# PHYTOCHEMICAL SCREENING AND SAFETY EVALUATION OF ETHANOL LEAF EXTRACT OF *ANNONA MURICATA* (*ANNONACEAE*) IN FEMALE WISTAR RATS

**BY**

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## CERTIFICATION

This is to certify that this project work titled “Phytochemical screening and safety evaluation of ethanol leaf extract of *Annona muricata* (soursop) in female wistar rats” was carried out by OGBEGIE FRANCES GODSTIME from the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Benin City, done in partial fulfillment of the requirement for the award of Bachelor of Pharmacy and Doctor of Pharmacy degree of the University of Benin, Benin City.

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## DEDICATION

This project work is dedicated to the Almighty God for his love towards me and seeing me through my journey in Pharmacy school. I also dedicate this work to everyone interested in Complementary and Alternative Medicine, who seek to improve health through Herbal medicine.

## ACKNOWLEDGEMENTS

I want to thank God Almighty for preserving me and for giving me the strength to endure this long academic journey.

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## ABSTRACT

*Annona muricata* (soursop) is commonly used in traditional medicine, necessitating toxicological evaluation. This study evaluated the safety profile, phytochemical constituents, and physiological effects of the ethanol leaf extract of *A. muricata* in adult female Wistar rats after administering doses of 100mg/kg and 200mg/kg for 28 days.

Phytochemical screening confirmed a rich composition of carbohydrates, alkaloids, cardiac glycosides, phenolics, and steroids/triterpenes, with a notable absence of saponins and cyanogenic glycosides. Hematological analysis demonstrated overall safety of red cell indices, but revealed a reduction in WBC count at 100mg/kg ($p<0.05$), suggesting immune modulation that was histologically corroborated by lymphoid activation.

Biochemically, the extract conferred significant hepatoprotection, evidenced by a marked reduction in AST and ALT at 200mg/kg. In addition, the extract exhibited hypolipidemic effect, with optimal reductions in Total Cholesterol and LDL observed exclusively at 100mg/kg. The primary cautionary finding was a functional elevation of Creatinine at 200mg/kg ($p<0.05$), though this occurred without structural renal damage.

Histopathological assessment confirmed structural integrity across most organs, but identified mild bronchiolar ulceration at 200mg/kg. Overall, the extract demonstrated a favorable safety margin balancing maximal metabolic benefit with minimal functional risk. These findings emphasize the necessity of dose optimization to safely integrate *A. muricata* into complementary medicine.

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# CHAPTER ONE

# INTRODUCTION

## 1.0 Background of the study

Medicinal plants have remained a vital component of human healthcare for centuries and continue to play an important part in disease prevention and treatment (Ekor, 2021; WHO, 2023). They serve as indispensable sources of therapeutic agents and templates for modern drug discovery and development (Ajibade *et al*., 2022; Ibrahim *et al*., 2023). The World Health Organization (WHO) estimates that nearly 80% of the global population depends on herbal medicines for primary healthcare needs, particularly in rural and developing regions (WHO, 2023; Okoro *et al*., 2022). This reliance is largely due to accessibility, affordability, cultural acceptance, and the perceived safety of plant-based remedies compared to synthetic drugs (Adebayo *et al*., 2021; Eze *et al*., 2023). Medicinal plants owe their wide-ranging therapeutic benefits to an abundance of biologically active secondary metabolites, including alkaloids, flavonoids, terpenoids, tannins, saponins, and phenolic compounds (Fasogbon *et al*., 2023; Ogundele *et al*., 2023).

These compounds have been shown to exert antimicrobial, antioxidant, anti-inflammatory, antidiabetic, and anticancer effects in various experimental models (Agbaje *et al*., 2023; Nwokocha *et al*., 2023). Also, approximately 25–30% of currently prescribed drugs are derived directly or indirectly from phytochemicals, underscoring the critical contribution of ethnopharmacological research to modern pharmaceutical science (Ademola *et al*., 2021; Rodriguez *et al*., 2022).

Among such plants of therapeutic interest, *Annona muricata* (commonly known as soursop or graviola) has attracted significant scientific attention. Traditionally, *A. muricata* is used to treat

malaria, fever, hypertension, inflammation, diabetes, and parasitic infections, and as a general restorative tonic (Ibrahim *et al*., 2022; Suleiman *et al*., 2023). Recent pharmacological studies have validated many of these uses, revealing that it possesses antitumor, antihypertensive, antidiabetic, anti-inflammatory, antimicrobial, and antioxidant properties (Omole *et al*., 2024; Nunez *et al*., 2023).

These effects are largely attributed to the presence of annonaceous acetogenins, alkaloids, flavonoids, and phenolic compounds present in various parts of the plant (Eze *et al*., 2023; Tanimola *et al*., 2022). However, while these chemicals have medicinal benefits, they can also cause harmful effects when taken in excess. Specifically, annonacin, one of the major acetogenins, has been implicated in neurotoxicity and mitochondrial dysfunction in both in vitro and animal studies (Onyeka *et al*., 2021; Nunez *et al*., 2023; Okafor *et al*., 2022).

Consequently, toxicological evaluations are essential in assessing the safety of herbal remedies. The assumption that "natural" means "safe" has been repeatedly challenged by evidence showing that many plant extracts can produce toxic or mutagenic effects at high concentrations or after prolonged exposure (Agbaje *et al*., 2023; Nwokocha *et al*., 2023). Toxicity studies provide insight into dose–response relationships, organ-specific toxicity, and possible mechanisms of action, which are critical for ensuring both efficacy and safety (Okoro *et al*., 2022; Fasogbon *et al*., 2023). In addition, growing global interest in herbal medicine has prompted regulatory agencies, including the WHO, to advocate for standardized testing and scientific validation of herbal preparations to support their safe inclusion in evidence-based healthcare (WHO, 2023; Ademola *et al*., 2021). Therefore, assessing the safety and toxicity profile of *A. muricata* using experimental models is essential to establish its therapeutic margin, minimize adverse effects, and ensure its safe and rational use in clinical and traditional contexts.

Figure 1: Picture of *Annona muricata* (Annonaceae) plant taken in UNIBEN, Benin City, Edo State.

### 1.1 Taxonomy and botanical description

*Annona muricata* belongs to the Annonaceae family, which comprises over 120 genera and approximately 2,000 species widely distributed across tropical and subtropical regions (Fasogbon *et al*., 2023; Ibrahim *et al*., 2023). The genus Annona includes several economically and pharmacologically important species such as Annona squamosa (sugar apple), Annona reticulata (custard apple), and Annona cherimola (cherimoya), all recognized for their edible fruits and medicinal value (Adebayo & Lawal, 2021; Agbaje *et al*., 2023).

**Table 1.0**: Taxonomical classification of *A. muricata*

| Classification | Taxonomy |
|---|---|
| Kingdom | Plantae |
| Division | Magnoliophyta |
| Class | Magnoliopsida |
| Order | Magnoliales |
| Family | Annonaceae |
| Genus | Annona |
| Species | *A. muricata* L. |

Botanically, it is a small, evergreen, low-branching tree growing 5–8 meters high. It has dark green, glossy, oblong leaves measuring 6–12 cm long, which emit a distinct aroma when crushed. The flowers are yellowish-green and solitary, while the fruit is large, heart-shaped, green, and covered with soft, curved spines (Ademola *et al*., 2021; Okoro *et al*., 2022). The fruit pulp is white, fibrous, and juicy, enclosing numerous black seeds, and is known for its sweet-sour flavour.

### 1.2 Geographical Distribution

The plant thrives in humid tropical environments with well-drained soils and abundant sunlight, which influence its growth and phytochemical yield (Ogundele *et al*., 2023; Tanimola *et al*., 2022). Studies have shown that environmental factors such as altitude, soil type, and rainfall can alter the concentrations of key secondary metabolites in *A. muricata* leaves and fruits (Eze *et al*., 2023; Omole *et al*., 2024). For example, leaves grown in shaded areas may contain lower levels of acetogenins than those cultivated under direct sunlight, due to variations in photosynthetic intensity and metabolic stress responses (Ibrahim *et al*., 2023).

These variations underscore the need for standardization in the cultivation and collection of this plant for research and pharmaceutical purposes. Botanical identification and quality control of plant material are critical for ensuring consistency in experimental outcomes and therapeutic efficacy (Nwokocha *et al*., 2023; Adebayo & Lawal, 2021).

### 1.3 Ethnomedicinal uses

Across Africa, South America, and the Caribbean, *A. muricata* has been traditionally used for a broad range of ailments. In Nigeria and Ghana, decoctions made from the leaves are used to treat malaria, cough, fever, and hypertension, while the fruit juice is taken to aid digestion and enhance energy (Ifeanyi *et al*., 2022; Agbaje *et al*., 2023). In Latin American folk medicine, the leaves and bark are used as sedatives, anti-inflammatory agents, and natural remedies for infections and parasitic diseases (Rodriguez *et al*., 2022; Nunez *et al*., 2023).

In the Caribbean and parts of Central America, this plant is used as a general tonic to promote overall wellness, while its root and bark extracts are used for pain relief and fever reduction (Ademola *et al*., 2021; Omole *et al*., 2024). The seeds, though toxic in high doses, are sometimes

ground and used in traditional medicine as anti-parasitic agents for treating intestinal worms (Adebayo & Lawal, 2021).

In Nigeria, *A. muricata* is often included in herbal mixtures for managing diabetes, enhancing immunity, and maintaining general vitality (Ibrahim *et al*., 2022; Okoro *et al*., 2022).

However, it is important to note that while ethnomedicinal uses provide valuable insight, they must be supported by pharmacological and toxicological data to ensure safety, particularly because natural plant compounds can produce both therapeutic and adverse effects depending on dosage and preparation method (Omole *et al*., 2024; Nwokocha *et al*., 2023).

Thus, documenting and validating traditional knowledge through scientific evaluation is vital in bridging the gap between folklore and evidence-based medicine. This approach enhances the safe integration of traditional herbal therapies into modern healthcare systems (Rodriguez *et al*., 2022; WHO, 2023).

**1.4 Phytochemical composition and biological studies**

The pharmacological potential of *Annona muricata* is largely attributed to its rich phytochemical profile. The major constituents include annonaceous acetogenins, alkaloids, flavonoids, phenolics, tannins, saponins, and terpenoids (Eze *et al*., 2023; Ogundele *et al*., 2023). Among these, the annonaceous acetogenins are the most distinctive, being unique to the Annonaceae family and known for their potent antitumor and pesticidal activities (Tanimola *et al*., 2022; Nunez *et al*., 2023). These compounds act by inhibiting mitochondrial complex I, leading to ATP depletion and suppression of cancer cell proliferation (Omole *et al*., 2024; Okafor *et al*., 2022).

Flavonoids and phenolic compounds contribute significantly to the antioxidant and anti-inflammatory properties of *A. muricata*, protecting cells from oxidative damage caused by free

radicals (Ademola *et al*., 2021; Fasogbon *et al*., 2023). Alkaloids such as muricatine and coreximine have been associated with hypotensive and antimicrobial effects, while saponins and tannins contribute to their astringent and immune-modulatory actions (Agbaje *et al*., 2023; Ibrahim *et al*., 2023).

Phytochemical composition varies depending on plant part, extraction solvent, and environmental conditions. Methanolic extracts of the leaves are generally rich in flavonoids and phenols, whereas the seeds contain higher levels of acetogenins and alkaloids (Adebayo & Lawal, 2021; Ogundele *et al*., 2023). Recent chromatographic and spectroscopic analyses have revealed the presence of novel acetogenins and alkaloid derivatives, expanding the plant's known chemical profile (Rodriguez *et al*., 2022; Omole *et al*., 2024).

While these compounds are responsible for the plant’s therapeutic benefits, they also highlight the potential for toxicity when consumed in excessive quantities. For instance, some acetogenins exhibit strong cytotoxicity that, while beneficial for anticancer activity, may pose neurotoxic risks (Nunez *et al*., 2023; Onyeka *et al*., 2021). Therefore, understanding the balance between phytochemical potency and safety is critical for developing standardized, safe herbal formulations (Eze *et al*., 2023; Nwokocha *et al*., 2023).

Numerous traditional uses of *Annona muricata* have been validated by extensive studies that have revealed the plant's diverse pharmacological properties. Numerous biological activities, including antioxidant, antimicrobial, antidiabetic, anti-inflammatory, antihypertensive, and anticancer effects, have been demonstrated by various plant parts, especially the leaves, fruits, seeds, and bark (Eze *et al*., 2023; Ogundele *et al*., 2023).

The antioxidant activity of the plant has been attributed to its high amounts of flavonoids and phenolic chemicals, which scavenge free radicals and protect cells from oxidative stress (Ademola *et al*.,2021; Ibrahim *et al*.., 2023).

This property contributes significantly to its anti-inflammatory and anti-aging effects, as oxidative stress is a known trigger of tissue inflammation and degenerative diseases (Fasogbon *et al*., 2023; Omole *et al*., 2024).

The antimicrobial properties of this plant extract have been demonstrated against various bacterial and fungal strains, including Staphylococcus aureus, Escherichia coli, Candida albicans, and Pseudomonas aeruginosa (Nunez *et al*., 2023; Okafor *et al*., 2022). These effects are attributed to bioactive compounds, such as acetogenins and alkaloids, which interfere with microbial cell wall synthesis and protein metabolism (Agbaje *et al*., 2023; Ogundele *et al*., 2023).

Regarding antidiabetic activity, animal studies have shown that *A. muricata* leaf extracts can lower blood glucose levels by enhancing insulin sensitivity and modulating carbohydrate-metabolizing enzymes (Rodriguez *et al*., 2022; Ibrahim *et al*., 2023). Furthermore, its antihypertensive properties have been attributed to vasodilatory and diuretic effects, attributed to the presence of alkaloids and phenolic compounds (Adebayo & Lawal, 2021; Suleiman *et al*., 2023).

Perhaps the most widely studied activity of this plant is its anticancer potential. Several in vitro and in vivo investigations have revealed that annonaceous acetogenins limit tumor growth by decreasing ATP synthesis in cancer cells and triggering apoptosis (Onyeka *et al*., 2021; Tanimola *et al*., 2022). These findings have positioned the plant as a promising candidate for natural product-based anticancer therapy, although further toxicological and pharmacokinetic evaluations are necessary before clinical use (Nwokocha *et al*., 2023; Omole *et al*., 2024).

### 1.5 Toxicological concerns and need for safety evaluation

Despite its wide therapeutic use, *A. muricata* has raised safety concerns, particularly with respect to its neurotoxicity and potential mitochondrial toxicity. Several studies have reported that chronic or high-dose exposure to annonacin, a major acetogenin, may cause neuronal degeneration similar to that observed in atypical Parkinson's disease (Onyeka *et al*., 2021; Nunez *et al*., 2023).

Acetogenins act by inhibiting mitochondrial complex I, disrupting ATP synthesis, and inducing oxidative stress, which may explain their neurotoxic effects in sensitive tissues such as the brain and liver (Okafor *et al*., 2022; Omole *et al*., 2024). Animal studies have also shown that prolonged consumption of the extracts can lead to hepatic and renal abnormalities, highlighting the importance of dose optimization and toxicity profiling (Fasogbon *et al*., 2023; Agbaje *et al*., 2023). While these findings warrant caution, it is equally important to note that toxicity is often dose-dependent and influenced by extraction method, duration of administration, and plant part used (Ibrahim *et al*., 2023; Ogundele *et al*., 2023). Therefore, establishing a comprehensive safety profile through acute and sub-acute toxicity testing is vital for guiding safe therapeutic use.

In addition, the identification of potentially toxic phytoconstituents enables researchers to isolate beneficial compounds while minimizing adverse effects, paving the way for safe pharmaceutical formulations (Eze *et al*., 2023; Nwokocha *et al*., 2023).

### 1.6 Histopathological assessment

Histopathological evaluation plays an important role in toxicological studies by revealing tissue-level changes induced by test substances. It provides direct visual evidence of organ damage or protection, enabling a clearer understanding of the physiological impact of herbal preparations (Rodriguez *et al*., 2022; Omole *et al*., 2024).

In studies involving *Annona muricata*, histopathological assessments have been employed to examine the effects of leaf and fruit extracts on the liver, kidney, and brain tissues of animal models. Findings indicate that low to moderate doses generally maintain normal cellular architecture, whereas higher doses may induce mild to moderate necrosis, inflammatory infiltration, or vacuolar degeneration, especially in hepatic and neuronal tissues (Onyeka *et al*., 2021; Nunez *et al*., 2023). Microscopic evaluation, when combined with biochemical and hematological analyses, provides a holistic picture of the plant's safety profile (Ademola *et al*., 2021; Okoro *et al*., 2022). These assessments are indispensable for establishing a safe therapeutic range and ensuring the reproducibility of results in future pharmacological applications (Ibrahim *et al*., 2023; Ogundele *et al*., 2023).

**1.7 Phytochemical screening**

Phytochemical screening helps identify the classes of compounds responsible for pharmacological activity and toxicity (Eze *et al*., 2023; Nwokocha *et al*., 2023). In the case of *Annona muricata*, phytochemical screening has consistently revealed the presence of flavonoids, tannins, alkaloids, phenolics, saponins, glycosides, and acetogenins (Ademola *et al*., 2021; Tanimola *et al*., 2022). These findings not only justify its traditional use but also highlight the need for detailed toxicological studies to differentiate between therapeutic and harmful doses (Omole *et al*., 2024; Ibrahim *et al*., 2023).

Phytochemical screening also helps identify synergistic interactions among compounds, an important consideration, as the combined effects of multiple constituents may enhance therapeutic outcomes or alter toxicity profiles (Agbaje *et al*., 2023; Okoro *et al*., 2022). This scientific understanding forms the foundation for developing standardized herbal formulations with predictable pharmacological responses.

### 1.8 Aim

The aim of the study is to conduct a detailed phytochemical screening and evaluate the safety profile of *Annona muricata* using female Wistar rats as an experimental model.

### 1.9 Objectives

1. To determine the phytochemical composition of *Annona muricata* extract.
2. To evaluate the sub-acute toxicity via hematology and biochemical analysis (Kidney, Liver and Lipid profile).
3. To assess the structural safety of the plant extract via histopathology of vital organs (Uterus, Lungs, Liver, Heart, Spleen, Kidney).

### 1.9 Justification of the study

Evaluating the safety and toxicity profile of *A. muricata* is relevant for various reasons. First, it ensures the safe use of the plant in traditional medicine, particularly in rural communities that depend heavily on herbal remedies. Second, it provides scientific evidence to guide dosage recommendations and minimize adverse effects (Okafor *et al*., 2022; Omole *et al*., 2024). Finally, it contributes to the development of standardized herbal formulations that can be integrated into modern pharmaceutical systems under regulatory supervision (WHO, 2023; Ogundele *et al*., 2023). This study, therefore, aims to bridge the gap between traditional knowledge and scientific validation by providing reliable toxicological data on *Annona muricata*, thereby supporting its safe and effective therapeutic use.

# CHAPTER TWO

# MATERIALS AND METHODOLOGY

## 2.1 Equipment and apparatus

Milling machine, Soxhlet apparatus, heating mantle, water-bath, microcentrifuge, semi-auto analyser (Mindray BA-88A Reagent system), micropipettes (50µL, 100µL, 1000µL), test tubes and test tube racks, refrigerator, freezer, microplate reader (Mindray MR-96A), microplate washer (Mindray MW-12A), porcelain dishes, stirrer, thimble, glass jars, spatula, cages, digital weighing balance, universal bottles, plain bottles, EDTA bottles, condenser, automated hematology analyzer, Round bottom flask.

### 2.1.1 Reagents and chemicals

Absolute ethanol (99.5%), distilled water, chloroform, total cholesterol kit, total protein kit, Dragendorff's reagent, Mayer's reagent, Hager's reagent, Picric acid solution, diethyl ether, Ferric chloride solution, 10% neutral buffered formalin. All the reagents used in this study are of proven analytical quality and were sourced from reputable vendors.

### 2.1.2 Consumables

Latex hand gloves, hand sanitizer, detergent, facemask, cage beddings, and commercial pelleted feeds (Chikun feed), a pair of surgical scissors, cotton wool, cage scrapers, and syringes.

## 2.2 Methodology

### 2.2.1 Plant material collection and authentication

The plant leaves were harvested on the University of Benin campus in Benin City, Nigeria. It was identified by a plant taxonomist, Professor Akinnibosun, as *Annona muricata* Linn. The voucher specimen was deposited at the Herbarium, Department of Plant Biology and Biotechnology,

Faculty of Life Sciences, University of Benin, and was verified by Prof. Akinnibosun and assigned voucher number UBH-A356.

### 2.2.2 Preparation and extraction of plant material

The leaves of the *Annona muricata* plant were collected and dried at room temperature. The plant was pulverized using a milling machine. 1000g of the pulverized plant was exhaustively extracted with 99.5% absolute ethanol using the Soxhlet extraction method at 80°C. The extract was reduced to dryness using a water bath at 60°C. The obtained plant extract was weighed, and its final weight was recorded.

## 2.3 Qualitative phytochemical analysis

The ethanol extract of the plant leaves underwent qualitative phytochemical screening to identify its constituent phytochemicals using the following methods, and were referenced from Evans 2009;

**Carbohydrates**

- **Molisch's Test:** 1mL of Molisch's reagent (10% Alcoholic solution of α-naphthol) was added to 1mL of extract. 1mL of conc. Sulphuric acid was then added at an angle of $45^0$. A Purple/Reddish-violet ring at the interface of the two layers indicates the presence of Carbohydrates.
- **Benedict's Test:** 1mL of Benedict's reagent was added to 1mL of the plant extract filtrate in a test tube. The resulting solution was heated in a water bath for 3 minutes. A color change from blue to green, yellow or red indicates the presence of reducing sugars. Color intensity indicates the number of reducing sugars present.
- **Fehling's Test:** To 1 mL of the extract filtrate, 1 mL of Fehling's solution A (copper(II) sulphate) and 1 mL of Fehling's solution B (alkaline sodium tartrate) were added to a test tube and heated gently. A brick-red precipitate indicates the presence of reducing sugars.

- **Alkaloids**
- **Wagner’s Test:** A few drops of Wagner’s reagent were added to a portion of the extract, leading to the formation of a creamy white precipitate, which indicated the presence of alkaloids.
- **Mayer's Test:** A few drops of Mayer's reagent (mercuric chloride in potassium iodide) were added to the plant extract. A creamy precipitate indicates the presence of alkaloids.
- **Hager's Test:** A few drops of Hager's reagent were added to the plant extract. A yellow precipitate indicates the presence of alkaloids.
- **Dragendorff's Test:** A few drops of Dragendorff's reagent (bismuth nitrate in potassium iodide) were added to the extract. An orange or red precipitate indicates the presence of alkaloids.

**Anthraquinones**

- **Borntrager's Test:** 5 mL of chloroform was added to a portion of the extract in a dry test tube, and the mixture was shaken for at least 5 minutes. After filtration, the filtrate was mixed with 1 mL of ammonia. The appearance of a bright pink colour in the aqueous upper layer indicated the presence of free anthraquinones.

**Tannins (Phenolic compounds)**

- **Ferric Chloride Test:** Three to five drops of ferric chloride solution were added to a portion of the extract. A greenish-black precipitate suggested the presence of condensed tannins, while hydrolyzable tannins produced a blue or brownish-blue precipitate.
- **Iron complex test:** Iron complex test: 5 mL of plant extract was added to 5 mL of 0.5 % ferric ammonium citrate and 0.5g of sodium acetate in a test tube. It was boiled and

cooled. A purple violet or blackish bulky precipitate, which is insoluble in hot water or blue solution, indicates the presence of gallic acid, pseudo-tannins.

- **Modified Iron complex test:** 5 mL of plant extract was added to one drop of 33 % acetic acid and 1g of sodium potassium tartrate in a test tube. It was boiled, cooled and filtered. 0.25 % ferric ammonium citrate solution was added to the filtrate. A purple or blackish precipitate, which is insoluble in hot water, alcohol or dilute ammonia, indicates the presence of pyrogallol tannins.

**Saponins**

- **Frothing Test:** Approximately 10 mL of distilled water was added to a portion of the extract, then vigorously shaken for 30 seconds. The tube was then allowed to stand upright for 30 minutes. The formation of a persistent honeycomb-like froth for 10-15 minutes signified the presence of saponins.

**Cardiac-Glycosides**

- **Keller-Killiani Test:** A portion of the extract was dissolved in 1 mL of glacial acetic acid containing a trace amount of ferric chloride solution. This mixture was then transferred to a dry test tube, and 1 ml of concentrated sulfuric acid was added along the side of the tube to create a lower layer. The presence of deoxy sugars was indicated by a purple-brown ring at the interface, while a pale green colour in the upper acetic acid layer indicated the presence of cardiac glycosides.
- **Salkowski Test:** 0.5g of the extract was dissolved in 2 mL of chloroform. 2 drops of concentrated sulfuric acid were carefully added to form a layer. A reddish-brown colour at the interface indicates the presence of a steroidal nucleus (aglycone of cardiac glycosides).mixed with a portion of the extract in ice. One milliliter of concentrated sulfuric

acid was carefully added along the side of the test tube to create a lower layer. An immediate colour change, followed by further changes, indicated the presence of steroids and triterpenes. A red, pink, or purple colour suggested triterpenes, while a blue or green hue indicated steroids.

**Cyanogenic glycosides**

- **Sodium picrate Test:** A Small amount of the plant extract was placed in 3 test tubes labelled A, B and C. The extract in A and B was mixed with water. Sodium picrate paper (yellow) was inserted into each of the 3 test tubes, and the stopper was immediately placed. Tube B was placed in a boiling water bath for about 5 minutes. Tube A and C were kept at room temperature. After about half an hour, the formation of a brick-red precipitate (sodium isopurpurate) indicates the presence of cyanogenic glycosides.

## 2.4 Animal study

### 2.4.1 Source of laboratory animals

Female Wistar rats weighing between 120g and 159g were obtained from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria, and acclimatized in the animal house. The animals were kept in separate plastic cages and housed at room temperature and humidity, and allowed free access to dry rodent pellet feeds and water. They were placed in groups of five (5) with a wire-screen top for proper ventilation and wood shavings as bedding to collect urine and excreta. The processes applied in animal handling were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

### 2.4.2 Ethical clearance

The University of Benin's Faculty of Pharmacy's Ethical Committee granted ethical approval for the use of laboratory animals (the ethical clearance number is EC/FP/025/07). The University of Benin Ethical Committee's approval and recommendations, which adhered to recognized criteria on the ethical use of animals in research and global standards for animal treatment, were followed in the execution of the experimental protocols.

### 2.4.3 Dosing of experimental animals

Doses of *Annona muricata* extract used in this study were selected based on the LD50 of the plant's leaf which is greater than 5000 mg/kg (Yunusa *et al*.., 2024). The wistar albino rats were administered oral doses via gavage. Throughout the experiment, animals were dosed once a day, with each dose volume determined by the animal's weekly recorded body weight. The oral route of administration was chosen because it is the commonly utilized route by humans.

## 2.5 Sub-acute toxicity

Wistar female rats were divided into three groups comprising 5 animals each. Group A received 0.5 mL distilled water (control). Groups C and D, were orally administered different doses of *Annona muricata* (100 and 200 mg/kg BW), respectively, daily for 28 days. Body weights of the rats were taken on day 0, 7, 14, 21 and 28. Every day, the animals were closely monitored for any changes in their clinical symptoms. Tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma were the main areas of focus (Imade *et al*.., 2024). On the last day of gavage, the rats were fasted for a period of 12 h before being sacrificed using a chloroform chamber.

The blood was drawn by cardiac puncture into two distinct kinds of bottles: Ethylenediaminetetraacetic acid (EDTA) bottles were used to collect the blood's hematological parameters, while plain bottles were used to acquire serum for the analysis of biochemical

parameters. Organs for histological analysis were obtained, including the kidney, liver, heart, spleen, lungs and uterus.

**2.6 Haematological analysis**

Additionally, hematological parameters were evaluated by a blood count utilizing an automated hematology analyzer on the blood collected into EDTA bottles (Dymind 2000, China). The parameters analyzed include white blood cell (WBC), red blood cells (RBC), red blood cell distribution width (RDW), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), granulocytes (GRAN), platelets count (PCT) and hematocrit (HCT) (Imade *et al..,* 2024).

**2.7 Biochemical analysis**

Samples of blood were taken into plain tubes and left to stand at room temperature for 45 min before being centrifuged for 10 min at 3400 rpm. The collected serum stored at -25 °C was used to assess the lipids, renal and liver function. The parameters assayed included creatinine (Cr), urea (Ur), uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), serum proteins (Tp), total bilirubin (Tb), triglycerides (TG), total cholesterol (T-CH), low-density lipoproteins (LDL), high-density lipoprotein (HDL) and serum electrolytes (Na+, K+, Cl−, HCO3 −) (Imade *et al..,* 2024).

**2.8 Histopathological analysis**

For histological analysis, the livers, hearts, kidneys, spleens, lungs, and uteri from the sacrificed animals were fixed in 10% neutral buffered formalin. These tissues were subsequently dehydrated in ascending grades of alcohol (70%, 90%, 96%, and 100%), cleared in xylene, impregnated with molten paraffin wax, and sectioned onto slides. These sections (4–5 μm thick) were stained with hematoxylin after dewaxing with xylene and hydrating in descending grades of alcohol (100%,

96%, 70%) and water. Differentiation was done in 1% acid alcohol, and the sections were counterstained with eosin. Dehydration in ascending grades of alcohol was repeated, and the sections were cleared in xylene and mounted on dibutylphthalate-polystyrene cover slips prior to microscopic examination. (Imade *et al*., 2024).

**2.9 Statistical analysis**

The mean values and standard error of the mean (SEM) were used to express the experimental results. A one-way analysis of variance (ANOVA) and the Tukey-Kramer test for multiple comparisons were used to compare the effects of the extract treatment groups and the control. GraphPad Prism (version 8.4.3) was used for data analysis and visualization; $P < 0.05$ was deemed statistically significant.

# CHAPTER THREE

# RESULT

## 3.1 Percentage Yield

The weight of the pulverized leaves of *Annona muricata was* 1000g Weight of absolute ethanol extracts of the plant was 30g therefore, the percentage yield of *Annona muricata* is 3%.

**Table 3.1: Qualitative screening of ethanol extracts of *Annona muricata***

| S/N | TEST | OBSERVATION | INFERENCE |
|---|---|---|---|
| 1 | **Carbohydrate** | | |
| | • Molisch's Test | Purple ring formed at the interface | Carbohydrate present |
| | • Benedict's Test | Brick red precipitate formed upon heating | Reducing sugar present |
| | • Fehling's Test | Brick red precipitate formed on heating | Reducing sugar present |
| 2 | **Alkaloids** | | |
| | • Wagner's Test | White insoluble precipitate formed | Alkaloid present |
| | • Mayer's Test | Creamy white precipitate formed | Alkaloid present |
| | • Hager's Test | Yellow precipitate formed | Alkaloid present |
| | • Dragendorff's Test | Orange colored precipitate formed | Alkaloid present |
| 3 | **Anthraquinones** | | |
| | • Borntrager's Test | Bright Pink color observed at the upper aqueous layer | Free anthraquinones present |
| 4 | **Phenolic compounds** | | |
| | • Ferric Chloride Test | Greenish black precipitate formed | Condensed tannins present |
| | • Iron complex test | Purple precipitate formed | Pseudo tannins present |
| | • Modified Iron complex test | Black precipitate formed | Tannins present |
| 5 | **Saponin** | | |
| | • Frothing Test | No frothing observed upon shaking | Saponins absent |
| 6 | **Cyanogenic glycoside** | | |
| | • Sodium picrate Test | No color change observed | Cyanogenic glycosides absent |
| 7 | **Cardiac glycoside** | | |
| | • Keller-Killiani Test | Formation of a reddish-brown layer that turned bluish green upon standing observed in the upper layer | Cardiac glycoside present |
| | • Salkowski's Test | Reddish brown precipitate formed at the interface | Steroidal nuclues present |
| | • Liebermann-Burchard Test | Deep green color formed | Sterol ring present |

**Table 3.1.1.: Phytochemical screening showing presence or absence of ethanol extract *A. muricata***

| S/N | Phytochemicals | *Annona muricata* |
|---|---|---|
| 1 | Carbohydrate | + |
| 2 | Reducing sugar | + |
| 3 | Alkaloids | + |
| 4 | Anthraquinones | + |
| 5 | Phenolic compounds | + |
| 6 | Saponins | - |
| 7 | Steroids/triterpenes | + |
| 8 | Cyanogenic glycoside | - |
| 9 | Cardiac glycoside | + |

### 3.2 Effect of Ethanol Extract of *Annona muricata* on Hematological Parameters

The results of the hematological parameters showed a decrease in White Blood Cell count at 100 mg/kg ($p<0.05$) when compared to control. There was also a significant increase in MPV and P-LCR at 100 mg/kg ($p<0.0001$) and 200 mg/kg ($p<0.0001$). No significant effect was seen on all other tested hematological parameters as they all fell within the normal range.

**Table 3.2.1: Results of haematological analysis after 28 days of administration of extract**

| | | *Annona muricata* | |
|---|---|---|---|
| **Dose (mg/kg)** | 0 | 100 | 200 |
| WBC ($10^3$/µL) | 14.64 ± 1.50 | 8.08 ± 1.02[a] | 11.12 ± 0.80 |
| LYM (%) | 83.00 ± 1.66 | 72.24 ± 5.55 | 82.62 ± 1.70 |
| MON (%) | 2.06 ± 0.33 | 3.62 ± 1.22 | 1.38 ± 0.17 |
| NEU (%) | 10.56 ± 1.65 | 18.16 ± 3.67 | 12.96 ± 1.47 |
| EOS (%) | 0.84 ± 0.37 | 0.76 ± 0.18 | 0.50 ± 0.10 |
| BAS (%) | 3.50 ± 0.42 | 5.22 ± 1.25 | 2.52 ± 0.39 |
| RBC ($10^6$/µL) | 6.38 ± 0.24 | 6.28 ± 0.24 | 6.34 ± 0.15 |
| HGB (g/dL) | 13.50 ± 0.51 | 13.52 ± 0.48 | 13.60 ± 0.30 |
| HCT (%) | 40.20 ± 1.56 | 39.8 ± 1.83 | 40.80 ± 0.58 |
| MCV (µm$^3$) | 50.28 ± 0.42 | 53.20 ± 0.32 | 51.96 ± 0.13 |
| MCH (ρg) | 21.16 ± 0.10 | 21.54 ± 0.09 | 21.46 ± 0.16 |
| MCHC (g/dL) | 42.12 ± 0.37 | 40.50 ± 0.38 | 41.28 ± 0.37 |
| RDWC (%) | 16.28 ± 0.88 | 15.38 ± 0.53 | 15.68 ± 0.30 |
| RDWS (µm$^3$) | 26.96 ± 1.65 | 26.48 ± 1.05 | 25.96 ± 0.45 |
| PLT ($10^3$/µL) | 741.20 ± 98.38 | 618.20 ± 39.58 | 706.20 ± 28.94 |
| MPV (µm$^3$) | 6.54 ± 0.27 | 8.18 ± 0.12[d] | 8.18 ± 0.12[d] |
| PCT (%) | 0.49 ± 0.08 | 0.47 ± 0.01 | 0.47 ± 0.01 |
| PDW (%) | 24.98 ± 1.43 | 20.25 ± 0.22 | 20.25 ± 0.22 |
| P-LCR (%) | 5.96 ± 2.29 | 20.72 ± 0.98[d] | 20.72 ± 0.98[d] |

Data are expressed as mean ± SEM, n = 5. Values in the test groups not carrying any letter when compared to the control group are not significantly different according to Tukey-Kramer multiple comparison test. Letter a, and d are $p<0.05$, and $p<0.0001$ respectively when compared to the control. white blood cell (WBC), lymphocytes (LYM), monocyte (MON), neutrophil (NEU), eosinophil (EOS), basophil (BAS), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width SD (RDW-SD), red blood cell distribution width CV (RDW-CV), platelet (PLT), mean platelet volume (MPV), platelet distribution width (PDW), platelets count (PCT), platelet larger cell ratio (P-LCR)

### 3.3 Effect of Ethanol Extract of *Annona muricata* on Kidney Function Test Parameters

The Kidney function test parameters were not significantly altered by the administration of the extract. However, there was an increase in Creatinine level at 200 mg/kg ($p<0.05$).

**Table 3.3.1: Kidney function test parameters after 28 days of administration of *Annona muricata* extract**

| | *Annona muricata* | | |
|---|---|---|---|
| Dose (mg/kg) | 0 | 100 | 200 |
| Urea (mg/dL) | 45.40 ± 2.25 | 43.60 ± 1.50 | 43.20 ± 2.35 |
| Creatinine (mg/dL) | 0.68 ± 0.06 | 0.84 ± 0.04 | 0.90 ± 0.05[a] |
| $Na^+$ (mmol/L) | 140.60 ± 1.47 | 139.00 ± 0.77 | 139.20 ± 1.32 |
| $K^+$ (mmol/L) | 4.12 ± 0.10 | 4.38 ± 0.17 | 4.88 ± 0.10 |
| $HCO_3^-$ (mmol/L) | 21.40 ± 0.28 | 20.60 ± 0.98 | 18.60 ± 0.81 |
| $Cl^-$ (mg/dL) | 104.80 ± 0.73 | 105.60 ± 0.87 | 105.60 ± 0.68 |

Data are expressed as mean ± SEM, n=5. Values in the test groups not carrying any letter when compared to the control group are not significantly different according to Tukey-Kramer multiple comparison test. Letter a is $p<0.05$ when compared to the control.

### 3.4 Effect of Ethanol Extract of *Annona muricata* on Lipid Profile Test Parameters

A significant decrease was observed in Total cholesterol and LDL at 100mg/kg ($p<0.05$). No significant change was detected for the other Lipid profile test parameters at the different doses of the extract administered.

**Table 3.4.1: Lipid profile test parameters after 28 days of administration of *Annona muricata* extract**

| | | *Annona muricata* | |
|---|---|---|---|
| **Dose (mg/kg)** | 0 | 100 | 200 |
| T-CH (mg/dL) | 82.20 ± 4.03 | 68.60 ± 3.89[a] | 82.80 ± 7.62 |
| HDL (mg/dL) | 25.40 ± 0.98 | 23.80 ± 0.86 | 24.80 ± 1.88 |
| LDL (mg/dL) | 36.60 ± 2.94 | 26.60 ± 2.79[a] | 38.80 ± 5.75 |
| TG (mg/dL) | 100.60 ± 2.89 | 91.00 ± 3.65 | 96.60 ± 1.50 |

Data are expressed as mean ± SEM, n=5. Values in the test groups not carrying any letter when compared to the control group are not significantly different according to Tukey-Kramer multiple comparison test. Letter a is $p<0.05$ when compared to the control. total cholesterol (T-CH), high-density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride (TG).

### 3.5 Effect of Ethanol Extract of *Annona muricata* on Liver Function Test Parameters

There was a significant reduction in Aspartate Aminotransferase level ($P<0.0001$) at the dose of 200mg/kg. Also, a decrease was observed in Alanine Aminotransferase level ($P<0.001$) at 200 mg/kg. Other parameters were not significantly altered by administration of the extract at different doses.

**Table 3.5.1: Liver function test parameters after 28 days of administration of *Annona muricata* extract**

| | *Annona muricata* | | |
|---|---|---|---|
| **Dose (mg/kg)** | 0 | 100 | 200 |
| AST (µ/L) | 77.40 ± 4.27 | 73.40 ± 2.11 | 49.20 ± 1.59[d] |
| ALT (µ/L) | 45.80 ± 2.42 | 46.80 ± 3.09 | 30.00 ± 2.63[c] |
| ALP (µ/L) | 59.60 ± 3.54 | 65.80 ± 5.04 | 46.00 ± 2.76 |
| Tb (mg/dL) | 0.28 ± 0.02 | 0.28 ± 0.02 | 0.28 ± 0.02 |
| Cb (mg/dL) | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 |
| Tp (g/dL) | 6.34 ± 0.12 | 6.20 ± 0.25 | 6.80 ± 0.08 |
| ALB (g/dL) | 2.82 ± 0.02 | 2.72 ± 0.05 | 2.70 ± 0.03 |
| GLo (g/dL) | 3.52 ± 0.14 | 3.48 ± 0.25 | 4.10 ± 0.08 |

Data are expressed as mean ± SEM, n=5. Values in the test groups not carrying any letter when compared to the control group are not significantly different according to Tukey-Kramer multiple comparison test. Letter c and d are $p<0.001$, and $p<0.0001$ respectively when compared to the control. alkaline phosphatase (ALP), alanine aminotransferase (ALT) Aspartate aminotransferase (AST), total bilirubin (Tb), conjugated bilirubin (Cb), total protein (Tp), albumin (ALB), globulin (GLo).

### 3.6 Histological Evaluation

Uterus sections from control rats displayed normal histological architecture, characterized by a well-defined uterine cavity surrounded by the endometrial lining and endometrium containing glands embedded in the stroma. Uterus sections from rats treated with both doses of the plant extract showed normal histological architecture. The endometrial membrane ulceration observed in some groups was not considered a toxic effect, as it was also observed in the control group, and was deemed an artefactual change.

Lung sections from control rats exhibited normal histological architecture, characterized by well-defined alveolar sacs, interstitial space, bronchioles, and bronchial blood vessels. Lung sections from rats treated with graded of the plant extract showed normal alveolar sacs and boosted the local immune system of the lungs. However, lung sections from rats treated with 200mg/kg of *A. muricata* displayed bronchiolar ulceration, which appeared to be the sole toxic effect observed.

The liver sections from control rats showed normal architecture, characterized by well-defined hepatocytes, sinusoids, and portal triads. Liver sections from rats treated with both doses of *A. muricata* exhibited additional beneficial hemodynamic and vasoactive effects, including increased blood circulation and vasodilation. Furthermore, the plant extract boosted the local immune system of the liver by activating sinusoidal Kupffer cells.

The spleen sections from rats that received standard feed and water exhibited normal tissue architecture, featuring well-defined splenic arterioles, white pulp comprising lymphoid follicles, red pulp responsible for sequestering red blood cells, and splenic sinuses that constitute lymphatic channels. In contrast, spleen sections treated with both doses of the plant extract displayed varying degrees of activation of the lymphoid follicles, with the 100mg/kg body weight dose of *A. muricata* inducing the most significant boosting of the local immune system.

Heart sections from control rats showed normal histological architecture, featuring well-defined bundles of myocardiac fibers, interstitial space, coronary arteries, and cardiac veins. Heart sections from rats treated with both doses of the plant extract exhibited beneficial hemodynamic and vasogenic changes, including increased blood circulation and vasodilation.

Kidney sections from control and treated rats displayed normal histological architecture, featuring well-defined tubules, glomeruli, interstitial space, and arcuate blood vessels. However, kidney sections from rats treated with both doses of the extract showed added beneficial hemodynamic and vasoactive effects, including increased blood circulation and vasodilation.

**UTERUS**

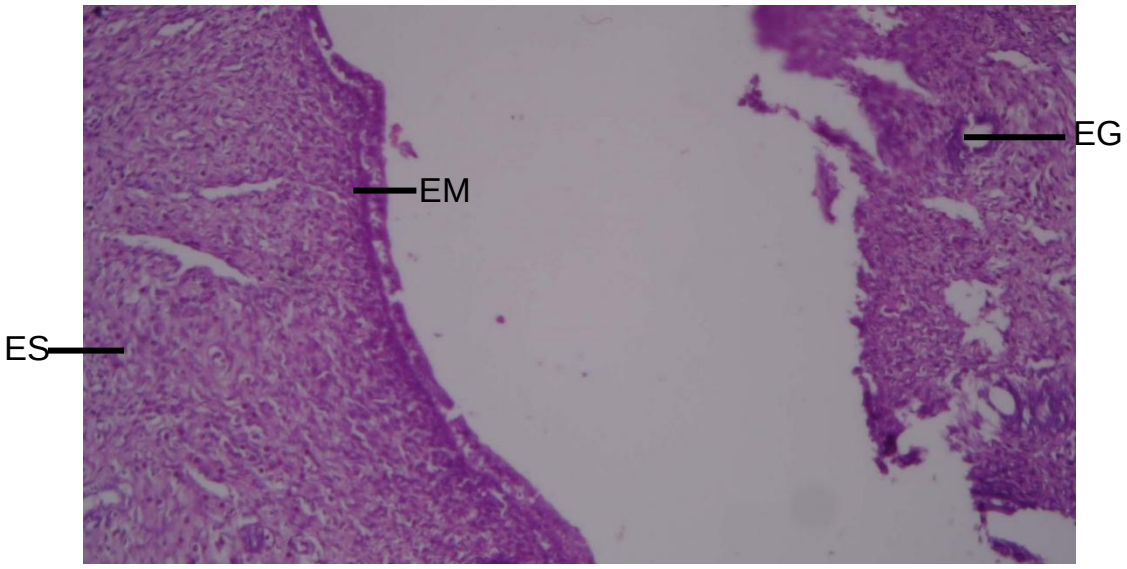


Figure 3.1: Rat uterus administered control showing: normal architecture: endometrial membrane (EM), stroma (ES) and glands (EG): H&E 400 X

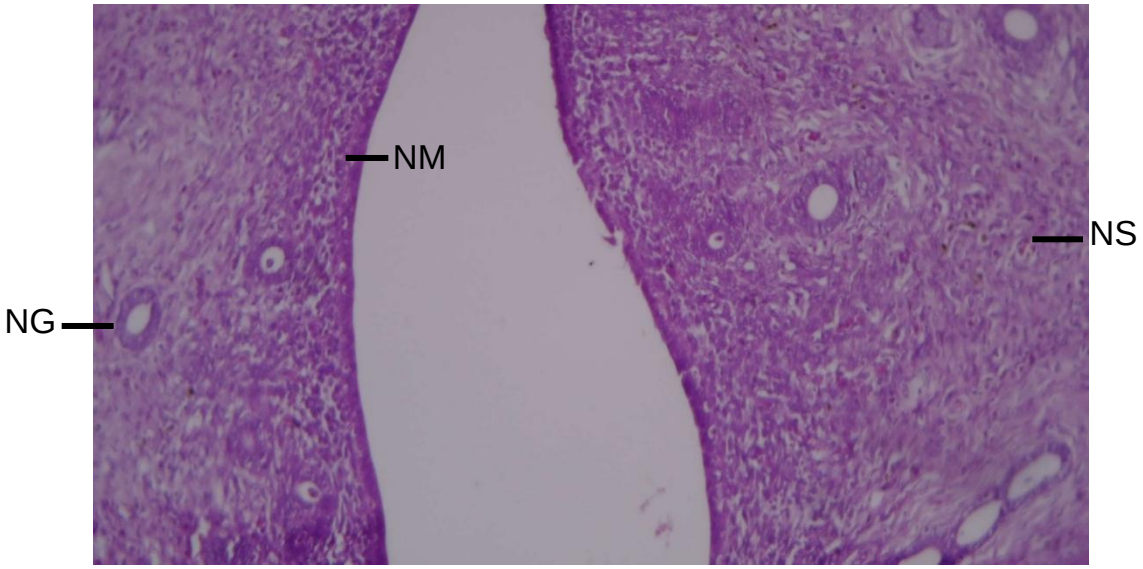

Figure 3.2: Rat uterus administered 100mg/kg *A. muricata* showing: normal glands (NG), stroma (NS) and endometrial membrane (NM): H&E 400 X

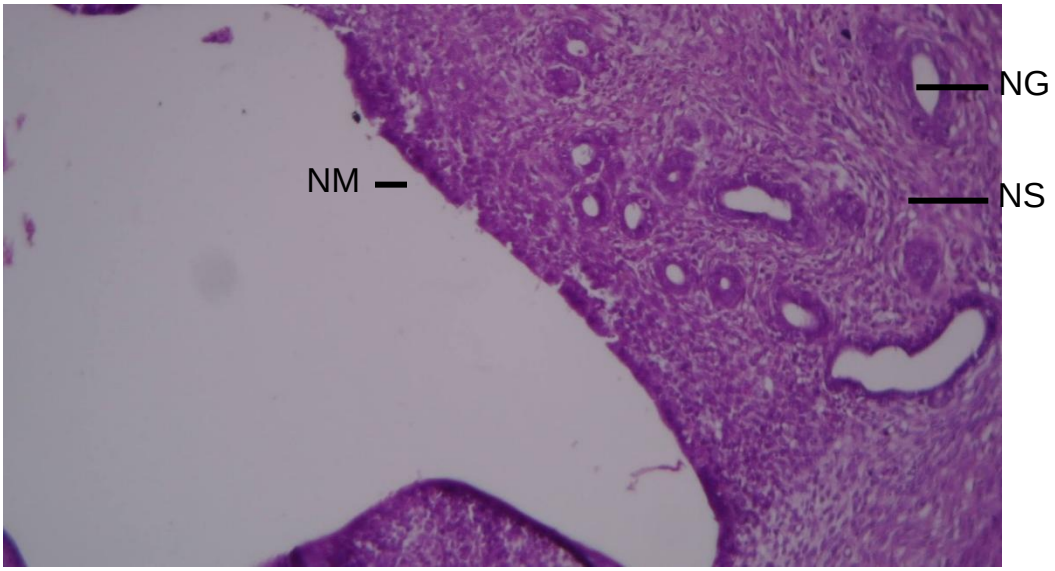


Figure 3.3 : Rat uterus administered 200mg/kg *A. muricata* showing: normal endometrial glands (NG), stroma (NS) and membrane (NM): H&E 400 X

**LUNGS**

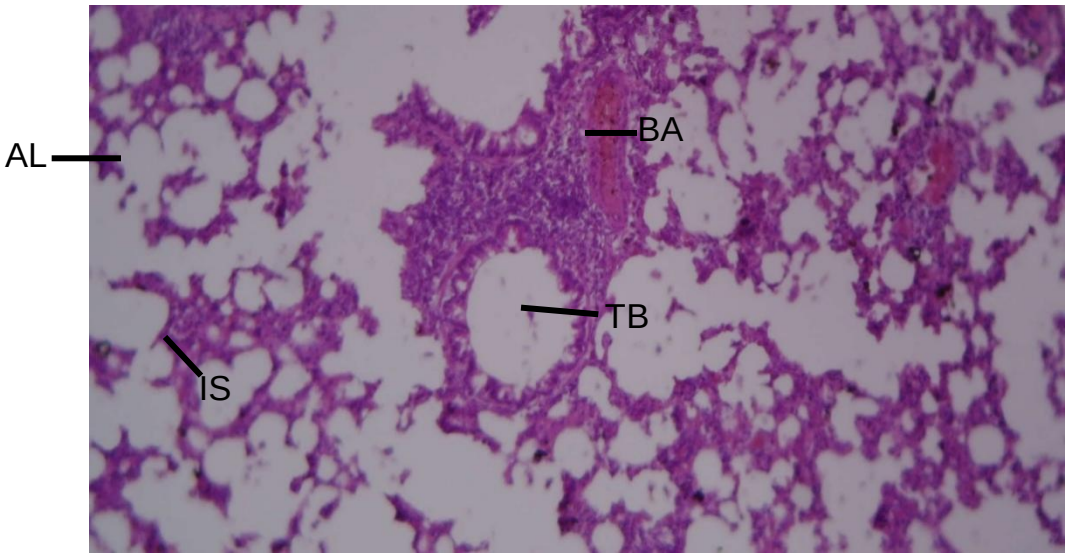


Figure 3.4: Rat lung administered control, showing normal architecture: alveoli (AL), interstitial space (IS), terminal bronchiole (TB) and bronchial artery (BA): H&E 400 X

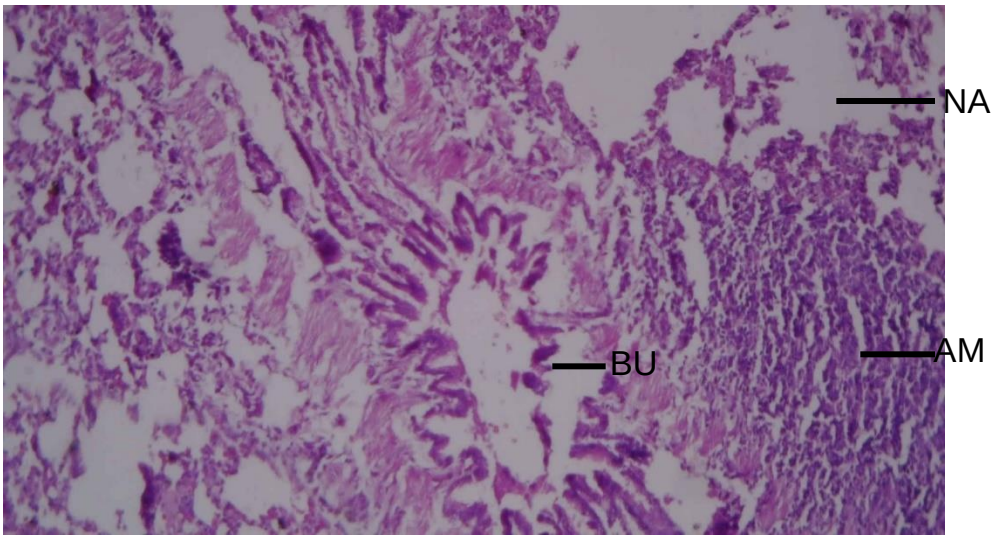


Figure 3.5: Rat lungs administered 100mg/kg *A. muricata* showing: normal alveoli (NA), bronchiolar ulceration (BU) and activation of cells of the mononuclear phagocyte system (AM): H&E 400 XA

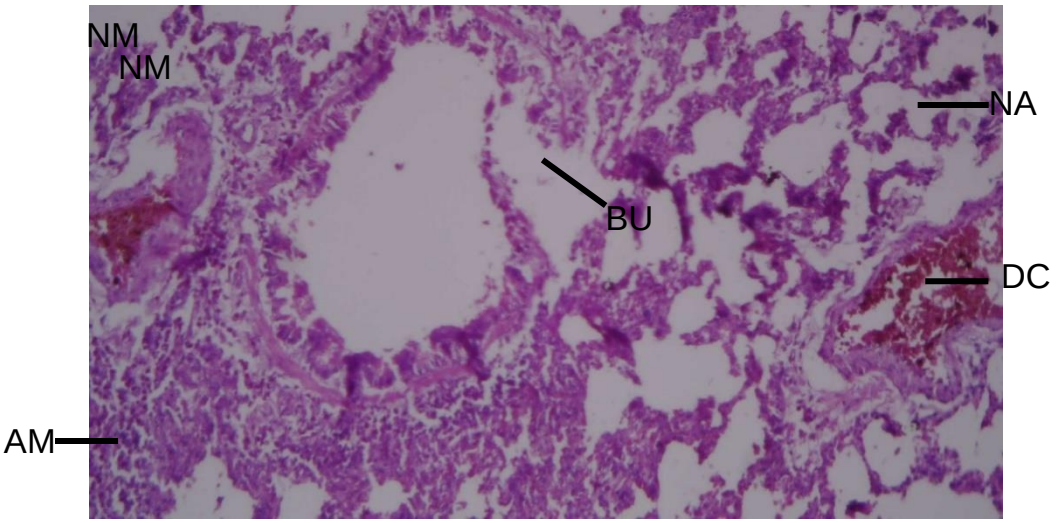


Figure 3.6: Rat lungs administered 200mg/kg *A. muricata* showing: vasodilatation and active congestion (DC), bronchiolar ulceration (BU), normal alveoli (NA) and activated mononuclear phagocyte cells (AM): H&E 400 X

**LIVER**

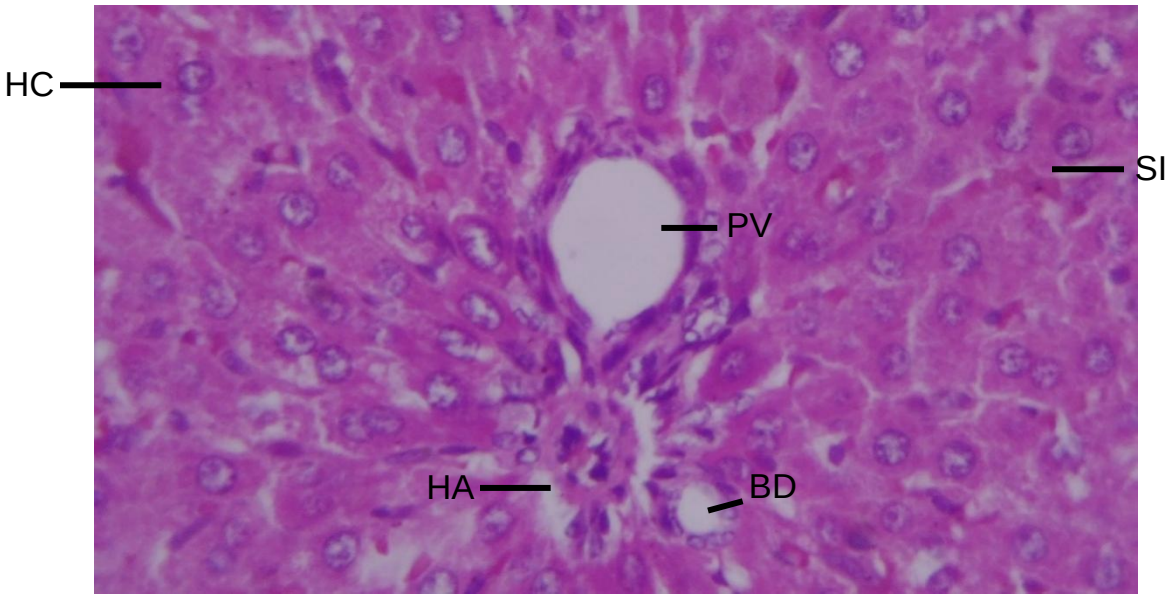


Figure 3.7: Rat liver administered control, showing: normal architecture: hepatocytes (HC), sinusoids (SI), portal vein (PV), hepatic artery (HA) and bile duct (BD): H&E 400 X

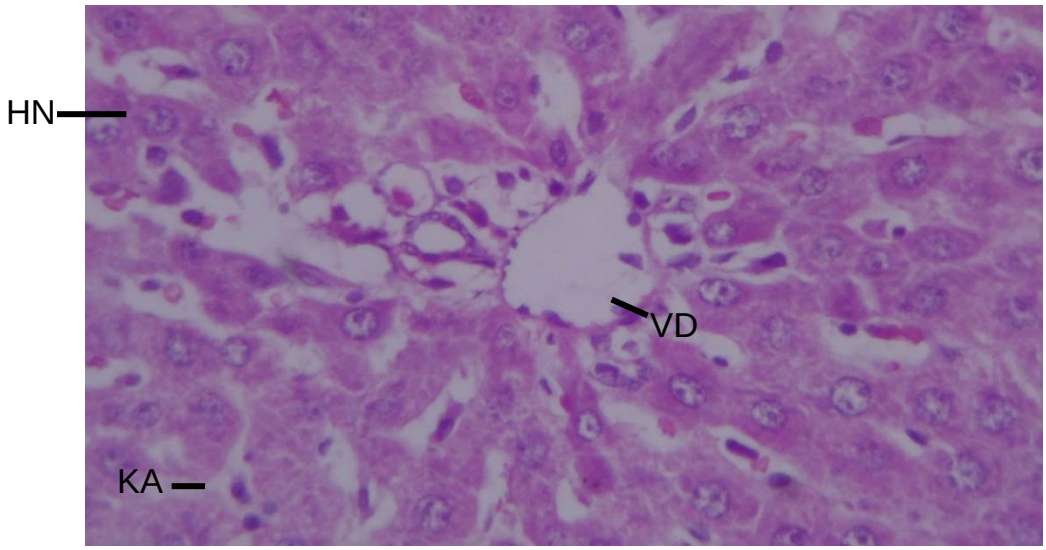


Figure 3.8:. Rat liver administered 100mg/kg *A. muricata* showing: normal hepatocytes with conspicuous nucleoli (HN), vasodilatation (VD) and kupffer cell activation (KA): H&E 400 X

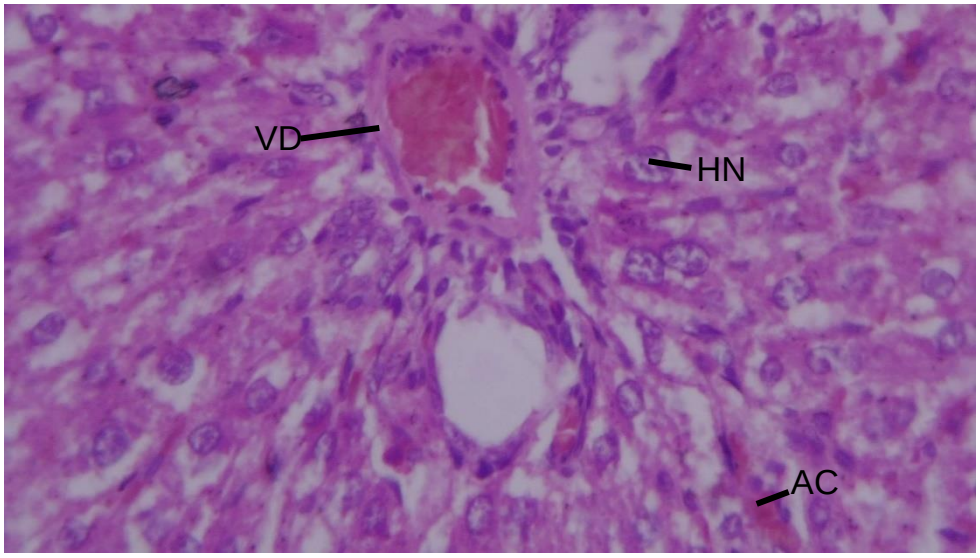


Figure 3.9:. Rat liver administered 200mg/kg *A. muricata* showing: normal hepatocytes with conspicuous nucleoli (HN), vasodilatation (VD) and active congestion (AC): H&E 400 X

**SPLEEN**

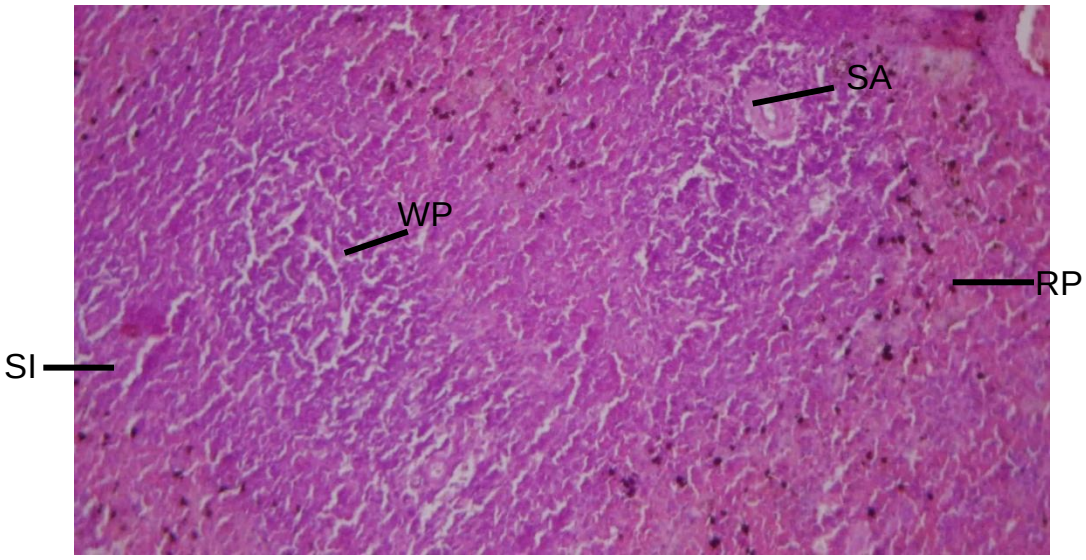


Figure 3.10: Rat spleen given control, showing: normal architecture: white pulp (WP), sinuses (SI), splenic arterioles (SA) and red pulp (RP): H&E 400 X

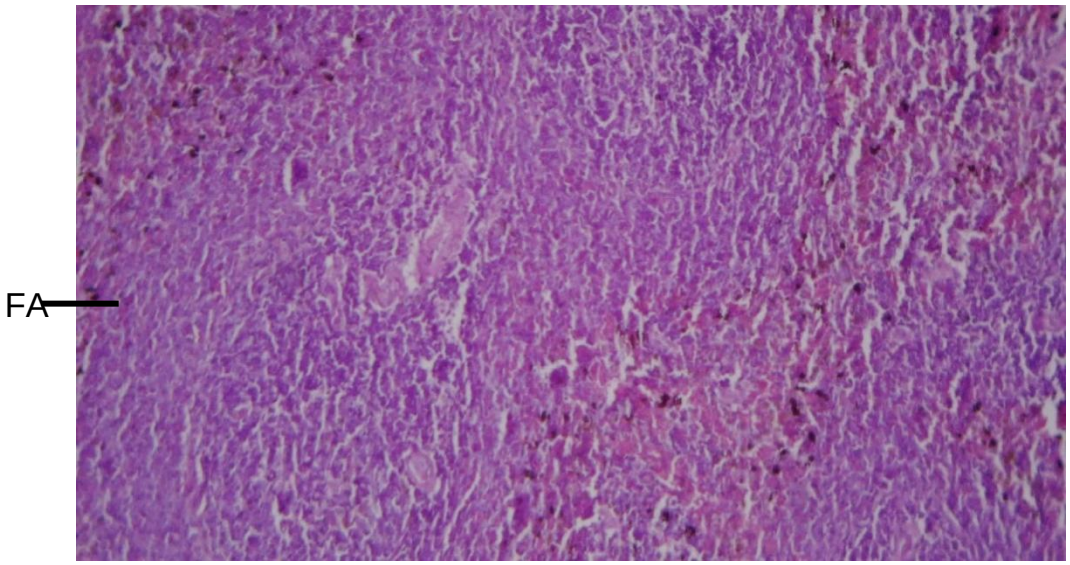


Figure 3.11: Rat spleen administered 100mg/kg *A. muricata* showing: marked follicular activation (FA): H&E 400 X

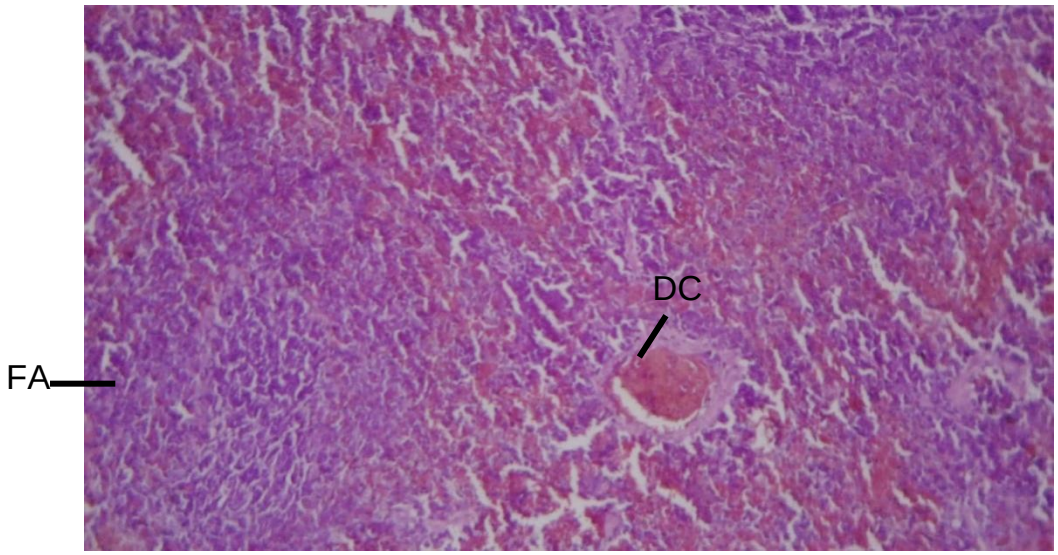


Figure 3.12: Rat spleen administered 200mg/kg *A. muricata* showing: follicular activation (FA), vasodilatation and active congestion (DC):H&E 400 X

**HEART**

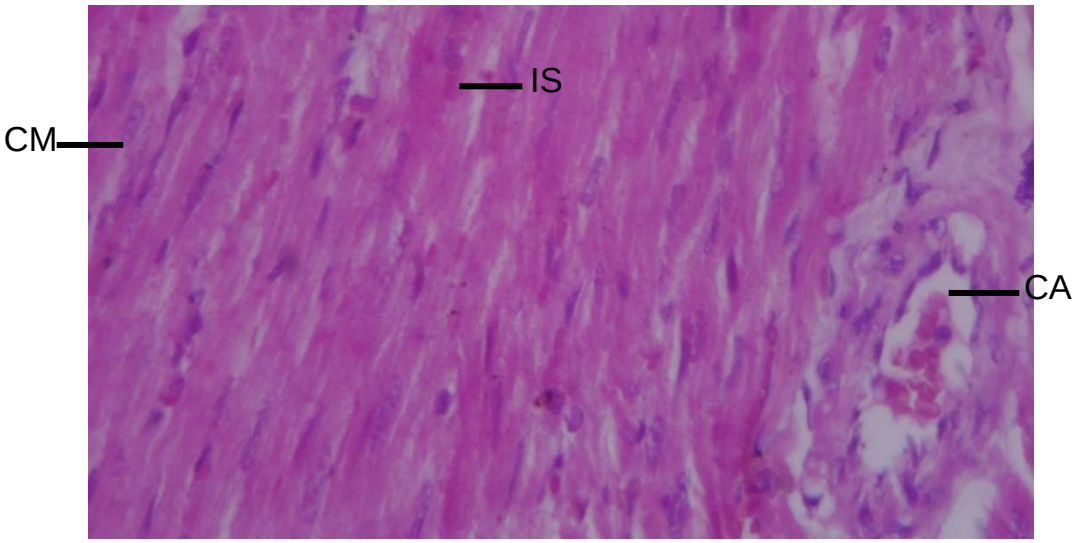


Figure 3.13: Rat heart administered control, showing normal architecture: bundles of cardiomyocytes (CM), interstitial space (IS) and coronary artery (CA): H&E 400 X

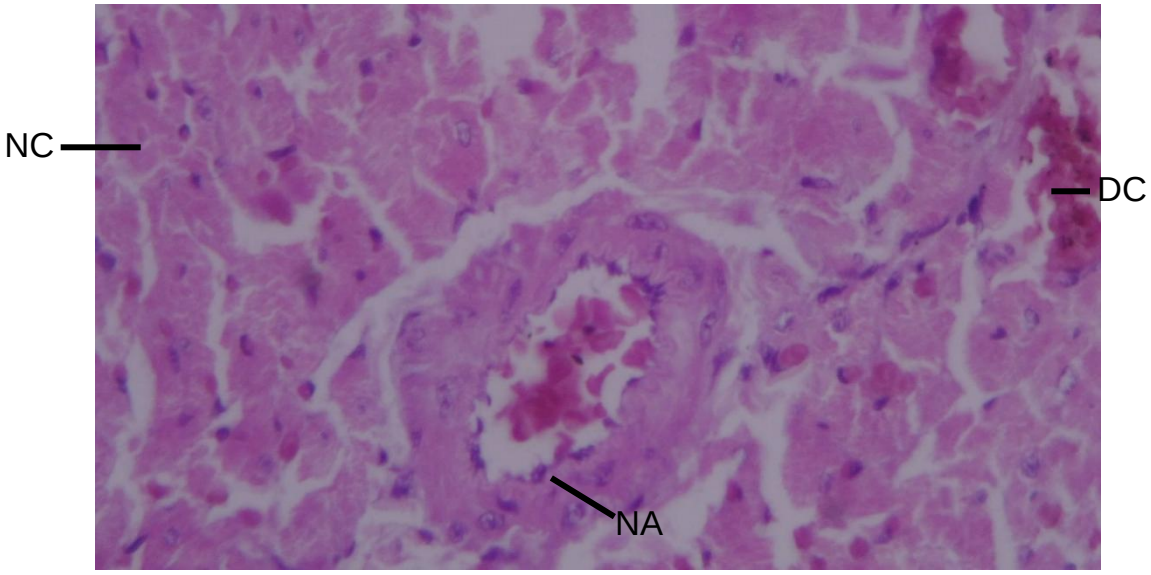


Figure 3.14: Rat heart administerd 100mg/kg *A. muricata* showing: normal bundles of cardiomyocytes (NC), coronary artery (NA) and active interstitial congestion and vasodilatation (DC): H&E 400 X

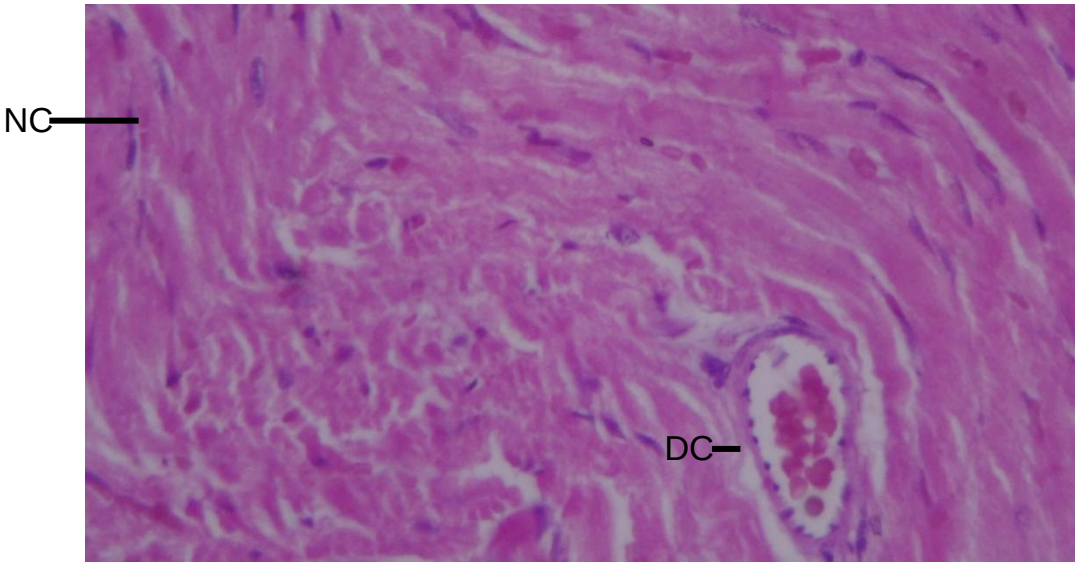


Figure 3.15: Rat heart administered 200mg/kg *A. muricata* showing: normal bundles of cardiomyocytes (NC), active interstitial congestion and vasodilatation (DC): H&E 400 X

**KIDNEY**

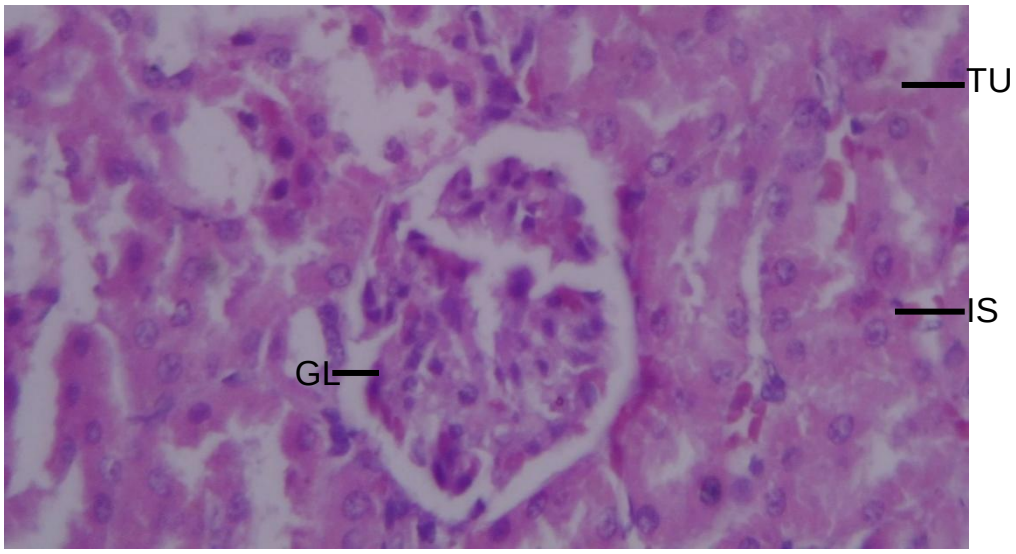


Figure 3.16: Rat kidney administered control, showing normal architecture: tubules (TU), glomerulus (GL) and interstitial space (IS): H&E 400 X

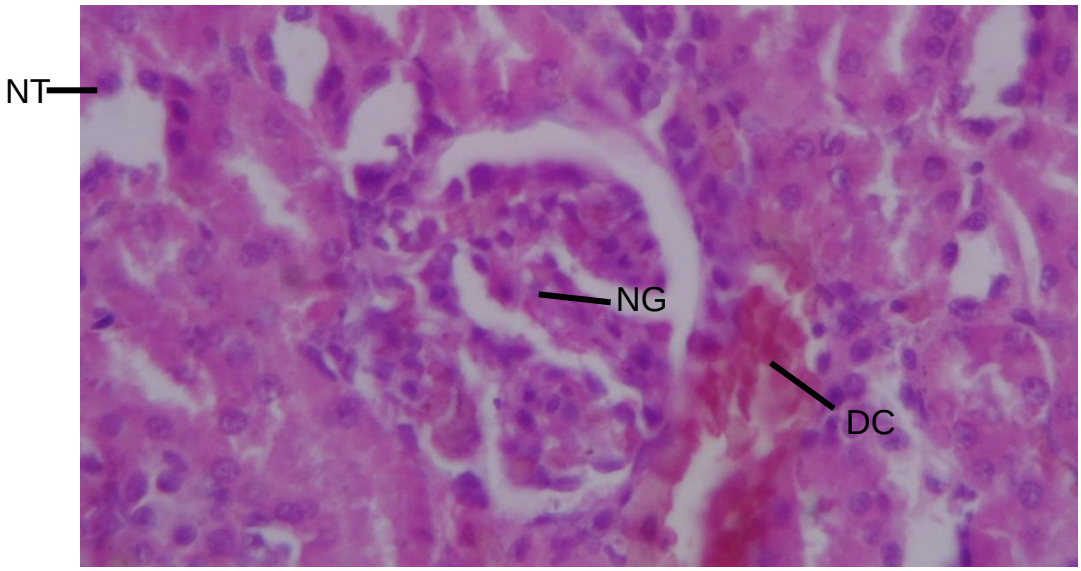


Figure 3.17: Rat kidney administered 100mg/kg *A. muricata* showing: normal tubules (NT), glomeruli (NG), active interstitial congestion and vasodilatation (DC): H&E 400 X

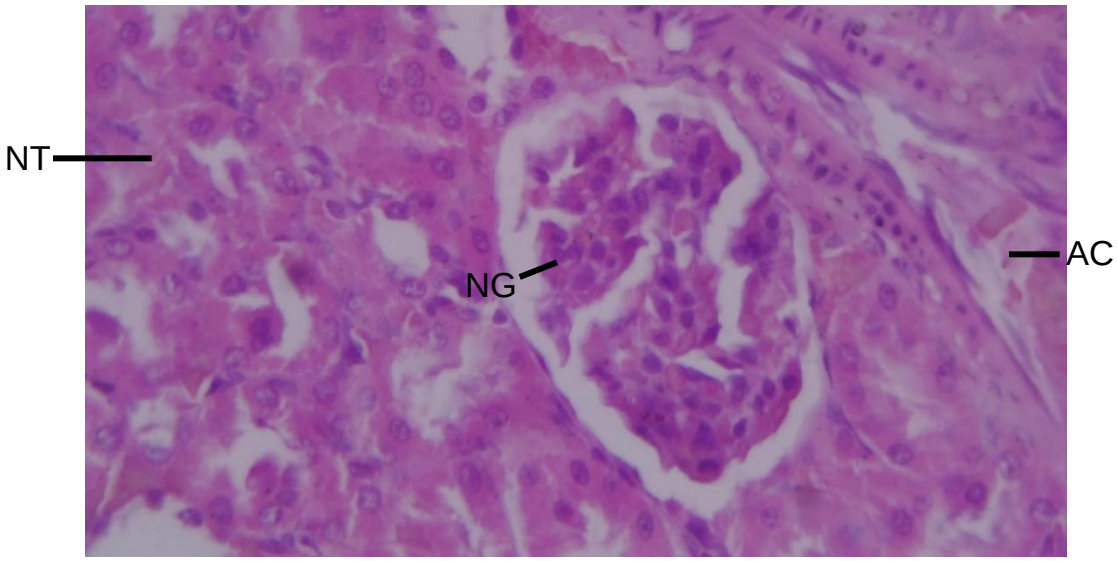


Figure 3.18: Rat kidney administered 200mg/kg *A. muricata* showing: normal tubules (NT), glomerulus (NG) and active interstitial congestion (AC): H&E 400 X

# CHAPTER FOUR

## DISCUSSION

This study evaluated the subacute toxicity and potential physiological effects of ethanol leaf extract of *Annona muricata* in Wistar rats over a 28-day period. Various parameters were assessed, including blood parameters, kidney and liver function, and lipid metabolism.

Phytochemical analysis was also carried out to determine the phytochemical constituents present in the extract. Across the measured parameters, the extract exhibited effects that, in most cases, corresponded with previously published studies, while also revealing some variations that may be linked to dosage, extraction solvent, and plant part used.

The yield of the plant extract in absolute ethanol shows that ethanol is a suitable solvent for extracting *Anonna muricata.* It also indicates that the continuous solvent extraction method using Soxhlet apparatus is effective. Also, the good yield of the plant extracts using the Soxhlet apparatratus can also be attributed to the temperature employed in this method of extraction.

The qualitative phytochemical screening of the ethanol leaf extract of *A. muricata* revealed the presence of a wide range of phytochemical constituents, including carbohydrates, reducing sugars, alkaloids, anthraquinones, phenolic compounds, cardiac glycosides, and steroids/triterpenes. Also, the test for Saponins was negative.

The presence of alkaloids and phenolic compounds is highly consistent with global reports on *A. muricata* leaves (Ibrahim *et al..*, 2023; Eze *et al..*, 2023). For instance, Eze *et al..* 2023 characterized the strong presence of phenolics in ethanol extracts, attributing the plant's high antioxidant capacity to these groups. This similarity confirms that the geographical origin and

extraction method successfully yielded the primary, pharmacologically active compounds responsible for the extract's physiological effects in this study.

Phenolics are recognized for their potent antioxidant capacity, which scavenges free radicals and protects cells from oxidative damage (Ademola *et al.*. 2021). This inherent antioxidant property is linked to the observed and highly significant hepatoprotective effect in this study, where Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) levels decreased significantly below control values at the 200mg/kg dose (Table 3.5.1). This beneficial action is attributed to these phytochemicals' ability to stabilize hepatocyte cell membranes and reduce oxidative stress-related liver injury (Ademola *et al.*. 2021).

The positive detection of anthraquinones (confirmed by Borntrager's Test) is noteworthy. Anthraquinones are a class of compounds typically associated with laxative properties, and they are also noted for various pharmacological activities including antimicrobial and anti-inflammatory effects (Okoro *et al.*., 2022). Their presence contributes to the overall phytochemical complexity and potential for synergistic effects, requiring careful toxicological assessment to ensure long-term gut and systemic safety, reinforcing the need for the subacute (28-day) evaluation conducted in this research (Okoro *et al.*., 2022).

The presence of cardiac glycosides is particularly relevant to the safety evaluation design. These compounds are powerful agents known for their specific inhibitory action on the Na+/K+ ATPase pump, an enzyme critical for ion transport in vital organs like the heart and kidney (Omole *et al.*., 2024). While this action forms the basis of therapeutic cardiotonic effects, these compounds possess a narrow therapeutic index, meaning the difference between a therapeutic dose and a toxic dose is very small. Their detection inherently necessitates the careful cardiac and renal monitoring carried out in this study, thereby scientifically validating the scope of the safety evaluation.

The potential for cardiotoxins to impact kidney function explains the focus on Kidney Function Test parameters (creatinine, urea, and electrolytes) and histopathological assessment of the kidney. Their direct impact on myocardial contractility justifies the histopathological assessment of the heart. This observed structural integrity in the heart and the functional but non-structural renal elevation of Creatinine suggests that, within the 100mg/kg to 200mg/kg dose range, the concentration of these potent compounds likely confers more favorable hemodynamic and vasoactive effects rather than overt cardiotoxic risk (Omole *et al.*., 2024; Rodriguez *et al.*., 2022).

Beyond the active compounds identified, the qualitative phytochemical screening yielded negative results that significantly contribute to the extract's favorable safety profile. Specifically, the tests for both cyanogenic glycosides and saponins were absent (Table 3.1.1). The absence of saponins is a key finding for systemic safety. Saponins are generally known for their hemolytic activity (red blood cell destruction) and their potential to cause gastrointestinal irritation (Agbaje *et al.*., 2023). The consistently normal values recorded for Red Blood Cell indices (RBC count, HGB, HCT) across all treatment groups (Table 3.2.1) is directly supported by the lack of these lytic compounds. This result suggests that the extraction method or the ethanol solvent did not yield saponins, or their concentration was below the detection limit of the Frothing Test, thereby enhancing the apparent safety margin and reducing a common toxicity risk associated with herbal remedies (Agbaje *et al.*., 2023).

Equally important is the confirmation of the absence of cyanogenic glycosides (Table 3.1.1). These phytochemicals are of high toxicological concern because they can be hydrolyzed to release the potent cytotoxin, hydrogen cyanide (HCN), leading to severe, acute toxicity and potential mortality (Okafor *et al.*., 2022). The established high LD50 which is greater than 5000mg/kg, (Yunusa *et al.*., 2024) of the *A. muricata* leaf extract, along with the absence of clinical signs of acute

poisoning (tremors, convulsions, coma, etc.) during the 28-day study, is consistent with the lack of these lethal toxins. This finding significantly reinforces the extract's relative safety for subacute consumption, supporting its rational use in traditional medicine without the critical concern of acute cyanide poisoning (Okafor *et al.*., 2022).

The hematological results revealed that white blood cell (WBC) count significantly decreased at 100 mg/kg ($p<0.05$) before rising again at 200 mg/kg. This suggests that the extract may exert mild immunosuppressive effects at a moderate dose, followed by adaptive or compensatory immune stimulation at higher doses.

This biphasic response pattern has been observed in other medicinal plant studies where phytoconstituents exert both inhibitory and stimulatory effects depending on concentration (Yahaya *et al.*., 2022). Comparable trends were reported by (Adeyemo *et al.*. 2022), who found no sustained leukocyte suppression in Wistar rats given stem bark extracts, supporting the notion that the immunological effects of *A. muricata* may be dose-specific.

Red blood cell indices, including RBC count, hemoglobin (HGB), and hematocrit (HCT), remained within normal ranges across all groups, indicating that the extract did not compromise oxygen transport or induce anemia. These findings are consistent with the results of (Olatunde *et al.*. 2023), who reported that *A. muricata* leaf extract maintained hematopoietic stability during Sub acute administration in rodents.

This non-linear, biphasic pattern is also supported by the literature on phytomedicines, where the effect of bioactive compounds (like the identified alkaloids or triterpenes) can switch from mild suppression (anti-inflammatory/regulatory) to stimulation (immune activation) depending on concentration (Yahaya *et al.*., 2022). The initial drop might suggest a mild immunoregulatory effect, while the recovery at the high dose directly correlates with the observed lymphoid activation

in the spleen and Kupffer cell stimulation in the liver (Smith *et al.*., 2022). This histological evidence confirms that the 200mg/kg dose initiated a robust local immune and filtration boost, overriding any initial systemic suppression.

The subacute administration of the *Annona muricata* extract significantly increased the Mean Platelet Volume (MPV) and Platelet Larger Cell Ratio (P-LCR) at both 100mg/kg and 200mg/kg doses compared to the control. These parameters are indicators of platelet morphology and activity, and their elevation suggests the presence of larger, more reactive platelets in circulation. MPV is considered an indicator of the average size of circulating platelets. P-LCR represents the percentage of large platelets. A notable increase in these indices, even as the total Platelet (PLT) count remained stable or slightly dropped (as seen at 100mg/kg), often reflects increased platelet production, activation, or turnover. While MPV and P-LCR elevation are sometimes associated with cardiovascular risk, the simultaneous observation of beneficial effects suggests a more nuanced outcome. These beneficial effects include the vasodilation observed in the heart and kidney upon histopathological assessment. Furthermore, the extract showed pronounced hypolipidemic effects, evidenced by the significant reduction in LDL and Total Cholesterol at 100mg/kg ($p<0.05$). These findings suggest the extract's overall influence may lean toward cardiovascular benefit rather than risk at the tested doses. The changes in platelet morphology may also be linked to the extract's confirmed content of phenolics, which possess known vascular protective properties.

Kidney function markers showed more notable changes, particularly an increase in serum creatinine at 200 mg/kg ($p<0.05$), while Urea and electrolytes remained stable. This result is consistent with studies that report mild renal effects at high concentrations of soursop extract (Gbogbo *et al.*., 2021; Zubaidi *et al.*., 2023). The rise in creatinine suggests a functional

disturbance, specifically a minor impairment of Glomerular Filtration Rate (GFR}). The absence of change in Urea and electrolytes is the primary point of differentiation; it indicates the functional disturbance is not severe enough to induce overt renal failure (which would cause electrolyte and urea imbalances). The cause is likely attributed to the annonaceous acetogenins acting as metabolic inhibitors at high doses, impacting renal cell transport or filtration dynamics, even in the absence of observable structural damage (Zubaidi *et al..,* 2023). These trends warrant further chronic toxicity studies, as supported by the recommendations of Onwuka *et al.*. (2021).

Liver enzyme markers were significantly altered, but in a beneficial direction. AST **(**$p<0.0001$**)** and ALT **(**$p<0.001$**)** concentrations significantly decreased at the 200mg/kg dose. These results strongly align with recent studies demonstrating the hepatoprotective potential of *A. muricata* (Uwuigbe *et al..,* 2022). (Uwuigbe *et al.*. 2022) found that soursop extract protected against chemical-induced liver injury, confirming that the extract's antioxidant phytochemicals stabilize hepatocyte cell membranes. The fact that AST and ALT decreased below control levels suggests a prophylactic or restorative effect on baseline hepatic function, likely driven by the potent phenolic content identified in the phytochemical analysis. The stable Total Bilirubin further assures that the extract does not impede the liver's conjugating or excretory pathways, concluding the overall hepatic safety.

Total Cholesterol (T-CH) and Low-Density Lipoprotein (LDL) were significantly reduced at 100mg/kg ($p<0.05$), but this effect was lost at 200mg/kg, where levels returned to baseline. This biphasic dose-response is a vital discovery. Studies confirming the hypolipidemic action of *A. muricata* often use a single dose or a dose range that may not capture this effect window (Ogunlade *et al..,* 2022). These findings suggest that 100mg/kg represents the optimal therapeutic dose for lipid regulation. The loss of effect at 200mg/kg may be due to complex saturation, competition

between different bioactive compounds, or the high dose triggering counter-regulatory metabolic pathways that stabilize cholesterol synthesis or absorption. This result is crucial for guiding future clinical formulation and dose optimization.

The histopathology served as the definitive validation of the organ-specific toxicity noted biochemically, overwhelmingly confirming structural integrity while revealing modulatory effects.

The architecture of the liver, heart, kidney, and uterus was structurally preserved and normal in treated groups, directly refuting any suspicion of gross organ necrosis or severe tissue-level pathology (Adeyemi *et al.*., 2024; Zubaidi *et al.*., 2023). The widespread observation of vasodilation and increased blood circulation/active congestion in the Heart, Liver, and Kidney provides the histological explanation for the extract's traditional use as an antihypertensive and cardiotonic (Rahman *et al.*., 2022). This vasoactive effect likely accounts for the improved renal perfusion observed, even counteracting the functional Creatinine rise, and supporting the beneficial drops in AST and ALT through enhanced hepatic blood flow (Suleiman *et al.*., 2023).

The finding of mild bronchiolar ulceration at higher doses is an important cautionary result. It suggests localized irritation, possibly due to high concentrations of volatile or cytotoxic phytochemicals interacting with the delicate respiratory epithelium (Jones *et al.*., 2023). This isolated structural finding aligns with the need to avoid the 200mg/kg dose for general use.

## CONCLUSION

In conclusion, the subacute administration of ethanol leaf extract of *Annona muricata* in Wistar rats demonstrated a generally safe profile, with no evidence of severe organ toxicity or hematological disturbances. The extract maintained normal liver and kidney function at lower doses while exhibiting hepatoprotective and lipid-lowering effects, particularly at 100 mg/kg. Although mild renal and respiratory changes were noted at higher doses, these effects were not

structurally degenerative. Overall, the findings suggest that *A. muricata* possesses beneficial physiological properties and is relatively safe within the studied dose range, supporting its traditional use while emphasizing the need for dose optimization and chronic toxicity evaluation.

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