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Research

Effect of *Cyathula prostrata* leaf ethanol extract on spermatogenesis in male Wistar rats

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Abstract

There is a globally noticeable decline in male fertility over time, often due to changing lifestyle habits such as food, work, alcoholism, medicaments amongst others. *Cyathula prostrata* is among the folkloric plants allegedly used in the management of male infertility. This study examines the potentials of *Cyathula prostrata* on spermatogenesis via sperm count, sperm motility and morphology as markers of male fertility. Six groups of six male Wistar rats were administered graded doses (0 – 300 mg/kg body weight) of *C. prostrata* leaf ethanol extract over 56 days. The semen was analyzed for sperm parameters, blood samples were analyzed for hormones, antioxidants, hepatic and renal functions and organs (testes, kidney, liver, and heart) of the rats were collected and subjected to histological assessment. Increased testicular levels of antioxidants: SOD, Catalase, glutathione S-transferase glutathione peroxidase and hormones; FSH and LH were observed. Enhanced spermatogenesis was observed in testicular sections in groups of rats administered *C. prostrata* extract (200 – 300 mg/kg body weight). Hepatic enzymes and renal functions remained significantly unaltered. Oral administration of *Cyathula prostrata* leaf extract (250 – 300 mg/kg body weight) has potentials of enhancing bodily functions linked to spermatogenesis.

Keywords: Cyathula prostrata, spermatogenesis, sperm, hormones, antioxidant, hepatic, renal

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Introduction

Fertility is the ability to establish a clinical pregnancy among fertile partners having unprotected sex (Zegers-Hochschild et al., 2017). Reproductive ability in males entail the production of semen containing quality spermatozoa in the adequate quantity, together with the desire and ability to mate (Oyeyemi et al., 2008). Conversely, infertility is the inability to conceive after 12 months of sexual practice without using contraception (Bashir et al., 2009). Infertility is one of the problems of human society (Dalouchi et al., 2014), and about 10-15% of couples have experienced some forms of infertility problems, of which 50% of these problems are due to male factors (Henkel et al., 2005, Kashani et al., 2012, Bianchi et al., 2018). Male infertility is not a defined clinical syndrome, (Poongothai et al., 2009, Kefer et al., 2009), it is a collection of different conditions with variety of etiologies and varying diagnosis. In men, oligozoospermia characterized by abnormally low concentrations of spermatozoa in the semen (Filipponi and Feil, 2009), asthenozoospermia characterized by low sperm motility (Wang et al., 2009), teratozoospermia characterized by the presence of spermatozoa with abnormal morphology (De Braekeleer et al., 2015) and azoospermia characterized by the absence of sperm in the ejaculate (Wosnitzer and Goldstein, 2014) amongst others are the main causes of infertility, account for 20%-25% of cases. Other factors including drug treatment, chemotherapy, toxins, oxidative stress, air pollutions and insufficient vitamins intake may hinder spermatogenesis and quality sperm production (Khaki et al., 2009, Makker et al., 2009, Poongothai et al., 2009, Dalouchi et al., 2014).

Spermatogenesis is a continuous process in which undifferentiated spermatogonia are differentiated into spermatocytes and spermatids through two rounds of meiotic division which finally produces mature spermatozoa (Luk et al., 2014). Semen analysis plays a key role in the diagnosis of male infertility, it assesses the formation and maturity of sperm as well as how the sperm interacts in the seminal fluid (Organisation, 1999, Poongothai et al., 2009, Murray et al., 2012, Kumar and Singh, 2015). Spermatogenetic failure, including oligospermia and azoospermia are two of the leading causes of male infertility (Bener et al., 2009). Several factors can interfere with spermatogenesis and reduce sperm quality, quantity and production (Khojasteh et al., 2016). Factors such as drug treatment, chemotherapy, toxins, air pollution and insufficient vitamins intake may pose harmful effects to spermatogenesis and normal sperm production (Khojasteh et al., 2016).

Different treatments like drugs, surgery, hormonal and laboratory methods are available to treat infertility (Sinclair, 2000, Dabaja and Schlegel, 2014). Owing to reasons such as cost, scarcity and adulteration of orthodox medicines, quite a number of people now patronize herbal remedies as therapy for ailments in most parts of Nigeria and indeed other parts of the World at large (Ojekale et al., 2015, Ajuogu et al., 2020). *Cyathula prostrata* of the *Amaranthaceae* family is an annual, branched herb or shrub with a height of approximately 1 metre or more, found in tropical Africa, America and Asia (Nagar, 2011). Its medicinal applications include its use for treatment of sores, articular rheumatism, dysentery, wounds and urethral discharges (Burkill, 1995, Richard et al., 2017). Mostly, oral tradition amongst traditional herbal practitioners suggest male fertility enhancing properties of the plant Some medicinal properties of the plant have

been affirmed and documented, viz; antimicrobial, anti-inflammatory, antihypertensive and antioxidant activity (Oladimeji et al., 2014, Sonibare and Olatubosun, 2015, Ojekale et al., 2016). This study was designed to investigate the effect of *C. prostrata* leaf extract on spermatogenesis in male wistar rats.

2.0 Materials and methods

2.1 Collection and authentication of plant materials

The whole plant of *C. prostrata* was collected from the farmland surrounding the Department of Botany, University of Lagos (UNILAG), Lagos State. The plant was authenticated by the Herbarium section of the Botany Department of the University of Lagos and issued a voucher number of LUH 6498.

2.3 Processing of *Cyathula prostrata*

The leaves of *C. prostrata* were thoroughly washed, separated and air-dried. The dried leaves were reduced to coarse form by shredding and further pulverized to very fine powder particles with an electrical 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 (Ojekale et al., 2016).

2.4 Extraction of *Cyathula 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 (Vijayakumar Mayakrishnan, 2014).

2.5 Experimental animals

Forty-eight adult (48) male Wistar rats weighing between 80 - 120 g were obtained from a private breeder in the University of Ibadan and used for this study. The animals were sorted and divided into 6 groups (n=6), housed in cages and allowed to acclimatize in the animal house of the Department of Biochemistry, Lagos State University for 8 days before the commencement of extract administration which lasted for 56 days due to the duration of spermatogenesis in rats (De Kretser et al., 1998). Experiment was conducted with approval of Institutional Research and ethics guidelines for animal experiments.

2.6 Experimental design

The dosage selection was based on the previous work (Ojekale et al., 2016) and information from review of literature (Etuk and Muhammad, 2009, Mohammadi et al., 2013). Animals in group A (control) were given equivalent volume of distilled water. All the animals were allowed access to food and water *ad libitum* throughout the duration of the study.

Group A: Control, distilled water (1 mL/kg body weight)
Group B: 50 mg/kg bodyweight of the *C. prostrata* leaf ethanol extract per os.
Group C: 100 mg/kg bodyweight of the *C. prostrata* leaf ethanol extract per os.
Group D: 200 mg/kg bodyweight of the *C. prostrata* leaf ethanol extract per os.
Group E: 250 mg/kg bodyweight of the *C. prostrata* leaf ethanol extract per os.
Group F: 300 mg/kg bodyweight of the *C. prostrata* leaf ethanol extract per os.

On the 56^{th} day, the animals were fasted overnight, and the weight of each rat was measured on the 57^{th} day. The animals were

sacrificed under ketamine anesthesia (Akaahan et al., 2016)

2.7 Collection of blood samples

Blood samples were collected through cardiac puncture using 2 ml sterilized syringes. The collected blood was allowed to clot and serum was obtained after centrifugation at 3000 rpm (Techmel and Techmel 412B, USA) for 15 minutes. Serum obtained was used in hormones, liver and kidney function analyses.

2.8 Epididymis sperm count, viability and motility

The testes and epididymis from the rats were carefully isolated after dissection and weighed using a digital (Mettler, USA) weighing balance independently. From the epididymis, sperm cells were extracted, mounted on a slide and their motility were assessed immediately under the microscope (Ceti, UK) fitted with XLI image capture software version 12 at ×10 objective (Ojekale et al., 2015). The motility assessment was expressed as percentage motile forms. The slides were stained with carbol fuschin and the sperm number and morphology examined (Etuk and Muhammad, 2009).

2.9 Hormonal analyses

The serum from the blood samples collected from different animals were used for hormone analyses. The hormones (testosterone, follicle stimulating hormone, and luteinizing hormone) were analyzed using LifeSpan BioSciences, Inc. (Seattle, USA) immunoassay enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's procedures.

2.10 Antioxidant assay

The testicular homogenate (rat testicles homogenized (in ice) in 0.25M Sucrose solution using a teflon tipped homogeniser) was used in assaying for different antioxidant parameters using spectrophotometric methods. Protein concentration of testicular homogenate was determined by means of the Biuret method as described by (Gornal et al., 1949), . Super oxide dismutase (SOD) activity was determined by the method of (Misra and Fridovich, 1972), Catalase activity was determined by the method of (Clairborne, 1995). The method of (Mitchell et al., 1974) was used in estimating the level of reduced glutathione (GSH). Glutathione-S-transferase activity was determined according to (Habig et al., 1974), lipid peroxidation was determined according to (Varshney and Kale, 1990). Glutathione reductase activity was determined as described.(Carlberg and Mannervik, 1975) Glucose -6-phosphate dehydrogenase was determined (Beutler, 1984). The method of (Jollow et al., 1974)was followed in estimating the level of reduced glutathione (GSH). The method of (LaDue and Wroblewski, 1955)was used to assay for lactate dehydrogenase.

2.11 Liver function test

The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), direct bilirubin, lactate dehydrogenase and total protein were measured in the serum for the determination of liver function using Randox kits (Randox laboratories, UK). Each parameter was evaluated using the manufacturer's procedure.

2.12 Renal function test

The levels of serum creatinine and urea were measured for the determination of kidney function using Randox kits (Randox laboratories, UK). Each parameter was evaluated using the manufacturer's procedure.

2.13 Weight of organs

dissection of animals across each group. The organs were blotted with filter paper and weighed independently using an Adam digital balance (USA) and compared to the control group to determine if there was any difference in the organ's weight.

2.14 Histology

The heart, kidney, liver and testes of the sacrificed rats were harvested and used for histological analysis. The was done as described by (Ojekale et al., 2015). 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 5µm 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.

The heart, kidney, liver and testes of experimental animals were isolated after

2.14 Statistical analyses

The data obtained from this study are expressed as mean ± standard error of mean (Mean ± SEM) and Mean difference were analyzed using one-way analysis of variance (ANOVA). Further comparison between groups were performed using Tukey test. A p value less than 0.05 was considered significant. All statistical analyses were carried out using GraphPad Prism 7.

3.0 RESULTS

Results showing sperm parameters (morphology, motility and count) responses to oral administration of aqueous extract of *C. populnea* are presented in Table 1. In the parameters determined, there were no significant differences ($p < 0.05$) observed across parameters and extract concentrations.

Table 1: Showing sperm indices

Parameters		**Control**	**50**	**100**	**200**	**250**	**300**
			(mg/kg bw)				
Morphology (%)	**normal sperm cells**	73.3±3.3	76.7±6.7	86.7±3.3	80.0±0.0	83.3±3.3	80±0.0
	abnormal sperm cells	26.7±3.3	23.3±6.7	13.3±3.3	20±0.0	16.7±2.3	20±0.0
Sperm motility (%)		63.3±12.0	76.7±6.7	83.3±3.3	76.7±3.3	83.3±3.3	83.3±3.3
Sperm count ($x10^6$)		207±66.9	116.3±1.7	184.0±159.1	334.3±131.6	244±25.2	205.7±49.2

Legend: kg (kilogram); mg (milligram); bw (body weight)

Values are expressed as means ± SEM, n = 3

The results presented in table 2 are for blood concentration of hormones in the experimental animals. The oral administration of graded doses of aqueous extract of *C prostrata* did not result in any significant difference in the blood concentration of both follicle stimulating and luteinizing hormones. Significant differences were observed in the blood concentration of follicle stimulating hormone in a concentration dependent (except 50mg/kg bw) manner.

Table 2: Showing blood levels of hormones associated with spermatogenesis

Parameters	**Control**	**50mg/kg bw**	**100mg/kg bw**	**200mg/kg bw**	**250mg/kg bw**	**300mg/kg bw**
Follicle stimulating hormone (ng/dl)	20.8±1.9	49.9±33.9	108.5±6.7*	87.2±8.7	116.3±12.9*	126.3±4.3*
Luteinizing hormone (ng/mL)	10.0±0.6	16.4±2.4	11.2±3.4	8.4±0.3	9.9±1.7	14.2±0.4
Testosterone (ng/dl)	4.6±0.5	4.3±1.5	5.3±1.4	7.2±1.2	4.3±1.5	3.5±0.4

Legend: kg (kilogram); mg (milligram); bw (body weight)

Values are expressed as M ± SEM, n = 3; values with symbol (*) are significantly different *(*$p < 0.05$*)* from the control group.

Table 3 shows that oral administration of graded doses of *C prostrata* caused significant($p < 0.05$) differences in testicular homogenate concentration of Superoxide dismutase, Glutathione S-transferase and Glutathione peroxidase. The effect on the other antioxidants measured was only significant at specific dosages (G6PD at 200mg/kg bw., Glutathione reductase at 50mg/kg bw.) There was no significant ($p < 0.05$) difference observed in testicular malondialdehyde concentrations.

Oral administration of graded doses of *C prostrata* to groups of experimental rats did not effect any significant change along concentration gradient or in the liver and kidney enzymes or metabolites analysed as presented in table 4

There were no observed significant *(*$p < 0.05$*)* difference (table 5) in the organ (heart, liver, kidney, testis) weights of the experimental animals administered with oral graded doses of *C. prostrata* leaf ethanolic extract.

Table 3. Showing results of antioxidant assay (rat testis)

Parameters	**Control**	**50mg/kg bw**	**100mg/kg bw**	**200mg/kg bw**	**250mg/kg bw**	**300mg/kg bw**
Superoxide dismutase (u/mg protein)	114.4±2.4	132.4±10.4	73.0±1.7*	74.6±0.0*	152.6±3.3*	74.6±0.0*
Catalase (u/mg protein)	7150±207.2	1820±31.9*	7877±179.3	993.4±39.9*	3848±2731	2765±120.1
Glutathione S-transferase (u/mg protein)	49.0±1.5	27.5±5.2	34.6±9.3	21.8±1.8*	23.3±3.3*	22.4±0.4*
Glucose-6-phosphate dehydrogenase (u/l)	500.5±37.2	482.8±93.0	479.4±38.5	205.6±48.6*	347.1±11.5	411.3±48.6
Glutathione reductase (u/mg protein)	71.9±2.2	23.9±2.8*	27.8±14.8	51.3±15.9	30.4±7.1	70.5±7.4
Glutathione peroxidase (u/mg protein)	28.7±0.9	14.2±0.6*	13.6±0.6*	7.7±0.9*	8.6±1.6*	5.1±0.3*
Malonyl dialdehyde (u/mg protein)	0.3±0	0.5±0.1	0.4±0	0.3±0	0.6±0.2	0.4±0

Legend: kg (kilogram); mg (milligram); bw (body weight), u (enzyme or international unit)
Values are expressed as means ± SEM, n = 3; values with symbol (*) are significantly different from the control group.

Table 4: Showing result of liver and kidney function analyses

Parameters	**Control**	**50mg/kg bw**	**100mg/kg bw**	**200mg/kg bw**	**250mg/kg bw**	**300mg/kg bw**
Alkaline phosphatase (u/L)	92.9±2.1	151.6±18	243±18.7*	124.2±25.3	101.8±7.5	122.6±12.0
Total protein (mg/dl)	8.0±0.2	12.2±1.3	10.8±0.8	12.2±1.0	12.9±1.1	11.3±0.6
Alanine aminotransferase (u/L)	57.8±3.7	49.7±3.7	50.1±3.7	53.3±6.7	51.1±0.8	53.2±2.2
Aspartate aminotransferase (u/L)	155.3±9.3	181.4±11.5	169.5±10.8	176.7±18.8	177.5±5.4	171.4±0.8
Bilirubin direct (conjugate) (mg/dl)	3.4±0.2	3.0±0.4	2.9±0.6	2.7±1.1	2.4±0.7	2.8±0.2
Bilirubin total (mg/dl)	7.4±0.4	5.7±0.7	5.0±1.1	3.6±0.2	4.9±0.5*	5.5±0.5
Lactate dehydrogenase (u/L)	140.0±9.6	151.8±25.5	151.0±27.1	160.7±40.7	116.6±4.2	79.0±2.3
Albumin (g/dl)	28.2±2.0	28.4±5.4	25.2±1.4	36.1±0.8	18.9±6.6	30.1±7.7
Globulin (mg/dl)	11.1±0.1	8.1±2.4	7.2±1.1	12.0±0.8	4.0±3.1	9.4±3.7
Creatinine (mg/ml)	0.2±0	0.8±0.4	1.4±0.4	1.1±0.1	0.9±0.4	1.1±0.4
Urea (mM/L)	2.4±0.2	2.5±0.2	3.3±0.2	2.8±0.4	2.8±0.1	2.6±0.3
Uric acid (mM/L)	0.36±0.03	0.38±0.03	0.49±0.02	0.42±0.01	0.42±0.02	0.40±0.04

Legend: kg (kilogram); mg (milligram); bw (body weight)

Values are expressed as M ± SEM, n = 3; values with symbol (*) are significantly different from the control group.

Table 5; Showing weights of different organs excised from the experimental animals

Weight of Organ	**Control**	**50mg/kg bw**	**100mg/kg bw**	**200mg/kg bw**	**250mg/kg bw**	**300mg/kg bw**
Heart (g)	0.78±0.08	0.72±0.03	0.72±0.03	0.72±0.03	0.73±0.03	0.77±0.04
Liver (g)	2.14±0.21	2.74±0.88	3.28±0.56	5.59±0.64	5.51±0.20	5.58±0.11
Kidney (g)	1.39±0.17	1.15±0.03	1.13±0.02	1.15±0.12	1.07±0.06	1.16±0.11
Testes (g)	1.18±0.05	1.11±0.11	0.99±0.05	1.15±0.09	1.11±0.03	1.13±0.09

Legend: kg (kilogram); mg (milligram); bw (body weight)
Values are expressed as M ± SEM, n = 3

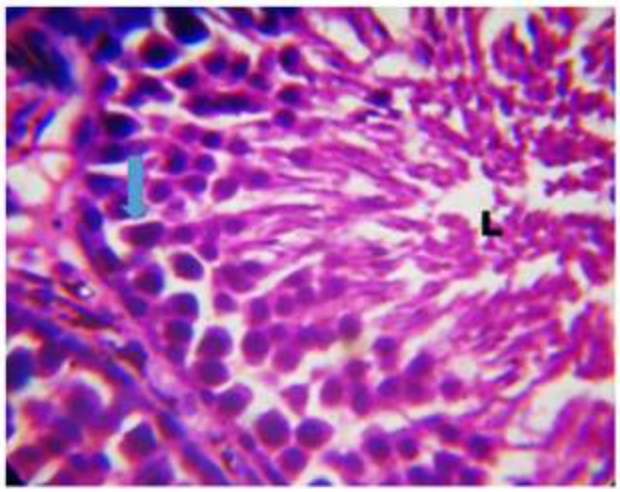

Fig. 1. Micrograph from testis in GRP. F animal showing seminiferous tubules with well-preserved epithelium. Appearances suggest fertility enhancement. Stage 7 slide showing all cells of the germ series in normal arrangement. Arrow: Sertoli cell; L: Lumen. H&E × 1000.

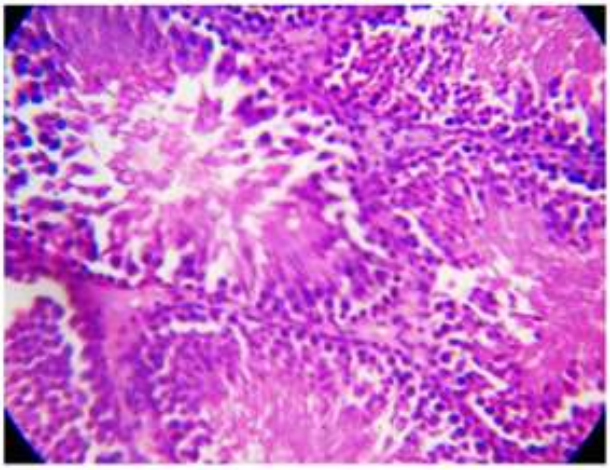

Fig. 2. Micrograph from testis in GRP. E animal showing seminiferous tubules with well-preserved epithelium, and cells of all germ series. Features suggest enhanced fertility. H&E × 400.

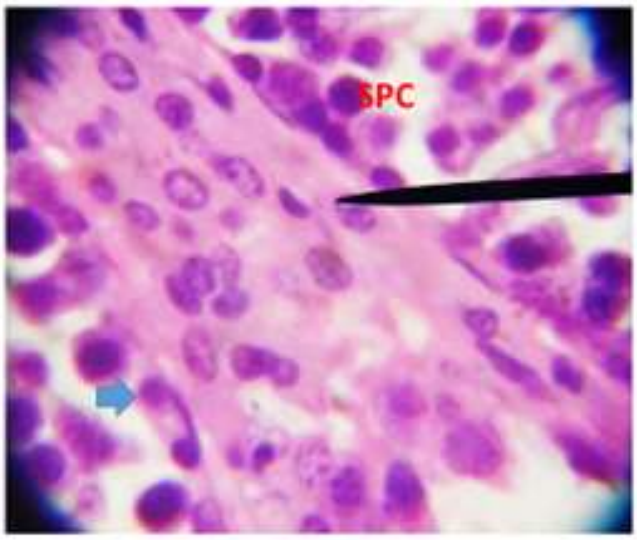

Fig. 3. Micrograph from testis in GRP. F2 animal showing seminiferous tubules with sloughing of germ cells and cell atrophy. Arrowhead: Pale spermatogonium; Black arrow-Leydig cell; SPC-Primary spermatocyte. H&E × 1000.

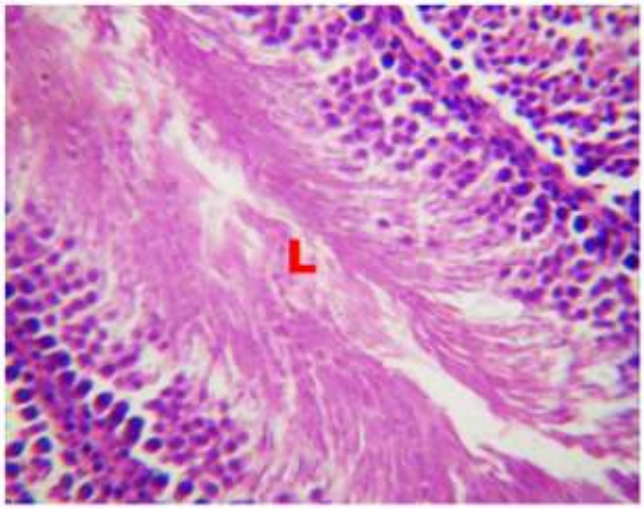

Fig. 4. Micrograph from testis in GRP. E animal showing seminiferous tubules well preserved - epithelium, appearances suggest fertility enhancement. Lumen is filled with sperm tail. L-Lumen. H&E × 400.

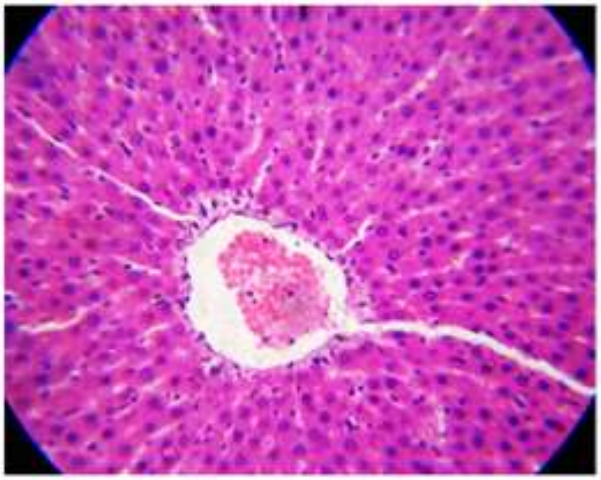

Fig. 5. Micrograph from liver in GRP. E animal showing well preserved histology-classic liver lobule. The center of the picture has clotted blood in a central vein. H & E × 400.

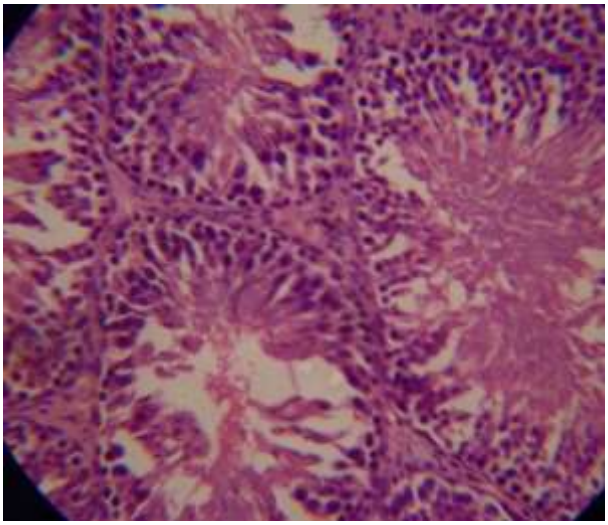

Fig. 6. Micrograph from testis in GRP. C animal showing seminiferous tubules fairly well-preserved. All germ layers are visible and tissue architecture is normal. Minimal sloughing is also seen. H&E × 400.

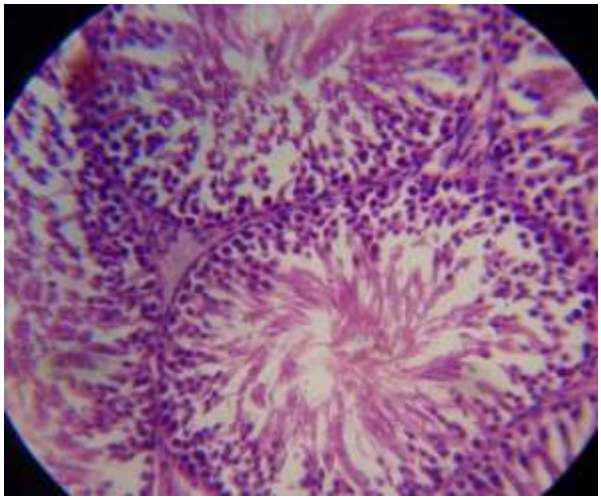

Fig. 7. Micrograph from testis in GRP. A. (Control) animal showing seminiferous tubules well preserved. All germ layers are visible and tissue architecture is normal. H&E × 400.

4.0 Discussion

This current study examined the effect of oral administration of *C. prostrata* ethanolic leaf extract on sperm indices in male Wistar rats. Total sperm count, motile sperm count and morphologic features have been reported as the indices of fertility in males (Etuk and Muhammad, 2009). The results obtained from this study showed no significant ($p < 0.05$) difference in all comparisons made. Although, there were slight increases in the sperm count of the test groups compared to the control group, these were of no statistical significance. Total counts were lowered in group 2 animals which received the lowest dose of the extract which raises the possibility that at lower doses, components of the extract may suppress spermatogenesis. This is a possibility that calls for further studies. Micrographs from testes of animals exposed to higher doses of the extract (groups E and F) showed histological evidence suggestive of enhanced spermatogenesis, with close packing of the tubules with germ cells in all compartments. (Figs. 1 and 4). Histology of

other organs examined in this study, liver, kidney and heart were largely normal indicative of the largely nontoxic nature of the leaf extract at doses of even up to 200g/kg bw in agreement with earlier reports (Kannappan and Sundaram, 2009, Ogbonnia et al., 2016)

Hormonal analyses showed no significant increase in LH and testosterone hormone concentration, however the concentration of FSH at 100, 250 and 300 mg/kgbw were significantly higher than that of the control group. The increase in sperm indices may be due to the increase in FSH hormone concentration, as the regulation of spermatogenesis is under the control of these hormones and androgen receptors (AR) of Sertoli cells (Alphonse et al., 2017). The results of sperm indices obtained were slightly similar to the work of (Nantia et al., 2009, Alphonse et al., 2017) where the plant extract of *Zanthoxylum macrophylla* increased the semen indices in male wistar rats significantly.

This study results suggests that the leaf extract of *C. prostrata* possess antioxidant activities, as some of the antioxidant parameters investigated were significantly higher in the test groups. These suggest that the increase in antioxidant activities might have been elicited as a result of *C. prostrata* extract administration which may be due to the presence of certain phytochemicals in the plant extract (Ojekale et al., 2016). The antioxidant potentials of the plant as observed in this study is in agreement with previous works (Oladimeji and Akpan, 2014, Oladimeji et al., 2014, Sonibare and Olatubosun, 2015). ROS activity has been clearly shown to be deleterious to the integrity of sperm. Normal sperm samples have been reported to be negatively affected when they were cultured with samples containing high ROS levels in the same incubator (Agarwal et al., 2014).

Liver and kidney function analyses showed no significant difference between the test groups and the control group, indicating no severe damage done to any of the organs. In conclusion, the result from the antioxidant assay suggest that *C. prostrata* extract could be a potent antioxidant source which could favor the production of quality sperm cells during the process of spermatogenesis.

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