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Reproductive parameters of male rats treated with *Pavetta crassipes* K.Schum. aqueous leaf extract

[Parámetros reproductivos de ratas macho tratadas con extracto acuoso de hoja de *Pavetta crassipes* K.Schum.]

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Abstract

Context: There is a growing concern about induction of male infertility resulting from medicinal plant use especially in malaria treatment. *Carica papaya* and *Azadirachta indica* are plants with documented antimalarial and anti-fertility effects. *Pavetta crassipes* leaves are reported to have antimalarial effects but there is no information about its reproductive toxicology.

Aims: To evaluate the effects of *P. crassipes* aqueous leaf extract on male reproductive parameters.

Methods: Healthy adult male rats were used for this study. The extract was administered orally at 400, 800 and 1600 mg/kg for two and four weeks, respectively while the control group received 10 mL/kg distilled water. Blood samples were collected for serum hormonal assay. The testes and epididymis were excised for histological examination and seminal fluid analysis.

Results: Serum testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels reduced significantly in all treated groups compared to control but this effect was reversed after the respective recovery periods except FSH and LH levels in the group treated with 1600 mg/kg for four weeks. The control group had normal testicular histology while varying degrees of reversible histological changes occurred in the treated groups except persistent irregular basal lamina at 800 mg/kg in the two-week treated group while persistent irregular basal lamina and tubular atrophy occurred at 1600 mg/kg in the four-week treated group. Sperm count reduction at 800 and 1600 mg/kg compared to control (86 x 10^6 mL^{-1}) in the groups treated for four weeks was irreversible.

Conclusions: The aqueous leaf extract of *Pavetta crassipes* has the potential to cause deleterious effects on male reproductive function in Wistar rats.

Keywords: hormone; infertility; reproduction; sperm-count; testosterone.

Resumen

Contexto: Existe una creciente preocupación por la inducción de infertilidad masculina resultante del uso de plantas medicinales, especialmente en el tratamiento de la malaria. *Carica papaya* y *Azadirachta indica* son plantas con efectos antipalúdicos y antifertilidad documentados. Se informa que las hojas de *Pavetta crassipes* tienen efectos antipalúdicos, pero no hay información sobre su toxicología reproductiva.

Objetivos: Evaluar los efectos del extracto acuoso de hoja de *P. crassipes* sobre los parámetros reproductivos masculinos.

Métodos: Se utilizaron ratas macho adultas sanas para este estudio. El extracto se administró por vía oral a 400, 800 y 1600 mg/kg durante dos y cuatro semanas, respectivamente, mientras que el grupo de control recibió 10 mL/kg de agua destilada. Se recogieron muestras de sangre para el análisis hormonal sérico. Los testículos y el epidídimo se extirparon para examen histológico y análisis de fluido seminal.

Resultados: La testosterona sérica, la hormona estimulante del folículo (FSH) y la hormona luteinizante (LH) se redujeron significativamente en todos los grupos tratados en comparación con el control, pero este efecto se revirtió después de los respectivos períodos de recuperación, excepto los niveles de FSH y LH en el grupo tratado con 1600 mg/kg para cuatro semanas. El grupo de control tenía histología testicular normal, mientras que en los grupos tratados se produjeron grados variables de cambios histológicos reversibles, excepto la lámina basal irregular persistente a 800 mg/kg en el grupo tratado de dos semanas, mientras que la lámina basal irregular persistente y la atrofia tubular ocurrieron a 1600 mg/kg en el grupo tratado de cuatro semanas. La reducción del recuento de espermatozoides a 800 y 1600 mg/kg en comparación con el control (86 x 106 mL^{-1}) en los grupos tratados durante cuatro semanas fue irreversible.

Conclusiones: El extracto acuoso de la hoja de *Pavetta crassipes* tiene el potencial de causar efectos nocivos sobre la función reproductiva masculina en ratas Wistar.

Palabras Clave: hormona; esterilidad; recuento de esperma; reproducción; testosterona.

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INTRODUCTION

Pavetta crassipes K.Schum. (*Rubiaceae*) is a tropical plant found in West and Central Africa. It is used traditionally as food and for the treatment of fever, schistosomiasis, mental illness, convulsions, pain, hookworms and microbial infections, the leaves and roots serve as aphrodisiac when chewed (Amos et al., 1998; Abubakar et al., 2007; Ibekwe et al., 2012; Bello et al., 2014). Some neuropharmacological effects of the aqueous leaf extract and fractions have been reported in previous studies (Bariweni and Ozolua, 2017).

Male reproduction is a complex process involving the testes, epididymis, hormones and sex glands. The testes perform spermatogenesis and steroidogenesis, which are vital for procreation. Although it is equipped with a powerful intrinsic defence system, it is very vulnerable to assault (D'Cruz et al., 2010).

There is a growing concern about induction of male infertility resulting from the use of medicinal plants especially in the treatment of malaria (Raji et al., 2005). *Carica papaya* and *Azadirachta indica* are plants with documented antimalarial and antifertility effects (Lohiya et al., 1994; Raji et al., 2005). *Pavetta crassipes* is reported to have antimalarial effects (Sanon et al., 2003; Weniger et al., 2004; Elhadj et al., 2010), but there is no information about its reproductive toxicology. This research was aimed at determining the effects of *P. crassipes* aqueous leaf extract on male reproductive parameters.

MATERIAL AND METHODS

Chemicals

Diethylether (BDH, Hamburg, Germany), radioimmunoassay kit (AccuBind ELISA Kits, California, USA) were obtained from Rovet Chemicals, Benin-city, Nigeria. Other reagents used were of analytical grade.

Vegetal material

Collection of plant material and preparation of the aqueous leaf extract is described in Bariweni and Ozolua (2017). The leaves of *Pavetta crassipes* were collected from Suleja, Abuja, Nigeria, in the month of April 2015 and authenticated by Mr. Ibrahim Muazzam, a taxonomist at the National Institute for Pharmaceutical Research and Development, Abuja, a voucher specimen was deposited for reference purposes with code NIPRD/H/6865. The sun-dried leaves were pulverized, and 250 g extracted by decoction. The resultant extract was dried and stored in a refrigerator until used.

Animals

Adult male Wistar rats weighing between 180 and 190 g were obtained from the animal house, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Nigeria. Ethical approval (NDU/PHARM/PCO/AEC/015) was obtained from the Animal Ethics Committee of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria. The animals were fed with standard rodent chow (Livestock Feeds Plc, Nigeria) and had free access to water. All animals were handled in accordance with EU directive (2010/63/EU) for animals.

Experimental design

Seventy (80) male rats were divided into 16 groups (5 rats per group). Groups 1 and 2 served as control and received 10 mL/kg distilled water alone for two and four weeks respectively. Groups 3 – 5 received 400 mg/kg, 800 mg/kg and 1600 mg/kg of *P. crassipes* aqueous leaf extract for two weeks. Groups 6-8 were also treated with 400, 800 and 1600 mg/kg of the aqueous extract respectively but were allowed to recover for two weeks and served as the respective recovery groups. Groups 9 – 11 were treated with 400, 800 and 1600mg/kg of

P. crassipes aqueous leaf extract respectively for four weeks while groups 12 – 14 served as their respective recovery groups. Groups 15 and 16 also served as recovery groups for the distilled water treated rats. The vehicle and extract were administered daily via the oral route. After each treatment and recovery period the animals were sacrificed under diethylether anesthesia, blood sample was collected from the abdominal aorta into non-heparinized bottles for serum hormonal analysis. The testes and epididymis were excised for histological examination and seminal fluid analysis respectively.

Sperm characteristics and count

The epididymis from each rat was removed and sperm squeezed into a universal bottle containing 2 mL of normal saline and mixed. The sperm was mounted on a binocular microscope stage (Olympus BH, Japan, Tokyo), at x10 for observation and count. Sperm motility was expressed as percent motile sperm of the total sperm count. Sperm count was done using the hemocytometer and expressed as million/ml (García et al., 2015).

Histological assessment

After sacrificing the rats, the testes were excised, rinsed in normal saline, blotted with Whatmann's number 1 filter paper, observed for visible damage, weighed and fixed in 10% formalin and processed after 24 h using automatic tissue processor (Leica TP 1020, Meyer Inc. Houston, Texas), embedded with paraffin wax (Lodha Petro, Mumbai, India) using embedding panel (Leica EG 1160, Meyer Inc. Houston, Texas), and at 4 µm using rotary microtome (Leica RM 2125, Meyer Inc. Houston, Texas). The sections were then stained with hematoxylin and eosin (Merck, Darmstadt, Germany) (Adefemi et al., 2003). Photomicrographs of the stained tissue sections were produced using a digital microscope (Olympus® DP27, Olympus BH, Japan, Tokyo) at x 400 magnification.

Serum hormonal assay

Blood samples collected was allowed to clot and then centrifuged at 5000 rpm (Labline LMC-3000, Biosan, Riga, Latvia) to separate the serum from the clotted blood samples. The serum samples were assayed for testosterone, luteinizing hormone, follicle stimulating hormone and estrogen using immunoassay techniques aided by commercial kits (AccuBind ELISA Kits, California, USA). Analyses were carried out according to the manufacturer's instructions. (Engvall et al., 1980; Tietz, 1995).

Statistical analysis

Results are presented as mean ± SEM and analyzed using One-way ANOVA followed by Dunnet's multiple comparison (Graphpad prism 6 software, San Diego, California, USA) and results considered significant when $p<0.05$ and $p<0.01$ with respect to the control group.

RESULTS

Sperm characteristics and count

Following two weeks treatment with the extract, sperm viability and motility was reduced. This effect of the extract on motility was reversed after two weeks. Although sperm viability improved after the recovery period, it was significantly lower than the control (Fig. 1). For the four weeks treated group, sperm viability and motility significantly reduced in the treated groups compared with the distilled water treated groups, however these effects were reversed after the recovery period. Sperm with abnormal cells were significantly increased in the 400 mg/kg treated group but was reversed after the recovery period (Fig. 2).

In Fig. 3, the results show that sperm counts were significantly reduced in the groups treated for two weeks. However, these effects were reversed after the recovery period. For the four week treated groups (Fig. 4), sperm count was significantly reduced, recovery occurred only in the 400 mg/kg treated group. The distilled water treated recovery groups presented no significant changes compared with the normal control.

The effect of oral administration of *P. crassipes* aqueous leaf extract on the rat testis for two weeks,

four weeks and their respective recovery groups are shown in Figs. 5 and 6. Varying degrees of changes in the treatment groups including interstitial tissue proliferation, irregular basal lamina shape and reduction in the amount of spermatozoa and Leydig cells occurred in both two and four week treated groups. Recovery occurred in both treatment groups except persistent irregular basal lamina (Figs. 5E and 6G) and tubular atrophy (Fig. 6G). The histology of recovery groups administered with distilled water (Figs. 5H and 6H) were not different from the normal control groups (Figs. 5A and 6A) and showed normal basal lamina, seminiferous tubules and numerous spermatozoa.

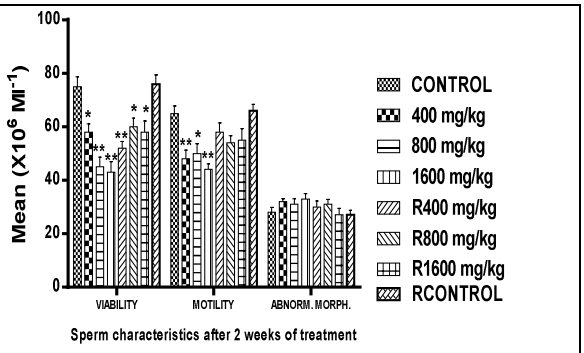

Figure 1. Effect of aqueous leaf extract of *Pavetta crassipes* on sperm viability, motility and morphology after two weeks treatment.

Data represents mean ± SEM for the treatment groups, n=5. *p<0.05, **p<0.01 respect to the control groups (administered with distilled water). Sperm viability and motility were significantly reduced for the treatment groups. Motility was reversed after recovery period.

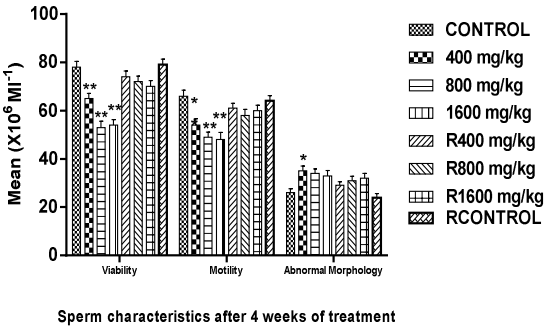

Figure 2. Effect of aqueous leaf extract of *Pavetta crassipes* on sperm viability, motility and morphology after 4 weeks treatment.

Data represents mean ± SEM for the treatment groups, n=5. *p<0.05, **p<0.01 respect to the control groups (administered with distilled water). Sperm viability and motility were significantly reduced for the treatment groups but was reversed after recovery period. Sperm with abnormal morphology increased in the 400 mg/kg group but it was reversed after recovery.

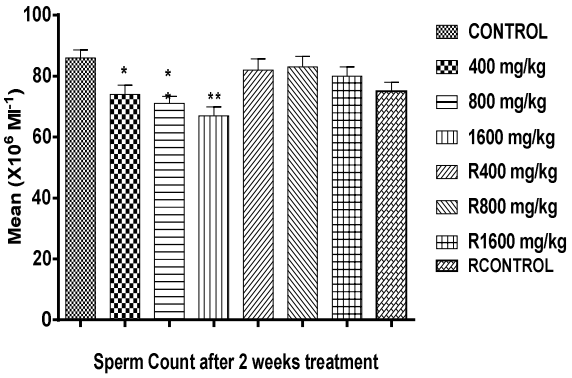

Figure 3. Sperm counts of rats treated for two weeks with aqueous leaf extract of *Pavetta crassipes*.

Data represents mean ± SEM for the treatment groups, n=5. *p<0.05, **p<0.01 respect to the control groups (administered with distilled water. Sperm count was significantly reduced for the treatment groups, but reversal occurred after recovery period.

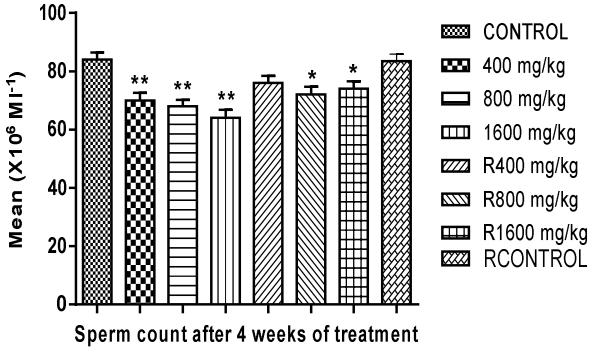

Figure 4. Sperm counts of rats following four weeks treatment with aqueous leaf extract of *Pavetta crassipes*.

Data represents mean ± SEM for the treatment groups, n=5. *p<0.05, **p<0.01 respect to the control groups (administered with distilled water. Sperm count was significantly reduced for the treatment groups, reversal occurred only in the 400 mg/kg treated group after the recovery period.

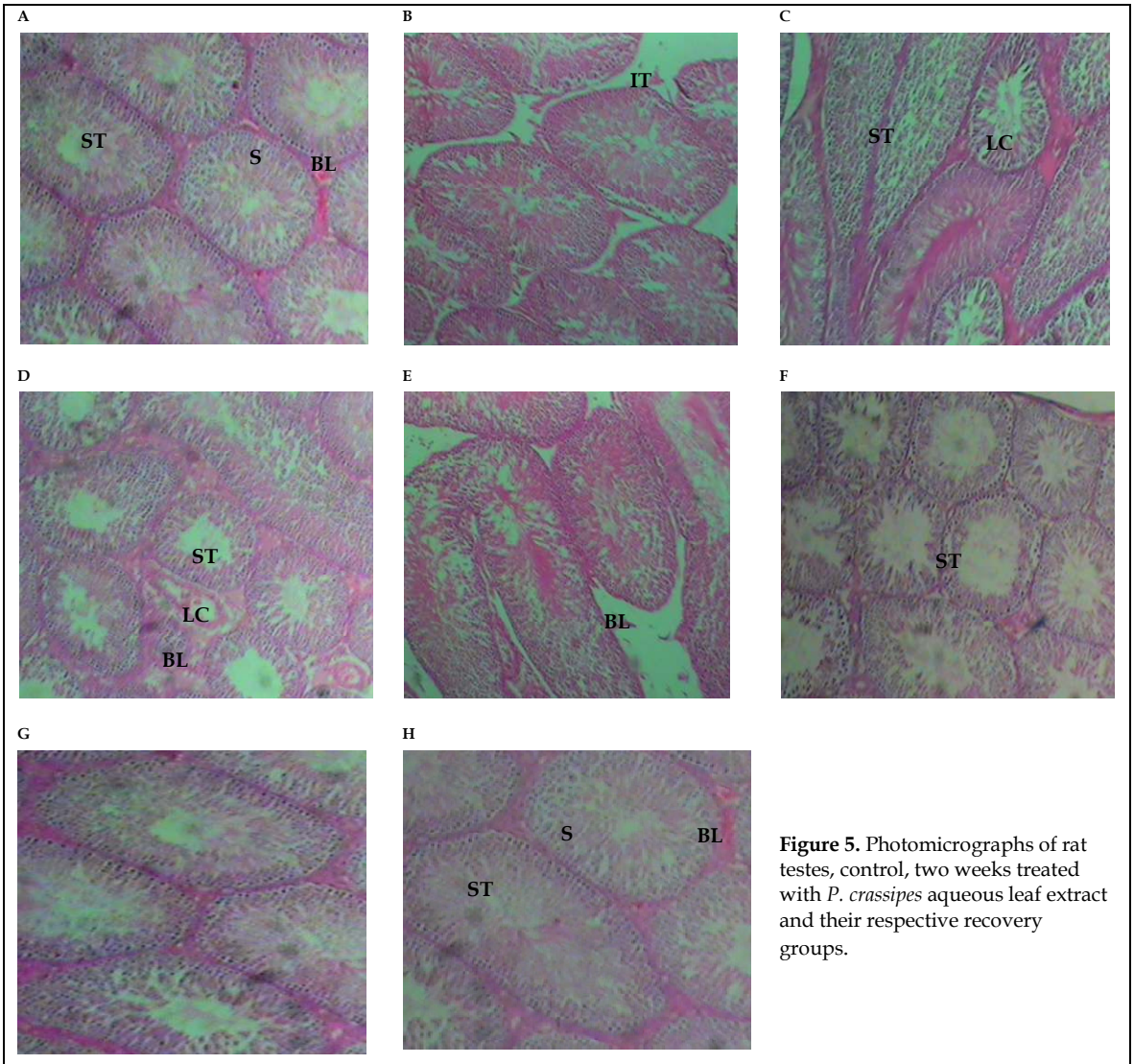

Figure 5. Photomicrographs of rat testes, control, two weeks treated with *P. crassipes* aqueous leaf extract and their respective recovery groups.

The controls [administered with distilled water **(A)** and **H** (RC)] show normal seminiferous tubule (ST) with numerous spermatozoa (S) and basal lamina (BL) with regular shape. The treated groups show changes to varying degrees. (**B**, 400 mg/kg) show increased interstitial tissue (IT) and irregular basal lamina. (**C**, R400) depicts recovery with reduced IT and connective tissue embedded with leydig cells (LC). (**D**, 800 mg/kg) show ST with scanty spermatozoa, leydig cells (LC) surrounded by irregular shaped basal lamina (BL). (**E**, R800) shows recovery with increased spermatozoa but with irregular basal lamina. (**F**, 1600 mg/kg) an almost empty seminiferous tubule. (**G**, R1600) recovery is shown here with reduced white space in the seminiferous tubule (ST).

Serum hormonal assay

The results (Table 1) show the effect of *P. crassipes* aqueous extract on serum testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) following treatment with the extract for two and four weeks, respectively. Serum testosterone levels reduced significantly in both groups, but this effect was reversed after the respective recovery periods. The levels of FSH and LH significantly increased during the treatment period and reversal also occurred after withdrawal of the extract except in the group treated with 1600 mg/kg for four weeks. The serum levels of evaluated hormones in the recovery groups administered with distilled water were not significantly different from the control groups for both treatment periods.

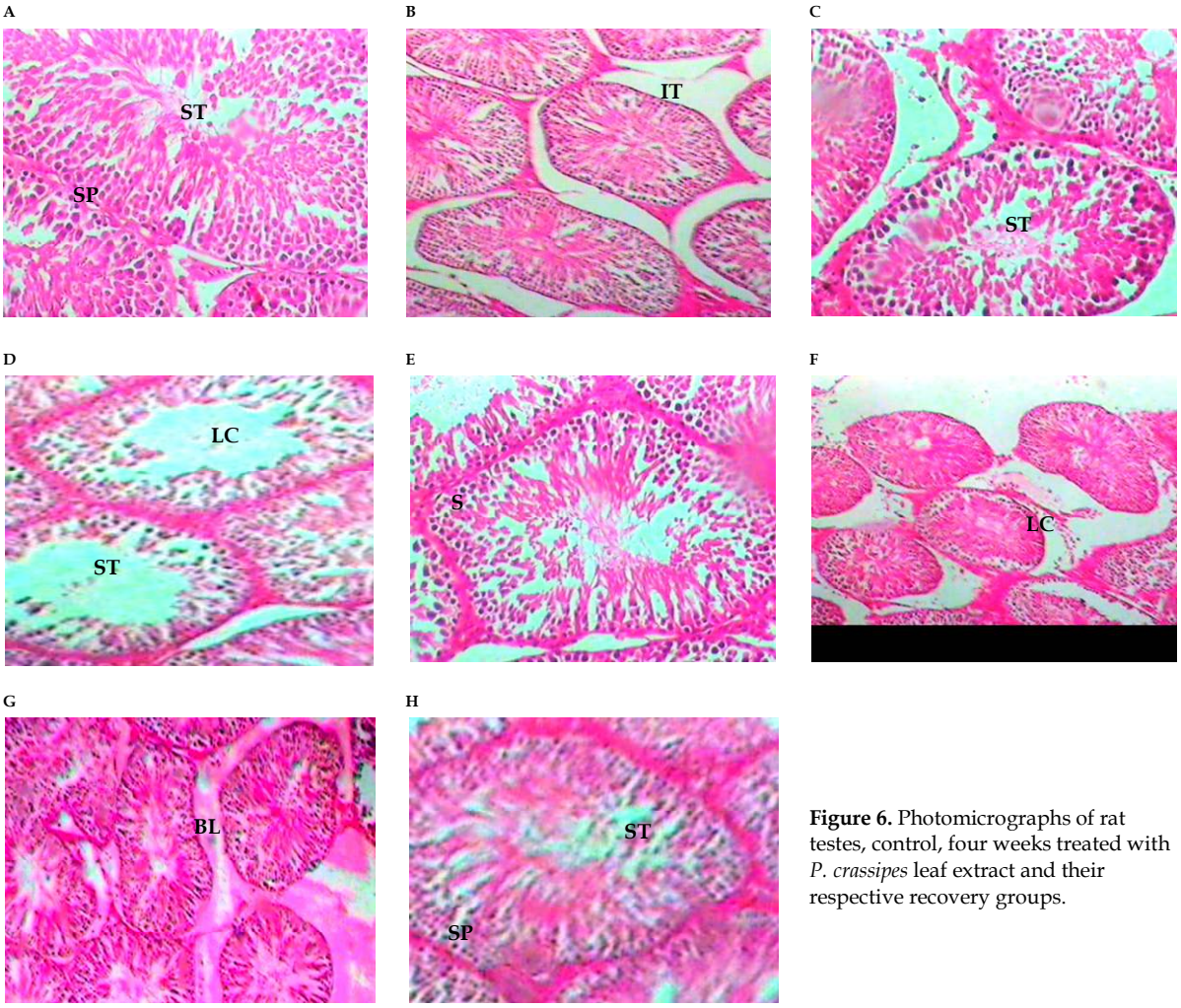

Figure 6. Photomicrographs of rat testes, control, four weeks treated with *P. crassipes* leaf extract and their respective recovery groups.

The control groups [administered with distilled water (**A**) and **H** (RC)] show normal seminiferous tubule (ST) with numerous spermatozoa (SP) with regular shaped cells. The treated groups show varying degrees of changes in the cells. (**B**, 400 mg/kg) show increased interstitial tissue (IT) and irregular basal lamina. (**C**, R400) depicts recovery with reduced IT and connective tissue embedded with leydig cells (LC). (**D**, 800 mg/kg) show ST with scanty spermatozoa, leydig cells (LC) surrounded by irregular shaped basal lamina (BL). (**E**, R800) shows recovery with increased spermatozoa but with irregular basal lamina. (**F**, 1600 mg/kg) shows tubular atrophy. (**G**, R1600) little recovery is shown here with normal seminiferous tubules and spermatogonia cells.

DISCUSSION

Spermatogenesis is a dynamic process which takes place within the seminiferous tubules of the testis. Spermatogenesis depends largely on the structure and physiology of sertoli cells and endocrine regulation by testosterone (D'Cruz et al., 2010). Phytochemicals from various plants have been reported to induce reversible spermatogenetic effects on animal species (Raji et al., 2005). In this study, the photomicrograph of the groups treated with the plant extract, shows an increasing interstitial space within the seminiferous tubules and the surrounding connective tissues as the dose administered increases resulting in wider lumen. These effects appear reversible although not completely as the lumen of seminiferous tubules in the recovery groups are narrower than the treatment groups. Destruction of interstitial cells, leading to a reduced number of spermatozoa and mild to moderate degeneration of seminiferous tubular epithelium as seen in this study may result from the presence of polyphenolic compounds in the extract (Ibekwe et al., 2012; Bariweni and Ozolua, 2017).

Table 1. Effect of two and four-weeks treatment with *P. crassipes* aqueous leaf extract (PCE) on serum hormones.

Treatment	Dose (mg/kg)	Parameters					
		Two weeks			Four weeks		
		Testosterone (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)	Testosterone (ng/mL)	FSH (mIU/mL)	LH (mIU/mL)
Control	10 mL/kg	1.84 ± 0.10	14.52 ± 0.30	14.26 ± 0.20	1.86 ± 0.08	15.01 ± 0.11	14.18 ± 0.20
PCE	400	1.42 ± 0.12**	17.16 ± 0.40**	16.20 ± 0.30**	1.52 ± 0.10*	15.94 ± 0.14**	15.20 ± 0.21**
	800	1.28 ± 0.10*	17.94 ± 0.20**	17.64 ± 0.30**	1.46 ± 0.20*	16.04 ± 0.14**	16.23 ± 0.16**
	1600	1.26 ± 0.09**	18.04 ± 0.20**	17.98 ± 0.21**	1.44 ± 0.10**	16.42 ± 0.13**	17.00 ± 0.18**
PCE recovery	400	1.80 ± 0.11	14.92 ± 0.30	14.54 ± 0.21	1.84 ± 0.11	14.92 ± 0.14	14.54 ± 0.14
groups (R)	800	1.78 ± 0.14	15.02 ± 0.18	14.82 ± 0.18	1.82 ± 0.08	14.60 ± 0.12	14.68 ± 0.08
	1600	1.74 ± 0.16	15.42 ± 0.20	15.04 ± 0.20	1.79 ± 0.06	14.28 ± 0.16**	14.92 ± 0.06*
RControl	10 mL/kg	1.86 ± 0.09	14.68 ± 0.22	14.24 ± 0.18	1.84 ± 0.10	14.96 ± 0.11	14.42 ± 0.16

Mean ± S.E.M. for the treatment groups, n=5, *p<0.05, **p<0.01 when compared to distilled water administered control. FSH- follicle stimulating hormone; LH-luteinizing hormone. The recovery control group (RControl) was also administered with distilled water.

Spermiogenesis is the final stage of spermatogenesis and comprises polarization of the spermatids, formation of acrosomal cap and flagellum, cytoplasmic remodeling and elongation of the nucleus (Lebelo and Van Der Horst, 2016). Spermatids resulting from spermiogenesis are morphologically mature but immotile when released into the lumen of the seminiferous tubule. Synthesis and secretion of various proteins by the epididymis resulting in the attainment of various morphological, biochemical and motile properties is a vital process that occurs during transportation of spermatids through the seminiferous tubule (D'Cruz et al., 2010). Reduced sperm count altered sperm morphology or reduced motility is suggestive of alterations in spermatogenesis, spermiogenesis or disruption of epididymal environment. Alkaloids have been reported to alter the morphology or reduce sperm motility (Raji et al., 2005, Yuan et al., 2012), by interacting with adrenergic systems, which modulate cell surface signaling systems responsible for sperm motility (Wang et al., 2009). The phytochemical analysis of the extract revealed the presence of alkaloids (Bariweni et al., 2018). Alkaloids are lipid soluble and can permeate sperm cell membranes to elicit its effects, which may account for the alteration in morphology and motility as seen in this study. The reduction in sperm count, viability and motility in the treatment groups after two and four weeks respectively could have resulted from alterations in spermatogenesis, spermiogenesis or disruption of epididymal structure and function. However, these effects appear to be reversible as seen with the recovery groups with the exception of sperm viability in the group treated for two weeks.

Steroidogenesis is a complex process which is essential in male reproduction. This process is governed by endocrine factors in the hypothalamo-pituitary-testicular axis with follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone as the key players. Leydig cells otherwise known as interstitial cells are the major cells responsible for the synthesis of testosterone. Disruption of interstitial cell structure or function would lead to reduced steroidogenesis and consequent reduction in serum testosterone levels as seen in this study. Testosterone levels on the other hand serve as negative feedback to FSH and LH release. A reduction in serum testosterone levels may lead to an increased release of FSH and LH, this could account for the significant increase in serum FSH and LH as seen in this study. Testosterone and FSH are necessary for spermatogenesis to occur. A reduction in testosterone levels may impair spermatogenesis resulting in reduction in

sperm count. *Carica papaya and Azadirachta indica* are plants with established antimalarial and antifertility effects. The aqueous leaf extract of *Azadirachta indica* administered to male mice for 28 days at a dose of 200 mg/kg damaged the seminiferous tubules, resulting reduced spermatogenesis and altered sperm morphology. These effects were however reported to revert back to normalcy after 42 days of withdrawal of the treatment (Lohiya et al., 1994). *Carica papaya* leaf extract at 100 mg/kg reduced the number of sertoli and leydig cells resulting in impaired steroidogenesis and spermatogenesis (Raji et al., 2005). These findings are not different from the results from our study. Alkaloids have been reported to cause a reduction in testosterone levels in rats (Raji et al., 2005), alkaloids present in the extract (Ibekwe et al., 2012, Bariweni et al., 2018) may be responsible for this effect.

Although the deleterious effects of the extract are not completely reversed as seen in the recovery groups for both two and four weeks, the results suggest that the deleterious effects may not be permanent. The reversibility of the effects of the extract on reproductive parameters may be due to metabolism and excretion of the extract and the presence of flavonoids (Bariweni and Ozolua, 2017), which are known antioxidants. Further studies may be required to characterize and quantify the alkaloid content responsible for these effects in male Wistar rats and other animal species.

CONCLUSIONS

The aqueous extract of *Pavetta crassipes* has the potential to cause deleterious effects on male reproductive function in Wistar rats, however some effects appear to be reversible on withdrawal of the extract. Although the effects of the extract on human reproductive function are unknown, this extract may be a suitable candidate for male contraception. It is also recommended that although the plant has aphrodisiac properties, prolonged use at high doses should be avoided as it may have adverse effects on male reproductive function.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Contribution	Bariweni MW	Yibala OI	Alade TO	Karibo DF
Concepts or ideas	x			x
Design	x			
Definition of intellectual content	x			
Literature search	x			x
Experimental studies	x	x	x	x
Data acquisition	x	x	x	x
Data analysis	x	x	x	x
Statistical analysis	x			x
Manuscript preparation	x	x	x	x
Manuscript editing	x			x
Manuscript review	x	x	x	x

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