Original Research Article

Ethanol Extract of *Xylopia aethiopica* (African Negro Pepper) Fruit adversely perturbed Semen Qualities in Male Wistar Rats

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ABSTRACT

Aim: This study was designed to assess the effect of *Xylopia aethiopica* fruit on the sperm qualities of male Wistar rats.

Methodology: The fruits of *Xylopia aethiopica* were air-dried and extracted by Soxhlet extractor using ethanol as solvent. The median lethal dose (LD_{50}) of the extract was assessed using standard method. Thirty adult Wistar rats were divided into five groups of six rats each. Animals in groups 1, 2, 3, and 4 were treated with 130, 259, 389 and 518 mg/kg body weight of *X. aethiopica* fruit extract respectively, while those in group 5 received normal animal feeds and water only. The administration was done once daily for 28 days via oral route. At the end of 28 days treatment, animals were sacrificed under ether anaesthesia in a desiccator after an overnight fast. The cauda epididymis were separated from both testes and tinged with 2 mL of normal saline then teased the cauda epididymis of each rat. The suspension was mixed through a metallic net to avoid any other tissue contamination. This suspension was used for the determination of the sperm parameters.

Results: Ethanol extract of *Xylopia aethiopica* fruit was observed to significantly perturbed sperm parameters of animals after 28 days of treatment. Sperm count and motility were significantly reduced by *Xylopia aethiopica* fruit in a dose-dependent manner when compared with those of the control group (*P<0.05)*. Administration of *Xylopia aethiopica* fruit increased sperm mortality and abnormality when compared with the control animals (P<0.05). Seminal pH was decreased by ethanol extract of *Xylopia aethiopica* fruit administration when compared with those in control animals (P<0.05).

Conclusion: The findings of this study revealed that ethanol extract of *Xylopia aethiopica* fruit adversely perturbed sperm quality of Wistar rats. This might not automatically translate to same effect in human. However, men interested in child-bearing should minimize its consumption.

Keywords: Potent contraceptive; sperm qualities; *Xylopia aethiopica* fruit consumption

1. INTRODUCTION

Infertility is defined as the inability to achieve pregnancy after 12 months of unprotected intercourse [1]. Male infertility is found in 50% of infertile couples [2]. According to Speroff and Fritz [3], 55% of the reasons for infertility are found to be male-related and 35% to be female-related, while 10% constitutes infertility of unknown origin [3]. Some of the etiologies of declining male fertility can be related to falling androgen levels, decreased sexual activity, alterations in sperm quality, especially, motility, morphology, and DNA integrity [4]. Gonadotropin releasing hormone (GnRH) secreted by the hypothalamus elicits the release of gonadotropins i.e. follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland [5]. LH is a glycoprotein that regulates testosterone synthesis by the extra-

tubular Leydig cells. The other gonadotropic hormone, FSH controls spermiocytogenesis and spermiogenesis by affecting both the germinal epithelium and Sertoli cells [6]. The levels of these hormones are under negative feedback control by the gonads [7]. Testosterone is responsible for normal growth, development of male sex organs, and maintenance of secondary sex characteristics. A high intra-testicular level of testosterone is an absolute prerequisite for sperm production, and function. Testosterone improves sperm motility and epididymis function [8]. Failure of pituitary gland to secrete FSH and LH will result in disruption of testicular function leading to infertility [9].

Semen is an organic fluid that contains spermatozoa. It is secreted by the gonads (sexual glands) and other accessory sex organs of male, and can fertilize female ova. In humans, semen contains several components besides spermatozoa: proteolytic and other enzymes as well as fructose which is the major energy source of spermatozoa, and provide a medium through which they can move or "swim" [10]. Male infertility can be assessed through semen analysis and hormonal profile [11].

Male impotence also called erectile dysfunction (ED) is a common medical condition that affects the sexual life of millions of men worldwide [12,13]. Erectile dysfunction is defined as the inability of a man to achieve and maintain an erection sufficient for naturally satisfactory intercourse.
Sexual dysfunction is a serious medical and social symptom that occurs in 10-52% of men and 25-63% of women [14]. It is the repeated inability to achieve normal sexual intercourse male impotence (or) erectile dysfunction is a significant problem that may contribute to infertility [15]. Erectile dysfunction is adversely affected by diabetes mellitus, antihypertensive, antipsychotic, antidepressant therapeutic and antimalarial drugs [16].

Generally, herbal medicines are perceived to be a safer source of medicine, this perception emanates from the idea of ‘‘green is safe’’ [17,18]. As such, not much research and clinical studies are conducted to assess the safety or toxicity of most herbal preparations in use as is done in the case of orthodox medicines. The story is no different in the case of *Xylopia aethiopica* fruit. Despite its extensive use in traditional medicine and although a remarkable number of *in vitro* and animal studies have been conducted to confirm its therapeutic uses, not much has been done in assessing the safety or toxicity of most of its bioactive constituents. Conversely, it is worthy of note that some research has been conducted in this regard. In a test for acute toxicity using the brine shrimp (*Artemia salina*) bioassay, it was observed that the hexane extract of *Xylopia aethiopica* dried fruits had low toxicity with LC_{50} of 0.30 ng/mL whilst xylopic acid and its derivative, deacetyl xylopic acid both showed an LC_{50} of 0.50 ng/mL. Again, in qualitative/semi quantitative test for toxicity, the Hippocratic test on rats was used in a 5 day follow-up period after a single intraperitoneal (i.p.) injection. Here, the hexane extract, xylopic acid and deacetyl xylopic acid all showed no toxicity at a dose of 20 mg/kg body weight [19]. In another research, the essential oil from the fruits of *X. aethiopica* was shown to be toxic to *Artemia salina* at concentrations ranging from 10 to 1000 µg/mL [20]. Koba *et al*. [21] investigated the *in vitro* cytotoxicity of essential oil from *X. aethiopica* fruits. The cytotoxicity was evaluated using the human epidermal cell line HaCaT. Here, it was observed that at concentrations in the range of 50-1500 µg/mL, the tested essential oil did not show any cytotoxicity but rather induced a significant increase in cell viability (up to 130%), suggesting their potential as cytoprotectors or antioxidants. At higher concentrations ranging from 1600 to 3000 µg/mL, a similar toxicity profile was recorded. Xylopic acid isolated from the dried fruits of *Xylopia aethiopica*, in a study conducted by Woode *et al*. [22], when administered at doses of 10, 30 and 100 mg/kg to male albino rats, caused visible cytotoxic activity by clearing all matured spermatozoa, germ cells and other cell in the seminiferous tubules when compared with the control group. These effects were however reversed when the treated rats were allowed a two-week treatment free period of recovery. These findings therefore suggest that xylopic acid possesses reversible spermatotoxic and antifertility effects at the doses tested. An ethanolic extract of a combination of equal quantities of *Alstonia congensis* bark and *Xylopia aethiopica* fruits have been investigated for acute and sub-acute toxicity [23]. In the acute toxicity study, there were no observable changes in the behaviour and sensory nervous system responses. Also no adverse gastrointestinal effects were observed in male and female mice used in the experiment. At a 20.0 g/kg dose, all the mice that received the

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extract survived beyond the 24 hours of observation. It was therefore inferred that the median acute toxicity value (LD_{50}) of the extract must be above 20.0 g/kg body weight. Although not entirely representative, these findings to some extent show that ethanolic extract of *Xylopia aethiopica* fruits is relatively safe [23].

Xylopia aethiopica is known to have myriads of chemical constituents with diverse therapeutic and pharmacological properties. These compounds, most of which have been isolated and characterized, include saponins, sterols, carbohydrates, glycosides, mucilage, acidic compounds, tannins, balsams, cardiac glycosides, volatile aromatic oils, phenols [24,25], alkaloids, rutin and fixed oils [26,27]. The plant also contains vitamins A, B, C, D, and E, and proteins together with high amounts of minerals like copper, manganese and zinc [25,27]. The effect of on body weight and glucose concentration of animals has been reported [28]. The fruit has also been reported to induce dyslipidemia [29], hepatotoxicity [30] as well as renal toxicity [21]. This study was designed to assess its effect on the sperm qualities of male Wistar rats.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plant Materials

The fruits of *Xylopia aethiopica* were obtained from new market in Aba, Abia State and were identified and authenticated by Prof. (Mrs) Margaret Bassey of the Department of Botany and Ecological Studies, University of Uyo with the voucher number UU/PH/4e. The plant was deposited in the Herbarium of the Department of Pharmacognosy and Natural Medicine, University of Uyo, Akwa-Ibom State, Nigeria.

2.2 Extraction of Plant Materials

The extraction was carried out in the Post-graduate Laboratory of Department of Pharmacognosy and Natural Medicine, Faculty of Pharmacy, University of Uyo, Nigeria. It was carried out according to the method described by Ogbuagu et al. [32]. The fruits were washed under running tap water to remove contaminants and air-dried. The plant material was pulverized using laboratory blender to provide a greater surface area. The pulverized plant material was macerated in 250 mL of 99.8% ethanol (Sigma Aldrich) contained in round bottom flask, which was then attached to a Soxhlet extractor coupled with condenser and heating mantle (Isomantle). It was then loaded into the thimble, which is placed inside the Soxhlet extractor. The side arm is lagged with glass wool. The mixture was heated using the heating mantle (Isomantle) at 60 °C and as the temperature increases it begins to evaporate, moving through the apparatus to the condenser. The condensate then drips into the reservoir containing the thimble. Once the level of solvent reaches the siphon it pours back into the flask and the cycle begins again. This continues until it is exhaustively extracted. The process runs for a total of 13 hours. Once it was set up, it was left to run without interruption as long as water and power supply were not interrupted. The equipment was turned on and off and overnight running was not permitted, and the time split over a number of days. The extract was poured into 1000 mL beaker and concentrated to dryness in water bath (A3672-Graffin Student Water Bath) at 35 °C. The total weight of the marc (residue) and the concentrated extract were recorded, these processes took several days. The dried extract was preserved in the refrigerator at 4°C for further analysis.

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

The median lethal dose (LD_{50}) of the extract was estimated using albino mice according to the method described by Airaodion et al. [33]. This method involves two phases:

In Phase one, five groups containing five mice each weighing between 20 g and 27g were fasted for 18 hours. They were respectively administered 1000 mg/kg, 2000 mg/kg, 3000 mg/kg, 4000 mg/kg and 5000 mg/kg body weight intraperitoneally (i.p) and were observed for physical signs of toxicity and mortality for 24 hours. A dosage of 1000 mg/kg recorded 0% mortality while 2000 mg/kg, 3000 mg/kg 4000 mg/kg and 5000 mg/kg recorded 100% mortality within 24 hours. Based on the value of phase one, phase two was conducted.

In Phase two, twenty-five albino mice weighing between 20 - 27g were grouped into five of five mice per group and were fasted for 18 hours. Each group was administered 1200 mg/kg, 1400 mg/kg 1600 mg/kg, 1800 mg/kg and 2000 mg/kg body weight intraperitoneally (i.p) and was observed for physical signs of toxicity and

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mortality within 24 hours. 1200 mg/kg recorded 0% mortality while 1400 mg/kg, 1600 mg/kg, 1800 mg/kg and 2000 mg/kg recorded 100% mortality within 24 hours. The LD_{50} was calculated as geometrical means of the maximum dose producing 0% (a) and the minimum dose producing 100% mortality (b).

$$LD_{50} = \sqrt{ab}$$

2.4 Experimental Design

Thirty adult male Wistar rats obtained from the University of Uyo, Nigeria were used for this study. They were acclimatized for seven days before the commencement of the experiment. They were weighed and divided into five groups of six rats each. Groups A, B, C, D served as the experimental groups, while group E served as the control. Animals in group A were administered 130 mg/kg body weight (10% of LD_{50}) of *X. aethiopica* fruit extract, those in group B were administered 259 mg/kg body weight (20% of LD_{50}) of *X. aethiopica* fruit extract, those in group C were administered 389 mg/kg body weight (30% of LD_{50}) of *X. aethiopica* fruit extract, those in group D were administered 518 mg/kg body weight (40% of LD_{50}) of *X. aethiopica* fruit extract, while those in group E (control) received normal feeds and water only. The administration was done once daily for 28 days via oral route. At the end of 28 days treatment, animals were sacrificed under ether anaesthesia in a desiccator after an overnight fast. The cauda epididymis were separated from both testes and tinged with 2 mL of normal saline then teased the cauda epididymis of each rat. The suspension was mixed through a metallic net to avoid any other tissue contamination. This suspension was used for the determination of the sperm parameters.

2.5 Determination of Sperm Qualities

2.5.1 Determination of Sperm Count

Sperm count was determined using the haemocytometer method [34]. A 1:20 dilution from each well-mixed sample was prepared by diluting 50 µL of liquefied semen with 950 µL diluent. The latter was prepared by adding 50 g of sodium carbonate ($NaHCO_3$), 10 mL of 35% (v/v) formalin and, 0.25 g of trypan blue or 5 mL of saturated aqueous gentian violet to distilled water and the solution was made up to a final volume of 1000 mL. Both chambers of the haematocytometer are scored and the average count is calculated,

2.5.2 Determination of Sperm Motility

Sperm motility was determined according to the method described by Larsen *et al*. [35].The sample was thoroughly mixed and an aliquot was immediately removed, allowing no time for the spermatozoa to settle out of suspension. The sample was remixed before removing a replicate aliquot. For each replicate, a wet preparation approximately 20 μm deep was prepared. The sample was allowed to stop drifting (within 60 seconds). The slide was examined with phase-contrast optics at ×200 or × 400 magnifications.

2.5.3 Determination of Sperm Abnormality

Abnormality of spermatozoa was determined according to the method described by Airaodion *et al*. [36]. A film of semen was prepared on slide. These films on slide were fixed in methanol. The slides were stained in eosine for 40 minutes. The films were washed in tap water and after drying, the slides were examined under the microscope to see abnormality of spermatozoa.

2.5.4 Determination of Sperm Mortality

Sperm mortality was determined as the difference between sperm motility and abnormality.

2.5.5 Determination of Seminal pH

Seminal pH was determined using pH paper in the range 6.0 to 10.0 according to the method described by Airaodion *et al*. [37].The sample was thoroughly mixed and a drop was evenly spread on the pH paper**.** The colour of the impregnated zone became uniform after about 30 seconds and the colour was compared with the calibration strip to read the pH, and the corresponding value was recorded.

2.6. Statistical Analysis

Results are expressed as mean ± standard deviation. The levels of homogeneity among the groups were assessed using One-way Analysis of Variance (ANOVA) followed by Tukey's test. All analyses were done using Graph Pad Prism Software Version 5.00 and P values < 0.05 were considered statistically significant.

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3. RESULT

3.1 Median Lethal Dose (LD_{50}) Result

The physical signs of toxicity observed in the animals included excitation, paw licking, increased respiratory rate, decreased motor activity, gasping and coma which was followed by death. In the first phase of the median lethal dose determination, no mortality was recorded in the group treated with 1000 mg/kg body weight of *X. aethiopica* fruit extract. However, 100 % mortality was recorded in the groups treated with 2000, 3000, 4000, and 5000 mg/kg body weight of *X. aethiopica* fruit extract respectively. Similarly, in the second phase of medial lethal dose determination, no mortality was recorded in the group treated with 1200 mg/kg body weight of *X. aethiopica* fruit extract while 100% mortality was recorded in the groups treated with 1400, 1600, and 1800 mg/kg body weight of *X. aethiopica* fruit extract respectively as presented in table 1.

The median lethal dose (LD_{50}) was calculated as geometrical means of the maximum dose producing 0% (a) and the minimum dose producing 100% mortality (b).

$LD_{50} = \sqrt{ab}$

Where a = 1200 mg/kg

b = 1400 mg/kg

LD_{50}= 1296.15 mg/kg

3.2: Effect of ethanol extract of *Xylopia aethiopica* fruit on Sperm Parameters of Animals after 28 days of Treatment

Ethanol extract of *Xylopia aethiopica* fruit was observed to significantly perturbed sperm parameters of animals after 28 days of treatment, as presented in Figs. 1-5. Sperm count and motility were significantly reduced by *Xylopia aethiopica* fruit in a dose-dependent manner when compared with those of the control group (*P<0.05)*. Administration of *Xylopia aethiopica* fruit increased sperm mortality and abnormality when compared with the control animals (P<0.05). Seminal pH was decreased by ethanol extract of *Xylopia aethiopica* fruit administration when compared with those in control animals (P<0.05).

Table 1: The Median Lethal Dose (LD_{50}) of *Xylopia aethiopica* Fruit Extract

Study Phase/ (Animal)	Dosage of Extract (mg/kg) b.w	No of Mice per Group	No. of Death Recorded	% Mortality
PHASE ONE				
I	1000	5	0	0
II	2000	5	5	100
III	3000	5	5	100
IV	4000	5	5	100
V	5000	5	5	100
PHASE TWO				
I	1200	5	0	0
II	1400	5	5	100
III	1600	5	5	100
IV	1800	5	5	100

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V	2000	5	5	100

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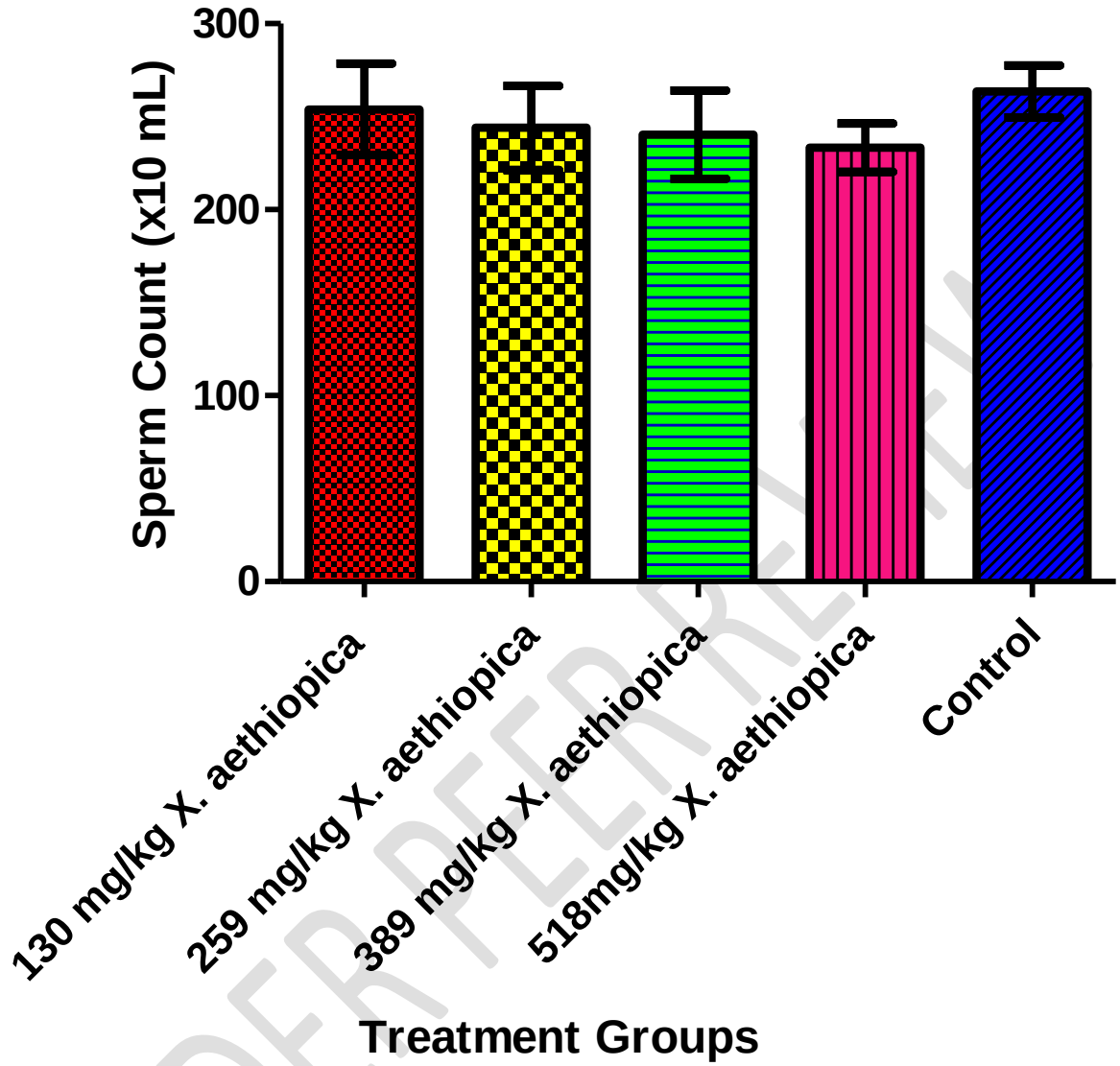

Fig. 1: Effect of *X. aethiopica* fruit extract on Sperm Count of Animals after 28 days of Treatment

Each bar represents mean ± SD with n = 6.

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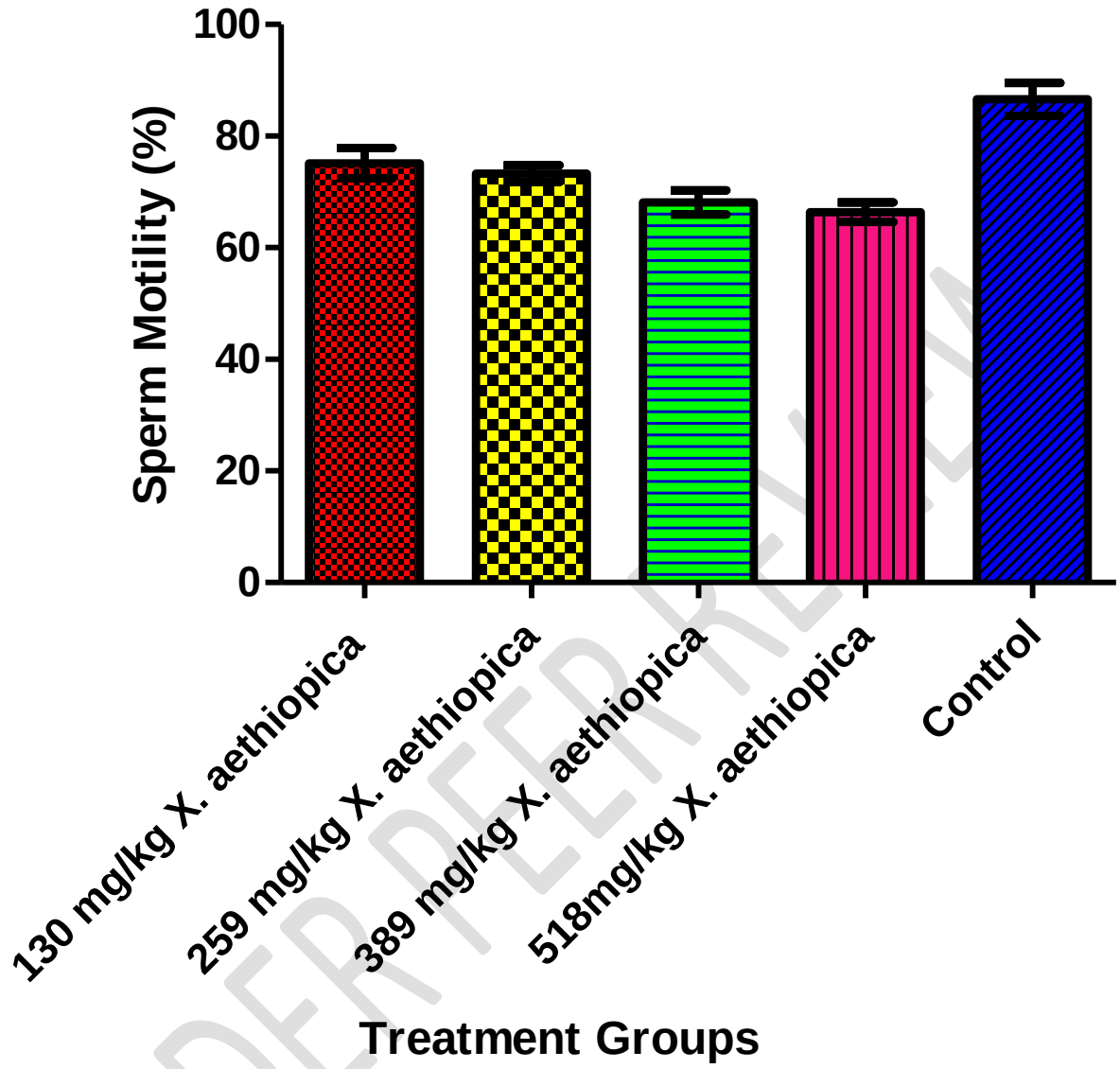

Fig. 2: Effect of *X. aethiopica* fruit extract on Sperm Motility of Animals after 28 days of Treatment

Each bar represents mean ± SD with n = 6.

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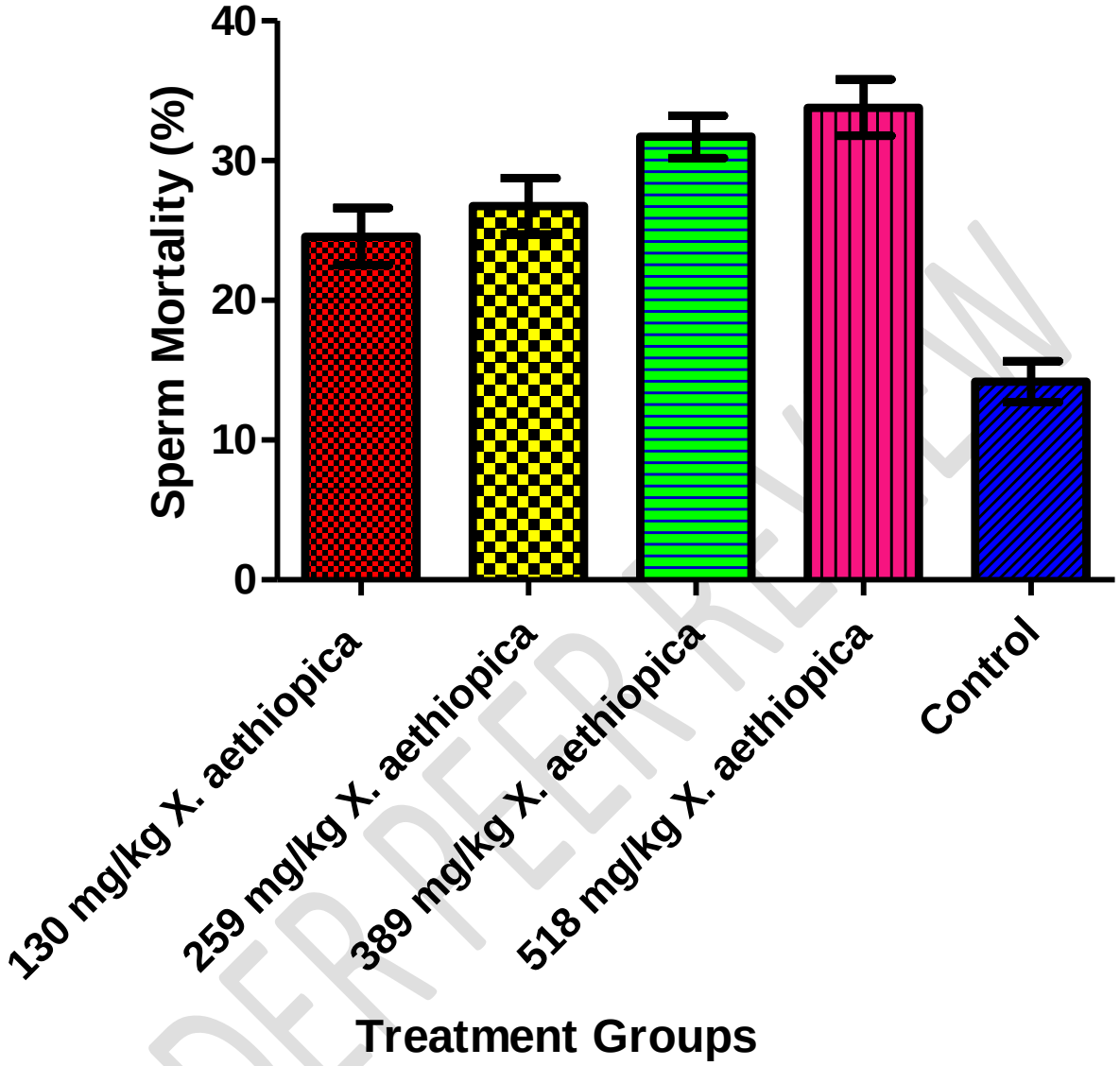

Fig. 3: Effect of *X. aethiopica* fruit extract on Sperm Mortality of Animals after 28 days of Treatment

Each bar represents mean ± SD with n = 6.

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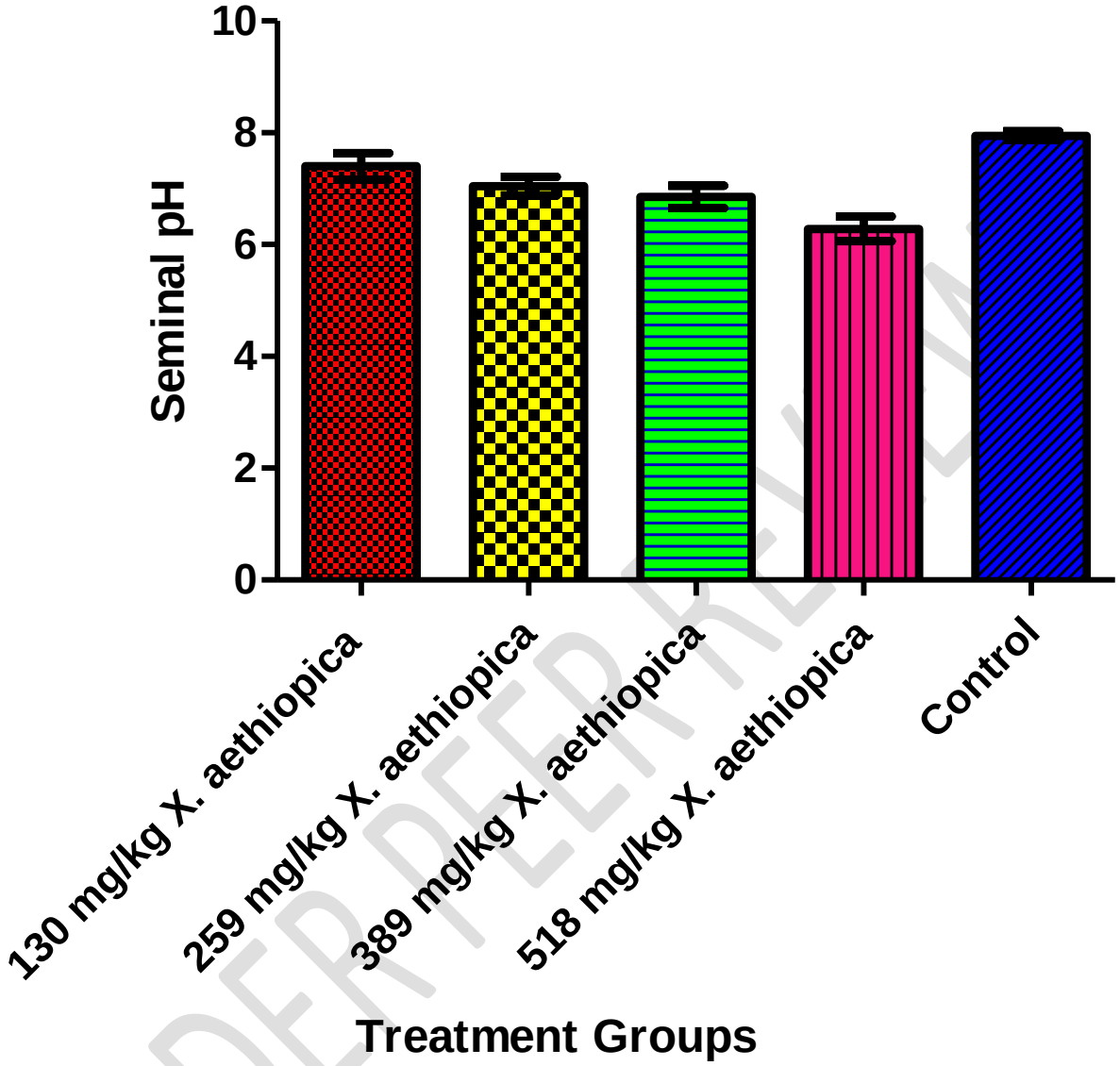

Fig. 4: Effect of *X. aethiopica* fruit extract on Seminal pH of Animals after 28 days of Treatment

Each bar represents mean ± SD with n = 6.

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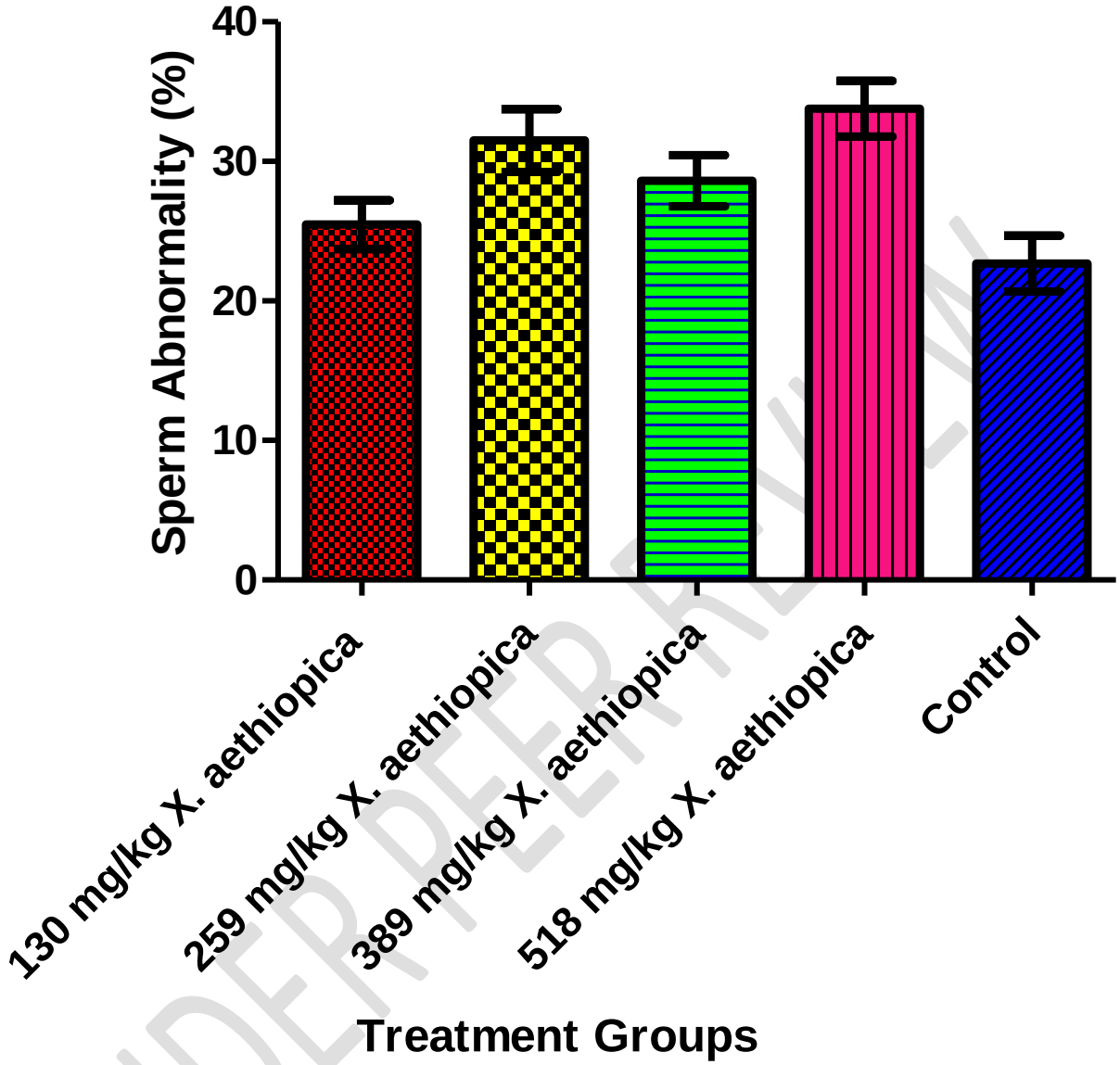

Fig. 5: Effect of *X. aethiopica* fruit extract on Sperm Abnormality of Animals after 28 days of Treatment

Each bar represents mean ± SD with n = 6.

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DISCUSSION

The acute toxicity study of the plant extracts recorded 100% mortality at a dose of 1400 mg/kg bodyweight and above (table 1). This shows that the fruit of *Xylopia aethiopica* might be highly toxic. The physical signs of toxicity observed in the animals included excitation, paw licking, increased respiratory rate, decreased motor activity, gasping and coma which was followed by death.

In this study, it could be clearly demonstrated that sperm count declined significantly (*P<0.05*) when animals treated with *Xylopia aethiopica* fruits were compared with the control groups at all doses of treatment (Fig. 1). This suggests that fruit extract of *Xylopia aethiopica* interfered with steroid hormone biosynthesis, which resulted in impaired spermatogenesis [38]. Disturbance in steroid hormone biosynthesis as well as spermatogenesis might affect the seminal quality of animals. This agreed with the decrease in sperm count of animals treated with *Xylopia aethiopica* fruits reported by Uyovwiesevwa *et al*. [39] and Abarikwu *et al*. [40] respectively. The decrease in sperm count observed in this study is dose-dependent. This indicates that consumption of *Xylopia aethiopica* fruit at high doses will lead to significant reduction in sperm count and thus infertility potential of male animals. Nwangwa, [41] and Eze [42] had both reported the antifertility effects of ethanolic extract of *Xylopia aethiopica* on male reproductive organ of Wistar rats. The result of this present study is in consonance with their respective findings.

There was a significant (*P<0.05*) decrease in sperm motility of animals treated with ethanol extract of *Xylopia aethiopica* fruit when compared with the control group at all doses in this study (Fig. 2). The reduced sperm motility observed in this study might be an indicator that *Xylopia aethiopica* fruit extract has the ability to reduce the ATPase activity in all tissue of the animals [43]. This causes suppression of energy metabolism. If ATPase activity is decreased, it could suppress the motility rate of sperm, as ATP is the main energy source of sperm and it is directly related to sperm motility [44]. Ogbuagu et al. [45] recently reported that ethanol extract of *Xylopia aethiopica* fruit induced oxidative stress in Wistar rats. The inhibitory motility observed in the sperm of rats treated with ethanol extract of *Xylopia aethiopica* fruit in this study could also be associated with oxidative stress induced by the consumption of the extract. The sperm is particularly vulnerable to lipid peroxidation because of the molecular anatomy of its plasma membrane. The increased oxidative stress can damage the sperm membrane leading to reduced motility. This agreed with the work of Kalender and Yel [46] as well as that of Sachder and Davies [47]. The inhibitory motility observed in the sperm of rats treated with ethanol extract of *Xylopia aethiopica* fruit in this study is dose-dependent. This indicates that consumption of *Xylopia aethiopica* fruit at high doses might cause a significant reduction in sperm motility and consequently infertility in male animals.

In this study, ethanol extract of *Xylopia aethiopica* fruit was observed to increase the number of abnormal spermatozoa when compared with those of the control animals after 28 days of treatment (Fig. 3). Increased abnormality of spermatozoa in *Xylopia aethiopica* treated-animals might be as a result of damage of Sertoli cells [48]. For normal testicular function, Sertoli cells plays vital role in maintaining conducive environment for spermatogenesis. Damage to the Sertoli cells might affect the maturation process of spermatozoa, culminating in increased abnormality of sperms observed in this study. This result corresponded to the findings of Adienbo *et al*. [49] who reported a significant increase in sperm abnormality in animals treated with *Xylopia aethiopica* fruit for 30 days.

The seminal pH reflects the balance between the pH values of the different accessory gland secretions, mainly the alkaline seminal vesicular secretion and the acidic prostatic secretion. In this study, seminal pH was observed to decline when animals treated with fruit extract of *Xylopia aethiopica* were compared with the control animals after 28 days of treatment (Fig 4). This might be that *Xylopia aethiopica* fruit extract affects the normal pH range of treated animals. If the pH is decreased, the medium of seminal plasma becomes acidic which in turn makes sperms highly fragile, thus leading to higher rate of mortality [50].

A significant increase was observed in sperm mortality of animals treated with ethanol extract of *Xylopia aethiopica* fruit when compared with the control group (Fig. 5). This might be attributed to the significant (*P<0.05*) decrease in seminal pH in experimental animals. Low pH of epididymal fluid of bovine has been reported to result in increased rate of mortality of spermatozoa [43,51]. The exact mechanism by which *Xylopia aethiopica* fruit reduced sperm count is unknown, but it has been suggested that it contain a compound called xylopic acid which possibly cross the blood testes barrier to exert harmful effects on the seminiferous tubules of the testes.

The antifertility effect of ethanol extract of

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Xylopia aethiopica fruit observed in this study agreed with the findings of Nnodim *et al*. (2012) who studied the effect of *Xylopia aethiopica* fruits extract on the sperm production and testicular oxidative status in male Wistar rats. Adienbo *et al*. [49] had previously reported the impairments in testicular function indices in male Wistar rats following the administration of *Xylopia aethiopica* fruit extract. Similarly**,** Nnodim *et al*. [52], reported the adverse effect of *Xylopia aethiopica* fruit on male reproductive hormones. *Xylopia aethiopica* fruit had also been reported to produce negative impact on female reproductive hormones of animals [53]. In fact, Adienbo *et al*., [49] has recommended *Xylopia aethiopica* fruit as a potent contraceptive.

5. CONCLUSION

The findings of this study revealed that ethanol extract of *Xylopia aethiopica* fruit adversely perturbed sperm quality of Wistar rats. This might not automatically translate to same effect in human. However, men interested in child-bearing should minimize its consumption.

CONSENT

It is not applicable.

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COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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