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Toxicity of *Xylopia aethiopica* fruit extract: An estrous cycle and histological study

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ABSTRACT

Xylopia aethiopica is commonly used in central and west Africa to treat various ailments and as food additives with the highest consumption rate among women. The study evaluated the effects of *Xylopia aethiopica* fruit extract (XAFE) on estrous cycle duration and the histology of kidney, liver, uterine horn, and ovaries in Wistar rats. Thirty-five female rats were used for the study (15 for estrous cycle and 28-day repeated toxicity). In the estrous cycle study, the rats were allocated into three groups (n=5) and received distilled water, and XAFE at 100mg/kg & 300mg/kg respectively for 28 days. The estrous cycle of each rat was evaluated from the vaginal fluid daily. For the 28-day repeated toxicity, twenty rats were distributed into four groups (n=5) and received distilled water, and XAFE at 100mg/kg, 200mg/kg, and 300mg/kg respectively for 28 days. All the rats were euthanized afterward. The uterine horns, ovaries, kidneys and livers were dissected and processed for light microscopy. Treatment with XAFE significantly increased estrous cycle duration (7.18±0.17 & 7.22±0.24) relative to the control (5.40±0.10). The ovary of rats treated with XAFE at 300mg/kg and 200mg/kg showed degenerating follicles and mild distortion of the endometrial epithelium respectively. Pyknosis and mild renal tubular distortion were observed in the liver and kidney respectively of rats treated with 300mg/kg XAFE. In conclusion, prolonged consumption of XAFE alters the development of ovarian follicles thereby prolonging estrous cycle duration. This could lead to infertility in females by delaying or preventing conception.

Keywords: Estrous cycle, follicles, *Xylopia aethiopica,* ovaries, uterine horn

INTRODUCTION

Xylopia aethiopica (Dunal A. Rich) is a medicinal plant native to the rainforest and savannah regions of Africa and Asia. The plant is called kimba in Hausa, eru in Yoruba and, uda in Igbo [1]. It is widely consumed in traditional medicine by women to ease childbirth, induce placental discharge, prevent nausea, terminate an unwanted pregnancy, treats menstrual abnormalities,
relieve pain and promote healing after birth [2, 3, 4, 5]. *Xylopia aethiopica* (*X. aethiopica*) is equally used in African cuisine (especiall y in Nigeria), because of its pharmacological properties such as; analgesic, anti-inflammatory, anti-allergic, anti-allodynic, antispasmodic, anti-hyperalgesic, anti-depressant, antioxidant, and hypoglycemic effects [6, 7, 4, 8].

The use of plants for medicinal, mythological purposes, and solving problems related to ill-health has been practiced in African and Asian countries for many years [9]. Some plants possess chemical compounds that have the ability to interfere with the normal reproductive cycle which may lead to infertility [10]. The reproductive system is sensitive to several factors such as lifestyle radiation, drugs, herbs, and toxicants [11]. Exposure to any of these and other factors could lead to functional alterations in adults [12]. Any interference to the normal functioning of the organs of reproduction may affect the ability of the animal to reproduce [13]. In Nigeria, there are several medicinal plants with anti-fertility properties. Yet the scientific evaluation of the fertility-regulating abilities of these plants is not fully explored.

The toxicity of herbal medicines has alerted many regulatory establishments to develop and implement a set of guidelines for assessing, and preventing the toxicity associated with herbal medicines. Hence, sub-acute toxicity testing involves the determination of the effect of the test compound upon repeated administration [14]. Health hazards associated with the consumption of food additives are of high concern to consumers, nutritionists, and toxicologists as well as possible toxic effects of the use of food additives among women [15]. Therefore, exploring the possible side effect of natural products on the reproductive system as well as some vital organs of the body would be important. The present study evaluated the effects of prolonged consumption of *X. aethiopica* fruit extract on estrous cycle duration as well as the histology of kidney, liver, uterine horn, and ovaries in Wistar rats.

Methods

***Chemicals*:** Methanol and methylene blue was bought from Sigma Aldrich (St. Louis, USA). Ketamine hypochlorite injection was obtained from the University of Maiduguri Clinic while hematoxylin and eosin stains were purchased from BDH Chemical Ltd (Poole, England).

Plant materials and extraction: Dried fruits of *X. aethiopica* were bought from Monday Market, Maiduguri, Nigeria. It was authenticated by a botanist at the Department of Biological Sciences, University of Maiduguri (UM/HAH/2018/001). Aqueous extraction was carried out as described by Adienbo *et al*. [8]. The fruits were pulverized and 330 g of the powder was soaked in three litres of distilled water. The mixture was refluxed for two hours in a continuous extraction (soxhlet) apparatus and the solution was filtered with a thimble to remove the debris. The filtrate was concentrated to powder using a rotary evaporator.

Animals: Thirty-five female rats (150-200g) were used for the study (15 for estrous cycle and 28-day repeated toxicity). They were purchased from the National Veterinary Research Institute Vom, Nigeria. The rats were housed in plastic cages at the Animal

house of the Department of Biochemistry, University of Maiduguri. They were allowed to acclimatize to the laboratory condition for two weeks. They were fed with grower mash (Vital Feed, Nigeria) and water *ad libitum*. The rats were weighed weekly throughout the period of the study. Dissections were performed under ketamine hypochlorite anesthesia.

Estrous cycle study: The modified method of Ngadjui et al. [16] was used for the estrous cycle study. Fifteen female Wistar rats were randomly allocated into three groups of five rats each. Group, I (control) was orally administered distilled water for 28 days while groups II and III received 100 mg/kg and 300 mg/kg body weight of XAFE respectively. Vaginal fluid was collected daily (9-10 am) and smears were made on glass slides. The smear was fixed in methanol, stained with methylene blue, and examined microscopically. The stages of the estrous cycle were classified as either proestrus, estrus, metestrus, and/or diestrus based on the number and proportion of certain cell types (squamous epithelial, regular, cornified, non-nucleated, and/or irregular cells, as well as leukocytes) according to the method described by Byers et al. [17]. The duration of the cycle of each rat was recorded in days.

Repeated dose 28-day oral toxicity study: A repeated dose 28-day oral toxicity study was carried out according to the method described by Yimam et al. [18] and the Organization for Economic Cooperation and Development [19], test guideline-407 with minor modifications. Briefly, twenty female Wistar rats were randomly distributed into four groups (n = 5). Group I served as control and received distilled water, group II received XAFE at 100 mg/kg, and group III received XAFE at 200 mg/kg, while group IV received XAFE at 300 mg/kg body weight respectively, for 28 consecutive days. All the rats were euthanized 24 hours after the last dose. The uterine horns, ovaries, kidneys and livers were dissected out, weighed, and fixed in Bouin's fluid (uterine horns and ovaries) and 10% formalin (kidneys, and livers) and processed for histological study.

Organ index: The organ index of each rat was calculated as follows; organ weight (g)/body weight (g) × 100%.

Histological study: The organs were dehydrated in graded alcohol (70%, 90%, & 100% ethanol) for 5 minutes each, embedded in paraffin wax, and sectioned at 5µm with a rotary microtome (Leica RM2125 Rotary Microtome). The sections were stained with Hematoxylin and Eosin (H&E) and mounted with DPX. Micrographs were taken using a microscope camera (AmScope, UK) at x100, x200 and x400 magnifications.

Statistical analysis: Estrous cycle and organ index data were analyzed with GraphPad Prism 7 (GraphPad, USA). One-way ANOVA and Dunnett multiple comparison test were conducted and the results were presented as Mean ± standard error of the mean (SEM). $P<0.05$ was considered statistically significant.

RESULTS

Estrous cycle and organ index: Treatment with XAFE at 100 mg/kg (7.18±0.17) and 300 mg/kg (7.22±0.24) significantly increased the estrous cycle duration relative to the control (5.40±0.10) at $P<0.05$ (Figure 1). The ovary and uterine horn indices were not significantly changed ($P>0.05$) in rats that received XAFE at 100 mg/kg, 200 mg/kg, and 300 mg/kg compared to the control (Figure 2). The right kidney index of rats treated with 100 mg/kg and 200 mg/kg XAFE were significantly higher ($P<0.05$) relative to the control (Figure 3). However, the left kidney and liver indices were not significantly

changed (P>0.05) in XAFE treated rats relative to the control (Figure 3).

Histology: The ovaries of control rats and the rats treated with 100 mg/kg and 200 mg/kg of XAFE showed normal corpus luteum with follicles at different stages of development (Figure 4a-4c). The ovaries of rats treated with 300 mg/kg of XAFE showed degenerating follicles (Figure 4d). The uterine horns of control rats and the rats treated with 100 mg/kg and 300 mg/kg of XAFE showed normal endometrial mucosa lined by simple columnar epithelium. It also showed normal endometrial glands and connective tissues (Figure 5a-b, & 5d). Mild distortion of the endometrial epithelium was observed in rats that received 200 mg/kg XAFE (Figure 5c). The liver of control rats and 100 mg/kg XAFE treated rats showed normal hepatocytes (Figure 6a-b). The liver of rats treated with XAFE at 200 mg/kg showed mild lymphocytes infiltration while the liver of rats treated with XAFE at 300 mg/kg showed pyknosis (Figure 6c-d). The kidneys of the control rats as well as the rats treated with 100 mg/mg and 200 mg/kg XAFE showed normal renal tubules and glomeruli (Figure 7a-c), while the kidneys of rats that received XAFE at 300 mg/kg showed mild distortion of renal tubules (Figure 7d).

FIGURES

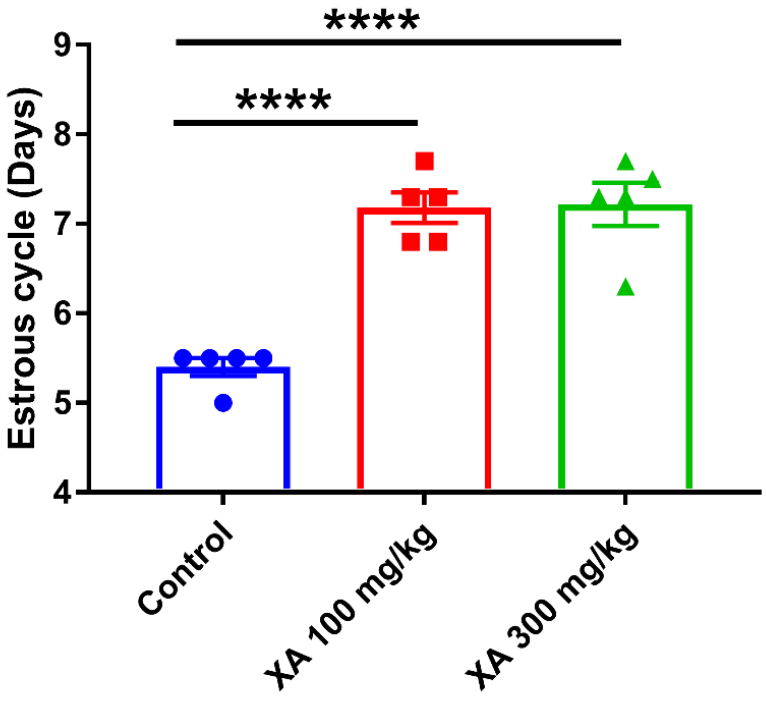

Figure 1: Estrous cycle duration in rats treated with *Xylopia aethiopica* fruit extract. Values are presented as the Mean±standard error of the mean. **** indicates a significant no difference with control at P<0.001. XA= *Xylopia aethiopica.*

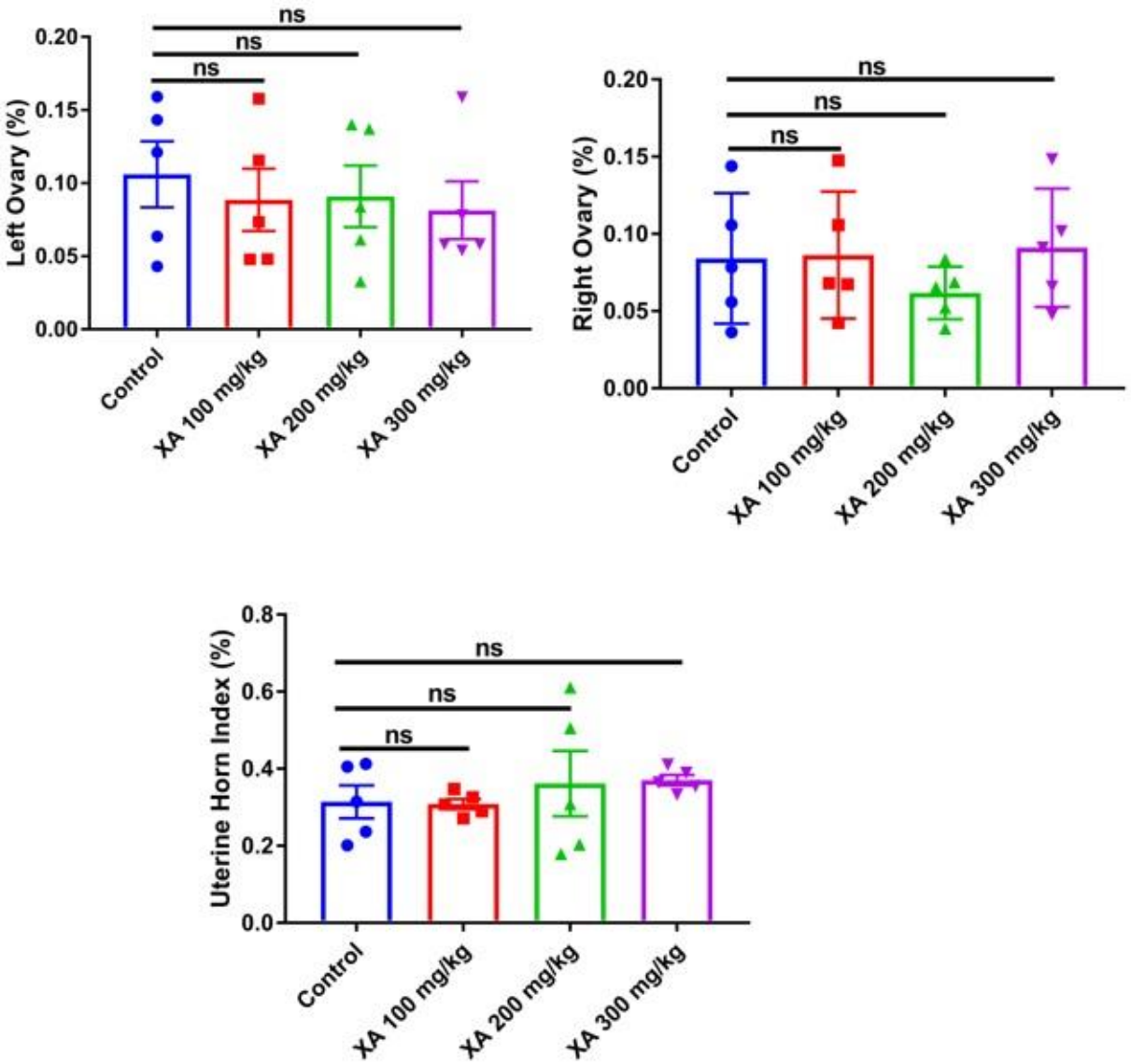

Figure 2: The ovary and uterine horn indices of rats treated with *Xylopia aethiopica* fruit extract. Values are presented as Mean±stanthe dard error of the mean. ns= non-significant change, XA= *Xylopia aethiopica.*

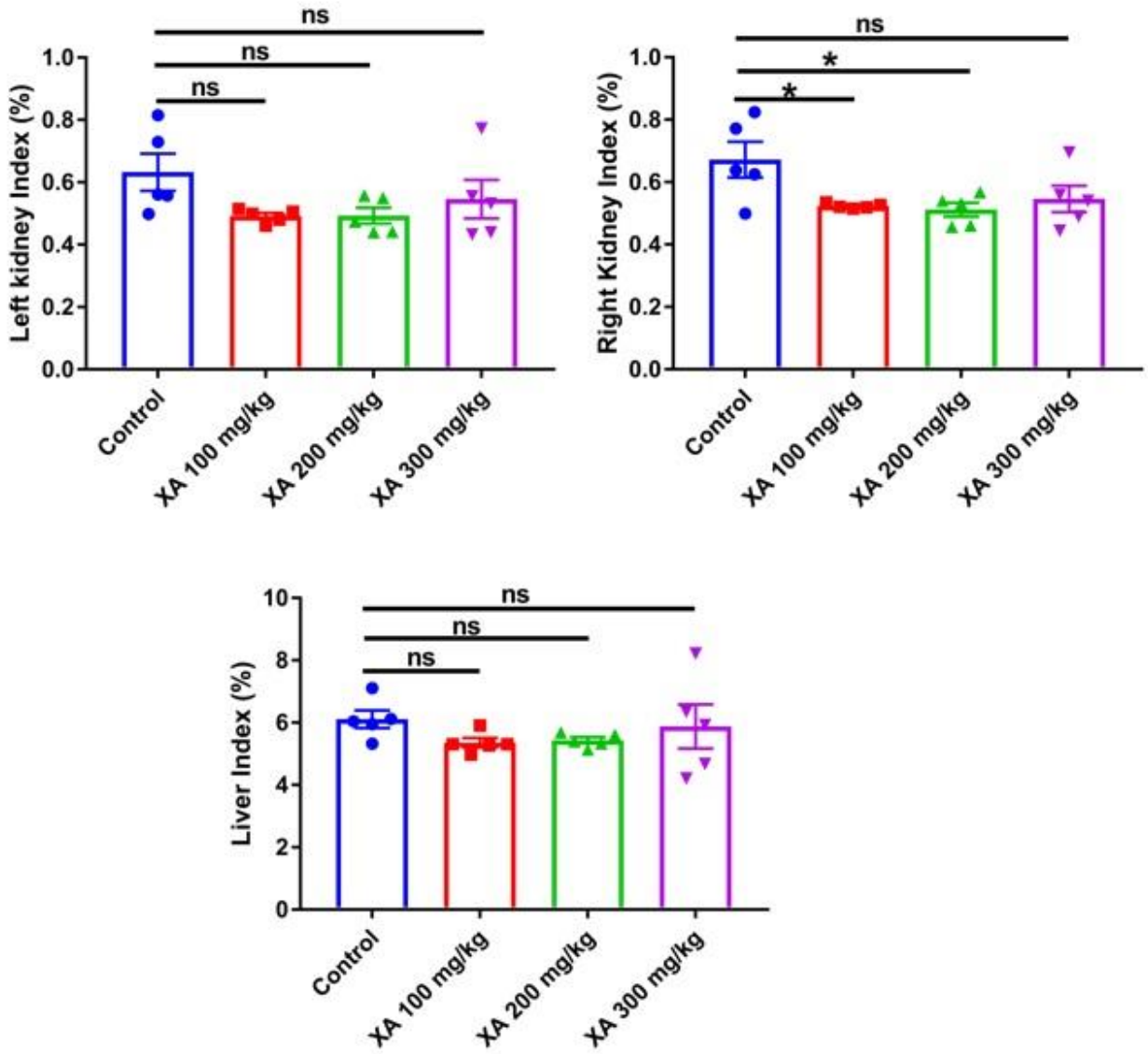

Figure 3: The kidney and liver indices of rats treated with *Xylopia aethiopica* fruit extract. Values are presented as the Mean±standard error of the mean. * indicates a significant difference with control at P<0.05. ns= non-significant change, XA= *Xylopia aethiopica.*

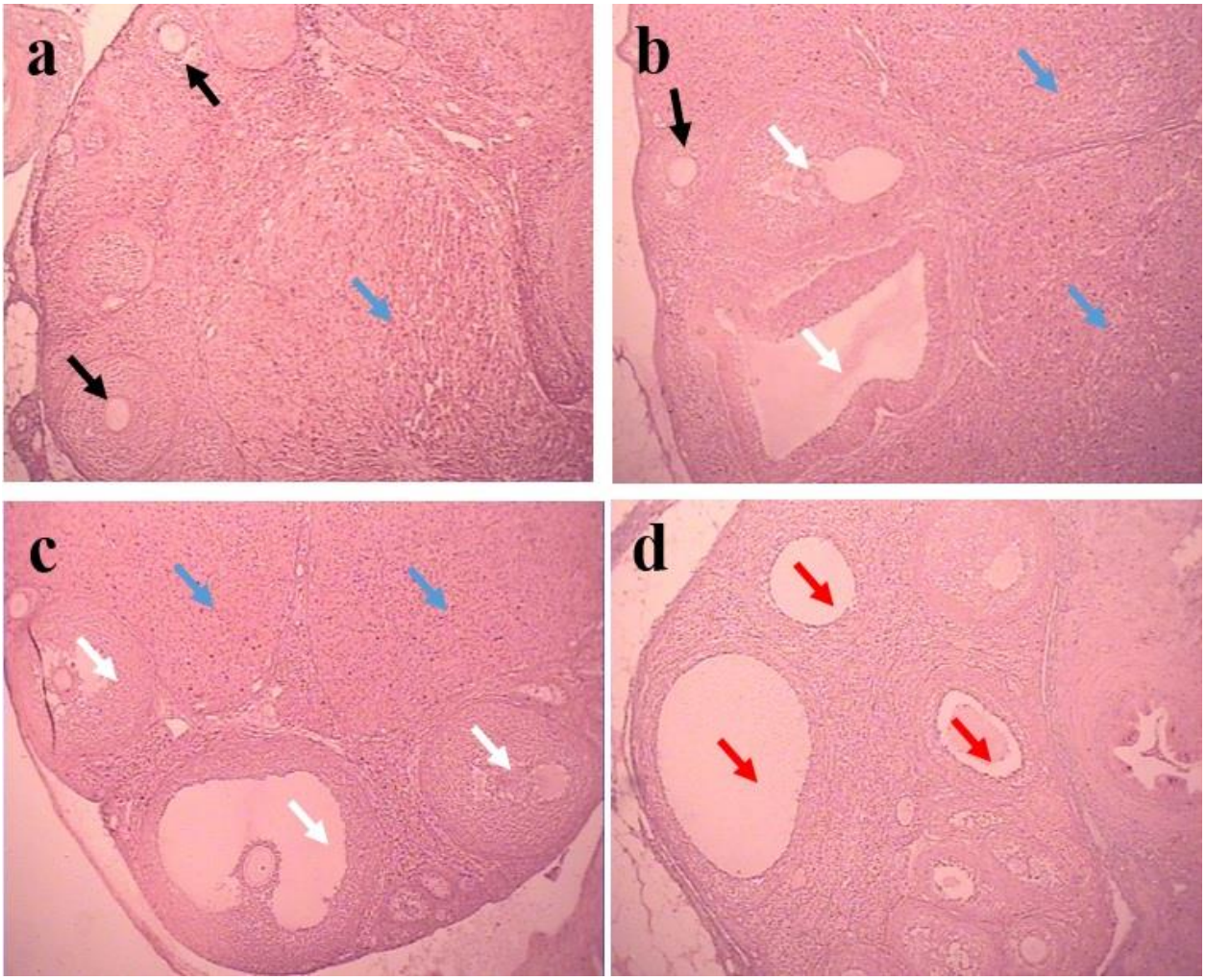

Figure 4: The histology of the ovary of rats treated with *Xylopia aethiopica* fruit extract shows corpus luteal (blue arrows), primary follicles (black arrows), secondary follicles (white arrows), and degenerating follicles (red arrows). a=control, b=treated with 100mg/kg *Xylopia aethiopica,* c=treated with 200 mg/kg *Xylopia aethiopica*, and d=treated with 300 mg/kg *Xylopia aethiopica.* H&E stain X100 magnification.

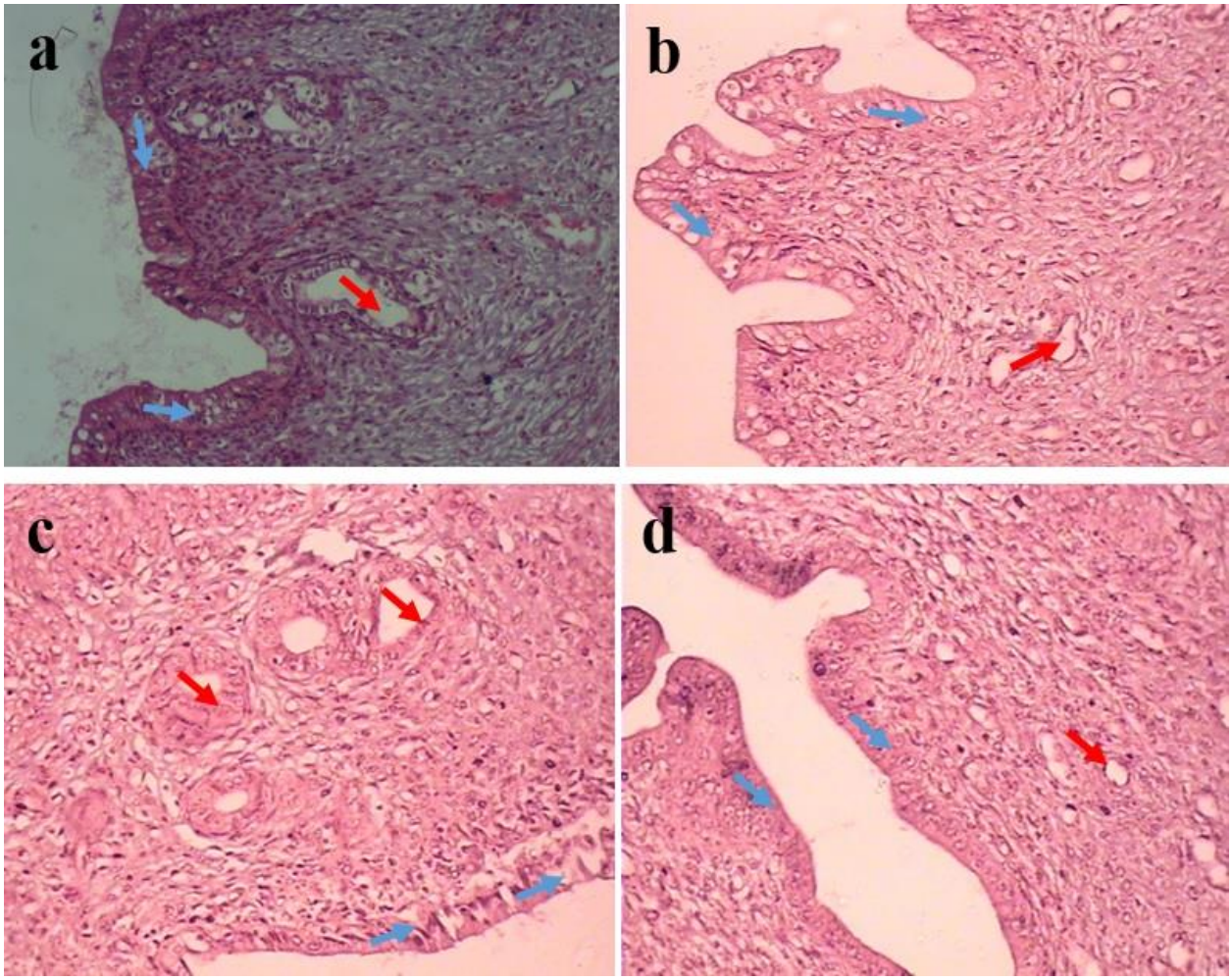

Figure 5: Uterine horn histology of *Xylopia aethiopica* fruit extract treated rats showing endometrial epithelia (blue arrows), and endometrial glands (red arrows). a=control, b=treated with 100mg/kg *Xylopia aethiopica,* c=treated with 200 mg/kg *Xylopia aethiopica,* and d=treated with 300 mg/kg *Xylopia aethiopica.* H&E stain X400 magnification.

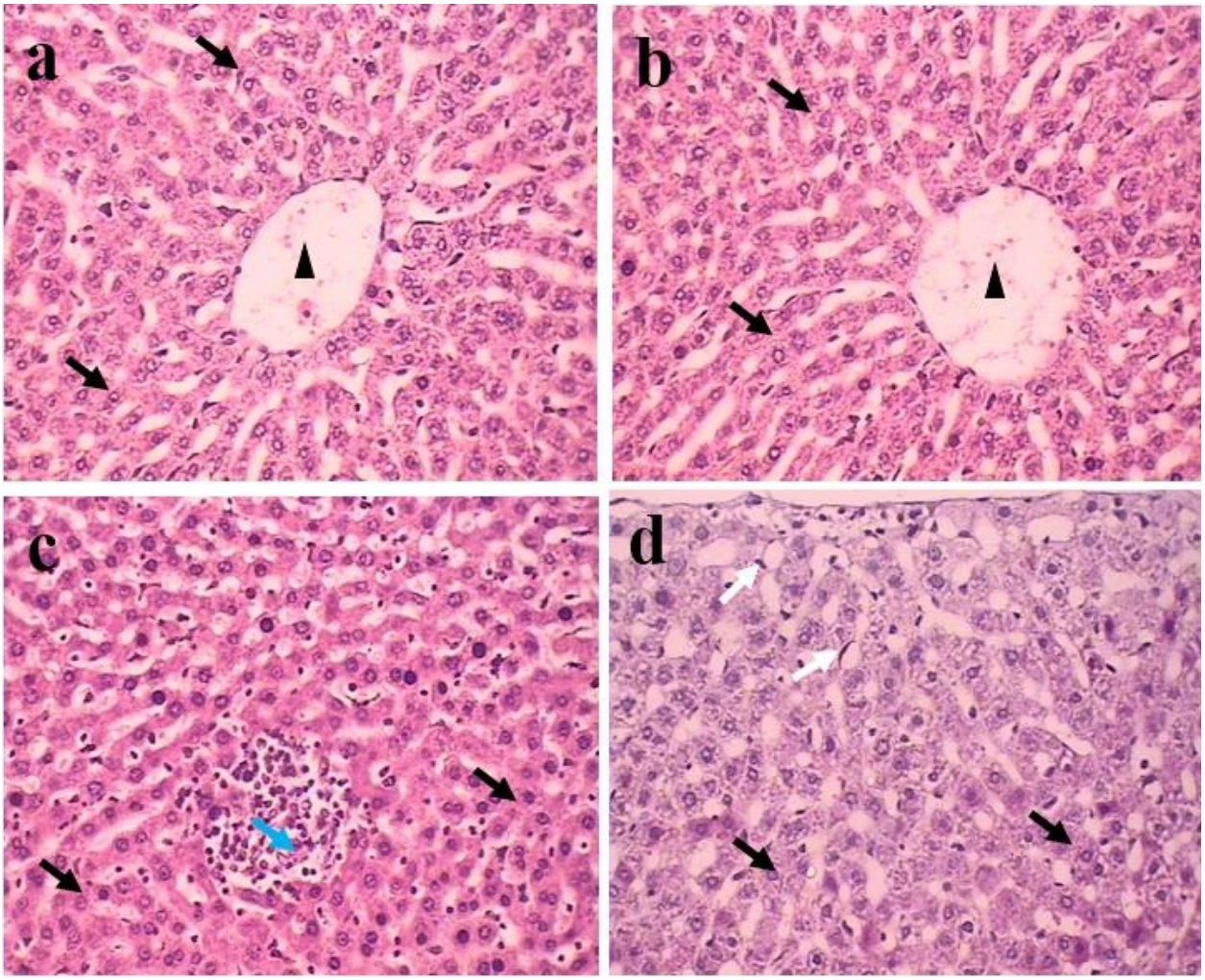

Figure 6: The histology of the liver of rats treated with *Xylopia aethiopica* fruit extract shows central vein (arrowhead), lymphocytes (blue arrow), hepatocytes (black arrows), and pyknosis (white arrows). a=control, b=treated with 100mg/kg *Xylopia aethiopica,* c=treated with 200 mg/kg *Xylopia aethiopica,* and d=treated with 300 mg/kg *Xylopia aethiopica.* H&E stain X200 magnification.

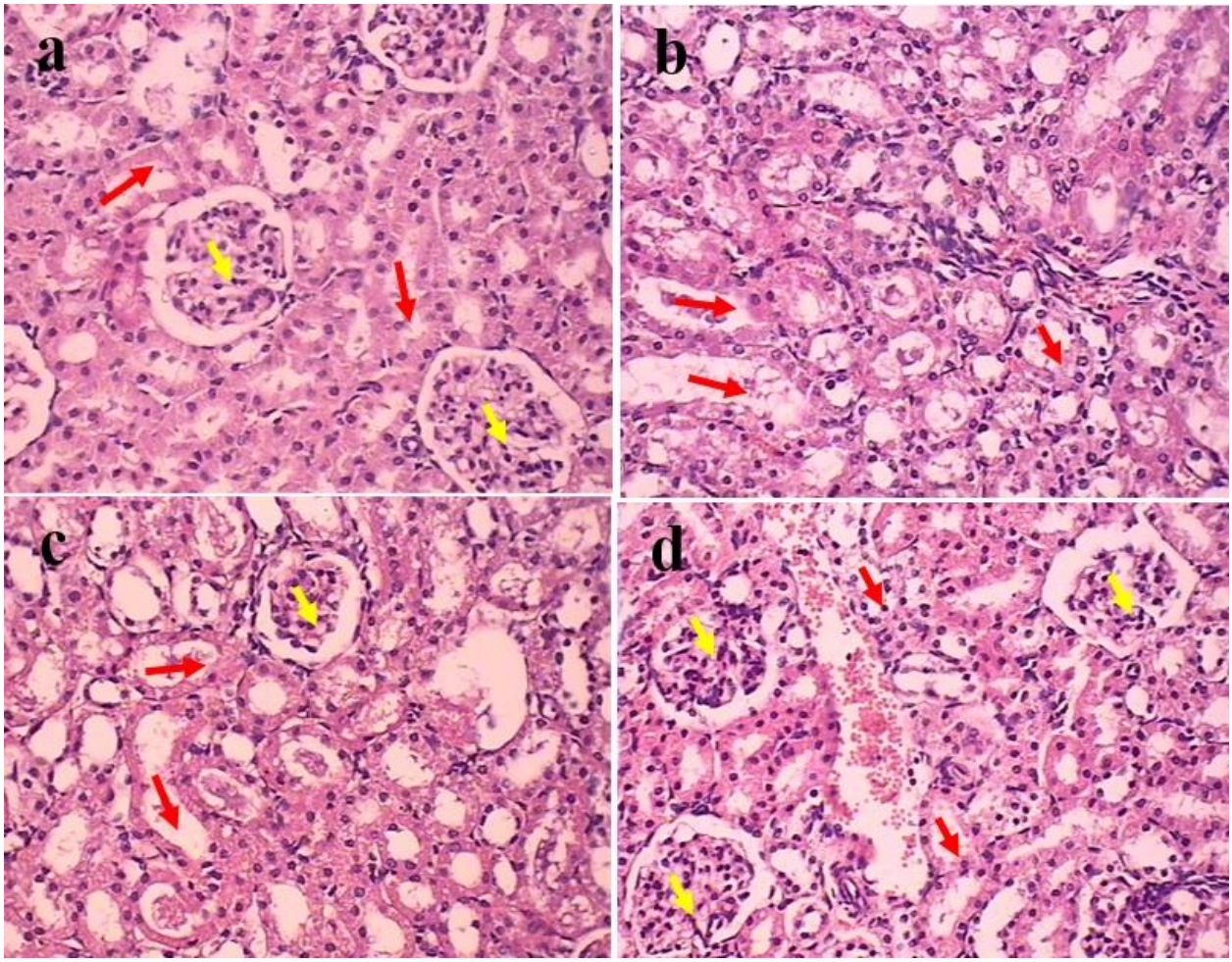

Figure 7: Kidney histology of *Xylopia aethiopica* fruit extracts treated rats showing glomerulus (yellow arrows), and renal tubules (red arrows). a=control, b=treated with 100mg/kg *Xylopia aethiopica,* c=treated with 200 mg/kg *Xylopia aethiopica*, and d=treated with 300 mg/kg *Xylopia aethiopica.* H&E stain X200 magnification.

DISCUSSION

The current study revealed that prolonged administration of XAFE increased estrous cycle duration and prevented the growth and development of ovarian follicles with mild effects on the uterine horn, liver and kidney histology. These findings suggest that prolonged consumption of XAFE in females could affect ovulation and/or conception.

The estrous cycle is a reproductive cycle involving histological, physiological, and biochemical changes within the ovary which leads to the maturation and ovulation of pre-ovulatory follicles, influenced by ovarian and extra-ovarian hormones [20]. Hence, the estrous cycle is initiated after puberty in almost all female mammals with a placenta. Also, the estrous cycle is a principal tool used in determining the reproductive cycle and reproductive toxicology studies [21]. According to Westwood [22], the normal average length of the estrous cycle of rats was 4.8 days. However, this study showed that the aqueous fruit extract of *X. aethiopica* significantly ($p<0.05$) prolonged estrous cycle from (5.40±0.10) in the control to (7.18±0.17) and (7.22±0.24) in rats treated with XAFE at 100 mg/kg and 300 mg/kg respectively. This is in agreement with the findings of Onyebuagu and Agbai [23] on the effect of *X. aethiopica* who reported that *X. aethiopica* has a negative effect on female reproductive organs. Previous studies reported that *Salacia lehmbachii* stem bark and *Alstonia scholaris* leaves also prolonged the duration of the estrous cycle in rats. These plants have similar phytochemicals to *X. aethiopica* [24, 25]. Byers et al. [17] documented that many drugs and chemicals interfere with the reproductive function of the female and affect the estrous cycle [26].

Goldman et al. [27] reported that substance intake can alter the estrous cycle, by either a persistent estrus and/or diestrus phase or an irregular cycle pattern of extended duration. The phytochemical constituents of *X. aethiopica* fruit have been shown to be alkaloids, flavonoids, and saponins [28, 5, 29, 30]. Some of the phytochemicals have been implicated to cause disorders in the estrous cycle in animals [31]. Dande et al. [32] and Onuka et al. [33] reported that saponins have the potential to increase the length of the estrous cycle which may have a negative effect on ovulation. In addition, the study by Stafford [34], has revealed that alteration in the hypothalamic-pituitary-ovarian (HPO) axis leads to disruption of ovarian function. Also, the antigonadotrophic effects of alkaloids, flavonoids, and saponins have been implicated in irregularity in the estrous cycle [35].

The biological basis of analyzing the body weight and organ-to-body weight ratio is that the organ weight may vary in proportional to the total body weight. Hence, they are normally investigated to determine whether the size of the organ has changed, particularly in relation to the weight of the whole animal as an indicator of an adverse effect of toxins [36]. The non-significant change in the ovarian, uterine horn, and liver indices that were observed in the current study signifies that XAFE does not affect the growth and development of the organs. The non-significant reduction in ovarian weight of rats that received XAFE could be the reason for what was observed in the histology of ovaries that received 100 mg/kg and 200 mg/kg of the extract, which demonstrated developing follicles, matured follicles, and corpus luteal while rat that received 300 mg/kg displayed developing follicles but no matured follicles. Since inflammation is comparable to ovulation, the anti-inflammatory potential of flavonoids might have inhibited the cyclo-oxygenase (COX-2) enzyme which is responsible for ovulation [37]. A Study by Stafford [34] has revealed also that alteration in the HPO axis leads to disruption of ovarian function. Also, studies have reported the disrupting effect of various

plant extracts on folliculogenesis [38]. This corresponds to a report by Onyebuagu and Agbai [23] that *X. aethiopica* has a negative influence on ova. Also, this agrees with Onuka et al. [33] that *X. aethiopica* has the potentials of delaying ovulation by the action of saponin.

Consequently, the significant decline in the weight of kidneys observed in the present study might be due to the effect of oral administration of the aqueous fruit extract of *X. aethiopica* on the kidney, which resulted in a mild distortion of epithelium in the glomeruli of kidney tissue at 300mg/kg of the extract. In addition, the non-significant reduction in liver weight observed might be due to the effect of *X. aethiopica* fruit extract of on the liver tissue resulting in aggregation of lymphocytes at 200 mg/kg and presence of pyknotic nuclei at 300mg/kg body weight of the extract. This damage to liver tissue could be a result of prolonged exposure to *X. aethiopica* fruit extract [39]. The study conducted by Chris-Ozoko et al. [40] also report that aqueous fruit extract of *X. aethiopica* caused mild to acute hepatitis and interstitial nephritis in the liver and kidney respectively.

CONCLUSION

Findings from the present study revealed that prolonged consumption of *X. aethiopica* fruit extract alters the development of ovarian follicles thereby prolonging estrous cycle duration with a mild effect on endometrial epithelium. This could lead to infertility in females as it might delay or prevent conception.

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