A 28-DAY REPEATED ORAL TOXICITY STUDY OF ETHANOL ROOT EXTRACT OF MORINGA OLEIFERA IN RODENTS

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NOVEMBER, 2025

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BEING A PROJECT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHARMACY BY THE DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY, UNIVERSITY OF BENIN, BENIN CITY, EDO STATE.

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CERTIFICATION

This is to certify that this work was carried out by **WINNER OSARUESE EHIMA** in the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City in partial fulfillment for the award of the Doctor of Pharmacy degree.

_____ _____

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HEAD OF DEPARTMENT

DEDICATION

I dedicate this work to Almighty God who made it all possible and also to my ever supportive parents, and to my supervisor, friends, I am deeply grateful for everything.

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All thanks and praise to God Almighty for His grace and mercy for the successful completion of this project. I am deeply grateful to my supervisor, Professor Ighodaro Igbe for his fatherly support, kindness, scholarly advice, direction and assistance which greatly contributed to the completion of this project.

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ABSTRACT

Moringa oleifera is a medicinal plant that has found massive application in traditional herbal medicine practice though, the toxicological assessment of the root extract has not been well known. This study aimed to evaluate the sub-acute impacts of the oral administration of ethanol root extract of *Moringa oleifera* on body weight, organ weight index, and histopathology of male Wistar rats.

Twenty male wistar rats were randomly assigned to four groups (n = 5). Group I (control) received distilled water, while Groups II, III, and IV received 150 mg/kg, 300 mg/kg, and 600 mg/kg of *Moringa oleifera* extract, respectively, via oral administration for 28 days. Body weights were taken in a weekly schedule, major organs (heart, kidney, liver, lungs and spleen) were harvested, weighed, preserved for histopathological investigations and organ weight index was calculated. Data were analyzed using GraphPad Prism, with a statistical significance level of $p < 0.05$.

The extract caused continuous body weight gain in all the treatment groups and there were no significant differences between them with the control showing that the metabolic and physiological functioning was preserved. The organ weight index of the liver, kidney, heart, spleen and the lungs were normal and did not show any hypertrophy or atrophy based on the doses. The histopathological examination showed minimal hepatic steatosis in the control group, and moderate steatosis at 600 mg/kg, no necrosis or inflammation. There was normal tissue in the kidneys, heart and spleen. The lungs of the rats treated with 300 mg/kg and 600 mg/kg, however, had diffuse alveolar damage that was characterized by intra-alveolar edema, hyaline membrane deposition, and neutrophilic infiltration which is a phenomenon that indicates dose-related pulmonary sensitivity.

In general, the extract showed relative systemic safety at both low and moderate doses, although the development of diffuse alveolar damage at 300 mg/kg and 600 mg/kg means that the respiratory risk may occur at higher exposures. More experiments and mechanistic research are

also advised in order to completely prove the safety of *Moringa oleifera* root extract in the long term

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND

The relationship between toxicology and herbal medicine has existed in an almost inseparable historical paradigm. In essence, the two fields began as a succession of the earliest experimental studies on the nature of the plant, mineral, and animal substances, and toxicology is frequently referred to as one of the oldest branches of science. Modern commentaries suggest that early toxicology dates back to the ancient world, where the initial trials with botanicals were used to define the most suitable remedies versus poisons (Hendrickson, 2021).

The Ebers Papyrus (c. 1500-BCE) of Egypt provides some evidence of medicinal and hallucinogenic uses of opium poppy, mandrake, and castor oil, and hints that dosage is critical: a small dose can work wonders, but a large dose can be fatal (Ezrari, 2025). A medical practitioner named Hippocrates (460370 1st -century) of Greece promoted careful observation and dosage in medical practice; this later was extended by Dioscorides (1st -century) with his De Materia Medica listing a whole host of medicinal and toxic plants throughout the Roman Empire (notably in the modern context of herbal toxicity 2022).

Paracelsus (1493-1541), who is commonly regarded as the founder of modern toxicology, claimed that everything was a poison; only the quantity of a poison is a medicine and the reverse. This is the basis of dose-response relationship that is the focus of contemporary toxicological investigation (Hendrickson, 2021).

Elsewhere, with the world medical traditions also flourishing: Ayurvedic texts listed plants and their levels of toxicity, and Chinese texts like the Ayurvedic-based Chinese Classics of Medicine (Thousand and One Nights): used medicinal herbs classified into those with therapeutic and toxic properties. Also African folk medicine kept broad pharmacopeials and oral traditions of safe and unsafe botanicals (Ezrari, 2025).

In this larger context of history, one species of family Moringaceae, a member of the family, has acquired a considerable amount of attention, i.e. *Moringa oleifera*. It is native to the Indian subcontinent and is currently spread all over Africa, Asia, and South Americas both as food and medicine. Recent studies have also been carried out on the nutrition and medicinal qualities of its leaves, seeds and roots (Hossain, 2022).

However, the history of the plant of the *M. oleifera* is still rather unexplored, which is mainly explained by the safety issues. Samely, traditionally, as stimulants, abortifacients, or drugs to address rheumatism, modern research suggests that some ingredient, especially the root bark, can have some toxic elements (WebMD, 2023).

In the 19th and 20th century pharmacological boom, chemists strived to discover the active components of a variety of therapeutic plants such as *M. oleifera*. As there was increased interest in natural therapeutics, scientists studied antioxidant, antimicrobial and anti-inflammatory activity during which safety profiles were also evaluated. Even though several researchers can state the befits and the safety of leaves, seeds, and pods, roots are understudied due to the perceived toxicity (Mughal *et al*, 2024).

In the recent few decades, toxicology has established itself as an essential tool that is indispensable when it comes to establishing the safety of herbal medicines. The regulatory agencies accentuate pre-clinical and clinical examination of botanical preparations safety (Toxicological advances of traditional medicine 2022). Sub-acute and repeated-dose oral toxicity animal tests are especially important in finding out whether herbal extracts can cause them to accumulate organ damage or result in systemic toxicity with time (Hendrickson, 2021).

1.2 TOXICOLOGY

Toxicology is the science studying how chemicals, physical agents or biological substances affect living organisms and the environment; and how to prevent or treat the toxic effects (Hendrickson, 2021). It draws on pharmacology, physiology, biochemistry, molecular biology and pathology to understand how toxicants act and when they become harmful.

In toxicology the most important concept is the dose-response relationship which has long been known: “only the dose makes the poison” (Hendrickson, 2021). Toxicological research aims to determine safe exposure limits, identify target organs, assess human risk, and inform regulation of drugs, herbal, chemical products and pollutants (Toxicity and safety assessment of herbal medicines 2025). We typically categorize tests by exposure duration: acute, sub-acute, sub-chronic and chronic — each providing additional information on short-term and long-term safety.

1.2.1 ACUTE TOXICITY STUDIES

Acute toxicity examines adverse effects manifesting within 24hrs of one or more doses. It is frequently used when evaluating the toxic profile of a compound (Hendrickson, 2021). The objective is to determine the median lethal dose (LD_{50}) — the dose causing death in 50% of test animals — which helps compare substances and set dose ranges for further studies (Hendrickson,

2021). Acute tests also help identify target organs, observe clinical signs (e.g., sedation, tremor, diarrhea) and elucidate possible mechanisms of action. While animal models (mice, rats) have been standard, there is increasing movement toward cell culture and computational models to reduce animal use (Toxicological advances of traditional medicine 2022).

In the acute testing of herbal medicines such as *M. oleifera*, one must investigate whether phytochemicals like alkaloids, tannins and saponins may be harmful at high doses — enabling development of safer repeated-dose studies.

1.2.2 SUB-ACUTE TOXICITY STUDIES

Sub-acute investigations involve administration of a compound daily for 14-28 days and are designed to detect cumulative effects that may be missed in acute tests. A dose that appears harmless initially may cause organ damage or metabolic disturbances when taken daily (Gad 2014 → update needed: recent studies indicate importance of 28-day repeated dose studies in herbs 2024). Typical parameters include:

• Body weight — signaling systemic toxicity or metabolic imbalance.

• Relative organ weights — especially liver, kidneys, heart, spleen, lungs to identify organ-specific toxicity.

• Hematology — RBC/WBC counts, hemoglobin, platelets to monitor blood/immune system effects.

• Biochemistry — liver enzymes (ALT, AST, ALP, bilirubin) and kidney markers (urea, creatinine, electrolytes) to detect early organ dysfunction (Hendrickson 2021).

Modern guidelines (e.g., updated preclinical herbal toxicity frameworks) standardise 28-day repeated dose oral rodent studies in herbal safety assessment (Mugale, 2024). In the case of *M. oleifera*, a sub-acute study is particularly relevant because the herb is commonly used daily in

supplements or folk remedies. Prolonged exposure may lower the threshold at which phytochemicals such as spirochin become harmful (Hossain, 2022).

1.2.3 SUB-CHRONIC TOXICITY STUDIES

Sub-chronic studies are administered at a daily dose over a period of approximately 90 days (3 months) (Yu *et al.,* 2022). Their objectives are to locate No Observed Adverse Effect Level (NOAEL) and plot dose-response curves to safe human limits. These researches provide a closer examination of cumulative and even reversible impacts. They usually add:

• Histopathology – damage can be examined by microscopy of tissues.

• Enzyme examination – assess alterations in metabolism.

• Endocrine testing – prolonged exposure may disrupt hormone concentrations.

Sub-chronic information plays a crucial role in drug development since it provides information about whether a compound is safe to use on a daily basis, or whether the effects are dose-dependent, and whether toxicity vanishes with discontinuation.

Sub-chronic trials, as in the case of *Moringa oleifera* root extract, may reveal the risk of long-term usage on liver, kidneys, or reproductive health, all of which was implied in earlier reports (Adane *et al.,* 2023)

1.2.4 CHRONIC TOXICITY STUDIES

Chronic toxicity involves the examination of damage caused by constant or repeated exposure over a long period (typically 6 to 12 months) in animal models (Obaiah *et al.,* 2024). These are the most encompassing tests and are normally performed on long-term human products such as medicines, food additives and herbal supplements.

Chronic studies aim to:

• Determine cumulative toxicity and permanent organ injury.

• Evaluate carcinogenicity.

• Find reproductive or developmental toxicity.

• Set long-term NOAEL values.

They include large groups of animals, numerous dose levels, broad biochemical, hematological, histopathological and clinical observations. They are time- and cost-consuming and are usually done only when something appears promising or there is anticipated high human exposure (Yu *et al.,* 2022).

Chronic testing is reduced in herbal medicine; however, it is necessary for plants such as *Moringa oleifera* which are taken frequently. It informs us that the risk of organ damage, mutation, or cancer increases with continuous use.

1.3 PARAMETERS USED IN EVALUATION OF TOXICITY

When testing various substances in toxicology, several parameters are employed to determine the safety profile of the test substances. These parameters provide us with systemic and organ-specific effects of repeated occurrence of xenobiotics, herbal extracts, and pharmaceutical agents. We tend to examine physical appearances, biochemical tests, hematological tests, and histopathological tests. These combined provide a holistic view of the physiological effect of a substance and assist in determining the possible toxic effects of the substance (Li *et al.,* 2021).

1.3.1 BODY WEIGHT

One of the least complex, although very sensitive, signs of toxicity in an animal study is body weight change. A high reduction in body weight gain compared to control animals can be an

indication of low food and water consumption, poorer absorption of nutrients, or direct toxicity on metabolism (Adane *et al.,* 2023). On the contrary, abnormal weight gain may be an indication of fluid retention, or hypertrophy of the organs.

Body weight alterations come in particularly handy in sub-acute and sub-chronic research, where the cumulative effects of a compound might manifest without any observable clinical effects. Growth and metabolism are coupled with general well-being; thus alterations in body weight tend to be an early indicator of systemic toxicity (Yu *et al.,* 2022).

1.3.2 ORGAN WEIGHT INDEX

When an organ is expressed in relation to the body weight (organ-to-body weight ratio), the weight of the organ would assist in estimating possible target organ toxicity. Some toxicants target certain organs (e.g., hepatotoxins which target the liver, nephrotoxins which target the kidneys). Changes in relative organ weights may indicate hypertrophy, atrophy, congestion, or edema of tissues (Li *et al.,* 2021). For example:

• Hepatic weight gain could indicate enzyme induction, fatty alterations or inflammation.

• The weight of the spleen can be reduced and this represents immunosuppression.

• Alterations of kidney weight can be indicative of nephrotoxicity.

In this way, organ weight indices are supplementary to histopathology and provide quantitative data on possible organ-specific toxicity (Li *et al.,* 2021).

1.3.3 CLINICAL BIOCHEMISTRY

Functional evidence of toxicity in blood serum or plasma is provided by biochemical markers,

which may be detected before structural alteration is apparent. They are particularly useful in the evaluation of the liver, kidneys, and metabolism.

1.3.3.1 Liver Function Test

The liver is key in the process of metabolism and detoxification. Damage to hepatocytes frequently appears in the form of hepatotoxicity manifested by increased serum enzymes:

• Alanine aminotransferase (ALT) – liver-specific.

• Aspartate aminotransferase (AST) – also occurs in heart and muscle; increased as a result of liver damage.

• Alkaline phosphatase (ALP) – increased in the case of cholestasis or bile duct obstruction.

• Total bilirubin – elevated when bile pigments are not capable of being conjugated or secreted.

The malfunction of these parameters are crucial indicators of liver damage caused by drugs or plants (Adane *et al.,* 2023).

1.3.3.2 Renal Function Test

The kidney remove waste products of metabolism. Nephrotoxicity may result in the build-up of waste products in the blood:

• Serum creatinine – high levels indicate poor glomerular filtration.

• Blood urea nitrogen (BUN) – elevated in renal failure or elevated protein breakdown.

• Electrolytes (Na^+, K^+, Cl^-, HCO_3^-) – imbalances indicate tubular dysfunction.

Frequent observation of these renal indices offers a practical assessment of nephrotoxicity in toxicity research (Adane *et al.,* 2023).

1.3.3.3 Blood Glucose Test

Xenobiotics which interfere with insulin secretion, hepatic breakdown of glucose or the integrity of pancreatic β-cells make glucose homeostasis sensitive. Hyperglycaemia can either signify insulin resistance or hypoglycaemia can either be a sign of surplus insulin secretion or depleted hepatic glycogen. It is necessary to monitor glucose since many plant extracts have potential hypoglycaemic or hyperglycaemic effects (Li *et al.,* 2021).

1.3.4 HEMATOLOGICAL PARAMETER

Hematological determinations provide essential information regarding the impact of a test substance on blood-forming organs and immunity. The parameters that are usually measured include:

• Red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Hct) – a decreased amount might indicate anaemia or bone marrow suppression.

• White blood cell (WBC) count and differential – Increased counts can indicate inflammation or infection; decreased counts may indicate immunotoxicity.

• Platelet count – thrombocytopenia may lead to bleeding risk; thrombocytosis may indicate inflammatory processes.

Blood cells are not very long-lived; hence hematological parameters are highly sensitive to toxic insults and thus they are good indicators of systemic toxicity (Li *et al.,* 2021).

1.3.5 HISTOPATHOLOGY

Histopathological examination is a type of microscopic analysis of fixed and stained tissue of vital organs (liver, kidney, heart, spleen, lungs, testes / ovaries). It is regarded as the gold standard for confirming organ-toxicity.

This analysis gives firsthand evidence of structural changes such as:

• Cell degeneration (fatty changes, necrosis).

• Inflammatory reactions (immune cell infiltration).

• Scarring or fibrosis of tissues.

• Neoplastic alterations (pre-cancerous or cancerous lesions).

Biochemical findings are only validated by histopathology and assist in detecting the exact site and mechanism of toxicity (Yu *et al.,* 2022). In the case of herbal extracts such as *Moringa oleifera*, gross morphology (organ weights, serum biochemistry) may be insufficient because subtle tissue alterations that may be missed in these methods would be detected through histological analysis.

1.4 HERBAL MEDICINE

Herbal medicine simply refers to the use of plant components such as the roots, leaves, bark, seeds and flowers to prevent or treat illnesses (Krsnik *et al.,* 2024). It continues to be a global health practice, particularly in low- and middle-income nations (Salm *et al.,* 2023). However, when not taken with caution or used for prolonged periods, it may actually lead to toxicity (Balkrishna *et al.,* 2024).

1.5 TAXONOMY

Moringa oleifera Lam. is a drought-resistant perennial tree that is part of the family Moringaceae. That family has only one genus (Moringa) with around 13 to 14 species growing in tropical and subtropical areas of Africa and Asia (Malpotra *et al.,* 2025). The most widely used and researched species is *M. oleifera* because of its nutritional, medicinal and industrial applications (Zhang *et al.,* 2023). It is a multipurpose plant: food, medicine, water filter, and more.

On a taxonomic level it belongs to:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Brassicales

Family: Moringaceae

Genus: Moringa

Species: *Moringa oleifera* Lam. (Zhang *et al.,* 2023)

1.5.1 COMMON NAMES

The list of other names for this plant is extensive.

In English: drumstick tree, horseradish tree, ben oil tree.

In Nigeria: “Zogale” (Hausa), “Ewe igbale” (Yoruba), “Okwe oyibo” (Igbo).

In India: Sahijan or Munaga;

In Philippines: Malunggay (Zhang *et al.,* 2023).

1.5.2 SCIENTIFIC CLASSIFICATION

Domain: Eukaryota;

Kingdom: Plantae;

Phylum: Tracheophyta;

Class: Magnoliopsida;

Order: Brassicales;

Family: Moringaceae;

Genus: Moringa;

Species: *Moringa oleifera* Lam. (Zhang *et al.,* 2023).

1.5.3 DISTRIBUTION AND HABITAT

Moringa oleifera grows in the sub-Himalayan regions (north India, Pakistan, Bangladesh, Afghanistan) and has been dispersed across Africa, the Middle East and the Americas. It is now widely cultivated in tropical and subtropical regions (Zhang *et al.,* 2023). It thrives in semi-arid tropical/sub-tropical conditions, withstands drought, adapts to sandy or loamy soils, tolerates annual rainfall of 250-3000 mm and temperatures ~25-35 °C (Malpotra *et al.,* 2025).

1.5.4 DESCRIPTION

M. oleifera is a fast-growing deciduous tree, reaching 10-12 m or more in height. It has greyish-white bark with vertical crevices and a deep root system for drought resilience (Zhang *et al.,* 2023). It has tripinnate feathery foliage, sweet-scented white/cream flowers in loose bunches, and slender triangular pods (“drumsticks”) ~30-50 cm long with winged seeds inside. The seed oil (“ben oil”) is clear, odourless, and used in food, cosmetics, and industry (Malpotra *et al.,* 2025). The roots are thick, long and pungent (hence “horseradish tree”), but also require careful safety evaluation because of potential toxicity (Zhang *et al.,* 2023).

1.5.5 CHEMICAL CONSTITUENTS

M. oleifera contains numerous phytochemicals that underlie its nutritional and medicinal roles. For example:

- Leaves: rich in vitamins (A, C, E, K, B-complex), minerals (Ca, K, Fe, Mg), proteins, amino acids, flavonoids (quercetin, kaempferol), phenolic acids, tannins, saponins, alkaloids (Malpotra *et al.,* 2025).
- Seeds: oil (ben oil), proteins, antimicrobial peptides.
- Pods: good sources of protein, vitamin C, carbohydrates.
- Roots: alkaloids (e.g., sphingoalkaloids), glucosinolates, flavonoids, tannins, saponins — with safety concerns at high doses (Zhang *et al.,* 2023).
- Bark: tannins and sterols (Malpotra *et al.,* 2025).

  Overall, *M. oleifera* is highly nutritious but its phytochemicals may be toxic in high concentrations, thus requiring further toxicological research (Zhang *et al.,* 2023).

1.5.6 TRADITIONAL USES AND MEDICINAL USES

- Leaves: used for diabetes treatment, immunity boosting, malnutrition, hypertension. Leaf extracts have been confirmed for antioxidant, anti-inflammatory and antimicrobial activities (Zhang *et al.,* 2023).
- Seeds: used as natural water coagulants and in antimicrobial folk applications.
- Pods: eaten as vegetables; used for digestive and reproductive issues.
- Roots: traditionally used as stimulants, abortifacients and for rheumatism/rheumatic conditions, snakebites, circulation problems; but root use is controversial due to toxicity concerns (Zhang *et al.,* 2023).

- Bark/gum: used for diarrhoea, dysentery, infections.

  Some modern pharmacology supports leaves/seeds for anti-diabetic, hepatoprotective, cardioprotective or antimicrobial roles; but root extracts especially need careful safety verification (Zhang *et al.,* 2023).

1.6 STUDY PROBLEM

The use of herbal medicine is also a massive contribution to the health-care sector worldwide and particularly in developing nations, where up to 80 % of the population use traditional medicines to meet their daily health-care requirements (WHO Africa 2022).

Moringa oleifera is a medicinal plant that has attracted a lot of attention due to its nutritional and therapeutic value. Its leaves and seeds have been researched and consumed without major issues, whereas its roots remain disputable (RSC Advances 2023).

Moringa oleifera roots are also traditionally applied in various communities as stimulants, abortive agents, for snakebites, rheumatism, digestive issues and snakebites again (Popoola & Obembe 2013 → update: recent review emphasises the need for safety-assessment of roots (Comprehensive Review, 2024)).

Nonetheless, phytochemical research has discovered that the roots possess compounds (such as spirochin-type alkaloids) which may be neuro- and cardiotoxic (older studies) and modern reviews flag them as under-studied for safety (RSC Advances, 2023).

This leaves a huge gap in our knowledge: whereas the nutritional and medicinal merits of the leaves and seeds of *Moringa oleifera* are well determined, the safety profile of the root extract remains not well known especially when subject to repeated exposure. Now that most herbal

medicines are used in chronic doses, we require systematic sub-acute and repeated-dosing oral toxicity studies (Comprehensive Review, 2023).

With no substantive evidence, the consistent use of *Moringa oleifera* root preparations might pose latent health hazards, especially to liver, kidney and blood — which are frequent targets of plant alkaloids. Thus the research problem presented by this study is the absence of toxicological evidence of repeated oral intake of ethanol root extract of *Moringa oleifera*.

1.7 RELEVANCE OF STUDIES

This study is important due to several practical reasons:

- Traditional Medicine Scientifically Validated

  Although *Moringa oleifera* root is widely used in folk medicine, its safety issues remain a concern due to the phytochemical compositions. This study will provide experimental evidence that either supports or warns against its further application and provide the evidence-based authority to support the traditional practices (Balkrishna *et al.,* 2024).

- Public Health Implications

  Herbal medicines are often considered safe because they are ‘natural’. However, many plant-based products have been associated with liver, kidney, or blood toxicity (Li *et al.,* 2021). This study allows us to protect population health by determining the sub-acute and repeated-dose toxicity of the *Moringa oleifera* root extract.

- Pharmacological Research

  Identifying the most affected organ systems with long-term exposure to root extract will lead to further mechanism-of-action research. It also lays the groundwork for

standardizing, establishing safe doses, and future clinical trials (Comprehensive Review, 2023).

- Regulatory Importance

  The outcomes will provide guidance to health regulators (e.g., in Nigeria and global agencies) for herbal medicines control—focusing on safety, quality, and effectiveness in the herbal drug market (RSC Advances, 2023).

- Value Added to Toxicological Literature

  Currently, there is limited information about the sub-acute and 28-day repeated oral toxicity of *Moringa oleifera* root extract. This research contributes to the toxicological database and fills a significant gap (Comprehensive Review, 2023).

1.8 OBJECTIVES OF STUDY

1.8.1 GENERAL OBJECTIVE

To determine the repeated-dose toxicity of the oral ethanol root extract of *Moringa oleifera* in sub-acute and 28-day period in rodents.

1.8.2 SPECIFIC OBJECTIVES

1. To determine the impact of repeated administration of *Moringa oleifera* root extract on body weight in rodents.

2. To identify alterations in the relative organ weights (liver, kidney, spleen, heart, and lungs) after 28 days of oral dosing.

CHAPTER TWO

2.0 MATERIALS AND METHOD

2.1 MATERIALS AND REAGENTS

Measuring cylinder, 200ml Pyrex beaker, 1ml, 2ml, 5ml and 10ml syringes (Agary Pharmaceuticals Ltd, Nigeria), oral-gastric tube, standard animal feed and water, Chloroform, 10% formyl saline, 95%v/v ethanol, distilled, animal cages, mortar and pestle, spatula, acacia gum, cotton wool, methylated spirit, universal containers, dissecting set, feed troughs, weighing balance.

2.2 PLANT COLLECTION, AUTHENTICATION AND EXTRACTION

The plant was collected at Obe Quarters, Benin City, Edo State, Nigeria and identified by Prof. Akinnibosun Henry Adewale at the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin (UNIBEN), Benin City, Edo State, Nigeria, with the voucher number UBH-M340.

The roots were collected, washed, chopped into bits, shade-dried for 14 days and further oven-dried at 60°C for 45 minutes after which they were pulverized using British milling machine. A Soxhlet extraction procedure was used to extract the bioactive compounds from 1170g of the powdered *Moringa oleifera* root. The Soxhlet extraction of the roots was carried out as follows;

The powdered roots were placed in a thimble that is made from thick cellulose fabric, which was carefully loaded into the main chamber of the Soxhlet extractor. The extractor was placed onto a round-bottomed flask containing 95% ethanol as the extraction solvent and then equipped with a condenser. The solvent was heated for several minutes to about 90°C to reflux. As the solvent

vapor travelled up to a distillation arm, into the condenser, the condensed vapor dripped slowly back down into the chamber holding the solid material. The chamber containing the solid material slowly fill with the warm solvent and once nearly full, it was automatically emptied by a siphon side arm, with the solvent returning to the distillation flask.

After extraction, the ethanol extract was then gathered and concentrated using a rotary evaporator to eliminate excess solvent. The concentrate was then dried by placing it in an oven. The final dried extract weighed 30.4g, resulting in a 2.6% yield, which indicates effective extraction of constituent from the solved. The extracted compound was collected and the non-soluble portion of the extracted solid in the thimble was discarded. The extract was then weighed and store in an airtight container in a refrigerator**.**

2.3 EXPERIMENTAL ANIMALS

Twenty Adult male albino rats weighing between 130–180 g were used for the experiment. The animals were procured from the Animal House of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria. The rats were acclimatized to the laboratory conditions for a period of two weeks before the commencement of the study. During acclimatization, they were maintained under standard environmental conditions (12-hour light/dark cycle, temperature 22 ± 2 °C, and relative humidity 50–60%) and were provided with standard commercial feed and tap water ad libitum.

All experimental procedures were conducted in compliance with internationally accepted guidelines for the care and use of laboratory animals (OECD, 2008; National Research Council, 2011). Ethical approval for this study was obtained from the Faculty of Pharmacy Ethical Committee on Animal Use, University of Benin, Benin City, Edo State, Nigeria.

2.4 STUDY DESIGN

Following a two-week acclimatization period, the animals were randomly assigned into four groups, each consisting of 5 male albino rats.

- Group I (control) rats were administered 10 ml/kg of distilled water.
- Group II rats were administered 150 mg/kg of the *Moringa oleifera* extract.
- Group III rats were administered 300 mg/kg of the *Moringa oleifera* extract.
- Group IV rats were administered 600 mg/kg of the *Moringa oleifera* extract.

Extract stock solutions of concentrations; 50mg/ml (for Group II), 100mg/ml (for Group III) and 200mg/ml (for Group IV) were prepared using Acacia gum (5%) as solubilizing agent. Using the dose of the extract for each group and the weights of the rats, the volume of the extract stock solutions to be administered to each rat in the different groups were calculated.

A single dose of the extract (group II-IV) and distilled water (group I) were administered daily for 28 days with the aid of an orogastric tube.

The initial weight of the rats was recorded at the start of the experiment (Day 0), and then measured again on Days 7, 14, 21, and 28 after treatment began. All weights were taken with a weighing balance.

Throughout the 28-day period, the rats were meticulously observed for signs of toxicity, behavioral changes, and mortality. Every observation was carefully documented.

At the end of the 28 days treatment period, the rats were sacrificed under chloroform anesthesia and, the selected organs (the liver, heart, kidney, spleen and lungs) were meticulously excised, and extraneous connective tissues were carefully removed. The organs were then rinsed with

sterile 0.9% saline solution to remove residual debris and gently pat-dried with Whatman filter paper and precisely weighed using an electronic analytical balance. The organ weight index was then calculated using the formula given below;

Organ weight index= Organ weight/ Body weight.

These organs which include liver, spleen, kidney, lungs and heart were collected and assayed for histopathology assay.

Histopathology

Spleen, liver, lungs, heart and kidney were fixed immediately in 10% formalsaline for routine histopathological examination. The tissues were embedded in molten paraffin wax and then sectioned, stained with haematoxylin and eosin and were examined under light microscope. Photomicrographs of the microscopic sections were taken with the help of a photomicroscope (Motic, Canada) provided with motic images plus 2.0 software.

CHAPTER THREE

3.0 RESULT

3.1 Effect of sub-acute oral treatment with ethanol extract of *Moringa oleifera* root on body weight

Sub-acute treatment with *Moringa oleifera* root extract at all doses had no significant ($p > 0.05$) difference in the pattern of weight gain in the rats when compared to the control (Table 3.1).

3.2 Effect of sub-acute oral treatment with ethanol extract of *Moringa oleifera* root on organ weight index

Sub-acute administration of *Moringa oleifera* root extract at all doses did not significantly alter the organ weight index in rats compared to the control ($p > 0.05$) (Table 3.2).

Treatment groups		Day 0	Day 7	Day 14	Day 21	Day 28
Control		164.50±3.33	193.44±6.20	194.06±8.65	195.90±10.25	199.78±14.06
MO mg/kg	150	157.96±7.66	188.74±10.48	189.58±9.60	200.36±9.70	213.40±13.67
MO mg/kg	300	134.44±3.10	168.64±4.77	177.08±4.27	182.34±7.90	197.80±8.81
MO mg/kg	600	143.50±3.47	174.04±2.31	181.96±6.42	198.78±7.91	213.57±11.82

Table 3.1. Effect of sub-acute oral treatment with ethanol extract of *Moringa oleifera* root on the body weight of male albino rats.

Values are expressed as mean ± SEM (n=5); Control group received 10 ml/kg distilled water

MO = *Moringa oleifera*

Table 3.2. Effect of sub-acute oral treatment with ethanol extract of *Moringa oleifera* root on the organ weight index of male albino rats.

	Treatment *Moringa oleifera*			
	control	150 mg/kg	300 mg/kg	600 mg/kg
HEART	0.0036±0.0002	0.0032±0.0001	0.0036±0.0002	0.0036±0.0002
LUNGS	0.0074±0.0006	0.0069±0.0003	0.0078±0.0005	0.0064±0.0005
LIVER	0.0368±0.0018	0.0337±0.0020	0.0412±0.0008	0.0358±0.0006
KIDNEY	0.0032±0.0002	0.0031±0.0001	0.0034±0.0002	0.0034±0.0002
SPLEEN	0.0051±0.0004	0.0043±0.0007	0.0054±0.0005	0.0037±0.0002

Values are expressed as mean ± SEM (n=5), Control group received 10 ml/kg distilled water

3.3 Histopathological effect of administration of *Moringa oleifera* root extract on Wistar rats.

Sub-acute administration of *Moringa oleifera* root extract shows at 150 mg/kg, normal lung morphology was obtained; but at 300 mg/kg and 600 mg/kg, diffuse alveolar damage, which was characterized by edema and inflammatory process was obtained.

3.3.1 Histopathological effect of administration of *Moringa oleifera* root extract on Wistar rats in liver tissues.

Sub-acute administration of *Moringa oleifera* root extract in liver tissues shows at 150 mg/kg, 300mg/kg and 600mg/kg normal hepatocytes morphology was obtained.

3.3.2 Histopathological effect of administration of *Moringa oleifera* root extract on Wistar rats in kidney tissues.

Sub-acute administration of *Moringa oleifera* root extract in kidney tissues shows at 150 mg/kg, 300 mg/kg and 600 mg/kg normal glomeruli containing normal mesangium, blood vessels and epithelium. Normal kidney morphology was obtained.

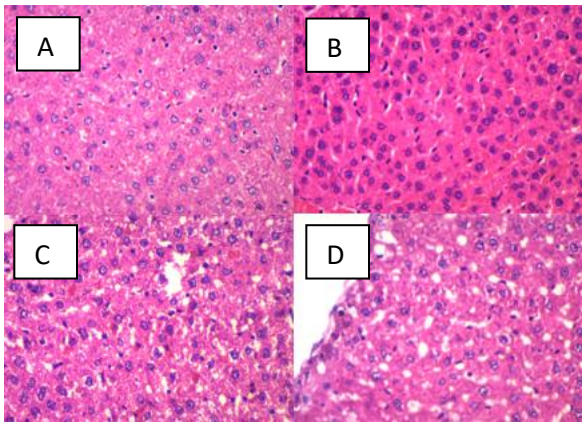

Fig 3.1. A (Control) shows hepatocytes with eosinophilic cytoplasm surrounding a centrally placed normochromic nuclei; B (150 mg/kg) shows hepatocytes with eosinophilic cytoplasm surrounding a centrally placed normochromic nuclei with indistinct nucleoli; C (300 mg/kg) shows hepatocytes with eosinophilic cytoplasm containing microvacuoles (ballooning degeneration), the cytoplasm surrounds a centrally placed nuclei; D (600 mg/kg) shows hepatocytes with eosinophilic cytoplasm containing microvacuoles (ballooning degeneration), the cytoplasm surrounds a centrally placed nuclei.

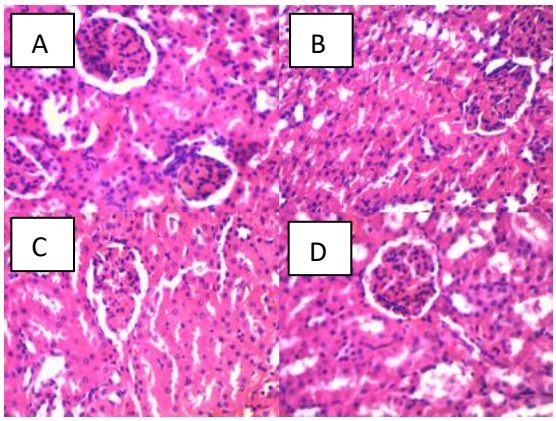

Fig 3.2. A (Control) shows normal glomeruli containing normal mesangium, blood vessels and epithelium; B (150 mg/kg) shows normal glomeruli containing normal mesangium, blood vessels and epithelium; C (300 mg/kg) shows normal glomeruli containing normal mesangium, blood vessels and epithelium; D (600 mg/kg) shows normal glomeruli containing normal mesangium, blood vessels and epithelium.

3.3.3 Histopathological effect of administration of *Moringa oleifera* root extract on Wistar rats in spleen tissues.

Sub-acute administration of *Moringa oleifera* root extract in spleen tissues shows at 150 mg/kg, 300 mg/kg and 600 mg/kg normal white pulp containing predominantly lymphocytes and normal red pulp containing predominantly red blood cells. Normal splenic tissue morphology was obtained.

3.3.4 Histopathological effect of administration of *Moringa oleifera* root extract on Wistar rats in heart tissues.

Sub-acute administration of *Moringa oleifera* root extract in heart tissues shows at 150 mg/kg, 300 mg/kg and 600 mg/kg myocytes with peripherally placed nuclei surrounded by eosinophilic cytoplasm showing normal myocytes morphology.

3.3.5 Histopathological effect of administration of *Moringa oleifera* root extract on Wistar rats in lung tissues.

Sub-acute administration of *Moringa oleifera* root extract shows at 150 mg/kg, normal lung morphology was obtained; but at 300 mg/kg and 600 mg/kg, diffuse alveolar damage, which was characterized by edema and inflammatory process was obtained.

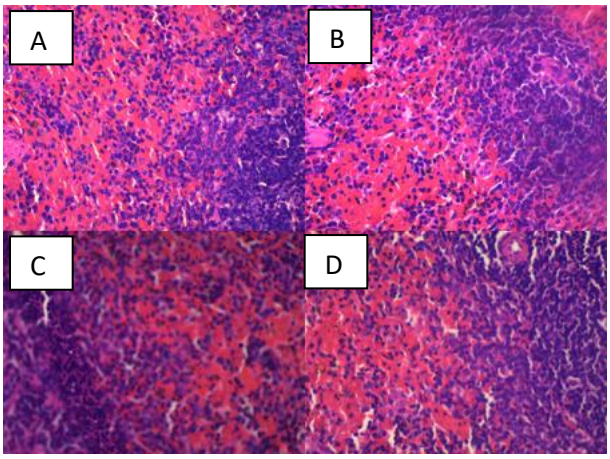

Fig 3.3. A (Control) shows a normal white pulp containing predominantly lymphocytes and normal red pulp containing predominantly red blood cells; B (150 mg/kg) shows a normal white pulp containing predominantly lymphocytes and normal red pulp containing predominantly red blood cells; C (300 mg/kg) shows a normal white pulp containing predominantly lymphocytes and normal red pulp containing predominantly red blood cells; D (600 mg/kg) shows a normal white pulp containing predominantly lymphocytes and normal red pulp containing predominantly red blood cells.

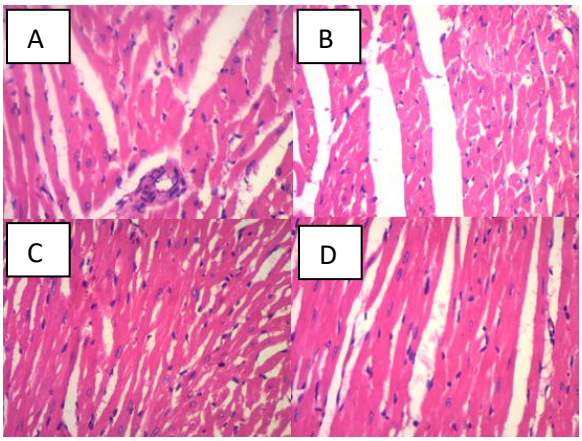

Fig 3.4. A (Control) shows myocytes with peripherally placed nuclei surrounded by eosinophilic cytoplasm; B (150 mg/kg) shows myocytes with peripherally placed nuclei surrounded by eosinophilic cytoplasm; C (300 mg/kg) shows myocytes with peripherally placed nuclei surrounded by eosinophilic cytoplasm; D (600 mg/kg) shows myocytes with peripherally placed nuclei surrounded by eosinophilic cytoplasm.

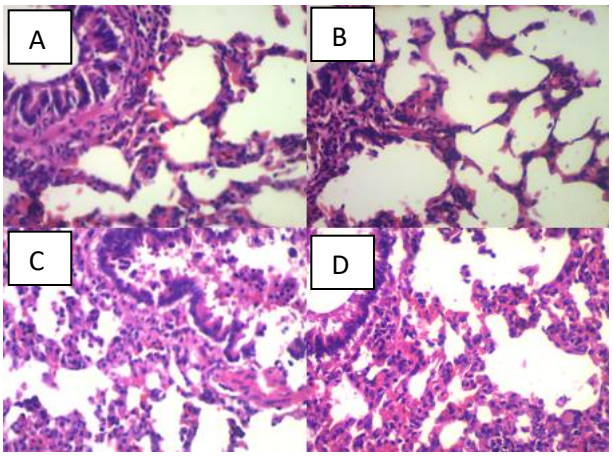

Fig 3.5. A (Control) shows a normal airway and alveoli sacs that are separated by the interstitium; B (150 mg/kg) shows a normal airway and alveoli sacs that are separated by the interstitium; C (300 mg/kg) shows the alveoli with intra-alveolar edema, hyaline membrane deposits and some collapsed alveoli. There are neutrophilic aggregates within the airway; D (600 mg/kg) shows the alveoli with intra-alveolar edema, hyaline membrane deposits and some collapsed alveoli. There are neutrophilic aggregates within the airway.

CHAPTER FOUR

4.1 DISCUSSION

This study evaluated sub-acute (28 days) oral administration of ethanol extract of roots of the plant, *Moringa oleifera*, on body weight, organ weight index, and the histopathological assessment of wistar male rats. All these parameters are very important determinants of the systemic toxicity, physiologic tolerance and particular organ-specific responses under long exposure. This findings complement the growing body of toxicological findings concerning the medicinal use of the *Moringa oleifera* species, namely roots, which are traditionally eaten but that have not been studied adequately in comparison with the leaves.

During the 28 days of treatment, the rats treated with 150 mg/kg, 300 mg/kg, and 600 mg/kg of extract showed gradual and consistent growth in body weight comparable to the control group.

Current metabolic functioning, good appetite, and proper physiological adjustment of drugs the administered substance are credible indicators of weight gain in toxicological studies. Lack of weight loss indicates that the extract was not severe enough to cause systemic toxicity resulting in the inability to feed, absorb nutrients, or metabolize energy, which again is consistent with previous studies that found that the preparations of *Moringa oleifera* root extract did not induce physiological distress or impair nutrient assimilation in the male rats during the various dosing regimens (Pareek *et al.,* 2023; Pop *et al.,* 2022).

Organ-weight measurements were also in physiological reference ranges of liver, kidneys, lungs, heart, and spleen among all dosage groups (Michael *et al.,* 2007; Alimba *et al.,* 2024). The evaluation of the relative organ weight index is necessary in the detection of the organ-specific toxicity, as considerable variations may tell about the inflammation, hypertrophy, congestion, or atrophy. The weight of liver, kidneys, spleen, lungs and heart in this study did not show any abnormal physiological intervention and did not depend on the dose. None of the organs exhibited dose-dependent hypertrophy or atrophy, which are normally used to measure injury, inflammation, or degenerative pathology of the cells (Mezencev, 2024). Therefore, the constant organ-weight indices indicate that the extract did not impose any severe structural load and did not cause the compensatory increase of the size of the organs (Palomino-Pacheco *et al.,* 2024).

A greater level of detail on tissue-level reactions was obtained with more thorough histopathological examination. In the control group, liver sections had very mild steatosis, and in the rats receiving the drug 600 mg/kg obtained mild steatosis. The appearance of microvesicular fatty changes that are without necrosis, inflammation and fibrosis indicates low grade metabolic response, as opposed to severe hepatocellular injury. The process would be mild steatosis which

tends to be reversible, possibly representing an adaptation to physiological conditions and temporary changes in lipid metabolism, but not toxin. Kidneys of cured rats endowed well-preserved glomeruli, normal mesangial morphology and well-organized tubular epithelium, no signs of tubular necrosis, distortion of glomeruli or interstitial inflammation. These Resolutions can be viewed as evidence of the maintained integrity of the renal organ and the fact that the extract did not affect the renal architecture of the organ in the sub-acute exposure.

Similarly, the heart and the spleen had normal microscopic structures, with no degeneration of the myocytes, depletion of lymphoid cells, congestion, and inflammatory infiltration. This is a demonstration that the extract did not trigger any cardiotoxic and immunotoxic effects, at the doses administered. Nevertheless, a significant variation in the lungs of rats, which received 300 mg/kg and 600 mg/kg, was detected. The lung tissue showed a diffuse alveolar destruction of characteristics of intra-alveolar edema, formation of hyaline membranes, neutrophilic inflammation, and partial alveolar destruction (Erjefalt *et al.,* 2022). Acute lung injury has a characteristic of diffuse alveolar damage which is an indication of pulmonary sensitivity at increased doses. This tendency is also very suggestive because lungs are especially susceptible to oxidative or inflammatory stress due to xenobiotics. Although it could not be established working with histology what exactly caused the injury directly, it could be due to a direct toxic effect on the lungs, aspiration during gavage, systemic inflammatory response, or unidentified infection. However, the fact that such lesion is found at 300 mg/kg and 600 mg/kg is evidence that there is a possible dose-related risk to the lungs, which should be investigated further.

In general, the body weight, organ weight index, and histopathological results demonstrates that the tolerability of the whole extract derived of the ethanol root of the plant is relatively high at

low and moderate concentrations, and the systemic and organ-specific integrity is not lost. The main issue is found at the doses (300 mg/kg and 600 mg/kg), which showed occurring diffuse alveolar damage, which references potential respiratory damage. In these findings, the potential of the root extract is sensitive with the root extract at high dosage and there is the need to conduct further research such as chronic dose exposure, oxidative stress biomarkers, microbiological screening, and mechanistic studies to clearly understand the safety profile of the root extract of the *Moringa oleifera*

CHAPTER FIVE

5.1 CONCLUSION

The sub-acute (28 days) toxicity study indicates that oral administration of 150 mg/kg, 300 mg/kg, and 600 mg/kg of *Moringa oleifera* ethanol root extract is not unpleasant in male wistar rats. There was no negative impact on body weight or body organ weight index, which suggests that systemic health and organ stability remained unchanged in all the treatment groups. The study also revealed that the histopathological examination indeed proved the normal structure and integrity of the liver (except slight steatosis at 600 mg/kg) kidneys, spleen, and heart, indicating that no serious toxicity was seen in these organs during the study.

There was however the manifestation of diffuse alveolar damage in the lungs at 300 mg/kg and 600 mg/kg and this points to the possibility of lung depreciation in a higher dosage. Though the cause of the situation needs additional investigation, this observation implies that the respiratory system would be especially susceptible to the concentrated or prolonged usage of the root extract.

Overall, *Moringa oleifera* root extract seems to be safe at low and moderate doses but creates the possibility of lung-related toxicity at very high doses. More research works can be added in the future such as the use of biochemical assays, oxidative stress markers, microbiological tests, and chronic toxicity to fully determine the safety margin of the extract and to better understand the mechanisms that explain the witnessed effects on the lungs.

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APPENDIX I

CALCULATION OF DOSES

ETHANOL ROOT EXTRACT

Prepared dose => 150 mg/kg

Stock solution => 50 mg/ml

Weight of rat =>148 g

If 1000 g will require 150mg of the extract,

The dose of the extract the rat will receive would be:

$\frac{148 \times 150}{1000} = 22.2$ mg

Amount of stock required will be;

If 50 mg is in 1 ml,

22.2 mg will be in $\frac{22.2 \times 1}{50}$ = 0.44 ml

Similar calculations were carried out for all the rats used in the experiment and at all the different doses.

APPENDIX II

The measures of association of the raw data were calculated using the following formulas;

Mean = $\sum x/\underline{n}$

Standard error of mean (S.E.M) = $S.D/\sqrt{n}$

Where n = number of observations

X= response

S.D = standard deviation = $\sqrt{\sum(x-x')^2/n-1}$

Where x'= mean