EFFECTS OF AQUEOUS EXTRACT OF Allium *cepa* (ONION) ON CIPROFLOXACIN-INDUCED TESTICULAR TOXICITY IN WISTAR RATS

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NIGERIA

MAY, 2015

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BY

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M.Sc/MED/3602/2009-2010

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF REQUIREMENTS FOR THE AWARD

OF A

MASTER DEGREE IN HUMAN ANATOMY.

DEPARTMENT OF HUMAN ANATOMY,

FACULTY OF MEDICINE

AHMADU BELLO UNIVERSITY, ZARIA

NIGERIA

MAY, 2015

DECLARATION

I, ALIYU, Abdullahi Taiye hereby declare that this thesis titled **"Evaluation of the Effects of A*llium cepa* (onion) Extract on Ciprofloxacin-induced Testicular Toxicity in Wistar Rats"** was performed by me in the department of Human Anatomy, under the supervision of Prof. S.A. Ojo (DVM, M.Sc., Ph.D, FCVSN) and Dr (Mrs) J. N. Alawa (B.Sc., M.Sc., Ph.D.). The information derived from the literature has been duly acknowledged in the text and a list of the references is provided. No part of this work has been presented for another degree or diploma at any other institution.

Abdullahi Taiye ALIYU	**…….**	**……..**
Name of student	**Signature**	**Date**

CERTIFICATION

This thesis titled **"Evaluation of the Effects of A*llium cepa* (onion) Extract on Ciprofloxacin-induced Testicular Toxicity in Wistar Rats"** by Abdullahi Taiye ALIYU meets the regulations governing the award of the degree of Masters of Science of the Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated
to
My late parents, Alhaji Aliyu Jabata and Alhaja Amina Kawu-Olobi Jabata and my late brother Habeeb Aliyu Jabata asking Allah to forgive them all and grant them Jannatu-l-firdaus, Ameen.

ACKNOWLEDGMENTS

First of all, I thank the Almighty Allah, the All-wise and the All-knowing for giving me the opportunity and strength to conclude my M.Sc. programme.

My profound gratitude goes to my major supervisor, Prof. S.A. Ojo, a great mentor, an outstanding scholar and an indefatigable academic for guidance, support and commitment that he offered me in making this work a success. Sir, the unwavering confidence you had in me as well as your determination in ensuring I did not give up until the final draft of this thesis was completed is sincerely appreciated. I am greatly honoured to be supervised by you. May Almighty God shower you with His care and blessings, grant you sound health and continue to uplift you.

I also appreciate the efforts of my minor supervisor, Dr. (Mrs) J.N. Alawa, a versatile and an energetic researcher, and an academic with a difference for her keen interest in my work and her contributive role in the designing, shaping and improving the work, right from its inception to its conclusion. Ma, your unusual passion for quality work and your valuable criticisms have really assisted in making this work a reality. May Almighty God crown your present and future endeavors with success.

My special regards goes to the Head, the department of Human Anatomy, Prof. S.S. Adebisi, for his role in ensuring the smooth running of the academic programmes in the department.

I appreciate the efforts of my lecturers, Prof. S.S. Adebisi, Dr. B. Danborno, Dr. A.O. Ibegbu, Dr. W.O. Hamman, Dr. J.N. Alawa, Dr. D.T. Ikymbe, Dr. A.U. Umana, and Mr. S.A. Musa for the quality of knowledge of anatomy they exposed me to.

My thanks also go to Dr. A.A. Buraimoh, our vibrant PG coordinator and Dr. A.U. Dahiru for the encouragement and concern they showed towards me in the completion of this program.

I also want to register my appreciation to Mr. Peter Akpulu and Mr. Bamidele for the technical assistance rendered in processing my tissue slides. Dr. M.O. Samaila, a consultant histopathologist in the department of Pathology, ABUTH deserves my gratitude for the interpretation of my slides despite her tight schedule.

To the staff of protozoology and entomology laboratory, Department of parasitology, Faculty of Veterinary Medicine, in persons of Mr. Baale, Sis. Kate, and Mal. Hussein, I say thank you for ensuring that the aspect of fluorescent microscopy of this work was achieved.

Mal. Y.Y. of department of public health, faculty of Veterinary Medicine, Hajia Jameela and Dr. (Mrs) Bola, both of Biotechnology centre, Mal. Bashir of the Multi-user laboratory, Mal. Aliyu Mansur of department of Biochemistry, Sadiya Ahmadu and Fareeha Muzaffar of the department of Human Anatomy, All deserve my gratitude for their role in running successfully my caspase-3 activity kit.

My appreciation and thanks go to my course mates: Ali abdussalam(The late), Jibril Murtala, Zubairu Rayyanu, Danladi Jibrin, Abel Agbon, Elvis Godam Tams, Dr. Anyawu Lofty-John, Rukayya Salahudeen, Zainab Dauda, and Vera Afuwai for the support and solidarity they showed in ensuring the completion of this programme. I wish you all successful undertakings in your future endeavours.

Many friends and associates who assisted in achieving this feat also deserve to be mentioned. Saheed Ibraheem (the doctor in the making), Yusuf Shuaib, AbdulAzeez Balogun, Mal. Sulyman Shehu (Abu 'Ubaidah), Yusuf Ayoola Bello, Adepoju Shamsudeen, AbdulMujeeb Aremu, Mal. Ahmad Bala, AbdulHakeem Miko, Mal. Umar-Faruoq Mahe Abubakar and Mal. Muhammad Ahmad of BUK who made his room available for me to stay throughout this study period and many others who have contributed in one way or the other, they are all appreciated.

My brothers and sisters have contributed immensely towards the actualization of this progamme. Abu AbdurRahman, Abu AbdulHakeem, Abu Asiya, Abu AbdusSalaam (The late), Khadijah, Hafsah (Kehinde), Asmau (Taiye kekere) and others whom Allah used as pillars and shock absorbers for me from financial and emotional hardship throughout my stay in Zaria are highly acknowledged. I ask Allah to reward them with what is better than what they have offered me.

To my inestimable jewel, Ummu Taymiyyah Bint Abdullah Bn Idris and my precious sprouts; Taymiyyah, Unaysah and Ali (Ibn Al-Madeeni), I say big thank you for the love, care and sacrifice you put up whenever I was away to Zaria for this programme. May Allah shower His love and mercy on you all.

To my late parents, Al-Imam Aliyu Jabata and Alhaja Aminat Aliyu Jabata and my late brother, Habeeb Aliyu Jabata, the last two of whom died while I was running this programme. I ask Allah to reward all of them abundantly and engulf them all with His mercy, forgive them their shortcomings, take care of those they left behind, count me as one of their 'Hasanaat' (good deeds) and grant them the highest level of paradise (Firdaus).

ABSTRACT

This study was carried out to investigate the effects of *Allium cepa* (onion extract) on ciprofloxacin induced testicular toxicity. Twenty five adult male Wistar rats weighing between 150g – 250g assigned randomly to five groups (A-E) were used for this study. The control group (A) received 1 ml of distilled water daily while group B was given 22.86 mg/Kg body weight(b.w) of ciprofloxacin. Group C was administered 22.86 mg/Kg b.w. of ciprofloxacin plus 100 mg/Kg b.w. of onion extract. Group D received the same dose of ciprofloxacin plus 200 mg/Kg b.w. onion extract while group E was given 100 mg/Kg b.w. of onion extract only. The experiment lasted for 25 days to cover two seminiferous cycles in rats and the route of administration was oral.

Semen samples from the epididymis were collected at the end of the experimental period and used for the analysis of sperm count, sperm motility and sperm viability. Blood samples were also collected for the determination of haematological parameters and the blood serum was used for the determination of antioxidant biomarkers. The haematological parameters *i.e* red blood cell count (RBC), heamoglobin count (Hb), Hematocrit (HCT), white blood cell count (WBC) and platelet count (PLT) were studied. Superoxide dismutase (SOD), catalase (CAT) and Malondialdehyde (MDA) were the antioxidant biomarkers investigated. Testicular protein concentration and caspase-3 activity were also determined.

The animals were sacrificed and the testes removed and used for histopathological studies, Flourescence microscopy was also employed to study the DNA integrity of the sperm cells following administration of ciprofloxacin and onion extract.

The results obtained revealed that weights of the testes were comparable in all the groups. *Allium cepa* administration led to a significant ($p<0.05$) decrease in body weights in a dose-dependent manner. It was also observed that administration of ciprofloxacin significantly

($p<0.05$) decreased sperm count, motility and viability, and SOD activity. Similarly, administration of ciprofloxacin caused a significant increase ($p<0.05$) in MDA level, testicular apoptosis (as marked by caspase-3 activity level) and DNA fragmentation. Rats given ciprofloxacin and 100 mg/Kgb.w of onion extract showed significant($p<0.05$) increase in sperm parameters with less testicular apoptosis and DNA fragmentation compared to the group given only ciprofloxacin. However, administration of 200 mg/Kgb.w of onion extract to ciprofloxacin-given rats potentiated the toxic effect of ciprofloxacin on sperm parameters, testicular apoptosis and DNA fragmentation.

Histological studies showed that ciprofloxacin caused testicular damage characterized by disruption of spermatogenesis. Onion extract at low dose was observed to protect against the damage caused by ciprofloxacin administration.

In conclusion, ciprofloxacin induced caspase-dependent apoptosis in the testis and this was ameliorated by the low dose of *Allium cepa* extract as the high dose of the extract was found to exacerbate the effect of ciprofloxacin, onion extract was also found to be apoptogenic both at low and high doses.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
Bw	Body weight.
BSA	Bovine serum albumin.
CAT	Catalase
DEVD-pNA	Asp-Glu-Val-Asp-pNitroanilide (Caspase 3 substrate)
DNA	Deoxyribonucleic acid
HCT	Haematocrit
LDL	Low density lipid
LPO	Lipid Peroxidation
MDA	Malondialdehyde
OS	Oxidative Stress
PLT	Platelet
RBC	Red blood cell
ROS	Reactive oxygen species
RNA	Ribonucleic acids
SOD	Superoxide dismutase
ST	Seminiferous tubules
$t_{\frac{1}{2}}$	Half life
WBC	White blood cell

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Spermatogenesis is the sequence of events by which spermatogonia are transformed into mature sperm (Moore and Persaud, 2008).

Proliferation and differentiation of the male germ cells and the intratesticular and extratesticular mechanisms of regulation of spermatogenesis can be disturbed at every level (Holstein *et al*., 2003). Drugs, hormones and their metabolites, different toxic substances or x-radiation may reduce or destroy spermatogenesis (Holstein *et al.*, 2003, Sheiner *et al*., 2003). Among the drugs that have been reported to disturb spermatogenesis is ciprofloxacin (Grabe *et al.,* 1986).

Ciprofloxacin is a synthetic antibacterial agent that has been approved in more than 100 countriesworld-wide (Wolfson and Hooper, 1985). It belongs to the family of fluoroquinolones with a very broad spectrum against microbial pathogens, especially Gram negative infectious diseases. Ciprofloxacin is well absorbed orally and induced its antibacterial action mainly by inhibition of DNA gyrase, which is equivalent to topoisomerase II in mammalian cell (Akasaka *et al., 1998*:Liu and Wang, 1999).

Ciprofloxacin is routinely used by fertility specialists to treat bacterial infections occurring prior to *in vitro* fertilization treatment, or when high concentrations of leukocytes are present in the semen of their patients (Herbold *et al*., 2011).

In vivo genotoxicity studies have shown that ciprofloxacin is safe for therapeutic use (Herbold *et al*., 2011). However, other studies have demonstrated ciprofloxacin to significantly impair both testicular function and structure. Administration of ciprofloxacin

significantly reduced sperm count, motility and daily sperm production in rats (Grabe *et al.*, 1986).

In healthy rats, ciprofloxacin caused obvious histological damage associated with a decrease in testicular volume and sperm concentration (Abd-Allah *et al*., 2000). Khaki *et al*., (2008) reported that oral administration of ciprofloxacin resulted in increase in germ cell apoptosis in the testes of male rats. Weyers *et al*., (2002) reported that there is an increased peroxide radical generation in the testis following ciprofloxacin treatment in mice. This increase induces DNA single-strand breaks and chromosomal aberrations as demonstrated by *in vitro* genotoxicity studies (Sanchez *et al*., 2005: Itoh *et al*., 2006). In addition, previous study showed that caspase-3, which has an important role in apoptosis, could be activated by ciprofloxacin (Olivia *et al*., 2002: Zhang *et al*., 2003).

Allium cepa (onion) has a beneficial effect on disease treatment worldwide and has been used since ancient times as a medicinal and food source (Khaki *et al*., 2009). *Allium cepa* is commonly used in our daily diet and has been source of much interest because of its antithrombotic, hypolipidaemic, hypotensive, diaphroretic, antibiotic, antidiabetic, antiatherogenic and anticancer medicinal properties (Augusti,1996).

The biological action of onion is ascribed to organosulfur and phenolic compounds it contains (Khaki *et al.,* 2009). Research has shown that onion contains exogenous and endogenous antioxidants such as selenium, glutathione, vitamins A, B, and C, and flavonoids such as quercetin and isorhamnetin (Markham, 1982).

These antioxidants protect DNA and other important molecules from oxidation and damage, which would otherwise induce apoptosis, and could improve sperm health parameters, increasing the rate of fertility in men (Yang *et al.,* 2006).

Researchers were able to show the androgenic activity of *Allium cepa* juice as well as its ability to recover spermatogenesis in *Toxoplasma gondii* infected rats (Khaki *et al.,* 2009).

1.2 Statement of research problem

Onion is one of the most important aphrodisiac foods. As an aphrodisiac, it increases libido and strengthens the reproductory organs (Kumar *et al.,* 2010).*Allium cepa* (Onion) extract has also been reported to improve sperm parameters in rats (Khaki *et al*., 2009). Despite this documented androgenic activity of onion, little is known about its mechanism of action. As a result, unravelling the mechanisms of action through which onion improves male reproductive function is the problem this work set out to answer.

1.3 Justification of the study

Antibiotics are commonly prescribed for many disease conditions. Fertility specialist clinics also prescribe ciprofloxacin for the treatment of genitourinary infections. This will exacerbate the infertility conditions of the male as studies have demonstrated that ciprofloxacin significantly impairs both testicular structure and function. As such, discovery of drugs that will inhibit the anti-fertility effect of ciprofloxacin without affecting its antibiotic activity is needed. *Allium cepa* may serve as a potential candidate for this.

1.4 Significance of the study

This work is significant as it seeks to elucidate the mechanism of action of *Allium cepa* on testicular toxicity due to ciprofloxacin treatment theknowledge of which canprovide a better understanding of its therapeutic potentials particularly as a sperm boosting agent.

1.5 Hypothesis

Allium cepa extract will protect against testicular toxicity due to ciprofloxacin administration in male Wistar rats.

1.6 Aim and objectives of the study

1.6.1 Aim of the study

The aim of this study is to investigate the effects of the *Allium cepa*extract on ciprofloxacin-induced testicular toxicity in Wistar rats.

1.6.2Objectives of the study

The objectives of the study are to investigate the effects of

i. *Allium cepa* extracton sperm apoptosis using acridine orange and fluorescence microscopy.
ii. *Allium cepa* extract on changes in the testis histology and sperm parameters due to ciprofloxacin treatment.
iii. *Allium cepa* extract on testicular apoptosis induced by ciprofloxacin using caspase-3 activity assay.
iv. *Allium cepa*extract on oxidative stress indicators.
v. *Allium cepa* extract and ciprofloxacin on haematological parameters.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Spermatogenesis

Spermatogenesis is the process by which a complex, interdependent population of germ cells produces spermatozoa. Mammalian spermatogenesis can be divided into two phases (Rodriguez *et al*., 1997). The first phase of spermatogenesis starts after birth. It is characterized by the sequential appearance of cells within the seminiferous tubules, which corresponds to each stage of the germinal cell. The second phase starts during puberty (ongoing phase), characterized by the concomitant presence of stem cells and early spermatogonia to fully differentiated spermatozoa (Rodriguez *et al*., 1997). Spermatogenesis is a chronological process that takes about 60 days in rats and 72 days in man to complete (Hess, 1990). During this period, the immature germ cellscyclically develop into highly specialized spermatozoa (Rodriguez *et al*., 1997).

The balance between germ cell proliferation, differentiation and apoptosis is critical to control spermatogenesis. During establishment of spermatogenesis at the puberal age, early germ cells apoptotic wave occurs, aimed at removing abnormal germ cells and maintaining a proper ratio between maturing germ cells and Sertoli cells (Koji, 2001).

Spertmatogonia that are dormant in the seminiferous tubules of the testis since the fetal period begin to increase in number at puberty. After several mitotic divisions, the spermatogonia grow and are transformed into primary spermatocytes. Each primary spermatocyte undergoes the first meiotic division to form two haploid secondary spermatocytes. The secondary spermatocytes undergo a second meiotic division to form four haploid spermatids. The spermatids are gradually transformed into four mature sperms by the process known as spermiogenesis. When spermiogenesisis is complete, the sperms enter the lumina of the seminiferous tubules(Moore and Persaud, 2008).

Sertoli cells lining the seminiferous tubules support and nurture the germ cells and may be involved in the regulation of spermatogenesis. Sperms are transported passively from the seminiferous tubule to the epididymis, where they are stored and become functionally mature. The epididymis is continous with the ductus deferens which transport the sperm to the urethra (Moore and Persaud, 2008).

Mature sperms are free swimming; actively motile cells consisting of a head and a tail. The head contains the haploid nucleus the anterior two-third of which is covered by the enzyme-containing acrosome. The enzyme facilitates the dispersion of the follicular cells of the corona radiata and zona pellucida during fertilization. The tail of the sperm consists of three segments: middle piece, principal piece and end piece. The tail provides the motility of the sperm that assists its transport to the site of fertilization. The middle piece of the tail contains mitochondria, which provide adenosine triphosphate for activity (Moore and Persaud, 2008).

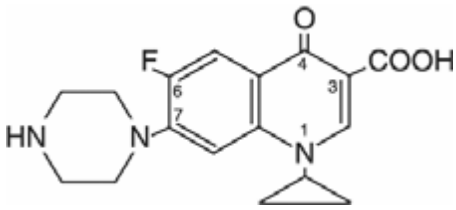
2.2 Ciprofloxacin

Ciprofloxacin is a synthetic chemotherapeutic antibiotic of the fluoroquinolone drug class. Ciprofloxacin has 12 FDA-approved human uses and other veterinary uses. It is a second-generationfluoroquinolone (FQ) antibacterial agent (Fatai et al., 2013).

The application of FQs is in treatment of urinary, skin, gastrointestinal, respiratory, bone and joint infections, as well as sexually transmitted diseases (Sarkozy, 2001).

2.2.1 Chemistry

Ciprofloxacin is1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid. Its empirical formula is C17H18FN3O3 and its molecular weight is 331.4. It is a faintly yellowish to light yellow crystalline substance and its chemical structure is as follows:



The quinolones bear both the acidic group (carboxylic acid) and the basic group (tertiary amine). This association gives them amphoteric properties. Their solubility is low, except between pH 6 and 8. Within this range, they have low water solubility and are prone to precipitate under more acidic conditions. It is apparently due to this property that crystalluria has been observed in man and animals (Sarkozy, 2001).

2.2.2 Anti-microbial activity

Ciprofloxacin has *in vitro* activity against a wide range of gram-negative and gram-positive microorganisms. The bactericidal action of ciprofloxacin results from inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, and recombination. DNA gyrase is the major target in gram-negative bacteria and topoisomerase IV is the major target for gram-positive bacteria (Blondeau *et al.,* 2004).

FQs accumulate within bacteria very rapidly, so that a steady-state intrabacterial concentration is obtained withina few minutes (Smith, 1986).

2.2.3 Spectrum of activities

In general, the fluoroquinolones have an excellent activity against *Enterobacteriaceae*, fastidious gram-negative bacteria and *Pseudomonas aeruginosa*, good to moderate activity against staphylococci, mycobacteria, chlamydia, mycoplasma and ureaplasma: and little or no activity against streptococci (particularly group D streptococci), enterococci, and anaerobic bacteria. The post-antibiotic effect of fluoroquinolones has been shown to be 4–8 h against *Escherichia coli*, *Klebsiella*, *Serratia*, and *Pseudomonas aeruginosa* (Sarkozy, 2001).

Ciprofloxacin has the broadest spectrum of activity against all gram-negative bacteria and streptococci tested compared to other FQs (Sarkozy, 2001). The activity of ciprofloxacin against *Chlamydia trachomatis*, *Mycoplasma hominis* and *Ureaplasma urealyticum* was found to be at least twice as high as that other FQs (Sarkozy, 2001).

2.2.4 Pharmacokinetics

2.2.4.1 Absorption

Ciprofloxacin is absorbed primarily from the duodenum and jejunum when administeredorally to monogastric animals.In man, the absolute bioavailability is approximately 70% with no substantial loss by first pass metabolism (Wolfson and Hooper, 1985). Bioavailability is lower in ruminants although themechanism for this anecdotal observation has not been determined. Bioavailability from parenteral injection sites is nearly 100% for all fluoroquinolones(Sarkozy, 2001).

2.2.4.2 Distribution

One of the most attractive pharmacokinetic characteristicsof fluoroquinolones is their large volume of distribution.After oral administration, ciprofloxacin is widely distributed throughout the body.The binding of ciprofloxacin to serum proteins is 20 to 40% which is not likely to be high enough to cause significant protein binding interactions with other drugs. Tissue concentrations often exceed serum concentrations in both men and women, particularly in genital tissue including the prostate (Sarkozy, 2001). Ciprofloxacin is present in active form in the saliva, nasal and bronchial secretions, mucosa of the sinuses, sputum, skin blister fluid, lymph, peritoneal fluid, bile, and prostatic secretions. Ciprofloxacin has also been detected in lung, skin, fat, muscle, cartilage, and bone. The drug diffuses into the cerebrospinal fluid (CSF); however, CSF concentrations are generally less than 10% of peak serum concentrations. Low levels of the drug have been detected in the aqueous and vitreous humor of the eye (Sarkozy, 2001).

2.2.4.3 Metabolism

Four metabolites have been identified in human urine which together account for approximately 15% of an oral dose. The metabolites have antimicrobial activity, but are less active than unchanged ciprofloxacin (Sarkozy, 2001).

2.2.4.4 Excretion

The excretion of fluoroquinolones is primarily via the kidney and secondarily via the liver. High urinary concentrations are achieved due to glomerular filtration and to probenecid-sensitive tubular secretion. The urinary excretion of ciprofloxacin is virtually complete within 24 hours after dosing. The renal clearance of ciprofloxacin, which is approximately 300 mL/minute, exceeds the normal glomerular filtration rate of 120 mL/minute. Thus, active tubular secretion would seem to play a significant role in its elimination. Excretion is decreased in individuals suffering from the renal failure and fluoroquinolones should be used in such patients with caution (Sarkozy, 2001).

The serum elimination half-life ($t_{1/2}$) of ciprofloxacin in man with normal renal function is approximately 4 hours. Approximately 40 to 50% of an orally administered dose is excreted in the urine as unchanged drug while its elimination $t_{1/2}$ is 2-4 h in dogs and horses, (Sarkozy, 2001).

2.2.5 Adverse effects

With few exceptions, the adverse effects of fluoroquinolones are not of severe consequence when compared tothe beneficial features they exhibit. The target tissues are the juvenile cartilage, central nervous system, urinary tract and digestive tract. Some skin eruptions were also observed in man. Embryonic losses in female monkeys exposed to very high doses were described (Sarkozy, 2001).

Toxicity of the fluoroquinolones is mild at therapeutic doses, and generally consists of gastrointestinal disturbances such as nausea, vomiting and diarrhoea. At slightly higher doses,

CNS signs of dizziness, restlessness, headache, depression, somnolence or insomnia may be seen (Sarkozy, 2001).

Studies related to the cytotoxic effects of ciprofloxacin revealed that this antibacterial agent inhibits the growth of various cultured mammalian cells. Cellular DNA damage and chondrotoxicity were reported to be induced by the ciprofloxacin (Zobeiri *et al.,* 2012).

It has been reported that quinolones have some toxic effects on the central nervous, cardiovascular and gastrointestinal systems, and that they also lead to chondrotoxicity, reproductive and developmental toxicity, genotoxicity, carcinogenicity and phototoxicity (Ikbal *et al.,* 2004).

2.2.6 Male reproductive toxicity

Ciprofloxacin is routinely used by fertility specialists to treat bacterial infections occurring prior to *in vitro* fertilization treatment, or when high concentrations of leukocytes are present in the semen of their patients (Herbold *et al*., 2011).Quinolones concentrations in prostatic fluid, prostatic tissue and in seminal fluid are found tobe relatively high in comparison to the corresponding plasma concentration. Ciprofloxacin is concentrated several folds in ejaculate and seminal fluid (Vartan *et al.,* 2003).

Semen concentrations were half of those observed in the serum shortly after ciprofloxacin administration, butthey were 10 times higher than serum concentrations in 12 h and 24 h after dosing.Ciprofloxacin concentrations in expressed prostatic secretion after oral administration of 500 mg ciprofloxacin in human volunteers ranged from 0.9–15 μg/ml, indicating pronounced diffusion of ciprofloxacin into the prostatic fluid (Sarkozy, 2001).

In vivo genotoxicity studies have shown that ciprofloxacin is safe for therapeutic use (Herbold *et al*., 2011). However, other studies have demonstrated ciprofloxacin to significantly impair both testicular function and structure. Administration of ciprofloxacin significantly reduced sperm count, motility and daily sperm production in rats (Grabe *et al.,*

1986). In healthy rats, ciprofloxacin caused obvious histological damage associated with a decrease in testicular volume and sperm concentration (Abd-Allah *et al*., 2000).

Khaki et al. (2008) reported that prolonged (60 consecutive days) administration of therapeutic dose of ciprofloxacin led to increase in germ cell apoptosis and marked reduction in sperm count, sperm motility and viability in rats. Abd-Allah *et al*. (2000) reported that ciprofloxacin treatment for 15 days in rats caused in a marked reduction in sperm count and motility.

Impairment in testicular function and structure has also been proven to be caused by ciprofloxacin (Zobeiri *et al.,* 2012).

Weyers *et al*. (2002) reported that there is an increased peroxide radical generation in the testis following ciprofloxacin treatment in mice (Weyers *et al.*, 2002). This increase induces DNA single-strand breaks and chromosomal aberrations as demonstrated by *in vitro* genotoxicity studies (Sanchez *et al*., 2005; Itoh *et al*., 2006). In addition, previous study showed that caspase-3, which has an important role in apoptosis, could be activated by ciprofloxacin (Olivia *et al*., 2002; Zhang *et al*., 2003).

2.3. Onion(*Allium cepa***)**

2.3.1 Description

Onions are perennials that are cultivated for food worldwide.Onion which derived its name from the Latin *Onio* and French *Oignon*, has been described as the dynamite of all natural foods. It is a tunicated bulb, compressed, round, or oblong in figure, invested with a shining,thin, dry membrane, of a reddish or white colour. (Kumar *et al*., 2010)

Onion is the best widely cultivated species of the genus *Allium* and it is widely distributed in the temperate regions.(Ogunlade *et al*., 2012)

2.3.2 Scientific classification

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Asparagales

Family: Alliaceae

Genus: *Allium*

Species: *A. cepa*

Edible Parts: Flowers, Leaves, Root, Seed (Kumar et al., 2010).

2.3.3 Chemical composition

Active ingredients in onions include phenolic compounds (flavonoids, antocyanins, phenolic acids and flavonols), organosulphur compounds, vitamins and some minerals (Ogunlade et al., 2012).

Researchhas alsoshownthatonioncontainsexogenousandendogenousantioxidantssuchasselenium,glutathione, vitaminsA, B, andC, and flavonoidssuch as quercetinand isorhamnetin (Griffiths et al.,2002).

2.3.4 Action and medical uses

Theonion(*Allium cepa*)haslongbeenusedintraditionalmedicine,isoneoftheimportant*Allium*speciescommonlyused inourdailydiet, andhasrecentlybeenthesourceofmuchinterestbecauseofitsantithrombotic,hypolipidaemic,hypotensive,diaphoretic, antibiotic,antidiabetic,antiatherogenic,andanticancermedicinalproperties (CummingsandBingham,1998; Endre*et al.*,1990).

The pharmacological evidence for the use of onions as an anti-asthmatic, anti-hypertensive, anti-hyperglycemic, anti-hyperlipidemic and anti-tumor agent has been reported (Augusti, 1996).

Quercetin is one of the well-studied flavonoids and is particularly high in onions. Quercetin is thought to be protective against coronary heart disease, stroke, and certain cancers. The organosulfur compounds in onions are believed to possess anti-inflammatory, anti-allergic, anti-microbial, and anti-thrombotic activity by inhibition of cyclooxygenase and lipoxygenase enzymes (Block *et al.*, 1997).

2.3.5 Anti-bacterial activity

Onions have been shown to possess antibacterial and antifungal properties (Hughes and Lawson, 1991;Augusti, 1996).

Onion oil has been shown to be highly effective against gram positive bacteria.(Zohri et al., 1995).Organosulfur compounds were cited as protective agents by researchers finding antibacterial effects of onion extract against oral pathogenic bacteria (Kim,1997).

2.3.6 Anti-fungal activity

Onion oil has been shown to be highly effective against dermatophytic fungi, and growth and aflatoxin production of *Aspergillus* fungi genera (Zohri et al., 1995). Welsh onion extracts have been proven to be more inhibitory toward aflatoxin production than the preservatives sorbate and propionate at values near 6.5 (Fan and Chen, 1999).

2.3.7 Anti-oxidant activity

Onions are good sources of dietary phytochemicals with proven antioxidant properties and ability to modulate the detoxification systems (El-Demerdash *et al.*, 2005).

Phenolic acids in onions, such as caffeic, chlorogenic, ferulic, sinapic, p-coumaric acids, vanillic, syringic and p-hydroxybenzoic appear to be active antioxidants (Ogunlade *et al*.,

2012). Its vitamins, especially vitamin C have a protective function against oxidative damage and a powerful quencher of singlet oxygen, hydroxyl and peroxyl radicals (Ogunlade *et al*., 2012).

Flavonoids have been widely studied for their antioxidative effects(Rice-Evans *et al*., 1995; Hertog and Katan, 1998). Onions are known to contain the flavonoids quercetin and kaempferol (Bilyk *et al.*, 1984; Rhodes and Price, 1996).

The mechanisms of action quercetin include free radical scavenging, chelation of transition metal ions, and inhibition of oxidases such as lipoxygenase(de Groot and Rauen, 1998; Suzuki *et al*., 1998; Lean et al., 1999).

The antioxidative effects of consumption of onions have been associated with a reduced risk of neurodegenerative disorders (Shutenko *et al*., 1999), many forms of cancer (Hertog and Katan, 1998; Kawaii *et al.*, 1999), cataract formation (Sanderson *et al.*, 1999), ulcer development (Suzuki *et al*., 1998), and prevention of vascular and heart disease by inhibition of lipid peroxidation and lowering of low density lipoprotein (LDL) cholesterol levels (Frèmont *et al*., 1998; Aviram *et al*., 1999; Kaneko and Baba, 1999).

2.3.8 Androgenic activity

Onion is one of the most important aphrodisiac foods. As an aphrodisiac, onion stands second only to garlic. It increases libido and strengthens the reproductory organs (Kumar *et al.*, 2010).Khaki *et al.*, (2009) reported that *Allium cepa* juice was able to recover spermatogenesis in *Toxoplasma gondii* infected rats.

Onion juice was also found to boost sperm parameters such as sperm counts, sperm motility, and sperm viability (Khaki *et al.*, 2008).Izawa,*et al*(2008) showed that administration of onion and quercetin alleviated the effects of male reproductive toxicity induced by diesel exhaust particles (DEPs)

Research has shown that onion contains exogenous and endogenous antioxidants such as selenium, glutathione, vitamins A, B, and C, and flavonoids such as quercetin and isorhamnetin (Markham, 1982).

These antioxidants protect DNA and other important molecules from oxidation and damage, which would otherwise induce apoptosis, and could improve sperm health parameters, increasing the rate of fertility in men (Yang *et al.,* 2006).

2.4Testis

2.4. 1. Gross anatomy of the testis

The testes are the male gonads. They are paired ovoid male reproductive glands that produce the male germ cells (sperms, or spermatozoa) and male hormones, primarily testosterone (Moore *et al*., 2010).

The testis in young healthy males has a volume of 15 to25 mL and a longitudinal length of 4.5 to 5.1 cm (Winter and Faiman, 1972). The testes are suspended in the scrotum by the spermatic cord, with the left testis (testicle) usually suspended (hanging) more inferiorly than the right testis (Moore *et al.,* 2010).

They have a tough fibrous outer surface, the tunica albuginea that thickens into a ridge on its internal, posterior aspect as the mediastinum of the testis. From this internal ridge, fibrous septa extend inward between lobules of minute but long and highly coiled seminiferous tubules in which the sperms are produced. The seminiferous tubules are joined by straight tubules to the rete testis (L. *rete*, a net), a network of canals in the mediastinum of the testis (Moore *et al.,* 2010).

The surface of each testis is covered by the visceral layer of the tunica vaginalis, except where the testis attaches to the epididymis and spermatic cord. The tunica vaginalis is a closed peritoneal sac partially surrounding the testis, which represents the closed-off distal

part of the embryonic processus vaginalis. The visceral layer of the tunica vaginalis is closely applied to the testis, epididymis, and inferior part of the ductus deferens. The parietal layer of the tunica vaginalis, adjacent to the internal spermatic fascia, is more extensive than the visceral layer and extends superiorly for a short distance into the distal part of the spermatic cord. The small amount of fluid in the cavity of the tunica vaginalis separates the visceral and parietal layers, allowing the testis to move freely in the scrotum (Moore *et al.,* 2010).

A capsulemade up of three distinct layers surrounds the parenchyma ofthe testis: the tunica vaginalis, tunica albuginea, and the tunicavasculosa. The testes receive their blood supply from the testiculararteries, the cremasteric arteries, and the deferentialarteries. The testicular artery arises from the abdominal aortajust below the renal artery and becomes a component of thespermatic cord above the internal inguinal ring as well as beingintimately associated with a network of anastomotic veins,which eventually form the pampiniform plexus. A counter currentheat exchange in the spermatic cord provides blood to thetestis that is 2 to 4°C lower than the rectal temperature in a normal male (Agger,1971).

The testicular arteries penetrate the tunicaalbuginea and then travel inferiorly along the posterior surfaceof the testicle and eventually ascend onto the anterior surfacewith several branches that course into the parenchyma. The medial and lateralaspects of the superior pole have the lowest density of superficialvessels compared with the inferior and anterior portions of the testicle(Goldstein and Schlegel,2013).

2.4.2Histology of the testis

The testis is divided into compartments by septa, whichare projections of the tunica albuginea. Each septum separatesthe seminiferous tubules, as well as the interstitial tissue whichis composed of Leydig cells, blood vessels, lymphatics, mastcells, nerves, and macrophages(Goldstein and Schlegel,2013).

The seminiferous tubules are highly convoluted and lined by a stratified epithelium which consists of two distinct populations of cells:

- Cells in various stages of spermatogenesis and spermiogenesis referred to as spermatogenic series.
- Non-spermatogenic cells called sertoli cells which support and nourish the developing spermatozoa.

In the interstitial spaces between the tubules, endocrine cells called Leydig cells are found either singly or in groups in the supporting tissue (Burkitt *et al.,* 1993).

In active seminiferous tubules, spermatogonia, the germ cells, are found in the basal layer of the seminiferous epithelium where they divide by mitosis giving rise to further stem cells(Type A spermatogonia), and to spermatogonia committed to meiosis

(Type B spermatogonia). Spermatogonia Type A are characterised by a large round or oval nucleus with condensed chromatin; nucleoli, which are associated with the nuclear membrane and a nuclear vacuole may be prominent. Spermatogonia Type B have dispersed chromatin, centrally located nucleoli, and no nuclear vacuole. Both types of spermatogonia have sparse poorly stained cytoplasm (Burkitt *et al.,* 1993).

Spermatogonia Type B enter the first stages of meiotic division when they become known as primary spermatocytes. Primary spermatocytes are readily recognised by their copious cytoplasm and large nuclei containing coarse clumps or thin threads of chromatin. In man, the first meiotic division cycle takes approximately three weeks to complete, after which time the daughter cells become the secondary spermatocytes. The secondary spermatocytes rapidly undergo the second meiotic division and are therefore much less commonly seen. The gametes thus produced, called spermatids, proceed through the long maturation process known as spermiogenesis to become recognisable as spermatozoa. During this process, the

nuclei of the spermatids become smaller, more condensed and less granular until they assume the small pointed form of spermatozoa (Burkitt *et al.,* 1993).

During the development process, the cells of the spermatogenic series are supported by Sertoli cells, the nuclei of which are usually found towards the basement membrane of the seminiferous tubule. The Sertoli cell nucleus is typically triangular or ovoid in shape with a prominent nucleolus and dispersed chromatin. Although not evident by light microscopy, Sertoli cells have extensive cytoplasm which ramifies throughout the whole germinal epithelium enclosing all the cells of the spermatogenic series (Burkitt *et al.,* 1993)

The basal layer of germinal cells is supported by a basement membrane which is surrounded by a lamina propria containing several layers of spindle-shaped fibromyocytes (myoid cells). These cells are responsible for the production of collagen and elastin fibres in the lamina propria whilst their contractile activity aids movement of spermatozoa along the tubules (Burkitt *et al.,* 1993)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Experimental animals

Twenty five adult male Wistar rats were obtained from the Department of Pharmacology, Ahmadu Bello University, Zaria. They were kept and maintained under laboratory conditions of temperature, humidity, light and free access to food and water. The animals were allowed to acclimatize for two weeks prior to the commencement of administration of the drug and the extract.

3.1.2 Drugs and reagents

Ciprofloxacin (500 mg/tablet) produced by Fidson Health Care Ltd. was obtained from a reputable pharmaceutical store (Royce Medicare Ltd) in Samaru, Zaria, Kaduna State. Acridine orange and ethidium bromide mixture were bought from Sigma Aldrich Co. UK. Caspase-3 activity kit was ordered from Abcam inc. UK. All other chemicals used were of analytic grade.

3.1.5 Plant materials

20 Fresh mature *Allium cepa* bulbs of Sokoto local variety weighing 200g were bought at Samaru market, Zaria, Kaduna State, Nigeria. The botanical identification and authentication of the plant sample was done at the herbarium section, Department of Biological sciences, Ahmadu Bello University, Nigeria with voucher number 423.

3.1.6 Equipment

The equipment used include:Digital weighing balance (VIC 303), light Microscope (Olympus), Fluorescent microscope (Zeiss, Germany), Dissecting set, Microcentrifuge, Micro pipettes and Spectrophotometer.

3.2 Methods

3.2.1Preparation of onion extract

Aqueous extract of *Allium cepa* bulbs were obtained using the method described by Azu et al. (2007).Briefly, the bulbs were thoroughly washed in water and blended and left to stand for 24 hrs so as to release the active contents. The juice obtained was then squeezed out using clothing mesh and the crude extract was stored at a temperature of 4˚C for the experiment.

3.2.2 Experimental procedures

Twenty five adult male Wistar rats eight weeks old were used for this study. The animals were randomly divided into a control group (A) and four experimental groups (B-E) of five animals each. All the animals were weighed before and at the end of the administration of the drug and extract. The experiment lasted for a period of twenty five days. Twenty five day duration was chosen to cover two seminiferous cycles in rat. The drug and the extracts were administered orally using distilled water as the vehicle via gastric intubation as stipulated in the table below:

Table 3.1: Administration of extract and ciprofloxacin

Animal groups	Treatment (Dosage/Kg body /weight/once daily)
A	1 ml of distilled water
B	Ciprofloxacin(22.86 mg)
C	Ciprofloxacin(22.86 mg) + onion extract (100 mg)
D	Ciprofloxacin(22.86 mg) + onion extract(200 mg)
E	Onion extract(100mg)

LD_{50}: Onion extract>3000mg/kgbw, $LD_{50:}$ Ciprofloxacin >2000 mg/Kgbw

The dose for ciprofloxacin which is an equivalent of double human therapeutic dose was the one found to be effective at inducing testicular toxicity by Elias and Nelson (2012), while that of onion was adopted from the work of Ogunlade *et al*. (2012).

3.2.3 Tissue preparation.

At the end of the experiment, the animals were anesthetized with diethyl ether and sacrificed.The testes were carefully dissected, washed in normal saline solution (0.9%), blotted and weighed.

3.2.4 Testicular histology

The testes were immediately fixed in Bouin's fluid for 12 h. They were dehydrated with varying percentagesof ethanol. The tissue wasthen cleared in xylene for 6 h, infiltrated with molten paraffin wax for 2 h in an oven at 63^{o}C. Thin sections (5 μm)were cut, stained with hematoxylin and eosin, and viewed under light microscope.

3.2.5 Histomorphometric Studies

The diameters and the heights of the seminiferous epithelium were measured in 20 tubular sections per animal at x 100 magnification and the digitised images were analysed using digimizer image analysis software(Khaki. *et al.* 2009). The number of intact seminiferous tubules per slide was also counted.

3.2.6 Fluorescence microscopy.

The semen from the rats in the experimental and control groupswere stained with acridine orange dye according to the method of Halvaei *et al*.(2012). A smear was prepared from a 15 μl sample and then fixed in a 1:1 (v/v) ethanol–acetone solution for 30 min at 4 ºC. After drying at room temperature, the slides were placed in a dark jar containing acridine orange

dye for 5 min. at the same temperature. After washing the slides with distilled water, they were immediately viewed under 400-fold magnification using a flourescent microscope.

3.2.7 Sperm analysis

3.2.7.1 Progressive sperm motility

This was done immediately after the semen collection. Semen was squeezed from the caudal epididymis onto a pre-warmed microscope slide (27˚C) and 2 drops of warm 2.9% sodium citrate was added, the slide was then covered with a warm cover slip and examined under the microscope using ×400 magnifications. Ten fields of the microscope were randomly selected and the sperm motility of 10 sperms was assessedon each field. Therefore, the motility of 100 sperms was assessed randomly. Sperms were labelled as motile or immotile. The percentage of motile sperms was defined as the number of motile sperms divided by the total number of counted sperms (i.e. 100)(Mohammad-Reza *et al.*, 2005)

3.2.7.2 Sperm viability (Life/dead ratio)

This was done by adding 2 drops of warm Eosin/Nigrosin stain to the semen on a pre-warmed slide. A uniform smear was then made and air dried. The stained slide was immediately examined under the microscope using $\times 400$ magnifications. The live sperm cells were unstained while the dead sperm cells absorbed were counted and the percentage was calculated (Laing, 1979).

3.2.7.3 Sperm count

This was done by removing the caudal epididymis from the right testes and blotted with filter paper. The caudal epididymis was immersed in 5 mL formol-saline in a graduated test-tube and the volume of fluid displaced was taken as the volume of the epididymis. The caudal epididymis suspended in the 5 mL was then homogenized into a suspension from which the sperm count was carried out using the improved neubauer haemocytometer under the microscope.

3.2.8 Haematological analysis

Approximately 2 ml of blood was collected by cardiac puncture into ethylene diamine tetra-acetate (EDTA) bottles. Samples were analysed for haematologic parameter using (Sysmex K× 21) auto analyser(Sysmex k-21n: Sysmex Corporation, Japan).

The procedures followed was based on the instruction manual of Haematology Analyzer (Sysmex K× 21). Total red blood cell (RBC) count ($\times 10^3$/ul), hematocrit (HCT) ($\times 10^3$/ul), white blood cells (WBC) count ($\times 10^3$ /ul) and platelet (PLT) count($\times 10^3$/ul) were assessed.

3.2.9 Biochemical analysis

The following oxidative stress indicators were measured: Catalase activity, superoxide dismutase activity and lipid peroxidation.

3.2.9.1 Determination of catalase activity

Catalase activity was determined using the method described by Sinha(1972). This method is based on the reduction of dichromate in acetic acid to chromic acetate, when heated in the presence of hydrogen peroxide with the formation of perchromic acid as unstable intermediate.

0.9ml of distilled water was added to 0.1ml of microsome and mixed thoroughly. 2.5ml of phosphate buffer was put in a small conical flask; 0.5ml of microsome and 2.0ml of H_2O_2 were added and the stop watch started. The reaction mixture was thoroughly mixed and the reaction was stopped after every 60 seconds for 3 minutes with dichromate/acetic acid solution. The mixture was then heated in water bath for 10minutes at 80^0C. Absorbance was read at 570nm.Standard curve was obtained using absorbance obtained at various H_2O_2 concentrations. To determine the catalase activity, the quantity of H_2O_2 consumed was obtained from the graph of the catalase standard curve.

3.2.9.2 Determination of superoxide dismutase (SOD) activity

SOD activity was determined by a method described by Fridovich (1989). This method is based on the ability of SOD to inhibit auto-oxidation of adrenaline at pH 10.2.

0.1ml of microsome was diluted in 0.9ml of distilled water to make 1:10 dilution of microsome. An aliquot mixture of 0.20ml of the diluted microsome was added to 2.5ml of 0.05M carbonate buffer. The reaction started with the addition of 0.3 ml of 0.3mM adrenaline. The reference mixture contained 2.5ml of 0.05M carbonate buffer, 0.3ml of 0.3mM adrenaline and 0.20 ml of distilled water. Absorbance was measured over 30s up to 150s at 480nm. Percentage inhibition was calculated thus:

Increase in absorbance per minute = $(A_5-A_1)/2.5$

$$\% \text{ inhibition} = 100 - \frac{\text{increase in absorbance for substrate}}{\text{Increase in absorbance of blank}} \times 100$$

Where, A_1 and A_5 are the absorbance at 30s and 150s respectively.

3.2.9.3 Assessment of lipid peroxidation

Lipid peroxidation was measured according to the method described by Akanji *et al.* (2009). The main reagents used were:15% Trichloroacetic acid (TCA) solution and 0.37% Thiobarbituric acid (TBA) solution and 0.25N HCL. 1.5 ml of serum was treated with 2ml of (1:1:1) TBA-TCA-HCL reagent and placed in water bath for 1h at 90^{o}C. The mixture was cooled and centrifuged at 3000rpm for 5min at 4^{o}C. The absorbance of the pink supernatant was measured against a reference blank using spectrophotometer at 535nm. The Malondialdehyde (MDA) concentration was then calculated using the molar extinction coefficient of 1.56×10^5 cm^{-1} M^{-1} as:

MDA conc. = absorbance/1.56×10^5 cm^{-1} M^{-1}

3.2.9.4 Determination of protein concentration.

The protein concentration was determined using Bradford reagent. 50 mg of the testicular tissue of the treated and control groups were suspended in 100 µl of chilled lysis buffer and homogenized on ice until efficient lysis was achieved. The tissue plus the buffer was centrifuged for 1 min. in a microcentrifuge at 10,000×*g*. The supernatant was then transferred to a fresh tube and protein concentration was assayed for as thus;

Bradford reagent was added to a given volume of the supernatant in the ratio 4:1.The mixture was incubated at room temperature and the absorbance of the mixture was read at 595nm in a spectrophotometer using regular cuvettes.Using the absorbance obtained in this process the protein concentrations of the testicular tissues of both the treated groups and the control were extrapolated from the standard curve obtained for protein concentrations using Bovine serum albumin (BSA).

3.2.9.5 Determination of caspase 3- activity.

Caspase-3 activity was determined using the caspase -3 activity kit (Abcam Inc. Uk). Following the instructions from the kit, 100 µg proteins from the supernatant of each sample was diluted by 50 µl cell lysis buffer. Then 50 µl of 2X reaction buffer (containing 10 mM DTT) was added to each sample. This was followed by the addition of 4 mM DEVD-p-NA substrate to each sample. This was incubated at 37^0c for 1 hr. The absorbance of each sample was then read at 400 nm in a spectrophotometer using regular cuvettes. Increase in caspase-3 activity was then determined by comparing the result with the level of caspase-3 in the un-induced control.

3.3 Data analysis

The data obtained from haematological, biochemical and sperm analyses were analyzed using Statistical Package for Social Scientist (SPSS version 18). The results were expressed as mean ± standard error of mean. Significant differences among means of the groups were determined using one way analysis of variance (ANOVA) and where significant difference

existed, it was followed by Duncan's multiple range tests.Mean was considered significant when $p \leq 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 Morphological Studies

4.1.1 Weights of the body and testes

The line graph in Fig. 4.1 shows that there was a continuous increase in the weekly mean weights in all groups. However, in week 2 through week 3 animals in group E which received a low dose of onion only (100 mg/Kg b.w.) showed a decline in mean weight gain.

Also, results from Table 4.1 showed that all the animals used for this study showed a weight gain at the end of the experiment as indicated by the value of body weight differences. However, administration of22.86 mg/Kg b.w. ciprofloxacin plus 200 mg/Kg b.w. onion extract (group D)led to a significantly ($p<0.05$) lower percentage gain in weight compared to the control, and to group C which received a low dose of onion plus ciprofloxacin. Rats given 100mg/Kg b.w. of onion extract only(group E) alsoshowed a significantly ($p<0.05$) lower percentage in weight gain compared to control.

4.1.2 Sperm Parameters

Results from Table 4.2 show that administration of ciprofloxacin to rats in group B for 25 days significantly ($p<0.05$) reduced sperm count, sperm motility and sperm viability compared to the control. Administration of 100 mg/Kg b.w. of onion only (group E) caused no significant change in sperm parameters compared to the control. Moreover, administration of ciprofloxacin and 100 mg/Kg b.w. of onion extract (group C) showed ameliorative effect by increasing the sperm count and sperm motility compared to ciprofloxacin-only group (group B). This effect was not dose dependent as administration of 200 mg/Kg b.w. of onion extract exacerbated the effect of ciprofloxacin(groups D).

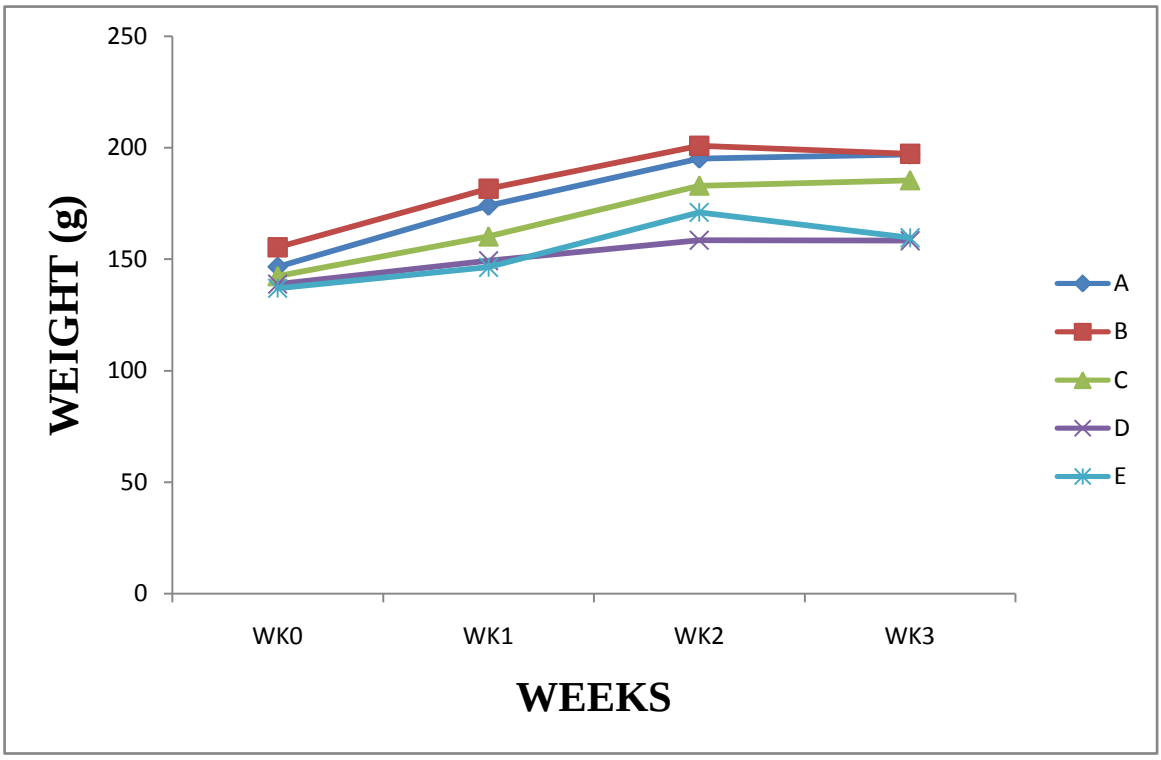


Fig. 4.1: A line graph showing the weekly mean weights of adult rats administered ciprofloxacin and onion extract for 25 days.

Table 4.1: Weights of rats and testes following administration of ciprofloxacin and onion extract for 25 days.

Mean ± SEM						
Groups	Final body weight (g)	Initial body weight (g)	Body weight difference(g)	% change in weight	Testis weight (g)	Testis-body weight ratio x 10^{-3}
A – Control	197±10.8	146.6±6.7	50.4	34.4±7.84	1.2±0.053	6.09±0.0004
B -Ciprofloxacin (22.86 mg/Kgbw)	197.2±8.3	155.4±5.0	41.8	26.8±2.65	1.128±0.043	5.72±0.0003
C -Ciprofloxacin+ onion extract (100 mg/Kgbw)	185.4±2.3	142.4±6.7	43	30.2±4.93	1.012±0.063	5.46±0.0004
D - Ciprofloxacin+ onion extract (200mg/ kgbw)	158.3±4.9	138.8±4.8	19.5	14.04±3.3*	1.063±0.023	6.72±0.0003
E-onion extract (100 mg/kgbw)	159.6±5.7	137±6.22	22.6	16.50±4.7*	1.072±0.043	6.72±0.0004

* $p \leq 0.05$ compared to control

Table 4.2**:** Effect of *Allium cepa* extract and ciprofloxacin administration on sperm parameters of male Wistar rats for 25 days.

Groups	Sperm count(x10$^{6/}$ml)	Sperm motility (%)	Sperm viability(%)
A – Control	93.50±8.09	78.80±2.48	82.40±2.50
B - Ciprofloxacin (22.86 mg/Kgbw)	43.50±3.59*	31.20±4.73*	49.40±6.51*
C- Ciprofloxacin+onion extract (100mg /Kgbw)	75.63±3.87^{b}	56.25±3.07*^{,b}	66.25±5.54
D- Ciprofloxacin+onion extract (200mg/Kgbw)	38.00±3.66*	27.00±9.13*	48.00±13.93*
E- onion extract (100 mg/Kgbw	103.00±11.47	80.80±1.66	83.40±0.81

* $p \leq 0.05$ compared to control, ^{b} $p \leq 0.05$ compared to group B

4.2 Haematological Studies

Results from Table 4.3 showed that the administration of ciprofloxacin and onion extract for 25days caused no significant change in the levels of haematocrit, white blood cell count and platelet count in all experimental groups compared to the control. However, rats treated with ciprofloxacin and a high dose of onion extract (group D) had a significantly ($p<0.05$) higher level of red blood cell count (RBC) compared to the control.

Table 4.3: Effect of *Allium cepa* extract and ciprofloxacin on red blood cells count (RBC), haematocrit (HCT), white blood cell count (WBC) and platelets (PLT) of adult male Wistar rats after 25 day treatment.

Groups	RBC ($x10^3/\mu l$)	Mean ± SEM HCT ($x10^3/\mu l$)	WBC ($x10^3/\mu l$)	PLT ($x10^3/\mu l$)
A – Control	7.90±0.18	53.82±0.74	10.08±1.60	704.6±58.8
B - Ciprofloxacin (22.86 mg/Kgbw)	7.90±0.22	52.14±1.04	8.20±1.40	776.8±42.8
C -Ciprofloxacin+ onion extract (100mg/Kgbw)	7.94±0.20	53.34±0.83	10.82±0.98	848.0±86.8
D - Ciprofloxacin+ onion extract (200mg/Kgbw)	13.00±2.20*	51.20±1.69	13.00±2.20	805.5±116.3
E- onion extract (100 mg/kgbw)	11.22±1.50	53.28±2.39	11.12±1.41	810.0±47.8

* $p \leq 0.05$ compared to control

4.3 Biochemical Studies

4.3.1 Antioxidant Biomarkers

The antioxidant biomarkers studied in this work are superoxide dismutase (SOD), catalase (CAT) and Malondialdehyde (MDA). Results from Table 4.4 showed that ciprofloxacin administered rats (group B) had a significantly($p<0.05$) lower SOD level compared to the control. In the same vein, administration of ciprofloxacin with or without onion extract (groups B, C and D) led to a significant ($p<0.05$)increase in the levels of MDA compared to the control and group E which was given 100 mg/Kg b.w. of onion extract only. Rats which received 100 mg/Kg b.w. of onion extract only (group E) showed no significant change in the levels of SOD and MDA compared to the control. All the rats in the experimental groups (B-E) showed no significant change in the levels of CAT compared to the control.

4.3.2Protein Concentration

Results from fig.4.3, showed that there was no significant change in the level of testicular tissue protein of all the experimental groups (B-E) compared to the control. However, administration of ciprofloxacin only (group B) and ciprofloxacin plus high dose of onion extract (group D) led to a decrease in the protein content. On the other hand, rats given 100 mg/Kg b.w. of onion extract only (group E) and ciprofloxacin plus 100 mg/Kg b.w. of onion extract maintained the protein content of the testes close to that of control.

4.3.3Caspase-3 Activity

Results from fig 4.3, show that ciprofloxacin-administered rats (Group B) had a significantly ($p<0.05$) higher level of caspase-3 activity in their testes compared to the control. Likewise, administration of 100 mg/Kg b.w. of onionextract only (Group E) caused a fall in the level of caspase-3 activity compared to ciprofloxacin-only administered group (B). Also, administration of 100 mg/Kg b.w. of onion extract showed an ameliorative effect by reducing the level of caspase-3 activity in Group C compared to Group B. This is nevertheless not dose dependent as administrationof 200 mg/ Kg b.w. of onion extract exacerbated the effect of ciprofloxacin by increasing level of caspase-3 (Group D).

The fold increases of caspase-3 levels compared to the control are as follows: B (7.7), C (4.4), D (8.7) and E (3.9).

Table 4.4: Effect of *Allium cepa* extract and ciprofloxacin administration on serum level of SOD, CAT, and MDA of male Wistar rats for 25 days.

Mean ± SEM			
Groups	Antioxidant Biomarkers		
	SOD (U)	CAT(mmol/min)	MDA(µg/l)
A – Control	273.00±15.57	11.69±1.63	22.13±1.95
B - Ciprofloxacin (22.86 mg/Kgbw)	197.50±1.52*	10.13±1.54	30.13±1.20*
C -Ciprofloxacin+onion extract (100mg/Kgbw)	239.00±10.69	9.54±0.88	34.37±0.63*
D – Ciprofloxacin+onion extract (200mg/Kgbw)	240.00±5.77	9.93±0.83	27.72±1.60*
E- Onion extract (100 mg/Kgbw)	280.00±10.40	10.43±1.51	24.34±0.67

* $p \leq 0.05$ compared to control

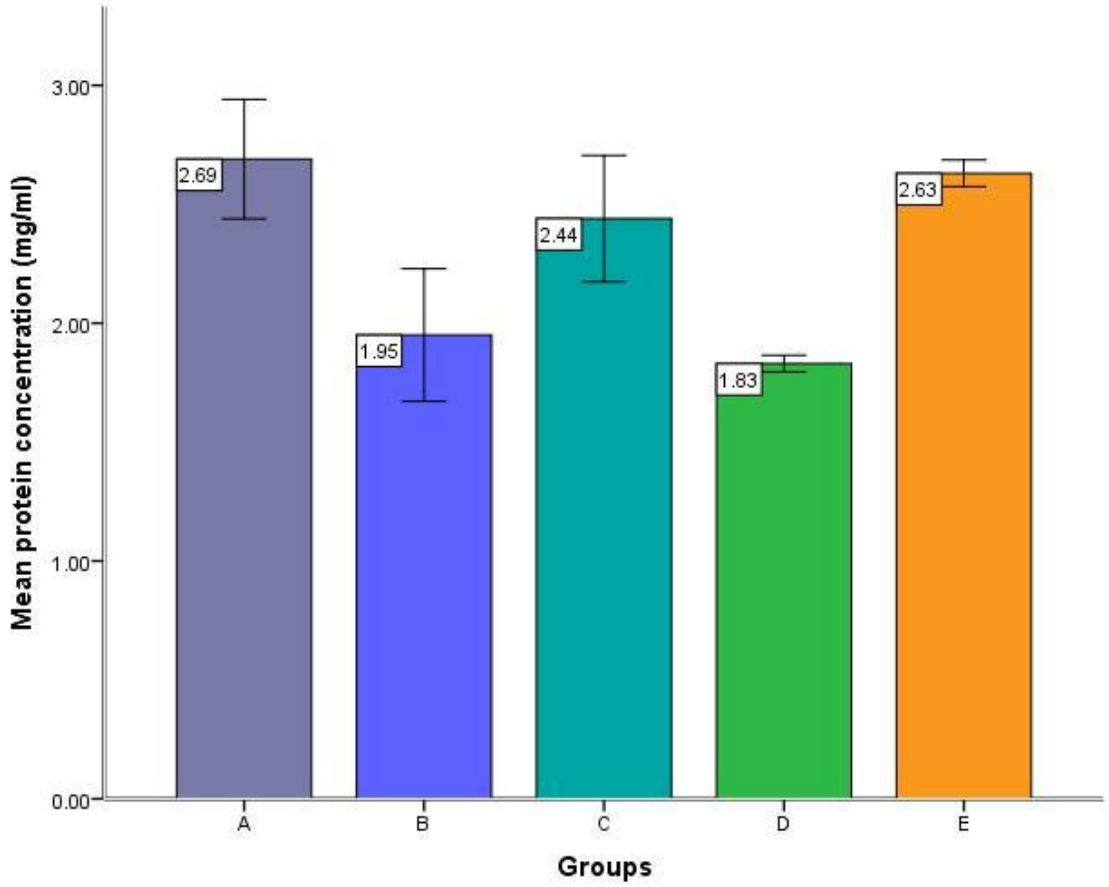


Fig 4.2: A chart showing the mean protein concentration in the testes of adult rats treated with ciprofloxacin and *Allium cepa*extract for 25 days.

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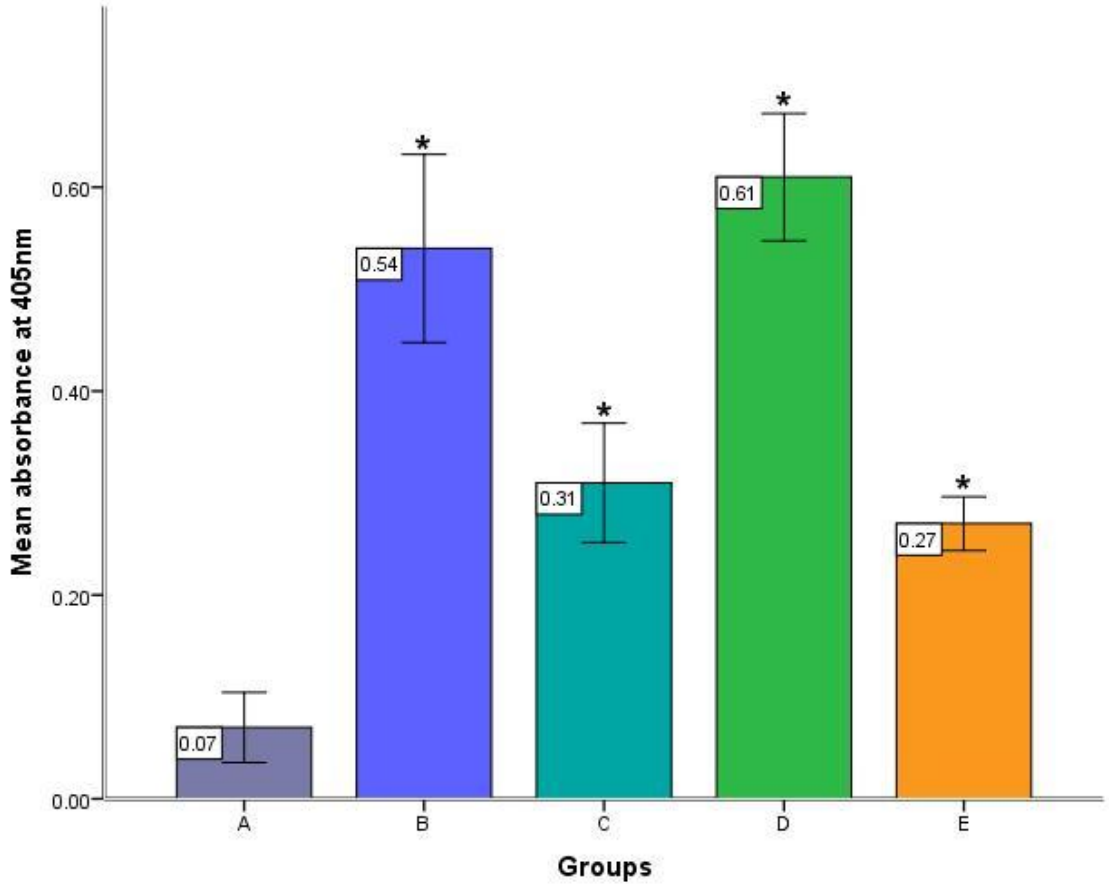


Fig 4.3: A chart showing the mean caspase-3 activity levels in the testes of adult rats administered ciprofloxacin and *Allium cepa* extract for 25 days.

*****$p < 0.05$ compared to control

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4.4 Histomorphometric studies

Table 4.5 showed the effect of ciprofloxacin and *Allium cepa* administration on the diameters, heights and number of intact seminiferous tubules. Rats administered 100 mg/Kg b.w. of onion extract only showed a significantly ($p < 0.05$) larger diameters and heights of the seminiferous epithelium compared to the control and ciprofloxacin-administered groups. Administration of ciprofloxacin with or without onion extract led to a massive loss of tubules as compared to the control.

Table 4.5: Effect of 25 day administration of *Allium cepa* extract and ciprofloxacin on histomorphometry of the seminiferous tubules of Wistar rats.

Group	Diameters of the seminiferous epithelium (μm)	Height of the seminiferous epithelium (μm)	Number of intact seminiferous tubules
A – Control	0.99 ± 0.047	0.30 ± 0.040	8.67 ± 0.880
B - Ciprofloxacin (22.86 mg/Kgbw)	0.76 ± 0.043	0.23 ± 0.045	3.78 ± 1.220**
C- Ciprofloxacin+ onion extract (100 mg/Kgbw)	0.84 ± 0.110	0.3 ± 0.070	5.67 ± 0.330**
D- Ciprofloxacin+ onion extract (200 mg/Kgbw)	0.78 ± 0.130	0.314 ± 0.015	1.78 ± 0.390**
E-Onion extract (100 mg/kgbw)	1.29 ± 0.056*,b	0.366 ± 0.013b	6.67 ± 0.330

$p < 0.05$ compared to control, **$p < 0.001$ compared to control, [b] $p < 0.05$ compared to group B,

4.5 Light Microscopic studies (H&E)

Plates I-V showed the photomicrographs of the testis of the control and the other four experimenta groups. Plate I showed the testis micrograph of the control with normal spermatogenic activities. The seminiferous tubule showed spermatogenic cells at different stages of development. However, administration of 22.86 mg/Kgb.w of ciprofloxacin caused damage in the seminiferous tubules which was characterized by aspermic lumen and disoriented elongated spermatids (plate II).

Administrationof 100 mg/Kgbw of onion extract tociprofloxacin-given rats led to an improved architecture of the testis with features close to that of the control (plate III). Administration of 200 mg/Kgbw of onion extract to ciprofloxacin-given rats potentiated the effect of ciprofloxacin by causing damage which was characterized by the presence of aspermic lumen. Rats administered 100 mg/Kgbw of onion extract only show seminiferous epithelium with a normal cyto-architecture(plate V).s

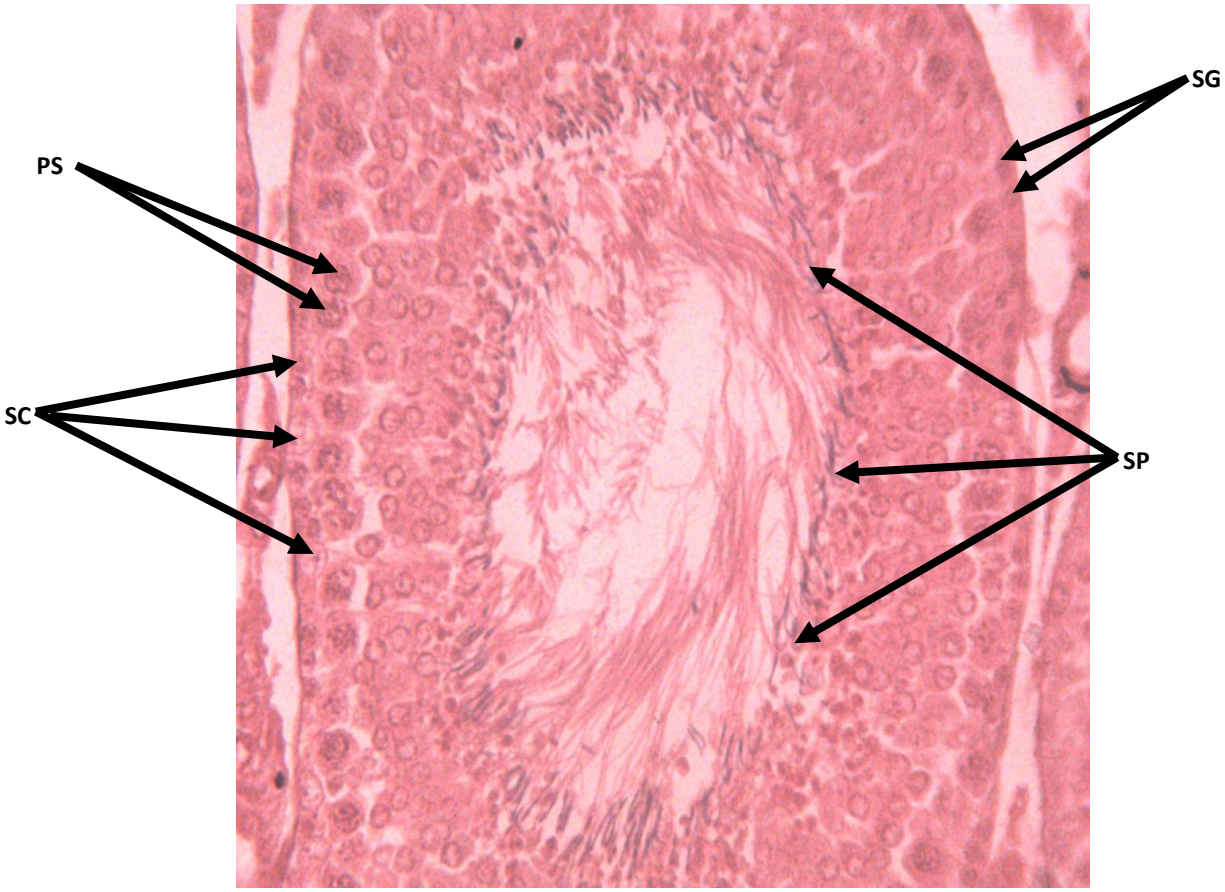


Plate I: Photomicrograph of testis from the control group Showingnormal spermatogenesis with Sertoli cells (SC),spermatogonia(SG),primary spermatocyte (PS) and spermatozoa (SP) seen in the seminiferous tubules (H&E x 250).

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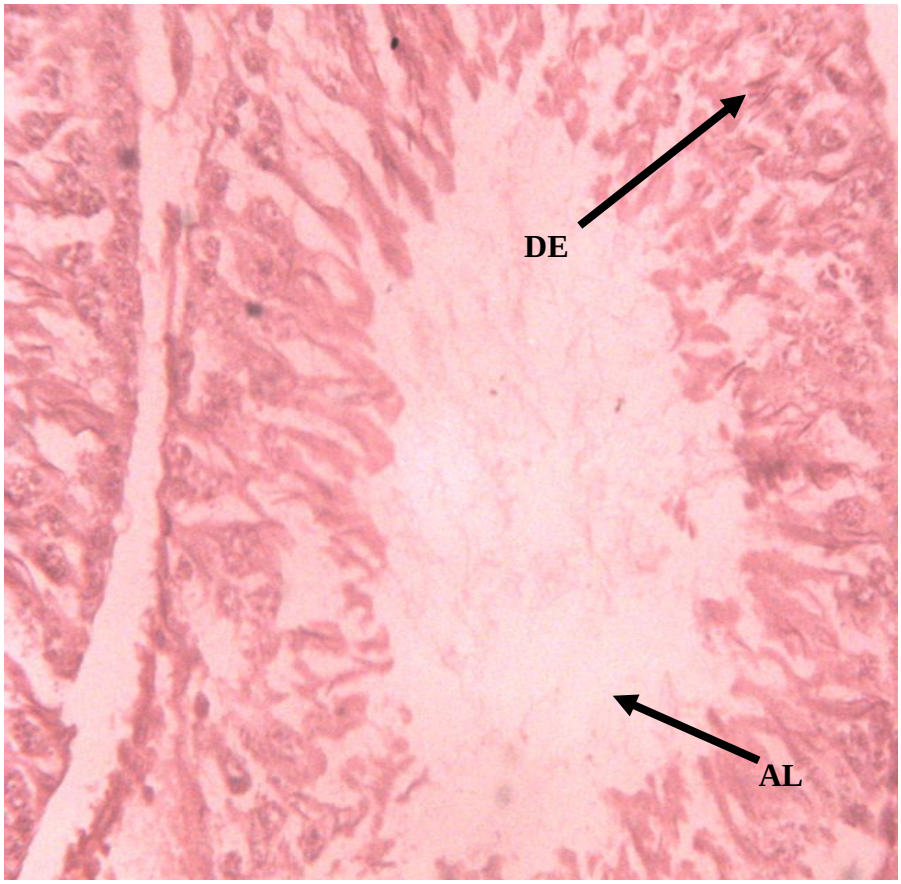


Plate II: Photomicrograph of the testis from a rat given 22.86 mg/Kg b.w. of ciprofloxacin with aspermic lumen (AL) and, disoriented elongated spermatids (DE)(H&E x 250).

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Plate III. Photomicrograph of the testis of a rat given 22.86 mg/kg b.wof ciprofloxacin and 100 mg/kg b.w of onion extract showing restoration of spermatogenicactivity with Sertoli cells (SC), primary spermatocyte (PS) andspermatozoa seen in seminiferous tubule(H&Ex 250).

43

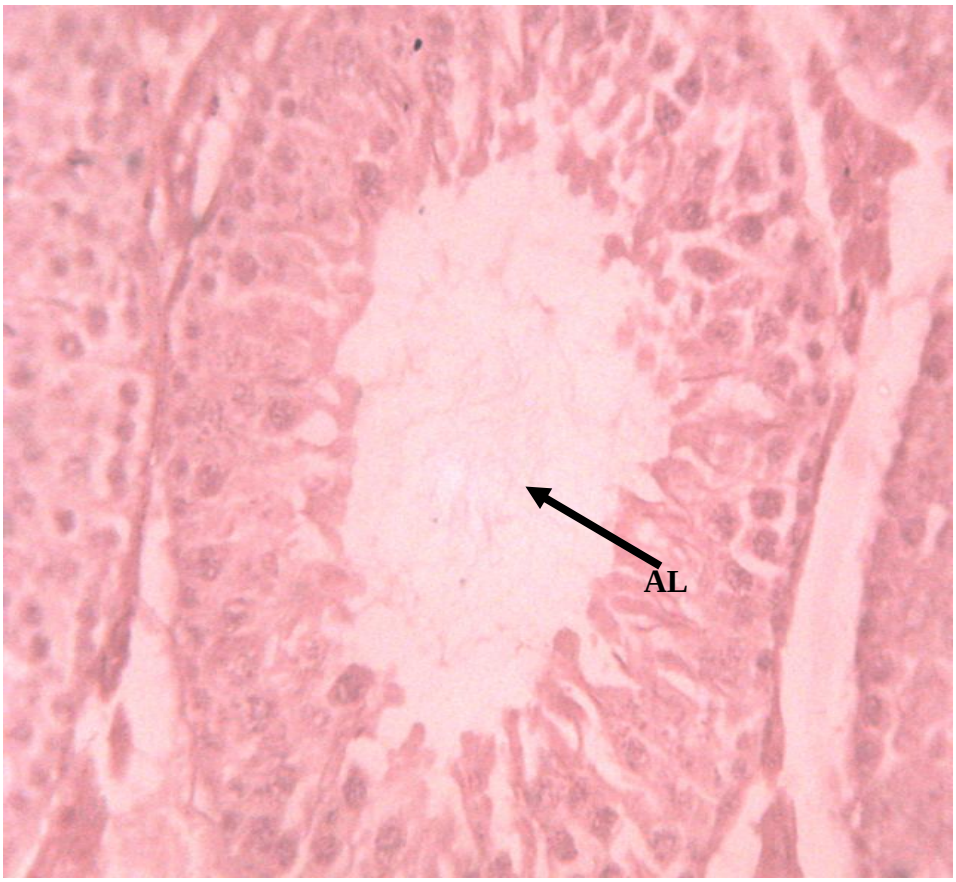


Plate IV: Photomicrograph of the testis of a rat given 22.86 mg/Kg b.w. ofciprofloxacin plus 200 mg/Kg b.w. of onion extract with aspermic lumen (AL)(H&E x 250).

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Plate V: Photomicrograph of testis from the rat given 100 mg/ Kg b.w of onion extract only showing normal spermatogenesis withspermatogonia(SG), primary spermatocyte (PS) and spermatozoa (SP) seenin the seminiferous tubule (H&E x 250).

4.6 Flourescence microscopic studies (Acridine orange staining).

Acridine orange(AO) staining is a flourescence staining which reflects sperm chromatin denaturation.The staining is used for the assessement of DNA fragmentation which is a marker of apoptosis. The dye gives rise to green flourescence when bound to intact DNA (double-stranded DNA) and red when bound to fragmented DNA (single-stranded DNA).The relative dominance of one flourescence over the other gives over-all DNA status of the sperm sample.

Examination of the photo micrograph in plateVII shows that administration of 22.86 mg/Kgb.w to rats for 25 days induced DNA damage in their sperm as demonstrated by the extensive area covered by red flourescence compared to that of the control (plate VI) which had only a patch of red flourescence.Administration of 100 mg/Kgb.w of onion extract was seen to be protective against DNA damage as indicated by the presence of substantial area with green flourescence (plateVIII) compared to that of ciprofloxacin-only administered rats (plate VII).Administration of 200 mg/Kgb.w of onion extract with ciprofloxacin aggravated the pro-apoptotic effect of the latter as only patches of green flourescence were observed to dot the pervasive and extensive red flourescence in the photomicrograph (plate IX).Treatment of rats with 100 mg/Kgb.w of onion extract induced no much DNA damage to their sperm as the flourescence pattern in the photomicrograph was comparable to that of the control (plate X).

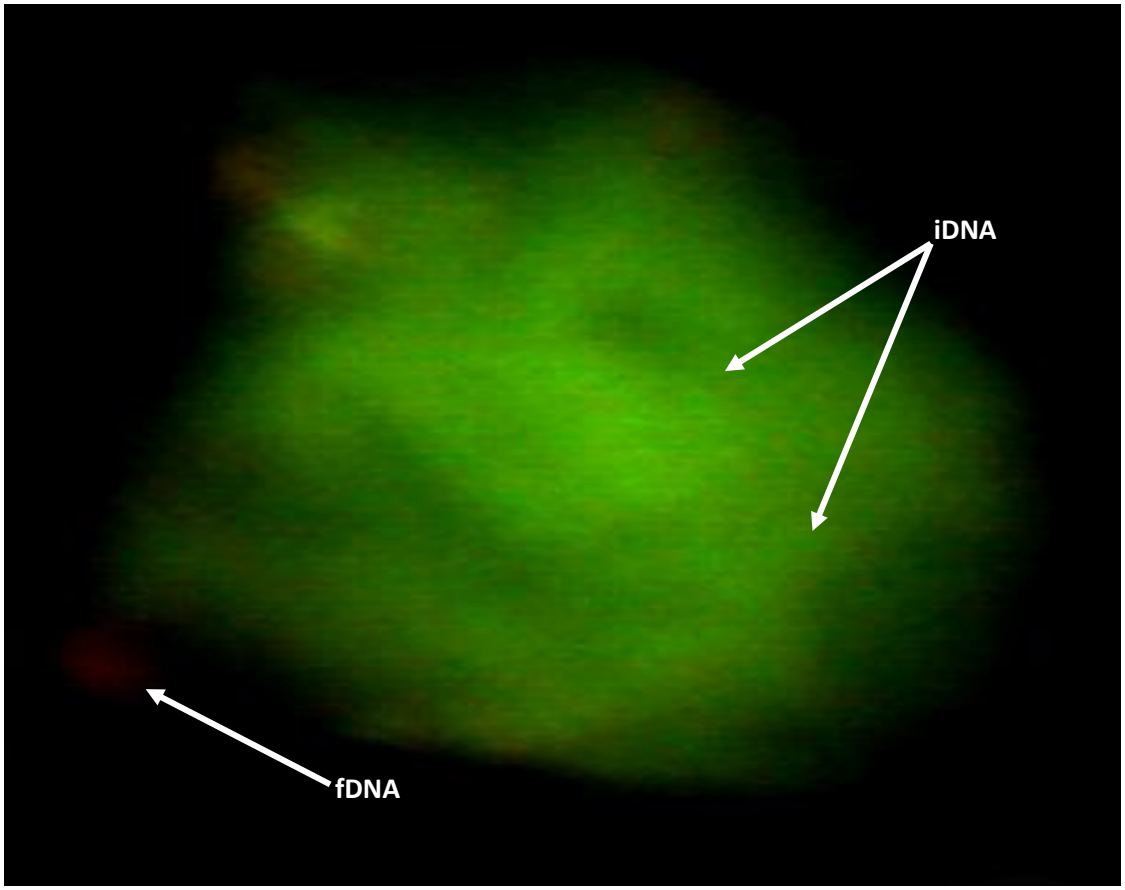


Plate VI: Photomicrograph of the sperm smear from a control showing an extensive region of intact DNA (iDNA) with a patch of fragmented DNA (fDNA). (AO X 400)

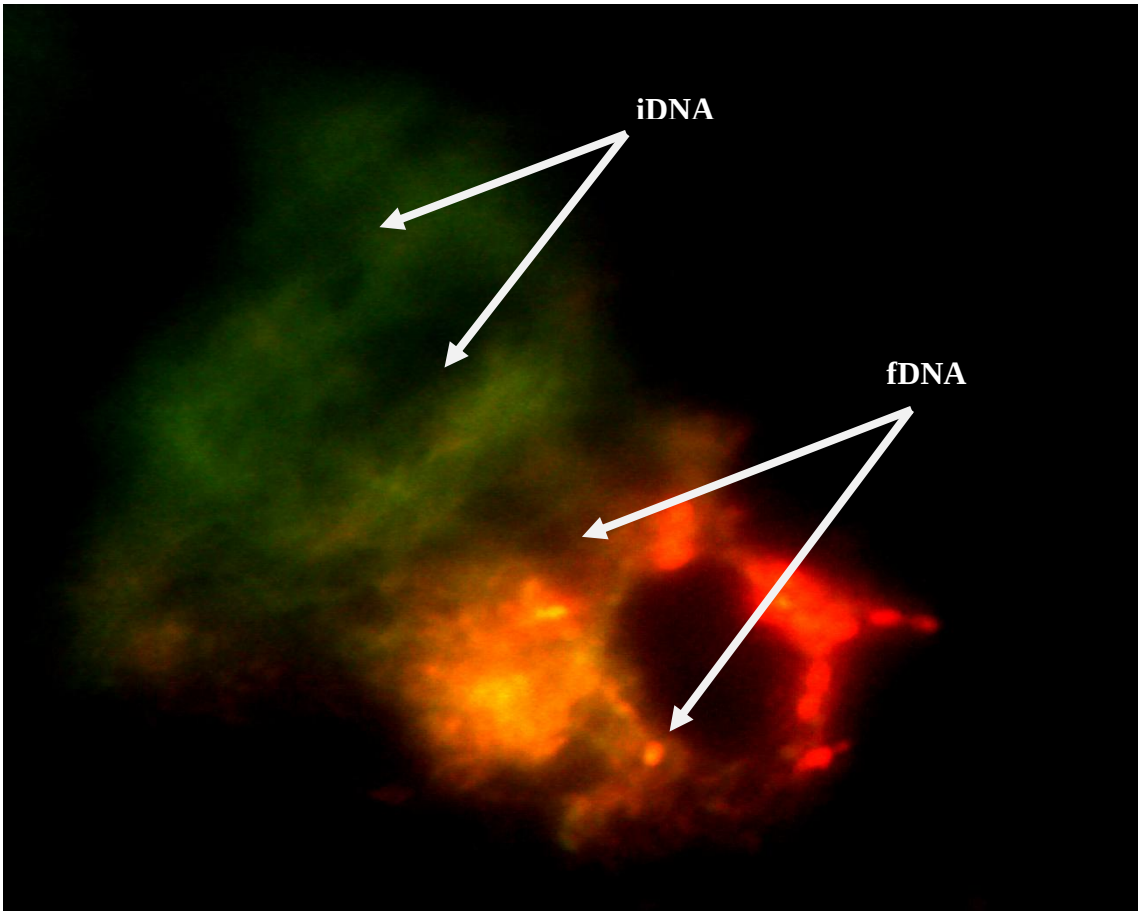


Plate VII: Photomicrograph of the sperm smear from a rat given 22.86mg/Kg b.w. of ciprofloxacin showing a highly extensive region of fragmented DNA (fDNA) compared to intact DNA (iDNA). (AO X 400)

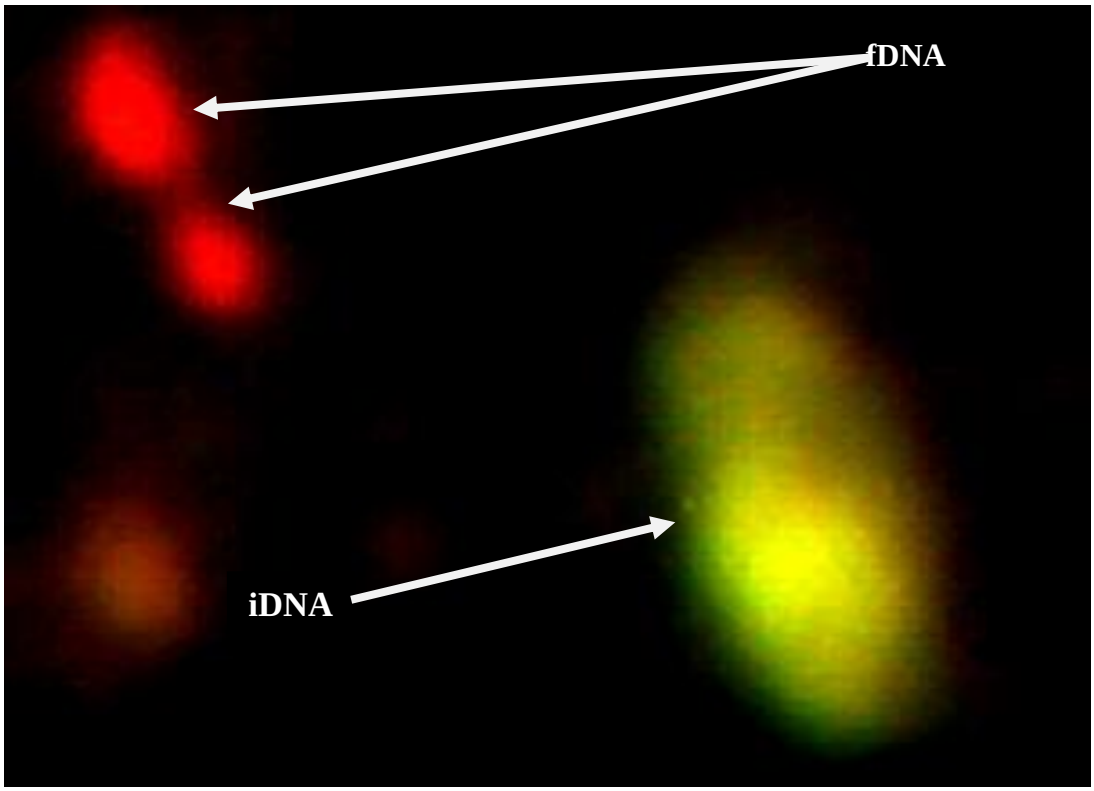


Plate VIII: Photomicrograph of the sperm smear from a rat given 22.86mg/Kg b.w. of ciprofloxacin and 100 mg /Kg b.w. of onion extract showing a dense region of fragmented DNA (fDNA)with a considerably prominent regions of intact DNA (iDNA). (AO X 400)

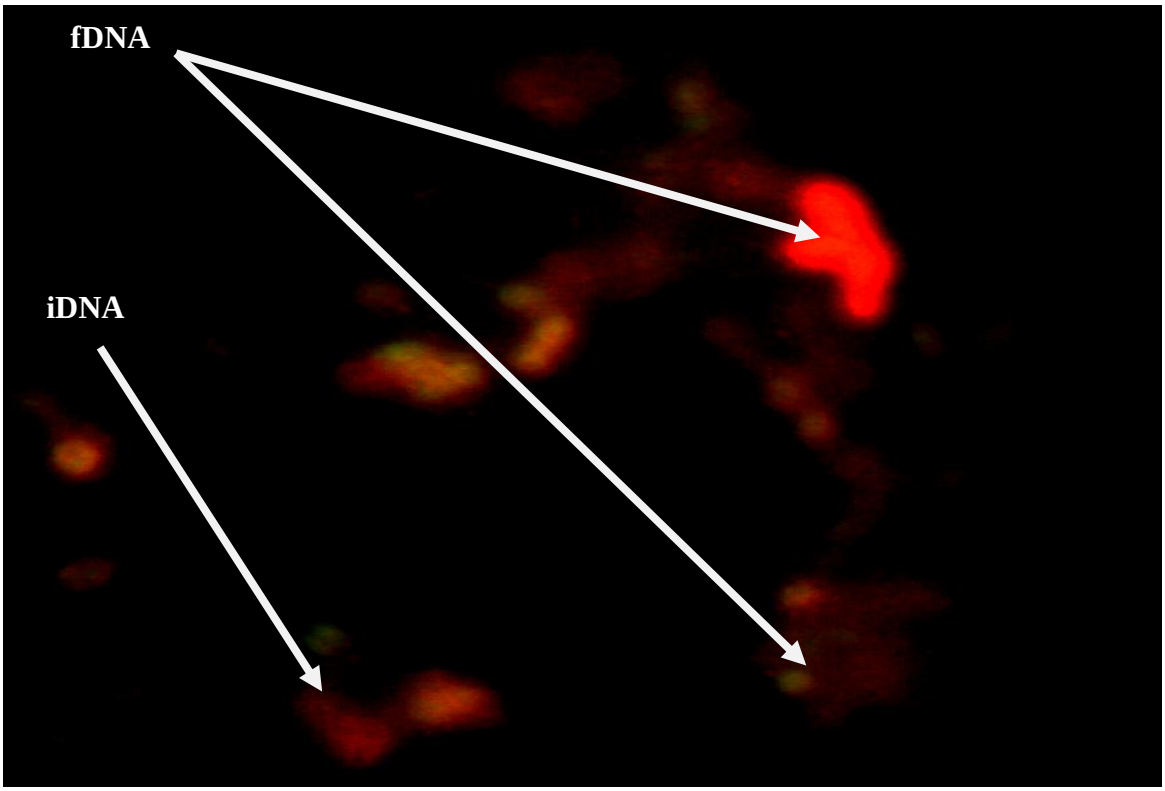


Plate IX: Photomicrograph of the sperm smear from a rat given 22.86mg/Kg b.w. of ciprofloxacin and 200 mg /Kg b.w. of onion extract showing a highly extensive region of fragmented DNA (fDNA) with patches of intact DNA (iDNA). (AO X 400)

50

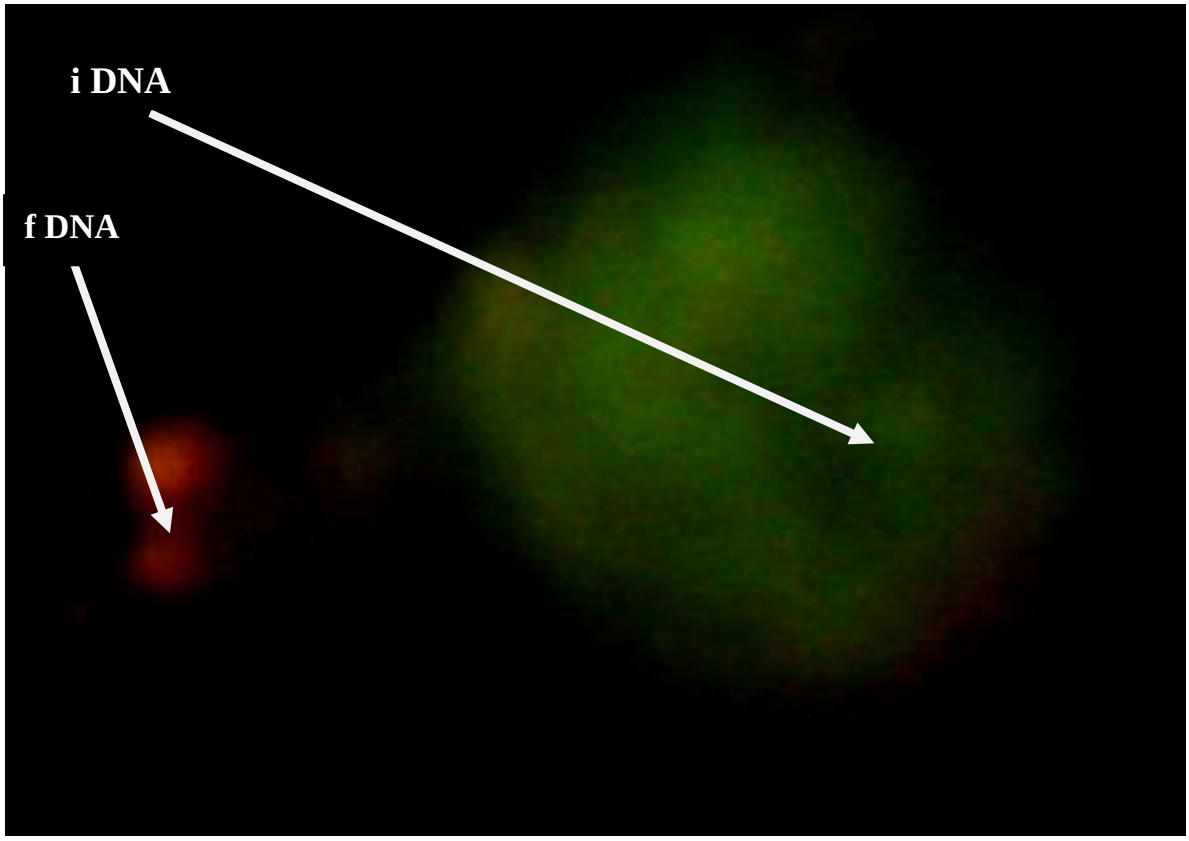


Plate X: Photomicrograph of the sperm smear from a rat given 100 mg /Kg b.w. of onion extract showing an extensive region of intact DNA (iDNA) with a small region of fragmented DNA (fDNA). (AO X 400)

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CHAPTER FIVE

5.0 DISCUSSION

The present study which aimed at evaluating the effects of *Allium cepa*extract on ciprofloxacin-induced testicular toxicity explored the effects of the extract on different morphological and biochemical parameters which included body and testis weights, sperm, anti-oxidant and haematological parameters, caspase-3 activity, DNA status and testicular cyto-architecture.

In this study, it was observed that there was a continuous increase in the weekly mean weights of the animals in all the groups. It was also observed that ciprofloxacin-administered rats had no significant gain in weight compared to the control. This result shows that ciprofloxacin has no negative effect on the food consumption and nutrient utilization. This finding is in tandem with the result reported of Abu -Aita *et al.* (2011).

Allium cepa-administered rats showed a significant ($p<0.05$) decrease in percentage weight change. This observation agrees with the work ofSung *et al.* (2011) and Wang *et al.* (2012).They reported that onion extract significantly reduced body weight, white adipose tissue weight and adipocyte size of the treated mice compared to high –fat induced control mice. This may be due to the inhibition of fatty acid synthase which in turn suppresses lipid accumulation in the body(Sung*et al., 2011*).

The testis weights did not differ significantly from the control. This observation is in line with the result obtained by Izawa *et al*. (2008)who reported no significant change in the weight of the testis between the *Allium cepa* administered rats and the control. This confirms the low caloric and protein content of *Allium cepa*(Ige and Akhigbe, 2012). However, the result goes contrary to the one reported by Khaki *et al*. (2009) and Elias and Nelson (2012).

The latter reported a significant decrease in testicular weights of the ciprofloxacin treated rats while the former reported a significant increase in testicular weights of *Allium cepa* treated rats. The dissimilarity observed in this study may be due to the duration of ciprofloxacin and onion extract administration.

In this study, no significant increase or decrease in the levels of red blood cell count (RBC), white blood cell count (WBC), hematocrit (HCT) and platelet count (PLT) were observed with the exception of group D which received 28.86 mg/Kg bw +200 mg/Kg bw of onion extract where the RBC count was significantly higher than control. This could signify the presence of a blood disease (Fatai *et al*., 2013). Non- significant changes observed in all these parameters indicate that neither ciprofloxacin nor onion induces anaemia (in the case of RBC and HCT), compromises immune system (in the case of WBC) or stimulates haemostasis (in the case of PLT) (Oyedeji *et al.*, 2013).

Oxidative stress results from excessive biosynthesis or intake of pro-oxidants, impaired biosynthesis of antioxidants or a combination of both (Mathur *et al.*,2011). Balancing pro-oxidant and anti-oxidants is vital for normal testis function and sperm fertilization ability (Mathur *et al*., 2008). Thus excessive generation of reactive oxygen species can cause cellular damage and impair sperm structure and function (Mathur *et al*., 2011).

In this study, anti-oxidant enzyme activities of sodium dismutase (SOD), catalase (CAT) and Malondialdehyde (MDA, a lipid peroxidation index) levels were assessed. Ciprofloxacin treatment caused a significant increase in MDAand a significant decrease in CAT activity. This observation could be responsible for the poor sperm quality associated with ciprofloxacin-treated rats since reactive oxygen species (ROS) have been proposed to have a role in gametogenesis (Mathur,2011).

A decrease in the serum level of CAT indicates oxidative stress as CAT is known to convert hydrogen peroxide produced in a cell to oxygen and water thus, completing the detoxification process initiated by SOD (Hancock *et al.,* 2001). This result is in consonance with the work of Weyers *et al*. (2002), who observed that there was an increase in peroxide radical generation following ciprofloxacin treatment in mice. Vartan *et al*. (2003), Khaki *et al.* (2008) and Abu- Aita *et al*. (2011) reported similar observations. Onion extract was found to maintain the level of CAT close to the control when administered together with ciprofloxacin. The anti-oxidant property of onion extract observed in this study agrees with the work of Khaki *et al*. (2009),Ige and Akhigbe(2012).

In the present study, low dose of onion extract maintained the protein content of the testis close to that of the control while treatment with ciprofloxacin with or without high dose of onion extract led to a non-significant decrease in the protein content compared to the control. This result is in disagreement with the work of Fatai *et al.* (2013), who observed a statistically significant increase in the level of serum total protein concentration of rat following administration of ciprofloxacin. The discrepancy in the result could be as a result of the different dosage of ciprofloxacin used.

In the present work, High dose of onion extract was found to potentiate the toxic effect of ciprofloxacin on sperm count, sperm motility and sperm viability. This observation agrees with the work of Abd-Allah *et al.* (2000), Vartan *et al.* (2003), Abu-Aita *et al.* (2011) and Elias and Nelson (2012) who reported that ciprofloxacin administration produced significant reduction in sperm count, sperm motility and sperm viability.

This observation could be due to the effect of ROS generation induced by ciprofloxacin administration which ultimately leads to an alteration in the micro-environment of the caudal epididymis (Rahim *et al.*, 2010) considering the fact that fluoroquinolones including

ciprofloxacin were detected in the seminal fluid, prostate, vesicle gland, tissues of testis and epididymis in human (Elias and Nelson 2012).

Moreover, this could also be due to ability of ciprofloxacin to permeate the blood-testis barrier with resultant alteration in the micro-environment of seminiferous tubules, since it has been reported that decrease in sperm motility caused by chemical agents was due to the ability of those agents to permeate blood-testis barrier (Oyedeji *et al.*, 2013). On the other hand, a low dose of onion extract was found to be effective in improving sperm qualities. This observation is in accordance with the work of Khaki *et al.* (2008), Ola-Mudathir *et al.* (2008), Izawa *et al.* (2008), Ige and Akhigbe (2012), and Ghalelikandi *et al.* (2012). The above cited workers reported remarkable roles of onion in improving sperm parameters in adult rat testis when it is administered alone or in combination with extracts or chemicals with oxidative properties.

The anti-oxidative and ameliorative effects of*Allium cepa* extract on ciprofloxacin induced-testicular toxicity could be through one the following mechanisms.

One, the phytochemical screening of onion shows that it contains various minerals which include magnesium (2.9 mmol/L), calcium (0.7 mmol/L), sodium (7.2 mmol/L) and flavonoids such as quercetin (0.35 mg/mil) (Kiviranta *et al.*, 1989). It also known that oral absorption of ciprofloxacin is drastically decreased by antacids containing magnesium and aluminium (Sarkozy, 2001). Taken together, *Allium cepa* extract may probably protect the testis and epididymis from the damaging effect of ciprofloxacin by reducing its absorption.

Two, Izawa *et al*. (2008) reported that onion and quercetin had a stabilizing effect on sertoli cells as shown by immunohistochemical staining of the Sertoli cell tubuline by monoclonal anti-tyrosinated tubulin antibody. As the endocrine requirement for spermatogenesis in vertebrate is as a result of action of reproductive hormone such as FSH and testosterone on

Sertoli cells (Griswold, 1998), it follows then that onion could protect against any damage that could be caused to the cytoskeletal protein in the Sertoli cells.

Furthermore, in rats, an androgen-binding protein secreted by the Sertoli cells into the lumen of seminiferous tubules under FSH stimulation is transported to the epididymis where it accumulates at concentrations higher than those found in the testis. This leads to a high local concentration of androgens essential for maturation of epididymal spermatozoa (Almeida *et al.* 2000).

It is also possible and plausible that anti-oxidants in onion such as vitamin C may protect sperm from oxidative damage by modulating testicular free radical production and/or stimulating testicular androgenesis as vitamin C is recognized to be essential for testicular differentiation, integrity and steroidogenic functions (Khaki *et al.*, 2009).

Apoptosis is a mode of cellular death based on a genetic mechanism that induces a series of cellular, morphological and biochemical alteration leading the cell to suicide (Vaux and Korsmeyer, 1999). Testicular apoptosis monitors germ cell population to the support capacity of Sertoli cells (Mathur *et al.,* 2011). Toxicants induce massive germ cell death either by increasing the expression of apoptosis related proteins or by oxidative imbalance (Mathur *et al.*, 2011).

Caspases (Cysteinyl aspartate-specific proteinases) play a central role in the regulation of apoptosis in mammalian seminiferous tubules (Said *et al.,* 2004). About 14 caspases have been implicated in the mammalian apoptotic pathway cascade. Among these, caspase-3 is considered to be a major executioner protease and therefore serves as an important marker of apoptosis (Olivia *et al.*, 2002; Zhang *et al.*, 2003; Said *et al.*, 2004).

The effect of onion extract and ciprofloxacin on caspase-3 activity in the testis was investigated in the present study. Ciprofloxacin administration induced a significant increase in caspase-3 activity compared to the control with high dose of onion extract exacerbating this effect. This could be due to the effect of free radicals generated by ciprofloxacin which led to a rise in the level of caspase-3. This is in agreement with the work of Olivia *et al.* (2002): Zhang *et al.* (2003) who reported that caspase-3, a biomarker of apoptosis could be activated by ciprofloxacin.

Low dose of onion extract was found to be more effective in protecting testis against the pro-apoptotic effect of ciprofloxacin .Furthermore, Though rats treated with low dose of onion extract were found to have the least fold increase in caspase-3 activity among the treated groups, the level was still significantly higher than that of the control. This result resonates with the one reported by Park *et al.* (2013), who reported an increase in caspase-3 activity level of MDA-MB-453 cancer cell line following treatment with *Allium cepa*extract.

Nevertheless, this observation could be explained as thus: In one study, *Allium cepa* extract was reported to cause a significant reduction in serum testosterone level (Ige and Akhigbe, 2012). In another report, flavonoids were implicated as anti-fertility agents (Iranloye and Owokunle, 2008). Thakare *et al.* (2009) also reported that ethanolic extractof *Allium cepa* Linn showed anti-fertility activity in rat. Taken together, reduction in the level of testosterone following treatment with onion extract could have triggered apoptosis and consequently increased the level of caspase-3 as it was reported that examination of the testicular tissue revealed that germ cell apoptosis resulting from a reduced intratesticular testosterone is caspase-3 dependent(Kim *et al.*,2001).

The findings obtained from histopathological studies showed that ciprofloxacin induced testicularchanges in the seminiferous tubules which was characterized by the aspermic lumen

and disoriented elongated spermatids,an effect that was potentiated by the high dose of onion extract. This observation agrees with the work of Abd-Allah *et al.* (2000), Demir *et al.*(2007), Khaki *et al.* (2008), Alyl *et al.*(2009), Abu-Aita *et al*. (2011) and Elias and Nelson (2012), who reported various degrees of testicular damage following administration of ciprofloxacin to experimental animals. Abu-Aita *et al.* (2011) reported that the testis of rat administered with ciprofloxacin showed marked necrosis of spermatogonia cells, lining seminiferous tubules associated with incomplete spermatogenesis.

Low dose of onion extract was found to attenuate these changes by restoring of spermatogenesis and protecting the germ cells. This ameliorative effect of onion extract on testicular damage was also reported by Khaki *et al*. (2009) and Ige and Akhigbe *et al.* (2012).

Results from histomorphometric studies indicated that administration of ciprofloxacin led to massive loss of seminiferous tubules and that onion extract at low dose significantly increased the diameters and the heights of the seminiferous. This finding runs contraryto the result obtained by Khaki et al. (2009) who reported that there was no significant change in the diameters of seminiferous tubules following administration of onion extract. The discrepancy between the results could be as a result of different dosesof onion extract used.

Fluorescence microscopic studies revealed that ciprofloxacin induced severe DNA fragmentation in the spermatozoa as about half of the sperm DNA in the ciprofloxacin-only administered rats were found to be apoptotic. The effect was aggravated by high dose of onion extract. This finding concurs with the result obtained by Zobeiri *et al*, (2012) who reported that ciprofloxacin induced detrimental effect on sperm chromatin packing and consequently elevated DNA damage. This result corroborated the earlier finding that ciprofloxacin induced caspase- 3 activity as DNA fragmentation is a late phase event of caspase-dependent apoptosis. It is also supported by the fact that ciprofloxacin can generate

free radicals which could also trigger cellular apoptosis. However, low dose onion extract was found to attenuate this effect.

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CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

The major findings of this present work are as follows:

1. *Allium cepa*extract at low dose showed ameliorative effect on ciprofloxacin- induced testicular toxicity through the following mechanisms:

i. Reduction of oxidative stress through increase in anti-oxidants i.e.catalase.

ii. Maintenance of testicular protein profile.

iii. Reduction in the level of testicular apoptosis as measured by caspase – 3 activity.

iv. Prevention of DNA fragmentation as measured by acridine orange flourescence.

2. *Allium cepa*extract at high dose was found to exacerbate the deleterious effect of ciprofloxacin on the testis.
3. *Allium cepa*extract at both low and high doses was found to reduce body weight- a property that can be exploited in treating obesity related diseases. This is yet to be investigated.

6.2 Conclusion

Ciprofloxacin induced caspase-dependent apoptosis in the testis and this was ameliorated by the low dose of *Allium cepa*extract as the high dose of the extract was found to exacerbate the effect of ciprofloxacin.

6.3 Recommendation

Further studies should be carried out in the following areas to unravel some of the observations pointed out in this work:

1. Hormonal assay should be carried out to ascertain the role of onion extract on testosterone level .
2. Immuno-histochemical staining of Sertoli cells using anti-tyrosinated tubulin antibody to ascertain the role of onion extract in stabilizing these cells.
3. Other Flourescent dyes such as aniline blue, chromomycin A3 and propidium iodide should also be employed study to the DNA integrity of the sperm cells following administration of ciprofloxacin and onion extract
4. The mechanism through which onion extract brings about reduction in body weight should also be investigated.

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APPENDIX

Table 5: Showing mean weekly weight values and percentage change in weight (**%ΔWt)** of the albino rats subjected to different treatments

Treatment groups	**Week0**	**Week1**	**Week2**	**Week3**
A	146.6±6.7^{a}	174.0±6.7^{a}	195.2±10.6^{a}	197.0±10.8^{a}
B	155.4±5.0^{a}	181.6±7.2^{a}	200.8±7.2^{a}	197.2±8.7a
C	142.4±6.7^{a}	160.2±3.9^{ab}	183.0±3.2^{ab}	185.4±2.3^{a}
D	138.8±4.8^{a}	149.3±9.3^{b}	158.5±6.2^{c}	158.3±4.9^{b}
E	137.0±6.2^{a}	146.4±8.7^{b}	171.0±5.2^{bc}	159.6±5.7^{b}

NOTE: Values are expressed as mean ± SEM (Standard of the mean). Means having different superscripted alphabets down a column are significantly different at $P<0.05$