REPRODUCTIVE TOXICOLOGICAL EFFECTS OF AQUEOUS EXTRACT OF *Acanthus montanus* (Nees) T. Anderson IN WISTAR RATS.

BY

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PG/LSC1605837

DEPARTMENT OF PLANT BIOLOGY AND BIOTECHNOLOGY

FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN

BENIN CITY.

SEPTEMBER, 2023.

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A PROJECT REPORT WRITTEN AND SUBMITTED TO THE DEPARTMENT OF PLANT BIOLOGY AND BIOTECHNOLOGY, FACULTY OF LIFE SCIENCES, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (M.SC.) OF PHYTOMEDICINE, UNIVERSITY OF BENIN, BENIN CITY, NIGERIA.

SEPTEMBER, 2023.

CERTIFICATION

This is to certify that this project work was carried out by **Paul Oborogheneruru OJOBA** in the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.

…… ……

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Prof. H. O. Shittu **Date**
(Postgraduate Coordinator)

…… ……..

(External examiner) **Date**

DEDICATION

This work is dedicated to the Almighty God.

ACKNOWLEDGEMENTS

I want to start by expressing my sincere gratitude to God for giving me the courage, knowledge, and resolve to take on this challenge. Throughout this journey, your grace has served as a consistent source of inspiration and direction for me.

I sincerely thank my supervisor, Prof. MacDonald Idu, for his unwavering guidance, insightful feedback, and mentorship. His expertise and support have been instrumental in shaping the direction and quality of this project. I am also thankful to Prof. D. E Vwioko, the Head of the Department, for his continuous support and for creating an environment conducive to learning and research. My appreciation also extends to Prof. H. O Shittu, the Postgraduate Coordinator, for his assistance, patience, and understanding throughout my postgraduate studies.

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To my parents, your unwavering love, encouragement, and sacrifices have been the foundation of my achievements. I am eternally grateful for your unwavering support.

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ABSTRACT

Acanthus montanus, commonly referred to as "beer's breech," "alligator plant," or "mountain thistle," is a potent medicinal plant that belongs to the Acanthaceae family. It holds significant importance in ethnomedicine. Throughout Nigeria's history, it has been utilized for managing a diverse array of health issues including wounds, gonorrhea, heart failure, and more. *Acanthus montanus* is rich in phytochemicals, including alkaloids, saponins, flavonoids, tannins, glycosides, and terpenoids. These compounds exhibit diverse biological and pharmacological properties, encompassing analgesic, anti-inflammatory, immunological, anti-fertility, antidiabetic, hepatoprotective, and hepatocurative activities.

This study investigated the reproductive toxicological effects of aqueous leaf extracts of *Acanthus montanus* on male and female Wistar rats. Extracts were administered at varying doses (200 mg/kg, 400 mg/kg, and 800 mg/kg) and 0.5 ml of distilled water as control. The body and organ weights (testes, penis, uterus, ovaries) demonstrated no significant deviations from the control group. Toxicological assessments revealed no adverse impacts on lipid metabolism, as evidenced by lipid profile assays. Hormonal analyses affirmed that the extracts maintained endocrine equilibrium, with hormone levels within normal ranges in all make treated groups, while the female groups exhibited varying level of fluctuations in their hormonal levels. Antioxidant assays disclosed noteworthy antioxidant effects, particularly at the highest dose (800 mg/kg), reflecting the potential of *Acanthus montanus* extracts to combat free radicals and uphold cellular integrity. Histological evaluation of reproductive organs unveiled no notable structural changes, indicating the extracts' non-induction of tissue damage or morphological aberrations. In conclusion, aqueous leaf extracts of *Acanthus montanus*, across various dosages, exhibited negligible impact on body and organ weights, reflecting safety. Moreover, they displayed antioxidant properties without compromising reproductive health or lipid metabolism. This underlines the promising prospects of *Acanthus montanus* extracts as natural antioxidants in the realms of reproductive health and oxidative stress management. While these findings are encouraging, further research across diverse animal models and potentially human subjects is imperative for a comprehensive understanding of the plant's benefits and mechanisms.

CHAPTER ONE

INTRODUCTION

1.0 MEDICINAL PLANTS

Some plants are often referred to as medicinal plants because they are known to be useful in the prevention or treatment of ailments; they are not frequently or indiscriminately consumed as their non-medicinal counterparts, as they may cause harm, because they are regarded as "reservoirs" of crude drugs, with an unspecified dosage and method of administration (Schulz *et al*., 2001). They can also be found as wild plant species that grow naturally and exist without the intervention of humans, or as domestic species that result from deliberate and careful human operations such as breeding, selection, and subsequent management (Calixto, 2000). They are the world's most abundant bioresource of traditional and modern medicines, nutraceuticals, food supplements, folk remedies, pharmaceutical intermediates, and chemical entities for synthetic pharmaceuticals (Nafiu *et al.*, 2017). *Acanthus montanus,* which belongs to the Acanthaceae family, stands out as one of these distinctive medicinal plants.

The Acanthaceae is a big flowering plant family with over 4300 species and 346 genera worldwide (Mabberley, 2008). Most of these taxa are tropical shrubs, herbaceous plants, and climbing vines, with certain representatives exhibiting epiphytic growth forms, contributing to its recognition as one of the twelve most diverse families within the realm of flowering plants. Its primary distribution hubs encompass tropical, subtropical, and temperate zones in Indonesia and Malaysia, the African continent, Brazil, and Central America, with occasional occurrences within Asian territories. *Acanthus* (family Acanthaceae) is a genus of flowering plants belonging to the major group angiosperms (flowering plants), with more than 29 species found in the tropical and subtropical regions of the world, including *Acanthus montanus* (Mabberley, 2008).

1.1 JUSTIFICATION OF STUDY

The investigation into the reproductive toxicological effects of natural compounds is of significant importance due to their potential impact on human and animal health. *Acanthus montanus*, a plant indigenous to certain regions, has been traditionally used for its medicinal properties. However, there has been only minimal research conducted to comprehensively understand the potential effects *of Acanthus montanus* leaf aqueous extract on the reproductive system. This study seeks to address this gap by examining the reproductive toxicological effects of *Acanthus montanus* leaf aqueous extract on male and female Wistar rats.

1.2 AIM OF STUDY

The study was aimed at evaluating the reproductive toxicological effect of *Acanthus montanus* leaves in Wistar rats.

1.3 OBJECTIVES OF STUDY

The aims of this research encompassed several key objectives, which were to:

- evaluate the effects of *Acanthus montanus* leaf aqueous extract on the body and organ weight in Wistar rats.
- determine the effects of *A. montanus* leaf aqueous extract on the lipid profile of Wistar rats
- investigate the effects of *A. montanus* leaf aqueous extract on hormonal levels in Wistar rats
- evaluate the *in vivo* antioxidant properties of *A. montanus* leaf aqueous extract in Wistar rats.
- evaluate the histopathological effects of *A. montanus* leaf aqueous extract in Wistar rats.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Acanthus montanus* (Nees) T. Anderson

2.1.1 Origin and Geographical Distribution of *Acanthus montanus*

The genus "*Acanthus*" is derived from the Greek word *"akantha"* meaning spine and refers to the toothed edges on some species' leaves, while the specific epithet *"montanus"* means pertaining to mountains. *Acanthus* is a hardy perennial with attractive lobed foliage and tall, erect racemes of two-lipped flowers with bright bracts. (Burkill, 1985).

Acanthus montanus (Nees) T. Anderson, belonging to the family Acanthaceae, is a diminutive shrub characterized by its limited branching and a pliable stem. It is widely distributed across diverse geographical regions, including Africa, the Balkans, Romania, Greece, and the Eastern Mediterranean, and is commonly referred to as "Bear's breeches, mountain thistle, or alligator plant". It is known in Edo as àgámobo, in Igbo as àgámeebu or ogwudurun-washishi, in Ijaw as èdulèè memen, and in Yoruba as ahun ékùn, and in Germany as *Gebirgs-acanthus*. In some areas, it is extensively available in some parts of Europe and Africa. (Burkill, 1985).

2.1.2 Botanical description of *Acanthus montanus*

Acanthus montanus is a perennial herb with thinly branching basal clusters of glossy, dark green leaves that are oblong to lance-shaped. It grows up to 30 cm in length, 180 cm tall, and 61 cm in, with silver marks and wavy borders on the leaves. It enjoys shady areas with deep watering on occasion, although it may also endure hot, dry conditions. The foliage displays serrated margins adorned with diminutive spines, featuring leaves that range from shallow to deeply lobed in structure. The upper leaf surface is characterized by a dark, glossy green coloration, while the lower leaf surface exhibits a pale green hue. The plant produces elongated, upright spikes bearing pink to reddish-hued flowers. It's great for slopes because of

its aggressive roots. They are found in the high forests and are geographically scattered over Africa and parts of the world. (Dressler *et al.*, 2014).

2.1.3 Taxonomic classification

Kingdom – Plantae

Division – Tracheophyta

Class – Magnoliopsida

Order – Lamiales

Family – Acanthaceae

Genus – *Acanthus*

Species – montanus

Binomial name: *Acanthus montanus* (Nees) T. Anderson

Source: GBIF, 2021.

Plate 2.1: *Acanthus montanus* plant

2.1.4 Folkloric uses of *Acanthus montanus*

Acanthus montanus has been used traditionally for several therapeutic purposes; In Nigeria, the paste of the young twigs with sugar has been applied to hasten suppuration, decoction of the young leaves and twigs has also been taken for indigestion and as vermifuge and emmenagogue. The leaves are taken by post-natal mothers in southern Nigeria to ensure health and vitality. The roots are used for the treatment of furuncles, leaves for boils on the fingers, and also for cough. (Igoli *et al.,* 2005). The central segment of both the branches and leaves is administered in the form of a heated poultice to treat fully developed abscesses. Additionally, leaves that have undergone decoction are utilized as a laxative and are also

employed in the therapeutic management of conditions such as gonorrhea, syphilis, wounds, and boils. Also used for treating cardiac dysfunctions and hepatitis. (Okenwa and Jude, 2014).

The leaves together with *Ananas comosus* and *Costus spp*. are crushed in water and used to treat urogenital infections, urethral discomfort, endometritis, urinary illness, cystitis, and leucorrhoea in the Democratic Republic of Congo (Didie, 2005). Bathing with the roots might help reduce aches and pains (Ibe and Nwufo, 2005). The leaves or foliage of the plant have been reported to exert spasmolytic, analgesic, anti-inflammatory, and antipyretic activities, according to documented pharmacological reports. It has been established that saponins and the gammaceranes- acanthusol and its 3-O—D-glucopyranoside (Anam, 1997) have been isolated from the plant. In Nigeria, the Igede people of Benue State (Igoli *et al*., 2004) and the Enugu-Ezike community of Enugu State employ the root poultice to treat furuncles. The furuncle, commonly known as a boil, is the most frequent type of abscess, and pyogenic organisms including *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been linked to it (Sleigh *et al.,* 2001). The root poultice is applied to hasten "boil ripening" in local use, which is a lay term for increased pus formation in a boil, which is thought to signify that the illness has been overcome.

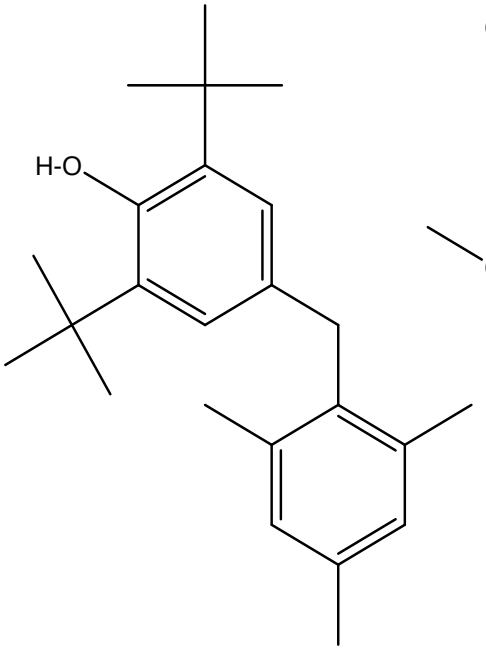
2.1.5 Phytochemistry of *Acanthus montanus*

Phytochemistry is coined from the word “Phytochemicals”. Hence it is the study of phytochemicals. They are biologically active compounds present in plants that are derived from the roots, leaves, stems, flowers, fruits, and seeds. They emerge as byproducts of both the primary and secondary metabolic pathways within the plant. These compounds hold significant importance in facilitating the plant's growth and development, as well as in deterring other plants, animals, insects, and microbial pests and pathogens. They also help

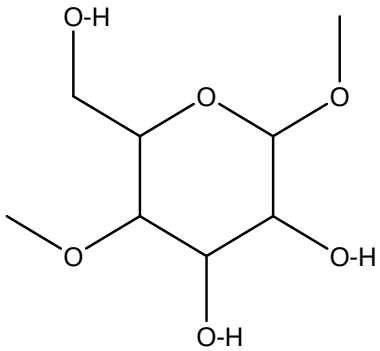
plants and protect them from disease and damage caused by environmental hazards like pollution, UV, stress and draught. (Vishnu *et al.*, 2019)

The phytochemical screening of the root extract of *Acanthus montanus* yielded an abundance of alkaloids, flavonoids, steroids, carbohydrates, saponins, tannins, glycosides, and terpenoids. (Odoh and Ezugwu, 2013 ; Orakwue *et al.*, 2012 and Okoli *et al.*, 2008).

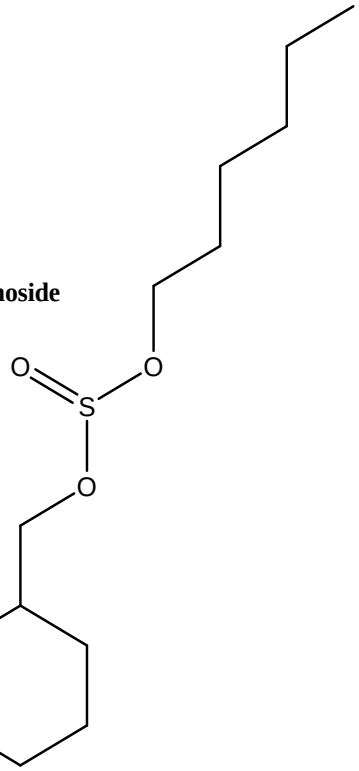
(Okenwa & Jude, 2014) reported that the Gas chromatography-mass spectrometry (GC-MS) analysis of ethanol leaf extract yielded nine compounds; 2,6-bis(1,1-dimethylethyl)-4-methyl phenol (13.68 %), alpha-methyl 4-methylmannoside (8.41 %), sulfurous acid cyclohexylmethylhexyl ester (5.67 %), allyl(2-tetrahydrofuryl methoxy)dimethylsilane (3.86 %), N,N-dimethylvaleramide (18.62 %), hexadecanoic acid methyl ester (16.12 %), 11-octadecenoic acid methyl ester (19.03 %), docosane (5.85%) and 2,6,10,15-tetramethyl heptadecane (8.76%). As shown in figures 1a and 1b.



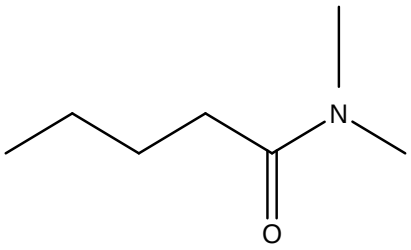
[1] 2,6-bis (1,1-dimethylethyl)-4-methyl phenol



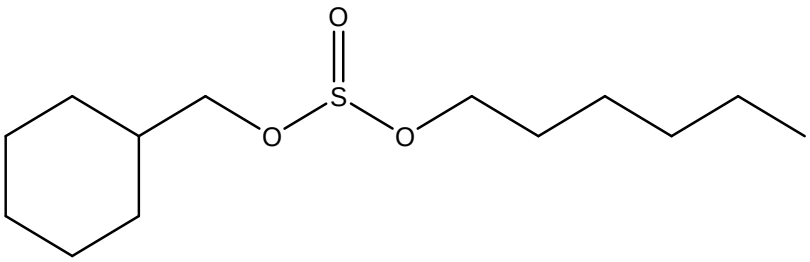
[2] Alpha-methyl 4-methylmannoside



[3] Sulfurous acid cyclohexylmethyl hexyl ester

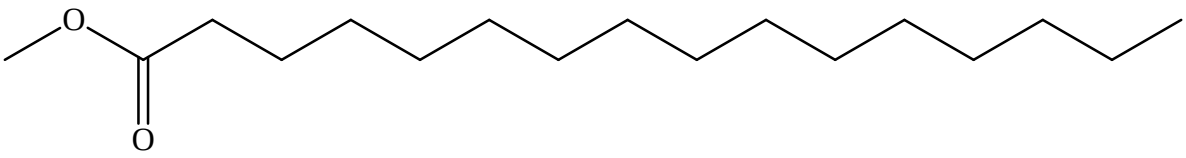


[5] N,N-Dimethylvaleramide

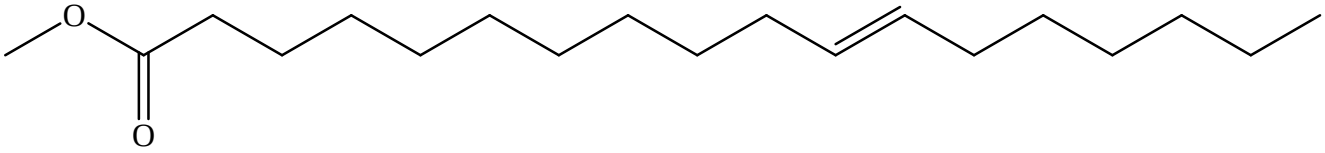


[4] Allyl (2-tetrahydrofurylmethoxy) dimethylsilane

Figure 1.1a: Chemical compounds obtained from the GC-MS analysis of *Acanthus montanus*

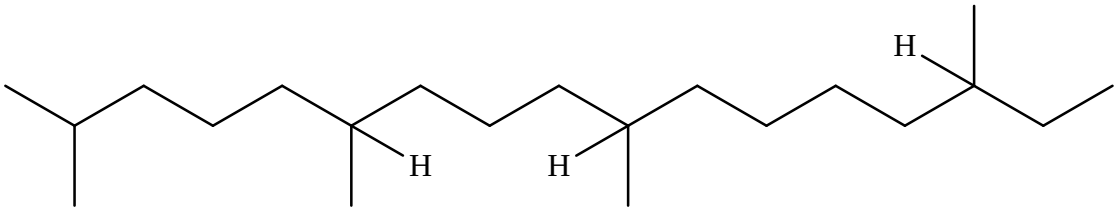


[6] Hexadecanoic acid methyl ester



[7] 11-Octadecenoic acid methyl ester

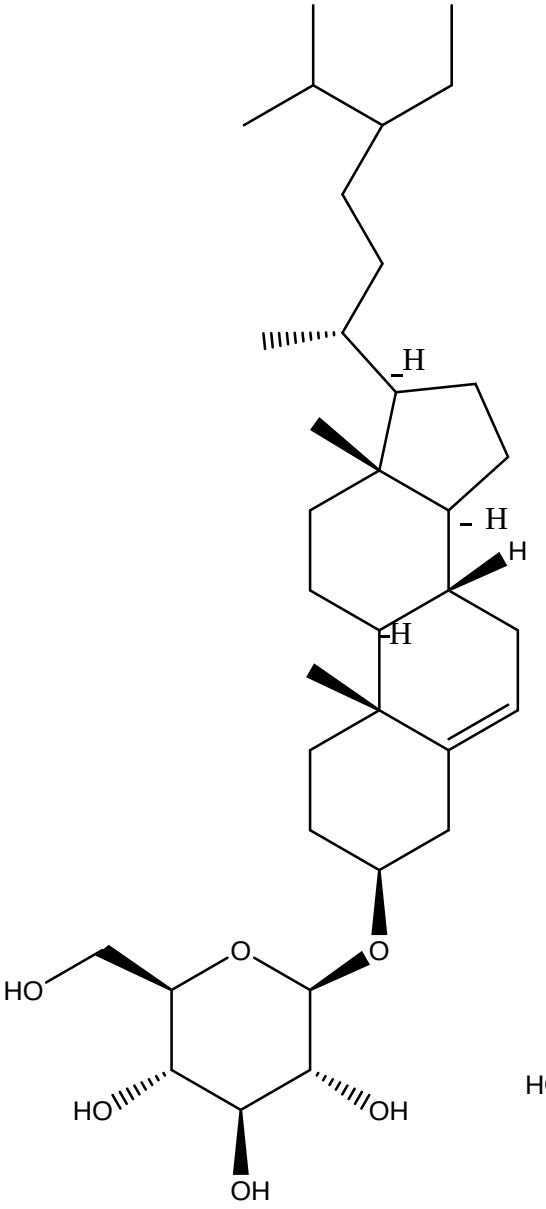
[8] Docosane



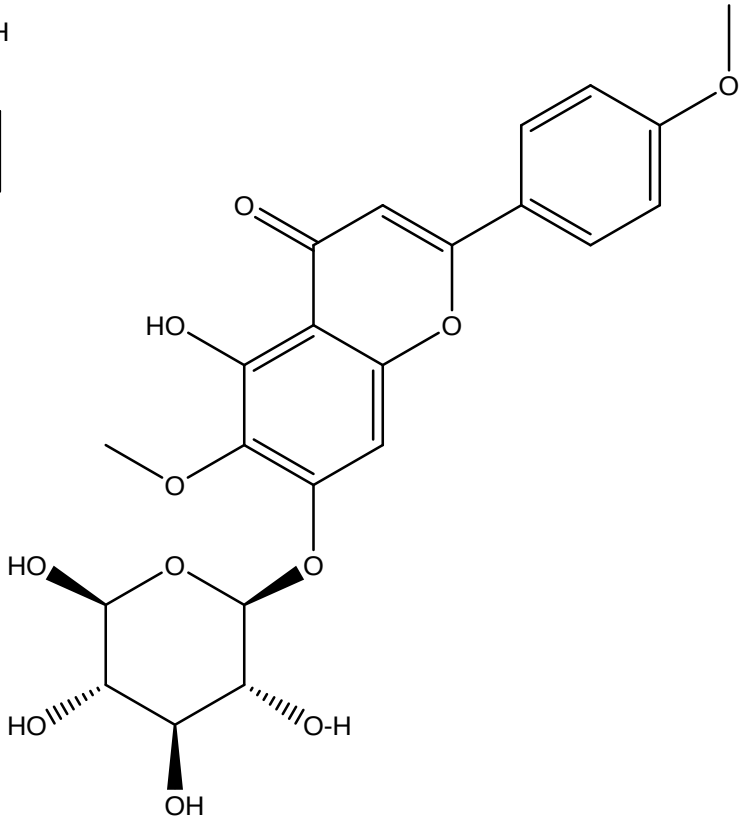
[9] 2, 6, 10, 15-Tetramethylheptadecane

Figure 1.1b: Chemical compounds obtained from the GC-MS analysis of *Acanthus montanus*.

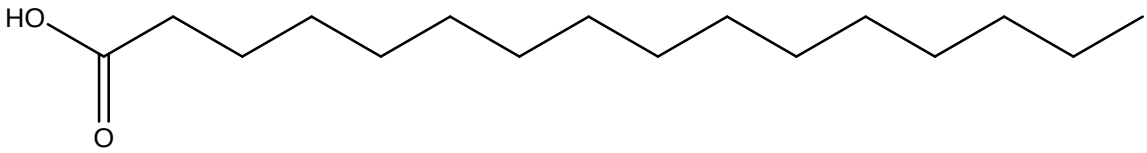
Further studies on the alcoholic extract of the aerial parts yielded nine compounds; β-sitosterol-3-O- β –D-glucoside, palmitic acid, linaroside, shikimic acid, protochatecuic acid, homoplantagenin, blepharin and acetoside as shown in figures 1.2a, b, and c (Elham *et al.*, 2012).



[1] β-sitosterol-3-O-β-D-glucoside

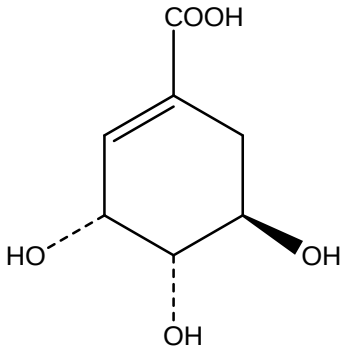


[3] Linaroside

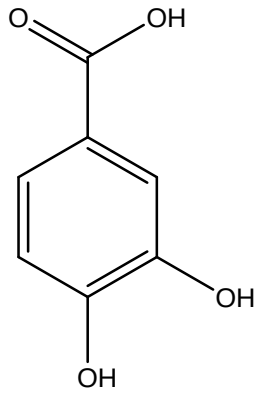


[2] Palmitic acid

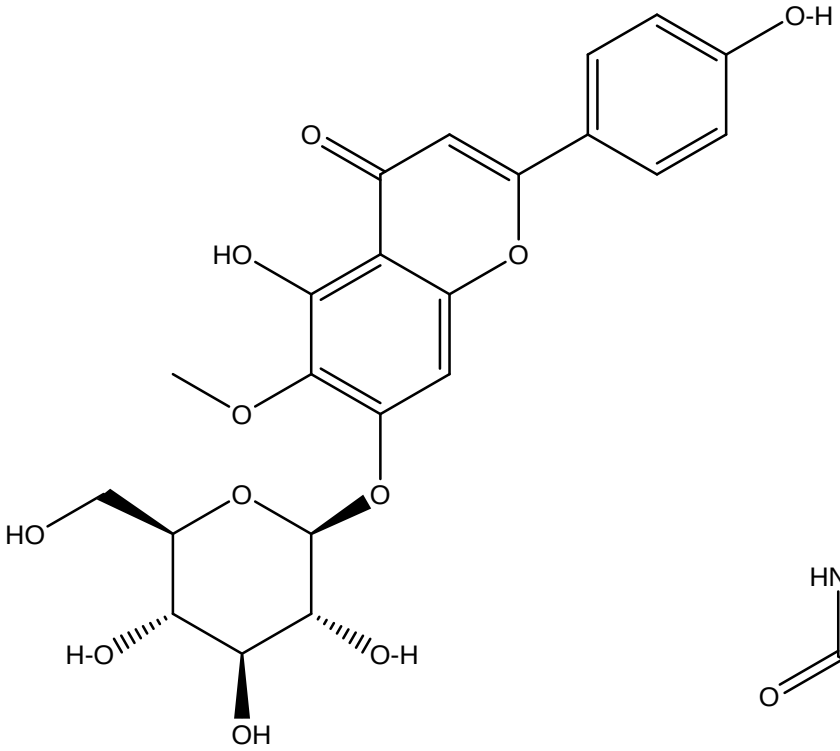
Figure 1.2a: Chemical compounds obtained from the alcohol extract of the aerial parts of *Acanthus montanus*.



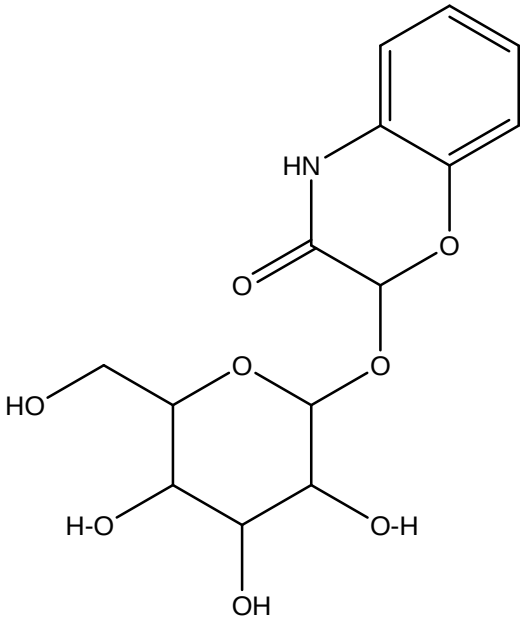
[4] Shikimic acid



[5] Protocatechuic acid

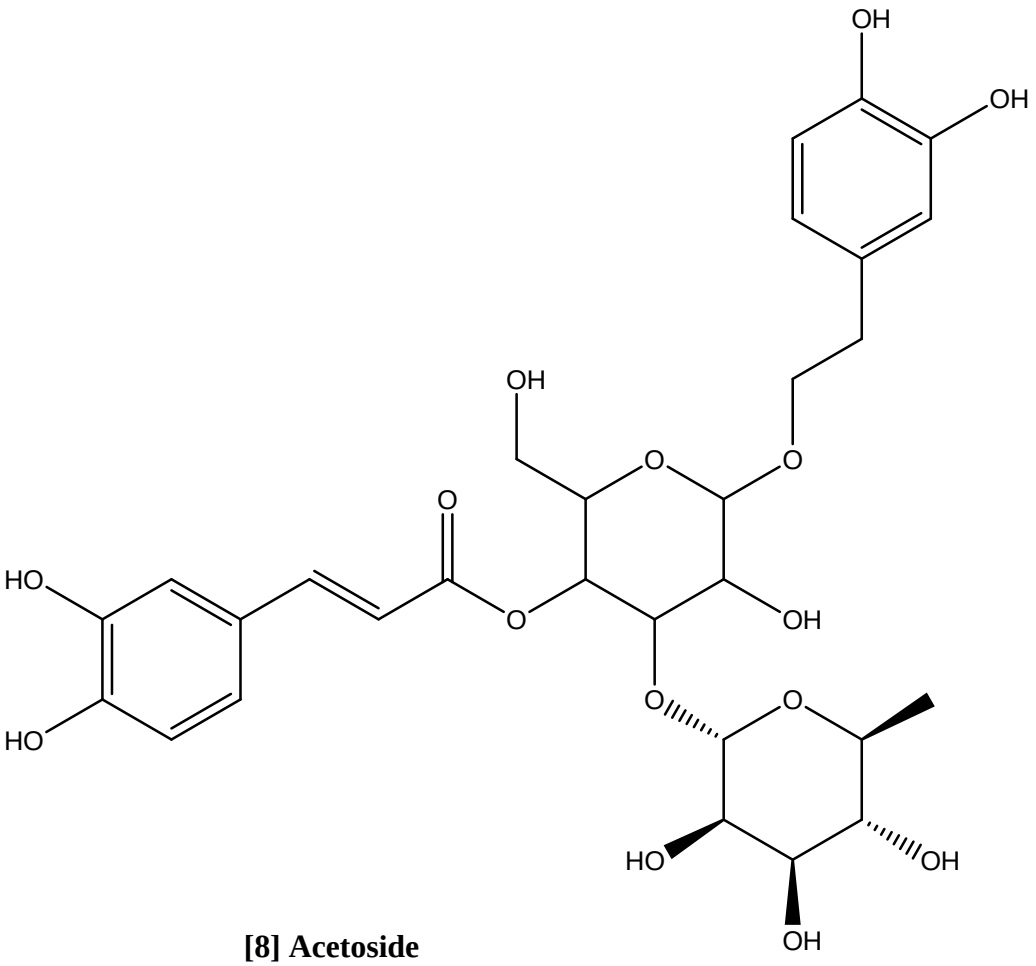


[6] Homoplantagenin



[7] Blepharin

Figure 1.2b: Chemical compounds obtained from the alcohol extract of the aerial parts of *Acanthus montanus*



[8] Acetoside

Figure 1.2c: Chemical compounds obtained from the alcohol extract of the aerial parts of *Acanthus montanus*

2.1.6 Reported Biological Activities of *Acanthus montanus*

Idu *et al.* (2022) reported the diverse folkloric benefits of *Acanthus montanus* parts that have been scientifically demonstrated and justified, some of these include:

Analgesic study

The analgesic properties of the methanol leaf extract of *A. montanus* were evaluated using a variety of pain assessment methods in both rats and mice. For rats, the cold-water tail flick test was employed, while for mice, the tail immersion, tail clip, acetic acid-induced writhing, and formalin pain tests were conducted. The results revealed a noticeable and statistically significant ($p < 0.05$) increase in pain tolerance 60 minutes after treatment with doses of 200 and 400 mg/kg of the extract in the tail flick, tail immersion, and tail clip assays. Importantly, it should be noted that the effects observed with the extract in these tests were considerably lower ($p < 0.05$) than those observed with morphine (10 mg/kg). Furthermore, the administration of the extract at doses ranging from 100 to 400 mg/kg demonstrated a dose-dependent reduction in writhing and significantly ($p < 0.001$) suppressed both phases of the formalin pain test. However, the first phase displayed a weaker impact compared to the second phase. These results suggest that the analgesic effect of the *Acanthus montanus* methanol extract operates through modulation of both the central and peripheral nervous systems (Adeyemi *et al.*, 2004).

Anti-microbial, anti-inflammatory, and immunological activities

Experiments were conducted to test the antimicrobial, anti-inflammatory, and immunological effects of a furuncle treatment used in ethnomedicine. The aqueous root extract exhibited moderate antibacterial effects against prevalent pathogens found in boils, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The *A. montanus* aqueous root extract

considerably suppressed the development of the microorganisms and significantly decreased topical acute edema in the mouse ear (57%). It effectively suppressed the formation of sudden paw edema in rats, and this inhibition was not influenced by the dosage used. but was ineffective in decreasing the vast edematous response to formaldehyde arthritis. It also prevented acetic acid-induced vascular permeability in rats, as well as heat and hypotonicity-induced hemolysis of ox RBCs. At the 800 mg/kg dose, it also raised the leukocyte and neutrophil total counts, as well as a substantial dose-related rise in the total number of macrophages. At 800 mg/kg dose, the extract considerably increased the number of macrophages with ingested *Candida albicans* and significantly) prevented delayed hemolytic transfusion responses (DHTR) in a dose-dependent manner. Alkaloids and sugars were found in abundance in the extract, but saponins, glycosides, and terpenoids were only found in negligible levels. An oral and intraperitoneal LD_{50} of more than 5000 mg/kg was determined in an acute toxicity test. The extract increased the total leukocyte and neutrophil counts and caused a significant increase in macrophages. (Okoli *et al*., 2008).

Anticonvulsant evaluation

Acanthus montanus, Alchornea laxiflora, Hyptis spicigera, Microglossa pyrifolia, Piliostigma reticulatum, and *Voacanga africana,* were evaluated for their anticonvulsant and sedative effects using different mouse models. These models induced convulsions or altered behavior through methods like maximal electroshock (MES), N-methyl-D-aspartate (NMDA), pentylenetetrazol (PTZ), isonicotinic hydrazide acid (INH), picrotoxin (PIC), and strychnine (STR), and they also assessed diazepam-induced sleep. *Acanthus montanus* demonstrated protective effects in 66.6% of mice against MES, PIC, and STR-induced convulsions, as well as 83.3% protection against PTZ-induced convulsions. *Alchornea laxiflora* provided protection in 75% and 87.5% of mice in the STR and NMDA tests, respectively, at a dose of 120 mg/kg. *Hyptis spicigera* offered complete protection in 100% of mice against STR-

induced convulsions and 87.5% protection against PTZ-induced convulsions at a dose of 160 mg/kg. *Microglossa pyrifolia* exhibited protection ranging from 50% to 100% of mice against convulsions. *Piliostigma reticulatum* showed protection in 62.5% to 100% of mice against convulsions and turning behavior. *Voacanga africana* demonstrated protection in 62.5% to 87.5% of mice against convulsions and turning behavior. All of the plants, except *A. laxiflora,* also had sedative properties, significantly increasing the total duration of diazepam-induced sleep (Bum *et al*., 2009).

TOXICITY STUDIES

The study evaluated the effects of *A. montanus* extract on both male and female albino rats. A total of 90 rats, comprising 45 males and 45 females, were divided into 18 groups, each consisting of five rats. To differentiate between male and female rats, alphabetic and numerical identifiers were used. The control groups, labelled as A and 1, were provided with regular rat chow. The experimental groups, labelled as B and 2, C and 3, D and 4, E and 5, received different doses (200, 400, 600, and 800 mg/kg) of the aqueous extract of *A. montanus* leaves. Similarly, groups F and 6, G and 7, H and 8, I and 9, were treated with different doses (200, 400, 600, and 800 mg/kg) of the methanol extract of *A. montanus*. The results from the test groups indicated a significant increase that was dose-dependent in liver enzyme levels, along with significant decreases in total protein and albumin levels. However, no significant differences in total and direct bilirubin levels when compared to the untreated group. Creatinine levels showed a significant increase in the test groups, while urea levels decreased. Furthermore, histopathological examination of the liver and kidney tissues in the groups administered 800 mg/kg of aqueous extract and 400, 600, and 800 mg/kg of methanol extract revealed mild to widespread abnormalities. With increased dosage, *A. montanus* leaves

may cause renal and hepatic impairment; therefore, they should be detoxified before use. (Iwueke *et al*. 2021).

Acute Toxicity Study

The root extract has an oral and intraperitoneal LD_{50} of more than 5000 mg/kg in an acute toxicity investigation (Okoli *et al.* 2008). It was also reported that the aqueous leaf extract of *Acanthus montanus* is non-toxic when given orally and acutely at therapeutic dosages (< 200 mg/kg). The oral acute toxicity of *A. montanus* aqueous leaf extract on Wistar rats was investigated by Paulin *et al.*, (2007) where the rats were given single doses of 0, 500, 1000, 2000, 4000, and 8000 mg/kg and then examined for 7 days for behavioural changes and mortality before being euthanized. At doses up to 4000 mg/kg, the results demonstrated that a single oral dose of aqueous extract did not cause significant changes in general behaviour or death. Animals given 8000 mg/kg dosages had a diminished reactivity to pinch, and their water and food intakes were significantly reduced. The relative organ weights and body weight growth were not considerably different. In comparison to the control, serum total proteins and transaminase activities increased significantly in rats given 8000 mg/kg, but serum total proteins and transaminase activities remained unchanged. Histological investigation revealed no disease in the liver or lungs, but the 8000 mg/kg treated rats' kidneys showed degenerative alterations, which were confirmed by a rise in creatinine. The results indicated that the plant did not exhibit any harmful effects in rats when administered at doses commonly used by the general population.

Sub-acute toxicity

The aqueous extract of *Acanthus montanus* was tested for sub-acute toxicity in female Wistar rats at doses of 0, 125, 250, 500, and 1000 mg/kg/day for 30 days and found to have no effect on hematological, biochemical, or oxidative stress markers. However, nephrotoxic and

hypercreatinine effects were detected at dosages more than 500 mg/kg, which is below the nominal human value. (Djami *et al*., 2011).

Safety / Embryotoxicity studies

Nana *et al*., (2008) investigated the effects of methanol/methylene chloride leaf extract from *A. montanus* on pregnant Wistar rats and identified the substance(s) required for these effects. The animals were given doses of 0, 250, 500, and 1000 mg/kg orally from days 6 to 15 of pregnancy (kg day). On day 20, they were either sacrificed or permitted to deliver and nurture. Several factors were evaluated. Multiple parameters were evaluated as the F1 generation offspring were allowed to produce the F2 generation. There was no toxicity in the mother or the organs, according to the findings. Embryotoxicity was seen during organogenesis as a decrease in fetal body weight, crown-rump, and tail lengths, and decreased ossification of extremity bones. However, these indicators of growth retardation were visible before day 5, and the treated pups' parameters returned to normal after delivery. For the F_1 and F_2 generations, all other factors were negligible. The extract's main chemical component was sitosterol, and its significance in these findings could not be overstated. This plant's MeOH/CH_2Cl_2 extract is embryotoxic at high dosages during pregnancy and a year after, although this did not appear after 5 days of post-natal survival. Sitosterol could potentially have a significant impact on the extract's effects, thus making it suitable for pregnant patients.

Antifertility / Fetotoxic activities

The estrous cycle of Wistar rats was observed before, during, and after oral administration of distilled water (control) and *A. montanus* aqueous extract (62.5, 125, 250, 500, and 1000 mg/kg/day). The pregnant rats were subjected to the extract dosage either during the initial 1 to 6 days (pre-implantation) or from day 6 to 15 (post-implantation) of gestation, and they were euthanized on either day 8 or 20 of pregnancy. In addition, ovariectomized rats were

administered the aqueous extract at doses of 500 and 1000 mg/kg/day, either with or without externally administered estrogen and/or progesterone. Subsequently, uterine weight and decidua count were assessed. The extract exhibited a reversible delay in the metestrous stage and, in rare instances, the diestrous stage of the estrous cycle, regardless of the dosage. However, it had no discernible effect on uterine wet weight or decidua count, indicating an absence of both estrogenic and progestational properties. Notably, the extract led to pre-implantation losses of 36.8 ± 6.5% ($p < 0.05$) at 1000 mg/kg/day, but none of the doses resulted in post-implantation losses. The extract also caused fetal growth to be slowed. (Asongalem *et al.,* 2008).

Hepatoprotective study

(Patrick-Iwuanyanwu & Wegwu 2008) revealed that the ethanol and aqueous extracts of the leaf and stem of *A. montanus* may prevent liver damage induced by Carbon tetrachloride (CCl_4) in rats.

Uroko *et al.* (2019) explored the potential hepatocurative effects of the methanol extract obtained from *Acanthus montanus* leaves in mice suffering from acetaminophen-induced liver failure. Their research findings demonstrated that the extract did not result in any deleterious reactions or fatalities among the mice. Additionally, the extract effectively reduced the enzymatic activities such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), along with reducing total bilirubin concentrations in rats afflicted with acetaminophen-induced liver failure. Moreover, it notably increased concentrations of total protein, albumin, and direct bilirubin, and also contributed to the improvement of liver histomorphology, all of which had been adversely affected by acetaminophen toxicity. These research findings underscore the hepatocurative potential of

the methanol extract derived from *A. montanus* leaves, highlighting its ability to reverse liver failure and alleviate the detrimental hepatotoxic effects.

Relaxant activity

The effects of *A. montanus* methanol extract on several smooth muscle preparations were investigated in this study. The extract induced relaxation in the rabbit jejunum and inhibited spontaneous contractions in a manner that depended on its concentration. In the case of the guinea pig taenia coli, the extract caused a concentration-dependent rightward shift in the concentration-response curves of CaCl2. The findings pointed to the presence of a non-specific smooth muscle relaxant. (Adeyemi *et al*., 1999).

Anthelmintic efficacy

The *in-vitro* egg hatch and larval growth suppression assays were used to assess the anthelmintic efficacy of crude aqueous leaf extract of *A. montanus* against strongylid nematodes of small ruminants. The parasitological analysis of fecal samples taken via rectum from sheep and goats was carried out using the McMaster counting technique, which yielded 700 eggs per gram (E.P.G.) of feces. With a yield of 13.01 % w/w, the crude aqueous leaf extract of *A. montanus* was extracted by cold water extraction. At a dosage of 25 mg/ml of extract, an egg hatch assay demonstrated a 91.75 % reduction in egg hatch. At a dosage of 200 mg/ml, the extract showed a 100 % inhibitory effect, comparable to 3.125 mg/ml albendazole activity. However, 0 % inhibition was observed in the distilled water control. The extract inhibited larval growth by 67.02 % and 85.26 % respectively, in a larval growth inhibition experiment on day 1 at 25 mg/ml and 200 mg/ml doses. On day 2, all concentrations of the extract produced 100 % inhibition with the exception of 25 mg/ml which produced 88.30 %. On day 2, albendazole, on the other hand, demonstrated 100 % larval suppression at all dosages. On day 3, the 25 mg/ml dosages completely (100 %) inhibited the larvae. At 200

mg/ml, the extract inhibited larval growth by 92.63 % which was comparable to the standard anthelminthic (albendazole) at 12.50 mg/ml (92.28 %). These findings demonstrated that the ethnomedicinal claim of *A. montanus'* anthelminthic action had a pharmacological basis. (Adamu *et al*., 2010).

Anti-diabetic study

Acanthus montanus roots were subjected to methanol extraction, then subsequent fractionation using n-hexane, petroleum ether, ethyl acetate, diethyl ether, and chloroform. In a preliminary investigation, the ethyl acetate fraction (EAF) was evaluated for its effects on alloxan-induced diabetic rats. Preliminary phytochemical analyses and acute toxicity assessments via intraperitoneal administration were conducted in mice. Thin-layer chromatography (TLC) was employed in an attempt to separate the constituents of the EAF.

The anti-diabetic study revealed a significant dose-dependent reduction in blood sugar levels in both normoglycemic and hyperglycemic rats. Intraperitoneal administration of EAF at doses of 100, 200, and 300 mg/kg to alloxan-induced diabetic rats resulted in substantial decreases in blood sugar levels (21.91%, 38.12%, and 49.20%, respectively), albeit lower than the sugar-lowering effect of glibenclamide (51.78%). Similarly, EAF (100, 200, and 300 mg/kg) significantly reduced blood sugar levels in normal rats by 19.20%, 27.80%, and 40.74%, respectively, compared to glibenclamide's effect of 49.94%. Phytochemical analysis revealed the presence of secondary metabolites such as alkaloids, flavonoids, glycosides, steroids, saponins, tannins, and terpenoids in the extract. An acute toxicity test in mice, conducted using Lorke's methodology, demonstrated the safety of the extract at a dose of 5000 mg/kg, with no reported fatalities. Overall, the study's findings suggested that the EAF derived from *A. montanus* roots possesses a significant and dose-dependent hypoglycemic effect in normoglycemic and alloxan-induced diabetic rats. This provides a biological/pharmacological basis for the traditional use of *A. montanus* root in folk medicine

for diabetes treatment. Among the chromatographic solvent systems investigated, chloroform: ethyl acetate (6:4) yielded the best resolution and the highest number of spots in the EAF (Odoh and Ezugwu, 2013).

Anti-bacterial activity

The leaves of *Acanthus montanus* are used to cure gonorrhea, syphilis, wounds, and boils in traditional herbal therapies in the South-Eastern region of Nigeria and other West African countries. Hypertension, cardiac dysfunctions, hepatitis, and heart disorders are all treated with *A. montanus* in Ayurvedic medicine. Using the Gas Chromatography-Mass Spectrometry (GC/MS) technology, the chemical constituents of the ethanol extract of *A. montanus* leaves were analyzed, and nine substances were found, including 2,6-bis(1,1-dimethylethyl)-4-methyl phenol (13.68 %), allyl(2-tetrahydrofuryl methoxy)dimethylsilane (3.86 %), sulfurous acid cyclohexylmethyl hexyl ester (5.67 %), alpha-methyl 4-methylmannoside (8.41 %), hexadecanoic acid methyl ester (16.12 %), 11-octadecenoic acid methyl ester (19.03 %), docosane (5.85 %), N,N-dimethylacetamide (18.62 %) and 2,6,10,15-tetramethyl heptadecane (8.76 %). *Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus, Escherichia coli, Salmonella typhi,* and *Proteus mirabilis* were all susceptible to the extract. The Disc Diffusion Technique was used to determine the susceptibility of each test microorganism to the extract. The presence of these bioactive chemicals in the leaves of *A. montanus* could explain its antibacterial properties as well as its use in Nigerian herbal medicine for the treatment of illnesses and infections. (Okenwa and Jude, 2014).

Effect of Processing on Biochemical Composition of Leaves

The evaluation focused on the biochemical composition of *Acanthus montanus* leaves following various processing methods. The initial analysis of the raw vegetables revealed that

they contained 59.15% moisture, 1.85% crude protein, 2.32% fat, 3.76% fiber, 2.04% ash, and 34.65% total carbohydrates per 100 grams. Additionally, the raw vegetables contained 5.35 g/100 g of saponins, 4.04 g/100 g of alkaloids, 1.10 g/100 g of tannins, 3.53 g/100 g of flavonoids, 2.87 g/100 g of phenols, and 1.27 g/100 g of anthocyanins. The content of essential nutrients such as calcium, magnesium, potassium, vitamin A, vitamin C, and titratable acidity was also determined. Subsequent processing, including boiling or a combination of boiling and sun-drying, resulted in significant reductions ($p < 0.05$) in protein, lipid, fiber, ash, saponins, alkaloids, tannins, phenols, anthocyanins, calcium, magnesium, potassium, vitamin A, vitamin C, and titratable acidity. The boiled samples exhibited increased moisture content but decreased total carbohydrate content, while the boiled + sun-dried samples showed decreased moisture content. However, the total carbohydrate content increased significantly ($p < 0.05$) in the boiled + sun-dried leaves, and the sun-dried vegetables experienced reductions in moisture, saponin, alkaloid, vitamin A, and vitamin C content ($p > 0.05$). There were no significant differences ($p > 0.05$) in lipid, calcium, potassium, and ash levels, but there were significant increases ($p < 0.05$) in protein, crude fiber, total carbohydrates, tannins, flavonoids, phenols, and anthocyanins. It is noteworthy that sun drying 2maintained or enhanced the release of certain bioactive compounds in *A. montanus* leaves. Furthermore, the reduced moisture content and increased titratable acidity in sun-dried vegetables could extend their shelf life by creating unfavorable conditions for microbial growth (Igwe and Eleazu, 2017).

Potent Insecticidal activity

From a phytochemical analysis of an alcoholic extract derived from the aerial parts, nine compounds were identified, with eight of them exhibiting varying levels of insecticidal activity. These compounds were evaluated against female *Aedes aegypti* adults at two different doses: 1.25 μg/mg and 0.63 μg/mg. Among the isolated compounds, β-sitosterol

glucoside (1) and palmitic acid (2) displayed the highest activity, with mortality rates of 100% and 90%, respectively, at a concentration of 1.25 µg/mg. Linaroside (3) exhibited 80% mortality, while acetoside (9) demonstrated 70% mortality. Protochatecuic acid (7) and homoplantagenin (6) had mortality rates of 40% and 30%, respectively, at this concentration. At the lower concentration of 0.63 µg/mg, compounds 1, 2, 3, and 6 still showed significant activity, with mortality rates of 90%, 80%, 70%, and 10%, respectively. In the study, acetone and Permethrin were used as negative and positive controls, respectively. Acetone resulted in 0% mortality, while Permethrin induced 100% mortality at the tested doses. The LD50 value for Permethrin was determined to be 4.9 x 10^-5. It is worth noting that prior research (Rahuman *et al.,* 2000) had reported the larvicidal effectiveness of palmitic acid against *Culex quinquefasciatus, Anopheles stephensi,* and *Aedes aegypti.* However, this study marks the first report of these compounds' adulticidal activity (Elham *et al.,* 2012).

Comparative Hypoglycemic Activities / Synergism of Combinational Formulation

The study was aimed at comparing the potential of crude leaf extracts from *Acanthus montanus* (ACMO), *Asystasia gangetica* (ASGA), *Emilia coccinea* (EMCO), and *Hibiscus rosasinensis* (HIRO), as well as their combinations, in reducing hyperglycemia in Type I diabetic rats. Hyperglycemia was induced by intraperitoneally injecting alloxan monohydrate at a dose of 120 mg/kg body weight in a phosphate buffer saline (PBS) solution with a pH of 7.4. Over a 14-day period, individual hyperglycemic rats (HyGR) received separate doses of ACMO, ASGA, EMCO, or HIRO, as well as their combined formulations (AAEH). Aqueous extracts (AQx) and ethanol extracts (ETHx) of the four herbal samples were prepared using established methods. The fasting blood glucose concentration (FBGC) of the rats, measured using the glucose oxidase spectrophotometric method, was assessed at regular 24-hour intervals for the entire 14-day duration. The results indicated that ETHx from the herbal samples exhibited a greater ability to reduce FBGC in HyGR compared to the aqueous

extracts. Among the combinations, AAEH displayed the highest FBGC reduction, lowering it by 53.55 ± 1.04%, while the AQx of EMCO showed the lowest FBGC reduction at 36.19 ± 0.88%. Notably, ethanol extracts of the herbal samples proved to be more effective than their aqueous counterparts in glycemic control and hyperglycemia treatment. Furthermore, the combination of herbal extracts demonstrated a synergistic enhancement of their therapeutic potential. Hence, the study highlighted the potential of ethanol extracts from these herbal samples in managing hyperglycemia and suggests that combining these extracts could further enhance their therapeutic effects (Ojiako *et al.*, 2015).

Spermatogenic effect

The effect of an aqueous extract of *A. montanus* leaves on spermatogenesis in Swiss albino mice was studied by Orlu and Obulor (2014). The extract enhanced spermatogenic activity reversibly regardless of dose, with 500mg/kg-1b.wt showing the best results. Regardless of the increase in the concentration of *A. montanus* extract, no substantial decrease in body weight or the weight of reproductive organs was observed, and the gonadosomatic index remained constant. The extract's ability to increase spermatogenic indices and overall spermatogenic yield suggests that it could be used as an herbal therapy for spermatogenic malfunction.

Anti-inflammatory activity

This study was aimed at investigating the anti-inflammatory properties of the aqueous extract from *Acanthus montanus* by inducing paw edema in mice using carrageenan. The administration of the extract at doses ranging from 100 to 400 mg/kg orally, diclofenac at 50 mg/kg orally, and the nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) at 100 mg/kg subcutaneously significantly reduced carrageenan-induced paw edema

in mice. Interestingly, at a dosage of 300 mg/kg, L-Arginine, a precursor of nitric oxide, significantly counteracted the anti-inflammatory effects of both the extract (reduction by 76.00%) and L-NAME (reduction by 85.00%). This effect was compared to diclofenac, a non-steroidal anti-inflammatory drug, and L-Nitro arginine methyl ester (L-NAME), a nitric oxide inhibitor. Diclofenac's anti-inflammatory action is unaffected by L-Arginine. These findings show that nitric oxide (NO) inhibition may be responsible for *Acanthus montanus* aqueous extract's anti-inflammatory properties. These results support the ethnopharmacological use of the plant in the treatment of diverse inflammatory ailments. (Foyet *et al.,* 2008).

Antimycobacterial study

The study examined crude extracts of six Cameroonian medicinal herbs for the *in vitro* antimycobacterial activity against the pathogenic H37Rv strain. *Acanthus montanus, Beilschemeidia obscura, Cissus petiolata, Enantia chlorantha, Urera repens,* and *Garcinia preussii* were all active, with minimum inhibitory concentrations (MICs) ranging from 31.25 g/ml to 250 g/ml. *B. obscura* was the most active at MIC 31.25. The MIC of *Acanthus montanus* was 62.5 g/ml, with a growth inhibition of 95.06 %. These findings imply that the anti-inflammatory properties of *Acanthus montanus* aqueous extract may be attributable to the suppression of nitric oxide (NO). (Nkenfou *et al.,* 2015).

Hypoglycemic, Antihyperlipidemic, and Hepatoprotective activities

Using established protocols, the effects of aqueous extracts of a polyherbal formulation (*Emilia coccinea, Acanthus montanus, Hibiscus rosasinensis,* and *Asystasia gangetica)* on serum glucose concentration, amylase activity, and lipid profiles of normal, diabetic, and liver-damaged rabbits were investigated. For 28 days, a mixture of the four plants' aqueous extracts was given orally in two doses: 120 mg/kg body weight and 240 mg/kg body weight. With no changes in amylase activity, the medication caused dose- and duration-dependent

significant ($p < 0.05$) decreases in serum glucose, total cholesterol, triacylglycerol, and LDL-cholesterol, as well as significant ($p < 0.05$) elevations in HDL-cholesterol concentrations. These findings support the crude drug's hypoglycemic, antihyperlipidemic, and hepatoprotective properties, and hence validate its use in ethnomedicine for diabetes control. No changes in amylase activity were seen at these doses. (Ojiako *et al*., 2015). Additional evaluation of the hypoglycemic effects revealed that the methanol extract displayed a higher level of inhibition on both α-amylase and α-glucosidase compared to the ethanol extract. The methanol extract exhibited inhibition on both α-amylase and α-glucosidase in both non-competitive and competitive manners, as indicated by the Lineweaver-Burk plot analysis. This suggests that the hypoglycemic action of *A. montanus* extracts could be due to the inhibition of these enzymes (amylase and glucosidase). The presence of phytochemicals in the extracts may have prompted this observation. (Anugweje *et al.,* 2012).

Hepato-Renal Protective / Polyherbal Leaf Formulation

The ability of single and polyherbal formulations of "*Acanthus montanus, Asystasia gangetica, Emilia coccinea,* and *Hibiscus rosa-sineneis"* leaf extracts to repair renal and hepatic damage in alloxan-induced diabetic rats was examined. The polyherbal formulation improved cellular stability and reversed glomerular shrinkage and the disrupted arrangement of tissue structures as well as blood biochemical indicators that suggested cellular integrity was restored. The findings from the study conducted by Ojiako *et al.* (2015) indicated that the individual and combined therapeutic approaches exhibited differing degrees of effectiveness in the restoration of kidney and liver damage in rats with hyperglycemia.

Contractile Ability on Rat Uterus

The uterine contractile activity of *A. montanus* extract was investigated in female Wistar rats using the *Ugo basile* organ bath model 4050. The uterus of the rats was significantly contracted by oxytocin and acetylcholine. Extract administration resulted in a dose-dependent ($p = 0.5$) reduction in oxytocin and acetylcholine-induced contractions. The methanol extract of *A. montanus* was found to have an anti-contractile effect on uterine smooth muscles in non-pregnant rats, corroborating its usage in the treatment of unplanned abortion. The absence of death at 5000 mg/kg of methanol extract indicates that the lethal dose is more than 5000 mg/kg, which could indicate that the plant is safe. (Okieimen *et al.,* 2018).

Biphasic Activity on Uterine Smooth Muscle

The effects of *Acanthus montanus* aqueous extract, fractions, and *Acanthus sulfate ester* (ASE) - a newly discovered sulfate ester – on the rat uterus were studied for the first time in the study. An organ bath containing an isolated unpregnant rat uterus suspended in *De Jalons* physiological solution and aerated with Carbogen (95% O_2 + 5% CO_2) was used in the *in vitro* study. Following equilibration, the extract, fraction, and ASE were individually introduced to the tissue and compared to standard uterine agonists in the absence and presence of standard antagonists. A two-channel *Ugo Basile* recorder was used to capture the responses. The extract displayed a biphasic activity, first relaxing spontaneous uterine contractions (IC_{50} = 3.00 mM) and then stimulating the same tissue (IC_{50} = 0.63 mM). Depending on the polarity of the extraction solvent, its percentage displayed both relaxation and contraction. The uterus was constricted by 100 % methanol fraction (IC_{50} = 150.0 M) and ASE (131.0 M). The IC_{50s} of the remaining fractions were inconsistent. PGF_2, acetylcholine, diazoxide, oxytocine, and histamine all had lower stimulatory actions. Phentolamine, prazosin, pyrilamine, indomethacin, and verapamil, but not atropine, inhibited the extract, methanol fraction, and ASE. Propranolol had little effect on the extract, whereas tetraethylammonium stimulated it

slightly. Quinacrine had no effect on the activity of the extract and MeOH fractions. The most powerful inhibitor was verapamil. Both external and intracellular calcium and potassium ionic channels were shown to be implicated in the mechanism of action of ASE as they are most likely connected. (Asongalem *et al.,* 2019).

Hepatocurative activity

In a study conducted by Uroko *et al.* (2019), the acute toxicity of a methanol extract derived from *Acanthus montanus* leaves was examined in mice. The results indicated the extract did not exert any harmful effects on the mice, and no fatalities were observed. Furthermore, the extract was evaluated in rats with acetaminophen-induced liver failure. It was found to reduce the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), as well as total bilirubin concentrations. Additionally, the extract significantly increased total protein, albumin, and direct bilirubin concentrations. It also improved liver histomorphology, which had been adversely affected by acetaminophen toxicity. These findings suggested that the methanol extract of *A. montanus* leaves contains hepatocurative properties capable of reversing liver failure and mitigating the hepatotoxic side effects associated with acetaminophen toxicity.

2.2 REPRODUCTIVE TOXICOLOGY

The use of herbal remedies for various health concerns, including reproductive health has increased in recent years. While traditional knowledge often supports the consumption of herbal medicines, it is essential to critically assess their safety and potential risks, particularly in the context of reproductive toxicology. Reproductive toxicological studies are essential in evaluating the effects of herbal remedies on reproductive systems and processes. Herbal remedies have been employed for generations to address reproductive health issues. However,

as societies evolve and healthcare practices advance, it becomes imperative to bridge the gap between traditional knowledge and contemporary scientific understanding. Reproductive toxicological studies provide a means to validate or refute the claims of traditional remedies within a modern scientific framework (Foster and Tyler, 2013). The reproductive system is highly sensitive to external influences, including chemicals present in herbal remedies. Substances with hormonal activity, such as phytoestrogens, can impact fertility, gestation, and developmental processes. Comprehensive reproductive toxicological studies are essential to assess whether herbal remedies contribute to reproductive harm or hormonal imbalances (McGuffin *et al.,* 2017). Several herbal compounds possess endocrine-disrupting potential, interfering with hormonal pathways critical to reproduction. Studies that elucidate the mechanisms of action and potential consequences of endocrine disruption are crucial for understanding the safety profile of herbal remedies (Diamanti-Kandarakis *et al.,* 2009). Herbal remedies can vary widely in composition and potency due to factors such as plant species, preparation methods, and storage conditions. These variations can influence their safety and efficacy. Reproductive toxicological studies provide a standardized approach to evaluate different preparations and ensure consistency in safety assessments (Gurib-Fakim, 2006). The use of herbal medicines for reproductive health is often driven by cultural practices and accessibility. Conducting reproductive toxicological studies reflects a commitment to public health and ethical responsibility, ensuring that individuals are informed about the potential risks and benefits of herbal remedies (WHO, 2000). Lastly, the utilization of herbal remedies for reproductive health warrants careful scrutiny through reproductive toxicological studies. These studies serve as a bridge between traditional wisdom and contemporary scientific knowledge, thus providing a methodological approach to evaluate the safety and potential risks associated with herbal remedies. By incorporating evidence-based

assessments, informed decisions that prioritize reproductive health and overall well-being can be made.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 PLANT COLLECTION AND AUTHENTICATION

Acanthus montanus fresh leaves were collected from Bolorunduro community, Akure, Ondo State. The plant was identified in the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Nigeria by Prof. MacDonald Idu and it was authenticated by Dr. H. A. Akinnibosun in the Herbarium Unit of the same department with voucher number **UBH-A45.**

3.2 PREPARATION AND EXTRACTION

Fresh leaves of *Acanthus montanus* were initially rinsed with distilled water and subsequently air-dried at room temperature within a sterile environment. These dried plant materials were then finely ground using a British mechanical grinder. A total of 1700 grams of powdered leaves were subjected to extraction using 3000 mL of distilled water via a 72-hour maceration process, following the aqueous extraction method. The resulting extracts were further concentrated in a semi-solid form using a regulated HH-S water bath from Science Tech Instruments, maintained at a constant temperature of 45 ºC. The percentage yield was determined using the formula: % yield = (extract weight / powdered sample weight) × 100 / 1 (Mukherjee, 2002).

3.3 EVALUATION FOR REPRODUCTIVE TOXICOLOGY

3.3.1 Experimental Animals

Forty (40) healthy Wistar rats, both male and female, were procured from the animal facilities at the University of Benin's Animal and Environmental Biology Department. These rats were accommodated in adequately ventilated cages under standard laboratory conditions, including

a 12-hour light/dark cycle and a controlled temperature of 23±2 ºC. They were provided with a standard diet and had unrestricted access to rat chow and water throughout the experiment, adhering to established protocols for animal care and handling during the study period.

3.3.2 Experimental Design

Four (4) groups of twelve (12) animals (6 males, 6 females) each received the following treatments:

- Group A: Distilled water (normal control)
- Group B: 200 mg/kg aqueous plant extract
- Group C: 400 mg/kg aqueous plant extract
- Group D: 800 mg/kg aqueous plant extract

The animals were acclimatized for fourteen (14) days prior to the administration of the extract for twenty-eight (28) days and were fasted overnight before sacrificing. The animals were sacrificed and the blood samples, along with the reproductive organs were isolated for analysis.

3.3.3 Determination of body and organ weight

The Wistar rats were weighed across the groups using Weigh 7001DX Multi-purpose Digital Scale on days 0, 7, 14, 21, and 28 (Eleazu *et al.*, 2013). The net change of body weight (difference between final body weight and initial body weight) was resolute for all animals. The various reproductive organs' weights were determined (Amna *et al.*, 2013). The relative liver, kidney, heart, lungs, spleen, and stomach weights were determined using the formula:

$$\text{Relative organ weight} = \frac{\text{Organ weight}}{\text{Bodyweight}} \times 100$$

3.3.4 Lipid profile

Total cholesterol levels were determined using the enzymatic method with wet reagent kits, following a modified version of the Trinder method (1969) as described by Benjamin *et al.*

(2020). In this process, 1000 µl of the reagent was dispensed into three separate test tubes labeled A, B, and C. Distilled water served as the standard solution, and 10 µl of each plasma sample was added to test tubes A, B, and C. After thorough mixing, the test tubes were allowed to stand for ten (10) minutes at 37°C to observe the color change. Subsequently, the absorbance of the blank, sample, and standard was measured at 500 nm using a UV spectrophotometer, following the method outlined by Ahuja and Ball, (2006). The cholesterol concentration was then calculated using the formula:

Cholesterol (mg/dl) = (Absorbance of sample / Absorbance of standard) × Concentration of standard (mg/dl).

For the determination of total triglycerides, an enzymatic method utilizing wet reagent kits was employed, with a modification of the Tietz method (1995). In this process, 1000 µl of reagent was dispensed into three test tubes labeled A, B, and C. The standard solution, consisting of distilled water, was added to test tube A, while plasma samples were pipetted into test tubes B and C. Test tube A served as the reagent blank. Following thorough mixing, the test tubes were allowed to stand at room temperature (25°C) for 10 minutes to observe the color change. The absorbance of the blank, sample, and standard was measured at 500 nm using a UV spectrophotometer, following the method outlined by Iyer and Patil (2011). The triglyceride concentration in the sample was calculated as:

Total Triglyceride (mg/dl) = (Absorbance of sample / Absorbance of standard) × Concentration of standard (mg/dl).

For the determination of high-density lipoprotein (HDL), the enzymatic method was utilized with a precipitating agent composed of 0.55 mmol/l phosphotungstic acid and 25 mmol/l magnesium chloride, pre-diluted in distilled water at a ratio of 4:1. 500 µl of reagent was pipetted into two separate test tubes labeled A and B. In test tube A, a standard solution and in test tube B, a plasma sample of 200 µl each was added. After thorough mixing, the test tubes

were left at room temperature for 10 minutes to observe the color change. Subsequently, the absorbance of the standard and sample was measured at 500 nm using the UV spectrophotometer, following the method outlined by Omodamiro and Nwankwo (2013). HDL concentration was calculated using the formula:

HDL (mg/dl) = (Absorbance of sample / Absorbance of standard) × Concentration of standard (mg/dl).

Low-density lipoprotein (LDL) was calculated using the following formula:

LDL (mg/dl) = Total cholesterol – Triglycerides / 5 - HDL.

3.3.5 Hormonal Assay

Determination of Serum Testosterone Concentration

The serum testosterone levels in the tested animals' plasma were determined following a standardized protocol as outlined in the manufacturer's manual (Gauthaman and Adaikan, 2008). This method relies on the competitive interaction between testosterone in the test plasma sample (serum) and testosterone reference standards.

The procedure involved the distribution of 10 µL of testosterone reference standards at concentrations of 0, 0.1, 0.5, 2.0, 6.0, and 18.0 ng/mL, along with serum samples that were diluted fivefold, into Goat Anti-Rabbit IgG-coated microtitre wells (96 wells). To each well, 100 µL of testosterone-HRP conjugate reagent (appearing blue) and 50 µL of rabbit anti-testosterone reagent were added (Neychev and Mitev, 2016). The resulting solution was thoroughly mixed for 30 seconds and then incubated at 37°C for 90 minutes. During this incubation period, the HRP-labelled testosterone competed with endogenous testosterone in the standard, sample, or quality control serum for binding sites on a specific testosterone antibody. Following incubation, the microtitre wells were washed five times with distilled water to remove unbound testosterone peroxidase conjugate. Subsequently, 100 µL of TMB

reagent was added to each well. The resulting solution was gently mixed for 5 seconds and incubated at room temperature for approximately 20 minutes until it turned blue (Neychev and Mitev, 2005). To stop the color change, 100 µL of Stop Solution (1N HCl) was added to each well, causing the color to shift from blue to yellow. The absorbance of the samples was measured within 15 minutes at 450 nm using a microtitre well reader. The intensity of the color formed was proportional to the amount of enzyme present and inversely related to the unbound testosterone in the sample (Gauthaman and Adaikan, 2008). The serum testosterone levels in the animals were determined from the calibration curve, which plots the concentration of the standard against the absorbance. The calculation was performed using the formula:

Testosterone concentration (ng/mL) = Cs × F,

Where Cs represents the corresponding testosterone concentration from the calibration curve, and F denotes the dilution factor.

Progesterone assay protocol

Before the assay commenced, all reagents were equilibrated to room temperature (between 18-25°C). In a swift manner, 50 µL of ready-to-use standards and appropriately diluted samples were dispensed into the respective wells within a 5-minute window. Following this, 100 µL of Progesterone Enzyme Conjugate Solution was added to each well, except for those designated as blanks, as outlined in the protocol by Akanni *et al.* (2017). Vigorous mixing ensued for 30 seconds, and the plate was then incubated at 37°C for a duration of 60 minutes. For optimal incubation, the wells could be covered with par film or placed in an appropriately sealed zip-lock bag. Following the incubation period, the contents of the wells were carefully discarded, and the plate was subjected to five rounds of washing with Wash Solution (approximately 250-300 µL per well). To eliminate any residual moisture, the plate was

inverted and firmly tapped against absorbent paper. Next, 100 µL of (TMB) Substrate Solution was introduced into all wells, adhering to the recommended pipetting sequence. The plate was subsequently incubated at room temperature, within the range of 18-28°C, for a duration of 10 minutes, without any agitation. To halt the reaction, 50 µL of Stopping Solution was added to each well in the same order as the Substrate Solution, and the contents were gently mixed. Finally, the absorbance was measured at 450 nm using a microwell reader to obtain the assay results.

Luteinizing hormonal assay

To initiate the assay, the required number of coated wells was securely placed within the holder. Subsequently, 50 µL of standards, specimens, and controls were carefully dispensed into their respective wells. Additionally, 100 µL of Enzyme Conjugate was introduced into each well, and thorough mixing was performed for a duration of 30 seconds, following the method outlined by Cicero *et al.* (2001). The mixtures within the wells were then subjected to incubation at room temperature (37°C) for a period of 2 hours. After incubation, the contents of the wells were removed by gently flicking the plate's contents into a waste container. Subsequently, the microtiter wells were rinsed with wash buffer, and this process was repeated five times. To ensure the complete removal of residual water droplets, the wells were tapped firmly onto absorbent paper or paper towels. Following the washing step, 100 µL of TMB solution was dispensed into each well and gently mixed for 10 seconds. The plate was then incubated at room temperature for 20 minutes in a dark environment. The reaction was subsequently halted by adding 50 µL (equivalent to one drop) of 2N HCl into each well, followed by a 30-second mixing step. A noticeable color change from blue to yellow was observed in the wells, and the optical density was measured at 450 nm using a microtiter well reader, in accordance with the method described by Gauthaman and Adaikan (2008).

Follicle-stimulating hormone

The reagents and samples were allowed to equilibrate to room temperature before use, and the samples underwent centrifugation again after thawing, as part of the preparation process for the assay. It was strongly recommended that all samples and standards be assayed in duplicate, and the reagents and samples should be prepared in accordance with the procedures outlined in the previous sections as per Tajuddin *et al.* (2006). First, the number of wells to be utilized was determined. Any remaining wells, along with the desiccant, were reinserted into the pouch and sealed using the Ziploc closure. Unused wells were stored at a temperature of 4°C. A Blank well, containing no solution, was also prepared. Subsequently, 50 µL of Standard or Sample was added to each well, and this process was duplicated for the standard. Following this, 50 µL of HRP-conjugate was introduced into each well, excluding the Blank well, followed by the addition of 50 µL of Antibody to each. Thorough mixing was performed, and the plate was then incubated for 60 minutes at 37°C. After the incubation period, each well was aspirated, and a washing step was carried out. This washing process was repeated two more times, totaling three washes in total. Wells were washed by filling each one with Wash Buffer (200 µL), utilizing various methods such as a squirt bottle, multi-channel pipette, manifold dispenser, or auto-washer. It was important to allow the wash buffer to stand for 10 seconds during each wash cycle, and the complete removal of the liquid at each step was crucial for optimal performance, as advised by Zamblé *et al.* (2008). Following the final wash, any remaining Wash Buffer was then removed through aspiration or decantation. The plate was then inverted and gently blotted against clean paper towels, following the procedure described by Neychev and Mitev (2016). Next, 50 µL of Substrate A and 50 µL of Substrate B were added to each well, mixed thoroughly, and incubated for 15 minutes at 37°C. The plate was kept in a location free from drafts and temperature fluctuations, and it was maintained in the dark during this incubation period. To conclude the

assay, 50 μL of Stop Solution was added to each well, and the plate was gently tapped to ensure thorough mixing, in accordance with the method outlined by Yakubu *et al.* (2008). Finally, the optical density of each well was promptly determined within 10 minutes using a microplate reader set to 450 nm.

3.3.5 Determination of Antioxidant Activity

The assessment of superoxide dismutase (SOD) activity followed the procedures outlined by Halliwell (1997). The underlying principle involves the inhibition of auto-oxidation with hematoxylin, resulting in an increase in absorbance at the 560 nm wavelength. This assay is conducted at pH 7.8, aiming to determine the percentage of SOD within a specified range. The SOD activity in the sample is quantified by measuring the amount of hematin present, as described by Lobo *et al.* (2010).

The essential principle underlying the assay is illustrated through the following equation:

$$O2 + HTH2\ SOD\ H2O2 + HT\ (Abs560\uparrow)$$

The procedure entails taking an aliquot mixture of plasma, which is 0.20 ml of the diluted microsome, and enclosing it using a 2.5 ml solution of 0.05 M carbonate buffer. The reactions are initiated by adding a 0.3 ml solution of 0.3 mM adrenaline, following Velavan (2011). For the standard, a combination of a 2.5 ml solution of 0.05 M carbonate buffer, 0.3 ml solution of 0.3 mM adrenaline, and 0.20 ml of distilled water is prepared. Absorbance is measured between 30 seconds to 150 seconds using a wavelength of 480 nm.

Calculations are performed as follows:

$$\text{Augment Absorbance/minute} = \%\ \text{inhibition} = 100 - (AAxs / 5.21 * 5A)$$

Where:

- As is the increased absorbance of the substrate

- Ab is the increased absorbance of the blank

1 unit of SOD activity corresponds to the total amount of SOD required to induce 50% inhibition of adrenaline oxidation to adrenochrome per minute, as described by Halliwell (1997).

The catalase activity assay was conducted in accordance with the method by Halliwell and Gutteridge (1995). The principle was based on catalase's ability to scavenge hydrogen peroxide, converting it into molecular oxygen and water. This activity was done by monitoring the decreased absorbance rate at a wavelength of 240 nm while measuring the consumption of H2O2 substrate spectrophotometrically, as specified by Kurutas (2016). The procedure involves adding 10 μL of tissue homogenate (containing 100-150 μg of protein) to a 2.8 ml solution of 50 mM potassium phosphate buffer (pH 7.0) within a 3 ml cuvette. The reaction is initiated by adding 0.1 ml of a freshly prepared 30 mM H2O2 solution, and the decomposition rate of H2O2 is measured at 240 nm wavelength over 300 seconds using a spectrophotometer, as detailed by McCall et al. (2000). The catalase activity is calculated in terms of H2O2 mole reduced per minute per milligram of protein, employing a molar loss coefficient of 0.041 Mm-1cm-1.

The assessment of malondialdehyde (MDA) activity follows the method described by Beckman and Koppenol (1996). The test principle revolves around the reaction of MDA with thiobarbituric acid (TBA), leading to the formation of an MDA-TBA2 adduct that exhibits strong absorption at 532 nm. The procedure entails centrifuging the treated blood culture, isolated from the supernatants after 24 hours of incubation, at 3000 rpm for 20 minutes. Then, 1300 μL of R1 is withdrawn from the micro-centrifuge tube. A 1 ml supernatant is diluted tenfold in Tris HCl, and further dilution of 200 μL of the supernatant in each culture is performed by adding 200 μL of distilled water and vortexing. Subsequently, 300 μL of R2 is added to all the test tubes, followed by vortexing, and the tubes are placed under incubation at

45°C for 40 minutes. After incubation, each tube is cooled on ice and centrifuged at 15000 g for 10 minutes at 4°C, as per Barcelos *et al.* (2011). The samples are then measured in a spectrophotometer at 586 nm.

Glutathione activity is measured by determining the first-order rate constant for the decomposition of tetra-butyl hydroperoxide, in accordance with the procedure described by Mates *et al.* (1999).

3.3.6 Histopathological analysis

The testes were preserved in Bouin's solution. After fixation, the organs were subjected to a dehydration process using 99.9% ethanol, followed by a series of ethanol washes at concentrations of 70% and 96%, concluding with a rinse in distilled water. Sections measuring 4 µm in thickness were then prepared and subjected to staining with hematoxylin and eosin dye. These stained tissue sections were subsequently examined under an optical photomicroscope (Leica MC170 HD, Leica Biosystems, Germany) at a magnification of X 400, as per the method described by Drury and Wallington (2013).

3.4 Statistical Analysis

The results are presented as the mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism version 6.0. Group data were also compared using a one-way analysis of variance (ANOVA) with subsequent post hoc analysis conducted with the Kruskal-Wallis test. Statistical significance was established at $p < 0.05$.

CHAPTER FOUR

RESULTS

4.1 BODY AND ORGAN WEIGHT

The results obtained from the body and organ weight in the male Wistar rats showed no significant difference in the graded doses (200, 400, and 800 mg/kg) of *Acanthus montanus* aqueous leaf extract when compared with the untreated group showed that it causes no pathological effect across the studied organs as shown in Table 4.1.

Table 4.1: Effect of *Acanthus montanus* leaf aqueous extract on the body and organ weight in reproductive toxicology of male Wistar rats

Groups	**Dose mg/kg**	**Day 0 (g)**	**Day 7 (g)**	**Day 14 (g)**	**Day 21 (g)**	**Day 28 (g)**	**Testes (g)**	**Penis (g)**
Control (DW)	0.5 ml	143.70±6.57	150.00±12.12	155.30±19.67	156.00±26.50	154.00±27.51	2.30±0.10[a]	0.33±0.03[a]
A. montanus	200	146.70±2.85	156.70±3.93	160.70±1.20	178.70±9.68	154.30±0.67	2.67±0.22[b]	0.43±0.03[b]
A. montanus	400	157.70±6.98	168.00±7.21	176.30±11.22	184.30±9.91	176.00±12.53	2.47±0.22[b]	0.33±0.03[a]
A. montanus	800	155.70±4.98	170.70±0.82	184.70±6.57	191.30±6.33	188.70±4.91	2.67±0.12[b]	0.37±0.03[b]

p-value < 0.05 showed the level of significance, Superscript showed no significant difference, n=5

Table 4.2: Effect of *Acanthus montanus* leaf aqueous extract on the body and organ weight in reproductive toxicology of female Wistar rats

Groups	Dose mg/kg	Day 0 (g)	Day 7 (g)	Day 14 (g)	Day 21 (g)	Day 28 (g)	Ovary (g)	Uterus (g)
Control (DW)	0.5 ml	138.00±7.00	140.30±7.80	146.00±7.21	151.30±9.33	147.30±8.84	0.20±0.00[a]	0.30±0.00[a]
A. montanus	200	181.70±5.21	185.00±2.08	191.30±3.38	192.30±4.70	188.70±3.84	0.17±0.03[b]	0.23±0.13[a]
A. montanus	400	202.70±6.67	202.70±5.36	199.00±2.65	202.30±3.38	195.30±3.48	0.23±0.03[a]	0.73±0.22[b]
A. montanus	800	227.70±6.94	223.00±10.00	230.00±9.29	229.00±8.02	225.00±11.06	0.17±0.03[b]	0.73±0.07[b]

P-value < a 0.05, Superscript showed no significant difference, n=5

4.2 LIPID PROFILE

The indexes evaluated in the lipid profile assay of the male Wistar rats were Total Cholesterol, Triglycerides, Very Low-Density Lipoproteins, High-density Lipoproteins, and Low-Density Lipoproteins. *Acanthus montanus* aqueous extract at graded doses (200, 400, and 800 mg/kg) maintained a normal physiological state of the TC, TAG, HDL, and LDL concentrations within normal ranges when compared with the control as shown in Table 4.2.

Table 4.3: Effect of *Acanthus montanus* leaf aqueous extract on lipid profile in reproductive toxicology of male Wistar rats

Groups	**Dose mg/kg**	**Cholesterol (mg/dl)**	**Triglycerides (mg/dl)**	**HDL(mg/dl)**	**LDL(mg/dl)**	**VLDL(mg/dl)**
Control (DW)	0.5 ml	78.67±0.29^{a}	219.40±0.05^{a}	16.71±0.20^{a}	58.46±0.20^{a}	43.89±0.01^{a}
A. montanus	200	75.00±1.93^{b}	227.00±0.83^{b}	19.16±1.79^{b}	48.77±3.83^{b}	45.39±0.17^{a}
A. montanus	400	73.03±1.11^{b}	225.40±1.37^{b}	18.45±0.51^{b}	46.41±1.83^{b}	45.07±0.27^{a}
A. montanus	800	73.78±0.75^{b}	224.40±1.31^{b}	20.84±1.12^{b}	49.74±0.67^{b}	44.88±0.26^{a}

P-value < 0.05, HDL----- High-density lipoprotein, LDL----- Low-density lipoprotein, VLDL--- Very low-density lipoprotein. Superscript showed no significant difference, n=5

Table 4.4: Effect of *Acanthus montanus* leaf aqueous extract on lipid profile in reproductive toxicology in female rats

Groups	Dose mg/kg	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Control (DW)	0.5 ml	78.42±0.07^{a}	213.30±0.88^{a}	16.08±0.13^{a}	53.84±0.33^{a}	42.66±0.18^{a}
A. montanus	200	71.45±2.60^{b}	211.70±0.99^{b}	17.04±0.43^{a}	34.22±8.98^{b}	42.35±0.20^{a}
A. montanus	400	73.24±1.13^{b}	212.70±1.13^{a}	17.34±0.27^{b}	48.05±0.70^{b}	42.53±0.23^{a}
A. montanus	800	69.77±0.87^{b}	213.10±0.82^{a}	17.31±0.24^{b}	44.45±0.68^{b}	42.63±0.17^{a}

P-value < a 0.05, HDL-----High density lipoprotein, LDL----- Low density lipoprotein, VLDL--- Very low-density lipoprotein, Superscript showed no significant difference, n=5

4.3 HORMONAL ASSAY

The effect of *Acanthus montanus* leaf aqueous extract (at 200 mg/kg, 400 mg/kg, and 800 mg/kg doses) across all groups of male rats when compared against the control group in the testosterone assay revealed a significant increase in testosterone levels in the extract groups, as shown in Figure 4.1.

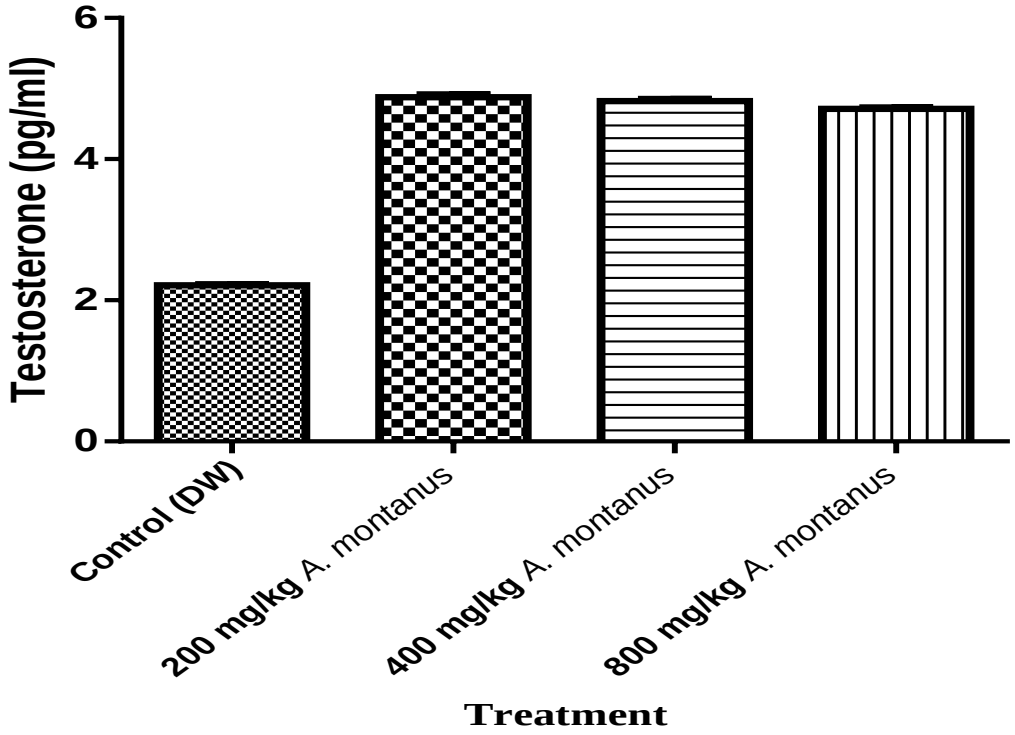


Figure 4.1: Effect of *Acanthus montanus* leaf aqueous extract on Testosterone in reproductive toxicology in male rats

Figure 4.2. revealed the *Acanthus montanus* leaf aqueous extract had a significant increase in the follicle-stimulating hormonal levels across all groups of male rats particularly at 400 and 800 mg/kg when compared to the normal control.

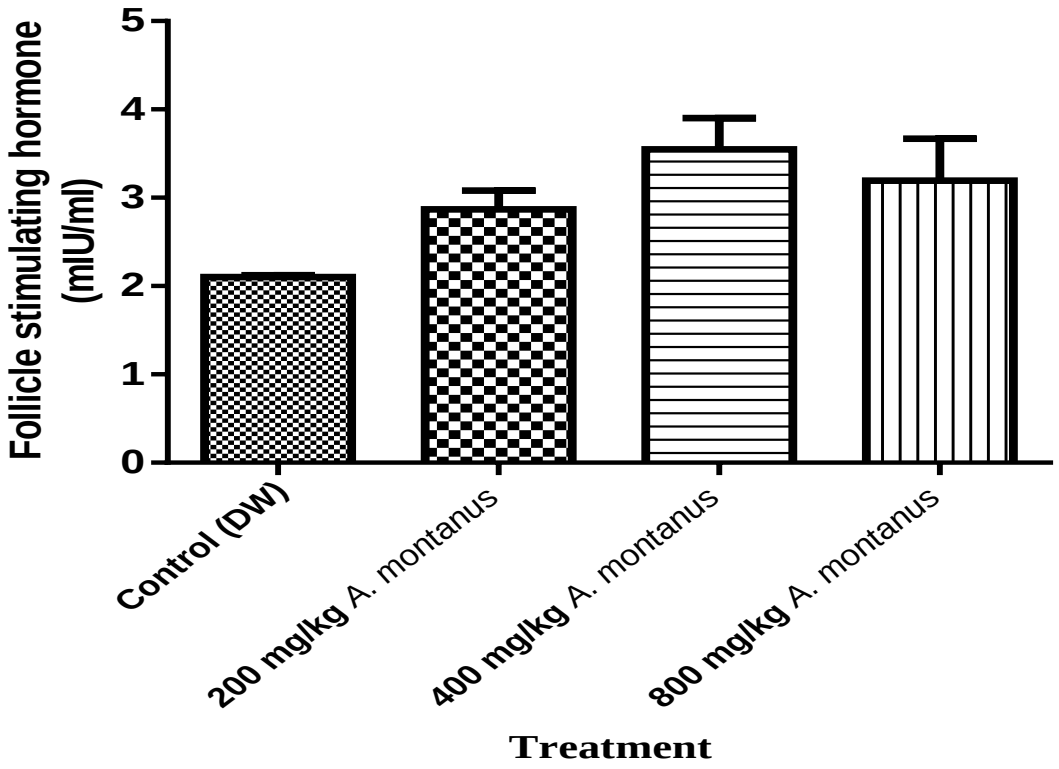


Figure 4.2: Effect of *Acanthus montanus* leaf aqueous extract on follicle-stimulating hormone in reproductive toxicology in male rats.

The effect of *Acanthus montanus* leaf aqueous extract had a high level of significance across all groups particularly at 200 mg/kg and 800 mg/kg in luteinizing hormonal assay in male animals when in comparison with the control as shown in Figure 4.3.

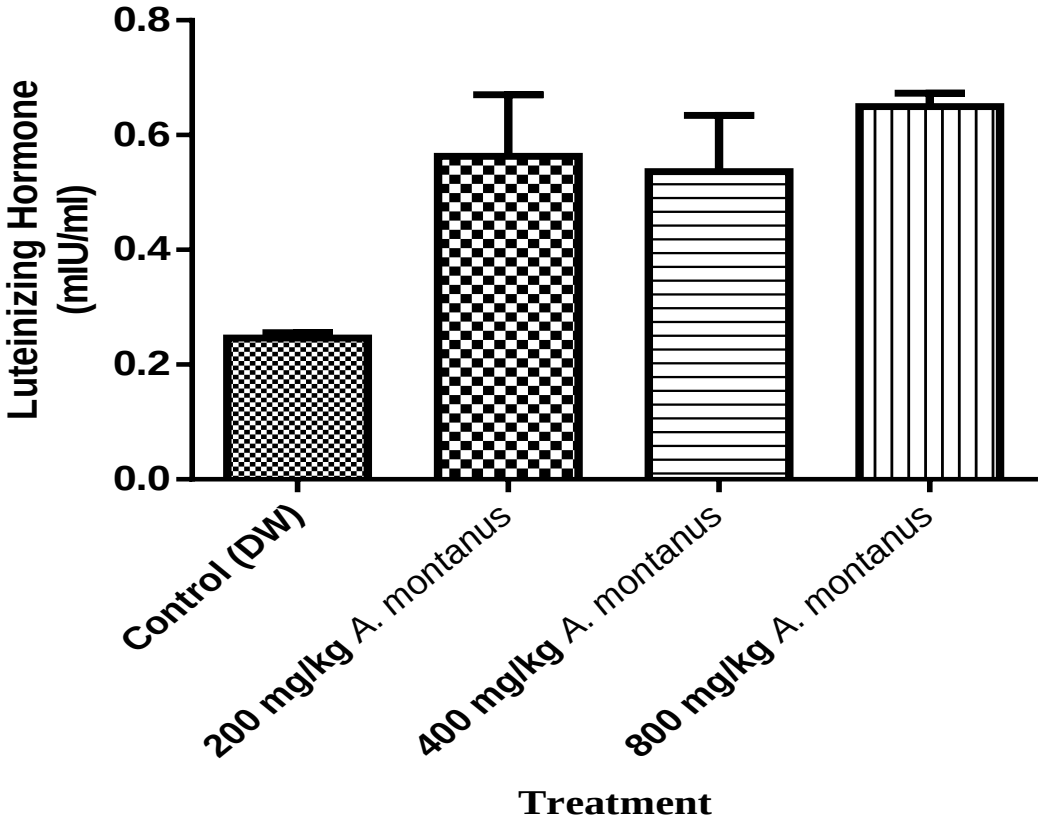


Figure 4.3: Effect of *Acanthus montanus* leaf aqueous extract on luteinizing hormone in reproductive toxicology in male rats.

Results obtained from Figure 4.4 had significant increase across all groups especially at 200 and 800 mg/kg of *Acanthus montanus* leaf aqueous extract in progesterone assay of the male animals when compared with the control.

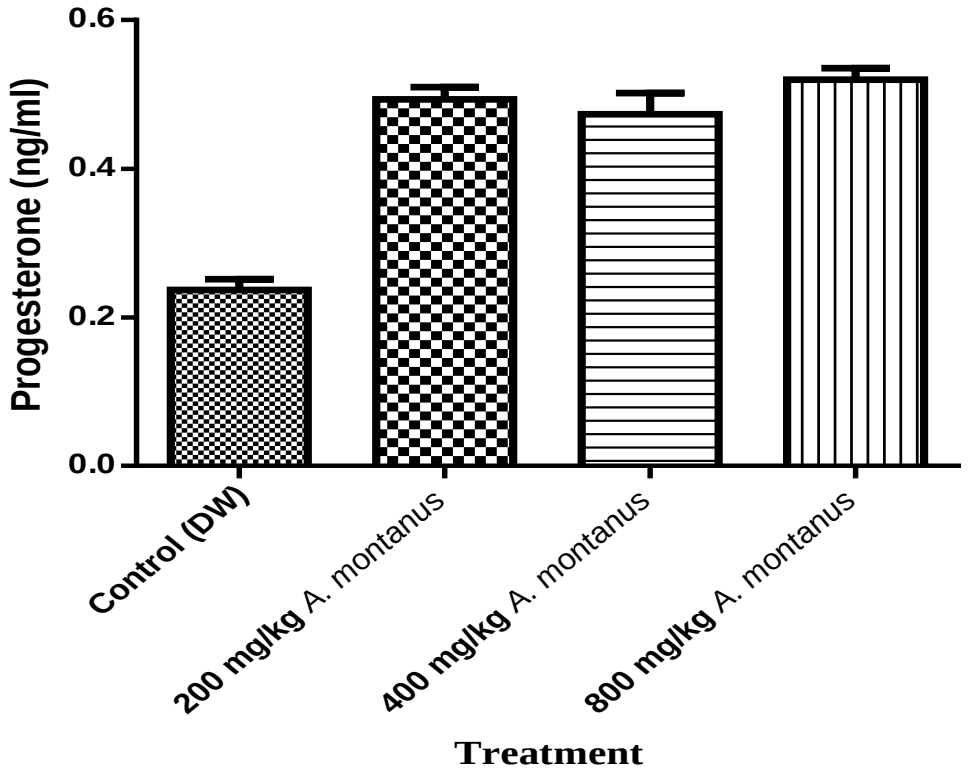


Figure 4.4: Effect of *Acanthus montanus* leaf aqueous extract on progesterone in reproductive toxicology in male rats.

This study investigated in the female animals graded doses (200, 400 and 800 mg/kg) of *Acanthus montanus* leaf aqueous extract, showed a significant increase across the doses in serum oestrogen when compared with the control as shown in Figure 4.5.

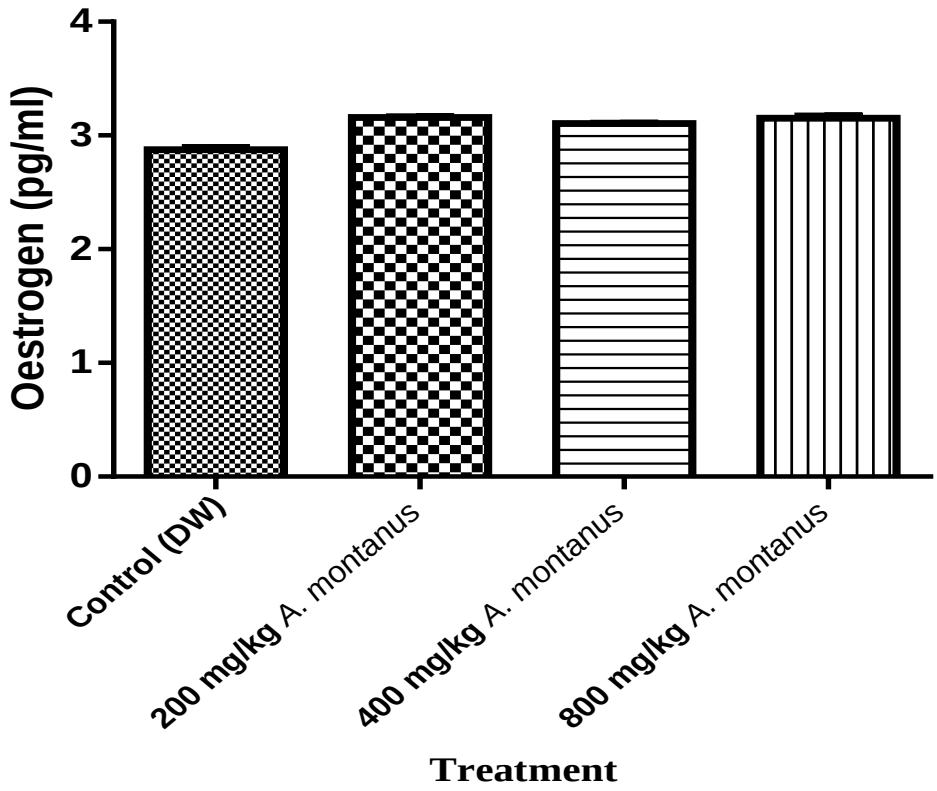


Figure 4.5: Effect of *Acanthus montanus* leaf aqueous extract on oestrogen in reproductive toxicology in female rats

Figure 4.6 showed significant decrease in follicle stimulating hormonal levels of the female animals at graded doses (200, 400 and 800 mg/kg) of *Acanthus montanus* aqueous leaf extract, when compared with the control.

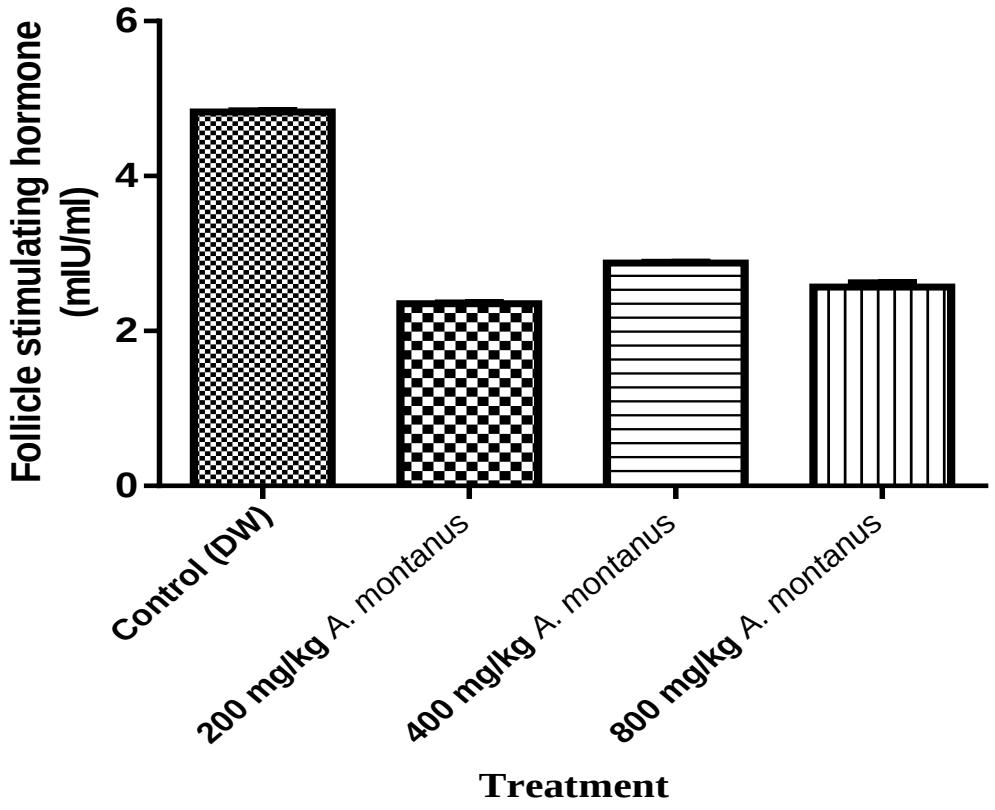


Figure 4.6: Effect of *Acanthus montanus* leaf aqueous extract on follicle stimulating hormone in reproductive toxicology in female rats.

Figure 4.7 showed significant decrease in luteinizing hormonal levels of the female animals at graded doses (200, 400 and 800 mg/kg) of *Acanthus montanus* aqueous leaf extract, when compared with the control.

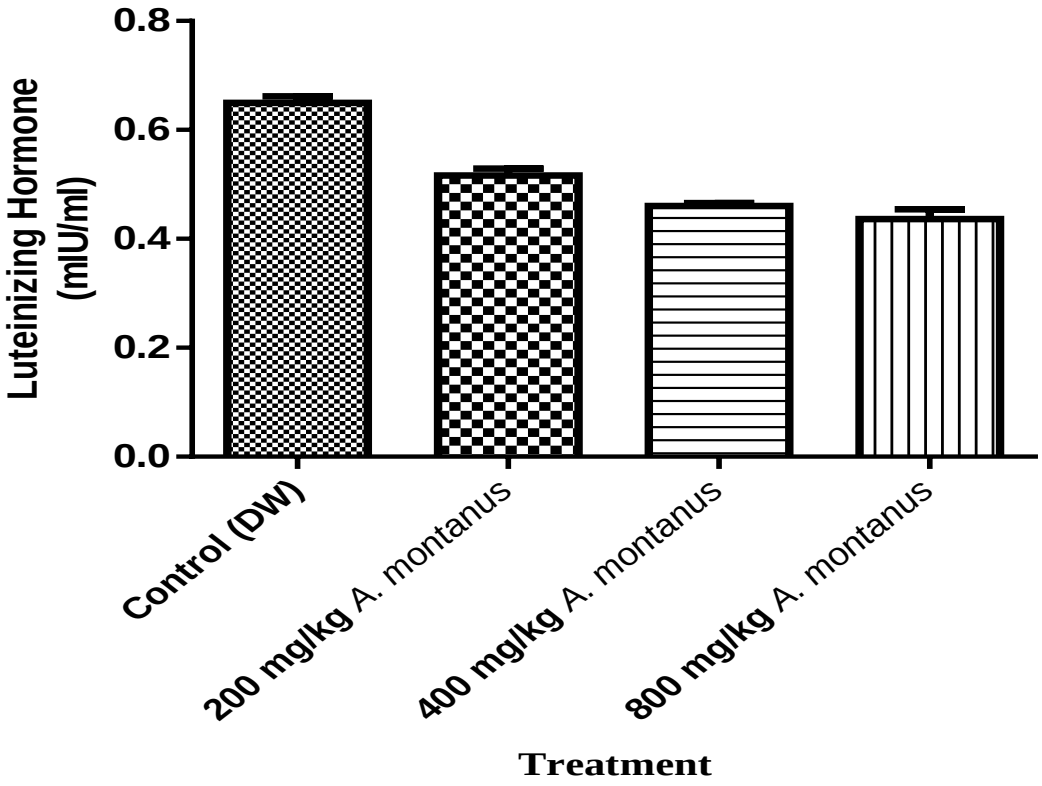


Figure 4.7: Effect of *Acanthus montanus* leaf aqueous extract on luteinizing hormone in reproductive toxicology in female rats.

Results obtained from Figure 4.8 showed a slight increase at 200 mg/kg but a significant increase at 400 mg/kg and 800 mg/kg of *Acanthus montanus* leaf aqueous extract in the progesterone hormonal assay of the female animals when compared with the control group.

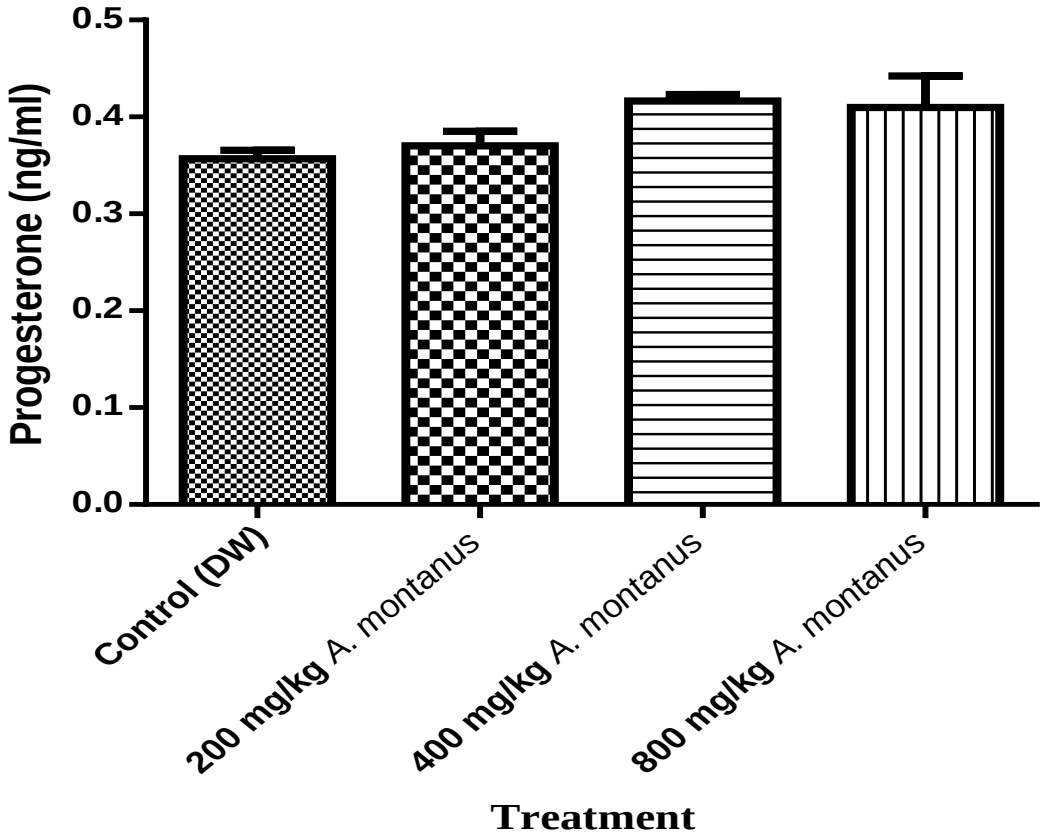


Figure 4.8: Effect of *Acanthus montanus* leaf aqueous extract on progesterone in reproductive toxicology in female rats.

4.4 ANTIOXIDANT ASSAY

The *Acanthus montanus* leaf aqueous extract exhibited significant antioxidant effects in male rats across different dosage groups (200 mg/kg, 400 mg/kg, and 800 mg/kg) compared to the control group (0.5 ml distilled water) as shown in Table 4.5.

Table 4.5: Effect of *Acanthus montanus* leaf aqueous extract on *in-vivo* antioxidant in reproductive toxicology in male rats

Groups	**Dose mg/kg**	**Malondialdehyde (x10-3 mmole/ml)**	**Superoxide dismutase (U/ml)**	**Catalase (U/ml)**	**Glutathione peroxidases (U/ml)**
Control (DW)	0.5 ml	63.85±0.66[a]	3.18±0.01[a]	193.80±0.16[a]	103.40±0.77[a]
A. montanus	200	35.90±0.31[b]	5.61±0.02[b]	201.60±1.03[b]	120.50±0.53[b]
A. montanus	400	36.45±0.69[b]	5.71±0.11[b]	200.20±2.04[b]	120.30±0.81[b]
A. montanus	800	37.00±1.50[b]	6.07±0.18[b]	196.30±0.57[b]	119.70±0.16[b]

P-value < a 0.05,

Table 4.6 also indicated significant antioxidant effects of the *Acanthus montanus* leaf aqueous extract in male rats at various dosages (200 mg/kg, 400 mg/kg, and 800 mg/kg) in comparison to the control group (0.5 ml distilled water).

Table 4.6: Effect of *Acanthus montanus* leaf aqueous extract on *in-vivo* antioxidant in reproductive toxicology in female rats

Groups	**Dose mg/kg**	**Malondialdehyde (x10-3 mmole/ml)**	**Superoxide dismutase (U/ml)**	**Catalase (U/ml)**	**Glutathione peroxidases (U/ml)**
Control (DW)	0.5 ml	63.19±0.29[a]	3.18±0.01[a]	194.20±0.59[a]	104.00±0.21[a]
A. montanus	200	32.79±0.67[b]	6.42±0.02[b]	196.90±0.84[b]	111.20±0.31[b]
A. montanus	400	32.07±1.03[b]	6.09±0.45[b]	198.10±0.92[b]	112.10±0.46[b]
A. montanus	800	31.18±0.49[b]	6.94±0.34[b]	197.70±0.77[b]	111.70±0.55[b]

P-value < a 0.05,

4.5 HISTOPATHOLOGICAL STUDY

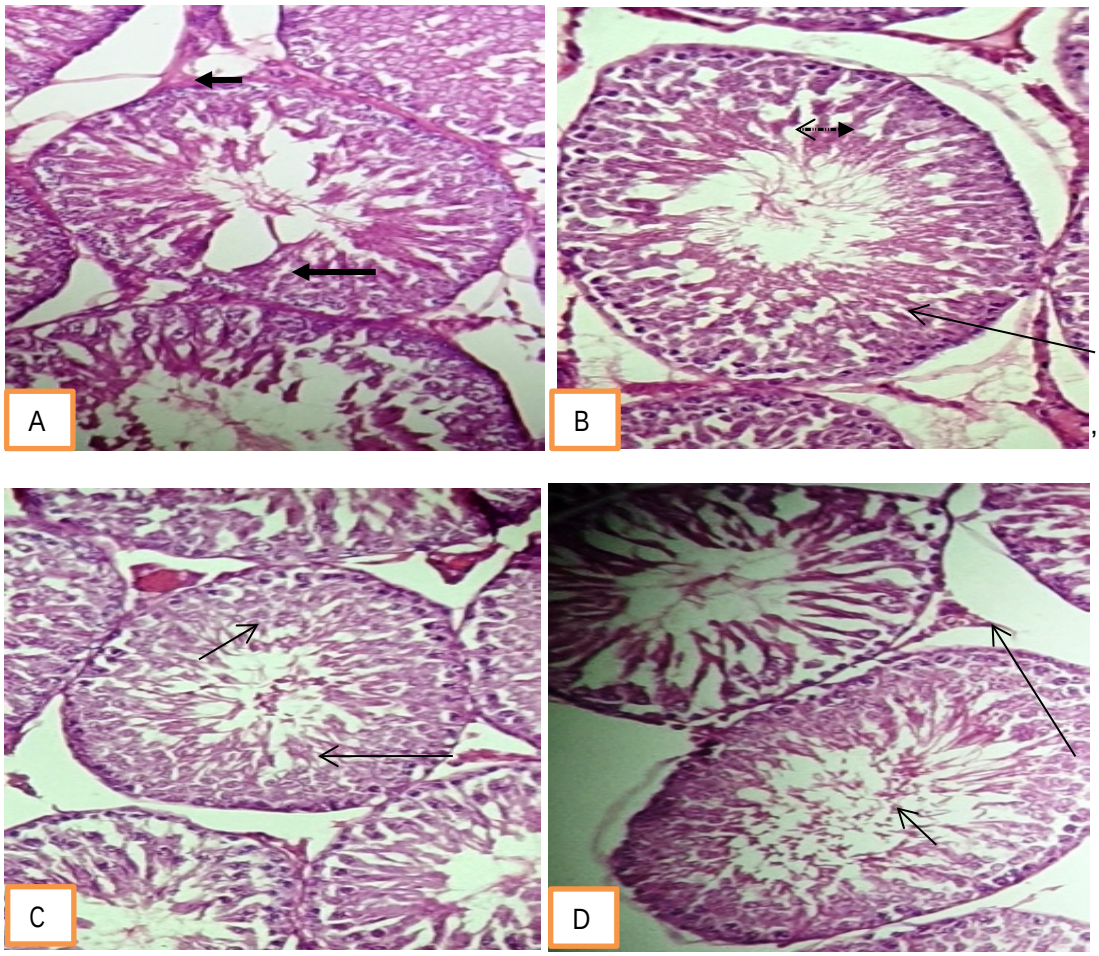


Plate 1: Effect of *Acanthus montanus* leaf aqueous extract on the testes in reproductive toxicology in male rats

A. **Control Testis:** sections show seminiferous tubules that are circular and thickening in outline (long arrow) and pyknotic Sertoli cells Leydig cells and other cells of spermatogenic series (short arrow).
B. **200 mg/kg *A. montanus* Testis:** sections show seminiferous tubules that are circular in outline (long arrow) and pyknotic Sertoli cells Leydig cells and other cells of spermatogenic series with mild edema (short arrow).
C. **400 mg/kg *A. montanus* Testis**: sections show seminiferous tubules that are circular in outline (long arrow) and pyknotic Sertoli cells Leydig cells and other cells of spermatogenic series (short arrow) with a congested vessel (arrowhead).
D. **800 mg/kg *A. montanus* Testis:** sections show seminiferous tubules that are circular in outline (long arrow) and pyknotic Sertoli cells Leydig cells and other cells of spermatogenic series (short arrow).

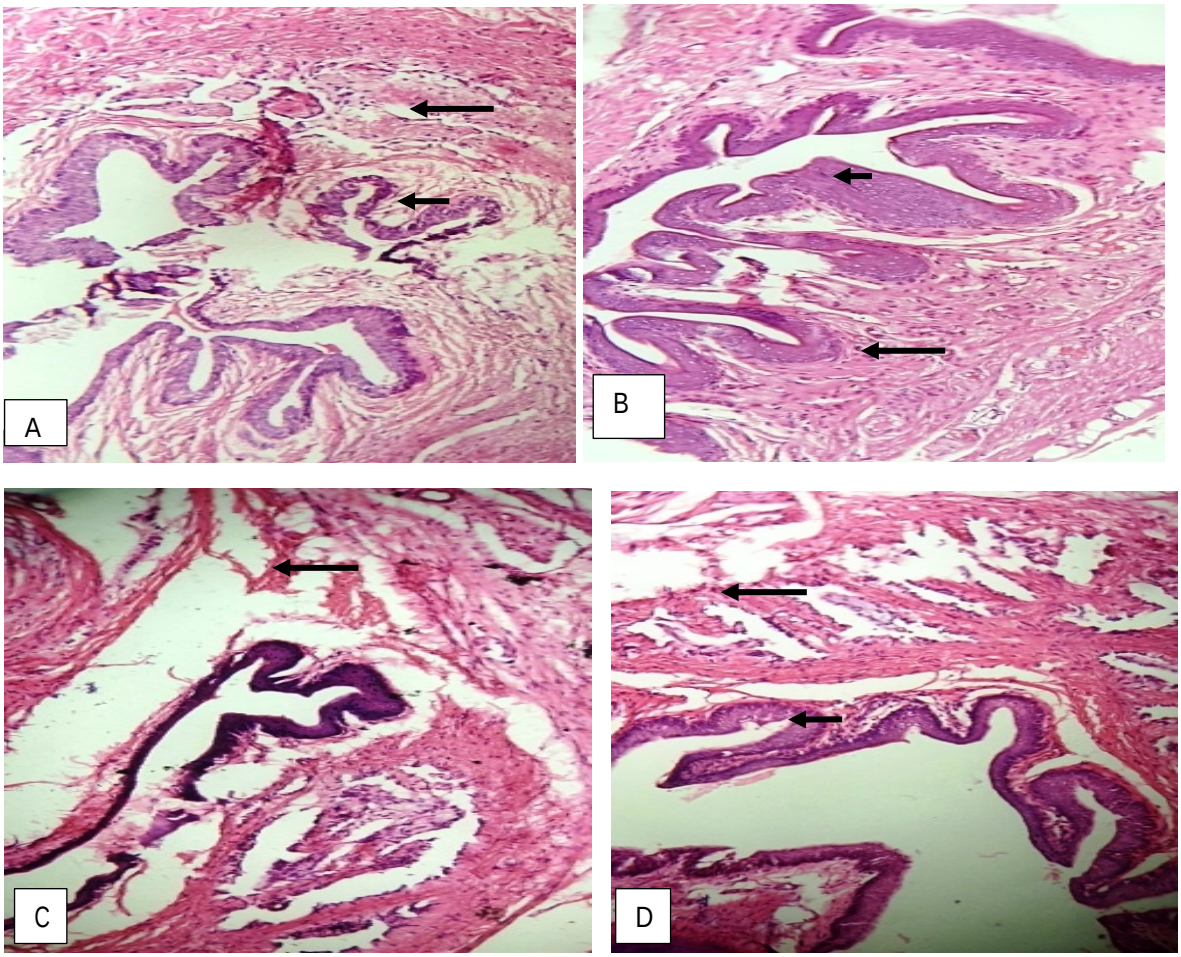


Plate 2: Effect of *Acanthus montanus* leaf aqueous extract on the penis in reproductive toxicology in male rats.

A. **Control Penis:** sections show well-outlined less thickened smooth muscles and connective tissue fibres (long arrow).and prominent urethra lined by stratified columnar epithelium (short arrow).

B. **200 mg/kg *A. montanus* Penis:** sections show smooth muscles and connective tissue fibres (short arrow).and urethra lined by proliferating stratified columnar epithelium (long arrow).

C. **400 mg/kg *A. montanus* Penis:** sections show unremarkable smooth muscles and connective tissue fibres (long arrow) and thickened urethra lined by proliferating stratified columnar epithelium (short arrow).

D. **800 mg/kg *A. montanus* Penis:** sections show well outlined less thickened smooth muscles and connective tissue fibres (long arrow).and prominent urethra lined by stratified columnar epithelium (short arrow).

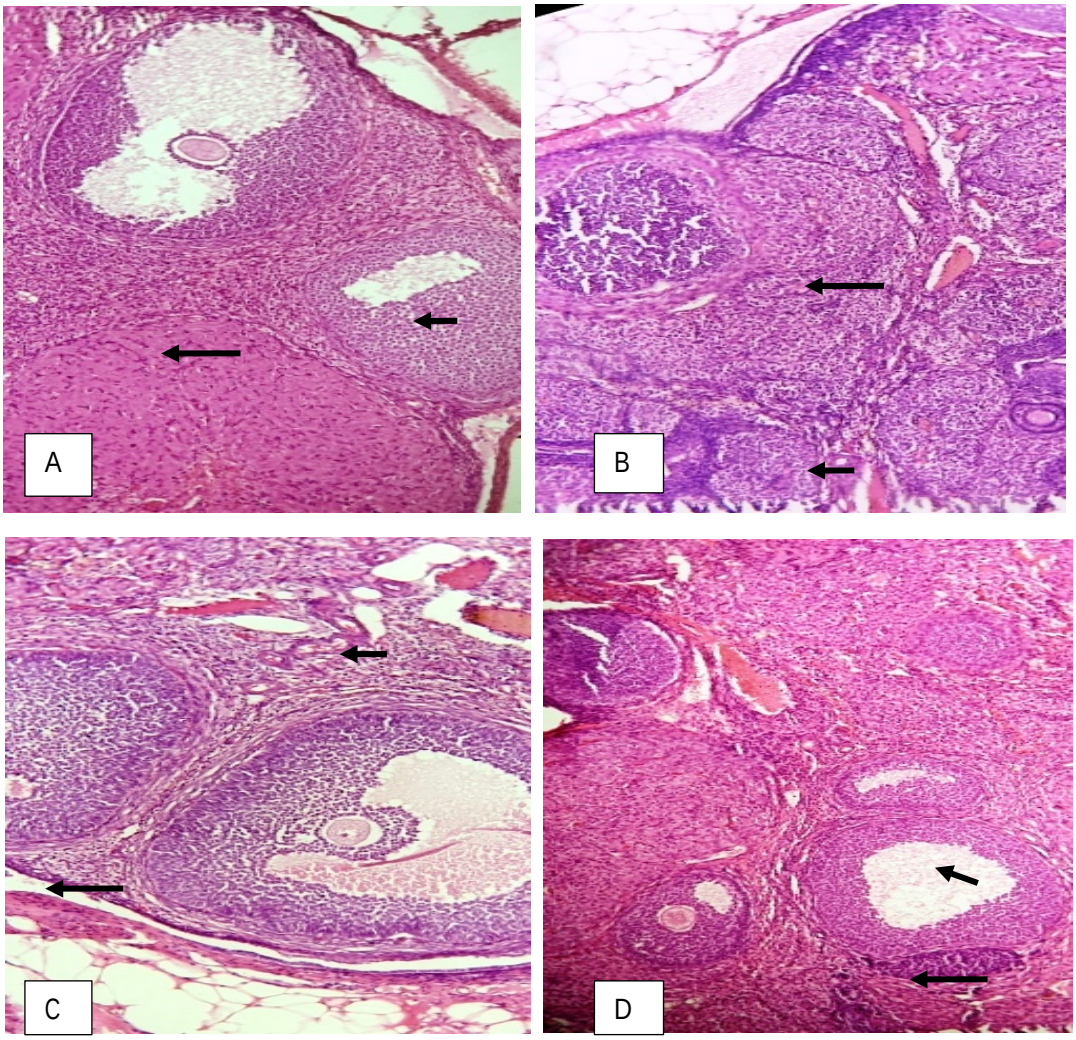


Plate 3: Effect of *Acanthus montanus* leaf aqueous extract on the ovaries in reproductive toxicology in female rats.

A. In the **control group**, the ovary exhibited numerous prominent corpus luteum (short arrow). Furthermore, there was a thick germinal epithelium and zona granulosa with a corona and oocyte positioned eccentrically (long arrow).

B. In the group treated with **200 mg/kg of *A. montanus***, the ovary displayed unremarkable corpus luteum (short arrow) alongside a thick germinal epithelium, zona granulosa, and noticeable fibrosis with a corona and unremarkable oocyte (arrow).

C. The ovary in the **400 mg/kg *A. montanus*** group revealed a large corpus luteum (long arrow). The germinal epithelium and zona granulosa were unremarkable, with a corona and a large oocyte positioned eccentrically (short arrow).

D. In the **800 mg/kg *A. montanus* group**, the ovary displayed a large corpus luteum (long arrow). There was thick germinal epithelium and zona granulosa with a corona and oocyte located eccentrically to varying degrees (short arrow).

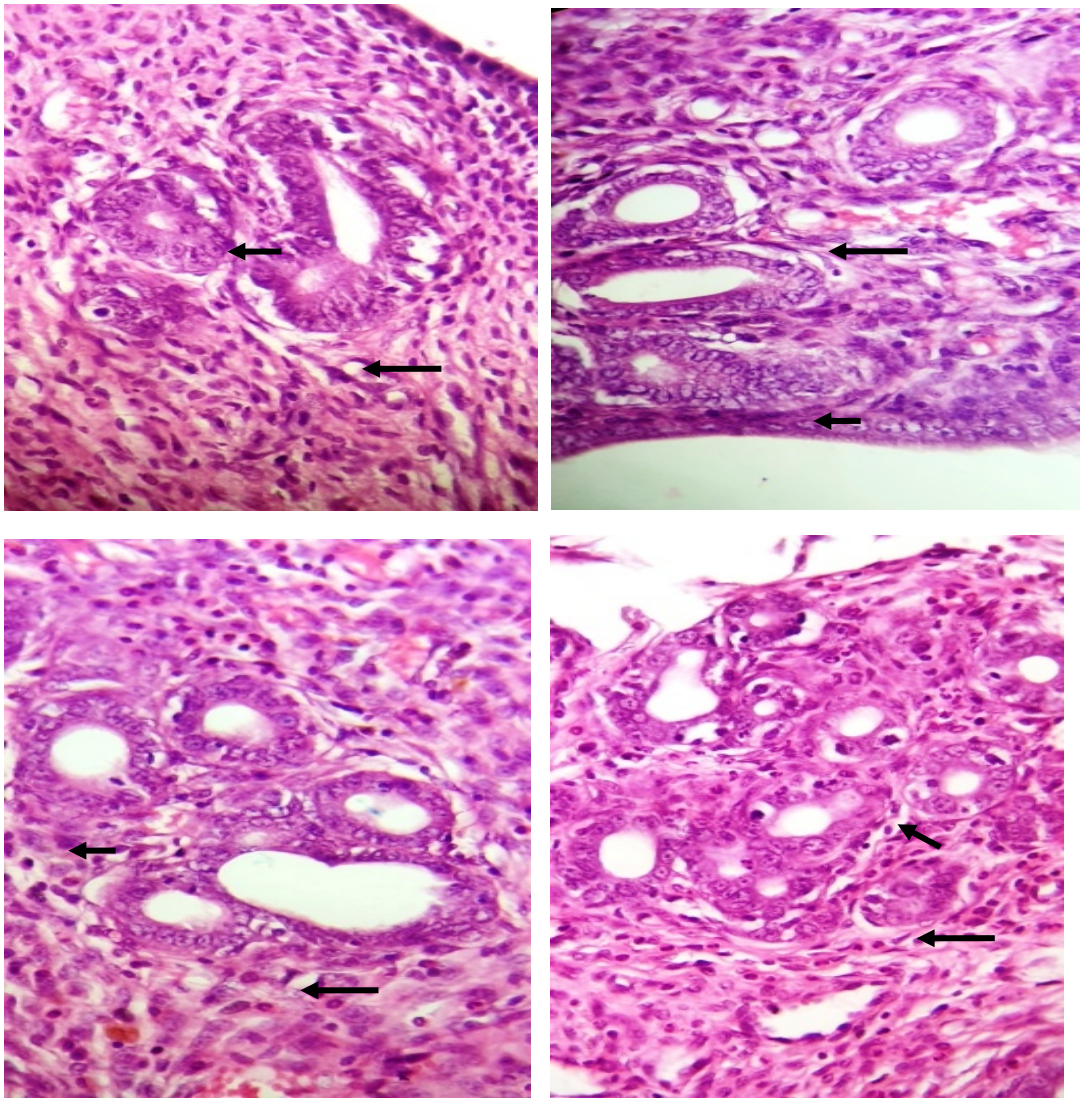


Plate 4: Effect of Acanthus *montanus* leaf aqueous extract on the uterus in reproductive toxicology in female rats.

A. **Control uterus** revealed prominent endometrial glands (short arrow) embedded in the lamina propria (long arrow), there is a visible close-up of simple columnar epithelium and lamina propria.

B. **200 mg/kg *A. montanus* uterus** revealed endometrial glands with mild proliferative changes (short arrow) embedded in the lamina propria (long arrow), there is a visible close-up of simple columnar epithelium and lamina propria

C. **400 mg/kg *A. montanus* uterus** showed endometrial glands (short arrow) embedded in the lamina propria (arrowhead), there is a visible close-up of simple columnar epithelium and pyknotic lamina propria

D. **800 mg/kg *A. montanus* uterus** revealed unremarkable endometrial glands (short arrow) embedded in the lamina propria (long arrow) and a visible close up of simple columnar epithelium.

CHAPTER FIVE

DISCUSSION

Body and Organ Weight

The results in Table 4.1 illustrated the effect of *Acanthus montanus* leaf aqueous extract on body and organ weight in a 28-day reproductive toxicological study involving male Wistar rats. Various extract doses were administered, and a control group received distilled water. The rats' body weight gradually increased over the study, while testes weight remained stable and penis weight consistent in the control group. Rats receiving 200 mg/kg of the extract displayed similar body weight trends. However, their testes weight significantly increased by day 21, suggesting potential effects on testicular growth, accompanied by gradual penis weight gain. In the 400 mg/kg group, body weight steadily increased, with notable testicular growth by day 14, implying an impact on testicular development; penis weight remained steady. The 800 mg/kg group exhibited consistent body weight gain and significant testes growth by day 7, suggesting a potential influence on testicular development; penis weight increased over 28 days. Overall, *Acanthus montanus* leaf extract exhibited dose-dependent effects on male Wistar rat body and organ weight, particularly influencing testicular and penile growth at higher doses (400 mg/kg and 800 mg/kg).

Table 4.2 evaluates the effect of *Acanthus montanus* leaf aqueous extract on body and organ weight in a reproductive toxicology study involving female Wistar rats over a 28-day period. The control group received 0.5 ml of distilled water (DW). Over the 28-day period, the body weight of the rats gradually increased. The weight of the ovaries and uterus remained relatively consistent during the study. In the 200 mg/kg group of *A. montanus* leaf extract the body weight increased over time, similar to the control group. The weight of the ovaries showed a slight decrease, but the change was not statistically significant. The weight of the uterus remained relatively unchanged. The body weight initially increased in the 400 mg/kg

group of *A. montanus* leaf extract, but by day 14, there was slight decrease. The ovaries' weight increased significantly by day 14, but this increase was not sustained, as the weight decreased by day 21. The uterus weight showed a significant increase by day 28. The rats in the 800 mg/kg group of *A. montanus* had their body weight initially increased and then stabilized. The ovaries' weight remained relatively unchanged, while the uterus weight showed a significant increase by day 28. In summary, the *Acanthus montanus* leaf aqueous extract appears to have varying effects on the body and organ weight of female Wistar rats, depending on the dose administered. The extract's impact on body weight was inconsistent across groups. The ovaries' weight showed fluctuations, with a significant increase at the 400 mg/kg dose. The uterus weight increased notably at both the 400 mg/kg and 800 mg/kg doses. These findings indicated that the extract might have dose-dependent effects on the reproductive organs of male and female rats, suggesting potential influences on penile, testicular, ovarian, and uterine function. This result was in consent with Murunga *et al.* (2016) report on grapefruit components to ameliorate reproductive toxicological organ weight loss. Similarly, visceral organs such as the testes, penis, uterus, and ovary of animals isolated, showed no damage or weight loss across treated when compared with the control as shown in Table 4.1 and 4.2 (Witthawaskul *et al.*, 2003; Benjamin and MacDonald, 2019).

Lipid Profile

The results in Table 4.3 and 4.4 present the effect of *Acanthus montanus* leaf aqueous extract on the lipid profile in a reproductive toxicology study using male and female Wistar rats. Cholesterol levels in the control groups were 78.67 mg/dl and 78.42 mg/dl. Rats administered *A. montanus* extract at all doses showed decreased cholesterol levels compared to the control. The reduction in cholesterol suggests that the extract might have potential cholesterol-lowering effects. Also, the triglyceride levels in the male control group were 219.40 mg/dl.

Rats in the extract groups exhibited slightly lower triglyceride levels than the control. This reduction implies a potential influence of the extract on triglyceride metabolism. For the female group, Triglyceride levels in the control group were 213.30 mg/dl. There were minor fluctuations in triglyceride levels among the groups, without a clear consistent pattern. Further investigation is needed to determine the extract's impact on triglyceride metabolism in female Wistar rats. High-density lipoproteins (HDL) are referred to as "good cholesterol. HDL levels in the control groups were 16.71 mg/dl and 16.08 mg/dl. *A. montanus* extract displayed higher HDL levels across all doses. Elevated HDL levels suggest a positive impact on cardiovascular health by promoting the removal of excess cholesterol from the bloodstream. LDL levels (bad cholesterol) in the control group were 58.46 mg/dl and 53.84 mg/dl. The extract groups showed reduced LDL levels compared to the control; lower LDL levels are associated with decreased risk of cardiovascular diseases. The VLDL levels, representing very low-density lipoprotein, showed minor variations without a clear pattern across groups. The lack of consistent changes in VLDL levels warrants further investigation. *Acanthus montanus* leaf aqueous extract appears to elucidate beneficial effects on the lipid profile of Wistar rats. The extract was associated with lower cholesterol and triglyceride levels, elevated HDL levels, and reduced LDL levels. These alterations in the lipid profile suggest potential cardiovascular benefits of the extract, indicating possible implications for heart health. The regulation of TC, TAG, and LDL levels at normal values with an increase in HDL suggested an ameliorative effect of hyperlipidemia, as similarly reported in Murphy *et al*. (2007), and Nissen *et al*. (2011). The activities are a result of increased inhibition of intestinal absorption of cholesterol, intrusion lipoprotein synthesis, and increased hepatic expression of LDL receptors and their protective role, leading to an increase in LDL removal from the blood to augment catabolism and cholesterol degradation in the body (Brown and Goldstein, 1981).

Hormonal Assay

The observed effect of *Acanthus montanus* leaf aqueous extract on testosterone levels in male rats in Figure 4.1 aligns with similar studies conducted at different doses (200 mg/kg, 400 mg/kg, and 800 mg/kg). In comparison to the control group, the extract groups exhibited a substantial and significant increase in testosterone levels. This outcome is consistent with findings from previous research hormones (Gordon *et al.,* 1976; MacDonald *et al.,* 2021), indicating that the extract possesses the capacity to enhance testosterone production or regulation. These parallel findings highlight the potential and reliability of the extract's impact on testosterone levels. The significant increase across various doses underscores a dose-dependent relationship, strengthening the notion that the extract plays a role in positively modulating testosterone levels.

The findings depicted in Figure 4.2 provided compelling evidence that *Acanthus montanus* leaf aqueous extract induces a substantial increase in follicle-stimulating hormone (FSH) levels across all groups of male rats. Notably, the extract's effect was particularly pronounced at the 400 mg/kg and 800 mg/kg dosage levels when compared to the normal control. The elevated FSH levels in response to the extract's administration suggested that it had the potential to influence the endocrine system's regulation of reproductive processes. FSH is a key hormone responsible for stimulating the growth and development of the testes and supporting the production of sperm (Gordon *et al.*, 1976; MacDonald *et al.,* 2021). The extract's capacity to significantly enhance FSH levels implies its ability to interact with hormonal pathways that control male reproductive function. The dosage-dependent effect, with the most substantial increase observed at the 400 mg/kg and 800 mg/kg doses, underscores the extract's potency in influencing FSH secretion (Muthusami and Chinnaswamy, 2005).

Figure 4.3 demonstrated a remarkable and statistically significant impact of the extract on luteinizing hormone (LH) levels in male animals. This effect is particularly pronounced in the 200 mg/kg and 800 mg/kg dosage groups when compared with the control group. The notable increase in LH levels across these dosage groups implies that the extract may have the potential to influence the male endocrine system's regulation of reproductive processes. The Luteinizing hormone plays a crucial role in stimulating the production of testosterone in the testes and supporting spermatogenesis, making it a pivotal hormone for male reproductive function (Gordon *et al.*, 1976; MacDonald *et al.,* 2021). The high level of statistical significance underscores the robustness of the extract's impact on LH levels, reinforcing the notion that it has a substantial influence on hormonal pathways. The dose-dependent pattern, with the most significant effects observed at the 200 mg/kg and 800 mg/kg doses, further supports the extract's potency in enhancing LH secretion.

Progesterone, often associated with female reproductive processes, also plays a role in male reproductive physiology. While its function in males is less understood compared to females, an increase in progesterone levels could imply regulatory roles in male reproductive health or interactions with other hormonal pathways (Gordon *et al.*, 1976; MacDonald *et al.,* 2021). The findings from Figure 4.4 revealed a substantial and statistically significant increase in progesterone levels among male animals following the administration of the plant’s extract. This effect is particularly pronounced in the 200 mg/kg and 800 mg/kg dosage groups when compared to the control group. The significance of the results underscores the extract's potential to modulate progesterone levels in male animals. The dose-dependent pattern, with the most significant effects observed at the 200 mg/kg and 800 mg/kg doses, suggests a potential threshold effect or optimal dosage range for eliciting this hormonal response.

A, montanus leaf aqueous extract also revealed a slight yet statistically significant increase in serum estrogen levels across the different doses when compared to the control group as shown

in Figure 4.5. The observed increase in serum estrogen levels suggests that the extract has the potential to modulate estrogen production or regulation in female animals. Estrogen plays a critical role in female reproductive health, including menstrual cycles, fertility, and various physiological processes (Mathur *et al.,* 2002). The slight but significant increase in estrogen levels across the doses suggests a dose-dependent relationship, where higher dosages may lead to more noticeable hormonal changes. However, the degree of increase observed in this study implies that the extract's influence on estrogen levels might be subtle. However, the implications of this finding are multifaceted. While the increase in estrogen levels is noteworthy, it's essential to consider the balance of hormonal regulation and potential downstream effects. Modulating estrogen levels, even subtly, could impact various aspects of female reproductive health, including menstrual cycles and fertility. *Acanthus montanus* was found to significantly prolong the metestrous and diestrous stages, both key parts of the rat estrous cycle's luteal phase. This disruption was reversible within about 9 days after extract administration ceased, indicating the potential reversibility of its effects. The normal development of follicles depends on a balance between ovarian and extraovarian hormones (Valsala and Karpagaganapathy, 2002). Ovarian estrogen prompts histamine release, facilitating decidualization and preparation of tissues for embryonic implantation. Imbalances in this hormonal equilibrium can lead to irregular ovarian function, estrous cycle disruptions, and potential fertility problems.

The result shown in Figure 4.6 indicates a notable and statistically significant decrease in follicle-stimulating hormone (FSH) levels among female animals exposed to graded doses (200, 400, and 800 mg/kg) of the extract, as compared to the control group. This reduction in FSH levels suggests that the extract has the potential to impact the regulation of FSH, a crucial hormone in female reproductive physiology. FSH is central to ovarian follicle development and plays a key role in supporting the reproductive processes of females

(Muthusami and Chinnaswamy, 2005). The observed decrease in FSH levels across the various doses highlights the extract's capacity to influence hormonal pathways involved in female reproductive health. However, alterations in FSH levels can have cascading effects on ovarian function, menstrual cycles, and fertility. MacDonald *et al.,* 2021). Nevertheless, further research is needed to identify and understand the underlying mechanisms through which the extract interacts with the endocrine system and leads to decreased FSH levels.

Figure 4.7 revealed a marked and statistically significant decrease in luteinizing hormone (LH) levels among female animals treated with graded doses (200, 400, and 800 mg/kg) of the extract, as compared to the control group. The observed decrease in LH levels signifies the extract's potential to influence the hormonal regulation of LH, a key hormone in female reproductive processes. LH is pivotal in triggering ovulation, stimulating the release of mature eggs from ovarian follicles, and supporting the corpus luteum (Muthusami and Chinnaswamy, 2005). The significant decrease in LH levels across the doses emphasizes the extract's ability to modulate hormonal pathways relevant to female reproductive function (MacDonald *et al.,* 2021).

As shown in Figure 4.8 varying degrees of progesterone hormone level changes in female rats treated with the extract at different doses were evaluated and the results. Specifically, there is a slight increase at the 200 mg/kg dose, but a more pronounced and statistically significant increase at the 400 mg/kg and 800 mg/kg doses when compared to the control group. The observed pattern suggests that the extract has the potential to influence progesterone production or regulation in female animals as Progesterone plays a central role in various reproductive processes, including preparing the uterine lining for implantation and supporting pregnancy. The significant increase at the 400 mg/kg and 800 mg/kg doses underscores the extract's potency in affecting progesterone levels. This dose-dependent effect may indicate an optimal range at which the extract can elicit a more significant hormonal response as also

reported by MacDonald *et al.,* (2021). This result is important to consider because altered progesterone levels can impact the menstrual cycle, fertility, and overall reproductive health of females.

Tables 4.5 and 4.6 presented the results of the effect of *A. montanus* extract on *in-vivo* antioxidant markers in male and female rats. These markers include Malondialdehyde (MDA), Superoxide Dismutase (SOD), Catalase, and Glutathione Peroxidase (GPx). Malondialdehyde (MDA) is a marker of lipid peroxidation, an oxidative stress indicator (Patlevič, *et al.,* 2020). The extract's effect on MDA levels indicated its potential in the reduction of oxidative stress as it led to a significant decrease in MDA levels at all doses (200 mg/kg, 400 mg/kg, and 800 mg/kg) when compared to the control group. This suggests that the extract has antioxidant properties that help mitigate lipid peroxidation and oxidative damage (Igwe and Eleazu, (2017). Superoxide Dismutase (SOD) is an enzyme that combats superoxide radicals, a type of reactive oxygen species (ROS) (Elahi and Kong, 2021). The extract's effect on SOD levels demonstrates its potential to enhance antioxidant defense mechanisms. The SOD levels increased significantly in the extract groups thereby indicating that the extract boosts the body's ability to counteract ROS (Igwe and Eleazu, (2017). Catalase is also an enzyme that breaks down hydrogen peroxide, another type of ROS (Dey *et al.,* 2021). The extract's effect on Catalase levels revealed its impact on hydrogen peroxide detoxification. The extract led to a notable increase in Catalase levels at all doses, suggesting improved antioxidant capacity in the treated rats. Glutathione Peroxidase (GPx) is also an antioxidant enzyme that neutralizes hydrogen peroxide and lipid peroxides. The extract's effect on GPx levels reflects its potential to enhance cellular antioxidant defense. The extract resulted in significant increases in GPx levels across all doses, indicating improved protection against oxidative stress (Shukla *et al.,* 2021).

The results from the histological analyses of male and female reproductive organs in response to *Acanthus montanus* leaf aqueous extract have important implications for reproductive health, especially when in comparison with the control groups. The observed changes suggest potential effects on fertility, reproductive function, and overall well-being In Plate 1 showing the testes the control exhibited thickened seminiferous tubules and pyknotic Sertoli cells, indicating possible perturbations in spermatogenesis. The dosages (200 mg/kg, 400 mg/kg, 800 mg/kg) of *A. montanus* extract all showed circular tubules with varying degrees of pyknosis in Sertoli cells. This could signify a disruption in sperm development and maturation, potentially impacting fertility. In the control penis of Plate 2, the smooth muscles and epithelium appeared normal but at higher dosages (400 mg/kg, 800 mg/kg), there were histological changes suggesting increased cell proliferation in the lining of the urethra. Further histological results in Plate 3 revealed the control ovary showed numerous corpus luteum and healthy ovarian structures, while the treated ovaries (200 mg/kg, 400 mg/kg, 800 mg/kg) displayed varying degrees of corpus luteum changes, germinal epithelium, and zona granulosa alterations. These modifications may indicate potential disruptions in ovulation and hormonal balance, which can affect fertility (Eleazu *et al*., 2013). The Uterus (Plate 4) revealed prominent endometrial glands were observed in the control. While the treated uterine tissues (200 mg/kg, 400 mg/kg, 800 mg/kg of the extract) showed changes in endometrial glands and epithelium. The alterations suggest potential effects on uterine health, which could impact reproductive success.

These histological changes, when compared to the untreated control groups, suggest that *Acanthus montanus* leaf aqueous extract may induce dose-dependent histomorphological alterations in reproductive organs. These alterations in the testes, penis, ovaries, and uterus could have significant implications for reproductive health. Possible disruptions to spermatogenesis, penile structure, ovarian function, and uterine health might impact fertility,

sexual function, and reproductive outcomes. Fuji *et al.* (2006). Further research is essential to understand the mechanisms behind these observed changes and their potential consequences on overall reproductive well-being.

CONCLUSION

In conclusion, the reproductive toxicological effect of *Acanthus montanus* aqueous extract on male and female Wistar rats is evident from the comprehensive analysis of the results and discussion. Further research is essential to elucidate the mechanisms of actions behind the observed changes and their potential consequences on overall reproductive well-being.

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