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Evaluation of the Effect of Date Palm [*Phoenix dactylifera*] Fruit Extract on Methotrexate-Induced Reproductive Toxicity in Male Rats

N. C. Azubuike [a*] and C. C. Opoh [a]

[a] Department of Medical Laboratory Sciences, Faculty of Health Sciences and Technology, College of Medicine, University of Nigeria, Enugu Campus, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author NCA conceptualized the study, designed the protocol, performed the investigations in the study, critically reviewed the literature, performed the statistical analysis, proof-read the first manuscript draft, and wrote the final manuscript. Author CCO managed the literature searches, collected data, and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Background: *Phoenix dactylifera* L. in the *Palmae* family (*Arecaceae*) is commonly known as Date palm. Its fruit is popularly consumed as food and also used as a herbal remedy for fertility enhancing effects in males.
Aim: The present study was designed to explore the effect of *P. dactylifera* fruit extract (PDFE) on methotrexate (MTX)-induced reproductive toxicity in male rat models.
Study Design: An experimental study which lasted for 19 days.
Place and Duration of Study: Department of Medical Laboratory Sciences and Animal House, College of Medicine, University of Nigeria, Enugu Campus, between April 2019 and November 2019.
Methodology: Sixteen (16) rats were divided into four groups (n=4) namely: Normal control (NC), MTX control, MTX+200mg/kg PDFE and MTX+400mg/kg PDFE. PDFE was administered by oral gavage, once daily, for 10 days. MTX injection (20mg/kg i.p.) was administered to rats in the three test groups on the fifth day. All the rats were sacrificed on Day 11 and the testes and epididymides of all rats were excised and histologically processed for light microscopical examination. Caudal epididymal sperm concentration was also determined.

**Corresponding author: E-mail: nkiruka.azubike@unn.edu.ng;*

Results: MTX induced a marked depletion and degeneration of spermatogenic series in the testicular tissues of MTX-control rats whereas PDFE treatment offered some cyto-architectural preservation in a dose-dependent manner. Similarly, a decreased number of spermatozoa within the lumen of epididymal tubules was observed with MTX treatment compared to the normal control and PDFE-treated rats. Treatment with PDFE caused a slight improvement in caudal epididymal sperm concentration although not statistically significant ($p>0.05$) when compared with the controls.
Conclusion: Data from the present study has revealed that oral treatment with *P. dactylifera* fruit extracts ameliorates the deleterious effects of methotrexate-induced reproductive toxicity in rats.

Keywords: Phoenix dactylifera; methotrexate; reproductive toxicity; testis; epididymis; caudal sperm concentration.

1. INTRODUCTION

Methotrexate (MTX) is a folic acid antagonist and has a wide use for the treatment of malignant tumors and some non-neoplastic diseases. The mechanism of action of MTX is mediated through the inhibition of folic acid metabolism thereby resulting to cellular folate deficiency and subsequent death of the cells [1]. Although the use of MTX has been employed in the treatment of certain cancer types, it however induces profound side effects including acute organ toxicity [2,3]. Previous studies involving animals have used MTX to induce testicular damage, reduce number of sperm cells and damage sperm [4,5].

Oxidative stress plays a major role in testicular damage caused by the use of MTX due to generation of free oxygen radicals [6] and this invariably leads to the disruption the testicular microanatomical structure and germ cells [7]. Consequently, compounds with antioxidant properties have been reported to protect the testicular tissue from deleterious effects of MTX-induced oxidative stress [4,8].

Date palm (*Phoenix dactylifera*), (family: Arecaceae) is majorly grown for its edible sweet fruit. It is grown in South Asia, Middle East and Africa having naturalized in the subtropical and tropical parts of the world. The fruit contains high concentrations of phenolic compounds such as flavonoids and anthocyanins as well as other phytoconstituents [9]. Previous researchers have documented its antioxidant, sex hormone modulating, gastroprotective, nephroprotective, hepatoprotective, anticancer, anti-inflammatory and antimicrobial effects [10,11].

In Nigeria, there is a growing interest in the use of date palm fruits for the enhancement of male fertility and good preservation of the gonads. However, there is paucity of scientific data describing the protective effect of date palm fruits against MTX-induced reproductive toxicity in male rats. Hence it is pertinent to explore on a readily available, natural and cheap plant product which could serve as a supplementation to protect the reproductive organs of males undergoing chemotherapy with MTX. The present study, therefore, was carried out to investigate the effects of date palm fruit extract against methotrexate-induced injury on the testes and epididymis of albino rats.

2. MATERIALS AND METHODS

2.1 Drugs and Chemical

Ham's F-10 fluid was purchased from SIGMA® (Sigma –Aldrich®, St. Loius USA). Methotrexate (Ebewe Pharma®, Unterach, Austria) was purchased from a local Pharmaceutical store. All other reagents and chemicals were of analytical grade.

2.2 Procurement of Plant Material

Samples of the date palm fruits [*Phoenix* dactylifera] were purchased from Ogbete Main Market in Enugu metropolis, Enugu State, Nigeria. An expert at the herbarium section of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, authenticated the specimen. The date fruits were split manually to separate the fruit from the seeds and then air-dried under shade for one week. The acquired flesh of the fruits was finely-grinded using a gasoline-powered grinding machine and eventually stored in an air-tight container.

2.3 Preparation of Crude Aqueous Extract

Two hundred (200) grams of the powdered plant sample was extracted by soaking in 2000mls of cold distilled water. The mixture was homogenized for about 5 min using a wooden

stirrer and left for 24 hours. Thereafter, with the aid of a muslin cloth, the homogenate was filtered to obtain a resultant filtrate which was evaporated under vacuum for concentration, transferred into a leak-proof container labelled as PDFE (*Phoenix dactylifera* Fruit Extract) and eventually stored in the refrigerator at 2 – 8^{o}C until needed.

2.4 Laboratory Animals

Sixteen (16) adult male albino Wistar rats (weighing between 110 – 130g) of approximately 3 months old were used. The animals were procured from the Animal House of the College of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, Nigeria. Animal housing was at the Animal facility of the Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, University of Nigeria, Enugu Campus. The rats were kept in clean wire-mesh cages and the facility was under standard environmental conditions of temperature (25±2^{o}C) and light (12 h light/dark cycle). The rats were adequately fed with commercially available rat chow and clean water *ad libitum*. They were allowed to acclimatize for one week before the commencement of the studies.

2.5 Experimental Protocol

The rats were randomly divided into four groups (A – D) (n = 4) namely: normal control, MTX-control, MTX+200mg/kg PDFE and MTX+400mg/kg PDFE respectively. Rats in groups C and D received 200mg/kg and 400mg/kg body weight of PDFE, respectively, once daily via oral gavage for 11 days (Days 1 - 11). MTX was dissolved in physiological saline and an intraperitoneal dose of 20mg/kg body weight (6) was administered to rats in groups B – D once on Day 5. Rats in group A received no treatment and thus served as the normal control.

2.6 Epididymal Sperm Concentration

The animals were sacrificed on Day 12 under diethyl ether anaesthesia at 24hrs after the last administration. The two cauda epididymides (left and right) were excised; several incisions (1mm) were made in one of the cauda epididymis which was suspended in 1ml of Ham's F- 10 solution (Sigma Aldrich). After 10 minutes of incubation at 37^{o}C, the haemocytometer method was employed for the determination of sperm concentration [12]. Testes of all the rats were also excised, and together with the other cauda epididymis from each animal, were preserved and processed for histological studies for light microscopical examination.

2.7 Histopathological Studies

The testes and epididymides were placed in tissue cassettes and fixed in 10% formal saline prior to further histological processing using the Automatic Tissue Processor. The tissues were later embedded in paraffin wax and sectioned with the Rotary Microtome (Heitz 150, Cambridge model) at 3 - 5µm thickness. The routine staining technique (Hematoxylin and Eosin) for the demonstration of general tissue morphology was used to stain the sections for light microscopical examination as previously described [13]. The Olympus Binocular microscope with in-built lighting system was used to examine the sections and their photomicrographs were taken using an AmScope eyepiece microscope-digital-camera.

2.8 Statistical Analysis

Data obtained from the caudal epididymal sperm concentration were expressed, where appropriate, as mean ± S.E.M. of four rats per group. The Statistical Package for Social Sciences [SPSS] software (SPSS, Chicago, IL; version 23.0) was employed for statistical analysis. Data were subjected to one-way analysis of variance (ANOVA), followed by students' t-test to determine the differences among the groups. Value of $p<0.05$ was the level to be considered statistically significant.

3. RESULTS AND DISCUSSION

The present study evaluated the effect of treatment with extract of *P. dactylifera* on methotrexate-induced reproductive toxicity. On the testis histology of the normal control rats, histoarchitectural features consistent with normal tissues were observed (Fig. 1a). However, treatment with MTX only, caused degeneration and depletion of spermatogenic series in the seminiferous tubules of treated rats (Fig. 1b). There is marked evidence of incomplete spermatogenesis which is revealed by absence of mature germ cells within most of the seminiferous tubular lumens. This finding is consistent with previous reports which demonstrated the adverse effects of MTX on testicular tissue [4,5].

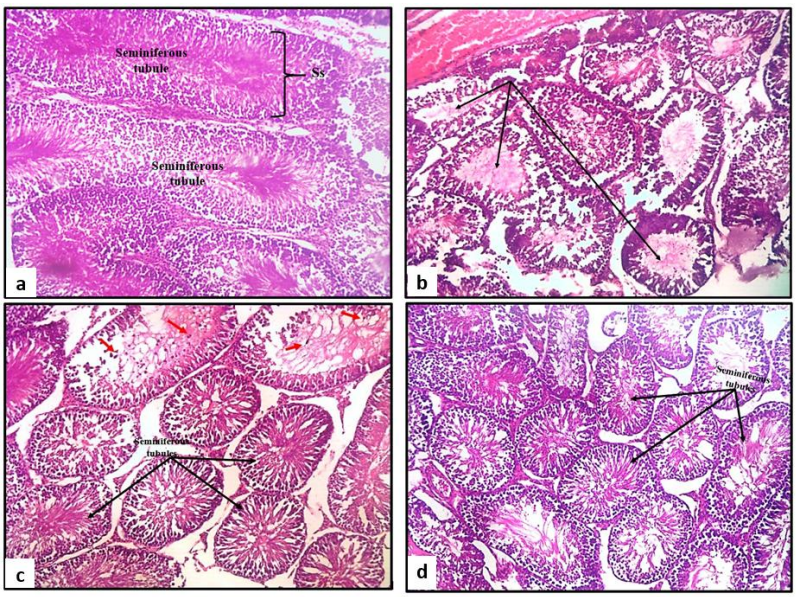

Fig. 1. Photomicrographs of Testis sections from normal control [a], MTX-Control [b] and MTX+200mg/kg PDFE (c) and MTX+400mg/kg PDFE [d] -treated rats [Stain: H&E; Mag.: x400]

a: *Normal histomorphology of the seminiferous tubular epithelium and interstitium is observed. Spermatogenic series (ss) show varying stages of sperm development in the tubules (arrows).* ***b:*** *degeneration and depletion of spermatogenic series. Marked evidence of incomplete spermatogenesis is revealed by absence of mature germ cells within most of the seminiferous tubular lumens (arrows).* ***c:*** *The general features reveal moderate preservation of the tissue. However, presence of immature sperm cells (red arrows) within the lumen of few seminiferous tubules is noted.* ***d:*** *There is evidence of marked tissue preservation from damage exerted by MTX as most seminiferous tubules reveal presence of normal cells in spermatogenic series*

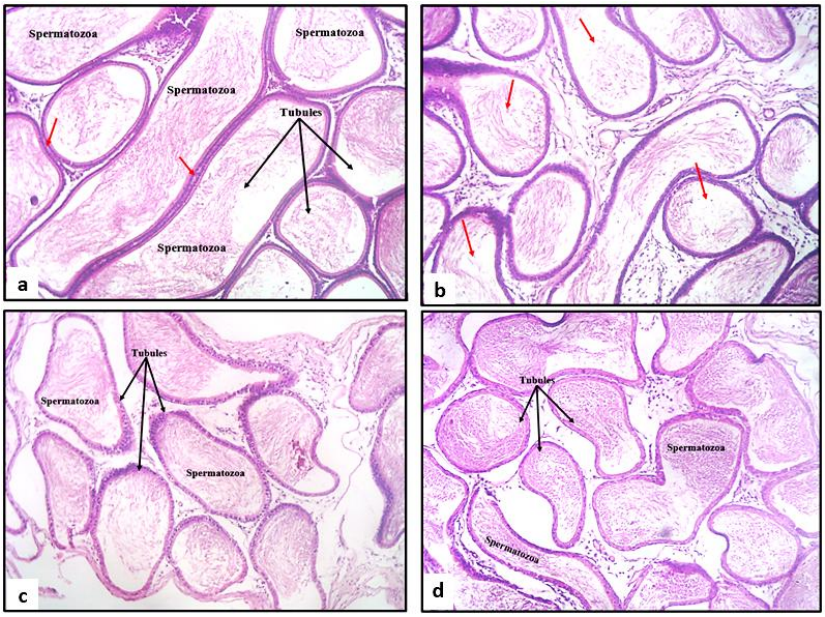

Fig. 2. Photomicrographs of Epididymis sections from normal control [a], MTX-Control [b], MTX+200mg/kg PDFE (c) and MTX+400mg/kg PDFE [d] -treated rats [Stain: H&E; Mag.: x400]

a: *Normal histoarchitecture is observed. The tubules lined by columnare epithelium (red arrows) appear normal.* ***b****: Section reveals decreased number of spermatozoa within the epididymal tubular lumen (arrows) when compared with normal control.* ***c:*** *Preserved tissue structure and increased number of spermatozoa are observed when compared to MTX-control.* ***d:*** *General features appear normal and increased number of sperm cells is observed*

Testicular structure and germ cell damage resulting from MTX use is mostly attributed to generation of reactive oxygen species (ROS) leading to oxidative stress [6]. It is well established that oxidative stress occurs as a result of an inbalance between the antioxidant reserve system and ROS. Once this ROS generation becomes uncontrolled, it leads to ininfertility and sperm abnormalities. Common reports due to MTX treatment in male reproductive system are decreased sperm cell numbers, vacuolization, disorganization of seminiferous tubules and sperm DNA damage (6, 14). Therefore, the need to preserve the germinal cells of patients undergoing treatment with MTX, is pertinent in order to avoid infertility.

Upon treatment with the extract of *P. dactylifera,* a dose-dependent preservation of the testicular tissue and germ cells were observed (Fig. 1c and 1d). Similar reports on the preventive and therapeutic effects of the extract has been documented against various agents used to induce testicular oxidative stress [15,16]. This ameliorative effect may be attributed to the phytoconstituents in the plant material. Dates have been shown to contain steroidal components [17], high phenolic compounds such as flavonoids [18,19] and other phytochemicals which all help to increase sperm health and protect testicular functions [20].

Table 1 reveals the findings from caudal epididymal sperm concentration from PDFE treatment groups in comparison with the controls. A decrease in the sperm concerntration of MTX-control rats was observed. Conversely, increased levels was noted upon PDFE treatment when compared with controls. These changes were, however, not considered statistically significant ($p>0.05$). These findings are consistent with the histopathological findings on the caudal epididymis (Fig. 2).

The microscopical examination revealed normal histoarchitecture of the epididymal tissues in the control and treatment groups (Fig. 2a – 2d). However, mild changes are noted in the numbers of spermatozoa within the lumen of the epididymal tubules. A slight decrease is observed in the MTX-treated group (Fig. 2b), whereas a somewhat increase in normally dispersed clumps of spermatozoa is noted upon PDFE-treatments (Fig. 2c and 2d). Although these changes were not statistically significant from the results of the caudal edididymal spermatozoa concentrations (Table 1), the pattern of changes observed is suggestive of the ameliorative effect of the extract against MTX-effects. Perhaps the plausible reason for the non-significant effect is due to the short duration of the present study (11 days), as it is known that the spermatogenic cycle in albino rats takes up to 48 – 52 days [20] for a testicular effect to be fully reflected on the caudal epididymis.

Table 1. Effects of *Phoenix dactylifera* fruit extract on caudal epididymal sperm concentration of albino rats with Methtotrexate-induced testicular damage

Treatments	Caudal epididymal sperm concentration ($x10^6$/ml)
Vehicle (Control)	89.50±9.71
MTX-Control	76.75±10.96
200mg/kg PDFE	98.50±12.55
400mg/kg PDFE	101.25±7.97
F-ratio	1.119
Sig.	0.380 (p>0.05)

Data expressed as mean ± SEM; n=4. Level of statistical significance is set at $p < 0.05$ when compared to the control group.
MTX= Methotrexate; PDFE = Phoenix dactylifera Fruit Extract

4. CONCLUSION

Findings from the present study further confirms the potential of methotrexate in exerting testicular toxicity, thus suggesting the need to preserve the reproductive organs of patients on MTX-therapy. The use of the fruit extract of *P. dactylifera* was observed to possess ameliorative potential against MTX-induced toxicity by its ability to preserve the testicular tissue architecture and spermatozoa concentration. Future studies may explore on the gonadoprotective effect of the extract and its bioactive principles for their potential use in maintaining testicular histoarchitectural integrity against toxicants. More so, the determination of the bioactive principle(s) responsible for the observed effects and their possible mode of action may be established.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by

the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate Institutional ethics committee. Animal housing and handling protocols were performed in strict accordance to guidelines describing the use of rats for research.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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