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Effect of ethanolic rind extract of *Citrullus lanatus* on hypothalamic-pituitary-gonadal axis structures, sexual behaviour and hormones of female Wistar rats

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Abstract
Background: Female sexual disorder is rarely considered, even thou females experience sexual dysfunction more than males.
Aim: Hence, this work was aimed at evaluating the effect of *Citrullus lanatus* ethanolic rind extract (CERE) on structures of hypothalamic-pituitary-gonadal (HPG) axis, hormones and sexual behaviour of adult female Wistar rats.
Methods: Female Wistar rats of 10 weeks' old were randomized into 4 groups (n=7). Groups I-III received oral saline (1 mL/kg), CERE (500 and 1000 mg/kg) daily for 14 days. Group VI received weekly hormonal treatment. Thereafter sexual behavioural test with sexually experienced male rats in unilevel pacing chamber was conducted. Female sexual behavioural parameters were scored. Blood was collected for hormonal assay. The animals were euthanized, thereafter ovaries, uterus, and hypothalamus were harvested for histological examination.
Results: CERE enhanced libido by increasing significantly (P<0.05) solicitation, hops and darts, time spent in the male compartment with corresponding decrease in the time spent in the female compartment relative to control group. Hormonal assay revealed significant increase in serum Luteinizing and Follicle Stimulating Hormone levels of CERE treated rats. Histological findings showed improved histo-architecture of the uterine, ovarian and hypothalamic sections.
Conclusion: CERE has aphrodisiac properties, therefore may be used for the treatment of women with hypoactive sexual desire.

Keywords: *Citrullus lanatus,* female sexual dysfunction, female sexual hormones, ovaries, uterus

Introduction
Female sexual dysfunction has been recognized as a medical disorder and unlike the male sexual disorder which has been largely researched [1], the female sexual disorder is rarely considered [1]; therefore, there is an urgent need to search for a natural remedy for this menace. Currently western remedies for female sexual dysfunction has progressed from hormonal therapy through pelvic physiotherapy and counselling to the use of sex toys and vestibulectomy in some cases [2]. In the less developed countries, the high cost of these treatments with the common traditional believe that females are not meant to enjoy sex has forced many patients to rely on medicinal herbs for treatments. Some of these sexual performance problems can be treated or managed to about 70% to 90%, with these natural substances called aphrodisiacs [3]. In Nigeria, one of the herbs that has been acclaimed to have sex enhancing potential is watermelon (*Citrullus lanatus).* It is grown for its edible fruit, which has a smooth hard green rind and a juicy, sweet flesh, usually deep red in colour.
The essence of marriage in humans is procreation and/or sexual fulfillment of both partners [4], but the repeated inability of couples to perform sexual activities effectively is one of the reasons most marriages fail. Ethnopharmacological relevance demonstrated that watermelon supplementation can improve aortic hemodynamics in patients with prehypertension, suggesting that watermelon has a potent vasodilator [5]. It has been reported to have tocolytic properties [6], and as such can be used to relax the smooth muscles of the copulatory organs. It has been established that *Citrullus lanatus* contains high amount of citrulline which is a precursor for nitric oxide synthase; that plays an important role in vasodilation [7]. During sexual arousal there is increased blood flow to the vagina, clitoris, vestibular bulbs and labial walls [8]. Genital vasocongestion and vaginal canal lubrication occurs during sexual arousal as a result of increased blood flow through the vaginal arteries which arise from the uterine,

hypogastric, hemorrhodial and clitoral vessels [8]. The increased blood flow causes engorgement of the vaginal wall which causes an increase in pressure that forces plasma out of the subepithelial vascular bed through the vaginal epithelium via intraepithelial spaces into the vaginal canal [8], thereby causing vaginal wetness.

Although several studies have established the aphrodisiac effect of *Citrullus lanatus* flesh, seed and rind extracts on adult male wistar rats [9-11] and sex enhancing propensity of *Citrullus lanatus* seed extract on female Wistar rats [12], there is lack of documented scientific literature on the sex enhancing property of the rind extract *Citrullus lanatus* on sexual behaviors of adult female Wistar rat.

Materials and Method

Plant material collection and identification

The fruits of *Citrullus lanatus* (watermelon) were collected from a local farm in Plateau State (Jos). The freshly collected *Citrullus lanatus* was identified at the Department of Plant Science and Biotechnology, University of Nigeria Nsukka.

Plant extraction and phytochemical analysis

The watermelon fruits were cleaned, the seeds removed, shade dried to constant weight and grinded into powder. Three hundred gram (300g) portion of the powder was extracted with 70% (v/v) ethanol in a Soxhlet apparatus for 24 h using a vacuum rotary evaporator, the filtrate was concentrated to complete dryness and finally stored in a refrigerator at 4^0C for the experiment. Phytochemical screening was carried out to qualitatively assess the phytoconstituents of the seed extract using the standard laboratory procedures of precipitation and coloration reaction as described by [13-15], to identify the secondary metabolites present.

Animal Procurement

Twenty-eight (28) female (160g-200g) and male (200g-250g) adult wistar rats of 12-13 weeks' old were used. They were kept in a well-ventilated house in a cage with optimum condition (temperature 23 ± 2^{0C} photoperiod; 12hours natural light/dark cycle). They were acclimatized for 2weeks. The animals were handled according to the guidelines for animal research in National Institute of Health guidelines for care and use of laboratory animals. The animals were fed growers mash manufactured by Top Feed Nigeria Limited and allowed water *ad libitum.*

Animal Grouping and Experimental Design

The female rats were randomly assigned into 4 groups of 7 animals each. The ethanolic seed extract was reconstituted and administered by oral gavage. The treatment of the animals and duration of the study was adopted from the method of Khadabadi and Bhajipale [16].

Table 1: Animal Grouping and Drug Administration

Groups (n=7)	Treatment	Dosage /Kg body weight
I (normal saline)	Normal saline	1mL
II (CERE Low dose)	Ethanolic rind extract	500mg
III (CERE High dose)	Ethanolic rind extract	1000mg
D IV positive control)	Ethinyl estradiol	100 µg/animal, orally. 48hrs to sexual behavioral test.
	Progesterone	500 µg/animal SC injection, 6hrs before the test.

SC = Subcutaneous, CERE = *Citrullus lanatus* ethanolic rind extract

Sexual behavior for the female rats

A unilevel pacing chamber (60 L × 40 W × 40 H cm) was used. The chamber was bisected by a transparent Plexiglas wall containing 2 small holes permitting the female to enter or exit the half of the cage in which the male was confined. The holes were too small for the male rats to pass through, thus allowing the female to control or "pace" the sexual interaction. A hormonally primed female rat was placed in one half of the chamber and a stimulus male was introduced into the other half after 5 minutes. Appetitive and consummatory behaviors were evaluated. For each test, the presence or absence of hops and darts displayed by the female was be noted. Solicitations was scored as a headwise orientation to the male, followed by an abrupt run away regardless of whether the female remained in the male's side of the chamber [17].

Scoring of Pacing Behaviour

Frequency of solicitation darts and hops, mount, intromission and ejaculatory were all noted.

Mean time spent in the male and female compartments

Finally, the lordosis quotient was scored as the total number of lordosis/total number of mounts (L/M ratio).

Whenever the female displayed kicking, boxing, or rolling over, she was considered to be resistive to the male's attempt to mount.

Preparation of male Rats used for mating

To become sexually experienced, male rats received 4 training test sessions (twice a week for 2 weeks) with non-experimental receptive females, i.e. the females used for the training of the males were not used for the rest of the study. Only males displaying at least 2 ejaculations during the 4 training test sessions were included in the final experiment. Immediately prior to the mating test with the experimental females, males were given brief access to fully receptive, non-experimental females (each given 100 µg Estrogen 48 h and 1mgP6h prior to the mating test to ensure full receptivity) [18]. Only males displaying active mounting behaviour were used with the experimental females.

Blood Collection and Hormone Assay

On the last day of the experiment, 1ml of blood was collected from each of the 24 rats. The blood samples were immediately placed in a centrifuge (Centurion Scientific Ltd., UK) and centrifuged for 5 minutes at 12,000 revolutions per minute. The serum was quickly decanted into a test tube and stored immediately in a chest freezer (Haier Electrical Appliances Inc., Philippines). The concentration of the serum testosterone, FSH and LH were determined shortly after using ELISA Kit.

Histological Study

Twenty- four hours after the last administration, the animals were euthanized by intra peritoneal injection of ketamine 50 mg/kg and xylazine 10 mg/kg. The brain, gonads and uterus were carefully dissected out, fixed in 10% formol saline for a maximum of 48 hours. Thereafter, was dehydrated through grades of alcohol, cleared with xylene, infiltrated and embedded in paraffin and then sectioned (5 µm) using a rotary

microtome. Histological staining with using hematoxylin/ eosin (uterus and ovary) was carried out to examine the general histo-architecture and integrity of the cells. While staining of the hypothalamus with crystal fast violet was done to examine the staining intensity of the Nissl substances. Sections were studied under the light microscope (Olympus Microscope CX41, Tokyo, Japan) at 40 and 100 magnifications. Slides of all the groups were studied and photographed.

Statistical analysis

SPSS version 22 was used for the statistical study. Data were expressed as mean ± standard deviation. Significant difference was analysed using one-way analysis of variance (ANOVA) and student's t-test. Differences were considered statistically significant at $P<0.05$.

Results

Result of phytochemical analysis

The phytochemical analysis of the ethanolic rind extracts of *Citrullus lanatus* revealed high presence of citrulline, lycopene and flavonoid, slight presences of Saponins and phenols and trace amount of alkaloid and steroids but tannins, steroids, cardiac glycoside and anthraquinone were not detected. (Table 4.1).

Table 2: Result of the qualitative analysis of the *Citrullus lanatus* rind extract

Phytochemical	*Citrullus lanatus* Rind
Saponin	++
Alkaloid	+
Flavonoid	+++
Tannin	-
Phenol	++
Cardiac Glycoside	-
Steroid	-
Anthraquinone	-
Citrulline	+++
Lycopene	+++

Key: - = Not detected ++ = Moderate concentration + = Low concentration +++ = High concentration

There were increased solicitation, hops/darts and lordosis; decreased mean time spent in the female cage in both 1st and 2nd weeks when compared to the negative control. (Table 3)

Table 3: Showing the result of the effects of rind extract of *Citrullus lanatus* on sexual behavior of adult female rats

Parameters	Duration	Group I (Normal saline)	Group II (CERE 500 mg/kg)	Group III Cere 100 mg/kg)	Group IV (ED & P; 100 and 500 µg/ µg/animal respectively)
No of Solicitation	WK 1	3.0± 0.7	3.5± 1.4	5.5±3.5	28.5± 27.6
	WK 2	8.5 ± 6.4	6.0± 4.2	9.0± 1.4	34.5 ± 2.6
No of Hops/darts	WK 1	3.0± 0.0	10.5± 9.1	10.5± 5.0	52.0 ± 5.7
	WK 2	13.0± 0.0	13.0± 1.4	23.0±1.4	61.5± 44.5
Mean time in Female Comp. (sec)	WK 1	149.5± 96.0	134.3±12	143.2± 1.7[a]	49.5± 34.7
	WK 2	135.3± 44.6	120± 13.1 [ab]	113.5±64.1[ab]	0.0 ± 0.0
Mean time in male comp. (sec)	WK 1	63.0± 52.0	73.7± 1.2	112±28.8	1512.7± 1.8
	WK 2	267.1±103.4	259.8± 55.5	286.5±201.5	1802.5± 3.5
Lordosis	WK 1	0.00	0.00	0.00	7.0± 4.9
	WK 2	0.00	0.00	3.0 ±10	24.0± 4.2
Lordosis Quotient	WK2	0.00	0.00	0.00	71.5±30.3
	WK 2	0.00	0.00	0.00	93± 9.1

Values expressed as mean ±SD, n=7, P value ≤ 0.05. **a**- significant when compared with negative control
b- Significant when compared with positive control. CERE = *Citrullus lanatus* ethanolic rind extract. ED = Ethinyl estradiol. P = Progesterone

At the end of the observatory period, Testosterone, FSH, and LH in the serum of the animals treated with the extract increased in levels in comparison with their respective control values. Significant differences ($P< 0.05$) were noted only in the FSH values. (Table 4)

Table 4: Showing the result of the effects of ethanolic rind extract of *Citrullus lanatus* on sexual hormone levels of female rats

Parameter	Group I (Normal saline)	Group II (CERE 500 mg/kg)	Group III CERE 100 mg/kg)	Group IV (ED & P; 100 and 500 µg/ µg/animal respectively)
Testosterone (ng/ml)	0.1± 0.0	0.2 ± 0.7	0.1± 0.0	0.6 ± 0.5
LH miu/ml	2.3 ± 0.7	6.6 ± 1.1	10.4 ± 0.6	12.5 ± 0.0
FSH miu/ml	4.9 ± 1.3	11.8± 2.8	20.8 ± 3.8 [a]	26.5 ± 3.5[a]

Values expressed as mean ±SD, n=7, P value ≤ 0.05. **a**- significant when compared with normal control. **b**- Significant when compared with negative control. CERE = *Citrullus lanatus* ethanolic rind extract. ED = Ethinyl estradiol. P = Progesterone

Result of the effect of rind extract of *Citrullus lanatus* on Histo-architecture uterus, ovaries and hypothalamus of female Wistar rats

Effect of *Citrullus lanatus* rind extract on the uterus of adult female Wistar rats

The uterine sections of rats in all the groups showed normal histological uterine features. All groups showed normal Uterine microanatomy of Endometrial stroma (St) Myometrium (M) cryptic glands (G) endometrial lumen (L) lined by a secretory epith (arrows), a perimetrium (P) and some dilated blood vessels (V). CERE (low and high doses) revealed more secretion filled uterine glands and thicker endometrial wall compared with the control group, while the hormone treated group had the best histoarchitecture as compared with other groups as it shows numerous secretion-filled cryptic glands and very thicker endometrial relative to other groups.

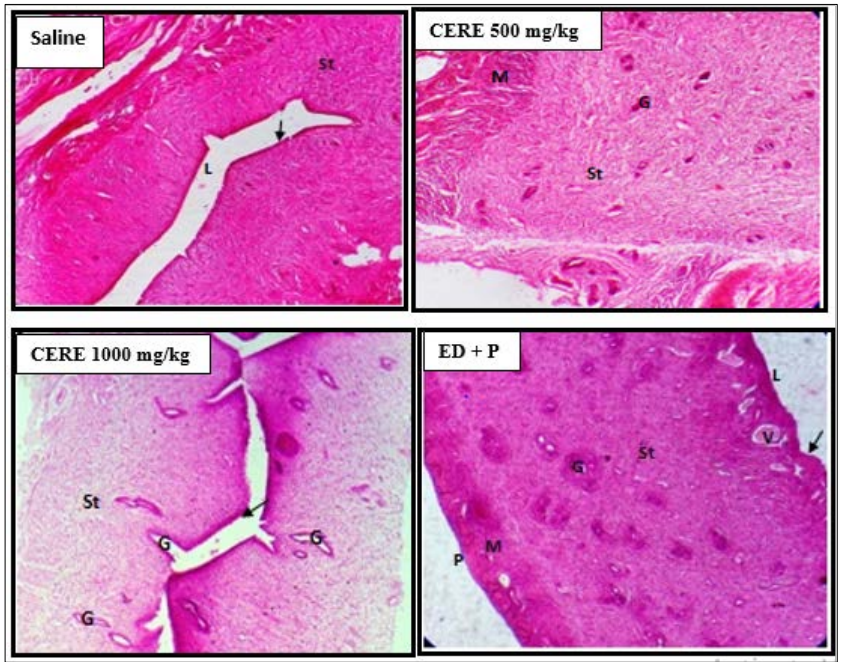

Fig 1: Photomicrograph of uterine section treated with normal saline, CERE (low and high doses) and hormone. St = Endometrial stroma, M = Myometrium, G = cryptic glands, L = endometrial lumen, arrows = Secretory epith, P = perimetrium, V = Dilated blood vessels, CERE = *Citrullus lanatus* ethanolic rind extract. ED = Ethinyl estradiol. P = Progesterone. H&E X400

Effect of *Citrullus lanatus* rind extract on the ovaries of adult female Wistar rats

The ovarian sections of rats in all the groups showed normal ovarian microanatomy with ovarian follicles (F) at different stages of development, embedded in the ovarian stroma (ST), in all the groups. The number of ovarian follicles (F) and large follicles (LF) seen in the CERE and hormone treated groups where higher relative to the control.

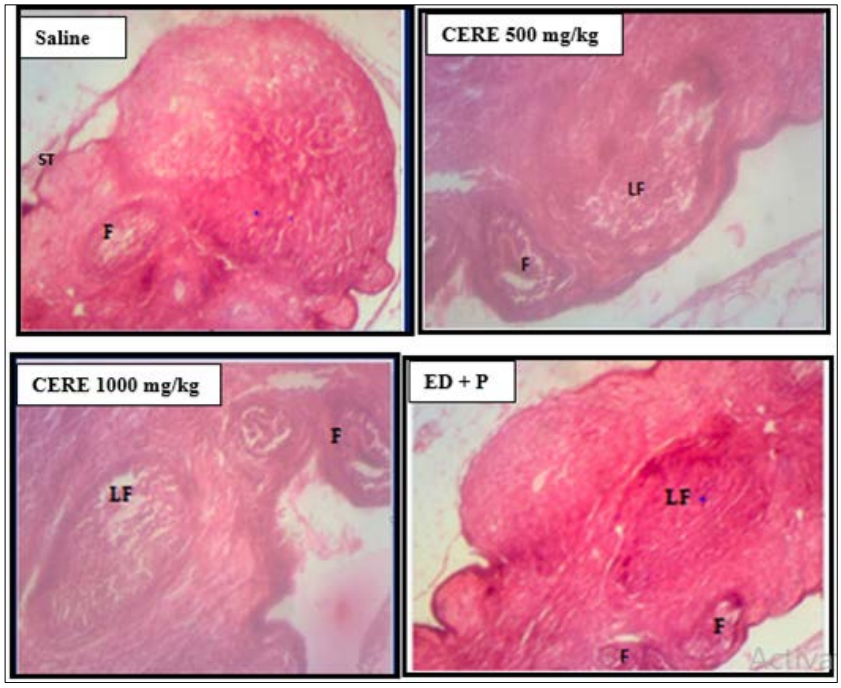

Fig 2: Photomicrograph of Ovarian Section Showing normal ovarian microanatomy with F = ovarian follicles, ST = ovarian stroma, LF = large follicle. CERE = *Citrullus lanatus* ethanolic rind extract. ED = Ethinyl estradiol. P = Progesterone. (H&E X400)

Effect of *Citrullus lanatus* rind extract on the hypothalamus of adult female Wistar rats

Neural tissue sections of the hypothalamus of the rats in all groups showed normal perikarya and neuroglia cells (P). However, there was slight increase in the stain intensity in the CERE treated groups indicative of increased neurosecretory activity when compared with the control group. The nissil staining of the high dose treated group was more intense compared with the low dose suggesting more neurosecretory activities in with increased dose. It also showed hypertrophic perikaya, without vacoulation (yellow arrow heads). The staining of the control group is less intense relative to the other groups. This is indicative of the less secretory activity. The nissil staring of the hormone treated group registered the most prominent staining intensity compared to the rest. It also showed few hypertrophic perikarya with prominent nucleoli (black arrows) and hyperchromatic perikarya, without cytoplasmic vacuolation (Yellow arrowheads).

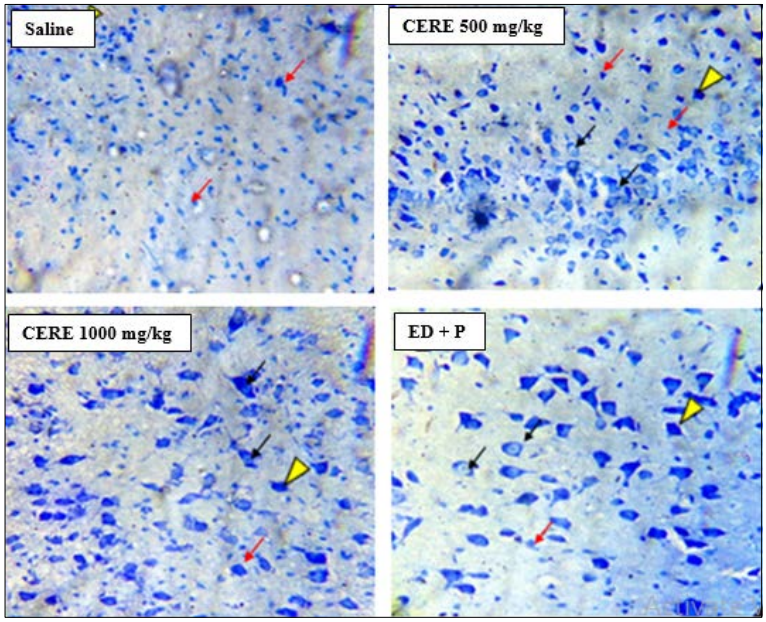

Fig 3: Photomicrograph of Neural tissue section of part of the paraventricular nucleus of the hypothalamus in Nissl staining. Perikarya and neuroglia cells = Red arrows, Prominent nucleoli = black arrows, hyperchromatic perikarya without cytoplasmic vacuolation (Yellow arrowheads), *Citrullus lanatus* ethanolic rind extract. ED = Ethinyl estradiol. P = Progesterone. CFV X400.

Discussion

In thisc study the phytochemical analysis of the ethanolic rind extracts of *Citrullus lanatus* revealed high presence of citrulline, lycopene and flavonoid; slight presences of Saponins and phenols; trace amount of alkaloid but complete absence of tannins, steroids, cardiac glycoside and anthraquinone. Similar result to this was observed by Oseni and Okoye [19]. The higher content of flavonoid, citrulline and lycopene in the rind extract could be the reason for the higher aphrodisiac activity observed in this study. These phytochemical contents of *Citrullus lanatus* rind extract may justify the claim for their remarkable therapeutic and aphrodisiac activities, and its wide usage in African Traditional Medicine. The result of the hormonal analysis of the female rats revealed an increase in the Luteinizing Hormone and Follicle Stimulating Hormone levels in the serum but surprisingly, there was no effect on serum testosterone level across the group. This showed that the extract had no effect on the testosterone level in all the rats. It could be an indication that GnRH-LH signalling was affected, because estrogen is converted to testosterone in females through this GnRH-LH signaling pathway [20].

Female sexual behavioural activity is separated into appetitive (proceptivity) and consummatory phases [21]. These sexual activities are similar to the arousal in women, which may be classified into genital arousal known as potency and psychological arousal known as libido or motivation [17]. According to Pfaus and Scepkowski [21], the psychological arousal in women could be very close to proceptivity. Solicitation, hops and darts are the indices of appetitive precopulatory sexual behaviors [21], while lordosis and lordosis quotient are the indices for consummatory sexual behavior. The female rat has to alert/arouse the males rats that she is ready for mating, and she does that through solicitation, hops and darts. No male initiates copulations with a lordotic (passively receptive) female, but a darting female provokes copulations in about 90% of the males [21].

In this experiment, an increase in appetitive sexual behavior was observed as evidenced by the high frequency in solicitations, hops and darts in the experimental groups when compared with the control group. The rats in the hormone treated rats were found to exhibit the highest frequencies in solicitations, hops and darts. Generally, elevated sex hormone level enhances sexual behavior in humans [17]; this correlates with the result of the hormonal assay in this work. This finding might have been brought about by the high serum FSH and LH observed in hormonal study. This in turn may be due to steroidal saponins content of *Citrullus lanatus*. The regulation of female sexual motivation in rats by ovarian hormones has been demonstrated previously in numerous experiments [17]. Nitric Oxide (NO) is considered to play an important role during the process of sexual excitement in females [22]. The high abundance of NO in the rind extract of *Citrullus lanatus* could have played a role in relaxing the smooth muscles of the female rat sexual organs [6], leading to increased blood flow; thus the enhanced appetitive sexual activity observed in this study. This observation correlates with Pfaus *et al.*, study on "Appetitive and Consummatory Sexual Behaviors of Female Rats in Bilevel Chamber" [23].

There was no effect on consummatory sexual behavior (lordosis and lordosis quotient) which was evidenced by the complete absence of lordosis in the CERE (low dose, 500mg/kg bw) and (high dose, 1000mg/kg bw) groups during the first week, but in the second week, few lordosis was observed in the rats that received high dose (1000mg/kg bw) of CERE. This effect was highest in the group administered with the hormones; estrogen and progesterone. Usually, when there is an appetitive sexual activity in rats without complementary consummatory activities, it implies that the hormonal factors that brought about the appetitive activity was not strong enough to bring about consummatory activitie [23]. In this study, standard drug was able to elicit both appetitive and consummatory activities, while the extract was able to bring about only the appetitive sexual behavioral activities. Similar result was previously reported [16, 24].

Female rats that are not sexually motivated spend less time in direct contact with the male (completely inside the male compartment), but spend more time in her compartment and in close proximity to (but out of reach of) the male (at the doorway of the apparatus, where she can easily escape into her own compartment as the male approaches) [24], and vice versa. In this study, the females spent more time in the doorway of the apparatus when they are not sexually motivated and excited, more time in their own compartment and less time with the male. In this study, there was an increase in time spent in the male compartments, time spent at

the doorway and a corresponding decrease in the time spent in the female compartment, in all the CERE treated groups. This shows that the rats in the hormone treated group were highly motivated sexually.

The histological examination of the ovarian sections of the female rats showed normal ovarian microanatomy with ovarian follicles (F) at different stages of development, embedded in the ovarian stroma (ST), but with fewer ovarian follicles when compared with the positive control group (D), which was found to have the highest number of ovarian follicles. The nissil stained neural section of the hypothalamus of the female rats in control group showed perikarya and neuroglia cells (Red arrows) in parts of the paraventricular nucleus of the hypothalamus. The normal group showed normal neurosecretory activity evidenced by the intensity of the nissil stain. Neural sections of the rats in the hormone treated group, increased neurosecretory activity evidenced by the very high intense of Nissl staining, when compared with the control group. This pattern of result observed in the female rat histological study gives credence to the high level of sexual activity found in the sexual behaviour of the rats in CERE and hormone treatment groups. This could be attributed to the effect of the phytochemicals found in the rind extract. The rind extract contains high amount of citrulline and lycopene. These two phytochemicals have been found to be essential in the production of nitric oxide (NO), which is integral in relaxing the smooth muscles of the blood vessel for proper blood flow to the heart and genital areas in both males and female [8], this in turn helps to maintain healthy sexual response. These phytochemicals could have contributed to the enhanced blood flow to the genital area, causing vasocongestion and wetting of the vagina, and ultimately lead to improved sexual responses seen in the treated rats in this study. The female's sexual behaviour is strongly dependent on peripheral gonadal steroids that have both peripheral and central nervous system (CNS) effects. Steroids act on the brain to induce sexual receptivity and all associated behaviours (proceptive, receptive and pacing behaviours).

Conclusion

From the findings in the study, it has been observed that *Citrullus lanatus* ethanolic rind extract has aphrodisiac properties therefore may be used for the treatment of women with sexual dysfunction, particularly hypoactive sexual desire (desire deficit). This effect was also observed to be dose dependent.

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