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Effect of *Cucurbita pepo* (Pumpkin) seed extracts on the cyclicity and reproductive hormones of female Wistar rats

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ABSTRACT

Introduction: Despite the traditional use of *Cucurbita pepo* seed in pregnancy, its effects on female reproduction remain scarce. This study evaluated the impacts of n-hexane, dichloromethane (DCM), and aqueous ethanol extracts of *C. pepo* seed on the cyclicity and reproductive hormones of female Wistar rats.

Methods: Ten groups of four rats received seed extracts or tween 80 orally for 21 days: A (control)= 0.5 mL tween 80 (vehicle); B, C, & D= 142.86, 285.71, and 428.57 mg/kg nHE; E, F, & G= 142.86, 285.71, 428.57 mg/kg of DCM; and H, I, & J= 142.86, 285.71, 428.57 mg/kg of aqueous ethanol extracts, respectively. Vaginal cytology monitored the estrous cycle daily, and blood samples were obtained for follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen, and progesterone at various oestrus cycle phases.

Results: Compared to the control, the estrous cycle phases did not change significantly ($P>0.05$). FSH levels significantly increased ($P<0.05$) with DCM and aqueous ethanol extracts of *C. pepo* seed during proestrus and estrus phases compared to the control. A significant ($P<0.05$) increase in LH was observed with n-hexane, DCM, and aqueous ethanol extracts during all estrous cycle phases compared to the control. All extracts significantly increased estrogen levels ($P<0.05$) during all phases. DCM and aqueous ethanol extracts reduced substantially the estrus-phase progesterone.

Conclusion: *Cucurbita pepo* may stimulate the hypothalamic-pituitary-gonadal axis in female reproduction. Further studies should be conducted using various phytoestrogen compounds to gain useful knowledge about the effectiveness, safety, and long-term effects of *C. pepo* seed extracts in regulating hormonal balance.

Implication for health policy/practice/research/medical education:
This article provides scientific evidence of the stimulatory effect of *Cucurbita pepo* seed on the hypothalamic-pituitary-gonadal axis in female reproduction. Thus, this could inform healthcare practitioners to assess natural dietary supplements as part of a holistic female reproductive health strategy.

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Introduction

Traditional medicinal plants have continued to play a vital role in managing fertility-related problems, especially in developing countries where the populace has limited access to, or cannot afford, conventional fertility therapies. According to the World Medicines Situation 2011 report, about 70 to 95% of the population in developing countries use traditional medicine (1). Modern healthcare and medicine are often available to a limited population because either access is poor or the facilities are too expensive for rural dwellers to afford. Hence, traditional medicine might be the only accessible and affordable primary healthcare choice for rural African communities (2).

Some of these traditional medicinal plants have been reported to play a significant role during pregnancy, childbirth, postpartum care, female fertility, amenorrhea, birth control, and delivery interventions (3-6). It is known that African women in some rural areas primarily depend on certain herbal medicines for their reproductive health

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needs (7). Some of these medicinal plants include *Aloe vera* L. (8), *Allium sativum* L. (9), *Cucurbita pepo* L. (9,10), and *Garcinia kola* (9,11).

Specifically, *Cucurbita pepo* (Pumpkin), a member of the family *Cucurbitaceae,* stands out as a plant with significant relevance in women's health. High prevalence of *C. pepo* use among African pregnant women has been documented, with no reported adverse effects during pregnancy (12,13). Within Africa, *C. pepo* is acknowledged by its local names such as 'Ugbogulu' in South Eastern Nigeria.

Cucurbita pepo is recognized for its potential effects on female reproductive parameters (14). The study by Ivoh Grace et al (14), specifically reported that the *C. pepo* seed might function as a potential hormone replacement therapy agent, exhibiting the ability to increase estrogen levels consistently throughout the menstrual cycle, unaffected by specific phases. Its traditional applications in folk medicine are diverse, ranging from the treatment of colds to the alleviation of aches and addressing benign prostatic hypertrophy, as highlighted by Shokrzadeh et al (15). Ethnopharmacological studies across various regions underscore the significance of *C. pepo*, attributing to it a multitude of therapeutic effects, including anti-inflammatory, antiviral, analgesic, anti-urinary disorders, anti-ulcer, antidiabetic, and antioxidant properties (16,17). Traditional medicinal practices, particularly within the Chinese and Ayurveda systems, have extensively utilized different parts of the plant, including its fruits and seeds, emphasizing its versatile pharmacological potential (18). However, in spite of all these reported findings on *C. pepo* seed, there is paucity of information on its effect on the cyclicity and reproductive hormones in females. Therefore, this study aims to bridge this gap as well as serve as a foundation for investigating the effect of *C. pepo* seed on female reproductive health. It also seeks to inform researchers, health care practitioners as well as the general public about the potential impact of *C. pepo* seed on female reproductive health.

Materials and Methods

Plant material and authentication

Fresh *C. pepo* (pumpkin) fruits were bought from central market Choba, Port Harcourt, Nigeria. It was packaged in a polythene bag and transported to the Department of Pharmacology, University of Port Harcourt. Authentication of the plant species was done at the University Herbarium by Dr Ekeke, with voucher specimen (Ref No; UPH/PSB/2021/071) available at the University Herbarium.

Preparation of extracts

Fresh *C. pepo* fruits were dried for four weeks to remove the seeds. Grinding the deshelled seeds into a fine powder was followed by cold maceration in N-hexane, dichloromethane (DCM), and 70% aqueous ethanol. Extraction was repeated for 72 hours with solvent replacement every 24 hours. The powder (500 g) was macerated in 1.5 litres of n-hexane for 24 hours. The solution was filtered by Muslim cloth and Whatman's No.1 paper. The residue was steeped and filtered twice more, then concentrated with a rotary evaporator. In an evaporating dish, the concentrated solution was dried over a water bath. DCM extraction was done using dried Marc from the previous extraction. A 500 g dry Marc was soaked in 1.5 L of DCM for 24 hours. Similar methods were used to mix, concentrate, and dry the filtrates after three extractions. Again, 500 g of dried Marc from the DCM extraction was used for the aqueous ethanol extraction. A 24-hour maceration in 1.5 L of 70% aqueous ethanol was followed by filtering, concentration, and drying.

Preliminary phytochemical screening

This followed Harborne (19) and Houghton & Raman (20) methods. Qualitative investigations identified phytochemical components in *Cucurbita pepo* seed extracts in n-hexane, DCM, and aqueous ethanol (21).

Alkaloid test

In a steam bath, 2 g of *C. pepo* seed extracts were mixed with 5 mL of 5% HCL and filtered. For alkaloid assays, the filtrate was separated into four test tubes (A-D): The test tube A received 2–3 drops of Meyer's reagent. A cream-white precipitate implied the presence of alkaloids. B. The test tube B received 2–3 drops of Dragendorff's reagent. Pink or crimson precipitate indicated alkaloids. The test tube C received 2–3 drops of Hager's reagent. Yellow precipitate indicated alkaloids. The test tube D received 2–3 drops of D. Wagner's reagent. A reddish-brown precipitate indicated alkaloid content.

Tannin test

Two grams of each extract were combined with 10 mL of distilled water, boiled, and filtered. Three drops of 5% ferric chloride reagent were applied to filtrate. Tannin was verified by a blue-black or blue-green precipitate.

Shinoda reduction flavonoid test

To do this procedure, 0.2 g of n-hexane, DCM, and aqueous ethanol extracts in 10 mL of distilled water was heated and filtered. Four magnesium fills and 2–3 drops of pure HCl were added. Flavonoids appeared orange to red crimson (22).

Saponin frothing test

In this experiment, 0.2 g of extracts in 10 mL of distilled water in a test tube were heated and shaken. It stood for nearly 30 minutes after shaking. Consistent honey-comb froth implied saponins.

Phlobatanin test

The same as previous tests, 0.2 g extracts were stirred with 10 mL distilled water, heated, and filtered. Then,

the filtrate was boiled with 1% hydrochloric acid. Red precipitate suggested Phlobatanin.

Carbohydrate tests

Molisch's test: A test tube containing 0.2 g of extracts was added 5 mL of distilled water, stirred, and heated. A 45-degree slanted test tube was progressively filled with 2 mL of concentrated H_2SO_4. The deep violet interface ring indicated carbohydrates.

Fehling's solution test: A boiling water bath was used to combine 2 mL of Fehling's solution A and B with 2 mL of the extract solution. Reducing sugars were indicated by a brick-red precipitate at the test tube bottom.

Check for anthraquinones

An amount of 0.2 g of extract was placed in a 100 mL conical flask and 20 mL chloroform was added. Then, it was stirred occasionally and heated in a water bath (~40 ˚C). The mixture was cooled after filtering into a clean test tube. One milliliter of 10% ammonia solution was added into 2 mL of filtrate and stirred. Observation of brilliant pink or violet color in the ammoniac layer signified the presence of anthraquinones (19).

Triterpenoids/steroids tests

Lieberman-Burchard test: Ten milliliters of chloroform was added to 0.2 g extracts, stirred, and filtered. The extract was dissolved in 1 mL acetic acid. After immersing the solution test tube in ice, 1 mL of concentrated sulphuric acid was gently poured on the side to layer. Steroid nuclei were violet to blue-green, while triterpenoid nuclei were pink-red (20).

Salkowski test: Ten milliliters chloroform was added into 0.2 g of extracts, stirred, and then filtered. Carefully pouring 2 mL of concentrated sulfuric acid made a film. Reddish brown coloration and the interface signified the presence of a steroidal ring (20).

Cardiac glycoside test

Keller-Killiani deoxy-sugar test: Ten milliliters of chloroform was added to 0.2 g of extract, mixed, and filtered. One milliliter glacial acetic acid with a trace of ferric chloride solution was added to the filtrate and underplayed with 0.4 mL concentrated sulphuric acid. The presence of a reddish-brown ring at the interface signified the presence of deoxy-sugar (21).

Kedde lactone ring test: A 0.2 g extract was mixed with 10 mL chloroform and filtered. The filtrate was treated with 1 drop of 2% 3,5-dinitrobenzoic acid and 20% sodium hydroxide. Cardenolides have lactone rings, making the solution violet (22).

Animals

Adult female Wistar rats obtained from the animal house of the Department of Pharmacology, University of Port Harcourt, Nigeria, were used for the study. Prior to the study, the experimental animals were acclimatized for two weeks. The animals were handled humanely in line with the Ethics and Regulation for the use of experimental animals as stipulated by NHMRC (23). Clean drinking water and commercial feed from Top Feeds Nigeria were given to them *ad libitum.*

Experimental design

The European Medicine Agency's HMPC Committee recommends administering *Cucurbita pepo* to 70 kg adults at a dosage range of 10-30 g (24). This study employed dosages of 10 g/70 kg, 20 g/70 kg, and 30 g/70 kg adult, resulting in 142.86 mg/kg, 285.71 mg/kg, and 428.57 mg/kg for low, medium, and high doses of the extracts of *Cucurbita pepo* seeds in animal usage (14). Female Wistar rats were randomly divided into 10 groups of four animals each for the following treatment:

- Group A (Control): 0.5 mL of 20% Tween 80.
- Group B: 142.86 mg/kg of n-hexane extract
- Group C: 285.71 mg/kg of n-hexane extract
- Group D: 428.57 mg/kg of n-hexane extract
- Group E: 142.86 mg/kg of DCM extract
- Group F: 285.71 mg/kg of DCM extract
- Group G: 428.57 mg/kg of DCM extract
- Group H: 142.86 mg/kg of aqueous ethanol extract
- Group I: 285.71 mg/kg of aqueous ethanol extract
- Group J: 428.57 mg/kg of aqueous ethanol extract

The treatments were begun during the oestrus part of the cycle and were given orally via oral gavage, daily for 21 days.

Vaginal cytology

Vaginal cytology was conducted in the mornings to assess the phases of the estrous cycle in the experimental animals. The pipette smear technique, as outlined by Obinna and Kagbo (25), was employed. A dropping pipette was used to wash the animals' vaginal walls with a few drops of normal saline (0.9% NaCl). Lavage containing vaginal wall cells were placed on a grease-free microscope slide and examined under a light microscope with a 10x objective lens. Estrous cycle phases were determined by the presence of distinct cells, such as irregular anucleated cells (cornified cells) indicating estrus, round and nucleated cells (epithelial cells) indicating proestrus, small round cells (leucocytes) indicating diestrus, and leucocytic cells with cornified and/or epithelial cells indicating metestrus (26). The estrous cycle stages of the experimental animals were fully determined using this approach.

Blood collection for hormonal analyses

Starting from day 18 of the administration period, blood samples were collected from the ten experimental groups (groups A-J) according to the phases of the estrous cycles. The orbital bleeding technique, following the protocols outlined by Obinna & Kagbo (24) and Parasuraman et al

(27), was employed. A microhematocrit plain tube was inserted into the orbital plexus of the medial canthus of the eye to draw blood into sterile bottles. After settling for 30–45 minutes to coagulate, blood samples were centrifuged at 3000 rev/min for 15 minutes. After harvesting, the sera were placed in clean bottles, stored in firmly sealed micro-centrifuge tubes, and stored at -20 °C for ELISA hormonal analysis. The hormonal investigation examined LH, FSH, estrogen, and progesterone levels to see how *C. pepo* seed extracts affected female reproductive hormones of the female Wistar rats.

Hormonal assay

Serum concentrations of luteinizing hormone, follicle-stimulating hormone, estrogen, and progesterone were determined using the Microplate Enzyme Immunoassay method with AccuBind ELISA Microwells. The assay kit, a product of Monobind Inc., USA, was employed, and the assay procedure strictly followed the manufacturer's manual. Following the assay run, the optical density measurements were employed to determine the concentration of each hormone.

Statistical analysis

Statistical analyses were conducted using SPSS 21, with data presented as mean ± SEM. The assessment was performed through a one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. The significance level was established at $P<0.05$.

Results

Preliminary phytochemical screening of extracts of *Cucurbita pepo* seeds

Table 1 shows the phytochemical screening for the presence of various chemical constituents using standard tests and procedures. The findings show the presence of fixed oils, triterpenoids/steroids, and trace protein in all three extracts (n-hexane, DCM, and aqueous ethanol) of *C. pepo* seeds.

Effects of *Cucurbita pepo* seed extracts on estrous cycle

Figures 1-3 respectively show the impact of n-hexane, DCM, and aqueous ethanol extracts of *C. pepo* seed on the different phases of the estrous cycle in female Wistar rats over a 21-day treatment period. None of the extracts demonstrated significant alteration in the phases of the estrous cycle when compared with the control group.

Effects of *Cucurbita pepo* seed extracts on female sex hormones according to the phases of the estrous cycle

Follicle-stimulating hormone and luteinizing hormone

Table 2 shows that administering n-hexane, DCM, and aqueous ethanol extracts of *C. pepo* seed significantly increased serum FSH levels during proestrus, estrus, and diestrus phases in treatment groups H (142.86 mg/kg of aqueous ethanol extract), I (285.71 mg/kg), and G (428.57 mg/kg) relative to the normal control. In addition, treatment groups H, I, and G had significantly higher blood LH levels during all periods of the estrus cycle than the control (Table 3).

Estrogen and progesterone

Table 4 shows that serum estrogen levels increased significantly across the test groups during the estrous cycle. The test groups G and H had high estrogen levels throughout the estrous cycle phases, while the I and E test groups had a significant increase in serum estrogen levels during the proestrus, metestrus, and diestrus phases, relative to the normal control. Compared to the normal control, test group D had higher estrogen levels during proestrus, estrus, and metestrus, while groups J, F and C had significantly increased estrogen levels during the

Table 1. Preliminary phytochemical screening of *Cucurbita pepo* seed extracts

Tests	Phytochemical elements	N-hexane extract of *C. pepo* seed	Dichloromethane extract of *C. pepo* seed	Aqueous ethanol extract of *C. pepo* seed
Dragendorff's reagent Wagner's reagent, Meyer's reagent, Hager's reagent	Alkaloid	Negative	Negative	Negative
Shinoda reduction test	Flavonoid	Negative	Negative	Negative
Filter paper test	Fixed oils	Positive	Positive	Positive
Frothing test	Saponins	Negative	Negative	Negative
Keller–Killiani's test Kedde test	Cardiac glycosides	Negative	Negative	Negative
Lieberman's test, Salkowski's test	Triterpenoids/steroids	Positive	Positive	Positive
Molisch test, Fehling's test	Carbohydrate	Negative	Negative	Negative
Phlobatannin test	Phlobatannin	Negative	Negative	Negative
Borntrager's test	Anthraquinone	Negative	Negative	Negative
Ninhydrin test	Protein	Trace	Trace	Trace
Ferric chloride	Phenols	Negative	Negative	Negative

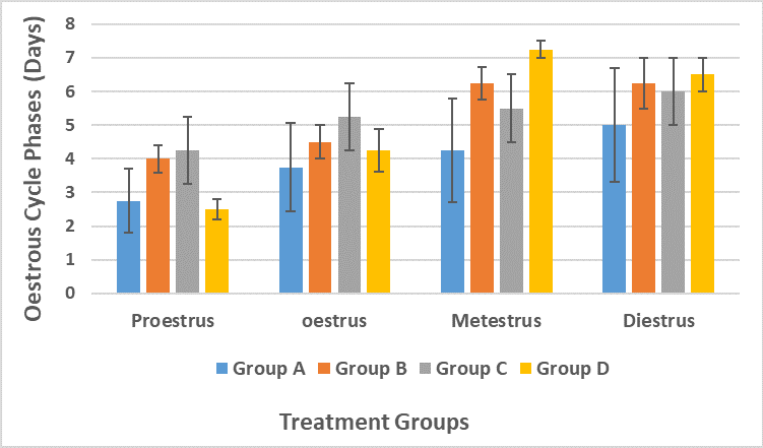

Figure 1. Effect of n-hexane extract of *Cucurbita pepo* seed on estrous cycle phases. Results are presented as mean ± SEM for 4 rats in each group. Experimental groups [Groups B, C, and D: 142.86, 285.71, and 428.57 mg/kg respectively of n-hexane extract of *C. pepo* seed] are compared with Group A (Normal control: 0.5 ml 20% tween 80). There was no significant difference at a 95% confidence interval (P>0.05; One-way analysis of variance [ANOVA] followed by Tukey's post hoc test).

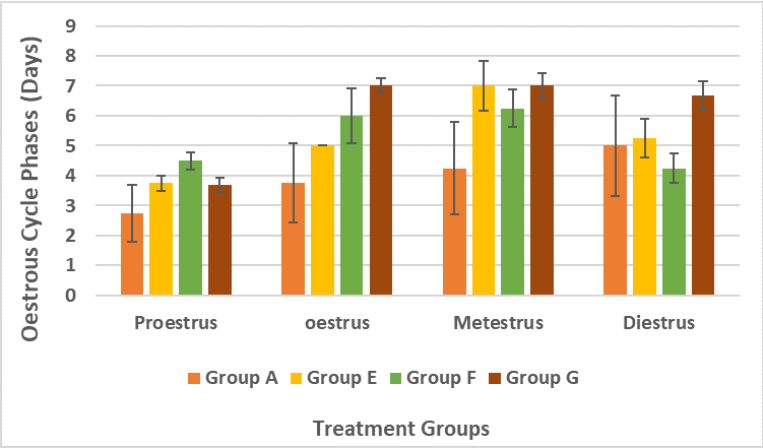

Figure 2. Effect of dichloromethane (DCM) extracts of *Cucurbita pepo* seed on estrous cycle phases. Results are presented as mean ± SEM for 4 rats in each group. Experimental groups (Groups E, F, and G respectively received 142.86, 285.71, and 428.57 mg/kg of DCM extract of *C. pepo* seed) were compared with group A (Normal control: 0.5 mL 20% tween 80). No significant difference was seen at a 95% confidence interval (P>0.05) as determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

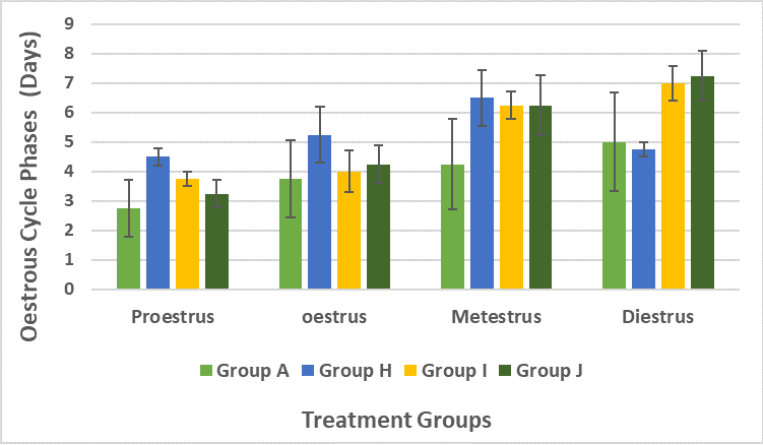

Figure 3. Effect of aqueous ethanol extracts of *Cucurbita pepo* seed on estrous cycle phases. Results are presented as mean ± SEM for 4 rats in each group. Experimental groups [Groups H, I, and J respectively received 142.86, 285.71, and 428.57 mg/kg of aqueous ethanol extract of *Cucurbita pepo* seed] were compared with group A (Normal control: 0.5 mL 20% tween 80). No significant difference was seen at a 95% confidence interval (P>0.05) as determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

Table 2. Effect of *Cucurbita pepo* seed extracts on follicle-stimulating hormone levels at different phases of the estrous cycle

Groups	Estrous phases			
	Proestrus (IU/L)	Oestrus (IU/L)	Metestrus (IU/L)	Diestrus (IU/L)
Group A	0.27±0.04	0.21±0.02	0.28±0.07	0.32±0.05
Group B	0.20±0.18	0.25±0.03	0.26±0.06	0.26±0.02
Group C	0.20±0.23	0.32±0.04	0.34±0.07	0.16±0.00
Group D	0.24±0.17	0.46±0.09	0.30±0.04	0.31±0.02
Group E	0.26±0.02	0.38±0.06	0.31±0.04	0.25±0.02
Group F	0.29±0.05	0.23±0.01	0.31±0.05	0.29±0.05
Group G	0.51±0.06*	0.48±0.04	0.52±0.01	0.49±0.11
Group H	0.60±0.05*	0.52±0.10*	0.51±0.14	0.67±0.15*
Group I	0.42±0.01	0.51±0.02*	0.38±0.06	0.42±0.05
Group J	0.27±0.01	0.31±0.09	0.34±0.05	0.28±0.04

Results are indicated as mean ± SEM. Statistical evaluation was done by one-way ANOVA followed by Tukey's post-hoc test. *P<0.05 was considered significant versus the normal control (Group A). B, C, and D: 142.86, 285.71, and 428.57 mg/kg of n-hexane extract of *C. pepo*, respectively; E, F, and G: 142.86, 285.71, and 428.57 mg/kg of dichloromethane extract of *C. pepo*, respectively; I, J, and K: 142.86, 285.71, and 428.57 mg/kg of aqueous ethanol extract of *C. pepo*, respectively.

Table 3. Effect of *Cucurbita pepo* seed extracts on luteinizing hormone levels at different phases of the estrous cycle

Groups	Estrous phases			
	Proestrus (IU/L)	Oestrus (IU/L)	Metestrus (IU/L)	Diestrus (IU/L)
Group A	0.28±0.03	0.32±0.04	0.38±0.08	0.37±0.04
Group B	0.30±0.09	0.34±0.04	0.38±0.05	0.31±0.02
Group C	0.33±0.05	0.38±0.04	0.49±0.02	0.27±0.01
Group D	0.29±0.02	0.72±0.05*	0.37±0.09	0.32±0.03
Group E	0.41±0.06	0.48±0.04	0.38±0.03	0.33±0.02
Group F	0.35±0.04	0.37±0.01	0.33±0.02	0.42±0.05
Group G	0.53±0.08	0.63±0.08	1.05±0.08*	0.78±0.11
Group H	1.55±0.08*	1.19±0.03*	1.40±0.16*	1.14±0.23*
Group I	0.95±0.11*	0.87±0.18*	0.90±0.14	0.87±0.09*
Group J	0.35±0.04	0.40±0.09	0.32±0.02	0.36±0.06

Results are indicated as mean ± SEM. Statistical evaluation was done by one-way ANOVA followed by Tukey's post-hoc test. *P<0.05 was considered significant versus the normal control (Group A). B, C, and D: 142.86, 285.71, and 428.57 mg/kg of n-hexane extract of *C. pepo*, respectively; E, F, and G: 142.86, 285.71, and 428.57 mg/kg of dichloromethane extract of *C. pepo*, respectively; I, J, and K: 142.86, 285.71, and 428.57 mg/kg of aqueous ethanol extract of *C. pepo*, respectively.

estrus, metestrus, and diestrus phases. Compared to the controls, the treatment groups G and J had significantly lower blood progesterone levels during estrus (Table 5).

Discussion

This study conducted a preliminary phytochemical screening of *C. pepo* seed extracts, revealing the presence of steroids/triterpenoids, fixed oil, and trace protein. In contrast, Malgwi et al (28) found steroids, triterpenes, polysaccharides, glycosides, cardiac glycosides, saponins, tannins, flavonoids, and alkaloids in aqueous *C. pepo* L. seed extract. Chonoko and Rufai (29) found steroids, flavonoids, and alkaloids in *C. pepo* seed ethanol extract in a phytochemical screening. However, the presence of fixed oils in our study contrasts with their results, which included flavonoids and alkaloids that were absent in our study. These differences suggest that extraction procedures and plant sources may affect phytochemical compositions (28,29). This study found no significant estrous cycle changes with the three *C. pepo* seed extracts. This supports the findings by Grace et al (14), who reported that the extracts of *C. pepo* seed might not impact the cyclicity of female Wistar rats.

DCM and aqueous ethanol extracts of *C. pepo* seeds also significantly affected blood FSH and LH levels across the estrous cycle in different treatment groups. These findings show that these extracts affect the anterior pituitary, which synthesizes and secretes gonadotropin. The extracts may activate the anterior pituitary gland and hypothalamus by raising serum FSH. Folliculogenesis and follicle development require gonadotropins from the anterior pituitary gland and hypothalamus, especially during proestrus. Similarly, the marked elevation in serum LH levels may be associated with the process

Table 4. Effect of *Cucurbita pepo* seed extracts on estrogen hormone levels at different phases of the estrous cycle

Groups	Estrous phases			
	Proestrus (IU/L)	Oestrus (IU/L)	Metestrus (IU/L)	Diestrus (IU/L)
Group A	45.25±2.32	45.00±0.41	41.00±4.76	49.00±0.82
Group B	51.75±6.02	45.75±1.55	53.50±7.33	42.75±1.93
Group C	64.00±3.74	54.00±3.24	57.00±0.71	69.00±3.27*
Group D	64.25±2.63*	68.50±4.33*	64.00±3.08*	60.50±1.04
Group E	65.50±2.25*	56.25±5.36	62.00±0.41*	64.00±1.73*
Group F	61.50±5.78	55.50±1.55	61.75±2.78*	59.00±3.11
Group G	75.25±3.07*	67.75±1.84*	73.00±0.00*	66.25±2.53*
Group H	76.75±6.63*	70.00±4.02*	64.75±5.09*	77.50±6.14*
Group I	65.00±0.91*	54.75±4.17	68.50±1.84*	62.75±1.03*
Group J	63.25±2.06	65.25±3.02*	54.75±1.03	56.25±2.10

Results are indicated as mean ± SEM. Statistical evaluation was done by one-way ANOVA followed by Tukey's post-hoc test. *P<0.05 was considered significant versus the normal control (Group A). B, C, and D: 142.86, 285.71, and 428.57 mg/kg of n-hexane extract of *C. pepo*, respectively; E, F, and G: 142.86, 285.71, and 428.57 mg/kg of dichloromethane extract of *C. pepo*, respectively; I, J, and K: 142.86, 285.71, and 428.57 mg/kg of aqueous ethanol extract of *C. pepo*, respectively.

Table 5. Effect of *Cucurbita pepo* seed extracts on progesterone hormone levels at different phases of the estrous cycle

Groups	Estrous phases			
	Proestrus (IU/L)	Oestrus (IU/L)	Metestrus (IU/L)	Diestrus (IU/L)
Group A	16.28±0.49	21.18±0.39	21.95±1.50	23.50±1.52
Group B	22.53±3.97	24.03±2.61	20.43±5.15	22.85±6.14
Group C	20.83±6.91	18.10±1.87	22.20±4.68	33.80±7.34
Group D	20.25±1.29	17.88±2.62	19.40±2.59	18.80±1.43
Group E	13.43±2.66	16.90±1.95	14.45±1.53	20.25±3.43
Group F	13.15±1.58	13.10±0.61	12.45±1.70	16.00±1.53
Group G	17.30±4.07	8.68±0.18*	16.10±1.51	19.45±2.94
Group H	17.38±1.33	17.33±0.57	16.05±1.72	16.33±1.81
Group I	21.25±0.28	13.23±2.97	18.23±1.94	17.35±2.32
Group J	10.55±1.01	9.38±1.64*	12.68±0.31	13.90±2.05

Results are indicated as mean ± SEM. Statistical evaluation was done by one-way ANOVA followed by Tukey's post-hoc test. *P<0.05 was considered significant versus the normal control (Group A). B, C, and D: 142.86, 285.71, and 428.57 mg/kg of n-hexane extract of *C. pepo*, respectively; E, F, and G: 142.86, 285.71, and 428.57 mg/kg of dichloromethane extract of *C. pepo*, respectively; I, J, and K: 142.86, 285.71, and 428.57 mg/kg of aqueous ethanol extract of *C. pepo*, respectively.

of ovulation, as it has been reported that a surge of LH before ovulation is a necessary prerequisite for ovulation to occur in a normally cycling animal (30). In the same line, the increase in LH at all the phases of the estrus cycle may equally be associated with the stimulatory effect of the extract on the hypothalamic-pituitary axis. According to Martin (31), the pulses of LH secreted by the anterior pituitary gland are evoked by pulses of GnRH secreted by the hypothalamus.

In normal cycling rats, FSH levels increase during estrous, causing follicle formation and maturation. These follicles produce androgens from theca cells, which aromatase converts into estrogen in granulosa cells (32). Elevated estrogen inhibits FSH and increases LH release. Ovulation is caused by the residual FSH and LH spike before ovulation (33). At the burst follicle, LH helps produce the corpus luteum, which secretes estrogen and progesterone. These ovarian hormones stop LH and FSH production. Non-fertilization causes corpus luteum regression and decreases ovarian hormone release. This reduction increases FSH secretion, starting a new cycle (30,34).

From the results, the aqueous ethanol extract that increased LH levels at all phases of the cycle may have inhibited the tonic centre of the hypothalamus, which is sensitive to negative feedback, while overly enhancing the surge centre, which responds to positive feedback (35). This may explain why peripheral LH increases throughout the oestrus cycle, regardless of phase. Evans and Ganjam (36) found that the anterior pituitary glands in males lack GnRH surges due to a diminished hypothalamic GnRH surge centre, unlike that of the females who have well-developed tonic and surge centres (especially before ovulation).

In our previous study, as documented by Anyanwu et al (37), we analyzed *C. pepo* seed extracts using GC-MS. It was discovered that all three extracts (n-hexane, DCM, and aqueous ethanol) contained linoleic acid, a bioactive molecule. Linoleic acid stimulates LH release via a unique PKC gonadotrope cell mechanism (38). This may explain the significant increase in LH in the treatment groups. Study results showed that all three extracts at varied doses boosted estrogen levels in test animals. At 142.86 mg/kg, the aqueous ethanol extract had considerable estrogenic action, the same as the n-hexane and DCM extracts, which had a significant influence on serum estrogen levels at 428.57 mg/kg. Based on these observations, *C. pepo* seed may be said to contain phytoestrogens. This supports Lestari & Meiyanto (39) and Patel et al (40) findings that pumpkin seed oil is high in phytoestrogens and sterols. Phytoestrogens, a diverse group of plant-derived compounds, mimic or modulate endogenous estrogen and are involved in reproduction, bone remodeling, skin, cardiovascular, nervous, immune systems, and metabolism (41,42).

Consistent with our findings, Shahin et al (43) found that oral *Cimicifuga racemosa*, a phytoestrogen, boosted estradiol and LH levels in female patients, thus offering beneficial effects in both women and men (44). Based on their chemical structures, Moreira et al (45) divided these chemicals into six groups, including isoflavones, coumestans, stilbenes, and lignans. These compounds resemble 17β estradiol in structure (42). Phytoestrogen rings have hydroxyl groups similar to those of 17β estradiol (44). According to Mortensen et al (46), phytoestrogens induce estrogenic effects by weakly binding to ERα and ERβ receptors and competing with 17β estradiol for the receptor's ligand binding region. More so, Anyanwu et al (37) suggested that GC MS-identified bioactive chemicals in all three extracts might contribute to the effects. These molecules are stearic acid, 9, 12 octadecadienoic acid, and n-hexadecanoic acid.

In 2004, Liu and colleagues found that linoleic acid can bind to estrogen receptors and displace estradiol, showing its potential (47). Similarly, Hu et al (48) found that stearic and linoleic acids had estrogen-like actions. This finding thus, provides further evidence of the potential estrogenic properties of these compounds present in the extracts. However, the observed bioactive component differences may explain the variation in *C. pepo* seed extract doses that increased estrogen hormone levels. These differences may have been caused by solvent extraction power. The extraction solvent affects extract yield, physiologically active component availability, and plant material pharmacological activity (49). This shows that n-hexane may extract bioactive chemicals less than the other solvents in the study. The less substantial effect on female sex hormones in this study may have been due to fewer bioactive components in the n-hexane extract of *C. pepo* seeds.

The decline in the peripheral progesterone level associated with the highest doses of DCM and aqueous ethanol extracts (428.57 mg/kg) at the oestrus phase may be linked to the estrogenic property of the extracts, which may have resulted to the inhibition of the luteinization of the granulosa of ruptured follicle, a process by which granulosa is converted from estrogen secretion to progesterone (50).

Conclusion

In female rats, n-hexane, DCM, and aqueous ethanol extracts of *C. pepo* seeds did not alter the normal pattern of the estrous cycle. The extracts increased the FSH and LH levels at different times. In addition, all extracts in all estrus cycle phases increased estrogen levels, but DCM and aqueous ethanol extracts reduced progesterone during estrus phase. These findings indicate that the extracts may have an effect on the anterior pituitary, leading to the activation of the female reproductive hypothalamic-pituitary-gonadal axis. It is recommended that additional comparison research be conducted using various phytoestrogenic compounds to gain useful knowledge about the effectiveness, safety, and long-term effects of *C. pepo* seed extracts in regulating hormonal balance.

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Authors' contributions

Conceptualization: Chinwe Fiona Anyanwu.
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Conflict of interests

All authors declare no existing conflict of interest

Ethical considerations

Approval for the study was duly obtained from the Research Ethics Committee of the Centre for Research Management and Development, University of Port Harcourt, with Ref. No: UPH/CEREMAD/REC/MM83/038. The rats were handled humanely in line with the Ethics and Regulation for the use of experimental animals as stipulated by NHMRC (23).

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