RAT SARCOMA (RAS) AND WILMS TUMOR 1 (WT1) EXPRESSION PATTERN IN MALE PRE- LEUKEMIC ALBINO WISTAR RAT ADMINISTERED AQUEOUS EXTRACT OF *VERNONIA AMYGDALINA*.

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BENIN CITY

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BEING A PROJECT SUBMITTED TO THE DEPARTMENT OF MEDICAL LABORATORY SCIENCE IN PARTIAL FULFILLMENT FOR THE REQUIREMENTS OF THE AWARD OF BACHELOR DEGREE IN MEDICAL LABORATORY SCIENCE (BMLS), UNIVERSITY OF BENIN, BENIN CITY, NIGERIA.

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APRIL, 2024

CERTIFICATION

This is to certify that this seminar work was carried out by **IDADA AISOSA DANIEL** with the matriculation number **BMS1802456** under the supervision of **DR. A.I. ARUOMAREN** in Partial fulfillment for the award of Bachelor of Medical Laboratory Science (BMLS) Degree of the Department of Medical Laboratory Science, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin City, Edo State**.**

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EXTERNAL EXAMINER DATE

DEDICATION

I dedicate this seminar work to God Almighty for making this seminar work a huge success and also to my lovely family for their love and support.

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ABSTRACT

Vernonia Amygdalina is a plant renowned for processing several bioactive compounds, some of which may hold promising medicinal properties. Delving into its impact on leukemia presents an opportunity to uncover valuable insights regarding its therapeutic applications in leukemia-related conditions. The objective of this study was to determine the effects of Vernonia Amygdalina leaves extract on RAS and WT1 leukemia gene in male albino Wistar rats. A total of twenty (20) male adult albino Wistar rats were selected into four (4)M groups. The groups were the control group,benzene:2-propanol group, 250mg/kg of Vernonia Amygdalina group, andbenzene:2-propanol with 250mg/kg Vernonia A mygdalina group. RNA extraction and semi-quantitative polymerase chain reaction (PCR) were used to isolate RNA from rat bone marrow, and complementary DNA was synthesized and was subsequently amplified using polymerase chain reaction. Gel electrophoresis was used to determine the bands of the genes while peripheral blood film and full blood count were carried out by manual and automated methods respectively. The findings revealed that; for WBC (White Blood Cells),there was no significant difference (p=0.881) in group 3 (8.90±3.60) and group 4 (7.03±1.16) when compared to group 1 (8.27±1.91) and group 2 (7.97±0.70).Also, in N(Neutrophils),there was no significant difference (p=0.149) in group 3 (2.50±0.50) and group 4 (6.00±2.04) when compared to group 1(10.00±0.58) and group 2 (9.00±2.65).L (Lymphocytes)also showed no significant difference (p=0.183) in group 3 (91.00±1.00) and group 4 (88.00±2.71) when compared to group 1(82.00±1.53) and group 2 (85.67±2.60).The M (Monocytes)also showed no significant difference (p=0.523) in group 3 (5.00±0.00) and group 4 (5.00±0.91) when compared to group 1 (6.67±1.76) and group 2 (4.33±0.33). E (Eosinophils)showedno significant difference (p=0.987) in group 3 (1.50±0.50) and group 4 (1.33±0.33) when compared to group 1 (1.33±0.33) and group 2 (1.33±0.33). The RBC (Red Blood Cells)also showed no significant difference(p=0.531) in group 3 (6.32±0.49) and group 4 (6.53±0.21) when compared to group 1 (6.02±0.78) and group 2 (6.99±0.24).Hb (Hemoglobin)also showed no significant difference (p=0.226) in group 3 (12.95±0.25) and group 4 (13.30±0.38) when compared to group 1 (12.50±1.40) and group 2 (15.03±0.64).PLT (Platelets)showed no significant difference (p=0.649) in group 3 (1010.50±252.50) and group 4 (27536.50±26837.19) when compared to group 1 (1356.00±705.72) and 2 (1111.67±193.69), except for a notable outlier in group 4 (27536.50±26837.19). This study concludes that Vernonia Amygdalina did not have any significant effect on leukemia gene RAS and WT1 expression. These results suggest that Vernonia amygdalina could not influence the expression of the leukemia gene RAS and WT1.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Vernonia amygdalina which is a small shrub with dark green leaves and rough barks grows predominantly in tropical Africa but has been adapted in many parts of West Africa (Igile *et al*., 1994). They are mostly found in various countries across the continent, and its geographical distribution includes regions such as West Africa, Central Africa, and parts of East Africa. It is a perennial shrub or small tree also known as bitter leaf, and can reach heights of up to 3 meters (Burkill, 1997). It is a plant with various phytochemicals that contribute to its medicinal properties and they include sesquiterpenoids (Okeke and Okoli, 2016), alkaloids (Izevbigie *et al*., 2004), flavonoids (Erukainure *et al*., 2018), tannins (Nwaoguikpe *et al*., 2014) and steroids (Ogundipe *et al*., 2015). The leaves are taken as green leafy vegetable. Its richness in minerals and vitamins has made it important to humans (Sobukola *et al*., 2007). Specifically, bitter leaf can be found in countries like Nigeria (Udoh *et al*., 2014), Ghana (Alabi *et al*., 2005), Cameroon (Dongmo *et al*., 2013), Uganda (Kakudidi, 2004) and others where the climate is suitable for its growth (Farombi and Owoeye, 2011). The plant has adapted to diverse ecosystems within the tropical region. It's important to note that while *Vernonia amygdalina* is accustomed to Africa, it has also gained attention and interest in other parts of the world for its potential medicinal properties, leading to cultivation in regions beyond its native habitat. It is commonly found in various states across Nigeria, it may not be equally distributed in every single state. The specific presence and abundance of bitter leaf can vary within each state due to factors such as local climate conditions, soil types, and cultivation practices (Oboh *et al*., 2012). While it is widely used in Nigerian cuisine and traditional medicine, its distribution may be influenced by regional variations. They can be used against fevers and are commonly used instead of quinine in African

countries including Nigeria (Masaba, 2000). The leaves are used traditionally to make women fertile (Adedapo *et al*., 2014), as purgative (Kupcham, 1971). It has been used widely to cure several parasitic ailments such as amoebic dysentery and schistosomiasis (Huffman *et al*., 1996), helminthosis (Nabukenya *et al*., 2014), it is also needed for hiccups, typhoid fever (Fadimu *et al*., 2014), yellow fever, (Ene and Atawodi, 2012), stomach-ache, convulsions (Tugume *et al*., 2016), boils, burns, diabetes (Ajibesin *et al*., 2008), jaundice (Simbo, 2010), inflammatory diseases (Ogbole *et al*., 2010), candidiasis (Mustapha *et al*., 2013), cancer, viral diseases (Koubé *et al*., 2016), bacterial infection, gastrointestinal (GIT) disorders, liver diseases, kidney problems, nausea (Atangwho *et al*., 2012). The plant leaves, root and twig are used for treating wounds, venereal diseases and hepatitis (Nwanjo, 2005; Erasto *et al*., 2006). The leaves are also needed for breast milk improvement in nursing mothers (Kankara *et al*., 2015), treating fever in poultry (Nalubega *et al*., 2012), helminthosis in livestock (Nabukenya *et al*., 2014). More so, the aqueous extract of the leaves is commonly needed for the treatment of diabetes, induced abrosia nausea, emesis, loss of appetite, dysentery and other gastrointestinal tract problems (Adedapo *et al*., 2014), scabies, headache, stomach-ache, joint pain related to AIDS, gingivitis and tooth ache which is due to its antimicrobial activity (Akah and Okafor,1992; Alabi *et al*., 2005; De Boer *et al*., 2005; Fasuyi, 2006; Innocent and Deogracious, 2006) by herbalist and naturopathic doctors for their patients.

1.2 Justification of Study

Vernonia amygdalina contains phytochemicals such as Sesquiterpenoids (Okeke and Okoli, 2016), Alkaloids (Izevbigie *et al*., 2004), Flavonoids (Erukainure *et al*., 2018), Tannins (Nwaoguikpe *et al*., 2014) and Steroids (Ogundipe *et al*., 2015) and these phytochemicals

contribute to the medicinal properties and potential health benefits associated with bitter leaf consumption (Iweala *et al*., 2017). The phytochemical screening of bitter leaf (Vernonia amygdalina) generally reveals the presence of various bioactive compounds, some of which may have therapeutic properties. In Rat sarcoma (RAS), some preliminary research indicates bitter leaf extracts can inhibit growth of certain cancer cell lines, but the molecular mechanisms are not fully understood and have not been linked to the RAS pathway (Yeap *et al*., 2010; Awah *et al*., 2012) and also while bitter leaf shows anti-cancer potential, there is no data establishing effects on oncogenic RAS signaling in cancer cells specifically while in Wilms Tumor 1 (WT1) gene there are no reports elucidating the effects of Vernonia amygdalina constituents on WT1 expression levels or transcriptional activity and several studies demonstrate cytotoxicity and anti-proliferative actions of bitter leaf extracts against leukemia cell lines (Awah *et al*., 2012; Asare *et al*., 2013). However the mechanisms of these anti-leukemic effects have not been tied to disruption of WT1 function.The anti-leukemic and erythropoietic effects of Vernonia amygdalina have been attributed to its bioactive compounds, such as sesquiterpene lactones and flavonoids, which may modulate cell signaling pathways involved in cancer cell death and erythropoiesis (Alara *et al*., 2017). A clinical study involving leukemia patients suggested that bitter leaf extract, in combination with conventional chemotherapy, may improve treatment outcomes and reduce side effects. However, further research is needed to confirm these findings (Yedjou *et al*., 2008).

1.3 Aim of Study

The aim of this study is to determine the effect of *Vernonia amygdalina* aqueous leaf extract on some epigenetic markers (RAS and WT1) in male preleukaemic Albino Wistar rats.

1.4 Specific Objectives

a. To determine the effect of the effect of *Vernonia amygdalina* aqueous leaf extract on Rat sarcoma (RAS) in male preleukaemic Albino Wistar rats.

b. To determine the effect of the effect of *Vernonia amygdalina* aqueous leaf extract on Wilms Tumor 1 (WT1) in male preleukaemic Albino Wistar rats.

c. To determine the effect of *Vernonia amygdalina* aqueous leaf extract on some haematological parameters in male preleukaemic Albino Wistar rats.

1.5 Research Questions

a. Does *Vernonia amygdalina* aqueous leaf extract have any effect on Rat sarcoma (RAS) in male preleukaemic Albino Wistar rats?

b. Does *Vernonia amygdalina* aqueous leaf extract have any effect on Wilms Tumor 1 (WT1) in male preleukaemic Albino Wistar rats?

c. Does *Vernonia amygdalina* aqueous leaf extract have any effect on haematological parameters in male preleukaemic Albino Wistar rats?

1.6 Research Hypothesis

Null Hypothesis (H_0)

a. *Vernonia amygdalina* aqueous leaf extract does not have any effect on DNA RAS, WT1 and haematological parameters in male preleukaemic Albino Wistar rats.

Alternate Hypothesis (H_A)

b. *Vernonia amygdalina* aqueous leaf extract has an effect on Rat sarcoma (RAS), Wilms Tumor 1 (WT1),and haematological parameters in male preleukaemic Albino Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and Distribution of Vernonia amygdalina.

Vernonia amygdalina (bitter leaf) is a versatile plant that has several uses. It is a leafy vegetable that originated in tropical Africa and is widely distributed across sub-Saharan Africa. *Vernonia*

amygdalina is thought to have originated in the tropical rainforest regions of sub-Saharan Africa based on the diversity of Vernonia species found in those areas (Erasto *et al*., 2006). Several studies have traced the domestication and early cultivation of bitter leaf to southeastern Nigeria, western Cameroon, and possibly other areas of West Africa (Burkill, 1985; Okwu and Njoku, 2009). Today, *Vernonia amygdalina* grows commonly as a wild or cultivated crop across much of sub-Saharan Africa including Nigeria, Cameroon, Gabon, DR Congo, Angola, Uganda, Tanzania, Malawi, Zimbabwe and others (Erasto *et al*., 2006; Burkill, 1985).It has been introduced and naturalized in parts of tropical Asia including India and Malaysia as well (Okwu & Njoku, 2009). As an important leafy vegetable in African cuisine, bitter leaf has also been taken globally by immigrants leading to cultivation in Europe, the Americas, and the Caribbean (Lim, 2012). It is a source of edible leafy vegetable. *Vernonia amygdalina*, or bitter leaf, is renowned for its rich nutritional profile. They were been used as medicinal products of plant origin. The leaves contain several medically active compounds like flavonoids, saponins, alkaloids, and tannins that exhibit antimicrobial, anti-parasitic, anti-tumor, anti-inflammatory and antioxidant properties (Ezekwesili *et al*., 2011). Bitter leaf extracts from the leaves demonstrate antidiabetic activities by lowering blood glucose levels as demonstrated in rat models (Adeneye, 2008). Bitter leaf extracts have also shown hepatoprotective effects on liver tissue damaged by toxins like carbon tetrachloride, indicating its potential in managing liver disorders (Hamzah *et al*., 2018). Bitter leaf is very high in vitamin C content with values ranging from 148-246mg per 100g in the fresh leaf, one of the highest for leafy greens (Udosen, 1995; Igile *et al*., 1994) and it also provides vitamin A, with values reported around 11,300 IU per 100g of leaf (Akubugwo *et al*., 2007). These vitamins play very important roles in immune function, skin health, and metabolism, Minerals; It contains very important minerals such as calcium, potassium,

magnesium, and iron. Iron, calcium and phosphorous are present in good amounts (Akubugwo *et al*., 2007). These minerals are vital for bone health, electrolyte balance, and oxygen transport in the body, Antioxidants; Bitter leaf is rich in antioxidants, including flavonoids and polyphenols. Bitter leaf contains proteins that contribute to the overall protein intake in the diet. Proteins are necessary for different bodily functions, including repair of tissue and support of immune system. Values ranging from 4.65-9.3g per 100g of leaf have been reported (Akubugwo *et al*., 2007; Igile *et al*., 1994), Fiber; High fiber content in bitter leaf aids in digestion and contributes to a healthy gastrointestinal system. Fiber also helps in the regulation of blood sugar levels and also promotes that feeling of fullness with reported values of 12-14g per 100g of leaf material (Udosen, 1995; Igile *et al*., 1994), Carbohydrates; Total carbohydrates range from about 11-19g per 100g, mostly in the form of insoluble fiber (Akubugwo *et al*., 2007; Igile *et al*., 1994). Phytochemicals; Bitter leaf contains phytochemicals like flavonoids, terpenoids, steroids, and alkaloids that may have antioxidant and medicinal effects and health benefits also with anti-inflammatory and anti-microbial properties (Farombi, 2003). The stem bark exhibits antiplasmodial activity against malaria parasite species like Plasmodium berghei in mice models, validating its traditional use to treat malaria (Ajaiyeoba *et al*., 2006). The methanolic extract of the flowers showed the highest antioxidant activity in assays compared to extracts from other parts, due to high phenolic content. This establishes its medicinal efficacy as an antioxidant agent (Moyo *et al*., 2013). The crude methanolic root extracts have exhibited trypanocidal properties in an in vitro model against Trypanosoma brucei gambiense, proving beneficial in treating trypanosomiases (Bizimana *et al*., 2006). It's important to note that the nutritional content may vary based on factors such as plant variety, growing conditions, and preparation methods. It can help in food breakdown and help one lose weight because of nutrients like zinc, iron, fiber, and carbohydrate contained in it. The

antioxidant contained in the Bitter leaf is necessary in positive gastric effects that give protection from stomach ulcers. A study in the International Journal of Reproductive Biomedicine has shown that it may have a positive effect on sperm quality. Flavonoids is present, which have powerful antioxidant effects that can help with treating high fevers. Study showed its use in traditional medicine for the treatment of the bacterial infection typhoid fever. It was also used to treat Osteoporosis because it contains traces of vitamin K which helps the body to keep healthy bones and prevents the weakening of bone tissue. Bitter leaf improves metabolic function as it contains Vitamin B1, which is also known as thiamine which helps in the metabolism of lipids, amino acids, and also glucose in the human body

Figure 2.1: Leaves of Vernonia amygdalina (Udochukwu *et al*., 2015).

Table 1: Some species of Vernonia amygdalina according to the International Plant Genetic Resources Institute (IPGRI)

S/N	Species	Description
1	Vernonia amygdalina variety amygdalina	This is the typical variety of bitter leaf. It has dark green leaves with pointed tips (Ezuruike and Prieto, 2014).
2	Vernonia amygdalina Subspecies. kilimanjari	This variety has ash-gray colored young shoots and leaf stalks. The leaves may sometimes appear silver-grey (Ezuruike and Prieto, 2014).
3	Vernonia amygdalina Subspecies. kilimanjari	This is named after Mt. Kilimanjaro area. It has less bitter leaves used as vegetable (Tengnäs*et al*., 2005).
4	Vernonia schimperi	This species is closely related to V. amygdalina but has a more spreading growth form. It has higher drought

		tolerance (Chadare*et al*., 2008).

2.2 Nutritional Properties of *Vernonia amygdalina*

The most constituent of fresh *Vernonia amygdalina* leaves was water, which is composed of 83.0% of their weight. Fresh leaves had protein of 1.3% and ash of 0.5%. Protein present in the leaves were lower and (van Gastel and van den Wijingaart, 1997; Madamba, 2006). Fresh leafy vegetables have low protein mostly in the enzyme form, rather than acting as a storage pool, as in grains and nuts (Wills *et al.*, 1998). On a fresh weight basis, mineral content was as follows: Phosphorus contained 61.55µg g-1 of fresh (540.0µg g -1) and for cowpea (90.0µg g -1). One hundred g of fresh leaves contained 0.47% of the recommended daily allowance (RDA) for this element. 0.0082µg g-1 of Selenium was present in fresh leaves. Thus 1.5% of the RDA for this element (0.82µg) was present in 100g leaves, about one-tenth of its content in foods considered good sources of the mineral –Iron content was 4.71µg g -1 (3.1-4.7% of the RDA for this mineral was present in 100g of fresh leaves). It is important to note however, that the nutritional value of

vegetables do not only depends on the amount of nutrients in the product, but also depends on the amount consumed in the food. Since vegetables are usually eaten with other dietary components, some of which may be good sources of the minerals under examination, this vegetable could be of help in completing the minerals available from these.

2.3 Phytochemicals of *Vernonia amygdalina*

The phytochemical studies of *Vernonia amygdalina* revealed that it contains biochemical constituents such as saponins (Idu *et al*., 2012), alkaloids (Okeke *et al*., 2018), tannins (Idu *et al*., 2012), flavonoid (Ezejiofor *et al*., 2017), phenolic compounds (Osuagwu *et al*., 2018) and terpenoids (Ezejiofor *et al*., 2017). These phytochemicals exhibit pharmacological and biochemical activities and also beneficial to human wellbeing (Omale and Okafor, 2008). Several study were conducted in isolating and characterizing some bioactive compounds from *Vernonia amygdalina* leaf extracts (Farombi and Owoeye, 2011; Erasto *et al.,* 2007; Kiplimo *et al*., 2011; Toyang and Verpoorte, 2013). The study of the phytochemicals showed the isolation of flavonoids, saponins, alkaloids, tannins, phenolics, terpenes, steroidal glycosides, triterpenoids, and sesquiterpene lactones (Luo *et al*., *2017*; Quasie *et al*., 2016). The finding of the study was in accordance with the report that *Vernonia amygdalina* leaves contains 0.87% of flavonoid, 0.37% of tannins, 2.15% of saponins and 2.13% of alkaloids (Atangwho *et al*., 2009) while another report showed 0.47% flavonoids, 2.78% Alkaloids, 0.64% saponins and 0.74 tannins (Ndukwe *et al*., 2013). Alkaloids are known to exhibit some metabolicroles and developmental control in living system (Edeoga *et al*., 2006). Alkaloids helps plants to drive predators and parasites. Flavonoids are expressed in plants during microbial infection giving their antimicrobial

activity (Kujumgiev *et al*., 1999). Saponins usually react with the cholesterol rich membranes of cancer cells, which will limit their growth and viability (Roa *et al*., 1995). Steroids are relevant in pharmacy because they contain sex hormones and also used for production of drugs (Okwu, 2001). Terpenoid have been found to be useful in the prevention and therapy of several diseases, including cancer. Terpenoid are also known to possess antimicrobial, antifungal, anti-parasitic, antiviral, anti-allergenic, antispasmodic, anti-inflammatory and immune mudulatory properties (Rabi and Bishayee, 2009; Ugwu *et al*., 2013). These phytochemicals give the medicinal actions and potential health benefits associated with bitter leaf consumption (Iweala *et al*., 2017). However, it's crucial to know that the toxicity of bitter leaf or its phytochemicals can be dose-dependent and excessive consumption may have adverse effects (Izevbuwa *et al*., 2013).

2.4 Haematological properties of *Vernonia amygdalina*

Vernonia amygdalina has been shown to be rich in Iron (Fe) (Ezejiofor *et al*., 2017). This high amount or concentration of iron in the extract is crucial for the formation of hemoglobin in red blood cells. Adequate iron in take is essential in preventing and treating iron-deficiency anemia. Also, it also contained antioxidants which may help preserve blood cells from oxidative stress, potentially supporting overall hematological health (Okeke *et al*., 2018). In terms of antianaemic effect, study has shown the leaf extract has an antianaemic effect comparable to the standard drug pyridoxine, significantly increasing RBCs, hemoglobin and hematocrits in anemic patients (Iwalewa *et al*., 2003). Also in the increases of white Blood Cells (WBCs) research has shown that bitter leaf extract increase WBC count in chickens infected with Salmonella bacteria, stimulating the immune system response (Eledayelu *et al*., 2013). Study show it increased platelet count and coagulation time in rabbits, suggesting potential coagulant properties (Ojiako

and Nwanjo, 2006). Also bitter leaf powder modulated hematological parameters in rats, increasing RBCs, hematocrit, hemoglobin and platelets, while reducing WBCs (Egedigwe, 2010). The leaf extracts are used as adjuvants in cancer therapy to help manage anemia and low blood cell counts associated with chemotherapy and radiation (Hensel *et al*., 2001). Also, by modulating blood parameters and stimulating the liver, bitter leaf helps in detoxification of the body and cleansing the blood (Igile *et al*., 1994). Conversely, the leaf has also exhibited platelet boosting and coagulant properties. This indicates it could be useful as a hemostatic to stop bleeding (Ojiako and Nwanjo, 2006). The leaf extracts have demonstrated anticoagulant potential and ability to dissolve blood clots. This suggests beneficial effects in thrombosis prevention (Nwanjo, 2005).

2.5 Anticancer Activity of *Vernonia amygdalina*

Researchers have shown that *Vernonia amygdalina* is edible (Farombi, 2003) and has been taken in large amounts with no case of toxicity, and with scientific evidence for anticancer action makes it an exclusive choice for cancer patients. Bitter leaf extracts have also shown to induce apoptosis (programmed death of cell) in various human cancer cell lines including breast, liver, colon, leukemia and pancreatic cancer cells. This demonstrates its anticancer activity (Izevbigie *et al*., 2004; Arsad *et al*., 2013). Studies have also demonstrated the ability of aqueous extract of bitter leaf to inhibit proliferation of cancer cells. The extracts suppress cancer cell growth and division (Ojiako *et al*., 2015*;* Yeap *et al*., 2010). Research shows bitter leaf extracts exhibit cytotoxic effects against cancer cells, decreasing cell viability. The cytotoxic phytochemicals likely include flavonoids, saponins and alkaloids (Ojiako and Nwanjo, 2006; Erasto *et al*., 2007). Also, the antioxidant compounds in bitter leaf such as flavonoids scavenge free radicals and may

confer some protection against cancers (Igile *et al*., 1994). Bitter leaf combined with chemotherapy drugs was shown to improve anticancer efficacy in breast cancer cells compared to chemo alone (Eleyinm *et al*., 2006).

2.6 Antioxidative Activity of *Vernonia amygdalina*

Iwalokun reported the antioxidant action of an aqueous extract of *Vernonia amygdalina* leaves against acetaminophen-induced hepatotoxicity and oxidative stress in mice. The antioxidant mechanism of *Vernonia amygdalina* has been backed by the recent studies of Adesanoye and Farombi and in this study, *Vernonia amygdalina* protected against carbon tetrachloride-induced liver injury by inducing antioxidant and phase 2 enzymes (Adesanoye *et al*., 2010). The antioxidant activity of *Vernonia amygdalina* has been attributed to the presence of flavonoids, as reported (Igile *et al*., 1994).After the determination of the antioxidant activity of the three flavones, it was shown that luteolin showed greater activity than the others. It can be said that the antioxidant properties of *Vernonia amygdalina* can be as a result of the presence of these flavonoids. The importance of this antioxidant component has been shown in neurotoxic studies since it has been confirm that flavonoids can traverse the blood brain barrier (Youdim *et al*., 2003).

2.7 Anti- Diabetic Activity of *Vernonia amygdalina*

Vernonia amygdalina, commonly known as bitter leaf, has been studied for its potential anti-diabetic properties. Research indicates that the plant exhibits hypoglycemic effects, which could be beneficial for managing diabetes (Ezejiofor *et al*., 2011). Study was carried out on the impact

of *Vernonia amygdalina* on diabetes-associated complications. The findings indicated potential protective effects against oxidative stress and inflammation, contributing to its anti-diabetic properties (Ademiluyi *et al*., 2013). These studies provide preliminary evidence supporting the anti-diabetic activity of *Vernonia amygdalina*. The major bioactive compounds in *Vernonia amygdalina* responsible for its anti-diabetic activity appear to be flavonoids, saponins, tannins, terpenes, and glycosides. These compounds exert hypoglycemic effects through mechanisms like increasing insulin secretion, inhibiting glucose absorption, and boosting antioxidant defenses (Ong *et al*., 2016).

2.8 Antimicrobial effect of *Vernonia Amygdalina*

Several studies have demonstrated the antibacterial and antifungal properties of extracts from various parts of *Vernonia amygdalina*, including the leaves, roots and stem and have also explored its effectiveness against different microorganisms. In terms of bactericidal activity, study have shown the antibacterial activity of *Vernonia amygdalina*, revealing notable effects against various bacterial strains (Okigbo and Anuagasi, 2009). Also studies have shown the presence of antifungal Properties and the research investigated demonstrated its efficacy against selected fungal species (Nwodo *et al*., 2011). Another study showed its antiparasitic effects, this study explored the antiparasitic properties of *Vernonia amygdalina*, shedding light on its potential in combating parasitic infections (Ekundayo and Onwuliri, 2008). The leaf extracts have also exhibited antifungal effects against Candida albicans, Aspergillus niger and other fungi according to research. The antifungal activity was attributed to phytochemicals like flavonoids,

saponins and alkaloids (Akinpelu and Onakoya, 2006). The root extracts displayed broader and more potent antibacterial effects compared to the leaves (Erasto *et al*., 2015). The major bioactive compounds responsible for the antimicrobial effects include vernodalin, vernolide and vernodalol. These phytochemicals are thought to act through mechanisms such as cell membrane disruption, inhibition of protein synthesis and DNA damage in microbes (Erasto *et al*., 2006).

2.9 Adverse effects of *Vernonia Amygdalina*

According to research, high doses of aqueous leaf extracts of *Vernonia amygdalina* (above 800 mg/kg) resulted in significant hepatotoxicity in rats. Lower doses did not cause liver damage (Akah and Okafor, 1992). The leaf extract has low toxicity when injected (Asuzu and Anaga, 1992). It was also reported that there were several side effects associated with *Vernonia amygdalina* including gastrointestinal upsets like diarrhea and abdominal pain, as well as headaches and dizziness at high doses (Azuzu and Chineme, 2013). The bitterness and unpleasant taste of leaves due to compounds like vernoniosides is a major constraint in use of *Vernonia amygdalina* preparations (Igile *et al*., 1994).

2.10 Leukaemia

Leukaemia is a form of cancer is produced in the blood-forming tissues of the body, particularly the bone marrow and lymphatic system. It is associated with the rapid production of abnormal white blood cells, that are vital for the immune system's action. Symptoms may include fatigue,

bleeding easily, swollen lymph nodes, and weight loss (Manisha, 2012). Treatment options for leukaemia often involve chemotherapy, radiation therapy, targeted therapy, or stem cell transplantation, depending on the type and stage of the disease. While advances in medical science have improved survival rates, leukaemia remains a serious and potentially life-threatening illness, requiring ongoing research and innovative treatments (Pollyea *et al*., 2014).

Leukemia encompasses a diverse group of malignancies, with a prevalence that varies among different populations. Common types include Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML), Chronic Lymphocytic Leukemia (CLL), and Chronic Myeloid Leukemia (CML) (Pui *et al* ., 2008; Döhner *et al*., 2015).

2.10.1 Risk Factors of Leukaemia

The root causes of different types of Leukaemia are unknown. There are many root causes and risk factors for any individual to develop Leukaemia and proliferate into a fatal disease stage. Thesefactors are as follows:

- Prior chemotherapy
- Ionizing radiation
- Smoking and alcohol consumption
- Work involving chemicals
- Family History and Age (Lin *et al*., 2020).

2.10.2 Acute Lymphoblastic Leukaemia

Acute lymphoblastic leukaemia (ALL) is the most noticed cancer in the pediatric age group, resulting to approximately 25–30% of all childhood malignant disorders. The annual incidence

of acute lymphoblastic leukaemia in the United States is approximately 4.6 cases per 100,000 between the ages 0–14 years, with a peak incidence at age 2–5 years. The risks of nonionizing radiation, chemicals, infections, hydrocarbons and pesticides have been evaluated as a cause. Acute lymphoblastic leukemia (ALL) is a cancer that starts from white blood cells called lymphocytes in the bone marrow. ALL is mostly experienced in children between the ages of 2 and 5, though adults can also be affected by ALL. The exact causes are unknown but may involve abnormal chromosome changes, radiation exposure, smoking, or exposure to certain chemicals. Some people have a genetic predisposition. The signs and symptoms include fatigue, easy bleeding/bruising, frequent infections, bone pain, and enlarged lymph nodes, spleen, or liver (Abdel-Aziz and El-Mougy FS, 2019). Diagnosis is made by blood tests, bone marrow biopsy, lumbar puncture, and imaging tests. The cells are examined under a microscope to determine if they are lymphoblasts and to classify the type of ALL (Bain, 2005). Treatment involves chemotherapy, targeted drugs, radiation therapy, stem cell transplants, and/or immunotherapy. The goal is to achieve a complete remission, after which maintenance chemotherapy may be given to prevent relapse (Pui and Evans, 2006). The prognosis is generally better in children than adults, and overall 5-year survival rates are around 90% in children and 40-60% in adults (Terwilliger and Abdul-Hay, 2017).

2.10.3 Acute Myeloid Leukaemia (AML)

Acute myeloblastic leukaemia (AML) has two stages of occurrence, during early childhood and later in adults. The age for the newly diagnosed patients with AML is 66 years. The etiology of

AML is unclear. Epigenetic changes like promoter silencing by hypermethylation of the p15/INK4b and other genes in the pathogenesis of AML, have been noticed. It is asssociated with the rapid growth of abnormal white blood cells that forms in the bone marrow and affect the formation of normal blood cells (De Kouchkovsky and Abdul-Hay, 2016). Signs and symptoms of AML include bleeding easily or bruising, weight loss, fever, and increased risk of infections. The exact cause is unknown but risk factors include smoking, previous chemotherapy, and exposure to high levels of radiation (Deschler and Lübbert, 2006). Diagnosis involves blood tests and bone marrow biopsy to examine features of the leukemia cells under the microscope. These features help classify the AML into subtypes (Arber *et al*., 2016). Treatment depends on the patient's age and health but usually involves chemotherapy, bone marrow transplant, targeted therapy drugs, and supportive care. Newer immunotherapies are also being used. The goal of treatment is to achieve remission (Döhner *et al*., 2017).

2.10.4. Chronic Lymphoblastic Leukaemia

CLL is the most common leukaemia that affect adults in Western Countries (Schriever and Huhn, 2003). It accounts for 25% of all leukaemia cases and affects about 3 in every 100,000 persons in the United States Inhibition of the programmed cell death (apoptosis) (Yee and O'Brien, 2006). It is the only leukaemia not related to exposure to ionizing radiation, drugs or chemicals. But it was noticed that the occurence of CLL in the relations of the CLL patients is very high indicating that CLL occurs in 5-10% of patients (Yee and O'Brien, 2006). CLL patients presented night sweats, weight loss and fatigue as well as lymphadenopathy, splenomegaly and hepatomegaly. During the process of normal B-cell maturation.

2.10.5 Chronic Myeloid Leukaemia

Chronic myeloid leukaemia is a disease that represents 14% of all leukaemias and 20% of adult leukaemias worldwide. The incidence is 1.6 cases per 100,000 adults every year. It was proven that the age at diagnosis is 65-67 years (Cardama and Cortes, 2006; Lee, 2000). On the other hand, CML is rare in children (Lee, 2000). It cannot be preventable and also not inheritable since there is no known hereditary, familial, geographic, ethnic, or economic association with CML. It can be said that chemical exposure or genetic predisposition can induce CML (Cardama and Cortes, 2006).

2.11 RAS and WT1

RAS and WT1 are two genes that have been imputed in the formation of certain types of leukemia:

- RAS genes (HRAS, KRAS, NRAS) encode signaling proteins that regulate cell growth and differentiation. Unnecessary changes in RAS genes can lead to constitutive activation of proliferation pathways and contribute to leukemogenesis. They are found mutated in around 20-30% of acute myeloid leukemia (AML) cases (Bowen *et al*., 2005; Mendoza *et al*., 2011; Neubauer *et al*., 2008)
- WT1 encodes a transcription factor that plays an important role in normal hematopoiesis and cell development. Aberrant WT1 expression can contribute to impaired differentiation of hematopoietic cells (Cilloni *et al*., 2009; Przybylski *et al*., 2008)

- There seems to be some interplay between RAS signaling and WT1 expression. One study found that oncogenic RAS cooperates with WT1 overexpression to induce an aggressive AML phenotype in a mouse model. The co-occurrence of RAS mutations and high WT1 expression is linked to higher rates of relapse and shorter survival times in AML patients.
- WT1 mutations are more frequent in acute lymphoblastic leukemia (ALL), found in around 10-15% of cases. There too, WT1 mutations are associated with worse outcomes. The cooperation between mutant RAS and WT1 may be one mechanism promoting leukemogenesis in some cases of AML and ALL.

2.11.1 RAS (Rat sarcoma)

The RAS (Rat sarcoma) and WT1 (Wilms Tumor 1) genes are both implicated to the forming of leukemia, particularly acute myeloid leukemia (AML). The relationship between RAS and WT1 involves complex molecular interactions that contribute to leukemogenesis. RAS refers to a family of small GTPase proteins that play a central role in controling proliferation of cell, differentiation, and cell survival.. When mutated, RAS proteins get stuck in their activated forms, driving uncontrolled cell growth that can lead to cancer development. RAS, standing for Rat Sarcoma, refers to a family of oncogenes that play a crucial role in cell signal transduction pathways. This family includes three main members: H-RAS, K-RAS, and N-RAS. These proteins are necessary for regulating various cellular processes, such as cell growth, differentiation, and survival. Mutations in RAS genes, particularly in the K-RAS isoform, are frequently implicated in various cancers, including colorectal, lung, and pancreatic cancers. Here are some key points about RAS:

- There are 3 main isoforms of RAS in humans that are products of HRAS, KRAS, and NRAS genes (Pylayeva-Gupta *et al.,* 2011). These homologous proteins have a high degree of sequence and functional similarity.
- RAS proteins function as signal switches by going between inactive GDP-bound and active GTP-bound conformational states (Wennerberg et al., 2005). This action is facilitated by guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs).
- The most common oncogenic mutations occur at hotspots G12, G13, and Q61, which impair GTP hydrolysis activity of RAS, locking it into the active GTP-bound form (Pylayeva-Gupta *et al.,* 2011).
- Oncogenic RAS mutations are present in ~30% of all human cancers, with particularly high frequencies in pancreatic (90%), colorectal (55%), and lung (35%) cancers (Cox *et al.,* 2014).

2.11.2 WT1 (Wilms' tumor 1)

WT1, or Wilms' tumor 1, is a tumor suppressor gene found on chromosome 11p13. It encodes a transcription factor that have an important role in normal kidney development and is related to the development of Wilms' tumor, a pediatric kidney cancer. WT1 stand for Wilms' Tumor 1, a transcription factor and tumor suppressor protein that plays important roles in cell growth, differentiation, and apoptosis during normal development. Here are some key points about WT1:

- WT1 was originally identified and named for its association with Wilms' tumor, a pediatric kidney cancer resulting from loss of WT1 function (Call *et al.,* 1990).

- During normal embryonic development, WT1 regulates formation of tissues such as kidneys, gonads, spleen through transcriptional control of differentiation pathways (Scholz and Kirschner, 2011).
- In normal adult tissue, WT1 is highly expressed in podocytes of the kidney, Sertoli and granulosa cells, hematopoietic stem cells, mesothelial cells and myeloid progenitors (Yang *et al.,* 2007).
- Depending on cellular context and interacting proteins, WT1 can either promote or suppress transcription of genes involved in cell proliferation (e.g. PDGFA, EGFR) and apoptosis (e.g. BCL2, Bak) (Toska and Roberts, 2014).
- Dysregulation of WT1 through overexpression or mutations can contribute to leukemogenesis and poorer prognosis in cancers like acute myeloid leukemia (Virappane *et al.,* 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out in the University of Benin, Benin City, Edo State, Nigeria. The University of Benin (UNIBEN) is a public research University located in Benin City, Edo State, Nigeria. It was founded in the year 1970 and is one of Nigeria's foremost institutions of higher education.

3.2 Study Population

In this study, animal (rats) model was used. A total of twenty (20) male rats of the Albino Wister strain were purchased from the animal holdings from the department of Anatomy, University of Benin, Benin City, Nigeria. The rats were housed at the animal house, department of Anatomy, University of Benin. Animals were exposed to 12 hours dark and light cycles with access to feed and water ad libitum. The rats were allowed to acclimatize for two weeks for the commencement of the study.

3.3 Identification of the *Vernonia Amygdalina*

The Vernonia Amygdalina leaves were harvested around University of Benin environs, Benin City, Edo State, Nigeria. It was further transported to the department of Plant Biology and biotechnology where it was identified and authenticated, then given a voucher number UBH-V342.

3.4 Ethical Consideration

Ethical approval was obtained from Research Ethics Committee on animal subjects from Edo State Ministry of Health, Benin City (Ref Number: HA/737/23/B/200600149, issued on 14th, December, 2023).

3.5 Experimental design

Twenty (20) male adult albino wistar rats were selected into a control group 1 and positive control group 2 and also two experimental groups 3 and 4. Each group consisted of 5 rats each. Afterwards, they were fed for 1 month following the established feeding routine. Group 1 was the control group was the control group which was fed with normal standardized feed and water while group 2 were given grower mash and water and administed with 0.2ml intravenous injection of benzene:2-propanol: water mixture (1:5:5 v/v) per body weight of the rat to induce leukemia. Group 3 were given grower mash and wateradministered with 250mg/kg body weight of *Vernonia amygdalina* for 14 days. Group 4 were given grower mash and water, administered with 0.2ml intravenous injection of benzene:2-propanol: water mixture (1:5:5 v/v) per body weight of the rat and treated with 250mg/kg body weight of *Vernonia amygdalina*for 14 days.

3.6 Sacrifice of the Animals

At the end of the experimental period, the animals were grossly observed for general physical characteristics and were weighed using a weighing balance. A midline incision was made through the ventral wall of the rats after cervical dislocation.

3.7 Collection of Samples

At the end of the experimental period, the animals were grossly observed for general physical characteristics. A midline incision was made through the ventral wall of the rats after cervical dislocation. Five milliliters (5ml) of blood were collected from each rat and placed in a plain container. Samples were stored in refrigerator at 4℃ for proper clot retraction. The sample were then spun to obtain a clear serum. The serum was then stored at-20℃ freezer. Bone marrow samples were obtained from the experimental animals by opening of the femur cavity after which a sterile forceps was used to obtain the marrow. The bone marrow was placed in an Eppendorf container containing trizol.

3.8 Laboratory Analysis

3.8.1 Crude Extraction

Bitter leaves were harvested in Ekosodin community in Ovia North East Local Government of Edo state, Nigeria. It was identified in department of plant biology and biotechnology university of Benin as *Vernonia amygdalina*. It was air dried under room temperature for about two weeks. After drying the leaves were pulverized with a British grinding machine into powdered form. The powdered form was weighed and soaked with distilled water at ratio of 1gram to 10ml of distilled water. The pulverized powder were soaked for about 24hrs with constant stirring. After 24hrs soaking they were filtered and the residue were discarded and the filtrate were

concentrated with water bath at about 45°c. Thereafter the concentrated extract was preserved in the refrigerator.

3.8.2 RNA Extraction and Semi-Quantitative Polymerase Chain Reaction (PCR)

RNA was isolated from the bone marrow with TRIzol Reagent (ThermoFisher Scientific) and converted to cDNA usingProtoScript First Strand cDNA synthesis Kit (NEB). PCR amplification of Ras and WT1 RNA was done using oneTaq® 2X Master Mix (NEB).

3.8.3 Procedure

After sacrificing the animals, the tissues were imbedded in 0.3mL of TRIzol reagent inside an empendoff tube for proper tissue preservation. The tissues were homogenized using a plastic pestle. RNA lyase buffer was then added to the homogenate to further break down the tissue cell membranes, after which it was spun at 10000 rpm for 10 minutes. The supernatant which contains the RNA was carefully removed and placed in a separate empendoff tube. The RNA precipitating buffer was then centrifuged at 10000 rpm for 30 minutes. The supernatant was carefully removed remaining the RNA precipitate at the bottom of the tube. RNA wash buffer was added and centrifuged again at 10000 rpm for 5 minutes. This step was repeated 3 times to remove excess solutions and buffers previously added. Nuclease free water was added to break the RNA in a pellet form and the phosphodiester bond of the RNA. The solution also has a nuclease inhibitor, which removes other DNA or RNA contaminants from the medium. The RNA was then quantified using 1qa UV spectrophotometer at 260nm.

3.8.4 Complementary DNA (Cdna) Synthesis.

All component of the cDNA kit was added to the RNA following manufacturer's instruction, these components included random primer, oligonucleotide, primer or deoxynucleotides, reverse transcriptase buffer. After adding all components to the RNA, the mixture was then incubated at 42℃ in a thermocycler for 1 hour. Then the temperature was increased to 75℃ to denature the reverse transcriptase. Thereafter all the RNA was converted to Cdna. For RQ-PCR on rats sample, the expression levels of Ras and WT1 were normalized to the levels of GADPH housekeeping gene.

3.8.5 Gene Amplification

An equal volume of both forward and reverse primer was added, PCR mix (master mix), taq polymerase and magnesium was also added. The mixture will be placed in a thermo-cycler for amplification. The thermo-cycler programmed for 30 cycles.

3.8.6 Gel Electrophoresis

After the PCR process, the DNA gel loading dye was added to the mixture. The agarose gel was prepared by dissolving 1% of agarose gel in TBE buffer. The gel was then allowed to solidify and then the sample was loaded and the gel connected for electrophoresis. Thereafter, a snap shot will be taken. The image will then be transferred to ImageJ. The intensities of the bands from agarose gel electrophoresis will be quantified using ImageJ software.

3.9. Full Blood Count (FBC) and Peripheral Blood Film (PBF)

FBC and PBF were both performed by automated method and manually, respectively. The FBC was performed using the FBC machine with five part and was then recorded while the PBF was performed using the hand i.e. drawing a thin film on a clean grease free glass slide.

3.9.1 Full Blood Count

Procedure

The blood samples collected were put in a well labeled EDTA container and mixed thoroughly to prevent clotting. The blood samples were then analyzed using automated hematology analyzer or Full Blood Count machine. This machine counts and characterizes different types of blood cells, including red blood cell (RBCs), white blood cells (WBCs) and platelets. It also provided quantitative measurements such as hemoglobin concentration, hematocrit, red cell indices (mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration) and results were recorded.

3.9.2 Peripheral Blood Film

Procedure

The blood samples collected were put in a well labeled EDTA container and mixed thoroughly to prevent clotting. A small drop of blood was collected from the sample and placed near one end of the clean grease free glass slide. Another slide which was used as a spreader was held at an angle of 45◦ to the first slide. The edge of the spreader was then used to spread the drop of blood along the length of the first slide, creating a thin, even smear. The blood smear is allowed to air dry completely at room temperature. This is important so as to avoid heat or excessive airflow as it can distort the blood cells. It was then heat fixed by putting the slide on a hot plate as this will

help preserve the morphology of the blood cells and prevent them from deteriorating during staining. After drying, it is stained using Giemsa stain which is a Romanowsky stain for 5mins and then rinse with buffer for 15mins. This will help visualize different components of the blood cells under a microscope. The slide is allowed to air dry before heat fixing again. The stained blood smear was then examined under a microscope and carefully observed the morphology and characteristics of various blood cells including, red blood cells (RBCs), white blood cells (WBCs).

3.10 Statistical Analysis

Data obtained from this research was presented and analysed using graph pad prism (version 8.02, California, USA). Analysis of variance (ANOVA) was used to compare means and results was expressed in Mean$\pm$Standard error of mean. $p<0.05$ was considered sifgnificant.

CHAPTER FOUR

4.0 RESULT

Researchers has previously reported the toxicological effect of benzene on haemopoietic tissues, although there is paucity of information on its effect on RAS and WT1 genes. In this study, DNA methylation genes (RAS and WT1) were analyzed in the bone marrow of male albino rats.

Table 4.1 shows the Full Blood Count result on the effect of *Vernonia amygdalina.* In WBC (White Blood Cells), there was no significant difference (p=0.881) in group 3 (8.90±3.60) and group 4 (7.03±1.16) when compared to group 1 (8.27±1.91) and group 2 (7.97±0.70). Also, in N(Neutrophils), there was no significant difference (p=0.149)in group 3 (2.50±0.50) and group 4(6.00±2.04) when compared to group 1(10.00±0.58) and group 2 (9.00±2.65).L (Lymphocytes)also showed no significant difference (p=0.183) in group 3 (91.00±1.00) and group 4 (88.00±2.71) when compared to group 1 (82.00±1.53) and group 2 (85.67±2.60).The M (Monocytes)also showed no significant difference (p=0.523) in group 3 (5.00±0.00) and group 4 (5.00±0.91) when compared to group 1 (6.67±1.76) and group 2 (4.33±0.33).E (Eosinophils) showed no significant difference (p=0.987) in group 3 (1.50±0.50) and group 4 (1.33±0.33) when compared to group 1 (1.33±0.33) and group 2 (1.33±0.33). The RBC (Red Blood Cells)also showed no significant difference (p=0.531) in group 3 (6.32±0.49) and group 4 (6.53±0.21) when compared to group 1 (6.02±0.78) and group 2 (6.99±0.24).Hb (Hemoglobin)also showed no significant difference (p=0.226) in group 3 (12.95±0.25) and group 4 (13.30±0.38) when compared to group 1 (12.50±1.40) and group 2 (15.03±0.64). HCT (Hematocrit) also indicate no significant difference (p=0.482) in group 3(36.70±3.20)and group

4 (39.48±1.09) when compared to group 1 (37.50±4.11) and group 2 (43.07±3.12). MCV (Mean Corpuscular Volume) showed no significant difference (p=0.079) in group 3 (58.15±0.65) and group 4 (59.70±0.55) when compared to group 1 (62.80±1.65) and group 2 (59.07±1.01). The MCH (Mean Corpuscular Hemoglobin) showed no significant difference (p=0.688) in group 3 (20.55±1.15) and group 4 (20.40±0.55) when compared to group 1 (20.87±0.55) and group 2 (21.50±0.78). MCHC (Mean Corpuscular Hemoglobin Concentration) showed no significant difference (p=0.685) in group 3 (35.45±2.45) and group 4 (52.24±18.67) when compared to group 1 (33.30±0.72) and group 2 (36.40±1.05) except for a notable outlier in group 4 (52.24±18.67). PLT (Platelets) showed no significant difference (p=0.649) in group 3 (1010.50±252.50) and group 4 (27536.50±26837.19) when compared to group 1 (1356.00±705.72) and 2(1111.67±193.69), except for a notable outlier in group 4 (27536.50±26837.19). The Peripheral Blood Film (PBF) result indicated that in plate 4.1 white blood cells (WBCs), leukocytes appeared adequate in number with relative lymphocytosis; large lymphocytes (+++), small lymphocytes (++), hyper segmental neutrophils (+) and no lymphoblast seen, in the red blood cells (RBCs), erythrocytes showed normocytic normochromic cells (++), polychromatic cells (++), nucleated RBCs (+) and stomatocytes (+) while for the platelets, it appeared adequate in number and normal in size and distribution. In plate 4.2 white blood cells (WBCs), leukocytes appeared adequate in number with relative lymphocytosis, small lymphocytes (++) lymphocytes (+) and no atypical cell seen while in red blood cells, erythrocytes showed normocytic monochromic cells (+++), few polychromatic cells and target cells. The platelet also appeared adequate in number and normal in size. In plate 4.3, white blood cells, leukocytes appeared adequate in number with absolute lymphocytosis, large lymphocytes (++), small lymphocytes (++), others (+) and no atypical cell was seen. In red blood cell,

erythrocytes showed normocytic normochromatic cells (+++), polychromatic cells (++), stomatocytes (+) and few crenated and target cells. Platelets also appeared normal in size and number. In plate 4.4 white blood cell (WBCs), leukocytes appeared adequate in number with preponderance of lymphocytosis large lymphocytes (++), small lymphocytes (+) and others (+). For red blood cells (RBCs), erythrocytes showed normocytic normochromic cells (+++), stomatocytes (+), polychromatic cells (+), crenated cells (+), ovalocytes (+) and macrocytic cells (+). The platelet also appeared adequate in number and normal in size.

Figure 4.1 shows the PCR and agarose gel analysis expression pattern of RAS. There was significant increase ($p<0.01$) in RAS expression in the group administered the induction regiment (benzene + isopropanol), induction regiment +250mg/kg of *Vernonia amygdalina* aqueous extract and 250mg/kg of Vernonia amygdalina aqueous leaf extract administered only group when compared to control. In addition, the experimental group that received the induction regiment also had a significantly higher ($p<0.01$) RAS when compared to the group that received induction regiment +250mg/kg of *Vernonia amygdalina* aqueous leaf extract and 250mg/kg of *Vernonia amygdalina* aqueousextract administered only group. In Figure 4.2, there was no significant increase ($p<0.01$) in WT1 mRNA expression in the group administered the induction regiment (benzene + isopropanol), induction regiment treated with250mg/kg of *Vernonia amygdalina* aqueousextract and 250mg/kg of *Vernonia amygdalina* aqueousextract administered only group when compared to control. Also, the group administered the induction regiment treated with 250mg/kg of *Vernonia amygdalina* aqueousextract had a significantly higher ($p<0.001$) WT1 mRNA expression when compared to the group administered the induction regiment (benzene + isopropanol) and 250mg/kg of *Vernonia amygdalina* aqueous extract administered only group.

Table 4.1: Full Blood Count on the Effect of *Vernonia Amygdalina.*

	Group 1	Group 2	Group 3	Group 4	F	P values
White Blood Cells ($X10^9$/L)	8.27±1.91	7.97±0.70	8.90±3.60	7.03±1.16	0.219	0.881
Neutrophils (%)	10.00±0.58	9.00±2.65	2.50±0.50	6.00±2.04	2.347	0.149
Lymphocytes (%)	82.00±1.53	85.67±2.60	91.00±1.00	88.00±2.71	2.069	0.183
Monocytes (%)	6.67±1.76	4.33±0.33	5.00±0.00	5.00±0.91	0.811	0.523
Eosinophils (%)	1.33±0.33	1.33±0.33	1.50±0.50	1.33±0.33	0.042	0.987
Red Blood Cells ($X10^9$/L)	6.02±0.78	6.99±0.24	6.32±0.49	6.53±0.21	0.793	0.531
Hemoglobin (g/dL)	12.50±1.40	15.03±0.64	12.95±0.25	13.30±0.38	1.796	0.226
Hematocrit (L/L)	37.50±4.11	43.07±3.12	36.70±3.20	39.48±1.09	0.902	0.482
MCV (fL)	62.80±1.65	59.07±1.01	58.15±0.65	59.70±0.55	3.297	0.079
MCH (pg)	20.87±0.55	21.50±0.78	20.55±1.15	20.40±0.55	0.507	0.688
MCHC (g/L)	33.30±0.72	36.40±1.05	35.45±2.45	52.24±18.67	0.512	0.685
Platelets ($X10^9$/L)	1356.00±705.72	1111.67±193.69	1010.50±252.50	27536.50±26837.19	0.571	0.649

Values are expressed as mean±SEM. $p \leq 0.05$- Significant; $p \geq 0.05$.

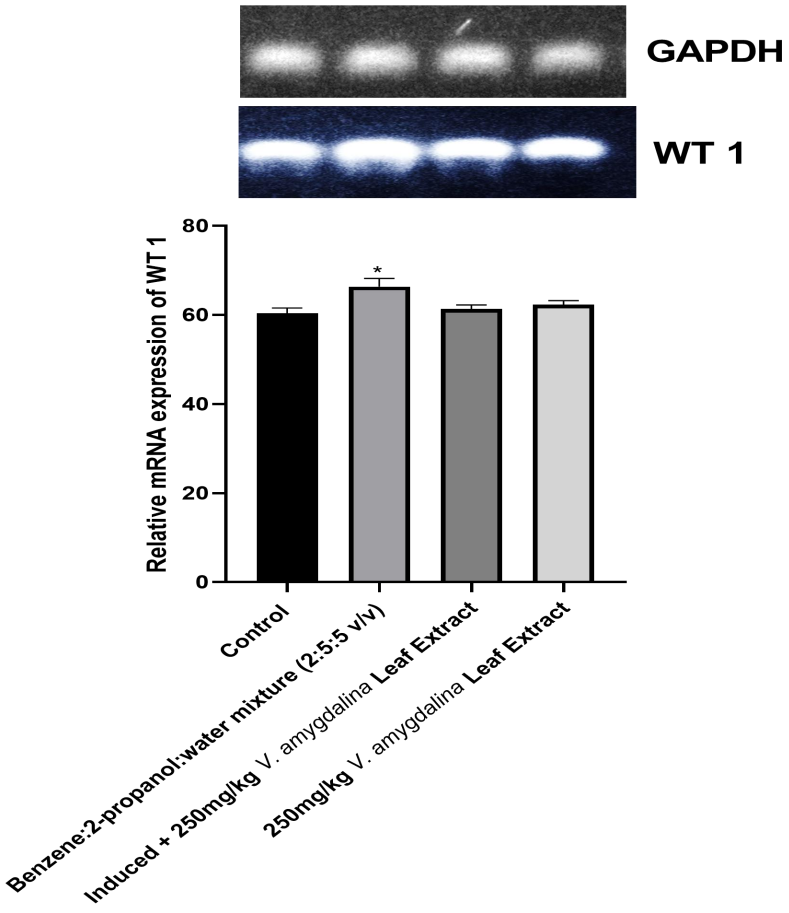


Figure 4.1: PCR and agarose gel analysis of WT1 mRNA from bone marrow of male rats induced with benzene and administered 250mg/kg *of Vernonia amygdalina* aqueous extract. Error bar represents mean±SEM. Statistical significance represented by (*p<0.05, **p<0.01, ***p<0.001)

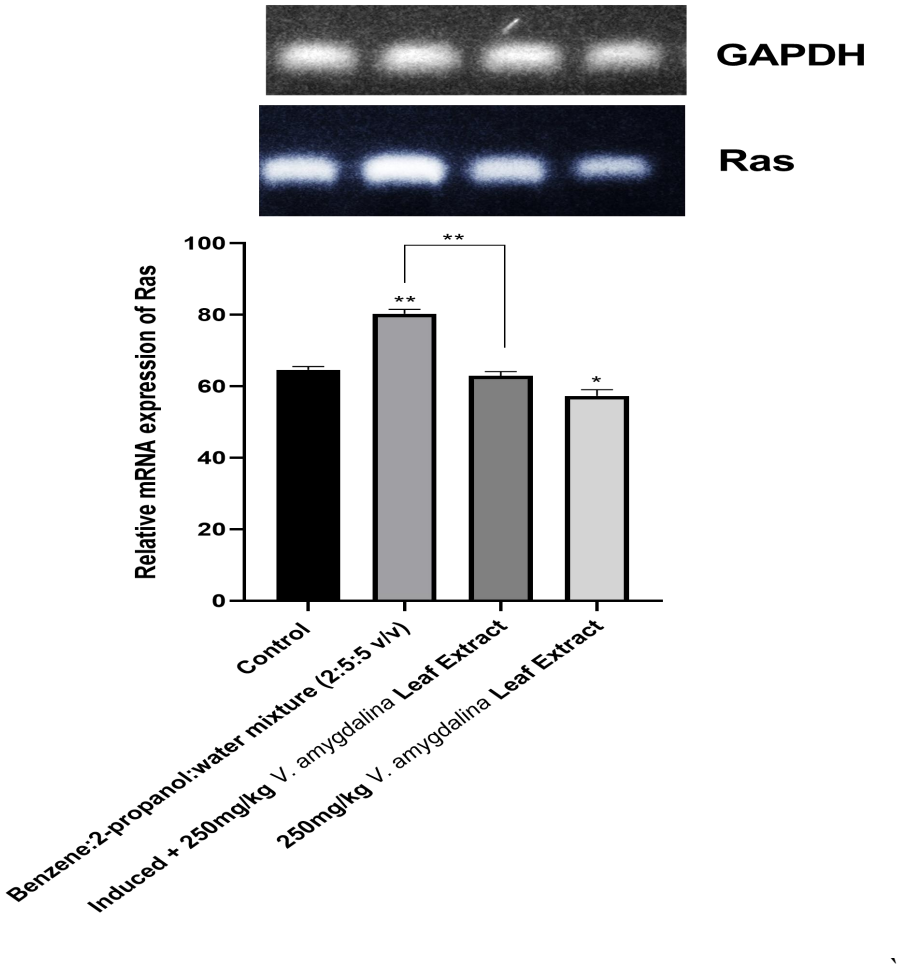
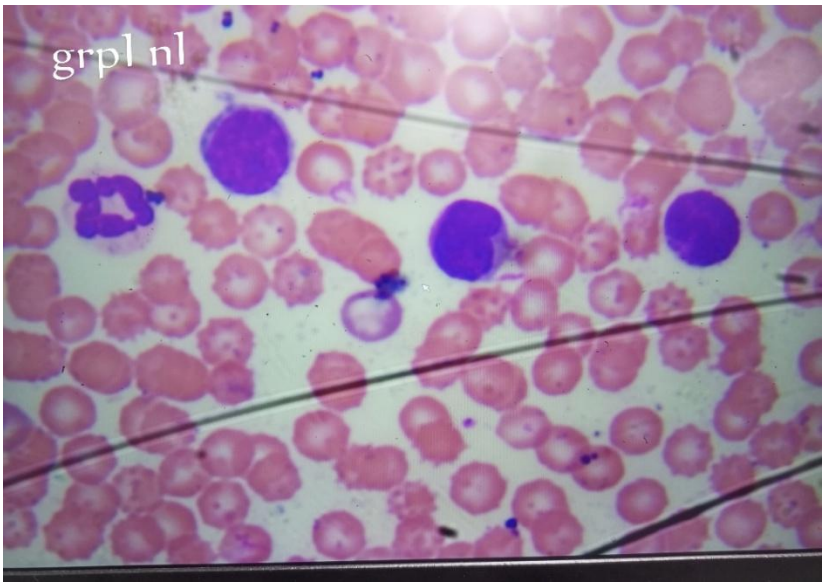


Figure 4.2: PCR and agarose gel analysis of Ras mRNA from bone marrow of male rats induced with benzene and administered 250mg/kg of *Vernonia amygdalina* aqueous extract. Error bar represents mean±SEM. Statistical significance represented by (*p<0.05, **p<0.01, ***p<0.001)

A



B

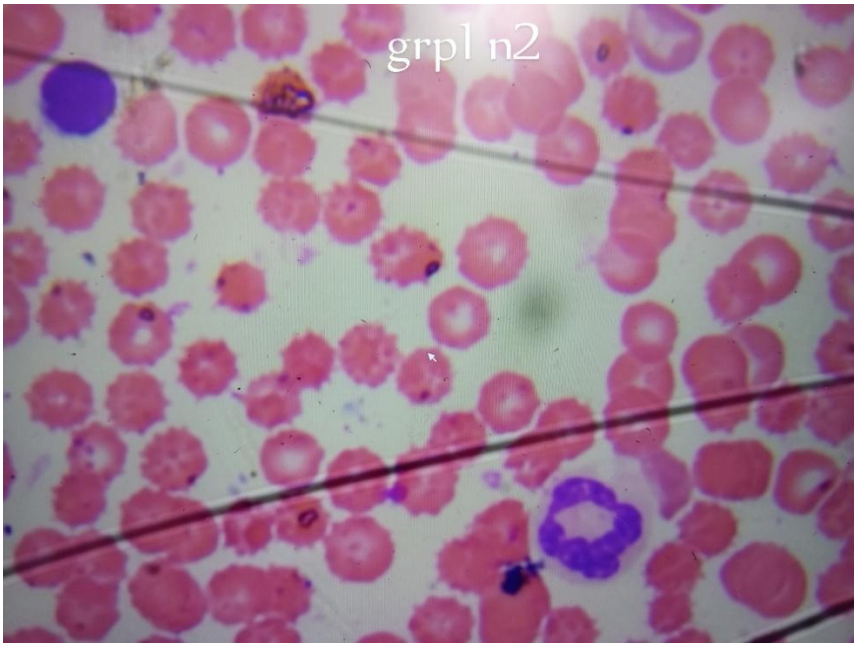
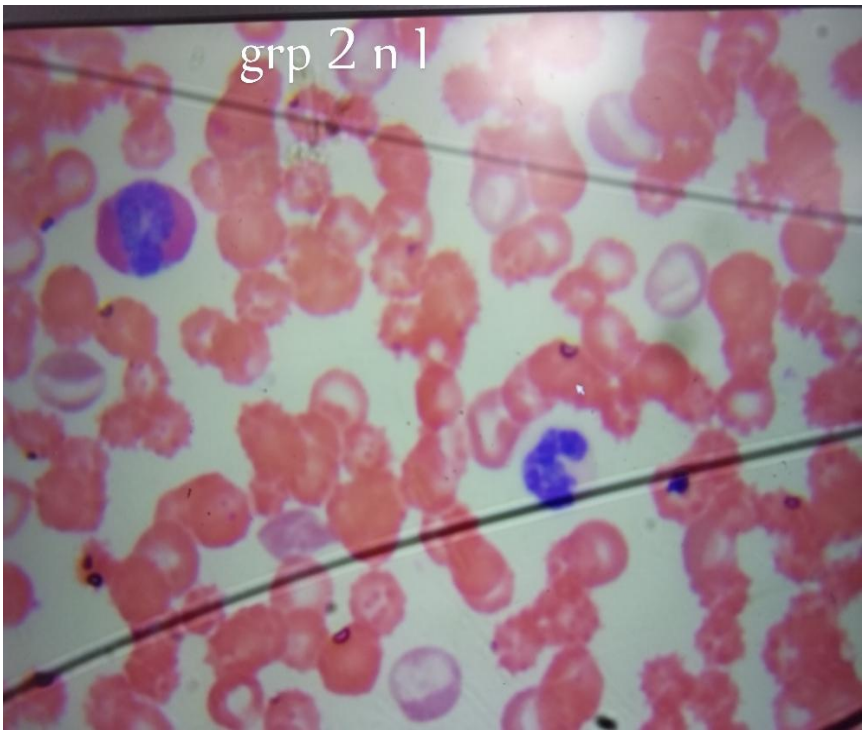


Plate 4.1: Panel A and Brepresents the blood picture stained with Leishman stain of male albino wistar rats.

A 

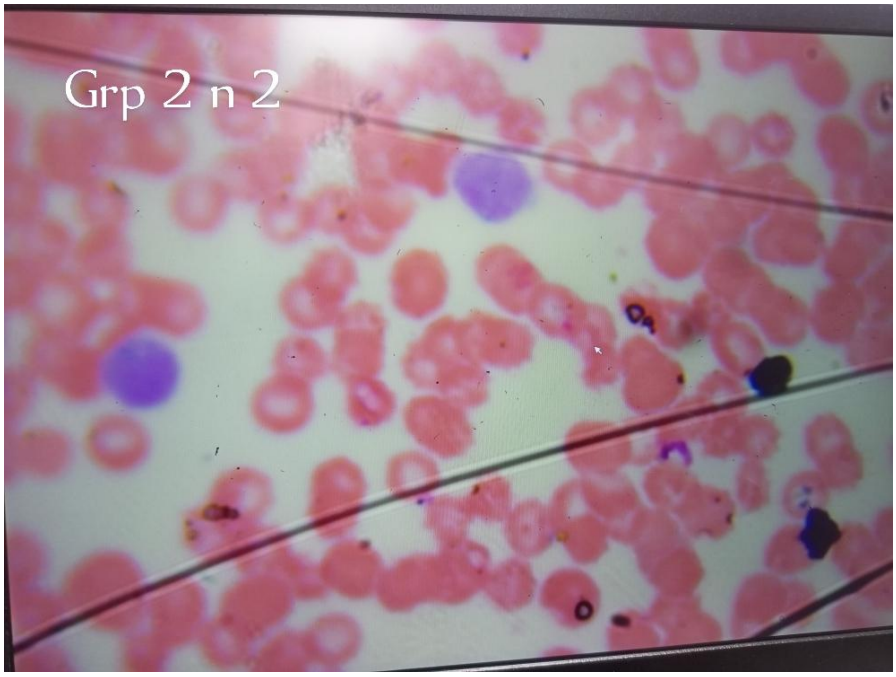
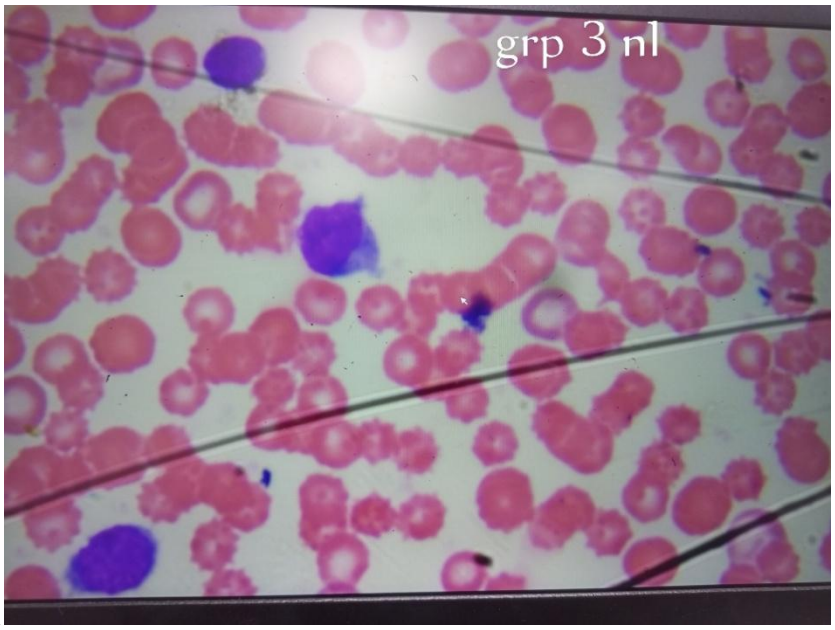
B 

Plate 4.2: Panel A and B represents the blood picture of male albino wistar rats administered with benzene and isopropanol

A



B

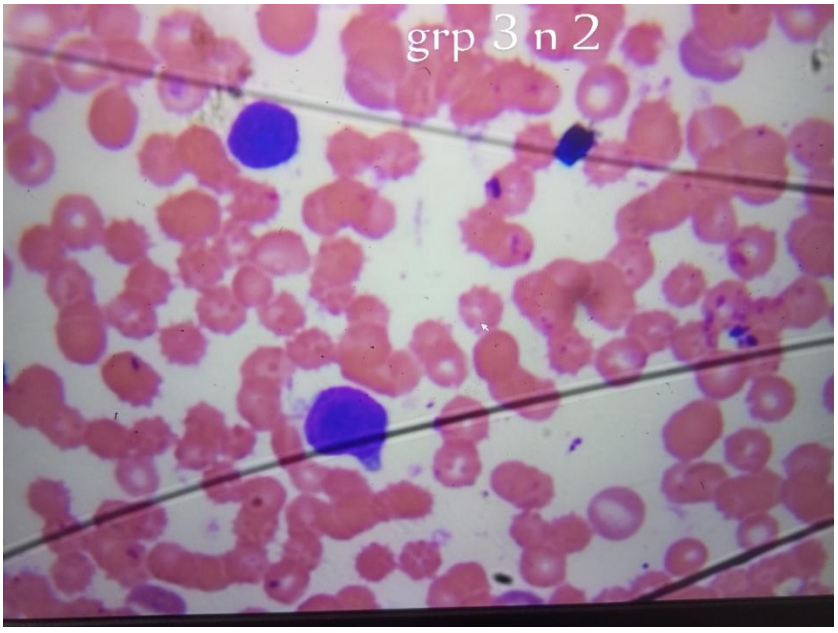
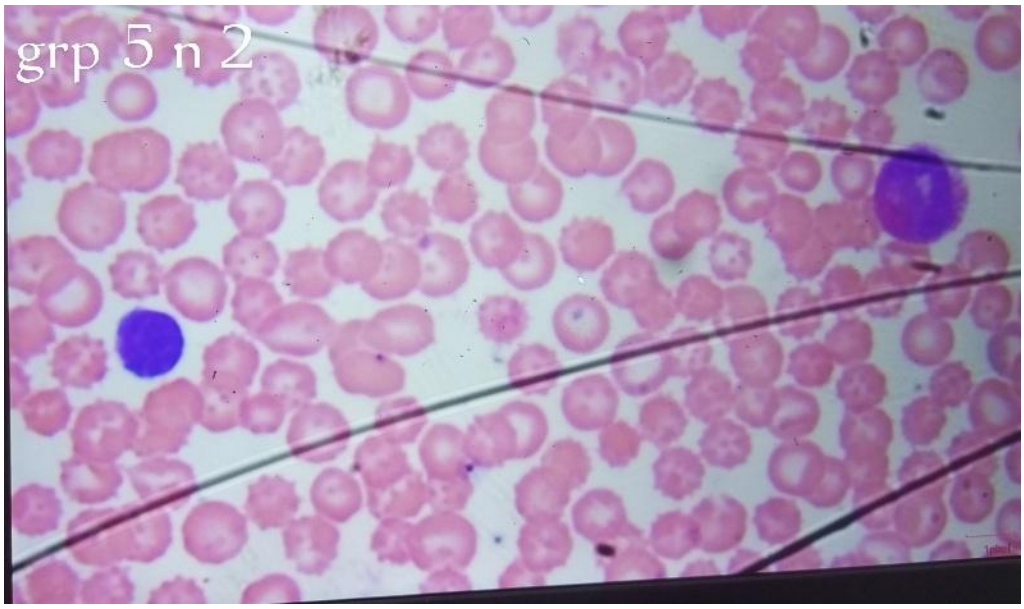


Plate 4.3: Panel A and B represent the blood picture of male albino wistar rats administed with the 250mg/kg of *Vernonia amygdalina* aqueous extract. Leucocyte appear normal with absolute lymphocytosis. RBC showed normocytic normochromic cells, poly chromatic cells, stomatocytes with few crenated and target cells.

A



B

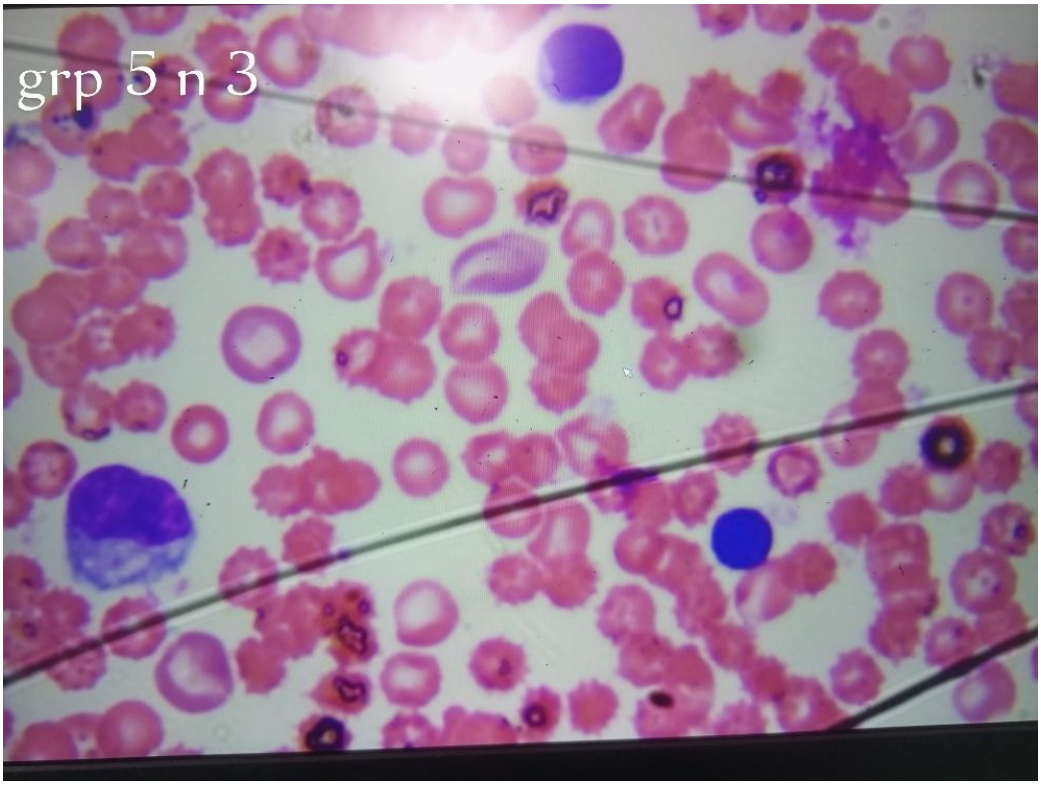


Plate 4.4: Panel A and B represent the blood picture of male albino wistar rats administed with the induction regiment (benzene + isopropanol) and 250mg/kg of *Vernonia amygdalina* aqueous extract. The blood picture showed preponderance of lymphocytes: large and small lymphocyte and no atypical cell seen. RBC showed normocytic normochromic cells, macrocytic cells, stomatocytes , target cells and crenated cells. Platelets appear normal in size and number.

CHAPTER FIVE

5.0 DISCUSSION

The objective of this study was to determine the effect of *Vernonia amygdalina* on RAS and WT1 gene expression in relation to leukemia. In recent years, natural compounds from medicinal plants have gained significant attention for their potential to manage or cure leukemia. One of such plant which is the *Vernonia amygdalina* has been investigated for its potential effect on leukemia. *Vernonia amygdalina* is knownfor its rich phytochemical composition which includes sesquiterpenoids, flavonoids, tannins, steroids and alkaloids, suggesting a potential role in influencing leukemia related parameters(Idu *et al*., 2012; Okeke *et al*., 2018; Idu *et al*., 2012; Ezejiofor *et al*., 2017; Osuagwu *et al*., 2018; Ezejiofor *et al*., 2017).

The RAS gene is a family of genes that make proteins involved in cell signaling pathways that control cell growth and cell death. Mutated (changed) forms of the RAS gene may be found in some types of cancer. These changes may cause cancer cells to grow and spread in the body. he RAS genes, particularly NRAS (Neuroblastoma RAS) and KRAS (Kirsten RAS), play an important role in the development and progression of certain types of leukemia. NRAS mutations are found in approximately 10-15% of AML cases and are associated with a poor prognosis (Papaxoinis *et al*., 2021) also KRAS mutations, although less frequent, can also occur in AML (Tzoupras *et al*., 2022). NRAS and KRAS mutations are found in approximately 25-30% of JMML cases and are thought to play a crucial role in the pathogenesis of this childhood leukemia (Niemeyer, 2022). NRAS mutations are present in approximately 30% of CMML cases, while KRAS mutations are less common (Patnaik *et al.,* 2018). Mutant RAS proteins are constitutively active, leading to sustained activation of downstream signaling pathways, such as the RAF/MEK/ERK (MAPK) pathway and the PI3K/AKT pathway, promoting uncontrolled cell proliferation, survival, and differentiation (Seifert *et al.,* 2020). The presence of RAS mutations

can influence treatment decisions and prognosis in leukemia patients (Hu *et al.,* 2021). Targeted therapies aimed at inhibiting the RAS signaling pathway or its downstream effectors, such as MEK inhibitors, have been explored as potential treatment options for RAS-mutated leukemias, although with limited success (Papaxoinis *et al.,* 2021; Tzoupras *et al.,* 2022). When Ras function is not properly regulated, hyper-proliferation can occur resulting in developmental disorders and cancer. Specific mutations at codons 12, 13, or 61 in the Ras genes is associated with tumors. The WT1 (Wilms' Tumor 1) gene plays an important role in the development and progression of various types of leukemia. WT1 is over expressed in a majority of AML cases, ranging from 60% to 90%, and its expression levels correlate with leukemic burden and poor prognosis (Bergmann *et al.,* 1997; Summers *et al*., 2007). WT1 over expression is associated with specific cytogenetic abnormalities, such as t (15;17) and inv(16), in AML (Bergmann *et al*., 1997). WT1 is over expressed in a significant proportion of ALL cases, particularly in B-cell ALL (Miwa *et al.,* 1992; Loeb *et al.,* 2003). WT1 over expression is linked to a poor prognosis and drug resistance in ALL patients (Miwa *et al.,* 1992; Loeb *et al*., 2003). WT1 is over expressed in CML, particularly in the blast crisis phase, and its expression levels correlate with disease progression and poor prognosis (Cilloni *et al.,* 2003; Berbec *et al*., 2021). WT1 acts as a transcriptional regulator, modulating the expression of various target genes involved in cell proliferation, survival, and differentiation (Yang *et al.,* 2007). WT1 overexpression in leukemia is thought to contribute to leukemogenesis by promoting cell growth, inhibiting apoptosis, and maintaining an undifferentiated state (Yang *et al*., 2007; Prose *et al*., 2022). WT1 has been explored as a potential target for immunotherapy, such as WT1 peptide vaccines and adoptive T-cell therapies, in various types of leukemia (Anguita *et al.,* 2022). WT1 expression levels are

also used as a minimal residual disease (MRD) marker for monitoring disease status and treatment response in leukemia patients (Cilloni *et al.,* 2003; Bergmann *et al.,* 1997).

5.1 CONCLUSION

Data from this study has shown that *Vernonia amygdalina* did not have any significant effect on leukemia gene RAS and WT1 expression. These results suggest that *Vernonia amygdalina* could not influence the expression of the leukemia gene RAS and WT1.

5.2. RECOMMENDATIONS

I will recommend that more research should be carried out on the effect of *Vernonia amygdalina* on leukemia as this will help in combating the disease more effectively.

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PROTOCOL NUMBER	**HA/737/23/B/200600149**
TITLE OF RESEARCH PROPOSAL	**EFFECT OF VERNONIA AMYGDALINA AND ALLIUM SATIVUM AQUEOUS LEAF EXTRACT ON SOME EPIGENETIC MAKERS AND GENE EXPRESSIONS OF KIT,FLT3,RAS AND WIT IN MALE PRELEUKEMIC ALBINO WISTAR RATS.**
PRINCIPAL INVESTIGATOR (S)	**DR.ARUOMAREN AUSTIN IROGHAMA**
DATE CONSIDERED	**14ST DECEMBER 2023**
DECISION OF THE COMMITTEE	**APPROVED**

THIS APPROVAL DATES 14/12/2023 TO 13/12/2024 IF THERE IS DELAY IN STARTING THE RESEARCH, PLEASE INFORM THE EDO SMoH R&EC SO THAT THE DATES OF APPROVAL CAN BE ADJUSTED ACCORDINGLY

REMARK

DR (MRS) Omonyemen Bosede BELLO *(CHAIRMAN)*

SIGNATURE & DATE.....14/12/2023

SUPERVISOR(S)

DECALARATION BY INVESTIGATOR(S)

PROTOCOL NUMBER (please quote in all enquiries)

Note that no participant accrual or activity related to this research may be conducted outside of the dates. All informed consent forms used in this study must carry the E&RC assigned number and duration of your research. No changes are permitted in the research without prior approval of the E&RC except in circumstances outlined in the Code. The E&RC reserves the right to conduct compliance visit to your research site without previous notification.

Signature & Date.....