EFFECTS AND CHARACTERIZATION OF SOME MEDICINAL PLANTS ON LEUCOCYTE FUNCTIONS IN BENZENE -INDUCED LEUKEMIC RATS

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY (Ph.D.) DEGREE IN MEDICAL LABORATORY SCIENCE (HAEMATOLOGY AND TRANSFUSION SCIENCE), UNIVERSITY OF BENIN, NIGERIA.

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CERTIFICATION

This research work titled 'Effects and characterization of some medicinal plants on leucocyte functions in benzene-induced leukemic rats'' submitted to the School of Postgraduate Studies, University of Benin, Benin City, Edo State, Nigeria, for the award of Doctor of Philosophy in Haematology and Blood Transfusion Science is an original research carried out by AKINOLA, Rasheed Olabisi in the Department of Medical Laboratory Science, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin City, under the supervision of Prof. O.I Ajayi. The research work has not been submitted previously in whole or in part to qualify for any other academic award.

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DEDICATION

This thesis is dedicated to Almighty God who make plants for medicine (Ezekiel 47:12, Rev 22:2), and to all leukemia patients globally.

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LIST OF ABBREVIATIONS

AL	Acute leukaemia
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myelogenous Leukaemia
AML	Acute Myelogenous leukemia
ANOVA	Analysis of Variance
CCAC	Canadian Council of Animal Care
CD	Cluster of Differentiatia
CLL	Chronic Lympholytic Leukemia
CML	Chronic Myelogenous Leukemia
CML	Chronic Myeloid Laekemia
CT	Computed Tomography
DIC	Disseminated Intravasuclar Coagulation
DNA	Deoxyribnonucleic Acid
EMEA	European Medicines Agency
FAB	French America British
FBC	Full Blood Count
FDA	Food and Drug Administration
GSH	Reduced Glutathione
HGB	Haemoglobin
IHC	Immunolusto Chemistry
IMRT	Intensity Modulated Radiation therapy

K3EDTA	Tripotasium Ethylene Diamina Tetraacetic Acid
LAUTECH	Ladoke Akintola University of Technology
LD	Lethal Dose
MCH	Mean Corpsular Haemoglobin Concentration
MCV	Mean Corpsular Volume
NIR	Near Infrared Light
NIR	Near Visible Infrared
PCV	Packed Cell Volume
PDT	Photodynamic Therapy
RBC	Red Blood Cell
RNA	Ribonucleic Acid
SOD	Superoxide Diutacesm
TCM	Traditional Chinese Medicine
WBC	White Blood Cell
WHO	World Health Organization

ABSTRACT

Medicinal plants have been used in virtually all cultures as a source of medicine. Assurance of the safety, quality, and efficacy of medicinal plants and herbal products has now become a key issue in industrialized and in developing countries. The widespread use of herbal remedies and healthcare preparations is described in the Vedas and the Bible. Medicinal plants have been used for thousands of years to flavor and conserve food, to treat health disorders and to prevent diseases including epidemics.This study seeks to evaluate possible ameliorative and inhibitory effects of some medicinal plants on benzene-induced leukemic rats. In this study, leukemia was experimentally induced in albino rats by Benzene chromosolv at 0.2ml at 1:10 dilution water ½ propanol 50/50 in water daily via tail vein for 4 weeks. The rats were divided into 6 main groups consisting of 6 rats per group. They were administered with the extracts of four different plants viz: *Ricinus communis*, Rosy periwinkle, *Psorospermum febrifugum*, and *Azadiractha indica* separately after the determination of the LD_{50} for 4 weeks after induction. The LD_{50} of each of the extracts were *Ricinus communis* 340mg/kg, Rosy periwinkle 30mg/kg, *Psorospermumfebrifugum 548mg/kg, and Azadiractha indica 40mg/kg. The animals were there after* weighed and sacrificed, blood samples were collected into lithium heparin and EDTA bottles for laboratory analysis of Full Blood Count (FBC), leucocyte functions assay and antioxidant profiles using standard methodologies. The animals Albino rats were initially weighed prior to plant extracts administration and induction with Benzene chromosolv. Results show a statistically significant decrease in final weight in all groups (pre and post treated with the extracts and a statistically significant increase in White Blood Cell Count (WBC) count in benzene induced rats ($P<0.05$, respectively) compared with non-induced controls. The induced leukemia was the lymphocytic type. These values reduced significantly with the post treated animals especially with *Ricinus communis* ($P<0.05$, respectively). Also, there was statistically significant increase in percentage viability index in Benzene induced rats compared with control and extracts treated rats ($P<0.05$ respectively). However, there was no significant different between the control and treated rats. Furthermore, there was statistically significant decreases in percentage chemotactic index, percentage phagocytic index and percentage respiratory burst in Benzene induced rats in the pretreated rats' extracts with concomitant increase in the rats pretreated with *Ricinus communis* compared with the control group. This is suggesting ameliorative effect in the leucocyte functions by all the extracts with more efficacies in rats treated with *Ricinus communis* when compared with controls. The anti-oxidanats enzymes exhibited higher activities in all the pretreated groups compared with those post-treated after induction with all the extracts as well. Ultimately, the TGF β-1 gene was significantly expressed in the *Ricinus communis* treated group and *Psorospermum febrifugum* but with a high value exhibited by the later. In conclusion, the extracts of *Psorospernumfebrifugum*, *Ricinus communis*, *Azadiractha indica* and *Rosy periwinkle* demonstrates ameliorative effects in the treatment of leukemia especially with *Ricinus communis*. The extracts equally showed a significant anti-oxidant enzymes activity in pretreated animals while TGF β-1 gene was significantly upregulated in animals in the leukemic rats treated with *Ricinus communis,* Resulting in massive apoptosis of the leukemic cells. This study has shown vividly that *Ricinus communis* is more effective and showed profound efficacy among the four extracts in the treatment of Benzene induced albino rats. It is recommended that extracts of *Ricinus communis* should

be evaluated further for its pharmacological and therapeutic efficacies in the management of leukemia.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Herbal medicine (HM) is the fulcrum of complementary and alternative medicine which in relevant times is increasing by wide spread popularity all over the world and gradually streaming towards integration into the mainstream healthcare systems (Bent, 2008).

Herbal Medicine included preparation of biologically active natural products that consist largely of herbs or herbal materials, some recipes may contain material such as Fungal and Bee products, as well as minerals (kadin, beton), ash shells, insects and animal parts, and are used for the maintenance of health and managements of various diseases (Shinde *et al.,* 2016.)

Leukemia is a hetorogenous group of hematological malignancies that arise from the dysfunctional production of developing leukocyte. It is classified as either acute or chronic based on the rapidity of proliferation and as myelocytic or lymphocytic based on the cell of origin (Adithya *et al.,*2016). The exact cause of leukemia is unkown, (Hutter, 2010). A combination of generic factors and environmental (non-inheritted) factors are believed to play a role. Risk factors include smoking, ionizing radiation, Petrochemicals, (such as benzene), prior chemotherapy, and down syndrome (Hutter, 2010). People with a family history of leukemia are also at risk. There are four main types of leukemia Acute lymphoblatrc (All), acute myeloid leukemia (AML), chromnic lymphocytic leukemia (cll) and chronic myeloid (cml) as well as a number of less common types (WHO 2014). Leukemia and lymphomas both belong to a broader group of tenows that affects the

blood, bone marrow, and lymphoid system, known as tenuous of the hematopoietic and lymphoid tissues (Vardiman, 2009). Symptoms may include bleeding and bruising problems, fever and increased risk of normal blood cells.

Diagnosis is typically made by blood tests or bone marrow biopsy (Hutter, 2010). Leukemia is characterized by the following infections, bleeding, red spots on the skin, night sweats, weight loss, swollen lymphonodes, Joint pain, bones pain, hepatosplenomegaly, weakness and fatigue (Alan *et al.,*2018). The detail clinical diagnosis of leukemia case include blood tests, physical examination, lumber puncture, biopsies, flow Cytometry and imaging techniques.

In Nigeria, reports have shown increase incidence rate of leukemia in a retrospective study of the pattern of leukemia incidence conducted by Omotr and Imieve (2006). A recent report by Dapus *et al*. (2017) has also revealed high incidence rate of leukemia diseases among the adult in Jos, North central, Nigeria. Thus, alternative therapies such as the use of plant derived products may be more effective with low side effect in the management and treatment of leukemia and its associated conditions.

Therefore, this study is to investigate the effect of some indigenous medicinal plants (*Azadiracthaindica,Ricinus communis, Psorospermumfebrifugum and Rosy periwinkle*) on leucocyte functions in Benzene induced leukemic Albino rats.

1.2 Statement of the Problem

The use of medicinal plant is very wide spread in many parts of the world because it is commonly considered that herbal drugs are cheaper and safer as compared to synthetic drugs and maybe used without or with minimum side effects. It is believed that anticancer effect of plants developed by suppressing cancers stimulating enzymes, repairing DNA, stimulating production of antitumor enzymes in cells, increasing body immunity and inducing antioxidant effects. Nowadays more than 60% of anticancer compounds that are useful for cancer patients are obtained from herbal, marine and microorganism's sources.

The positive effect of plants in curing diabetics, fertility and sterility, thyroid disorders, anaemia and psychological disorders has been established (kootie, *et al.,* 2017). Thus, finding plants that can replace or serve as possible alternative to synthetic chemotherapy and cure for leukemia with less cytotoxic effect in our locality is necessary.

1.3 Aim

This study seeks to evaluate possible ameliorative and inhibitory effects of some medicinal plants on benzene-induced leukemic rats.

1.4 Research Questions

1. Are there any changes on leucocyte functions in leukemia?
2. Do the extracts from the chosen medicinal plants have any ameliorative or preventive effects on these indices?
3. Do the extracts have antioxidant properties on non-leukemic rats as well as leukemia?
4. What are the mechanisms of action of the effectives extract(s)?
5. Do any of the extracts prevent the development of the leukemia after induction?

1.5 Specific objectives of the Study

1. To investigate leucocyte functions in benzene-induced leukemicWistar rats using full blood count, chemotaxis assay, phagocytic test and viability test.
2. To observe possible anti leukemia effect of the medicinal plants (*Rosy periwinkle, Ricinuscommunis, Azadirachtaindica*and*psorospenumfebrifugum*) on leukemia..
3. To investigate any anti-oxidative properties of the extracts of *Rosy periwinkle*, *psorospenumfebrifugum, Azadirachtaindica*and*Ricinuscommunis.*
4. To determine which of the extracts have pronounced result on the estimated parameters.
5. To theoretize specific mechanism of action of the most effective extracts.

1.6 Justifications and Rationale

The natural therapies present in alternative medicine are age-old as compared to western medicine for treatments such as antibiotics and surgeries. According to physicians, most alternative medicine therapies started with chemical impression of scientific research. The medicines are safe and involve natural substances. One primary objective of alternative medicine is to relieve people from depending largely on drug usage and help them manage their lives naturally.

Nevertheless, the public pay high prices for orthodox medicines because of cost of experimental techniques through research and development. Another common perception is that orthodox medicine which is scientifically based is more reliable, safer, and more effective. This notion may be wrong because drugs once thought to be safe are often withdrawn from the market for causing severe side effects and fatalities. In view of the

fact that cancers are actually own cells that have lost the ability to control the growth. And sothe body has a very difficult time distinguishing cancerous cells from normal cells. And by the same way, it is very difficult for us to design drugs that will destroy cancer cells. Hence finding medical plants extract that ameliorate or inhibit cancerous cells will make a great impact.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of Leukemia

Leukemia was first documented approximately 150 years ago. In the years since, several advances have been made in understanding the mechanism of leukemia. Medical advances have developed better ways of diagnosing, treating, tracking cases, and classifying leukemia in recent years. However, being able to link the development of this disease to environmental, development, and physical factors still remains largely a mystery (Lackritz *et al*., 2001).

Leukemia is a type of cancer involving the blood forming cells, mostly white blood cells. When a person has leukemia their white blood cells do not mature fully and their body rapidly produces immature white blood cells. These cells are different from normal cells and are unable to perform the functions mature cells do. Eventually these leukemic cells replace normal cells in the bone marrow of an individual. Often this uncontrolled proliferation can cause the leukemia cells to spill over into the blood stream and eventually enter vital organs. This decreases the amount of room available for normal /mature cells like red blood cells, non-malignant white cells and platelets. There are several types of leukemia track type affects a different type of white blood cell and involves a different level of white blood cells maturity (Lackritz *et al.,* 2001).

2.1.2 Types Of Leukemia

There are four types of leukemia: Acute myeloid leukemia (AML), Chronic myeloid leukemia (CML), Acute lymphocytic leukemia (ALL), and Chronic lymphocytic leukemia (CLL) **(**Cancer Treatment Center of America, 2014**)**

(i) **Acute Myeloid Leukemia (AML)** is type of cancer that is fast growing and found in the blood and bone marrow of an individual. AML is the most common type of Leukemia; it develops from the bone marrow making blast cells that do not fully mature and then these immature cells develop into white blood cells. Due to these cells being immature they are unable to defend the body against infections. The bone marrow may also produce abnormal red blood cells and platelets. These abnormal cells will outnumber the normal cells in time due to their ability to rapidly produce. (Cancer Treatment Center of America, 2014). AML is also known as acute myelogenous leukemia, acute granulocytic leukemia or acute lymphocytic leukemia, is a fast-growing form of cancer of the blood and bone marrow.

(ii) Chronic Myeloid leukemia (CML)

Also known as chronic myelogenous leukemia, chronic Myeloid leukemia (CMC) is a form of cancer that affects the bone marrow and blood marrow and then, overtime, spreads to the blood.

(iii) Acute Lymphocytic Leukemia (ALL)

Acute Lymphocytic leukemia (ALL), also called acute lymphocytic leukemia and acute lymphoid leukemia is a blood cancer that results when abnormal white blood cells (leukemia cells) accumulate in the bone marrow.

(iv) Chronic Lymphocytic Leukemia (CLL)

Chronic Lymphocytic Leukemia (CLL) is a typically slow-growing cancer which begins in lymphocytes in the bone marrow and extends into the blood. It can also spread to lymph nodes and organs such as the liver and spleen.

2.2 Classification of Leukemia

All types of leukemia develop when bone marrow cells undergo mutations known as leukemia changes. While healthy cells grow and divide at the rate necessary to replace old and damaged cells, leukemic changes can dramatically speed up that process.

Over time, the resulting accumulation of mutant cells can outnumber the healthy white blood cells, red blood cells and platelets in the body, leading to leukemia.

Basically, leukemia is classified in two different ways. First, there are two main types of leukemia, classified as acute or chronic, based on its speed of progression. With acute leukemias, immature blood cells (blasts) do not function properly and multiply at a very rapid place. Chronic leukemias involve mature blood cells functioning normally for a period of time before becoming cancers and replicating themselves at a slightly accelerated pace. The second way in which the condition is classified is by the types of cells affected. “Lymphocytes” means that the cancer affects the lymphoid cells (lymphocytes), which form the lymphatic tissue that makes up the body’s immune system, whereas “myelogenous” means the leukemia affects the myeloid cells, which make-up the blood forming tissue that produces red blood cells, white blood cells and platelets producing cells (Daley *et al.,* 1990).

There are four main types of leukemia, based on whether they are acute or chronic, and mycloid or lymphocytic.

- Acute lymphocytic leukemia – the most common form of leukemia in children
- Acute myelogenous leukemia – the most common form of leukemia overall, affecting both adults and children.
- Chronic lymphocytic leukemia – the most prevalent form of chronic leukemia in adults.
- Chronic myelogenous leukemia – mainly affects and may produce few or no symptoms for months or even years.

Others rare types of leukemia include having cell leukemia, myelodysplastic syndromes and myeloproliferative disorders.

The WHO classifications are based on a combination of clinical, morphologic, immunophenotypic, and genetic features. Other commonly used classification systems include the French – American – British (FAB) system which is based on the morphology of the abnormal leucocytes. FAB classification has been replaced by the newer World Health Organization (WHO) classification.

2.2.1 Classification of Acute Leukemia

Acute leukemia is a proliferation of immature white blood cells in the bone marrow (blasts) that may also involve peripheral blood or solid organs. The percentage of bone marrow blasts cells required for a diagnosis of acute leukemia has traditionally been set arbitrarily at 30% or more. However, more recently proposed classification systems have lowered the blast cells percentage when certain morphologic and cytogenetic features are present.

Acute leukemia can be classified in many ways;

1. By morphology and cytochemistry supplemented by immunophenotyping, as proposed by French – American – British (FAB) group (Bennet *et al.,* 1976).
2. Proposed World Health Organization Classification of Acute leukemia (Harris *et al.,* 1999).
3. By immunophenotyping alone as proposed by the European Group for the immunological classification of leukemia (EGIL) (Bene *et al.,* 1995).

The traditional classification of acute leukemia used criteria proposed by the French – American – British Cooperative Group (FAB), using 30% bone marrow blast cells cutoff (Bennet *et al.,* 1985). This classification system originally distinguished different leukemia types by morphological features and cytochemical studies, particularly myeloperoxide (or sudan black B) and non-specific esterase staining. It was revised to include leukemia types that could only be accurately identified with the addition of immunotyping or electron microscopic studies (Bennet *et al.,* 1991).

Although the FAB classification failed to distinguish immunophenotypic groups of acute lymphoblastic leukemias, did not recognize the significance of myelodysplatic changes in acute myeloid leukemias or cytogenetic abnormalities in either leukemia type, and resulted in some subcategories of little clinical significance, this system provided very clear guidelines for classification. In addition, some distinct leukemia subtypes, particularly acute promyelocytic leukemia and acute myeloid leukemia with abnormal eosinophils, were found to correlate with specific cytogenetic aberrations and hold unique clinical features, and those remain in recently proposed classification systems.

Acute myelogenous leukemia (AML) was based on how leukemia blasts the predominant cell in the disease process, recapitulate normal hematopoiesis. Are blasts in a given case myeloblasts, monoblasts, megakaryoblasts etc., and areminimally, or moderately differentiated. The original classification scheme proposed by the French - American - British (FAB) Cooperative Group divides AML into 8 subtypes (M0 to M7) and ALL into 3 subtypes (L1 to L3). Although AML blasts evolve from common myeloid precursors, the 8 subtypes differ in degree of maturation. M0 designated AML with minimal morphologic or cytochemical differential, M1 - M2 AML with minimal or moderate granulocytic differentiation (APL), M4 AML with mixed myelomonocytic differentiation, M5a and M5b monoblastic leukemia with minimal or moderate differentiation, M6a myeloid leukemia with dysplastic background erythropoiesis, M6b acute erythroblasts leukemia, M7 acute megakaryoblastic leukemia. The FAB classification of ALL includes 3 subtypes (L1 to L3), which are differentiated based on morphology, including cell size, prominence of nucleoli, and the amount and appearance of cytoplasm. Approximately 75 of adult ALL cases have blasts with the B - cell phenotype, and 25% have blasts with the T-cell phenotype. The FAB classification of ALL and AML is based on morphology and cytochemical staining of blasts (Chesone*t al.,* 1990).

The classification schemes by the World Health Organization (WHO) require the additional evaluation of the leukemic blasts by molecular analysis evaluation of the leukemic blasts by molecular analysis and flow cytometry (Harri *et al.,* 1997; BrunangeloFalini *et al.,*2010; Sachdeva*et al.,* 2006). The results of these 4 methods of evaluation (i.e morphology, staining, molecular analysis, flow cytometry) not only

differentiate ALL from AML, but also categorizes the new classification of AML as proposed by WHO, knowing the subtype of a patient's leukemia helps in predicting the clinical behavior of the disease and the prognosis, and in making treatment recommendations. This classification also improves the reproducibility of diagnosis and stresses the heterogeneity of the subtypes of AML and ALL (Vardiman*et al.,* 2009). Recent advances in molecular biology have shown that various subtype of AML and ALL behave differently and should not be treated similarly. For example, the identification of M3 AML (acute promyelocytic leukemia) is crucial because it is associated with disseminated intravascular coagulation (DIC), and retinoic acid, in addition to chemotherapy, is the treatment of choice. The two most significant differences between the FAB and the WHO classification are: Patient with recurring clonal cytogenetic abnormalities should be considered to have AML regardless of the blast's percentage. (Arber *et al.,* 2008; Weinberg *et al.,* 2009).

The world health organization (WHO) classification has changed the grouping of ALL to reflect increased understanding of the biology and molecular pathogenesis of the diseases. In addition to discerning the L1 - L3 terms, the new classification characterizes these heterogeneous diseases based upon immunophenotype into 3 basic categories: Precursors B- cells ALL, precursor T- cells ALL, and mature B- cells ALL (Burkitt lymphoma/leukemia) (Jaffe *et al.,* 2001).

Table 2.1: Classification of Cell

CELL TYPE	ACUTE	CHRONIC
Lymphocytic leukemia (or lymphoblastic)	Acute lymphoblastic leukemia (All)	Chronic lymphocytic leukemia (CLL)
Myclogenous leukemia (Myelobid or non-lymphocytic)	Acute Myelogenous leukemia (AML or Myelobastic)	Chronic Myelogenous leukemia (CMK)

(Annie *et al.,* 2013)

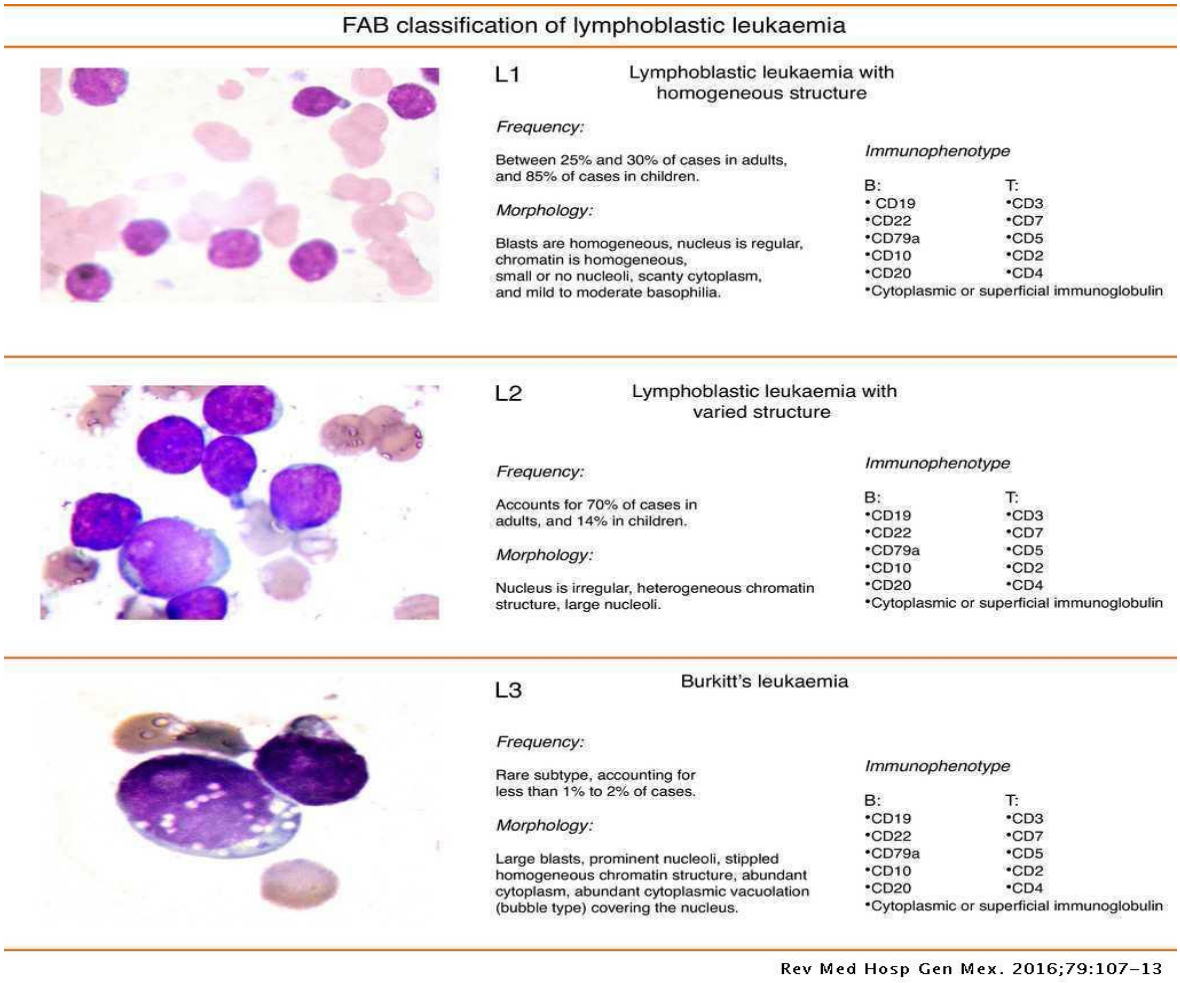

Figure 2.1: Classification of Lymphoblastic Leukaemia

Table 2.0: *French – American – British Classification*

FAB subtype	Name
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

(Arber *et al.,* 2008; Weinberg *et al.,* 2009)

2.3 Incidence of Leukemia

Globally, an estimated 300,000 new cases of Leukemia (2.8% of all new cancer cases) are diagnosed each year (Wan *et al*., 2011). Age specific incidence rates fall gradually from age 0 – 4 and remain stable through childhood and early adulthood, rates rise sharply from around age 55 – 59. The highest rates are in the 85 to 89 age group for female and males. Incidence rates are significantly lower in females than male in most age groups. Five countries with the highest number of Leukemia in females were China with 27384 cases, the United States with 17225 cases, India with 12913 cases, Russia with 5903 cases, and Germany with 4767 cases. The ASUR of leukemia per 100,000 people in the world was 4.7 (in men was 5.6 Sand in women was 3.9) (WCRI, 2019*)*.

In Africa the estimated prevalence of AML is 1% Department of Community Medicine and Primary Care, College of Medicine, University of Lagos. Africa is expected to witness the highest increase in AML cases over the next 10 years. National prevalence of AML in Nigeria is not available; however, studies report that AML accounts for between

19% and 24% of Leukemia that were managed in hospitals. Department of Hematology and Blood Transfusion, Faculty of Clinical Science, College of Health Sciences, University of Jos, Nigeria. However, the incidence of All is higher in those of European descent than in those of Asia and African descent.

In Nigeria, of 146 leukemic patients evaluated, 30 – 1% had CML, 23.3% had All, 22.6% had AML and 21.2% had AML has a global incidence of 351,965 in 2012 with an age-standardized rate of 4-7 per 100,000, a 5-year prevalence of 1.5% and a Male-female ratio of approximately 1.4 (union for international cancer control 2014).Its incidence appears to be on the increase in developed countries(Abiola *et al.,* 2016)

The estimated prevalence of AML in Africa is 1% (Hughes*et al.,*2017). National prevalence of AML in Nigeria is not available; however, studies report that AML accounts for between 19% and 24% of Leukemia that were managed in Hospitals (Jatan*et al.,*2017). Although these cancers are relatively rare, they have a disproportionally large effect on overall cancer survived statistics. It is the most common type of leukemia in adults, yet continues to have the lowest survival rate of all leukemias (Ward *et al.,* 2018).

2.3 Background of Leukemia Treatment

In the 1904s and 1950s treatment of leukemia was based on single agent chemotherapy. In the 1960s multi-agent chemotherapy began dramatically in an eased survivorship in the 1970s and 1980s pre-symptomatic therapy for central nervous system leukemia also improved treatment.

2.3.1 Induction Therapy

Induction Therapy is the initial chemotherapy designed to bring about a remission i.e. to clear the blood and bone marrow of immature white cells (blast cells, blasts) and about a complete remission, or complete response (Ferraraf *et al.,* 2006)

2.3.2 Radiation Therapy (Radiotherapy)

In the early 20th century, leukemia was regarded as an incurable chronic disease. Around 1897, after the discovery of radiation, studies showed that x-rays could reduce the size of tumour. It was discovered that daily doses of radiation over several weeks would greatly improve therapeutic response (American Cancer Society). Shortly after becoming widely used, radiation was shown to be a cause as well as a cure for leukemia.

2.3.3 Chemotherapy

Until after World War II there were no effective treatments for leukemia. One of the most important treatments for cancers, chemotherapy, actually developed from an agent of chemical warfare used by the Germans during War I, mustard gas, which attacks rapidly dividing white blood cell. In the 1940s, there were new treatments such as Aminoprotein, first used by Sidney Ferber of Boston to treat acute childhood leukemia. Aminoprotein is a compound related to folic acid that can be found in ALL patients in remission Aminoprotein prevents the DNA replication in tumor cells used rational drug design to create 6 – mercaptopurin, the first truly affection leukemia drug by 1950, the pair had combined diamino purines and thioguanine, which disrupted DNA synthesis by replacing adenine and guanine (George-Hitchings *et al.,*1905 – 1998; GertrdeEcion *et al.,* 1999)

2.3.4 Deoxyribonucleic Acid (DNA)

The greatest advances of the discovery of Deoxyribonucleic Acid (DNA) by James Waston and Frances Crick. This led to a greater understanding of a detailed mechanism of cancer. (American Cancer Society).

2.3.5 Herbal Medicine

Medicinal Plants are rich source of novel drugs that forms the ingredients in traditional systems of medicine, modern medicines, nutraceuticals, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs. WHO pointed out that more than 80% of world's population depends on plants to meet their primary health care needs. Treatment of cancer usually involves a combination of surgery, radiation therapy and chemotherapy but despite these therapeutic options. Cancer remains associated with high mortality. Various cancer and cancer-related conditions have been treated for ages by local herbalists (Sofowora *et al.,*2008) and many plants have been reported useful in the management of such conditions. As chemotherapy destroys the normal cells along with cancer cells, biological active components from plants are significant and important source of new drugs that are likely to lead to new drugs that will likely lead to new acid better treatments for cancers. Plants have produced many anticancer drugs such as taxanes and vincristine and still serve as a veritable source of new products through the use of standard bioassay methods. According to an estimate, 50% of breast cancer and 37% of prostate cancer patients use herbal products (Richardson *et al.,* 2009).

2.4 Leukocyte Functions

One blood cell that are characterized by its colorlessness (absence of hemoglobin) so they are referred to as white cells. Their main functions are body defense and found in the peripheral blood within the range of (4 – 11 x 10^9/c). A secondary granule with materials that help them to fight pathogen, there are three cells which are granulocytes neutrophils, eosinophils and basophils (Harlands *et al.,* 2007)

Also, the white blood cells (leucocytes) are divided into two broad groups, the phagocyte and the immunocyte. (Granulocyte which include three type of cell – neutrophil, eosinophil and basophil-together with monocyte comprise the phagocyte. Only mature phagocyte cells and lymphocytes, and the precursor cell and plasma cell, which make up the immunocyte population. The function of the phagocyte infection is closely connected with two soluble protein systems of the body immunoglobin and complement. These proteins also involved in blood cell destruction in number of diseases. (Hoff brand *et al.,* 2011)

Basically, Monocytes have a longer lifespan than many white blood cells and help to break down bacteria. Lymphocytes create antibodies to fight against bacteria, viruses and other potentially harmful invaders. While neutrophils kill and digest bacteria and fungi. Both eosinophils and basophils play active pathogenic roles in the inflammation associated with allergic disorders. Both types of cells share a majority of their cell surface structures, and because of those common surface molecules, both cells can be stimulated with a single ligand simultaneously (Hoffbrand *et al.,* 2013)

Knowledge concerning leukocyte functions has increased enormously over the last two decades largely because techniques have become available to assess them. It has

provided insights into many disease entities, as well as severe combined immunodeficiency disease and chronic granulomatous disease, new entities have been discovered and described in which subtle disturbances in white blood cell function are sufficient to cause disease.

All white blood cells or leucocytes are involved in minimum system function, however, the specific roles and function vary for each blood type (Prinyakupt and Phempitiya, 2015)

Diapedesis (also called Extravasations or leukocyte migration to sites of injury or infection is medicated by Pathogen- associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) prevent on microbes and damaged tissue, respectively.

Local inflammatory cell, such as macrophages and mast cells, detect pathogen-associated molecular patterns (DAMPs) and release lytokines as a signal for leukocytes to migrate out of circulation. Histamine and heparin released by per vascular mast cells aid in opening intracellular junctions between the capillary endothelial cells. Furthermore, endothelial cells secrete chemoattracts and express surface markers, including selections, interbrain and cellular adhesion molecules (CAMs) on their lumen that cause leukocyte adhesion, rolling, or rest, and eventual migration into the affected tissues (Borriello *et al.,* 2017).

Assessment of the Evolution of Cancer Treatment Therapies

Cancer therapy has been characterized throughout history by ups and downs, not only due to the ineffectiveness of treatment and side effects, but also by hope and the reality of

complete remission and cure in many cases(Manuel *et al.,*2011). Within the therapeutic arsenal, alongside surgery in the case of solid tumors, are the antitumor drugs and radiation that have been the treatment of choice in some instances. In recent years, immunotherapy has become an important therapeutic alternative, and is now the first choice in many cases. Nanotechnology has recently arrived on the scene, offering nanostructures as new therapeutic alternatives for controlled drug delivery, for combining imaging and treatment, applying hyperthermia and providing directed target therapy, among others. These therapies can be applied either alone or in combination with other components (antibodies, peptides, folic acid, etc.).

In addition, gene therapy is also offering promising new methods for treatment. Chemotherapy, Surgery and radiotherapy are most common types of cancer treatments available nowadays. The history of chemotherapy began in the early 20th century, but its use in treating cancer began in the 1930s. The term "chemotherapy" was coined by the German Scientist Paul Ethrlich, who had a particular interest in alkylating agents and who came up with the term to describe the chemical treatment of disease. During the first and second World Wars, it was noticed that soldiers exposed to mustard gas experienced decreased levels of leukocytes. These lead to the use of nitrogen mustard as the first chemotherapy agent to treat lymphomas, a treatment used by Gilman in 1943.

In the following years, alkylating drugs such as cyclophosphamide and chlorambucil were synthesized to fighter Cancer (Gilman *et al.,* 1946). Kite and Farber designed folate antagonists such as aminopterin and amethopterim, leading to the development of methotrexate, which is in 1948 achieved leukemia remission in children (Farber*et al.,* 1948). Elion and Hitchings developed a drug for solid tumors, 5-fluorouracil (5-fu),

which is up to now an important chemotherapy agent against colorectal, head and neck Cancer (Heidelberger *et al.,*1957). The 1950s saw the design of corticosteroids, along with the establishment of the cancer Chemotherapy National Service Centre in 1955, whose purpose was to test cancer drugs. At that time, monotherapy drugs only achieved brief responses in some types of *Cancers* (Pearson *et al.,*1948). By 1958, the first Cancer to be cured with chemotherapy, chorio carcinoma, was reported (*Li et al.,*1958).

During the 1960s, the main targets were hematologic Cancers. Better treatment was developed, with alkaloids from vinca and ibenzmethyzin (procarbazine) applied to leukemia and Hodgkin's disease (Johnson *et al.,*1963). In the 1970s, advanced Hodgkin's disease was made curable with chemotherapy using the MOMP protocol *(DeVita and Moxleyet al.,*1966), which combined nitrogen custard with vincristine, methotrexate and prednisone, and the MOPP protocol (Devita and Serpick*et al.,*1967) containing procarbazine but no methotrexate. Patients with diffuse large B-cell lymphoma were treated with the same therapy and, in 1975, a cure for advanced B-cell lymphoma was reported using protocol C-MOPP, which substituted cyclophomide for nitrogen mustar. (Devitaet *al.,*1975)

Surgery and radiotherapy were the basic for solid tumor treatment into the 1960s. This led to a plateau in curability rates due to uncontrolled micrometastases. There was some promising publication about the use of adjuvant chemotherapy after radiotherapy or surgery in curing patients with advanced cancer.

There was significant progress in 1978 when higher cure rates of metastatic germ cancer were achieved by combining cisplatin, bleomycin and vinblastine (Einhorn, 1981; Donohue *et al.,*1981).The experience with polychemotherapy drugs act against tumor

cells in different phases of their cellular cycle. One of these solid tumor drugs was CMF (Cytoxan, methotrexate and fluoroucil), a standard therapy for treating breast cancer for over 30 years. Understanding of molecular changes in cancer cells quickly developed after the 1970s. As a consequence, numerous drugs with various mechanisms of action were introduced during the 1980s. Subsequent advances and developments led to liposomal therapy, which places drugs inside liposomes (vesicles made of lipid bilayers), decreasing some of the side effects of chemotherapy such as cardiotoxicity. Examples of liposomal drugs include liposomal doxorubicin and daunorubicin, one of the first steps in nanotechnology – based approaches. The 1990s sparked the beginning of targeted chemotherapy by screening for specific critical molecular targets. These advances in modern chemotherapy and studies on genetics and molecular biology contributed to the ongoing decline in death rates. Data from the genome sequence suggested that many dysfunctions associated with cancer could be due to the abnormal function of some proteinkinases.

The current pharmacological trend has been to developed kinase inhibitors (Manning *et al.,*2002) The first tumors targeted with drugs approved by FDA (Food and Drug Administration) and the EMEA (European Medicines Agency) were renal cell cancer, hepatocellular cancer and gastrointestinal stromal tumors. In recent years, numerous specific tumors have been tested with various kinase inhibitors and there is tend towards combining chemotherapy with these new targeted therapies.

Chemotherapy

Is curative in some types of advanced cancer, including acute lymphoblastic and acute myelogenous leukemia, Hodgkin's and non-Hodgkin's lymphoma, germ cell cancer, small cell lung cancer, ovarian cancer and choriocarcinima.

In pediatric patients, curable cancers include acute leukemia, Burkitt's lymphoma, Wilms' tumor and embryonal rhabdomyosarcoma. Although treatment is not always curative for these cancers, there has been significant improvement in progression – free and overall survival. Another modality of treatment is neoadjuvant therapy, which aims to reduce the size of the primary tumor and prevent micrometastases. This type of treatment improves on move conservative surgical techniques in preserving the functionality of important organs. Neoadjuvant chemotherapy is indicated for anal, breast, lung, gastroesophageal, rectal, bladder, and head and neck cancer, as well as some sarcoma. There are many cancers for which adjuvant chemotherapy has been established with curative effect, and with the new effective drugs and combinations the curability rates are expected to rise even more. Since 1990, the incidence and mortality of cancer have been declining and despite the increase in the elderly population, mortality rates for the United States declined from 2005 to 2007 (Devita, 2008; Chu *et al.,* 2008),

In 1890, Halsted performed the first radical mastectomy, believing that cancer would be more curable if surgical techniques were more aggressive, thus avoiding regional recurrences. He had many followers at that time, but thanks to advances in chemotherapy, radiotherapy, biology and technology, the outlook now is quite different. Radical surgery has been replaced by less extensive operations.

The turn of the 20th century marked the beginning of development of cancer surgery techniques, with the first abdominoperineal resection performed in 1908 by Miles, the first lobotomy being performed in 1912 and the first radical hysterectomy performed by Wertheim in 1906, all carried out under oncological criteria. Additionally, in 1904, Young made the first radical suprapubicprostectomy. Modern surgery has changed significantly, with Halstedian techniques replaced by non-invasive procedures such as laparoscopic colectomy (for the removal of colon Cancer, Videothoracoscopy, radiofrequency ablation and radiosurgery techniques such as Cyberknife (Sherwood *et al.,* 2007). Breast – conserving surgery with sentinel – node removal has been used to improved esthetic results and avoids lymphedema (Singletary *et al.,*2001).

Another example of conservative surgery is the use of laryngoscopic laser surgery in early larygeal cancer (Genden *et al.,* 2007). The most recent development is the Da Vinci, a robotic system for the removal of cancer from prostate and kidney.

The discovery of x-rays and radiation by Beequerel and Rontgen in the late 19th century was the first step towards radiation treatment. Marie Curie's work greatly contributed to the development of radiotherapy. The first cancer case cured exclusively by radiation occurred 1898. After World War II, technological progress allowed charged particles to be propelled through a vacuum tunnel called linac, or linear accelerator, 1960, Ginzton and Kalplan began to use a rotational linear radiotherapy called "clinae 6" which was used to concentrate X-rays more deeply thereby they not affecting the skin as much. The development of modern computers enabled three - dimensional X-ray therapy, such as intensity - modulated radiation therapy (IMRT) using mapping information from Computed Tomography (CT) Scans. This provides a three - dimensional reconstruction,

which helps avoid toxicity. Since the contours of the tumor are targeted and separated from healthy tissues. In 2003, a specific type of IMRT was developed called the Tomo Therapy System. This treatment uses CT- guided IMRT technology that directs the radiation source by rotating it around the patient, which makes the morphological limits of a tumor easier to trace with the beam (Hall *et al.,*2006). Another significant trend is the use of charged particle radiotherapy with proton or helium ions for specific types of patients with melanoma of the urea tract. It is also used as adjuvant therapy for skull base chondroma, chondrosarcoma and spine (usually cervical).

In summary, the lines of development have been fractionated dose delivery, technological advances in X-ray production and delivery and improvement of computer - based treatment planning.

Immunotherapy

The concept of immunotherapy in medicine incorporates the use of components of the immune system, including antibodies (Abs), cytokines, and dendritic cells, to treat various illnesses, such as cancer, allergies, and autoimmune and infectious diseases. Immunotherapy also includes the use of vaccines for the prevention to clinical practice, offering much more specificity, higher efficacy, directed therapy, less toxicity, lower secondary effects and better tolerance.

Although immunotherapy can be used for several illnesses (macular degeneration, autoimmune diseases, etc.) in the case of cancer, the aim of immunotherapy is to kill tumor cells (either directly or indirectly) or to help patients' immune systems destroy tumors. Surgery, chemotherapy and radiotherapy are not specifically directed to tumor cells and may also affect healthy tissue. Antibiotics can provide specificity and lower

toxicity, the first evidence of this potential came in 1982 when a patient suffering from lymphoma responded to treatment using a mouse mab directed specifically against his tumor B lymphocytes, (Miller *et al.,* 1982).

Photodynamic Therapy

Photodynamic therapy (PDT) is a technology that uses a photosensitizer that is activated upon exposure to visible or near infrared (NIR) light, and transfers energy to molecular oxygen, thereby generating reactive oxygen species (e.g. singlet oxygen, free redicals, peroxides). The subsequent oxidation of lipids, amino acids and proteins includes cell death (Chatterjee *et al.,* 2008).

FDA- approved photosensitizers absorb in the visible spectral regions below 700nm, where light penetrate only a few millimeters into the skin. PDT is therefore limited to treatment of certain types of skin cancer and its effectiveness for other tumors is not yet apparent (Faster *et al.,*2010). PDT is usually performed as an outpatient procedure and may be repeated and used in combination with other therapies, such as surgery, radiation and chemotherapy (National Cancer institute, 2011).

Hyperthermia

Hyperthermia, as an anticancer therapy, consists of heating a tumor to inhibit proliferation of cancer cells with the aim of destroying or rendering them more sensitive to the effects of conventional protocols of radiation and chemotherapy. In fact, hyperthermia is currently used as an adjunct therapy to radiotherapy and/or chemotherapy. When cells are heated beyond their normal temperature, they can become sensitized to conventional therapeutic agents such as radiation and chemotherapy. When high temperature is used, typically above 43°C, the heat causes irreparable damage and results

in tumor cell death in a process known as thermal ablation. The success of local thermal ablation consists of destroying the entire tumor mass without damaging adjacent vital structures. This requirement is particularly important for patients with limited reserves of tissue function.

Hyperthermia treatments make use of microwaves, ultrasounds and radio frequency, which can be focused and used locally to target the tumor. A significant advantage of thermal technology is that it is minimally invasive. Mild heat increases blood flow in the tumor, allowing chemotherapy to exert greater effect on cancer cells. By depressing the metabolic activity of target cells, heat also reduces the oxygen demand in the tumor and tumor tissue oxygenation increases, which makes hyperthermia one of the most potent radio sensitizers available (Griffin *et al.,* 2010). Results from clinical trials conducted under quality assurance guidelines have shown hyperthermia to be beneficial in the treatment of several types of solid tumors, including breast cancer, melonoma, sarcoma and locally advanced cervical cancer, with reports demonstrating improved overall survival, as compared to patients who only receive radiotherapy or chemotherapy (Hurwitz *et al.,* 2010.)

Gene Therapy

Gene therapy aims to treat diseases by introducing DNA, RNA, small interfering RNA and antisense oligonucleotides into specific target cells or tissues to restore missing functionality and to eradicate pathogenic dysfunction. The therapeutic gene material is delivered to specific target cells using efficient vectors that aim to sustain stable, regulated gene expression without creating unwanted side effects. Viral carriers, organic cationic compounds, recombinant proteins and inorganic nanoparticles are the four kinds

of carriers currently being explored for gene delivery applications (Nazarali*et al.,* 2008). All of them show advantages and disadvantages, but none of them fulfill the criteria for an ideal vector. Indeed, viruses can be regarded as nanoparticles due to their dimensions, regular geometries and well - characterized surface properties. The most widely used viral vectors for gene transfer include adenoviruses, herpes simplex - 1 viruses, retroviruses and lentiviruses (Raty*et al.,* 2008).

Viruses are very efficient carriers; however, some of them have limited DNA cargo capacity, cause immunogenicity and toxicity and their manufacture is rather expensive. The concept of gene therapy was initially envisioned in the 1970s, but due to the cumbersome nature of the testing required to design and produce effective and safe vectors, gene therapy systems were not fully developed until the early 1980s. The FDA has not yet approved a human gene therapy product for sale, although gene related research is growing rapidly and many clinical trials are ongoing. Most of these are in phase I or II and are aimed at dose determination and toxicity assessment.

Oncolytic Viruses have also been engineered that exploit tumor cells characteristics by replicating them in these target cells as a method for improving the dissemination of biological agents in solid tumors(Alemany*et al.,* 2000.)

For the delivery therapeutic genes encoding proteins with cytotoxic or anti-angiogenic actions, transcriptional targeting using relatable promoters has been explored as a way of restricting transgene expression to an optimal therapeutic window.

To date, there are two gene therapy products available on the market for clinical use, both of which have been approved for cancer treatment in China. Since 2004 China has been

the only country in the world where gene therapy is licensed for practice. These products are adenoviral vectors marketed under the brand names Gendicine™ and Oncorine™ (Peng *et al.,* 2005).

Leukemia

Is a form of cancer that affects blood forming tissues such as bone marrows and it associate with uncontrolled growth of white blood cells (leucocytes) (Hutter*et al.,*2010). Under normal circumstances, blood forming or hematopoietic cells of the bone marrow make leukocytes to defend the body against infectious organisms such as viruses and bacteria. If bone leukocytes are damaged and remain in an immature form, they become poor infection fighters, multiply excessively and do not die off as they should under normal physiological environment.

This condition results in excessive level of functional or immature leukocytes and is called leukemia (Bench *et al.,* 2005).

Over production of Leukemia cells compromise physiological functionality of several other circulatory cells such as oxygen-carrying potential of red blood cells (erythrocytes) and blood clotting associated platelets. Furthermore, continuous production of Leukemia cells overwhelms the bone marrow, enter the blood stream, and eventually invade other parts of the body, such as the lymph nodes, spleen, liver, central and peripheral nervous system in this way, pathogenesis of leukemia is quite different from other cancers, which usually begins in major organs and ultimately spread to the bone marrow (Greaves and Wredemann, 2002).

Leukemia is one of the leading causes of morbidity and mortality worldwide. The incidence of leukemia is highest among white and lowest among American Indians and Alaskan natives (WHO, 2005).

Traditional Medicine is the knowledge, skills and practices based on the theories beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement and treatment of physical and mental illness (WHO, 2017). There are many different systems of traditional medicine, and the philosophy and practices of each are influenced by the prevailing conditions, environments, and geographic area within which it first evolved (WHO, 2005), however, a common philosophy is a holistic approach to life, equilibrium of the mind, body and the environment, and the emphasis on health rather than disease. Generally, the focus is on the overall condition of the individual, ratherthan on the particular ailment or disease from which the patient is suffering and the use of herbs is a core part of all systems of traditional medicine (Engebretson, 2002, Conboy*et al.,* 2007; Righton 2007; Schmidt *et al.,* 2008).

Traditional Chinese Medicine (TCM) is an important example of how ancient and accumulated knowledge is applied in a holistic approach in present day health care.

Over the past 100 years, the development and production of chemically synthesized drugs have revolutionized health care, developing countries still rely on practitioners of herbal medicine for their primary care. In Africa up to 90% and in India 70% of the population depend on traditional medicine to help most of their health care needs. In China, traditional medicine account for around 40% of all health care delivery and more than 90% of general hospitals in China have units of traditional medicine (WHO, 2005).

However, use of traditional medicine is not limited to developing countries and during the past two decades public interest in natural therapies has increased greatly in industrialized countries, with expanding use of ethno-botanicals, in the United State in 2007, about 38% of adults and 12% of children were using some form of traditional medicine (Ernst *et al.*, 2005; Blooms *et al.,* 2008).

The Anti-Leukemic Activity of Traditional Medicinal Plants

Many ethnomedicinal plants have been recommended to treat leukemia disease in the conventional medicine system, conventional medicine pursues the use of natural ingredients, thereby offering reinforcements to the cells regularly to revitalize the normal cell for cancer therapy.

Phytochemicals

Phytochemicals are chemicals derived from medicinal plants and they are usually active ingredients and are mainly secondary metabolites found in plants. Plants synthesize phytochemicals for many reasons, including to protect themselves against insect's attack and plant diseases. The compounds found in plants are of many kinds, but most can be grouped into four major biosynthetic classes: Alkaloids – Phenylpropanols, Polyketides and terpenoids (Welters *et al.,* 2014).

The following four plants were selected for study in this research work

1. Castor oil plant (*RicinusCommunis)*
2. *Azadiracthaindica* (Neem)
3. *Rosy periwinkle*
4. *Psorospernumfebrifugum*

Castor Oil Plant (*RicinusCommunis*)

- A wind-pollution flowering plant of the euphorbia ceae family
- Traditionally used as a laxative and for induction of labour.
- Ricin, a toxic protein of the castor bean, has been used as a biological agent and its immunogenicity has been employed in the development of animus vaccine (a defensive biological agent).
- Castor oil is extracted by pressing the seed
- Is a colourless to pale yellow liquid with sharp taste and odour.
- Its boiling point is 313^0c and density 961kg/m^3

  (*Doan et al.,*2004; O'connell *et al.,*2002)

AzadiracthaIndica **(Neeem)**

A plant in the mahogany family, it grows in tropical regions.

- It is noted for its drought resistance.
- Almost every part of the neem free is used in traditional medicine and modern medicine as insecticides, insect repellant, antimalarial, ant diabetics worm expeller, antiulcer and antifungi, as well as anticancer properties (Subapriya *et al.,* 2005; Bose *et al.,* 2007)

Rosy Periwinkle (Catheranthusroseus)

It is a perennial flowering herb that grows to about 2 feet fall. Rosy Periwinkle has been historically used by:

- Indians to treat wasp stings.
- Chinese to treat malaria and diabetes
- Jamaicans to treat diabetes.

(Duke *et al.,1985;* Dobelis *et al.,*1986)

Christmas Berry (*Psorospernum Febrifugum*)

Locally called Legunoko (Yoruba Southwest Nigeria)

- It is a flowering plant species in the genus *Psorospernum*it grows in open woodland over a wide range of altitude.
- It has about 28 distinct species.

Widely used in African traditional medicine for treatment of fevers and skin related problems. Modern research has shown the presence of various medically active compounds (Burkil *et al.,* 2004; Ruffo *et al.,* 2002)

2.4.1 Interpretation of the Full Blood Count

The full blood count (FBC) is perhaps the single most common investigation performed in medical patients. It has the potential, when interpreted carefully and in relation to the clinical history, to assist in diagnosis and management. Clinicians are after alerted to the presence of a primary hematological disorder. (Mike-Leach *et al.,*2014).

It is a series of blood test that provides information about the components of blood including red blood cells, white blood cells, and platelets. Automated machines rapidly count the cell types. The CBC test results can help diagnoses and determine these severity (Braunwald *et al.,* 2001).

Blood count of various types have been used for clinical purposes since the 19th century. Automated equipment to carry out complete blood counts was developed in the 1950s and 1060s (Verso *et al.,* 1962).

2.4.2 Total white blood cell: All the white cell types are given as a percentage and as an absolute number per liter. Iron deficiency anemia shows up as a low RBC count. Hemoglobin, the amount of hemoglobin in the blood, expressed in grams per deciliter (g/dl). A low level of hemoglobin is a sign of anemia,(Dungdale *et al.,* 2012).

2.4.3 Hematocrit: The proportion of blood volume occupied by red blood cells, is typically about three times of hemoglobin concentration measured in g/dl. for example, if the hemoglobin is measured at 17g/dl, that compares with a hematocrit of 51%. (Barr*et al.,* 2007).

2.4.4 Hematocrit or Packed Cell Volume (PCV)

This is the fraction of whole blood volume that consists of red blood cells (David and Dugdale, 2012). Red blood cell indices include. Mean corpusular Volume (MCV) the average volume of the red cell, measured in femolitres.

2.4.5 Mean CorpusularHaemoglobin (MCH)

The average volume amount of hemoglobin per red cell, in pictograms, mean corpuscular haemoglobin concentration (MCHC) the average concentration of haemoglobin in the cells, Red blood cells distribution width (ROW) the variation in cellular volume of the RBC population (Dugdale *et al.,* 2012). A complete blood count with differentials / platelet with all include: Neutrophil granulocytes: May indicate bacterial infection. May also be raised in acute viral infections. Because of the segmented be raised in acute viral infection. Because of the segmented appearance of the nucleus, neutrophils are sometimes referred to as 'seg'. The nucleus of less mature neutrophils be not segmented, but has a bind or roof like shape, less mature neutrophils those that are recently been released from the bone marrow into the blood stream are known as "bands" or "stabs", stab is a German term for rod.

2.4.6 Lymphocytes

Higher with some viral infections such of glandular fever and also raised in chronic lymphocytic leukemia (CLL) can be decreased by the infection. In young children under age 8, lymphocytes are more common than neutrophils.

2.4.7 Monocytes

May be raised in bacterial infection, tuberculosis, malarial, Rocky Mountain Spotted fever, monocytic leukemia, chronic ulcerative colitis and regard enteritis, Eosinophil granulocyte: Increased in paralytic infections, asthma, or allergic reaction.

Basophil granulocytes may be increased in bone marrow related conditions such as leukemia lymphoma, a manual count will also give information about other cells that are not normally present in peripheral blood, but may be released in certain disease processes. Platelets numbers are given, as well as information about their size and the range of sizes in the blood (Dugdale *et al.,*2012).

2.5 Leucocyte Functions and Assessments

2.5.1 Chemotaxis

Chemotaxis enables microorganisms to move according to chemical gradients. *Miguel et al,* 2018. Chemataxis is based on the action of chemosensory pathways. Signalling is initiated by the recognition of signals (or chemo effectors) at chemoreeptors that form a tenary complex with the sensor keinale Che A and the adaptor protein Che W. Signal binding to the chemoreceptor creates a molecular stimulus that modulates Che A autophosphorylation and consequently transphosphyorylation of the response regulator Che Y (Wadhams and Armifage, 2011; Sourjik and Wingreen 2012; Porkinson, *et al.,* 2015.

In addition to taxis towards chemical gradients, many microorganisms perform energy taxis, a process that is also meditated by chemoreceptors and permits microorganisms to move towards optimal condition for metabolic activity (Scheweinitzer and Jossenhaas 2010).

2.5.2 Chemoattractants and Chemorepellent

Chemoattractants and Chemorepellents are substances that not of organic origin with chemo inducing impact in cells with motility. The ligands from chemotaxis generate chemical concentration gradients that microorganisms, eukaryotes and prokaryotes could move to or away from respectively (Xu *et al.,* 2016).

2.5.3 Methods Used to Study Chemotaxis

During the past 40 years of chemotaxis studies, experimental methods became more sophisticated and precise. However, new methods usually do not replace the old methods but rather extend them and provide insights into particular features of chemotaxis at different scales.

2.5.4 Swarm Plate Assay

One of the first methods to quantitatively study the chemosensitive behavior was the swarm plate assays. A petiduh containing metabolizable attractant, salts needed for growth and soft agents (a low enough can contraction so that the bacteria can (Swim) is inoculated in the center with the bacteria. As the bacterial grow, they consume the local supply of attractant, thus creating a gradient, which they follow to form a ring surrounding the inoculum (Adler *et al.,* 1966). Measuring the diameter of the swarm ring after a fixed time gives an estimate of chemotactic efficiency of the bacteria.

2.5.5 Phagocytosis

Phagocytosis is the process by which phagocytes ingest foreign material killing or bacteria destruction is closely associated with and directly follows upon phagocytosis(Venden-Brock *et al.,*2016). Phagocytosis is an important process for nutrition on unicellular organisms, while in multicellular organisms it is found in

specialized cells called phagocytes. Phagocytosis consists on recognition and ingestion of particles larger than 0.5m into a plasma membrane derived besides, known as phagosome (Gordon *et al.,* 2016)

In mammal's professional phagocytes (i.e., Macrophages, dendritic cells (DCs), angrambcytes) derived from common mybid progenitor cell. Specific combinations of indirective events instruct differentiation of these common progenitors to their mature progeny (*Gordon et al.,* 2016).

The process of phagocytosis involves several phases;

(i) detection of the particle to be ingested

(ii) activation of the internalization process

(iii)formation of a specialized vacuole called phagosome and

(iv)phagosome maturation.

Macrophages and neutrophils are attracted to the point of inflammation or infestation by the chemical combination released by the offending agents (bacterial) and infected tissues or by the microorganism and the complement cascade of the circulatory system. Neutrophils has the ability to accidentally ingest particles they find on their navigation path. For phagocytosis to be complete, the particle and the phagocytes must be seen in adhesion to one another which is basically determined by the chemical nature of the particle. If there is failed attempt for phagocyte to the bacterium for onward phagocytosis. Encapsulated bacteria such as Bacillus anthracic are more difficult to engulf as the phagocyte attempt to push them off and when they accidentally get sucked on the lining of a blood vessel, they might be ingested with ease (surface phagocytosis). Some bacteria might not be engulfed until the coating of their surface with special antibodies. These

types of antibodies are important in initiating immunity. Neutrophils have been linked with binding to oxoeicosanoid receptor (Powell and Rokochi, 2015).

2.5.6 Respiratory Burst Activity (Oxidative Burst)

Is the rapid release of reactive oxygen species (superoxide anion and hydrogen peroxide) from different types of cells. (Ping Yeng *et al.,*2016). Usually, it denotes the release of these chemicals from immune cells, such as neutrophils and macrophages because they are infected by different bacteria or fungi. It is a crucial reaction that occurs in phagocytes to degrade internalized particles and bacteria. NADPH Oxidase, an enzyme family widely expressed in many types of cells produces superoxide, which spontaneously recombines with other molecules to produce reactive free radicals. To combat infection, immune cells use NADPH Oxidase to reduce O^{2-} to an oxygen free radical and then H_2O_2. Neutrophils and monocytes use myeloperoxidase to further combine H_2O_2 with Cl^- to produce hypochlorite, which plays a role in destroying bacteria. There are many sources of reactive oxygen species in living cells, such as mitochondria and Xanthine Oxidase (Benna *et al.,*2017).

CD_{10} (Nep0072ilysin)

Neprilysin, also known as membrane metallo-endopeptidase (MME), neutral endopeptidase (Nep), cluster of differentiation 10 (CD10), and common acute lymphoblastic leukemia antigen (CALLA) is an enzyme that in humans is encoded by the MME gene. Neprilysin is a Zinc dependent metalloprotease that clears peptides at the amino side of hydrophobic residues and inactivates several peptides hormones including glucagon, enkephalins, substance P, neutotensin, oxytocin, and bradykinin.

Neprilysin is expected in a wide variety of tissues and is particularly abundant in kidney. It is also a common acute lymphocytic leukemia antigen that is an important cell surface marker in the diagnosis of human acute lymphocytic leukemia (ALL). This protein is present on leukemia cells of pre-B phenotype, which represent 85% of cases of ALL Hematopoietic progenitors expressing CD10 are considered "common lymphoid progenitors", which means they can differentiate into T, B or natural killer cells (Kohn *et al.,*2012).

CD10 is of use in hematological diagnosis since it is expressed by early B, pro-B and pre-B lymphocytes, and by lymph node germinal centres(Singh*et al.,*2011). Hematologic diseases in which it is positive include ALL, angioimmunoblastic T cells lymphoma, Burkitt lymphoma (variable), follicular centre cells (70%) hairy cell leukemia (10%), and myeloma (Some). It tends to be negative in acute myeloid leukemia, chronic lymphocytic leukemia, mantle cell lymphoma, and marginal zone lymphoma (Papandreou and Nanus *et al.,* 2010).

CD_{20}

Is a membrane – embedded surface molecule which plays a role in the development and differentiation of B – cells into plasma cells. A B cells marker, is a nonglycosylated 33-37 KDaphosphoprotein member of the MS4A family, which forms a tetraspan membrane – bound protein. The extracellular portion of CD20 is 44 amino acids, in length and provides the docking site for anti CD20 MAbs binding (Morcos et *al.,* 2019). CD20 expression is initiated at the pre-B cell stage of development and remains present until terminal differentiation into a plasma cell. The biological activity of CD20 is not fully

elucidated; however, it is thought to act as an ion channel and a store – operated Ca^{2+} channel (O'Keefe *et al.,* 2018; Li *et al.,* 2015).

CD20 is also thought to function as a modulator of cell growth and differentiation, and to initiate intracellular signals. Hyper cross – linking of MAb bound to CD20 with a secondary Ab result in the mobilization of Ca^{2+} from intracellular stores (Jak*et al.,* 2011). Although the function of CD20 is not fully understood, it clearly has dynamic cellular activity and remains an ideas specific therapeutic target for B cell malignancies. CD20 is a transmembrane protein, originally identified as a B-cell surface marker involved in Ca^{++} channeling, B-cell activation, and profiling, CD20 has been identified as one of the top 22 genes in melanoma that defines the aggressive nature of the disease (Bittner *et al.,* 2000). CD20 is expressed in a vast majority of mature B-cell neoplasms and some cases of B-lymphoblastic leukemia/lymphomas, plasma-cell myelomas, Hodgkin lymphomas, T-cell neoplasms, and AMLs.

A positive CD20 represent an aberrant immunophenotype, but the presence of monoclonal T cells receptor (TCR) gene rearrangements and negatively for immunoglobulin heary chain gene rearrangement indicate that the tumor is a T cell lymphoma.

TGF-BETA 1

Transformation Growth Factor- Beta (TGF β)

TGF β is a secreted cytokine, which intricately controls a plethora of physiological and pathological process during development and concinogenesis. It is part of family of pleiropic and multifunctional regulatory cytokines. It has effects on cell growth,

development and differentiation, as well as playing important roles in wound repair and immune responses.

Receptor binding of the TGF- β super familyoflit and results in activation of Amad proteins, leading to signal transduction and regulation of gene expression (Schimere and Hill, 2007)

Pathway genes for TGF- β have been found to be universally present across metazoan species (Vizzzini *et al.,* 2016) and TGF- βlike proteins have been identified in a number of invertebrate species including C. savidnyi and C. intestinalis. (Vizzini *et al.,* 2016, Schmierer and Hill, 2007; Corporea*u et al.,*2011)

TGF- βis a key cytokine secreted by powanchymal cells also by infiltrating cells, (e.gLymphocyte monocytes/macrophages and platelits) TGF- β production by irradiated cell is increased after exposure to a stress such as irradiation through various mechanisms, including activation of the ERK-Specific pathway in fibroblastic or exposure to inflammatory cytokines such as TNF x via activation of Activator protein- I transcription factor (Sulliva*n et al.,* 2009.

TGF- βi plays a role in regeneration of damaged tissues and organs, bones formation, metastasis), and the generation of histopathological lesions like fibrosis (*ota, et al.,* 2013)

TGF- β, usually stimulates the proliferation of certain cell types of mesenchymal origin such as hepatic stellate cells, fibroblasts and osteoblasts both in vitro and vivo (Ibelgaufts, 2007)

TGF- β in Leukemia.

TGF- β is a potent inhibitor of human myeloid leukemia cells (Zuckerman *et al*., 2007) in acute promyelocytic leukemia (APL) t (15:17) translocation in which the retinoic acid receptor x (RARx) gene on 17q12 fuses with a nuclear regulation factor PML on 15q12 results in the fussionprotein PML-RARx. (Jones *et al.,* 2009). In clronic myeloid leuccemia (CML) t (9:22) the so-called Philadelphia chromosome results in the formation of BCR-ABL fussion gene. The fussion proteins is an acute tyrosine kinase which enhances resistance of malignant cells to TGF- β induced growth inhibition and apoptosis(Roseep*et al.,* 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This is an experimental Research study design which was carried out in four places and phases.

Phase One include preparation of the selected plants extracts, induction of leukemia in the albino rats using established method by Akanni *et al.*, 2012 and confirmation of leukemia using full blood count and CD_{10} and CD_{20}.

Phase Two includes, assessment of anti-oxidants activities of the extracts.

Phase Three is the assessment of leucocyte functions using chemotaxis assay, viability assay and phagocytic assessment.

Phase Four is assessing the possible mechanisms of action of the efficacious plant.

3.2 Study Centres

a. The Faculty of Pharmacy, OAU, Ile-Ife

b. The Plant Science Department, OAU, Ile-Ife

c .Animal House, Lauthec University, Osogbo

d. University of Benin Teaching Hospital3.2 Ethical Clearance

Approval for the study was obtained from the Research Ethics Committee of the Osun State Ministry of Health, Osogbo, Osun State. This was carried out in strict compliance with the guidelines for the care and use of animals for research which is in line with that set by World Health Organization .

3.3 Materials

Wistar rats, aqueous extract of *Ricinuscommunis*, *Rosy periwinkle*, *Azadirachtaindica*, and *psorospermumferbrigum,* wooden cages, oral cannular, and hand gloves. K_2EDTA bottles, Heparin bottles, Needles and syringes, Razor blades, methylated spirit, water, commercial poultry feed, permanent marker measuring cylinder, distilled water and Whatman Filter paper.

3.4 Wistar Rats

The Wistar rat is an out bred stock, used in all fields of medical and biological research. Its longevity and high rate of spontaneous tumors make it an ideal choice of ageing studies. It is an wistar strain, easy-to-handle, it is however slower learner than long Evans rat. The albino rat has been a top choice for many leading Pharmaceutical, chemical and contract research organizations due to the many advantages if offers, such as smaller body size and high rates of survival (Weber *et al.,* 2011)

3.5 Reagents

Distilled water, Benzene chromosolv, 5 flourousil (Anti-cancer agent).

3.6 Equipment

The major equipment's that were used in this study are as follows: weighing balance and bench centrifuge, automatic pipette, micropipette, microtiter plate reader SYSMEXKX – 2IN

3.6. Methodology

3.6a Collection of the Plant Materials

The fresh leaves of *PsorospermFebrifugum*, *Rosy Periwinkle*,*Azadiractha India* and seeds of *RicinusCommunis* used were collected from open vegetation and surrounding

field of college of Basic Medical Science (LAUTECH), Osogbo, Osun State. The plants were transported in different clean polythene bags to ObafemiAwolowo University, Ile-Ife for plant identification and authentication by a botanist at the Department of Plant Science, ObafemiAwolowo University, Ile-Ife, Osun State.

PREPARATION OF PLANT EXTRACTS

200g of dried powder of, Rosy periwinkle, *Psorosperm febrifugum, Azadiractha indica* leave were subjected to successive extraction with water and 200g of *Ricinuss communis* seeds were subjected to successive extraction with hexane in boxhlet apparatus. The extract was concentrated under reduced pressure in rotary evaporator. The yields of the different plant species were labelled and recorded and stored in a clean glass bottle for further phytochemical analysis using were established protocols and methods.

DETERMINATION OF ACUTE TOXICITY (LD_{50})

Healthy Wistar rats were fasted overnight, but with free access to water. Animals were randomly divided in five groups (n=5/group). The first group (control group) received orally distilled water. Groups 2,3,4 and 5 were orally treated with Ricinus communus in doses of 10mg, 100mg, 1000mg, 1600mg, 2900mg/kg and 5000mg/kg respectively. Animals were observed for general behavioral and body weight changes hazardous symptoms and mortality for a period of 14 days after treatment.

LD_0 = The highest dose that gave mortality.

D_{100} = The smallest dose that gave mortality

The acute toxicity study of the plant extracts was carried out using the method of Lorke (1983).

Step 1 **A** **B** **C**

111	111	111

10mg/kg 100mg/kg 1000mg/kg

Step 2 **D** **E** **F**

1 1 1

1600mg/kg 2900mg/kg 5000mg/kg

LD_{50} = x

D_o = The highest dose that gave no mortality

D_{50} = The smallest dose that gave no mortality

1:10

is in 10ml (To be prepared stock solution)

Sample 1: ***Ricinus Communis***

Step 1 111 111 111

10mg/kg 100mg/kg 1000mg/kg

Step 2 1 1 1

1600mg/kg 2900mg/kg 5000mg/kg

D_0 = 1000mg/1kg

D_{100} = 2900mg/1kg

LD_{50} =

LD_{50}= $\sqrt{DO\ x\ D100}$

= $\sqrt{100mg/kg\ x\ 2900mg\ /\ kg}$

= 1702mg/kg

The dose to be administered for the routine experiment is $1/10^{th}$ the value of LD_{50}.

$\frac{1}{10}\ of \frac{1702}{1}$

= 170.2mg/kg

= 170mg/kg (Normal Dose)

V = $\frac{Dose\ mg}{kg}$ x $\frac{weight\ g}{concentration}$

1g of extract in 10ml in distilled water 1000mg in 10ml.

= 1000mg/ 10ml

= 100 mg/ml

Low dose = ½ of normal dose (170mg/kg)

= ½ x 170mg/kg

= 85mg/kg Group A

High dose = 2 xnormal dose.

= 2 x 170mg/kg

= 340mg/kg Group B

Ricimus Communis

Low dose – 85mg/kg.

High dose = 340mg/kg

Sample 2 ***Rosy Periwinkle***

Step 1 111 111 111

10mg/kg 100mg/kg 1000mg/kg

Step 2 **1** 1 1

1600mg/kg 2900mg/kg 5000mg/kg

$LD_{50} = \sqrt{DO \; x \; D100}$

$LD_{50} = \sqrt{100 \; x \; 1000 \; (mg/kg)}$

= 316mg/kg

$\frac{1}{10}$ th of 316mg/kg

=30mg/kg

Low dose = 15mg / kg

High dose = 60mg/kg

Sample 3: ***Psorospermum febrifugum***

STEP 1

111	111	111
10mg/kg	100mg/kg	1000mg/kg

STEP 2

1	1	1
1600mg/kg	2900mg/kg	5000mg/kg

$LD_{50} = \sqrt{Do\ x\ D100}$

D_0 = 5000mg/kg

D_{100} = 6000mg/kg.

= $\sqrt{5000mg/\ kg\ x\ 6000\ mg/kg}$

= 5477mg/kg

= 5.5g/kg

$\frac{1}{10}$th 5477mg/kg

= 547.7 mg/k

548mg/kg

Low dose ½ x 548mg/kg

= 274mg/kg

High dose 2 x 548 = 1096mg/kg

Sample 4: ***Azadiractha indica***

STEP 1

111	111	111
10mg/kg	100mg/kg	1000mg/kg

STEP 2

1	1	1
1600mg/kg	2900mg/kg	5000mg/kg

$LD_{50} = \sqrt{Do\ x\ D100}$

$= \sqrt{100\ x\ 1600}$

$= \sqrt{16000 mg/kg}$

= 400mg/kg

$\frac{1th}{10}$ of 400 mg/kg = 40mg/kg

Low dose ½ x 40mg/kg

= 20mg/kg

High dose 2 x 40 = 80mg/kg

= 80 mg/kg

Sample 1

Low dose = 85mg/kg GP A

High dose = 340 mg/kg GP B

Sample 2

Low dose = 15mg/kg

High dose = 60mg/kg

Sample 3

Low dose = 274mg/kg

High dose = 1096mg/kg

Sample 4

Low dose = 20mg/kg

High dose = 80mg/kg

In the first phase, nine Wister rats were randomly divided into three groups of the rats per group and were given 10mg, 100mg and 1000mg extract per kg body weight orally (via cannula) respectively and replicate for each of the four plant extracts.

The Wister rats were observed four sighs of advance effects and death for 24 hours and then weighed daily four 14 days.

In the second phase of the study, the procedure was repeated using three Wister rates randomly divided into three groups of one rat each, given 1600mg, 2900mg and 5000mg extract/kg body weight, respectively. The rats were also observed for signs of toxicity, mortality and weighed for 14 days. Tijani *et al*., (1986). The procedure was carried out with control group given distilled water. The procedure was done for the four extracts.

Plant extract	**LD $_{50}$**
Ricimus Communis	*340mg/kg*
Rosy Periwinkle	*30mg/kg*
Psorospermum febrifugum	*548mg/kg*
Azadiratha indica	*40mg/kg*

CHOICE OF DOSAGE OF EXTRACT ADMINISTRATION

Based on 10ml/kg volume selection, required dose volume for a 100g rat can be calculated as follows:

$$\frac{100g}{1000g} \times 10ml = 1ml$$

NB: 1kg = 1000g

Based on 20ml/kg volume selection required dose volume for a 100g rat can be calculated as follows

$$\frac{100g}{1000g} \times 20ml = 2ml$$

Dosage calculation and preparation of stock solution of guide plant extract (with selected doses, 200mg/kg and 400mg/kg

For a rat weighing 120g can be calculated as follows:

Step 1 Dosage Calculation

Body weight of animal = 120g

$$\text{Dosage in mg} = \frac{\text{Body weight of animal (g)}}{1000} \times \text{dose (mg)}$$

$$\text{Dosage in mg } \frac{120g}{1000g} \times 200(mg) = 240mg$$

Step 2 Dissolution of dose in a suitable vehicle for oral administration from the OECDS guidelines, 120g rat requires 24mg of the circle plant extract which should be constituted in not more than 1.2 ml of normal saline

:. 120g = 24mg = 1.2ml of normal saline

Bulk volume of the stock solution required for large number of animal can be calculated by multiplying both sides by a constant value as follows:

24mg = 1.2 ml

40 x 24mg = 40 x 1.2ml

$$960mg \text{ of circle plant extract will be dissolved in 48ml of N/saline} = \frac{960mg}{48ml} = 20mg/ml$$

In lorkes method of determination of oval acute toxicity (LD_{50}) of a suspected toxic substance is divided into two places phases, phase I & phase III, if there is no death of the animals when administered 10mg/kg, 100mg/kg and 1000mg/kg respectively in places. The phase III is expected to have 200mg/kg, 3000mg/kg, 4000mg/kg and 5000mg/kg respectively. The LD_{50} will be geometric mean of 100 x 1000.

3.6b Animal Grouping

Thirty-six Albino rats weighing between 80 – 120mg were randomly allocated into six groups labelled (A-F), each group comprises of 6 rats. This grouping was for each plant extract making a total of 144Albinorats for the study.

GROUP A: Received distilled water (0.5m/kg) + normal saline (0.5ml/kg) orally for the four weeks.

GROUP B: Were given benzene chromosolv (0.2ml at 1:10 dilution water ½ propanol) 50/50 v/v in water with daily via tail vein for 4 weeks.

GROUP C: Received aqeous herbal extract (10mg/kg body weight) via gastric garage daily using oral cannular for 4 weeks. Thus, this group is further subdivided into group C_a- *Azadirathaindica* (10mg/kg body weight) C_b-*Psorospernumfebrifugum*, C_c-*RicimusCommunim* and C_d- Rosy Periwinkle).

GROUP D: Was administered (5 – fluorouracil, which is an, antineoplastic, antimetabolite, is effective in the palliative management of carcinoma).

GROUP E: Were pretreated with aqeous extracts of all the medicinal plants separately for four weeks prior to the administration of benzene chromosolor (0.2ml at 1:10 dilution (water / 2 - propanol) 50/50 v/v in water) for 4 weeks. Thus this group was further

subdivided to group E_a – Azadiractha indica, (10mg/kg body weight), E_b – *Psoropernum febrifugum*, E_c – *Rcinus communis,* E_d – *Rosy periwinkle*).

GROUP F Were given benzene chromosolv (0.2ml at 1:10 dilution water ½ - propanol) 50/50 v/v in water 7 daily via tail vein for 4 weeks being treated with aqueous extract (10ml at 1:10 dilution water / 2-propanol) for another 4 weeks. The rats were sacrificed by cervical dislocation at the end of treatment period.

The experimental design was the same for all extracts i.e. *Ricinuscommunis, Psorosperumfebrifugum,* and *Rosy Periwinkle*.

3.6c Animal Treatment

Experimental procedures involving the animal and their care was conducted in conformity with international, national and institute guidelines for the care of laboratory animal in biomedical research promulgated by the Canadian Council of Animal Care (CCAC) 1985). The Animals were allowed to acclimatize for a period of one week before the commencement of the experiment. The animals were maintained at room temperature 28^0c (±2) with 12 hours light/dark cycle and also allowed unrestricted access to water and rat feed.

Prior to any procedure on the animals, they were weighed and the weight recorded. They were allowed to fast for 24hrs before the experiments.

3.6d Administration of Benzene Solution to Induce Leukemia

Benzene chromosolv, a product of sigma - Aldrich (lot STED 71354 > 99.9%) was diluted in water for injection at a concentration of 1ml of the benzene chromosolv to 9ml of water. Exactly 0.2ml was administered through the vein at the tail 48 hourly for 4 weeks (Huff *et al.,*2007; Akanni*et al*., 2014; Akanni*et al*., 2012).

3.6e Administration of Plant Extract

Exactly 1ml of the extracts was administered orally once daily by garage with the aid of cannular to the groups based on the experimental design over a period of 4 weeks. Assessment of induction and confirmation of leukaemia using full blood count.

3.6f BLOOD SAMPLE COLLECTION.

After specified period of time the rats in each of the groups were re-weighed to determine their final weight. The blood sample was collected from their tail vein using 1mlsyringe for haematological parameters for both automation (SYSMEX KX-2IN) and manual techniques. Film appearance and morphological changes were observed and compared among the groups. The blood features were captured using digital microscope.

3.7. Estimation of Haematological Parameters using Sysmex KN-2IN auto-analyser

Haematological parameters were assessed by flow cytometry (direct current method) using suitable cell packs according to the manufacturers' specification for the desired cell population on the SYSMEX K X – 2IN auto-analyser.

Operating Principle of Automated Hematology Analyser.

The impedance when a particle in a conductive liquid passes through a small aperture. The number of pulses correlates to the C and PLT).

3.8. Differential White Blood Cell Count

The Leishman staining technique was used to complement the automation estimation result. The technique was applied as described. Bain and Lewis, (2004).

The stained slides were allowed to dry and examined using the battlement method with an oil immersion objective (x 100). One hundred consecutive leucocytes indicating various types encountered were counted and recorded.

3.9. Experimentation

Phase 1

3.9.1 Preparation of Plant Extracts

Two hundred grams (200g of dried powder of, Rosy periwinkle, *Psorospermfebrifugum, Azadiracthaindica* leave were subjected to successive extraction with water and 200g of *Ricinusscommunis* seeds were subjected to successive extraction with hexane in boxhlet apparatus. The extract was concentrated under reduced pressure in rotary evaporator. The yields of the different plant species were labelled and recorded and stored in a clean glass bottles for further phytochemical analysis using were established protocols and methods.

3.9.2 Determination of Acute Toxicity (LD_{50})

The acute toxicity study of the plant extracts was carried out using the method of Lorke (1983).

Step 1	**A**	**B**	**C**
	111	111	111
	10mg/kg	100mg/kg	1000mg/kg
Step 2	**D**	**E**	**F**
	1	1	1
	1600mg/kg	2900mg/kg	5000mg/kg

LD_{50} = x

D_o = The highest dose that gave no mortality

D_{50} = The smallest dose that gave no mortality

1:10

is in 10ml (To be prepared stock solution)

Sample 1: ***RicinusCommunis***

Step 1 111 111 111

10mg/kg 100mg/kg 1000mg/kg

Step 2 1 1 1

1600mg/kg 2900mg/kg 5000mg/kg

D_0 = 1000mg/1kg

D_{100} = 2900mg/1kg

LD_{50} =

LD_{50}= $\sqrt{DO\ x\ D100}$

= $\sqrt{100mg/kg\ x\ 2900mg\ /\ kg}$

= 1702mg/kg

The dose to be administered for the routine experiment is 1/10th the value of LD_{50}.

$\frac{1}{10}\ of\ \frac{1702}{1}$

= 170.2mg/kg

= 170mg/kg (Normal Dose)

V = $\frac{Dose\ mg}{kg}$ x $\frac{weight\ g}{concentration}$

1g of extract in 10ml in distilled water 1000mg in 10ml.

= 1000mg/ 10ml

= 100 mg/ml

Low dose = ½ of normal dose (170mg/kg)

= ½ x 170mg/kg

= 85mg/kg Group A

High dose = 2 xnormal dose.

= 2 x 170mg/kg

= 340mg/kg Group B

RicimusCommunis

Low dose – 85mg/kg.

High dose = 340mg/kg

Sample 2 ***Rosy Periwinkle***

Step 1 111 111 111

10mg/kg 100mg/kg 1000mg/kg

Step 2 **1** 1 1

1600mg/kg 2900mg/kg 5000mg/kg

$LD_{50} = \sqrt{DO \; x \; D100}$

$LD_{50} = \sqrt{100 \; x \; 1000 \; (mg/kg)}$

= 316mg/kg

$\frac{1}{10}$ th of 316mg/kg

=30mg/kg

Low dose = 15mg / kg

High dose = 60mg/kg

Sample 3: ***Psorospermumfebrifugum***

STEP 1 111 111 111

10mg/kg 100mg/kg 1000mg/kg

STEP 2 1 1 1

1600mg/kg 2900mg/kg 5000mg/kg

$LD_{50} = \sqrt{Do \; x \; D100}$

D_0 = 5000mg/kg

D_{100} = 6000mg/kg.

= $\sqrt{5000mg/\ kg\ x\ 6000\ mg/kg}$

= 5477mg/kg

= 5.5g/kg

$\frac{1}{10}$th 5477mg/kg

= 547.7 mg/k

548mg/kg

Low dose ½ x 548mg/kg

= 274mg/kg

High dose 2 x 548 = 1096mg/kg

Sample 4: ***Azadiracthaindica***

STEP 1

111	111	111
10mg/kg	100mg/kg	1000mg/kg

STEP 2

1	1	1
1600mg/kg	2900mg/kg	5000mg/kg

LD_{50} = $\sqrt{Do\ x\ D100}$

= $\sqrt{100\ x\ 1600}$

= $\sqrt{16000mg/kg}$

= 400mg/kg

$\frac{1th}{10}$ of 400 mg/kg = 40mg/kg

Low dose ½ x 40mg/kg

= 20mg/kg

High dose 2 x 40 = 80mg/kg

= 80 mg/kg

Sample 1

Low dose = 85mg/kg GP A

High dose = 340 mg/kg GP B

Sample 2

Low dose = 15mg/kg

High dose = 60mg/kg

Sample 3

Low dose = 274mg/kg

High dose = 1096mg/kg

Sample 4

Low dose = 20mg/kg

High dose = 80mg/kg

In the first phase, nine Wister rats were randomly divided into three groups of the rats per group and were given 10mg, 100mg and 1000mg extract per kg body weight orally (via cannula) respectively and replicate for each of the four plant extracts.

The Wister rats were observed four sighs of advance effects and death for 24 hours and then weighed daily four 14 days.

In the second phase of the study, the procedure was repeated using three Wister rates randomly divided into three groups of one rat each, given 1600mg, 2900mg and 5000mg extract/kg body weight, respectively. The rats were also observed for signs of toxicity,

mortality and weighed for 14 days. Tijani *et al*., (1986). The procedure was carried out with control group given distilled water. The procedure was done for the four extracts.

Plant extract	**LD $_{50}$**
Ricimus Communis	*340mg/kg*
Rosy Periwinkle	*30mg/kg*
Psorospermumfebrifugum	*548mg/kg*
Azadirathaindica	*40mg/kg*

3.9.3 Blood Film

Principle

The basic component (cytoplasm) of the cell is stained by acidic dye (eosin) while the basic stain (methylene blue) is attached to the acidic part of the cell (the nucleic acid and nucleoproteins) of the nucleus. A pH to the alkaline side accentuates the azure component (methylene blue) at the expense of the eosin and a pH to the acidic side accentuates the acidic component (eosin) at the expense of methylene blue.

Procedure

Thin blood film was made by placing a drop of blood in the center line of a slide about 1 or 2cm from one end of a clean, grease free slide. A smooth edge spreader with a narrow breadth to the slide was placed at an angle of 450c to the slide.

The spreader was moved back to the drop of the blood and as the blood move along the edge, the film was spread by a rapid smooth and forward to the other end of the slide to form a tail end. They were labelled by affixed labels.

The films were dried in the air and placed on a staining rack, then stained with Leishman's Stain.

Staining Method

The thin films were placed on the staining rack and flooded with Leishman's Stain, allowed to study for 2 minutes to fix the smears, the double-diluted with the buffer solution (PH 6.9) and the diluted stain was allowed to stay for 10 minutes. The slides were then gently washed with a stream of buffered distilled water.

The back of the slide was wiped clean and carefully arranged on a slide rack to dry.

A drop of immersion oil was placed on the film when placed under the microscope. It was examined toward the tail using the oil immersion objective (x 100). Dacie and Lewis *et al.,* 2009.

Phase 2

Assessment of Anti-oxidants activities of the extract using

- ✓ **Catalase**
- ✓ **Reduced Glutathione (GSH)**
- ✓ **Superoxidase (SOD) activities**

3.10.1 CATALASE (Spectrophometric method) (Hadwan 2018)

PROCEDURE

1. 25ul of sample was placed into a test tube.
2. 75ul of Assay Buffer was added to the test tube.
3. 25ul of the colorimetric Assay substrate solution was added to the test tube
4. Mixed by inversion and incubated for 15 minutes.
5. 825ul of the stop solution was added and mixed.

6. 10ul aliquot of the mixture removal and added to another test tube. 1ul of the chromogen reagent was added and mixed well.
7. Left for 15minutes at room temperature for color development.
8. 1ul of the resulting solution was transferred to a cuvette and absorbance measured at 520nm.

3.10.2 REDUCED GLUTATHIONE (Spectrophotometric Method) (Giustani *et al.*, 2011)

1. 50ul of standard reagent was placed into a clean glass tube.
2. 50ul of sample was placed into a clean glass tube.
3. 400ul GSH dilution buffer was added to the glass tubes each.
4. 10ul GSH chromogen added to the glass tubes. Mixed well.
5. Transfer 400ul to a cuvette and read at 412nm within minutes.

3.10.3 SUPEROXIDE DISMUTASE (SOD(Spectrophotometric Method)Boisani et al; 2001

1. 50ul of sample was put in a clean cuvette.
2. 1.0ul of SOD Assay Buffer was added to the cuvette.
3. 1.0ul of SOD chromogen solution was added to the cuvette and mixed.
4. The absorbance was read immediately at 420nm.

Phase3– Leucocyte Functional Assessment

Assessment of leucocyte functions using chemotaxis assay / Migration assay, phagocytosis function tests, viability test (Trypan blue test).

3.11.1 Viability Test (Trypan Blue) or Cell Exclusion Test

Principle: The reactivity of trypan blue is based on the fact that the chromospore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable.

PROCEDURE

1. Obtain cells suspension from whole blood as described in Appendix 1.
2. Place 0.5 ml of a suitable cell suspension (dilute cells in complete medium without serum to an approximate concentration of 5 X 10^6 to 10^6 X 10^6 cells per ml) in a screw cap test tube.
3. Add 0.1 ml of 0.4% Trypan blue stain (Sigma Aldrich). Mix thoroughly.
4. Allow to stand 5 min at 15 to 30^{o}C (room temperature).
5. Fill a haemocytometer as for cell counting
6. Under a microscope, observe if non-viable are stained and viable cells excluded the stain (Freshney, 1987).

TEST PROCEDURES

Cell preparation or isolation of peripheral blood leucocytes.

To achieve quality and appropriate cell concentration.

1. 5ml of heparinized blood sample was centrifuged at 300rpm for 30 minutes.

2. The plasma (uppermost layer) was separated

3. Carefully, the Buffy coat (interphase) between the supernatant plasma and the red cell deposits aspirated

4. The Buffy coat was suspended in 5ml Hank's balanced salt solution (HBSS) without phenol and centrifuged again at 3000 rpm for 5 minutes.

5. The supernatant was discarded. The final cell concentration was adjusted to 5 – 10 x 10^6 cells/ml by counting using standardhaemocytometer.

3.11.2 CHEMOTAXIS ASSAY

Principle: The chemotaxis assay evaluates the migration of neutrophils through permeable supports (Boyden Chamber) that contain 5.0 m pore polyster membrane.

PROCEDURE

1. From the cell's suspension above

2.Migration Chambers was put in 24-well plate (lower chamber)

3. 100serum free medium (HBSS) was added in chamber (upper chamber)

4. 200 cell (5 - 10 x 10^6) cells/ml was placed in serum – free medium in the chamber (upper

chamber)

5. 750 culture mediums added in lower chamber (containing chemo attractant)

6. It was incubated at 37^0c, 5% Co_2 incubator for 1 – 2 hours

7. The insert chamber (upper chamber) removed

8. The cells in the lower chamber (migrated cells) counted.

The final count in cells/ml was obtained by first dividing the total count by 0.1 (chamber depth) then divided the result by the total surface area counted. ThenChemotactic index = x 100

3.11.3 Phagocytosis Function Test

This is the test used to determine the phagocytic capability of leucocytes by measuring their ability to phagocytize heat inactivate) E. Cole.

PROCEDURE

Based on modified version of the method introduced by Lehrer and Cline (Noah *et al*., 1995).

1. 100 of leucocyte suspension at a concentration of 1 x 10^6 cells/ml will be nix with 100E. cols (3 x 10^8 cells/ml suspension)
2. Then, incubate at (37^0c in a water bath for 1 hour
3. After 1 hour, cells would be fixed in 4% formoldehyde, and stained with methylene blue.
4. Examined microscopically for dead and live *E. Coli* within the leukocytes.
5. Counted 200 cells

The phagocytic index – (phagocytic activity or % phagocytic cells i.e. number of cells with engulf bacteria x % of cells with more than one bacterium per cell).

3.11.4 Nitro Blue Tetrazolium Test (NBT) (Roos and Boer 2013)

Phagocytic leukocytes, when appropriately stimulated, consume oxygen and produce susperoxide (O_2) in a process often referred to as the respiratory burst (Robinson, 2008).

Principle: Leucocytes ingest the dye, nitrobluetetrazolium, and in the presence of reactive oxygen species, the yellow colored NBT compound is converted to the purple - blue formazan compound (Abeer, 2013).

Phase 4 – Molecular Studies

Molecular assay of TGF-β1 gene expression.

3.12.1 DNA processing (Wu *et al*., 2004)

DNA was extracted from peripheral blood leukocytes in the DNA shield by phenol-chloroform method and then subjected to polymerase chain reaction followed by restriction fragment analysis (PCR-RFLP) for identification of mutations resulting in hemoglobin S. The presence of TGF-β1 polymorphism yields specific products.

Test procedure:

1. 400µl of Genomic lysis buffer was added to 100 µl of blood sample; it was completely mixed by vortexing for 4-6 seconds and allowed to stand for 5-10 minutes at room temperature.
2. The mixture was transferred into a collection tube in the Zymo-spin IICR column and was centrifuged for 1 minute at 10,000g.
3. The spin column was transferred into a new collection tube, 200 µl of DNA Pre washed buffer was added and centrifuged for 1 minute at 10,000g.
4. 500 µl of g-DNA wash buffer was added to the spin column and centrifuged for 1 minute at 10,000g.
5. The column was transferred into a micro centrifuge tube. 50 µl of DNA elution buffer was added and incubated for 2-5 minutes at room temperature and then centrifuged at top speed for 30 seconds to elute the DNA.

3.12.2 Protein Expression by the TGF- β1 gene (Torres *et al*., 2016)

3.12.2.1Polymerase Chain Reaction

Principle: Polymerase chain reaction identifies a small fragment of the DNA section which is used as the template for primers that initiate the reaction leading to the production of numerous copies of the DNA molecule by specific proteins known as polymerase enzyme which strings individual DNA blocks together to form long molecular strands. Polymerases require a supply of DNA building blocks, primers and a longer DNA molecule as a template for constructing the strand.

Test procedure

1. Following the DNA extraction, the primers sense 5'-CCGCTTCTGTCCTTTCTAGG-3'and antisense 5'-AAAGCGGGTGATCCAGATG-3' were utilized for the screening.
2. The reaction mix consisted of 1X reaction buffer, $MgCl_2$ (5mM), dNTPs (0.4mM), 0.8mM of each primer, 1 unit of Taq polymerase and 12ng/uL of DNA.
3. The cycling conditions applied comprised DNA template was heated to 95^{o}C for 5min to break the hydrogen bonds that enables the holding of DNA strands together in a helix.
4. The mixture was cooled to 50-70^{o}C, allowing the primers to anneal to their complementary sequence in the template DNA.
5. This was followed by 35cycles of 95^{o}C for 30s, 60^{o}C for 30s and 72^{o}C for 30s, and a final extension of 7min at 72^{o}C.

3.12.2.2TGF-β1 genotyping using Restriction Fragment Length Polymorphism

Principle: The RFLP functions as a molecular marker specific to a single restriction enzyme combination and is based on the comparison of restriction digestion profiles of

DNA samplesisolated from different individuals. The RFLP takes advantage of the recognition sequences of restriction enzymes that corresponds to the normal allele or the mutated allele of a gene.

Test procedure:

1. The amplicon (amplified DNA product) is added to the Bsu36I restriction enzyme and incubated overnight.
2. The 406bp DNA fragment was restricted by the Bsu36I which cleaved the DNA at the cleavage site to generate two fragments 223bp and 183bp.
3. The digested fragments were analyzed using an agarose gel electrophoresis; the agarose gel was placed into the electrophoresis chamber, completely sub-merged and covered with electrophoresis buffer.
4. The samples were loaded into the well and electrophoresis was performed, the agarose gel and casting tray was carefully removed from the electrophoresis chamber.
5. The gel was gently placed on flat surface and moistened with few drops of electrophoresis buffer and the unprinted side of the Ethidium Bromide was gently placed on the agarose gel and the gel stained for 3-5 minutes.
6. The staining card was thereafter removed and the gel visualized using a mid-range ultraviolet transilluminator (300nm) separating the fragments on the basis of their length, forming different bands.
7. The DNA appeared as bright orange bands on a dark background separate.
8. The bands obtained from PCR-RFLP were analyzed using the GelCompare II version 2.5 (Applied Maths, Sint-Martens-Latem, Belgium).

3.15 Data Analysis

Standard statistical software - graph pad prism version 2.9.10 was used. Data generated for each group were presented as mean (± SEM). Statistical significance difference among the groups was calculated with one-way ANOVA at 95% confidence limit. ($P < 0.05$) was considered statistically significant.

CHAPTER FOUR

RESULTS

Table 4.1: the summary of the Immuno-histochemistry confirmation of the leukaemia induced by Benzene. CD_{10} and CD_{20} were positive in all Benzene induced groups. However, CD_{10} was negative after treatment with all the extracts while CD_{20} was negative with the groups treated with conventional drug and those pre-treated with the extracts before induction with Benzene. They are both positive in Lymphoma and Acute Lymphoid Leukemia. CD_{20} is more specific for CLL.

Figure 4.1a, 1b and 1c: There was a no significant difference between the initial (Pre-treated, Figure1a) weights of the control animals and the benzene induced group, however, after the induction (Figure 1b), the weight of the benzene induced leukaemic animals reduced significantly ($P<0.05$) but increased significantly (Figure 1c)with all extracts after the treatment ($P<0.05$, respectively).

Figure 4.2a, 2b and 2c: There wasno significant change(though with some observable decreases) in the PCV of all the pre-treated animals (2a) with different extracts after induction compared with controls ($P>0.05$, respectively)while the in rats treated with *P. febrifugum* and *R. periwinkle*showed significant increases (2b) compared with values from other groups and standard drugs ($P<0.05$, respectiovely). However, only*P. febrifugum*exhibited a significant increase when pre and post treated groups were compared with the benzene induced group (2c) and control rats respectively ($P<0.05$).

Figure 4.3: Therewas no significant differences in haemoglobin levels amongst rats treated with benzene only, *A. indica* only, *P. febrifugum* only, *R. communis* only and *R. periwinkle* only when compared with control rats respectively ($P>0.05$, respectively).

Figure 4.4a and 4b: There was a significant increase (4a) in total white blood cells (WBC) level among Benzene induced rats and controls which showed a significant lymphocytic preponderance (4b) ($P<0.05$, respectively).

Table 4.1: Summary of CD10 and CD20 Leukemia

Test	Pre-treated		Post-treated		Conclusion
	CD_{10}	CD_{20}	CD_{10}	CD_{20}	
Control	Negative	Negative	Negative	Negative	Negative
B	Positive	Positive	___	Positive	Induction successful
C	Negative	Positive	___	Positive	Ameliorated
D	Negative	Negative	___	Negative	Ameliorated
E	Negative	Negative	___	Negative	Induction not successful

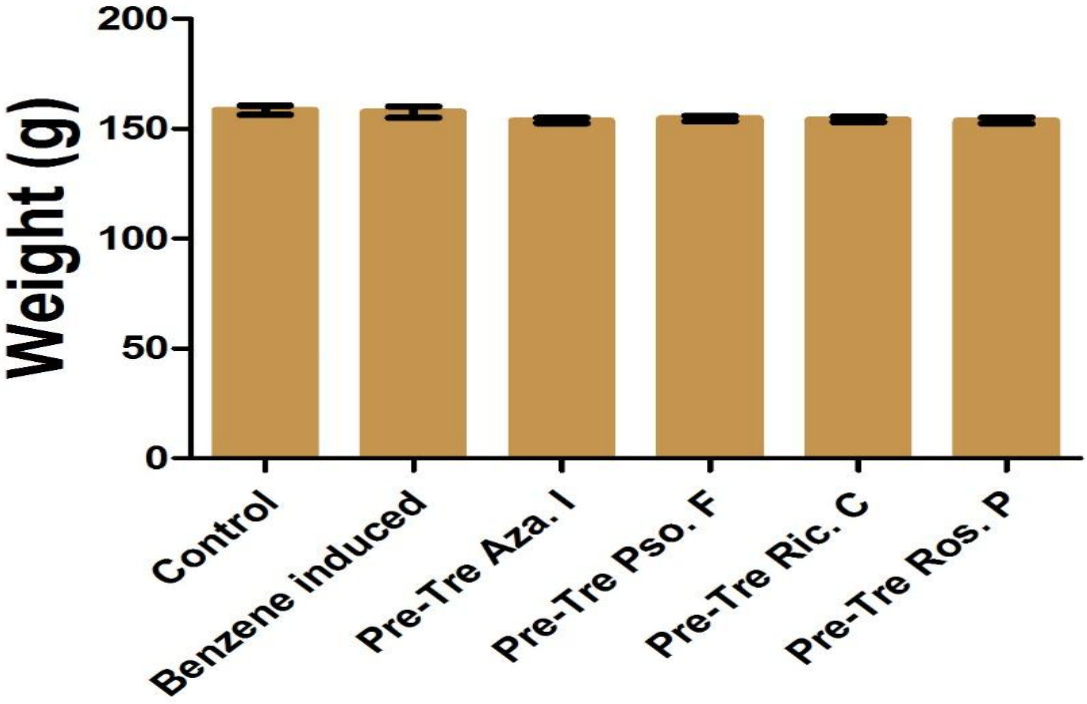

Figure 4.1a: Comparison of Initial Weight in Control, Benzene induced and Pre-treated of all the plant extracts

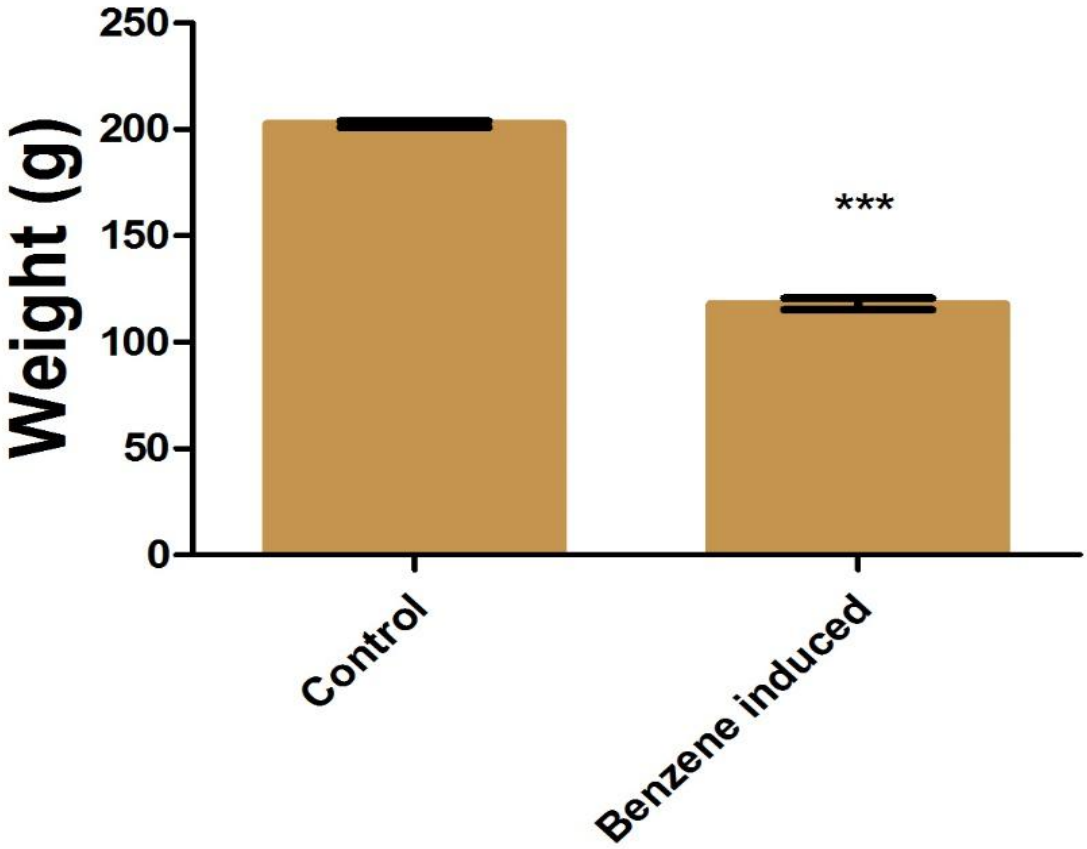

Figure4.1b: Comparison of final weight in control and benzene induced rats

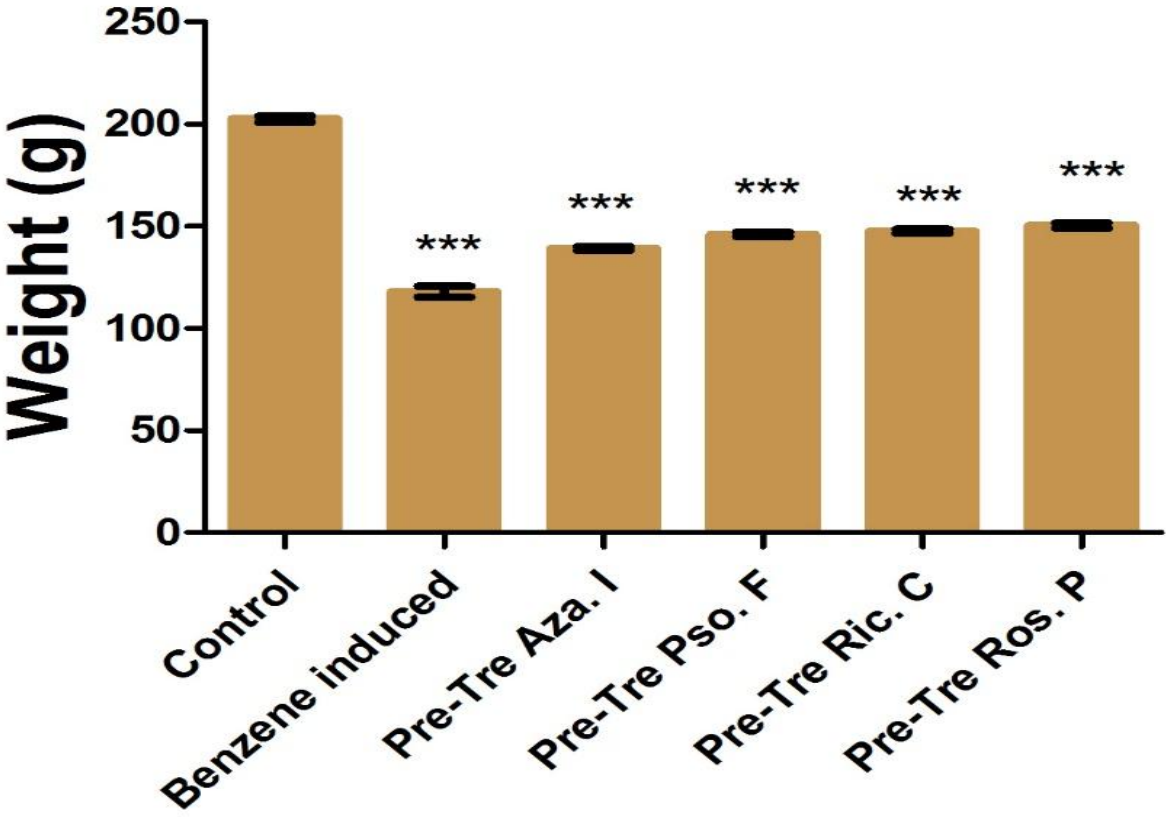

Figure 4.1c: Comparison of Final Weight in Control, Benzene induced and Pre-treated with all the plant extracts

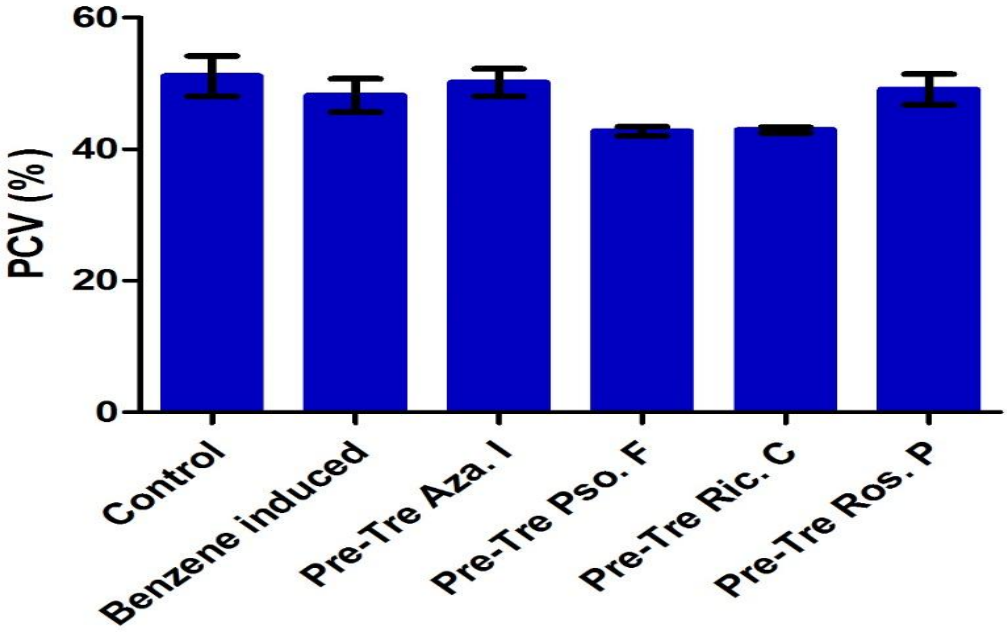

Figure 4.2a: Comparison of PCV in Control, Benzene induced and Pre-treated with all the plant extracts

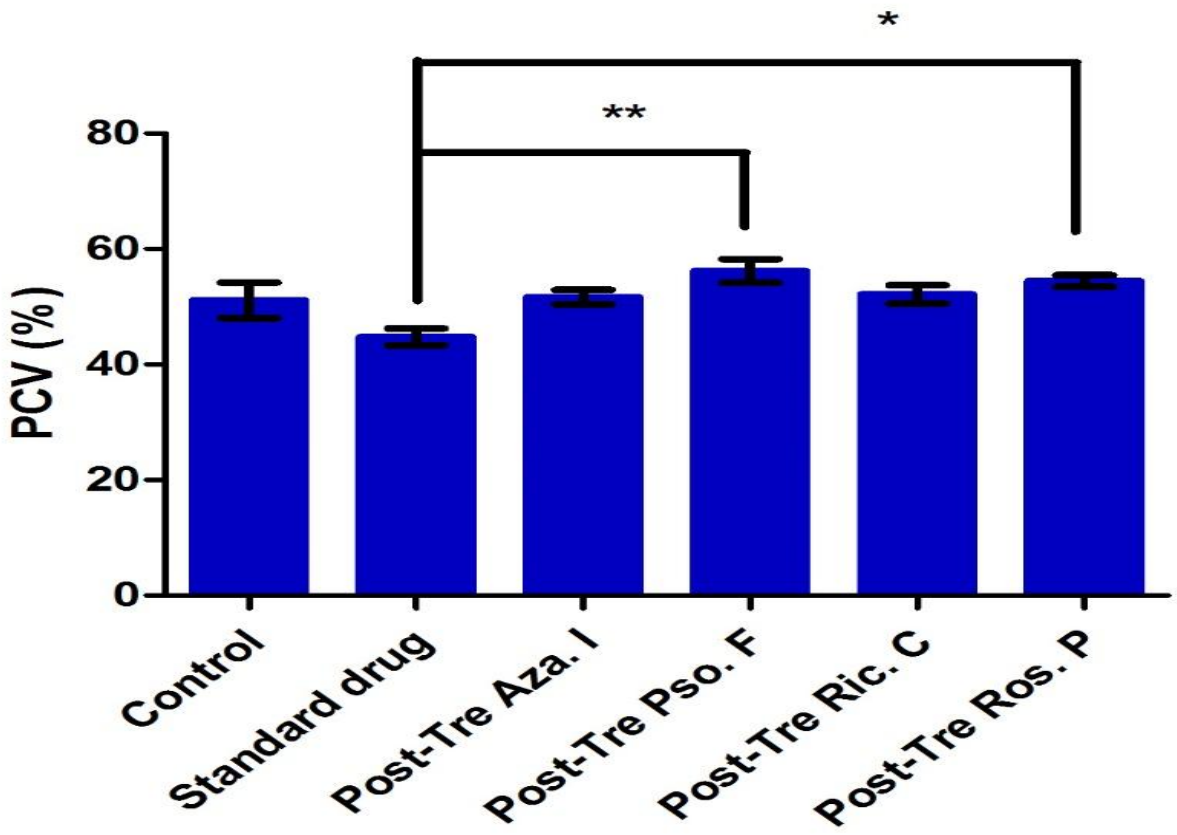

Figure4.2b: Comparison of PCV in Control, Standard drugs and post-treated with all the plant extracts

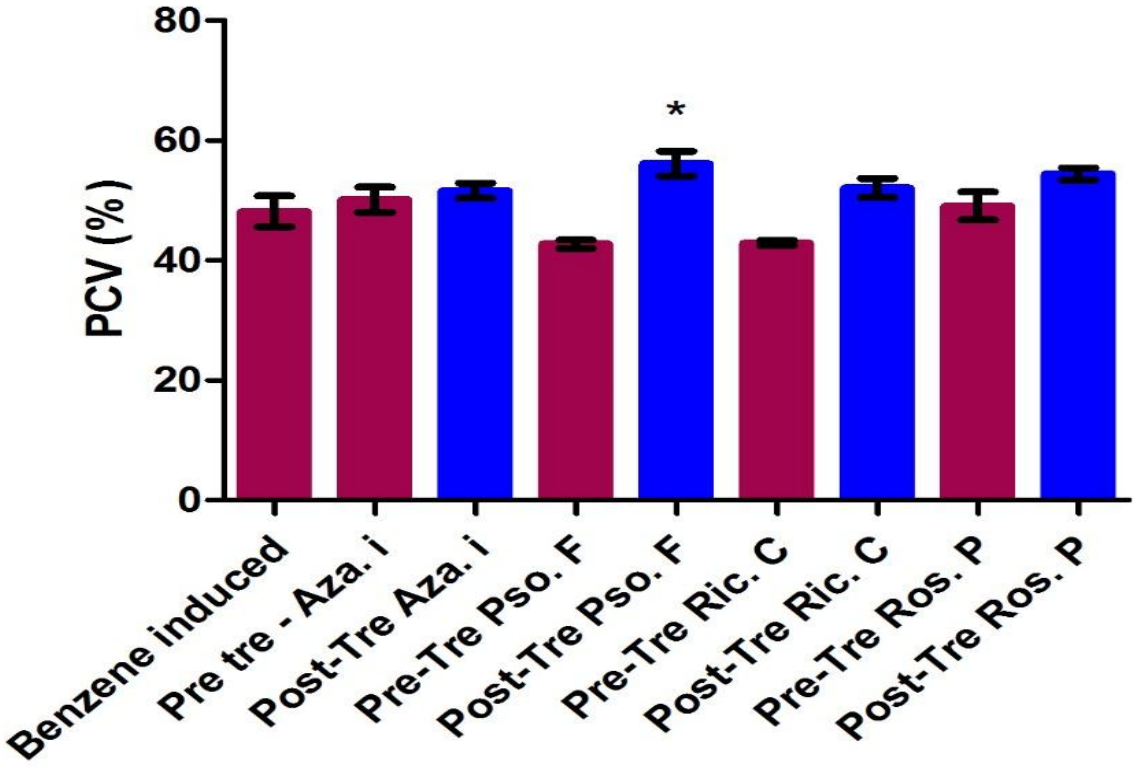

Figure4.2c:Comparison of PCV in Benzene induced, pre-treated and post-treated

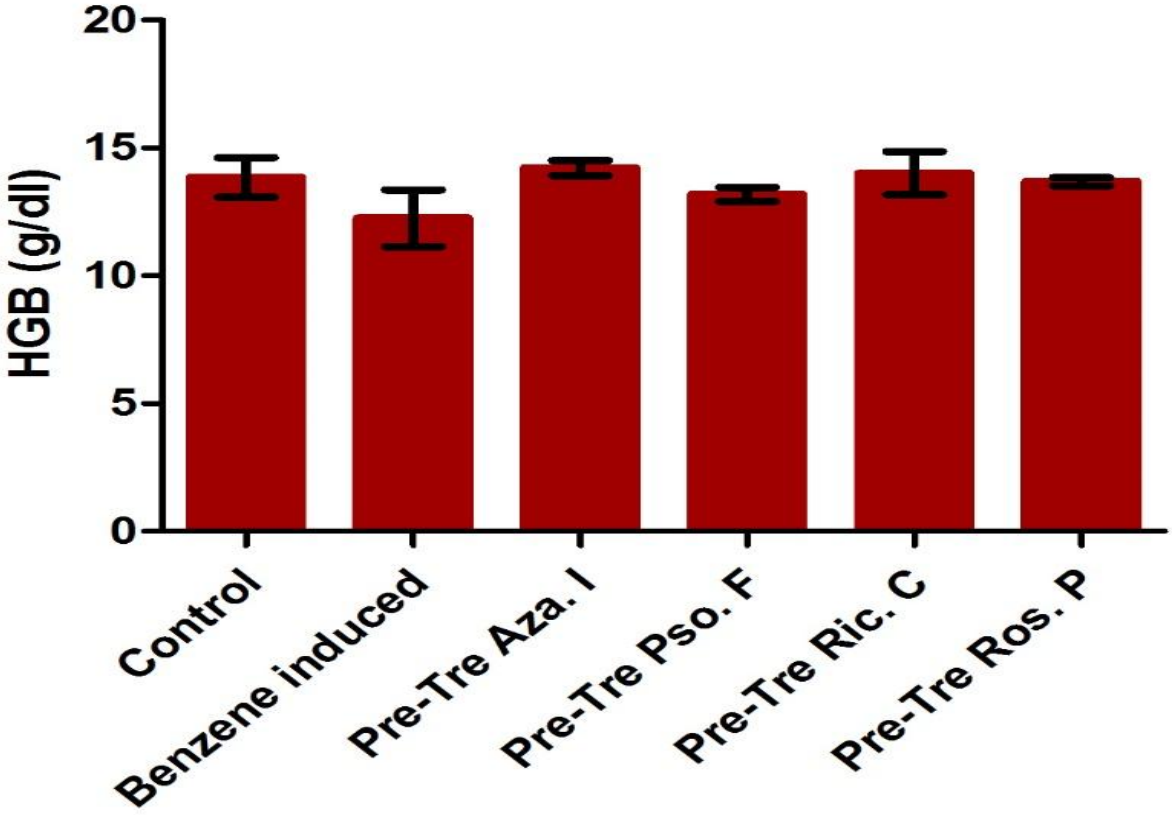

Figure 4.3: Comparison of HGB in Control, Benzene induced and Pre-treated with all the plant extracts

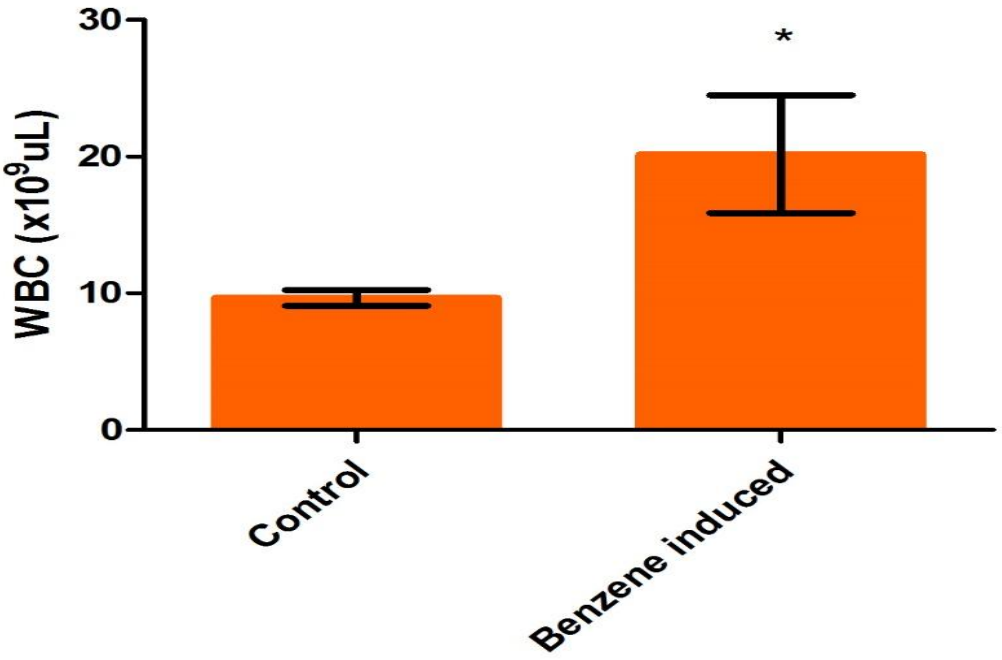

Figure. 4.4a**:** WBC in control and Benzene induced

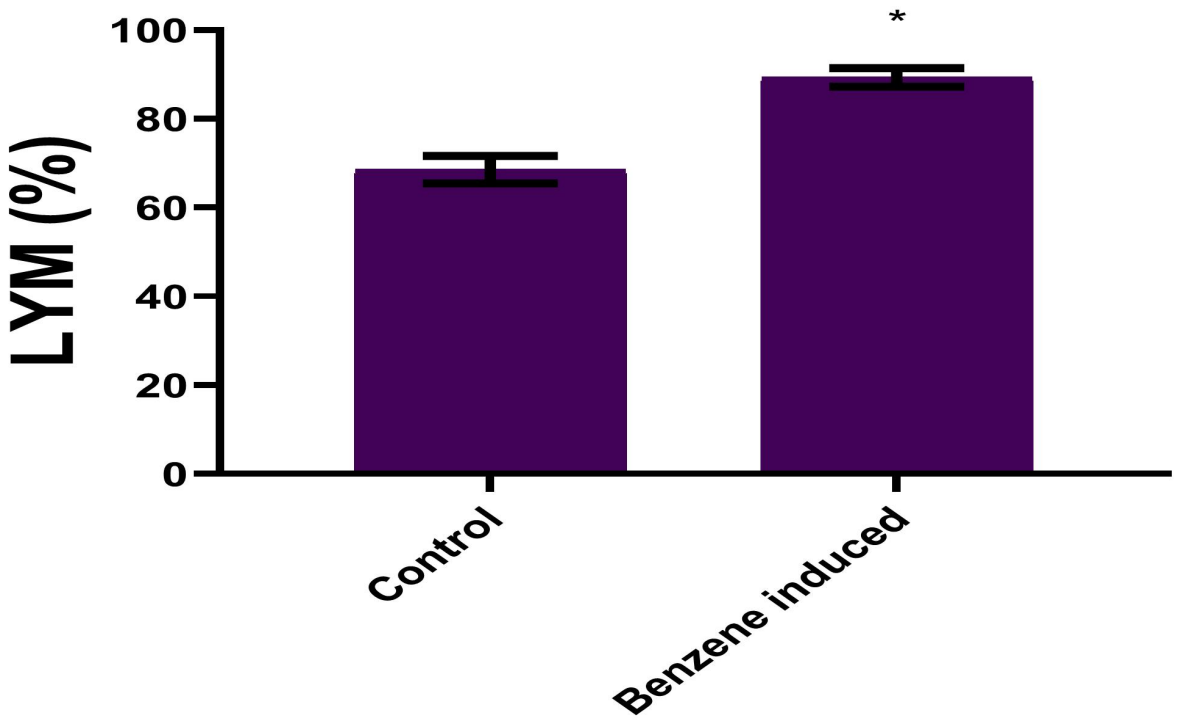

Figure. 4.4b**:** WBC in control and Benzene induced

Summary of Observations in Phase One

1. There was a significant loss in weight after a successful induction of Leukemia
2. The lost weight were significantly reversed with the administration of the different extracts
3. There was a significant increase in the total white blood cell count on the induced animal with a predominance of lymphocytes
4. There were histochemical confirmation of successful induction of chronic lymphocytic leukemia with Benzene induction process.

Phase Two Results

Assessment of the Antioxidant Functions of the Extract

Figure 4.5: there were significant increase in the SOD activities in the animals treated with *R. communis* after induction while *A. indica* exhibited a significant reduction ($P<0.05$, respectively) when compared with pre-treated controls. However, there was no significant difference in the other groups treated with *P. febrifugum* and *R. periwinkle*

Figure 4.6: There were graded significant increases in the gluthathione activities as demonstrated by all the extracts in the treated animals after induction ($P<0.05$, respectively)when compared with pre-treated controls. This is in the order of R.communis>>P. febrigum>> *P. periwinkle >>A indica*

Figure 4.7: There were significant reductions in the catalase activities in the post treated animals with all the extracts ($P< 0.05$, respectively) except *R.communis.*

Figure 4.8: There were significant reductions in the Hydroxyl radicals' activities in the post treated animals with all the extracts when compared with the pre-treated groups ($P< 0.05$, respectively).

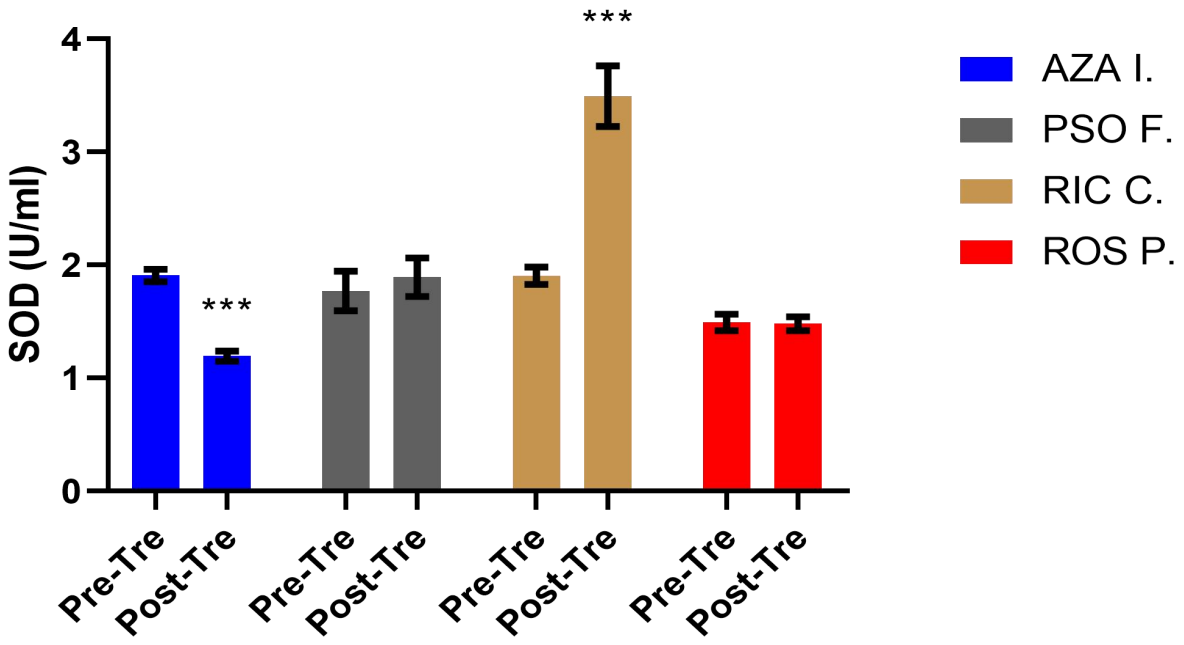

Figure 4.5: Showing SOD concentration in both pre and post treated groups

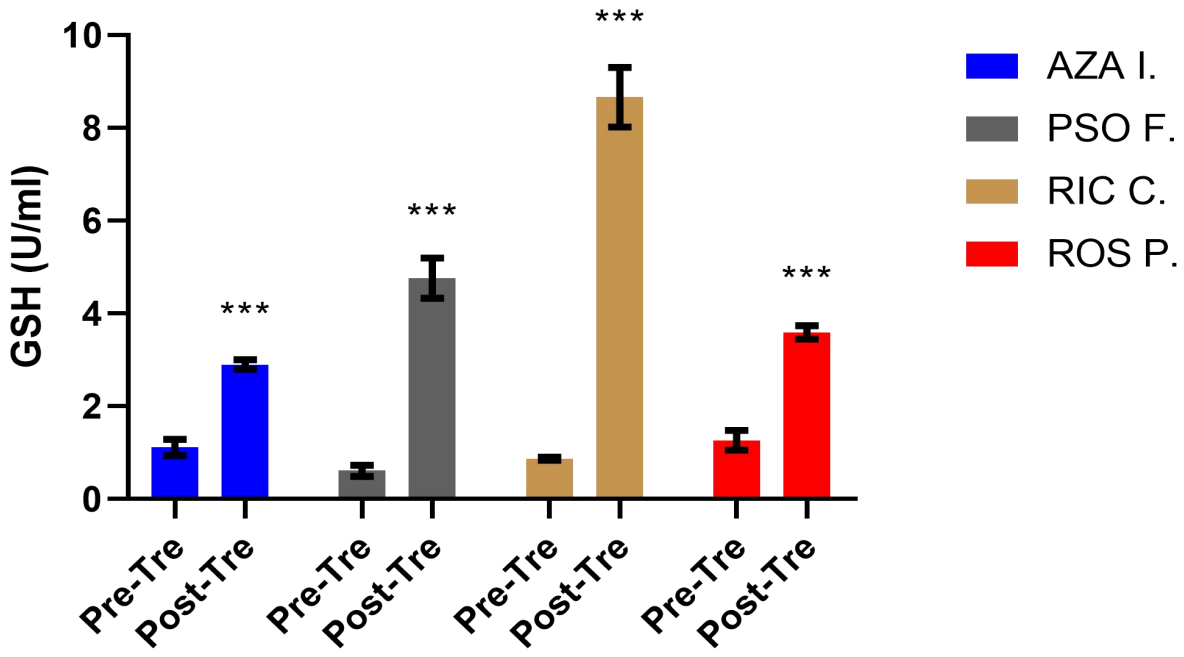

Figure 4.6: Showing GSH concentration in both pre and post treated groups

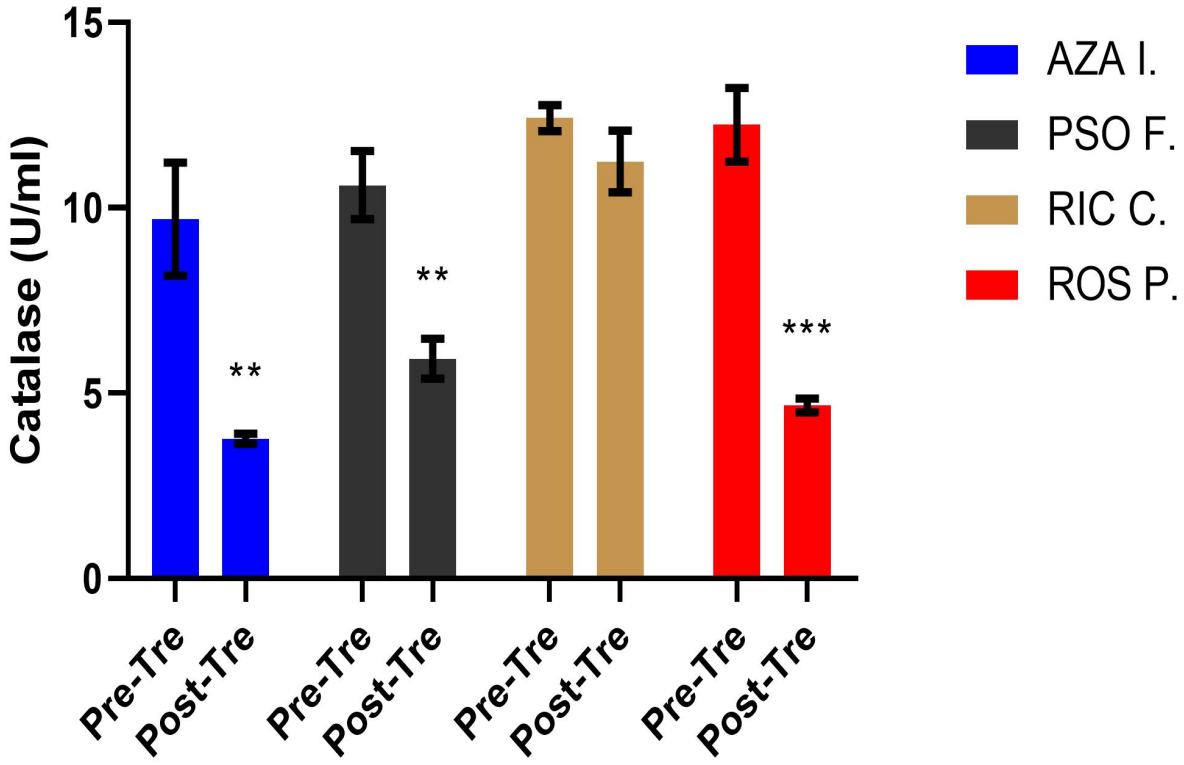

Figure 4.7: Showing Catalase in pre and post treated Benzene induced leukemic rats

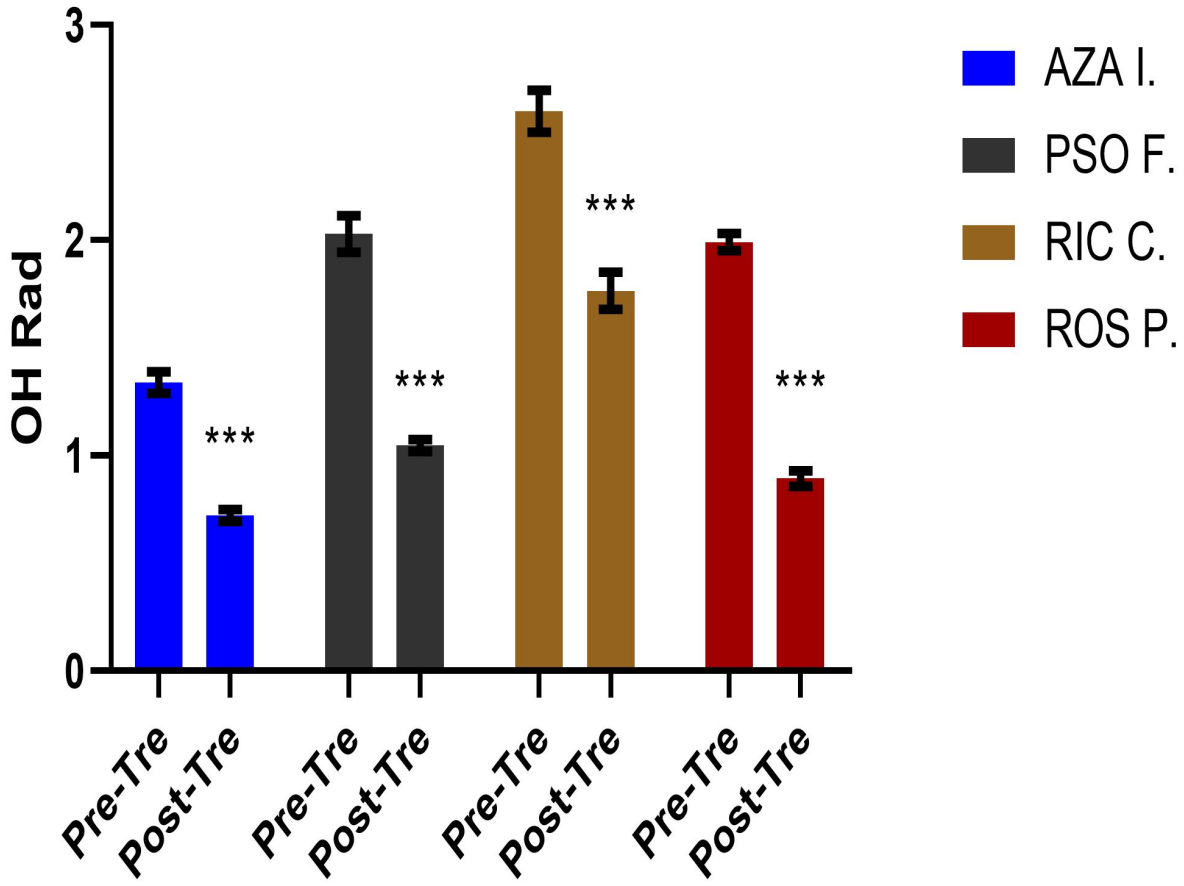

Figure 4.8: Showing OH Radical in pre and post treated Benzene induced leukemic rats

Summary

Ricinus communis is a more effective antioxidant than all other extracts with more pronounced effects on: SOD, GSH, OH and Catalase activities compared with other extracts.

Phase 3 Results

Assessment of the effects of the extracts on Leucocyte functions.

Figure 4.9: Shows total white blood cell counts in benzene induced rats and rats pre-treated with extracts before induction. There reductions in the counts in all the pretreated groups but significantly different with *R.periwinkle* only ($P<0.05$). This showed the preventive actions of the extracts

Figure 4.10: Shows the comparison of total white blood cell counts in benzene induced rats, rats treated with standard drugs, pre-treated and post-treated animals with extracts before and after induction. There were significant reductions in the counts in animals treated with standard drugs and *R.communis* only ($P<0.05$, respectively) while all other reductions were not statistically significant.

Figure 4.11: shows comparison of combined effects of some of the extracts of white blood cell count. There was no significant synergistic effect observed in all the groups compared.

Figure. 4,12: The synergistic comparison effect of lymphocyte count did not show any significant synergy between the extracts.

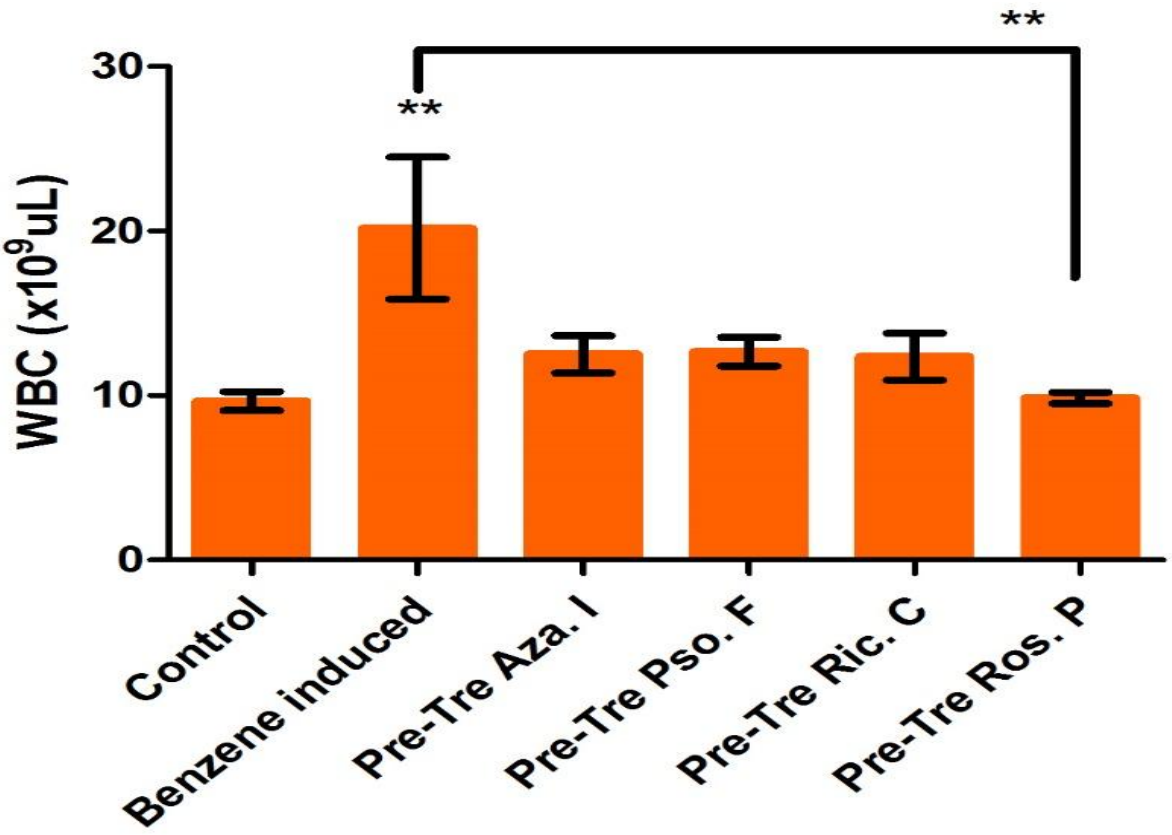

Figure 4.9: Comparison of WBC in Control, Benzene induced and Pre-treated with all the plant extracts

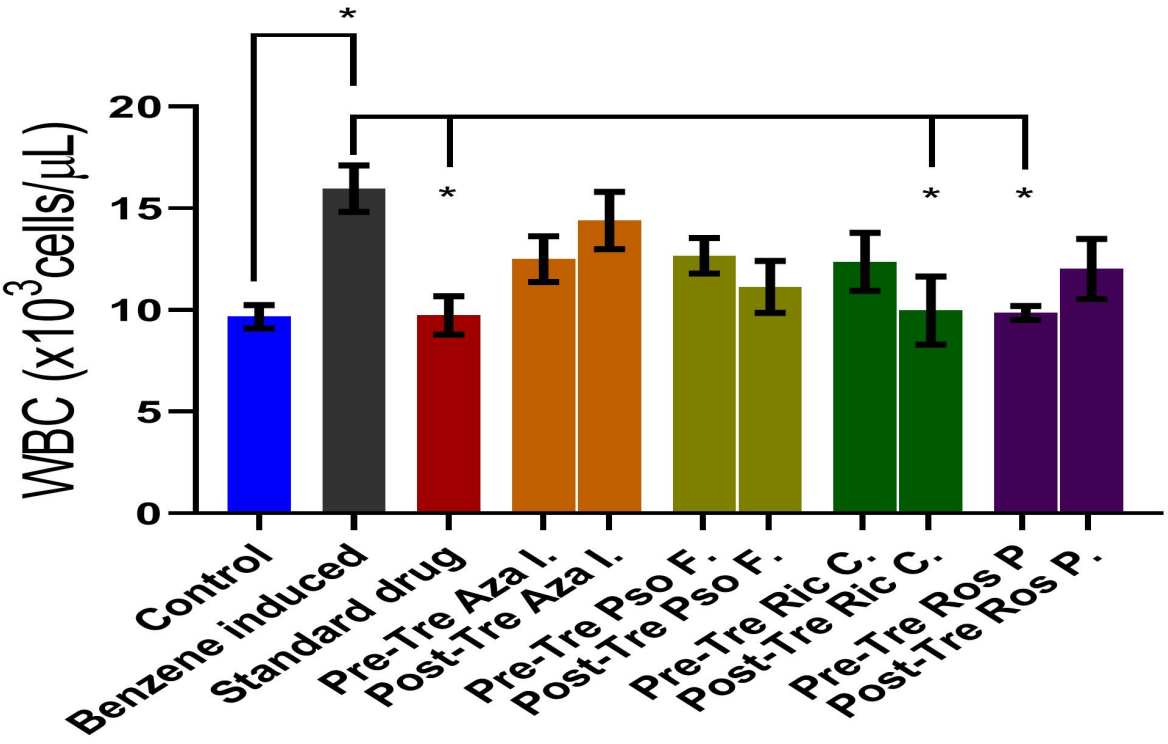

Figure. 4.10: WBC cell values in pre and post treated benzene induced leukemic rats

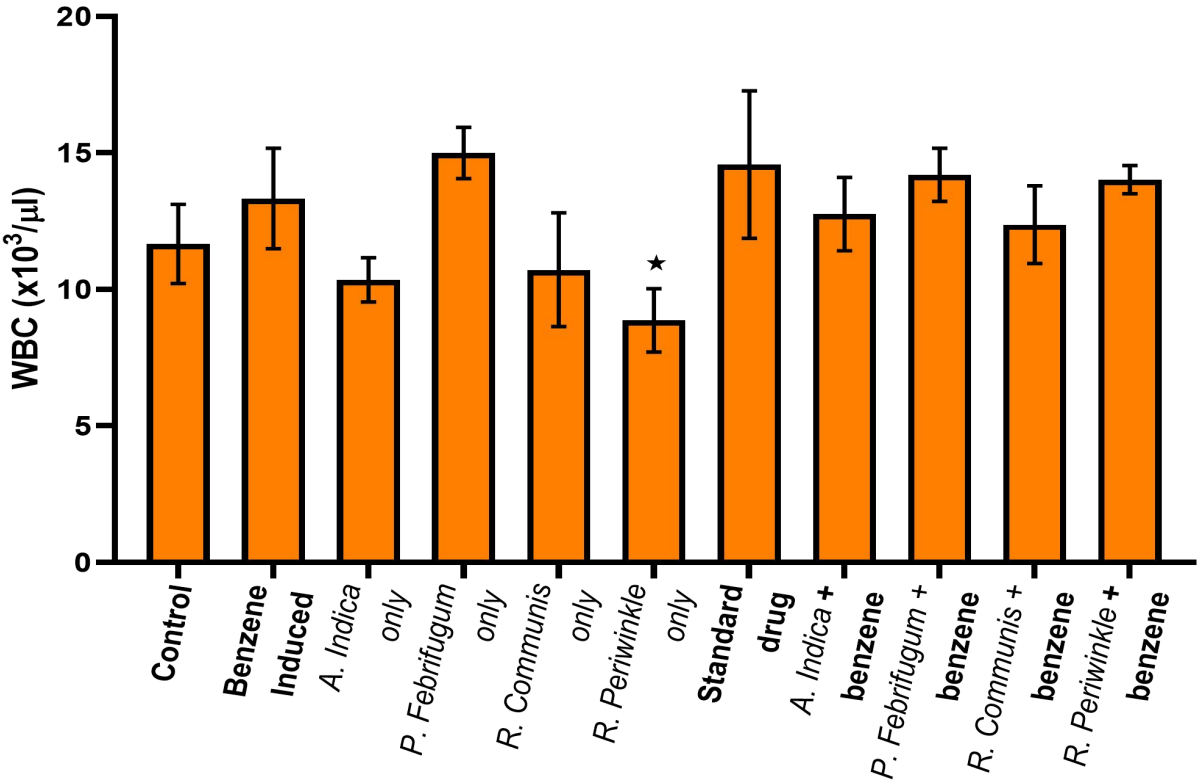

Figure 4.11: WBC count level in benzene induced rats pre-treated with single and combined doses of the selected extracts (Synergy).

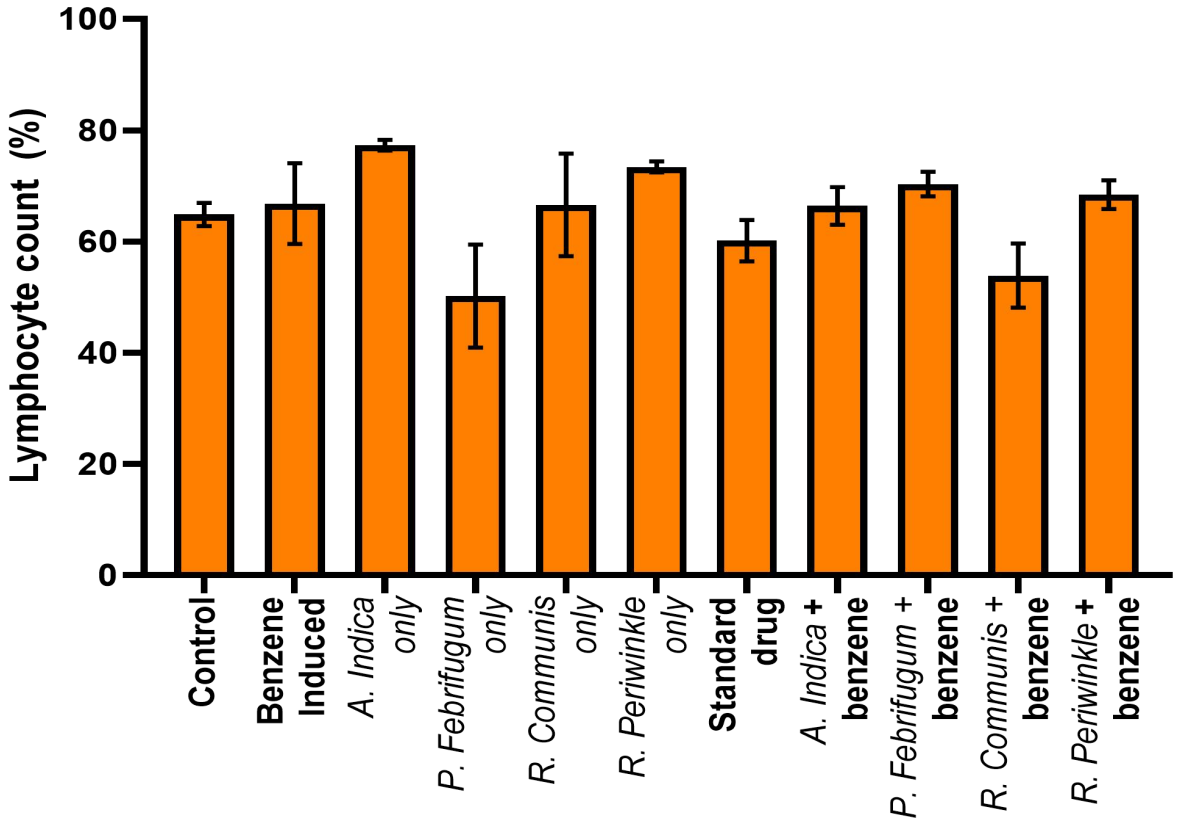

Figure 4.12: Lymphocytes count in benzene induced rats pre-treated with single and combined doses of the extracts.

Figure 4.13: This shows the significant effectiveness Ricinus communis and Rosy periwinkle only in preventing the surge of lymphocytes in the induction of leukaemia with benzene ($P<0.05$, respectively). The other extracts however did not show any significant change when compared with control.

Figure 4.14: Comparison between control, Benzene induced leukaemic rats, standard drug with pre and post treated animals shows the significant reductions of lymphocytes with the extracts in the order *R periwinkle>>R.communis>>A. Indica >>P. febrigum. This shows that R. communis and R. Periwinkle are more effective in the amelioration of the leukaemia.*

Figure 4.15: This shows the comparison of Neutrophils in control, Benzene induced and pre-treated rats with all the extracts. There were significant increased differential population of Neutrophils due to the suppression of the lymphocytes with *R communis and P. febrigum* ($P<0.05$, respectively). *R. communis* however, showed a greater efficacy in the prevention of leukaemia.

Figure 4.16: Comparison between control, Benzene induced leukaemic rats, standard drug with pre and post treated animals shows the significant increase in the differential population of neutrophils with the extracts in the order *R. communis >>R periwinkle>>A. Indica >>P. febrigum*. This shows that *R. communis and R. Periwinkle* are more effective in the amelioration of the lymphoblastic leukaemia.

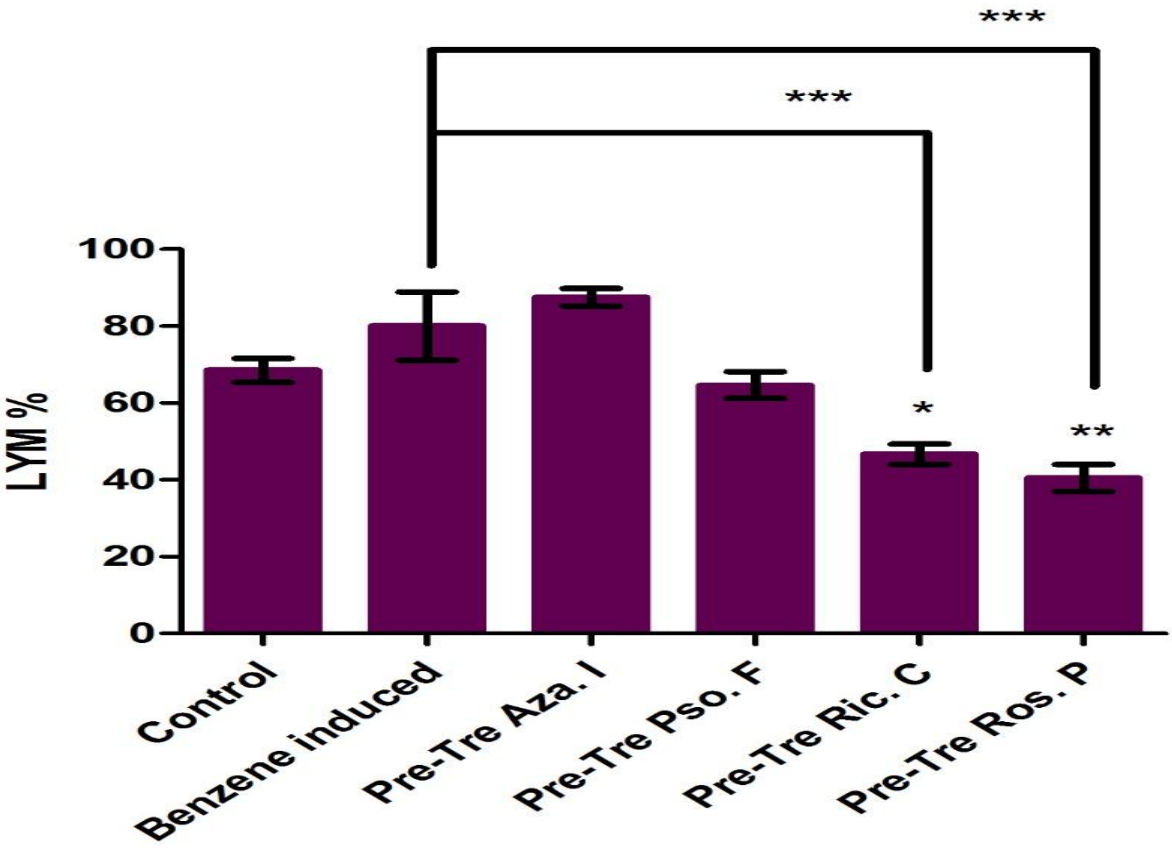

Figure 4.13: Lymphocytes count in Control, Benzene induced and pre-treated groups with all the plant extracts.

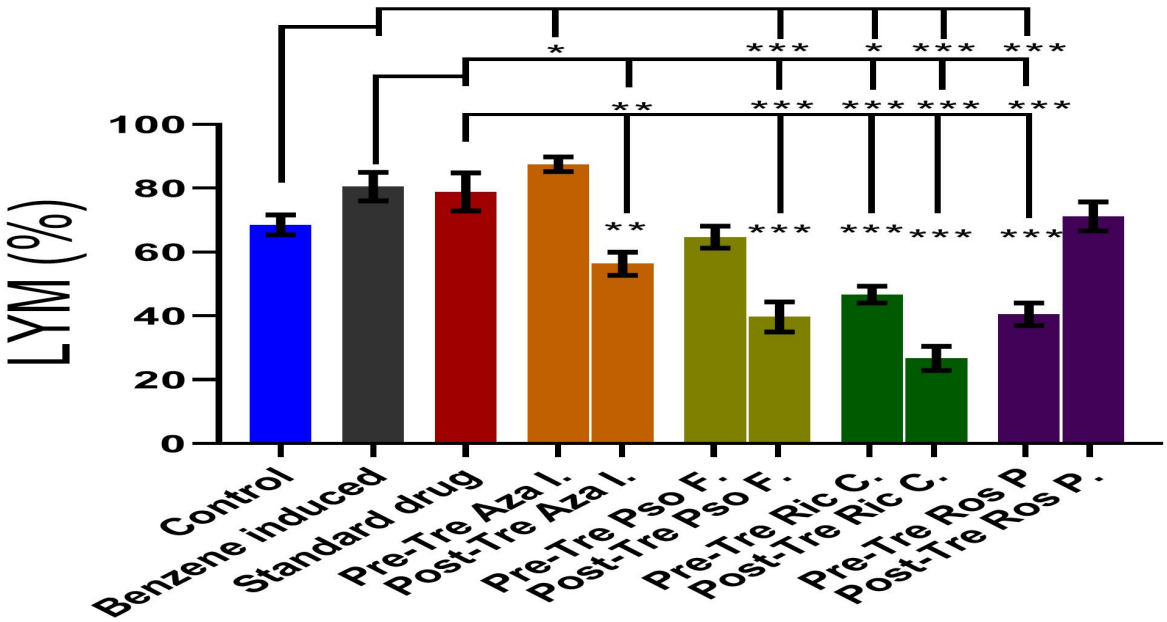

Figure. 4.14: Joint comparison of lymphocyte count (%) in pre and post treated benzene induced leukemic rats.

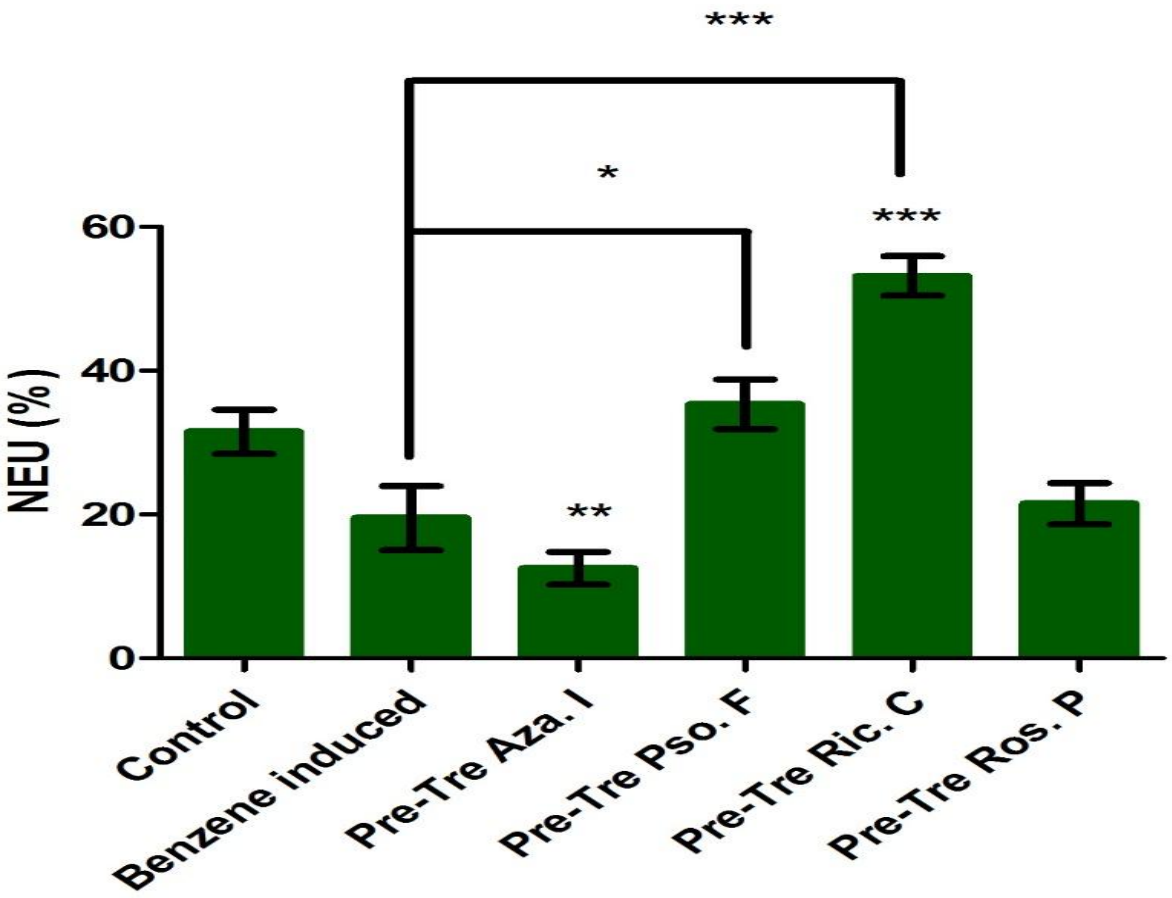

Figure 4:15: Comparison of Neutrophils in Control, Benzene induced and pre-treated rats with all the plant extracts

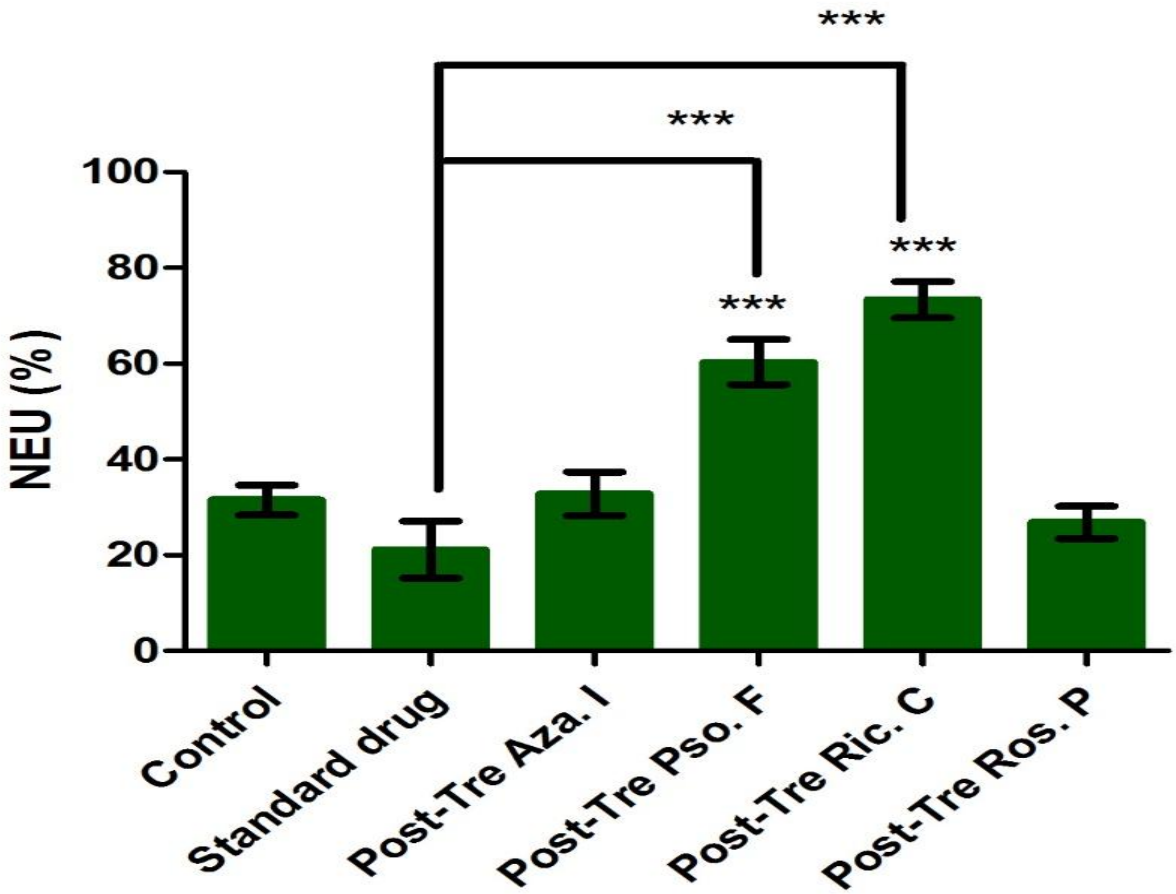

Figure 4.16: Comparison of Neutrophils in Control, Standard drug and post-treated with all the plant extracts

Figure 4.17: This shows the percentage viability index in benzene induced rats pre-treated with extracts. There was a significant decrease ($P<0.05$) in the viability index of white cells in benzene induced leukemic rats compared with those pre-treated with the extracts before induction. All the extracts pre-treated animals exhibited no change in their viability indices indicating a preventive action of the extracts in leukaemia induced by Benzene.

Figure 4.18: This shows the percentage chemotatic index in benzene induced rats pre-treated with extracts. There was a significant decrease ($P<0.05$) in the chemotatic index of white cells especially the granular cells in benzene induced leukemic rats compared with those pre-treated with the extracts before induction. All the extracts pre-treated animals exhibited no change in this index indicating a preventive action of the extracts in leukaemia induced by Benzene

Figure 4.19: Results show phagocytic index in benzene induced rats pre-treated with extracts. There was a significant reduction in the phargocytic of white cells in the leukaemic rats compared with the controls and animals pre-treated with with the extracts. However, the animals pre-treated *R. communis* showed an enhanced increased in phargocytic activity. This indicates a more effectiveness in the prevention of benzene induced leukaemia.

Figure 4.20: Results show respiratory burst in benzene induced rats pre-treated with extracts. There was a significant decrease with the benzene induced group compared with the control ($P<0.05$). However, there were comparable significant increases with all the extracts except with R. periwinkle that a slight reduction in its activity.

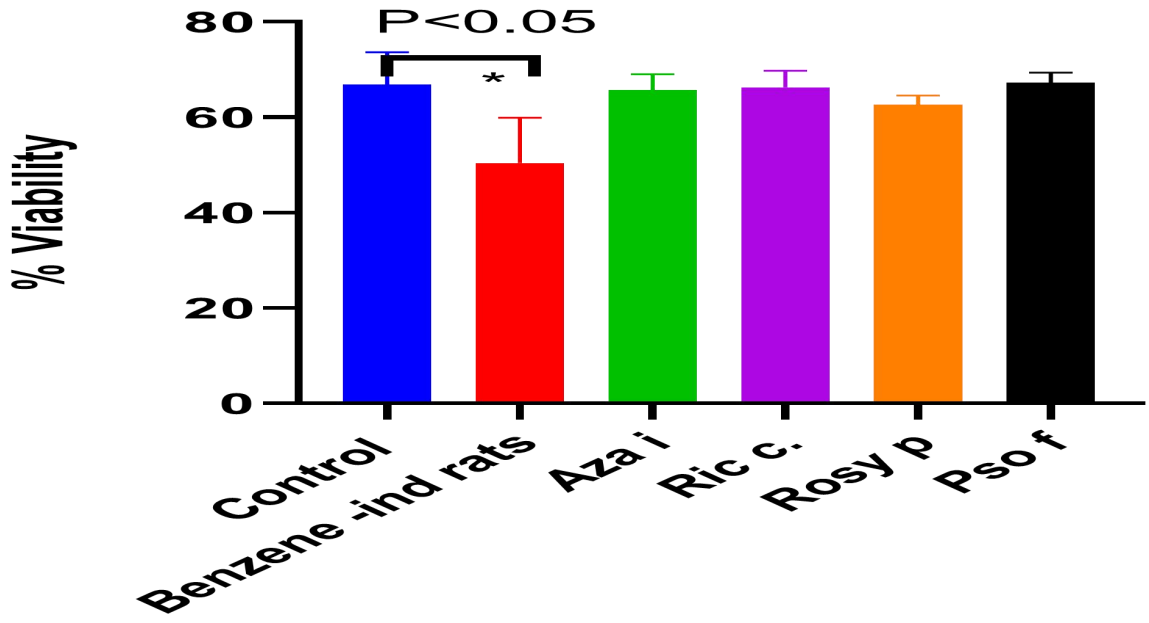

Figure 4.17: Shows Viability index in benzene induced rats pre-treated with extracts

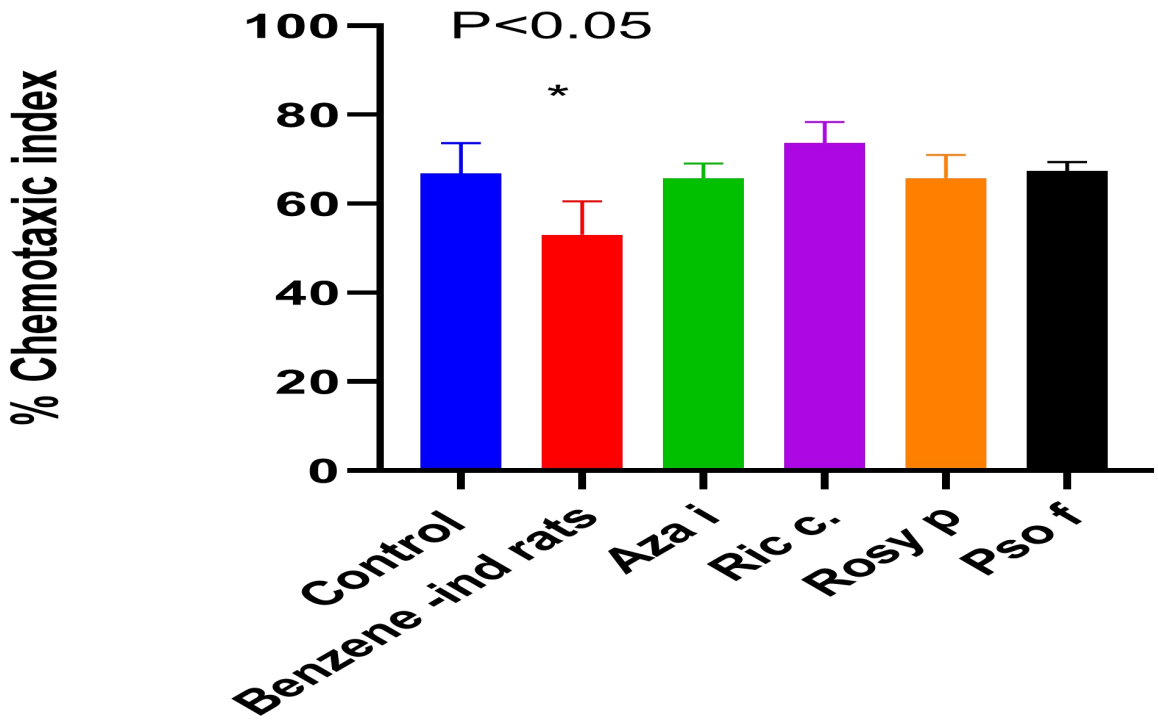

Figure 4.18: Comparison of Viability index in benzene induced rats pre-treated with extracts

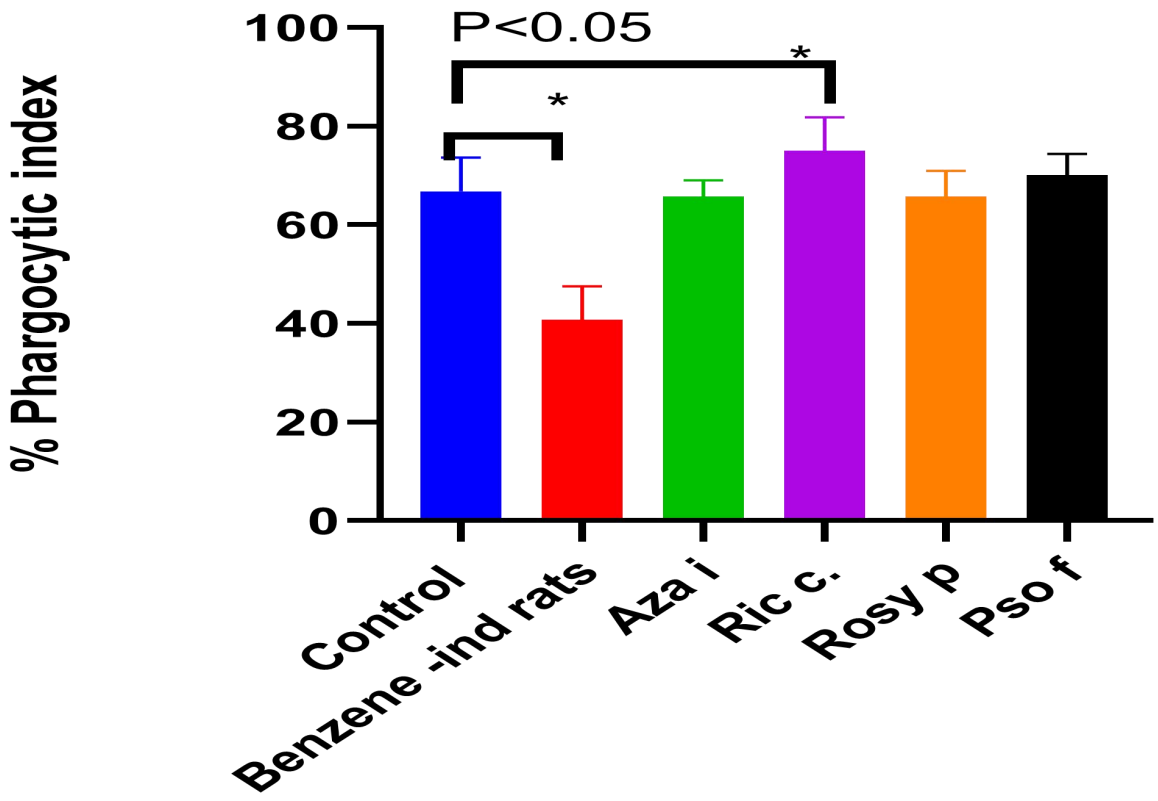

Figure4.19: Results show phagocytic index in benzene induced rats pre-treated with extracts

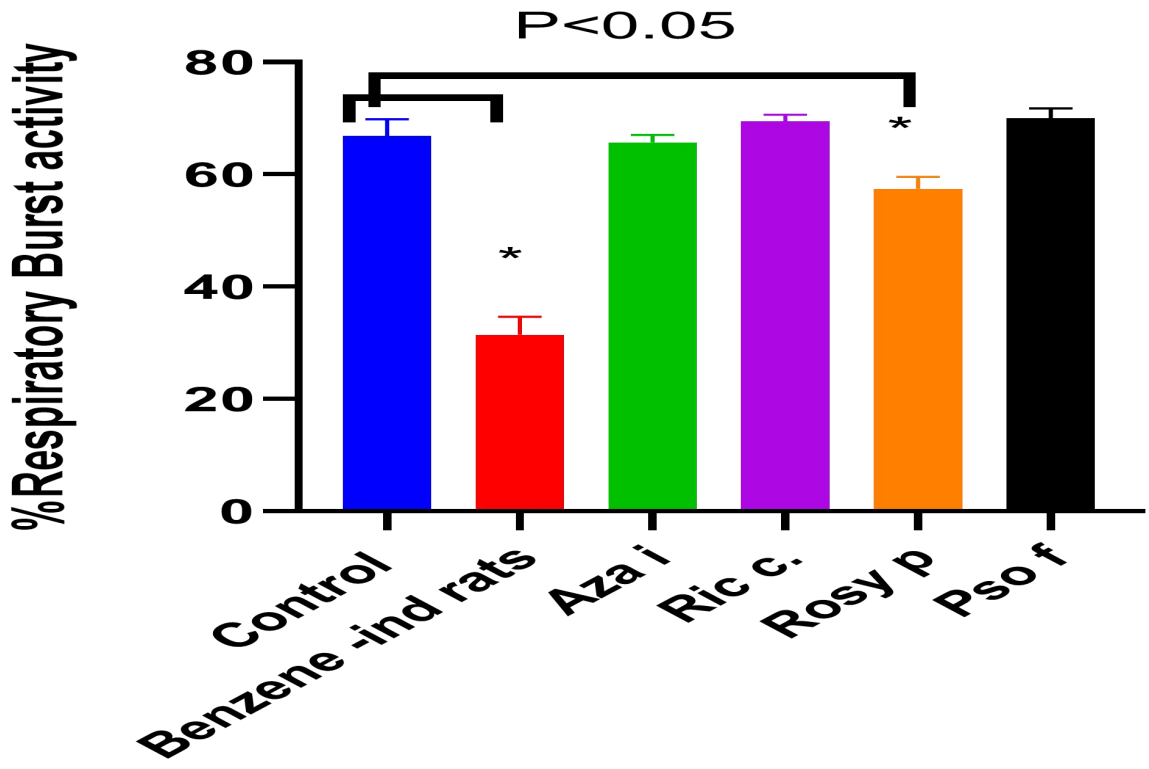

Figure 4.20: Results show respiratory burst in benzene induced rats pre-treated with extracts.

Figure 4.21: Results show the relationship between viability index and white blood cell count. There was a positive but no significant correlation between Viability index and total white blood cell count ((R =0.07539; P>0.05). This could be interpreted as that the total number of leucocytes does not predict the viability of the individual cell.

Figure 4.22: Results show the relationship between chemotactic index and total white blood cell count. There was a negative but no significant correlation between chemotactic index and total white blood cell count. This could be interpreted as that increase in total number of leucocytes may not determine the chemotactic actions of the individual cell.

Figure 4.23: Results show the relationship between phagocytic activity and white blood cell count. There was a positive but no significant correlation between Phagocytosis and total white blood cell count. This could be interpreted as that the total number of leucocytes does not predict the phagocytic ability of the individual cell involved in such action.

Figure 4.24: Results show the relationship between Respiratory burst activity and white blood cell count. There was a negative but no significant correlation between Respiratory burst activity and total white blood cell count. This could be interpreted as that the total number of leucocytes does not predict the ability of the individual cell in carrying out destructive action of ingested substances.

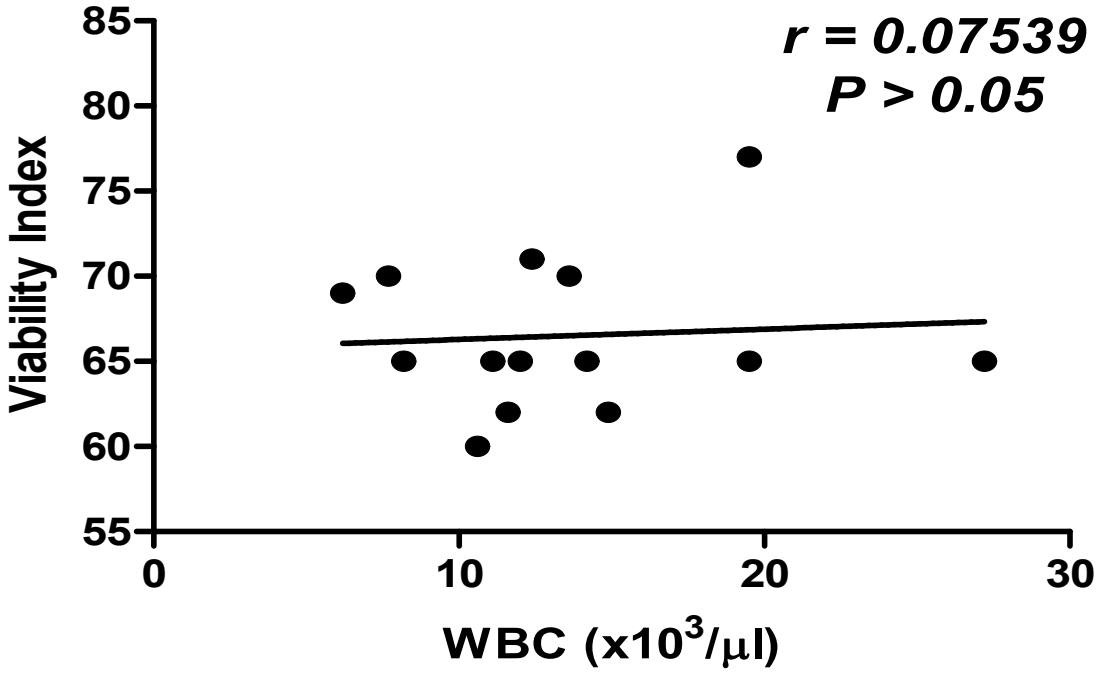

Figure 4.21: Results show the relationship between viability index and white blood cell count.

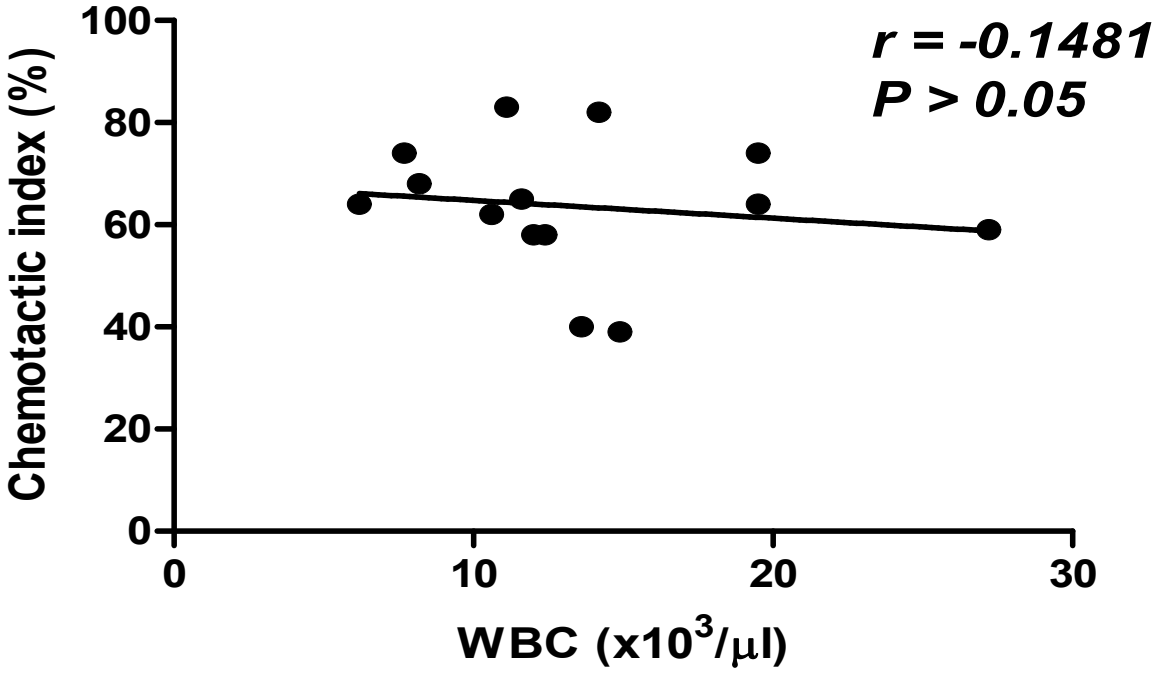

Figure 4.22: Results show the relationship between chemotactic index and white blood cell count.

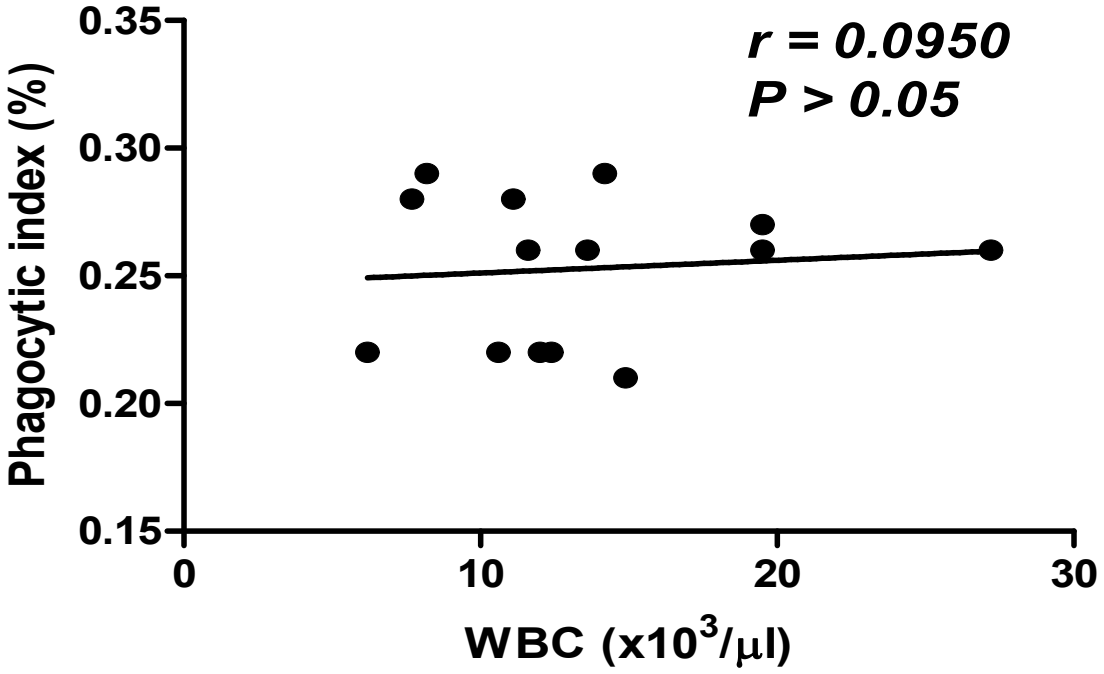

Figure 4.23: Showing the relationship between respiratory burst and white blood cell count.

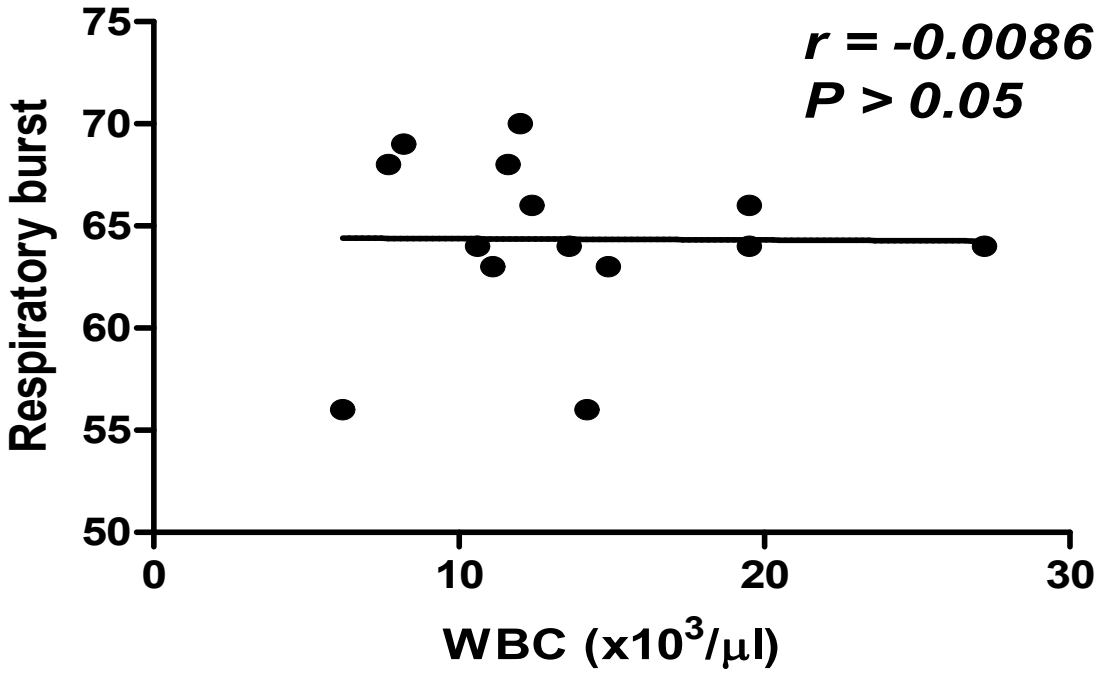

Figure 4.24: Relationship between Respiratory burst and White blood cell count

SUMMARY OF PHASE 3 STUDIES

1. Leukemic state is associated with decreased functions of cellular immunity – Viability of all white cells are decreased as well as chemotaxis, respiratory and phargocytic functions
2. Treatment with the extracts improved all the leucocyte functions respectively
3. Rosy periwinkle however showed a limited activity in respiratory function reversal
4. Ricinus communis showed a massive ability in the improvement of phargocytic function
5. There were positive correlations of white blood cells with Viability and phargocytic functions while it showed negative correlations with chemotaxis and respiratory burst activities.

Phase 4 Results

Molecular Studies and Mechanism of Action

FIGURE 4. 25: There was a significant increase in the TGF-β1 gene protein expression in the *Ricinus communis* treated group when compared with control, standard drug, *Rosy periwinkle* and Benzene –induced leukaemia group ($P<0.05$, respectively). TGF-β1 was up-regulated with Ricinus communis treatement than with Rosy periwinkle. This indicates that the *Ricinus communis* extract is capable of restoration of cellular functions and regulating the excessive proliferation of cells as well as controlling the cellular apoptosis

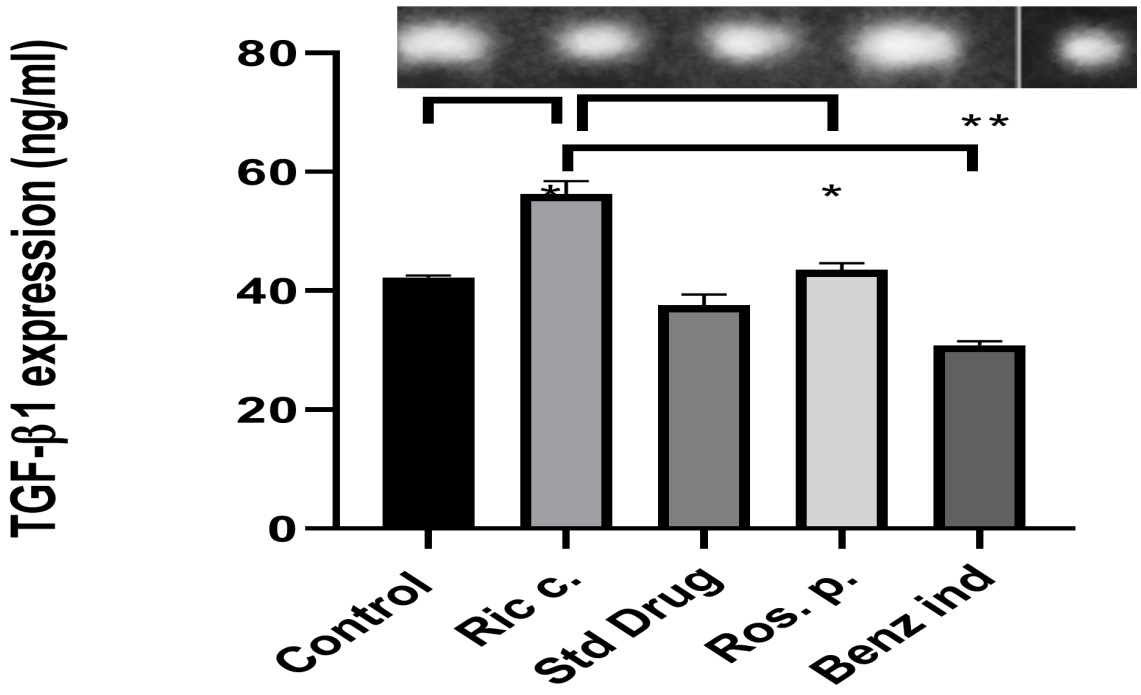

Figure 4.25: Comparison of TGF-β1 Expression in the selected efficacious extracts compared with control, Benzene –induced rats as well as standard drug.

CHAPTER FIVE

DISCUSSION AND CONLUSION

The hallmark of a cancer cell is its ability to multiply in an uncontrolled and continuous manner. Therefore, the ability to prevent cellular proliferation is a mainstay of anticancer treatment modalities. Globally, an estimated 300, 000 new cases of leukemia (2.8% of all new cancer cases) are diagnosed each year (Wang *et al.,* 2014). The prevalence and incidence of cancer in Nigeria seems to be low compared with developed nations around the world due mainly to lack of data or under-reporting of cases (Abutu *et al.,* 2019). Better understanding of the mechanisms that govern and control cell proliferation and apoptosis is important in understanding and controlling the cancer disease such as acute myeloid leukemia. Monocytes and macrophages obtained from myeloid cells, have the ability to proliferate or die depending on their environment and the cell controlling signals they encounter (Basu *et al.,* 2008). However, medicinal plants have long been considered as legitimate and prospective materials for discovery of anticancer agents (Nasir *et al.,* 2021), thus, with the traditional age-long consumption of plants as food, herbs and a source of medicine.Thisstudy selected four plants used as herbs: *Ricinus communis (Rc), Rosy periwinkle (Rp)Psorospermfebrifugum (Pf) and Azadirachta indica (Ai)* in our locality and designed its protocols to evaluate their in vivo anticancer potentials in benzene induced rats, the most efficacious and its possible mechanism of action.

This study therefore has demonstrated the successful induction of leukemia in Wistar rats injected intravenously with (0.2 ml/rat, 48 hourly) benzene chromasolv for four weeks as previously described by Akanni *et al.,* 2017. Benzene induced leukocytosis as previously

reported by Akanni *et al*, (2014) and Al-Fartosi *et al*, (2015). The mechanism of Benzene induced leukaemia as previously postulate dmechanism as described by McHale *et al*., (2012) involves an interference of benzene with key molecular and cellular factors through induction of genetic or epigenetic/chromosomal abnormalities and genetic instabilities in a hematopoietic stem cell to generate leukaemic stem cell and subsequent myeloid leukaemogensis. Though, this postulation is still undergoing elucidation.

This study however, using Immuno-histochemistry CD_{20} confirmation and other standard diagnostic procedures concluded a chronic lymphocyticleukaemiatype.which is characterized by the appearance of normal mature non-granulocytes in blood smear, high lymphocyte ratio and relatively low blood platelet count (Ravandi *et al.,* 2017). This however, was in accordance with previous reports of Mekonnen *et al*., (2007) and Steriti, (2017) who reported that benzene have deleterious effects on hematopoiesis, hematological and cellular immunity parameters.CD_{20} expression is initiated by at the pre-B cell stage of development and remains present until terminal differentiation into plasma cell (Middleton *et al*., 2016).

The comparison between the initial weight before the leukemia induction of the animals and the weight after leukemia was induced showed significant weight loss in the final weight compared with the initial weight. The weight loss was observed in all animal groupings induced as compared to the negative control group. The reason for the weight loss can be linked to rapid usage of body energy supply by cancerous cells due to rapid growth of the cancer cells as suggested by Wu *et al.* (2013).

Also, there was no significant difference in both packed cell volume and Hemoglobin concentration in induced animals that were pre-treated with all the extracts

before induction (P>0.05, respectively). This implies the possibility of the extract in preventing anaemia which is a hallmark in leukaemias but when the test animals were compared with controls, standard drug (flouracil) treatment and treatment with the different extracts after induction, there was a significant decrease in PCV with the standard drug group (P<0.05, respectively). However, *P. febrigum*and*R. periwinkle* showed significant increase in the mean values of the PCV (P<0.05, respectively). This indicates possible haematinic activities of the extracts

As part of specific objectives to understand the mechanisms of action of the extracts and also to identify the most efficacious, antioxidant activities were studied in pre and post treated animals as compared with the control group. There were significant increases in the SOD activities in the animals treated with *R. communis* after induction while *A. indica* exhibited a significant reduction (P<0.05, respectively) when compared with pre-treated controls. However, there was no significant difference in the other groups treated with *P. febrifugum*and *R. periwinkle.* These indicatethat only *R. communis* is capable of amplifying the activities of superoxide dismutase which was significantly reduced in the leukaemic animals. Pre-treating the animals before induction also inhibited its reduction. Increase in SOD concentration prevents damage to tissues, breakdown potentially harmful oxygen radicals in the cells. This was in line with the study by Hayyau *et al., (2016).*

Also, there were graded significant increases in the gluthathione reductase activities as demonstrated by all the extracts in the treated animals after induction (P<0.05, respectively) when compared with pre-treated controls. This is in the order of *R.communis>> P. febrigum>> P. periwinkle >> A indica*. It is noteworthy here that Pre-

treating the animals before induction did not enhance the reduction of gluthathione reductase activity but the post-treated animals responded positively in the order shown above. *R. communis*again proved to be a stronger antioxidant stimulator especially with glutathione challenge. Increase in glutathione reductase activity helps in preventing damage to important cellular component caused by reactive oxygen. This is in accordance with the work of Estrela *et al*., (2006) which observed elevated glutathione levels in various types of tumors.

Furthermore, catalase activities were significantly reduced in leukemic rats as well as pre-treated groups but was again significantly enhanced by *R. communis*both at pre and post experimental stages ($P< 0.05$, respectively). This confirms the antioxidant capabilities of the compound as an enhancer of the most antioxidant enzymes.

Hydroxyl radical activities were highly expressed in the leukaemic rats and the pre-treated groups but there were significant reductions in the Hydroxyl radicals' activities in the post treated animals with all the extracts when compared with the pre-treated groups ($P< 0.05$, respectively). Surprisingly, *A. indica* exhibited the most enhancing ability here.

In all, *Ricinus communis* proved to be a more effective antioxidant than all other extracts with more pronounced effects on: SOD, GSH, OH and Catalase activities.

Leucocyte functional activities were also assessed in benzene induced leukaemic, pre and post treated rats compared with the control animals. There were reductions in the total white cell counts in all the pretreated groups but significantly decreased with *R.periwinkle* only ($P<0.05$). This showed the preventive actions of the extracts while standard drug (flouracil) and *R.communis*only showed significant reductions in the counts

impost treated animals (P<0.05, respectively), all other reductions with other extracts were not statistically significant. This proves further, an enhanced activity of *R. communis* over the other extracts.

Various combinations of the extracts were tested on both pre-treated and post-treated animals, there was no synergistic effect recorded, this indicate a probable antagonistic action of the extracts against each other, therefore, the practice of mixing them for treatment should be discouraged.

The outcome of the induction process in this study was a proven chronic lymphocytic leukemia with higher numbers of lymphocytes in the peripheral blood film. The effect of the extracts in ameliorating this was tested with the various extracts, the results showed significant effectiveness of *Ricinus communis* and *Rosy periwinkle* only in preventing the surge of lymphocytes (P<0.05, respectively). The other extracts however did not show any significant change when compared with control.However,Comparison between control, Benzene induced leukaemic rats, standard drug with pre and post treated animals shows the significant reductions of lymphocytes with the extracts in the order *R periwinkle>> R. communis >>A. Indica >>P. febrigum.* This shows that *R. Periwinkle*and*R. communis* are more effective in the amelioration of the leukaemia.

Leukemic state is associated with decreased functions of cellular immunity – Viability of all white cells are decreased as well as chemotaxis, respiratory burst activity and phargocytic functions. Treatment with the extracts improved all the leucocyte functions respectively. However, *Rosy periwinkle* showed a limited activity in respiratory function

improvedment while *Ricinus communis* showed a massive ability in the improvement of phargocytic function.

All the extracts recorded various degrees of improvement in leucocyte viability tests.Cell viability is the quantification of the number of live cells and is usually expressed as a percentage of the control (Kroemer *et al*., 2009). Cell viability assay is a measure of cellular activity and is used to determine the number of healthy cells in a sample. It reports on the overall health of a population of white cells. Leucocyte Viability test is usually carried out to ascertain how viable the harvested leucocytes are before other functions are carried out.

Respiratory burst is an essential component of innate immunity that enables phagocytic cells to remove or eliminate microbes and in the process there is the release or Reactive oxygen species (ROS) and the excessive production of ROS can result in the induction of oxidative stress in cells (Gorski and Ktosowska, 2012). The chemotaxis and phargoctic functions are indicative of the ability of polymorphonuclear leucocytes to migrate and cause ingestion of invading organisms or dead cells for physiological effectiveness.

There were positive correlations of white blood cells with Viability and phargocytic functions while it showed negative correlations with chemotaxis and respiratory burst activities. This means that the total number viability of white blood cells in circulation logically determines the amount of viable ones. This is in line with the observations of Kroemer *et al*., (2009) while the migration and ingestion organisms may depend on inherent ability of individual cells.

Molecular Studies

Finally, there was a significant increase in the TGF-β1 gene protein expression in the *Ricinus communis* treated group when compared with control, standard drug, *Rosy periwinkle* and Benzene –induced leukaemia group ($P<0.05$, respectively). TGF-β1 was up-regulated with *Ricinus communis*treatement than with *Rosy periwinkle*. This indicates that the *Ricinus communis* extract is capable of restoration of cellular functions and regulating the excessive proliferation of cells as well as controlling the cellular apoptosis. TGF-β1 is a secreted protein that performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation, and apoptosis. In humans, TGF- β1 is encoded by the TGF- β1gene (Ghadami*et al*., 2000), during hematopoiesis, the TGF-β signaling pathway is a potent negative regulator of proliferation while stimulating differentiation and apoptosis when appropriate. In hematologic malignancies, including leukemias, myeloproliferative disorders, lymphomas, and multiple myeloma, resistance to these homeostatic effects of TGF-β develops. Mechanisms for this resistance include mutation or deletion of members of the TGF-β signaling pathway and disruption of the pathway by oncoproteins. These alterations define a tumor suppressor role for the TGF-β pathway in human hematologic malignancies (Dong and Blobe, 2006).Chen et al., (1998) reported that TGF beta 1 was found to significantly inhibit the proliferation of leukemia cells of HL-60, K562 and DAMI cell lines. They concluded that,TGF beta 1 may act as an inhibitory autocrine factor in the proliferation of leukemia cells and the mechanism by

which TGF beta 1 inhibits the growth of leukemia cells involves its down-regulating expression of positive cytokines and receptors and up-regulating TGF beta 1 expression in leukemia cells. This is in line with the observations in this study.

In summary, Lymphocytic leukamia was successfully induced in rats using Benzene chromoslov at 10mg/kg body weight daily for 4 weeks. The extracts of *Psorospernumfebrifugum*, *Ricinus communis*, *Azadirachta indica* and *Rosyperiwinkle* demonstrated varying preventive and ameliorative effects on the induced animals, with leukemia especially with *Ricinus communis*. The extracts equally showed significant antioxidant enzyme activities in post treated Wistar rats while TGF- β1 gene protein was significantly up-regulated in rats treated with *Ricinus communis* resulting in massive apoptosis of the leukemia cells. This study has shown clearly that *Ricinus communis* is more effective and showed profound efficacy among the four extracts used in this study.

Figure 5.1. Schema of mechanism of action of *Ricinus communis* in the treatment of leukaemia

Benzene induces leukaemia through established alterations of the antioxidant pathways and induces an chronic lymphocytic response, suppressing the leucocyte functional activities and down-regulate Tissue growth factor- β1 (TGF-β1) gene. These creates a state of leukaemia and predisposes the animal to massive infections and generalized nodular development. The treatment of the animals with the extracts especially that of *Ricinus communis* improves the antioxidant defenses, enhancesleucocyte functions and up-regulate TGF-β1 gene. This regulates cellular apoptosis and reduce excessive cellular proliferation.

5.2 Conclusions

The extracts of *Psorospernumfebrifugum*, *Ricinus communis*, *Azadirachta indica* and *Rosyperiwinkle* demonstrated varying preventive and ameliorative effects on the induced animals, with leukemia especially with *Ricinus communis*.

The extracts equally showed significant antioxidant enzyme activities in post treated animals while the TGF β1 gene protein was significantly up-regulated in animals after treatment with *Ricinus communis*resulting in massive apoptosis of the leukaemic cells.

Leucocyte functions were significantly enhanced after treatment with the extracts and especially with that of *Ricinus communis*.

Ricinus communis has shown a mechanistic ability to reverse leukocytosis, improve leucocyte functions, and enhance antioxidant activities as well as up-regulating TGF-β1 gene response in leukemic rats as observed in this study.

It is recommended that extracts of *Ricinus communis* should be evaluated further for its pharmacological and therapeutic efficacies in the management of lymphoid leukaemia.

5.3 Research Findings

1. Benzene successfully induced significant leukocytosis with histochemical features in keeping with lymphoblastic leukaemia in rats
2. There was a significant loss in weight after a successful induction of Leukemia
3. The lost weights were significantly reversed with the administration of the different extracts
4. Total white blood cell count was significantly reduced in leukaemic rats treated with *Rosy periwinkle*and *Ricinus communis* only
5. Lymphocyte count reduced significantly in all leukaemic rats treated with all extracts, but, with a profound effect seen with *Ricinus communis* in the order: *Ricinus communis >>Psorospernumfebrifugum>> Rosy periwinkle>>Azadirachta indica*
6. Leukemic state is associated with decreased functions in cellular immunity – Viability of all white cells are decreased while chemotaxis, respiratory function as well as phargocytic functions of polymorphonuclear cells were all decreased.
7. Treatment with the extracts improved all the leucocyte functions respectively. *Rosy periwinkle* however showed a limited activity in respiratory function reversal while *Ricinus communis* showed a massive ability in the improvement of phargocytic function

8. There were positive correlations of white blood cells with Viability and phargocytic functions while it showed negative correlations with chemotaxis and respiratory burst activities

9. *Ricinus communis* is a more effective antioxidant than all other extracts with more pronounced SOD, GSH, OH and Catalase activities compared with other extracts

10. There were preventive effects of the extracts on the development of leukaemia after induction in pre-treated rats with extracts in the order: *Rosy periwinkle >> Ricinus communis >>Psorospernumfebrifugum>>Azadirachta indica*

11. Ricinus communis exhibited a more significant expression of TGF-β1 protein in Benzene –induced leukemic rats compared with that of Rosy periwinkle

5.4 Contributions to Knowledge

1. Total white blood cell count was significantly reduced in leukaemic rats treated with *Rosy periwinkle and Ricinus communis* only
2. Lymphocyte count reduced significantly in all leukaemic rats treated with all extracts, but, with a profound effect seen with *Ricinus communis* in the order: Ricinus communis *>>Psorospernumfebrifugum>> Rosy periwinkle >>Azadirachta indica*
3. Leukemic state is associated with decreased cellular immunity functions – Viability of all white cells are decreased while chemotaxis, respiratory function as well as phargocytic functions of polymorphonuclear cells were all decreased
4. Ricinus communis is a more effective antioxidant than all other extracts with more pronounced SOD, GSH, OH and Catalase activities compared with other extracts
5. There were preventive effects of the extracts on the development of leukaemia after induction in pre-treated rats with extracts in the order: *Rosy periwinkle >> Ricinus communis >>Psorospernumfebrifugum>>Azadirachta indica*
6. *Ricinus communis* exhibited a more significant expression of TGF-β1 protein in Benzene –induced leukemic rats compared with that of Rosy periwinkle

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APPENDIX

Figure1.1.1: RicinusCommunis Plant

Figure 1.1.2: RicinusCommunis Fruits

Figure 1.1.3: RicinusCommunis Seeds

Figure 1.2: AzadiracthaIndica

Figure 1.3: Rosy Periwinkle

Figure 1. 4: Christmas Berry

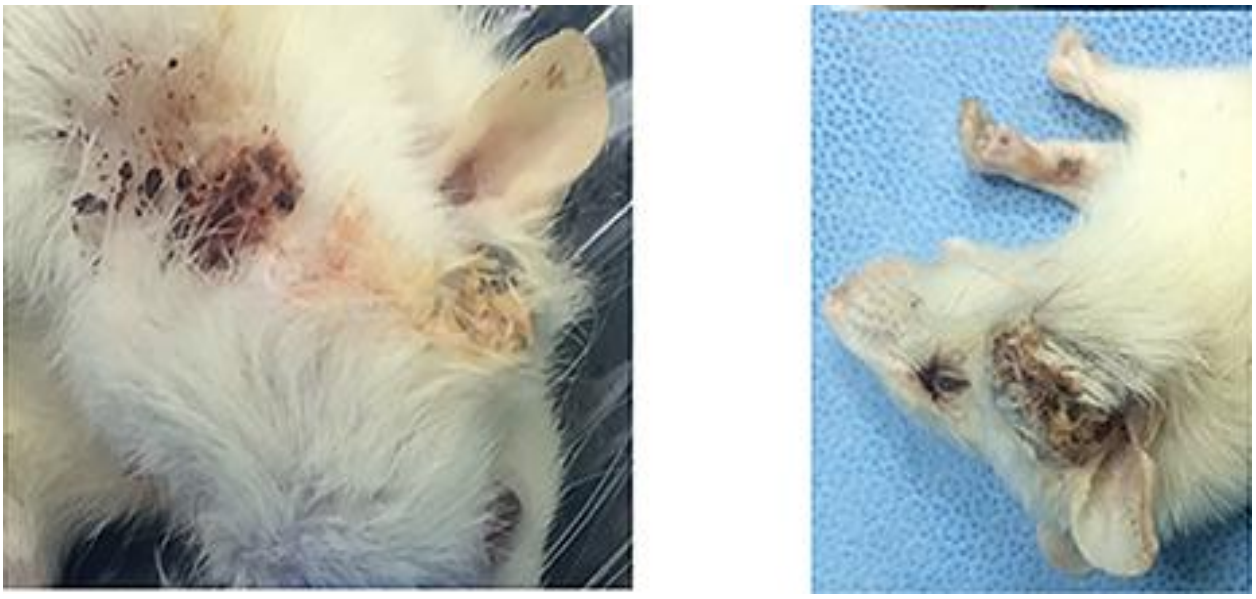


Figure 3.2 Effect of Benzene chromosolv on the animals showing Tumor

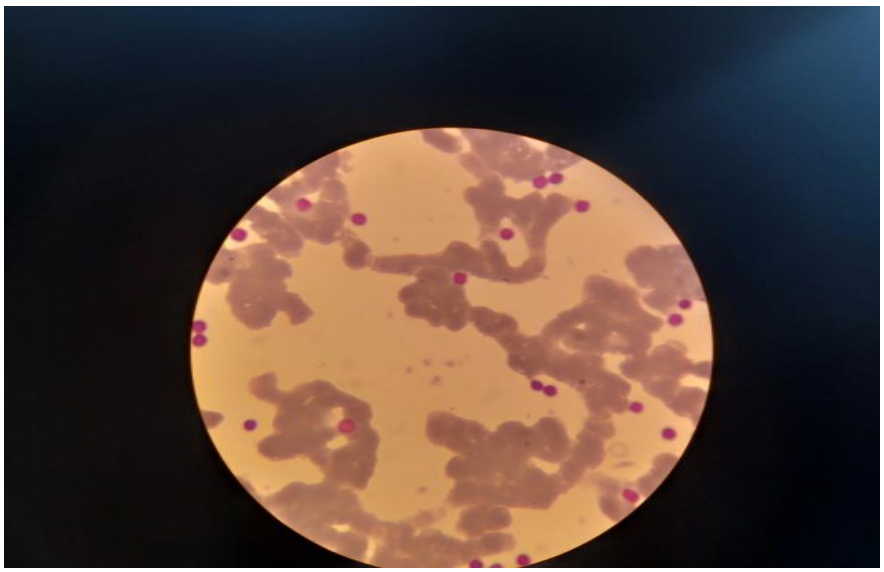


Plate 1: Photomicrograph of differential count showing lymphocytosis

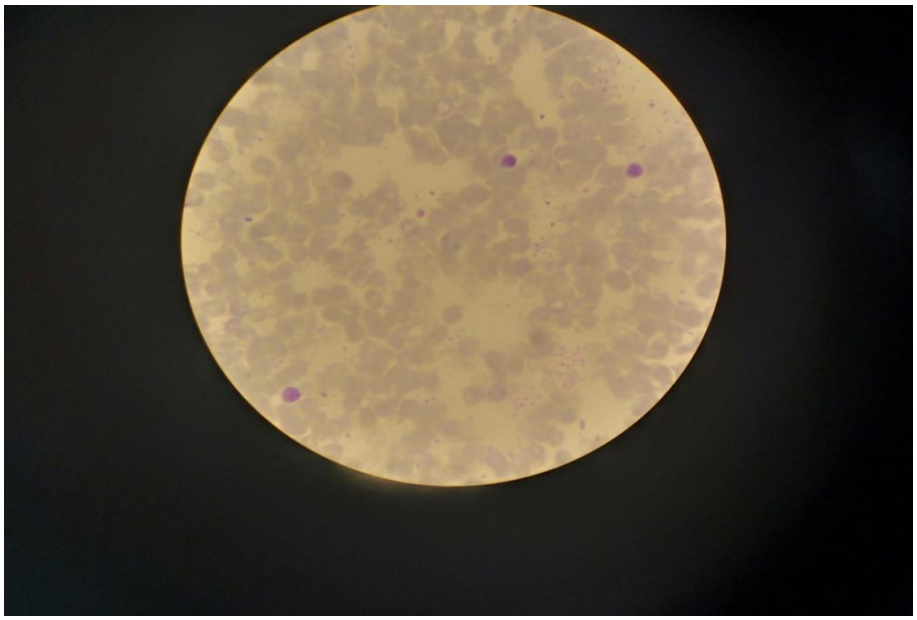
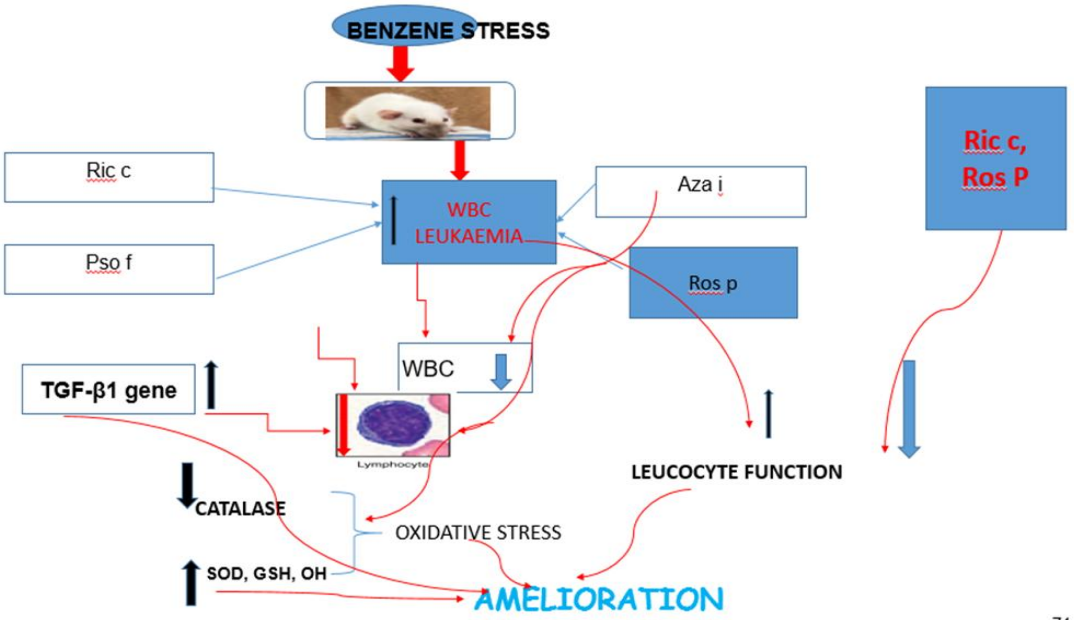
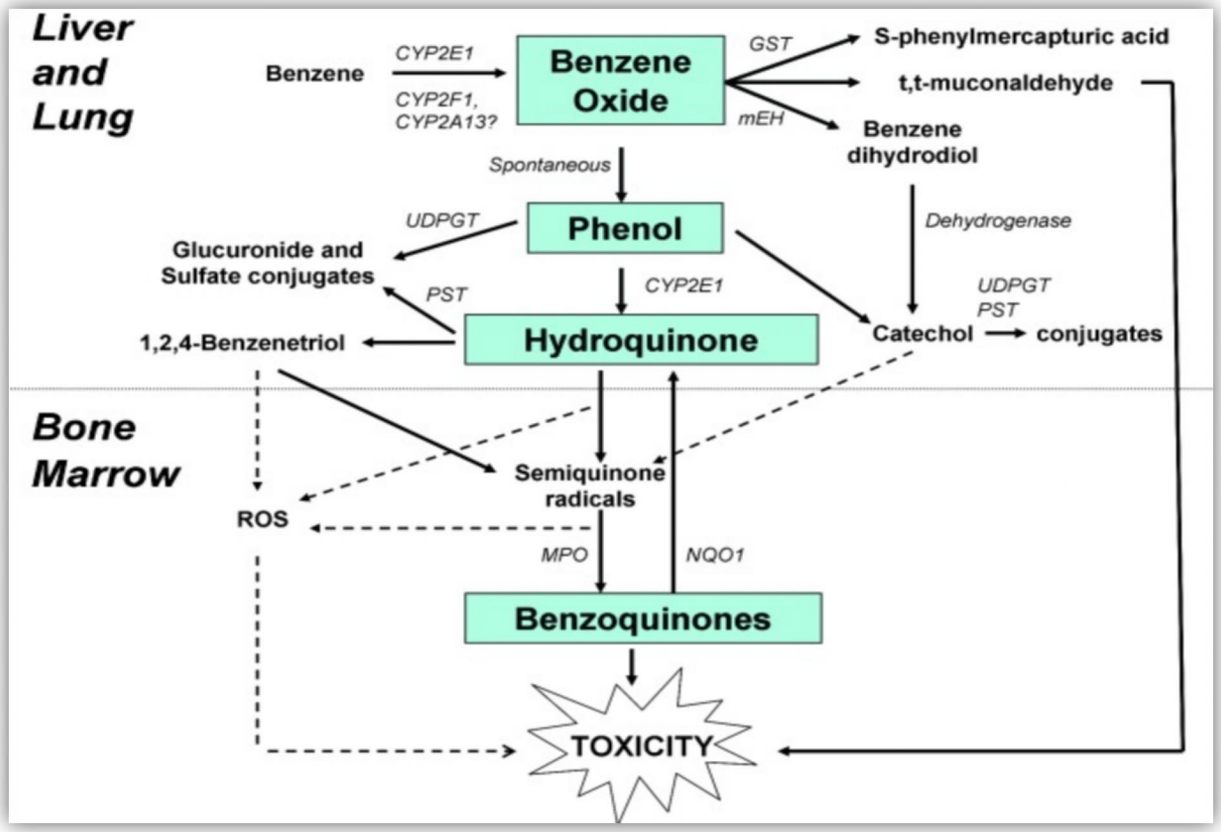


Plate 2: Showing blood film after treatment with the extracts

SUGGESTED MECHANISM OF ACTION OF RICINUS COMMUNIS OF BENZENE INDUCED LEUKEMIC RATS



Schematic diagram of mechanism of action *of Ricinus Communis* in the treatment of leukaemia.



BENZENE INDUCED LEUKEMIA - MECHANISM

Figure 2.2: Simplified metabolic scheme for benzene showing site– specific metabolism from liver to bone marrow including major pathways and metabolizing enzymes leading to toxicity.Steinmaus et al; 2008.