Original Research Article

Alterations in Female Reproductive Hormones of Wistar Rats sequel to the Administration of *Xylopia aethiopica* Fruit

ABSTRACT

Aim: This study was aimed at investigating the effect of *Xylopia aethiopica* fruit on the reproductive hormones of female 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 female 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. Reproductive hormones were assay using ELISA techniques.

Results: A non-significant increase was observed in the serum concentration of follicle stimulating hormone (FSH) when animals treated with 130 mg/kg body weight of *Xylopia aethiopica* extract were compared with those in the control group. Increase in the dose of *Xylopia aethiopica* extract resulted in a decrease in serum FSH levels. Administration of the extract for 28 days led to a dose-dependent decrease in the serum level of luteinizing hormone (LH). The result of this study indicates the extract decreased serum level of progesterone in female rats at low doses of 130 mg/kg and 259 mg/kg when compared with those in the control group. However, increase in the dose of the extract increases the serum progesterone concentrations. Administration of *Xylopia aethiopica* extract to animals for 28 days led to a dose-dependent decrease in the serum level of estrogen. This decrease was only significant ($P<0.05$) when the estrogen levels of animals treated with 389 mg/kg and 518 mg/kg of *Xylopia aethiopica* extract were respectively compared with those in the control group. Conversely, *Xylopia aethiopica* extract increases serum levels of prolactin in a dose-dependent manner.

Conclusion: The effect of *Xylopia aethiopica* extract on female reproductive hormones observed in this study showed that the extract might be a potent contraceptive. Plant products as contraceptive will be more acceptable for economic reasons and for the fact that they are associated with minimum side effects than synthetic agents.

Keywords: Contraceptive; female reproductive hormones; infertility; *Xylopia aethiopica* fruit

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1. INTRODUCTION

Xylopia aethiopica has a great patronage in both nutrition and ethnomedicine. The plant which also known as African Negro pepper, is popular among traditional medicine practitioners and traditional birth attendants (TBA) who utilize the fruit preparations to cause the discharge of placental after a woman has giving birth [6]. A preparation of the stem bark or fruit is helpful in the management of bronchitis, stomach aches, asthma, and dysenteric conditions [1]. The seed extract is helpful as a vermifuge for roundworms [2]. Several postnatal women eat the aqueous preparation of the fruit for its perceived antiseptic properties. Some of the women have been reported to sometimes come to the hospitals with characteristics which suggest complications in organ [3]. Medicinal plant extracts with a therapeutic property has the tendency of wrong prescription and sometimes, overdosed. The fact that *Xylopia aethiopica* is a natural product does not automatically confers on it safety and might be risky to its consumers.

Chemical ingredients of the plant are perceived to be useful in preventing and managing cancerous tumors [4]. *Xylopia aethiopica* fruit is known to have alkaloids, terpenoids, flavonoids, and organic oils [5,6].

Fig. 1. *Xylopia aethiopica* Fruit [7]

Xylopia aethiopica is characterized with numerous chemical components with various medicinal potentials [8]. 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 [9,10], alkaloids, rutin and fixed oils [11,12]. 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 [10,12]. The impact of the fruit on body weight and glucose concentration of animals has been reported [13]. The fruit has also been reported to induce dyslipidemia [14], hepatotoxicity [15], renal toxicity [16] as well as oxidative stress [17]. Recently, Ogbuagu et al. [18] reported that the fruit extract of *Xylopia aethiopica* adversely perturbed sperm qualities in male Wistar rats. This study was therefore aimed at investigating its effect on the reproductive hormones of female Wistar rats.

2. MATERIALS AND METHODS

2.1 Collection and Authentication of Plant Materials

The fruits of *Xylopia aethiopica* were sourced from a market in Aba, Abia State. They were identified and authenticated by Prof. Margaret Bassey of Botany and Ecological Studies Department, University of Uyo. It was assigned a voucher number of UU/PH/4e and 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

Extraction of the plant was carried out in the Post-graduate Laboratory of Department of Pharmacognosy and Natural Medicine, Faculty of Pharmacy, University of Uyo, Nigeria. It was extracted based on the outlined method in Ogbuagu et al. [7]. The fruits were rinsed under flowing tap water to eliminate contaminants and air-dried. The plant material was milled by laboratory blender. The pulverized plant material was macerated in 250 mL of 99.8% ethanol (Sigma Aldrich) contained in a flask attached to a Soxhlet extractor coupled with condenser and heating mantle (Isomantle). It was then poured into the sample holder (thimble) and inserted in the apparatus. The side arm is lagged with glass wool. The mixture was heated using the heating mantle (Isomantle) at 60 °C and as the temperature rises it starts to evaporate, going via the extractor to the condenser. The condensate dripped into the reservoir housing the thimble. As soon as the solvent gets to the siphon it emptied itself into the flask and the process repeats itself. The process goes on until it is exhaustively extracted. The process runs for a total of 13 hours. As soon as it was set up, it was allowed to run without interruption as long as water and power supply were not interrupted. The apparatus was switched on and off and overnight running was not allowed, and the time for the complete process split over some 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 noted. Several days was spent on the entire process. The evaporated extract was kept in the refrigerator until when the need for it arise.

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

The median lethal dose (LD_{50}) of the extract was determined using albino mice according to the

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method described by Airaodion et al. [19]. 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 treated with 1000 mg/kg, 2000 mg/kg, 3000 mg/kg, 4000 mg/kg and 5000 mg/kg body weight via intraperitoneal (i.p) route and were monitored for visible 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 and 27g were grouped into 5 of 5 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 computed as geometrical means of the maximum dose yielding 0% mortality (a) and the minimum dose yielding 100% death (b).

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

2.4 Experimental Design

Thirty female Wistar rats used in this study were purchased from the University of Uyo, Nigeria. They were allowed to acclimatize for seven days prior to the start of the treatment. The weights were determined and were separated 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 exposed to 130 mg/kg body weight (10% of LD_{50}) of *X. aethiopica* fruit extract, those in group B were treated with 259 mg/kg body weight (20% of LD_{50}) of *X. aethiopica* fruit extract, those in group C were exposed to 389 mg/kg body weight (30% of LD_{50}) of *X. aethiopica* fruit extract, those in group D were treated with 518 mg/kg body weight (40% of LD_{50}) of *X. aethiopica* fruit extract, while those in group E (control) received normal animals feeds and water only. The treatment was done once daily for 28 days via oral route. After 28 days treatment, the animals were sacrificed under ether anaesthesia in a desiccator after an overnight fast. Blood was taken from the rats through cardiac puncture.

2.5 Determination of Female Reproductive Hormones

2.5.1 Determination of Serum Follicle Stimulating Hormone (FSH) Concentrations

Serum follicle stimulating hormone (FSH) concentration was determined using enzyme-linked immunosorbent assay (ELISA) technique [20].

Principle of the Assay

The FSH quantitative test is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay system utilizes a mouse monoclonal anti-α-FSH antibody for solid phase (microtiter wells) immobilization and another mouse monoclonal anti-β-FSH antibody in the antibody enzyme-horseradish peroxidase-(HRP) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in FSH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 45-minute incubation at room temperature, the wells are washed with water to remove unbound-labeled antibodies. A solution of TMB Reagent is added and incubated at room temperature for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of Stop Solution and the color is changed to yellow and measured spectrophotometrically at 450 nm. The concentration of FSH is directly proportional to the color intensity of the test sample.

Assay Procedure

The desired number of coated wells was secured in the holder. 50 µL of standard, sample, and control was dispensed into appropriate wells. 100 µL of enzyme conjugate reagent was added to each well and were thoroughly mixed for 30 seconds before incubating at room temperature (18-25°C) for 45 minutes. The incubation mixture was removed by flicking plate contents into a waste container. The microtiter wells were rinsed and flicked 5 times with deionized water. The wells were stroke sharply onto absorbent paper to remove

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all residual water droplets.100 µL of TMB Reagent was added to each well and was gently mixed for 10 seconds. The mixture was incubated in the dark at room temperature for 20 minutes and 100 µL of stop solution was added to each well and gently mixed for 30 seconds. The blue colour was observed to have changed to yellow completely. The optical density of the mixture was read at 450 nm with a microtiter plate reader within 15 minutes.

Calculation of Results

The average absorbance value (A450) was calculated for each set of reference standards, controls and samples. A standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in mg/dL on a linear graph paper, with absorbance on the vertical or Y-axis, and concentration on the horizontal or X-axis. The corresponding concentration of FSH in mg/dL was determined using the mean absorbance value for each sample from the standard curve.

2.5.2 Determination of Serum Luteinizing Hormone (LH) Concentration

Serum luteinizing hormone (LH) concentration was determined using enzyme-linked immunosorbent assay (ELISA) technique.

Principle of the Assay

The LH ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay (ELISA). The assay system utilizes mouse monoclonal anti-α-LH for solid phase (microtiter wells) immobilization, and a mouse monoclonal anti-β-LH antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the LH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 45 minute incubation at room temperature, the wells are washed with water to remove unbound-labeled antibodies. A solution of TMB Reagent is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of stop solution, and the color is changed to yellow and measured spectrophotometrically at 450 nm. The concentration of LH is directly proportional to the color intensity of the test sample.

Assay Procedure

The desired number of coated wells was secured in the holder. 50 µL of standards, samples, and controls were dispensed into appropriate wells. 100 µL of enzyme conjugate reagent was added to each well and gently mixed for 30 seconds before incubating at room temperature (18-25°C) for 45 minutes. The incubation mixture was removed by flicking plate contents into sink. The microtiter wells were rinsed and flicked 5 times with deionized water. The wells were sharply stroke onto absorbent paper to remove all residual water droplets. 100 µL of TMB reagent was added to each well and gently mixed for 10 seconds before incubating in the dark at room temperature for 20 minutes. 100 µL of stop solution was added to each well gently mixed for 30 seconds. The blue color was observed to have changed to yellow color completely. The optical density of the mixture was read at 450 nm with a microtiter plate reader within 15 minutes.

Calculation of Results

The average absorbance value (A450) was calculated for each set of reference standards, controls and samples. A standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in mg/dL on a linear graph paper, with absorbance on the vertical or Y-axis, and concentration on the horizontal or X-axis. The corresponding concentration of LH in mg/dL was determined using the mean absorbance value for each sample from the standard curve.

2.5.3 Determination of Serum Progesterone Concentration

Serum progesterone concentration was determined using enzyme-linked immunosorbent assay (ELISA) technique.

Principle of the Test

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled antigen (present in standards, controls and patient samples) and an enzyme-labeled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic

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reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed is inversely proportional to the concentration of progesterone in the sample. A set of standards is used to plot a standard curve from which the amount of progesterone in patient samples and controls can be directly read.

Assay Procedure

The desired number of coated wells was secured in the holder. 25 µL of standards, samples and controls were dispensed into appropriate wells. 100 µL of working progesterone-HRP conjugate reagent was added to each well, followed by 50 µL of anti-progesterone reagent to each well. The mixture was thoroughly mixed for 30 seconds and incubated at room temperature (18-25°C) for 90 minutes. The microwells were rinsed and flicked 5 times with deionized water. 100 µL of TMB reagent was added to each well and gently mixed for 10 seconds before incubating at room temperature (18-25°C) for 20 minutes. After the incubation, 100 µL of stop solution was added to each well and gently mix 30 seconds. The absorbance was read at 450 nm with a microtiter well reader within 15 minutes.

Calculation of Results

The average absorbance value (A450) was calculated for each set of reference standards, controls and samples. A standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in ng/dL on a linear graph paper, with absorbance on the vertical or Y-axis, and concentration on the horizontal or X-axis. The corresponding concentration of progesterone in ng/dL was determined using the mean absorbance value for each sample from the standard curve.

2.5.4 Determination of Serum Estrogen Concentration

Serum estrogen concentration was determined using enzyme-linked immunosorbent assay (ELISA) technique.

Principle of the Assay

In the estrogen ELISA Assay Kit, the 17β-Estradiol (antigen) in the sample competes with the antigenic 17β-Estradiol conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti 17β-Estradiol coated on the microplate (solid phase). After incubation, the bound/free separation is performed by a simple solid-phase washing. Then the enzyme HRP in the bound-fraction reacts with the substrate (H_2O_2) and the TMB Substrate and develops a blue color that changes into yellow when the Stop Solution (H_2SO_4) is added. The colour intensity is inversely proportional to the 17β-Estradiol concentration in the sample. 17β-Estradiol concentration in the sample is calculated through a calibration curve.

Assay Procedure

The desired number of microtiter coated wells was secured in the holder. 25µL of specimens were dispensed into the appropriate wells. 100µL of estradiol–HRP conjugate reagent was dispensed into each well. 50 µL of antiestradiol (E_2) reagent was added to each well and was thoroughly mixed for 30 seconds, and then incubated at room temperature for 90 minutes. The microwells were rinsed and flicked 5 times with deionized water. 100 µL of TMB reagent was added to each well and gently mixed for 10 seconds, then incubated at room temperature for 20 minutes. The reaction was stopped by adding 100 µL of stop solution into each well and then mixed gently for 30 seconds to ensure that all blue colours changed to yellow completely. Absorbance was read at 450nm with a microtiter well reader within 15 minutes using microplate Reader machine (MR – 9620A).

Calculation of Results

The average absorbance value (A450) was calculated for each set of reference standards, controls and samples. A standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in mg/dL on a linear graph paper, with absorbance on the vertical or Y-axis, and concentration on the horizontal or X-axis. The corresponding concentration of estrogen in mg/dL was determined using the mean absorbance value for each sample from the standard curve.

2.5.5 Determination of Serum Prolactin Concentration

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Serum prolactin concentration was determined using enzyme-linked immunosorbent assay (ELISA) technique [20].

Principle of the Assay

The ELISA test is performed as an indirect solid phase sandwich-type immunoassay. Microwells are coated with anti-monoclonal prolactin followed by blocking the unreacted sites to reduce non-specific binding. Prolactin Antigens present in calibrators and patient samples bind to the coated antibody. The Antigen-Antibody complex is reacted with enzyme (HRP) labeled anti-monoclonal prolactin conjugate resulting in the monoclonal prolactin antigen being sandwiched between the solid phase antibody and the enzyme conjugate. The enzyme converts added substrate (TMB) to form a colored solution. The intensity of color change, which is proportional to the concentration of antibodies present in the samples is read by a microplate reader at 450 nm. Results are expressed in nanogramm per milliliter (ng/mL)

Assay Procedure

All reagents were allowed to stand at room temperature prior to the assay. 25 μL of Prolactin standards, control, and samples were pipetted into appropriate wells and 100 μL of Enzyme Conjugate was added to all wells. The plate was covered and incubated for 60 minutes at room temperature (18–26 °C). Liquid were removed from all wells and the wells were washed three times with 300 μL of buffer, and blotted on absorbent paper. 100 μL of TMB substrate were added to each well, and incubated for 15 minutes at room temperature.50 μL of Stop Solution was added to each well, and the plate was shaken gently to mix the solution. The absorbance of the mixture was read on ELISA Reader at 450 nm within 15 minutes after adding the stopping solution.

Calculation of Results

The average absorbance value (A450) was calculated for each set of reference standards, controls and samples. A standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration in ng/dL on a linear graph paper, with absorbance on the vertical or Y-axis, and concentration on the horizontal or X-axis. The corresponding concentration of prolactin in ng/dL was determined using the mean absorbance value for each sample from the standard curve.

2.6 Statistical Analysis

Data were subjected to analysis of variance using Graph Pad Prism. Results were presented as Mean ± Standard Error of the Mean (SEM). One-way analysis of variance (ANOVA) was used to compare the mean, followed by Tukey's post hoc test. Differences between means were considered to be significant at $p<0.05$.

3. RESULTS

3.1 Median Lethal Dose (LD_{50}) Result

The visible signs of toxicity of *X. aethiopica* fruit extract observed in this study are excitation, decreased motor activity, paw licking, increased respiratory rate, gasping and coma which could be followed up by death. In the first phase of the median lethal dose determination, no death was observed in the group administered 1000 mg/kg body weight of *X. aethiopica* fruit extract. However, all the animals died in the groups exposed to 2000, 3000, 4000, and 5000 mg/kg body weight of *X. aethiopica* fruit extract respectively (Table 1). In the same vein, in the second phase of medial lethal dose determination, no death 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 computed as geometrical average of the maximum dose yielding 0% death (a) and the minimum dose yielding 100% death (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 Female Reproductive Hormones of Animals after 28 days of Treatment

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A non-significant increase was observed in the serum concentration of follicle stimulating hormone (FSH) when animals treated with 130 mg/kg body weight (low dose) of *Xylopia aethiopica* extract were compared with those in the control group (P = 0.05), as presented in Table 2. Increase in the dose of *Xylopia aethiopica* extract resulted in a decrease in serum FSH levels. This decrease was significant when the FSH levels of animals treated with 389 mg/kg and 518 mg/kg body weight of ethanol extract of *Xylopia aethiopica fruit* were respectively compared with those in the control group (P<0.05). Administration of ethanol extract of *Xylopia aethiopica fruit* to animals for 28 days led to a dose-dependent decrease in the serum level of luteinizing hormone (LH). This decrease was only significant (P = 0.01) when the LH levels of animals treated with 389 mg/kg and 518 mg/kg of *Xylopia aethiopica* extract were respectively compared with those in the control group. The result of this study indicates that extract of *Xylopia aethiopica* decreased serum level of progesterone in female rats at low doses of 130 mg/kg and 259 mg/kg. The decrease was only significant (P<0.05) at the lowest dose of 130 mg/kg when compared with those in the control group. However, increase in the dose of the extract increases the serum progesterone concentrations. The increase became significant (P = 0.02) when the concentration of serum progesterone in animals treated with 518 mg/kg of *Xylopia aethiopica* extract were compared with those in the control group. Administration of *Xylopia aethiopica* extract to animals for 28 days led to a dose-dependent decrease in the serum level of estrogen. This decrease was only significant (P<0.05) when the estrogen levels of animals treated with 389 mg/kg and 518 mg/kg of *Xylopia aethiopica* extract were respectively compared with those in the control group. Conversely, *Xylopia aethiopica* extract increases serum levels of prolactin in a dose-dependent manner. The increase was however nonsignificant (P>0.05) when the prolactin levels of animals treated with 130 mg/kg (low dose) of *Xylopia aethiopica* extract were compared with those in the control group.

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

LD_{50}= 1296.15 mg/kg

Table 2. Effect of ethanol extract of *Xylopia aethiopica* fruit on Female Reproductive Hormones of Animals after 28 days of Treatment

Group	A	B	C	D	E	
Dose of extract (mg/kg)	**130**	**259**	**389**	**518**	**Control**	**P Value**

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FSH (mg/dL)	20.52±2.15	16.84±3.08	11.47±2.26	9.33±1.42	18.77±2.26	0.05
LH (mg/dL)	31.89±7.08	29.03±3.82	24.41±3.33	20.72±3.06	34.98±5.07	0.01
Pg (ng/dL)	11.03±2.62	16.67±3.27	23.55±2.99	28.22±4.01	19.71±2.13	0.02
Estrogen (mg/dL)	29.11±3.38	26.45±4.10	24.06±2.44	20.98±3.04	33.02±7.25	0.04
Prolactin (ng/dL)	25.09±2.35	30.27±4.05	32.33±3.62	38.02±3.84	20.14±4.04	0.05

Values are presented as Mean±S.D, where n = 6. Values are statistically significant at p value ≤ 0.05

Legend: FSH = Follicle Stimulating Hormone, LH = Luteinizing Hormone, Pg = Progesterone

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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 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.

The results of the effect of ethanol extract of *Xylopia aethiopica* fruit on female reproductive hormones of animals after 28 days of treatment are presented in table 2**.** Maturation of pre-ovulatory follicles and ovulation are under the combined and balanced influences of ovarian and extra ovarian hormones. Imbalances or alterations in these hormones lead to irregularity in the ovarian functions and duration of estrous cycle [21]. These hormonal imbalances might be caused by numerous chemical agents contained in plant extracts [22]. The balance of sexual hormones in humans is very important [23]. To produce a successful fertility in the first half of the menstrual cycle, estrogen levels rise and cause the growth and increase of uterine cells. In response to the FSH hormone, an ovum begins to grow inside one of the ovaries. On day 14 of a 28-day cycle, an ovary is released in response to the LH hormone. And the increase in progesterone levels occurs in the second half of the menstrual cycle, which results in an increase in the thickness of the lower limb [24]. In this study, a nonsignificant increase was observed in the serum concentration of follicle stimulating hormone (FSH) when animals treated with 130 mg/kg body weight (low dose) of ethanol extract of *Xylopia aethiopica* fruit were compared with those in the control group. Increase in the dose of Xylopia *aethiopica* extract resulted in decrease in serum FSH levels. This decrease was significant (P<0.05) when the FSH levels of animals treated with 389 mg/kg and 518 mg/kg body weight of *Xylopia aethiopica* extracts were respectively compared with those in the control group. Follicle stimulating hormone is the central hormone of mammalian reproduction, essential for gonadal development and maturation at puberty as well as gamete production during the fertile phase of life [25]. It stimulates the growth and maturation of ovarian follicles by acting directly on the receptors located on the granulose cells [22]. The reduction in the levels of FSH by the extract of *Xylopia aethiopica* fruit at high doses might hamper folliculogenesis and delay maturation of the follicle in the pre-ovulatory phase [26]. The decrease in the serum levels of FSH at these doses is similar to the findings of Nnodim *et al.* [27] who reported a decrease in serum FSH when animals were treated with high doses of *Xylopia aethiopica* fruit extract. It is possible that the extract at these doses might have exerted its effect on the anterior pituitary or the hypothalamus since the secretion of stimulating hormone is regulated by the gonadotropic releasing hormone secreted by the hypothalamus [28]. The reduction observed in the level of this hormone might adversely affect conception in female animals. The principal function of FSH is to stimulate gametogenesis and follicular development in females [29]. In females, FSH acts on immature follicular cells of the ovary and induces development into mature follicle and oocyte capable of steroidogenesis. Steroidogenic functions in females depend on other hormonal factors such as luteinizing hormone which stimulates androgen formation in gonads while FSH stimulates the conversion of androgens to estrogens [30]. The decrease in circulating FSH observed in this study could also be traced to the net effect of synthesis and clearance. Since steroids reduce FSH synthesis and extracts of *Xylopia aethiopica* fruit contains

steroids [7], it might be suggestive that steroids could also possibly be responsible for decrease in circulating FSH level observed in this study.

Lutenizing hormone (LH) stimulates secretion of sex steroids from the gonads [31] In females, ovulation of mature follicles in the ovary is induced by a surge of luteinizing hormone secretion during the pre-ovulatory periods. Several authors have demonstrated that LH release surges at the pre-estrous stage were responsible for ovulation [22, 26]. Any substance capable of inhibiting this release could provoke disruption of ovulation by decreasing the number of mature follicles or induce an estrous cycle disruption at rest [32,33]. The significant decrease observed in the level of serum LH in this study (especially at high doses) might indicate an inhibitory effect of the extract on the release of LH which may trigger disruption of ovulation. This might result in impairment of estrous cycle, hamper conception and normal reproduction in females. It is therefore possible that *X. aethiopica* fruit contains anti-gonadotropic substance which might affect the estrous cycle and hamper reproduction in females [34].The reduction in the serum level of LH observed in this study is dose-dependent.

Progesterone which is produced in the ovaries, placenta, and adrenal glands, helps to regulate the monthly menstrual cycle, prepare the body for conception and pregnancy as well as stimulate sexual desire [35]. The hormone also encourages the growth of milk-producing glands in the breast during pregnancy. High progesterone levels were believed to be partly responsible for symptoms of premenstrual syndrome (PMS), such as breast tenderness, feelings of bloat and mood swings. The feedback inhibition of Gonadotropin-releasing hormone (GnRH) secretion by estrogens and progesterone provides the basis for the most widely-used form of contraception. Such feedback inhibition of GnRH prevented the mid cycle surge of LH and ovulation [36]. The result of this study indicates that extract of *X. aethiopica* decreased serum level of progesterone in female rats at low doses of 130 mg/kg and 259 mg/kg. The decrease was more significant ($P<0.05$) at the lowest dose of 130 mg/kg when compared with those in the control group. The reduction in the levels of serum progesterone by *Xylopia aethiopica* extract at these doses might produce consequential effect on conception in females; impede ovulation which might result in annovulation and sequelae [22]. Alkaloids had equally been reported to inhibit the synthesis of cellular progesterone [37]. Therefore, the reduced level of progesterone by *Xylopia aethiopica* may not be unconnected with the alkaloidal component of the extract. This result is consistent with the findings of Onuka *et al.,* [38] who reported an increase in the level of progesterone when animals were treated with low doses of *Xylopia aethiopica* fruit extracts. However, increase in the dose (388.5 and 518 mg/kg) of the extract in this study increased the serum progesterone concentrations. The increase became significant ($P<0.05$) when the concentration of serum progesterone in animals treated with 518 mg/kg of *Xylopia aethiopica* extract were compared with those in the control group. The phytochemical evaluation of *Xylopia aethiopica* fruit revealed that it contains alkaloids, saponin, tanins, Coumarin, phlobatannins, anthraquinones, steroids, flavonoids, cardiac glycosides and so on [7]. Yun *et al.,* [39] cited in Egba *et al.,* [26] had reported that high dose of extract of *Xylopia aethiopica*, containing saponins lowered serum androgens and 17β-estradiol, but elevated progesterone levels, suggesting that saponins might modulate steriodogenesis in the ovary. High level of progesterone has antiestrogenic effect on the myometrial cell, decreasing their excitability, their sensitivity to oxytocin, and their spontaneous electrical activity while increasing their membrane potential [40]. The increase observed in the serum concentration of progesterone in this study sequel to administration of high dose of *Xylopia aethiopica* fruit extract was in line with the findings of Nnodim *et al.,* [27] who investigated the effects of *Xylopia aethiopica* fruits on reproductive hormonal level in rats. Decrease in serum levels of FSH and LH had been reported to increase progesterone concentration in Wistar rats [41]. Therefore, the increase observed in the concentration of progesterone in this study following the administration of high dose of *Xylopia aethiopica* might be due to the reduction in the concentrations of FSH and LH observed in this study. Increase in serum progesterone concentrations had been speculated to indirectly predispose animals to teratogenicity and carcinogenesis [42]. Thus, the increase observed in the serum level of progesterone in this study at high doses suggested that extracts of *Xylopia aethiopica* might indirectly predispose

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its consumers to teratogenicity and carcinogenesis when consumed in high dosage.

Estrogen stimulates the growth of the uterine lining, causing it to thicken during the pre-ovulatory phase of the cycle. It is well established that estrogen is directly responsible for growth and development of reproductive organs. In synergy with follicle stimulating hormone, estrogen stimulates granulose cell proliferation during follicular development. Plants with estrogenic properties can directly influence pituitary action by peripheral modulation of luteinizing hormone and follicle stimulating hormone, decreasing secretion of these hormones and blocking ovulation. Thus, the reduction in the serum concentration of estrogen observed in this study might be attributed to a decreased aromatase activity or substrate supplementation during estrogen synthesis [43]. Consequently, such decrease in estrogen levels may hamper ovulation, preparation of the reproductive tract for zygote implantation and the subsequent maintenance of pregnancy state [43]. Kadohama *et al.,* [44] had reported that several plant alkaloids inhibit aromatase activity. Thus, it is possible that the phytochemical content of extract of *Xylopia aethiopica* may disrupt endocrine activity and possibly induce hormonal imbalance or disorders such as anti-fertility and contraception in hormone dependent organs like the ovary and mammary glands. The findings in this study have important implications for female contraceptive development. The reduction in the serum level of estrogen observed in this study is consistent with the report of Adienbo *et al.,* [45] who observed a dose dependent decrease in the number of pregnant females in all the test groups when they studied the contraceptive efficacy of hydro-methanolic fruit extract of *Xylopia aethiopica* in male albino rats.

In the context of this study, ovaries are central in the synthesis of estrogen and could be altered by the extract. During the process of folliculogenesis, thecal and granulosa cells are involved in the estrogen synthesis. Theca cells can not directly produce estrogen and therefore in growing follicles, androgens are released from the thecal cells and transported to the granulosa cells where P_{450} aromatase enzyme converts androgens to estrone and 17-beta estradiol [46]. In addition to saponin, flavonoids are one of the major phytochemical contents of the *Xylopia aethiopica* fruit extract. Studies have shown that saponins and flavonoids inhibit aromatase enzyme in human preadipocyte [47] and inhibition of aromatase enzyme has been shown to reduce estrogen production throughout the body to nearly undetectable levels [48]. To a large extent, estrogen from androgen conversion contributes to the estrogen pool. Moreover androgen production from the thecal cells is largely under the control of LH from the pituitary [49]. Therefore, the decrease in LH observed in this study could impair androgen production and inhibit the conversion of androgens into estrogens in the granulosa cells resulting in the reduction of estrogen level. This result is consistent with the findings of Onuka *et al.,* [38] who reported a decrease in estrogen concentration sequel to *Xylopia aethiopica* administration. FSH has also been reported to stimulate the conversion of androgens to estrogens [30]. The decrease in the level of estrogen might also be attributed to the observed decrease in the concentration of serum FSH in this study. Estrogen production by the ovary or corpus luteum occurs as a result of interplay of different endocrine glands and enzymes [50]. It is possible that *Xylopia aethiopica* fruit was able to reduce estrogen concentration by interfering with one of the steps or enzymes that lead to its production.

The enhanced level of prolactin observed in this study might be attributed to the effect of the extract probably acting as a dopamine antagonist. The elevated level of prolactin in this study justifies the folklore use of the plant in stimulating lactation. The result of this study is consistent with the findings of Onyebuagu *et al.,* [34], Anacletus *et al.,* [51] and Ehigiator and Adikwu [52] who respectively reported an increase in serum prolactin level when they administered different doses of *Xylopia aethiopica* extracts on female Wistar rats. The increase serum prolactin was as a result of increased synthesis and secretion of prolactin in the anterior pituitary in response to hormonal factors such as thyrotropin-releasing hormone and oxytocin, physiological signals such as sleep, stress, pregnancy and tactile signals during weaning, whereas, reduction in the circulating prolactin can be traced to the activity of prolactin-inhibiting hormone, dopamine and tissue-specific prolactin clearance by ovary, liver, kidney and mammary gland during lactation [53]. The concentration of prolactin in the serum at any time is therefore influenced by the factors inducing synthesis, those inhibiting

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synthesis and turnover rate at the target cells [54]. These hormonal imbalances observed in this study might be caused by numerous chemical agents contained in the extract. Yakubu *et al.* [22] and Benie *et al.* [32] independently reported that phytochemical screening has revealed many bioactive as well as toxic agents of plant extract that could affect the regulation of estrous cycle, conception and reproduction. Alkaloids and flavonoids have been shown to reduce plasma concentrations of estrogen [54]. Therefore, the presence of these phytochemicals in *Xylopia aethiopica* fruit may account for the alterations in the levels of the circulating hormones observed in this study. Our previous study [18] showed that extracts of *Xylopia aethiopica* fruit reduced sperm qualities in Wistar rats and might result in infertility in male rats. This is suggestive that the fruit has the propensity to induce infertility in both male and female rats.

5. Conclusion

The effect of *Xylopia aethiopica* extract on female reproductive hormones observed in this study showed that the extract might be a potent contraceptive. Plant products as contraceptive will be more acceptable for economic reasons and for the fact that they are associated with minimum side effects than synthetic agents.

CONSENT

It is not applicable.

REFERENCES

1. Burkill HM. Brief description and details of the uses of over 4,000 plants. Royal botanical garden, kew; 2004.
2. Dalziel JM. The useful plants of Tropical West Africa.Crown overseas Agents Colonies, London. 1975;461.
3. Ezekwesili C, Nwodo O, Eneh F. Ogbunugafor H. Investigation of the chemical composition and biological activity of *Xylopia aethiopica* Dunal (Annonacae).African Journal of Biotechnology. 2010;9(43):7352-7356.
4. Del-Rio A, Obdulio BG, Castillo J, Main FR, Ortuno A. Uses and properties of Citrus flavonoids. Journal of Agricultural Food Chemistry. 1997;45:4505-4515.
5. Iwu MM. Handbook of Africa Medical Plant.CRCPress, London. 1993;435.
6. Shanmugam P, Anura W, Nedra KD, Gavin C, Wijesundara DSA, Raymond A, Veranja K. Antioxidant constituents from Xylopia championii. Pharmaceutical Biology. 2008;46(5):352-355.
7. Ogbuagu EO, Ogbuagu U, Unekwe PC, Nweke IN, Airaodion AI. Qualitative determination of the phytochemical composition of ethanolic extract of *Xylopia aethiopica* fruit. Asian Journal of Medical Principles and Clinical Practice. 2020; 4(3):1-5
8. Nwafor A, Adienbo MO, Egwurugwu JN. *In vivo* effects of *Xylopia aethiopica* on hemorheological parameters in Guinea pigs. African. Journal of Applied Zoology/ Environmental Biology. 2009;11:79-81.
9. Esekhiagbe M, Agatemor MMU, Agatemor C. Phenolic content and antimicrobial potentials of *Xylopia aethiopica* and Myristica argentea. Macedonian Journal of Chemistry and Chemical Engineering. 2009;28(2):159-162.
10. John-Dewole O, Agunbiade S, Alao O, Arojojoye O. Phytochemical and antimicrobial studies of extract of the fruit of *Xylopia aethiopica* for medicinal importance. Journal of Biotechnology and Pharmaceutical Research. 2012; 29(6):118-122.
11. Asekun O, Kunle O. The chemical constituents of the fruit essential oil of *Xylopia aethiopica* (Dunal) A. Rich from Nigeria.Journal of Essential Oil Bearing Plants. 2004;7(2):186-189.
12. Nwaichi E, Igbinobaro O. Effects of some selected spices on some biochemical profile of Wister albino rats. American Journal of Environmental Engineering. 2012;2(1):8-11.
13. Ogbuagu EO, Nweke IN, Unekwe PC, Airaodion AI, Ogbuagu U. Weight gain reduction and hypoglycemic effects of *Xylopia aethiopica* fruit extract on Wistar

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rats. International Journal of Research and Reports in Hematology. 2020;5(3):1-8.

14. Ogbuagu EO, Unekwe PC, Airaodion AI, Nweke IN, Ogbuagu U. Hypolipidemic propensity of ethanolic extract of *Xylopia aethiopica* fruit in Wistar rats. Asian Journal of Research in Cardiovascular Diseases. 2020;3(5):1-11.

15. Ogbuagu EO, Unekwe PC, Airaodion AI, Nweke IN, Ogbuagu U. Hepatotoxic effect of *Xylopia aethiopica* fruit in Wistar rats. International Research Journal of Gastroenterology and Hepatology. 2021; 4(1):1-16

16. Ogbuagu EO, Airaodion AI, Ogbuagu U, Nweke IN, Unekwe PC. Nephrotoxicity of ethanol extract of *Xylopia aethiopica* fruit in wistar rats. International Journal of Advances in Nephrology Research. 2021;4(1):1-16.

17. Ogbuagu EO, Ogbuagu U, Airaodion AI, Uche CL, Ezirim EO, Nweke IN, Unekwe PC. Effect of Ethanol Extract of *Xylopia aethiopica* Fruit on Oxidative Stress Indices of Wistar Rats. Asian Journal of Immunology. 2022;6(1):40-51.

18. Ogbuagu EO, Airaodion AI, Ogbuagu U, Ezirim EO, Nweke IN, Unekwe PC. Ethanol Extract of *Xylopia aethiopica* (African Negro Pepper) Fruit adversely perturbed Semen Qualities in Male Wistar Rats. International Journal of Research and Report in Gynaecology. 2022;7(2):43-52

19. Airaodion AI, Ekenjoku JA, Ogbuagu EO, Okoroukwu VN, Ogbuagu U. *Carica papaya* leaves might cause miscarriage. Asian Research Journal of Gynaecology and Obstetrics. 2019;2(2):1-9.

20. Tanv S, Harkaran S.Estimation of serum prolactin levels and determination of prevalence of hyperprolactinemia in newly diagnosed cases of subclinical hypothyroidism. Journal of family medicine and primary care. 2018;7(6): 1279.

21. Shivalingappa H, Satyanaranyan ND, Purohit MG, Sahranabasappa, A. &Patil, S. B. (2002).Effect of ethanol extract of *Rivea hypocraterifomis* on the estrous cycle of the rat. *Journal of Ethnopharmacology.*82: 11-17.

22. Yakubu, M. T., Akanji, M. A., Oladiji, A. T., Olatinwo, A. O., Adesokan, A. A., Yakubu, M. O., Owoyele, B. V., Sunmonu, T. O. & Ajao, M. S.(2008). Effect of *Cnidoscolousaconitifolius*(Miller) I.M. Johnston leaf extract on reproductive hormones of female rats. *Iranian Journal of Reproductive Medicine.*6(3): 149-155

23. Farahbod, F. &Soureshjani SH. Medicinal herbs affecting gonadotropin hormones in women: An updated systematic review. International Journal of Life Science and Pharma Research. 2018;8(1):20-28.

24. Vlckova R, Sopkova D, Andrejcakova Z, Valocky I, Kadasi A, Harrath AH. Dietary supplementation of yucca (*Yucca schidigera*) affects ovine ovarian functions. Theriogenology. 2017;88:158-165.

25. Ogbuagu EO, Airaodion AI. Tiger Nut (*Cyperus esculentus* L.) Boosts Fertility in Male Wistar Rats. *Asian Research Journal of Gynaecology and Obstetrics.* 2020;3(3): 8-18

26. Egba SI, Omodamiro OD, Obike JC, Ali ES. Influence on some female fertility hormonal response in Wistar albino rats: Possible contraceptive role for methanol leaf extract of *Ocimum gratissimum. Journal of Chemical and Pharmaceutical Research*. 2015;7(5):889-898.

27. Nnodim JK, Nwanjo HU, Okolie NJ, Opara AU, Nwosu DC, Okoroiwu I, Dike J, Okorie, H, Nwadike CN, Uduji HI. Effects of *Xylopia aethiopica* Fruits on reproductive hormonal level in rats.*Global Journal of Medicinal Plant Research,* 2013;1(1): 29-31.

28. Airaodion AI, Ngwogu AC, Megwas, AU, Ekenjoku JA, Ngwogu KO. Effect of common household insecticides used in Nigeria on rat male reproductive hormones. *Int. J. Res. Rep. Gynaecol.*, 2019;2(1):1-8.

29. Litwack G, Schmidt RJ. Biochemistry of hormones II: polypeptide hormones. In Textbook of Biochemistry with Clinical Correlations, 5th edn (Devlin, T.M., ed.), pp. 905-958. John Wiley and Sons, Inc., New York. 2001.

30. Omotuyi IO, Ologundudu A, Onwubiko VO, Wogu MD, Obi FO. *Hibiscus sabdariffa*Linn anthocyanins alter circulating reproductive hormones in rabbits (*Oryctolaguscuniculus*). *Journal of Diabetes and Endocrinology*. 2010;1(3):36-45

31. Airaodion AI, Ogbuagu EO, Ekenjoku JA,

Okoroukwu VN, Ogbuagu U, Airaodion, EO. Antifertility effect of ethanolic leaf extract of *Carica papaya* in male Wistar rats. *Merit Research Journal of Medicine and Medical Science.* 2019;7(10):374-381.

32. Benie T, Duval J, Thieulant ML. Effects of some traditional plant extract on rat oestrous cycle compared with clomid. *Phytotherapy Research*. 2003;17(7): 748-755.
33. Zahradnik HP, Hanjalic-Beck A, Groth K. Non-steroidal anti-inflammatory drugs and hormonal contraceptives for pain relief from dysmenorrhea: a review. *Contraception*. 2010;81(3): 185-196.
34. Onyebuagu P., Aloamaka CP, Igweh JC. *Xylopia aethiopica* Lowers Plasma Lipid Precursors of Reproductive Hormones in Wistar Rats. Int. J. Herbs and Pharm. Res. 2013; 2(4): 48-53.
35. Montaserti A, Pourheydar M, Khazaei M, Ghorbani R. Anti-fertility effects of *Physalisalkekengi*alcoholic extract in female rat. *Iranian J Reprod Med* 2007; 5: 13-16.
36. Norris DO, Carr JA. Vertebrate Endocrinology. Academic press. 2013; P. 126.
37. Gocze PM, Porpaczy Z, Freeman, D. A. Effects of alkaloids in cigarette smoke on human granulose cell progesterone synthesis and cell viability. *Gynecol Endocrinol.* 2006;10:223-228
38. Onuka AE, Okechukwu NC, Maxine KM. A Comparative Study between *Xylopia aethiopica* Dried Fruit Extract and Ibuprofen Inhibiting Effects on Some Reproductive Hormones Irrespective of the Estrous Cycle. *International Journal of Complementary & Alternative Medicine.* 2017;8(5):1-6.
39. Yun YW, Yuen BH, Moon YS. Effects of superovulatory doses of pregnant mare serum gonadotropin on oocyte quality and ovulatory and steroid hormone responses in rats. *Biol. Pharm. Bull.* 1987;26(11):1574-1580.
40. Al-Shemary NNA, Mousa SA, Muslim ZZ. Histological and hormonal study about the effect of aqueous extract of *Ocimum gratissmum* on female reproductive system in albino mice. *Journal of Pharmaceutical Science and Research*. 2018;10(4): 765-767.
41. Chinnappan SM, George A, Ashok G, Choudhary YK. Effect of herbal extract *Eurycomalongifolia* (Physta®) on female reproductive hormones and bone biochemical markers: an ovariectomised rat model study. *BMC Complementary Medicine and Therapies*. 2020;20(31):1-9.
42. Oze G, Onyeze GO, Ojiako O, Abanobi S, Ozims S, Ohiri A. Raised serum estrogen and progesterone concentrations in female rats treated with extract of Alstoniaboonei (De wild). *International Research Journal of Biochemistry and Bioinformatics*. 2012;2(6):135-141.
43. Hsia SM, Yeh CL, Kuo YH, Wang PS, Chiang W. Effects of Adlay (*Coixlachryma*-jobi L. var. ma-yuenStapf.) Hull extracts on the secretion of progesterone and estradiol *in vivo* and *in vitro*. *Experimental Biology and Med*ine. 2007232:1181-1194.
44. Kadohama N, Shintani K, Osawa Y. Tobacco alkaloid derivatives as inhibitors of breast cancer aromatase.*Cancer Lett*; 2013;75: 175-182.
45. Adienbo OM, Nwafor A, Ogbomade RS. Contraceptive efficacy of hydro-methanolic fruit extract of *Xylopia aethiopica* in male albino rats. *International journal of Advanced Biological and Biomedical Research.* 20131(7): 718-727
46. Cui J, Shen Y, Li R. Estrogen synthesis and signaling pathways during ageing: from periphery to brain. Trends Mol Med. 2013;19(3): 197-209.
47. Golan M, Avitan A, Qutob M, Dweik H. Abu-Lafi S. Quillaja saponins inhibit tilapia aromatase activity *in vitro*. Cybium. 2008;32: 80-82.
48. Stadtlander T, Levavi-Sivan B, Kerem Z, Dweik H, Qutob M. Effects of a saponin fraction extracted from *Trigonellafoenum-graecum*L. 2012.
49. Balunas MJ, Su B, Brueggemeier RW, Kinghorn AD. Natural products as aromatase inhibitors. *Anticancer Agents Med Chem*. 2008;8(6): 646-682.
50. Daramola OI, Akindele OO, Bolarinwa AF, Yinusa-Raji, Y. Effects of *Telfairia Occidentalis* Seed Oil on Female Reproductive Functions in Wistar Rats.*Nigerian Journal of Physiological Sciences.* 2015;30:95-101.
51. Anacletus FC, Nwauche KT, Wopara I. Hyperprolactin activity of aqueous extract of *Xylopia aethiopica* on female wister albino rats. *Research Journal of*

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Pharmaceutical Dosage Forms and Technology. 2015;7(3):103-105.
52. Ehigiator BE, dikwu E. Toxicity study of ethanolic stem bark extract of *Xylopia aethiopica* on fertility indices of male rats: An experimental study. International Journal of Reproductive BioMedicine. 2019;18(1):265–274.
53. Wass JA, Shalet SM. Oxford Textbook of Endocrinology and Diabetes. Oxford: Oxford University Press. 2002.
54. Bianco F, Basini G, Grasselli F. The plant alkaloid Sanguinarine affects swine granulose cell activity. *Reproductive Toxicology*. 2006;21(3): 335-340.
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