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Original Research Article

The Effect of Ethanolic Extract of *"Laurus nobilis"* (Bay Leaves) on the Reproductive Characteristics of Male Wistar Rats

Victor, P. D[1*], Krukru, E. E[1], Okpara, P. E[2], Ajie, P. C[1], Reuben, E[2], Amadi-Ikpa, H. A[1], Wami-Amadi, C. F[2], Otto, B. J[2], Dan-Jumbo, D[2], Nkpurukwe, C. I[2]

[1]Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Nigeria
[2]Department of Human Physiology, Faculty of Basic Medical Sciences, College of Medical Sciences, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Nigeria

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***Corresponding author:** Victor, P. D
Department of Human Anatomy, Faculty of Basic Medical Sciences, College of Medical Sciences, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Nigeria

Abstract

Background of the study: Erectile dysfunction may be responsible for a reasonable amount of male infertility. Erectile dysfunction can lead to male infertility. Male infertility is due to any problem in the male reproductive system including decreased sexual desire, barrenness, premature ejaculation, etc. ***Method***: 20 male Wistar rats and 5 female Wistar rats were used for this study. Male animals were grouped into control, Viagra, high-dose Bay leaves, and low-dose Bay leaves. The extract was administered for 21 days. The animals were acclimatized for one week. Extracts were administered for three weeks. A sexual behavioural study was carried out on the animals in the dark for three days. 24 hours after the last day of the sexual behavioural study, the animals were sacrificed and the testes were harvested and sent for histological studies; semen was collected for semen analysis. ***Results***: Data were expressed as mean ± SEM. The mean difference between the tested groups and the control was tested using a one-way ANOVA. Values were considered statistically significant when P is ≤ 0.05. Mount frequency increased significantly in high-dose groups of both premature plant and bay leaves treated animals. ***Conclusion***: This implies that the plant extract used for the present study increases sexual function (libido and potency).

Keywords: Infertility, Semen analysis, testes, Histological studies.

INTRODUCTION

Male sexual dysfunction is of varied etiologies and this includes personal lifestyles (chronic alcohol abuse, cigarette smoking), androgen deficiency, aging population, psychological disorders, side effects-hypertensive, central agents, psychiatric medications, antiulcer, antidepressants and anti-androgens, and chronic medical conditions like diabetes, hypertension, and pulmonary cancer (Yakubu *et al*., 2007). Erectile dysfunction has been estimated to affect about 150 million men worldwide- wide and its prevalence increased with age (Aytac *et al*., 2008). It is estimated that Nigeria has about 12 million infertile persons (Giwa-Osagie *et al*., 1990). Erectile dysfunction may be responsible for a reasonable amount of male infertility. Erectile dysfunction can lead to male infertility. Male infertility is due to any problem in the male reproductive system including decreased sexual desire, barrenness, premature ejaculation, etc (Kothari, 1994).

Infertility and problems of impaired fecundity have been a concern through ages and are also significant clinical problems today which affect 8-12% of couples worldwide. Of all infertility cases, approximately 50% are due to male factors (WHO, 2018). Infertility is a condition with psychological, economic, and medical implications (WHO 2018). The problem of infertility in both males and females leads to inability to procreate this could result from any of the following: diabetes, eating disorders including anorexia, nervosa and bulimia, excessive alcohol use, exposure to environmental toxins such as pesticides, over-exercising, radiation therapy or other cancer treatments. Many other factors such as infection, genetic disorders,

and environment can also cause infertility (Ejikeman, 2002). Infertility has significant negative social impacts on the lives of infertile couples and this had led to violence, divorce, social stigma, emotional stress, depression, anxiety, and low self-esteem.

Herbal healers in the West Bank area of Palestine have a wide range of herbal remedies used in cases of infertility in males and females. Unfortunately, most of them lack scientific evidence of pharmacological or toxicological nature. Some other native plants such as black cohosh had been used but it has side effects such as nausea, headache, rash, etc. Also, agnus castus had been used and it also has side effects such as nausea and headache. Plants such as Shatavari (asparagus) had been used also but have side effects such as fast heart rates, itchy skin, and dizziness. Hence, the need to get plant extracts capable of treating infertility with minimal or no side effects.

METHODS AND MATERIALS

MATERIALS

25 adult male Wistar rats, 18 mice, 5 female Wistar rats, bay leaves, ethanol, xylene, tissue paper, filter paper, a packet of a clean microscope slide, water, oral catheter, aluminum foil, test tube, tab water, cotton wool, dissection board, laboratory coats, 2 beakers, disposable gloves, stopwatch, weighing balance, 5 liters of distilled water, rat cage with gauge, bags of growers feed and sodium hydrogen salt.

METHODS

Experimental Location

The experiment was carried out in the animal house of the department of human anatomy, faculty of basic medical sciences, Rivers State University.

Type and duration of the study

It was an experimental study. The study was carried out from October 2022 to December 2022.

Experimental protocol

25 adult male rays weighing 160-180g were obtained from the animal house unit department of Pharmacology, Faculty of Basic Clinical Science: Rivers State University, Port Harcourt. They were housed in wire cages with wire gauze in a well-ventilated house with 12 hours of natural light and 12 hours of darkness. The animals were provided with commercial feed and clean water. They were allowed to acclimatize for two weeks before the commencement of the experiment.

Plant Material

The leaves of *laurel nobilis* (bay leaf) were brought from the market and taken to the department of plant science and biotechnology for identification and authentication. A voucher specimen was deposited in the department.

Extraction of Bay leaf (Laurus nobilis) by Cold Maceration

The ground leaf sample was weighed, and about 5000g of the ground sample was measured and soaked in 5 litres of absolute ethanol for 48 hours with intermittent agitation and gyration, a process known as cold maceration. After 48 hours the solution was filtered twice; first with a chess cloth to remove large particles and then with filter paper to remove finely ground particles. The collected filtrate (extract) was concentrated using a thermo-regulated water bath at mild temperatures (from 35 - 40 °C) until complete dryness was achieved, leaving a paste (crude extract).

LD50 Studies

The LD50 studies were determined using Lorke's method. This method involves two phases.

Phase 1

9 mice were used for the present study. Animals were grouped into 3 groups with each group consisting of 3 animals. Group 1 received 500mg/kg, group 2 received 1000mg/kg, and group 3 received 2000mg/kg of extract. After two hours if no death is recorded, phase 2 will be carried out.

Phase 2

Nine mice were used; animals were grouped into three groups each containing three animals. Group 4 received 3000mg/kg, group 5 received 4000mg/kg, and group 6 received 5000mg/kg of extract. The animals were observed for 24 hours.

Experimental design/ setup

20 male Wistar rats and 5 female Wistar rats were used for this study. Male animals were grouped into control, Viagra, high-dose Bay leaves, and low-dose Bay leaves. The extract was administered for 21 days. The animals were acclimatized for one week. Extracts were administered for three weeks. A sexual behavioural study was carried out on the animals in the dark for three days. 24 hours after the last day of the sexual behavioural study.

Animal Grouping/Administration

Group 1- Normal saline (0.5 ml)
Group 2- Viagra
Group 3- High dose bay leaves (1500mg/kg)
Group 4- Low dose bay leaves (500mg/kg)

Procedure for Sexual Behavior Test

STEP l

Female Wistar rats were brought to heat (oestrus phase) using the method described by Szechtman *et al*., (1981). Female rats allow mating only during the oestrus phase. 500mg/kg body weight of estradiol valerate suspension was administered orally to the female Wistar rats 48 hours prior to the pairing and 5mg/kg body weight of progesterone were injected

subcutaneously 6 hours before the commencement of the experiment.

STEP II

Sexual behavioral test was carried out 2-3 hours daily for three days before the end of the experiment. This test was carried out in the dark under dim red light because Wistar rats are most active at night.

The receptivity of the female Wistar rats was confirmed by exposing the rats to the male rats before the commencement of the experiment. Only 10 most receptive female rats will be used. The receptive females firmly raise their hind limb quarters and tails to accept male sexual advances.

STEP III

The male Wistar rats were introduced into different cages and allowed to acclimatize for 10 minutes. One receptive female Wistar rat was introduced gently into the same cage (1 male: 1 female). Females that failed to accept males were substituted with another artificially warmed female. The test was terminated if the male doesn't show sexual interest. The observation of male sexual behavior commences immediately after the female is introduced into the cage containing the male. Male sexual behaviors were recorded on a digital camera. The frequencies were observed carefully and recorded manually with the aid of a stopwatch.

STEP IV

The following sexual behaviors were observed and recorded. These behaviours include:

Mount frequency(MF)

This is defined as the number of times the male rat attempts to lift up its forelimbs and body over the hind quarter of the female rat with the intention of mating the female. It is known as the number of mounts before ejaculation.

Intromission frequency (IF)

The number of times the male rat attempts vaginal penetration/Intromission before ejaculation.

Ejaculation frequency (EF)

The number of times of ejaculation (characterized by rhythmic contraction of the posterior abdomen).

Mount Latency (ML)

The time interval between introduction of a female Wistar rat and the first mount attempted by the male Wistar rat.

Ejaculation frequency (EF)

The time interval between first Intromission of a series to ejaculation, which is characterized by longer, deeper pelvic thrusting and slow dismount followed by a period of reduced activity.

Intromission latency (IL)

The time between addition of a receptive female into the arena and first Intromission.

Sperm Analysis

Semen Collection

The testis together with the epididymis' was excised. The distal epididymis was then cut open to expose the semen for collection by applying a little pressure on the caudal epididymis to squeeze the semen into a microscope slide. Two drops of 2.9% sodium citrate were added and the slide was covered with a cover slip for examination and evaluation under the microscope. The sperm cells were classified into non-motile, sluggish, or actively motile. The percentage of motile sperm cells was calculated by dividing the number of motile sperm cells by the total number of counted sperm cells following the method described by Osuchukwu (2016). The sperm count was carried out using the Neubauer Haemocytometer.

Sperm Motility

Semen was squeezed from the caudal epididymis onto a pre-warmed microscope slide (27°C) and two drops of warm 2.9% sodium citrate were added, the slide was then covered with a warm cover slip and examined under the microscope using X400 magnification. Ten fields of the microscope were randomly selected and the sperm motility of 10 sperms was assessed on each field. Therefore, the motility of 100 sperms was assessed randomly. Sperms were labeled as motile, sluggish, or immotile. The percentage of motile sperm cells was defined as the number of motile sperms divided by the total number of counted sperms (i.e. 100).

Sperm viability (Life/dead ratio)

This was done by adding two drops of warm Eosin/Nigrosin stain to the semen on a pre-warmed slide, a uniform smear was then made and dried with air; the stained slide was immediately examined under the microscope using x400 magnification. The live sperm cells were unstained while the dead sperm cells absorbed the stain. The stained and unstained sperm were counted and the percentage was calculated.

Sperm morphology

This was done by adding two drops of warm Walls and Ewas stain (Eosin/Nigrosin stain can also be used) to the semen on a prewarmed slide, a uniform smear was then made and air-dried; the stained slide was immediately examined under the microscope using x400 magnification Five fields of the microscope were randomly selected and the types and number of abnormal spermatozoa were evaluated from the total number of spermatozoa in the five fields; the number of

abnormal spermatozoa was expressed as a percentage of the total number of spermatozoa.

Sperm count

This was done by removing the caudal epididymis from the right testes and blotting with filter paper. The caudal epididymis was immersed in 5 ml formol-saline in a graduated test-tube and the volume of fluid displaced was taken as the volume of the epididymis. The caudal epididymis and the 5 ml formol-saline were then poured into a mortar and homogenized into a suspension from which the sperm count was carried out using the improved Neubauer haemocytometer under the microscope.

Histology of the Testes

After weighing the testes, they were immediately fixed in Bouin's fluid for 12 hours and the Bouin's fixative was washed from the samples with 70% alcohol. The tissues were then cut in slabs of about 0.5 cm transversely and the tissues were dehydrated by passing through different grades of alcohol: 70% alcohol for 2 hours, 95% alcohol for 2 hours, 100% alcohol for 2 hours, 100% alcohol for 2 hours and finally 100% alcohol for 2 hours. The tissues were then cleared to remove the alcohol; the clearing was done for 6 hours using xylene. The tissues were then infiltrated in molten Paraffin wax for 2 hours in an oven at 57 ^{O}C, thereafter the tissues were embedded. Serial sections were cut using rotary microtone at 5 microns (5 µm). The satisfactory ribbons were picked up from a water bath (50-55 ^{O}C) with microscope slides that had been coated on one side with egg albumin as an adhesive and the slides were dried in an oven. Each section was deparaffinized in xylene for 1 minute before being immersed in absolute alcohol for 1 minute and later in descending grades of alcohol for about 30 seconds each to hydrate it. The slides were then rinsed in water and immersed in an alcoholic solution of hematoxylin for about 18 minutes. The slides were rinsed in water, then differentiated in 1% acid alcohol and then put inside running tap water to blue and then counterstained in alcoholic eosin for 30 seconds and rinsed in water for a few seconds, before being immersed in 70%, 90% and twice in absolute alcohol for 30 seconds each to dehydrate the preparations. The preparations were cleared of alcohol by dipping them in xylene for 1 minute. Each slide was then cleaned, blotted, mounted with DPX and coverslip, and examined under the microscope. Photomicrographs were taken at x40, x100 and x400 magnifications

Statistical Analysis

Data were expressed as mean ± SEM. The mean difference between the tested groups and the control was tested using a One-Way ANOVA. Values were considered statistically significant when $p \leq 0.05$. The significant difference was assessed using the LSD Posthoc test. Computer software package SPSS version 25 was used.

RESULTS

In the present study, mount frequency increased significantly in the high-dose group of bay leaves treated animals when compared to the control (Table I). Intromission frequency increased significantly (0.00) in high-dose bay leaves when compared to the control (Table I).

Mount latency and Intromission latency decreased significantly when compared to the control in bay leaf-treated rats (Table I).

Ejaculation frequency increased significantly in bay leaves treated animals (Table I). Post-ejaculation interval was reduced in both premature plantain and bay leaves treated animals (Table I).

Sloughing off of some Sertoli cells was observed in high-dose treated animals (plate IV).

Table I: Effect of Bay Leaves on Sexual Behaviour of Wistar rats

GROUP	Control Mean ± SEM	Viagra Mean ± SEM	Low Dose Bay Leaves Mean ± SEM	High Dose Bay Leaves Mean ± SEM	F	p-value
MF (n)	2.0 ± 1.0[a]	14.0 ± 1.0*	3.5 ± 0.5[a]	5.0 ± 0.6*[a]	45.0	0.00
ML (s)	17.5 ± 2.5	12.5 ± 2.5	13.0 ± 1.0	11.0 ± 0.6*	3.02	0.03
IF (n)	4.0 ± 1.0[a]	16.0 ± 1.0*	4.5 ± 0.3[a]	7.3 ± 0.3*[a]	59.91	0.00
IL (s)	21.5 ± 1.5[a]	13.5 ± 1.5*	13.5 ± 0.5	18.0 ± 1.7*	5.58	0.05
EF (n)	1.0 ± 0.0[a]	4.5 ± 0.5*	2.0 ± 0.3	4.3 ± 0.5*[a]	28.18	0.00
EL (s)	115.0 ± 15.0	146.5 ± 36.5	97.0 ± 1.0	111.3 ± 1.2	1.41	0.34
PEI (s)	391.0 ± 6.0[a]	241.5 ± 65.5*	322.3 ± 0.5	361.3 ± 6.4[a]	5.19	0.05

Key

a= significant compared to control

* = significant compared to Viagra

MF = Mount frequency, ML = Mount latency, IF = Intromission frequency, IL = Intromission latency, EF = Ejaculation frequency, EL = Ejaculation latency, PEI = Post ejaculatory interval.

Table II: Effect of Bay Leaves on Semen Analysis

GROUP	Control Mean ± SEM	Viagra Mean ± SEM	Low Dose Bay Leaves Mean ± SEM	High Dose Bay Leaves Mean ± SEM	F	p-value
Normal	82.5 ± 2.5	80.0 ± 10.0	87.5 ± 2.5	70.0 ± 2.9	2.51	0.17
Abnormal	17.5 ± 2.5	20.0 ± 10.0	12.5 ± 2.5	60.0 ± 0.0	2.51	0.17
Viability	87.5 ± 2.5	82.5 ± 7.5	87.5 ± 2.5	60.0 ± 0.0	2.38	0.19
Sperm count	550.0 ± 50.0	550.0 ± 150.0	600.0 ± 100.0	316.7 ± 72.65	2.10	0.22
Ph	8.0	8.0	8.0	8.0	-	-
Volume	0.35 ± 0.05	0.30 ± 0.10	0.20 ± 0.10	0.17 ± 0.67	1.76	0.41
Appearance	milky	milky	Milky	milky	-	-
Viscosity	normal	normal	Normal	normal	-	-

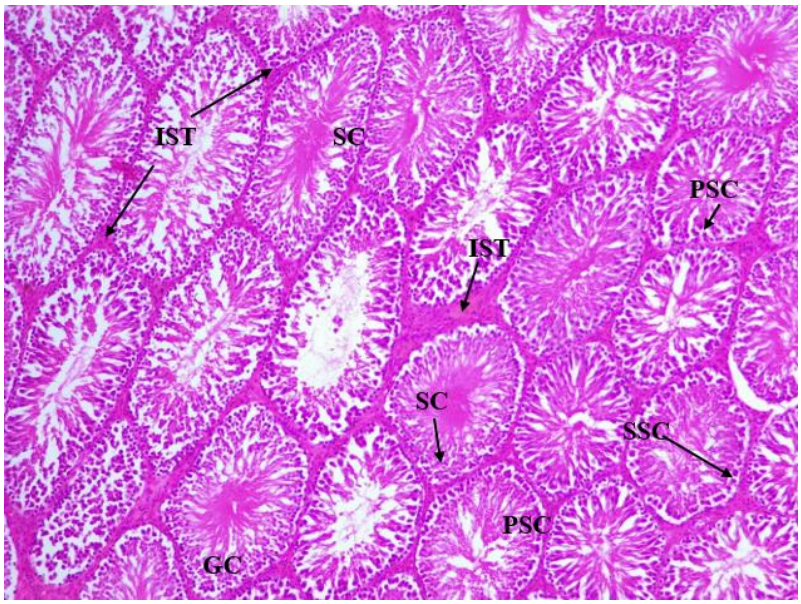

Plate 1: Photomicrograph showing section of seminiferous tubules of tissues of wistar rats. Group 1 (received distilled water). Section showed Sertoli cells (SC) with germinal cells (GC), primary spermatid (PSC), secondary spermatids (SSC) and interstitial tissues with Leydig cells (LC). H&E×100

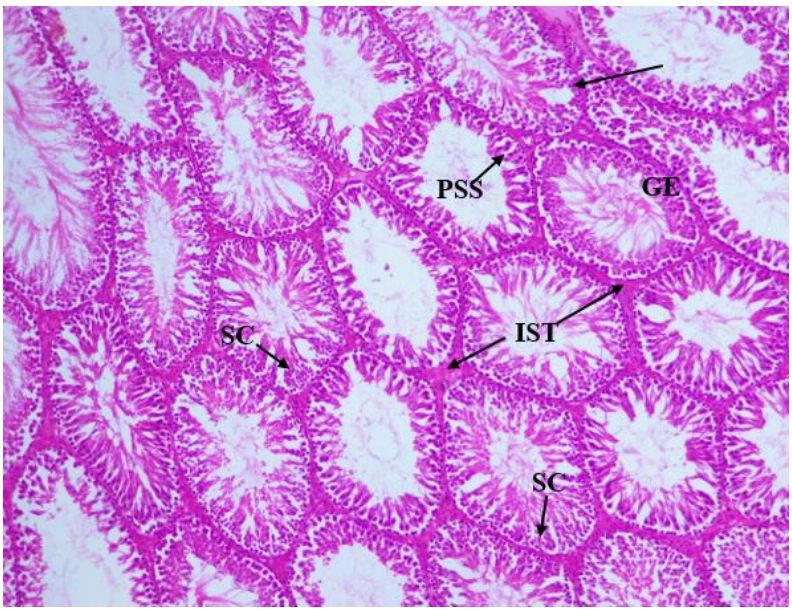

Plate ll: Photomicrograph showing section of seminiferous tubules of testis. Group ll (Viagra group). Section displayed testicular Sertoli cells (SC), germinal epithelium (GE), primary spermatids and secondary and mature spermatocytes (PSS) and interstitium with Leydig cells. H&E×100

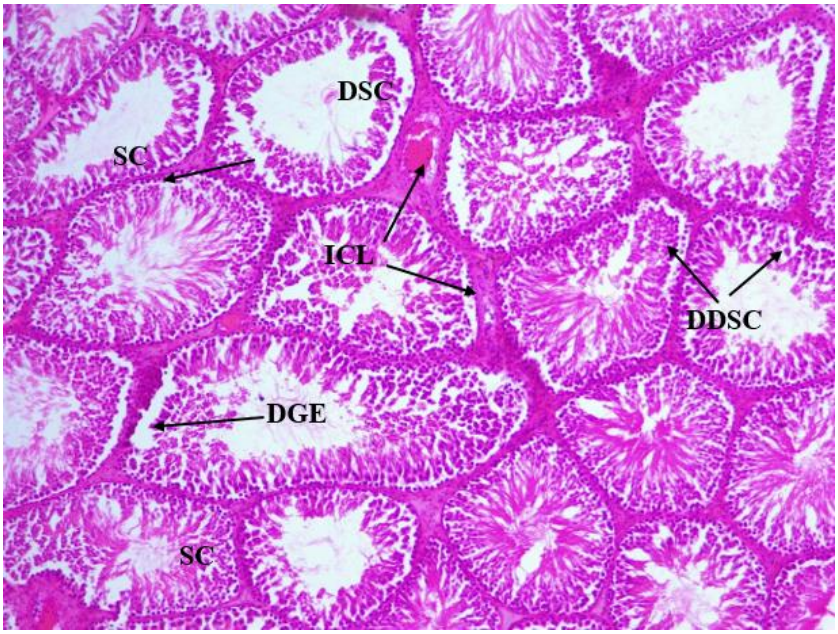

Plate lll: Photomicrograph showing section of seminiferous tubules of testes. Group lll (Low dose Bay leaves). Section showed distorted testicular Sertoli cells (DSC), germinal epithelium (DGE), primary spermatids, and secondary and mature spermatocytes (DSS) and interstitial lesions (ICL). H&E×100

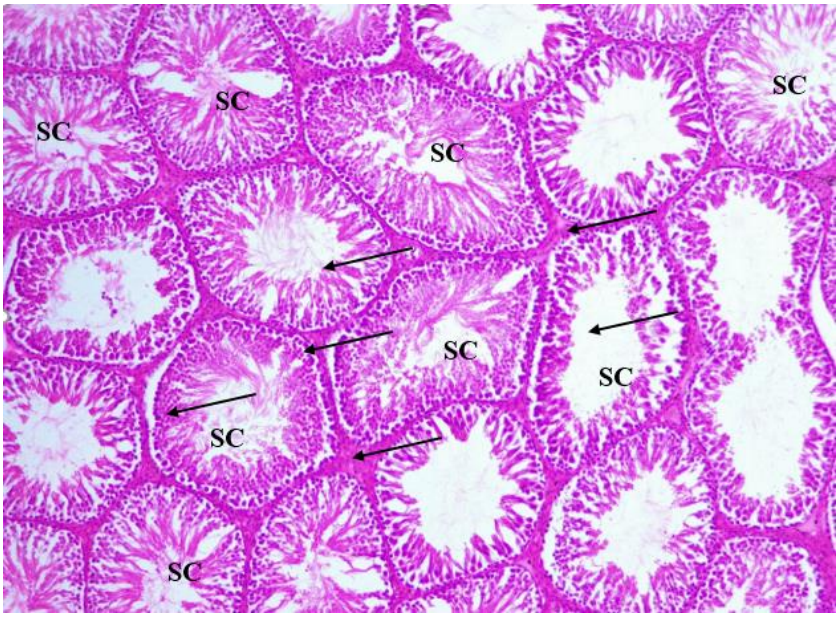

Plate IV: Photomicrograph showing section of seminiferous tubules of testes. Group IV (High dose bay leaves). Section showed sloughing off of some Sertoli cells, germinal epithelium, primary spermatids, secondary and mature spermatocytes. H&E×100

DISCUSSION

In the present study, mount frequency increased significantly in high–dose group of the bay leaf-treated animals. This implies that the plant extract used for the present study increases sexual function (libido and potency). This study agrees with Tajuddin *et al*., (2004) who stated that an increase in mount frequency indicates sexual function.

Intromission frequency increased significantly (0.00) in high-dose bay leave when compared to the control. This suggests that bay leaves extract activated penile erection. This result agrees with Yakubu and Akanji, (2010). They suggested that an increase in Intromission frequency suggests activation of erection.

Mount latency and Intromission latency decreased significantly when compared to the control in bay leave-treated rats. This implies that bay leaves are likely to enhance sexual appetite/motivation and arousal. This agrees with Yakubu and Afolayan (2009) and Fouche *et al*., (2015), who stated that plant extracts capable of decreasing mount and Intromission latencies enhance sexual appetite and motivation.

Ejaculation frequency increased significantly in bay leaves treated rats. This implies that these plants are likely to have aphrodisiac effects. This agrees with Fouche *et al*., (2015). They stated that increase in ejaculation frequency indicates enhanced aphrodisiac effect of the plant.

Ejaculation frequency increased significantly in bay leaves treated rats. This implies that this plant is likely to have aphrodisiac effects. This agrees with (Fouche *et al*., 2015). They stated that an increase in ejaculation frequency indicates an enhanced aphrodisiac effect of the plant.

Ejaculation latency was prolonged in the present study. This indicates that the plant exerts some aphrodisiac effect i.e. the extract and standard drug prolonged the duration of coitus (an indicator of an increase in sexual motivation). The result obtained from the sexual behavioral test in the present study agrees with wattanathorn *et al*., (2012), who stated that prolongation of ejaculation latency suggests a prolonged duration of coitus, which is an indicator of increased sexual motivation. It also indicates enhanced copulatory performance.

Post ejaculatory interval in the present study reduced indicating that the plant extract sustained increased sexual activity. This result agrees with Yakubu (2006), who stated that a reduction in a post-ejaculatory interval is an indication of a sustained increase in sexual activity.

CONCLUSION

Results from the present study have revealed that Bay leaves are likely to increase sexual function (libido and potency), activate penile erection, and sustain increased sexual activity.

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