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Original Research Article

Ethanol Leaf Extract of *Cyathula prostrata* Enhances Spermatogenesis in Wistar Rats Via Expression of AKAP4, DAZL and PRM2

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ABSTRACT

Cyathula prostrata (Linn.) Blume-a member of the amaranth family-is reportedly used in traditional medicine as a fertility enhancer in males. This work investigated *C. prostrata* extract effects on the expression of testes-specific genes; deleted in azoospermia-like (DAZL), protamine 2 (PRM2) and A-kinase anchoring protein-4 (AKAP4). Normal adult male Wistar rats (n=48) were sorted into 6 categories of eight animals each. Rats orally received graded doses of *C. prostrata* ethanol leaf extract over a 72-day period; control animals received distilled water. Testes of rats were harvested and subjected to histological examination. RNA extracted from animal testes were purified, and the genes amplified using RT-PCR. Semen indices, including count, morphology and motility were assessed using microscopy. *C. prostrata* extracts improved sperm count and motility in rats, relative to the control. Morphological assessment revealed an increase in the population of normal cells and a decrease in abnormal cell numbers in all treatment groups, compared to the control. DAZL gene exhibited a consistently high expression across all groups. Higher doses of *C. prostrata* (200-300 mg/kg bw), however, caused a downregulation in the expression of AKAP4 and PRM2 genes. Micrographs of testes from animals showed normal (50 and 100 mg/kg bw groups), enhanced (250 mg/kg bw group) and deleterious (200 and 300 mg/kg bw groups) spermatogenic changes. *C. prostrata* exhibits a positive effect on spermatogenesis by preserving the expression of testes-specific genes and enhancing count, motility and morphology of sperm cells, thereby suggesting its suitability for use as a fertility enhancer in males.

Keywords: *Cyathula prostrata*, AKAP4, DAZL, PRM2 and spermatogenesis.

Introduction

The ability of man to maintain optimal sexual performance, especially with decline in age is important for both reproduction and sexual satisfaction.[1] Unfortunately, there is a rise in the incidences of sexual dysfunctions globally, indicating a serious challenge for male fertility and sexual performances.[2] Sexual dysfunction is a problem that can manifest at any period (excitement, arousal, orgasm and resolution) of the sexual response cycle, consequently preventing an individual or couple from experiencing sexual satisfaction.[2-4] In males, sexual dysfunctions could take the form of low libido as well as ejaculation and erectile abnormalities.[5] Globally, approximately 30 million men are affected by one or more forms of sexual dysfunction.[6] About 15% of couples worldwide face sexual dysfunction problems, with nearly 40-50% resulting from male related factors.[2] The problem of male sexual dysfunction cannot be overemphasized with the global prevalence of erectile dysfunction (one form of male sexual dysfunction) expected to reach about 300 million men in 2025.[7]

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Sexual dysfunction represents a serious challenge to the psychological, emotional and social well-being of affected individuals.[8,9] The challenges arising from sexual frailty include, but are not limited to, low self-esteem, depression and conjugal dissatisfaction; which may eventually lead to divorce.[9,10] Sexual dysfunctions may stem from a variety of factors including psychogenic (anxiety and depression), vasculogenic (atherosclerotic penile arterial disease, occlusion), endocrine/metabolic (diabetes mellitus, hypogonadism, hyperprolactinemia, renal failure), neurogenic (spinal cord injury, multiple sclerosis) and iatrogenic (antidepressant, thiazide diuretics, non-selective α/β2 blockers).[2,11]
Conventional evaluation of sexual dysfunction could involve the assessment of hormones (testosterone, follicle-stimulating hormone (FSH), thyroid, and luteinizing hormone (LH)) and semen alongside a detailed physical examination.[5,12,13] However, there is a growing interest in employing functional indicators of spermatogenesis for the assessment of male sexuality.[14] Testicular enzymes such as alkaline phosphatase (ALP), lactate dehydrogenase (LDH), acid phosphatase (ACP) and γ-glutamyl transferase (GGT) have been reported to play key roles in spermatogenesis and sperm maturation.[6,15,16] Also, distinct mRNAs exclusively expressed by spermatogenic cells at different stages of maturation can serve as excellent molecular markers for spermatogenesis and indicators of sexuality.[17-19] The intricate process of spermatogenesis involves the meiotic transformation of spermatogonia (proliferative germ cells in the testis) into spermatids which subsequently metamorphosize into spermatozoa.[20]
In order to ensure a successful outcome for males with impaired reproductive abilities, proper diagnosis must be matched with effective management of the condition. Owing to concerns arising from the use of drugs such as toxicity, adverse reaction and resistance,

medicinal plants are receiving more global attention in the fight against several health problems including male sexual dysfunction.[21] Several studies have reported that plants possess aphrodisiac properties and enhance semen quality and hormonal status in males with sexual dysfunction.[6,22-24] The usage of medicinal plants as aphrodisiacs may have certain advantages such as affordability, accessibility and lower side effects over conventional aphrodisiacs.[6]

Cyathula prostrata (Linn.) Blume-a member of the amaranth family-is an annual and branched shrub which grows to about one meter in height.[25] *C. prostrata* is native to many tropical countries, including Ghana, Cote d'Ivoire, Cameroon and Nigeria.[23] It is also naturally occurring in subtropical areas such as China and Australia.[26] It is well-known for its numerous therapeutic benefits, including anti-inflammatory and antioxidant,[27] antidiabetic[28] and antimicrobial.[29] *C. prostrata* is also used as a local remedy for enhancing male sexual performance.[30] Despite the plethora of research assessing the pharmacological activities of this plant, there is no evidence of its impact on the expression of genes crucial to spermatogenesis.

In this study, the spermatogenesis status of rats administered *C. prostrata* leaf ethanol extracts were evaluated for the expression of three stage-specific gene transcripts in the testes alongside histopathological examination. These testis-specific genes were chosen based on their expression stage; deleted in azoospermia-like (DAZL) gene expression starts at the spermatogonial level,[31,32] protamine 2 (PRM2) gene expression is a marker for early spermatid and mature sperm presence,[18] while A-kinase anchoring protein-4 (AKAP4) gene expression occurs solely in round and elongated spermatids.[33] AKAP4, a member (X-linked) of the AKAP4 gene family, codes for the most abundant protein present in the sperm fibrous sheath of all mammalian organisms.[34]

Materials and Methods

Collection and authentication of plant materials

C. prostrata plant was obtained in July, 2019 from the farmland surrounding the Department of Botany, University of Lagos, Lagos State. Plant sample was authenticated by the Herbarium section of the Department of Botany, University of Lagos and issued a voucher number of LUH 6498.

Processing of C. prostrata

The leaves of *C. prostrata* were thoroughly washed, separated and air-dried. The dried leaves were shredded into pieces and further crushed into powder using an electric blender (Phillips, UK). The powdered leaves samples obtained were weighed using a digital weighing scale (Mettler, USA) and stored separately in airtight containers until needed.[30]

Extraction of C. prostrata leaves

The powdered leaves (100 g each) were extracted with 1000 mL of 60% ethanol using a Soxhlet extractor. The ethanol extract was filtered using Whatman filter paper 1 to get clear filtrates. The filtrates obtained were concentrated using a water bath (PioWay, PRC) at 40°C for 84 hours until a dark semisolid material was obtained. After concentrating, the semisolid materials obtained were stored at 4ºC, until use.[35]

Experimental animals

Forty-eight (48) adult normal male Wistar rats (with weights between 80 -120 g) were acquired in July, 2019 from a private breeder in the University of Ibadan and sorted into 6 categories (n=8 per group). Animals were housed in cages and allowed to acclimatize in the animal house of the Department of Biochemistry, Lagos State University for 8 days prior to oral administration of extracts which lasted for 72 days due to the duration of spermatogenesis in rats.[36] Experiment was conducted with approval of Institutional Research and ethics guidelines for animal experiments (ethical approval number: AREC/2019/012).

Animal grouping

Dosage of extracts administered to animals in each group was based on a previous study [30] and data from review of literature.[37,38] Control animals (group A) were given equal amount of distilled water. Rats across all groups were supplied food and water *ad libitum* all through the investigation period.

Group A: Control, distilled water (1 mL/kg body weight)

Group B, C, D, E and F: received 50, 100, 200, 250 and 300 mg/kg body weight of the *C. prostrata* leaf ethanol extract per os (orally) respectively.

Sample collection and preparation

Rats from six experimental groups (A to F) were sacrificed by cervical dislocation and testes harvested. Testes were rinsed in phosphate-buffered saline (PBS) pH 7.5, and immediately suspended in 5mLs of RNA later solution. All testes were stored in RNA later solution at 4°C prior to RNA extraction.

Sperm count, viability and motility assessment

The testes from the rats were carefully isolated after dissection and weighed independently using a digital weighing balance (Mettler, USA). Further, sperm cells were extracted from testes and mounted on a slide for immediate assessment of motility (expressed as percentage motile forms) under the microscope (Ceti, UK) fitted with XLI image capture software version 12 at x10 objective.[39] Sperm number and morphology were examined following staining of slides with carbol fuschin.[37]

Histology

The testes of sacrificed rats were collected and used for histological analysis. This was done as described by Ojekale *et al.* [39] Briefly, the organs were cut into 0.5 cm thick slabs and fixed in Bouin's fluid for 72 hours. They were then passed through graded alcohol, cleared in xylene, embedded in molten paraffin and blocked out. Serial sections of 5 µm thick were cut from these blocks and stained with haematoxylin and eosin stains. They were examined under light microscopes (CETI, UK) at magnifications ranging from x400 to 1000.

RNA extraction

RNA extraction was carried out on testes within 7 days of harvest. RNA was extracted using Total RNA purification Kit (Jena Bioscience, Germany). For each sample, 300 µl of lysis buffer (2-Mercaptoethanol added) was added to 50 mg of fresh testis tissue and homogenised using a glass pestle and mortar. An additional 200 µl of lysis buffer was introduced into the homogenised samples and vortexed. Then, the mixture was centrifuged at 10,000g for 10 min and the supernatant (lysate) released into a micro centrifuge tube. Isopropanol (300µl) was added to the lysate to isolate and precipitate RNA from the aqueous phase. The isolated RNA was washed twice by ethanol (75%) and purified. RNA was eluted with 50 µL elution buffer. RNA was stored at -20°C. RNA purity was assessed by gel electrophoresis (Cleaver Scientific, UK) and viewed with a UV transilluminator.

cDNA synthesis

The first-strand cDNA synthesis reaction mixture was prepared by using 5µl of the isolated RNA template, 1 µL of 5 µM Oligo (dT) primer, 0.5 µL of 500 µM dNTPs, 2 µL of 10X reverse transcriptase reaction buffer, and 1 µL of 10 U/µL of Firescript RT, 0.5 µL of 1 U/µL of RiboGrip RNase inhibitor in a total volume of 20 µL. The cDNA reactions occurred at 60°C for 30 min and enzyme activation at 85°C for 5min using Firescript RT cDNA synthesis kit (Solis Biodyne, EU). cDNA was stored at -20°C.

RT-PCR

One-step PCR amplification was carried out in a 25 µL reaction mixture, composed of 5 µL of the isolated RNA template, 0.3uL of each primer (0.6µM) (Stab Vida, Portugal), 1 µL of 400µM dNTPs (Thermos Scientific), 2 µL of Qiagen One-step RT-PCR Enzyme mix, 5µL of 1X Qiagen One-step RT-PCR buffer. Thermal cycling was

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performed at 60°C for 30min for reverse transcription, followed by an initial PCR activation at 95°C for 15min and then 30 cycles of denaturation at 94°C for 35 secs, annealing at 55°C for 35 secs and elongation at 72°C for 1 min. Negative (water) controls were included in all PCR amplifications. PCR amplicons were run on a 2% agarose gel prepared using Tris Borate EDTA (TBE) running buffer, stained with Red safe dye and visualized by UV transillumination.

The primer sequences

New primers for amplification of testes specific genes DAZL, AKAP4, PRM2 and β-Actin were designed for this study. Protein coding (cDNA) sequences for each gene was obtained from the ensemble genome browser. This sequence was used as template for primer selection using NCBI primer blast, melting temperature and estimated PCR product size are indicated in the screen shots in the appendix section. All primers were further validated using Bisearch tool.

Statistical analysis

The data obtained from this study are expressed as mean ± standard error of mean (Mean ± SEM). Variations in mean between groups were determined by one-way analysis of variance (ANOVA) and Dunnett test using GraphPad Prism 7.

Results and Discussion

In this current study, sperm indices were assessed, viz., morphology, count and motility (Table 2). Morphological assessment showed that all doses of *C. prostrata* ethanol leaf extracts increased the population of normal sperm cells. In contrast, abnormal cell numbers fell in all treatment groups, relative to the control. Although sperm motility increased across all groups, it appeared independent of *C. prostrata* extract concentration. However, a concentration-dependent increase in sperm count was recorded across the groups. This is consistent with the findings of Adelakun *et al* [40] on the aqueous crude extract of *Solanum nigrum.* Improvement in count, motility and morphology of sperm cells as noted in this study, may have resulted from the activity of phytochemical constituents inherent in plant extracts.[41] Increase in total count and motility may also be attributed to improved functionality of the epididymis[42] or elevated testosterone and gonadotropins (FSH and LH) activity, which enhances spermatogenesis.[43] Previous studies on *Rutacha lepensis* [24] and *Moringa oleifera* [22] have also revealed the positive effects of plant extracts on sperm count and production. Moreover, a recent study by Ajuogu *et al* [23] reported a significant improvement in total sperm count (but not morphology and motility) in buck rabbits treated with *C. prostrata.* RNA Isolates from animal testes with the best quality (Figure 1) were used for the RT-PCR procedure. Overall, the expression of testes specific genes (DAZL, AKAP4, and PRM2) in animals fed with 50 and 100 mg/kg of *C. prostrata* leaf ethanol extracts appeared in a similar fashion to the control group (Figure 2). This indicates that a very significant portion of seminiferous tubules from the testes in these groups contain cells in all stages of maturation in the spermatogenic series including spermatogonia, spermatids and matured spermatozoa.[44-46] However, the expression of these genes (with the exception of DAZL) diminished at all concentrations of extract beyond 100 mg/kg, indicating the presence of largely immature germ cells possibly mostly primary and secondary spermatogonia in the seminiferous tubules of animals associated to these groups.[45] Further, the most pronounced downregulation of all testes specific genes was observed at the highest extract concentration (300 mg/kg). This suggests that minimal quantity of spermatogenetic cells at all stages will be found in the seminiferous tubules of rats belonging to this group. This trend also suggests that lower (but not higher) concentrations of the extract preserved the existence of germ cells including spermatogonia (related to DAZL) as well as spermatids and mature sperms (related to PRM2 and AKAP4) in the testes of treated animals. It is possible that ample levels of secondary metabolites resulting from higher concentrations of the extract might have triggered a negative interaction with testes specific genes.

Micrographs obtained from testes of animals exposed to the highest extract dose (300 mg/kg bw) showed histological evidence suggestive of considerable spermatogenic damage in form of sloughing and germ cell loss (Figure 8). Sloughing was also observed in animals fed with 200 mg/kg bw of extract but to a lower extent (Figure 6). However, histoarchitecture of animals administered with 250 mg/kg bw of extract revealed enhanced spermatogenesis (Figure 7), while the presence of normal seminiferous tubules characterised the micrographs of testes derived from animals exposed to 50 and 100 mg/kg bw of *C. prostrata* (Figures 4 and 5), similar to the control (Figure 3).

Table 1: The Primer sequences

Gene	Primer sequence	Expected band size (bp)
β-Actin	Forward: 5'-ACTCTTCCAGCCTTCCTTC-3'	171
	Reverse: 5'- ATCTCCTTCTGCATCCTGTC-3'	
DAZL	Forward: 5'- CTCAGGAGGTTATTGGTTTTG3'	396
	Reverse: 5' GAGAAGTGAAATGCTTGTAGTC3'	
AKAP4	Forward: 5'- GAACAAGTGTGGAGAGAAGC-3'	383
	Reverse: 5'- CAAGAAGAGCACTGACCAGA-3'	
PRM2	Forward :5' TGGAGGACTATGGGAGGACA -3'	200
	Reverse: 5'ATAGTGCCACCTGCATTTCC-3'	

Table 2: Sperm indices

Parameters	Control	50	100 (mg/kg bw)	200	250	300
Morphology (%)						
normal sperm cells	70 ± 0.0	83.3 ± 3.3 ***	76.7 ± 6.7 ***	80 ± 0.0 ***	83.3 ± 3.3 ***	80 ± 0.0 ***
abnormal sperm cells	30 ± 0.0	16.7 ± 3.3 ***	23.3 ± 6.7 ***	20 ± 0.0 ***	16.7 ± 3.3 ***	20 ± 0.0 ***
Sperm motility (%)	70 ± 0.0	76.7 ± 8.8 ***	90 ± 0.0 ***	80 ± 0.0 ***	83.3 ± 3.3 ***	80 ± 0.0 ***
Sperm count ($x10^6$)	160.7 ± 36.1	108.7 ± 9.2 ***	290.3 ± 159.6 ***	379.3 ± 104.4 ***	378 ± 55.2 ***	427 ± 87.6 ***

Legend: kg (kilogram); mg (milligram); bw (body weight). Values are expressed as means ± SEM, n = 3.
Values across groups with asterisk are statistically different from the control at $p < 0.05$.

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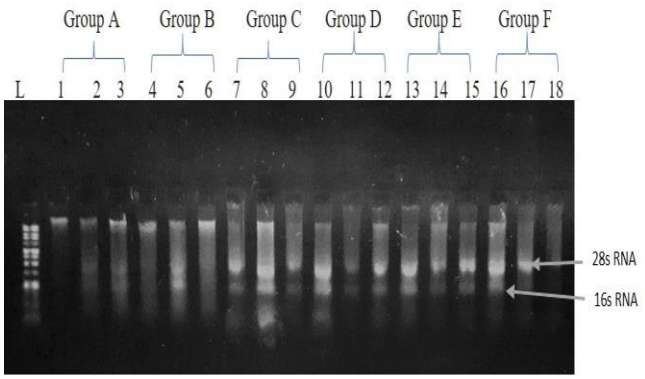

Figure 1: Total RNA quality check by agarose gel electrophoresis.

L is molecular ladder, Isolates with the best quality was used for the RT-PCR procedure. The bands revealed the presence of 16s and 28s RNAs.

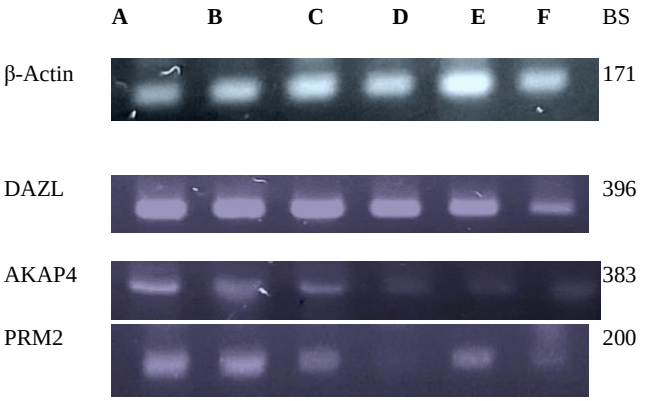

Figure 2: RT-PCR results of DAZ, AKAP4 and PRM2 genes in testes samples of *C. prostrata* treated and control rats.
The reference gene, β-actin, was used as the positive control. Lanes 1 to 6 represent experimental groups A to F. BS: the band size for each gene.

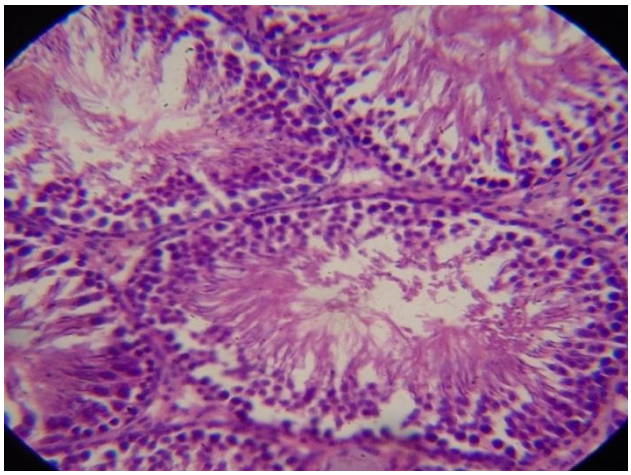

Figure 3: Micrograph from testis in GRP A (Control) animal, showing normal seminiferous tubules. All cell types of the germ series are visible and lumen are filled with mature sperm tail. H&E × 400.

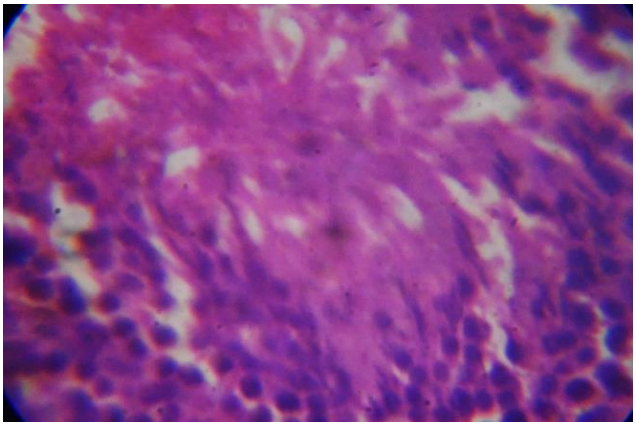

Figure 4: Micrograph from testis in GRP B animal, showing a seminiferous tubule with normal and dense cell population. Ad-luminal area is well populated with spermatozoids suggesting adequate spermiogenesis. H&E × 1000.

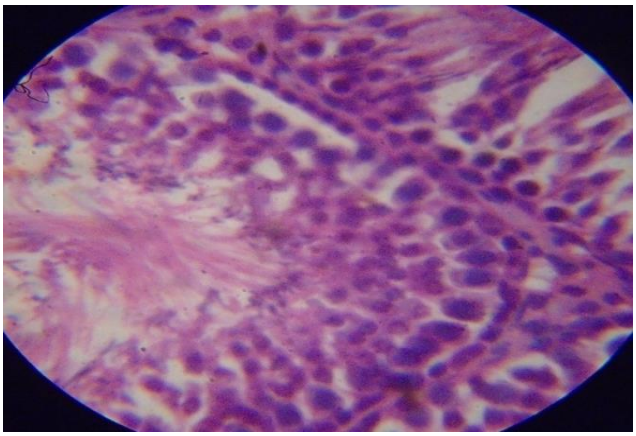

Figure 5: Micrograph from testis in GRP C animal, showing two normal tubules at different stages of spermatogenesis. Blue arrow: dark spermatogonia on the basement. H&E × 1000.

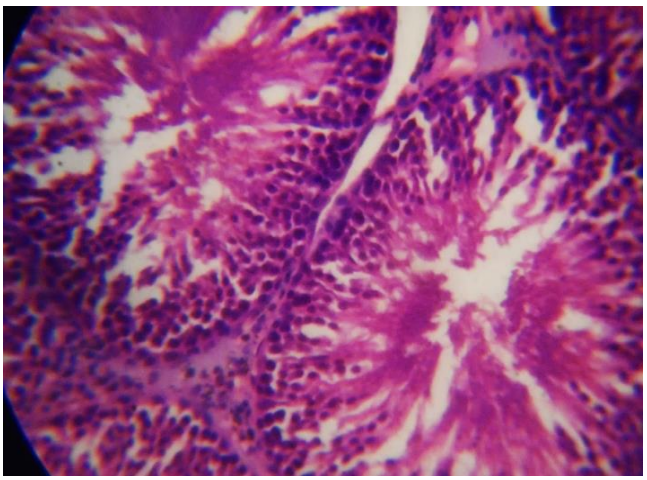

Figure 6: Micrograph from testis in GRP D animal, showing two seminiferous tubules with normal germ cell complement but having spaces indicating some sloughing and loss of cell-to-cell adhesion especially around the ad-luminal area (Blue arrow). H&E × 400.

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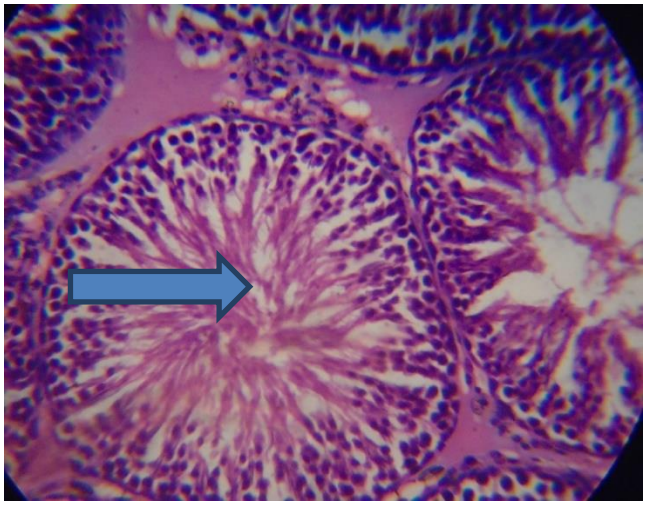

Figure 7: Micrograph from testis in GRP E animal showing seminiferous tubules with features suggesting enhanced fertility. All germ layers are visible but some amount of interstitial edema is visible (Blue arrow). H&E × 400.

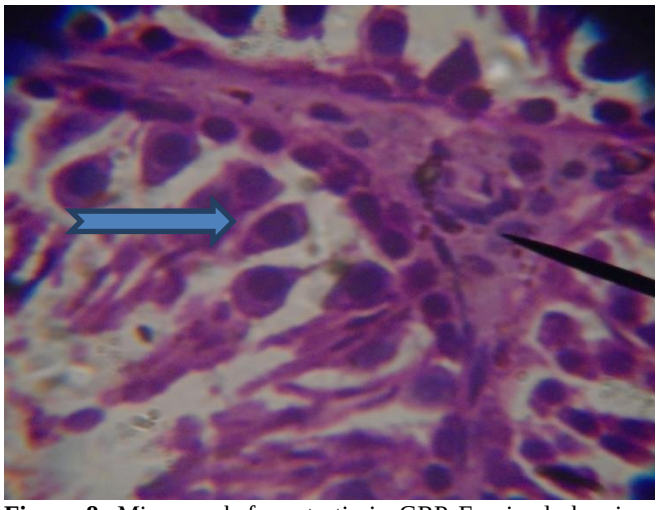

Figure 8: Micrograph from testis in GRP F animal showing two seminiferous tubules with considerable sloughing and germ cell loss especially in the basal areas. Blue notched arrow: Sertoli cell; Blue arrow: Leydig cell in interstitium. H & E, × 1000.

Conclusion

C. prostrata exhibits a positive effect on spermatogenesis by preserving the expression of testes-specific genes and enhancing count, motility and morphology of sperm cells; thereby suggesting its suitability for use as a fertility enhancer in males. There is ample room for further studies to elucidate the mechanism of action of *C. prostrata* on testes-specific genes in order to ascertain its spermatogenic efficacy.

Conflict of interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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