Investigating Anti-leukemic Role of Copper Dithiocarbamate Using Wnt and HIF 1 Alpha Pathway Markers in AML Rat Model

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A thesis submitted in the partial fulfillment of requirements for the Degree of

Master of Philosophy in Biochemistry/Molecular Biology

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بسم الله الرحمن الرحيم

"Read! In the Name of your Lord Who has created (all that exists). He has created man from a clot (a piece of thick coagulated blood). Read! And your Lord is the Most Generous. Who has taught the writing by the Pen? He has taught the man that which he knew not"

[Quran, 96: 1-5]

DECLARATION

This is certified that the thesis entitled **"Investigating Anti-leukemic Role of Copper Dithiocarbamate Using Wnt and HIF 1 Alpha Pathway Markers in AML Rat Model"** is my own effort and thesis is my own composition produced under supervision towards the award of **Master of Philosophy in Biochemistry/Molecular Biology** and that, to the best of my knowledge, no part of this thesis has been previously presented elsewhere by anyone for any other degree. All references herein have been duly acknowledged.

Zia Un Nisa

DEDICATION

Firstly, I dedicate this thesis to Allah Almighty with endless gratitude, for the guidance, strength, and power of mind, protection, skills and the healthy life has given me.

And lastly, this study is wholeheartedly dedicated to my beloved parents, who have been my source of inspiration and gave me strength when I thought of giving up, who continually provided their moral, spiritual, emotional and financial support.

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Acknowledgments

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I further extend my utmost gratitude to my beloved Family, especially my **Parents** whose sacrifice and unconditional support have helped make my career possible, who inspired and supported me through this most valued and challenging educational journey of my life. I am thankfull to Allah Almighty for blessing me with such an adorable and supporting family.

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List of Abbreviations

Abs	Absorbance
ACS	American Cancer Society
ALP	Alkaline Phosphatase
ALT	Alanine Transferase
AML	Acute myeloid leukemia
DDTC	Diethyldithiocarbamate
DMSO	Dimethyl Sulfoxide
DOXO	Doxorubicin
CuDTC	Copper Dithiocarbamate
EDTA	Ethylene Diamine Tetra Acetic acid
EDS	Energy Dispersive Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
mg/dl	Miligrams per deciliter
RBCs	Red Blood Cells
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
WBCs	White Blood Cells
XRD	X-ray Diffraction Spectroscopy
EDS	Energy Dispersive Spectroscopy

Abstract

Acute leukemia's are typically life-threatening affliction in which cancerous alteration occurs in the early progenitors or haemopoietic stem cell with a high mortality rate. There is both inability of haemopoietic stem cell to differentiate and excessive proliferation in stem cell compartment causing buildup of non-functional cells known as myeloblasts. The clinical and biological discrepancy towards therapy resistance in this haematological disorder makes treatment complex that highlight the need to explore and develop novel therapeutic compounds that must show better efficacy and low toxicity then currently available conventional chemotherapeutic drugs. In present study, anti-leukemic potential of a novel dithiocarbamate derivative named copper dithiocarbamate (CuDTC) was investigated as an individual drug and in combination with known chemotherapy (Doxorubicin) in benzene-induced leukemic rat model. The compound was first characterized by XRD and Scanning electron microscopy (SEM). To investigate antileukemic potential of the test compound, it was administered to benzene induced leukemic rat model. For this purpose, different parameters were analyzed including haematological, morphological, biochemical (LFTs, RFTs, lipid profile and LDH) and relative mRNA expression of the well-known proliferative biomarkers of Wnt and HIF-1α pathway: *AXIN*, *GSK3β*, *LEF*, *HIF-1α*, *HSP90* and *PTEN* in samples obtained from different experimental groups. It was revealed that, in isolation CuDTC showed remarkable recovery of cellular indicators comparative to leukemic conditions. Blood cell counts, hepatic and renal biomarkers of compound treated leukemic rats were significantly improved. The relative expression of *AXIN*, *GSK3β*, *LEF*, *HIF-1α*, *HSP90* and *PTEN* was also recovered significantly to normal in the CuDTC treated group comparative to benzene induced leukemic rats. However, it was observed that expression of aberrant proliferative genetic markers in combination therapy did not show significant improvements. Therefore, we conclude that CuDTC as individual therapeutic drug may serve as an exceptional therapeutic agent to treat benzene induced leukemia at haematological, biochemical, morphological as well as relative expression level against AML, whereas, further research is needed to assess the efficacy of the co-administration of CuDTC and traditional therapy against leukemia.

1. Introduction

1.1 Cancer

Cancer is a primary health issue worldwide with the most common cause of mortality across the globe. It is estimated that due to demographic shifts the global cancer burden is predicted to be 47% increase from 2020, which is likely to be 28.4 million cases by 2040. However, with rising risk factors coupled with globalization and developing economy this could be worsened further (Siegel *et al*., 2022). Overall, 74% fatalities worldwide are caused by noncommunicable diseases (NCDs) having cancer at the top most (World Health Organization, 2022). Cancer is a group of disorders marked by unrestricted cellular growth followed by a multistep mechanism that typically evolves from a precancerous lesion to a malignant tumor causing normal cells transformation into tumor cells (Hirschey *et al*., 2015). Normally, balance between growth of cells and death is maintained by cell cycle whereas, cancer cells take control of their fate by dysregulating the cell cycle. When this efficient system fails abnormal and aberrant cells proliferate and expand even when they are not needed (Kahriman., 2021). These disrupted biological pathways result in increased levels of growth factor concentrations and expression of survival protein receptors on cancer cell surfaces. Following these, mutations in surrounding cells seen which results in more growth factors release causing cancer cells to multiply and disseminate other parts of the body called metastasis (Erdogan & Webb, 2017).

1.2 Factors that Cause Cancer

There are many factors that underlie the multistep developmental pathway of cancer growth. Most commonly, clonal expansions and mutations in a population of cells lead to this evolutionary process (Merlo *et al*., 2006). Mutations in cancer related genes alters the cell behavior, leading to increased proliferation and lack of cooperation in cells population. The accumulation of these genetics variations are therefore what, that characterizes tumor formation (Michor *et al*., 2004). Mutations that primarily concerned with cancer causation typically occurs in three types of genes, proto-oncogenes, DNA repair genes and tumor suppressor genes respectively. DNA repair genes as name indicates these are responsible to correct errors that commonly occurs during DNA replication, but when mutations acquired in these genes the mistakes during replication of DNA remains uncorrected. With each

clonal division these mistakes become mutations that eventually leads to cancer development (Nowell, 1976). Similarly, mutations in tumor suppressor genes cause uncontrollable proliferation of cells. Proto-oncogenes that are responsible for division and growth of cells in appropriate manner when mutated transformed into oncogenes, which are cancer causing genes upon mutation, this whole process incite neoplastic activity (Vogelstein & Kinzler, 2004) as shown in figure 1.1.

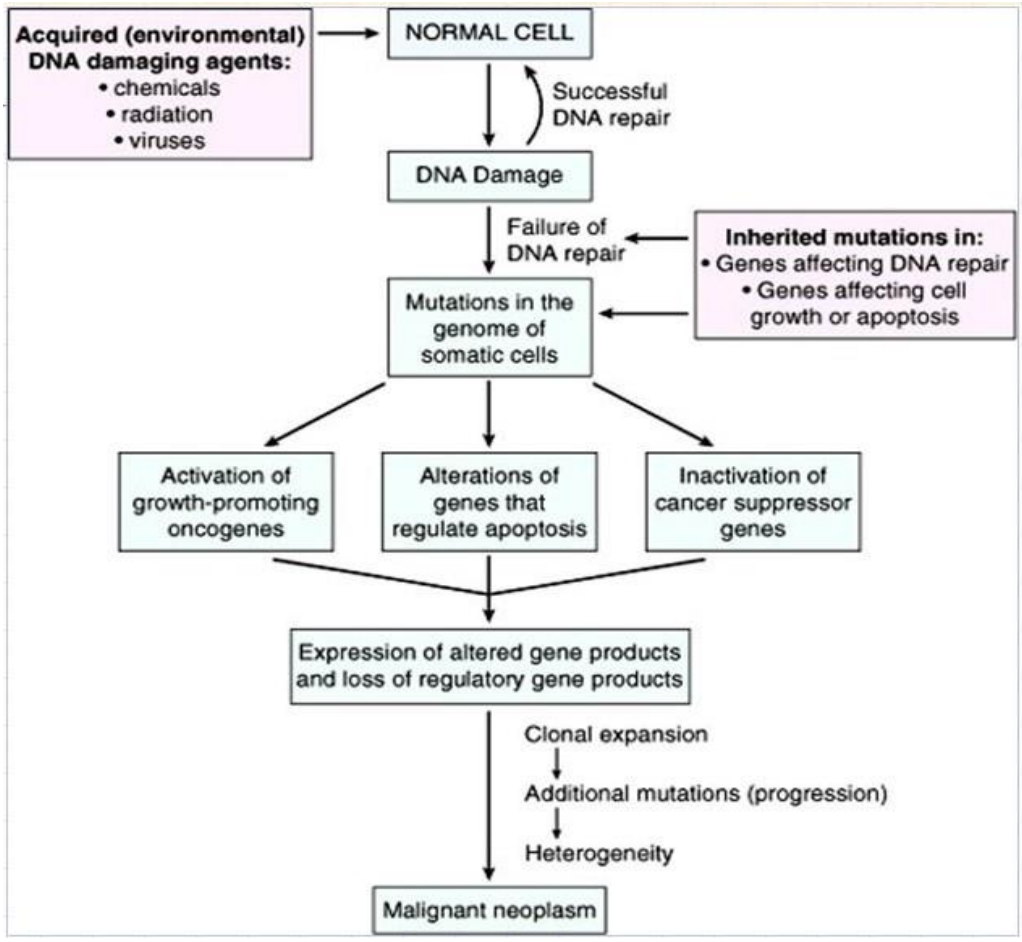

Figure 1.1: General etiology and pathogenesis of cancer (Cancer Research UK)

Apart from intrinsic factors there are some external or extrinsic factors that lead to cancer causation and development. It includes infectious agents like viruses, Tobacco smoke, radiations (UVB cause 90% of skin cancers) and chemicals (i.e. benzene, polychlorinated biphenyls etc).

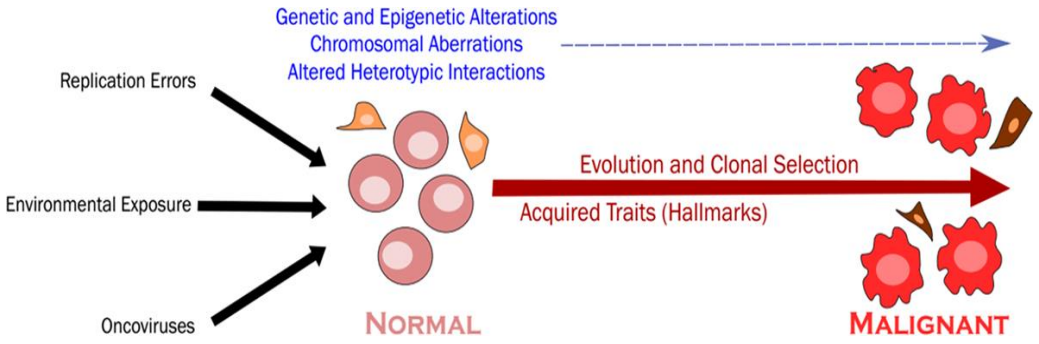

Figure 1.2: Causes of Cancer (Fouad & Aanei, 2017)

1.3 Stages of Cancer Progression

Genetically altered cells undergo number of aberrant alterations before becoming malignant neoplasm. At first after attaining mutation, cells undergo clonal expansion and rapid increase in number of cells observed the stage called hyperplasia. At this stage morphology of cells remains normal. Further divisions lead them to another stage called dysplasia, as name indicates dys means 'bad' and plasia means 'growth'. Dysplastic cells are moderately differentiated and appear abnormal under microscope. After this, cells attain another stage called anaplasia in which complete lack of differentiation occur, cells show marked pleomorphism, nucleus to cytoplasmic ratio greatly increases from normal 1:4 or 1:6 to 1:1 showing extremely hyperchromatic nuclei. Anaplastic cells also called neoplastic cells leads to development of neoplasm. Neoplastic cells staying at their primary site called cancer In situ. When these cells invade neighboring tissues and enter other body organ or disseminate termed as metastasis that poses a bad prognosis leading to increased risk of mortality in cancer patients (Clark, 1991).

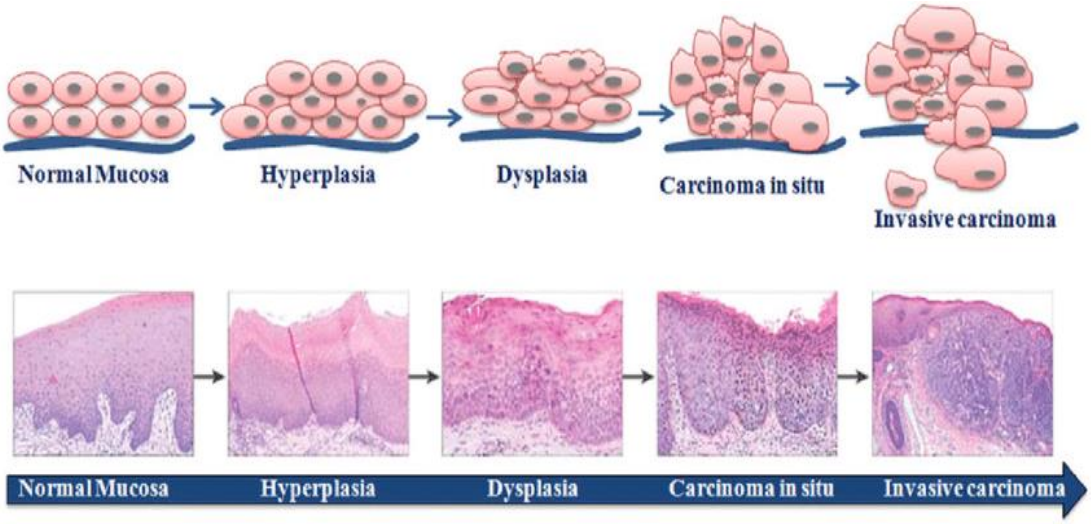

Figure 1.3: Stages involved in cancer progression (American Cancer Society)

1.4 Hallmarks of Cancer

Normally, the cells in human body divides for a limited number of divisions followed by a process of senescence in which cells no longer capable of dividing but are still metabolically active and alive. There are number of cell cycle check points that control growth and division of normal cells. When these checkpoints are damaged either because of extrinsic or intrinsic factors or the cell's DNA become damaged these molecular brakes stop cells from further dividing until it repaired. Normal cell must overpass all these checkpoints to become a cancer cell (Williams & Stoeber, 2012). During cancer progression, cancer cells must attain six basic biological characteristics called hallmarks of cancer which are considered the baseline for explaining the complication of metastatic tumor. These factors include growth signals self-sufficiency, insensitivity towards anti-growth signals, evasion from cell death, limitless replicative potential, angiogenesis and ability to metastasize and invade (Weinberg & Hanahan, 2000). During last two decades two "evolving" cancer hallmarks including evasion of the immune system and reprogramming of energy metabolism has been added, along with two assisting characteristics, which include tumor promoting inflammation and genomic instability. Gene mutations that regulate these cellular traits are seen in almost every cancer and from the basis to understand and develop targeted treatment against molecular origins of cancer (Hanahan & Weinberg, 2011).

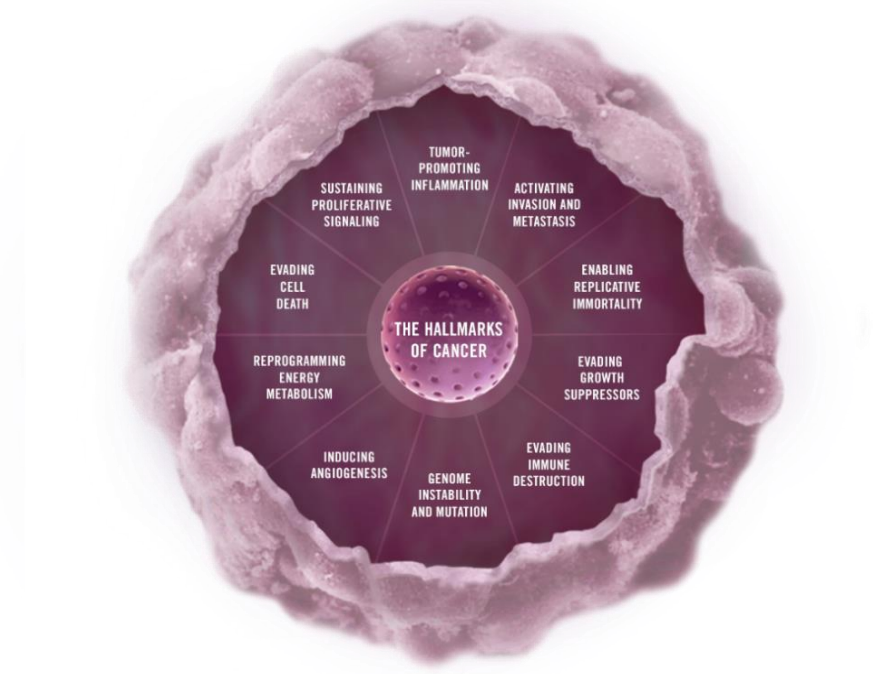

Figure 1.4: Hallmarks of Cancer (Research Gate Scientific Images)

1.5 General Classification of Cancer

As for the classification concerned, there are different types of cancer. It may develop anywhere in the body and named as, for the part of body from where it gets started. For example lung cancer remain lung cancer until it metastasizes. But in general there are two major categories in which it classify.

1.5.1 Haematologic or blood cancers

As for the name indicates haeme means ‘blood’, these are blood cancers including leukemias, lymphomas, myeloproliferative disorders and multiple myeloma (Hochman *et al*., 2018).

1.5.2 Solid tumor cancers

Solid tumors are tumor of any other tissue or body organs usually non cystic. They are named referring to the anatomical location, tissue or organ in which these tumors arise or originate. Most common are breast, colon, lung, prostate etc (Gargalionis *et al*., 2022).

1.6 Leukemia (Blood Cancer)

Leukemias are defined as group of pathologic cellular proliferative neoplasms characterized by uncontrolled growth of pluripotent stem cells in bone marrow. Production of all types of blood cells carried out from a single progenitor cell called hematopoietic stem cell from bone marrow through a series of complex molecular and biological mechanisms that cause their proliferation and differentiation to different and mature cell lineages from this single progenitor. There are two major cell lineages myeloid and lymphoid (figure 1.5) each includes successive differentiation steps until mature cells form. Oncogenic mutations in these progenitor cells underlie the cause of specific blood cell cancer. Thus, interruptions in normal self-controlled process of haematopoiesis results in abnormal maturation, malevolent proliferation and buildup of various cells in the hierarchy of hematopoietic cells.

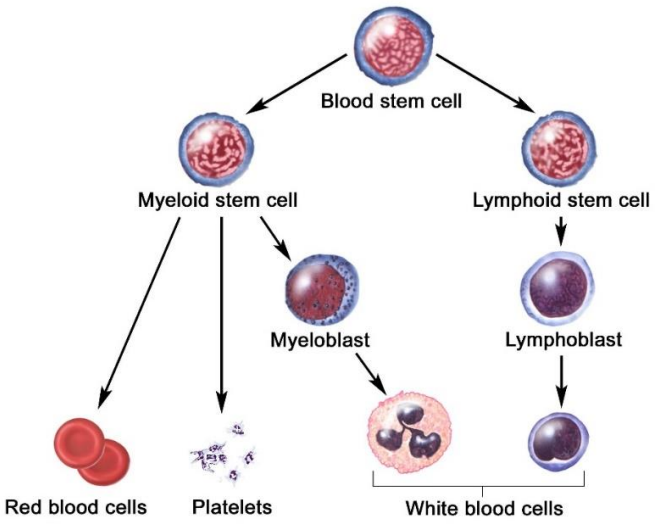

Figure 1.5: Demographic representation of myeloid and lymphoid cell lineages (National Cancer Institute NCI)

1.7 Types of Leukemia

Leukemias can be divided into chronic and acute leukemias, which depends on the clinical presentation of the disease along maturation state of the malignant cells in the peripheral blood and bone marrow. Usually acute leukemias are of rapid onset and comprise of acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) whereas, chronic leukemias are slow in onset accidently diagnosed and comprise of chronic lymphoid leukemia (CLL)

and chronic myeloid leukemia (CML). Among all newly diagnosed cases of leukemia AML accounts for high mortality rate which shows its aggressive behavior comparable to other leukemias.

Table 1.1: Types of Leukemia and their potential cause, incidence and symptoms

	Type of Leukemia	**Genetic Abnormality**	**Oncogene Involved**	**Incidence & Symptoms**	**References**
1	**Acute Myeloid Leukemia (AML)**	t (8;21) t (15;17) Mutation	*ETO/AML1* *PML, RαRα* *FLT3-ITD*	• In adults it is most common type of acute leukemia. • Upon cytogenetic analysis 60% of cases show abnormal karyotype. • Bone marrow failure is the characteristic feature caused by infiltration of blast cells in marrow. • Impaired central nervous system. • Disseminated intravascular coagulation (DIC) and symptoms of marked anaemia.	(Steffen et al., 2005) (Alcalay et al., 2001)
2	**Acute lymphoblastic leukemia (ALL)**	t (12;21) t (9;22) t (4;11)	*TEL/AML1* *BCR/ABL1* *AF4/MLL*	• Most common malig-nancy of childhood. • Lymphoblasts buildup in bone marrow. • Bone marrow hyper-cellular with >20% blasts. • Bone marrow failure, organ infiltration with normochromic normo-cytic anaemia including thrombocytopenia occur.	(Zuckerman & Rowe, 2014) (De Keersmaecker et al., 2005)

3	**Chronic myeloid leukemia (CML)**	t (9;22)	*BCR/ABL1*	• Clonal disorder of pluripotent stem cell. • Presence of characteristic Philadelphia chromosome (Ph). • BCR/ABL fusion protein show enhanced activity of tyrosine kinase. • Account for 15% leukemias. • Symptoms include hyper-metabolism and spleno-megaly.	Chereda & Melo, 2016) (Warmuth et al., 1999)
4	**Chronic lymphocytic leukemia (CLL)**	17p deletion 11q 22-23 deletion	*p53* *ATM*	• Disease occurs in older age most oftenly after 50 years. • Caused by chromosomal abnormalities i.e. deletions. • Humoral immunity compromised because of weak surface expression of immunoglobulin on B-cells. • Impaired apoptosis and lymph nodes enlargement occur.	(Delgado et al., 2020) (Galton, 1966)

1.8 Acute Myeloid Leukemia (AML)

Acute leukemia's are typically life-threatening affliction in which cancerous alteration occurs in the early progenitors or haemopoietic stem cells (Warren & Rossi., 2009). There is both inability of haemopoietic stem cell to properly differentiate and over proliferation in compartment of stem cell causing buildup of dysfunctional cells known as myeloblasts (Stone *et al*., 2004). Mutations that are commonly detected in case of cancer broadly divided into two groups. One refers to 'driver mutations' gave growth advantage to cancer cell and usually affect clinical outcomes of resulting disease. Other type of mutations called 'passenger mutations' which may already present in the cell from which cancer arose and

do not give any growth advantage to cancer cells. Collectively, AML genome contains 10 mutations on average within protein coding genes. So far, large number of AML 'driver mutations' identified, in which most common within *DNMT3A*, *NPM1*and *FLT3* genes (Kishtagari *et al*., 2020). These usually occurs on one allele for the gene and may be of 'gain of function' or 'loss of function' mutations. On cytogenetic analysis, karyotypic abnormalities were found in 60% of cases and others with normal karyotype have mutations which are detected by molecular methods and each have different prognostic significance.

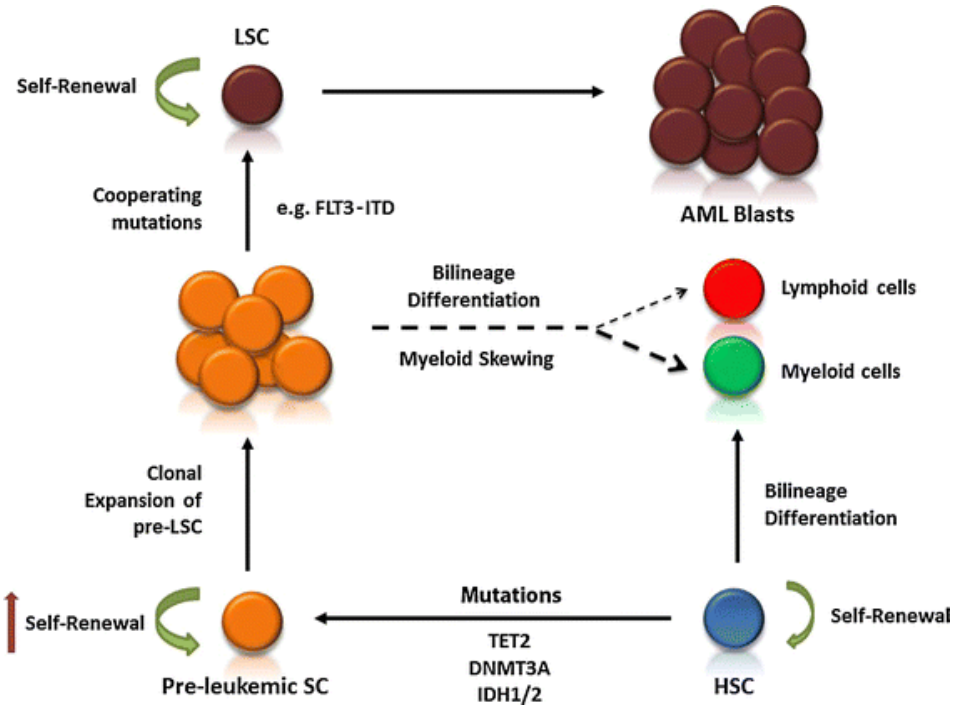

Figure 1.6: Step wise mutations that occurs in haematopoietic stem cell for the development of fully transformed leukemic stem cells (Chan & Majeti, 2013)

1.9 Benzene as a Carcinogen

Benzene refers to hydrocarbon which is aromatic in nature having (C_6H_6) molecular formula. It is an organic compound and have high potential of carcinogenesis. Two main sources from which it produces a long ago are coal since 1849 and petroleum since 1941, considered as one of the largely diffused and produced product in industrial compounds. In both humans and animals, prolong exposure to benzene has serious toxic and deleterious effects on bone marrow. For about 90 years, toxicity of benzene for hematopoietic system

in humans has been known. Benzene hematotoxicity causes decrease in production of all hematopoietic cell lines including WBCs, RBCs and platelets indicating the clinical finding of pancytopenia (Morimoto & Wolff, 1980).

1.10 Benzene as an inducer of Acute Myeloid Leukemia

Delore and Borgomano in 1928, for the very first time reported direct association of prolong exposure of benzene leading to lymphoblastic leukemia in a worker who exposed for a period of five years (Delore & Borgomano, 1928). To start its toxicity cycle, first benzene is metabolized into toxic metabolites. Liver is the major organ where its primary metabolism take place and it is converted into number of different phenolic and opened-ring compounds along with other conjugates. These metabolites then acts on stem cells, progenitor cells and also on early blast cells and inhibit their proliferation, differentiation and maturation. There is evidence that they also affect stromal cells of the microenvironment which play a crucial role for the differentiation and maturation of marrow cells (Kawasaki *et al*., 2009).

1.11 Toxicodynamics and Toxicokinetics of Benzene in AML

Major target of benzene toxicity is bone marrow that cause carcinogenesis after its metabolism into toxic metabolites. There are many different modes of entry of benzene into body depending upon source of exposure, from oral consumption to absorption through skin. But, clinically well-defined and most common mode of benzene entry is inhalation through lungs. Once it gain excess to body, benzene gets oxidized into benzene oxide by action of cytochromes mainly CYP2E1 in the liver and lungs (figure 1.7). Resulting benzene oxide along with its tautomer oxepin hydrolyzed into benzene dihydrodiol by the action of epoxide hydrolase or rearranges to phenol which either further metabolized by cytochromes into hydroquinone and 1,4-Benzoquinone or eliminated in the urine. In liver benzene dihydrodiol then further breaks down in the presence of dihydrodiol dehydrogenase into toxic metabolites mainly catechol, reactive monoaldehydes and E, E- muconic acid (Goldstein, 1977).

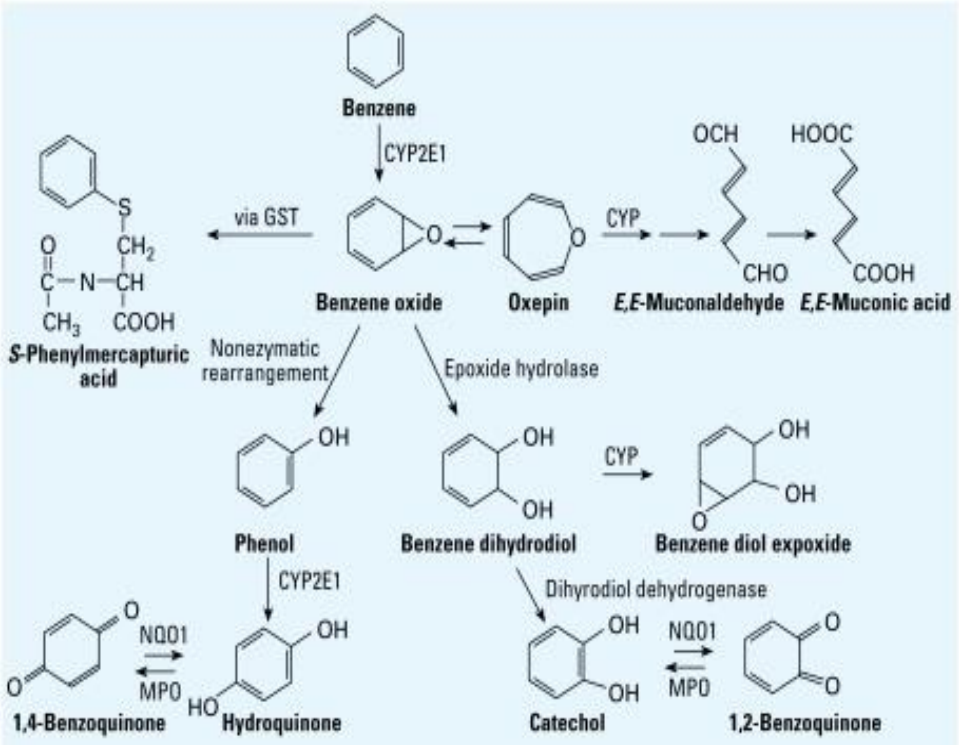

Figure 1.7: Metabolism of benzene into toxic metabolites that cause AML (Rappaport et ai., 2009)

All these are toxic to bone marrow and hematopoietic stem cells (HSCs). They trigger redox reactions by producing reactive oxygen species, causes genotoxicity at cellular level leading to formation of protein adducts that alter cellular progression, disrupt cellular activities and seed in the development of acute myeloid leukemia AML (Bette Meek & Klaunig, 2010; Bowen, 2006) as shown in figure 1.8.

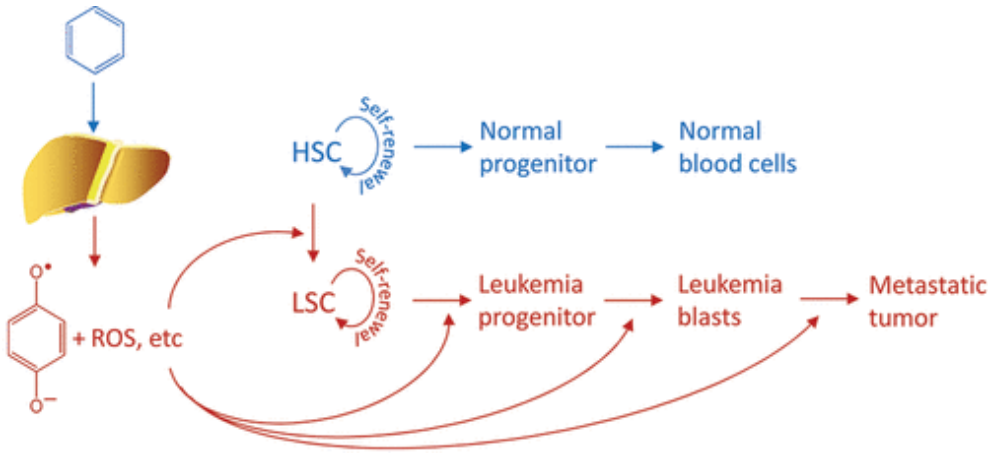

Figure 1.8: Step wise acquisition of acute myeloid leukemia by benzene metabolites (NCI)

1.12 Investigations for Diagnosis of Acute Myeloid Leukemia

Diagnosis of AML refers to >20% blast cells presence in the bone marrow. Several different methods can be utilized for diagnosis of acute myeloid leukemia but evaluation of complete blood cell counts and microscopic study of blood smears remains gold standard. Blood films typically shows variable number of blast cells with increased total white cell counts. Hypercellular bone marrow found, which contains many leukemic blast cells. Typical morphological changes include hyperchromatic nuclei, increased nucleus to cytoplasmic ratio, reduced cytoplasmic content and distortion in shape of nucleus found (Bennett *et al*., 1976). Based on differences in blast cell morphology AML can be subdivided into 9 different types.

For morphological studies, most commonly used stains for staining of blood films are Romanowsky stains among which May-Grunwald Giemsa and Leishman stains are the most frequently used. These are comprised of Eosin Y and Azure B dyes. Azure B interacts with acidic (anionic) components of the cell i.e. DNA and stain them blue while Eosin Y interacts with basic (cationic) components i.e. various proteins and stain them red showing differential staining of different cellular compartments which reveals subtle difference in their morphology (Rowley, 1973).

Blood counts and morphological analysis alone is not always sufficient to make definitive diagnosis in case of AML. Other investigations including 'myeloid immunophenotyping' $CD13^+$, $CD33^+$ and TdT^- should be performed. Cytogenetic and molecular analysis be utilized to check genetic abnormalities and mutations which are used to diagnose and classify the disease. Prognosis and therapy responses are also monitored using these techniques. In persons owing to benzene induced hepatic cytotoxicity, changes in hepatic (ALT, ALP and AST) and renal markers (Uric acid, LDH) can be used to detect and monitor AML (Delaunay *et al*., 2014). As in case of over proliferative cells like leukemia proapoptotic genes expression is suppressed while on other hand proliferative genes expression is increased which provides key evidence to differentiate between normal and leukemic cells (Köhler *et al*., 2002).

1.13 Treatment of AML

Several different treatment approaches used to treat AML which includes chemotherapy, immunotherapy, radiation therapy and surgery. The aim of treatment is to bring complete remission which is defined as presence of <5 % blasts in bone marrow with normal peripheral blood cell counts. This state is then consolidated with intensive therapy so that hopefully help in eliminating the disease. Specific therapy given to AML patients is primarily determined by the clinical status, age as well as genetic abnormality found in patient. In younger otherwise fit patients treatment is primarily by using intensive chemotherapy, usually given in three or four blocks and most common drugs used are cytosine arabinoside and daunorubicin/doxorubicin (Roboz, 2011).

But all chemotherapeutic drugs till to date are myelotoxic indicating that they have limited selectivity among leukemic and normal marrow or body cells that results in bone marrow failure from chemotherapy. Other side effects caused by chemotherapy includes severe anaemia, hair loss, nausea, vomiting and fatigue (Meisenberg, 2015). To limit these side effects naturopathy in combination with standard anticancer drugs are being used for decades to enhance effectiveness in AML treatment.

Another difficult aspect to treat cancer effectively is the evolution of treatment resistance, which can be reduced by inventing new drugs that are specifically inhibitors of tumors having specific type of mutations i.e. *FLT3*. Monoclonal immunoconjugates targeted against specific myeloid CD (cluster of differentiation) markers may also be promising in some cases (Kumar, 2011). In poorly prognostic and relapsed patients stem cell transplantation either allogenic or autologous is considered preferred choice to cure, enhance and extend the quality of life of patients for many years. In case of relapse of promyelocytic variant Arsenic trioxide is useful for treatment. Arsenic trioxide blocks *ERK* and *AKT* pathways, with no or minimal effects on normal cells (Su *et al*., 2015). However, in patients under 60 years of age, AML prognosis improving steadily and approximately one-third of patients achieve long term cure. In patients over 70 years of age only 10% just achieve long term remission. Nevertheless, always there remains a need to develop more specific drugs against AML with high sensitivity and specificity for cancer cells that targets specific cellular functions i.e signaling pathways with least side effects to normal cells of the body.

1.14 Doxorubicin

Doxorubicin (Adriamycin) belongs to a class of antibiotics (anthracycline) and have been implicated in treatment of number of malignances including hematological and solid tumors for more than 30 years. It has been obtained from a cultured fluid of the actinobacterium *Streptomyces peucetius* and later synthesized commercially. IUPAC name is 3-amino-2,3,4-trideoxy-L-fucosyl moiety, comprising of aglycones and sugar moieties (Tacar *et al*., 2013). Intravenous mode of action is commonly used in clinical settings.

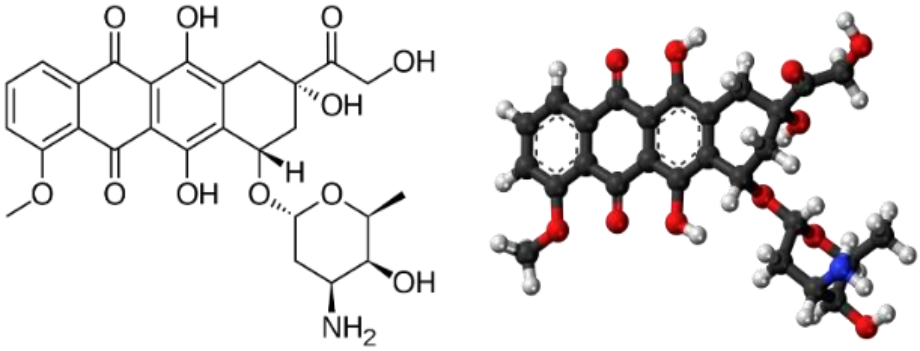

Figure 1.9: Structure of Doxorubicin (Ajaykumar, 2020)

1.14.1 Mode of Action of Doxorubicin

So far many different mechanisms of action have been proposed on the cytostatic and cytotoxic effects of doxorubicin. These includes 1) inhibition of macromolecules synthesis by intercalating with DNA bases, 2) production of ROS leading to subsequent initiation of DNA damage, 3) peroxidation of lipids, 4) disruption in strand separation of DNA and helicase activity and 5) Causing direct damage to DNA by inhibiting topoisomerase II. All these modes of actions ultimately induce apoptotic cell death. Doxorubicin intercalates with DNA bases and inhibit topoisomerase II enzyme that is involved in DNA replication thus interfering with resealing of DNA strands and broke DNA strands for replication leading to apoptosis of the cancer cell. In some other cases quinone structures permit doxorubicin to play a role as electron acceptors during redox reactions that involves cytochromes, this electrons addition converts quinones to semiquinone free radicals which cause free radical mediated injury to DNA. ROS mediated mechanism of action proves to be effective in number of malignancies. There has been subsequent evidence found that doxorubicin may bind to mitochondrial DNA as well as nuclear DNA and is a potent anticancer drug so far but have many side effects (Micallef & Baron, 2020).

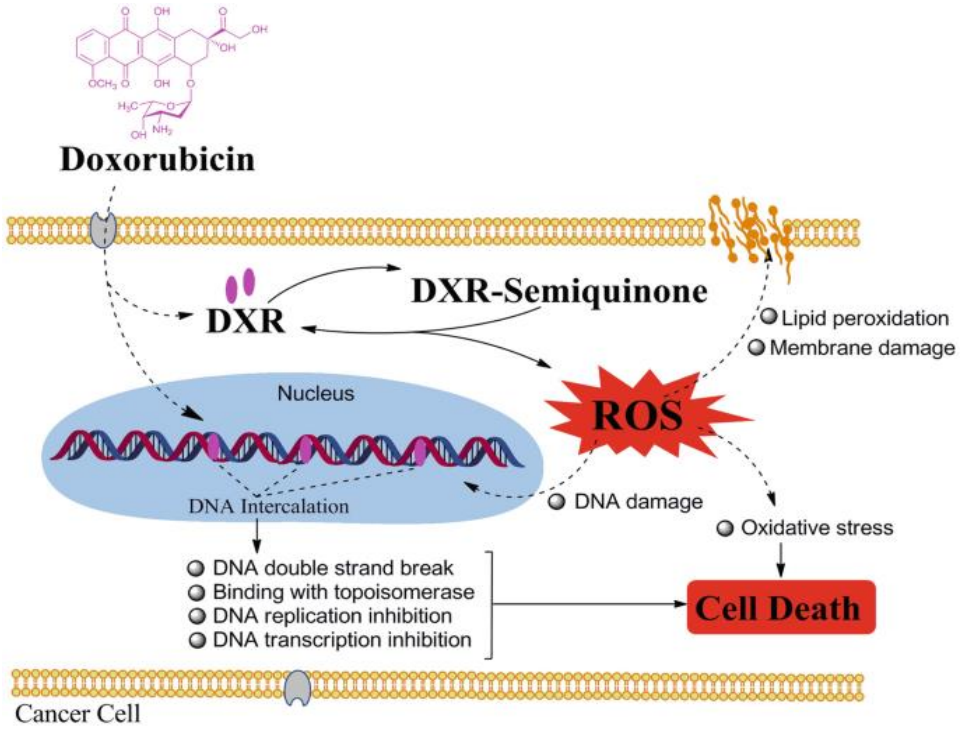

Figure 1.10: Mechanism of action of doxorubicin inducing DNA damage that leads to cell death of the cells (Hrestha et al., 2019)

1.15 Organometallic Compounds

Metal complexes made up of organic groups and metal atoms called organometallic complexes comprises of vast group of substances played a crucial role in the development of organometallic species of biological and medical interest (Van Staveren & Metzler-Nolte, 2004). As they are sub-group of metal complexes so their properties are similar to those of metal complexes, which are used to large extent as catalyst or cofactors in different biological processes, which necessitate the use of metal atoms to operate optimally (Top *et al.*, 1985). Rosenberg in 1960-1970 discovered a novel antitumor agent named cisplatin that provided an impulse to boost the discovery of other metallodrugs that forms the basis for the emergence of chemotherapy (Chavain & Biot, 2010). In organometallic complexes carbon atom along with metal atom forms one or more chemical bonds. Biocompatibility of organometallic compounds towards its target biomolecule is boosted when it is conjugated with another metal atom or biomolecule, this property render them more effective in case of their medicinal activity (Albada & Metzler-Nolte, 2016).

1.16 Characterization of Synthetic Compound

Following techniques are used to characterized organometallic compound.

- (XRD) X-ray diffraction analysis
- Scanning Electron Microscopy (SEM) with (EDS) Energy Dispersive X-ray Spectroscopy

1.16.1 XRD

X-ray powder diffraction is a highly sensitive, reliable, non-destructive technique commonly used for quantitative and qualitative analysis of crystalline compounds with effective resolution (Chauhan & Chauhan, 2014). In XRD, when X-rays thrown to a crystal the rays diffracts in a way that corresponds to the structure of that specific crystal and plots intensity of the pattern of diffraction against angle of the detector 2θ. Based on the pattern of diffraction, the recognition of materials and compounds is done by using database as there is a distinct diffraction pattern of each material (Sharma, 2000). It follows Bragg's law which states association between different parameters including incident X-rays wavelength, incident angle and spaces between planes of atoms of crystal lattice (Skoog, 2007). Calculation of inter particle spacing is done by using Bragg's equation.

Bragg's equation: $2d\sin\theta = n\lambda$

Where'd' refers to inter planar spaces, order of reflection is denoted by 'n' and 'λ' wavelength of incident rays (Sarkar et al., 2013).

1.16.2 SEM with Energy Dispersive X-ray Spectroscopy

SEM is a type of electron microscopy, one of the most versatile technique used for microstructure morphology analysis and characterization of chemical or elementalcomposition of a substances. It uses beam of high energy electrons to take images of the sample surface by scanning it, that depends upon the surface morphology of the material. Three-dimensional, in-depth images with high magnification and high resolution is taken, which is defined as the least possible minimum distance or space by which two substances or structures can be separated and still, they appear as two distinct entities under

electron microscopy (Zhou *et al*., 2006). The elemental composition of individual components or constituents can be determined with closely related technique, energy dispersive X-ray spectroscopy (EDS). It also operates by using high energy beam of electron but the resulting spectra which is obtained is based on emission of characteristic X-rays rather than diffraction. Emission of X-rays is a phenomenon in which electrons from inner-shall that is actually low energy level transits themselves to high energy levels i.e. K to L or so on and the hole created in inner shells is filled by electron that has high energy level resulting in the emission of characteristic X-ray having energy that is between those two energy levels. As these characteristic X-rays have different but specific energy that corresponds to different but specific element present in the compound and the percentage of the content of elements in the compound is determined by collective intensity of the peaks (Shindo & Oikawa, 2002).

1.17 Copper Dithiocarbamate (CuDTC)

Copper dithiocarbamate, consists of copper as a central metal atom, linked with amine-1-carbodithionic acid (derivative of dithiocarbamate) and a suitable metal carrier, is actually an organometallic compound synthetically made by using primary or secondary amines, in which nature of cation used in complex determine its solubility either in water or organic solvent. IUPAC name of copper dithiocarbamate is copper; N,N-diethylcarbamodithioate and readily soluble in dimethyl sulfoxide (DMSO). NMR spectroscopic and X-ray crystallographic studies of the compound has been done once it prepared (Bala *et al*., 2018). Because of lipophilic nature dithiocarbamate complexes are usually well known as they may bind with large number of metals as chelators, which further emphasizes their biological significance. They form stable complexes with nearly all transition elements because of their chelating ligand property (Hogarth, 2012). As an alternative option in anticancer treatment, chelators gain too much attention for last decade and their demand continuously increasing day by day because of harsh side effects of current chemotherapy. Dithiocarbamate complexes in this regard have revolutionized anticancer therapeutics because of their immense biological activities (Wang *et al*., 2016). Thus, there is need to develop novel dithiocarbamate derivatives (anticancer drugs based on metals) against treatment of cancer with high specificity and sensitivity and their underlying molecular pathways which are probing by these compounds.

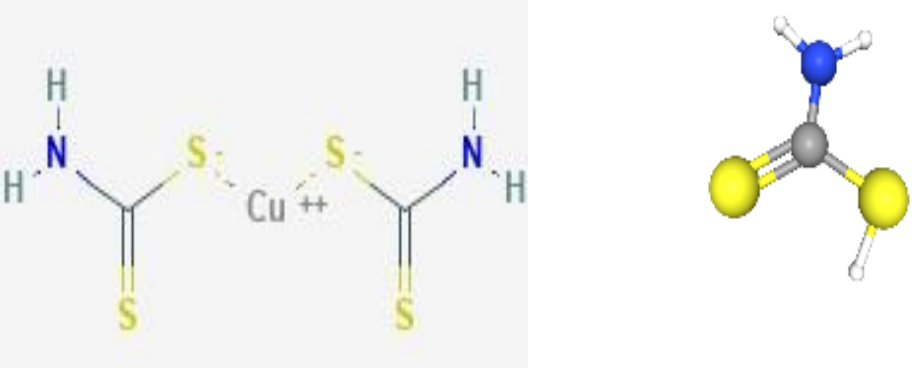

Figure 1.11: Molecular structure of Copper Dithiocarbamate (CuDTC)

1.17.1 Different Therapeutic and Biological Properties of CuDTC

Diverse array of dithiocarbamate complexes have been developed so far, having cytotoxic and antimicrobial properties which include antibacterial, antiviral and antifungal properties. There metal chelating and cytotoxic activity make them efficient against cancer treatment and becoming promising therapy from pharmaceutical point of view (Hogarth & Onwudiwe, 2021).

1.17.2 Pharmacokinetics of CuDTC in Cancer

Cytotoxic property of metal dithiocarbamate complexes made them effective anti-cancerous agents however, there cytotoxicity do not exhibit severe side effects as do conventional chemotherapy i.e. cisplatin induce nephrotoxicity (Shiri et al., 2016). Unlike traditional chemotherapy mechanism of action of copper diethyldithiocarbamate complex in cancer cells have pleiotropic effects on metabolism of cells and do not show intercalation with DNA directly (Roner *et al*., 2011)). Metal atoms when forms complexes with dithiocarbamate exhibit powerful oxidative activity which forms the basis of pharmacodynamics of copper dithiocarbamate in tumor cells that are usually rich in copper (Chan *et al*., 2016). Two well-known mode of actions of Cu(DTC) to induce cell death in tumor cells are degradation by proteosome and the other one oxidative stress. Several evidences have shown that Cu(DTC) seemed to be potential inhibitor of super oxide dismutase enzyme commonly referred as SOD-1, which is actually strong antioxidant enzyme (Shazia, 2010). It neutralizes free radicals from cells to protect them from oxidative damage however, when SOD-1 is inhibited, it results in frequent rise in free radical levels in the cell i.e. superoxide radical (Insecticide, 2012). These elevated levels of free radicals by Cu(DTC) in tumor cells

ultimately responsible for stimulation of pro-apoptotic genes (bax and bak) messenger RNA expression, that leads to subsequent apoptosis of the cells (Clarke & Sdler, 2012). Inhibition of SOD also directly associated with free radical induced damage to mitochondrial membrane, causing release of cytochrome c and cell death of cancer cells making SOD an effective target to cause selective killing in tumor cells (Cattaruzza *et al*., 2011).

1.17.3 Signaling Pathways through which Metal Dithiocarbamate Complexes Interact

Well known cytotoxic and anti-oxidant properties of dithiocarbamate containing compounds has made them effective against treatment of cancer. In spite of this, it is utmost necessary to investigate under lying molecular pathways through which these compounds directly interact with cellular machinery of the cell to perform their anti-proliferative, pro apoptotic and selective cytotoxic function in cancer cells. NF-κB signaling pathway has been prominently studied in this regard as dithiocarbamate complexes are considered to be potential inhibitors of this pathway (Cvek *et al*., 2007). Well known published literature also exists. As dithiocarbamate forms complexes with metal atoms especially zinc and copper this property increased (Verhaegen *et al*., 1995). However, It is postulated that these complexes may also interact with markers of other proliferative pathways i.e. HIF1α and Wnt pathway and cause their inhibition in cancer cells by down regulating or up regulating their negative and positive regulatory components.

1.18 Wnt Signaling Pathway

Wnt signaling pathway plays a crucial role in the development and homeostasis of many tissues and organs in the body. It is involved in the regulation of cell proliferation, differentiation, cell movement and gene expression. The pathway is also important for the regulation of stem cell self-renewal and differentiation, for tissue repair and regeneration (Duchartre *et al*., 2016). During development, Wnt signaling pathway helps to control cell fate decisions, patterning and morphogenesis. It has been implicated in a variety of diseases, including cancer, bone disorders, and neurological disorders (Acebron *et al*., 2014). Apart from normal physiological functions aberrant Wnt signaling can contribute to carcinogenesis in many different ways which include self-renewal of cancer stem cells in the heart of tumor, cell proliferation, angiogenesis, metastasis and improving survival of cancer cells (Jung & Park, 2020; Martin-Orozco *et al*., *2019*).

Primarily, two major pathways involved in wnt signaling are canonical and non-canonical pathways. Wnt signaling starts with binding of wnt ligands on the surface of the cell with receptors (Frizzled family receptors) and co-receptors (LRP5/6) low-density lipoprotein receptor-related proteins 5,6. There are 19 secreted glycoproteins that mainly constitute family of wnt ligands that take part in several wnt signaling pathway, playing their function as paracrine or in autocrine manner. Glycosylation of wnt ligands in the endoplasmic reticulum and acylation by O-acetyltransferase Porcupine (PRCN) is important for stimulation of both canonical and non-canonical pathways. These lipidated wnt ligands then transported to extracellular space of the cell in the form of vesicles which are enclosed by membranes like exosomes (Taciak *et al*., 2018).

In canonical pathway, ultimate goal is to activate β-catenin (protein) which is the crucial regulator of this pathway. At different locations in cell it found in several forms, it remains bounded with E-cadherin at cytoplasmic membrane. In the formation of cytoskeleton β-catenin connects actin filaments through binding with α-catenin. Normally, levels of β-catenin controlled strictly in the cytoplasm of the cell. Upon nuclear translocation, it causes transcription of several genes and chromatin remodeling (Song *et al*., 2014).

Wnt canonical signaling cascade begins with binding of ligands with frizzled receptors FDZ and co-receptors LRP, this complex (Wnt, FDZ, Dishevelled protein and LRP) in turn activates wnt/β-catenin, which translocates in nucleus and forms complex with T- cell factor/lymphoid enhancer factor (*TCF/LEF*) comprises of family of transcription factors that activates specific genes transcription which give growth advantages to the cell including proliferation, differentiation, decision of the fate of the cell and maintain self-renewal capacity (Nusse, 2012) (Figure 1.12).

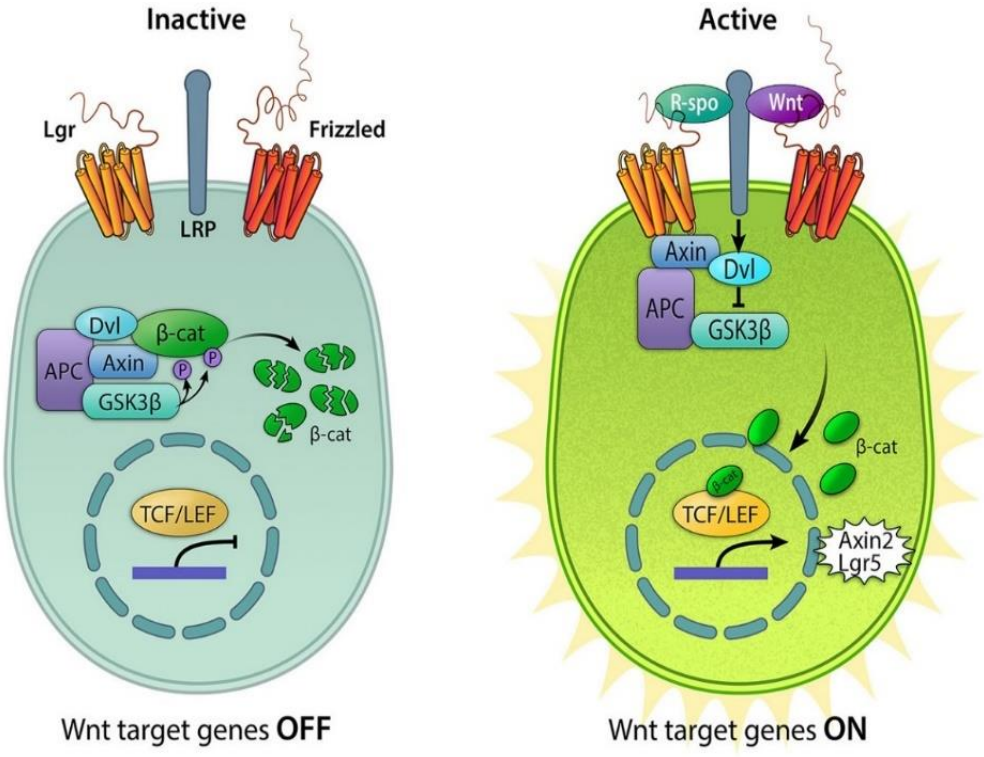

Figure 1.12: Canonical Wnt signaling pathway, Active and Inactive state (Mikesch et al., 2007)

In non-canonical pathway activation also begins with ligand binding with frizzled receptors but co-receptor used are *ROR2* and *RYK*. In this case effector molecules which are small G-Proteins and c-jun amino terminal kinase are *DVL*-dependent and *NLK* (Nemo like kinase) and *NFAT* (nuclear factor of activated T cells) are effectors of the Ca^{2+}-dependent non-canonical pathway. In tumorigenesis, reorganization of the cytoskeleton during metastasis of the tumor small G proteins play essential role while *NLK* impart their role in inhibition of canonical pathway by phosphorylating transcription factor *TCF/LEF* (Mikesch *et al*., 2007).

When there is no canonical wnt signaling, β-catenin master protein of the pathway, is followed by formation of complex with *AXIN* and *APC* and is phosphorylated at amino terminal commonly known as degradation box by glycogen synthase kinase 3β and casein kinase 1α that tagged it for degradation which is mediated by proteasome formation after polyubiquitination of β-catenin (Luis *et al*., 2012).

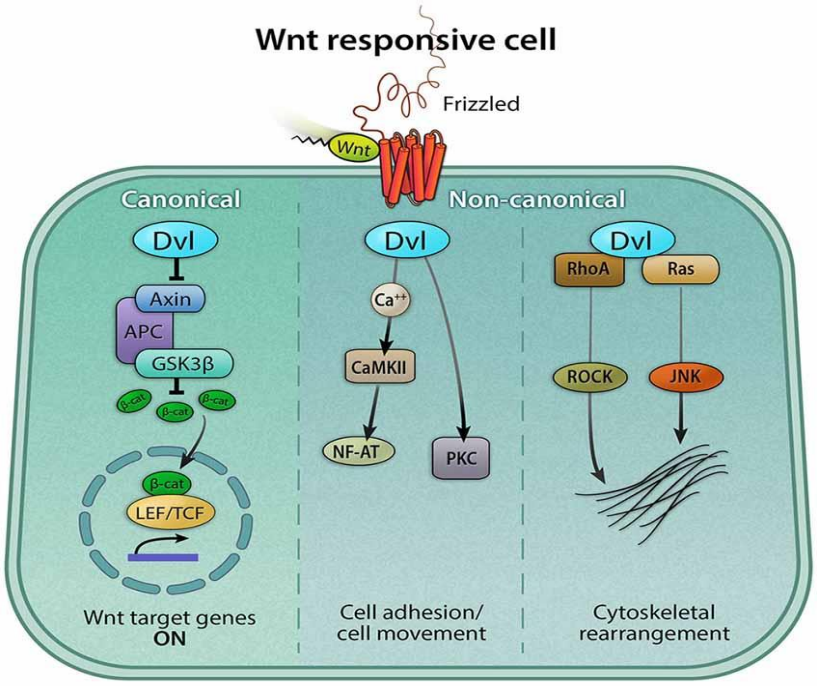

Figure 1.13: Non-canonical Wnt signaling pathway (Luis et al., 2012)

1.18.1 Intracellular inhibitors of Wnt Pathway

AXIN, APC, GSK3β and *CK1α* are the major intracellular inhibitors of the wnt pathway as they form complex with β-catenin leading to its degradation. They negatively regulate wnt pathway and maintains proper balance of wnt signaling, thus ensuring normal development. Any genetic alteration or mutation in these regulators may take part in cancer stem cell production, showing evidence of aberrant wnt signaling in several malignant disorders especially in leukemia's (Hayat *et al*., 2022).

1.19 HIF 1α Signaling Pathway

In 1991, Semenza and co-workers discovered HIF 1 in their study that was conducted on erythropoietin gene, which actually encodes hormone namely erythropoietin produced by the kidneys (90%) and (10%) from liver for the production of red blood cells in response to low oxygen levels, a condition called hypoxia (Semenza *et al*., 1991). Study of the *EPO* gene revealed presence of specific cis-DNA sequences (5'-RCGTG-3', where R refers to either A or G) in 3' flanking region, which are essential for transcriptional activation of *EPO* gene only in response to hypoxia and named as hypoxia response elements (HREs). Further studies found that activation of several different regulatory genes is initiated only, when a specific protein binds to HREs region of the *EPO* gene, which was named as hypoxia

inducible factor (HIF-1) (Goldberg *et al*., 1988). HIFs act as heterodimers and have two subunits namely α which is oxygen-dependent and β subunit which is independent of oxygen. As α subunit plays crucial role in response to oxygen levels and in its active state cause transcription of many genes so it is commonly referred as HIF-1α (Wenger *et al*., 2005).

The HIF-1α pathway is a complex network of molecular interactions that primarily involved in hypoxic (low oxygen) conditions providing cells with an adaptive mechanism in hypoxic environment. To thrive in low oxygen conditions it induces the transcription of genes that help cells to survive. It can also interact with other transcription factors, such as NF-κB and AP-1, to further regulate gene expression. The HIF-1α pathway is important for many physiological processes, including angiogenesis, cell growth, differentiation, and apoptosis (Minet *et al*., 2001). Dysregulation of the pathway has been implicated in the development of cancer, cardiovascular diseases, and other diseases.

1.19.1 Regulation of HIF-1α Pathway in Normoxia

Under normoxia, (PHDs) prolyl hydroxylases, a set of enzymes strictly regulates HIF-1α levels. At specific prolyl residues HIF-1α is hydroxylated by PHDs and are easily recognized by a specific tumor suppressor gene (VHL) von Hippel Lindau, which is a E3 ligase and cause proteosome mediated degradation of HIF-1α (Semenza, 2014). Another negative regulation of HIF-1 during normal levels of oxygen is VHL independent suppression. As in hypoxia, HIF-1 is transcriptionally activated by cooperated binding of C-TAD domain of HIF-1 with co-activator CBP/p300. But normally when oxygen tension is normal in the cells, there is an oxygen dependent hydroxylation by factor inhibiting (FIH-1) at asparagine residue that cause blockage of interaction between two domains as a result subsequent inhibition of genes which are mediated by HIF-1α (Lando *et al*., 2002).

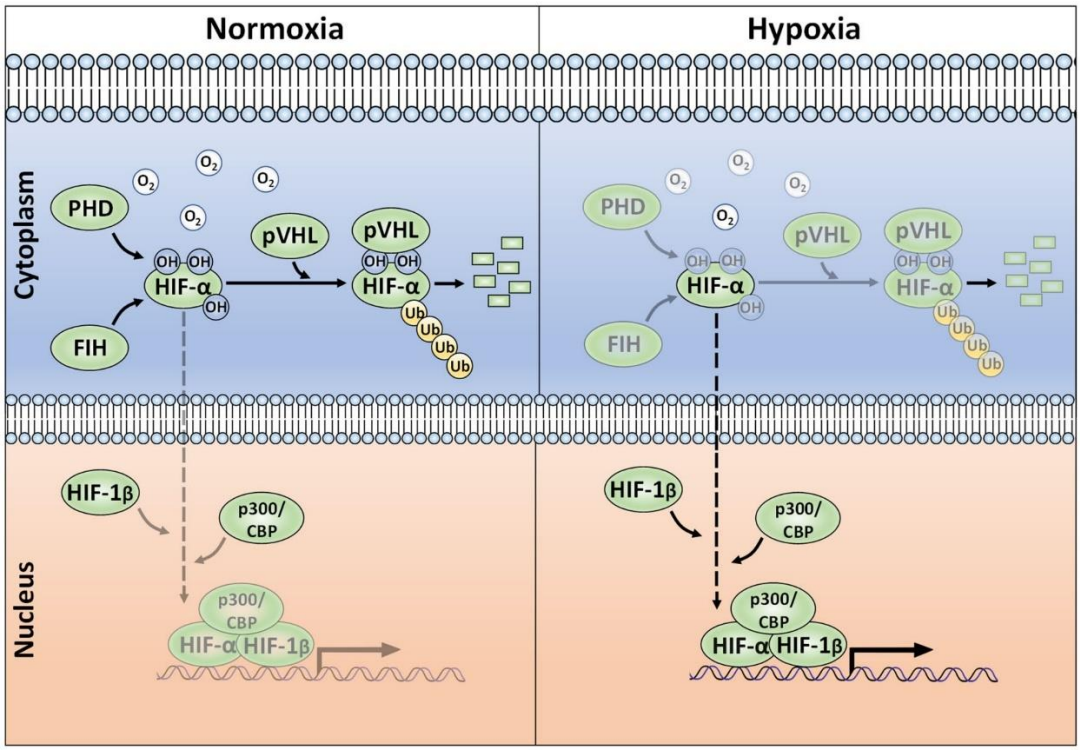

Figure 1.14: pVHL tumor suppressor gene mediated regulation of HIF-1α, during hypoxia and normoxia (Albanese et al., 2021)

1.19.2 Regulation of HIF-1α Pathway in Hypoxia

In hypoxic periods reduced activity of PHD and FIH may stabilized HIF-α that results in, its nucleus translocation where it dimerizes with HIF-β. HIFα/β complex binds within target genes that contain hypoxia responsive elements in their promotor regions promoter regions followed by elevated expression of genes that play their part in signaling pathway (Wenger *et al*., 2005; Semenza, 2014) .

There has been increasing evidence that hypoxia play a vital role in cancer metabolism and dormancy. Hypoxia itself is an independent entity that can even cause initiation and progression of cancer along with increasing stemness activity. There are two main processes that are induced by HIF and is responsible for metastasis. One is (EMT) epithelial mesenchymal transition and the other one is cancer stem cells (CSC) production. This was proved by a study performed using cell line culture, when hypoxia was induced in them they showed high levels of mRNA expression of proteins including vimentin, N-cadherin and fibronectin (that physically take part in process of EMT), along with reduced E-cadherin

mRNA expression. CD44 known marker of stem cell, also have elevated expression in these cell line cultures (Pezzuto & Carico, 2018).

1.19.3 Oncogenic regulation of HIF-1α Pathway in Tumors

One of the most important intracellular negative regulator of HIF-1α is tumor suppressor gene *p53*. It was observed that during normoxia, *p53* normally binds with HIF-1α and cause its ubiquitination which is mediated by *Mdm2* resulting in HIF-1α proteasomal degradation (Bae *et al*., 2002). As in more than 50% of human cancers *p53* is mutated, resulted in loss of negative regulation of HIF-1α. It revokes *Mdm2* mediated HIF-1 degradation, which is associated with high levels of HIF-1α expression in tumor cells (Ravi *et al*., 2000).

Second well-known oncogenic regulator of HIF-1α is Hsp90 that may increase or elevate HIF-1α, which is independent of oxygen levels of the cells. It was suggested that binding of Hsp90 with HIF-1α induces changes in its structure causing its dimerization with HIF-1β triggering stabilization and transactivation of HIF-1α (Isaacs *et al*., 2002; Gradin *et al*., 1996).

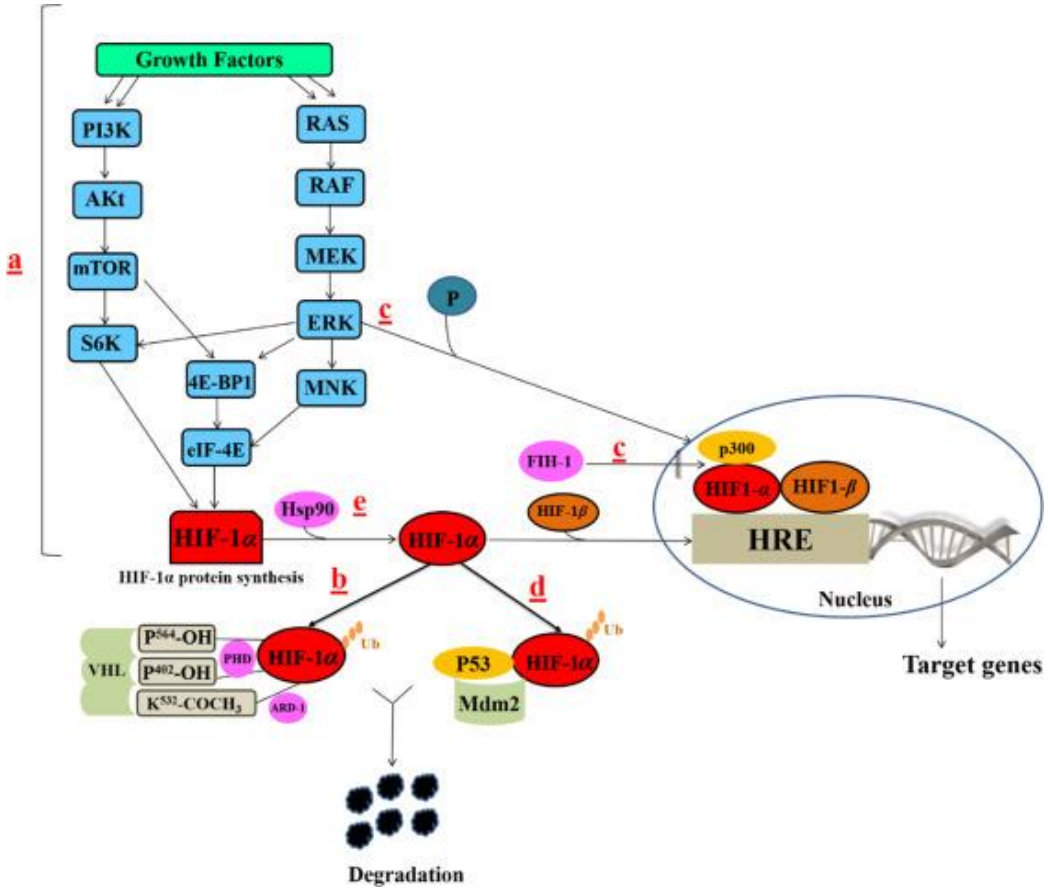

Figure 1.15: Illustration of different levels of HIF-1α Pathway where regulation occur (Georgina 2015)

Some other well studied inducers include activation of PI3K pathway directly up-regulate HIF-1α levels (Semenza, 2002). This activation can be reversed by a tumor suppressor gene called *PTEN* which cause phosphorylation of PI3K and downstream inhibition of HIF-1 (Gingras *et al*., 2001). Another proliferative pathway, *RAS/RAF* activation by certain growth factors also directly elevates HIF-1 expression causing transcription and translation of under lying proteins products (Conrad *et al*., 1999).

Aims and Objectives

Aim of this study was to determine the anti-leukemic potential of CuDTC in combination with conventional therapy which is Doxorubicin. The main objectives of the study were:

- To characterize synthetic compound CuDTC a derivative of dithiocarbamate, an organometallic compound.

- To establish acute myeloid leukemic rat model by intravenous administration of benzene.
- To evaluate and compare the effects of benzene, Doxo, CuDTC and CuDTC+Doxo treatments on haematological & Biochemical parameters in leukemic rat model.
- To determine the relative mRNA expression of biomarkers of HIF-1α and Wnt pathway, in treated and leukemic rats.

2. Materials & Methods

The study mentioned below was approved by the Bioethics Committee (BEC) of Quaid-i-Azam University, Islamabad. All assays (in-vivo and in-vitro) were conducted under standard settings, using optimized protocols in Molecular Cancer Therapeutics Laboratory at QAU, Islamabad. Different tools and molecular biology techniques were used to get desired results.

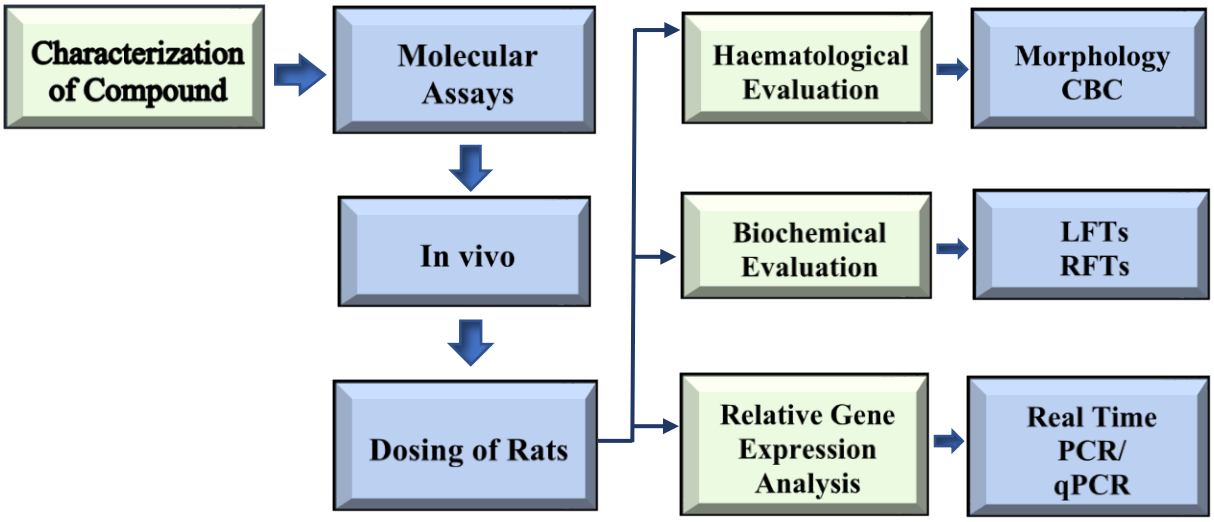

Figure 2.1: Study Design

2.1 Techniques used for Compound Characterization:

Collectively, XRD, FTIR and SEM were carried out for characterization of compound. Structural confirmation of Copper Dithiocarbamate (CuDTC) was done by XRD, FTIR techniques. Whereas, SEM with EDS was performed to check the elemental composition of the CuDTC.

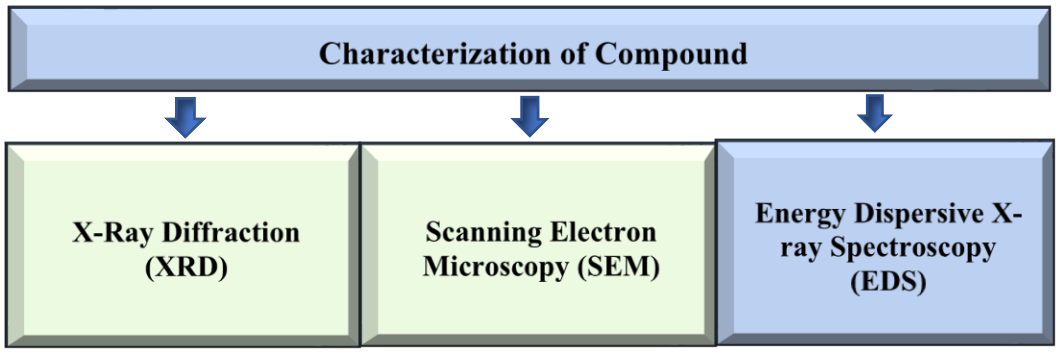

Figure 2.2: Different techniques used for characterization of CuDTC

2.1.1 XRD

X-ray spectrophotometer model (Bucker D8 Advance) was used for XRD analysis of compound, available in Department of Chemistry, Quaid-i-Azam University, Islamabad. Instrument was operated at radiation of Cu-Kα, λ=1.54Å, at voltage of 40KV and 30mA current respectively. XRD works by irradiating the material by incident X-rays. It gives the primary information about the identification of compound (nature, size of particle) based on their diffraction pattern.

2.1.2 SEM with EDS

SEM is a highly powerful and advanced technique used for high resolution microscopy of materials with a good depth of the field. Type of electron microscope that uses electrons beam to image the sample surface by scanning it. It depends upon the surface morphology of the material. The elemental composition of individual components or constituents can be determined with closely related technique, energy dispersive X-ray spectroscopy (EDS) in conjunction with SEM. For SEM/EDS analysis of the compound facility at National University of Science and Technology (NUST) was used.

2.2 Experimental Strategy

To perform in vivo studies, Sprague Dawley rats were used, which were purchased from National Institute of Health Sciences (NIH), Islamabad. Rats were kept under proper conditions in the Primate Facility of the Faculty of Biological Sciences QAU. For experiment from total, 15 rats were divided into five groups, which were numbered as group 1,2,3,4 & 5 respectively, having 3 rats each group. All the handling guidelines as

instructed by the Ethical Committee of Quaid-i-Azam, University were strictly followed throughout the experiment.

Table 2.1: Dosing and mode of administration of drugs among different groups.

No	Group	No of Rats	Dosage Concentration	Mode of Administration
1	Normal	3	Control	-
2	Benzene	3	1ml on alternate days for three weeks	Intravenous
3	Doxorubicin	3	0.625mg/ml	Intravenous
4	CuDTC	3	0.05mg/ml	Intravenous
5	CuDTC + DOXO	3	0.05mg/ml + 0.625mg/ml	Intravenous

2.2.1 Benzene Administration

Standard ratio of (1:2:1) was used for preparation of Benzene injections by mixing benzene, isopropanol and water. To make stock solution, fixed proportion of 0.5ml benzene, 1ml isopropanol and 0.5ml water were used. From this stock 100µl was injected intravenously to rats group 2,3,4 & 5 at alternative days for three weeks respectively.

2.2.2 Doxorubicin Administration

Doxorubicin is chemotherapeutic drug, purchased from Actavis, Italy. Stock solution for doxorubicin administration was made by dissolving 2.75mg of DOXO in 1ml of water. Standard dose of doxorubicin (300µl of 0.625mg/ml) was given to rats of group 3 (after induction of leukemia) for three weeks on alternative days to treat leukemia.

2.2.3 Compound Administration

For administration of compound stock solution was prepared by dissolving 1mg of compound in 0.1% DMSO. From stock 200µl solution was taken and further diluted with 1.9ml of 0.1% DMSO so that the working concentration of 0.05mg/ml is maintained.

After leukemia induction in group 4, 100µl of this solution was administered intravenously to group 4 rats. In case of group 5, in which combination of known chemotherapeutic drug (DOXO) and test compound (CuDTC) was used, 300µl of 0.625mg/ml doxorubicin and 100µl of the working solution was administered on alternate days for three weeks.

2.3 Animal Dissections

After accomplishment of all doses, animal dissections were performed. Rats were dissected following the rules and guidelines provided by the Institutional Animal Care and Use Committee of QAU. Following steps were followed:

i. At first chloroform treatment to render the rats unconscious.

ii. Laying down of the unconscious rat on a dissecting board.

iii. Abdominal cavity was cut by using sterile surgical tools.

iv. To collect essential organs i.e. liver, heart and kidney body cavities of rats were exposed.

2.3.1 Collection and Storage of Blood

Blood was drawn directly from heart by using heparinized syringes. For Haematological evaluation lavender top EDTA Ethylene Diamine Tetra Acetic Acid (anticoagulant) vials commercially available were used and for Biochemical evaluation yellow top gel vials also known as serum tubes were used. Serum tubes were then centrifuges at 3000rpm for 15 minutes for serum separation and collection. To avoid risk of hemolysis serum tubes were stored at -40°C and EDTA tubes at -4°C before further use.

2.4 Preparation of Blood Smears for Morphological Analysis

Fresh blood was used to make blood smears for to study morphology of blood cells. To make slides a small drop of blood was placed on the slide, which is approximately one cm from one end of the slide. At an angle of 45°, spreader was placed in front of the blood drop slightly moved back so that it touches the drop of blood so that blood will spread along the margin in contact with slide. Then, the spreader was pushed forward with a rapid, smooth and straight movement along the length of the slide. Smears prepared was allowed to dry in the air. Fixation was done by dipping them in chilled methanol for 3 minutes.

2.4.1 Giemsa Staining of Blood Smears

To stain, smears were covered with 10% giemsa staining solution for 10 minutes. After that the slides were washed with tap water and air dried. These were then examined under oil emersion lens (100X lens) using compound microscope.

2.5 Complete Blood Counts

For haematological evaluation blood complete picture is considered as the baseline test. It provides information about total counts of different blood cells including WBCs, RBCs and Platelets. Blood CP was performed by using Automated Blood Analyzer at Rawalpindi Institute of Urology and Transplantation (RIUT). For this 100µl blood stored in EDTA tube was sent to RIUT.

2.6 Estimation of Hepatic Enzymes Activity (LFTs)

Microlab 300 Auto analyzer (Merk) was used to perform biochemical assays. Parameters that were analyzed include Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline Phosphatase (ALP),.

2.6.1 Alkaline Phosphatase (ALP)

ALP assay was performed following all the guidelines and protocols provided by AMP diagnostic kits. Control and standards were measured before estimation of samples to check the validity of results, kits as well as instrument. In an Eppendorf tube, 1000µl of Reagent 1 was taken followed by addition of 20µl of sample (serum) and incubated for 5 minutes at 37°C. After completion of incubation 250µl of Reagent 2 was added. Then absorbance was measured using Microlab 300 at the wavelength of 420nm. Total three readings were taken collectively at 1-minute intervals. Mixture of all reagents excluding sample was taken as "blank". Following equation was used to calculate enzyme activity.

At 37°C ALP activity in U/L = A/min* 2757

2.6.2 Alanine Aminotransferase (ALT)

LTA diagnostic kit was used to perform AST estimation and the assay protocol provided by the kit was used. To start first in an eppendrof tube 1ml of Reagent A (Tris buffer 100mM pH 7.15) was taken followed by addition of 125µl sample, vortexed for mixing and

incubated at room temperature for 5 minutes. Then 250µl of Reagent B (Alpha ketoglutarate 12mM, LDH 1700U/L, L-alanine500mM, NADH0.18mM) was added and the initial absorbance was taken immediately by using auto analyzer. Three consecutive measurements were taken after 1-minute intervals to determine the change in absorbances. Enzyme activity was calculated by using following equation.

At 37°C ALT/GPT activity U/L = A/min*1746

2.6.3 Aspartate Transferase (AST)

For AST estimation, protocol provided by the LTA diagnostic kit was followed. Working Reagent was prepared by adding 1ml of reagent in cuvettes for each sample and control separately followed by 3 minutes incubation at 37°C. Then, 100µl of sample (serum) was added into respective cuvettes except blank and mixed gently. Spectrophotometer was maintained at zero and the absorbance of blank, control and samples were taken at a wavelength of 340nm. Collectively, total three readings were taken with 1-minute intervals. Average absorbance per min (ΔA/min) was calculated that was then multiplied by factor 1746 given in kit literature to get results in U/L. Following equation was used.

AST activity U/L = ΔA/min*1746

2.7 Estimation of Renal Markers (RFTs)

Microlab 300 Autoanalyzer (Merk) was used for estimation of uricase activity in experimental samples.

2.7.1 Uric Acid Estimation

Amplex® Red Uric acid/ Uricase Assay kit (Thermofisher Scientific) was used for estimation of uric acid levels. Working solution of the reagents were prepared according to the protocol mentioned in kit literature. Uric acid was taken as positive control that provided in kit. In a 96 well microplate containing control and samples, 50µl of working solution was added in each well followed by 30 minutes incubation at 37°C at dark place to protect from light source. The instrument was resetted at zero and absorbance was taken at the wavelength of 560nm for three times at 1-minute interval each. Following formula was used to calculate results.

(Sample Abs/ Standard Abs) * Standard Conc = mg/dl

2.8 Serum Lipid Profile Levels

For estimation of lipid profile, serum separated from blood that was collected from different experimental groups was sent to Rawalpindi Institute of Urology and Transplantation (RIUT).

2.9 Estimation of Lactate Dehydrogenase Levels

For LDH levels facility at the Rawalpindi Institute of Urology and Transplantation was utilized. Serum was sent to RIUT for this purpose.

2.10 Analysis of Relative Gene Expression

2.10.1 RNA Extraction by TRIzol® Reagent

To study relative expression of different genes, TRIzol reagent RNA extraction method was used to extract total RNA from whole blood. For extraction of RNA from Bone marrow samples same method was used. The protocol used for this was as follow:

i. 1ml of whole blood was taken from EDTA tube to eppendrof tube and centrifuged at 3000rpm for 15 minutes at 24°C.

ii. Supernatant was discarded and interphase was collected in a separate microcentrifugation tube.

iii. Then, 1ml Trizol reagent was added, vigorously shaked and incubated for 20 minutes at -20°C.

iv. 0.2ml chloroform and mixture ratio (24:1) of isoamyl alcohol was added and again kept for incubation at room temperature for 10 minutes.

v. After incubation, centrifugation was done for 15 minutes at 13000rpm, while setting the temperature at 4°C. The mixture separated into three layers, first the lower phenol-chloroform red phase, second interphase and third upper colorless aqueous phase.

vi. 500µl from the upper colorless phase that contained RNA was taken into separate clean RNase free tube.

vii. RNA precipitation from aqueous phase was done by adding 500µl isopropanol followed by room temperature incubation for 10 minutes and centrifugation at 13000rpm for 10 mins at 4°C.

viii. RNA precipitate was observed the seemed like a gel or white pellet at the bottom and side of the tube. Supernatant was discarded and RNA pellet was washed by adding 1mL of 75% ethanol followed by centrifugation at 4°C for 5 minutes at 8000rpm.

ix. Ethanol was discarded and RNA pellet was air dried for 10 minutes at room temperature, then resuspended in RNase free water (20µl) and incubated for 10 to 15 minutes at 55-60°C.

2.10.2 Quantification of RNA

Nanodrop quantification and 1% Agarose Gel Electrophoresis was performed for quality assurance of extracted RNA. Reagents used for RNA extraction are given below in the table 2.2.

Table 2.2: Reagents used for RNA Extraction

No.	Chemical Name	Quantity
1	TRIZOL	1ml/cycle
2	Isopropanol	200µl/cycle
3	Chloroform	200µl/cycle
4	75% Ethanol	2ml/cycle
5	RNase Free Water	20µl/cycle

2.10.3 cDNA Synthesis

As to perform qPCR for relative gene expression studies total RNA acquired from above mentioned process was subjected to cDNA synthesis by using RevertAid First Strand cDNA Synthesis Kit (Thermofisher Scientific). The literature provided along kit was followed. All RNA extractions were concentrated at 1.5µg or 1500ng. All the components of kit were placed on ice throughout the process. As it is mandatory that the purification of RNA should be performed in RNase free environment to avoid RNase contamination. All the plasticware,

glassware and reagents were made RNase free since, the efficiency of the whole process depends solely on the quality and quantity of RNA templates and even a minute amount of RNase cab degrade RNA and affect the length of the newly transcribed RNA. Reagents were briefly centrifuged after thawing. Two mixtures were prepared to synthesize cDNA from RNAs. First RNA primer mixture was prepared by adding 1.5µg template RNA from each sample along with 1µl Random Hexamer primer and total reaction volume was raised upto 12µl by using nuclease free water (as shown in table 2.3). This mixture was then cooled on ice for 2 minutes followed by incubation at 65°C for 5 minutes.

Table 2.3: Reagents required for RNA mixture synthesis

No	Component Name	Amount/Volume
1	Random hexamers	1µl
2	Template/Total RNA	0.1-0.5µg (recommended 1µg)
3	Nuclease free water	Upto 12µl

cDNA Synthesis mixture was then prepared (as shown in table 2.4). 8µl from this mixture was added in each RNA primer mixture tube. These samples were then incubated for 1 hour at 42°C after gentle shaking and centrifugation. After cDNA synthesis reaction was terminated by incubating cuvettes at 85°C for five minutes. These were briefly centrifuged and chilled on ice. cDNA that was synthesized stored at -20°C for further use.

Table 2.4: Reagents required for cDNA synthesis mixture

No	Chemical Name	Amount/Volume
1	RevertAid M-MuLV Reverse Transcriptase	(200 U/ul) 1ul
2	10mM dNTP mix	2µl
3	5X Reaction Buffer	4µl
4	RiboLock RNase Inhibitor	(20U/µl) 1µl

2.10.4 Conventional PCR Amplification

There were two major purpose to run conventional PCR. First was to check the annealing temperature of Primer sequences that were developed or designed online by using Integrated DNA Technology tool (as shown in table 2.7). Second reason was to check whether cDNA was synthesized properly or not. It was expected that by combining the components of the reaction mixture in 200µl PCR tubes (Axygen, USA) the desired transcript might be amplified. (Table 2.5) Listed all the reagents that were used in the 25µl reaction mixture. After preparation of reaction mixture vortexed the tubes shortly and briefly centrifuged for 30 secs at 8000rpm.

Table 2.5: Reagents and their quantities used for conventional PCR

No	Reagents	Quantity
1	cDNA Sample	1µl
2	Forward Primer (0.4µM)	1µl
3	Reverse Primer (0.4µM)	1µl
4	$MgCl_2$ (25mM)	1.5µl
5	dNTPs (10mM)	0.5µl
6	Taq DNA polymerase (5U/µl)	0.2µl
7	10X Taq Buffer with $(NH4)_2SO_4$	2.5µl
8	DNase free distilled water	17.3µl
	Total Reaction Volume	**25µl**

Reaction tubes were loaded in the thermocycler (Biometra, Germany) and the standard PCR cycles were processed (as shown in table 2.6). After PCR amplification 2% Agarose gel was used visualized amplified products. A standard marker called ladder was used along with amplified products to confirm the estimated size of the products.

Table 2.6: Cycle steps involved in Conventional Polymerase Chain Reaction

No	Steps	Temperature	Duration
1	Initial denaturation	95°C	10 minutes
2	Denaturation	95°C	1 minute - (40 cycles)
3	Annealing 40 (cycles)	60°C	1 minute - (40 cycles)
4	Extension	72°C	45 seconds - (40 cycles)
5	Final Extension	72°C	10 minutes

2.10.5 Agarose Gel Electrophoresis

As gel electrophoresis is used for the separation and visualization of DNA, RNA or other charged molecules by passing gel matrix through electric current. Here gel electrophoresis was performed for the visualization of PCR products. 2% agarose gel was prepared as follow:

i. In a conical flask 0.8g of agarose powder was added in 40ml of 1xTBE buffer (prepared by mixing 4ml of 10x TBE in 36ml of D/W).

ii. The mixture was heated in a microwave oven for 2 minutes at high power and observed whether the clear solution was produced and agarose granules dissolved properly. The solution was cooled to 70°C and 6µl of tracking dye (Ethidium Bromide) was added.

iii. Molten agar was then poured into gel casting tray and the sample combs were appropriately placed in it.

iv. The gel was allowed to cool for 20-30 minutes and after solidification and hardening of the gel sample combs were removed carefully to avoid well floor to be damaged.

v. Gel was placed in Biometra gel tank filled with 1x TBE running buffer. Before loading into the gel, amplified products were briefly centrifuged and tapped.

vi. Total of 10µl sample was loaded (4µl of loading dye Bromophenol blue & 6µl of the amplified product). Gel was run at a voltage of 110V for 25-30 minutes.

vii. Visualization of bands on gel was done by using gel documentation system and the results were recorded by using Gene Snap Software.

2.11 Primer Designing

qPCR Primers were specifically designed by using qPCR tool Integrated DNA technologies (https://eu.idtdna.com/scitools/Applications/RealTimePCR/). NM numbers of the desired genes were searched from gene database (NCBI). Single hit primers were selected by verification of the primers specifically via In-silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr). Specific primers selected were ordered from Macrogen, Korea. Primers were received in Lyophilized from and nuclease free water was used to resuspend them for use. Table 2.7 shows the list of Primers used for qPCR.

Table 2.7: Primers used for qPCR

No	Primers Name	Primer Sequence (5'- 3')	Tm°C	Amplicon Size (bp)
1	B-Actin-F B-Actin-R	5'CTCACGGTGTTGCCAAAATG3' 5'GCCTTGATCCTTTGGTTATTCG 3'	58.9	70 – 120
2	AXIN – F AXIN - R	5'CAGCCTTCTCATCTCTTCATCC3' 5'GTGATTTTGTCCTCTGCTTGG 3'	55	200
3	GSK3β -F GSK3β-R	5'ACCTGCCCTCTTCAACTTTAC3' 5'CACGGTCTCCAGCATTAGTATC3'	58.4	200
4	LEF – F LEF - R	5'TGAAATGCCCACCTTCTACC3' 5'AAGTGTCCCCTGAAAGTGAAG3'	54.8	200
5	HIF1α – F HIF1α - R	5'GGTGGATATGTCTGGGTTGAG3' 5'AGGGAGAAAATCAAGTCGTGC3'	54.9	200
6	HSP90-F HSP90-R	5'CTCTGCGTATTTGGTTGCTG3' 5'CTTTGTTCCACGACCCATTG3'	54.3	200
7	PTEN – F PTEN - R	5'CCACAAACAGAACAAGATGCTC3' 5'ATCACAAAGACTTCCATTTTCCAC3'	54	200

2.12 Real Time PCR

For quantification of amplified cDNA and analysis of relative gene expression MIC qPCR by Bio Molecular Sciences (BMS) was used. Following steps were taken to proceed qPCR.

2.12.1 Determination of Primer Efficiency

To analyze the efficiency of the Primers, dilutions of cDNA were made from each sample. PCR tubes pooled by 1µl of cDNA of all samples were further diluted three times. 10-fold dilutions were prepared as 10x, 100x and 1000x and were run in triplicates for each candidate gene.

2.12.2 Real Time PCR Reagents

Table 2.8 shows the complete list of RT-PCR reagents.

Table 2.8: The Reagents and their quantities used for qRT-PCR

No	Reagents	Quantity
1	Syber Green Master Mix	2µl
2	cDNA	5µl
3	Forward Primer	1µl
4	Reverse Primer	1µl
5	Nuclease free water	1µl
	Total Reaction Volume	**10µl**

2.13 Data Analysis

2.13.1 Double Delta CT Analysis

qRT-PCR data was analyzed by using double delta CT analysis software. Cq values of the reference and target genes were used to determine their relative expression.

2.14 Statistical Analysis

Graph Pad Prism software was used for statistical analysis of the obtained data. Descriptive statistics of the data was done and obtained results were plotted against mean ± Standard deviation (SD). One-way analysis of variance (ANOVA) was applied to check the

comparative effect of different treatments among different rat groups. Tukey's post-hoc test was used for separation of groups. At 95% confidence interval p-value <0.05 was considered statistically significant.

3. Results

3.1 Characterization of Copper Dithiocarbamate

Characterization of the compound was done by using different techniques which includes XRD and SEM with EDS to check proper synthesis, functional groups distribution, structural morphology and elemental composition of the compound.

3.1.1 XRD (X ray Diffraction) Spectroscopy

In XRD diffraction pattern of CuDTC different peaks were observed from 20°-70° which indicate metal ion bridges the S-atom of one dithiocarbamate to the next molecule in the chemical structure between two dithiocarbamate ligands. The XRD pattern of Cu conjugated dithiocarbamate (Figure 3.1) presents three significant peaks at 28-to 29°, 34°, 48° and 67° indicating square-planar structure of CuDTC crystal with overall pseudo-octahedral coordination geometry.

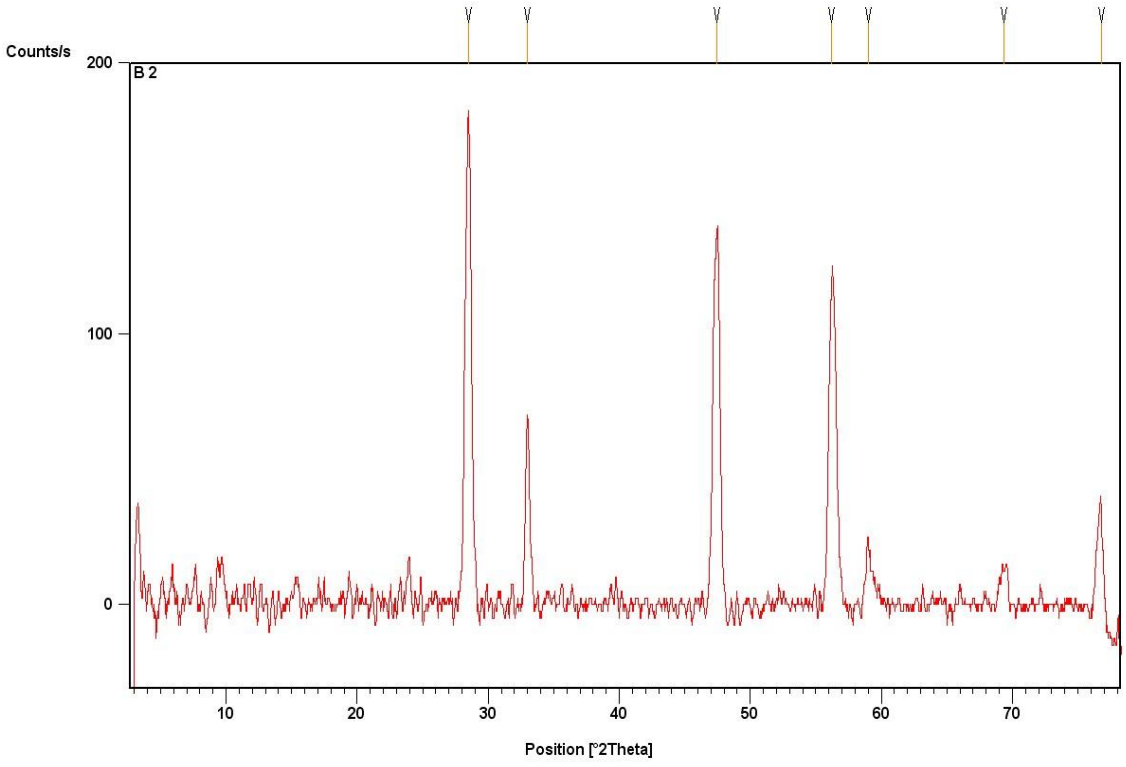

Figure 3.1: XRD pattern of Copper Dithiocarbamate (CuDTC)

3.1.2 SEM (Scanning Electron Microscopy)

SEM being highly powerful and versatile technique was performed to get 3D, In depth images of the compound to ensure its proper synthesis and structure of the complex. Images were collected at different magnifications at 1000X and 2500X respectively. Particle size was determine to be 10µm. SEM images clearly showed complex formation of metal atom with dithiocarbamate complex.

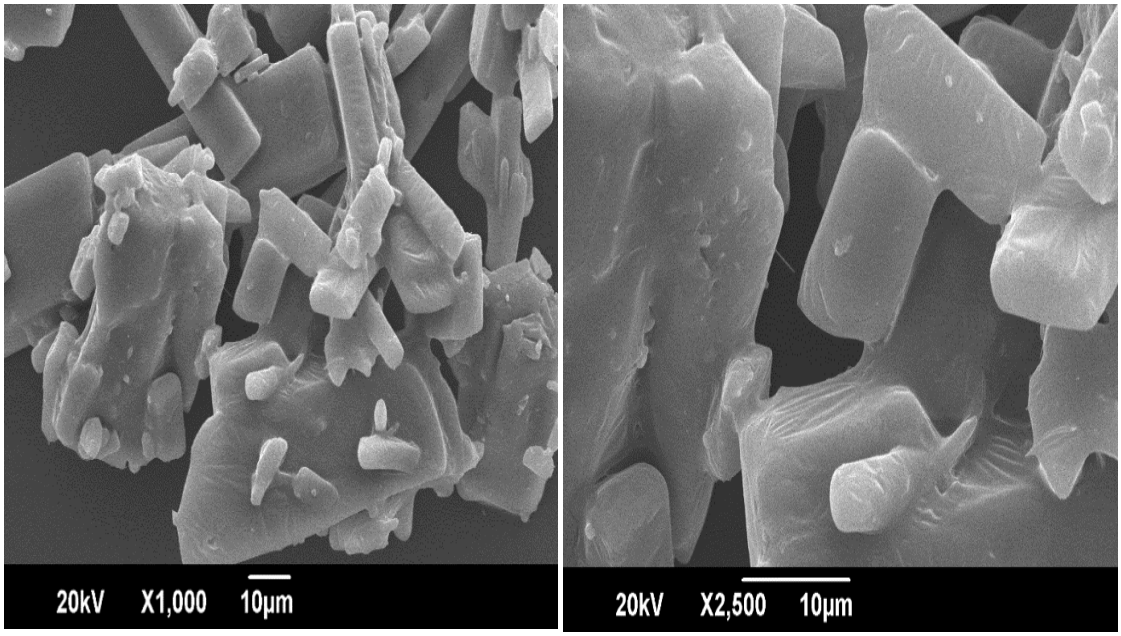

Figure 3.2: Scanning Electron Microscopic Images of Copper Dithiocarbamate Complex

3.1.4 EDS (Energy Dispersive X ray) Spectroscopy

Energy dispersive spectra was obtained, performed in conjunction with SEM. Elemental composition of the complex was determined by using the intensity of individual peaks in the graph (Fig. 3.4).

Table 3.1: Individual Elements and their percentages by weight and atomic size in composition of Copper Dithiocarbamte

Element	Weight%	Atomic %
C K	66.9	80.3
N K	7.0	7.2
O K	5.1	4.6
S K	14.4	3.4
Cu K	6.7	1.5

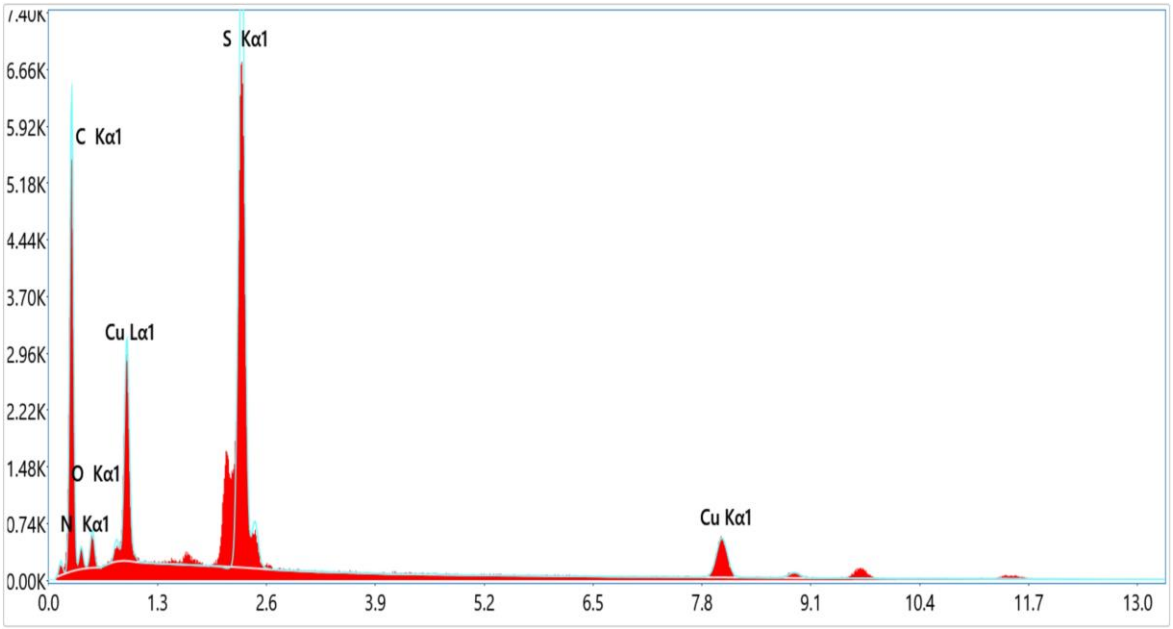

Figure 3.3: Energy Dispersive Spectroscopic Image of Copper Dithiocarbamate

3.2 Haematological Profile

Blood complete picture was obtained to check the effect of test compound on different types of blood cells in experimental rat groups.

3.2.1 White Blood Cells

Total white blood cells count was analyzed among different experimental groups. In group 2 rats, overall WBCs count was significantly increased which indicates successful induction of leukemia in group 2. After leukemia induction, Doxo treatment to group 3, although did reduction in WBCs count but not shown significant using one-way Anova analysis. CuDTC treatment in group 4 leukemic rats showed significant decrease in white blood cell counts in comparison to benzene group. Combination therapy (CuDTC+Doxo) showed best results and restored WBC counts to normal as were in normal group rats. Significance was shown in CuDTC alone and in combination with Doxo, both demonstrate reduction in WBCs counts against benzene.

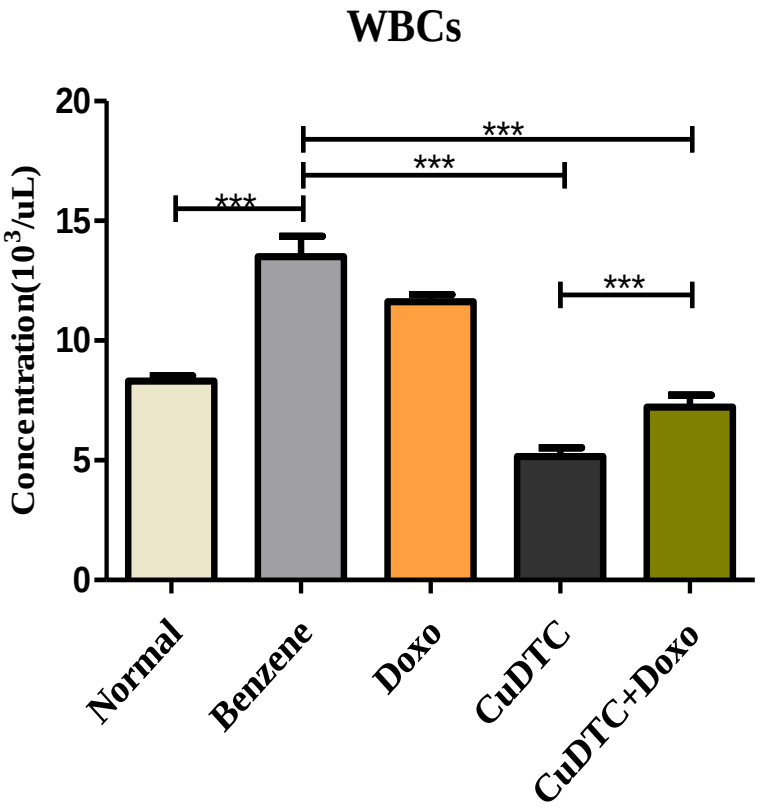

Figure 3.4: Comparison of WBCs count in different experimental groups

3.2.2 Neutrophils

There was significant increase in neutrophils with respect to normal group, which clearly justifies elevated levels of myeloid lineage cells as indicative of myeloid leukemia. Doxorubicin treatment in group 3 slightly reduced neutrophils count but not as significant to CuDTC and coadministration of CuDTC with Doxo. CuDTC alone showed the best possible results against benzene group, which were nearly as in normal rats group. There was significant difference observed in CuDTC group and Doxo, which indicates that CuDTC did better reduction in neutrophil counts even against conventional therapy (doxorubicin).

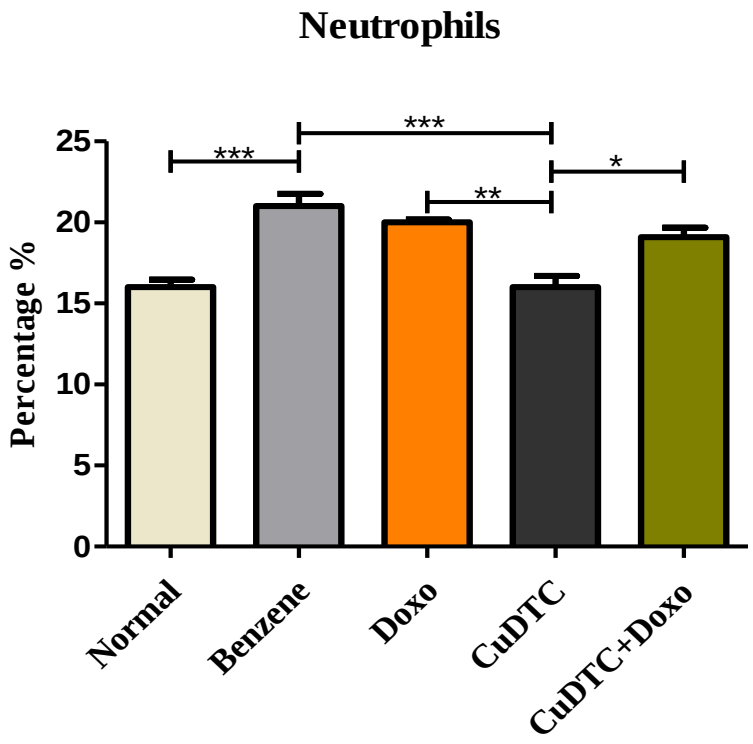

Figure 3.5: Comparison of Neutrophils count in different experimental groups

3.2.3 Lymphocytes

Suppression of lymphocyte count was observed in leukemic rats as compared to normal group indicates over proliferation of myeloid lineage in AML. Doxorubicin treated group 3 showed further decrease in lymphocyte count however, treatment with compound significantly restore back this aberrant reduction to normal and compound in conjunction with traditional therapy showed same results as compound alone. While CuDTC and CuDTC in conjunction with Doxo both showed significant improvement against Doxo and benzene treated groups, as in these groups lymphocyte count was nearly as in normal rats group.

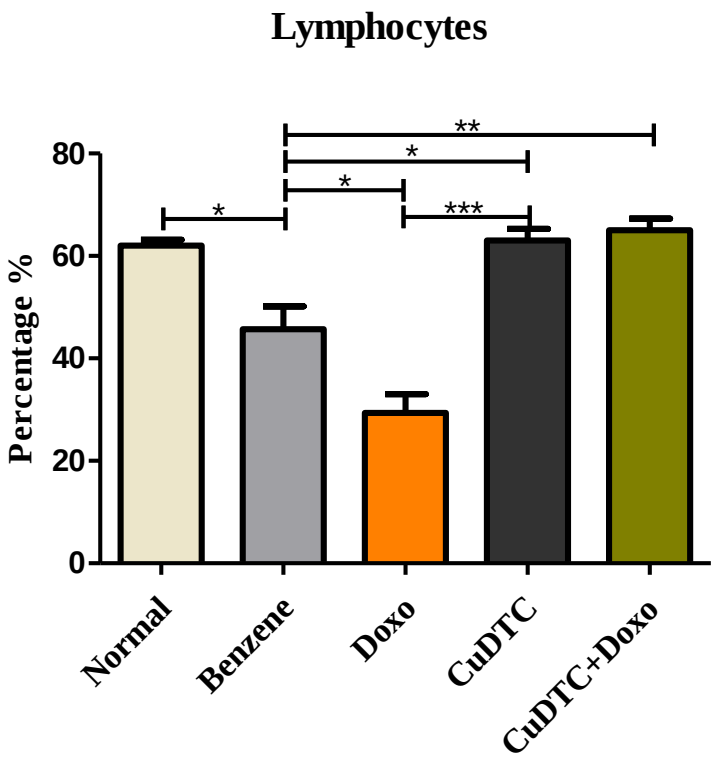

Figure 3.6: Comparison of Lymphocytes count in different experimental group

3.2.4 Red Blood Cells

Red blood cells count was significantly decreased in group 2 (leukemic rats). Upon treatment with Doxo in group 3, there was negligible increase in RBCs count. In group 4 treatment with CuDTC reverted back the level of RBCs however, in group 5 treatment with combination therapy CuDTC along with Doxo showed the highest possible improvement in restoring RBCs count to normal levels. As results shows, against RBCs count, combination therapy showed higher efficacy then CuDTC alone.

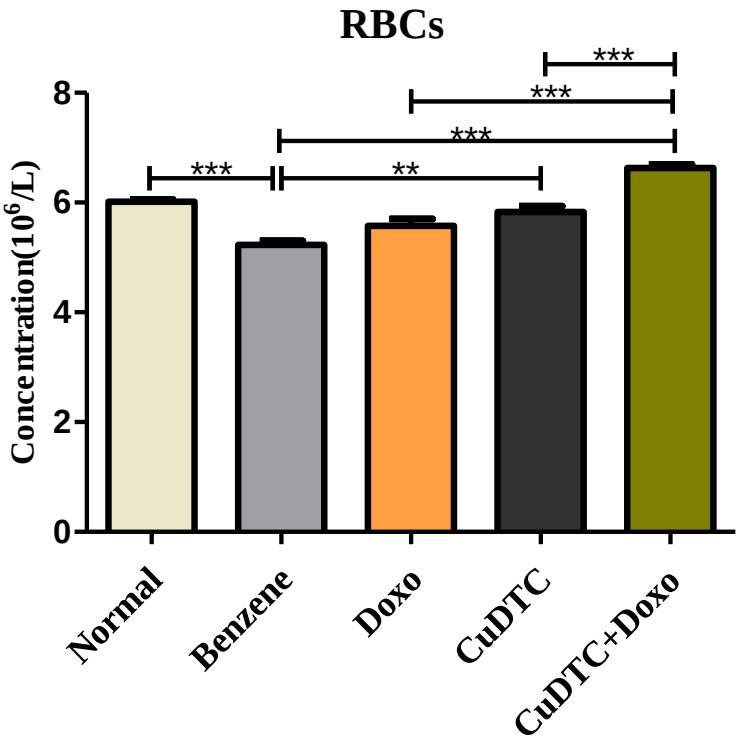

Figure 3.7: Comparison of RBCs counts in different experimental groups

3.2.5 Hemoglobin

There was significant reduction in hemoglobin levels observed in group 2 leukemic rats. Doxorubicin treatment in group 3 did not show increase in hemoglobin. While, CuDTC treatment in group 4 exhibited significant increase in hemoglobin as compared to Doxo and benzene however, combined treatment of CuDTC and Doxo in group 5 showed best possible results and restored back hemoglobin levels even greater than normal. Against hemoglobin levels, combination therapy showed excellent efficacy then CuDTC alone.

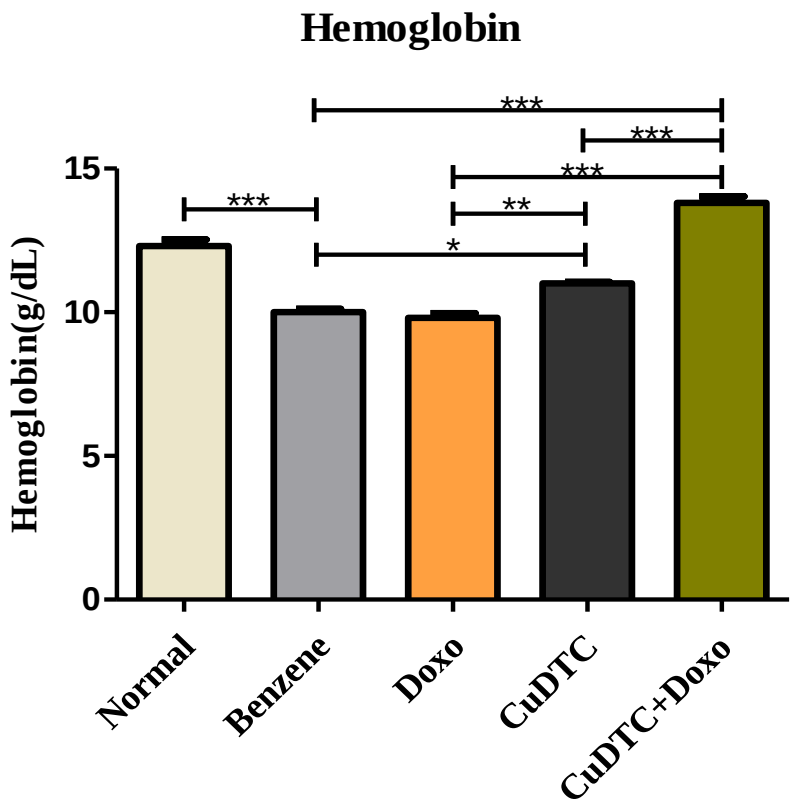

Figure 3.8: Comparison of Hemoglobin levels in different experimental groups

3.2.6 Platelets

As thrombocytopenia considered the characteristics feature of acute myeloid leukemia, complete blood picture showed reduction in number of platelets in group 2 benzene induced leukemic rats. Doxo treatment in group 3 also showed slight increase but still thrombocytopenic picture comparative to normal group 1. CuDTC treated group 4 showed increase in platelets count then benzene treated group. However, there was substantial rise observed in group 5, treated with coadministration of CuDTC and Doxo indicative of significant efficacy of combination therapy in increasing platelets to normal levels then CuDTC alone.

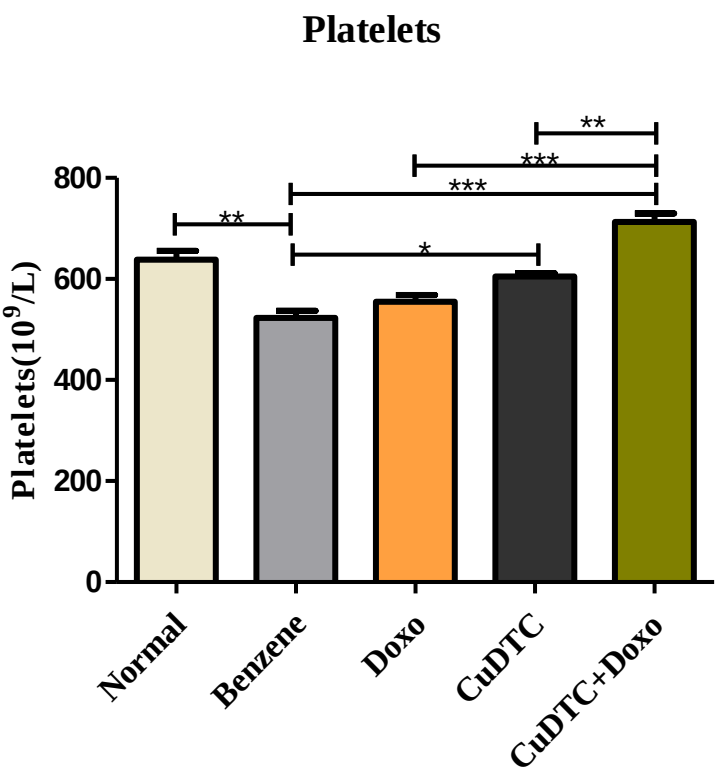

Figure 3.9: Comparison of Platelets count in different experimental groups

3.3 Microscopic Examination of Blood cells

Morphological analysis of blood cells of untreated, normal group showed normal morphology as shown in (Fig. a). Red blood cells had their intact biconcave shape with normochromic, normocytic morphology and normal count. White blood cells were well differentiated, presence of multilobed nuclei indicative of their normal structure and function. Nucleus to cytoplasm ratio was normal that usually lies between 1:4 to 1:6, with granular cytoplasm. Benzene treated group 2 showed presence of blast cells indicative of successful induction of leukemia in rats. Undifferentiated WBCs (blast cells) with greater nucleus to cytoplasm ratio 1:1 were observed as illustrated in (Fig. b) with abnormal function. Red blood cells count was reduced significantly in benzene treated group 2 with distortion of normal biconcave shape of RBCs. Anisocytosis (unequal size) and poikilocytosis (unequal shape) was observed in RBCs morphology indicative of anaemia as a consequence of leukemia. (Fig. c) showed doxorubicin treated group morphology, in which restoration of WBC morphology was observed with presence of stab shaped nuclei in cells with improved morphology of RBCs however, RBCs count showed slight increase as chemotherapy considered as the secondary cause of anaemia. In CuDTC treated group (Fig. d) normal blood cells morphology restoration was profoundly observed with normal count, size and shape of RBCs and presence of multilobed nuclei of WBCs (Neutrophils). Meanwhile, blood smear morphology from coadministration of CuDTC+Doxo was virtually similar to morphological picture of normal group (Fig. e). Displayed normochromic, normocytic RBCs picture with significant increase in count. Well differentiated, mature WBCs having multilobed nuclei, with normal nucleus to cytoplasm ratio was observed. In regaining of normal blood cells morphology, combination therapy showed best efficacy in comparison to Doxo and CuDTC treatment alone.

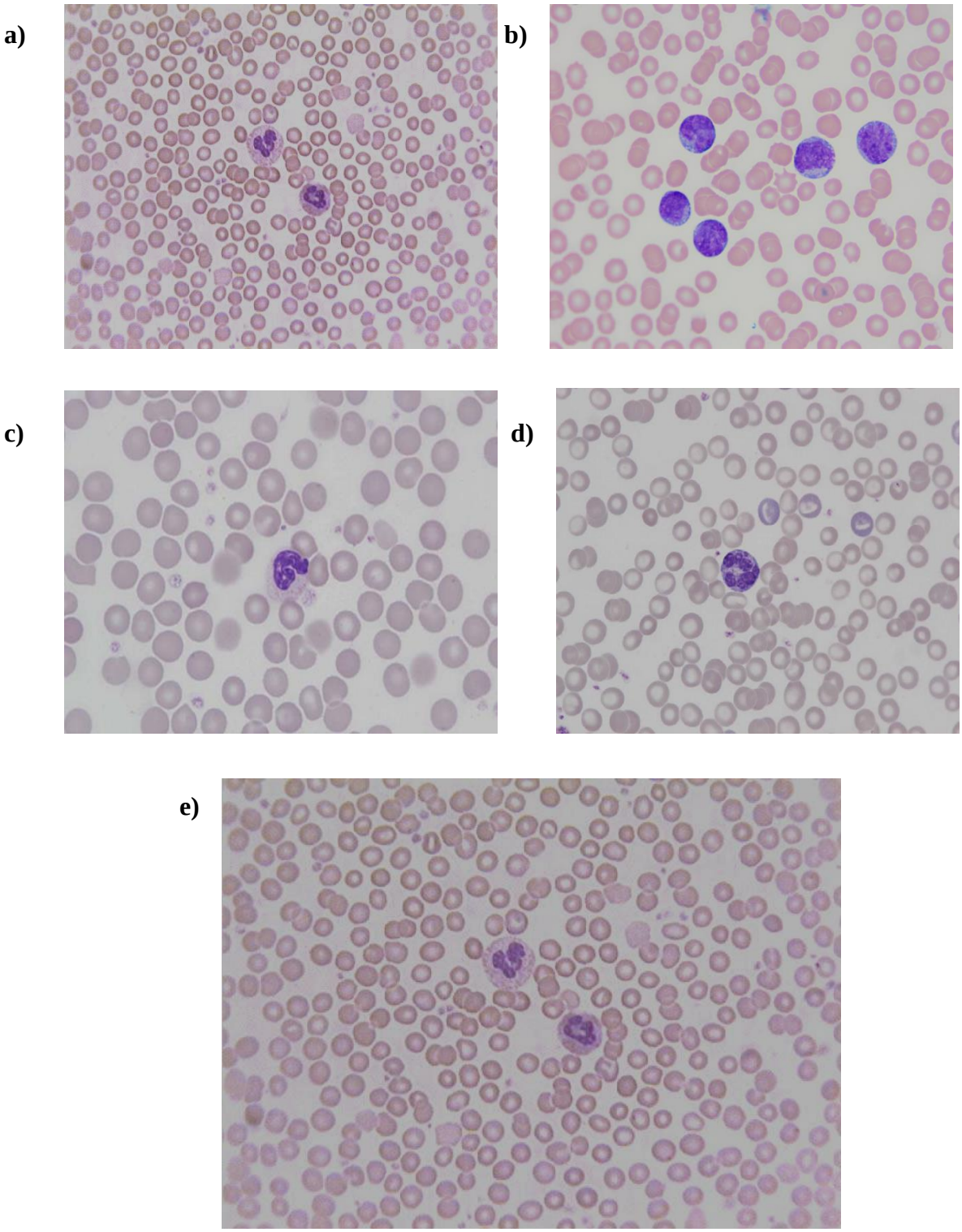

Figure 3.10: Microscopic Examination of blood cells morphology a) Normal, b) Benzene treated, c) Doxorubicin treated, d) CuDTC treated, e) CuDTC + Doxorubicin treated

3.4 Biochemical Evaluation

3.4.1 Estimation of Hepatic Enzymes

To check the effects of leukemia and different compound treated groups on liver enzymes, biochemical assessment was done which include commonly well-known markers ALT, ALP and AST.

a) Alanine Aminotransferase (ALT)

Normal or control group represented normal levels of ALT, while there was significant rise seen in benzene treated group 2 as compare to normal group. However, these abnormally elevated levels of ALT was substantially reduced by the treatment of doxorubicin which is known anticancer drug. Upon treatment with CuDTC being test compound further decreases ALT levels even lower than normal, against Benzene and Doxo groups. However, combination therapy treatment in group 5 did significant rise in ALT which were virtually identical to normal group indicating that coadministration of compound and conventional therapy showed best efficacy in sustainment of ALT levels to normal.

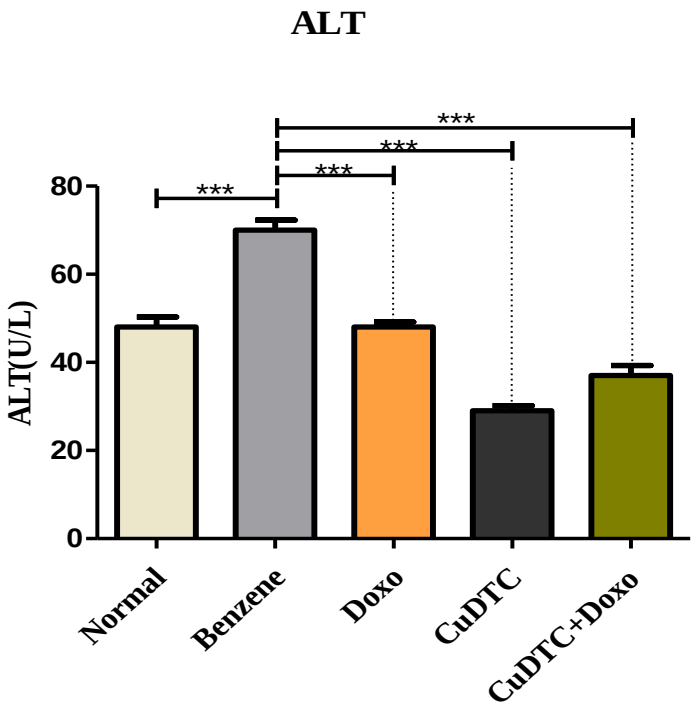

Figure 3.11: Comparison in Serum ALT levels in different study groups.

b) Alkaline Phosphatase (ALP)

It was observed that ALP levels significantly raised in group 2, benzene treated leukemic rats in comparison to normal or control group 1, indicative of high cell turn over or wear and tear as usually noticed in malignant disorders. Group 3 treated with Doxo even did not show significant reduction in ALP levels as anticancer therapy as an indicative of cytotoxicity associated with high levels of abnormal cell deaths leads to increased cell turn over in body. While treatment with both test compound CuDTC in group 4 and combination therapy in group 5, remarkably recovered back ALP levels towards normal showing good efficacy of both regimens against abnormal levels of ALP.

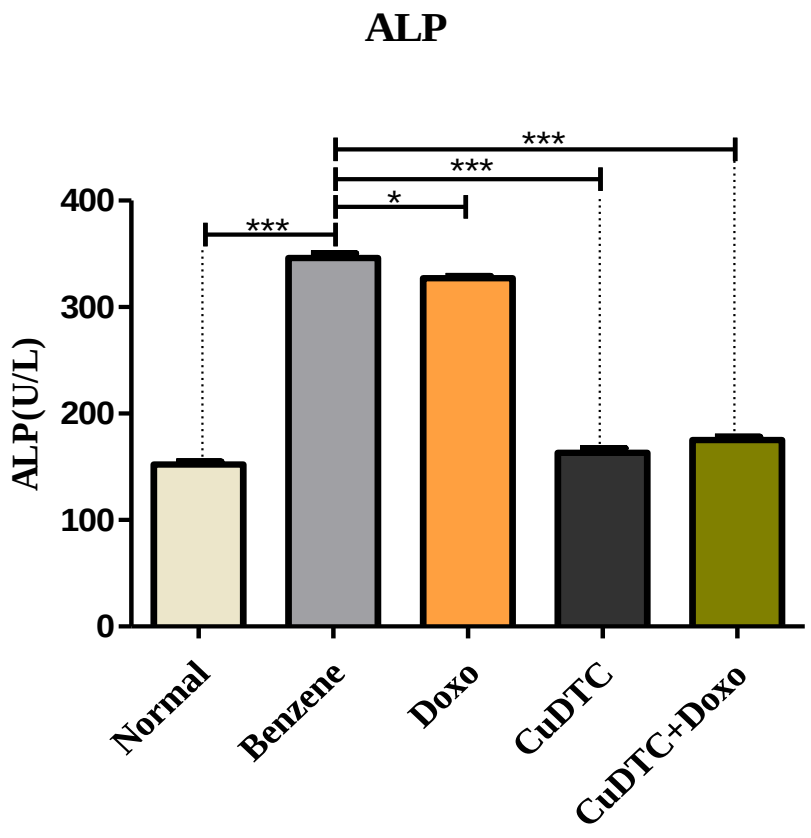

Figure 3.12: Comparison of Serum ALP levels in different study groups

c) Aspartate Aminotransferase (AST)

There was significant difference seen in normal and benzene treated group, as serum AST levels were much higher in benzene treated group against normal or control group. Doxorubicin treatment in group 3 showed same levels of AST as in benzene treated group 2. However, treatment with CuDTC in group 4 predominantly reduced high expression levels of AST in comparison to Benzene and Doxo treated groups. Likewise, combination therapy also showed significant reduction against both group 3 and 4, again indicating that in restoration of normal AST levels both CuDTC alone and in combination gave best possible efficacy which were almost same as in normal rat group 1.

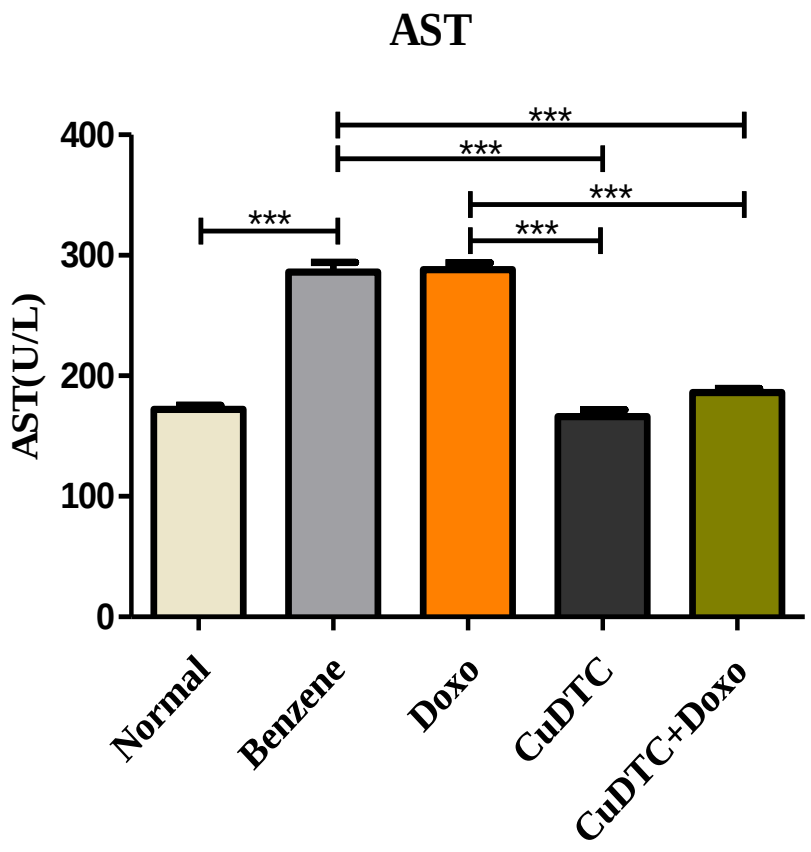

Figure 3.13: Comparison of Serum AST levels in different study groups

3.4.2 Estimation of Renal Biomarkers

a) Uric Acid

In serum uric acid levels rapid elevation was observed in benzene treated leukemic rats when compared to normal group as indicated by significance test ($p<0.05$). Upon treatment with Doxo in group 3 and CuDTC treatment in group 4 both showed noticeable reduction in serum uric acid levels against benzene group. However, combination therapy in group 5 presented remarkable recovery in uric acid levels which were nearly similar with normal group indicating better effectiveness of combination therapy against CuDTC and Doxo alone.

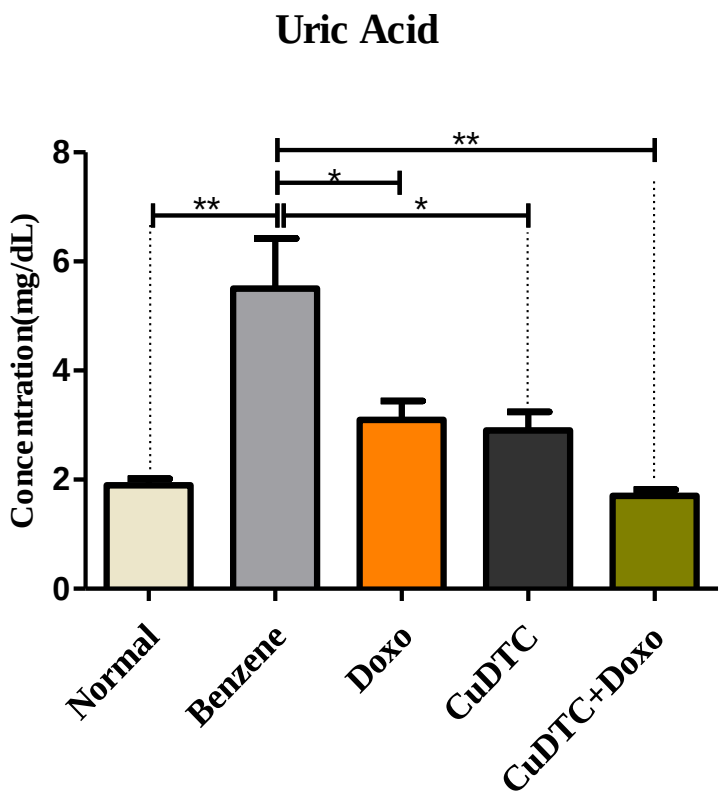

Figure 3.14: Serum Uric Acid levels in different experimental groups

3.4.3 Serum Lactate Dehydrogenate (LDH)

Serum LDH levels were significantly raised in benzene treated rats in comparative to normal or control group. However, it was observed that LDH levels drastically increased in group 2 treated with Doxo, whose possible justification is that as traditional chemotherapy associated with cytotoxicity and caused cell death by generating ROS production indicative of high cell turn over which in turn directly proportional to serum LDH levels. While CuDTC treatment in group 4 did significant reduction against benzene and Doxo groups, but coadministration of CuDTC and Doxo showed better reduction, showing identical results in both normal and combination treatment.

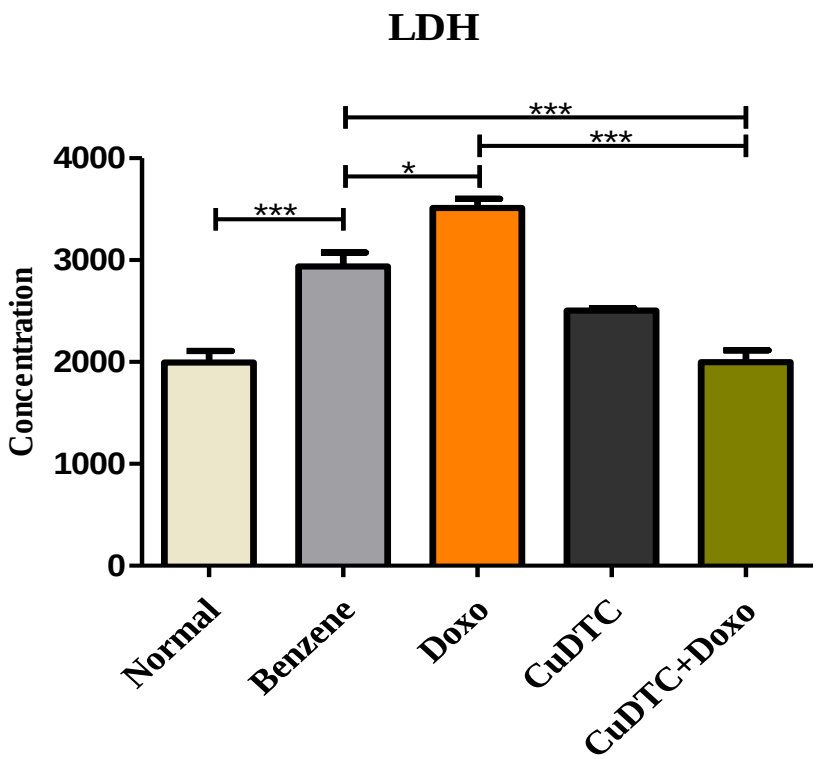

Figure 3.15: Serum LDH levels in different experimental groups

3.4.4 Serum Lipid Profile

a) Cholesterol Levels

Serum cholesterol levels were significantly raised in benzene treated group 2 when compared with normal or control group 1. Noticeable reduction in cholesterol levels observed in group 3 Doxo treated group in comparison to benzene treated group but there was no significant change seen in level of cholesterol in CuDTC treated group. However, combination treatment CuDTC along with Doxo in group 5 substantially reduced serum cholesterol levels towards normal and showed effective results against any other treated group.

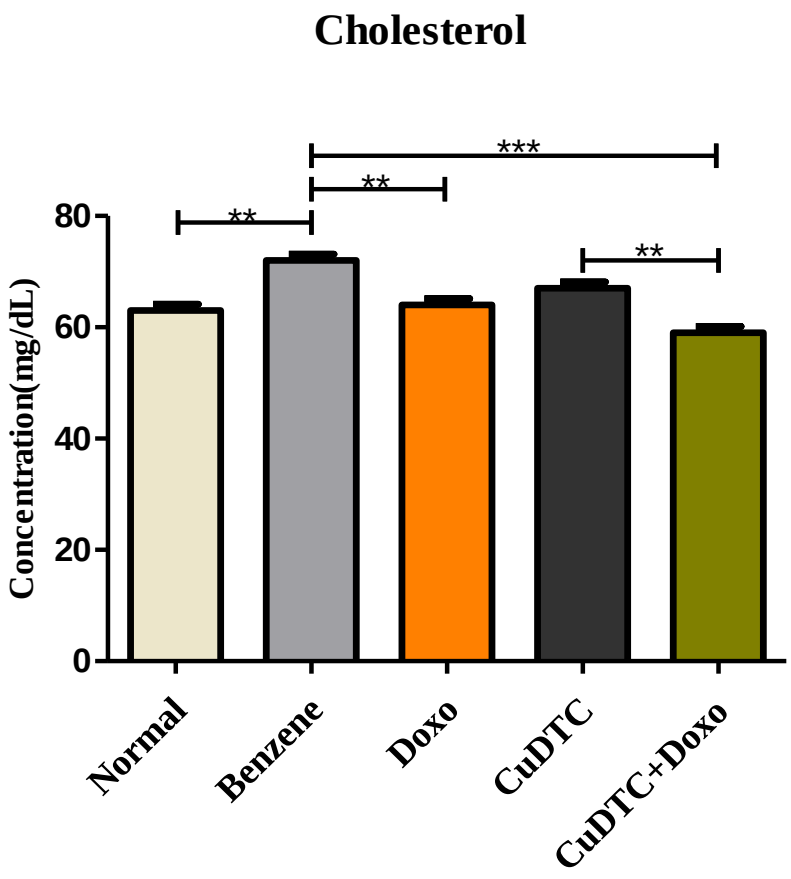

Figure 3.16: Serum Cholesterol levels in different experimental groups

3.5 Analysis of Relative Gene Expression of Biomarkers of Wnt Pathway

3.5.1 *AXIN*

First group showed relative expression levels of *AXIN* in normal group rats respectively. It was observed that relative expression of *AXIN* considerably reduced in group 2 benzene treated rats. Treatment with Doxo in group 3 even more reduced levels of *AXIN* however, in group 4 compound CuDTC treatment substantially restored *AXIN* expression which is more like as normal group rats. While combination treatment in group 5 also maintained normal relative gene expression of *AXIN* with comparatively benzene and Doxo group but slightly reduced then normal levels.

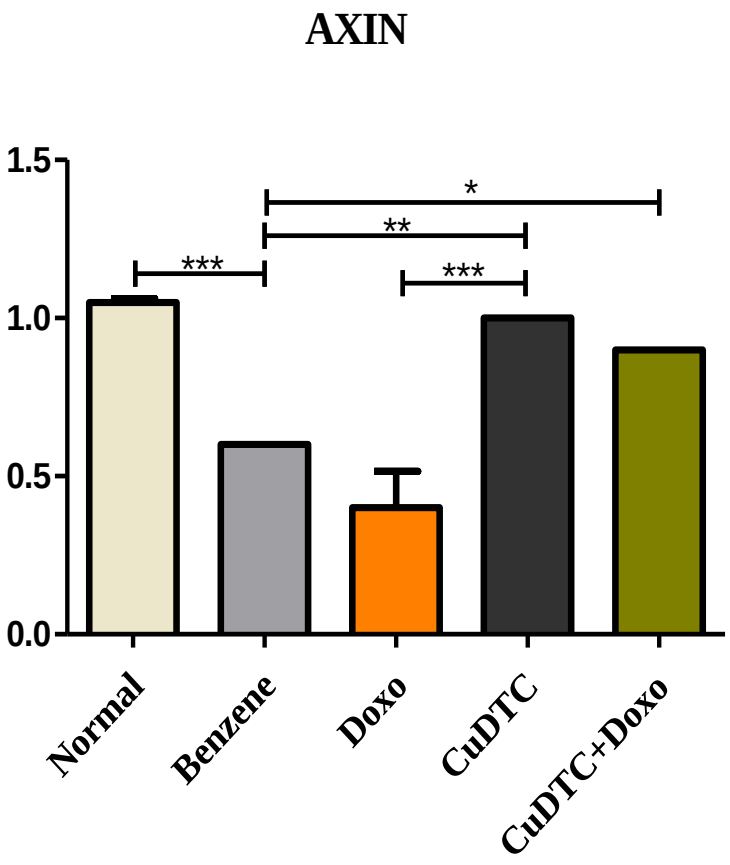

Figure 3.17: Comparison of change in relative expression of AXIN in different experimental groups

3.5.2 GSK3β

It was shown that GSK3β relative expression substantially reduced in benzene treated group 2 rats as compared to normal group rats. Doxorubicin treatment in group 3 showed further reduction in its levels. While treatment with CuDTC in group 3, relative expression of GSK3β considerably raised against Doxo group but there is no significance difference observed against benzene treated group. However, coadministration of compound CuDTC+Doxo showed best possible expression levels of GSK3β as likely to be normal group comparative to benzene, Doxo and CuDTC alone treated groups.

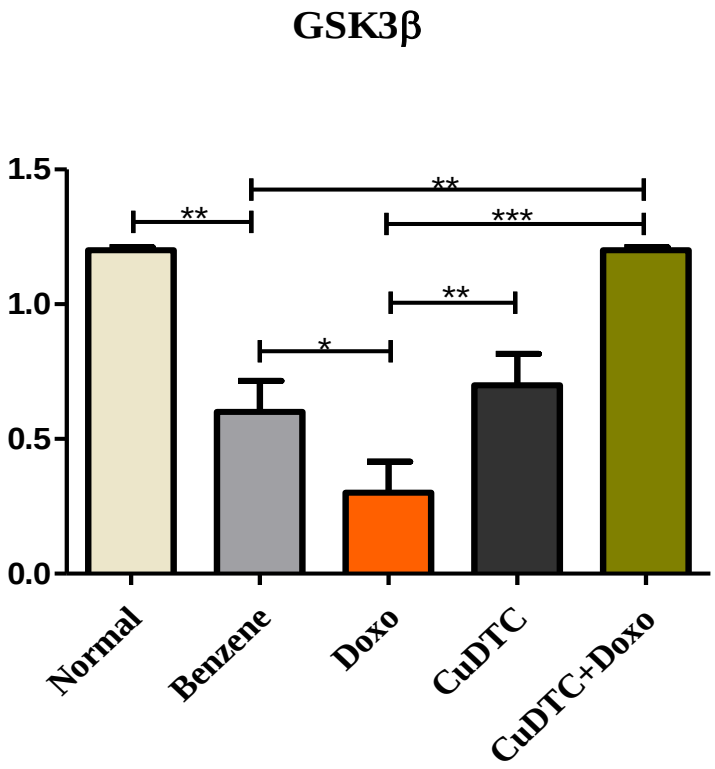

Figure 3.18: Comparison of change in relative expression of GSK3β in different experimental groups

3.5.3 *LEF*

Relative expression of *LEF* in benzene treated group 2 rats showed rapid reduction in comparison to normal group rats. Doxo treatment in group 3, leukemic rats showed marked reduction in expression levels of *LEF* even against benzene treated group. However, compound CuDTC treatment in group 4, restored back expression levels of *LEF* showing best efficacy among all other treated groups. In group 5, combination treatment did not show any therapeutic significance against other groups.

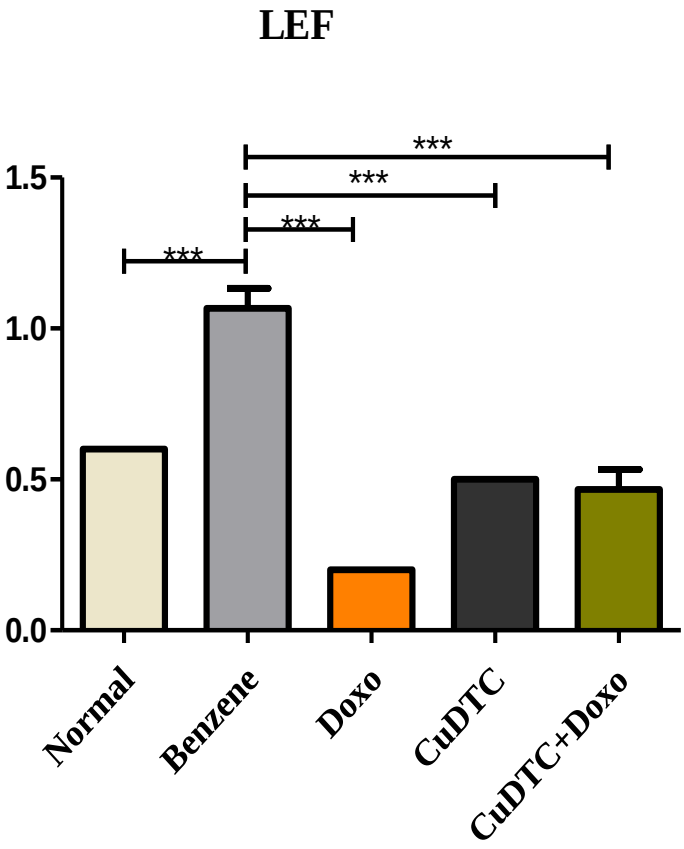

Figure 3.19: Comparison of change in relative expression of LEF in different experimental groups

3.6 Analysis of Relative Gene Expression of Biomarkers of HIF-1α Pathway

3.6.1 HIF-1α

HIF-1α is a crucial biomarker of HIF-1α signaling pathway. Relative expression of HIF-1 was significantly reduced in benzene treated group 2 rats as compared to normal group rats. This expression was increased in group 3 Doxo treated rats. In group 4, treatment with test compound CuDTC further showed considerable elevation in relative expression of HIF-1 levels which is likely to be similar as in normal group rats and significant as compared to both benzene and Doxo treated group. However, in group 5 combination treatment showed dramatic reduction in HIF-1 expression levels.

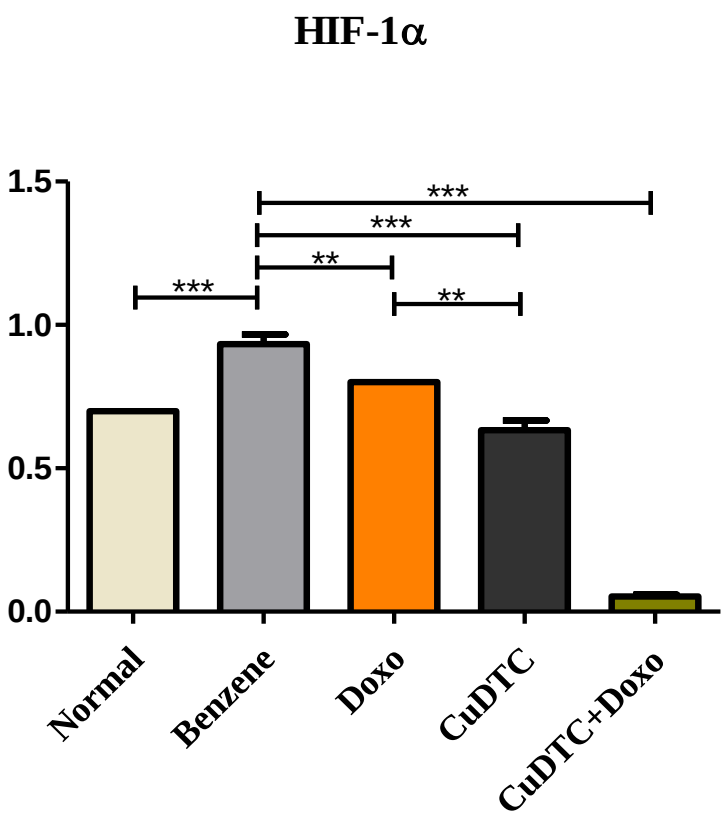

Figure 3.20: Comparison of change in relative expression of HIF-1α in different experimental groups

3.6.2 HSP90

Relative expression levels of HSP90 significantly raised in group 2, benzene treated rats as compared to normal group 1, as HSP90 is an independent inducer of HIF-1α levels in cancer cells. Doxo treated group 3 showed substantial decrease even more than normal group rats. In group 4, compound CuDTC treatment cause significant elevation in HSP90 expression (as compared to abnormally decreased levels in Doxo) which is likely to be similar in normal group rats showing good efficacy as compared to all other treated groups. Co-administration of compound and conventional therapy showed considerable rise in relative expression of HSP90 levels.

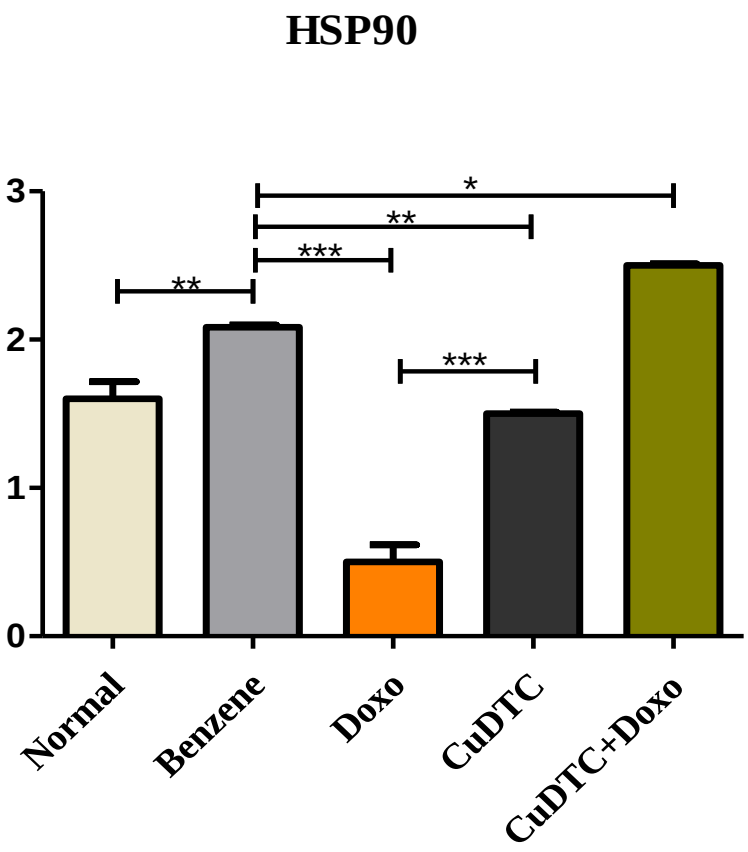

Figure 3.21: Comparison of change in relative expression of HSP90 in different experimental groups

3.6.3 *PTEN*

Relative expression levels of *PTEN* (tumor suppressor gene) significantly reduced in group 2, benzene treated rats as compared to normal group 1. Doxo treated group 3 showed substantial rise in relative expression of *PTEN*. In group 4, compound CuDTC treatment cause significant elevation in *PTEN* expression even more than normal group rats. Co-administration of compound and conventional therapy showed considerable reduction in relative expression of *PTEN* levels even lower than normal group. Combination therapy showed good efficacy against expression of *PTEN* levels.

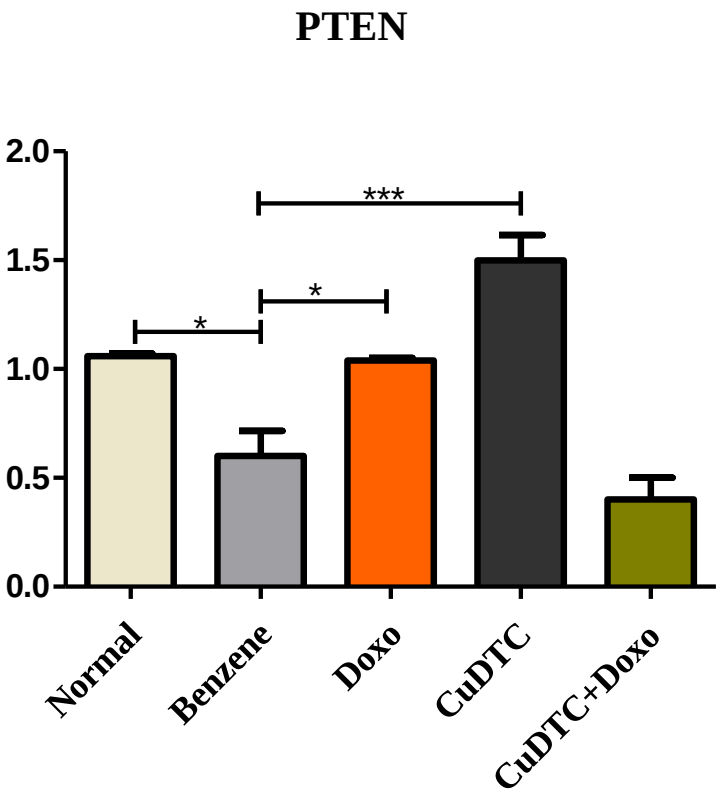

Figure 3.22: Comparison of change in relative expression of PTEN in different experimental groups

4. Discussion

Conventional chemotherapies have currently been demonstrated to be ineffective against cancer due to multidrug resistance and a wide range of negative side effects including nausea, vomiting, fatigue, hair loss, loss of appetite, mouth sores, and increased risk of infection, that severely impair one's quality of life. Despite advancements in therapy high global mortality and morbidity rates of cancer still poses a severe threat. New therapeutic approaches with more efficacy and fewer side effects are needed to fight the growing challenges in the treatment of leukemia (Murray & Dyson, 2020). Organometallic compounds are becoming more and more significant in this field because of their distinctive biological and physio-chemical characteristics, which can be reinforced when combined with other organic molecules to create innovative therapeutics against cancer (Ong & Gasser, 2020).

Organometallic compounds are being developed as potential anti-cancer drugs due to their unique chemical structures, which allow them to interact with multiple targets in the body and inhibit cancer cell growth. Additionally, they may interact with proteins and enzymes involved in signaling pathways and interfere with their function. These have been shown to induce apoptosis, or programmed cell death, and inhibit the proliferation of cancer cells in vitro. They have also been tested in animal models with promising results. While organometallic compounds are still in the early stages of development, they may hold potential for the treatment of cancer in the future (Chavain & Biot, 2010).

This study was designed to investigate antileukemic activity of Copper dithiocarbamate and its potent efficacy in combination with conventional chemotherapy, which is doxorubicin in this case (Tisato *et al*., 2010) as acute myeloid leukemia positioned among most common kinds of cancer having very short survival rate (Kirtonia *et al*., 2020). AML was induced experimentally in rats by intravenously administration of benzene for 10 days, and than substantiated by blood cell counts and their morphological analysis. To see either benzene effectively induce leukemia or not, blood cell count parameters of benzene treated leukemic rats were compared with the untreated control rat’s blood cell parameters. In benzene-induced leukemic rats, there was significant increase in blast cells and distorted red blood cells morphology was observed after standard differential Giemsa staining of blood smear slides.

The total count of white blood cells in group 2 leukemic rats were significantly elevated indicating abnormal functioning of bone marrow in which mutation was caused after benzene administration as benzene is best known for its toxicity for HSCs (haematopoietic stem cells). Because of mutation that impairs bone marrow normal function leading to aberrant explosive production of myeloid lineage cells that were immature, undifferentiated characterized by increased nucleus to cytoplasm ratio and functionally abnormal, clearly indicating induction of acute myeloid leukemia by benzene administration. Doxorubicin treatment in group 3 leukemic rats, showed reduction in levels of WBCs counts but not much significant against benzene and normal groups. While CuDTC treatment in group 4 rats did dramatic reduction in WBCs levels which is even more than normal group levels. However, coadministration of compound and Doxo in group 5 showed best possible results in which WBCs counts were restored back to normal levels as in control group rats indicating that compound and Doxo performed synergistic action on WBCs counts restoring them back to normal levels.

As, a large body of literature exists on characteristic diagnosis of different types of leukemias. Due to uncontrollable proliferation of myeloid lineage bone marrow becomes infiltrated leading to reduction in other cell lineages including RBCs, Platelets and lymphoid cells (D'Andrea & Reddy, 2014). Distorted RBCs morphology with presence of anisocytosis (unequal size) and poikilocytosis (unequal shape) seems to be characteristic feature of Leukemia. Severe anaemia and thrombocytopenia is associated with consequence of Acute myeloid leukemia. RBC counts, hemoglobin and platelet counts were significantly reduced in group 2 benzene treated rats indicating presence of anaemia and thrombocytopenia as a consequence of leukemia. In group 3, treatment with standard chemotherapy doxorubicin did not show considerable elevation in levels of these three parameters which stems the fact that side effects of chemotherapy include anaemia and thrombocytopenia. Compound CuDTC treatment in group 4 showed significant improvements in RBC, haemoglobin and platelet levels however, combination treatment produced a more improved and substantial response in improving overall haematological profile indicating best possible efficacy and synergistic effect of both treatments. In the benzene-induced leukemic rats, neutrophils levels increased, indicating AML with reduction in lymphocyte count indicating reduced production of lymphoid lineage due to impaired bone marrow functioning. During diagnosis, higher lymphocyte count is associated with shorter remission and survival rate (Park et al.,

2018). Doxorubicin treatment in group 3 cause slight reduction in neutrophils while slight elevation in lymphocytes count. As it was associated with severe side effects and cytotoxicity which was recovered by CuDTC treated group 4 and combined therapy treated group 5 in which neutrophil and lymphocyte counts nearly becomes normal indicating good efficacy of both regimens for improving haematological parameters against leukemic pathogenicity.

Hepatic biochemical markers analysis in serum of leukemic rats group 2 revealed significant rise in ALT, AST, and ALP levels, consistent with previous researches (Jumaah *et al*., 2021) as infiltration of reticuloendothelial system organs in the course of leukemias leads to obstruction of these organs consequently results in leakage of different metabolic enzymes into blood stream (Akinlolu *et al*., 2018). CuDTC treatment in group 4 and combination therapy in group 5, on the other hand, exhibited a remarkable decrease in these hepatic markers, however group of rats treated with combined therapy recovered more significantly. Levels of transaminases rise due to leukemic infiltration, indicating liver damage, Jaundice and cholestasis may also cause a disruption in hepatic profile in the leukemic group (Akinlolu *et al*., 2018).

LDH levels were substantially higher in Group 2 benzene treated leukemic rats as compared to untreated (control) group 1. Elevated LDH levels are associated with high cell turnover, leukemic cell growth, increased cell metabolism and as well as activation of hypoxia in evasion of cell death (apoptosis) (Transfus *et al*., 2017). Increased LDH levels also directly correlates with high rate of anaerobic glycolysis as name indicates it is independent of oxygen supply, known as Warburg effect (Vaupel et al., 2019). In group 3 treatment with doxorubicin revealed significant rise in LDH levels indicative of high cell turn over as doxorubicin is a prooxidant and cause death of cells by ROS generation. However, compound treatment in group 4 did significant reduction in LDH levels but combination therapy in group 5 revealed best possible results likely to be similar as in normal group rats.

In order to achieve chronic proliferation and drug resistance all leukemic cells require reprogramming of their cholesterol metabolism. They require cholesterol to meet their demand for rapid proliferation, thus they increase its synthesis and absorption from GIT (Zhao *et al*., 2019). In leukemic rats (Group 2) cholesterol levels were considerably higher but significantly lower in Groups 3 treated with Doxorubicin. CuDTC administration in

group 4 did not show significant recovery, however in group 5 combination treatment recovers more significantly as did normal levels in control rats respectively. Since tumor load promotes cell lysis and purines are catabolized into uric acid, an elevated blood uric acid level in group 2 leukemic rats shows aggressiveness and cellular turnover of leukemic cells (Rasool et al., 2015), this rise was substantially reduced in group 3,4 and 5 treated with Doxo, CuDTC and CuDTC+Doxo respectively.

When there is no canonical wnt signaling, β-catenin master protein of the pathway, is followed by formation of complex with *AXIN* and *APC* and is phosphorylated at amino terminal commonly known as degradation box by glycogen synthase kinase 3β and casein kinase 1α that tagged it for degradation which is mediated by proteasome formation after polyubiquitination of β-catenin.

AXIN, *GSK3β* are the major intracellular inhibitors of the wnt pathway as they form complex with β-catenin leading to its degradation. They negatively regulate wnt pathway and maintains proper balance of wnt signaling, thus ensuring normal development. Any genetic alteration or mutation in these regulators may take part in cancer stem cell production, showing evidence of aberrant wnt signaling in several malignant disorders especially in leukemia's. There was significant reduction observed in relative expression levels of *AXIN* and *GSK3β* in benzene treated leukemic group than normal group as indicative of increased activation of Wnt pathway in leukemic cells. While compound treatment in group 3 significantly raised expression of *AXIN* recovering back to normal levels as compared to combination treatment in group 5 which did reduction but comparatively lower than normal indicating better efficacy of CuDTC alone treatment in regulating relative expression of *AXIN* gene. Unlikely *AXIN*, restoration back of normal expression by *GSK3β* was achieved by combination therapy in group 5 which were nearly as in normal rats group. CuDTC alone treatment also showed significant elevation but less in comparison to combined therapy.

Higher relative expression of *LEF* (leukocyte enhancer factor) is directly associated with aberrant activation of Wnt signaling pathway and transcription of proliferative and growth beneficiary genes. Which gave growth advantage to tumor cells and help in maintaining self-renewal capacity of cancer stem cells in the heart of tumor. Expression of *LEF* was considerably elevated in group 2 benzene treated rats again indicative of increased

translocation of β-catenin into nucleus and transcription of downstream effector genes. Doxorubicin in group 3 showed significant reduction even more than normal. While both CuDTC and combination treatment showed good efficacy in maintaining normal base line expression levels of *LEF* in group 4 and 5 respectively.

The HIF-1α pathway is a complex network of molecular interactions that primarily involved in hypoxic (low oxygen) conditions providing cells with an adaptive mechanism in hypoxic environment. To thrive in low oxygen conditions it induces the transcription of genes that help cells to survive. Dysregulation of the pathway has been implicated in the development of cancer, cardiovascular diseases, and other diseases (Isaacs *et al*., 2002). HIF-1α expression in group 2 benzene treated leukemic rats was significantly raised indicating aberrant activation of HIF pathway. Treatment with traditional therapy in group 3 did reduction in expression levels but not as CuDTC alone did. CuDTC showed considerable recovery of HIF-1α relative expression levels as nearly as normal expression in control group but combination did not show significant improvement as levels were reduced too much indicating abnormal decrease in levels.

Relative expression of Hsp90 showed marked elevation in leukemic rats (group 2) as one of the oncogenic regulator of HIF-1α is Hsp90 that may increase or elevate HIF-1α, which is independent of oxygen levels of the cells. It was suggested that binding of Hsp90 with HIF-1α induces changes in its structure causing its dimerization with HIF-1β triggering stabilization and transactivation of HIF-1α (Isaacs *et al*., 2002; Gradin *et al*., 1996). CuDTC treatment substantially reduce Hsp90 expressions to normal in group 4 but combination therapy showed dramatic elevation even more then control group rats.

PI3K pathway directly up-regulate HIF-1α levels (Semenza, 2002). This activation can be reversed by a tumor suppressor gene called *PTEN* which cause phosphorylation of PI3K and downstream inhibition of HIF-1 (Gingras *et al*., 2001). Significant reduction in *PTEN* (tumor suppressor gene) expression was observed in benzene treated leukemic rats group 2. CuDTC administration in group 4 showed remarkable rise in *PTEN* levels indicating *PTEN* dependent phosphorylation of PI3K and downstream inhibition of HIF-1α pathway.

Conclusion

The study presented herein has concluded that copper dithiocarbamate as novel derivative of dithiocarbamate has sufficient anti-leukemic potential as it minimizes the deleterious effects of AML in rat model (induced by benzene administration) based on the morphological, biochemical evaluation and relative gene expression analysis using qPCR in this study. Both compound and combination therapy showed good efficacy against traditional therapy in improving haematological profile, enzymatic parameters and relative expression of biomarkers of Wnt signaling pathway. However, combination therapy appears ineffective when using HIF -1α pathway biomarkers to examine relative gene expression. It is suggested that to assess the efficacy of combination therapy against biomarkers of HIF-1α pathway more research is needed to unmask underlying molecular mechanisms.

1. References

Acebron, S. P., Karaulanov, E., Berger, B. S., Huang, Y. L., & Niehrs, C. (2014). Mitotic wnt signaling promotes protein stabilization and regulates cell size. *Molecular Cell*, *54*(4), 663-674.

Adeyemi, J. O., & Onwudiwe, D. C. (2020). The mechanisms of action involving dithiocarbamate complexes in biological systems. *Inorganica Chimica Acta*, *511*, 119809.

Ajibade, P. A., Fatokun, A. A., & Andrew, F. P. (2020). Synthesis, characterization and anti-cancer studies of Mn (II), Cu (II), Zn (II) and Pt (II) dithiocarbamate complexes-crystal structures of the Cu (II) and Pt (II) complexes. *Inorganica Chimica Acta*, *504*, 119431.

Akinlolu, A. A., Salau, B. A., Odewabi, A., Biliaminu, S. A., & Abdulazeez, I. M. (2018). Plasma hepatic enzymes as biopredictors of type, metastasis, and prognostication of hematological malignancies. *Journal Of Medical Sciences*, 38(1), 24.

Albanese, A., Daly, L.A., Mennerich, D., Kietzmann, T., Sée, V. (2021**)**. The Role of Hypoxia-Inducible Factor Post-Translational Modifications in Regulating Its Localisation, Stability, and Activity. *Int. J. Mol. Sci*, *22*, 268.

Alcalay, M., Orleth, A., Sebastiani, C., Meani, N., Chiaradonna, F., Casciari, C., ... & Pelicci, P. G. (2001). Common themes in the pathogenesis of acute myeloid leukemia. *Oncogene*, *20*(40), 5680-5694.

Alvarez-Ordóñez, A., & Prieto, M. (2012). Technical and methodological aspects of fourier transform infrared spectroscopy in food microbiology research. *Fourier Transform Infrared Spectroscopy In Food Microbiology* (pp. 1-18). Springer, Boston, MA.

As Insecticide, B. C. (2012). The Effectiveness of Organotin (IV) Benzylisopropyldithiocarbamate Compounds as Insecticide Against Aedes Aegypti Linn (Diptera: Culicidae) in Laboratory. *American Journal Of Applied Sciences*, *9*(8), 1214-1218.

Awang, N., Baba, I., Yamin, B. M., Othman, M. S., & Kamaludin, N. F. (2011). Synthesis, characterization and biological activities of organotin (IV) methoxyethyldithiocarbamate complexes.

Bae, M. K., Ahn, M. Y., Jeong, J. W., Bae, M. H., Lee, Y. M., Bae, S. K., ... & Kim, K. W. (2002). Jab1 interacts directly with HIF-1α and regulates its stability. *Journal Of Biological Chemistry*, *277*(1), 9-12.

Balamurugan, K. (2016). HIF-1 at the crossroads of hypoxia, inflammation, and cancer. *International Journal Of Cancer*, *138*(5), 1058-1066.

Bennett, J. M., Catovsky, D., Daniel, M. -T, Flandrin, G., Galton, D. A. G., Gralnick, H. R., & Sultan, C. (1976). Proposals for the Classification of the Acute Leukaemias French-American-British (FAB) Co-operative Group. British Journal of Haematology, 33(4), 451–458.

Berthomieu, C., & Hienerwadel, R. (2009). Fourier transform infrared (FTIR) spectroscopy. *Photosynthesis Research*, 101(2–3), 157–170.

Bette Meek, M. E., & Klaunig, J. E. (2010). Proposed mode of action of benzeneinduced leukemia: Interpreting available data and identifying critical data gaps for risk assessment. Chemico-Biological Interactions, 184(1–2), 279–285.

Bowen, D. T. (2006). Etiology of Acute Myeloid Leukemia in the Elderly. Seminars in Hematology, 43(2), 82–88.

Bukhtoyarov, O. V., & Samarin, D. M. (2015). Pathogenesis of cancer: Cancer reparative trap. *Journal Of Cancer Therapy*, *6*(05), 399.

Cao, R., Pérez, L. G., Moya, R., & Díaz, A. (2000). Interaction of Copper (II) Dithiocarbamates with Hydrogen Peroxide. *Revista De La Sociedad Química De México*, *44*(2), 158-162.

Cattaruzza, L., Fregona, D., Mongiat, M., Ronconi, L., Fassina, A., Colombatti, A., & Aldinucci, D. (2011). Antitumor activity of gold (III)-dithiocarbamato derivatives on prostate cancer cells and xenografts. *International Journal Of Cancer*, *128*(1), 206-215.

Chan, K. M., Raja, N. F., & Hami, A. (2016). Synthesis, characterization and antitumor activity of new organotin (IV) methoxyethyldithiocarbamate complexes.

Chan, S.M., Majeti., R. (2013). Role of DNMT3A, TET2, and IDH1/2 mutations in pre-leukemic stem cells in acute myeloid leukemia. *Int J Hematol* 98, 648–657.

Chauhan, A., & Chauhan, P. (2014). Powder XRD technique and its applications in science and technology. *J Anal Bioanal Tech*, *5*(5), 1-5.

Chavain, N., & Biot, C. (2010). Organometallic complexes: new tools for chemotherapy. *Current Medicinal Chemistry*, *17*(25), 2729-2745.

Chereda, B., & Melo, J. V. (2016). The biology and pathogenesis of chronic myeloid leukemia. *Chronic Myeloid Leukemia* (pp. 17-39). Springer, Cham.

Clark, W. H. (1991). Tumour progression and the nature of cancer. *British Journal Of Cancer*, *64*(4), 631-644.

Clarke, M. J., & Sadler, P. J. (Eds.). (2012). *Metallopharmaceuticals II: Diagnosis And Therapy (Vol. 2)*. Springer Science & Business Media.

Conrad, P. W., Freeman, T. L., Beitner-Johnson, D., & Millhorn, D. E. (1999). EPAS1 trans-activation during hypoxia requires p42/p44 MAPK. *Journal Of Biological Chemistry*, *274*(47), 33709-33713.

Cvek, B., & Dvorak, Z. (2007). Targeting of Nuclear Factor-κB and Proteasome by Dithiocarbamate Complexes with Metals. Current Pharmaceutical Design, 13(30), 3155–3167.

De Keersmaecker, K., Marynen, P., & Cools, J. (2005). Genetic insights in the pathogenesis of T-cell acute lymphoblastic leukemia. *Haematologica*, *90*(8), 1116-1127.

Delaunay, J., Mazur, G., Minden, M., Wierzbowska, A., Jones, M. M., Berrak, E., & Kantarjian, H. M. (2014). Relationship between baseline white blood cell count and renal and hepatic function in older patients with acute myeloid leukemia. Leukemia Research Reports, 3(1), 17–20.

Delgado, J., Nadeu, F., Colomer, D., & Campo, E. (2020). Chronic lymphocytic leukemia: From molecular pathogenesis to novel therapeutic strategies. *Haematologica*, *105*(9), 2205.

Delore, P., and Borgomano, C. (1928). Leucemie aigue au cours de l'intoxication benzenique. Sur l'origine toxique de certaines leucemies aigues et leurs relations avec les anemies graves. *J. Med. Lyon*, 9, 227-233.

derivative; however, there was a lack of correlation between ROS generation and antiproliferative activity. *Molecules*, *21*(8), 1088.

Diethyldithiocarbamate and Its Assessment for Cancer Therapy. *Pharmaceutics*, *14*(3), 640.

Duchartre, Y., Kim, Y.M., Kahn, M. (2015). The Wnt signaling pathway in cancer. *Crit Rev Oncol Hematol*, 99, 141-9.

Erdogan, B., & Webb, D. J. (2017). Cancer-associated fibroblasts modulate growth factor signaling and extracellular matrix remodeling to regulate tumor metastasis. *Biochemical Society Transactions*, *45*(1), 229-236.

Fouad, Y. A., & Aanei, C. (2017). Revisiting the hallmarks of cancer. *American Journal of Cancer Research*, *7*(5), 1016.

Futreal, P. A., Coin, L., Marshall, M., Down, T., Hubbard, T., Wooster, R., ... & Stratton, M. R. (2004). A census of human cancer genes. *Nature Reviews Cancer*, *4*(3), 177-183.

Galton, D. A. G. (1966). The pathogenesis of chronic lymphocytic leukemia. *Canadian Medical Association Journal*, *94*(19), 1005.

Gargalionis, A. N., Papavassiliou, K. A., & Papavassiliou, A. G. (2022). Mechanobiology of solid tumors. *Biochimica Et Biophysica Acta (BBA)-Molecular Basis Of Disease*, 166-555.

Georgina, N., Masoud, Wei. Li. (2015). HIF-1α pathway: role, regulation and intervention for cancer therapy. *Acta Pharmaceutica Sinica B*, 5 (5), 378-389.

Gewirtz, D. (1999). A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochemical Pharmacology*, *57*(7), 727-741.

Gingras, A. C., Raught, B., & Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. *Genes & Development*, *15*(7), 807-826.

Goldberg, M. A., Dunning, S. P., & Bunn, H. F. (1988). Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science*, *242*(4884), 1412-1415.

Goldstein, B. D. (1977). Benzene toxicity: a critical evaluation: hematotoxicity in humans. *Journal Of Toxicology And Environmental Health. Supplement*, *2*, 69-105.

Gradin, K., McGuire, J., Wenger, R. H., Kvietikova, I., Fhitelaw, M. L., Toftgård, R. U. N. E., ... & Poellinger, L. (1996). Functional interference between hypoxia and dioxin signal

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646-674.

Hayat, R., Manzoor, M., & Hussain, A. (2022). Wnt signaling pathway: A comprehensive review. *Cell biology international*, *46*(6), 863-877.

Hirschey, M. D., DeBerardinis, R. J., Diehl, A. M. E., Drew, J. E., Frezza, C., Green, M. F., ... & Team, T. V. (2015). Dysregulated metabolism contributes to oncogenesis. *Seminars In Cancer Biology*, 35, 129-150.

Hochman, M. J., Yu, Y., Wolf, S. P., Samsa, G. P., Kamal, A. H., & LeBlanc, T. W. (2018). Comparing the palliative care needs of patients with hematologic and solid malignancies. *Journal Of Pain And Symptom Management*, *55*(1), 82-88.

Hogarth, G. (2012). Metal-dithiocarbamate complexes: chemistry and biological activity. *Mini Reviews In Medicinal Chemistry*, *12*(12), 1202-1215.

Hogarth, G., & Onwudiwe, D. C. (2021). Copper Dithiocarbamates: Coordination Chemistry and Applications in Materials Science, Biosciences and Beyond. *Inorganics*, *9*(9), 70.

Hrestha, B., Pokhrel, A.R., Darsandhari, S., Parajuli, P., Sohng, J.K., Pandey, R.P. (2019). Engineering *Streptomyces peucetius* for Doxorubicin and Daunorubicin Biosynthesis. In: Arora, D., Sharma, C., Jaglan, S., Lichtfouse, E. (eds) Pharmaceuticals from Microbes. *Environmental Chemistry For A Sustainable World*, vol 26. Springer, Cham.

Isaacs, J. S., Jung, Y. J., Mimnaugh, E. G., Martinez, A., Cuttitta, F., & Neckers, L. M. (2002). Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1α-degradative pathway. *Journal Of Biological Chemistry*, *277*(33), 29936-29944.

Ismail, A. A., van de Voort, F. R., & Sedman, J. (1997). Fourier transform infrared spectroscopy: principles and applications. *Techniques And Instrumentation In Analytical Chemistry*, 18, 93-139. Elsevier.

Jumaah, H. M., Yenzeel, J. H., & Mehdi, M. G. (2021). Evaluation of Some Biochemical Parameters and Hormones in Patients with Acute Myeloid Leukemia in Iraq. *Iraqi Journal Of Science*, 1460-1466.

Jung, Y. S., & Park, J. I. (2020). Wnt signaling in cancer: therapeutic targeting of Wnt signaling beyond β-catenin and the destruction complex. *Experimental & Molecular Medicine*, *52*(2), 183-191.

Kahriman, ş. Y. Cancer metastasis. *Health & Science 2021-iii*, 69.

Kalf, G. F., & Snyder, C. A. (1987). Recent advances in the metabolism and toxicity of benzene. *CRC Critical Reviews In Toxicology*, *18*(2), 141-159.

Kawasaki, Y., Hirabayashi, Y., Kaneko, T., Kanno, J., Kodama, Y., Matsushima, Y., ... & Inoue, T. (2009). Benzene-induced hematopoietic neoplasms including myeloid leukemia in Trp 53-deficient C57BL/6 and C3H/He mice. *Toxicological sciences*, *110*(2), 293-306.

Kirtonia, A., Pandya, G., Sethi, G., Pandey, A. K., Das, B. C., & Garg, M. (2020). A comprehensive review of genetic alterations and molecular targeted therapies for the implementation of personalized medicine in acute myeloid leukemia. *Journal Of Molecular Medicine*, 98(8), 1069–1091.

Kishtagari, A., Levine, R. L., & Viny, A. D. (2020). Driver mutations in acute myeloid leukemia. *Current Opinion In Hematology*, *27*(2), 49-57.

Köhler, T., Schill, C., Deininger, M. W., Krahl, R., Borchert, S., Hasenclever, D., ... & Niederwieser, D. (2002). High Bad and Bax mRNA expression correlate with negative outcome in acute myeloid leukemia (AML). *Leukemia*, *16*(1), 22-29.

Kumar, C. C. (2011). Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. *Genes & cancer*, *2*(2), 95-107.

Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., Bruick, R. K. (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev*, 16, 1466-1471

Luis, T. C., Ichii, M., Brugman, M. H., Kincade, P., & Staal, F. J. (2012). Wnt signaling strength regulates normal hematopoiesis and its deregulation is involved in leukemia development. *Leukemia*, *26*(3), 414-421.

Malaguarnera, L., Pilastro, M. R., DiMarco, R., Scifo, C., Renis, M., Mazzarino, M. C., & Messina, A. (2003). Cell death in human acute myelogenous leukemic cells induced by pyrrolidinedithiocarbamate. *Apoptosis*, *8*(5), 539-545.

Martin-Orozco, E., Sanchez-Fernandez, A., Ortiz-Parra, I., & Ayala-San Nicolas, M. (2019). WNT signaling in tumors: the way to evade drugs and immunity. *Frontiers In Immunology*, *10*, 2854.

Meisenberg, B. R. (2015). The financial burden of cancer patients: time to stop averting our eyes. Support Care Cancer, 23(5), 1201–1203.

Merlo, L. M., Pepper, J. W., Reid, B. J., & Maley, C. C. (2006). Cancer as an evolutionary and ecological process. *Nature Reviews Cancer*, *6*(12), 924-935.

Micallef, I., & Baron, B. (2020). Doxorubicin: An overview of the anti-cancer and chemoresistance mechanisms.

Michor, F., Iwasa, Y., & Nowak, M. A. (2004). Dynamics of cancer progression. *Nature Reviews Cancer*, *4*(3), 197-205.

Mikesch, J. H., Steffen, B., Berdel, W. E., Serve, H., & Müller-Tidow, C. (2007). The emerging role of Wnt signaling in the pathogenesis of acute myeloid leukemia. *Leukemia*, *21*(8), 1638-1647.

Minet, E., Michel, G., Mottet, D., Raes, M., & Michiels, C. (2001). Transduction pathways involved in Hypoxia-Inducible Factor-1 phosphorylation and activation. *Free Radical Biology and Medicine*, *31*(7), 847-855.

Morimoto, K., & Wolff, S. (1980). Increase of Sister Chromatid Exchanges and Perturbations of Cell Division Kinetics in Human Lymphocytes by Benzene Metabolites. *Cancer Research*, 40(4), 1189–1193.

Murray, B. S., & Dyson, P. J. (2020). Recent progress in the development of organometallics for the treatment of cancer. *Current Opinion In Chemical Biology*, *56*, 28-34.

Natelson, E. A. (2007). Benzene-induced acute myeloid leukemia: A clinician's perspective. *American Journal Of Hematology*, *82*(9), 826-830.

Naz, F., Shi, M., Sajid, S., Yang, Z., & Yu, C. (2021). Cancer stem cells: a major culprit of intra-tumor heterogeneity. *American Journal Of Cancer Research*, *11*(12), 5782.

Newland, L. W., & Daun, K. A. (1982). Handbook of environmental chemistry.

Nowell, P. C. (1976). The Clonal Evolution of Tumor Cell Populations: Acquired genetic lability permits stepwise selection of variant sublines and underlies tumor progression. *Science*, *194*(4260), 23-28.

Nusse, R. (2012). Wnt signaling. *Cold Spring Harbor Perspectives in Biology*, *4*(5), a011163.

Park, Y., Lim, J., Kim, S., Song, I., Kwon, K., Koo, S., & Kim, J. (2018). The prognostic impact of lymphocyte subsets in newly diagnosed acute myeloid leukemia. *Blood Research*, 53(3), 198–204.

Paun, R. A., Dumut, D. C., Centorame, A., Thuraisingam, T., Hajduch, M., Mistrik, M., ... & Tabrizian, M. (2022). One-Step Synthesis of Nanoliposomal Copper Diethyldithiocarbamate and Its Assessment for Cancer Therapy. *Pharmaceutics*, *14*(3), 640.

Pezzuto, A., & Carico, E. (2018). Role of HIF-1 in cancer progression: novel insights. A review. *Current Molecular Medicine*, *18*(6), 343-351.

Rappaport, S. M., Kim, S., Lan, Q., Vermeulen, R., Waidyanatha, S., Zhang, L., ... & Smith, M. T. (2009). Evidence that humans metabolize benzene via two pathways. *Environmental Health Perspectives*, *117*(6), 946-952.

Rasool, M., Farooq, S., Malik, A., Shaukat, A., Manan, A., Asif, M., ... & Hussain, A. (2015). Assessment of circulating biochemical markers and antioxidative status in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients. *Saudi Journal Of Biological Sciences*, *22*(1), 106-111.

Ravi, R., Mookerjee, B., Bhujwalla, Z. M., Sutter, C. H., Artemov, D., Zeng, Q., ... & Bedi, A. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α. *Genes & Development*, *14*(1), 34-44.

Roboz, G. J. (2011). Novel approaches to the treatment of acute myeloid leukemia. *Hematology 2010, the American Society of Hematology Education Program Book*, *2011*(1), 43-50.

Roner, M. R., Carraher Jr, C. E., Shahi, K., & Barot, G. (2011). Antiviral activity of metal-containing polymers—Organotin and cisplatin-like polymers. *Materials*, *4*(6), 991-1012.

Rowley, J. D. (1973). A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature, 243(5405), 290–293.

Sarkar, S., Horn, G., Moulton, K., Oza, A., Byler, S., Kokolus, S., & Longacre, M. (2013). Cancer development, progression, and therapy: an epigenetic overview. *International Journal Of Molecular Sciences*, *14*(10), 21087-21113.

Semenza, G. L. (2002). Signal transduction to hypoxia-inducible factor 1. *Biochemical Pharmacology*, *64*(5-6), 993-998.

Semenza, G. L. (2014). Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annual Review Of Pathology: Mechanisms Of Disease*, *9*, 47-71.

Semenza, G. L., Nejfelt, M. K., Chi, S. M., & Antonarakis, S. E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3'to the human erythropoietin gene. *Proceedings Of The National Academy Of Sciences*, *88*(13), 5680-5684.

Semenza, G. L., Nejfelt, M. K., Chi, S. M., & Antonarakis, S. E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3'to the human erythropoietin gene. *Proceedings Of The National Academy Of Sciences*, *88*(13), 5680-5684.

Sharma BK (2000) Instrumental Methods of Chemical Analysis. Krishna Prakashan Media, Meerut, India, 514.

Shazia, R., Muhammad, I., Anwar, N., Haji, A., & Amin, A. (2010). Transition metal complexes as potential therapeutic agents. *Biotechnology And Molecular Biology Reviews*, *5*(2), 38-45.

Shindo, D., & Oikawa, T. (2013). Analytical electron microscopy for materials science. *Springer Science & Business Media*.

Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2021). Cancer statistics, 2021. *Cancer J Clin*, *71*(1), 7-33.

Skoog, D. A., Holler, F. J., & Crouch, S. R. (2017). Principles Of Instrumental Analysis. *Cengage Learning*.

Skrott, Z., & Cvek, B. (2012). Diethyldithiocarbamate complex with copper: the mechanism of action in cancer cells. *Mini Reviews In Medicinal Chemistry*, *12*(12), 1184-1192.

Smith, M. T., Yager, J. W., Steinmetz, K. L., & Eastmond, D. A. (1989). Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. *Environmental Health Perspectives*, *82*, 23-29.

Snyder, R., Witz, G., & Goldstein, B. D. (1993). The toxicology of benzene. *Environmental Health Perspectives*, *100*, 293-306.

Song, X., Wang, S., & Li, L. (2014). New insights into the regulation of Axin function in canonical Wnt signaling pathway. *Protein & cell*, *5*(3), 186-193.

Staal, F. J., Famili, F., Garcia Perez, L., & Pike-Overzet, K. (2016). Aberrant Wnt signaling in leukemia. *Cancers*, *8*(9), 78.

Steffen, B., Müller-Tidow, C., Schwäble, J., Berdel, W. E., & Serve, H. (2005). The molecular pathogenesis of acute myeloid leukemia. *Critical Reviews In Oncology/Hematology*, *56*(2), 195-221.

Stone, R. M., O'Donnell, M. R., & Sekeres, M. A. (2004). Acute myeloid leukemia. *ASH Education Program Book*, *2004*(1), 98-117.

Su, M., Alonso, S., Jones, J. W., Yu, J., Kane, M. A., Jones, R. J., & Ghiaur, G. (2015). All-trans retinoic acid activity in acute myeloid leukemia: Role of cytochrome P450 enzyme expression by the microenvironment. PLoS ONE, 10(6), 1–14.

Taciak, B., Pruszynska, I., Kiraga, L., Bialasek, M., & Król, M. (2018). Wnt signaling pathway in development and cancer. *J Physiol Pharmacol*, *69*(2), 185-196.

Tisato, F., Marzano, C., Porchia, M., Pellei, M., & Santini, C. (2010). Copper in diseases and treatments, and copper-based anticancer strategies. *Medicinal Research Reviews*, *30*(4), 708-749.

Top, S., Jaouen, G., Vessieres, A., Abjean, J. P., Davoust, D., Rodger, C. A., ... & McGlinchey, M. J. (1985). Chromium tricarbonyl complexes of estradiol derivatives: differentiation of. alpha. -and. beta. -diastereoisomers using 1-and 2-dimensional NMR spectroscopy at 500 MHz. *Organometallics*, *4*(12), 2143-2150.

Van Staveren, D. R., & Metzler-Nolte, N. (2004). Bioorganometallic chemistry of ferrocene. *Chemical Reviews*, *104*(12), 5931-5986.

Vaupel, P., Schmidberger, H., & Mayer, A. (2019). The Warburg effect: essential part of metabolic reprogramming and central contributor to cancer progression. *International Journal Of Radiation Biology*, 95(7), 912–919.

Verhaegen, S., McGowan, A. J., Brophy, A. R., Fernandes, R. S., & Cotter, T. G. (1995). Inhibition of apoptosis by antioxidants in the human HL-60 leukemia cell line. Biochemical Pharmacology, 50(7), 1021–1029.

Vogelstein, B., & Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nature Medicine*, 10(8), 789–799.

Walaa Fikry, M. E. (2017). Lactate dehydrogenase (LDH) as prognostic marker in acute leukemia quantitative method. *J. Blood. Disord. Transfus*, *8*, 1-8.

Wang, L., He, X., Bi, Y., & Ma, Q. (2012). Stem cell and benzene-induced malignancy and hematotoxicity. *Chemical Research In Toxicology*, *25*(7), 1303-1315.

Wang, T., Fu, Y., Huang, T., Liu, Y., Wu, M., Yuan, Y., ... & Li, C. (2016). Copper ion attenuated the antiproliferative activity of di-2-pyridylhydrazone dithiocarbamate

Warmuth, M., Danhauser-Riedl, S., & Hallek, M. (1999). Molecular pathogenesis of chronic myeloid leukemia: implications for new therapeutic strategies. *Annals Of Hematology*, *78*(2), 49-64.

Warren, L. A., & Rossi, D. J. (2009). Stem cells and aging in the hematopoietic system. *Mechanisms Of Ageing And Development*, *130*(1-2), 46-53.

Weinberg, R. A., & Hanahan, D. (2000). The hallmarks of cancer. *Cell*, *100*(1), 57-70.

Wenger, R. H., Stiehl, D. P., & Camenisch, G. (2005). Integration of oxygen signaling at the consensus HRE. *Science's STKE*, *2005*(306), re12-re12.

Williams, G. H., & Stoeber, K. (2012). The cell cycle and cancer. *The Journal Of Pathology*, *226*(2), 352-364.

World Health Organization (WHO) said on Sept 21 (2022).

Zhao, L., Zhan, H., Jiang, X., Li, Y., Science, H. Z.-B., & 2019, undefined. (2019). The role of cholesterol metabolism in leukemia. *Medcentral.Net*, 1(1), 44–49.

Zhou, W., Apkarian, R., Wang, Z. L., & Joy, D. (2006). Fundamentals of scanning electron microscopy (SEM). *Scanning Microscopy For Nanotechnology* (pp. 1-40). Springer, New York, NY.

Zuckerman, T., & Rowe, J. M. (2014). Pathogenesis and prognostication in acute lymphoblastic leukemia. *F1000prime Reports*, *6*.