THE EFFECT OF COMBINED DRUG TREATMENT ON HL60 CELLS

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
ATILIM UNIVERSITY

HANAN SALEM SAID ALJADI

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

JUNE 2019

THE EFFECT OF COMBINED DRUG TREATMENT ON HL60 CELLS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
ATILIM UNIVERSITY

BY

HANAN SALEM SAID ALJADI

IN PARTIAL FULFILLMENT 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.

_____

Prof. Dr. Şeniz Özalp Yaman
Head of Department

This is to certify that we have read the thesis "THE EFFECT OF COMBINED DRUG TREATMENT ON HL60 CELLS" submitted by "HANAN.S.S.ALJADI" 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 Co-Supervisor	Assoc. Prof. Dr. S. Belgin İşgör Supervisor

Examining Committee Members:

Asst. Prof. Dr. Pembegül Uyar Arpacı _____
Biotechnology. Dept., Selçuk University

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

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

Date: June 28, 2019

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: HANAN.S.S.ALJADI

Signature:

ABSTRACT

THE EFFECT OF COMBINED DRUG TREATMENT ON HL60 CELLS

HANAN.S.S.ALJADI

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

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

Co-Supervisor: Assoc. Prof. Dr. Yasemin G. İşgör

June 2019, 52 Pages

Previous studies have demonstrated the advantages of using the combination of drugs in the treatment of various cancers by increasing the effectiveness of one drug due to the synergistic effect of another drug and overcome drug resistance. In this study different drugs alone and in combinations, were used. These drugs are Doxazosin Mesylate which is selective α1- adrenergic receptor used as antihypertensive drug and recently has been used antitumor drug. Genistein, is a natural anticancer drug used to treat different type of cancers and SU6656 which is a strong anticancer drug with high toxicity on both healthy and cancer cells which was used in this study as control for the toxicity. This study is aimed to evaluate the effect these drugs whether alone or in combination, on cell growth of human leukemia cells (HL60) and their effect on antioxidant enzymes; Glutathione-S-transferase (GST), Superoxide Dismutase (SOD) in addition to Protein Tyrosine Kinase (PTK) of these cells. Firstly, human leukemia cell lines treated with different concentrations of each drug as single treatment and then subjected to different concentrations of combined drugs. The Cell viability assay was done by using the trypan blue assay to know the effect of each drug alone and in combination on cell growth. The results revealed that doxazosin mesylate had less toxic effect on the growth of human leukemia cells (HL60) compared to genistein

and SU6656. While in combination 7.5µM of doxazosin mesylate with 0.312 µM genistein, the cytotoxic effect of anticancer drug genistein was a significantly increased. SU6656 was more toxic on human leukemia cells. The effect of the drugs that used in this study whether alone or in combination on the activity of antioxidant enzymes GST and SOD in addition to the activity of PTK enzyme were measured and human leukemia cells used as the source of these enzymes. Enzymatic assay results have shown that Doxazosin mesylate and genistein inhibit the GST activity, whether alone or in combination, while SU6656 induced the GST activity. Doxazosin mesylate and SU6656 induced the activity of SOD enzyme while genistein alone or in combination with doxazosin mesylate inhibit the SOD activity of HL60 cells. The activity of protein tyrosine kinase was induced by doxazosin mesylate whether alone or in combination with genistein. While genistein alone at higher doses induced PTK activity and at lowest doses PTK activity was inhibited. SU6656 alone and in combination with doxazosin mesylate inhibited PTK activity.

Keywords: HL60; Doxazosin Mesylate; Genistein; SU6656; Antioxidant Enzymes; Glutathione-S-Transferase; Superoxide Dismutase; Protein Tyrosine Kinase.

ÖZ

BİRLEŞİK İLAÇ TEDAVİSİNİN HL60 HÜCRELERİ ÜZERİNE ETKİSİ

HANAN.S.S.ALJADI

Yüksek Lisans, Kimya Mühendisliği ve Uygulamalı Kimya Anabilim Dalı

Danışman: Doç. Dr. S. Belgin İşgör

Eş -Danışman Doç. Dr. Yasemin G. İşgör

Haziran 2019, 52 sayfa

Önceki çalışmalar, başka bir ilacın sinerjistik etkisine bağlı olarak bir ilacın etkinliğini artırarak ve ilaca karşı direncin üstesinden gelmekle birlikte, çeşitli kanserlerin tedavisinde ilaç kombinasyonunun kullanılmasının avantajlarını göstermiştir. Bu çalışmada tek başına ve kombinasyon halinde farklı ilaçlar kullanılmıştır. Bu ilaçlar, antihipertansif ilaç olarak kullanılan ve son zamanlarda antitümör ilaç olarak kullanılan selektif adrenerjik reseptör olan Doxazosin Mesilat'tır. Bir diğer ilaç, Genistein, farklı kanser türlerini tedavi etmek için kullanılan doğal bir antikanser ilacıdır. Son olarak hem sağlıklı hem de kanser hücrelerinde yüksek toksisiteye sahip güçlü bir antikanser ilacı olan SU6656 aynı zamanda bu çalışmanın toksisite kontrolü olarak kullanılmıştır. Bu çalışmada tek başına veya kombinasyon halinde kullanılan ilaçların insan lösemi hücrelerinin (HL60) hücre büyümesi ve antioksidan enzimler Glutatyon-S-transferaz (GST) ve Superoksit Dismutaz (SOD) üzerindeki etkilerini araştırıp bu hücrelerin Protein Tirozin Kinazın (PTK) aktivitesi üzerine de etkileri de çalışılmıştır. Bu çalışmada ilk olarak, insan lösemi hücre hatları kullanılarak, her ilacın farklı konsantrasyonları tek olarak ve daha sonra farklı kombine ilaç konsantrasyonları tabi tutularak ilaç etkileri araştırıldı., her ilacın kendisi ve ilaç kombinasyonunun hücre canlılılığı üzerine etkisi tripan mavisi metodu kullanılarak yapıldı. Doksazosin mesilatın, genistein ve

SU6656 ile karşılaştırıldığında insan lösemi hücrelerinin (HL60) büyümesinde daha az toksik etkisi olduğu görüldü. Ortaya çıkan sonuçlar göstermiştir ki, 0.312 uM genistein ile 7.5 uM doksazosin mesilat kombinasyonunda, antikanser ilaç genisteinin sitotoksik etkisi önemli ölçüde artmıştır. SU6656'nın insan lösemi hücrelerinde daha toksik etkisi olduğu bulunmuştur. Bu çalışmada kullanılan ilaçların etkisinin PTK enziminin aktivitesine ek olarak GST ve SOD antioksidan enzimlerinin aktivitesinin ölçülmesi bu enzimlerin kaynağı olarak kullanılan insan lösemi hücrelerinin kullanılmasıyla gerçekleşmiştir. Enzim sonuçları, Doxazosin mesilat ve genisteinin, tek başına veya kombinasyon halinde GST aktivitesini inhibe ettiğini, SU6656'nın ise GST aktivitesini indüklediğini göstermiştir. Doksazosin mesilat ve SU6656, SOD enziminin aktivitesini indüklerken, tek başına doxazosin mesilat ile kombinasyon halinde genisten, HL60 hücrelerinin SOD aktivitesini inhibe etmiştir. Protein tirozin kinazın aktivitesi, tek başına ya da genistein ile kombinasyon halinde doksazosin mesilat ile indüklenmiştir. Genistein sadece daha yüksek dozlarda PTK aktivitesini ve en düşük dozlarda PTK aktivitesini indüklemiştir. Tek başına SU6656 ve doksazosin mesilat ile kombinasyon halinde PTK aktivitesini inhibe etmiştir.

Anahtar Kelimeler: HL60; Doksazosin Mesilat; Genistein SU6656; Antioksidan Enzimler; Glutation-S-transferaz; Superoksit Dismutaz; Protein Tirozin Kinaz.

ACKNOWLEDGEMENT

Praise be to Allah and the lord of the worlds who gave us the strength and knowledge to complete this work.

I would like to express my gratitude to my academic supervisor Assoc. Prof. Dr. S. Belgin İşgör who gave me this opportunity to be one of her students. She gave me moral support and guided in different matters regarding the topic, she had been very kind and patient while suggesting me the outlines of this thesis. I thank her for her overall support.

Secondly, I would like to express my sincere thanks and appreciation to my academic co-supervisor Assoc. Prof. Dr.Yasemin G.İşgör for her continuous support, her patience, and her friendship. I was fortunate to have the chance to work with her.

Last but not the least, I would like to express my special thanks and appreciation to my husband and my kids for their patience and for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. There are no words to describe my sincere thanks and appreciation to my parents for giving birth to me in the first place and supporting me spiritually throughout my life.

Finally, I would like to express my thank to my friend Eklas Alarabi. Who participated me this stage of my academic life.

TABLE OF CONTENTS

ABSTRACT iii

ÖZ v

ACKNOWLEDGEMENT vii

TABLE OF CONTENTS viii

LIST OF TABLES xi

LIST OF FIGURES xii

LIST OF ABBREVIATIONS xiv

CHAPTER 1 1

INTRODUCTION 1

1.1. Drug Combination Used for Cancer Treatment 1

1.2. Cell lines and Their Importance 3

1.3. Cytotoxicity Test 3

1.4 . Drugs Used in The Study 4

1.4.1. Doxazosin Mesylate 4

1.4.2. Genistein 5

1.4.3. SU6656 6

1.5. The Role of Reactive Oxygen Species on Cancer Progression 7

1.6. Antioxidant Enzymes 7

1.6.1. Glutathione-S-Transferase Enzyme (GST) 8

1.6.2. Superoxide Dismutase Enzyme (SOD) 11

1.7. Tyrosine Kinase Enzyme (TK) 12

1.8. Aim of Study 13

CHAPTER 2..... 14

MATERIALS AND METHODS..... 14

2.1. Materials:…… ..... .14

2.2. Methods… 15

2.2.1. Culturing of Human Leukemia Cell Lines (HL60) 15

2.2.2. Treatment of HL60 cells with drugs..... 15

2.2.3. Trypan Blue Assay 17

2.2.4. Preparation of Cell Homogenates..... 17

2.2.5. BCA Protein Assay..... 18

2.2.6. Enzyme Assays..... 19

2.2.6.1. Glutathione-S-Transferase (GST) Enzyme Assay 19

2.2.6.2. Superoxide Dismutase (SOD) Enzyme Assay..... 20

2.2.6.3. Protein Tyrosine Kinase (PTK) Enzyme Assay..... 21

CHAPTER 3..... 23

RESULTS AND DISCUSSION 23

3.1. Cell Viability Results 23

3.1.1 . The Effect of Doxazosin Mesylate, Genistein and Their Combinations on HL60 Cell Viability 23

3.1.2. The Effect of Doxazosin Mesylate, SU6656 and Their Combinations on HL60 Cell Viability..... 24

3.2. BCA Protein Assay Results 25

3.2.1. Amount of Protein (mg/ml) in HL60 Samples after Administration of Drugs Alone..... 25

3.2.2. Amount of Protein (mg/ml) in HL60 Samples after Administration of Drugs Combination 27

3.3 . The Results of Effect of Drugs on The Enzyme Activity of HL60 cell lines ……29

3.3.1. The Effect of Drugs on GST Activity of HL60 Cells 29

3.3.2 The Effect of Drugs in Combination on GST Activity of HL60 Cells 31

3.3.3. The Effect of Drugs on SOD Activity of HL60 Cells 33

3.3.4. The Effect of Drugs in Combination on SOD Activity of HL60 Cells 35

3.3.5. The Effect of Drugs on PTK Activity of HL60 Cells 37

3.3.6. The Effect of Drugs in Combination on PTK Activity of HL60 Cells 40

DISCUSSION 42

CONCLUSION 46

REFERENCES 47

LIST OF TABLES

Table 2.1 Concentrations of drugs used……..15
Table 2.2 Concentrations of Doxazosin with Genistein 16
Table 2.3 Concentrations of Doxazosin with SU6656.....16
Table 2.4 Glutathione-S-Transferase (GST) Assay mixture.....19
Table 2.5 Superoxide Dismutase (SOD) Assay mixture.....21
Table 2.6 Protein Tyrosine Kinase (PTK) Assay mixture.....22
Table 3.1 Protein content in HL60 samples treated with Doxazosin.....26
Table 3.2 Protein content in HL60 samples treated with Genistein.....26
Table 3.3 Protein content in HL60 samples treated with SU6656.....27
Table 3.4 Protein content in HL60 samples treated with Doxazosin and Genistein……..28
Table 3.5 Protein content in HL60 samples treated with Doxazosin and SU6656…..…….....28

LIST OF FIGURES

1.1 Chemical structure of Doxazosin Mesylate…… 4

1.2 Chemical structures of Genistein……. 5

1.3 Chemical structure of SU6656……. 6

1.4 The quadruple structure of human GSTP ……..……... 8

1.5 Role of GSH in carcinogenesis …..…… 10

1.6 Dismutation reaction .....……..... 11

2.1 Trypan blue assay……. 17

2.2 Microplate and BCA Protein Assay Kit …….. 18

2.3 Superoxide Dismutase (SOD) Reaction……..……..… 20

3.1 Screening of Doxazosin and genistein on HL60 Cells…….. 23

3.2 Screening of Doxazosin and SU6656 on HL60 cells ……..……. 24

3.3 BSA Standard calibration curve……..……..... 25

3.4. a The effect of different concentrations of Doxazosin mesylate on GST enzyme of HL60 cells…….....….. 30

3.4. b The effect of different concentrations of Genistein, SU6656 on GST enzyme of HL60 cells……..... 30

3.5. a The effect of diverse concentrations of combined drugs Doxazosin mesylate with Genistein on GST enzyme of cells HL60……..... 31

3.5. b The effect of diverse concentrations of combined drugs Doxazosin mesylate with SU6656 on GST enzyme of cells HL60 ……... 32

3.6. a The effect of different concentrations of Doxazosin mesylate on SOD enzyme of HL60 cells……..…. 33

3.6. b	The effect of different concentrations of Genistein on SOD enzyme HL60 cells……...….....	34
3.6.c	The effect of different concentrations of SU6656 on SOD enzyme of HL60 cells…….	34
3.7. a	The effect of diverse concentrations of combined drugs Doxazosin mesylate with Genistein on SOD enzyme of HL60 cells…….....	36
3.7. b	The effect of diverse concentrations of combined drugs Doxazosin mesylate with SU6656 on SOD enzyme of HL60 cells…….....	36
3.8. a	The effect of different concentrations of Doxazosin mesylate on PTK enzyme of HL60 cells …….……	38
3.8. b	The effect of different concentrations of Genistein on PTK enzyme of HL60 cells…….....	38
3.8.c	The effect of different concentrations of SU6656 on PTK enzyme of HL60 cells …….....	39
3.9. a	The effect of diverse concentrations of combined drugs Doxazosin mesylate with Genistein on PTK enzyme of HL60 cells…….	41
3.9. b	The effect of diverse concentrations of the combined drugs Doxazosin mesylate with SU6656 on PTK enzyme of HL60 cells…….....	41

LIST OF ABBREVIATIONS

NCI	National Cancer Institute
MDR	Multi Drug Resistance
ROS	Reactive Oxygen Species
GST	Glutathione-S-transferase
GSH	Glutathione reduced form
CDNB	1-Chloro-2,4-dinitrobenzen
SOD	Superoxide Dismutase
XOD	Xanthine Oxidase
NBT	Nitro Blue Tetrazolium Chloride
PTK	Protein Tyrosine Kinase
TMBZ	Tetramethylbenzidine
HRP	Horse Reddish Peroxidase
BSA	Bovine Serum Albumin
DOX	Doxazosin
GEN	Genistein

CHAPTER 1

INTRODUCTION

Cancer has been acknowledged as a cluster of illnesses that involve abnormal development of cells that may cause metastasis [1]. Cancer is a complex disease where it exists in different types with various causes, making it difficult to diagnose and treat.

To understand the mechanism of cancer, scientific researches depend on use certain models such as cancer cells, these cells have the ability to continue to grow and develop over time under certain circumstances in a laboratory [2]. And provide a fundamental study of cellular and genetic levels that involved in cancer and helps to explore new anticancer drugs in addition to understanding the mechanism of chemotherapeutic drugs and problems that may face it such as drug resistance [3].

1.1. Drug Combination Used for Cancer Treatment

Drug combination is applied by using one or more drugs in order to have a better clinical response. Drug combinations are also used to overcome or reduce drug resistance [4]. According to NCI (National Cancer Institute) dictionary cancer terms monotherapy is defined as "the therapy that uses one type of treatment such as radiation or surgery alone". The monotherapy has been successful in treating many human diseases [5]. The use of a single drug, which usually targets a single protein, is therefore not be able to effectively treat complex diseases. That is why the use of one or more drugs simultaneously to treat a disease, could potentially improve the efficacy due to their synergistic effect [4]. The design of the chemotherapeutic combination requires understanding of numerous principles such as toxicity, non-cross resistance, and increased efficacy in killing cancer cells. Specific drug combination strategies are applied to achieve optimum effectiveness [6].

One of the previous studies was conducted by Chang and co-workers to evaluate the effect of two drugs genistein and terazosin. Genistein, which is soy isoflavone has the ability to prevent the growth of different types of cancer cells and terazosin, a class of α-adrenoreceptor antagonist. The effect of these two drugs combinations on human prostate cancer cells (DU-145) were studied. The results showed that genistein alone was more effective to inhibit cell growth, whereas terazosin had no significant toxic effect on the prostate cancer cells. On the other hand, the combination of genistein with a nontoxic dose of terazosin resulted in a significant increase in the antitumor activity of genistein on these cells [7].

In another study, the effect of genistein (GE) and tamoxifen (TAM) combination was evaluated. In that study GE, TAM alone and their combinations were applied to liver hepatocellular carcinoma, hepG2, cells. The MTT result showed that., GE and TAM inhibited cell proliferation and triggered apoptosis in the HepG2 cell lines. From this result, it can be concluded that the drug combination in this study had a great effect on the liver functions [8].

A research was conducted on Benign Prostatic Hyperplasia on the long-term effects of α_1-blocker (doxazosin), α-5 reductase (finasteride) and their combination on quality of life in men. The results showed that, combination therapy has been found to considerably decrease the incidence of urinary retention and lower the risk of surgical treatment, leading to a greater reduction in the volume of prostate and a higher increase in the maximum urinary flow rate relative to monotherapy [9].

Combined administration of chemotherapy drugs is often used to overcome drug resistance. Furthermore, the cancer cells are not subjected to the same proportion and dose of chemotherapy owing to the variations between physicochemical and pharmacokinetic characteristics of drugs [10].

1.2. Cell lines and Their Importance

Cell culture and the establishment of cell lines have played an important role in the study of specific cell physiology, pathophysiology and differentiation processes. It allows studying step-by-step changes in the cell's structure, biology, and genetic make-up under controlled environments [11].

Cell lines provide a pure cell population that is valuable as it provides a consistent sample and reproducible outcome. They have been used in the production of vaccines, the testing of drug metabolism, cytotoxicity and biological compound synthesis [11].

HL60 cell line of human promyelocytic cancer is used widely for experimental studies. It was originally gathered from a 36-year-old female with acute promyelocytic leukaemia at the National Cancer Institute (NCI). The cells are largely similar to promyelocytes. Several reagents may cause differentiation into cells similar to granulocytes, others into cells similar to monocytes/macrophages [12],[13].

1.3. Cytotoxicity Test

One of the most significant indices for in vitro biological assessment research is cytotoxicity. In vitro, chemicals like drugs and pesticides have distinct processes of cytotoxicity such as cell membrane destruction, inhibition of protein synthesis, irreversible receptor binding, etc. Nowadays, to determine the cell death induced by these damages, short-term cytotoxicity and cell viability these tests are needed to be inexpensive, reliable and reproducible [14].

Cell viability is described as the sum of viable cells in a test and cell proliferation. It is a basic marker for cell survival or death after exposure to poisonous/toxic agents within the activity of certain genes, proteins, and pathways.

Cell toxicity (Cytotoxicity) and proliferation tests are more often than not utilized for drug Screening to recognize whether the test particles have cell proliferation impacts or coordinate cytotoxic impacts .

There are many methods based on diverse cellular activities such as the action of the enzyme, the penetrability of the cell membrane, cell adherence and action of nucleotide take-up.

These procedures can essentially be categorized into particular classifications such as ATP assay, sulforhodamine B assay, and metabolic activity methods[15].

1.4 . Drugs Used in The Study

The drugs that are decided to be used in this study are Doxazosin Mesylate, Genistein and SU6656.

1.4.1. Doxazosin Mesylate

Doxazosin (DXZ) is frequently used as an antihypertensive remedy as well as treating benign prostatic hyperplasia. This medication is a quinazoline compound classified as one of selective α-adrenergic blockers. Benzene, a pyrimidine and two fused aromatic rings, which are well known for their biological activity due to their many pharmacophores, create quinazoline [16]. The structure of the DXZ is shown in Figure 1.1.

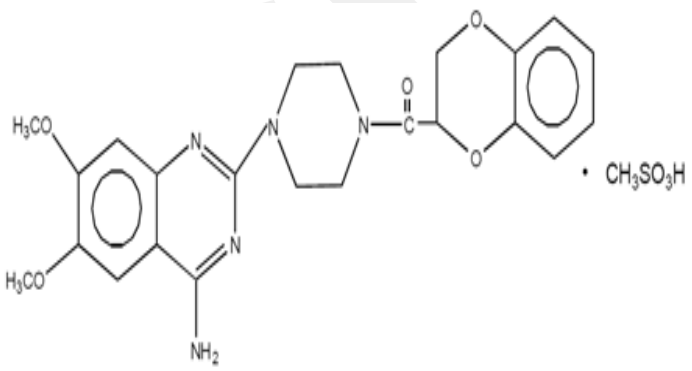

Figure 1.1 Chemical structure of Doxazosin Mesylate.

Doxazosin has recently been used as a prospective anti-cancer drug, which accounts for apoptotic activity over α1-adrenoceptor-independent-mechanism [17]. As an apoptosis inducer, DXZ was created as a strong cell progress inhibitor with antiangiogenic impact on several tumour cell lines. Doxazosin can induce apoptosis in prostate cancer cells, endothelial and malignant cells, cardio-myocytes, cardio-myoblasts breast cancer cells, bladder smooth muscle cells, urothelial cells, pituitary adenoma cells, colon cancer cells and HeLa cells [18].

Previous studies reported that, in breast cancer cell lines (MDA-MB-231and MCF7), DXZ had induced apoptosis through the inhibition of both epidermal growth factor receptor (EGFR) and a nuclear transcription factor (NF-kB), which are related to the creation of new vessels. In vitro research examination, which utilizes glioblastoma cell lines, (DXZ) promotes antiproliferative effects in addition to induce cell death as well as inhibiting cell proliferation through caspase-three activation and cell cycle arrest at the G0 and G1 stage [16].

1.4.2. Genistein

Genistein (Figure 1.2), chemically named as (4',5,7-trihydroxyisoflavone) or else as 5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one. Moreover, Genistein is a naturally arising as soybean isoflavone glycoside with a heterocyclic diphenolic structure similar to estrogen, it is deliberated to be a strong anticancer agent[19].

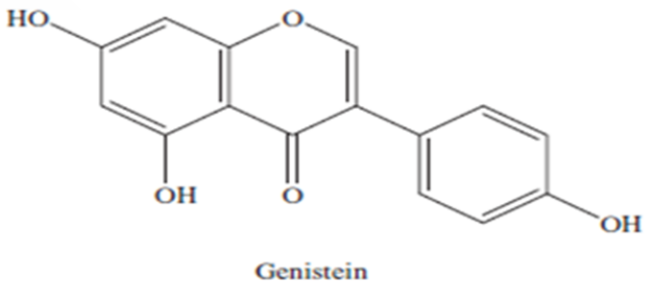

Figure 1.2 Chemical structure of Genistein.

Genistein helps to treat various malignancies such as breast tumor, prostate tumor, leukemias and many carcinomas. It also activates the arrest of cell cycle growth, apoptotic cell death by inactivation of NF-πB, caspase 3 gene stimulation in prostate tumor cells, and prevents the proliferation of tumor cells[19]. Previous studies have also submitted that, Genistein has ability to prevent the growth of tumor cells, by inhibiting protein tyrosine kinases, topoisomerases I and II, and articulates the genes of mRNAs that stay linked to the cell cycle in different cell types[19].

1.4.3. SU6656

SU6656 is chemically named as 2-oxo-3-(4,5,6,7-tetrahydro-1 H-indol-2-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonicaci diethylamide. SU6656 is a strong inhibitor of the Src family of tyrosine kinase (SFK) enzyme that controls signaling pathways. It was created as a selective SFK inhibitor and released as a strong driven DNA synthesis inhibitor of Platelet-derived growth factor. It also has cross-reactivity with certain other kinases such as the Aurora B kinases that regulate the ploidy of cells and the centrosome number [20]. The chemical structure of SU6656 is shown in Figure 1.3.

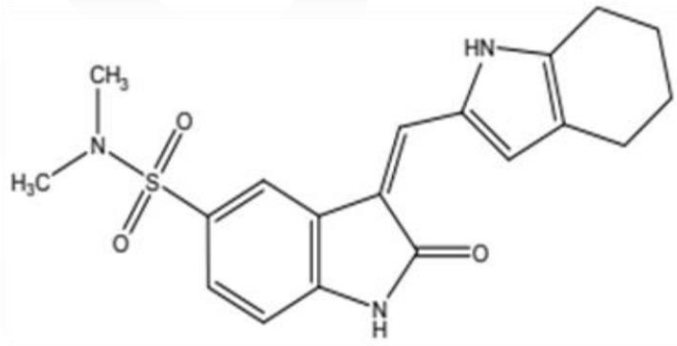

Figure 1.3 Chemical structure of SU6656.

Concurrent use of SU6656 before fractional irradiation stimulates blood vessel destruction in the cancer cell and delay its growth [21]. In addition, due to the increased anti-angiogenic effect of irradiation, SU6656 is used as an effective agent in the treatment of drug resistant. SU6656 is used as a tool to investigate the role of Src family kinases in cellular signal transduction mechanisms [20].

1.5. The Role of Reactive Oxygen Species on Cancer Progression

Enzymatic and non-enzymatic structures produce reactive oxygen species (ROS) in eukaryotic cells. These species are radicals such as superoxide ($O2^{\bullet-}$), hydroxyl ($OH^{\bullet}$) and peroxyl ($RO_2^{\bullet}$) or nonradical, for instance, hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2). They play significant roles in cell physiology and pathophysiology [22],[23].

Reactive oxygen species at low concentrations are essential in the process of cytokine signaling and regulation, growth factor and hormone action, intracellular activity, immunomodulation, and apoptosis. In addition to they have a strong function by enhancing the body's immunity and making it more disease-resistant [23],[24].

While physiological levels are essential to cell survival, overproduction of reactive oxygen species is harmful to cells and induce oxidative damages which is regarded to be key factors in the growth of several diseases, such as neurodegenerative diseases, cardiovascular disorders, and cancer [22].

1.6. Antioxidant Enzymes

Antioxidant enzymes are a group of enzymes such as Superoxide Dismutase, Catalase, Glutathione Peroxidase, and Glutathione-S-Transferase which are produced in the human body and act as a primary defense against reactive oxygen species by inducing cell damage at high concentrations. These enzymatic antioxidants work by giving their own electrons to reactive oxygen species and make them more stable and less reactive or inactive species[23].

1.6.1. Glutathione-S-Transferase Enzyme (GST)

GST enzymes have a catalytic capacity to catalyze the tripeptide glutathione conjugation to electrophiles such as carcinogens, natural toxins and exogenous drugs. This response typically leads to less toxicity than the initial compounds [25]. Furthermore, GST isoenzymes indicate extra catalytic activities depend on GSH such as decreasing H_2O_2 in relation to isomerizing several unsaturated compounds [26].

GST enzymes (GST; EC 2.5.1.18) are found in organisms as various as microbes, insects, plants, birds, fish and mammals. These enzymes are a large group of proteins separated into two families. Cytosolic or soluble GSTs are the first family and 16 members have been recognized in humans such as Alpha (α), Mu (μ), Pi (π), Sigma (σ), Theta (θ), Zeta (ζ) and Omega (ω)[26]. Mitochondrial GSTs are the second family, branded as "membrane-associated proteins engaged in the metabolism of eicosanoids and glutathione" and are structurally dissimilar to cytosolic GSTs. However, they still maintain the capacity to catalyze glutathione (GSH) conjugation to electrophiles [27]. The soluble glutathione-S-transferase has been determined by X-ray crystallography with many classes. Figure 1.4 shows the human quadruple structure GSTP1-1[28].

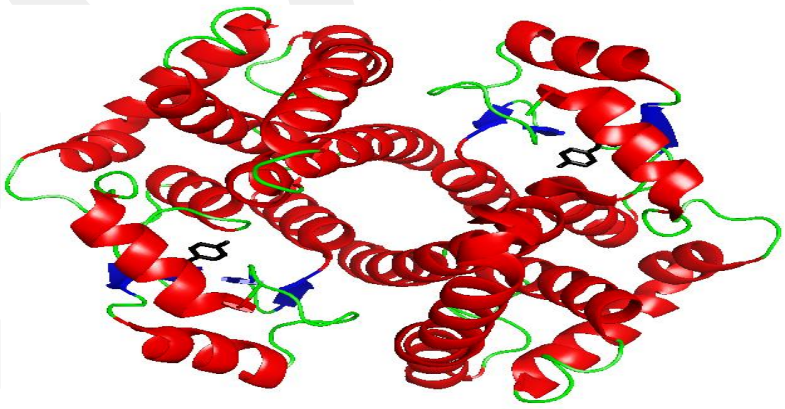

Figure 1.4 The quadruple structure of human GSTP [28].

The high level of cytosolic glutathione-S-transferase is discovered in the human body's liver, whereas in the kidney, lung, and intestine reduced concentrations are discovered.

High concentrations of GSTP are used as markers for a number of cancers [29]. Whereas in different types of cancer, for example, breast cancer, the overexpression of class GSTP has been recognized which can be used as a predictive factor for these diseases.

Glutathione has been shown to regulate development of carcinogenesis in various ways. Its protective function in cell conversion is to detoxify carcinogens such as reactive oxygen species or xenobiotics which are generated as result of UV exposure, chemical agents, climate, inflammation and nutrition. This detoxification is mediated by its antioxidant function or by Phase II metabolism responses which catalyzed by GST. High expression rates of several GSH enzymes leading to change its function in transformed cells [30]. Due to glutathione inhibit apoptosis and redox regulation of thiol groups leading to transformation and immortalization of cells. In addition, abnormal concentrations of several reactive oxygen species and reactive nitrogen species in tumor cells control post-translational changes through S-glutathionylation or S-nitrosylation of redox-sensitive transcription factors and proteins shown to be engaged in tumorigenesis (Figure 1.5).

Glutathione contributes to cell resistance to anticancer drugs by either apoptosis inhibition or deficiency in GSH-conjugation to detoxify chemotherapeutic drugs. Thus, the strategy of therapy depends on glutathione depletion by either extrusion or metabolism have been shown to sensitize cells to anti-cancer treatment [30].

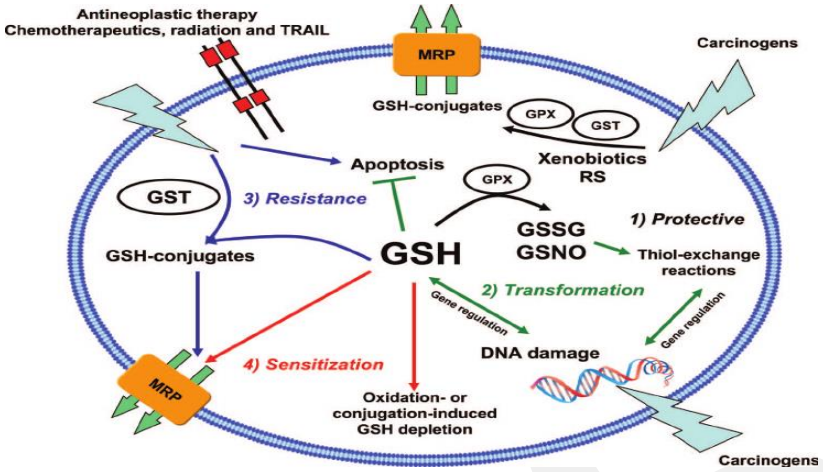

Figure 1.5 Role of GSH in carcinogenesis [30].

Although glutathione transferase's detoxifying capacity to maintain cells against pathogens, it may decrease the effectiveness of certain anti-cancer drugs, such as electrophilic alkylating agents, which are substrates for glutathione-S-transferases. Due to its protective mechanism inside the cancer cell towards anticancer medicines, high glutathione-s-transferase activity can cause problems for the patient during chemotherapy. The therapeutic significance of chemotherapeutic can therefore be accomplished if glutathione-S-transferase activity is inhibited [31]. The improvement of GST inhibitors can therefore generate a beneficial reaction to overcome multi-drug resistance (MDR) and enhance the effectiveness of antitumor drugs [28].

1.6.2. Superoxide Dismutase Enzyme (SOD)

Superoxide dismutases are an endogenous enzyme acts as a primary line defence against reactive oxygen species to protect the cell [32]. Superoxide dismutase reducing superoxide anion level, which is causing cell damage at the high concentration by converting this anion to molecular oxygen and hydrogen peroxide. This reaction, as shown in Figure 1.6 is followed by alternative oxidation-reduction of metal ions that is found on the active site of superoxide dismutases [33].

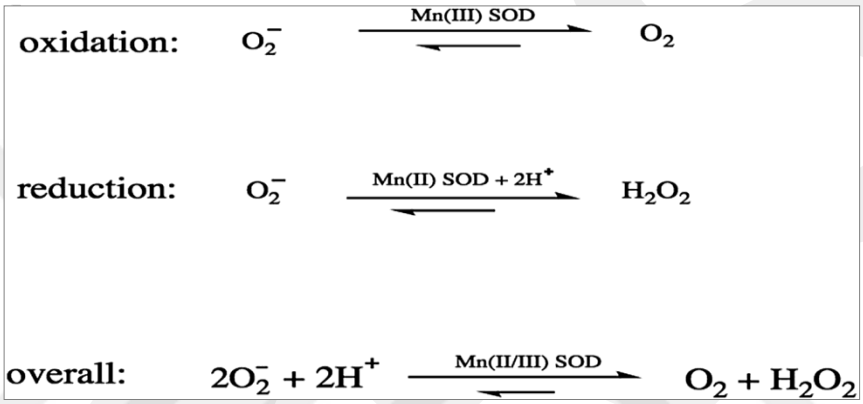

Figure 1.6 Dismutation reaction [34].

The activity of Superoxide dismutase needs to metal cofactor which is bounded on its active site and according to the type of these metal cofactors, superoxide dismutase divided into three families:

- (Cu, Zn-SOD) Copper, zinc superoxide dismutase exists in eukaryotes and localized mainly in the cytosol.
- (Fe-SOD) Iron superoxide dismutase exists in prokaryotes and chloroplasts in some plants
- (Mn-SOD) Manganese superoxide dismutase exists in prokaryotes and mitochondria of eukaryotes [32].

The various classes of superoxide dismutase are coded by a diverse group of genes, for example, copper, zinc superoxide dismutase is coded by the SOD1 gene, manganese superoxide dismutase is coded by the SOD2 gene and the eukaryotic extracellular Cu-Zn SOD is coded by the SOD3 gene. Which have been found in substantial amount in all human tissues[32].

They have different functions, manganese superoxide dismutase (SOD2) due to it is existing in mitochondria, it's considered to be important for life more than others[35]. SOD1 and SOD2 are closely associated with cancer, SOD1 is acting by reducing reactive oxygen species (ROS) level. When it decreases, leading to accumulation of ROS, which causing DNA damage and cancer progression. Previous studies showed that the highest levels of SOD2 were detected in late stages of different cancers such as lung cancer, while in the early stage of some cancers its levels were low such as breast cancer. For this reason, it has been used as a marker to detect different types of cancers[36].

1.7. Tyrosine Kinase Enzyme (TK)

Tyrosine kinase is a subclass of protein kinases that have controlling roles in everyone feature of cell biology [37]. Tyrosine kinases play the main role in the stimulation of signal transduction cascades through inducing the transport of a phosphate group from adenosine triphosphate (ATP) to tyrosine residuum in the cell . By Phosphorylation of tyrosine residues, numerous functions are regulated, for instance, the activity of the enzyme, cellular localization, signal transduction and interactions between proteins [38].

There are two categories of tyrosine kinase enzyme first, receptor tyrosine kinases (RTK), which have an extracellular ligand-binding domain, a single-pass transmembrane domain, and an intracellular kinase domain, and the second one are cytoplasmic non-receptor tyrosine kinases (n-RTK), which are multi-domain proteins that are regularly connected to the internal plasma membrane[39].

The Src family kinases are a cluster of genes that express the code for cytoplasmic non-receptor protein kinases that have the main role in intracellular signaling paths and regulate cell growth. This family of kinases is controlled by various kinds of receptors, as well as receptor tyrosine kinases, cytokine receptors, G-protein coupled receptors, steroid hormone receptors, and integrin receptors. These genes are classical proto-oncogenes that are frequently mutated or overexpressed in cancers and uncontrolled cell proliferation are produced [40],[38].

Both classes of tyrosine kinase (RTK) and (n-RTK) are involved in different signaling baths and the dysregulation in these kinases have a key role in carcinogenesis. Due to the complexity of these proteins, researchers have focused their attention to inhibit these kinases to treat cancer [38].

1.8. Aim of Study

The main objective of this study is to evaluate the effect of different drugs Doxazosin mesylate, Genistein and SU6656 whether alone or in combination, on cell growth of human leukemia cells (HL60) and their effect on antioxidant enzymes Glutathione-S-transferase (GST) and Superoxide Dismutase (SOD) in addition to Protein Tyrosine Kinase (PTK) of these cells.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials:

All the chemicals and reagents that are used in this study are freshly prepared and all are with high quality in order to obtain the best results.

HL60 cell line from ATCC (American Type Culture Collection)**,** Doxazosin Mesylate (Pfizer), SU6656 (Sugen), Genistein (Sigma), RPMI-1640 medium (1X) (HyClone), Pen-Strep solution (Penicillin 10,000 units/ml, streptomycin 10mg/ml) and L-glutamine solution (200mM, 29.2 mg/ml) (BI biological industry), Bovine serum albumin (BSA) (Thermoscientific). NaCl (Sigma-Aldrich), KCl (Fluka), Sodium Phosphate dibasic anhydrous (Fisher Scientific), Potassium Phosphate Monobasic (KH_2PO_4) (Riedel-deHaen), DDT (Fluka), Reduced form of glutathione (GSH) (Fluka), 1-chloro-2,4 Dinitrobenzene (CDNB) (Fluka). Pure Ethanol , Potassium Phosphate Dibasic (K_2HPO_4) (Riedel-deHaen), Sodium Carbonate Anhydrous (Na_2CO_3) (Fisher Scientific), Sodium Hydrogen Carbonate NaH_2CO_3 (MERCK), Xanthine (Sigma-Aldrich), NBT (Nitro blue tetrazolium) (Fisher scientific), Xanthine Oxidase (Calbiochem), EDTA (Sigma), Tween 20 0.05% (MERCK), 2-Mercaptoethanol (Fisher Scientific), Universal Tyrosine kinase Assay kit (Takara, Japan), 1N Sulfuric acid solution .

2.2. Methods

2.2.1. Culturing of Human Leukemia Cell Lines (HL60)

HL60 cells were grown in 250ml of RPMI 1640 medium complemented with 2.5 ml of L-Glutamine solution, 2.5 ml of Pen/Strep solution and 25ml of bovine serum albumin. Culture were kept in an incubator at 37 $^{\circ}$C with humidified atmosphere of 5% CO_2 and 95% air. Sub-culturing was done every 2-3 days.

2.2.2. Treatment of HL60 cells with drugs

HL60 cell lines have been treated with different concentrations of the drugs: Doxazosin, Genistein and Su6656 either alone or in combinations in this study. The concentrations are optimized previously and then used. The concentrations of the drugs used in this study are given in Table 2.1.

Table 2.1 Concentrations of drugs used.

Drugs	**Concentrations (µM)**					
Doxazosin	7.5	3.75	1.875	0.625	0.312	0
Genistein	7.5	3.75	1.875	0.625	0.312	0
SU6656	-------	3.75	1.875	0.625	0.312	0

HL60 treated with the combination of Doxazosin mesylate with Genistein, and Doxazosin mesylate with SU6656 separately, and the concentrations that are used in this study are given in the Tables 2.2 and 2.3 respectively.

Table 2.2 Concentrations of Doxazosin with Genistein .

Doxazosin (µM)	**Genistein (µM)**
0	0
0	0.005
0	0.05
0.05	0
0.05	0.005
0.05	0.05
0.5	0.05

Table 2.3 Concentrations of Doxazosin with SU6656.

Doxazosin (µM)	**SU6656 (µM)**
0	0
0	0.005
0	0.05
0.05	0
0.05	0.005
0.05	0.05
0.5	0.05

2.2.3. Trypan Blue Assay

To determine the amount of viable cells, present in a cell suspension, the trypan blue dye exclusion test is used. The method is based on principle that live cells with intact cell membrane exclude certain dyes such as trypan blue, ezine, or propidium, while dead cells do not. A viable cell will have a clear cytoplasm, while dead cells have a blue cytoplasm under microscope [41].

In this assay for 50µl of cell suspension equal components of 0.4% trypan blue dye was added to achieve a 1:2 dilution and hemocytometer was used to count the cells under microscope as shown in Figure 2.1.

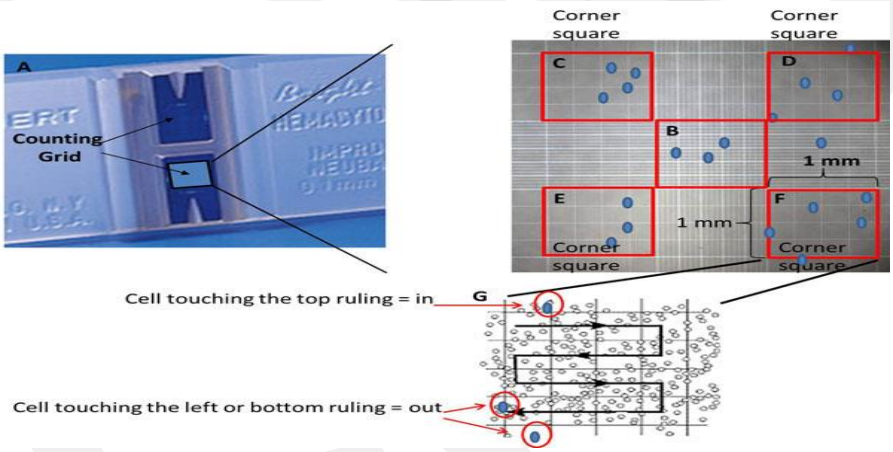

Figure 2.1 Trypan blue assay.

2.2.4. Preparation of Cell Homogenates

To prepare tissue homogenates, phosphate-buffered saline (1X PBS) have a final concentration of 137mM NaCl, 10mM phosphate, 2.7mM KCl, and at pH 7.4 with 1mM of DDT are used. The cells are homogenated by using sonication and then centrifuged at 6000 rpm at 4˚C for 10 min. The pellets are collected and stored at -80 ºC.

2.2.5. BCA Protein Assay

The BCA protein assay is used to quantify complete protein in a sample. The principle of this method is that proteins can reduce Cu^{+2} to Cu^{+1} in an alkaline solution and cause a purple color to form from bicinchoninic acid. Four amino acid residues that are present in protein molecules, including cysteine or cystine, tyrosine and tryptophan, primarily cause copper to decrease [42].

In this assay, calibration curve and operating reagents are prepared for BCA standards. In each microplate well, 20µl of each sample was introduced and 200 µl of working reagent was added evenly. The plate covered and incubated for 30 minutes, the spectrophotometer measured the wavelength at 562nm. Figure 2.2 shows the microplate and the kit used in the assay.

Figure 2.2 Microplate and BCA Protein Assay Kit.

2.2.6. Enzyme Assays

2.2.6.1. Glutathione-S-Transferase (GST) Enzyme Assay

The activity of GST enzyme is determined by evaluating the rate of conjugation reaction that occurs between (GSH) Glutathione reduced form and 1-chloro-2,4-dinitrobenzene (CDNB), which is proportional to the increase in absorbance at 340nm.

In this study, the cytosol of HL60 cell line treated with different concentrations of combination drugs and drugs alone are used as a source of GST enzyme. In this assay, 200µL, 200mM of potassium phosphate buffer pH 6.5 was added to each well of microplate, 15µL, 200mM of glutathione-reduced form, 15µL, 50mM of CDNB and then 20µL of cytosol was added and the total volume of each reading was 250µL as shown in Table 2.4. The protocol was optimized previously [43].

Table 2.4 Glutathione-S-Transferase (GST) Assay mixture.

Reagents	**Volume added per well**
Potassium Phosphate buffer pH 6.5 (200 mM)	200µL
GSH (200 mM)	15 µL
CDNB (50 mM)	15µL
Cytosol	20µL

2.2.6.2. Superoxide Dismutase (SOD) Enzyme Assay

Superoxide Dismutase (SOD) catalyze the dismutation of the reactive oxygen species into elemental oxygen (O_2) and peroxides, which is further converted into water. In the assay as shown in figure 2.3. Superoxide ions which are produced from the transformation of xanthine to uric acid and hydrogen peroxide by xanthine oxidase (XOD) catalyzes the transformation of NBT to NBT-diformazan which absorbs light at 560nm.

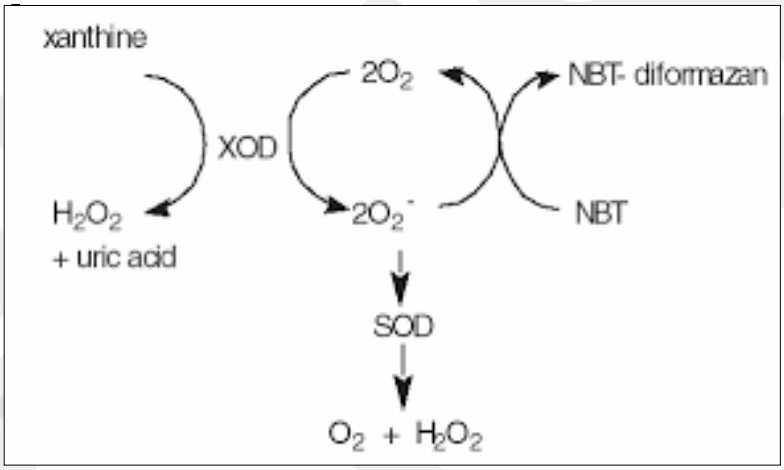

Figure 2.3 Superoxide Dismutase (SOD) Reaction.

In this assay, 10µL of cytosol with different concentrations was added to each well of microplate then 60 µL of sodium carbonate buffer pH 10.1 with EDTA, 75µL of Xanthine, 10µL of nitro blue tetrazolium (NBT), 80 µL of extra buffer are added consequently. After 2 min, incubation in dark 15µL of xanthine oxidase was added with the final volume of 250µL. The assay was followed by kinetic measurement at 560nm. The reagent mixture used in SOD assay is presented in Table 2.5.

Table 2.5 Superoxide Dismutase (SOD) Assay mixture.

Reagents	Volume added per well	Concentration
Cytosol	10 µL	----------
Sodium carbonate buffer pH 10.1 with EDTA	60 µL	200 mM with 0.6 mM EDTA
Xanthine	75 µL	0.3 mM in 2.5 mM NaOH
NBT	10 µL	25 mM
Extra buffer	80 µL	200 mM with 0.6 mM EDTA
Incubation for 2 minutes		
XOD	15 µL	3.64 U/ml

2.2.6.3. Protein Tyrosine Kinase (PTK) Enzyme Assay

In this assay (40µL) of cytosol was added to each well of the microplate, then (10µL) of 40 mM of ATP-2Na added and incubated for 30 minutes in an incubator at 37°C. After incubation, the sample solution removed and the walls washed four times by washing buffer at pH 7.4, 100 µL of the blocking solution was added to each well and incubated again for 30 min. After that blocking solution discarded from each well and wells are washed again, Microplate was dried on paper towel. Then 50 µL of anti-phosphotyrosine (PY20) -HRP solution added into each well and incubated for 30 min at 37 °C.

Antibody solution removed and the wells were washed again. 100 µL of HRP substrate solution (TMBZ) added into each well, and incubated for 15 min. After this incubation blue color was observed, 100 µL of stop solution (1N sulfuric acid) was added into each well and the color changed to yellow, the absorbance was measured at 450nm. The reagent mixture used in PTK assay was designated in Table 2.6.

Table 2.6 Protein Tyrosine Kinase (PTK) Assay mixture.

Reagents	**Volume added per well**
Cytosol	40 µL
ATP-2Na 40 mM	10 µL
Incubation for 30 minutes at 37°C	
Blocking solution	100 µL
Incubation for 30 minutes at 37 °C	
anti-phosphotyrosine (PY20)– HRP solution	50 µL
Incubation for 30 minutes at 37 °C	
HRP substrate solution (TMBZ)	100 µL
Incubation for 15 minutes at 37 °C	
1N sulfuric acid (stop solution)	100 µL

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cell Viability Results

3.1.1 . The Effect of Doxazosin Mesylate, Genistein and Their Combinations on HL60 Cell Viability

As mentioned under " Method", HL60 samples subjected to different types and concentrations of drugs and cell viability of cells were tested on those samples and the results are given in Figures 3.1 and 3.2 respectively. Due to the results it has shown that doxazosin mesylate at the highest concentration (3.75µM) caused 65% of cell death while genistein caused 60 % of cell death at the same concentration. On the other hand, the percentage of cell death was found to be 80% at (3.75µM) of doxazosin-genistein combination. In contrast genistein alone which caused 42% of cell death, doxazosin alone was found to be nontoxic on the cells, where it caused 6% of cell death at (0.312µM) concentration as shown in Figure 3.1.

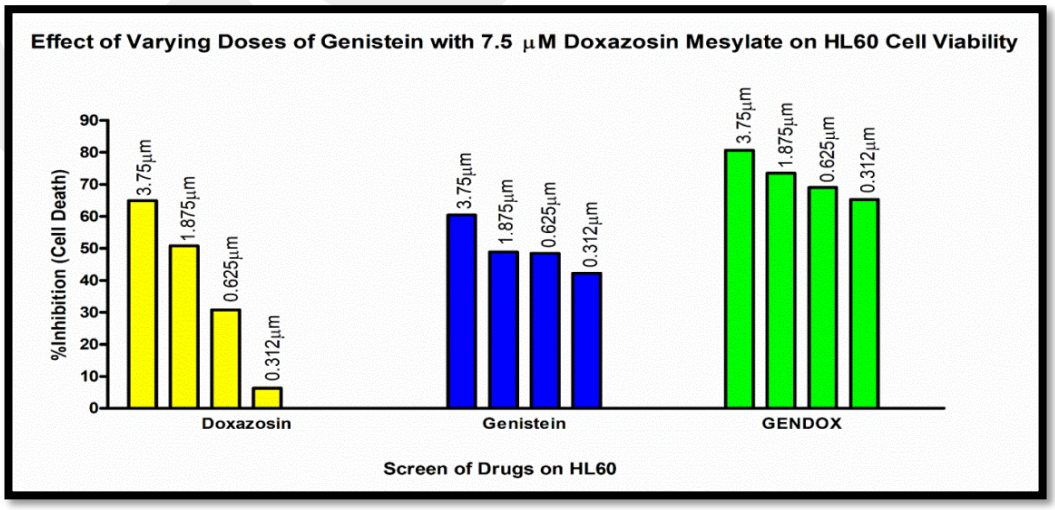

Figure 3.1 Screening of Doxazosin and genistein on HL60 Cells.

3.1.2. The Effect of Doxazosin Mesylate, SU6656 and Their Combinations on HL60 Cell Viability

After cell viability analysis SU6656 was found to be more toxic on HL60 cells by causing 96% of cell death at high concentration (3.75 µM) and 85% of cell death at low concentration (0,312 µM) compared to doxazosin alone which was less toxic on cancer cells. Doxazosin and SU6656 combination resulted in a slight increase in cell toxicity, as compared with SU6656 alone. The toxicity of drug combination was found to be much higher than that of doxazosin alone, even for its highest concentration as shown in Figure 3.2.

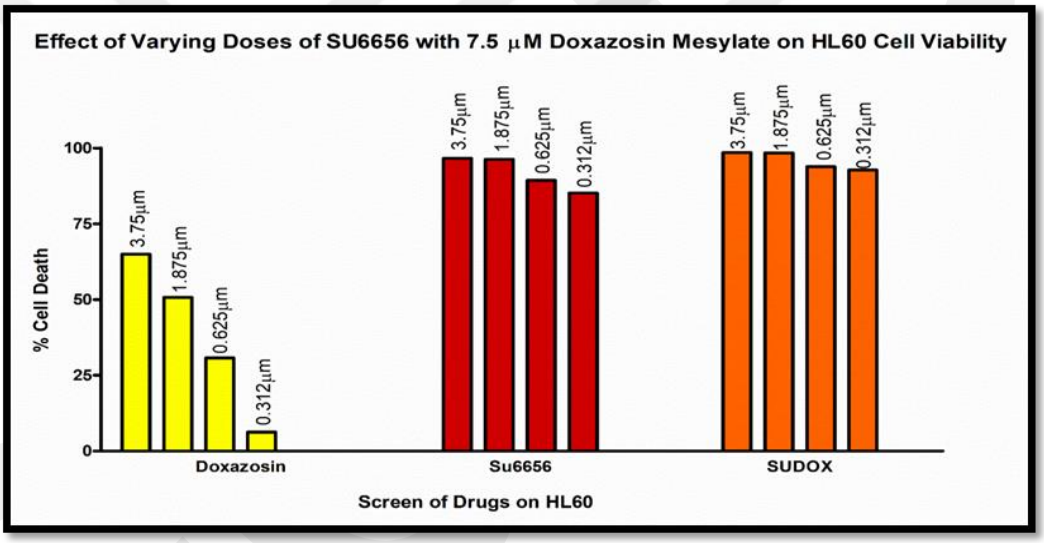

Figure 3.2 Screening of Doxazosin and SU6656 on HL60 cells.

3.2. BCA Protein Assay Results

In this method protein amount (mg/ml) in HL60 samples were determined by using BCA assay (Bicinchoninic Acid) the principle of this assay is detecting the reduced copper in alkaline solution resulted in purple color formation and the absorbance was measured at 562nm by using bovine serum albumin (BSA) as standard. The standard calibration curve (Figure 3.3) for BSA was used to quantify the protein amount in the samples by comparing the absorbance of standard calibration curve with the absorbance of the samples.

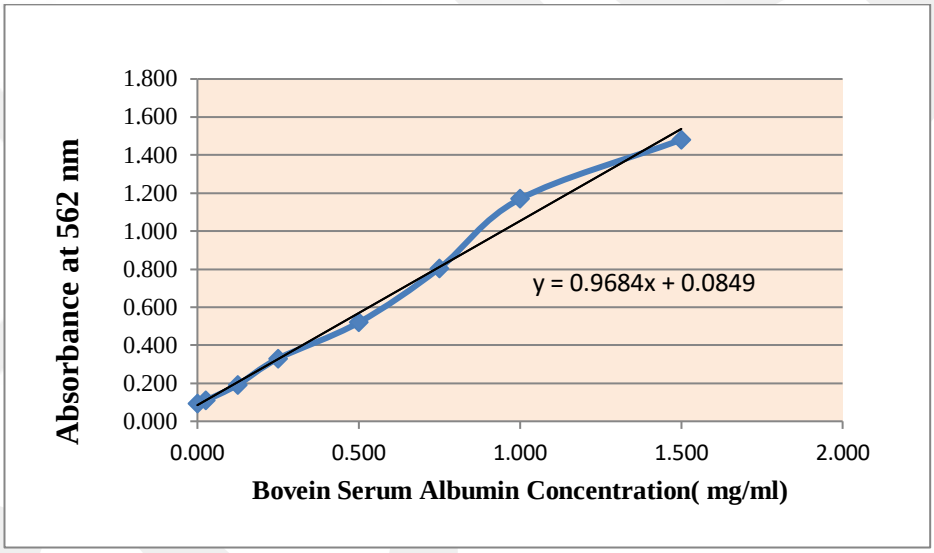

Figure 3.3 BSA Standard calibration curve.

3.2.1. Amount of Protein (mg/ml) in HL60 Samples after Administration of Drugs Alone

The concentration of proteins in HL60 samples treated with different concentrations of doxazosin mesylate, genistein and SU6656 have shown below in Tables 3.1,3.2,3.3 respectively in addition to the concentration of protein in HL60 without treatment (control).

The results showed that the amount of protein in HL60 samples increased after administration of doxazosin and genistein as a drug alone with different concentrations. While after administration of SU6656 the amount of protein found to be decreased at 0.625µM doses compared to control (Table 3.3).

Table 3.1 Protein content in HL60 samples treated with Doxazosin.

Doxazosin (µM)	**Protein Content (mg/ml)**
7.5	12.35
3.75	17.39
1.875	12.38
0.625	13.29
0.312	13.94
0	10.51

Table 3.2 Protein content in HL60 samples treated with Genistein

Genistein (µM)	**Protein Content (mg/ml)**
7.5	3.014
3.75	2.919
1.875	3.321
0.625	2.983
0.312	4.086
0	2.326

Table 3.3 Protein content in HL60 samples treated with SU6656.

SU6656 (µM)	Protein Content (mg/ml)
3.75	7.794
1.875	8.480
0.625	5.769
0.312	7.560
0	7.738

3.2.2. Amount of Protein (mg/ml) in HL60 Samples after Administration of Drugs Combination

The concentration of proteins in HL60 samples treated with different concentrations of combined drugs have studied. The protein content of HL60 cells treated with doxazosin and genistein is presented in Table 3.4 and that of doxazosin with SU6656 in Table 3.5.

The results showed that the amount of protein in the samples which treated with a combination of doxazosin and genistein decreased except in one combination (0.05µM GEN + 0.05µM DOX) the amount of protein was increased. While the amount of protein in the samples that treated with a combination of doxazosin with SU6656 was decreased compared to control.

Table 3.4 Protein content in HL60 samples treated with Doxazosin and Genistein.

Doxazosin (μM)	**Genistein (μM)**	**Protein Content (mg/ml)**
0	0	10.51
0	0.005	8.195
0	0.05	5.796
0.05	0	9.702
0.05	0.005	8.600
0.05	0.05	13.67
0.5	0.05	7.050

Table 3.5 Protein content in HL60 samples treated with Doxazosin and SU6656.

Doxazosin (μM)	**SU6656 (μM)**	**Protein Content (mg/ml)**
0	0	10.51
0	0.005	8.742
0	0.05	8.338
0.05	0	9.702
0.05	0.005	9.400
0.05	0.05	8.996
0.5	0.05	8.135

3.3 . The Results of Effect of Drugs on The Enzyme Activity of HL60 cell lines

3.3.1. The Effect of Drugs on GST Activity of HL60 Cells

This assay was done on the basis of the ability of GST to induce conjugation reaction between glutathione reduced form (GSH) and CDNB. Whereas the activity of this enzyme is directly proportional with this reaction and the absorbance was measured at 340nm as it was explained under "method" section.

The source of glutathione-S-transferase enzyme was human leukemia cells, which was treated with different type and concentrations of drugs where the control was HL60 cells without treatment.

Doxazosin mesylate showed overall decrease in the activity of GST enzyme. The highest decrease of the enzyme activity was observed after using 3.75 µM of the drug, as the activity of the enzyme was 0.029 Unit/ml with respect to control (0.0553 Unit/ml). The results are given in Figure 3.4.a.

The activity of the enzyme was decreased as well to (0.030 Unit/Ml) through the addition of genstein by using both 0.05µM and 0.005µM drug doses sequentialy. However, SU6656 showed a dose-dependent effect on the enzyme activity. By adding 0.005µM dose of SU6656, the activity was increased to (0.067 Unit/mL). On the other hand, by adding 0.05µM of the SU6656 drug, the activity decreased to (0.029 Unit/mL). All the results were analyzed with respect to control value (0.0553 Unit/ml) (Figure 3.4.b).

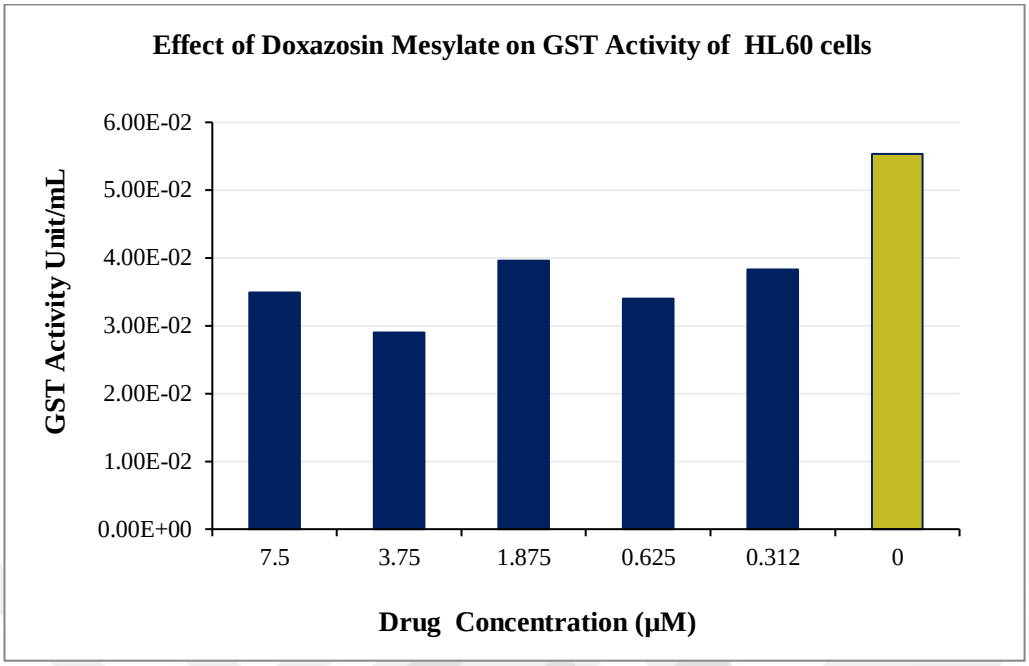

Figure 3.4.a The effect of different concentrations of Doxazosin mesylate on GST enzyme of HL60 cells.

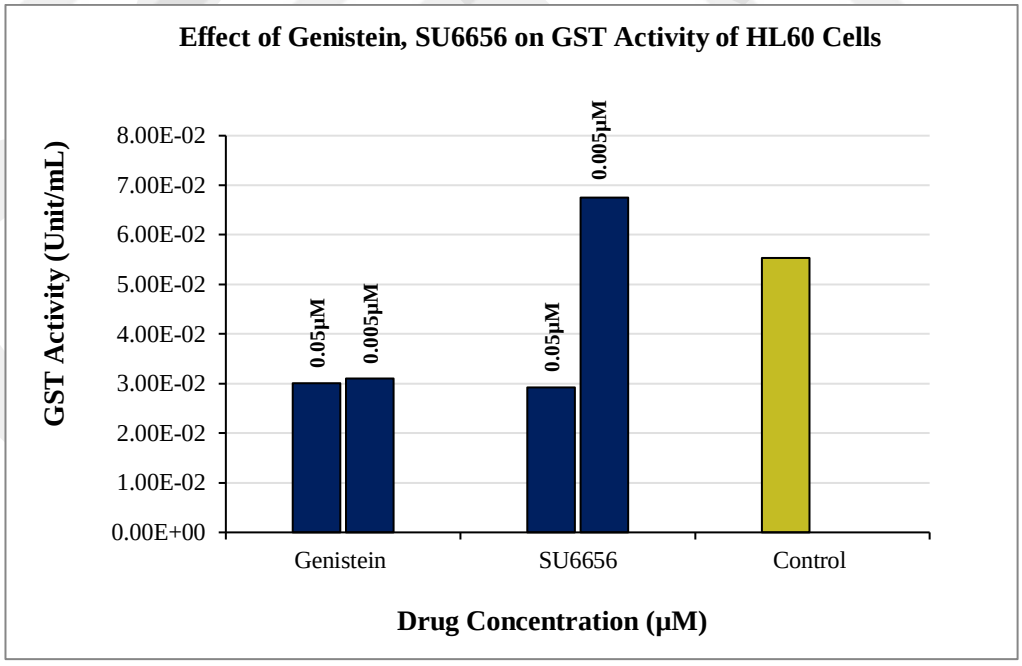

Figure 3.4.b The effect of different concentrations of Genistein, SU6656 on GST enzyme of HL60 cells.

3.3.2 The Effect of Drugs in Combination on GST Activity of HL60 Cells

The combination of doxazosin mesylate with genistein at different concentrations used in this study as mentioned in Table 2.2 under methods, resulted in the decrease of GST enzyme activity compared to the control as shown in Figure 3.5.a.

The administration of SU6656 together with doxazosin mesylate at different concentrations that are given in Table 2.3 under methods section, resulted decrease in the GST enzyme activity too. The only 0.005µM SU6656 concentration alone resulted in the increase of the GST activity to (0.067 Unit/mL)which is higher than the control. All the results were analyzed with respect to control value (0.0553Unit/mL) (Figure 3.5.b).

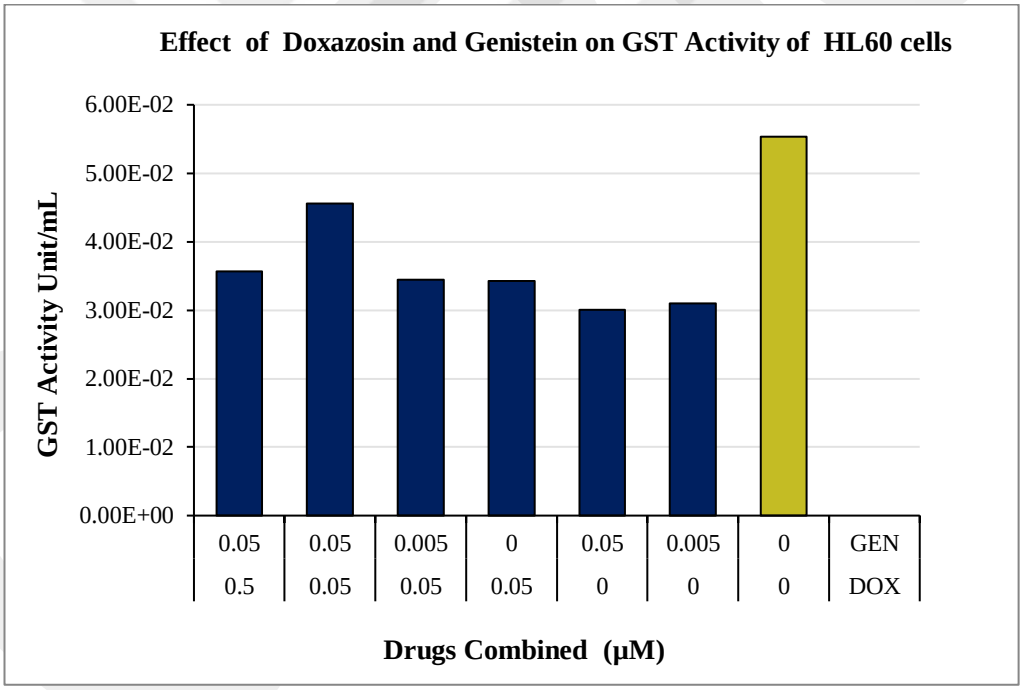

Figure 3.5.a The effect of diverse concentrations of combined drugs Doxazosin mesylate with Genistein on GST enzyme of cells HL60.

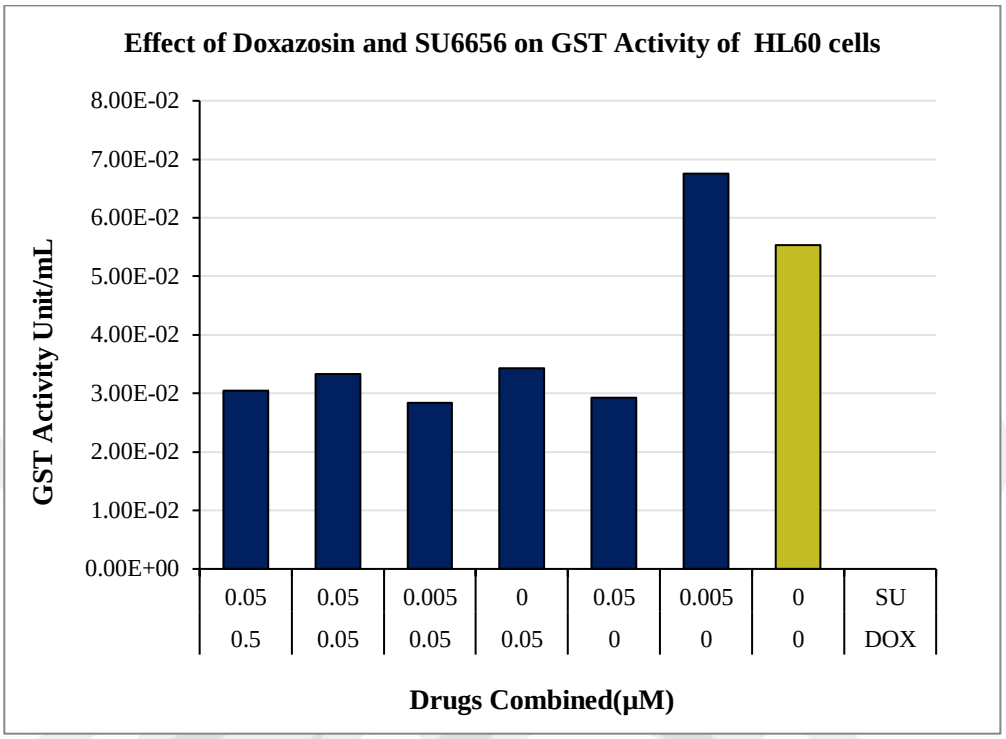

Figure 3.5.b The effect of diverse concentrations of combined drugs Doxazosin mesylate with SU6656 on GST enzyme of cells HL60.

3.3.3. The Effect of Drugs on SOD Activity of HL60 Cells

The activity measurement of this enzyme is based on suppressing of the reduction reaction of nitroblue tetrazolium (NBT) which is generated by superoxide radicals. The HL60 cells were used as superoxide dismutase enzyme source and its activity tested by using different drug and drug combinations at varying concentrations compared to the control.

According to the principle of this assay, the activity of SOD enzyme is high when the NBT diformazan formation is inhibited. Doxazosin showed an increase in the SOD enzyme activity at different concentrations more than control (Figure 3.6.a).

Genistein resulted in decrease in SOD activity except at low concentration (0.005µM) than control (Figure 3.6.b). On the other hand, SU6656 drug resulted in a significant increase in the enzyme activity at lowest doses more than high dose 3.75µM that was used. All the results were analyzed with respect to control (0.0534 Unit/mL) (Figure 3.6.c).

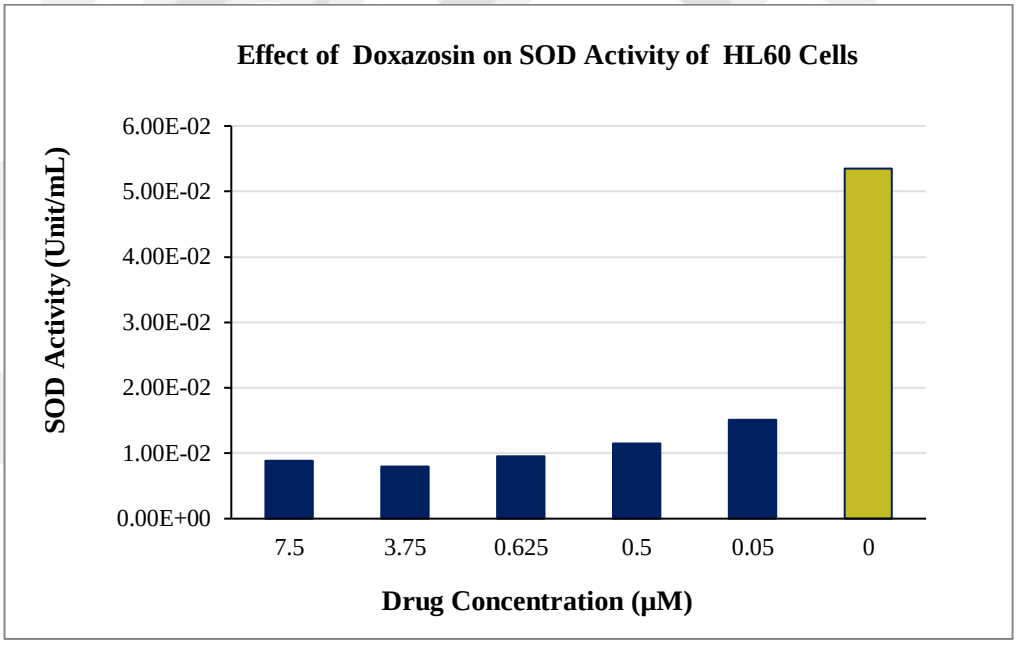

Figure 3.6.a The effect of different concentrations of Doxazosin mesylate on SOD enzyme of HL60 cells.

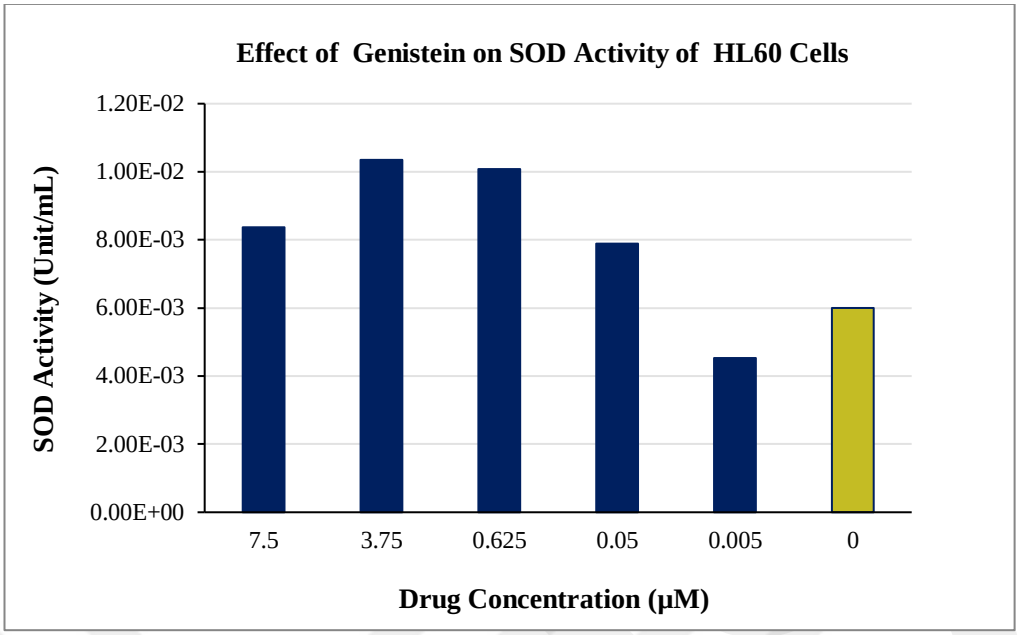

Figure 3.6.b The effect of different concentrations of Genistein on SOD enzyme HL60 cells.

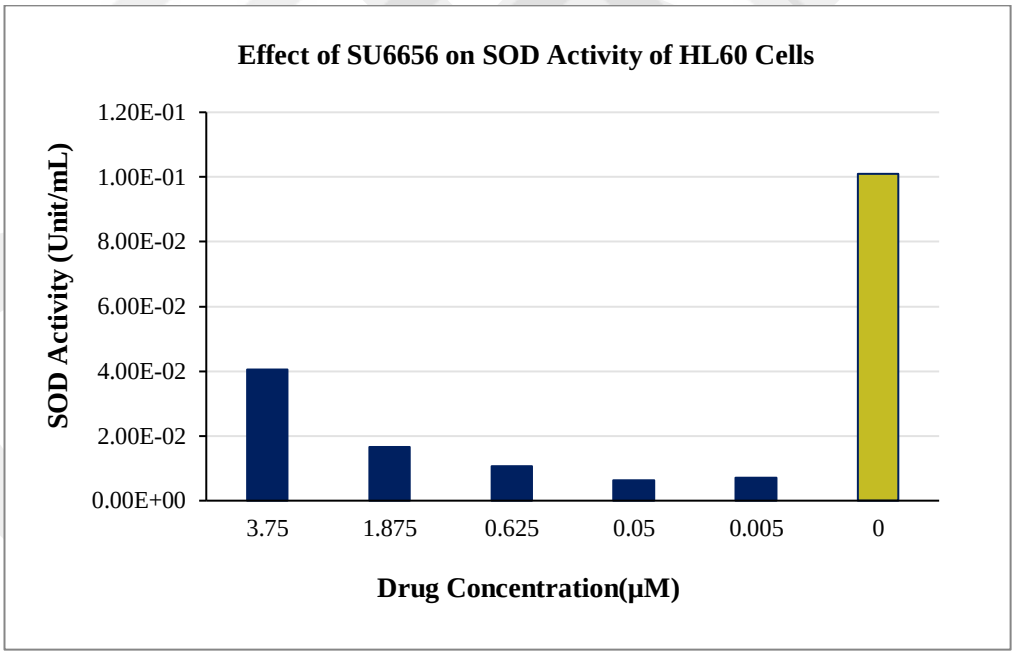

Figure 3.6.c The effect of different concentrations of SU6656 on SOD enzyme of HL60 cells.

3.3.4. The Effect of Drugs in Combination on SOD Activity of HL60 Cells

The combination of Genistein with doxazosin mesylate at dose (0.005µM GEN + 0.05µM DOX) showed decrease in SOD activity when compared to other doses of combination and control. The other dose of drug combination (0.05µM GEN + 0.05µM DOX) showed slight decrease in enzyme activity when compared to control.

On the other hand the drug combination at (0.05µM GEN+ 0.5µM DOX) dose showed the activity of SOD not affected when compared to control. Doxazosin at 0.05µM resulted in highest decrease in SOD activity compared to genistein, which showed less decrease in SOD activity at the same dose. While the highest increase in SOD activity was observed at 0.0045 Unit/mL When genistein administered at low dose 0.005µM compared to control (Figure 3.7.a). All the results were analyzed with respect to control value (0.006 Unit/mL).

The decrease of SOD activity was observed at different concentrations of SU6656 with doxazosin mesylate (Figure 3.7.b). The highest decrease of SOD activity was observed at 0.00948Unit/mL, using a dose of (0.005µM SU+ 0.05µM DOX) and the minimum decrease was at 0.0069 Unit/mL by using a dose (0.05µM SU + 0.5µM DOX).

SU6656 when administered alone at 0.05µM showed that, the SOD activity was not affected by this dose, while the activity of SOD was slightly decreased when it was administered at low dose 0.005µM compared to control. All the results were analyzed with respect to control value (0.006 Unit/mL).

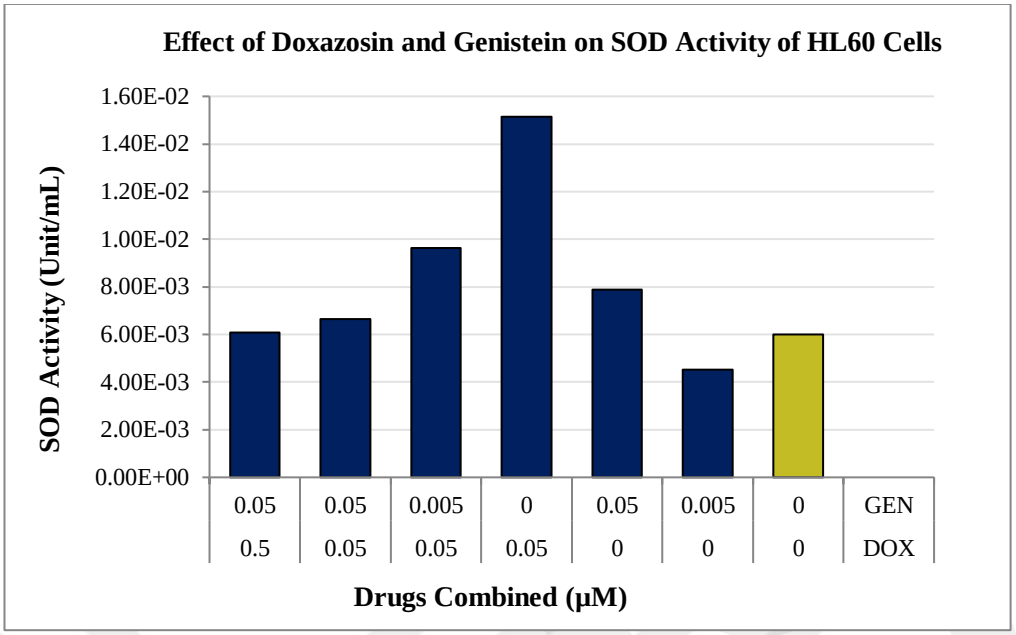

Figure 3.7.a The effect of diverse concentrations of combined drugs Doxazosin mesylate with Genistein on SOD enzyme of HL60 cells.

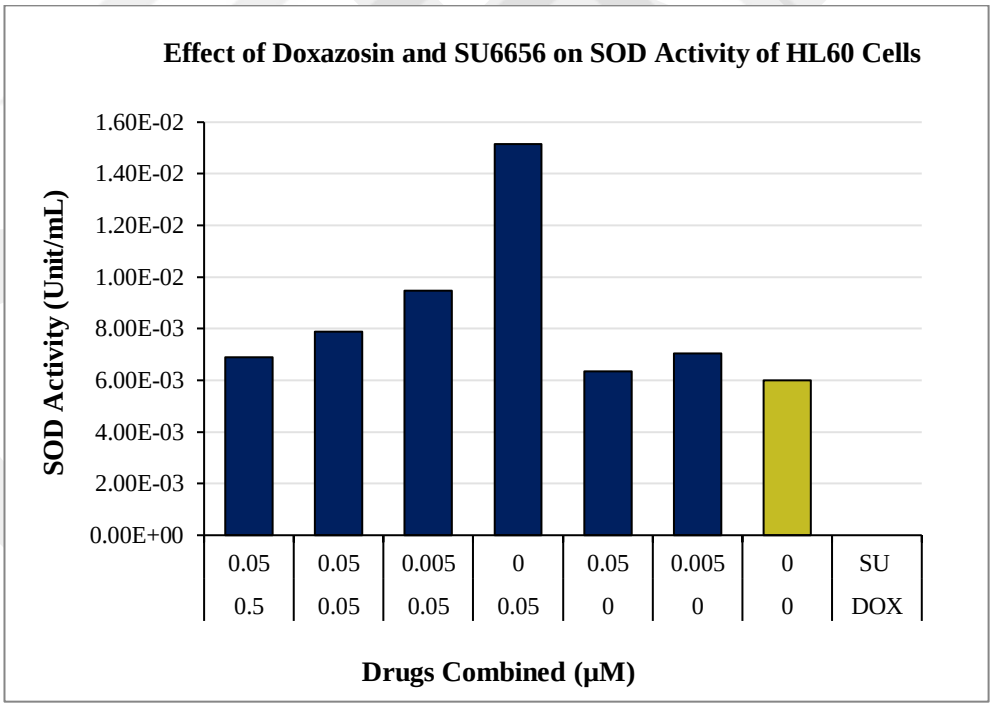

Figure 3.7. b The effect of diverse concentrations of combined drugs Doxazosin mesylate with SU6656 on SOD enzyme of HL60 cells.

3.3.5. The Effect of Drugs on PTK Activity of HL60 Cells

The activity of the PTK enzyme in the sample determined by using PTK standard, which supplied in assay kit by comparing its absorbance with the absorbance of the samples. The effect of different drugs concentrations on PTK enzyme in HL60 samples was tested and given in the figures below. Doxazosin mesylate with different concentrations resulted in the increase of PTK activity. The highest increase in enzyme activity was observed using a 0.312µM dose, the activity of enzyme was 0.0579 Unit/mL (Figure 3.8.a**)**.

The administration of genistein using both doses of 7.5µM and 3.7µM have resulted in the increase of PTK activity to (0.0487 Unit/mL) compared to control. While when it was administered at both doses of 1.875µM and 0.625µM the activity of the enzyme decreased. The slight increase of PTK activity observed when genistein administered at the lowest dose 0.312µM (Figure 3.8.b**).**

The activity of PTK enzyme was decreased to (0.0244 Unit/mL) when SU6656 was administered at lowest dose 0.312µM. The administration of SU6656 at high doses of 3.75µM and 1.875µM resulted in the decrease of PTK activity to (0.030Unit/mL). The administration of SU6656 at 0.625µM dose had no effect on the PTK activity **(**Figure 3.8.c**)**. All the results were analyzed with respect to control value (0.0334 Unit/mL).

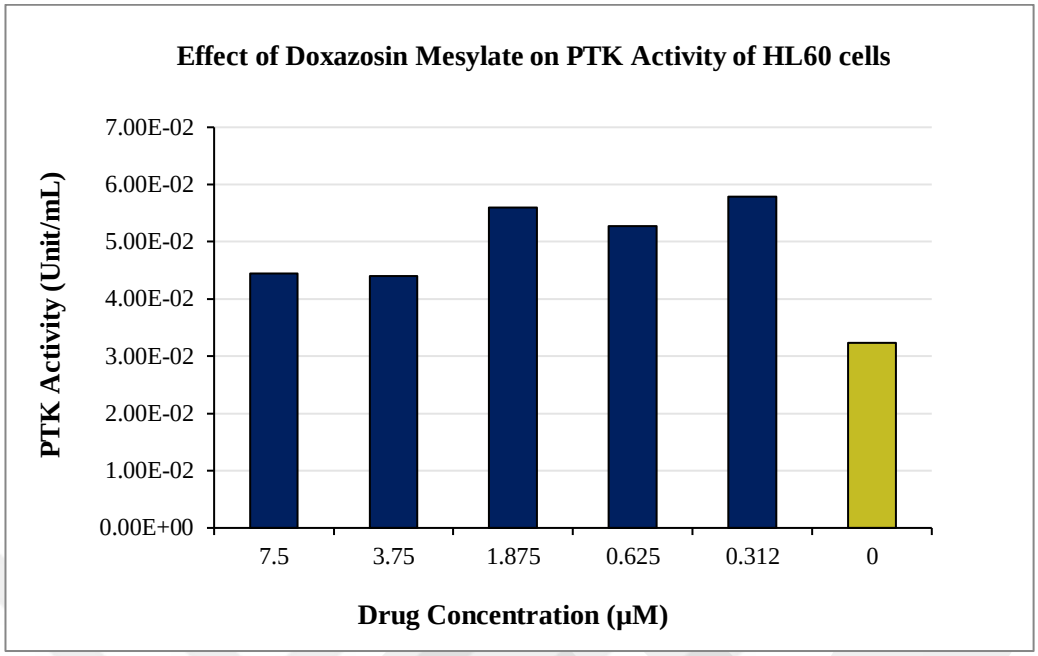

Figure 3.8.a The effect of different concentrations of Doxazosin mesylate on PTK enzyme of HL60 cells.

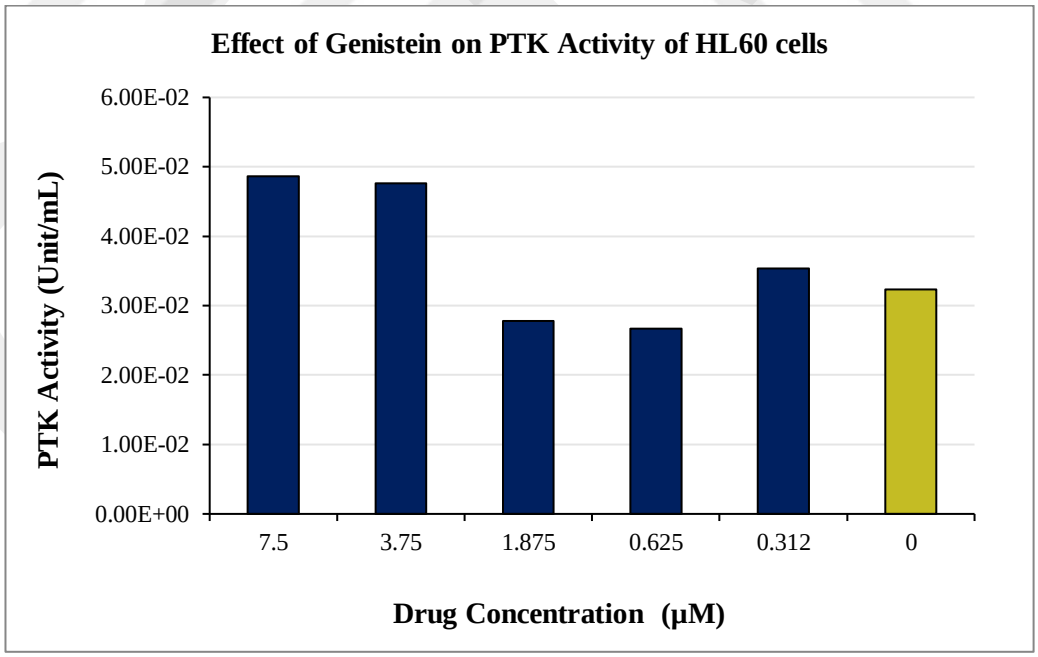

Figure 3.8.b The effect of different concentrations of Genistein on PTK enzyme of HL60 cells.

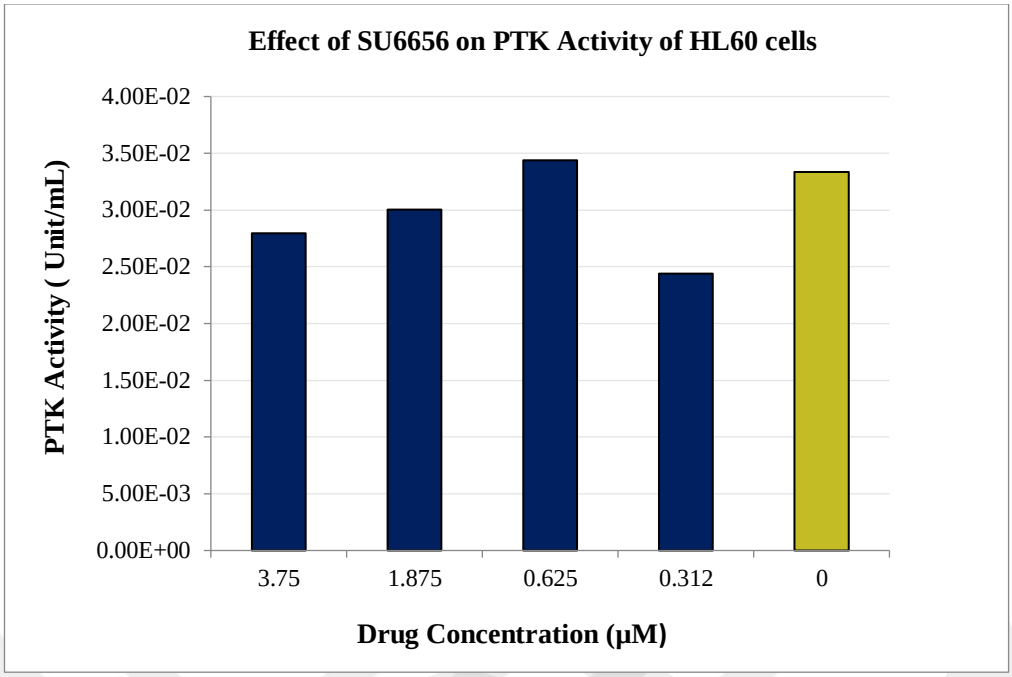

Figure 3.8.c The effect of different concentrations of SU6656 on PTK enzyme of HL60 cells.

3.3.6. The Effect of Drugs in Combination on PTK Activity of HL60 Cells

Genistein with doxazosin mesylate at different concentrations resulted in the increase of PTK activity. Where the highest increase of enzyme activity was 0.104 Unit/mL, which was observed using the doses of (0.05µM GEN + 0.05µM DOX) and the lowest increase observed using the dose (0.05µM GEN + 0.5 µM DOX) compared to control (Figure 3.9.a). The administration of genistein at low dose 0.005µM resulted in higher increases in PTK activity more than when it was administered at 0.05 µM dose. All the results were analyzed wiht respect to control value (0.0323 Unit/mL).

The effect of SU6656 in combination with doxazosin mesylate varies by doses, where the highest decrease in PTK activity was at (0.05µM SU + 0.5µM DOX) dose. The lowest decrease was observed at (0.05µM SU + 0.05µM DOX) dose compared to control (0.139 Unit/mL).

While a combined form of SU6656 with doxazosin mesylate was applied by using doses 0.005µM and 0.05µM, the PTK activity was slightly increased. SU6656 at 0.05µM concentration showed decrease in PTK activity while at 0.005µM resulted in an increase in PTK activity as shown in Figure 3.9.b.

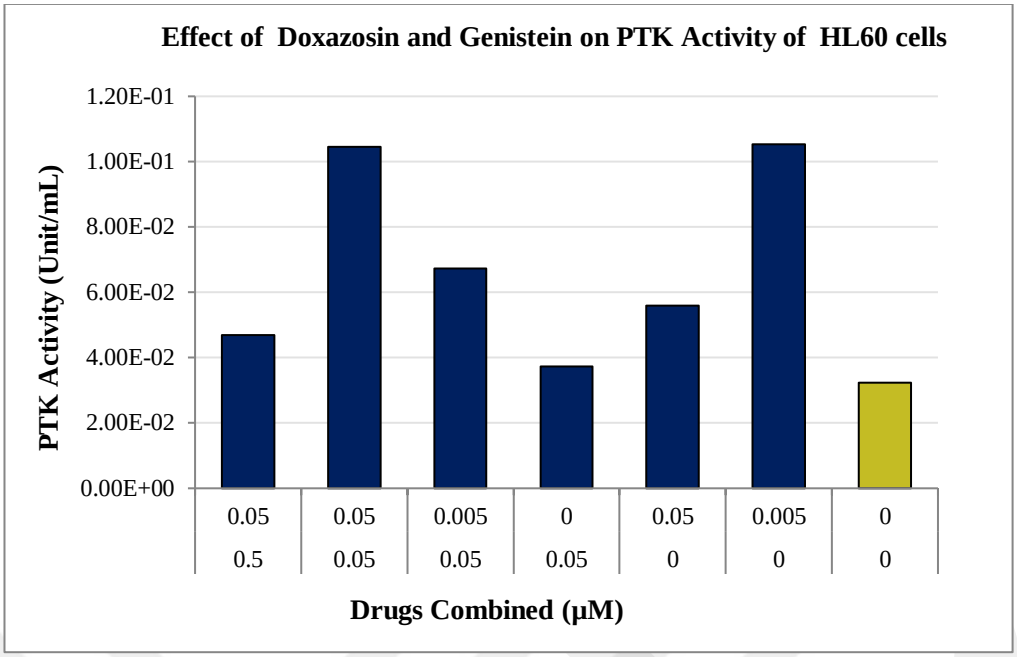

Figure 3.9.a The effect of diverse concentrations of combined drugs Doxazosin mesylate with Genistein on PTK enzyme of HL60 cells.

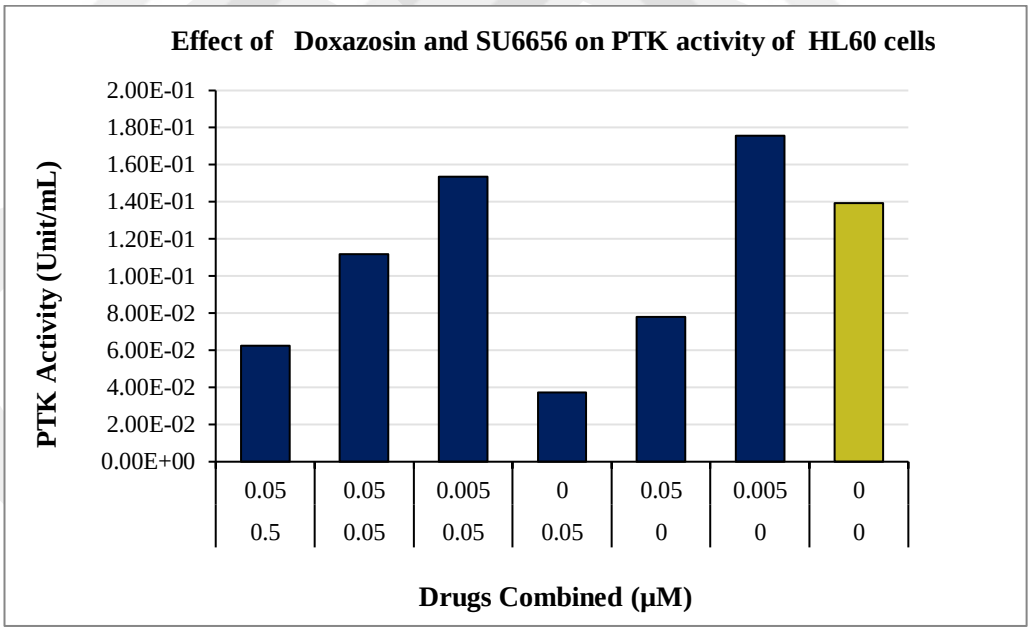

Figure 3.9.b The effect of diverse concentrations of the combined drugs Doxazosin mesylate with SU6656 on PTK enzyme of HL60 cells.

DISCUSSION

In this study, different of drugs (Doxazosin mesylate, Genistein and SU6656) were used either alone and in combination to assess their effect on enzymes (GST, SOD and PTK) of human leukemia cell lines (HL60) in addition to evaluate their effects on human leukemia cell growth. HL60 were grown in RPMI 1640 medium supported with protein sources and antibiotics to avoid contamination. Firstly, human leukemia cell lines treated with different concentrations of each drug as a single treatment to evaluate the effect of each drug on these cell lines and then subjected to different concentrations of combined drugs. In this experimental study human leukemia cell lines used as a source for glutathione-S-transferase enzyme, superoxide dismutase enzyme and protein tyrosine kinase enzyme.

One of the studies that is related to the combination of drugs were performed by Kee-lung Chang 2009. In that study the effect of genistein and terazosin either alone or in combination on prostate cancer cell line DU-145 were tested. The results showed that genistein alone was more effective to inhibit cell growth than terazosin, which had non-toxic effect on prostate cancer cell line. While genistein in combination with non-toxic dose of terazosin the percentage of cell growth inhibition was significantly increased [7].

In another study (Masumeh 2017) the effect of genistein and tamoxifen on hepatocellular carcinoma cell line was tested alone and in combination and the results showed that combined form of genistein and tamoxifen potentially prevent cell growth of hepatocellular carcinoma and induce apoptosis[8].

The effect of doxazosin mesylate either alone or in combination with the chemotherapeutic drug (Adriamycin) on prostate cancer cell lines DU145 and PC-3 were tested by C. CAL and co-workers. The results showed that doxazosin mesylate alone had a cytotoxic effect on prostate cancer cell lines and its

cytotoxic effect was significantly increased when administered in combined form with Adriamycin[44] .

The effect of genistein on the activities of antioxidant enzymes SOD, CAT and GPx in prostate cancer cell lines LNCAP and PC-3 were tested by SUZUKI and co-workers. The results showed that genistein resulted significantly increase in GPx activity at 100µM dose. On the other hand, genistein at the same dose showed no significant difference in the activity of SOD and CAT compared to control [45].

According to the previous studies Doxazosin mesylate and Genistein showed cytotoxic effects on cancer cell lines. In addition to SU6656 which is a strong inhibitor to cancer cells, in this study, the results showed that doxazosin mesylate was less toxic on human leukemia cells compared to other drugs where it caused 65% cell death at highest dose 3.75 µM and only 6% cell death at the lowest dose 0.312 µM. Genistein alone was having a cytotoxic effect on human leukemia cells where it caused 42 % of cell death at low dose 0.312 µM. While its cytotoxic effect of the same dose was significantly increased when administered in combination with 7.5µM of doxazosin mesylate as shown in Figure 3.1 under results section. Su6656 alone was found to be more toxic to HL60 cells than doxazosin at their different doses. While in combination with doxazosin, SU6656 resulted in a slight increase in cell toxicity, as compared with its toxicity when administered alone (Figure 3.2).

The activity of GST enzyme in human leukemia cells was tested with different drugs used in this study either alone and in combination. The results showed that doxazosin mesylate alone resulted in a decrease in the GST activity of HL60 cells at different doses. Where there was no significant difference in their effect on GST enzyme, relative to each other and compared to control. This result might be due to the low toxicity of doxazosin mesylate on HL60 cells as demonstrated by cell viability results. Genistein at different concentration either alone or in combination inhibit the activity of GST enzyme. According to previous studies it has concluded that due to the protective function of the GST enzyme it may reduce the effectiveness of anticancer drug in cancer cells [31].

So, to improve the efficacy of anticancer drug the inhibition of GST activity enzyme in cancer cell is needed. Again, in this study, the activity of GST enzyme was inhibited by SU6656 either alone or in combination. Where it was no significant difference in their effect on GST enzyme relative to each other compared to control (Figure 3.5.b), except at the lowest dose 0.005µM when it was administered alone the GST activity was may due to phase I enzymes are activated by the drug.

The effect of different drugs that were used in this study either alone and in combination on the activity of SOD enzyme in human leukemia cells was tested and the results showed that doxazosin mesylate induced the activity of SOD enzyme at different doses. According to the principle of SOD assay that was used in this study, the enzyme activity is high when the NBT diformazan formation inhibited through this result doxazosin mesylate stimulated the activation of SOD enzyme to scavenge free radicals that was found at high levels in cancer cells.

On the other hand, Genistein resulted in inhibition of SOD activity at different doses where the maximum inhibition of SOD activity was observed at 3.75µM and 0.625µM doses compared to control. While in combination with doxazosin the maximum inhibition was observed at combination dose (0.005µM GEN + 0.05µM DOX). For the other combination doses were no significant difference in their effect relative to control. According to Kong due to high levels of reactive oxygen species in cancer cell and after administering of anticancer drugs will generate free radical these overproductions of free radical suppress the capacity of SOD enzyme [46]. SU6656 alone significantly induce the activity of SOD enzyme at different doses due to the high toxicity of this drug. But in combination with doxazosin resulted in a decrease in SOD activity at different doses of combination where was this effect directly proportional with decreasing the doses of drug combination.

The activity of protein tyrosine kinase (PTK) in human leukemia cells was tested by using different drugs either alone and in combination and the results showed that doxazosin mesylate resulted in an increase in PTK activity at different doses. This result might be related to the increase in protein amount at different concentrations of doxazosin mesylate administered to human leukemia cells. Or doxazosin mesylate may has a catalytic effect on phosphorylation mechanism. Besides, Genistein alone at higher doses 7.5µM and 3.75µM resulted in stimulation of PTK activity, but at 1.875µM and 0.625µM doses the activity of PTK inhibited and then slightly stimulated at the lowest dose 0.312µM. In combination with doxazosin, genistein showed an increase in PTK activity where it was maximum (0.05µM GEN + 0.05µM DOX) compared to other doses and control (Figure 3.9.a). This difference might be related to the concentrations of drugs or to the amount of protein in each sample. According to Fabrizio Spinozzi and co-workers, genistein was tested for its effect on PTK enzyme in T leukemia cells and the results showed that genistein found to be a strong natural inhibitor of protein tyrosine kinase [47]. SU6656 is known to be a strong inhibitor to tyrosine kinase and the results in this study demonstrated that SU6656 alone or in combination with doxazosin resulted in inhibition of PTK activity. The difference between doses to inhibit the enzyme might be related to the difference in the amount of proteins in each sample.

CONCLUSION

Doxazosin mesylate showed less toxic effect on human leukemia cell lines compared to genistein and SU6656. Genistein had cytotoxic effect on HL60 cells and this effect significantly increased when administered in combination with doxazosin mesylate, in addition to genistein whether alone or in combination with doxazosin caused inhibition to the activity of GST enzyme and this effect is needed to enhance the efficacy of chemotherapeutic drugs that may alter due to the high activity of GST enzyme and resulting in drug resistance. According to GST enzyme and cell viability results, doxazosin mesylate has a synergistic effect and more effective when used in combination with genistein to overcome drug resistance. On the other hand, genistein was a good inhibitor to SOD enzyme whether alone or in combination with doxazosin mesylate and it can be used to treat some cancers that is related to high levels of SOD enzymes. The defense response of human leukemia cells toward the drugs are different, according to the type and concentration of the drugs that are used.

REFERENCES

[1] Delbridge, A. R., Grabow, S., Strasser, A., & Vaux, D. L. (2016). Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. Nature reviews Cancer, 16(2), 99.

[2] Ishiguro, T., Ohata, H., Sato, A., Yamawaki, K., Enomoto, T., & Okamoto, K. (2017). Tumor-derived spheroids: relevance to cancer stem cells and clinical applications. Cancer science, 108(3), 283-289.

[3] Ferreira, D., Adega, F., & Chaves, R. (2013). The importance of cancer cell lines as in vitro models in cancer methylome analysis and anticancer drugs testing. In Oncogenomics and cancer proteomics-novel approaches in biomarkers discovery and therapeutic targets in cancer. IntechOpen.

[4] Chen, H., & Li, J. (2018, November). DrugCom: Synergistic Discovery of Drug Combinations Using Tensor Decomposition. In 2018 IEEE International Conference on Data Mining (ICDM)(pp. 899-904). IEEE.

[5] Lehár, J., Krueger, A. S., Avery, W., Heilbut, A. M., Johansen, L. M., Price, E. R., ... & Lee, M. S. (2009). Synergistic drug combinations tend to improve therapeutically relevant selectivity. Nature biotechnology, 27(7), 659.

[6] Batra, H., Pawar, S., & Bahl, D. (2018). Curcumin in combination with anti-cancer drugs: A nanomedicine review. Pharmacological research.

[7] Chang, K. L., Cheng, H. L., Huang, L. W., Hsieh, B. S., Hu, Y. C., Chih, T. T., ... & Su, S. J. (2009). Combined effects of terazosin and genistein on a metastatic, hormone-independent human prostate cancer cell line. Cancer letters, 276(1), 14-20.

[8] Sanaei, M., Kavoosi, F., Atashpour, S., & Haghighat, S. (2017). Effects of genistein and synergistic action in combination with tamoxifen on the HepG2 human hepatocellular carcinoma cell line. Asian Pacific journal of cancer prevention: APJCP, 18(9), 2381.

[9] Kang, D., Hu, C., Fu, Y., & Wang, D. (2017). Combination of α-blocker and 5α-reductase inhibitor for treatment of benign prostatic hyperplasia. Clinical & Investigative Medicine, 40(5), 200-210.

[10] Tian, J., Min, Y., Rodgers, Z., Wan, X., Qiu, H., Mi, Y., ... & Roche, K. (2017). Nanoparticle delivery of chemotherapy combination regimen improves the therapeutic efficacy in mouse models of lung cancer. Nanomedicine: Nanotechnology, Biology and Medicine, 13(3), 1301-1307.

[11] Kaur, G., & Dufour, J. M. (2012). Cell lines: Valuable tools or useless artifacts.

[12] Manda-Handzlik, A., Bystrzycka, W., Wachowska, M., Sieczkowska, S., Stelmaszczyk-Emmel, A., Demkow, U., & Ciepiela, O. (2018). The influence of agents differentiating HL-60 cells toward granulocyte-like cells on their ability to release neutrophil extracellular traps. Immunology and Cell Biology, 96(4), 413-425.

[13] Benghuzzi, H., & Tucci, M. (2017). The HL-60 Cells Response to Various Particle Sizes of Tetracalcium Phosphate Ceramic Delivery System. Key Engineering Materials, 720.

[14] Aslantürk, Ö. S. (2018). In Vitro Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages (Vol. 2, p. 64). InTech.

[15] Li, X., Jin, Y., Mu, Z., Chen, W., & Jiang, S. (2017). MicroRNA-146a-5p enhances cisplatin-induced apoptosis in ovarian cancer cells by targeting multiple anti-apoptotic genes. International journal of oncology, 51(1), 327-335.

[16] Krai, J., Beckenkamp, A., Gaelzer, M. M., Pohlmann, A. R., Guterres, S. S., Filippi-Chiela, E. C., ... & Beck, R. C. R. (2017). Doxazosin nanoencapsulation improves its in vitro antiproliferative and anticlonogenic effects on breast cancer cells. Biomedicine & Pharmacotherapy, 94, 10-20.

[17] Jamal, A., Shahzadi, L., Ahtzaz, S., Zahid, S., Chaudhry, A. A., ur Rehman, I., & Yar, M. (2018). Identification of anti-cancer potential of doxazocin: Loading into chitosan based biodegradable hydrogels for on-site delivery to treat cervical cancer. Materials Science and Engineering: C, 82, 102-109.

[18] Hazzah, H. A., EL-Massik, M. A., Abdallah, O. Y., & Abdelkader, H. (2013). Preparation and characterization of controlled-release doxazosin mesylate pellets using a simple drug layering-aquacoating technique. Journal of Pharmaceutical Investigation, 43(4), 333-342.

[19] Kim, I. G., Kim, J. S., Lee, J. H., & Cho, E. W. (2014). Genistein decreases cellular redox potential, partially suppresses cell growth in HL-60 leukemia cells and sensitizes cells to γ-radiation-induced cell death. Molecular medicine reports, 10(6), 2786-2792.

[20] Prakash, C. R., Theivendren, P., & Raja, S. (2012). Indolin-2-ones in clinical trials as potential kinase inhibitors: a review. Pharmacology & Pharmacy, 3(01), 62.

[21] El-Rashedy, A. A., & El-Din, A. A. M. (2018). Drug design of Src Kinase inhibitor: An overview.

[22] Hecht, F., Pessoa, C. F., Gentile, L. B., Rosenthal, D., Carvalho, D. P., & Fortunato, R. S. (2016). The role of oxidative stress on breast cancer development and therapy. Tumor biology, 37(4), 4281-4291.

[23] Kunwar, A., & Priyadarsini, K. I. (2011). Free radicals, oxidative stress and importance of antioxidants in human health. J Med Allied Sci, 1(2), 53-60.

[24] Gupta, R. K., Patel, A. K., Shah, N., Chaudhary, A. K., Jha, U. K., Yadav, U. C., ... & Pakuwal, U. (2014). Oxidative stress and antioxidants in disease and cancer. Asian Pac Cancer Prev, 15, 4405-4409.

[25] Kanwal, R., Pandey, M., Bhaskaran, N., MacLennan, G. T., Fu, P., Ponsky, L. E., & Gupta, S. (2014). Protection against oxidative DNA damage and stress in human prostate by glutathione S-transferase P1. Molecular carcinogenesis, 53(1), 8-18.

[26] Amato, R. J., Stepankiw, M., & Ochuwa, N. S. (2014). Molecular-targeted Therapy for Renal Cell Carcinoma. In Renal Disease in Cancer Patients (pp. 115-127). Academic Press.

[27] Tew, K. D., & Townsend, D. M. (2012). Glutathione-s-transferases as determinants of cell survival and death. Antioxidants & redox signaling, 17(12), 1728-1737.

[28] Ruzza, P., & Calderan, A. (2013). Glutathione transferase (GST)-activated prodrugs. Pharmaceutics, 5(2), 220-231.

[29] Zhang, J., Grek, C., Ye, Z. W., Manevich, Y., Tew, K. D., & Townsend, D. M. (2014). Pleiotropic functions of glutathione S-transferase P. In Advances in cancer research (Vol. 122, pp. 143-175). Academic Press.

[30] Franco, R., Schoneveld, O. J., Pappa, A., & Panayiotidis, M. I. (2007). The central role of glutathione in the pathophysiology of human diseases. Archives of physiology and biochemistry, 113(4-5), 234-258.

[31] Morrow, C. S., Smitherman, P. K., Diah, S. K., Schneider, E., & Townsend, A. J. (1998). Coordinated Action of Glutathione S-Transferases (GSTs) and Multidrug Resistance Protein 1 (MRP1) in Antineoplastic Drug Detoxification MECHANISM OF GST A1-1-AND MRP1-ASSOCIATED RESISTANCE TO CHLORAMBUCIL IN MCF7 BREAST CARCINOMA CELLS. Journal of Biological Chemistry, 273(32), 20114-20120.

[32] Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine, 54(4), 287-293.

[33]Younus, H. (2018). Therapeutic potentials of superoxide dismutase. International journal of health sciences, 12(3), 88.therapy. Tumor biology, 37(4), 4281-4291.

[34] McIsaac, A. R., & Mazziotti, D. A. (2017). Ligand non-innocence and strong correlation in manganese superoxide dismutase mimics. Physical Chemistry Chemical Physics, 19(6), 4656-4660.

[35] Borrelli, A., Schiattarella, A., Bonelli, P., Tuccillo, F. M., Buonaguro, F. M., & Mancini, A. (2014). The functional role of MnSOD as a biomarker of human diseases and therapeutic potential of a new isoform of a human recombinant MnSOD. BioMed research international, 2014.

[36] Che, M., Wang, R., Li, X., Wang, H. Y., & Zheng, X. S. (2016). Expanding roles of superoxide dismutases in cell regulation and cancer. Drug discovery today, 21(1), 143-149.

[37] Roskoski Jr, R. (2015). Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors. Pharmacological research, 94, 9-25.

[38] Hojjat-Farsangi, M. (2016). Targeting non-receptor tyrosine kinases using small molecule inhibitors: an overview of recent advances. Journal of drug targeting, 24(3), 192-211.

[39] Shah, N. H., Amacher, J. F., Nocka, L. M., & Kuriyan, J. (2018). The Src module: an ancient scaffold in the evolution of cytoplasmic tyrosine kinases. Critical reviews in biochemistry and molecular biology, 53(5), 535-563.

[40] Juan, W. C., & Ong, S. T. (2012). The role of protein phosphorylation in therapy resistance and disease progression in chronic myelogenous leukemia. In Progress in molecular biology and translational science (Vol. 106, pp. 107-142). Academic Press.

[41] Strober, W. (2015). Trypan blue exclusion test of cell viability. Current protocols in immunology, 111(1), A3-B.

[42] He, F. (2011). BCA (bicinchoninic acid) protein assay. Bio protocol, 101.

[43] Isgor, Y. G., & Isgor, B. S. (2011). Kinases and glutathione transferases: selective and sensitive targeting. Frontiers in Biology, 6(2), 156-169.

[44] Cal, C., Uslu, R., Gunaydin, G., Ozyurt, C., & Omay, S. B. (2000). Doxazosin: a new cytotoxic agent for prostate cancer? BJU international, 85(6), 672-675.

[45] Suzuki, K., Koike, H., Matsui, H., Ono, Y., Hasumi, M., Nakazato, H., ... & Yamamoto, T. (2002). Genistein, a soy isoflavone, induces glutathione peroxidase in the human prostate cancer cell lines LNCaP and PC-3. International Journal of Cancer, 99(6), 846-852.

[46] Kong, Q., Beel, J. A., & Lillehei, K. O. (2000). A threshold concept for cancer therapy. Medical hypotheses, 55(1), 29-35.

[47] Spinozzi, F., Pagliacci, M. C., Migliorati, G., Moraca, R., Grignani, F., Riccardi, C., & Nicoletti, I. (1994). The natural tyrosine kinase inhibitor genistein produces cell cycle arrest and apoptosis in Jurkat T-leukemia cells. Leukemia research, 18(6), 431-439.