Phenolic Extract of *Crithmum maritimum L.* prevents Benzene induced leukemia in Balb/c mice

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I certify that this research work titled "*Phenolic Extract of Crithmum maritimum L. prevents Benzene induced leukemia in Balb/c mice"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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Contents

1 Chapter 1 Introduction 16

1.1 Cancer Statistics in the U.S., 2019 16

1.2 Statistics of Leukemia in the U.S., 2019 17

1.3 Cancer Statistics in Pakistan, 2018 18

1.4 Cases of Leukemia in Pakistan, 2019 20

1.4.1 Reported cases of Leukemia in Shaukat Khanum Memorial Cancer Hospital & Research Center, Pakistan Report from Dec. 1994-2017 20

1.5 Hematopoietic system 20

1.6 Anatomy and Physiology of Bone Marrow 22

1.6.1 Types of Bone Marrow 22

1.7 Hematotoxicity 23

1.8 Few agents responsible for hematotoxicity 23

1.9 Injury Due to Environmental Pollutants 24

1.10 Leukemia and History 24

1.11 Benzene-induced leukemia 25

1.12 The biochemical mechanism behind benzene metabolism 25

1.13 Mechanism of Action of Benzene 26

1.14 The mechanism behind benzene-induced leukemia 27

1.15 Signal transduction pathway in LSC and HSC 29

1.16 Problem Statement 31

1.17 Aim of the Study 31

2 Chapter 2 Literature Review 32

2.1 Benzene metabolism and toxicity in leukemia 32

2.2 Leukemia and signaling cascades 33

2.3 Chemotherapy for various types of leukemia 35
2.4 Ethnomedicine for leukemia 37
2.5 Animal models of benzene induced leukemia 39
2.6 Crithmum maritimum L. 40
3 Chapter 3 Methodology 42
3.1 Materials 42
3.2 Conditions for protocol 42
3.3 Grouping for protocol 43
3.4 Dosage 43
3.5 Drug administration route 44
3.6 Protocol for plant extract preparation of *Crithmum maritimum L.* 44
3.7 Protocol 45
3.7.1 Acclimatization period 45
3.7.2 Development of leukemia mice model 45
3.7.3 Collection of the blood sample 45
3.7.4 Treatment of developed leukemic mice model 46
3.7.5 Sacrification 46
3.7.6 Sample storage 47
3.8 Techniques 47
3.8.1 Complete blood count 47
3.8.2 Histology with H&E staining 47
3.8.3 Flow cytometry 47
3.9 Observations during protocol 48
4 Chapeter 4 Results 49
4.1 Weight Analysis 49

4.2 Histopathology Results 50
4.3 Histopathology of Liver 50
4.3.1 Control group 50
4.3.2 Group treated with Benzene 51
4.3.3 Group treated with the standard drug: Cyclophosphamide 52
4.3.4 Group treated with phenolic extract of Crithmum maritimum L. 53
4.4 Histopathology of Spleen 54
4.4.1 Control Group 54
4.4.2 Group treated with Benzene 55
4.4.3 Group treated with the standard drug: Cyclophosphamide 56
4.4.4 Group treated with phenolic extract of Crithmum maritimum L. 57
4.5 Histopathology of Kidney 58
4.5.1 Control group 58
4.5.2 Group treated with Benzene 59
4.5.3 Group treated with the standard drug: Cyclophosphamide 60
4.5.4 Group treated with phenolic extract of Crithmum maritimum L. 61
4.6 Complete Blood count 62
4.6.1 White blood cell counts 62
4.6.2 Red blood cell counts 62
4.6.3 Thrombocytes counts 63
4.6.4 Lymphocytes counts 63
4.6.5 Monocytes count 64
4.6.6 Granulocytes count 64
4.6.7 Hemoglobin 65
4.6.8 Hematocrit 65

4.7 Flow cytometry results 66
5 Discussion 67
6 Conclusion 69
7 References 70

List of figures

Figure 1: (Cancer et al., 2019) Number of new cancer cases of different types of cancer reported in Pakistan in 2018 18

Figure 2: Adapted from (Ramaiah et al., 2017), Diagrammatic representation and Lineage differentiation of Hematopoiesis through a lineage model, that varies with species. Transcription Factors; stimulatory cytokines; Cells circulating; Tissues are shown in the above 21

Figure 3: Adapted from (PDQ Adult Treatment Editorial Board, 2002), Red and Yellow marrow 22

Figure 4: Adapted from (Snyder, 2012) Metabolic Pathway of benzene metabolism 26

Figure 5: Adapted from (Smith et al., 2011) Pathways involved in the induction of leukemia through benzene 28

Figure 6: (Seke Etet et al., 2012), Graphical representation of normal signaling pathway in HSC. 29

Figure 7: (Seke Etet et al., 2012), Graphical representation of leukemic stem cell signaling pathway 30

Figure 8: Adapted from (Luis, Ichii, Brugman, Kincade, & Staal, 2012), Wnt signaling cascade 33

Figure 9: Adapted from (Scholl, Gilliland, & Fröhling, 2008), Abnormal Signaling Cascade in AML 34

Figure 10: Adapted from (Abd Hamid, M Bunnori, Adekunle, & Ali, 2015), Representation of Geometry and Structural formula of calixarene 35

Figure 11: Intra-peritoneal position for injecting the drug to Balb/c mice 44

Figure 12: Adapted from (Parasuraman, Raveendran, & Kesavan, 2010), an illustration of collection of blood sample form tail of a mouse 46

Figure 13: Physical condition of mice being observed after protocol 48

Figure 14: Weight analysis of Control Group, Benzene Group, Standard Drug i.e. Cyclophosphamide & Phenolic Extract Group i.e. PE of Crithmum maritimum 49

Figure 15: Histology of normal liver cells 400x 50

Figure 16: Histology of normal liver cells 400x 50

Figure 17: Histopathology of the liver treated with Benzene 400x 51
Figure 18: Histopathology of the liver treated with benzene 40x 51
Figure 19: Histopathology of the group treated with standard drug 400x 52
Figure 20: Histopathology of the group with standard drug 40x 52
Figure 21: Histopathology of the group treated with P.E of Crithmum maritimum 400x 53
Figure 22: Histopathology of the group treated with P.E of Crithmum maritimum 40x 53
Figure 23: Spleen of control group 400x, showing lymphocytes 54
Figure 24: Normal histology of spleen from the control group, 40x 54
Figure 25: Histopathology of spleen 400x 55
Figure 26: Histopathology of spleen 40x 55
Figure 27: Histopathology of the group treated with standard drug 400x 56
Figure 28: Histopathology of the group treated with standard drug 40x 56
Figure 29: Histopathology of the group treated with P.E of Crithmum maritimum 400x 57
Figure 30: Histopathology of the group treated with P.E of Crithmum maritimum 40x 57
Figure 31: Histology of normal kidney cells 400x 58
Figure 32: Histology of normal kidney cells 40x 58
Figure 33: Histopathology of the kidney treated with Benzene 400x 59
Figure 34: Histopathology of the kidney treated with Benzene 40x 59
Figure 35: Histopathology of the group treated with standard drug 400x 60
Figure 36: Histopathology of the group with standard drug 40x 60
Figure 37: Histopathology of the group treated with P.E of Crithmum maritimum 400x 61
Figure 38: Histopathology of the group treated with P.E of Crithmum maritimum 40x 61
Figure 39: Pie graph of WBC's 62
Figure 40: Pie graph of RBC's 62
Figure 41: Pie graph of Platelets 63
Figure 42: Pie graph of Lymphocytes 63
Figure 43: Pie graph of monocytes 64
Figure 44: Pie graph of granulocytes 64
Figure 45: Pie graph of Hemoglobin 65
Figure 46: Pie graph of Hematocrit 65
Figure 47: Rate of apoptosis in all groups 66

List of tables

Table 1: Adapted from (Facts, 2019), Estimated Deaths and New Cases of Different Cancers in U.S. 2019 16

Table 2: Adapted from (Facts, 2019), Number of Estimated death and new cases of leukemia by sex, in the U.S, 2019 18

Table 3: Adapted from (Cancer et al., 2019), Facts and figures of new and death cases of various types of cancer in Pakistan, 2018 19

Table 4: Adapted from (Generalić Mekinić et al., 2016), Values obtained from anti-oxidation assay by different methods of Eos and ethanolic extract 41

List of acronyms and abbreviations

HSC	Hematopoietic stem cells
LSC	Leukemic stem cells
AML	Acute myeloid leukemia
PTEN	Protein Tyrosin Phosphate
Wnt	Wingless related-integration site
IC_{50}	Inhibitory concentration 50
FA group	Formaldehyde group
BZ group	Benzene group
NMR	Nuclear Magnetic Resonance
PDGF	Platelet-Derived Growth Factor
DDT	Dichloro diphenyl trichloroethane
CYP2E1	Cytochrome P450 2E1
ROS	Reactive Oxygen Species
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ECM	Extracellular matrix
EO	Essential Oils
ALL	Acute lymphocytic leukemia
CML	Chronic myeloid leukemia
CLL	Chronic lymphocytic leukemia

Abstract

Benzene is an organic compound and it has been classified as Class A carcinogen. At the start of the 20th century, it has been widely used in many industrial and commercial products e.g. printing, shoe industry, rubber industry, etc. Benzene has been reported for causing various diseases including aplastic anemia, leukemogenesis and hematological malignancies in humans. Benzene toxic metabolites (1.4 benzoquinone) in the liver or in the bone marrow can lead to traumatic bone marrow injury and hepatotoxicity, subsequently cause Leukemias. In benzene-induced leukemia, oxidative DNA Damage and chromosomal mutations occur by the change in function of Topoisomerase II. Deviant activation of STAT proteins and dysregulation of STAT pathway is associated with leukemogenesis. Current chemotherapeutics for the treatment of leukemia have been limited by high cost and severe side effects. Therefore there is an urgent need for the discovery of new biologically active compounds for treatment of Leukemia. Among various medicinal plants, *Crithmum maritimum L*. belongs to the family of Apiaceae and has been used traditionally for the treatment of various ailments including obesity, scurvy, Urinary tract infections, gastrointestinal problems, and Kidney problems. To validate the ethno pharmacological and anti-leukemic effects of phenolic extracts of *Crithmum maritimum L.* against benzene induced leukemia, extract of *Crithmum maritimum L.* has been prepared and tested on Leukemic model of Balb/c mice. Cytotoxicity of the extract against leukemia has been evaluated through a number of experiments. Annexin V assay in flow cytometry has been used to detect the apoptotic effect of *Crithmum maritimum L*. on Leukemic cells in Balb/c strain comparative to FDA approved Chemotherapeutic drug Cyclophosphamide. Annexin V has the affinity to bind with phosphatidylserine and ability to detect apoptotic cells by binding with phosphatidylserine, by flow cytometry. Translocation of phosphatidylserine (PS) from the inner region to outer layer of plasma membrane renders some alterations in plasma membrane due to early stages of apoptosis, due to which PS opens up at cell surface.

Keywords Hematopoiesis, Benzene, Cyclophosphamide, Flow cytometry, apoptosis, phenols

1 Chapter 1 Introduction

1.1 Cancer Statistics in the U.S., 2019

New cases of cancer that are supposed to be diagnosed are greater than 1.7 million. New estimated death cases due to cancer among Americans are 606,880, which predicts 16,60 deaths/day (Facts, 2019).

Sr.No.	Cancer Type	Estimated New Cases	Estimated Cases of Death
1.	Breast Cancer	268,600	42,260
2.	Childhood Cancer	11,060	1,190
3.	Colon Cancer	101,420	51,020
4.	Rectum Cancer	44,180	51,020
5.	Renal Cancer	73,820	14,770
6.	Liver Cancer	42,030	31,780
7.	Lung Cancer	31,780	142,670
8.	Lymphoma	82,310	1,000
9.	Pharynx cancer	53,000	10,860
10.	Ovarian Cancer	22,530	13,980
11.	Pancreatic Cancer	56,770	45,750
12.	Prostate Cancer	174,650	31,620
13.	Skin Cancer		7,230
14.	Thyroid Cancer	52,070	2,170
15.	Urinary bladder cancer	80,470	17,670
16.	Cervical Cancer	13,170	4,250
17.	Endometrial Cancer	61,880	12,160

Table 1: Adapted from (Facts, 2019), Estimated Deaths and New Cases of Different Cancers in U.S. 2019

1.2 Statistics of Leukemia in the U.S., 2019

Estimated new leukemia cases in the U.S. are 61,780 in number. More than sixty thousand people are supposed to be diagnosed with leukemia in 2019. Leukemia has various types' i.e. acute myeloid leukemia, acute lymphocytic leukemia, chronic myeloid leukemia, and chronic lymphocytic leukemia. People who are above 20 years of age, mostly diagnosed with CLL (37%) and AML (32%). Whereas, in children and among teenagers (0-19 years of age) 74% of cases are of AML.

Incidence Rate: As far as the incidence rate of leukemia is concerned, there is a trend of stability in cases of CLL from 2006-2015, but there is an increase of 0.7% in ALL, 1.8% for CML, and 3,7% for AML, per year.

Death Rate: An estimation of death cases supposed to happen in 2019 in the U.S. is 22,840.

Mortality Pattern: In comparison to incidence value, death rate there was stability in death rate for AML from 2007-2016. There was a decline of 1% per year for CLL and AML. For CLL, there was a reduction in the death rate by 3% per year.

Survival: Survival pattern varies with the type of leukemia as well as with age according to each year (Facts, 2019).

	Estimated New Cases			**Estimated Deaths**		
	Both Sexes	Male	Female	Both sexes	Male	Female
Leukemia	61,780	35,920	25,860	22,840	13,150	9,690
Acute lymphocytic leukemia	5,930	3,280	2,650	1,500	850	650
Chronic lymphocytic leukemia	20,720	12,880	7,840	3,930	2,220	1,710
Acute						

myeloid leukemia	21,450	11,650	9,800	10,920	6,290	4,630
Chronic myeloid leukemia	8,990	5,250	3,740	1,140	660	480
Other leukemia‡	4,690	2,860	1,830	5,350	3,130	2,220

Table 2: Adapted from (Facts, 2019), Number of Estimated death and new cases of leukemia by sex, in the U.S, 2019

1.3 Cancer Statistics in Pakistan, 2018

173 937 were reported in new cases due to cancer and 118 442 were deaths reported in 2018, due to 2018 (Cancer, Method, & Country-specific, 2019).

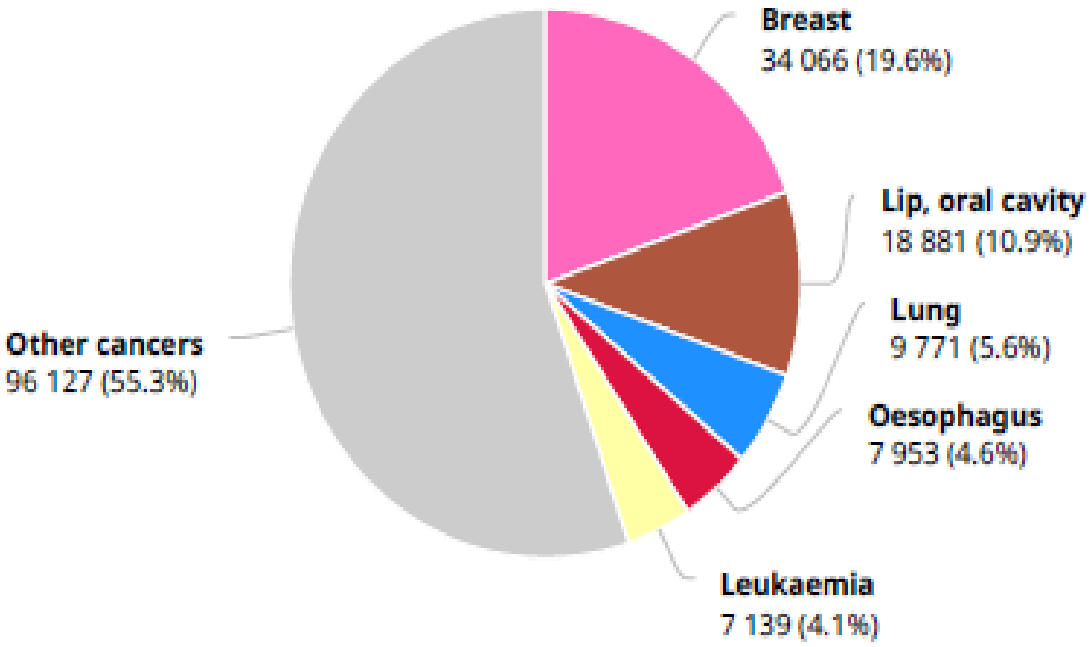

Figure 1: (Cancer et al., 2019) Number of new cancer cases of different types of cancer reported in Pakistan in 2018

	New Incidents	Cases of deaths
Site of Cancer	**No.**	**No.**
Breast	34,066	17,158
Lip and oral cavity	9,771	13,351
Lung	18,881	9,260
Oesophagal	7,953	7,555
Leukemia	7,139	4,945
Non-Hodgkin lymphoma	5,876	4,818
Cervical or Uterine	5,601	3,861
Bladder	4,610	2,614
Prostate	4,552	3,417
Ovarian	4,504	3,326
Hepatic	4,381	4,222
Throat	4,365	2,442
Brain	4,299	4,033
Gastric	4,154	3,923
Gall bladder	3,312	2,189
Colon	3,016	2,181
Rectum	2,839	2,430
Renal	2,209	1,329
Thyroid	1,971	434
Hodgkin lymphoma	1,597	908
Multiple myelomas	1,406	952
Pancreatic	1,070	1,008
Salivary glands	906	549
Melanoma	428	313

Table 3: Adapted from (Cancer et al., 2019), Facts and figures of new and death cases of various types of cancer in Pakistan, 2018

1.4 Cases of Leukemia in Pakistan, 2019

According to the World Health Organization GLOBOCAN 2018, Leukemia has been characterized among the 5th most common types of cancers prevalent in Pakistan. Approximately 7,139 new cases and 4,945 deaths have been reported in Pakistan due to leukemia in Pakistan (Cancer et al., 2019).

1.4.1 Reported cases of Leukemia in Shaukat Khanum Memorial Cancer Hospital & Research Center, Pakistan Report from Dec. 1994-2017

Among the top ten malignant cancers reported, leukemia was on 2nd number with a total count of 5,089 among 84,803 of the total cases of cancers of various types reported. In adults, both male and females who are above 18 years of age, 2,883 were of leukemia out of 76,092 reported cases for cancers whereas, in children, number of leukemia cases were 1,778 of acute lymphoblastic leukemia and 213 of acute myeloid leukemia among 8,711 reported child patients. Within 39,663 female cancer patients, 963 females were diagnosed with leukemia. In males, 36,429 were total cased reported of various cancers, among them 1920 cases were of leukemia (Mahmood et al., 2017).

1.5 Hematopoietic system

The hematopoietic system consists of circulating blood cells and tissues that form blood and is one of the major organs of the body and is the most extensive one. The primary function of the hematopoietic system is hematopoiesis, the formation of mature and highly specialized blood cells that are involved in the Respiratory system, Immune System and homeostasis of the body in the form of erythrocytes (RBCs), Leukocytes and platelets respectively. Blood cells play a major role in respiration, defense system and in balancing the body. Eventually, the Hematopoietic system is highly modulated, multiplex and potent process, which can be compromised by any xenobiotics (Ramaiah, Bounous, & Elmore, 2017).

Cells within the Hematopoietic system along with their precursors are:

- Red Blood Cells (RBCs): originated from proerythroblasts
- Platelets/Thrombocytes
- Leukocytes (WBCs): originated from Myeloblast
- Granulocytes (neutrophils, eosinophils, basophils)

- Monocytes: originated from monoblast
- Macrophages: originated from megakaryoblast
- Lymphocytes: originated from lymphoblast (Corey & Blatt, 2011).

Red Blood Cells: the Basic function of RBCs is the exchange of gases from the lungs to the body tissues.

Platelets: They prevent vascular damage by the formation of a homeostatic plug, also help in wound healing and triggers an inflammatory process.

Monocytes: Their main function is to remove necrotic debris and pathogens, later turned into macrophages.

Lymphocytes: They have a defensive mechanism against various diseases.

Leukocytes: are also called as White Blood Cells, consists of further three types of cells: Eoisnophills, Neutrophil, and Basophils (Gwaltney-Brant, 2014).

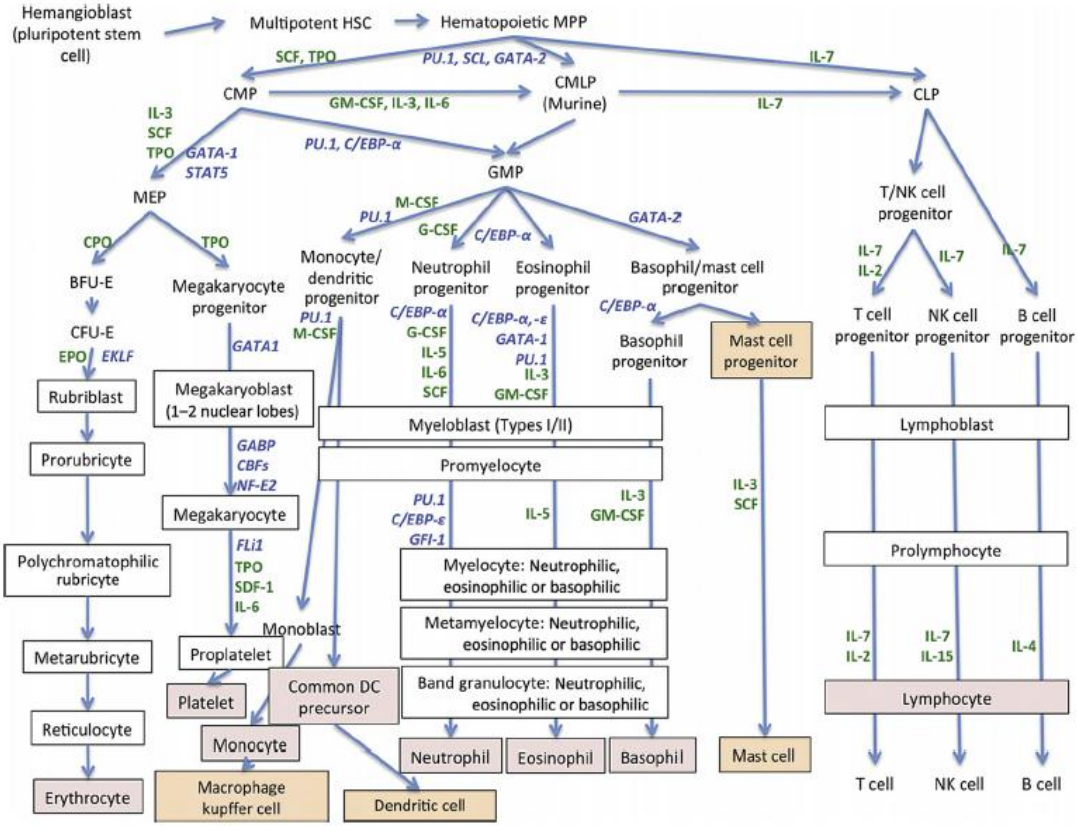

Figure 2: Adapted from (Ramaiah et al., 2017), Diagrammatic representation and Lineage differentiation of Hematopoiesis through a lineage model, that varies with species. Transcription Factors; stimulatory cytokines; Cells circulating; Tissues are shown in the above

1.6 Anatomy and Physiology of Bone Marrow

Bone marrow is the production site of hematopoietic stem cells (Corey & Blatt, 2011). Bone Marrow is the major organ for hematopoiesis and is the initial lymphoid tissue in the body. The mainstream multipotent progenitor stem cell gives rise to lymphoid and hematopoietic lineages (Ramaiah et al., 2017).

1.6.1 Types of Bone Marrow

1. **Yellow bone marrow:** In this marrow, the number of adipocytes increased, medullary spaces of long bones filled with fat cells with age, so it turns out yellow and less active.
2. **White bone marrow:** Intracellular matrix and stromal cells are present due to starvation or decay of cells.
3. **Red bone marrow:** Red blood cells dominate in this marrow, turning it into the active bone marrow. Proportionally, red marrow comprises more of the body's weight and volume in a young child as compared to an adult. It is contained in the medullary cavities of bones (Corey & Blatt, 2011).

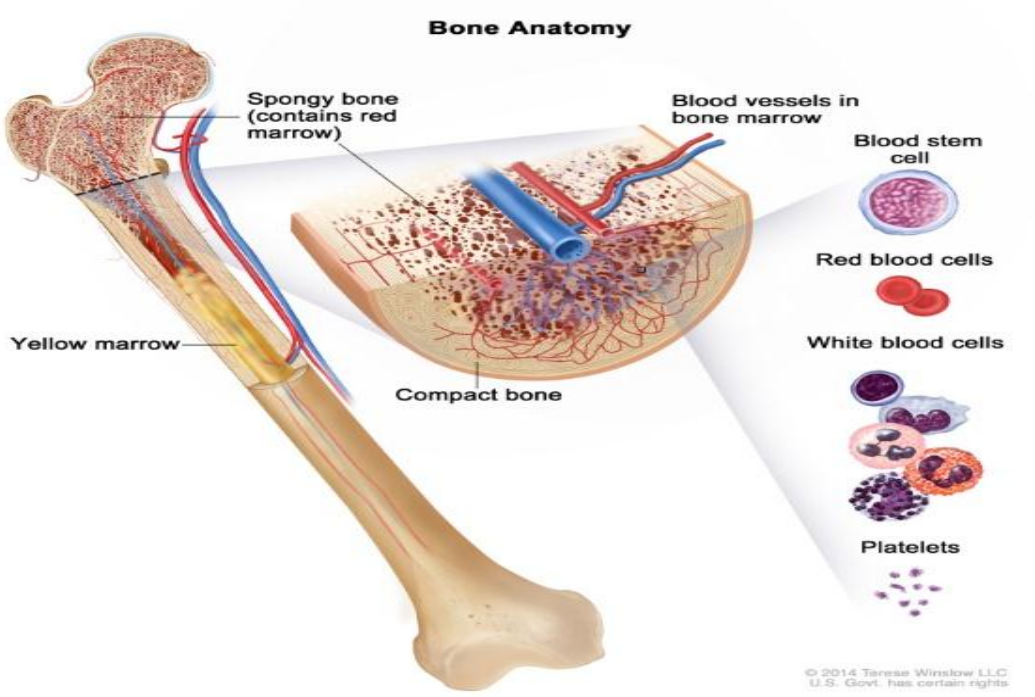

Figure 3: Adapted from (PDQ Adult Treatment Editorial Board, 2002), Red and Yellow marrow

1.7 Hematotoxicity

The hematopoietic system is uniquely susceptible to toxins, and the most affected organs from this toxicity are kidney and liver. Slight injury in blood calls can affect the oxygenation of tissues, homeostasis, and immunity of the body. Hematotoxicity is described as the noxious effect of any foreign agent or toxin on blood-forming tissues and on blood cells. It could happen via direct injury or injury to other body systems that can cause it, indirectly. Consequences of hematotoxicity can severely affect the whole range of hematologic cells, the bone marrow, and blood cells. For analyzing toxicity, evaluation of bone marrow is necessary because any reaction such as inflammation, hemorrhage demands a high supply of cells (Ramaiah et al., 2017). Hematotoxicity leads to the malfunction in lineages of different cell types like myeloid and lymphoid lineages, which cause mutations in blood cells within the bone marrow and in blood too. Sometimes that chemical that is causing hematotoxicity and sometimes its metabolite is responsible for toxicity in the bone marrow that disturbs the whole hematopoiesis (Ramaiah et al., 2017).

1.8 Few agents responsible for hematotoxicity

Following are few agents responsible for hematotoxicity in the bone marrow and in progenitor cells of the hematopoietic system:

- Cytotoxic/alkylating agents
- Nucleotide Antagonist
- Antibiotics
- Antifungals
- Antivirals
- Antiparasitics
- Hormones and hormones antagonists
- Central Nervous System drugs
- Environmental toxicants
- Agents toxic for specific molecular regions

1.9 Injury Due to Environmental Pollutants

Benzene and Benzene derivatives such as DDT, CCL_4, Benzopyrene, etc act as mutagenic. They can cause toxicity by the alkylation of proteins. They can introduce oxidative stress among progenitor cells. They can also mutate Growth factors which lead to inhibition of them, causing hematotoxicity at the targeted site. They can also trigger oxidation and reduction cycles. They can also impair the immune system by various signaling mechanisms. The most widely studied hepatotoxicants are Benzene and its metabolites. They are polycyclic, organic and aromatic hydrocarbons. They can cause genotoxicity and are cytotoxic to the progenitors and stem cells of hematopoiesis. They can be mutagenic at any level among myeloid and lymphoid lineages in bone marrow or in circulation cells in the blood. Benzene can cause hematotoxicity even at very low doses. It can cause various blood disorders like leukemia, Anemia, Pancytopenia, Acute Myeloid Leukemia, Myelodysplastic syndrome, and many more (Ramaiah et al., 2017). Benzene has been used in many industries such as shoe, plastics, petroleum industry, dyes, etc. in the US and worldwide. Concentrations even less than 1 ppm are toxic at an occupational and environmental level for humans.

1.10 Leukemia and History

In 1827, a French surgeon, Alfred Velpeau described a case of a patient with abdominal swelling, weakness, and fever. After his death, an autopsy was performed and results have shown enlarged spleen and liver, Lemak and Freireich reported this in 1991. In 1837, at the University of Paris Alfred Donné had knowledge of three types of blood cells Platelets, Red and white cells. White cells were considered as mucous globules and platelets were termed as small globules. A blood sample of a patient sent to him, who was considered to be leukemic because he eas having more white cells in his samples than other cells. In 1844, Donné reported hematological abnormalities as Leukemia, because he had many samples with an excess of mucous globules in their blood. In 1845, two papers focused on the reason behind an excessive increase in white cells in this disease. John Hughes, who was a Scottish physician and classmate of Donné, suggested that such a rise in white cells may be due to some infection, but after an autopsy, there was no evidence of any infection. Professor Rudolph Virchow, a Pathologist at the University of Wurzburg, proposed it as the disorder of white blood cells and termed it as “leukämie”. In 1854, the work of Professor Julius Vogel at the University of Giessen in Medicine department

implemented that Leukemia is truly a disease when he reported his observation on twenty-five patients. He characterized leukemia as a chronic but slowly developing disease that leads to death. In 1857, the first case of acute leukemia was reported by Nickolaus Friedreich. Pioneer of Hematology, Ernst Neumann explained that blood cells produced from bone marrow and disease of bone marrow were leukemia. The term of Myelogenous leukemia was formulated by him (Snyder, 2012).

1.11 Benzene-induced leukemia

The high concentration of Benzene triggered the generation of Reactive oxygen species and cytotoxicity in HL60 cell lines. Metabolism of Benzene takes place in the Liver and lungs also, through cytochrome-P450. CYP2E1 is present in the liver, oxidized benzene to an oxide of benzene. CYP2F1 and CYP2A13 enzymes are present in Human Lungs and are highly in an active state to metabolize even at very small doses of benzene in the lungs. Chromosomal abnormalities are also the target of benzene hematotoxicity. Associated chromosomal aberrations in AML includes mutations at 5q, 7q, +8 and t (8; 21). Aneuploidy in chromosomes was also found to be initiated by benzene. Inhibition of topoisomerase II is the other mechanism through which mutation in chromosomes is caused by benzene to induce leukemia in stem cells. Increased cleavage at DNA strands is also done when benzene is exposed to cells in the bone marrow. Benzene toxicity leads to mutations at the genetic level causing point mutations at a locus of glycoprotein in human erythrocytes has been observed on prolonged exposure to Benzene. The generation of oxidative stress and errors in DNA-repair system are two main mechanisms behind mutations induced by benzene in DNA. Along with the generation of radicals of oxygen, an increase in the number of mitochondrial DNA is also caused by exposure to benzene (Mchale, Zhang, & Smith, 2012)**.**

1.12 The biochemical mechanism behind benzene metabolism

Following are the main events happening underneath the benzene metabolism and toxicity to bone marrow:

- Benzene metabolized to metabolites of benzene oxide
- The reaction of benzene metabolites with targeted cells in bone marrow

- Formation of mutated bone marrow cells
- Clonal proliferation of mutated cells
- Formation of leukemic cells

1.13 Mechanism of Action of Benzene

Cytochrome P450 metabolizes benzene within the liver, as the liver is the primary site of metabolism for benzene. The initial metabolite of benzene is benzene oxide, which is metabolized by Cyt P450 2E1.

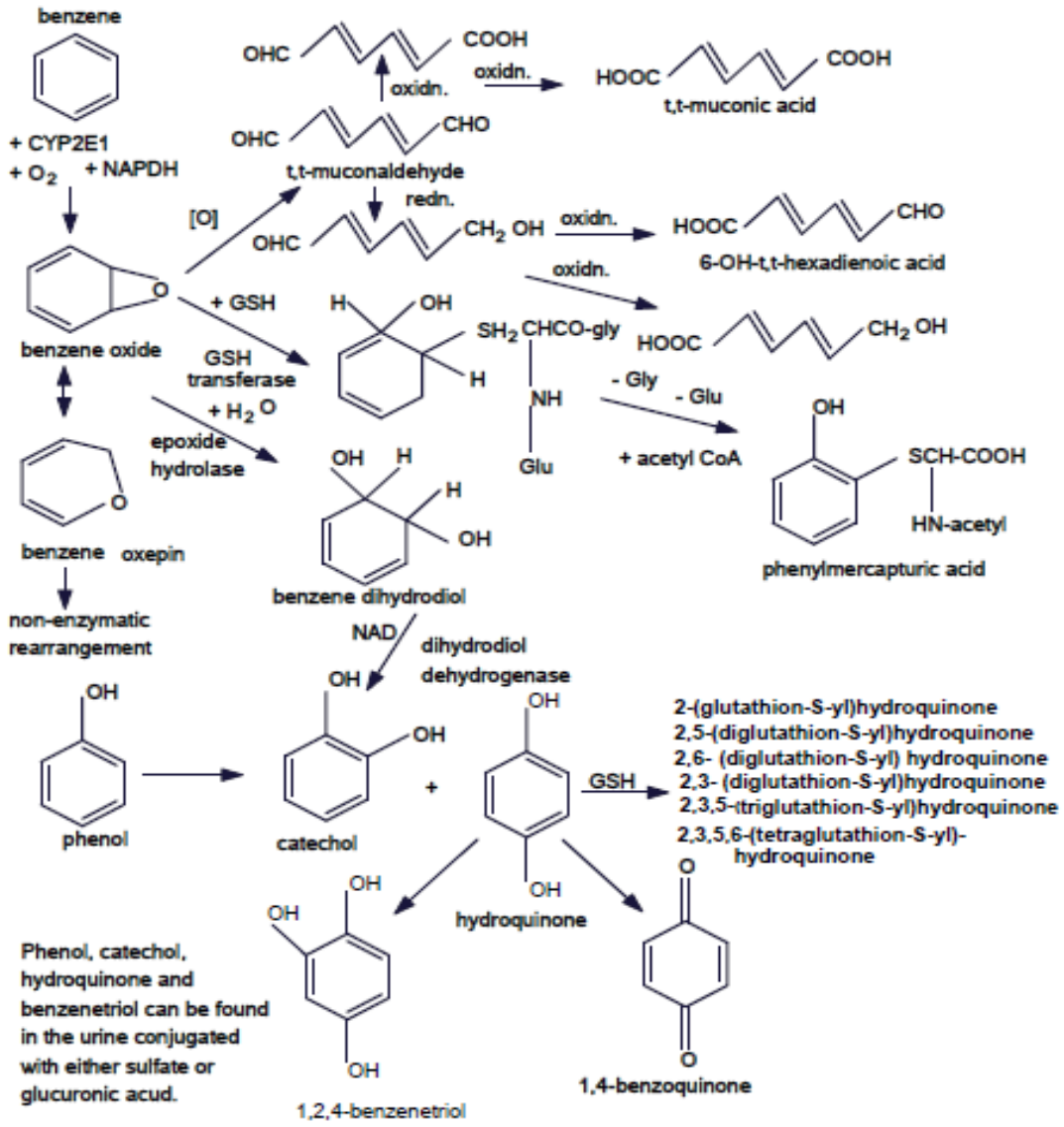

Figure 4: Adapted from (Snyder, 2012) Metabolic Pathway of benzene metabolism

This benzene oxide is further converted to a phenol, which further metabolized to 1,2,4-trihydroxy benzene, catechol and hydroquinone and after this, they are converted to more actively toxic form. Oxidation of hydroquinone leads to the production of 1,4 benzoquinone. Peroxidase hydrolase enzyme can also transform benzene oxide to Dihydrodiol benzene. Aldehyde metabolites are also formed due to the opening of the benzene ring. (Snyder, 2012)(Bette Meek & Klaunig, 2010) Phenylmercapturic acid is also excreted when glutathione conjugate with benzene oxide. Conjugates of hydroquinone and glutathione are supposed to be the most active, biologically. These conjugates produce ROS. 1,4 benzoquinone and aldehyde metabolite i.e. *tt*-muconaldehyde inhibit erythropoiesis (Snyder, 2012).

After the metabolism of benzene, its metabolites react with precursor stem cells present in the bone marrow. After their reaction, it may lead to direct or indirect mutations. Direct mutations are caused when benzene metabolites interfere with the genome of an organism and mutate DNA. Initiation of DNA damage by oxidative stress, changes in topoisomerase II interruptions in DNA repair system and disruptions in the normal functioning of protein at cellular levels are characterized as indirect mutations. After these mechanisms of mutations either direct or indirect, now the cells are being mutated and get proliferated. While proliferation to other sites, they disturb the functionality of the normal cell, as well as a normal apoptotic pathway required to kill the mutated cell, is also compromised that leads to an overgrowth of mutated cells. Methylation of bone marrow precursor cells by benzene cause changes in differentiation state of stem and progenitor cells. After methylation, additional chromosomal aberrations are needed to convert large mutated cells into neoplasm (Bette Meek & Klaunig, 2010).

1.14 The mechanism behind benzene-induced leukemia

Gene or maybe a set of many genes gets mutated by reactive benzene metabolites and causes the initiation of leukemia. Theses mutated genes most possibly are related to differentiation state and cell proliferation of Stem cells of humans. Genetic mutations cause aberrations in chromosomes due to deletions, inversions, translocation, and aneuploidy. Abnormal mitotic recombinations, alterations in epigenetics or abnormalities in genes are in the result of mutations at the genetic level. Prolonged exposure to benzene can induce more active aberrations at the genomic level. HSC remained in quiescence before mutations, but after they get mutated they entered into a cycle. The generation of leukemic stem cells started when aryl hydrocarbon receptor become

active after exposure to benzene. Impaired immunosurveillance allows leukemic cells to escape from the defense system of the body. Cell signaling pathways involved in hematopoiesis also get altered due to the toxicity of benzene causing induction of leukemia through benzene (Smith, Zhang, McHale, Skibola, & Rappaport, 2011).

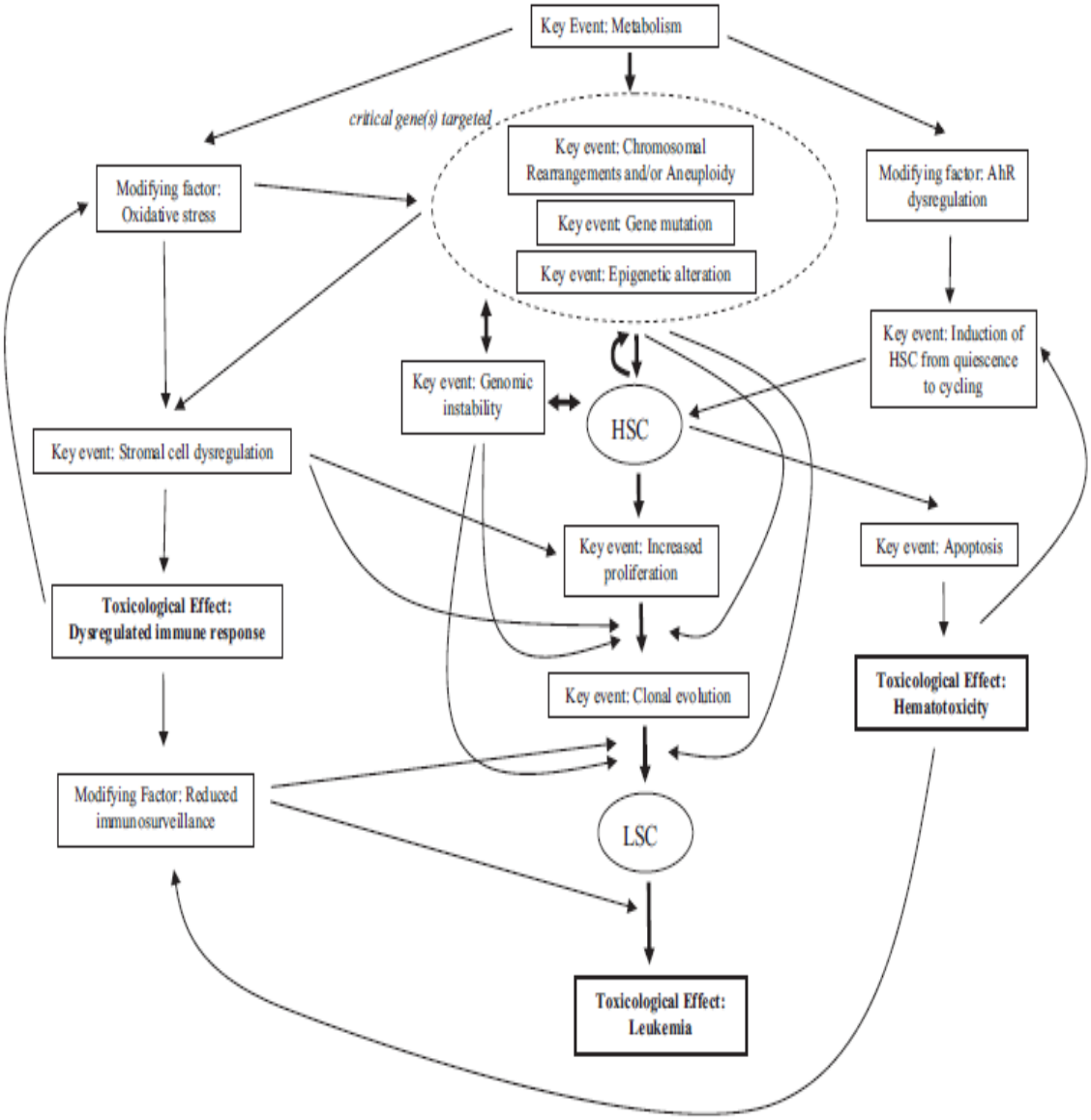

Figure 5: Adapted from (Smith et al., 2011) Pathways involved in the induction of leukemia through benzene

1.15 Signal transduction pathway in LSC and HSC

Translocation in chromosomes of BCR-ABL, AML1-ETO or modifications in tyrosine kinase receptors i.e. cKIT and FL3 and in RAS proceeds to the origination of leukemia cells. Downstream signal transduction follows in mostly leukemias (Seke Etet, Vecchio, & Nwabo Kamdje, 2012).

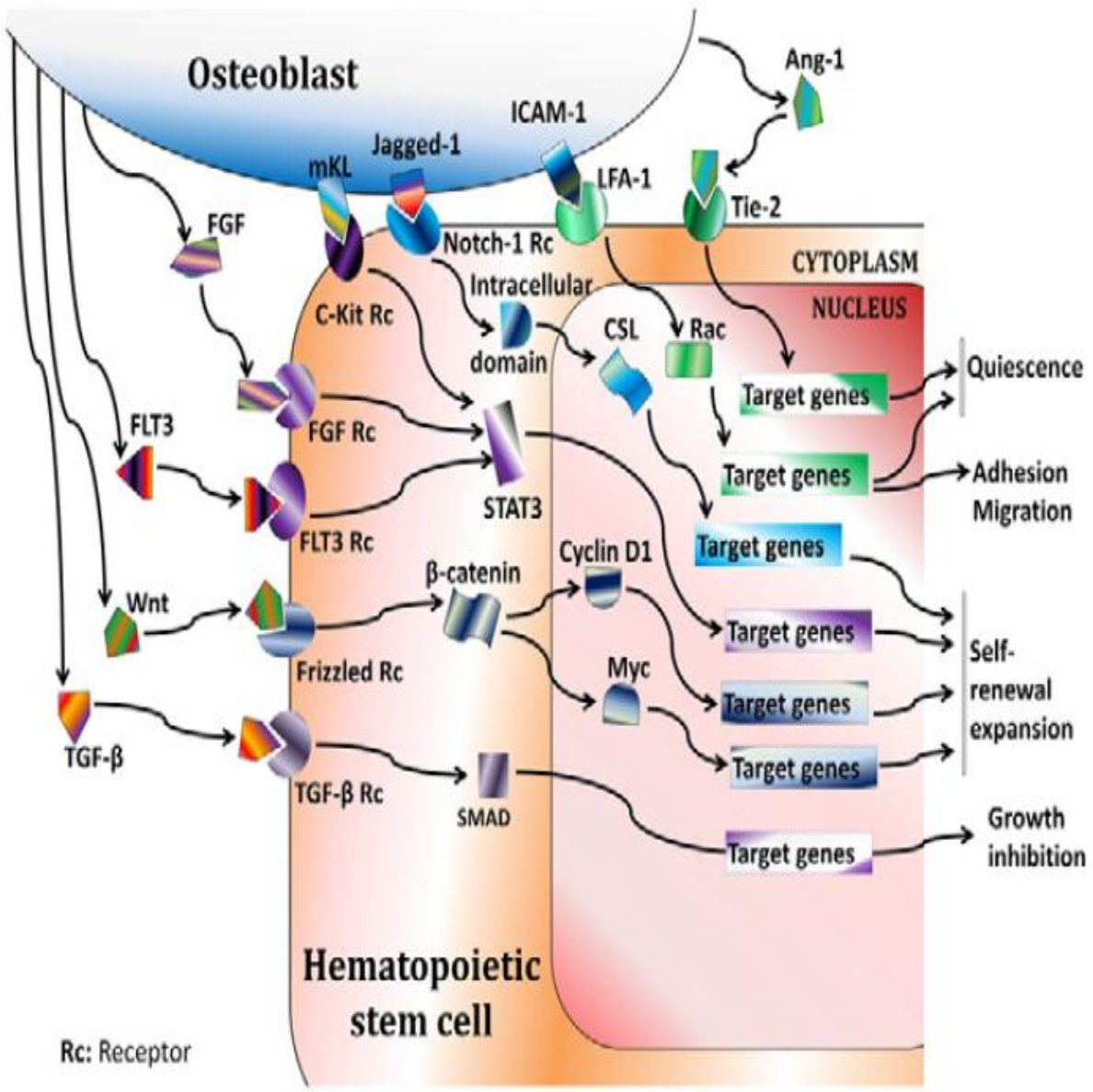

Figure 6: (Seke Etet et al., 2012), Graphical representation of normal signaling pathway in HSC.

Downstream targets are an abnormal trigger of NF-kB by RAS. BCR-ABL onco-gene stops apoptosis of leukemic cells, the action of its downstream target NF-kB. Along the BCR-ABL pathway, various other pathways have also been reported which are RAS, PI3-K/Akt, c-MYC and STAT5 pathways. Cytoskeletal proteins i.e. F-actin and proteins for cell adhesion i.e. FAK, vinculin, and paxilin. Activation of Rho GTPases due to BCR-ABL followed association ECM proteins and HSCs. JAK/STAT5 is also a downstream activation triggered by BCR-ABL (Seke Etet et al., 2012).

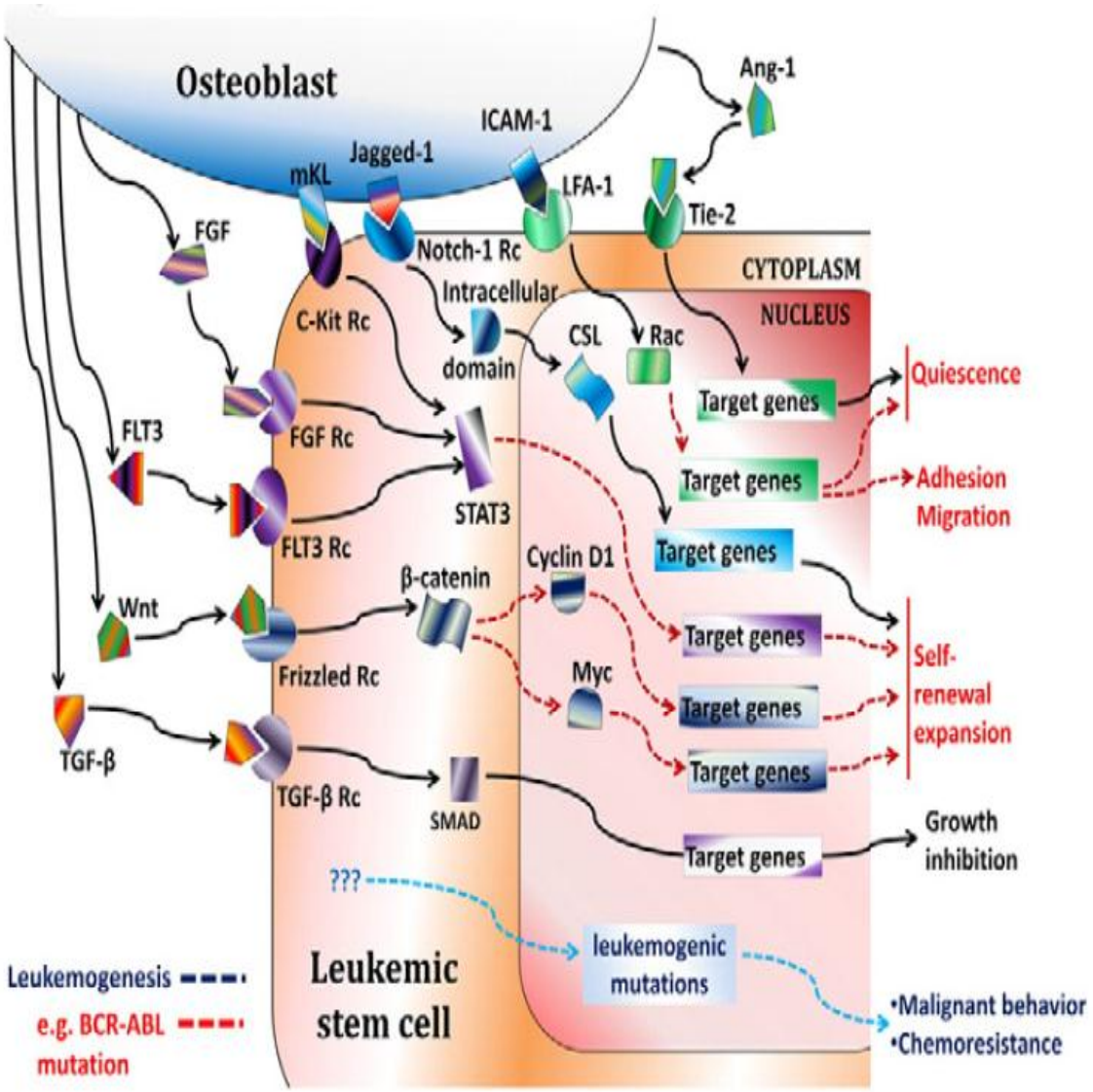

Figure 7: (Seke Etet et al., 2012), Graphical representation of leukemic stem cell signaling pathway

1.16 Problem Statement

Consequences of benzene induced leukemia at very low concentrations and to analyze the anti-leukemic effect of *Crithmum maritimum L.* against benzene induced leukemia**.**

1.17 Aim of the Study

The aim of this study was to:

- Check the efficacy of *Crithmum maritimum L.* against benzene induced leukemia in Balb/c mice.
- Identify *Crithmum maritimum L.* as a novel treatment for benzene induced leukemia.
- Comparative analysis of *Crithmum maritimum L.* with *the* commercially available drug for the treatment of leukemia i.e. Cyclophosphamide.
- Check the effectiveness of herbal therapy with relevance to chemotherapy.

2 Chapter 2 Literature Review

2.1 Benzene metabolism and toxicity in leukemia

Noxious effects of benzene promote desolation in bone marrow at the site of production of SCs, as well as promotes leukemogenesis. Results of this toxicity not only disturb the structures of Hematopoietic stem cells but also interferes in their functional properties that lead to an on-set of leukemia. Every single metabolite of benzene is provoked to toxicity, involved in the metabolic process of benzene. According to studies, it has been revealed that every by-product of benzene is responsible for the initiation of toxicity. The primary site of the metabolic reaction of benzene is the liver. At the liver, benzene is transformed into the opened-ring structure and other products which are hydroxylated form of benzene. After this primary metabolism in the liver happens, the by-products then move towards bone marrow and phase of secondary metabolism starts in here. Primary and secondary metabolism follows damage to cellular macromolecules and that damage assist in creating oxidative stress at the cellular level. Every single metabolite or by-product after metabolism of benzene follows up the activation of multiple biological targets, which is not clear yet that which metabolite has which target for toxicity. Ongoing studies will reveal biological targets for toxicity by benzene by-products (Snyder & Hedli, 1996).

Benzene has been known as a carcinogen for inducing toxicity in the hematopoietic system. Methylation of the PTEN promoter is a typical example of the silencing of tumor suppressor genes transcriptionally. PTEN methylation and benzene have a dose-dependent relationship between them. The expression of methylated PTEN mRNA decreases with an increase in the dose of benzene and vice versa. Hematotoxicity caused by benzene is followed up by an increase in methylation of PTEN mRNA. Toxicity due to benzene causes methylation of PTEN mRNA, which is a response mechanism behind hematotoxicity (Yang et al., 2014).

Suppression of myeloid stem cells and blood cells at the chromosomal level leads to the onset of AML and toxicity at the pluripotent stem cell level and at progenitor origination site. Cells exposed to benzene undergone aneuploidies. Monosomy at chromosomes number 7 and trisomy at 8^{th} chromosomes has been detected in benzene exposed cells. Monosomies at chromosomes 7 and 8 have been observed instead of trisomy. Benzene worked in a dose-dependent manner in here. The dose of less than 10 ppm happens to follow more monosomy than greater than 10 ppm.

These monosomies can work as biomarkers for toxicity induced by benzene and for various other leukemogenesis (L. Zhang et al., 2012).

2.2 Leukemia and signaling cascades

The homeostatic environment is important and necessary for HSCs for balancing in their regeneration process by themselves and for their modifications to other cell types. Homeostasis is also important for their response to injuries and toxicities caused by foreign particles. Production at large scale of blood cells is sustainable when the destination and counts of progenitors evolved from HSCs is balanced and maintained well. Wnt signaling plays an important role in maintain homeostasis of HSCs and progenitors because they reside in niches.

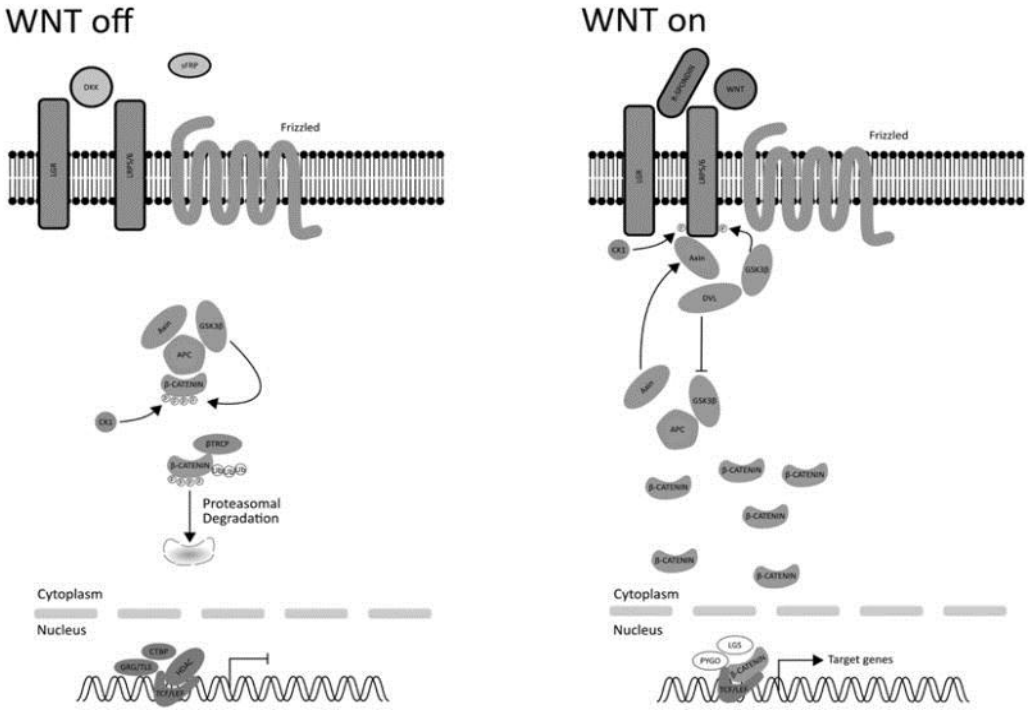

Figure 8: Adapted from (Luis, Ichii, Brugman, Kincade, & Staal, 2012), Wnt signaling cascade

Wnt proteins and its signaling pathway are responsible for causing many leukemias. Wnt pathway is also involved in the homeostasis of HSCs according to studies. The fate of the Wnt pathway is totally dependent on gradients present in Wnt signaling. When Wnt is off, the production of b-catenin is maintained by its degradation by proteasomes. There is the presence of a destruction complex, that includes proteins for degradation of b-catenin as well as scaffolding proteins axin 1 and 2 and APC, a tumor suppressor protein is there for the destruction of b-

catenin. On the other hand, when Wnt is on and binds with the frizzled receptor and Lrp5 and Lrp 6, it will initiate the other cascade. This complex disables the marked sites at b-catenin for destruction and makes it stable. B-catenin in its dephosphorylated form is moved to the nucleus where it binds with transcript factor Lef/tcf and I this complex leads to Wnt genes activations. Overexpression of b-catenin and mutations in them causes cancer at the HSC niche (Luis, Ichii, Brugman, Kincade, & Staal, 2012).

CML is caused by malignancy in Hematopoietic stem cells and they are converted to leukemic stem cells. Imatinib is an anti-cancer drug used against the treatment of CML. When patients treated with imatinib, they left with CML because of dormant leukemic cell and they become chemo resistive. Many pathways responsible for leukemogenesis and chemoresistance i.e. wnt, notch, hedgehog, BMI-1, PTEN, Alox5, FoxO, etc (Seke Etet et al., 2012).

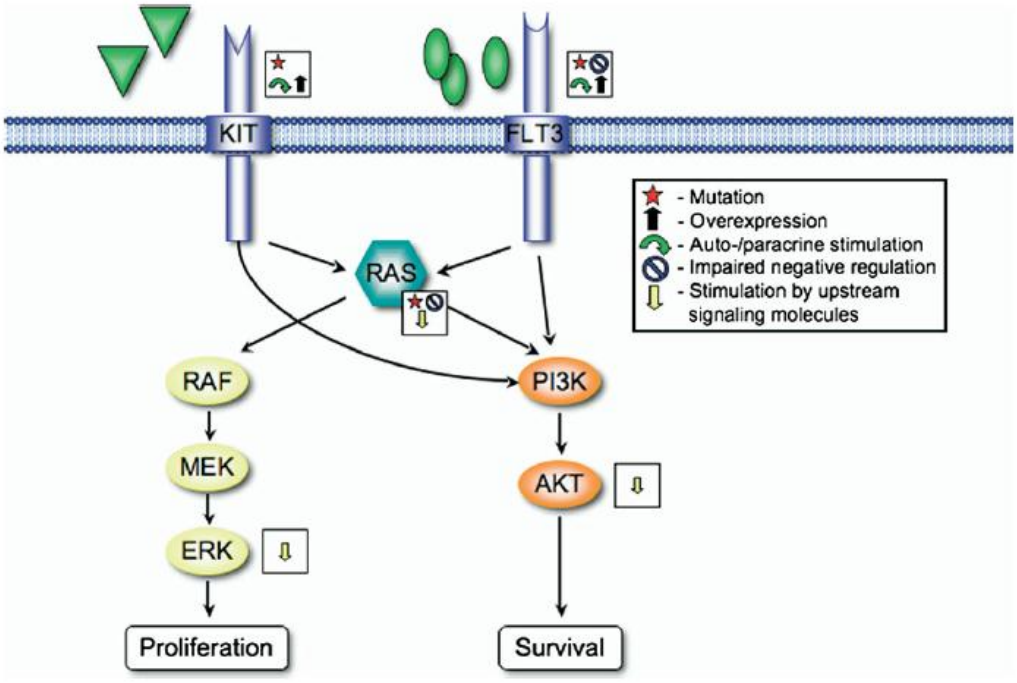

Figure 9: Adapted from (Scholl, Gilliland, & Fröhling, 2008), Abnormal Signaling Cascade in AML

In AML, abnormal transduction of signals increases the rate of survival and increased proliferation of mutated progenitors of stem cells. For therapy of leukemia, this downstream target should also be marked for modifications and terminations. More intense genetic studies such as the sequencing of DNA, analyzing copies of DNA, and profiling of transcriptomes are

very important for the analysis of downstream activated target to study leukemogenesis. The study of pathogenesis happening in leukemia can be used for therapeutics (Scholl, Gilliland, & Fröhling, 2008).

2.3 Chemotherapy for various types of leukemia

Abnormalities in Topoisomerase II provoke leukemia. For the treatment of leukemia, targeting the topoisomerase II by poisoning it with various drugs is an effective therapy. 6 antineoplastic agents i.e. teniposide, idarubicin, etoposide, doxorubicin, mitoxantrone, and doxorubicin. These drugs are approved to be used for the treatment of leukemia in the U.S. Topoisomerase inhibitors are the most effective agents against neoplastic agents in leukemia. But these inhibitors have some severe toxicities like loss of hair, myelosuppression, risk of developing non-lymphocytic leukemia, infections and sometimes heart failure. Some cancers who have fewer concentrations of topoisomerase are resistant to inhibitors of topoisomerase. Alterations at binding sites topoisomerase also produce resistant leukemic cells. If neoplasm is MPR or MDR positive then it will show less effectiveness to topoisomerase inhibitors (Hande, 1998).

Calixarene is the macromolecule of the third generation with supramolecular geometry and having host-guest chemistry.

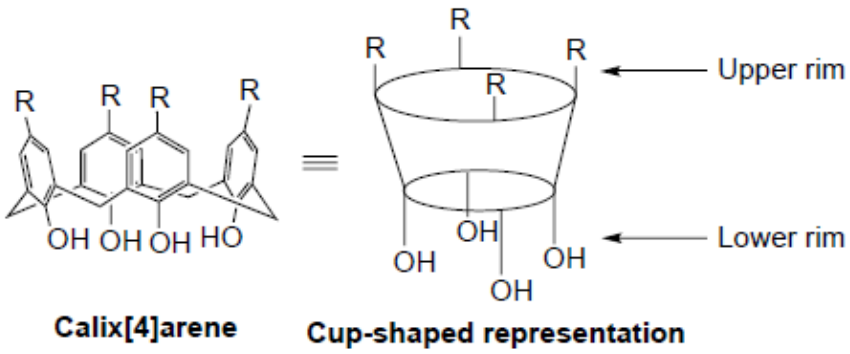

Figure 10: Adapted from (Abd Hamid, M Bunnori, Adekunle, & Ali, 2015), Representation of Geometry and Structural formula of calixarene

Calixarenes works as an anticancer by inhibition of enzymes by inhibiting the activity of some phosphatases. It also stops angiogenesis to tumor cells by inhibiting PGDF receptor phosphorylation, which is involved in angiogenesis. By suppressing oncogenes through calixarene, it reacts as an anti-cancerous agent. This agent also treats cancer, by up-regulation of

tumor-suppressing genes. They also worked as drug carriers or they have the capacity to mutualize or let other drugs to penetrate within, so they work in mutualism with other anti-cancerous drugs and make them stable at the site of action. When anti-cancer drug penetrated into calixarene, after reaching the site of action, it needs to be released. The mechanism of its release is totally based on the pH of the tumor cell. Tumor cells have 5.7-7.8 pH, whereas the pH of normal cells is 7.4. So, for sustained release of the drug, pH is an important factor and is the most common method for release of the drug. Caliaxerens have also been explored as photosensitizers for treatment of leukemia, as it remains within the cell and kills it by various method or destroy blood vessels that supply for to the tumor cell, thus killing the cell by starving the cell (Abd Hamid, M Bunnori, Adekunle, & Ali, 2015).

Abnormalities in signaling cascades lead to the formation of malignancies. P13K, AKT, and mTOR pathways are involved in the various cellular process like the translation of messager RNA, Succession of the cell cycle, suppression of autophagy and apoptosis, and transcription of genes. Inherent initiation of this cascade not only proliferate malignant cells but also develops chemoresistance in leukemia. mTOR has two complexes mTOR1, mTOR2. P13K cellular pathway is initiated, when various growth factors attached and phosphorylate the surface of the receptor at the attachment site like FLT3-L or IGF attaches. Cytokines are also involved in this activation. These pathways are multi-step and with the presence of a negative feedback loop due to which chemosensitivity exists. Leukemia ad various other cancers have multiple stimuli involved in the oncogenesis of leukemia. An agent is needed who works against this type of oncogenesis (Dinner & Platanias, 2016).

Resistant of leukemia cells against conventional chemotherapy is the major issue that initiates the disease again, and then ultimately death of the patient. Recently it has been discovered that DNTs has the capability to target blast cell in leukemia. Observations have been made on patient-derived models of leukemia as well as in vitro. But some leukemic cells are even resistant to DNT therapy initially. Daunorubicin has been used along with DNTs against KG1a, stem cells like cell lines of AML and this combination allow the cytotoxic activity of DNT to work (Chen, Lee, Kang, Minden, & Zhang, 2018).

2.4 Ethnomedicine for leukemia

The use of plants as ethnomedicine is known to form hundreds of years. For various medicinal purposes, 350,000 plants have been used so far. WHO has confirmed that 80% of the people especially from rural areas are using medicinal plants. The reason behind their frequent use, is they are more effective, they have less toxicity as compared to chemo, they have fewer side effects than synthetic compounds and also due to advancements in science and technology. Various anticancerous drugs used in chemotherapy are extruded from these naturally occurring compounds and plants like paclitaxel, vincristine, etoposide, topotecan, vinblastine, etc. Various plants have various phytochemicals for the cure of disease (Kong, Goh, Chia, & Chia, 2003).

Over 90,000 terrestrial and marine has been tested on cell lines of leukemia by NCI. Many chemotherapeutic agents used in the treatment of cancer are originated from plants e.g vinca alkaloid has been evolved from periwinkle Madagascar plant. Pacific yew plant has given paclitaxel and many more. Murine and wild plants have been tested. Families like Chrysobalanaceae, Fabaceae, Fagaceae, Aquifoliaceae, and marine plant extract Porifera has been reported selectively for the treatment of leukemia (Cragg, Newman, & Yang, 2006).

Many medicinal plants compounds could not be synthesized manually because of the complex phytochemical structures present in plants used as medicine. Extract of *Moringa oleifera* has been tested against ALL and AML. This extract induced death to leukemic cells 70-80% of the cells of AML and ALL. The antioxidant effect of M. oleifera depends on dose-dependent. Flavonoids and phenols are responsible or this radical scanning activity. *Allamanda catharica* has also been reported for the treatment of p338 leukemia in mice. Allamadin derivatives are involved in killing leukemia cells. The extract of the plants was prepared in hot water and ethanol fractions (Khalafalla et al., 2010).

The alcoholic extract of the *Anona glabra* has been prepared and for validation of its anti-cancerous effects against leukemia. The cytotoxic activity of this alcoholic extract is not against normal lymphocytes of humans. Extract of *Annona glabra* was highly toxic against leukemic cell lines who are drug-resistant and have strong resistance from multiple drugs. Cytotoxic values has been tested against Adriamycin. The cytotoxic values of this extract have been less than Adriamycin. The most potent site for cytotoxic activity of this plant is seeds. This extract work in a concentration-dependent mechanism. Sensitive leukemic cells and resistant cells undergo necrosis and apoptosis after exposure with seed extract of this plant. Up-regulation of WAF1 and

p21 arrests the cell cycle at the G1 and G0 phase to induce apoptosis in leukemia cells. *A. glabra* belongs to the family of Annonaceae (Cochrane, Nair, Melnick, Resek, & Ramachandran, 2008). B-CLL still needs more innovative and advance treatment modules. A naturally occurring product Honokiol is potent against neoplasm and angiogenesis. B-CLL tumor cells derived from the patients of B-CLL and they have been checked by honokiol because they possess resistance against apoptosis. Honokiol worked against resistant tumor cells of B-CLL by caspase-dependent apoptosis cascade. Honokiol didn't affect the normal mononuclear cells. Apoptosis followed by honokiol initiate cleavage of PARP and also activated caspases 3,8 and 9. The mechanism of honokiol is the up-regulation of BAX a protein associated with BcL2 and down-regulate the expression of a survival protein i.e. Mcl-1. Interleukin 4 has also been used for treatment in B-CLL and make them resistant against treatment. But when honokiol is used with IL-4, honokiol inhibited the effect of IL-4 by apoptosis. Cytotoxicity of other chemotherapeutic agents like chlorambucil, fludarabine**,** or cladribine has been enhanced when used with honokiol (Frank, Battle, & Arbiser, 2005).

The activity of plant extract *Centaurea albonitens* has been tested against various types of leukemic cell lines also tested in mutualism with vincristine. This plant extract has been used traditionally and is the latest one. The efficiency of this extract has been tested and it has been discovered that it arrest cell cycle at phase G1/G0. Dosage plays an important role in the cytotoxic effect of this plant extract. The anti-cancerous activity of vincristine has been increased when low doses of *Centaurea albonitens* has been given against B-ALL cell lines. Vincristine and *Centaurea albonitens extract* worked in a synergistic manner. As well as used to enhance the activity of chemotherapy agents (Bahmani et al., 2018).

Leukemic stem cells have not been targeted by conventional chemotherapy. Faulted initiation of mediators like osteopontin and other signaling cascades form leukemia. Curcumin has anti-leukemic effects against leukemia by inhibiting their growth and by activation of the apoptotic pathway leading to the death of leukemic cells. Curcumin has been used in comparison with Daunorubicin and its effect has been checked against bone marrow cells. Cytotoxic effect of curcumin has been achieved by inhibition of OPN with small interfering RNA. With an increase in expression on OPN mRNA, there is the possibility of increased chemosensitivity (ZahedPanah et al., 2017).

2.5 Animal models of benzene induced leukemia

A combination of benzene and formaldehyde has been used to check the toxic effect on the spleen and to check the defensive response of the body against these pollutants by using mouse mode using Balb/c strain. The dosage used for FA was 3 mg/m^3 for two weeks for 8 hours per day. Benzene was also given with a dosage of 150mg per KG by weight per day. Benzene was dissolved in corn oil. There were 5 groups for this experimentation. Control group, Corn Oil group, BZ group, FA group, and BZ+FA combined group. FA was given as an inhalant whereas benzene was given intragastrically. There are some assays and parameters to check the effect of FA and Benzene toxicity. Weight analysis, Complete blood count Cellularity of Bone marrow, reactive oxygen species, western blot has been performed to evaluate the effect of toxicity of benzene and formaldehyde (Wei et al., 2017).

Long term exposure to benzene at elevated levels of concentrations can impair the human defense system. Benzene has been given at various concentrations to check the effect of its metabolism and toxicity in the bone marrow. Dosage of different concentrations such as 1 ppm, 5 ppm, 10 ppm, 100 ppm, and 200 ppm of benzene has been checked on B6C3Fl/CrlBR mice. 82-86 days old mice used for this experimentation. The mode of administration used was through gavage. The exposure of benzene was for 8 weeks. High concentrations had a more tragic effect on B and T Cells' lymphatic system whereas fewer concentrations had fewer harmful effects. Apoptosis caused by benzene in lymphocytes was more in 100 and 200 ppm conc. As compared to the control group and groups with fewer concentrations. Counting of nucleated cells, immunostaining, flow cytometry using Annexin V staining, lymphocytes count in the spleen, and the apoptotic assay was used to check the apoptotic mechanism (Farris et al., 1997).

To discover the toxicity of benzene in the hematology system, a low conc. of 20mg per cubic meter had tested on Balb/c mice. Benzene had been given for three months and after that mice kept alive for ninety days to check the long term effects of benzene. During experimentation, the frequency of micronucleus of reticulocytes in peripheral blood, comet assay, analysis of peripheral blood and bone marrow examined. Even after treatment of 90 days, benzene exposed groups had more DNA strand breaks and a number of micronuclei. The myelodysplastic syndrome gets observed in the bone marrow (J. Zhang, Liang, Fan, Yin, & Pu, 2010).

Benzene induced genotoxic effects on exposure in Balb/c male mice. Cimetidine has been used as treatment therapy not even against benzene-induced leukemia but also against clastogenic

effects of radiotherapy. Eight-week-old mice of weight 22-24 grams of balb/c strain were used. Benznen with dosage of 200, 400 and 600 mg per kg was given to the mice and sacrificed after 24, 48 and 72 hours. Before giving a dose of the benzene treatment dose of 10, 15 and 3o mg per kg was also given before 1 or 2 hours of benzene administration, Benznen was given as an i.p injection. Mie was sacrificed by cervical dislocation. The detection of micronuclei by micronuclei assay had been used to check the effects (Mozdarani & Kamali, 1998).

2.6 Crithmum maritimum L.

Lipid extract of *Crithmum maritimum* leads to the extraction of three compounds and they are bioactive in nature. The first one is C17 polyacetylene metabolites, Second is falcarinol and falcarindiol and the third one is O-geranylvanillin they were isolated. brine shrimp assay for lethality was used to isolate these compounds. First, two compounds were more cytotoxic than the third one. This plant has been used for many purposes like use in culinary, it works well for digestion and urine issues, also used as diuretics (Cunsolo, Ruberto, Amico, & Plattelli, 1993).

Falcarindiol was isolated from the chloroform extract by the bioassay-guided fractionation process. Apiaceae family mostly have the falcarinidol, which is a polyacetylene compound. Microplate bio-assay was used to determine the activity of this compound and its structure was investigated by using the NMR technique. The antibacterial activity was investigated against M. luteus and B. cereus with MIC of 50 micro-gram pr ml. The cytotoxic effect of this extract was also been tested on IEC-6 cells and they gave IC_{50} value of 20 micro molars after 48 hours of exposure. Various other phytochemicals present in it determine it that it can be used as in various food products, in pesticides and also works an antibiotic (Meot-Duros et al., 2010).

Aerial parts of *Crithimum maritimum L.* have been used for the extraction of essential oils. 0.17% and 0.19% yield was obtained from hydrodistillation. Samples were taken from Antalya and Mersin. Analysis of samples has been carried out by Gas chromatography and mass spectrometry. 19 and 21 compounds were recognized from the essential oils. The percentage of monoterpenes was 64% in both samples and phenols with a percentage of 30 and 28 were present. The anti-bacterial activity has been checked and it is considered to be used in antibiotics. The essential oil has been tested against 8 randomly selected bacterias and found to be active mostly against gram-positive bacteria (Senatore, Napolitano, & Ozcan, 2000).

Crithmum maritimum has been tested for the phytochemical present in it and also for the biological compounds present in it. Stem, leaves, and flowers were used for samples. EO . was prepared by hydro-distillation. The method of ethanolic extraction was used for non-volatiles. A dominant compound found in EO and ethanol extract were Limonene, with a percentage of 57-74% and in dry weight, it is 0.7-8.1 mg per gram. Antioxidant activity was determined by three methods which are Ferric Reducing/Antioxidant Power (FRAP) assay, Briggs–Rauscher (BR) method and DPPH. Crithmum maritimum has antioxidative activity, cholinesterase inhibiting activity, antimicrobial activity, and vasodilatory activity (Generalić Mekinić et al., 2016).

	Sample	FRAP (μmol Fe^{2+}/L)	DPPH (inhibition %)	BR (min)
Essential oils	*Flowers*	22.0 ± 2.3	2.8 ± 0.0	0.3 ± 0.1
	Stems	42.0 ± 2.3	2.8 ± 0.2	0.8 ± 0.0
	Leaves	8.4 ± 2.4	2.6 ± 0.2	0.6 ± 0.0
Ethanolic extracts	*Flowers*	16065.6 ± 95.3	61.0 ± 3.8	49.2 ± 0.1
	Stems	3009.4 ± 39.8	13.0 ± 0.7	1.7 ± 0.3
	Leaves	17335.0 ± 276.0	61.8 ± 3.8	68.0 ± 1.0
Referent compounds	*Chlorogenic acid*	370.2 ± 6.9	91.6 ± 0.7	114.3 ± 2.5
	Limonene	42.9 ± 2.8	2.7 ± 0.2	0.3 ± 0.0

Table 4: Adapted from (Generalić Mekinić et al., 2016), Values obtained from anti-oxidation assay by different methods of Eos and ethanolic extract

3 Chapter 3 Methodology

3.1 Materials

Following are the materials used:

- Plant extract of *Crithmum maritimum L.*
- Balb/c mice strain
- Disposable Insulin syringes 01 mL
- EDTA vials
- Benzene (RDH-Germany)
- Cyclophosphamide injection 50 mL
- 10% Formalin solution
- Xylazine
- Ketamine
- Phenol
- Dibasic and Tribasic salts
- Histology jars 30ml
- Face mask
- Nitrile examination glove
- Distilled water
- Dissection kits

3.2 Conditions for protocol

- 1 Week Acclimatization Period
- Temperature 25°C ± 2°C
- 12-hrs light-dark cycle
- Free access to food and water
- Woodchip Bedding (Changed after 2nd day)

3.3 Grouping for protocol

20 Balb/c mice 7-8 weeks old were bought from National Institute of Health Sciences, Islamabad. Following groups has been made:

Groups	**Size**	**Label**
Group: 1	5	Control
Group: 2	5	Benzene
Group: 3	5	Benzene + Cyclophosphamide
Group: 4	5	Benzene + Crithmum maritimum Phenolic Extract

3.4 Dosage

Following doses has been used in the protocol:

Groups	**Drug Dose**	**Duration**
Group 1	Control group	Saline 70mg/kg for 8 weeks
Group 2	Benzene = 70mg/kg Solvent: Olive Oil	For 3 Weeks, 5 Consecutive Days/week

Group 3	Cyclophosphamide = 30mg/kg Solvent: DMSO	For 4 Weeks, From 5th Week of Protocol, 1 Dose After 48 hours
Group 4	Crithmum maritimum P.E= 70mg/kg Solvent: DMSO	For 4 Weeks, From 5th Week of Protocol, 5 Consecutive Days/week

3.5 Drug administration route

Intra-peritoneal route of administration has been used for administration.

Figure 11: Intra-peritoneal position for injecting the drug to Balb/c mice

3.6 Protocol for plant extract preparation of *Crithmum maritimum L.*

The protocol used for extract preparation is as follows:

1. 5 g of dry powder of *Crithmum maritimum L*. is obtained.
2. 50 mL of pure phenol is mixed with dry powder.
3. Magnetic stirring is carried out by mixing both, for 24 hours in a magnetic stirrer.
4. Then, after 24 hours extract was kept to settle down for the next 24 hours at 4°C.

5. Whatman N°4 filter is used for the filtration of extract.
6. Purified extract kept into the oven for drying.
7. The extract was stored at 4°C until analysis.

3.7 Protocol

The protocol followed in the experiment includes the following steps and procedure:

3.7.1 Acclimatization period

Balb/c mice were divided into four groups and weighted after buying from NIH Islamabad and the acclimatization period was 1 week, given to them for adjustments to environmental conditions and temperature.

3.7.2 Development of leukemia mice model

Before starting the induction, mice were weighed again and their weight was noted. Carcinogen used for the development of mice model is Benzene bought from Sigma Aldrich Co. Benzene was dissolved in olive oil. 1% stock solution of benzene and olive oil was prepared (v/v). The dosage used for the development of the leukemia mice model is 70mg/kg. This group is also called a negative control group. Benzene has been given to 3 groups of mice consecutively for the first five days of 2^{nd}, 3^{rd} week and 4^{th} week. After the induction period, mice were weighed again and noted. For confirmation of the development of leukemia mice model, blood was collected from the tail vein of mice in EDTA vials for complete blood profiling for analysis of cell counts.

3.7.3 Collection of the blood sample

For the complete blood count test, blood has been collected from 3 mice of every group. For the collection of blood from the tail vein of mice, mice need to be anesthetized for a short period of time. Xylazine and ketamine are used in combination as anesthetics. A combination of Xylaz 30µl and Ketamax 1ml, dissolved in 9 ml of distilled water. There are certain methods that do not need anesthesia but tail vein method requires anesthesia because animals should not be stressed and methods used for blood sample collection should be least painful.

Following steps has been involved in the collection of a blood sample:

1) Mice were kept into the restrainer.
2) The temperature maintained was 24 to 27 °C. and mice were made comfortable.

3) For visibility of the tail vein, the tail was dipped into warm water.
4) A needle of 23 G was used for insertion into a vein and blood is collected in the syringe.

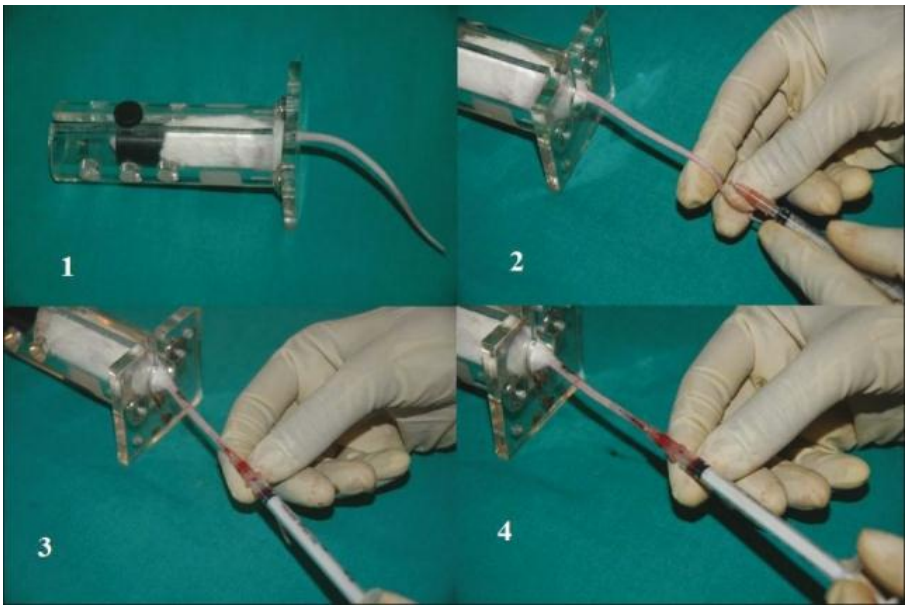

Figure 12: Adapted from (Parasuraman, Raveendran, & Kesavan, 2010), an illustration of collection of blood sample form tail of a mouse

3.7.4 Treatment of developed leukemic mice model

The next step is the treatment of developed leukemia mice model with prepared extract of *Chrithmum maritimum L.* 3rd group of mice is called an experimental group. *Chrithmum maritimum L.* plant extract was dissolved in 15% DMSO and the stock solution was prepared with a final concentration of 1%. 3rd group of mice was treated with the prepared extract with the dosage of 70mg/kg of *Crithmum maritimum L.* and treated for 4 weeks started from 5th week of protocol till 8th week. The dose was given for five days of a week whereas 6th and 7th day were rest days. The 4th group of mice was called a positive control group. Cyclophosphamide i.e. a chemo drug used for the treatment of blood cancer is used in comparison with plant extract, with the dosage of 30 mg/kg. 5% stock solution of cyclophosphamide was prepared and injected to mice. After treatment, mice were weighed again and noted.

3.7.5 Sacrification

After induction and treatment, mice were sacrificed using standard surgical procedure by abdominal incision.

3.7.6 Sample storage

After scarification, liver, spleen, and kidneys of 3 mice from every group were stored in a 10% formalin solution for histological analysis whereas organs of rest of 2 mice from each group were stored in cryopreservation at -70°C.

3.8 Techniques

Following are the techniques used for analysis:

1. Complete blood count
2. Histological analysis with H&E staining
3. Flow Cytometry

3.8.1 Complete blood count

Mice blood samples were collected and analyzed into an MCL-KT-6200 digital hemocytometer analyzer and results were obtained.

3.8.2 Histology with H&E staining

Slides of blood, liver, and spleen of H&E stain were prepared and organ embedded paraffin plates were obtained from Ali pathology lab, Islamabad. Slides were examined under a Labomed LB-200 Binocular Biological Microscope. Images were captured with magnification 40x, 100x and 400x by using Pixel Pro software for a Labomed biological microscope.

3.8.3 Flow cytometry

3.8.3.1 Standard Protocol for Separation of MnC

1) Collect around 3mL blood from the donor (after informed consent) in an EDTA tube.
2) Dilute the blood with PBS (pH=7.4) at a 1:1 ratio.
3) Take the equal volume of Lymphocyte Separation Medium (D = 1.077 g/ml, at +20°C) in Falcon tube. (For instance: for 6mL blood use 6mL medium in 15mL Falcon tube). Load the diluted blood on the surface of the medium slowly (avoid mixing of blood in medium).
4) Centrifuge the preparation (blood on medium as in step 3) at 1200g for 20 min at 20°C. (**Note:** Swing rotor centrifuge is recommended).
5) Collect the ring (mononuclear cells) at the interface of lymphocyte separation medium and plasma by a Pasteur pipette.
6) Wash (centrifuge) the collected cells twice with 10mL PBS (pH=7.4) 20°C at 300g.

7) Resuspend the pellet in 0.5mL PBS (pH=7.4).

3.8.3.2 Cell staining

1×10^6 cells were transferred to FACS tubes. 10ul Annexin V-FITC was added followed by 5ul addition of Propidium Iodide (PI) and vortexed. Cells were incubated at room temperature for 15 minutes. 400ul Annexin V binding buffer was added.

3.8.3.3 Flowcytometry

Stained cells were analyzed on BD FACscan (MAKE, USA). Annexin V-FITC stained cells were acquired on FL-1 and PI stained cells were acquired on FL-3.

3.9 Observations during protocol

Following observations were made during the protocol:

- Weight Loss
- Bruises & Scars
- Dizzy mice
- Swelling in Abdomen
- Loss of Appetite
- Pleural Effusion

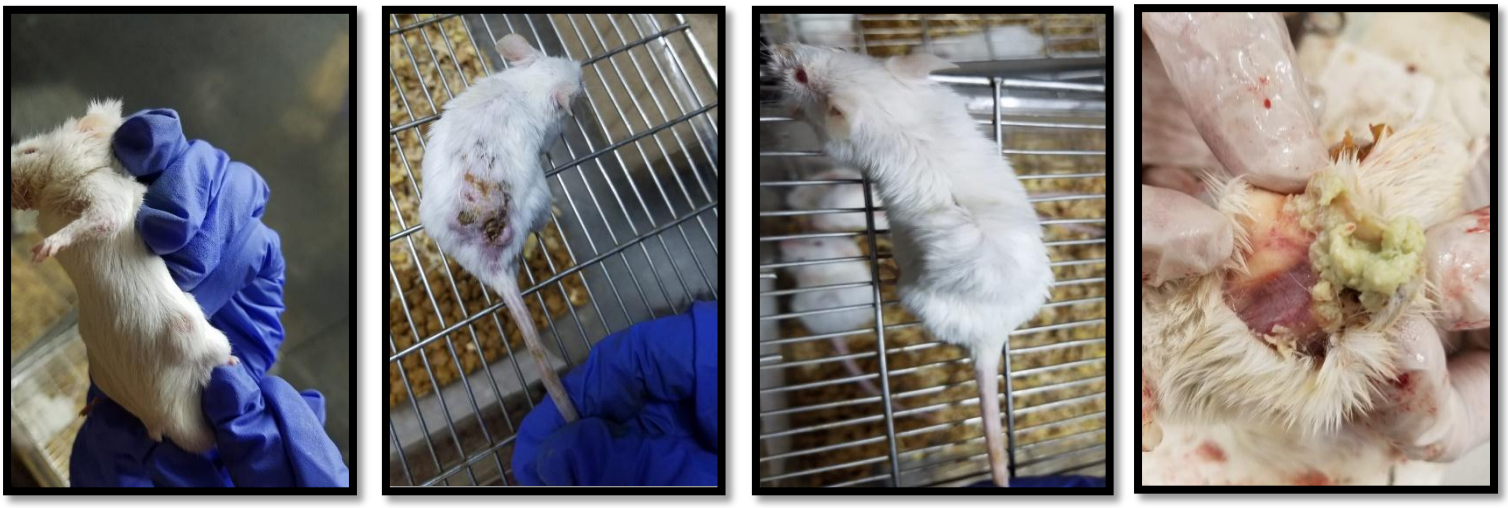

Figure 13: Physical condition of mice being observed after protocol

4 Chapeter 4 Results

4.1 Weight Analysis

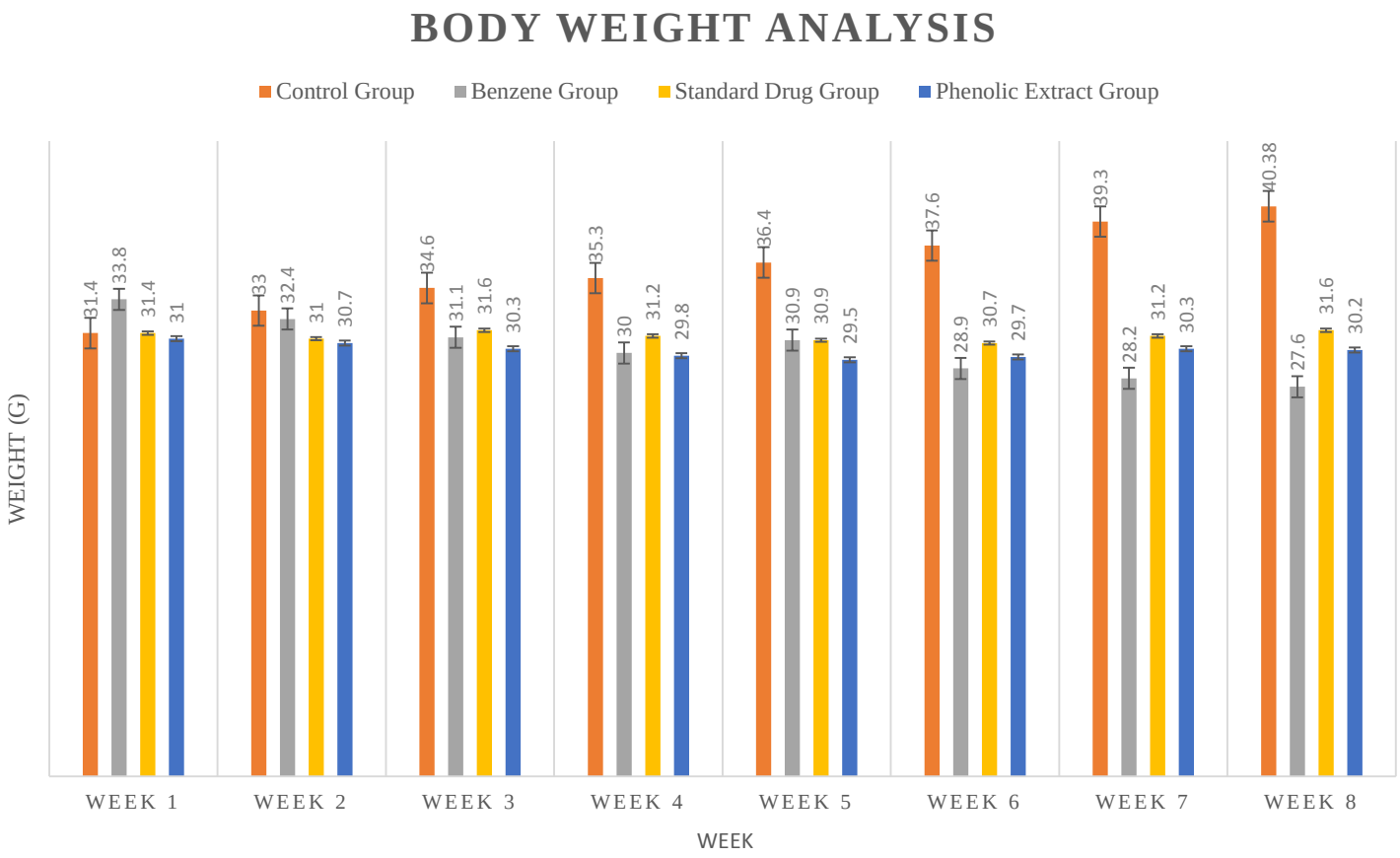

Figure 14: Weight analysis of Control Group, Benzene Group, Standard Drug i.e. Cyclophosphamide & Phenolic Extract Group i.e. PE of Crithmum maritimum

Weight of the control group has been increased with the passage of time. Weight of benzene treated group started decreasing from week 2 and it started decreasing gradually until week 8. The weight of the group to whom the standard drug has been given showed up and down patterns. With the administration of benzene, it has been decreasing till week 4, then after treatment, it has been decreasing gradually and after 6th week, the weight has been started increasing gradually. Weight of Crithmum maritimum PE treated group has been decreasing till a week and then slightly increasing in 3rd week of treatment.

4.2 Histopathology Results

4.3 Histopathology of Liver

4.3.1 Control group

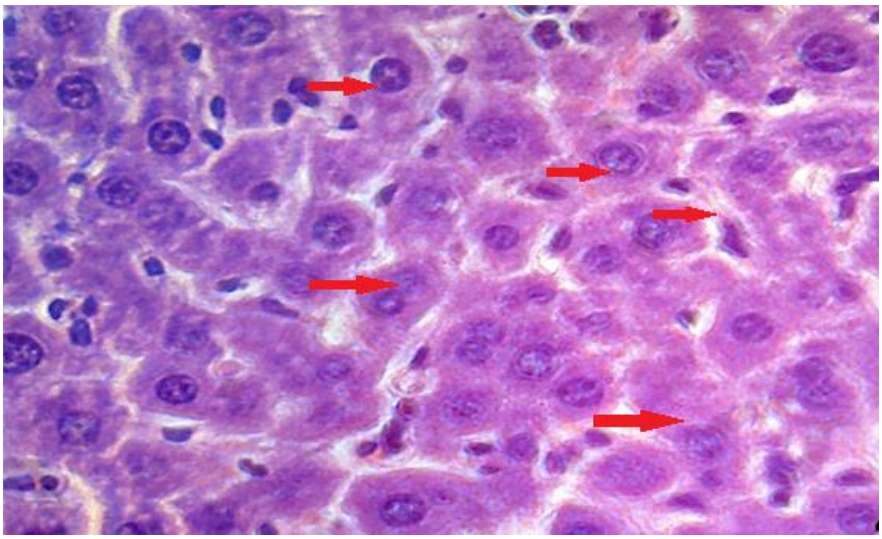

Figure 15: Histology of normal liver cells 400x

Hepatocytes are arranged in one cell layer thick plates separated by sinusoid, hepatocytes, Nucleus with multi nucleoli, Cytoplasmic glycogen is present.

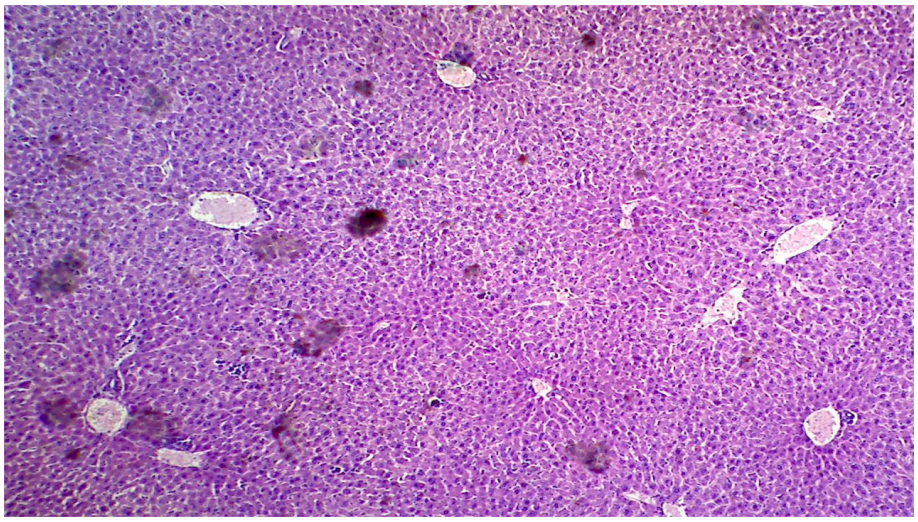

Figure 16: Histology of normal liver cells 400x

Liver from a normally fed animal, hepatocytes with 'feathery' cytoplasm containing glycogen.

4.3.2 Group treated with Benzene

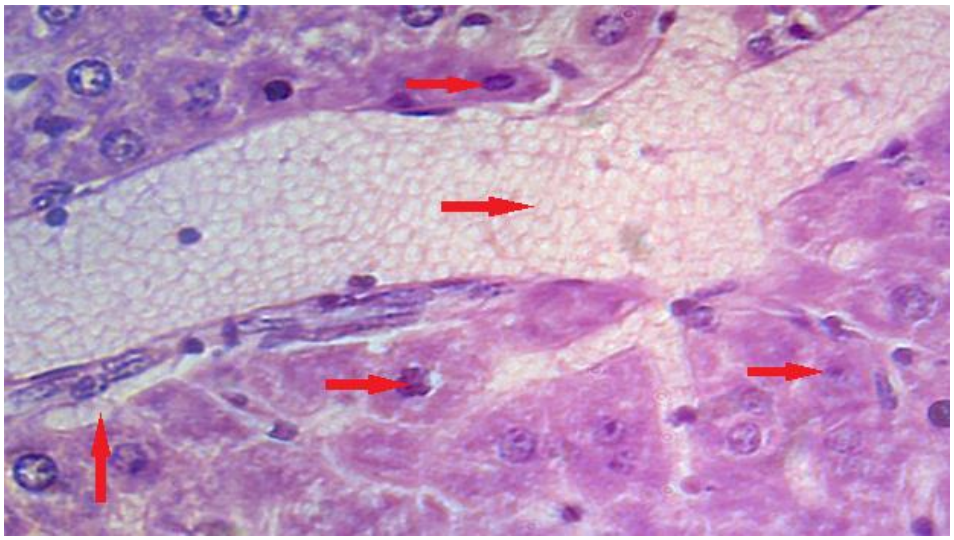

Figure 17: Histopathology of the liver treated with Benzene 400x

Apoptotic Cells and Necrosis, Primary sclerosing cholangitis, Extramedullary hematopoiesis is commonly seen in mouse livers and can include megakaryocytes, which should not be mistaken for polyploid or neoplastic cells.

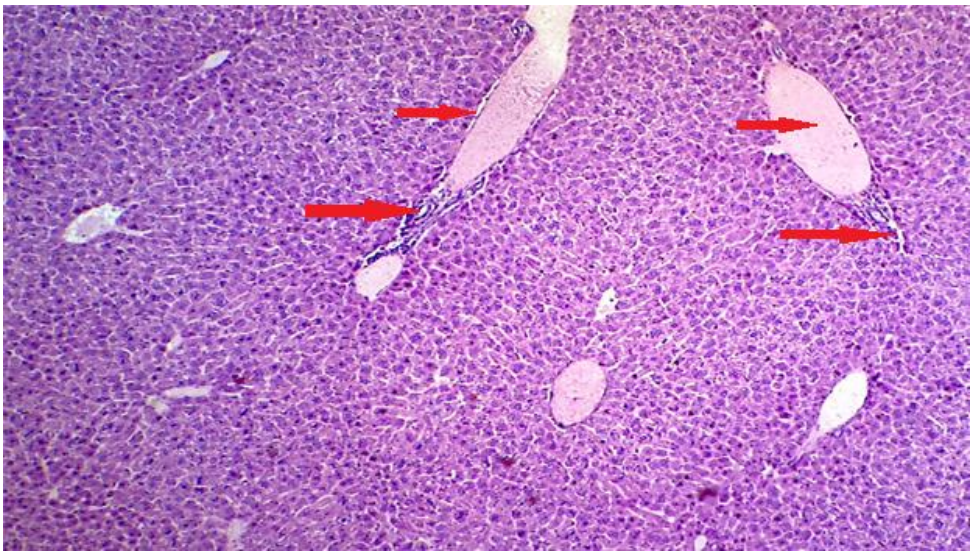

Figure 18: Histopathology of the liver treated with benzene 40x

Increased number of lymphocytes in portal inflammation, Massive hepatic necrosis.

4.3.3 Group treated with the standard drug: Cyclophosphamide

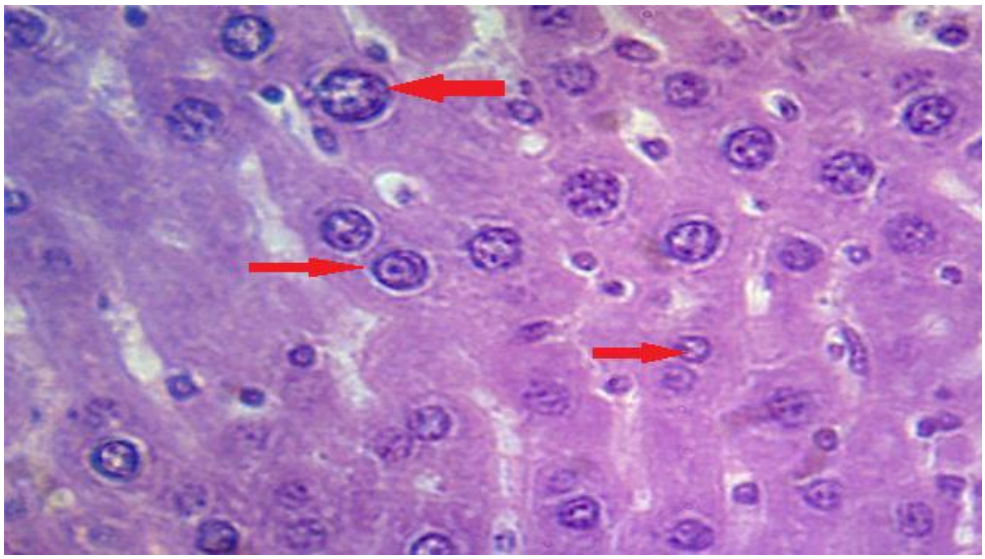

Figure 19: Histopathology of the group treated with standard drug 400x

Most of the cell is normal hepatocytes, Variable sized nuclei as seen in this section and binucleate nuclei (thick arrow) are common and increase with age in mice, liver enlargement

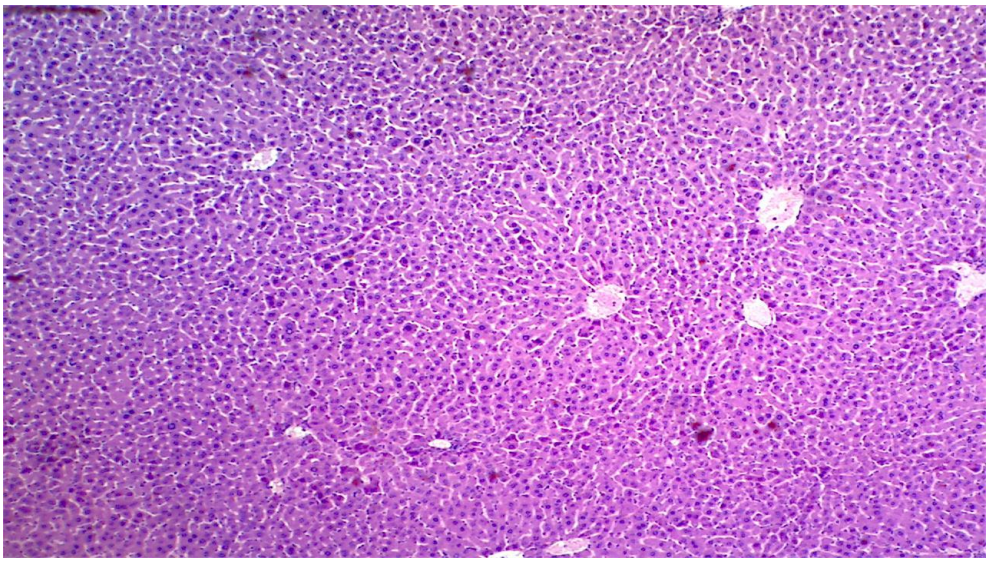

Figure 20: Histopathology of the group with standard drug 40x

Hepatocytes are normal in their morphology.

4.3.4 Group treated with phenolic extract of Crithmum maritimum L.

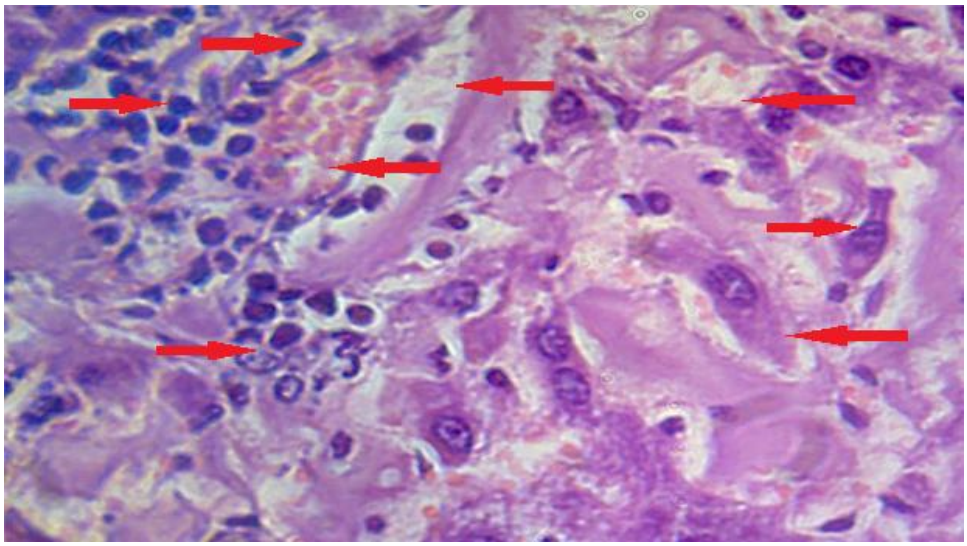

Figure 21: Histopathology of the group treated with P.E of Crithmum maritimum 400x

Hematopoiesis predominantly dark staining erythrocyte precursors, Lymphocytes, Necrosis, Glycogen depletion, Apoptosis.

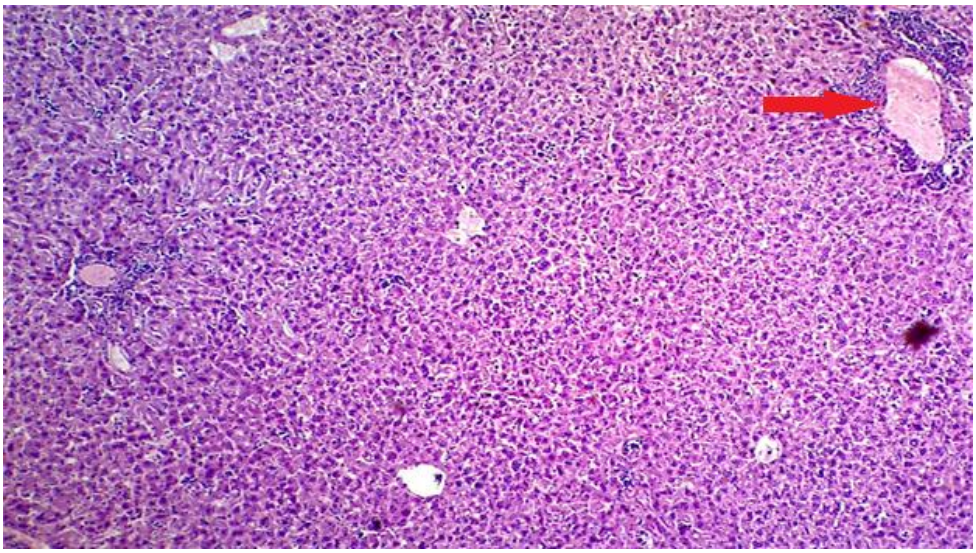

Figure 22: Histopathology of the group treated with P.E of Crithmum maritimum 40x

Hematopoiesis predominantly dark staining erythrocyte precursors.

4.4 Histopathology of Spleen

4.4.1 Control Group

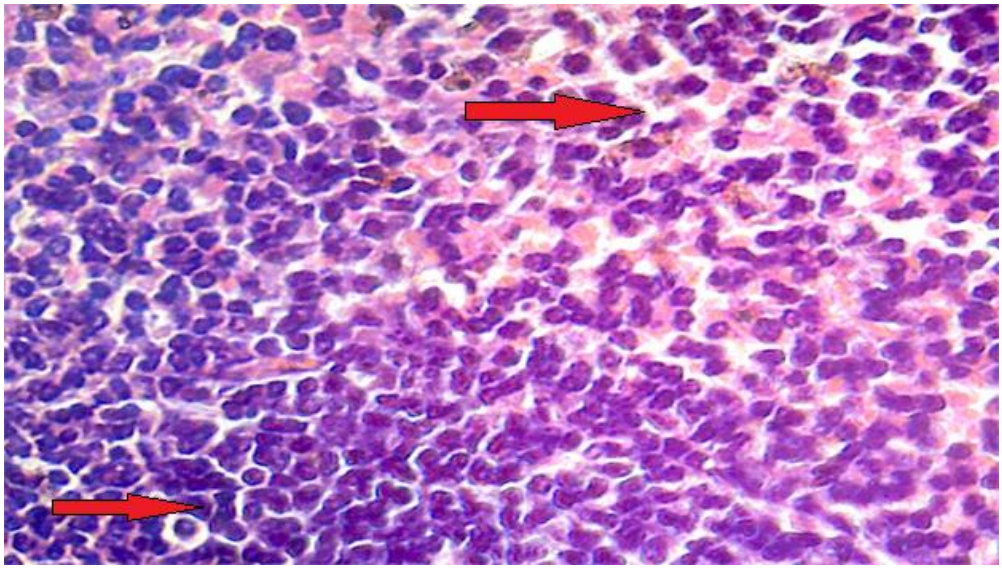

Figure 23: Spleen of control group 400x, showing lymphocytes

Marginal zone of lymphocytes evolved from B-lymphoid follicle, in the outer rim there are loosely arranged lymphocytes whereas in the mental zone there are tightly packed lymphocytes are present.

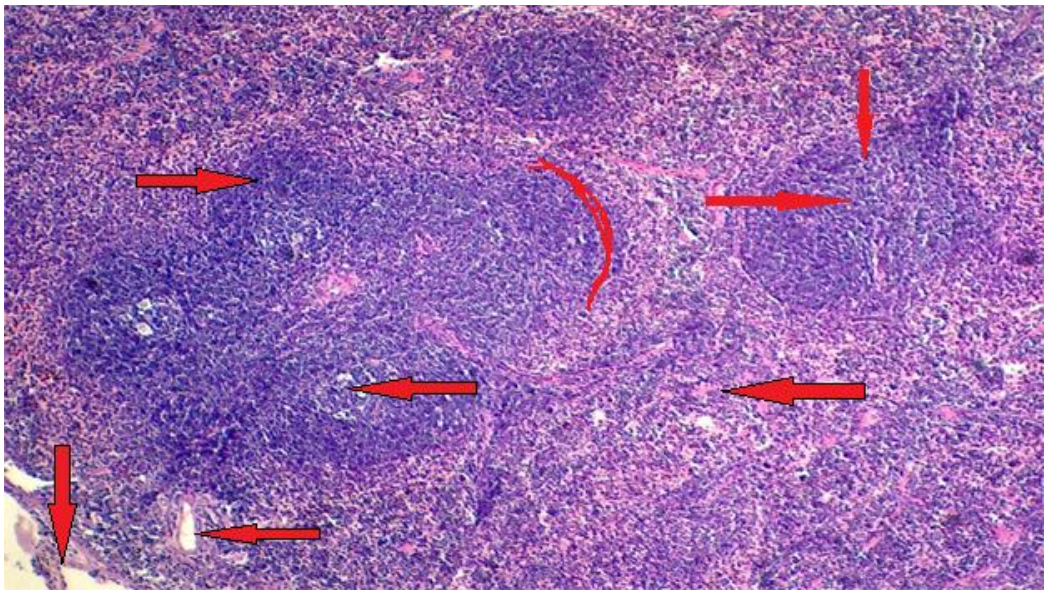

Figure 24: Normal histology of spleen from the control group, 40x

There are two types of pulps present in Spleen, one is white pulp which consists of a central arterial in the middle, B cells and T cells. Red pulp surrounds white pulp. On the other side of red and white pulp, the capsule is present whereas a central artery is also present.

4.4.2 Group treated with Benzene

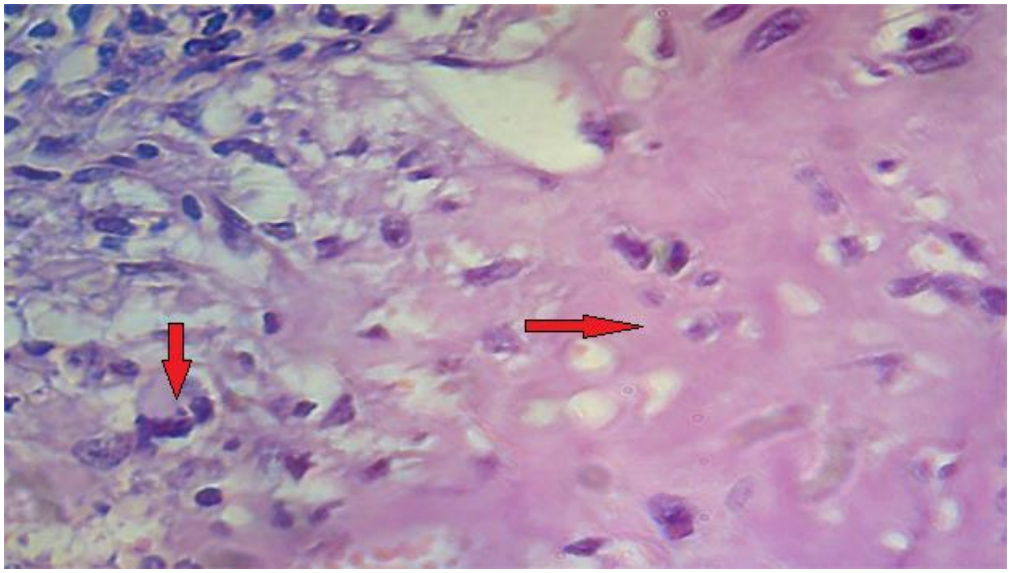

Figure 25: Histopathology of spleen 400x

Amyloid deposition can be seen in the spleen. A megakaryocyte can be seen in the spleen. Spleen shows loss of normal structure. Asplenia can also be seen.

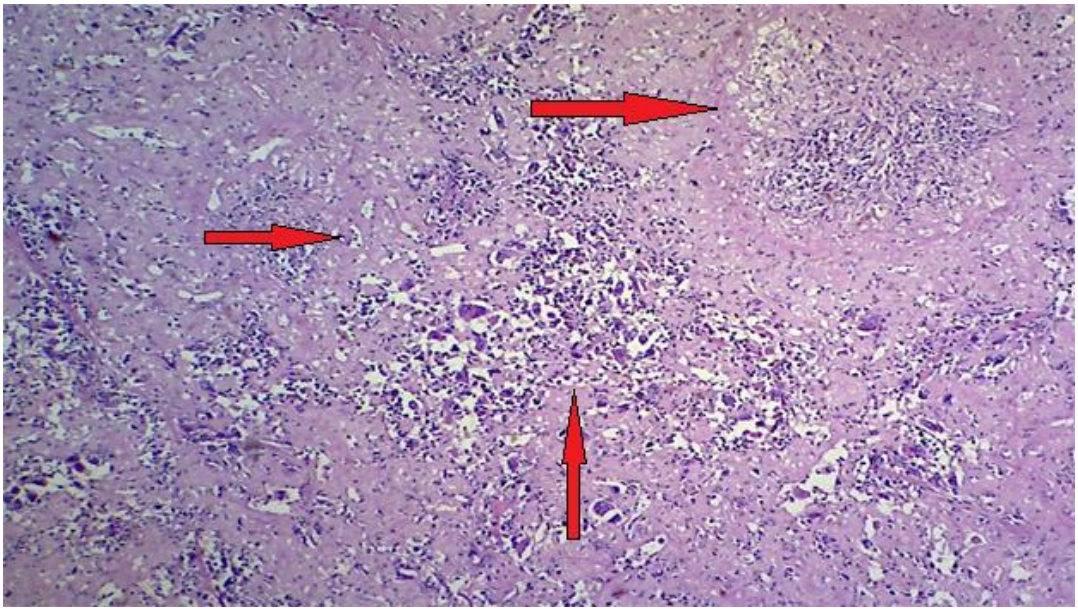

Figure 26: Histopathology of spleen 40x

Necrotizing granulomatous lymphadenitis is seen in the spleen. White pulp and red pulp are not normal in their structure. Asplenia can also be seen in spleen.

4.4.3 Group treated with the standard drug: Cyclophosphamide

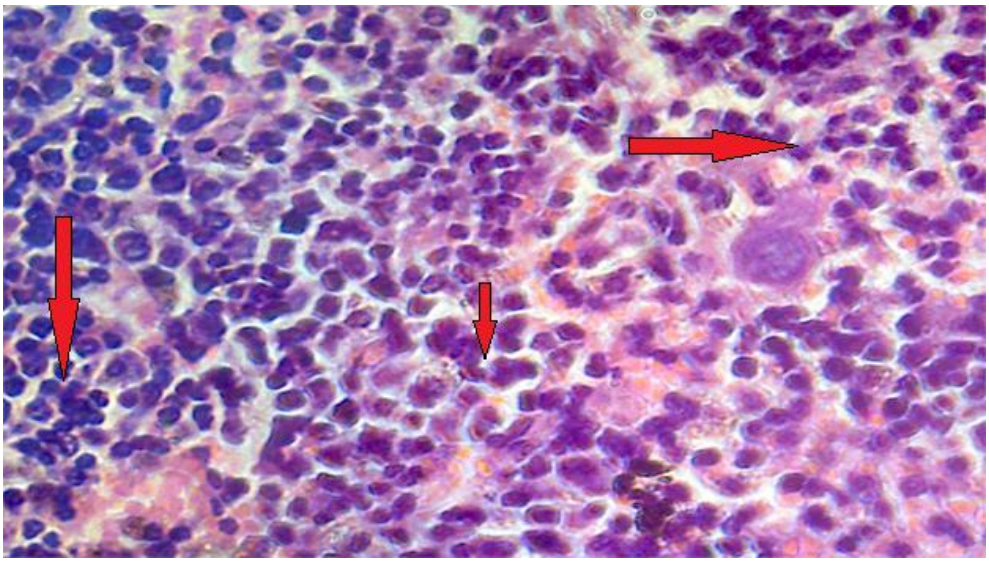

Figure 27: Histopathology of the group treated with standard drug 400x

Hyperplasia of lymphocytes and extramedullary hematopoiesis can be seen.

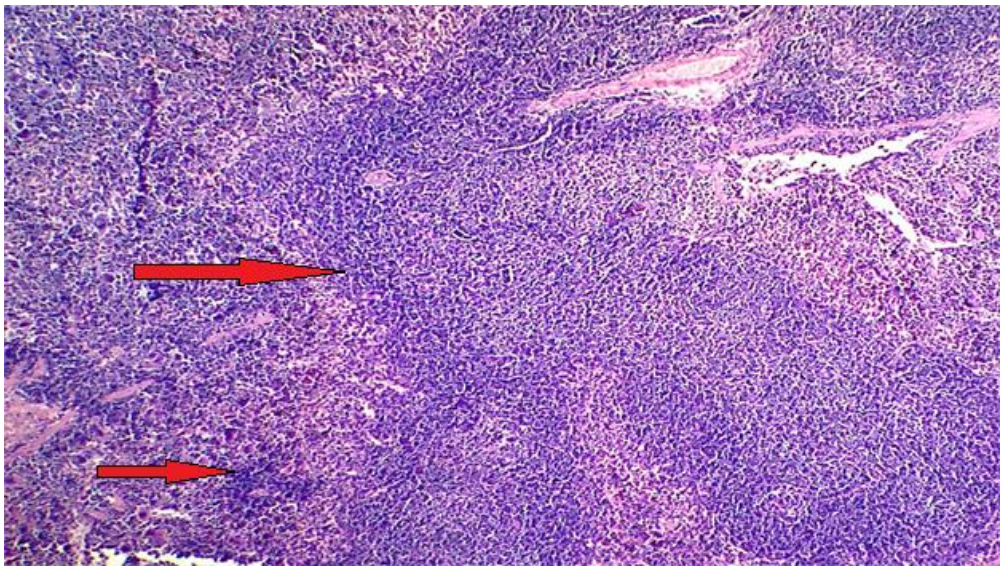

Figure 28: Histopathology of the group treated with standard drug 40x

An enlarged spleen is observed is seen with hyperplasia and extramedullary hematopoiesis. Splenomegaly is also observed.

4.4.4 Group treated with phenolic extract of Crithmum maritimum L.

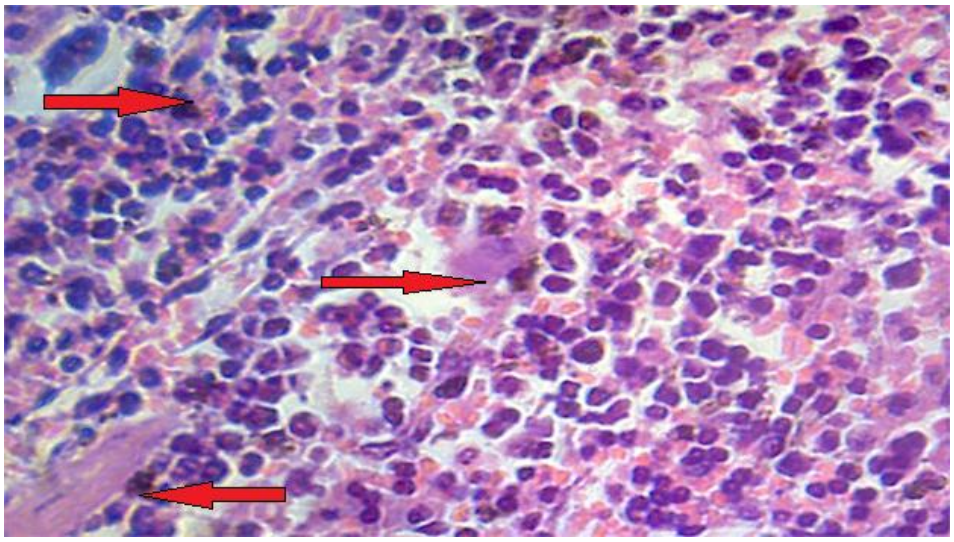

Figure 29: Histopathology of the group treated with P.E of Crithmum maritimum 400x

Deposition of amyloid is seen. Extramedullary hemopoiesis is also observed. Hyperplasia is also observed. Normal structure of the spleen is observed.

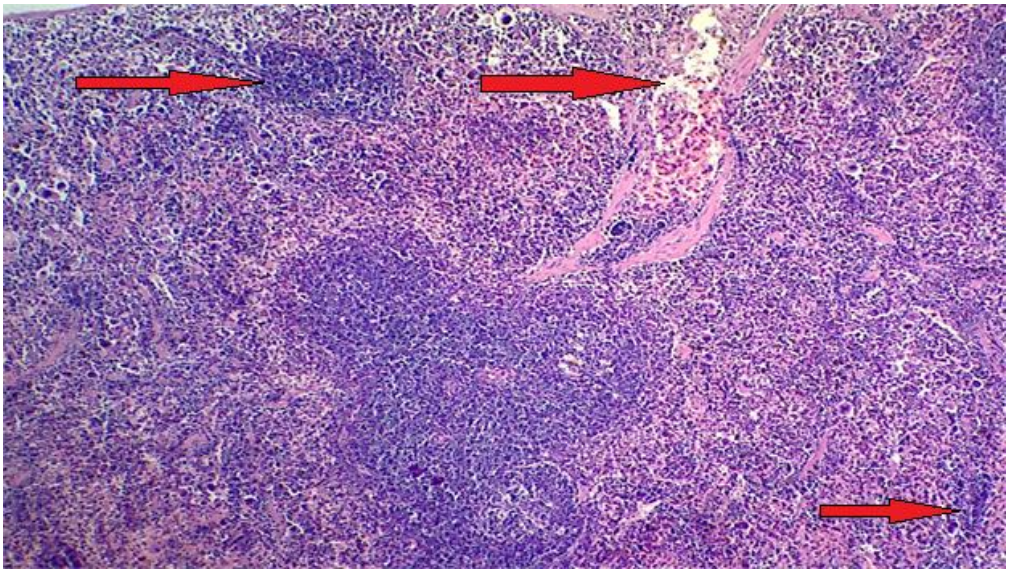

Figure 30: Histopathology of the group treated with P.E of Crithmum maritimum 40x

Amyloid deposition, extramedullary hematopoiesis and hyperplasia is seen the normal structure of spleen is also observed.

4.5 Histopathology of Kidney

4.5.1 Control group

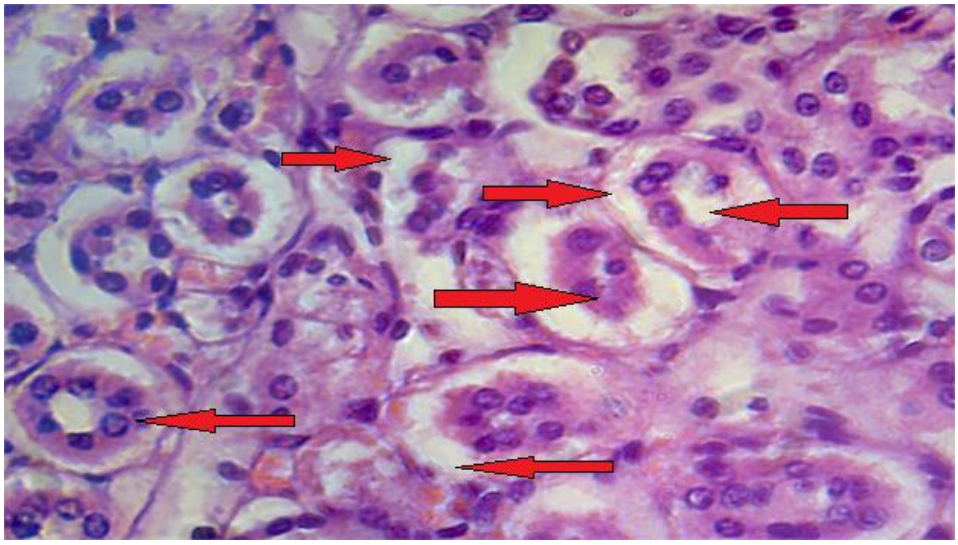

Figure 31: Histology of normal kidney cells 400x

Renal corpuscles, Bowman's capsule, space of Bowman, Renal proximal tubule, Renal distal tubules can be observed in this image.

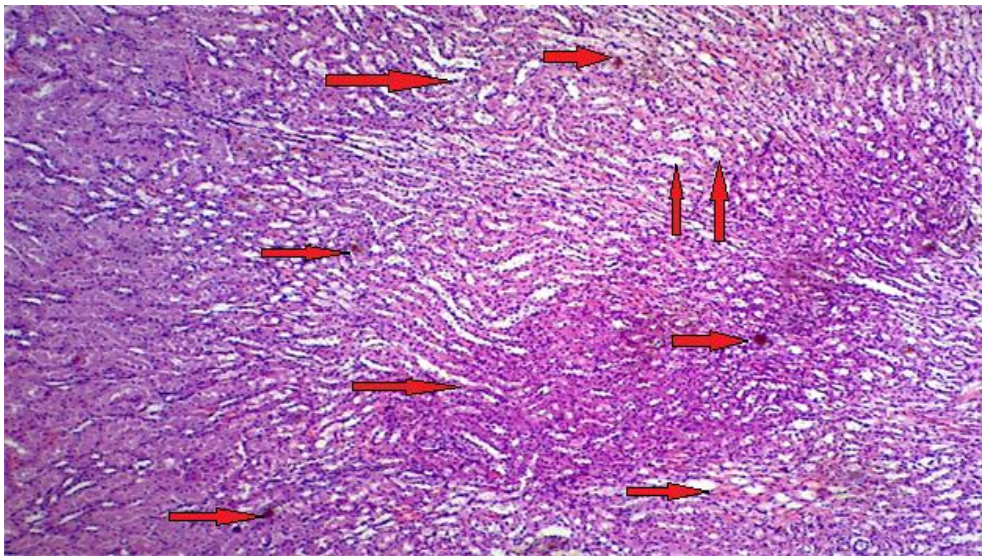

Figure 32: Histology of normal kidney cells 40x

Glomerulous, Proximal tubule, Distal tubule can be observed in this image.

4.5.2 Group treated with Benzene

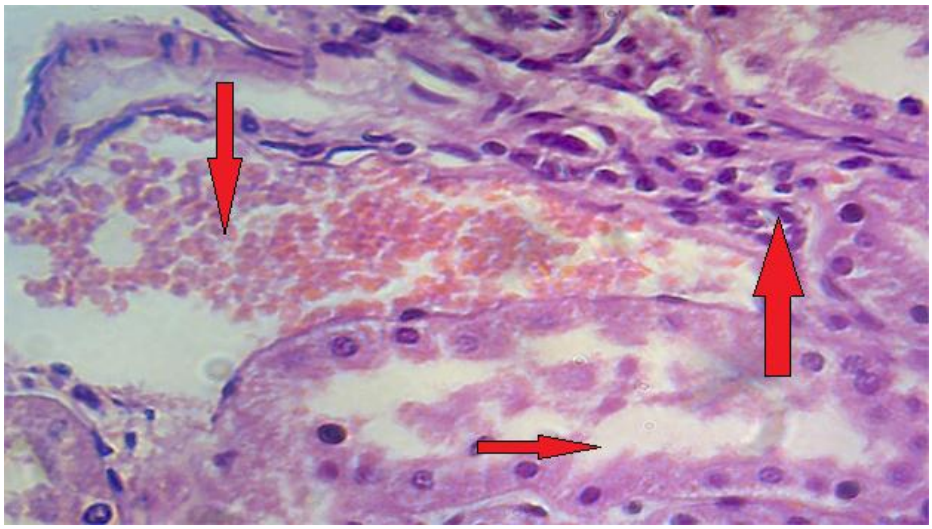

Figure 33: Histopathology of the kidney treated with Benzene 400x

Vascular congestion can be observed in picture whereas disorganization along with necrosis and apoptosis is alose observable. Clusters of darkly stained nuclei are also celled as hemangioblastoma.

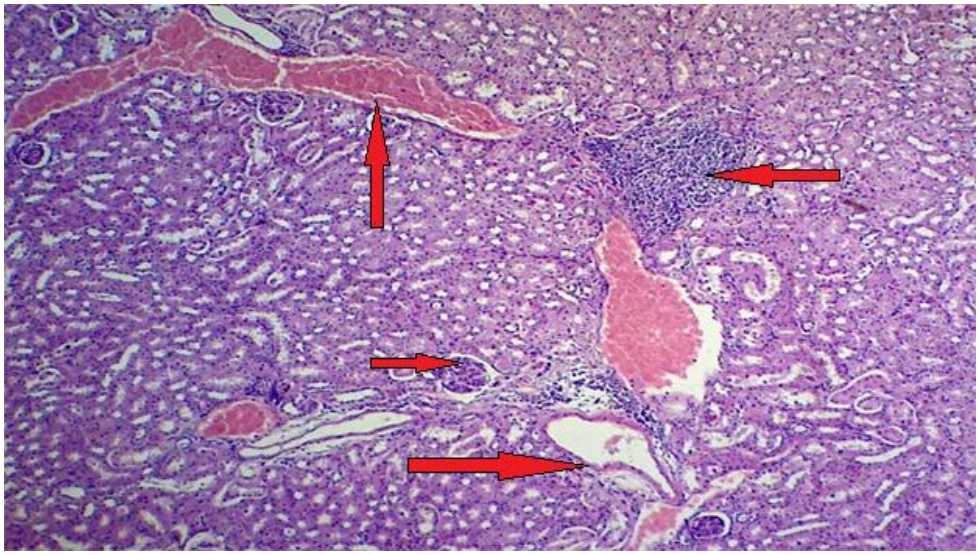

Figure 34: Histopathology of the kidney treated with Benzene 40x

Vascular congestion can be seen. Atrophy in the renal corpuscle and in the epithelial layer is also observable. Widened walls and degenerative renal corpuscle is observable. There are many apoptotic changes observable in the above slide.

4.5.3 Group treated with the standard drug: Cyclophosphamide

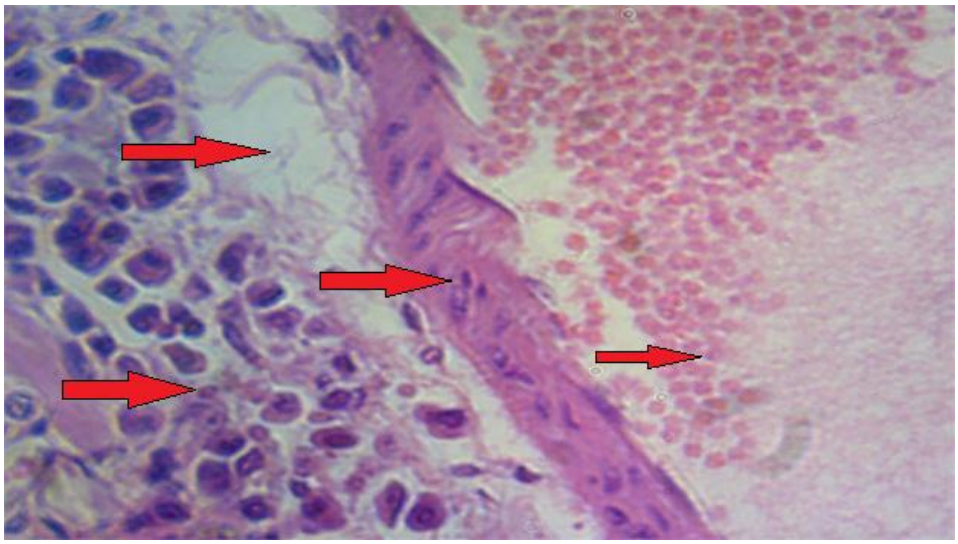

Figure 35: Histopathology of the group treated with standard drug 400x

Necrosis, apoptosis and vascular congestion are observable.

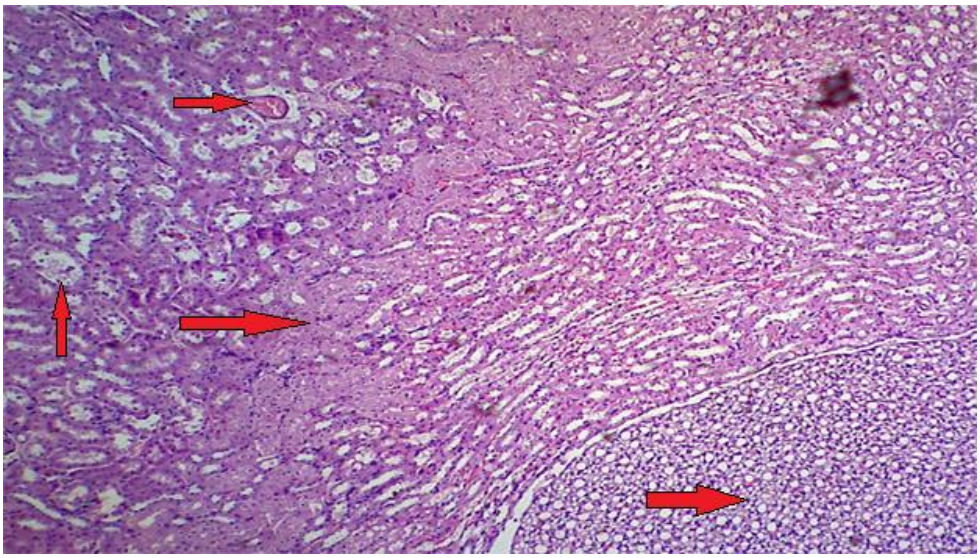

Figure 36: Histopathology of the group with standard drug 40x

Most of the cell is normal in their histology whereas at some points apoptotic and necrotic cell is observable. Medullary rays can also be seen.

4.5.4 Group treated with phenolic extract of Crithmum maritimum L.

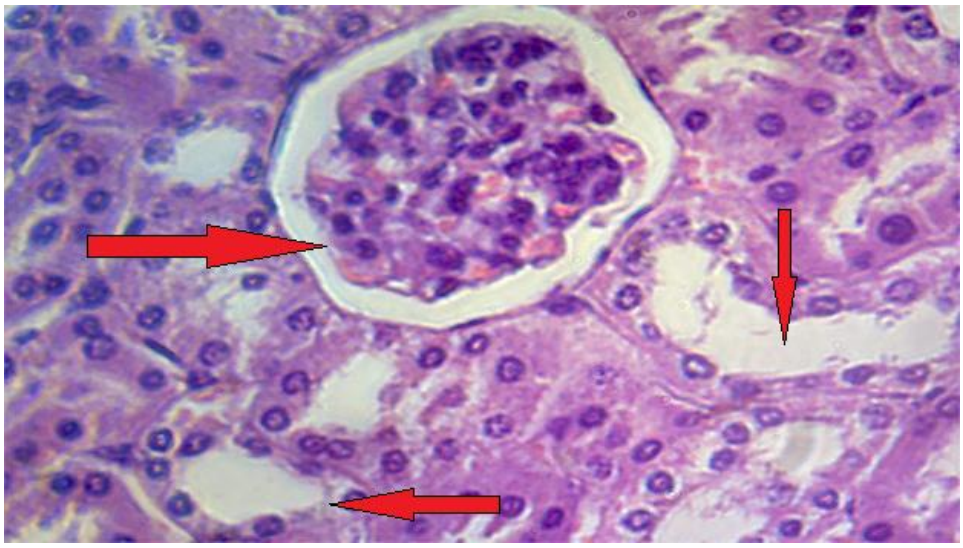

Figure 37: Histopathology of the group treated with P.E of Crithmum maritimum 400x

The normal glomerulus, proximal and distal tubules are observable.

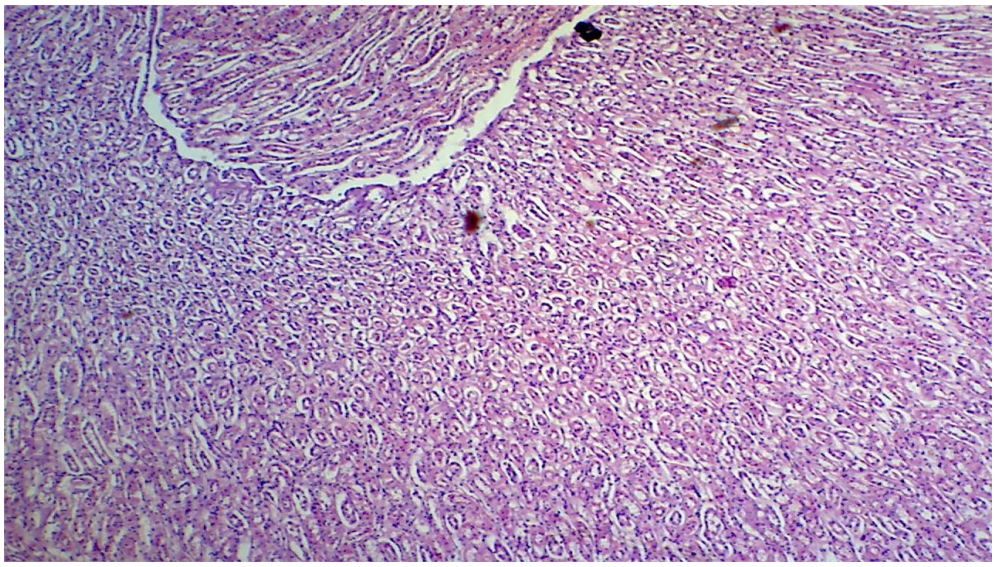

Figure 38: Histopathology of the group treated with P.E of Crithmum maritimum 40x

The normal histology of this group is observable. No abnormality is seen in this slide. All cells are normal in their structure.

4.6 Complete Blood count

4.6.1 White blood cell counts

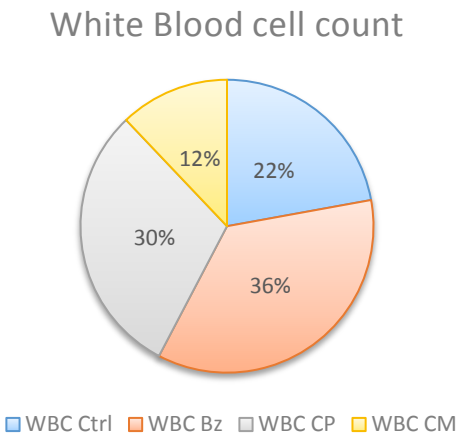

Figure 39: Pie graph of WBC's

The white blood cell count of the control group is 27%. WBC count of benzene treated group is 36% and is elevated from the control group. The count of cyclophosphamide treated group is 30% less than benzene treated group but still more than the control group. In an extract-treated group, WBC count is 12% which is very less than the control group.

4.6.2 Red blood cell counts

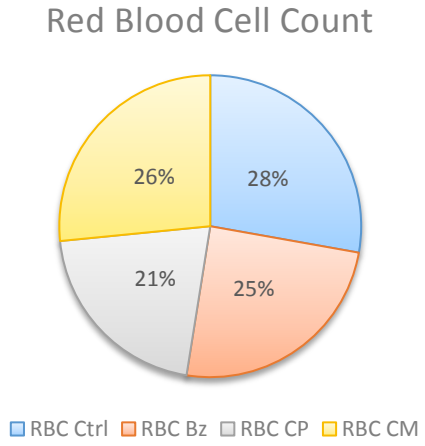

Figure 40: Pie graph of RBC's

The red blood cell count of the control group is 28%, comparable to benzene treated group of which count is 25%. There is a decrease in red cells of benzene treated group. There is also a decrease to 21% in the group which has been treated with standard drug i.e. cyclophosphamide. The count of red cells in the extract-treated group is 26%, less than the control group, but more than the benzene treated group and standard drug group.

4.6.3 Thrombocytes counts

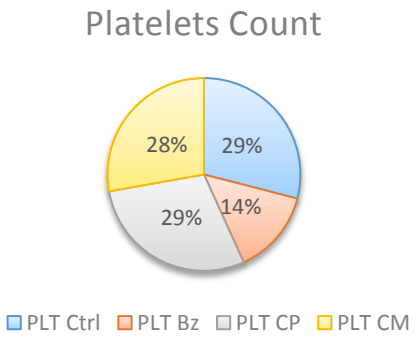

Figure 41: Pie graph of Platelets

Platelets/Thrombocyte count of the control group and the group treated with the standard group has a percentage of 29 i.e. the same in both groups. 14% in the group that has been treated with benzene whereas comparatively in the group that has been treated with extract has the highest percentage of 28. It is decreased in benzene treated group. After treatment, the count has been increasing in the cyclophosphamide group and extract-treated group.

4.6.4 Lymphocytes counts

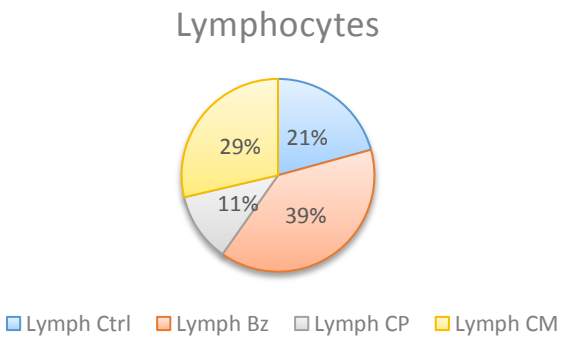

Figure 42: Pie graph of Lymphocytes

The lymphocytes count of the control group is 21% whereas it is highest in benzene treated group with 39%. The lowest percentage is in the group with standard drug cyclophosphamide and it is 29% in the extract-treated group. Lymphocyte count in benzene treated group is highly increased than other groups. Treated groups with cyclophosphamide and extract has less number of lymphocytes.

4.6.5 Monocytes count

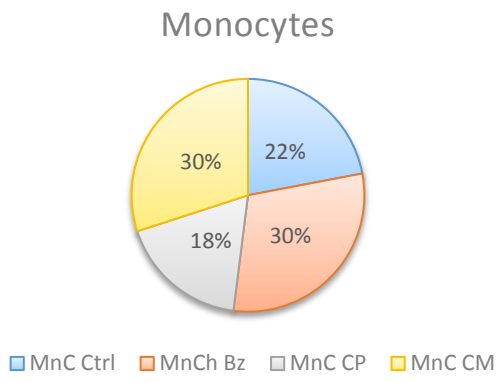

Figure 43: Pie graph of monocytes

Monocytes count is the same in the benzene treated group as well as in the extract-treated group i.e. 30%. It is less in the Control group with 22% and is lesser in the group that is treated with standard drug.

4.6.6 Granulocytes count

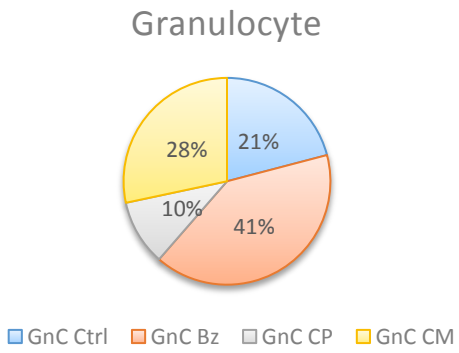

Figure 44: Pie graph of granulocytes

Granulocytes have been increasing in the group treated with benzene with a percentage of 41% whereas 21% is in the control group. Granulocyte count is 10% in the cyclophosphamide group and is lesser than the control group. In the extract-treated group, I am slightly higher that is 28% than the control group.

4.6.7 Hemoglobin

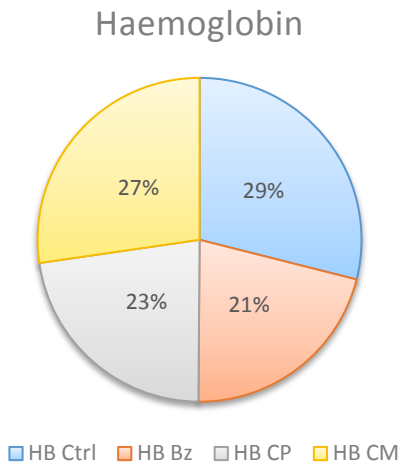

Figure 45: Pie graph of Hemoglobin

HB level of the control group is 26% where is it is low in benzene treated group i.e. 21%. It is the less in the standard drug group with a percentage of 23% where it is 27% in the extract-treated group.

4.6.8 Hematocrit

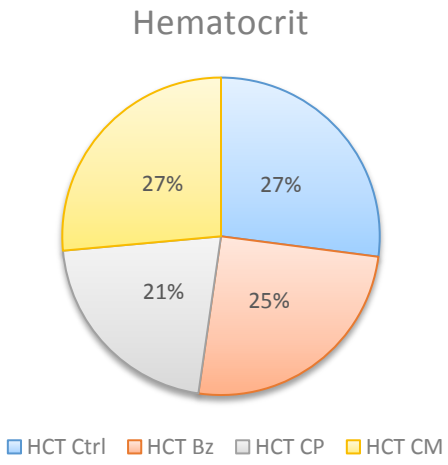

Figure 46: Pie graph of Hematocrit

HC level is low in benzene treated group i.e. 25%. It is 27% of the control group. In the cyclophosphamide treated group, it is 21% less than the control group. It is 27% in the extract-treated group.

4.7 Flow cytometry results

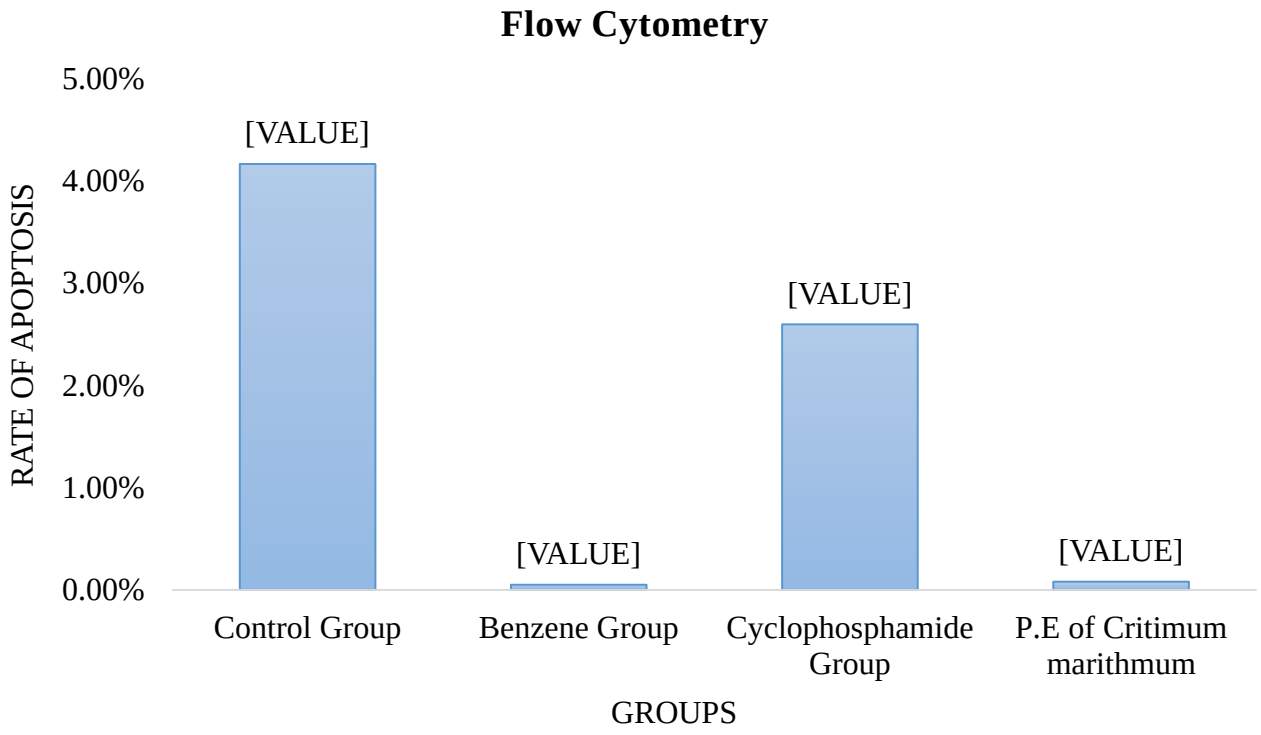

Figure 47: Rate of apoptosis in all groups

Flow cytometry has shown rate of apoptosis i.e. early and late apoptosis in each group. 4.17% has been observed in control group. 0.05% in 2nd group which has leukemia. 3rd group has rate of apoptosis of 2.60 %, comparatively less than control group. In the 4th group, that has been treated with extract has percentage of 0.08 apoptosis.

5 Discussion

Benzene induces leukemia in Balb/c mice, by metabolism of its derivatives i.e benzoquinone and so many others. Benzene has been converted to its toxic metabolites in the liver by CYTP450 and causes toxicity and hematotoxicity by interfering with precursor cells in bone marrow. After toxicity in hematopoietic stem cells converted to leukemic stem cells and then converted to mature immature types of blood cells. Then these blood cells are then transferred to body organs of whole body and cause malignancies where located. Treatment of leukemia with novel plant extract Critimum maximum L. in phenol fraction has been tested on Balb/c strain. The apoptotic activity has been tested by flow cytometry. In weight analysis, weight of the mice treated with plant extract increases very slow with treatmen phase whereas in cyclophosphamide treated group, increase in ratio of weight is good. The weight of the group, which is kept under normal conditions and not treated with any kind of agent, kept on increasing slowly with the passage of time. In the benzene treated group, weight of the mice increases gradually in the first and the second week, whereas in 3^{rd} and 4^{th} week it started decreasing very slowly, as benzene metabolizes very slowly instead of rapid action of metabolism and is highly effective at very small concentration.

Histology of the control group shows normal liver, normal kidney, and normal spleen cells. In benzene treated group, which is basically a leukemic model for studies, shows proliferation of cells, excess of lymphocytes, medullary hematopoiesis, abnormal cells in this group. Nephrotoxicity, hepatotoxicity, and enlargement of organs i.e. liver, spleen, and kidney have been observed in this group. The group treated with a standard chemo drug i.e. cyclophosphamide, organs have started phase of recovery, some dead leukemic cells, some cells are normal and healthy, Half of the cells of organs have normal histology and half have leukemia symptoms. In extract-treated group, extract shows some activity in curing cells of organs and extracts stops the activity of leukemia cells to affect organs.

In complete blood count, there is an increase in white leukocytes in leukemia due to abnormal production at bone marrow by leukemic precursor cells. Due to the abnormal growth of leukocytes, other cells also get disturbed. There is decrease in number of erythrocytes observed in leukemic group, in the standard drug-treated group, and in-plant extract-treated group. A decrease in count of erythrocytes causes anemia. Severe Thrombocytopenia has been in leukemic

group but mild thrombocytopenia has been observed in the extract-treated group. There is no thrombocytopenia observed in with cyclophosphamide. Hight lymphocytosis has been observed in leukemia model of balb/c mice. Mild lymphocytosis has also been analyzed in group treated with plant extract whereas group treated with cyclophosphamide has lymphocytopenia i.e. decrease in lymphocytes count. Granulocytes and monocytosis have been seen in leukemia model. Group treated with cyclophosphamide has monocytopenia whereas in plant extract-treated group monocytosis is present. Granulocytopenia has also been analyzed in with cyclophosphamide and granulocytosis in plant extract-treated cells. Hematocrit and Hb are also disturbed due to decrease in erythrocytes.

Flow cytometry is a technique which is known for analysis of apoptotic cell in early and late phase. Some changes happen at cell surface when cell is marked for apoptosis. Phosphatidylserine translocates out at the outermost surface of cell when cell is marked fo apoptosis. Annexin V is used in flow cytometry is a Calcium-dependent protein and has the affinity to bind with PS. At FLH-1 chamber, annexin v is present and at FLH-3 Propidium iodide is present. Annexin V binds when cell is marked for apoptosis and detected when PS comes out on the cell membrane whereas PI binds when cell becomes porous and leaky (CHOUDHURY, MOHANIA, & DINAND, 2013). 10,000 cells acquired from the population for sampling. Forward scattered and side scattered graphs show gated cells. Cells are divided into four quadrants i.e. lower left, lower right, upper left, upper right. In lower left quadrant alive cells are gated, in lower right, cells in early phase of apoptosis are gated, in upper left quadrant late apoptotic cells are gated and in upper right quadrant dead cells are gated. Cells are classified on the basis of annexin +ve, annexin –ve, PI +ve, and PI –ve. In flow cytometry, FSC and SSC are forme on the basis of cell's complexity or granularity and on the basis of size (Hingorani, Deng, Elia, Mcintyre, & Mittar, 2011).

In the control group, normal apoptosis rate is observed which is 4.17% and 94% cells are alive and remaining are dead cells. In benzene treated group, very minute ratio is observed for apoptosis i.e. 0.05% and 99% cells are alive. Cyclophosphamide is used as a standard chemotherapeutic drug for the treatment of leukemia. Cyclophosphamide shows good apoptotic activity with percentage of 2.6 and 96% cells are alive. The herbal extract is used in comparison to a standard chemo drug i.e extract from *Critimum maritime L.* plant. The extract tested have shown apoptosis to LSC and is responsible for antioxidant activity. 0.08% cells are in late

apoptosis because extract shows slow activity with no adverse side effects. Because of solubility in DMSO, DMSO interferes with anti-oxidant activity of the phenolic extract.

6 Conclusion

The results have shown the antioxidant activity of *Critimum maximum L.*, Phenolic extract contains carboxylic acid group and phenolic extract of *Critimum marithmum L.* have many anti oxidants such as terpenes, chlorogenic acid, rutin, quercitin and various vitamins which are responsible for anti oxidant activity of plant. The Activiy of extract stopped the growth of leukemia stem cell, did not let them proliferate more. Weight analysis have shown decrease in group at mid of induction protocol, whereas in the end of treatment it becomes stabilized and balancd. The results from Complete blood count have also shown that there is improvement in mice with leukemia. In flow cytometer, rate of apoptosis, has been observed which states that plant has curing capacity for leukemia but at slower rates in comparison to chemo drugs. Plants ave various phytochemicals to fight with cancerous cells and the activity of radical scavenging has been observed through different assays i.e. HPLC, BCB method etc. Glutathione assay can be used to identify the signal transduction pathway of *Critimum marihmum L.*

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