INVESTIGATION INTO ANTI-CANCER ACTIVITY OF D-LIMONENE IN CHRONIC MYELOID LEUKEMIA

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy (Pharmacology)

by

Shah Bhavini Bharatkumar

Enrollment No. 139997390009

under supervision of

Dr. Anita A. Mehta

Department of Pharmacology, L. M. College of Pharmacy, Ahmedabad.

GUJARAT TECHNOLOGICAL UNIVERSITY

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ABSTRACT

Chronic myeloid Leukemia (CML), a myeloproliferative malignancy of blood has attained importance nowadays because of its increased incidence. Treatment with tyrosine kinase inhibitors (TKIs) therapy has transformed the outcomes of patients with CML but resistance and associated adverse effects has lessened its efficacy. Natural products have been known for its enormous antioxidant and anticancer activities. D-Limonene, a monoterpene obtained from citrus fruits has been known for its potential chemopreventive and chemotherapeutic effects in various cancers. However, the underlying mechanism is still poorly understood. The aim of present study was to evaluate the effect of D-limonene in CML.

In this study, *in vitro* effect of D-Limonene on K562 cells and mouse primary hepatocytes were evaluated by MTT assay. D-Limonene has shown significant dose dependent reduction in percentage of cell viability of K562 cells (IC_{50}=3.6± 0.23 mM and 3.29± 0.19 mM at 24 and 48 h respectively) comparable with Doxorubicin. Treatment with D- Limonene of mouse primary hepatocytes did not show significant change on percentage of cell viability. However, significant reduction in viability of primary hepatocytes was noted with Doxorubicin (IC_{50}= 14.13 μM) treatment.

The *in vivo* effect of D-limonene (0.5, 1.0 and 1.5 g/kg, orally) was also evaluated on chemically immunocompromised K562 implanted xenograft C57BL/6 mice by subcutaneous and tail vein injection model for 14 and 17 days respectively. In subcutaneous injection model, D-Limonene treatment showed significant improvement in body weight as compared to disease control (DC) group. In haematological parameters, D-Limonene has shown significant improvement in WBC and NEU count as compared to DC group. In tumor, D- Limonene treatment showed significant dose dependent reduction in tumor volume in K562 implanted xenograft mice as compared with DC group.

In tail vein injection model, treatment with D-Limonene has shown significant improvement in body weight as compared to DC group. In haematological parameters, significant reduction

in WBC and NEU count were noted with D-Limonene treatment in all treatment groups as compared to the DC group. The change in LYM count, RBC count and Hb content was non-significant with D-Limonene at 0.5 g/kg; however, at doses 1.0 and 1.5 g/kg, significant increase in RBC count and Hb content was observed as compared to the DC group. In biochemical parameters, significant dose dependent reduction in serum SGPT, SGOT, ALP, lipid peroxidation and nitrite production were observed in D-Limonene treated group as compared to DC group, whereas significant increase in superoxide dismutase level was observed with D-Limonene treatment as compared to DC group.

We have also evaluated the effect of D-Limonene on angiogenesis by using CAM assay. D-Limonene (1, 5 and 10 µg/implant) has shown significant dose-dependent reduction in number of blood vessels on CAM as compared to implant soaked with the vehicle. Reduction in number of blood vessels on CAM was highest with D-Limonene at concentration 10 µg/implant. Further, we have evaluated the effect of D-Limonene on expression of VEGF protein by western blotting and MMP-2 and 9 proteins by gelatin zymography. D-Limonene (0, 1, 2, 3 and 4 mM) has shown significant dose dependent reduction in the expression of VEGF, MMP-2 and 9 proteins as compared to proteins from untreated cells. The inhibition of expression of VEGF, MMP-2 and 9 was highest at concentration of 4mM.

The *in vitro* antioxidant effect of D-Limonene (25, 50, 100, 200 and 400 µM) was evaluated by DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging, superoxide radical scavenging and nitric oxide radical scavenging assays in comparison with Trolox. D-Limonene has shown concentration dependent reduction in free radical formation in all assays except iron chelating assay. Further, the antioxidant activity of D-Limonene on K562 cells was also evaluated by lipid peroxidation assay in which, D-Limonene has significantly reduced the production of malondialdehyde production in dose dependent manner.

In conclusion, D-Limonene has significantly inhibited the *in vitro* growth of K562 cells without producing toxicity to normal hepatocytes. Moreover, it has inhibited the *in vivo* progression of CML in K562 cell implanted tumor xenograft model, which further supports its *in vivo* efficacy. It has inhibited angiogenesis by inhibiting the expression of VEGF and invasion by inhibiting MMP-2 and 9, which confirms it involvement in regulation of downstream pathways involved in angiogenesis and metastasis of CML. Apart from this D-

Limonene has presented appreciable antioxidant property in cell free system and on K562 cells as well, which has also important role in progression of CML and makes it promising molecule for the treatment of CML.

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As it is a prolonged journey, maybe I have forgotten few names to consider, but nonetheless, they remain a core part of this mammoth task, and I seek forgiveness for the same and offer my kind respect to every one of them.

Thanks to one & all…

Bhavini Bharatkumar Shah

A mother is someone who can take the place of many,
but no one can take her place!

Dedicated to my mother, father, almighty and family

Table of Content

List of Abbreviations

ABTS	2,2'-Azino-Bis(3-Ethylbenzothiazoline-6-Sulphonic Acid)
ALP	Alkaline Phosphatase
AP	Accelerated Phase
BC	Blast Crisis
BCR-ABL	Breakpoint Cluster Region-Abelson
CAM	Chick Chorioallantoic Membrane
CCyR	Complete Cytogenetic Response
CHR	Complete Hematologic Response
CLPs	Common Lymphoid Progenitors
CML	Chronic Myeloid Leukemia
CMP	Common Myeloid Progenitor
CP	Chronic Phase
CT	Computed tomography
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
ECM	Extra cellular matrix
ECs	Endothelial Cells
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FISH	Fluorescent In Situ Hybridization
FRAP	Ferric Reducing Anti-Oxidant Power
GMLP	Granulocyte-Macrophage-Lymphocyte Progenitor
GMPs	Granulocyte Macrophage Progenitors
Hb	Haemoglobin
HIF1	Hypoxia-Inducible Factor 1
HSCs	Haemopoietic Stem Cells

IARC	International Agency for Research on Cancer
IFN-α	Interferon-Alfa
JAK2–STAT	Janus kinase 2–signal transducer and activator of transcription
LSCs	Leukemic Stem Cells
MAPK	Mitogen Activated Protein Kinase
MMPs	Matrix Metalloproteinases
MMR	Major Molecular Response
MPPs	Multi-Potent Progenitors
mTOR	Mammalian (or mechanistic) Target of Rapamycin
MTT	3-(4,5-Dimethythiazol- 2-yl)-2,5-Diphenyl Tetrazolium Bromide
MVD	Microvessel Density
NF- κB	Nuclear Factor kappa B
NOX	NADPH oxidase
PARP	Poly ADP ribose polymerase
Ph	Philadelphia
PI3K	Phosphatidylinositol 3 Kinase
PLT	Platelet
RBC	Red Blood Cell
RPMI-1640	Roswell Park Memorial Institute Medium-1640
RQ-PCR	Reverse Transcriptase Quantitative-Polymerase Chain Reaction
SGOT	Serum Glutamic Oxalocetic Transaminase
SGPT	Serum Glutamic Pyruvate Transaminase
SOD	Superoxide Dismutase
TKIs	Tyrosine Kinase Inhibitors
TPTZ	2, 4, 6- Tripyridyl-S-Triazine
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell
WHO	World Health Organization

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Chapter-1

Introduction

CHAPTER-1

Introduction

Cancer is a heterogeneous group of disease that encompasses more than two hundred diseases sharing common characteristics of uncontrolled proliferation and invasion to other parts of the body. This fundamental abnormality is reflected in several aspects of cell behaviour that distinguish cancer cells from normal cells. Cancer may be either benign-slow growing that doesn't spread to other tissues and are usually harmless or malignant-very rapidly growing that has capability to invade the surrounding tissues. In 2018, about 18.1 million new cases and 9.6 million cancer related deaths were reported worldwide by GLOBOCAN database from IARC (International Agency for Research on Cancer) (1). It has been projected for around 24.12 million new cases and over 13.02 million deaths by 2030. In India, 1.16 million new cases and 0.8 million deaths were reported in the year 2018 and it has been projected with 1.55 million new cases and 1.06 million cancer related death by 2030 (2).

Normally, the cells present in a living organism are specific for certain function and are under the strict observation of complicated signals that control the cellular proliferation and apoptosis. However, alteration of these signals or responsiveness of cells to these signals may promote the discrepancy in cell division, adhesion, invasion and apoptosis that upshot the formation of massive tissue named as tumor or neoplasm.

The signals that direct the cell growth and division in-turn are modulated by intricate genetic control systems. These control systems are responded by numerous stimuli or signals for cell growth-promotion, inhibition and apoptosis (3). Mutation or the permanent alteration of nucleotide sequence in DNA is one of the contributors in modulating these cellular signals. Mutation resulting from insertion, deletion or duplication of nucleotide may alter the homology of genomic sequence. Mutation of proto-oncogenes or tumor suppressor genes, promotes the development of this multifaceted disease (4). Proto-oncogenes usually encrypt the proteins that are commonly involved in cell growth and

differentiation. Mutation triggers the transformation of proto-oncogenes to oncogenes, the genes with carcinogenic potential, which governs the destiny of cells for apoptosis or survival. Tumor suppressor genes or anti-oncogenes, normally protects the cell from one step on the track of cancer. These genes repair the DNA damage, control the cellular growth and guide the cell when to undergo proliferation or apoptosis. Hence, impairment in regulation of either gene drives the cell toward uncontrolled proliferation and cancer development (5). However, it is estimated that only 5-10% of malignancy are triggered by inherited traits such as mutation, the enduring 90-95% are contributed by environmental factors. The majority of cancers are apparently sporadic in nature and outcome owing to exposure of carcinogens such as certain chemicals and UV radiation. Certain oncogenic viruses such as Human Papillomaviruses (HPV), Hepatitis B and C, Epstein-Barr and Human Immunodeficiency Virus (HIV) interfere with crucial pathways of the cells and affect continuum of carcinogenesis.

Despite infrequent incidence, mutation of genes, which regulates the activity of Hematopoietic stem cells (HSCs), has been considered as one of the major offenders in development of blood malignancy. In particular, mutation of HSCs has been associated with altered cellular responses and conversion of normal stem cells to the cancerous myeloid progenitor, which allows it to multiply more rapidly than the normal and contribute in prevalence of myeloid type of leukemia (6, 7).

Chronic myeloid leukemia (CML) is a myeloproliferative disease of the hematopoietic stem cell (HSC), characterized by the presence of Philadelphia chromosome (Ph) or BCR-ABL oncogene and increased production of the myeloid cells. In the 19^{th} century, the disease was first described independently and virtually concurrently by John H Bennett in Scotland and Rudolph Virchow in Germany, based on autopsy interpretations (8).

Among all malignancies, the global incidence of blood malignancy is 6% and CML accounts of about 18-20% of all leukaemia affecting adults. In 2018, 0.24 million new cases and 0.18 million cancer related deaths were estimated by CML worldwide. Researchers estimate that by 2030, nearly 0.3 million new cases and 0.23 million deaths will be projected with CML. India accounts for 5.7 % of new cases and 5.6 % of deaths of world population related to CML. Although CML is not amongst the leading sites of cancer worldwide, its increasing incidence is a cause of concern globally (2, 9).

The common risk factors for CML are age, gender and radiation exposure (10). It can appear at any age but the prevalence greatly upsurges in the older population with median age of 50-60 years and rare in children. To some extent, males are more prone to get affected by the disease than females (ratio 1.7:1), regardless of significant ethnic or geographical predisposition. Studies involving survivors of the atomic bombs in Japan, has recognized that increased incidence of ionizing radiation as a chief offender in development of the disease. CML is often asymptomatic, abnormal routine blood count leads to diagnosis of the disease. Common indications are lethargy, weight loss, unusual bleeding, sweats, anaemia, and splenomegaly (11).

CML is a slow-growing blood cancer, which begins in the bone marrow, but often moves into the blood. Most of the patients are diagnosed in the chronic or stable phase, characterized by expansion of the myeloid cell compartment. In this phase of CML, cellular differentiation and functions are maintained to some extent. After a time point chronic phase progresses to accelerated phase, which is associated with leucocytosis and basophilia resulting in worsening of symptoms. Accelerated phase shift in blast crisis, resembling an acute leukemia of myeloid (70%), or lymphoid (20%–30%), or undifferentiated phenotype that conveys a poor prognosis (12, 13).

In chronic phase of the disease, the complete blood count presents increased granulopoiesis with predominance of neutrophils along with slight increase in eosinophils and basophils. The biochemistry also correlates of myeloid hyperplasia by increased uric acid and lactate dehydrogenase levels. The bone marrow morphology shows granulocytosis with relatively depressed erythropoiesis. Presence of marrow fibrosis is noted in the accelerated phase. This phase can be defined exclusively based on karyotypic clonal evolution, with blood and marrow morphology. In blast crisis, bone marrow shows features that would be expected in acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL), but there may be morphological pieces of evidence to the origin of leukemia, such as eosinophilia or basophilia (14, 15).

In BCR-ABL fusion gene, the ABL or ABL1 gene is the human homologue 1 of the Abelson murine leukemia virus and BCR is breakpoint cluster region. The Ph chromosome is an outcome of reciprocal translocation t(9;22)(q34;q11), of ABL gene from chromosome 9 to BCR gene on chromosome 22. This translocation fuses the sequence from ABL gene, from chromosome 9q34 downstream of BCR on chromosome 22q11, generating two

pathognomonic fusion genes, BCR–ABL on the 22q- (Ph) chromosome, and ABL–BCR, on the derivative 9q+. However, the latter one does not have any evidence for functional relevance in the disease and only BCR–ABL gene is supposed to be responsible for the leukemic process. Further, this translocation generates abnormally truncated chromosome 22 with BCR–ABL oncoprotein, a major hallmark of CML that comprises numerous important domains of its parental BCR and ABL normal counterparts. Depending on site of break in chromosome 22, variable parts of BCR are preserved in the Bcr-Abl fusion gene, which leads to the formation of proteins with different molecular weights such as p190 $^{BCR-ABL}$, p210$^{BCR-ABL}$ and p230 $^{BCR-ABL}$ kDa. Among these, p210$^{BCR-ABL}$ has been reported as an important because of its differential ability to transfer HSCs to critical differentiation specific pathways (16). BCR-ABL gene is also present in approximately 25 % of acute lymphoblastic leukemia (ALL) cases. However, presence of BCR-ABL in CML defines the disease whereas in ALL, it defines poor prognosis (17).

The BCR-ABL gene comprises of several important domains of its parent BCR and ABL counterparts that endow it with specific properties of leukemogenesis. The dimerization of domain encoded by the amino-terminus of BCR, leads to constitutively activated tyrosine kinase of the ABL portion. Subsequently, BCR-ABL oncoprotein interferes with a series of signal transduction pathways, including the phosphatidylinositol 3 kinase (PI3K), the Janus kinase 2–signal transducer and activator of transcription (JAK2–STAT) and the mitogen activated protein kinase (MAPK) pathways. These pathways, exaggerated cellular proliferation, diminished response to apoptotic stimuli, reduced adherence to the bone marrow stroma, and exacerbated genomic instability of myeloid cells (18).

The advancement of the disease is not solely dependent on enhanced cell proliferation and survival, but on increased motility and active invasion of the leukemic cells through blood vessel and matrix barriers. The mechanism by which BCR-ABL induces disease progression is not completely understood, however, the gene has been identified to play a decisive role in the regulation of angiogenesis, motility and invasion of leukemic cells. Various studies have proposed the association of BCR-ABL and vascular endothelial growth factor (VEGF), a cytokine involved in angiogenesis and capillary leakage. Many studies have confirmed the overexpression of VEGF in neoplastic myeloid cells and in other leukemia (19). In particular, the role of VEGF has been elaborated as a major autocrine growth factor for leukemic cells and has been implicated in leukemia-associated angiogenesis (20). Activation of PI3K and mTOR pathway, activates the downstream

pathways that upsurge the expression of VEGF. In CML, the expression of VEGF was found to be increased within the bone marrow and peripheral blood with advancement of the disease (21).

Furthermore, BCR-ABL gene is also found to be involved in regulation and expression of MMPs (matrix metalloproteinases) group of enzymes. MMPs are endopeptidases, capable of degrading the extracellular matrix proteins. MMPs are not only implicated in normal processes but also in pathological processes, such as tumor invasion and metastasis (22, 23). In CML, stimulation of angiogenesis by angiogenic factors including VEGF and MMPs plays an important role in the pathogenesis of the disease. Among all MMPs, MMP-2 and MMP-9 are abundantly involved in the invasive phenotype of various malignant neoplasias, however, their role in CML pathogenesis and progression has become much more complex and a focus of attention (24, 25). Studies have reported that, BCR/ABL oncogene induces the TGF-β1 which further upregulate the production of MMP-9 by PI3K/Akt/NF-κB/MMP9 signaling pathway in CML (24, 26). Moreover, it is found that VEGF stimulated the secretion of MMP-2 and 9 in BCR-ABL positive CML cells, suggesting its important role in the pathogenesis of the disease (27).

BCR-ABL is also involved in generation and accumulation of oxidative stress in CML. Oxidative stress is a pervasive condition associated with overproduction of reactive oxygen species (ROS), generated as an outcome of normal cellular processes, environmental stresses, and UV irradiation. In cancer, chronic exposure of oxidative stress induces alteration in genomic stability, epigenetic events, subsequent gene expression, cell proliferation and apoptosis, tumor neovascularization, invasion and metastasis (28). Particularly in CML, an imbalance between the free radical generation and the antioxidant defence system has been observed which modulate the signal transduction and cell proliferation pathways. It was confirmed that BCR-ABL fusion protein is associated with augmented levels of ROS and tyrosine kinase activity in hematopoietic cells compared with their non-transformed parental counterparts (29). BCR-ABL also reported promoting the chromosomal aberration induced by ROS and repair of ROS-dependent DNA double-strand breaks (30, 31). It has also increased self-mutagenesis via increased ROS and has developed resistance to chemotherapy (32). Apart from this, Leukemic cells also exhibit atypical activities of antioxidant enzymes compared to the normal cells (33). Therefore, there is a need of the treatment that can lower ROS through targeting directly the ROS-producing enzymes or would supplement the antioxidative potential of cells (34).

Patients with CML were anciently treated with busulfan, Hydroxyurea and interferon -alfa as an effective treatment available for CML. However, persistence of BCR-ABL transcripts in some patients restricted its superiority for the treatment (35, 36).

In early 1990s, the researchers have identified that the BCR-ABL gene controls the activity of tyrosine kinase receptor and provided the rationale for the development of specific tyrosine kinase inhibitors for therapeutic use (8). Treatment with Tyrosine kinase inhibitors (TKIs) therapy has revolutionized and transformed the prognosis of patients with CML over the last 20 years. Discovery of imatinib was proved a new paradigm in oncology, with implications much beyond the realm of CML (37). Later, the Food and Drug Administration (FDA) approved the other TKIs such as nilotinib, dasatinib and ponatinib for the treatment of chronic phase of CML. The TKIs has shown profound benefit in survival and quality of life for both adult and pediatric patients by interfering with the tyrosine kinase receptors and signal transduction pathway of cell proliferation and thereby blocking proliferation of the malignant clone (38, 39).

Imatinib has been used as frontline therapy for treatment of CML, with stable major molecular response (MMR) in nearly 60% of patients, whereas poorly tolerated in about 20% and no response in remaining 20% of patients (40). Despite of its efficacy, inferior response or loss of response with imatinib has been noted in majority of patients nowadays. It might be due of failure in attaining adequate blood levels, insufficient cellular uptake by reduced activity of the organic cation transporter 1 (OCT-1) influx pump and resistance induced by great range of kinase domain mutations, resulting in poor tyrosine kinase inhibition compared to nilotinib and dasatinib. The side effect comprises excessive fluid retention, gastrointestinal disturbance including nausea and diarrhoea, muscle cramps, organ toxicities (41).

Nilotinib is used as second-line therapy for CML. It is structurally similar to imatinib and binds to the kinase domain of BCR–ABL, with greater affinity than imatinib (42). Nilotinib is less vulnerable to kinase domain mutations, although some major mutations are as frequent as imatinib. A significant concern related to the use of nilotinib is the occurrence of hyperglycemia, hypercholesterolemia and vascular events, including peripheral arterial occlusion, coronary artery disease and cerebrovascular disease. Therefore, it is definitely an issue to keep in mind when selecting nilotinib for a patient with risk for diabetes and atherosclerosis and other cardiovascular complications.

Dasatinib shares similar potency to nilotinib but has a relatively short half-life, suggesting more frequent daily dosing. The numbers of kinase domain mutations are similar to imatinib and dasatinib. The incidence of pleural effusions and pulmonary arterial hypertension are the major drawbacks of the drug. Ponatinib is a third generation TKIs that blocks mutated BCR-ABL and used as a drug of choice in patients that are resistant to other TKIs (43). However, it has been also reported to produce hypertension and arterial thrombotic events (44, 45). The associated risk or side effects with TKIs is irreversible and reserves its use for patients harbouring the resistance to the therapy.

Failure of chemotherapy, suggested the requirement of allogeneic stem cell transplantation therapy for complete eradication of the disease. However, absence of availability of either suitable donor or identical siblings has reduced its contribution for the improvement of life expectancy in CML patients. Further, because of the cost concern, it is still out of reach for the most (46).

Nowadays, Natural products are gaining importance for treatment in wide variety of human diseases because of efficacy, safety and cost-effectiveness. From anciently, the use of plants and its secondary metabolites have been reported for its potential antibacterial, antidiabetic, immunomodulatory and antifungal activities. Apart from all these activities, plants have been also reported for its potent antioxidant and anticancer properties (47). Antioxidants or free radical scavengers controls the oxidative stress by scavenging or neutralizing free radicals and portray a main line of defence, regulating an optimum state of health.

Generally, endogenous antioxidants balance the level of ROS and prevent the occurrence of chronic degenerative diseases in the body. However, the endogenous antioxidants are insufficient at some level and create a requirement from exogenous sources, to accomplish the needs. Plants have attracted considerable attention as an exogenous source of antioxidants and have been reported to induce beneficial effects on human health and disease prevention (48).

Apart from antioxidant activity, plants have been also known for its potential anticancer activities. About 25% of currently used drugs in the world are isolated from plant derived secondary metabolites and their derivatives and over 3000 species of plants have been known for its anticancer properties. It reduces the side effects and resistance associated

with malignancy and emphasises its use as a source of medicines for the treatment of cancer. Secondary metabolites such as alkaloid, Flavonoids, Glycosides, Tannins, Resins and volatile oil show considerable activity for treatment of all type of cancers including leukemia and lymphoma (49).

Enormous studies have documented the efficacy of essential oils and their chemical constituents as a source of new bioactive molecule against treatment of various cancers. Monoterpenes, non-nutritive secondary metabolite found in the essential oils of citrus fruits and other plants have exhibited its anti-tumor activity in various types of cancers. Monoterpenes avert the process of carcinogenesis at both, initiation and promotion stages and have been reported for treating early and advanced cancers (50, 51).

D-Limonene ((R)-1-methyl-4-(1methylethenyl) cyclohexene), a monocyclic monoterpene abundantly found in volatile oils of citrus fruits such as orange, lemon, grapefruit and other plants. It has been widely used as a flavour and fragrance enhancer because of its pleasant citrus fragrance and listed in the Code of Federal Regulations as generally recognized as safe (GRAS) for a flavouring agent and can be found in common food items such as fruit juices, baked goods, soft drinks, and ice creams (52).

The Pharmacokinetic profile indicates D-Limonene is rapidly and almost completely taken up from the gastrointestinal tract in humans as well as in animals orally and has a half-life of approximately 24 h in rat (53, 54). Oral administration of [^{14}C] D-Limonene in rats has shown that D-Limonene was highly distributed in tissues such as liver, kidneys, and blood. About 25–30% of an oral dose of D-Limonene was found in urine as D-limonene-8,9-diol and its glucuronide in humans and 7–11% was eliminated as perillic acid and its metabolites (55).

D-Limonene has been reported for its antioxidant, anti-inflammatory and gall-stone dissolving property (56-58). It has also been used to relieve heartburn, because of its potential for gastric acid neutralization property. Beside this, D-limonene has well-established chemopreventive and chemotherapeutic activity against many types of cancers. It has inhibited neoplasia in both *in vivo* animal models and *in-vitro* cell growth (59). It is found that oral administration of D-Limonene exhibited anticancer activity by inhibiting the growth of rodent pancreatic, mammary and gastric carcinogenesis (60-64). These

observations suggest that it can be viewed as a conceptually promising agent in cancer therapy of CML.

From all previous study and published literature evidence, we have aimed the following objectives:

1. To study the effect of D-Limonene on K562 (CML) cells and on primary hepatocytes by MTT assay.
2. To study the effect of D-Limonene in immunocompromised C57BL/6 mice by subcutaneous and tail vein injection model of CML.
3. To identify the possible mechanisms of D-Limonene involved in CML using,
    - Chick chorioallantoic membrane (CAM) assay of angiogenesis.
    - Expression of angiogenic factor VEGF by western blotting
    - Expression of tissue invasion and metastasis marker MMPs by gelatin zymography.
4. To evaluate the *in vitro* antioxidant effect of D-Limonene.

Chapter-2

Review of Literature

CHAPTER-2

Review of Literature

2.1 Cancer

2.1.1 Introduction

Cancer is the most dreadful disease of the century in which normal cell suddenly become a "rebel," breaking the rules and dividing recklessly. It denounces and destroys normal tissue and organization of the body, transforming them to similar outrageous behaviour. More than 100 types of cancers have been identified among which Breast, Lung, Colorectal, Prostate, Ovary, Liver and Blood are the leading site for cancer development with a higher rate of incidence (65).

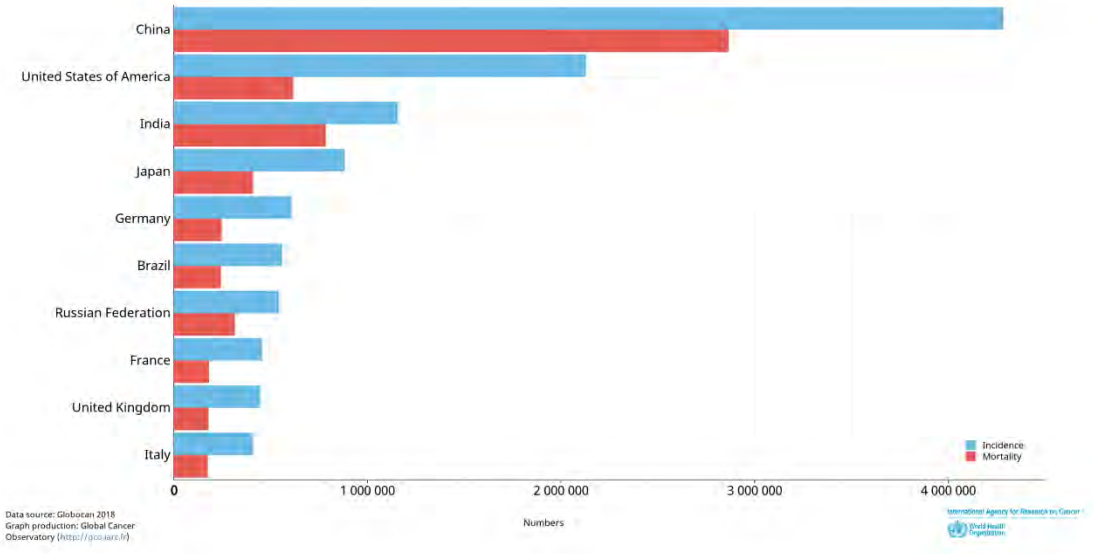


FIGURE 2.1: Estimated number of new cases and deaths related to cancer in different countries (66).

In GLOBOCAN 2018, an estimated 18.1 million new cancer cases and 9.6 million of cancer related deaths have been reported. Incidence rate of cancer worldwide are projected around 23.6

million and deaths from cancer to rise to over 13.1 million in 2030. India has been ranked third, for the estimated new cases and death related to cancer (Fig. 2.1) (66). Some cancers may develop as an outcome of intrinsic, unavoidable cellular transformations but about 70 to 90% of cancers have been reported due to extrinsic – environmental factors. Intrinsic transformation includes mutations - due to random errors in DNA replication, whereas extrinsic factors are environmental factors that affect mutagenesis rates such as diet, lifestyle, autoimmune diseases, carcinogens and radiations may induce genetic alterations during cell division to increase the risk for cancer development (67). Cancer transitions are extremely striking in emerging economies, often ascribed as an outcome of the westernized lifestyle (68).

The uncontrolled proliferation of cancerous cells within a normal tissue exhibits a diverse set of phenotypic abnormality including loss of differentiation, increased ability to infiltrate or invasiveness, motility and angiogenesis (18). These phenotypic abnormalities emerge as an outcome of stepwise accumulation of genetic mutation that liberates cancerous cells from homeostatic mechanism that govern normal cell proliferation. Multiple genetic alterations in human cancers strongly indicate the accumulation of alterations during tumor progression (38).

2.1.2 Classification of cancer

The tumors are divided into two types such as benign and metastatic. Benign tumor is restricted to the area where it grows as the cell adhesion molecules hold the cells together keeping it localized to the area where it grows. Whereas, cells of malignant tumor grow and divide more rapidly than a benign tumor, do not respond to apoptotic signals and even infiltrate the nearby tissue. Along with the progression of malignant tumor, the invading cells enter the circulatory system eventually forming secondary tumors; the process is called metastasis. Further, Cancer is often named based on the type of cells from which it has been originated, using -carcinoma, -sarcoma or -blastoma as a suffix. The classification includes:

1. **Carcinoma**: Cancers arising from epithelial cells. Example includes cancer of breast, prostate, lung and colon.
2. **Sarcoma**: Cancers originated from connective tissues, developing from mesenchymal cells outside of bone marrow. Example includes cancer of nerve, bone and cartilage.

3. **Lymphoma and Leukemia**: Cancers arising from haematopoietic stem cells.
4. **Blastoma**: Cancer originated from embryonic tissue or immature precursor cells. More common in children than adults.
5. **Germ cell tumor**: Cancers derived from pluripotent cells, presenting in testicle or ovary. Example includes seminoma and dysgerminoma (47).

Further, the expansion of malignancy occurs in three different stages (69):

1) **Initiation**: By the mutation of a single cell.
2) **Promotion**: Stimulation of mutated cells for faster growth.
3) **Progression**: Additional mutation in the tumor resulting in malignancy.

Genetic factors comprise alterations in any one of the following (70):

1) **Oncogenes**: Responsible for the unchecked growth of the cells that ought to die.
2) **Tumor suppressor genes**: Tells the cells when not to multiply.
3) **Suicide genes**: Regulates the programmed cell death or apoptosis.
4) **DNA repair genes**: Initiate a cell to repair a damaged DNA.

2.2 Haematopoiesis and Leukemia

Pluripotent stem cells are known for their capacity of self-renewal and multilineage differentiation. The HSCs or blood-forming stem cells have been isolated from progenitors present in the bone marrow. Haematopoiesis is the restricted differentiation of HSCs, and production of new mature blood cells. In bone marrow, HSCs account for 0.05% of bone marrow cells (71). Terminal differentiation and shorter life span of mature blood cells urge to maintain the haemopoietic system in a steady state through the proliferation and differentiation of haemopoietic stem and progenitor cells (72). In an adult human, it generates 2 million cells in every second and around a trillion cells in a day (73).

The classical model of haematopoiesis (Fig. 2.2) suggests that HSCs differentiate into multi-potent progenitors (MPPs) and increase their proliferative rate while losing capacity of self-renewal (74). MPPs further differentiate into common myeloid progenitor (CMP) and

granulocyte-macrophage-lymphocyte progenitor (GMLP). The formed progenitors are more lineage-committed and are more defined based on the expression of different transcription factors and surface markers. Progenitors are named depending on their potential to differentiate into distinct mature cell types. The familiar types of progenitor are:

1. **Common lymphoid progenitors (CLPs)**: These cells can differentiate into B-cells, T-cells, and NK-cells. These cells are important for immune function of the body.
2. **Granulocyte macrophage progenitors (GMPs)**: These give rise granulocytes, monocyte and dendritic cells. They too have a specific role against infections.
3. **Megakaryocyte erythrocyte progenitors (MEPs):** These are precursors for erythrocytes and thrombocytes are responsible for oxygen transport and process of clotting respectively.

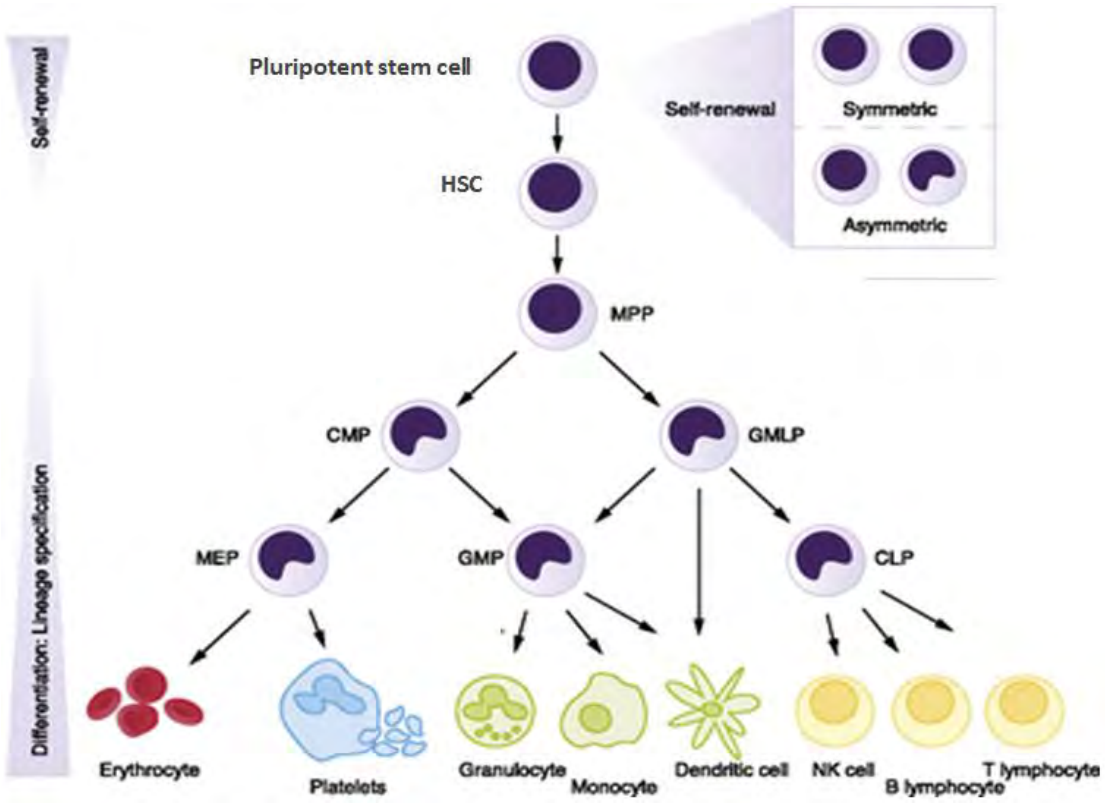


FIGURE 2.2: Haematopoietic hierarchy for the development of mature blood cells from HSCs (75).

Many studies have demonstrated important pathways associated with the conversion of normal stem cells to cancer cells with maintaining ability to self-renewal. However, the event of transformation is unknown but evidence indicates that certain types of leukemia are the outcome of mutations that accumulate in HSCs.

"Leukemia" was derived from the Greek words "leukos" means white and "haima" means blood, "white blood". It can be acute or chronic depending morphological and clinical characteristics of the leukemic cells. Leukemia is a heterogeneous group of haematological malignancies of bone marrow that produce an increased amount of abnormal or immature white blood cells and suppresses the production of normal blood cells. This abnormal cell interferes with the function of bone marrow and replaces it. The cells have ability to invade in other organs and eventually can cause death if remain untreated. In 2018, 2.4 million new cases of leukemia have been reported with 3.2 million deaths worldwide (66).

The exact root of leukemia is unknown but the combination of genetic factors and environmental factors are believed to play an important role in the development of the disease. Smoking, ionizing radiation, heredity, some chemicals (such as benzene), and prior chemotherapy are the ordinary risk factors for leukemia.

Leukemia may be of myeloid or lymphoid origin, both belong to a broader group of malignancies that affects bone marrow and blood. It has been divided into four main types:

1. Acute Lymphoblastic or Lymphocytic Leukemia (ALL)
2. Chronic Lymphoblastic or Lymphocytic Leukemia (CLL)
3. Acute Myelogenous or Myeloid Leukemia (AML)
4. Chronic Myelogenous or Myeloid Leukemia (CML)

Acute leukemia is related to increased production of immature blood cells and common in children, while chronic is related to relatively mature, but abnormal cells and common in adults or older ones. Other less common types of leukemia include hairy cell, T-cell prolymphocytic, T-cell prolymphocytic, large granular lymphocytic and adult T-cell leukemia (76, 77).

2.3 Chronic Myeloid Leukemia

2.3.1 Introduction

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder affecting the HSCs of bone marrow. CML arises from a translocation between the BCR (region q11) gene on chromosome 22 and the ABL1 (region q34) gene on chromosome that creates an elongated chromosome 9 and a truncated chromosome 22. This reciprocal chromosomal translocation forms oncogenic fusion gene BCR-ABL t(9;22)(q34;q11) on chromosome 22 called Philadelphia (Ph) chromosome (Fig. 2.3), which encodes for the consequent formation of a unique BCR-ABL protein product (78). This protein has constitutive tyrosine kinase activity that drives uncontrolled proliferation of hematopoietic stem cells and development of haematological malignancy (79).

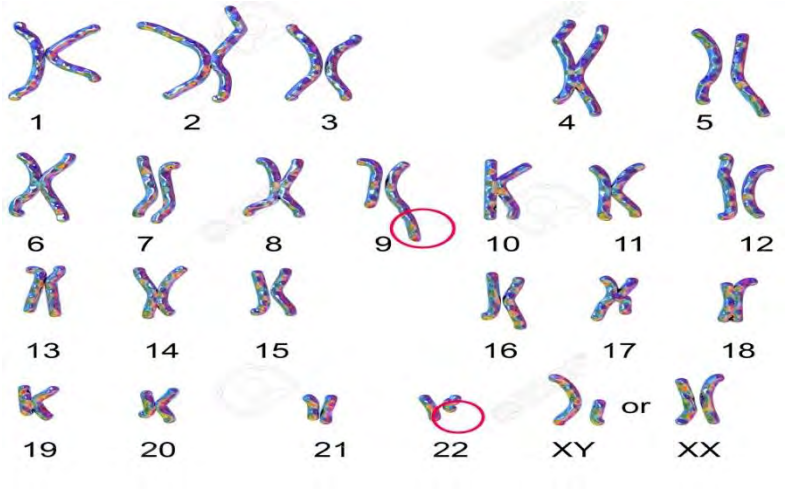


FIGURE 2.3: Illustration of Philadelphia chromosome karyotype in male or female (80).

2.3.2 History of CML

In 1845, a pathologist Rudolf Virchow has first reported the disease in patients with splenomegaly, hepatomegaly, anaemia, granulocytosis and purulent blood. Later Neumann found that the disease has been originated from the bone marrow which has changed its colour from red to dirty green-yellow (81). In 1959, Nowell and Hungerford identified chromosome change associated with leukemia and named it “Philadelphia (Ph) chromosome”, that was a breakthrough in cancer biology and CML (82, 83). In 1973, Rowley had described about the

reciprocal chromosomal translocation between the long arms of chromosomes 9 and 22 in the Philadelphia chromosome (84). In later years, involvement of proto-oncogene ABL on chromosome 9 and the previously unidentified BCR gene on chromosome 22 was revealed and deregulated Abl tyrosine kinase was identified as a pathogenic factor for the development of CML (85). Daley in 1990, reported the ability of BCR-ABL to transform primary myeloid cells and induce a CML-like disease in mice and confirmed that the constitutively active BCR-ABL tyrosine kinase was the underlying pathogenic factor in the development of CML (86).

2.3.3 Epidemiology

The global incidence of blood malignancy is 6% and CML accounts of about 20% of all leukemia affecting adults. CML incidence rates are 1.5-2.8 cases per 100,000 inhabitants (87). In India, the incidence rates are 0.8 to 2.2 per 100,000 population (88). Although CML is not amongst the leading sites of cancer worldwide, its increasing incidence is a cause of concern globally. Researchers estimate that by 2020, nearly 2 lakh people worldwide will be diagnosed with CML (9).

2.3.4 Stages of CML

Stages of CML can be helpful in selection of treatment option and for prediction of survival. Being a blood malignancy, staging of CML is quite different from other malignancy. Unlike other cancers, the staging depends on type and number of cells in the bone marrow and blood. Other consideration includes complete blood count, age of the patient, size of spleen and liver.

Chronic phase (CP): Patients in the chronic phase typically have less than 10% of immature cells or blasts in their blood or bone marrow samples with mild symptoms. Nearly, 40% of patients are asymptomatic and diagnosed accidentally by abnormal blood count. Patients may have symptoms such as fatigue, weight loss and night sweats. This phase usually has a long latency, typically of 4-5 years of onset, and majority of the cells in peripheral blood are neutrophils. Most of the patients are diagnosed in the chronic phase and usually respond to standard therapies (77).

Accelerated phase (AP): The patients in the accelerated phase have more than 15% but less than 30% blasts or promyelocytes, 20% or more basophils, low platelet counts (100 x 1,000/mm^3 or less) in their blood samples. This phase is considered a transient phase, lasts for 6-9 months. The patient may develop acute leukemia with severe infections, bleedings and symptoms of anemia. Treatment response is poor in this phase as compared to the chronic phase.

Blast phase or blast crisis (BC): In this phase, 30% or more blast cells have been noted in bone marrow and blood samples of a patient. The blasts also show lymphoid phenotype and the disease transforms from myeloid to lymphoid side. The blast cells form large clusters in the bone marrow and may spread to tissues and organs beyond the bone marrow. If remain untreated, the patient may survive only for 3-6 months (89).

2.3.5 Etiology and risk factors

The etiology of the disease is unknown, but it has been directed that ionizing radiation can contribute to disease development, since the occurrence of CML increases in persons exposed to high doses of ionizing radiation (90). The incidence of CML amplified after treatment with ^{131}I for thyroid carcinomas (91, 92).

CML is the disease of middle age or elderly age of people with a median age of 50-60 years, very rare in children. The male to female ratio is of 1.2-1.7 indicating that males are affected more frequently than females (10, 93).

2.3.6 Signs and symptoms (78, 94)

The patients with CML are often asymptomatic; although some may have developed the following signs and symptoms:

- Fever and fatigue
- Night sweats and malaise
- Bone pain due to a packed bone marrow
- Enlarged spleen and liver
- Loss of appetite due to abdominal fullness or satiety

- Abdominal pain
- Weight loss

2.3.7 Diagnosis and clinical manifestations

The complete blood count of peripheral blood shows an increased number of white blood cell (WBC) counts, mainly due to segmented neutrophils and neutrophils in different stages of maturation such as myelocytes, metamyelocytes. Basophilia is invariably present and many may develop eosinophilia as well. Most of the patients may have mild anaemia. The platelet count is generally normal or increased in chronic phase. The bone marrow is packed due to increased numbers of neutrophils and their precursors and spleen are enlarged. Depending on the stage of disease, blasts may vary from 5% to 15-19% and sometimes to 25-30% in chronic, accelerated and blastic phase respectively. CML can also be diagnosed with performing the bone marrow aspiration and biopsy. A needle is used for aspiration and to remove a sample of the fluid containing bone marrow cells. A bone marrow biopsy is used to remove a small amount of solid tissue using a needle. The cells, tissues, and organs are evaluated for the diagnosis of the disease.

The presence of Ph chromosome or BCR-ABL gene product is spotted in blood and bone marrow cells by conventional cytogenetic testing such as FISH and RQ-PCR. In fluorescent in situ hybridization (FISH) method, the alteration in fluorescence is noted at the point of colocation or fusion. This analysis can be performed on either metaphase or interphase cells. In RQ-PCR (Reverse transcriptase quantitative-polymerase chain reaction), the presence of BCR-ABL fusion gene and other molecular abnormalities can be detected (95, 96).

Flow cytometry is also done to identify cases with unrecognized progression to lymphoid blast crisis by their phenotypic features, while conventional karyotyping may identify additional cytogenetic abnormalities such as cytogenetic clonal evolution. Sometimes, computed tomography (CT) scan and ultrasound examination are also used to examine the effect in other parts of the body and to measure the size of the spleen in people with CML (97).

2.3.8 Pathophysiology of CML

In CML, the Ph chromosome has been formed as a consequence of reciprocal translocation t(9;22)(q34;q11) in long arms of chromosome 9 and 22 in hematopoietic stem cells (Fig. 2.4). The Abelson murine leukemia oncogene (c-ABL) on chromosome 9 fuses with the Breakpoint cluster region (BCR) gene on chromosome 22 and forms a fusion oncogene BCR-ABL, located on truncated chromosome 22 called Ph chromosome. Although, the reason is unknown but genomic instability could be speculated as an underlying cause for the formation of Ph chromosome (98).

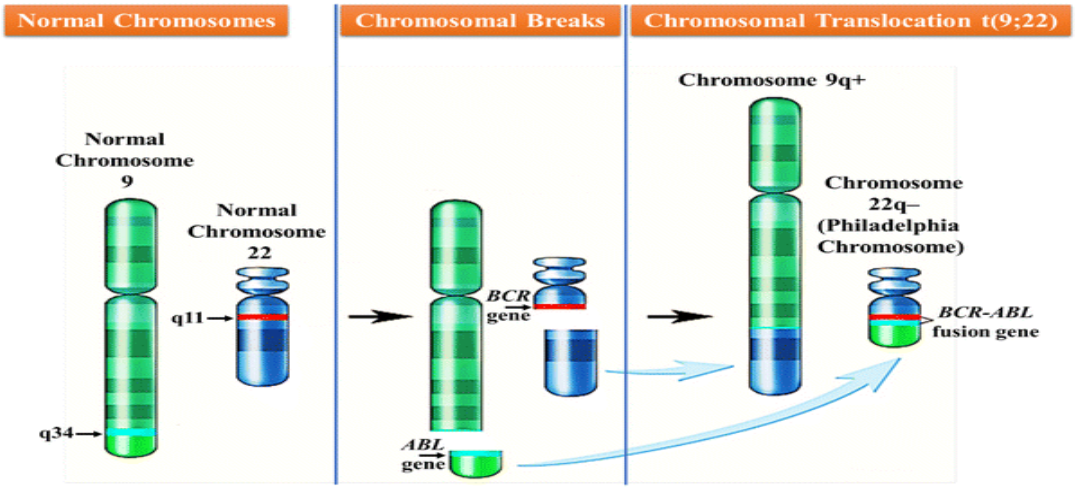


FIGURE 2.4: Reciprocal translocation of BCR-ABL gene and formation of Philadelphia (Ph) chromosome (98).

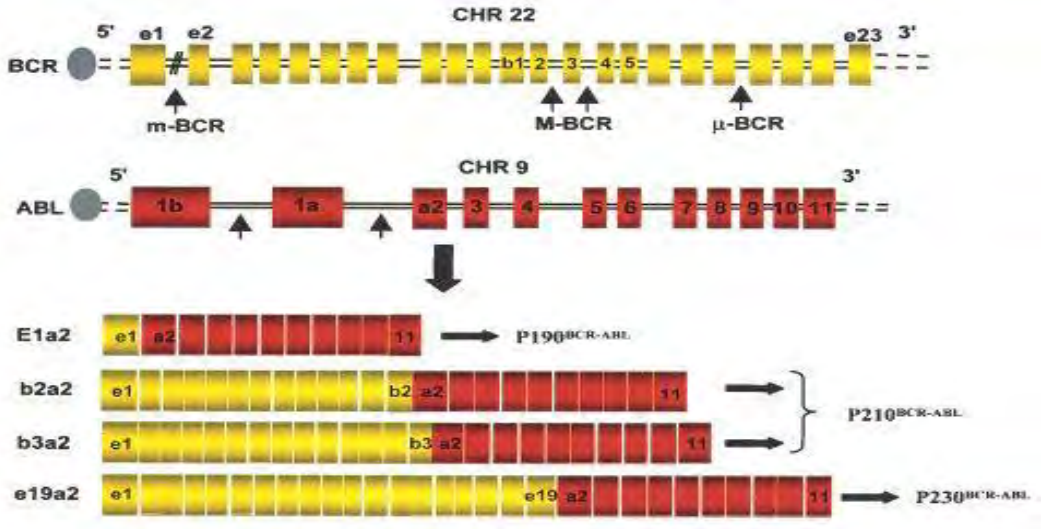


FIGURE 2.5: Different breakpoint regions identified on Ph chromosome resulting the formation of chimeric proteins (99).

The t(9;22)(q34;q11) indicates breaks happening in region (3), band (4), sub-band (1) of ABL gene on the long arm (q) of chromosome 9 and region (1), band (1), sub-band (2) of BCR gene on the long arm (q) of chromosome 22. This BCR-ABL oncogene commonly encoded in a protein, depending on the precise location of the breakpoints in the BCR and ABL genes the molecular weight of the BCR-ABL protein can be of different sizes such as 190 kDa, 210 kDa or 230 kDa (Fig. 2.5). The ABL gene contains 11 exons among which the first exon has two variants: 1a and 1b, which derive from alternative splicing. The breakpoint in the ABL gene usually occurs before exon 2 (1a).

The ABL gene codes a constitutively expressed, non-receptor tyrosine kinase having a molecular mass of 145 kDa. The ABL exons a2 to a11 were put next to the 5' part of BCR known as major breakpoint cluster region (M-bcr) of the BCR gene, located between exon b1 to b5 and spreads over 5.8 kb (100). Two fusion transcripts, b2a2 and b3a2 were formed, translated into a chimeric protein of 210 kD named p210BCR-ABL (101). In BCR-ABL positive CML, 95% of cells have either b2a2 or b3a2 transcripts and in 5 % alternative splicing causes the expression of both fusion products (102). In the minor breakpoint cluster region (m-bcr), the breakpoint upshot in a fusion transcript called e1a2, gives escalation to protein of 190 kD (p190BCR-ABL) which is common in adults and children with Ph-positive ALL (103). There is also breakpoint in the micro breakpoint cluster region (µ-bcr) which creates another fusion transcript (e19a2), translated into a protein with 230 kD (p230BCR-ABL) which is associated with neutropenic CML and some rare cases of CML (104-107).

The proteins are constitutively active tyrosine kinase that phosphorylates multiple substrates along with tyrosine residue and activates certain pathways such as PI-3k, RAS, RAF, JNK, MYC and STAT etc., leading to enhanced proliferation, inhibition of apoptosis and abnormal cellular adhesion (Fig. 2.6). This malignant clone has ability to multiply, suppress and replace normal haematopoiesis (108). Transfer of BCR-ABL1 gene in mice, also developed CML-like disease (86).

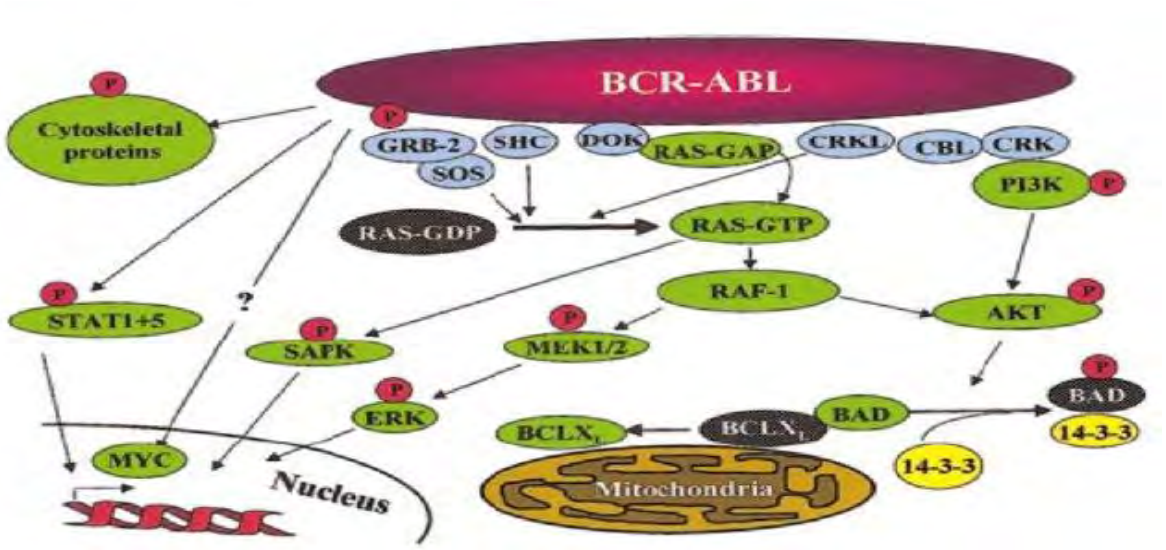


FIGURE 2.6: Downstream signaling pathways of BCR-ABL oncogene in CML (99).

There is also a risk of progression of CML into AML and ALL and due to possibility of aberrations and duplication of the Ph chromosome. Translocation on Ph chromosome is also found in varying degree that is 2-20 % in ALL and AML patients.

2.3.9 Role of VEGF in CML

Angiogenesis is the formation of new blood vessels from the pre-existing vessels, which plays an important role in embryonic development, wound healing, tumor growth and metastasis. As the tumour grows, the central tumour cells become hypoxic. Angiogenesis increases the growth of the tumour beyond a few millimeters in diameter, because of successful establishment of an

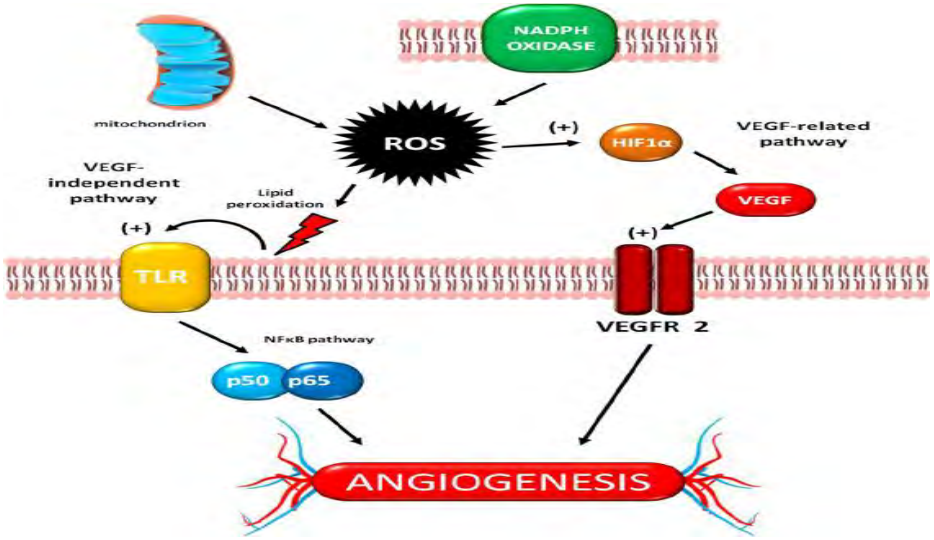


FIGURE 2.7: Role of ROS in regulation of VEGF and angiogenesis (109).

efficient blood supply. The tumour initiates its own blood supply called “angiogenic switch”, which further supplies oxygen and nutrients by the vasculature and are crucial for cell function and survival. Angiogenesis activates the cascade of consecutive processes emanating from microvascular endothelial cells which proliferate and degrade the endothelial basement membrane of parental vessels, migrate, and penetrate into host stroma and initiate a capillary sprout (109).

The recruitment of endothelial cells during angiogenesis and the formation of vascular tumors are governed by the breakdown of basement membranes. The event occurs under the control of numerous activating factors that were shown to be overexpressed in certain cancers (110).

Progression of tumor growth is characterized by induction of proangiogenic factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and VEGF receptors (VEGFR) in the growing endothelial cells (ECs). VEGF is one of the most important angiogenesis growth factor that stimulates permeability, proliferation, migration and tube formation of ECs. Moreover, it also encourages the mobilization of bone marrow-derived endothelial progenitor cells. Stimulus such as hypoxia, growth factors, cytokines and oxidative stress can upsurge VEGF expression in tumor cells, which is associated with exaggerated micro-vessel counts and poor prognosis in many cancers (111-113).

Beside a key factor in angiogenesis, VEGF is also a pivotal regulator in haematological malignancies that stimulates the proliferation and self-renewal of myeloid progenitors. ROS, predominantly hydrogen peroxide is formed by Nox family of enzymes, mediates the signal transduction by human VEGFR-2 and several redox signaling pathways (Fig. 2.7) that lead to induction of transcription factors and genes involved in angiogenesis (114).

There are increasing evidences indicating that VEGF also plays an important role in the development and progression of CML. The numbers of blood vessels were measured in patients with acute and chronic leukemia and myelodysplastic syndromes, the highest number of blood vessels and largest vascular area were found in patients with CML. Further, bone marrow biopsies and the plasma levels of vascular endothelial growth factor (VEGF) and other related growth factors were determined in which highest plasma level of VEGF was noted in patients with CML, suggesting the role of vascularity and angiogenic factors in CML and leukemogenic

process (19). In addition, the concentration of VEGF in bone marrows was found to inversely correlate with the prognosis in patients having chronic phase of CML (115).

Angiogenesis plays an important role in the growth, progression and metastasis of solid tumors. Certain types of leukemia also shares similar profile to solid tumors, where the bone marrow has been reported to have high VEGF expression and microvessel density (MVD). However, the exact mechanism triggering VEGF expression in hematolymphoid tumors is unknown; different mechanisms similar to those observed in solid tumors are anticipated. In leukemia, NF-κB is activated by the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. The pathway is crucial for the several aspects of cell growth, survival and apoptosis. PI3K/Akt activation has been associated in both the pathogenesis and the progression of neoplasms, which further activate the VEGF secretion and endothelial cell activities in leukemic cells (116).

In another study, the immunohistochemistry of bone marrow section from patients in different phases of CML was performed to identify the expression of VEGF protein. The patients with CP-CML show the distribution of VEGF pattern parallel to that of normal marrow. VEGF was abundantly expressed in myeloid progenitors and megakaryocytes and less abundantly in mature granulomonocytic cells, whereas erythroid cells did not show VEGF expression. In BC-CML, myeloblasts expressed significant amounts of VEGF. The significant mRNA expression of VEGF was detected in leukemic cells in myeloid BC-CML, whereas in lymphoid BP-CML it was absent, suggesting higher expression of VEGF in immature myeloid cells in CML. The magnitude of VEGF expression depends on the phase of the disease and the cell type intricate in disease progression (117).

Number of studies has established the incidence of high VEGF levels in samples from patients with leukemia. The prognostic significance of VEGF expression has been investigated in chronic and acute myeloid leukemia. In patients with CML, a cellular VEGF protein level was highly expressed in all phases and too has significant influence on survival. However, the mechanism behind this observation is unclear (118).

In patients with CML, very high MVD has been noted in the bone marrow. Moreover, the plasma levels of VEGF have also been shown to be significantly higher in CML patients compared with normal controls which correlate with bone marrow vascularity (119). Besides

its role as an essential regulator of angiogenesis, VEGF triggers growth, survival, and migration of leukemic cells and plays a pivotal role in hematopoiesis. Therefore dysregulation of VEGF expression and signaling pathways plays an important role in the pathogenesis and clinical features of hematologic malignancies (120).

Some studies have suggested the role of BCR-ABL gene in the expression of critical effector molecules, including pro-angiogenic growth factors. However, little was known about the mechanisms and signaling pathways contributing to BCR-ABL induced expression of angiogenic growth factors in CML cells. It was tempting to speculate the BCR-ABL dependent VEGF production in CML cells. Mayerhofer *et al*. have suggested the role of BCR-ABL in induction of VEGF production in CML cells through a pathway involving PI3-kinase and the mammalian target of rapamycin, mTOR (21). Moreover, it was also reported that HIF-1α was involved in BCR/ABL-dependent expression of VEGF in Ba/F3 cells in which BCR/ABL was found to induce HIF-1α mRNA expression in Ba/F3 cells (121).

Apart from VEGF, a number of additional angiogenic growth factors have been reported to be produced by leukemic cells in patients with CML and thereby contribute to the pathogenesis of CML (122). The expression of BCR /ABL in murine FL5·12 cells was found to be related to an up-regulated expression of VEGF and matrix metalloproteinase-2 (MMP-2) (123).

2.3.10 Role of MMPs in progression of CML

Metastasis is an event associated with approximately 90% of deaths related to human cancers. Collagen is the major structural protein of all tissues and the chief obstacle of migration of tumour cells. Degradation of collagen by collagenolytic enzymes, has been postulated a pivotal role in facilitating the dissemination of cancer.

During cell migration, components of the basement membrane and extracellular matrix (ECM) are rearranged and degraded, leading to invasion of malignant cells to surrounding tissue and in systemic circulation. The proteinases secreted from tumor cells itself has tendency to rearrange and degrade the ECM (124). Matrix metalloproteinase (MMPs) are the group of family of closely related metal-dependent endopeptidases, especially proteinases that has been associated with categorized by their substrate specificity and expression patterns many cancers (27). Since

collagen represents the major structural protein of all tissues and the chief obstacle of migration of tumour cells, it has long been postulated that collagenolytic enzymes play a pivotal role in facilitating the dissemination of cancer. The expression and activity of MMPs are amplified in almost every type of human cancers, which further correlates with advanced tumour stage, increased invasion and metastasis and poor survival. The MMPs are secreted as inactive zymogens (pro MMPs) and are kept inactive by an interaction between a cysteine-sulfhydryl group in the pro-peptide domain and the zinc ion bound to the catalytic domain. Activation requires the proteolytic removal of the pro-peptide domain. Endogenous inhibitors known as tissue inhibitor of metalloproteinases (TIMPs) carry out the extracellular regulations of MMPs. Besides TIMPs, cytokines and growth factors also play an important role in the modulation of MMP secretions in different tissue especially during inflammation and wound healing (Fig. 2.8).

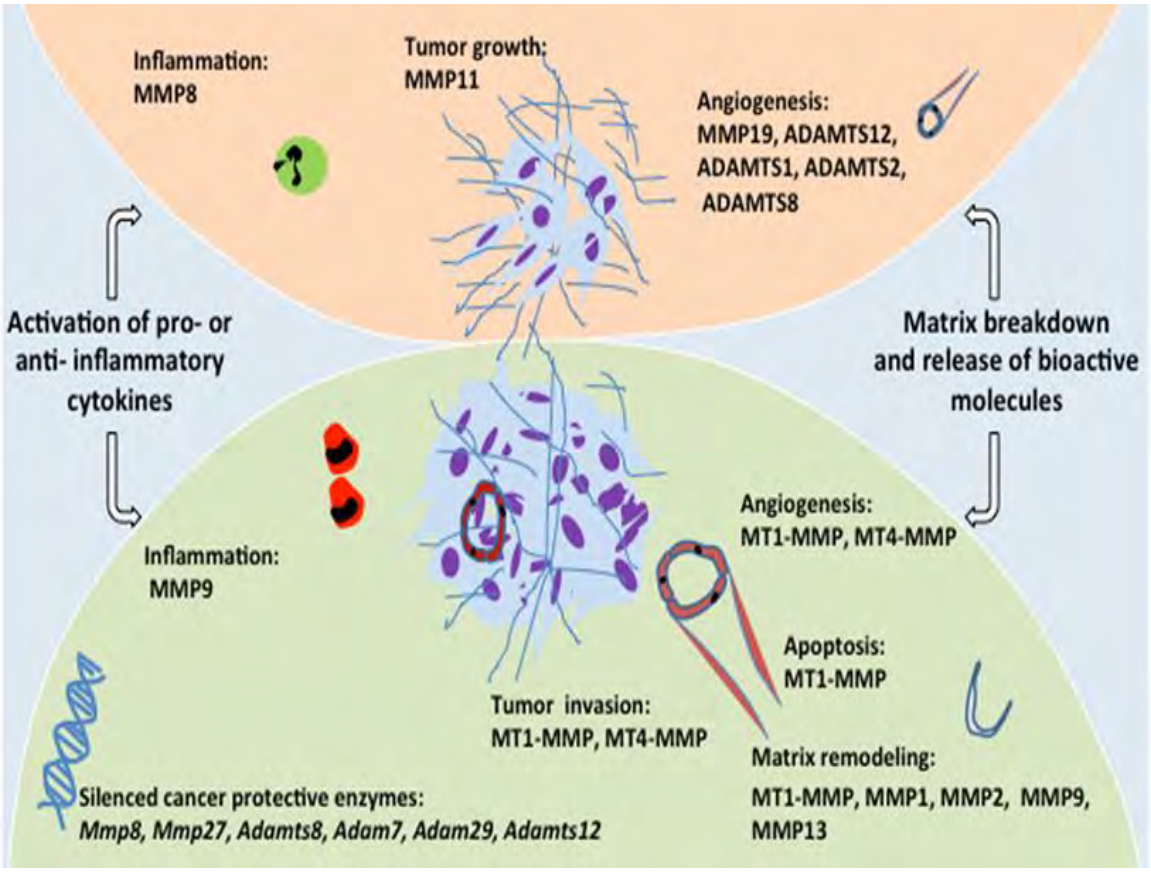


FIGURE 2.8: Role of matrix metalloproteinases (MMPs) in tumor development (125).

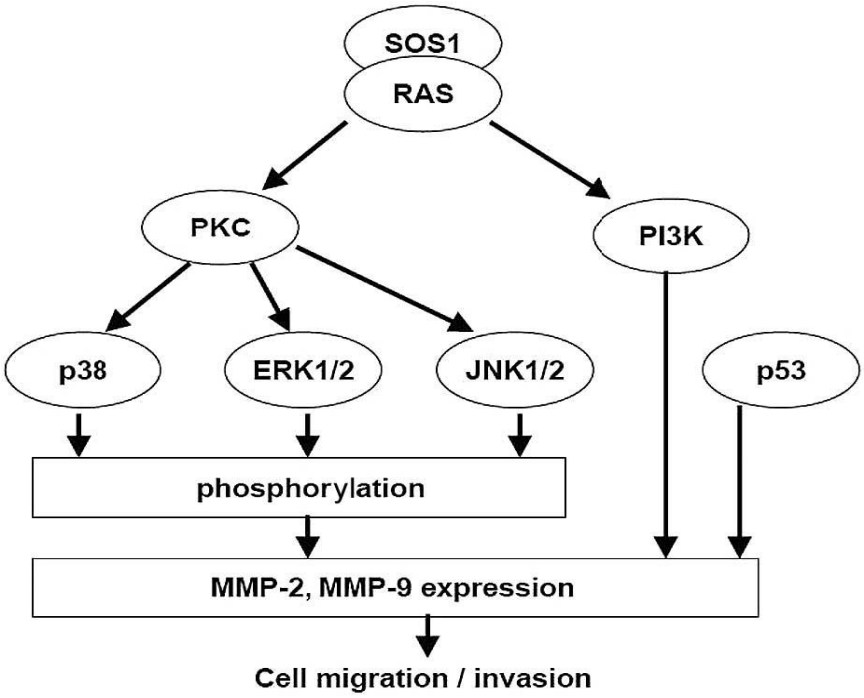


FIGURE 2.9: Pathways involved in cell migration and invasion (126).

Among all types particular note are the gelatin-degrading MMPs, of which MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are known to play a key role in cancer cell invasion and metastasis via the degradation of type IV collagen, a key component of the basement membrane (127). MMP-9 is involved in the release of extracellular matrix bound or cell-surface–bound cytokines, such as vascular endothelial growth factor, the promoter of angiogenesis (Fig. 2.9) (126).

In CML, an excess egress of leukemic cell blasts from the bone marrow into peripheral blood can transfer invasion into various organs and tissues. The cell surface implication between MMP-2/MMP-9 and integrins has been suggested to be involved in the growth and progression of CML (128). Moreover, several lines of evidence indicate the role of MMP-9 in CML pathogenesis (129, 130).

Studies have reported that, BCR/ABL oncogene induces the TGF-β1 which further upregulate the production of MMP-9 by PI3K/Akt/NF-κB/MMP9 signaling pathway in CML (24, 26). Moreover, it is found that VEGF stimulated the secretion of MMP-2 and 9 in BCR-ABL positive CML cells, suggesting its important role in the pathogenesis of the disease (27).

2.3.11 BCR-ABL and production of reactive oxygen species and genomic instability

The biological effects of Reactive oxygen species (ROS) vary extensively in different cells and include modulation of signaling pathways by altering the activity of cellular signaling. ROS contributes to several cellular functions and is known to play an important role in the regulation of cell proliferation, signal transduction, gene expression and maintenance of genomic integrity (Fig. 2.10).

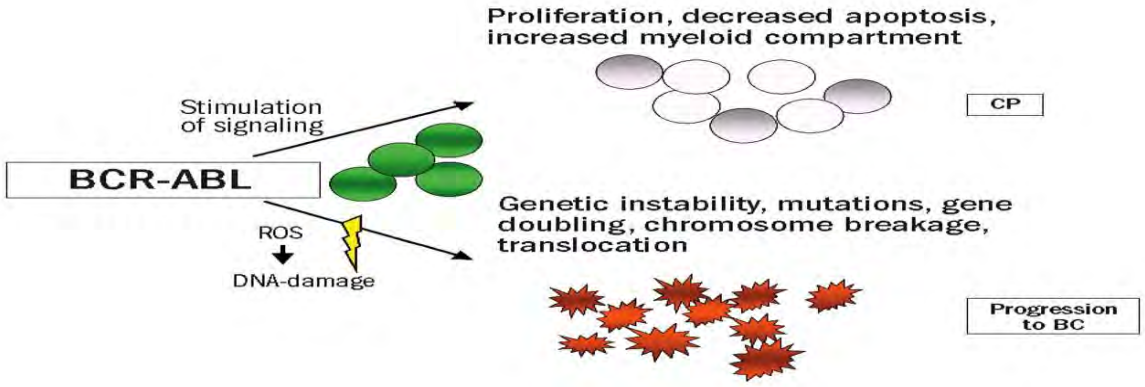


FIGURE 2.10: Role of ROS in progression of CML from CP-CML to BC of CML by promoting genetic instability (131).

Genomic instability can be defined as, the extent of DNA mutations and chromosomal rearrangements in the genome. In certain conditions, there is a causal relationship between the exposures to DNA-damaging factors with the involvement of genomic instability in cancer, which is unknown. ROS have capability to damage not only DNA, but also the proteins that repair the DNA, leading to increased genomic instability. The elevated level of ROS can be related to many cancers; however, it is hard to determine whether ROS belong to the reasons or consequences of the disease. Particularly, the ROS damages the DNA repair gene and increases accumulation of DNA damage upon the next cellular divisions leading to genomic instability with increasing the probability of acquiring a mutation in cancer-related genes. Deficiency of DNA repair would results in loss of control over intracellular ROS, leading to an elevated level of these species in cancer. Beside this, in certain cancers including CML, the source of ROS can attribute to some cancer-specific features. BCR-ABL was identified to stimulate ROS production in mouse-derived hematopoietic cells (29). Therefore, BCR-ABL, the root of CML, induces ROS production leading to oxidative stress, characteristic for countless cancers. As this

stress was induced by hydrogen peroxide in non-transformed hematopoietic cells, numerous events typical for BCR-ABL expressing cells happened. Consequently, that important work proposes two decisive facts. Firstly, CML can be introduced by anti-apoptotic and proliferative signaling of BCR-ABL, but the expansion of the disease can be related to oxidative stress prompted by BCR-ABL. Secondly, the progression of CML can be influenced by chemicals that modulate the ROS levels, known as antioxidants (132).

Tyr177 residue on the BCR-ABL protein was identified as a major regulatory site in ROS production pathway, of BCR-ABL-expressing cells. The overproduction of ROS in BCR-ABL positive cells was also found to be dependent on the PI3K/mTOR signaling pathway and PI3K activation itself, suggesting possible underlying mechanisms of mutual relationship between the activity of the BCR-ABL kinase and ROS production, which can be noteworthy for cancer biology and therapeutic targeting of the kinase in CML and other malignancies (133).

Further, leukemia progenitor cells (LPCs) have been reported to contain higher levels of ROS and oxidative DNA damage compared with their normal counterparts. ROS-induced genomic instability in CML-CP start in leukemic stem cells (LSCs) and continue in LPCs. Leukemia cells are tolerant to DNA damage due to BCR-ABL facilitated protection from apoptosis and modulation of the response to DNA damage. Moreover, nuclear localization of FOXO3a, a critical facilitator of resistance to physiologic oxidative stress, contained in the nuclei of LSCs in a murine model of CML-CP, proposing another mechanism of defence from the deadly effects of ROS (134). In some studies, PI3K has been reported to stimulate ROS in BCR-ABL leukemia cells (133).

2.3.12 Consequences of increased ROS in CML

ROS such as superoxide radicals ($^{\cdot}O_{2}^{-}$), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) are the generator of endogenous DNA damage and double stranded break. These radicals are thought to be formed through the electron transport reaction of mitochondria in which O_2 accepts a single electron, resulting in $^{\cdot}O_2$. Oxygen radicals are also produced by a family of NADPH oxidases (NOXs) of which 7 different isoforms (NOX1, 2, 3, 4, 5 Duox1/2) have so far been recognized (135).

The consequences of increased ROS in myeloid leukemia drop into 2 major categories—the first are the outcomes of nonspecific oxidative damage to biomolecules, the second are more specific effects developing from over activation of ROS signaling pathways. Nonspecific oxidative damage is the outcome of persistent elevated ROS levels and the exhaustion of antioxidant defences of cell. This promotes lipid peroxidation, increased intracellular pH and most significantly, oxidative DNA damage producing single- and double-stranded breaks in DNA via direct or indirect activation pathways. The arrangement of such an environment is tremendously favourable for tumorigenesis, and supports selection of those clones that are able to survive and flourish. Increased ROS may also have apparent effects in myeloid disease by altering the signaling in pathways that are regulated by ROS, particularly pro-proliferative and/or survival pathways (136).

ROS can be important for imatinib resistance, a first line agent for CML treatment by accumulation of genetic changes in blast phase, which suggest the role of ROS in CML progression (137). Further, it is reported that activated growth factor receptors increase the relative levels of intracellular ROS (138).

2.3.13 Experimental models of CML

Cell culture: Immortalized leukemic cell lines are extensively used in research and provide a system that is easy to work with, renewable, and has the benefit of carrying experiments at multiple sites using the same cell models. All available CML cell lines were established from peripheral blood or bone marrow of patients in advanced BC, but there are no cell lines that fully represent CML CP. Beside this, the cell lines may have the acquisition and selection of new genetic alterations and genetic drift because of extensive sub-culture which is an another drawback (139).

In this study, the K562 cell line was selected for all *in vitro* assays. The K562 cells are the CML cell line, derived from the pleural effusion of a patient in terminal blast crisis. The cells have been reported to carry the Philadelphia chromosome marker and were considered to represent the outgrowth of a CML clone. It has strong reactivity with erythrocytes and erythroid precursor cells, therefore it is known as human erythroleukemic cell line (140, 141).

Animal models of CML: The BCR/ABL oncogene has shown to deregulate tyrosine kinase transform cytokine-dependent lymphoid and myeloid hematopoietic cell lines to become independent of cytokine for survival and proliferation. *In vitro* studies on cell lines has demonstrated the involvement of BCR/ABL oncogene in many constitutively activated cell signaling pathways, several of which were demonstrated to be required for transformation. The animal models are needed to understand the pathophysiology of leukemia. The process of haematopoiesis is essential to be studied in the context of the living organism to glean the wealth of information.

BCR/ABL is associated with two different human leukemia such as B-cell acute lymphoblastic leukemia (B-ALL) and chronic myeloid leukemia (CML). B-ALL is characterized by a profound differentiation arrest in B-lymphoid expansion and overgrowth of marrow and hematopoietic organs with proliferating immature blasts. In contrast, CML is associated with increased production of neutrophils, with preservation of differentiation, and gradual evolution to acute leukemia in blast crisis.

The lack of an accurate *in vitro* model of BCR/ABL-induced CML, reflects the difficulty in establishing *in vitro* myelopoiesis; none of the cell line have ever been derived from chronic-phase CML patients. In the absence of an accurate model for CML-like leukemia, the involvement of BCR/ABL was indeed the principal cause of this disease was in some doubt. *In vivo* models are compulsory to understand the essential mechanisms and pathways through which BCR/ABL induces leukemia. It has great utility as platforms for testing of new therapies for the disease.

There are four distinct approaches to the modeling of Ph-positive leukemia in the laboratory mouse, *Mus musculus*. The models include:

I. Usage of BCR/ABL transgenic mice
II. Retroviral transduction of BCR/ABL into murine bone marrow followed by transplantation
III. Xenotransplantation of primary human CML cells in immunodeficient mice
IV. The propagation of BCR/ABL-transformed hematopoietic cell lines in mice

Usage of BCR/ABL transgenic mice and retroviral transduction of BCR-ABL into murine bone marrow by transplantation techniques are highly advanced techniques that lacks the feasibility at ordinary level. It also requires the advanced skills and expertise because of its complexity and need of retroviral vectors for successful transduction.

Xenotransplantation of human CML cells into immunodeficient mice: The severe combined immunodeficient (SCID) mouse lacks the functional B and T cells owing to a mutation. Normal and malignant, both human hematopoietic cells can engraft SCID mice. The persistent natural killer (NK) cell mediated immunity in host, creates relative trouble in establishing human myelopoiesis in SCID mice. It has inspired the use of strains lacking NK activity, such as bg/nu/xid mice and non-obese diabetic/LtSz scid/scid (NOD/SCID) mice, which are profoundly immunodeficient, with impairment of macrophage and NK function with superiority to SCID mice for engraftment of human myeloid cells. The NOD/SCID model system holds great promise for elucidating and understanding the characteristics of the clonogenic leukemia cell in CML. It helps for testing new therapeutic approaches for CML. It is plausible, that xenotransplantation might produce beneficial prognostic information when performed serially with samples from the same CML patient. However, variation in engraftment limits its usefulness. Secondly, it is necessary to be seen whether recipient mice engrafted with chronic-phase CML cells accurately recapitulate the pathophysiology of human CML, as the recipients do not appear to have circulating human myeloid cells or dissemination of human cells to liver or other extramedullary sites. Moreover, the recipients are not being able to efficiently support, long-term human normal or CML-derived myelopoiesis. Factors other than host immune responses, such as the marrow microenvironment, may account for this.

In this study, C57BL/6 strain of mice was selected and the immunosuppression was done with the combination ketoconazole, cyclosporine and cyclophosphamide. The particular species was used for subcutaneous xenograft and tail vein injection model of CML because of its high take rate, easy availability, reduced mortality and low costs. Further, the usage of nude mice may not accurately reflect true disease progression because they lack of immune cells which play a critical role in tumorigenesis.

Propagation of BCR/ABL-transformed hematopoietic cell lines in mice: Hematopoietic cytokine-dependent cell lines are derived from *in vitro* culture of bone marrow cells from mouse,

and are undeniably dependent on particular cytokines for survival and proliferation. Upon cytokine withdrawal, the cells undergo apoptosis. On introduction into immunodeficient or syngeneic immunocompetent mice by subcutaneous or intravenous injection, the cells fail to form tumors or induce leukemia, because the ambient levels of cytokine *in vivo* are insufficient to allow cell survival.

Intravenous injection of BCR/ABL-positive cytokine-independent cells into syngeneic recipient results in cell proliferation and fatal leukemia like disease. The mice exhibit all-encompassing infiltration of marrow, spleen, liver, and often other organs with cells that phenotypically resemble the input cells and express BCR/ABL protein, with the cause of death due to either bone marrow failure or severe anaemia. Use of immunodeficient mice serves merely as 'living incubators' for the autonomous growth of BCR/ABL transformed cells, and the disease process therefore does not represent acute leukemia. In addition, the model has the advantage that the cells hired are permanent cell lines that can be manipulated and extensively characterized *in vitro*, and tested for *in vivo* leukemogenicity (142, 143).

2.3.14 Conventional Treatment modalities of CML

Stem cell transplantation: Allogeneic stem cell transplantation therapy is required for complete eradication of the disease. Allogeneic stem cell transplantation is considered as the first line treatment for CML in children as a best curative therapy. The procedure includes administration of high (marrow ablative) doses of chemotherapy with or without radiation (pre-transplant conditioning) to eradicate leukemia cells, shadowed by the infusion of healthy hematopoietic stem cells to restore haematopoiesis. However, transplantation related mortality, lack of bone marrow donors or identical siblings and the age of patient limit its availability. It has reduced its contribution for the improvement of life expectancy in CML patients. Rigorous chemotherapy followed by autologous stem cell transplantation might also lengthen the survival period, and few patients may reach significantly extended periods of Philadelphia chromosome (Ph) negativity. Further, because of the cost concern, it is still out of reach for the most (46).

Chemotherapy: The symptoms of CML were being controlled with oral chemotherapy since 1950s. However, different treatment strategies emerged later, which permit a choice between two major aspects of the treatment:

1. Usage of mild chemotherapy for palliation and symptomatic relief: In this, the drugs are given to the patients for symptomatic relief, without attempting to cure them. The drugs include busulfan, hydroxyurea and interferon-alfa (IFN- α) in low dose.

2. Intensive chemotherapy for tumour regression or Ph negativity: This alternative is used to prolong the time to blast crisis, occasionally with the anticipation of curing the patient. The dreadful side effects and increased initial mortality resulting from such therapy must be pondered against the reduced long-term survival associated with symptomatic treatment.

Treatment response: There are some factors that have been identified at diagnosis and during treatment, which can aid in predicting the outcome of treatment (Table 2.1).

TABLE 2.1: Treatment response in CML

Hematologic response	
Complete	B-Platelets < 450x109/L B-Leukocytes < 10x109/L B-Basophils <5 % No immature cells in peripheral blood No palpable spleen
Cytogenetic response	**% Ph positive cells**
Complete	0 %
Partial	1 – 35 %
Minor	36 % – 65 %
Minimal	66 – 95 %
No	>95 %
Molecular response	**BCR-ABL transcripts**
Complete	No detectable transcripts
Major	3-log reduction

Busulfan: Patients with CML were anciently treated with Busulfan as the first alkylating agent to be effective in CML. Before Busulfan, whole body or splenic radiation was effective palliation for CML. Busulfan mainly acts on the primitive stem cell and its effects persisted even after several weeks of discontinuation of the therapy. Despite of standard dosage of the drug, some patients have developed permanent marrow hypoplasia or marrow suppression. It has developed irreversible gonadal failure, pulmonary and marrow fibrosis, skin pigmentation and hypoadrenalism in all patients, which has limited its use in treatment of CML (144, 145).

Hydroxyurea: After Busulfan, Hydroxyurea or a hydroxycarbamide, a ribonucleotide reductase inhibitor was selected, that inhibited the DNA synthesis. It was used for the treatment due to its cytotoxicity and rapidity of action with added low adverse effect profile. The side effect includes anorexia, skin atrophy, alopecia, macrocytosis, megaloblastic changes in the marrow, and rarely, skin or mucosal ulcers. It worked on relatively late myeloid progenitors compared to Busulfan, which accounts for the prompt recovery of the leukocyte count and has reduced the splenomegaly [95]. Despite of its superiority over Busulfan, Hydroxyurea failed to eradicate the Ph clone and disease progression.

Interferon-alfa (IFN-α): Later, Interferon-alfa (IFN-α), a naturally occurring cytokine came in to the picture in the early 1980s. It was the only drug, capable to induce Ph negativity without inducing myelosuppression, therefore it as considered as the treatment of choice for newly diagnosed CP-CML patients. There was substantial evidence of prolonged survival by postponing blastic phase. Complete hematologic response rate was seen in up to 80% of cases, and major cytogenetic responses > 66% in up to 38% of selected, newly diagnosed patients. Several clinical trials suggested the superiority of interferon-alpha over busulfan and/or hydroxyurea therapy for the treatment of CML. However, persistence of BCR-ABL transcripts in some patients restricted its superiority for the treatment (36).

Tyrosine kinase inhibitors (TKIs): Perhaps, one of the most exciting incidences in the treatment of CML is the development of tyrosine kinase inhibitors. In early 1990s , the researchers have identified that the BCR-ABL gene controls the activity of tyrosine kinase receptor and provided the rationale for the development of target specific tyrosine kinase inhibitors for therapeutic use (8). Treatment with Tyrosine kinase inhibitors (TKIs) therapy has revolutionized and transformed the prognosis of patients with CML over the last 20 years.

Discovery of Imatinib was proved a new paradigm in oncology, with implications much beyond the realm of CML (37). Later, the other TKIs such as Nilotinib and Dasatinib were approved by the FDA, for the treatment of chronic phase of CML. The TKIs has shown profound benefit in survival and quality of life for both adult and paediatric patients by interfering with the tyrosine kinase receptors and signal transduction pathway of cell proliferation and thereby blocking proliferation of the malignant clone (38, 39).

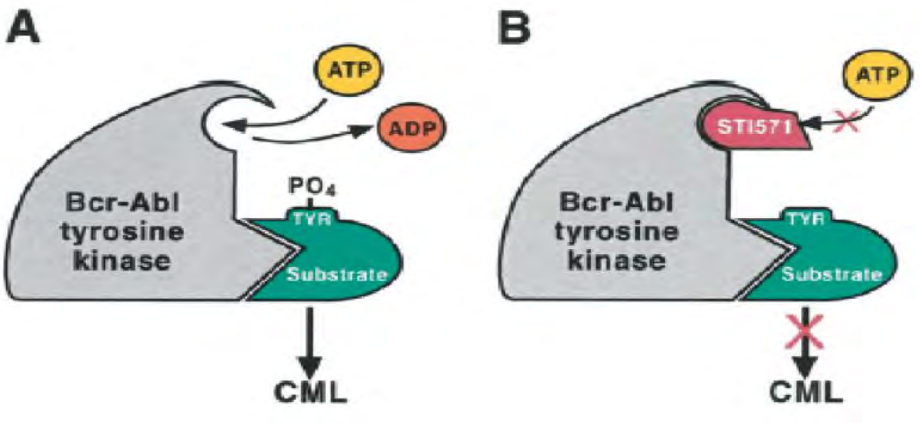


FIGURE 2.11: Mechanism of action of tyrosine kinase inhibitors (8).

(i) Imatinib: Imatinib or STI-571 has been used as a frontline therapy for treatment of CML, with stable major molecular response (MMR) in nearly 60% of patients, whereas poorly tolerated in about 20% and no response in remaining 20% of patients (40). Imatinib substantially decreases the number of CML cells in, resulting in a complete hematologic response (CHR) in almost all and a complete cytogenetic response (CCyR) in the vast majority of patients. Despite of its efficacy, inferior response or loss of response with Imatinib has been noted in majority of patients nowadays. It might be due of failure in attaining adequate blood levels, insufficient cellular uptake by reduced activity of the organic cation transporter 1 (OCT-1) influx pump and resistance induced by great range of kinase domain mutations, resulting in poor tyrosine kinase inhibition compared to Nilotinib and Dasatinib. The side effect comprises excessive fluid retention, skin rash, musculoskeletal pain, gastrointestinal disturbance including nausea and diarrhoea, muscle cramps, organ toxicities (41).

(ii) Nilotinib: It is used as second-line therapy for CML. It is structurally similar to Imatinib and binds to the kinase domain of BCR–ABL, with greater affinity than Imatinib (42).

Nilotinib is less vulnerable to kinase domain mutations, although some major mutations are as frequent as Imatinib. A significant concern related to the use of Nilotinib is the occurrence of hyperglycaemia, hypercholesterolemia and vascular events, including peripheral arterial occlusion, coronary artery disease and cerebrovascular disease. So, it is definitely an issue to keep in mind when selecting Nilotinib for a patient with risk for diabetes and atherosclerosis and other cardiovascular complications.

(iii) Dasatinib: It is an oral dual kinase inhibitor which has in the arrived the clinics in 2003. Unlike imatinib, dasatinib appears to inhibit the enzymatic activity of the BCR–ABL1 oncoprotein irrespective of the position of the BCR–ABL1 activation loop, and it targets a much broader range of tyrosine kinases. Dasatinib is more promiscuous than imatinib and nilotinib and was distinguished to overcome most mechanisms of resistance to imatinib, except T315I mutation. Dasatinib shares similar potency to nilotinib but has a relatively short half-life, suggesting its more frequent administration in daily doses. The numbers of kinase domain mutations are similar of Imatinib and Dasatinib. The incidence of pleural effusions and pulmonary arterial hypertension are the major drawbacks of the drug.

(iv) Bosutinib: It is approved since 2013 for treatment of CML patients in CP, AP and BC phases and who are unresponsive to treatment with imatinib, dasatinib or nilotinib (146). Bosutinib inhibits BCR-ABL as well as other tyrosine kinases. It inhibits many mutations except the T315I mutation. Elevated liver enzymes and gastrointestinal problems including diarrhoea, vomiting and nausea are the most frequent side effects of bosutinib (147, 148).

(v) Ponatinib: It is the latest third generation TKIs that blocks mutated BCR-ABL and used as drug of choice in patients that are resistant to other TKIs (43, 149). It is the only TKI with activity against the T315I mutation. However, it has been also reported to produce hypertension and arterial thrombotic events (44, 45).The associated risk or side effects with TKIs is irreversible and reserves its use for patients harbouring the resistance.

2.3.15 Failure of Chemotherapy

BCR-ABL dependent and independent mechanisms have been proposed for failure of chemotherapy (150). The first reason is reduced supply of drug to the target or it may not reach the target because of poor patient adherence, poor absorption, enhanced elimination by liver and kidneys, decreased drug influx or increased drug efflux. Second, the target may get mutated

which may lead to a conformational change in the BCR-ABL protein that partially or totally hinders the binding of TKI to the target. The most bothersome point mutation is the Threonine 315 Isoleucine mutation (T315I) that makes the cells completely resistant to imatinib, nilotinib and dasatinib (151).

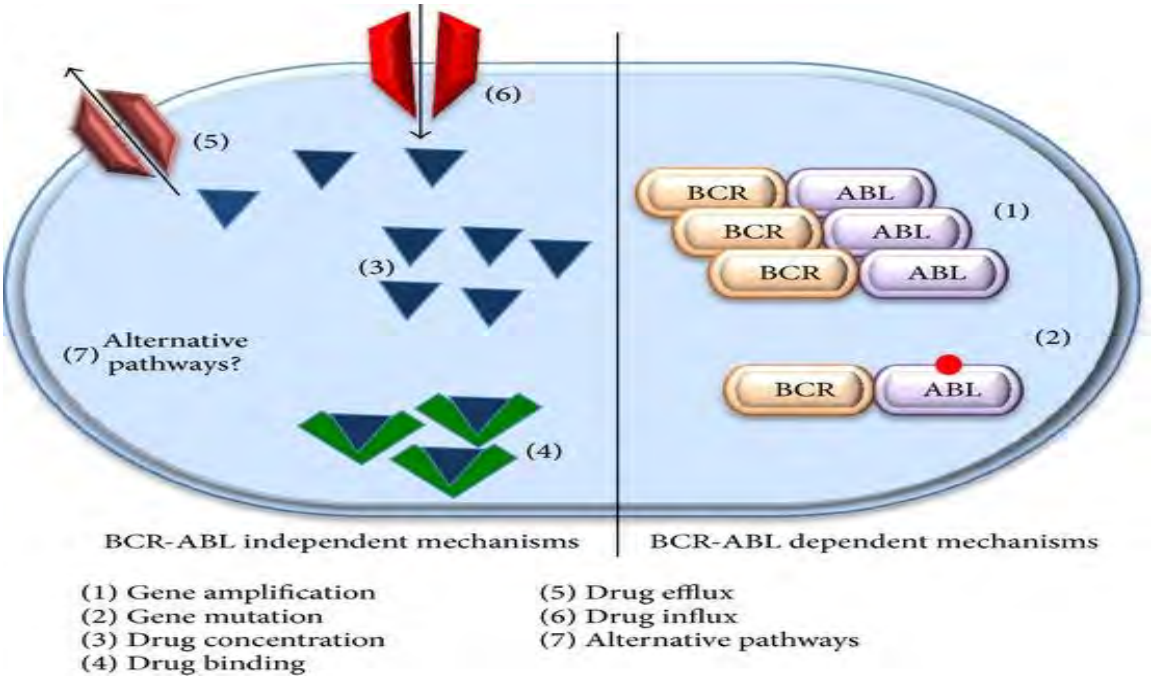


FIGURE 2.12: Possible mechanisms for failure of chemotherapy (150).

2.3.16 Prognosis and survival

The prevalence of CML continues to increase in the rapidly aging population. Additional improvement in survival is expected. In the pre-TKI era, CML was associated with poor prognosis and a short survival time, while a small number of younger patients were cured by bone marrow transplantation, the remaining were associated with considerable treatment related mortality and morbidity. With the discovery of the TKI imatinib in the early 2000s, the survival and quality of life has rapidly increased and is currently indicating a 10-year relative and overall survival (OS) of nearly 83.3 %. Almost half the patients (48.3%), randomly assigned to imatinib has completed the study treatment with imatinib and 82.8% had a complete cytogenetic response (152).

Discontinuation of TKIs in patients CML has shown remission of disease. In a trial Stop Imatinib (STIM), in patients on imatinib with ongoing complete molecular response for longer than 2 years who then stopped treatment, the risk of relapse was investigated. 100 patients had a median follow-up of 50 months and were watched closely for indication of molecular relapse. Largely, 61% experienced relapse at a molecular level, with 95% of the events occurring within 7 months after stoppage of imatinib (153). At 48 weeks after stopping nilotinib, 98 patients (51.6%) remained in MMR or better (154).

2.4 Plants as anticancer agents

Based on observation and experience, humans have cured their majority of diseases using plants, roots, stems, or fruits since ancient eras. Natural products are gaining importance for treatment in a wide variety of human diseases because of efficacy, safety and cost effectiveness. The usage of plants and its secondary metabolites have been reported for its potential antibacterial, antidiabetic, immunomodulatory and antifungal activities. Apart from all these activities, plants have been also reported for its potential antioxidant and anticancer properties (47).

Nevertheless, conventional therapy is divided between one derived from plants, and those synthesized or modified in laboratories. In certain diseases like cancer, the use of conventional medicine does not offer the expected outcomes in such cases the usage of plants as an alternative therapy is adequate and successfully applied (70). Plants contain a large number of bioactive metabolites with anticancer effects that acts through different mechanisms of action, affecting different stages of carcinogenesis. Therefore, their use could be effective for chemoprevention and complementary treatment. About 25% of currently used drugs in the world are isolated from plant derived secondary metabolites and their derivatives and over 3000 species of plants have been known for its anticancer properties. Secondary metabolites such as alkaloid, Flavonoids, Glycosides, Tannins, Resins and volatile oil show considerable activity for treatment of all type of cancers including leukemia and lymphoma (49). Similarly, some mechanisms of action of the metabolites can also favour the outcome to the modern chemotherapeutic agents and reduce the resistance associated with chemotherapy, number or intensity of side effects, by improving the quality of life of the cancer patients. Enormous studies have documented the efficacy of essential oils and their chemical constituents as source of new bioactive molecule against

treatment of various cancers. The use of bioactive extracts or metabolites derived from plants in suitable doses and form could be an important stratagem for the prevention and treatment of cancer. Therefore, a greater number of in vitro, in vivo, and clinical investigations are obligatory to describe the mechanisms of action of the metabolites, which would allow choosing the right metabolite in treatment of cancer (47, 69).

Plants have been also known as potent antioxidants or free radical scavengers. Generally, endogenous antioxidants balance the level of ROS and prevent the occurrence of chronic degenerative diseases in the body. However, the endogenous antioxidants are insufficient at some level and create a requirement from exogenous sources, to accomplish the needs. Plants have attracted considerable attention as an exogenous source of antioxidants and have been reported to induce beneficial effects on cancer prevention and progression (48).

2.5 Terpenoids in treatment of cancer

Essential or volatile oils are natural, complex, volatile and aromatic secondary metabolites of plant that synthesized by the secretory cells of aromatic plants. Over 300 different compounds could be identified, the main group is composed of terpenes and terpenoids, the others include aromatic and in a lower extent aliphatic compounds plants (155). Terpenoids are formed of five-carbon isoprene units $(C_5H_8)_n$ called isoprenoids, are plant secondary metabolites along with alkaloids and flavonoids. Terpenoids are evolutionarily the oldest of this group and at present, they represent a highly diversified group of small molecules synthesized by plants (156). They are subdivided based on the number of isoprene units present in it such as monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$) and tetraterpenes, which contain eight units of isoprene such as carotenoids. Monoterpenes, non-nutritive secondary metabolite found in the essential oils of citrus fruits and other plants have exhibited its anti-tumor activity in various types of cancers. Monoterpenes avert the process of carcinogenesis at both, initiation and promotion stages and have been reported for treating early and advanced cancers (50, 51).

Some monoterpenes have been reported for its anticancer activity by inhibiting the NF-κB signaling (155). More studies were done to identify the effect of six monoterpenes such as carvacrol, thymol, carveol, carvone, eugenol and isopulegol on cell cycle progression, which

has demonstrated that carvacrol and carveol stopped the cell cycle progression in S phase; thymol and isopulegol stopped it in G0/G1 phase whereas carvone and eugenol, no effect on cell cycle (157).

TABLE 2.2: Monoterpenoids as an inhibitor of NF-kB signaling (155).

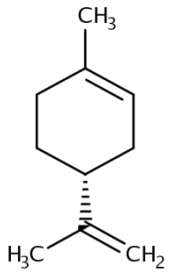
Monoterpene	**NF-kB inhibition**	**Therapeutic indications**
Aucubin	IκBα degradation	Inflammation, hepatotoxicity, cancer (158)
Catalposide	IκBα degradation	Intestinal inflammation (159)
Genipin	IκBα degradation	Cancer cell growth, inflammation (160)
D-Limonene	DNA binding	Lymphoma, growth and metastasis of gastric cancer (161-163)
Perillyl alcohol	DNA binding	Lymphoma, mammary and pancreatic tumors (60, 164)
α-Pinene	p65 translocation	Inflammation (165)

2.6 D-Limonene

2.6.1 Introduction

D-Limonene is a monocyclic monoterpene which is abundantly found in volatile oils of citrus fruits such as orange, lemon, grapefruit and others plants. It can be easily obtained by steam distillation. Limonene is named because it was isolated from the peels of lemon. The D-isomer of Limonene is more common in nature and commonly used as compared to its L-counterpart. It has been widely used as a flavour and fragrance enhancer because of its pleasant citrus fragrance and listed in the Code of Federal Regulations as generally recognized as safe (GRAS) for a flavouring agent and can be found in common food items such as fruit juices, baked goods, soft drinks, and ice creams (52). D-Limonene has been reported for its potential chemopreventive and antitumor activities. However, the mechanism of action has not yet fully elucidated.

2.6.2 Structure and chemical characteristics



Chemical name	: ((R)-1-methyl-4-(1methylethenyl) cyclohexene
Formula	: $C_{10}H_{16}$
Mol. Wt.	: 136.238 g/mole
Colour	: Clear, colourless
Odour	: Citrus

FIGURE 2.13 : Structure of D-Limonene (52).

2.6.3 Pharmacokinetic of D-Limonene

The Pharmacokinetic profile indicates, D-Limonene is rapidly and almost completely taken up from the gastrointestinal tract in humans as well as in animals orally. In ADME studies using radioactive ^{14}C rats, the highest concentration of D-Limonene in blood was obtained 2 h after oral administration, most occurred in the serum fraction. About 75-90 % of administered dose was recovered from urine, 5% from faeces and 2% from expired CO_2 within 48 h. In rats with bile duct cannulation, about 25% of the dose was excreted in bile within 24 h (166).

In phase-I clinical trial, D-limonene after single and repetitive dosing for extended periods as long as 1 year in patients with advanced breast cancer did not show any toxicity (54). Oral administration of [^{14}C] D-Limonene in rats has shown that D-Limonene was highly distributed in tissues such as liver, kidneys, and blood. About 25–30% of an oral dose of D-Limonene was found in urine as D-limonene-8,9-diol and its glucuronide in humans and 7–11% was eliminated as perillic acid and its metabolites (55).

In healthy human volunteers, D-Limonene at dose 100 mg/kg has indicated major peaks of dihydroperillic acid, perillic acid and limonene-1,2-diol without any graded toxicity. The chemopreventive activity of limonene during initiation can be attributed to the induction of phase I and phase II enzymes, with resulting carcinogen detoxification (167). Along with D-Limonene its metabolites also hamper the tumor progression by inhibition of p21-dependent

signaling and may induce apoptosis via induction of the transforming growth factor beta-signaling pathway.

2.6.4 Pharmacological activity of D-Limonene

D-Limonene has been reported for antioxidant, antimicrobial, anti-inflammatory and cholesterol gall-stone dissolving property (56-58, 168, 169). It has also been used to relieve heartburn, because of its potential for gastric acid neutralization property.

Dabbah et al. have reported the antimicrobial property of D-Limonene against *Salmonella senftenberg, Escherichia coli, Staphylococcus aureus and Pseudomonas species* (170). D-Limonene has been reported for inhibiting the growth of all yeasts and Gram-positive cocci, while lacking efficacy against Gram-negative bacterial strains. Winniczuk and Parish have reported the antifungal activity of D-Limonene (171).

D-Limonene exerted anti-oxidant effect and protected the lymphocytes cells from oxidative stress by modulation of H_2O_2 level in *in vitro* studies (172). It has also reduced the levels of lipid peroxidation by-products and increased antioxidant enzymes in streptozotocin (STZ)-induced diabetic rats (173).

Yoon et al. have reported the effect of D-limonene on the production of pro-inflammatory cytokines and inflammatory mediators in RAW 264.7 macrophages. D-limonene significantly inhibited lipopolysaccharide (LPS)-induced NO and prostaglandin E_2 production in RAW 264.7 macrophage cells. Apart from these, D-limonene significantly decreased the expression of other cytokines such as TNF-α, IL-1β, and IL-6 in a dose-dependent manner (174).

Oral administration of D-Limonene in TNBS (2,5,6-trinitrobenzene sulfonic acid)-induced colitis in rats has and significantly reduced serum concentrations of TNF-α and IL-6 compared to untreated TNBS-colitis rats. The anti-inflammatory effect was mediated through TNFα-induced NF-κB translocation in fibroblast cultures (56).

2.6.5 Anticancer activity of D-Limonene

Kaji et al. have evaluated the effect of D-limonene on N-nitrosomorpholine (NNM) induced hepatocarcinogenesis in male Sprague-Dawley rats. D-Limonene has significantly inhibited the hepatocarcinogenesis suggesting its anti-proliferative and apoptosis inducing activity (175). The effect of D-Limonene was also checked in N-nitrosodiethylamine (NDEA) alone and along with phenobarbital (PB) induced hepatocarcinogenesis in AKR mice, which has shown significant inhibition of c-myc and c-jun both at mRNA and oncoprotein levels (176).

In vitro studies on breast cancer cells has shown D-Limonene and related monoterpenes have significantly inhibited the cell proliferation by inhibiting cell cycle progression and expression of cyclin D1 gene (177). In colon cancer, D-Limonene suppressed the viability of cancer cells in a dose-dependent manner by activating caspase-3 and -9 and PARP cleavage. It has increased expression of Bax protein and cytosol cytochrome c from mitochondria and a decreased bcl-2 protein. In addition, D-Limonene has decreased the levels of p-Akt (Ser473), p-Akt (Thr308) and p-GSK-3β (Ser9), suggesting its role in apoptosis via the mitochondrial death pathway and the suppression of the PI3K/Akt pathway (178, 179). In human leukemia cells, D-Limonene has induced apoptosis by mitochondrial death pathway (180).

In vivo studies has presented that dietary administration of D-limonene enhances tumor latency during the promotion or progression stage of N-methyl N-nitrosourea (NMU)- and 7,12-dimethylbenz-(a) anthracene (DMBA)-induced carcinogenesis in rats (181-183). In addition, they inhibit post-translational modification of signal transduction proteins, resulting in G1 cell cycle arrest as well as differential expression of cell cycle- and apoptosis-related genes. It inhibits post-translational isoprenylation of small GTP-binding proteins including $p21^{ras}$, such inhibition may alter signal transduction and gene expression leading to apoptosis, cellular redifferentiation and tumor regression (157). Oral administration of D-Limonene in patients with breast cancer resulted in significant changes in several metabolic pathways and significantly reduced the tissue level cyclin D1 expression (62, 184, 185).

D-Limonene has significantly reduced the growth of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinoma by increasing apoptosis and decreasing DNA synthesis in Wistar rats (161). Beside this, D-Limonene has also inhibited the growth and metastasis of

human gastric cancer, implanted in nude mice by reducing tumor weight, apoptotic index, microvessel density and VEGF expression (64).

Nakaizumi et al. have reported the anticancer effect of D-Limonene in N-nitrosobis (2-oxopropyl) amine induced pancreatic carcinoma in Syrian golden hamsters by inhibition of cell proliferation pathway (186).

The combination treatment of D-Limonene with anticancer drug improves the sensitization of anticancer drug and reduces the dose-related toxicity of anticancer drug. Rabi and Bishayee reported that D-Limonene has improved the treatment outcome of prostate cancer refractory to docetaxel. It has enhanced the antitumor effect of docetaxel against prostate cancer cells without being toxic to normal prostate epithelial cells through the modulation of proteins involved in mitochondrial pathway of apoptosis (187). Combination of D-Limonene treatment with suboptimal doses of 4-hydroxyandrostrenedione on NMU-induced mammary tumour in rats has shown significant regression of tumor as compared to individual 4-hydroxyandrostrenedione treatment in 75 % of rats (188).

D-limonene has also been reported for modulating the immune response in BALB/c mice with lymphoma. It has increased the survival of lymphoma-bearing mice, delayed hypersensitivity reaction to 2,4-dinitrofluorobenzene (DNFB), phagocytosis and microbicidal activity. *In vitro* studies have indicated that D-limonene increases NO production in peritoneal macrophages obtained from tumor-bearing mice (162, 189).

In studies involving *in vitro* percutaneous absorption of tamoxifen, D-Limonene has significantly increased the permeability coefficient of tamoxifen in comparison with the control through porcine epidermis (190).

These observations suggest that D-Limonene can be viewed as a conceptually promising agent in cancer therapy of CML.

Chapter-3

Materials and Methods

CHAPTER-3

Materials and Methods

3.1 Drugs and chemicals

D-Limonene, anti-VEGF antibody, beta-actin primary antibodies and peroxidase conjugated secondary antibodies were obtained from MP Biomedicals Solon, OH, USA. Cell culture medium Roswell Park Memorial Institute medium (RPMI)-1640, Dulbecco's Modified Eagle Medium (DMEM), Trypsin, Fetal Bovine Serum (FBS), HEPES, Trypan Blue, 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Trypsin, Collagenase II, sodium bicarbonate, Dimethyl sulfoxide (DMSO) and trypan blue were purchased from Himedia, Mumbai, India. Antibiotic-antimycotic solution was obtained from Gibco, Grand Island, NY, USA. Doxorubicin, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2carboxylic acid), DPPH, DMSO, ABTS, potassium persulfate, Griess reagent, TPTZ were purchased from Sigma Aldrich, St. Louis, MO, USA. Cyclosporine (Sandimmune) from Novartis, Basel, Switzerland and Ketoconazole (Nizral) from Johnson & Johnson, New Brunswick, New Jersey, USA. Cyclophosphamide (Endoxan) was purchased from Baxter, Halle, Germany. Ampoxin was purchased from Unichem laboratories Ltd., Mumbai, India. SGOT, SGPT and ALP kits were brought from Erba, Transasia Bio-medicals Ltd., Baddi, Solan, India. Sterilized gelatin sponge was obtained from Alinda healthcare Pvt Ltd, Ahmedabad, India. All other chemicals and solvents used for the experiment were of analytical grade and procured from S. D. Fine Chemical Ltd, Mumbai, India.

3.2 Cell characteristics and culture condition

K562 is a suspension cell line with spherical shape and presence of bcr:abl fusion gene. Cells bear some proteomic resemblance to undifferentiated granulocytes, monocytes and erythrocytes. The diameter is about 20 µ with mean doubling time of 12 h (191).

Primary Hepatocytes derived from mice is an adherent cell line, spherical in shape and presents in bunches. It becomes elongated and flat and in shape after attachment to the

surface. Cells produce cellular products albumin, human transforming growth factor alpha and mouse TGF alpha with mean doubling time of 37 h (192).

K562 cells were obtained from NCCS (National Centre for Cell Science, Pune, India) and primary hepatocytes were isolated from mouse in our laboratory. K562 and primary hepatocytes were maintained in RPMI-1640 and DMEM medium respectively, supplemented with 10% FBS with 1 % antibiotic-antimycotic solution. Cells were maintained in an incubator at 37˚C supplemented with 5% CO2 atmosphere. Cells used in the study were in the logarithmic growth phase.

3.3 Estimation of cytotoxic activity of D-Limonene on K562 cells by MTT assay

Principle

MTT assay is a colorimetric assay that measures the viability of cells by its metabolic activity. The mitochondrial succinate dehydrogenase, NAD(P)H-dependent oxido-reductase enzymes present in viable cells causes reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to insoluble purple coloured crystals of formazan. The cells are then treated with an organic solvent (e.g. DMSO- Dimethyl sulfoxide) to solubilise the crystals of formazan. The intensity of purple colour is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the absorbance is a measure of the viability of the cells (193).

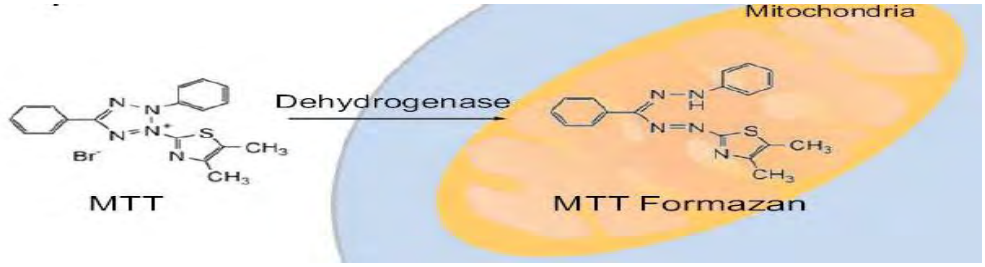


FIGURE 3.1: Principle of MTT assay (194).

Procedure

1. K562 cells were seeded in sterile filtered RPMI-1640 containing 10% FBS with 2.0 g/l sodium bicarbonate in a 96-well plates with cell density 10^4/well.

2. Cells were treated with D-Limonene at concentration 1, 2, 4 and 8 mM and Doxorubicin as a standard at concentration of 0.4, 0.8, 1.6 and 3.2 µM along with control.

3. Cells were incubated at 37º C in 5% (v/v) CO_2 for 24 h and 48 h by keeping final volume 200 µl with medium RPMI-1640 containing 10% FBS.

4. After 24 h and 48 h of incubation 20 µl of MTT (5 mg/ml) solution was added to each well & the plate was incubated at 37º C for 3 hr.

5. After 3 h of incubation the 96 well plate was centrifuged at 3000 rpm for 7 min for settlement of cells and media was aspirated from each well.

6. 100 µl of DMSO was added to each well and the readings were noted in ELISA plate reader at 570 nm after 1 h.

Calculation:

% viability = (Absorbance of treated cells – Absorbance of blank cells) / (Absorbance of controlled cells – Absorbance of blank cells) × 100

Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula:

% cytotoxicity = 100 – % cell viability (194)

3.4 Isolation of primary hepatocytes from mouse

Principle

Primary cell isolation is required for generation of primary cell cultures, which are useful as an *in-vitro* tool for illumination of disease mechanisms. Liver is one of the highly perfused organs of the body. Different species can be used for isolation of hepatocytes from liver. Isolation of primary hepatocytes provides an opportunity to study pharmacological and toxicological effects of drug on such cells.

In the first step of primary hepatocyte isolation, the liver is subjected to non-recirculating perfusion with calcium chelators or calcium free buffer. It causes irreversible separation of desmosomal cell contacts. In the second step, liver is perfused with collagenase to dissolve

the extra cellular matrix. In this step the calcium is added back to ensure maximal enzyme activity of collagenase. This optimal treatment dissociates the liver completely within 10 – 15 min, that is, sufficiently rapid to avoid the need for continuous oxygenation during perfusion.

Procedure

Flush the tubing of peristaltic pump with 70% alcohol followed by autoclaved water. Suspension buffer, Perfusion buffer and Collagenase buffer were prepared. 6 week old mice was anesthetised with an intraperitoneal injection (ketamine 100 mg/kg body and xylazine 5 mg/kg). Abdominal cavity of animal was opened and portal vein was exposed. Place loose ligatures around the portal vein. Cannulate the portal vein with perfusion buffer making a small incision and allow perfusate efflux by making another incision in vena cava. When liver was blanched, it was dissected out and perfusion buffer was drained out. The liver was treated with collagenase II buffer. In the second step transfer the liver to suspension buffer containing petri-dish. The Gilsson's capsule was dissected and cloudy suspension was formed. The suspension was filtered through cell strainer and centrifuged (50 g for 10 min) 3 times with wash buffer (195-197). The cells were transferred to sterile filtered DMEM culture media containing 10% FBS with 1 mM sodium pyruvate and 2.2 g/l sodium bicarbonate. The cell viability was determined using trypan blue method and the hepatocytes were seeded in culture flask maintained at 37 °C and 5 % CO_2 for 24 hours. The non-adherent hepatocytes were discarded with culture media.

3.5 Estimation of cytotoxicity of D-Limonene on mouse primary hepatocytes by MTT assay

1. Mouse primary hepatocyte were cultured in DMEM media containing FBS and maintained in incubator at 37 °C in a humidified atmosphere of 5% CO_2.

2. After 24 h incubation, the cells were treated with D-Limonene at concentration 2, 4, 8, 16 and 32 mM and Doxorubicin as a standard at concentration of 2, 4, 8, 16 and 32 µM along with control for 48 h and the MTT assay was executed by same protocol as described in section 3.3.

3.6 *In vivo* study of D-Limonene by tumor xenograft model in immunocompromised C57BL/6 mice

Experimental animals and housing conditions

Healthy male C57BL/6 mice 4-6 week of age weighing 25-30 g were procured from Mahaveera Enterprises, Hyderabad, India. Mice were housed under standard laboratory conditions with a temperature of 25±2 ˚C, relative humidity of 60±5% and 12 h light: dark cycle in individually ventilated cage. The mice were fed with autoclaved balanced rodent food pellet and ampoxin (0.1 µg/ml) by drinking water, provided *ad libitum*. Mice were cared for and used in accordance with the CPCSEA guidelines and all animal experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (PERD/IAEC/2017/002) prior to initiation of the experiment.

3.6.1 *In vivo* subcutaneous injection tumor xenograft model

Study protocol: Healthy 4-6 week old C57BL/6 male mice weighing 30-35 g were selected. Mice were immunocompromised by treatment with ketoconazole 10 mg/kg orally and cyclosporine 30 mg/kg intraperitoneally for 7 days. Cyclophosphamide 60 mg/kg subcutaneously was injected on days 3 and 1 before tumor cell injection (198). For tumor cell implantation all mice were divided into 5 groups viz., Normal control, Disease control, Treatment group (T1, T2 and T3). Except normal control group all groups had n=10 and for normal control n=6 animals for the experiment. After the achievement of immunosuppression all mice were injected with K562 (5×10^6 cells in 0. 1 ml) cancer cell line mixed with equal volume of matrigel (199). The subcutaneous injection of K562 cells was given in right shoulder blade region. After the one week of tumor cell implantation, treatment with D-Limonene was started at doses 0.5, 1.0 and 1.5 mg/kg orally for 14 days. Tumor growth was observed at the site of injection. Tumor volume was measured externally by digital calliper using following formula:

Volume (mm^3) = (A) × (B^2)/2,

Where, A was the largest and B the smallest diameter (mm) (198).

Body weight, haematology, biochemistry and tumor tissue histopathology were done at the end of the study.

Experimental Design: C57BL/6 male mice were divided into five groups and each group was contained ten mice. except normal control (n=6) group.

i. **NC -** Normal control (Vehicle treated mice)

ii. **DC -** Disease control (Immunocompromised + K562 (5×10^6) cells treated mice)

iii. **T1 -** D-Limonene 0.5 mg/kg (Immunocompromised + K562 (5×10^6) cells + D-Limonene treated mice)

iv. **T2 -** D-Limonene 1.0 mg/kg (Immunocompromised + K562 (5×10^6) cells + D-Limonene treated mice)

v. **T3** – D-Limonene 1.5 mg/kg (Immunocompromised + K562 (5×10^6) cells + D-Limonene treated mice)

Evaluation parameters

a. **Body weight:** Changes in body weight were observed before and after D-Limonene treatment and the percentage reduction in body weight was calculated.

b. **Blood collection and complete blood count:** Mice were anaesthetized with isoflurane and blood was collected from retro-orbital plexus of mice in heparinized centrifuge tubes for assessment of complete blood count. The complete blood count was done by an automated haematology analyzer (VetScan HM-5; Abaxis Inc.,Union City, CA, USA) before starting the experiment, after 7 days of immunosuppression and at the end of experiment (200).

c. **Biochemical parameters:** Blood samples were collected from retro-orbital plexus of mice in clean and dry centrifuge tubes under isoflurane anaesthesia. Blood was collected before the initiation of experiment, after immunosuppression and at the end of treatment. Blood was allowed to clot for 30 min. at the room temperature and centrifuged at 3000 rpm for 15 min. Serum was collected and stored at -20 ºC (201).

1) Determination of SGOT (Serum glutamic oxalocetic transaminase)

Principle

Serum glutamic oxaloacetic transaminase converts L- Aspartate and α-Ketoglutarate to Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with 2, 4, Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produced a brown coloured complex whose intensity is measured. The calibration curve is plotted using a Pyruvate standard. The activity of SGOT is read from the calibration curve.

L-Aspartate	SGOT	Oxaloacetate
+	⟶	+
α Ketoglutarate	pH 7.4	L-Glutamate

Oxaloacetate	Alkaline	2,4, Dinitrophenyl
+	⟶	Hydrazone
2, 4, DNPH	Medium	(Brown coloured complex)

Procedure

0.5 ml of buffered substrate in test tube was taken and incubated at 37^{o}C for 3 minutes. 0.1 ml of test serum was added in test tube by keeping blank. Mixed well and incubated the test tube at 37 ^{o}C for 1 hr. 0.5 ml of DNPH colour reagent was added in test tubes and allowed to stand at room temperature for 10 minutes. 5.0 ml of working NaOH solution was added in all test tubes and allowed to stand at room temperature for 10 minutes. The absorbance of test against blank was read on UV/Visible spectrophotometer at 505±10 nm.

2) Determination of SGPT (Serum glutamic pyruvate transaminase)

Principle

Serum glutamic pyruvic transaminase converts L-alanine and α-ketoglutarate to Pyruvate and Glutamate. The formed Pyruvate reacts with 2, 4, Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produced a brown coloured complex and its intensity is measured. The reaction does not obey Beer's law and hence a calibration

curve is plotted using a Pyruvate standard. The activity of SGPT is read from this calibration curve.

L-Alanine + α Ketoglutarate —SGPT, pH 7.4→ Puruvate + L-Glutamate

Pyruvate + 2, 4, DNPH —Alkaline Medium→ 2,4, Dinitrophenyl Hydrazone (Brown coloured complex)

Procedure:

0.5 ml of buffered substrate was added in different test tubes and incubated at 37ºC for 3 minutes. 0.1 ml of test serum was added in test tubes by keeping blank. Mixed well and incubated the test tube at 37 ºC for 30 minutes. 0.5 ml of DNPH colour reagent was added in test tubes. Mixed well and allowed to stand at room temperature for 20 minutes. 5.0 ml of working NaOH solution was added in all test tubes and allowed to stand at room temperature for 10 minutes. The absorbance of test against blank was read on UV/Visible spectrophotometer at 505±10 nm.

3) Determination of ALP (Alkaline phosphatase)

Principle

Alkaline Phosphatase at an alkaline pH hydrolyses di sodium Phenylphosphate to form phenol. The phenol formed reacts with 4-Aminoantipyrine in the presence of Potassium Ferricyanide, as an oxidising agent, to form a red coloured complex. The intensity of the colour formed is directly proportional to the activity of Alkaline Phosphatase present in the sample.

Di Na. Phenylphosphate + H_2O —ALP→ Phenol + di Na. Hydrogen Phosphate

Phenol	Alkaline Medium	Red
+	⟶	coloured
4-Aminoantipyrine	K_3 Fe(CN)$_6$	complex

Procedure

Different test tubes were taken and labelled as standard, blank and test. 1.05 ml of distilled water was added in blank test tube. In remaining test tubes 1.00 ml of distilled water was added. 1.0 ml buffer reagent and 0.1 ml substrate reagent was added in all the four test tubes. Mixed well and allowed to stand at 37 ºC for 3 minutes. 0.05 ml of serum and phenol standard was added in the test tube labelled as test and standard respectively. Mixed well and allowed to stand at 37 ºC for 15 minutes. 1.0 ml of colour reagent was added in all the test tubes mixed well and the absorbance was measured of the Blank (Abs B), standard (Abs. S) and Test (Abs. T) against distilled water at 510 nm.

- Calculate the ALP in sample by using following formula:

$$ALP = \frac{\text{Abs. T - Abs. C}}{\text{Abs. S - Abs. B}} \times 10$$

d. Tumor growth: Tumor growth was observed throughout the study and the tumor volume was measured on alternate day by digital calliper. At the end of study, the tumors were excised and compared on a scale.

e. Histopathological analysis: At the end of experiment, the mice were sacrificed by cervical dislocation and tumors were excised. The tumors were stored in 10% neutral buffered formalin and treated with haematoxylin and eosin stains. The tumor blocks were prepared by paraffin embedding. The slides were prepared and observed under 100x and 400x magnification and photo documented by optical microscopy (IX 51; Olympus, Tokyo, Japan) equipped with a digital camera (TL4) for confirmation of presence of tumor cells (198).

3.6.2 *In vivo* tail vein injection tumor xenograft model

Study Protocol

Immunosuppression of C57BL/6 mice was done by combination of ketoconazole, cyclosporine and cyclophosphamide as described in study protocol for the subsection 3.6.1. Tail vein injection with K562 (approx. 5 x 10^7 in 0.2 ml) was given and the blood was collected from retro-orbital plexus weekly to confirm the development of the disease. The blood was analyzed for the presence of circulating blast cells. Giemsa staining of peripheral blood smear was performed for identification of blast cells. Complete blood count was done to find out change in number of blood cells (202). After the disease induction mice were divided in 5 groups. Treatment with D-Limonene at doses 0.5, 1.0 and 1.5 mg/kg was given orally for 17 days in treatment group T1, T2 and T3 respectively. Body weight was measured weekly, haematological, biochemical and histopathological parameters were analyzed at the end of study.

Experimental design

C57BL/6 male mice were divided into five groups and each group was contained ten mice, except normal control (n=6) group. The experimental design and grouping was same as mentioned in experimental design of subsection 3.6.1. The K562 (5×10^7) cells were injected by tail vein injection instead of K562 (5×10^6) by subcutaneous injection.

Evaluation parameters

a. **Giemsa differential staining:** To determine the induction and progression of model, differential WBC staining of blood smear was performed. In this technique, a drop of blood was placed on a glass slide and thinly spread over it with another glass slide by keeping 30 º angles to each other. The smear was air dried and stained with Giemsa stain. The smear was washed with water. After washing, the slides were observed at 100X and 400X magnification under the microscope (203).

b. **Body weight:** Changes in body weight were observed before and after D-Limonene treatment and the percentage reduction in body weight was calculated.

c. **Blood collection:** Blood collection and blood cell count determination was done according to method discussed in subsection 3.6.1 before starting the experiment, after

immunosuppression, at every week of tumor cell transplantation for determination of disease induction and progression and at the end of experiment.

d. **Biochemical parameters**: Serum collection was done for evaluation of biochemical parameters such as SGOT, SGPT and ALP by method discussed in subsection 3.6.1.

e. **Histopathological analysis:** It was performed as discussed in subsection 3.6.1

f. **Estimation of serum oxidative stress markers:** The *in vivo* antioxidant activity of D-Limonene was determined by estimation of lipid peroxidation, nitrire and superoxide dismutase levels in serum.

Estimation of serum anti-lipid peroxidation effect (204-206)

Principle

Lipid peroxidation is the degradation of lipids, useful marker for oxidative stress. In this process free radicals take away electron from lipids of cell membrane causing cell damage. Polyunsaturated lipids are susceptible to an oxidative attack, typically by reactive oxygen species, resulting in a well-defined chain reaction with the production of end products such as malondialdehyde (MDA), which can be detected spectrophotometrically by formation of pink colour with thiobarbituric acid (TBA).

Procedure

The 0.5ml of serum was taken by centrifugation of blood at 3000 rpm for 15 min. from different treatment group. Lipid peroxidation was initiated by adding 100 µl of 1mM ferric chloride. The reaction was stopped by adding 2ml of ice cold 0.25N HCL containing 0.38% TBA, 15 % TCA, and 0.2 ml of 0.05% butylated hydroxyl toluene. These reaction mixtures were heated for 60 min at 90ºC then cooled and centrifuged at 7000 rpm for 15min. The absorbance of supernatant was measured at 532nm against blank, which contained all reagents except serum. The anti-lipid peroxidation effect was calculated by the following formula,

Concentration of the test= Abs (test) – Abs (blank) / 1.56 x 1000000

Estimation of serum nitrite (207, 208)

Principle:

The amount of nitrite in the serum indicates the production of nitric oxide (NO) is determined by colorimetric assay based on Griess reagent. Nitric oxide is oxidized to nitrite and/or nitrate with the help of oxygen. The measurement of concentration of nitrate/nitrite or of total nitrate and nitrite (NO) is routinely used in estimation of NO production. Griess reagent may contain 0.2% naphthyl ethylenediamine dihydrochloride (NEDD), and 2% sulphanilamide in 5% phosphoric acid. nitrites form a diazonium salt. When the nitrite reacts with azo dye agent (N-alpha-naphthyl-ethylenediamine) and sulphanilamide it forms azo salts which can be measured by measuring the absorbance of solution.

Procedure

Nitric oxide production in the serum was determined using Griess reagent [0.1% N-(1-naphthyl) ethylenediaminedihydrochloride, 10% sulphanilamide in 2.5% phosphoric acid]. Serum or sodium nitrite (50 µl) as standard was used for the detection and mixed with solution containing vanidium trichloride and Griess reagent mixed in equal quantity (50 µl). This mixture was incubated at room temperature. Absorbance was measured at 550 nm. The standard curve was plotted and concentration of test sample was determined by using equation of line. Serum nitrite was calculated using following equation:

$$\text{Nitrite } (\mu M) = \frac{\text{Amount of Nitrite in sample obtained from standard Nitrite curve}}{\text{Sample volume } (\mu L)}$$

Estimation of Superoxide dismutase (208, 209)

Principle

SOD estimation is done by epinephrine method. In presence of xanthine oxidase epinephrine is oxidized to adrenochrome and oxygen is generated from this reaction. Amount of formed adrenochrome is introduced per O_2. Auto oxidation rate of epinephrine and sensitivity of the auto oxidation to inhibition by SOD were raised as pH was increased from 7.8-10.2. The auto oxidation of epinephrine occurs by at least two distinct pathways, one of them is free radicals chain reaction including O_2 and thus inhabitable from SOD. One unit of SOD

activity was defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline at 26°C.

Procedure

EDTA 0.1 ml ($1x10^{-4}$ M), epinephrine, 1 ml ($3 x10^{-3}$ M) and carbonate buffer, 0.5 ml (pH 9.7) were added to the 0.1 ml of serum. The optical density of adrenochrome formed from the reaction between above chemicals was measured at 480 nm for 3 min and at an interval of 30 sec. The unit of enzyme activity was expressed in the terms of U/ml of serum. One unit of enzyme activity is defined as the 50% concentration of enzyme required for the production of chromogen in one minute under the stable assay condition.

Calculation

Rate = (Final OD-Initial OD)/3 min.

% of Inhibition= [(blank OD-R)/Blank OD] x 100

Enzyme unit (U) = (% of inhibition/50) x common dilution factor.

3.7 Effect of D-Limonene on angiogenesis

3.7.1 Chick chorioallantoic membrane (CAM) assay

Chick eggs: Post laying chick eggs were purchased from Jesal Agro Ltd., chhataral, kalol, Gandhinagar at the time of experiment. Chick eggs were maintained in an egg incubator (A. P. Poultry Equipments, Hyderabad, Telangana, India) at 37 ºC and 60% humidity under the sterile condition.

Study protocol

The experiment was performed in aseptic or sterile area. The egg incubator was sterilised with the use of 70 % IPA (isopropyl alcohol). Chick eggs were maintained at 37º C and 60 % humidity in egg incubator for 48 h. On the 3rd day of incubation, eggs were sterilized and an acute pole was created for false air sac over the CAM with 16-G or 18-G hypodermic needle and 1-ml syringe. A small window of 2x2 cm was made in the shell of the egg by removing the upper shell of the egg with forceps. The viability of chick embryos was assessed by monitoring rhythmic contraction and relaxation movement of the heart. The window was sealed with transparent adhesive tape and the eggs were returned to the

incubator. On the 8th day the transparent tape was removed and 1 mm^3 gelatin sponges pre-soaked with D-Limonene at concentration of 0, 1, 5 and 10 µg/implant (1 implant/egg, n=12 for each treatment) were implanted on the CAM of eggs. The eggs were sealed again and returned to incubator for incubation. On the 12th day of incubation, the eggs were observed under the microscope (Olympus) under 100X for counting number of blood vessel surrounding implants (210).

3.7.2 Estimation of VEGF expression by western blot analysis (211)

Gel preparation: The stacking and resolving gels were prepared as follows:

TABLE 3.1: List of chemicals and quantity required for gel preparation.

	Resolving Gel (10 %)	**Stacking Gel (6 %)**
Mili Q water	2.05 ml	3.05 ml
Acrylamide (30 %)	1.65 ml	0.65 ml
Tris Buffer (1.5 M, pH-8.8)	1.25 ml	-
Tris Buffer (1 M, pH-6.8)	-	1.25 ml
SDS (10 %)	50 µl	50 µl
Ammonium Persulfate (100 mg/ml)	50 µl	50 µl
TEMED	2.5 µl	2.5 µl

Sample preparation

1. The K562 cells were treated with D-Limonene at concentration 0, 1, 2, 3 and 4 mM for 24 h and the protein samples were prepared.
2. The protein samples were mixed with 4X sample buffer and centrifuged at 13,000 rpm for 10 seconds.
3. Samples were heated in boiling water at 95-100 ºC for 10 min. and centrifuged as mentioned in above step.
4. Approximately 30 µl of sample were loaded in each lane and electrophoresed.

Electrophoresis and transfer of gel

1. The gel was run with 1X running buffer at 75V for 10-15 min. for proper stacking and later speeded up to 100V till the dye reached to the bottom of the gel.
2. Gel casting apparatus was disassembled and the stacking gel was cut.
3. 1X transfer buffer kept at 4 ºC and was used for the transfer of gel to the PVDF membrane by semidry method.
4. The transfer clamp was arranged and PVDF membrane was put first and then gel in between filter pads.
5. The transfer was done at 25V and 1.3A for 20 min.

Blocking and incubation with primary and secondary antibody

1. The blocking of PVDF membrane was done with 2% BSA in 1X TBS for 1 h on rocker shaker at room temperature.
2. Blocking buffer was removed and the membrane was incubated with primary antibody against VEGF and beta-actin (diluted in 0.2% BSA in 1X TBS) and kept overnight at 4 ºC on rocking platform.
3. On next day, the primary antibody was removed and the membrane was washed with 1X TBST 3 times at the interval of 10 min.
4. After the 3rd wash the membrane was incubated with peroxidase conjugated secondary antibody (diluted in 0.2% BSA in 1X TBS) for 1 h at room temperature.
5. The membrane was again washed with 1X TBST 3 times at the interval of 10 min.

Development of Blot

1. After washing, Western Blot luminol reagent solution A and B were mixed in ratio of 1:1 and poured over the membrane.
2. The images of the blot were visualised on chemidoc image system.

3.7.3 Estimation of MMP-2 and 9 expression by gelatin zymography (212)

Preparation of gel

1. Separating gel: Mix 2.2 ml of distilled water, 0.625 ml of 1% gelatin, 2 ml of 30 % acrylamide–bis-acrylamide solution, 1.25 ml of separating gel buffer stock, 31 µl of 20% SDS; and 20 µl of 10% APS.

2. Stacking gel: Mix 1.375 ml of distilled water, 0.25 ml of acrylamide–bis-acrylamide stock, 0.25 ml of stacking gel buffer stock, 12 µl of 20% SDS, and 10 µl of 10% APS

3. Add 5 µl of TEMED to the separating gel solution to initiate polymerization.

4. Immediately, pipet separating gel solution into each cassette avoiding the formation of bubbles.

5. Let the gel polymerize for at least 1 h at room temperature.

6. Add 2 µl of TEMED to the stacking gel solution, swirl rapidly, and pipet the solution on top of the polymerized separating gels until it reaches the top of the front plate.

7. Insert the appropriate combs (usually 10 wells) rapidly into the liquid stacking gel, making sure that no bubbles remain trapped under the comb. Let the stacking gel polymerize at room temperature (about 30–60 min).

Sample preparation

1. The K562 cells were seeded in 6 well plate at density of 2 million cells per 2 ml of culture media in each well.

2. The cells were treated with D-Limonene at concentration of 0, 1, 2, 3 and 4 mM for 24 h in incubator at 37 °C in a humidified atmosphere of 5% CO2.

3. After the 24 h of incubation the cells were washed with ice cold PBS and centrifuged at 200g for 7 min. at 4 ºC.

4. The pellets were resuspended with 200 µl of ice cold lysis buffer and incubate it on ice for at least 15 min.

5. The suspension was centrifuged at 16,000g for 12 min at 4°C in a microcentrifuge tube. The supernatant was collected and protein concentration was determined by Bradford method.

6. Mix the sample with 4X sample buffer without heating. Load 30 µl of sample in each lane.

Electrophoresis and development of gel

1. The comb was removed gently from the stacking gel and the cassette was placed into the electrophoresis unit filled with 1X running buffer.

2. Samples were loaded in each well and the gel was run at constant voltage. To avoid heating the gel was run at 125 V, and current approx. 30–40 mA/gel until the bromophenol blue tracking dye reaches the bottom of the gel.

3. The gel was removed from the cassette and placed in a plastic tray containing 100 ml of renaturing solution for 30 min at room temperature with gentle agitation.

4. Incubate the gel for an approx. 16 h in developing buffer at 37 °C for in a closed tray.

5. Decant the developing buffer and stain the gel with staining solution for 1 h.

6. Destaining of gel was done with destaining solution until areas of gelatinolytic activity appear as clear sharp bands over the dark blue background.

3.8 *In vitro* antioxidant activity of D-Limonene

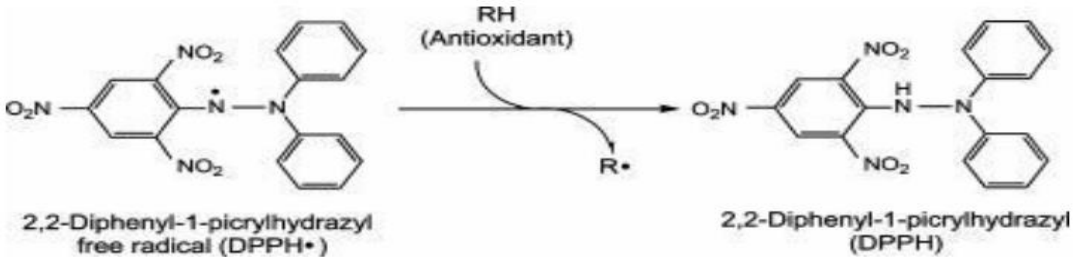
3.8.1 Test solution preparation

Test solutions of D-Limonene at concentration of 25, 50, 100, 200 and 400 µM were prepared by adding 0.1% Tween 80. Trolox was used as a standard at concentration of 10, 25, 50, 100 and 250 µM. The stock solution of Trolox 1 mM was prepared in absolute ethanol and working solution of Trolox (10, 25, 50, 100 and 250 µM) were made from the stock solution by dilution with required amount of double distilled water. Absorbance was measured by Thermo Scientific Multiskan GO, Waltham, Massachusetts, United States.

3.8.2 Free radical scavenging activity by DPPH method (213, 214)

Principle

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay involves determination of the hydrogen atom donating or free radical scavenging capacity of compound to be evaluated for its antioxidant activity. DPPH free radical (DPPH•) accepts hydrogen from an antioxidant, so the antioxidant effect is directly proportional to the disappearance of DPPH• in test samples. DPPH• shows a strong absorption maximum at 517 nm (purple) in UV spectrometer, whereas absorption of hydrogen from an antioxidant turns the colour to yellow from purple followed by formation of DPPH. The antioxidant effect can be easily evaluated by reduction of absorbance at 517 nm.



(Purple colour) (Yellow colour)

FI**GURE 3.2:** Principle of free radical scavenging activity by DPPH assay.

Procedure

Different concentration of D-Limonene and Trolox were prepared and stored at -20 ºC until needed. To this 3 ml of methanolic solution of DPPH was added, this mixture was incubated at 37 °C for 30 min. A blank was prepared in the similar way and absorbance was measured at 517 nm. Free radical scavenging activity was expressed as the percentage inhibition calculated by using the following formula,

$$\% \text{ of scavenging radical} = \text{Absorbance of control} - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

3.8.3 Azobis-ehtylbenzthiazoline sulfonic acid (ABTS) assay (214, 215)

Principle

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay also known as Trolox equivalent antioxidant capacity (TEAC) assay, is used to measure the antioxidant activity. It reacts with sodium persulfate and converted to its radical cation which is blue in colour detected at 734 nm. Reaction with antioxidants makes the ABTS radical to stable colourless form which can be detected by spectrophotometrically.

Procedure

This assay was used for determination of cationic radical scavenging capacity of D-Limonene and Trolox. Initially, the stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol. Test solutions (150 µl) were allowed to react with 2850 µl of the ABTS solution for 2 h in a dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. Results are expressed as percentage inhibition using equation described in DPPH method.

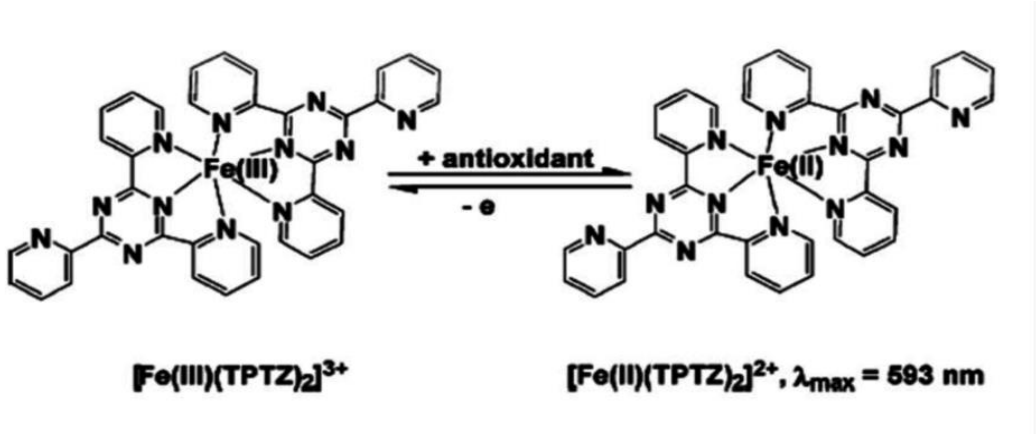
ABTS	+	Ammonium persulfate	→	ABTS$^{\bullet}$		
(colourless)				(Blue/Green)		
ABTS•	+	AOH	→	ABTS	+	AO
(Blue/Green)		Antioxidant		(colourless)		

3.8.4 Ferric reducing anti-oxidant power (FRAP) assay (214, 216)

Principle

Ferric reducing antioxidant power (FRAP) assay, involves reduction of Fe3+ to Fe2+ by the use of antioxidants as reductants in a colorimetric reaction. Conversion of Ferric (Fe3+) to ferrous (Fe2+) ion causes formation of a coloured ferrous-probe complex from a colourless ferric-probe complex. Antioxidants acts as reducing agents and donates electrons to free

radicals to stabilize them and minimize the damage caused by free radicals to other cellular components.



FI**GURE 3.3:** Principle of Ferric reducing anti-oxidant power by (FRAP) assay.

Procedure

This assay was preferred to assess the biological ferrous supplying action and ferric induced oxidative damage attenuating capacity of D-Limonene and Trolox. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2$-$3H_2O$ and 16 ml C_2H_4O2), pH 3.6, 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3$-$6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3$-$6H_2O$ solution and then warmed at 37 ^oC before using. Test solutions (150 µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the coloured product [ferrous tripyridyltriazine complex] were then taken at 593 nm. Results were expressed as percentage inhibition using equation described in DPPH method.

3.8.5 Iron chelating assay (217, 218)

Principle

Iron chelating assay involves chelation of free iron by antioxidants. Phenanthroline can quantitatively chelate with Fe2+ and form a deep red coloured complex. This reaction is limited by the presence of other chelating agents and results in a reduction of the red colour of the phenanthroline-Fe2+ complex. The antioxidants form a coordinate complex with the metal ions and arrest the oxidation reaction by inhibiting transfer of electrons.

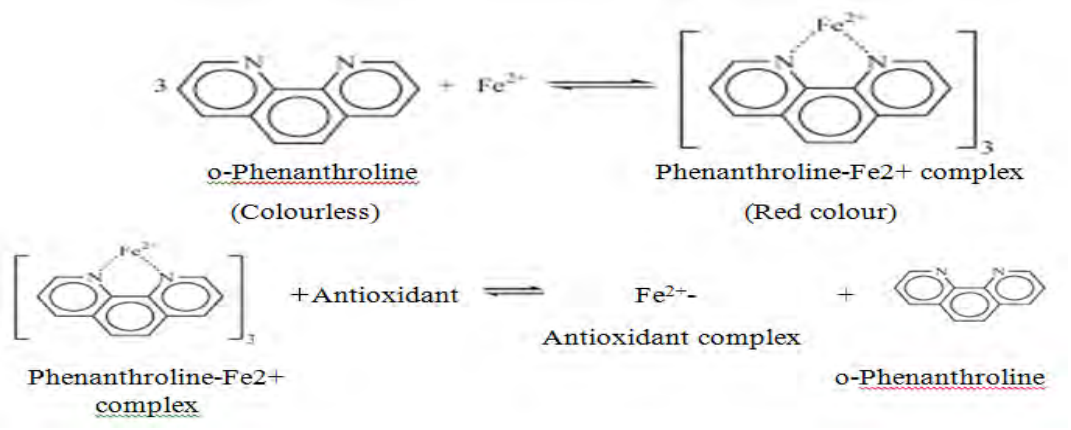


FIGURE 3.4: Principle of iron chelating assay.

Procedure

This assay was performed to assess indirect anti-oxidant activity of compounds or extracts. The stock solutions included ferric chloride (0.2 mM in double distil water) and phenanthroline (5 mM in double distil water). Test solutions of D-Limonene and Trolox (600 µl) were mixed with 1200 µl ferric chloride and 1200 µl phenanthroline. Then the absorbance was taken at 510 nm using the spectrophotometer. Results were expressed as percentage inhibition using equation described in DPPH method.

3.8.6 Hydroxyl radical scavenging assay (218, 219)

Principle

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the antioxidant test compounds for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system (Fenton reaction). The hydroxyl radical attacks epoxyribose, which eventually results in Thiobarbituric Acid Reacting Substances (TBARS) formation, which is estimated using a spectrophotometer.

Procedure

The most damaging oxidative radical, hydroxyl radical, was determined using thiobarbituric acid assay. The stock solutions included hydrogen peroxide (1 mM), ascorbic acid (1 mM), EDTA (1 mM), ferric chloride (0.2 mM), trichloroacetic acid (2.8%), thiobarbituric acid (1%) and deoxyribose (28 mM in phosphate buffer 7.4). The working solutions were prepared by mixing hydrogen peroxide (100 µl), ascorbic acid (100 µl), EDTA (200 µl),

ferric chloride (500 µl) and deoxyribose (100 µl). Test solutions of D-Limonene and Trolox (500 µl) were allowed to react with 1000 µl of working solution for 60 min in the dark condition. Later, they were incubated with 100 µl of trichloroacetic acid and 100 µl of thiobarbituric acid for 20 min at 100 ^{o}C. Readings of the colored product were then taken at 546 nm. Results were expressed as percentage inhibition using equation described in DPPH method.

3.8.7 Superoxide radical scavenging assay (218, 220).

Principle

This assay involves the conversion of Nitroblue Tetrazolium (NBT) to NBT diformazan by superoxide radical. Superoxide anion reduces highly water-soluble tetrazolium salt to a water-insoluble formazan dye by utilisation of SOD. The rate of the reduction affects the formation of formazan and its absorbance intensity.

Procedure

Superoxide is a preliminary by product of oxidative phosphorylation in each living cell. The stock solutions included alkaline DMSO (10 part of DMSO and 1 part of 5mM NaOH) and nitro blue tetrazolium (1 mg/ml in double distil water). Test solutions of D-Limonene and Trolox (600 µl) were mixed with 2 ml alkaline DMSO and 200 µl nitro blue tetrazolium. Then the absorbance was taken at 560 nm using the spectrophotometer. Results were expressed as percentage inhibition using equation described in DPPH method.

3.8.8 Nitric oxide radical scavenging assay (218, 221)

Principle

Sodium nitroprusside at physiological pH spontaneously generates nitric oxide. Nitric oxide interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent. This generated nitric oxide can be measured by the Griess reaction. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide.

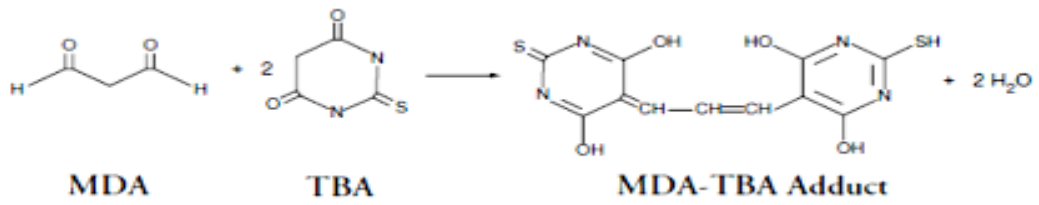
Procedure

This assay was performed to ultimately determine nitric oxide scavenging power of D-Limonene and Trolox. The stock solutions included sodium nitroprusside (5 mM in phosphate buffer solution pH 7.4) and Griess reagent (250 ml, prepared solution from). Test solutions (1200 µl), sodium nitroprusside (600 µl) were mixed and were allowed to react with 2000 µl of Griess reagent for 30 min in the dark condition. Readings of the colored product were then taken at 546 nm. Results were expressed as percentage inhibition using equation described in DPPH method.

3.9 *In vitro* lipid peroxidation assay of D-Limonene on K562 cells (222)

Principle

Lipid Peroxidation levels were determined by measuring malondialdehyde (MDA) level. Since MDA is known to be one of the most abundant aldehydes formed as a product of lipid peroxidation. Malondialdehyde (MDA) forms a 1:2 adduct with thiobarbituric acid and produces the adduct that can be measured by spectrophotometrically.



FI**GURE 3.5:** Principle of lipid peroxidation assay.

Procedure

1. For each determination, 1 x 10^7 cells were treated with 0.5 mM, 1 mM and 2mM concentration of D-Limonene for 2 h followed by 75 µM H_2O_2 for 2 h.

2. The cells were collected by centrifugation at 200 x g for 10 min and washed twice with PBS and the pellet was resuspended in 1ml of 1.15 % potassium chloride and homogenize.

3. The cellular protein was precipitated by mixing homogenates with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8 % thiobarbituric acid.

4. The mixture was brought to final volume of 4ml with water and heated to 95 ºC for 120 min, cooled and 5 ml of mixture of n-butanol and pyridine (15:1 v/v) was added to each sample and shaken vigorously.

5. Centrifugation was done at 625g for 10 min. supernant was collected and absorbance was measured at 535 nm.

6. Inhibitory activity towards lipid peroxidation was expressed as equivalent of MDA.

3.10 Statistical analysis

Data were expressed as the Mean ± SEM for the number of animals in each group. Graphs was plotted and statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test. The statistical analysis of data will be performed by using Graph pad prism software (version 5.0, Graph Pad software Inc, USA). A p value of 0.05 and 0.001 or less was considered to be statistically significant.

Chapter-4

Results

CHAPTER-4

Results

4.1 Characteristics of K562 cells and primary hepatocytes

The K562 cells were maintained in RPMI-1640 and mouse primary hepatocytes were maintained in DMEM culture media containing 10 % FBS (Fig. 4.1).

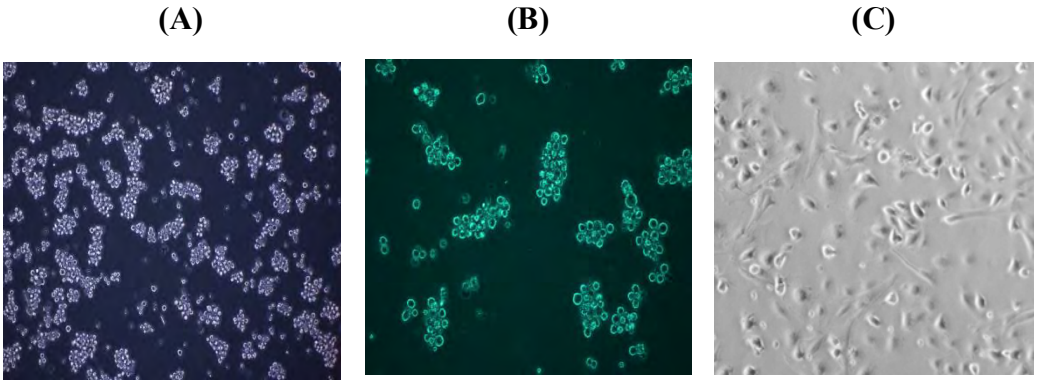


FIGURE 4.1: Morphology of K562 cells and mouse primary hepatocytes. K562 cells (A) 100x and (B) 200x magnification, mouse primary hepatocytes (C) 200x magnification.

4.2 Effect of D-Limonene on K562 cells by MTT assay

D-Limonene at concentration of 1, 2, 4 and 8 mM shows significant reduction in percentage of cell viability of K562 cells as compared to untreated cells at 24 h (IC_{50}=3.6 ± 0.23 mM) and 48 h (IC_{50}=3.29 ± 0.19 mM) of treatment. Doxorubicin at concentration 0.4, 0.8, 1.6 and 3.2 µM shows significant reduction in percentage cell viability of K562 cells at 24 h (IC_{50}= 1.6 ± 0.18 µM) and 48 h (IC_{50}= 0.95 ± 0.28 µM) of treatment. However, no significant difference was observed in IC_{50} value at 24 h and 48 h of treatment. The reduction in percentage of cell viability of Doxorubicin was comparable with D-Limonene (Fig. 4.2 and 4.3).

TABLE 4.1: MTT assay of D-Limonene on K562 cells at 24 h and 48 h.

Concentration (mM)	D-Limonene treatment on K562 cells	
	24 hour	48 hour
	% cell viability	% cell viability
0	100.00 ± 8.13	100.00 ± 6.08
1	75.90 ± 5.01***	73.36 ± 3.92***
2	56.37 ± 4.15***	51.65 ± 3.28***
4	32.82 ± 2.70***	28.32 ± 3.57***
8	15.27 ± 5.48***	11.73 ± 3.27***

Cell viability was calculated as a percentage of untreated cells (100%). Data shown are the means ± SD from 3 separate experiments in quadruplicate.

TABLE 4.2: MTT assay of Doxorubicin on K562 cells at 24 h and 48 h.

Concentration (μM)	Doxorubicin treatment on K562 cells	
	24 hour	48 hour
	% cell viability	% cell viability
0	100.00 ± 8.33	100.00 ± 6.96
0.4	77.40 ± 4.50***	65.27 ± 3.09***
0.8	58.59 ± 4.92***	37.66 ± 3.69***
1.6	41.11 ± 4.04***	8.35 ± 2.79***
3.2	19.69 ± 2.94***	4.27 ± 2.98***

Cell viability was calculated as a percentage of untreated cells (100%). Data shown are the means ± SD from 3 separate experiments in quadruplicate.

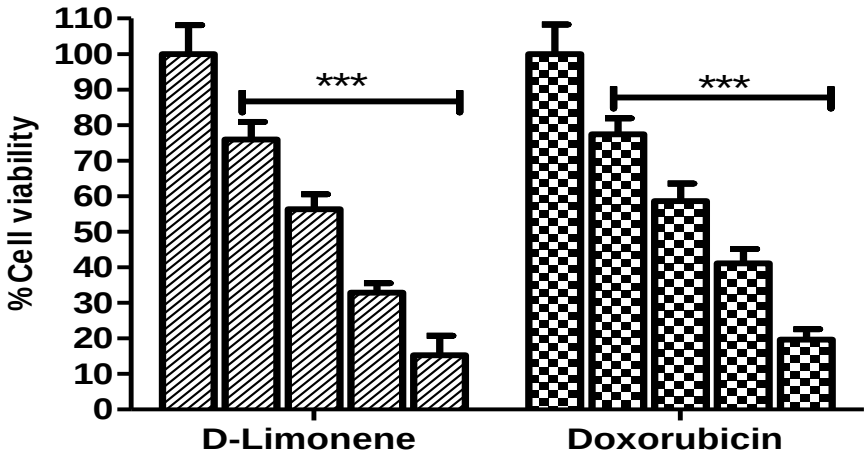


FIGURE 4.2: MTT assay of D-Limonene and Doxorubicin on K562 cells at 24 h of treatment. Data shown are the means ± SD from 3 separate experiments in quadruplicate. ***p<0.001 compared to untreated cells.

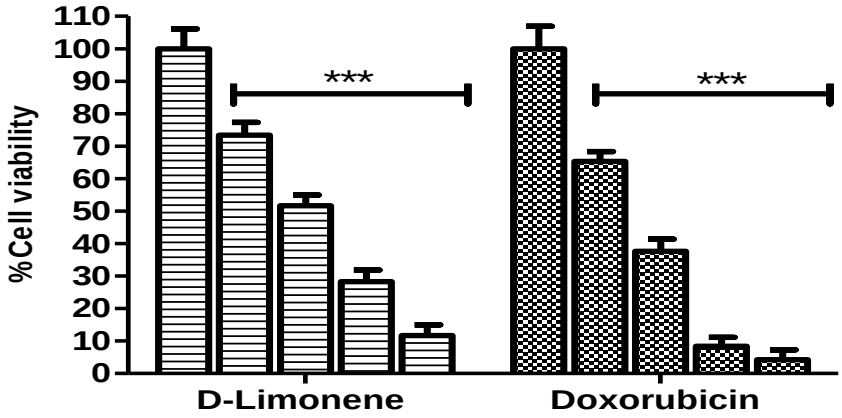


FIGURE 4.3: MTT assay of D-Limonene and Doxorubicin on K562 cells at 48 h of treatment. Data shown are the means ± SD from 3 separate experiments in quadruplicate. ***p<0.001 compared to untreated cells.

4.3 Effect of D-Limonene on primary hepatocytes by MTT assay

Treatment with D-Limonene at concentration of 2, 4, 8, 16 and 32 mM did not show any reduction in percentage cell viability of normal mouse primary hepatocytes compared to untreated cells by MTT assay at 48 h of treatment. However, Doxorubicin at concentration of 2, 4, 8, 16 and 32 µM for 48 h shows significant reduction in percentage of cell viability (IC_{50}= 14.13 ± 0.19 µM) of mouse primary hepatocytes as compared to untreated cells (Fig. 4.4).

TABLE 4.3: MTT assay of D-Limonene and Doxorubicin on mouse primary hepatocytes at 48 h of treatment.

D-Limonene		**Doxorubicin**	
Concentration (mM)	**% cell viability**	**Concentration** (µM)	**% cell viability**
0	100.00 ± 2.89	0	100.00 ± 6.26
2	85.13 ± 6.58	2	71.18 ± 5.66***
4	84.43 ± 6.63	4	59.98 ± 6.68***
8	84.91 ± 4.49	8	47.30 ± 5.32***
16	87.68 ± 3.91	16	36.40 ± 5.81***
32	85.45 ± 3.72	32	26.89 ± 5.70***

Data shown are the means ± SD from 3 separate experiments in quadruplicate. ***p<0.001 compared to untreated cells.

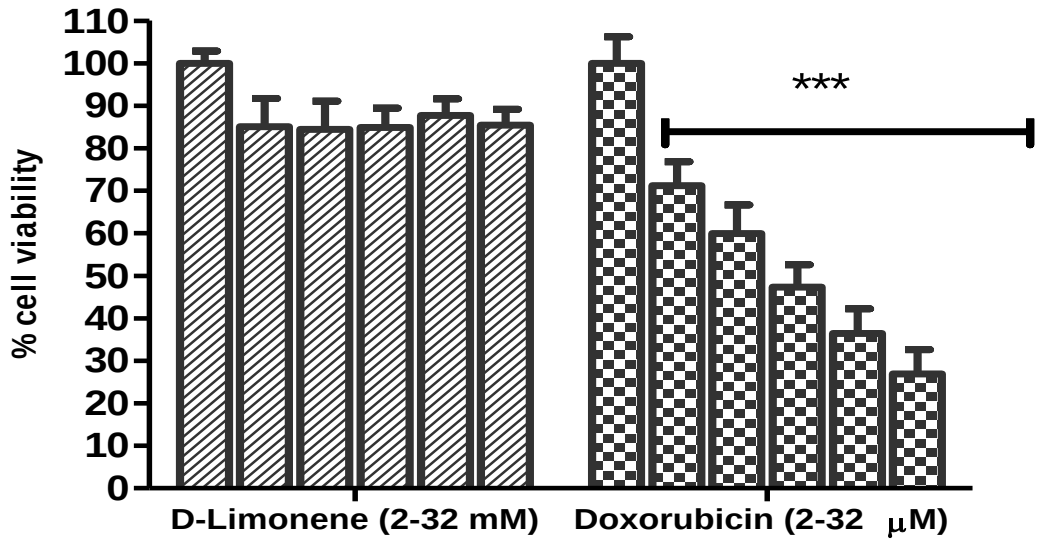


FIGURE 4.4: MTT assay of D-Limonene and Doxorubicin on mouse primary hepatocytes. Data shown are the means ± SD from 3 separate experiments in quadruplicate. ***$p<0.001$ compared to untreated cells**.**

4.4 Estimation of *in vivo* effect of D-Limonene by subcutaneous and tail vein injection of K562 cells in C57BL/6 mice

4.6.1 Effect of D-Limonene on subcutaneous K562 tumor xenograft model of CML in immunocompromised C57BL/6 mice.

a. Body weight: In subcutaneous K562 xenograft model, changes in body weight were observed before and after D-Limonene treatment. At the end of 14 days treatment with D-Limonene, disease control group has shown significant reduction in body weight as compared to normal control group. However, D-Limonene treated groups have shown significant increase in body weight as compared to disease control group. The percentage reduction in body weight was also calculated at the end of treatment. Disease control group has shown significant increase in percentage reduction of body weight as compared to normal control group, whereas D-Limonene treatment has reduced the percentage reduction as compared to disease control group (Fig. 4.5).

TABLE 4.4: Body weight of C57BL/6 mice before and after D-Limonene treatment for 14 days and percentage changes in body weight at the end of treatment.

Treatment Group	Body weight Before treatment (g)	Body weight after treatment (g)	% reduction in body weight (%)
NC	28.4 ± 1.15	28.6 ± 1.17	-0.9 ± 3.35
DC	28.71 ± 0.99	19.2 ± 0.86 ###	34.8 ± 3.7 ###
T1	28.00 ± 1.06	24.17 ± 0.91 **	13.67 ± 0.89 ***
T2	27.50 ± 0.80	24.66 ± 0.55 ***	10.14 ± 1.74 ***
T3	27.00 ± 0.58	25.00 ± 0.37 ***	7.14 ± 2.77 ***

NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). ###p< 0.001 compared to normal control group, **p<0.01 and ***p<0.001 compared to disease control group.

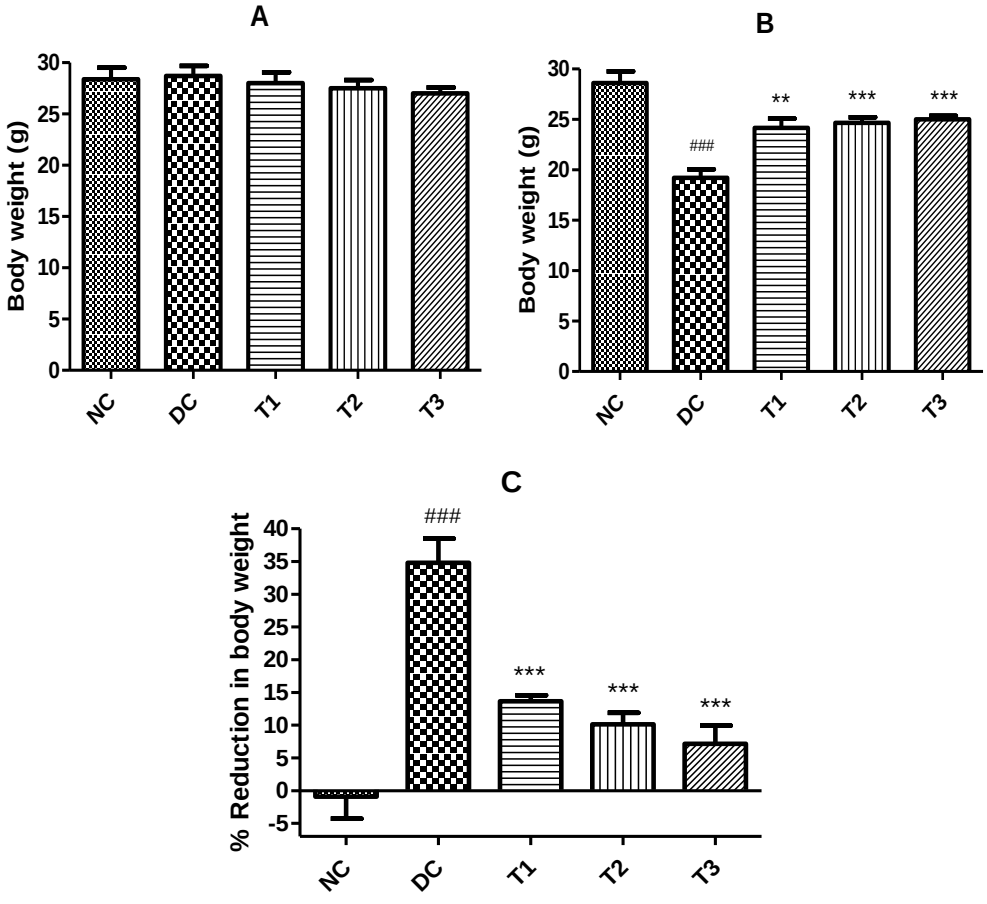


FIGURE 4.5: Changes in body weight with D-Limonene treatment. (A) Body weight before D-Limonene treatment, (B) Body weight after D-Limonene treatment and (C) Percentage reduction in body weight at the end of D-Limonene treatment in subcutaneous xenograft model of CML. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). ###p< 0.001 compared to normal control group, **p<0.01, ***p<0.001 compared to disease control group.

b. Haematological parameters: Changes in haematological parameters were observed after 1 week of immunosuppression. Haematology of C57BL/6 mice has shown significant reduction in WBC, NEU and LYM count after one week of immunosuppression as compared to week 0 or before immunosuppression. However, changes in RBC, Hb and PLT were non-significant (Fig. 4.6).

After D-Limonene treatment, significant reduction in WBC, NEU and LYM count was observed in disease control group as compared to normal control group whereas significant increase in WBC count was noted in all treatment groups as compared to disease control group. NEU count was significantly increased with D-Limonene treatment at dose 1.0 (T2) and 1.5 mg/kg/d (T3) as compared to disease control group. No significant changes were observed in LYM, RBC, Hb and PLT in other groups (Fig. 4.7).

TABLE 4.5: Changes in haematological parameters at week 0 (before immunosuppression) and week 1 (after immunosuppression).

	Week 0 (before immunosuppression)	**Week 1 (after Immunosuppression)**
WBC (10^3/µl)	9.07 ± 0.45	3.76 ± 0.30 ***
NEU (10^3/µl)	3.81 ± 0.43	0.91 ± 0.11 ***
LYM (10^3/µl)	6.41 ± 0.93	1.71 ± 0.52 **
RBC (10^6/µl)	8.78 ± 0.19	8.17 ± 0.71
Hb (g/dl)	12.93 ± 0.41	12.13 ± 0.34
PLT (10^5/µl)	9.44 ± 0.55	9.69 ± 0.68

WBC= white blood cell count, NEU=neutrophil count, LYM=lymphocyte count, RBC=red blood cell count, Hb=haemoglobin content, PLT=platelet count. Data are represented as mean ± SEM (n=10). **p<0.01, ***p<0.001 compared to week 0.

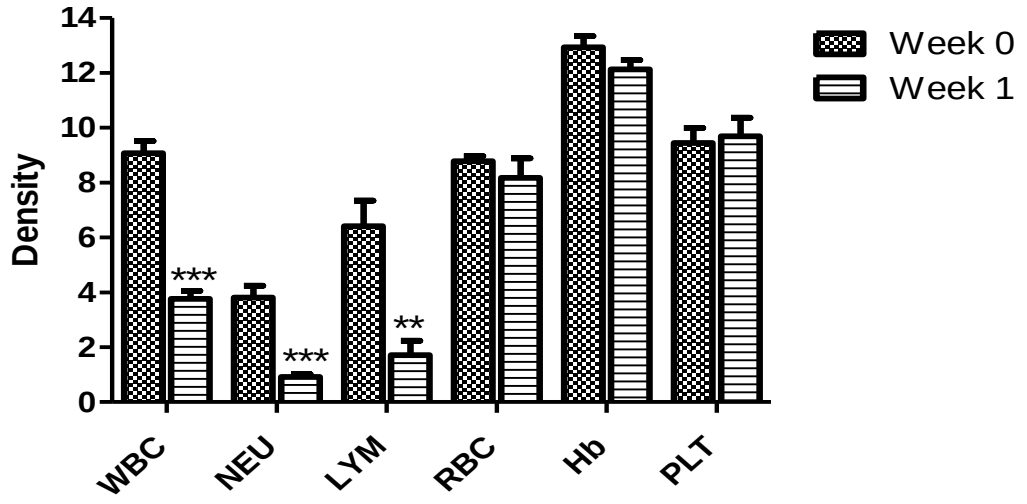


FIGURE 4.6: Changes in haematological parameters at week 0 (before immunosuppression) and week 1 (after immunosuppression). WBC= white blood cell count, NEU=neutrophil count, LYM=lymphocyte count, RBC=red blood cell count, Hb=haemoglobin content, PLT=platelet count. Data are represented as mean ± SEM (n=10). **p<0.01, ***p<0.001 compared to week 0.

TABLE 4.6: Haematological parameters at the end of D-Limonene treatment for 14 days in C57BL/6 mice.

Treatment group	NC	DC	T1	T2	T3
WBC (10^3/µl)	9.44 ± 0.56	5.26 ± 0.52 ##	8.06 ± 0.24 *	8.31 ± 0.58 *	9.07 ± 0.47 **
NEU (10^3/µl)	3.48 ± 0.42	1.78 ± 0.49 #	2.62 ± 0.27	3.8 ± 0.14 **	2.81 ± 0.31 **
LYM (10^3/µl)	7.65 ± 0.35	5.14 ± 0.64 #	6.42 ± 0.41	7.21 ± 0.46	7.09 ± 0.58
RBC (10^6/µl)	8.89 ± 0.71	7.05 ± 0.51	8.39 ± 0.75	8.26 ± 0.27	8.03 ± 0.29
Hb (g/dl)	12.4 ± 0.51	11.00 ± 0.15	12.10 ± 0.47	12.7 ± 0.51	12.46 ± 0.33
PLT (10^5/µl)	9.77 ± 0.42	9.09 ± 0.44	9.34 ± 0.29	9.68 ± 0.59	9.74 ± 0.17

WBC=white blood cell count, NEU=neutrophil count, LYM=lymphocyte count, RBC=red blood cell count, Hb=haemoglobin content, PLT=platelet count. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). #p<0.05 and ##p< 0.01 compared to normal control group and *p<0.05 and **p<0.01 compared to DC group.

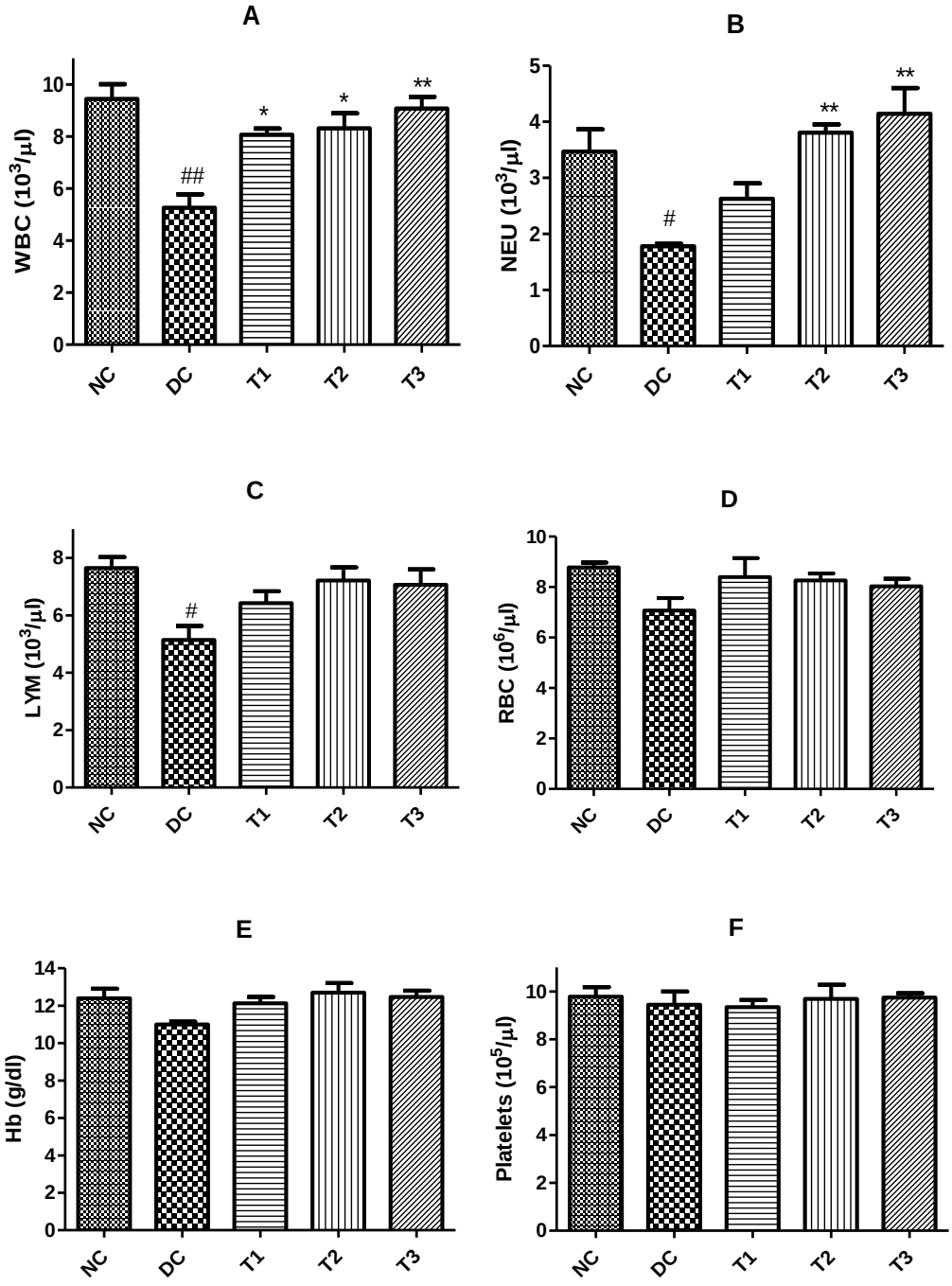


FIGURE 4.7: Haematological parameters at the end of D-Limonene treatment for 14 days in C57BL/6 mice. (A) WBC=white blood cell count, (B) NEU=neutrophil count, (C) LYM=lymphocyte count, (D) RBC=red blood cell count, (E) Hb=haemoglobin content, (F) PLT=platelet count. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). #p<0.05 and ##p< 0.01 compared to normal control group and *p<0.05 and **p<0.01 compared to DC group.

c. Biochemical parameters: Animals after one week (week 1) of immunosuppression did not show significant changes in serum SGOT, SGPT and ALP level as compared to week 0 (Fig. 4.8). The changes in serum SGOT, SGPT and ALP level were also non-significant in all groups followed by D-Limonene treatment in C57BL/6 mice (Fig. 4.9).

TABLE 4.7: Changes in biochemical parameters at week 0 (before immunosuppression) and week 1 (after immunosuppression).

	Week 0 (before immunosuppression)	Week 1 (after immunosuppression)
SGOT(IU/L)	56.15 ± 2.64	59.53 ± 3.73
SGPT(IU/L)	33.66 ± 2.96	29.66 ± 4.76
ALP(IU/L)	96.66 ± 8.37	86.00 ± 6.35

SGOT=serum glutamic oxaloacetic transaminase, SGPT= serum glutamic pyruvic transaminase and ALP=alkaline phosphatase. Data are represented as mean ± SEM (n=10).

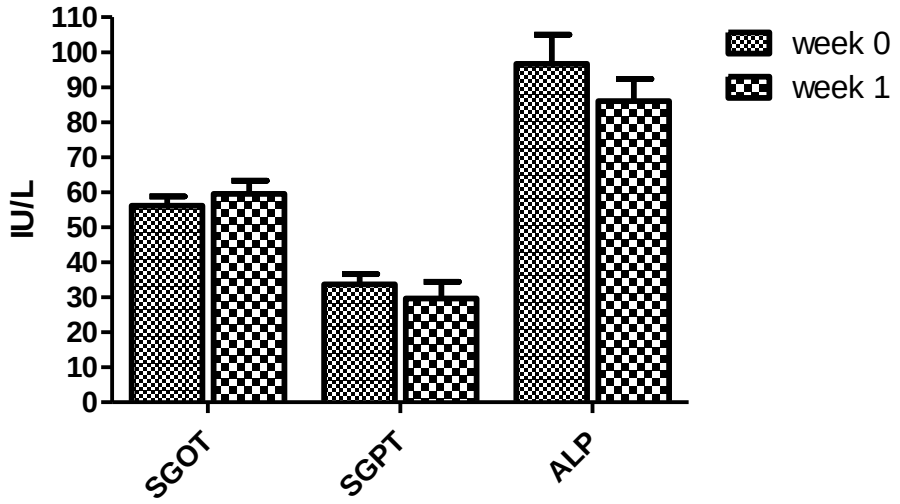


FIGURE 4.8: Changes in biochemical parameters at week 0 (before immunosuppression) and week 1 (after immunosuppression). SGOT=serum glutamic oxaloacetic transaminase, SGPT= serum glutamic pyruvic transaminase and ALP=alkaline phosphatase. Data are represented as mean ± SEM (n=10).

TABLE 4.8: Changes in biochemical parameters at the end of D-Limonene treatment.

Treatment Group	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)
NC	60.13 ± 3.63	30.45 ± 4.39	83.51 ± 3.04
DC	53.66 ± 4.88	35.66 ± 2.03	83.00 ± 5.10
T1	57.00 ± 2.08	31.66 ± 4.63	85.33 ± 6.98
T2	54.33 ± 1.76	30.33 ± 1.45	80.00 ± 5.03
T3	52.00 ± 2.30	32.33 ± 3.71	85.66 ± 8.76

SGOT=serum glutamic oxaloacetic transaminase, SGPT= serum glutamic pyruvic transaminase and ALP=alkaline phosphatase. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10).

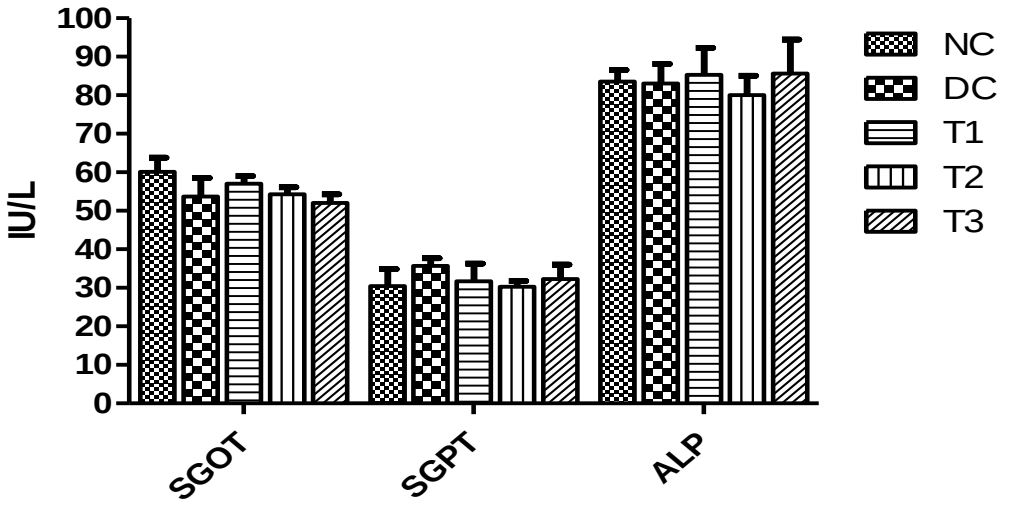


FIGURE 4.9: Changes in biochemical parameters at the end of D-Limonene treatment. SGOT=serum glutamic oxaloacetic transaminase, SGPT= serum glutamic pyruvic transaminase and ALP=alkaline phosphatase. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10).

d. Tumor growth: After D-Limonene treatment, significant reduction in tumor volume was observed in 1.0 and 1.5 g/kg/d treatment groups as compared to disease control group. However, the reduction in tumor volume in 0.5 g/kg/d D-Limonene treated group was non-significant (Figure 4.10). Tumor mass was excised at the end of study from mice, shows regression of tumor in all treatment group (Figure 4.11 and 12).

TABLE 4.9: Effect of D-Limonene treatment on tumor volume in C57BL/6 mice.

Day	Tumor volume (mm^3)			
	DC	**T1**	**T2**	**T3**
0	108.81 ± 17.64	106.93 ± 19.59	102.30 ± 17.98	107.88 ± 15.43
2	137.76 ± 17.43	117.90 ± 21.5	106.07 ± 19.84 #	103.82 ± 18.59 #
4	155.81 ± 12.54	150.37 ± 20.18	111.37 ± 14.22 ##	98.15 ± 14.36 ##
6	252.12 ± 18.38 ***	172.95 ± 15.73 ** ###	120.93 ± 20.18 ###	77.01 ± 17.90 ###
8	291.06 ± 16.61 ***	220.28 ± 22.50 *** ###	118.45 ± 13.64 ###	74.76 ± 20.36 * ###
10	369.16 ± 26.95 ***	248.17 ± 18.75 *** ###	122.34 ± 16.05 ###	60.31 ± 21.09 ** ###
12	480.53 ± 26.41 ***	332.58 ± 20.99 *** ###	134.47 ± 19.31 ###	46.32 ± 19.88 *** ###
14	510.60 ± 47.90 ***	377.71 ± 24.21 *** ###	140.89 ± 19.95 ###	40.80 ± 20.59 *** ###

NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 compared to Day 0 of respective groups. #p<0.05, ##p<0.01 and ###p<0.001 compared to disease control group on respective day.

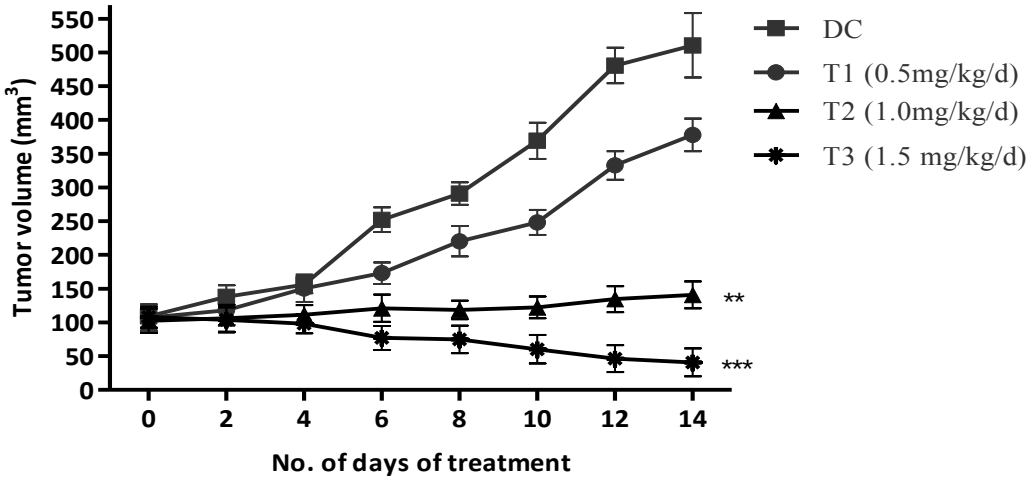


FIGURE 4.10: Effect of D-Limonene treatment on tumor volume in C57BL/6 mice. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). **p<0.01 and ***p<0.001 compared to disease control group (overall treatment).

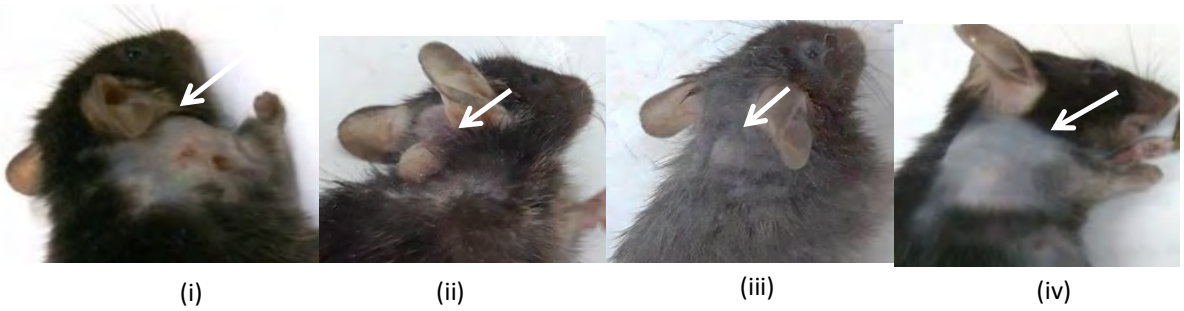


FIGURE 4.11: Effect of D-Limonene on regression of tumor mass in C57BL/6 mice. The tumor mass was presented by an arrow in each group. (i) DC, (ii) T1 (iii) T2 and (iv) T3. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group.

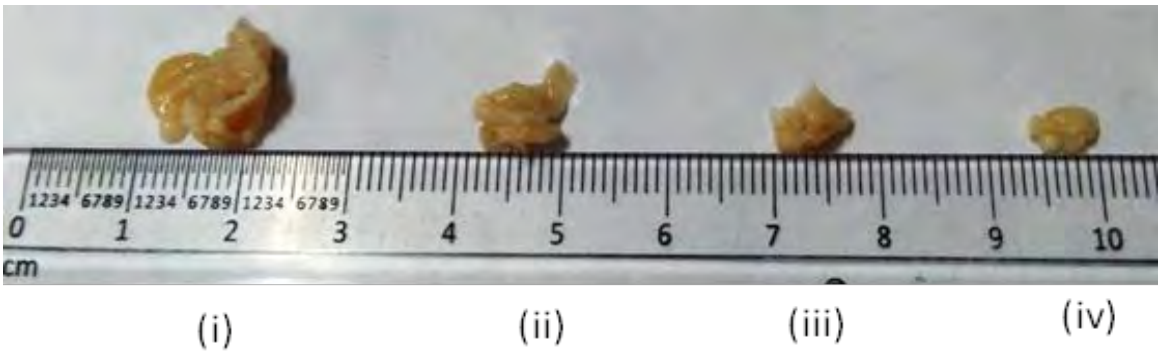


FIGURE 4.12: Tumors were excised at the end of treatment from K562 tumor implanted xenograft C57BL/6 mice (i) DC, (ii) T1 (iii) T2 and (iv) T3. DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group.

e. Histopathological analysis: At the end of D-Limonene treatment, haematoxylin and eosin stain of all excised tumor tissue under 100x and 400x magnification shows presence of malignant cells. However, the density of malignant cells was reduced with D-Limonene treatment in dose dependent manner as compared to disease control group. The malignant cells were characterised by cells with large and irregular nuclei and scant cytoplasm. The malignant cells were indicated by arrows (Figure 4.13 and 4.14).

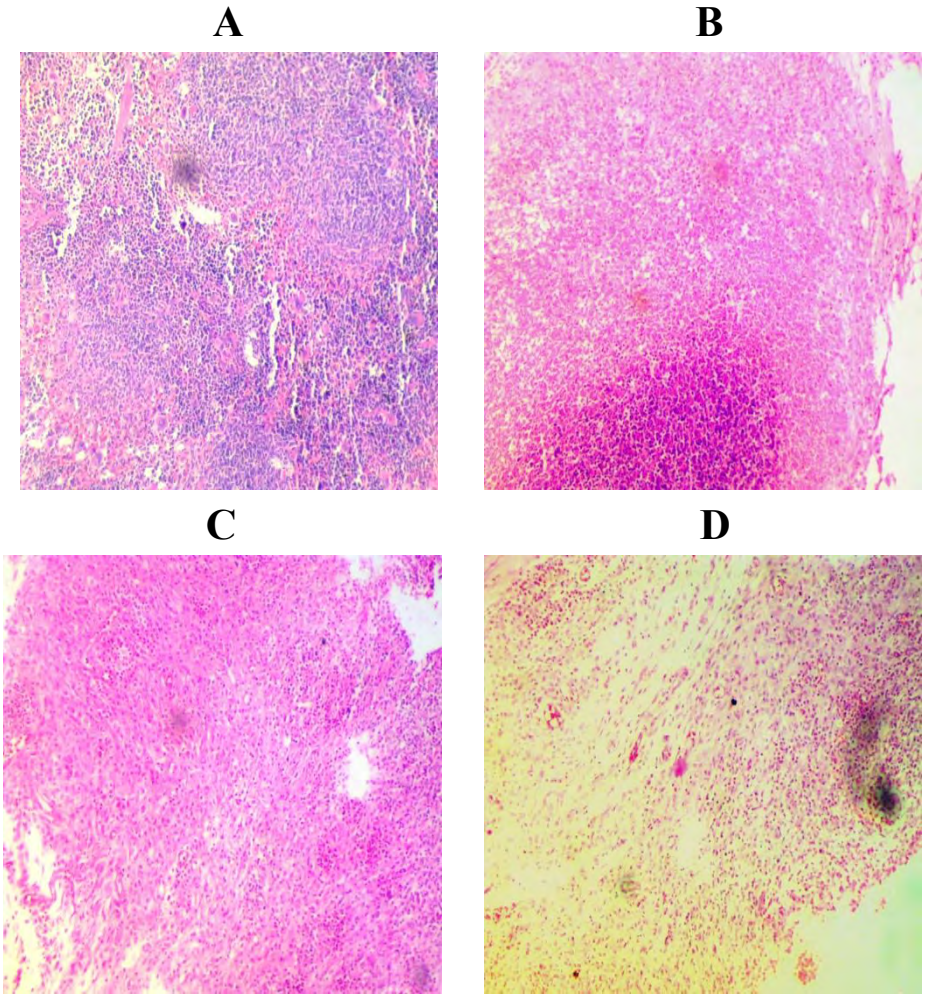


FIGURE 4.13: Histopathology of tumor tissue at the end of D-Limonene treatment at 100x magnification. The H&E-stained section of K562 xenograft tumor under microscope shows presence of blast cells. (A) Disease control, (B) D-Limonene 0.5 g/kg/d, (C) 1.0 g/kg/d and (D) 1.5 g/kg/d treated groups.

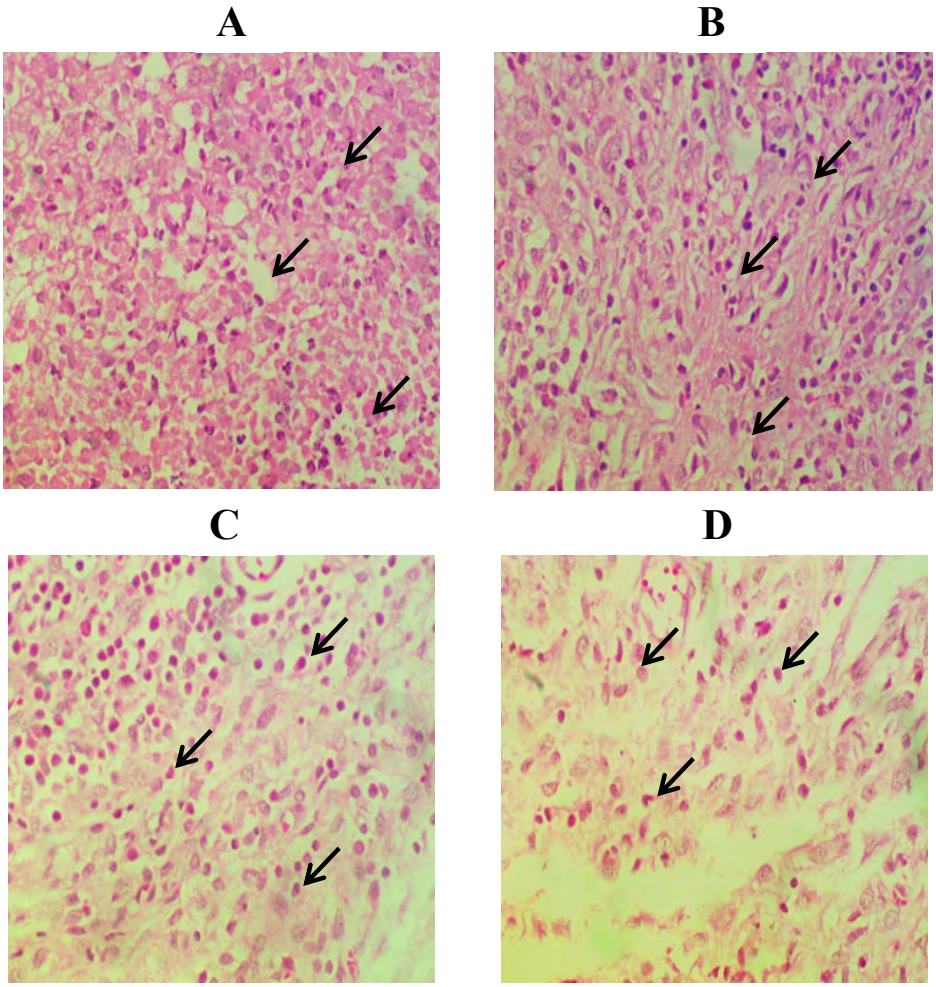


FIGURE 4.14: Histopathology of tumor tissue at the end of D-Limonene treatment at 400x magnification. The H&E-stained section of K562 xenograft tumor under microscope shows presence of blast cells with large nucleus represented by arrow. (A) Disease control, (B) D-Limonene 0.5 g/kg/d, (C) 1.0 g/kg/d and (D) 1.5 g/kg/d treated groups.

4.4.2 Effect of D-Limonene on K562 tail vein injection model of CML in immunocompromised C57BL/6 mice.

a. Giemsa differential staining for determination of model establishment: The Giemsa staining of peripheral blood smear under microscope at 100x and 400x magnification shows presence of circulating blast identified by large and irregular nuclei and comparative larger diameter (≈ 28μ) of the cells than other white blood cells (≈ 4-7 μ). The blast cell has been presented by arrows (Fig. 4.15).

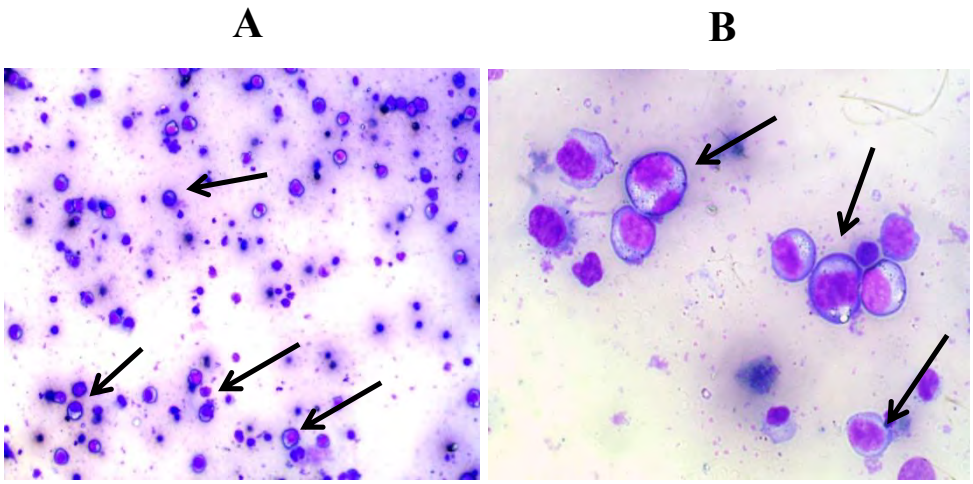


FIGURE 4.15: Circulating blast cells in peripheral blood smear followed by Giemsa staining. The blast cells are indicated by arrows under (A) 100x and (B) 400x magnification.

b. Body weight: Mice were checked for body weights weekly throughout the study and the changes in body weight was represented as percentage reduction in body weight. As the disease progressed, significant reduction in body weight was noted in K562 cells injected C57BL/6 mice as compared to normal control group (Fig. 4.16). At the end of study, mice treated with vehicle or disease control group has shown significant reduction in body weight as compared to normal control group. Whereas, mice treated with D-Limonene orally for 17 days has significant increase in body weight as compared to disease control group in all treatment groups (Fig. 4.17).

TABLE 4.10: Percentage reduction in body weight during disease induction and progression in tail vein injection model of CML.

Week	Body weight (g)	% reduction in body weight (%)
0	31.57 ± 0.95	-
1	29.57 ± 0.84	6.28 ± 0.94
2	27.42 ± 0.86 *	13.10 ± 1.2 **
3	25.85 ± 0.83 **	18.04 ± 1.76 ***
4	23.57 ± 1.13 ***	25.43 ± 2.64 ***

Data are represented as mean ± SEM (n=10). *p<0.05, **p<0.01, ***p<0.001 compared to week 0 or untreated group.

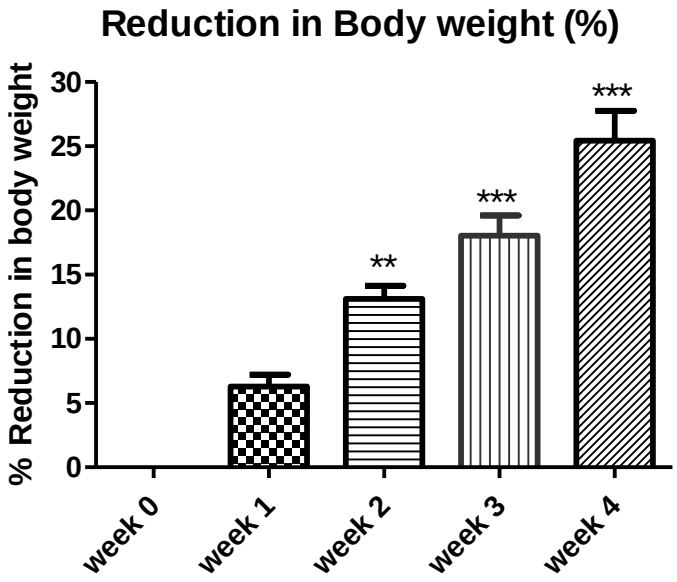


FIGURE 4.16: percentage reduction in body weight during disease induction and progression in tail vein injection model of CML. Data are represented as mean ± SEM (n=10). *p<0.05, **p<0.01, ***p<0.001 compared to week 0 or untreated group.

TABLE 4.11: Body weight of C57BL/6 mice before and after D-Limonene treatment for 17 days and percentage changes in body weight at the end of treatment.

Treatment Group	Body weight Before treatment (g)	Body weight after treatment (g)	% reduction in body weight (%)
NC	28.8 ± 1.15	29.2 ± 1.11	-1.6 ± 3.04
DC	23.67 ± 1.33	17.83 ± 0.60 ###	23.95 ± 3.00 ###
T1	22.17 ± 1.25	22.50 ± 0.43 ***	-2.87 ± 5.13 ***
T2	22.67 ± 0.84	23.83 ± 0.48 ***	-5.68 ± 3.33 ***
T3	22.67 ± 0.84	24.17 ± 0.48 ***	-7.17 ± 3.53 ***

Data are represented as mean ± SEM (n=10). ###p< 0.001 compared to normal control group, ***p<0.001 compared to disease control group.

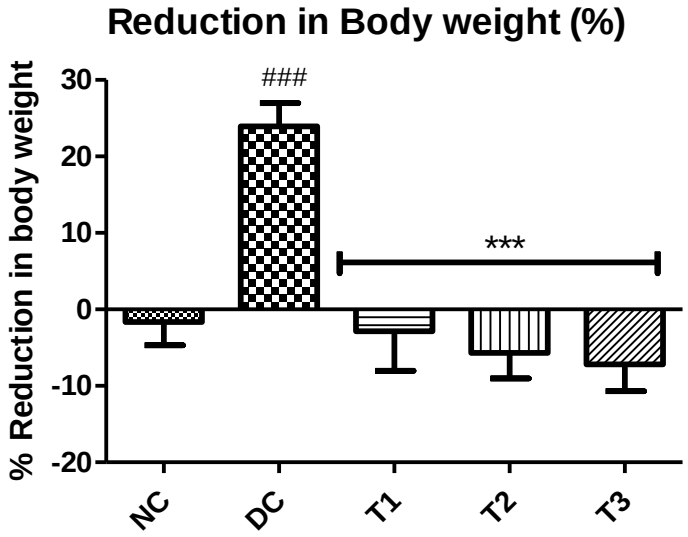


Figure 4.17: Reduction in body weight after D-Limonene treatment for 17 days in C57BL/6 mice. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). ###p< 0.001 compared to normal control group, ***p<0.001 compared to disease control group.

c. Haematological parameters: Immunosuppression and tail vein injection of K562 cells in C57BL/6 mice has shown significant changes in haematological parameters as compared to week 0 or untreated group. Immunosuppression for one week has shown significant reduction in WBC, NEU and LYM count as compared to week 0 or untreated group whereas as changes in RBC, Hb and PLT counts were non-significant. After the tumor cell injection, significant increase in WBC and NEU count was noted at week 4 as compared to week 0, whereas significant reduction in LYM and RBC count and Hb content was observed. The changes in PLT count was non-significant (Fig. 4.18). At the end of D-

Limonene treatment, significant reduction in WBC and NEU count was observed in all treatment groups as compared to disease control group, whereas significant increase in LYM count, RBC count and Hb content were observed at D-Limonene 1.0 and 1.5mg/kg as compared to disease control group. No significant changes in PLT count were observed (Fig. 4.19).

TABLE 4.12: Changes in haematological parameters of C57BL/6 mice during model establishment.

Treatment group	Week 0	Week 1	Week 2	Week 3	Week 4
WBC (10^3/µl)	8.93 ± 0.84	3.16 ± 0.30 ***	5.43 ± 0.76	15.06 ± 0.49 ***	17.38 ± 0.69 ***
NEU (10^3/µl)	3.75 ± 0.37	0.71 ± 0.31 ***	3.77 ± 0.16	3.28 ± 0.58	4.38 ± 0.11 *
LYM (10^3/µl)	8.12 ± 0.31	1.68 ± 0.37 ***	3.77 ± 0.16 **	3.27 ± 0.58 **	4.37 ± 0.11 **
RBC (10^6/µl)	10.98 ± 0.31	10.52 ± 0.37	9.77 ± 0.46	7.27 ± 0.58 *	6.38 ± 0.61 **
Hb (g/dl)	13.18 ± 0.61	12.72 ± 0.47	12.58 ± 0.86	10.27 ± 0.58 *	9.38 ± 0.31 *
PLT (10^5/µl)	8.08 ± 0.73	9.00 ± 0.91	9.34 ± 0.40	8.91 ± 0.25	8.10 ± 0.34

WBC=white blood cell count, NEU=neutrophil count, LYM=lymphocyte count, RBC=red blood cell count, Hb=haemoglobin content, PLT=platelet count. Data are represented as mean ± SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 compared to week 0.

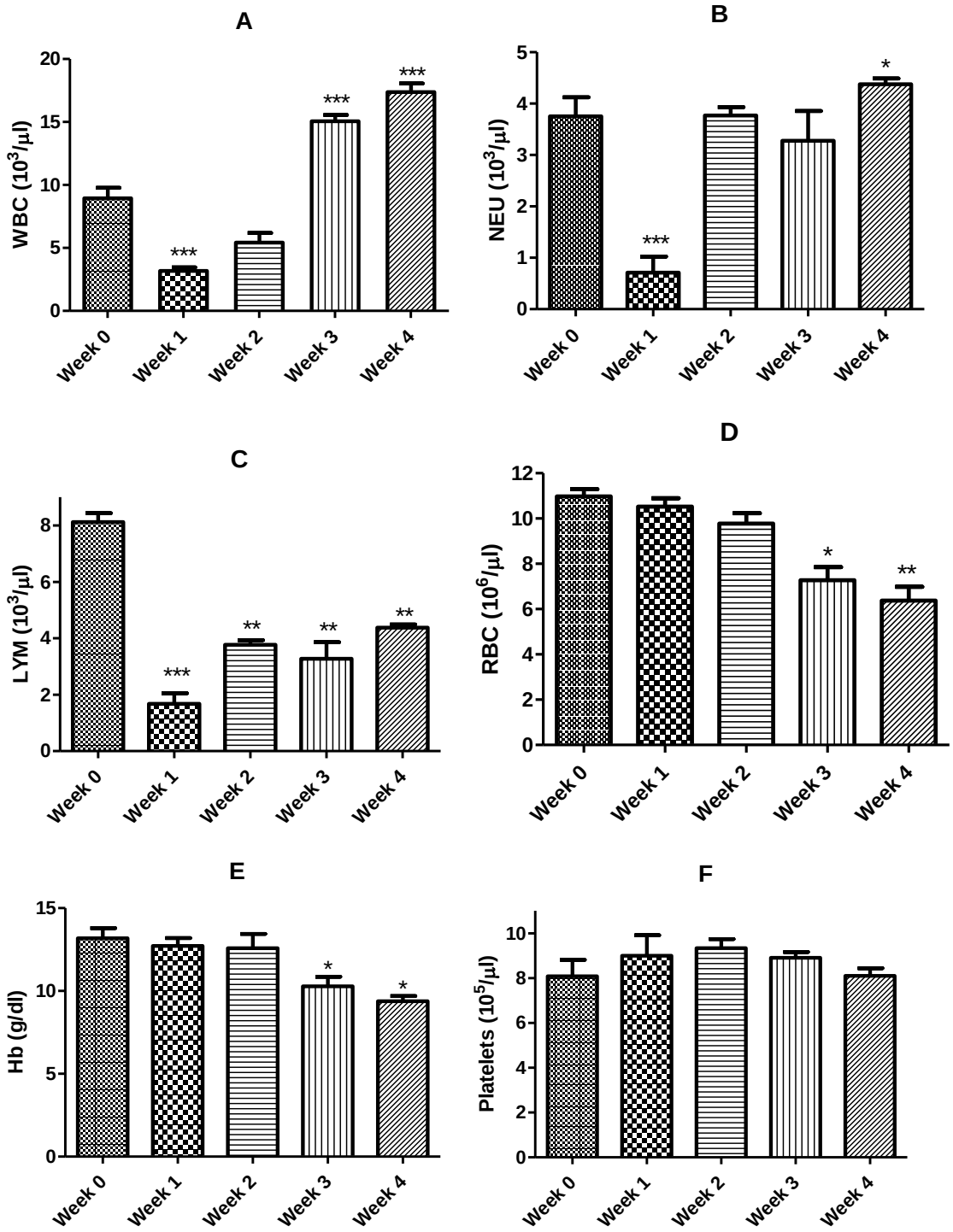


FIGURE 4.18: Changes in haematological parameters of C57BL/6 mice during model establishment. WBC=white blood cell count, NEU=neutrophil count, LYM=lymphocyte count, RBC=red blood cell count, Hb=haemoglobin content, PLT=platelet count. Data are represented as mean ± SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 compared to week 0.

TABLE 4.13: Haematological parameters at the end of D-Limonene treatment for 17 days in C57BL/6 mice.

Treatment group	NC	DC	T1	T2	T3
WBC (10^3/µl)	10.53 ± 0.66	24.75 ± 0.55 ###	15.74 ± 0.78***	13.73 ± 0.76***	11.48 ± 1.39 ***
NEU (10^3/µl)	3.81 ± 0.43	5.67 ± 0.38 ###	4.87 ± 0.46 *	2.71 ± 0.43 **	2.56 ± 0.27 **
LYM (10^3/µl)	6.41 ± 0.31	4.73 ± 0.39 ##	6.24 ± 0.22 *	7.18 ± 0.43 **	6.48 ± 0.27 **
RBC (10^6/µl)	10.78 ± 0.19	6.23 ± 0.38 ###	6.74 ± 0.43	8.06 ± 0.48 *	9.48 ± 0.27 **
Hb (g/dl)	12.9 ± 0.41	7.23 ± 0.56 ###	6.74 ± 0.77	8.06 ± 0.68 *	9.47 ± 0.22 **
PLT (10^5/µl)	9.84 ± 0.55	8.92 ± 0.62	9.64 ± 0.19	7.94 ± 0.55	8.27 ± 0.27

WBC=white blood cell count, NEU=neutrophil count, LYM=lymphocyte count, RBC=red blood cell count, Hb=haemoglobin content, PLT=platelet count. DC is disease control group. T1, T2 and T3 are D-Limonene treated group at dose 0.5, 1.0 and 1.5 g/kg/d respectively. Data are represented as mean ± SEM (n=10). ###p< 0.001 compared to normal control group and *p<0.05, **p<0.01 and ***p<0.001 compared to DC group.

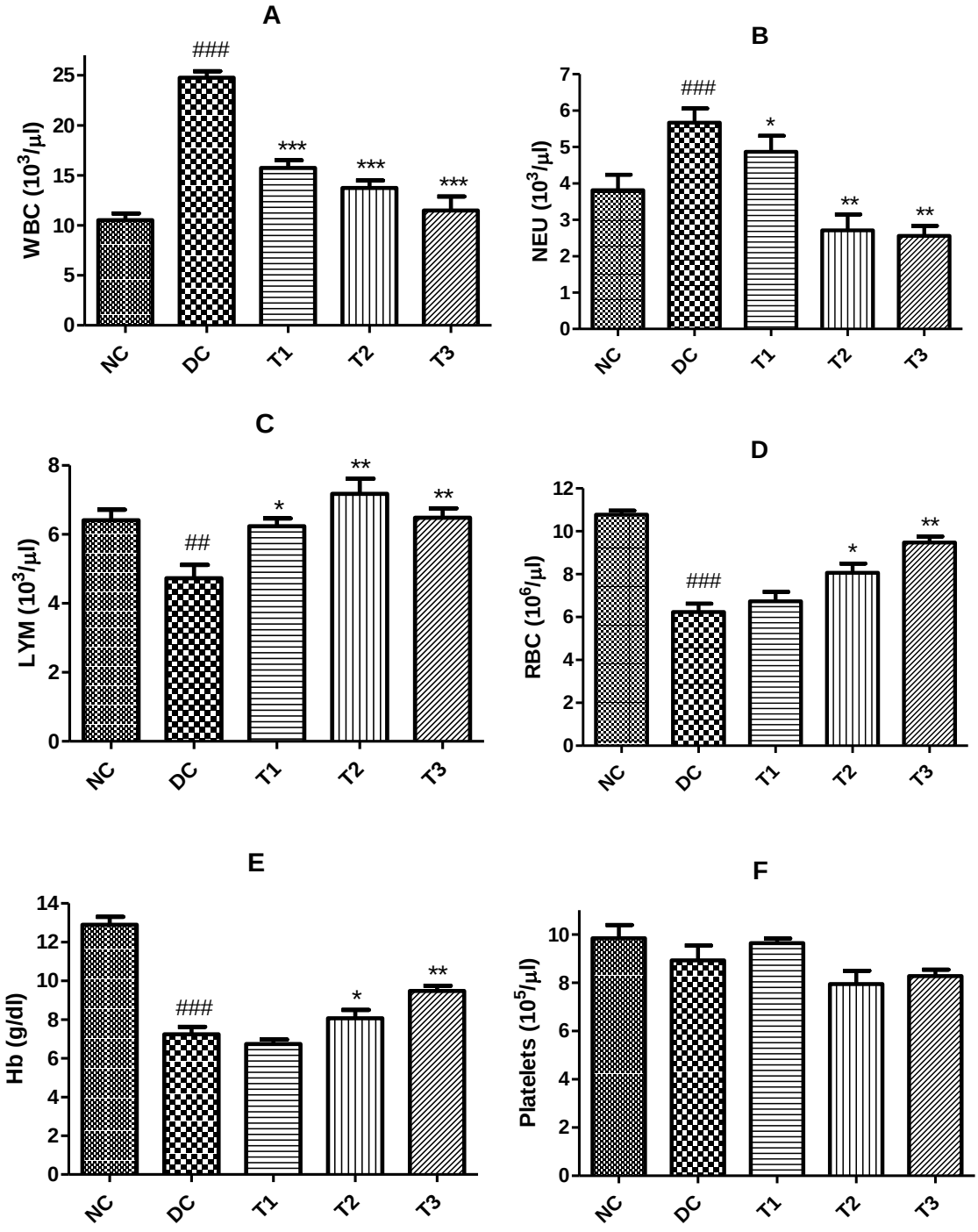


FIGURE 4.19: Haematological parameters at the end of D-Limonene treatment for 17 days in C57BL/6 mice. WBC=white blood cell count, NEU=neutrophil count, LYM=lymphocyte count, RBC=red blood cell count, Hb=haemoglobin content, PLT=platelet count. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). ###p< 0.001 compared to normal control group and *p<0.05, **p<0.01 and ***p<0.001 compared to DC group.

d. Biochemical parameters: Serum biochemical parameters were observed before immunosuppression, after immunosuppression and after disease induction. No significant changes in SGOT, SGPT and ALP level were observed after immunosuppression.

However, significant increase in SGOT and ALP levels were noted in week 4 after disease progression as compared to untreated group, changes in SGPT level were non-significant (Fig. 4.20). In treatment groups, significant increase in SGOT, SGPT and ALP level were noted in disease control group as compared to normal control group. D-Limonene treatment at 1.0 and 1.5 g/kg/d has shown significant reduction in all parameters as compared to disease control group. D-Limonene at 0.5 g/kg/d has shown significant reduction in SGOT and SGPT level as compared to disease control group, whereas changes in ALP were non-significant (Fig. 4.21).

TABLE 4.14: Effect of Immunosuppression and tumor cell injection on biochemical parameters.

	Week 0	**Week 1**	**Week 4**
SGOT(IU/L)	57.66 ± 1.76	56.00 ± 5.56	80.66 ± 2.33 **
SGPT(IU/L)	30.66 ± 3.17	31.33 ± 1.85	42.00 ± 4.04
ALP(IU/L)	84.00 ± 7.57	83.00 ± 6.24	119.33 ± 5.2 *

SGOT=serum glutamic oxaloacetic transaminase, SGPT=serum glutamic pyruvic transaminase and ALP=alkaline phosphatase. Data are represented as mean ± SEM (n=10). *p<0.05 and **p<0.01 compared to week 0.

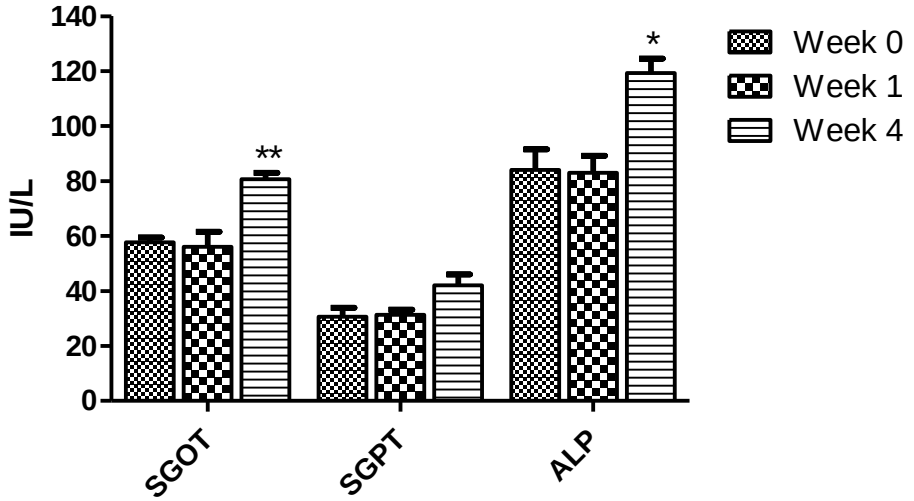


FIGURE 4.20: Effect of Immunosuppression and tumor cell injection on biochemical parameters. SGOT=serum glutamic oxaloacetic transaminase, SGPT=serum glutamic pyruvic transaminase and ALP=alkaline phosphatase. Data are represented as mean ± SEM (n=10). *p<0.05 and **p<0.01 compared to week 0.

TABLE 4.15: Effect of D-Limonene on biochemical parameters at the end of treatment.

Treatment Group	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)
NC	60.00 ± 4.56	32.00 ± 5.16	93.53 ± 3.46
DC	87.66 ± 2.60 ##	52.30 ± 4.33 ##	127.00 ± 6.42 ##
T1	55.00 ± 4.04 **	31.66 ± 1.76 **	103.33 ± 2.96
T2	56.00 ± 2.08 **	28.33 ± 1.76 **	94.00 ± 7.09 *
T3	58.66 ± 6.11 **	31.33 ± 1.20 **	84.33 ± 4.80 **

SGOT=serum glutamic oxaloacetic transaminase, SGPT=serum glutamic pyruvic transaminase and ALP=alkaline phosphatase. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). ##p<0.01 compared to NC whereas *p<0.05 and **p<0.01 compared to DC.

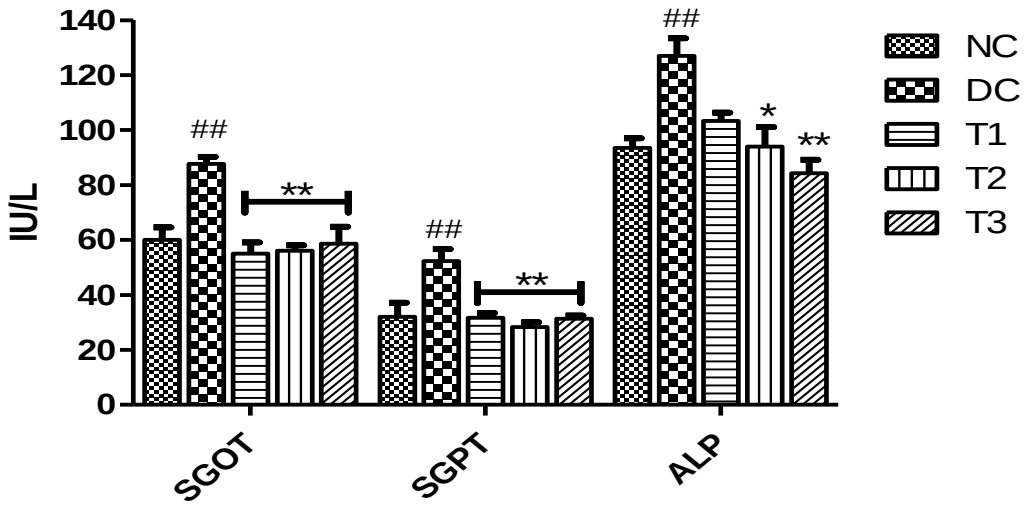


FIGURE 4.21: Effect of D-Limonene on biochemical parameters at the end of treatment. SGOT=serum glutamic oxaloacetic transaminase, SGPT=serum glutamic pyruvic transaminase and ALP=alkaline phosphatase. NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). ##p<0.01 compared to NC whereas *p<0.05 and **p<0.01 compared to DC.

e. Histopathological analysis: Haematoxylin and eosin stain of liver tissue was performed and the stained sections were observed under 100x and 400x magnification. Increase in inflammatory cell infiltration was noted in disease control group and D-Limonene 0.5 g/kg/d treated group as compared to normal control group. No significant changes in the histopathology of liver were observed with D-Limonene 1.0 and 1.5 g/kg/d treated groups (Fig. 4.22 and 4.23).

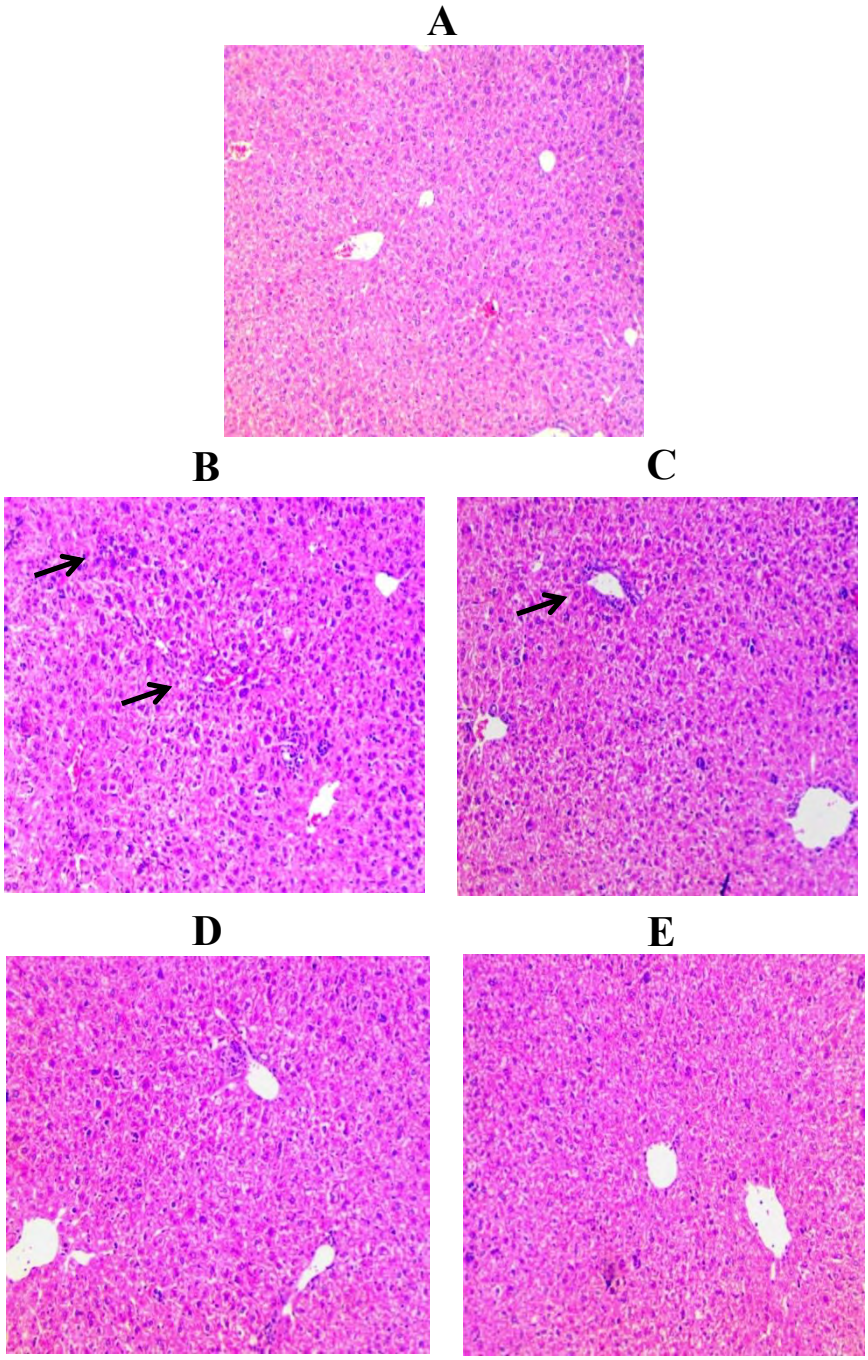
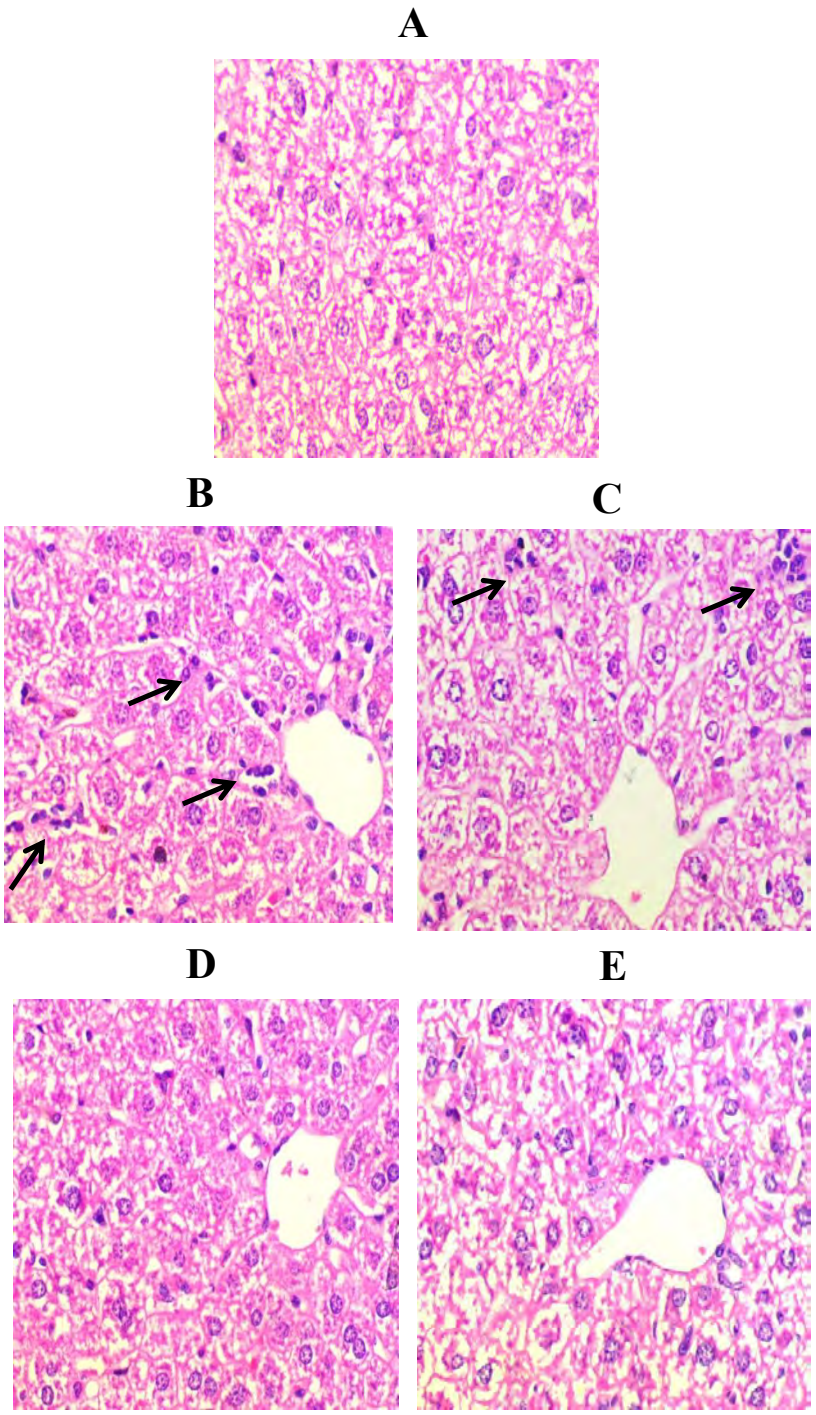


FIGURE 4.22: Histopathology of liver tissue at the end of D-Limonene treatment under 100x magnification. (A) Normal control, (B) Disease control, (C) D-Limonene 0.5 g/kg/d, (D) 1.0 g/kg/d and (E) 1.5 g/kg/d treated groups.



F**IGURE 4.23:** Histopathology of liver tissue at the end of D-Limonene treatment under 400x magnification. (A) Normal control, (B) Disease control, (C) D-Limonene 0.5 g/kg/d, (D) 1.0 g/kg/d and (E) 1.5 g/kg/d treated groups.

f. Estimation of serum oxidative stress markers: At the end of D-Limonene treatment, remarkable changes in serum oxidative stress markers such as MDA, SOD and nitrite were observed in all treatment groups. There was significant increase in serum lipid peroxidation or malondialdehyde and nitrite production was observed in disease control group as compared to normal control group, whereas significant reduction was observed with 1.0 and 1.5 g/kg/d of D-Limonene treatment. In serum superoxide dismutase assay, significant reduction in SOD level was observed in disease control group as compared to normal control group, however significant increase was noted with D-Limonene at 1.0 and 1.5 g/kg/d as compared to disease control group. No significant changes in serum oxidative stress markers were observed with D-Limonene at 0.5 g/kg/d (Fig. 4.24).

TABLE 4.16: Effect of D-Limonene treatment on serum oxidative stress markers at the end of 17 days in C57BL/6 mice.

Treatment Group	MDA (µmole/ml of serum)	SOD (units/ml)	Nitrite (µM)
NC	1.33 ± 0.18	47.50 ± 3.86	15.33 ± 1.30
DC	2.60 ± 0.09 ###	15.00 ± 2.38 ###	20.00 ± 0.93 #
T1	2.35 ± 0.07	23.50 ± 1.56	17.16 ± 0.70
T2	1.92 ± 0.10 **	27.75 ± 1.03 *	15.50 ± 0.67 *
T3	1.06 ± 0.08 ***	36.50 ± 1.55 ***	11.16 ± 0.70 ***

MDA= malondialdehyde, SOD= superoxide dismutase, NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). #p<0.05, ##p<0.01 and ###p<0.001 compared to NC whereas *p<0.05, **p<0.01 and ***p<0.001 compared to DC.

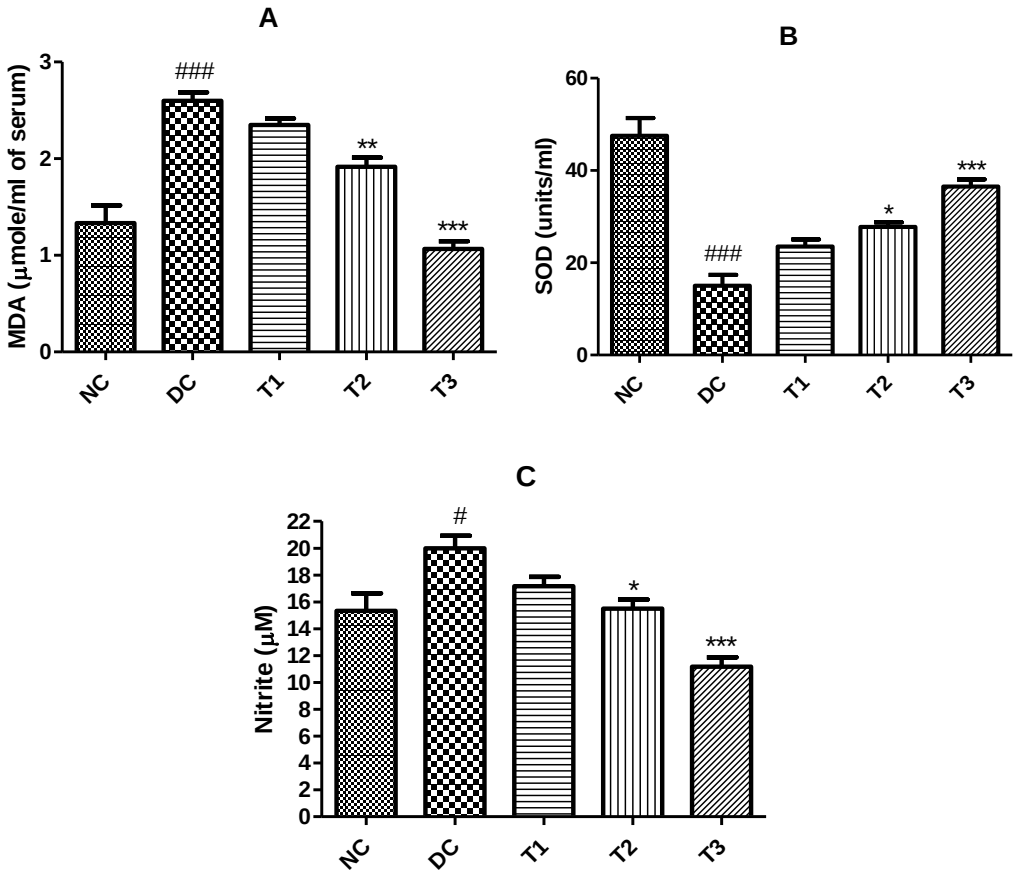


FIGURE 4.24: Estimation of serum oxidative stress markers at the end of D-Limonene treatment for 17 days in C57BL/6 mice. MDA= malondialdehyde, SOD= superoxide dismutase, NC= normal control, DC=disease control, T1=0.5 g/kg/d, T2=1.0 g/kg/d and T3= 1.5 g/kg/d D-Limonene treated group. Data are represented as mean ± SEM (n=10). #p<0.05, ##p<0.01 and ###p<0.001 compared to NC whereas *p<0.05, **p<0.01 and ***p<0.001 compared to DC.

4.5 Effect of D-Limonene on chick chorioallantoic membrane (CAM) assay

On 12th day of incubation, D-Limonene treatment has shown significant dose-dependent reduction in number of blood vessels on CAM as compared to implant soaked with the vehicle or D-Limonene at 0 µg / implant (Fig. 4.25).

TABLE 4.17: Effect of D-Limonene on angiogenesis by chick chorioallantoic membrane (CAM) assay.

D-Limonene (µg / implant)	Number of blood vessels
0	10.2 ± 0.47
1	8.2 ± 0.53 *
5	3.9 ± 0.50 ***
10	0.5 ± 0.22 ***

Data are expressed as mean ± SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 compared to 0 µg / implant.

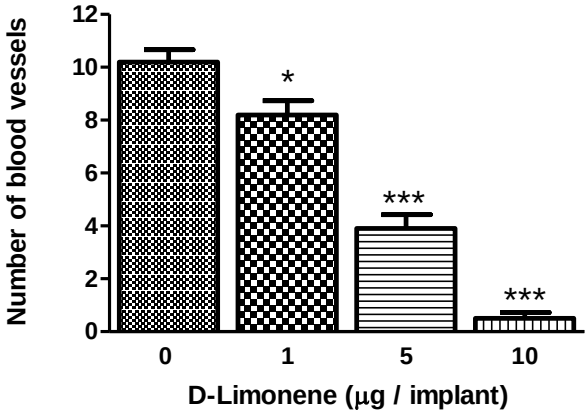


FIGURE 4.25: Chick chorioallantoic membrane (CAM) assay representing the effect of D-Limonene on angiogenesis. Data are expressed as mean ± SEM (n=10). *p<0.05, **p<0.01 and ***p<0.001 compared to 0 µg / implant.

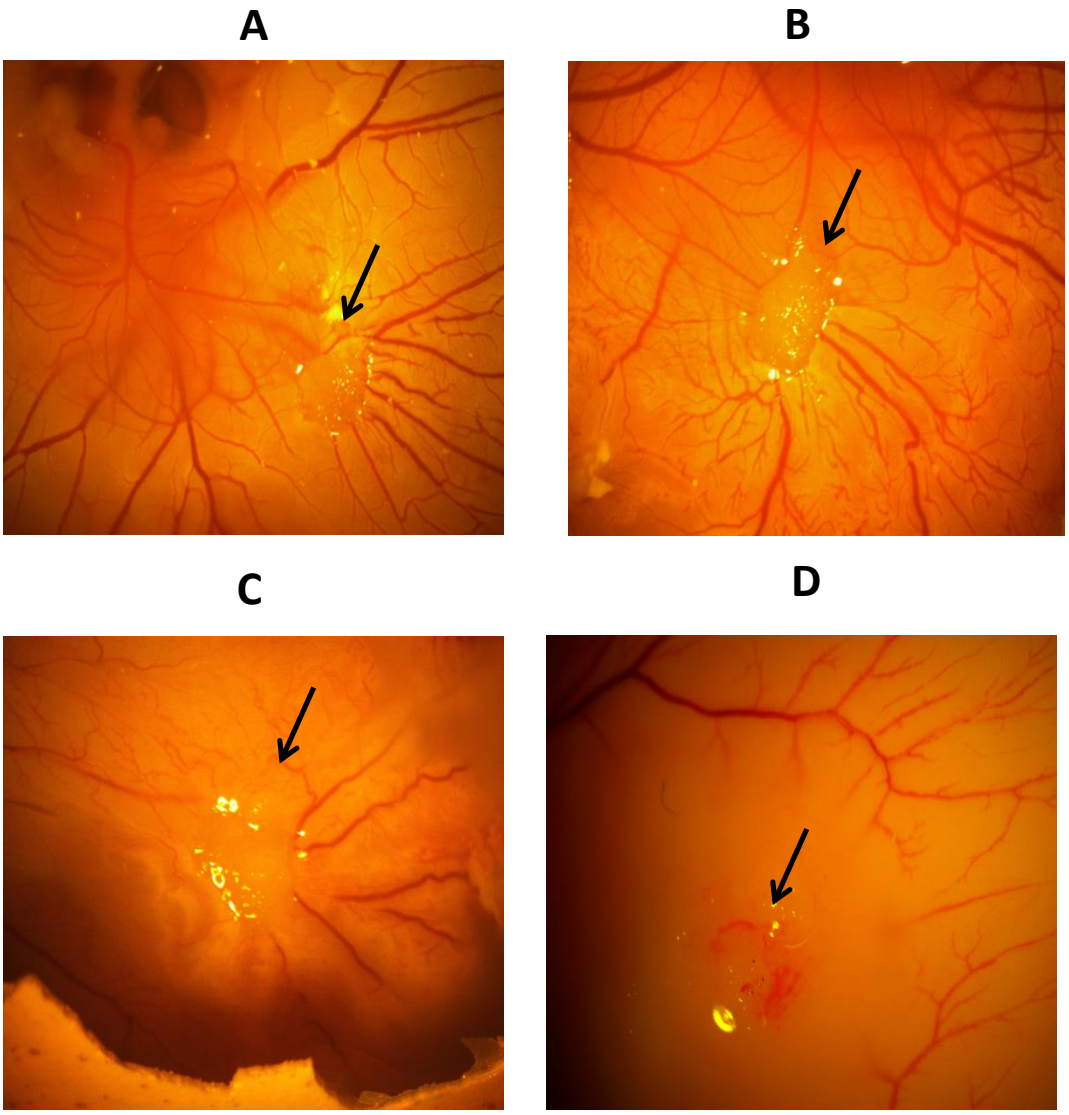


FIGURE 4.26: Representative images of angiogenesis on chick chorioallantoic membrane (CAM) under microscope at 100x magnification. The gelatin sponge soaked with vehicle and D-Limonene are indicated by arrows on 12th day of incubation. D-Limonene treatment was given as (A) 0 µg/implant (B) 1 µg/implant, (C) 5 µg/implant and (D) 10 µg/implant.

4.6 Effect of D-Limonene on expression of VEGF protein by Western blot analysis

In western blot analysis, treatment with D-Limonene changes the relative expression for VEGF protein as compared to β-actin. D-Limonene at concentration of 3 and 4 mM has shown significant reduction in relative expression of VEGF as compared to vehicle treated or 0 mM D-Limonene treated cells. However, the reduction in relative VEGF expression was non-significant with D-Limonene at 1 and 2 mM concentration (Fig. 4.27).

TABLE 4.18: Effect of D-Limonene on percentage relative fold changes in expression of VEGF protein by western blotting.

D-Limonene (mM)	**% relative fold change in VEGF expression (%)**
0	1.00
1	0.93 ± 0.06
2	0.85 ± 0.07
3	0.21 ± 0.08 ***
4	0.11 ± 0.07 ***

Data are expressed as mean ± SD from experiments in triplicate. ***p<0.001 compared to vehicle treated or 0 mM D-Limonene treated cells.

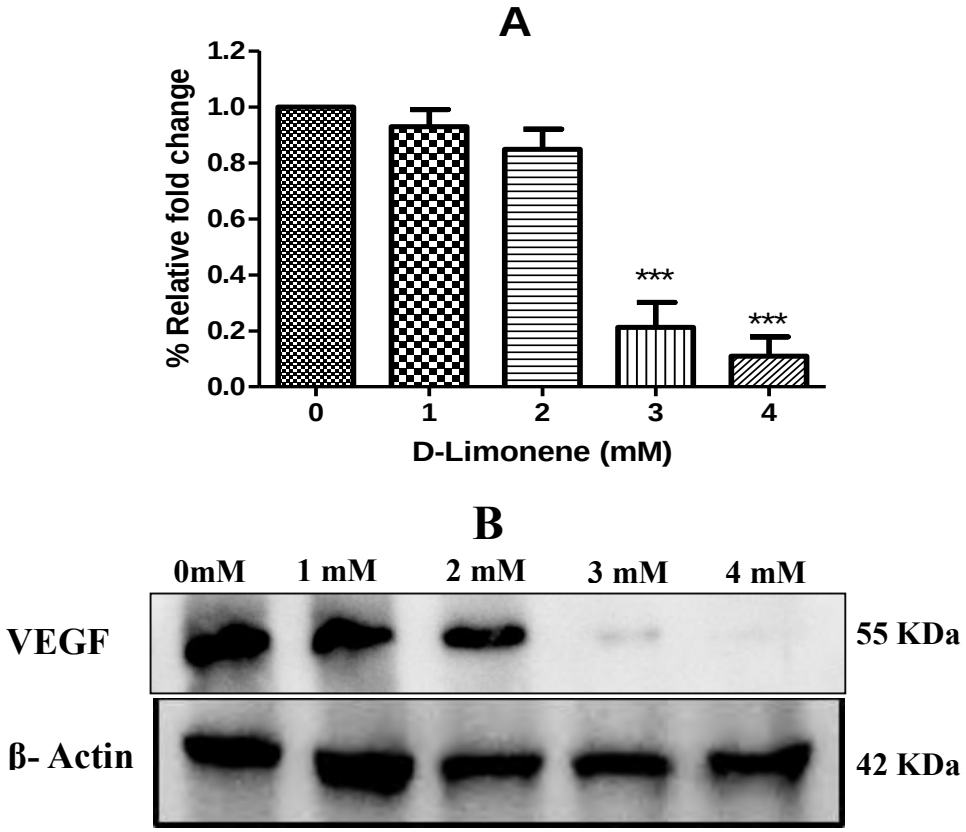


Figure 4.27: Effect of D-Limonene on expression of VEGF protein by Western blot analysis. **(A)** Effect of D-Limonene on percentage relative fold changes of VEGF protein. Data were expressed as mean ± SD from experiments in triplicate. ***p<0.001 compared to vehicle treated or 0 mM D-Limonene treated cells. (B) Representative images from western blot analysis of VEGF and β-actin protein.

4.7 Effect of D-Limonene on expression of MMP-2 and 9 proteins by gelatin zymography

In gelatin zymography, K562 cells treated with D-Limonene has shown significant dose dependent reduction in expression of matrix metalloproteinase (MMP)-2 and 9 levels in all treatment groups as compared to untreated cells or D-Limonene 0 mM (Fig. 4.28).

TABLE 4.19: Effect of D-Limonene on percentage relative fold changes of MMP-2 and MMP-9 protein by gelatin zymography.

D-Limonene (mM)	% relative fold change in protein expression (%)	
	MMP-2	MMP-9
0	1.00	1.00
2	0.74 ± 0.04 **	0.7 ± 0.02 **
3	0.59 ± 0.03 ***	0.54 ± 0.02 ***
4	0.38 ± 0.04 ***	0.27 ± 0.04 ***

Data are expressed as mean ± SD from experiments in triplicate. **p<0.01 and ***p<0.001 compared to vehicle treated or 0 mM D-Limonene treated cells.

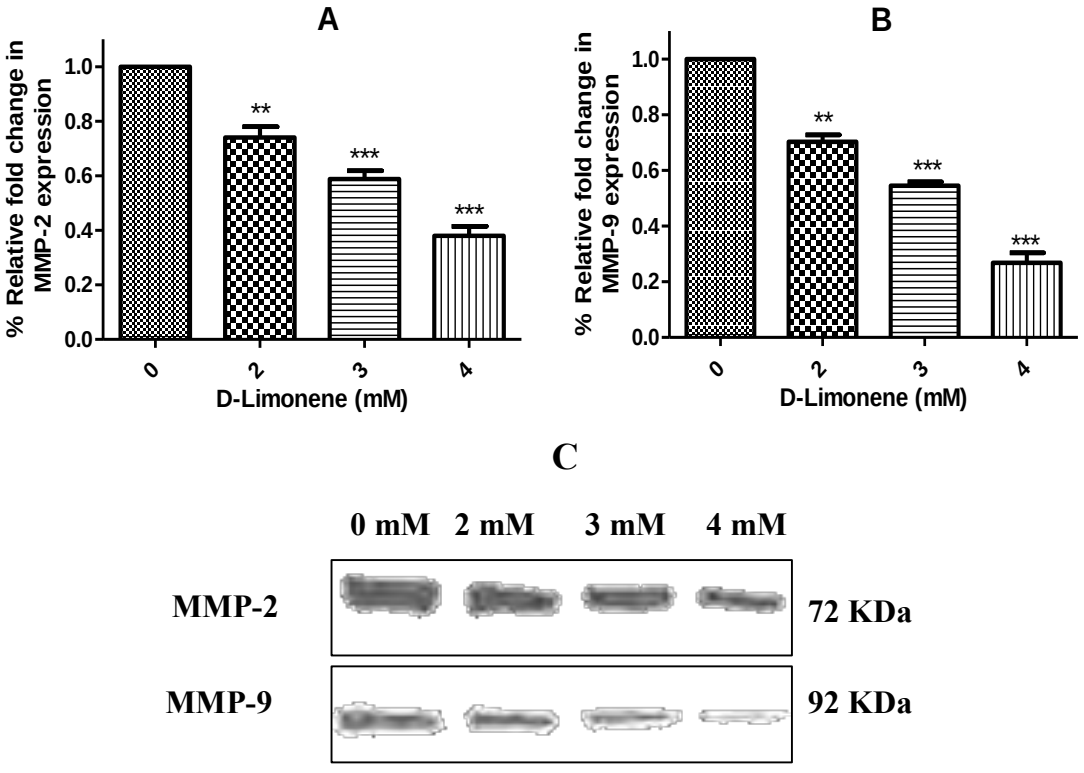


Figure 4.28: Effect of D-Limonene on expression of MMP-2 and 9 proteins by gelatin zymography. Effect of D-Limonene on percentage relative fold changes of (A) MMP-2 and (B) MMP-9 protein. Data are expressed as mean ± SD from experiments in triplicate. **p<0.01 and ***p<0.001 compared to vehicle treated or 0 mM D-Limonene treated cells. (C) Representative images from gelatin zymography of MMP-2 and 9.

4.8 Estimation of *in vitro* antioxidant activity of D-Limonene

The *in vitro* antioxidant activity of D-Limonene was determined in comparison with Trolox as standard by using DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging, superoxide radical scavenging and nitric oxide radical scavenging assays.

4.8.1 Effect of D-Limonene on DPPH assay

D-Limonene has shown appreciable concentration dependent reduction in free radical formation in comparison with standard Trolox. The IC_{50} values of D-Limonene and Trolox were 384.73 µM and 153.30 µM respectively (Fig. 4.29).

TABLE 4.20: Free radical scavenging activity of D-Limonene and Trolox by DPPH assay.

D-Limonene		Trolox	
Conc. (µM)	**% scavenging activity**	**Conc. (µM)**	**% scavenging activity**
25	7.38 ± 0.22	10	2.60 ± 0.19
50	13.42 ± 0.24	25	10.52 ± 0.28
100	18.88 ± 0.20	50	18.12 ± 0.39
200	24.95 ± 0.08	100	43.29 ± 0.54
400	53.06 ± 0.17	250	75.77 ± 0.11
IC_{50}	**384.73 µM**	**IC_{50}**	**153.30 µM**

Data are presented as mean ± S.D. from 3 separate experiments in quadruplicate.

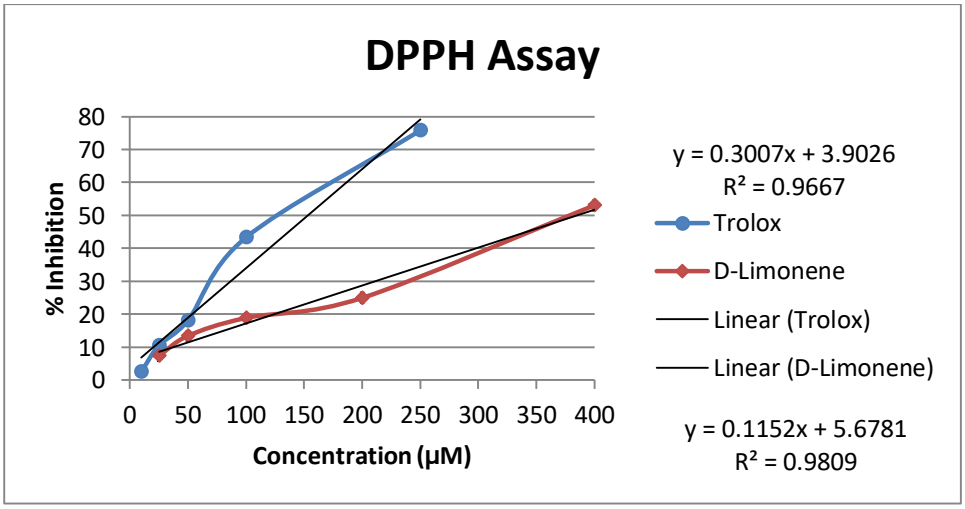


FIGURE 4.29: Antioxidant activity of D-Limonene and Trolox by DPPH assay

4.8.2 Effect of D-Limonene on ABTS assay

D-Limonene (IC_{50}= 603.23 µM) has shown appreciable concentration dependent reduction in free radical formation in comparison with Trolox (IC_{50}= 203.37 µM) as a standard (Fig. 4.30).

TABLE 4.21: Free radical scavenging activity of D-Limonene and Trolox by ABTS assay.

D-Limonene		Trolox	
Conc. (µM)	**% scavenging activity**	**Conc. (µM)**	**% scavenging activity**
25	6.05 ± 0.22	10	5.28 ± 0.07
50	13.60 ± 0.24	25	10.59 ± 0.16
100	24.83 ± 0.20	50	24.34 ± 0.14
200	26.47 ± 0.08	100	37.03 ± 0.15
400	34.27 ± 0.17	250	55.80 ± 0.20
IC_{50}	**603.23 µM**	**IC_{50}**	**203.37 µM**

Data are presented as mean ± S.D. from 3 separate experiments in quadruplicate.

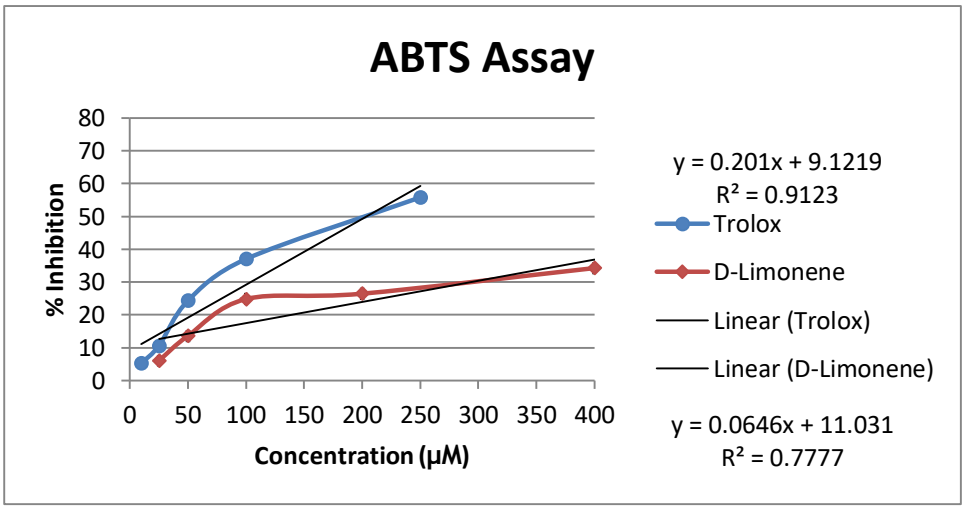


FIGURE 4.30: Antioxidant activity of D-Limonene and Trolox by ABTS assay.

4.8.3 Effect of D-Limonene on FRAP assay

FRAP assay of D-Limonene was performed against Trolox as standard. D-Limonene (IC_{50}= -589.85 µM) has shown appreciable concentration dependent reduction in free radical formation by FRAP assay in comparison with Trolox (IC_{50}= -171.73 µM) (Fig. 4.31).

TABLE 4.22: Free radical scavenging activity of D-Limonene and Trolox by FRAP assay.

D-Limonene		Trolox	
Conc. (µM)	**% scavenging activity**	**Conc. (µM)**	**% scavenging activity**
25	0.19 ± 0.95	10	-2.24 ± 0.30
50	-3.59 ± 0.30	25	-9.79 ± 0.37
100	-6.84 ± 0.12	50	-17.01 ± 3.27
200	-11.33 ± 0.23	100	-48.11 ± 0.26
400	-34.73 ± 0.22	250	-77.50 ± 0.30
IC_{50}	**-589.85 µM**	**IC_{50}**	**-171.73 µM**

Data are presented as mean ± S.D. from 3 separate experiments in quadruplicate.

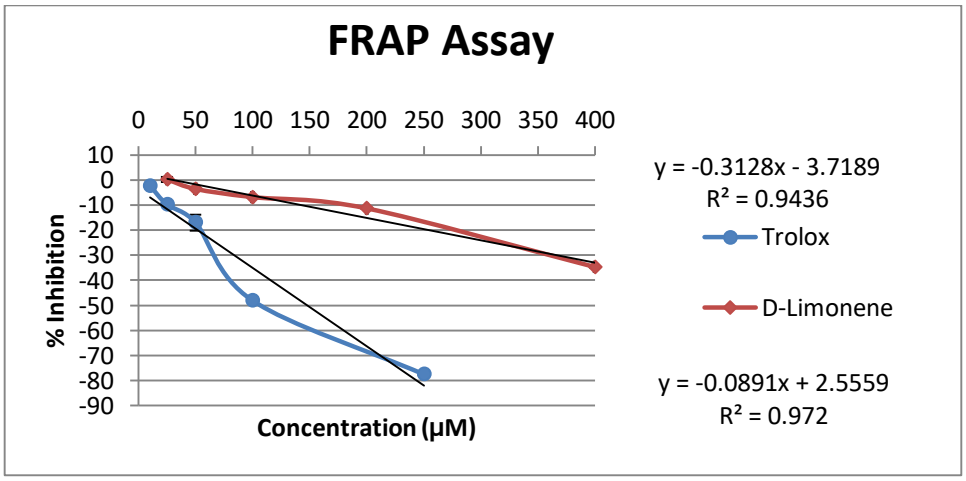


FIGURE 4.31: Antioxidant activity of D-Limonene and Trolox by FRAP assay.

4.8.4 Effect of D-Limonene on iron chelating assay

In iron chelating assay D-Limonene was used with Trolox as a standard. However, D-Limonene did not show any percentage inhibition in iron chelating assay in comparison with Trolox. The IC_{50} value for D-Limonene and Trolox were -18475.5 µM and 225.96 µM respectively (Fig. 4.32).

TABLE 4.23: Free radical scavenging activity of D-Limonene and Trolox by iron chelating assay.

D-Limonene		Trolox	
Conc. (µM)	**% scavenging activity**	**Conc. (µM)**	**% scavenging activity**
25	0.14 ± 0.12	10	1.05 ± 0.06
50	0.00 ± 0.10	25	2.86 ± 0.69
100	-0.47 ± 0.06	50	6.88 ± 0.12
200	-0.14 ± 0.06	100	29.30 ± 0.08
400	-1.01 ± 0.06	250	53.21 ± 0.14
IC_{50}	**-18475.5 µM**	**IC_{50}**	**225.96 µM**

Data are presented as mean ± S.D. from 3 separate experiments in quadruplicate.

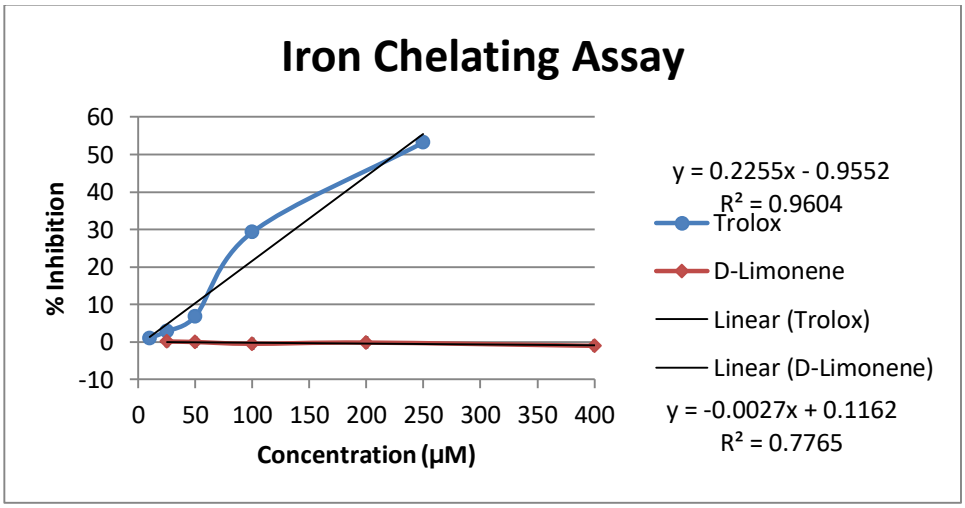


FIGURE 4.32: Antioxidant activity of D-Limonene and Trolox by iron chelating assay.

4.8.5 Effect of D-Limonene on hydroxyl radical scavenging assay

Hydroxyl radical scavenging assay of D-Limonene has shown appreciable concentration dependent radical scavenging activity. The antioxidant activity of D-Limonene (IC_{50}= 442.75 µM) was compared with Trolox (IC_{50}= 146.37 µM) as a standard (Fig. 4.33).

TABLE 4.24: Free radical scavenging activity of D-Limonene and Trolox by hydroxyl radical scavenging assay.

D-Limonene		Trolox	
Conc. (µM)	**% scavenging activity**	**Conc. (µM)**	**% scavenging activity**
25	5.46 ± 0.13	10	8.91 ± 0.05
50	11.38 ± 0.75	25	18.86 ± 0.11
100	19.87 ± 0.11	50	30.46 ± 0.12
200	26.87 ± 0.12	100	44.31 ± 0.23
400	44.69 ± 0.17	250	73.02 ± 0.09
IC_{50}	**442.75 µM**	**IC_{50}**	**146.37 µM**

Data are presented as mean ± S.D. from 3 separate experiments in quadruplicate.

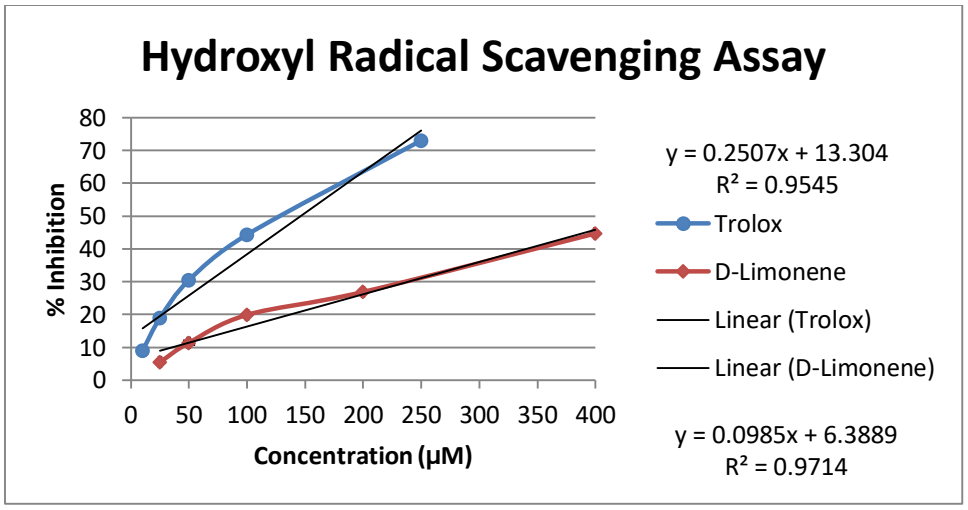


FIGURE 4.33: Antioxidant activity of D-Limonene and Trolox by hydroxyl radical scavenging assay.

4.8.6 Effect of D-Limonene on superoxide radical scavenging assay

D-Limonene (IC_{50}= 692.89 µM) has shown appreciable concentration dependent reduction in superoxide radical formation by superoxide radical scavenging assay in comparison with Trolox (IC_{50}= 105.25 µM) as a standard (Fig. 4.34).

TABLE 4.25: Free radical scavenging activity of D-Limonene and Trolox by superoxide radical scavenging assay.

D-Limonene		Trolox	
Conc. (µM)	**% scavenging activity**	**Conc. (µM)**	**% scavenging activity**
25	3.01 ± 0.12	10	15.23 ± 0.17
50	8.67 ± 0.16	25	24.01 ± 0.14
100	13.57 ± 0.15	50	49.10 ± 0.12
200	21.47 ± 0.14	100	57.49 ± 0.17
400	29.02 ± 0.04	250	87.78 ± 0.06
IC_{50}	**442.75 µM**	**IC_{50}**	**105.25 µM**

Data are presented as mean ± S.D. from 3 separate experiments in quadruplicate.

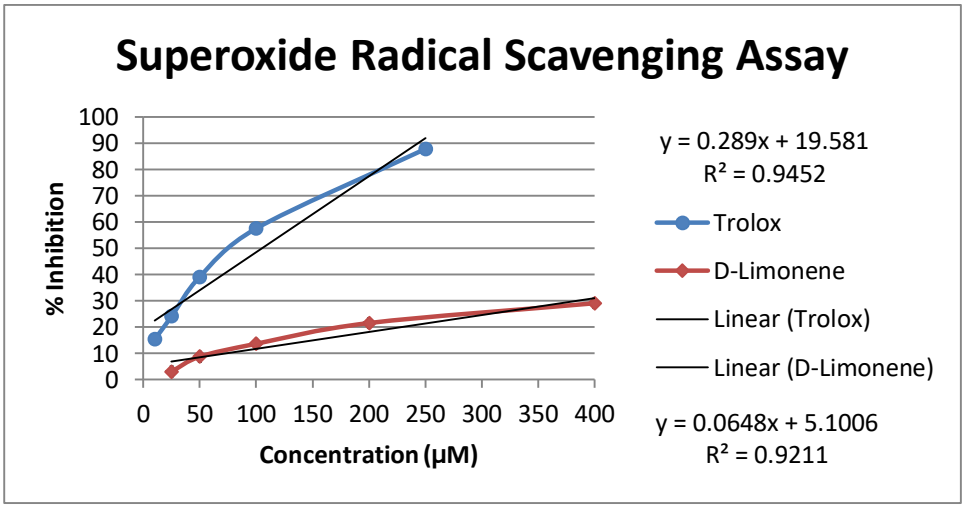


FIGURE 4.34: Antioxidant activity of D-Limonene and Trolox by superoxide radical scavenging assay.

4.8.7 Effect of D-Limonene on nitric oxide radical scavenging assay

The nitric oxide radical scavenging assay of D-Limonene was performed by using Trolox as standard. D-Limonene (IC50= 415.65 µM) has shown appreciable concentration dependent reduction in nitric oxide radical formation in comparison with Trolox (IC_{50}= 99.79 µM) as a standard (Fig. 4.35).

TABLE 4.26: Free radical scavenging activity of D-Limonene and Trolox by nitric oxide radical scavenging assay.

D-Limonene		Trolox	
Conc. (µM)	**% scavenging activity**	**Conc. (µM)**	**% scavenging activity**
25	1.09 ± 0.06	10	10.52 ± 0.05
50	8.87 ± 0.28	25	26.95 ± 0.19
100	16.47 ± 0.21	50	43.75 ± 0.13
200	30.20 ± 0.15	100	57.19 ± 0.10
400	44.76 ± 0.26	250	91.95 ± 0.06
IC_{50}	**415.65 µM**	**IC_{50}**	**99.79 µM**

Data are presented as mean ± S.D. from 3 separate experiments in quadruplicate.

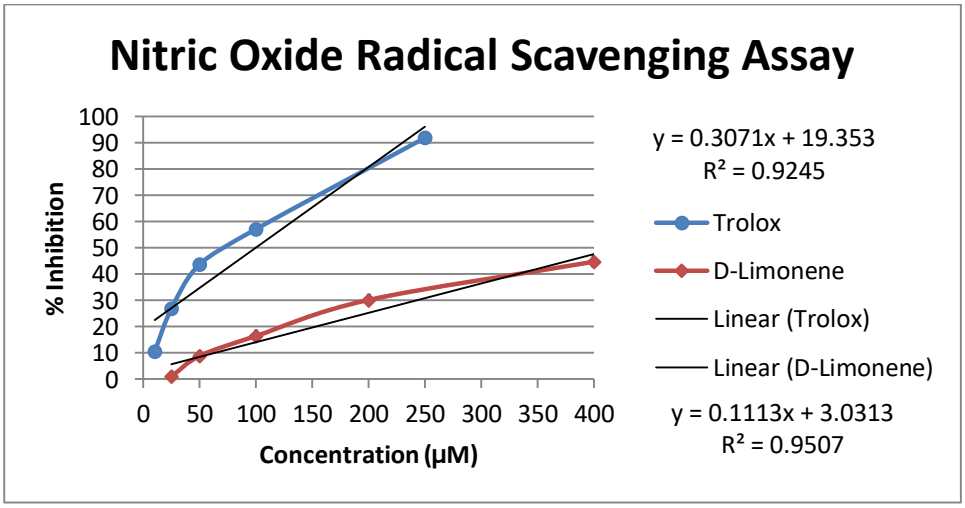


FIGURE 4.35: Antioxidant activity of D-Limonene and Trolox by nitric oxide radical scavenging assay.

4.9 Effect of D-Limonene on *in vitro* lipid peroxidation assay on K562 cells

In *in vitro* lipid peroxidation assay, D-Limonene has shown significant reduction in malondialdehyde production on K562 cells as compared to untreated cells. The reduction of malondialdehyde production was in concentration dependent manner (Fig. 4.36).

TABLE 4.27: *In vitro* lipid peroxidation assay of D-Limonene on K562 cells.

Concentration (mM)	MDA (nM/10^7 cells)
0	**6.40 ± 0.10**
0.5	**5.56 ± 0.08 *****
1	**3.12 ± 0.07 *****
2	**2.35 ± 0.06 *****

Data are presented as mean ± S.D. from 3 separate experiments in quadruplicate. ***p<0.001 compared to untreated cells.

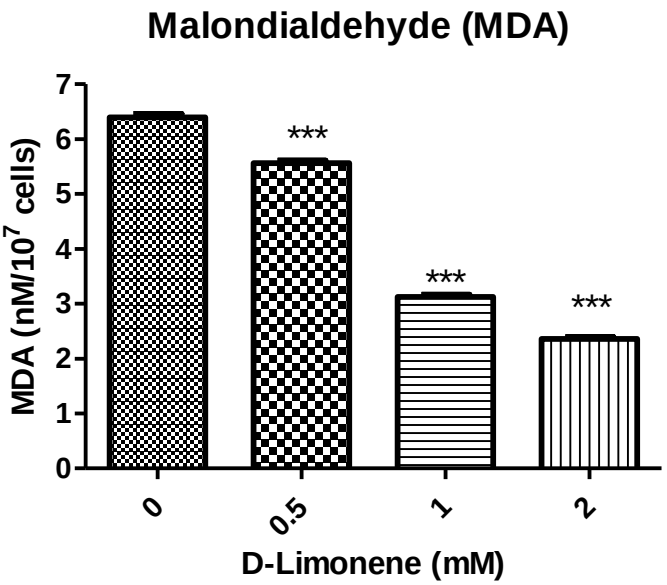


FIGURE 4.36: Effect of D-Limonene on *in vitro* malondialdehyde production from K562 cells by lipid peroxidation assay. ***p<0.001 compared to 0mM or untreated cells.

Chapter-5

Discussion

CHAPTER-5

Discussion

Chronic myeloid or myelogenous leukemia (CML) is a type of myeloproliferative disorder characterized by an excessive formation of myeloid blood cells, especially granulocytes with presence of Philadelphia (Ph) chromosome. The Ph chromosome is formed by the acquired genetic mutation of haematopoietic stem cells. The reciprocal translocation occurs at(9;22) that generates BCR-ABL fusion gene, which is responsible for generation of BCR-ABL oncoprotein that activates tyrosine kinase signaling [1, 2]. The fusion protein leads to constitutive activation of tyrosine kinase receptor, forcing the cell to divide uncontrollably [3]. Apart from its tyrosine kinase activity, BCR–ABL has been known to cause DNA damages and impairment of DNA repair, which leads to an accumulation of mutations, deletions, and chromosomal aberrations in the course of CML.

Plants and its secondary metabolites have been reported for its potential antibacterial, antidiabetic, immunomodulatory, antifungal, antioxidant and anticancer properties [4]. D-Limonene is a monocyclic monoterpene secondary metabolite of plant, abundantly found in volatile oils of citrus fruits has been reported for its antioxidant, antimicrobial, anti-inflammatory and cholesterol gall-stone dissolving property. It has exhibited its anti-carcinogenic activity by either inhibiting cell proliferation or enhancing the apoptosis of cells.

An anticancer effect of particular drug can be evaluated by various preclinical screening methods such as *in vitro* human cancer cell lines, *in vivo* tumor xenograft model, or genetically engineered mouse model [5]. For initial screening of drug, direct use of animal systems is highly unethical and are under strict regulations [6]. Therefore, *in vivo* evaluation of anticancer drugs usually ensued by *in vitro* cellular assays. Cellular screening mainly comprises of permanent human tumor cell lines as it is the most suitable test system in terms of management, because of its immortality and quick reproducibility [7]. In our study, we have used K562 cells lines for all *in vitro* experiments. The K562 cells are the first human

immortalized erythroleukemia type of myelogenous leukemia cell line. The cells were first derived from the pleural effusion of a 53 year old female, from blastic phase. The particular cell line was selected for performing all *in vitro* experiments as the cells were considered to represent the outgrowth of a CML clone. It has been reported to carry the BCR-ABL gene, the marker of CML. Being an erythroleukemic type of cells, it also bears some proteomic resemblance to undifferentiated granulocytes and erythrocyte. Because of all these characteristics, K562 is the ideal cell line to study the CML [8].

In preliminary screening, compound that reduces the cell growth or viability of cancer cell line can be seen as a promising anticancer agent. For the determination of cell growth inhibition various accepted methods such as MTT assay, sulforhodamine B (SRB) assay, propidium iodide (PI) assay, and luciferase assay are available that utilizes certain dyes by live cell; however, selection of a particular method depends on factors such as requirement of minimum number of cells, sensitivity, speed, and ease of handling or simplicity. MTT assay is a colorimetric assay in which the yellow coloured tetrazole is converted in the purple coloured formazan. This conversion is NADH, NADPH, and other oxidized substrates dependent, which is an outcome of mitochondrial activity, seen in the viable cells. So, the intensity of purple colour relates with the percentage cell viability. Variation in formazan production correlates with a cell line-specificity and the length of drug exposure.

In this study, the MTT assay of D-Limonene has shown significant dose dependent reduction in percentage cell viability of K562 cells at 24 and 48 h of treatment as compared to untreated cells. The cytotoxicity of D-Limonene was compared with the available anticancer drug doxorubicin as a standard, as it reduces the percentage cell viability of K562 cells [9, 10]. Furthermore, the reduction in % cell viability is a measure of sensitivity of drug to particular cancer cell line [11]. Therefore, the dose dependent reduction in percentage cell viability of K562 cells by D-Limonene indicates its sensitivity against K562 cells.

Discovery of anticancer drugs has been a major breakthrough in the treatment of cancer. Unfortunately, these drugs also exert their effect on normal cells because of lack of target specificity that gives rise to organ toxicities. Being a major detoxifying or metabolising organ, liver always suffer from high doses of toxicants or anticancer drugs and develops hepatotoxicity. After diarrhoea, hepatotoxicity is the second common cause of imatinib and other TKIs discontinuation [12]. On histopathological examination of liver tissues, imatinib has been reported for inducing hepatic necrosis and mild cholestasis. Clinically it is involved

in cytolytic hepatitis type of hepatotoxicity [13-16]. Therefore, we have decided to evaluate the *in vitro* effect of D-Limonene on liver cells or hepatocytes. For that, the mouse primary hepatocytes were isolated. It constitutes a valuable biological tool to understand the functional properties or alterations occurring in the liver, as it can retain the key properties of liver cells such as the induction of cytochrome P450 gene expression by drugs like phenobarbital [17, 18]. To check the effect of D-Limonene on the liver, MTT assay with higher concentration of D-Limonene and doxorubicin on mouse primary hepatocytes was performed. Doxorubicin was used as a standard, as it induces hepatotoxicity by persuading the necrosis of liver tissues and because of its availability [19-21]. After 48 h of treatment, doxorubicin treatment has shown significant reduction in percentage cell viability of mouse primary hepatocytes as compared to untreated cells by MTT assay. However, D-Limonene treatment up to concentration as high as 32mM did not produce any significant changes in % cell viability of mouse primary hepatocytes, which suggest its relatively low hepatotoxicity as compared to doxorubicin.

Thus, in vitro studies have demonstrated that D-Limonene reduces the viability of K562 cells without affecting on mouse primary hepatocytes.

Although the *in vitro* studies is the fastest, economic and effective to screen the anticancer activity, there is always need to check the *in vivo* anticancer activity of these drugs. Many factors such as physiochemical properties, pharmacokinetics and toxicity, may significantly affect the absolute effectiveness of the anticancer agent. Moreover, *in vitro* studies are inadequate in the evaluation of the off-target effects, which may contribute to the toxicity. Therefore, we have aimed to investigate the *in vivo* effect of D-Limonene in tumor xenograft model of CML in mice.

Despite the wide variety of available animals, mice are ideal animals to develop the human cancer models because of its genetic and physiologic similarity to humans, small size, reliable breeding, ease of handling and relatively short life span. Moreover, the spontaneous leukemia development is very rare in rats as compared to mouse and it also takes longer time to develop, after tumor cell implantation [22]. In literature, use of nude or SCID mice have been reported for the development of CML, because of absence of T cell and B cells mediated immune response as it can develop the chances of tumor rejection. However, absence of immune cells also limits the usage of these strains as it fails to reflect true disease progression because of critical role of immune cells in tumorigenesis. Beside this, some

studies have also reported the use of C57BL/6 mice as it develops the tumor with 80-100 % take rate [23, 24]. Therefore, in our study, we have selected the C57BL/6 mice for both the animal models. Among all available models of CML, we have selected two tumor xenograft models of CML, by subcutaneous and tail vein injection model because of its availability, versatility, reduced mortality and cost-effectiveness as compared to other available models. In our first *in vivo* model of CML, we have started with subcutaneous tumor xenograft model because it ideally replicates the interactions between the subcutaneously transplanted leukemia cells with adjacent stromal tissue and immune cells as compared to *in vitro* studies and have been shown to closely maintain the histopathological, cytological, and biochemical characteristics typical of original tumor [25].

During tumor progression, unintentional reduction in body weight is common with all cancer. CML has been also associated with marked reduction in body weight, this reduction is may be because of disease associated anorexia and increased metabolic activity [26]. In our study, body weight was checked throughout the study, as a measure of disease progression. Significant changes in body weight were noted before and after assigning the respective treatments in all groups. At the end of study, data showed significant reduction in body weight in disease control group as compared to normal control group, which may be because of tumor progression. It may increase the disease-associated anorexia and discomfort in the animals. However, D-Limonene treatment has shown significant increase in body weight as compared to disease control group, which may be because of its protective effect against tumor progression [27].

On the first day, all animals were immunocompromised for one week by the combination of ketoconazole, cyclosporine and cyclophosphamide, as immunosuppression is needed for the successful implantation of K562 tumor cell and to avoid tumor rejection because of active immune system.

Cyclosporine was selected for the protocol of immunosuppression because it is a potent immunosuppressant drug that suppresses the immune system by selectively inhibiting the activation of T-cells, by binding with cytosolic protein cyclophilin of the T-cells. This binding inhibits calcineurin, which is responsible for activating the transcription of interleukin 2 (IL-2), which has an important role in T-cell activation and proliferation [28, 29]. However, individual cyclosporine was not sufficient for maximum tumor development [30]. The combination of cyclosporine with cyclophosphamide has shown effective tumor

development with 100% take rate in mouse osteosarcoma [30]. Cyclophosphamide is an alkylating agent that binds with DNA and inhibits further DNA replication. Besides its anticancer activity, it also enhances the tumor success rate by reducing the immune response followed by inhibiting neutrophils, B- cells, T cells and natural killer cells [31]. Ketoconazole was used in the immunosuppression protocol because of its antifungal action, as during immunosuppression the chances of fungal infection development increases with cyclosporine and cyclophosphamide treatment. Ketoconazole decreases the fungal cell wall synthesis by inhibiting ergosterol synthesis, a key component of fungal cell wall. Further, it reduces the metabolization of cyclosporine by inhibiting cytochrome P450 enzyme and prolongs its duration of action [32].

After the one week of immunosuppression, haematological data showed significant reduction in WBC, NEU and LYM count as compared to week 0 or untreated group, which was an indicator of immunosuppression and confirms the successful establishment of immunosuppression. Administration of cyclosporine has been reported as popular immunosuppressive agent in childhood aplastic anaemia, whereas cyclophosphamide has been reported for inducing neutropenia in nude rat xenografted tumor models [33, 34]. Hurd and Guiliano have also reported the cyclophosphamide induced suppression of B and T lymphocytes [35]. However, changes in RBC, Hb and PLT were non-significant because of immunosuppression, which confirms that the immunosuppression was only because of drugs and not because of other physiological conditions. The ketoconazole and ampoxin, a combination of amoxicillin and clavulanic acid was used as prophylactic to avoid fungal and bacterial infections respectively [36].

At the end of treatment, the haematological parameters showed significant reduction in WBC, NEU and LYM count in disease control group as compared to normal control group in K562 cells implanted immunocompromised C57BL/6 mice, whereas changes in RBC, Hb and PLT was non-significant. However, in D-Limonene treated groups significant increase in WBC count in all treatment, whereas significant increase in NEU count at dose 1.0 (T2) and 1.5 mg/kg/d (T3) group was noted as compared to disease control group. The changes in LYM, RBC, Hb and PLT were non-significant in all D-Limonene treatment groups. In literature, immunosuppression has been reported as a major contributor for the development and progression of many cancers. It reduces the ability of immune cells to fight against cancer cells [37, 38]. At the end of treatment, reduced WBC, NEU and LYM count in disease

control group was may be because of immunosuppression done prior the injection of K562 cells, which further supports the environment for tumor progression.

D-Limonene treatment showed dose dependent improvement in WBC and NEU count in all treatment groups, which may be because of immunomodulatory effect of D-Limonene. Raffel and Kutanhave reported the immunomodulatory effect of D-Limonene on haematological parameters in Balb/c mice. D-Limonene treatment has significantly increased the WBC count whereas no changes in Hb content were observed. Moreover, immunosuppression is one of the major struggles with available chemo and radiotherapies, in such condition immunomodulation by D-Limonene may reduce the immune system related problems in cancer patients [39, 40].

Liver function tests such as SGOT, SGPT and ALP are the most common predictive test for liver abnormalities. Murakami and Shimizu has reported mild to moderate alteration in liver function associated with CML [41]. Therefore, we performed these biochemical tests to find out effect of disease progression on liver function. In biochemical tests, no significant changes in SGOT, SGPT and ALP levels were observed in disease control and D-Limonene treated group, suggesting subcutaneous K562 cell implantation does not have any significant effect on liver function. Moreover, these biochemical tests also represents the safety of D-Limonene on liver function, in all treatment groups [42].

During the 14 days of treatment, disease control group has shown significant increased tumor mass and volume, whereas D-Limonene treated groups has shown dose dependent reduction in it, which supports the *in vivo* anticancer activity of D-Limonene in subcutaneous K562 implanted xenograft model. In our study, the reduction in tumor volume or mass may be because of cytotoxicity of D-Limonene to K562 cells, which inhibit the proliferation of K562 cells and ultimately reduces the further tumor growth.

Histopathological examination of tumor tissue showed presence of malignant cell in all treatment groups. The tumor cell invasion in adjacent stromal tissues with presence of angiogenesis, confirmed that the tumor development was not the mere result of simple hyperplasia but because of malignant and invasive characteristics of K562 cells. Among all groups, increased density of blast cells was noted in disease control group, which may be because of uninterrupted proliferation of K562 cells. However, dose dependent reduction in blast cell density was noted with all D-Limonene treatment groups, which may be because

of cytotoxicity of D-Limonene on K562 cells. Further, D-Limonene also possess the immunomodulatory activity that increases the WBC and NEU count and may contribute in tumor regression [39, 40, 43].

The subcutaneous tumor xenograft model is good model per se in evaluating cytotoxic or cytostatic drugs; in which D-Limonene treatment has shown dose dependent reduction in tumor mass. However, subcutaneous xenograft model lacks the physiological changes that occur because of tumor cell transplantation, as the tumor cells were implanted on limited area. Therefore, to evaluate the physiological of effect of tumor cells, we have evaluated the tail vein injection model of CML [44]. In this model, the K562 tumor cells were implanted in tail vein of immunocompromised mice and the disease progression was observed by performing Giemsa differential staining of peripheral blood [45, 46].

In tail vein injection tumor xenograft model, the animals were immunocompromised by the one week protocol for immunosuppression, similar to one used for subcutaneous xenograft transplantation, as it is mandatory to achieve successful tumor cell transplantation without rejection [23, 24]. After the tail vein injection of K562 cell in immunocompromised C57BL/6 mice, Giemsa stain of peripheral blood smear showed marked increase in circulating blast cells under the microscope on 4^{th} week, which confirms the successful establishment of the model. Wang et al. has also reported that tail vein injection of K562/A02 cell in SCID mice has significantly increased the number of circulating blast cells, which is the characteristic of successful establishment of CML model [47].

In tail vein injection model, changes in body weight showed similar results as it was seen in subcutaneous model. Significant reduction in body weight was observed in all groups during four weeks of model induction as compared to initial week. The reduction in body weight was may be because of successful induction and progression of disease [26]. After confirmation of model establishment, the changes in body weight were again measured before and after giving respective treatments in all groups. At the end of study, the disease control group continued the reduction in body weight, whereas animals treated with D-Limonene showed significant increase in body weight as compared to disease control group, which may be because of its anticancer activity that suppresses the disease progression.

In haematological parameters, one week of immunosuppression shows similar results as it was seen in subcutaneous model. At the end of 4^{th} week, significant increase in WBC and

NEU count whereas reduction in LYM and RBC count and Hb content was observed in all animals as compared to initial week. No change in PLT count was noted. Complete blood count is the common diagnostic test for leukemia, whereas increase in total WBC count is the shared characteristic of all four types of leukemia. Increase in granulocytes count differentiates myeloid leukemia from lymphoid leukemia because of expansion of Ph chromosome; molecular marker of CML is predominantly in the granulocyte compartment of the myeloid lineages. CML increases total WBC count by increasing leucocytosis from HSC. In laboratory test, it also shows marked increase in granulocytes count. Further, the BCR-ABL gene directs the activity of HSC more towards leucocytosis; therefore sometimes reduction in RBC and PLT count can be seen with disease progression. Reduction in RBC may be the reason for reduction in Hb content [48-51]. At the end of study, disease control group shows significant increase in WBC and NEU count, and reduction in LYM, RBC count and Hb content as compared to normal control group, whereas D-Limonene treatment shows significant reduction in WBC and NEU count, and increase in LYM count, RBC count and Hb content as compared to disease control group. Changes in platelet count werenonsignificant. As the animals are treated with tail vein injection of K562 cells, it has capacity to metastasise in bone marrow and may affect the proliferation of HSC [47]. D-Limonene has the cytotoxicity against K562 cells, which may further reduce the proliferation of K562 cells in all treatment groups.

In biochemical parameters, no significant changes were observed after one week of immunosuppression, similar to subcutaneous model. However, at the end of four week of model induction, significant increase in SGOT and ALP was noted in all animals as compared to initial week. These changes may be because of CML related alteration in liver physiology [52]. At the end the treatment, disease control group showed significant increase in SGOT, SGPT and ALP level as compared to normal control group, which may be associated with disease progression. However, the values for increased level of SGOT, SGPT and ALP were falling under the normal range for mice. D-Limonene treatment has reduced the elevated levels of SGOT, SGPT and ALP, which may be because of normalisation of liver physiology. Further, to identify the liver function related abnormality, histopathological analysis of liver was performed. In CML patients, histopathology of liver has shown necrosis or infiltration of inflammatory cells, further in blast crisis, it has also been associated with liver enlargement and increased level of ALP [41]. In our study, marked increase in necrosis or infiltration of inflammatory cells was noted in histopathology of

disease control group and D-Limonene at 0.5 g/kg/d treated group as compared to normal control group, which may be associated with circulating blast of K562 cells. Treatment with D-Limonene at 1.0 and 1.5 g/kg/d did not produce any changes in histopathology of liver, which may be because of antiprolieferative effect of D-Limonene on K562 cells.

Malondialdehyde (MDA) is an end product of free radical induced-lipid peroxidation and it is used as a surrogate marker of oxidative stress induced damage, whereas superoxide dismutase is an enzyme that catalyzes breakdown of superoxide radicals into ordinary molecules [53]. Serum nitrite is also a free radical that damages the cellular components. Ciarcia et al. has reported marked decrease in superoxide dismutase (SOD) level whereas significantly higher malondialdehyde and nitric oxide levels in CML cells as compared to normal peripheral blood cells [54].

In our study, significant reduction in MDA and nitrite production was noted with D-Limonene treatment 1.0 and 1.5 g/kg/d as compared to disease control group, which may be because of its free radical scavenging activity. However significant increase in SOD level was observed with D-Limonene treatment 1.0 and 1.5 g/kg/d as compared to disease control group, which may be because of protective effect of D-Limonene on antioxidant enzyme level [55].

Thus, D-Limonene treatment has reduced the in vivo growth of K562 cells and tumor progression in both, subcutaneous and tail vein injection model of CML.

Angiogenesis is the formation of new blood vessel from the pre-existing vessels, which plays an important role in tumor growth and metastasis. The imbalance in angiogenic or anti-angiogenic factors may contribute in the development and progression of CML [56, 57]. The chick chorioallantoic membrane (CAM) assay is a rapid and simple alternative to *in vivo* studies for the evaluation of anti-angiogenic compounds. It reduces the requirement for animal experiments and assesses the anti-angiogenic efficacy more efficiently and objectively of a test drug [58]. In CAM assay, D-Limonene has shown significant dose dependent reduction in number of blood vessels formation in all treatment groups as compared to vehicle treated group. The reduction in blood vessel formation may be because of anti-angiogenic effect D-Limonene on CAM. However, the mechanism remains unclear. Therefore, to identify the mechanism for inhibition of angiogenesis by D-Limonene, we have performed the western blotting and gelatin zymography to identify the expression of

angiogenic factors such as vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) respectively.

In literature, high expression of VEGF was reported in all phases of CML and it has also significant influence on survival [59-61]. Apart from VEGF, the BCR /ABL oncogene in murine FL5·12 cells was found to be related with an up-regulated expression of matrix metalloproteinase-2 (MMP-2). Primary CML cells secrete VEGF and suggests the expansion/activation of endothelium, which plays a role in progression from chronic to accelerated and blastic phases of CML. Increased plasma levels of VEGF and other angiogenic factors has been associated with and increased marrow vascularity in patients with CML. It has been recognized that migration of proliferating endothelial cells requires a regulated degradation of the basement membrane and surrounding ECM, which involves the production of ECM-degrading proteinases such as MMPs [62]. The cell surface implication between MMP-2/MMP-9 and integrins has been suggested to be involved in the growth and progression of CML [63]. Moreover, it is found that VEGF stimulated the secretion of MMP-2 and 9 in BCR-ABL positive CML cells, suggesting its important role in the pathogenesis of the disease [62]. Consistently, ROS have also been known to increase the production of the angiogenic factor such as VEGF and MMP [64, 65].

In our study, treatment of K562 cells with D-Limonene has shown significant dose dependent reduction in expression of VEGF protein as compared to untreated cells in western blotting. In gelatin zymography, significant reduction in expression of MMP-2 and 9 were noted with D-Limonene treatment as compared to untreated cells. In literature, it has been reported that the angiogenic growth factors produced in CML patients, are directly triggered by disease specific oncogene BCR-ABL [66]. Thus, the reduction in expression of VEGF and MMPs followed by D-Limonene treatment was may be because of down regulation of BCR-ABL expression or inhibition of downstream signalling pathway. However, further studies are needed to elaborate the complicated signalling pathway.

Moreover, oxidative stress is the outcome of imbalance between the production of reactive oxygen species (ROS) and the defensive antioxidants that are present in the biological system to detoxify these ROS or repair ROS mediated damage [67]. In CML, increased levels of ROS have been associated with presence of BCR-ABL fusion protein as compared with their non-transformed cells [68, 69]. Koptyra and Falinski has stated that BCR-ABL causes DNA damage by stimulating ROS resulting in mutations of kinase domain which

plays a major role in development of resistance of given therapy and contribute to progression of CML [70]. In myeloid malignancies, ROS generated by malignant cells may confer a proliferative advantage on the malignant population, but may also freely diffuse into neighbouring normal cells and hinders the normal cell proliferation in a paracrine manner. If such an environment persisted, it could potentially disturb normal haematopoiesis [53, 69].

Free radicals or ROS plays a crucial role in the pathogenesis of various diseases; whereas antioxidants have a major role in prevention of such diseases. BCR/ABL has also been reported for its capacity to mimic some signaling events induced by activated growth factor receptors in CML. Literature suggests the activation of growth factor receptors coincides with an increase of intracellular ROS levels, whereas antioxidants or antioxidant enzymes have been shown to reduce tyrosine phosphorylation events in cells stimulated with these growth factors [71]. More than one antioxidant assay must be selected to check the antioxidant activity of any compound because antioxidant acts by more than one mechanism, and single assay cannot capture the complete mechanism. In our study, we have evaluated the *in vitro* antioxidant effect of D-Limonene by using DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging, superoxide radical scavenging and nitric oxide radical scavenging assay. D-Limonene has shown appreciable antioxidant activity in all the assays except iron chelating assay in comparison with trolox as a standard, a water soluble analogue of vitamin E. In all these assays, the antioxidant activity of D-Limonene may be attributed to the presence of methylene group, which strongly activates the antioxidant activity of monoterpenes. Hydrogen abstraction from methylene group produces the carbon centered radical, which is resonance stabilised and have been proposed to be interconverted through the conjugation by double bond. Therefore, the antioxidant activity may be the attribute of the presence of methylene group and double bond in the chemical structure of D-Limonene [72, 73]. However, in iron chelating assay absence of antioxidant activity was seen with D-Limonene which may be because of inability of D-Limonene to trap iron chemically [74].

After completion of *in vitro* antioxidant assays in cell free system, we have also evaluated the effect of D-Limonene on lipid peroxidation of K562 cells. Lipid peroxidation is the oxidative degradation of lipids by the generation of ROS, which is evaluated by quantifying malondialdehyde production. Large amount of ROS may lead to increase in malondialdehyde production [75]. In our study, D-Limonene treatment has shown significant dose dependent reduction in malondialdehyde production on K562 cells as

compared to untreated cells. The reduction in malondialdehyde production was may be because of antioxidant or free radical scavenging activity of D-Limonene, which reduces the ROS induced lipid peroxidation or malondialdehyde formation in K562 cells.

Thus, limonene has inhibited the angiogenesis by decreasing the expression of VEGF, MMP-2 and 9. Further, D-Limonene has shown good antioxidant activity in cell free system as well as on K562 cells.

Chapter-6

Summary & Conclusion

CHAPTER-6

Summary and Conclusion

Summary:

- Treatment with D-Limonene has reduced the percentage of cell viability of K562 cells in dose dependent manner at 24 and 48 h of treatment without producing cytotoxicity to primary hepatocytes up to the concentration of 32mM as compared to Doxorubicin.
- Treatment with D-Limonene has shown dose dependent reduction in tumor development and progression in subcutaneous and tail vein injection model of CML in immunocompromised K562 cells implanted C57BL/6 mice.
- In CAM assay of angiogenesis, treatment with D-Limonene has shown significant dose dependent manner inhibition of angiogenesis.
- Further, D-Limonene has also shown significant dose dependent reduction in the expression of VEGF, MMP-2 and MMP-9 protein in K562 cells by western blot analysis and gelatin zymography respectively.
- Besides this, D-Limonene has shown appreciable *in vitro* antioxidant activity in cell free medium and in K562 cells.

Conclusion:

D-Limonene reduces the viability of K562 cells without affecting mouse primary hepatocytes. It has also reduced the growth of K562 cells and tumor progression in both, subcutaneous and tail vein injection model of CML. This protective action might be attributed to inhibition of

the angiogenesis by decreasing the expression of VEGF, MMP-2 and 9 and also due to its promising antioxidant activity.

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Appendices

Appendix-A

Approval certificate from Institutional Animal Ethics Committee (IAEC) for animal experiments

PERD/IAEC/2017/002

B. V. Patel Pharmaceutical Education & Research Development (PERD) Centre

Certificate

This is to certify that the project title **"Investigation into anti-cancer activity of D-Limonene in Chronic Myeloid Leukemia"** has been approved by the IAEC.

Dr. Manish Nivsarkar	Dr. R. J. Verma
Name of Chairman/ ~~Member Secretary~~ IAEC:	Name of CPCSEA nominee:

Signature with date

19/4/17

Chairman/ Member Secretary of IAEC:

19/4/2017

CPCSEA nominee:

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

8

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SHORT COMMUNICATION

WILEY

Anti-leukemic and anti-angiogenic effects of D-Limonene on K562-implanted C57BL/6 mice and the chick chorioallantoic membrane model

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Abstract

Background: D-Limonene, a monoterpene from citrus fruit has been found to have chemopreventive and chemotherapeutic activities in various types of cancers. In this study, we evaluated the in vivo effect of D-Limonene on a K562-induced model of chronic myeloid leukemia (CML) in C57BL/6 mice.

Method: The tail vein injection model of K562 cells in immunocompromised C57BL/6 mice was developed and evaluated for characteristics of the disease. The mice were treated with D-Limonene and evaluated for haematological parameters. We also evaluated the effect of D-Limonene on angiogenesis using the chick chorioallantoic membrane (CAM) assay.

Results: In a complete blood count, a significant dose-dependent reduction in white blood cell, neutrophil and lymphocyte counts, but an elevation in red blood cell count and haemoglobin content was observed with D-Limonene treatment compared to the disease control or untreated group. In the CAM assay, D-Limonene produced a significant dose-dependent reduction in number of blood vessels in treatment groups compared to the vehicle-treated group.

Conclusion: These studies suggest promising anti-leukemic and anti-angiogenic effects of D-Limonene in the treatment of CML.

KEYWORDS
angiogenesis, C57BL/6 mice, chick chorioallantoic membrane, chronic myeloid leukemia, D-Limonene

1 | INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the presence of the Philadelphia chromosome. Reciprocal translocation t(9;22)(q34;q11) generates the Bcr-Abl chimeric gene which leads to constitutive activation of the tyrosine kinase domain.[1] First line treatment with tyrosine kinase inhibitors (TKIs) has an excellent response in CML patients, with improved life expectancy. However, resistance to a currently used TKI, imatinib, has developed in the chronic (35%), accelerated (45%) and blastic (90%) phases of the disease. This is associated with a poor prognosis in CML patients, and has increased the need for new drug for the treatment of the disease. Natural products are now rapidly emerging

and appear promising for the development of new anti-cancer drugs.[2,3]

D-Limonene, a monocyclic monoterpene present in citrus fruits, has been found to have chemopreventive and chemotherapeutic activity against various types of cancers.[4-9] Oral administration of D-Limonene inhibited the growth of rodent pancreatic, mammary, and gastric carcinogenesis and exhibited anticancer activity.[7,8,10-13] In our previous studies, we reported that D-Limonene inhibited the growth of K562 cells *in vitro* without producing any toxicity in primary hepatocytes isolated from the mouse.[14] In this study, we evaluated the in vivo effect of D-Limonene in immunocompromised C57BL/6 mice.

The chick embryo model offers a number of unique advantages for studying the complex, multistep process of tumor cell metastasis. It serves as a naturally immunodeficient host capable of sustaining grafted tissues and cells, without restrictions, because of its underdeveloped lymphoid system.[15,16] The chorioallantoic membrane (CAM) provides a uniquely supportive environment for primary tumor formation, and is a source of angiogenesis, the formation of new blood vessels from an existing vasculature.[17] Angiogenesis has a crucial role in growth, metastasis and dissemination of tumors.[18] The imbalance between pro- and antiangiogenic factors causes pathogenesis and progression of hematologic neoplasias.[19] Increases in a number of angiogenic factors can significantly increase the number of vessels in the bone marrow of patients with CML.[20,21] The present study aimed to evaluate the effect of D-Limonene on angiogenesis using an in vivo chick CAM assay.

2 | MATERIALS AND METHODS

2.1 | Materials

D-Limonene (MP Biomedicals, Solon, OH, USA); RPMI-1640 and fetal bovine serum (FBS) (Himedia, Mumbai, India); antibiotic-antimycotic solution (Gibco, Grand Island, NY, USA); cyclosporine (Sandimmune, Novartis, Basel, Switzerland); ketoconazole (Nizral, Johnson & Johnson, New Brunswick, NJ, USA); cyclophosphamide (Endoxan, Baxter, Halle, Germany); ampoxin (Unichem Laboratories Ltd, Mumbai, India); sterilized gelatin sponge (Alinda Healthcare Pvt Ltd, Ahmedabad, India).

2.2 | Cell and culture conditions

The human CML cell line (K562) was obtained from NCCS (National Centre for Cell Science, Pune, India).[22,23] Cells were cultured in RPMI-1640 containing 1% antibiotic-antimycotic solution supplemented with 10% FBS and maintained in an incubator (Forma stericycle CO_2 incubator, Thermo Scientific, Waltham, MA, USA) at 37°C with a 5% CO_2 atmosphere.

2.3 | Experimental animals and housing conditions

Healthy C57BL/6 (Mahaveera Enterprises, Hyderabad, India) male mice weighing 25-33 g were housed under standard laboratory conditions (12:12 h light:dark cycle, relative humidity 60 ± 5%, temperature 25 ± 2°C) in individually ventilated cages. The mice were fed with autoclaved balanced rodent food pellets and Ampoxin (0.1 μg/mL in drinking water) was provided *ad libitum*. All the experiments were carried out in strict accordance with guidelines set by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. The experimental procedures were reviewed and approved by the institutional animal ethics committee (PERD/IAEC/2017/002) prior to initiation of the experiments.

2.4 | In vivo establishment of K562 cells in C57BL/6 mice and treatment with D-Limonene

Immunosuppression of C57BL/6 mice was achieved with a combination of ketoconazole (10 mg/kg, orally), cyclosporine (30 mg/kg, intraperitoneally) and cyclophosphamide (60 mg/kg, subcutaneously).[24] K562 cells (approx. 5×10^7) were introduced by tail vein injection. Blood was collected weekly from the retro-orbital plexus to confirm the development of the disease. The blood was analyzed for the presence of circulating blast cells by Giemsa staining of a peripheral blood smear and a complete blood count.[25] Following an increase in the number of blast cells in the peripheral blood smear, treatment with D-Limonene (0.5, 1.0 and 1.5 g/kg, n = 10) orally for 17 days was initiated in al groups except the disease control or untreated group. Body weight and hematological parameters (total white blood cells count [WBC], neutrophil count [NEU], lymphocytes count [LYM], red blood cells count [RBC], hemoglobin content [Hb] and platelet count) were analyzed.

2.5 | Chick CAM assay

Fertilized chick eggs were procured from Jesal Agro Ltd, Gandhinagar, Gujarat, India at the time of experiments. Chick eggs were incubated at 37°C and 60% humidity in an egg incubator (A. P. Poultry Equipments, Hyderabad, Telangana, India) in sterile conditions. On the third day of incubation, eggs were sterilized with isopropyl alcohol and an air sac was made by gently aspirating 2 ml albumin, which is enough to bring the membrane down.[15] A small window of 2 × 2 cm was made in the shell of the egg and the viability of the chick embryos was assessed by monitoring rhythmic contraction of the heart. The window was sealed with transparent adhesive tape and the eggs were incubated in the incubator. On the eighth day, the transparent tape was removed and 1 mm^3 gelatin sponges presoaked with D-Limonene at concentrations of 1, 5 and 10 μg per implant (1 implant per egg, n = 12 for each treatment) or with vehicle were implanted on the CAM of the eggs. The eggs were resealed and returned to the incubator. On the 12th day of incubation, the eggs were observed under the microscope (Olympus) at 10× magnification for counting the number of blood vessels surrounding the gelatin sponge implants.[26]

2.6 | Statistical analysis

Statistical analysis was performed by using GraphPad Prism software (version 5.03, Graph Pad Software Inc., La Jolla, CA, USA). Data are presented as means ± SEM. Significant differences were determined by one-way ANOVA followed by Bonferroni multiple comparisons. $P < 0.05$ was considered to be statistically significant.

3 | RESULTS AND DISCUSSION

Chronic myeloid leukemia is a condition in which an acquired mutation of genes controls the proliferation and development of cells present in the blood and bone marrow.[27] The Philadelphia chromosome resulting from this mutation has the ability to cause proliferation of myeloblasts.[28] Imatinib and other TKIs are losing their effectiveness in the treatment of CML due to developed resistance. Adjuvant therapy or a combination of drugs may be a better alternative in the treatment of disease.[1,29]

3.1 | Determination of model establishment and growth of K562 cells in C57BL/6 mice

We selected the tail vein model among the various available models for CML. Immunosuppression was achieved with ketoconazole, cyclosporine and cyclophosphamide.[24,25] After 1 week of immunosuppression, a significant reduction in WBC, NEU, and LYM counts was noted in a complete peripheral blood count, compared to 0 weeks; however, no significant change was observed in RBC count and Hb content, which confirmed the successful achievement of immunosuppression. The C57BL/6 mice were injected with K562 cells via tail vein injection followed by immunosuppression and the peripheral blood was examined weekly for circulating blasts using Giemsa staining. The blast cells were differentiated from normal blood cells under the microscope by the larger diameter of the cells (≈28 μm), the presence of prominent nuclei, reduced cytoplasm and a higher nuclear/cytoplasmic ratio, which confirms the establishment of model.[29] By the fourth week after the transplantation of K562 cells in C57BL/6 mice, Giemsa differential staining of peripheral blood smears showed large numbers of blast cells in the disease control or K562 treated group compared with untreated group.[29] As the disease progressed, changes in haematological parameters were noted. By the fourth week after K562 cell injection, significant increases in WBC, NEU, and LYM counts were observed, compared to 0 weeks, whereas significant reductions in RBC count and Hb content were seen, confirming the establishment of the model (Figure 1). No significant changes in platelet count and other parameters were noted.

3.2 | Effect of D-Limonene on the growth of K562 cells in C57BL/6 mice

In CML, the disease progression leads to an increase in the number of granulocytes, which is the reason for the increased numbers of total WBCs, NEUs, and LYMs. However, the significant reduction in RBC count and Hb content is because of a reduced ability to generate new RBCs from bone marrow.[28] D-Limonene treatment at doses of 0.5, 1.0 and 1.5 g/kg for 17 days in immunocompromised K562 cell-injected C57BL/6 mice produced significant dose-dependent changes in haematological parameters compared to disease control or untreated (DC) group. The treatment also produced a significant

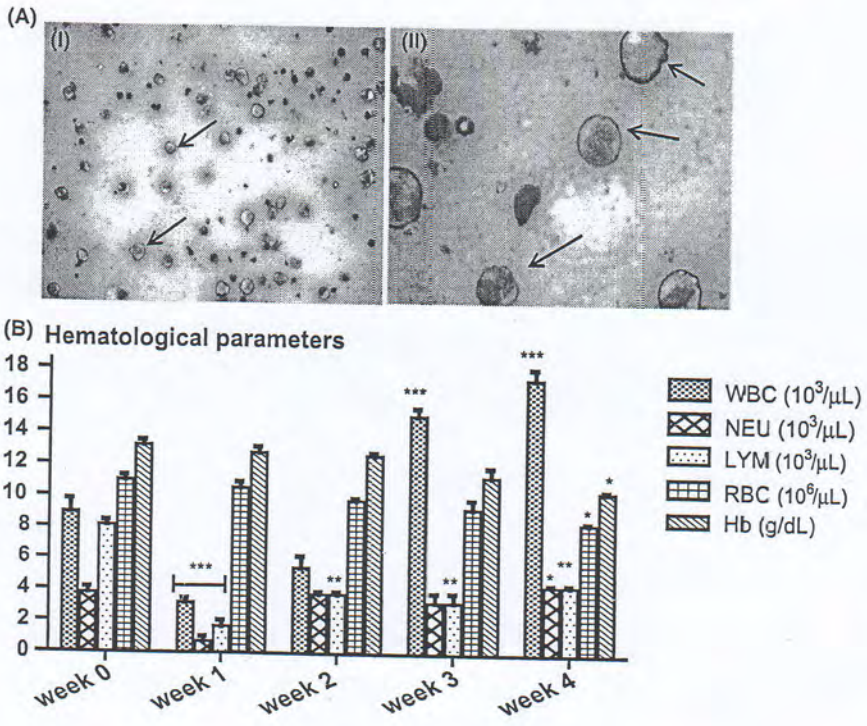

FIGURE 1 Determination of model establishment and growth of K562 cells in C57BL/6 mice. A, Differential staining of peripheral blood of C57BL/6 mice with Giemsa stain showing the presence of circulating K562 blast cells at 10× (i) and 40× (ii) magnification, which confirms the establishment of the CML model. B, Haematological parameters show significant reductions in WBC, NEU, and LYM counts 1 wk after tail vein injection of K562 cells compared to week 0. No significant changes were observed in RBC count and Hb content. Four weeks after tail vein injection, significant increases in WBC, NEU, and LYM counts and reductions in RBC count and Hb content were noted compared to week 0. Data shown are the means ± SEM (n = 10), $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs week 0

reduction in WBC and NEU counts compared to the DC group. The change in LYM count was not significant with 0.5 g/kg D-Limonene, but at doses of 1.0 and 1.5 g/kg a significant dose-dependent reduction was observed compared to the DC group. Changes in RBC count and Hb content were non-significant with D-Limonene treatment (Figure 2). D-Limonene at a dose of 1.5 g/kg produced the greatest effect on disease progression by reducing WBC, NEU, and LYM counts and increasing RBC count and Hb content compared to other treatment group, which suggests it has potential for use in the treatment for CML.

3.3 | Effect of D-Limonene on angiogenesis using the CAM assay

Angiogenesis has been linked with the growth, dissemination, and metastasis of various tumors. Angiogenesis plays an important role

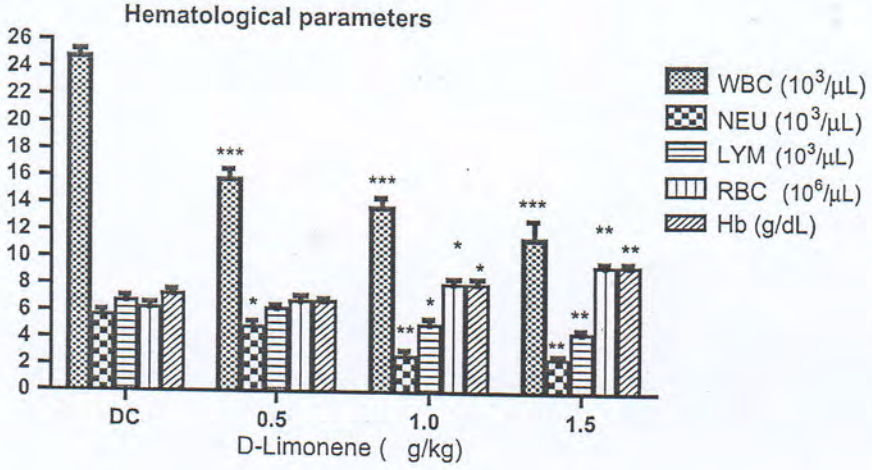

FIGURE 2 D-Limonene treatment in immunocompromised K562-implanted C57BL/6 mice produced significant changes in haematological parameters. D-Limonene at a dose of 0.5 g/kg significantly decreased the WBC and NEU counts, whereas RBC count and Hb content were non-significantly increased. At doses of 1.0 and 1.5 g/kg D-Limonene significantly reduced WBC, NEU, and LYM counts and increased RBC count and Hb content. Data shown are the means ± SEM (n = 10), $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ vs DC

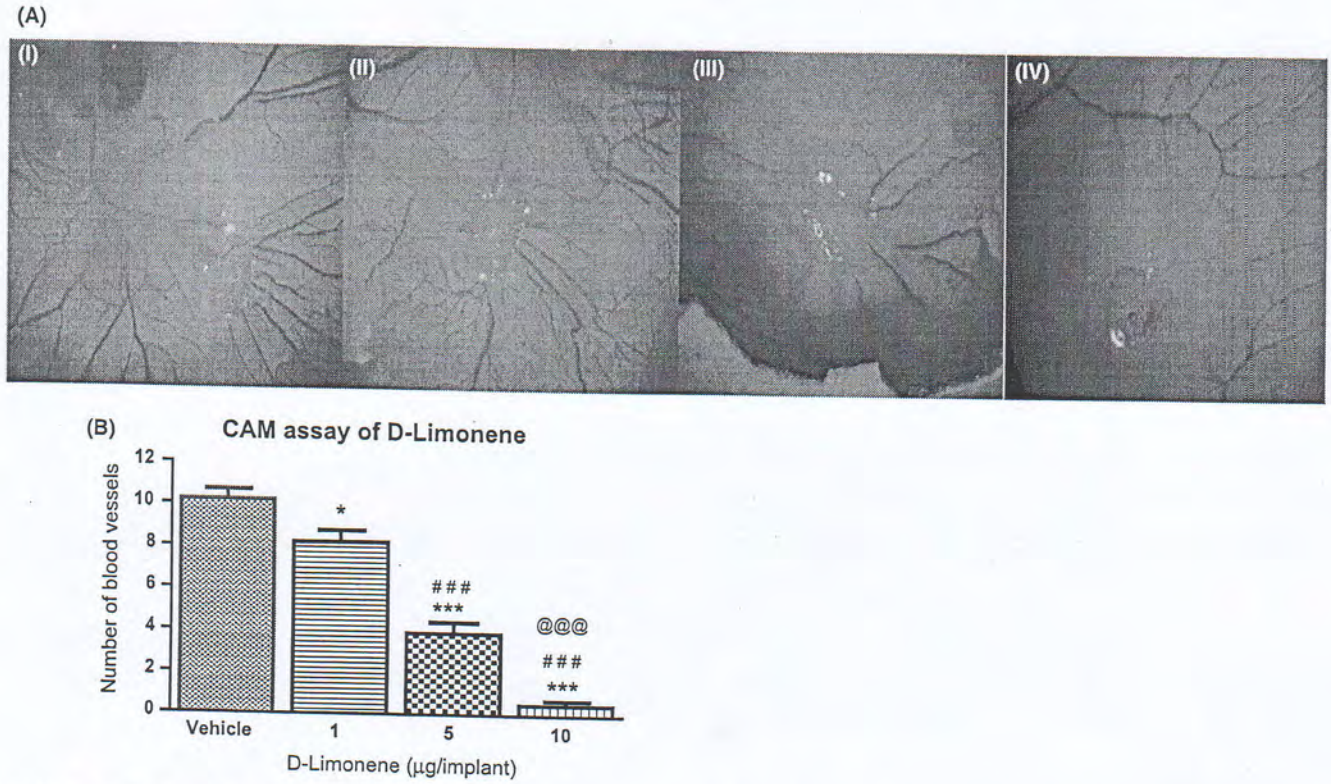

FIGURE 3 Effect of D-Limonene on angiogenesis using the CAM assay. A, Images show the reduction in number of blood vessels formed on the CAM membrane at 10x magnification. The gelatin sponges were soaked with vehicle (i), and D-Limonene at 1 μg per implant (ii), 5 μg per implant (iii) and 10 μg per implant (iv). B, D-Limonene treatment groups at concentrations of 1, 5, and 10 μg per implant induced significant concentration-dependent reductions in the number of blood vessels compared to the vehicle-treated group. The highest reduction was observed with highest concentration of 10 μg per implant. Values are expressed as means ± SEM (n = 10). $*P < 0.05$, $***P < 0.001$ vs vehicle-treated implant, $^{###}P < 0.001$ vs 1 μg per implant, $^{@@@}P < 0.001$ vs 5 μg per implant

in the progression of CML by affecting the vascularity of bone marrow or leukemic cells. CAM assays have been widely used to study the effect of various compounds on angiogenesis because of their simplicity, cost-effectiveness and reproducibility.[19] We evaluated the effect of D-Limonene on angiogenesis using the CAM assay.

On the fourth day after implantation of gelatin sponges soaked with D-Limonene at concentrations of 1, 5 and 10 μg per implant, a significant dose-dependent reduction in the number of blood vessels on CAM was seen compared to CAM implanted with vehicle. The reduction in the number of blood vessels on CAM was highest when D-Limonene was applied at 10 μg per implant (Figure 3).

Additional studies will be needed to confirm the effect of D-Limonene on the expression of various angiogenic and anti-angiogenic factors.

4 | CONCLUSION

In conclusion, we have confirmed that D-Limonene produces a significant dose-dependent reduction in the progression of CML by reducing the growth of K562 blasts in K562 cells implanted into C57BL/6 mice. In CAM assays, D-Limonene also inhibited angiogenesis in a concentration-dependent manner, which suggests its importance as a promising anticancer therapy for CML.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

Anita A. Mehta, Manish Nivsarkar, Kiranj Chaudagar, and Bhavini Shah conceived and designed the study. Bhavini Shah and Ruma Baksi carried out the experimental work and data analysis. All authors contributed to revising the manuscript. All authors gave final approval for publication.

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Research Article

In vitro evaluation of antioxidant activity of d–limonene

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Abstract

Objective: Reactive oxygen species (ROS) and free radicals are involved in pathogenesis of cancer. D-Limonene, a monoterpene present in citrus fruit has been reported for its potential anticancer activities. The aim of present study was to evaluate the *in vitro* antioxidant effect of D-Limonene. **Material and methods:** Test solutions with different concentration of D-Limonene and Trolox (standard) were prepared. Six different *In vitro* antioxidant assays such as DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging and superoxide radical scavenging assay were performed for evaluation of antioxidant activity of D-Limonene. **Results:** D-Limonene has shown appreciable antioxidant activity in comparison with Trolox. The IC_{50} of D-Limonene in DPPH (384.73 µM), ABTS (603.23 µM,), FRAP (-589.85 µM), iron chelating (-18475.5 µM), hydroxyl radical scavenging (442.75 µM) and superoxide radical scavenging assay (692.89 µM) was comparable with Trolox the IC_{50} value of Trolox DPPH (153.30 µM), ABTS (203.37 µM,), FRAP (-171.73 µM), iron chelating (225.96 µM), hydroxyl radical scavenging (146.37 µM) and superoxide radical scavenging assay (105.25 µM) respectively. **Conclusion:** In vitro antioxidant assays has shown concentration dependent reduction in free radical formation by D-Limonene in comparison with Trolox in all assays except iron chelating assay which suggests its promising role for cancer treatment.

Keywords: Antioxidant; D-Limonene; Reactive oxygen species (ROS)

Introduction

Cancer is one of the dreadful diseases which is associated with the key characteristics of uncontrolled proliferation and metastasis (Fidler, 2003). Cancer is the second leading cause of mortality, responsible for 8.8 million deaths representing nearly 1 in 6 deaths globally in 2015. According to WHO, In India nearly 1 to 1.4 million new patients is diagnosed with incidence of cancer every year, among them 0.54 million dies because of the delayed diagnosis of the disease (WHO, 2018).

Free radicals and reactive oxygen species (ROS) are the molecules that are generated by normal cellular processes, environmental stresses, and UV irradiation. It causes tissue injury by reacting with cellular components. Overproduction of ROS is involved in the pathogenesis of several diseases including diabetes, atherosclerosis, premature aging, neurodegenerative diseases, inflammation and cancer (Collins, 1999). Reactive oxygen species encompasses singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydroxyl radical (^-OH) and hydrogen peroxide (H_2O_2). ROS produces base pair modification, rearrangement of DNA sequence, miscoding of DNA lesion, gene duplication and the activation of oncogenes (Waris and Ahsan, 2006). Elevated levels of ROS and down regulation of endogenous antioxidant enzymes and ROS scavengers are associated with cellular proliferation, apoptosis, senescence which are implicated in the development of cancer (Agosteinelli and seiler, 2006).

Antioxidants are the chemical trap that inhibits the oxidation, especially one used to counteract the deterioration of stored food products. Numerous antioxidants are commercially available for its use in foods. However, natural antioxidants are preferred over synthetic antioxidants because of its safety and functional and sensory properties. Monoterpenes are the major components of essential oils of many plants, known for its natural antioxidants potential. It was demonstrated that reactive oxygen species can influence the growth and death of cancer cells (Miguel, 2010; Baratta et al., 1998).

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D-Limonene, a monocyclic monoterpene present in citrus fruits is colorless oil sparingly soluble in water with a sweet orange smell. Commercially, it is mostly obtained from waste orange peel and has been reported for its chemopreventive and chemotherapeutic activity against various types of cancers (Kaji et al., 2001; Lu X-G et al., 2004; Miller et al., 2013; Nakaizumi et al.,1997; Uedo N, 1999). D-Limonene is listed in the Code of Federal Regulation as generally recognized as safe (GRAS) for a flavoring agent and is used as an additive in foods, soaps and perfumes (Ciriminna et al., 2014).

In our previous studies, we have reported that D-Limonene has inhibited the growth of K562, chronic myeloid leukemia cells *in vitro* without producing any toxicity on primary hepatocytes isolated from the mouse (Shah et al., 2018).

Since antioxidants can act through various mechanisms, the detection of such activity must be evaluated using various antioxidant assays. The objective of the present study was to evaluate the *in vitro* antioxidant effect of D-Limonene by DPPH, ABTS, FRAP, Iron chelating, Hydroxyl radical scavenging and Superoxide scavenging assay.

Materials and methods

Materials

D-limonene (MP Biomedicals Solon, OH, USA), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2carboxylic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), dimethyl sulfoxide (DMSO), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6sulfonic acid)), potassium persulfate, Griess reagent, TPTZ (2,4,6-tripyridyl-s-triazine), ferric chloride hexahydrate, sodium acetate trihydrate, hydrochloric acid, sulphuric acid, hydrogen peroxide, were purchased from Sigma Aldrich (St. Louis, MO, USA).

Instrument

Thermo Scientific Multiskan GO , Waltham, Massachusetts, United States.

Test solution preparation

Test solutions of D-Limonene at concentration of 25, 50, 100, 200 and 400 µM were prepared by adding 0.1% Tween 80. Trolox was used as a standard at concentration of 10, 25, 50, 100 and 250 µM. The stock solution of trolox 1 mM was prepared in absolute ethanol and working solution of trolox (10, 25, 50, 100 and 250 µM) were made from the stock solution by dilution with required amount of double distilled water.

Diphenylpicrylhydrazyl (DPPH) assay

DPPH assay of D-Limonene and Trolox at different concentrations was performed with some modifications. 10 ml of stock solution was mixed with 45 ml of methanol. The methanolic solution of DPPH was mixed with the test solutions and allowed to react for 24 h in the dark. Blank solutions were prepared in the similar way and the absorbance was measured at 517nm (Brand-Williams et al., 1995, Thaipong et al., 2006). Scavenging activity was expressed as the percentage inhibition calculated by using the following formula:

$$\% \text{ Inhibition } = \frac{\text{Absorbance of control} - \text{ Absorbance of sample X 100}}{\text{Absorbance of control}}$$

Azobis-ehtylbenzthiazoline sulfonic acid (ABTS) assay

The stock solutions of ABTS and potassium persulfate were prepared according to standard assay method. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS solution with 60 mL methanol. Test solutions were allowed to react with ABTS solution for 2 h in a dark. The absorbance was taken at 734 nm. Results were expressed as % inhibition using equation described in DPPH method (Arnao et al., 2001; Thaipong et al., 2006).

Ferric reducing anti-oxidant power (FRAP) assay

The stock solutions of acetate buffer of pH 3.6, TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in HCl, and $FeCl_3$-$6H_2O$ solution were prepared. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3$-$6H_2O$ solution and then warmed at 37°C before using. Test solutions were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark. Readings of the colored product [ferrous tripyridyltriazine complex] were taken at 593 nm. Results were expressed as % inhibition using equation described in DPPH method (Benzie and Strain 1996; Thaipong et al., 2006).

Iron chelating assay

The stock solutions of ferric chloride were prepared based on standard assay method. Test solutions were mixed with 1200 µl of ferric chloride and 1200 µl of phenanthroline solution. The absorbance was taken at 510 nm using the spectrophotometer. Results were expressed as % inhibition using equation described in DPPH method (Alam et al., 2013; Dinis et al., 1994).

Hydroxyl radical scavenging assay

The stock solutions of hydrogen peroxide, ascorbic acid, EDTA, ferric chloride, trichloroacetic acid, thiobarbituric acid and deoxyribose were prepared. The working solutions were prepared by mixing hydrogen peroxide (100 µl), ascorbic acid (100 µl), EDTA (200 µl), ferric chloride (500 µl) and deoxyribose (100 µl). Test solutions (500 µl) were allowed to react with 1 ml of working solution for 60 min in the dark. Later, they were incubated with 100 µl of

trichloroacetic acid and 100 µl of thiobarbituric acid for 20 min at 100°C. Readings of the colored product were then taken at 546 nm. Results were expressed as % inhibition using equation described in DPPH method (Alam et al., 2013; Ottolenghi, 1959).

Superoxide radical scavenging assay

The stock solutions of alkaline DMSO and nitro blue tetrazolium were prepared according to standard procedures. Test solutions were mixed with 2 ml of alkaline DMSO and 200 µl nitro blue tetrazolium. Then the absorbance was taken at 560 nm using the spectrophotometer. Results were expressed as % inhibition using equation described in DPPH method (Alam et al., 2013; Meyer and Isaksen, 1995).

Statistical analysis

In order to evaluate the data, a linear regression ($p< 0.05$) was used. Data were expressed as the mean ± SD of three independent experiments carried out in triplicate.

Results

Effect of D-Limonene on DPPH assay

DPPH assay of D-Limonene (IC_{50}= 384.73 µM) has shown appreciable concentration dependent reduction in free radical formation in comparison with Trolox (IC_{50}= 153.30 µM) as a standard. The percentage inhibitions for DPPH assay are given in (Figure 1).

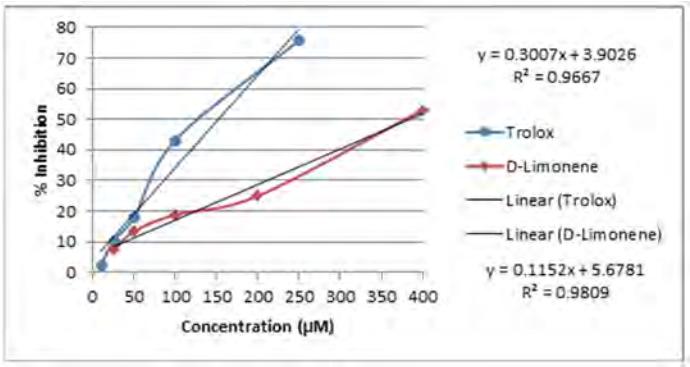

Figure 1. Antioxidant activity of D-Limonene and Trolox by DPPH assay

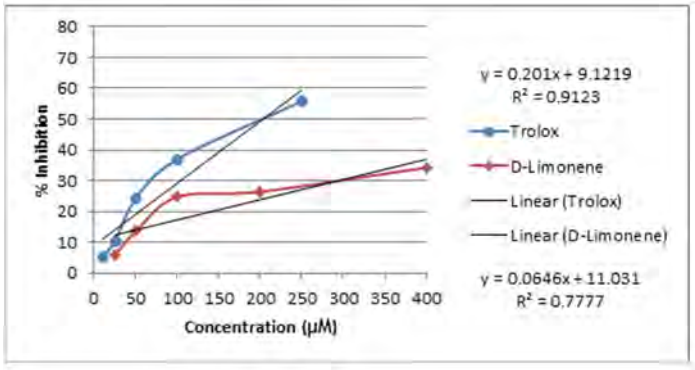

Figure 2. Antioxidant activity of D-Limonene and Trolox by ABTS assay

Effect of D-Limonene on ABTS assay

D-Limonene (IC_{50}= 603.23 µM) has shown appreciable concentration dependent reduction in free radical formation by ABTS assay in comparison with Trolox (IC_{50}= 203.37 µM) as a standard. The percentage inhibitions for ABTS assay are given in (Figure 2).

Effect of D-Limonene on FRAP assay

D-Limonene (IC_{50}= -589.85 µM) has showed appreciable concentration dependent reduction in free radical formation by FRAP assay in comparison with Trolox (IC_{50}= -171.73 µM) as a standard. The percentage inhibitions for FRAP assay are given in (Figure 3).

Effect of D-Limonene on Iron chelating assay

D-Limonene (IC_{50}= -18475.5 µM) did not show any % inhibition in iron chelating assay in comparison with Trolox (IC_{50}= 225.96 µM) as a standard. The percentage inhibitions for iron chelating assay are given in (Figure 4).

Effect of D-Limonene on Hydroxyl radical scavenging assay

D-Limonene (IC_{50}= 442.75 µM) has showed appreciable concentration dependent reduction in free radical formation by hydroxyl radical scavenging assay in comparison with Trolox (IC_{50}= 146.37 µM) as a standard. The percentage inhibitions for hydroxyl radical scavenging assay are given in (Figure 5).

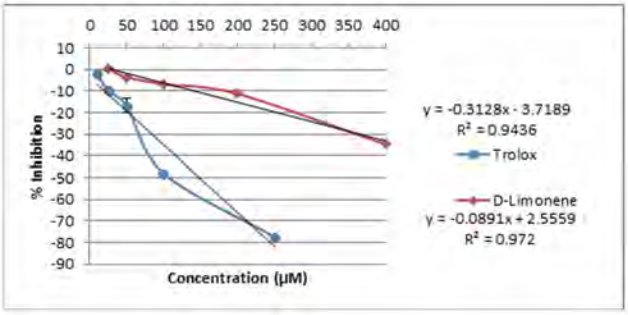

Figure 3. Antioxidant activity of D-Limonene and Trolox by FRAP assay

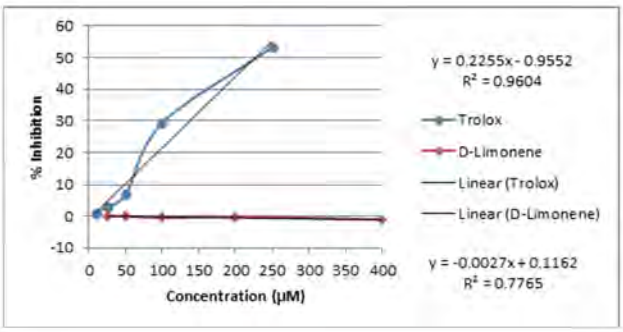

Figure 4. Antioxidant activity of D-Limonene and Trolox by iron chelating assay

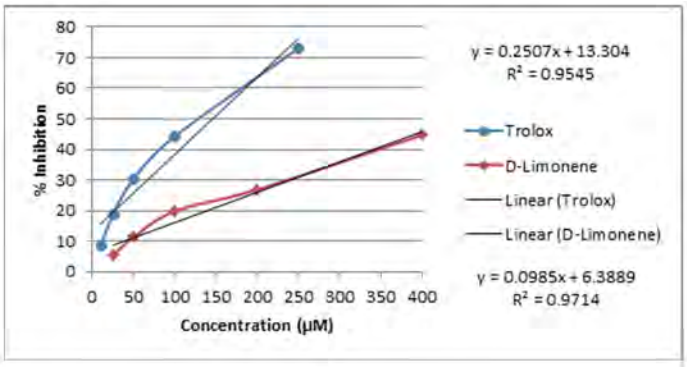

Figure 5. Antioxidant activity of D-Limonene and Trolox by Hydroxyl radical scavenging assay

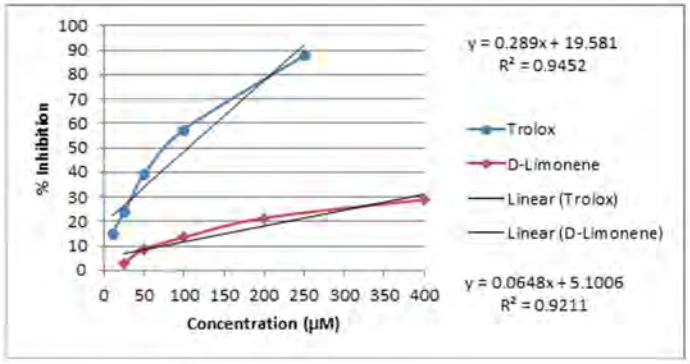

Figure 6. Antioxidant activity of D-Limonene and Trolox by Superoxide radical scavenging assay

Effect of D-Limonene on Superoxide radical scavenging assay

D-Limonene (IC_{50}= 692.89 µM) has showed appreciable concentration dependent reduction in free radical formation in by superoxide radical scavenging assay comparison with Trolox (IC_{50}= 105.25 µM) as a standard. The percentage inhibitions for superoxide radical scavenging assay are given in (Figure 6).

Discussion

In humans, about 1–3% of the O2 consumed by the body is converted into superoxide and other ROS which may also damage DNA, proteins, or lipids. These deleterious effects are found to be responsible for the development of diseases like CVD and cancer (Halliwell, 1996). Because of their safety natural antioxidants are the only alternative to synthetic antioxidants in counteracting the free radicals. Monoterpenes from essential oils promote health partly via their antioxidant and free radical scavenging effects. It protects the cellular components against free radical induced damage. But due to their diverse chemical structures, they are likely to possess different antioxidant capacities (Dorman et al., 2000; Grassmann, 2005).

D-Limonene is a major component of oils obtained from citrus plants such as orange, lemon and grapefruit. It is commonly used as an additive in foods, soaps and perfumes (Whysner and Williams, 1996) . It has been used to prevent gastric diseases, such as to dissolve gallstones and is also suggested to exert antiproliferative effects in various cancer cell types (Kaji et al., 2001; Lu X-G et al., 2004; Miller et al., 2013; Nakaizumi et al.,1997; Uedo, 1999) .

In our study, we have evaluated the *in vitro* antioxidant activity of D-Limonene by six different antioxidant assays such as DPPH, ABTS, FRAP, iron chelating, hydroxyl radical scavenging and superoxide radical scavenging assay. Except iron chelating assay in all antioxidant assay, D-Limonene has appreciably reduced the ROS formation by different mechanisms. In these assays, D-Limonene has reduced the level of free radicals in test solutions in concentration dependent manner; however the IC_{50} of D-Limonene was higher than that of standard Trolox in all assays.

In DPPH assay of D-Limonene, DPPH radical was converted to stable form by donation of hydrogen atom. D-Limonene donated hydrogen atom to DPPH radical and the antioxidant effect was determined by the disappearance of DPPH radical in test samples which was directly proportional to antioxidant activity of D-Limonene. In ABTS assay, D-Limonene reacted with ABTS radical cation formed by mixture of ABTS and sodium persulfate. D-Limonene has shown appreciable antioxidant activity by reducing the level of ABTS radical in concentration dependent manner. In FRAP assay, reduction of Fe3+ to Fe2+ was occur with D-Limonene treatment which has increased by conversion of Ferric (Fe3+) to ferrous (Fe2+) ion. Coloured ferrous-probe complex was formed from a colourless ferric-probe complex which was determined by increased absorbance of the test solution. D-Limonene has also shown reducing effect and donated electrons to free radicals to stabilize them which was indicated in hydroxyl radical scavenging assay of D-Limonene. Superoxide scavenging assay involves conversion of nitroblue tetrazolium to diformazan by superoxide radical. D-Limonene has stabilized the superoxide anion formation in concentration dependent manner. However, in iron chelating assay, no change in % inhibition was observed with increased concentration of D-Limonene which suggests its reduced chelating efficacy (Brand-Williams et al., 1995; Thaipong et al., 2006).

Conclusion

We confirmed the concentration dependent antioxidant activity of D-Limonene by *in vitro* antioxidant assays. Besides the anticancer activity, D-Limonene has shown appreciable concentration dependent antioxidant activity

by reducing the free radical formation in all assays except in iron chelating assay which makes it a promising molecule for treatment of cancer.

Acknowledgments

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Declaration of conflict of interest

No conflict of interest.

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Original article

D-limonene possesses cytotoxicity to tumor cells but not to hepatocytes

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ABSTRACT

Introduction: K562, human chronic myeloid leukemia cell line shows presence of constitutively active BCR–ABL gene. D-limonene, monoterpenes obtained from essential oils of citrus fruits have exhibited its antitumor activity in various types of cancers. Murine xenograft models are used for estimation of in vivo effect of D-limonene in K562 tumor xenograft model.

Aim: The aim of present study was to evaluate the in vitro and in vivo effect of D-limonene on primary hepatocytes and K562 tumor implanted C57BL/6 mice respectively.

Material and methods: Effect of D-limonene on growth of K562 cells and mouse primary hepatocytes was determined in vitro by MTT assay. The in vivo effect of D-limonene was also determined on chemically immunocompromised K562 tumor xenografted C57BL/6 mice.

Results and discussion: In vitro dose dependent and time dependent studies of D-limonene shows significant reduction in viability of K562 cells. Dose dependent studies of doxorubicin and D-limonene treatment for 48 h shows significant reduction in viability of primary hepatocytes with doxorubicin whereas, the reduction was non significant with D-limonene. D-limonene treatment for 14 days also shows dose dependent reduction in tumor volume in K562 tumor xenograft C57BL/6 mice.

Conclusions: D-limonene inhibited the growth of primary hepatocytes in-vitro and also inhibited in vivo K562 tumor growth in C57BL/6 mice, which suggests safety and efficacy of D-limonene in the treatment of chronic myeloid leukemia.

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1. INTRODUCTION

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder characterized by the presence of the Philadelphia (Ph) chromosome, resulting from translocation of abl proto-oncogene on chromosome 9 and the bcr gene on chromosome 22, t(9;22)(q34:q11) and generation of a chimeric BCR–ABL gene, a constitutively active tyrosine kinase that expresses P210BCR–ABL fusion protein of 210kDa.[1,2] Imatinib and other tyrosine kinase inhibitors (TKIs) are used as first-line treatment of CML, has increased the survival rate in patients with fewer side effects as compared with treatments such as interferon and hydroxyurea.[3–5] Imatinib and other TKIs eventually seems to stop working caused by changes in the genes of the CML cells which known as resistance.[6–8] However, the emergence of resistance to first-line therapy in CML patients leads to search for new molecule for the treatment of the disease.[9,10]

Monoterpenes, non-nutritive dietary components found in the essential oils of citrus fruits and other plants have exhibited its antitumor activity in various types of cancers.[11,12] D-limonene ((R)-1-methyl-4-(1methylethenyl) cyclohexene), a monocyclic monoterpene abundantly found in volatile oils of citrus fruits such as orange, lemon, grapefruit and others plants has inhibited neoplasia in animal models and cell growth in-vitro.[13–15] Oral administration of D-limonene inhibited the growth of rodent pancreatic, mammary and gastric carcinogenesis and exhibited anticancer activity.[16–20]

2. AIM

The aim of present study is to evaluate the in vitro effect of D-limonene on primary hepatocytes and in vivo effect on K562 cells implanted C57BL/6 xenograft mice.

3. MATERIAL AND METHODS

3.1. Material

D-limonene was purchased from MP Biomedicals Solon, OH, USA. Cell culture medium Roswell Park Memorial Institute medium (RPMI)-1640, Dulbecco's modified Eagle medium (DMEM), trypsin, fetal bovine serum (FBS), HEPES, MTT, collagenase, sodium bicarbonate and trypan blue were obtained from Himedia, Mumbai, India. Antibiotic-antimycotic solution was obtained from Gibco, Grand Island, NY, USA. Doxorubicin was purchased from Sigma-Aldrich, St Louis, USA. Cyclosporine (Sandimmune) from Novartis, Basel, Switzerland and Ketoconazole (Nizral) from Johnson&Johnson, New Brunswick, New Jersey, USA. Cyclophosphamide (Endoxan) was purchased from Baxter, Halle, Germany. Ampoxin was purchased from Unichem laboratories Ltd., Mumbai, India. Rodent diet was obtained from VRK Nutrition, Pune, India.

3.2. Cell and cell culture

Human CML cell line K562 was purchased from National Centre for Cell Science (Pune, India, NCCS). Cells were maintained in RPMI-1640 medium supplemented with 10% FBS with 1% antibiotic-antimycotic solution. Cells were maintained in an incubator at 37°C supplemented with 5% CO_2 atmosphere. Cells used in the study were in the logarithmic growth phase.

3.3. Experimental animals and housing conditions

Healthy 4–6-week-old C57BL/6 male mice weighing 25–30 g were purchased from Mahaveera Enterprises, Hyderabad, India. Mice were housed under standard laboratory conditions (with a relative humidity of 60% ± 5% and a temperature of 25°C ± 2°C and 12 h : 12 h light and dark cycle) in individually ventilated cage. The mice were fed with autoclaved balanced rodent food pellet and Ampoxin (0.1 μg/mL) by drinking water was provided ad libitum. Mice were cared for and used in accordance with the CPCSEA guidelines and all animal experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (PERD/IAEC/2016/001) prior to initiation of the experiment.

3.4. Cell viability (MTT) assay of K562 cells

Effect of D-limonene on the viability of K562 cells was evaluated in time and dose dependent manner using 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assay. K562 cells (104 cells/well) were seeded in 96 well plate containing RPMI 1640+10% FBS in presence of different concentrations of D-limonene. The cells were treated with D-limonene at concentration of 1 mM, 2 mM, 4 mM and 8 mM and doxorubicin as a standard at concentration of 0.4 μM, 0.8 μM, 1.6 μM and 3.2 μM for 24 h and 48 h. After the treatment, 20 μl of MTT (5 mg/mL) was added to each well and the cells were incubated at 37°C for another 4 h. The amount of 100 μL of dimethylsulfoxide (DMSO) was added in each well followed by removal of culture medium by centrifugation (200 g for 7 minutes). Then, the optical density (OD) was measured at 570 nm by a microplate reader (EL800 BioTek Instruments, Inc.) Experiments were carried out in quadruplicate.

3.5. Primary culture preparation of mouse hepatocytes

Mouse was anaesthetized and abdomen was opened to cannulate the portal vein with perfusion buffer.[21] Gradually the perfusate flow was increased to wash out the blood from the liver (up to 20 mL/min) making an incision in vena cava to allowing perfusate efflux. The liver was blanched and was dissectedout; perfusion was continued with warm collagenase (type II) buffer (0.05%).The liver was transferred to the petri dish containing ice-cold suspension buffer containing calcium and magnesium). Gilsson's capsule was dissected and suspension was filtered through cell strainer. Debris were discarded and washed with wash buffer (perfusion buffer containing calcium) by centrifugation (50 g for

5 minutes) and resuspension.[22,23] Cell viability was determined using trypan blue method and cells were cultured in DMEM containing 10% FBS and maintained in an incubator at 37°C supplemented with 5% CO_2 atmosphere.

3.6. Cell viability (MTT) assay on primary mouse hepatocytes

The primary hepatocytes of mouse were seeded in 96 well plate containing DMEM+10% FBS. Treatment with doxorubicin and D-limonene was given at different concentration after 24 h of plating. The cells were treated with doxorubicin at concentration of 2 μM, 4 μM, 8 μM, 16 μM and 32 μM and D-limonene with concentration of 2 mM, 4 mM, 8 mM, 16 mM and 32 mM for 48 h. The cytotoxicity of D-limonene and doxorubicin on primary hepatocytes was compared by addition of MTT as described above and absorbance was measured. Experiments were carried out in quadruplicates.

3.7. Tumor xenograft model

Healthy male C57BL/6 mice were immunocompromised by oral administration of ketoconazole 10 mg/kg and intraperitoneal cyclosporine 30 mg/kg every day for 7 days. Cyclophosphamide was given at a dose of 60 mg/kg subcutaneously on days 3 and 1 before tumor cell injection in all groups of animals. K562 cell suspension of 0.1 mL (approximately 5×10^6 cells) was injected subcutaneously into the right shoulder blade of mice.[24] After achieving 100 mm^3 of tumor volume, mice ($n = 8$) were treated with vehicle and D-limonene (0.5 g/kg, 1.0 g/kg and 1.5 g/kg) orally for 14 days and tumor growth was observed at the site of injection. Tumor volume was measured externally by digital caliper using following formula:[25]

$$\text{Volume } (mm^3) = \frac{A \times B^2}{2}$$

where A was the largest and B the smallest diameter (mm).

At the end of the study, the tumors were excised and histopathological analysis of excised tumors was performed.

3.8. Histopathological analysis

All mice were sacrificed by cervical dislocation. The tumors were excised and maintained in 10% neutral buffered formalin and stained with hematoxylin and eosin. The slides were observed at 100× and 400× magnification and photodocumented by optical microscopy (IX 51; Olympus, Tokyo, Japan) equipped with a digital camera (TL4) for confirmation of presence of tumor cells.

3.9. Statistical analysis

All data were represented as mean + SD. Statistical analysis was performed by one-way ANOVA followed by Bonferroni comparison using Graphpad Prism software (v. 5.0, Graph Pad software Inc, USA). P value of 0.05 and 0.001 or less was considered to be statistically significant.

4. RESULTS

4.1. Effect of D-limonene on viability of K562 cells

MTT assay of D-limonene at concentration of 1 mM, 2 mM, 4 mM and 8 mM shows significant reduction percentage of cell viability of K562 cells as compared to untreated cells at 24 h and 48 h of treatment. However, no significant difference was observed in reduction of percentage of cell vi-

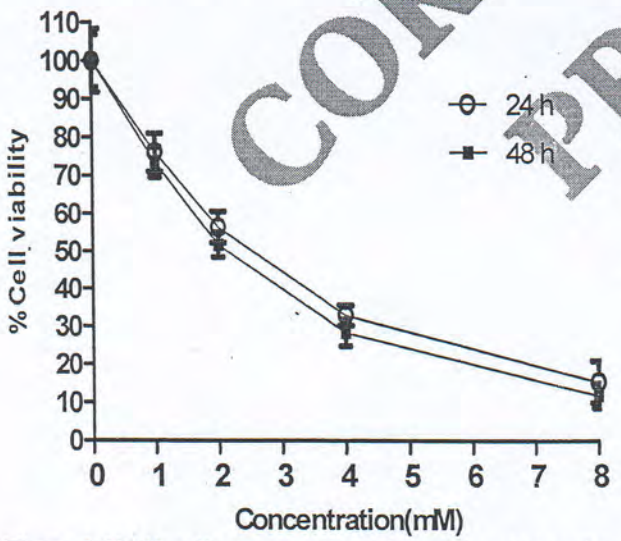

Figure 1. K562 cells were exposed to varying concentrations of D-limonene for 24 h and 48 h. The percentage of cell viability was measured by MTT assay. Cell viability was calculated as a percentage of untreated cells (100%). Data shown are the means ± SD from 3 separate experiments in quadruplicate.

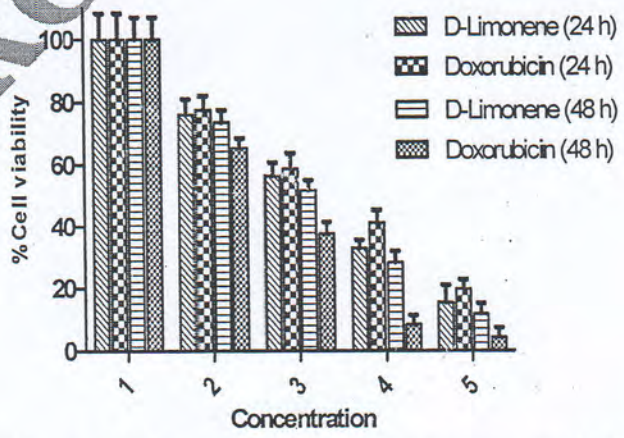

Figure 2. K562 cells shows reduction in percentage of cell viability after treatment with D-limonene and doxorubicin for 24 h and 48 h. D-limonene was used at concentration of 1 mM, 2 mM, 4 mM and 8 mM and doxorubicin was used at concentration of 0.4 μM, 0.8 μM, 1.6 μM and 3.2 μM. The percentage of cell viability was measured by MTT assay. Data shown are the means ± SD from 3 separate experiments in quadruplicate.

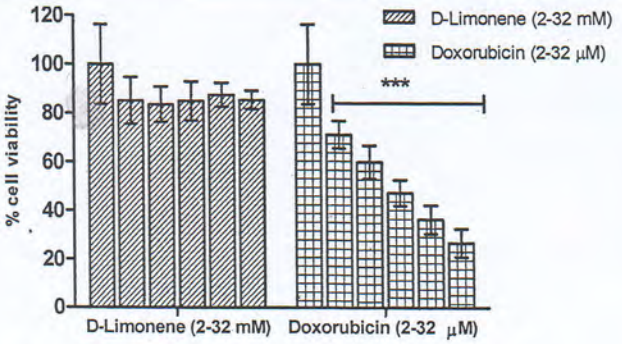

Figure 3. MTT assay of primary hepatocytes from mouse shows reduction in percentage of cell viability after treatment with doxorubicin at concentration of 0 mM, 2 mM, 4 mM, 8 mM, 16 mM and 32 mM. No significant reduction in % of celll viability was observed with D-Limonene at concentation of 2 mM, 4 mM, 8 mM, 16 mM and 32 mM. Data shown are the means ± SD from 3 separate experiments in quadruplicate.

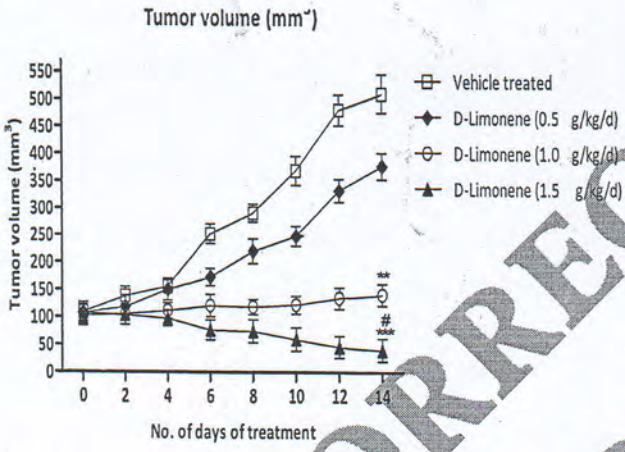

Figure 4. Graph shows the mean tumor volume of K562 xenograft. D-limonene treatment suppresses the tumor growth. K562 xenograft mice were treated with vehicle and with D-limonene at concentration of 0.5 g/kg a day, 1 g/kg a day and 1.5 g/kg a day orally for 14 days. A tumor growth curve was recorded every 2 day. Comments: Mean ± SD (n = 8); * P < 0.05 vs. vehicle treated group; # P < 0.05 vs. D-limonene (0.5 g/kg a day) group. No treatment was given to the vehicle treated group.

ability of K562 cells at 24 h and 48 h of treatment. The IC50 value of D-limonene was 3.6 mM and 3.29 mM for 24 h and 48 h of treatment respectively (Figure 1). The reduction in percentage of cell viability of doxorubicin at concentrations of 0.4 μM, 0.8 μM, 1.6 μM and 3.2 μM was comparable with D-limonene at 24 h and 48 h of treatment (Figure 2). The IC50 value of doxorubicin was 1.6 μM and 0.95 μM at 24 h and 48 h, respectively.

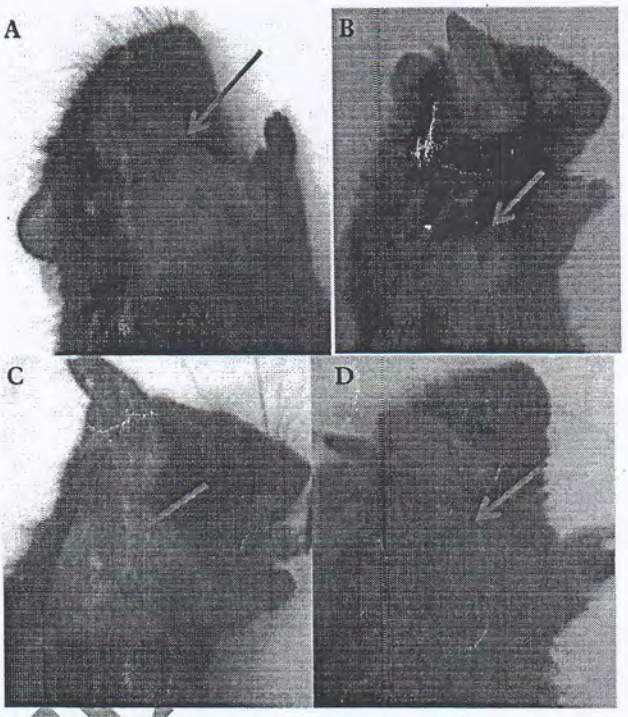

Figure 5. Immunocompromised C57BL/6 mice having K562 tumor xenograft as shown by arrow. Mice were treated with different concentrations of D-limonene, shows significant dose dependent regression of tumor. (A) Vehicle treated, (B) D-limonene 0.5 g/kg a day, (C) 1.0 g/kg a day and (D) 1.5 g/kg a day orally for 14 days. No treatment was given to the vehicle treated group.

4.2. Effect of D-limonene on cell viability of primary mouse hepatocytes

Treatment with D-limonene at concentration of 2 mM, 4 mM, 8 mM, 16 mM and 32 mM for 48 h did not show any reduction in percentage of cell viability of normal mouse primary hepatocytes compared to untreated cells by MTT assay. However, doxorubicin at concentration of 2 μM, 4 μM, 8 μM, 16 μM and 32 μM for 48 h shows significant reduction in percentage of cell viability (IC50 = 14.13 μM) of mouse primary hepatocytes as compared to untreated cells (Figure 3).

4.3. Tumor xenograft model shows regression of the malignant tumor

Immunocompromised K562 cell injected C57BL/6 mice showed palpable tumor on 3rd day of tumor cell implantation. Increase in mean tumor volume was seen with the vehicle treated group every day. D-limonene treatment at daily dose of 0.5 g/kg, 1.0 g/kg and 1.5 g/kg for 14 days shows dose dependent reduction in mean tumor volume as compared to vehicle treated group (Figure 4).

However, significant reduction in tumor volume was observed only at dose of 1.0 g/kg a day and 1.5 g/kg a day with D-limonene treatment as compared to vehicle treated group. The reduction in tumor size was observed in immu-

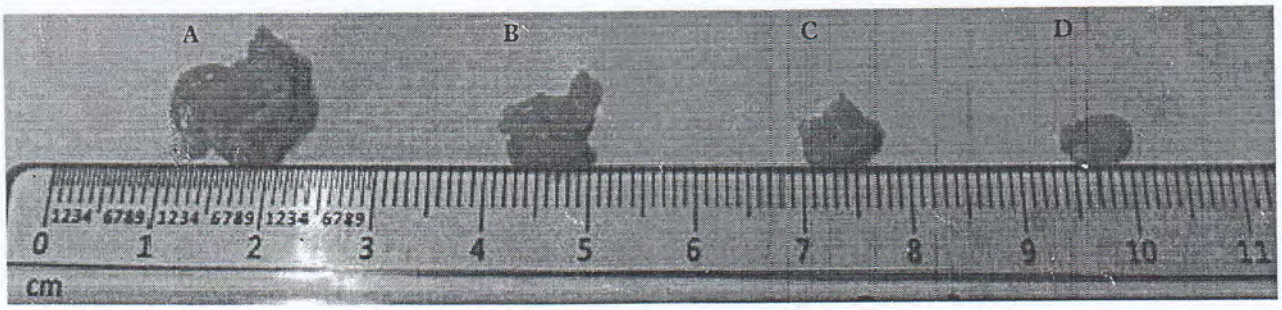

Figure 6. Tumors were excised at the end of 14 days from K562 tumor implanted xenograft C57BL/6 mice. (A) Vehicle treated, (B) D-limonene 0.5 g/kg a day, (C) 1.0 g/kg a day and (D) 1.5 g/kg a day orally for 14 days. D-limonene treated group at concentration 1.5 g/kg a day, shows more reduction in the tumor size as compared to 0.5 g/kg a day and 1.0 g/kg a day. No treatment is given to vehicle treated group.

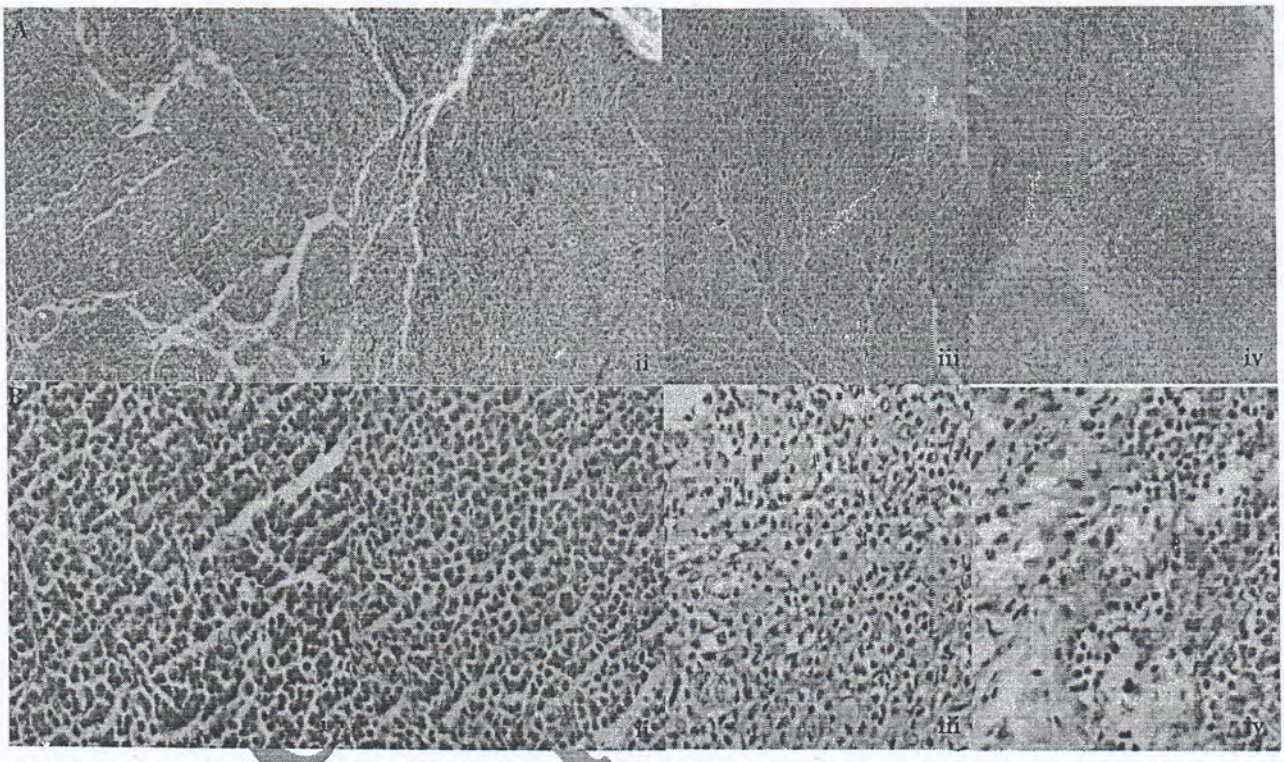

Figure 7. Light microscopy observation of HE-stained section of K562 xenograft tumor at (A) 100× and (B) 400× magnification. (i) Vehicle treated, (ii) D-Limonene 0.5 g/kg a day, (iii) 1.0 g/kga day and (iv) 1.5 g/kga day orally for 14 days. Cells with large and irregular nuclei and scant cytoplasm are characteristic of malignant cells.

nocompromised C57BL/6 mice with D-limonene in dose dependent manner at the end of 14 days treatment (Figure 5). The weight of excised tumors was significantly reduced in the D-limonene treatment groups, compared to vehicle-treated group in dose dependent manner (Figure 6).

4.4. Histopathological analysis

Histopathological data shows the presence of malignant tumor when stained with standard hematoxylin and eosin. The stained section of K562 xenograft shows a characteristic of malignant cells, large and irregular nuclei and scant cytoplasm at magnification of 100× and 400× (Figure 7). It also shows invasion of malignant cells in adjacent stromal tissue.

5. DISCUSSION

Terpenes have been reported for its multiple biological effects, including antiviral, antibacterial, anti-inflammatory, antioxidant and anti cancer activity. D-limonene, a monocyclic monoterpene obtained from citrus fruits, has shown in vitro cytotoxic effect on various cancer cell lines along with K562 cells.[14,15] It has also inhibited DMBA-NMU induced mammary carcinoma in rats.[26,27]

Phase I clinical trials in patients with advanced cancer, D-limonene has shown well tolerated and proven clinical activity.[28] D-limonene in combination with docetaxel has improved the sensitivity of hormone refractory prostate

cancer cells, without producing toxicity to normal prostate epithelial cells.[29,30] The combined beneficial effect could be through the modulation of proteins involved in mitochondrial pathway of apoptosis.

In our study, the cell viability assay of D-limonene has shown significant reduction in the percentage of cell viability in concentration dependent manner which suggests its cytotoxic action on K562 cell line. Anthracycline antibiotic, doxorubicin is one of the most common and widely used anticancer agents and is still a milestone in the therapy of many carcinomas. Similarly with available anticancer treatment, doxorubicin therapy is mostly accompanied by severe side effects based on its systemic toxicity, especially cardiotoxicity and hepatotoxicity.[31–33] In our study, we have evaluated the effect of D-limonene on isolated normal primary hepatocytes from mice by using doxorubicin as a standard. Doxorubicin showed significant reduction in percentage of cell viability of primary hepatocytes in a concentration dependent manner. Treatment with D-limonene did not show significant reduction in percentage viability of primary hepatocytes. This may be attributed to its antioxidant activity.[34]

Previous studies suggest, D-limonene shows regression of tumor in in-vivo animal models.[19,20] Immunocompromised K562 tumor xenograft model was used to evaluate the in vivo efficacy of D-limonene at different doses. Immunosuppression was achieved with combination treatment of cyclosporine, ketoconazole and cyclophosphamide in C57BL/6 mice with 100% take rate.[24] Significant increase in tumor volume was observed after 2–3 days of K562 cell implantation. D-limonene treatment showed significant reduction in tumor volume in K562 tumor xenograft mice as compared with vehicle treated group in dose dependent manner. The significant reduction in tumor weight of excised tumors was also observedwith D-limonene treatment.

The histological studies of tumor tissue confirmed the presence of malignant tumor cells. It also showed invasion of tumor cells into adjacent stromal tissue. However, the density of the malignant cells was reduced in the dose dependent manner with D-limonene treatment.

6. CONCLUSIONS

In conclusion, the results from the present in vitro study suggests that D-limonene do not show toxicity on normal primary hepatocytes isolated from mouse as compared to doxorubicin. The in vivo studies on K562 tumor implanted C57BL/6 mice, D-limonene shows regression of tumor growth in dose dependent manner. Further studies need to elucidate the mechanism of D-limonene in CML before its clinical application.

Conflict of interest

None.

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