EFFECTS OF FRACTIONSFROM CRUDE METHANOLIC EXTRACT OF*AZADIRACHTA INDICA* (NEEM)LEAF ON SPERMIOGRAM, TESTOSTERONE PROFILE, TESTES HISTOLOGY AND HAEMATOLOGYOF NEW ZEALAND WHITE RABBIT BUCKS

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN THERIOGENOLOGY

**DEPARTMENT OF THERIOGENOLOGY AND PRODUCTION,
AHMADU BELLO UNIVERSITY,
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FEBUARY, 2021

DECLARATION

I declare that the work reported in this dissertation, titled **'Effects of fractions fromcrude methanol extract of*Azadirachta indica* (neem)leafon spermiogram, testosterone profile, testes histology and haematology of New Zealand white rabbit'** was carried out by me in the Department of Theriogenelogy and Production, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria. The information derived from literature has been duly acknowledged in the text and in the list of references provided. No part of this dissertation has been presented for another degree or diploma at any instituition.

Mohammed Saifullah UMAR

……. .....

(Name of Student) Signature Date

CERTIFICATION

This dissertation, entitled **'EFFECTS OF FRACTIONS FROMCRUDE METHANOLICEXTRACT OF*AZADIRACHTA INDICA* (NEEM) LEAF ONSPERMIOGRAM, TESTOSTERONE PROFILE, TESTES HISTOLOGY AND HAEMATOLOGY OF NEW ZEALAND WHITE RABBIT BUCKS',** by Mohammed Saifullah UMAR meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this project to my parents,late Alhaji Umar Sade Jibia and Hajia Hadiza Bashir Gado.

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Abstract

The effects of different fractionsfrom methanolic extract of *Azadirachta indica* (neems) leaves on spermatogenesis, testicular histology, testosterone concentration and haematological parameters in New Zealand white rabbits were evaluated in this study. Crude extract of the powdered leaves of neem (*Azadirachta Indica*) was extracted using methanol and further fractionated using hexane, chloroform, ethylacetate and butanol. Twenty five matured New Zealand white rabbit bucks wererandomly assigned to five groups (A, B, C, D, and E). Group A rabbits served as control and were administered only distilled water, while groups B, C, D and E, rabbits representing the hexane, chloroform, ethylacetate and butanol groups, respectively, were administered fractions from the respective solventextracts at similar dose rate of 300mg/kg. Semen samples were collected using artificial vagina weekly for fourteen weeks and were immediately evaluated for parameters such as volume, colour, motility, concentration, and percentage live ratio. Five millilitres of blood sample was collected from each rabbit buck through venipuncture of the ear vein three times during the experimental period, that was at the beginning, middle and end of the experiment for determination of haematological parameters. Serum sample was also harvested from the collected blood for determination of testosterone concentration. Two rabbit bucks from each group were humanely sacrificed at the end of the experiment for testicular histology. Data collected were analysed using graph pad prism software version 5.0 and P values, ≤ 0.05 were considered statically significant between treatment groups and the control. No significant difference ($P \geq 0.05$) was observed in mean sperm motility between the control group (90.00±2.04%) and treated groups B (84.33±5.36%), D (90.00±4.40%) and E (95.00±0.00%) at first week. However, significant ($P < 0.05$) decrease in sperm motility was recorded from the treatment group C that receive chloroform fraction

(00.68±1.64%) when compared with the control group (90.00±2.04%) at the end of the experiment.Although, no significance differences ($p>0.05$) were observed in semen volume between the treatment groups and the control group. However, numerical decrease in semen volume that is not significant ($P>0.05$) was observed in group C which was treated with chloroform fraction. Significant difference ($P<0.05$) in sperm concentration and sperm abnormalities were observed between the control group and the treatment groups. No significant ($P>0.05$) difference were observed in testosterone concentration between the various treatment group and the control group, however, there was gradual decrease in plasma concentration of testosterone values in the treatment groups when compared with the control group. Significant differences ($P<0.05$) in haemoglobin concentration, packed cell volume, red and white blood cell counts were observed between the treatment groups and the control. There was a significant decrease ($P<0.05$) in red blood cell count in the treatment groups; hexane fraction ($5.70\pm0.07\times10^{12}$/L), Chloroform extract ($5.28\pm0.02\times10^{12}$/L), ethylacetate fraction ($5.52\pm0.00\times10^{12}$/L) and butanol fraction ($4.81\pm0.00\times10^{12}$/L) compared with the control ($6.54\pm0.00\times10^{12}$/L), however, the values are within the normal haematological range values for the New Zealand white rabbit. Testicular degeneration, depletion and vacuolation of spermatogenic cell layers were observed in treatment group with chloroform fraction. However, other treated groups did not show difference from the control with intact and prominent seminiferous tubules and spermatogenic layers at different stages of division. Further studies need to be conducted to validate this findings and also to isolate specific antifertility agent(s) from chloroform fraction.

The study showed that chloroform fraction of crude methanolic *Azadirachta indica* (neem) leaf extract was detrimental to spermatogenesis, which significantly reduced gross semen motility

and testicular histology, while butanol, ethyl acetate, and hexane fractions had no significant effect on semen characteristics, histology and androgen profile of New Zealand white rabbit bucks.All the fractions from crude methanol neem leaf extract significantly reduce the PCV and RBC count but had no significant effect on testosterone concentration in the New Zealand white rabbit bucks.

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LIST OF ABBREVIATIONS

ROS----------Reactive Oxygen Specie

GnRH----------Gonadotrophin Releasing Hormone

LH----------Luteinising Hormone

FSH----------Follicle Stimulating Hormone

CL----------Corpus Luteum

Mg----------Milligram

Kg----------Kilogram

EDTA------Ethylene-Diamine-Tetra acetic Acid

LD_{50}---------- Median Lethal Dose

CNS---------- Central Nervous System

DNA---------- Deoxyribonucleic Acid

RNA----------Ribonucleic Acid

SOD---------- Superoxide Dismutase

GPx---------- Glutathione Peroxidase

PUFAs---------- Poly-unsaturated Fatty Acids

CAT---------- Catalase

ABU----------Ahmadu Bello University

NARICT----------National Institute of Chemical Research Technology

USA----------United States of Ameriaca

RBC----------Red Blood Cell

WBC----------White Blood Cell

PCV----------Packed Cell Volume

MCV----------Mean Corpuscular Volume

MCHC--------Mean Corpuscular Haemoglobin Concentration

ANOVA-------Analysis of Variance

SEM----------Standard Error of Mean

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Neem (*Azadirachta indica* Juss) is a fast growing evergreen popular tree found commonly in India, Africa and America (Pandey *et al.*, 2014). It is mainly evergreen but sometimes shed its leaves during the dry season (Orwa *et al.*, 2009; Puri, 1999). Neem has a deep taproot system and is a mycorrhizal-dependent species. The bark is grey, becomes fissured and flakes in old trees and secretes Sticky fetid sap exudates from old trees in humid climates (Orwa *et al.*, 2009; Puri, 1999). The branches are numerous and spreading. The leaves are alternate, petiolated, clustered at the end of the branches, unequally pinnate, glabrous and dark glossy green at maturity, 20-40 cm in length and bearing 10-20 leaflets (FAO, 2015). Neem was 'discovered' in the western world in 1959 when a German entomologist noticed that it was the only green standing tree after a swarm of locusts swept through the Sudan (Eureka and Kaushik, 2016). It is known with different names by different ethnicity and region in Nigeria: Dongoyaro (Hausa) Kiswahili: mwarubaini (muarobaini). English: neem, Indian lilac, French: azadira d'Inde, (Parotta, 2001). The tree is variously known as '*Divine Tree*' '*Heal All*', 'Nature's *Drugstore*' '*Village Pharmacy'* and '*Panacea for alldiseases*' in Indian Ayurvedic tradition. In East Africa it is known as *Muarubaini* (*Swahili*), which means the 'tree of the 40', as it is supposed to cure 40 different diseases. (Eureka and Kaushik, 2016).

Neem extract is composed of a complex mixture of molecules, including normal hydrocarbons, phenolic compounds, terpenoids, alkaloids, and glycosides (Sarawaneeyaruk*et al.*, 2015; Hossain

et al., 2013). The chemical constituents found in the leaves of *A. indica* are nimbin, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, nhexacosanol and amino acid, 7-sdesacetyl-7-benzoylazadiradione,7-sdesacetyl-7-benzoylgedunin, 17-hydroxy azadiradione and nimbiol (Kokate*et al.,* 2010, Hossain*et al.,* 2013).

The leaves and bark of neem tree are freely consumed and are primary ingredients in medicinal preparations for treatment of acute malaria. Some anti-malarial agents have been reported to have anti-fertility actions (Ekaluo *et al*., 2011). Neem leaf extract (NLE) have been reported to exhibit anti-inflammatory, anti-hyperglycemic, anti-ulcer, immunomodulatory, antiviral, anti-fungal, anti-bacterial, nematicidal, anti-malarial, insecticidal, anti-mutagenic and anti-oxidant properties (Biswas *et al.,* 2002, Sharma *et al.,* 2003, Siddiqui *et al.,* 2004, Udeinya *et al.,* 2004, Wandscheer *et al.,* 2004, Sithisaran *et al.,* 2005 and Subapriya and Nagini 2005). Anti-helminthic activity of neem leaf extract has also been reported in ruminants (Al-Rofaai *et al.,* 2012). One of the bioactive fractions of neem leaf (nimbolide) has anti-cancer property (Kumar *et al.,* 2013). The ethanolic neem leaf extract is reported to induce abnormal head morphology and reduces mean sperm count in murine (Khan and Awasthi, 2003). The aqueous neem leaf extract is said to inhibit motility and viability of human spermatozoa under in vitro culture conditions (Khillare and Shrivastav, 2003). Neem oil has been used as herbal vaginal contraceptive in human (Sharma *et al.,* 2003). Ogbuewu *et al*.(2008), reported lowest sperm motility in rabbit fed a neem leaf up to 15% of the diet composition with mild depressive effect on spermatogenesis, semen quality and seminiferous tubule diameter when compared with the other group on 5% and 10% of the diet.

Awasthy (2001) reported that administration of ethanolic crude extracts of Neem to adult male mice causes sperm DNA and chromosome strand breakage, spindle disturbances and deregulation of genes responsible for sperm morphology while a progressive linear decrease in the percentage of sperm motility was observed with motility falling to absolute at the end of study period. Al-Hashemi and Hossain*et al*. (2016) reported that different organic solvent crude extracts of neem leaf (butanol, ethyl acetate, chloroform and hexane) posses variable antioxidant properties with the highest antioxidant activity found in the butanol extract and the lowest in the hexane (Al Hashemi and Hossain*et al*., 2016). These differences may be linked with their differences in polarities. Garg *et al*. (1994) reported that strong antifertilityactivity of crude organic solvents extract of neem leaf is present in non-polar or weaklypolarconstituents, extractable with hexane.

1.2 Statement of research problem

The efficiency of animal production is dependent upon the optimum utilisation of feed for growth, development and reproduction**(**Sucharita *et al*., 2016). Competitive pressure of consumption of soybean and vegetablesby both human and rabbit (Ogbuewu *et al.,* 2010) has led to investigation of other available, cheaper and non conventional sources of nutrient to rabbit (Mahmud *et al*., 2015). Neem leaf based diet have been shown to increase rabbit carcass weight and quality (Wasanthakumar *et al.,* 1999a; Wasanthakumar *et al.,* 1999b, Gowda *et al.,* 2000 Ogbuewu, 2008 and Ogbuewu *et al.,* 2010). Ogbuewu *et al*. (2008) reported excellent carcass quality in rabbit fed up to 15% neem leaf diet composition.

However, using several crude extract, there has been several reports indicating neem leaf extact to affect fertility of rabbit buck fed neem leaf based diet. Adekeye *et al*. (2013), reported that

methanolic neem bark extract causes significant distortion in histology of testicular tubular epithelium with a reduction in interstitial cell of leyding double tail sperm morphology, reduced sperm motility and decrease in Serum testosterone levels. Methanolic neem leaves extract are also reported to cause decrease in sperm motility, concentration, and morphology, in rabbit (Lisanti*et al*., 2016).The ethanolic neem leaf extract has been reported to induce abnormal head morphology and reduces means sperm count in rats (Khan and Awasthi, 2003).Garg *et al*., (1994) reported that using different crude organic solvents on neem extraction yielded phyto constituents that are distributed according to the polarity of the solvent. It is possiblethat the different phyto constituents contained in different organic solvent extract could be investigated to access which among them will cause possible toxicity associated with the infertility in neem leaf (Garg *et al*., 1994).

1.3 Justification

Reproductive inefficiency is the most limiting constraint to efficient animal production in the tropics (Gbadamosi and Egbunike, 1999). Despite the advantages of neem leaf as cheap, 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. Early reports have shown that, most predominate active compounds that were in the neem leaves are distributed according to the polarity of the organic solvents (Hossain *et al*., 2013). Khan *et al*. (1987) also reported that neem leaf extract has biological effect in low polar solvent over the high polar solvent extract. Therefore, there is need to identify and characterise the active fraction responsible for antifertility and for the purpose of standardisation and development of a suitable non conventional based livestock feed with minimal or no effect on reproduction. The rational

approach to meet such requirement is to sequentially extract the leaf with different crude preparations of organic solvents based on their polarity and testing their biological activity at each step and then fractionate the most active fraction from the crude extract (Upadhyay *et al*., 1993).

Information on neem leaf extract phytoconstituents solubility and their effect on spermatogenesis, testosterone concentration, and histology of testes using different crude fractions is limited to the best of our knowledge.

This study was, therefore, designed to evaluate and compare the effect of different crude organic solvents (ethtyl acetate, butanol, chloroform and hexane) extracts of neem leaf on spermiogram, testosterone concentration, histology of testes and haematology of New Zealand white rabbit.

The study hopefully will help in bulding awareness and facilitating the use of neem leaf as inexpensive, simple and natural useful products. Promoting environment friendly technology approach in integrated livestock feed management.

1.4 Aim of the study

To study the effect of fractions from crude methanolneem leaf*Azadirachta indica* extract on spermiogram, testes histology, testosterone profile and haematological parameters of New Zealand white rabbits.

1.5 Objectives of the Study

Determine the effect of fractions from crude methanolic neem leaf extracts on:

I. Semen characteristics,

II. Histopathological changesin testes,

III. Haematological and

IV. Testosterone profile of New Zealand white rabbits.

1.6 Research Questions

I. Dofractionsfromcrude methanol neem leaf extracts haveeffects on semen characteristics, histology of testes and haematological parameters of New Zealand white rabbit bucks?

II. What is/are the effects of differentfractions from crudemethanol neem leaf extract on semen characteristics, histology of testes and haematological parameters of New Zealand white rabbit?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Antifertility Effects ofNeem Leaves

It is a usual practice in India among pupils and saints in shrines to take drinks made from fresh green leaves of neem because it was believed to suppress libido (desire for sex) (Bansal *et al.,* 2010). Neem has been reported to contain a vast number of biologically active compounds, which are chemically diverse and have enormous therapeutic potentials. (Kabeh and Jalingo, 2007). Its chemistry dates back to 1880-1890 when influenced by its folk-lore medicinal values after the isolation of active principles and Siddiqui (1942) was the first to report the isolation of three products namely nimbin, nimbidin and nimbinin from its oil. In 1959 the antifertility property of neem components in rats and human were reported (Sharma and Saksena, 1959). In this study addition of aqueous solution of sodium nimbidinate salt to semen of rat and human being resulted in 100% death of sperm cells in 30minutes period. Male rats when tested for fertility after feeding neem oil have been found to result in a significant loss of reproductive function (Khare *et al.,* 1984) with no inhibitions of spermatogenesis both in volume and duration demonstrating that probably it induces functional sterility without interfering with structural integrity of the testis or the process of spermatogenesis. Another report has given contrasting results in which administration of neem bark ethanol extract and seed oil resulted to azoospermia in rats (Purohit and Dexit, 1940). Aladdakatti *et al.* (2001) shows that dried leaf powder of *Azadirachta indica* (neem) has antiandrogenic effect and causes changes in the histology (intracellular spaces and vacuolization in sertoli cells and defect in mitochondrial sheath of late spermatid) and biochemical parameters of testis of adult albino rats. Upadhyay *et al.* (1993) observed a long-term contraceptive effect of single intra-vas deferens administration of neem oil in male rats and found to be an alternative approach to vasectomy with no

inflammatory/obstructive changes in epididymis and vas deferens. Purified neem seed extract called praneem cause termination of pregnancy by bleeding and a decrease in progesterone levels in rats, baboons and monkeys (Mukherjee *et al.,* 1990). Aqueous extract of old and tender leaves of *Azadirachta indica* (neem) are reported to immobilise and cause100% mortality of human spermatozoa with no morphological changes in sperm head, mid- piece and tail (Khillare and Shrivastav, 2003).Aqueous and methanol extract of dried neem seed extract did not demonstrate antiimplantation effect in female rats (Dhawan *et al.,* 1980). However, methanolic extract of the neem seed extract was reported to cause abortion in rats (Tewari *et al.,* 1986).At lower doses, (100mg/kg) ethanol extract of dried neem leaf extract did not cause antispermatogenic activity (Lai *et al*., 1986) however, there was loss of libido in 100% of the males (Choudhary *et al*., 1990), Shaikh*et al*. (1993) also reported antispermatogenic activity of the dried leaf extract at 20mg/kg in male rats.Oral administration of neem seed oil has also been reported to inhibit fertilization and development of the ova (Juneja and Williams, 1993) while intravaginal application application was spermicidal in baboon, monkey and rabbit (Talwar *et al*., 1995).

Similarly intravaginal application of neem seed oil was also reported to prevent pregnancy in rats, monkeys and women (Sinha *et al*., 1984). Neem oil did not inhibit ovulation in rabbit (Khare *et al*., 1984) but it does prevent pregnancy (Prakash and Mathur, 1986).

Both the precoital and postcoital antifertility effects of vaginal application of neem oil have been subsequently confirmed in rhesus monkeys (Bardhan*et al.,* 1991). It has been reported that the application of neem oil intravaginally before sexual intercourse prevented pregnancy without adverse effect on vagina, cervix and uterus (Sinha *et al.,* 1984). The active components of neem oil have been found to be absorbed through the vaginal mucosa into circulation and exerted

antifertility effects in addition to direct spermicidal effect. According to another report (Khare *et al.,* 1984) neem oil did not inhibit ovulation activity in rabbits. Prakash and Mathur (1986) reported that neem oil prevent pregnancy and does not produce any side effects.

2.2 Taxonomical Classification of *Azadirachta indica*

Order- Rutales

Suborder- Rutinae

Family- Meliaceae

Subfamily- Melioideae

Tribe- Melieae

Genus- Azadirachta

Species- Indica (Pankaj *et al.,* 2011).

Azadirachta indica (*A.indica*) commonly known as Neem, belongs to the family of Meliaceae and has been used in ayurvedic treatment for more than 4000 years ago (Pankaj *et al.,* 2011) and its usage was recorded around 4500 years ago (Khatkar *et al.,* 2013). Besides that the plant is regarded as a village dispensary in India (Asif, 2013).

Neem is native to east India and Burma and grows much in South East Asia (SEA) and West Africa and it is cultivated in Pakistan, Peninsular Malaysia, Singapore, Philippines, Australia (Hashmat *et al.,* 2012). Plantation of Neem in small scale in Europe and United States of America has shown success (Kumar *et al.,* 2013).

A neem tree is about 12-18m in height with a circumference up to 1.8-2.4m (Bempah *et al.,* 2011). It is a flowering plant which will produce flower by 3-5 years of age (Bempah *et al.*,2011) The flowers are 4-7mm in length and 6-10mm in width (Sultana *et al.,* 2011), has a jasmine like

odour and white in colour (Bempah *et al.*, 2011). The leaves are dark green in colour up to 30cm in length (Bempah *et al.,* 2011) and has 3 lobed stigmata and seeded drupes (Jafari *et al.,* 2013). The fruit of Neem is about 2cm long with white kernels and when mature its able to produce 50kg of fruit yearly (Bempah *et al.,* 2011). The branch of Neem is dense with up to 10cm in length and has a dark brown bark (Sultana *et al.,* 2011). Furthermore, Neem tree is able to adapt in very dry condition (Bempah *et al.,* 2011, Sultana *et al.,* 2011) with temperature of up to 120°C and minimal rain fall of 18 inches per year (Kumar *et al.,* 2013). The plant has been reported to grow well in calcareous soil with the pH up to 8.5 (Debashri and Tamal, 2012).

2.3 Active Principle and Constituents of Neem Leaf

Different part of neem tree, such as thestems, branches, leaves, flowers and fruits, produce different phytochemicals (Singh, 2008). The chemical composition of *A. indica* is very complex as it contains remarkably diverse array of phytochemicals, such as terpenoids, flavonoids, coumarins, carbohydrates, proteins, fatty acids and their esters and hydrocarbons (Singh, 2008). However, harvesting, processing and storage conditions can influence the presence or absence of some phytochemicals in accordance with their differential growth. According to Siddiqui (1942), the isolation of chemical constituents, namely nimbin, nimbinin and nimbidin, from neem oil, with the emergence of new isolation methods and improved analytical techniques for structure elucidation led to identification of many important phytochemicals previously unknown (Govindachari, 1992). The chemical compound of neem is very diverse and there are reports of more than 300 phytochemicals isolated and characterised (Akhila and Rani 1999). The crude extracts prepared with water, chloroform, ethanol, butanol, ethyl acetate and hexane have shown

the presence of numerous phytochemicals. These phytochemicals are grouped based on the presence or absence of isoprene units as isoprenoids and nonisoprenoids (Fig. 1)

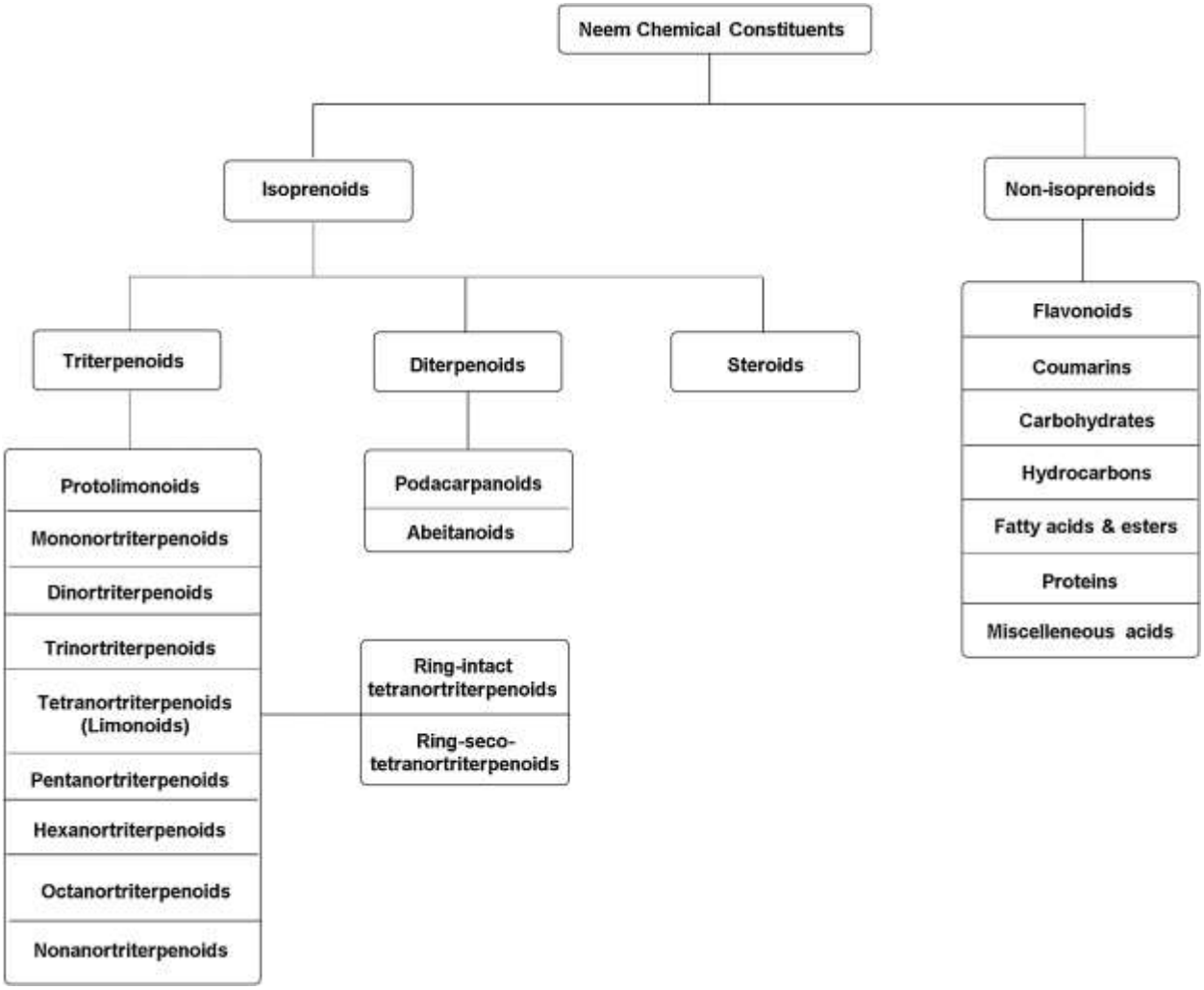


Fig. 1: chemical constituents of neem (Singh, 2008).

Neem isoprenoids are categorized into three classes, namely diterpenoids, triterpenoids and steroids. However, triterpenoids represent the major class since more than 180 triterpenoids have been isolated from the different parts of neem tree (Akhila and Rani, 1999). The neem triterpenoids are subdivided into classes based on the removal of carbon atom either from the side chain or from the ring skeletal structure of the parent compound apotirucallol (Singh, 2008). The derived compounds are referred to as "nor compounds" based on IUPAC nomenclature recommendations to indicate the loss of carbon atom from the parent structure (Giles, 1999).

Neem triterpenoids are categorized into protolimonoids, mononortriterpenoids, dinortriterpenoids, trinortriterpenoids, tetranortriterpenoids, pentanortriterpenoids, hexanortriterpenoids, octanortriterpenoids and nonanortriterpenoids. Protolimonoids have C-8 side chain with all the 30 carbons similar to parent triterpenoid structure apotirucallol.

2.3.1 Protolimonoids

Neem protolimonoids are considered as precursors to limonoids. The mononor, dinor, trinor, tetranor, pentanor, hexanor, octanor and nonanor triterpenoids are represented by the loss of 1, 2, 3, 4, 5, 6, 8 and 9 carbons, respectively, from the parent triterpenoid apotirucallol structure (Siddiqui*et al.*, 1988).

2.3.2 Tetranortriterpenoids

Also known as limonoids are the most important and well-studied class of triterpenoids and about one-third of phytochemicals isolated from neem belong to this class (Akhila and Rani, 1999). Limonoids are oxygenated triterpenes bearing a furan ring, which is formed by the loss of 4 carbon atoms from the side chain of the protolimonoid (Tan and Luo, 2011). Limonoids are

categorized into two classes based on their skeletal ring structure, namely ring intact limonoids and ring seco limonoids. As the name suggests in ring intact limonoids, rings A, B, C and D are intact, whereas in ring seco limonoids, there is a cleavage of one or more rings of the steroidal skeleton (Tan and Luo 2011, Giles, 1999). Azadirone, azadiradione, isonimolide, azadirachtin, salannin, nimbolide, gedunin and 7-deacetyl-7 benzoylepoxyazadiradione are few representative limonoids of both the classes. Azadirachtin, nimolide and gedunin have been extensively studied for their anti-cancer properties (Akhila and Rani, 1999).

2.3.3 Azadirachtin

Is a ring-C seco limonoid with 16 stereogenic centers, is the most studied limonoid and due to its structural complexity, it took 18 years to solve the structure. Moreover, due to its unique structure and wide range of biological properties, it was referred to as "scientific gold mine" (Morgan, 2009). Due to their wide range of bioactivities, such as anti-inflammatory, anti-cancer and immune modulatory activity, there is continued interest towards the identification and isolation of limonoids. Gualtieri *et al.* (2014) have recently reported the isolation of 8 limonoids from neem leaves.

2.3.4 Diterpenoids

Represent another important class of neem isoprenoids that are well characterised. These compounds are derived from 4-isoprene units and their biosynthetic precursor is 20-carbon diterpene alcohol geranylgeraniol. However, due to chemical modifications occurring at later stages of biosynthetic pathway, numerous diterpenoids with wide variety of chemical structures are generated. There are more than 20 diterpenoids isolated from different parts of neem tree.

Neem diterpenoids mainly belong to two classes, namely podacarpanoids (margolone) and abeitanoids (sugiol) (Ara *et al.,* 1989). Apart from triterpenoids and diterpenoids, several important steroids which are previously known, such as cholesterol, b-sitosterol and stigmasterol, have also been isolated from neem tree (Singh, 2008).

In addition to isoprenoids, neem tree is a rich source for number of non-isoprenoids. Various classes of non-isoprenoids include flavonoids (quercetin, catechin and nimbaflavanone), coumarins (scopoletin), isocoumarins (margocetin), acids and their derivatives (nimbochalcin, nimbocetin, gallic acid and tiglic acid), styryl derivatives (a-hexyl cinnamaldehyde and b-asarone) hydrocarbons (octadecane and nonadecane), fatty acids and their derivatives (arachidic acid, stearic acid and oleic acid), carbohydrates and proteins (D-glucose, L-arabinose and D-glucosamine), thiols (dipropyl sulfide) (Puri, 1999; Singh, 2008).

2.4 Biological Activities of Neem Plant

2.4.1 Antibacterial Activity

Thakurta *et al.* (2007) reported that methanolic extract of *A.Indica* (neem) leaves has significant antibacterial, antisecretory and antihemarrhagic activity against vibrio cholera. The different neem leaf fractions such as hexane, chloroform and methanol extracts of *Azadirachta indica* showed antibacterial activity against *Escherichia coli,Klebsiella pneumoniae, Proteus vulgaris, Micrococcus luteus, Bacillus subtilis Enterococcus faecalis* and *Streptococcus faecalis* (Thakurta *et al.,* 2007) It was reported that methanol extract was the most effective, followed by chloroform and hexane extract showed low antibacterial activity (Koona and Budida, 2011). Neem Oil from leaves, seeds and bark is reported to have a wide spectrum of antibacterial

activity against gram positive and gram negative microorganisms which include *M.tuberculosis* and streptomycin resistant strains (Chopra *et al.,* 1956). The phytochemical constituents like alkaloids, saponings, steroids, tannis, crude glycosides and flavonoids of neem plants were tested for antibacterial activity against pathogenic strains of *E.coli, Corynebacterium bovis* and *Staphylococcus aureus*. (Faiza *et al.,*2009). The findings of Faiza *et al*. (2009) was also supported by Hymete *et al.*(2005) who linked flavonoids compounds among other phytochemicals of neem leaf to have antimicrobial activity. Hafiza *et al.*(2002) reported that crude saponins also prevent the growth of the microbes. WineeSurabhilal *et al.* (2013) compared metabolic extract and acetonic extracts of leaves of *Azadirachta indica* against two different bacterial strains that is,*Eshcherichia coli* and *B. subtilus* and it was reported that methanolic plant extracts showed maximum antibacterial activity as compared to acetonic extracts. Mahmood *et al.,* (2011) reported antibacterial effects of crude extracts of neem seed against pathogens involved in the infection of eyes and ear. Irshad (2011) reported that neem extracts of acetone showed maximum antibacterial activity as compared to pure, Ethanol, acetone and methanol extracts of neem. Neem seed oil gives bactericidal activity against 14 pathogenic bacterial strains (Baswa *et al.,* 2001). The crude aqueous extracts of *A. Indica* (Neem) shows better antimicrobial activity against some pathogenic bacterial strains (Srinivasan*et al.,* 2001). Siswomihardjo *et al.* (2007) reported that ethanolic extracts of neem leaves and bark poses antibacterial activity against *streptococcus mutans* with neem bark extracts having higher antibacterial properties than the leaves extracts.

2.4.2 Antifungal Activity

Venugopal and Venugopal (1999) reported that the aqueous and ethanolic extracts of *Azadirachta indica* leaves have antidermatophytic activity against dermatophytes with ethanolic extract showing more activity as compared to aqueous extract. Antifungal characteristics of methanolic and acetone extracts of *Azadirachta indica* against two different fungal strains i.e. *Aspergillus niger* and *Aspergillus fumigatus* was reported by WineeSurabhila*et al.* (2013). The seed and leaf extracts of *Azadirachtaindica* (Neem) were also shown to have antifungal activity against dermatophytes *Trichophyton* and *Epidermatophyton floccosum* (Ranganathan *et al.,* 1996). It has been reported that aqueous ethanolic and ethyl acetone extracts of *Azadirachta indica* neem leaves has broader spectrum antifungal activity against the growth of few human pathogens such as *Aspergillus flavus, Candida albicansAspergillus terreus, Aspergillus fumigates, Aspergillus niger,* and *Microsporum, gypseum* (Mahmood *et al.,* 2011). Gedunin, a compound isolated from neem seed oil, has also been reported to have antifungal activity (Rao *et al.,* 1977). The compounds of Sulphur such as cyclic tetrasulphide and trisulphide isolated from the stem distillate of fresh, matured neem leaves has antifungal activity against *Trichophyton mentagrophytes* (Pant *et al.*, 1986).

2.4.3 Antimalarial Activity

The addition of 5% neem oil (*Azadirachta indica*) has been reported to control breeding of anopheles stephensi and aedes aegypti in water overhead water tanks (Dua *et al.,* 1995). Nimbolide isolated from neem extracts has been reported to have antimalarial activity by preventing the growth of plasmodium falciparum (Rojanapo *et al.,* 1985). Gedunin isolated from neem seed oil has been reported to show antimalarial activities (Khalid *et al.,* 1989). Both

aqueous and alcohol extracts of bark and leaves of neem are effective antimalarial agents, particularly on chloroquine resistant strains (Badam *et al.,* 1987; Kabeh and Jalingo, 2007).

2.4.4 Antitumour and Antiviral Activity

It has been reported that limonoids and polysaccharides found in neem seed oil, bark and leaves reduce tumours and revealed effectiveness against lymphocytic leukemia (Biswas *et al.,* 2002). Mitotic inhibition activity by the leaf extract has pronounced antiviral effect on small pox, fowl pox, polio and herpes simplex virus (Hassan *et al.,* 2010). Aqueous extracts of neem leaf and also some fractions neem oil (Nim-76) showe antiviral activity against HIV and Polio Viruses (Hassan *et al.,* 2010).

2.4.5 Anti-inflammatory, Antipyretic and Analgesic Activities

The chloroform extract of stem bark has been reported to be effective against carrageenin – induced paw aedema in rat and mouse ear Inflammation (Biswas *et al.,* 2002). Inflammatory stomatitis in children is treated by the bark extract while Antipyretic activity has been reported in neem oil (Dua *et al.,* 1995). A methanol extract of the leaves showed antipyretic effect when it is administrated into male rabbits (Biswas *et al.,* 2002).

2.4.6 Antiulcer Activity

Nimbidin, a coumpound from neem oil, has been reported to prevent acetylsalicylic acid, indomethacin, serotonin-induced gastric lesions as well as cysteamine induced duodenal ulcers (Pillai *et al.,* 1978, Pillai and Santhakumari, 1984). Leaf extract of *Azadirachta indica* (neem) has been reported to cause inhibition of mucus depletion and cell defragmentation (Dharmani and Palit, 2006).

2.5 Plants with anti-fertility properties in male

Plants that have antifertility activity in male may act through affecting sperm motility, concentration and viability (Mishra *et al.,* 2012) or through either one or combination of the following:

1. Antispermatogenic agents which suppress sperm production.

2. Prevention of sperm maturation.

3. Prevention of sperm transport through vas deferens.

4. Prevention of sperm deposition. (Sharma *et al.,* 2001b).

2.5.1 Spermicidal agents

Spermicidal agents are defined as drugs that have the ability to immobilize or kill the sperm upon contact. An ideal spermicide should immediately and irreversibly produce immobilization of the sperm, non- irritating to the vaginal and penile mucosa, not have adverse effects on the developing fetus, free from long term topical and systemic toxicity and should not be systemically absorbed. Therefore, one of the oldest approaches for achieving spermicidal action has been to modify vaginal pH(Mishra *et al.,* 2012). The acidic pH of lemon juice may immobilise sperm by denaturing dyein ATPase. Saponins are natural surfactants widely occurring in many plants (*Phytolacca dodecadra, Calendula officinalis* and *Acacia caesia*) are reported to have spermicidal action. A common lipid bilayer, which contains external, internal and transmembrane proteins, is fundamental feature of the plasma membrane of the sperm. Saponin molecules interact with this lipid bilayer, affect the glycoproteins of the cellular

membrane and modify the ionic transport across the membrane, leading to surface changes (Sharma *et al.,* 2001a). Saponins isolated from *Sapindus mukorosii* (reetha) are derivatives of hederagenin. At higher concentrations of saponins (1-50 mg/mL) spermatozoa represented marked disruption, vacuolation, vesiculation, erosion of the membrane covering the head region and Coiling of the tail (Dhar *et al.,* 1989).These saponins was formulated as contraceptive cream named 'CONSAP'.

Table 2.1: Plants with Spermicidal activity

Botanical name	Family	Plant part extract	References
Acacia auriculiformis	Fabaceae	Acaciaside A and B	Pakrashi *et al.* (1991)
Achyranthes Aspera	Amaranthaceae	50% ethanolic extract	Chauhan and Agarwal (2008)
Aegele marmelos	Rutaceae	Leaves extract	Remya *et al.* (2009)
Albizzia lebbek	Fabaceae	Lebbekanin-E	Varshney *et al.*(1979)
Albizzia procera	Fabaceae	Oleanolic acid saponin and proceric acid saponin mixture	Banerji *et al.*(1979)
Allium sativum	Lilliceae	Bulb Crude aqueous extract	Chakrabarti *et al.*(2003)
Nagallis arvensis	Primulaceae	Whole plant	Kamboj and Dhawan (1982)
Azadirachta indica	Meliaceae	Leaves Alcoholic extract	Kabeh and Jalingo (2007)
Cestrum parqui	Solanaceae	leaf extract	Pankajakshy and Madambath(2009)
Chenopodium album	Chenopodiaceae	seed extract	
Chrysophyllum albidum	Sapotaceae	Root and bark ethanolic extract	Yakubu *et al.*(2007)
Colebrookia oppositifolia	Lamaceae	Leaf extract	Gupta *et al.*(2001)
Juniperus phoenicea	Cupressaceae	Fruit extract	Setty *et al.*(1977)
Molluga pentaphylla	Molluginacea	Fruit extract	Rajasekaran *et al.*(1993)
Quassia amara	Simaraubaceae	Stem extract	Raji and Bolarinwa, (1997)
Sapindus mukorosii	Sapindaceae	Saponins	Dhar *et al.*(1989)

Sarcostema acidum	Asclepiadaceae	Stem extract	Sharma *et al.*(2002)
Terminalia chebula	Combretaceae	Fruit extract	Srivastava *et al.*(2010)
Tinospora cordifolia	Menispermaceae	Stem extract	Gupta and Sharma (2010)
Trigonella foenumgraecum	Fabaceaedried	seed extract	Shomaf and Quadan (2007)
Zizyphus Mauritiana	Rhamnaceae	Aqeous bark extract	Dubey *et al.*(2011)

2.5.2 Antispermatogenic activity

Spermatogenesis is a complex process which comprises of Spermatocytogenesis, Spermatidogenesis and Spermiogenesis (Mandal and Das, 2010). In spermatocytogenesis, a diploid spermatogonium, divides mitotically, producing two diploid intermediate cells called primary spermatocytes. Each primary spermatocyte undergoes meiosis I to produce two haploid secondary spermatocytes. Spermatidogenesis is the creation of spermatids from secondary spermatocytes. Spermiogenesis, includes the differentiation of spermatids into mature sperm (Mandal and Das, 2010). Spermiogenesis incorporate polarization of the spermatid, formation of acrosomal cap and flagellum, cytoplasmic remodeling elongation of the nucleus, develop a thickened mid- piece and begin to grow a tail (Ifeanyi *et al.*, 2009). Antispermatogenic activity is indicted by decrease in number of spermatocytes, spermatids and spermatozoa while Increase in cholesterol level and Sudanophilic lipid accumulation indicates inhibition in the steroidogenesis (Ifeanyi *et al.*, 2009). The extract of *Cannabissativa* was reported to induce increased lipid peroxidation in the testis, along with concomitant decrease in the levels of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Sailani and Moeini, 2008). Damage to the basement membrane, shrinkage of the seminiferous tubules, scanty cytoplasm and shrunken nuclei in the germinal epithelium, and complete arrest of spermatogenesis was also reported (Mandal and Das, 2010).

Joshi *et al.* (1996) and Choudhary *et al.* (1990) reported that the aqueous leaf extract of *Azadirachta indica* damaged the seminiferous tubules, germinal epithelium, marginal condensation of chromatin in round spermatids, degeneration of germ cells and the derangement of germ cell. Coutinho (2002) also reported that gossypol from Gossypium species causes spermatogenesis inhibition in rabbits.

It has also been reported that extract of *Carica papaya* inhibit sperm concentration, membrane damage in the acrosome, bent mid piece, coiled tail, detached head and arrest of spermatogenesis (Lohiya *et al.*,1999). Piperine, an alkaloid extracted from the fruits and roots of *Piper longum*, has been shown to cause damage to the germ cells, increase in lipid peroxidation in testis and epididymis, inhibition in antioxidant enzymes, activation of caspase 3 and Fas apoptotic protein (D'Cruz *et al.*,2008). The extract of *Vinca rosea* has been reported to cause changes in the seminiferous tubule by a decline in the percentage of primary spermatocytes (Bustos-Obregon and Lopez, 1973). *Ocimum sanctum* has also been reported to cause to antifertility by targeting spermatogenesis (Kasinathan *et al.,* 1972). *Curcuma longa extract* has been shown to cause reduction in the diameter of seminiferous tubules, and loosening of the germinal epithelium, in male Wistar rats (Mishra and Singh, 2009b).

Table 2. 2: Antispermatogenic activity

Botanical name	Family	Plant extract	References
Aegle marmelos	Rutaceae	Methanolic Leaf-extract	Chauhan and Agarwal (2008)
Albizzia lebbeck	Fabaceae	Methanolic Pods extract	Gupta *et al.*(2006)
Allium sativum	Amaryllidaceae	Bulb-Crude Aq extract	Hammami *et al.*(2008)
Azadirachta indica	Meliaceae	Leaves extract	Ifeanyi *et al.*(2009)
Bacopa monnieri	Scrophulariaceae	Leaves extract	Singh and Singh, (2009)
Barleria prionitis	Acanthaceae	Methanolic Root-extract	Verma *et al.*(2005)
Cannabis sativa	Cannabinaceae	Alcoholic Root- extract	Sailani and Moeini(2008)
Citrullus colocynthis	Cucurbitaceae	Ethanolic root extract	Mali*et al.*(2001)
Citrullus colocynthis	Cucurbitaceae	Fruit-50% ethanolic extrct	Chaturvedi*et al.*(2003)
Colebrookia oppositifolia	Lamiaceae	Leaves-Ethanolic extract	Onyeka*et al.*(2012)
Crotalaria juncea	Fabaceae	Seeds-Petroleum, ether,benzene and ethanol extracts	Vijaykumar*et al.*(2004)
Cuminum cyminum	Apiaceae	Seed-Ethanolic extract	Sharma*et al.*(2001a)
Curcuma longa	Zingiberaceae	50% ethanolic extract	Purohit(1991)
Hibiscus rosasinensis	Malvaceae	Bark-Methanolic extract	Reddy*et al.*(1997)
Lepidium meyenii	Brassicaceae	Root-Ethanolic Extract	Gonzales*et al.*(2001)
Leptadenia hastate	Asclepiadaceae	Leaves and stem-Aqueous extract	Bayala*et al.*(2011)
Momordica charantia	Cucurbitaceae	Seeds-Petroleum ether, benzene and alcohol extracts	Naseem *et al*.(1998)
Mondia whitei	Apocynaceae	Root bark-Aqueous extract	Watcho*et al.*,(2005)
Morinda lucida	Rubiaceae	Leaf-Leaf extract	Yinusa*et al.*(2005)
Mucuna Urens	Cannabaceae	Seed-Ethanol extract	Etta*et al.*(2009)
Ocimum sanctum	Lamiaceae	Leaf-Aqueous extract	Leigh and Fayemi(2008)
Parkinsonia aculata	Fabaceae	Stem bark-Ethanolic crude extract	Gupta*et al.*(2007)
Piper nigrum	Piperaceae	Fruit-Dry powder-Mice	Mishra and Singh(2009)
Rosmarinus officinalis	Lamiaceae	Fruit-Methanolic extract	Yakubu*et al.*(2008)
Ruta graveolens	Rutaceae	Leaf-Alcoholic extract-Rat	Khouri and El-Akawi (2005)
Sarcostemma	Asclepiadaceae	Stem-70% methanolic	Verma*et al.*(2002)

acidum		extract	
Semecarpus anacardium	Anacardiaceae	Fruit-Ethanolic extract	Sharma *et al*.(2003)
Tacoma stans	Bignoniaceae	Leaves-Ethanolic extract	Mathur*et al*.(2010)
Terminalia bellirica	Combretaceae	Fruit-Alcoholic extract	Rao(1989)
Thevetia peruviana	Apocynaceae	Stem bark-Methanol extract	Gupta *et al*.(2011)

Table 2. 3: Plants that affect Sertoli cells

Botanical name	Family	Plant part extract	References
Aegle marmelos	Rutaceae	Methanol leaves	Chauhan and Agarwal(2008)
Albizzia lebbeck	Mimosaceae	Bark methanolic extract	Gupta *et al.*(2006)
Azadirachta indica	Meliaceae	Leaf powder	Aladakatti and Ahamed(2005)
Barleria prionitis	Ccanthaceae	Root extract	Gupta*et al.*(2000)
Dendrophthoe falcate	Loranthaceae	Methanolic extracts	Gupta *et al.*(2007)
Thespesia populnea	Malvaceae	Leaf extract	Krishnamoorthy and Vaithinathan (2003)
Tinospora cordifolia	Menispermaceae	Methanolic extracts	Gupta and Sharma(2008)
Tripterygium wilfordii	Celastraceae	Leaf extract	Lan *et al.*(1992)

2.5.4 Plants that affect Leydig cells

Leydig cells are polyhedral in shape; display a large prominent nucleus, an eosinophilic cytoplasm and numerous lipid-filled vesicles. The neurones of the hypothalamus synthesize and secrete gonadotropin-releasing hormone, which induces the production and release of LH and FSH from the pituitary gland. LH causes the synthesis of testosterone in the Leydig cells of the testis (Dufau *et al.*, 1984). Numerous plant products are known to target Leydig cells and hinder their functions as methanolic extract of *Sarcostemma acidum* decrease in the number of mature Leydig cells and an increase in the degeneration of Leydig cell population (Venma *et al.*, 2002), 50 percent ethanolic extracts of the roots of *Martynia annua* caused Leydig cell atrophy and a significant reduction in the serum concentration of LH and testosterone (Mali *et al.*, 2002), methanolic extract of *Tinospora cordifolia* reduce stem cell nuclear area and mature Leydig cell numbers, (Gupta and Sharma, 2003), leaves extracts of *Azadirachta indica* exhibited a decrease in the number of Leydig cells and their nuclear diameter, indicating androgen deficiency and Atrophy of the Leydig cells (Aladakatti and Ahamed, 2005), seed extracts of *Carica papaya* caused pronounced hypertrophy of pituitary gonadotrophs and degeneration of Leydig cells (Udoh *et al.*, 2005), Palmitine hydrochloride (isolated from the roots of *Berberis chitria*) reduce mature and immature Leydig cells (Gupta and Dixit, 1989), Ethanolic extracts of *Colebrookea oppositifolia* decrease in the nuclear and cytoplasmic surface area of Leydig cells (Gupta *et al.*, 2001) and the flower extract of *Malvaviscus conzattii* caused (Chakraborty and Pakrashi, 1991).

Table 2 .4 Plants exhibit Effects on Leydig cells

Botanical name	Family	Plant part extract	Reference(s)
Albizia. lebbeck L	Fabaceae	methanolic bark extract	Gupta*et al.*(2006)
Azadirachta indica	Meliaceae	leaf extracts	Aladakatti and Ahamed(2005a)
Berberis chitria	Berberidaceae	roots extract	Gupta and Dixit(1989)
Calotropis procera	Asclepiadaceae	root extract	Gupta *et al.*(1990)
Colebrookea oppositifolia	Lamiaceae	Ethanolic extract	Gupta *et al.*(2001)
Garcinia cambogia	Cluciaceae	Ethanolic Seeds extract	Oluyemi *et al.*(2007)
Malvaviscus conzattii	Malvaceae	flower extract	Chakraborty and Pakrashi (1991)
Martynia annua	Martyniaceae	Ethanolic root extract	Mali*et al.*(2002)
Ocimum sanctum	Lamiaceae	Leaf powder	Singh(1997)

2.5.6 Effect on motility

The passage of the sperm through three segments of the epididymis-caput, corpus and cauda is very essential for the final maturation of the sperm (Bedford, 1979). The synthesis and secretion of various proteins by the epididymis, as well as the attainment of various morphological, biochemical and motility properties during the passage through the epididymis are fundamental for the full fertilizing capabilities of spermatozoa. Methanol seed extracts of *Abrus precatorious* has been reported to decreased cauda epididymal sperm motility of albino rats (Ratnasooriya *et al.,* 1991)

The methanol seed extract of *Abrus precatorious* reduce motility of spermatozoa due to the decline in cAMP and enhanced production of reactive oxygen species (Ratnasooriya *et al.,*1991). The ethanolic extracts of *Azadirachta indica* has been reported to interfered with sperm DNA and caused chromosome strand and spindle breakage, and deregulation of genes responsible for sperm morphology and sperm motility, with motility falling to absolute zero(Awasthy, 2001). *Piperine* has been reported to cause a reduction in rat sperm motility, viability and concentration (D'Cruz and Mathur, 2005). Allitridum, an active principle from garlic, has been reported to inhibit sperm motility and complete immobilization of rat, hamster and human spermatozoa (Qian *et al.,* 1986). Leaves extract of *Ocimum sanctum* has been reported to decrease sperm count, and motility. The effects were found to be reversible upon withdrawal of treatment for 2 weeks (Ahmed *et al.,* 2002).

Table 2. 5: Plants effect on sperm motility

Botanical name	Family	Plant extract	References
Abrus precatorius	Fabaceae	Seed Dry extract	Ratnasooriya *et al.*(1991)
Aegle marmelos	Rutaceae	Leaf-Methanolic extract	Shyam *et al.*(2012)
Aegle marmelos	Rutaceae	Leaf and ethanol extract	Chauhan andAgarwal (2008)
Bursera fagaroides	Burseraceae	Cortex/ethanoic extract	Huacuja *et al.*(1990)
Carica papaya	Caricaceae	Seeds Chloroform extract	Lohiya*et al.*(2008)
Carica papaya	Caricaceae	Seeds Chloroform extract	Pathak *et al.*(2000)
Cyclamen persicum	Primulaceae	Saponins compounds	Primorac *et al.*(1985)
Echinops echinatus	Asteraceae	Root/50% alcoholic extract	Chaturvedi *et al*(1995)
Gossypum herbaceum	Malvaceae	Gossypol compound	Chang *et al.*(1980)
Hedera nepalensis	Araliaceae	Nepalin compound	Pant *et al.*(1988b)
Lagenaria	Cucurbitaceae	Whole fruit and ethanol extract	Saba *et al.*(2009)
Pentapanax leschenaultii	Araliaceae	Fruit/ethanolic extract	Pant *et al.*,(1988b)
Phytolacca dodecandra	Phytolaccaceae	BuOH extract of berries	Chang *et al.* (1980)
Primula vulgaris	Primulaceae	Saponins compounds	Primorac *et al.*(1985)

2.5.7 Effect on Testosterone

Spermatogenesis is regulated by different hormones which are secreted by the hypothalamus, pituitary gland and testis (Sofikitis *et al.,* 2008).The major male sexual hormone Testosterone is synthesized and secreted by Leydig cells under the control of the pituitary gonadotrophin luteinizing hormone (LH).

Numerous plant products are known to target enzymes which are utilised in synthesis of androgens. Gossypol caused a dramatic decrease in histochemical stain for 3-β-HSD, proving the direct inhibitory effect on leyding cells secretion there by reducing the level of testosterone (Paz and Homonnai, 1984). The crude methanol extract of *Quassia amara* has been reported to cause reduction in the levels of testosterone, LH and follicle-stimulating hormone (Raji and Bolarinwa, 1997). *Mentha piperita labiatae* and*Mentha spicata labiatae* has been shown to increase FSH and LH levels and decreased total testosterone level (Akdogan *et al.,* 2004). Suppression of the activities of steroidogenic enzymes including the P450 side-chain cleavage enzyme,3β-hydroxysteroid dehydrogenase,17α-hydroxylase,20α -hydroxylase and 17 β-hydroxysteroid dehydrogenase, was observed when primary mouse Leydig cells were incubated with varying concentrations of crude *Toona sinensis* (Poon *et al.,* 2005). Dose-dependent decrease in the enzyme activity of 3α, 3β, 17β- hydroxysteroid dehydrogenases and degeneration of Leydig cells were reported when *Abrus pecatorius* was administered to male rats (Sinha and Mathur, 1990).

2.5.7 Plants with Effect on Testosterone

Botanical name	Family	Plant extract	References
Abelmoschus Abelmoschus	Malvaceae	Fruit and methanolic extract	Olatunji *et al.*(2009)
Abrus precatorius	Fabaceae	Dry extract from seed	Sinha and Mathur (1990)
Aegle marmelos	Rutaceae	Leaf and ethanol extract	Chauhan and Argawal(2008)
Albizia lebbeck L	Fabaceae	Bark and methanolic extract	Gupta *et al.*(2006)
Allium sativum	Lilliceae	Crude extract	Hammami *et al.*(2008)
Bulbine natalensis	Asphodelaceae	Stem and aqueous extract	Yakubu and Afolayan (2009)
Chromolaena odoratum	Asteraceae	Leaves and aqueous extract	Yakubu *et al.*(2007)
Curcuma longa L	Zingiberaceae	Crude alcoholic extract	Ashok and Meenakshi(2004)
Juniperus phoenicea	Cupressaceae	Cones and ethanol extract	Shkukani *et al.*(2007)
Dendrophthoe falcate	Loranthaceae	Stem methanol extract	Gupta and Kachhawa(2007)
Psoralea corylifolia	Leguminosae	Crude extract	Takizawa *et al.*(2004)

2.6. Biological Effects of Neem Leaf Phytoconstituents

2.6.1Saponins

They are steroid or triterpene glycoside compounds found in a variety of plants (Frater, 2014). And are chemically referred to as triterpene and steroid glycosides. They consist of nonpolar and polar aglycones coupled with one or more monosaccharide moieties with soap-like behaviour in aqueous solutions (Gemede and Nagussie, 2014).

2.6.2Distribution of Saponin

The saponins are majorly found in the plant kingdom with a diverse group of compounds, varying in structure, physicochemical properties and biological effects. Traditionally, saponins has been reported to be used traditionally as detergents, as piscicides (fish poison) and molluscicides, in addition to their industrial applications as foaming and surface active agents (Cockburn *et al*., 2009). Saponins are extremely toxic and hemolytic to fish and cold-blooded animals (Gemede and Nagussie, 2014).

2.6.3 Sources of Saponin:

Saponins have been reported to be present in more than 100 plant families (Sparg *et al*., 2004) and in a few marine sources, such as fish and sea cucumber species (Sparg *et al*., 2004). Saponins can be classified into the two groups: pentacyclic triterpenoid saponins and steroidal saponins. The steroidal saponins are mainly found in monocotyledons plants (such as in the family's Agavaceae, Dioscoreaceae and Liliaceae), while triterpenoid saponins mostly are present in dicotyledons plants (Fabaceae, Araliaceae and Caryophyllaceae) (Sparg *et al*., 2004). saponins are sometimes further classified into 12 main classes, as follows: cycloartanes, lanostanes, cucurbitanes dammaranes, tirucallanes, lupanes, hopanes, oleananes, 23-nor oleananes, taraxasteranes,ursanes, and steroids (Vincken *et al*., 2007).

2.6.4 Effects of saponins on reproduction

Saponins have been reported to have abortifacient, antizygotic and anti-implantation on cattle (Tewary *et al*., 1973; Stolzenberg *et al*., 1976). Saponins from *Gutierrezia spp* (broom weed), *Agave lecheguilla*, and commercial pharmaceutical- grade saponins caused abortion or death or both in rabbits, goats and cows (Dollahite *et al*., 1962). Saponins isolated from the crude extract of *Gledi-tischia horrida*, *Costus speciosus* and *Phytolacca dodecandra* caused sterility in mice (Chou *et al*., 1971). Quin and Xu,(1998) reported that saponin rich in butanol extract of *Mussaenda pubescens* was capable of terminating pregnancy in rats. Saponins were found to be extremely strong stimulators of luteinising hormone release from cultured hypophysial cells (El Izzi *et al*., 1992) but their action was neutralised in the presence of serum indicating a passive membrane-permeabilising effect (Benie *et al*., 1990).

2.6.5 Anticardiovascular activity

Ingestion of saponincontaining food has been reported to decrease cholesterol levels in thebloodstream and as a results decrease the risk ofcardiovascular diseases (Lee *et al*., 2013). It has also beenreported that ginseng saponins decrease bloodcholesterol levels in rabbits by increasingcholesterol excretion through bile acid formation

(EL-Farok *et al*., 2013). Elekofehinti *et al*.(2012) showed that consumption of saponin from *Solanumanguivifruit* lead to reduction in the risk of hyperlipidemic symptoms and heart diseases.

2.6.7 Isolation and Identification of Saponins

There are two basic extraction techniques in saponin extraction which include the conventional and the green technologies. The conventional extraction techniques are maceration, Soxhlet, and reflux extraction, where the green technologies are microwave-assisted, ultrasound-assisted and

accelerated solvent extraction (Faizal and Geelen 2013). The conventional extraction is based on the solubility of solute from plant materials into solvent. So, it often utilises a large quantity of solvent to extract the desired solute and sometimes is aided with elevated temperature by heating, and mechanical stirring or shaking. The green extraction method is involved less hazardous chemical synthesis, safer chemicals, energy efficiency and pollution prevention (Arif *et al.,* 2009).

2.6.8 Effects of saponin of on cell membrane permeability

The large number of the biological effects of saponins has been associated to their action on permeability of cell membranes. They have a specific ability to form pores in membranes. Saponins have a lytic action on erythrocyte membranes. The haemolytic action of saponins is believed to be the result of the affinity of the aglycone moiety for the phospholipids present in the cell membrane with which they form insoluble complexes(Surana *et al*., 2008) The amount of glycosides required for permeabilisation is much lower for cholesterol-rich lipid layers than cholesterol-free membranes (Gogelein and Huby, 2017).

2.7.1 Alkaloids

Are alkaline, low-molecular-weight nitrogen-containing compounds with numerous pharmacological effects on vertebrates (Matsuura and Fett-Neto,2013). These metabolites can be divided into different classes according to their precursor (e.g., indole alkaloids are alkaloids derived from tryptophan), encompassing more than 20 different classes (e.g., pyrrolidine

alkaloids, tropane alkaloids, piperidine alkaloids, pyridinealkaloids, quinolizidine alkaloids, and indole alkaloids, among others) (Yang and Stöckigt,2010).The function of alkaloids and other secondary metabolites in plants is mainly defensive in nature and hence improve plant reproductive rates,either by improving defenses against biotic and abiotic stresses or by affecting pollinators and seed/fruit parasite. Defensive strategies include predator repellence by toxicity or bitterness taste ordamage repair by antioxidant system (Vilariño and Ravetta,2008; Matsuura and Fett-Neto,2013). Flowervisitors can be attracted by stimulant properties of some alkaloids, whereas visit duration can be controlledby nonlethal toxicity (Irwin *et al*. 2014). This and among many other functions lead tosignificant improvement in survival rates for plants and, at the same time, provide important pharmacologicalactivities for the human therapeutic potentials, such as antioxidant compounds, antitumoral drugs,analgesics, anti-inflammatories, and stimulants (Yang and Stöckigt,2010).To minimize self-intoxication risk, defense compounds are often stored in the vacuole or apoplastic compartment, showing limited metabolic activity (Mithöfer and Boland,2012).More than 20 different classes (e.g., pyrrolidine alkaloids, tropane alkaloids, piperidine alkaloids, pyridine alkaloids, quinolizidine alkaloids, and indole alkaloids, among others) (Yang and Stöckigt 2010).To minimize self-intoxication risk, defense compounds are often stored in the vacuole orapoplastic compartment, showing limited metabolic activity (Mithöfer and Boland,2012).

2.7.2 Mechanisms of Action of Alkaloids

Toxicity may be associated with enzymatic alterations affecting physiological processes, inhibition of DNA synthesis and repair mechanisms by intercalating with nucleic acids, or affecting the nervous system (Mithöfer and Boland 2012) for example, Taxines are calcium channel antagonists, therefore, increases cytoplasmic calcium (Wilson *et al*. 2001).

Pyrrolizidine alkaloid toxic effects are mainly due to their biotransformation into strong reactive pyrrole structures by oxidases from the mammalian liver. The reactive pyrroles act by alkylating nucleic acids and proteins (Cushnie *et al*. 2014). Alkaloid mechanisms of action as antibacterial agents differ among alkaloid classes (Yang and Stöckigt 2010; Lee *et al*. 2014). Isoquinolines, such as berberine, sanguinarine, protoberberine, and benzophenanthridine, inhibit cell division. The phenanthridine isoquinoline alkaloid ungeremine acts by inhibiting nucleic acid synthesis; pergularinine and tylophorinidine, which are indolizidine alkaloids, inhibit nucleic acid synthesis as well, by targeting dihydrofolate reductase (Cushnie *et al*. 2014).

2.7.3 Flavonoids

Belong to a group of natural substances that occur in many plants and its product such as fruit, vegetables, grains, bark, roots, stems, and flowers, tea (Middleton, 1998) Flavonoids has been classified into four main classes based on their molecular structure (Rice-Evans, 1996). Among the 4 main groups of flavonoids is flavonoid example is quercetin, which is found in abundance in onions, apples, broccoli, and berries. The second group is the flavanones, such as narigin that is mainly found in citrus fruit. Flavonoids belonging to the catechins are mainly found tea and red wine (de Groot and Reuen, 1998), whereas anthocyanins are found in strawberries and other berries, grapes, wine, and tea (Middleton, 1998).

An important effect of flavonoids is the scavenging of oxygen-derived free radicals. In vitro experimental systems also showed that flavonoids possess antiinflammatory, antiallergic,antiviral, and anticarcinogenic properties (Middleton, 1998)

2.7.4 Antioxidative effects of flavonoids

The best-described property of almost every group of flavonoids is their capacity to act as antioxidants (Middleton, 1998). The flavones and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species (Grace 1994; Reuen, 1998). Body cells and tissues are continuously damaged by free radicals and reactive oxygen species, which are produced during normal oxygen metabolism or are induced by exogenous damage (de Groot and Rauen, 1998; Grace, 1994). The mechanisms and the cascade of events by which free radicals interfere with cellular functions are not fully understood, but one of the leading events seems to be lipid peroxidation, which results in cellular membrane damage. This cellular damage causes a shift in the net charge of the cell, changing the osmotic pressure, leading to swelling and eventually cell death. Free radicals causes influx of various inflammatory mediators, leading to a general inflammatory response and tissue damage (Halliwell, 1995). The natural antioxidant-defense mechanisms of the body include enzymes such as superoxide dismutase, catalase, and glutatione peroxidase, but also nonenzymatic counterparts such as glutathione, ascorbic acid, and tocopherol (Halliwell, 1995).

Flavonoids such as quercetin, reduce ischemia-reperfusion injury by interfering with inducible nitric-oxide synthase activity (Shoskes, 1998). Nitric oxide is produced by several different types of cells, including endothelial cells and macrophages. Although the early release of nitric oxide through the activity of constitutive nitric-oxide synthase is important in maintaining the dilation of blood vessels (Huk *et al*., 1998), the oxidative damage is principally due to concentrations of nitric oxide produced by macrophages. Nitric oxide reacts with free radicals, thereby producingthe highly damaging peroxynitrite (Shoskes 1998). Nitric oxide injury takesplace for the most part through the peroxynitrite route because peroxynitrite can directly oxidize LDLs

(low density lipoprotein), resulting in irreversibledamage to the cell membrane (Huk *et al*., 1998). When flavonoids are used asantioxidants, free radicals are scavenged and therefore can nolonger react with nitric oxide, resulting in less damage (Shutenko *et al*., 1998).

The xanthine oxidase pathway has been implicated as an important route in the oxidative injury to tissues, especially after ischemia-reperfusion (Sanhueza *et al*., 1992). The metabolism of xanthine to uric acid is done by dehydrogenase and xanthine oxidase. Xanthine dehydrogenase is the form of the enzyme present under physiologic conditions, but its configuration is changed to xanthine oxidase during ischemic conditions. Xanthine oxidase is a source of oxygen free radicals (Chang *et al*., 1993). During the reoxygenation, xanthine oxidase reacts with molecular oxygen, thereby releasing superoxide free radicals (Chang *et al*.,1993). Quercetin and silibin, inhibit xanthine oxidase activity, thereby resulting in decreased oxidative injury (Shoskes, 1998;Chang *et al*., 1993; Lio *et al*., 1986).

Cos *et al.* (1998) reported that luteolin (3_, 4_5, 7- tetrahydroxyflavone) was reported to be the most potent inhibitor of xanthine oxidase.

Leukocyte immobilization is another major mechanism responsible for the formation of oxygen-derived free radicals in endothelial layer. May be due to immobilization and firm adhesion of leukocytes to the endothelial wall, so also the release of cytotoxic oxidants and inflammatory mediators and further activation of the complement system. Physiologically, leukocytes circulate freely along the endothelial wall (Friesenecker *et al*., 1994). However, during ischemia and inflammation, endothelium derived mediators and complement factors may cause adhesion of the leukocytes to the endothelial wall, thereby immobilising them and stimulating degranulation of the neutrophil. As a result, oxidants and inflammatory mediators are released, resulting in injury

to tissues (Ferrandiz *et al*., 1996). Oral administration of a purified micronized flavonoid fraction was reported to decrease the number of immobilised leukocytes during reperfusion (Friesenecker *et al*., 1994). The decrease in the number of immobilized leukocytes by flavonoids may be related to the decrease in total serum complement and is a protective mechanism against inflammation-like conditions associated with reperfusion injury (Friesenecker *et al*., 1995). Some flavonoids can inhibit degranulation of neutrophils without affecting superoxide production (Ferrandiz *et al*., 1996). The inhibitory effect of some flavonoids on mast cell degranulation was shown to be due to modulation of the receptor-directed Ca^{2+} channels in the plasma membrane (Bennett *et al*., 1981).

2.7.5FlavonoidsInteraction with other enzyme systems

The major effects of flavonoids may be the result of radical scavenging and interaction with various enzyme systems (Nelson *et al*., 1992). When reactive oxygen species are in the presence of iron, lipid peroxidation results (Nelson *et al*., 1992). Quercetin has been reported to have iron-chelating and iron-stabilizing properties (Ferrali *et al*., 1997) and also protect the cells by direct inhibition of lipid peroxidation (Sorata *et al*., 1984). Flavonoids reduce complement activation thereby decreasing the adhesion of inflammatory cells to the endothelium (Friesenecker *et al*., 1995) and in general resulting in a diminished inflammatory response. Flavonoids also reduce the release of peroxidase there by inhibits the production of reactive oxygen species by neutrophils

(Middleton and Kandaswami 1992). Flavonoids also causes the inhibition of the metabolism of arachidonic acid (Ferrandiz and Alcaraz, 1991). This feature gives flavonoids anti-inflammatory

and anti thrombogenic properties (Ferrandiz and Alcaraz 1991). The release of arachidonic acid is a starting point for a general inflammatory response (Nelson *et al*., 1992).

2.7.6 Toxicity of flavonoids

Formica and Regelson (Formica and Regelson 1995) reported that quercetin poses both toxic and mutagenic properties. Studies conducted in laboratory animals (Ertrurk *et al*., 1984, Starvic 1984). Dunnick and Hailey (1992) reported that high doses of quercetin result in the formation of tumors in mice. However, long-term studies, shows no carcinogenicity (Zhu *et al*., 2001). In contrast with the potential mutagenic effects of flavonoids in earlier studies, several other reports indicated that flavonoids, such as quercetin, seem to be antimutagenic in rats (Plakas *et al*., 1985; Kato *et al*., 1984).

2.8 Reproduction in rabbits

In male the oval shaped testes within the scrotum remains in communication with the abdominal carvity, where they were at birth. The testicle descends at about two months (Hoffman and Smith, 2005).

In the rabbit doe, ovulation does not occur spontaneously, but has to be induced through a neurohormonal reflex, which is initiated during mating and occur 10-16 hours after mating (Hafez, 1993). There have been some common incidences of failure to ovulate which appears to vary with the season of the year (Adams, 1972). When using artificial insemination, in the absence of a male, ovulation has to be induced by artificial method which include treatment with luteinizing hormone, human chorionic gonadotrophin injection (HCG), copper salts or other hormonal means(Adams, 1972). The ovulation induction method most commonly used is an intramuscular injection of Gonadotrophin releasing hormone or its synthetic analogues (Foote *et*

al. 1963), since they are molecules with a smaller molecular weight (gonadoreline is a decapeptide and busereline a nonapeptide), they can also be absorbed subcutaneously or across mucus layers (Camier *et al.* 1989; Donnez *et al.* 1989). This has been shown to induce ovulation in rabbit does with results similar to that obtained by natural mating. Alternatively, lutenising hormone or human chorionic gonadotrophin can be used to induce ovulation in rabbit does (Foote *et al.* 1963), however, the repeated injection of these hormone can result in failure of the does to ovulate due to antibody formation (Adams, 1972). Gonadotrophin releasing hormone can be repeatedly injected without antibody formation (Adams, 1981). Vasectomised males has been used to induce ovulation in does prepared for artificial insemination (Khalifa, 1994; Khalifa *et al.* 2000), but the method is time –consuming and some females refuse to mate, but ovulate when given luteinising hormone or gonadotrophin releasing hormone.

Giojalas *et al.*, (2004), comparing human and rabbit spermatozoa, suggested that the timing and duration of the capacitation is programmed according to egg availability in the oviduct: long in period ovulators, such as rabbits. Indeed, Brackett *et al.* (1982) reported that invitro capacitation of raw rabbit semen is long and difficult whereas percoll selected spermatozoa (without granules) show a faster rate of capacitation. This circumstance maximizes the possibilities that an ovulated egg would meet spermatozoa in the best functional state.

2.8.1 Gonad development and puberty in rabbit bucks:

In mammals, gonad differentiation is initiated early during foetal life. Genital crest arising from the adjacent mesonephros are colonized by germ cells (Nathalie *et al.*, 2013). Somatic cells and

germ cells then differentiate in a coordinated manner: somatic cells differentiate into cells expressing steroids or cells acting as nursing or supporting cells for germ cells. At the same time, germ cell differentiate and acquire ability to enter meiosis (Nathalie *et al.,* 2013).

Rabbit gestation is 30days, though short but still enough to enable regular and systematic sampling through out foetal life. Meiosis occurs at birth in females and not during life, as in the case of numerous other mammalian species (Nathalie *et al.,* 2013). 15days interval is required between the appearance of genital crest and the onset of meiosis in rabbits, while this interval is only 3 days long in the mouse. The gonads begin to differentiate on the 16^{th} day after fertilisation. After birth the testes develop less quickly compared to the rest of the body. From the age of five weeks they begin to grow very rapidly. Accessory glands undergo a similar development, but at a more even rate and are les precocious (Nathalie *et al.,* 2013). Spermatogenesis begins between 40 and 50 days. The testicular tubes become active at about 84 days. The first spermatozoa are present in the ejaculate at about 110 days (Hoffman and Smith, 2005; Kemppainen, 2007; Smith and Boyer, 2008).

Sexual maturityis reached at 32 weeks by New Zealand White rabbits in temperate climates (Lombardi and Fernandez, 2003; Rodel and Bora, 2004). However, a young buck in the same conditions can be used for reproduction from the Age of 20 weeks. Indeed the first manifestation of sexual behavior appears at days 60 to 70 when the rabbit makes its first attempt at ridding. Coitus may occur for the first time at about 100 days, but the viability of the spermatozoa is very week or nil in the first ejaculate. So first mating should be timed for age 135 to 140 days (Kemppainen, 2007; Smith and Boyer, 2008).

The onset of puberty varies from breed to breed, but environmental conditions in the rabbitry also play a role. Particularly feeding, which is even more important than climate (Lombardi and Fernedez, 2003; Rodel and Bora, 2004).

Does generally reach puberty when they have grown up to 70 to 75 percent of their mature body weight. However, it is usually preferable to wait until they reach 80 percent of their mature weight before breeding them. These relative weights should not be considered absolute thresholds for all rabbits, but rather limits applicable to the population as a whole. Sexual behavior appears long before the ability to ovulate and bear a litter(Nathalie *et al.,* 2013).

2.8.2 Male reproductive system

Vertebrate male reproductive endocrine system commonly comprise of hypothalamus, anterior pituitary gland and testes, which form the hypothalamic-pituitary-gonadal system (HPG). The male reproductive tract consists of testes, epididymis and ductus deferens (Emmanuele and Emmanuele, 1998; Foley, 2001). The major function of male reproductive tract is to produce spermatozoa and hormones. The anterior pituitary gland produces two hormones: follicle stimulating hormone (FSH) and luteinising hormone (LH) (Emmanuele and Emmanuele 1998).

The hypothalamic-pituitary-gonadal axis is a critical part in the development and regulation of a number of body system such as reproductive and immune systems. Fluctuations in the hormones produced by each gland and have various wide spread and local effects on the body (Emmanuele and Emmanuele, 1998).

This axis control development, reproduction and aging in animals. The hypothalamus produces gonadotrophin-releasing releasing hormone (GnRH). The anterior portion of the pituitary gland

produces luteinising hormone and follicle stimulating hormone (FSH), and the gonads produce estrogen and testosterone (Emmanuele and Emmanuele, 1998).

In oviparous organism (e.g fish, reptiles, amphibians, and birds), the HPG axis is commonly referred to as the hypothalamus-pituitary-gonadal-liver axis (HPGL-axis) in females. Many egg-yolk and chorionic proteins are synthesized heterologously in the liver, which are necessary liver proteins are vitelogenin and choriogenin (Emmanuele and Emmanuele, 1998).

The hypothalamus is located in the brain and secretes GnRH (Millar *et al.,* 2004). GnRH travels down the anterior portion of the pituitary through the hypophyseal portal system and binds to receptor on the secretory cells of the adenohypophysis (Charlton, 2008). In the response, to GnRH stimulation these cells produce luteinizing hormone and follicle stimulating hormone, which travels in the blood stream (Vadakkadath and Atwood, 2005).

These two hormones play an important role in communicating with the gonads. In females, LH and FSH, act primarily to activate the ovaries to produce estrogen and inhibin and regulate the menstrual cycle and ovarian cycle. Estrogen forms negative feedback loop by inhibiting the production of GnRH in the hypothalamus. Inhibin act to inhibit activin, which is peripherally produced hormone that positively stimulates GnRH-producing cells (Meethal *et al.,* 2009). Follistain, which is also produced in all body tissue, inhibits activin and gives the rest of the body more control over the axis. In males Luteinizing hormone stimulates the interstitial cells located in the testes to produce testosterone, and follicle stimulating hormone plays a role in spermatogenesis. Only small amount of estrogen are secreted in males. Recent research has shown that a neurosteroid axis exist, which helps the cortex to regulate the hypothalamus production of GnRH (Meethal *et al.,* 2009). One of the most important functions of the HPG is to

regulate reproduction by controlling the uterine and ovarian cycle (Katja and Mariec, 2007). In females, the positive feedback loop between estrogen and luteinizing hormone help to prepare the follicle in the ovary and uterus for ovulation and implantation. When the egg is released, the ovary begins to produce progesterone to inhibit the hypothalamus and the anterior pituitary thus stopping the estrogen-luteinizing hormone positive feedback loop. If conception occurs, the fetus will over the secretion of progesterone; therefore the mother cannot ovulate again. If conception doesn't occur, decreasing excretion of progesterone will allow the hypothalamus to restart secretion of GnRH (Katja and Mariec, 2007). These hormones levels also control the uterine (menstrual) cycle causing the proliferation phase in preparation for ovulation, the secretory phase after ovulation, and menstruation when conception does not occur. The activation of HPG axis in both males and females during puberty also causes individuals to acquire the secondary sex characteristics (Katja and Mariec, 2007).

In males, the production of GnRH, LH and FSH are similar, but the effect of these hormone are different (Veldhuis *et al.,* 2009). FSH stimulates sustentacular cells to release androgen binding protein, which promote testosterone binding. LH binds to interstitials cells, causing them to secrete testosterone. Inhibin is produced by spermatogenic cells, which, also through inactivating activin, inhibits the hypothalamus. After puberty, these hormones levels remain relatively constant (Veldhuis *et al.,* 2009).

Although males remain fertile until death, the activation of the HPG axis deceases. As males age, the testes begin to produce less testosterone, leading to a condition known as post-pubertal hypogonadism (Veldhuis *et al.*, 2009). Post-pubertal hypogonadism results in progressive muscle

mass decrease, increase in visceral fat mass, loss of libido, impotence, decreased attention, increased risk of fractures, and abnormal sperm production.

Semen is a mixture of spermatozoa suspended in the seminal plasma. It is a representative of the combined secretions from different locations by the epididymis and various gland, such as the seminal vesicles, bulbourethral gland and prostate, which, at the time of ejaculation, are combined (Boiti *et al.,* 2005). The relative contribution of these different glands vary between species (Morrel, 2011). The accessory sex gland secretions act as vehicle for the sperm cells, as nutrient and as an aid to neutralising acidity in female's genitals passages (Biljani, 2012; Edmondson *et al.,* 2012). Spermotogenesis is a precise organised process by which normal diploid cells turn into sperm which occurs within semeniferous tubule of the testes (Nuti, 2012). Three distinct phases can be divided in the process of mammalian spermatogenesis which are: prolioferation of diploid spermatogonia, meiosis of spermatocytes into haploid spermatids, and differentiation of haploid spermatids during spermiogenesis (Chung*et al*. 2009). Round spermatids are differentiated into elongated spermatozoa with midpiece and tail at the final phase of spermiogenesis.

As a result of extensive chromatin remodeling and compaction during spermiogenesis, spermatozoa with highly condensed nucleus are formed (Chung, 2009). Spermatozoa are released into seminiferous tubules, spermatogonia and immature spermatocyte gather at the basal compartment of the seminiferous tubule. The seminiferous tubule is the main compartment of the testes and also the site of sperm cells formation and maturation (Jones, 1999; Chung*et al.,* 2009).

After completing mitosis and meiosis, haploid spermatids enter spermiogenesis, a process of physical and physiological maturation. During spermiogenesis, spermatids acquire tail and

motility with a develop mid-piece which connects the head compartment with the tail. This physical maturation process mostly happens in seminiferous tubule. Sperm maturation and storage occurs in the epididymis (Jones, 1999). Epididymis also forms the blood epididymal barrier which allows the epididymis to regulate and modify the luminal fluid contents. Different epididymal fluid contents in caput epididymis, corpus epididymis and cauda epididymis change the surface of the sperm plasma membrane into fully matured condition and provide spermatozoa with motility and capacity for fertilisation as the immotile immature spermatozoa transits through the epididymis. Therefore, it is shown that luminal microenvironment of the epididymis is vital for successful sperm maturation (Foley, 2001). Finally, after a series of morphological changes, mature spermatozoa are stored in a quiescent state.

The estimated duration spermatogenesis in the rabbit depends on the point chosen as the onset of spermatogenesis. If spermatogenesis is considered to begin with the first of the series of spermatogonial divisions leading to the production of primary spermatocyte then about four cycle of the seminiferous epithelium or 4× 10.9 = 43.6 days are required. However, if one assumes that spermogenesis start with the formation of spermatogonial stem cell and that the life span of these stem cells is one cycle, or 51.8 days (Swierstra and Foote, 1965). Since the duration of spermatogenesis is about 7-8 weeks, a minimum of 10 weeks is recommended to determine the effect of certain exogenous factors (Boiti *et al.,* 2005).

Rabbit semen consist of two main parts which are the gelatinous and the fluidy parts. Following ejaculation, the gelatinous part fills the lumen of the vagina after ejaculation (Engle, 1926). Ejaculated rabbit semen is heterogeneous mixture consisting of seminal plasma in which the mature spermatozoa are suspended along with numerous membrane surrounded granules

(Eghianruwal and Anika, 2011). These vesicular formations, which have a predominantly round shape and a diameter ranging from 0.5-4 um, are produced and secreted by the anterior of the prostate (pro-prostate) as shown by their presence in a vasectomised rabbit bucks (Cardinali *et al.,* 2007). Similar multi lamellar granules have also been isolated from human semen and from semen of other animals' species as prostasomes and prostasomelike vesicles, respectively (Arienti *et al.,* 1998; Kravets *et al.,* 2000; Ghaoui *et al.,* 2004; Sicilliano *et al.,* 2008). The physiological roles of these prostatic secretory granules (PSGs) have been thoroughly debated and several hypothesis have been put forward (Kravets *et al.,* 2000). In rabbits, prostatic secretory granules are able to inhibit spermatozoa capacitation, delaying the acrosome reaction, and they promote spermatozoa motility during the first few hours after mating (Cardinali *et al.,* 2007; Castellini *et al.,* 2008).

The shape of rabbit spermatozoa is similar to that of other mammals. The dimensions of the ovoid head are about 7×4×0.5 cm. the length of the tail is 45 cm. the acrosomal cap is situated on the top of the head for three-quarters of its extent and presents an increase along its edge. The longitudinal section of the rabbit spermatozoa head shows a very compact nuclear chromatin surrounded by the acrosomal complex (Boiti *et al.,* 2005).

2.9Methods of Semen Collection

There are several methods of semen collection but the most recent methods are; the artificial vagina, electro-ejaculator and the rectal massage. Abdominal massage, penile and cloacal massage could also be used (Morrel, 2011). The use of any particular method depends on the type and condition of specie. The ideal method of semen collection will be the one which is safe for the sire and the collector (Morrel, 2011), and where the semen obtained is a real

representative of a normal ejaculate, free of contamination and should eventually be protected from thermal shock.

2.9.1 The artificial vagina

This is more quick method of semen collection in animals. The artificial vagina consist of a rubber casing, a rubber liner, two rubber bands, rubber funnel, graduated test tube, protective jacket and a two way valve. When the artificial vagina is coupled, the space between the rubber linner and rubber casing is filled with glycerol, after which the coupled artificial vagina is inserted into hot water at temperature of 40-55^{oC}; the warm glycerol stimulates the vagina of the doe. A latex rubber collection cone is placed in the artificial vagina and a graduated collection tube is place on the end of the cone. Lubrication with non perfumed petroleum jelly on the end where intromission of the penis occurs is essential (Dorji, 2012). The rabbit doe is restrained firmly in place allowing for the male to exhibit his courting behaviour. The buck is allowed few false mounts, after which the person with artificial vagina collects the ejaculate by directing the penis into the artificial vagina. The test tube containing the ejaculate is then carefully handled against foreign bodies, contamination and direct sunlight and cold tempreture (Nuti, 2012).

2.9.2 Electro ejaculator

This is used by inserting a probe or electrode in the male's rectum stimulating the ampula and sacral nerves plexus of the reproductive tract (Carter *et al.*, 1990; Dorji, 2012). The electro ejaculator could either be operated only on battery or battery-cum-electric transistorised circuits. This method is used where artificial vagina is not practical in semen collection from males for artificial insemination purposes, situations like when the male is extremely slow in serving the

artificial vagina or physical incapable of mounting. The experience with the use of electro ejaculator is usually aversive (Dorji, 2012).

2.9.3 Rectal massage

This is achieved by massaging the vesicular glands and ampullae of the ductus deferens through the rectal wall (Barth and Oko, 1989; Morell, 2011). Semen is then collected by gentle massage resulting into stimulation and protrusion of the glans penis from the prepuce (Sekoni, 1990). This method has a rapid semen collection characteristics when animals cannot mount, but contamination with urine may occur (Morell, 2011),

2.9.4 Abdominal massage/ digital massage

Massaging the back of a bird and stroking gently towards the tail with the thumb and forefinger, stimulates erection of the copulatory organ. Gentle pressure is exerted on the ejaculatory ducts and semen is collected into a calibrated tube. This method is most preferred in poultry (Burrows and Quinn, 1937; Morell, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Animals and Management

A total of twenty five (25) adult male New Zealand white rabbits, having an average weight of 3.1kg, were purchased and used for the experiment. The rabbits (plate I) were kept in the animal house of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria, Nigeria. The animals were examined, screened and dewormed with ivermectin and treated against coccidia parasite using triple sulphur. The rabbits were fed with pelleted diet made from chicken commercial *top feed*^{R} growers and water was provided *ad libitum.* Animals were allowed to acclimatise for two weeks prior to commencement of the experiment under strict observation and during which the bucks were trained for semen collection and base-line data was established for two weeks. Blood samples were collected trice through ear venipuncture and two ejaculate each were obtained from the rabbit within 14days of acclimatization.

3.2 Ethical Clearance

Ethical clearance was obtained for the use of rabbits in this study from Ahmadu Bello University Zaria Committee on Animal Use and Care (ABUCAUA/2018/009).

3.3 Collection and Preparation of Neem Leaf Samples:

Fresh mature healthy neem(*Azadiracta indica,* L.)leaves were collected from the Botanical Garden of Biological Science Department, Faculty of Life Sciences, Ahmadu Bello University Zaria, Samaru Main Campus. Identification was confirmed by a Taxonomist at herbarium, Department of Botany, Faculty of Life Sciences, Ahmadu Bello University Zaria with identification number of V/N: 90015. The leaves were collected and washed properly, air dried at room temperature and then made into powder with 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).

3.4 Extraction Procedure of Neem Leaf Crude Extracts

The powder 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 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 water (200 mL). Then it was extracted successively with different organic solvents with increasing polarity such as hexane, chloroform, ethyl acetate and butanol to obtain hexane (43.7 g), ethyl acetate (73.63 g), chloroform (57.23 g) and butanol (36.81 g) and residual methanol fractions (146.90 g), respectively. All fractions were filtered separately through Whatman No. 41 filter paper to remove particles. The particle free fractions was evaporated completely by using rotary evaporator (Yamato, Rotary Evaporator, model-RE 801) under reduced pressure to obtain dry fractions from the methanol crude extracts. The residue left in the

separatory funnel was re-extracted twice follow the same procedure and filtered. The combined extracts were concentrated and dried by using rotary evaporator under reduced pressure.

3.5 Determination of Acute Toxicity (LD_{50}) of Neem Leaf Crude Methanol Extract

Median lethal dose (LD_{50}) of crude methanolic neem leaf extract was carried out as a basis for the doses used in the experiment. This was done using the method described by Lorke (1983). The trial was carried out in two phases. In phase I, nine rabbits were divided into three groups (I, II and III)of three rabbits each. The group I, II and III rabbits were, each, dosed with 10, 100 and 1000 mg/kg of crude methanolic neem leaf extract, respectively. The animals were observed for 48 hours for clinical signs of toxicity and death. Thereafter, the period of observation was extended to two weeks. Based on the result obtained, the second phase of the test was conducted as follows: Three rabbits individually caged and numbered I, II and III were administered the reconstituted crude methanolic neem leaf extract at a dose of 1600 mg/kg, 2900 mg/kg and 5000 mg/kg, respectively. The LD_{50} was then determined based on the dose-response (death) as described by Lorke (1983) as follows:

LD_{50} was calculated as the geometric mean of 2900 and 5000 using the formula below

$LD_{50} = \sqrt{(D_o \times D_{100})}$

Where LD_{50} = Median lethal dose

D_0 = Highest dose that gave no mortality (5000 mg/Kg body weight 0/1)

D_{100} = Lowest dose that produced mortality (65000 mg/Kg body weight 1/1)

$= \sqrt{5000 \times 65000}$

$= \sqrt{325000000}$

LD_{50}= 3808 mg/Kg body weight.

3.6Animal Grouping and Treatment

The experimental animals were randomly allocated to five (5) groups; A, B, C, D and E, comprising of five (5) rabbits each. Group A served as the negative control administered only distilled water. Group B, C, D and E served as the experimental group and were treated with n-hexane, chloroform, ethylacetate, and n- butanol fraction of methanolic neem leaf extract, respectively, for 16weeks at a dose of 300 mg/kg across all the groups.

3.7 Administration of the Extract

The different fractions of the methanolic crude leaf extract obtained were dissolved in distilled water at a concentration of 300mg/kg. The rabbits were dosed orally at approximately the same time each day using a graduated syringe and stainless steel intubation canula.

3.8 Semen Collection and Evaluation

Prior to the commencement of the experiment, semen samples were collected and evaluated weekly for two weeks to establish a base-line data. During the experimental period, semen samples were collected using artificial vagina (plate III) that was specifically designed for rabbits. The rabbit bucks were teased with a mature doe before semen collection to ensure optimum semen quality. Semen sample was collected in the morning between 8:00 to 10:00 a.m with the aid of artificial vagina, made similar to Herbert and Adejumo (1995). The artificial vagina was pre warmed for 10-15 minutes in warm water at 40-60^{o}C from thermo flask,

thereafter dried with serviette. The AV was then lubricated with glycerol. Semen collection was done once weekly for twelve weeks.

Semen volume was read off the collection tube and recorded in millilitres. Semen colour was evaluated as milky, creamy and watery and graded 1, 2 and 3, respectively,as described by Zemjanis (1970).

3.8.1 Sperm motility

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 percentage of motile sperm cell was broadly categorised as: very good (100-81%), good (80-61%), fair (60-41%) and poor (40-20%).

3.8.2 Concentration

Concentration of spermatozoa was determined using Neubaeur haemocytometer, which has 25 large squares and constitutes or equal to 400 small squares. 25µl of the semen sample was aspirated using micropipette and diluted with 5ml of 3% NaCl inside a test tube. A cover slip was placed on the haemocytometer and two drops of diluted semen was added under the cover slip on each side of the haemocytometer. The haemocytometer was then placed in a pre-wetted chamber and lid was closed and left for 5minutes. Sperm cells were counted diagonally from top left to right bottom in the large 5 squares or a total of 80 small squares and examined at x 40 magnification under the microscope.

The semen concentration was calculated as described by Rekwot *et al*. (1997):

Concentration (sperm cells/ml) = Average count per square× Dilution factor $\times 10^6$

3.8.3 Percentage live sperm cells

This was determined as described by Rekwot *et al*. (1997). One drop of semen was mixed with two drops of eosin-negrosin 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 colourless). One hundred stained and unstained sperm cells were counted using a light microscope (x40 magnification) and percentage for each group was estimated.

3.8.4 Sperm abnormalities

This was determined as 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 light microscope. One hundred sperm cells were counted per slide using hand counter and five categories of cells were recorded as: normal cell, free head, free tail, coiled tail and bent tail.

3.9 Histological Processing

This was carried out as 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 venisection. Individual testes were dissected and placed in Bouin`s solution and taken to the laboratory. Samples were refrigerated at 4^0C 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.

3.9.1 Haematological and Testosterone Analyses

Five millilitres (5ml) of blood was collected from each rabbit buck three times during period of the study. That is: beginning (first week), middle (seventh week) and end (Twelveth week) of the experiment between 7 AM – 9 AM using 25G syringes through venipucture of the ear vein. The blood sample was collected into sterile sample bottles, containing Ethelene-Di-amine-Tetra Acetic acid (EDTA) for the evaluation of haematological parameters and no anti-coagulant for testosterone assay. The haematological parameters of Packed Cell Volume (PCV), haemoglobin (Hb) concentration, Red Blood Cell (RBC), platelet, absolute and differential leucocyte counts were determined as described by Dacie and Lewis (1991). Erythrocytic indices of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) was calculated from the value of PCV, Hb concentration and RBC count as described by Schalm *et al.* (1975), while testosterone values were assayed using the Enzyme -Linked Immunosorbent Assay (ELISA) technique, according to the manufacturers manual instruction (monobind Inc. Lake Forest, CA 92630, USA).

3.9.2 Data Analyses

Data collected on semen characteristics (colour, volume, pH and spermatozoa concentration) and haematological parameters and were expressed as means ± standard error of mean (SEM) while data on motility and percent live / dead ratio of spermatozoa were expressed as percentages and mean ± SEM. Repeated measure one-way analysis of variance (ANOVA), with Tukey's multiple comparison tests was done and GraphPad Prism software (Version 5.0) was used for the analysis. P-values ≤ 0.05 were considered statistically significant.



Plate I: Housing of the animal during experimental period



Plate II: *Azadirachta indica* (neem) leaves

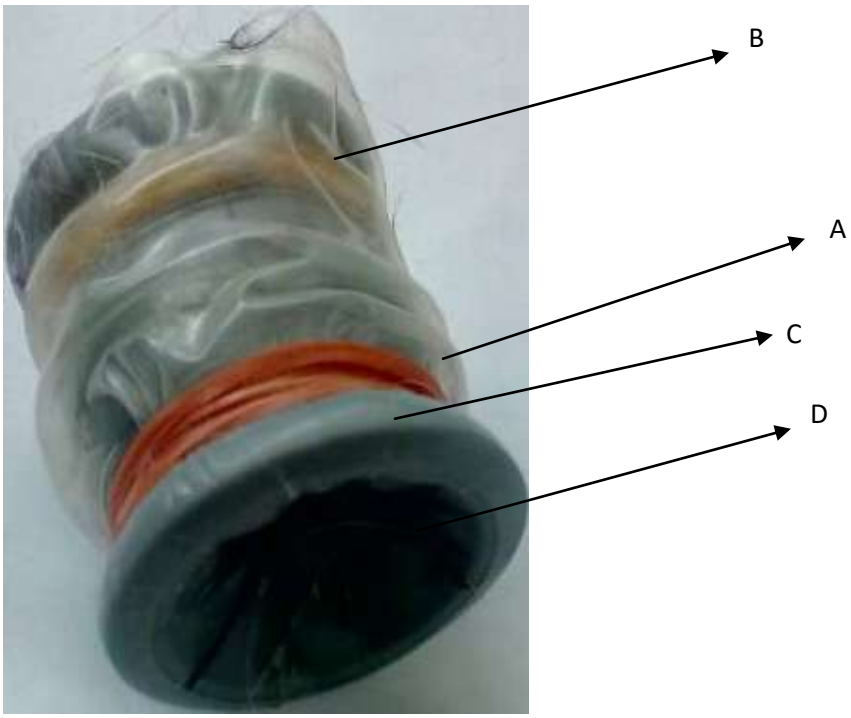


Plate III: locally design Artificial Vagina (AV) for rabbits.

A. Rubber liner fixed on both ends by rubber bands
B. Rubber bands
C. Plastic cylinder
D. Expanded glycerol within the rubber liner

CHAPTER FOUR

4.0 RESULTS

4.1 Phytochemical Screening of Fractions from Crude Methanolic Neem Leaf Extract

The phytochemical screening of different fractions from crude methanolic neem leaf extract showed the presence of various primary and secondary metabolites (Table 4.1)

4.2 Acute Oral Toxicity

4.2.1 Physical observations

No clinical signs were observed during the first phase of the acute oral toxicity study. That is, at dose levels of 10mg, 100mg, and 1000 mg/kg and those treated with the extract at the dose levels of 2500mg and 5500 mg/kg weight. In second phase of the trial, rabbits at a dose level of 6500 mg/kg body weight of the extract show signs of dyspnea, depression and recumbency (Tables 4.2 and 4.3).

Table 4.1: Qualitative phyto chemical 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

Table 4.2: 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 4.3: 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

4.3 Effect of Fractions from Crude Methanol Neem Leaf Extracts on Semen Characteristics

Semen volume (Figure 4.1) showed no significant difference ($P>0.05$) between the control group (A) and groups B, C, D and E administered n-hexane, chloroform, ethylacetate and n-butanol respectively.

Figure 4.2 shows the Mean (± SEM) values of semen pH of New Zealand white rabbit administered fractions from crude methanol neem leaf extract. No significant difference ($P>0.05$) between the control group (A) and experimental groups (B, C, D and E).

Semen gross motility (Figure 4.3) differed significantly ($P<0.05$)the control group and treatment group C administered chloroform fraction from crude methanol neem leaf extract. Gross sperm motility in group C was observed to decreases from the first week of the experiment to the last week with the lowest value of 00.00 at the end of the study period.

Semen concentration significantly differed ($P<0.05$) between the control group A and group C (chloroform fraction). However, no significance diference ($P>0.05$) was observed between treatment groups B, D and E (Figure 4.4).

The percentage live spermatozoa of New Zealand white rabbit administered chloroform fraction (group C) shows significant decrease ($P<0.05$) compared with the control (Figure 4.5). The percentage dead spermatozoa was significantly ($P<0.05$) different between the control and group C treated with chloroform fraction. However, no significant difference ($P>0.05$) was observed between treatment groups B, D and E and the control (Figure 4.6)

Semen morphological abnormalities significantly differed($P<0.05$) between the control group A and treatment group C. An increased number of free head and free tails was observed in the group treated with chloroform fraction (Figure 4.7). However, no significant difference ($P>0.05$) was observed between treatment groups B, D and E and the control.

Testosterone value did not differ significantly ($P>0.05$) between the treated groups (B, C, D. and E) and the control (group A). (Fig. 4.8)

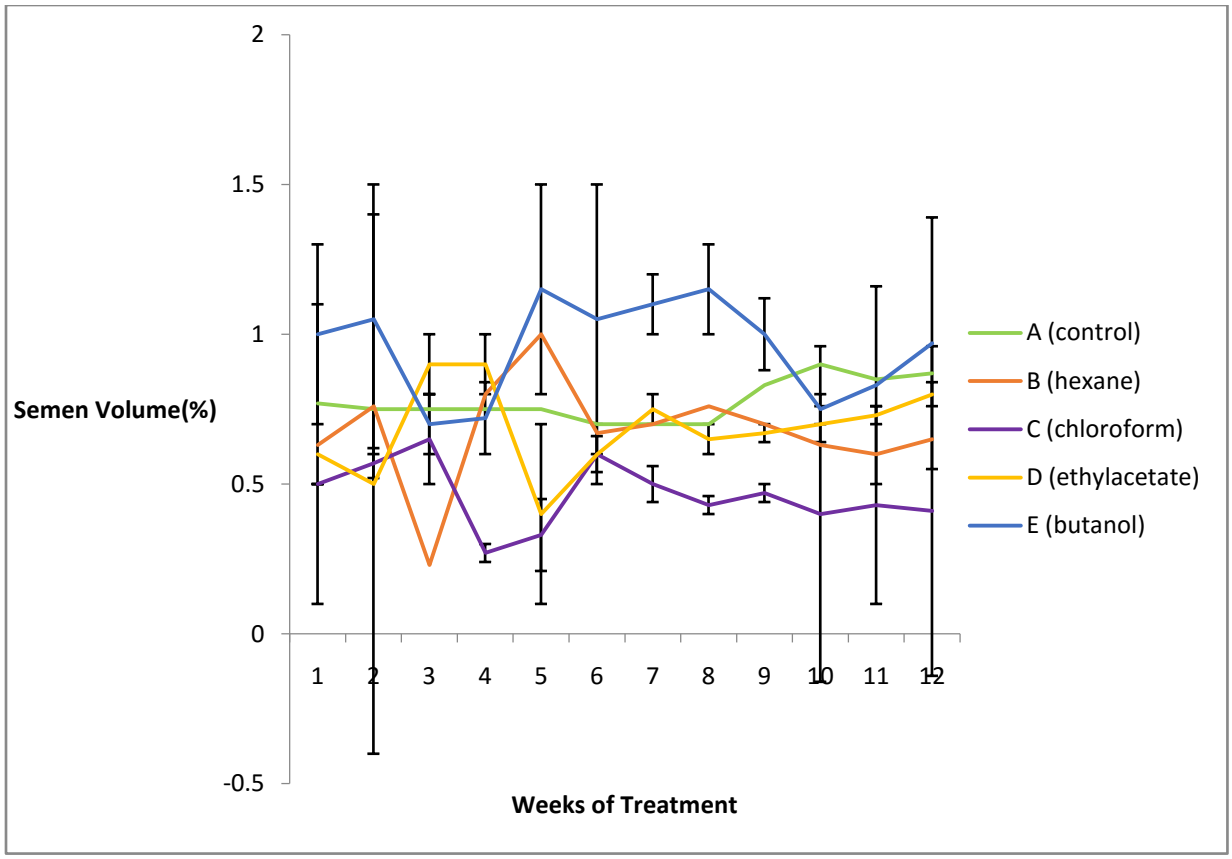


Figure 4.1: Mean(± SEM)semen volume of New Zealand white rabbit administered different fractions from methanolic neem leaf extract.

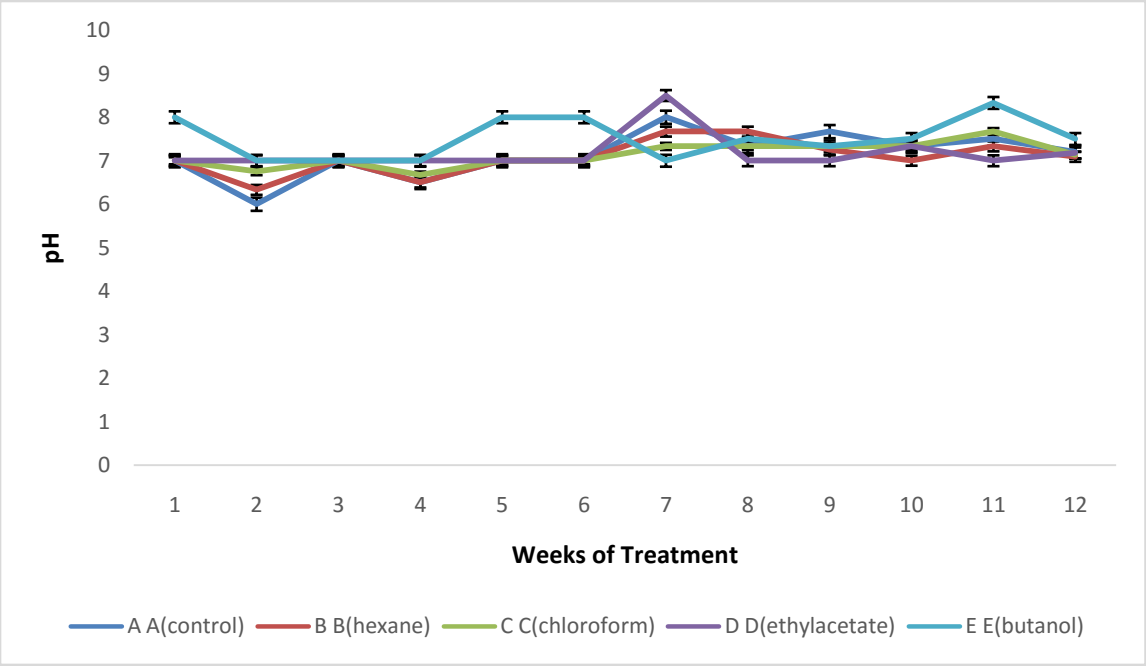


Figure 4.2: Mean (± SEM)semen pH of New Zealand white rabbit administered different fractions from methanolic neem leaf extract.

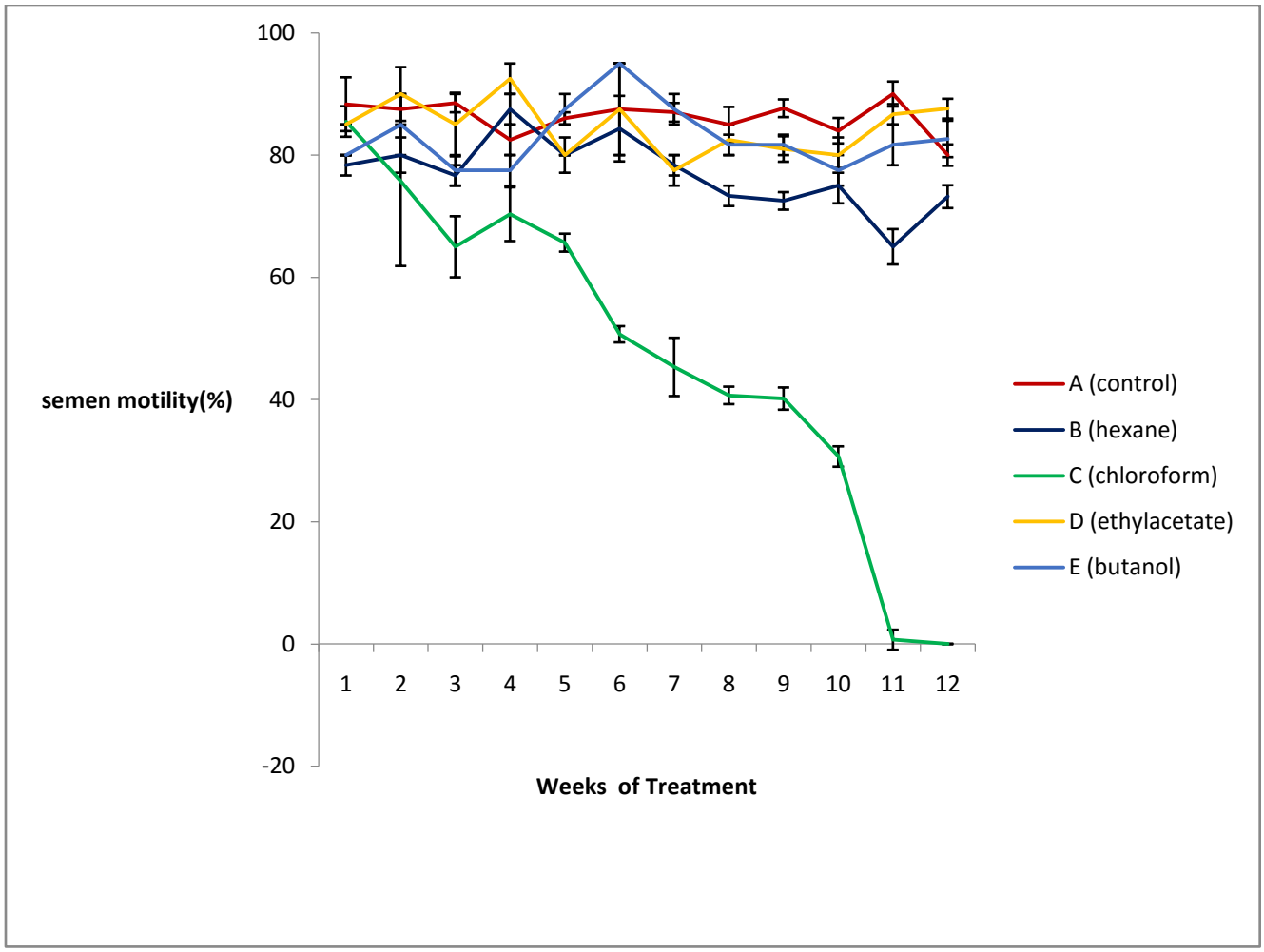


Figure 4.3: Mean (± SEM) sperm motility of New Zealand white rabbit administered different fractions from methanolic neem leaf extract.

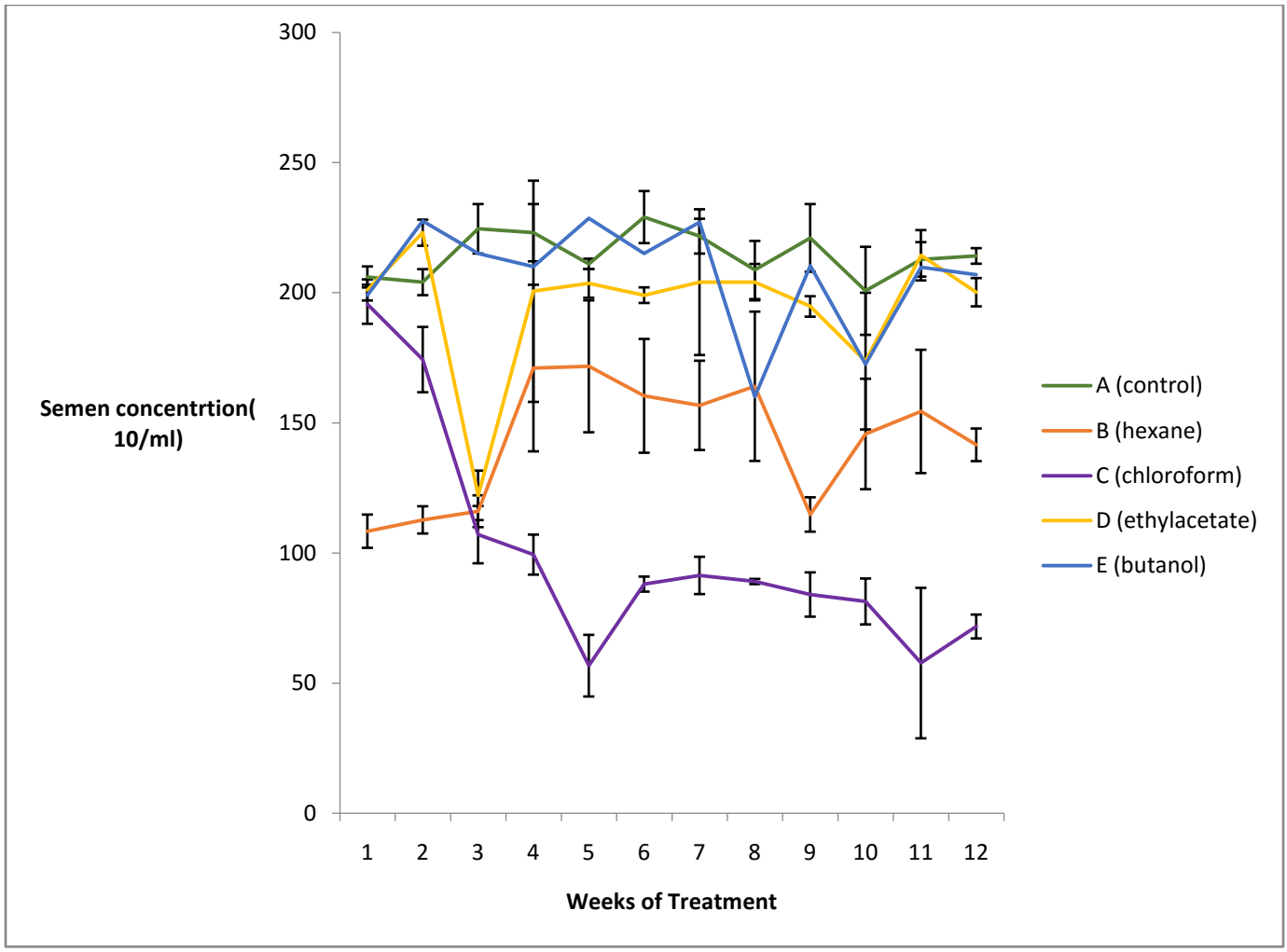


Figure 4.4: Mean (± SEM) semen concentration of New Zealand white rabbit administered different crude fractions from methanolic neem leaf extract.

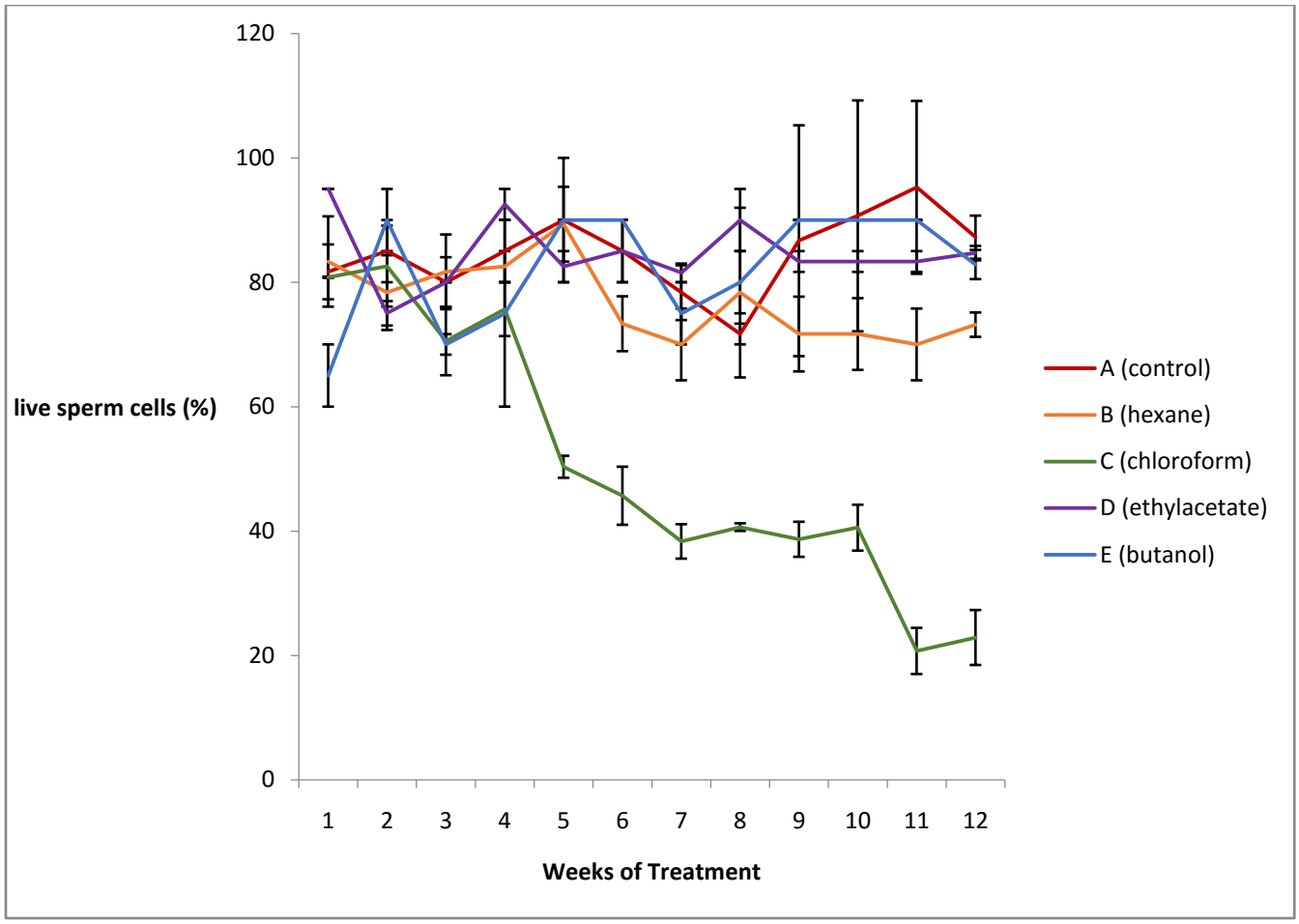


Figure 4.5: Mean(± SEM) live sperm cells of New Zealand white rabbit administered crude fractions from methanolic neem leaf extract.

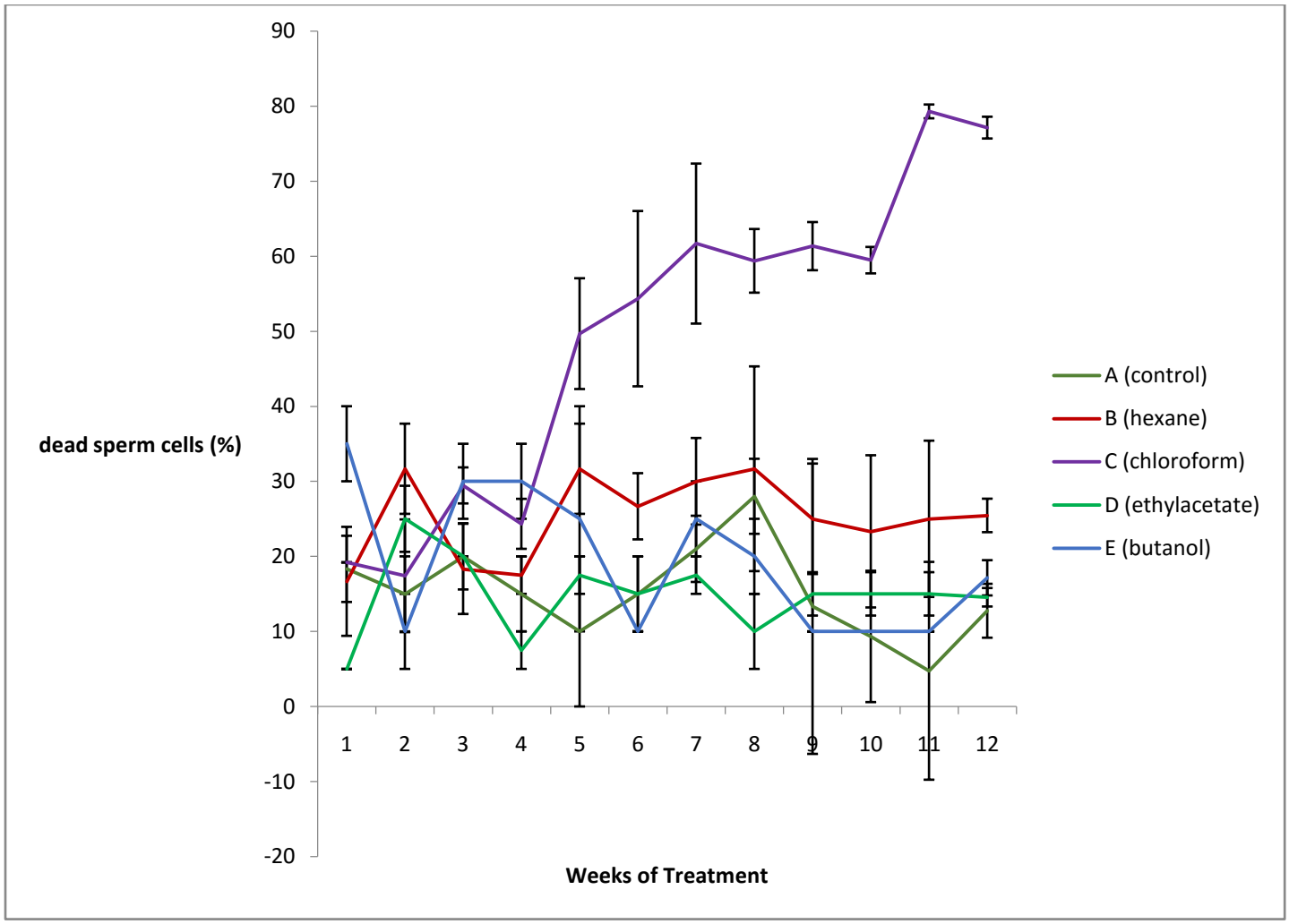


Figure 4.6: Mean (± SEM) Dead sperm cells of New Zealand white rabbit administered crude fractions from methanolic neem leaf extract

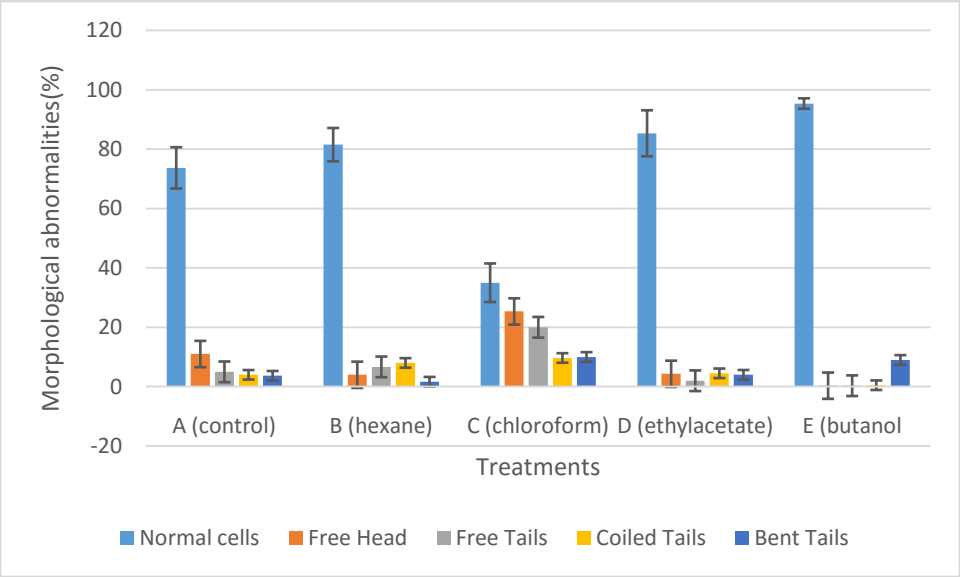


Figure 4.7: Mean (±SEM) spermatozoa morphological abnormalities (%) of rabbit bucks administered crude fractions from methanolic neem leaf extract

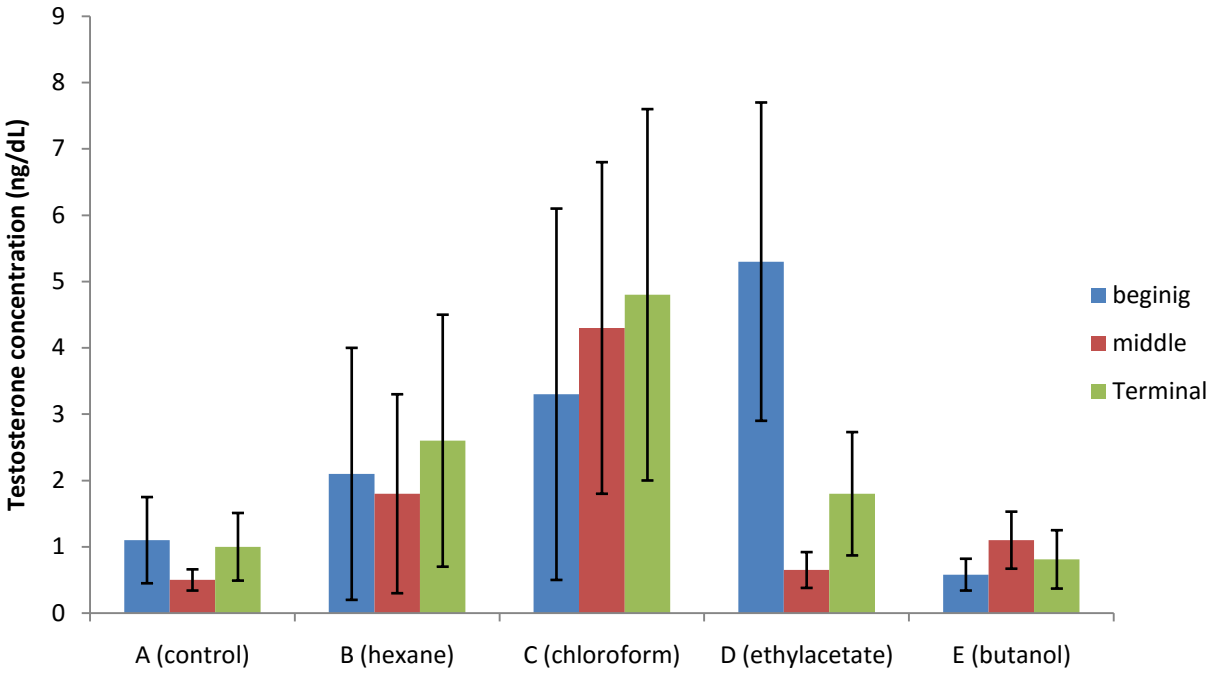


Figure 4.8: Mean (± SEM) values of serum testosterone concentration (ng/dL) of New Zealand White rabbit bucks administered crude fractions from methanolic neem leaf extract.

4.4 Effect of Fractions from Crude Methanol Neem Leaf (*Azadirachta indica*) Extracts on Haematological Parameters

The mean (± SEM) packed cell volume (PCV), haemoglobin concentration (Hb) white blood cells count (WBC), red blood cells, neutrophils and lymphocytes are presented in Table 4.3

Both the PCV and RBC values were significantly ($p < 0.05$) lower in all groups that were administered with the various fractions from the crude methanolic extract of neem leaves compared to the control values. Values of Hb, WBC, granulocytes and lymphocyte in all groups did not differ significantly ($p < 0.05$) with control values.

The platelet counts were significantly ($p < 0.05$) lower in all extract administered groups compared to the control values.

Table 4.3: Mean (±SEM) values of haematological parameters of rabbit bucks (n = 5 per group) treated with different crude organic solvent extract of *Azadirachta indica* leaf extract.

Parameters	A (water)	B (n-hexane)	C (chloroform)	D (ethylacetate)	E (n-butanol)
P C V	48.90±0.00[a]	43.26±0.02[b]	44.42±0.04[b]	43.26±0.02[b]	34.38±0.07[b]
R B C	06.54±0.00[a]	5.70±0.07[b]	5.28±0.02[b]	5.52±0.00[b]	4.81±0.00[b]
Haemoglobin	12.60±0.00 [b]	11.34±0.02[b]	12.40±0.00[b]	11.46±0.02[b]	13.84±0.02[b]
W B C	10.30±0.00[a]	9.34±0.49[a]	9.68±0.32[a]	9.34±0.49[a]	9.00±0.61[a]
Granulocyte	52.40±0.00 [b]	41.70±0.24[b]	35.82±0.17[b]	41.34±0.10[b]	28.98±0.07[b]
Lymphocyte	35.40±0.00[a]	41.38±2.87[a]	41.70±3.67[a]	41.98±2.62[a]	49.88±5.56[a]
Platelets	114.00±0.00[a]	301.00±0.00[b]	337.00±0.00[b]	301.00±00[b]	301.00±00[b]

$P<0.05$, [a, b]: Means with different superscripts on the same row are significantly different.

4.5 Effect of Fractions from Crude Methanol Neem Leaf Extract on Testes Histology

Normal histological artitechture was observed in the control group administered distilled water with intact seminisferous tubules, interstitial cells and layers of spermatogenic cells (Plate IV). However, expoliation of germ cell layers and mild depletionof spermatogenic cell layers were observed in chloroform fraction treated group (Plate VI), while ethyl acetate (Plate VII), hexane (plate V) and butanol (Plate VIII) fractions treated groups did not show appreciable changes compared to the control.

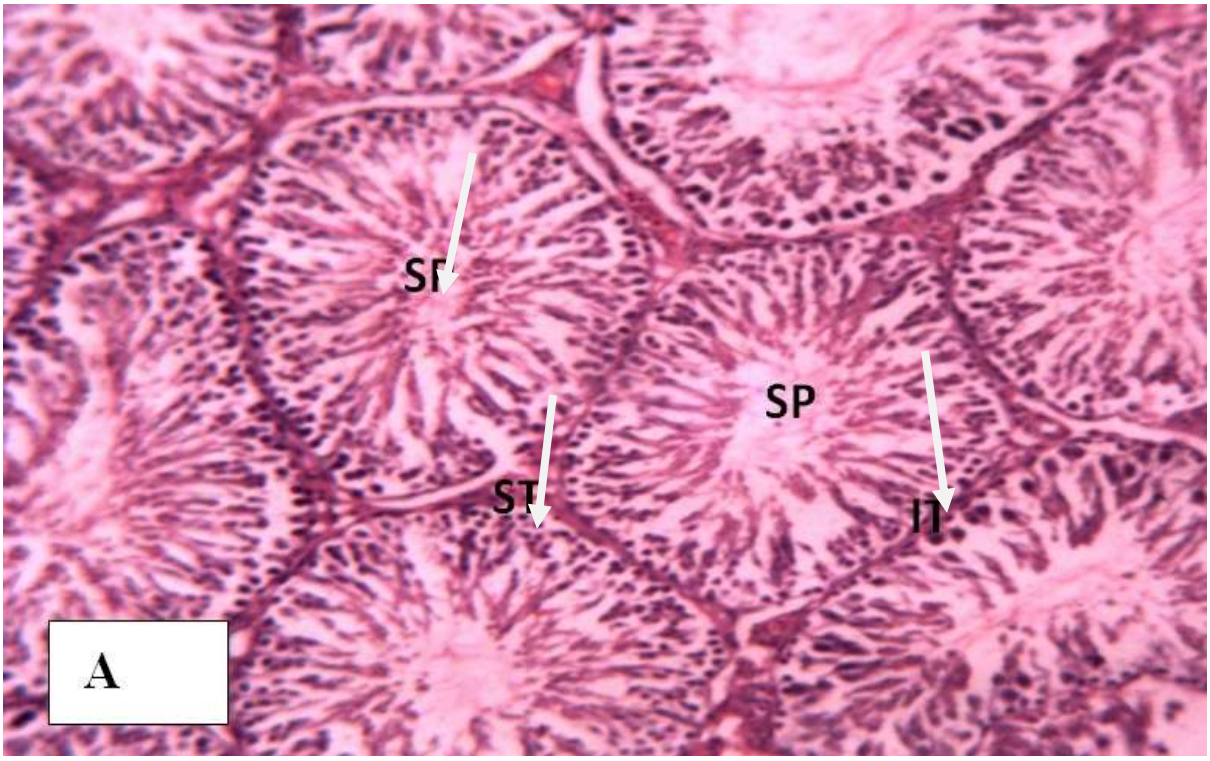


Plate IV: Photomicrographs of the testis of New Zealand white rabbit buck (Group A) showing normal histology of the testis of New Zealand white rabbit buck in the control group. Note the intact seminiferous tubules (**ST)** with thick layer of spermatogenic cells (**SP**) and interstitial cells (**IT**)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

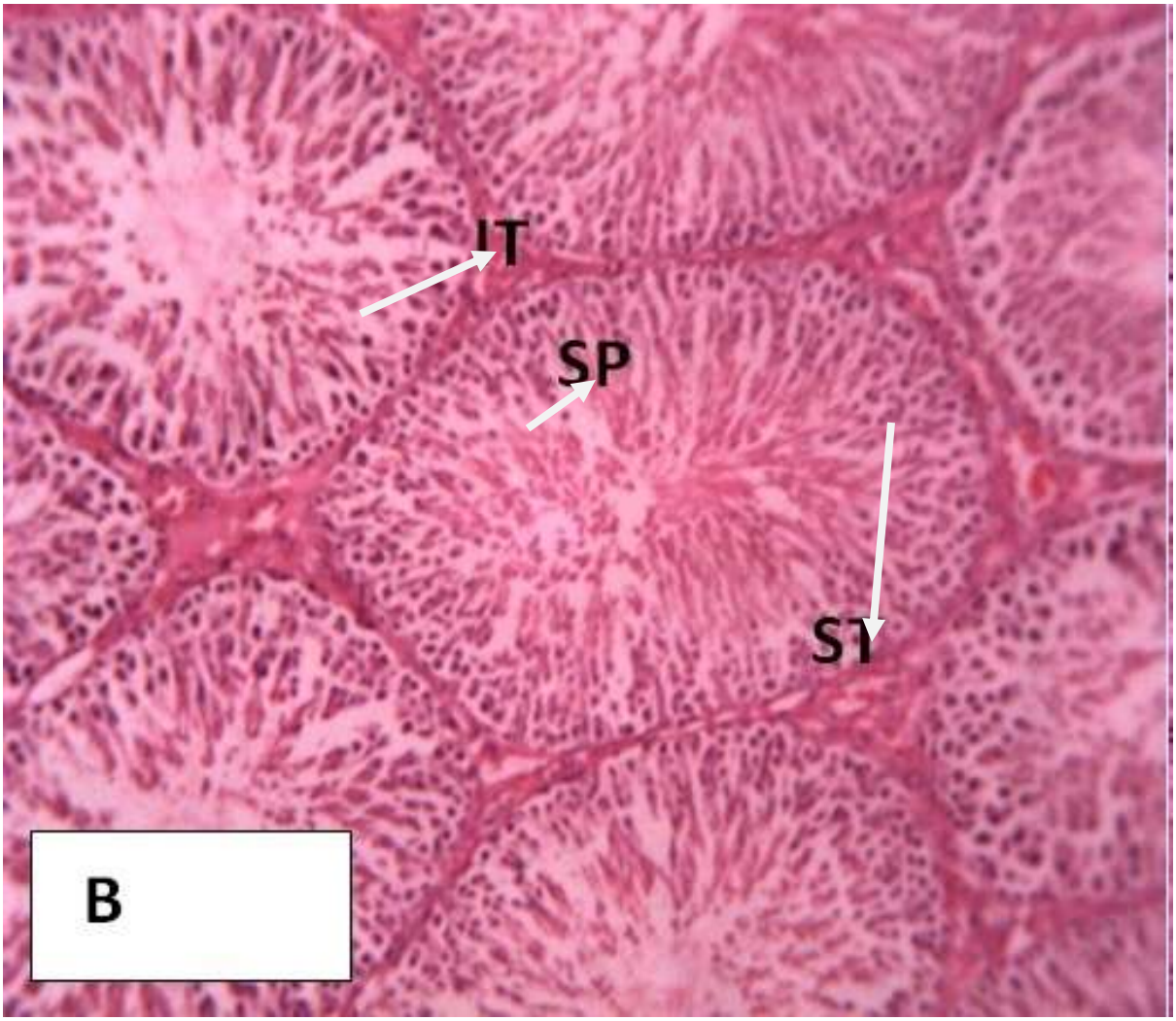


Plate V: Photomicrographs of the testis of New Zealand white rabbit buck administered hexane fraction of crude methanolic neem leaf extract (Group B)showingseminiferous tubules filled with spermatogenic cells (**SP**), leydig cells within the interstitial space (**IT**) and prominent seminiferous tubule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

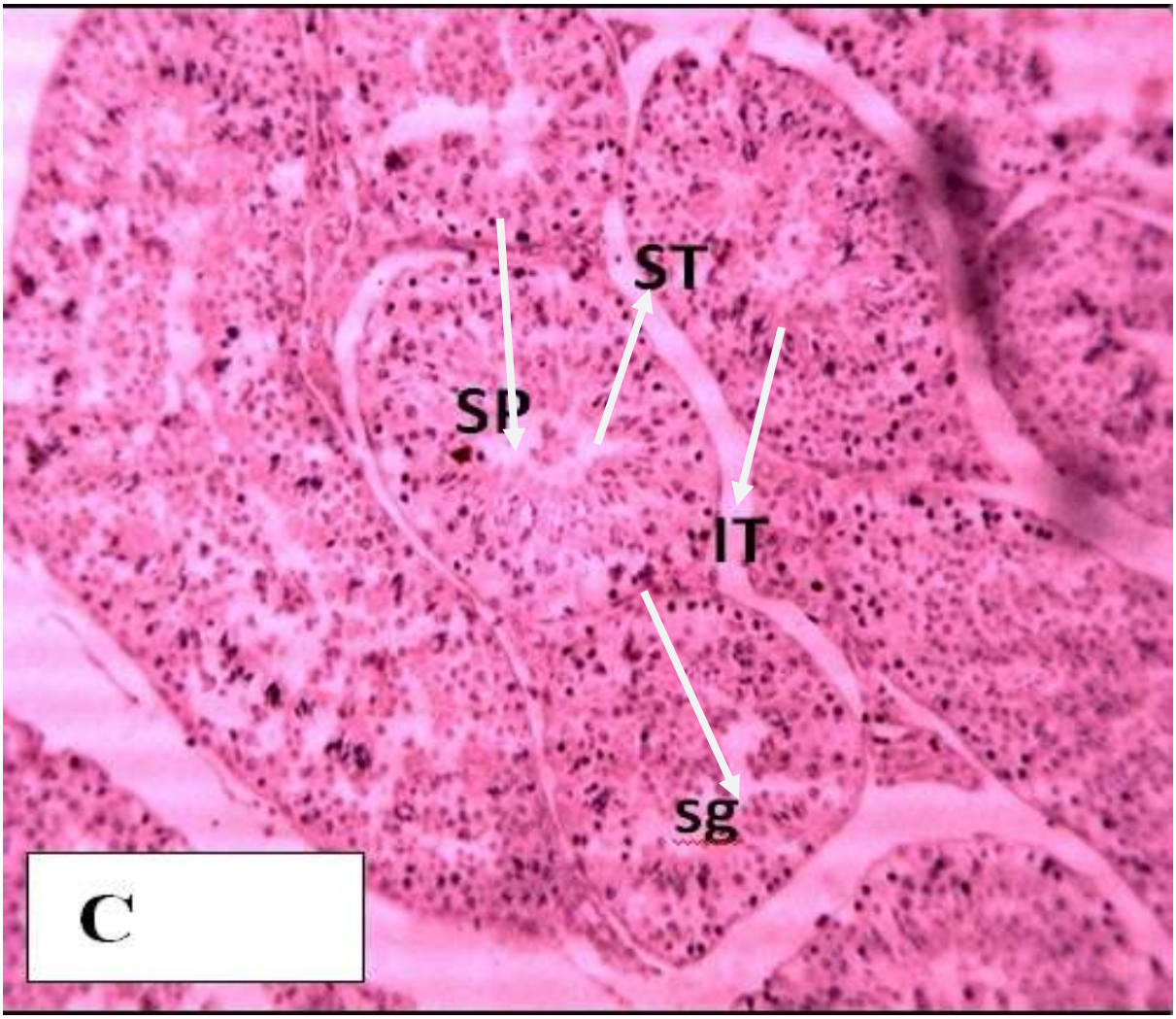


Plate VI: Photomicrograph of the testis of rabbit buck administered chloroform fraction of crude methanolic neem leaf extract (Group C) showing exfoliation of germ cell layers in the interstitial cell space (**IT**), mild depletion (vacuolation) of spermatogenic cell layer (**sg**) and prominent seminiferous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

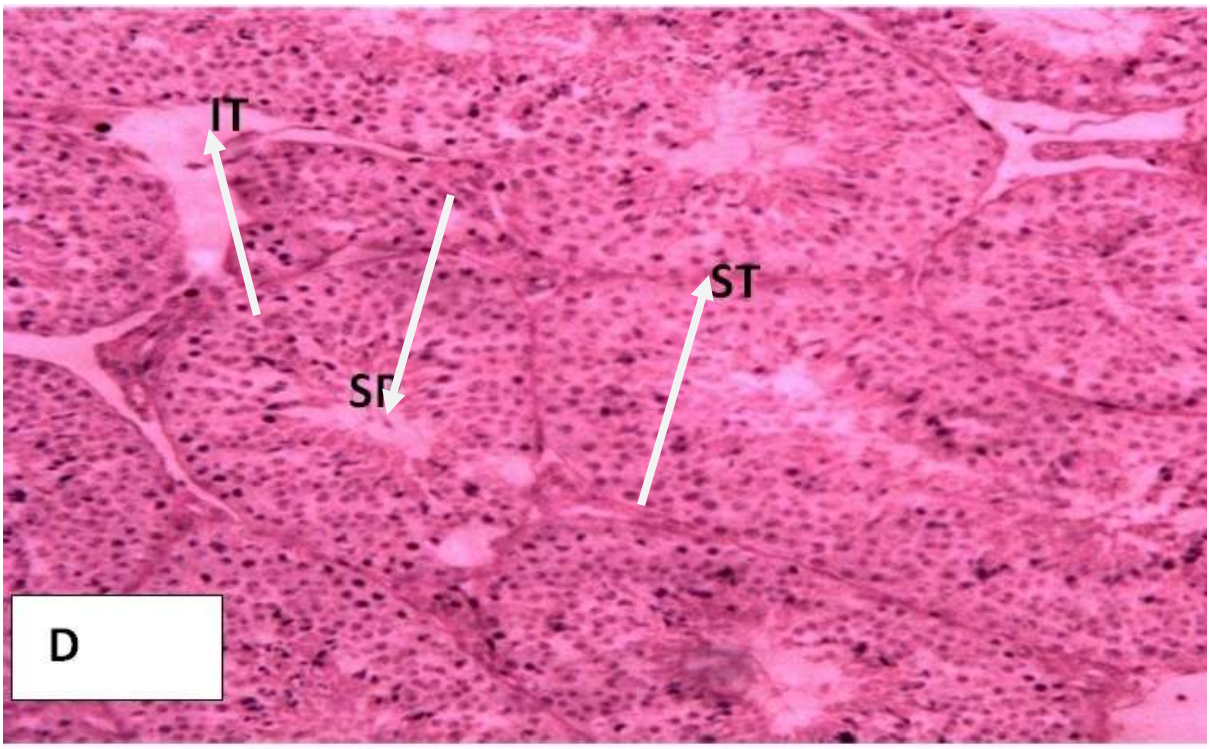


Plate VII: Photomicrograph of the testis of rabbit buck administered ethyl acetate fraction of crude methanolic neem leaf extract (Group D) showingseminiferous tubules (**ST**) filled with spermatogenic cells layers at different stages of spermatogenesis (**SP**) and mild loss of interstitial layer (**IT**)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

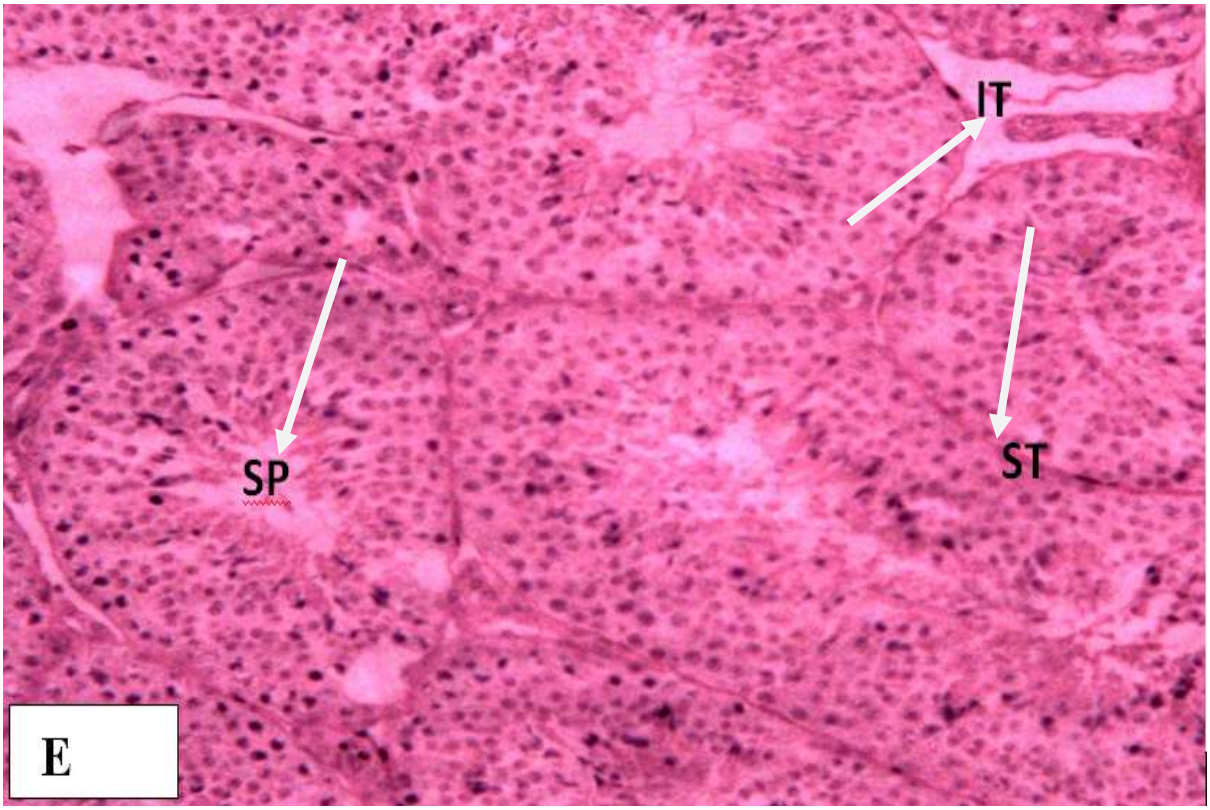


Plate VIII: Photomicrograph of the testis of New Zealand white rabbit buck administered butanol fraction of crude methanolic neem leaf extract (Group E) showing mild depletion of spermatogenic cell layer (**SP**) and the interstitial cells (**IT**) with prominent seminiferous tubules (**ST**) (H&E x100).

5.0 CHAPTER FIVE

DISCUSSION

The different biological activities of plant extracts on animals depend on the plant`s secondary metabolite and their regular consumption (Galeane *et al.,* 2017). Different organic solvents extracts were prepared from crude methanol neem (*Azadirachta indica*) leaf extract to obtain fractionsof hexane, chloroform, ethyl acetate and butanol by maceration procedure. The different fractions from methanolic neem leaf crude extract showed various composition of plant secondary metabolite. Phytochemical analysis of individual fraction shows that all the fractionswas positive for glycosides, steroids, triterpenes and tanins. All the fractions from the methanolic crude extracts are negative for anthraquinones except for hexane fraction. Chloroform fraction was negative for flavonoids. However, all the remaining three fractions used in this study were positive for flavonoids. Mossini and Kemmelmaier (2005) and Galeane *et al*., (2017) reported both the presence of terpenes and phenolics compounds in neem leaf using different organic solvents. This is similar to the findings in the present study. Although flavanoids were not detected in the chloroform fraction, Hossaini *et al* (2013) reported that the highest amount of flavonoids was found in hexane fraction followed by chloroform, ethyl acetate and n-butanol fractions with increasing order of concentration. The pH of semen of New Zealand white rabbit administered fractions from methanolic neem leaf did not differ from the control group post administration of fractions from methanolic neem leaf crude extracts for 14weeks of treatment. Lisanti *et al*. (2016) reported similar findings in mice following administration of aqueous leaf and seed extract of *Azadirachta indica*. No statistical significant differencewas observed in mean sperm motility between the control group and treated groups B, D and E. The

highest mean sperm motility was recorded from the control group followed by group receiving ethyl acetate fraction, n-hexane, and n-butanol fractions.The probable cause may be because the process of separation or purification during extraction probably changed the toxicity proportion of the phytoconstituents as earlier reported by Lisanti *et al*. (2016) in quantitative and qualitative manner. As a result, even after reconstitution, biological activity did not reappear to be the same as the whole leaves.

However, a significant decrease in sperm motility was recorded from the treatment group C that received chloroform fraction ofcrudemethanolic leaf extract 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 first week of treatment with chloroform fraction as compared with the control group.A numberof phytochemical componentshave been isolated from neem leaves and many of the secondarycompounds of the leaves have been identified, purified and some havebeen tested for their biological effects on mammals (Atawodi and Atawodi,2009).Majority of plant-derivedspermicides were attributed to triterpens obtained from saponins of several structural types, and phenol compounds (Aladakatti and Jadaramkunti, 2015). 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 spermatozoa motility may be caused by the presence of saponins in the chloroform fraction of the methanol crude extract. Saponinmay decrease spermatozoa motility and viability gradually to absolute zero (Joshi *et al*., 2011). Nimbitin, azadirachatin and salanin are alkaloids of *Azadirachta indica* whose individual effects contribute to the general biological properties of the plant (Iyare and Obaji, 2017). Kumbar *et al*. (2012) also reported similar findings that nimbolide, which is an isoprenoid of

neem leaf, immobilised and killed 100% of rat spermatozoa and suggested that isoprenoid of the leaf is highly polar in nature and causes sperm death.Al-jadidi and Hossaini (2015) linked azadirachtin, which is the major alkaloid found in ethylacetate, chloroform and hexane fractions of the methanolic crude extract to be associated with decreasing sperm motility and concentration. Aladakatti *et al.*(2001) and Aladakatti and Ahamed(2005)also reported similar results. The decrease in motility seen in the current study is probably cytotoxic, similar to the reports of Awasthy (2001), who reported sperm motility to decreases 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 theplasma membrane or by way of its synergism with blockage of somebiochemical pathway like energy utilisation (Aladakatti and Jadaramkunti 2015).The probable cause of the absolute decrease in motility might be due to cellular damage caused by chloroform fraction changing the osmotic pressure, leading to swelling and eventually cell death(Halliwell 1995). An increased number of dead spermatozoa was also observed especially at weeks 11 and 12 in group C. This findings is similar to the findings of Ghosh *et al*. (2017) who related the death of the spermatozoa to reduction in hypo-osmotic swelling of the sperm indicating that chloroform fraction extract may probably cause injury to the sperm plasma membrane. Invitro screening of most plant extracts for their spermicidal properties also indicated that it involves either loss of cellular membrane integrity or arrest the motility as an end point (Abu *et al*., 2011). Several other reports have shown sperm immobilisation properties of some plants like *Achyranthes aspera* and *Stephania hermandifolia* (Paul *et al*., 2006), *Ruta graveolens* (Harat *et al*., 2008),*Cestrum parqui* (Souad*et al*., 2007), *Allium sativum*(Chakrabarti*et al*., 2003) and *Carica papaya* (Lohiya*et al*., 2000).The non-significant change in semen gross motility in

ethylacetate and butanol fractions of crude methanolic neem leaf extract can be attributed toquercetin and ß-sitosterol, which are polyphenolic flavonoids purified from ethylacetate and butanol solvents of fresh neem leaves(Mahmood*et al*., 2011).Al-Hashemi and 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 was found in all the fractions with the exception of chloroform fraction in the current study.

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 group 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 neem leaf extract in mice, rats and mice, respectively. However, Mohan *et al*. (1997) reported significant decrease in semen volume and sperm concentration following feeding broiler cock with water washed neem seed kernel meal.

There was significantly lower sperm concentration between the control group and group C that was treated with chloroform fraction. Sperm concentration is one of the most sensitive tests for spermatogenesis since it gives the cumulative results of all stages in sperm production and it is correlated with fertility (Meistrich *et al*., 1982). Chemicals acting during the spermatogonials phase (early phase of cell multiplication) will probably have a disproportionately greater effect on sperm output than chemicals acting during the spermatid phase (Sharpe, 1991). The decrease in sperm concentration can also be related 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 whichsaponin 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. Shavah (1990) also reported a reduction in sperm concentration and motility in mice. Santra and Manna (2009), andLisanti *et al*. (2016), also reported similar results in rat.

There was significant difference in sperm morphological abnormalities in rabbits treated with chloroform fraction ofmethanolic neem leaf crude extracts compared with the control group receiving only distilled water. Increased free head and bent tail sperm morphological abnormalities wereobserved. This was contrary to the report of Mohan *et al.*, (1997), Ekaluo, *et al*. (2011)and Lisanti *et al*. (2016) who reported no significant difference in sperm head abnormalities in chicken, rats and mice, respectively.

Motility development, transformation of spermatozoa tail structures, morphological change of acrosome, disappearance of cytoplasmic droplet, and plasma membrane transformation occur at the process of sperm maturation in epididymis. Since spermatozoa comes from the changes in spermatogenic cell in seminiferous tubules during spermatogenesis, abnormality in sperm takes place in epididymis. Bansal *et al*. (2010) reported no morphological abnormalities in the sperm head, mid-piece and tail when comparing aqueous old and tender neem leaf extract.

Androgen is essential for most of the stages of spermatogenesis, meiosis in particular. Sperm production cannot proceed optimally to completion, without continuous androgen supply (Aladakatti *et al.*, 2006). Therefore, any interference of testosterone production will lead to atrophy of the organs and impairment of spermatogenesis. Although there is no statistical

significant difference in the various treatment groups and the control group, results from currentstudy shows regular decrease in plasma concentration of testosterone values in the treatment group from each neem leaf crude extract when compared with the control group. The highest testosterone value was recorded from the group D receiving ethylacetate fraction of neem leaf extract at the beginning of the experiment.

Group C receiving treatment of chloroform fraction of neem leaf extract had numerical high values compared with the control group. However, these values were not statistically significant ($P>0.05$) when compared with the control group. The findings from this study are contrary to that of Ekaluo *et al*. (2011) who reported a significant decrease in serum testosterone levels in rats treated with aqueous leaf extract of neem. Serum testosterone level decreased significantly when compared with the control group. These decreases might be as a result of drastic reduction of interstitial cell of leydig in the entire treated groups (Adekeye*et al*., 2013). It is also reported that any changes in circulating androgen would affect the internal microenvironment of the testis and thereby lead to alteration in sperm motility and metabolism (Upadhyay*et al*., 1993). A potent volatile crude extract of neem; Nim-76, have inhibited spermatozoa motility in rats and humans mainly by spermicidal effect and no alterations in hormone values were observed (Riar *et al.*, 1990; Riar*et al*., 1991).

Neem leaves, like most other tropical tree leaves, contain bioactive compounds (Kausik *et al*., 2002; Akpan *et al.,* 2008) which may affect nutrient utilisation. These bioactive compounds may also alter the haematological and serum biochemical parameters of animals. There was significant decrease in haemoglobin concentration, packed cell volume, red and white blood cell count. Although the decrease were statistically significant, the blood indices showed normal

physiological ranges as established by Kronfield and Mediway (1975); Mitruka and Rawnsley (1977) and Hewitt *et al*. (1989). There was a significant decrease in red blood cell count, pack cell volumeand haemoglobin concentration in all the treatment group. However, the values are within the normal haematological range values for the New Zealand white rabbit (Unigwe *et al*., 2016). The results from the present study agrees with the findings of Sadre *et al*. (1984), Gowda *et al*. (1998) and Biu *et al*. (2009) that neem preparations fed to laying hens significantly reduced the content of haemoglobin, erythrocyte count and packed cell volume. Lather *et al*.(2002) noticed a decrease in haemoglobin, packed cell volume mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration whereas there was increase in total leucocyte count on feeding neem seedcake at different levels in broiler chicks. Renu *et al*.(2003) reported that neem leaf extract has immune potentiating effect in broiler chicks.

The current finding, however, disagree with Saini (2004), who stated that there was significant increase in body weight, haemoglobin, packed cell volume and total erythrocyte count due to neem leaf extract (NLE) feeding within seven days.

The decrease in Hb and RBC observed in this study could suggest that fractions of methanol neem leaf crude extracts might not support erythropoiesis. However, it was only a slight decrease in all the hematological parameters observed when compared with the control group but still within the normal range values (Kronfield and Mediway, 1975; Mitruka and Rawnsley, 1977; Hewitt *et al*., 1989).

Afolabi *et al*. (2010) posited that changes in haematological parameters are often used to determine stresses due to nutrition and other factors. Numerous research efforts have been

directed at the optimal utilisation of neem leaf in the feeding and medication of farm animals (Sokunbi and Egbunike, 2000; Oforjindu, 2006; Esonu*et al*., 2006).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Summary and Conclusion

The semen characteristics, testosterone profile, haematological parameters and histology of testes of New Zealand White rabbit administered methanolic neem leaf crude extract were observed for 14 weeks at a dose rate of 300 mg/kg body weight (98days). On termination of the adminstration, the histological changes, semen abnormalities, testosterone concentration, and haematological changes of New Zealand white rabbit were evaluated.

From the finding of this work, the following conclusions were made:

1. Of all the neem leaf crude extracts evaluated, chloroform fraction of methonolic neem leaf crude extract significantly reduce gross semen motility while n-butanol, ethyl acetate, and n- hexane fractions had no significant difference on semen motility.
2. Semen volume of New Zealand white rabbit was not significantly affected by individual fractions of methanolic neem leaf crude extract.
3. Chloroform fraction of neem leaf crude extract significantly decreased semen concentration, while other fractions induced non-significant changes in on sperm concentration.

4. Of the sperm morphological abnormalities examined, sperm head and tail abnormalities were significantly higher in New Zealand white rabbit receiving chloroform fraction.
5. Testosterone concentration showed no significant difference in New Zealand white rabbit administered fractions from methanolic neem leaf crude extracts.
6. Of the haematological parameters evaluated, all fractions from methanolic neem leaf crude extract significantly reduced the packed cell volume, haemoglobin concentration, red blood cell and white cell concentration.
7. Chloroform fraction induced testicular degeneration in the testis of New Zealand white rabbit of the experimental group by inducing depletion and vacuolation of spermatogenic cell layers.
8. Chloroform neem leaf crude extract may cause infertility and testicular toxicity in New Zealand white rabbit.
9. Chloroform neem leaf crude extract may be associated with the infertility reported after feeding neem containing diet.

6.2 Recommendations

1. Feature studies should be conducted to analyse and isolate specific compounds of chloroform fraction to determine its antifertility effect.

2. Further studies should be carried out to determine the mechanism of toxicity of the chloroform fraction of *Azadirachta indica* leaf on testicular parenchyma.

3. Chloroform neem leaf fraction could be further studied for possible isolation of antifertlity agents.

6.3 Contributions to Knowledge

1. Oral administration with chloroform fraction of crude methanolic extract of neem (300mg/kg) induced testicular degeneration in the testes of New Zealand white rabbits by causing depletion and vacuolation of spermatogenic cell layers.
2. Chloroform neem leaf crude extract may cause infertility and testicular toxicity in New Zealand white rabbits.

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APPENDICE

Appendix I

Means (± SEM) semen volume (ml) of New Zealand white rabbit administered crude fractions from methanolic neem leaf extract (n=5).

Week	A (water)	B (n-hexane)	C (chloroform)	D(ethylacetate)	E(n-butanol)
1	0.77±0.89[a]	0.63±0.17[a]	0.50±0.00[a]	0.60±0.50[a]	1.00±0.30[a]
2	0.75±0.05[a]	0.76±0.15[a]	0.57±0.05[a]	0.50±0.90[a]	1.05±0.45[a]
3	0.75±0.05[a]	0.23±0.03[a]	0.65±0.15[a]	0.90±0.10[a]	0.70±0.10[a]
4	0.75±0.05[a]	0.80±0.20[a]	0.27±0.03[a]	0.90±0.10[a]	0.72±0.12[a]
5	0.75±0.05[a]	1.00±0.25[a]	0.33±0.12[a]	0.40±0.30[a]	1.15±0.35[a]
6	0.70±0.01[a]	0.67±0.12[a]	0.60±0.06[a]	0.60±0.10[a]	1.05±0.45[a]
7	0.70±0.06[a]	0.70±0.05[a]	0.50±0.06[a]	0.75±0.05[a]	1.10±0.10[a]
8	0.70±0.06[a]	0.76±0.09[a]	0.43±0.03[a]	0.65±0.05[a]	1.15±0.15[a]
9	0.83±0.09[a]	0.70±0.04[a]	0.47±0.03[a]	0.67±0.03[a]	1.00±0.12[a]
10	0.90±0.06[a]	0.63±0.03[a]	0.40±0.56[a]	0.70±0.06[a]	0.75±0.05[a]
11	0.85±0.06[a]	0.60±0.06[a]	0.43±0.33[a]	0.73±0.33[a]	0.83±0.33[a]
12	0.87±0.07[a]	0.65±0.05[a]	0.45±0.35[a]	0.73±0.34[a]	0.92±0.42[a]

P> 0.05; [a, b]= means with different superscripts on the same row are significantly different.

Appendix II

Means (± SEM) values of pH of New Zealand white rabbit administered crude fractions from Methanolic neem leaf extract.(n=5)

Week	A (water)	B (n-hexane)	C (chloroform)	D(ethylacetate)	E(n-butanol)
1	7.00±0.00[a]	7.00±0.00[a]	7.00±0.00[a]	7.00±0.00[a]	8.00±1.00[a]
2	6.00±0.00[a]	6.33±0.33[a]	6.75±0.25[a]	7.00±0.00[a]	7.00±0.00[a]
3	7.00±0.00[a]	7.00±0.00[a]	7.00±0.00[a]	7.00±0.00[a]	7.00±0.00[a]
4	6.50±0.00[a]	6.50±0.50[a]	6.67±0.33[a]	7.00±0.00[a]	7.00±0.00[a]
5	7.00±0.00[a]	7.00±0.00[a]	7.00±0.00[a]	7.00±0.00[a]	8.00±1.00[a]
6	7.00±0.00[a]	7.00±0.00[a]	7.00±0.00[a]	7.00±0.00[a]	8.00±1.00[a]
7	8.00±0.56[a]	7.67±0.67[a]	7.33±0.33[a]	8.50±0.50[a]	7.00±0.00[a]
8	7.33±0.33[a]	7.67±0.67[a]	7.33±0.33[a]	7.00±0.00[a]	7.50±0.50[a]
9	7.67±0.33[a]	7.25±0.25[a]	7.33±0.33[a]	7.00±0.00[a]	7.33±0.33[a]
10	7.33±0.33[a]	7.00±0.00[a]	7.33±0.33[a]	7.33±0.33[a]	7.50±0.50[a]
11	7.50±0.50[a]	7.33±0.33[a]	7.67±0.67[a]	7.00±0.00[a]	8.33±0.67[a]
12	7.20±0.13[a]	7.09±0.11[a]	7.13±0.98[a]	7.18±0.11[a]	7.50±0.50[a]

P>0.05

Appendix III

Means (± SEM) values of semen gross motility of New Zealand white rabbit administered crude fractions from methanolic neem leaf extract.

Week	A (water)	B (n-hexane)	C (chloroform)	D(ethylacetate)	E(n-butanol)
1	88.33±4.40 [a]	78.33±1.67[a]	85.50±2.50 [a]	85.00±0.00[a]	80.00±0.00[a]
2	87.50±2.50 [a]	80.00±2.89[a]	75.75±13.90[b]	90.00±4.40[a]	85.00±5.00[a]
3	88.50±1.50 [a]	76.67±1.67[a]	65.00±5.00[b]	85.00±5.20[a]	77.50±2.50[a]
4	82.50±2.50 [a]	87.50±2.50[a]	70.33±4.40[b]	92.50±2.50[a]	77.50±2.50[a]
5	86.00±1.00 [a]	80.00±2.89[a]	65.67±1.47[b]	80.00±0.00[a]	87.50±2.50[a]
6	87.50±7.50 [a]	84.33±5.36[a]	50.83±1.33[b]	87.50±7.50[a]	95.00±0.00[a]
7	87.00±1.53 [a]	78.33±1.67[a]	45.33±4.77[b]	77.50±2.50[a]	87.50±2.50[a]
8	85.00±2.89 [a]	73.33±1.67[a]	40.67±1.43[b]	82.50±2.50[a]	81.61±1.67[a]
9	87.67±1.45 [a]	72.50±1.44[b]	40.15±1.82[b]	81.00±2.08[a]	81.67±1.67[a]
10	84.00±2.08 [a]	75.00±2.89[a]	30.67±1.67[b]	80.00±2.89[a]	77.50±2.50[a]
11	90.00±2.04 [a]	65.00±2.89[b]	00.68±1.64[b]	86.67±1.67[a]	81.67±3.33[a]
12	80.00±1.75 [a]	73.20±1.87[a]	00.00±0.00[b]	87.60±1.62[a]	82.65±2.98[a]

P-value=0.0001, [a, b]: Means with different superscripts on the same row are significantly different

Appendix IV

Means (± SEM) values of sperm concentration (10^6/ml) of New Zealand white rabbit administered crude fractions from Methanolic neem leaf extract. (n=5).

Week	A (water)	B (n-hexane)	C (chloroform)	D(ethylacetate)	E (n-butanol)
1	206.00±4.04[a]	108.33±6.39[b]	195.50±7.50[b]	201.00±4.05[a]	199.00±21.00[a]
2	204.00±5.00[a]	112.67±5.23[b]	174.25±12.54[b]	223.00±5.02[a]	227.50±18.50[a]
3	224.50±9.50[a]	116.00±6.11[b]	107.00±11.00[b]	209.00±9.50[b]	215.00±17.00[a]
4	223.00±11.00	171.00±32.00[a]	99.33±7.69[b]	200.50±42.50[a]	210.00±16.54[a]
5	211.00±2.00[a]	171.67±25.34[a]	56.67±11.89[b]	203.50±5.50[a]	228.50±3.50[a]
6	229.00±10.00	160.33±21.86[a]	88.00±2.89[b]	199.00±3.00[a]	215.50±3.50[a]
7	221.67±6.69[a]	156.67±17.15[a]	91.33±7.17[b]	204.00±28.00[a]	227.00±5.50[a]
8	208.67±11.17	164.00±28.68[a]	89.00±1.00[b]	204.00±7.00a	160.00±48.00[a]
9	221.00±13.0[a]	114.75±6.60[b]	84.00±8.50[b]	194.67±3.93[a]	210.33±6.69[a]
10	200.67±16.91	145.67±21.22[a]	81.33±8.82[b]	173.67±26.26[a]	172.50±50.50[a]
11	212.75±6.61[a]	154.33±23.69[a]	57.66±28.90[b]	214.33±9.67[a]	209.67±16.25[a]
12	214.07±2.99[a]	141.51±6.27[b]	71.71±4.59[b]	200.14±5.43[a]	206.86±7.15[a]

$P<0.005$, [a, b]: Means with different superscripts on the same row are significantly different.

Appendix V

Means (± SEM) values of live spermatozoa (%) of New Zealand white rabbit administered crude fractions from methanolic neem leaf extract. (n=5).

Week	A (water)	B (n-hexane)	C (chloroform)	D(ethylacetate)	E(n-butanol)
1	81.67±4.41[a]	83.33±7.26[a]	80.80±0.10[a]	95.00±0.00[a]	85.00±5.00[a]
2	85.00±5.00[a]	78.33±6.01[a]	82.59±6.51[a]	75.00±1.96[a]	90.00±5.00[a]
3	80.00±0.00[a]	81.67±6.01[a]	70.55±5.50[b]	80.00±4.02[a]	70.00±1.67[b]
4	85.00±5.00[a]	82.50±2.50[a]	75.67±4.33[b]	92.50±2.50[a]	75.00±15.00[b]
5	90.00±10.0[a]	89.33±6.01[a]	50.33±1.77[b]	82.50±2.50[a]	90.00±0.00[b]
6	85.00±5.00[a]	73.33±4.41[a]	45.67±4.67[b]	85.00±5.00[a]	90.00±0.00[a]
7	78.33±4.41[a]	70.00±5.77[a]	38.33±2.77[b]	81.50±1.50[a]	75.00±5.00[a]
8	71.67±1.67[a]	78.33±13.64[a]	40.63±0.61[b]	90.00±5.00[a]	80.00±5.00[a]
9	86.67±18.56	71.67±6.01[a]	38.67±2.82[b]	83.33±1.67[a]	90.00±0.00[b]
10	90.67±18.56	71.67±5.77[a]	40.54±3.69[b]	83.33±1.67[a]	90.00±0.00[b]
11	95.25±13.9[a]	70.00±5.77[a]	20.72±3.71[b]	83.33±1.67[a]	90.00±0.00[a]
12	87.24±3.47[a]	73.18±1.96[a]	22.88±4.42[b]	84.68±1.16[a]	82.85±2.35[a]

$P<0.05$, [a, b]: Means with different superscripts on the same row are significantly different.

Appendix VI

Means (± SEM) values of dead spermatozoa (%) of New Zealand white rabbit administered crude fractions from methanolic neem leaf extract.

Week	A (water)	B (n-hexane)	C (chloroform)	D(ethylacetate)	E(n-butanol)
1	18.33±4.41[a]	16.67±7.26[a]	19.20±00.01[b]	5.00±0.00[a]	35.00±5.00[a]
2	15.00±5.00[a]	31.67±6.01[a]	17.41±07.51[a]	25.00±4.40[a]	10.00±5.00[a]
3	20.00±0.00[a]	18.33±6.01[a]	29.45±02.40[b]	20.00±4.41[a]	30.00±5.57[a]
4	15.00±5.00[a]	17.50±2.50[a]	24.33±03.33[b]	07.50±2.50[a]	30.00±5.00[a]
5	10.00±10.00	31.67±6.01[a]	49.67±07.38[b]	17.50±2.50[a]	25.00±15.00[a]
6	15.00±5.00[a]	26.67±4.41[a]	54.33±11.68[b]	15.00±5.00[a]	10.00±0.00[a]
7	21.67±4.41[a]	30.00±5.77[a]	61.67±10.66[b]	17.50±2.50[a]	25.00±5.00[a]
8	28.33±5.00[a]	31.67±13.64[a]	59.37±04.24[b]	10.00±5.00[a]	20.00±5.00[a]
9	13.33±19.65	25.00±7.36[a]	61.33±03.21[b]	15.00±2.89[a]	10.00±0.00[b]
10	09.33.±08.75	23.33±10.14[a]	59.46±01.76[b]	15.00±2.89[a]	10.00±0.00[b]
11	04.75±14.51	25.00±10.41[a]	79.28±00.92[b]	15.00±2.89[a]	10.00±0.00[a]
12	12.75±3.59[a]	25.45±2.23[a]	77.12±01.45[b]	14.55±1.23[a]	17.14±2.35[a]

$P<0.05$, [a, b]: Means with different superscripts on the same row are significantly different.

Appendix VII

Mean (±SEM) Spermatozoa morphological abnormalities (%) of rabbit bucks administered crude fractions from methanolic neem leaf extract.

Parameters	A (water)	B (hexane)	C (chloroform)	D (ethylacetate)	E (butanol)
Normal cells	76.57±6.96[a]	79.66±5.61[a]	35.00±6.49[b]	85.33±7.75[a]	89.84±1.76[a]
Free Head	11.00±1.00[a]	4.00±1.00[a]	25.33±0.88[b]	4.17±2.96[a]	0.33±0.33[b]
Free Tails	5.00±0.58[a]	6.67±3.33[a]	20.00±0.58[b]	2.00±2.00[a]	0.33±00.33[a]
Coiled Tails	4.00±2.00[a]	8.00±3.79[a]	09.67±0.33[b]	4.50±4.50[a]	0.50±0.50[a]
Bent Tails	3.67±1.76[a]	1.67±0.33[a]	10.00±2.00[b]	4.00±4.00[a]	9.00±6.00[a]

P>0.05