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Research Paper

# Morphometrical and Histological Analyses of the Epithelial Lining of Male Reproductive System in Wistar Rats Following Administration of Neem Leaves Extract

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## ABSTRACT

**Background:** Neem leaves (*Azadirachta indica* L.) have been used for many therapeutic purposes and medicinal applications. The extract of this plant has been used in both male and female genders as a traditional agent to prevent early pregnancy. In this study, the effect of this extract was investigated histologically and morphometrically on the germinal epithelia of the seminiferous tubules, epididymis and secretory epithelia of rats' prostate glands.

**Methods:** Twenty male albino rats were divided into four groups of five each and administered the extract at a concentration of zero, 100, 200 or 400 mg/kg of the body weight for 50 consecutive days. These rats were sacrificed and the male reproductive organs were removed, weighed and processed for routine histological examinations. The micrographs were analyzed and the structural changes in the epithelial lining and morphometric analyses were recorded, which included measuring the epithelial thickness in the seminiferous tubules, epididymis and secretory prostatic epithelia.

**Results:** The extract was found to reduce the rats' weight; decreased both the weight and dimension of the testes; reduced the number of germinal epithelial lining cells in the seminiferous tubules of the testes, the epididymal and prostatic secretory epithelial cells.

**Conclusion:** The histological alterations were most significant in response to the treatment with the extract at 200 mg/kg of the rats with the greatest damages observed in the epithelial lining. The deleterious effects of the extract were found to be dose-dependent and this corroborates the use of this extract as a contraceptive in animal models, and potentially in humans.

**Keywords:** Epididymis, Epithelia, Morphometry, Prostate gland, Seminiferous tubules

## Introduction

Neem leaves (*Azadirachta indica* L.) have been widely used in traditional medicine for fertility regulation for a long time [1]. In addition, the *A. indica* leaves have been shown to possess numerous effects, such as antibacterial, antifungal, anti-inflammatory, immunomodulatory, antihyperglycemic, antiulcer, antimutagenic, antimalarial, antiviral, and antioxidant properties [2]. Some studies have explored the effect of this plant's extract on the male and female reproductive systems as a potential contraceptive agent. In Gambia and Ghana, *A. indica* leaves are used as tea drinks to prevent pregnancy [2].

Experimental studies on the effects of *A. indica* extract on the female reproductive system have shown that it induces reactive oxygen species (ROS) and results in mitochondria-mediated apoptosis both in granulosa cells and in follicular oocytes. These alterations can lead to the deterioration of oocyte quality, thereby limiting the reproductive outcomes [1]. In male animals, it has been found to cause gross changes in the reproductive organs and reduce the amount, motility, and morphology of the spermatozoa [3]. Also, it has affected the structure and function of the testes, causing a decrease in the quality of spermatozoa [3]. Further, the use of the *A. indica* has been explored as a means of contraception in the human populations in developing countries. This is mainly because public health care clinics and medical professionals are not readily available to provide advice to individuals on family planning methods.

**Aim of the study:** In many parts of the world where *A. indica* extract is used as a contraceptive, no specific dosage is followed as the plant leaves are commonly collected and boiled to obtain a concoction which is ingested by ordinary people without a knowledge of its effects. This study was aimed at examining the effect of several concentrations of the aqueous extract of *A. indica* on the histology and morphometry of the testes, epididymis and prostate glands in male rats, specifically to compare the differences in the thickness of the epithelial layer that lines the tubules of these organs.

## Materials and Methods

**Plant Collection, Authentication and Extraction:** The extraction method used in this study was done according to the technique described by a previous study [4]. The plant leaves were collected from an *A. indica* tree in the botanical garden of the University of Maiduguri, Jere Local Government, Borno State, Nigeria. The leaves collection was carried out both in the early morning and after the sunset. This was suggested by an expert [5] who identified the period as the best time to collect the plant's materials because the photosynthesis is inactive and the chemical constituents are properly preserved in the leaves. The plant was verified and authenticated by a botanist from the Department of Biological Sciences, University of Maiduguri, Nigeria. After collection, the leaves were carefully spread in the shade and dried well with constantly turning them over to prevent decays. This process was continued until the leaves were completely dried over four days. Subsequently, the *A. indica* leaves were crushed mechanically into a fine powder. The powder obtained was then dissolved in 1.5 liters of distilled water and let to stand at room temperature for 24 hours. It was then sieved, the filtrate evaporated in an oven overnight at 40°C, and the dried extract was weighted.

**Experimental Animals:** The male adult albino rats (n=20) were randomly selected for the present study. They were divided into four groups of five rats each as follows: Control (group A) and treatment groups (B, C & D). The mean body weight of the rats before starting the study was 212±60 grams. The rats were housed in the animal house of the Anatomy Department, Faculty of Medical Sciences, University of Maiduguri, for two weeks to get acclimatized before beginning the experiments. The extract was administered to the rats for a period of 50 consecutive days over seven weeks. This time period was selected because the process of spermatogenesis in rats takes a minimum of 50-54 days [6]. Therefore, this cycle was the reference point for testing the effect of the extract on the spermatogenic series in the rats' testes, and the associated histological changes in the epididymis and seminal vesicles.

During this period, the animals were closely monitored, and standard environmental conditions and the light and dark cycle were maintained. The animals were fed the growers feed supplied by Vital Feed Company in Borno State, Nigeria. The rats were housed in plastic cages with stainless steel mesh covers, and the floors were padded with saw dust which were renewed daily.

**Animal grouping:** The animals were divided into four groups of five rats each as follows:

Group A: Received equal amounts of distilled water.

Group B: Received 100 mg/kg of *A. indica* extract.

Group C: Received 200 mg/kg of *A. indica* extract.

Group D: Received 400 mg/kg of A. indica extract.

**Administration of the extract to the animals:** The animals were weighed just before starting the extract administration and measurements were recorded weekly to observe changes in the weight following ingestion of the extract. The extract was administered once daily for a period of seven consecutive weeks.

**Sacrificing the animals:** The rats were humanely sacrificed using Ketamine injection (Cargill Inc., Orlando, FL, USA) which was injected intraperitoneally into each animal to induce sleep. Both testes of each rat were harvested by dissecting the abdomino-pelvic region and the tissues were weighed immediately. The length and

**Table 1.** Bilateral weights of the rats' testes, mean body weight, and testes index

| Groups | Right Testes Weight (g) | Left Testes Weight (g) | Mean Testes Weight (g) | Mean Body Weight (g) | Testes Index (%) |
|---|---|---|---|---|---|
| A | 1.18±0.4 | 1.20±0.2 | 2.40 | 260.0 | 0.92 |
| B | 1.23±0.3 | 1.27±0.2 | 2.50 | 242.0 | 1.03 |
| C | 0.86±0.5 | 1.04±0.4 | 1.90 | 235.4 | 0.81 |
| D | 0.92±0.3 | 0.72±0.6 | 1.60 | 224.6 | 0.71 |

breadth of the testes were recorded, using a vernier caliper and the right testis of each rat was finally preserved in Bouin's solution. The epididymis and prostate glands were also dissected and preserved for further tissue processing and histological examinations.

**Tissue preparation:** The testes, epididymis and seminal vesicles were removed from the preservative and washed to remove excess fixative. Graded series of alcohol was used to dehydrate the tissues followed by embedding them in paraffin wax. Tissue sections were made at a thickness of 5µ, using a Leica microtome, model RM 2125RTS (Leica Biosystems; Deer Park, IL, USA). The tissue sections were then mounted on a glass slide and stained with Eosin and Hematoxylin. Tissue sections were examined by an expert animal pathologist under a microscope at the magnifications of X40, X100 and X400.

**Histomorphometric analyses:** The images of the histological sections were obtained, using an Amscope light microscope (MBJX-ISCOPE, Los Angeles, CA, USA), equipped with a built-in digital camera (M500, X64, V.3.7) using 10X and 40X objective lenses. These images were further analyzed using an Image J software (1.53a Wayne Rasband, National Institutes of Health, USA, Java 18.0-112). A standardized ocular micrometer was used to measure points of interest in the histological slides for morphometric analyses.

The morphometric analyses were made, using the computerized image analysis system Image J. The stage micrometer was used to calibrate Image J, using the *same* objective and pixel resolution as the micrographs were being measured. The following parameters were measured in each micrograph:

- The germinal layer thickness (µm) in the testicular wall measured as the length of a line drawn from the basal membrane of the seminiferous tubule to the luminal epithelia.
- The epithelial wall thickness (µm) of the seminiferous tubule measured as the distance from the basal lamina of the seminiferous tubule to the apical epithelia.
- Epithelial thickness of the prostate gland measured in µm as the length from the basal lamina to the apical border of the columnar epithelial cells of the prostate glands.

**Statistical analyses:** The data obtained from the above measurements were analyzed, using GraphPad Prism 9.1.0 and one-way analysis of variance (ANOVA), and expressed as means±SEM and percentages followed by Dunnet's post-hoc test for multiple comparisons. The level of statistical significance was set at $P<0.05$.

**Ethical consideration:** During this research, the authors adhered to the ethical guidelines set by the University of Maiduguri's Research and Ethics Committee, and also the guidelines of the U.S. National Institutes of Health (NIH) for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978), and also conformed to the Directive 2010/63/EU. The study protocol for this research project was approved by the Ethics Committee of the Department of Human Anatomy, University of Maiduguri, Nigeria (Code #: UM/HA/UGP 19.20073-099).

## Results

**Effect of the extract on the testicular weight**: Administration of *A. indica* extract during the experimental period showed a reduction in the testicular weight as the concentration of the extract given increased. In group D, the rats' testes had the least mean testicular weight (1.6 g) compared to that of the animals in the control group (2.4 g). The testicular index was calculated by dividing the mean testicular weight by the mean body weight, multiplied by 100. The values obtained also showed that the extract caused a reduction in the weight of the testes relative to the animals' mean body weight (Table 1).

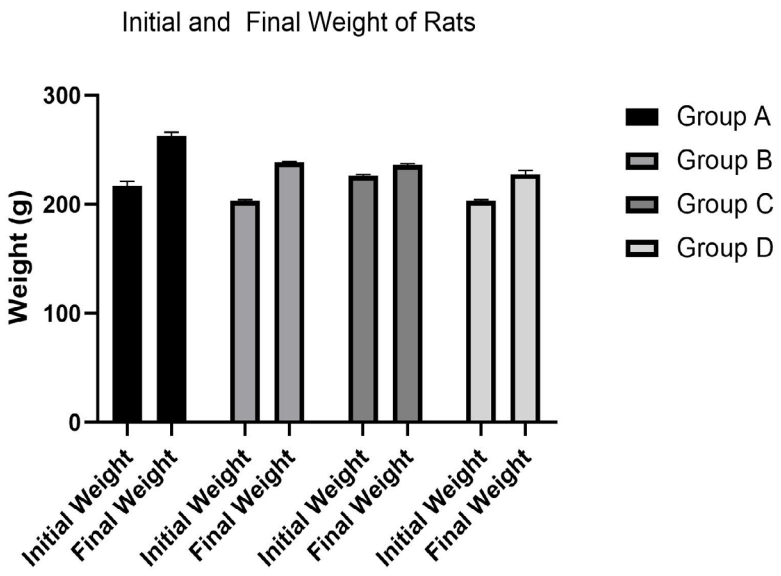


**Figure 1.** Initial and final weights of the rats in groups A to D

**Effect of the extract on the rats' weight:** The rats in all groups showed weight gains at the end of the experimental period with the highest gain observed in group A (46 g). In groups B, C and D, there were also increases in the body weights as follows: 35 g (group B), 10g (group C) and 24 g (group D), respectively. (Figure 1).

**Effect of the extract on the testicular dimension:** The administration of the extract reduced the testicular length insignificantly in the groups that received it as compared to those of the control group (Figure 2). In all groups, there were variations in the length of the right and left testes. In groups A and B, the right testes were longer than the left ones, but in groups C and D, the left testes were longer than the right ones. The testicular width had the most gains in group C as compared to those in other groups (Figure 3).

**Morphometric analysis of the effect of the extract on the thickness of testes, epididymis and prostate epithelial lining**: The administration of the extract caused an insignificant increase in the thickness of the

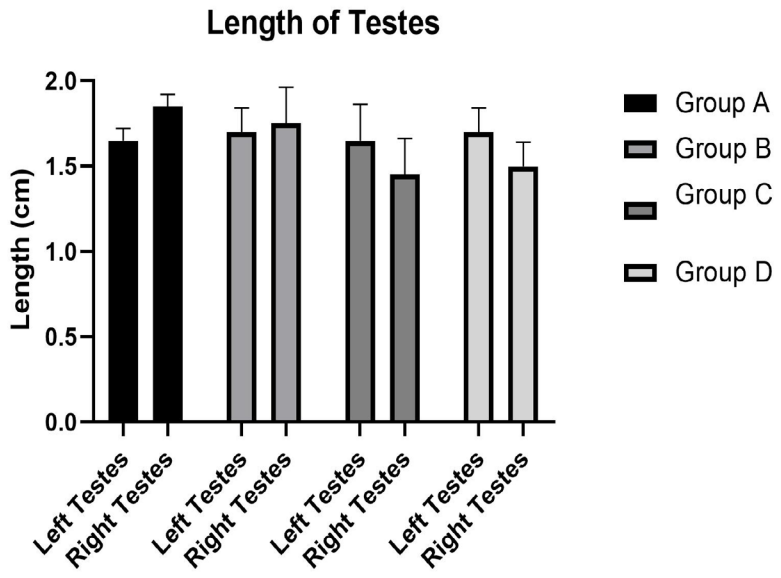


**Figure 2.** Length of the left and right testes in groups A to D

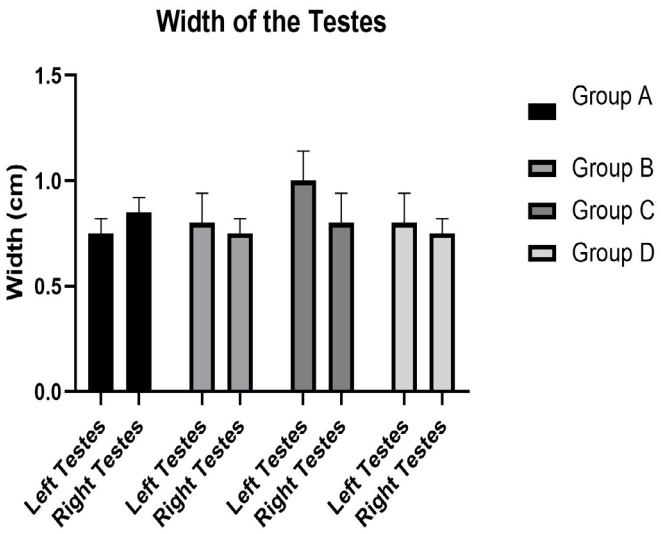


**Figure 3.** Width of the left and right testes in groups A to D

germinative layer of the seminiferous tubules of the rats' testes in group C (119 µ) compared to those of group A (110 µ). There was an insignificant decrease in the seminiferous tubular epithelial lining in group D (105 µ) compared to that of group A (Figure 4).

The epididymal thickness was similar in all groups with all the values for the experimental groups decreased insignificantly compared to that of the control group (Figure 5). The epithelial lining of the prostate glands showed a significant decrease ($P<0.05$) in group B compared to that of group A. There was a significant increase ($P<0.05$) in the prostatic epithelia of the rats in group C while an insignificant increase was observed in the prostatic epithelia of the rats in group D (Figure 6).

**Histological analyses:** The testes of the rats in group A showed normal testicular architecture. The seminiferous tubules had the typical germinal epithelia, and the underlying smooth muscle and myoepithelial cells appeared normal. There was a series of cells from the basal lining, extending to the lumens. Mature sperm cells were also observed in the lumens. The interstitial spaces contained typical connective tissue and blood vessels (Figure 7A). The germinal epithelia of the seminiferous tubules became increasingly compact as the concentration of the given extract increased among the treatment groups (Figure 7 B-7D). In group D, the lining was the flattest and the lumens were more abundant than those in other groups. The seminiferous tubules in group D were elongated as compared to the circular seminiferous tubules observed in other

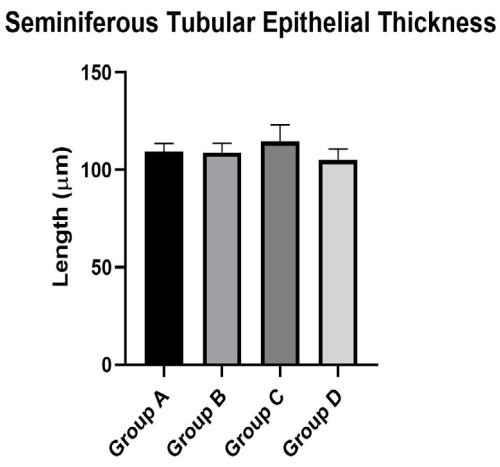


**Figure 4.** Thickness of the epithelial lining in the seminiferous tubules of groups A to D

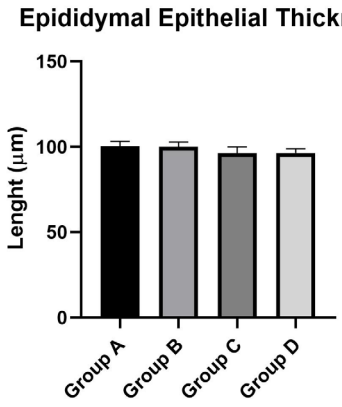


**Figure 5.** Thickness of the epididymis epithelia in groups A to D

groups. The interstitial spaces were also sparse in the groups as the seminiferous ducts filled up the spaces in between. This contrasted with that of the control group where there were abundant interstitial spaces.

The germinal epithelial series differed in all treated groups compared to those observed in the controls. The spermatogonia were seen resting on the basement membrane in the control group, and were recognizable by the eosinophilic nuclei along with Sertoli cells which were larger and had more intensely stained nuclei and cytoplasms. Spermatocytes were recognized by their darkly stained nuclei placed above the basal lining of the seminiferous tubules. The spermatids were observed by their eosinophilic nuclei and shrunk cytoplasm located closer to the lumens. The sperm cells were located in the apical region of the seminiferous tubules and had flagella, which were eosinophilic and whip-like in appearance (Figure 8A). The groups that had been treated with the extract showed gradual reductions in the number of cells in the germinal lining, with the sperm cells located in the lumens (Figure 8B-8D). The reduction in the number of germinal cells were the most marked in group D with few spermatocytes and matured sperm cells (Figure 8D).

The micrographs of the epididymis in the control group showed simple columnar epithelia resting on the basement membranes together with supporting smooth muscle fibers. The lumens contained sperm cells and the interstitial spaces contained connective tissue and blood vessels (Figure 9A). The groups treated with the extract showed changes in the epithelial lining as the concentration of the extract increased (Figure 9B-9D). In group C, the epithelial cells were distorted and had darkly stained nuclei with clear cytoplasm, which appeared devoid of the organelles (Figure 10C). In the control group, the epithelial lining of the simple columnar cells was intact in the epididymis (Figure 9A). In groups

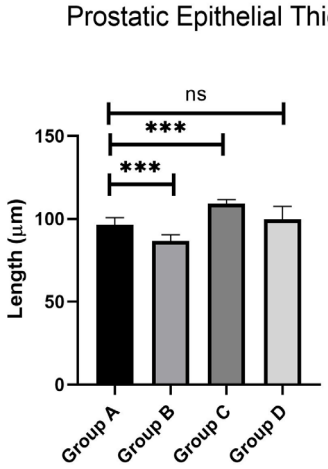


**Figure 6.** Thickness of the prostatic epithelial lining in groups A to D

Ns=Not significant, ***Significance level, P<0.05

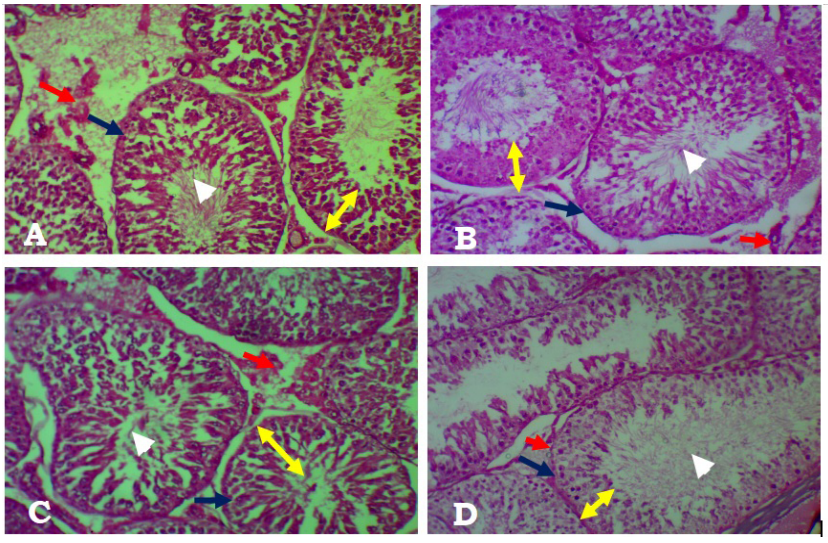


**Figure 7.** Seminiferous tubules of the testes

A) Seminiferous basement membranes (blue arrows); B) Germinal epithelia (yellow arrows); C) Lumens with spermatozoa (white arrows); and D) blood vessels in the interstitial spaces (red arrows). H & E staining at 100x magnification.

B and D, there were apical border of cilia, which were most prominent in the group that had received the highest dose of the extract, with the myoepithelial cells arranged at the base of the epithelial lining (Figure 10D).

The prostate gland micrographs in both the control and treated groups are presented in Figures 11 and 12, A-D. The control group had simple columnar epithelia, which lined the prostatic alveolar glands. They were highly secretory with the Corpora amylacea (prostatic secretion) observed in the lumens. The surrounding connective tissue had rich vascular supply with smooth muscle fibers (Figures 11A,B and 11D). The prostatic tissue in group C had cuboidal epithelia that lined the prostatic alveolar glands, with little secretory product in the lumens. The interstitial spaces were sparse and very few blood vessels were observed in them (Figure 11C). In groups A, B and D, the epithelial cells were columnar with ciliated brush borders and alveolar sacs contacting each other. The nuclei were located at the base of the epithelia and the highly stained nuclei were granular and eosinophilic in appearance. The staining was more intense

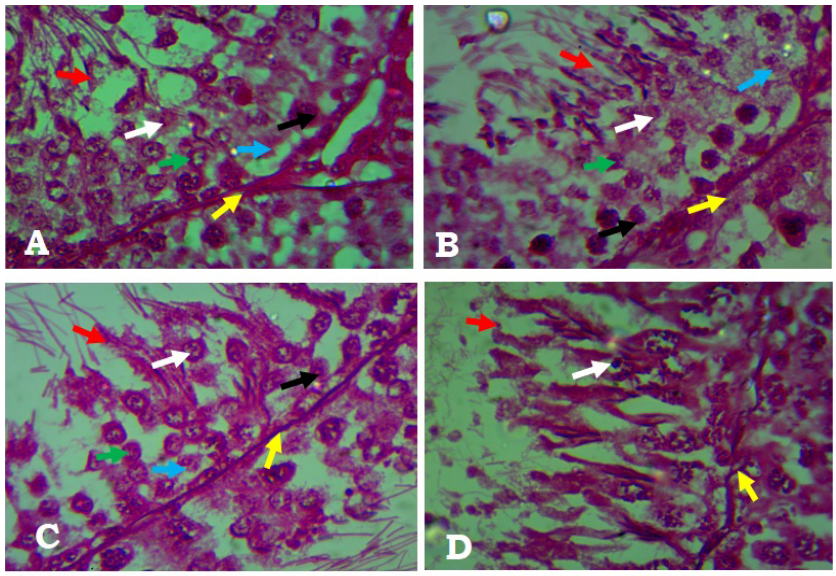


**Figure 8.** Germinative series

A) Basement membrane (yellow arrows) underlying the basal layers of Sertoli cells (blue arrows), B) spermatogonia (black arrows), C) mature spermatocytes (green arrows), and D) spermatids (white arrows). The mature spermatozoa (red arrows) were situated near the luminal surfaces. H & E staining at 400x magnification.

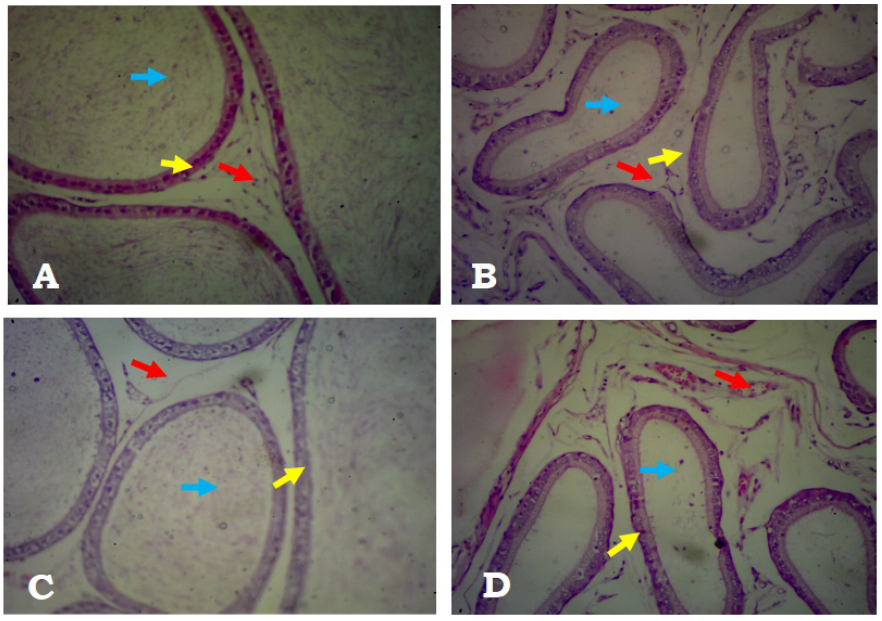


**Figure 9.** Epididymis thickness in all groups (yellow arrows)

The epididymis lumens are shown by blue arrows while the interstitial spaces are marked with red arrows. H & E staining at 100x magnification.

and granular in group B (Figure 12B). The cells in group C were cuboidal and had an underlying layer of smooth muscle fibers with the myoepithelial cells situated at the base of the epithelial cells (Figure 12C).

## Discussion

Many plant extracts are used by people as contraceptives in some developing countries with little or no access to healthcare to prescribe contraceptives. This is potentially helpful as a traditional method of birth control. The leaves from the plant, *A. indica*, are among the local means of birth control used for this purpose. In the treated groups, administering the extract reduced the weight of all animals. It also decreased the weight and dimension of the testes in all treated groups, compared to those in the controls. The reason for the testicular weight loss could be attributed to the reduction in the number

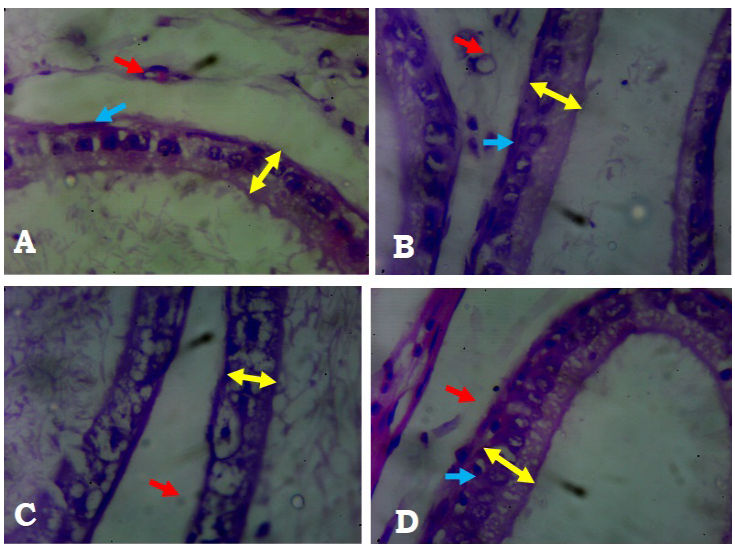


**Figure 10.** The magnified view of epididymis where the epithelial layer thickness is identified with yellow arrows

In group C, the epithelial layer consisted of distorted and pyknotic columnar cells. The myoepithelial cells (blue arrows) were situated near the basal membrane, associated with the epithelial lining. The interstitial spaces (red arrows) in groups A, B and D were vascular. H & E staining at 400x magnification.

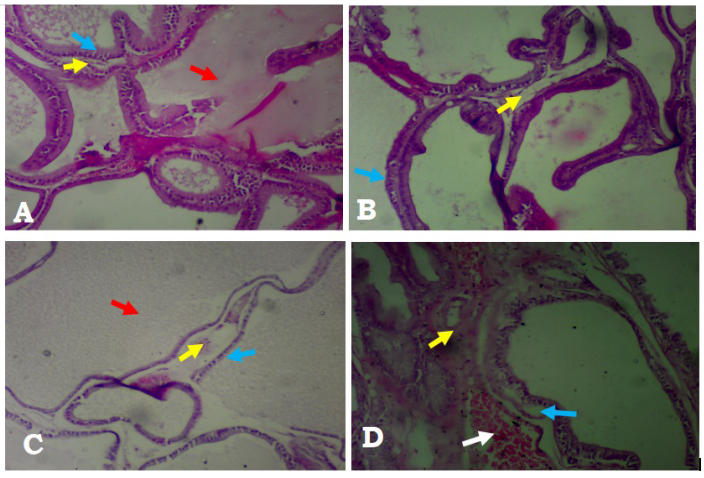


**Figure 11.** Micrographs of the prostate glands with the acini lined with epithelial cells (blue arrows)

These were eroded and shrunk in group C. The prostate stroma (yellow arrows) contained blood vessels, and there were bleeding spots in group D (white arrows). In group A, the prostatic secretions were stained in the acini lumens (red arrows). H & E staining at 100x magnification.

of germinal and sperm cells. This was consistent with the findings reported by other studies where *A. indica* extract had decreased the weight of accessory sex glands in rats, such as the seminal vesicles and ventral prostates together with reduced serum testosterone levels [3, 7, 8].

The morphometric analyses showed that the germinal lining of the seminiferous tubules was the longest in group C, which may be attributed to the disorganization of the epithelial lining. The epithelial thickness in the epididymis was similar in all groups, while the prostatic epithelia had significantly decreased as observed during the histological examinations in this group.

We administered several concentrations of *A. indica* extract to the male Wistar rats to determine the histological effects in the epithelial lining of the testicular seminiferous tubules, epididymis and prostate glands. These linings were also measured in thickness to determine changes in the epithelial layer following the extract administration. At the end of the experimental period, it was observed that at the lowest dose of the extract (100 mg/kg), there was little or no histological changes in the epithelial lining of the organs as they remained intact. However, the Sertoli calls were slightly distorted and contained lightly stained cytoplasm, which could be attributed to damages to the intracel-

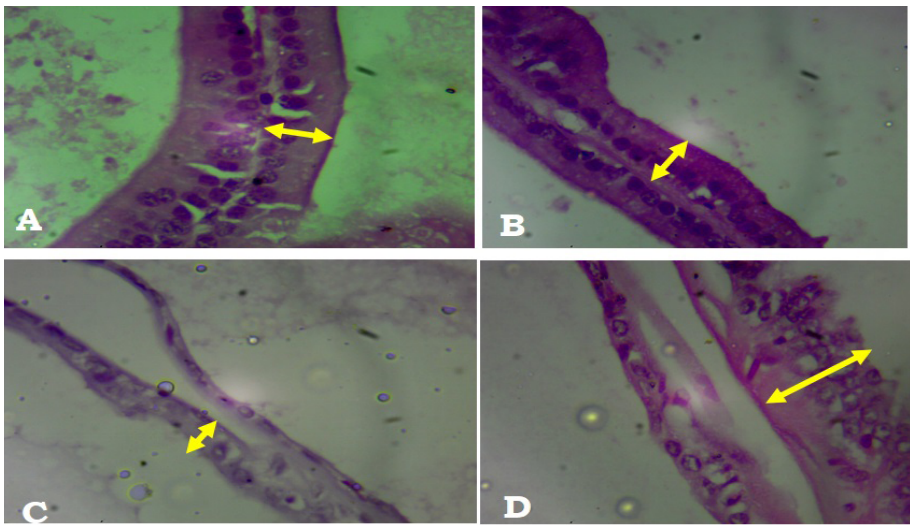


**Figure 12.** Micrographs of the secretory epithelia of the rats' prostate glands (yellow arrows)

This layer was eroded in group C, with the columnar epithelial cells distorted and disorganized. H & E staining at 400x magnification.

lular organelles. This is in agreement with the findings reported by a previous study [9] that determined a dose of 100 mg/kg of the extract, administered orally, caused intracellular abnormalities and vacuolization within Sertoli cells. It also caused reduced cytoplasmic inclusions in Leydig cells and disruption in the final stages of the spermatids' development.

The administration of the extract at a concentration of 200 mg/kg caused the most severe histological damages in the tissues examined. At this concentration, the extract reduced the germinal epithelia in the seminiferous tubules with few sperm cells, compared to those noted in other groups. The epithelia of the epididymis and prostate glands were also distorted and consisted of cuboidal cells instead of the columnar epithelia as found in other groups. These findings suggest that at this concentration, cellular and intercellular damages may occur in the epithelial lining [2]. The findings also imply that the extract may cause damages to the seminiferous tubules, leading to chromatin condensation, and germinal cell degeneration. Hence, the disruptions in the spermatogenesis, impaired motility, morphology, and reductions in number of spermatozoa in the male rats.

In the group that received the extract at the highest dose (400 mg/kg), there was wearing away of the germinal epithelia and increased number of basal cells in the epididymis. The findings are suggestive of the replacement of columnar epithelial cells and increased interstitial bleeding in the prostate glands, and reduction in the secretory products of the prostate glands. Our results are consistent with those of a previous study that used the extract at a dose of 500 mg/kg. That study reported atrophy in the seminiferous tubules with widened spaces among cells, with the Leydig cells being degenerate. Also, the number of Leydig cells and the core dimensions were found to be significantly shrunk [3].

Also, the *A. indica* leaves have been found by studies to cause morphological changes in the heads of rats' spermatozoa, and other sperms' parameters along with ultrastructural changes in the testes [3, 9-11]. Recently, it has been shown that the powdered leaves also affect the fructose levels in the vas deferens fluid [3]. Further, studies have indicated that the administration of the *A. indica* leaf extract decreases the concentration of luteinizing hormone and causes histological alterations in the anterior pituitary. This can affect the testosterone secretion in male rats by influencing spermatogenesis [9-12].

## Conclusions

Neem leaves (*A. indica*) have been used by male and female individuals in old cultures to prevent pregnancy. The current study investigated the effects of the aqueous extract of *A. indica* on the treated animals' total weight, and the weight of the testes, epididymis and prostate glands. These were followed by morphometric analyses of the epithelial lining of the affected organs, and the observed changes as a result of the extract administration. We found that the extract reduced the animals' total weight, and the weight and dimension of the testes. It also shrunk the germinal epithelial lining of the seminiferous tubules, epididymal epithelia and the prostatic secretory epithelial cells. Finally, the effects of the extract were found to be dose-dependent. Our findings potentially corroborate the application of the *A. indica* extract as a contraceptive agent. The safety and efficacy of this proposed method of birth control warrant future research.

## Ethical Considerations

### Compliance with ethical guidelines

The Authors adhered to the ethical guidelines set by the University of Maiduguri Research and Ethics Committee, and the National Institutes of Health (NIH) guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and conformed to the Directive 2010/63/EU. The present research was approved by the Ethics Committee, Department of Human Anatomy, University of Maiduguri (Code #: UM/HA/UGP 19.20073-099).

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This study was funded solely by the authors.

### Authors' contributions

All authors contributed fairly equally to all aspects of this research project. Also, they reviewed and approved the final draft of the manuscript prior to submission for publication.

### Conflict of interest

The Authors declare no conflict of interests with any internal or external entities.

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