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

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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 suggest 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 lanostan and cyclopentanoperhydrophenanthrene 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 oleracea 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°C).

The methanolic extract was then preabsorbed with silical gel and placed in the oven at a reduced temperature (50°C) overnight and then subjected to open column chromatography on silical gel (F254, 50-200 mesh, E. Merck) for fractionation. The solvents (mobile phases) were hexane (non-polar), ethylacetate (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:

<table>
<thead>
<tr>
<th>Hexane</th>
<th>Ethylacetate</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% (50 mL) : 0% (0 mL)</td>
<td>10% (5 mL) : 90% (45 mL)</td>
<td>0% (0 mL) : 100% (50 mL)</td>
</tr>
<tr>
<td>90% (45 mL) : 10% (5 mL)</td>
<td>20% (10 mL) : 80% (40 mL)</td>
<td>50% (25 mL) : 50% (25 mL)</td>
</tr>
<tr>
<td>80% (40 mL) : 20% (10 mL)</td>
<td>30% (15 mL) : 70% (35 mL)</td>
<td>40% (20 mL) : 60% (30 mL)</td>
</tr>
<tr>
<td>70% (35 mL) : 30% (15 mL)</td>
<td>40% (20 mL) : 60% (30 mL)</td>
<td>50% (25 mL) : 50% (25 mL)</td>
</tr>
<tr>
<td>60% (30 mL) : 40% (20 mL)</td>
<td>50% (25 mL) : 50% (25 mL)</td>
<td>30% (15 mL) : 70% (35 mL)</td>
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<tr>
<td>50% (25 mL) : 50% (25 mL)</td>
<td>40% (20 mL) : 60% (30 mL)</td>
<td>20% (10 mL) : 80% (40 mL)</td>
</tr>
<tr>
<td>40% (20 mL) : 60% (30 mL)</td>
<td>30% (15 mL) : 70% (35 mL)</td>
<td>10% (5 mL) : 90% (45 mL)</td>
</tr>
<tr>
<td>30% (15 mL) : 70% (35 mL)</td>
<td>20% (10 mL) : 80% (40 mL)</td>
<td>0% (0 mL) : 100% (50 mL)</td>
</tr>
</tbody>
</table>

The methanolic extract was then preabsorbed with silical gel and placed in the oven at a reduced temperature (50°C) overnight and then subjected to open column chromatography on silical gel (F254, 50-200 mesh, E. Merck) for fractionation. The solvents (mobile phases) were hexane (non-polar), ethylacetate (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:
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_r value)
were then pooled or bulked together, this then reduced the number
of fractions to five (fractions 1, 2, 3, 4, 5)

R_r = distance compound has moved from origin
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) spectrophotometry. 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 1H-NMR spectra was recorded at 200MHz and the 13C-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 1H-NMR and 13C-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 olereaceae
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 (2790°C and 20%Na citrate) was added, the slide was then covered with a warm cover slip
and examined under the microscope using ×400 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 ×400 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 Wallis and Ewas stain
(Eosin/Nigrosin stain can also be used) to the semen on a
pre-warmed slide, a uniform smear was then made and air-dried; the
stained slide was immediately examined under the microscope using
×400 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-saline 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.

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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 π → π* transition and n → π* transition.

The IR spectrum of compound 29 (Figure 2) shows signals at 2911.93 cm⁻¹ corresponding to C=O stretching vibrations, 1712.74 cm⁻¹ for C=O 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=O stretching vibrations.

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

The 13C-NMR of compound 29 (tetracyclic steroid) shows peaks at 179.8 ppm. The chemical shift of a CHO carbon also shows the characteristic fingerprint of tetracyclic triterpenes, there is also one prominent olefinic proton.

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

<table>
<thead>
<tr>
<th>S/No</th>
<th>δH (ppm)</th>
<th>Multiplicity</th>
<th>δC (ppm)</th>
<th>Multiplicity</th>
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<tbody>
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<td>Doublet</td>
<td>36.386</td>
<td>Triplet</td>
</tr>
<tr>
<td>2</td>
<td>1.50</td>
<td>Singlet</td>
<td>26.242</td>
<td>Triplet</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>72.098</td>
<td>Doublet</td>
</tr>
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<td>Triplet</td>
</tr>
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<td>-</td>
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</tr>
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<tr>
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</tr>
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<td>12</td>
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<td>Triplet</td>
</tr>
<tr>
<td>13</td>
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<td>-</td>
<td>39.990</td>
<td>Singlet</td>
</tr>
<tr>
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<td>Singlet</td>
<td>56.984</td>
<td>Doublet</td>
</tr>
<tr>
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<td>Doublet</td>
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</tr>
<tr>
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</tr>
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<td>Quartet</td>
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</table>

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

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

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

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

Figure 4: 13C-NMR of compound 29 (tetracyclic steroid)
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.

![Image of testosterone levels](image)

**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.

![Image of sperm characteristics](image)

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

![Image of sperm count](image)

**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 0.75 mg/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 marmelos 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 Sarcothema 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-adrenohypophysial – 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