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Ethanolic Extract of *Xylopia aethiopica* (African Negro Pepper) Fruit Adversely Perturbed Semen Qualities in Male Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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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 ethanolic 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.

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Results: Ethanolic 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 ethanolic extract of *Xylopia aethiopica* fruit administration when compared with those in control animals (P<0.05).

Conclusion: The findings of this study revealed that ethanolic 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 has been defined to be the inability to get pregnant after one year of unprotected intercourse [1]. Infertility in male has been discovered in 50% of infertile couples [2]. Speroff and Fritz [3] have observe that 55% of the causes of infertility are discovered to be male-related while only 35% are said to be female-related, while the remaining 10% are infertility of unidentified origin [3]. Some of the causes of decreasing male fertility could be traced to declining levels of androgen, reduced sexual activity, perturbations in semen qualities, especially, motility, morphology, and DNA integrity [4]. Gonadotropin releasing hormone (GnRH) evokes the distribution of gonadotropins i.e. follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland [5]. Luteinizing hormone is a known glycoprotein that controls the synthesis of testosterone through the extra-tubular Leydig cells. The second gonadotropic hormone, follicle stimulating hormone regulates spermiogenesis as well as spermiocytogenesis by altering Sertoli cells as well as germinal epithelium [6]. The concentrations of these sex hormones are subjected to negative feedback regulation by the gonads [7]. Testosterone is responsible for normal growth and development of male sexual organs, as well as the maintenance of secondary sex characteristics. A high intra-testicular concentration of this hormone is a basic requirement for the synthesis of sperm and its function. Testosterone enhances the motility of sperm as well as epididymis function [8]. Inadequate secretion of FSH and LH by the pituitary gland could lead to interference in the function of the testicles which can contribute to infertility [9].

Semen is an organic fluid with spermatozoa as its constituent. It is released by sexual glands as well as other accessory sex organs of male, and can fertilize the ova of the female. In humans, semen has different constituents outside spermatozoa [10]. Infertility in male could be examined by analyzing semen and hormonal profile [11].

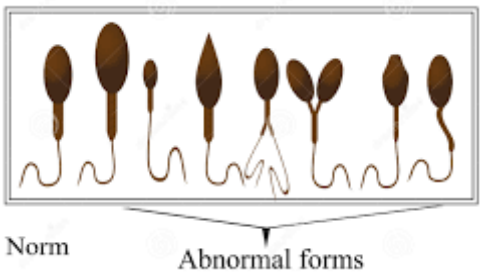

Fig. 1. Sperm morphology

Male impotence popularly known as erectile dysfunction (ED) is a frequent health condition which influence the sexual behaviour of several of men all over the world [12,13]. ED is the incapability of a man to attain and sustain an erection good enough for natural satisfactory intercourse.

Sexual dysfunction is a severe medical and social sign that occurs in 10-52% of men and 25-63% of women [14]. It is the iterated failure to get usual sexual intercourse. ED is a common medical issue that may add to infertility cases [15]. ED is negatively impacted by by some medications such as antihypertensive, antidiabetic agents, antipsychotic, antidepressant therapeutic and antimalarial drugs [16].

Literarily, medications from plant sources are believed to have less side effect when compared with orthodox drugs. This belief comes from the conception of ‘‘green is safe’’ [17,18]. Owing to this perception, medicinal plants are utilized without recourse to their toxicity. The situation is

the same with *Xylopia aethiopica* fruit. "Despite its use as traditional medication and some animal studies that have been carried out to ascertain its therapeutic uses, not much has been done in evaluating its safety or toxicity of most of its bioactive constituents. However, a qualitative/semi quantitative test for toxicity has been reported" [19]. "In a related study, the essential oil of the fruits of *X. aethiopica* was reported to be toxic to *Artemia salina* at concentrations between 10 and 1000 µg/mL" [20]. Koba et al. [21] investigated "the *in vitro* cytotoxicity of essential oil from *X. aethiopica* fruits. The test was carried out using the human epidermal cell line HaCaT. In that study, it was identified that at concentrations between 50 and 1500 µg/mL, the examined essential oil did not portray any cytotoxicity instead it caused a noticeable rise in the viability of cells (up to 130 %), indicating their ability to be cytoprotectors or antioxidants. At higher concentrations between 1600 and 3000 µg/mL, a close result was reported." "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 significant cytotoxicity by removing all matured spermatozoa, germ cells and other cell in the seminiferous tubules when compared with their control group. However, these impacts were alleviated when the experimental animals were given 14 days to recover. Therefore, their results predict that xylopic acid contains reversible spermatotoxic and antifertility effects at the experimented dosages." "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, no significant changes in the behaviour and sensory nervous system responses were observed. Similarly, no negative gastrointestinal impacts were noticed in both male and female mice used in the study. At a dose of 20.0 g/kg, all the treated mice survived after the one day of observation. Therefore, it was concluded that the median acute toxicity value (LD_{50}) of the extract must be more than 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 characterized with numerous chemical components with various medicinal potentials. The chemical components of this plant have been investigated to 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 has also be known to contain vitamins such as vitamin A, vitamin B, vitamin C, vitamin D, and vitamin E, and proteins as well as several minerals such as copper, manganese and zinc [25,27]. The impact of the fruit 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 [31]. This present study focused on examining its impact on the oxidative stress biomarkers of 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% ethanolic (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 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 methanolic. 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.

3. RESULTS

3.1 Median Lethal Dose (LD_{50}) Result

The visible symptoms of toxicity seen in the experimental 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 ethanolic extract of *Xylopia aethiopica* fruit on Sperm Parameters of Animals after 28 days of Treatment

Ethanolic extract of *Xylopia aethiopica* fruit was observed to significantly perturbed sperm parameters of animals after 28 days of treatment, as presented in Figs. 2-6. 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 ethanolic extract of *Xylopia aethiopica* fruit administration when compared with those in control animals (P<0.05).

4. 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 visible symptoms of toxicity seen in the animals were excitation, paw licking, increased respiratory rate, decreased motor activity, gasping and coma and 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. 2). This could be an indication that *Xylopia aethiopica* fruit extract 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 independently reported the adverse impact of ethanolic fruit extract of *Xylopia aethiopica* on male sex organ of Wistar rats. The result of this present study is in consonance with their respective findings.

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
V	2000	5	5	100

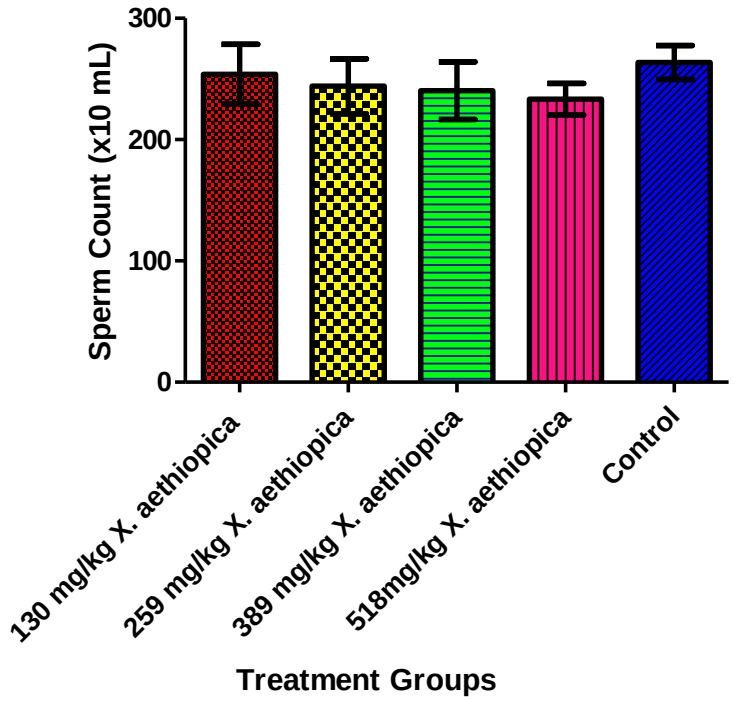

Fig. 2. Effect of *X. aethiopica* fruit extract on Sperm Count of Animals after 28 days of Treatment

Each bar represents mean ± SD with n = 6.

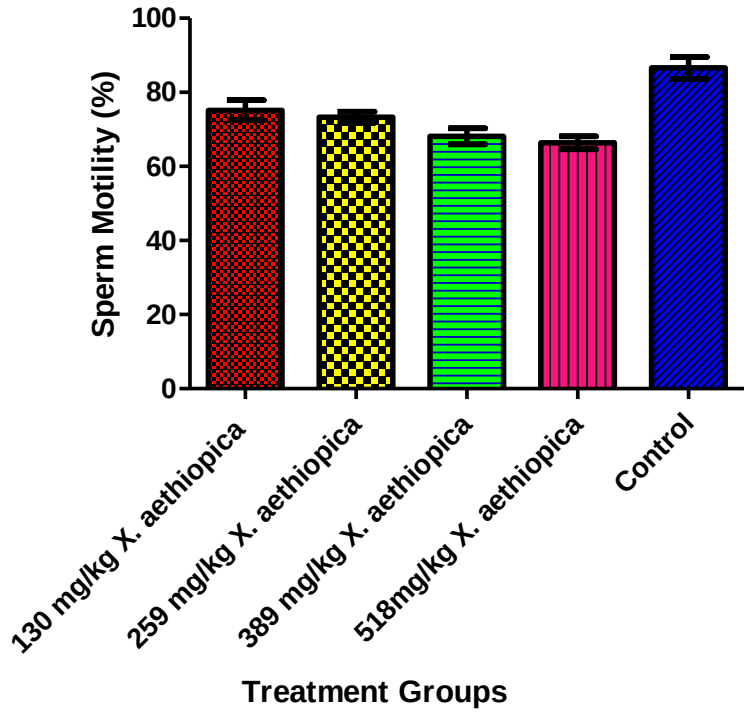

Fig. 3. Effect of *X. aethiopica* fruit extract on Sperm Motility of Animals after 28 days of Treatment

Each bar represents mean ± SD with n = 6

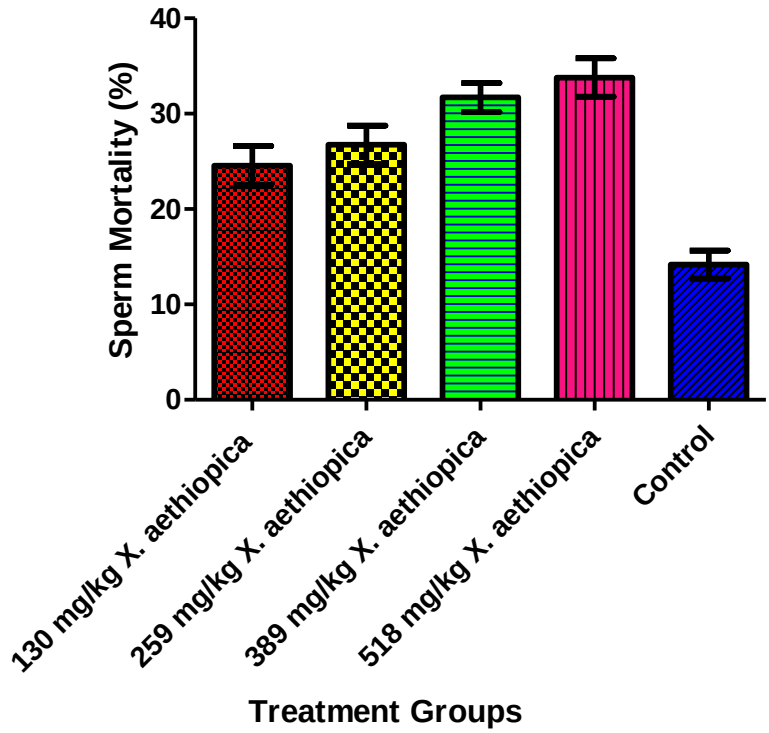

Fig. 4. Effect of *X. aethiopica* fruit extract on Sperm Mortality of Animals after 28 days of Treatment

Each bar represents mean ± SD with n = 6.

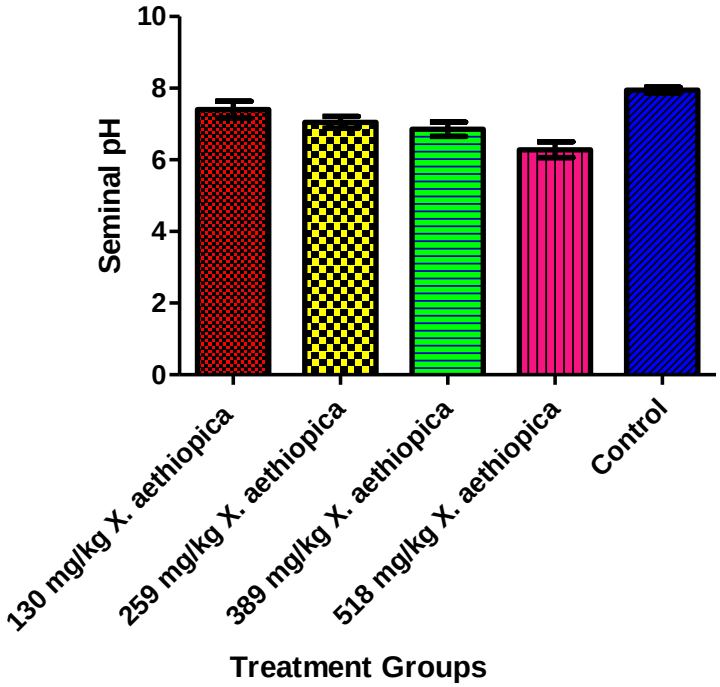

Fig. 5. Effect of *X. aethiopica* fruit extract on Seminal pH of Animals after 28 days of Treatment
Each bar represents mean ± SD with n = 6

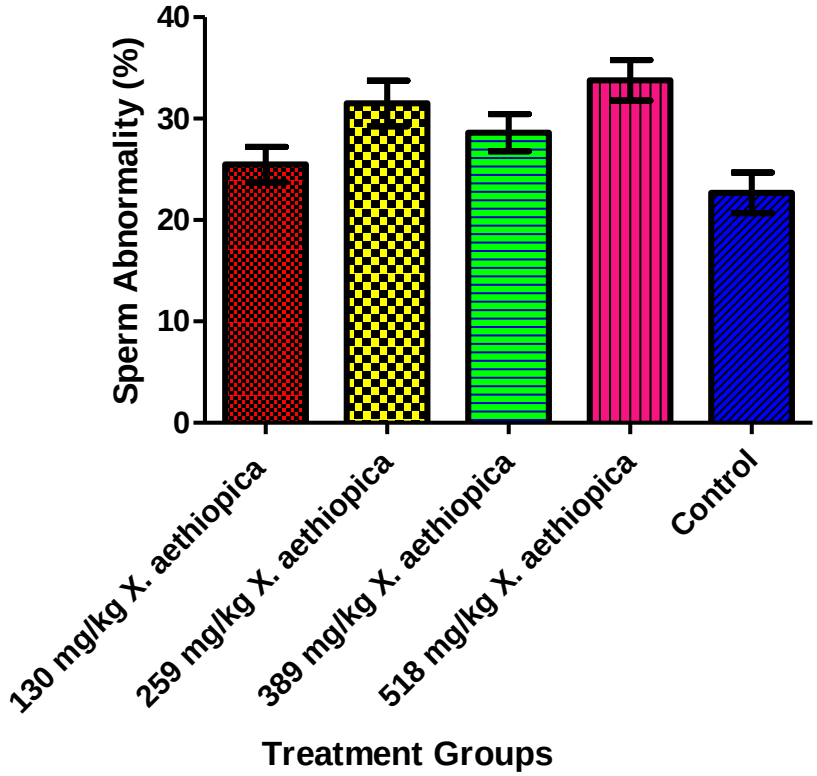

Fig. 6. Effect of *X. aethiopica* fruit extract on Sperm Abnormality of Animals after 28 days of Treatment
Each bar represents mean ± SD with n = 6

There was a noticeable (*P<0.05*) decline in sperm motility of animals treated with ethanolic extract of *Xylopia aethiopica* fruit when compared with the control group at all doses in this study (Fig. 3). 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 ethanolic extract of *Xylopia aethiopica* fruit induced oxidative stress in Wistar rats. The inhibitory motility seen in the sperm of animals exposed to ethanolic 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 seen in the sperm of rats administered ethanolic 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, ethanolic 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. 4). 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 observed a noticeable elevation in sperm abnormality in animals treated with *Xylopia aethiopica* fruit for 30 days.

The seminal pH shows the balance between the pH values of the various accessory gland secretions, majorly the alkaline seminal vesicular secretion and the acidic prostatic release. 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. 5). 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 ethanolic extract of *Xylopia aethiopica* fruit when compared with the control group (Fig. 6). 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 reported that it contain a compound called xylopic acid which possibly cross the blood testes barrier to adversely perturbed the seminiferous tubules of the testes.

"The antifertility effect of ethanolic extract of *Xylopia aethiopica* fruit observed in this study agreed with the findings of Nnodim et al. [52] who studied the impact of fruit extract of *Xylopia aethiopica* on the production of sperm 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 negative 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 ethanolic 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.

DISCLAIMER

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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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