Original Research Article

Effect of Hydromethanolic Fruit Pulp Extract of *Azanza garckeana* (*Malvaceae*) on Sexual Behavior, Sex Hormones and Histology of Female Wistar rats

ABSTRACT

Aim: To evaluate the effect of hydromethanolic fruit pulp extract of *Azanza Garckeana*on sexual behavior, sex hormones and histology of female Wistar rats.

Study design: Twenty-five female Wistar rats and five (5) male Wistar rats were used for the study, the female animals were randomly divided into 5 groups (n= 5): Group I served as normal control and received 1ml/kg distilled water, group II served as positive control and received 100µg estradiol valerate and 50µg progesterone 48 hr and 5hr respectively before the sexual behavioral test, while group III, IV and V received 250mg/kg, 500mg/kg and 1000mg/kg body weight of the extracts respectively for 14days.

Place and Duration of study: Department of Human Physiology (animal house), Ahmadu Bello University, Zaria, between September 2021 and November 2021.

Methodology: Sexual behavioral tests were performed with sexually experienced male rats 1:1 after the treatment period in a unilevel pacing chamber and the process videotaped for proper scoring. Proceptive behaviors and Lordosis was extrapolated as index for the level of proceptivity and receptivity respectively in the female animals. The proximity time of the female to the male was also recorded. At the end of the experiment, the rats were anaesthetized with combined doses of ketamine and diazepam injection 0.4mg/kg. Blood sample was collected for hormonal assays which were done using ELISA kit. The ovaries were dissected out and fixed in 10% formalin for histological examination. Phytochemical screening was also carried out on the extract to determine the presence and absence of secondary metabolites.

Results: The hormonal assay result revealed significant ($p = .000$) increase in the serum Estradiol levels across the treatment groups but no significant change in progesterone level except at 500mg/kg dose where it was significantly increased when compared to both negative and positive control. Receptivity and proceptivity in the extract treated groups and positive control groups increased significantly when compared with the negative control group, indicating enhanced sexual behaviour. The proximity time also significantly increased ($p = .000$) in the extract treated groups when compared to negative control. The histological sections of ovaries revealed increased follicle development in the treated groups when compared to negative control. The presence of flavonoid and saponin in the fruit pulp extract could be the reason for the high aphrodisiac activity.

Conclusion: This study has demonstrated that *A. garckeana fruit extract* possesses significant aphrodisiac potentials in female Wistar rats.

Keywords: *Azanza garckeana, sexual behavior, histology, estrogen, progesterone, lordosis quotient, paracopulatory behavior*

1. INTRODUCTION

"The World Health Organization (WHO) defines sexual health as a state of physical, emotional, mental and social well-being in relation to sexuality, not merely the absence of disease, dysfunction or infirmity" [1]. "Good sexual health requires a positive and respectful approach to sexuality and sexual relationships, as well as having pleasurable and safe sexual experiences" [2]. "Sexual relationship is amongst the most important social and biological relationships in human life as it has proven to enhance an individual's quality of life and well-being" [3]. "Sexual and other physiologic functions diminish with advancing age" [4]. "Apart from aging people, healthy young people who are unable to carry out sexual act efficiently or entirely, normally suffer from psychogenic, organic, or mixed etiologies leading to sexual dysfunction" [5]. "Sexual dysfunction refers to difficulties that occur during the sexual response cycle that prevent one from experiencing satisfaction from sexual activity" [6]. Female sexual dysfunction (FSD) may manifest as apparently unrelated emotional disorders that could degrade one's quality of life and destroy relationships, amongst family, social sphere and even the workplace [7], these problems make some individuals resort to using sexual enhancing substances.

Over a long period of time, people have devised ways to increase sexual desire and improve sexual health. This has led to the discovery and use of substances called aphrodisiacs [8]. The term aphrodisiac has been used for substances that enhance sexual activity and are helpful in treating sexual disorders [9]. Aphrodisiacs are defined as drugs, food, scents or devices that can arouse or increase sexual drive or boost libido, most aphrodisiacs carry out their function either by altering the level of specific neurotransmitters or specific sex hormones such as progesterone, estrogen and testosterone in the body [10]. "The use of plants or their products to treat sexual disorders or improve on sexual performance has a long history in most countries, and their investigations in animals have proven that they are effective in improving sexual desire and sexual behavior in animals" [11]. "*Azanza garckeana* plant locally called Goron tula (Hausa, Nigeria), variously it is called snot appel (Afrikaans), chinga, mukole (Bemba), azanza, tree hibiscus, snot apple, quarters, wild hibiscus, African chewing gum (English), muneko (Lozi), mukole (Lunda), uxhakuxhaku (Ndebele), mkole (Nyanja), mutohwe (Shona), mtobo (Swahili), muneko (Tongan) and morajwa (Tswana)" [12]. "*Azanza garckeana* grows naturally in semi-arid areas" [13], "in

Nigeria *Azanza garckeana* is found abundantly in Tula, a town in Kaltungo Local Government Area of Gombe State, Nigeria. It is also found around Kankiya, Katsina State and Daggish Kali hills of Zah district, Michika local government area of Adamawa State" [14].

"Among the Tula people of North-Eastern Nigeria, ripened fruits are consumed as an aphrodisiac and for treatment of infertility in men. Other parts of the plants have been formidably studied with numerous reports of isolated compounds" [15], "in Zimbabwe, an infusion of the roots is dropped into the ear to treat earache or orally as an antiemetic or to treat cough, chest pain, menstruation and in large doses as an abortifacient" [16,17]. "A tea of the stems and leaves is taken to treat liver problems. A poultice of the pounded fruit is applied to abscesses to sufficiently thin out inflamed tissue encompassing the infected cavity or draw pus to a head so the abscess may rupture" [18]. "The fruit of this important ethno-medicinal plant has not been previously investigated with view of isolating its medicinal principles" [19]. This study aimed at investigating the effect of *A. garckeana* fruit extract on sexual behavior (paracopulatory behaviors and lordosis quotient), sex hormone levels (Estradiol and progesterone) and histology of female Wistar rats.

2. MATERIALS AND METHODS

Experimental Animals

A total of thirty seven(37) female Wistar rats age between 2-3 months (110-140 g) and five (5) sexually experienced male Wistar rats (200-300 g) were used for the research. Twenty five out of the thirty seven female Wistar rats were used for the main study and the remaining twelve for acute toxicity study. The animals were obtained from animal house of Physiology department, Ahmadu Bello University (ABU), Zaria. The animals were housed in plastic cages and kept at room temperature, they were fed with standard animal feed and water *ad libitum*. The study was carried out in accordance with the principles of laboratory animal care and standard experimental procedure.

Fruit pulp sourcing and extraction

Fresh fruit of *Azanza garckeana* was sought from Kaltungo local government of Gombe state and confirmed taxonomically in the Department of Botany, A.B.U Zaria.Voucher number ABU07276. The fruit pulp was air dried after removal of the seeds and pulverized. The pulverized sample was macerated in 70% aqueous methanol and allowed to stand for 72 hours and then filtered. The resulting filtrate was concentrated using rotary evaporator and evaporated to dryness on a water bath at 50°C.The dried extract was stored properly in a container before further experiments.

$$\%\text{yield} = \frac{\text{weight of dry extract (g)}}{\text{weight of dry fruit (g)}} \times 100\% \quad \text{yield} = \frac{278.5}{1000} x\ 100 = 27.8\%$$

Preliminary phytochemical screening tests.

Phytochemical screenings of *A. garckeana* was carried out to detect the presence and absence of secondary metabolites using procedures described by Trease and Evans [20].

Methods

Determination of the Estrous Phase

The phases of the estrous were determined using vaginal smear according to the method of Long and Evans [21]. This was done based on cytology of vaginal smears obtained daily in the morning (9 – 10am) and viewed under the microscope as shown in figure 1. Vaginal secretions were collected using a 1ml syringe filled with 0.02ml of normal saline (0.9% NaCl) by inserting the tip into the rat vagina but not deeply. The vaginal fluid was placed on glass slides. A different glass slide for each cage, one drop was collected with a clean tip from each rat and viewed unstained under a light microscope with 10 and 40 x objective lenses. Rats in their estrous phases were used for the experiment.

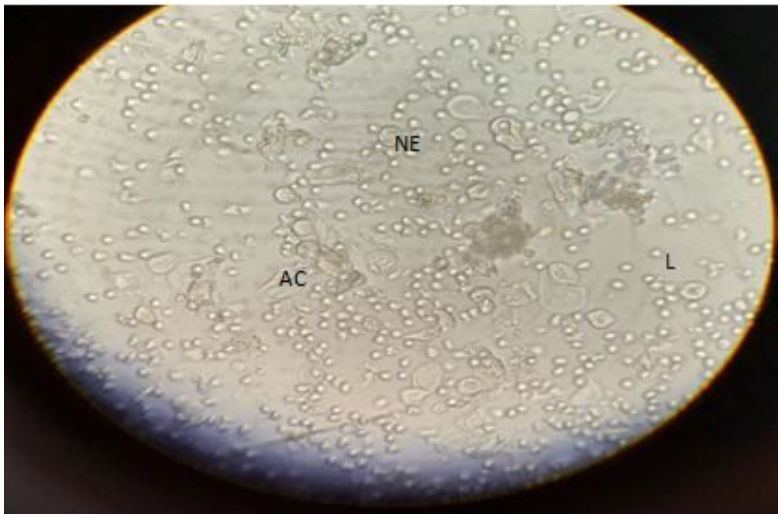


Figure 1: Vaginal secretion viewed with x40 magnification showing all cell types indicating Metestrus. NE= nucleated epithelial cells AC= anucleated cornified cell L= leucocytes.

Experimental Design

Twenty-five (25) adult female Wistar rats were randomly divided into 5 groups of five (5) animals each (n = 5) as shown in Table 1.

Table 1: Experimental design

Groups (n=5)	Treatment	Dosage/ Kg body weight
I	Distilled water	1ml
II (positive control) [22]	Estradiol valerate	100µg 48hours before testing orally
	Progesterone	50µg five (5) hours before testing orally
III	Fruit pulp extract	250mg
IV	Fruit pulp extract	500mg
V	Fruit pulp extract	1000mg

The five sexually experienced male animals were used for the sexual behavior study using 5: 1 ratio.

Assessment of Sexual Behavior

A unilevel pacing chamber (60 L × 40 W × 40 H cm) was used. The chamber was bisected by a transparent Plexi-glass wall containing 3 small holes permitting the female to enter or exit the half of the cage in which the male was confined (Figure 2). The holes were too small for the male rats to pass through, thus allowing the female to control or "pace" the sexual interaction. During the first two weeks of the laboratory work the animals (female and male) were habituated separately to the pacing chamber twice a week for two weeks. To become sexually experienced, male rats received 4 training test sessions (twice a week for 2weeks) with non-experimental receptive females. On the day of the study, experimental female rat (in their estrous phase) were placed in one half of the chamber and a stimulus male was introduced into the other half after 5 minutes. Appetitive and consummative behaviors were evaluated for a period of 30 minutes per female animal. All frequencies of hopping, darting, and ear wiggling during the test was added and divided by the length of the test to establish a standard measure of proceptive behavior [23]. Lordosis quotient was calculated by dividing the number of lordosis responses by the total number of mounts and multiplied by 100.

$$Lq = \frac{NL}{Nm} \times 100$$

Lq: lordosis quotient NL: number of lordosis response Nm: total number of mounts

Figure 2: Copulatory unilevel pacing chamber.

Assessment of Hormonal levels

After oral administration of the extract for fourteen (14) days, rats were anaesthetized with combined doses of ketamine and diazepam injection 0.4mg/kg given intra-peritoneally. Each rat was dissected to expose the heart and blood samples were collected in plain bottles by cardiac puncture. The blood sample was centrifuged and the serum obtained was used for hormonal analyses [24]. The serum level of estradiol and progesterone was analyzed using enzyme linked immunosorbent assay (Elisa) kits by the method of Tietz [25] following the manufacturer's manual. The principle of the immunoassays follows the typical competitive binding scenario. Competition occurs between unlabeled hormone (present in standards, controls and samples) and an enzyme-labelled hormone (conjugate) for a limited number of antibody binding sites on the microwell plate.

Histological studies

At the end of the behavioral study, the ovarieswere carefully dissected out and was fixed in 10% formalin and processed for embedding. The ovarian tissue was carefully sectioned using a rotary microtome, stained and the histological architecture examined [26].

2.1 Statistical Analysis

The data obtained from the study is expressed as mean ± Standard error of mean (SEM). The significant differences among the groups were assessed using one-way analysis of variance (ANOVA) followed by Turkey's *post hoc* test. *P* values < 0.05 was considered statistically significant. All data were analyzed using IBM SPSS Version 23.0.

3. RESULTS

3.1. The phytochemical constituent detected in *A. garckeana* fruit extract are shown in table 2.

Table 2: Phytochemical screening results for hydromethanolic fruit pulp extract of *A. garckeana*

S/N	Phytoconstituents	Test	Inferences
1	Alkaloids	Dragendorff test	
2	Cardiac Glycosides	Keller-Kiliani test	+
3	Saponins	Frothing test	+
4	Phenolic compounds	Lead acetate test	+
5	Tannins	Ferric Chloride test	+
6	Steroids	Salkowiski test	+
7	Carbohydrates	Molisch test	+
8	Flavonoids	Shinoda test	+
9	Terpeniods	Liebermann Burchard test	+
10	Anthraquinones	Bontragers test	

Keys: + PRESENT

3.2:Serum estradiol and progesterone hormone levels in female Wistar rats treated with *A. garckeana* fruit extract.

There was no significant difference noted in the level of estradiol hormone between the three doses of the extract. However, a significant increase (*p =0.000*) was observed in the groups administered with the standard drug (positive control), 250mg/kg extract, 500mg/kg extract and 1000mg/kg extract when compared to the control group that received distilled water. Although the rise in the level of estrogen at the higher dose of 1000mg/kg is not up to that of the lower doses of 250mg/kg and 500mg/kg of the extract (table 3). The level of estradiol in the groups treated with the extract was not significantly different from that of the positive control group (table 3).

There was no significant difference in progesterone hormone levels in the groups administered 250mg/kg and 1000mg/kg of the extract when compared to both positive and negative control groups. However, there was a significant increase *p =0.000* in the group administered 500mg/kg extract when compared to the control group and the positive control as shown in table 3.

Table 3: Effect of ***A. garckeana*** **fruit extract** on the serum level of estradiol and progesterone hormone in female Wistar rats

Groups	Estradiol level	Progesterone level
Negative control	41.80±6.61[a]	8.92±0.68 [a]
Positive control	108.40±3.91[b]	7.86±0.93 [a]
250mg/kg Extract	104.40±2.30[b]	7.54±0.34[a]
500mg/kg Extract	112.40±4.72[b]	11.50±0.39[b]
1000mg/kg Extract	70.00±1.58[b]	6.86±0.38[a]

Data represented mean ± SEM, values with different super superscript letters [a,b] = $p< 0.05$ show the level of significance between the groups. Positive control= Combined estrogen and progesterone (Estradiol valerate 100µg and progesterone 50µg).

3.3: Effect *A. garckeana* fruit extract on the proceptive (soliciting) behavior of female Wistar rats

A significant (*p =0.000*) increase in the proceptive behavior was observed in the groups administered the standard drug, 250mg/kg extract, 500mg/kg extract and 1000mg/kg dose of extract when compared to the negative control group that received distilled water (figure 3). The level of proceptivity amongst all the treated groups including the positive control was not statistically different.However, administration of 250mg/kg extract resulted in a higher level of proceptive behavior when compared to the positive control that received the standard drug as show in figure 3.

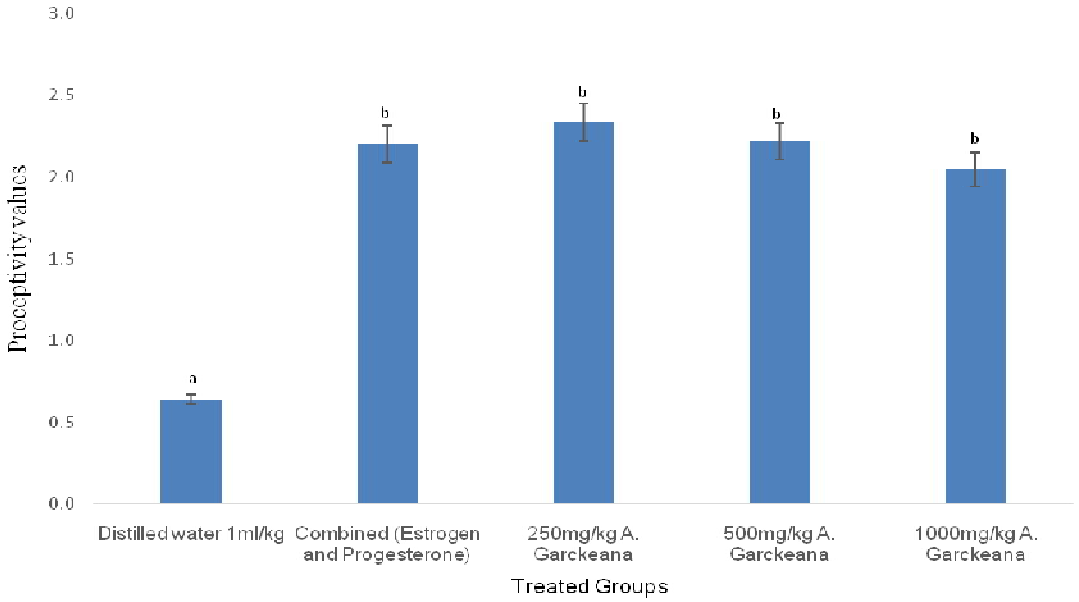


Figure 3: Effect of *A. garckeana* fruit extract on proceptive behavior of female Wistar rats.

Data represented mean ± SEM, values with error bars having different super superscript letters a,b = $p < 0.05$ are significantly different. Combined estrogen and progesterone (Estradiol valerate 100µg and progesterone 50µg).

3.4: Effect of *A. garckeana* fruit extract on the receptivity of female Wistar rats using the lordosis quotient as the measure of evaluation.

A significant ($p = 0.000$) increase in the receptivity was observed in the group administered 1000mg/kg dose of *A. garckeana* when compared to the normal control, positive control and 250mg/kg *A. garckeana* extract treated group respectively (figure 4). A significant ($p = 0.000$) increase in the receptivity was also observed in the 250mg/kg and 500mg/kg extract treated group when compared with the positive and normal control (figure 4).

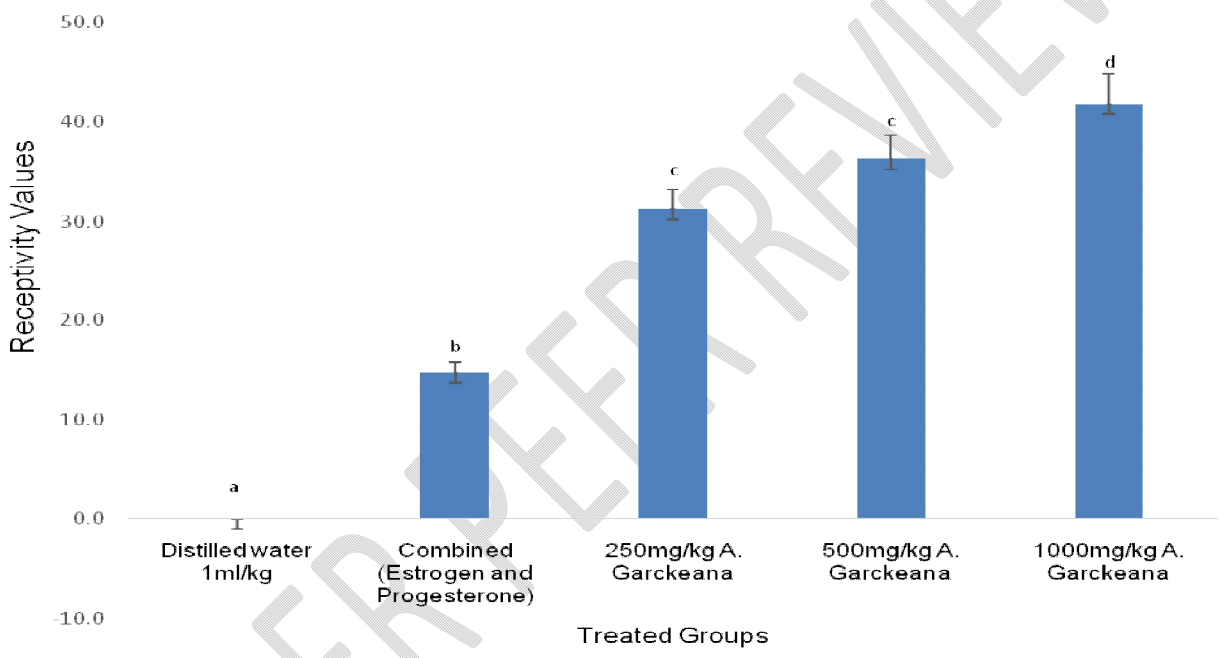


Figure 4: Effect of ***A. garckeana*** **fruit extract** on receptive behavior of female Wistar rats. Data represented as mean ± SEM, values with error bars having different super superscript letters a,b,c,d = $p < 0.05$ are significantly different.

3.5: Effect of *A. garckeana* fruit extract on the time spent in the male chambers by the female Wistar rats (proximity time).

The result obtained showed an increase in time spent in the male chamber in the groups administered the standard drug, 250 mg/kg, 500 mg/kg and 1000mg/kg dose of extract when compared to the negative (normal) control group that received distilled water as shown in table 4. The time spent increased significantly in the 250 mg/kg and 1000 mg/kg dose of the extract when compared to positive control and normal control, with the increase at the 1000 mg/kg dose significantly higher than the dose of 250mg/kgof the extract (Table 4).

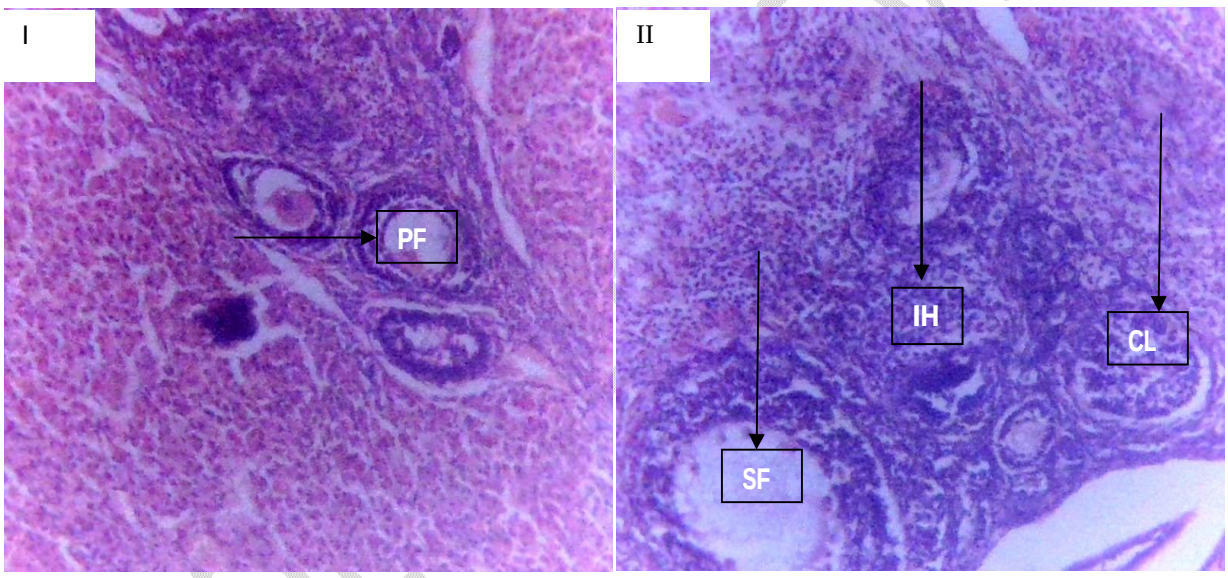
Table 4: Effect of *A. garckeana* fruit extract on the time spent in the male chambers by the female Wistar rats

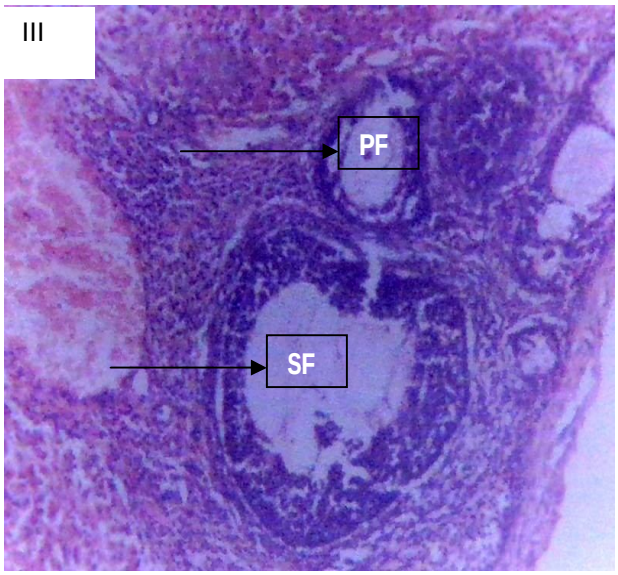
Groups	Time spent in male chamber (Proximity time)
Negative control	171.60±4.72[a]
Positive control	271.80±41.35[b]
250mg/kg Extract	318.80±10.52[c]
500mg/kg Extract	202.40±4.39[a]
1000mg/kg Extract	357.60±6.58[d]

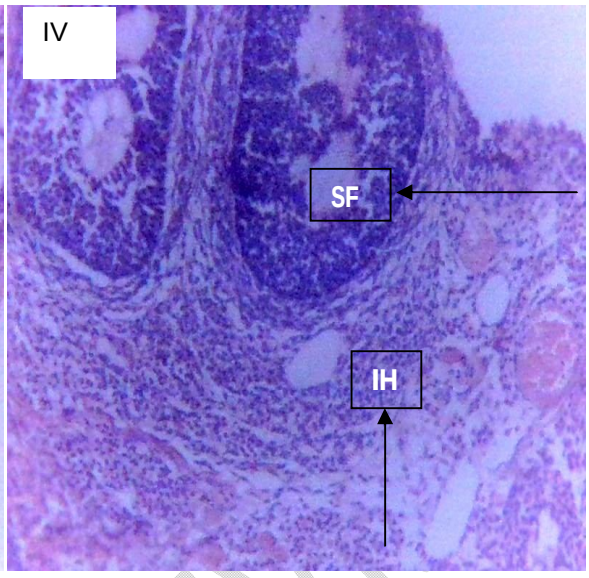
Data represented mean ± SEM, values with different super superscript letters a,b,c,d = $p < 0.05$ show the level of significance between the groups. Positive control= Combined estrogen and progesterone (Estradiol valerate 100µg and progesterone 50µg).

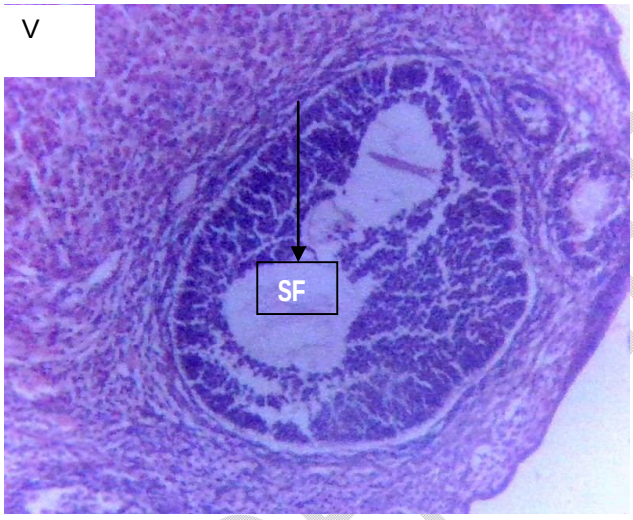
3.6: Effect of *A. garckeana* fruit extract on histo-architecture of the ovary in female Wistar rats

Plate I showed slight hyperplasia of the inflammatory cells (IH) i.e cumulus oophurus, 65% primary follicle (PF), 20% secondary follicle(SF) and 15% corpus luteum (CL) in the negative control group, Plate II showed slight IH, 40% PF, 35% SF and 15% CL in the positive control group, the group administered with 250mg/kg dose of extract showed no hyperplasia of the inflammatory cells, 50% PF, 30% SF and 20% CL as shown on plate III, the 500mg/kg dose of extract group showed normal ovary with 60% PF, 25% SF and 25% CL on plate IV. The 1000mg/kg dose of extract group result showed a normal ovary with 55% PF, 35% SF and 10% CL as shown on plate V. The percentages signify the number of a type of cell compared to the total number of all the cells present in the ovary.









Photomicrograph sections of the ovary using H & E X250 magnification

Plate I: Normal control

Plate II: Positive control (combined estrogen and progesterone)

Plate III: 250mg/kg extract treated group

Plate IV: 500mg/kg extract treated group

Plate V: 1000mg/kg extract treated group

IH: hyperplasia of the inflammatory cells. SF: Secondary follicle. PF: primary follicle. CL: corpus luteum

4. DISCUSSION

The phytochemical analysis of the *A. garckeana* fruit extract revealed the presence of cardiac glycosides, saponins, phenolic compounds, tannins, steroids, carbohydrates, flavonoids and terpeniods. Similar result to this was reported by Ochokwu *et al*., [18] but anthraquinones and alkaloids were not detected in this study as in contrast with the stated research.

The results on the effect of *A. garckeana* fruit extract on the level of estradiol obtained from this study revealed that the level of estradiol in the groups treated with the extract was not significantly different from that of the positive control group, however it showed a significant increase in the level of estradiol in the three doses of the extract tested when compared to normal control. The significant increase in estradiol may be as a result of increase in gonadotropin released by direct stimulation of accessory sex organs of the female Wistar rats by the male Wistar rats. Estrogen is known to increase sexual desire [27], and "it also acts in a feedback mechanism, influencing the production of follicle stimulating hormones (FSH) from the pituitary gland. FSH in turn promotes the development of the immature ovarian follicles, which increases the production of estrogen from the ovary" [28]. Increase in estradiol levels could also be related to the saponin present in the extract which is believed to be an estrogen precursor [29].

The results on the effect of *A. garckeana* fruit extract on the level of progesterone obtained from this study revealed a decrease in the level of progesterone in the groups administered 250mg/kg and 1000mg/kg doses of the extract compared to the normal control while there was a significant increase at the dose of 500mg/kg as compared to control, making the 500mg/kg dose more potent for increasing the level of progesterone hormone. However, the decrease at 250mg/kg and 1000mg/kg could be as a result of inhibition of luteinizing hormone of the anterior pituitary by the high level of estrogen in the extract treated groups. The increase in progesterone level observed at 500mg/kg at estrus phase could be due to potent positive feedback mechanism on the pituitary to release gonadotropic hormones which stimulate granulosa cells to increase production of progesterone. It could also be as a result of an increase in follicular numbers observed at that dose from the histological study in this work.

The results on the effect of *A. garckeana* fruit extract on proceptive (paracopulatory) behavior shows a significant increase in the level of proceptive behavior in the positive control, 250mg/kg, 500mg/kg and 1000mg/kg treated group when compared to the normal control group. The level of proceptive behavior noted in the three extract treated groups as well as the positive control was not significantly different. Generally, elevated sex hormone level enhances sexual behavior in humans [30]. "This finding maybe as a result of the increase in the level of estrogen in the groups treated with the extract as compared to control obtained in this study. This in turn may be due to the steroidal saponin content of *A. garckeana,* the regulation of female sexual motivation in rats by ovarian hormones has been demonstrated previously in numerous experiments" [30].

The results obtained from this study on the effect of *A. garckeana* extract on the female rat receptivity revealed a significant dose-dependent increase in receptivity in the three doses of the extract when compared to both negative and positive control groups, indicating that 1000mg/kg dose of the extract resulted in the highest level of sexual receptivity. This might be as a result of the estrogenic property or presence of phytochemical constituents (flavonoids and saponin) in the extract.

The time spent in the male chamber by the female rat increased significantly in the groups administered with 250mg/kg and 1000mg/kg doses of the extract when compared to normal and positive control. This is as a result of the high level of the sex steroid hormone estrogen which increased the ability of the female rat to stay in close proximity to the male rats and subsequently promote the copulatory act. The increase in the time spent at 500mg/kg dose of the extract when compared to normal control was not statistically significant, this could be related to the increased progesterone hormone level noted in this group.

"The presence of flavonoid and saponin in the fruit extract could be the reason for the high aphrodisiac activity i.e increased sexual behavior and sex hormone levels observed in the extract. Steroidal saponins have been recorded to play a role in enhancing sexual behavior by either binding to hormone receptors, which may result in conformational change that will enhance the physiological function of the hormones or bind to enzymes that are involved in the synthesis of such hormones and thus enhance its production" [31]. Saponins foundin the leaf *Tribulis terrestris* L. have been used as an aphrodisiac agent in rats [32].

These phytochemical contents of *A. garckeana* may justify the claim for their remarkable aphrodisiac activities, and its usage in treatment of diseases.

The results from the histological studies on the effect of *A. garckeana* fruit extract on the histo-achitecture of the ovary showed an increase in the proliferation and growth of the ovarian follicle from primary follicle to secondary follicle then subsequently to corpus luteum (shown by the percentages of each type of follicle) in all three doses of the extract when compared to control. This corroborates with the significant increase in estrogen level in the extract treated groups when compared to control. Estrogen acts in a feedback mechanism where it influences the production of follicle stimulating hormones (FSH) from the pituitary gland. FSH in turn promotes the development of the immature ovarian follicles which increases the production of estrogen from the ovary [28].

CONCLUSION

This study has demonstrated that *A. garckeana* fruit extract possesses significant aphrodisiac potentials in female Wistar rats evidenced by the increased sexual behavior, sex hormone levels, histological changes in the ovaries, and increased proximity time of the extract treated animals.

ETHICAL APPROVAL

Ethical approval was sought and gotten from A.B.U committee on animal use and care with the approval number ABUCAUC/2021/121.The study was carried out in accordance with the principles of laboratory animal care and standard experimental procedure.

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