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# EFFECTS OF *PHYLLANTHUS AMARUS* ON EPIDIDYMAL SPERM CHARACTERISTICS, TESTOSTERONE LEVELS AND HISTOLOGY OF REPRODUCTIVE ORGANS OF MALE RATS

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## Abstract

The effects of crude aqueous (AE) and methanolic (ME) extracts of the aerial parts of *Phyllanthus amarus* (*PA*) on male reproductive system of experimental animal models rats were investigated in this study. Six groups of adult male albino Wistar rats were used for the study (n=6). Rats in groups I and II [Controls] were administered with water and 3% Tween 80, while groups II and III received 200 mg/kg b.wt. of AE and ME respectively, and groups III and VI received 400 mg/kg b.wt. of AE and ME respectively for 42 days. Caudal epididymal sperm count showed a significant increase ($p<0.05$) in all the treatment groups when compared with the controls. Sperm motility revealed no significant difference ($p>0.05$) in all treatment groups except an increase in group treated with 400 mg/kg ME ($p<0.05$). Testosterone assay showed a significant increase ($p<0.05$) in groups treated with 200 mg/kg AE and 400 mg/kg ME when compared with the controls. Histologically, the testes showed increased cellularity of the seminiferous tubules and the epididymal ducts are filled with spermatozoa with no pathological change. Mild histological alteration of the vas deferens and seminal vesicles was observed. The ability of the extracts to increase serum testosterone levels and epididymal sperm concentration supports the folkloric use of this plant in the treatment of male infertility.

**Keywords**: *Phyllanthus amarus,* Spermatogenesis, Testosterone, Fertility, Histology

## Introduction

*Phyllanthus amarus* Schum & Thonn [*Euphorbiaceae*] is a plant with a height of about 30-60 cm. It is found in India, China, Phillippines, Cuba, Guam, and from Sierra Leone to Southern Nigeria, Equitorial Guinea, and everywhere in tropical Africa (1). It blooms with many flowers and has a rigid, short and prostate stem. Its anti-viral, anti-inflammatory, and hepatoprotective, radioprotective , chemoprotective, hypoglycemic, cholagogue, vulerary, diuretic and antioxidant activities have been documented (2,3). Extracts have also shown anti-carcinogenic and anti-mutagenic activity in-vivo and in-vitro (4). Major constituents in *P. amarus* include tannins, flavonoids, lignans, elligitannins have been isolated (5). Traditionally, *P. amarus* is used widely and all parts of the plant are employed therapeutically (6). It is used in Ayuverdic medicine for the treatment of many liver disorders (7). It has been used to improve the function of the circulatory, digestive and skeletal system (8).

Infertility is a major public health problem with up to 40% of the cases among couples being due to male factors (9). Many plant materials have been demonstrated to have varying degrees of aphrodisiac and fertility enhancing properties (10,11). The use of plants in traditional medicine for the prevention and treatment of various diseases is diverse. Parallel to the increasing interest in the many benefits they offer is the increasing concern on the associated risks with their use. Safety and quality of most herbal products cannot be assured and many are unregulated. It is of great importance for traditional medicine practitioners to demonstrate the efficacy of their medicines.

In Nigerian traditional medicine practice, there is a claim that the decoction of the aerial parts of *Phyllanthus amarus* can be used to boost sperm count in males. However, there is paucity of scientific information to authenticate this claim. The present research, therefore, was carried out to investigate the effects of consumption of crude extracts of *Phyllanthus amarus* aerial parts on serum testosterone levels, some epididymal sperm profile and histology of the reproductive organs of adult Male Albino Wistar rats.

## Methods

**Drugs and Chemical**

Ham's F-10 fluid, Methanol and Tween 80 were purchased from SIGMA® (Sigma –Aldrich®, St. Loius USA). All other reagents and chemicals were of analytical grade.

**Plant Materials Collection**

Fresh samples of the aerial parts [leaves and stem] of *Phyllanthus amarus* were collected from various sites in Enugu metropolis in Enugu state, Nigeria. The plant specimen was authenticated by experts at the herbarium section of Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. The plant materials were air-dried under shade and crushed into powder using a gasoline powered grinding machine.

**Preparation of Plant extracts**

**Crude Aqueous Extraction:** Four hundred (400) grams of the powdered sample of the plant was extracted by soaking in 2400mls of distilled water, homogenized for about 6 min using a wooden stirrer and left overnight. Thereafter the homogenate was strained through muslin cloth and the resultant filtrate was stored in the refrigerator (4±2°C) until required. The extractive value of the aqueous extract (AE) was 100mg/ml.

**Crude Methanol Extraction:** One thousand eight hundred (1800) grams of powdered aerial parts of *P. amarus* was placed in a container and 8 litres of 80% methanol was added to it. The container was well stoppered and shaken intermittently for a period of 72hrs. The extract was filtered using Whatmann filter paper and the resultant filtrate was evaporated to dryness on a rotary evaporator and the residue stored in the refrigerator (4±2°C) until required. The methanol extract (ME) gave a yield of **12.7%** W/W. The ME was reconstituted in 3% Tween 80 to get a concentration of 200mg/ml prior to use.

**Laboratory animals**

Thirty-six (36) adult Wistar albino male rats approximately 3 months old and weighing between 180 – 240g were used in this study. These animals were procured from the Animal House of Department of Physiology, Faculty of Basic Medical Sciences and housed at the Animal House of the College of Medicine, University of Nigeria, Enugu Campus. They were kept in clean wire-mesh cages which had openings beneath for easy exit of rats'

faeces and prevention of coprophagy. The facility was under standard environmental conditions of light (12 h light/dark cycle) and temperature (25±2°C). They were fed with commercially available rat pellets (Guinea Feed®, Benin, Nigeria) and clean water *ad libitum*. The animals were allowed to acclimatize for two weeks prior to the commencement of the studies. They were under veterinary supervision and were handled in accordance to institutional protocols and the guiding principles for the care and use of animals for scientific research (12).

**Experimental protocol**

The rats were randomly divided into six groups (I - VI) (n = 6). Rats in groups I and II (Controls) received 2ml/kg of 3% Tween 80 and Distilled water respectively. Rats in groups III and IV received 200mg/kg body weight (b.w.) of AE and ME respectively, while those in groups V and VI received 400 mg/kg b.w. AE and ME respectively. A single dose of the extracts was administered via an oral cannula daily for 6 weeks. The control groups were treated similarly.

**Testosterone assay**

At the end of the sixth week (Day 42), blood was obtained from each rat via the retro-orbital sinus. Each blood sample was spun at 2500 revolution per minute for 10 minutes in an angle –head desktop centrifuge at room temperature to obtain clear sera. Serum samples were assayed for testosterone in batches with the control sera at both physiological and pathological levels by standard quantitative ELISA technique with Microwell kits from Syntro Bioresearch Inc. California, USA. Analyses were carried out according to the manufacturer's instructions.

**Epididymal sperm count and motility**

The animals were sacrificed and the male organs were removed. The cauda epididymis was excised; several incisions (1mm) were made in the cauda epididymis which was suspended in 1ml of Ham's F-10 solution (obtained from Sigma Aldrich). After 10 minutes incubation at 37°C, sperm concentration and motility were determined by haemocytometer method (13). The rest of the organs – (testes, epididymis, vas deferens and seminal vesicle) were excised and preserved in 10% formal saline and processed for histological studies.

**Gross and histopathological studies**

The excised tissues were necropsied and cut up into smaller pieces (about 3mm thick) and fixed in 10% formal saline. Further histological processing was done using the Automatic Tissue Processor. The tissues were embedded and subsequently sectioned at 5μm using the Rotary Microtome (Heitz 150 Rotary Microtome, Cambridge model). Sections were stained according to Haematoxlin and Eosin (H and E) technique as described by Baker and Silverton (14). The sections were examined using Olympus Binocular microscope with in-built lighting system and their photomicrographs were taken using an eyepiece microscope-digital-camera (AmScope).

**Statistical Analysis**

Data obtained in the present study were expressed, where appropriate, as mean ± S.E.M. of six rats per group. Statistical Package for Social Sciences [SPSS] software program (SPSS, Chicago, IL; version 20.0) was used for the analyses. Data were subjected to one-way analysis of variance (ANOVA). This was followed by Student's *t*-test to determine the statistical significance of the differences in the parameters among the groups. The level of significance was considered at $p<0.05$.

## Results

**Effect of *P. amarus* on serum testosterone levels**

Statistically significant increase in Testosterone levels ($p<0.05$) was observed only in groups treated with 200mg/kg b.wt AE (Table 1) and 400mg/kg b.wt ME (Table 2) when compared with their respective controls.

**Effect *P. amarus* extracts on Epididymal sperm count and motility**

Treatment with AE produced a significant increase ($p < 0.05$) in Sperm concentration of both AE-treatment groups when compared with the control (Table 1). No significant change was observed in the Sperm motility upon AE treatment.

Treatment with ME also produced a significant increase ($p < 0.05$) in Sperm concentration for both ME-treated animals when compared with the control (Table 2). The Sperm motility was significantly improved ($p<0.05$) in 400mg/kg ME group when compared with the control.

**Gross and histological findings**

Macroscopical features of the excised organs revealed no obvious abnormal changes. Upon

microscopy, histoarchitectural features consistent with normal tissues were observed in the testis, epididymis, vas deference and seminal vesicles of control rats (Figures 1A, 2A, 3A and 4A respectively). Testes sections from rats treated with AE and ME showed increased cellularity of the seminiferous tubules with no observable pathological change [Figures 1B and 1C]. Increased numbers of spermatozoa were observed in the epididymal tubules of both AE and ME- treated rats [Figure 2B and 2C respectively] when compared with the control [Figure 2A]. Mildly dilated luminal epithelium of the vas deferens of ME-treated rats were observed [Figure 3B] with no observable pathological changes. No histoarchitectural alteration was observed in the seminal vesicles of experimental animals (Figure 4B) when compared with control (Figure 4A).

## Discussion

To our knowledge, this is the first study demonstrating the effects of daily oral administration of extracts of *Phyllanthus amarus [PA]* aerial parts (stem and leaves) for up to 6weeks on the reproductive system of male albino Wistar rats. The decoction in Nigerian folklore medicine is used to improve sperm count in sub-fertile males (15). Interestingly, the present study has shown that oral delivery of the extract results in significant increase in cauda epididymis sperm density and motility with also a raise in the serum peripheral testosterone levels. The observed effects in this study maybe a direct or indirect action of a single or combination of its phytochemical principles.

Testosterone (T) is produced in the interstitial cells of the Leydig and is essential for growth and division of the germ cell epithelium in forming sperm (16,17). The significant increase in the serum peripheral T levels was upon *PA* treatment for 6 weeks in the present study correlates with a previous report by Obianime and Uche [18] in guinea pigs. Steroids, being one of the phytochemical constituents of this plant [18,19], are well known precursors in the synthesis of hormones. Low serum testosterone levels are related to low sexual desire, and an increase in serum T levels results in resumption of sexual activity (20). Sexual behavior and erection are androgen dependent which may be acting both centrally and peripherally [21]. Improvement in sexual function and libido have been demonstrated upon testosterone supplementation [22]. Bankole *et al.,* (19) has demonstrated that the ethanol extract of *PA* exerted a positive effect on penile erection properties in male guinea pigs. Although the sexual behaviour indices were not evaluated in this study, the raised testosterone level seems to justify the traditional use of *PA* as an aphrodisiac (15).

More so, the *PA* extracts contain phytoestrogens in the form of lignans (phyllanthin and hypophyllanthin) and flavonoids (quercetin and astragalin). Phytoestrogens are plant-derived, non-steroidal compounds possessing estrogenic activity (both structurally and functionally). They mimic endogenous hormones at the hormone receptor, exerting agonistic or antagonistic effects, or at key enzymes of hormone metabolism, affecting the level of active steroids (23).

Studies have shown that flavonoids bind to estrogen receptor site on cell membranes in order to prevent over proliferation of these cells in response to estrogen (24). Flavonoids block the enzymes that produce estrogen, and they do this by blocking estrogen synthetase (aromatase), a key enzyme used in the estrogen biosynthesis (25). Aromatase is an enzyme required for conversion of androgens (testosterone and androstenedione) to estrogens (Estradiol and Estrone respectively). By inhibiting aromatase, the body produces less estrogens and maintains higher testosterone levels. This mechanism may be responsible for the efffect observed in the present study.

Lignans have been shown to be moderate or weak inhibitors of human estrogen synthetase (aromatase) (25). The lignans present in *PA* are almost insoluble in aqueous medium but it has been shown that maximum amounts have been obtained by extracting with methanol (26). The increased serum testosterone levels as observed in the the present study may suggest a combined effect of the flavonoids and lignans present in the extract.

The dose- dependent significant increase in epididymal sperm concerntration may also be attributed to the effects of quercetin, one of the bioactive constituents of *PA*. Taepongsorat *et.al.,* (27) reported that sperm production can be increased by quercetin treatment in male rats. A possible mechanism to this observed effect could be that the bioactive component may have acted

through the sex organs (i.e. stimulating the testis or epididymis) or through a hypothalamus-pituitary-testis-axis (i.e. stimulating testosterone secretion) (28). The improved sperm quality (motility and concentration) as observed in this study disagrees with the results of a previous reports [18,29]. In their studies, gradual inhibition of fertility potential in male mice and guinea pigs with a decline in epididymal sperm profiles were observed. A possible explanation for this disagreement may be due to different rodent species and plant parts used, as the present study involved the use of rats and the entire aerial parts of *PA*.

The significant increase in epididymal sperm count as observed in our study of 42 days when compared with the control, requires further investigations since actual sperm cycle in rats takes between 48 - 52 days (30). *PA* may have acted by enhancing spermatogenesis or by increasing daily sperm production (31).

Histologically, the sloughing/bulging in of the seminiferous tubular epithelium into the lumen in the *PA* ME-treated groups suggests an increased activity of the tissue. These testicular changes and the increased numbers of stored sperm cells in epididymis as observed upon microscopical evaluation suggest increased activity of spermatogenic cells and improved sperm production. The various reproductive organs studied [testis, epididymis, seminal vesicles and vas deferens] revealed no obvious pathological feature. This does not agree with the work of Adedapo *et al.* (32) reporting varying degrees of severe testicular lesions with reduction of seminiferous tubular diameter following the administration of the aqueous extract of *P. amarus* (dose of 400mg/kg) for 14 days. The differences with our results may be cannot be explained but the short duration of treatment in their study may be consequential. Moreover, no other data could be found in literature reporting such a drastic effect on male reproductive organs after treatment with *PA*.

The mechanism(s) that may have precipitated the increased epididymal sperm count with no significant difference in serum testosterone levels as observed in rats that received 400mg/kg b.w. of AE of *PA,* was not elucidated in this study. The fact that the serum testosterone levels remained unaffected at the end of the experiment may suggest that the improvement in spermatogenesis observed is due to an effect exerted at the testicular level. Intratesticular testosterone concentration actually determines the extent of spermatogenesis and not basically serum peripheral testosterone levels (33).

The increased epididymal sperm quality might be a result of the antioxidant activity of *PA* extracts on the epididymis [34]. *PA* is rich in flavonoids (5) and hence would serve as a good source of natural antioxidants. The antioxidant effects leading to the quality of the stored sperm reserve may be considered to have occurred due to the retention of fluid and sperm in the epididymis as well as the dilation of the epididymal lumen (27).

In conclusion, considering all parameters investigated upon treatment with doses of *P. amarus*, it is noteworthy that positive reproductive effects (improved sperm quality and sex organ function) were observed. The indication is that *PA* might have acted directly or indirectly by stimulation of the sex organs. Based on our results, the use of *PA* as alternative drug for modifying impaired sexual functions and enhancing fertility may be considered in males with cases of hypotestosteronemia and low sperm count. The mechanisms of action need to be further investigated. However, the effects of *PA* treatment on daily sperm production being a better indicator of sperm output should be conducted.

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**Table 1:** Effects of *Phyllanthus amarus* crude aqueous extract on spermatozoa indices and serum testosterone levels in male albino rats compared with the corresponding controls.

| GROUPS | Parameters | | |
|---|---|---|---|
| | **Sperm Motility (%)** | **Sperm Counts ($x10^6$/ml)** | **Serum Testosterone Levels (ng/ml)** |
| **Control – Distilled water** | 63.00±1.00 | 60.88±0.77 | 2.87±0.14 |
| **200mg/kg *AEPA*** | 58.83±2.30 | 74.17±3.47* | 5.53±0.75* |
| **400mg/kg *AEPA*** | 62.67±2.11 | 95.92±6.29* | 3.50±1.31 |
| F-ratio | 1.498 | 17.974 | 2.532 |
| Sig. | 0.255 | 0.000 | 0.113 |
| Data expressed in mean ± SEM; *p<0.05 when compared to the control group | | | |

**Table 2:** Effects of *Phyllanthus amarus* crude methanol extract on spermatozoa indices and serum testosterone levels in male albino rats compared with the corresponding controls.

| TREATMENT GROUPS | Parameters | | |
|---|---|---|---|
| | **Sperm Motility (%)** | **Sperm Counts ($x10^6$/ml)** | **Serum Testosterone Levels (ng/ml)** |
| **Control – 3% Tween 80** | 61.50±1.06 | 64.50±2.56 | 3.00±0.69 |
| **200mg/kg *MEPA*** | 65.00±1.46 | 114.92±13.27* | 6.03±1.39 |
| **400mg/kg *MEPA*** | 67.67±1.23* | 145.00±12.90* | 6.68±1.28* |
| F-ratio | 6.027 | 14.208 | 2.865 |
| Sig. | 0.012 | 0.000 | 0.088 |
| Data expressed in mean ± SEM; *p<0.05 when compared to the control group | | | |

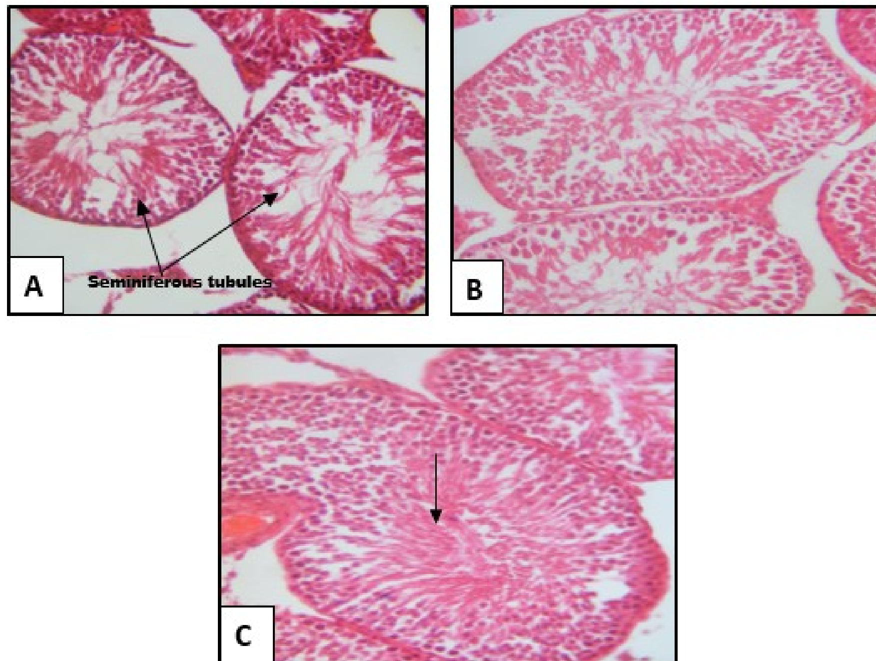


**Figure 1:** Photomicrographs of Testis sections from control [A], *AEPA*-treated [B] and *MEPA*-treated rats [C]. **A**: Normal histomorphology of the seminiferous tubular epithelium and interstitium is observed. **B**: No observable histoarchitectural alteration; features being comparable with control. C: Sperm bundles are formed by the sloughing in of the seminiferous tubular lumen (arrow). Hypercellularity of the tubules are noted. [Stain: H&E; Mag. X400]

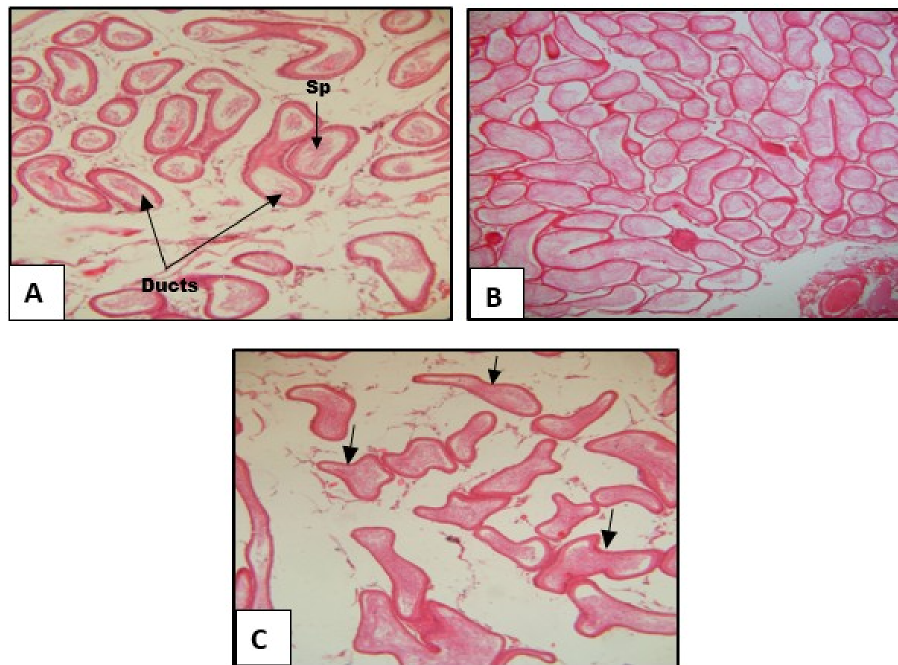


**Figure 2:** Photomicrographs of Epididymis sections from control [A], *AEPA*-treated [B] and *MEPA*-treated rats [C]. **A**: Normal histomorphology of the epididymal ducts and luminal spermatozoa (Sp) is shown. **B**: Ducts show no obvious histoarchitectural alteration. However, increased spermatozoa in lumen of ducts is observed. **C**: Ducts appear markedly distended (arrows); increased spermatozoa within the lumen is also observed. [Stain: H&E; Mag. x40]

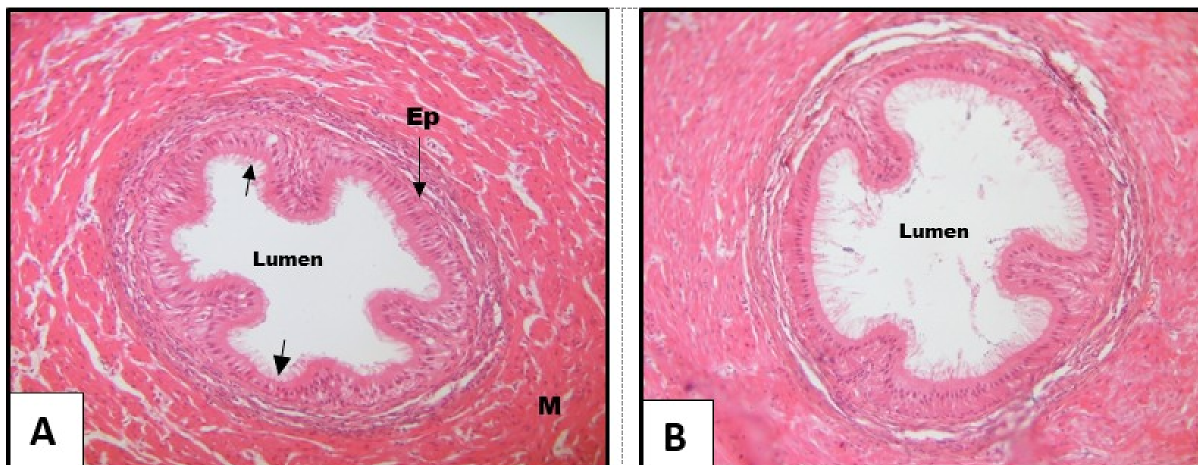


**Figure 3:** Photomicrographs of Vas deferens sections from control [A], and *MEPA*-treated rats [B]. **A**: Normal histomorphology of the muscular wall (M), epithelial lining (E) and stereocilia (arrows) observed. **B**: Intact tissue with no obvious histopathological change is noted; however, the lumen appears slightly dilated. [Stain: H&E; Mag. x400]


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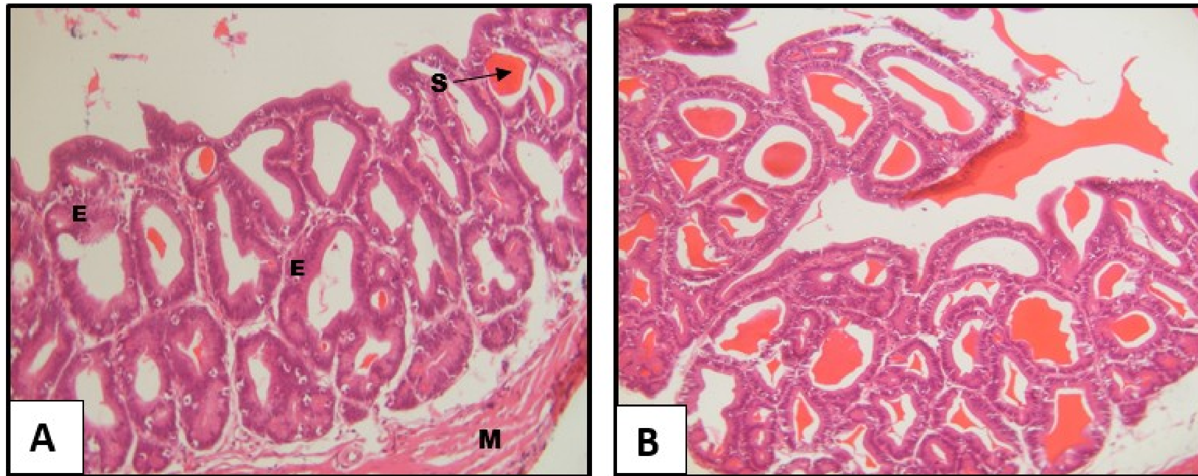


**Figure 4:** Photomicrographs of Seminal vesicles sections from control [A], and *MEPA*-treated rats [B]. **A**: Normal muscular wall (M), epithelial lining (E) and luminal secretions (S) is shown. **B**: No obvious histopathological alteration is observed. [Stain: H&E; Mag. X100]