farklı konsantrasyonlarda Genistein ve 7.5 µM Doksazosin mesilat ile kombinasyon halinde verilmesi ile, kontrolle karşılaştırıldığında. GST aktivitesinin kademeli olarak azaldığı görülmüştür. Diğer taraftan, hücreler farklı konsantrasyonlarda SU6656 ve 7.5 µM Doxazosin konsantrasyonları ile muamele edildiğinde, en yüksek GST aktivitesi en yüksek SU6656 konsantrasyonunda bulunmuştur. K562 hücreleri üzerine 7.5 µM Doxazosin uygulandığında, GST aktivitesi, kontrol ile karşılaştırıldığında azalmıştır. Ancak GST aktivitesinin, 0.5 µM Doxazosin'de arttığı görülmüştür. Deneysel sonuçlara göre K562 hücrelerinin farklı konsantrasyonlarda Genistein ve 7.5µM doksazosin ile ayrı

ayrı muamele edildikten sonra SOD aktivitesinin kontrole göre tüm konsantrasyonlarda arttığı bulunmuştur.

Doxazosin 7.5µM'i değişken konsantrasyonda genisteinle birlikte uygulanırken, sonuç, kombine ilaçların, formazan oluşumunu, her bir ilacın tek başına kullanıldığı sonuçtan 10 kat daha fazla arttırdığını gösterdi.

Hücreler, değişken Adriamisin konsantrasyonuyla birlikte 7.5 µM doksazosin ile işlemden geçirilirken, SOD aktivitesi artmıştır.

Protein tirozin kinaz sonucuna göre, K562 hücreleri üzerindeki PTK enziminin aktivitesinin, 7.5 µM Doksazosin ile muamele edildikten sonra veya tek başına ve kombinasyon halinde değişken konsantrasyon Genistein kullanarak azaldığını göstermiştir.

K562 hücrelerinde PTK aktivitesi Adriamisin kullanılarak artmıştır. 7.5 µM Doxazosin ve değişken SU6656 konsantrasyonunda kombinasyon halinde PTK aktivitesi azalmış ve K562 hücreleri 7.5 µM Doxazosin ve değişken konsantrasyondaki Genistein ile muamele edildiğinde PTK aktivitesi azalmıştır.

Sonuç olarak, Doxazosin mesilatın, Genistein ve SU6656'dan daha az toksik olduğu gösterilmiştir. Doksazosin kullanıldıktan sonra PTK aktivitesi azalmıştır. Bununla birlikte, Genistein, ilaç direncini azaltma avantajı olan GST enzim aktivitesi için güçlü bir inhibitör olarak düşünülebilir. Öte yandan, sonuçlara göre Adriamisin, K562 hücreleri üzerinde en güçlü toksik etkiye sahiptir, ancak Doxazosin; Genistein ve SU6656 ile Adriamycin'in birlikte kullanıldığında toksisitesini azaltmıştır.

**Anahtar Sözcükler:** Hücre çizgileri, K562, Kemoterapi, ilaç direnci, Genistein. SU6656, Adriamisin, Doksazosin mesilatlar, Antioksidan Enzimler, Glutatyon-S-Transferaz (GST), Superoksit Dismutaz (SOD), Protein tirozin kinaz (PTK).

## ACKNOWLEDGMENT

First of all, I am very thankful for Allah who gives me the opportunity to finish my master degree.

Foremost, I would like to express my sincere gratitude to my supervisors Assoc. Prof. Dr. S. Belgin İşgör and Assoc. Prof. Dr. Yasemin G. İşgör for their continued support for my master study and research, for their patience, motivation, enthusiasm, and their door was always open whenever I ran into trouble or had a question about my research. They have consistently allowed this paper to be my own work but steered me in the right direction whenever they thought I needed it.

My sincere thanks also go to my friends and classmates during my study Ms, Hanan Aljadi thank you for being a part of my life you are such a nice girl who gives me support and happens.

Finally, I must express my very profound gratitude to my parents and my family specially my husband for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| GST | - | Glutathione-S-Transferase |
| SOD | - | Superoxide Oxide Dismutase |
| PTK | - | Protein Tyrosine Kinase |
| GSH | - | Reduced Glutathione |
| NBT | - | Nitro Blue Tetrazolium Chloride |
| ROS | - | Reactive Oxygen Species |
| CDNB | - | 1 -Chloro-2,4-Diitrobenzene |
| HRP | - | Horse Reddish Peroxidase |
| EDTA | - | Ethylene Diamine Tetra Acetic Acid Disodium Salt Dihydrate |
| NBT | - | Nitro Blue Tetrazolium Chloride |
| XOD | - | Xanthine Oxidase |
| ROS | - | Reactive Oxygen Species |

# CHAPTER 1

# INTRODUCTION

Cancer remains a major health issue universally [1]. The criticalness of investigating modern anti-cancer agents, new formulations, and modern drug delivery strategies is described in the viewpoint of the genuine adverse impacts of regularly used antitumor drugs, as well as tumor cells' developing resistance to standard chemotherapy. Numerous cancers such as Chronic Myeloid Leukaemia, CML initially respond to cancer treatment but along these lines create chemotherapy of resistance and disadvantage. Subsequently, it is critical to creating new successful anticancer drugs, with more cancer cell targeting normal cell sparing[2].

## 1.1. Cell Lines

A cell line is a permanently developed cell culture when given the suitable medium and space will proliferate forever. Cells vary in that they become immortalized from their strains. It enables studying step-by-step changes in the cell's structure, biology, and genetic make-up under controlled conditions [3].

K562 is one of the most frequently used cell lines. K562 cells were commercially available and the purchased sample was originated from the pleural effusion of 53-year-old woman in terminal blast crisis with chronic myelogenous leukemia. It is a significant tool for studies into malignant hematopoiesis and tumour pathogenesis [4].

Apoptosis is a significant mechanism in regulating K562 cells and can be induced via the changes in the metabolic state of the cells [5].

### 1.2. Drug Combinations

Combination chemotherapies play an important role in clinical cancer treatment. Searching for new treatments for cancer is time-consuming. The number of therapeutic drug combinations that can be tested on is limited due to the cumbersome, time-consuming sophisticated tool [6].

In nature, cancer cells are mutagenic, posing difficulties for standard monotherapies that only affect individual molecular objectives. As a consequence of mutation, cancer cells also create drug resistance over time, resulting in a loss of drug effectiveness. In some cases, because of the buffering effects of complex biological systems, the effectiveness of these therapies is further reduced [7]. Prostate cancer is discovered mainly in elderly males, and surgery, radiotherapy, chemotherapy, and hormone therapy are usually involved in treatment. Drug combinations may enhance control over tumor growth and may reduce the necessity of radiation therapy [6].

### 1.3. Cytotoxicity

Cell cytotoxicity relates to the ability to destroy living cells by using certain chemicals or mediator cells. A cytotoxic agent is any material that kills cells, including cancer cells. These agents can prevent dividing and growing cancer cells.

Degree of cytotoxicity of a substance may differ and depends heavily on the assays used to assess it. A cytotoxic compound causes a short-term loss in cell viability by triggering cell death or causing a significant decline in cell survival [8].

Cytotoxic assays can be classified into [9]

- Enzyme Release Assays
- Cell Viability Assays
- Cell Survival Assays
- Assays Based on Altered Cell Permeability

### 1.4. Chemotherapeutic Drugs

There is a wide range of anticancer drugs nowadays in the market; the drugs have been chosen by looking at the result of previous studies using anticancer drugs [10]. In addition, drugs that are used in this study are widely used in clinics and they have a big market share.

#### 1.4.1. Doxazosin

Doxazosin mesylates (DXZ), 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(1,4-Benzodioxan-2-Ylcarbonyl) piperazine methane sulfonate (Figure 1), is one of the quinazoline-based alpha 1-adrenergic receptor antagonists [11]. The commercial name of DXZ is Cardura, with the chemical formula of $C_{23}H_{25}N_5O_5$ [12].

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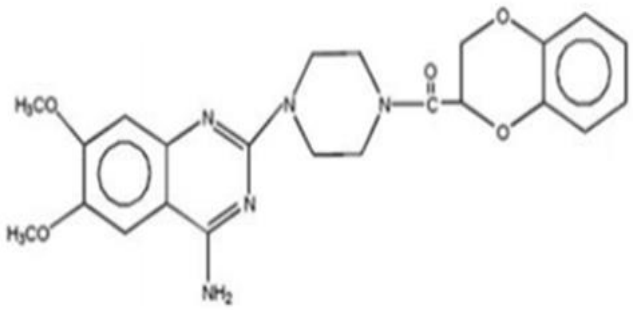


Figure 1.1 The structure of (DXZ).

DXZ induces apoptosis in the cell line of breast cancer (MCF7) by inhibiting both the receptor of epidermal growth factor (EGFR) and the nuclear transcription factor (NF-kB) engaged in angiogenesis [13]. Additionally, DXZ has the capacity to suppress angiogenic reaction as well as human endothelial cell growth through interference with the features of Vascular Endothelial Growth Factor, VEGF and Fibroblast Growth Factor, FGF-2 so it is considered as an antiangiogenic agent used to treat advanced prostate cancer [14]. Doxazosin has antihypertensive action by inhibiting post-synaptic alpha-1 adrenoceptors of the vascular smooth muscle. This inhibits the effect of catecholamines on vasoconstriction [14].

The success of DXZ is the use of β-adrenoceptor antagonists, diuretics, calcium channel antagonists and angiotensin-adapting enzyme inhibitors in monotherapy with uncontrolled hypertension. [15].

### 1.4.2. Genistein

The molecular formula of the Genistein is C15H10O5. A naturally arising as soybean isoflavone, it has a natural polyphenolic structure similar to estrogen [16]. Having a powerful anti-tumor property. There are multiple mechanisms of Genistein as an anti-cancer agent like inhibition of tyrosine kinase and angiogenesis inhibition which are observed in various cancer cells [17].

Genistein described as a compound of phytoestrogens, they discovered in the latter study that estrogens are suggested to cause breast cancer through their genotoxic metabolites and through stimulation of cell growth and proliferation through estrogen receptor (ER). As a result, inhibition of the manufacturing of estrogen by aromatase enzyme inhibition as well as inhibit estrogen action via block estrogen receptor is a common way for genistein to treat breast cancer [18].
The antioxidant mechanism of genistein is reliant on donates hydrogen atom from the phenolic hydroxyl group. In human prostate cancer cell anti-oxidant enzyme such as glutathione peroxidase their expression increases by genistein.

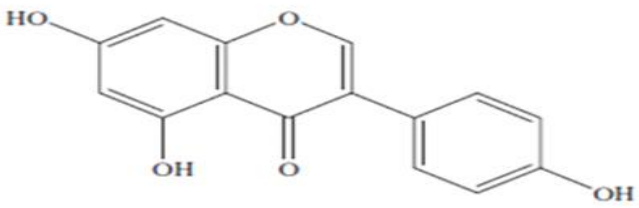


Figure 1.2 The structures of Genistein.

### 1.4.3. SU6656

SU6656's chemical formula is C19H21N3O3S has molecular weight 371.45 g/mol. It is a selective Platelet-derived growth factor receptor Tyrosine kinase inhibitor.

SU6656 was used as an analytical tool to examine the role of Src family kinases in cellular signal transduction processes. Su6656 was reported to increase fat oxidation in relation to reducing body weight [19].

Su6656 is competitive inhibition of ATP [19]. This compound is useful in elucidating the roles of Src family members in a variety of cell activities including receptor internalization, motility, apoptosis, differentiation, oncogenesis, cell cycle control, and axonal growth [20].

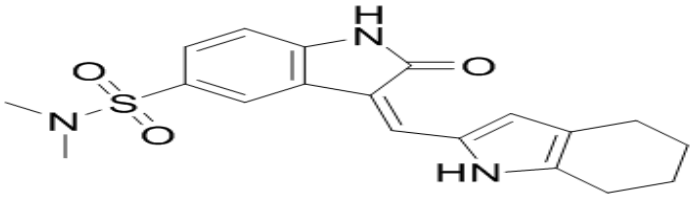


Figure 1.3 The chemical structure of SU6656.

### 1.4.4. Doxorubicin (DOX)

Doxorubicin (DOX) or Adriamycin is a Quinone-containing antibiotic used to treat a wide range of cancers, including breast cancer and cancer of the prostate [21]. DOX clinical use is correlated with enhanced danger of heart failure or cardiomyopathy. Children treated with DOX for leukemia developed heart failure years after DOX chemotherapy was finished. Evidence indicates that DOX-induced cardiotoxicity is caused by oxygen radicals produced by DOX redox activation [22].

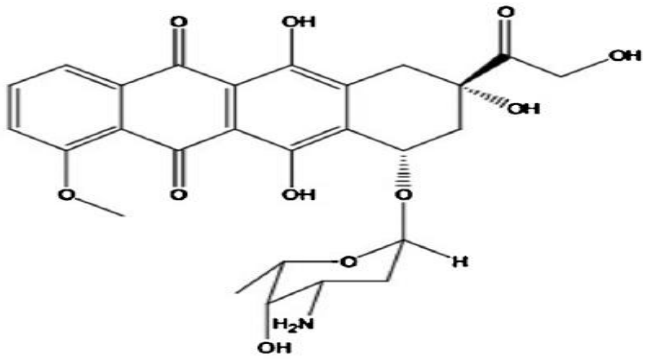


Figure 1.4 The chemical structure of doxorubicin (DOX).

Doxorubicin is oxidized to semiquinone, an unstable metabolite, which is converted back to doxorubicin in a process that releases reactive oxygen species. Reactive oxygen species can lead to membrane damage, DNA damage, and oxidative stress. Alternatively, doxorubicin can enter the nucleus and poison topoisomerase-II, also resulting in DNA damage and cell death [23].

### 1.5. Antioxidant Process and Oxidative Stress

Oxidative stress is defined as an imbalance condition between reactive oxygen species and antioxidant enzyme. Reactive oxygen/nitrogen species are depicted by superoxide anion radical, hydroxyl, alkoxyl, lipid peroxyl and peroxynitrite [24].

Apoptosis is the main growth and progression mechanism for normal cells. Cancer cells prevent and continue to propagate apoptosis. ROS generation and oxidative stress mediate apoptosis [25].

Oxidative stress on protein can be both irreversible and reversible protein oxidative modifications. Irreversible modifications mainly include protein carbonylation and tyrosine nitration [26]. The various biochemical responses involving oxygen lead to reactive toxic intermediates that can cause harm to DNA. Since oxidative DNA damage can trigger mutations and mutations are known to cause cancer, a great deal of attempts has been made to explore the role of oxidative DNA damage in carcinogenesis [27].

When an antioxidant destroys a free radical, this antioxidant itself becomes oxidized. Therefore, the antioxidant sources must be constantly restored in the body.

Thus, while in one particular system, an antioxidant is effective against free radicals in other systems, the same antioxidant could become ineffective. In addition, in certain circumstances, an antioxidant may even act as a pro-oxidant, for example, it can generate toxic ROS [27].

### 1.5.1. Antioxidant Defense System

Increased concentrations of ROS are cytotoxic, whereas decreased concentrations are required to control a few critical physiological processes including cell differentiation, apoptosis, cell proliferation, and pathways for redox-sensitive signal transduction increased concentrations of ROS, in any case, lead to DNA damage including cell death and mutations. The cells contain a huge amount of antioxidants to decrease or repair the harm initiated by ROS and to control the signaling pathways that are redox-sensitive. The SODs transform superoxide radical into hydrogen peroxide and molecular oxygen, whereas catalase and peroxidase change hydrogen peroxide into water [28].

#### 1.5.1.1. Glutathione S-Transferase (GST)

Glutathione-S-Transferase (GST) (EC2.5.1.1.8) defines a group of detoxification enzymes using glutathione in various biotransformation reactions that contribute to the conversion of many compounds, including therapeutic drugs, carcinogens and oxidative stress products (Figure 7). Glutathione-S-transferases (GSTs) are essential enzymes in a variety of species to detoxify a variety of xenobiotics [29]. The enzyme protects cells by combining thiol group of glutathione with the electrophilic center of xenobiotics [30], as well as mutagenic, carcinogenic and poisonous chemicals [31].

The multifunctional of GST enzymes are correlated with chemotherapy drug resistance. As a result of that glutathione-S- transferase being a target for the novel study of treatment cancer to minimize the resistance while decreasing the side effect.

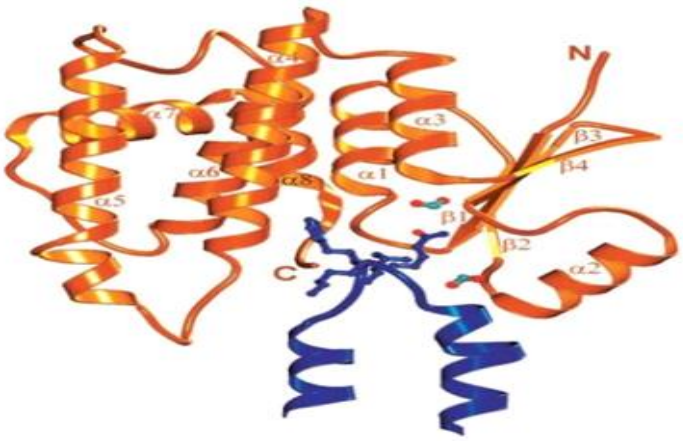


Figure 1.5 Glutathione S-transferase.

### 1.5.1.2. Superoxide Oxide Dismutase (SOD)

The human body containing complex anti-oxidant defense system, which is based on enzymatic and non- enzymatic antioxidants. The actions of these antioxidants are important in order to decrease the ROS concentration thus decline the damaging effect on the cells.

The enzymatic antioxidant molecules are classified as first, second, third and fourth according to their response against free radicals. Superoxide dismutase is the first line defense antioxidants that catalyze the transformation of the superoxide anion into hydrogen peroxide and molecular oxygen [32].

According to the metal co-factor and it is position in the cells, there are three types of SOD were identified.

- copper and zinc-containing dismutase (Cu/Zn SOD, SOD-1) found in the cytosol
- manganese-containing dismutase (Mn-SOD, SOD-2) found in the mitochondria, which is reported to be the antioxidant enzyme with the strongest anti-tumor effect [33].
- extracellular dismutase (Ec-SOD, SOD-3) found in the extracellular matrix

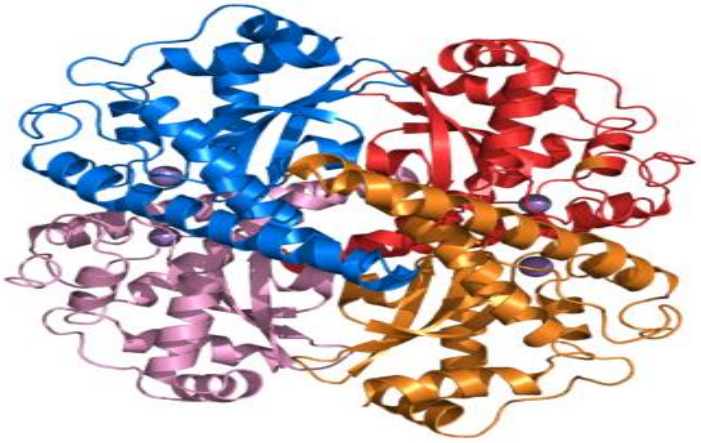

Figure 1.6 the 3D staging of superoxide dismutase (SOD).

### 1.5.1.3. Tyrosine Kinase Enzyme

Cell proliferation and morphogenesis beside apoptosis (cell death) must be monitoring intensively to maintain the normal tissue patterning. Consequently, any change or altering in the cellular signal transduction pathway of these processes may enhance cell growth, inhibited apoptosis, in addition, to enhance cell invasion ended with cancer [34].

Many of these signals are controlled by the tyrosine kinase receptor. Tyrosine kinases are enzymes that catalyze the transition of a phosphate group from adenosine triphosphate to target proteins. In various ordinary cellular regulatory procedures, they play a significant role. Kinases of tyrosine can be categorized as kinases of the receptor protein and kinases of the protein that are not receptor. Receptor tyrosine kinases are membrane-spanning cell surface proteins that play critical roles in transducing extracellular signals to the cytoplasm [35] (Pawson et al., 2002). As an inactive enzyme, receptor tyrosine kinase establishes in the plasma membrane in the absence of ligand. Once binding with the ligand, receptor dimerization enhanced and the confirmation changed that lead to kinase activation thus transphosphorylation of the receptor act on tyrosine residues specifically. That is known as phosphotyrosine residues which are important in signal transduction from the plasma membrane to the cells [34].

Tyrosine kinases play a critical part in modulating the signaling of growth factor. Activated forms of these enzymes can cause the proliferation and development of tumor cells by inducing anti-apoptotic impacts.

Besides activation by growth factors, activation of protein kinase by somatic mutation is a prevalent tumor genesis mechanism. There are roughly 60 recognized receptor tyrosine kinases, separated into some 20 subfamilies as described by receptor and/or ligand [36]. Overexpression of different type pf PTKs, for instance, epidermal growth factor receptor (EGFRs), platelet-derived growth factor receptor (PDGFRs), insulin growth factor receptor (IGFRs), vascular endothelial growth factor receptor (VEGFRs) and fibroblast growth factor receptor ( FGFRs) directly may lead to breast cancer [37].

### 1.6. Aim of This Study

The aim of this study is to analyze the effect of Doxazosin, Genistein, Adriamycin, and SU6656 drugs on K562 cells. In this study, drugs were administered alone and also their different combinations separately on the K562 cells. The treated cells then analyzed on the basis of their Glutathione-S- transferase, superoxide dismutase and PTK activities.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Materials

Genistein (Sigma) , SU6656 (Sugen company) , Doxazosin (Cardura, Pfizer company), Doxorubicin (HyClone), K562 Cell line (American Type Culture Collection , ATCC), Potassium phosphate monobasic ($KH_2PO_4$) (Riedel. De Hoen), Sodium phosphate dibasic anhydrous ($Na_2HPO_4$) (Fisher Scientific), Potassium chloride (KCL) (Fluka), Dithiothreitol (DTT) (Fluka), Sodium Chloride (NaCl) (Sigma Aldrich). The reduced form of Glutathione (GSH) and 1-Chloro-2,4-Dinitrobenzene (CDNB) (Fluka), Pure Ethanol, monopotassium phosphate ($KH_2PO_4$) and Dipotassium phosphate ($K_2HPO_4$) (Riedel. de Haen). Xanthine (Sigma–Aldrich), Xanthine Oxides (Calbiochem), Nitro Blue Tetrazolium Chloride (NBT) (Fisher Scientific), Ethylene Diamine tetra acetic acid (EDTA) (Sigma), Sodium Carbonate Anhydrous ($Na_2CO_3$) (Fisher Scientific), Sodium Hydrogen Carbonate ($NaHCO_3$) (Merck). Bovine Serum Albumin (BSA) (Thermo Scientific). Tween 20 (Merck), 2-Mercaptoethanol ($C_2H_6O_5$) (Fisher scientific), universal tyrosine kinase Assay Kit (Takara, Japan), Sulfuric acid (1-N $H_2SO_4$). RPMI-1640 Medium (1x) (Hyclone), Trypsin Edta (Lonza), L-glutamine solution and Pen–Strep Solution (BI biological industry).

## 2.2 Methods

### 2.2.1 Culturing of K562 Cell Line:

The K562 cells were grown in tissue culture flasks T75 or T25 containing RPMI-1640 medium with 10% Fetal Bovine Serum, FBS, 10% L-glutamine and 0.1% penicillin-streptomycin. The flasks containing cells are stored in a $CO_2$ incubator at 37°C for their growth. All experiments were carried out under aseptic conditions by using 70%. Ethanol (Figure 2.1).

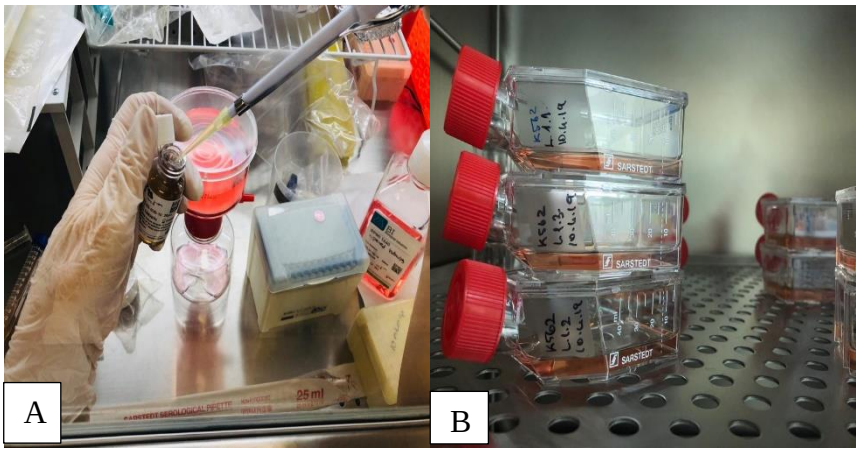


Figure 2.1 A) Cell medium preparation. B) Cell Lines incubation.

### 2.2.2 Drugs Concentration Used to Treat K562 Cell Line

K562 cell lines were used in order to screen the effect of the tested drugs in the laboratory. Doxazosin Mesylate only used in single concentration. However, for both Genistein and Adriamycin, four different concentrations were used in the plates (Table 2.1).

Table 2.1 Concentrations of the drugs used.

| Drug | Concentrations (µM) | | | |
|---|---|---|---|---|
| **Doxazosin Mesylate** | **7.5** | | | |
| **Adriamycin** | **3.75** | **1.875** | **0.625** | **0.312** |
| **Genistien** | **3.75** | **1.875** | **0.625** | **0.312** |

### 2.2.3 K562 Cell Line Treated with a Different Combination of Drugs

After K562 cell line was cultured, it was treated with a different concentration of Combination of drugs (Table 2.2,2.3,2.4,).

Table 2.2 K562 cells treated by Combination of Doxazosin Mesylate with varying concentrations of SU6656

| The concentration of Doxazosin Mesylate (µM) | Concentrations of SU6656 (µM) |
|---|---|
| 7.5 | 3.75 |
| 7.5 | 1.875 |
| 7.5 | 0.625 |
| 7.5 | 0.312 |

Table 2.3 K562 cells treated by Combination of Doxazosin Mesylate with varying concentrations of Genistein

| Concentration of Doxazosin Mesylate (µM) | Concentrations of Genistein (µM) |
|---|---|
| 7.5 | 3.75 |
| 7.5 | 1.875 |
| 7.5 | 0.625 |
| 7.5 | 0.312 |

Table 2.4 K562 cells treated by Combination of Doxazosin Mesylate with varying concentrations of Doxorubicin

| Concentrations of Doxazosin Mesylate (µM) | Concentrations of Doxorubicin (µM) |
|---|---|
| 0 | 0.05 |
| 0 | 0.005 |
| 0.5 | 0 |
| 0.05 | 0.05 |
| 0.05 | 0.005 |

### 2.2.4 Preparing the Cell Homogenization

To prepared tissue homogenates phosphate-buffered saline (PBS) should have a final concentration of 137mM NaCl, 10 mM phosphate, 2.7 mM Kcl with 1 Mm of DDT at PH 7.4 is used. The cells are homogenates by using sonication and then centrifuged at 6000 rpm at 4 °C for 10 min. the pellets are collected and stored at-80°.C

### 2.2.5 Determination of Protein Content of K562 Cell Lines by Using BCA Protein Assay

BCA assay is a colorimetric assay used to detect and quantitate the protein amount. The purple-colored product has absorbance at 560nm. This assay was done by using bovine serum albumin (BSA), as a protein standard. Working reagent ( WR) was prepared by mixing 50 parts from BCA Reagent A and 1 part from BCA Reagent B and then 200µl from this mix was added to 20µl of the sample in each well. After 30 minutes incubation at 37°C absorbance was read at 560 nm as an endpoint (Figure 2.2) [38].


Figure 2.2 BCA Protein Assay Kit.

### 2.2.6 Enzyme Assays

#### 2.2.6.1 Glutathione- S-Transferase (GST) Assay

The assay mixture was optimized before in the laboratory [10] and applied by adding 200uL of 200Mm potassium phosphate buffer at pH 6.5, 15uL of 200mM GSH, 20uL cytosol of K562 as enzyme source and 15ul of 50mM CDNB at a total volume of 250uL. The reaction was followed kinetically at 340 nm (Table 2.5).

Table 2.5 The Reaction component of GST Activity Assay

| Reagents | Volume added per well |
|---|---|
| Potassium phosphate buffer (200 mM ) | 200 uL |
| GSH ( 200 mM ) | 15 uL |
| Enzyme with different drug concentration | 20 uL |
| CDNB ( 50 mM ) | 15uL |

### 2.2.6.2 Superoxide Dismutase (SOD) Assay

The microplate assay of SOD that was used in this study was optimized before in the laboratory [10].

The reaction media contains 10 uL of K562 cell cytosol that was treated by different concentration and combinations of drugs as enzyme source, 60uL, 200 mM sodium carbonate buffer, pH 10 containing 10 mM EDTA, 75uL, 0,3 mM Xanthine, 10ul, 25 mM of nitro blue tetrazolium (NBT), and 80uL extra buffer was added to complete the final volume into 250uL separately. After 2 min incubation, Xanthine Oxidase (XOD) was added lastly. The reaction was followed at 560 nm kinetically. The SOD assay components are given in (Table 2.6).

Table 2.6 The components of Superoxide Dismutase activity assay

| Reagent | Volume added per well |
|---|---|
| Cytosol | 10 uL |
| 200 mM sodium carbonate buffer PH 10.1 with 0.6 mM EDTA | 60 uL |
| 0.3 mM xanthine | 75 uL |
| 25 mM Nitro blue tetrazolium (NBT) | 10 uL |
| Extra buffer | 80 uL |
| Incubation for 2 mints | |
| 3.64 U/ml Xanthine oxidase XOD | 15 uL |

### 2.2.6.3 Protein Tyrosine Kinase (PTK) Assay

The principle of this assay is dependent on the phosphorylation of peptide substrate (tyrosine) by the action of protein tyrosine kinase enzyme that uses adenosine triphosphate as a source of a phosphate group (universal protein kit, TAKARA).

First of all PTK control was dissolved in 100 µl of double-distilled water. kinase reacting solution was prepared by adding 2-mercaptoethanol only before use. the final concentration should be 10mM, 400µl from it was added to PTK control the total volume was 500µl.

By using a serial dilution of the starting concentration the standard curve was prepared.

anti-phosphotyrosine HRP prepared by dissolving in 5.5ml of distilled water, the blocking solution and kinase reacting buffer they are used directly without prepare, ATP-2Na is dissolved in 0.55 ml of distilled water.

Secondary prepared samples are diluted with kinase reacting buffer more than 5 times.

40µl of the sample was added to each well and 10µl of 40 mM ATP-2Na was added to it 30 mint incubated at 37℃.

The wells are washed 4 times by using Washing buffer and extra solution should be removed. 100µl of blocking solution was added to each well incubate again for 30mint at 37℃, before adding the antibody solution the extra blocking solution should be removed, 50 µl of Anti-phosphotyrosine (PY20) – HRP was added and incubated at 37℃ for 30mint. The excesses solution was removed and the wells washed with washing buffer 4 times and then 100µl of HRP substrate solution (TMBZ) was added and incubated for 15 mints at 37℃. Finally, 100 µl of stop solution was added to stop color development and read at 450nm as it shown in (Table 2.7).

The activity was calculated on the basis of the prepared standard curve.

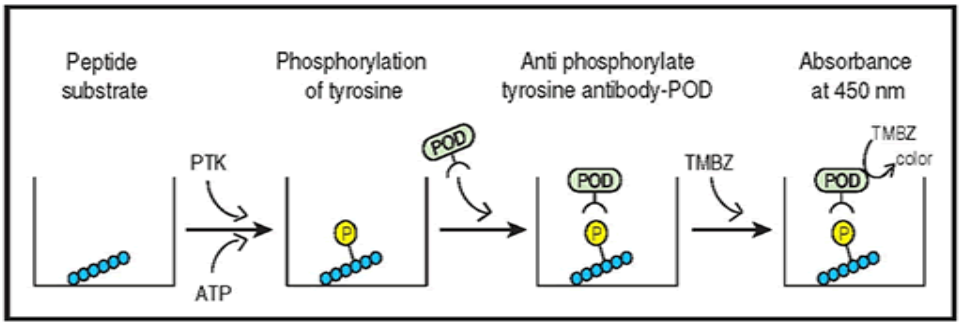


Figure 2.3 Tyrosine kinase assay.

Table 2.7 The component of Tyrosine kinase assay

| **Reagents** | **Volume added per well** |
|---|---|
| **Cytosol** | 40 µL |
| ATP-2Na 40 mM | 10 µL |
| Incubation for 30 minutes at 37℃ | |
| Blocking solution | 100 µL |
| Incubation for 30 of PTK at 37℃ | |
| anti-phosphotyrosine HRP solution | 50 µL |
| Incubation for 30 minutes at 37℃ | |
| HRP substrate solution (TMBZ) | 100 µL |
| Incubation for 15 minutes at 37℃ | |
| 1N-$H_2SO_4$ (stop solution) | 100 µL |

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 The Result of Determination of Protein Content of K562 Cell Lines by Using BCA Protein Assay

The protein amount in mg/ml of K562 cell line treated with different concentrations of genistein was measured and the results are given in (Table 3.1). The result shows that the protein content change was not in the same order in the concentration change. For example, the lowest protein content was determined at the highest drug concentration while the highest protein content belongs to the second-highest concentration (1.857 μM) of Genistein.

Table 3.1 The amount of protein (mg/ml) of K562 treated with Genistein.

| Genistein (μM) | Protein content (mg/ml) |
|---|---|
| 3.75 | 0.4535 |
| 1.857 | 0.7358 |
| 0.625 | 0.6218 |
| 0.312 | 0.5986 |

The amount of protein (mg/ml) of K562 cell line treated with Doxazosin was measured and the result showed that the amount of the protein is decreased compared to control (Table 3.2).

Table 3.2 The amount of protein (mg/ml) in K562 cell line treated with Doxazosin.

| Doxazosin (µM) | Protein content (mg/ml) |
|---|---|
| 7.5 | 0.3124 |
| 0 | 0.7222 |

The amount of protein (mg/ml) of K562 cell line treated with different concentrations of Doxazosin and Genistein combinations were also measured. After the cell was treated with different concentrations of Genistein with 7.5 µM concentration of doxazosin the BCA result showed that the highest protein amount was observed at the highest concentration of Genistein. Consequently, the amount of the protein is decreased by decreasing the Genistein concentration as it is shown in (Table 3.3).

Table 3.3 The amount of protein (mg/ml) of K562 cell line treated with doxazosin and Genistein

| Doxazosin Mesylate(µM) | Genistein(µM) | Protein content (mg/ml) |
|---|---|---|
| 7.5 | 3.75 | 0.9862 |
| 7.5 | 1.875 | 0.8219 |
| 7.5 | 0.625 | 0.7286 |
| 7.5 | 0.312 | 0.7534 |

The BCA assay used to measure the amount of protein (mg/ml) of K562 cell line treated with the combination of different concentrations of SU6656 with 7.5 µM of doxazosin showed that the protein content is decreased compared to control may be due to the toxicity of SU6656 (Table 3.4). But the result also showed no significant difference in protein amount between the concentrations.

Table 3.4 The amount of protein (mg/ml) of K562 cell line treated with doxazosin and SU6656

| Doxazosin Mesylate(µM) | SU6656 (µM) | Protein content (mg/ml) |
|---|---|---|
| 7.5 | 3.75 | 0.2628 |
| 7.5 | 1.875 | 0.2718 |
| 7.5 | 0.625 | 0.2906 |
| 7.5 | 0.312 | 0.2587 |

### 3.2: Effect of Drugs Treatment on GST Enzyme Activity of K562 Cell Line

The GST activity of K562 cells treated with different concentrations of genistein was followed. The result showed that the GST activity of the cell is directly proportional to the concentration of the drug used meaning that, increase in the concentration of the drug ended up with an increase in GST activity ( Figure 3.1.A).

When the Doxazosin administrated alone (7.5 µM) the activity was measured as 0.03504 U/ul which is less than control (Figure 3.1.B). But when 0.5 µM Doxazosin is administered the GST activity of K562 cell line was higher than the control (Figure 3.2.B).

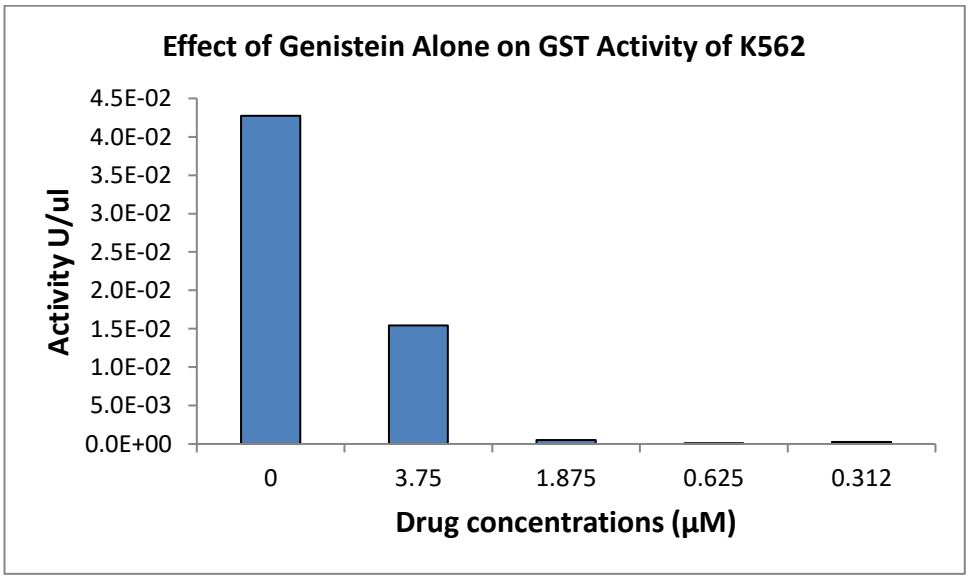


Figure 3.1.A The Effect of Genistein Alone on GST Activity.

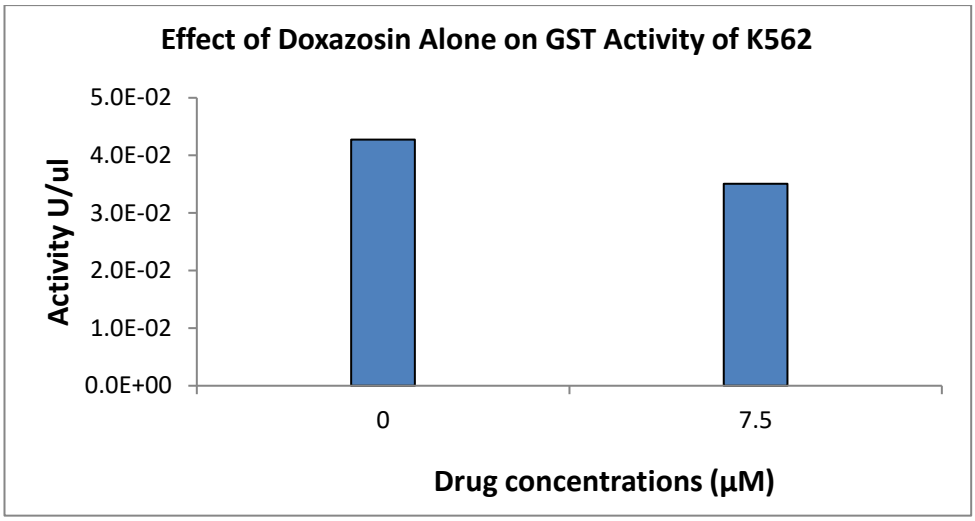


Figure 3.1.B The Effect of Doxazosin Alone on GST Activity.

After the cell line was treated with a combination of 7.5 µM doxazosin and different concentration of the genistein, it has shown that the activity of GST enzyme was decreased gradually compared to control (Figure 3.2.A).

On the other hand, after the cells treated with a combination of 7.5 µM of doxazosin and various concentration of SU6656, the highest GST activity (0.08491u/ul) measured with 7.5 µM of Doxazosin and 3.75 µM SU6656 combinations (Figure 3.2.B).

While after the cells treated with different concentration of doxazosin and Adriamycin, the GST activity on K562 cells was almost the same as the control. While by using 0.5µM doxazosin and 0 Adriamycin the GST activity was the highest relative to the control (Figure 3.2.C).

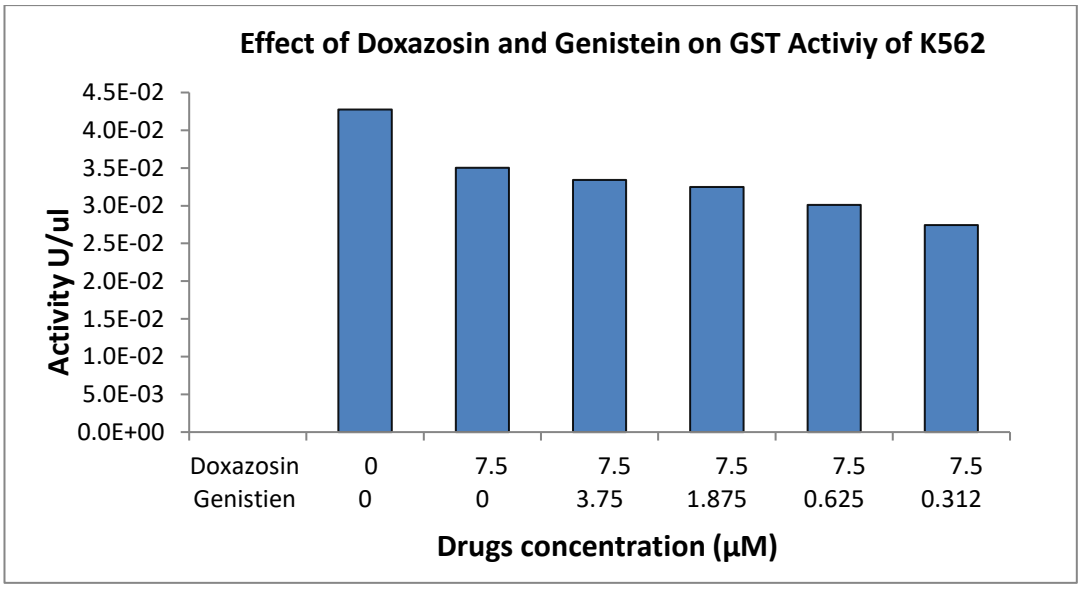


Figure 3.2.A The Effect of Doxazosin and Genistein on GST Activity.

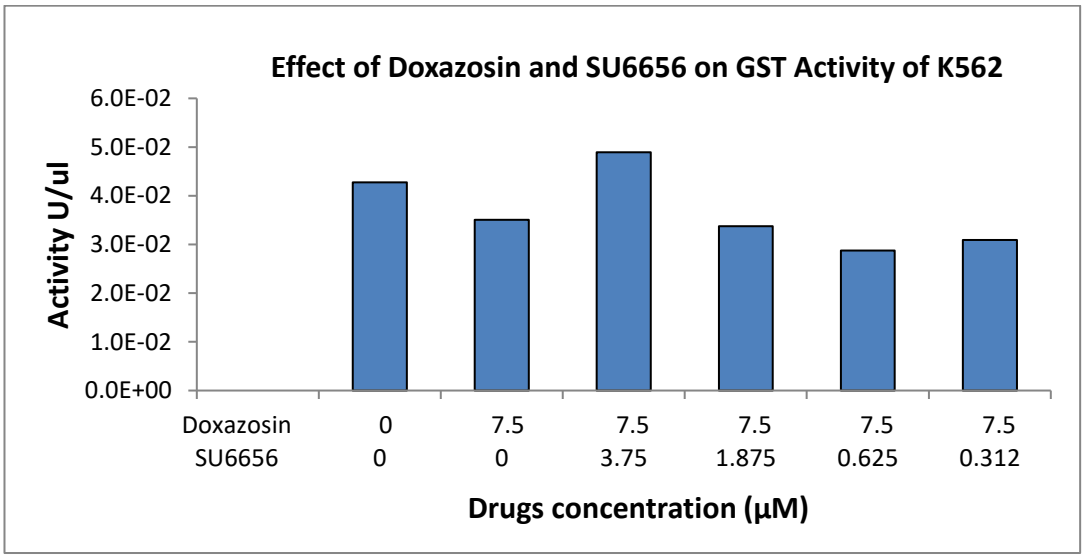


Figure 3.2.B The Effect of Doxazosin and SU6656 on GST Activity.

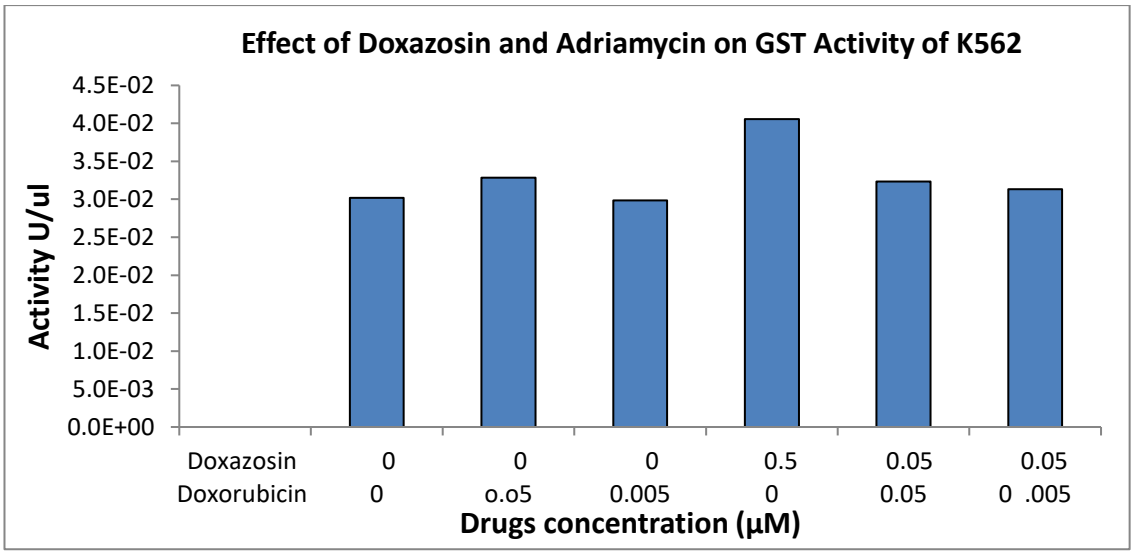


Figure 3.2.C The Effect of Doxazosin and Adriamycin on GST Activity.

### 3.3: The Effect of Drugs Using Different Concentrations on Superoxide Dismutase Enzyme

The SOD activity of K562 cells treated with different concentration of Genistein, Adriamycin, and 7.5 µM of doxazosin were tested.

The SOD activity of the cells treated with different concentrations of drugs alone studied separately. According to the experimental result, it is found that the SOD activity of K562 did not show any correlation to concentration. In other words, the SOD activity shows variation with variable concentrations of Genistein (Figure 3.3.A**)**. When it is administered to K562 cells the doxazosin (7.5 µM) alone, the SOD activity increased relative to its control (Figure 3.3.B**)**.

With Adriamycin, the SOD activity decreased when using the lowest concentration of Adriamycin while its highest two concentrations showed a increase in SOD activity relative to its control (Figure 3.3.C**)**.

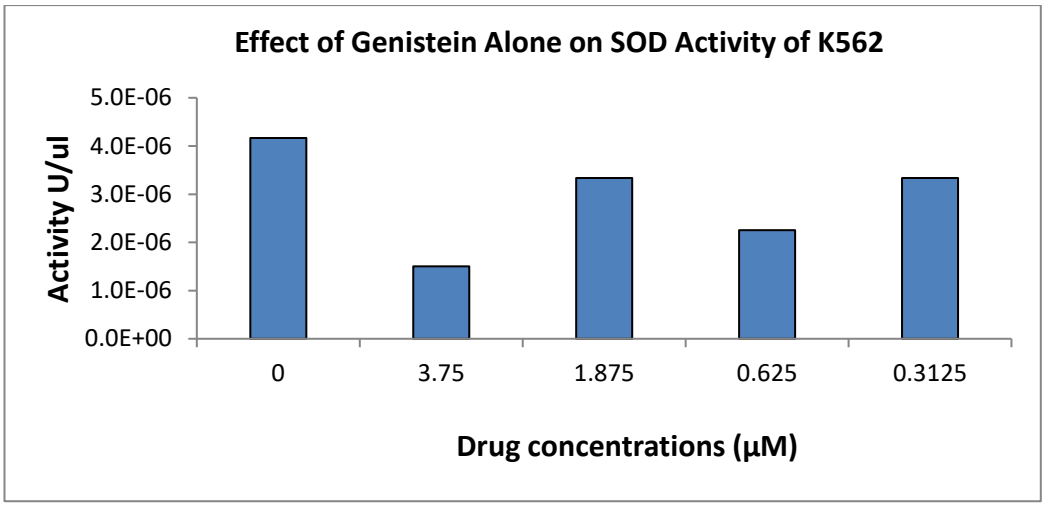


Figure 3.3.A The Effect of Genistein Alone on SOD Activity.

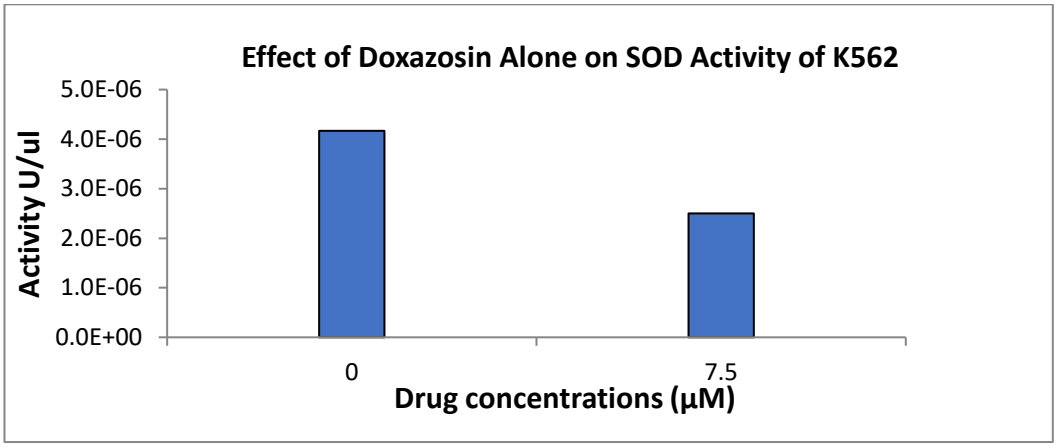


Figure 3.3.B The Effect of Doxazosin Alone on SOD Activity.

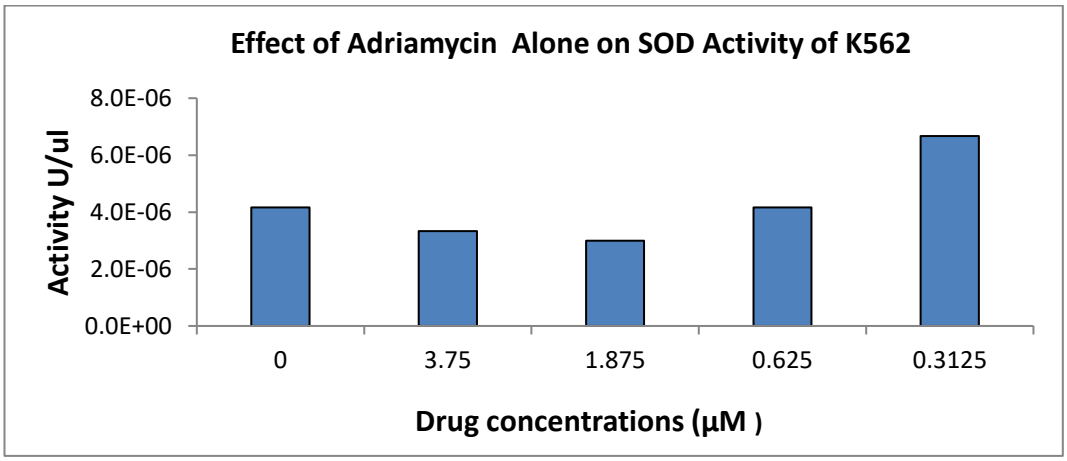


Figure 3.3.C The Effect of Adriamycin Alone on SOD Activity.

The effect of combinations of drugs on SOD activity of K562 cells were also studied. The combinations of doxazosin (7.5 µM) and variable concentrations of genistein resulted in a significant decrease in SOD activity relative to both control and doxazosin alone (Figure 3.4.A). When the doxazosin (7.5 µM) administered with different concentrations of SU6656 the result showed that the SOD activity varies. The SOD activity was the same as a control for the highest and lowest concentrations of SU6656 but increases almost the same rate in the rest of the concentrations of SU6656.

The result showed that the SOD activity responded to the SU6656 concentrations. at 7.5 µm Doxazosin concentration, the SOD activity changed with the change in SU6656 concentrations. At the highest concentration of SU6656, the SOD activity was measured the same as the control as it is observed for the lowest concentration (0.3125 µm) of SU6656. Between the highest and lowest concentrations, the SOD activity responded directly with the concentration of SU6656 meaning that increase in concentration caused an increase in formazan dye formation but a decrease in SOD activity (Figure 3.4.B).

Whereas by using a combination of doxazosin and Adriamycin to treat K562, the SOD activity increased while by using 0.05 µM of Adriamycin the SOD activity was lowest relative to the control. (Figure 3.4.C).

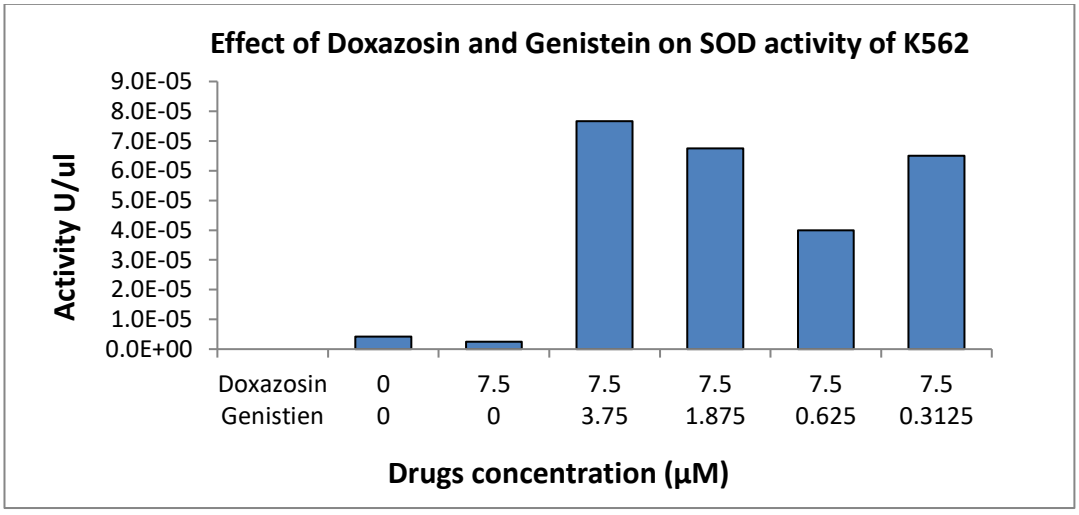


Figure 3.4.A The Effect of Doxazosin and Genistein on SOD Activity.

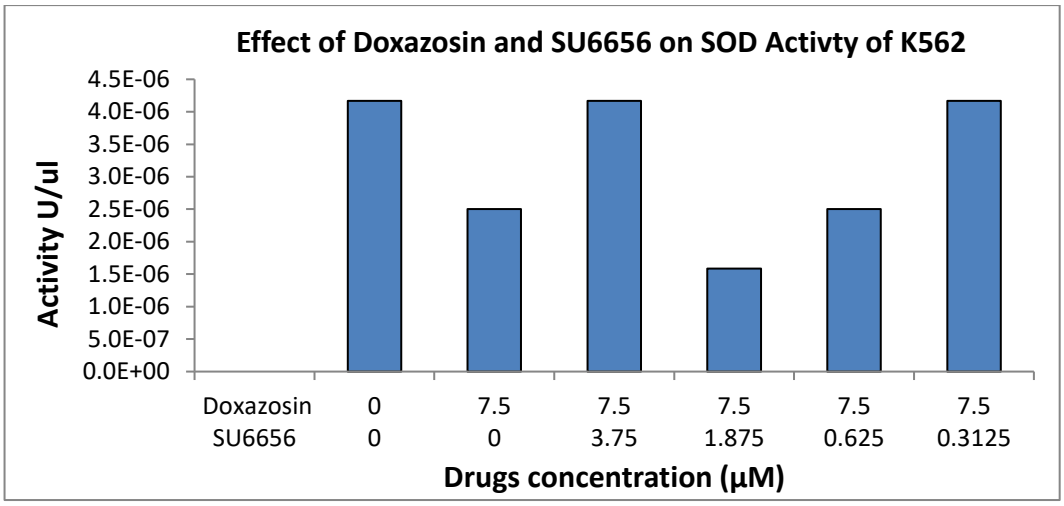


Figure 3.4.B The Effect of Doxazosin and SU6656 on SOD Activity.

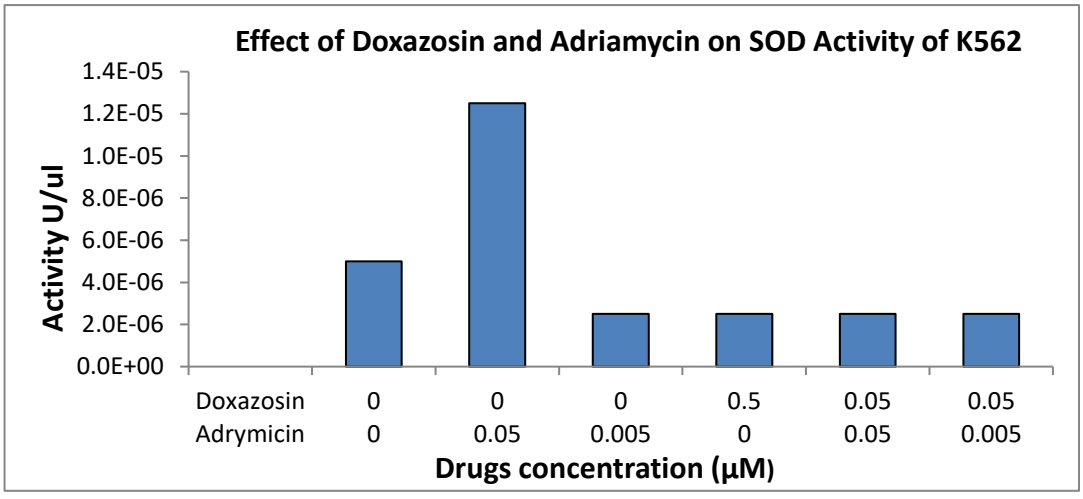


Figure 3.4.C The Effect of Doxazosin and Adriamycin on SOD Activity.

### 3.4: The Effect of Drugs Using Different Concentrations on PTK Enzyme Activity

PTK standard Activity is used according to universal tyrosine kinase assay kit to calculate the Protein tyrosine kinase enzyme activity by using a standard titration curve for PTK enzyme (Figure 3.5). The effect of drug treatment on the PTK activity of K562 cells was calculated then relative to the standard calibration curve of PTK enzyme mentioned above.

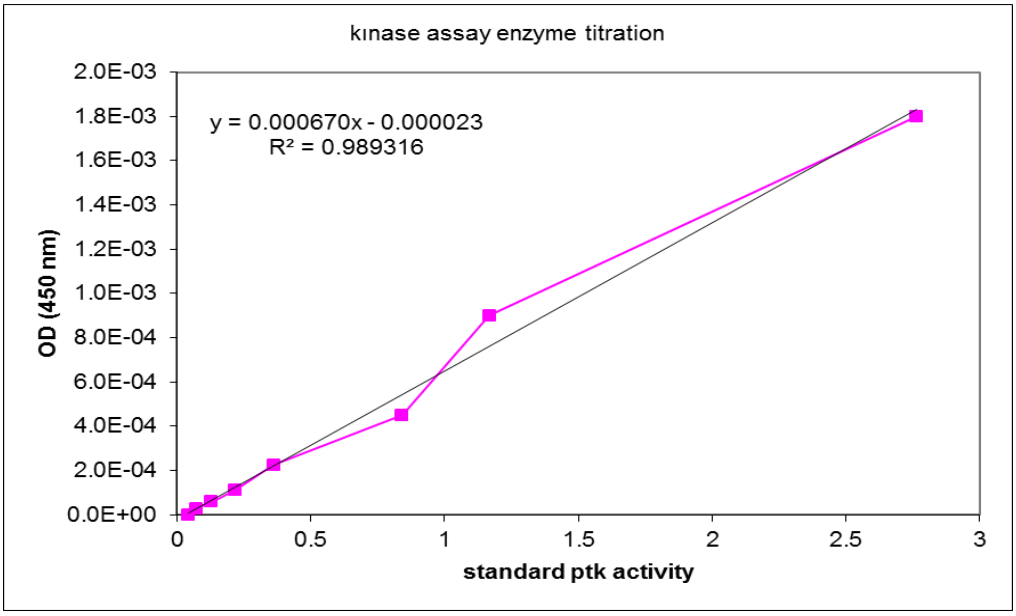


Figure 3.5 PTK Standard Activity

As it was mentioned under "Method" Protein tyrosine kinases catalyze the phosphorylation of tyrosine protein when HRP substrate solution (TMBZ) is bound to the phosphate group on protein tyrosine; they form a complex that has a color, which is absorbed at 450nm.

The result showed that PTK activity was decreased when the cells were treated with genistein and doxazosin separately relative to control (Figure3.5.A) (Figure 3.5.B) The highest PTK activity was observed with doses of genistein (3.75 µm).

However, the effect of Adriamycin on PTK activity was different than genistein and doxazosin it increased the enzyme activity 20 times relative to the control (Figure 3.5.C).

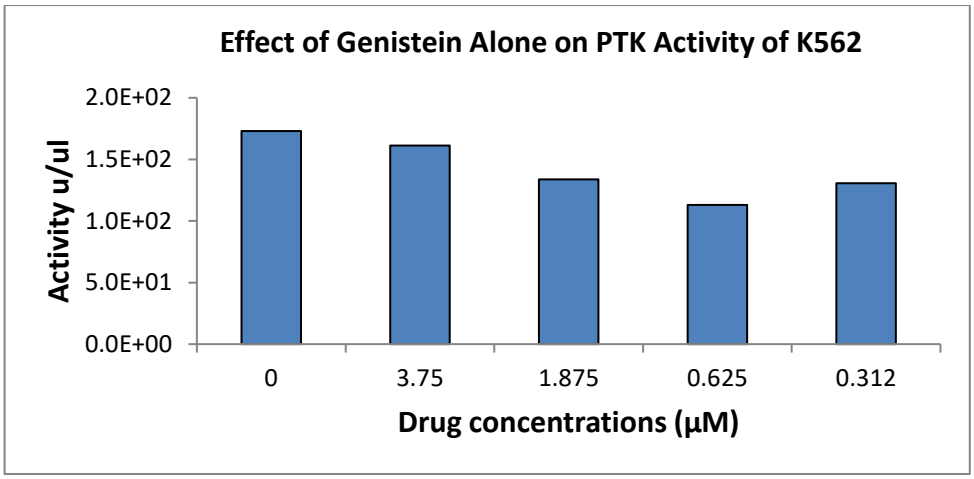


Figure 3.5.A The Effect of Genistein Alone on PTK Activity.

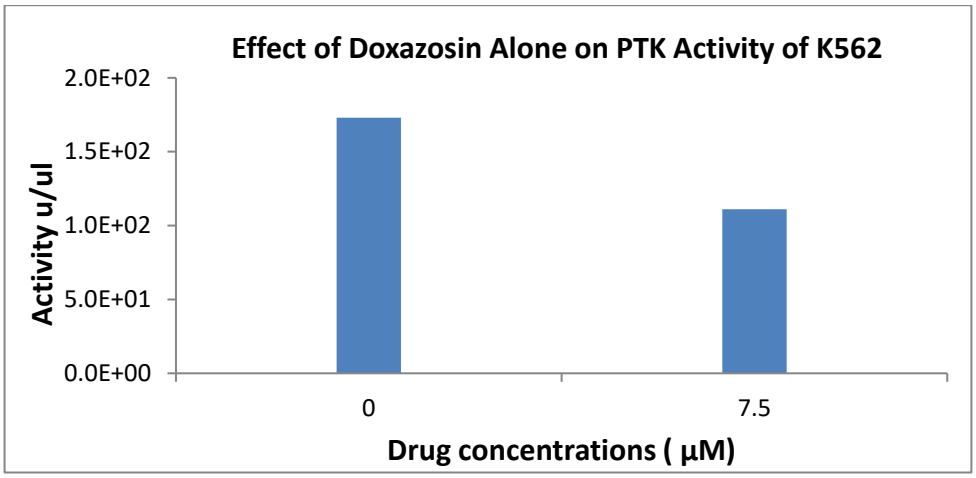


Figure 3.5.B The Effect of Doxazosin Alone on PTK Activity.

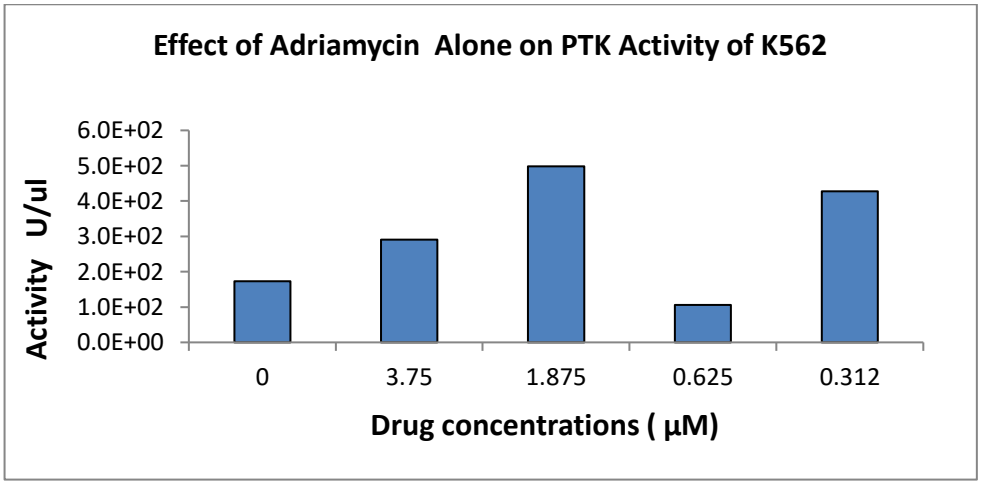


Figure 3.5.C The Effect of Adriamycin Alone on PTK Activity.

When the K562 cell line was treated with different concentrations of Doxazosin and Genistein in combination, the enzyme activity was decreased at all concentrations(Figure 3.6.A). Besides, when the cell was treated with different drug concentrations of SU6656 and Doxazosin combinations the PTK enzyme activity was decreased also than in their control but at 0.625 µM of SU6656, the activity is reached again to the value of the control (Figure 3.6.B).

Then the effect of doxazosin and Adriamycin combination on PTK activity showed that. PTK activity at equal concentrations (0.05µM) of both was as same as the control. While at 0.05µM doxazosin and 0.005µM Adriamycin the PTK activity was higher than the value of the control (Figure 3.6.C).

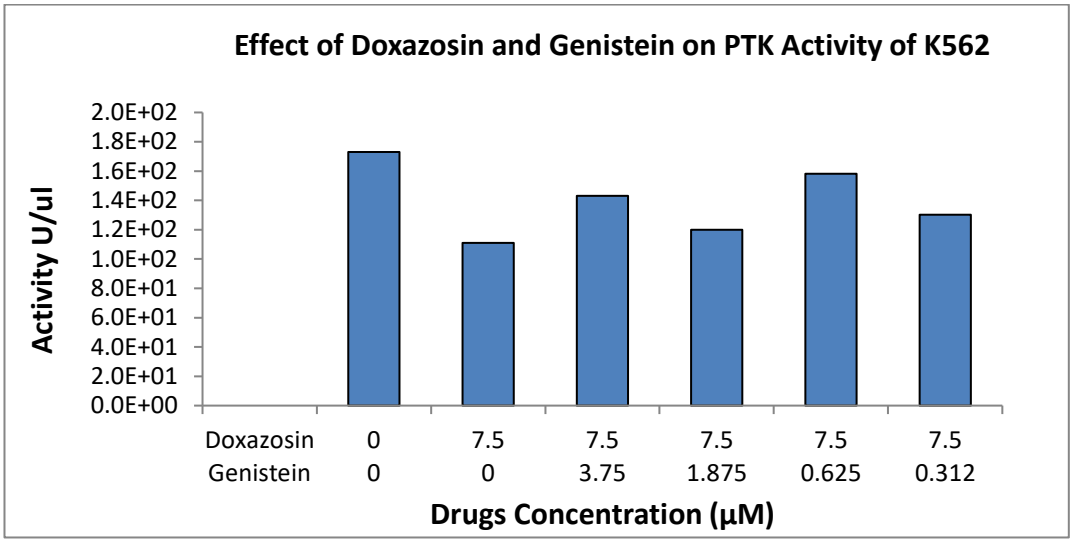


Figure 3.6.A The Effect of Doxazosin and Genistein on PTK Activity.

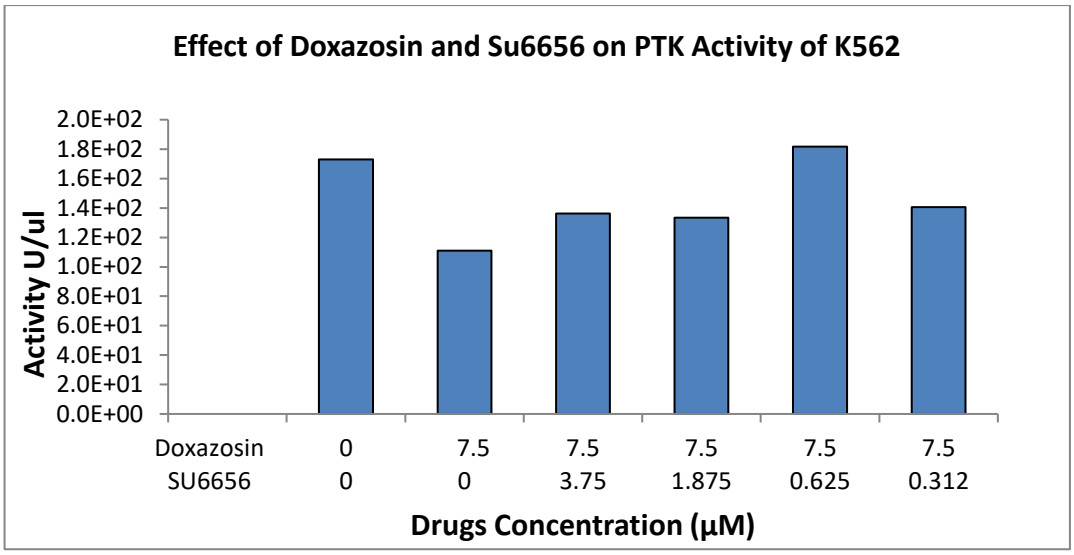


Figure 3.6.B The Effect of Doxazosin and SU6656 on PTK Activity.

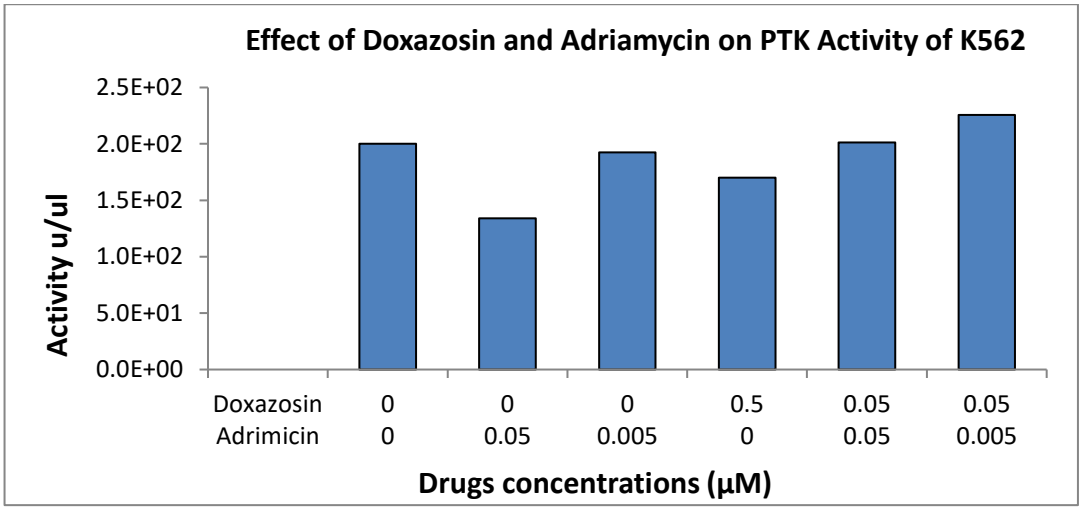


Figure 3.6.C The Effect of Doxazosin and Adriamycin on PTK Activity.

# DISCUSSION

The findings showed that the Genistein, Doxazosin, Adriamycin and SU6656 drugs either alone or in combinations have different effects on the antioxidant enzymes Glutathione-S -transferase ( GST), Superoxide dismutase (SOD ) of K562 cell line, in addition to Protein Tyrosine Kinase (PTK) as one of the enzymes that have a direct relation with cancer.

40 to 50% of pharmaceutical drugs are a failure which is related to the toxicity of these drugs [9]. While by using a combination of drugs it is possible to promote each drug in that combination without increasing the dose of single-drug thereby lower toxicity and fewer side effects.

B-blockers and α1-blockers are considered one of the main treatments available in the market to treat hypertension they can be used as monotherapy or in combination with diuretics, calcium channel antagonists [15]. For most patients, combination therapy may be required to achieve and to maintain target blood pressure levels.

Reserpine is an effective but poorly tolerated drug. In dosages of 0.10 to 0.50 mg/day, reserpine causes a significant fall in blood pressure, either as monotherapy or in combination with diuretics and/or hydralazine. That means combining drugs is not always a better solution to increase the efficacy of the targeted drug [15]. Combination therapy of finasteride and doxazosin is an effective strategy for benign prostatic hyperplasia (BPH) patients with enlarged prostate volume and symptomatic lower urinary tract symptoms (LUTS) compared with monotherapy [39].

In this study different combination of drugs with variable concentrations are used. And also different colorimetric assays are used to see the effect of this combination on the antioxidant enzyme of K562 cells.

Recent studies showed that by using a combination of Genistein and Docetaxel which is a conventional anticancer drug. On breast cancer cell line (MCF7) in order to minimize the toxicity of Docetaxel and promote their effect, there is a synergistic cytotoxic potential against hormone-dependent breast cancer cell line with ED50 of 0.880 but antagonistic effect against hormone-independent breast cancer cell line with ED50 1.409 the difference was so significant [18].

Using of Imatinib which is tyrosine-protein kinase inhibitors and has strong anti-cancer activity against BCR-ABL oncogene, with the platinum (Pt) based anti-cancer drug, on chronic myelogenous leukemia K562 in order to reduce drug resistance correlates with Imatinib, the result showed the combination treatment induces apoptosis and DNA damage. Which is properly related to the synergistic effect of both [40].

In the literature, it was also found that the treatment of cells with SU6656 increased GST activity. While in combination with Doxazosin, the enzyme activity inhibited almost 50 %.

Moreover, Genistein alone inhibited GST about 57% but in the presence of Doxazosin, the percent is lower. Consequently, it may be suggested that the Doxazosin is enhancing the effect of an anti-cancer drug [41].

This study showed that using of Genistein alone on K562 cells, Glutathione –S– Transferase has shown to be decreased in the activity with all drug doses, thereby genistein may consider as potent inhibitor agent for GST enzyme activity. Due to the fact that the drug resistance is related to the GST activity, the response of Genistein on GST activity can be concluded that the use of this drug is an advantage in cancer treatment. On the other hand, DOX administration alone on K562 cells again ended up with a decrease in GST activity relative to control. But when the drugs DOX and GEN administered together, it is observed that the GST activity was decreased too. But when their rates are compared the decrease in GST activity of drugs alone was higher than that of their combinations (Figure 3.2.A). This result concluded that both drugs may have oxidant property and just need to be converted to more soluble form caused the GST activity of K562 cells higher.

While after the cells treated with a combination of 7.5 µM of doxazosin and various concentration of SU6656, the highest GST activity was at the highest concentration of SU6656 (3.75 µM ) relative to the control, which is probably related with the high toxicity of SU6656. Unfortunately, the effect of doxazosin on the response of SU6656 cannot be concluded because in this study there is no SU 6656 alone due to cell culture problem in vitro.

While after the cells treated with different concentration of Doxazosin and Adriamycin, the GST activity on K562 cells was almost the same as the control. It is probably because that doxazosin has a synergistic effect on Adriamycin due to the fact that Adriamycin generates a high level of free radical. But the GST activity by using this combination is not increased.

In the literature it was also reported that primary leukemia cells were established from patient suffering refractory acute leukemia is used on non-irradiated mice to study the effect Quercetin and Adriamycin (doxorubicin) on T cell acute lymphoblastic leukaemia (T-ALL), the result showed that SOD enzyme activity significantly enhanced by using this combination also the survival of non-irradiated mice with T cell acute lymphoblastic leukemia improved by high dose of quercetin and Adriamycin compared with either treatment alone [42].

Skeletal muscle cells were treated with 0, 20, 40, and 80 µmol/L genistein in 50 µmol/L $FeSO_4H_2O_2$ for 24h and the result showed that Genistein increased the activity of Superoxide dismutase and glutathione peroxidase significantly.

After K562 cells treated with different concentration of Genistein and 7.5µM Doxazosin separately. The SOD activity increased at all concentrations relative to the control. when administrated together doxazosin 7.5µM with variable concentration of genistein, the result showed that combined drugs increase the formazan formation 10 times much higher than the result in which each drug used alone. Thus it is concluded that drug-drug interaction may either increase the radical formation that SOD of K562 cannot oxidize them into $H_2O_2$ or they may inhibit the SOD activity.

This result can also be related to cell viability of cells. If the drug combination has a cytotoxic effect on the cell, the SOD cannot be expressed and the production of the

radical couldn't be used by the enzyme. In order to be sure cytotoxicity, further analysis must be performed.

When the SU6656 was administered at highest dose 3.75µM the whole measured SOD activity was blocked (Figure 3.4.B), this effect was observed from where a decrease in SOD activity was observed with a decrease in SU6656 concentration where doxazosin concentration was constant.

By treating the cells with 7.5µM doxazosin and different concentration of Adriamycin, the SOD activity was increased at all concentration. On the other hand, by using Adriamycin alone the SOD activity was decreased gradually by decreasing in the Adriamycin concentration especially at 0.312 µM of Adriamycin the activity was the lowest. This result was considered to be the result of a possible increase in phase I enzymes.

Protein tyrosine kinase is categorized into receptor and non- receptor protein tyrosine kinases, recent studies show that doxazosin is a strong angiogenic agent in breast cancer cells because of it is inhibitory action on NF-Kb, VEGFR, EGFR and FGF-2 which are receptor protein tyrosine kinase [15]. Genistein also reported as tyrosine-protein kinase inhibitor in some cancer cells [43].

In the literature, SU6656 was also reported as selective Src family tyrosine kinase inhibitor which is a selective inhibitor for a platelet-derived growth factor (PDGF) important for blood vessels formation [44].

In this study, it has shown that after the cells treated with doxazosin, genistein separately or in combination, the PTK activity decreased relative to the control. By using variable concentrations of SU6656 and 7.5 µM of doxazosin in combination, the PTK activity on K562 cells was decreased. While the PTK activity increased by using Adriamycin whether alone or in combination with 7.5 µM doxazosin. But at 0.625µM of Adriamycin alone, the PTK activity decreased it is probably related to phase I metabolism of action or the effect of the drug on cell viability.

# CONCLUSION

The behavior of the K562 cancer cell line changes according to the type of the drugs and the difference in the drug doses used. The same drug has a different effect on the anti-oxidant enzyme whether it was used in non-combination or combination form.

The result showed that Doxazosin mesylate is less toxic than Genistein and SU6656. The activity of PTK decreases after using Doxazosin. However, Genistein can be considered as a potent inhibitor for GST enzyme activity which is an advantage to reduce drug resistance. On the other hand, according to the result, Adriamycin has the most strong toxic effect on the K562 cells.

Besides, Doxazosin decreases the toxicity of Genistein and SU6656 and Adriamycin when they used in combinations.

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