PHOENIX DACTYLIFERAAQUEOUS EXTRACT IMPROVE TESTICULAR INTEGRITY FOLLOWINGGOSSYPOL TOXICITY IN ADULT WISTAR RATS (RATTUS NORVEGICUS)

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ABSTRACT

Background: The importance of traditional medicine in solving health problems is on the increase at global level since a lot of side effect is associated with modern medicine. Gossypol an active compound found in cotton seed oil and other parts of the cotton plant has been linked to male infertility by causing oxidative damage to the testes. Therefore, the ability of aqueous extract of date palm (Phoenix dactylifera)
which is known for its antioxidant activities, to protect the testis against gossypol induced testicular damage in male wistar rats was evaluated.

**Method:** Thirty Five (35) adult male wistar rats were divided into 7 groups of 5 rats each (n=5). Group 1 the control group received 0.1 ml of phosphate buffer solution orally; group 2 received 15mg/kg body weight of gossypol; group 3 received 200mg/kg body weight of vitamin E, group 4 received 40mg/kg body weight of aqueous extract of phoenix dactylifera; group 5 received 15mg/kg of gossypol and 40mg/kg of P. dactylifera; group 6 received 15mg/kg of gossypol and 200mg/kg of vitamin E; and group 7 received 30mg/kg of gossypol for period of 56 days administration. Sperm were obtained from the epididymis for analysis and the testes processed for histological observation (using H/E stains) and enzyme activities (MDA and SOD) respectively following abdominal incision.

**Result:** Significant increase in sperm motility, counts and viability were observed in the vitamin E and phoenix dactylifera treated group, significant increase in lipid peroxidation (MDA activity), superoxide dismutase (SOD) activity and alteration in the differentiation of spermatogonia (spermatogonia behavior) was observed in gossypol toxicity, however, vitamin E and phoenix dactylifera extract demonstrated reduced MDA activity and improved sperm characteristic.

**Conclusion:** The differentiation of spermatogonia observed at different stages and improved sperm characteristic in Phoenix dactylifera aqueous extract treated rats demonstrated its antioxidant and fertility enhancing effects against, free radical generating activity of gossypol at minimal dose.

**Keywords:** Gossypol, infertility, antioxidant, testes, sperm characteristic and free radicals.
BACKGROUND:

Infertility is a major clinical problem, affecting people medically and psychosocially. Male reproductive capacity was found to be the cause in no less than 50% of infertile couples (WHO, 2000). The testes are the primary reproductive organs in the male. It is essential in the reproductive system as it is concerned with the development of healthy sperm cells, an important pre-requisite for fertility and procreation among couples (Khan et al., 2010) and in the production of hormones mainly testosterone. Within each testis, there are almost 200 million of seminiferous tubules, and these structures account for 80-90% of the testicular mass (Greenspan and Gardner, 2001). The germinall epithelium of seminiferous tubules of the testis is one of the most proliferating tissues in the body capable of producing millions of spermatozoa every hour (Guyton and Hall, 2002; Ganong, 2003).

Among the numerous causes of male infertility, oxidative stress (OS) has been identified as one factor that affects fertility status and this has led to its study in recent times. In order to sustain life, oxygen is essential as physiological levels of reactive oxygen species (ROS) are necessary to maintain normal cell function. Conversely, breakdown products of oxygen such as ROS can be detrimental to cell function and survival (de Lamirande and Gagnon, 1995). The generation of ROS by spermatozoa has been proposed to occur in two ways which are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the level of the sperm plasma membrane (Aitken et al., 1992), and NADPH-dependent oxidoreductase (diphorase) at the mitochondrial level (Gavella and Lipovac, 1992) following exposure to cytotoxic agents. Cytotoxic effects are seen when oxidative stress is generated and the antioxidant defense system is inefficient (Zhang et al., 2011). Many substances organic and inorganic have been found to cause oxidative stress in cells some of these substances include atrazine, cadmium, dimethoate and gossypol.

Cottonseed oil is the oil obtained from cotton seeds. It is now packaged in different brands for human consumption. The oil is used in the production of biscuits, crackers, doughnuts and other pastries; in this case, the oil replaces butter fat (Orhevba and Efomah, 2012). The oil contains a naturally occurring yellow pigment known as gossypol. Gossypol is a natural defense compound produced by the plants against pests and diseases (The EFSA, 2008). It is a polyphenolicbinaphthalene carcinogenic pigment which is reputed to induce infertility in man (Gu et al., 2000; Coutinho, 2002). Gossypol is also known to increase erythrocyte osmotic fragility (Risco et al., 2002) and cause hypokalaemia (Yu and Chang, 1998), among other side effects. As a male contraceptive the mode of action of gossypol includes inhibition of acrosin and aryl sulphase (Yuan and Shi, 2000), impairment of ATP production in spermatozoa and the arrest of spermatogenesis (Coutinho, 2002).
The incidence of sexual inadequacy in human males has led to the development of various treatment options, some of these options maybe expensive alongside side effects that may be detrimental to general health as a result, the use of synthetic antioxidants is being restricted these days therefore there is a more growing trend in the search for antioxidants of natural origin which have fewer side effects at the same time less expensive (Cicero et al., 2001; Adimoelja, 2000). Medicinal plants have been known to provide valuable therapeutic agents, both in modern and in traditional medicine (Krentz and Bailey, 2005). The use of date palm as herbal medicine has been popular worldwide especially in the Asian and African countries (Bahmanpouret et al., 2006). The date palm (*Phoenix Dactylifera*) is a monocotyledon of the family of the Palmae, one of the genera of which is the Coryphoideae, of which one species is Phoenix Dactylifera (Barreveld, 1993). Date palms have been cultivated in the Middle East since at least 6000 BC.

Reports have indicated that Date palm contain flavonoid components (Mahran et al., 1985 and Abbas and Ateya, 2011) that have positive effects on sperm quality (Vayalil, 2002 and Kostyuk et al., 2004). The scavenging properties of date palm is said to be the main important effects on the sperm parameters. Several studies have also shown their functional health benefits on sperm parameters and reproductive system of adult rats (Bahmanpouret et al., 2006) and their protective effect on cadmium induced male infertility in rats (Wafaa et al., 2012).

Therefore to determine whether Date palm could attenuate gossypol induced testicular damage, this study was designed to examine the antagonistic actions of Date palm on biochemical and histological alterations in male rat testes induced by gossypol. Gossypol the active component in cotton seed oil has been studied and confirmed as an infertility agent. It exerts this effect by causing oxidative stress. *Phoenix dactylifera* has been known for its medicinal properties alongside many other positive properties mainly its antioxidant effects.

**MATERIALS AND METHODS**

**Animal Source and Handling**

A total of thirty five (35) Wistar rats with average weight between 120 – 150g were procured from the Animal Holding Facility of Bingham University, Karu, Nasarawa, Nigeria and were acclimatized in the control room for 4 weeks. The rats were fed with standard diet (growers mash); water was given ad libitum and maintained under standard conditions the animal room was maintained at normal room temperature under day/night cycles. The rats were grouped into Seven (7) groups of five (5) rats each.

**Extract Preparation**
Cotton seed was obtained from Dengi market, Plateau state, Nigeria. The seeds were pounded to powder using a mortar and pestle and the cotton seed oil was extracted using the solvent extraction method described by Orheva and Efomah, 2012. The experimental process involved the following; collection of seeds, cleaning of seeds, drying, cooling, size reduction, weighing of the crushed seeds, solvent extraction, weighing of the cottonseed cake, recovery of solvent, and recovery of crude cottonseed oil. The samples collected were properly cleaned in order to remove any foreign materials. They were oven dried in the laboratory at a temperature of 130°C, to a moisture content of 12%. This was done because the lesser the moisture content, the more the oil yield (Taiwo et al., 2008). The seeds were then crushed into powder using Thomas Willey mill (Model ED-5). Twelve (12 g) of the crushed sample was weighed and mixed with 5 ml of N-hexane. The mixed sample was placed on a filter paper and the filter paper was then properly folded and inserted into the assembled soxhlet apparatus. The weight of the filter paper and sample was recorded. One hundred and fifty milliliters (150 ml) of the solvent (N-hexane) was measured using a measuring cylinder and then poured into a five hundred milliliters (500 ml) round bottom flask which is the lower part of the soxhlet apparatus. This was now heated with a heating mantle at 60°C for 6 hours. As the solvent boiled, it evaporated into the reflux condenser and this hot solvent vapor was cooled by the surrounding water which flowed continuously through the soxhlet arrangement. The cooled solvent then condensed back into the portion of the soxhlet containing the folded sample and this facilitated the extraction of the oil from the sample. The extracted solution in the round bottom flask was a combination of oil and solvent. The sample left after the oil had been removed was subjected to hot pressing using hydraulic press to remove the bulk of the oil remaining in the press cake (Orheva and Efomah, 2012).

Gossypol was extracted from the cotton seed oil using 70% cold acetone. This is because the decomposition rate of gossypol is lower in acetone than in other organic solvents such as methanol, chloroform, ethanol, and acetonitrile (Nomeir and Abou-Dounia, 1982).

Date palm fruits were obtained from Masaka market in Nassarawa state. The seeds were removed from the fruit and the pulp was pounded to powder using mortar and pestle. 435 g of the powder was soaked in 4350 ml of distilled water for 24 hours. The solution was then filtered using fine sieve and the residue was discarded. The filtrate was evaporated at 60°C using water bath till a thick semisolid paste with a fruity smell was obtained. This was dissolved in phosphate buffer solution before administering to the rats. An aqueous extract was selected because most of the antioxidant components in dates are extracted in water (Vayalil, 2002; Al-Farsi et al., 2005b).
The administration of the extracts was totally by gavage. The appropriate concentrations of Phoenix dactylifera, Vitamin E and Gossypol were administered using metal oro-pharyngeal cannula once daily for 56 days. The control group received 0.1 ml phosphate buffer. Group 2 received 15mg/kg body weight Gossypol only, Group 3 received 200mg/kg body weight Vitamin E only, Group 4 received 40mg/kg body weight Phoenix dactylifera only, Group 5 received 15mg/kg body weight Gossypol + 40mg/kg body weight Phoenix dactylifera, Group 6 received 15mg/kg body weight Gossypol + 200mg/kg body weight Vitamin E and Group 7 received 30mg/kg body weight Gossypol.

**Animal Sacrifice**

The animals were sacrificed by cervical dislocation about 24 hours after the last administration and the testes were excised. The left testes of each animal were fixed in Bouin’s fluid for histological analysis using hematoxylin and eosin (H/E) while the right testes was homogenized in 5% sucrose solution for enzyme Histochemistry. Spermatozoa were obtained from the epididymis for semen analysis using a counting chamber.

**Semen Analysis**

The caudal epididymis was dissected out; several incisions (about 1mm) were made on the caudal epididymis which was suspended in 1ml of normal saline solution according to Chowdhury et al., 1986, for sperm motility and morphology. The sperm concentrations were determined by fixing the sperm in 10% Formo-saline in ratio 1:9. The counting was done using the improved nebular haemocytometer (Chowdhury et al., 1984).

**Histological Preparation**

The organs were cut in slabs of about 0.5 cm thick transversely and fixed in 10% buffered formalin for 24 hours and processed routinely for paraffin embedding. 5μ sections were obtained with rotatory microtome and processed for Hematoxylin and Eosin (H/E) according to Pearse, 1980 and Orcein – Modified Taezer- Unna (1891) methods respectively.

**Enzyme Biochemistry**

Excised testicular tissues were put in Lao style mortar containing 5% sucrose solution and were homogenized thoroughly. Tissue homogenates were collected in 5ml plain serum bottles for enzyme assay), Superoxide Dismutase (SOD) and Malondialdehyde (MDA). Enzyme activity of SOD was assayed according to the method described by Misra and Fridovich, (1972). The concentration of malondialdehyde was quantified according to the procedure described by Reilly and Aust (1999) for assessment of lipid peroxidation.

**Test Statistics**

Data were statistically evaluated using the ANOVA test with Medcalc 3rd ed., 2000 and were expressed as Mean ± SEM. A value of \( P < 0.05 \) was considered to indicate a significant difference between groups, hence indicating the level of significance.

Results

Table I: semen analysis

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Motility (%)</th>
<th>Sperm cell count (Million/ml)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1</td>
<td>85.0 ± 4.46</td>
<td>6.4 ± 5.9</td>
<td>71.0 ± 4.0</td>
</tr>
<tr>
<td>GROUP 2</td>
<td>16.0 ± 2.9*</td>
<td>26.6 ± 3.2*</td>
<td>42.0 ± 9.1</td>
</tr>
<tr>
<td>GROUP 3</td>
<td>77.0 ± 3.0</td>
<td>62.8 ± 3.5</td>
<td>72.0 ± 3.7</td>
</tr>
<tr>
<td>GROUP 4</td>
<td>67.4 ± 2.5*</td>
<td>65.0 ± 3.9</td>
<td>78.4 ± 5.1</td>
</tr>
<tr>
<td>GROUP 5</td>
<td>64.0 ± 2.4*</td>
<td>58.4 ± 1.2</td>
<td>82.0 ± 3.7</td>
</tr>
<tr>
<td>GROUP 6</td>
<td>70.0 ± 4.4</td>
<td>73.2 ± 0.9</td>
<td>76.0 ± 2.4</td>
</tr>
<tr>
<td>GROUP 7</td>
<td>52.0 ± 7.3*</td>
<td>43.6 ± 0.9*</td>
<td>70.0 ± 4.4</td>
</tr>
</tbody>
</table>

\( P < 0.05 \) level of significance

The gossypol treated groups showed significant decrease in sperm motility and sperm count when compared to the control group. Although the group treated with date palm only and the group treated with gossypol and date palm showed a significant decrease in sperm motility when compared to the control group, the date palm treated group and the gossypol and date palm group showed a significant increase sperm motility (\( P < 0.05 \)), sperm count (\( P < 0.05 \)) and viable sperm viability (\( P < 0.05 \)) when compared to the gossypol treated group.

Table III: The level of lipid peroxidation in groups using MDA activities

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MDA (x10^-8 units/mg protein) Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>GROUP 2</td>
<td>4.7 ± 1.0</td>
</tr>
<tr>
<td>GROUP 3</td>
<td>0.8 ± 0.2*</td>
</tr>
<tr>
<td>GROUP 4</td>
<td>1.8 ± 0.1*</td>
</tr>
<tr>
<td>GROUP 5</td>
<td>1.9 ± 0.2*</td>
</tr>
<tr>
<td>GROUP 6</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>GROUP 7</td>
<td>5.0 ± 0.2*</td>
</tr>
</tbody>
</table>

MDA= Malondialdehyde
SEM= standard error of mean
\( P < 0.05 \) * significant
From the table above, the group treated with vitamin E only (group 3) and date palm only (group 4) showed a significant decrease in malondialdehyde levels when compared with the control group (group 1). Group treated with Gossypol and date palm (group 5) also showed a significant decrease in MDA level when compared with the control group. The group treated with gossypol (30mg/kg) showed a significant increase in MDA level when compared to the control group. After a concomitant administration of date palm and gossypol, there was a significant reduction in testicular MDA compared to Gossypol treated groups.

Table IV: The activity of Superoxide Dismutase (SOD).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>SOD (UNITS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN ± SEM</td>
<td></td>
</tr>
<tr>
<td>GROUP 1</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>GROUP 2</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>GROUP 3</td>
<td>3.5 ± 0.7*</td>
</tr>
<tr>
<td>GROUP 4</td>
<td>5.1 ± 0.3*</td>
</tr>
<tr>
<td>GROUP 5</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>GROUP 6</td>
<td>4.8 ± 0.9*</td>
</tr>
<tr>
<td>GROUP 7</td>
<td>1.6 ± 0.2*</td>
</tr>
</tbody>
</table>

SOD = Superoxide dismutase
SEM= Standard error of mean
P < 0.05 * significant

Groups treated with date palm (group 4), gossypol 30mg/kg (group 8) and those treated with gossypol and date palm (group 5) showed a significant increase in SOD activity when compared to the control group. However group treated with gossypol 30mg/kg showed significant decrease in SOD activity when compared to the date palm treated group.
**Histological Analysis**

**Figure I:** Micrograph of the testes of Group 1, H/E stain, X400. The white arrows indicate the spermatogonia while the black arrow indicates the differentiating cells, the blue arrow indicates the Leydig cells. The micrograph demonstrated normal histology of the seminiferous tubule.

**Figure II:** Micrograph of the testes of Group 2 gossypol low dose H/E X400. White arrow shows damaging basement membrane. Black arrow shows spaces indicating decreased cell count. Reduce spermatogonia differentiation from the germinal epithelium into a mature spermatocytes and de-arrangement in the spermatogonia along the germinal epithelium was observed.
Figure III: Micrograph of the testes of Group 4, Phoenix dactylifera only H/E stain, X400. White arrow indicates interstitial tissue. Blue arrow indicates basement membrane. Black arrow indicates differentiating cells. Proper alignment or arrangement in the pattern of the spermatogonia behavior and the various developmental stages of spermatogonia were well expressed.

Figure IV: Micrograph of the testes of Group 3, Vitamin E only H/E stain, X400. White arrow indicates germinal epithelium. Black arrow indicates differentiating cells.
**Figure V:** Micrograph of the testes of Group 6 Gossypol + Vitamin E, H/E stain, X400. White arrow indicates spermatogonia. Blue arrow indicates basement membrane. Black arrow indicates differentiating cells. This group shows normal spermatogenesis.

**Figure VI:** Micrograph of the testes of group 5 Date palm + gossypol H/E X400. White arrow indicates interstitial cells. Black arrow indicates decreased differentiating cells. Various developmental stages were expressed from germinal epithelium, however, intra-spermatogonia spaces were large and scattered spermatogonia cells were observed.
Figure VII: Micrograph of the testes of group 8 Gossypol high dose H/E X400. White arrow indicates basement membrane. Black arrow indicates interstitial tissue. This group show decreased sperm count as seen by the decreasing number of differentiating cells.

Discussion

Gossypol is a proven testicular toxin and male contraceptive (Yu and Chan, 1998; Yuan and Shi, 2000); its mechanism of action has been reported to include inhibition of acrosomal enzymes (Yuan and Shi, 2000), affinity for extracellular and intracellular proteins (Wang et al., 1992), and inhibition of spermatogenesis and sperm motility (Kalla and Vasudev, 1980; Ridley and Blasco, 1981; Coutinho, 2002).

The present study was designed to evaluate the protective potential of date fruit extract as an antioxidant-rich nutraceutical on gossypol induced testicular toxicity. From the results obtained, it was observed that the group treated with gossypol (30mg/kg) only showed a significant decrease in body weight of rats after the period of experiment when compared to the control group. This is consistent with the work done by Sakesena et al., 1981 where rabbits treated with gossypol exhibited a decrease in appetite and body weight. However there was a significant increase in body weight in the group treated with date palm only.

In this present study, animals exposed to gossypol showed a significant decrease in sperm count and sperm motility. The animals exposed to gossypol 15mg/kg (group 2) and gossypol 30mg/kg (group 8) showed a significant reduction in sperm count and motility (p<0.05), this is in line with the reports of Kalla and Vasudev (1980) and Shi et al., (1981) on the inhibitory effects of gossypol on these sperm parameters; the deleterious effect of gossypol on sperm motility was reported to be due to its ability to deplete ATP in spermatozoa. Kalla and Vasudev (1980), concluded that gossypol was capable of inducing spermatogonial damage, thereby arresting spermatogenesis this is in agreement with the reduction in sperm count observed in this work.
It was observed that there was a significant increase in level of peroxidation of the lipid layer of cell membrane as revealed by the significant increase in malondialdehyde (MDA) activities in the groups administered gossypol only. Malondialdehyde has been considered the biomarker of lipid peroxidation, which leads to impairments of the cell’s functions as well as genotoxicity and carcinogenesis (Korchazhkina et al., 2003).

In table 3, there was a significant increase in MDA levels in group 8 (30mg/kg gossypol) when compared to the control group. Group 5 (40mg/kg date palm + 15mg/kg gossypol) showed significant decrease in MDA levels when compared to the groups treated with gossypol only, thereby suggesting that date palm played a role in maintaining testicular integrity.

Superoxide dismutase is the most predominant enzymatic antioxidant found in sperm cells (Makker et al., 2009). It is a natural scavenger of reactive oxygen species and superoxide radicals (Zhang et al., 2011) and does this by combining with active oxygen free radicals specifically superoxide ions in order to prevent lipid peroxidation of the cell membrane and damaging metabolites’ formation (Zhang et al., 2011). The gossypol treated groups showed significant lower SOD activity compared to the group treated with date palm only.

Also, the group treated with date palm extract and gossypol showed a significant increase in SOD activity compared with the gossypol only treated group this is consistent with the research carried out by Akunna et al., 2012 in which the group where date palm was treated with atrazine showed a significant increase in SOD activity compared to the atrazine treated group. Therefore the result obtained in this study suggest that date palm plays a role in preventing SOD inhibition by gossypol hence improving the antioxidant status of the cells in order to prevent oxidative stress. In H/E staining, the spermatogonia stained more densely than the differentiating cells. Testes from the control group and date palm treated groups of rats exhibited typical features of seminiferous epithelium where the darkly stained germinal epithelium lie immediately above the basement membrane, different stages of the differentiating cells with the most mature cells located towards the lumen of the tubule.

However, in the groups treated with gossypol only, it was observed that there was impaired spermatogenesis evident by the presence of fewer spermatogonia when compared to the control group, this corresponds with the sperm analysis in which these groups showed the lowest sperm count supporting Randelet et al., (1992) where they reported that at effective doses of gossypol, there was decreased sperm motility and decreased sperm counts as a result specific mitochondrial damage in the tails of spermatozoa, in addition to this, the enzyme assay of this group suggests that the antioxidant mechanism of the testis was suppressed by gossypol evident by high levels of malondialdehyde indicating lipid peroxidation. Immunoassay is a bioanalytical method.
which involves the quantitation of an analyte by a reaction between an antigen (analyte) and an antibody.

**Conclusion**

This study shows that despite series of data relating gossypol to reproductive toxicity, aqueous extract of Date fruit (Phoenix dactylifera) was able to maintain the integrity of the testes and prevent the damage caused by gossypol evidenced by the improved sperm characteristics, histo-architecture of the testes and reduction in lipid peroxidation in the testes of male wistar rats.

**References**


