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# Ethanolic Seed Extract of Cucurbita Pepo (Pumpkin) Protects the Testis from Azadirachta Indica (Neem) Induced Damage

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**ABSTRACT**

*Cucurbita pepo* seeds are highly nutritional, containing a wide array of healthy compounds and free-radical scavenging antioxidants that have been found useful in promoting good health. The aim of this study was to investigate the effect of methanolic seed extract of *Cucurbita pepo* (pumpkin) on *Azadirachta Indica* (neem) leaf induced testicular damage in adult male Albino Wistar rats. Twenty adult male albino wistar rats weighing between 110-230g were used, and assigned into 5 groups (I-V) of 4 animals each. Group I served as control. Groups II received 500mg/kg of ethanolic seed extract *Cucurbita pepo* (ESECP) for 5 weeks. Group III received 500mg/kg of methanolic leaf extract of *Azadirachta indica* (MLEAI) for 2 weeks and then 500mg/kg of ESECP for 3 weeks. Group IV received a co-administration of 500mg/kg of MLEAI and 500mg/kg of ESECP for 5 weeks, while Group V received 500mg/kg of for 5 weeks. All extracts were administered orally, once a day. Twenty four hours after the last administration, the rats were anesthetised and blood samples and testes were obtained for further investigation. Results showed significant increases in serum testosterone levels, improvements in sperm parameters (count, motility, and morphology) and better testicular microarchitecture in ESECP treated rats when compared to rats treated with MLEAI alone. This reveals that ethanolic seed extract of *Cucurbita pepo* exhibits both protective and ameliorative activities against *Azadirachta indica* induced testicular damage in adult male albino wistar rats.

**Keywords:** Testosterone, Sperm count, Sperm motility, Sperm morphology

## 1. Introduction

*Azadirachta indica* (Neem) locally called Dogoyaro in Nigeria, belongs to the mahogany family (Obi, 2004). Its bark, leaves, roots, and seeds have been used for medicinal purposes. These parts contain compound that have proven antiseptic, antiviral, antipyretic, anti-inflammatory, antifungal and antiulcer properties (Aftab and Sial, 1999). In males, concoctions derived from Neem leaf have been used as powerful spermicidal agent (Sadre *et al*., 1983). Observations on experimental animals have shown that the aqueous leaf extract of neem has reduction effects on serum concentration of testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH/ICSH) and prolactin (Joshi *et al.,* 2011; Kasturi *et al.,* 2002). Other studies reported adverse effects on motility, morphology, and number of spermatozoa in the cauda epididymidis, level of fructose in the seminal vesicle, and on litter size (Mishra and Singh, 2005).

*Cucurbita pepo* (Pumpkin) belongs to genus *Cucurbita* of the family *Cucurbitaceae* that includes cucumbers and squash. Pumpkin fruits come in variable sizes, colours, shapes and weights. Trace elements such as zinc, vitamins (carotenoids, tocopherol) and other substances like proteins, phytosterols, polly-unsaturated fatty acids and antioxidants are naturally present in pumpkin that are important to human health (Sabudak, 2007; Stevenson *et al.,* 2007). It has been suggested, that extracts derived from *Cucurbita pepo* (Pumpkin) may help protect human sperm from damages caused by chemotherapy and autoimmune diseases (Elfiky *et al.,* 2012). Other health benefits of pumpkin seeds include its use as an anti-inflammatory, antiviral, analgesic, urinary disorders, anti-ulcer, antidiabetic and antioxidant (Wang, 2001; Smith, 1997). Pumpkin seeds also contain L-tryptophan, which helps promote sleep and fight depression (Shona, 2014).

Due to the increased incidences of malaria and its increased resistance to generic drugs, intake of concoctions derived from neem leafs to treat such conditions as well as and other ailments in the developing world is on the rise. However, public enlightenment on *Azadirachta indica's* (neems') antifertility property has been passively heeded, thus the need to explore cheaper and readily accessible fruits and food items that may confer protective and ameliorative effect on the testes during neem treatment. Limited research has been done on the effect of methanolic seed extract of *Cucurbita pepo* (pumpkin) on neem leaf induced testicular damage in adult male Albino Wistar rats, hence the essence of this work.

## 2. Materials and Method

### 2.1 Animal Procurement, Care and Treatment

Twenty (20) adult male wistar rats weighing between 110-230g were purchased from Best farm in Okofia, Nnewi South L.G.A, Anambra State. The animals were kept in the research section of the Animal House of the Department of Anatomy, Faculty of Basic Medical Sciences Nnamdi Azikiwe University, Nnewi Campus where they were used for the research that lasted for 35 days. They were housed in well ventilated stainless steel rat cages and sawdust bedding, under normal temperature (27-31°C), and allowed to acclimatize over a period of two (2) weeks. They were fed with grower's mash (Top feeds, Nigeria Ltd), and distilled water *ad libitum* throughout the duration of the experiment.

### 2.2 Procurement and Preparation of Methanolic Leaf Extract of *Azadirachta Indica* (Neem)

Fresh young leaves of *Azadirachta indica* were collected from neem trees within Nnamdi Azikiwe University, Nnewi Campus, Anambra State. The leaves were washed, shed dried and milled into powder using local grinder. 50g of the dried leaves was macerated in 95% methanol for 48hours. It was then filtered Whatman No 1 filter paper. The filtrate was concentrated using a rotatory evaporator and was further dried using a laboratory oven at 45°C into a semi solid form. The extract was preserved in a refrigerator for further usage.

### 2.3 Procurement and Preparation of Ethanolic Seed Extract of *Cucurbita Pepo* (Pumpkin)

500 grams of white *Cucurbita pepo* (pumpkin) seeds were obtained from Onitsha Main Market in Anambra State, Nigeria and shed dried. The dried seeds were ground into powder and soaked in 70% ethanol in a specific separating funnel for 24 hours with frequent stirring. Ethanolic extract was obtained following the modified method of Abd El-Ghany *et al.*, (2010). The resultant extract was filtered and concentrated using a rotary evaporator (Heidolph.VV2000, Germany). The residue was lyophilized using a vacuum freeze dryer (Tilburg, Holland; 145Fm-RB) and the final extract refrigerated for further use.

### 2.4 Experimental Design and Protocol

Before the onset of administration, the rats were weighed and randomly assigned into five experimental groups (I-V) of four animals each. Group I served as control and received only animal feed (growers mash, Premier Feeds Mills Co. Ltd, Plateau State, Nigeria) *ad libitum* throughout the duration of the experiment. Groups II received 500mg/kg of ethanolic seed extract *Cucurbita pepo* (pumpkin seed) extract through the duration of the experiment. Group III received 500mg/kg of methanolic leaf extract of *Azadirachta indica* (Neem leaves) for two (2) weeks after which it was withdrawn and rats fed with 500mg/kg of ethanolic seed extract of *Cucurbita pepo* (Pumpkin) for three weeks. Group IV received a co-administration of 500mg/kg of methanolic leaf extract of *Azadirachta indica* (Neem) and 500mg/kg of ethanolic seed extract of *Cucurbita pepo* (Pumpkin) for 5 weeks. Group V received 500mg/kg of methanolic leaf extract of *Azadirachta indica* (Neem) through the duration of the experiment. All extracts were administered once a day via oral route.

### 2.5 Termination of Experiment, Blood Sample Collection, and Organ Extraction

Extract administration lasted for 35 days (7 weeks). 24 hours after the last administration, the animals were weighed. 2mls of blood were collected from three rats in each group via cardiac puncture after anaesthetizing with chloroform. Sera obtained were assayed for testosterone hormone using the Microwell enzyme linked immunoassay (ELISA) technique; using analytical grade reagents (Syntron Bioresearch Inc., USA). The abdominal cavity was afterwards opened up through a midline abdominal incision to expose the reproductive organs, the testes were detached and cleared free of the surrounding tissues, weighed with an electronic weighing

balance and the epididymis was afterwards quickly excised for semen analysis. The testes were then fixed in freshly prepared Bouin's fluid for further histological studies.

### 2.6 Sperm Count Assessment

This was carried out as described by Saalu *et al*., (2011) using a modified method of Yokoi and Mayi (2004). The epididymis was minced with Anatomic scissors in 5mL physiologic saline, placed in a rocker for 10 minutes, and allowed to incubate at room temperature for 2 minutes. After incubation, the supernatant fluid was diluted 1:100 with solution containing 5g sodium bicarbonate and 1 mL formalin (35%). Total sperm number was determined by using the new improved Neubauer's counting chamber (haemocytometer). Approximately 10 µL of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and allowed to stand for 5 minutes. This chamber was then placed under a binocular light microscope using an adjustable light source. The ruled part of the chamber was then focused and the number of spermatozoa counted in five 16-called squares, when multiplied by $10^6$.

### 2.7 Assessment of Sperm Motility

This was carried out using a modified method of Sonmez *et al*. (2005). This was evaluated both subjectively and using a computer-assisted semen analyzer (CASA) (Sperm Vision Minitube™ of America, Inc., 2002). For the analysis, a 300-µl aliquot of the thoroughly but gently mixed semen sample was placed into an open 3-ml tube. The tube was kept in a 35°C water bath (Grants Instruments Ltd., Cambridge, UK) for 5 min before semen analyses. A 5-µl aliquot was placed on a pre-warmed 38°C microscope slide, covered with a coverslip (24 × 24 × 1.5 mm) and the proportions of total motile spermatozoa were recorded in percentage.

### 2.8 Assessment of Sperm Morphology

The sperm cells were evaluated using a modified method of Zemjanis (1977) with the aid of light microscope at x 400 magnification. Semen sample were taken from the original dilution for motility and diluted 1:20 with 10% neutral buffered formalin. One hundred sperm cells from the sample were scored for morphological abnormalities using phase-contrast optics, spermatozoa were categorized. In this study a spermatozoon was considered abnormal morphologically if it had rudimentary tail, round head, and detached head; and was expressed as a percentage of morphologically normal sperm.

### 2.9 Tissue Processing and Photomicrography

For histological studies, sections of the testis were, fixed, and passed through dehydration, clearing, infiltration, embedding, sectioning and staining processes. The tissues were fixed in freshly prepared boin's fluid. Dehydration of the fixed tissues was carried out in different grades of 50%, 70%, 90% and absolute alcohol. The tissues were cleared in xylene for hours after which infiltration was due in molten paraffin wax at a temperature of $60^0$c for two hours each in two changes and stained using Hematoxylin and eosin method.

### 2.10Data Analysis

Differences between the mean sperm count, sperm morphology (sperm head abnormality) and sera testosterone levels of the control and treatment groups were compared for statistical significance by one-way analysis of variance (ANOVA) and post hoc Scheffe's test using SAS 9.2 Enterprise Guide 4.3 software (SAS Institute Inc., Cary, North Carolina, USA). Data was expressed as means ± standard deviation (SD) and were considered statistically significant when $P < 0.05$.

## 3. RESULT

The oral $LD_{50}$ of ethanolic seed extract of *Cucurbita pepo* (Pumpkin) and methanolic leaf extract of *Azadirachta indica* (Neem) in Albino Wistar rats was calculated to be 3,872.98 mg/kg and 3,872.98 mg/kg respectively. Results obtained showed statistically significant ($P < 0.05$) differences in sperm count and motility as well as serum levels of testosterone in the test groups when compared with the control group. Ethanolic seed extract of *Cucurbita pepo* (Pumpkin) caused increase is sperm count and motility as well as serum levels of testosterone

when compared to rats treated with methanolic leaf extract of *Azadirachta indica* (Neem), as shown in Tables 1 and 2.

**Table 1**: Effect of ethanolic seed extract of *Cucurbita pepo* (pumpkin) and methanolic leaf extract of *Azadirachta indica* (neem) on sperm count, motility and morphology after 35 days.

| S/N | GROUP | Sperm count ($\times 10^6$/dL) ±S.E.M | Sperm motility (%) ±S.E.M | Sperm Cell Morphology (%) ±S.E.M | |
|---|---|---|---|---|---|
| | | | | Normal | Abnormal |
| 1 | I | 16.0 ± 1.00 | 90.0 ± 1.00 | 85.0 ± 0.10 | 5.0 ± 0.20 |
| 2 | II | 42.0 ± 6.50* | 77.50 ± 2.50* | 85.5± 0.50 | 5.0± 4.00 |
| 3 | III | 37.0± 20.00* | 62.50 ± 2.50* | 91.0 ± 1.00 | 7.0 ± 1.00 |
| 4 | IV | 28.0± 35.00* | 75.0 ± 5.00* | 91.0 ± 1.00 | 7.5± 0.50 |
| 5 | V | 11.0 ± 1.00* | 37.50 ± 2.50* | 68.0 ± 0.00* | 32.0 ± 0.00* |

*=p<0.05

Table 1, shows that there was significant increases in sperm count, percentage of motile sperm, and also a higher percentage of normal sperm cell, in groups II, III, and IV, when compared with group V.

**Table 2:** Effect of ethanolic seed extract of *Cucurbita pepo* (pumpkin) and methanolic leaf extract of *Azadirachta indica* (neem) on Serum Testosterone levels after 35 days.

| S/N | Groups | Serum Testosterone Mean(ng/ml) ± SEM |
|---|---|---|
| 1 | Group I (Control) | 1.70 ± 0.10 |
| 2 | Group II | 4.65 ± 0.35 |
| 3 | Group III | 5.10 ± 1.50 |
| 4 | Group IV | 7.70 ± 0.70 |
| 5 | Group V | 1.60 ± 0.20 |

*=p<0.05

Table 2, shows that there was statistically significant increases in serum Testosterone levels in groups II, III, and IV, when compared with control and the group that received only 500mg/kg of methanolic leaf extract of *Azadirachta indica* (group V).

3.1 Histological findings

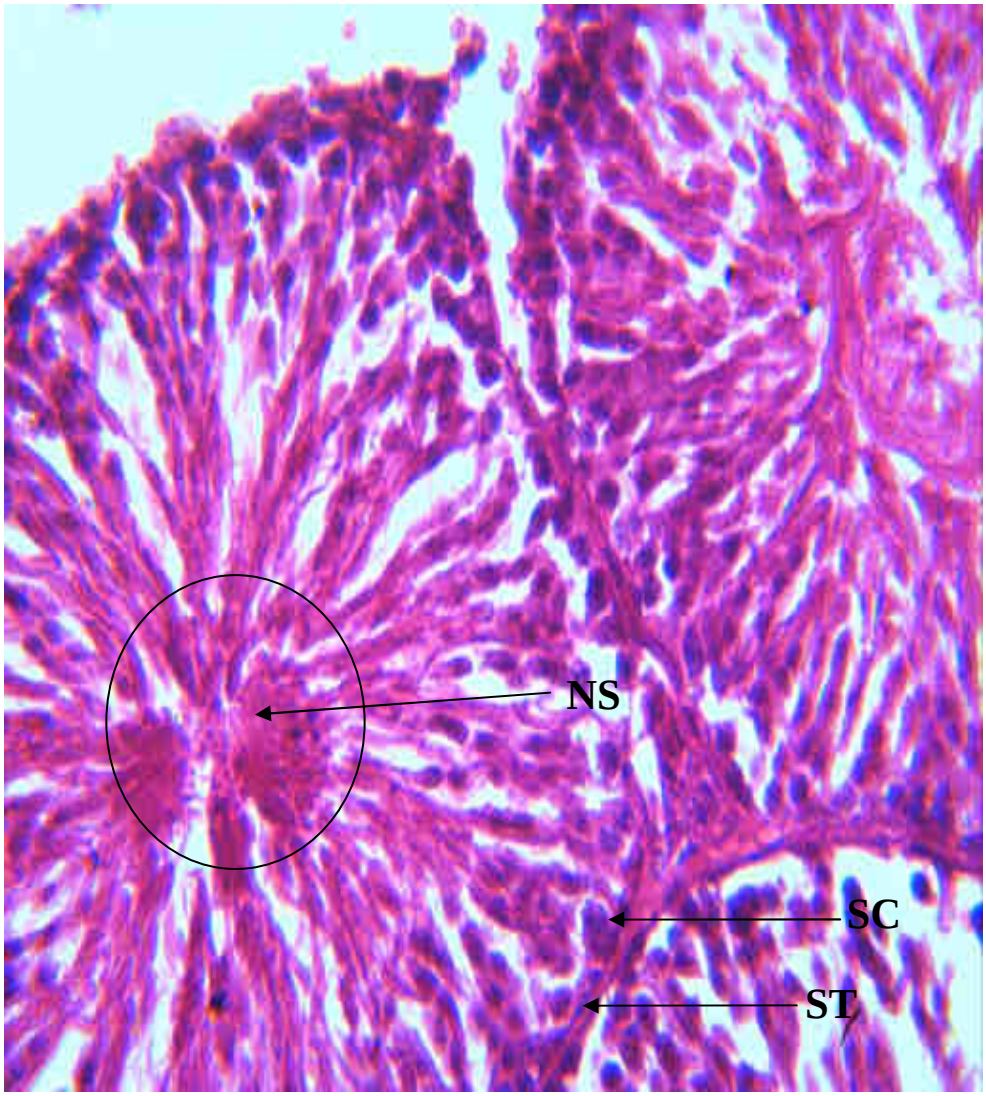


Plate 1: Photomicrograph of sections of testis (x400)(H/E) of control group showing normal testicular architecture with seminiferous tubules (ST) that are lined with sertoli cell (SC) and normal spermatogenesis(NS).

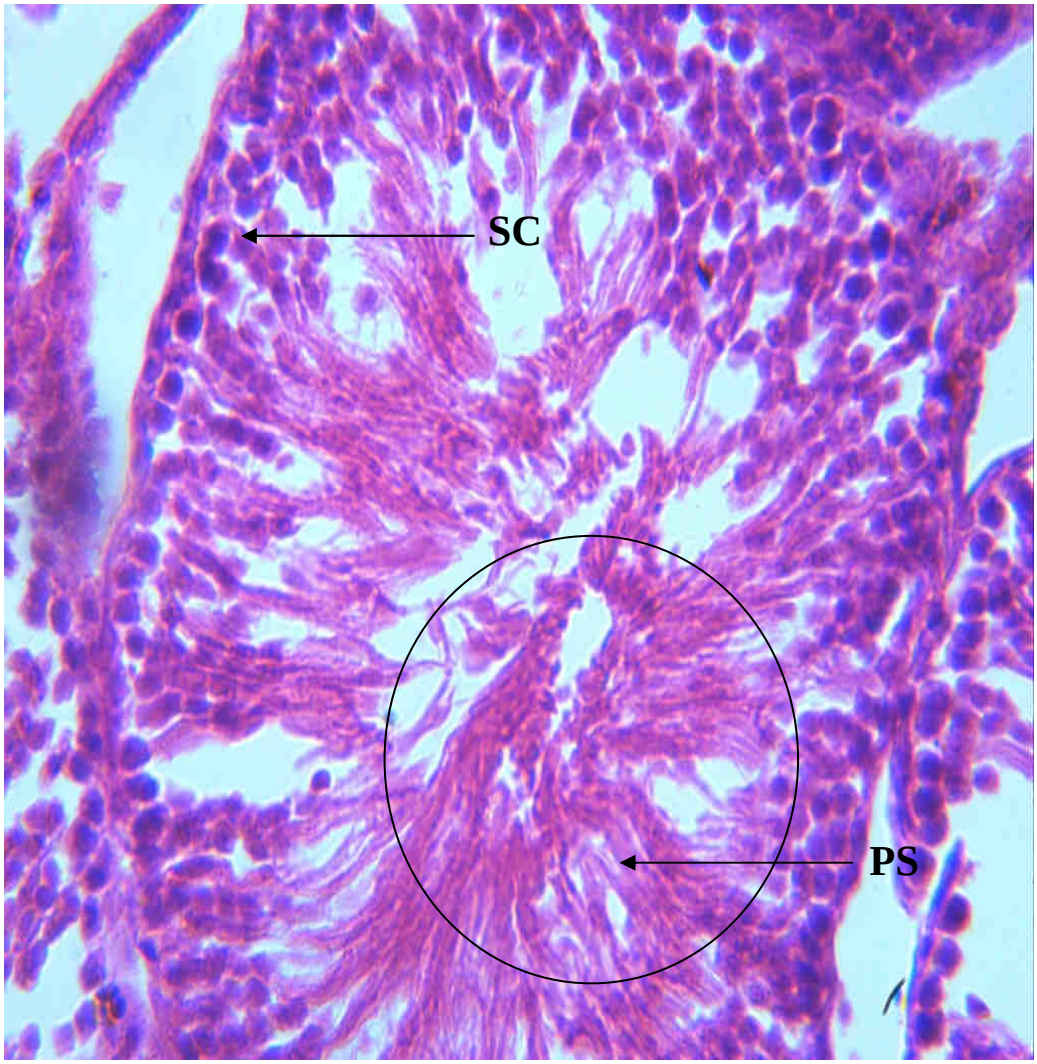


Plate 2: Photomicrograph of sections of group of testis administered with 500mg/kg of ethanolic seed extract of *Cucurbita pepo* (x400)(H/E) showing production of sperm cells (PS) with normal with seminiferous tubule that are lined with sertoli cell (SC).

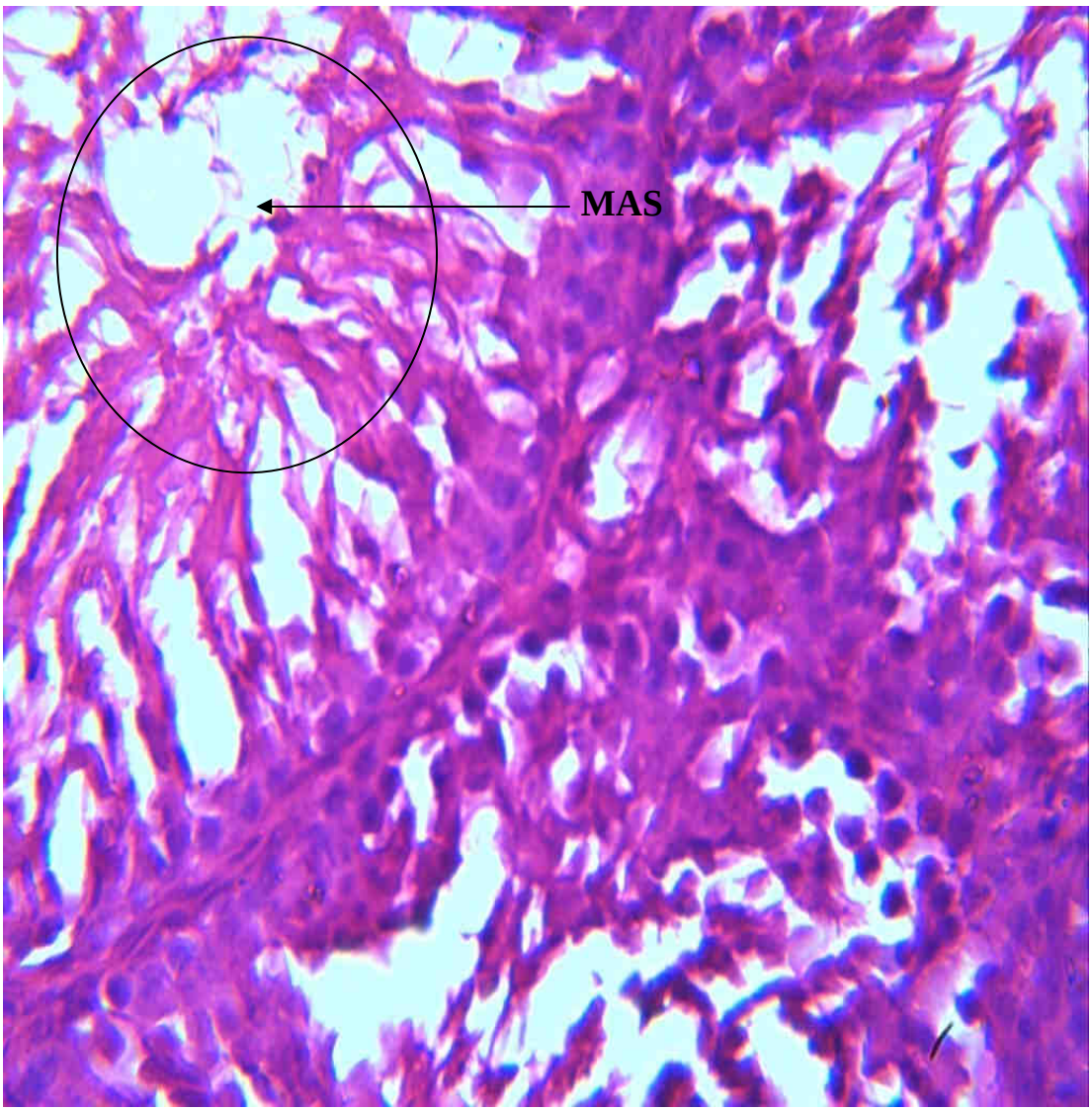


Plate 3: Photomicrograph of section of group administered 500mg/kg of methanolic leaf extract of *Azadirachta indica* for two weeks after which was withdrawn and rats fed with 500mg/kg of ethanolic seed extract of *Cucurbita Pepo* for three weeks (x400) (H/E) showing moderate restoration of the damaged testicular tissue with mild arrest of spermatogenesis (MAS).

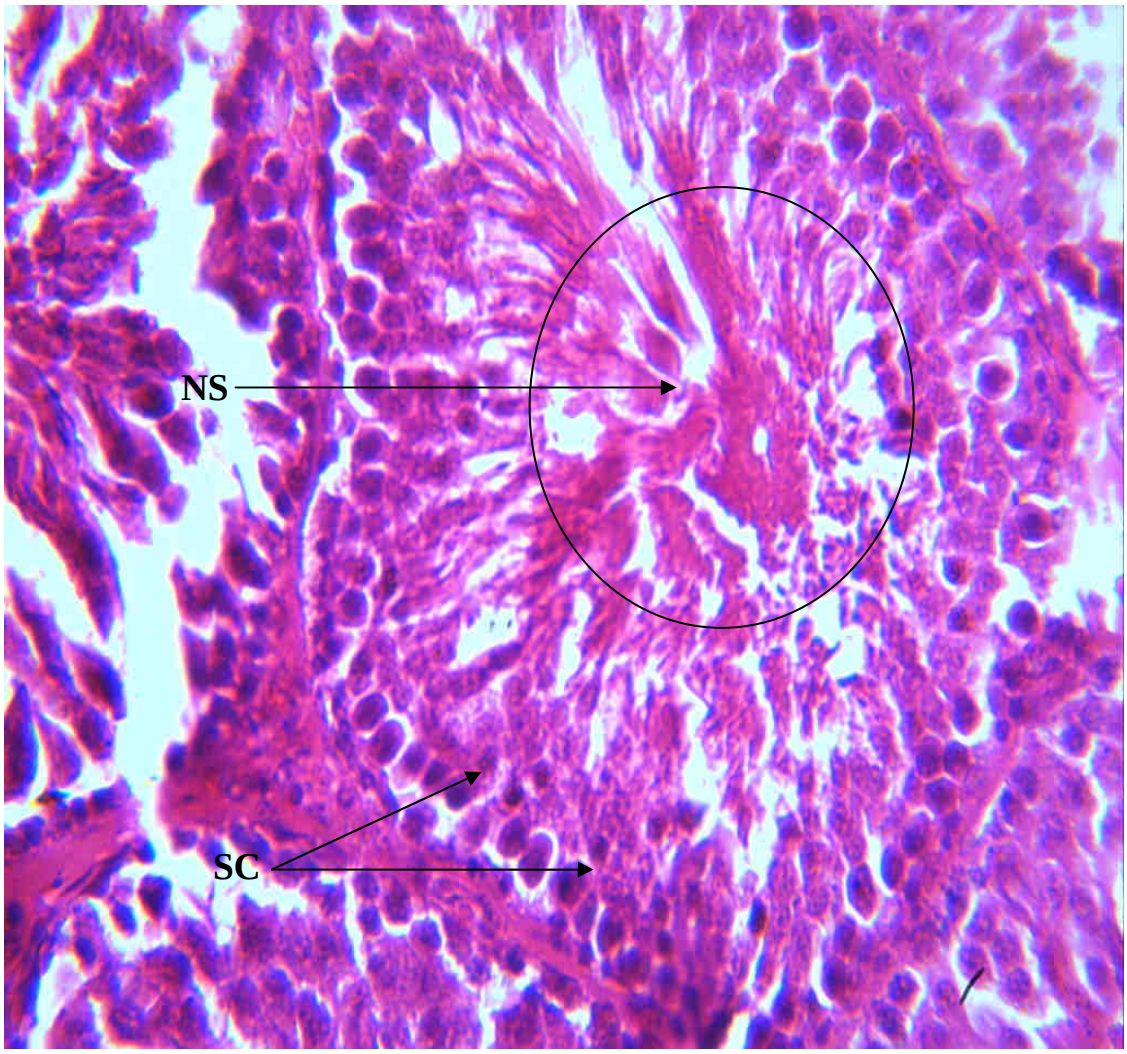


Plate 4: Photomicrograph of section of group co-administered with 500mg/kg of methanolic leaf extract of *Azadirachta indica* and 500mg/kg of ethanolic seed extract of *Cucurbita Pepo* throughout the duration of experiment (x400)(H/E), showing normal spermatogenesis (NS) normal testicular architecture with seminiferous tubules that are lined with sertoli cells (SC).

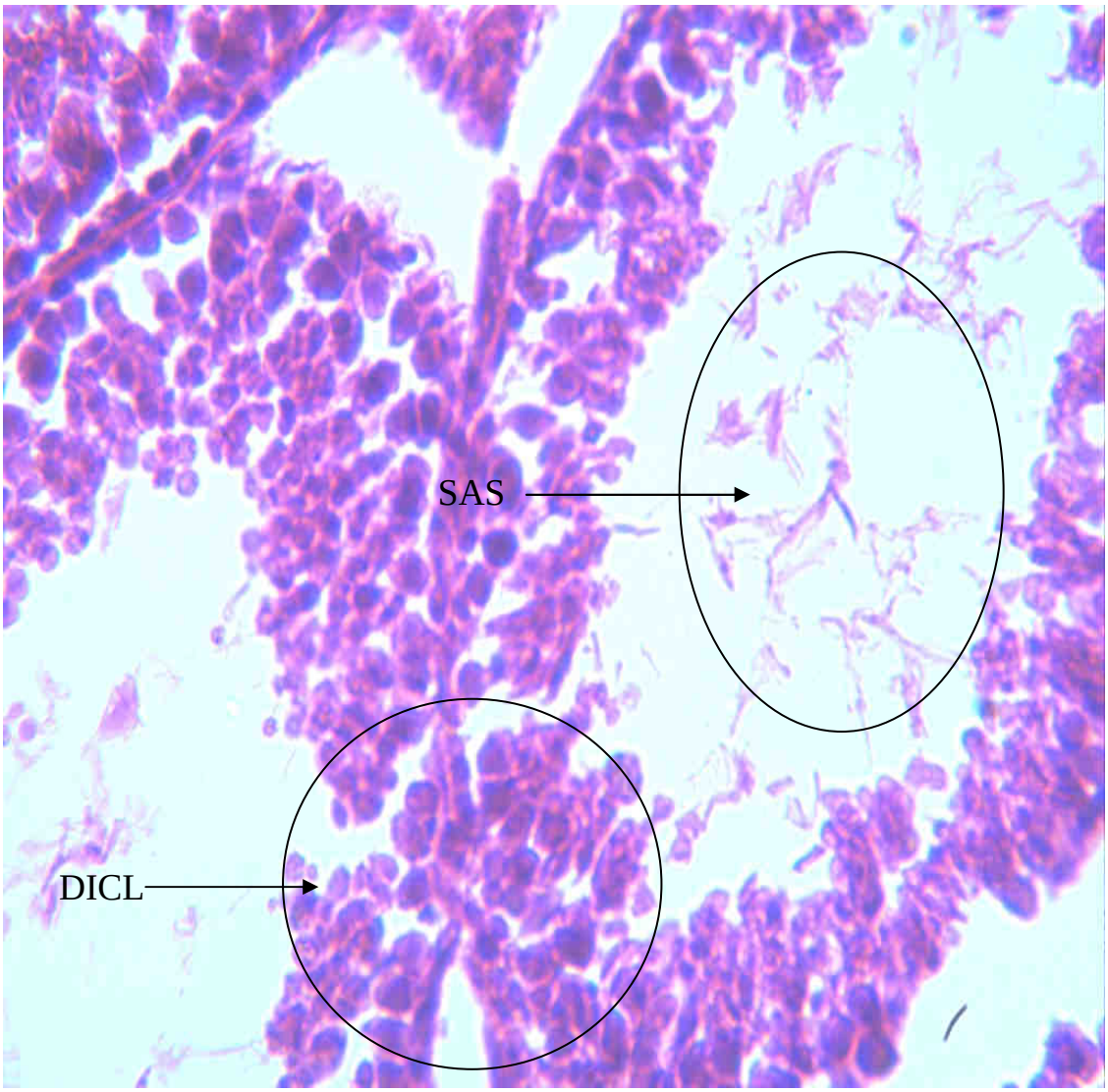


Plate 5: Photomicrograph of group five animals that serve as positive control section of testis administered with 500mg/kg of methanolic leaf extract of *Azadirachta indica* throughout the duration of the experiment (x400)(H/E) shows moderate to severe damaged testicular tissue with degeneration of interstitial cell of the leydig (DICL) and sever arrest of spermatogenesis (SAS).

## 4. Discussion

Despite the benefits of neem, its consumption has been linked to male infertility (Joshi *et al.*, 2011; Kasturi *et al.*, 2002). To remedy the cases of infertility caused by neem and other substances, drug formulations are continually been tested with the purpose of ameliorating these conditions. The principal goal of this study was to investigate the effects of ethanolic seeds extract of *Cucurbita pepo* (Pumpkin seeds) on methanolic leaves extract of *Azadirachta indica* (Neem leaves) induced testicular damage in adult male albino wistar rats.

Results obtained from this study showed that leaf extract of *Azadirachta indica* (neem) negatively affected the testicular function in adult male Wistar rats. This was manifested in the group that was administered 500mg/kg of methanolic leaf extract of *Azadirachta indica* only throughout the duration of the experiment. This led to lower sperm count, reduced sperm motility and sperm quality (Morphology), as well as decreased serum levels of testosterone when compared to control. There was also moderate to severe damages in testicular microarchitecture with degeneration of interstitial cell of the leydig (cells responsible for testosterone production), scanty sertoli cells and severe arrest of spermatogenesis visible in the testicular histology as seen in their respective micrographs. These findings correlate with those of Ekaluo *et al.,* (2010), Sathiyaraj *et al.,* (2010), and Kalyan and Chanchal (2009) who documented similar effects such as dose related reduction in the testicular sperm count, epididymal sperm count and motility and abnormal sperm count, seminiferous tubules with intraepithelial vacuolation, loosening of germinal epithelium, occurrence of giant cells, mixing of germ cell types in stages of spermatogenesis, degenerated appearance of germ cells, with adverse effects on morphology and number of spermatozoa in the cauda epididymidis following treatment of experimental rats with neem leaf extracts. This could be as a result of Neem leaf extract being able to induce morphologic apoptotic changes such as shrinkage, membrane leakage, and cytoplasmic fragmentation prior to degeneration of sperm cells, and its ability to suppress activities of Leydig cells thus inhibiting testosterone production, which in turn slows down spermatogenesis.

Experimental animals that received only ethanolic seed extract of *Cucurbita pepo* (Pumpkin) throughout the duration of the experiments showed an increase in the number of sperm count, percentage of normal sperm cells as well as higher serum levels of testosterone when compared to control and neem fed rats and seen in their respective histological slides. These improvements in testicular parameters when compared with control agrees with findings of Minisy *et al.,* (2017), Aghaei *et al.,* (2014), and Abd El-Ghany *et al.,* (2010). According to Abd El-Ghany *et al.,* (2010), pumpkin seeds improve sexual health status. The increase in sperm count, and percentage of normal sperm cells seen in pumpkin seed treated group when compared to control and neem leaf fed rats obtained in this current study could be as a result of high antioxidant contents as well as vitamins E and A, and Carotenoids present in pumpkin seeds that have the potentials of breaking down the oxidative chain reaction and play a very significant role in increasing the body's capacity to fight free radical-induced oxidative stress, and therefore improve the process of spermatogenesis (Mohammadi *et al.,* 2013). The presence of unsaturated fatty acids such as omega 3, 6, and 9, as well as proteins in pumpkin seeds and its oil are also reported to promote dehydrogenase activity which is key in testosterone production. This in turn increases spermatogenesis, and secretory activity of the accessory sex glands (Aghaei *et al.,* (2014). Higher serum levels of testosterone obtained in pumpkin treated group may have been caused also by the high content of zinc present in pumpkin seeds which is important as it helps stimulate testosterone production in men (Aghaei *et al.,* (2014). Xanthopoulou *et al.,* (2009) documented that pumpkin seed extract confers its protective and improvability effects possibly due to its high antioxidant constituents as well as its free radical scavenging ability.

In the group that were initially treated with neem extract, the improved testicular parameters observed in this research after administration of pumpkin seed extract such as increase in the number of sperm count, increase in percentage of normal sperm cells and higher serum levels of testosterone when compared to control and neem fed rats suggests that that pumpkin not only has protective and curative effects, but also has soothing effect on the testis as evidenced in their respective histological slides. Pumpkin seed extracts have high antioxidant constituents such as flavonoids that not only scavenge free radicals that potentiate testicular damage but also have been reported to have great effects on semen quality (Das *et al.,* 2004). Flavonoids over the years have been employed in the treatment of reproductive diseases in men and women involving hormone inbalance (Qin *et al.,* 2000). Seeds of Pumpkin also have great concentrations of squalene, a triterpene produced by humans, and animals; and a precursor of steroid hormones such as testosterone (Katharina, 2012). Triterpene also is a precursor of vitamins D. Vitamin D has been reported to increase testosterone levels in both humans and experimental animals (Katharina, 2012). These findings are in agreement with that of Aghaei *et al.,* (2014) that reported the protective effect of pumpkin seed extract against cyclophosphsmide induced testicular damage due to significant increase in total antioxidant capacity level due its high antioxidant content in pumpkin seed extract administered.

Statistically higher percentages of motile sperm observed in experimental groups initially treated with neem extract and then pumpkin seed extract when compared to neem-alone treated group showed that there were improvements following pumpkin seed extract administration. This could be as a result of not only the abundant phytochemicals present in the seed but also L-Carnitine and L-acetyl-carnitine in particular (important forms vitamin O made in the body from amino acids and also abundantly found in pumpkin seeds). The initiation of sperm motility has been closely related to an increase of L-carnitine in the epididymal lumen and L-acetyl-

carnitine in sperm cells (Matalliotakis *et al.,* 2000). This agrees with findings of Bin *et al.,* (2015) who documented that L-carnitine contributes to the inhibition of cell apoptosis and the modulation of autophagy in protecting cyclophosphsmide induced testicular injury while improving sperm parameters especially sperm motility.

## 5. Conclusion

The result of this study showed that ethanolic seed extract of *Cucurbita pepo* (Pumpkin seeds) not only exhibited protective and antioxidant activities against methanolic extract of *Azadirachta indica*– induced testicular damage in adult male albino wistar rats but also provided curative properties.

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