Academic Sciences Asian Journal of Pharmaceutical and Clinical Research

Vol 6, Suppl 2, 2013 ISSN - 0974-2441

Research Article

EFFECT OF ISOLATED TETRACYCLIC STEROID CONSTITUENT OF *Portulaca oleracea* ON REPRODUCTIVE PARAMETERS IN MALE RATS

K.O. OYEDEJI [1], A.F. BOLARINWA[2], I.A. OLADOSU[3]

[1]Department of Physiology, College of Medicine and Health Sciences, Afe Babalola University, Ado-Ekiti, Nigeria,[2]Department of Physiology, College of Medicine, University of Ibadan, Ibadan, Nigeria,[3]Department of Chemistry, Faculty of Science, University of Ibadan, Ibadan, Nigeria.

Received: 6 March 2013, Revised and Accepted: 22 March 2013

ABSTRACT

The effect of oral administration of isolated tetracyclic steroid constituent of *Portulaca oleracea* at doses of 0.50 mg/kg BW and 0.75 mg/kg BW on reproductive parameters in male albino rats were investigated. The isolated compound was administered on daily basis for 25 days and blood samples were collected for hormonal assay, semen analysis was also carried out.

Treatment of rats with 0.50 mg/kg BW and 0.75 mg/kg BW caused significant ($p<0.05$) decrease in testosterone levels, sperm motility and sperm count as well as significant ($p<0.05$) increase in the percentage of abnormal sperm cells relative to their respective controls.

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

Keywords: Tetracyclic steroid, Sperm count, Sperm motility, Testosterone, Albino rats.

INTRODUCTION

Steroids are drugs that mimic certain natural hormones in the body that regulate and control how the body works and develops. Hundreds of distinct steroids were found in plants, animals, and fungi. All steroids are made in cells either from the sterols lanosterol (animals and fungi) or from cycloartenol (plants). There are two main groups of natural steroids-anabolic steroids and corticosteroids.

Health risk can be produced by long-term use or excess of anabolic steroids (Barrett-Connor, 1995). These effects include harmful changes in cholesterol levels, acne, high blood pressure, liver damage, dangerous changes in the structure of the left ventricle of the heart (De Piccoli *et. al.,* 1991).

It has been reported that steroids have protective effects against NMDA-induced seizures and lethality in mice (Budziszewska *et. al.,* 1998). Steroids have been reported to increase aggressiveness in mice and rats (Kostowski et. al., 1970) as well as induced polycystic ovaries in rats (Stener-Victorin *et. al.,* 2003), but have no effect on in utero development in rats (Sprando *et. al.,* 2004). Steroids have also been reported to induce sleep in rats (Mendelson *et. al.,* 1983).

However, due to paucity of information from literature on the effect of tetracyclic steroid on reproductive parameters in male albino rats, this study therefore aims at investigating the effect of isolated tetracyclic steroid 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.

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-gauze 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 (50^0C).

The methanolic extract was then preabsorbed with silical gel and placed in the oven at a reduced temperature (50^0C) 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 below:

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)

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 29 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.

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.

Infrared (IR) analysis

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

Nuclear Magnetic Resonance (NMR) analysis

The ^{1}H-NMR spectra was recorded at 200MH2 and the ^{13}C-NMR spectra at 50MHz 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 and ^{13}C-NMR shifts were 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 Miller and Tainter (1994). 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 tetracyclic steroid.
Group II rats received 0.75 mg/kg BW of tetracyclic steroid.
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) (Mohammad-Reza *et al.*, 2005).

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 (Laing, 1979).

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 (Laing, 1979). 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 5 ml 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 5 ml 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

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.

Spectral Analyses

The characterized name of compound 29 that was sent for spectral analyses is tetracyclic steroid. The chemical identity and the structural elucidation of this compound was obtained based on the spectroscopical analyses.

The UV spectrum of compound 29 (Figure 1) shows absorbance at 316 nm, 256 nm, 238 nm and 196 nm, this corresponds to $\pi \rightarrow \pi^*$ transition and $n \rightarrow \pi^*$ transition.

The IR spectrum of compound 29 (Figure 2) shows signals at 2911.93 cm^{-1} corresponding to C-H stretching vibrations, 1712.74 cm^{-1} for C=0 stretching vibrations, 1650.27 cm^{-1} for C=C stretching vibrations, 1462.15 cm^{-1} for C-H deformations and 1022.04 cm^{-1} for C=0 stretching vibrations.

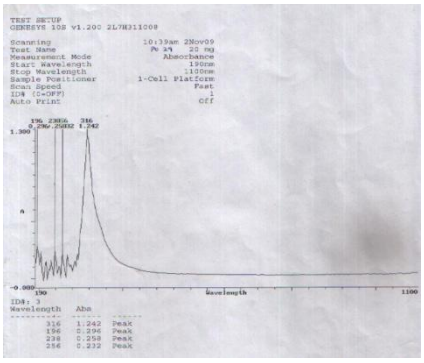


Figure 1: UV spectrum of compound 29 (tetracyclic steroid)

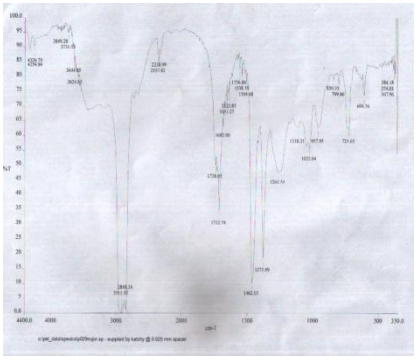


Figure 2: IR spectrum of compound 29 (tetracyclic steroid)

Further justification to the structure of compound 29 was obtained from the NMR spectra of compound 29 (Figures 3 and 4).

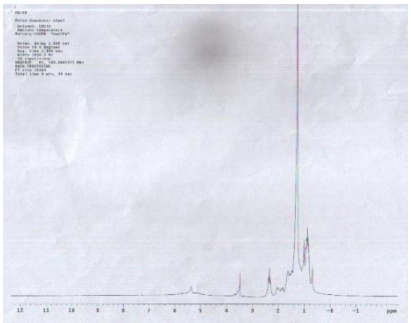


Figure 3: ^{1}H-NMR spectrum of compound 29 (tetracyclic steroid)

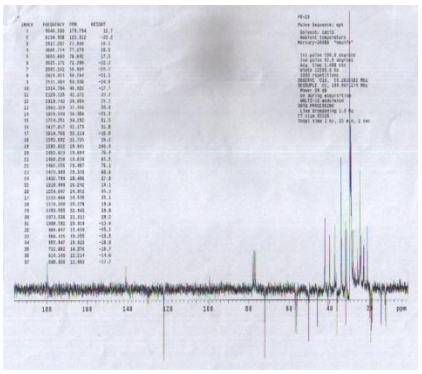


Figure 4: ^{13}C-NMR of compound 29 (tetracyclic steroid)

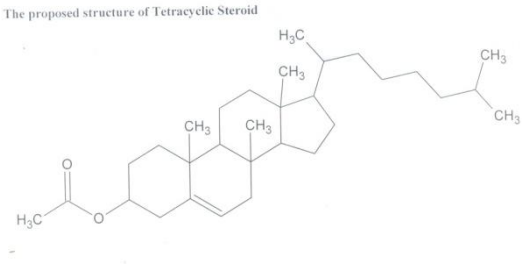
The ^{1}H-NMR of compound 29 indicated the presence of 7 methyl groups, characteristics fingerprint of tetracyclic triterpenes, there is also one prominent olefinic proton.

The ^{13}C-NMR also corroborated the proposed structure. Signals at 21.31 corresponds to a methyl group attached to a carbonyl functional group, other methyl signals are seen at 22.9 (q), 22.9 (q), 19.0 (q), 14.4(q), 12.2(q), 19.0 (q) indicating 6 methyls groups. The presence of olefinic carbons are indicated by the ^{13}C-NMR corresponding to the signals at 122.0 (s) and 136.0 (d). The carbonyl carbon also shows the characteristics ^{13}C-NMR at 179.8 ppm. The clustering of signals within 20 and 50 is a characteristics fingerprint of a steroid. The signals at 72.1 ppm corresponds to a CHO assigned to C-3. Details of the ^{1}H-NMR and ^{13}C-NMR of compound 29 is presented in Table 1.

All these facts point to the proposed structure as tetracyclic steroid.

Table 1: ^{1}H-NMR and ^{13}C-NMR chemical shift (δ) data of compound 29 (tetracyclic steroid)

^{1}H-NMR			^{13}C-NMR		
S/No	**δH (ppm)**	**Multiplicity**	**S/No**	**δC ppm)**	**Multiplicity**
1	1.90	Doublet	1	36.386	Triplet
2	1.50	Singlet	2	26.242	Triplet
3	-	-	3	72.098	Doublet
4	2.00	Singlet	4	37.456	Triplet
5	-	-	5	122.012	Singlet
6	5.35	Singlet	6	136.012	Doublet
7	2.00	Singlet	7	39.990	Triplet
8	-	-	8	32.175	Singlet
9	2.08	Singlet	9	46.036	Doublet
10	-	-	10	36.386	Singlet
11	1.90	Doublet	11	19.255	Triplet
12	1.50	Singlet	12	39.990	Triplet
13	-	-	13	39.990	Singlet
14	2.00	Singlet	14	56.984	Doublet
15	1.00	Doublet	15	24.952	Triplet
16	1.90	Doublet	16	26.242	Triplet
17	1.70	Singlet	17	56.984	Doublet
18	-	-	18	34.292	Doblet
19	1.00	Doublet	19	36.386	Triplet
20	1.30	Doublet	20	26.242	Triplet
21	1.30	Doublet	21	26.242	Triplet
22	1.30	Doublet	22	37.456	Triplet
23	1.30	Doublet	23	22.942	Doublet
24	1.00	Doublet	24	22.942	Quartet
25	-	-	25	22.900	Quartet
26	1.00	Doublet	26	19.012	Quartet
27	0.82	Multiplet	27	14.376	Quartet
28	2.00	Singlet	28	12.214	Quartet
29	1.00	Doublet	29	19.012	Quartet
30	-	-	30	179.794	Singlet
31	2.00	Singlet	31	21.311	Quartet



The proposed structure of tetracyclic steroid is shown above

Effect on Hormonal Levels

The effect of tetracyclic steroid 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 5.

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

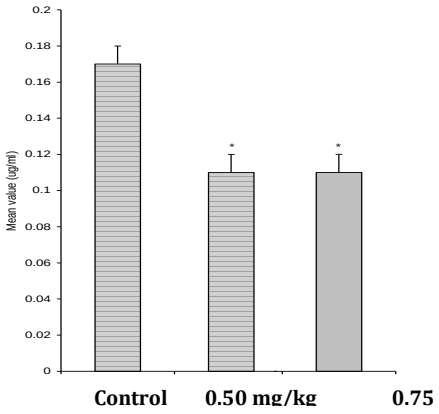


Figure 5: Effect of 25 days treatment with tetracyclic steroid on plasma level of testosterone (n=5, *p<0.05)

Effect on Sperm Characteristics

The effect of tetracyclic steroid 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 6 and 7.

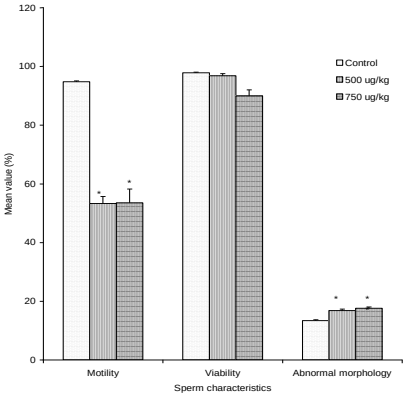


Figure 6: Spermogram showing the effect of tetracyclic steroid on sperm characteristics after treatment of rats for 25 days(n=5, *p < 0.05)

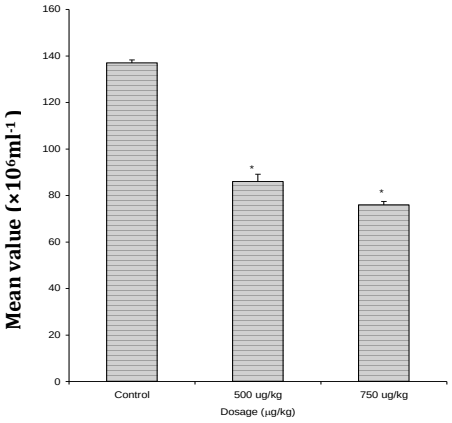


Figure 7: Spermogram showing the effect of tetracyclic steroid on sperm count after treatment of rats for 25 days(n=5, *p < 0.05)

Treatment of rats with 0.50 mg/kg BW and 0.75 mg/kg BW of tetracyclic steroid caused significant ($p<0.05$) decrease in sperm motility relative to the control. Treatment of rats with 0.50 mg/kg BW and 0.75 mg/kg BW of tetracyclic steroid caused no significant ($p>0.05$) changes in sperm viability relative to the control. Treatment of rats with 0. 50 mg/kg BW and 0.75 mg/kg BW of tetracyclic steroid caused significant ($p<0.05$) increases in the percentage of abnormal sperm cells relative to the control. Treatment of rats with 0.50 mg/kg BW and 0. 75mg/kg BW of tetracyclic steroid caused significant ($p<0.05$) decreases in sperm counts relative to the control.

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.

Tetracyclic steroid caused significant decrease in testosterone levels. Similar report was given by Das *et al.* (2009) in rats treated with *Aegle mermelos* extract. This decrease in testosterone levels could indicate that the extract inhibit the mechanism intervening in the process of hormone synthesis in the Leydig cells.

The andrological results also show that treatment of rats for 30 days with tetracyclic steroid caused significant decrease in sperm motility. Similar report was given by Verma *et al* (2002) in rats treated with *Sarcotemma acidum* extract. This suggests that the isolate (tetracyclic steroid) 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 (Baldessarini, 1980) and thus, creating a different microenvironment in the inner part of the wall of the seminiferous tubules from that in the outer part (Bloom and Fawcett, 1975).

There was a statistically non-significant decrease in sperm viability as well as a significant increase in the percentage of morphologically abnormal sperm cells induced after treatment of rats with the isolate. This could be due to the ability of tetracyclic steroid 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 (William, 2000; Bowman and Rand, 1985).

Sperm count is considered to be an important parameter with which to assess the effects of chemicals on spermatogenesis (Reddy *et al.*, 2006). 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 tetracyclic steroid 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 (Christensen, 1975). Similar report was given by Krishnamoorthy *et al.* (2007) in *Terminalia chebula* extract treated rats.

In conclusion, this study has shown that isolated tetracyclic steroid 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 tetracyclic steroid during the treatment period.

REFERENCES

1. Baldessarini RJ (1980): In drugs and the treatment of psychiatriod disorders.The pharmacological basis of therapeutics Ed. By Goodman and Gilman. Macmillan Pub. Co. Inc. pp. 301-417.
2. Barrett-Cornnor E. (1995): Testosterone and risk factors for cardiovascular disease in men. *Diabetes Metab* 21(3):156-61.
3. Bloom W, Fawcett DW (1975): Male reproductive system. In the textbook of Histology. Saunders Company, Philadelphia.
4. Bowman WC, Rand MJ (1985): The reproductive system and drugs affecting the reproductive systems. Textbook of pharmacology, 2nd edition, 20:1-8.
5. Budziszewska B, Siwanowicz J, Leskiewicz M. *et. al.,* (1998): Protective effects of neurosteroids against NMDA-induced seizures and lethality in mice. Eur Neuropsychopharmacol. 8 (1):7-12.
6. Christensen AC (1975): Leydig cell: In: Handbook of Physiology, edited by P.O. Greep and E.B. Astwoods. Washington D C American Physiological Society.
7. Das UK, De D, Chatterjee K, Mallick C, Bera TK, Ghosh D (2009): Antigonadal effect induced by hydro-methanolic extract of leaf of *Aelgle mermelos* in male rat: Effect of hCG co-administration. Journal of Medicinal Plants Research Vol. 3(10), Pp728 – 735.
8. De Piccoli B, Giada F, Benettin A, Sartori F, Piccolo E (1991): Anabolic steroid use in body builders: an echocardiographic study of left ventricle morphology and function. *Int J Sports Med* 12(4):408-12.
9. Kostowski W, Rewerski W, Piechocki T. (1970): Effects of some steroids on aggressive behaviour in mice and rats. Neuroendocrinology, vol. 6, No 5-6.
10. Krishnamoorthy P, Viathinathan S, Rani V, Bhuvaneswari A (2007): Effect of *Terminalia chebula* fruit extract on lipid peroxidation and antioxidative system of testis of albino rats. African Journal of Biotechnology, Vol. 6 (16), Pp 1888 – 1891.
11. Laing JA (1979): Fertility and infertility in domestic animals. 3rd edition 1979 Bailliere Tindall, a division of Cassell Lt.
12. Mendelson WB, Martin JV, Wagner R *et. al.,* (1983): Sleep induction by an adrena steroid in the rats. *Psychopharmacology* 93 (2): 226-229.
13. Mohammad – Reza P, Farzaneh D, Taherch TK, Zoherb PP (2005): The effects of hydroalcholic extract of *Actinidia chinensis* on sperm count and motility, and blood levels of estradiol and testosterone in male rats. Achieves of Iranina Medicine, Volume 8, Number 3, 211-216.
14. Reddy PS, Pushpalatha T, Reddy PS (2006): Reduction of spermatogenesis and steroidogenesis in mice after fentin and fenbutatin administration. Toxicol. Lett. 2006; 166: 53 – 59.
15. Sprando RL, Collins TFX, Black TN et. al., (2004): Effects of androstenedione on in utero development in rats. *Food and Chemical Toxicology* 42:917-924.
16. Stener-Victorin E, Lundeberg T, Cajander S. *et. al.,* (2003): Steroid-induced polycystic ovaries in rats. *Reproductive Biology and Endocrinology* 1:33.
17. Verma PK, Sharma A, Annu M, Prachi S, Gupta RS, Joshi SC, Dixit VP (2002): Effect of *Sarcostemma acidum* stem extract on spermatogenesis in male albino rats. J. Androl. 4(1): 43 – 47.
18. William KW (2000): Hormones and Hormone antagonists. In: Remington, The Science and Practise of Pharmacy, vol 11, 20th edition 77: 1390-1391.