

ORIGINAL ARTICLE

Effect of Xylopic Acid on Sex Hormones and Spermatogenesis in Male Rats

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Abstract: *Background:* Fruit extracts of *Xylopic acid* has been shown to possess antimicrobial, cardiovascular, diuretic, cytotoxic, anti-proliferative, and spermatogenic activity. These activities are attributed to the kaurane derivatives of which Xylopic acid (XA) is a major component. It is worth stating that no study has examined the effects of XA on spermatogenesis and reproductive function. *Objective:* The present study was thus intended to evaluate the effect of XA on serum sex hormone levels and spermatogenesis in male rats. *Methods:* Xylopic acid was administered orally to male Sprague-Dawley rats at the doses of 10, 30 and 100 mg kg⁻¹ for 28 days. Blood was collected into eppendorf tubes from the saphenous veins of animals on day 7 of the treatment and on day 28 after which the rats were euthanized to removed testes and other organs. *Results and Discussion:* Xylopic acid did not cause any changes in body weight, but significantly decrease testicular and epididymal weight ($P < 0.01$). Sperm motility, viability, and epididymal sperm counts of rats administered with XA for 28 days were significantly reduced ($P < 0.01$). Serum testosterone levels were significantly reduced ($P < 0.01$). There were various degrees of damage to the seminiferous tubules. Reversal of these changes, however, occurred after two weeks of recovery. *Conclusion:* Xylopic acid thus possesses anti-androgenic and spermatotoxic properties the mechanism of which may involve direct effect on germ cells and other cells in the testes.

Keywords: Spermatogenesis, anti-androgen, xylopic acid, Sex hormones

Introduction

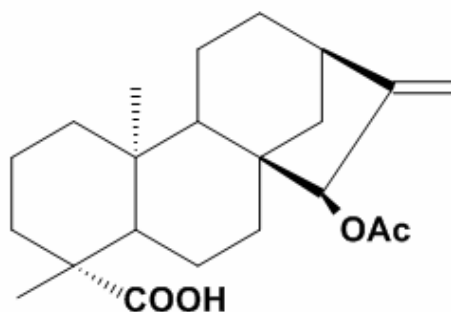
Xylopic acid, also called African guinea pepper or Negro pepper, is an angiosperm of the Annonaceae family, and grows predominantly in humid forest zones of West Africa. The fruit of *Xylopic acid* is used as a cough remedy, as a carminative, as a post-partum tonic, as an antimalarial and to treat uterine fibroid and amenorrhea [1]. Traditional medicine practitioners and birth attendants use decoction of the seeds to induce placental discharge postpartum [1]. The fruit has been shown to have antimicrobial, antifungal activity as well as possess haematopoietic activity, immune boosting effect, androgenic and spermatogenic activity [2-5]. These activities of *Xylopic acid* is attributed to the kaurane derivatives in the extract [6-7]. Xylopic acid [15 β -acetoxy(-)-kaur-16-en-19-oic acid; Fig. 1], a diterpenes and other kaurane derivatives obtained upon extraction of the fruits of *Xylopic acid* with petroleum ether has been showed to possess cardiovascular and diuretic activity [8].

Kaurane a class of diterpenes namely kauranoic and XA [9], are compounds of rigid tetracyclic skeleton and they form intermediates in the biosynthesis of the plant growth hormones such as gibberellins [10]. Antimicrobial, anti-parasitic, insect anti-feedant, anti-HIV and anti-inflammatory activities have been reported for different kauranes[10]. Although antimicrobial, cardiovascular, diuretic, cytotoxic, androgenic and spermatogenic activities have been reported for various extracts of this plant [5,8,11], it is worth stating that no study have examine the effects of XA on spermatogenesis and reproductive function in rats. The present study was thus intended to evaluate the effect of XA, a major constituent of the fruits of *Xylopiya aethiopia* on serum sex hormone levels, spermatogenesis and testicular histology in male rats.

Material and Methods

Plant material: The dried fruits of the *Xylopiya aethiopia* [Dun.] A. Rich, were bought from the Central Market, Kumasi and authenticated in the Department of Pharmacognosy, KNUST, Kumasi, Ghana and a herbarium has been deposited there as well (voucher number FP/08/76).

Extraction of XA: XA was extracted using the method described by Ekong and Ogan, [12.] The dried fruits (0.36 kg) were pulverized and soaked in petroleum ether (40-60°C) for three days. The petroleum ether extract was drained and concentrated using rotary evaporator at 50°C. Ethyl acetate was added to the concentrate to facilitate crystallization of XA. Crystals formed after three days were washed with petroleum ether 40-60°C repeatedly. XA was purified using recrystallization by dissolving it in ethanol. The resulting solution was filtered and left to stand for three days to recrystallized, yielding 1.41% (5.1 g) of XA. The structure of XA is as shown below.



Molecular weight: 360.487 g mol⁻¹

Molecular formula: C₂₂H₃₂O₄

Structure of Xylopic Acid 15β-acetoxy-(-)-kaur-16-en-19-oic acid]

Purity of the isolated xylopic acid was determined using High Performance Liquid Chromatography (HPLC). The chromatograph consisted of LC-10AT Shimadzu pump with programmable absorbance detector (783A Applied Biosystems) and Shimadzu CR501 chromatopac. Phenomenex hypersil 20 micron C18 200 × 3.20 mm column was used.

The mobile phase consisted of methanol and water (9:1) eluted isocratically at 0.5 ml min^{-1} . Portions of $20 \mu\text{l}$ of a suitable concentration of pure XA were loaded and injected into the column after dissolving in the mobile phase at 60°C . The eluent was monitored at 206 nm . Portions of the XAE and XA were loaded and injected. The peak(s) were noted as component(s) of the XAE and XA.

Animals: All experiments were performed with male albino rats (90 days old) weighing $150\text{--}200 \text{ g}$ bought from Noguchi Memorial Institute for Medical Research, University of Ghana, Accra and kept at the Animal House Facility of the Department of Pharmacology, KNUST, Kumasi. The animals were allowed to acclimatize to the laboratory condition (Temperature $24\text{--}25^\circ\text{C}$ and 12 hour light-dark cycle) for two week before commencement of the experiment with free access to solid pellet diet (GAFCO Trading company, Tema) and water *ad libitum* throughout the study. Prior permission was obtained from the ethical committee of the Pharmacology Department, KNUST. All the animals were treated according to the National Institute of Health Guidelines for the care and use of laboratory animals (NIH, Department of Health and Human Services Publication no. 85-23, revised 1985).

Drugs and chemicals: Testosterone, follicle-stimulating hormone (FSH), prolactin (PRL) and luteinizing hormone (LH) assay kits were obtained from HySkill Diagnostics (Bahlingen, Germany). Testosterone propionate was a gift from Abeth Consult limited (Ghana), Cyproterone acetate was obtain from Bayer Australia Ltd (Australia).

Experimental design: Male Sprague Dawley rats were divided into four groups of six animals each. Group I served as the control and were given distilled water (vehicle for the XA). Groups II, III and IV rats were given XA orally at the dose of $10, 30$ and 100 mg kg^{-1} respectively for 28 days. The blood was collected into eppendorf tubes from the saphenous veins of animals on day 7 of the treatment and on day 28 after which the rats were euthanized to removed testes and other organs.

Semen collection and analysis: The left testis was removed along with its epididymis and fatty tissues trimmed off. Caudal epididymis was separated from the testis and lacerated to collect semen onto a microscope slide for sperm characteristic evaluation according to methods describe by Lucio *et al.*, [13] and WHO [14]. Briefly, two drops of warm 2.9% sodium citrate was added to the semen on the microscope slide and examined under the microscope for progressive sperm motility. Sperm viability was evaluated with the eosin–nigrosin stain technique. Semen was mixed with two drops of the stain. A thick smear was prepared and air-dried. The stained slides were examined under the microscope. Viable (live) sperm cells appeared unstained while the non-viable (dead) sperm absorbed the stain. The viable and non-viable sperm were counted and the percentage of each calculated. Cauda epididymis from the right side was placed in a petri dish containing 10 ml of phosphate buffered saline (PBS) pre-warmed to $35\text{--}37^\circ\text{C}$ and split with surgical blade to open the epididymal duct to release its contents.

The petri dish was then swirled to achieve a uniform sperm suspension from which a sperm count was carried out in the improved Neubauer haemocytometer. Total sperm concentration was calculated as describe by by Lucio *et al.*, [13] and WHO [14].

Estimation of serum Prolactin, FSH, LH and Testosterone: Serum FSH, LH and testosterone concentrations were estimated by the enzyme-linked immunosorbent assay (ELISA) using NoviWell™ assay kits (HySkill Diagnostics, Bahlingen, German). The blood was centrifuged at 500 g for 15 min and serum was collected and stored at -20°C until assayed. Assays were carried out as instructed by the manufacturer.

Histology: Testicular tissues of control and treated rats were removed and weighed, fixed in Bouin's fluid for 6 h before being transferred into 10% formalin for histological evaluation. After dehydration with varying percentage of ethanol, tissues were cleared in xylene and embedded in molten wax. Thin sections were cut (5 µm), stained with haematoxylin and eosin, and then analyzed microscopically.

Statistical analysis: Results were expressed as mean ± SD. The significance of difference between the means was determined by one-way analysis of variance (ANOVA) with Newman-Keuls's as *post-hoc* test. Difference between subject factors (time and dose treatment) was analyzed using two-way analysis of variance as a within-subject factor followed by Bonferroni's as *post hoc* test. In all statistical tests, a value of P<0.05 was considered significant. All analysis was performed using Sigma Plot for Windows, Version 11.0, (Systat Software, Erkrath, Germany; www.systat.com).

Results

Finger print of XAE and XA in HPLC: Several peaks were observed after loading XAE indicating the presence of several compounds in the fruits as shown in figure 1. A single peak was observed for XA indicating the presence of a single compound (Figure 2).

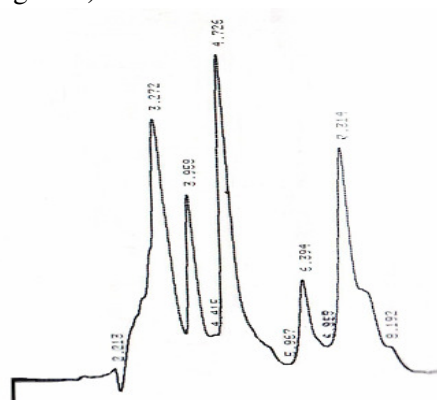


Figure-1: HPLC finger print of the extract showing peaks of the various compounds in the extract.

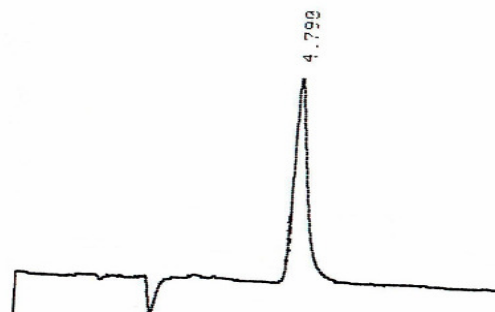


Figure-2: Chromatogram of XA showing a single peak corresponding to the isolated XA.

Determination of some properties of xylopic acid: Melting point of the crystals (XA) was determined to be 260-261°C. The crystals were also sparingly soluble in petroleum ether, ethanol, methanol, ethyl acetate and soluble in chloroform.

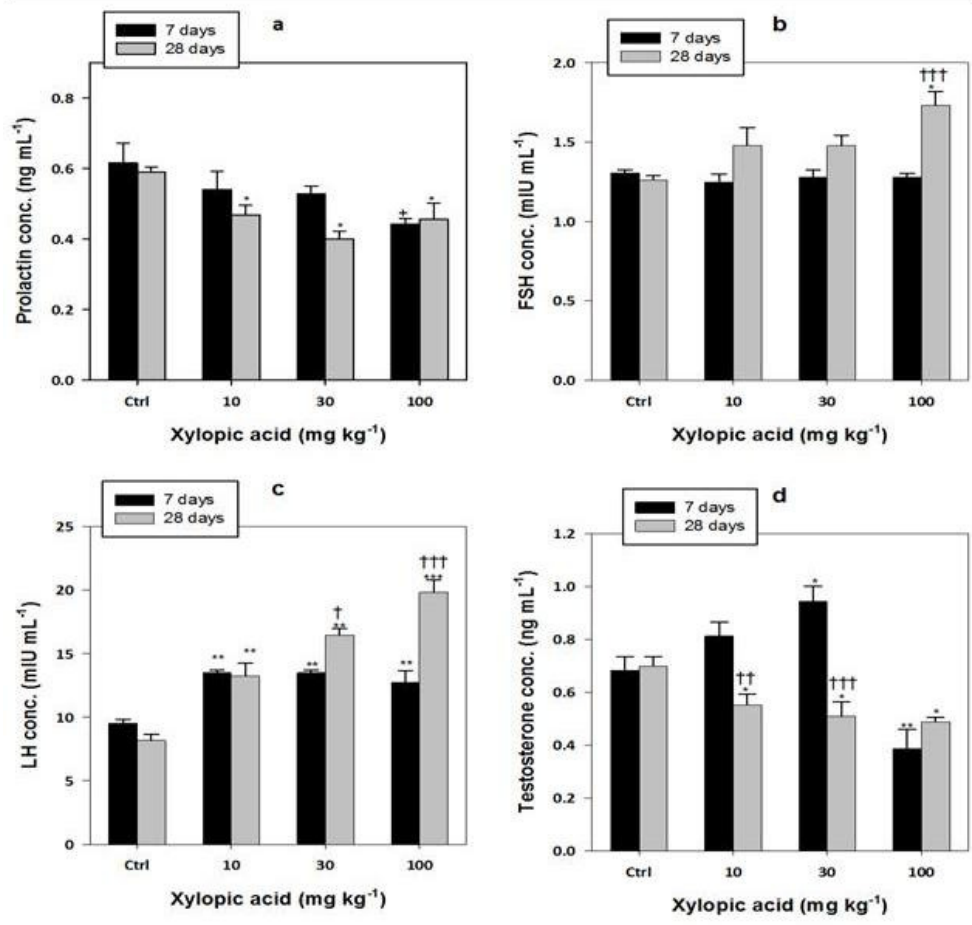
Effect of XA on body and organ weight of male rats: XA treatment show no significant change in animal body weight compared with the control. However, the weight of the testis and epididymis both showed a dose dependent reduction in the treated animals ($p < 0.001$) compare with control animals. The seminiferous tubular diameter also showed a significant ($P < 0.001$) reduction in all treated rats compared to control rats as shown in table 1.

| Table-1: Effect of XA on animal body weight, and reproductive organ weight | | | | |
|---|------------------------------|--------------------------|-----------------------------|--|
| Treatment Group | change in body wt (g) | testicular wt (g) | wt of epididymis (g) | Seminiferous tubular D (μm) |
| Control | 70.7 \pm 17.7 | 0.96 \pm 0.2 | 0.2 \pm 0.0 | 135.2 \pm 18.9 |
| 10 mg kg ⁻¹ | 69.4 \pm 9.19 | 0.825 \pm 0.2 | 0.19 \pm 0.0 | 115.5 \pm 11.8* |
| 30 mg kg ⁻¹ | 76.9 \pm 4.9 | 0.6 \pm 0.04** | 0.14 \pm 0.01*** | 106.6 \pm 6.6*** |
| 100 mg kg ⁻¹ | 68.9 \pm 11.0 | 0.6 \pm 0.05*** | 0.12 \pm 0.01*** | 83.28 \pm 11.5*** |
| Data are express as mean \pm SD, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls <i>post hoc</i>), wt = weight and D = diameter. | | | | |

Effect on Prolactin, FSH, LH and Testosterone: Serum prolactin concentration was significantly reduced ($P < 0.05$) when rats were treated with XA (10, 30 and 100 mg kg⁻¹) as compared to the controls group for both 7 days and 28 days. Treatment for 7 days showed no significant change in the serum FSH level compare with the control group. However, a significant increase was noted in FSH when treatment was continued for 28 days ($P < 0.05$).

Duration of treatment had significant [$F_{3, 16} = 22.78$; $P = 0.002$] incremental effect on serum FSH at 100 mg kg⁻¹ dose level. Luteinizing hormone levels showed a significant dose dependent increased after 7 days of treatment ($P < 0.05$) as well as 28 days ($P = 0.001$). Two-way analysis of variance showed that duration had significant effect [$F_{3, 16} = 19.62$; $P = 0.0004$] as well as concentration [$F_{3, 16} = 15.96$; $P < 0.0001$] on LH levels. Generally, there was a significant ($P < 0.05$) increased in serum testosterone levels in rats treated with XA (10 and 30 mg kg⁻¹) for 7 days (Fig 3). However, continuous treatment for 28 days significantly ($P = 0.05$) decreased serum testosterone levels at all dose levels compare with the control groups. Concentration and duration of dosing both significantly decrease serum testosterone level in rats [$F_{3, 12}$; $P = 0.0004$] as shown in fig 3.

Figure-3: Effect of XA on reproductive hormones in male rats. Results are presented as means \pm SD. * $P \leq 0.05$, ** $P \leq 0.01$, * $P \leq 0.001$ compared to respective controls (one-way ANOVA followed by Newman-Keuls *post hoc*); † $P \leq 0.05$, †† $P \leq 0.01$, ††† $P \leq 0.001$ (two-way repeated measures ANOVA followed by Bonferroni's *post hoc*).**



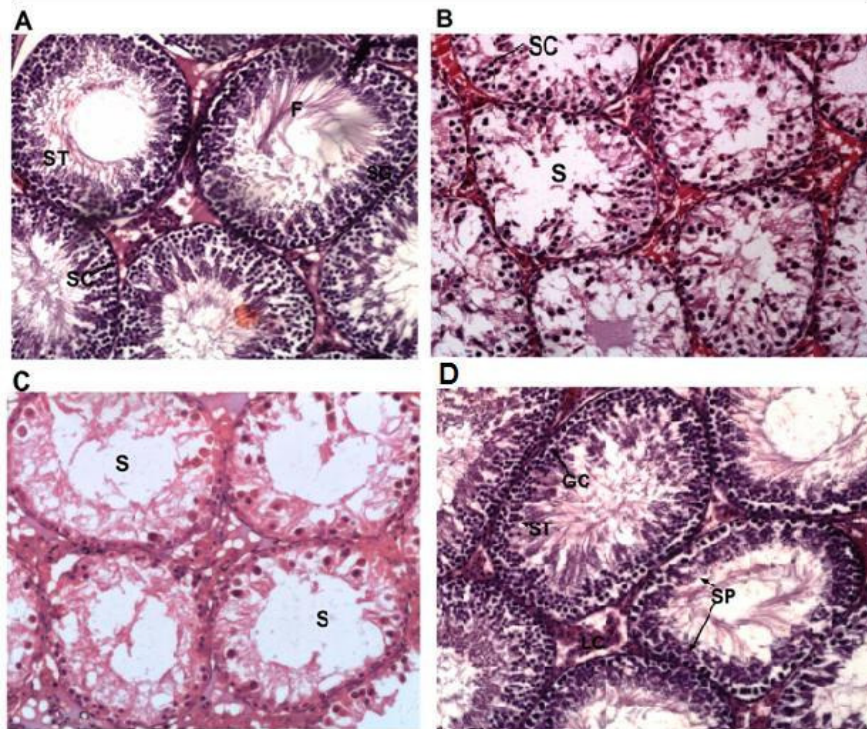
Effect of XA on sperm count, sperm motility and viability: Administration of XA significantly decreased epididymal sperm count ($F_{3, 16} = 69.16, p < 0.001$) dose-dependently in treated rats compared with control group. The treated animals also exhibited a significant decrease in sperm motility ($F_{3, 16} = 43.95, P < 0.001$) and sperm viability ($F_{3,16} = 15.68, P < 0.001$) compared with the control group (Table 2).

| Parameter | Control | 10 mg kg ⁻¹ | 30 mg kg ⁻¹ | 100 mg kg ⁻¹ |
|------------------------------------|------------------|------------------------|------------------------|-------------------------|
| Sperm count (X10 ⁶ /ml) | 129.8 \pm 13.1 | 93.0 \pm 6.1*** | 64.3 \pm 1.7*** | 55.8 \pm 1.82*** |
| Sperm motility (%) | 75.2 \pm 3.6 | 50.6 \pm 8.3*** | 13.2 \pm 4.3*** | 0.0 \pm 0.0*** |
| Viability (%) | 69.0 \pm 2.6 | 47.6 \pm 5.7*** | 15.6 \pm 2.9*** | 0.0 \pm 0.0*** |

Data are express as mean \pm SD, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared to espective controls (one-way ANOVA followed by Newman-Keuls *post hoc*)

Histology of the testis: Histological analysis of the testes of rats in the control showed normal morphological appearance with seminiferous tubules showing all the various stages of spermatogenesis (Fig 4). Administration of XA (10, 30 and 100 mg kg⁻¹) caused visible cytotoxic activity by clearing all matured spermatozoa, germ cells and other cell in the seminiferous tubules compared with the control rats (Fig 4). The treated group showed a depleted germ cell layers, disorganization of the normal regular layering of the various stages of spermatogonia and spermatocytes as shown in fig 4. Regeneration of germinal epithelium and restructuring of the germinal interstitium occurred in the recovery rats as shown in fig 4.

Fig-4: Light photomicrograph showing testes of A) control rat seminiferous tubules showing various stages of spermatogenesis; B) testes of rat treated with XA (30 mg kg⁻¹) showing irregular shape seminiferous tubules; C) testes of rats treated with XA 100 mg kg⁻¹ showing seminiferous tubules with various degrees of degeneration. D) Testis of rats from the recovery group showing various stages of spermatogenesis. H&E stain, x 250. LC, Leydig cell; GC, Germinal cell; ST, Spermatids; SP, Spermatozoa; SG, spermatogonia; F, Flagellae; S, Space; Sc, Sertoli cell.



Discussion

In testis, a significant proportion of the weight is associated with spermatogenic function [15]; hence the suppression of testicular weight observed in this study following XA treatment might result from the large changes in the sperm count and content of spermatids and spermatozoa seen in the present study as evident in the results of the histology which showed a marked reduction of these cells in the seminiferous tubules (Fig. 4).

The significant reduction in the reproductive organ weights of the rats in this study may be attributed to the decrease in testosterone levels. It has been shown in rat that Leydig cell number per testis increases in parallel with testicular weight following birth, accompanied by increases in testosterone level [16].

Gonadotropins and testosterone are the prime regulators of germ cell development. The successful and complete male germ cell development is dependent on the balanced endocrine interplay of hypothalamus, pituitary and the testis [17]. FSH binds with receptors in the sertoli cells and stimulates spermatogenesis. LH stimulates the production of testosterone in Leydig cells, which in turn may act on the Sertoli and peritubular cells of the seminiferous tubules and stimulates spermatogenesis [18]. It has been hypothesized that an elevation of intratesticular testosterone contributes to the maintenance of testicular atrophy and that agents reducing the intratesticular testosterone levels stimulate the recovery of spermatogenesis [19]. This hypothesis could be related to the result obtained in the present study where there was an increase in the serum testosterone level after administration of 30 mg kg⁻¹ body weight of xylopic acid for 7 days. This level however, reduced with the administration of higher doses of the X.A (100 mg kg⁻¹). The dose dependent reduction in serum testosterone level when duration of treatment was extended from 7 days to 28 days has further shown that xylopic acid could be acting as testosterone antagonist. The increased serum LH and FSH levels observed may probably be due to suppression of negative feed-back inhibition of anterior pituitary which may secondarily increase the level of these hormones as seen in the 28 day treated rats [20].

The finding of this study has also demonstrated that the number of Leydig cells decreased in the rats treated XA. This could be the cause of testosterone shortage, which is needed along with the Sertoli cells to carry out spermatogenesis [21]. There are a number of probable mechanisms for the anti-gonadal activities of XA; it may exert a direct inhibitory action on the testis; or it may affect the pituitary, causing changes in gonadotrophins concentrations and thus subsequent spermatogenic impairment; or they may change the concentration of neurotransmitter. Studies have shown that XA exhibits cytotoxicity and antiproliferative activity in human cervical cancer cells [22]. The spermatotoxicity and the dose dependent degeneration of the seminiferous tubules as well as reduction in tubular diameter in the testis of rats treated with XA in the present study as shown in Fig 4 is thus in conformity with the earlier studies indicating the cytotoxicity of XA [22-23]. The decrease in epididymal sperm count and high number of morphologically abnormal sperm indicates interference of XA with testicular spermatogenesis as observed in the histological section of the testis. Decrease in sperm motility and viability suggests alteration of sperm maturation in the epididymides [24]. Reduced number of spermatozoa, malformed spermatozoa or their reduced motility and viability are the leading causes of disturbed fertility or infertility in patients [25]. Among plant based contraceptives, inhibition of male fertility after administration of natural substances has been related to decreased spermatozoa density [26].

Seminiferous tubular cytoarchitecture were, however, restored to normal in the recovery groups. In conclusion, the results of the present study suggested that XA possesses reversible antifertility, spermatotoxic and anti-androgenic properties the mechanism of which may involve direct effect on germ cells and other cells in the testes and a possible Hypothalamic-pituitary-gonadal axis involvement.

Acknowledgements

The authors would like to thank Mr. Thomas Ansah, Chief Technician of the Pharmacology Laboratory, Department of Pharmacology, KNUST/Kumasi for his invaluable technical assistance.

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