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Effects of Aqueous Leaf Extract of *Thunbergia alata* (Boxer Ex Sims) on Reproductive Parameters in Male Wistar Rats

Nandala Christopher

Faculty of Science of Kampala International University Uganda

ABSTRACT

Leaves have been documented to enhance sexual activity, but the parameters they affect remain unknown. This study was to determine the effect of an aqueous leaf extract on reproductive parameters in male Wistar rats. Twenty-five male Wistar rats (150–200 g, n = 5) were grouped as follows: Group A: 1 mL of distilled water Negative control, Group B: Sildenafil citrate 25 mg/kg b.w. Positive control: Group C: aqueous leaf extract at a dose of 250 mg/kg b.w.; Group D: aqueous leaf extract at a dose of 500 mg/kg b.w.; and Group E: aqueous leaf extract at a dose of 1000 mg/kg b.w. These rats were orally administered with different test agents daily for 30 days and thereafter sedated with diethyl ether, sacrificed, and the testis and epididymis removed. Sperm variables examined microscopically include serum sex hormones. It was indicated that there was a significant p = 0.006 increase in the mean testosterone levels of the animals treated with 500 mg/kg and 1000 mg/kg as compared to those treated with sildenafil. Significant increases in sperm count were seen in the animals treated with 1000 mg/kg. Regarding the mean levels of follicle-stimulating hormone, the study notes that there was a significant (p <0.001) increase in the mean follicle-stimulating hormone levels in the animals that were treated with 500 mg/kg and 1000 mg/kg. A significant (p = 0.006) and (p = 0.013) increase in sperm motility was noted between the animals treated with 1000 mg/kg and the animals that were treated with distilled water and 250 mg/kg, respectively. The study also noted a significant (p = 0.025) and (p <0.001) increase in sperm viability between the animals treated with 500 mg/kg and 1000 mg/kg of those that received distilled water. Furthermore, there was also a significant (p = 0.001) and (p = 0.013) increase in sperm viability in the animals treated with 1000 mg/kg and those that received sildenafil and 250 mg/kg, respectively.

Keywords: Sexual dysfunction, Ejaculation, Sperm viability, Stimulating hormone.

INTRODUCTION

Sexual dysfunction (SD) is generally described as the inability to have a satisfying sexual encounter on several occasions due to a disruption of a typical sexual response cycle consisting of libido, erection, orgasm, ejaculation, and detumescence [1]. Male sexual dysfunction or impairment (MSD) refers to an impairment or delay in any of the phases or steps that constitute sexual orientation, sexual function, or behavior [2]. The above steps are affected by issues ranging from vascular, intra-cavernosal nitric oxide system, and androgens, hence generating statistics showing that about 10% of males in all age groups suffer from sexual dysfunction due to either one or more of the mentioned factors [3]. Erectile dysfunction, on the other hand, refers to the inability of an individual to maintain an erection sufficient to permit satisfactory sexual intercourse [4]. Infertility is usually defined as the inability of a couple to conceive after one year of unprotected, frequent sexual intercourse, and an estimation of about 30% of reported infertility problems are attributed to male factors [5]. The decline of male fertility is of utmost importance, as evidence points to a progressively declining sperm concentration (count) over the past 35 years globally and, additionally, a 1.2% deterioration in free serum testosterone [6]. About 8.9% to 68.7% of infertile men reported a decrease in sexual satisfaction along with hypoactive sexual desire as the most prevalent types of sexual dysfunction, and in addition, 20–30% of adult men globally are reported to show some form of sexual dysfunction [7]. Male infertility is an occurrence with a wide range of factors that affect spermatogenesis and sperm quality [8]. Fundamentally, the assessment of male infertility is centered around the analysis of semen

quality, which is determined by sperm morphology, sperm concentration, and motility [9]. The release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the anterior pituitary gland is controlled by a conventional negative feedback loop involving hypothalamic gonadotrophin-releasing hormone (GnRH) [10]. FSH aids in the process of spermatogenesis in the testis, while LH is involved in testosterone secretion. Theoretically, an erection is initiated by erectile stimuli such as sight, smell, touch, psychological thoughts, and hearing. Stimulation of sensory receptors in the glans penis sends nerve impulses through the pudendal nerve, which enter the spinal cord and activate the parasympathetic pathways. Activation of parasympathetic nerves supplying the penis stimulates the release of nitric oxide (NO) from cavernous nerves and penile endothelial cells. NO causes vascular relaxation, which increases blood flow to the cavernosal sinusoids, resulting in a rise in intra-cavernosal pressure. The intra-cavernosal pressure and volume are greater than those of the sub-tunicalvenular plexus, compressing the veins between the tunica albuginea and the peripheral sinusoids. Veno-occlusion prevents blood from leaving the body, resulting in tumescence (penile erection) [11]. For the treatment of SD, drug therapy, which includes the utilization of testosterone replacement for conditions involving insufficiency of androgens [12], plus standard pharmaceutical medications have been offered, including Tadalafil, Sildenafil, and Vardenafil. Other interventions include surgical techniques involving urethral suppositories, suction devices, and penile implants [13]. These techniques, however, are costly, difficult to obtain, and have major side effects such as hurting in both the penis and testes, urethral burning, bleeding, implant extrusion, and infections, to mention but a few. As a result, medicinal plants have been employed as an alternative, with evidence of their use as plants (tonics) that stimulate libido or improve sexual performance [14]. A wide range of factors, such as androgen shortages, lifestyle changes, and drug side effects, to mention but a few, are all etiologic factors for sexual dysfunction [15]. Different treatments, such as psychological counseling, medication, and surgical approaches, have been used as remedies for sexual dysfunction. Unfortunately, some of these treatments are prohibitively expensive and possess many adverse side effects, like discomfort in the penis, testes, urethral burning, pain and bleeding, implant extrusion, and infections [14]. As a result, there is a need to look for herbal alternatives that improve libido, sexual potency, and sexual pleasure but are also less expensive, more widely available, faster acting, and have fewer side effects. The United States National Health and Social Life Survey (NHSLS) by 2020 reported estimations of about 31% of men who suffer from an issue related to sexual dysfunction during their lifetime. In Uganda, the major form of sexual dysfunction reported is erectile dysfunction (ED), and its prevalence among patients attending primary care clinics stands at 57.4% [16]. ED is linked to a variety of psychological issues, including decreased quality of life, depression, anxiety, relationship problems, and marital conflict [17]. Due to stigma, patients who suffer from ED usually cannot come out to seek remedies, leading to marital problems and family breakups [18]. According to a survey, ED is one of the most important variables affecting men's relationships, with 12 percent to 28 percent saying it is one of the most important elements affecting their relationships [19]. PDE-5 Inhibitors Sildenafil, tadalafil, verdenafil, avanafil, mirodenafil, and lodenafil [20] have been used as medical therapies in the management of ED; however, most people in Uganda use herbal plants like Termininalia cattapa seeds, Mondia whitei, Citropsis articulata, and Cola acuminate, which have also shown efficacy in the management of sexual dysfunction. Therefore, this study is meant to determine the effects of the aqueous leaf extract of *T. alata* on reproductive parameters in male Wistar rats.

Methodology

Study design

The study design was experimental, using an animal model to evaluate the effect of an aqueous extract of T. alata on reproductive parameters in male Wistar rats. The experiments were carried out in the Department of Physiology, Kampala International University, Western Campus, Uganda, and other reputable laboratories in Uganda between August 2022 and April 2023.

Area of Study

The study will be conducted in the Physiology Department Laboratories at Kampala International University, Western Campus, Uganda.

Study Sample

The study sample involved twenty-five (25) male Wistar rats at the age of three months, weighing between 150 and 200 grams, which were randomly allocated to five different groups. The rats were of the same age and weighed at the point of collection for uniformity.

Sampling Techniques

Simple random sampling was used to sample the Wistar rats. This involved randomly assigning numbers to each of the rats. Rats were assigned to their respective groups when the corresponding number of rats was picked from an opaque bag.

Plant collection and identification

Fresh leaves of *T. alata* plants were collected from the wild at Mabira Forest Reserve, Nagojje, Mukono District, Central Uganda. Also, some herbarium specimens were prepared by collecting a fresh shoot with leaves, kept and transported in a cool dry paper bag, and taken for identification in the Herbarium Unit, College of Natural Sciences, Makerere University, Uganda, for identification and voucher number deposition.

Plant extraction

The aqueous leaf extract of *T. alata* was obtained using the hot maceration method [21]. Before being dried in a 20 °C oven, fresh leaves were thoroughly rinsed in clean water to remove any dirt particles. With a mortar and pestle, the dried leaves were pounded to a coarse powder, and then 1000 g of the crushed material was extracted with double-distilled water (2 L) and boiled for one hour. This was done to mimic the treatments used by herbalists to treat sexual dysfunction [14]. The mixture was filtered with cotton wool before being finely filtered with Whatman No. 1 filter paper. The filtrate was concentrated in a rotary evaporator, and the resulting concentrate was dried in a 30-degree Celsius oven until it reached a semi-solid residue. The extract was weighed and kept at 4°C in the refrigerator.

Care of experimental animals

At the age of three months and weighing between 150 and 200 grams, twenty-five Wistar rats (25 males) were procured from the Animal House of the School of Medicine, Mbarara University, Uganda. The sexual parameters as well as the LD50 were determined using Wistar rats, both male and female. They were housed in four-animal cages with a 12-hour light/dark cycle. The animals acclimatized for seven days while being fed a regular pellet diet and having unfettered access to water [22].

Acute toxicity

Alata's LD50 was determined using the acute toxic class method, which employed three female non-pregnant rats aged 8–12 weeks [23]. Wistar rats weighing 150–200 g were chosen for the investigation using random sampling. For acute oral toxicity testing, the OECD-423 guidelines were followed. Before being weighed, the animals were fasted overnight and given only water. The groups were subsequently administered leaf extract at a dose of 300 mg/kg body weight orally, and they will be monitored for 14 days. For a total of 14 days, each animal was checked individually over the first 30 minutes after medication, then frequently for the next 24 hours, with attention in the first four hours. The animals were observed for toxic symptoms like behavioral changes, aberrant movement, salvation, convulsions, and death for 72 hours. The acute toxicity dose was determined to be >2000 mg/kg.

Experimental procedure and treatment protocol

Twenty-five healthy male rats were used and maintained under standard environment conditions and fed with a standard pellet diet and water *ad libitum*[22]. After a week of adaptation, the rats were randomly divided into five groups A, B, C, D and E (n=5) and these rats were orally administered with different test agents daily for 30 days as follows;

Group A: 1 mL of distilled water (Negative control)
Group B: Sildenafil citrate 25 mg/kg b. w. (Positive control) (standard drug)
Group C: Aqueous leaf extract of T. alata at a dose of 250mg/kgb. w.
Group D: Aqueous leaf extract of T. alata at a dose of 500mg/kgb. w.
Group E: Aqueous leaf extract of T. alata at a dose of 1000mg/kgb. w.

Table 1: Grouping of experimental animals

GROUP	Number of Rats(n)	TREATMENT
Group A	5	1 mL distilled water (Negative control)
Group B	5	Sildenafil citrate at a dose of 25mg/kg b.w. (Positive control)standard drug
Group C	5	Aqueous leaf extracts of T. alata a dose of 250mg/kgb. w.
Group D	5	Aqueous leaf extract of T.alata at a dose of 500mg/kgb. w
Group E	5	Aqueous leaf extract of T. alata at a dose of 1000mg/Kgb. w.

Sildenafil (Viagra) was the first pharmacologically authorized treatment for impotence, and its availability has led to millions more couples seeking treatment for erectile dysfunction. As a result, Viagra will be employed as the positive control drug in this investigation. Because distilled water does not affect the experimental group, it will be utilized as a negative control. The sample size of Wistar rats was determined using the resource equation method [24]. It states that:

E= Total number of animals- Total number of groups

$E = 30 - 5$

$E = 25$; Where E is the degree of freedom of analysis of variance (ANOVA)

Evaluation of Sexual behaviour in the animals (Non-competitive method)

The non-competitive method [21] was deployed where male rats had four training test sessions (twice a week for two weeks) with non-experimental receptive females to become sexually experienced; the females used in this training were employed in the rest of the study. The final experiment involved males who had at least two ejaculations during the four training test sessions. Female rats were given estradiol benzoate (100g/kg) and progesterone (5mg/kgb.w.) subcutaneously 48 hours before the experiment and 4 hours before the males were exposed to make the females fully receptive [21]. Each male was allowed to acclimate to the test chamber (60 L × 40 W × 40 H cm) before the exam. The animals were watched for three hours and their conduct graded. Males were placed in glass cages one by one. A female was introduced into the arena using a non-competitive approach after 15 minutes of acclimation. During a 15-minute observation period at the start of the first hour, the number of mounts were recorded. The test was ruled negative if the male did not mount during the next 15 minutes. The female was separated from the male for 100 minutes. At the third hour, the female will be introduced again, and the number of mounts were counted for 15 minutes. All studies were carried out during the day, between 0900 and 1200 h, at room temperature (26-27 °C). This was done after a period of30 days. The following parameters will be determined: mount and intromission frequencies (number of mounts and intromissions preceding the first ejaculation[21].

Determination of the reproductive parameters

The animals were sedated with Diethyl ether and decapitated, and the testis and epididymis were carefully removed via an abdominal incision. After that, the testes were detached from the epididymis and put in 2.0 mL normal saline (0.9 per cent NaCl, 37°C) on a pre-warmed petri plate. The cauda epididymis was minced with a sharp razor blade. To separate the tissue from the sperm, the liquid was gently mixed to homogenize it before being passed through a nylon mesh.

Determination of sperm count

A 20-fold (1:19v/v) dilution of 500 L of sperm suspension in formaldehyde fixative [10 per cent formalin in phosphate-buffered saline (PBS)] was used to count sperm. Then, using a Pasteur pipette, transfer 10 mL of the diluted solution into a Neubauer hemo cytometer by gently pressing the pipette tip against the lower edge of one of the chambers at the V-shaped groove. Allowing capillary action to fill the chamber by slowly depressing the plunger of the pipette. Fill the chamber halfway with the solution and set aside for 7 minutes. A hemocytometer with 250 small squares was used to count and assess the sperm that had settled. At least 200 spermatozoa were counted in the updated Neubauer chamber's central grid, row by row until a complete row (five massive squares) was analyzed.

Determination of sperm motility

In a nutshell, a volume of 10µL of semen was placed on a clean glass slide and then covered with a clean cover slip. Allowing the weight of the coverslip to spread the sample. The solution was analyzed under a magnification of ×400 using a light microscope (Olympus CX41 Tokyo). Then the percentage motility was assessed as progressive (PR), non-progressive (NP), and immotile (IM) movements of sperms of at least 200 spermatozoa. The per cent motility was determined by the progressive (PR) under a microscope according to WHO guidelines.

Determination of sperm viability

Briefly, sperm viability was assessed by nigrosin-eosin staining method. One drop of 50µl of semen and mixed with an equal volume of eosin– nigrosin suspension (1% aqueous solution of eosin-y- EIRR117A and 10% aqueous solution of nigrosine-A1817) mixed thoroughly in a microcentrifuge. The mixture then placed on a glass slide and observed under 400X magnification. The percentage of alive (without stain) and dead (red) cells determined by at least counting 200 cells [25].

Hormonal assay evaluation

The animals were euthanized after receiving the final dosage of the extract and test agents, and whole blood obtained through heart puncture and stored in a non-heparinized vacutainer spinning at 2500rpm for 10 minutes at 4°C, with serum collected for the hormonal assay.

Determination of serum testosterone concentration

The ELISA kits (BiolegendInc) were used by the manufacturer's manual. In a nutshell, 10 µl of standards, 10 µlof specimens, and 10µl of controls were placed in the appropriate wells after acquiring the required number of coated wells. To each well, add 100 µl testosterone-horseradish peroxidase (Testosterone-HRP) Conjugate Reagent. Then pour 50µl of rabbit anti-testosterone reagent into each well and stir thoroughly for 15 seconds. Before being disposed of, the incubation mixture warmed in a rubbish receptacle for 70 minutes at 37°C.Deionized water was used to rinse and flick the microtiter wells seven times. The wells were aggressively shaken onto absorbent paper to remove any leftover water droplets. After that, 100 µl of tetramethylbenzidine (TMB) reagent was pipetted into each well, swirled for 5 seconds, and allowed to sit at room temperature for 15 minutes before being stopped with 100µl of stop solution. It was then mixed for 25 seconds to completely change the blue colour to yellow. A microtiter well reader was used to measure the absorbance at 450 nm within 10 minutes.

Calculation

The mean absorbance of the appropriate reference standard plotted versus its concentration in ng/ml, with absorbance values on the vertical (Y) axis and concentrations on the horizontal (X) axis, with absorbance values on the vertical (Y) axis and concentrations on the horizontal (X) axis. The calibration curve was used to calculate the testosterone concentration in ng/ml based on the mean absorbance values of each sample. Because the samples were diluted, a dilution factor was used in the final computations. For each set-off reference standard, control, and sample, the mean optical density value (OD450) calculated.

Principle

To give a constant level of rabbit anti-Testosterone, the Testosterone ELISA used a technique of competitive binding of testosterone in the sample and testosterone-horseradish peroxidase (HRP) conjugates. Then warmed for 60 minutes the goat anti-rabbit IgG-coated wells, controls, samples, testosterone standards, testosterone-HRP conjugate reagent, and rabbit anti-testosterone reagent, as well as the goat anti-rabbit IgG-coated wells, controls, and testosterone-HRP conjugate reagent. During incubation, a predetermined amount of HRP labelled testosterone competes with testosterone in the standard, sample, or quality control serum for a restricted number of binding sites on the testosterone-specific antibody. As a result, as the concentration of testosterone in the rat sample grows, the amount of testosterone-HRP immunologically associated with the well decreases. After the unbound testosterone-peroxidase conjugate was removed, TMB Reagent was added and the wells were rinsed, resulting in a blue tint. After the colour development was stopped, the spectrophotometric absorbance at 450 nm was determined. The amount of enzyme in the sample was proportional to the amount of enzyme in the sample, and the colour intensity was proportional to the amount of unlabeled testosterone in the sample. The standard concentration was shown against the absorbance in a calibration curve. The standard curve was used to compute the testosterone concentration in both the test sample and the controls that run concurrently with the standards and this was done by an automated reader.

Determination of serum follicle-stimulating hormone level

The ELISA Kits (Biolegend Inc.) were used by the manufacturer's manual by Nielsen *et al.*, 2001. In that order, the anti-FSHHRP conjugate working solution was made, wash buffer, and the required number of well strips. 20 µl of each calibrator, control, and test sample was added twice to each labelled well, followed by 80 µl of assay buffer. At 25° C, heated for 20 minutes on a plate shaker (exactly 300 rpm). Three times with 300 µl of diluted wash buffer, each well was washed. Then, filled each well with 90 mL of the conjugate working solution, warmed for 15 minutes at 25 ° C with a plate shaker (150 rpm to be precise), and washed the wells as before. At 5-minute intervals, 70µl TMB substrate was injected into each well. The mixture was heated for 20 minutes on a plate shaker at 25 ° C. (or until the calibrator turned dark blue to the desired OD). Within 10 minutes of the Stop Solution addition, 30 mL of Stop Solution was added to each well at 5-minute intervals, and the plate is spectrophotometrically measured at 450 nm with a microplate reader.

Calculations

The mean absorbance of each unknown copy calculated by subtracting the mean optical density value of the "0" standard from the mean absorbance values of the standard, control, and serum sample necessary test. The calibration curve is plotted on a log sheet with the average optical density on the Y axis and the calibration

material concentration on the X axis. Unknown values read directly on the calibration curve at wavelengths standard. The readings were determined by an automated reader.

Principle

The usual two-step process which involves the sandwich method was followed. A monoclonal antibody specific for FSH was immobilized on the microplate, and the test included radish peroxidase and a second monoclonal antibody acting on a separate site of FSH (HRP). FSH from the test sample and the standards were bound to the plate, then cleaned and warmed to room temperature with the HRP conjugate. The enzyme-substrate was added after the second wash. The enzymatic reaction stopped by adding a stopping solution. The absorbance was measured using a microtiter plate reader. The degree of colour produced by the enzymatic reaction was determined by the amount of FSH present in the test sample. A calibration curve was built using a set of standards, from which the amount of FSH in rat test samples and controls was estimated spectrophotometrically.

Determination of serum luteinizing hormone level

The technique described by Nielsen *et al.*, [26] was used to assess serum luteinizing hormone. In a nutshell, 50 µl of standards, test samples, and controls were inserted correctly in designated wells in the coated wells stacked in a rack. Each well received 100 µl of Enzyme Conjugate Reagent, which was stirred carefully for 20 seconds before being left at room temperature (25°C) for 30 minutes. Shaking the plate contents into the sink removed the incubation mixture. The microtiter wells were shaken 5 times with distilled water after the mixture had been washed with water. The wells were placed directly on the paper towel to remove any remaining water droplets. This was followed by adding 100 µL of Tetramethylbenzidine Reagent (TMB) to each well and mixing gently for 10 s. The mixture was left at room temperature for 30 min, then 100 µl of stop solution was added to each well to stop the reaction. It was slowly mixed for 20s to make sure that all the blue turns completely yellow. The absorbance then read at 450 nm using a microtiter plate reader within 10 min.

Calculation

Mean absorbance value (A450) was analyzed for each set of reference standards, controls and test samples. The corresponding concentration of LH in mlU/ml from the standard curve determined using the mean absorbance value for each test sample. This was carried out by an automated reader to generate results.

Principle

This test employed a two-step or sandwich approach, with one highly specific monoclonal antibody immobilized on the microplate and the other specific to LH conjugated with radish peroxidase horseradish peroxidase horseradish peroxidase horseradish (HRP). Both the sample and the standard bound to the plate were then washed before HRP Conjugate was used to warm it up. Following the second wash, the enzyme-substrate was introduced. By providing a stopping solution, the enzyme reaction will be halted. Microtiter plate readers were used to assess absorbance. The amount of LH present in the sample influenced the colour intensity of the enzymatic reaction. A standard curve was created using a set of standards that were used to read the amount (concentration) of LH in test and control samples directly. The readings were carried out by an automated reader which generated the values.

Statistical analysis

The data was statistically evaluated using Microsoft Excel and IBM's SPSS (Statistical Package for Social Science) version 22 applications (IBM Corporation, Armonk, New York, USA). All data were expressed as means ± standard errors of the mean (SEM). To establish the level of significance between the control and treatment groups, a one-way analysis of variance (ANOVA) was used, followed by a Tukey test. At $p<0.05$, a difference was declared significant.

Ethical Consideration

Ethical Permission and approval were sought from the Research Ethics Committee (REC) of Kampala International University- Western Campus, Uganda before the commencement of this research study. The care and handling of the animals was done in accordance with the guidelines of National Institute of Health Guide for the Care and Use of Laboratory Animals, 8th edition (2011). In compliance with the internally accepted 3Rs (Replacement, Reduction, and Refinement) principles of animal experimentation, the appropriate guidelines were applied. The animals (Wistar rats) which were used in the study are necessary and given the required scientific results (replacement). Adequate and proper number of animals were used according the prescribed number for each protocol to achieve the set objectives of the study (Reduction). Animals were adequately trained, restrained, sedated or anaesthetized during tissue collection procedures to minimize the risk of injury, distress and/or pain to the animal (refinement). Also, the principal investigator acquired skills in restraining laboratory animals, administration of plant extract, drugs, tissue/sample collection techniques as well conducting procedures under

the guidance of a specialist with sufficient knowledge and experience of those procedures to alleviate pain on the animals and enhance proper sample collection (Refinement).

RESULTS

Percentage plant yield

Thunbergia alata extraction was done by macerating 1000g of pulverized *T. alata* leaf powder in 2000ml of distilled water and the results are shown in Table 2 below:

Table 2: Percentage Yield

Solvent	Weight of *T. alata* powder (g)	Weight of Crude Extract (g)	Percentage Yield (%)
Aqueous	1000	72	7.2

Thunbergia alata leaf extract powder that was used in the study yielded 72 g of the crude extract, which was then used in subsequent tests.

Acute toxicity of *T. alata*

Table 3: Signs of toxicity observed with *T. alata* administration

Extract Dose	Number of animals	Signs of toxicity
300 mg/ kg body weight	03	Piloerection, hyperactivity
2000 mg/ kg body weight	03	Piloerection, defecation, urination, irritation, hyperactivity

After the rats were given a single dose orally of 300 and 2000 mg/kg body weight, there were no significant signs of mortality, general behavior, mortality or gross differences of appearance in internal organs during the testing period which don't seem to go against the toxicity signs stated by Acute toxic class method. The results for the acute toxicity test of *T. alata* using the OECD – 423 guidelines are as shown in Table 3 above. *Thunbergia alata* aqueous leaf extract was found not to be toxic at the highest acceptable dose tested, and the lethal dose was classified as LD_{50}>2000 mg/ kg body weight. No death of animal was registered at the highest dose administered.

Table 4: Percentage of body weight and percentage relative organ weight of the animals

	Percentage relative organ weights (g/ 100 g)		
Parameters	**Control**	**300 mg/ kg of T. alata**	**2000 mg/ kg of T. alata**
Weight	125.133 ± 0.829	132.203 ± 1.439 [a]	126.393 ± 0.530 [b]
Liver	3.713 ± 0.104	3.630 ± 0.053	4.547 ± 0.088 [a, b]
Spleen	0.297 ± 0.012	0.287 ± 0.009	0.413 ± 0.009 [a, b]
Kidney	0.763 ± 0.009	0.827 ± 0.015	1.220 ± 0.057 [a, b]

The values are expressed as mean ± SEM (standard error of the mean); a = $p < 0.05$ vs control group; b = $p < 0.05$ vs 300 mg/ kg group. The study results in Table 4 above indicate that there was a significant increase in the weights of the animals receiving 300 mg/kg of T. alata compared to the control group. Furthermore, there was a significant decrease in the weights of the animals receiving 2000 mg/ kg of T. alata compared to the300 mg/kg of.T. alata group. The relative organ weights of the liver, spleen and kidney of the animals that received 2000 mg/ kg of *T.* alata were found to have significantly increased compared to those in both the control group and the 300 mg/ kg of T. alata group.

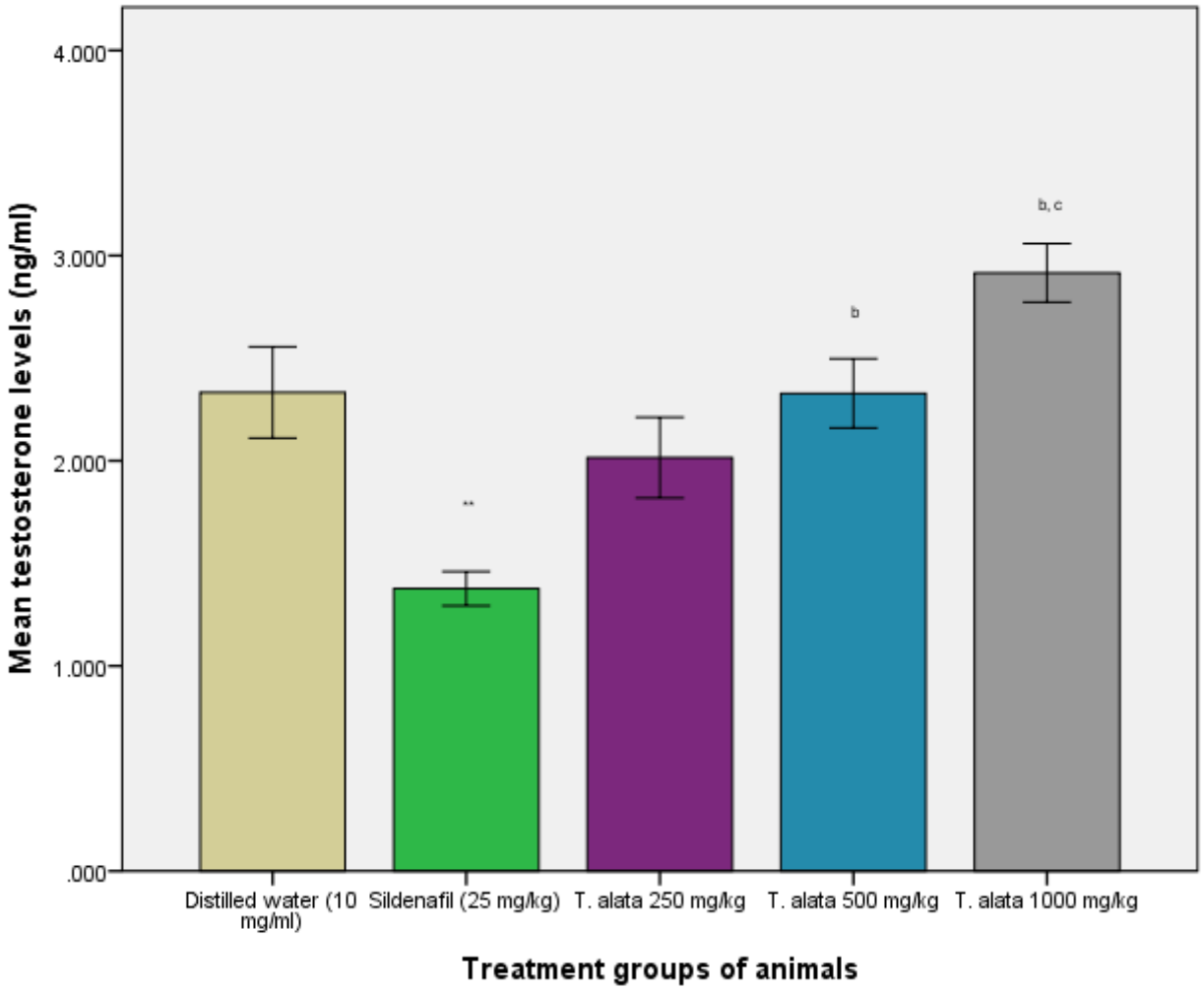

Figure 1: following mean testosterone levels (ng/ml). Results are expressed in Mean ± SEM. n = 5. * = p < 0.05 vs distilled water, b = p < 0.05 vs sildenafil, c = p < 0.05 vs *T. alata* 250 mg/kg

Findings in figure 1 indicate that the animals treated with 1000 mg/ kg of *T. alata* had the highest mean testosterone hormone levels while the animals treated with sildenafil had the lowest. The study results also indicate that animals treated with sildenafil were found to have significantly (p = 0.006) decreased mean testosterone levels compared to animals treated with distilled water. There was also a significant (p = 0.006) and (p <0.001) increase in the mean testosterone levels of the animals treated with 500 mg/kg and 1000 mg/kg of *T. alata* respectively as compared to those treated with sildenafil. Furthermore, there was a significant (p = 0.010) increase in the mean testosterone hormone levels of the animals treated with 1000 mg/kg of *T. alata* compared to those treated with 250 mg/kg of *T. alata.*

Effect of *T. alata* on serum follicle-stimulating hormone levels

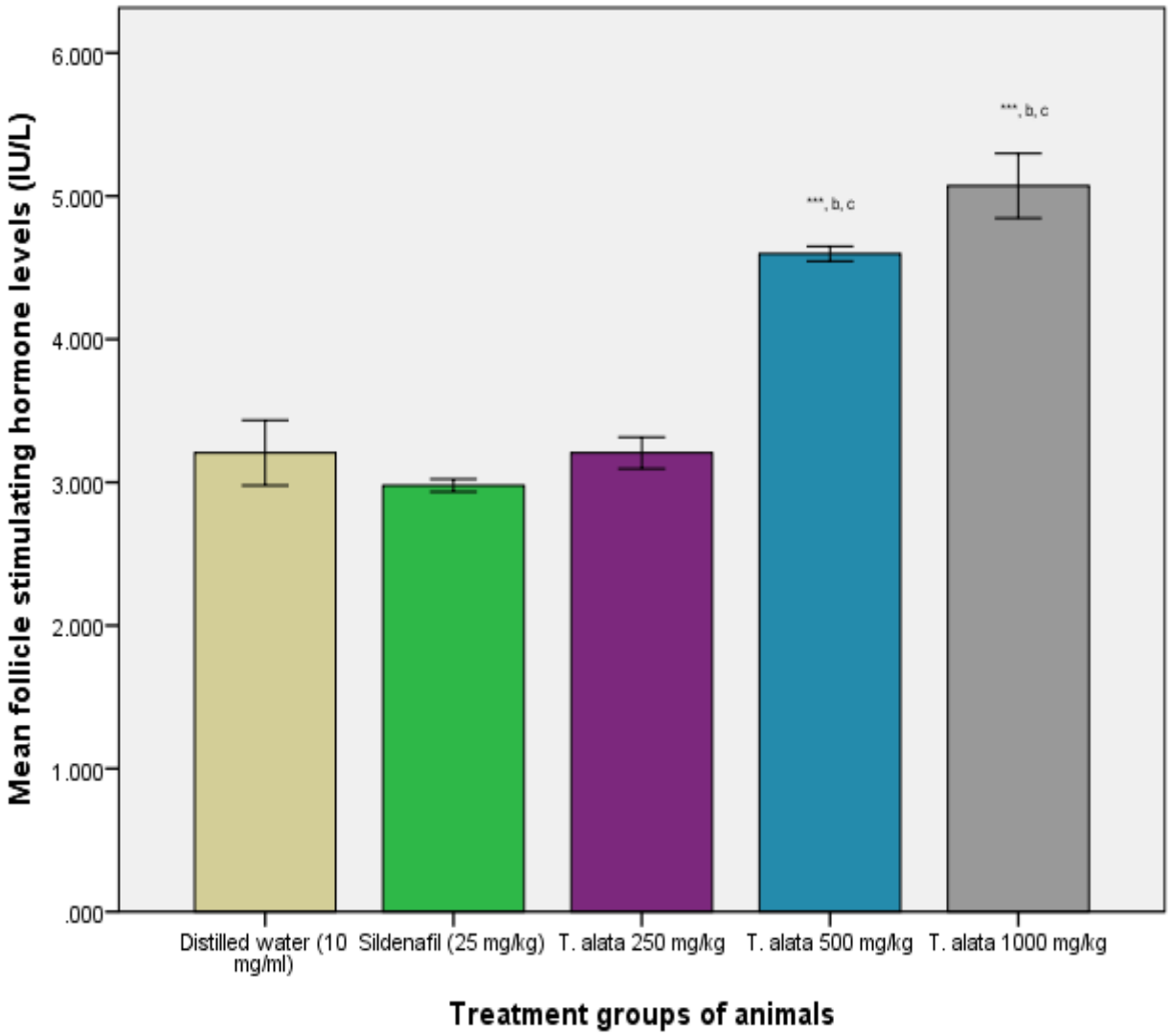

Figure 2: shows mean follicle stimulating hormone levels (IU/L). Results are expressed in Mean ± SEM. n = 5. * = p < 0.05 vs distilled water, b = p < 0.05 vs sildenafil, c = p < 0.05 vs *T. Alata* 250 mg/kg

The study results indicate that the animals treated with 1000 mg/ kg of *T. alata* had the highest mean follicle-stimulating hormone levels while the animals treated with sildenafil had the lowest. Regarding the mean levels of follicle-stimulating hormone, the study notes that there was a significant (p <0.001) increase in the mean follicle-stimulating hormone levels in the animals which were treated with 500 mg/ kg and 1000 mg/kg of *T. alata* compared to those that received distilled water, sildenafil and 250 mg/ kg of *T. alata*, respectively.

Effect of *T. alata* on luteinizing hormone levels

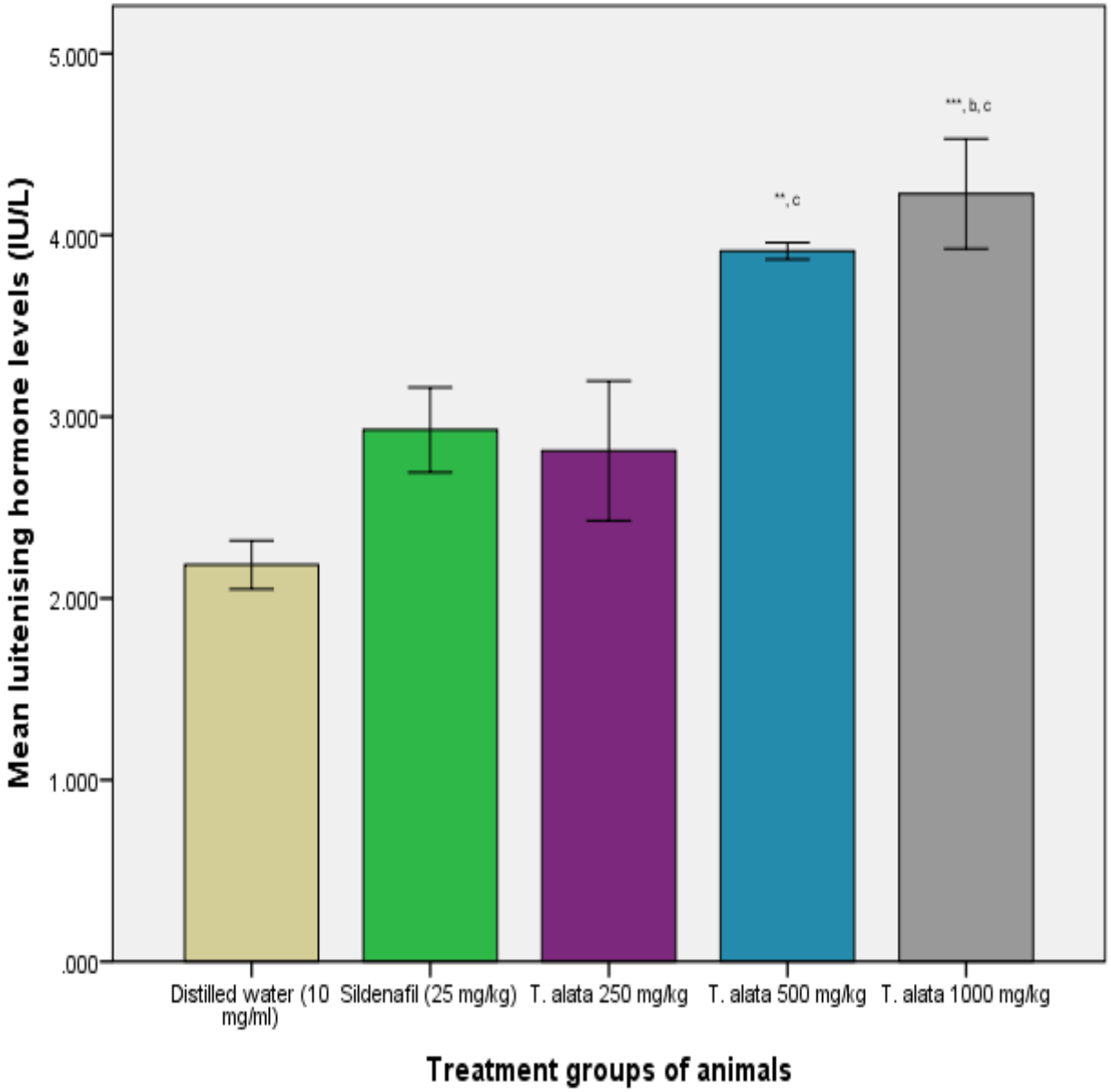

Figure 3: shows mean luteinising hormone levels (IU/L). Results are expressed in Mean ± SEM. n = 5. * = p < 0.05 vs distilled water, b = p < 0.05 vs sildenafil, c = p < 0.05 vs *T. alata 250* mg/kg

Study results from Figure 3 above indicate that the animals treated with 1000 mg/ kg of *T. alata* had the highest mean luteinizing hormone levels while the animals treated with distilled water had the lowest.On the levels of luteinizing hormone, the study noted that there was a significant ($p = 0.001$) and ($p < 0.001$) increase in the mean luteinizing hormone levels in the animals that were treated with 500 mg/ kg of *T. alata* compared to those that received distilled water, and 250 mg/ kg of *T. alata* ($p = 0.040$). Significant increases in the mean luteinizing hormone levels were also seen in the animals that were treated with 1000 mg/kg of *T. alata* as opposed to those that received distilled water, sildenafil ($p = 0.012$) and 250 mg/kg of *T. alata* ($p = 0.006$) respectively.

Effect of *T. alata* on sperm motility, sperm count and sperm viability in male Wistar rats

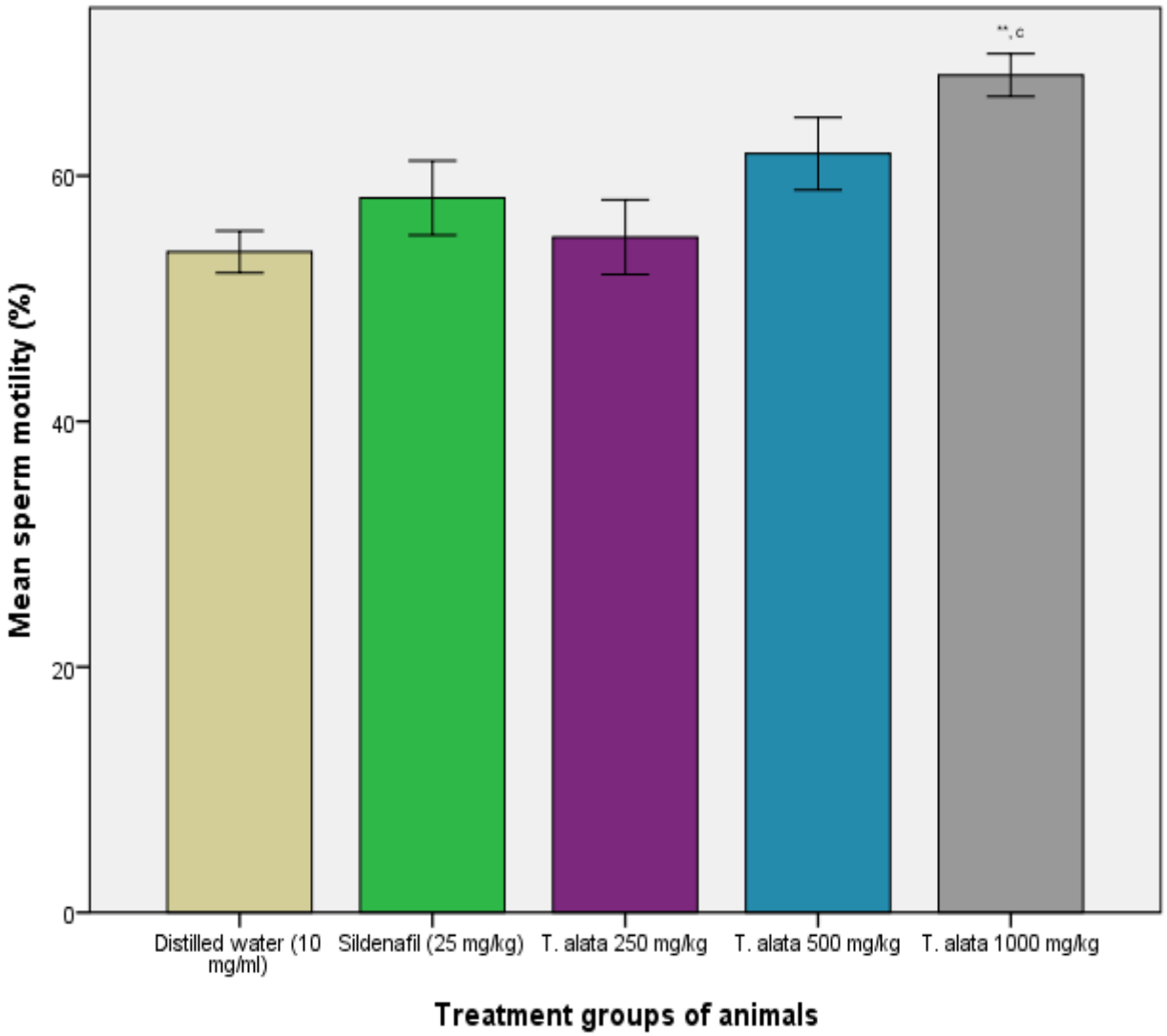

Figure 4showsthe mean percentage of sperm motility. Results are expressed in Mean ± SEM. n = 5. * = p < 0.05 vs distilled water, c = p < 0.05 vs *T. alata* 250 mg/kg

The study results note that the mean sperm motility was highest in the animals treated with 1000 mg/kg of *T. alata* and lowest in animals treated with distilled water. A significant ($p = 0.006$) and ($p = 0.013$) increase in sperm motility was noted between the animals treated with 1000 mg/kg of *T. alata* and the animals that were treated with distilled water and 250 mg/kg of *T. alata* respectively.

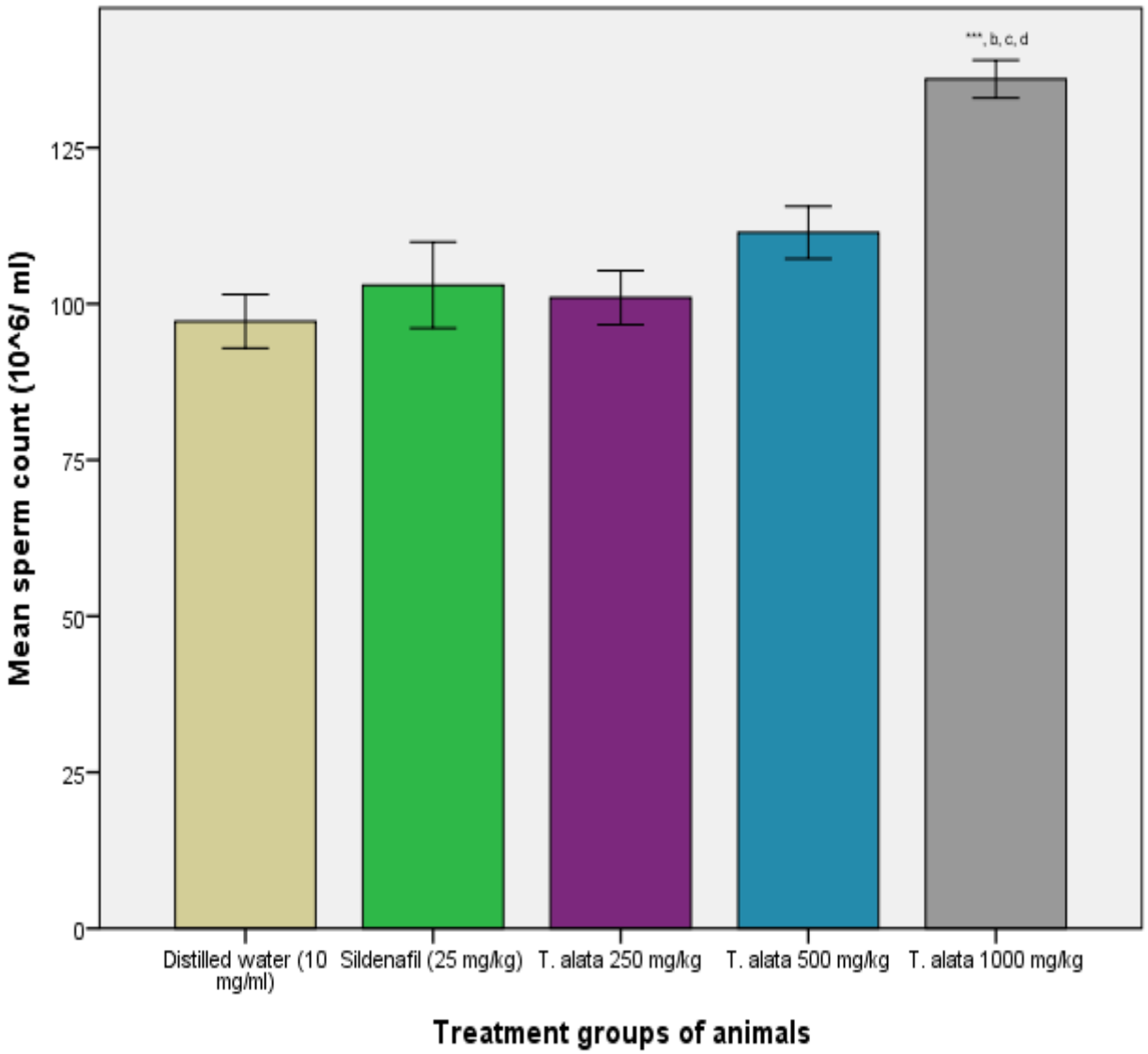

Figure 5showsthe mean sperm count. Results are expressed in Mean ± SEM. n = 5. * = p < 0.05 vs distilled water, b = p < 0.05 vs sildenafil, c = p < 0.05 vs *T. Alata*250 mg/kg, d = p < 0.05 vs *T. Alata*500 mg/kg

According to the study results in Figure 5, the mean sperm count was highest in animals treated with 1000 mg/ kg of and lowest in animals treated with distilled water. Significant (p <0.001), (p = 0.001), (p <0.001) and (p = 0.012) increases in sperm count were seen in the animals which were treated with 1000 mg/kg of T. alata as compared to those treated with distilled water, sildenafil, 250 mg/ kg and 500 mg/ kg of T. alata respectively.

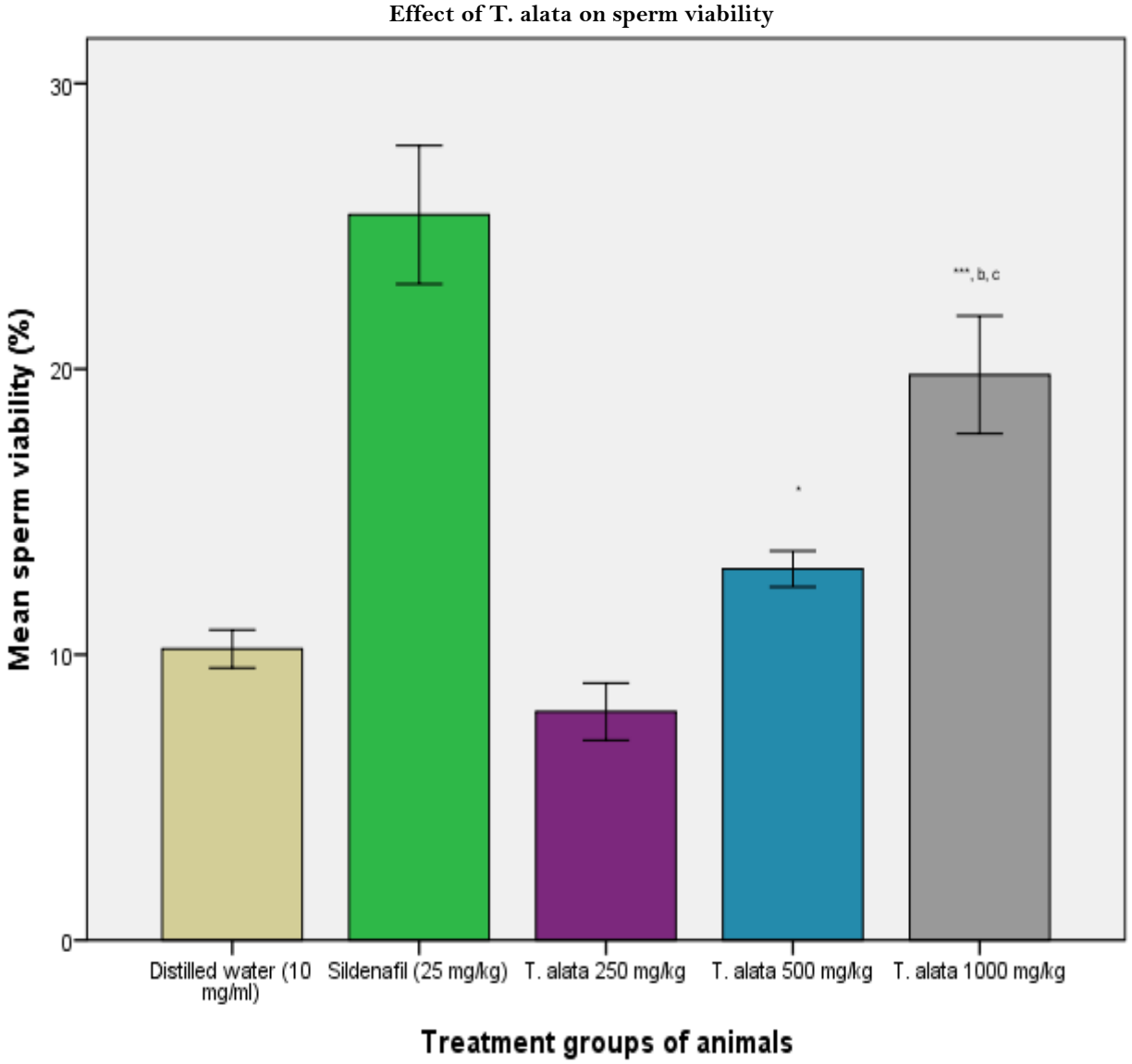

Figure 6: shows the mean percentage of sperm viability. Results are expressed in Mean ± SEM. n = 5. * = p < 0.05 vs distilled water, c = p < 0.05 vs *T. alata* 250 mg/kg

The study findings indicate that animals treated with sildenafil had the highest mean sperm viability while those treated with 250 mg/kg of *T.* alata had the lowest. The study also noted a significant (p = 0.025) and (p <0.001) increase in sperm viability between the animals treated with 500 mg/ kg and 1000 mg/ kg of *T. alata* with those that received distilled water. Furthermore, there was also a significant (p = 0.001) and (p = 0.013) increase in sperm viability in the animals treated with 1000 mg/kg of *T. alata* and those that received sildenafil and 250 mg/ kg of *T. alata* respectively.

Effect of *T. alata* on mounting and intromission frequency in male Wistar rats

Effect of *T. alata* on mounting frequency

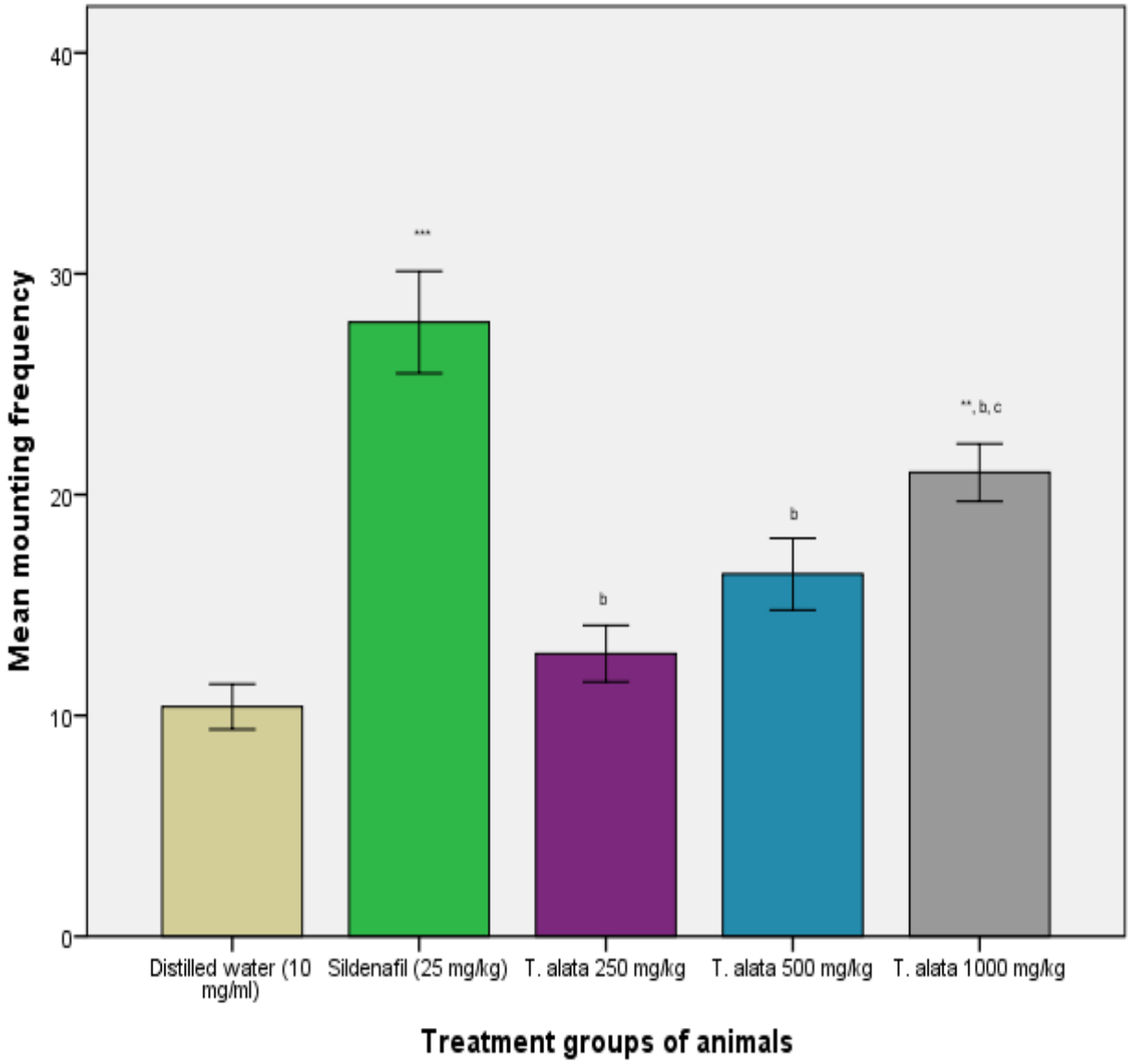

Figure 7: shows the mean mounting frequency. Results are expressed in Mean ± SEM. n = 5. * = p < 0.05 vs distilled water, c = p < 0.05 *vsT. alata* 250 mg/kg

According to the study results, the mounting frequency was highest in the animals that were treated with sildenafil and lowest in those treated with distilled water. The study results indicate that there were significant (p <0.001) and (p = 0.001) differences between the animals treated with distilled water, and those treated with sildenafil and 1000 mg/ kg of T. alata respectively. Significant (p <0.001), (p <0.001) and (p = 0.044) differences in the mean mounting frequency were also reported between the animals treated with sildenafil and those treated with 250 mg/ kg, 500 mg/ kgand 1000 mg/ kg of T. alata. There was also a significant (p = 0.011) increase in the mean mounting frequency of the animals treated with 250 mg/ kg of T. alata and those treated with 1000 mg/kg of T. alata.

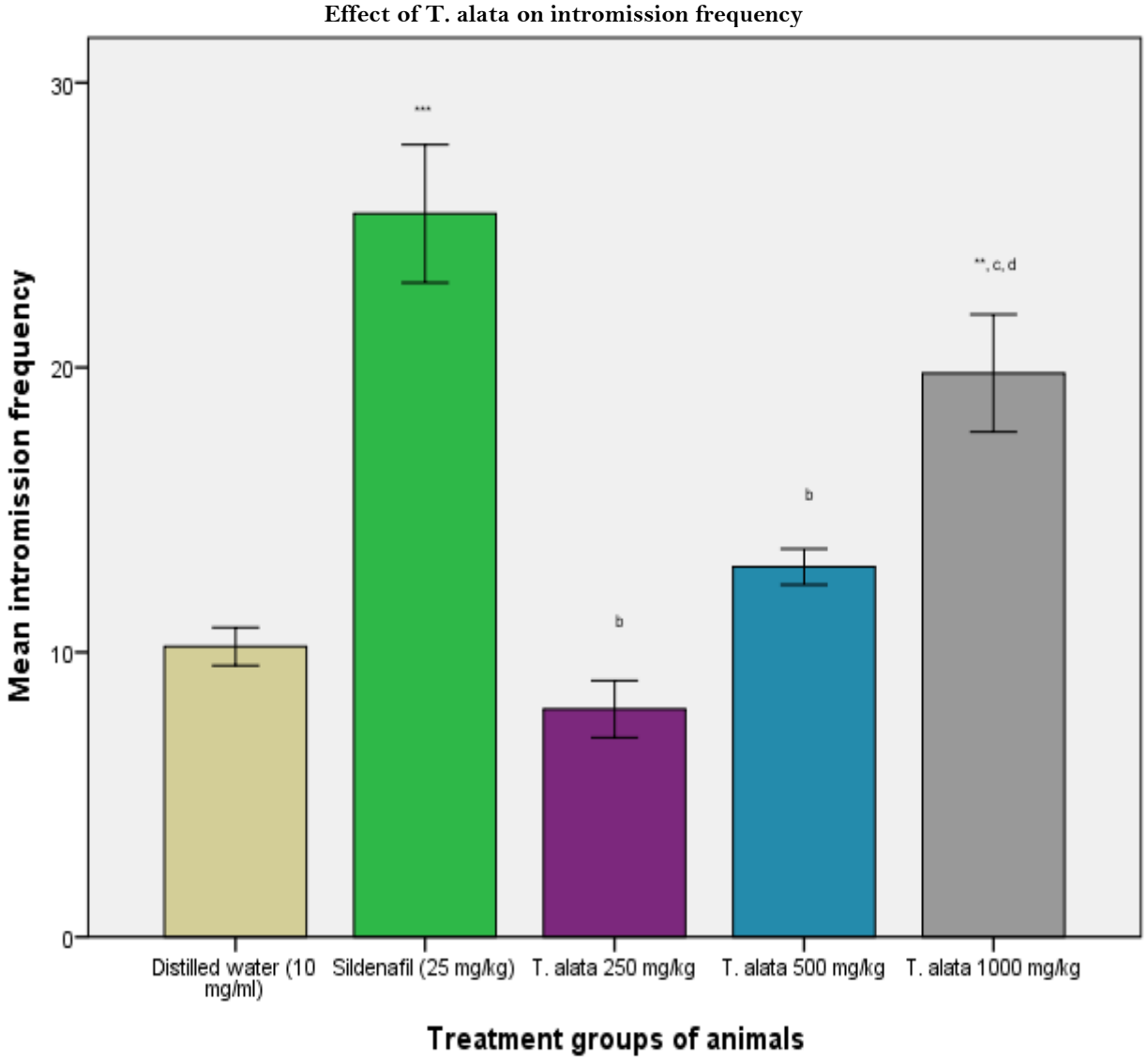

Figure 8: shows mean intromission frequency. Results are expressed in Mean ± SEM. n = 5. * = p < 0.05 vs distilled water, b = p < 0.05 vs sildenafil, c = p < 0.05 *vs T. alata* 250 mg/kg, d = p < 0.05 vs *T. alata* 500 mg/kg

Intromission frequency was noted to be highest in the animals which were treated with sildenafil and lowest in those treated with 250 mg/ kg of T. alata. A significant (p <0.001) and (p = 0.002) decrease in the mean intromission frequency was noted among the animals treated with distilled water and those treated with sildenafil and 1000 mg/ kg of T. alata respectively. The study also noted a significant (p <0.001) and (p <0.001) decrease in the mean intromission frequency between animals treated with sildenafil and those treated with 250 mg/kg and 500 mg/ kg of T. alata, respectively. It was also noted that there was a significant (p <0.001) and (p = 0.039) increase in the intromission frequency in animals treated with 1000 mg/ kg of T. alata and those treated with, 250 mg/ kg and 500 mg/ kg of T. alata.

DISCUSSION

Many plants have been used as potential aphrodisiacs by local communities and cultures in Uganda [14] some of which have been shown to affect the hypothalamus-pituitary-gonadal [27]. However, published literature suggesting the use of *T. alata* as a potential agent to alleviate premature ejaculation did not provide scientific evidence to support this reasoning [28]. To scientifically understand the basis of the claims made by local communities, we investigated the effects of aqueous leaf extract of T. alata on male reproductive parameters in male Wistar rats. T. alata contains Flavonoids, Saponins, Glycoside steroids, and phenolic substances such as caffeoylmalic acid, feruloylmalic acid, and tannins [28]. The effects of these bioactive substances on reproductive parameters involve various pathways [29]. In this study, treatment of male rats with aqueous leaf extract of *T. alata* significantly enhanced the selected reproductive parameters and sexual behaviour of male rats at 500 mg/kg and 1000 mg/kg compared to the control group (distilled water). Clinical toxicity symptoms, such as salivation, weight loss, and change in the appearance of hair and salivation, were not observed during any period of the experiment. Similarly, no mortality or behavioural, neurological, or autonomic profile changes were observed in rats treated with up to the highest dose of 2000 mg/kg body weight. This reveals that short-term use for this purpose is safe. The increment in Mounting and Intromission Frequency following the administration of aqueous leaf extract of *T. alata* at a dose of 1000 mg/kg body weight suggests enhanced libido [29]. This increase in libido could have been due to anterior pituitary hormones and serum testosterone, consequently triggering the dopaminergic pathway, thereby increasing sexual desire and behaviour [29]. Mount and intromission frequency are valid indicators of libido, vigour, and potency [21]. The number of mounts reflects sexual desire and motivation, while intromission frequency reflects erection efficiency and activation of ejaculatory reflexes [29]. The intromission frequency increased due to the coordinated action of the penile muscles to maintain adequate erection, which serves as a strong indicator that the mechanism of penile erection is activated [21]. On this account, one may suggest that the aqueous leaf extract could increase potency by sustaining erection, thus elevating the number during intromission [30, 31].The presence of alkaloids and saponins in phytochemical analysis could be the causative agents of penile erection via different mechanisms, such as inducing vasodilation of blood vessels (ergogenic effect) [31] or by activation of acetylcholine-induced nerve stimulation, leading to an increase in the second messenger cyclic guanosine monophosphate [30]. The results obtained in this study show that the aqueous leaf extract of *T. alata* can enhance sexual competence and conform to traditional claims [14]. Furthermore, the sexual behaviour exhibited in this study could be due to the bioactive substances in T. alata, such as flavonoids, which modulate the synthesis of neurotransmitters in the brain responsible for arousal, including serotonin and dopamine [32]. Studies have reported that neurotransmitters of the limbic system responsible for reward, such as dopamine and serotonin for depression, play a critical role in sexual desire and behaviour [33]. Flavonoids have been reported to modulate the dopaminergic pathway in the central nervous system, consistent with the notion that T. alata is effective in enhancing sexual activity [29]. .Notably, there was a marked increase in the weight of the sex glands, primarily the cauda epididymis, at different doses in groups III, IV, and V after 30 days which could suggest an improvement in glandular function, perhaps because of an increase in the availability of androgens influenced by the aqueous leaf extract of *T. alata*, thereby enhancing the secretory function of the gland [34]. Indeed, as shown in earlier studies, these organs require the presence of sex hormones like Testosterone, FSH and LH to grow and proliferate [35]. A marked increase in the sperm count of the cauda epididymis which is the natural reservoir of sperms may be because maturation and transit of spermatozoa takes place here so there's marked enrichment of nutritive substances [35]. A Study by Koloko *et al.*, [29] outlined the presence of substances such as Lactoferrin, Lipocalin, and Glutathione 5 peroxidase which are present in epididymal fluid and are essential components for the physiological functioning and morphology of sperm. It is therefore coherent to suggest that these substances could play a role in sperm maturation; nevertheless, more research is needed to precisely identify the proteins and their role. Shah *et al.*, [35] further showed that a marked increase in sperm motility and viability with a progressive increase in the dosage of the extract, which is a good indicator of the fertilization potential of spermatozoa. Sperm concentration increased markedly in the dose groups compared to the control group, which suggests that the physiological formation of spermatozoa is influenced by hormones of the hypothalamic-pituitary-testicular axis [36]. The increase in the sperm count, good viability and morphology score in the 1000 mg/kg dosage group as compared to the negative control could be due to the presence of polyphenols in the aqueous extract which has been reported as anti-oxidative properties which impede the formation of Reactive Oxygen species (ROS) and studies by Desai *et al.*, [36] have identified Caffeoylmalic acid as a strong scavenger for ROS hence the recorded increases sperm concentration. Flavonoids have also been documented to be strong antioxidative agents that improve oxidative-related testicular damage and stimulate

androgenesis, essentially promoting spermatogenesis and testicular integrity[37]. The administration of the aqueous leaf extract of T. alata on spermatogenesis could be linked to its ability to enhance the secretory functions of the male sex organs through hormonal feedback mechanisms[38]. This mechanism involves the hypothalamus, pituitary gland, and gonads, which secrete gonadotropin-releasing hormone synthesized in the hypothalamus, inducing the secretion of FSH and LH from the anterior pituitary gland[39]. FSH stimulates spermatogenesis by acting on Sertoli cells, whereas LH activates Leydig cells to cause testosterone release. Although the direct action of the T. alata leaf extract on the hypothalamus-pituitary-gonad axis is not known, it is evident that the increase in testosterone in the dose groups induced an increase in sperm production [40].

CONCLUSION

The study shows that the aqueous leaf extract of *T. alata* appears to improve selected reproductive parameters and in male wistar rats. The aphrodisiac potential of the extract was pronounced at a dose of 1000mg/kg bw where the animals showed the highest scores in relation to selected parameters. Overall, the efficacy of the treatment was found to be optimum at 30days and the efficacy of the plant was due to presence of bioactive substances like Flavonoids, Alkaloids, Saponins and glycoside phenols which act either centrally or by peripheral means. This has demonstrated that the results from this study can be extrapolated to humans which may result in changes observed in male wistar rats.

RECOMMENDATIONS

The current results show that the aqueous leaf extract of *T. alata* enhanced the selected reproductive parameters through various mechanisms either by central or peripheral means, however, the exact mechanisms and pathways that were involved remain unknown. To carry out the isolation and characterization of the aphrodisiac principle(s) in the plant extract. To determine the actual mechanism of action of the crude extract (aqueous and ethanolic) and elucidate the pathway(s) involved. To carry out experiments involving the potential of extracting certain reproductive ailments like testicular toxicity.

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