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RESEARCH ARTICLE

SUB-CHRONIC TOXICITY STUDY OF METHANOL LEAF EXTRACT OF *ANTHOCLEISTA GRANDIFLORA* ON THE REPRODUCTIVE FUNCTIONS AND HAEMATOLOGICAL PARAMETERS OF MALE WISTER RATS

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ABSTRACT

Medicinal plants are an important source of therapeutic compounds, but their safety profiles require thorough scientific evaluation. *Anthocleista grandiflora*, commonly used in African traditional medicine, is reputed for diverse medicinal applications, yet little is known about its toxicological effects. This study investigated the sub-chronic influence of its methanol leaf extract on reproductive and haematological parameters in male Wistar rats. Acute toxicity was assessed using the modified Lorke's method, while sub-chronic evaluation involved daily oral administration of 200, 400, and 800 mg/kg body weight for 28 days. At the end of the treatment period, blood, vas deferens, and testes were collected for hematological profiling, serum testosterone assays, semen analysis, and histopathological examination. The acute toxicity test revealed no mortality, with the median lethal dose (LD_{50}) estimated at greater than 5000 mg/kg, suggesting a broad safety margin. Sub-chronic administration did not produce significant alterations ($P>0.05$) in serum testosterone concentration, sperm count, sperm motility, or testicular weight when compared with controls. However, a mild but non-significant improvement in sperm morphology was observed. Hematological values remained within normal physiological limits, indicating no risk of anemia, immunosuppression, or coagulation impairment at the tested doses. These findings suggest that methanol leaf extract of *A. grandiflora* is relatively safe at sub-chronic doses and does not exert adverse effects on male reproductive function or hematological integrity. Nonetheless, extended studies are required to explore possible long-term consequences, dose-dependent variations, and underlying mechanisms of action.

KEYWORDS

Medicinal plants; Anthocleista grandiflora; Methanol extract; Toxicity; Reproduction; Haematology; Wistar rats.

1. INTRODUCTION

Medicinal plants and herbs have been utilized by humans since ancient times for the treatment of various ailments such as respiratory tract infections, skin diseases, diarrhea, and the common cold (Subramanian et al., 2018; Njan et al., 2019). They have also made remarkable contributions to modern pharmacology, with nearly 25% of current drugs originating from plant sources . In recent years, the global demand for herbal products has risen significantly (Thomford et al., 2015). The World Health Organization (WHO) estimates that nearly 80% of the world's population relies on medicinal plants to meet their healthcare needs, both physical and psychological, with a much higher dependence observed in African countries (Mahomoodally, 2013; Akinyemi, 2000; Sofowora et al., 2013; Abd El-Ghani, 2016). This increasing trend is linked to a preference for natural health remedies, the search for effective therapies for diseases with limited treatment options, and conditions such as HIV infection where current therapies are inadequate (Tang et al., 2017; Rahman et al., 2017; Thomford et al., 2015). Furthermore, concerns about adverse effects of synthetic drugs, misconceptions that herbs are inherently safe, and the high cost of conventional medicine and healthcare facilities have further encouraged the use of medicinal plants (Karimi et al., 2015; Njan et al., 2019; Liu et al., 2016; Njan et al., 2019; Mpinga et al., 2013; Njan et al., 2019).

Anthocleista grandiflora, commonly referred to as the "forest fever tree," is a tall and slender evergreen species reaching 6–30 meters in height, with large terminal leaves that can grow up to 100 × 50 cm. It thrives in medium to low elevation rainforests of Zimbabwe, Swaziland, and north-eastern South Africa. The plant bears cream-colored bisexual flowers, while its roots, stems, bark, leaves, and flowers are traditionally considered medicinal, although it is not used as food (Sunday et al., 2022).

In Nigerian traditional medicine, different parts of *A. grandiflora* are widely applied for therapeutic purposes (Onwusonye et al., 2018). Its leaves and bark are employed in malaria treatment, while the bark is also used for diarrhea, hypertension, diabetes, and venereal diseases. Additionally, the bark has applications in managing epilepsy, hepatitis, and fever, with in vitro studies showing antiviral activities of public health importance (Boon, 2010).

Male reproduction is a highly coordinated process involving the testes, epididymis, hormones, and accessory sex glands. Fertility is largely dependent on spermatogenesis and steroidogenesis within the testes, but the reproductive system remains vulnerable despite its strong protective mechanisms (D'Cruz et al., 2010).

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Hematological parameters are valuable indicators of physiological status and toxicological effects following exposure to xenobiotics (Joshi et al., 2002). Blood serves as a critical medium for transporting oxygen and nutrients while eliminating metabolic wastes (Cheeke and Shull, 1999; Mpinga et al., 2013). It constitutes about 7% of body weight and comprises erythrocytes, leukocytes, thrombocytes, and plasma (Austin and Perkins, 2006). Hematopoiesis originates from hematopoietic stem cells (HSCs) and progenitor cells, which generate all mature blood cell lineages. Each blood cell type plays essential roles in maintaining normal physiological balance. For example, erythrocytes transport oxygen from pulmonary circulation to peripheral tissues and facilitate carbon dioxide removal (Jagger et al., 2001; Mpinga et al., 2013). Leukocytes function as immune cells, providing both innate and adaptive defense, and are classified into neutrophils, eosinophils, monocytes, lymphocytes, and basophils (Mosmann and Coffman, 1989; Mpinga et al., 2013). Platelets contribute not only to hemostasis but also to vascular repair, thrombosis, inflammation, atherosclerosis, and even tumor progression (Harrison, 2005).

Assessment of hematological indices provides insights into how foreign agents, including plant extracts, may alter blood composition (Ashafa et al., 2009). Hence, this study was designed to evaluate the effects of sub-chronic oral administration of the methanol leaf extract of *Anthocleista grandiflora* on hematological and reproductive functions in male Wistar rats.

2. Materials and Methods

2.1 Experimental Animals

Male Wistar rats weighing between 155–230 g were used for this study. The animals were obtained from the Animal House of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria, and housed in the same facility throughout the experiment. They were kept in clean, well-ventilated cages with daily bedding changes, maintained under a 12-hour light/dark cycle, and at an ambient temperature of 26–27 °C. Before the commencement of the experiment, the rats were allowed a two-week acclimatization period. During this time, they were given unrestricted access to tap water and fed with standard pelletized grower feed (Vital Feed Ltd, Jos, Nigeria).

2.2 Plant Material and Extraction

Fresh leaves of *Anthocleista grandiflora* were collected from Igbanke village in Orhionwon Local Government Area, Edo State, Nigeria. The plant was identified and authenticated by a taxonomist at the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, where a voucher specimen (UBH-W44) was deposited for reference.

The leaves were separated from the stems, rinsed with clean water to remove dust and debris, and air-dried at room temperature until constant weight was achieved. The dried leaves were pulverized into fine powder using a mechanical grinder and stored in an airtight amber container. Eight kilograms (8 kg) of powdered material were soaked in 3 L of 99.9% methanol for 72 hours with intermittent shaking. The mixture was filtered using a porous sieve lined with wire gauze. The filtrate was concentrated using a water bath at 40 °C to obtain a brownish-green, jelly-like crude extract, which was stored in a sterile glass container under refrigeration until required. The final yield was weighed and the percentage recovery was determined.

2.3 Oral Median Lethal Dose (LD_{50}) Determination

The acute toxicity of the methanol leaf extract of *A. grandiflora* was determined using the modified Lorke method (Lorke, 1983). In the first phase, nine mice (mixed sexes) were divided into three groups (n = 3) and administered 10, 100, and 1000 mg/kg of extract orally. The animals were observed for 24 hours for signs of mortality or toxicity. Since no deaths occurred, the study progressed to phase two, in which fresh groups of mice were individually treated with 2000, 3000, 4000, and 5000 mg/kg of extract. The animals were monitored for immediate signs of toxicity and death for three days, followed by 30 minutes of daily observation for the next eleven days (Ozolua et al., 2010). Gross toxicological symptoms were monitored and the LD_{50} was calculated as follows;

$LD_{50} = (D_0 + D_{100})$.

Where: D_0 = Highest dose that gave no mortality

D_{100} = Lowest dose that produced mortality.

2.3.1 Experimental Design

As no deaths were recorded at 5000 mg/kg, three graded doses (200, 400, and 800 mg/kg) were selected for the sub-chronic study, following the approach of (Bautista et al., 2004; Ozolua & Uwaya, 2013; Prasanth et al., 2015).

2.4 Reproductive Toxicity Studies

Twenty (20) male rats (155–230 g) were randomly allocated into four groups (n = 5). Group I served as the control and received distilled water daily, while Groups II, III, and IV were treated with 200, 400, and 800 mg/kg of the methanol leaf extract of *A. grandiflora* respectively, administered orally for 28 days. These doses were chosen based on the LD_{50} results. At the end of the study, animals were fasted overnight and euthanized under halothane anesthesia. Blood was collected via the abdominal aorta using a 5 ml syringe (Monoject Pharmaceutical Ltd., Nigeria) and dispensed into plain vacutainer bottles without anticoagulants (BD Vacutainer®, Plymouth, UK) (Ozolua et al., 2009). The testes were carefully excised through laparotomy.

2.4.1 Determination of Testosterone Levels

Blood samples were left to clot in plain bottles and centrifuged at 3000 rpm for 10 minutes using a tabletop centrifuge (Alpin Medical, England) (40). The clear serum was separated with Pasteur pipettes and stored at –20 °C in labeled plain bottles until analysis. Serum testosterone concentration was determined using commercial ELISA kits (Randox Laboratory Ltd, UK), following the manufacturer's protocol. Absorbance was read at 450 nm and serum levels were calculated from a standard calibration curve.

2.4.2 Semen Collection and Analysis

At the end of treatment, animals were sacrificed, and orchidectomy was performed using the open castration method. A midline pre-scrotal incision was made to expose and excise the testes. Spermatozoa were collected from the vas deferens, which was ligated, cut, and transferred into sterile Petri dishes containing 6 µl of normal saline. The vas deferens was teased to release sperm cells. A drop of sperm suspension was mounted on a clean glass slide, covered with a cover slip, and examined microscopically for sperm count, motility, and morphology (Oyedeji et al., 2013).

2.4.3 Sperm Motility

Sperm suspensions were mounted on grease-free slides and examined at ×20 and ×40 magnifications. Motility was classified and expressed as percentages of progressively motile, non-progressive, and immotile spermatozoa (Brazil et al., 2004; WHO, 2010; Ibeh et al., 2018)

2.4.4 Sperm Count

Semen samples were diluted at a ratio of 1:100 with a solution containing 5 g $NaHCO_3$, 25 mg eosin, and 1 ml of 35% formalin in 100 ml distilled water. Ten microliters of diluted semen were charged into the counting chambers of an improved Neubauer hemocytometer and allowed to stand for 5 minutes. Counts were performed at ×40 magnifications, expressed as million sperm/ml of semen, and repeated twice for accuracy.

2.4.5 Sperm Morphology

Sperm smears were prepared on grease-free slides, air-dried, and stained with Improved Eosin–Leishman stain for 15 minutes. After rinsing and drying, slides were examined at ×400 magnification. At least 30 fields per slide were assessed, and spermatozoa were categorized as normal or abnormal, expressed as percentages (Ibeh et al., 2018; Ibeh et al., 2017).

2.5 Hematological Analysis

Hematological parameters including white blood cell (WBC) count, platelet count (PC), packed cell volume (PCV), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), monocytes (MON), lymphocytes (LYM), and granulocytes (GRAN) were analyzed using an automated hematology analyzer at the University of Benin Teaching Hospital (UBTH), Benin City, Nigeria.

2.6 Statistical Analysis

All results were expressed as mean ± standard error of mean (SEM). Differences between treated and control groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test in SPSS (version 20). A p-value of <0.05 was considered statistically significant.

3. RESULTS

3.1 Acute Toxicity Test

Table 1: Results of acute toxicological test of methanol seed extract of *A. grandiflora* on mice

Dose mg/Kg	Number of deaths/number of rats	Mortality (%)	Signs of toxicity
10	0/3	0	Nil
100	0/3	0	Nil
1000	0/3	0	Nil
2000	0/3	0	Nil
3000	0/3	0	Nil
4000	0/3	0	Nil
5000	0/3	0	Nil

The animals were observed for 72hrs and then for additional 11 days

Table 2: Effect of oral administration of methanol leaf extract of *A. grandiflora* on the reproductive functions of male Wister rats on the 28th day.

Group	Sperm Count ($x10^6$sperm/mm^3)	PM	NPM	Immotile	Percentage Morphology	Testosterone (ng/ml)
Control	744.2±45.47	70.80±4.33	17.40±1.94	11.80±2.78	95.80±1.16	0.5708±0.0396
200 mg/kg	879.6±28.92	77.40±3.29	15.40±1.50	11.00±3.11	98.60±0.24*	0.7038±0.2027
400 mg/kg	836.8±73.50	72.40±3.78	18.80±2.31	8.80±2.39	98.70±0.53*	1.4310±0.2676
800 mg/kg	693.4±40.18	67.60±3.36	22.20±1.93	10.20±1.77	99.08±0.04*	1.0850±0.4435

Values are presented as Mean ± SEM, n=5. Data analyzed by one-way ANOVA followed by turkey multiple test comparison. Statistically, all parameters analyzed were not significant when compared to the control except for morphology at $p<0.05$. PM=Progressive motility. NPM=Non-progressive motility. % M=Percentage morphology.

Table 3: Effect of oral administration of methanol leave extract of *A. grandiflora* on weight of spleen and testis of male Wister rats on the 28th day.

Group	Weight of Spleen(g)	Weight of Testis(g)
Control	0.7260±0.0343	1.386±0.067
200 mg/kg	0.6435±0.0327	1.389±0.077
400 mg/kg	0.6429±0.066	1.244±0.042
800 mg/kg	0.7830±0.055	1.415±0.003

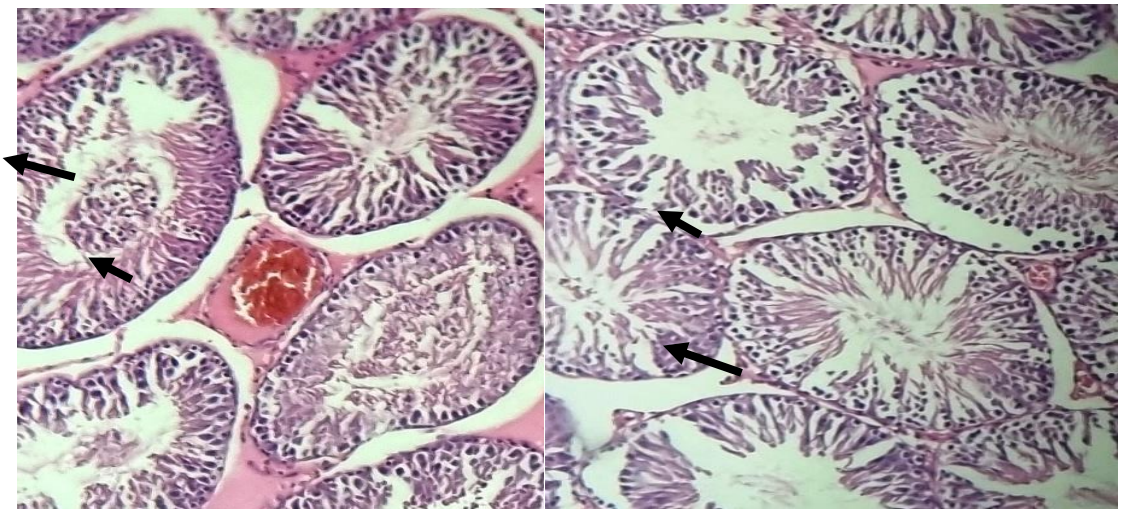
Values are presented as Mean ± SEM, n=5. Data analyzed by one-way ANOVA followed turkey multiple test comparison. Statistically, all parameters analyzed were not significant when compared to the control at $p>0.05$.

Table 4: Mean values of the effect of a 28-day oral dosage of methanol leaf extract of *Anthocleista grandiflora* on hematological parameters in male Wister rats.

Parameters	Control	200 mg/kg	400 mg/kg	800 mg/kg
PLT (10^3/µl)	651.6±42.08	687.4±51.93	665.6±81.42	728.4±40.24
WBC (10^3/µl)	12.60±0.811	10.26±0.958	10.58±0.868	14.08±0.761
LYM (10^3/µl)	10.16±0.630	8.200±0.745	8.780±0.745	11.48±0.530
MON (10^3/µl)	1.740±0.183	1.520±0.142	1.480±0.131	1.960±0.289
GRAN (10^3/µl) RBC(10^{-6}/µl)	0.70±0.26 6.912±0.198	0.58±0.16 6.754±0.096	0.38±0.06 6.604±0.218	0.64±0.13 6.996±0.183
HGB (g/dl)	15.68±0.34	15.02±0.25	14.76±0.41	14.60±0.40
HCT (%)	38.14±0.92	36.88±0.77	36.60±0.91	36.22±1.11
PCT(%)	0.528±0.003	0.525±0.030	0.456±0.056	0.567±0.025
MCV (μm^{-3})	55.22±0.513	54.58±0.453	55.48±0.7453	68±0.6644
MCH (pg)	22.60±0.288	22.24±0.156	22.40±0.221	20.90±0.301
MCHC(g/dl)	41.14±0.593	40.74±0.215	40.30±0.378	40.34±0.503

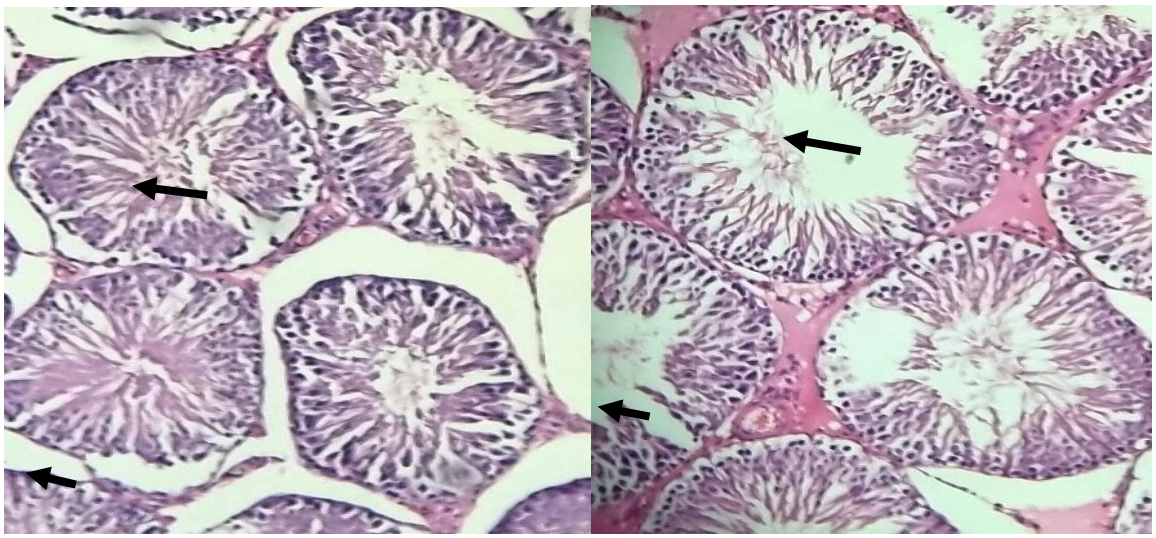
Data are presented as mean ± S.E.M, n = 5. P<0.05: Not significantly different from control group.

WBC=White blood Cell; LYM=Lmphocyte; MON=Monocyte; GRAN=Granulocyte; RBC=Red blood cell; HGB= Heamoglobin; HCT=Heamatocrit; PLT=Platelet; MCV=Mean Corpuscular Volume; MCH=Mean Corpuscular Heamoglobin; MCHC=Mean Corpuscular Heamoglobin Concentration; PCT=Procalcitonin.



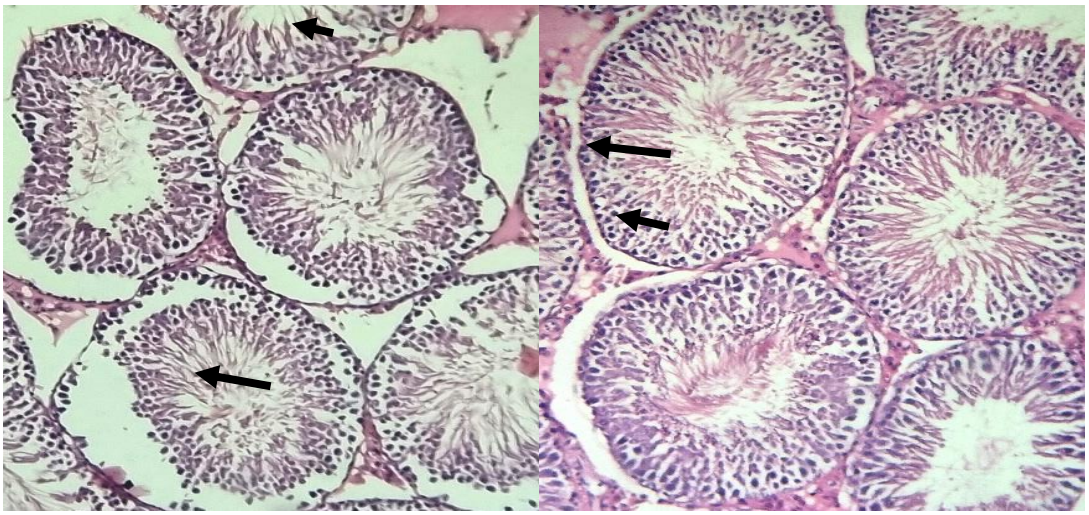
Testis sections reveal seminiferous tubules that appear not so prominent in outline (long arrow) and pyknotic Sertoli cells and other cells of spermatogenic series with visible leydig cells and prominent oedematous changes (short arrow).

(400mg/Kg) Testis (X400 MAGNIFICATION)



Testis sections reveal thickened seminiferous tubules that appear round in outline (long arrow) and pyknotic Sertoli cells and other cells of spermatogenic series with congested visible leydig cells (short arrow).

(800mg/Kg) Testis (X400 MAGNIFICATION)



Testis sections reveal thickened seminiferous tubules that appear not so prominent in outline (long arrow) and pyknotic Sertoli cells and other cells of spermatogenic series with visible leydig cells and mild oedematous changes (short arrow).

Figure 1: Photomicrographs showing the effects of methanolic extract of *Anthocleista grandiflora* leaves on testicular and spleen histology (2a–d). Haematoxylin and Eosin staining x400.

4. Discussion

This study evaluated the sub-chronic effects of methanol leaf extract of *Anthocleista grandiflora* on male reproductive function and hematological indices in Wistar rats. Parameters assessed included serum testosterone, sperm characteristics, testicular histology, and blood profile as an indicator of systemic health (Muyibi et al., 2000; Njan et al., 2019).

Toxicological investigations are crucial for determining the safety profile of medicinal plant extracts. They help define toxicity thresholds, identify sensitive species, detect target organ effects, and provide essential data for risk assessment in both acute and long-term exposures. The toxicity of plants may vary depending on the organ sampled, phytochemical composition, storage form, and seasonal changes (Jaouad et al., 2004). The median lethal dose (LD_{50}) remains a standard index for estimating acute toxicity (Oyedeji et al., 2013).

Table 1 presents the findings of the acute toxicity study of the methanol leaf extract of *A. grandiflora* in mice. Oral administration of extract doses ranging from 10 mg/kg to 5000 mg/kg did not result in mortality in any of the test groups. No observable signs of toxicity were detected at all dose levels. The animals were monitored closely for 72 hours following administration and subsequently for an additional 11 days, during which no adverse behavioral or physiological changes were observed. This indicates that the extract is relatively safe, consistent with reports that substances with LD_{50} values above 5000 mg/kg are considered practically non-toxic (Ahmed, 2015; Ihekwereme et al., 2018).

Testosterone plays a central role in spermatogenesis and the maintenance of male reproductive functions (Mahomoodally, 2013; Prathima et al., 2017). It is regulated by luteinizing hormone (LH), produced by the anterior pituitary, with feedback control exerted by Leydig cells (Njan et al., 2019). Disruption of this endocrine axis by toxicants can impair fertility. Findings from this study showed that serum testosterone was elevated in extract-treated groups, although not significantly different from the control ($P < 0.0001$). This increase may suggest a stimulatory effect of the extract on Leydig cells, leading to enhanced testosterone biosynthesis.

Semen analysis provides direct insight into male fertility potential (Khatun et al., 2018). Core parameters include sperm count, motility, viability, and morphology (Prasanth et al., 2015). The present study demonstrated that extract administration had no adverse effects on sperm parameters, except for a significant improvement in sperm morphology, where the proportion of normal spermatozoa was higher across all treatment groups ($P < 0.05$). At lower doses (200 and 400 mg/kg), sperm counts were slightly increased compared with controls, though not significantly ($P > 0.05$), whereas a decline was observed at the highest dose (800 mg/kg). Similarly, sperm motility improved at lower doses but decreased at 800 mg/kg. These findings suggest a possible dose-dependent effect, with lower doses potentially enhancing epididymal function and membrane stabilization, possibly through antioxidant activity (Njan et al., 2019). Since progressive motility is critical for natural and assisted fertilization, the observed increase at lower doses indicates a potential fertility-promoting effect of the extract (WHO, 2010). The observed improvement in sperm morphology, particularly the reduction in abnormal tail structures, further suggests that the extract did not disrupt spermatogenesis.

Changes in reproductive organ weight, especially the testes, serve as sensitive indicators of testicular toxicity (Mangelsdorf et al., 2003). Alterations in testicular weight may reflect damage to seminiferous tubules, interstitial edema, or impaired spermatogenesis (Sellers et al., 2007). In this study, extract administration had no significant impact on testicular weight or semen indices compared to controls ($P < 0.05$), further supporting its non-toxic nature at the tested doses.

Evaluation of hematological indices is equally important since blood reflects systemic health and hematopoietic activity (Obianime et al., 2010). White blood cells and their differentials provide insight into immune status, with elevations typically indicating immunological responses (Aprioku & Igbe, 2017). Extract administration caused a non-significant increase in WBC and lymphocyte counts at 800 mg/kg, suggesting a possible immune-stimulatory effect. Other parameters such as RBC, Hb, and PCV remained unaffected, implying no risk of anemia. Platelet counts also showed a mild, non-significant increase across treatment groups, indicating that coagulation functions were not compromised.

Overall, findings from this study demonstrate that methanol leaf extract of *A. grandiflora* is relatively safe at the tested doses, with no significant toxic effects on hematological parameters, reproductive hormones, or semen quality. At low doses, the extract may even enhance testosterone levels, sperm motility, and morphology, suggesting a possible fertility-supporting role.

5. Conclusion

This study demonstrated that sub-chronic oral administration of methanol leaf extract of *Anthocleista grandiflora* did not produce significant toxicological effects on reproductive indices or hematological parameters in male Wistar rats. The absence of mortality in the acute toxicity test at doses up to 5000 mg/kg further suggests that the plant extract possesses a wide margin of safety. While minor variations in sperm parameters and immune-related indices were observed, these changes were not statistically significant, indicating that the extract may be relatively safe within the tested therapeutic range.

Given the ethnomedicinal importance of *A. grandiflora* and its reported use in managing conditions such as malaria, hypertension, and diabetes, the findings of this study provide preliminary evidence supporting its potential role as a safe herbal remedy. However, further studies involving chronic exposure, phytochemical characterization, and mechanistic evaluations are warranted to confirm its long-term safety and potential pharmacological benefits.

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