Original Research Article

Oestrogenic Activity of Aqueous Ethanol Extract of *Cucurbita Pepo* (Pumpkin) Seed in Female Wistar Rats

ABSTRACT

This study aimed to investigate the effect of aqueous ethanol extract of *Cucurbita pepo* seed (AEECPS) on the reproductive hormones of female rats. A total of 16 healthy non-pregnant female wistar rats divided into 4 groups of 4 rats each, for a 12-day treatment by oral gavage as follows: A (Control) = 0.5 ml 20% tween 80 (Vehicle); B, C & D = 142.86, 285.71, and 428.57mg/kg AEECPS respectively. Vaginal cytology examination was done daily to determine the oestrous cycle phases. At the end of the treatment, blood sample was collected according to the four phases of the oestrous cycle for hormonal assay - FSH, LH, Oestrogen and Progesterone. *C.pepo* seed extract caused a significant ($P<0.05$) increase in only the oestrogen level at the proestrus, oestrus, metestrus and diestrus phases, and also produced a significant ($P <0.05$) decrease in progesterone at the oestrus stage, though only at the dose of 285.71mg/kg relative to the control. There was a significant ($P <0.05$) decrease in the diestrus phase in group B (142.86mg/kg AEECPS) treated rats when compared with the control. *Cucurbita pepo* seed may have estrogenic properties, given the significant increase in oestrogen concentration throughout the phases of the oestrous cycle. This finding may be considered for clinical trial in post-menopausal females as hormone replacement therapy due to its oestrogenic activity.

Keywords: Aqueous ethanol, Cucurbita pepo. Oestrous cycle, post-menopausal, female hormones

INTRODUCTION

Hormones are chemical substances that are produced and secreted by endocrine glands into the blood stream (1). Hormones play a vital role in the health of humans and hormonal imbalance in females can cause several conditions such as menopause, infertility and some other symptoms like hot flashes, neck or shoulder stiffness (2).

"Hormonal imbalance in females has been a challenge in women globally. Each year, the number of menopausal women increases by approximately 47,000, and it is estimated that by 2030 there will be approximately 1.2 billion menopausal women" (3). "Eighty percent of these women present with complaints related to the hormonal changes associated with this condition (genitourinary symptoms, vasomotor symptoms, cognitive symptoms), but medical assistance is sought in only 25% of the cases" (4). Furthermore, due to pharmacological side effects and high cost of most of the orthodox medicine, many women resort to natural remedies especially herbal formulations (5,6).

Although *Cucurbita pepo* has been reported to have anti-hypercholesterolemic, anti-hypertensive, anti-inflammatory, anti-parasitic, anti-tumor, anti-oxidant, anti-diabetic, anti-carcinogenic, anti-bacterial, intestinal and anti-inflammatory activities (7–9), as well as immense nutraceutical benefits (10), studies on its effect on the female reproductive hormones remains scarce. This study was therefore aimed to evaluate the effect of aqueous ethanol extract of *Cucurbita pepo* seed on reproductive hormones of female wistar rats.

MATERIALS AND METHOD

Plant Material

Fresh *Cucurbita pepo* (Pumpkin) fruits were purchased from a local market at Choba, Port Harcourt in Rivers State. Identification and authentication of the plant were done by Dr. Chimezie Ekeke in the Department of Plant Science and Biotechnology, University of Port Harcourt with voucher specimen Ref No UPH/PSB/2021/071. Soon after authentication, the seeds were removed from the fruits, air dried under room temperature for one month, after which, they were deshelled, and then ground into fine powder.

Preparation of Aqueous ethanol extract

The extraction process was carried out as illustrated by (11). The aqueous ethanol extract of *Cucurbita pepo* seed (AEECPS) was obtained by exhaustive extraction for a period of 72hours with fresh replacement of solvent at 24 hours' interval. A 680g of the powdered deshelled seeds of *Cucurbita pepo* was macerated in 70 percent aqueous ethanol (70% hydro-alcohol) at room temperature in a macerating glass jar and filtered after 24hours. A fresh replacement of the

solvent was done after every 24 hours. The combined aqueous ethanol extracts were filtered using Whatman NO.1 filter paper. The filtrate was concentrated using rotary evaporator and water bath. The extract obtained was used for the study.

Animals

Sixteen healthy sexually mature female Wistar rats weighing between 150g to 250g were purchased from the animal house of the Department of Pharmacology, University of Port Harcourt were used for the study. The rats were handled humanely in line with the Ethics and Regulation for the use of experimental animals as stipulated by National Health and Medical Research Council (12). They were housed separately and had access to commercial feed and water *ad libitium*, and were allowed to acclimatize for two weeks prior the study.

Experimental Design

The committee on Herbal medicinal product (HMPC) of the European medicine Agency recommended that *Cucurbita pepo* be administered to a 70kg adult human at a dose range of 10-30g (13). Based on this, the doses used for the study were 10g/70kg, 20g/70kg and 30g/70kg adult, which when calculated for animal use are 142.86mg/kg, 285.71mg/kg and 428.57mg/kg respectively.

The female rats were randomly divided into 4 groups of four animals each for the following treatment.

Group A (Control) – 0. 5ml of 20% tween 80

Group B - 142.86mg/kg of AEECPS

Group C - 285.71mg/kg of AEECPS

Group D - 428.57mg/kg of AEECPS

All treatments were administered daily by oral gavage for 12 days. The rats were weighed every three days, and doses adjusted according.

Vaginal Cytology

The phases of the oestrous cycle of the experimental animals were assessed daily (in the mornings) using the pipette smear technique illustrated by Organisation for the Economic Co-operation and Development (OECD), (14) and Obinna & Kagbo (15). A few drops of physiologic saline (0.9% NaCl) contained in a dropping pipette was inserted into the vagina of the animals and used to wash the vaginal walls. The lavage containing cells lining the vaginal wall was released on a grease-free microscope slide and observed under light microscope at 10x objective lens. The phases of oestrous cycle were established depending on the various distinct cells ranging from round and nucleated cells (epithelial cells), irregular anucleated cells (cornified cells) to the small round cells (leucocytes). Proestrus phase was identified by the predominance of epithelial cells; oestrus phase was mainly characterized by cornified cells; metestrus phase described by the presence of leucocytic cells; cornified and/or epithelial cells, and diestrus by mainly leucocytes.

Blood Collection for Hormonal Assay

Collection of blood samples from the rats according to the oestrous cycle phases was done by ocular puncture (16). About 4mls of blood samples were collected in a plain bottle from each rat using a micro capillary tube. The serum separated from the clotted blood after centrifuging was used for hormonal assay.

Hormonal Assay

Determination of the serum concentration for luteinizing hormone, follicle stimulating hormone, oestrogen and progesterone was done using Microplate Enzyme Immunoassay using Accu-bind ELISA Microwells. The kit used is a product of Monobind Inc., USA. The procedure for running the assay is as stated in the manufacturer's manual. After running the assay, the optical density obtained was used to find the concentration of each hormone.

Statistical Analysis

The data were represented as mean ± SEM. Statistical level of significance was determined by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test. The significance level was set at $p<0.05$.

RESULTS

3.1 Effect of AEECPS on Oestrous Cycle

The charts in figures 1- 3 shows that AEECPS had no significant ($p>0.05$) effect on the Proestrus, oestrus and metestrus phases of the oestrous cycle in comparison with the control (group A).

Figure 4 charts showed there was no significant ($p>0.05$) effect on the diestrus phase of AEECPS treatment groups C and D rats relative to the Control (group A). However, there was a significant ($p<0.05$) decrease in the diestrus phase of only group B rats when compared with the normal control (group A).

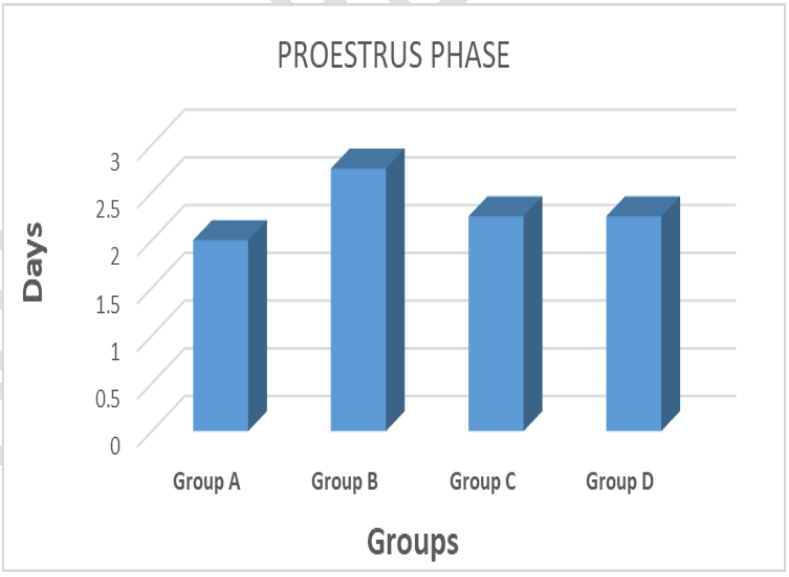

Figure 1: Effect of Aqueous Ethanol extract of *C. pepo* seed on Proestrus Phase of the Oestrous Cycle of test rats.

Values are given as mean ± SEM for 4 rats in each group. Test groups (Groups B, C and D given 142.86mg/kg, 285.71mg/kg and 428.57mg/kg doses of Aqueous Ethanol extract of *C. pepo* seed respectively) are compared

with Group A (Control given 0.5ml 20% tween 80). No significant difference at a 95% confidence interval (p >0.05). P: statistical level of significance as determined by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test.

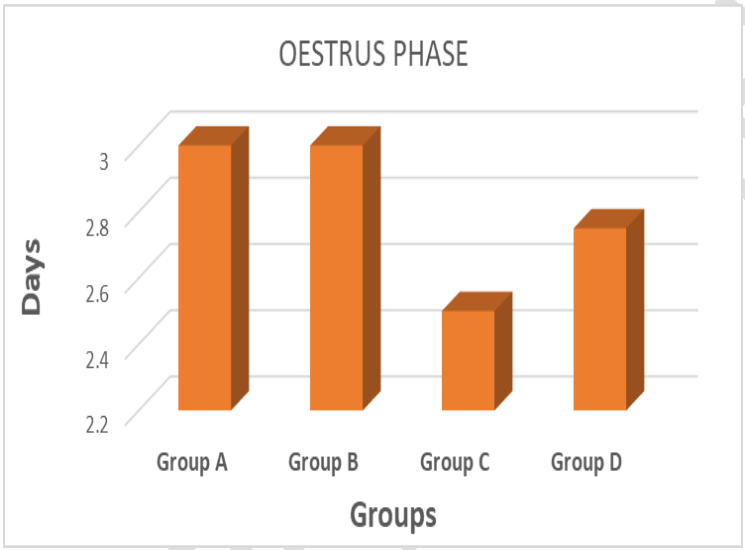

Figure 2: Effect of Aqueous Ethanol extract of *C. pepo* seed on Oestrus Phase of the Oestrous Cycle of test rats.

Values are given as mean ± SEM for 4 rats in each group. Test groups (Groups B, C and D given 142.86mg/kg, 285.71mg/kg and 428.57mg/kg doses of Aqueous Ethanol extract of *C. pepo* seed respectively) are compared with Group A (Control given 0.5ml 20% tween 80). No significant difference at a 95% confidence interval (p >0.05). P: statistical level of significance as determined by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test.

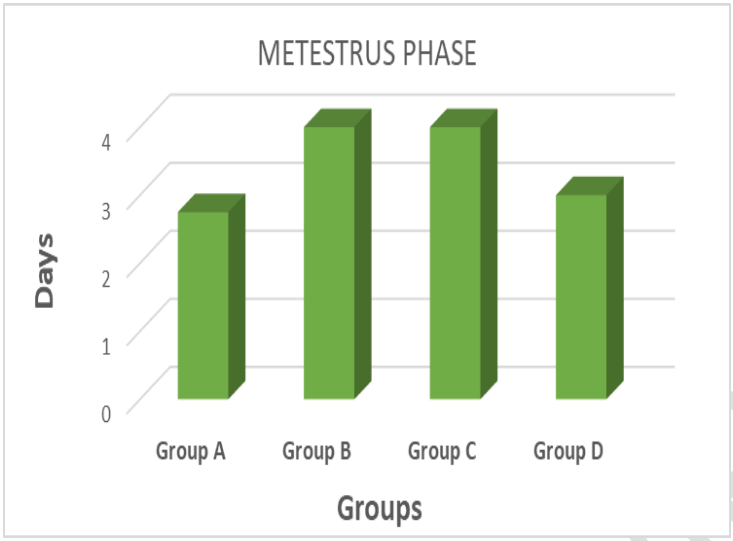

Figure 3: Effect of Aqueous Ethanol extract of *C. pepo* seed on Metestrus Phase of the Oestrous Cycle of test rats.

Values are given as mean ± SEM for 4 rats in each group. Test groups (Groups B, C and D given 142.86mg/kg, 285.71mg/kg and 428.57mg/kg doses of Aqueous Ethanol extract of *C. pepo* seed respectively) are compared with Group A (Control given 0.5ml 20% tween 80). No significant difference at a 95% confidence interval ($p > 0.05$). P: statistical level of significance as determined by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test.

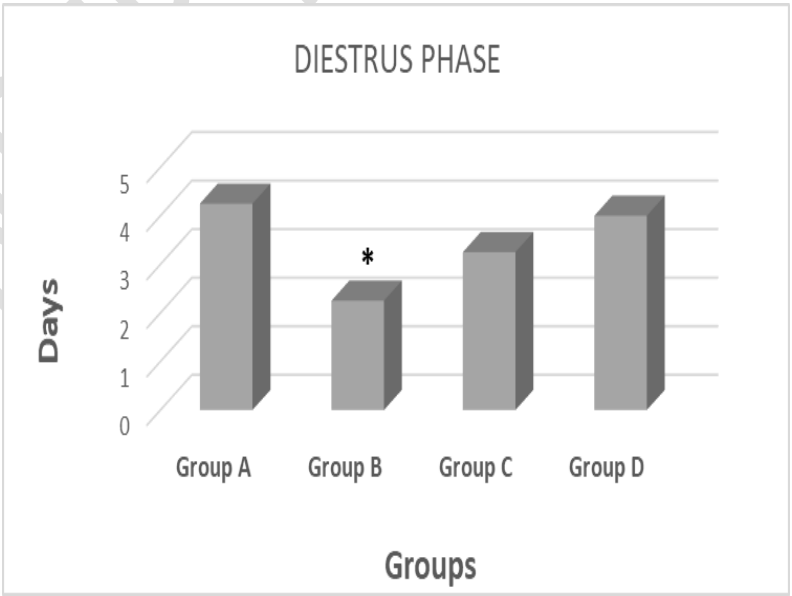

Figure 4: Effect of Aqueous Ethanol extract of *C. pepo* seed on Diestrus Phase of the Oestrous Cycle of test rats.

Values are given as mean ± SEM for 4 rats in each group. Test groups (Groups B, C and D given 142.86mg/kg, 285.71mg/kg and 428.57mg/kg doses of Aqueous Ethanol extract of *C. pepo* seed respectively) are compared with Group A (Control given 0.5ml 20% tween 80) * indicate significant difference at $p<0.05$, compared to Group A. P: statistical level of significance as determined by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test.

3.2 Effect of AEECPS on Female Sex Hormones according to the Phases of the Oestrous Cycle

Tables 1-4 summarized the effects of AEECPS on female sex hormones at different phases of oestrous cycle of female rats treated for 12 days.

At the proestrus and metestrus phases, there was no significant ($p>0.05$) difference in the mean serum level of FSH, LH, Oestrogen and Progesterone in all the AEECPS treated groups except in Group C (285.71mg/kg) which showed a significant ($p<0.05$) increase in only the oestrogen level at the both phases in comparison with the control (Group A) (tables 1 and 3).

There was no significant ($p>0.05$) variation in the mean serum levels of FSH, LH, Oestrogen and progesterone in AEECPS treated groups B (142.86 mg/kg) and D (428.57mg /kg). Only Group C treated rats (285.71mg/kg) showed a significant ($p<0.05$) increase in the oestrogen level with a significant ($p<0.05$) decrease in the progesterone level relative to the control (group A). However the FSH and LH levels of group C rats were not significantly ($p>0.05$) changed (table 2).

FSH, LH, oestrogen and progesterone levels of all the AEECPS treated groups were not significantly ($p>0.05$) altered in comparison with the Control (group A), with the exception of the oestrogen level of Group D rats which showed a significant ($p<0.05$) increase (table 4).

Table 1: Effect of aqueous ethanol extract of *Curcubita pepo* seed on Female Sex Hormones at the Proestrus phase of the Oestrous Cycle.

Animal	Female Sex Hormones

	FSH (IU/L)	LH (IU/L)	Oestrogen (ng/ml)	Progesterone (pg/ml)
Group A (Normal Control - 0.5ml of 20% Tween 80)	0.54±0.05	0.49±0.10	67.00±0.00	20.63±2.81
Group B (142.86mg/kg of Aqueous ethanol extract of *C. pepo*)	0.59±0.12	0.46±0.10	67.00±2.68	20.08±3.25
Group C (285.71mg/kg of Aqueous ethanol extract of *C. pepo*)	0.61±0.09	0.59±0.09	76.25±2.10*	17.83±3.59
Group D (428.57mg/kg of aqueous ethanol extract of *C. Pepo*)	0.52±0.04	0.43±0.03	67.50±2.63	19.78±1.68

Values are given as mean ± SEM for each group. * indicate significant difference at $p<0.05$, compared to Group A (Control); P: statistical level of significance as determined by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test.

Table 2: Effect of aqueous ethanol extract of *Curcubita pepo* seed on Female Sex Hormones at the Oestrus phase of the oestrous cycle.

Animal	Female Sex Hormones			
	FSH (IU/L)	LH (IU/L)	Oestrogen (ng/ml)	Progesterone (pg/ml)
Group A (Normal Control - 0.5ml of 20% Tween 80)	0.50±0.12	0.55±0.09	63.25±0.48	27.68±3.27
Group B (142.86mg/kg of Aqueous ethanol extract of *C. pepo*)	0.75±0.10	0.40±0.07	64.25±1.44	22.60±1.85
Group C (285.71mg/kg of Aqueous ethanol extract of *C. pepo*)	0.86±0.18	0.58±0.11	73.50±1.19*	14.48±2.35*
Group D (428.57mg/kg of aqueous ethanol extract of *C. Pepo*)	0.48±0.09	0.42±0.01	67.25±3.57	20.48±0.76

Values are given as mean ± SEM for each group. ***** indicate significant difference at p<0.05, compared to Group A (Control); P: statistical level of significance as determined by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test.

Table 3: Effect of aqueous ethanol extract of *Curcubita pepo* seed on Female Sex Hormones at the Metestrus phase of the Oestrous Cycle

Animal	Female Sex Hormones			
	FSH (IU/L)	**LH (IU/L)**	**Oestrogen (ng/ml)**	**Progesterone (pg/ml)**
Group A (Normal Control - 0.5ml of 20% Tween 80)	0.77±0.11	0.56±0.13	63.25±0.63	22.73±4.93
Group B (142.86mg/kg of Aqueous ethanol extract of *C. pepo*)	1.05±0.08	0.65±0.10	67.00±0.71	24.28±3.59
Group C (285.71mg/kg of Aqueous ethanol extract of *C. pepo*)	0.61±0.05	0.67±0.06	74.25±2.50*	17.88±2.00
Group D (428.57mg/kg of aqueous ethanol extract of *C. Pepo*)	0.54±0.80	0.41±0.05	66.25±2.53	20.80±4.78

Values are given as mean ± SEM for each group. ***** indicate significant difference at p<0.05, compared to Group A (Control); P: statistical level of significance as determined by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test.

Table 4: Effect of aqueous ethanol extract of *Curcubita pepo* seed on Female Sex Hormones at the Diestrus phase of the Oestrous Cycle

Animal	Female Sex Hormones			
	FSH (IU/L)	**LH (IU/L)**	**Oestrogen (ng/ml)**	**Progesterone (pg/ml)**
Group A (Normal Control - 0.5ml of 20% Tween 80)	0.67±0.08	0.59±0.12	65.75±1.43	25.48±4.05
Group B (142.86mg/kg of Aqueous ethanol extract of *C. pepo*)	0.76±0.11	0.40±0.03	64.25±0.95	25.73±5.22
Group C (285.71mg/kg of Aqueous ethanol extract of *C. pepo*)	0.80±0.11	0.69±0.06	77.75±2.75*****	20.08±3.04
Group D (428.57mg/kg of aqueous ethanol extract of *C.*	0.71±0.18	0.50±0.14	63.50±1.94	21.93±2.87

***Pepo*)**

Values are given as mean ± SEM for each group. ***** indicate significant difference at $p<0.05$, compared to Group A (Normal Control). P: statistical level of significance as determined by one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test.

DISCUSSION

"Hormone Replacement Therapy (HRT) involves the administration of synthetic oestrogen and progestogen to replace a woman's depleting hormone levels and thus alleviate menopausal symptoms" (17). From the results of this study, the oestrogen level was increased in all the phases of the oestrous cycle at the medium dose of 285.71mg/kg after 12 days with no corresponding changes in the oestrous cycle phases. This shows that *Cucurbita pepo* seed may have oestrogenic properties with the potential to increase the peripheral oestrogen level, without necessarily altering the normal cyclicity (oestrous / menstrual cycle) of the individual.

As a potential hormone replacement therapy agent, the *Cucurbita pepo* seed as shown in the results can increase the oestrogen level all through the cycle, the phases notwithstanding. This finding is supported by the review done by Lestari & Meiyanto (10), which revealed that pumpkin seeds have estrogen-like effects and as such were reported to be rich in polyunsaturated fatty acids such as Palmitic acid, stearic acid, oleic acid and linoleic acid. This report is in tandem with findings on the Gas Chromatography Mass Spectroscopic (GCMS) analysis of aqueous ethanol extract of *Cucurbita pepo* seed by Anyanwu *et al* (18) who reported that the bioactive compounds, Palmitic acid, stearic acid, and linoleic acid were present in the extract, amongst others. Findings by Liu *et al* (19) identified linoleic acid as an oestrogen receptor ligand capable of displacing estradiol from estrogen receptors, hence the possible estrogenic activity of *Cucurbita pepo* seed. According to Rosano *et al* (20), "oestrogen hormones play a key role in the menstrual cycle, reproduction, modulation of bone density, and cholesterol transport in the body".

The decrease in the progesterone level at the oestrus phase is a normal physiologic occurrence in a normal female with regular cycle. It is known that in a normal cycling female, basically, during the growth and maturation of follicles which occurs at the proestrus phase of the oestrous cycle, there is usually a rise in the FSH concentration which triggers the development and maturation of

follicles in animals. According to Miller (21), "the FSH released in response to GnRH secretion from the hypothalamus initiates the ovarian follicular development and the theca cells of these follicles synthesize androgens which are converted to estrogen in the granulosa cells by the enzyme, aromatase". "As the level of oestrogen increases, inhibition of FSH occurs with an attendant stimulation of LH release" (22). "The residual FSH together with the pre-ovulatory surge of LH triggers ovulation, after which the LH also stimulates the development of corpus luteum from the ruptured follicle by luteinization of the granulose" (16,23). "The cells of the corpus luteum secrete large quantity of progesterone and little of oestrogen which accounts for the high level of progesterone associated with the luteal phase. Hence low progesterone level is frequently associated with the follicular phase, made up of proestrus and oestrus phases" (16).

From the result, the reduction in the number of diestrus phase further explains the oestrogenic activity of the *C.pepo* seed, evidenced by increased oestrogen level with a corresponding low progesterone level. This suggests that *C.pepo* seed may shorten the luteal phase, period of progesterone dominance, in order to enhance the follicular phase, the period of oestrogen dominance.

"Generally, the oestrous cycle is classified into four phases. These four phases are also grouped into two, the follicular phase made up of proestrus and oestrus phases, and the luteal phase which comprise metestrus and diestrus phases. The proestrus phase is the period of rapid follicular growth which precedes the onset of oestrus; the oestrus phase is the heat period when ovulation occurs and is usually seen as the fertile period since mating is allowed only in this phase; the metestrus phase is the period of early corpus luteum development and the diestrus phase, the period of mature corpus luteum activity, which usually ends with the regression of the corpus luteum. The oestrous cycle thus is synchronized by the female sex hormones since all the phases of the estrous cycle occur as a result of the cyclical increase and decrease of these hormones" (24).

CONCLUSION

From the findings of this study, it can be concluded that aqueous ethanol extract of *Cucurbita pepo* seed may have estrogenic properties, given the significant increase in oestrogen concentration throughout the phases of the oestrous cycle.

Ethics Approval

The research was reviewed and approved by Research Ethics Committee, University of Port Harcourt, in accordance with the principles guiding the use and handling of experimental animals. Reference number: UPH/ CEREMAD/REC/MM83/038.

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