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Fractional extracts of *Azadirachta indica* leaf affect spermiogram, testosterone profile, and testis histology of rabbit bucks

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Abstract

The effect of fractions from a crude extract of *Azadirachta indica* leaves on spermatogenesis, testicular histology and testosterone concentration of New Zealand White rabbits were evaluated in this study. Twenty-five matured male New Zealand White rabbits were used for this study and were randomly assigned to five groups (A, B, C, D, and E). Group A served as the control and was administered distilled water (0.5ml); while groups B, C, D and E served as the hexane, chloroform, ethyl acetate, and butanol treated groups, respectively at the same dosage of 300 mg/kg. Semen samples were collected using an artificial vagina weekly for twelve weeks and were evaluated for volume, colour, motility, concentration, percentage live-dead ratio and morphological abnormalities. A blood sample (2ml) was also collected from each buck through venipuncture of the ear vein three times at regular intervals for the determination of testosterone concentration. Two bucks from each group were humanely sacrificed at the end of the experiment for testicular histology. Significantly lower ($p<0.05$) sperm motility, higher dead sperm cells, sperm abnormalities, degenerative changes, depletion and vacuolation of spermatogenic cell layers were observed in treatment group C at the end of the experiment. The present study has shown that the chloroform fraction of methanolic crude *Azadirachta indica* (neem) leaves extract is detrimental to sperm cells and testicular histology.

Keywords: *Azadirachta indica,* Butanol fraction; *Spermiogram; Neem*; hexane fraction; Neem, Rabbit buck

Introduction

Humans and rabbits have been competing for available food materials (such as soybean and vegetables) since the last decade (Ogbuewu *et al.,* 2010). It is also without a doubt that the Coronavirus pandemic adversely affected the supply and price of global food commodities, thereby aggravating the food competition to an all-time high between the human race and livestock population, especially rabbits (Nasereldin *et al*. 2021). Therefore, an investigation into other available, cheaper and non-conventional sources of nutrients to rabbits has become the next alternative (Mahmud *et al*., 2015). Consequently, neem leaf-based diets have been reported to increase rabbit carcass weight and quality

(Wasanthakumar *et al.,* 1999a; Wasanthakumar *et al.,* 1999b, Gowda & Sastry*,* 2000 Ogbuewu, 2008; Ogbuewu *et al.,* 2010a). Ogbuewu *et al*. (2008), for example, reported excellent carcass quality in rabbits fed up to 15% neem leaf diet composition. Neem (*Azadirachta indica* Juss) is a fast-growing evergreen popular tree found commonly in Africa and India (Pandey *et al.,* 2014). It is known by different names by different ethnicity and region in Nigeria: Dogonyaro (Hausa) Indian lilac (Parotta, 2001). Neem extract comprises a complex mixture of molecules, including normal hydrocarbons, phenolic compounds, terpenoids, alkaloids and glycosides (Hossain *et al*., 2013a; Sarawaneeyaruk, 2015). Among the chemical constituents found in the leaves of *A. indica* are; nimbin, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, nhexacosanol, amino acids, 7-sdesacetyl-7-benzoylazadiradione,7-sdesacetyl-7-benzoylgedunin, 17-hydroxy azadiradione and nimbiol (Kokate *et al*., 2010; Hossain *et al.*, 2013b).

Despite the advantages of neem leaf as a cheaper, available and non-competitive source of feed with human being, its utilisation in animal feed is still limited because of the reported cases of infertility (Khan & Awasthy, 2003, Adekeye *et al*., 2013, Lisanti *et al*., 2016). Earlier reports on the antifertility characteristics of the leaves were based on the methanol or ethanol crude extracts. Hossain *et al*. (2013a) have shown that most predominate active compounds present in the leaves were distributed according to the polarity of organic solvents. There is a dearth of information on the solubility of the active chemical compound of the leave and its effects on male reproduction. Therefore, the major objective of this study was to sequentially extract the leaf material with different preparations of organic solvents based on their polarity and test their effects on spermatogenesis, testicular histology and testosterone concentration of rabbit bucks.

Materials and Methods

Experimental animals and management

A total of twenty-five adult male New Zealand white rabbits having an average weight of 3.1 kg were purchased from National Animal Production Research Institute (NAPRI) and used for the experiment. The rabbits were kept in the animal house of Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria, Nigeria. The animals were examined, screened and dewormed with ivermectin and treated against coccidia parasite using sulphurdimidine. The animals were fed with a pelleted diet made from commercially available growers rabbit feed (Top feed^{R}), and potable water was provided *ad libitum.* Animals were allowed to acclimatise for two weeks prior to the commencement of the experiment, during which the bucks were trained for semen collection, and baseline data were established. Blood samples were collected thrice through ear venipuncture, and two ejaculates each were obtained from the rabbit within 14 days of acclimatisation. Ethical clearance was sought for the use of rabbits in this study from Ahmadu Bello University Zaria Committee on Animal Use and Care (ABUAUC).

Extraction procedure

Fresh mature healthy neem (*Azadiracta indica,*) leaves were collected from the Botanical Garden, Department of Biological Science, Faculty of Life Sciences, Ahmadu Bello University Zaria. Identification was confirmed by a Taxonomist at hibereum, Department of Botany Faculty of Life Sciences, Ahmadu Bello University Zaria with identification number of V/N: 90015. The leaves were washed properly, air-dried at room temperature and then made into powder using a mechanical grinder to obtain fine powdered material. This was then subjected to extraction in a Soxhlet apparatus using methanol as described by Akpantah *et al.* (2003). The powdered samples (1.5kg) were extracted with methanol solvent (2L) by using Soxhlet extractor for 72 h. After complete extraction, the methanol solvent was evaporated using a rotary evaporator (Yamato, Rotary Evaporator, model-RE801) under reduced pressure to obtain methanol crude extract (364.5 g). The methanol crude extract from *Azadirachta indica was* suspended in 200 ml of water. Then it was extracted successively with different organic solvents having an increasing polarity, that is; hexane, chloroform, ethyl acetate and butanol to obtain hexane (43.7 g), ethyl acetate (73.63 g), chloroform (57.23 g), butanol (36.81 g) and residual methanol fractions (146.90 g). All fractions were filtered separately through Whatman No. 41 filter paper to remove particles. The particle free fractions were subjected to distillation using rotary evaporator to obtain dry fractions from the methanol crude extracts. The residue left in the separatory funnel was subjected to extraction processes twice followed by filtration. The combined extracts were concentrated and dried by using rotary evaporator under reduced pressure.

Animal grouping

The experimental animals were randomly allocated to five (5) groups; A, B, C, D and E, comprising five rabbits each. Group A served as the control administered distilled water at 0.5ml /kg. Group B, C, D and E, served as the experimental groups and were treated with n-hexane, chloroform, ethylacetate, and n-butanol fraction of methanolic neem leaf extract for 12 weeks at a dose of 300 mg/kg across all the groups. The rabbits were dosed orally at approximately the same time each day using a graduated syringe and stainless steel intubation cannula.

Sample collection and evaluation

Semen samples were collected using an artificial vagina that was locally designed for rabbits (Herbert & Adejumo, 1995). The rabbit bucks were teased with a matured doe before semen collection for optimum semen yield. Semen samples were collected in the morning between 8:00 to 10:00 a.m. using the artificial vagina (AV). Semen collection was done once weekly for twelve weeks. Semen volume was read off the calibrated collection tube and recorded in millilitres. The gross motility of the semen was examined immediately after collection based on the swirling motion observed from a drop of semen on a pre-warmed clean glass slide under a light microscope at x10 magnification. The concentration of spermatozoa was determined using Neubauer haemocytometer as described by Zemjanis (1970).

The percentage live-dead ratio of the sperm cells was determined using the method described by Rekwot *et al*. (1997). One drop of semen was mixed with two drops of eosin-nigrosin stain. A thin sperm smear was made on a clean, grease-free glass slide and air-dried. This technique was based on the principle that eosin-nigrosin penetrates and stains dead sperm cells (stained pinkish), while live sperm cells repel the stain (remained translucent). One hundred stained and unstained sperm cells were counted using a light microscope at (x40 magnification), and the percentage for each group was estimated. The sperm morphological abnormalities were determined using the method described by Koonjaenak (2006). A thin semen smear stained with eosin-negrosin was prepared on a clean, grease-free glass slide. The smear was air-dried and observed under a light microscope. One hundred sperm cells were counted per slide using a hand counter, and five categories of cells were recorded as a normal cell, free head, free tail, coiled tail and bent tail.

Histological processing

This was carried out using the method described by Akpantah *et al.* (2003). At the end of the study, two rabbit bucks from each group were randomly selected and humanely sacrificed by jugular venipuncture. Individual testis were dissected and placed in Bouin`s solution and taken to the laboratory. Samples were refrigerated at 4^{o}C for five days (for fixation) for histological slide preparation. After fixation, the tissues were dehydrated (using 90% alcohol and chloroform), infiltrated in liquid paraffin and embedded in paraffin blocks (Leica E. G. 1160 Germany). Using rotary microtome, sections were cut at 5 microns thickness. Each section was stained with haematoxylin and eosin (H&E) using standard staining procedures, according to Luna (1968). Slides were prepared from these tissues and examined under light microscopy. Lesions observed were recorded. Pictures of the slides were taken after optical focus using a digital camera (Casio®, EX-Z80, 8.1 MP, S/N 44315714B, China).

Testosterone analyses

Two millilitres (2 ml) of blood were randomly collected from three rabbit bucks three times at regular intervals from each group during the study. Collections were done at the beginning (first week), middle (seventh week) and end (Twelveth weeks) of the experiment between 7 a.m and 9 a.m. using 25G needle. The blood sample was collected through venipuncture of the auricular vein into sterile, non heparinised sample bottles. Testosterone values were assayed using the Enzyme-Linked Immunosorbent Assay (ELISA) technique, according to the manufactures manual instruction (monobind Inc. Lake Forest, CA 92630, USA).

Statistical analysis

Data collected were expressed as means ± standard error of the mean (SEM), and percentages. Repeated measure one-way analysis of variance (ANOVA), with Tukey's multiple comparison tests using GraphPad Prism software (Version 5.0), was adopted for the analysis. P-values ≤ 0.05 were considered statistically significant.

Results

The different fractions from methanolic neem leaf crude extract showed the various components of plant secondary metabolite (Table 1). During the acute oral toxicity study, no death and clinical signs were observed (Tables 2 and 3). The LD50 was above 5000mg/kg. Figure 1 shows the mean (± SEM), semen

Table 1: Qualitative phytochemical screening of fractions from crude methanolic neem leaf extract

S/No	Constituents	chloroform	hexane	ethylacetate	n-butanol	test
1	Carbohydrate	-	+	+	+	Molish
2	Anthraquinones	-	+	-	-	Bontragers
3	Glycosides	+	+	+	+	Fehling
4	Cardiac glycosides	+	+	+	+	keller-killiam
5	Saponins	+	-	+	+	Froth
6	Steroids	+	+	+	+	Lieberman
7	Triterpenes	+	+	+	+	Lieberman
8	Tanins	-	-	+	+	ferric chloride
9	Flavoids	-	+	+	+	shinoda
10	Alkaloids	+	+	+	+	Dragendorff

Key:
+ presence
- Absence

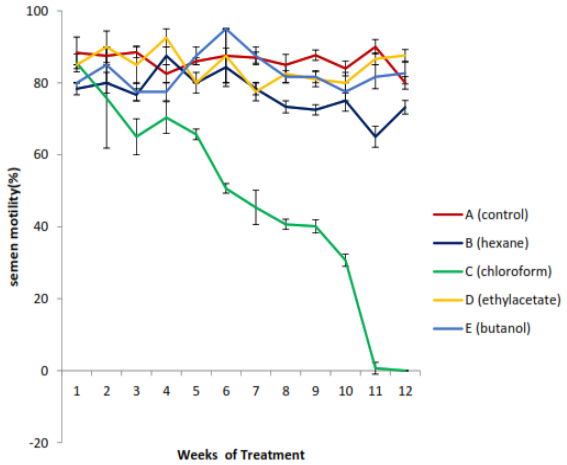

Figure 1: Mean (± SEM) values of semen gross motility of New Zealand white rabbit administered crude fractions from methanol neem leaf extract
P-value=0.0001

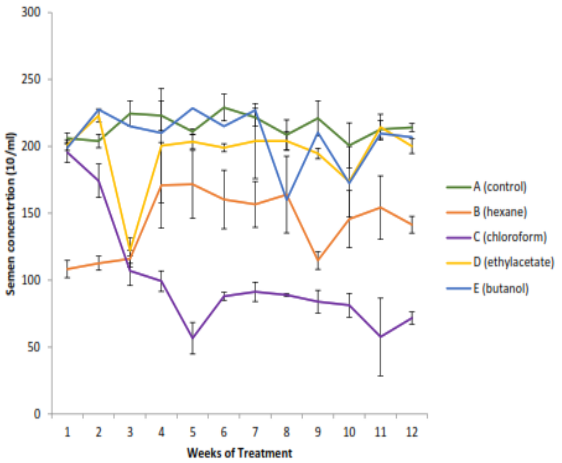

Figure 2: Means (± SEM) values of sperm concentration (10^6/ml) of New Zealand white rabbit administered crude fractions from Methanol neem leaf extract. (n=5)
P<0.005

gross motility, Sperm concentration (Figure 2), percentage live spermatozoa (Figure 3), percentage sperm abnormalities (Figure 4), and testosterone concentration (Figure 5) of New Zealand white rabbit administered fractions of crude methanol neem leaf extract.

Table 2: Summary of results from phase I median lethal dose (LD_{50}) evaluation

Group	Dose (mg/Kg)	No. dead/No. dosed
1	10	0/3
2	100	0/3
3	1000	0/3

Table 3: Summary of results from phase II median lethal dose (LD_{50}) evaluation

Group	Dose (mg/Kg)	No. dead/No. dosed
1	2900	0/1
2	5000	0/1
3	**65000**	1/1

Testicular histology indicates normal testicular histo architecture in the control group (Plate I). Intact seminiferous tubules with thick layer of spermatogenic cells at different stage of development (Plate II). However, exfoliation of germ and their regular consumption (Galeane *et al.,* 2017).

Results from the present study showed the presence of terpenes and phenolic compounds in all the fractions. Mossini & Kemmelmeier (2005) and Galeane *et al*. (2017) reported similar findings in neem leaf using different organic solvents. Although flavonoids were not detected in the chloroform

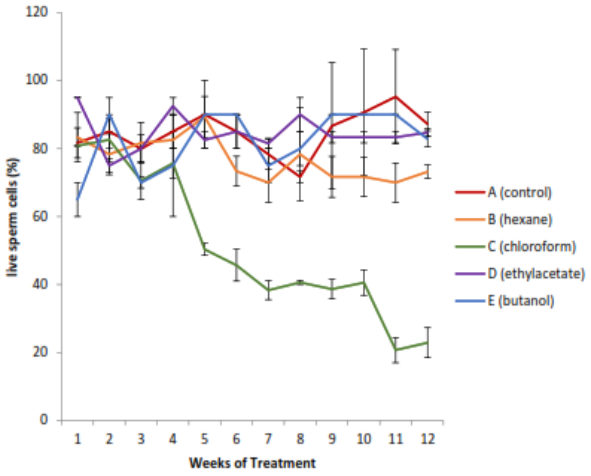

Figure 3: Means (± SEM) values of live spermatozoa (%) of New Zealand white rabbit administered crude fractions from methanol neem leaf extract. (n=5)
P<0.05

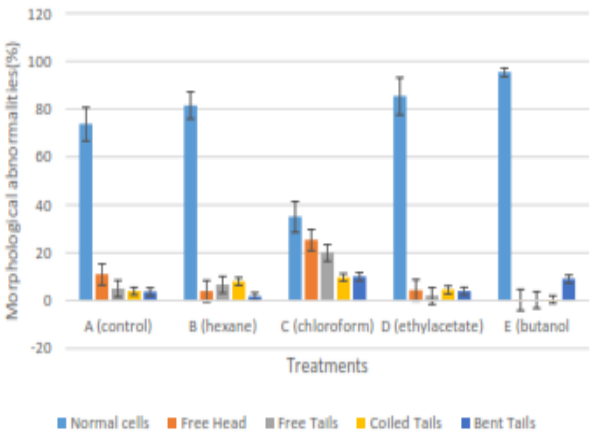

Figure 4: Mean (±SEM) Spermatozoa morphological abnormalities (%) of rabbit bucks administered crude fractions from methanol neem leaf extract
P<0.05

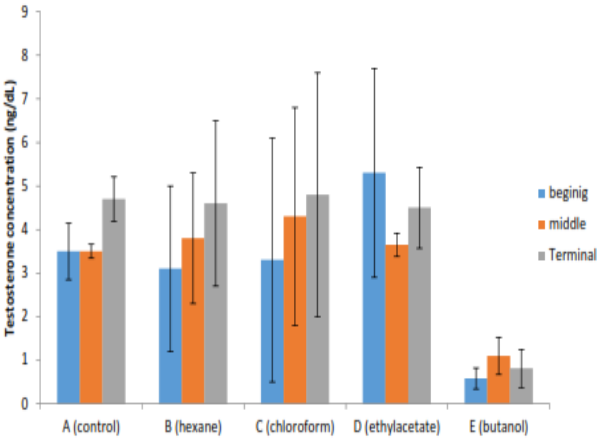

Figure 5: Mean (± SEM) values of Testosterone concentration (ng/dL) of New Zealand White rabbit bucks administered crude fractions from methanolic neem leaf extract

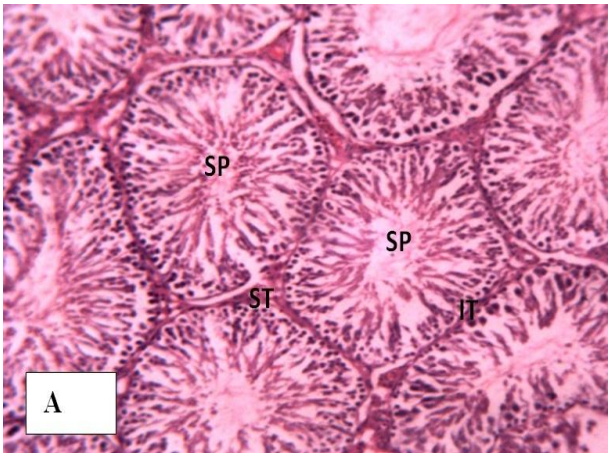

Plate I: photomicrographs of the testis of New Zealand white rabbit buck (Group A) showing normal histology of the testis of New Zealand white rabbit buck in the control group.
Note the intact seminiferous tubules (**ST)** with a thick layer of spermatogenic cells (**SP**) and interstitial cells (**IT**) (H&E x 100)

fraction, Hossain *et al* (2013b) reported that the highest amount of flavonoids was found in hexane fraction followed by chloroform, ethyl acetate and n-butanol fractions in increasing order of concentration. The semen pH of New Zealand white rabbit administered fractions from methanolic neem leaf did not differ from the control group. This is similar to the work of Lisanti *et al*. (2016), who reported similar findings in mice semen following administration of aqueous leaves and seed extract of *Azadirachta indica*.

Highly statistical significantly lower sperm motility was recorded for group C (chloroform fraction) at the end of the study when compared with the control group and other treated groups B, D and E. A progressive decrease in gross motility was observed from the second week of treatment with chloroform fraction as compared with the control
group. Majority of plant-derived spermicides was attributed to triterpens obtained from saponins of several structural types, and phenol compounds (Aladakatti *et al*., 2005). Although, the cascade of events leading to the continual decrease in semen

motility in group C (chloroform fraction) was not fully understood, the decrease in spermatozoal motility may be caused by the presence of saponins in the chloroform fraction of the methanol crude extract. Saponin has been reported to decrease spermatozoa motility and viability gradually to obsolute zero (Joshi *et al*., 2011). Nimbitin, azadirachatin and salanin are cell layers was observed in group C (Plate III), and mild loss of interstitial layers in groups D and E (Plate IV and V) was observed at the end of the experiment.

Discussion

The different biological activity of plant extract on animals depends on the secondary plant metabolite alkaloids of *Azadirachta indica* whose individual effects contribute to the general biological properties of the plant (Jafari *et al*., 2015). Kumbar *et al*. (2013) reported that nimbolide which is an isoprenoid of neem leaf, immobilise and kill 100% rat spermatozoa and suggested that isoprenoid of leaf is highly polar in nature and causes sperm death. Most of plant

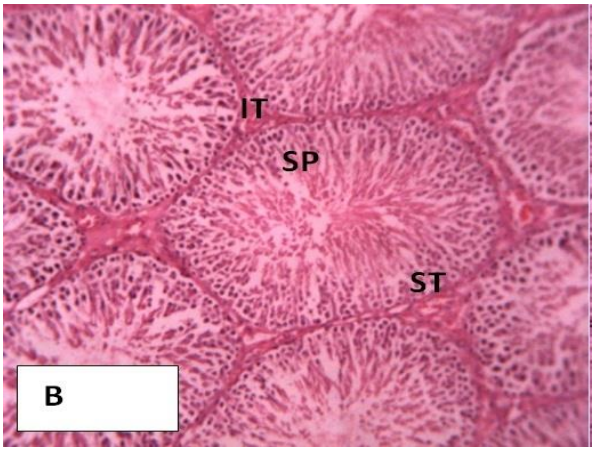

Plate II: Photomicrographs of the testis of New Zealand white rabbit buck administered hexane fraction of crude methanolic neem leaf extract (Group B) showing seminiferous tubules filled with spermatogenic cells (**SP**), leydig cells within the interstitial space (**IT**) and prominent seminiferous tubule (**ST**) (H&E x 100)

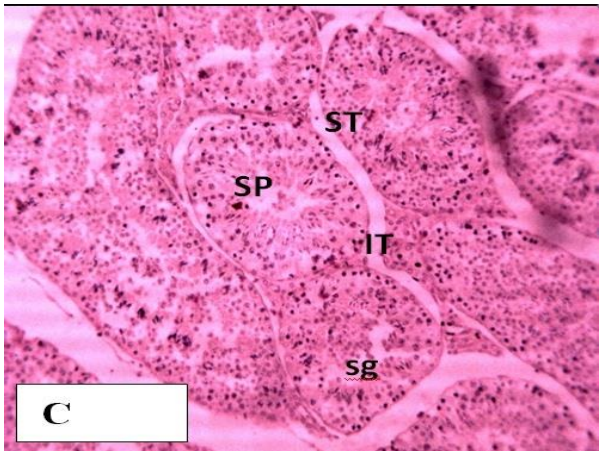

Plate III: Photomicrograph of the testis of rabbit buck administered chloroform fraction of crude methanolic neem leaf extract (Group C) showing exfoliation of germ cell layers in the interstitial cell space (**IT**), mild depletion (vacuolation) of spermatogenic cell layer (**sg**) and prominent seminiferous tubule (**ST**) (H&E x100)

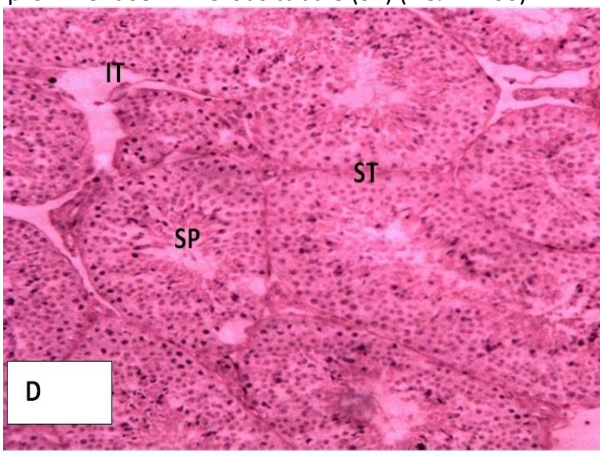

Plate IV: Photomicrograph of the testis of rabbit buck administered ethyl acetate fraction of crude methanolic neem leaf extract (Group D) showing seminiferous tubules (**ST**) filled with spermatogenic cells layers at different stages of spermatogenesis (**SP**) and mild loss of interstitial layer (**IT**) (H&E x100)

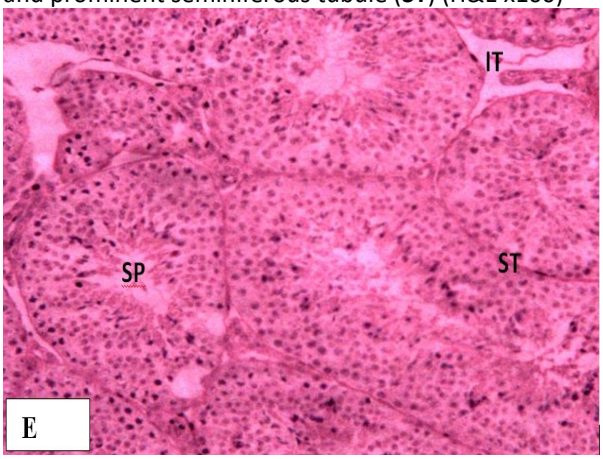

Plate V: Photomicrograph of the testis of New Zealand white rabbit buck administered butanol fraction of crude methanolic neem leaf extract (Group E) showing mild depletion of spermatogenic cell layer (**SP**) and the interstitial cells (**IT**) with prominent seminiferous tubules (**ST**) (H&E x100)

spermicidal compounds act on the sperm surface by disrupting the plasma membrane. Al-jadidi & Hossain (2015) reported that ethyl acetate, chloroform, and hexane fractions of the methanolic crude extract contain a significant amount of alkaloids. Azadirachtin, which is the major alkaloid found in neem leaf, has also been associated with decreasing sperm motility and concentration (Aladakatti *et al.,* 2001; Aladakatti & Ahamed, 2005).

This decrease in motility is probably cytotoxic and is similar to Awasthy (2001) report, who reported sperm motility to decrease linearly with various concentrations of neem leaf extract with motility falling to absolute zero. Azadirachtin-A has been reported to cause sperm-immobilising effect by either direct structural and functional modulation of the plasma membrane or by way of its synergism with blockage of some biochemical pathway like energy utilisation (Aladakatti *et al.* 2005). Therefore, the probable cause of the absolute decrease in motility from this study might be due to cellular damage caused by chloroform fraction changing the osmotic pressure, leading to swelling and eventually cell death. Also, an increased number of dead spermatozoa was observed especially at weeks 11, and 12 in Group C. This is similar to the findings of Ghosh *et al*. (2017), who related the death of the spermatozoa to the reduction in hypo-osmotic swelling of the sperm, indicating that chloroform fraction extract may probably cause injury to the sperm plasma membrane. Also, *In vitro* screening of most plant extracts for their spermicidal properties indicated that it involves either loss of cellular membrane integrity or suppression of motility as an endpoint (Abu *et al*., 2011). Al-Hashemi & Hossain (2016) reported that the highest antioxidant activity among neem leaf crude extracts was found majorly with butanol fraction and lowest with chloroform fraction. This can be ascribed to the presence of flavonoids (Alabi *et al.*, 2011) that is found in all the fractions with the exception of chloroform fraction.

A decrease in semen volume was observed in group C receiving chloroform fraction neem leaf crude extract, but the decrease was not statistically significant. The insignificant difference observed between the control group and the four treatment groups in semen volume agrees with the work of Alabi *et al*. (2011), Khanavi *et al*. (2007), and Lisanti *et al*. (2016), who reported no significant difference in semen volume after administration of methanolic and aqueous neem leaf extract in mice, rats and mice respectively. However, Mohan *et al*. (1997) reported a significant decrease in semen volume and sperm concentration following feeding broiler cock with water washed neem seed kernel meal.

A significant decrease was observed in sperm concentration between the control group and group C. The decrease in sperm concentration can also be correlated with the significant decrease in gross semen motility. The mechanism by which the chloroform fraction causes a decrease in sperm concentration may be similar to the mechanism by which saponin causes suppression of cell proliferation of tumour cells (Chen *et al*., 1998). This is similar to the report of Mohan *et al.* (1997), who reported significant reduction in sperm concentration of broiler cock after feeding neem seed kernel meal. Chen *et al*. (1998) also reported a reduction in sperm concentration and motility in mice. Santra & Manna (2009); Lisanti *et al*. (2016), also reported similar results in rat.

There was significant increase in sperm morphological abnormalities in group C compared with the control group. Increase in free head and bent tail sperm morphological abnormalities were observed. This is contrary to the report of Mohan *et al*. (1997), Ekaluo *et al*. (2011) and Lisanti *et al*. (2016), who reported no any significance difference in sperm head abnormalities in chicken, rats, and mice, respectively. These differences might be due to variation in specie and also part of the plant used. While the present study utilises the leaf component, the above studies reported the use of the seed kernel meal, aqueous leaf and seed extract in chicken, rats and mice respectively.

Androgen is essential for most of the stages of spermatogenesis, meiosis in particular. Sperm production cannot proceed optimally to completion, without continued androgen supply (Aladakatti *et al*. 2006). Therefore, any interference in testosterone production will lead to atrophy of the organs and impairment of spermatogenesis. Although, there is no any statistically significant different in concentration of testosterone in the various treatment groups and the control group, result from this study shows irregular increase and decrease in plasma concentration of testosterone values in the treatment groups from each neem leaf crude extract when compared with the control group. The findings from this study is contrary to that of Ekaluo *et al*. (2011), who reported a highly significant decrease in serum testosterone levels in rats treated with aqueous leaf extract of neem. Reason may probably be due to the differences in polarity of the extract used and therefore, will affect the effects of the various phytoconstituents of each extract. Serum

testosterone level decreased significantly when compared with the control group.

In conclusion, among the four neem leaf fractions used, chloroform neem leaf fraction shows higher level of adversity to the fertility parameters such as sperm motility, semen volume, concentration and morphological abnormalities.

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Conflict of interest

There is none to be declared

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