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Research Article

EFFECT OF ISOLATED ERGOSTEROL CONSTITUENT OF *PORTULACA OLERACEA* ON REPRODUCTIVE PARAMETERS IN MALE RATS

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ABSTRACT

Objective: This study was designed to evaluate the reproductive effect of isolated ergosterol constituent of Portulaca oleracea on reproductive parameters in male albino rats.

Methods: The isolated compound was administered on daily basis for 25 days at doses of 0.50 mg/kg and 0.75 mg/kg and blood samples were collected for hormonal assay, semen analysis was also carried out. Data were analysed using ANOVA at $p<0.05$.

Result: Treatment of rats with 0.50 mg/kg BW and 0.75 mg/kg BW caused decrease in testosterone levels, sperm motility and sperm count as well as increase in the percentage of abnormal sperm cells relative to their respective controls.

Conclusion: These findings on the reproductive parameters suggests that isolated ergosterol constituent of *Portulaca oleracea* has deleterious effect on reproductive functions in male albino rats.

Keywords: Ergosterol, Sperm count, Sperm Motility Testosterone, Albino rats.

INTRODUCTION

Ergosterol is a sterol found in fungi and occasionally in grasses [1] and in plant flowers [2]. Ergosterol is a biological precussor to vitamin D_2. It is turned into viosterol by ultraviolet light, and is then converted into ergocalciferol, a form of vitamin D also known as D_2. For this reason, when yeast and fungi are exposed to ultraviolet light, significant amount of vitamin D_2 are produced.

Ergosterol has been reported to have antitumor effect [3] and it has been widely used to quantify biomas in several studies of soils and mycorrhizal systems [4] and to determine biomass associated with decaying leaves in freshwaters [5]. Its antioxidant and anti-inflammatory effects has been reported [6]. It has also been reported to suppress cell growth [7].

However, due to paucity of information from literature on the effect of ergosterol on reproductive parameters in male albino rats, this study therefore aims at investigating the effect of isolated ergosterol constituent of *Portulaca oleracea* on these aforementioned parameters.

MATERIALS AND METHODS

Experimental Animals

Adult male albino rats weighing between 150 g and 250 g bred in the Pre-clinical Animal House of the College of Medicine, University of Ibadan were used. They were housed under standard laboratory conditions with a 12 hours daylight cycle and had free access to feed and water; and were acclimatized for two weeks to laboratory conditions before the commencement of the experiments. All experiments were carried out in compliance with the recommendations of Helsinki's declaration on guiding principles on care and use of animals.

Plant Material

Fresh specimens of *Portulaca oleraceae* were collected from the Botanical Garden of the Forestry Research Institute of Nigeria, Jericho, Ibadan, and was authenticated in the above named institute where a voucher specimen (No FHI 108334) was deposited.

Table 1:

Hexane		Ethylacetate		Methanol
100% (50 mL)	:	0% (0 mL)		
90% (45 mL)	:	10% (5 mL)		
80% (40 mL)	:	20% (10 mL)		
70% (35 mL)	:	30% (15 mL)		
60% (30 mL)	:	40% (20 mL)		
50% (25 mL)	:	50% (25 mL)		
40% (20 mL)	:	60% (30 mL)		
30% (15 mL)	:	70% (35 mL)		
20% (10 mL)	:	80% (40 mL)		
10% (5 mL)	:	90% (45 mL)		
0% (0 mL)	:	100% (50 mL)	:	0% (0 mL)
	:	90% (45 mL)	:	10% (5 mL)
		80% (40 mL)	:	20% (10 mL)
		70% (35 mL)	:	30% (15 mL)
		60% (30 mL)	:	40% (20 mL)
		50% (25 mL)	:	50% (25 mL)
		40% (20 mL)	:	60% (30 mL)
		30% (15 mL)	:	70% (35 mL)
		20% (10 mL)	:	80% (40 mL)
		10% (5 mL)	:	90% (45 mL)
		0% (0 mL)	:	100% (50 mL)

Twenty-one fractions were obtained after the column chromatographic procedure.

Extraction, Fractionation and Isolation of Constituents of *Portulaca oleracea*

About 3.2 kg of air-dried specimen of *Portulaca oleracea* was cold-extracted in methanol for 72 hours. The mixture was filtered using a wire-guaze and a sieve with tiny pores (0.25 mm) and concentrated at room temperature by exposing the extract for six days. The resulting solution was then placed in the oven at a reduced temperature (50 ^{0}C).

The methanolic extract was then preabsorbed with silical gel and placed in the oven at a reduced temperature (50 ^{0}C) overnight and then subjected to open column chromatography on silical gel (F_{254}, 50-200 mesh, E. Merck) for fractionation. The solvents (mobile phases) were hexane (non-polar), ethylacetate (partially polar) and methanol (polar). The gradients of the mobile phases involved hexane with an increasing percentage of ethylacetate (hexane/ethylacetate mixture) and then ethylacetate with an increasing percentage of methanol (ethylacetate/methanol mixture) as shown in Table 1:

Thin Layer Chromatography (TLC)

The 21 fractions were spotted on precoated plates of silica gel GF_{254} (20 x 20, 0.5 mm thick; E. Merck) using capillary tubes. The spotted TLC plates were developed in a tank that contained a mixture of ethylacetate/methanol (9:1) as the mobile phases.

The TLC plates were then examined under the ultraviolet (UV) light at a wavelength of 365 nm and the well-defined spots of the components were then revealed by the UV light. Fractions with similar relative fronts or retention or retardation factors (R_f value) were then pooled or bulked together, this then reduced the number of fractions to five (fractions 1, 2, 3, 4, 5)

$$R_f = \frac{\text{distance compound has moved from origin}}{\text{distance of solvent front from origin}}$$

The TLC analysis of all the fractions indicated fraction 2 as the fraction that contains many components.

This fraction 2 was further subjected to open column chromatography and eluted using hexane and chloroform (Hexane: Chloroform 50:50) as mobile phases to produce another 46 fractions (Isolated compounds). Isolated compound 7 upon standing overnight gave regular – shaped crystals which were separately washed with hexane and sent for UV, IR and NMR analyses.

Spectroscopy

The quantitative estimation of the isolated compound was obtained by the ultraviolet (UV) spectrophotomety. The infrared and the nuclear magnetic resonance (NMR) analyses were to identify the nature and to obtain the formulae of the isolated compounds.

(i)Ultraviolet (UV) analysis

The UV spectra of the isolated compound was recorded in Chloroform in Genysis 32010 (thermoelectron coupling) spectrophotometer at the Central Research Laboratory, Ladoke Akintola University of Technology, Ogbomoso.

(ii)Infrared (IR) analysis

The IR spectra of the isolated compounds were recorded in Nujol on Spectrum II BX FTIR (Perkin Elmer) spectrophotometer at the Central Research Laboratory, University of Ibadan.

(iii)Nuclear Magnetic Resonance (NMR) analysis

The ^{1}H-NMR spectra was recorded at 200MHZ in CDCl3 on a Varian-Mercury nuclear magnetic resonance spectrophotometry using tetramethysilane (TSM) as an internal standard at the Central Science Laboratory, Obafemi Awolowo University, Ile-Ife.

The ^{1}H-NMR shifts was calculated for the isolated compound using the Advanced Chemistry Development (ACD) software for further confirmation of the structure of the isolated compound.

Acute Toxicity Test of the Isolated Compound

The acute toxicity test of the isolated compound of *Portulaca oleracea* was evaluated in albino mice as described by [8]. Fifteen adult male mice weighing between 20-22g were divided into five mice per group for the isolate. Three doses of the isolate: 0.5 mg/kg BW, 2.5 mg/kg BW and 5 mg/kg BW were orally given to the animals. The control group mice (n=5) received 0.2 ml of distilled water. The animals were observed for seven days for behavioural changes and mortality.

Experimental Design

Fifteen animals were randomly divided into three groups with each group consisting of five rats. The three groups were subjected to the following oral daily treatments for 25 days:

Group I rats received 0.50 mg/kg BW of ergosterol.

Group II rats received 0.75 mg/kg BW of ergosterol.

Group III rats received 0.5 ml of distilled water as the control group.

Collection of Blood Samples

Blood samples were collected through the medial cantus into EDTA bottles for hormonal assay.

Hormonal Assay

Plasma samples were assayed for testosterone using the enzyme-linked immunosorbent assay (ELISA) technique using the Randox kit.

Semen Collection

The testes were removed along with the epididymides. The caudal epididymides were separated from the testes, blotted with filter papers and lacerated to collect the semen.

Semen Analysis

Progressive Sperm Motility: This was done immediately after the semen collection. Semen was squeezed from the caudal epididymis onto a pre-warmed microscope slide (27 ^{0}C) and two drops of warm 2.9 % sodium citrate was added, the slide was then covered with a warm cover slip and examined under the microscope using X400 magnification. Ten fields of the microscope were randomly selected and the sperm motility of 10 sperms was assessed on each field. Therefore, the motility of 100 sperms was assessed randomly. Sperms were labelled as motile, sluggish, or immotile. The percentage of motile sperms was defined as the number of motile sperms divided by the total number of counted sperms (i.e. 100) [9].

Sperm viability (Life/dead ratio): This was done by adding two drops of warm Eosin/Nigrosin stain to the semen on a pre-warmed slide, a uniform smear was then made and dried with air; the stained slide was immediately examined under the microscope using x400 magnification. The live sperm cells were unstained while the dead sperm cells absorbed the stain. The stained and unstained sperm were counted and the percentage was calculated [10].

Sperm morphology: This was done by adding two drops of warm Walls and Ewas stain (Eosin/Nigrosin stain can also be used) to the semen on a prewarmed slide, a uniform smear was then made and air-dried; the stained slide was immediately examined under the microscope using x400 magnification [10]. Five fields of the microscope were randomly selected and the types and number of abnormal spermatozoa were evaluated from the total number of spermatozoa in the five fields; the number of abnormal spermatozoa were expressed as a percentage of the total number of spermatozoa.

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

Statistical Analysis

The mean and standard error of mean (S.E.M.) were calculated for all values. Comparison between the control and experimental groups was done using one-way analysis of variance (ANOVA) with Duncan's Multiple Range Test. Differences were considered statistically significant at $p<0.05$.

RESULTS

(i) Acute Toxicity

No mortality and changes in behaviour were observed in all the treated and control groups. Hence lower doses of the isolated compound were used for this study.

(ii) Spectral Analyses

The characterized name of compound 7 that was sent for spectral analyses is ergosterol. The chemical identity and the structural elucidation of this compound was obtained based on the spectroscopical analyses.

The UV spectrum of compound 7 (Figure 1) shows absorbance at 205 nm, 238 nm, 268 nm, 352 nm which is indicative of the presence of chromophore.

The IR spectrum of compound 7 (Figure 2) shows signals at 2832.74 cm^{-1} corresponding to C-H stretching vibrations, 1747.93 cm^{-1} for C= O. stretching vibrations, 1453.94 cm^{-1} for C-H deformations, 1469.97 cm^{-1} for C-H deformations, and 1376.97 cm^{-1} for C-H deformations.

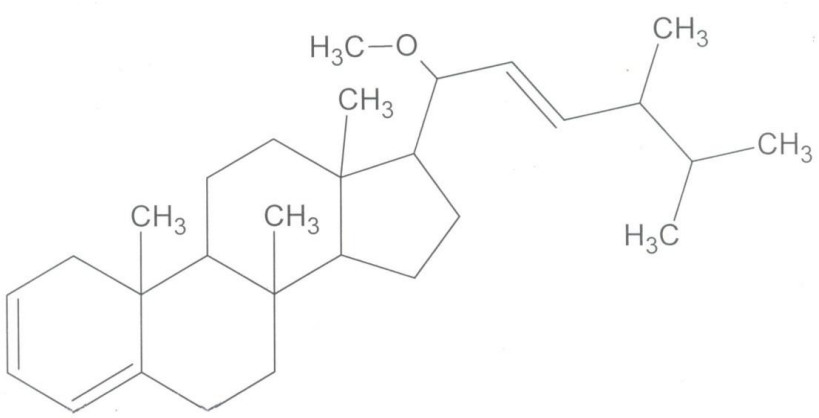
Further justification to the structure of compound was obtained from the ^{1}H-NMR spectrum of compound 7 (Figure 3). Details of the ^{1}H-NMR of compound 7 is presented in Table 1.

All these facts point to the proposed structure as ergosterol.

Table 1: ^{1}H-NMR chemical shift (δ) data of compound 7 (Ergosterol)

S/No	δH (ppm)	Multiplicity	J (MH_Z)
1	2.02	Singlet	-
2	5.64	Singlet	-
3	5.66	Singlet	-
4	5.38	Multiplet	-
5	-	-	-
6	1.84	Singlet	-
7	1.50	Singlet	-
8	-	-	-
9	2.36	Triplet	20
10	-	-	-
11	1.10	Singlet	-
12	1.50	Singlet	-
13	-	-	-
14	2.02	Triplet	20
15	1.10	Singlet	-
16	2.10	Singlet	-
17	1.82	Singlet	-
18	3.48	Doublet	10
19	4.20	Double doublet	20
20	4.36	Double doublet	20
21	2.10	Singlet	-
22	1.62	Singlet	-
23	0.98	Singlet	-
24	0.90	Singlet	-
25	0.90	Singlet	-
26	-	-	-
27	0.90	Singlet	-
28	0.91	Singlet	-
29	0.98	Singlet	-

The proposed structure of ergosterol is shown below:



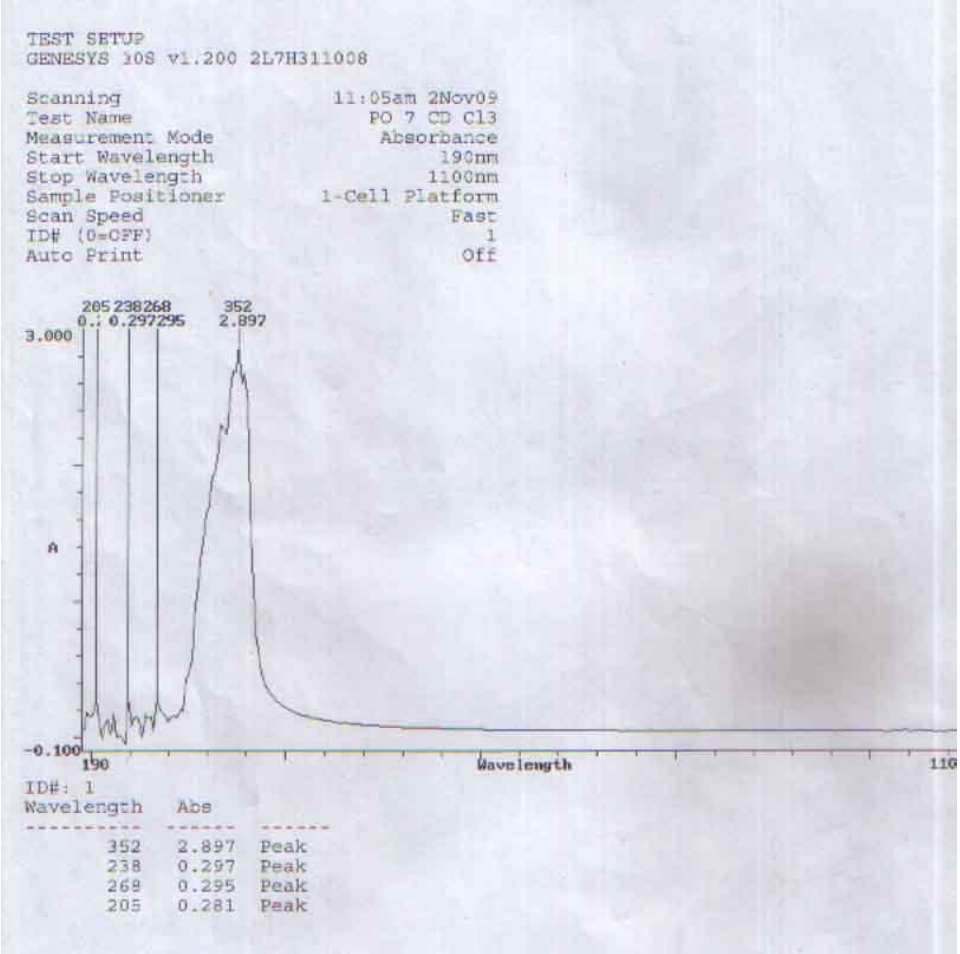


Fig. 1: UV spectrum of compound 7 (ergosterol)

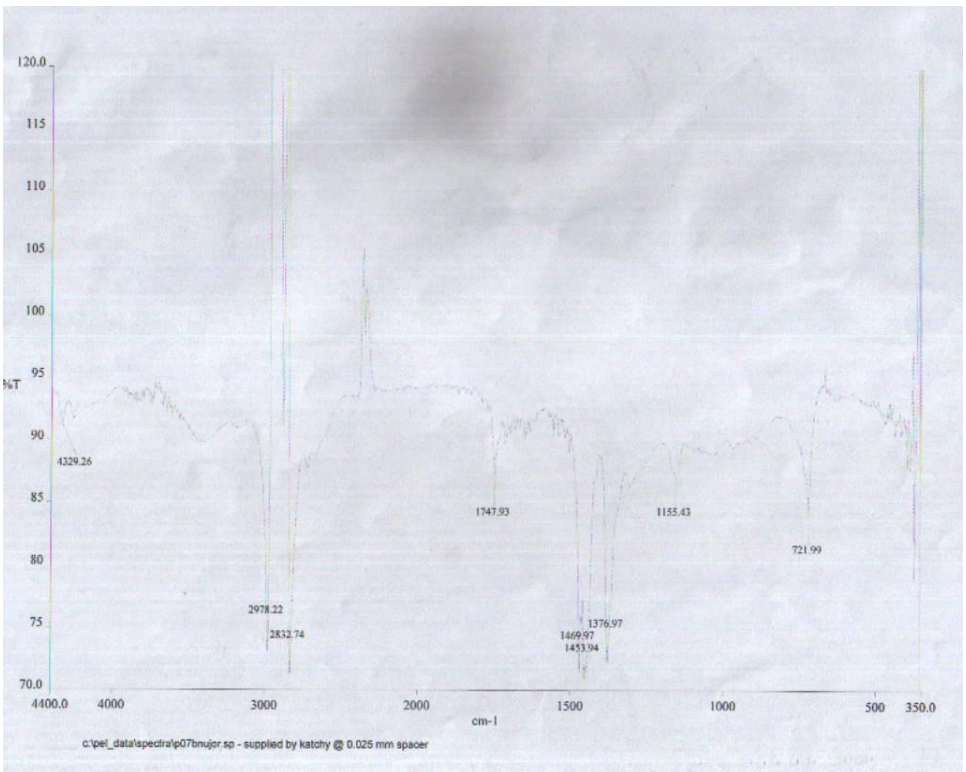


Fig. 2: IR spectrum of compound 7 (ergosterol)

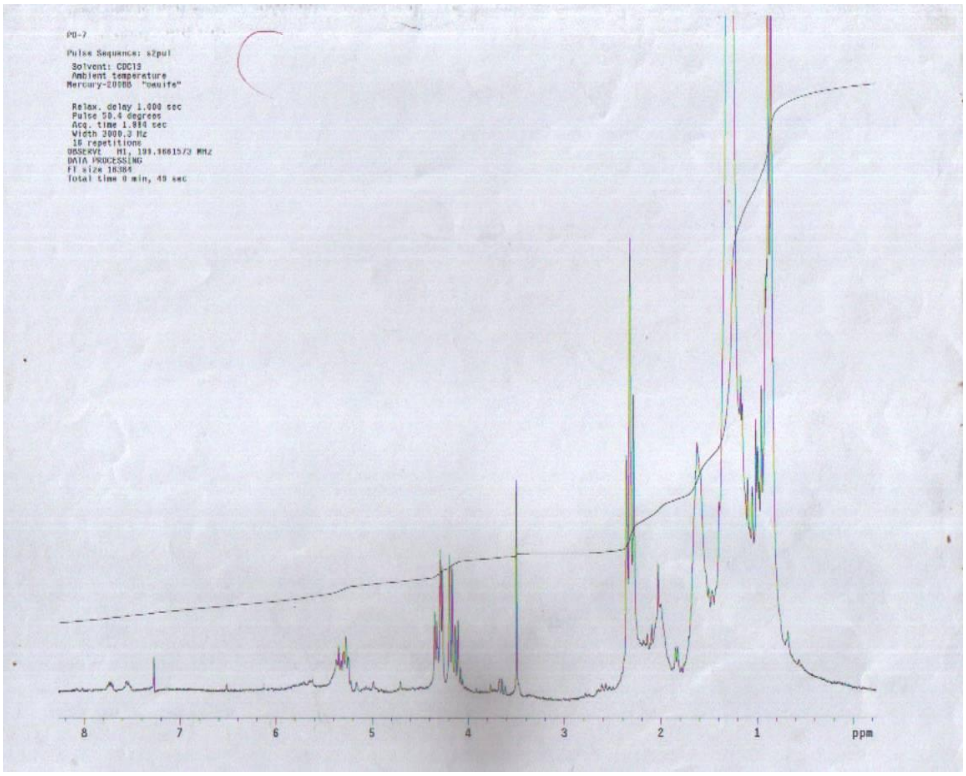


Fig. 3: ^{1}H-NMR spectrum of compound 7 (ergosterol)

(iii) Effect on Hormonal Levels

The effect of ergosterol at doses of 0.50 mg/kg BW and 0.75 mg/kg BW on testosterone levels of albino rats after treatment of rats for 25 days is shown in Figure 4.

Treatment of rats with 0.50 mg/kg BW and 0.75 mg/kg BW of ergosterol caused significant ($p<0.05$) decrease in the testosterone levels relative to the control.

(iv) Effect on Sperm Characteristics

The effect of ergosterol at various doses on sperm characteristics and sperm counts of albino rats after treatment of rats for 25 days are shown respectively in the spermograms of Figures 5 and 6.

Treatment of rats with 0.50 mg/kg BW and 0.75 mg/kg BW of ergosterol caused significant decrease in sperm motility relative to the control. Treatment of rats with 0.50 mg/kg BW of ergosterol caused insignificant ($p>0.05$) change in sperm viability relative to the control. Treatment of rats with 0.50 mg/kg BW of ergosterol caused no significant ($p>0.05$) change in the percentage of abnormal sperm cells relative to the control, while 0.75 mg/kg BW of ergosterol caused significant ($p<0.05$) increase in the percentage of abnormal sperm cells relative to the control. Treatment of rats with 0.50 mg/kg BW of ergosterol caused significant ($p<0.05$) decrease in sperm count relative to the control, while 0.50 mg/kg BW of ergosterol caused non-significant ($p>0.05$) change in sperm count relative to the control.

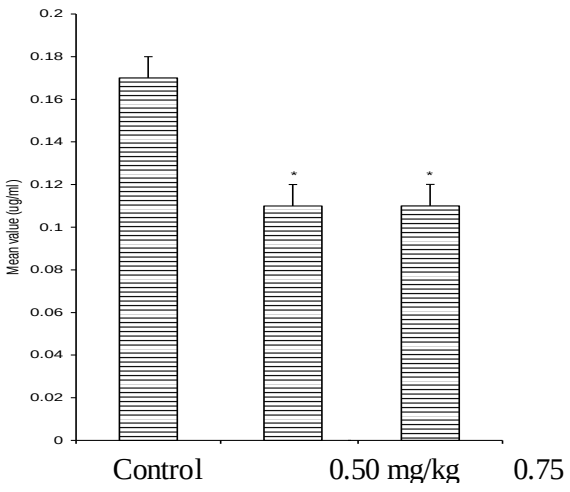


Fig. 4: Effect of 25 days treatment with ergosterol on plasma level of testosterone (n=5, *p<0.05)

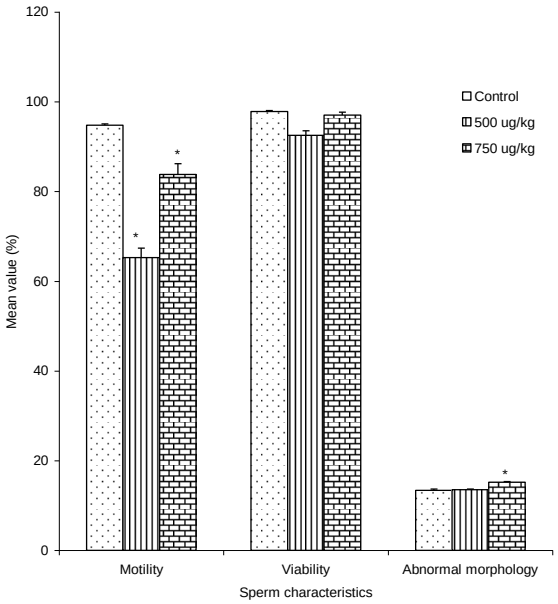


Fig. 5: Spermogram showing the effect of ergosterol on sperm characteristics after treatment of rats for 25 days(n=5, *p < 0.05)

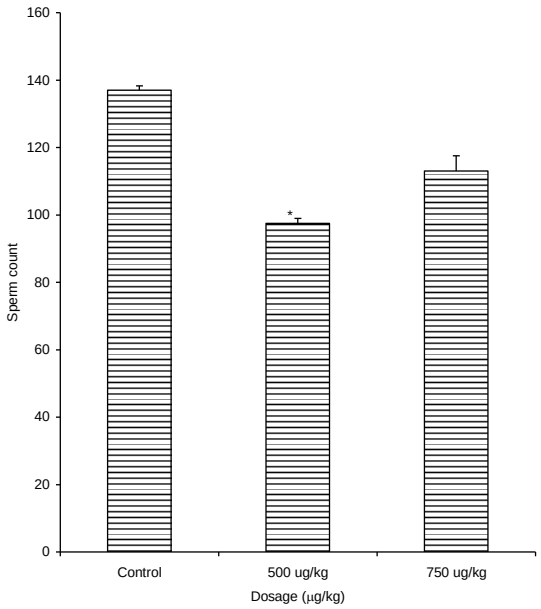


Fig. 6: Spermogram showing the effect of ergosterol on sperm count after treatment of rats for 25 days(n=5, *p < 0.05)

DISCUSSION

It was observed that the highest dose of the isolated compound caused no mortality or behavioural changes in all the treated animals which indicates that the isolate has wide safety margins.

The isolate (ergosterol) caused significant decrease in testosterone levels. Similar report was given by [11] and [12] in rats treated with *Aegle mermelos* and *Carica papaya* extracts respectively. This decrease in testosterone levels could indicate that ergosterol inhibit the mechanism intervening in the process of hormone synthesis in the Leydig cells.

The andrological results show that treatment of rats for 30 days with the extract significant decrease in sperm motility. Similar report was given by [13] in rats treated with *Sarcotemma acidum* extract. This suggests that ergosterol was able to permeate the blood-testis barrier with a resultant alteration in the microenvironment of the seminiferous tubules, since it has been reported that the decrease in sperm motility caused by chemical agents was due to their ability to permeate the blood-testis barrier [14] and thus, creating a different microenvironment in the inner part of the wall of the seminiferous tubules from that in the outer part [15].

There was a statistically non-significant decrease in sperm viability and significant increase in the percentage of morphologically abnormal sperm cells induced after treatment of rats with ergosterol. This could be due to the ability of the isolate (ergosterol) to either interfere with the spermatogenic processes in the seminiferous tubules, epididymal functions or activities of testosterone on hypothalamic release factor and anterior pituitary secretion of gonadotropins which may result in alteration of spermatogenesis ([16]; [17]). Contrary result was reported by [18] in *Ficus carica* extract treated rats.

Sperm count is considered to be an important parameter with which to assess the effects of chemicals on spermatogenesis [19]. Spermatogenesis is influenced by the hypothalamic-

adenohypophysial – Leydig cell system relating gonadotrophin releasing hormone, leutinizing hormone and androgen. This implies that the decrease in sperm count caused by ergosterol in the treated rats might be as a result of decrease in plasma level of testosterone, because this hormone has been reported to be important in the initiation and maintenance of spermatogenesis [20]. Similar report was given by [21] in *Terminalia chebula* extract treated rats.

In conclusion, this study has shown that isolated ergosterol constituent of *Portulaca oleracea* could have some toxic potentialities on the reproductive functions of male albino rats. However, its effect on human reproductive functions are unknown; nevertheless, considering these findings in animal model, it is recommended that men with infertility or reproductive problems should abstain from taking ergosterol during the treatment period.

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