

**ACUTE AND SUB ACUTE TOXICITY STUDY OF MIRACLE SEED ULTIMA® IN
MALE WISTAR RATS**

BY

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DEPARTMENT OF MEDICAL BIOCHEMISTRY

COLLEGE OF POSTGRADUATE STUDIES

UNIVERSITY OF BENIN

BENIN CITY

APRIL, 2026

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MALE ALBINO WISTAR RATS**

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**A THESIS WRITTEN IN THE DEPARTMENT OF MEDICAL BIOCHEMISTRY AND
SUBMITTED TO THE COLLEGE OF POSTGRADUATE STUDIES IN PARTIAL
FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF
MASTER OF SCIENCES IN MEDICAL BIOCHEMISTRY**

SUPERVISED BY

DR. JOHN. C. ANIONYE

APRIL, 2026

CERTIFICATION OF THESIS

We certify that this work was written and presented by **EHIMIYEN OMOSE SANDRA (MRS)** with matriculation number **PG/BMS0901273** in the department of Medical Biochemistry, School of Basic Medical Sciences, University of Benin, Benin City.

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DEDICATION

This thesis is dedicated to God Almighty, the source of all knowledge and my ever-present help. To my lovely husband (my Odogwu) Engr. Afeinsumu Ehimiye, thank you for being my greatest inspiration, and my awesome children Valerie, Olivia and Ebenezer (The great 3), thank you for standing by mummy always.

ACKNOWLEDGEMENT

My greatest recognition goes to God Almighty for wisdom, knowledge, good health, guidance and provision. My lovely husband Engr. Afeinsumu Ehimiye for your love, financial and moral support that can never be overlooked, and my awesome children Valerie, Olivia and Ebenezer you are all treasured by mummy always.

I wish to thank my lovely parents, Mr. and Mrs. Isreal Itua for their love, encouragement and support all these years. To my younger siblings and their family, Mr. and (Barr) Mrs. Otubor, Dr. and Mrs. Dom-Eichie and my niece Itohan Dom-Eichie, Mr. and (Barr) Mrs. Ehiorogie, and Oselenjodamen Itua, thank you all for your concern and support. To my in-laws Deacon and Pastor Mrs. Robinson Ehimiye, Wilson Ehimiye and Anderson Ehimiye thank you for your support and love.

Also, I wish to appreciate my P.G coordinator Dr. Mrs. Eluehike for her motherly role to me in the department, My friend since my undergraduate days Mrs. Ada Ukuwuonwo- Ediale thank you for always being there for me. A very big thank you to Dr Rawlings and Mr Daniel for their guidance and support through out the course of this research work. To my course mates Msc Mbc 2024/2025 thank you all for being so amazing.

Finally, I wish to acknowledge my project supervisor Dr. J.C. Anionye, for the topic, encouragement and the confidence bestowed upon me to carryout this research. God bless you all.

Table Of Contents

Title Page.....	ii
Certification Of Thesis	iii
Author's Statement	iv
Dedication.....	V
Acknowledgement	Vi
Table Of Contents	Vii
List Of Tables	X
List Of Figures	Xi
Abstract	Xii
Chapter One	1
Introduction	1
1.1 Background Of The Study	1
1.2 Statement Of The Problem.....	9
1.3 Justification Of The Study	11
1.4 Aim Of Study	12
1.5 Objectives Of Study	12
Chapter Two	14
Literature Review.....	14
2.1 Concept Of Toxicity Studies	14
2.1.1 Overview Of Toxicity Studies	14
2.1.2 Classification Of Toxicity: Acute, Subacute, Subchronic, And Chronic Exposures	15
2.1.2.1 Acute Toxicity-----	15
2.1.2.2 Sub Acute Toxicity-----	15
2.1.2.3 Sub Chronic Toxicity-----	16
2.1.2.4 Chronic Toxicity-----	16
2.1.3 Systemic Vs Organ-Specific Effects	17
2.1.3.1 Systemic Toxicity -----	17
2.1.3.2 - Organ Specific Toxicity -----	17
2.1.4 The Role Of Preclinical Toxicity Testing In Drug And Nutraceutical Development...	18

2.1.4.1 Pharmaceutical Development-----	18
2.1.4.2 Nutraceutical And Polyherbal Formulation-----	18
2.1.4.3 Estabilsing Safety Margins-----	19
2.1.5 Regulatory Frameworks And International Harmonization In Toxicity Evaluation	19
2.1.5.1 Global Regulatory Requirements And Oecd Guidelines-----	20
2.1.5.2 Sector Specific Variations-----	20
2.1.6 The Necessity Of Tiered Toxicity Testing Strategies In Safety Assessment	20
2.1.6.1- Step Wise Approach -----	21
2.1.6.2- Integration Of New Methodologies-----	21
2.1.6.3 Reduction And Refinement (3rs)-----	21
2.2 Acute Toxicity Studies.....	222
2.2.1 Purpose Of Acute Toxicity Studies.....	222
2.2.2 Lorke Method Principles	24
2.2.2.1 Phased Dosing Strategy-----	24
2.2.3 Observational Parameters And Ld ₅₀ Calculation	25
2.2.3.1 Ethical Consideration-----	25
2.2.4 Common Endpoints In Acute Toxicity Studies	26
2.3 Sub-Acute Toxicity Studies	30
2.3.1 Definition And Rationale.....	30
2.3.2 Study Design Principles.....	31
2.3.3 Multi-Parameter Assessments.....	32
2.3.3.1 General Observation And Growth Indicators-----	32
2.3.3.2 Hematology-----	33
2.3.3.3 Clinical Biochemisty -----	34
2.3.3.4 Liver Function Evulation -----	34
2.3.3.5 Kindney Function Evaluation -----	36
2.3.3.6-Lipid Profile Evaluation-----	37
2.3.3.7 Fasting Blood Glucose-----	39
2.3.3.8 Fistopathocosy-----	39
2.3.4 Noael Determination	40

2.4 Phyto-Chemistry And Pharmacology Of Miracle Seed Ultima®	41
2.4.1 Composition Overview.....	41
2.4.2 Content Of Miracle Seed Ultima.....	43
2.4.2 Detailed Phytochemical Profile -----	44
2.4.2.1 Zingiber Officinale (Ginger) Constituents-----	44
2.4.2.2 Allium Sativum (Garlic) Constituents-----	45
2.4.2.3 Vernonia Amygdalina And Moringa Oleifera Constituents-----	46
2.4.3 Pharmacological Mechanisms Of Action	46
2.4.3.1 Antioxidant Defense And Oxidative Stress Mitigation-----	46
2.4.3.2 Anti - Inflammatory And Immunodulatory Activity-----	47
2.4.3.3 Metabolic Regualtion And Anti Diabetic Effects-----	48
2.4.3.4 Potential Therapeutic Benefits	49
2.5 Safety Concerns In Herbal Extracts.....	51
2.5.1 Reported Toxicities In Similar Formulations: Alkaloids And Saponins.....	52
2.5.2 Examples Of Dose-Dependent Effects In Rodent Models	53
2.6 Previous Studies On Related Herbal Products	54
2.6.1 Toxicological Studies On Individual Components	54
2.6.1.1 Acute And Sub Acute Toxixity Assessments Of Nisella Sativa-----	54
2.6.1.2 Acute And Sub Acute Toxicity Assessments Of Moringa Deifera -----	55
2.6.1.3 Acute And Sub Acute Toxicity Assessments Of Ziagiber Officinale-----	56
2.6.1.4 Acute And Sub Acute Toxixity Assesments Of Allium Satium-----	57
2.6.1.5 Safety Profile Of Honey As A Vehicle-----	58
2.6.2 Key Findings From Individual Component Studies.....	58
2.6.2.1 General Safety Low Toxicity Profile Observed At Therapeutic Does -----	58
2.6.2.2 Dose Dependent Effects Mild Adverse Effects Reported Only Of High Dose posures-----	59
2.6.2.3. No Observed Adverse Effectr Level(Noael Data Where Available -----	59
2.6.2.4 Species Specific Variations In Toxicological Responses-----	60
2.6.3 Research Gaps And Study Justification.....	60

2.6.3.1 Limited Available Research On Multiherb Combinations Compared To Individual Ingredients Studies-----	60
2.6.3.2 Absence Of Toxicity Data Specific To Multi Component Blends Like Miracle Seed Ultima -----	61
2.6.3.3 Limited Investigation Of Oxidative Strees Markers In Existing Safety Studies----	61
2.6.3.4 Lack Of Compretnsive Hematological And Biochemical Profiling In Combination Products-----	62
2.6.3.5 Need For Standerdized Toxicological Assessment Of Synergistic Herbal Materices---	62
Chapter Three	63
Materials And Methods.....	63
3.1 Materials	63
3.1.1 Test Substance	63
3.1.2 Product Characterization.....	63
3.2 Methods.....	66
3.2.1 Qualitative Phytochemical Screening	66
3.2.1.1 Test For Tannis -----	66
-- 3.2.1.2 Test For Saponins-----	66
3.2.1.3. Test For Flavonoids-----	66
3.2.1.4 Test For Terpenoids-----	67
3.2.1.6 Test For Coumarins-----	67
3.2.1.7 Test For Anthraquinones-----	67
3.2.1.8 Test For Cardiac Glycoside-----	68
3.2.1.9 Test For Steroids-----	68
3.2.10 Test For Phenols-----	68
3.2.2 Quantitative Phytochemical Assay.....	68
3.2.2.1 Determination Of Alkaloids Content-----	68
3.2.2.2 Determination Of Tannins Content-----	69
3.2.2.3 Determination Of Flavonoids Content-----	69
3.2.2.4 Determination Of Cardiac Gycosides Content-----	70
3.2.2.6 Determination Of Terpenoid Content-----	70

3.2.2.7 Determination Of Total Coumarin-----	70
3.2.3 Proximate Analysis.....	71
3.2.3.1 Determination Of Moisture Content -----	71
3.2.3.2 Determination Of Ash Content -----	71
3.2.3.2 Determination Of Crude Fibre-----	72
3.2.3.4 Determination Of Carbohydrate Content -----	72
3.2.3.5 Determination Of Crude Protein -----	73
3.2.3.6 Determination Of Crude Fat-----	74
3.2.3.7 Determination Of Ethanol Concentration-----	75
3.2.4 Elemental Screening.....	76
3.2.5 In-Vitro Antioxidant Capacity	77
3.2.5.1 Dpph (2,2 Diphenyl-1-picrylhydrazyl Radical Scavenging Assay)-----	77
3.2.5.2 Ferric Reducing Antioxidant Power(FRAP) Assay -----	77
3.3 Toxicity Study Of Miracle Seed Ultima®.....	77
3.3.1 Study Design	77
3.3.2 Acute Toxicity Studies Phase One	78
3.3.2.1 Experimental Animals-----	78
3.3.2.2 Preparation Of Test Doses -----	79
3.3.2.3 Experimental Design-----	79
3.3.2.4 Dose Administration -----	80
3.3.2.5 Observations-----	80
3.3.2.6 Determination Of LD ₅₀ (Median Lethal Doses -----	81
3.3.3 Acute Toxicity Studies Phase Two	82
3.3.3.1 Dose Administration -----	82
3.3.3.2 Observation -----	82
3.3.4 Miracle Seed Ultima® Sub-Acute Toxicity Studies	82
3.3.4.1 Preparation Of Test Doses -----	83
3.3.4.2 Dose Preparation Calculation-----	83
3.3.4.3 Animal Acclimatization And Housing-----	83

3.3.4.4 Experimental Design And Animal Grouping-----	84
3.3.4.5 Administartion Of Test Substance -----	85
3.3.5. Daily Quantity Of Feed Consumed:.....	87
3.3.6 Weekly Body Weight:	87
3.3.7observations (Symptoms, Signs And Mortality):.....	88
3.3.8 Blood Sample Collection And Preparation:.....	88
3.3.9. Biochemical And Hematological Analyses	89
3.3.10 Relative Organ Weight:	89
3.4 Hematological Analyses	89
3.4.1 Hemoglobin Concentration.....	90
3.4.2 Red Blood Cell (Rbc) Count.....	91
3.4.3 Packed Cell Volume (Hematocrit)	93
3.4.4 Mean Corpuscular Volume (Mcv)	94
3.4.5 Mean Corpuscular Hemoglobin (Mch)	95
3.4.6 Mean Corpuscular Hemoglobin Concentration (Mchc)	95
3.4.7 Differential White Blood Cell Count	98
3.4.8 Platelet Count	101
3.5. Biochemical Analysis	102
3.5.1 Assessment Of Status Of Glucose Metabolism	102
3.5.2. Assessment Of The Lipid Profile	103
3.5.3. Assessment Of Kidney Function Status And Uric Acid	107
3.5.4. Assessment Of Liver Function Status	113
Chapter Four	122
Results	122
Chapter Five	153
Discussion, Conclusion And Recommendations.....	153
5.1 Discussion	153
5.2 Conclusion.....	159
5.3 Recommendations.....	160
References -----	161
Appendix	184

LIST OF TABLES

Table 4.1: Qualitative Phytochemical Screening of MIRACLE SEED ULTIMA Sample	124
Table 4.2: Quantitative Phytochemical Composition of MIRACLE SEED ULTIMA Sample..	125
Table 4.3: Relative Abundance of Phytochemicals in MIRACLE SEED ULTIMA	126
Table 4.4: Quantitative Elemental Composition of Miracle Seed Ultima®	127
Table 4.5: Antioxidant Activity of MIRACLE SEED ULTIMA Compared with Ascorbic Acid	130
Table 4.6: Proximate Composition of MIRACLE SEED ULTIMA (per 100 g, n = 3)	131
Table 4.7: External observation of Male Wistar Rats treated with Miracle Seed Ultima® in Acute Toxicity Study.....	134
Table 4.8: Internal (Gross Pathology) of Male <i>Wistar</i> Rats treated with Miracle Seed Ultima® in Acute Toxicity Study.	135
Table 4.9: External observation of Male <i>Wistar</i> Rats treated with Miracle Seed Ultima® in Phase two Acute Toxicity Study.....	136
Table 4.10: Internal (Gross Pathology) of Male <i>Wistar</i> Rats treated with Miracle Seed Ultima® in Phase two Acute Toxicity Study.....	137
Table 4.11: Effect of Miracle Seed Ultima® on Liver function parameters.....	144
Table 4.12: Effects of Miracle Seed Ultima® on Lipid profile parameters.....	145
Table 4.13: Effects of Miracle Seed Ultima® on Kidney function parameters	146
Table 4.14: Effects of Miracle Seed Ultima® on Fasting Blood Glucose parameter.....	157
Table 4.15: Effect of Miracle Seed Ultima on Hematology parameters.....	151
Table 4.16: Effects of Miracle Seed Ultima® on Initial, final body weight and various tissues of male <i>Wistar</i> rats.	152

LIST OF FIGURES

FIGURE 1: Two bottles of Miracle Seed Ultima

ABSTRACT

The widespread use of herbal supplements in Sub-Saharan Africa has created an urgent need for safety evaluation of commercially available polyherbal formulations. Miracle Seed Ultima® (MSU) is a popular liquid extract marketed for immune enhancement, fertility improvement, and metabolic regulation, yet it lacks comprehensive toxicological data to support its safety claims. This study investigated the acute and subacute toxicity profile of MSU in male albino *Wistar* rats to establish its safety. The acute toxicity assessment was conducted using the Lorke method, which involved administering MSU at doses ranging from 10 mg/kg to 5000 mg/kg. Animals were observed for 14 days for mortality, behavioral changes, and clinical signs of toxicity. The subacute toxicity study followed OECD guideline 407, where rats received daily oral doses of 100 mg/kg, 300 mg/kg, and 1000 mg/kg for 28 days. Body weight, food consumption, and clinical signs were monitored throughout the study period. Blood samples were collected for hematological analysis and biochemical assessment of liver function (ALT, AST, ALP, total protein, albumin, bilirubin), kidney function (urea, creatinine, electrolytes), lipid profile (total cholesterol, triglycerides, LDL, HDL, VLDL), and fasting blood glucose. Phytochemical analysis identified high concentrations of cardiac glycosides (310.59 ± 2.36 mg/g), steroids (174.22 ± 5.01 mg/g), flavonoids (151.82 ± 3.58 mg/g), coumarins (113.27 ± 4.81 mg/g), and alkaloids (93.81 ± 7.86 mg/g). Elemental analysis indicated high levels of calcium ($6,304.00 \pm 2.08$ mg/kg), potassium ($5,252.33 \pm 1.20$ mg/kg), and magnesium ($3,704.67 \pm 2.40$ mg/kg), with no detectable levels of lead or cadmium, suggesting low contamination risk. In vitro antioxidant tests showed moderate DPPH radical scavenging at $55.77 \pm 3.67\%$ and FRAP reducing activity at $51.95 \pm 1.31\%$, with notably strong nitric oxide scavenging at $88.15 \pm 2.21\%$, comparable to ascorbic acid ($92.38 \pm 0.41\%$). Acute toxicity tests at doses up to 5,000 mg/kg showed no mortality or clinical signs over 14 days, classifying MSU as practically non-toxic ($LD_{50} > 5,000$ mg/kg). However, kidney cysts were observed in some animals at higher doses. Subacute exposure (100, 300, 1,000 mg/kg/day for 28 days) revealed dose-dependent hepatocellular stress, with elevated serum ALT at 300 mg/kg (135.00 ± 8.70 U/L) and 1,000 mg/kg (200.56 ± 22.20 U/L). Conversely, kidney function improved, with decreased plasma urea at 1,000 mg/kg (68.62 ± 2.90 mg/dL) versus control (102.72 ± 5.70 mg/dL), suggesting nephroprotective effects. All hematological parameters and metabolic markers remained normal, indicating a No Observed Adverse Effect Level (NOAEL) of 100 mg/kg/day. This study provides the first comprehensive toxicological evaluation of Miracle Seed Ultima®, demonstrating that the formulation has acceptable safety at moderate doses but may cause mild liver stress at higher doses. The findings suggest that MSU can be safely used at doses up to 100 mg/kg, but long-term users should undergo periodic liver function monitoring, particularly those with pre-existing liver conditions. These results provide essential baseline data to inform regulatory decisions, guide clinical use, and protect consumer safety while supporting the continued use of this traditional herbal formulation.

CHAPTER ONE INTRODUCTION

1.1 Background of the Study

The utilization of natural products derived from plants, microbes, and minerals has constituted the foundation of therapeutic interventions throughout human history. In contemporary healthcare, particularly within developing nations, there is a substantial reliance on traditional medicine, with approximately 80% of the population in these regions depending on herbal remedies for their primary health care needs (Mugale *et al.*, 2024; Etaware *et al.*, 2025). This global surge in the consumption of herbal and seed-based supplements is driven by a prevalent perception that natural products are inherently safe and devoid of the adverse effects often associated with synthetic pharmaceuticals (Mugale *et al.*, 2024; Jitäreanu *et al.*, 2023). In West Africa, the demand for herbal medicine has witnessed a significant upward trajectory, where over 70% of the population relies on it despite historical efforts to prioritize conventional medicine (Naatu *et al.*, 2024). This reliance is often underpinned by cultural acceptance, accessibility, and the perceived efficacy of these treatments for various ailments, ranging from infectious diseases to chronic metabolic disorders (Owusu *et al.*, 2025; Mutombo *et al.*, 2023). However, the widespread commercialization of these products, often categorized as "wellness" supplements, requires a rigorous scientific evaluation of their safety profiles, as the lack of standardized toxicity data remains a critical gap in public health protection (Mugale *et al.*, 2024).

One such formulation gaining prominence in this landscape is Miracle Seed Ultima® (MSU), a polyherbal liquid extract positioned as a therapeutic agent for immune enhancement, fertility support, and metabolic regulation. The formulation is primarily composed of *Nigella sativa* (Black Seed), supported by *Allium sativum* (Garlic), *Moringa oleifera* (Moringa), and *Zingiber officinale*

(Ginger), and honey. The therapeutic claims associated with MSU are extensive, claiming to improve hematological parameters, protect renal integrity, and modulate the hypothalamic-pituitary-gonadal (H-P-G) axis to support reproductive function . The biological rationale for these effects lies in the rich phytochemical profile of its constituents. *Nigella sativa* contributes thymoquinone, a quinone known for upregulating endogenous antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), while *Allium sativum* provides organosulfur compounds like allicin, which exhibit antimicrobial and cardiovascular benefits. Furthermore, the inclusion of *Moringa oleifera* introduces phenolic acids and flavonoids, and *Zingiber officinale* contributes gingerols and shogaols, which act as gastric stimulants. While these bioactive constituents offer potential health benefits, their concentration and interaction within a herbal blend warrant careful toxicological scrutiny.

The biochemical complexity of herbal formulations like MSU poses significant challenges regarding safety and toxicity. Although secondary metabolites such as alkaloids, phenolics, terpenoids, and saponins are responsible for therapeutic efficacy, many of these compounds possess a narrow therapeutic index and can exert toxic effects at higher concentrations (Mugale *et al.*, 2024; Hossain *et al.*, 2025). For instance, alkaloids, while pharmacologically active in the central nervous system, are frequently implicated in cases of toxicity due to their interaction with neurotransmitter receptors and potential to induce physiological disturbances (Debnath *et al.*, 2018). Similarly, while phenolic compounds and flavonoids are celebrated for their antioxidant capacity and ability to scavenge free radicals, they can exhibit pro-oxidant behaviors under specific physiological conditions or at elevated doses, potentially leading to cellular damage rather than protection (Hossain *et al.*, 2025). The liver, being the primary site of xenobiotic metabolism, is particularly vulnerable to damage from these bioactive compounds. The metabolic activation of

phytochemicals can lead to the formation of reactive intermediates that induce oxidative stress, mitochondrial injury, and hepatocellular necrosis (Mugale *et al.*, 2024; Jităreanu *et al.*, 2023). Furthermore, the kidneys, tasked with the elimination of metabolic byproducts, are susceptible to nephrotoxicity manifested by tubular necrosis and alterations in filtration markers such as urea and creatinine (Mugale *et al.*, 2024; Owusu *et al.*, 2025). Therefore, the evaluation of hepatic and renal integrity is important when assessing the safety of any potent polyherbal formulation.

Despite the established use of the individual ingredients of MSU in ethnomedicine, the safety profile of the combined extract remains largely empirical rather than evidence-based. The concept of combinatorial chemistry in herbal medicine suggests that the interaction between multiple plant extracts can result in synergistic, additive, or antagonistic effects, altering the pharmacokinetic and pharmacodynamic properties of the final product (Mayoka *et al.*, 2025). Current literature indicates that a significant proportion of commercially available herbal products lack adequate regulatory toxicity studies, with data suggesting that approximately 50% of plant extracts may exhibit hepatotoxicity and 30% nephrotoxicity in preclinical models (Mugale *et al.*, 2024). In Ghana, a study of herbal products revealed that less than half met quality control standards, with issues ranging from adulteration to a lack of information on composition and adverse effects (Owusu *et al.*, 2025). This regulatory gap highlights the urgent need for systematic preclinical toxicity studies to bridge the divide between traditional usage and modern scientific validation (Etaware *et al.*, 2025). The rigorous determination of the No-Observed-Adverse-Effect Level (NOAEL) and the evaluation of acute and sub-chronic toxicity are essential steps in establishing the safety margins for human consumption (Mugale *et al.*, 2024).

The integration of advanced toxicological assessments into the evaluation of herbal medicines is increasingly becoming a standard requirement for global acceptance. Scientific validation involves a multi-stage process including phytochemical analysis, preclinical studies, and clinical trials to confirm pharmacological efficacy and safety (Etaware *et al.*, 2025). In the context of MSU, the presence of potent bioactives such as thymoquinone and allicin necessitates an investigation into their potential accumulation and systemic effects over time. Thymoquinone, for example, has been shown to modulate inflammatory pathways via the inhibition of NF- κ B, a key transcription factor in inflammatory signaling (Hirsa *et al.*, 2025). While this mechanism contributes to anti-inflammatory activity, the modulation of such critical signaling pathways must be evaluated for potential off-target effects and long-term safety (Hossain *et al.*, 2025). Additionally, the use of honey as a vehicle in MSU may influence the absorption and bioavailability of these lipophilic compounds, further complicating the toxicokinetic profile of the mixture. Understanding these interactions is crucial for defining the therapeutic window and preventing potential iatrogenic diseases resulting from herb-drug interactions or metabolic disturbances (Mugale *et al.*, 2024).

To address these safety concerns, *in vivo* toxicity studies utilizing rodent models serve as the gold standard for preclinical safety assessment. The use of *Wistar* albino rats in toxicological research is well-established due to their anatomical, physiological, and biochemical similarities to humans, which allows for a reasonable extrapolation of data (Aragie *et al.*, 2025; Jitäreanu *et al.*, 2023). Specifically, male *Wistar* rats are often selected to minimize physiological variations associated with hormonal cycles in females, thereby ensuring homogeneity in metabolic responses (Aragie *et al.*, 2025). Guidelines established by the Organization for Economic Cooperation and Development (OECD), such as OECD 423 for acute toxicity and OECD 407/408 for repeated dose toxicity, provide a standardized framework for these evaluations (Jitäreanu *et al.*, 2023; Aragie *et*

al., 2025). These protocols facilitate the assessment of lethal doses (LD50), identification of target organ toxicity, and monitoring of hematological and biochemical parameters (Aragie *et al.*, 2025). By subjecting MSU to these rigorous testing standards, it is possible to generate robust data regarding its safety, thereby validating its traditional use and supporting its integration into the formal healthcare sector (Mutombo *et al.*, 2023).

The biological rationale for this study is further reinforced by the need to understand the impact of MSU on specific organ systems involved in metabolism and excretion. The liver and kidneys are the primary organs responsible for the detoxification and elimination of xenobiotics, making them the most common targets for toxic injury by plant extracts (Mugale *et al.*, 2024). Biochemical analysis of serum enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) provides critical insight into hepatocellular integrity (Aragie *et al.*, 2025). Similarly, serum urea and creatinine levels serve as indicators of renal function. Histopathological examination of these tissues complements biochemical data by revealing structural alterations such as necrosis, inflammation, or cellular vacuolation that may not be immediately apparent through blood analysis alone (Aragie *et al.*, 2025; Mugale *et al.*, 2024). Furthermore, given the claims of MSU regarding hematopoiesis and fertility, the evaluation of hematological indices and reproductive organs is necessary to verify efficacy and rule out cytotoxic effects on rapidly dividing cells (Aragie *et al.*, 2025).

The prevalence of herbal medicine use in Sub-Saharan Africa, particularly for the management of chronic and infectious diseases, underscores the public health importance of verifying the safety of popular formulations like MSU. In the Bono region of Ghana, for example, 92.7% of respondents in a recent study reported using herbal medicine, citing effectiveness and safety as

primary reasons (Owusu *et al.*, 2025). However, this high prevalence of use is often accompanied by a lack of regulatory compliance, with many products failing to meet registration standards or lacking expiry dates and contraindication warnings (Owusu *et al.*, 2025). This disconnect between high consumer trust and low regulatory oversight creates a significant public health risk. By conducting a comprehensive toxicity evaluation of MSU, this study aims to provide the scientific evidence required to support regulatory compliance, enhance consumer safety, and foster the development of evidence-based herbal medicine policies (Owusu *et al.*, 2025; Mayoka *et al.*, 2025).

In addition to safety, understanding the phytochemical composition of MSU provides a basis for elucidating its mechanisms of action. The presence of alkaloids, for instance, suggests potential neuroactive or physiological effects that require monitoring for signs of central nervous system stimulation or depression (Debnath *et al.*, 2018). The diverse chemical profile of alkaloids, ranging from the pyridine group to the isoquinoline group, is associated with a wide spectrum of biological activities, including analgesic, anti-inflammatory, and antimicrobial effects (Debnath *et al.*, 2018). However, the potential for alkaloids to interact with neurotransmitter systems or metabolic enzymes like CYP450 necessitates a thorough evaluation of behavioral and metabolic changes in test animals (Debnath *et al.*, 2018; Mayoka *et al.*, 2025). Similarly, the high concentration of unsaturated fatty acids and antioxidants in seed oils, such as those from *Nigella sativa*, suggests a role in modulating lipid profiles and reducing oxidative stress, which is relevant to the metabolic support claims of MSU (Hossain *et al.*, 2025; Marappan *et al.*, 2025).

Furthermore, the global context of natural product research highlights the importance of transitioning from basic extraction and screening to more sophisticated evaluations of safety and

efficacy. While Africa possesses immense biodiversity and a rich tradition of ethnomedicine, the continent's contribution to global drug discovery has been limited by a lack of infrastructure and systematic research (Mayoka *et al.*, 2025). Successful models from countries like China and India demonstrate that the integration of traditional medicine into mainstream healthcare requires rigorous clinical and preclinical validation (Mayoka *et al.*, 2025; Etaware *et al.*, 2025). By applying modern toxicological methods to African herbal formulations, researchers can elevate these products from folk remedies to standardized therapeutics. This approach not only ensures patient safety but also adds value to the natural product industry, potentially opening avenues for economic growth and international trade (Marappan *et al.*, 2025; Mayoka *et al.*, 2025).

The evaluation of MSU also aligns with the growing recognition of dietary phytochemicals in disease prevention and management. Compounds such as quercetin, catechins, and various phenolic acids, which are likely present in the plant-based components of MSU, have been extensively studied for their role in preventing chronic diseases through epigenetic modifications and modulation of metabolic pathways (Hossain *et al.*, 2025). However, the therapeutic potential of these compounds is often limited by bioavailability issues and the risk of toxicity at high doses (Hossain *et al.*, 2025). Therefore, establishing the dose-response relationship and the safety margins of MSU is critical for determining its viability as a long-term health supplement. This study will contribute to the body of knowledge regarding the safe use of bioactive seed extracts, supporting the broader goal of harnessing dietary phytochemicals for health promotion while mitigating risks (Hossain *et al.*, 2025; Hirsu *et al.*, 2025).

The use of *in vivo* models allows for the assessment of systemic effects that *in vitro* assays cannot fully capture. While *in vitro* and *in silico* methods are valuable for screening and identifying

potential mechanisms, they lack the complexity of a living organism where absorption, distribution, metabolism, and excretion (ADMET) processes occur simultaneously (Tan *et al.*, 2025; Jităreanu *et al.*, 2023). For a complex mixture like MSU, where the interaction between ingredients may alter toxicity profiles, the whole-organism approach provided by the *Wistar* rat model is indispensable. Monitoring clinical signs, body weight changes, and food consumption in these animals provides a holistic view of the test substance's impact on general health and well-being (Aragie *et al.*, 2025). These parameters, combined with detailed hematological and biochemical analyses, facilitate the detection of subtle toxicological effects that might otherwise go unnoticed.

Ultimately, the justification for this study lies in the importance to validate the safety of Miracle Seed Ultima® amidst its growing popularity and the broader context of increasing herbal medicine consumption in West Africa. The formulation's reliance on potent botanical ingredients with known bioactive profiles presents both therapeutic promise and toxicological risks. By conducting a systematic preclinical toxicity evaluation using established rodent models and adhering to international guidelines, this research aims to generate the empirical data necessary to define the safety profile of MSU. This endeavor not only serves to protect public health by identifying potential hazards but also contributes to the scientific validation of African traditional medicine, fostering its integration into modern healthcare systems and supporting the development of safe, effective, and standardized natural health products (Etaware *et al.*, 2025; Mutombo *et al.*, 2023). Through this rigorous scientific inquiry, the study seeks to bridge the gap between traditional knowledge and evidence-based medicine, ensuring that the benefits of natural products can be harnessed responsibly and effectively.

1.2 Statement of the Problem

The growing worldwide use of herbal medicines and seed-based supplements has become a major trend in modern healthcare, particularly in Sub-Saharan Africa where cultural tradition and lower costs drive their popularity. However, there's a serious mismatch between how these products are advertised as "safe" natural remedies and what science actually knows about their potential dangers. Miracle Seed Ultima® (MSU) is a perfect example of this problem: it's a liquid herbal extract sold as a cure-all for boosting immunity, improving fertility, and regulating metabolism. While the product claims to combine the health benefits of black seed, garlic, moringa, and ginger in honey, there's a striking lack of solid scientific research proving it's actually safe for people to use (Mugale *et al.*, 2024). The widespread belief that natural products can't be harmful ignores a basic scientific fact: around 150,000 plant species contain toxic substances that can cause serious health problems (Mugale *et al.*, 2024). The uncontrolled use of such powerful mixtures without proper testing creates a real public health risk, making it essential to carefully study their safety and potential to damage specific organs.

The concern about MSU comes from its complex chemical makeup. The formula is packed with plant compounds; alkaloids, saponins, flavonoids, and phenolic acids which can be beneficial but also have narrow safety ranges and known toxic effects (Hossain *et al.*, 2025). Alkaloids are common in plants and affect the nervous system, but they often cause poisoning by interfering with brain chemicals, potentially triggering seizures and paralysis at high doses (Debnath *et al.*, 2018). Similarly, saponins found in seeds act like detergents that can break down cell walls, destroying red blood cells and making the intestinal lining too permeable (Mugale *et al.*, 2024). Even phenolic compounds and flavonoids, usually praised for fighting cell damage, can actually promote damage instead of preventing it under certain conditions or when taken in large amounts

(Hossain *et al.*, 2025). The way these active ingredients might work together or against each other in the herbal blend makes safety prediction even harder, as these interactions can unexpectedly change how each compound is absorbed, moves through the body, breaks down, and leaves the system potentially making the whole mixture more toxic (Mayoka *et al.*, 2025; Jitäreanu *et al.*, 2023).

Liver damage is a major worry since the liver processes most substances we consume. When plant chemicals are broken down in the liver, they can create harmful byproducts that kill liver cells, block bile ducts, and cause fat buildup (Mugale *et al.*, 2024). Earlier studies on similar seed oils found that taking them by mouth significantly raised liver damage markers (ALT and AST enzymes) and showed visible cell injury and blood vessel problems under the microscope (Aragie *et al.*, 2025). Because MSU contains powerful compounds like thymoquinone and allicin that affect how enzymes work, taking too much could overload the liver's ability to detoxify, leading to drug-induced liver damage (Mugale *et al.*, 2024). The kidneys are also at risk from toxic plant byproducts building up. Getting rid of toxic alkaloids and saponins can damage kidney tubes, cause inflammation, and throw off kidney function tests (Mugale *et al.*, 2024; Aragie *et al.*, 2025). The complete absence of information about how MSU affects kidney health is a critical gap, especially for people who already have kidney disease.

How MSU affects metabolism is also poorly understood. Although it's sold to support healthy metabolism, the compounds in it that affect blood sugar and fats need careful study. Black seed ingredients, for example, can change how insulin works and affect cholesterol levels; but taking the wrong amount or combining it with certain foods could cause blood sugar to drop dangerously low or create unhealthy fat levels in some people (Hossain *et al.*, 2025). The honey base adds extra concerns about calories and blood sugar spikes, which might actually be harmful for diabetic

patients despite the product's health claims. MSU also lacks basic safety standards. There's no established lethal dose to classify how poisonous it is, and no safe dose level has been determined for long-term use (Mugale *et al.*, 2024). Without these measurements, users and doctors have no way to know the difference between a helpful dose and a dangerous one. This problem is made worse by studies from Ghana showing that many herbal products don't meet quality standards and fail to list warnings or side effects (Owusu *et al.*, 2025).

Long-term toxicity from regular use is another major concern that single-dose tests can't catch. Studies looking at repeated use over weeks or months are needed to find delayed problems like blood disorders, reproductive damage, and slow organ breakdown (Aragie *et al.*, 2025). Since MSU is marketed to improve fertility, checking its effects on reproduction is especially important some plant extracts have been shown to damage testicles or disrupt hormones (Mugale *et al.*, 2024).

Finally, not understanding how MSU actually causes harm at the cellular level makes it impossible to create proper safety guidelines. Figuring out whether it damages cells through oxidative stress, inflammation, or direct poisoning is crucial for predicting how it might interact with medications or who shouldn't use it (Hirsa *et al.*, 2025; Jităreanu *et al.*, 2023). There's an urgent need to test both single-dose immediate effects and repeated-dose cumulative risks in properly designed animal studies to identify quick reactions and long-term dangers, providing the scientific proof needed for regulation and consumer safety (Owusu *et al.*, 2025; Naatu *et al.*, 2024).

1.3 Justification of the Study

This study is necessary because we urgently need to confirm whether Miracle Seed Ultima® (MSU) is actually safe for people to use. The research will provide solid scientific evidence to

challenge unsupported health claims and help regulatory agencies like NAFDAC in Nigeria and other international organizations understand the real risks of widely-used herbal supplements. By testing MSU in animal models using both single-dose and repeated-dose approaches, the study will determine how different amounts affect the body. This will help us identify safe dose levels and spot when harmful effects like mild liver stress or kidney changes start to appear, which can guide proper dosage advice for traditional medicine users. The research follows well-established testing protocols: the Lorke Method for acute oral toxicity to find the lethal dose and identify immediate warning signs, and OECD guideline 407 for 28-day repeated-dose testing to track effects that build up over time. Using these standard methods ensures the results are scientifically sound and relevant for regulating herbal products across different countries. Beyond just safety testing, this study will deepen our understanding of how complex herbal mixtures work in the body. It will investigate the mechanisms behind potential harm, such as how plant chemicals might trigger oxidative stress and which biological markers indicate organ damage. This addresses important gaps in current knowledge about how multiple plant ingredients interact with each other interactions that could affect the body's balance, metabolism, and long-term health, especially in vulnerable groups of people

1.4 Aim of Study

To investigate the acute and subacute toxicity of Miracle Seed Ultima® in male albino *Wistar* rats.

1.5 Objectives of Study

- i. To evaluate the qualitative and quantitative phytochemical constituents of the drug.
- ii. To carry out proximate and elemental analysis and evaluate the in vitro antioxidant capacity of *Miracle Seed Ultima*®.
- iii. To determine the median lethal dose (LD₅₀) of *Miracle Seed Ultima*® using the oral route.

- iv. To assess clinical signs of toxicity, body weight, and behavioral changes following repeated oral administration for 28 days.
- v. To evaluate biochemical and hematological parameters indicative of hepatic, renal, hematopoietic function and lipid profile.

CHAPTER TWO

LITERATURE REVIEW

2.1 Concept of Toxicity Studies

2.1.1 Overview of Toxicity Studies

Toxicology is the scientific discipline dedicated to understanding the adverse effects of chemical substances, including pharmaceuticals, environmental pollutants, and plant-based products, on living organisms (Jatoth *et al.*, 2024). At its core, toxicity assessment seeks to characterize the interaction between a toxicant a specific chemical agent and the biological system, particularly at the cellular and molecular levels, to determine the potential for harm (Jatoth *et al.*, 2024). A foundational principle in toxicology, historically attributed to Paracelsus, asserts that all substances possess the potential to be poisonous; the differentiation between a remedy and a poison is determined solely by the dosage (Jatoth *et al.*, 2024; Saleema *et al.*, 2017). Consequently, the primary objective of toxicity assessment is not merely binary classification of a substance as toxic or non-toxic, but the elucidation of the dose-response relationship to establish safe exposure limits (Jatoth *et al.*, 2024). Toxicity studies are mandatory for public health protection because exposure to chemical agents may induce severe adverse effects ranging from systemic dysregulation to failure of vital organs such as the liver, kidneys, heart, and lungs (Jatoth *et al.*, 2024). These assessments provide a primary safety profile, particularly for novel pharmaceutical agents and polyherbal formulations (Jatoth *et al.*, 2024). Modern toxicity testing adheres to rigorous guidelines, such as those established by the Organisation for Economic Co-operation and Development (OECD), ensuring standardized methodologies for hazard characterization (Gothe *et al.*, 2023). Contemporary assessments have evolved to incorporate the 3Rs principle

Replacement, Reduction, and Refinement aiming to minimize animal usage while maintaining scientific validity (EMA, 2009; OECD, 2008).

2.1.2 Classification of Toxicity: Acute, Subacute, Subchronic, and Chronic Exposures

Toxicity is categorized based on the duration and frequency of exposure to the test substance. This time-based classification is important for regulatory bodies to determine appropriate testing strategies for hazard classification and risk assessment.

2.1.2.1 Acute Toxicity

Acute toxicity refers to adverse effects occurring following the oral, dermal, or inhalation administration of a single dose of a substance, or multiple doses given within a 24-hour period (Gothe *et al.*, 2023; OECD, 2001). The historical objective of acute systemic toxicity testing was to identify the median lethal dose (LD50), the statistically derived single dose expected to cause death in 50% of test animals (OECD, 2001). However, current guidelines such as OECD 420 (Fixed Dose Procedure), 423 (Acute Toxic Class Method), and 425 (Up-and-Down Procedure) prioritize "evident toxicity" over lethality as an endpoint to refine the process (Gothe *et al.*, 2023; Strickland *et al.*, 2023). Data from these studies allow substances to be ranked according to the Globally Harmonised System (GHS) of Classification and Labelling of Chemicals (Gothe *et al.*, 2023). For example, substances with an LD50 greater than 2000 mg/kg are typically classified as Category 5 or unclassified, indicating a low acute toxicity hazard (Al-Afifi *et al.*, 2018; OECD, 2001).

2.1.2.2 Sub-acute Toxicity

Subacute toxicity studies involve repeated exposure to a substance, typically for a duration of 28 days (Al-Afifi *et al.*, 2018). These studies, governed by guidelines such as OECD 407, investigate

effects on a broad variety of potential targets, including the nervous, immune, and endocrine systems (OECD, 2008). Subacute assessments provide data on cumulative toxic effects not apparent in acute studies, such as alterations in body weight gain, food consumption, and organ weight variations (Bemidinezhad *et al.*, 2023). Crucially, they facilitate the determination of the No-Observed-Adverse-Effect Level (NOAEL), a key parameter for calculating starting doses for human clinical trials (Al-Afifi *et al.*, 2018; OECD, 2008).

2.1.2.3 Sub-chronic Toxicity

Sub-chronic toxicity testing generally extends for 90 days in rodents, representing approximately 10% of the animal's lifespan (EMA, 2009; Gothe *et al.*, 2023). This duration allows for the identification of latency in toxic effects and assesses the reversibility of toxicity following a recovery period. Subchronic studies are particularly relevant for identifying target organ toxicity and establishing dose regimens for chronic studies (Bemidinezhad *et al.*, 2023). Regulatory frameworks often utilize subchronic data to designate toxic substances if LD50 values fall below specific thresholds (Strickland *et al.*, 2023).

2.1.2.4 Chronic Toxicity

Chronic toxicity studies involve repeated exposure over a significant portion of the test animal's lifespan, typically ranging from 6 months to 2 years (EMA, 2009). These evaluations are essential for assessing carcinogenic potential and long-term cumulative effects that may not manifest during shorter exposure periods (Jitäreanu *et al.*, 2022). While acute and subacute studies focus on immediate hazards, chronic studies ensure safety during prolonged therapeutic use or lifetime exposure to environmental chemicals (Bemidinezhad *et al.*, 2023). For marketing authorization of

pharmaceuticals intended for long-term use (greater than 3 months), chronic toxicity studies of 6 months in rodents and 9 months in non-rodents are generally required (EMA, 2009).

2.1.3 Systemic vs Organ-Specific Effects

Toxicological effects are broadly distinguished as systemic, affecting the general physiological status of the organism, or organ-specific, targeting discrete tissues.

2.1.3.1 Systemic Toxicity

Systemic toxicity encompasses adverse effects occurring at sites distant from the route of entry or influencing the whole organism (Strickland *et al.*, 2023). Indicators of systemic toxicity in preclinical studies include mortality, significant alterations in body weight, and changes in food and water consumption (Al-Afifi *et al.*, 2018; Bemidinezhad *et al.*, 2023). For instance, cachexia or a failure to gain weight in treated animals compared to controls suggests a systemic disturbance in metabolism or nutrient absorption (Bemidinezhad *et al.*, 2023). In acute testing, systemic toxicity is often quantified via the LD50 or LC50, providing a metric for the relative potency of a substance (Strickland *et al.*, 2023).

2.1.3.2 Organ-Specific Toxicity

Organ-specific toxicity involves damage to specific tissues, most commonly the liver (hepatotoxicity), kidneys (nephrotoxicity), heart (cardiotoxicity), and nervous system (neurotoxicity) (Jatoth *et al.*, 2024). The liver and kidneys are primary targets due to their central roles in metabolism and excretion. Toxicity in these organs is evaluated through biochemical analysis of serum markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) for liver function, and urea and creatinine for kidney function alongside histopathological examination (Al-Afifi *et al.*, 2018; Bemidinezhad *et al.*,

2023). For example, significant elevations in serum ALP may indicate hepatobiliary alterations even in the absence of histopathological lesions (Bemidinezhad *et al.*, 2023).

Neurotoxicity and immunotoxicity are also critical organ-specific endpoints. OECD Guideline 407 emphasizes the assessment of neurotoxic potential through functional observational batteries, evaluating sensory reactivity, grip strength, and motor activity (OECD, 2008). Similarly, immunotoxicity is assessed by examining lymphoid organs such as the thymus, spleen, and lymph nodes, both by weight and histopathology (OECD, 2008). Identifying target organs during subacute and subchronic studies is vital for monitoring potential adverse effects in subsequent human clinical trials (EMA, 2009).

2.1.4 The Role of Preclinical Toxicity Testing in Drug and Nutraceutical Development

Preclinical toxicity testing acts as a mandatory gatekeeper in the development of pharmaceuticals and nutraceuticals, ensuring potential risks are identified prior to human exposure.

2.1.4.1 Pharmaceutical Development

For pharmaceuticals, the International Council for Harmonisation (ICH) Guideline M3(R2) dictates that nonclinical safety studies must support the conduct of human clinical trials and marketing authorization (EMA, 2009). These tests characterize toxic effects regarding target organs, dose dependence, and reversibility. This information is utilized to estimate an initial safe starting dose for human trials, often derived from the NOAEL in the most sensitive animal species (EMA, 2009). The duration of preclinical toxicity studies must correlate with the proposed duration of human clinical trials; for instance, a two-week repeated-dose toxicity study in two species is generally required to support a clinical trial of up to two weeks (EMA, 2009).

2.1.4.2 Nutraceuticals and Polyherbal Formulations

In the domain of traditional medicine and nutraceuticals, toxicity testing is critical to validate safety claims. While traditional medicines are often perceived as safe due to historical usage, scientific validation is required to rule out adverse effects such as hepatotoxicity and nephrotoxicity (Jatoth *et al.*, 2024). Recent incidents, such as the contamination of cough syrups with diethylene glycol, underscore the necessity of rigorous toxicity testing for plant-based products (Jatoth *et al.*, 2024). Studies on formulations like "Hydaljss08" and "Immunoboost-Tab" demonstrate that products intended for immune stimulation must undergo OECD-compliant acute toxicity testing to rule out lethality and establish LD50 values (Jatoth *et al.*, 2024). Furthermore, testing distinguishes between intrinsic toxicity (caused by bioactive compounds like pyrrolizidine alkaloids) and extrinsic toxicity (caused by contaminants like heavy metals or pesticides) (Jitäreanu *et al.*, 2022).

2.1.4.3 Establishing Safety Margins

Preclinical data enable the calculation of safety margins. For example, in developing hepatoprotective herbal formulations, researchers utilize acute and subacute data to calculate human equivalent doses based on body surface area normalization factors (Bemidinezhad *et al.*, 2023). If a substance shows no mortality or significant adverse effects at limit doses (e.g., 2000 mg/kg), it suggests a wide margin of safety for therapeutic use (Bemidinezhad *et al.*, 2023).

2.1.5 Regulatory Frameworks and International Harmonization in Toxicity Evaluation

Toxicity evaluation operates within a global regulatory framework designed to ensure the safety of chemicals, pharmaceuticals, and consumer products. Harmonization is facilitated by the International Cooperation on Alternative Test Methods (ICATM), involving regulatory bodies from Brazil, Canada, China, the European Union, Japan, South Korea, Taiwan, and the United States (Strickland *et al.*, 2023).

2.1.5.1 Global Regulatory Requirements and OECD Guidelines

Different sectors impose varying requirements for acute systemic toxicity data. Pharmaceutical regulations rely heavily on ICH guidelines, particularly M3(R2) (Strickland *et al.*, 2023; EMA, 2009). Conversely, industrial chemical and pesticide sectors often utilize the GHS for hazard classification. The cornerstone of international harmonization is the OECD Guidelines for the Testing of Chemicals. Guidelines such as OECD 420, 423, and 425 are internationally accepted specifications for safety testing (Gothe *et al.*, 2023). Data generated using these methods in one member country are accepted in others under the Mutual Acceptance of Data agreement, reducing duplicative testing (Strickland *et al.*, 2023). For instance, the Acute Toxic Class Method (OECD 423) is a stepwise procedure using minimal animals to classify substances into toxicity categories defined by fixed LD50 cut-off values (OECD, 2001; Gothe *et al.*, 2023).

2.1.5.2 Sector-Specific Variations

Despite harmonization, sectoral differences persist. For medical devices, ISO 10993 standards are recognized globally, though specific applications differ; the US FDA requires acute systemic toxicity information for surface devices contacting breached skin, while other jurisdictions may focus on implants (Strickland *et al.*, 2023). In the cosmetics sector, the EU, India, and Taiwan have banned animal testing, necessitating non-animal alternatives or weight-of-evidence approaches, whereas China still requires animal data for certain categories of "special" cosmetics (Strickland *et al.*, 2023; Jitäreanu *et al.*, 2022).

2.1.6 The Necessity of Tiered Toxicity Testing Strategies in Safety Assessment

Tiered testing strategies are essential to optimize resource allocation, minimize animal usage, and provide a logical progression of safety assessment.

2.1.6.1 Stepwise Approach

Assessment typically begins with acute toxicity testing to establish immediate hazards and lethality ranges (LD50). If a substance proves to be of low toxicity (e.g., LD50 > 2000 mg/kg), extensive testing at lower doses may be unnecessary (OECD, 2001; Gothe *et al.*, 2023). Following acute evaluation, subacute (28-day) studies identify target organs and establish the NOAEL (OECD, 2008). If findings raise concerns, or if the substance is intended for long-term use, subchronic (90-day) and chronic studies are triggered (Al-Afifi *et al.*, 2018; EMA, 2009). This structure prevents unnecessary long-term studies on highly toxic substances rejected early in development.

2.1.6.2 Integration of New Methodologies

Modern tiered strategies integrate "Omics" technologies; transcriptomics, proteomics, and metabonomics to elucidate toxicity mechanisms at the molecular level. Toxic-transcriptomics can reveal changes in gene expression related to DNA damage before overt pathological signs appear (Jitäreanu *et al.*, 2022). These molecular insights serve as early biomarkers, refining decision-making for subsequent in vivo testing.

2.1.6.3 Reduction and Refinement (3Rs)

Tiered strategies align with the 3Rs principle. Regulatory bodies in Canada, the EU, and the US encourage "waiving" or "bridging" studies where data from similar compounds (read-across) suggest low risk (Strickland *et al.*, 2023). Guidelines like OECD 423 and 425 are explicitly designed as animal reduction methods, using small numbers of animals and stopping rules to limit the total number required for hazard classification (Gothé *et al.*, 2023; OECD, 2001). The ultimate goal is to integrate in silico, in vitro, and in vivo methods to provide comprehensive safety assessment while adhering to ethical standards (Jitäreanu *et al.*, 2022; Strickland *et al.*, 2023).

2.2 Acute Toxicity Studies

Acute toxicity studies represent the essential initial phase in the toxicological assessment of novel chemical entities, pharmaceutical agents, or polyherbal formulations, providing fundamental data on inherent hazards and safety limits following acute, short-term exposure (Ainyanbhor *and* Onoagbe, 2024; Amarasiri *et al.*, 2025; Erhirhie *et al.*, 2018). Acute toxicity is defined as adverse effects that manifest immediately or shortly after a single administration or multiple administrations within a 24-hour period (Enegide *and* Arome, 2013; Zwickl *et al.*, 2024).

2.2.1 Purpose of Acute Toxicity Studies

The objectives of acute toxicity evaluations are centered on quantifying the immediate hazard posed by a substance and informing the design of subsequent, more extended safety testing protocols (Agatemor *and* Nwodo, 2021).

2.2.1.1 Establishing the Median Lethal Dose (LD₅₀)

The primary traditional focus of acute toxicity testing is the establishment of the median lethal dose (*LD*₅₀) (Agatemor *and* Nwodo, 2021; Enegide *and* Arome, 2013; Irinmwiniwa *et al.*, 2023). The *LD*₅₀ is the statistically derived dose of a substance administered via a specified route that is expected to result in the mortality of 50% of the tested animal population (Erhirhie *et al.*, 2018; Zwickl *et al.*, 2024). This quantitative endpoint serves as a measure of the substance's acute poisoning potential, where toxicity is inversely proportional to the *LD*₅₀ value (Arya *and* Bist, 2022; Irinmwiniwa *et al.*, 2023).

While the classical LD50 test, which historically utilized up to 100 animals across five dose groups, was suspended by the Organization for Economic Co-operation and Development (OECD) in 2002 (Erhirhie et al., 2018; Zwickl et al., 2024; Arya and Bist, 2022), the estimation of this parameter remains crucial for initial hazard classification. Modern alternatives, such as the Fixed Dose Procedure (OECD 420), Acute Toxic Class method (OECD 423), and the Up-and-Down Procedure (OECD 425), focus on establishing toxicity categories and minimizing animal usage while retaining the core LD50 concept (Arya and Bist, 2022; Erhirhie et al., 2018; Zwickl et al., 2024).

2.2.1.2 Identifying Immediate Toxic Signs and Target Organs

Acute studies aim to observe and record dose-dependent non-lethal and lethal adverse effects, noting the time of onset, duration, severity, and degree of recovery in surviving subjects (Enegide and Arome, 2013; Erhirhie et al., 2018). These observations are vital for hazard identification and communication (Ainyanbhor and Onoagbe, 2024). Furthermore, post-mortem examinations (necropsy and histopathology) are conducted to identify specific target organs that exhibit pathological changes or morbidity following exposure, such as the liver and kidney, which are often susceptible due to their roles in metabolism and excretion (Ainyanbhor and Onoagbe, 2024; Saleh et al., 2021;).

2.2.1.3 Dose Selection for Subsequent Studies

The data derived from acute toxicity testing, including the estimated LD₅₀ and the doses producing observable toxic signs, are instrumental in establishing appropriate dose levels for subsequent prolonged studies, such as the 28-day repeated dose oral toxicity study (sub-chronic toxicity)

(Agatemor *and* Nwodo, 2021; Amarasiri *et al.*, 2025;). This ensures that effective but non-toxic doses are tested in longer-term evaluations, thereby estimating the safety margin of the pharmacological agent (Enegide *and* Arome, 2013).

2.2.2 Lorke Method Principles

The Lorke method, introduced by D. Lorke in 1983, represents a practical and cost-effective approach to acute toxicity testing that explicitly addresses the need for reduction in animal numbers compared to classical methodologies (Agatemor *and* Nwodo, 2021; Enegide *and* Arome, 2013; Lorke, 1983).

2.2.2.1 Phased Dosing Strategy

Lorke's methodology typically employs a maximum of 13 animals to obtain adequate information on acute toxicity and the LD₅₀ (Lorke, 1983; Agatemor *and* Nwodo, 2021). The process is executed in two critical stages:

- i. **Phase 1 (Range-Finding):** This preliminary phase utilizes a small cohort of animals (e.g., nine Swiss mice, divided into three groups of three) (Agatemor *and* Nwodo, 2021; Mba *et al.*, 2020). These groups are administered widely separated doses of the test substance, typically increasing by a factor of ten, such as 10 mg/kg, 100 mg/kg, and 1000 mg/kg body weight (Agatemor *and* Nwodo, 2021; Mba *et al.*, 2020). The goal is to determine the range of doses that causes no toxicity, some toxicity, or total mortality (Erhirhie *et al.*, 2018).
- ii. **Phase 2 (Narrowing the Lethal Range):** Based on the mortality and toxicity observations from Phase 1, the doses for Phase 2 are strategically selected to narrow the lethal dose range (Mba *et al.*, 2020). Typically, four specific doses are tested on four new groups of

animals (often one animal per dose in the original Lorke method, although the application for *Yagari* and *N. imperialis* used three groups of three, or three groups of one, administered higher doses like 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg) (Agatemor *and* Nwodo, 2021; Mba *et al.*, 2020; Lorke, 1983).

2.2.3 Observational Parameters and LD₅₀ Calculation

Animals are observed intensively for clinical signs of toxicity and mortality (Mba *et al.*, 2020; Agatemor *and* Nwodo, 2021). The observation period is typically 24 hours post-administration in each phase, although monitoring often extends to 48 hours or longer to detect delayed effects (Mba *et al.*, 2020; Ainyanbhor *and* Onoagbe, 2024). The final LD₅₀ is calculated using the established formula:

$$LD_{50} = \sqrt{D_0 \times D_{100}}$$

where D₀(M₀) is the highest dose resulting in 0% mortality, and D₁₀₀ (M₁) is the lowest dose resulting in 100% mortality (Agatemor *and* Nwodo, 2021; Enegide *and* Arome, 2013). If no mortality is observed even at the highest tested dose (e.g., 5000 mg/kg body weight), the substance is classified as practically non-toxic, and the LD₅₀ is stated as greater than the limit dose (Agatemor *and* Nwodo, 2021; Mba *et al.*, 2020; Ainyanbhor *and* Onoagbe, 2024).

2.2.3.1 Ethical Considerations

The core strength of the Lorke method, along with other OECD-approved alternatives (OECD 420, 423, 425), lies in its adherence to the 3Rs principles; Reduction, Refinement, and (eventual) Replacement; by significantly reducing the number of animals required (Lorke, 1983; Erhirhie *et*

al., 2018; Arya and Bist, 2022). The shift from mandatory LD₅₀ determination to observing explicit signs of toxicity (evident toxicity) is a major refinement aimed at minimizing animal distress and maximizing the utility of the data obtained (Erhirhie *et al.*, 2018; Zwickl *et al.*, 2024).

2.2.4 Common Endpoints in Acute Toxicity Studies

The evaluation of acute toxicity encompasses several crucial endpoints that define the substance's toxicological profile and determine its safety classification.

2.2.4.1 Mortality

Mortality is the definitive endpoint for calculating the LD₅₀ (Erhirhie *et al.*, 2018). For substances suspected to have low toxicity, a limit test at a high dose (e.g., 2000 mg/kg or 5000 mg/kg) is performed (; Ainyanbhor and Onoagbe, 2024; Erhirhie *et al.*, 2018). For instance, the methanol extracts of *Napoleonae imperialis* and the aqueous/methanol root extracts of *Tetracera alnifolia* showed no mortality up to 5000 mg/kg body weight, classifying them as safe with low risk of acute intoxication (Mba *et al.*, 2020; Ainyanbhor and Onoagbe, 2024). Similarly, the polyherbal formulation *Yagari* was found not noxious up to 5000 mg/kg (Agatemor and Nwodo, 2021). The observation period usually extends up to 14 days following the single dose to capture delayed lethality (Ainyanbhor and Onoagbe, 2024; Zwickl *et al.*, 2024).

2.2.4.2 Behavioral Changes

Systematic monitoring of behavioral and physical signs is critical for identifying non-lethal toxic effects (Ainyanbhor and Onoagbe, 2024; Arya and Bist, 2022). These observations help deduce

potential mechanisms or systems targeted by the substance (Erhirhie *et al.*, 2018). Observed parameters include:

- **Neurological Indicators:** Nervousness, dullness, tremors, convulsions, hyperesthesia, incoordination, muscle spasms, and the loss of righting reflex (Ainyanbhor *and* Onoagbe, 2024; Agatemor *and* Nwodo, 2021).
- **Autonomic and General Signs:** Changes in breathing, salivation, lacrimation, diarrhea, changes in appetite or water intake, sluggishness, lethargy, and weight changes (Ainyanbhor *and* Onoagbe, 2024; Arya *and* Bist, 2022). For the *Tetracera alnifolia* study, the absence of signs such as tremor, convulsion, or reduced water intake confirmed the extract's low acute toxicity (Ainyanbhor *and* Onoagbe, 2024).

Gross Pathology and Histopathology

At the conclusion of the observation period, animals are subjected to necropsy. Organs are examined grossly for visible lesions and measured to determine the relative organ/body weight ratio (Ainyanbhor *and* Onoagbe, 2024; Amarasiri *et al.*, 2025). Significant changes in organ weight can serve as a primary indicator of toxicity (Ainyanbhor *and* Onoagbe, 2024).

For microscopic evaluation, excised tissues (commonly liver, kidney, and sometimes heart, spleen, and small intestine) are fixed (e.g., in 10% neutral buffered formalin), processed, and stained with Haematoxylin and Eosin (HandE) (Agatemor *and* Nwodo, 2021; Ainyanbhor *and* Onoagbe, 2024; Amarasiri *et al.*, 2025). Histopathology assesses the anatomical architecture for cellular damage, such as necrosis, cellular vacuolization, congestion, hemorrhages, glomerular congestion, and inflammatory cell infiltrations (Ainyanbhor *and* Onoagbe, 2024; Amarasiri *et al.*, 2025).

Generally, safe polyherbal formulations show normal tissue architecture in vital organs (Agatemor and Nwodo, 2021; Amarasiri *et al.*, 2025). However, certain observations, such as kidney congestion (*Annona squamosa*) or mild portal congestion (*Tetracera alnifolia* aqueous extract) may be noted even when overall LD₅₀ suggests low toxicity (Saleh *et al.*, 2021; Ainyanbhor and Onoagbe, 2024). The finding of normal cellular structures (e.g., normal hepatocytes, sinusoids, and glomeruli) confirms the absence of acute toxic effects on the cellular level (Amarasiri *et al.*, 2025).

Preliminary Biochemical Indicators

To assess functional impairment, biochemical parameters indicative of key organ function are typically analyzed, even in acute studies.

- i. **Liver Function:** Serum enzyme activities, specifically Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and Alkaline phosphatase (ALP), are assayed. Elevated levels suggest hepatocellular injury or cholestasis (Ainyanbhor and Onoagbe, 2024; Agatemor and Nwodo, 2021; Saleh *et al.*, 2021). Plasma total protein and albumin concentrations are also assessed, as a decrease may indicate compromised liver synthetic function (Ainyanbhor and Onoagbe, 2024). For *Tetracera alnifolia* root extracts, while low doses were safe, doses up to 5000 mg/kg significantly increased AST, ALT, and ALP activities, correlating with decreased plasma protein and albumin, suggesting potential slight hepatotoxicity at high acute exposure (Ainyanbhor and Onoagbe, 2024).
- ii. **Renal Function:** Plasma concentrations of blood urea nitrogen (BUN) and creatinine are primary indicators of renal filtration capacity (Ainyanbhor and Onoagbe, 2024). Electrolyte balance (sodium, potassium, chloride, bicarbonate) may also be monitored

(Ainyanbhor *and* Onoagbe, 2024). Insignificant differences in these markers compared to controls indicate normal renal function (Ainyanbhor *and* Onoagbe, 2024; Amarasiri *et al.*, 2025).

- iii. **Haematological and Oxidative Stress Markers:** A comprehensive panel of haematological parameters, including Packed Cell Volume (PCV), Haemoglobin (Hb) concentration, and White Blood Cell (WBC) counts, is typically evaluated to monitor effects on the blood components and immune system (Agatemor *and* Nwodo, 2021; Ainyanbhor *and* Onoagbe, 2024). Furthermore, preliminary assessment of oxidative stress biomarkers such as Catalase (CAT), Superoxide Dismutase (SOD), and Malondialdehyde (MDA) concentration may be included. For instance, some extracts show increased CAT activity following acute exposure, suggesting potential immediate antioxidant benefits (Ainyanbhor *and* Onoagbe, 2024).

2.3 Sub-acute Toxicity Studies

2.3.1 Definition and Rationale

The assessment of subacute toxicity, typically conducted over a period of 28 days, represents a critical phase in the non-clinical safety evaluation of chemical substances. Unlike acute toxicity studies, which evaluate the adverse effects occurring within a short timeframe following a single exposure, subacute studies are designed to investigate the health hazards likely to arise from repeated exposure over a limited period (OECD, 2025). The primary rationale for this approach is to distinguish between immediate toxicological responses and cumulative effects that may only manifest after continuous dosing. While acute toxicity testing establishes the lethal dose range and immediate symptomatic responses, it often fails to identify effects on specific target organs that require time to develop, such as endocrine disruption or cumulative tissue damage (OECD, 2025; Hanif *et al.*, 2025).

The 28-day repeated dose study is standardized internationally under the Organisation for Economic Co-operation and Development (OECD) Test Guideline 407 (OECD, 2025). This guideline provides a framework for characterising the toxicological profile of a substance, identifying target organs, and establishing a dose-response relationship. Data derived from these studies are essential for bridging acute findings to chronic safety profiles. By assessing parameters such as hematology, clinical biochemistry, and histopathology after four weeks of exposure, researchers can predict potential hazards in longer-term subchronic (90-day) or chronic studies and determine appropriate dose levels for those extensive evaluations (OECD, 2025). Furthermore, recent updates to these guidelines have incorporated parameters to screen for neurotoxicity, immunotoxicity, and endocrine-mediated effects, thereby broadening the scope of hazard identification (OECD, 2025).

2.3.2 Study Design Principles

The design of a subacute toxicity study requires careful consideration of animal models, dosage strategies, and administration routes to ensure scientific validity and animal welfare. The preferred species for these studies is the rat, although other rodent species may be used with justification (OECD, 2025). Young, healthy adult animals are selected, typically dosing begins shortly after weaning (before nine weeks of age), with stringent controls on weight variation to ensure homogeneity (OECD, 2025). Groups usually consist of at least ten animals (five males and five females) per dose level to allow for statistical analysis of sex-dependent toxicological responses (OECD, 2025).

Dose selection is a strategic process often informed by the results of acute toxicity tests or range-finding studies. The highest dose is generally selected to induce observable toxic effects without causing mortality or severe suffering, while the lowest dose aims to elicit no observable adverse effects, thereby establishing a safety margin (OECD, 2025). Intermediate doses are spaced at two- to four-fold intervals to elucidate the dose-response curve (OECD, 2025). For instance, in the safety evaluation of sodium dehydroacetate, doses were set at 50, 100, and 200 mg/kg body weight based on preliminary acute data (Du *et al.*, 2023).

The route of administration must reflect the intended pathway of human exposure. Oral administration is most common, achieved via gavage, diet, or drinking water (OECD, 2025). Gavage ensures precise dosing but is technically demanding and can induce stress-related physiological changes, such as elevated corticosterone levels, or lead to "gavage-related reflux," where the substance is inadvertently aspirated into the respiratory tract (Taylor *et al.*, 2025). To mitigate confounding factors associated with gavage, such as esophageal irritation or aspiration pneumonia, strict adherence to volume limits (typically 1 mL/100g body weight) and technical

competence is required (OECD, 2025; Taylor *et al.*, 2025). The observation period extends through the 28-day dosing phase, often supplemented by a 14-day recovery period for satellite groups (high dose and control) to assess the reversibility, persistence, or delayed onset of toxic effects (OECD, 2025; Yamashita *et al.*, 2024).

2.3.3 Multi-Parameter Assessments

The OECD 407 framework requires detailed multi-parameter assessments across clinical observation, clinical pathology, and necropsy (OECD, 2025).

2.3.3.1 General Observations and Growth Indicators

Test subjects undergo daily monitoring to detect signs of illness, death, and observable clinical changes including alterations to their skin, coat, eyes, and mucous membranes (Du *et al.*, 2023; OECD, 2008; OECD, 2025). Observable symptoms like raised fur, excess salivation, or loose stools require documentation (Kharchoufa *et al.*, 2020; OECD, 2025). More thorough clinical evaluations occur weekly at minimum, focusing on abnormalities in movement patterns, body positioning, autonomic nervous system responses, convulsive movements, and unusual or repetitive behaviors (OECD, 2008; OECD, 2025). Testing of functional capabilities including responses to sensory stimuli, hand strength, and locomotive function typically takes place during week four of the exposure period (OECD, 2008; OECD, 2025).

Weight is tracked weekly or more frequently (Du *et al.*, 2023; OECD, 2008; OECD, 2025). Weekly measurements also include feed intake and, when relevant, fluid intake (OECD, 2008; OECD, 2025). Alterations in weight and feed consumption serve as important warning signs, with notable decreases potentially indicating toxic effects (Du *et al.*, 2023; Shende and Kakadiya, 2022). As an example, female rats receiving 100mg/kg and 200 mg/kg doses of DHA-S demonstrated substantial reductions in both weight and food intake (Du *et al.*, 2023).

2.3.3.2 Hematology

Hematological parameters provide insight into potential effects on the hematopoietic and immune systems (Baig *et al.*, 2022; Won *et al.*, 2021; Yang *et al.*, 2023). Measured endpoints include Red Blood Cell (RBC) count, Hemoglobin (HGB) concentration, Hematocrit (HCT) value, Platelet (PLT) count, and total and differential White Blood Cell (WBC) counts (Du *et al.*, 2023; Won *et al.*, 2021; Yang *et al.*, 2023).

- i. **Red Blood Cell Indicators (RBC, HGB, HCT):** The measurement of red blood cell count, hemoglobin levels, and hematocrit values reflects the blood's capacity to transport oxygen. Reduced values in these markers signal anemia, which can stem from blood loss, red cell breakdown, or impaired bone marrow function (Du *et al.*, 2023; Kharchoufa *et al.*, 2020). For instance, notable declines in hemoglobin and hematocrit levels in DHA-S-treated rats pointed to anemia, potentially caused by inadequate nutrition or direct damage to bone marrow (Du *et al.*, 2023). In contrast, elevated red cell counts may arise from fluid depletion or insufficient oxygen availability (Kharchoufa *et al.*, 2020).
- ii. **White Blood Cell Indicators (WBC and Differential):** Total white blood cell counts and differential analysis (including neutrophils, lymphocytes, monocytes, eosinophils, and basophils) evaluate immune system toxicity and inflammatory responses (Shende and Kakadiya, 2022). Reduced white blood cells or lymphocytes suggest compromised immunity or bone marrow damage, as documented in rats receiving elevated DHA-S doses (Du *et al.*, 2023). Elevated white blood cell counts generally reflect inflammation, infection, or physiological stress (Bak *et al.*, 2024; Yang *et al.*, 2023).
- iii. **Platelet and Clotting Function:** Platelet counts evaluate the cellular aspects of blood clotting. Low platelet levels heighten bleeding susceptibility and may indicate bone

marrow toxicity or platelet destruction in circulation (Kharchoufa *et al.*, 2020; Du *et al.*, 2023). Clotting tests, including Prothrombin Time and Activated Partial Thromboplastin Time, measure the clotting mechanism's functionality. Extended clotting times suggest insufficient clotting factors (produced by the liver) or vitamin K inadequacy, frequently indicating liver impairment or disruption of clotting processes (Du *et al.*, 2023; Bak *et al.*, 2024). Markedly extended PT and APTT values were essential findings in establishing DHA-S toxicity (Du *et al.*, 2023).

Changes in these measurements provide vital information about the animals' physiological condition and disease status (Shende and Kakadiya, 2022; Fan *et al.*, 2022). For specific substances like Haloxylon scoparium extract, substantial platelet count reductions occurred at intermediate dosage levels (Kharchoufa *et al.*, 2020).

2.3.3.3 Clinical Biochemistry

Clinical biochemistry assesses organ integrity and metabolic disturbances (OECD, 2025). Mandatory assessments cover liver function, renal function, and essential metabolic parameters (Won *et al.*, 2021; Yang *et al.*, 2023).

2.3.3.4 Liver Function Evaluation

The liver serves as the main organ for processing and eliminating foreign substances, making it particularly vulnerable to chemical damage (Ribeiro *et al.*, 2020; Yang *et al.*, 2023). Assessment of liver health includes measuring enzymes released when cells are damaged and tracking substances that the liver produces or eliminates.

- i. **Transaminase Enzymes (ALT and AST):** Alanine aminotransferase and aspartate aminotransferase typically exist within cells. Elevated blood levels of these enzymes signal compromised liver cell membrane integrity or cell death (Ribeiro *et al.*, 2020; Shende and

Kakadiya, 2022). ALT is located predominantly in liver cell cytoplasm and serves as a highly specific indicator of liver damage (Ribeiro *et al.*, 2020; Al-Awthan *et al.*, 2018). In contrast, AST occurs across multiple tissues including cardiac muscle, skeletal muscle, and renal tissue; therefore, elevated AST alone is less definitive for liver injury (Ribeiro *et al.*, 2020). Marked increases in both enzymes appeared in mice exposed to *Chrysobalanus icaco* extract, indicating liver toxicity (Ribeiro *et al.*, 2020).

- ii. **Alkaline Phosphatase (ALP):** This enzyme relates to bile duct function. Raised blood ALP concentrations generally suggest liver or bile duct damage, blocked bile flow, or compromised bile elimination (Kharchoufa *et al.*, 2020; Du *et al.*, 2023). For instance, rats receiving high-dose *Haloxylon scoparium* extract showed substantial ALP increases, reflecting bile system impairment (Kharchoufa *et al.*, 2020).
- iii. **Total Bilirubin (TB):** Bilirubin forms from heme degradation. The liver processes bilirubin for removal from the body. Raised total bilirubin levels suggest either excessive red blood cell breakdown or compromised liver uptake, processing, or elimination capabilities (Ribeiro *et al.*, 2020; Yang *et al.*, 2023). Significant TB elevations occurred in male rats given curcumol, indicating possible liver excretion problems (Yang *et al.*, 2023).
- iv. **Albumin (ALB) and Total Protein (TP):** The liver is the sole source of albumin production. Decreased blood albumin levels may reflect diminished liver production capacity, long-term liver disease, or protein loss through the kidneys (Ribeiro *et al.*, 2020; Al-Awthan *et al.*, 2018). Total protein changes typically follow albumin patterns but may also indicate hydration levels or inflammatory protein production (Yang *et al.*, 2023).

2.3.3.5 Kidney Function Evaluation

Kidney toxicity is assessed by measuring nitrogen-containing waste materials that the kidneys typically filter and eliminate.

- i. **Urea / Blood Urea Nitrogen (BUN):** Urea represents the main end product of protein breakdown. Raised blood urea levels indicate decreased kidney filtration capacity, damage to kidney tissue, or factors preceding kidney function such as fluid depletion (Ribeiro *et al.*, 2020; Al-Awthan *et al.*, 2018). For example, substantial urea increases were detected in female rats exposed to sodium dehydroacetate (DHA-S), pointing to kidney damage (Du *et al.*, 2023).
- ii. **Creatinine (CRE/CREA):** Creatinine results from the breakdown of creatine phosphate in muscle tissue. The kidney filtering units freely remove it without reabsorption. Consequently, blood creatinine serves as a more precise and dependable measure of kidney function and filtration capacity compared to urea (Ribeiro *et al.*, 2020; Du *et al.*, 2023). Raised creatinine concentrations are clear indicators of kidney impairment or failure (Al-Awthan *et al.*, 2018; Yang *et al.*, 2023).
- iii. **Sodium (Na⁺), Potassium (K⁺), and Chloride (Cl⁻):** The kidneys control the elimination and retention of these electrolytes. Significant alterations in blood concentrations may signal damage to kidney tubules or hormonal disruptions (such as aldosterone imbalances) (Yang *et al.*, 2023; Bak *et al.*, 2024). In studies examining curcumol toxicity, dose-related elevations in Na⁺ and Cl⁻ were documented, indicating disruptions in electrolyte balance potentially associated with kidney effects (Yang *et al.*, 2023). Calcium (Ca) and inorganic phosphorus (P) are additionally tracked to evaluate mineral processing and kidney regulation (Shang *et al.*, 2022; Bak *et al.*, 2024).

2.3.3.6 Lipid Profile Evaluation

Lipid profile testing in subacute toxicity research is essential for assessing the metabolic condition of test animals and identifying possible disturbances in fat metabolism triggered by foreign compounds. These measurements offer descriptive insights into lipid metabolism abnormalities, which frequently appear before observable tissue damage in organs like the liver and heart (Liyanagamage *et al.*, 2020).

- i. **Total Cholesterol (TC):** Total cholesterol reflects the combined cholesterol circulating in blood, including cholesterol attached to different lipoproteins. In toxicity research, significant total cholesterol changes signal metabolic disturbance. Raised total cholesterol may indicate problems with liver lipid handling, bile duct blockage, or development of metabolic syndrome (Liyanagamage *et al.*, 2020; Yamashita *et al.*, 2024). On the other hand, reduced cholesterol concentrations might reflect serious liver impairment (decreased production), poor absorption, or inadequate nutrition (Yang *et al.*, 2023). For instance, research evaluating Curcumol documented a notable total cholesterol reduction at particular doses, demonstrating the substance's impact on lipid balance (Yang *et al.*, 2023).
- ii. **Triglycerides (TG):** Triglycerides are compounds formed from glycerol and three fatty acids, representing the principal components of body fat and the major energy storage form. Blood triglyceride concentrations serve as a responsive indicator of liver metabolic impairment (Kharchoufa *et al.*, 2020). Foreign substances that interfere with fatty acid breakdown or release can cause triglyceride buildup in the liver, termed hepatic steatosis. For example, marked triglyceride elevations in rats exposed to Haloxylon scoparium extract were associated with potential liver metabolic dysfunction (Kharchoufa *et al.*, 2020). Increased circulating triglycerides frequently correspond with tissue examination

findings of cytoplasmic vacuoles in liver cells, representing accumulated lipid droplets (Ribeiro *et al.*, 2020). Alternatively, substantial triglyceride decreases may indicate the lipid-lowering capacity of a test compound, which is therapeutically beneficial in metabolic disease models but requires safety evaluation in healthy animals (Liyanagamage *et al.*, 2020).

- iii. **High-Density Lipoprotein Cholesterol (HDL-C):** Commonly called "good cholesterol," HDL facilitates reverse cholesterol transport, transferring lipid molecules from body tissues to the liver for elimination. Decreased HDL-C may compromise this protective function, while increased levels are typically considered a beneficial, safe physiological response (Liyanagamage *et al.*, 2020).
- iv. **Low-Density Lipoprotein Cholesterol (LDL-C):** LDL delivers cholesterol to body tissues. Raised LDL-C indicates possible cardiovascular toxicity and metabolic dysfunction. In numerous rodent studies, LDL-C is computed using the Friedewald formula ($\text{LDL-C} = \text{TC} - \text{HDL-C} - \text{TG}/5$) instead of direct analysis (Liyanagamage *et al.*, 2020).
- v. **Very Low-Density Lipoprotein Cholesterol (VLDL-C):** VLDL carries internally produced triglycerides. Like LDL, VLDL concentrations are typically calculated. Changes in VLDL concentrations closely reflect triglyceride variations and are important for evaluating liver lipid export function (Liyanagamage *et al.*, 2020). In research with chitin nanofibers, decreases in VLDL and chylomicrons were documented, indicating specific effects on lipid absorption and transport mechanisms (Yamashita *et al.*, 2024).

2.3.3.7 Fasting Blood Glucose

Fasting blood glucose is a required measurement in OECD Test Guideline 407 (OECD, 2008). Changes in glucose concentrations can reflect pancreatic toxicity, hormonal disruption, or substantial metabolic stress (OECD, 2025). For instance, notable glucose reductions in blood were found in female rats given high doses of sodium dehydroacetate, linked to poor nutrition and loss of appetite (Du *et al.*, 2023). In contrast, during safety testing of antidiabetic herbal preparations, glucose concentrations are tracked to confirm the substance does not cause dangerously low blood sugar in healthy test subjects (Liyanagamage *et al.*, 2020).

Endocrine Markers (Optional)

TG 407 allows for the optional measurement of thyroid hormones (T3, T4, and TSH) if effects on the pituitary-thyroid axis are indicated (OECD, 2025). Withametin administration, for instance, caused high T3 and T4 levels and low TSH, indicative of hyperthyroidism (Baig *et al.*, 2022).

Gross Necropsy and Organ Weights

All animals are subjected to a detailed gross necropsy. Key organs including the liver, kidneys, adrenals, testes, epididymides, prostate, thymus, spleen, brain, and heart are weighed wet to avoid drying artifacts (OECD, 2025). Organ weights are analyzed both as absolute values and relative to body weight (organ-to-body weight ratio). Changes in relative organ weight are often sensitive indicators of toxicity, such as hypertrophy (swelling), hyperplasia (cell proliferation), or atrophy (degeneration) (OECD, 2025). For instance, an increase in relative liver weight often accompanies hepatic enzyme induction or fatty liver changes (Won *et al.*, 2021).

2.3.3.8 Histopathology

Tissues are fixed (commonly in 10% formalin) and processed for microscopic examination. Full histopathology is initially performed on the control and high-dose groups. If treatment-related

changes are identified, examinations are extended to lower dose groups (OECD, 2025). This evaluation correlates functional changes (biochemistry/hematology) with structural damage. Common findings include inflammatory cell infiltration, necrosis, fibrosis, or cellular degeneration. In the assessment of withametin, histopathology revealed renal tubular degeneration and hepatic sinusoidal congestion, which correlated with elevated serum creatinine and liver enzymes (Baig *et al.*, 2022). Similarly, pancreatic acinar cell atrophy observed in bromochlorophene-treated rats provided the structural basis for the observed metabolic disturbances (Won *et al.*, 2021). The examination of reproductive organs (testes, ovaries) is crucial for detecting potential endocrine-disrupting effects, such as the degeneration of seminiferous tubules or changes in follicular development (Hanif *et al.*, 2025; OECD, 2025).

2.3.4 NOAEL Determination

The ultimate quantitative outcome of a subacute toxicity study is the determination of the No-Observed-Adverse-Effect Level (NOAEL). The NOAEL is defined as the highest dose level at which no statistically significant or biologically relevant adverse treatment-related findings are observed compared to controls (OECD, 2025).

Determining the NOAEL requires distinguishing between "adverse effects" and non-adverse adaptive responses. An adverse effect is a functional or structural change that causes impairment or increases susceptibility to other insults. For example, in the study of bromochlorophene, the NOAEL was established at 250 mg/kg body weight because higher doses (500 and 1000 mg/kg) caused significant increases in cholesterol and triglycerides accompanied by histopathological lesions in the pancreas (Won *et al.*, 2021). Conversely, in the evaluation of curcuminol, despite statistically significant changes in certain hematological and biochemical parameters (such as electrolyte fluctuations), the absence of histopathological damage led researchers to conclude these

changes were not toxicologically relevant at the tested doses, although a definitive NOAEL was not explicitly capped by toxicity in that specific context (Yang *et al.*, 2023).

The NOAEL derived from 28-day studies is fundamental for human risk assessment. It serves as the point of departure for calculating reference doses (RfD) or acceptable daily intakes (ADI) by applying uncertainty factors (typically a factor of 100) to account for inter-species differences and intra-species variability (OECD, 2025). Furthermore, the NOAEL aids in selecting dose levels for subsequent subchronic (90-day) or chronic toxicity studies, ensuring that long-term evaluations are conducted at scientifically appropriate exposure levels (Baig *et al.*, 2022).

2.4 Phyto-chemistry and Pharmacology of Miracle Seed Ultima®

2.4.1 Composition Overview

Miracle Seed Ultima® is a complex blend of multiple plant-based ingredients formulated to work together through their combined biochemical effects. This supplement relies on the collective pharmacological properties of various plant compounds in a natural base. Rather than targeting a single pathway like conventional drugs, it uses diverse plant chemicals to influence multiple bodily systems simultaneously, including antioxidant defense, metabolism, and immune function. (Shukla *et al.*, 2015).



Figure 1.1: Bottles of Miracle Seed Ultima

2.4.2 Content of Miracle Seed Ultima

- i. ***Nigella sativa* (Black Seed):** Often distinguished as the "Miracle Seed," *N. sativa* serves as the foundational bioactive component. Pharmacologically, it is renowned for its therapeutic efficacy in managing dyslipidemia and diabetes mellitus through the modulation of carbohydrate digestion and absorption. Its biochemical profile is dominated by quinones, specifically thymoquinone, which drives its potent organo-protective properties (El-Saadony *et al.*, 2024).
- ii. ***Vernonia amygdalina* (Bitter Leaf):** Included for its metabolic regulatory properties, *V. amygdalina* fractions have been documented to exhibit significant chemopreventive effects. Its inclusion is critical for modulating enzymatic antioxidant systems, specifically glutathione reductase and glutathione-S-transferase, thereby reinforcing the formulation's capacity to mitigate oxidative stress (Onyibe *et al.*, 2021; Edo *et al.*, 2024).
- iii. ***Allium sativum* (Garlic):** A member of the Amaryllidaceae family, garlic is integrated for its rich organosulfur profile. It is established in ethnopharmacology for treating hypertension, hypercholesterolemia, and hyperglycemia (Tudu *et al.*, 2022). Its inclusion provides a broad spectrum of antimicrobial and cardiovascular benefits, mediated by compounds such as alliin and allicin (El-Saadony *et al.*, 2024).
- iv. ***Moringa oleifera* (Moringa):** This plant acts as a dense source of essential micronutrients, phenolics, and flavonoids. It contributes to the formulation's ability to inhibit carbohydrate-hydrolyzing enzymes (α -amylase and α -glucosidase), which is critical for postprandial glucose control (Jikah & Edo, 2023).

- v. ***Zingiber officinale* (Ginger):** Utilized for its pungent oleoresin containing gingerols and shogaols, ginger acts as a bio-enhancer and anti-inflammatory agent. Its constituents modulate inflammatory pathways by inhibiting cytokines such as TNF- α and enzymes like COX-2 (Almatroodi *et al.*, 2021; Edo *et al.*, 2025).
- vi. **Pure Honey:** This natural solvent functions as an Anupana or carrier vehicle. Beyond its sweetening properties, honey is employed to enhance the bioavailability of lipophilic phytochemicals within the matrix, facilitating efficient absorption in the gastrointestinal tract.

2.4.2 Detailed Phytochemical Profile

The biological efficacy of Miracle Seed Ultima® is derived from a complex mixture of bioactive secondary metabolites. These compounds are classified into organosulfur compounds, phenolic compounds, alkaloids, and terpenes, each contributing distinct yet complementary biochemical activities.

2.4.2.1 *Zingiber officinale* (Ginger) Constituents

The rhizome of *Z. officinale* contributes a diverse array of volatile and non-volatile compounds. The oleoresin contains the pungent principles, primarily gingerols and their dehydration products, shogaols (Edo *et al.*, 2025).

- i. **Gingerols and Shogaols:** The most abundant bioactive constituent is 1-gingerol (1-[4'-hydroxy-3'-methoxyphenyl]-5-hydroxy-3-decanone). Upon drying or thermal processing, gingerols undergo dehydration to form shogaols, such as 1-shogaol, which are thermodynamically more stable and possess potent pharmacological properties

- (Ajanaku *et al.*, 2022; Edo *et al.*, 2025). Other homologues identified include, 2-,3-, and 4-gingerols and their corresponding shogaols (Edo *et al.*, 2025).
- ii. **Paradols and Zingerone:** Hydrogenation of shogaols yields paradols (e.g.,1-paradol), while retro-aldol degradation of gingerols produces zingerone. These compounds are noted for their robust antioxidant capacities (Ajanaku *et al.*, 2022).
 - iii. **Terpenes:** The volatile oil fraction is rich in sesquiterpenes, particularly α -zingiberene, which constitutes a significant portion of the essential oil, alongside β -sesquiphellandrene and ar-curcumene (Ajanaku *et al.*, 2022).

2.4.2.2 *Allium sativum* (Garlic) Constituents

Garlic is a reservoir of organosulfur compounds, which are responsible for its characteristic odor and biological activity. Approximately 184 diverse secondary metabolites have been identified in garlic, including 70 organosulfur compounds (El-Saadony *et al.*, 2024).

- i. **Alliin and Allicin:** The primary cysteine sulfoxide in intact garlic is alliin (S-allyl-L-cysteine sulfoxide). Upon tissue disruption, the enzyme alliinase hydrolyzes alliin to form allicin (diallyl thiosulfinate), a highly unstable and reactive compound responsible for antimicrobial effects (Tudu *et al.*, 2022).
- ii. **Oil-Soluble Organosulfur Compounds:** Due to the instability of allicin, it rapidly decomposes into oil-soluble polysulfides, including diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS). These polysulfides are crucial for cardiovascular protection and antithrombotic activity (El-Saadony *et al.*, 2024).

- iii. **Water-Soluble Compounds:** Aged preparations or aqueous extracts typically contain S-allyl cysteine (SAC), which is chemically stable and contributes significantly to antioxidant activity (Tudu *et al.*, 2022).
- iv. **Flavonoids and Saponins:** *A. sativum* also contains significant quantities of flavonoids (quercetin, kaempferol) and saponins, which contribute to its immunomodulatory and anti-inflammatory profile (El-Saadony *et al.*, 2024).

2.4.2.3 *Vernonia amygdalina* and *Moringa oleifera* Constituents

- ***Vernonia amygdalina*:** This botanical is characterized by a high content of saponins and alkaloids, which are implicated in its antidiabetic and chemopreventive properties (Edo *et al.*, 2024). It also contains sesquiterpene lactones and flavonoids (luteolin, apigenin) that modulate enzymatic antioxidant systems (Edo *et al.*, 2023).
- ***Moringa oleifera*:** Moringa provides a dense profile of phenolic acids (chlorogenic acid, gallic acid) and flavonoids (quercetin, kaempferol) (Ansari *et al.*, 2024). It is also a source of essential amino acids and micronutrients that support hematopoiesis (Jikah & Edo, 2023).

2.4.3 Pharmacological Mechanisms of Action

The therapeutic benefits of Miracle Seed Ultima® are mediated through the modulation of specific biochemical pathways by its constituent phytochemicals. The formulation addresses pathology through antioxidant defense, anti-inflammatory signaling, and metabolic regulation.

2.4.3.1 Antioxidant Defense and Oxidative Stress Mitigation

Oxidative stress, characterized by an imbalance between reactive oxygen species (ROS) and endogenous antioxidants, is a central pathogenomic factor in diabetes and renal toxicity.

- i. **Enzymatic Upregulation:** The flavonoids and phenolic acids present in *Moringa* and *Vernonia* have been shown to upregulate the activity of antioxidant enzymes, specifically Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPx) (Edo *et al.*, 2024). *Zingiber officinale* constituents, particularly gingerol, significantly enhance renal SOD and CAT levels while reducing lipid peroxidation markers like malondialdehyde (MDA) in diabetic models (Almatroodi *et al.*, 2021).
- ii. **Radical Scavenging:** The organosulfur compounds in *Allium sativum*, such as SAC and allicin, exhibit direct free radical scavenging activity (Tudu *et al.*, 2022). Furthermore, -shogaol from ginger activates the Nrf2 signaling pathway, a master regulator of antioxidant gene expression, thereby enhancing cellular resistance to oxidative injury (Ajanaku *et al.*, 2022).
- iii. **Lipid Peroxidation Inhibition:** By reducing MDA levels, the formulation protects cellular membranes from oxidative damage. Studies indicate that *Nigella sativa* and *Zingiber officinale* extracts effectively lower MDA in hepatic and renal tissues, preserving organ architecture (Almatroodi *et al.*, 2021).

2.4.3.2 Anti-Inflammatory and Immunomodulatory Activity

Chronic low-grade inflammation is a hallmark of metabolic syndrome. The formulation targets inflammatory pathways through multiple mechanisms.

- i. **NF-kappaB Inhibition:**-Gingerol and-shogaol have been demonstrated to inhibit the nuclear factor-kappa B (NF-κB) pathway. This inhibition suppresses the expression of pro-inflammatory cytokines, including Tumor Necrosis Factor-alpha

(TNF- α), Interleukin-1 β (IL-1 β), and Interleukin-6 (IL-6) (Almatroodi *et al.*, 2021; Kim *et al.*, 2025).

- ii. **Enzyme Inhibition:** Phytochemicals from ginger and garlic synergistically inhibit the activity of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), thereby reducing the production of prostaglandins and nitric oxide, which are key mediators of inflammation (Shukla *et al.*, 2015).
- iii. **Immunomodulation:** Bioactive fractions of *Zingiber officinale*, particularly when fermented or combined with probiotics, have shown the capacity to enhance the phagocytic activity of macrophages and modulate the production of nitric oxide (NO) in a dose-dependent manner (Kim *et al.*, 2025). *Allium sativum* constituents also stimulate T-lymphocyte proliferation and macrophage activation, bolstering innate immunity (El-Saadony *et al.*, 2024).

2.4.3.3 Metabolic Regulation and Anti-Diabetic Effects

The formulation addresses hyperglycemia and dyslipidemia through distinct biochemical interventions.

- i. **Carbohydrate Hydrolysis Inhibition:** Flavonoids and tannins from *Moringa oleifera* and *Vernonia amygdalina* inhibit α -amylase and α -glucosidase, the enzymes responsible for carbohydrate digestion. This inhibition retards glucose absorption and reduces postprandial hyperglycemia (Ansari *et al.*, 2024; Edo *et al.*, 2025).
- ii. **Insulin Sensitization and Mimicry:** *Allium sativum* extracts have been observed to increase serum insulin levels and improve insulin sensitivity, potentially by stimulating pancreatic β -cells (Eidi *et al.*, 2006). Similarly, -gingerol facilitates the

translocation of Glucose Transporter type 4 (GLUT4) to the cell membrane in skeletal muscle, enhancing glucose uptake independent of insulin (Ansari *et al.*, 2024; Edo *et al.*, 2025).

- iii. **Lipid Metabolism:** The formulation exerts hypolipidemic effects by inhibiting HMG-CoA reductase (mediated by garlic bioactive compounds) and activating Peroxisome Proliferator-Activated Receptor gamma (PPAR- γ), which regulates fatty acid storage and glucose metabolism (Ansari *et al.*, 2024; El-Saadony *et al.*, 2024). Zingiber officinale has also been shown to reduce total cholesterol (TC), triglycerides (TG), and low-density lipoprotein (LDL) while increasing high-density lipoprotein (HDL) (Edo *et al.*, 2025).

2.4.3.4 Potential Therapeutic Benefits

Based on the phytochemical profile and pharmacological mechanisms of its constituents, Miracle Seed Ultima® offers several therapeutic applications.

- i. **Glucose Regulation:** The product acts as a hypoglycemic agent suitable for the management of diabetes mellitus. By inhibiting hepatic gluconeogenesis and enhancing peripheral glucose uptake via the upregulation of GLUT4 transporters (facilitated by Allium and Zingiber constituents), the formulation helps maintain glycemic homeostasis (Edo *et al.*, 2025; El-Saadony *et al.*, 2024). Studies on *Allium sativum* have demonstrated significant reductions in fasting blood glucose and improvements in insulin levels in streptozotocin-induced diabetic models (Eidi *et al.*, 2006).
- ii. **Hematopoiesis and Hematological Protection:** The product serves as a "Blood Tonic," improving erythropoietic parameters such as Packed Cell Volume (PCV) and Hemoglobin (Hb) concentration. This effect is attributed to the iron-rich profile of *Moringa oleifera* and

the cytoprotective properties of the antioxidant constituents, which extend the lifespan of erythrocytes by protecting their membranes from oxidative hemolysis (Abbaspour *et al.*, 2014). Additionally, *Nigella sativa* extracts have been linked to improved hematological indices in toxicological models (Ahmed *et al.*, 2016).

iii. **Nephroprotection and Organ Safety:** The formulation supports renal integrity by reducing lipid peroxidation in kidney tissues.

a. **Renal Integrity:** Administration of-gingerol has been proven to attenuate renal damage in diabetic models. It preserves glomerular architecture and reduces serum creatinine and blood urea nitrogen (BUN) levels by mitigating oxidative stress and inflammation within renal tissues (Almatroodi *et al.*, 2021).

b. **Anti-Fibrotic Activity:** Bioactive polyphenols from Hibiscus (taxonomically related to Vernonia in bio-activity context regarding anthocyanins) and garlic prevent renal epithelial-mesenchymal transition and fibrosis, thereby preserving kidney function under stress conditions (Sakhaei *et al.*, 2021). The reduction of urea and creatinine suggests an enhancement in glomerular filtration rate (GFR) and tubular function (El-Saadony *et al.*, 2024).

iv. **Reproductive Support:** The reduction of oxidative stress in the reproductive microenvironment helps improve fertility parameters. The modulation of the Hypothalamic-Pituitary-Gonadal (H-P-G) axis by antioxidant-rich botanicals like *Nigella sativa* supports reproductive health. Oxidative stress is a known cause of sperm DNA damage and reduced motility; therefore, the high antioxidant capacity of the formulation (driven by flavonoids and thymoquinone) offers protective effects for germ cells.

- v. **Antimicrobial Activity:** The product exhibits broad-spectrum antibacterial and antifungal effects. This is primarily driven by the organosulfur compounds in Garlic (Allicin) and the quinones in Black Seed. These compounds disrupt microbial cell membranes, inhibit essential enzymatic processes in pathogens, and prevent biofilm formation (El-Saadony *et al.*, 2024; Tudu *et al.*, 2022).

In conclusion, Miracle Seed Ultima® is a complex phytopharmaceutical alternative where the aggregate pharmacological effect likely exceeds the sum of its individual components. The formulation integrates the potent antioxidant and anti-inflammatory properties of *Nigella sativa* and *Zingiber officinale* with the metabolic regulatory capacities of *Allium sativum* and *Moringa oleifera*. Through the modulation of the Nrf2/NF-κB pathways, inhibition of carbohydrate-hydrolyzing enzymes, and enhancement of insulin signaling, the product offers a multiple approaches to managing oxidative stress, diabetes mellitus, and associated organ pathologies. The inclusion of honey as a vehicle further potentiates the bioavailability of these bioactive compounds, ensuring efficient therapeutic delivery.

2.5 Safety Concerns in Herbal Extracts

The toxicological evaluation of herbal medicinal products (HMPs), particularly those derived from seed extracts, is critical due to the presence of potent secondary metabolites which may pose significant safety risks, notably hepatotoxicity and nephrotoxicity (Mugale *et al.*, 2024). A comprehensive assessment necessitates the integration of dose-ranging studies, histopathological examinations, and mechanistic analyses focusing on oxidative stress pathways (Rosidah *et al.*, 2024; Quan *et al.*, 2020).

2.5.1 Reported Toxicities in Similar Formulations: Alkaloids and Saponins

Medicinal plants frequently contain secondary metabolites that are toxic to biological systems, including humans and animals (Mugale *et al.*, 2024; Dai and Mumper, 2010). Among the major classes of phytochemicals associated with organ toxicity, alkaloids and saponins present notable concerns:

2.5.1.1 Hepatotoxicity from Alkaloids

Alkaloids constitute one of the primary groups of phytochemicals linked to liver injury (Quan *et al.*, 2020). Pyrrolizidine alkaloids (PAs), exemplified by those found in plants such as *Symphytum*, *Senecio*, *Crotalaria*, and *Heliotropium*, are known to be hepatotoxic, genotoxic, cytotoxic, and phototoxic (Jitäreanu *et al.*, 2023). The consumption of PA-containing herbs has been linked to hepatic sinusoidal obstruction syndrome (HSOS) in patients (Quan *et al.*, 2020). Specific PA structures, such as retronecine, heliotridine, and otonecine, which contain a double bond, are responsible for genotoxicity through the formation of DNA adducts, potentially leading to tumour induction (Jitäreanu *et al.*, 2023).

The hepatotoxic mechanism often involves metabolism by cytochrome P450 (CYP450) enzymes, leading to the generation of an active intermediate (Quan *et al.*, 2020). This intermediate depletes hepatic glutathione (GSH) levels, causing mitochondrial dysfunction and the subsequent overproduction of reactive oxygen species (ROS), which ultimately contributes to liver injury (Quan *et al.*, 2020).

2.5.1.2 Nephrotoxicity from Saponins

Nephrotoxicity is frequently reported in toxicological evaluations of herbal extracts, accounting for approximately 30% of organ-specific toxicities observed in some reviews (Mugale *et al.*, 2024). Saponin compounds and their derivatives have been specifically implicated in toxic

hepatitis (Quan *et al.*, 2020). For instance, specific saponins identified in species such as *Albizia julibrissin*, *Dumasia truncata*, and *Melia azedarach* have been associated with liver toxicity (Quan *et al.*, 2020). While the sources do not specifically attribute all observed nephrotoxicity solely to saponins, many polyherbal mixtures (PHMs) contain bioactive compounds like flavonoids, alkaloids, and saponins that may induce organ-specific damage in the kidneys, which are crucial for detoxification and excretion (Mugale *et al.*, 2024).

2.5.2 Examples of Dose-Dependent Effects in Rodent Models

The dose-dependent effects of herbal extracts are frequently assessed in preclinical rodent models to establish safety profiles and potential therapeutic indices (Mugale *et al.*, 2024).

For extracts of *Chloranthus serratus* roots, stems, and leaves administered to Sprague Dawley rats over 14 days, the observed hepatotoxicity was clearly linked to the administered dose and the part of the plant extracted (Li *et al.*, 2021). Ethanol extracts of roots (ER, 4.14 g/kg/day), stems (ES, 3.20 g/kg/day), and leaves (EL, 1.16 g/kg/day) resulted in significantly increased liver indices across all treatment groups (Li *et al.*, 2021). The lethal dose (LD50) values were markedly different: >10.35 g/kg for ER, >8.05 g/kg for ES, and >2.90 g/kg for EL, demonstrating variability in toxicity thresholds based on the extraction yield and the part utilized (Li *et al.*, 2021).

Studies involving *Moringa oleifera* seed products also demonstrate dose-dependent and context-specific effects:

- *Moringa oleifera* seed extract, when co-administered with chemotherapy in obese mice, was found to worsen tumor progression, illustrating a critical herb-drug interaction related to dosage and combination therapy (Zunica *et al.*, 2021).
- *Moringa oleifera* seed oil (300 mg/kg) exhibited protective effects against dichlorvos-induced toxicity in male Wistar rats, suggesting a beneficial dose range (Adeoye *et al.*, 2022).

- Topical application of *Moringa oleifera* seed oil (1, 3, and 10 μ L) showed efficacy in reducing chronic skin inflammation in Swiss mice, indicating a dose-dependent anti-inflammatory potential (Cretella *et al.*, 2020.).

2.6 Previous Studies on Related Herbal Products

2.6.1 Toxicological Studies on Individual Components

2.6.1.1 Acute and subacute toxicity assessments of *Nigella sativa*

Nigella sativa, commonly referred to as black seed, has been subjected to various toxicological evaluations to ascertain its safety profile across different extraction methods and dosages. Research indicates that the toxicity profile is heavily dependent on the method of preparation and the concentration of bioactive constituents, particularly thymoquinone (TQ). In acute toxicity studies involving male Swiss albino mice, the median lethal dose (LD50) for intraperitoneally administered volatile oil, aqueous extract, and fixed oil were determined to be 1,853 mg/kg, 3,020 mg/kg, and 3,371 mg/kg, respectively (Mashayekhi-Sardoo *et al.*, 2020). Conversely, oral administration of aqueous, methanol, and chloroform extracts at doses as high as 21 g/kg did not result in mortality, suggesting a wide margin of safety for oral consumption of crude extracts (Mashayekhi-Sardoo *et al.*, 2020).

Further investigations into the subchronic toxicity of *Nigella sativa* oil (BCO) have focused on the concentration of TQ. Ramalingam *et al.* (2021) conducted a 90-day repeated dose oral toxicity study in *Wistar* rats using BCO containing 5% TQ. The study established a no-observed-adverse-effect level (NOAEL) of 0.1 mL/kg body weight, which corresponds to 94 mg/kg of oil or 5 mg/kg of TQ. At this dosage, no significant alterations were observed in body weight, food consumption, or organ weights (liver, kidney, spleen, and brain). Biochemical parameters, including liver

enzymes (AST, ALT, ALP) and renal markers (urea, creatinine), remained within physiological limits (Ramalingam *et al.*, 2021). However, Mashayekhi-Sardoo *et al.* (2020) noted that chronic administration of fixed oil at 2 mL/kg for 12 weeks in rats resulted in significant decreases in leukocyte and platelet counts, indicating mild hematological toxicity despite the absence of histopathological damage.

Comparative studies have also evaluated the specific organotropism of *Nigella sativa* forms. Algheshairy *et al.* (2025) demonstrated that *Nigella sativa* oil (NSO) and seeds (NSS) exert protective effects against chemically induced hepatorenal dysfunction. In their assessment, NSO was superior in restoring renal oxidative damage markers, such as glutathione peroxidase (GHPx) and catalase (CAT), while NSS provided enhanced hepatic protection, normalizing liver enzymes more effectively than the oil form (Algheshairy *et al.*, 2025).

2.6.1.2 Acute and subacute toxicity assessments of *Moringa oleifera*

Moringa oleifera leaves constitute a significant source of plant protein and secondary metabolites, necessitating rigorous safety evaluation. Palomino-Pacheco *et al.* (2024) evaluated the acute oral toxicity of *Moringa oleifera* leaf aqueous extract (MoAE) in Holtzman strain albino rats. A single dose of 2,000 mg/kg administered orally did not result in mortality or signs of toxicity over a 14-day observation period, classifying the extract as category 5 or unclassified (non-toxic) according to OECD guidelines. In a subsequent 28-day repeated dose study, daily administration of MoAE at doses of 100, 250, and 500 mg/kg did not induce significant changes in body weight gain or biochemical indicators, including alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) (Palomino-Pacheco *et al.*, 2024). Histopathological examination

of the liver, heart, kidney, and stomach revealed no structural alterations (Palomino-Pacheco *et al.*, 2024).

Further toxicological characterization of *Moringa oleifera* has involved fractionated components. Alves *et al.* (2024) investigated a saline leaf extract (LE) and a protein-rich fraction (PRF). While neither preparation exhibited cytotoxicity against human peripheral blood mononuclear cells, the saline extract administered at 2,000 mg/kg in mice caused a transient increase in serum ALT levels and mild leukocyte infiltration in the liver (Alves *et al.*, 2024). These findings suggest that while the aqueous extract and protein fractions possess a generally high safety profile, specific extraction methods concentrating certain metabolites, such as flavonoids like rutin, may induce mild, reversible hepatic alterations at high doses (Alves *et al.*, 2024).

2.6.1.3 Acute and subacute toxicity assessments of *Zingiber officinale*

The rhizome of *Zingiber officinale* (ginger) is widely utilized for its anti-inflammatory and antioxidative properties, yet high-dose exposure warrants toxicological scrutiny. La Torre *et al.* (2024) assessed the acute toxicity of the ethanolic extract of ginger rhizome in rats at doses of 300 and 2,000 mg/kg. While body weight variations were statistically insignificant, the high dose (2,000 mg/kg) induced histopathological changes, including moderate hepatic steatosis, mild lymphocytic portal inflammation, and mild infiltration in the renal tubules and glomeruli (La Torre *et al.*, 2024). Additionally, biochemical assays revealed statistically significant differences in parameters compared to the control group at this high dosage, indicating potential hepatorenal stress upon acute excessive consumption (La Torre *et al.*, 2024).

Conversely, comparative studies generally position ginger as having a higher safety threshold than other *Allium* species. Oladosu *et al.* (2025) reported that *Zingiber officinale* exhibits a higher LD50 (8,660 mg/kg) compared to garlic, suggesting a broader margin of safety. *In silico* ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiling of ginger compounds, such as 6-gingerol and shogaol, predicts favorable oral bioavailability and manageable metabolic transformation, although high concentrations may theoretically interact with metabolic enzymes (Oladosu *et al.*, 2025).

2.6.1.4 Acute and subacute toxicity assessments of *Allium sativum*

Allium sativum (garlic) contains potent organosulfur compounds contributing to both its therapeutic efficacy and toxicological potential. Acute toxicity evaluations have determined the LD50 of garlic extract to be approximately 4,472 mg/kg in mice, classifying it as moderately toxic at very high doses (Oladosu *et al.*, 2025). Clinical signs of toxicity observed at doses of 2,200 mg/kg and above include loss of appetite, depression, and partial paralysis (Oladosu *et al.*, 2025). Furthermore, Tudu *et al.* (2022) reviewed extensive toxicological data, noting that while ethyl acetate extracts showed no mortality at 5,000 mg/kg in some rodent studies, raw garlic and high concentrations of allicin can cause gastrointestinal disturbances and potential liver stress.

In aquatic models, which are highly sensitive to toxins, dietary supplementation with garlic oil (1%) was found to improve growth performance and mitigate the hematological toxicity induced by cypermethrin in Nile tilapia, suggesting a protective rather than toxic effect at nutritional doses (Öz *et al.*, 2024). However, subacute exposure to high doses of garlic aqueous extract in rodents has been associated with hepatocellular alterations (Oladosu *et al.*, 2025). *In silico* toxicity predictions for garlic constituents like allicin and diallyl disulfide suggest potential hepatotoxicity

risks, which correlate with *in vivo* findings of elevated liver enzymes at supra-therapeutic levels (Oladosu *et al.*, 2025).

2.6.1.5 Safety profile of honey as a vehicle

Honey serves as a common vehicle in polyherbal formulations, yet it is not devoid of toxicological risks primarily stemming from environmental contaminants and plant-derived toxins. Kulawik *et al.* (2025) provided a comprehensive overview of bioactive contaminants in honey, identifying pyrrolizidine alkaloids (PAs), grayanotoxins, and triptolide as potential phytotoxins transferred from forage plants. PAs, for instance, are hepatotoxic and potentially carcinogenic, while grayanotoxins can induce neurotoxic effects (Kulawik *et al.*, 2025).

Furthermore, environmental pollution contributes to the presence of heavy metals in honey. Naccari *et al.* (2025) analyzed honey samples for toxic metals and found that lead (Pb) levels frequently exceeded the maximum residue limit (MRL) of 0.1 mg/kg established by EU regulations. Despite this, the calculated Estimated Daily Intake (EDI) and Target Hazard Quotient (THQ) for these metals were below levels indicating adverse health effects for the general population (Naccari *et al.*, 2025). Additionally, honey may contain 5-hydroxymethylfurfural (HMF), a degradation product formed during heating or storage, which possesses potential genotoxic properties (Kulawik *et al.*, 2025). However, honey also provides essential microelements such as zinc, copper, and selenium, which contribute to antioxidant defense systems (Naccari *et al.*, 2025).

2.6.2 Key Findings from Individual Component Studies

2.6.2.1 General safety: low toxicity profiles observed at therapeutic doses

A synthesis of the available literature indicates that the individual components *Nigella sativa*, *Moringa oleifera*, *Zingiber officinale*, and *Allium sativum* exhibit low toxicity when administered at traditional or therapeutic dosages. For *Moringa oleifera*, oral administration up to 2,000 mg/kg did not result in mortality or signs of acute toxicity (Palomino-Pacheco *et al.*, 2024). Similarly, *Nigella sativa* fixed oils and aqueous extracts demonstrated a wide margin of safety, with some extracts showing no mortality even at 21 g/kg (Mashayekhi-Sardoo *et al.*, 2020). *Zingiber officinale* is generally regarded as safe, with *in silico* models confirming the drug-likeness and favorable pharmacokinetic properties of its major constituents (Oladosu *et al.*, 2025).

2.6.2.2 Dose-dependent effects: mild adverse effects reported only at high-dose exposures

Adverse toxicological effects are predominantly dose-dependent. *Nigella sativa* oil containing 5% TQ induced body weight loss at 2,000 mg/kg, necessitating a reduction in dosage for safety establishment (Ramalingam *et al.*, 2021). In the case of *Zingiber officinale*, a dose of 2,000 mg/kg was required to elicit histopathological changes such as hepatic steatosis and renal infiltration (La Torre *et al.*, 2024). For *Allium sativum*, toxicity signs such as disorientation and weakness were only observed at supratherapeutic doses exceeding 2,200 mg/kg (Oladosu *et al.*, 2025). These findings underscore that toxicity is rarely intrinsic to the botanical itself at consumption levels but emerges upon excessive loading.

2.6.2.3 No-observed-adverse-effect-level (NOAEL) data where available

Establishing the NOAEL is critical for regulatory safety assessments. Ramalingam *et al.* (2021) rigorously determined the NOAEL for thymoquinone-rich *Nigella sativa* oil (5% TQ) to be 0.1 mL/kg body weight/day (equivalent to 94 mg/kg body weight) in a 90-day subchronic study. This

dosage corresponds to a human equivalent dose of approximately 900 mg/day for an adult (Ramalingam *et al.*, 2021). For *Gancao Xiexin* decoction, a distinct polyherbal formulation, the NOAEL was established at 3.83 g/kg, providing a benchmark for the safety evaluation of complex herbal mixtures (Ru *et al.*, 2023).

2.6.2.4 Species-specific variations in toxicological responses

Toxicological responses often vary by species and sex. In the evaluation of *Gancao Xiexin* decoction, female rats exhibited higher sensitivity to hematological alterations, specifically mild anemia, compared to males at high doses (Ru *et al.*, 2023). Similarly, differences in metabolic handling of *Nigella sativa* components were noted, where *in vitro* cytotoxicity varied significantly depending on the cell line used (e.g., HepG2 vs. L6 muscle cells) (Mashayekhi-Sardoo *et al.*, 2020). *In silico* models used for *Allium sativum* and *Zingiber officinale* also highlighted that human oral absorption predictions might differ from rodent *in vivo* outcomes due to species-specific metabolic pathways (Oladosu *et al.*, 2025).

2.6.3 Research Gaps and Study Justification

2.6.3.1 Limited available research on multi-herb combinations compared to individual ingredient studies

While extensive data exists for individual herbs, there is a marked scarcity of toxicological evaluations for polyherbal formulations. Arsana *et al.* (2025) highlighted that despite the long history of traditional polyherbal use, empirical facts regarding their safety are often absent. The interaction between multiple herbs can lead to synergistic or antagonistic toxicological effects that are not predictable from single-component data. For instance, Oladosu *et al.* (2025) noted that

combining *Zingiber officinale* and *Allium sativum* resulted in an LD50 of 5,477 mg/kg, suggesting an interaction that modifies the toxicity profile compared to the individual ingredients. The lack of data on how these components behave when co-administered in a complex matrix represents a significant gap in the current literature.

2.6.3.2 Absence of toxicity data specific to multi-component blends like Miracle Seed Ultima®

Specific proprietary blends containing *Nigella sativa*, *Moringa oleifera*, *Zingiber officinale*, *Allium sativum*, and honey lack dedicated safety profiling in the scientific literature. Current safety assumptions are extrapolated from isolated studies of the ingredients, such as the safety of TQ-rich oil (Ramalingam *et al.*, 2021) or *Moringa* extracts (Palomino-Pacheco *et al.*, 2024). However, no study has evaluated the cumulative toxicity or potentiating effects of these specific ingredients when combined in a single formulation. Given that *Nigella sativa* oil can alter hematological parameters (Mashayekhi-Sardoo *et al.*, 2020) and *Allium sativum* can influence liver enzymes (Oladosu *et al.*, 2025), the concurrent administration requires empirical validation to rule out additive toxicity.

2.6.3.3 Limited investigation of oxidative stress markers in existing safety studies

While some studies assess basic liver and kidney function markers, there is a limited investigation into the oxidative stress mechanisms within toxicological evaluations of polyherbal mixtures. Algheshairy *et al.* (2025) emphasized the importance of measuring antioxidant enzymes (SOD, CAT, GHPx) and lipid peroxidation markers (MDA) to understand the underlying mechanisms of hepatorenal protection or injury. Many standard toxicity studies, such as those reviewed for *Nigella*

sativa (Mashayekhi-Sardoo *et al.*, 2020), focus primarily on gross pathology and basic serum biochemistry, often overlooking the redox status of tissues which serves as an early indicator of cellular stress.

2.6.3.4 Lack of comprehensive hematological and biochemical profiling in combination

products Comprehensive profiling of hematological and biochemical parameters in the context of polyherbal consumption remains inconsistent. While Ramalingam *et al.* (2021) provided a detailed analysis for *Nigella sativa* oil, and Arsana *et al.* (2025) evaluated platelet and white blood cell responses to "Usadha" polyherbal, data regarding the specific combination of black seed, moringa, ginger, garlic, and honey is non-existent. Ru *et al.* (2023) demonstrated that subacute toxicity could manifest as subtle hematological changes (e.g., decreased RBC and HGB) without overt clinical signs, underscoring the need for detailed hematological scrutiny in safety assessments of combined herbal products.

2.6.3.5 Need for standardized toxicological assessment of synergistic herbal matrices

Differences in the chemical makeup of herbal ingredients such as varying thymoquinone levels in black seed oil (Ramalingam *et al.*, 2021) or contaminants in honey (Naccari *et al.*, 2025); highlight the need for uniform safety testing of finished products. The honey carrier itself may contain pollutants, including lead and other heavy metals (Naccari *et al.*, 2025) as well as plant-derived toxins (Kulawik *et al.*, 2025), which are not considered when testing isolated plant extracts. As such, it is crucial to assess the complete formulation to verify that honey does not add toxicity and that the combination of ingredients stays within safe exposure limits established for individual components..

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Test Substance

The herbal product, Miracle Seed Ultima® was bought from a pharmacy shop opposite the University of Benin Teaching Hospital, Benin City, Edo State. As an inclusion criterion, the product was ascertained to have been ‘registered’ with the National Agency for Food, Drug Administration and Control (NAFDAC). The manufactured and expiry dates of the product were inspected and all were confirmed to be within the acceptable time frame.

3.1.2 Product Characterization

The product was purchased in its commercially available liquid form. The following product information was documented:

- Batch number: MSUL-240315-0847
- Manufacturing dates: 30th of June 2025
- Expiry date: 30th of June 2027

The product was inspected for physical characteristics, including color, odor, consistency, and the presence of any particulate matter or sediment. The bottles were examined to ensure intact seals and proper labeling. The product was stored at room temperature ($25 \pm 2^{\circ}\text{C}$) in a cool, dry place protected from direct sunlight, as recommended by the manufacturer, until use in the experimental study.

3.1.3 Equipment and Apparatus

The apparatus and equipment used in this study were obtained from certified laboratory suppliers and were in proper working condition at the time of use. Weighing and general instrumentation included an Analytical Weighing Balance (Mettler H-80, Germany; Adventurer OHAUS AR1530), an Electric Centrifuge (80-2 model, Tecmel, USA), a Water Distiller (B. Bran Sc. Inst. Company, England), a Water Bath, Desiccators and Muffle Furnace for ash content determination, and an Oven for moisture content determination. Animal cages were used for the housing of experimental animals throughout the study period.

General laboratory glassware and consumables used included measuring cylinders (Pyrex, England), micropipettes (Microlux, England), universal bottles, EDTA bottles, lithium heparin bottles, plain sample tubes, plastic sample containers, cuvettes (Pyrex, England), and filter papers (Whatman, England). Additional consumables included metal wire mesh, cotton wool, disposable gloves and nose masks, gentian violet, scissors, and cardboard papers.

Sample preservation was carried out using a refrigerator maintained at -4°C (Citizens PRC4246) and deep freezers operating at -20°C and -80°C (Citizens PRC4246). Spectroscopic and analytical measurements were performed using a UV/VIS Spectrophotometer (T70, PG Instruments Ltd., UK), a Microplate Spectrophotometric Reader/ELISA Reader (PG Instruments Ltd., UK), and an Atomic Absorption Spectrometer (AAS). Surgical and administration tools employed included oro-gastric gavage tubes, dissecting sets, and syringes of 2 ml and 5 ml capacities (Pyrex, England).

3.1.4 Reagents and Chemicals

All chemicals used in this study were of analytical grade and were primarily sourced from local vendors and laboratories within the University of Benin and Benin City. For qualitative and quantitative phytochemical screening, the solvents used included methanol (95% and 70%), ethanol, chloroform, benzene, petroleum ether, and acetone. Acids and bases employed included concentrated sulfuric acid (H_2SO_4), hydrochloric acid (HCl), glacial acetic acid, ammonium hydroxide, and sodium hydroxide (NaOH). Specific detection reagents used for phytochemical identification included Mayer's reagent, 1% ferric chloride (FeCl_3), aluminum chloride, potassium acetate, lead acetate, 4.77% disodium hydrogen phosphate (Na_2HPO_4), picric acid for Buljets reagent, acetic anhydride, as well as esculin and linalool standards. Distilled water was used throughout the phytochemical procedures.

For proximate analysis, digestion and extraction reagents included potassium sulphate (K_2SO_4) and copper sulphate (CuSO_4) as catalysts for the Kjeldahl nitrogen determination, alongside boric acid, methylene red indicator, and diethyl ether. Mineral and elemental analysis was carried out using a digestion acid mixture of nitric acid and perchloric acid, with acetylene and nitrous oxide serving as flame gases for the Atomic Absorption Spectrometer (AAS).

For in vitro antioxidant assays, the DPPH assay utilized 2,2-diphenyl-1-picrylhydrazyl (DPPH) dissolved in methanol, while the FRAP assay employed TPTZ (ferric-tripyridyltriazine) and ferric chloride. Biochemical and diagnostic assessments were conducted using a glucometer (Roche Diagnostics, Germany) for blood glucose determination. Organ function and lipid profile analyses were carried out using commercial diagnostic kits from Randox Laboratories (UK), which covered liver function markers including alanine aminotransferase (ALT), aspartate aminotransferase

(AST), alkaline phosphatase (ALP), total and direct bilirubin, total protein, and albumin; kidney function markers including urea and creatinine; and lipid profile parameters including total cholesterol, triglycerides, and HDL-cholesterol.

3.2 METHODS

3.2.1 Qualitative Phytochemical Screening

3.2.1.1 Test for Tannins

The required test tubes were labelled and arranged in a rank, it was carried out according to Harborne, 1973 and Sofowora, 1993 guide. 2ml of prepared sample solution was pipetted into test tube, few drops of 1% ferric chloride (FeCl_3) solution was added to the test tube containing sample, the mixture was gently shake for proper mixing, color change in the solution were observed. A blue-black or green precipitate indicates the presence of tannins, Blue-Black: Indicates hydrolyzable tannins and Green: Indicates condensed tannins.

3.2.1.2 Test for Saponins

This test was done according to Frothing guide, test tube needed were labelled according, 2 ml of prepared sample solution was pipetted into test tube, 5 ml of distilled water was added to the test tube then shake vigorously for about 30 seconds to 1 minutes, the test tube was allowed to stand for 10-15 minutes. A stable layer of foam on the surface of the solution indicated presence of Saponin.

3.2.1.3 Test for Flavonoids

This test was carried out according to Harborne, 1973 and Sofowora, 1993 guide, 2 mL of dilute sodium hydroxide (NaOH) solution was added to the test tube containing the sample, a few drops

of concentrated hydrochloric acid (HCl) was added and color change was observed, A yellow coloration indicates the presence of flavonoids.

3.2.1.4 Test for Terpenoids

This test was done according to Salkowski guide, 2 ml of prepared sample solution was pipetted into a clean test tube, 2 ml of chloroform was added to the test tube containing the sample solution, and 3 mL of concentrated sulfuric acid (H_2SO_4) was added to the test tube to form a layer.

3.2.1.5 Test for Alkaloids

This test was carried out using Mayer's guide as reported by Harborne, 1973 and Sofowora, 1993, 2 ml of sample was pipetted into a clean test tube, a few drops of dilute hydrochloric acid (HCl) to the sample to acidify it, few drops of Mayer's reagent was added to the sample solution tube, formation of a creamy white precipitate was observed which indicate presence of alkaloids.

3.2.1.6 Test for Coumarins

2 ml of sample was pipetted into a test tube, 1 ml of 10% sodium hydroxide (NaOH) solution was added, followed by addition of few drops of concentrated HCl. The appearance of greenish fluorescence under normal light was observed which indicate presence of Coumarin (Sofowora, 1993).

3.2.1.7 Test for Anthraquinones

2 ml of sample was pipetted into a test tube, 5 ml of distilled water was added to the test tube then shake thoroughly, the mixture was filtered to obtain aqueous extract, 2 ml of benzene was added and shake thoroughly, benzene layer was separated then equal volume of ammonia solution (10%)

was added, shake and allowed the layer to separate. A pink- violet coloration in the lower layer indicate the presence of anthraquinones.

3.2.1.8 Test for Cardiac glycoside

This test was conducted using Spectrophotometric Method (Keller–Killiani Test) Sarker, *et al.*, 2006. 1 ml of sample was pipetted into a test tube, 1 ml of glacial acetic acid containing traces of ferric chloride was added to the test tube, 1 mL of concentrated sulfuric acid was added slowly along the side of the test tube. A blue /green ring at the junction was observed which indicate the presence of deoxy sugar characteristic of cardiac glycosides.

3.2.1.9 Test for Steroids

Determination of steroid was conducted using Liebermann–Burchard Test (Skoog, *et al.*, 2007), 1 ml of sample was pipetted into a clean test tube followed by addition of 2 ml of chloroform, 2 ml of acetic anhydride then mix well, 1ml of concentrated sulfuric acid was added down the side of the test tube, a bluish green color was observed which indicate presence of steroids.

3.2.10 Test for Phenols

Phenol test was conducted using method described by Sofowora, 1993. 2 ml of sample was pipetted into a test tube, 2 drops of freshly prepared 1% ferric chloride solution was added then observe color change.

3.2.2 Quantitative Phytochemical assay

3.2.2.1 Determination of Alkaloids content

5 ml of sample was pipetted into a test tube, 100 of 10% sulfuric acid was added and stir for 2-3 hours to extract the alkaloids, it was mixture was filtered using a Buchner to obtain filtrate,

ammonium hydroxide solution (25%) was added dropwise while stirring until the pH reaches 9 – 10 (alkaline). The alkaloids were precipitated and collected using a pre-weighed filter paper, it was dry in an oven at 60°C until a constant weight is achieved then the dried alkaloid residue was weighed using an analytical balance (Harborne, 1998).

3.2.2.2 Determination of Tannins content

Tannins content determination was carried out using method described by Harborne, 1998. 1 ml of sample was measured into a conical flask, 100 mL of 70% methanol was added and heated in a water bath at 70°C for 10 minutes, the solution was filtered with filter paper to obtain the extract. Series of Tannins standard with known concentration was prepared and absorbance of sample and standard was measured at 700 nm using spectrophotometer. Calibration curve of tannic acid concentration against absorbance was plotted and use to determine sample tannin (Obadoni and Ochuko, 2001).

3.2.2.3 Determination of Flavonoids content

Flavonoids content was determined using Aluminum Chloride Colorimetric Method described by Chang *et al.*, 2002. Test tubes for sample and standard was setup in a rack, 1 ml of sample / standard solution into test tube, 1 ml of 10% aluminum chloride solution was added to all tubes, 1 mL of 1 M potassium acetate solution was also added and 3 ml of distilled water, the mixture was mix and incubated for 30 minutes at room temperature (in the dark), the absorbance of sample and standard was measured at 415 nm using a UV-Vis spectrophotometer. Methanol was used as blank.

3.2.2.4 Determination of Steroids content

Steroids content determination was carried out using Liebermann-Burchard Method, 1 ml of sample / standard was added to test tubes, 2 ml of acetic anhydride, 1 mL of concentrated sulfuric

acid was added down the sides of the tube to prevent splashing, the mixture was gently mix then it was allowed to stand in a water bath 25-30°C) for 30 minutes to develop the characteristic green-blue coloration. The absorbance was measured at 620 nm and chloroform was used as blank.

3.2.2.5 Determination of Cardiac Glycosides Content

Portion of the sample was soaked in 100 mL of 70 % ethanol for 2 h and then filtered. A solution of 5 mM lead acetate and 4.77 % disodium hydrogen phosphate (Na_2HPO_4) (10 mL) were added to the filtrate. Then, 10 mL of freshly prepared Buljets reagent (95 mL of 1 % picric acid + 5 mL of 10 % NaOH) was added. After an hour, the mixture was diluted with 20 mL of distilled water, and the absorbance was measured at 495 nm. (Harbone, 1980).

3.2.2.6 Determination of Terpenoid content

Terpenoids were extracted using petroleum ether in a separatory funnel. The extract was evaporated to dryness and dissolved in 5 mL of 95% methanol. 5 mL each of cell and media extracts were mixed to obtain a final volume of 10 mL. 1 mL of the methanol extract was used for determination of terpenoid content and absorbance was measured at 548 nm using 95% methanol as blank. The standard curve was prepared by using varying concentrations (1.29 μM -12.9 μM) of linalool as a Standard. (Harbone, 1980).

3.2.2.7 Determination of total coumarin content

The sample and standard (esculin) were dissolved in methanol: acetone (1:1, v/v) and transferred to a volumetric flask (25 mL). Aliquots of 0.25, 0.50, 0.75, 1.0 and 1.25 mL were diluted to 100 mL in the same solvent, yielding concentrations of 5.0, 10.0, 15.0, 20.0, 25.0 $\mu\text{g/mL}$. The absorbance of the reaction mixture was measured at 327nm. The determination of total coumarins

was calculated as esculin equivalents, based on the esculin linearity curve. Triplicate measurement was performed and the mean computed. (Vianna *et al.*, 2011).

3.2.3 Proximate Analysis

3.2.3.1 Determination of Moisture content

Moisture content was determined using Oven-drying method, 2 ml of the sample was weighed using analytical balance and recorded as initial weight, the dish containing the sample was placed into a preheated oven set to 105°C. The sample was dried for 4 hours, the sample was removed from the oven and immediately placed in a desiccator to cool for 25 minutes, the sample was further weigh and recorded as the final weight.

3.2.3.2 Determination of Ash content

Ash content was determined using the method of AOAC (2000).

Principle

The total ash complete is estimated by complete removal of organic material using ignition.

Procedure

One gram of samples was weighed in crucibles and ignited in a furnace at 500-600°C for 3 hours until it ashes completely. It was then cooled in a dessicator, cooled and weighed immediately at room temperature.

Calculation:

$$\% \text{Ash} = \frac{W_2 - W_0}{W_1 - W_0} \times 100$$

$$W_1 - W_0$$

3.2.3.3 Determination of crude fibre

This was determined by enzymatic-gravimetric method as described by A.O.A.C (2000)

Procedure

One gram of sample(w_0) was boiled in 200ml of sulphuric acid and boiled entirely for thirty minutes. The boiled sample was filtered through a muslin cloth which was rinsed with hot distilled water. 200ml of sodium hydroxide was added to the residue and allowed to boil for 30min, it was then rinsed with hot distilled water. It was also rinsed with hydrochloric acid. It was finally rinsed three times with petroleum ether. It was allowed to drain, dried in the oven, allowed to cool in a desiccator and weighed(w_1). The sample was ashed at 500 °C, for 90 minutes in a muffle furnace cooled in a desiccator and weighed(w_2).

calculation:

$$\% \text{crude fibre} = \frac{w_1 - w_2}{w_0} \times 100$$

w_0

3.2.3.4 Determination of carbohydrate content

Carbohydrate content was determined by obtaining the difference after adding up the % protein content, % fibre content and %lipid then subtracting from one hundred.

$$\% \text{ carbohydrate} = 100 - \% \text{lipid} + \% \text{protein} + \% \text{ash} + \% \text{fibre}$$

3.2.3.5 Determination of crude protein

Nitrogen was determined using the micro-Kjeldahl method (A.O.A.C 2000) and crude protein content will be subsequently calculated by multiplying the nitrogen content by a factor of 6.25

Principle

Proteins and other food components are digested with sulphuric acid in the presence of catalysts. Ammonium sulphate is produced from the total organic nitrogen in the food. Alkali is then used to neutralize the acid digest to produce ammonium which is steam distilled directly into a hydrochloric acid containing the indicator methylene red. Hydrochloric acid and ammonium react to produce ammonium chloride which is then titrated with sodium hydroxide. A blank determination is run to determine the nitrogen content of the reagents. The percentage nitrogen is calculated. The percent nitrogen multiplied by the conversion factor for that particular food produces the percent protein in a food.

Procedure

One milliliter of 4% CuSO_4 , H_2SO_4 and 0.8g of K_2SO_4 were placed in the micro kjeldahl flask. A specific quantity of the miracle seed ultima was added to the reagents in micro Kjeldahl flask. It was digested first at low temperature until frosting ceases, then at a high temperature, until the solution was clear, pale yellow or light blue. The flask was left to cool, and 4ml of distilled water was gradually added and content were distilled using a Kjeldahl distillation apparatus. Ten milliliters of 30% NaOH was used to liberate during distillation ammonium. Ammonium was collected in 0.01 M HCl (a drop of methylene red was added to the HCl.). The distillate was titrated with 0.01M NaOH. Nitrogen content in the sample was calculated from the volume of HCl neutralized by NaOH.

Calculations

%nitrogen= titre value (blank) – titre value (distillate) x 0.14

Weight of sample

Protein = % nitrogen x 6.25

% protein = protein x 100

Initial weight

3.2.3.6 Determination of Crude fat

Crude fat was determined using Soxhlet extraction A.O.A.C (2000)

Principle

The free lipid content consists of neutral fats (triglycerides) and free fatty acid was determined by extracting the dried and ground material with diethyl ether in a continuous extraction apparatus (Soxhlet extractor).

Procedure

The weight of an empty flask was determined. One gram of sample was wrapped and placed in an extraction thimble. The thimble was plugged with cotton wool to avoid loss of sample. The thimble was placed in the extractor. An already weighed, clean and dry soxhlet extractor flask was attached to bottom of the extractor. Petroleum ether (500mls) was poured into dry soxhlet flask and the heating mantle switched on so that the petroleum ether boiled. Heating continued for eight hours after which the solvent was siphoned completely into flask and taken to dryness by distillation.

The flask was removed dried to a constant weight. The cooled, weighed and the amount of extracted lipids was calculated from the difference between the weight before and after extraction.

Calculation:

Weight of empty porous thimble= w_0

Weight of thimble +ground sample= w_1

Weight of ground sample= $w_1 - w_0$

Weight of empty extraction flask= w_2

Weight of extraction flask + ether= w_3

$$\% \text{ lipid} = \frac{w_3 - w_2}{w_1 - w_0} \times 100$$

$$w_1 - w_0$$

3.2.3.7 Determination Ethanol Concentration

The spectrophotometric method for ethanol concentration was determined by solvent extraction and dichromate oxidation reaction (Malinee *et al.* 2019).

Firstly, ethanol in liquid sample was extracted by Tri-n butyl phosphate (TBP, Sigma Aldrich USA). 1 mL of TBP and 1 mL of aqueous sample were mixed in a microtube and then vortex vigorously for 1 min. the mixture was centrifuge at 3,420 xg for 5min to separate into two phases. Upper phase, TBP layer, was clear and transparent, while lower phase, water later, was turbid. Then 500uL of dichromate reagent (containing 10% w/v of $K_2Cr_2O_7$ in 5M of H_2SO_4), and vortex vigorously for 1 min. the mixture was set still for 10 min at room temperature to allow oxidation

product in lower phase to develop its color to blue-green. 100uL of the oxidation products were diluted with 900uL of deionized water. The optical density at 595nm (A595) of the tested sample was measured using a UV/Vis Spectrometer. The ethanol concentration in sample was estimated from the ethanol standard curve representing the relationship between A595 and the concentration of ethanol. (Malinee *et al.* 2019)

3.2.4 Elemental Screening

Elemental analysis was performed using Atomic Absorption Spectrometry (AAS) (AOAC, 2002). All the glass wares used were soaked in HCl-water solution overnight at 1:1. The glass wares after removal from solution were washed with detergent, rinsed with distilled water and then with deionised water. They were subsequently oven-dried. Portions the sample (0.5 mL) were seeded in a digestion tube and digested with 5 mL of mixed acids (nitric and perchloric acids). The digestion tube was heated until the dense white fume initially formed produced a clear homogenous solution. After the heating procedure the tube was cooled and small volume of deionised water was added. The solution was filtered into a 25 mL conical flask and the volume made up to the mark with deionised water. A reagent blank was prepared without the sample following the same procedure. Liquefied sample was aspirated, aerolized and mixed with acetylene and nitrous oxide and burned in a flame to release the individual atoms. The sample absorbs UV light at specific wavelengths and the ground state metal atoms in the sample are transitioned to higher state, thus reducing its intensity. The AAS measures the change in intensity and the intensity converts the light intensity into an absorbance related to the sample concentration by a computer-based software.

3.2.5 In-vitro Antioxidant Capacity

3.2.5.1 DPPH (2,2-Diphenyl-1-picrylhydrazyl) Radical Scavenging Assay

The DPPH radical is a stable free radical that changes color from purple to yellow upon reduction by antioxidants. The degree of decolorization indicates the scavenging potential of the sample.

DPPH radical scavenging assay was done according to method described by Blois, 1958. 0.1 Mm DPPH solution was prepared in methanol, 1 ml of the DPPH solution was mix with varying concentration (10- 100 µg/mL) of the sample, the mixture was incubated in the dark at room temperature for 30 minutes, absorbance was obtained at 517 nm of UV-Vis spectrophotometer.

3.2.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Antioxidants reduce Fe^{3+} -TPTZ (Ferric-tripyridyltriazine) complex to Fe^{2+} , which develops a blue color, measurable at 593 nm.

1 ml of FRAP reagent was mix with 100 µL of the sample, the mixture was incubated for 30 minutes at 37°C, the absorbance was measured at 593 nm.

3.3 Toxicity Study of Miracle Seed Ultima®

3.3.1 Study Design

This study was an experimental, in vivo laboratory-based toxicological investigation designed to assess the acute and sub-chronic toxicity of Miracle Seed Ultima, a dietary supplement, in male *Wistar* rats. The study follows internationally accepted guidelines established by the OECD (modified) for sub acute study and utilizing Lorke's method for acute toxicity and is structured to evaluate potential toxic effects after single and repeated oral administrations at varying dose levels

3.3.2 Acute Toxicity Studies Phase One

3.3.2.1 Experimental Animals

The animals used in this study were handled in strict accordance with international guidelines set by the Canadian Council on the Care and Use of Laboratory Animals in Biomedical Research (1984). Ethical approval for the study was obtained from the Research Ethics Committee of the College of Medical Sciences, University of Benin, before the research commenced (see approval certificate: CMS/REC/2025/870.) A total of 20 healthy male *Wistar* rats, weighing between 160 and 180g, were used for acute oral toxicity study. The rats were procured from the Anatomy Department, School of Basic Medical Sciences, University of Benin, Benin City, Nigeria. The rats were housed in standard polypropylene cages (5 rats per cage) with stainless steel wire mesh tops lined with clean wood shavings, which were replaced every 48 hours to maintain hygiene.

The animal house was maintained under controlled environmental conditions:

- Temperature: $25 \pm 2^{\circ}\text{C}$
- Relative humidity: 50-60%
- Light/dark cycle: 12 hours (lights on at 6:00 AM, lights off at 6:00 PM)
- Ventilation: Adequate air circulation with 15-20 air changes per hour

The animals were provided with pelleted grower mash (Jerrison Allison Allied Company Ltd, Benin City, Nigeria).

Clean drinking water was provided *ad libitum* in sterile water bottles. Food and water consumption were monitored daily by measuring the amount provided and subtracting the amount remaining

after 24 hours. The animals were observed daily during the acclimatization period of seven (7) for any signs of disease, stress, or abnormal behavior. Only healthy animals showing normal behavior, feeding patterns, and consistent weight gain were included in the study.

3.3.2.2 Preparation of Test Doses

The Miracle Seed Ultima® (MSU) commercial product was used directly without further extraction or processing to reflect real-world consumption patterns. Three different dose concentrations were prepared based on Lorke guidelines and doses used in previous studies:

1. **Low dose:** 10 mg/kg body weight
2. **Medium dose:** 100 mg/kg body weight
3. **High dose:** 1000 mg/kg body weight

Since the commercial product was in liquid form, appropriate volumes were calculated to deliver the desired doses based on the product concentration specified by the manufacturer. The product was diluted with distilled water when necessary to achieve a constant administration volume of 1 ml/kg body weight for all dose groups, ensuring consistency in gavage procedure.

3.3.2.3 Experimental Design

Twelve (12) male *Wistar* rats, weighing between 180–220 g, were used in this study. After a 7-day acclimatization period, the rats were weighed and randomly assigned to four (4) groups of three (3) rats each. Each group was structured to ensure that its average weight represented the overall weight range of all the rats, with an initial mean weight of 200 ± 0.8 g at the start of the experiment. The study lasted for fourteen days (14 days), during which the rats were fed ad libitum

based on basal diet consisted of standard pelleted starter mash. The animals in each group were marked for individual identification using gentian violet ink on different parts of the rat.

3.3.2.4 Dose Administration

The Miracle Seed Ultima® is administered in a single dose by gavage using a stomach tube.

Group 1 (Normal Control, n=3): Animals received standard rat chow and drinking water *ad libitum*. They received 1 ml/kg body weight of distilled water by oral gavage. No extract or treatment was administered.

Group 2 (Low Dose, n=3): Animals received 10 mg/kg body weight of MSU-Miracle Seed Ultima® commercial product by oral gavage.

Group 3 (Medium Dose, n=3): Animals received 100 mg/kg body weight of MSU-Miracle Seed Ultima® commercial product by oral gavage.

Group 4 (High Dose, n=3): Animals received 1000 mg/kg body weight of MSU-Miracle Seed Ultima® commercial product by oral gavage.

Prior to administration, the animals fasted overnight, weigh and gavage (Roll *et al.*, 1986).

3.3.2.5 Observations

Animals were observed individually after dosing once during the first 30 minutes and periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days. Signs of toxicity were noted and time the animal took to recovered. All observations are systematically recorded with individual records being maintained for each animal.

Observations considered in this study include changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and behavior pattern, convulsions, salivation, diarrhea, lethargy, sleep and coma (Roll *et al.*, 1986).

3.3.2.6 Determination of LD₅₀ (Median Lethal Dose)

The median lethal dose (LD₅₀) is a standard measure in toxicology that represents the dose of a substance causing death in 50% of test animals under controlled conditions. It is expressed in milligrams per kilogram of body weight (mg/kg). A lower LD₅₀ value indicates higher toxicity because less substance is needed to cause death in half the population, while a higher LD₅₀ suggests lower toxicity. The LD₅₀ is calculated using mathematical methods, such as finding the square root of the product of the highest non-lethal dose and the lowest lethal dose. This value helps classify substances by toxicity level and establish safe dosage ranges for further research or therapeutic use.

LD₅₀ Calculation

The median lethal dose (LD₅₀) will be calculated using the arithmetic method:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

Where:

D₀ is the highest dose that did not cause death

D₁₀₀ is the lowest dose that did cause death

3.3.3 Acute Toxicity Studies Phase Two

Based on the results obtained in Phase 1 especially in the absence of mortality, an additional four (4) rats were used in the second phase. The rats were divided into four groups of one rat each and administered higher oral doses as follows:

3.3.3.1 Dose Administration

The Miracle Seed Ultima® is administered in a single dose by gavage using a stomach tube. They were divided into three groups given different doses of 1600mg/kg, 2900mg/kg and 5000mg/kg as per Locke method. Prior to administration, the animals fasted overnight, weigh and gavage (Roll *et al.*, 1986).

3.3.3.2 Observations

Animals were observed individually after dosing once during the first 30 minutes and periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days. Signs of toxicity were noted and time the animal took to recovered. All observations are systematically recorded with individual records being maintained for each animal.

Observations considered in this study include changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and behavior pattern, convulsions, salivation, diarrhea, lethargy, sleep and coma (Roll *et al.*, 1986).

3.3.4 Miracle Seed Ultima® Sub-Acute Toxicity Studies

Sub-acute toxicity studies were conducted according to Organization for Economic Cooperation and Development guideline [OECD, (2008)] modified:

3.3.4.1 Preparation of Test Doses

The Miracle Seed Ultima® (MSU) commercial product was used directly without further extraction or processing to reflect real-world consumption patterns. Three different dose concentrations were prepared based on the manufacturer's recommended dosage and doses used in preliminary studies:

- i. **Low dose:** 100 mg/kg body weight
- ii. **Medium dose:** 300 mg/kg body weight
- iii. **High dose:** 1000 mg/kg body weight

Since the commercial product was in liquid form, appropriate volumes were calculated to deliver the desired doses based on the product concentration specified by the manufacturer. The product was diluted with distilled water when necessary to achieve a constant administration volume of 1 ml/kg body weight for all dose groups, ensuring consistency in gavage procedure.

3.3.4.2 Dose Preparation Calculation:

The volume of commercial product required for each dose was calculated using the following formula:

$$\text{Volume (ml)} = [\text{Dose (mg/kg)} \times \text{Body weight (kg)}] / \text{Product concentration (mg/ml)}$$

Fresh dose preparations were made every three days to ensure product stability and potency. The diluted preparations were stored in clean, labeled glass bottles at 4°C between administrations. Before each administration, the preparations were brought to room temperature and mixed thoroughly by gentle shaking to ensure homogeneity.

3.3.4.3 Animal Acclimatization and Housing

Twenty (20) male *Wistar rats*, weighing between 150- 180g, were obtained from the Animal House of the Department of Anatomy, University of Benin, and allowed to acclimatize to laboratory conditions for 7 days before the commencement of the experiment. During the acclimatization period, animals were housed in standard polypropylene cages (5 rats per cage) with stainless steel wire mesh tops and wood shaving bedding, which was changed every two days to maintain hygiene.

The animal house was maintained under controlled environmental conditions:

- Temperature: $25 \pm 2^{\circ}\text{C}$
- Relative humidity: 50-60%
- Light/dark cycle: 12 hours (lights on at 6:00 AM, lights off at 6:00 PM)
- Ventilation: Adequate air circulation with 15-20 air changes per hour

The animals were provided with pelleted grower mash (Jerrison Allison Allied Company Ltd, Benin City, Nigeria).

Clean drinking water was provided *ad libitum* in sterile water bottles. Food and water consumption were monitored daily by measuring the amount provided and subtracting the amount remaining after 24 hours. The animals were observed daily during the acclimatization period for any signs of disease, stress, or abnormal behavior. Only healthy animals showing normal behavior, feeding patterns, and consistent weight gain were included in the study.

3.3.4.4 Experimental Design and Animal Grouping

After the 7-day acclimatization period, the 20 male *Wistar rats* were randomly divided into four groups using the random number table method to minimize selection bias. The random allocation ensured that the average body weight across all groups was similar at the start of the experiment (approximately 130-185g). The animals in each group were marked for individual identification using gentian violet ink on different parts of the rat. The experimental groups were designated as follows:

Group 1 (Normal Control, n=5): Animals received standard rat chow and drinking water *ad libitum*. They received 1 ml/kg body weight of distilled water by oral gavage daily for 28 days. No extract or treatment was administered.

Group 2 (Low Dose, n=5): Animals received 100 mg/kg body weight of MSU-Miracle Seed Ultima® commercial product by oral gavage daily for 28 days.

Group 3 (Medium Dose, n=5): Animals received 300 mg/kg body weight of MSU-Miracle Seed Ultima® commercial product by oral gavage daily for 28 days.

Group 4 (High Dose, n=5): Animals received 1000 mg/kg body weight of MSU-Miracle Seed Ultima® commercial product by oral gavage daily for 28 days.

The dose levels were selected based on the manufacturer's recommended therapeutic dose (low dose), multiples of this dose (medium dose), and a higher dose to assess potential toxic effects (high dose). The duration of 28 days was chosen in accordance with OECD Guidelines 407 for repeated dose 28-day oral toxicity study in rodents (OECD, 2008), which is the standard protocol for sub-acute toxicity evaluation.

3.3.4.5 Administration of Test Substance

Route of Administration

The Miracle Seed Ultima® (MSU) commercial product was administered once daily by oral gavage. The oral route was selected as it reflects the natural route of human consumption and allows for systemic absorption of the bioactive constituents.

Each animal was gently but firmly restrained to minimize stress and prevent injury. The calculated dose volume, based on individual body weight recorded weekly, was drawn into a 1ml syringe attached to a curved stainless steel gavage needle appropriate for the rat's size. The needle was carefully inserted through the mouth into the esophagus following its natural curvature and gently advanced until it reached the stomach (approximately at the level of the last rib). The dose was slowly delivered into the stomach, each animal was observed for any signs of distress or respiratory difficulty.

The control group received an equivalent volume of distilled water via the same route and at the same time.

Dose Adjustment

Individual body weights were recorded weekly and dose volumes were adjusted accordingly to maintain consistent mg/kg dosing throughout the 28-day study. Weekly weighing also served to monitor the growth patterns and detect any potential adverse effects on body weight gain.

Duration of Treatment

The treatment was conducted daily for 28 days, consistent with internationally accepted sub-acute toxicity testing guidelines for rodents. All administrations were performed by the same trained personnel to ensure consistency and minimize procedural variations.

3.3.5. Daily Quantity of Feed Consumed:

The quantity of feed given to each group of rats daily was determined by subtracting the quantity of feed left the next morning from that given the day earlier. From the results, the average quantity consumed per day for the period of the study, by the rats, was determined. This quantity of feed consumed by each rat was assessed using a sensitive balance from the commencement of dosing (day 1), until the day of sacrifice (day 28) (Aniagu *et. al.*, 2005). The results was used to determine the quantity of feed consumed per week.

Feed Efficiency:

This was determined using the formula:

Feed efficiency= $\frac{\text{Weight gain or lost/day/rat}}{\text{Feed consumed/day/rat}} \times 100$ (Matos *et al.*, 2005).

Feed consumed/day/rat

3.3.6 Weekly Body Weight:

The body weight of each rat was assessed using a sensitive balance during the acclimatization period, once before commencement of dosing (day 1), once weekly during the dosing period, (day 7, 14, 21) and once on the day of sacrifice (day 28). (Aniagu *et. al.*, 2005). The results was used to determine the average weekly weight of the rats in each group, after which the weekly weight of

the bitters fed rats and the control rats was used to determine the weekly dose of the bitters to be given to the rats and the weight gain or lost weekly by the rats.

3.3.7 Observations (symptoms, signs and mortality):

The animals were observed for toxic symptoms such as weakness or aggressiveness, food refusal, loss of weight, diarrhoea, discharge from the eyes and ears, noisy breathing and other physiological changes including mortality, (Fielding and Metheron, 1991; Vijoyalakshmi *et al*, 2000).

Clinical signs that were assessed before dosing, immediately and 4hrs after dosing, include level of sedation, restlessness, changes in nature of stool, urine and eye colour, excretion of worms, diarrhoea, haematuria, uncoordinated muscle movements, etc. (Fielding and Metheron, 1991; Vijoyalakshmi *et al*, 2000).

3.3.8 Blood Sample Collection and Preparation:

The last dose of the drug was administered on morning of the 28th day. All meals were stopped by 7pm on the 28th day. After an overnight fast, blood samples were collected from the animals (following chloroform anaesthesia and sacrifice/opening up of the animals), using syringes and needles via cardiac puncture. Appropriate specimen bottles were used for collection of blood from each animal. Anticoagulant bottles containing EDTA for hematological analyses, lithium heparin for assay of parameters for liver function and kidney function statuses, fluoride oxalate for blood glucose test, while the plain bottle was used for other biochemical parameters like lipid profile tests. The samples were mixed by gentle inversion.

The samples were centrifuged at 4000r/min for 10mins to obtain plasma and serum respectively. The plasma and serum supernatants were separated into sterile plain bottles and used for assay of the required biochemical parameters.

3.3.9. Biochemical and Hematological Analyses

Hematology parameters measured include: White blood cell count, Red blood cell count, Hemoglobin concentration, Hematocrit, Mean Corpuscular Volume, Mean Corpuscular Hemoglobin, Mean Corpuscular Hemoglobin Concentration, Platelet Count

Clinical biochemistry parameters measured included:

- Liver function: ALT, AST, ALP, total bilirubin
- Renal function: Urea, creatinine and electrolytes
- Metabolic markers: Lipid profile, fasting blood glucose,

3.3.10 Relative Organ Weight:

After blood collection different organs namely the heart, liver, and kidneys of the respective rats from all groups was carefully dissected out and weighed (this weight was designated as the absolute organ weight). The relative organ weight was then calculated using the formula:

$$\text{Relative Organ Weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100 \quad (\text{Aniagu } et al., 2005).$$

3.4 HEMATOLOGICAL ANALYSES

Hematological parameters were determined within 2 hours of blood collection to ensure accuracy and prevent artifactual changes that can occur with prolonged storage. The analyses were performed at the department of biochemistry, University of Benin. Both manual methods using established protocols and standardized commercial kits were employed for comprehensive hematological evaluation. Hematological parameters assessed included hemoglobin concentration, red and white blood cell counts, hematocrit, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

3.4.1 Hemoglobin Concentration

Hemoglobin Concentration Determination

Hemoglobin concentration was determined using the cyan-methemoglobin method, which is the gold standard recommended by the International Committee for Standardization in Haematology.

Principle: Hemoglobin is oxidized to methemoglobin by potassium ferricyanide present in Drabkin's reagent. Methemoglobin then combines with potassium cyanide to form cyanmethemoglobin (also called hemiglobin cyanide), a stable colored compound that absorbs light maximally at 540 nm. The intensity of the color produced is directly proportional to the hemoglobin concentration in the sample.

Reagent: Drabkin's reagent containing:

- Potassium ferricyanide: 200 mg/L
- Potassium cyanide: 50 mg/L
- Potassium dihydrogen phosphate: 140 mg/L
- Distilled water to 1000 ml (pH 7.0-7.4)

Procedure:

- i. Drabkin's reagent (5.0 ml) was dispensed into a clean, dry test tube.
- ii. Exactly 20 μ l (0.02 ml) of well-mixed EDTA blood was added to the reagent using a calibrated micropipette to give a 1:251 dilution.
- iii. The mixture was mixed thoroughly by gentle inversion 8-10 times and allowed to stand for 5 minutes at room temperature (20-25°C) to allow complete conversion to cyanmethemoglobin.
- iv. The absorbance was measured at 540 nm using a UV-Visible spectrophotometer against a reagent blank (Drabkin's reagent without blood).

- v. A standard solution of cyanmethemoglobin with known hemoglobin concentration (provided in the commercial kit) was similarly processed, and its absorbance was measured.

Calculation:

Hemoglobin concentration (g/dL) = (Absorbance of sample / Absorbance of standard) ×
Concentration of standard (g/dL)

Normal reference range for male *Wistar* rats: 12-18 g/dL

3.4.2 Red Blood Cell (RBC) Count

RBC count was performed using the improved Neubauer hemocytometer method, a manual counting technique that provides accurate cell enumeration.

Principle: Red blood cells are diluted in an isotonic solution (Hayem's solution) that prevents hemolysis while maintaining cell integrity. The diluted cells are counted microscopically in a counting chamber of known volume, and the concentration is calculated based on the dilution factor and chamber dimensions.

Reagent - Hayem's Solution:

- Mercuric chloride: 0.25 g
- Sodium chloride: 0.50 g
- Sodium sulfate: 2.50 g
- Distilled water: 100 ml

Procedure:

- i. Well-mixed EDTA blood (20 µl) was drawn into a clean, dry red blood cell (RBC) pipette up to the 0.5 mark.

- ii. The external surface of the pipette was carefully wiped clean with tissue paper to remove excess blood.
- iii. The tip of the pipette was immediately immersed in Hayem's solution, and the solution was drawn up to the 101 mark, giving a 1:200 dilution.
- iv. The ends of the pipette were sealed with the index finger and thumb, and the pipette was gently rotated and shaken for 2-3 minutes to ensure thorough mixing of blood and diluent.
- v. The first 3-4 drops were discarded to clear the capillary tube of unmixed fluid.
- vi. A small drop of the diluted blood was placed on the counting chamber of an improved Neubauer hemocytometer.
- vii. A clean coverslip was carefully placed over the counting area, and the chamber was allowed to stand for 2-3 minutes to allow cells to settle.
- viii. The hemocytometer was examined under a light microscope using the 40× objective (high power).
- ix. Red blood cells were counted in the five small corner squares of the central ruled area (each small square = $1/25 \text{ mm}^2$).

Cells touching the top and left boundaries of the squares were included in the count, while those touching the bottom and right boundaries were excluded to avoid double counting. The count was performed in duplicate (both chambers of the hemocytometer), and the average was calculated. Counts differing by more than 10% were repeated.

Calculation:

The number of RBCs per microliter (μl) was calculated using the formula:

RBC count ($\times 10^6/\mu\text{l}$) = (Number of cells counted $\times$ Dilution factor) / (Area counted $\times$ Depth of chamber)

Where:

- Number of cells counted = Total cells in 5 small squares
- Dilution factor = 200
- Area counted = 5 small squares = $5 \times 1/25 = 1/5 \text{ mm}^2$
- Depth of chamber = 0.1 mm

Simplified formula:

$$\text{RBC count } (\times 10^6/\mu\text{l}) = N \times 10,000$$

Where N = average number of cells counted in 5 small squares

Normal reference range for male *Wistar* rats: $7\text{-}10 \times 10^6/\mu\text{l}$

3.4.3 Packed Cell Volume (Hematocrit)

Hematocrit (PCV) was determined using the micro hematocrit method, which provides a measure of the proportion of blood volume occupied by red blood cells.

Principle: When whole blood is centrifuged at high speed, the cellular components separate from the plasma based on density. Red blood cells, being the densest and most numerous, pack at the bottom of the tube. The height of the red cell column relative to the total blood column represents the hematocrit.

Procedure:

- i. Plain capillary tubes (75 mm length, 1 mm internal diameter) were filled with well-mixed EDTA blood by capillary action to approximately three-quarters full (about 50-60 mm).
- ii. One end of the capillary tube was sealed with plasticine (modeling clay) by pressing it firmly into the sealant.
- iii. The sealed tubes were placed in a microhematocrit centrifuge with the sealed end facing outward toward the periphery and the open end toward the center.

- iv. Tubes were balanced in the centrifuge to prevent vibration.
- v. The centrifuge lid was secured, and samples were centrifuged at 12,000 rpm for 5 minutes.
- vi. After centrifugation, three distinct layers were visible:
 - a. Bottom layer (red): Packed red blood cells
 - b. Middle layer (thin, whitish): Buffy coat containing white blood cells and platelets
 - c. Top layer (clear/straw-colored): Plasma
- vii. The tubes were read using a microhematocrit reader or a standard ruler with the scale positioned so that the bottom of the red cell column aligned with zero and the top of the plasma column aligned with 100.
- viii. The hematocrit was read at the junction between the red cell column and the buffy coat. Measurement was performed in duplicate, and the average was calculated. Values differing by more than 2% were repeated.

Calculation:

Packed Cell Volume (%) = (Height of red cell column / Total height of blood column) × 100

Normal reference range for male *Wistar* rats: 40-54%

3.4.4 Mean Corpuscular Volume (MCV)

MCV represents the average volume of individual red blood cells and was calculated from the hematocrit and RBC count values.

Principle: MCV provides information about the size of red blood cells and helps classify anemia (microcytic, normocytic, or macrocytic).

Formula: $MCV \text{ (fL)} = [\text{Hematocrit (\%)} \times 10] / \text{RBC count } (\times 10^6/\mu\text{l})$

The factor of 10 converts the units appropriately (1 fL = 10^{-15} L).

Example Calculation: If Hematocrit = 45% and RBC count = $8.0 \times 10^6/\mu\text{l}$, $\text{MCV} = (45 \times 10) / 8.0 = 56.25 \text{ fL}$

Normal reference range for male *Wistar* rats: 50-60 fL

Interpretation:

- $\text{MCV} < 50 \text{ fL}$: Microcytic (small red cells)
- $\text{MCV} 50\text{-}60 \text{ fL}$: Normocytic (normal-sized red cells)
- $\text{MCV} > 60 \text{ fL}$: Macrocytic (large red cells)

3.4.5 Mean Corpuscular Hemoglobin (MCH)

MCH represents the average amount of hemoglobin per red blood cell and was calculated from the hemoglobin concentration and RBC count.

Principle: MCH indicates the hemoglobin content of individual red blood cells and complements MCV in characterizing red cell morphology.

Formula: $\text{MCH (pg)} = [\text{Hemoglobin (g/dL)} \times 10] / \text{RBC count} (\times 10^6/\mu\text{l})$

The factor of 10 converts grams to picograms appropriately ($1 \text{ pg} = 10^{-12} \text{ g}$).

Example Calculation: If Hemoglobin = 14.5 g/dL and RBC count = $8.0 \times 10^6/\mu\text{l}$, $\text{MCH} = (14.5 \times 10) / 8.0 = 18.13 \text{ pg}$

Normal reference range for male *Wistar* rats: 17-24 pg

3.4.6 Mean Corpuscular Hemoglobin Concentration (MCHC)

MCHC represents the average concentration of hemoglobin in a given volume of packed red blood cells and was calculated from the hemoglobin concentration and hematocrit values.

Principle: MCHC reflects the hemoglobin concentration within red blood cells and is useful in distinguishing between different types of anemia.

Formula: $\text{MCHC (g/dL)} = [\text{Hemoglobin (g/dL)} / \text{Hematocrit (\%)}] \times 100$

Example Calculation: If Hemoglobin = 14.5 g/dL and Hematocrit = 45%, $MCHC = (14.5 / 45) \times 100 = 32.2 \text{ g/dL}$

Normal reference range for male *Wistar* rats: 30-36 g/dL

Interpretation:

- MCHC < 30 g/dL: Hypochromic (decreased hemoglobin concentration)
- MCHC 30-36 g/dL: Normochromic (normal hemoglobin concentration)
- MCHC > 36 g/dL: Hyperchromic (increased hemoglobin concentration, rare)

3.4.7 White Blood Cell (WBC) Count

Total WBC count was performed using the improved Neubauer hemocytometer method with Turk's solution, which lyses red blood cells while preserving white blood cells.

Principle: White blood cells are diluted in a solution (Turk's solution) that lyses red blood cells through the action of acetic acid, making white cells easier to visualize and count. The diluted white cells are counted microscopically in a counting chamber of known volume.

Reagent - Turk's Solution:

- Glacial acetic acid: 2 ml
- Gentian violet (1% aqueous solution): 1 ml
- Distilled water: 100 ml

The acetic acid lyses red blood cells, while gentian violet stains the nuclei of white blood cells, making them easier to identify.

Procedure:

1. Well-mixed EDTA blood (20 μ l) was drawn into a clean, dry white blood cell (WBC) pipette up to the 0.5 mark.
2. The external surface was carefully wiped clean.

3. The tip of the pipette was immediately immersed in Turk's solution, and the solution was drawn up to the 11 mark, giving a 1:20 dilution.
4. The pipette was sealed and gently rotated for 2-3 minutes to ensure thorough mixing and complete lysis of red blood cells.
5. The first 3-4 drops were discarded.
6. The counting chamber of an improved Neubauer hemocytometer was loaded with the diluted blood.
7. A clean coverslip was placed over the chamber, and the preparation was allowed to stand for 2-3 minutes for cells to settle.
8. The hemocytometer was examined under a light microscope using the 10× objective (low power).
9. White blood cells were counted in the four large corner squares of the ruled area. Each large corner square has an area of 1 mm² and is subdivided into 16 smaller squares.
10. All white blood cells within the four large corner squares were counted, including those touching the top and left boundaries but excluding those touching the bottom and right boundaries.
11. The count was performed in duplicate (both chambers), and the average was calculated. Counts differing by more than 10% were repeated.

Calculation: $\text{WBC count } (\times 10^3/\mu\text{l}) = (\text{Number of cells counted} \times \text{Dilution factor}) / (\text{Area counted} \times \text{Depth of chamber})$

Where:

- Number of cells counted = Total cells in 4 large corner squares
- Dilution factor = 20
- Area counted = 4 mm²

- Depth of chamber = 0.1 mm

Simplified formula: WBC count ($\times 10^3/\mu\text{l}$) = $N \times 50$

Where N = average number of cells counted in 4 large corner squares

Normal reference range for male *Wistar* rats: $6-12 \times 10^3/\mu\text{l}$

3.4.7 Differential White Blood Cell Count

Differential leukocyte count was performed to determine the relative percentages of different types of white blood cells (neutrophils, lymphocytes, monocytes, eosinophils, and basophils).

Principle: A thin blood film is prepared, stained with Leishman's stain (a Romanowsky-type stain), and examined microscopically. Different types of white blood cells are identified based on their morphological characteristics and staining properties, and their relative proportions are determined.

Reagent - Leishman's Stain: Commercial Leishman's stain powder (a mixture of eosin and methylene blue) dissolved in absolute methanol.

Procedure:

1. Blood Film Preparation:

A clean, grease-free glass slide was used.

A small drop of well-mixed EDTA blood (about 2 mm diameter) was placed near one end of the slide.

A spreader slide (another clean slide) was held at a 30-45° angle and backed into the blood drop until the blood spread along the edge.

The spreader slide was then pushed forward smoothly and rapidly to create a thin, even film.

The film was air-dried by waving it gently or allowing it to dry at room temperature.

Staining:

The dried blood film was placed on a staining rack. Leishman's stain (undiluted) was poured onto the film to cover it completely and left for 2 minutes to fix the cells. An equal volume of buffered distilled water (pH 7.2) was added to the stain on the film to cover it completely and left for 2 minutes to fix the cells.

An equal volume of buffered distilled water (pH7.2) was added to the stain on the slide and mixed by gentle rocking and mixed by gentle rocking. The diluted stain was left for 10 minutes to stain the cells. The stain was washed off gently with tap water or buffered distilled water. The slide was allowed to air dry completely.

Microscopic Examination:

The dried, stained film was examined under a light microscope. The film was first scanned using the 10× objective to assess overall quality and distribution of cells. The examination area was located using the 40× objective—the area where red blood cells are close together but not overlapping (the feather edge or tail of the film). The examination was then continued using the 100× oil immersion objective. A drop of immersion oil was placed on the film, and the oil immersion lens was carefully lowered into the oil.

White blood cells were systematically identified and counted across the film in a serpentine pattern (moving back and forth across the slide). A total of 100 white blood cells were counted and classified based on morphological characteristics:

Neutrophils:

- Size: 12-15 μm
- Nucleus: Multi-lobed (2-5 lobes), purple
- Cytoplasm: Pink with fine granules

Lymphocytes:

- Size: 7-12 μm (small) or 12-16 μm (large)
- Nucleus: Round or slightly indented, dark purple
- Cytoplasm: Scant, pale blue

Monocytes:

- Size: 15-22 μm (largest white cell)
- Nucleus: Kidney-shaped or horseshoe-shaped, purple
- Cytoplasm: Blue-gray with fine granules

Eosinophils:

- Size: 12-17 μm
- Nucleus: Usually bi-lobed, purple
- Cytoplasm: Filled with large, red-orange granules

Basophils:

- Size: 10-14 μm
- Nucleus: Obscured by granules
- Cytoplasm: Filled with large, deep purple-black granules

Each cell type was recorded using a differential counter (manual or electronic tally counter). The percentage of each cell type was calculated.

Calculation:

Percentage of each cell type = (Number of that cell type / Total WBC counted) $\times$ 100

Absolute count for each cell type = (Percentage $\times$ Total WBC count) / 100

Normal reference ranges for male *Wistar* rats:

Neutrophils: 9-34% (absolute: $0.54-4.08 \times 10^3/\mu\text{l}$)

Lymphocytes:65-85%(absolute: $3.90-10.20 \times 10^3/\mu\text{l}$)

Monocytes:0-5%(absolute: $0-0.60 \times 10^3/\mu\text{l}$)

Eosinophils:0-6%(absolute: $0-0.72 \times 10^3/\mu\text{l}$)

Basophils:0-2%(absolute: $0-0.24 \times 10^3/\mu\text{l}$)

3.4.8 Platelet Count

Platelet count was estimated using the direct microscopic method on stained blood films.

Principle: Platelets are counted in relation to red blood cells on a stained blood film, and the platelet count is estimated based on the red blood cell count.

Procedure:

- i. A well prepared and stained blood film (prepared as described in section 3.3.8) was examined under oil immersion ($100\times$ objective).
- ii. Ten fields with an even distribution of red blood cells were selected.
- iii. In each field, the number of platelets and red blood cells were counted.
- iv. The average number of platelets per field was calculated.

Calculation: Platelet count ($\times 10^3/\mu\text{l}$) = (Average number of platelets per field / Average number of RBCs per field) $\times$ RBC count ($\times 10^6/\mu\text{l}$) $\times 1000$

Alternative method (Rule of Thumb): If there are an average of 8-20 platelets per oil immersion field in an area where red cells are close but not touching, the platelet count is considered adequate ($150-400 \times 10^3/\mu\text{l}$).

Normal reference range for male Wistar rats: $500-1,300 \times 10^3/\mu\text{l}$

Note: Rat platelet counts are normally higher than human values.

3.5. BIOCHEMICAL ANALYSIS

Clinical biochemistry parameters measured included:

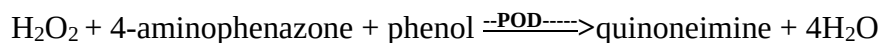
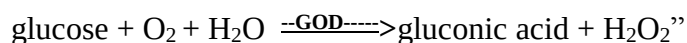
- Liver function: ALT, AST, ALP, total bilirubin
- Lipid profile: Total Cholesterol, Triglycerides, HDL, LDL, VLDL
- Renal function: Urea, creatinine and electrolytes
- Metabolic markers: fasting blood glucose

3.5.1 Assessment of status of glucose metabolism

a. Determination of the fasting blood glucose (FBG).

Principle:

The Randox glucose kit was used. The principle applied is the glucose oxidase method as described by Barham and Trinder (1972). Glucose is determined after enzymatic oxidation in the presence of glucose oxidase (GOD). The hydrogen peroxide formed reacts, under catalysis of peroxidase (POD), with phenol and 4-aminophenazone to form a red-violet quinoneimine dye indicator. Using a visible spectrophotometer set at 500nm, the absorbance of the dye which is equivalent to the plasma glucose concentration was read. Using the known absorbance and concentration of the standard, the concentration of glucose in the sample was then determined.



Procedure:

Three tubes were labelled standard, sample and reagent blank respectively. 20µl of the standard solution was pipetted into the tube labelled standard, 20µl of the sample solution was also pipetted

into the tube labelled sample, while nothing was put in the tube labelled reagent blank. 2000 µl of Reagent 1 was then added to each tube. Mixing, incubation for 10min at 37°C was done, after which the absorbance of the standard (A_{standard}) and the sample (A_{sample}) against the reagent blank was measured within 60 minutes using a spectrophotometer set at 500nm.

Calculation

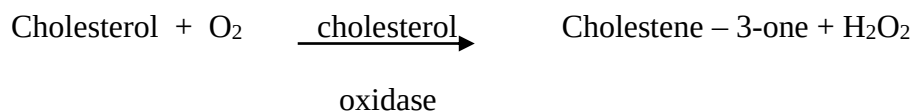
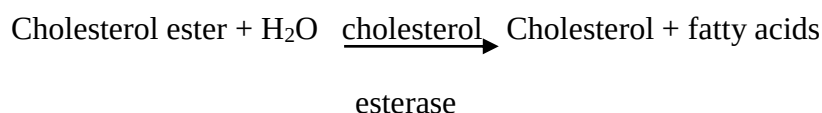
$$\text{Glucose concentration (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Standard conc. (mg/dl)}$$

3.5.2. Assessment of the lipid profile

1. Determination of Plasma Total Cholesterol (Trinder, 1969).

Assay Principle

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase (Trinder, 1969).



Procedure:

The following were pipetted into curvette:

	Reagent blank (µl)	Standard (µl)	Sample (µl)
Distilled H ₂ O	10µl	-----	-----
Standard	----	10µl	-----

Sample	-----	-----	10μl
Reagent	1000μl	1000μl	1000μl

The solution was mixed and incubated for 5 minutes at 37⁰c. The absorbance of the sample (A_{sample}) was measured against the reagent blank within 60 minutes.

Calculation:

Conc. Of Cholesterol in sample

$$= \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Conc. of standard (mg/dl)}$$

2.Determination of Plasma HDL-Cholesterol (Trinder, 1969; Tietz, 1990).

Assay Principle

Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL (high density lipoprotein) fraction, which remains in the supernatant, is determined (Tietz, 1990).

Procedure:

1. Precipitation

The following were pipetted into centrifuge tubes;

Sample/standard	500μl
Diluted precipitant (R1)	1000μl

The solution was mixed and allowed to sit for 10 minutes at room temperature, after which it was centrifuged for 10 minutes at 4,000rpm. The clear supernatant was separated off within two hours and the cholesterol content was determined by the CHOD-PAP method.

2. Cholesterol CHOD-PAP Assay

The following were pipetted into test tube;

	Reagent blank	Standard	Sample
Distilled H ₂ O	100μl	-----	-----
Standard supernatant	-----	100μl	-----
Sample supernatant	-----	-----	100μl
CHOL reagent	1000μl	1000μl	1000μl

The solutions are mixed for 5 minutes at 37⁰c. The absorbance of the sample (A_{sample}) and standard (A_{standard}) was measured against the reagent blank within 60 minutes at a wavelength of 500nm.

Calculation:

1. HDL Cholesterol

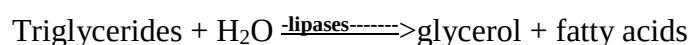
Concentration of HDL cholesterol in supernatant

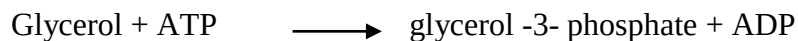
$$= \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Concentration of Standard}$$

3.Determination of Plasma Triglycerides (Trinder, 1969; Tietz, 1990).

Principle:

The triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase





Note: to correct for free glycerol, subtract 0.11mmol/l (10mg/dl) from the triglyceride value obtained.

Procedure:

The following were pipetted into test tubes:

	Reagent blank		Standard	Sample
Standard	-----	10µl	-----	
Sample	-----	-----	10µl	
Reagent R1	1000µl	1000µl	1000µl	

Solution was mixed and incubated for 5 minutes at 37⁰c, and the absorbance of the sample (A_{sample}) and standard (A_{standard}) was measured against the reagent blank within 60 minutes at wavelength of 500nm/546nm.

Calculation:

Triglyceride concentration= $\frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Standard Conc.}$

(mmol/l or mg/dl)

4.Determination of serum LDL-cholesterol (Trinder, 1969; Tietz, 1990).

LDL-cholesterol is calculated using the Friedewald equation as follows:

In mmol/l:

$$\text{LDL} = \text{Total Cholesterol} - \frac{\text{Triglycerides}}{2.2} - \text{HDL Cholesterol}$$

In mg/dl:

$$\text{LDL} = \text{Total Cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL Cholesterol}$$

5.Determination of serum VLDL-cholesterol (Friedewald *et al.*, 1972).

VLDL-cholesterol will be calculated using the Friedewald's formula:

In mmol/l:

$$\text{VLDL- cholesterol} = \frac{\text{Triglycerides}}{2.2}$$

In mg/dl:

$$\text{VLDL- cholesterol} = \frac{\text{Triglycerides}}{5}$$

3.5.3. Assessment of kidney function status and Uric Acid

a. Determination of plasma electrolytes: sodium (Na⁺), potassium (K⁺), chloride (Cl⁻) and bicarbonate (HCO₃⁻)

The plasma sodium, potassium, chloride and bicarbonate ion levels were determined using Ion Selective Electrode Device (Tietz *et. al.*, 1996).

Principle

An ion-selective electrode (ISE), also known as a specific ion electrode (SIE), is a transducer (or sensor) that converts the activity of a specific ion dissolved in a solution into an electrical potential, which can be measured by a voltmeter or pH meter. The ion selective membrane is a membrane across which only the intended ion can be transported. The transport of ions from a high concentration to a low one through a selective binding with some sites within the ion selective membrane creates a potential difference/reduction potential or voltage which is theoretically dependent on the logarithm of the ionic activity (Nernst equation). This explains the relationship of the ionic activity to the free energy change that is captured by the voltmeter (Tietz *et. al.*, 1996).

This potential difference/reduction potential (whose magnitude is in turn equivalent to the concentration of the ion generating it in the sample), is given by the Nernst equation:

$$E = E^{\circ} - \frac{N}{n} \times \log \frac{a_{\text{red}}}{a_{\text{ox}}} = E^{\circ} - \frac{0.0592\text{V}}{n} \times \log \frac{a_{\text{red}}}{a_{\text{ox}}}$$

where:

E = electrode potential of the half cell

E° = standard electron potential when $a_{\text{red}}/a_{\text{ox}} = 1$

n = number of electrons involved in the reduction reaction

$N = (R \times T \times \ln 10)/F$ (the Nernst factor if $n=1$)

$N = 0.0592V$ if $T = 298.15\text{ K}$ (25°C)

$N = 0.0615V$ if $T = 310.15\text{ K}$ (37°C)

R = gas constant ($28.31431\text{ Joules} \times \text{K}^{-1} \times \text{mol}^{-1}$)

T absolute temperature (unit: K, kelvin)

F = faraday constant ($= 96,487\text{ Coulombs} \times \text{mol}^{-1}$)

$\ln 10$ = natural logarithm of 10 = 2.303

a = activity

“ $a_{\text{red}}/a_{\text{ox}}$ = product of mass action for the reduction reaction” (Tietz *et. al.*, 1996).

A difference in ionic composition of the two solutions involved in the ISE device causes an electrical potential difference to develop across the ion selective membrane. A change in potential across the ion selective membrane are measured with respect to a reference electrode, the potential of which is constant. The change in potential difference between the reference electrode (E_{Ref}) and the ion selective electrode (E_{ISE}) for the sample is proportional with the potential difference (E_{Cell}) already known for a calibrated solution of known composition and quantity. Hence the unknown solution is therefore said to be the same in terms of composition and quantity as the calibrated solution (Tietz *et. al.*, 1996).

The basic formula of the net potential generated by the unknown solution is given for the galvanic cell as:

$$E_{\text{Cell}} = E_{\text{ISE}} - E_{\text{Ref}}(\text{Tietz } et. al., 1996).$$

The strength of this charge (E_{cell}) is directly proportional to the concentration of the selected ion.

Calculation of analyte concentration is done automatically by the ISE analyzer/device. It calculates the analyte concentration from the potential developed and recorded by the voltmeter and presents it as a digital number on the screen of the ISE analyser/device (Tietz *et. al.*, 1996; Burtiset *al.*, 2008).

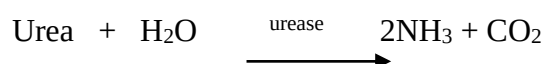
Procedure:

Depending on which electrolyte concentration is to be determined, with all the materials ready and the specific electrode with the ion selective membrane and electrode specific for the specific electrolyte installed in the ISE analyzer and with the electrode wire connected to one terminal of a galvanometer or voltmeter and the other terminal connected to the reference electrode, both electrodes are then immersed in the solution to be tested. The passage of the ion through ion selective membrane creates an electrical current, which registers on the galvanometer; and because the galvanometer has already been calibrated against standard solutions of varying concentration, the ionic concentration in the test solution can be automatically derived from the galvanometer reading. Calculation of analyte concentration is done automatically by the analyzer which calculates the analyte concentration from the potential developed and recorded by the voltmeter/galvanometer. The concentration of the sodium, potassium, chloride, and bicarbonate depending on which is being measured then appears on the display screen of the ISE analyzer as a digital number (Tietz *et. al.*, 1996; Burtiset *al.*, 2008).

b. Determination of plasma urea: Randox lab. kit, UK, was used (Weatherburn, 1967)

Principle:

Urea in plasma is hydrolyzed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction (Weatherburn, 1967)



$\text{NH}_3 + \text{hypochloric acid} + \text{phenol} \rightarrow \text{indophenol (blue compound)}$

The absorbance of the resulting solution (indophenol) is measured spectrophotometrically at a wavelength of 546nm. The absorbance (A) of the resulting solution being equivalent to the urea in the sample and its concentration was determined by the formula:

$$\text{Urea concentration} = \frac{A_{\text{Sample}}}{A_{\text{standard}}} \times \text{Standard conc. (mg/dl)}$$

Procedure:

Three cuvettes were labelled standard, sample and blank respectively. 10µl of the standard solution (CAL) was pipetted into the tube labelled standard, 10µl of the sample solution was also pipetted into the tube labelled sample, while 10µl of distilled water was put in the tube labelled reagent blank. 100 µl of the solution labelled Reagent 1 (a mix of 116mmol/l EDTA, 6mmol/l sodium nitroprusside and 1g/l urease), and 2.5ml of the solutions labelled Reagent 2 (diluted 120mmol/l

phenol) and Reagent 3 (a mix of 27mmol/l sodium hypochloride (diluted) and 0.14N sodium hydroxide) in the kit, was then added to each tube sequentially.

Each tube was mixed immediately and incubated at 37°C for 15mins.

The absorbance of the sample (A_{sample}) and standard (A_{standard}) were read against the blank at 546nm. (The colour of the reaction is known to be stable for at least 8 hours)".

Calculation:

Urea concentration = $\frac{A_{\text{Sample}}}{A_{\text{standard}}} \times \text{Standard conc. (mg/dl)}$

A_{standard}

c. Determination of plasma creatinine: Randox lab. kit, UK was used (Bartels and Bohmer, 1972).

Principle: Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The intensity of the complex formed is directly proportional to creatinine concentration (Bartels and Bohmer, 1972). The absorbance of the resulting solution is measured two times 2 minutes from each other spectrophotometrically at a wavelength of 492nm (Bartels and Bohmer, 1972). The change in absorbance of the standard and sample solutions is then determined and inputted with the known concentration of the standard into the prescribed formula "to determine the concentration of creatinine in the sample.

Procedure:

Three cuvettes were labelled standard, sample and blank respectively. 100µl of the standard solution was pipetted into the tube labelled standard, 100µl of the sample solution was also pipetted into the tube labelled sample, while 100µl of distilled water was put in the tube labelled reagent blank. 1000 µl of the solution labelled working reagent (Picric acid + Sodium Hydroxide) was

then added to each tube sequentially and the resulting solutions mixed. After 30 seconds the absorbance A_1 of the standard and sample were read after zeroing the spectrophotometer set at 492nm, with the blank. Exactly 2 minutes later the absorbance A_2 of standard and sample were read again.

Calculation:

$$\boxed{\Delta A}_{\text{sample}} = A_2 - A_1 \text{ (for sample)}$$

$$\Delta A_{\text{standard}} = A_2 - A_1 \text{ (for standard)}$$

Concentration of creatinine in plasma:

$$\frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{Concentration of standard (mg/dl)}.$$

3.5.4. Assessment of liver function status

a. Determination of plasma total protein: Radox lab. kit, UK, was used (Tietz, 1995).

Principle:

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a coloured complex which will be measured spectrophotometrically at a wavelength of 546nm (Tietz, 1995). The intensity of the coloured complex formed is proportional to the concentration of total protein in the sample” (Tietz, 1995).

The concentration of total protein is then calculated using the formula:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard (g/l)}$$

Absorbance of Standard

Procedure:

Three cuvettes were labelled standard, sample and blank respectively. 0.02ml of the standard solution (CAL) was pipetted into the cuvette labelled standard, 0.02ml of the sample plasma solution was also pipetted into the cuvette labelled sample, while 0.02ml of distilled water was put in the cuvette labelled reagent blank. 1ml of the solution labelled R1 (made up of a mixture of sodium hydroxide; Na-k-tartrate; potassium iodine and cupric sulphate, was then added to the standard and sample cuvettes sequentially and the R2: blank reagent (made up of a mixture of sodium hydroxide and Na-K-tartrate) and the resulting solutions mixed and incubated for 30 minutes at 25°C before readings were taken. The absorbance of the sample (A_{sample}) and of standard (A_{standard}) were measured against the reagent blank at a wavelength of 546nm” and 1cm light path.

Calculation:

$$\text{Total protein concentration} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Conc. of standard (g/l)}$$

b. Determination of plasma albumin: Randox lab. kit, UK, was used (Doumas *et al.*, 1971)

Principle:

The measurement of plasma albumin is based on its quantitative binding to the indicator 3,3,5,5-tetrabromo-m-cresol sulphonephthalein (bromocresolgreen, BCG). The albumin–BCG-complex absorbs maximally at 578nm, the level of absorbance being directly proportional to the concentration of albumin in the sample (Doumas *et al.*, 1971).

The concentration of albumin is then calculated using the formula:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard (g/dl)}$$

Absorbance of standard

Procedure:

Three cuvettes were labelled standard, sample and blank respectively. 0.01ml of the standard solution (CAL) was pipetted into the cuvette labelled standard, 0.01ml of the sample plasma solution was also pipetted into the cuvette labelled sample, while 0.01ml of distilled water was put in the cuvette labelled reagent blank. 3ml of the solution labelled R1, (composed of BCG concentrate, succinate buffer, bromocresolgreen, Brij 35 and preservative) was then added to each cuvette sequentially and the resulting solutions mixed and incubated for 20 minutes at 25°C before readings were taken. The absorbance of the sample (A_{sample}) and of standard (A_{standard}) were measured against the reagent blank at a wavelength of 578nm and 1cm light path.

Calculation:

The albumin concentration in the sample was calculated using the formula:

$$\text{Albumin concentration (g/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Concentration of standard (g/l)}$$

c. Determination of plasma total bilirubin: Randox lab. kit, UK, was used (Jendrassik and Grof, 1938).

Principle:

The colorimetric method used was based on that described by Jendrassik and Grof, (1938). Total bilirubin was determined in the presence of caffeine, which releases albumin bound to bilirubin, by the reaction with diazotized sulphanilic acid. Both this released bilirubin and direct (conjugated)

bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. The resulting solution was measured spectrophotometrically at a wavelength of 578nm (Jendrassik and Grof, 1938) with the level of absorbance being indirectly proportional to the concentration of bilirubin in the sample.

The concentration of bilirubin is then calculated using the formula:

$$\text{Total bilirubin in mg/dl} = 10.8 \times \text{absorbance of sample at 578nm}$$

Procedure:

Into two cuvettes labelled “Sample Blank” and “Sample”, 200µl of Reagent 1 (made up of a mix of sulphanilic acid and hydrochloric acid) was added, followed by 1 drop (50µl) of Reagent 2 (sodium nitrite) being added to the sample cuvette only. This was closely followed by the addition of 1000µl of Reagent 3 (caffeine and sodium benzoate) and 200µl of the sample to the two cuvettes. Mixing and incubating for 10 minutes at 25°C was done, followed by the addition of 1000µl of Reagent 4 (tartrate and sodium hydroxide) to the two cuvettes. Mixing and incubating for a further 5-30 minutes at 25°C was done. The absorbances of the sample against sample blank at 578nm were read.

Calculation:

$$\text{Total bilirubin in mg/dl} = 10.8 \times \text{absorbance of sample at 578nm}$$

d. Determination of plasma direct/conjugated and indirect/unconjugated bilirubin:

The Randox lab. kit, UK, was also used (Jendrassik and Grof, 1938).

Principle: The blue coloured complex formed by the reaction of the sample with diazotized sulphanilic acid is indicative of conjugated (direct) bilirubin alone, since caffeine has not been added (indirect bilirubin is only added to make up total bilirubin when caffeine breaks down the bilirubin-albumin complex to release free bilirubin). Its absorbance is measured spectrophotometrically at 546nm.

The direct bilirubin is calculated using the formula:

Direct bilirubin in mg/dl = $14.4 \times$ absorbance of sample at 546nm

While the indirectly reacting bilirubin (unconjugated bilirubin or indirect bilirubin) is calculated by subtracting the directly reacting bilirubin (direct bilirubin) from the total bilirubin value (Kirk, 2008).

Procedure:

Into two cuvettes labelled “Sample Blank” and “Sample”, 200µl of Reagent 1 (made up of a mix of sulphanilic acid and hydrochloric acid) was added, followed by 1 drop (50µl) of Reagent 2 (sodium nitrite) being added to the sample cuvette only. This was closely followed by the addition of 2000µl of 0.9% NaCl and 200µl of the sample to the two cuvettes. Mixing and incubating for 10 minutes at 25°C was done. The absorbances of the sample against sample blank at 546nm was then determined.

Calculation:

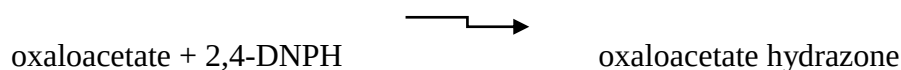
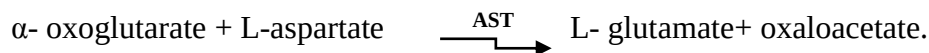
Direct bilirubin in mg/dl = $14.4 \times$ absorbance of sample at 546nm

The indirectly reacting bilirubin (unconjugated bilirubin or indirect bilirubin) is calculated by subtracting the directly reacting bilirubin (direct bilirubin) from the total bilirubin value:

Indirect bilirubin = total bilirubin – direct bilirubin (Kirk, 2008).

e. **Determination of plasma aspartate aminotransferase (AST), EC 2.6.1.57.** Randox lab. kit, UK, was used (Reitman and Frankel, 1957).

Principle:



Aspartate aminotransferase (AST) catalyses the reaction between α -oxoglutarate and L-aspartate to yield L-glutamate and oxaloacetate. AST activity is then measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-Dinitrophenylhydrazine (2, 4-DNPH).

The absorbance of the resulting solution will be measured spectrophotometrically at a wavelength of 546nm (Reitman and Frankel, 1957). AST activity in plasma is obtained by extrapolating it from a standard curve derived from known AST standard activities and their corresponding absorbance's.

Procedure:

Two test tubes were labelled "sample blank" and "sample". 0.1ml of the sample was put into the sample tube only, followed by the addition of 0.5 ml of Reagent 1 (made up of a mix of phosphate buffer, L-aspartate and α -oxoglutarate) into both tubes and 0.1ml of distilled water into the sample blank tube. The solutions were mixed and incubated for exactly 30 minutes at 37°C, then into the tubes was added 0.5ml of Reagent 2 (2, 4-dinitrophenylhydrazine). The solutions were mixed again and allowed to stand for exactly 20 minutes at 25°C, then 5.0ml of Sodium hydroxide was added.

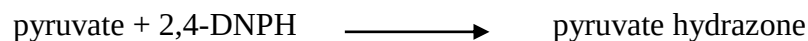
The solutions were mixed and the absorbance of Samples (A_{sample}) read against the sample blank after 5 minutes at a wavelength of 546nm, 1cm light path

Calculation:

The AST activity in plasma samples were obtained by extrapolating them from the standard curve derived from known standard AST activities and their corresponding absorbances f.

D.Determination of plasma alanine aminotransferase (ALT), EC 2.6.1.2: Randox lab. kit, UK, was used (Reitman and Frankel, 1957).

Principle:



Alanine aminotransferase (ALT) catalyses the reaction between α - oxoglutarate and L-alanine to yield L- glutamate and pyruate. Alanine aminotrasferase is measured by monitoring the concentration of pyrurate hydrazone formed with 2, 4-dinitrophenylhydrazine.

The absorbance of the resulting solution is measured spectrophotometrically at a wavelength of 546nm” (Reitman and Frankel, 1957). ALT activity in plasma is obtained by extrapolating it from a known standard curve derived from known standard ALT activity values and their absorbances.

Procedure:

Two test tubes were labelled “sample blank” and “sample”. 0.1ml of the sample was put into the sample tube only, followed by the addition of 0.5 ml of Solution R1 (a mix of phosphate buffer, L-alanine and α -oxoglutarate) into both tubes and 0.1ml of distilled water into the sample blank tube only. The solutions were mixed and incubated for exactly 30 minutes at 37⁰ C, then into the tubes was added 0.5ml of Solution R2 (2, 4 – Dinitrophenylhydrazine). The solutions were mixed again and allowed to stand for exactly 20 minutes at 25⁰C, then 5.0ml of sodium hydroxide was added. The solutions were mixed and the absorbance of samples (A_{sample}) read against the sample blank after 5 minutes at a wavelength of 546nm, 1cm light path

Calculation:

Using the absorbance results for the samples, the activity of ALT in plasma was obtained by extrapolating it from a standard curve of the known standard ALT activity levels and their corresponding absorbances (appendix III: G)

E.Determination of plasma alkaline phosphatase (ALP), EC 3.1.3.1: Randox lab. kit, UK, was used (DGKC, 1972).

Principle:

This is an optimized standard method according to the recommendations of the deutsche gesellschaft fur klinische chemie (DGKC). Alkaline phosphatase (ALP) enzyme reacts with disodium para-nitrophenylphosphate and liberates phenol which forms a yellow coloured complex at alkaline pH.



The absorbance of the resulting yellow solution is measured spectrophotometrically at a wavelength of 405nm (DGKC, 1972). The absorbance is again read after 1, 2, and 3 minutes at wavelength of 405nm, 1cm light path, at 25⁰C.

To calculate ALP activity, the following formula is used:

$$\text{IU/l} = 3300 \times \frac{\Delta A}{\Delta t} \text{ at } 405\text{nm/min};$$

Procedure:

Fresh distilled H₂O was aspirated and used as water blank. The following solutions were then pipetted into a test tube, Sample (0.05ml) and Reagent (3.00ml) made up of a mix of diethanolamine buffer, MgCl₂ and substrate: p-nitrophenylphosphate. After mixing, the initial absorbance was read and timer was simultaneously started. The absorbance was again read after 1, 2, and 3 minutes at wavelength of 405nm, 1cm light path, at 25⁰C.

Calculation:

To calculate ALP activity, the following formula was used:

$$\text{IU/l} = 3300 \times \frac{\Delta A}{\Delta t} \text{ at } 405\text{nm/min}$$

Where the mean change in absorbance per minute was represented by $\frac{\Delta A}{\Delta t}$

CHAPTER FOUR

RESULTS

This chapter presents the findings of the qualitative and quantitative phytochemical analysis of the MIRACLE SEED ULTIMA supplement. It also details the results of the Proximate analysis, the in-vitro antioxidant capacity of the supplement as well as the results of both acute and sub-acute toxicity studies.

Table 4.1 below outlines the results for the analysis aimed to determine both the presence and concentration of major bioactive constituents. Quantitative results are reported as mean $\pm$ standard error of the mean (SEM) in milligrams per gram (mg/g) of the sample. The screening indicated that steroids were present at very high levels (+++), cardiac glycosides and terpenoids at high levels (++) , while tannins, coumarins, flavonoids, and alkaloids were detected at low levels (+). Saponins, phenols, phlobatannins, and anthraquinones were absent (-)

Table 4.2 details the results of the quantitative phytochemical composition of Miracle Seed Ultima. The low SEM values indicate high precision and reproducibility of the measurements. Among the quantified phytochemicals, cardiac glycosides were the most abundant, followed by steroids, flavonoids, coumarins, alkaloids, terpenoids, and tannins.

Table 4.3 outlines the results for the relative abundance of the constituent phytochemicals in Miracle seed Ultima. These data indicate that cardiac glycosides constitute the highest amount of the total quantified phytochemicals, followed by steroids, flavonoids, coumarins, alkaloids, and terpenoids. Tannins constituted the least amount of phytochemicals present.

Table 4.4 presents the quantitative elemental composition of the MSU supplement. It measures pH and concentrations of essential minerals including calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), and heavy metals lead (Pb) and cadmium (Cd) in mg/kg. The supplement exhibited a slightly acidic pH of 4.20 ± 0.06 . Ca shows the highest mineral level at 6304.00 ± 2.08 mg/kg, followed by K at 5252.33 ± 1.20 mg/kg and Mg at 3704.67 ± 2.40 mg/kg. Na was present at 1566.00 ± 2.08 mg/kg. Trace elements including Fe (710.70 ± 0.40 mg/kg), Zn (587.17 ± 0.66 mg/kg), Mn (330.67 ± 0.42 mg/kg), and Cu (112.70 ± 0.25 mg/kg) were also detected at substantial levels. Heavy metals Lead (Pb) and Cadmium (Cd) were not detected (ND), indicating low contamination risk and compliance with safety standards. The values are provided as mean $\pm$ standard error of the mean (SEM), demonstrating high consistency with small SEM values (e.g., 2.08 for Ca, 0.40 for Fe), reflecting the uniformity and quality of the supplement formulation.

Table 4.1: Qualitative Phytochemical Screening of MIRACLE SEED ULTIMA Sample

Phytochemical Constituent	Presence in MIRACLE SEED ULTIMA
Tannins	+
Coumarins	+
Flavonoids	+
Cardiac Glycosides	++
Saponins	-
Steroids	+++
Terpenoids	++
Phenols	-
Phlobatannins	-
Alkaloids	+
Anthraquinones	-

Key: Negative (-) = Absent Positive (+) = Present (low) Positive (++) = Present (high) Positive (+++) = Present (very high)

Table 4.2: Quantitative Phytochemical Composition of MIRACLE SEED ULTIMA Sample

Phytochemical Constituent	Standard Used	Mean Concentration (mg/g)
Flavonoids	Quercetin Equivalent (QE)	151.82 ± 3.58
Terpenoids	Beta-Sitosterol (BSE) Equivalent	87.38 ± 3.57
Cardiac Glycosides	Securidaside Equivalent (SE)	310.59 ± 2.36
Steroids	Stigmasterol Equivalent (SE)	174.22 ± 5.01
Tannins	Tannic Acid Equivalent (TAE)	49.92 ± 2.66
Alkaloids	Atropine Equivalent (AE)	93.81 ± 7.86
Coumarins	Esculin Equivalent (EE)	113.27 ± 4.81

Table 4.3: Relative Abundance of Phytochemicals in MIRACLE SEED ULTIMA

Phytochemical Constituent	Concentration (mg/g)	Relative Abundance (%)
Cardiac Glycosides	310.59 ± 2.36	31.7
Steroids	174.22 ± 5.01	17.8
Flavonoids	151.82 ± 3.58	15.5
Coumarins	113.27 ± 4.81	11.5
Alkaloids	93.81 ± 7.86	9.6
Terpenoids	87.38 ± 3.57	8.9
Tannins	49.92 ± 2.66	5.1

Table 4.4: Quantitative Elemental Composition of Miracle Seed Ultima®

Parameter	Mean ± SEM
Ph	4.20 ± 0.06
Ca	6304.00 ± 2.08
Mg	3704.67 ± 2.40
Na	1566.00 ± 2.08
K	5252.33 ± 1.20
Fe	710.70 ± 0.40
Zn	587.17 ± 0.66
Mn	330.67 ± 0.42
Cu	112.70 ± 0.25
Pb	ND
Cd	ND

Note: ND = Not Detected

The antioxidant properties of MIRACLE SEED ULTIMA were evaluated using four complementary in vitro assays: DPPH radical scavenging activity, FRAP reducing activity, Nitric Oxide radical scavenging activity, and Total Antioxidant Capacity (TAC). The results are presented in Table 4.4. The DPPH radical scavenging assay quantifies the ability of a sample to donate electrons or hydrogen atoms to neutralize free radicals. MIRACLE SEED ULTIMA exhibited a DPPH radical scavenging activity of $55.77 \pm 3.67\%$, indicating moderate radical neutralization capacity under the test conditions. In comparison, the reference antioxidant, ascorbic acid, demonstrated a higher inhibition of $95.44 \pm 0.39\%$, consistent with its established strong antioxidant profile. The relatively lower inhibition by MIRACLE SEED ULTIMA reflects a measurable but moderate capacity to reduce DPPH radicals.

In the FRAP assay, which evaluates the reducing power of antioxidants by measuring their ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions, MIRACLE SEED ULTIMA recorded a reducing activity of $51.95 \pm 1.31\%$, whereas ascorbic acid achieved $89.25 \pm 0.67\%$. The FRAP results are consistent with the DPPH assay, showing that MIRACLE SEED ULTIMA possesses electron-donating properties capable of reducing oxidized intermediates, albeit at levels lower than the reference antioxidant.

The Nitric Oxide radical scavenging assay measures the sample's ability to inhibit nitric oxide radicals, which are implicated in oxidative stress. MIRACLE SEED ULTIMA showed a high scavenging activity of $88.15 \pm 2.21\%$, approaching the performance of ascorbic acid at $92.38 \pm 0.41\%$. This indicates strong potential in neutralizing nitric oxide-related free radicals.

The Total Antioxidant Capacity (TAC) assay assesses the overall antioxidant potential through the formation of a phosphomolybdenum complex. MIRACLE SEED ULTIMA demonstrated a TAC

of 0.194 ± 0.016 mg/mL, significantly higher than ascorbic acid's 0.020 ± 0.003 mg/mL, suggesting a robust cumulative antioxidant effect from its bioactive constituents.

All measurements were performed in triplicate, and results are expressed as mean $\pm$ SEM to reflect the reproducibility and precision of the assays. Across all assays, MIRACLE SEED ULTIMA demonstrated consistent antioxidant activity, confirming the presence of bioactive constituents with radical scavenging and reducing potential.

The proximate analysis of MIRACLE SEED ULTIMA was conducted to determine its nutritional composition and physicochemical characteristics. Three batches were analyzed, and the mean percentage values were computed. The results are presented in Table 4.6. The results show that MIRACLE SEED ULTIMA contains a high level of carbohydrates, which constitute 94.03% of the composition (on a dry weight basis), indicating that the solid content of the product is predominantly carbohydrate-based. Moisture content was very high at 97.10%, suggesting the formulation is primarily water-based with substantial water retention. Ash content, representing the total mineral content, was 2.73%. Crude protein, crude fibre, and crude fat were present in low quantities at 2.48%, 0.73%, and 0.02%, respectively. The proximate parameters for non-moisture components sum to approximately 100%, consistent with reporting on a dry weight basis.

Table 4.5: Antioxidant Activity of MIRACLE SEED ULTIMA Compared with Ascorbic Acid

Assay	MIRACLE ULTIMA (Value)	SEED Reference Antioxidant	Standard (Value)
DPPH radical scavenging	55.77 ± 3.67% Inhibition	Ascorbic acid	95.44±0.39% Inhibition
FRAP reducing activity	51.95 ± 1.31% Inhibition	Ascorbic acid	89.25±0.67% Inhibition
Nitric Oxide radical scavenging	88.15 ± 2.21% Inhibition	Ascorbic acid	92.38±0.41% Inhibition
Total Antioxidant Capacity (TAC)	0.194 ± 0.016 mg/mL	Ascorbic acid	0.020±0.003 mg/mL

Values are expressed as mean ± SEM (n = 3).

Table 4.6: Proximate Composition of MIRACLE SEED ULTIMA (per 100 g, n = 3)

Sample	Moisture	Crude	Crude	Crude	Ash	Carbohydrate
	Content (%)	Protein	Fibre	Fat (%)	(%)	(%)
		(%)	(%)			
MIRACLE	97.100	2.480	0.733	0.020	2.733	94.033
SEED						
ULTIMA						

This section outlines findings from the acute toxicity assessment of Miracle Seed Ultima® (MSU) in male *Wistar* rats via the Locke observation approach, focusing on behavioral patterns, physical indicators, and survival rates. After oral dosing with MSU at 10, 100, and 1000 mg/kg, subjects were observed closely for the initial 4 hours and periodically over 14 days.

At the end of fourteen (14) days Phase one Acute toxicity study of Miracle Seed Ultima® treated Male *Wistar* Rats, all the rats were active and alive, with a normal state of external observation and healthy condition when compared with group one (Control). (Table 4.7).

The internal gross pathology of tissues shows all tissue in normal state except few left kidney cysts observed in some test subjects in all groups, when compared with group one (Control). (Table 4.8)

All the rats were active and alive, with a normal state of external observation and healthy condition when compared with group one (Control). (Table 4.9).

The internal gross pathology of tissues shows all tissue in normal state when compared with group one (Control) (Table 4.10).

The daily feed and water consumption remained consistent and comparable to the normal control rats. Generally, all treatment groups maintained similar feed and water intake throughout the acute phase, with no significant differences ($p>0.05$) observed between them and the control.

The treatments did not cause a significant change in relative weight gain/loss in the rats when compared to the control group. Generally, the 10 mg/kg, 100 mg/kg, and 1000 mg/kg treatment groups maintained body weight progression within the range observed in the control group, with no statistically significant differences among the groups ($p>0.05$). Similarly, Miracle Seed Ultima did not cause a statistically significant change in feed efficiency in the rats when compared to the

control group. Generally, the 10 mg/kg, 100 mg/kg, and 1000 mg/kg treatment groups maintained feed efficiency within the range observed in the control group, with no significant differences among the groups ($p>0.05$).

The initial body weight of the rats (Day 0) was not significantly different across all groups ($p>0.05$). Over the course of the study, weekly body weight measurements showed no significant differences among the groups at Day 7 (Week 1) and Day 14 (Week 2) ($p>0.05$). Generally, all treatment groups (10 mg/kg, 100 mg/kg, and 1000 mg/kg) maintained body weights comparable to the control group.

Table 4.12 shows the body weight across the time duration of the study. The initial body weight of the rats (Day 0) was not significantly different across all groups ($p>0.05$) (Table 4.12). Over the course of the study, weekly body weight measurements showed no significant differences among the groups at Day 7 (Week 1) and Day 14 (Week 2) ($p>0.05$) (Tables 4.14). Generally, all treatment groups (10 mg/kg, 100 mg/kg, and 1000 mg/kg) maintained body weights comparable to the control group.

Table 4.7: External observation of Male Wistar Rats treated with Miracle Seed Ultima® in Acute Toxicity Study.

					Abnormal	General
Groups	Survival status	Skin/Fur	Eyes/Nostrils/ Mouth	Anus/Genitals	Discharge	condition
Control	Alive	Normal	Normal	Normal	No	Healthy
Control	Alive	Normal	Normal	Normal	No	Healthy
Control	Alive	Normal	Normal	Normal	No	Healthy
10mg/kg	Alive	Normal	Normal	Normal	No	Healthy
10mg/kg	Alive	Normal	Normal	Normal	No	Healthy
10mg/kg	Alive	Normal	Normal	Normal	No	Healthy
100mg/kg	Alive	Normal	Normal	Normal	No	Healthy
100mg/kg	Alive	Normal	Normal	Normal	No	Healthy
100mg/kg	Alive	Normal	Normal	Normal	No	Healthy
1000mg/kg						
g	Alive	Normal	Normal	Normal	No	Healthy
1000mg/kg						
g	Alive	Normal	Normal	Normal	No	Healthy
1000mg/kg						
g	Alive	Normal	Normal	Normal	No	Healthy

Table 4.8: Internal (Gross Pathology) of Male *Wistar* Rats treated with Miracle Seed Ultima® in Acute Toxicity Study.

Groups	Lungs	Heart	Liver	Kidneys	Spleen	Stomach/Intestine	Brain
Control	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Control	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Control	Normal	Normal	Normal	Normal	Normal	Normal	Normal
10mg/kg	Normal	Normal	Normal	Left Kidney cyst	Normal	Normal	Normal
10mg/kg	Normal	Normal	Normal	Left Kidney cyst	Normal	Normal	Normal
10mg/kg	Normal	Normal	Normal	Normal	Normal	Normal	Normal
100mg/kg	Normal	Normal	Normal	Left Kidney cyst	Normal	Normal	Normal
100mg/kg	Normal	Normal	Normal	Normal	Normal	Normal	Normal
100mg/kg	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1000mg/kg	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1000mg/kg	Normal	Normal	Normal	Left Kidney cyst	Normal	Normal	Normal
1000mg/kg	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Table 4.9: External observation of Male *Wistar* Rats treated with Miracle Seed Ultima® in Phase two Acute Toxicity Study.

Groups	Survival status	Skin/Fur	Eyes/Nostrils/Mouth	Anus/Genitals	Abnormal	General condition
					Discharge	
Control	Alive	Normal	Normal	Normal	No	Healthy
1600mg/kg						
g	Alive	Normal	Normal	Normal	No	Healthy
2900mg/kg						
g	Alive	Normal	Normal	Normal	No	Healthy
5000mg/kg						
g	Alive	Normal	Normal	Normal	No	Healthy

Table 4.10: Internal (Gross Pathology) of Male *Wistar* Rats treated with Miracle Seed Ultima® in Phase two Acute Toxicity Study.

Groups	Lungs	Heart	Liver	Kidneys	Spleen	Stomach/Intestine	Brain
Control	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1600mg/kg	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2900mg/kg	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5000mg/kg	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Group	Feed Consumed (g/day/rat)	Water Consumed (ml/day/rat)	Relative Weight Gain/Loss	Feed Efficiency (%)
Control	20.03 ± 0.00 ^a	47.67 ± 0.00 ^a	1.66 ± 2.16 ^a	12.97 ± 8.16 ^a
10 mg/kg	21.56 ± 0.00 ^a	49.35 ± 0.00 ^a	1.47 ± 1.52 ^a	6.56 ± 6.99 ^a
100mg/kg	21.43 ± 0.00 ^a	47.17 ± 0.00 ^a	3.051. ± 0.73 ^a	13.42 ± 3.81 ^a
1000mg/kg	19.70 ± 0.00 ^a	70.06 ± 0.00 ^a	2.95 ± 0.35 ^a	14.95 ± 1.80 ^a

Table 4.11: Daily Feed and Water consumption, Relative Weight Gain/Loss, and Feed Efficiency in Rats.

All research results are expressed as mean ± SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet on the same position differ significantly at 95% level of significance ($p < 0.05$) using Tukey's test of significance. Feed consumed represents the average daily feed intake per rat, water consumed represents the average daily water intake per rat, relative weight gain/loss is expressed as percentage change from initial body weight, and feed efficiency is calculated as the ratio of weight gain to feed consumed expressed as percentage.

Table 4.12: Body Weight of Rats in Acute Phase I (g) (Mean $\pm$ SEM)

Group	Day 0 (Start)	Day 7 (Week 1)	Day 14 (Week 2)
Control	161.00 $\pm$ 20.95 ^a	186.57 $\pm$ 20.67 ^a	184.41 $\pm$ 15.03 ^a
10 mg/kg	167.16 $\pm$ 8.16 ^a	185.85 $\pm$ 16.45 ^a	187.73 $\pm$ 14.13 ^a
100 mg/kg	150.35 $\pm$ 17.41 ^a	190.86 $\pm$ 15.51 ^a	193.03 $\pm$ 7.36 ^a
1000 mg/kg	152.70 $\pm$ 0.95 ^a	189.16 $\pm$ 17.37 ^a	193.98 $\pm$ 4.38 ^a

All research results are expressed as mean $\pm$ SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet on the same position differ significantly at 95% level of significance (p <0.05) using Tukey's test of significance.

This section outlines the key findings from a 28-day sub-acute toxicity evaluation of Miracle Seed Ultima® (MSU) in male *Wistar* rats, assessing its impact on hepatic and renal function, lipid metabolism, glucose homeostasis, and hematological indices. The study administered MSU at doses of 100 mg/kg (Group 2), 300 mg/kg (Group 3), and 1000 mg/kg (Group 4), with comparisons to a control group (Group 1). Results, presented in tabular format with statistical analyses, highlight dose-related physiological responses and provide insights into the supplement's overall safety.

As shown in the Table 4.13 below, the subacute administration of Miracle Seed Ultima® at the highest dose caused a significant increase ($p < 0.05$) in ALT levels in the rats when compared to the lower dose groups, indicating dose-dependent hepatocellular membrane stress. However, ALT levels at the highest dose were not significantly different ($p > 0.05$) from the control. ALP levels were not significantly altered ($p > 0.05$) across all treatment groups when compared to the control. Similarly, AST levels showed a trend toward elevation at the highest dose compared to the control, though this did not reach statistical significance ($p > 0.05$). Total protein levels were significantly decreased ($p < 0.05$) in the low dose group when compared to the control, while the medium and high dose groups were not significantly different from the control. Albumin levels remained unchanged ($p > 0.05$) across all groups. Total and direct bilirubin levels showed no significant differences ($p > 0.05$) across all treatment groups when compared to the control, suggesting that hepatic conjugation and excretory function were unaffected at all dose.

As shown in Table 4.14 below, the subacute administration of Miracle Seed Ultima® did not result in significant changes ($p > 0.05$) in total cholesterol levels across all treatment groups when compared to the control. Triglyceride levels were similarly unchanged ($p > 0.05$) across all dose

groups when compared to the control. LDL cholesterol levels showed no significant differences ($p>0.05$) across all treatment groups when compared to the control. HDL cholesterol levels likewise remained unchanged ($p>0.05$) across all groups when compared to the control. VLDL cholesterol levels showed no significant alterations ($p>0.05$) across all treatment groups when compared to the control.

As shown in the Table 4.15, the subacute administration of Miracle Seed Ultima® resulted in a significant decrease ($p<0.05$) in urea levels in all treatment groups when compared to the control, with the effect being more pronounced at the medium and high dose groups.

Creatinine levels were significantly decreased ($p<0.05$) in the low dose group when compared to the control, while the medium and high dose groups showed no significant difference ($p>0.05$) from the control. Potassium levels showed no significant differences ($p>0.05$) across all treatment groups when compared to the control. Sodium levels were significantly increased ($p<0.05$) in all treatment groups when compared to the control. Chloride levels however remained unchanged ($p>0.05$) across all treatment groups when compared to the control.

Table 4.16 outlines the results of Fasting Blood Sugar in the subacute administration of Miracle Seed Ultima®. There were no significant changes ($p>0.05$) in fasting blood glucose levels across all treatment groups when compared to the control.

Table 4.17 shows the daily feed and water consumption, daily weight change, and feed efficiency in rats treated with various doses of Miracle Seed Ultima during the sub-acute study period. The daily feed consumption per rat varied among the groups. The highest dose group (1000 mg/kg) recorded the highest feed intake (26.66 g/day/rat), while the lowest intake was observed in the 100

mg/kg group (23.87 g/day/rat). The control and 300 mg/kg groups demonstrated intermediate feed consumption at 25.46 and 26.30 g/day/rat, respectively. Overall, feed consumption did not follow a consistent dose-dependent pattern across the treatment groups. Generally, all groups maintained consistent feed intake within their respective groups throughout the measurement period. Daily water consumption per rat differed among the groups. The control group maintained the lowest intake (35.37 ml/day/rat), while the highest intake was observed in the highest dose group (1000 mg/kg) at 41.83 ml/day/rat. The 100 mg/kg and 300 mg/kg groups demonstrated equal intermediate water consumption at 39.36 ml/day/rat. Overall, Miracle Seed Ultima caused a dose-dependent increase in water consumption, with higher doses associated with higher intake. Generally, all groups maintained consistent water intake within their respective groups throughout the measurement period.

The sub-acute administration of Miracle Seed Ultima caused variable effects on body weight gain in the rats. The control group exhibited the highest daily weight gain (1.58 g), while the 1000 mg/kg group showed the lowest (0.76 g). Among the treatment groups, the 300 mg/kg group recorded the highest weight gain (1.30 g), followed by the 100 mg/kg group (0.98 g). However, no significant differences ($p > 0.05$) were observed among any of the groups, as all groups shared the same superscript designation.

Feed efficiency varied slightly among the groups during the study period. The control group maintained the highest feed efficiency (6.22%), while the treatment groups exhibited progressively lower values with increasing dose — 4.93% at 300 mg/kg, 4.11% at 100 mg/kg, and 2.84% at 1000 mg/kg. The 1000 mg/kg group was the only group that differed significantly ($p < 0.05$) from the control, as denoted by its differing superscript. Miracle Seed Ultima at the highest dose therefore

significantly reduced feed efficiency compared with the control, while the lower doses did not produce a statistically significant alteration.

Table 4.18 outlines the body weight of the rats from the start to the end of the experiment. The body weight of the rats at the start of the experiment (Day 0) was not significantly different among all groups ($p > 0.05$), indicating comparable baseline weights. Similarly, at the end of the first week (Day 7), no significant differences in body weight were observed among the groups ($p > 0.05$), suggesting that the treatments had not yet measurably affected weight gain. By the second week (Day 14), although the 1000 mg/kg group recorded a lower mean body weight (161.67 g) compared with the other groups, the differences were not statistically significant ($p > 0.05$). During the third week (Day 21), there was still no significant difference in body weight between the groups ($p > 0.05$), although the 300 mg/kg group showed a slightly higher mean weight (178.86 g). At the end of the fourth week (Day 28), the 1000 mg/kg group exhibited the lowest mean body weight (178.34 g) compared with the control (200.08 g) and the other treated groups, while the 300 mg/kg group recorded the highest mean weight among the treatment groups (191.69 g); however, these differences were not statistically significant ($p > 0.05$). Overall, all treatments maintained body weight within a comparable range to the control group throughout the study period, with no treatment causing a significant reduction or increase in weight gain.

Table 4.13: Effect of Miracle Seed Ultima® on Liver function parameters

Groups	ALT (U/L)	ALP (UI)	AST (U/L)	PROTEIN		Total	Direct
				(g/dl)	Albumin (g/dL)	Bilirubin (mg/dl)	Bilirubin (Mg/dl)
Control	147.35±12.9 ^a	34.67±0.6 ^a	133.56±9.0 ^a	4.06±0.1 ^a	1.63±0.0 ^a	0.48±0.0 ^a	0.37±0.0 ^a
100mg/kg	129.54±2.5 ^a	32.49±0.3 ^a	125.88±3.6 ^a	3.39±0.1 ^b	1.54±0.0 ^a	0.57±0.0 ^a	0.48±0.0 ^a
300mg/kg	135.00±8.7 ^a	33.71±1.9 ^a	136.37±11.3 ^a	3.63±0.2 ^a	1.57±0.0 ^a	0.60±0.0 ^a	0.48±0.0 ^a
1000mg/kg	200.56±22.2 ^{ac}	33.23±0.8 ^a	175.08±19.4 ^a	3.92±0.2 ^a	1.55±0.0 ^a	0.58±0.0 ^a	0.49±0.0 ^a

All research results are expressed as mean ± SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet on the same position differ significantly at 95% level of significance (p <0.05) using Tukey's test of significance. ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, AST: Aspartate aminotransferase.

Table 4.14: Effects of Miracle Seed Ultima® on Lipid profile parameters

TOTAL					
	CHOLESTEROL	TRIGLYCERIDES	LDL	HDL	VLDL
Groups	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)
Control	179.62±3.9 ^a	134.33±14.7 ^a	122.01±5.7 ^a	30.74±0.8 ^a	26.87±2.9 ^a
100mg/kg	174.67±3.3 ^a	115.08±10.5 ^a	122.00±2.8 ^a	29.65±0.9 ^a	23.02±2.1 ^a
300mg/kg	186.92±1.9 ^a	134.33±13.9 ^a	129.63±4.4 ^a	30.43±2.2 ^a	26.87±2.8 ^a
1000mg/kg	179.37±4.2 ^a	135.19±18.4 ^a	123.24±4.2 ^a	29.11±0.6 ^a	27.04±3.7 ^a

All research results are expressed as mean ± SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet on the same position differ significantly at 95% level of significance (p <0.05) using Tukey's test of significance. LDL: Low-density lipoprotein, HDL: High-density lipoprotein, VLDL: Very low-density lipoprotein.

Table 4.15: Effects of Miracle Seed Ultima® on Kidney function parameters

Groups	UREA	CREATININE	POTASSIUM	SODIUM	CHLORID
	(mg/dl)	(mg/dl)	(mmol/L)	(mmol/L)	E (mmol/L)
Control	102.72±5.7 ^a	2.79±0.1 ^a	0.88±0.1 ^a	32.95±0.9 ^a	23.84±0.9 ^a
100mg/kg	89.32±7.8 ^a	2.12±0.1 ^b	0.69±0.0 ^a	77.25±4.5 ^b	22.32±1.2 ^a
300mg/kg	74.85±6.3 ^b	2.29±0.1 ^a	0.69±0.0 ^a	72.19±6.6 ^b	22.09±1.6 ^a
1000mg/kg	68.62±2.9 ^b	2.29±0.2 ^a	0.74±0.0 ^a	67.55±8.2 ^b	24.93±0.6 ^a

All research results are expressed as mean ± SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet on the same position differ significantly at 95% level of significance (p <0.05) using Tukey's test of significance.

Table 4.16: Effects of Miracle Seed Ultima® on Fasting Blood Glucose parameter.

Group	GLUCOSE (mg/dl)
Control	72.60±5.5 ^a
100mg/kg	60.60±7.6 ^a
300mg/kg	75.60±3.6 ^a
1000mg/kg	70.40±7.1 ^a

All research results are expressed as mean ± SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet on the same position differ significantly at 95% level of significance ($p < 0.05$) using Tukey's test of significance.

Table 4.17: Daily Feed and Water Consumption, Weight Change, and Feed Efficiency in Rats

Group	Feed Consumed (g/day/rat)	Water Consumed (ml/day/rat)	Daily Weight Change (g)	Feed Efficiency (%)
Control	25.46 ± 0.00 ^a	35.37 ± 0.00 ^a	1.58 ± 0.11 ^a	6.22 ± 0.44 ^a
100mg/kg	23.87 ± 0.00 ^a	39.36 ± 0.00 ^a	0.98 ± 0.30 ^a	4.11 ± 0.68 ^a
300mg/kg	26.30 ± 0.00 ^a	39.36 ± 0.00 ^a	1.30 ± 0.30 ^a	4.93 ± 0.49 ^a
1000mg/kg	26.66 ± 0.00 ^a	41.83 ± 0.00 ^a	0.76 ± 0.28 ^a	2.84 ± 1.05 ^b

All research results are expressed as mean ± SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet on the same position differ significantly at 95% level of significance ($p < 0.05$) using Tukey's test of significance.

Table 4.18: Body Weight of the Rats in Sub acute Phase (g)

Group	DAY_0	DAY_7	DAY_14	DAY_21	DAY_28
Control	155.71±8.47 ^a	165.67 ± 7.75 ^a	179.58 ± 9.64 ^a	185.29±10.25 ^a	200.08±10.97 ^a
100mg/k g	155.37± 7.80 ^a	159.84 ± 8.51 ^a	165.96± 9.53 ^a	173.68± 11.03 ^a	182.82±11.81 ^a
300mg/k g	155.26± 7.41 ^a	157.41± 7.21 ^a	169.16± 6.70 ^a	178.86± 7.63 ^a	191.69±10.66 ^a
1000mg/ kg	154.55± 6.90 ^a	147.94±13.26 ^a	161.67 ± 8.71 ^a	169.32± 5.85 ^a	178.34±11.00 ^a

All research results are expressed as mean ± SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet differ significantly at 95% level of significance (p <0.05) using Tukey's test of significance.

As shown in the table 4.17 below, the subacute administration of Miracle Seed Ultima® did not result in significant changes ($p>0.05$) in total white blood cell count across all treatment groups when compared to the control. Red blood cell count, hemoglobin concentration, and hematocrit values showed no significant differences ($p>0.05$) across all treatment groups when compared to the control. Red blood cell indices, including mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, likewise remained unchanged ($p>0.05$) across all groups when compared to the control.

Platelet count showed no significant differences ($p>0.05$) across all treatment groups when compared to the control.

Table 4.18 below, the subacute administration of Miracle Seed Ultima® did not result in significant differences ($p>0.05$) in initial body weight across all treatment groups when compared to the control, confirming that the random allocation of animals was appropriate and that all groups were comparable at the commencement of the study. Final body weight likewise showed no significant differences ($p>0.05$) across all treatment groups when compared to the control.

Liver weight showed no significant differences ($p>0.05$) across all treatment groups when compared to the control. Left kidney weight similarly remained unchanged ($p>0.05$) across all treatment groups when compared to the control.

Heart weight showed no significant differences ($p>0.05$) across all treatment groups when compared to the control. Spleen weight likewise remained unchanged ($p>0.05$) across all treatment groups when compared to the control.

Table 4.19: Effect of Miracle Seed Ultima on Hematology parameters.

GROUP	WBC (10³/μL)	RBC (10⁶/μL)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT (10³/μL)
Control	12.22±1.6 ^a	7.05±0.3 ^a	14.46±0.4 ^a	42.22±0.7 ^a	60.24±1.8 ^a	20.50±0.3 ^a	34.20±0.6 ^a	682.20±103.8 ^a
100 mg/kg	12.04±2.7 ^a	6.94±0.2 ^a	13.82±0.5 ^a	39.88±1.4 ^a	57.18±1.5 ^a	19.72±0.5 ^a	34.60±0.4 ^a	1016.20±542.2 ^a
300 mg/kg	15.96±1.1 ^a	7.12±0.4 ^a	14.52±0.8 ^a	42.10±2.1 ^a	59.20±0.8 ^a	20.34±0.2 ^a	34.42±0.3 ^a	330.00±27.8 ^a
1000 mg/kg	12.02±1.9 ^a	6.98±0.2 ^a	13.58±0.4 ^a	38.66±1.2 ^a	55.50±1.4 ^a	19.40±0.3 ^a	35.10±0.5 ^a	540.40±130.8 ^a

All research results are expressed as mean ± SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet on the same position differ significantly at 95% level of significance (p <0.05) using Tukey's test of significance. WBC: White blood cell count, RBC: Red blood cell count, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelet count.

Table 4.20: Effects of Miracle Seed Ultima® on Initial, final body weight and various tissues of male *Wistar* rats.

Group	Initial Body Weight (G)	Final Body Weight (G)	Liver (G)	Left Kidney (G)	Heart(G)	Spleen (G)
Control	155.71±8.5 ^a	200.08±10.9 ^a	7.61±0.8 ^a	0.69±0.4 ^a	1.94±1.3 ^a	0.91±0.1 ^a
100mg/kg	155.37±7.8 ^a	182.83±11.8 ^a	6.79±0.7 ^a	0.59±0.0 ^a	0.68±0.0 ^a	0.93±0.0 ^a
300mg/kg	155.26±7.4 ^a	191.69±10.7 ^a	7.68±0.5 ^a	0.67±0.0 ^a	0.72±0.0 ^a	1.14±0.2 ^a
1000mg/kg	154.55±6.9 ^a	178.34±10.9 ^a	6.37±0.5 ^a	0.59±0.0 ^a	0.65±0.0 ^a	1.04±0.0 ^a

All research results are expressed as mean ± SEM (= standard error of the mean) of three determinations (n=3) and subjected to one-way analysis of variance (ANOVA). Means in the same column with different superscript alphabet on the same position differ significantly at 95% level of significance (p <0.05) using Tukey's test of significance.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

This study evaluated the acute and subacute toxicity of Miracle Seed Ultima® in male *Wistar* rats to establish its safety profile. The comprehensive phytochemical analysis revealed that cardiac glycosides dominate the formulation, followed by steroids, flavonoids, coumarins, alkaloids, terpenoids, and tannins. Cardiac glycosides possess narrow therapeutic indices and can strengthen heart contractions through inhibition of sodium-potassium ATPase, but accumulation beyond safe thresholds can cause serious arrhythmias (Fender *et al.*, 2024). Steroids provide anti-inflammatory effects by modulating prostaglandin and leukotriene production. Flavonoids act through multiple antioxidant mechanisms including direct free radical scavenging, metal chelation, and upregulation of endogenous antioxidant systems (Alsawaf *et al.*, 2022). Coumarins possess anticoagulant properties through interference with vitamin K-dependent clotting factor synthesis. Alkaloids contribute diverse pharmacological activities but can be toxic at high concentrations. Terpenoids offer antimicrobial and anti-inflammatory properties, while tannins provide antioxidant effects but can interfere with iron absorption. The absence of saponins, phenols, phlobatannins, and anthraquinones may reflect specific processing methods or ingredient combinations (Mugale *et al.*, 2024). This phytochemical profile is consistent with constituents of garlic, ginger, and moringa (Ajanaku *et al.*, 2022; Edo *et al.*, 2025).

The proximate analysis revealed very high moisture content indicating a water-based formulation, with carbohydrates dominating the dry matter. These carbohydrates likely serve as preservatives, improve palatability, and act as carriers for bioactive compounds. The low protein, fiber, and fat

contents confirm this is a refined liquid extract rather than a nutritional supplement. The elemental analysis showed calcium as the most abundant mineral, essential for bone health, muscle contraction, and nerve transmission. Potassium was present at high levels and plays critical roles in membrane potential, fluid balance, and cardiac function. The substantial potassium content has implications for individuals with kidney disease or those taking medications affecting potassium levels. Magnesium serves as a cofactor for enzymatic reactions in energy metabolism and insulin sensitivity. The trace elements iron, zinc, manganese, and copper function in hemoglobin synthesis, immune function, antioxidant defense, and various enzymatic reactions. Importantly, the toxic heavy metals lead and cadmium were not detected, indicating good quality control and low contamination risk (Jitäreanu *et al.*, 2022).

The antioxidant assessments showed moderate DPPH and FRAP activities but notably high nitric oxide scavenging capacity approaching ascorbic acid performance. Nitric oxide can react with superoxide to form peroxynitrite, causing oxidative damage to lipids, proteins, and DNA (Abdelwahab *et al.*, 2011). The strong nitric oxide scavenging suggests benefits in conditions with excessive nitric oxide production such as chronic inflammation. The superior total antioxidant capacity reflects cumulative effects of multiple compounds working synergistically (Alsawaf *et al.*, 2022). However, in vitro antioxidant activity does not always translate to in vivo benefits due to bioavailability, metabolism, and tissue distribution factors (Ansari *et al.*, 2024).

The acute toxicity assessment revealed no mortality at doses up to the limit tested, classifying Miracle Seed Ultima® as practically non-toxic (Al-Afifi *et al.*, 2018; OECD, 2001). This finding aligns with previous studies on similar herbal products (Adeoye *et al.*, 2022; Aragie *et al.*, 2025). However, kidney cysts observed in higher dose groups warrant consideration. These cysts can develop through tubular obstruction, altered cellular proliferation, or direct toxic injury to nephron

segments (Aminu *et al.*, 2023). The threshold nature appearing only at high doses emphasizes dose optimization importance. The unilateral left kidney involvement is unusual and may reflect anatomical variations in blood flow or filtration dynamics. Histopathological examination would be valuable to characterize these changes and assess reversibility potential.

General health indicators including feed and water consumption, daily weight change, feed efficiency, and body weight progression were monitored throughout the subacute study period as key parameters for detecting systemic toxicity (Al-Afifi *et al.*, 2018; OECD, 2008). Daily feed consumption did not follow a consistent dose-dependent pattern, with the 1000 mg/kg group recording the highest intake and the 100 mg/kg group the lowest. The absence of a significant reduction in feed intake across all groups suggests that Miracle Seed Ultima® did not adversely affect appetite or cause gastrointestinal discomfort severe enough to suppress feeding behaviour, which is an important indicator of general well-being (Du *et al.*, 2023). In contrast, water consumption exhibited a clear dose-dependent increase, with the 1000 mg/kg group consuming the most compared to the control group, while the 100 mg/kg and 300 mg/kg groups recorded equal intermediate values. This increase in water intake at higher doses may reflect a compensatory physiological response to the metabolic demands imposed by bioactive compounds in the formulation, or a mild osmotic effect from the higher doses (Aragie *et al.*, 2025; Baig *et al.*, 2022). Daily weight change was variable across groups, with the control group showing the highest gain and the 1000 mg/kg group the lowest; however, none of these differences reached statistical significance ($p > 0.05$), consistent with the absence of gross toxicity. Feed efficiency, which reflects the capacity of animals to convert feed into body mass, was significantly reduced only in the 1000 mg/kg group, while the 100 mg/kg and 300 mg/kg groups did not differ significantly from the control. This finding suggests that at the highest tested dose, the metabolic burden of repeated

exposure may impair anabolic efficiency, even without an overt reduction in feed intake (Yang et al., 2023). Body weight was monitored weekly throughout the 28-day study period, and no statistically significant differences were observed between any of the groups at any time point ($p>0.05$), from baseline (Day 0) through the final measurement (Day 28). Although the 1000 mg/kg group consistently recorded slightly lower mean body weights and the 300 mg/kg group tended to weigh slightly more than the other treated groups by Day 28, these trends did not achieve statistical significance, indicating that the treatments did not produce clinically meaningful suppression or promotion of body weight gain (Aragie et al., 2025; Du et al., 2023). Taken together, the preserved body weight and feed intake across groups, alongside only a significant reduction in feed efficiency at the highest dose, suggest that Miracle Seed Ultima® is generally well tolerated at the doses examined, with the 1000 mg/kg dose representing the threshold at which subtle metabolic strain begins to manifest.

The subacute study revealed elevated alanine aminotransferase at higher doses indicating mild hepatocellular stress, while normal aspartate aminotransferase and alkaline phosphatase suggest mild membrane disruption rather than widespread necrosis or cholestasis (Ainyanbhor and Onoagbe, 2024; Aragie et al., 2025; Aminu et al., 2023). This pattern is consistent with metabolic processing of phytochemicals generating reactive intermediates when detoxification capacity is exceeded (Acosta et al., 2023). The liver's cytochrome P450 enzymes catalyze oxidation reactions producing reactive oxygen species and electrophilic intermediates. When production exceeds neutralization by phase II enzymes or antioxidant systems, lipid peroxidation of hepatocyte membranes occurs, increasing permeability and enzyme leakage. Given the high concentrations of cardiac glycosides, steroids, and alkaloids, repeated high-dose exposure likely overwhelms hepatic

detoxification pathways. The preserved protein synthesis function suggests reversible stress rather than permanent damage.

Interestingly, decreased plasma urea at higher doses may indicate enhanced nitrogen metabolism, improved urea cycle function, or increased renal clearance rather than damage (Adeoye *et al.*, 2022). Bioactive compounds in moringa and garlic can improve kidney function by reducing inflammation and oxidative stress (Alsawaf *et al.*, 2022; Chen *et al.*, 2013). Decreased creatinine at lower doses with stable electrolytes supports absence of significant dysfunction. The acute study kidney cysts did not translate to functional impairment in the subacute evaluation, suggesting an acute response not progressing at lower doses. The improved markers may reflect antioxidant and anti-inflammatory properties of flavonoids, phenolic compounds, and terpenoids reducing oxidative damage in renal tissues (Alsawaf *et al.*, 2022; Bassong *et al.*, 2022). This may represent a hormetic dose-response where low to moderate doses provide beneficial effects while higher doses overwhelm cellular defenses (Debnath *et al.*, 2018). The minerals zinc, magnesium, and manganese serve important roles in renal protection as cofactors for antioxidant enzymes.

The unchanged lipid profile and fasting glucose indicate metabolic safety at tested doses, though the short duration may have limited detection of subtle effects requiring longer exposure (Chen *et al.*, 2013; Cretella *et al.*, 2020; Daoud *et al.*, 2025). The stable hematological parameters reflect normal bone marrow function without effects on erythropoiesis, leukopoiesis, or thrombopoiesis (Baig *et al.*, 2022; Du *et al.*, 2023). Normal red blood cell indices indicate adequate hemoglobin synthesis, proper erythrocyte maturation, and normal iron metabolism. The substantial iron content detected does not lead to overload or oxidative stress. Stable white blood cell counts suggest no immunosuppression or excessive inflammation. Normal platelets indicate no interference with

thrombopoiesis or coagulation despite coumarin presence. These findings reassure that Miracle Seed Ultima® does not disrupt blood cell production or immune function at appropriate doses.

The findings align with existing literature showing low acute toxicity for black seed, garlic, and moringa extracts (Adeoye *et al.*, 2022; Aragie *et al.*, 2025). Mild liver enzyme elevations match other seed oil and polyherbal product reports showing dose-dependent transaminase increases without hepatic failure (Ainyanbhor and Onoagbe, 2024; Aragie *et al.*, 2025). Kidney cysts, while less common, have precedent in studies showing renal congestion or mild structural changes at high doses that rarely translate to functional impairment at therapeutic ranges (Ainyanbhor and Onoagbe, 2024; Saleh *et al.*, 2021). Stable hematological and metabolic parameters contrast with some reports, likely reflecting formulation, dose, duration, and model differences (Du *et al.*, 2023).

Study limitations include the twenty-eight day duration which may not capture all long-term effects, exclusive use of male rats limiting understanding of sex-dependent differences (OECD, 2025), absence of histopathological examination which could elucidate kidney cyst nature and characterize subclinical hepatic changes, and lack of tissue oxidative stress markers that would provide mechanistic insights. From a clinical perspective, Miracle Seed Ultima® demonstrates acceptable safety at moderate doses but poses risks at very high doses. The therapeutic range should be carefully defined to maximize benefits while avoiding hepatocellular stress and renal changes. Consumers should strictly follow recommended dosages. Healthcare providers should monitor liver function in long-term users, especially those with pre-existing conditions or taking multiple medications. The high cardiac glycoside content warrants special attention in individuals with cardiovascular conditions or taking cardiac medications, as accumulation can cause serious arrhythmias. The substantial potassium content requires consideration in kidney disease patients or those taking medications affecting potassium. Potential herb-drug interactions should be

carefully assessed, particularly with medications undergoing hepatic metabolism or affecting cardiac function.

5.2 CONCLUSION

This study provides the first comprehensive toxicological evaluation of Miracle Seed Ultima®, addressing a critical safety data gap. The phytochemical analysis revealed a complex profile dominated by cardiac glycosides, followed by steroids, flavonoids, coumarins, alkaloids, terpenoids, and tannins, providing biochemical basis for therapeutic claims and dose vigilance requirements. The proximate analysis showed a water-based formulation with carbohydrates as the major solid component. The elemental analysis revealed substantial essential minerals and trace elements with no heavy metal contamination, indicating good quality control. The antioxidant assessments demonstrated moderate DPPH and FRAP activities but notably high nitric oxide scavenging capacity and superior total antioxidant capacity, suggesting multiple free radical neutralization mechanisms.

The acute toxicity assessment revealed favorable safety with no mortality at highest doses tested, classifying the product as practically non-toxic. However, kidney cysts at higher acute doses indicate potential renal structural effects warranting consideration in dose recommendations. The subacute assessment revealed dose-dependent effects, notably mild hepatocellular stress evidenced by elevated alanine aminotransferase at higher doses without corresponding aspartate aminotransferase or alkaline phosphatase changes, suggesting reversible membrane disruption rather than severe necrosis. Conversely, kidney function improved at moderate doses with decreased urea and creatinine suggesting renoprotective effects through antioxidant and anti-inflammatory mechanisms. Stable hematological parameters, lipid profiles, and glucose levels support overall safety at appropriate doses.

Based on these findings, Miracle Seed Ultima® can be considered relatively safe for short-term use at moderate doses, demonstrating acceptable safety margins when consumed according to guidelines, with adverse effects primarily at doses substantially exceeding therapeutic ranges. However, the high cardiac glycoside content, dose-dependent hepatic effects, structural kidney changes at high doses, and substantial potassium content necessitate caution and monitoring protocols for long-term users. This study provides essential baseline data to inform regulatory decisions, guide clinical use, and protect public health while allowing potential therapeutic benefits.

5.3 RECOMMENDATIONS

Based on these findings, several recommendations are proposed:

- i. Chronic toxicity studies extending beyond 28 days, ideally 90 days or longer, should be conducted to assess long-term safety and identify cumulative effects.
- ii. Comprehensive histopathological examination of all major organs should be performed to correlate biochemical changes with structural alterations at the cellular and tissue levels.
- iii. Efficacy studies should be conducted in rat models of diabetes, hypertension, and other oxidative stress-related diseases to validate the product's therapeutic claims.
- iv. Tissue oxidative stress markers including malondialdehyde, glutathione, and antioxidant enzyme activities should be measured to elucidate the biochemical mechanisms underlying observed effects.
- v. Studies should include female animals to assess sex-dependent differences in toxicity profiles arising from hormonal influences and metabolic variations.

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RESEARCH ETHICS COMMITTEE
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Date: 21ST December, 2025

Our Ref: CMS/REC/01/VOL.2/870

Re: EVALUATION OF BIOCHEMICAL AND TOXICOLOGICAL PROPERTIES OF MIRACLE SEED ULTIMA AND ITS THERAPEUTIC POTENTIAL IN WISTAR RATS FED WITH HIGH-FRUCTOSE DIET.

Name of Principal Investigator: ELEMENFOLO DANIEL EROMONSELE
Department of Medical Biochemistry,
School of Basic Medical Sciences
Other Investigator: MRS EHIMIYEN OMOSE SAMDRA

REC Approval No: CMS/REC/2025/870

This is to inform you that the research described in the submitted proposal, the Informed Consent Forms and other participant information materials have been reviewed and approved by the College Research Ethics Committee, University of Benin.

This approval dates from 21ST DECEMBER, 2025 to 22ND DECEMBER, 2026. In multi-year research, Endeavour to submit your annual report to the REC early in order to obtain renewal of your approval and avoid disruption of your research.

The National Code of Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the code including ensuring that all adverse events are reported promptly to the REC. No, changes are permitted in the research without prior approval by REC except in circumstances outlined in the code. REC reserves the right to conduct compliance visit to your research site without prior notice. Thank you.

PROF. F.A IMARHIAGBE
Chairman, REC

Promoting best ethical & scientific standard for research in Nigeria

**APPENDIX
APPENDIX I
LIVER FUNCTION TEST**

		Descriptives							
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
ALT (U/L)	Control	5	147.3460	29.02656	12.98107	111.3048	183.3872	112.26	188.51
	100mg/kg	5	129.5380	5.63068	2.51812	122.5466	136.5294	124.50	139.02
	300mg/kg	5	135.0060	19.59368	8.76256	110.6772	159.3348	111.68	156.94
	1000mg/kg	5	200.5580	49.66785	22.21214	138.8872	262.2288	147.16	255.35
	Total	20	153.1120	40.22297	8.99413	134.2871	171.9369	111.68	255.35
ALP	Control	5	34.6700	1.31881	.58979	33.0325	36.3075	33.08	36.23
	100mg/kg	5	32.4880	.59563	.26637	31.7484	33.2276	32.04	33.53
	300mg/kg	5	33.7140	4.17732	1.86816	28.5272	38.9008	30.69	40.87
	1000mg/kg	5	33.2340	1.69618	.75856	31.1279	35.3401	30.99	35.63
	Total	20	33.5265	2.31946	.51865	32.4410	34.6120	30.69	40.87
AST	Control	5	133.5620	20.14913	9.01096	108.5436	158.5804	115.75	163.50
	100mg/kg	5	125.8760	8.02179	3.58745	115.9156	135.8364	116.33	138.44
	300mg/kg	5	136.3740	25.15725	11.25066	105.1372	167.6108	93.07	159.04
	1000mg/kg	5	175.0840	43.40664	19.41204	121.1875	228.9805	127.97	223.94
	Total	20	142.7240	31.81020	7.11298	127.8364	157.6116	93.07	223.94
PROTEIN	Control	5	4.0600	.31804	.14223	3.6651	4.4549	3.63	4.42

	100mg/kg	5	3.3900	.26524	.11862	3.0607	3.7193	2.92	3.57
	300mg/kg	5	3.6340	.37334	.16696	3.1704	4.0976	3.44	4.30
	1000mg/kg	5	3.9160	.40041	.17907	3.4188	4.4132	3.62	4.62
	Total	20	3.7500	.41157	.09203	3.5574	3.9426	2.92	4.62
ALB	Control	5	1.6320	.11009	.04923	1.4953	1.7687	1.52	1.76
	100mg/kg	5	1.5380	.15271	.06829	1.3484	1.7276	1.35	1.72
	300mg/kg	5	1.5700	.08660	.03873	1.4625	1.6775	1.42	1.62
	1000mg/kg	5	1.5460	.10784	.04823	1.4121	1.6799	1.39	1.64
	Total	20	1.5715	.11366	.02542	1.5183	1.6247	1.35	1.76
T.BIL	Control	5	.4780	.03271	.01463	.4374	.5186	.43	.51
	100mg/kg	5	.5660	.08173	.03655	.4645	.6675	.43	.63
	300mg/kg	5	.6020	.06017	.02691	.5273	.6767	.53	.68
	1000mg/kg	5	.5840	.12700	.05680	.4263	.7417	.41	.76
	Total	20	.5575	.09043	.02022	.5152	.5998	.41	.76
D.BIL	Control	5	.3680	.04712	.02107	.3095	.4265	.33	.45
	100mg/kg	5	.4760	.09044	.04045	.3637	.5883	.33	.55
	300mg/kg	5	.4780	.04025	.01800	.4280	.5280	.43	.52
	1000mg/kg	5	.4860	.13069	.05845	.3237	.6483	.32	.68
	Total	20	.4520	.09283	.02076	.4086	.4954	.32	.68

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ALT (U/L)	Between Groups	15839.652	3	5279.884	5.670	.008
	Within Groups	14900.212	16	931.263		
	Total	30739.864	19			

ALP	Between Groups	12.534	3	4.178	.745	.541
	Within Groups	89.684	16	5.605		
	Total	102.218	19			
AST	Between Groups	7276.447	3	2425.482	3.248	.050
	Within Groups	11949.439	16	746.840		
	Total	19225.886	19			
PROTEIN	Between Groups	1.334	3	.445	3.773	.032
	Within Groups	1.885	16	.118		
	Total	3.218	19			
ALB	Between Groups	.027	3	.009	.664	.586
	Within Groups	.218	16	.014		
	Total	.245	19			
T.BIL	Between Groups	.045	3	.015	2.200	.128
	Within Groups	.110	16	.007		
	Total	.155	19			
D.BIL	Between Groups	.047	3	.016	2.168	.132
	Within Groups	.116	16	.007		
	Total	.164	19			

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) ID	(J) ID	Mean	Std. Error	Sig.	95% Confidence Interval	
			Difference (I-J)			Lower Bound	Upper Bound
ALT (U/L)	Control	100mg/kg	17.80800	19.30040	.793	-37.4108	73.0268
		300mg/kg	12.34000	19.30040	.918	-42.8788	67.5588
		1000mg/kg	-53.21200	19.30040	.061	-108.4308	2.0068
	100mg/kg	Control	-17.80800	19.30040	.793	-73.0268	37.4108
		300mg/kg	-5.46800	19.30040	.992	-60.6868	49.7508
		1000mg/kg	-71.02000*	19.30040	.010	-126.2388	-15.8012
	300mg/kg	Control	-12.34000	19.30040	.918	-67.5588	42.8788
		100mg/kg	5.46800	19.30040	.992	-49.7508	60.6868
		1000mg/kg	-65.55200*	19.30040	.017	-120.7708	-10.3332
	1000mg/kg	Control	53.21200	19.30040	.061	-2.0068	108.4308
		100mg/kg	71.02000*	19.30040	.010	15.8012	126.2388
		300mg/kg	65.55200*	19.30040	.017	10.3332	120.7708
ALP	Control	100mg/kg	2.18200	1.49737	.484	-2.1020	6.4660
		300mg/kg	.95600	1.49737	.918	-3.3280	5.2400
		1000mg/kg	1.43600	1.49737	.774	-2.8480	5.7200
	100mg/kg	Control	-2.18200	1.49737	.484	-6.4660	2.1020
		300mg/kg	-1.22600	1.49737	.845	-5.5100	3.0580
		1000mg/kg	-.74600	1.49737	.958	-5.0300	3.5380
	300mg/kg	Control	-.95600	1.49737	.918	-5.2400	3.3280
		100mg/kg	1.22600	1.49737	.845	-3.0580	5.5100
		1000mg/kg	.48000	1.49737	.988	-3.8040	4.7640
	1000mg/kg	Control	-1.43600	1.49737	.774	-5.7200	2.8480
		100mg/kg	.74600	1.49737	.958	-3.5380	5.0300
		300mg/kg	-.48000	1.49737	.988	-4.7640	3.8040
AST	Control	100mg/kg	7.68600	17.28398	.970	-41.7638	57.1358
		300mg/kg	-2.81200	17.28398	.998	-52.2618	46.6378
		1000mg/kg	-41.52200	17.28398	.117	-90.9718	7.9278

	100mg/kg	Control	-7.68600	17.28398	.970	-57.1358	41.7638
		300mg/kg	-10.49800	17.28398	.928	-59.9478	38.9518
		1000mg/kg	-49.20800	17.28398	.051	-98.6578	.2418
	300mg/kg	Control	2.81200	17.28398	.998	-46.6378	52.2618
		100mg/kg	10.49800	17.28398	.928	-38.9518	59.9478
		1000mg/kg	-38.71000	17.28398	.155	-88.1598	10.7398
	1000mg/kg	Control	41.52200	17.28398	.117	-7.9278	90.9718
		100mg/kg	49.20800	17.28398	.051	-.2418	98.6578
		300mg/kg	38.71000	17.28398	.155	-10.7398	88.1598
PROTEIN	Control	100mg/kg	.67000*	.21707	.032	.0489	1.2911
		300mg/kg	.42600	.21707	.243	-.1951	1.0471
		1000mg/kg	.14400	.21707	.909	-.4771	.7651
	100mg/kg	Control	-.67000*	.21707	.032	-1.2911	-.0489
		300mg/kg	-.24400	.21707	.681	-.8651	.3771
		1000mg/kg	-.52600	.21707	.113	-1.1471	.0951
	300mg/kg	Control	-.42600	.21707	.243	-1.0471	.1951
		100mg/kg	.24400	.21707	.681	-.3771	.8651
		1000mg/kg	-.28200	.21707	.577	-.9031	.3391
	1000mg/kg	Control	-.14400	.21707	.909	-.7651	.4771
		100mg/kg	.52600	.21707	.113	-.0951	1.1471
		300mg/kg	.28200	.21707	.577	-.3391	.9031
ALB	Control	100mg/kg	.09400	.07387	.592	-.1173	.3053
		300mg/kg	.06200	.07387	.835	-.1493	.2733
		1000mg/kg	.08600	.07387	.657	-.1253	.2973
	100mg/kg	Control	-.09400	.07387	.592	-.3053	.1173
		300mg/kg	-.03200	.07387	.972	-.2433	.1793
		1000mg/kg	-.00800	.07387	1.000	-.2193	.2033

T.BIL	300mg/kg	Control	-.06200	.07387	.835	-.2733	.1493
		100mg/kg	.03200	.07387	.972	-.1793	.2433
		1000mg/kg	.02400	.07387	.988	-.1873	.2353
	1000mg/kg	Control	-.08600	.07387	.657	-.2973	.1253
		100mg/kg	.00800	.07387	1.000	-.2033	.2193
		300mg/kg	-.02400	.07387	.988	-.2353	.1873
	Control	100mg/kg	-.08800	.05244	.366	-.2380	.0620
		300mg/kg	-.12400	.05244	.125	-.2740	.0260
		1000mg/kg	-.10600	.05244	.221	-.2560	.0440
	100mg/kg	Control	.08800	.05244	.366	-.0620	.2380
		300mg/kg	-.03600	.05244	.901	-.1860	.1140
		1000mg/kg	-.01800	.05244	.986	-.1680	.1320
	300mg/kg	Control	.12400	.05244	.125	-.0260	.2740
		100mg/kg	.03600	.05244	.901	-.1140	.1860
		1000mg/kg	.01800	.05244	.986	-.1320	.1680
	1000mg/kg	Control	.10600	.05244	.221	-.0440	.2560
		100mg/kg	.01800	.05244	.986	-.1320	.1680
		300mg/kg	-.01800	.05244	.986	-.1680	.1320
D.BIL	Control	100mg/kg	-.10800	.05394	.228	-.2623	.0463
		300mg/kg	-.11000	.05394	.215	-.2643	.0443
		1000mg/kg	-.11800	.05394	.169	-.2723	.0363
	100mg/kg	Control	.10800	.05394	.228	-.0463	.2623
		300mg/kg	-.00200	.05394	1.000	-.1563	.1523
		1000mg/kg	-.01000	.05394	.998	-.1643	.1443
	300mg/kg	Control	.11000	.05394	.215	-.0443	.2643
		100mg/kg	.00200	.05394	1.000	-.1523	.1563
		1000mg/kg	-.00800	.05394	.999	-.1623	.1463

1000mg/kg	Control	.11800	.05394	.169	-.0363	.2723
	100mg/kg	.01000	.05394	.998	-.1443	.1643
	300mg/kg	.00800	.05394	.999	-.1463	.1623

*. The mean difference is significant at the 0.05 level.

**Post Hoc
Homogeneous Subsets**

ALT (U/L)

Tukey HSD^a

ID	N	Subset for alpha = 0.05	
		1	2
100mg/kg	5	129.5380	
300mg/kg	5	135.0060	
Control	5	147.3460	147.3460
1000mg/kg	5		200.5580
Sig.		.793	.061

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

ALP

Tukey HSD^a

ID	N	Subset for alpha = 0.05
		1
100mg/kg	5	32.4880
1000mg/kg	5	33.2340
300mg/kg	5	33.7140

Control	5	34.6700
Sig.		.484

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

AST

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
100mg/kg	5	125.8760
Control	5	133.5620
300mg/kg	5	136.3740
1000mg/kg	5	175.0840
Sig.		.051

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

PROTEIN

Tukey HSD^a

ID	N	Subset for alpha = 0.05

		1	2
100mg/kg	5	3.3900	
300mg/kg	5	3.6340	3.6340
1000mg/kg	5	3.9160	3.9160
Control	5		4.0600
Sig.		.113	.243

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

ALB

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
100mg/kg	5	1.5380
1000mg/kg	5	1.5460
300mg/kg	5	1.5700
Control	5	1.6320
Sig.		.592

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

T.BILTukey HSD^a

ID	N	Subset for alpha = 0.05 1
Control	5	.4780
100mg/kg	5	.5660
1000mg/kg	5	.5840
300mg/kg	5	.6020
Sig.		.125

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

D.BILTukey HSD^a

ID	N	Subset for alpha = 0.05 1
Control	5	.3680
100mg/kg	5	.4760
300mg/kg	5	.4780
1000mg/kg	5	.4860
Sig.		.169

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

APPENDIX II

LIPID PROFILE

		Descriptives							
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
CHOL	Control	5	179.6160	8.94317	3.99951	168.5116	190.7204	168.59	188.46
	100mg/kg	5	174.6680	7.29072	3.26051	165.6154	183.7206	164.10	182.82
	300mg/kg	5	186.9240	4.21821	1.88644	181.6864	192.1616	182.05	193.59
	1000mg/kg	5	179.3860	9.34031	4.17711	167.7885	190.9835	171.80	195.51
	Total	20	180.1485	8.38771	1.87555	176.2229	184.0741	164.10	195.51
TRIGS	Control	5	134.3320	32.86483	14.69760	93.5249	175.1391	96.26	176.47
	100mg/kg	5	115.0820	23.56206	10.53727	85.8258	144.3382	94.12	142.25
	300mg/kg	5	134.3320	31.06974	13.89481	95.7538	172.9102	81.28	161.50
	1000mg/kg	5	135.1880	41.03806	18.35278	84.2325	186.1435	87.70	168.98

	Total	20	129.7335	31.26493	6.99105	115.1011	144.3659	81.28	176.47
LDL	Control	5	122.0120	12.83402	5.73955	106.0765	137.9475	107.75	138.61
	100mg/kg	5	122.0020	6.33085	2.83124	114.1412	129.8628	115.45	131.58
	300mg/kg	5	129.6260	9.84978	4.40495	117.3959	141.8561	120.07	142.52
	1000mg/kg	5	123.2400	9.29852	4.15842	111.6944	134.7856	110.42	131.75
	Total	20	124.2200	9.60534	2.14782	119.7246	128.7154	107.75	142.52
HDL	Control	5	30.7380	1.88699	.84389	28.3950	33.0810	29.18	33.46
	100mg/kg	5	29.6500	2.08130	.93078	27.0657	32.2343	28.02	33.07
	300mg/kg	5	30.4320	4.96885	2.22214	24.2624	36.6016	27.63	39.30
	1000mg/kg	5	29.1080	1.41185	.63140	27.3550	30.8610	27.63	31.13
	Total	20	29.9820	2.77718	.62100	28.6822	31.2818	27.63	39.30
VLDL	Control	5	26.8660	6.57254	2.93933	18.7051	35.0269	19.25	35.29
	100mg/kg	5	23.0160	4.71358	2.10798	17.1633	28.8687	18.82	28.45
	300mg/kg	5	26.8680	6.21222	2.77819	19.1545	34.5815	16.26	32.30
	1000mg/kg	5	27.0380	8.20808	3.67076	16.8463	37.2297	17.54	33.80
	Total	20	25.9470	6.25296	1.39820	23.0205	28.8735	16.26	35.29

ANOVA

Sum of Squares	df	Mean Square	F	Sig.

CHOL	Between Groups	384.041	3	128.014	2.150	.134
	Within Groups	952.679	16	59.542		
	Total	1336.720	19			
TRIGS	Between Groups	1433.552	3	477.851	.446	.723
	Within Groups	17138.874	16	1071.180		
	Total	18572.426	19			
LDL	Between Groups	199.900	3	66.633	.686	.573
	Within Groups	1553.089	16	97.068		
	Total	1752.989	19			
HDL	Between Groups	8.241	3	2.747	.318	.812
	Within Groups	138.301	16	8.644		
	Total	146.542	19			
VLDL	Between Groups	57.369	3	19.123	.446	.723
	Within Groups	685.521	16	42.845		
	Total	742.890	19			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) ID	(J) ID	Mean	Std. Error	Sig.	95% Confidence Interval	
			Difference (I-J)			Lower Bound	Upper Bound
CHOL	Control	100mg/kg	4.94800	4.88026	.744	-9.0145	18.9105
		300mg/kg	-7.30800	4.88026	.462	-21.2705	6.6545
		1000mg/kg	.23000	4.88026	1.000	-13.7325	14.1925
	100mg/kg	Control	-4.94800	4.88026	.744	-18.9105	9.0145
		300mg/kg	-12.25600	4.88026	.096	-26.2185	1.7065
		1000mg/kg					

	300mg/kg	1000mg/kg	-4.71800	4.88026	.770	-18.6805	9.2445
		Control	7.30800	4.88026	.462	-6.6545	21.2705
		100mg/kg	12.25600	4.88026	.096	-1.7065	26.2185
	1000mg/kg	1000mg/kg	7.53800	4.88026	.436	-6.4245	21.5005
		Control	-.23000	4.88026	1.000	-14.1925	13.7325
		100mg/kg	4.71800	4.88026	.770	-9.2445	18.6805
		300mg/kg	-7.53800	4.88026	.436	-21.5005	6.4245
		100mg/kg	19.25000	20.69956	.789	-39.9719	78.4719
		300mg/kg	.00000	20.69956	1.000	-59.2219	59.2219
TRIGS	Control	1000mg/kg	-.85600	20.69956	1.000	-60.0779	58.3659
		Control	-19.25000	20.69956	.789	-78.4719	39.9719
		300mg/kg	-19.25000	20.69956	.789	-78.4719	39.9719
	100mg/kg	1000mg/kg	-20.10600	20.69956	.767	-79.3279	39.1159
		Control	.00000	20.69956	1.000	-59.2219	59.2219
		100mg/kg	19.25000	20.69956	.789	-39.9719	78.4719
	300mg/kg	1000mg/kg	-.85600	20.69956	1.000	-60.0779	58.3659
		Control	.85600	20.69956	1.000	-58.3659	60.0779
		100mg/kg	20.10600	20.69956	.767	-39.1159	79.3279
	1000mg/kg	300mg/kg	.85600	20.69956	1.000	-58.3659	60.0779
		Control	.01000	6.23115	1.000	-17.8174	17.8374
		300mg/kg	-7.61400	6.23115	.623	-25.4414	10.2134
LDL	Control	1000mg/kg	-1.22800	6.23115	.997	-19.0554	16.5994
		Control	-.01000	6.23115	1.000	-17.8374	17.8174
		300mg/kg	-7.62400	6.23115	.622	-25.4514	10.2034
	100mg/kg	1000mg/kg	-1.23800	6.23115	.997	-19.0654	16.5894
		Control	7.61400	6.23115	.623	-10.2134	25.4414
		100mg/kg	7.62400	6.23115	.622	-10.2034	25.4514
	300mg/kg	Control	7.61400	6.23115	.623	-10.2134	25.4414
		100mg/kg	7.62400	6.23115	.622	-10.2034	25.4514

		1000mg/kg	6.38600	6.23115	.738	-11.4414	24.2134
		1000mg/kg Control	1.22800	6.23115	.997	-16.5994	19.0554
		100mg/kg	1.23800	6.23115	.997	-16.5894	19.0654
		300mg/kg	-6.38600	6.23115	.738	-24.2134	11.4414
HDL	Control	100mg/kg	1.08800	1.85945	.935	-4.2319	6.4079
		300mg/kg	.30600	1.85945	.998	-5.0139	5.6259
		1000mg/kg	1.63000	1.85945	.817	-3.6899	6.9499
	100mg/kg	Control	-1.08800	1.85945	.935	-6.4079	4.2319
		300mg/kg	-.78200	1.85945	.974	-6.1019	4.5379
		1000mg/kg	.54200	1.85945	.991	-4.7779	5.8619
	300mg/kg	Control	-.30600	1.85945	.998	-5.6259	5.0139
		100mg/kg	.78200	1.85945	.974	-4.5379	6.1019
		1000mg/kg	1.32400	1.85945	.891	-3.9959	6.6439
	1000mg/kg	Control	-1.63000	1.85945	.817	-6.9499	3.6899
		100mg/kg	-.54200	1.85945	.991	-5.8619	4.7779
		300mg/kg	-1.32400	1.85945	.891	-6.6439	3.9959
VLDL	Control	100mg/kg	3.85000	4.13981	.789	-7.9941	15.6941
		300mg/kg	-.00200	4.13981	1.000	-11.8461	11.8421
		1000mg/kg	-.17200	4.13981	1.000	-12.0161	11.6721
	100mg/kg	Control	-3.85000	4.13981	.789	-15.6941	7.9941
		300mg/kg	-3.85200	4.13981	.789	-15.6961	7.9921
		1000mg/kg	-4.02200	4.13981	.767	-15.8661	7.8221
	300mg/kg	Control	.00200	4.13981	1.000	-11.8421	11.8461
		100mg/kg	3.85200	4.13981	.789	-7.9921	15.6961
		1000mg/kg	-.17000	4.13981	1.000	-12.0141	11.6741
	1000mg/kg	Control	.17200	4.13981	1.000	-11.6721	12.0161
		100mg/kg	4.02200	4.13981	.767	-7.8221	15.8661

300mg/kg	.17000	4.13981	1.000	-11.6741	12.0141

Homogeneous Subsets

CHOL

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
100mg/kg	5	174.6680
1000mg/kg	5	179.3860
Control	5	179.6160
300mg/kg	5	186.9240
Sig.		.096

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

TRIGS

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
100mg/kg	5	115.0820
Control	5	134.3320
300mg/kg	5	134.3320

1000mg/kg	5	135.1880
Sig.		.767

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

LDL

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
100mg/kg	5	122.0020
Control	5	122.0120
1000mg/kg	5	123.2400
300mg/kg	5	129.6260
Sig.		.622

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

HDL

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
1000mg/kg	5	29.1080
100mg/kg	5	29.6500
300mg/kg	5	30.4320
Control	5	30.7380
Sig.		.817

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

VLDL

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
100mg/kg	5	23.0160
Control	5	26.8660
300mg/kg	5	26.8680
1000mg/kg	5	27.0380
Sig.		.767

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

APPENDIX III

KIDNEY FUNCTION TEST

		Descriptives							
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
UREA	Control	5	102.7200	12.64950	5.65703	87.0136	118.4264	81.48	113.92
	100mg/kg	5	89.3220	17.54631	7.84695	67.5354	111.1086	67.99	111.64
	300mg/kg	5	74.8460	14.10721	6.30894	57.3296	92.3624	65.71	99.47
	1000mg/kg	5	68.6240	6.63054	2.96527	60.3911	76.8569	63.76	80.05
	Total	20	83.8780	18.26420	4.08400	75.3301	92.4259	63.76	113.92
Creatinine	Control	5	2.7980	.29525	.13204	2.4314	3.1646	2.52	3.19
	100mg/kg	5	2.1240	.24440	.10930	1.8205	2.4275	1.75	2.32
	300mg/kg	5	2.2940	.28910	.12929	1.9350	2.6530	1.79	2.50
	1000mg/kg	5	2.2880	.39047	.17463	1.8032	2.7728	1.78	2.86
	Total	20	2.3760	.38471	.08602	2.1959	2.5561	1.75	3.19
K+	Control	5	.8840	.19334	.08646	.6439	1.1241	.66	1.11
	100mg/kg	5	.6860	.08173	.03655	.5845	.7875	.62	.78
	300mg/kg	5	.6880	.06907	.03089	.6022	.7738	.64	.80
	1000mg/kg	5	.7380	.10663	.04769	.6056	.8704	.63	.88
	Total	20	.7490	.13973	.03125	.6836	.8144	.62	1.11
Na+	Control	5	32.9520	2.07612	.92847	30.3742	35.5298	30.23	35.16
	100mg/kg	5	77.2480	10.26809	4.59203	64.4985	89.9975	66.33	90.70

CL-	300mg/kg	5	72.1880	14.71103	6.57897	53.9218	90.4542	52.27	85.55
	1000mg/kg	5	67.5480	18.42180	8.23848	44.6743	90.4217	42.66	85.08
	Total	20	62.4840	21.41288	4.78807	52.4625	72.5055	30.23	90.70
	Control	5	23.8420	1.94740	.87090	21.4240	26.2600	21.17	26.66
	100mg/kg	5	22.3180	2.64307	1.18202	19.0362	25.5998	18.42	24.71
	300mg/kg	5	22.0900	3.62738	1.62221	17.5860	26.5940	16.39	25.33
	1000mg/kg	5	24.9260	1.31169	.58661	23.2973	26.5547	22.94	26.13
	Total	20	23.2940	2.61026	.58367	22.0724	24.5156	16.39	26.66

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
UREA	Between Groups	3494.598	3	1164.866	6.555	.004
	Within Groups	2843.441	16	177.715		
	Total	6338.039	19			
Creatinine	Between Groups	1.280	3	.427	4.458	.019
	Within Groups	1.532	16	.096		
	Total	2.812	19			
K+	Between Groups	.130	3	.043	2.883	.068
	Within Groups	.241	16	.015		
	Total	.371	19			
Na+	Between Groups	6049.632	3	2016.544	12.120	.000
	Within Groups	2662.084	16	166.380		
	Total	8711.716	19			
CL-	Between Groups	26.830	3	8.943	1.394	.281
	Within Groups	102.626	16	6.414		

Total	129.456	19			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) ID	(J) ID	Mean	Std. Error	Sig.	95% Confidence Interval	
			Difference (I-J)			Lower Bound	Upper Bound
UREA	Control	100mg/kg	13.39800	8.43125	.412	-10.7240	37.5200
		300mg/kg	27.87400*	8.43125	.021	3.7520	51.9960
		1000mg/kg	34.09600*	8.43125	.005	9.9740	58.2180
	100mg/kg	Control	-13.39800	8.43125	.412	-37.5200	10.7240
		300mg/kg	14.47600	8.43125	.348	-9.6460	38.5980
		1000mg/kg	20.69800	8.43125	.106	-3.4240	44.8200
	300mg/kg	Control	-27.87400*	8.43125	.021	-51.9960	-3.7520
		100mg/kg	-14.47600	8.43125	.348	-38.5980	9.6460
		1000mg/kg	6.22200	8.43125	.880	-17.9000	30.3440
	1000mg/kg	Control	-34.09600*	8.43125	.005	-58.2180	-9.9740
		100mg/kg	-20.69800	8.43125	.106	-44.8200	3.4240
		300mg/kg	-6.22200	8.43125	.880	-30.3440	17.9000
Creatinine	Control	100mg/kg	.67400*	.19569	.016	.1141	1.2339
		300mg/kg	.50400	.19569	.085	-.0559	1.0639
		1000mg/kg	.51000	.19569	.081	-.0499	1.0699
	100mg/kg	Control	-.67400*	.19569	.016	-1.2339	-.1141
		300mg/kg	-.17000	.19569	.821	-.7299	.3899
		1000mg/kg	-.16400	.19569	.836	-.7239	.3959
	300mg/kg	Control	-.50400	.19569	.085	-1.0639	.0559

		100mg/kg	.17000	.19569	.821	-.3899	.7299
		1000mg/kg	.00600	.19569	1.000	-.5539	.5659
	1000mg/kg	Control	-.51000	.19569	.081	-1.0699	.0499
		100mg/kg	.16400	.19569	.836	-.3959	.7239
		300mg/kg	-.00600	.19569	1.000	-.5659	.5539
K+	Control	100mg/kg	.19800	.07759	.089	-.0240	.4200
		300mg/kg	.19600	.07759	.094	-.0260	.4180
		1000mg/kg	.14600	.07759	.274	-.0760	.3680
	100mg/kg	Control	-.19800	.07759	.089	-.4200	.0240
		300mg/kg	-.00200	.07759	1.000	-.2240	.2200
		1000mg/kg	-.05200	.07759	.907	-.2740	.1700
	300mg/kg	Control	-.19600	.07759	.094	-.4180	.0260
		100mg/kg	.00200	.07759	1.000	-.2200	.2240
		1000mg/kg	-.05000	.07759	.916	-.2720	.1720
	1000mg/kg	Control	-.14600	.07759	.274	-.3680	.0760
		100mg/kg	.05200	.07759	.907	-.1700	.2740
		300mg/kg	.05000	.07759	.916	-.1720	.2720
Na+	Control	100mg/kg	-44.29600*	8.15795	.000	-67.6360	-20.9560
		300mg/kg	-39.23600*	8.15795	.001	-62.5760	-15.8960
		1000mg/kg	-34.59600*	8.15795	.003	-57.9360	-11.2560
	100mg/kg	Control	44.29600*	8.15795	.000	20.9560	67.6360
		300mg/kg	5.06000	8.15795	.924	-18.2800	28.4000
		1000mg/kg	9.70000	8.15795	.642	-13.6400	33.0400
	300mg/kg	Control	39.23600*	8.15795	.001	15.8960	62.5760
		100mg/kg	-5.06000	8.15795	.924	-28.4000	18.2800
		1000mg/kg	4.64000	8.15795	.940	-18.7000	27.9800
	1000mg/kg	Control	34.59600*	8.15795	.003	11.2560	57.9360

CL-	Control	100mg/kg	-9.70000	8.15795	.642	-33.0400	13.6400
		300mg/kg	-4.64000	8.15795	.940	-27.9800	18.7000
		100mg/kg	1.52400	1.60177	.778	-3.0587	6.1067
		300mg/kg	1.75200	1.60177	.698	-2.8307	6.3347
		1000mg/kg	-1.08400	1.60177	.904	-5.6667	3.4987
	100mg/kg	Control	-1.52400	1.60177	.778	-6.1067	3.0587
		300mg/kg	.22800	1.60177	.999	-4.3547	4.8107
		1000mg/kg	-2.60800	1.60177	.392	-7.1907	1.9747
	300mg/kg	Control	-1.75200	1.60177	.698	-6.3347	2.8307
		100mg/kg	-.22800	1.60177	.999	-4.8107	4.3547
		1000mg/kg	-2.83600	1.60177	.322	-7.4187	1.7467
	1000mg/kg	Control	1.08400	1.60177	.904	-3.4987	5.6667
		100mg/kg	2.60800	1.60177	.392	-1.9747	7.1907
		300mg/kg	2.83600	1.60177	.322	-1.7467	7.4187

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

UREA

Tukey HSD^a

ID	N	Subset for alpha = 0.05	
		1	2
1000mg/kg	5	68.6240	
300mg/kg	5	74.8460	
100mg/kg	5	89.3220	89.3220
Control	5		102.7200
Sig.		.106	.412

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Creatinine

Tukey HSD^a

ID	N	Subset for alpha = 0.05	
		1	2
100mg/kg	5	2.1240	
1000mg/kg	5	2.2880	2.2880
300mg/kg	5	2.2940	2.2940
Control	5		2.7980
Sig.		.821	.081

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

K+

Tukey HSD^a

ID	N	Subset for alpha = 0.05
		1
100mg/kg	5	.6860
300mg/kg	5	.6880
1000mg/kg	5	.7380
Control	5	.8840
Sig.		.089

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Na+

Tukey HSD^a

ID	N	Subset for alpha = 0.05	
		1	2
Control	5	32.9520	
1000mg/kg	5		67.5480
300mg/kg	5		72.1880
100mg/kg	5		77.2480
Sig.		1.000	.642

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

CL-

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
300mg/kg	5	22.0900
100mg/kg	5	22.3180
Control	5	23.8420
1000mg/kg	5	24.9260
Sig.		.322

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

APPENDIX IV

GLUCOSE

Descriptives

GLUCOSE (mg/dl)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	72.60	12.260	5.483	57.38	87.82	58	90
100mg/kg	5	60.60	16.950	7.580	39.55	81.65	44	85
300mg/kg	5	75.60	8.019	3.586	65.64	85.56	65	85
1000mg/kg	5	70.40	15.773	7.054	50.81	89.99	51	87
Total	20	69.80	13.832	3.093	63.33	76.27	44	90

ANOVA

GLUCOSE (mg/dl)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	632.400	3	210.800	1.123	.369
Within Groups	3002.800	16	187.675		
Total	3635.200	19			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: GLUCOSE (mg/dl)

Tukey HSD

(I) ID	(J) ID	Std. Error	Sig.	95% Confidence Interval

		Mean Difference (I-J)			Lower Bound	Upper Bound
Control	100mg/kg	12.000	8.664	.526	-12.79	36.79
	300mg/kg	-3.000	8.664	.985	-27.79	21.79
	1000mg/kg	2.200	8.664	.994	-22.59	26.99
100mg/kg	Control	-12.000	8.664	.526	-36.79	12.79
	300mg/kg	-15.000	8.664	.341	-39.79	9.79
	1000mg/kg	-9.800	8.664	.676	-34.59	14.99
300mg/kg	Control	3.000	8.664	.985	-21.79	27.79
	100mg/kg	15.000	8.664	.341	-9.79	39.79
	1000mg/kg	5.200	8.664	.931	-19.59	29.99
1000mg/kg	Control	-2.200	8.664	.994	-26.99	22.59
	100mg/kg	9.800	8.664	.676	-14.99	34.59
	300mg/kg	-5.200	8.664	.931	-29.99	19.59

Homogeneous Subsets

GLUCOSE (mg/dl)

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
100mg/kg	5	60.60
1000mg/kg	5	70.40
Control	5	72.60
300mg/kg	5	75.60
Sig.		.341

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

APPENDIX V

BODY AND ORGAN WEIGHT

		Descriptives							
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Initial body weight (g)	Control	5	155.7080	18.94024	8.47033	132.1906	179.2254	134.77	183.29
	100mg/kg	5	155.3740	17.35355	7.76074	133.8267	176.9213	135.29	180.94
	300mg/kg	5	155.2640	16.57871	7.41422	134.6788	175.8492	135.80	179.53
	1000mg/kg	5	154.5520	15.33745	6.85912	135.5080	173.5960	136.01	176.29
	Total	20	155.2245	15.70019	3.51067	147.8766	162.5724	134.77	183.29
Final body weight (g)	Control	5	200.0760	24.53988	10.97457	169.6057	230.5463	171.17	238.20
	100mg/kg	5	182.8260	26.42042	11.81557	150.0207	215.6313	159.67	224.59
	300mg/kg	5	191.6920	23.83239	10.65817	162.1002	221.2838	167.76	225.73
	1000mg/kg	5	178.3380	24.58075	10.99284	147.8170	208.8590	139.12	198.85
	Total	20	188.2330	24.37276	5.44992	176.8262	199.6398	139.12	238.20
Liver(g)	Control	5	7.6100	1.71037	.76490	5.4863	9.7337	5.57	10.31
	100mg/kg	5	6.7940	1.53354	.68582	4.8899	8.6981	4.91	8.86
	300mg/kg	5	7.6840	1.22417	.54746	6.1640	9.2040	6.58	9.49
	1000mg/kg	5	6.3720	1.08594	.48565	5.0236	7.7204	4.78	7.26

	Total	20	7.1150	1.41308	.31597	6.4537	7.7763	4.78	10.31
Left Kidney(g)	Control	5	.6960	.09864	.04411	.5735	.8185	.56	.80
	100mg/kg	5	.5900	.04123	.01844	.5388	.6412	.55	.66
	300mg/kg	5	.6720	.10305	.04609	.5440	.8000	.51	.77
	1000mg/kg	5	.5940	.14223	.06361	.4174	.7706	.43	.79
	Total	20	.6380	.10586	.02367	.5885	.6875	.43	.80
Heart(g)	Control	5	1.9360	2.81598	1.25934	-1.5605	5.4325	.54	6.97
	100mg/kg	5	.6820	.08289	.03707	.5791	.7849	.59	.79
	300mg/kg	5	.7160	.11283	.05046	.5759	.8561	.64	.91
	1000mg/kg	5	.6520	.06870	.03072	.5667	.7373	.57	.75
	Total	20	.9965	1.40882	.31502	.3372	1.6558	.54	6.97
Spleen (g)	Control	5	.9140	.27144	.12139	.5770	1.2510	.64	1.34
	100mg/kg	5	.9320	.16843	.07533	.7229	1.1411	.71	1.18
	300mg/kg	5	1.1380	.47772	.21364	.5448	1.7312	.84	1.98
	1000mg/kg	5	1.0440	.18078	.08085	.8195	1.2685	.80	1.27
	Total	20	1.0070	.29162	.06521	.8705	1.1435	.64	1.98

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Initial body weight (g)	Between Groups	3.550	3	1.183	.004	1.000
	Within Groups	4679.876	16	292.492		
	Total	4683.426	19			
Final body weight (g)	Between Groups	1396.840	3	465.613	.753	.536
	Within Groups	9889.759	16	618.110		
	Total	11286.599	19			

Liver(g)	Between Groups	6.119	3	2.040	1.026	.407
	Within Groups	31.820	16	1.989		
	Total	37.939	19			
Left Kidney(g)	Between Groups	.044	3	.015	1.381	.284
	Within Groups	.169	16	.011		
	Total	.213	19			
Heart(g)	Between Groups	5.895	3	1.965	.988	.423
	Within Groups	31.816	16	1.989		
	Total	37.711	19			
(g) Spleen	Between Groups	.164	3	.055	.603	.623
	Within Groups	1.452	16	.091		
	Total	1.616	19			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) ID	(J) ID	Mean	Std. Error	Sig.	95% Confidence Interval	
			Difference (I-J)			Lower Bound	Upper Bound
Initial body weight (g)	Control	100mg/kg	.33400	10.81651	1.000	-30.6123	31.2803
		300mg/kg	.44400	10.81651	1.000	-30.5023	31.3903
		1000mg/kg	1.15600	10.81651	1.000	-29.7903	32.1023
	100mg/kg	Control	-.33400	10.81651	1.000	-31.2803	30.6123
		300mg/kg	.11000	10.81651	1.000	-30.8363	31.0563
		1000mg/kg	.82200	10.81651	1.000	-30.1243	31.7683
	300mg/kg	Control	-.44400	10.81651	1.000	-31.3903	30.5023
		100mg/kg	-.11000	10.81651	1.000	-31.0563	30.8363
		1000mg/kg	.71200	10.81651	1.000	-30.2343	31.6583
	1000mg/kg	Control	-1.15600	10.81651	1.000	-32.1023	29.7903

Final body weight (g)	Control	100mg/kg	-.82200	10.81651	1.000	-31.7683	30.1243
		300mg/kg	-.71200	10.81651	1.000	-31.6583	30.2343
		1000mg/kg	21.73800	15.72399	.527	-23.2487	66.7247
	100mg/kg	Control	-17.25000	15.72399	.696	-27.7367	62.2367
		300mg/kg	-8.86600	15.72399	.941	-53.8527	36.1207
		1000mg/kg	13.35400	15.72399	.830	-31.6327	58.3407
	300mg/kg	Control	-8.38400	15.72399	.950	-36.6027	53.3707
		100mg/kg	8.86600	15.72399	.941	-36.1207	53.8527
		1000mg/kg	-4.48800	15.72399	.992	-40.4987	49.4747
	1000mg/kg	Control	-21.73800	15.72399	.527	-66.7247	23.2487
		100mg/kg	-4.48800	15.72399	.992	-49.4747	40.4987
		300mg/kg	-13.35400	15.72399	.830	-58.3407	31.6327
Liver(g)	Control	100mg/kg	.81600	.89190	.797	-1.7358	3.3678
		300mg/kg	-.07400	.89190	1.000	-2.6258	2.4778
		1000mg/kg	1.23800	.89190	.524	-1.3138	3.7898
	100mg/kg	Control	-.81600	.89190	.797	-3.3678	1.7358
		300mg/kg	-.89000	.89190	.753	-3.4418	1.6618
		1000mg/kg	.42200	.89190	.964	-2.1298	2.9738
	300mg/kg	Control	.07400	.89190	1.000	-2.4778	2.6258
		100mg/kg	.89000	.89190	.753	-1.6618	3.4418
		1000mg/kg	1.31200	.89190	.477	-1.2398	3.8638
	1000mg/kg	Control	-1.23800	.89190	.524	-3.7898	1.3138
		100mg/kg	-.42200	.89190	.964	-2.9738	2.1298
		300mg/kg	-1.31200	.89190	.477	-3.8638	1.2398
Left Kidney(g)	Control	100mg/kg	.10600	.06502	.391	-.0800	.2920

			300mg/kg	.02400	.06502	.982	-.1620	.2100
			1000mg/kg	.10200	.06502	.423	-.0840	.2880
	100mg/kg	Control	300mg/kg	-.10600	.06502	.391	-.2920	.0800
			1000mg/kg	-.08200	.06502	.599	-.2680	.1040
	300mg/kg	Control	100mg/kg	-.00400	.06502	1.000	-.1900	.1820
			300mg/kg	-.02400	.06502	.982	-.2100	.1620
			1000mg/kg	.08200	.06502	.599	-.1040	.2680
	1000mg/kg	Control	1000mg/kg	.07800	.06502	.636	-.1080	.2640
			100mg/kg	-.10200	.06502	.423	-.2880	.0840
			300mg/kg	.00400	.06502	1.000	-.1820	.1900
			1000mg/kg	-.07800	.06502	.636	-.2640	.1080
Heart(g)	Control		100mg/kg	1.25400	.89185	.514	-1.2976	3.8056
			300mg/kg	1.22000	.89185	.536	-1.3316	3.7716
			1000mg/kg	1.28400	.89185	.494	-1.2676	3.8356
	100mg/kg	Control	100mg/kg	-1.25400	.89185	.514	-3.8056	1.2976
			300mg/kg	-.03400	.89185	1.000	-2.5856	2.5176
			1000mg/kg	.03000	.89185	1.000	-2.5216	2.5816
	300mg/kg	Control	100mg/kg	-1.22000	.89185	.536	-3.7716	1.3316
			300mg/kg	.03400	.89185	1.000	-2.5176	2.5856
			1000mg/kg	.06400	.89185	1.000	-2.4876	2.6156
	1000mg/kg	Control	100mg/kg	-1.28400	.89185	.494	-3.8356	1.2676
			300mg/kg	-.03000	.89185	1.000	-2.5816	2.5216
			1000mg/kg	-.06400	.89185	1.000	-2.6156	2.4876
(g)	Spleen	Control	100mg/kg	-.01800	.19051	1.000	-.5631	.5271
			300mg/kg	-.22400	.19051	.650	-.7691	.3211
			1000mg/kg	-.13000	.19051	.902	-.6751	.4151
			100mg/kg	.01800	.19051	1.000	-.5271	.5631

		300mg/kg	-.20600	.19051	.705	-.7511	.3391
		1000mg/kg	-.11200	.19051	.934	-.6571	.4331
	300mg/kg	Control	.22400	.19051	.650	-.3211	.7691
		100mg/kg	.20600	.19051	.705	-.3391	.7511
	1000mg/kg	1000mg/kg	.09400	.19051	.959	-.4511	.6391
		Control	.13000	.19051	.902	-.4151	.6751
		100mg/kg	.11200	.19051	.934	-.4331	.6571
		300mg/kg	-.09400	.19051	.959	-.6391	.4511

Homogeneous Subsets

Initial body weight (g)

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
1000mg/kg	5	154.5520
300mg/kg	5	155.2640
100mg/kg	5	155.3740
Control	5	155.7080
Sig.		1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Final body weight (g)

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
1000mg/kg	5	178.3380
100mg/kg	5	182.8260
300mg/kg	5	191.6920
Control	5	200.0760
Sig.		.527

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Liver(g)

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
1000mg/kg	5	6.3720
100mg/kg	5	6.7940
Control	5	7.6100
300mg/kg	5	7.6840
Sig.		.477

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Left Kidney(g)

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
100mg/kg	5	.5900
1000mg/kg	5	.5940
300mg/kg	5	.6720
Control	5	.6960
Sig.		.391

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Heart(g)

Tukey HSD^a

ID	N	Subset for alpha = 0.05 1
1000mg/kg	5	.6520
100mg/kg	5	.6820
300mg/kg	5	.7160
Control	5	1.9360
Sig.		.494

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Spleen (g)		
Tukey HSD ^a		
ID	N	Subset for alpha = 0.05 1
Control	5	.9140
100mg/kg	5	.9320
1000mg/kg	5	1.0440
300mg/kg	5	1.1380
Sig.		.650

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

APPENDIX VI

FEED EFFICIENCY

FEED EFFICIENCY

Descriptives

FEED_EFFICIENCY_ACUTE

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
CONTRO L	3	12.9667	14.12881	8.15727	-22.1312	48.0646
GROUP 1	3	6.5600	12.11433	6.99421	-23.5337	36.6537
GROUP 2	3	13.4167	6.59386	3.80696	-2.9634	29.7967
GROUP 3	3	14.9500	3.11630	1.79919	7.2087	22.6913
Total	12	11.9733	9.15942	2.64410	6.1537	17.7930

Descriptives

FEED_EFFICIENCY_ACUTE

	Minimum	Maximum
CONTROL	2.80	29.10
GROUP 1	-6.93	16.51
GROUP 2	8.95	20.99
GROUP 3	12.39	18.42
Total	-6.93	29.10

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
FEED_EFFICIENCY_ ACUTE	Based on Mean	2.987	3	8	.096
	Based on Median	.484	3	8	.702
	Based on Median and with adjusted df	.484	3	4.897	.708
	Based on trimmed mean	2.635	3	8	.121

ANOVA

FEED_EFFICIENCY_ACUTE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	123.704	3	41.235	.413	.748
Within Groups	799.141	8	99.893		
Total	922.845	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: FEED_EFFICIENCY_ACUTE

	(I) GROUP	(J) GROUP	Mean Difference (I- J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Tukey HSD	CONTRO L	GROUP 1	6.40667	8.16058	.859	-19.7264
		GROUP 2	-.45000	8.16058	1.000	-26.5831
		GROUP 3	-1.98333	8.16058	.995	-28.1164
	GROUP 1	CONTRO L	-6.40667	8.16058	.859	-32.5397
		GROUP 2	-6.85667	8.16058	.834	-32.9897
		GROUP 3	-8.39000	8.16058	.739	-34.5231
	GROUP 2	CONTRO L	.45000	8.16058	1.000	-25.6831
		GROUP 1	6.85667	8.16058	.834	-19.2764
		GROUP 3	-1.53333	8.16058	.997	-27.6664

	GROUP 3	CONTRO L	1.98333	8.16058	.995	-24.1497
	GROUP 1		8.39000	8.16058	.739	-17.7431
	GROUP 2		1.53333	8.16058	.997	-24.5997
LSD	CONTRO L	GROUP 1	6.40667	8.16058	.455	-12.4117
		GROUP 2	-.45000	8.16058	.957	-19.2683
		GROUP 3	-1.98333	8.16058	.814	-20.8017
	GROUP 1	CONTRO L	-6.40667	8.16058	.455	-25.2250
		GROUP 2	-6.85667	8.16058	.425	-25.6750
		GROUP 3	-8.39000	8.16058	.334	-27.2083
	GROUP 2	CONTRO L	.45000	8.16058	.957	-18.3683
		GROUP 1	6.85667	8.16058	.425	-11.9617
		GROUP 3	-1.53333	8.16058	.856	-20.3517
	GROUP 3	CONTRO L	1.98333	8.16058	.814	-16.8350
		GROUP 1	8.39000	8.16058	.334	-10.4283
		GROUP 2	1.53333	8.16058	.856	-17.2850

Multiple Comparisons

Dependent Variable: FEED_EFFICIENCY_ACUTE

		95% Confidence Interval	
	(I) GROUP	(J) GROUP	Upper Bound
Tukey HSD	CONTROL	GROUP 1	32.5397
		GROUP 2	25.6831
		GROUP 3	24.1497
	GROUP 1	CONTROL	19.7264
		GROUP 2	19.2764
		GROUP 3	17.7431
	GROUP 2	CONTROL	26.5831
		GROUP 1	32.9897
		GROUP 3	24.5997
	GROUP 3	CONTROL	28.1164
		GROUP 1	34.5231
		GROUP 2	27.6664
LSD	CONTROL	GROUP 1	25.2250
		GROUP 2	18.3683

	GROUP 1	GROUP 3	16.8350
		CONTROL	12.4117
		GROUP 2	11.9617
		GROUP 3	10.4283
	GROUP 2	CONTROL	19.2683
		GROUP 1	25.6750
		GROUP 3	17.2850
	GROUP 3	CONTROL	20.8017
		GROUP 1	27.2083
		GROUP 2	20.3517

Homogeneous Subsets

FEED_EFFICIENCY_ACUTE

		Subset for alpha = 0.05	
	GROUP	N	1
Tukey HSD ^a	GROUP 1	3	6.5600
	CONTROL L	3	12.9667

	GROUP 2	3	13.4167
	GROUP 3	3	14.9500
	Sig.		.739

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Descriptives

FEED_EFFICIENCY_SUBACUTE

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
CONTRO L	5	6.2240	1.00316	.44863	4.9784	7.4696
GROUP 1	5	4.1060	1.52418	.68163	2.2135	5.9985
GROUP 2	5	4.9320	1.09978	.49184	3.5664	6.2976
GROUP 3	5	2.8420	2.34715	1.04968	-.0724	5.7564
Total	20	4.5260	1.92619	.43071	3.6245	5.4275

Descriptives

FEED_EFFICIENCY_SUBACUTE

	Minimum	Maximum
CONTROL	5.11	7.70
GROUP 1	2.35	6.54
GROUP 2	3.76	6.27
GROUP 3	-.94	4.99
Total	-.94	7.70

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
FEED_EFFICIENCY_ SUBACUTE	Based on Mean	.976	3	16	.428
	Based on Median	.640	3	16	.600
	Based on Median and with adjusted df	.640	3	11.200	.605
	Based on trimmed mean	.913	3	16	.457

ANOVA

FEED_EFFICIENCY_SUBACUTE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	30.301	3	10.100	4.021	.026
Within Groups	40.192	16	2.512		
Total	70.494	19			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: FEED_EFFICIENCY_SUBACUTE

	(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Tukey HSD	CONTRO L	GROUP 1	2.11800	1.00240	.191	-.7499
		GROUP 2	1.29200	1.00240	.583	-1.5759
		GROUP 3	3.38200*	1.00240	.018	.5141

	GROUP 1	CONTRO L	-2.11800	1.00240	.191	-4.9859
		GROUP 2	-.82600	1.00240	.842	-3.6939
		GROUP 3	1.26400	1.00240	.599	-1.6039
	GROUP 2	CONTRO L	-1.29200	1.00240	.583	-4.1599
		GROUP 1	.82600	1.00240	.842	-2.0419
		GROUP 3	2.09000	1.00240	.200	-.7779
	GROUP 3	CONTRO L	-3.38200*	1.00240	.018	-6.2499
		GROUP 1	-1.26400	1.00240	.599	-4.1319
		GROUP 2	-2.09000	1.00240	.200	-4.9579
LSD	CONTRO L	GROUP 1	2.11800	1.00240	.051	-.0070
		GROUP 2	1.29200	1.00240	.216	-.8330
		GROUP 3	3.38200*	1.00240	.004	1.2570
	GROUP 1	CONTRO L	-2.11800	1.00240	.051	-4.2430
		GROUP 2	-.82600	1.00240	.422	-2.9510
		GROUP 3	1.26400	1.00240	.225	-.8610

	GROUP 2	CONTRO L	-1.29200	1.00240	.216	-3.4170
		GROUP 1	.82600	1.00240	.422	-1.2990
		GROUP 3	2.09000	1.00240	.053	-.0350
	GROUP 3	CONTRO L	-3.38200*	1.00240	.004	-5.5070
		GROUP 1	-1.26400	1.00240	.225	-3.3890
		GROUP 2	-2.09000	1.00240	.053	-4.2150

Multiple Comparisons

Dependent Variable: FEED_EFFICIENCY_SUBACUTE

95% Confidence Interval

	(I) GROUP	(J) GROUP	Upper Bound
Tukey HSD	CONTROL	GROUP 1	4.9859
		GROUP 2	4.1599
		GROUP 3	6.2499
	GROUP 1	CONTROL	.7499
		GROUP 2	2.0419
		GROUP 3	4.1319

	GROUP 2	CONTROL	1.5759	
		GROUP 1	3.6939	
		GROUP 3	4.9579	
	GROUP 3	CONTROL	-.5141	
		GROUP 1	1.6039	
		GROUP 2	.7779	
	LSD	CONTROL	GROUP 1	4.2430
			GROUP 2	3.4170
			GROUP 3	5.5070
GROUP 1		CONTROL	.0070	
		GROUP 2	1.2990	
		GROUP 3	3.3890	
GROUP 2		CONTROL	.8330	
		GROUP 1	2.9510	
		GROUP 3	4.2150	
GROUP 3		CONTROL	-1.2570	
		GROUP 1	.8610	
		GROUP 2	.0350	

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

FEED_EFFICIENCY_SUBACUTE

		Subset for alpha = 0.05		
	GROUP	N	1	2
Tukey HSD ^a	GROUP 3	5	2.8420	
	GROUP 1	5	4.1060	4.1060
	GROUP 2	5	4.9320	4.9320
	CONTRO L	5		6.2240
	Sig.		.200	.191

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

APPENDIX VII

RELATIVE WEIGHT GAIN/LOSS

Descriptives

REL_WEIGHT

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
CONTRO L	3	1.6600	3.73358	2.15558	-7.6147	10.9347
GROUP 1	3	1.4700	2.63272	1.52000	-5.0700	8.0100
GROUP 2	3	3.0500	1.26099	.72803	-.0825	6.1825
GROUP 3	3	2.9500	.61294	.35388	1.4274	4.4726
Total	12	2.2825	2.17256	.62716	.9021	3.6629

Descriptives

REL_WEIGHT

	Minimum	Maximum
CONTROL	-1.40	5.82
GROUP 1	-1.49	3.55

GROUP 2	2.21	4.50
GROUP 3	2.44	3.63
Total	-1.49	5.82

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
REL_WEIGHT T	Based on Mean	3.434	3	8	.072
	Based on Median	.799	3	8	.528
	Based on Median and with adjusted df	.799	3	4.613	.549
	Based on trimmed mean	3.126	3	8	.088

ANOVA

REL_WEIGHT

	Sum of Squares	df	Mean Square	F	Sig.

Between Groups	6.247	3	2.082	.365	.780
Within Groups	45.673	8	5.709		
Total	51.920	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: REL_WEIGHT

		(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Tukey HSD	CONTRO L	GROUP 1	GROUP 1	.19000	1.95092	1.000	-6.0575
			GROUP 2	-1.39000	1.95092	.889	-7.6375
			GROUP 3	-1.29000	1.95092	.909	-7.5375
	GROUP 1	CONTRO L	GROUP 1	-.19000	1.95092	1.000	-6.4375
			GROUP 2	-1.58000	1.95092	.848	-7.8275
			GROUP 3	-1.48000	1.95092	.871	-7.7275

	GROUP 2	CONTRO L	1.39000	1.95092	.889	-4.8575
		GROUP 1	1.58000	1.95092	.848	-4.6675
		GROUP 3	.10000	1.95092	1.000	-6.1475
	GROUP 3	CONTRO L	1.29000	1.95092	.909	-4.9575
		GROUP 1	1.48000	1.95092	.871	-4.7675
		GROUP 2	-.10000	1.95092	1.000	-6.3475
LSD	CONTRO L	GROUP 1	.19000	1.95092	.925	-4.3088
		GROUP 2	-1.39000	1.95092	.496	-5.8888
		GROUP 3	-1.29000	1.95092	.527	-5.7888
	GROUP 1	CONTRO L	-.19000	1.95092	.925	-4.6888
		GROUP 2	-1.58000	1.95092	.441	-6.0788
		GROUP 3	-1.48000	1.95092	.470	-5.9788
	GROUP 2	CONTRO L	1.39000	1.95092	.496	-3.1088
		GROUP 1	1.58000	1.95092	.441	-2.9188
		GROUP 3	.10000	1.95092	.960	-4.3988

GROUP 3	CONTROL	1.29000	1.95092	.527	-3.2088
	GROUP 1	1.48000	1.95092	.470	-3.0188
	GROUP 2	-.10000	1.95092	.960	-4.5988

Multiple Comparisons

Dependent Variable: REL_WEIGHT

		95% Confidence Interval	
	(I) GROUP	(J) GROUP	Upper Bound
Tukey HSD	CONTROL	GROUP 1	6.4375
		GROUP 2	4.8575
		GROUP 3	4.9575
	GROUP 1	CONTROL	6.0575
		GROUP 2	4.6675
		GROUP 3	4.7675
	GROUP 2	CONTROL	7.6375
		GROUP 1	7.8275
		GROUP 3	6.3475
	GROUP 3	CONTROL	7.5375

LSD	CONTROL	GROUP 1	7.7275
		GROUP 2	6.1475
		GROUP 1	4.6888
		GROUP 2	3.1088
		GROUP 3	3.2088
		GROUP 1	4.3088
	GROUP 1	CONTROL	4.3088
		GROUP 2	2.9188
		GROUP 3	3.0188
	GROUP 2	CONTROL	5.8888
		GROUP 1	6.0788
		GROUP 3	4.5988
	GROUP 3	CONTROL	5.7888
		GROUP 1	5.9788
		GROUP 2	4.3988

Homogeneous Subsets

REL_WEIGHT

			Subset for alpha = 0.05
	GROUP	N	1
Tukey HSD ^a	GROUP 1	3	1.4700
	CONTRO L	3	1.6600
	GROUP 3	3	2.9500
	GROUP 2	3	3.0500
	Sig.		.848

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Descriptives

REL_WEIGHT

N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound

CONTRO L	5	1.5840	.25560	.11431	1.2666	1.9014
GROUP 1	5	.9800	.36352	.16257	.5286	1.4314
GROUP 2	5	1.2980	.28908	.12928	.9391	1.6569
GROUP 3	5	.7580	.62552	.27974	-.0187	1.5347
Total	20	1.1550	.49486	.11065	.9234	1.3866

Descriptives

REL_WEIGHT

	Minimum	Maximum
CONTROL	1.30	1.96
GROUP 1	.56	1.56
GROUP 2	.99	1.65
GROUP 3	-.25	1.33
Total	-.25	1.96

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
REL_WEIGHT T	Based on Mean	1.166	3	16	.353
	Based on Median	.750	3	16	.538
	Based on Median and with adjusted df	.750	3	10.639	.546
	Based on trimmed mean	1.090	3	16	.382

ANOVA

REL_WEIGHT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.964	3	.655	3.894	.029
Within Groups	2.689	16	.168		
Total	4.653	19			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: REL_WEIGHT

	(I) GROUP	(J) GROUP	Mean Difference (I- J)	Std. Error	Sig.	95% Confidence Interval Lower Bound
Tukey HSD	CONTRO L	GROUP 1	.60400	.25929	.133	-.1378
		GROUP 2	.28600	.25929	.693	-.4558
		GROUP 3	.82600*	.25929	.027	.0842
	GROUP 1	CONTRO L	-.60400	.25929	.133	-1.3458
		GROUP 2	-.31800	.25929	.620	-1.0598
		GROUP 3	.22200	.25929	.827	-.5198
	GROUP 2	CONTRO L	-.28600	.25929	.693	-1.0278
		GROUP 1	.31800	.25929	.620	-.4238
		GROUP 3	.54000	.25929	.201	-.2018
	GROUP 3	CONTRO L	-.82600*	.25929	.027	-1.5678
		GROUP 1	-.22200	.25929	.827	-.9638
		GROUP 2	-.54000	.25929	.201	-1.2818

LSD	CONTRO L	GROUP 1	.60400*	.25929	.033	.0543
		GROUP 2	.28600	.25929	.286	-.2637
		GROUP 3	.82600*	.25929	.006	.2763
	GROUP 1	CONTRO L	-.60400*	.25929	.033	-1.1537
		GROUP 2	-.31800	.25929	.238	-.8677
		GROUP 3	.22200	.25929	.405	-.3277
	GROUP 2	CONTRO L	-.28600	.25929	.286	-.8357
		GROUP 1	.31800	.25929	.238	-.2317
		GROUP 3	.54000	.25929	.054	-.0097
	GROUP 3	CONTRO L	-.82600*	.25929	.006	-1.3757
		GROUP 1	-.22200	.25929	.405	-.7717
		GROUP 2	-.54000	.25929	.054	-1.0897

Multiple Comparisons

Dependent Variable: REL_WEIGHT

		95% Confidence Interval	
	(I) GROUP	(J) GROUP	Upper Bound
Tukey HSD	CONTROL	GROUP 1	1.3458
		GROUP 2	1.0278
		GROUP 3	1.5678
	GROUP 1	CONTROL	.1378
		GROUP 2	.4238
		GROUP 3	.9638
	GROUP 2	CONTROL	.4558
		GROUP 1	1.0598
		GROUP 3	1.2818
	GROUP 3	CONTROL	-.0842
		GROUP 1	.5198
		GROUP 2	.2018
LSD	CONTROL	GROUP 1	1.1537
		GROUP 2	.8357
		GROUP 3	1.3757
	GROUP 1	CONTROL	-.0543

		GROUP 2	.2317
		GROUP 3	.7717
GROUP 2		CONTROL	.2637
		GROUP 1	.8677
		GROUP 3	1.0897
GROUP 3		CONTROL	-.2763
		GROUP 1	.3277
		GROUP 2	.0097

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

REL_WEIGHT

			Subset for alpha = 0.05	
	GROUP	N	1	2
Tukey HSD ^a	GROUP 3	5	.7580	
	GROUP 1	5	.9800	.9800
	GROUP 2	5	1.2980	1.2980

	CONTRO L	5		1.5840
	Sig.		.201	.133

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound
START_WEE K	CONTRO L	3	161.0000	36.29049	20.95233	70.8494

	GROUP 1	3	167.1633	14.13478	8.16072	132.0506
	GROUP 2	3	150.3500	30.14711	17.40544	75.4604
	GROUP 3	3	152.7000	1.65227	.95394	148.5955
	Total	12	157.8033	22.14624	6.39307	143.7323
WEEK_1	CONTRO L	3	186.5733	35.80435	20.67165	97.6304
	GROUP 1	3	185.8467	28.49299	16.45043	115.0662
	GROUP 2	3	190.8567	26.85926	15.50720	124.1346
	GROUP 3	3	189.1567	30.09175	17.37348	114.4046
	Total	12	188.1083	26.09396	7.53268	171.5290
WEEK_2	CONTRO L	3	184.4133	26.03347	15.03043	119.7426
	GROUP 1	3	187.7333	24.47677	14.13167	126.9297
	GROUP 2	3	193.0333	12.75083	7.36169	161.3585
	GROUP 3	3	193.9833	7.59421	4.38452	175.1183
	Total	12	189.7908	16.99718	4.90666	178.9913

Descriptives

		95% Confidence Interval for Mean		
		Upper Bound	Minimum	Maximum
START_WEEK	CONTROL	251.1506	120.00	189.00
	GROUP 1	202.2761	152.20	180.29
	GROUP 2	225.2396	115.54	167.97
	GROUP 3	156.8045	151.10	154.40
	Total	171.8744	115.54	189.00
WEEK_1	CONTROL	275.5163	148.79	220.00
	GROUP 1	256.6272	154.53	210.24
	GROUP 2	257.5788	162.33	215.66
	GROUP 3	263.9087	171.35	223.90
	Total	204.6876	148.79	223.90
WEEK_2	CONTROL	249.0841	154.46	201.59
	GROUP 1	248.5370	159.47	201.87
	GROUP 2	224.7081	178.47	202.19

	GROUP 3	212.8484	186.72	201.87
	Total	200.5903	154.46	202.19

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
START_WEE K	Based on Mean	5.005	3	8	.030
	Based on Median	.629	3	8	.616
	Based on Median and with adjusted df	.629	3	4.326	.631
	Based on trimmed mean	4.331	3	8	.043
WEEK_1	Based on Mean	.113	3	8	.950
	Based on Median	.052	3	8	.983
	Based on Median and with adjusted df	.052	3	6.563	.983
	Based on trimmed mean	.105	3	8	.955
WEEK_2	Based on Mean	3.327	3	8	.077
	Based on Median	.239	3	8	.867

	Based on Median and with adjusted df	.239	3	4.871	.866
	Based on trimmed mean	2.720	3	8	.115

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
START_WEEK	Between Groups	538.273	3	179.424	.296	.828
	Within Groups	4856.741	8	607.093		
	Total	5395.014	11			
WEEK_1	Between Groups	48.371	3	16.124	.017	.997
	Within Groups	7441.470	8	930.184		
	Total	7489.841	11			
WEEK_2	Between Groups	183.725	3	61.242	.164	.918
	Within Groups	2994.219	8	374.277		
	Total	3177.944	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable		(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
START_W EEK	Tukey HSD	CONTR OL	GROUP 1	-6.16333	20.117 86	.989	-70.5878	58.2611
			GROUP 2	10.65000	20.117 86	.949	-53.7745	75.0745
			GROUP 3	8.30000	20.117 86	.975	-56.1245	72.7245
		GROUP 1	CONTR OL	6.16333	20.117 86	.989	-58.2611	70.5878
			GROUP 2	16.81333	20.117 86	.836	-47.6111	81.2378
			GROUP 3	14.46333	20.117 86	.887	-49.9611	78.8878
		GROUP 2	CONTR OL	-10.65000	20.117 86	.949	-75.0745	53.7745

		GROUP 1	-16.81333	20.11786	.836	-81.2378	47.6111
		GROUP 3	-2.35000	20.11786	.999	-66.7745	62.0745
		GROUP 3 CONTR OL	-8.30000	20.11786	.975	-72.7245	56.1245
		GROUP 1	-14.46333	20.11786	.887	-78.8878	49.9611
		GROUP 2	2.35000	20.11786	.999	-62.0745	66.7745
	LSD	CONTR OL GROUP 1	-6.16333	20.11786	.767	-52.5552	40.2285
		GROUP 2	10.65000	20.11786	.611	-35.7419	57.0419
		GROUP 3	8.30000	20.11786	.691	-38.0919	54.6919
	GROUP 1	CONTR OL	6.16333	20.11786	.767	-40.2285	52.5552
		GROUP 2	16.81333	20.11786	.428	-29.5785	63.2052
		GROUP 3	14.46333	20.11786	.493	-31.9285	60.8552

		GROUP 2	CONTR OL	-10.65000	20.117 86	.611	-57.0419	35.7419
			GROUP 1	-16.81333	20.117 86	.428	-63.2052	29.5785
			GROUP 3	-2.35000	20.117 86	.910	-48.7419	44.0419
		GROUP 3	CONTR OL	-8.30000	20.117 86	.691	-54.6919	38.0919
			GROUP 1	-14.46333	20.117 86	.493	-60.8552	31.9285
			GROUP 2	2.35000	20.117 86	.910	-44.0419	48.7419
WEEK_1	Tukey HSD	CONTR OL	GROUP 1	.72667	24.902 26	1.000	-79.0191	80.4725
			GROUP 2	-4.28333	24.902 26	.998	-84.0291	75.4625
			GROUP 3	-2.58333	24.902 26	1.000	-82.3291	77.1625
		GROUP 1	CONTR OL	-.72667	24.902 26	1.000	-80.4725	79.0191
			GROUP 2	-5.01000	24.902 26	.997	-84.7558	74.7358

		GROUP 3	-3.31000	24.902 26	.999	-83.0558	76.4358
	GROUP 2	CONTR OL	4.28333	24.902 26	.998	-75.4625	84.0291
		GROUP 1	5.01000	24.902 26	.997	-74.7358	84.7558
		GROUP 3	1.70000	24.902 26	1.000	-78.0458	81.4458
	GROUP 3	CONTR OL	2.58333	24.902 26	1.000	-77.1625	82.3291
		GROUP 1	3.31000	24.902 26	.999	-76.4358	83.0558
		GROUP 2	-1.70000	24.902 26	1.000	-81.4458	78.0458
	LSD	CONTR OL	GROUP 1	.72667	24.902 26	.977	-56.6980
GROUP 2			-4.28333	24.902 26	.868	-61.7080	53.1414
GROUP 3			-2.58333	24.902 26	.920	-60.0080	54.8414
GROUP 1		CONTR OL	-.72667	24.902 26	.977	-58.1514	56.6980

			GROUP 2	-5.01000	24.90226	.846	-62.4347	52.4147
			GROUP 3	-3.31000	24.90226	.898	-60.7347	54.1147
			GROUP 2 CONTR OL	4.28333	24.90226	.868	-53.1414	61.7080
			GROUP 1	5.01000	24.90226	.846	-52.4147	62.4347
			GROUP 3	1.70000	24.90226	.947	-55.7247	59.1247
			GROUP 3 CONTR OL	2.58333	24.90226	.920	-54.8414	60.0080
			GROUP 1	3.31000	24.90226	.898	-54.1147	60.7347
			GROUP 2	-1.70000	24.90226	.947	-59.1247	55.7247
			GROUP 1	-3.32000	15.79615	.996	-53.9048	47.2648
			GROUP 2	-8.62000	15.79615	.945	-59.2048	41.9648
			GROUP 3	-9.57000	15.79615	.927	-60.1548	41.0148
WEEK_2	Tukey HSD	CONTR OL	GROUP 1	-3.32000	15.79615	.996	-53.9048	47.2648
			GROUP 2	-8.62000	15.79615	.945	-59.2048	41.9648
			GROUP 3	-9.57000	15.79615	.927	-60.1548	41.0148

	GROUP 1	CONTR OL	3.32000	15.796 15	.996	-47.2648	53.9048
		GROUP 2	-5.30000	15.796 15	.986	-55.8848	45.2848
		GROUP 3	-6.25000	15.796 15	.978	-56.8348	44.3348
	GROUP 2	CONTR OL	8.62000	15.796 15	.945	-41.9648	59.2048
		GROUP 1	5.30000	15.796 15	.986	-45.2848	55.8848
		GROUP 3	-.95000	15.796 15	1.000	-51.5348	49.6348
	GROUP 3	CONTR OL	9.57000	15.796 15	.927	-41.0148	60.1548
		GROUP 1	6.25000	15.796 15	.978	-44.3348	56.8348
		GROUP 2	.95000	15.796 15	1.000	-49.6348	51.5348
LSD	CONTR OL	GROUP 1	-3.32000	15.796 15	.839	-39.7460	33.1060
		GROUP 2	-8.62000	15.796 15	.600	-45.0460	27.8060

	GROUP 1	GROUP 3	-9.57000	15.79615	.561	-45.9960	26.8560
		CONTR OL	3.32000	15.79615	.839	-33.1060	39.7460
		GROUP 2	-5.30000	15.79615	.746	-41.7260	31.1260
		GROUP 3	-6.25000	15.79615	.703	-42.6760	30.1760
	GROUP 2	CONTR OL	8.62000	15.79615	.600	-27.8060	45.0460
		GROUP 1	5.30000	15.79615	.746	-31.1260	41.7260
		GROUP 3	-.95000	15.79615	.954	-37.3760	35.4760
	GROUP 3	CONTR OL	9.57000	15.79615	.561	-26.8560	45.9960
		GROUP 1	6.25000	15.79615	.703	-30.1760	42.6760
		GROUP 2	.95000	15.79615	.954	-35.4760	37.3760

Homogeneous Subsets

START_WEEK			
		Subset for alpha = 0.05	
	GROUP	N	1
Tukey HSD ^a	GROUP 2	3	150.3500
	GROUP 3	3	152.7000
	CONTRO L	3	161.0000
	GROUP 1	3	167.1633
	Sig.		.836

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

WEEK_1

		Subset for alpha = 0.05	
	GROUP	N	1
Tukey HSD ^a	GROUP 1	3	185.8467
	CONTRO L	3	186.5733
	GROUP 3	3	189.1567
	GROUP 2	3	190.8567
	Sig.		.997

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

WEEK_2

		Subset for alpha = 0.05	
	GROUP	N	1

Tukey HSD ^a	CONTRO L	3	184.4133
	GROUP 1	3	187.7333
	GROUP 2	3	193.0333
	GROUP 3	3	193.9833
	Sig.		.927

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

[DataSet2]

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound
START_WEEK	CONTROL	5	155.7080	18.94024	8.47033	132.1906
	GROUP 1	5	155.3740	17.35355	7.76074	133.8267
	GROUP 2	5	155.2640	16.57871	7.41422	134.6788
	GROUP 3	5	154.5520	15.33745	6.85912	135.5080
	Total	20	155.2245	15.70019	3.51067	147.8766
WEEK_1	CONTROL	5	165.6720	17.31860	7.74511	144.1681
	GROUP 1	5	159.8440	19.03941	8.51468	136.2035
	GROUP 2	5	157.4180	16.13770	7.21700	137.3804
	GROUP 3	5	147.9440	29.66965	13.26867	111.1043

	Total	20	157.7195	20.55752	4.59680	148.0983
WEEK_2	CONTRO L	5	179.5820	21.56633	9.64475	152.8039
	GROUP 1	5	165.9640	21.31608	9.53284	139.4966
	GROUP 2	5	169.1640	14.99930	6.70789	150.5399
	GROUP 3	5	161.6860	19.48376	8.71340	137.4937
	Total	20	169.0990	19.15271	4.28268	160.1353
WEEK_3	CONTRO L	5	185.2940	22.92849	10.25393	156.8245
	GROUP 1	5	173.6780	24.68060	11.03750	143.0330
	GROUP 2	5	178.8560	17.07305	7.63530	157.6570
	GROUP 3	5	169.3180	13.08356	5.85114	153.0726
	Total	20	176.7865	19.33169	4.32270	167.7390
WEEK_4	CONTRO L	5	200.0760	24.53988	10.97457	169.6057
	GROUP 1	5	182.8260	26.42042	11.81557	150.0207
	GROUP 2	5	191.6920	23.83239	10.65817	162.1002
	GROUP 3	5	178.3380	24.58075	10.99284	147.8170
	Total	20	188.2330	24.37276	5.44992	176.8262

Descriptives

		95% Confidence Interval for Mean		
		Upper Bound	Minimum	Maximum
START_WEEK	CONTROL	179.2254	134.77	183.29
	GROUP 1	176.9213	135.29	180.94
	GROUP 2	175.8492	135.80	179.53
	GROUP 3	173.5960	136.01	176.29
	Total	162.5724	134.77	183.29
WEEK_1	CONTROL	187.1759	150.31	192.43
	GROUP 1	183.4845	144.25	192.15
	GROUP 2	177.4556	141.40	178.60
	GROUP 3	184.7837	109.09	175.65
	Total	167.3407	109.09	192.43
WEEK_2	CONTROL	206.3601	153.64	210.30
	GROUP 1	192.4314	137.81	195.17
	GROUP 2	187.7881	151.42	188.73
	GROUP 3	185.8783	137.12	181.08
	Total	178.0627	137.12	210.30

WEEK_3	CONTROL	213.7635	165.90	221.81
	GROUP 1	204.3230	150.64	210.32
	GROUP 2	200.0550	161.11	203.61
	GROUP 3	185.5634	158.16	184.64
	Total	185.8340	150.64	221.81
WEEK_4	CONTROL	230.5463	171.17	238.20
	GROUP 1	215.6313	159.67	224.59
	GROUP 2	221.2838	167.76	225.73
	GROUP 3	208.8590	139.12	198.85
	Total	199.6398	139.12	238.20

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
START_WEE K	Based on Mean	.061	3	16	.979
	Based on Median	.064	3	16	.978
	Based on Median and with adjusted df	.064	3	15.743	.978

	Based on trimmed mean	.058	3	16	.981
WEEK_1	Based on Mean	1.761	3	16	.195
	Based on Median	.760	3	16	.533
	Based on Median and with adjusted df	.760	3	13.153	.536
	Based on trimmed mean	1.658	3	16	.216
WEEK_2	Based on Mean	.170	3	16	.915
	Based on Median	.148	3	16	.929
	Based on Median and with adjusted df	.148	3	13.788	.929
	Based on trimmed mean	.172	3	16	.914
WEEK_3	Based on Mean	.470	3	16	.708
	Based on Median	.412	3	16	.746
	Based on Median and with adjusted df	.412	3	14.408	.747
	Based on trimmed mean	.433	3	16	.733
WEEK_4	Based on Mean	.041	3	16	.989
	Based on Median	.018	3	16	.997
	Based on Median and with adjusted df	.018	3	15.097	.997

Based on trimmed mean	.037	3	16	.990

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
START_WEE K	Between Groups	3.550	3	1.183	.004	1.000
	Within Groups	4679.876	16	292.492		
	Total	4683.426	19			
WEEK_1	Between Groups	817.035	3	272.345	.604	.622
	Within Groups	7212.585	16	450.787		
	Total	8029.620	19			
WEEK_2	Between Groups	873.392	3	291.131	.764	.531
	Within Groups	6096.311	16	381.019		
	Total	6969.703	19			
WEEK_3	Between Groups	710.508	3	236.836	.593	.629

	Within Groups	6390.064	16	399.379		
	Total	7100.573	19			
WEEK_4	Between Groups	1396.840	3	465.613	.753	.536
	Within Groups	9889.759	16	618.110		
	Total	11286.599	19			

Post Hoc Tests

Multiple Comparisons

Dependent Variable	(I) GROUP	(J) GROUP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound

START_W EEK	Tukey HSD	CONTR OL	GROUP 1	.33400	10.816 51	1.000	-30.6123	31.2803
			GROUP 2	.44400	10.816 51	1.000	-30.5023	31.3903
			GROUP 3	1.15600	10.816 51	1.000	-29.7903	32.1023
		GROUP 1	CONTR OL	-.33400	10.816 51	1.000	-31.2803	30.6123
			GROUP 2	.11000	10.816 51	1.000	-30.8363	31.0563
			GROUP 3	.82200	10.816 51	1.000	-30.1243	31.7683
		GROUP 2	CONTR OL	-.44400	10.816 51	1.000	-31.3903	30.5023
			GROUP 1	-.11000	10.816 51	1.000	-31.0563	30.8363
			GROUP 3	.71200	10.816 51	1.000	-30.2343	31.6583
		GROUP 3	CONTR OL	-1.15600	10.816 51	1.000	-32.1023	29.7903
			GROUP 1	-.82200	10.816 51	1.000	-31.7683	30.1243

LSD	CONTR OL	GROUP 2	-.71200	10.816 51	1.000	-31.6583	30.2343
		GROUP 1	.33400	10.816 51	.976	-22.5960	23.2640
		GROUP 2	.44400	10.816 51	.968	-22.4860	23.3740
		GROUP 3	1.15600	10.816 51	.916	-21.7740	24.0860
		GROUP 1	CONTR OL	-.33400	10.816 51	.976	-23.2640
		GROUP 2		.11000	10.816 51	.992	-22.8200
		GROUP 3		.82200	10.816 51	.940	-22.1080
		GROUP 2	CONTR OL	-.44400	10.816 51	.968	-23.3740
		GROUP 1		-.11000	10.816 51	.992	-23.0400
		GROUP 3		.71200	10.816 51	.948	-22.2180
		GROUP 3	CONTR OL	-1.15600	10.816 51	.916	-24.0860

WEEK_1	Tukey HSD	CONTR OL	GROUP 1	-0.82200	10.816 51	.940	-23.7520	22.1080
			GROUP 2	-0.71200	10.816 51	.948	-23.6420	22.2180
			GROUP 1	5.82800	13.428 13	.972	-32.5901	44.2461
			GROUP 2	8.25400	13.428 13	.926	-30.1641	46.6721
			GROUP 3	17.72800	13.428 13	.564	-20.6901	56.1461
		GROUP 1	CONTR OL	-5.82800	13.428 13	.972	-44.2461	32.5901
			GROUP 2	2.42600	13.428 13	.998	-35.9921	40.8441
			GROUP 3	11.90000	13.428 13	.812	-26.5181	50.3181
		GROUP 2	CONTR OL	-8.25400	13.428 13	.926	-46.6721	30.1641
			GROUP 1	-2.42600	13.428 13	.998	-40.8441	35.9921
			GROUP 3	9.47400	13.428 13	.893	-28.9441	47.8921

LSD	GROUP 3	CONTR OL	-17.72800	13.42813	.564	-56.1461	20.6901
		GROUP 1	-11.90000	13.42813	.812	-50.3181	26.5181
		GROUP 2	-9.47400	13.42813	.893	-47.8921	28.9441
	CONTR OL	GROUP 1	5.82800	13.42813	.670	-22.6384	34.2944
		GROUP 2	8.25400	13.42813	.547	-20.2124	36.7204
		GROUP 3	17.72800	13.42813	.205	-10.7384	46.1944
	GROUP 1	CONTR OL	-5.82800	13.42813	.670	-34.2944	22.6384
		GROUP 2	2.42600	13.42813	.859	-26.0404	30.8924
		GROUP 3	11.90000	13.42813	.389	-16.5664	40.3664
	GROUP 2	CONTR OL	-8.25400	13.42813	.547	-36.7204	20.2124
		GROUP 1	-2.42600	13.42813	.859	-30.8924	26.0404

			GROUP 3	9.47400	13.428 13	.491	-18.9924	37.9404	
			GROUP 3	CONTR OL	-17.72800	13.428 13	.205	-46.1944	10.7384
			GROUP 1		-11.90000	13.428 13	.389	-40.3664	16.5664
			GROUP 2		-9.47400	13.428 13	.491	-37.9404	18.9924
WEEK_2	Tukey HSD	CONTR OL	GROUP 1	13.61800	12.345 35	.693	-21.7023	48.9383	
			GROUP 2	10.41800	12.345 35	.833	-24.9023	45.7383	
			GROUP 3	17.89600	12.345 35	.489	-17.4243	53.2163	
		GROUP 1	CONTR OL	-13.61800	12.345 35	.693	-48.9383	21.7023	
			GROUP 2	-3.20000	12.345 35	.994	-38.5203	32.1203	
			GROUP 3	4.27800	12.345 35	.985	-31.0423	39.5983	
		GROUP 2	CONTR OL	-10.41800	12.345 35	.833	-45.7383	24.9023	

		GROUP 1	3.20000	12.34535	.994	-32.1203	38.5203
		GROUP 3	7.47800	12.34535	.929	-27.8423	42.7983
		GROUP 3 CONTR OL	-17.89600	12.34535	.489	-53.2163	17.4243
		GROUP 1	-4.27800	12.34535	.985	-39.5983	31.0423
		GROUP 2	-7.47800	12.34535	.929	-42.7983	27.8423
	LSD	CONTR OL GROUP 1	13.61800	12.34535	.286	-12.5530	39.7890
		GROUP 2	10.41800	12.34535	.411	-15.7530	36.5890
		GROUP 3	17.89600	12.34535	.166	-8.2750	44.0670
	GROUP 1	CONTR OL	-13.61800	12.34535	.286	-39.7890	12.5530
		GROUP 2	-3.20000	12.34535	.799	-29.3710	22.9710
		GROUP 3	4.27800	12.34535	.733	-21.8930	30.4490

		GROUP 2	CONTR OL	-10.41800	12.34535	.411	-36.5890	15.7530		
			GROUP 1	3.20000	12.34535	.799	-22.9710	29.3710		
			GROUP 3	7.47800	12.34535	.553	-18.6930	33.6490		
		GROUP 3	CONTR OL	-17.89600	12.34535	.166	-44.0670	8.2750		
			GROUP 1	-4.27800	12.34535	.733	-30.4490	21.8930		
			GROUP 2	-7.47800	12.34535	.553	-33.6490	18.6930		
		WEEK_3	Tukey HSD	CONTR OL	GROUP 1	11.61600	12.63929	.795	-24.5453	47.7773
					GROUP 2	6.43800	12.63929	.956	-29.7233	42.5993
					GROUP 3	15.97600	12.63929	.597	-20.1853	52.1373
GROUP 1	CONTR OL			-11.61600	12.63929	.795	-47.7773	24.5453		
	GROUP 2			-5.17800	12.63929	.976	-41.3393	30.9833		

		GROUP 3	4.36000	12.63929	.985	-31.8013	40.5213	
	GROUP 2	CONTR OL	-6.43800	12.63929	.956	-42.5993	29.7233	
		GROUP 1	5.17800	12.63929	.976	-30.9833	41.3393	
		GROUP 3	9.53800	12.63929	.873	-26.6233	45.6993	
	GROUP 3	CONTR OL	-15.97600	12.63929	.597	-52.1373	20.1853	
		GROUP 1	-4.36000	12.63929	.985	-40.5213	31.8013	
		GROUP 2	-9.53800	12.63929	.873	-45.6993	26.6233	
	LSD	CONTR OL	GROUP 1	11.61600	12.63929	.372	-15.1781	38.4101
			GROUP 2	6.43800	12.63929	.617	-20.3561	33.2321
			GROUP 3	15.97600	12.63929	.224	-10.8181	42.7701
GROUP 1		CONTR OL	-11.61600	12.63929	.372	-38.4101	15.1781	

			GROUP 2	-5.17800	12.63929	.687	-31.9721	21.6161
			GROUP 3	4.36000	12.63929	.735	-22.4341	31.1541
			GROUP 2 CONTR OL	-6.43800	12.63929	.617	-33.2321	20.3561
			GROUP 1	5.17800	12.63929	.687	-21.6161	31.9721
			GROUP 3	9.53800	12.63929	.461	-17.2561	36.3321
			GROUP 3 CONTR OL	-15.97600	12.63929	.224	-42.7701	10.8181
			GROUP 1	-4.36000	12.63929	.735	-31.1541	22.4341
			GROUP 2	-9.53800	12.63929	.461	-36.3321	17.2561
			GROUP 1	17.25000	15.72399	.696	-27.7367	62.2367
			GROUP 2	8.38400	15.72399	.950	-36.6027	53.3707
WEEK_4	Tukey HSD	CONTR OL	GROUP 3	21.73800	15.72399	.527	-23.2487	66.7247

	GROUP 1	CONTR OL	-17.25000	15.72399	.696	-62.2367	27.7367
		GROUP 2	-8.86600	15.72399	.941	-53.8527	36.1207
		GROUP 3	4.48800	15.72399	.992	-40.4987	49.4747
	GROUP 2	CONTR OL	-8.38400	15.72399	.950	-53.3707	36.6027
		GROUP 1	8.86600	15.72399	.941	-36.1207	53.8527
		GROUP 3	13.35400	15.72399	.830	-31.6327	58.3407
	GROUP 3	CONTR OL	-21.73800	15.72399	.527	-66.7247	23.2487
		GROUP 1	-4.48800	15.72399	.992	-49.4747	40.4987
		GROUP 2	-13.35400	15.72399	.830	-58.3407	31.6327
LSD	CONTR OL	GROUP 1	17.25000	15.72399	.289	-16.0834	50.5834
		GROUP 2	8.38400	15.72399	.601	-24.9494	41.7174

	GROUP 1	GROUP 3	21.73800	15.72399	.186	-11.5954	55.0714
		CONTR OL	-17.25000	15.72399	.289	-50.5834	16.0834
		GROUP 2	-8.86600	15.72399	.581	-42.1994	24.4674
		GROUP 3	4.48800	15.72399	.779	-28.8454	37.8214
	GROUP 2	CONTR OL	-8.38400	15.72399	.601	-41.7174	24.9494
		GROUP 1	8.86600	15.72399	.581	-24.4674	42.1994
		GROUP 3	13.35400	15.72399	.408	-19.9794	46.6874
	GROUP 3	CONTR OL	-21.73800	15.72399	.186	-55.0714	11.5954
		GROUP 1	-4.48800	15.72399	.779	-37.8214	28.8454
		GROUP 2	-13.35400	15.72399	.408	-46.6874	19.9794

Homogeneous Subsets

START_WEEK			
		Subset for alpha = 0.05	
	GROUP	N	1
Tukey HSD ^a	GROUP 3	5	154.5520
	GROUP 2	5	155.2640
	GROUP 1	5	155.3740
	CONTRO L	5	155.7080
	Sig.		1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

WEEK_1

		Subset for alpha = 0.05	
	GROUP	N	1
Tukey HSD ^a	GROUP 3	5	147.9440
	GROUP 2	5	157.4180
	GROUP 1	5	159.8440
	CONTRO L	5	165.6720
	Sig.		.564

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

WEEK_2

		Subset for alpha = 0.05	
	GROUP	N	

			1
Tukey HSD ^a	GROUP 3	5	161.6860
	GROUP 1	5	165.9640
	GROUP 2	5	169.1640
	CONTRO L	5	179.5820
	Sig.		.489

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

WEEK_3

			Subset for alpha = 0.05
		N	1
Tukey HSD ^a	GROUP 3	5	169.3180
	GROUP 1	5	173.6780
	GROUP 2	5	178.8560

	CONTRO L	5	185.2940
	Sig.		.597

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

WEEK_4

		Subset for alpha = 0.05	
	GROUP	N	1
Tukey HSD ^a	GROUP 3	5	178.3380
	GROUP 1	5	182.8260
	GROUP 2	5	191.6920
	CONTRO L	5	200.0760
	Sig.		.527

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

DATASET ACTIVATE WEEKLY_BODYWEIGHT_ACUTE.

DATASET CLOSE DataSet2.