EFFECTS OF *ICACINA TRICHANTHA* AQUEOUS LEAVES EXTRACT ON NRF-2 GENE IN ALUMINIUM CHLORIDE-INDUCED ANAEMIA IN ALBINO WISTAR RATS

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UNIVERSITY OF BENIN

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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 BACHELORS DEGREE IN MEDICAL LABORATORY SCIENCE (BMLS), UNIVERSITY OF BENIN, BENIN CITY, NIGERIA.

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CERTIFICATION

This is to certify that this Project was satisfactorily carried out by **AIGBEKAEN, EFFORT OSAWARU** with the matriculation number BMS2001145 under the supervision of **DR. (MRS.) P. A. OBAZELU** in the Department of Medical Laboratory Science, School of Basic Medical Sciences, University of Benin, Benin City, in partial fulfillment of the requirement for the Award of Bachelor of Medical Laboratory Science (BMLS) Degree.

DR. (MRS.) P.A OBAZELU **(Project Supervisor)**	**DATE**
DR. (MRS.) ZAINAB OMORUYI **(Head of Department)**	**DATE**
EXTERNAL EXAMINER	**DATE**

DEDICATION

I dedicate this seminar work to God Almighty for his strength and for the success of this work and his guidance throughout my course of study.

ACKNOWLEDGEMENT

I give thanks to almighty God, my creator who has granted me grace and strength to finish this seminar work. My profound gratitude goes to my supervisor **DR.(MRS) P.A OBAZELU** for her genuine concern, support and guidance throughout the course of this study.

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ABSTRACT

Aluminium chloride ($AlCl_3$) is known to induce oxidative stress, impairing erythropoiesis and redox homeostasis, which may contribute to anaemia and other haematological alterations. Nuclear factor erythroid 2–related factor 2 (NRF2) is a master regulator of antioxidant defense and cytoprotective genes, making it a key biomarker in toxin-induced oxidative stress. Evaluating the modulation of this gene by herbal extracts could provide valuable insights into their therapeutic potential. The aim of this study was to determine the expression of NRF2 gene in aluminium chloride-induced anaemia bearing Wistar rats treated with aqueous leaves extract of *Icacina trichantha*. Sixty (60) adult male albino Wistar rats were randomly divided into six (6) groups; A, B, C, D, E and F, representing control, aluminium chloride group, ferrous sulphate group, aluminium chloride + 100 mg/kg of *Icacina trichantha* leaf extract, aluminium chloride + 200 mg/kg of *Icacina trichantha* leaf extract, and aluminium chloride + 400 mg/kg of *Icacina trichantha* leaf extract, respectively. Blood samples were collected for haematological analysis using an ERMA haematology autoanalyzer, while NRF2 mRNA expression was quantified using polymerase chain reaction (PCR). Data obtained were analyzed using GraphPad Prism 8.0 software. Haematological parameters revealed no statistically significant differences across most groups ($p > 0.05$), although mean cell volume (MCV) (fL) was significantly reduced in group F (54.64±0.96) compared to group C (58.22±0.49) ($p < 0.05$), and mean cell haemoglobin (MCH) (pg) was significantly lower in group F (18.72±0.23) compared to group C (19.66±0.07) ($p < 0.05$). NRF2 expression was elevated in group B relative to the control, though not significantly, but was significantly higher compared to groups C, D, E, and F ($p < 0.05$). Treatment with *Icacina trichantha* extract across the different doses did not restore NRF2 expression to control levels. In conclusion, aluminium chloride administration induced NRF2 upregulation as an oxidative stress response, while treatment with *Icacina trichantha* aqueous leaf extract led to a significant reduction in NRF2 expression, suggesting a modulatory effect that warrants further mechanistic investigation.

CHAPTER ONE

INTRODUCTION

1.1. Background of Study

The utilization of medicinal plants as therapeutic agents is an age-old practice embraced by diverse cultures globally. Traditional medical systems have long depended on these plants for the treatment of numerous diseases. (Obazelu *et al*., 2025). These plants (such as *Icacina trichantha*) have been largely used due to their rich secondary metabolites such as alkaloids, flavonoids, saponins, tannins, and glycosides (Sofowora *et al*., 2013). With rising concerns about drug resistance and side effects from synthetic drugs, there is growing interest in natural remedies and plant-derived bioactive compounds (Petrovska, 2012). As such, researchers are increasingly investigating the pharmacological significance of crude plant extracts and their isolated compounds, aiming to develop novel treatments for both chronic and acute diseases (Bello *et al*., 2017). This scientific renaissance affirms the enduring relevance of traditional knowledge systems and highlights medicinal plants as promising leads in modern drug development.

Icacina trichantha, a tropical plant native to West and Central Africa, has long been employed in ethnomedicine for treating conditions such as fever, diarrhea, inflammation, and even diabetes. Commonly referred to as “Ewe gbegbe” in Yoruba, it is consumed either as food or medicine, with both its leaves and tubers being utilized (Alawode *et al*., 2018). Phytochemical screening of its parts has revealed the presence of flavonoids, saponins, phenols, tannins, and glycosides—substances well known for their antioxidative and anti-inflammatory properties (Onakpa *et al*., 2016). In vitro studies have demonstrated that aqueous and methanolic leaf extracts of *Icacina trichantha* have significant radical-scavenging capacity, with high total phenolic and flavonoid content contributing to DPPH inhibition and lipid peroxidation control (Alawode *et al*., 2018). In

vivo experiments further support its pharmacological benefits: administration of the tuber extract in rats significantly increased antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) while reducing malondialdehyde (MDA) levels, suggesting its ability to combat oxidative stress effectively (Onakpa *et al*., 2016). Additionally, its essential oils contain sesquiterpenes, phytol, oleic acid, and linoleic acid, which also contribute to its therapeutic potential (Olubomehin *et al*., 2024).

Nuclear factor erythroid 2–related factor 2 (NRF2), encoded by the NFE2L2 gene, is a pivotal redox-sensitive transcription factor involved in the cellular defense against oxidative stress (Hayes and Dinkova-Kostova, 2014). The NFE2L2 gene is located on the short arm of chromosome 2 (2q31.2) and spans approximately 11 exons, encoding a basic leucine zipper (bZIP) transcription factor composed of about 605 amino acids (Kerins and Ooi, 2018). NRF2 plays a central role in regulating the transcription of antioxidant and cytoprotective genes through interaction with the antioxidant response element (ARE) in the promoter regions of target genes (He *et al*., 2020).

Structurally, NRF2 contains seven functional domains (Neh1–Neh7) that contribute to its transcriptional regulation, stability, and interaction with other cellular proteins. Among these, the Neh2 domain is critical for binding to KEAP1 (Kelch-like ECH-associated protein 1), which serves as a cytoplasmic repressor by facilitating NRF2 ubiquitination and proteasomal degradation under homeostatic conditions (Hayes and Dinkova-Kostova, 2014). In response to oxidative or electrophilic stress, conformational changes in KEAP1 inhibit NRF2 degradation, allowing NRF2 to translocate into the nucleus, where it forms heterodimers with small Maf proteins and binds to AREs (Kerins and Ooi, 2018).

Activation of NRF2 signaling leads to the upregulation of a wide array of genes involved in antioxidant defense, including heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD), and glutathione peroxidase (GPx). These enzymes collectively work to mitigate oxidative damage, detoxify harmful electrophiles, and maintain redox homeostasis (Matte *et al*., 2015).

The NRF2 pathway is particularly important in erythroid cells, which are highly vulnerable to oxidative injury due to their role in oxygen transport and limited ability to regenerate damaged proteins. Studies have demonstrated that NRF2 supports erythropoiesis by regulating antioxidant defense during red blood cell development and protecting erythroid progenitor cells from oxidative insults (Matte *et al*., 2015).

Impairment of NRF2 signaling has been associated with increased oxidative stress, disrupted erythropoiesis, and hemolytic anaemia, particularly in aged or toxin-exposed organisms (Mbiandjeu et al., 2024). Conversely, pharmacological activation of NRF2 has shown potential in mitigating oxidative-stress-induced anaemia and restoring red blood cell integrity (Abdul-Aziz *et al*., 2015). Thus, NRF2 serves not only as a master regulator of redox balance but also as a key gene in hematological protection and therapeutic modulation.

Aluminium is a non-essential element that, when accumulated in biological tissues, induces significant oxidative damage. Aluminium chloride ($AlCl_3$), in particular, is frequently used to model oxidative stress and anaemia in experimental animals due to its toxic effects on haematological and antioxidant systems (Anacletus and Okerenta, 2016). It increases reactive oxygen species (ROS) generation and triggers lipid peroxidation, leading to the depletion of endogenous antioxidants like glutathione (GSH), SOD, and CAT (Olanrewaju *et al*., 2023). These disturbances impair erythropoiesis and destabilize red blood cell (RBC) membranes,

ultimately reducing RBC count, hemoglobin (Hb) concentration, and packed cell volume (PCV), which are hallmarks of anaemia (Igbokwe *et al*., 2020).

Mechanistically, $AlCl_3$ disrupts iron metabolism and heme biosynthesis while promoting oxidative DNA and protein damage (Garbossa *et al*., 1996). It also affects mitochondrial integrity and may trigger apoptosis in erythroid progenitor cells (Chmielnicka *et al*., 1994). The use of aluminium-induced anaemia models is, therefore, valuable for assessing the protective effects of antioxidants and evaluating new treatments for haematological disorders. Evidence from studies involving antioxidant co-therapy (such as vitamin C, quercetin, and plant extracts) demonstrates that such treatments can reverse aluminium-induced damage and restore normal hematological and oxidative balance (Bouasla *et al*., 2014).

1.2. Justification of Study

Anaemia remains a widespread health concern, particularly in developing countries, where nutritional deficiencies, chronic diseases, and environmental toxins such as aluminium contribute significantly to its burden. Aluminium chloride has been shown to induce oxidative stress and impair erythropoiesis, resulting in anaemic conditions. Oxidative damage caused by such agents disrupts the red blood cell membrane, impairs iron metabolism, and interferes with the genetic regulation of red cell formation.

Nuclear factor erythroid 2–related factor 2 (NRF2) is a critical transcription factor that regulates the expression of antioxidant defense genes and plays a key role in redox homeostasis and erythrocyte protection. Its impairment has been associated with increased oxidative damage, ineffective erythropoiesis, and the development of haemolytic anaemia. Despite the growing understanding of NRF2's importance in blood-related disorders, there is limited research

exploring natural agents that can modulate this pathway to restore red blood cell integrity in anaemic conditions.

Traditional medicine has long relied on medicinal plants for the treatment of anaemia and other systemic disorders. *Icacina trichantha*, known for its use in ethnomedicine, contains phytochemicals with antioxidant and anti-inflammatory properties. Preliminary studies on its leaves and tubers have shown evidence of free radical scavenging activity and possible protective effects on various organs. However, scientific validation of its effects on erythropoiesis and oxidative stress at the molecular level, particularly through the NRF2 pathway, is still lacking.

This study addresses a critical need to explore the potential of *Icacina trichantha* aqueous leaf extract in modulating NRF2 expression in aluminium chloride-induced anaemia. Investigating this interaction offers an opportunity to uncover the plant's molecular mechanism of action, particularly in relation to oxidative stress and red blood cell protection. The research also aims to provide scientific support for the traditional use of *Icacina trichantha* in managing anaemia, bridging the gap between ethnomedicine and evidence-based practice. Ultimately, the findings could lay the groundwork for the development of safe, affordable, and plant-derived therapeutic strategies for treating oxidative-stress-related blood disorders.

1.3. Aim of Study

This study aims to investigate the potential therapeutic effects of *Icacina trichantha* leave extracts on anaemia, and the expression of Nuclear factor erythroid 2-related factor 2 (NRF2) genes in albino Wistar rats.

1.4. Specific Objectives

a. To determine the effect of the *Icacina trichantha* aqueous leaves extract on Nuclear factor erythroid 2-related factor 2 (NRF2) gene expression in aluminium chloride-induced anaemia in albino Wistar rats.

b. To determine the effect of the *Icacina trichantha* aqueous leaves extract on some haematological parameters in aluminium chloride-induced anaemia in albino Wistar rats.

1.5. Research Questions

1. Does *Icacina trichantha* aqueous leaves extract have any effect on Nuclear factor erythroid 2-related factor 2 (NRF2) gene expression in aluminium chloride-induced anaemia in albino Wistar rats?

2. Does the aqueous leaves extract have any effect on haematological parameters in aluminium chloride-induced anaemia in albino Wistar rats?

1.6. Research Hypothesis

Null Hypothesis (H_0)

a. The *Icacina trichantha* aqueous leaves extract does not have any effect on Nuclear factor erythroid 2-related factor 2 (NRF2) gene expression in aluminium chloride-induced anaemia in albino Wistar rats.

b. The *Icacina trichantha* aqueous leaves extract does not have any effect on haematological parameters in aluminium chloride-induced anaemia in albino Wistar rats.

Alternate Hypothesis (H_A)

a. The *Icacina trichantha* aqueous leaves extract has an effect on Nuclear factor erythroid 2-related factor 2 (NRF2) gene expression in aluminium chloride -induced anaemia in albino Wistar rats.

b. The *Icacina trichantha* aqueous leaves extract has an effect on haematological parameters in aluminium chloride-induced anaemia in albino Wistar rats.

1.7. Scope of Study

The study was designed to cover the effect of *Icacina trichantha* aqueous leave extract on some haematopoietic genes, and haematological parameters.

CHAPTER TWO

LITERATURE REVIEW

2.1. Origin and distribution of *Icacina Trichantha*

Icacina trichantha is a scandent perennial shrub in the Icacinaceae family, commonly known as "false yam," characterized by a large, subterranean tuber and climbing stems that ascend up to 2 meters, adorned with broad, membranous, oblong-elliptical leaves and silky-hirsute clusters of small, cream-colored flowers that mature into velvety, ellipsoidal drupes (Kadiri *et al*., 2020). Native to the humid forest–savanna transition zones of West Africa, it is commonly found in southern Nigeria and neighboring regions, thriving in forest edges and fallow lands (Ekeke *et al*., 2021). Ethnobotanical studies report vernacular names such as "gẹgbẹ," "urumbia," or "eriagbo" among the Yoruba and Igbo communities, reflecting its integration into local food systems (Ekeke *et al*., 2021). In rural areas, the tuber is harvested, processed into flour, or cooked as porridge during periods of scarcity and famine, highlighting its nutritional and cultural importance (Umoh and Iwe, 2014). Nutritional and phytochemical analyses reveal significant carbohydrate content in the tuber (~73.6%), with leaves being particularly rich in protein, fiber, and minerals such as potassium, magnesium, calcium, and iron (Alawode, 2024). Chloroform extracts of the tuber peel have also yielded bioactive compounds, including undecane, dodecanoic acid derivatives, and triarachine, which exhibit antibacterial, antiviral, and antioxidant properties (Akoh and Mac-Kalunta, 2021). The genus *Icacina*, while distributed more broadly from Cape Verde through Central Africa to Angola, finds its most prominent traditional use and domestication in West Africa (Mbatchou and Dawda, 2012).

Figure 2.1. Leaves of *Icacina Trichantha* (Alawode, 2024).

Table 2.1. Some species of *Icacina* according to International Board for Plant Genetic Resources (IBPGR, 1992).

S/N	SPECIES
1	*Icacina trichantha*
2	*Icacina oliviformis*
3	*Icacina mannii*
4	*Icacina senegalensis*
5	*Icacina guessfeldtii*
6	*Icacina claessensii*
7	*Icacina schweinfurthiana*

Table 2.2. Taxonomy of *Icacina Trichantha* (Alawode, 2024).

Domain	Eukaryota
Kingdom	plantae
Class	Angiosperms
Order	Icacinales
Family	Icacinaceae
Genus	Icacina
Specie	Trichantha

2.2.1. Uses of Some Plant Parts of *Icacina trichantha*

The plant *Icacina trichantha* is a versatile and highly valued species in traditional African medicine and local communities, particularly in West and Central Africa. Its therapeutic and nutritional value is widely recognized, with various parts of the plant being utilized for different purposes. The plant is rich in bioactive compounds such as alkaloids, saponins, tannins, and flavonoids, which form the basis for its broad range of applications (Alawode *et al*., 2024). Traditional knowledge of its uses is now being validated by scientific studies that investigate the underlying pharmacological mechanisms.

2.2.2. Uses of the Leaves

The leaves of *Icacina trichantha* are a primary source of its medicinal properties. They are often used in traditional remedies due to their reported anti-inflammatory, antioxidant, and hematopoietic effects. The leaves are typically prepared as an aqueous extract, a method that is common in ethnomedicine. These extracts are believed to contain compounds that can mitigate various forms of toxicity and disease states.

For instance, studies have shown that plant extracts with antioxidant properties can help to alleviate blood toxicity by modulating cellular defense mechanisms. The leaves contain bioactive compounds that act as antioxidants, helping to protect cells from oxidative stress, a key factor in many disease conditions. Research has investigated the ability of plant-based extracts to combat conditions like anaemia by influencing antioxidant enzyme systems. For example, a study on a bi-herbal formula highlighted its antioxidant effects on glutathione peroxidase (GPx) and superoxide dismutase (SOD) gene expressions in phenylhydrazine-induced anaemia (Obazelu and Williams, 2024). This kind of research provides a scientific basis for the traditional use of plant leaves to boost the body’s defenses against diseases that involve oxidative damage.

In addition to their medicinal applications, the leaves have other cultural uses. For example, some traditions in Nigeria use the leaves as a wrapper for processed oil bean seeds or for coronation ceremonies. This demonstrates the plant's deep cultural significance beyond its therapeutic value.

2.2.3. Uses of the Root and Tuber

The underground tuber of *Icacina trichantha* is perhaps its most significant part, prized for both its nutritional and medicinal properties. This large, fleshy tuber is a critical food source, especially in times of famine or drought, due to its high starch and carbohydrate content (Alawode *et al.*, 2024). However, it contains anti-nutritional factors like hydrogen cyanide, which must be removed through processing, such as prolonged maceration and repeated washing, before consumption.

Medicinally, the tuber is used as an emetic to induce vomiting, a traditional first-aid treatment for poisoning and constipation. Its extracts have been studied for their protective effects on the liver and kidneys against carbon tetrachloride intoxication. The tuber is also believed to have aphrodisiac, purgative, and anti-malarial properties (Alawode *et al*., 2024). The use of its extracts in animal models has demonstrated a wide variety of pharmacological activities. Studies investigating how plant extracts can mitigate haematotoxicity and other forms of blood poisoning often focus on the effects of these extracts on gene expressions and some cellular pathways (Obazelu and Agbikimi, 2025). This aligns with the traditional use of the tuber for treating ailments related to blood toxicity.

The tuber also contains a high concentration of alkaloids, saponins, and flavonoids, which contribute to its potent biological activities (Alawode *et al*., 2024). These compounds are believed to be responsible for its effectiveness in treating conditions like rheumatism, malaria,

and toothaches in traditional medicine. The tuber's combustible nature also gives it an unusual use as a source of fierce heat when burned.

2.2.4. Uses of the Fruits and Seeds

The fruits of *Icacina trichantha* are also utilized, though less frequently than the leaves and tubers. The fruit is a drupe with a soft, sweet outer pulp that is edible. In some communities, the seeds are also consumed, but they require extensive preparation, including steeping in water for a week, to remove bitter and potentially toxic elements. After this process, the seeds are pounded into a flour that can be mixed with other starches to create a nourishing paste (Plants of the World Online, 2021).

2.3. Biochemical Constituents of *Icacina trichantha*

The biochemical makeup of *Icacina trichantha* is a complex and varied profile of both primary and secondary metabolites, which underlies its significant role in traditional medicine and its nutritional value (Otun *et al*., 2015). Numerous phytochemical screenings across different plant parts, including the leaves, roots, and tubers, have consistently identified a wide array of bioactive compounds. These include prominent classes such as alkaloids, flavonoids, saponins, tannins, and phenols (Shagal *et al*., 2013). The specific composition of these compounds can vary depending on the plant part and the type of solvent used for extraction, a fact that is central to the efficacy of various traditional preparations (Alawode, 2024).

Further in-depth analysis of these extracts has revealed specific compounds that contribute to the plant's efficacy. The leaves, for instance, are a rich source of fatty acids such as stearolic acid, oleic acid, and erucic acid (Otun *et al*., 2015). Additionally, the presence of phytol and gamma-sitosterol has been noted in the leaves, highlighting a diverse chemical makeup that varies depending on the part of the plant being examined (Otun *et al*., 2015). The large, fleshy tuber of

Icacina trichantha is especially significant for its high concentration of carbohydrates, primarily starch, which is why it is often used as a famine food in some regions (Alawode, 2024). In addition to nutritional components, the tuber contains other important phytochemicals, including steroids, reducing sugars, and cardiac glycosides (Shagal *et al*., 2013).

The therapeutic actions of these compounds, such as their anti-inflammatory, antioxidant, and anti-apoptotic effects, are central to the plant's medicinal properties. These biological activities often involve the modulation of key cellular pathways and gene expression, a focus of extensive research on plant-based remedies. For example, studies have investigated the effects of plant extracts on genes related to erythropoiesis, such as ETS variant-6 (ETV6) and Nuclear Factor Erythroid 2 (NFE2) (Obazelu and Gaius-Igboanugwo, 2025). Furthermore, other research has explored how these extracts can influence the expression of genes involved in cell adhesion and inflammation, such as Intercellular Adhesion Molecules 1 (ICAM-1) and Vascular Cell Adhesion Molecules 1 (VCAM-1) (Obazelu and Faluyi, 2023). This comprehensive biochemical profile, which includes both macro-nutrients and powerful secondary metabolites, provides a solid scientific foundation for its long-standing use in both nutrition and traditional medicine.

2.4. Phytochemicals of *Icacina trichantha*

The medicinal and nutritional properties of *Icacina trichantha* stem from a rich blend of biochemicals known as phytochemicals. Extensive analyses of the plant's leaves and tubers have identified several key classes of compounds, including alkaloids, flavonoids, tannins, saponins, and phenols (Shagal and Kubmarawa, 2013). The presence of these compounds underpins the plant's traditional uses for its antioxidant, anti-inflammatory, and antimicrobial effects. This phytochemical diversity contributes to the plant's ability to modulate various biological pathways (Otun *et al*., 2015).

Specifically, the leaves of the plant contain fatty acids like stearolic acid and erucic acid, which are believed to contribute to its medicinal value (Otun *et al*., 2015). The tuber, on the other hand, is a valuable source of starch and also contains steroids and cardiac glycosides (Shagal and Kubmarawa, 2013). Modern research has linked these constituents to the modulation of specific cellular processes. For instance, studies have shown that the plant's extracts can have a variable effect on the expression of interleukin-1β (IL-1β) and transforming growth factor-β (TGF-β), which are crucial genes in inflammatory responses (Obazelu and Abadaike-Elvis, 2024). Furthermore, the phytochemicals have been found to influence genes involved in programmed cell death, such as Caspase-1 and CYP11A1, highlighting their potential role in regulating cell fate and physiological balance (Obazelu and Ezeonyebuchi, 2025).

2.5. Antimicrobial activity of *Icania Trichantha*

The antimicrobial activity of *Icacina trichantha* is well-documented in various studies, supporting its traditional use in managing infections. Extracts from different parts of the plant, including leaves, roots, and tubers, have shown inhibitory effects against a range of pathogenic microorganisms (Timothy and Idu, 2011). The efficacy of the extracts is often dependent on the type of solvent used, with methanol and ethanol extracts generally exhibiting more potent activity than aqueous (water-based) extracts (Shagal and Kubmarawa, 2013).

Research has demonstrated that *Icacina trichantha* extracts possess a broad spectrum of antibacterial properties. They have shown effectiveness against both Gram-positive bacteria, such as *Staphylococcus aureus*, and Gram-negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa* (Timothy and Idu, 2011). The plant's antifungal activity has also been investigated, with extracts demonstrating a significant inhibitory effect on fungi like *Candida albicans* and species of *Fusarium* and *Aspergillus*, which are common causes of food spoilage

and illness (Otun *et al*., 2015). This broad activity validates its use in traditional medicine for treating infections and poisoning.

The antimicrobial properties are directly linked to the plant's rich phytochemical content. The presence of alkaloids, tannins, and saponins is particularly credited for these effects (Shagal and Kubmarawa, 2013). These compounds are known to interfere with the growth and survival of microorganisms by disrupting cell membranes, inhibiting essential enzymes, or denaturing microbial proteins. While the specific mechanisms are still being explored, this robust activity is consistent with a broader body of research showing that plant extracts can modulate genes that play a role in inflammation and host defense (Obazelu and Efosa, 2025). The synergistic action of these various phytochemicals provides a strong scientific basis for the plant's efficacy in fighting microbial pathogens.

2.6. Antioxidant Activity of *Icacina Trichantha*

The antioxidant capacity of *Icacina trichantha* is a key factor in its therapeutic potential, with studies confirming its ability to combat oxidative stress. Extracts from the plant's various parts, particularly the tuber, have been shown to possess significant antioxidant properties. In a study evaluating the tuber's methanol extract, researchers found a high antioxidant capacity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, with the extract showing a remarkable dose-dependent effect (Onakpa *et al.*, 2016). This was further supported by the FRAP (Ferric Reducing Antioxidant Power) assay, which confirmed the extract's strong reducing power, a hallmark of antioxidant activity. These in-vitro findings were also validated in-vivo, where the extract elevated levels of antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT**)** in rats, while depressing malondialdehyde (MDA), a marker of lipid peroxidation (Onakpa *et al.,* 2016).

The antioxidant effects of the plant's phytochemicals are crucial for its ability to regulate cellular health and manage various pathological conditions. These properties are often linked to the modulation of gene expression, which governs a cell's response to damage and stress. For instance, the plant's compounds can influence genes involved in inflammation and cellular adhesion, such as Interferon-Gamma (IFN-γ) and Intercellular Adhesion Molecule-1 (ICAM-1) (Obazelu and Osarinmwian, 2025). Furthermore, this protective activity extends to regulating cell death pathways. Studies have demonstrated that the phytochemicals can impact the expression of pro-apoptotic genes like Caspase 3 and Caspase 9, indicating a role in maintaining cellular balance and preventing uncontrolled cell death (Obazelu and Osazee, 2024). The combined action of these antioxidant and gene-modulating effects provides a comprehensive explanation for the plant's wide-ranging use in traditional medicine.

2.7. Nuclear Factor Erythroid 2-Related Factor 2 (NRF-2) Gene

The nuclear factor erythroid 2-related factor 2, or NRF2, is a master regulator of the cellular antioxidant and detoxification systems, making it a critical component of the body's defense against oxidative stress. It functions as a transcription factor that orchestrates a broad protective response to neutralize threats from reactive oxygen species and other harmful substances (Bhandari *et al*., 2021). The NRF2 pathway is considered the most important intrinsic defense mechanism against various forms of cellular damage, inflammation, and xenobiotic exposure (Saha *et al*., 2020).

Under normal physiological conditions, NRF2 is kept inactive in the cytoplasm by a protein called KEAP1. The KEAP1 protein acts as a repressor, tagging NRF2 for continuous degradation, which keeps its cellular levels low. However, in the presence of oxidative or electrophilic stress, KEAP1 undergoes a conformational change that causes it to release NRF2. This stabilization

allows NRF2 to translocate to the cell nucleus, where it binds to a specific DNA sequence known as the Antioxidant Response Element (ARE) (Saha *et al*., 2020). This binding event activates the transcription of a wide variety of cytoprotective genes.

The target genes of NRF2 include a suite of antioxidant and detoxification enzymes, and its activation has been shown to be a key mechanism through which plant extracts exert their protective effects. For example, research has demonstrated that such extracts can modulate genes crucial for cellular defense, such as Nuclear Factor Erythroid 2-Related Factor 2 (NRF-2) and Heme Oxygenase 1 (HO-1), which are central to the antioxidant response (Obazelu and Omoregie, 2024). This is consistent with findings showing that phytochemicals from plant sources can also influence genes associated with programmed cell death, such as Caspase 3 and Caspase 9, which are often activated by severe oxidative stress and cellular damage (Obazelu and Osazee, 2024). These studies highlight the therapeutic potential of targeting the NRF2 pathway and its downstream effects to combat a wide range of diseases driven by cellular stress and damage.

2.8. Molecular Structure and function of NRF2 gene

The NRF2 gene encodes the NRF2 protein, a transcription factor that is part of the basic region-leucine zipper (bZIP) family. The protein is composed of seven highly conserved domains called NRF2-ECH homology (Neh) domains (Neh1-Neh7) (Bhandari *et al*., 2021). These domains are essential for the protein's function and regulation:

a) **Neh1:** Contains the bZIP motif, which allows NRF2 to bind to DNA and form a dimer with other small proteins, a necessary step for gene transcription (Saha et al., 2020).

b) **Neh2:** This is the most crucial domain for NRF2 regulation. It contains two specific motifs, DLG and ETGE, that allow it to bind to its negative regulator, the KEAP1 protein. This binding is key to its degradation under normal conditions (Bhandari et al., 2021).

c) **Neh3-Neh5:** These domains act as transcriptional activation domains, meaning they recruit other proteins to help turn on gene expression once NRF2 is in the nucleus (Saha et al., 2020).

2.8.1. Function: The Cellular Defense Mechanism

The primary function of the NRF2 gene is to respond to and mitigate oxidative stress by controlling the expression of numerous genes that produce antioxidant and detoxifying enzymes (Obazelu and Omoregie, 2024). This process is a tightly regulated two-step mechanism:

1. **Inactivation:** Under normal, unstressed conditions, the NRF2 protein is located in the cell's cytoplasm. Here, it is bound to the KEAP1 protein, which constantly tags NRF2 for degradation by the proteasome. This ensures that NRF2 levels remain low when they are not needed (Bhandari *et al*., 2021).

2. **Activation:** When a cell is exposed to oxidative stress from toxins or inflammation, the KEAP1 protein releases NRF2. The now-stable NRF2 protein is free to travel into the nucleus. Once inside, it partners with other proteins and binds to a specific DNA sequence called the Antioxidant Response Element (ARE) in the promoter regions of target genes (Saha *et al*., 2020). This binding turns on the expression of a wide range of protective genes, including Heme Oxygenase 1 (HO-1) and NAD(P)H: Quinone Oxidoreductase 1 (NQO1), to neutralize harmful compounds and repair damage (Obazelu and Omoregie, 2024).

2.9. Role of NRF2 gene in erythropoiesis

The NRF2 gene plays a crucial and multifaceted role in erythropoiesis, the complex process by which hematopoietic stem cells differentiate into mature red blood cells (Mbiandjeu *et al.*, 2024). Its function is particularly vital in protecting developing red blood cell precursors from oxidative stress, a significant threat during their maturation.

Red blood cell maturation is a dynamic process characterized by significant metabolic changes. During this time, the cells are highly susceptible to damage from reactive oxygen species (ROS), which can interfere with cell differentiation and lead to premature cell death. NRF2 acts as a key protective mechanism, activating a suite of antioxidant genes that help these developing cells withstand oxidative damage. Research on NRF2-deficient mice, for instance, has shown that a lack of functional NRF2 leads to an age-dependent anaemia due to both reduced red blood cell lifespan and ineffective erythropoiesis (Mbiandjeu *et al*., 2024). This highlights NRF2's essential role in ensuring normal erythroid maturation and growth by limiting age-related oxidation.

2.9.1. NRF2 Functions in Erythropoiesis

The role of NRF2 in red blood cell development is not limited to general antioxidant defense; it also has several specific functions that are crucial for the production of healthy, oxygen-carrying red blood cells:

a) **Heme and Iron Metabolism:** NRF2 directly regulates genes involved in iron metabolism and the synthesis of heme, the molecule that binds to iron and forms the core of hemoglobin (Kerins and Ooi, 2018). By controlling the expression of genes such as Heme 2.9.1.2 Oxygenase 1 (HO-1), NRF2 helps manage the significant iron flux required for red blood cell production while simultaneously detoxifying byproducts of

heme degradation (Itoh et al., 1999). This delicate balance is vital, as free iron can catalyze the production of harmful free radicals.

b) **Protection Against Apoptosis:** Studies have shown that in the absence of NRF2, erythroid precursors are more susceptible to apoptosis (programmed cell death) (Mbiandjeu et al., 2024). This is because NRF2 helps activate protective mechanisms, such as autophagy, that counteract stress-induced cell death. Without NRF2, overactivation of stress responses can drive the cells towards apoptosis, leading to ineffective erythropoiesis and anaemia (Mbiandjeu et al., 2024).

c) **Globin Gene Regulation:** NRF2 has been shown to play a direct role in regulating globin gene expression, particularly the **gamma-globin gene**, which is responsible for producing fetal hemoglobin (Vinjamuri et al., 2018). NRF2 mediates chromatin looping to activate gamma-globin transcription, suggesting its potential as a therapeutic target for blood disorders like sickle cell anaemia.

2.10. Haematological Parameters

Blood provides a clear reflection of a person's physiological and overall health condition. Through its analysis, clinicians can identify metabolites and other internal constituents of the body (Nseabasi, 2014). Hematological parameters comprise measurable elements of blood, including hemoglobin (Hb), hematocrit (Hct), red blood cell (RBC) count, white blood cell (WBC) count, as well as indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). These values serve as standard clinical markers for health and disease, with their regulation in healthy individuals often influenced by genetic factors (Kelada *et al*., 2012). Historically, hematological

measurements have been essential for diagnosing and tracking treatment in a range of infectious diseases and cancers (Kone *et al*., 2017).

2.10.1. Red Blood Cells (RBCs)

Red blood cells, also referred to as erythrocytes, are essential for transporting gases and nutrients throughout the body. Mature erythrocytes have a biconcave, disc-like shape and are non-nucleated in mammals (Smith, 1987), a structure that enhances flexibility for navigation through blood vessels and increases surface area for efficient gas exchange—vital for their physiological role (Tirado, 2023). Each RBC has an average lifespan of about 120 days, during which it delivers oxygen from the lungs to peripheral tissues for metabolic processes such as ATP production and carries carbon dioxide from tissues back to the lungs for elimination. Normal RBC counts range from 4.5 to 5.5 × 10^12/L in males and 4.0 to 5.0 × 10^12/L in females (Cheesebrough, 2006)

2.10.2. Haemoglobin (Hb)

Haemoglobin is the oxygen-carrying protein found in RBCs, crucial for adequate tissue oxygenation. It is usually measured in grams per decilitre (g/dl) of whole blood, with normal values ranging from 14–18 g/dl in males and 12–16 g/dl in females (Williams et al., 2023). Low haemoglobin levels result in anaemia, whereas elevated levels, often due to increased RBC counts, are referred to as erythrocytosis.

2.10.3. Haematocrit (Hct) / Packed Cell Volume (PCV)

Haematocrit measures the proportion of blood volume occupied by RBCs, expressed as a percentage of the total blood volume (cells plus plasma). Normal values range from 40–54% in males and 36–48% in females (Williams et al., 2023). It is commonly determined using the micro-haematocrit centrifugation method.

2.10.4. Red Cell Indices

a) Mean Corpuscular Volume (MCV)

MCV reflects the average size of RBCs and is calculated by multiplying the haematocrit by 10, then dividing by the erythrocyte count. It is expressed in femtolitres (fl), with normal values averaging 87 ± 7 fl. MCV, along with Hb and Hct, is useful in classifying anaemia as microcytic (low MCV), normocytic (normal MCV), or macrocytic (high MCV) (Fischer & Fischer, 1983). It also contributes to determining the Red Cell Distribution Width (RDW).

b) Mean Corpuscular Haemoglobin (MCH)

MCH indicates the average amount of haemoglobin per RBC, with normal values around 29 ± 2 picograms (pg) per cell.

c) Mean Corpuscular Haemoglobin Concentration (MCHC)

MCHC measures the average concentration of haemoglobin in a given volume of packed RBCs and is expressed in g/dl or as a percentage. Normal values are approximately 34 ± 2 g/dl.

d) Red Cell Distribution Width (RDW)

RDW measures the variability in RBC size, a condition known as anisocytosis. It is expressed as a percentage, representing the coefficient of variation in RBC volumes. The typical reference range is 13 ± 1.5% (Evans & Jehle, 1991).

CHAPTER THREE

MATERIALS AND METHOD

3.1. Reagents

Only reagents of analytical grade were used in the course of this study.

3.2. Study Area

In this research, a rat model was employed. Sixty (60) Albino Wistar rats were obtained from the animal facility of the Department of Anatomy, University of Benin, Benin city, Nigeria. The rats were kept in the animal facility of the Department of Anatomy, University of Benin.

3.3. Identification of *Icacina Trichantha* leaves

Leaves of *Icacina trichantha* were collected on April 2nd, 2025, from the Ekosodin community in Ovia North East Local Government Area, Edo state, Nigeria. Authentication and identification of the leaves were carried out in the Department of Plant Biology and Biotechnology (PBB), Faculty of Life Science, University of Benin, with voucher number UBH-1185.

3.3.1 Processing of *Icacina Trichantha* Leaves

The process commenced with the removal of damaged and unhealthy leaves from the sample. The selected leaves were then thoroughly washed and drained. To ensure effective grinding the leaves were initially air-dried for two weeks. This was followed by further drying in a hot air oven at 50ºc for 24 hours to ensure they were fully dried and suitable for grinding.

Adequate drying was achieved to prepare the leaves for grinding. The grinding was performed using an industrial 1000A high-speed grinding machine.

3.3.2 Preparation of Plant Extract

A total of 1500grams of the pulverized plant material was macerated in 15litres of distilled water with continuous stirring for 24hours. The mixture was then filtered using Whatman nitrocellulose filter paper (0.45m pore size). The resulting filtrate was concentrated using a water bath maintained at 37°c, after which it was stored in an airtight container and refrigerated until needed (Obazelu and Olorunda, 2024).

3.4. Animal Care

The animals were housed in a well-ventilated room at the Department of Anatomy's animal holding unit, University of Benin, Benin city. They experienced alternating 12hour light and dark periods and were given free access to food and water. Acclimatization was carried out over a duration of two (2) weeks before the experiment began.

3.4.1. Inclusion criteria

- Apparently healthy Wistar rats weighing 150-200grams
- Male rats

3.4.2. Exclusion criteria

- Rats with excessive breathing
- Rats with reduced appetite
- Sick rats
- Rats weighing less than 150grams

3.5. Ethical considerations

Ethical clearance for the use of animal subjects was granted by the Research Ethics Committee on Animal Subjects, Edo State Ministry of Health, Benin city, under reference number HA/737/25/D/06180723, issued on 18th June, 2025.

3.6. Preparation of Aluminium Chloride and Ferrous Sulphate Drug Solution

3.6.1. Aluminium Chloride Solution

Aluminium chloride solution was prepared by mixing 0.1gram of aluminium chloride powder (manufactured by Guangdong Guanghua Sci-Tech co, LTD, Batch number T/CSTM 00071-2019) with 100millilitres of distilled water. Subsequently, 0.1millilitre of aluminium chloride solution was administered to each Wistar rat in the various test groups, with an average weight of 150grams (Obazelu and Olorunda, 2024).

3.6.2. Ferrous Sulphate Drug Solution

A solution of ferrous sulphate was prepared by dissolving 1000mg of the powdered drug in 50ml of distilled water. Each rat in group C, with an average body weight of 150g received 0.3ml of this solution orally every 48 hours over a period of 28 days.

3.7. Research design

3.7.1. Grouping of animals:

Sixty (60) adult Albino Wistar rats, weighing between 150g and 200g, were randomly assigned into six groups, with ten (10) rats per group. The groups were designated as group A, Group B, Group C, Group D, Group E, and Group F.

Group A: This group served as the control. The animals were provided with standard feed (produced by KARMA AGRIC FEEDS AND FOOD LIMITED, Oyo state) with unrestricted access to clean water.

Group B: This group was administered only aluminium chloride solution intraperitoneally

Group C: Animals in this group received aluminium chloride solution and were treated with the ferrous sulphate drug solution intraperitoneally.

Group D: Animals in this group were administered aluminium chloride solution intraperitoneally and treated with low dose of *Icacina trichantha* leave extract orally

Group E: Animals in this group were administered aluminium chloride solution intraperitoneally and treated with higher dose of *Icacina trichantha* leave extract orally

Group F: Animals in this group were administered aluminium chloride solution intaperitonialy and treated with the highest dose of *Icacina trichantha* leave extract orally

3.8. Dosage of Ferrous Sulphate Administered

40mg/kg.bw

40mg - 1000g

? - 150g (Mean weight of rats)

$= \frac{40 \times 150}{1000} = 6mg$

1000mg of ferrous sulphate powder = 50mls

6mg = x

$X = \frac{6 \times 50}{1000} = 0.3ml$ of 6mg/ml 48 hourly for 4 weeks

3.9. Extract Dosing

The dosage administered is calculated by;

Weight of the animal = g/kg

Dose of extract = mg/kg

Stock of extract = mg/ml

Volume to administer = $\frac{\text{Weight} \times \text{Dose of extract}}{\text{Stock}}$

40g of the extract was weighed

40g is equivalent to 40000mg

40g of the extract is dissolved in 400ml of distilled water

Concentration of extract = $\frac{40000mg}{400ml} = 100mg/ml$

Group A was the control group and was given only feed and water *ad libitum*

Group B was administered Aluminium Chloride solution intraperitoneally

Group C was administered 40mg/kg ferrous sulphate drug solution

Group D was administered 100 mg/kg *Icacina trichantha* leave extract orally

Group E was administered 200 mg/kg *Icacina trichantha* leave extract orally

Group F was administered 400 mg/kg *Icacina trichantha* leave extract orally

Calculating dose of extract for each group using;

Volume to administer = $\frac{\text{Weight} \times \text{Dose of extract}}{\text{Stock}}$ (Obazelu and Olorunda, 2024).

Group D

Average weight of 10 rats = 150kg

Dose = 100mg/kg

Stock = 100mg/ml

150g to kg = 0.150kg

Volume to administer = $\frac{\text{Weight} \times \text{Dose of extract}}{\text{Stock}}$

$= \frac{0.150 \times 100}{100} = 0.15\text{ml}$

Group E

Average weight of 10 rats = 150kg

Dose = 200 mg/kg

Stock = 100 mg/ml

150g to kg = 0.150kg

Volume to administer = $\frac{\text{Weight} \times \text{Dose of extract}}{\text{Stock}}$

$= \frac{0.150 \times 200}{100} = 0.3\text{ml}$

Group F

Average weight of 10 rats = 150kg

Dose = 400 mg/kg

Stock = 100 mg/ml

150g to kg = 0.150kg

Volume to administer = $\frac{\text{Weight} \times \text{Dose of extract}}{\text{Stock}}$

$= \frac{0.150 \times 400}{100} = 0.6\text{ml}$

3.10. Administered Doses of *Icacina Trichantha* Leave Extract

Group A (control) received only standard feed and clean water ad libitum. Group B (aluminium chloride treated group) received 0.1ml of aluminium chloride solution intraperitoneally every 48 hours for 28 days. Group C (ferrous sulphate drug solution treated group) received 0.1ml of aluminium chloride intraperitonially every 48 hours for 28 days and were treated with 0.3ml of 6mg/ml of ferrous sulphate 48 hourly for 28 days. Group D received 0.1ml of Aluminium Chloride solution intraperitoneally every 48 hours for 28 days and were treated with 0.15ml of 100mg/kg body weight of *Icacina trichantha* leave extract orally using a gavage tube every 24 hours for 28 days. Group E received 0.1ml of Aluminium Chloride solution intraperitoneally every 48 hours for 28 days and were treated with 0.3ml of 200mg/kg body weight of *Icacina trichantha* leave extract orally using a gavage tube every 24 hours for 28 days. Group F received 0.1ml of Aluminium Chloride solution intraperitoneally every 48 hours for 28 days and were treated with 0.6ml of 400mg/kg body weight of *Icacina trichantha* leave extract orally using a gavage tube every 24 hours for 28 days (Obazelu and Omoregie, 2024).

3.11. Physical Examination

3.11.1. Measurement of Body Weight

Body weight measurements were taken twice during the experiment, on day 0 and day 28, representing the initial and final weights, respectively. Each rat was individually weighed using a digital weighing scale. This involved gently removing the animals from their cages, placing them on the scale, and recording the readings while the animals remained still.

3.12. Animal Sacrifice and Sample Collection

At the end of the experimental period, the animals were assessed for general physical appearance and condition. Following anesthesia with chloroform and subsequent cervical dislocation, a middle incision was made along the ventral surface. Blood samples (5ml) were collected from each rat with the aid of a sterile syringe and transferred into an EDTA container (Ethylene Diamine Tetra-acetic Acid) for full blood count analysis. Bone marrow was collected by carefully opening the femur longitudinally to expose the marrow cavity. With the aid of sterile forceps, the marrow was extracted and placed into Eppendorf tubes containing Trizol reagent for molecular studies (Obazelu and Omoregie, 2024).

3.13. Laboratory Analysis

3.13.1 Haematological Profile

Full blood count parameters were assessed following sample collection using an automated three-part ERMA Haematology Auto analyzer PCE-210N (Diamond Diagnostic; Holliston, USA). Sample processing and analysis were carried out following the manufacturer's instructions strictly (Obazelu and Omoregie, 2024).

3.13.2. Detection Principle of Haematology Analyzer

The haematology analyzer operates by measuring and classifying each blood cell based on changes in electrical resistance that occurs as each cell moves through a small aperture sensor.

The blood sample is first mixed in a conductive solution. As individual blood cells (poor conductors of electricity) pass through the aperture, they cause an increase in electrical resistance. This resistance change corresponds to the cell's volume, following Ohm's law (U=RI), where voltage (U) increases with rising resistance (R), assuming a constant current (I).

The generated voltage signals are then amplified using a magnifying circuit, which also filters out background noise, allowing clean signals to be analyzed. Separate circuits are used for analyzing White Blood Cells (WBCs), platelets (PLTs), and Red Blood Cells (RBCs). A micro processing unit (MPU) interprets the signals, calculates the results, and produces histograms. Platelet counting is enhanced by an advanced liquid-electronic system that minimizes repetitive counting errors. In cases where red blood cells enter the platelet analysis zone, their signal pulses, being similar, can lead to potential overlap, which the system is designed to distinguish and correct (Obazelu and Omoregie, 2024).

3.13.3. Procedure

The blood sample was properly mixed and introduced into the analyzer through the sampling probe. An aliquot of 20 µL was collected into the system, and analysis was done immediately after.

3.13.4 Peripheral Blood Film

3.13.5. Preparation of Leishman Stain (Obazelu and Olorunda, 2024).

Stock Solution of Eosin Y

1. 1 gram of Eosin Y powder was added to a clean, dry glass staining dish.
2. 100ml of distilled water was added to the dish and the mixture was stirred until the powder was completely dissolved.
3. Solution was labelled as "Eosin Y stock solution."

Stock solution of Methylene blue:

1. 1 gram of Methylene blue powder was added to another clean, dry glass staining dish.
2. 100 ml of distilled water was added to the dish and the mixture was stirred until the powder is completely dissolved.

3. It was then labelled as "Methylene blue stock solution."

Working solution of Leishman stain:

1. 1 ml of the Eosin Y stock solution was added to a clean, dry staining jar.
2. 1 ml of the Methylene blue stock solution was also added to the same staining jar.
3. 98 ml of ethanol (95%) was added to the staining jar.
4. The contents of the staining jar were mixed thoroughly using a glass stirring rod and then allowed to ripen for three (3) days after which it was labelled as "Leishman stain working solution."

3.13.6. Procedure for Leishman Staining (Obazelu and Olorunda, 2024).

1. A drop of blood sample from the sacrificed animals was placed on a clean grease free glass slide.
2. Another clean grease free slide was placed at an angle of 45 degree to the drop of blood while allowing it to spread along the edges after which a smooth motion was applied to create a thin and even blood film. The film was allowed to air dry completely.
3. The film was flooded with the prepared working solution of Leishman stain for 2 minutes.
4. After 2 minutes the slide was buffered with twice the volume of stain using a Sorensen's buffer solution for 8 minutes.
5. After 8 minutes, slide was gently rinsed and allowed to dry after which a drop of immersion oil was place on it.
6. The prepared slide was placed on the microscope stage, and the peripheral blood film was examined using ×100 objective lens.
7. Different cellular components such as white blood cells, red blood cells, and platelets were observed for morphology and abnormalities.

3.14. Nuclear factor erythroid 2-related factor 2 (NRF2) mRNA Assay

3.14.1. Isolation of Total RNA

Total RNA was isolated from whole drosophila samples with Quick-RNA MiniPrep™ Kit (Zymo Research). The DNA contaminant was removed following DNAse I (NEB, Cat: M0303S) treatment. The RNA was quantified at 260 nm and the purity confirmed at 260 nm and 280 nm using A&E Spectrophotometer (A&E Lab. UK).

3.14.2. cDNA Conversion

One (1 µg) of DNA-free RNA was converted to cDNA by reverse transcriptase reaction with the aid of cDNA synthesis kit based on ProtoScript II first-strand technology (New England BioLabs) in a condition of 3-step reaction: 65 °C for 5 min, 42 °C for 1 h, and 80 °C for 5 min (Olumegbon et al., 2020).

3.14.3. PCR Amplification and Agarose Gel Electrophoresis

Polymerase chain reaction (PCR) for the amplification of NRF2 was carried out with OneTaqR2X Master Mix (NEB) using the following primers (Inqaba Biotec, Hatfield, South Africa). PCR amplification was performed in a total of 25 µl volume reaction mixture containing cDNA, primer (forward and reverse) and Ready Mix Taq PCR master mix. Under the following condition: Initial denaturation at 95 ◦C for 5 min, followed by 30 cycles of amplification (denaturation at 95 ◦C for 30 s, annealing for 30 s and extension at 72 ◦C for 60 s) and ending with final extension at 72 ◦C for 10 min. The amplicons were resolved on 1.0% agarose gel. The GAPDH gene was used to normalize the relative level of expression of each gene, and quantification of band intensity was done using “image J” software (Elekofehinti et al., 2020).

PRIMER SEQUENCES OF NRF2

Forward primer CACATCCAGACAGACACCAGT

Reverse primer CTACAAATGGGAATGTCTCTGC

GAPDH

Forward CTCCCTGGAGAAGAGCTATGA

Reverse AGGAAGGAAGGCTGGAAGA

3.15. Statistical Analysis

Data obtained from this research was presented and analyzed using GraphPad prism 8.0 (California, USA). Analysis of variance (ANOVA) was used to compare treatment groups of continuous variables. Tukey HSD *post hoc* was applied where a significant difference was observed in the ANOVA. A Bar chart was used to represent the mRNA gene expression patterns. A p value of ≤ 0.05 was considered statistically significant.

CHAPTER FOUR

RESULTS

4.0. Results

Table 4.1 shows the comparison of Mean±SEM of red blood cell count, haemoglobin concentration, haematocrit and red cell indices of all six groups: groups A, B, C, D, E and F, representing control, aluminium chloride group, ferrous sulphate group, aluminium chloride + 100mg/kg *Icacina trichantha* leave extract, aluminium chloride + 200mg/kg *Icacina trichantha* leave extract and aluminium chloride + 400mg/kg *Icacina trichantha* leave extract respectively.

There was no statistically significant change in Red Blood Cell count (RBC) (µL) across all the groups ($p>0.05$). There was no statistically significant change in Haemoglobin concentration (g/dL) across all the groups ($p>0.05$). There was no statistically significant change in Haematocrit across all the groups ($p>0.05$). Mean cell volume (MCV) (fL)was significantly lower in group F (54.64±0.96) when compared to group C (58.22±0.49) ($p<0.05$). Compared to group C (19.66±0.07), Mean cell haemoglobin (MCH) (pg) was significantly lower in group F (18.72±0.23) ($p<0.05$). There was no statistically significant change in Mean cell haemoglobin concentration (MCHC) (g/dL) across all the groups ($p>0.05$). There was no statistically significant change in RDW-SD (fL) across all the groups ($p>0.05$). There was no statistically significant change in RDW-CV (%) across all the groups ($p>0.05$).

Table 4.1: Mean Comparison of Red Blood Cell Count, Hemoglobin Concentration and Red Blood Cell Indices of Studied Groups

Parameters	Group A	Group B	Group C	Group D	Group E	Group F	f value	P value
RBC Count (x109/L)	8.09±0.12	7.74±0.21	7.65±0.21	8.18±0.06	7.84±0.31	8.05±0.29	0.8828	0.5083
Haemoglobin (g/dL)	15.33±0.24	14.84±0.32	15.06±0.36	15.5±0.22	15±0.59	15.1±0.61	0.3059	0.9043
HCT (L/L)	44.88±0.78	44.52±0.96	44.46±0.99	45.1±0.73	44.32±1.46	43.86±1.52	0.1445	0.9797
MCV (fL)	55.55±0.73	57.7±1.26	58.22±0.49	55.22±0.91	56.62±0.59	54.64±0.96^{c}	2.729	0.0446
MCH (pg)	18.9±0.07	19.16±0.24	19.66±0.07	18.9±0.23	19.06±0.18	18.72±0.23^{c}	3.049	0.0296
MCHC (g/dL)	34.1±0.37	33.3±0.39	33.8±0.19	34.32±0.31	33.78±0.36	34.36±0.46	1.289	0.3029
RDW-SD (fL)	36.35±1.24	37.18±1.09	37.18±1.09	35.9±1.24	35.04±1.1	33.74±0.81	1.513	0.2246
RDW-CV (%)	16.48±0.45	16.36±0.34	16.26±0.42	16.3±0.37	15.68±0.56	15.48±0.23	1.007	0.4361

Key: Table presented in mean±SEM. $p<0.05$ was considered significant. Superscript a represents significance with group A, b represents significance with group B, c represent significance with group C, d represents significance with group D, e represents significances with group E

RBC: Red Blood Cell

HCT: Haematocrit

MCV: Mean Cell Volume

MCH: Mean Cell Haemoglobin

MCHC: Mean Cell Haemoglobin Concentration

RDWS: Red Cell Distribution Width – Standard Deviation

RDWC: Red Cell Distribution Width – Coefficient of Variation

Table 4.2. shows the blood morphology among the studied groups namely; groups A, B, C, D, E and F, representing control, aluminium chloride group, ferrous sulphate group, aluminium chloride + 100mg/kg *Icacina trichantha* leaf extract, aluminium chloride + 200mg/kg *Icacina trichantha* leaf extract, and aluminium chloride + 400mg/kg *Icacina trichantha* leaf extract respectively.

All groups showed the presence of both small and large (atypical) lymphocytes. Small lymphocytes were highly present in group A while it was moderately present in the other groups. Atypical lymphocytes were highly present in the aluminium chloride induced group (Group B), moderately present in groups A, C and D and mildly present in groups E and F. Eosinophils was mildly present in group B only, Monocytes in groups C and F while Basophils in group C only. All groups showed the presence or normocytic and normochromic red blood cells although they were only mildly present in the aluminium chloride induced group (Group B) compared to the other groups which showed moderate and high presence of these red blood cells. Polychromatic cells were absent in all groups. Crenated red blood cells were mildly present in groups B and C. Platelets were highly present in all groups.

Table 4.2. Blood Morphology among the Studied Groups

	LYMPH% (Small)	LYMPH% (Large)	EOS%	MON%	BAS%	NORMOCT CELLS%	NORMOCM CELLS%	POLYCMT CELLS%	CRENATED CELLS%	PLT% Normal
GROUP A	+++	++	-	-	-	++	++	-	-	+++
GROUP B	++	+++	+	-	-	+	+	-	+	+++
GROUP C	++	++	-	+	+	++	++	-	+	+++
GROUP D	++	++	-	-	-	++	++	-	-	+++

LYMPH-Lymphocytes

EOS- Eosinophils

MON-Monocytes

BAS-Basophils

NORMOCT-normocytic cells

NORMOCM-Normochromic cells

POLYCMT-Polychromatic cells

PLT-Platelet.

- = absent, + = mildly present, ++ = moderately present and +++ = highly present.

Figure 4.1 shows the body weight of groups A, B, C, D, E and F, representing control, aluminium chloride group, ferrous sulphate group, aluminium chloride + 100mg/kg *Icacina trichantha* leave extract, aluminium chloride + 200mg/kg *Icacina trichantha* leave extract, and aluminium chloride + 400mg/kg *Icacina trichantha* leave extract respectively. The weight gain of group A increased at day 28 when compared to day 0. Groups B, C, D, E, and F maintained similar initial and final weight at the end of the experiment.

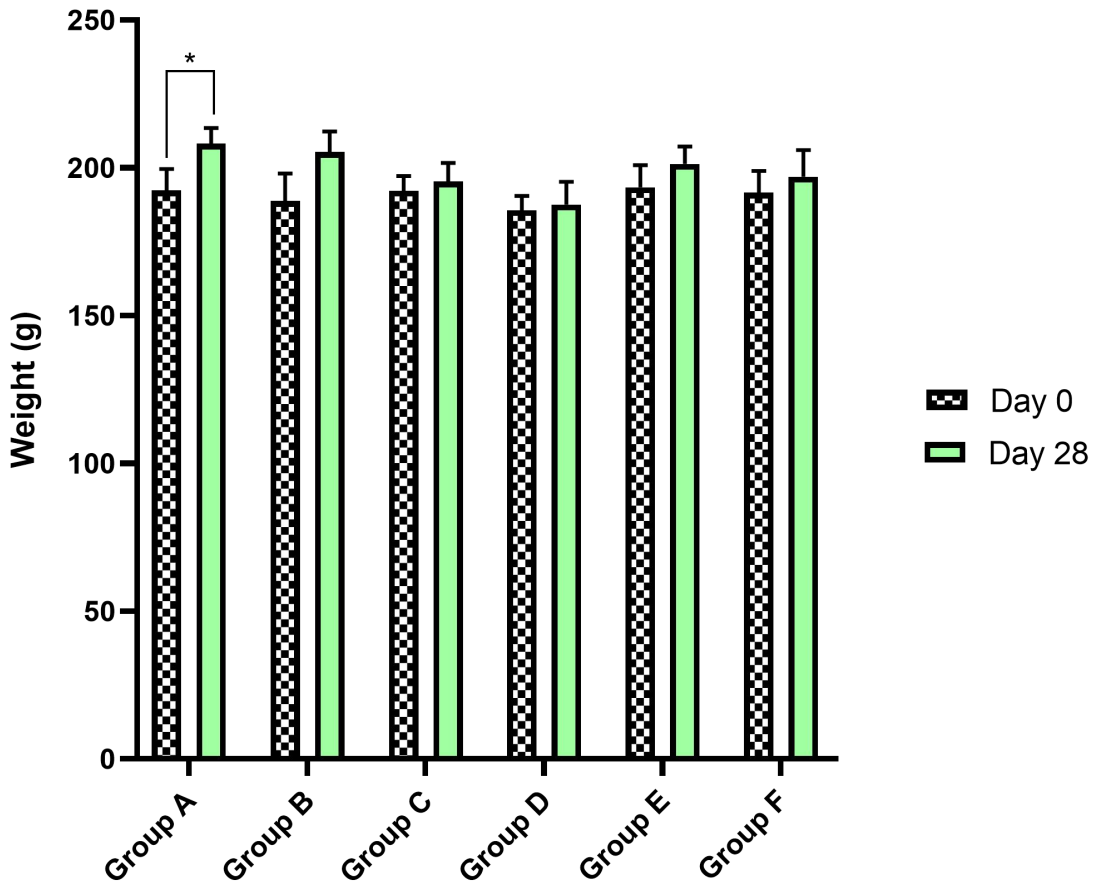

Figure 4.1: Initial and Final Body weights of Groups A, B, C, D, E and F measured at Day 0 and Day 28.

GROUP A: Control Group

GROUP B: Aluminium Chloride Group

GROUP C: Ferrous Sulphate Group

GROUP D: Aluminium Chloride + 100mg/kg *Icacina trichantha* leave extract

GROUP E: Aluminium Chloride + 200mg/kg *Icacina trichantha* leave extract

GROUP F: Aluminium Chloride + 400mg/kg *Icacina trichantha* leave extract

Figure 4.2 Illustrates the gene expression as represented by gel electrophoresis picture and internal control (Glycealdehyde-3-Phosphate Dehydrogenase {GADPH}) of mRNA expression of Nuclear factor erythroid 2-related factor 2 (Nrf2) of groups A, B, C, D, E and F, representing control, aluminium chloride group, ferrous sulphate group, aluminium chloride + 100mg/kg *Icacina trichantha* leave extract, aluminium chloride + 200mg/kg *Icacina trichantha* leave extract, and aluminium chloride + 400mg/kg *Icacina trichantha* leave extract respectively, illustrated on the bar chart. There was a significant decrease in the mRNA expression of Nrf2 of group D, E, and F when compared to group A ($p<0.05$). There was a significant decrease in the mRNA expression of NRF2 of groups D and F when compared to group C ($p<0.05$). Group B had a higher mRNA expression when compared to groups C, D, E, and F ($p<0.05$).

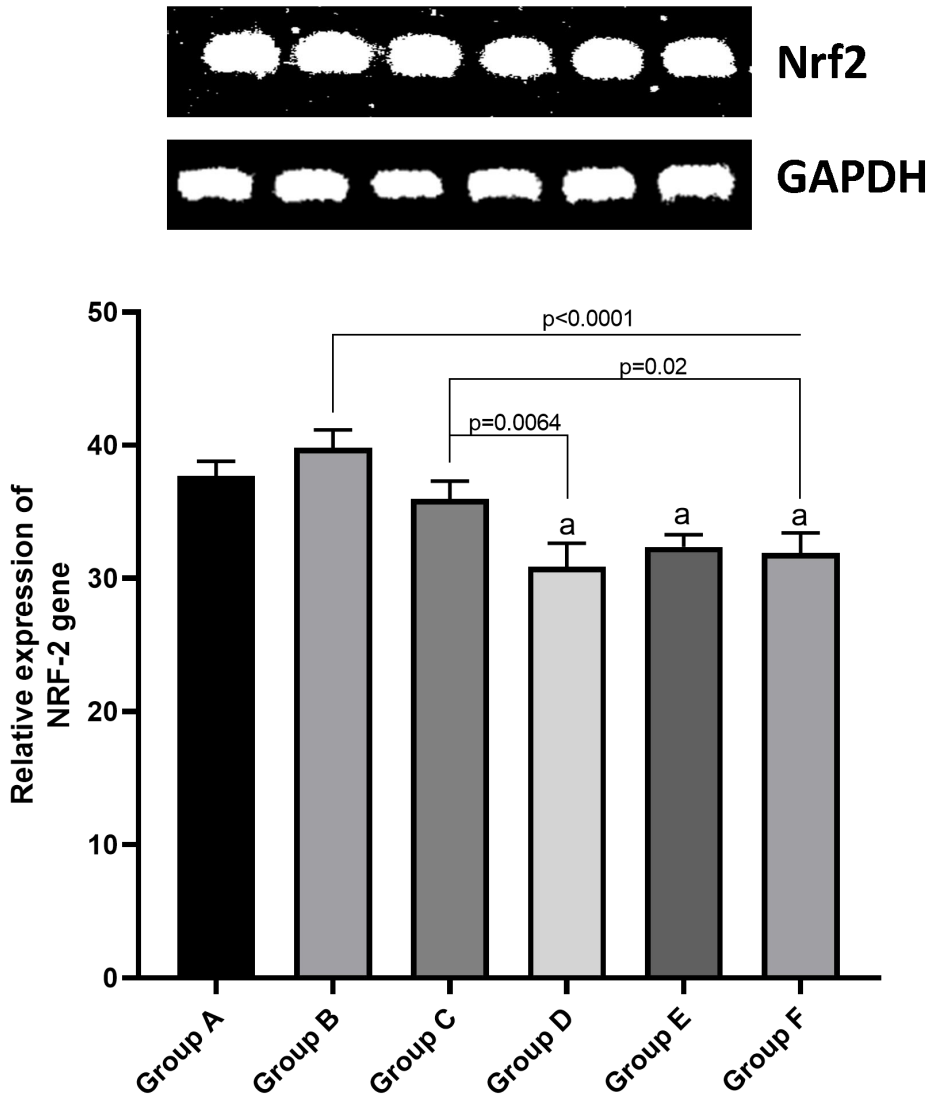

Figure 4.2:

(Figure shows mean±SEM. Error bar represents triplicate of each group. $p<0.05$ was considered significant. a represents significance with group A)

GROUP A: Control Group

GROUP B: Aluminium Chloride Group

GROUP C: Ferrous Sulphate Group

GROUP D: Aluminium Chloride + 100mg/kg *Icacina trichantha* leave extract

GROUP E: Aluminium Chloride + 200mg/kg *Icacina trichantha* leave extract

GROUP F: Aluminium Chloride + 400mg/kg *Icacina trichantha* leave extract

CHAPTER FIVE

DISCUSSION

Anaemia disrupts the body's ability to deliver oxygen effectively, often manifesting as fatigue and reduced functional capacity (Bhadra and Deb, 2020). Aluminium chloride ($AlCl_3$), widely prevalent in industrial settings, is known to provoke oxidative stress through generation of reactive oxygen species (ROS) and impairment of antioxidant systems ((Kadhim *et al*., 2024)). In toxicological models, such stress can lead to impaired erythropoiesis and red blood cell (RBC) damage. NRF2 is the key transcription factor orchestrating cellular antioxidant defense by inducing enzymes like HO-1, NQO1, and GSH-synthesizing proteins (Baird and Yamamoto, 2020). As such, NRF2 expression serves as a sensitive indicator of oxidative stress and cellular adaptive responses.

In this study, one control group was administered $AlCl_3$ alone to *induce oxidative stress without treatment*. This group (B) exhibited a modest, non-significant increase in NRF2 expression compared to untreated controls—implying an emerging, but not yet robust, oxidative defense response. Critically, this group also displayed no signs of anaemia, as their RBC count, hemoglobin (Hb), and hematocrit (HCT) remained unchanged. This suggests that under the study's exposure conditions, oxidative stress from $AlCl_3$ was present but insufficient to cause overt hematotoxicity, mirroring observations in early-phase exposures where compensatory mechanisms maintain erythropoiesis (Valko *et al*., 2007).

The treatment groups, including ferrous sulphate and various doses of *Icacina trichantha*, exhibited significantly reduced NRF2 expression relative to the $AlCl_3$-only group. This pattern indicates that the phytochemicals may have effectively lowered oxidative burden, reducing the

cellular need for NRF2 upregulation. Interestingly, literature supports such feedback regulation: moderate antioxidant interventions can suppress NRF2 activation by resolving oxidative triggers (Longobardi *et al*., 2024).

Despite changes in NRF2 expression, all groups (including those administered the extract) showed no significant alterations in RBC, Hb, HCT, MCHC, RDW-SD, or RDW-CV ($p > 0.05$). This contrasts sharply with studies where higher $AlCl_3$ doses or longer exposure durations led to normocytic anaemia and impaired iron metabolism (Zhang *et al*., 2011). The absence of hematological compromise here likely reflects sub-threshold exposure and effective antioxidant protection afforded by *Icacina trichantha*.

In the highest extract dose group (F), both MCV and MCH were significantly lower than in the ferrous sulphate group. These findings suggest a subtle trend toward microcytic, hypochromic red cells, possibly indicating interference with hemoglobin synthesis or red cell maturation at high phytochemical concentration. However, the staying power of RBC suggests these effects are early or minimal modifications, not full-blown anaemia.

The peripheral blood film examination provided additional insights into the haematological effects of aluminium chloride exposure and subsequent treatment with *Icacina trichantha* extract. All groups demonstrated the presence of small and atypical lymphocytes, although their distribution varied across treatments. Notably, atypical lymphocytes were most pronounced in the aluminium chloride–only group (Group B), suggesting that aluminium-induced oxidative stress may have triggered lymphocyte activation or morphological alteration, consistent with previous reports linking aluminium toxicity to immune dysregulation and abnormal lymphocyte morphology (Kumar and Gill, 2014). The reduced frequency of atypical lymphocytes in the

extract-treated groups may reflect a protective or modulatory effect of *Icacina trichantha* bioactive compounds on immune cells.

Red cell morphology showed predominantly normocytic and normochromic cells across all groups, though they were only mildly present in aluminium chloride Group, compared to the moderate-to-high presence observed in control and treated groups. This reduction in normal morphology in the aluminium chloride group further supports the toxin's potential to impair red cell integrity. The mild presence of crenated red blood cells in aluminium chloride Group and Ferrous Sulphate Group suggests membrane instability, possibly due to oxidative lipid peroxidation, while the absence of polychromatic cells across all groups indicates that there was no marked release of immature erythrocytes into circulation, implying that compensatory erythropoiesis was not strongly activated under these experimental conditions (Buttarello, 2016).

Interestingly, eosinophils appeared only in the aluminium chloride group, and monocytes and basophils were sporadically observed in a few groups (Ferrous Sulphate Group and Aluminium Chloride + 400mg/kg *Icacina trichantha* leave extract group), but these changes were not consistent across the experiment. Such sporadic findings may indicate non-specific immune responses rather than a defined effect of treatment. Platelets were consistently highly present in all groups, suggesting preserved megakaryocytic activity and platelet production despite aluminium exposure or treatment, which contrasts with reports of toxin-induced thrombocytopenia in more severe models (Igbokwe *et al*., 2019).

Overall, the PBF findings reinforce the haematological data by showing that aluminium chloride induced subtle but detectable alterations in blood cell morphology, particularly atypical lymphocyte proliferation and reduced normochromic, normocytic cells. Treatment with *Icacina*

trichantha extract appeared to partially restore normal morphology, supporting its protective role in maintaining erythrocyte and lymphocyte integrity under oxidative stress conditions.

Icacina trichantha is rich in flavonoids, tannins, and phenolic antioxidants (Alawode *et al*., 2018). These compounds may neutralize ROS directly and stabilize cellular redox environments independently of NRF2 pathways. Other botanical antioxidants, such as rutin, have been shown to activate NRF2 at moderate stress and downregulate it as oxidative levels normalize (Moratilla-Rivera *et al.,* 2023). The current reduction in NRF2 may thus reflect improved redox equilibrium via NRF2-independent activation or stabilization.

In similar studies, agents like melatonin alleviated $AlCl_3$-induced organ damage by activating NRF2 nuclear translocation and elevating downstream antioxidants (SOD, CAT, HO-1, NQO1) in spleen tissue (Liu *et al*., 2019). This contrasts with our model, where NRF2 mRNA declines but antioxidant protection appears maintained, implying potential post-translational stabilization or parallel pathways at play.

This study's principal limitation is the exclusive reliance on NRF2 mRNA levels without corroborating protein expression or nuclear translocation studies. Functional NRF2 roles are often regulated post-translationally, thus mRNA data alone may underrepresent actual activity. Additionally, no downstream antioxidant markers (HO-1, NQO1, SOD) or iron metabolism indicators (ferritin, serum iron) were measured, restricting full mechanistic insight. Finally, absence of reticulocyte data and erythropoietic bone marrow evaluation limits interpretation of early red cell production dynamics.

CONCLUSION

In aluminium chloride-induced oxidative stress, treatment with *Icacina trichantha* aqueous leaf extract significantly reduced NRF2 gene expression while largely preserving haematological stability. Red blood cell count, haemoglobin, haematocrit, and most indices remained unaltered, with only minor reductions in MCV and MCH at the highest extract dose, suggesting subtle microcytic changes rather than overt anaemia. Peripheral blood film (PBF) analysis further supported this, showing maintenance of normocytic, normochromic red blood cells across most groups, with only mild alterations such as crenated cells and increased atypical lymphocytes in the untreated aluminium chloride group. The extract-treated groups displayed fewer morphological abnormalities, underscoring a protective role against oxidative and structural red cell damage. Taken together, these findings suggest that *Icacina trichantha* exerts its beneficial effect primarily through direct antioxidant mechanisms that neutralize reactive oxygen species, thereby limiting the need for NRF2-mediated transcriptional activation. This dual action of biochemical stability and morphological preservation highlights its therapeutic potential as a supportive agent in toxin-induced oxidative stress.

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APPENDIX I

Prof. Akinnibosun Henry Adewale (FLS, MRSB; London)
Faculty of Life Sciences,
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P. M. B. 1154 Ugbowo, 300283 Benin City,
Edo State, Nigeria.

University of Benin

Department of Plant Biology and Biotechnology

Herbarium Unit

Faculty of Life Sciences

University of Benin, Benin City, Edo State

Plant Name: *Icacina trichantha* Oliv.

Family: Icacinaceae

Common Name: False Yam, Raynal

Voucher Number: UBH-I185

Student Name: Audu Winnifred Omoye

Plant Identification and Voucher Number Issued by:

03/04/2025

Prof. **Akinnibosun** Henry Adewale (FLS, MRSB; London, LMBOSON, MAEIAN; MFBAN, MECOSON; Nigeria)

APPENDIX

II

EDO STATE MINISTRY OF HEALTH
HEALTH RESEARCH ETHICS COMMITTEE

PROTOCOL NUMBER	**HA/737/25/D/05210723 (PLEASE QUOTE IN ALL ENQUIRIES)**
APPROVAL NUMBER	**HA/737/25/D/06180723**
TITLE OF RESEARCH PROPOSAL	**EFFECT OF AQUEOUS LEAVES EXTRACT OF *ICACINA TRICHANTHA* ON SOME GENES IN ALUMINIUM CHLORIDE-INDUCED ANAEMIA IN ALBINO WISTAR RATS**
PRINCIPAL INVESTIGATOR (S)	**OBAZELU PROGRESS ARHENRHEN**
DATE CONSIDERED	**18TH JUNE, 2025**
DECISION OF THE COMMITTEE	**APPROVED**

THIS APPROVAL DATES 18/06/2025 TO 18/06/2026. IF THERE IS A DELAY IN STARTING THE RESEARCH, PLEASE INFORM THE HREC EDO SMoH SO THAT THE DATES OF APPROVAL CAN BE ADJUSTED ACCORDINGLY

REMARK: Please kindly note that the HREC Edo SMoH seal authenticates this approval

DR (MRS.) OMONYEMEN B. BELLO (MBBS, MPH, FPHCM) *(CHAIRMAN)* **SIGNATURE & DATE**..... 23/6/25

SUPERVISOR(S) ..... Dr. Mrs. P.A. Obazelu .....

.....

ATTESTATION BY INVESTIGATOR(S)

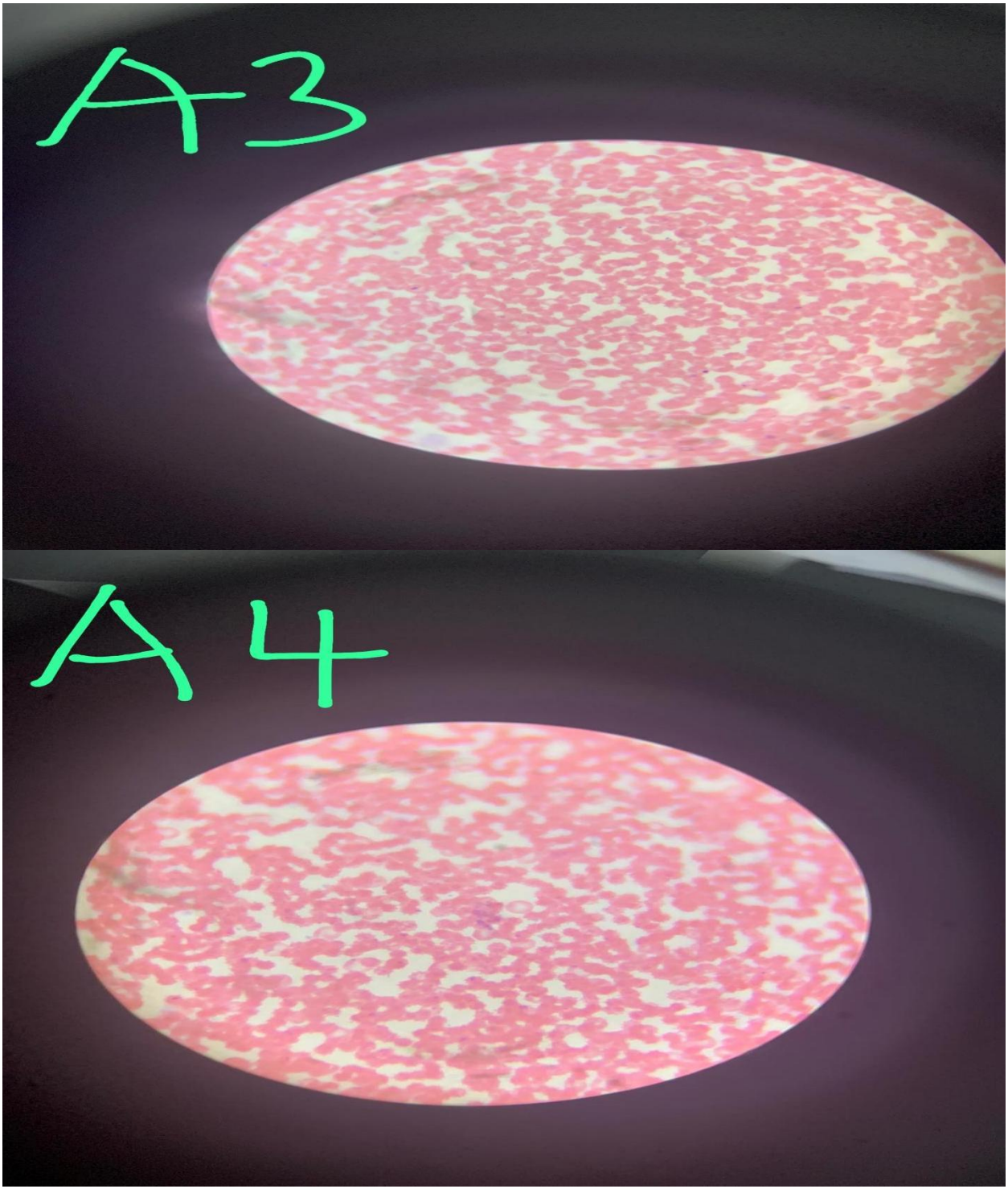
No participant accrual or activity related to this research may be conducted outside of the approval dates. All informed consent forms used in this study must carry the Edo SMoH HREC-assigned number and duration of your research. No changes are permitted in the research without prior approval of the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

Signature & Date..... 15/07/2025

edohrec@edostate.gov.ng

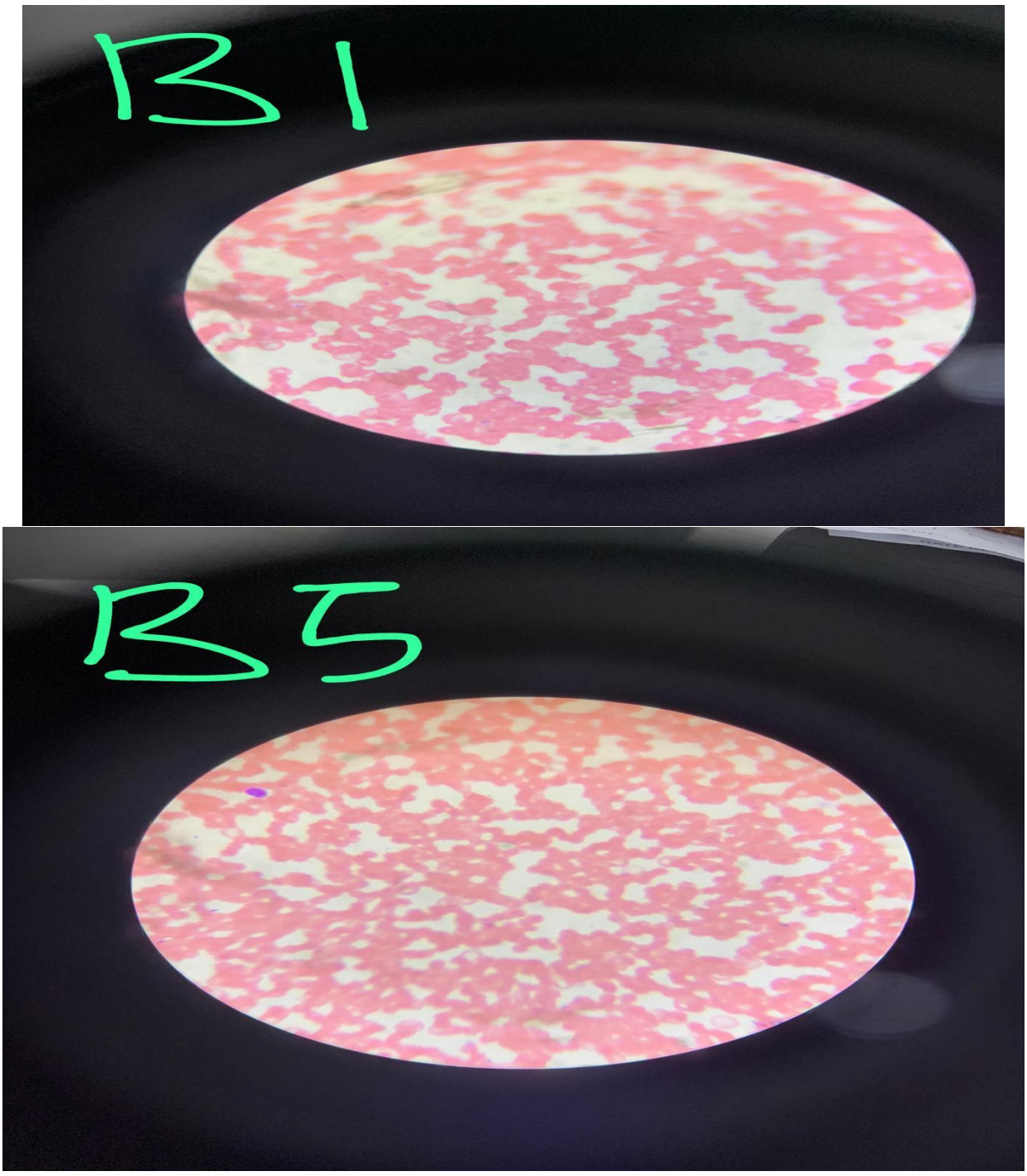
Room 16, Block D, 2nd floor, State secetariat building.

APPENDIX III



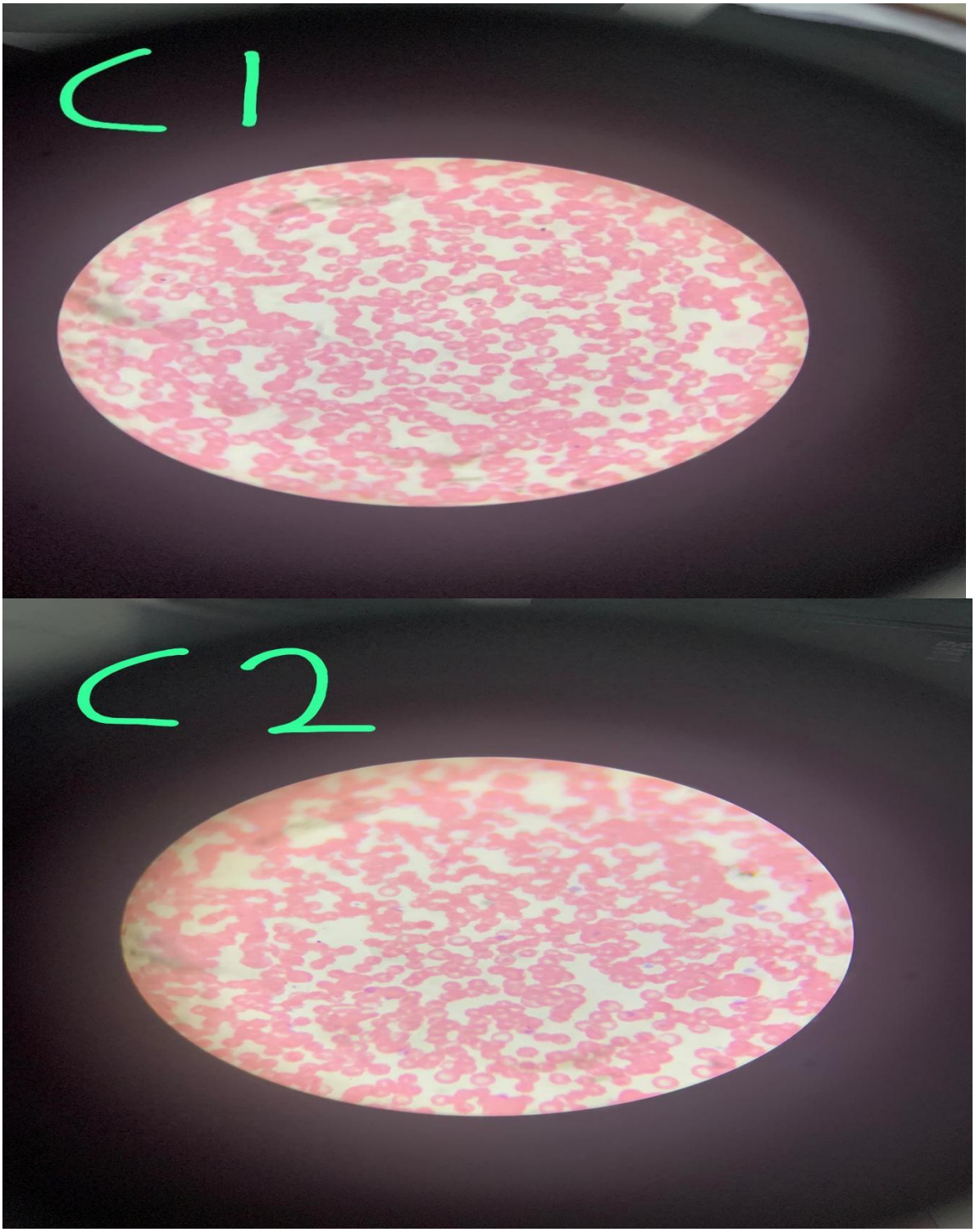
Slides Showing the Blood Cell Morphology of Group A Experimental Animals

APPENDIX IV



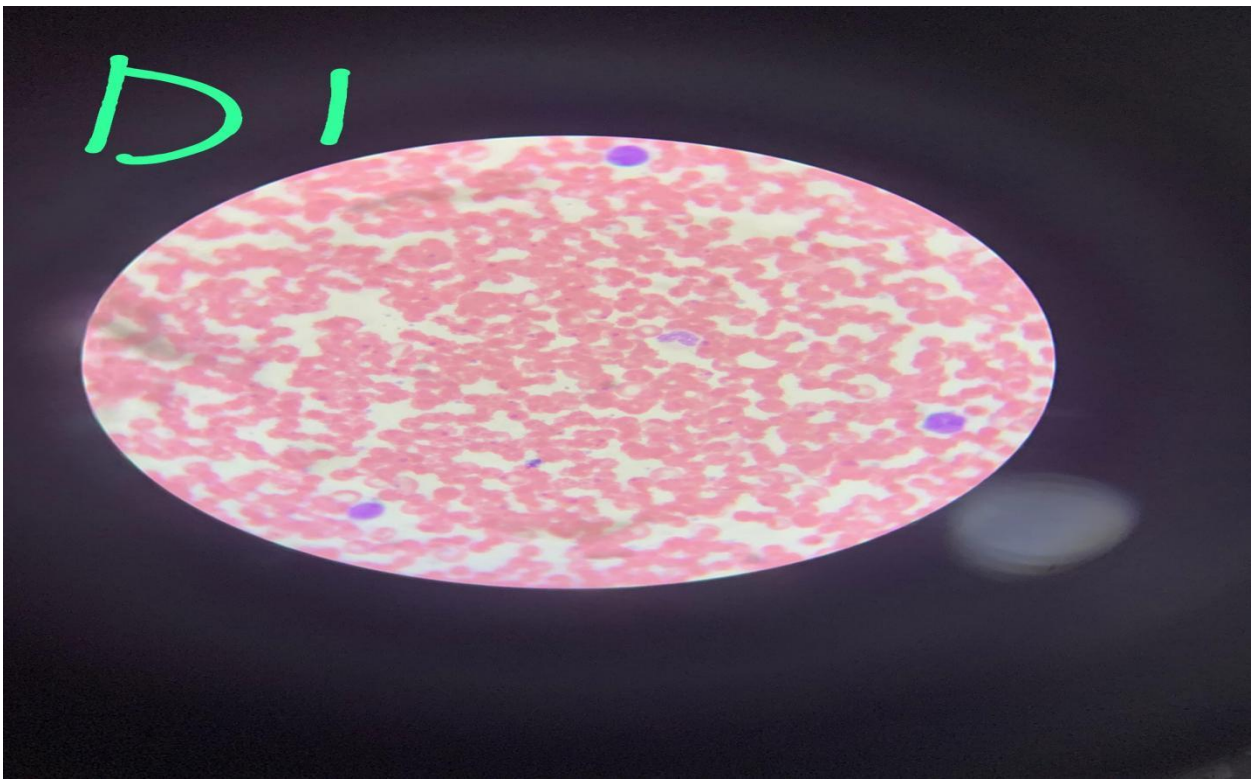
Slides Showing the Blood Cell Morphology of Group B Experimental Animals

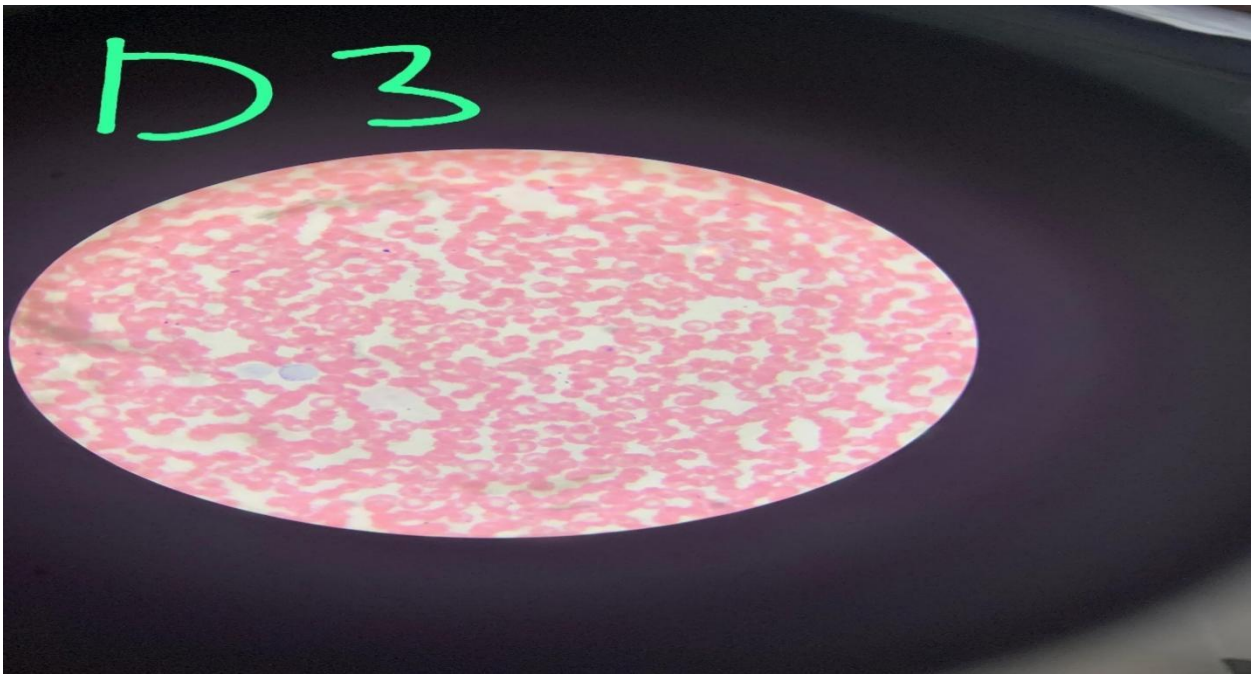
APPENDIX V



Slides Showing the Blood Cell Morphology of Group C Experimental Animals

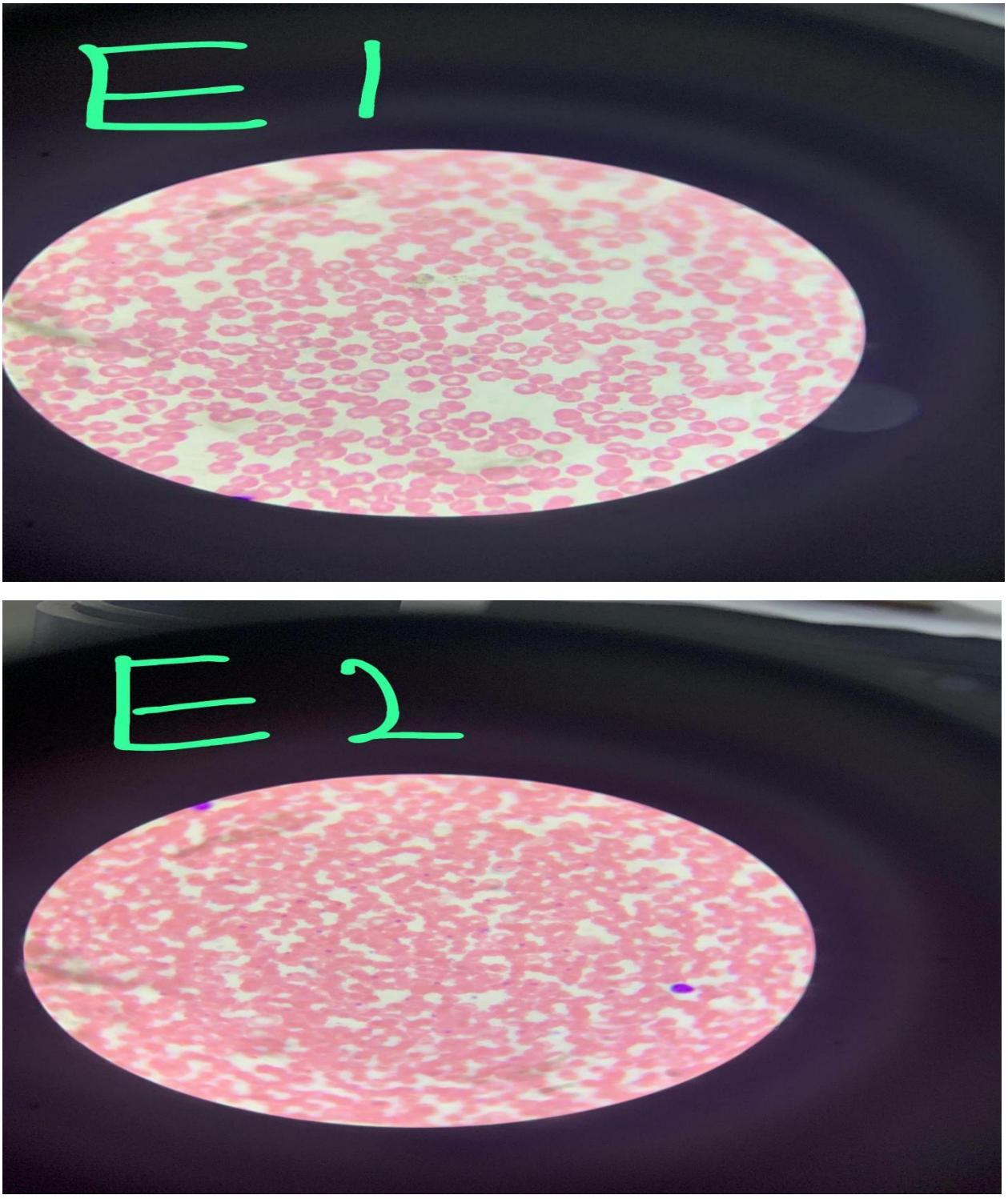
APPENDIX VI





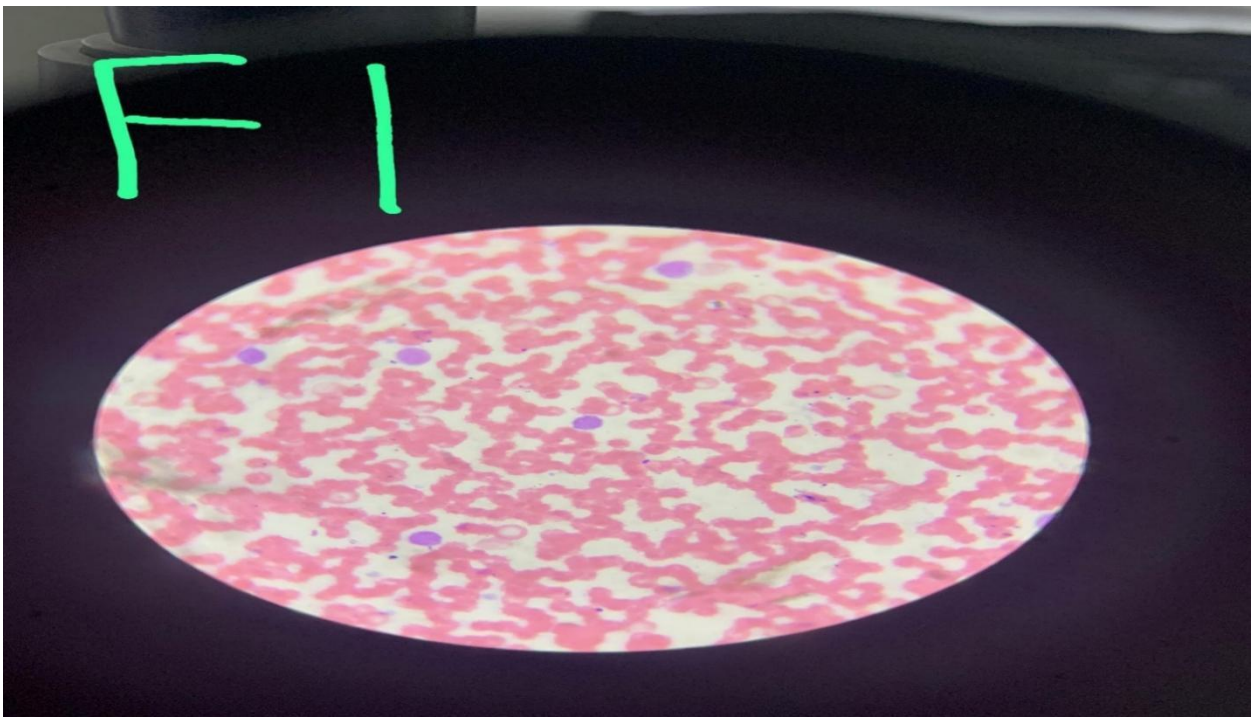
Slides Showing the Blood Cell Morphology of Group D Experimental Animals

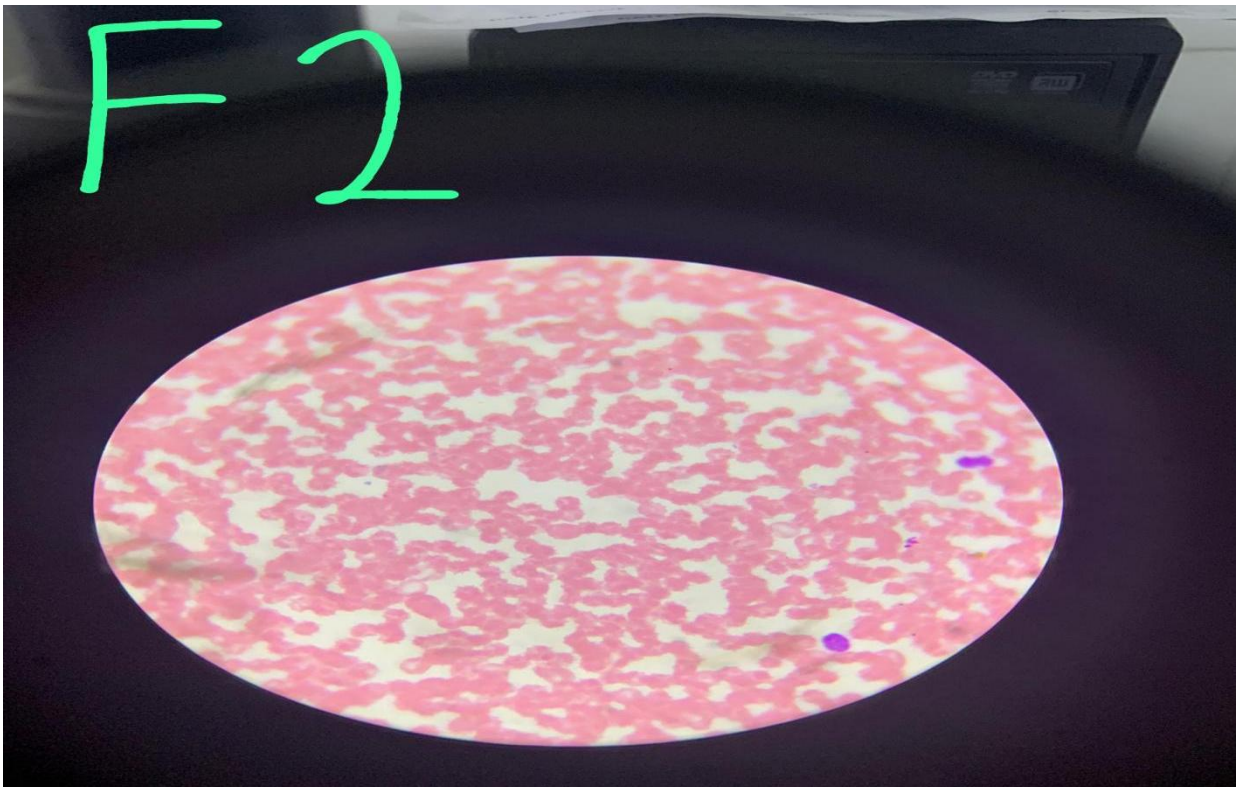
APPENDIX VII



Slides Showing the Blood Cell Morphology of Group E Experimental Animals

APPENDIX VIII





Slides Showing the Blood Cell Morphology of Group F Experimental Animals

APPENDIX IX

MATERIALS AND REAGENTS USED

MATERIALS USED

Forceps

Eppendorf Container

Gavage tube

Glass slides

Staining racks

Immersion oil

Microscope

Timer

Sorvall biofuge

Germany eppendor mastercycler

Germany Labnet Electrophoresis system

USA micro pipettes

Hisense Microwave

A & E

UV-visible

Spectrophotometer

Water Bath

REAGENTS USED

Trizol

Chloroform

Buffer solution

Distilled Water

Leishman Stain

Primers used were synthesized by Inqaba Biotec, South Africa.

Zymo DNA extraction kit.

Loading dye.

EZ-Vision.

TBE buffer.

Nuclease Free Water.

Agarose.

All purchased from Inqaba Biotec