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Effect of *Cucurbita pepo* (pumpkin) seed extracts on serum prolactin levels of non-lactating female Wistar rats

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ABSTRACT

Despite the traditional use of *Cucurbita pepo* seed to enhance milk production during lactation, its effect in non-lactating females remains scarce. The aim of this study was to investigate the effect of n-hexane (nHE), Dichloromethane (DCM) and Aqueous ethanol (Aq. Eth) extracts of *C. pepo* seed on serum prolactin levels of non-lactating female Wistar rats. A total of 44 non pregnant female rats weighing 190 g were randomly grouped into 11 groups of 4 rats, for treatment as follows: A (control): 0.5 mL 5% Tween 80 (vehicle); B (positive control): 10mg/kg Clomiphene Citrate, C, D & E: 142.86, 285.71, and 428.57 mg/kg of nHE; F, G & H: 142.86, 285.71, and 428.57 mg/kg of DCM; and I, J & K: 142.86, 285.71, and 428.57 mg/kg of Aq. Eth extracts of *C. pepo* seed. Vaginal cytology monitored the estrous cycle daily, and blood samples were obtained for serum prolactin at various oestrus cycle phases. There was no significant ($P>0.05$) variation in serum prolactin levels in rats treated with 142.86 mg/kg, 285.71 mg/kg, and 428.57 mg/kg of all three extracts of *C. pepo* during the Proestrus, estrus, and metestrus phases relative to the control. A significant ($P<0.05$) increase in serum prolactin levels was observed at the diestrus phase in rats treated with 142.86 mg/kg, 285.71 mg/kg, and 428.57 mg/kg of n-HE, 428.57 mg/kg of DCM and 142.86 mg/kg, 285.71 mg/kg of Aq. Eth extracts of *C. pepo* seed, relative to the positive control group. Rats treated with 142.86 mg/kg of Aq. Eth had a significant increase in serum prolactin in the diestrus phase, relative to the control. The findings of this study show that *C. pepo* seed extracts may exhibit a phase-specific effect within the estrous cycle of non-lactating female rats and as such may have potential applications in regulating prolactin levels.

KEYWORDS: N-Hexane, Dichloromethane, Aqueous ethanol, *Cucurbita pepo* seed, Prolactin

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INTRODUCTION

Cucurbita pepo is one of the fifteen species under the *Cucurbita* genus of the Cucurbitaceae family. It is a versatile plant that is widely used for its nutritional and medicinal properties. It is commonly known as pumpkin and has various other names such as marrow and vegetable marrow. Pumpkins are originally from North and Central America but are now cultivated worldwide. It is widely recognised as "kadoo" in Urdu and Hindi but referred to as "squash" in English (Adnan *et al.*, 2017). In Nigeria, it is called Gooji (Hausa), Ukoro (Igbo), Famfan (Kanuri), Agbadu (Tiv), and Elegede (Yoruba) (Daniel *et al.*, 2013). The plant is an annual creeper with stems that can reach up to 30 ft (9 m) in length and has large clusters of leaves that are rough, resembling melons. It possesses circular lobed leaves and large yellow-orange flowers that are pollinated by insects (Kesh & Yadav, 2023). The seeds and pulp of the plant are used for medicinal purposes. The seeds have a cooling effect and are similar to melons in nature. The seeds possess diuretic and stimulating properties and are effective in treating aching chests, bronchitis, fever, and reducing thirst. Additionally, they are beneficial for brain health and are utilised in the management of renal problems (Roth & Lindorf, 2013). Pharmacologically, it is utilised for many purposes such as reducing high cholesterol levels, lowering blood pressure, reducing inflammation, combating parasites, inhibiting tumour growth, preventing oxidative damage, managing diabetes, preventing cancer, fighting bacterial infections, and alleviating intestinal inflammation (Caili *et al.*, 2006; Hussain *et al.*, 2022). *C. pepo* contains many kinds of phytochemicals, including linoleic acids, oleic acid, alkaloids, flavonoids, and palmitic acid, which may contribute to its therapeutic effects (Adebayo *et al.*, 2013; Anyanwu *et al.*, 2022). Beyond its phytochemicals, *C. pepo* seeds are a valuable source of essential nutrients such as proteins, carbohydrates, minerals, and fats, making them beneficial for both human and animal health. This, along with a high mineral concentration, is beneficial for both humans and animals.

Traditionally, *C. pepo* seeds have been used to enhance milk production during lactation. This folkloric use has been

supported by scientific research demonstrating that the seeds can stimulate lactation and increase milk production in lactating female rats (Daniel *et al.*, 2013). Despite these findings, there is a paucity of information regarding the effect of *C. pepo* seed on serum prolactin levels of non-lactating female rats.

MATERIALS AND METHODS

Plant Material and Authentication

Fresh *Cucurbita pepo* (pumpkin) fruits were purchased from Choba central market, Port Harcourt, Nigeria. The plant was authenticated by Dr. Chimezie Ekeke of the University of Port Harcourt Plant Science Biotechnology Department and recorded in the University Herbarium with (Ref No; UPH/PSB/2021/071) available at the University Herbarium.

Preparation of Extracts

Fresh *C. pepo* fruits were obtained, and the seeds were collected after drying for four weeks. The deshelled seeds were then ground into fine powder, followed by cold maceration using three solvents: N-hexane, Dichloromethane, and 70% aqueous ethanol.

The extraction was carried out successively for 72 h with a fresh replacement of the solvent every 24 h. For the N-hexane extraction, 500 g of the powder was macerated in 1.5 L of n-hexane for 24 h. The resulting solution was filtered with Muslin fabric and Whatman's No.1 filter paper. The residue was then soaked and filtered twice more, and the filtrates were concentrated with a rotary evaporator. The concentrated solution was transferred to an evaporating dish and dried over a water bath.

A similar process was followed for Dichloromethane extraction using the dried Marc obtained from the previous extraction. The 500 g of the dried Marc was soaked in 1.5 L of dichloromethane for 24 h. The extraction was repeated three times, and the filtrates were combined, concentrated, and dried using the same method.

The procedure was repeated once more for the aqueous ethanol extraction, using 500 g of the dried Marc obtained from the Dichloromethane extraction. Each step involved maceration in 1.5 L of 70% aqueous ethanol for 24 h, filtration, concentration, and drying.

Animals

For this study, 44 female rats with regular cycles and an average weight of 180 g were used. The animals were procured from the Animal House of the Department of Pharmacology at the College of Health Sciences, University of Port Harcourt, Nigeria, and were acclimatised for two weeks before the commencement of the study. During the study, the animals had access to commercially sourced feed (Top Feeds Nigeria Limited) and clean drinking *ad libitum*.

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 Regulations for the use of experimental animals as stipulated by NHMRC (2008).

Experimental Design

Dosage used was as reported by Ivoh *et al.* (2023). Following acclimatization, the female rats were randomly grouped into eleven groups of four (4) animals each to be treated as follows:
Group A (Control) - 0.5 mL of 5% Tween 80.
Group B (positive control) - 10 mg/kg Clomiphene citrate
Group C - 142.86 mg/kg of N-hexane Extract
Group D - 285.71 mg/kg of N-hexane Extract
Group E - 428.57 mg/kg of N-hexane Extract
Group F - 142.86 mg/kg of Dichloromethane Extract
Group G - 285.71 mg/kg of Dichloromethane Extract
Group H - 428.57 mg/kg of Dichloromethane Extract
Group I - 142.86 mg/kg of Aqueous ethanol Extract
Group J - 285.71 mg/kg of Aqueous ethanol Extract
Group K - 428.57 mg/kg of Aqueous ethanol Extract

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

Vaginal Cytology

The oestrous cycle of the female Wistar rats was assessed by morning vaginal cytology. The pipette smear method reported by Ivoh *et al.* (2023) was used. A dropping pipette was used to wash the vaginal walls of the animals with a few drops of normal saline (0.9% NaCl). Lavage containing vaginal wall cells was placed on a grease-free microscope slide and examined under a light microscope with a 10x objective lens. Oestrous cycle phases were determined by the presence of distinct cells, such as irregular anucleated cells (cornified cells) indicating oestrous, 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. The oestrous cycle phases of the experimental animals were fully understood using this approach.

Blood Collection for Hormonal Analyses

Starting on day 21, blood samples were taken from each of the eleven groups using the orbital bleeding technique (Obinna & Kagbo, 2017; Ivoh *et al.*, 2023). The samples were taken at different times during the oestrous cycle. To do this, a microhaematrocrit plain tube was put into the orbital plexus of the medial canthus of the eye until it made contact with the orbital bone. The tube was then slightly bent and taken out to let the blood flow through it into the clean plain bottles. The blood samples were left still for 30 to 45 min so that the platelets could clot. After that, they were spun at 3000 rev/min for 15 min to get the sera. After the sera were collected, they were put into clean bottles, kept in microcentrifuge tubes with tight lids, and

kept at 20 °C until they were analysed. Later, the sera were used for ELISA hormonal research to check the amounts of Prolactin.

Hormonal Assay

The serum concentration of prolactin was measured using a Microplate Enzyme Immunoassay using the Colorimetric Accu-bind ELISA Microwells Prolactin Test System (Product Code: 4425-300) from Monobind Inc., Lake Forest, CA 92630, USA. The assay procedure strictly followed the manufacturer's manual. Following the assay run, the optical density measurements were employed to determine the serum concentration of prolactin hormone.

Statistical Analyses

Statistical analyses were performed in SPSS 21 and provided as mean±SEM. The assessment used one-way ANOVA and the Tukey post-hoc test. The significance threshold was set at $p<0.05$.

RESULTS

Tables 1 to 3 respectively show the results of the effect of graded doses of n-Hexane, Dichloromethane and Aqueous Ethanol extracts of *C. pepo* seed administration on serum prolactin levels of non-pregnant, non-lactating female Wistar rats at various phases of their oestrous cycle.

From the results obtained in Tables 1 and 2, it was observed that at the proestrus, estrus and metestrus phases of rats treated with nHE and DCM extracts of *C. pepo* seed at doses of 142.86 mg/kg, 285.71 mg/kg and 428.57 mg/kg had no significant variation in serum prolactin levels when compared to the vehicle control group. However, there was a significant increase in serum prolactin levels at the diestrus phase of rats treated with 142.86 mg/kg, 285.71 mg/kg and 428.57 mg/kg doses of nHE and 428.57 mg/kg of DCM extracts of *C. pepo* seed, relative to the positive control group (group B).

Table 3 shows that rats treated with 142.86 mg/kg, 285.71 mg/kg and 428.57 mg/kg of Aqueous ethanol extract of *C. pepo* seed had no significant variation in serum prolactin levels at the estrus, proestrus and Metestrus phases of the oestrous cycle compared to the vehicle control group A. However, a significant increase in serum prolactin levels was observed in the diestrus phase in rats treated with 142.86 mg/kg of Aq. Eth extract of *C. pepo* seed relative to both controls and in rats treated with 285.71 mg/kg of Aq. Eth extract relative to the positive control group B.

DISCUSSION

Prolactin, also known as lactotropin, is a protein hormone primarily associated with enabling mammals to produce milk. It plays a crucial role in lactation, breast tissue development, and milk production. Prolactin is an anterior pituitary hormone, first seen in pigeons to enhance crop milk production and thereafter termed for its main role in mammals to stimulate lactation (Riddle *et al.*, 1933; Horseman & Buntin, 1995; Gillam & Molitch, 2011). This study found that all three extracts of *Cucurbita pepo* seed at doses of 142.86 mg/kg, 285.71 mg/kg and 428.57 mg/kg had no significant ($P>0.05$) variation in serum prolactin levels in non-lactating female rats at proestrus, oestrous, and metestrus phases of their oestrous cycle when compared to the control group. This implies that *C. pepo* seed may not alter normal serum prolactin levels in normal cycling female rats at the follicular phases of their cycle. Studies have shown that in normal cycling non-pregnant, non-lactating female Wistar rats, serum prolactin levels are generally low. The hypothalamus provides a tonic inhibitory input through a "short-loop" negative feedback mechanism. Neuroendocrine

Table 1: Effect of n-hexane extract of *C. pepo* seed on prolactin level at different phases of Oestrous Cycle

Groups	Oestrous Phases			
	Proestrus (ng/mL)	Oestrus (ng/mL)	Metestrus (ng/mL)	Diestrus (ng/mL)
Group A (Normal Control)	1.28±0.11	0.96±0.00	1.38±0.31	0.86±0.16$^{\#}$
Group B (Positive Control)	0.89±0.12	0.89±0.14	0.95±0.06	0.00±0.00*
Group C (142.86 mg/kg n-hexane extract)	0.81±0.27	1.52±0.26	0.80±0.11	1.16±0.37$^{\#}$
Group D (285.71 mg/kg n-hexane extract)	1.39±0.50	1.09±0.26	1.26±0.46	1.44±0.04$^{\#}$
Group E (428.57 mg/kg n-hexane extract)	1.50±0.13	0.91±0.13	1.45±0.24	1.36±0.07$^{\#}$

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 as significant versus the normal control (Group A); $^{\#}p<0.05$ was considered significant versus the positive control (Group B) for 21 days

Table 2: Effect of dichloromethane extract of *C. pepo* seed on prolactin level at different phases of Oestrous Cycle

Groups	Oestrous Phases			
	Proestrus (ng/mL)	Oestrus (ng/mL)	Metestrus (ng/mL)	Diestrus (ng/mL)
Group A (Normal Control)	1.28±0.11	0.96±0.00	1.38±0.31	0.86±0.16$^{\#}$
Group B (Positive Control)	0.89±0.12	0.89±0.14	0.95±0.06	0.00±0.00*
Group F (142.86 mg/kg dichloromethane extract)	1.18±0.18	1.19±0.21	1.31±0.20	0.81±0.14
Group G (285.71 mg/kg dichloromethane extract)	0.87±0.19	0.76±0.05	1.03±0.17	0.80±0.10
Group H (428.57 mg/kg dichloromethane extract)	1.30±0.13	1.29±0.13	1.59±0.24	1.26±0.18$^{\#}$

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 as significant versus the normal control (Group A); $^{\#}p<0.05$ was considered significant versus the positive control (Group B) for 21 days

Table 3: Effect of aqueous ethanol extract of *C. pepo* seed on prolactin level at different phases of Oestrous Cycle

Groups	Oestrous Phases			
	Proestrus (ng/mL)	Oestrus (ng/mL)	Metestrus (ng/mL)	Diestrus (ng/mL)
Group A (Normal Control)	1.28±0.11	0.96±0.00	1.38±0.31	0.86±0.16#
Group B (Positive Control)	0.89±0.12	0.89±0.14	0.95±0.06	0.00±0.00*
Group I (142.86 mg/kg aqueous ethanol extract)	1.56±0.08	1.37±0.27	1.25±0.21	2.11±0.19*#
Group J (285.71 mg/kg aqueous ethanol extract)	1.24±0.08	1.42±0.12	1.25±0.14	1.33±0.03#
Group K (428.57 mg/kg aqueous ethanol extract)	0.76±0.03	1.11±0.08	0.92±0.29	0.78±0.02

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 as significant versus the normal control (Group A); #$p<0.05$ was considered significant versus the positive control (Group B) for 21 days

dopamine (NEDA) neurons in the periventricular region of the hypothalamus and arcuate nuclei regulate the release of prolactin (Phillipps *et al.*, 2020). Dopamine is released when these neurons fire faster with prolactin. Dopamine then acts on lactotroph dopamine D2 receptors via the pituitary portal blood channels to tonicly decrease prolactin secretion (Phillipps *et al.*, 2020).

The findings of this study also demonstrated a significant increase in serum prolactin levels at the diestrus phase of non-lactating female Wistar rats treated with 142.86 mg/kg, 285.71 mg/kg and 428.57 mg/kg of nHE extract, 428.57 mg/kg of DCM extract and 142.86 mg/kg, 285.71 mg/kg of Aq. Eth extracts of *C. pepo* seed. This implies that the effect of *C. pepo* seed on Prolactin secretion in female rats may be specific to the diestrus phase of the oestrous cycle and as such may be via a mechanism that is closely linked to the hormonal and physiological changes occurring during diestrus, which is characterized by low estrogen and progesterone levels. This finding is in tandem with that reported by Daniel *et al.* (2013) that aqueous *C. pepo* linn seed extracts significantly increased serum concentration levels of prolactin in lactating female rats, which emphasizes the potential of *C. pepo* seed to elevate prolactin levels, and is consistent with findings of this present study. However, unlike the latter study, our study found an increase in serum prolactin levels in the diestrus phase of non-lactating female Wistar rats. This ability of *C. pepo* seed extract to potentially increase prolactin levels in non-lactating female rats, suggests that the mechanism of action is not specific to lactation. The extract may be influencing prolactin secretion through a general mechanism that is not dependent on lactation status. This could be related to the antioxidant and anti-inflammatory effects of the extract, which are known to modulate the hypothalamic-pituitary-ovarian axis, thus increasing the concentration of serum prolactin (Bolzán *et al.*, 1995; Bolzán *et al.*, 1997; Roshankhah *et al.*, 2019).

However, the disparity in the doses of the extracts of *C. pepo* seed that elicited the significant effect on the prolactin hormone as shown in this study may not be unconnected with their inherent bioactive compounds which lies in the extracting power of the solvents used in the extraction process. It has been stated that the type of solvents used in the extraction process determines the extract yield, available biologically active compounds as well as the pharmacological activities of the plant materials (Pandey & Tripathi, 2014; Rasul, 2018; Abubakar & Haque, 2020). From this assertion, Dichloromethane may have less extracting power of bioactive compounds compared to the other extracting solvents used in this study. Secondly, it is also possible that the bioactive compounds of the Dichloromethane extract of *C. pepo* may not necessarily be less but the compounds may not have much effect on serum prolactin as shown in this study.

CONCLUSION

In conclusion, this study's findings demonstrated that *C. pepo* seed extracts may exhibit a phase-specific effect within the oestrous cycle of non-lactating female rats possibly via a mechanism related to its antioxidant and anti-inflammatory effect, which are known to modulate the hypothalamic-pituitary-ovarian axis, and as such may have potential applications in regulating prolactin levels.

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