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Reproductive Outcome in Female Wistar Rats Following Treatment with *Cucurbita pepo* (Pumpkin) Seed Extracts

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ABSTRACT

Due to healthcare shortages and high infertility costs, medicinal plants and herbal preparations with fertility properties are gaining popularity in developing countries. In this study, female wistar rats were treated with n-hexane (nHE), dichloromethane (DCM), and aqueous ethanol (Aq. Eth) extracts of *Cucurbita pepo* seed to investigate its effect onreproductive outcome in female wistar rats. 48 rats were randomly divided into 12 of 4 rats each for daily oral gavage treatment for 21 days: A (control) = 0.5ml 20% tween 80 (vehicle); B (positive control) = 10mg/kg clomiphene citrate; C, D, and E = 142.86, 285.71, and 428.57 mg/kg nHE; F, G, and H = DCM; and I, J, and K = Aq.Eth extracts. Group L (positive control 2): 8 days/10mg/kg clomiphene citrate. After treatment, rats mated 2:1 with males. Mating confirmation day was gestational day 0. On GD 20, animals were laparatomised and reproductive outcome was assessed by foetal weight, FCRL, litter size, implantation, and resorption sites. On reproductive outcome indices, all extracts exhibited no significant effect (p >0.05). Clomiphene citrate markedly reduced reproductive outcome indices. It can be concluded that *Cucurbita pepo* seed Cucurbita pepo seed had no effect on female rats' reproductive outcomes at the dosages and period utilised in this investigation. These findings can be starting point for further researchto ascertain if variations in duration or doses of *Cucurbita pepo* seed extracts may yield different outcomes.

Keywords: Fertility, *Cucurbita pepo,* Clomiphene citrate, foetus, outcome indices.

INTRODUCTION

Infertility is a complicated physiologic, psychologic, and economic issue (1). Infertility is the failure to conceive after a year of unprotected and uninterrupted sexual activity (2,3) or in the absence of any identified reproductive pathology (4). About 15 percent of couples suffer from infertility globally (5), andOne in six reproductive-age couples worldwide experience infertility (1,6). According to research, 20–50% of infertility is male, 40% is female, and 25% is idiopathic (2,5,7,8). Infertility in females can be caused by ovarian illnesses, cervical issues, congenital

malformations, endometriosis, hypothalamus-pituitary-ovarian axis dysfunction, tubal disorders, uterine pathologies, and systemic diseases can cause female infertility (2,9).

Due to healthcare shortages and high infertility treatment costs, many women in underdeveloped countries like Nigeria prefer herbal remedies (8,10–12) which are cheap and widely available. This has revived interest in fertility-boosting medicinal plants and herbs.

Curcubita pepo L. (Pumpkin), a native plant consumed in practically all of Eastern Nigeria, is used in reproductive health therapies (13,14). Therapeutically, it is anti-inflammatory, anti-hypercholesterolemia, anti-hypertensive, anti-parasitic, anti-carcinogenic, anti-oxidant, and anti-bacterial (15). Scientific studies carried out on *C. pepo* seed shows that it contains different categories of bioactive compounds such as linoleic, Palmitic, stearic and oleic acids (16), flavonoids and alkaloids (17) that may contribute to its therapeutic qualities.

Cucurbita pepo seed extract has been found to remarkably remediate the side effects of cyclophosphamide on male reproductive health, possibly by preventing oxidative stress (18). Oxidative stress associated with cyclophosphamide occurs as a result of generation of reactive oxygen species (ROS) within cells. This occurs through various pathways, including the metabolism of cyclophosphamide in the liver and other tissues. This metabolic process produces byproducts such as acrolein and phosphoramide mustard, which can generate ROS directly or indirectly through the activation of cellular pathways (19). The antioxidant properties of *Cucurbita pepo* seed may be attributed to innate compounds such as phenolic acids, tocopherols, and carotenoids, which counteract the oxidative damage induced by cyclophosphamide on sperm cells and reproductive tissues. By scavenging reactive oxygen species (ROS) and enhancing the activity of endogenous antioxidant enzymes, *Cucurbita pepo* seed extract may protect against lipid peroxidation, DNA damage, and mitochondrial dysfunction, thereby preserving sperm quality and fertility in the cyclophosphamide treated males (20). This research is further informed by the preventive benefits of Cucurbita pepo seed extract in men, driving us to investigate if females have comparable protective effects or other reproductive outcomes. This study addresses the research gap and examines the potential benefits as well as risks of using *Cucurbita pepo* for female fertility and fecundity.

Traditionally, *Cucurbita pepo* has been used to treat pregnancy associated nausea, motion sickness, and as supplement for nutritional deficiencies. Previous research suggest *Cucurbita pepo* is relatively safe in pregnancy (13,14,21). Despite its reported safety in pregnancy and potential protective benefits in men, the effect of *Cucurbita pepo* seed on female fertility remains under explored. This study examined the reproductive outcome in female Wistar rats following treatment with n-hexane, dichloromethane, and aqueous ethanol extracts of *Cucurbita pepo* seed as an indicator for fertility.

MATERIALS AND METHODS

The Plant Material and Authentication

Fresh pumpkin fruits (*Cucurbita pepo)* were purchased from central market Choba, in the city of Port Harcourt, Rivers state. It was put in a plastic bag and taken to the University of Port Harcourt's Department of Pharmacology. The plant species authentication was done in comparison with the voucher specimen (Ref No. UPH/PSB/2021/071) kept at the University Herbarium.

Preparation of the Extract

Fresh *Cucurbita pepo* fruits were cut open to get the hard seeds inside. The seeds were then dried in the shade at room temperature for four weeks and the shells were taken off. The *Cucurbita pepo* seeds that had been peeled were weighed out and then ground into a fine powder. This is how the extraction process was done, as shown by Harborne, (22). Each of the three solvents—1.5 litres of N-hexane, Dichloromethane, and 70% watery ethanol—was used for 72 hours of the cold maceration extraction method. All of the time, the liquid was changed every 24 hours. Three different types of extraction liquids were used, with N-hexane, dichloromethane, and watery ethanol being the least polar. 500g of Cucurbita pepo seed powder was macerated in 1.5L n-hexane for 24 hours. After 24 hours, they were mixed and filtered with a Muslim cloth and Whatman's No. 1 filter paper. After soaking the Marc in the same quantity of n-hexane for 24 hours, it was filtered with a Muslim cloth and Whatman's filter paper. Marc was macerated again in 1.5Litres of solvent for 24 hours (72 hours total) and filtered again. Add the filtrates and concentrate using rotary evaporator (Model No. RE-52A) at 450C in vacuo. In an evaporating dish, dry over a water bath (Digital thermostatic water bath, Jinotech instruments). Drying the marc to a consistent weight allowed the second solvent, dichloromethane, and the third solvent, 70% aqueous ethanol, to extract *C.pepo* seed extract.

Animals

The study employed adult female Wistar rats from the University of Port Harcourt Department of Pharmacology animal house. The experimental animals were acclimatized for two weeks before the study. They had unlimited access to commercial feed (Top Feeds Nigeria) and clean water.

Acute Oral Toxicity Study

To estimate the LD50 of the three extracts, 54 female rats were studied. N-hexane, dichloromethane, and aqueous ethanol extracts of Cucurbita pepo seed were tested for acute oral toxicity using approach byLorke, (23). Eighteen (18) animals were employed for each extract. In the first phase, three groups of three animals received oral gavage dosages of 10mg/kg, 100mg/kg, and 1000mg/kg N-hexane extract and were examined for 24 hours. 3 groups of 3 animals were administered extract at 1600mg/kg, 2900mg/kg, and 5000mg/kg by oral gavage in the second phase and watched for 24 hours for mortality and other toxicity signs. Same method with DCM and Aq. Eth extracts, 18 animals each.

Experimental Design

The *Cucurbita pepo* seed extract doses used for this study was as recommended by the Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency, (24). This study utilised 48 female rats with regular oestrous cycles and an average weight of 190g and 24 sexually mature male rats weighing 200g. Male rats were solely mated.

Female Wistar rats were randomly assigned to 12 groups of four for the following treatment:
Group A (Normal Control): 0.5ml of 20% Tween 80.
Group B (positive control): 10mg/kg of Clomiphene Citrate
Group C: 142.86mg/kg of N-hexane Extract
Group D: 285.71mg/kg of N-hexane Extract
Group E: 428.57mg/kg of N-hexane Extract
Group F: 142.86mg/kg of Dichloromethane Extract

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Group G: 285.71mg/kg of Dichloromethane Extract
Group H: 428.57mg/kg of Dichloromethane Extract
Group I: 142.86mg/kg of Aqueous ethanol Extract
Group J: 285.71mg/kg of Aqueous ethanol Extract
Group K: 428.57mg/kg of Aqueous ethanol Extract
Group L (positive control 2): 10mg/kg of Clomiphene Citrate

Using anoral gavage syringe, all treatments were given daily for 21 days except group L, which was given for 8 days. Every three days, animals were weighed and dosages adjusted.

Reproductive Outcome

After extract administration, two females and a male were caged overnight on day 21. The presence of sperms in vaginal aliquot or plug indicated mating. This was GD 0 (25,26).Anaesthetized and dissected, the rats' uteri were removed and incised at the greater horn curvature on gestational day 20 (GD 20). The reproductive outcome was examined according to Kagbo & Obinna, (27) using the following parameters:

- The total uterine implants and resorptions.
- The mean crown rump length of the pups.
- The mean litter size
- The mean pup weight at gestational day 20 (GD 20)

Statistical Analyses

The study used SPSS 21 to evaluate data as mean ± SEM, using one-way Analysis of Variance (ANOVA) and Tukey post-hoc test. We used a significance threshold of $p<0.05$.

RESULTS

Table 1: Effect of n-hexane extract of *C. pepo* seed on the Reproductive Outcome of female wistar rats

Groups	Foetal Weight(g)	FCRL (cm)	Litter size (No)	Implantation sites (No)	Resorption sites (No)
A	3.56±0.08#	3.67±0.07#	6.25±1.84#	6.50±1.66#	0.25±0.25
B	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	3.25±3.25
C	3.29±0.10#	3.65±0.06#	8.25±0.48#	8.25±0.48#	0.00±0.00
D	3.31±0.11#	3.71±0.05#	5.50±0.87#	7.00±1.22#	1.50±1.50
E	3.33±0.12#	3.68±0.08#	8.25±0.25#	8.25±0.25#	0.00±0.00

Results are given as Mean ± SEM fir each group. Statistical evaluation was done by one-way ANOVA, followed by Tukey's post-hoc test. Experimental groups are compared with group A (Normal Control) and group B (Positive Control – Clomiphene citrate). *$p<0.05$ was considered as significant versus the Normal control (Group A); #$p < 0.05$ was considered significant versus the positive control (Group B).

Table 2: Effect of Dichloromethane extract of *C. pepo* seed on the Reproductive Outcome of female Wistar Rats.

Groups	Foetal Weight(g)	FCRL (cm)	Litter size (No)	Implantation sites (No)	Resorption sites (No)
A	3.56±0.08#	3.67±0.07#	6.25±1.84#	6.50±1.66#	0.25±0.25

B	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	3.25±3.25
F	3.73±0.51#	3.83±0.24#	8.50±1.26#	8.75±1.25#	0.25±0.25
G	3.30±0.12#	3.66±0.05#	9.00±0.71#	9.00±0.71#	0.00±0.00
H	3.82±0.12#	3.81±0.03#	6.75±0.85#	7.00±0.71#	0.00±0.00

Results are given as Mean ± SEM for each group. Statistical evaluation was done by one-way ANOVA, followed by Tukey's post-hoc test. Experimental groups are compared with group A (Normal Control) and group B (Positive Control – Clomiphene citrate). *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).

Table 3: Effect of Aqueous ethanol extracts of *Cucurbita pepo* seed on Reproductive outcome of Female Wistar Rats

Groups	Foetal Weight(g)	FCRL (cm)	Litter size (No)	Implantation sites (No)	Resorption sites (No)
A	3.56±0.08#	3.67±0.07#	6.25±1.84#	6.50±1.66#	0.25±0.25
B	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	3.25±3.25
I	4.16±0.82#	3.94±0.23#	6.50±1.55#	6.50±2.40#	1.00±1.00
J	3.19±0.30#	3.54±0.16#	4.75±1.32	4.75±1.32	0.00±0.00
K	3.87±0.03#	3.97±0.04#	6.50±0.29#	6.50±0.29#	0.00±0.00

Results are given as Mean ± SEM for each group. Statistical evaluation was done by one-way ANOVA, followed by Tukey's post-hoc test. Experimental groups are compared with group A (Normal Control) and group B (Positive Control – Clomiphene citrate). *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).

Table 4: Effect of Clomiphene citrate administered for 21 days on GROUP B (Positive Control) rats

Animals	Foetal Weight(g)	FCRL (cm)	Litter size (No)	Implantation sites (No)	Resorption sites (No)	Remarks
1	-	-	-	-	-	No pup
2	-	-	-	-	-	No pup
3	-	-	-	-	-	No pup
4	-	-	-	-	13	No pup, 13 Pinpoint areas of resorption seen on the uterine horns

Table 5: Effect of Clomiphene citrate administered for 8 days on GROUP L (Positive Control 2)

Animals	Foetal Weight (g)	FCRL (cm)	Litter size (No)	Implantation sites (No)	Resorption sites (No)	Remarks
1	-	-	-	-	-	No pup
2	-	-	-	-	-	No pup
3	-	-	-	-	-	No pup
4	3.71	3.8	1	1	0	1 pup

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Acute Toxicity Study

The acute toxicity investigation showed no death, morbidity, or other harm at the levels utilised. This showed that all three extracts were safe at 5000mg/kg. However, the European Medicine Agency's Committee on Herbal Medicinal Products (HMPC) (24)recommended administering *C. pepo* to 70kg adults at 10–30g.Thus, this was employed in the research to provide n-hexane, dichloromethane, and aqueous ethanol extracts of Cucurbita pepo seed at 143mg/kg, 286mg/kg, and 429mg/kg.

Effect of n-Hexane Extract of *C.pepo* seed on Reproductive outcome of Female Wistar Rats

Table 1 shows that all the doses of n-hexane seed extract of *C. pepo* as used in this study caused no significant (P>0.05) variation in the reproductive outcome of treated female wistar rats relative to the normal control (group A) as assessed by foetal weight of the pups, foetal crown rump length of the pups, litter size of the rat, number of implantation as well as resorption sites on the rats' uterine horns.

Result findings in table 2 shows that the Dichloromethane extract of *Cucurbita pepo* seed had no significant (P>0.05) effect on the reproductive outcome (foetal weight, crown rump length, litter size, and number of implantation and resorption sites) of rats treated for 21 days compared to the control (group A). Aqueous ethanol extract of *Cucurbita pepo* seed at 142.86mg/kg, 285.71mg/kg, and 428.57mg/kg did not affect reproductive outcome (foetal weight, crown rump length, litter size, and number of implantation & resorption sites) in test rats compared to Group A. No pathology was found on the pups, placenta, or umbilical cord of the test groups (figures 1 and 2).

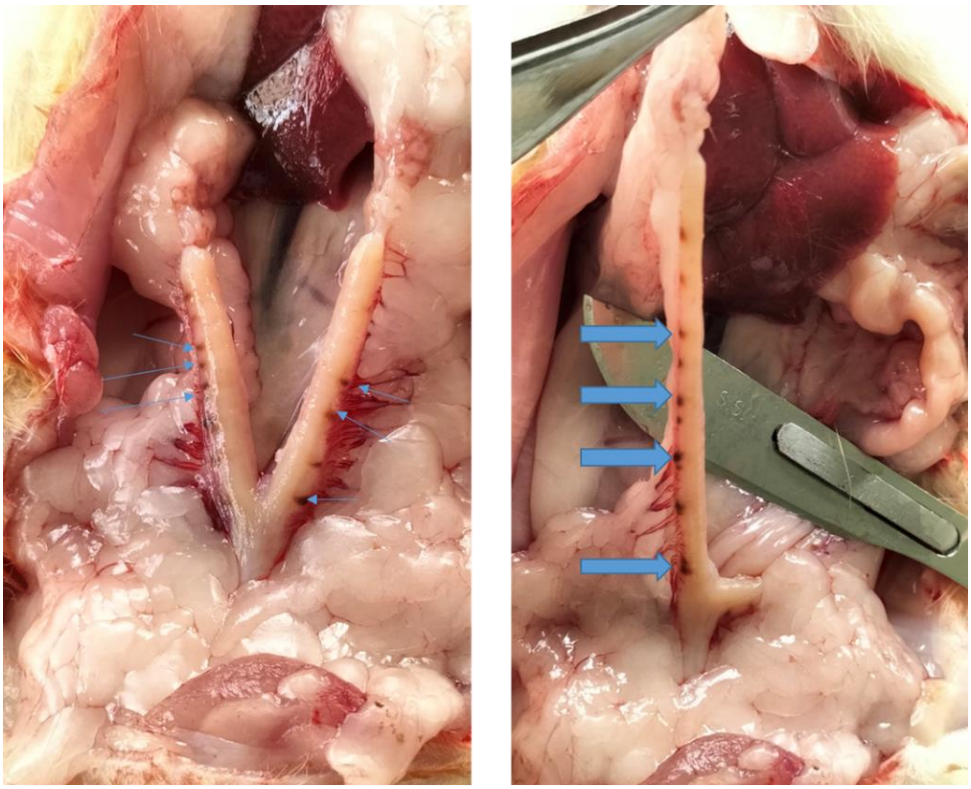

Figure 1: Picture of the uterine horns of clomiphene-treated rat (21 days duration) showing pinpoint resorption sites (arrows)

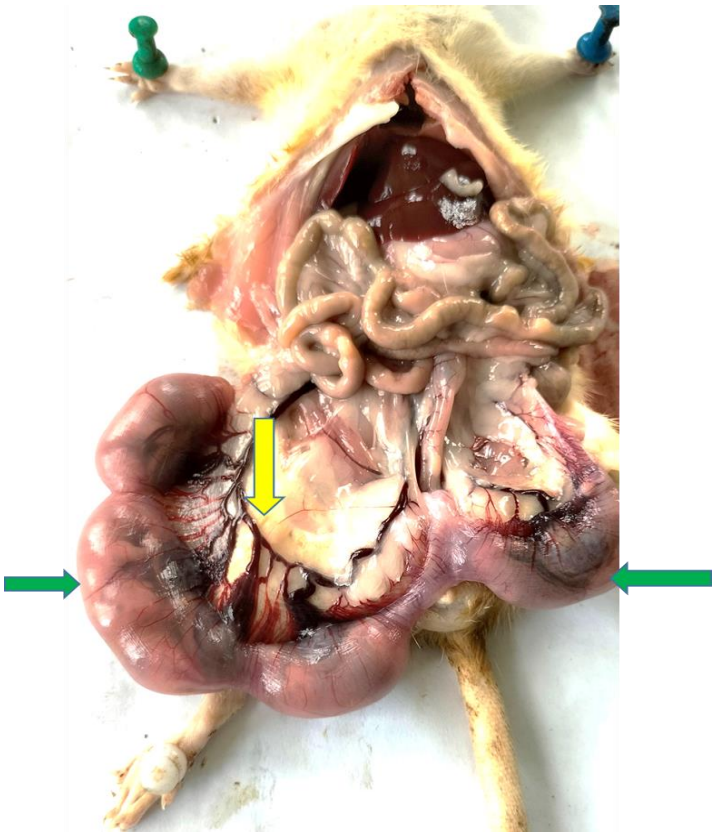

Figure 2: The picture of a laparatomised pregnant rat showing the gravid left and right uterine horns (green arrows), and the engorged blood vessels (yellow arrow) supplying the uterine horns and the placenta

Effect of Clomiphene Citrate on Reproductive Outcome of Female Wistar Rats

From the result, out of the four (4) clomiphene citrate treated-rats (positive control – group B) administered for 21 days, no pup was seen at GD 20 on laparotomy, even though mating was confirmed by presence of vaginal plug or sperm cells in vaginal smear in all, instead 13 pinpoint areas of resorption were seen on the two (2) uterine walls of just one (1) out of the four (4) rats. When four female rats (group L) were given clomiphene citrate for eight days (group L) and mating was verified, only one had a pup at gestation day 20 (GD 20) after laparotomy.

DISCUSSION

The study examined female Wistar rats administered with three different *Cucurbita pepo* seed extracts and clomiphene citrate as a positive control. This study found no significant influence of the three *Cucurbita pepo* seed extracts on reproductive outcome indices. In other researches, specifically those that examined the protective effects of *C. pepo* seed extract on male reproductive health following chemotherapy and the critical role the extract played in improving the functionality of the reproductive system in uranyl acetate-induced reproductive toxicity, a different narrative emerged (18,29). It is clear from those researches that *C. pepo* seed extract can mitigate the negative effects of chemotherapy, especially with regard to sperm parameters and epididymal histology. Similarly, Ofoego *et al*., (30)found that *C.pepo* seed extract improved testicular parameters in *Azadirachta Indica* (Neem)-induced reproductive toxicity. These varying responses of the male and female rats to *Cucurbita pepo* seed extracts may indicate intricate interactions between *Cucurbita pepo* seed extracts and the complex

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hormonal and physiological pathways governing reproductive health. However, the protective effects observed in the *C.pepo* seed extract study demonstratethe multifaceted nature of herbal remedies, revealing potential benefits in specific reproductive contexts.

It is important to note that, Clomiphene citrate, a selective oestrogen receptor modulator (SERM) that was used as the first treatment for infertility in women who were usually estrogenic and not ovulating (WHO group II), did not lead to a successful pregnancy. This study findings aligns with the concerns stirred up byHomburg, (31) regarding the effectiveness of Clomiphene citrate. The reported efficacy of Clomiphene citrate, as shown by a single live birth rate of around 25% for initial attempts, suggests that there is room for improvement(31). The disparities in ovulation and conception rates, together with instances of non-responsiveness to Clomiphene citrate, are acknowledged concerns that have spurred the investigation of other therapeutic alternatives (32,33). In contrast to the findings of Ogbuehi *et al.,* (34), our results showed that clomiphene citrate did not lead to a substantial increase in the number of pups born by the rats that were treated. This highlights the variety in how treatments can affect outcomes in different experimental settings. The successful beginning of pregnancy requires several factors, such as the release of a mature egg during ovulation, the presence of healthy sperm, the close proximity of the sperm and egg in the reproductive tract, the fertilisation of the egg, the transportation of the developing embryo into the uterus, and the implantation of the embryo into a well-prepared and healthy lining of the uterus. Any disruption in these processes might result in infertility (35). The choice of the three different solvents (n-hexane, dichloromethane, and aqueous ethanol) for the extraction of *Cucurbita pepo* seed compounds is crucial. It has been established that different solvents have different extracting powers which eventually affects the extract yield, availability of bioactive compounds or phytochemical constituents as well as the pharmacological activities of the plant material (36,37). Each of these solvents extract different types of compounds, and their varying polarities can influence which phytochemicals are obtained. According to Anyanwu *et al*., (16), only Palmitic, stearic, and linoleic compounds and their derivatives were bioactive compounds found in all three extracts. Research findings by Kim *et al*., (38) revealed that the major fatty acids found in *C.pepo* seeds grown in Korea were Palmitic, stearic, oleic and linoleic acids.

The essential fatty acids such as, linoleic acid and α-linolenic acid and their derivatives which balances female reproductive hormones and also aids in lubricating the mucous membrane (39), was found to be present in all three extracts (Aqueous ethanol, dichloromethane and n-hexane) of *Cucurbita pepo* seed. Linoleic acid (LA) and α-linolenic acid (ALA), present in the extracts of Cucurbita pepo seeds, could act as building blocks for the synthesis of important compounds like prostaglandins, which regulates hormone levels in the female reproductive system (39). These prostaglandins influence the production, release, and sensitivity of hormones like oestrogen and progesterone, essential for menstrual cycle regulation and fertility (40). Moreover, these essential fatty acids contribute to the structural integrity of mucous membranes in the reproductive tract, ensuring proper lubrication and protection against pathogens. This in turn contributes to sexual function and fertility, thus, highlighting the significance of these essential fatty acids in supporting reproductive well-being. However, the data from tables 1-3 indicate that there were no noticeable differences in reproductive outcomes, such as foetal weight, foetal crown-rump length (FCRL), litter size, and the number of implantation sites, as compared to the normal control group. The absence of importance in these reproductive outcome measures may indicate possible constraints linked to dose and

duration of therapy. This study, being the first of its type, establishes the foundation for future research in investigating the intricate processes that contribute to the protective benefits of pumpkin seed extract.

The localised regions of resorption identified in rats treated with clomiphene citrate for a period of 21 days (as shown in plate 1) might be associated with a reduction in the expression of CD98 on the endometrial epithelium. CD98, a glycoprotein of type II, plays a vital role in the transportation of amino acids and hormones. It is found in the tissues of the ovary, placenta, and testes. It has a crucial impact on the ability of the endometrium to receive and support implantation. The clomiphene citrate group had reduced CD98 expression, which might lead to diminished transfer of hormones and amino acids in the endometrial tissue, potentially leading to failure of implantation (41-44).

CONCLUSION

The non-significant effect on reproductive outcome indicators in treated rats showed that *Cucurbita pepo* seed extracts did not enhance female wistar rat fertility. Despite its extensive usage in infertility therapy, clomiphene citrate did not yield positive results. Our findings suggest that future research should focus on the role of CD98 in implantation failure to identify routes and molecular connections. For subsequent investigations on the impact of *C.pepo* seed on reproductive outcome, treatment techniques must be refined, including larger dose and longer duration of exposure.

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None

Conflict of Interest

The authors report no 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, (45).

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