MODULATORY ACTIVITY OF QUERCETIN ON REPRODUCTIVE FUNCTIONS IN ALCOHOL-AND NICOTINE-TREATED MALE WISTAR RATS

BY

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A Thesis in the Department of Physiology Submitted to the Faculty of Basic Medical Sciences in partial fulfillment of the requirements for the Award of the Degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

NOVEMBER, 2018

ABSTRACT

Infertility is a prevalent health condition and 50% of its prevalence is related to male factor. Exposure to testicular toxicants such as Alcohol (A) and Nicotine (N) contributes to male infertility via induction of oxidative stress. Antioxidants such as Quercetin (Q), have been reported to enhance fertility. However, there is paucity of information on the effects of quercetin in A- and N-induced reproductive toxicity. This study was carried out to examine the modulatory activity of quercetin on reproductive functions in A- and N-treated male Wistar rats.

Ninety male Wistar rats (180-200 g) were divided into two studies of forty-five animals each. Each study had nine groups (n=5) and treated orally as follows: Control (distilled water), Corn oil (CnO) (2 mL/kg, Corn oil), Q (30 mg/kg, quercetin), A (3 mL/kg, alcohol), N (1 mg/kg, nicotine), A+N (3 mL/kg, alcohol+1 mg/kg, nicotine), A+Q (3 mL/kg, alcohol+30 mg/kg, quercetin), N+Q (1 mg/kg, nicotine+30 mg/kg, quercetin), A+N+Q (3 mL/kg, alcohol+1 mg/kg, nicotine+30 mg/kg, quercetin) for 52 days. Forty-five untreated female rats were cohabited with the second 45 treated male rats in study two for mating. Experimental animals were sacrificed by cervical dislocation; blood samples were obtained for hormonal assay and selected organs (hypothalamus, pituitary, testis, seminal vesicle, epididymis) were harvested. Follicle Stimulatinf Hormone (FSH), Luteinizing Hormone (LH) and testosterone levels were assayed by microscopy. Immunoexpression of apoptotic factor (p53), antiapoptotic protein (Bcl2), proliferative factors (Ki67 and EGFR) and cell cycle regulator (CYD) were determined by Immunohistochemistry. Fragmentation of DNA was determined by Agarose gel electrophoresis. Data were subjected to descriptive statistics using ANOVA at $\alpha_{0.05}$.

Mature sperm, sperm count and motility significantly decreased ($P<0.05$) in A+N (50.0±2.7 %; 31.0±1.0×10^6 cell/mL; 34.0±1.1%) compared with control (86.0±2.5%; 96.0±1.9×10^6 cell/mL; 90.0±1.6%) A+Q (75.0±2.2%; 88.0±3.0×10^6 cell/mL; 80.0±3.5%), N+Q (75.0±2.0; 88.0±2.0×10^6 cell/mL ; 83.0±2.6%) and A+N+Q (76.0±1.8%; 70.0±2.2 10^6 cell/mL; 80.0±2.7%). Serum FSH, LH and testosterone reduced in A+N (2.6±0.6 mIU/mL; 1.6±0.2 mIU/mL; 1.2±0.4 ng/mL) compared with control (8.3±0.6 mIU/mL; 5.0±0.4 mIU/mL; 4.2±0.4 ng/mL) and A+N+Q (5.6±0.5 mIU/mL; 3.5±0.3 mIU/mL; 3.1±0.3 ng/mL). Testicular MDA significantly increased in A+N (165.0±3.03 µmol/mg protein), N+Q (102.0±4.0 µmol/mg protein), A+N+Q (104.0±6.0 µmol/mg protein) and control (96.0±0.98 µmol/mg protein). While

SOD and GSH reduced in A+N (79.6±4.6 U/mg protein; 136.0±5.5 mmol/mg protein) compared with control (121.0±0.7 U/mg protein; 354.0±16.6 mmol/mg protein) and A+Q (112.0±5.1 U/mg protein; 320.0±12.6 mmol/mg protein), N+Q (110.0±5.1 U/mg protein; 323.0±23.6 mmol/mg protein) and A+N+Q (112.0±4.8 U/mg protein; 315.0±9.1 mmol/mg protein), respectively. A+N rats did not produce offspring, but A+N+Q rats did. Apototic factor (p53), testicular DNA fragmentation increased in A+N, but not in A+Q, N+Q and A+N+Q. The Bcl2, Ki67, EGFR and cell cycle CYD were increased by Q supplementation.

Quercetin ameliorated alcohol- and nicotine-induced male reproductive dysfunction by increasing sex hormone levels and sperm quality via reduction in testicular oxidative stress. Quercetin also resulted in up-regulation of proliferative factors and down-regulation of apoptotic proteins.

Keywords: Quercetin supplementation, Male reproductive functions, Alcohol and nicotine administration, Apoptosis.

Word count: 494

DEDICATION

This research work is dedicated to:

The Almighty God (God of the whole universe, The Author of research).

My parents; Mr & Mrs James Olutunde Akintayo

My siblings; Estate Manager Joseph Akintayo, Miss Taiwo and Miss Kehinde Akintayo

AKNOWLEDGEMENTS

My heartfelt and sincere appreciation goes to my supervisor and my mentor Professor Y. Raji for his guide and care in making this research work worthwhile, undoubtedly you are a great man, words a lone aren't enough to show my candid appreciation. God bless you richly sir.

My profound gratitude goes to those who gave their candid support in order to ensure that this research work see the light of the day, I am forever grateful and may God reward you greatly. Special thanks to all the Professors in the Department (Professor A.R.A. Alada, Professor A.A. Fasanmade, Professor S.B. Olaleye, Professor E. Adewoye) and other members of staff. My gratitude also goes to the laboratory staff member. My sincere appreciation goes to Mr Jonathan Maduweke, a pathologist of The Immunohistopathological unit, National Hospital, Abuja, Nigeria, who really helped in the scoring of the Immunohistochemistry results. There are a lot of people who made the reality of this work to come to lime light. God has blessed my life with great and wonderful people who are exceptional in diverse ways. And to those who made a significant impact and contribute to my life; I cannot name them all. Also using this avenue to extend greetings to members of the Laboratory for Reproductive and Developmental Programming (LRPDP) unit.

To my family, my undoubtedly beautiful future wife and to my unborn generation I love you all.
To all well wishers thank you so much.

CERTIFICATION

I certify that this research study was carried out by Christopher Oloruntoba **AKINTAYO (153937), titled: Modulatory Activity of Quercetin on Reproductive Functions in Alcohol- and Nicotine-Treated Male Wistar Rats** under my supervision in the Department of Physiology, College of Medicine, University of Ibadan, Ibadan, Nigeria.

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A	Alcohol
ACP	Acid phosphatase
ADH	Alcohol dehydrogenase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
C_2H_5OH	Ethanol
CO_2	Carbon dioxide
CYD	Cyclin D
DNA	Deoxyribonucleic acid
DDH_2O	Double distilled water
E	Eosine
EBS	Estrogen binding site
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immune sorbent assay
FSH	Follicle stimulating hormone
GGT	Gamma glutamyl transferase
G6PDH	Glucose-6-phosphate dehydrogenase
GnRH	Gonadotropin releasing hormone
GSH	Reduced glutathione
H	Hematoxylin
H_2O	Water

Hb	Hemoglobin
HPG-axis	Hypothalamic-pituitary gonadal axis
ICSH	Interstitial cell-stimulating hormone
IL-6	Interluekin-6
Ki 67	Proliferative marker
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MEOS	Microsomal ethanol-oxidizing system
NAD	Oxidized Nicotinamide adenine dinucleotide
NADPH	Reduced Nicotinamide adenine dinucleotide
NF-KB	Nuclear factor kappa B
NO	Nitric oxide
O_2^-	Superoxide anion
$ONOO^-$	Peroxyl nitrite
PBS	Phosphate buffer saline
PCV	Packed cell volume
Q	Quercetin
RBC	Red blood cell
ROS	Reactive oxygen species
SGOT	Serum glutamate-oxalocetate
SOD	Superoxide dismutase
T	Testosterone
WBC	White blood cell

CHAPTER ONE

1.0 INTRODUCTION

The use of drugs and its abuse are rampant among reproductive age youths and this calls for alarm, thus there is a war against abuse in all its ramification by the government; there are two widely abuse drugs that are socially accepted and are used by the youths as a result of relative ease and these include alcohol and nicotine (Fawibe and Shittu, 2011). Present in certain beverages is alcohol which is regarded in many cultures as a food staple, it serves as a source of calories (empty), it is also know to be toxic as well as an additive drug (Lieber, 1991; Dunne and Katz, 2015; Lee *et al*., 2016).

Alcohol otherwise known as ethanol is quickly absorbed from the digestive system; about 20% absorption rate takes place in the stomach while 80% occurs in the proximal small intestine. In human, chronic alcoholics are often associated with impotence, loss of libido, premature or delayed ejaculation, sterility testicular atrophy and gynaecomastia (Boyden and Pamenter, 1983). Chronic alcohol exposure for a long period is associated with reduced libido, erectile dysfunction and gynecomastia (Buffum, 1983). Study suggests that alcohol's effects may be linked to reduced testicular production and increased metabolic clearance in the liver. Light alcohol ingestion has been reported not to appear to interfere with semen quality (Monoski, *et al.,* 2002), while excessive alcohol intake has deleterious effects on male fertility causing reduction in serum concentration of testosterone. In some culture alcohol is regarded as staple food (Lieber and DeCarli,1989b) and it has also been known to have embryotoxic effects; it can as well disrupts the testicular blood-barrier (Farghali *et al.,* 1991). Exposure to chronic alcohol can as well inhibit the development of the maturing germ cells and/or promotes degeneration of germ cells and enhances the occurrence of apoptosis (programmed cell death) (Zhu *et al.,* 2002). Studies on semen samples of alcoholics have decreased sperm count, impaired forward motility of morphologically normal spermatozoa and increased number of teratospermia as reported by Kucheria *et al.,* 1985, which are indicative of impaired reproductive functions.

However, nicotine which is one of the most illicit used drugs is an alkaloid from dried leaves of *Nicotiana tabacum* and *Nicotiana rustica* and lower quantities in tomatoes, potatoes, egg-plant, green pepper, and cocoa plant. Nicotine can be consumed in different form ranging from

smokeless tobacco products such as snuff and chewing tobacco but more often than not, it is consumed as smoked tobacco. Nicotine constitutes about 0.3 to 0.5% of the tobacco plant by dry weight, with biosynthesis taking place in the root and accumulating in the leave. It is a potent neurotoxin with a particular specificity to insect, hence, it is used widely as an insecticide in the past and currently nicotine derivative such as imidacloprid is still widely used (Belluzi *et al*., 2005). Nicotine is highly toxic and absorbed quickly through the respiratory tract, mouth mucosa and skin. The liver metabolizes approximately 80-90% of nicotine but the kidney and lungs are involved as well (Armitage *et al*., 1975). It is now considered to be one of the most insidiously addicting substances because most users of nicotine develop rapid tolerance for it and also have extremely long-lasting craving for it when trying to stop. In spite of the compelling evidence of serious health hazard involving multiple organ systems, about 71.5 million adults take nicotine in form of cigarette and smoking is associated with over 100,000 deaths per year in the United State (CDC, 2002).

Nicotine is considered the primary chemical in tobacco that is responsible for engendering tobacco use and independence (Harvey *et al.,* 2004). Although, it is clear that other compounds in tobacco and non-pharmacologic variables can play a role in development and maintenance of tobacco dependence. Nicotine produces addictive effects by altering neuropharmacological processes in the brain (Corrigall, 1999). For instance, it produces an increase in extracellular dopamine in nucleus accuben, an effect common with other drugs of abuse such as heroin and cocaine (Gerrits *et al*., 2002). Available pharmacotherapies for tobacco dependence involve administration of medication that substitutes for or modifies some aspect of these neuropharmacologic effects of nicotine (Fiore, 2000). However, despite the efficacy in promoting cessation, the vast majority of smokers fail to quit (Fiore *et al.,* 1994). In different studies, nicotine has been shown to be more addictive than cocaine, and even heroin (Stolerman and Jarvis, 1995).

Some effects of nicotine on different parts of the system have been documented ranging from cardiovascular, renal, and gastrointestinal to name a few. The association between smoking and various cancers, particularly lung cancer (Powell *et al.,* 2013) is well known. Nicotine has been shown to stimulate angiogenesis in different pathological setting including wound healing, tumor growth and vascularization of atherosclerotic plaque (Heeschen *et al.,* 2001). It is also well

known that nicotine does not affect vascularization but adversely influence follicular growth by an increase in apoptotic cell death (Bordel *et al.,* 2006; Nishioka *et al.,* 2014). Reduction in cerebral glucose metabolism by intravenous injection of nicotine has also been documented (Stapleton *et al*., 2003). It has also been noted that majority of people diagnosed with schizophrenia smoke tobacco. Estimated for the number of schizophrenics that smoke ranges from 75-90%. It was then argued that the increased level of smoking in schizophrenia may be due to a desire to self-medication with nicotine (Glynn *et al*., 1993).

The effect of nicotine on the cardiovascular system is multifactorial, reflecting activities of nicotine receptors centrally and on peripheral autonomic ganglia (Benowitz and Burbank, 2016). Some effects of nicotine on reproduction have been studied. Several epidemiological studies have revealed that a consistent and highly significant incidence of infertility (Sarokhani *et al*., 2017) as well as an increased risk of spontaneous abortion among smokers (Zhao *et al.,* 2017). The effects of nicotine administration on weight and histology of some visceral organs in female albino rats have been studied (Iranloye and Bolarinwa, 2005). The in vitro effects of nicotine and cocaine on sperm motility (Condorelli *et al.,* 2013; Samplaski *et al.,* 2015) and effects of nicotine on human luteal cells in-vitro have also been documented. Adverse effects of smoking include the fact that tobacco causes premature ejaculation and also affects penile function by vasoconstriction of blood vessels. However, this depends on individual sensitivity or susceptibility (Forsberg *et al*., 1979). On the other hand, risk of ulcerative colitis has been frequently shown to be reduced in smokers on a dose dependent basis and the effect is eliminated if the individual quits smoking (Zhai *et al.,* 2017). Smoking also appears to interfere with the development of Kaposi's sarcoma, breast cancer among women carrying the very high risk gene (Moses *et al.,* 2017). With regard to neurological diseases, a large body of evidence suggests that the risks of Parkinson Disease or Alzheimer's disease might be twice as has high for non-smokers than for smokers (Quik *et al.,* 2008; Lu *et al.,* 2017).

Studies have indicated that nicotine can be used to help adult suffering from autosomal dominant nocturnal frontal lobe epilepsy (Steinlein, 1995). Nicotine has also been shown to improve long term memory and learning (Grundey *et al.,* 2017). There are a lot of controversies on the effects of nicotine on changes in testosterone level due to smoking and the quality of semen (Dai *et al.,*

2015) reported that it has no effect on testosterone level. However, individual studies that are associated with alcohol alone, nicotine alone with altered male reproductive function in rats and humans have been reported. Nevertheless, the use of alcohol and nicotine remain the broad public health concern since over 140 million humans use alcohol and nicotine world-wide for a prolong period, abuse of these drugs (alcohol and nicotine) may result to infertility.

Infertility is a global health issues of which about 10-15% of couples are affected and 40% of the cases of fertility are linked to male partner (Kumar and Singh, 2015). There are a number of factors that could be responsible for this, and most essentially caused by reactive oxygen species (ROS) which could exert a direct effect or damage to the testis as well as the sperm; ROS contributes 30-80% of pathological cases (Agarwal *et al.,* 2003; Ahmadnia *et al.,* 2007). Macromolecules and membrane bound polyunsaturated fatty acid (PUFA) have been shown to be damaged by ROS in which it could as well alter the functions of the cells (Nicco and Batteux, 2017). Spermatozoa are abundant in having polyunsaturated fatty acids which when exposed to oxidative stress could be susceptible to damage, present in the germ cells in the testis are the enzymatic and non-enzymatic scavenger system as previously described (Asadi *et al.,* 2017) and these are proteins, vitamins and vital enzymes which could serve in preventing damage induced by oxidative stress. However, other preventive measures against free radical generation include nutritional antioxidant, many of which are micromolecules and non-nutrient components available in vegetables as well as in fruits. Many antioxidants also have the properties of chelating and they are phytoestrogens in nature and they also serve as free radical scavengers, modifiers of biological and enzymatic functions (Khanduja and Bhardwaj, 2003).

Nevertheless, of all bioflavonoids, quercetin (3, 3', 4'.5, 7-pentahydrateflavone) is one of the most widely distributed dietary polyphenolic compounds. It is present in red wine, tea and onions (Formica and Regelson, 1995). It has been showed that quercetin possesses anti-atherogenic, anti-inflammatory, anticoagulative, anti-hypertensive and anticarcinogenic properties (Kris-Etherton *et al*., 2004). Studies reported that quercetin is being used as an alternative drug for the treatment of infertility (Taepongsorat *et al*., 2008). Quercetin has also shown beneficial effects on sperm viability, motility and serum testosterone in diabetic rats (Khaki *et al.,* 2010). The study was carried out to investigate the effects of quercetin on reproductive functions in alcohol and nicotine-treated male Wistar rats.

1.1 Research Questions

The following research questions will provide a guide to achieving the aim and objectives of the study:

(i) In spite of the growing knowledge of adverse reproductive effects of alcohol or/and nicotine on reproduction, the mechanism by which these drugs impair male reproductive activities has not been fully elucidated, what will be the possible mechanism of action of alcohol and nicotine to alter reproductive activities?

(ii) What toxic effect does alcohol or/and nicotine have on various body organs (particularly, the testis)?

(iii) If alcohol and nicotine could alter reproductive function/activities, could quercetin be potent enough to ameliorate the negative effects caused by alcohol and nicotine?

(iv) If quercetin alleviates the adverse effects of alcohol and nicotine what could be the mechanism of action?

SIGNIFICANCE OF THE STUDY

Individual reports on alcohol and nicotine have shown their deleterious effects on reproductive functions as each affects fertility success rate and which have been comprehensively elucidated in several studies. Nicotine being a major chemical constituent of cigarette (Konstantinos and Farsalinos, 2015), cigarette is widely consumed all over the world (WHO, 2009) and there are about 1.1 billion of smokers in the world, 70% of whom are in low-income countries (Loddenkemper and Kreuter, 2015; WHO, 2015). Nigerians smoke about 17 billion sticks of cigarette and which could pose as a threat to their reproductive system (WHO, 2013). However, cigarette smoking is positively correlated with alcohol consumption (Zhang *et al.,* 2015). Consumption of alcohol and/or nicotine induces testicular injury via oxidative stress (Dosumu *et al.,* 2011; Oyeyipo *et al.,* 2012; Adaramoye and Arisekola, 2013). Alcohol and/or nicotine affect the three components of the male reproductive system, thereby affecting testosterone secretion and alter spermatogesis (Kuladip, 2010).

Moreover, quercetin as a plant-derived flavonoid with a powerful antioxidant properties (Gormaz *et al.,* 2015; Lee *et al.,* 2018) is present in vegetables, especially onions, fruits, apples, berries, wines and tea (Gormiz *et al.,* 2015), and it is also one of the most potent scavengers of reactive

oxygen species (Nicket *et al.,* 2011) and prevents redox imbalance in cells (Kostyuk *et al.,* 2011). It has been reported that quercetin improves sperm profile via its antioxidant potential (Kheki *et al*., 2010; Turk *et al*., 2012; Altintas *et al.,* 2014). However, both there is paucity of information on the combined effects of both alcohol and nicotine on reproductive functions at the molecular level to ascertain the mechanism of alcohol and/or nicotine induced reproductive dysfunction in animal model. Hence, this study seeks to investigate the antioxidant capacity of quercetin on alcohol and nicotine induced infertility in Wistar male rats.

This study is of importance in that its outcome/results will be beneficial;

(i) In contributing to knowledge as it will determine the observed adverse effect of alcohol and nicotine exposure in experimental rats.

(ii) In providing knowledge on the effect of both alcohol and nicotine on reproductive activities using rat model.

(iii) In showcasing the antioxidant potential of quercetin in ameliorating the hampering effects of alcohol and nicotine on reproductive activities in rats.

(iv) In providing a platform for further study on the effect of quercetin on alcohol and nicotine induced infertility in Wistar male rats.

AIM AND OBJECTIVES

AIM:

The study was carried out to investigate effects of Quercetin on reproductive functions in male Wistar rats treated with alcohol and nicotine.

SPECIFIC OBJECTIVES:

To investigate effects of quercetin on actions of alcohol and/or nicotine on:

1. Reproductive functions (sperm motility, sperm count, mature sperm)
2. Testicular redox status (oxidative stress parameter; Lipid peroxidation and antioxidant enzymes)
3. Testicular DNA Fragmentation

4. Testicular immunohistochemical expression of markers of apoptosis and cell proliferation.
5. Histology of selected organs like: hypothalamus, pituitary, testis, seminal vesicle, prostate, epididymis and liver.
6. Hypothalamic pituitary-testicular axis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MALE FERTILITY/INFERTILITY

Fertility simply means the ability to reproduce and fertility rate is the number of children that a couple gives birth to in contribution to the general population. It is quite different from fecundity (which is a potential to reproduce and this can be influenced by a number of factors such as production of gamete, process of fertilization and ability to carry the fetus to term. The word infertility is inability to reproduce, however, human fertility is associated with a number of factors ranging from nutritional status, lifestyle, sexual behaviour, psychic factor (emotion, instinct and timing) and way of life (culture and economy) respectively (Daumler *et al.,* 2016).

2.1.1 MALE FERTILITY

The hormonal changes in both male and female (men and women) is one of the determinant factors in which women can become pregnant and also determine when a man is most fertile (virile). An average menstrual cycle of a woman is 28 days, although, this can vary depending on a number of factors, it may be lesser or longer (lesser as low as 20 days and longer as 40 days) than normal average of 28 days. Men are tend to be fertile starting from the onset of puberty and to the rest of their life-span, they can produce and ejaculate semen at any time of the month; however, females are only fertile in about 2 to 4 days of their cycle each month (Reed and Carr, 2018).

2.1.2 INFERTILITY

According to the American Society of Reproductive Medicine, infertility is defined as a reproductive system disease in which the body lacks the capability to carry out the essential reproductive function. Although, it may look quite simple and natural when referring to conceiving a fetus or being pregnant, thus, the physiological process involved is a bit complicated and is dependent on a number of factors (Barrat *et al.,* 2017).

2.3 TESTICULAR DYSFUNCTION

2.3.1 PENILE DYSFUNCTION

Problems with the penis can cause pain and affect a man's sexual function and fertility (Fode *et al.,* 2012). Penile disorders include:

Erectile dysfunction

Erectile dysfunction increases with age, (Silber *et al.,* 2006) but fertility does not decline in men as sharply as it does in women. There have been examples of males being fertile at 94 years old, (Silber *et al*, 2006). Available evidence showed that advancement in age in men is linked to reduction in the volume of semen and sperm variables including motility and morphology as observed by Kidd *et al*., 2001. Studies show comparison among men who are in age range of between 30 and 50 above, revealed a relative reduction in the rate of pregnancy, varying between 23% and 38% (Kidd *et al*., 2001).

Production of spermatozoa could be associated to increase in age, there is a comparison between men in their 20-50 years of age who are capable of producing about 75% or sperms than men in their 50-80 years (Silber *et al*., 2004). People within the age bracket of 20-30 years could have about 90% of mature sperm in their seminiferous tubules, while about 50% of mature sperms are present in the seminiferous tubules of men between 40-69 years of age, and about 10% of sperm cells are located in men of 80 years of age (Silber, 2004). Preliminary study showed that people or children given birth to by aged parents are at the risk of health challenges. A study observed that children of men in their 40s or above are more likely to be autism syndrome about six times than children that are given birth to by their parents under the age of 30 (Reichenberg *et al.,* 2006). The increase in age of men has also been associated with schizophrenia in some studies (Malaspina *et al*., 2001, 2002; Sipos *et al*., 2004).

Other dysfunctions of the erectile tissues that are associated with men are inability to get erection or keep erection, a painful erection that doesn't disappear (priapism) and bending penis during erection which could be as a result of hard lump (plaque), inflammation of the skin covering the penis head (this is seen in uncircumcised men (Balanitis), penile cancer, inflammation of the

penis, hypospadia (a disorder that involves opening of the urethra under the penis), tightening of the penis foreskin which is prevalent in young babies and newborns. Over time it goes away even with no treatment or intervention. However, interference of phimosis with the act of urinating, the removal of penile foreskin could be an option (Sipos *et al.,* 2004).

Retraction of the uncircumcised penis may not go back to its unretracted position. This is known as paraphimosis. Thus, there may be impairment in the flow of blood to the penis and such individuals may experience swelling and pain. So lubricant could be applied by the physician on the foreskin to make an incision (small) in order to make the foreskin to be pulled forward. Circumcision could be an option if this doesn't work out.

- Ambiguous genitalia: this results when the baby is given birth to with undistinguished genitalia (that is the genitalia did not appear to be male or female). Male children born with this type of disorder seem to have a very small penis or their penis doesn't exist. They may however have testicular tissue and it could also be seen that such a child may posses both ovarian and testicular tissues (Sipos *et al.,* 2004).

- Micro penis: another type of penile disorder, in which the penis is formed but with an average size and this can be examined by standard measures

- Sexually transmitted diseases (STDs): STDs such as HIV/AIDS, HPV, Chlamydia, syphilis, hepatitis B, genital herpes, and gonorrhea. These diseases can be spread or transmitted via sexual intercourse from one person to another.

2.3.2 DISEASE OF THE ACCESSORY ORGAN

The prostatic gland helps in the production of semen; sperm containing fluid. The prostatic gland evelops specific tube that transports urine from the bladder away from the body. The size of the prostate gland in young men is about the walnut size. As one advances in age this increases and grows bigger. However, there could be a problem if the prostate gland becomes very large, which usually happens after about 50 years of age. The higher the age, the higher the chances of having prostatic gland problem.

Problems that are common or associated to accessory sex organ are:

- Inflammation of the prostate gland (prostatitis), which could be caused by bacteria infection.
- Enlargement of the prostate gland which otherwise known as Benign Prostatic Hyperplasia (BPH). This condition causes urinary dribbling during the night.
- Cancer associated with penile or prostatic cancer. This is a type of cancer that could be treated at early detection.

2.4. HISTORICAL BACKGROUND OF NICOTINE

Nicotine as an alkaloid belongs to the plants family of nightshade or Solanaceae, which comprises of about 0.6-3.0% of tobacco dry weight, and its biosynthesis occurring in the roots of the plant and gets accumulated in the leaves (Henningfield *et al*, 2006). One of its functions is to serve as chemical an antiherbivore that is majorly meant for insects; thus nicotine is said to be useful in the past as insecticide and presently the analogs of nicotine known to be imidacloprid is globally utilized (Henningfield *et al*, 2006). In Solanaceae family, nicotine is also present in minute concentration and is present in both tomatoes and eggplant species.

In a very minute concentration, about 1 mg of nicotine is yielded in an average cigarette, the chemical substance in cigarette functions as stimulant in animals and this could be one of the responsible factors for the smoking of tobacco dependence forming properties. Addiction to nicotine has been a difficult and hardest thing to quit or stop, thus behavioral and pharmacologically characteristics which determine the addiction of nicotine are basically related to the ones that determine drug addiction like those of cocaine and heroin (Lodgson *et al.,* 1994). The content of nicotine in cigarette is on the rise over a period of time and another study observed only 1.6% increase each year between 1998 and 2005 which was noted in majority of cigarette marketing firm (Gable, 2004).

2.4.1 NICOTINE HISTORICAL SURVEY

In the history of nicotine, *Nicotiana tabacum* is a tobacco plant that is named after nicotine, and this is given by a Portugal ambassador and a French scientist, Jean Nicot de Villemain, he sent two seeds of tobacco to Paris from Brazil in the 1560s and he therefore promoted the medicinal

use of nicotine (Glynn *et al.,* 1993). The isolation of nicotine was from tobacco plant and this were carried out by two scientists who originated from Germany; Posselt and Reiman and the chemical formula was described as well (Pictet and Crepieux, 1993).

2.4.2 THE CHEMISTRY OF NICOTINE

The hygroscopic nature of nicotine shows its miscibility in water in the form of its base. The properties of nicotine among which include boiling point, melting point and density constitute the following 247 ^{0}C, 79 ^{0}C and 1.01g/ml respectively. Nicotine has $C_{10}H_{14}N_2$ as it chemical formula and 162.23g/ml as its molecular mass (Svensson, 1987).

2.4.3 PHARMACOKINETICS OF NICOTINE

2.4.4 ABSORPTION OF NICOTINE

Nicotine is easily absorbed via the oral mucosa. The absorption of nicotine through the mucosa cavity is dependent on pH according to (Dewly *et al.,* 1997). After absorption of nicotine majorly from the nasal cavity, it is diffused via the mucosa membrane, skin and lungs respectively (Svensson, 1987).

2.4.5 DISTRIBUTION OF NICOTINE

The distribution of nicotine is majorly seen in the body tissues and its distributive volume is from 1 to 3 per kg. the administration of nicotine via various routes enters the brain and gets to the peripheral and specific organs in the body. The activity of the pattern in which it executes its effects in the cerebral stimulation depicts the parts of the brain it exerts its effects (Benovitz, 1990, 2002). Experimental study showed the action of nicotine administration on various body organs including the lungs, kidney, liver and as well as the animals' blood following treatment with nicotine. Around 1990s, there was a new development which showed the method in which radiotracer was used in re-examination of the distribution of nicotine around the body system.

Researchers also established that after the administration of lethal dose of nicotine, there was a high level of nicotine in the brain of guinea pigs in comparison with the other organs of the body (Wada *et al.,* 1989). There was also a similar report in the percentage of nicotine level in the brain of dogs and rabbits after administration of nicotine (Tsujimoto *et al.,* 1995). Another study

observed that the content of nicotine was higher in some certain organs in rabbits such as the kidneys after injecting subcutaneous dose of 5mg/kg nicotine. In the same study, the concentration or levels of nicotine were higher in the cerebral cortex, pancreas, skeletal muscle, spleen, ileum, ventricular muscle of the heart and the omental fat at about one hour of administration. The pattern of the degree of levels of nicotine after administration in dogs showed that the kidney has the highest level and the levels were reduced in the lungs, brain, ileum, heart, muscle and pancreas (Mishra *et al.,* 2015).

Nicotine distribution in the body was also determined using radio-labeled nicotine in different species it was noticed in some specific organs that radio-labeled nicotine was concentrated in these organs, specifically the brain (Benowitz *et al.,* 2009). Injection of intramuscularly or intravenously (S)-nicotine-methyl-^{14}C showed that aroud five minutes, a high concentration of nicotine was noted in the skeletal, in the blood and liver and higher concentration was noticed in the tissue than in the blood (Hasson and Schmiterlow, 1962; Hagiya *et al.,* 2010). Studies also showed that intraperitoneal administration of labeled ^{14}C nicotine after three hours showed that concentration was highest in the kidneys and next to these are the liver, heart, spleen, skeletal muscle and the lungs inclusive. Administration of ^{14}C nicotine was found to be more in the wall (mucosa lining) of the stomach compared to other tissues. Yamamoto and friends, 1967, injected 5mg/kg methyl-^{14}C-nicotine into mice and about five minutes after it was discovered that, the wet weight of nicotine (0.5 to 1 μg/g) was found in different parts of the brain especially the cerebellum (Tewari *et al.,* 2011). The specific site or route in which nicotine gets into the body system via tobacco smoke is through the respiratory tract (Laube *et al.,* 2017).

Spraying of solution of ^{14}C-nicotine straight into the trachea or autoradiogram of mice from mice that were sacrificed at two minutes interval showed huge concentration of the nicotine radioactivity in the lungs and the respiratory system and this showed that nicotine could easily traverse the central nervous system via this route (Middlekauff *et al.,* 2014). Radioactivity of labeled solution of nicotine also indicated a high persistency in the lungs at about fifteen minutes post-administration, larger amount was observed in the stomach and kidneys respectively, while reduction was noticed in the brain (Singh and Kathiresan, 2015). Moreover, the measurement of the administration of nicotine and its uptake from smoked tobacco has also been established (Beko *et al.,* 2017). The exposure of mice to nicotine extraction from the lungs and cigarette

smoke was carried out by researchers and another study also established that guinea pigs were exposed to 4g of cigar for up to 40 minutes, following intermittent ventilation of fresh air and it was observed that the same lungs had concentrated nicotine uptake level. (Farsalinos *et al.,* 2014).

2.4.6 METABOLISM OF NICOTINE

The predominant route of nicotine elimination is hepatic metabolism (Svensson, 1987). Although, a number of metabolites of nicotine have been identified, it is unclear whether any of these compounds contribute to the pharmacological effects of nicotine (Svensson, 1987). Nicotine is excreted unchanged in urine in a pH-dependent fashion with urinary pH less than 5, an average of 23% of the nicotine dose is excreted unchanged in urine. When urinary pH is maintained above 7.0, unchanged nicotine urinary excretion drops to 2% (Armitage *et al.,* 1975). After intravenous administration, nicotine exhibits bi-exponential decline in plasma. Total plasma clearance ranges from 0.92 to 2.43 L/mm. Non-renal blood clearance average 1.2L/min, indicating that nicotine elimination is dependent on hepatic blood flow (Zins *et al*., 1997). In addition, drugs that alter hepatic blood flow cause significant alteration in the systematic clearance of nicotine (Zhang *et al*., 1998).

2.4.7 PHARMACODYNAMICS OF NICOTINE

2.4.8 NICOTINE RECEPTORS

The effects of nicotine pharmacology is exerted on the central nervous system as well as the peripheral nervous system. Nicotine's action at the peripheral level is of great importance and has a reinforcing on nicotine self-administration. One of the actions of nicotine associated to the lungs is that of the relaxation of the skeletal muscles, arousal at the electrocortical level which is due to harbitual usage (Clark, 1985b; Rose *et al.,* 2003). Animal studies also showed the effect of neuropsychopharmacological activities of minute or few with normal exception and is mediated via the central sites of action. These effects are contributed by the reinforcing properties or actions of nicotine both in human and animals (Clark, 1985b). additionally, the antagonist effect of nicotine in human and rhesus monkey exposed to tobacco smoke or who are prone to smoking of tobacco proves that nicotine has a central site of reinforcement, although, the main site of

action in the body has been partially established but not fully elucidated (Stolerman *et al*., 1973; Deneris *et al*., 1991).

2.4.9 NICOTINIC PERIPHERAL RECEPTORS

Acetylcholine acts in different ways via the cholinergic receptor, thus, in mammals; the peripheral nervous system, muscarinic and nicotine exert various actions of acetylcholine. There are subdivisions of nicotine cholinergic receptors which are based on the sensitivity and their location of the nicotinic antagonists. The ganglionic type of receptors are located at the sensory nerve ending and autonomic ganglion in the adrenal gland (medulla). The transmission of the cholinergic receptor and location in the autonomic ganglion is blocked by by some compounds such as hexamethonium. Located at the muscular end plate are the neuromuscular type of receptors (C10) in which transmission process is selective by decamethonium and alpha bungoratoxin. In both the skeletal and muscular system, doses of nicotine administration at higher concentration will stimulate the nicotinic acetylcholine receptors (nAchRs) compared to that of the autonomic ganglion. The nAchRs of the neuromuscular is less sensitive that that of the nAchRs of the ganglion (Luetje and Patrick, 1991). It appears that doses of nicotine gotten from cigarette smoking did not affect the motor end plate of the muscle. However, systemic administration of selective antagonists-mecamylamine can easily pass into the central nervous system. Mecamylamine (a C6 antagonist) antagonizes nicotine action in the electrophysiological and behavioural experiments (Collins *et al.,* 1986). Studies have also showed to establish whether the centrally acting nicotinic actions could be blocked by neuromuscular antagonist according to (Swanson *et al.,* 1987). Nicotinic acetylcholine receptors occur in a family of physiologically essential ligand gated channels of active family in a classical excitatory neurotransmission and in some novel forms of signaling which is of neurochemical according to the report by Wada *et al.,* (1989). Nicotinic acetylcholine receptors perform peripheral and central functional roles; therefore, they are required for the nervous system functional regulation. Nicotinic acetylcholine receptors are also nicotine target receptors by acting in such a way similar to acetylcholine. Ganglionic nAchRs in humans including the subunits of the following, alpha1, beta1, gamma and delta have been showed to increase in number following chronic treatment with nicotine. The ganglionic receptor subunits that are located intracellularly and on cell surface have also been reported which include alpha 3, beta 4 and alpha 7 respectively

(Wada *et al.,* 1989). Hence, in terms of the half maximal potency by which nicotine carried out its effects differs in the subunits or subtypes of nAchRs.

Preliminary works on the mammalian muscle nAchRs types are of α1, β1 or γ in fetus and epsilon in adult subunits have been identified. Another ganglionic nAchRs in the vertebrate compose of α3, α5 and β04 subunits and of which another contains α7 subunit of ganglionic nAchRs. In the CNS of vertebra vertebrae, present is the α7subunit and also found in the chicks are the sub-types of nAchRs, thus, nAchRs have α8 or α8 plus and α7 subunits respectively There are different fundamental properties of nAchRs subunits in which a total gap in understanding in their physiological significance and genes of the diversity of nAchRs. Thus, each subtype or subunit of nAchRs possess an important sensitivity for both nicotine and different agents have been proposed (Swanson *et al.,* 1987).

2.5 PSYCHOACTIVE EFFECTS OF NICOTINE

The major psychoactive and potent chemical agent present in tobacco is purely nicotine (with its reinforcing activities), it has been shown to induce or activate both behavioural and locomotive sensitization in animals (Stolerman and Jarvis, 1995; Schoffelmeer *et al*., 2002), those behaviours mimic neuroplasticity which is limited to psychostimulant drugs. However, many different species of rodents (such as mouse, rat), dog, primate and human inclusive that are exposed to nicotine administration showed the reinforcing properties of the effects of nicotine (Hanson *et al*., 1979; Rasmussen and Swedberg, 1998; Rose *et al*., 2003). The release of epinephrine or adrenaline from the adrenal medulla and glucose from the liver and the feeling of calmness, alertness and relaxation and the feeling of mild euphoria which most tobacco users find appealing or fascinating have been attributed to be caused by nicotine (Rose *et al*., 2003). Also attributed to the effects of nicotine among many others include reduction of appetite, rise in metabolic activity; many people who smoke tobacco may lose weight due to the consequence of the above mentioned in which nicotine exerts (Ramos *et al*., 2004). In about few seconds, blood rich in nicotine reaches the lungs and gets to the brain thus leading to the stimulation of the release of chemical messengers which include beta endorphin, dopamine, norepinephrine and acetylcholine (Phillip, 2003). The release of these neurochemicals or chemical messengers such as acetylcholine results in enhancement of memory (memory enhancing effect), concentration,

pleasure, reduces anxiety, relaxation, thus, the effects produced by nicotine lasts about five minutes to two hours (Glynn *et al.,* 1993).

2.5.1 ADDICTION OF NICOTINE

Previous study revealed that, the effects of nicotine in the brain produce different numbers of outcomes majorly entails addictive effect which could be as a result of the fact that nicotine activates the reward pathways (a circuit in the brain) that regulates both euphoria and pleasure feelings (Stolerman and Jarvis, 1995). One of the key chemicals in the brain that is involved in feelings or desire to take drugs is dopamine, and research revealed that dopamine level increases in the reward circuit in the brain, therefore, nicotine acts as an intense addictive agent (Parker *et al*., 2004). In different studies, researchers revealed that the addictive effect of nicotine is more potent than the effect produced by both heroin and cocaine (Risner and Goldberg, 1983; Stolerman and Javis, 1995). Nicotine also has stimulatory effect on the brain involving in the synthesis of neurotransmitters and its effect is related to other drugs such that it causes down-regulation of dopamine production. When there is cessation to the exposure to nicotine, there is an unpleasant change it produces at the neural level in the brain and the body as well. Aside human studies, animal studies have showed the addictive effects of nicotine on the primates (Parker *et al*., 2004). Whenever administration of nicotine stops, mice suffers from behavioural changes associated with it (Rasmussen and Swedberg, 1998), more so, some higher animals like gorillas also cultivate the habit of cigarette smoking by learning from humans while watching them smoke and this has been proven to be a difficult task quitting cigarette smoking in gorillas according to the reports (Goldberg *et al*., 1981).

2.5.2 TOXICITY OF NICOTINE

Nicotine lethal dose in rats, mice and in adult humans are 50mg/kg, 3mg/kg and 40-60mg respectively (Okamoto *et al*., 1994), thus, nicotine is poisonous or deadly at these respective doses. Other drugs that are alkaloid in nature such as cocaine could be less deadly at a dose of 1000mg compared to nicotine (Okamoto *et al.,* 1994). The center for disease control and prevention has categorize nicotine to the classes of drugs belonging to carcinogens in that it possesses properties related or similar to carcinogens in the form of standalone as evaluated.

Studies also have indicated the development of cancer in living tissues following administration of nicotine, thus nicotine has also been established to be associated to birth defects and miscarriages . However, some neuropharmacological changes are also attributed the effects of nicotine which has been documented in infants suffering from 'sudden infant death syndrome' as reported (Machaalani *et al*., 2005). Studie have also showed that people who smoke cigarette are diagnosed to have schizophrenia and the number that has been estimated in the general populace is between 75-90% and this may be as a result of increase in smoking level which could be due to the desire to smoke, which is linked to nicotine self medication as observed by (Glynn *et al.,* 1993).

2.5.3 THERAPEUTIC USES OF NICOTINE

The therapeutic use of tobacco has been attributed to combat with the development of cancer of the breast in women with the high risk of the gene, in the treatment of allergic asthma (Wynder and Hoffman, 1965; Neerad *et al.,* 2008), this is given to patients in form of nasal spray in order to interfere with Kaposi,s sarcoma development. Although, the use of nicotine in the treatment of certain diseases is also related with health risk, hence, nicotine level can be regulated or controlled when given to patients via the nasal spray, dermal patches, inform of gums and lozengens in order to alleviate their dependence on nicotine (Pullan *et al*., 1994). The mode of action through which nicotine elucidate its therapeutic effect is not fully understood, but a possible mechanism of action may be via its action as an anti-inflammatory agent due to its vasoconstriction activity (Clair *et al*., 2011). People who smoke tobacco cigarette are prone to develop neurological disease and a high risk of developing Alzheimer or Parkinson disease which is twice high than in people who do not smoke (Warburton, 1992). Nicotine has also showed to lower acetylcholine level in the brain and this could be associated to the cholinergic stimulatory action of nicotine. In neurodegenerative disorder conditions like Alzheimer or Parkinson disease the acetylcholine level is reduced and dopamine effect is also lowered significantly as reported (Warburton, 1992)

2.6 THE CHEMISTRY OFALCOHOL

Alcohol is regarded as ethanol or is often used interchangeably with ethanol. However, in the chemistry of alcohol, the hydroxyl group (OH) which is the functional group that binds to carbon atom which is joined to another hydrogen or carbon atom. There are different classes of alcohol that ranges from simply acyclic with the general formula $CnH_2n_{+1}OH$, and ethanol with the general formula C_2H_5OH, which are present in alcoholic beverages, other types of alcohol include wood alcohol or methyl alcohol/methanol and isopropyl alcohol or propan-2-ol. (Vale, 2007).

2.6.1 SIMPLE ALCOHOL

The simple most important form of alcohol as otherwise called ethanol having C_2H_5OH and ethane backbone. For the past centuries, alcohol is used by humans for different purposes in distilled alcoholic beverages and in fermented forms. Alcohol boils at 78.4^0C which shows it is a flammable liquid used as a solvent in the industry, as fuel in cars, and in the chemical industry as raw material. In some countries among which United States of America is one of which alcohol is unpalatable because it contains certain addictives and there is a legal restriction and tax on consumption of alcohol or methanol and methylated spirit used among the youths is on the increase. However, the simplest form of alcohol which is methanol with general formula CH_3OH can be gotten from wood distillation , hence, its name wood alcohol, a clear liquid that boils at 64.7^0C is useful as fuel, raw material and also as solvent in the laboratory or chemical industry. Methanol is highly toxic, unlike ethanol, about 10 mls of methanol when sip can cause total blindness via destruction of the optic nerves, more so, about 30 mls could be highly fatal (Vale, 2007).

2.6.2 PHYSICAL AND CHEMICAL PROPERTIES OF ALCOHOL

One of the properties of attributed to alcohol include hanging and biting along with its odour through the nasal cavity. The boiling point of alcohol is higher than that of the ether and hydrocarbons. Alcohol has boiling point of 78.29^0C in comparison to that of hydrocarbon and hexane which is 69^0C, and Diethyl ether has a boiling point of 34.6^0C. Alcohol undergoes nucleophilic substitution reactions and also forms ester compounds. It undergoes oxidation

reaction to form carboxylicacids, ketone and aldehydes, also it can be dehydrated to give alkenes (Vale, 2007).

2.6.3 APPLICATIONS OF ALCOHOL

Alcohol has a wide application use, one of which is its usefulness as fuel and also utilized in the industry as beverage and is consumed by humans since pre-historical ages. It used as anti-freeze and about 50%v/v is used in this regard. The performance of fuel is increased in the induction of internal combustive engines in which alcohol is injected into the supercharger or turbocharger in the air intake that pressurizes the air. With this process the pressurized air is cooled providing a air charge that is denser which allows enough power and fuel efficiency. In the industry the application of is also used as reagents and/or solvents. Due to the property of alcohol to dissolve substances that are non-polar, it is also used in the field of medicine for the manufacturing of medical drugs, as perfumes and so on. It can as well be used as disinfectant inform of antiseptic before a patient is given injection along with iodine. Additionally, in some specimens, alcohol is also of significance in preservation.

2.6.4 TOXICITY OF ALCOHOL

The result of long term use of alcohol most especially in pregnant women could lead to fetal alcohol syndrome (FAS). When alcohol is consumed in large amount it leads to drunkenness/intoxication which also results in hangover when the effect goes off or wears off. Due to the regular consumption of alcohol and its doses, it can as well lead into failure of the respiratory system (acute consumption) or even death. Ethanol impairs judgment in humans and also can cause recklessness and irresponsive action. Alcohol could be very toxic in rat with the lethal dose of 10.3g/kg as reported by Gable, (2004).

2.6.5 ABSORPTION OF ALCOHOL

Alcohol can be absorbed along the whole length of the gastrointestinal tract; it can be estimated that 20% of absorption occurs in the stomach and 80% in the intestine (Pawan, 1972). Generally, absorption from the gastrointestinal tract is so rapid that within 15 minutes after taking/drinking alcohol, over half the dose is absorbed. Although, the absorption of alcohol from the human stomach is a passive process, it can be modified by different factors, among which are; the

concentration of ingested alcohol, beverage being consumed, amount of alcohol ingested, gastric emptying, and the simultaneous intake of food (Hogbnen *et al*., 1957; Cook and Birchall, 1969; Davenport, 1987). Furthermore, the absorption of alcohol from the stomach can be influenced indirectly by high concentrations of alcohol which can induce spasm of the pylorus (Ritche, 1970).

In contrast to the gastric absorption, alcohol absorption from the small intestine appears to be unrelated to the presence of food or to alcohol concentration (Ritche, 1970). Gastric emptying time is probably a prime factor contributing to the variable rates of absorption of ingested alcohol among individuals. Despites the rapid and virtually complete absorption of alcohol from the upper gastrointestinal tract, significant amounts of alcohol are found in the proximal jejunum and ileum after drinking alcohol. Studies by Halsted *et al*. (1973), suggested that alcohol found in the lower tract resulted from its passage from the vascular space into the gut lumen, rather than from transit along the intestinal tract. In many cases, sufficient amounts of alcohol are probably retained in both upper and lower intestine during frequent and prolonged drinking to contribute significantly to the intestinal dysfunction frequently occurring among alcoholics (Istvan *et al*., 1995).

2.6.6 DISTRIBUTION OF ALCOHOL

After oral administration of alcohol in men, peak concentration is obtained in the blood generally within 1-3 hours. Normally, the concentration of alcohol then begins to decline and the drug is cleared from the body within 8-12 hours after administration (Haggard *et al*., 1941). Absorbed alcohol becomes distributed in the total body water and it gains access into the brain tissue (Shi *et al*., 1998). The drug is found in similar concentration in the water of blood, urine, saliva and alveolar air (Jacobsen, 1952; Dubowski, 1963; Ritche, 1970). In fact alcohol has been used to measure the total body water in man (Pawan and Hoult, 1963; Pawan, 1965). In addition, its distributive properties have other utility, for example, the predicted ratio of the concentration of alcohol in the blood versus vitreous humor would be 0.79 based upon the ratio of water content of the two fluids. Felby and Olsen, (1969), reported that the ratio, to be 0.73 in human cadavers; such findings may be useful in forensic postmortem hemoconcentration.

Alcohol can readily traverse the placental barrier (Dilts, 1970; Idanpaan-Heikkila *et al*., 1971, 1972; Waltman and Iniquez, 1972). Akesson (1974), studied the distribution of ethanol for about 10 minutes. In maternal organs, high concentrations of metabolites of alcohol were found in the liver, pancreas, heart, and bone marrow. This distribution may be clinically significant since all these organs may be functionally and or biochemically affected by chronic alcohol consumption. For example, alcoholic myocardiopathy may be the result of disturbances of cardiac metabolism in alcoholics (such as triglycerides). In fetus, high concentration of radioactive metabolites were found in the liver and bones, the latter being especially interesting since skeletal defects have been reported to occur in malformed offspring among alcoholic women (Jones and Smith, 1973).

2.6.7 METABOLISM OF ALCOHOL

Approximately, 10% of a dose of ethanol is eliminated unchanged from the body via urine, sweat and expired air, the remainder is metabolized, principally in the liver (Thompson, 1986). The metabolism of alcohol results in oxidation of acetaldehyde, acetate, and ultimately to CO_2 and H_2O via the Krebs cycle. A large fraction of the carbon of ethanol becomes incorporated into the hepatic lipids. Minute amounts of alcohol are also converted to the corresponding glucuronide and sulfate conjugates. Several enzymes systems can catalyze the oxidation of alcohol to aldehyde in the body. At least three enzymatic systems appear to be capable of catalyzing alcohol oxidation to acetaldehyde: (1) A soluble, NAD dependent alcohol dehydrogenase (ADH). (2) An NADPH-dependent microsomal ethanol-oxidizing system (MEOS); and (3) A peroxidative system involving catalase and H_2O_2. Acetaldehyde, produced by the oxidation of alcohol is further metabolized to acetate in the presence of NAD-dependent mitochondria enzyme, acetaldehyde dehydrogenase (AcDH). This enzyme is present in OX liver (Racker, 1949), human liver (Kraemer and Deitrich, 1968), rat kidney (Butler, 1965) and monkey brain (Erwin and Deitrich, 1966).

Acetaldehyde has been demonstrated in both the blood and alveolar air after alcohol consumption, but the concentration in the body is generally quite low due to its rapid oxidation in the presence of AcDH (except after the administration of disulfuram which inhibits AcDH, apparently by comparing with NAD for the active site of that enzyme.

2.6.8 EXCRETION OF ALCOHOL

Alcohol is eliminated slowly from the body, compared to its rate of absorption from the GI tract. Therefore, drug accumulation and resultant inebriation can occur after relative short periods of only moderate consumption of alcohol beverages. Up to 95% of a low dose of ethanol is oxidized in the body to CO_2, which is then eliminated via the expired air. Lesser amounts of alcohol (about 2%) are eliminated unchanged via this route. After excessive consumption of alcohol, the amount that is eliminated unchanged in the breath can approach 10% of the dose.

In man, an additional portion of alcohol (up to 5-10% depending upon the dose) may be excreted unchanged in urine. In some species, the glucuronide or sulfate conjugate of alcohol may be excreted. Indeed, some alcohol has been shown to be eliminated in sweat, tears, bile and saliva. The drug tends to 'follow' the total body water.

2.6.9 TOLERANCE OF ALCOHOL

The absorption, distribution and excretion of alcohol do not appear grossly or consistently different between tolerant and non-tolerant animals. In contrast, many studies showed that the rate of alcohol metabolism increased upon chronic dosage, by mechanisms that are not resolved. It was reported that in rats treated chronically with alcohol, an enhanced rate of metabolism of alcohol occurred in vitro and in vivo (Israel *et al*., 1975). It has been reported that alcohol disappears from the blood of alcoholics faster than from the blood of non-alcoholics (Conney, 1967; Liber and Decarli, 1968; Rubin *et al*., 1968; Kater *et al*., 1969a).

When alcoholics abstain for a few week, the rate of disappearance of alcohol returns towards that observed in non-alcoholics control. Shah *et al*., (1972), found no correlation between ADH activity and the rate of ethanol disappearance from the blood. Based on studies involving human liver biopsy specimens they postulated that, in general, the activities of microsomal enzymes increased in prolonged drinking and this increased activity would gradually disappear with abstinence.

Moreover, the reproductive system comprises of three basic components which are the hypothalamus, anterior pituitary gland and the gonads (testes or ovaries). Interference of alcohol

with these components could results into loss of secondary sexual characteristics or secondary reproductive dysfunction. It has also been proven that alcohol causes impotence, (Emanuele and Emanuele, 1998). The Leydig cells of the testes are the site of male sex hormone production, testosterone (Lipsette *et al.,* 1980). Studies indicate that reduction in testosterone level is associated with heavy consumption of alcohol. The Sertoli cells are of significance in that they play a vital role in the maturation of sperm cells, serving as the nurse cells by nourishing the developing sperm (Emanuele and Emanuele, 1988). Both gonadotrophins (FSH and LH) production or secretion can be significantly decreased following exposure to alcohol, more so, it affects the activities of the anterior pituitary hormones, and also could alter the production of the hypothalamic hormone (GnRH) (Emanuele and Emanuele, 1998).

Several research studies have showed that alcoholic men have reduced production of testosterone and testicular shrinkage (atrophy of the testis) (Adler, 1992). Atrophy of the testes primarily results from reduction in the diameter of the seminiferous tubules and loss of spermatozoa (Van Thiel *et al.,* 1974), which could lead to impotence or infertility and also decreased secondary sexual characters in males (these include shift in deposition of fat from the abdomen to the hip arc, enlargement of the breast, reduced hair on the chest area and face). Lloyd and Williams, 1948, observed that about 72% of men having advanced cirrhosis had reduced sexual potency and libido respectively. Study by Van and colleagues, 1974, also observed prominent impotence among subjects, however, the impotence was frequently more in patients having greater liver damage than those with none. The prevalence of impotence which serves as a semi quantitative measures reported 22 men having cirrhosis, and out of them 18 could not ejaculate, of which they were simply impotent, and only 2 men were unable to produce sperm and only 1 man was having adequate sperm count.

The deleterious effects of alcohol on the function of the Leydig cell and production of testosterone were clearly shown in a study containing volunteers of young healthy males with functional liver in which they had over a period of four weeks (Gordon *et al.,* 1976). The effects of alcohol on metabolism of alcohol are different, for instance, comparing men having alcoholic liver disease with non alcoholic liver disease. Thus, testosterone levels in the blood and rate of production are decreased in both men, the clearance of testosterone metabolism only increases in

men that have no alcoholic liver disease. Men with liver alcoholic disease had reduced clearance of testosterone metabolism (Southren *et al.,* 1973).

Additional method in which testosterone levels could be reduced following alcohol administration or exposure is observed in the conversion of the precursor of testosterone or testosterone itself into estrogen, this is simply known as aromatization (Van Thiel *et al.,* 1974). More so, the testosterone immediate precursor (androstenedione) can be converted into estrone (which is a less potent estrogen). Thus, the process in the conversion of testosterone to estradiol could be enhanced regularly via alcohol consumption as seen in men. Studies have also showed that men with liver disease as a result of consumption of alcohol over prolong period of time have increased estrogen levels (Van Thiel *et al.,* 1974, 1978; Gordon *et al.,* 1978). In order to establish the mode of action by which alcohol causes testosterone reduction, researchers investigated the effects of alcohol on the testis in-utero and also analyzed the independent analysis of the testis. The experiment showed that the production of testosterone in isolated testis was significantly reduced as it was shown in studies in which the testis was intact in the animals (or animals with intact testes) (Badr *et al.,* 1977).

Studies have showed different mechanisms in which alcohol causes the suppression (Anderson *et al.,* 1983). For instance, researchers suggested that, acetaldehyde, a breakdown product of alcohol could be a factor contributing to the action of alcohol leading to reduced testosterone level, thus, some studies showed that acetaldehyde is essentially potent than alcohol itself in leading to the suppression of the release of testosterone (Badr *et al.,* 1997; Cobb *et al.,* 1978). Although, the suppression of the production of testosterone is not caused by acetaldehyde, as this was evident by the breakdown of alcohol to acetaldehyde which was mediated by the enzyme involved in the breakdown, these enzymes utilize cofactors which are essentially used by the enzymes in the prevention of the production of testosterone). (Ellingboe and Varanelli, 1979; Gordon *et al*., 1980). Disturbances in other hormonal systems also may contribute to the alcohol-induced suppression of testosterone levels. For example, the adrenal hormones cortisol and corticosterone (in rats) can suppress the reproductive system by inhibiting the ability of the Leydig cells to produce and release testosterone. Studies in human and animals found that alcohol exposure increased adrenal hormone levels, thereby interfering with reproductive functions (Rivier and Vale, 1998). More so, nitric oxide (NO), a gas found in all tissues, may

contribute to alcohol's toxic effects. Nitric oxide affects numerous biological processes, including widening of the blood vessels (i.e. vasodilation), the immune response, communication between cells of the nervous system, and hormone secretion (i.e. decreases testosterone secretion) (McCann and Rettori, 1996). In the testes (as well as in many other tissues), the gas (NO) is generated by an enzyme called nitric oxide synthase (NOS). Study has indicated that inhibition of the enzyme NOS by various substances can prevent the alcohol-induced decline in testosterone levels (Adams *et al*., 1993; Shi *et al*., 1998). Sertoli cells also may be an important target for alcohol's actions on the reproductive system. The mechanisms underlying alcohol's effects on the Sertoli cells have not yet been elucidated. It appears that alcohol may damage some of the proteins required for sperm cell production that Sertoli cells provide (Zhu *et al*., 1997).

With the development of sophisticated techniques that allow measuring even low hormone levels in the blood (i.e. radioimmunoassay), researchers now are able to assess changes in the normal levels of the hormones of the anterior pituitary gland (FSH and LH) following exposure to alcohol. These studies observed a relative reduction in the levels of testosterone. Most importantly, the LH blood level was not increased but decreased in other studies (Van Thiel *et al.,* 1974; Gordon *et al.,* 1976, 1978; Ching *et al.,* 1998; Adams and Cicero, 1991; Emanuele *et al.,* 1991, 1992). The result showed an unexpected outcome, because exposure to alcohol could alter the testicular function thereby decreasing the level of testosterone, which could have caused a rise in the levels of LH due to the feedback regulatory mechanism of the hypothalamic pituitary gonadal axis (HPG axis). Accordingly, with the absence or reduction of LH levels in many alcoholics could imply that alcohol acted both on the testes and as well on the hypothalamus and/or the anterior pituitary gland. Sequentially, studies showed that rats fed with alcohol had a reduced blood LH levels which could occur due to improvement in the production and secretion of LH from the anterior pituitary gland (Emanuele *et al.,* 1991).

Another concern is that could alcohol affect the release of LH from the anterior pituitary gland or the release of GnRH/LHRH from the hypothalamus. In order to address this, scientists removed animals anterior pituitary gland and then grown them in-vitro in the absence or presence of alcohol (Pohl *et al.,* 1987). The outcome of the study indicated that alcohol decreased the secretion of LH from the isolated pituitary gland, this suggests that alcohol reduces the level of

LH by acting on the anterior pituitary gland. Alcohol also affects the level of LH either by acting directly on the pituitary gland and/or hypothalamus. A study using Wistar rats showed that administration of alcohol significantly reduced the level of GnRH in blood vessels that link the hypothalamus with the pituitary gland (Ching *et al.,* 1998). Though, the exert mode of action in which alcohol causes infertility has not been fully elucidated, however, Emanuele and Emanuele, (1998), reported the deleterious effects of alcohol on the male reproductive system and suggested several mechanisms by which alcohol causes its deleterious effects . These mechanisms are associated with metabolism of alcohol, alcohol-related cell damage and other reactions hormones related with the consumption of alcohol. However, animal and epidemiological studies demonstrated that abuse of alcohol is associated directly with increase in the diseases of multiple organs like liver injury, neurological disorders, cardiovascular disease, while the mechanism of cellular injuries like damage to the DNA, protein modification and lipid peroxidation (Shi *et al.,* 2006).

At the level of the reproductive system, alcohol has shown to have a negative effect. Thus, researchers have investigated about the profound outcome of alcohol in the past decades, but there are some questions that are yet to be answered. Further research studies could target on investigating the ingestion of the effects of alcohol in earlier life during prepubertal and pubertal in which reproductive organs, majorly the testes are matured and are exposed to alcohol consumption. These investigations could be relevant in evaluating the way(s) and why teenagers consume alcoholic beverages, which is eventually illegal in some countries. More so, researchers must also learn the cellular mechanism in which alcohol induced its deleterious effects and effective intervention to alleviate or prevent those deleterious effects caused by alcohol.

2.7 QUERCETIN

Quercetin is otherwise known to be called 3, 3', 4', 5', 7-Pentahydroxyflavone, as one of the known antioxidants, is a non-steroidal plant (Moutsatsou, 2007) and is distributed in vegetables as well as in fruits as described in table 2.1 (Chen and Zhou, 2010). Quercetin, also known as bioflavonoid can as well be found in healing herbs, tea (Middleton, 1998) and wine (Yoshikawa *et al*., 1997).

One of the usefulness of quercetin as a flavonoid is seen in supplement (nutrition). Laboratory studies showed that it has anti-inflammatory and antioxidant properties (Davis *et al*., 2009; Stewart *et al*., 2008) and it is being investigated for a wide range of potential health benefits. Quercetin in plants is mainly present in its glycosylated forms, such as quercitrin (3-rhamnosyl-quercetin) or rutoside (3-rhamnosy-glucone and its glucosides are absorbed better than quercetin administered in nonglucosic forms (Olthof *et al*., 2000). In plants, quercetin occurs almost exclusively bound to one or more sugar molecules, so-called quercetin-β-glycosides. The dominant type of glycoside varies between plants. Apple, for example, contains predominantly galactosides, rhamnosides and arabinosides, while onion contains mainly glukosides (Hollman and Arts, 2000). The details of quercetin in selected foods are shown in table 2.1.

Table 2.1 Amount of quercetin in selected food (Mangels et al., 1993)

Plant	**Quercetin Concentration (mg/100mg)**
Broccoli Raw	**2.8**
Carrot Raw	**0.4**
Celery Raw	**3.5**
Powdery Cocoa Raw	**20.1**
Cranberries Raw	**14.0**
Lettuce Raw	**2.0**
Kale Raw	**5.1**
Lingonberries Raw	**11.3**
Onions Raw	**22.6**
Red Rioe Tomatoes	**0.5**

2.7.1 QUERCETIN EFFECTS

Quercetin possesses a wide know activities within cells and as an antioxidant it possesses chelating as well as radical scavenging properties (Bjeldanes and Change, 1997; Boots *et al.,* 2008). It also shows to have the potency against free radicals, functioning as scavenger of reactive oxygen species, and reacting nitrogen species among which are ONOO- and NO according to studies by Haenen and Bast, 1999; Heijnen *et al.,* 2001; Nicket *et al*., 2011, also protects and prevent against lipid peroxidation and against low-density lipoprotein (regarded asbad cholesterol) (Kleemann *et al.,* 2011; Leckey *et al.,* 2010). The resulting scavenging and antioxidant capacity displayed by quercetin could be linked to the availability of pharmacophores present in the molecule (Heijnen *et al*., 2002)

The mode of action of which quercetin displayed in combating cancer is through its antioxidant activity in which it causes the removal of oxidative stress and causing inactivation of carcinogenic enzymes and also serves as a competitive inhibitor in estrogen receptors and in modifying the pathways for signal transduction, and of transcription factors as well as in other proteins (Bors and Saran, 1987; Choi *et al.,* 2001; Chen *et al.,* 2005; Murakami et al., 2008).

Quercetin also exhibits anticancer properties by causing inactivation of apoptotic process, proliferation, fatty acid synthase, reducing the expressions of metalloproteinase 9 (MMP-9) and metalloproteinase 2 (MMP-2) respectively (Shen *et al*., 2003; Brusselmans *et al*., 2005; Vijayababu *et al*., 2006; Zhong *et al*., 2006; Daker *et al*., 2012). Quercetin has been shown to exert growth inhibitory activity on human breast (Scambia *et al*., 1993; Singhal *et al*., 1995), ovarian (Scambia *et al*., 1990), Leukaemic (Larocca *et al*., 1990), colon (Shiu-Ming, 1996) and nasopharyngeal carcinoma cells (cancer cells) (Zhong *et al*., 2008). Studies on quercetin inhibiting growth of nasopharyngeal carcinoma cells, ovarian and breast cancer as well as on leukemia in humans have been extensively investigated (Larocca *et al.,* 1990; Scambia *et al.,* 1990; Singhal *et al.,* 1995; Shiu-Ming Kuo, 1996; Zhong *et al*., 2006, 2008), all these were achieved through the release of cytokines IL-6, tumor necrotic factor, nitric oxide (NO) and so on (Kim *et al.,* 2007). Tan *et al*., 2003 also studied the effect of quercetin having the properties in inhibiting the steps in angiogenesis among which are inhibiting the tubular formation of human microvascular dermal endothelial cells which is dose dependent and also prevents migration and proliferation of these cells and in the suppression of vascular endothelial growth

factor receptor 2 (VEGFR2) and causes reduction of vascular endothelial growth factor (VEGF) respectively (Lin *et al.,* 2012). Quercetin has been shown to modulate the functions of the functions of the ovaries in which it could interfere with the steroidogenic activity of the cell and angiogenesis. The effects of production of progesterone could result from the steroidogenic enzyme inhibition. Quercetin has also been reported to accelerate progesterone release which has proapoptotic function and anti-proliferative activity and as part of the importance of quercetin, it is being used in reproductive function regulation (ovarian folliculogenesis, oocyte maturation and ovulation), including fertility and treatment of reproductive disorders. As for estrogen (E2) production, quercetin has a dose-dependent biphasion activity. Follicular development is strictly dependent on the angiogenic process, driven mainly by VEGF.

Quercetin displayed a strong inhibitory effect on VEGF (Hung, 2007) and inhibits proliferation, migration, and differentiation of endothelial cells in process of angiogenesis. This finding acquires particular relevance, since it is possible to speculate a possible negative influence of quercetin on ovarian physiology. These findings suggest that quercetin represents a potential modulator of ovarian functions (Santini *et al*., 2009). Via estrogen receptors quercetin induced cell cycle arrest and apoptosis in human breast cancer MCF-7 cells (Choi *et al*., 2001). Anti-proliferative activity of quercetin in human ovarian cancer cells is mediated by its interaction with type II estrogen binding site (type II EBS) (Markaverich *et al*., 1998). Quercetin might exert a protective effect against post-menopausal bone loss. Directly induce apoptosis of mature osteoclasts in dose-range effective for inhibiting bone resorption (Wattel *et al*., 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODOLOGY

3.1 NICOTINE PREPARATION

Nicotine hydrogen tartrate (95% Nicotine) with product number (26140) was obtained from BDH chemical Ltd Poole England. Nicotine dosage was freshly prepared with distilled water for the treated group of animals that were delivered at 1.0 mg/kg body weight. The working solution was stored in a brown glass bottle (at 4^0 C for no longer than 5 days in a refrigerator) in order to preserve the potency of the drug due to exposure to ultraviolet light. LD50 for nicotine is 50 mg/kg bw (rats) (Hayes, 1982).

3.2 ALCOHOL PREPARATION

Absolute ethanol was obtained from Sigma (C_2H_5OH) 99-100% with boiling point range (77-79^0C) was used in this study. 25mL of ethanol was measured with a measuring cylinder and was toped up with distilled water to the calibrated mark 100 on the measuring cylinder to make 25% ethanol. LD50 for alcohol is 7.06g/kg bw (Wiberg *et al.,* 1970).

3.3 QUERCETIN PREPARATION

Quercetin was purchased from Sigma Chemical Company St. Louis, MO, USA) and the stock solution was dissolved with corn oil (which was used as vehicle), mixed vigorously and stored in a dark brown bottle. The quercetin solution was freshly prepared each week. 30 mg/kg quercetin preparation: the quercetin supplement was weighed (about 1000mg), and 1000 mL of corn oil was added. Therefore, to get 1000mg in 100 ml= 100mg in 10mL = 10 mg/mL. for the stock, a 200g rat would take: 1000mg in 100ml. To determine the volume/dose of quercetin a 200g rat will take: x=200g X 10mg/1000g = 2mg. So, from the stock, a 200g rats would take: 2mg/100ml/1000mg = 0.2 mL. LD50 for quercetin is 160 mg/kg bw (Harwood *et al*., 2007).

3.4 EXPERIMENTAL ANIMALS

Ninety male (180-200g) and forty five female (160-180g) Wistar rats were purchased from the central animal house, University of Ibadan for the study and then acclimatized. They were

housed in clean, well ventilated cages. Rat feeds were purchased from Ladokun feeds, mokola market, Ibadan and water was made available *ad libitum*.

3.4.1 EXPERIMENTAL DESIGN

The animals used for the study and were divided into nine groups of five rats each.

Group 1: Animals that served as the control group and were given distilled water

Group 2: Animals in this group were given Corn oil alone at 2mL/kg body weight (vehicle), (Sato *et al*., 2000)

Group 3: Animals in this group were given supplementation of Quercetin at 30mg/kg body weight (Arias *et al.,* 2016).

Group 4: The Animals in this group were treated with Alcohol and were delivered at 3 mL/kg body weight as 25%v/v.

Group 5: Animals in this group were treated with Nicotine at 1.0 mg/kg body weight (Oyeyipo *et al.,* 2010).

Group 6: Animals in this group were treated with Alcohol and were delivered at 3 mL/kg body weight as 25%v/v + Nicotine delivered at 1.0 mg/kg body weight.

Group 7: The rats were treated with Alcohol and were delivered at (3 ml/kg body weight as 25%v/v) + Quercetin delivered at (30 mg/kg body weight).

Group 8: The rats in this group were treated with Nicotine and delivered at (1.0 mg/kg body weight) + Quercetin delivered at (30mg/kg body weight).

Group 9: The animals in this group were treated with Alcohol and delivered at (3 ml/kg body weight as 25%v/v) + Nicotine delivered at (1.0 mg/kg body weight) + Quercetin and delivered at (30mg/kg body weight). The control groups include groups1-), while the experimental groups consist of groups 4-9. However, the route of administration of the drugs used was via oral route and lasted for a period of 52 days.

3.4.2 BLOOD SAMPLES COLLECTION

After the completion of the experiment, on the last day of the study, the rats were anesthetized with Ether and samples of blood from the control and experimental animals were collected via orbital sinus vein through capillary tubes into plain sample bottles (Schalm *et al*., 1986), Blood samples were then allowed to clot at room temperature, after which they were then subjected to centrifugation (Union Laboratory England) at 10,000 rv in order to obtain serum for hormonal assay for respectively.

3.4.3 HEMATOLOGICAL PROFILE

Hematological profile were carried out in line with Mitruka and Rawnsley, (1997) for packed cell volume packed cell volume (PCV) and the procedure by Schalm *et al.,* (1975) was used to estimate Haemoglobin (Hb), Erythrocyte count (RBC) and Leukocyte count (WBC) were determined by scanning Giemsa's stained slides in the classic manner.

3.4.4 DETERMINATION OF TOTAL PROTEIN (EPIDIDYMAL AND SERUM)

Total protein determination was carried out using Randox kit formulated for biuret procedure as described by Ramnik (1999).

3.4.5 SACRIFICE AND TISSUE PREPARATION

At the end of the treatment period, after collection of blood samples animals were sacrificed, the gonad and epididymis from sacrificed rats were excised, prepared and taken for histological examination.

3.4.6 COLLECTION TECHNIQUES FOR PARAMETERS

3.4.7 EVALUATION OF FERTILITY

With the mincing of the caudal epididymis the sperm cells were obtained and diluted with physiological solution (normal saline) at room temperature of 37^0C and the examination for spermatozoa indices were carried out with the aid of light microscope at x40 objective as follows:

(a) SPERM COUNT

The evaluation of concentration of spermatozoa count was by Feustan *et al.,* (1989) protocol. Spermatozoa mixed in physiological solution (normal saline) in ratio 1:20, it was then mixed, a drop to two drops of sperm solution was then introduced unto the haemocytometer on each side of the counting chamber, after that it was allowed to stand for up to 5 minutes for sedimentation, and sperm cells were counted in five (5) large squares and expressed in millions.

(b) SPERM MOTILITY

Sperm motility was done by the method Linder *et al.,* (1986) protocol. This was modified and was done by mincing and a drop of the suspension of sperm was introduced unto the slide and examined under the microscope. The number of sperm cells that were non-motile were determined; it was then followed by counting of the total sperm cells.

(c) SPERM VIABILITY BY EOSIN STAIN

Sperm viability test was employed by adding onto prepared slide and then it was allowed to be left for 5 minutes to stand at room temperature (37^0C), after which, it was examined under the microscope. The live spermatozoa did not pick up eosin stain while the head of the dead sperm cells were stained. The sperm viability test was carried out in accordance to procedure employed by (Krzanowska *et al.,* 1995), and viability of spermatozoa was expressed as the total % of live spermatozoa.

(d) SPERM NUCLEAR MATURATION BY ANILINE-BLUE

Using Morel *et al.,* (2000) method, the procedure for evaluation of sperm nuclear maturation was carried out using aniline blue. The nuclei of the sperm that were stained with the blue coloration were considered not mature, while the nuclei of mature spermatozoa were unstained with aniline blue, that is did not pick up the stain. Thus, the percentage of immature sperm was calculated from the observation of one hundred sperm preparation from each group.

(e) SPERM MORPHOLOGY

A drop of Eosin stain was added to the sperm suspension and kept for 5 minutes at 37^0C spread gently to make a thin film. The film was air dried and then observed under the microscope for

changes in sperm morphology, according to the method of Feustan *et al.,* (1989). The criteria chosen for head abnormality were no hook, excessive hook, amorphous, pin and short head. For tail, the abnormalities recorded were, coiled flagellum, bent flagellum, bent flagellum tip. The results are the percentage overall abnormal form.

3.4.8 HISTOLOGICAL STUDY

The selected organs (hypothalamus, pituitary, liver, testis, prostate, epididymis and seminal vesicle) were harvested and were fixed in 10% v/v buffered formaldehyde and then dehydrated in ascending grades of ethanol in this manner, 70, 90 and 95% v/v. then cleaned in xylene, impregnated and embedded in paraffin wax. Sections of the tissues were cut at 5µm on a microtone machine. The sections were floated out on clean microscope slide, which had been albumenized to avoid detachment from slides during staining process. The slides were air-dried for two hours at 37^0C. Then they were stained and the slides were passed through 20-100% ethanol concentration for dehydration and cleaned with xylene. A mounting medium was put on the tissue sectioned. A thin glass-covered slip was placed on the mounting medium and underlying tissues were allowed to dry. This was later observed using a light microscope at X100 magnification and the photomicrographs were taken.

3.4.9 OXIDATIVE BIOMARKERS IN THE TESTIS

3.5 LIPID PEROXIDATION ASSAY

Lipid peroxidation was assayed by measuring the Thiobarbituric Acid Reactive Substances (TBARS) produced during lipid peroxidation. Reagent for lipid peroxidation include the following: 1 ml of Trichloroacetacetic acid (20% TCA), 0.25ml of Thiobarbituric acid reagent (200 mg TBA was diluted in 30 mL of distilled water and 30 mL of acetic acid), all in test tubes for each of the experimental and control groups, also added were 0.5 mL of testicular homogenate and 0.5 mL of normal saline, after which they were subjected to incubation for one hour at 95^0C and were centrifuged using centrifuge machine (for a period of 10 minutes at 3000 rpm. The supernatants were then collected and the absorbance was determined spectrophotometrically at 535nm. The concentration of the thiobarbituric acid reactive substances (TBARs) was then expressed as nmol of malondialdehyde/mm (MDA) according to Ohkawa *et al.,* (1979).

Calculation : TBARs activity = $\frac{\text{Absorbance x volume of mixture x 1000}}{\text{Estimation Coefficient of sample x mg protein}}$

3.5.1 ESTIMATION OF ANTIOXIDANT ENZYMES

(a) DETERMINATION OF REDUCED GLUTATHIONE (GSH)

Reduced glutathione (GSH) assay was carried out according to the method of Tietze, (1969). 0.1ml of tissue (testis) component was mixed with 0.1 ml of 1.0 mM of Reduced glutathione (GSH) and 0.1 mL of 1.0 mM CDNB (p- Chlorodinitrobenzoic acid) was added. To the resulting mixture, 2.70 ml of 100 mM phosphate buffer (pH 6.5) was added and the change read for five minutes in one minute interval at wavelength 340 nm. GSH was calculated using the formula:

$$\text{Activity (U/ml)} = \frac{[(\text{O.DTest} - \text{O.D Blank}) \times \text{Total Volume})]}{(\text{Estimation Coefficient}) \times (\text{Sample Volume})}$$

(b) DETERMINATION OF SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase (SOD) activity was assayed by the method described by Sum and Zigma, (1978). The reaction was carried out in 0.5m Sodium carbonate buffer pH 10.2 and was initiated by the addition of 3×10^{-4} epinephrine in 0.005N HCl. The absorbance was read at 320nm. SOD activity was calculated using the formula:

$$\text{Percentage Inhibition} = 100 - \frac{100 \times \text{Change in substrate absorbance}}{\text{Change in blank absorbance}}$$

(c) ESTIMATION OF SERUM HORMONAL LEVEL

The serum hormonal level of testosterone, LH and FSH concentrations were estimated using ELISA technique. The procedures of the hormonal assays were specified and carried out using the manufacturer's kits (Fortress Diagnostic Limited. Antrim Technology Pack, Atrim, BT41IQS. UK).

3.5.2 LIBIDO TEST

Libido test was employed by using the method described by Kamaldeep and Anupam,(2002) with a little modification. This was done by observing the mounting behavioural pattern displayed by the male Wistar rats. Virgin female Wistar rats were paired on the 52nd day of the experiment with the experimental male Wistar rats between 5-6pm for the observation of the behavioural mounting of libido orientation. The number of mount was recorded for 15 minutes and this process was done for both the control and treated groups.

3.5.3 FERTILITY STUDY

The total numbers of 45 female Wistar rats (virgin animals) were used for the aspect of fertility study. The method of Bolarinwa, (1993) was adopted. The female rats were cohabited with the experimental male Wistar rats from the 38th day of the experiment till the day treatment lasted. Positive mating index was taken as the presence of vaginal plug and this was taken as the pregnancy day one. The litter numbers delivered by the untreated female Wistar rats were recorded. The following formula was then employed in calculating the percentage fertility success rate in the present study as indicated below (Raji *et al*., 2006):

$$\%\text{Fertility Success} = \frac{\text{Female rats that became pregnant}}{\text{Female rats that were mated}} \times 100$$

3.5.4 SPERM CAPACITATION AND ACROSOME REACTION

PROCEDURE/PROTOCOL

The method of Carmen et al., (1995) was adopted and employed in rat sperm capacitation procedure in the present study. Specimen of sperm cells from the caudal epididymis of experimental and control Wistar rats were collected through teasing or by perfusion, and were quickly collected onto a pre-warmed modified sperm capacitation medium (SCM). The modified sperm capacitation medium constitutes the following: $NaHCO_3$ (24.9 mM), NaCl (110 Mm), $CaCl_2$ (2.4 mM), $MgCl_2$ (0.4 mM), glucose (5 mM), lactate (6.26 mM), KCl (2.7 mM), bovine serum albumin (BSA) (12 mg/mL). The concentration of sperm was then determined with hemocytometer (improved Neubauer). 1 ml of sperm capacitation medium was transferred into eppendorf tube and samples of sperm (100µl) from the experimental and control rats were

introduced into the sperm capacitation medium. It was incubation for about 1-3 hours at 37^0C, the sperm cells that were highly motile swum up (appeared) at the upper two-third layer of the Eppendorf tube. Thereafter, it was then transferred into a new Eppendorf tubes and was adjusted to a concentration of $2x10^6$ sperm/mL. This was an adopted method by Fraser and McIntyre, 1989; Li and Chen, 1996; Ain *et al.,* 1999) which was believed to cause induction of sperm capacitation and acrosome reaction. However, sperm aliquots were then obtained for the assessment of sperm capacitation and acrosome reaction from each of the experimental and control male Wistar rats. The percentage of motility exhibited by sperm cells and the pattern of movement i.e movement characteristics were examined at the end of the period of incubation time. The method of Feng *et al.,* (2007), was then employed in the staining stage (process) using coomassie brilliant blue (CBB) dye/staining technique after the sperm aliquots were removed for assessment of sperm acrosomal status from each group. Samples of sperm that were air-dried on glass slides were fixed with 5% paraformaldehyde in phosphate buffer saline (PBS) for a period of 15 minutes. They were then washed again with PBS and the slides were then stained with 0.25% CBB in 10% glacia acetic acid and 25% methanol. After which, it was then rinsed with water and dried and then covered-slip and placed (positioned) under the mounting media. The acrosomal region was stained in acrosome-intact sperm while unstained in the acrosome-reacted sperm. This test was done three times in order to observe the absence or presence of acrosome reaction in each slid and also determined were the percentage of acrosome reacted sperm.

Table 3.1: Chemical constituents of Sperm Capacitation Medium (SPM)

S/N	Chemical	mM	Mol. Weight	Mass / Liter
1	NaCl	110	58.44	6.43g
2	KCl	2.7	74.5	201 mg
3	$CaCl_2$	2.4	110.98	266 mg
4	$MgCl_2$	0.49	95.21	47 mg
5	NaH_2PO_4	0.32	119.98	38 mg
6	Glucose	5	180.16	900 mg
7	Sodium Lactate	6.26	112.06	701 mg
8	Pyruvate	0.125	86.06	11 mg
9	BSA	-	-	12 g

3.5.5 MEASUREMENT OF SERUM ELECTROLYTES

The serum level of electrolytes (Na^+, K^+, Cl^-, and Ca^{2+}) concentrations were carried out using ELISA technique according to a standardized method by the World Health Organization (WHO).

3.5.6 ESTIMATION OF BIOCHEMICAL PARAMETERS

Standard laboratory kits were purchased and the manufacturers' guide/protocol were strictly followed for the evaluation of Alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), Gamma-glutamyl transferase (GGT), Acid phosphatase (ACP), and Glucose-6-phosphate dehydrogenase (G6PDH).

(i) ESTIMATION OF PROSTATE SPECIFIC ANTIGEN (PSA)

The prostate specific antigen was estimated using Calbiotech, Inc (CBI) PSA ELISA Kit is intended for the quantitative measurement of PSA in animal / human serum and the protocol provided by the manufacturer's guide/owner's guide was strictly adhered to.

(ii) EVALUATION OF LIPID PROFILE

Serum lipid profile estimation such as total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL) were assayed using commercial standard kits (Randox laboratories^{TM} assay kits). Also, the concentration for vLDL, were determined from the values obtained from the above mentioned analysis.

3.5.7 LIVER FUNCTION TEST

Estimation of bilary level of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were assayed with standard commercial kits and the protocol was strictly followed using the manufacturer's guide.

3.5.8 EXTRACTION OF GENOMIC DNA FROM TESTIS (SALTING-OUT METHOD)

Materials

Pipettes and tips, 1.5 ml microfuge, Micropipettes, UV transilluminator, Electrophoretic apparatus, Eppendorf tubes

Reagents

Lysis Buffer Compositon

The Lysis Buffer composition include : 1.5% KCl (ice cold), 10% sucrose, 1% SDS, 0.8% Tween-80 and 20 mg/mL proteinase K

Salting out Method: using 6 M NaCl

Precipitation Reagent

The precipitation reagent used was 3M of Sodium acetate (measure at pH 5.2) and 95% Ethanol (chill prior to use).

Washing Solution

For the washing reagent, 70% Ethanol was used (chill prior to use).

Equipment Used

Incubator/water bath, Centrifuge,Vortex mixer.

Procedure for RBC Lysis

0.5- 1g of testicular tissue was cut and put in a in a clean tube, 4 lysis buffers were added and homogenized using Teflon homogenizer at 100 rpm for 10 min. The homogenate was then digested overnight by adding proteinase K (0.3 mg/mL) at 37^0C. It was then entrifuged for ten minutes at 8,000 revolution/min. The supernatant was transferred into a fresh eppendorf tube.
1/3 volume of 6 M NaCl was added and shaken vigorously. It was then centrifuged again at 8000 rpm and the supernatant was transferred to a fresh tube. 1/10 vol of 3M Na-acetate was added
Followed by addition of 2 volume of ice-cold absolute ethanol and kept in the freezer or ice overnight. It was then centrifuged at 8000 rpm for 10 min and the resulting DNA pellet was air dried. The DNA pellet was reconstituted in 50 µL TE buffer and the yielded DNA was purified.
This was then followed by DNA electrophoresis using 0.9% agarose gel pre-stained with ethidium bromide at 100mV for 1 hour. The photograph of gel under UV light was taken using a digital camera.

CONSTITUENTS OF 3M SODIUM ACETATE BUFFER, pH 5.2 (50 mL)

20.41 g of NaAcetate was measured into a measuring cylinder and 32 mL of Distilled water was added, mixed and glacial acetic acid was added and top up to 50 mL. It was sterilized by autoclaving at 121^oC for 15 min and stored at 4^oC.

CONSTITUENTS OF 0.5M EDTA BUFFER, pH 8.0 (50 mL)

9.305 g of Na_2EDTA and 1 g of NaOH were measured into measuring cylinder and mixed with 30 mL of distilled water and 50 mL of water was added and sterilized by autoclaving at 121^oC for 15 minutes and then stored at 4^oC.

CONSTITUENTS OF 6 M SODIUM CHLORIDE (50 mL)

17.5 g of NaCl was measured into a test tube and mixed with 30 mL of distilled and water was added to top it upto 50 mL and was stored at 4^0C.

CONSTITUENTS OF 1 M TRIS-HCl (50 mL)

6.06 g of Tris-Base was weighed and 40 mL of distilled water was added followed by 2.1 mL of Conc. HCl into a beaker. The solution was mixed and 50 mL of water was added. It was then sterilized by autoclaving at 121^{o}C for 15 minutes and stored at 4^{o}C.

ETHIDIUM BROMIDE (10 mg/mL) - Toxic

10 mg Ethidium bromide dye was measured and 1 ml of sterile water was added and gently mixed in a brown bottle or foiled Eppendorf tube and stored at room temperature.

BROMOPHENOL BLUE LOADING BUFFER (x4)

25 mg bromophenol blue and 4 g of sucrose were measured and added together into a test-tube, 10 mL of sterile water was added and mixed gentle and stored at 4^{0}C.

3.5.9 IMMUNOHISTOCHEMISTRY STUDY

Immunohistochemistry study was carried out by using the protocol of Hsu *et al., (*1981).

Principle:

After the tissues have been processed and sectioned, the process uses antibodies that bind to specific protein to form a complex. This binding is then visualized by using second step biotinylated antibody, followed by amplification with streptavidin horseradish peroxidase enzyme which converts the substrate diamino benzidine into a brown coloured precipitate at the site of reaction, observed as positive reaction.

Reagents used include: Xylene**,** graded ethanol (70%, 90%, 95%, 100%). Phosphate Buffered Saline: (8g of sodium chloride was dissolved in 1 liter of distilled water, and 1.44g disodium hydrogen phosphate, 0.24g potassium dihydrogen phosphate, and 0.2g potassium chloride were added and pH was adjusted to 7.4 using HCI). The stock solution used was 100 mM citric acid, Hydrogen peroxide (0.3% in methanol) and the Blocking reagent used was Egg Avidin. The primary antibody used was diluted with dilution factor or ratio of 1:50 - 1:100) and the secondary antibody was biotinylated anti-immunoglobulin antibody, Streptavidin horseradish peroxidase conjugate (1:200 dilution). The substrate reagent used was 5mg of Diaminobenzidine which was dissolved in 100 ml of PBS and 0.1 ml of 0.3% hydrogen peroxide was added.

Procedure:

3µ thick of testicular tissue was cut and embedded in paraffin, it was allowed to heat on hot plate for 1 hour at 70^0c. The sections were then hydrated and deparafinized in xylene, and were rehydrated through graded ethanol to distilled water (100%, 95%, 90% H_20). Sections were placed in pre-warmed 'Target Unmasking Fluid'citric acid solution with pH 6.0 (1:10 dilution) in a microwave at power 100 for 15 minutes. The sections were equilibrated by gently displacing hot citric acid with running tap water for 3 minutes. The peroxidase was blocked in tissue using peroxidase block containing 0.3%H_20_2 for 15 minutes. The excess waster was stabilized with PBS mixed with Tween 20 for 2 minutes. Egg Avidin Protein was used to block non-specific binding in humidified chamber for 15 minutes and was removed gently by washing for 2 minutes with PBS. The sections were incubated with primary antibody (*p53* Ab-6, EGFR Ab-I, BCl_2 Ab-3, KI67 Ab-1) in humid chamber for 45 minutes and was extensively washed with PBS for 3 minutes. It was then incubated with secondary biotinylated antibody for 45 minutes at room temperature followed by washing in PBS thrice .Polymer was added to initiate polymerization, by incubating with Streptavidin horseradish peroxidase system (Dako AS, Denmark) for 15 minutes. Thereafter, it was washed twice with PBS and peroxidase substrate DAB was added for 15 minutes and brown precipitate develops indicating positive reaction. It was washed with water and counterstained for 2 minutes with haematoxylin and dehydrated in graded ethanol and cleared with xylene. It was mounted with DPX and examined under light microscope at magnification x40.

3.6. EVALUATION OF IMMUNOHISTOCHEMISTRY RESULTS

To analyze for the expression of p53, Bcl-2, KI67, EGFR and CYD expression, the intensity of staining and quantity of cells stained were evaluated in proportion to the germ cell and testicular cytoarchitectural areas. Brown staining of the interstitial cells (Leydig cell) and seminiferous tubules of the testicular tissue were viewed under x40 magnification. Positive control slides were prepared from cell known to express the protein and control negative prepared by omitting the incubation state with the primary antibody. An expression index was also created to evaluate the

expression as described earlier (Ravi *et.al* 1996). This was done by classifying the protein expression into four categories based on the number of cells with positive expression.

Results were reported as positive when a complete or incomplete circumferential membrane staining was observed in at least 1% of the affected area. Staining was defined as immunostaining of testicular architectural areas above background level and scored as follows:

- 1^{+}=weak/mild expression: when a complete or incomplete circumferential membrane staining was observed in at least 1-10 % of the seminiferous area of the testes
- 2^{+}=moderate expression: when a complete or incomplete circumferential membrane staining was observed in at least 10-50 % of the testicular (interstitial) area
- 3^{+}=strong expression: when a complete or incomplete circumferential membrane staining was observed in more than 50% of the testicular area
- The absence of membrane staining or cytoplasmic staining was reported as negative.

The percentage of stained cells was assessed as follows: 1–10, 10–50, and >50% and percentage area of +ve immunoreactive cells was estimated quantitatively using ImageJ Analysis Software (USA).

3.6.1 Statistical analysis

Data were expressed as Mean ± SEM and the differences in means were compared by analysis of variance and Student's t-test where applicable. $P<0.05$ was considered statistically significant. The Statistical Package for Social Sciences (SPSS) software (version 22.0; SPSS Inc., USA) was used for data analysis.

CHAPTER FOUR

4.0 RESULTS

4.1. Effects of quercetin on alcohol and/or nicotine treatment on reproductive organ weights in male Wistar rats

The results in the Table 4.1 showed that there were no significant differences ($p>0.05$) in change in mean prostatic, epididymal, and seminal vesicular weights of all the treated groups when compared to the control group. However, the group treated with alcohol alone showed an increase in testicular weight in comparison to the other treated groups. There was a significant decrease ($p>0.05$) in changes in mean testicular weights of nicotine and alcohol plus nicotine treated groups when compared to the control group. However, there was a significant increase ($p<0.05$) in nicotine alone treated group when compared to the nicotine plus quercetin treated group.

Table 4.1: Effects of Quercetin on relative weights of reproductive organs of male Wistar rats treated with alcohol and nicotine.

Group	Prostate (%)	Epididymis (%)	Seminal vesicle (%)	Testis (%)
Control	0.33±0.04	0.65±0.03	0.26±0.03	1.17±0.08
Corn oil	0.34±0.03	0.64±0.07	0.25±0.04	1.18±0.04
Quercetin	0.35±0.02	0.65±0.06	0.27±0.03	1.20±0.08
Alcohol	0.38±0.03	0.67±0.09	0.33±0.03	1.52±0.06*
Nicotine	0.32±0.03	0.60±0.06	0.28±0.02	1.01±0.08*
Alcohol+ Nicotine	0.30±0.01	0.62±0.08	0.32±0.04	0.98±0.07*
Alcohol+ Quercetin	0.33±0.04	0.66±0.08	0.29±0.04	1.20±0.06[a]
Nicotine+ Quercetin	0.32±0.02	0.66±0.03	0.32±0.04	1.24±0.06[b]
Alcohol+ Nicotine+ Quercetin	0.34±0.04	0.64±0.05	0.30±0.05	1.27±0.04[a,b]

Values are presented as mean ±SEM, $^{*}p<0.05$ were considered significant compared to Control, $^{a}p<0.05$ significantly different from Alcohol, $^{b}p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly different from Alcohol+Nicotine.

4.2. Effects of quercetin on sperm profile of experimental rats treated with alcohol and/or nicotine

Table 4.2 showed that alcohol alone, nicotine alone and alcohol plus nicotine treatments showed a significant reduction ($p<0.05$) in sperm characteristics when compared to the control, vehicle, and quercetin groups. However, following the co-treatment of quercetin plus alcohol alone, nicotine alone and alcohol plus nicotine there were significant differences ($p<0.05$) in sperm parameters compared to alcohol, nicotine, and alcohol plus nicotine treated groups.

Table 4.2: Effects of quercetin on sperm profile of experimental rats treated with alcohol and/or nicotine .

Group	Sperm count (10^6/ml)	Mature sperm (%)	Sperm motility (%)	Abnormal sperm morphology (%)
Control	96.00±1.87	86.00±2.45	90.00±1.58	12.00±1.22
Corn oil	96.40±1.81	85.00±2.24	89.00±2.02	11.00±1.02
Quercetin	94.40±0.87	86.00±1.87	89.00±1.87	12.00±1.12
Alcohol	38.00±2.55*	56.00±2.92*	42.00±2.55*	45.00±1.11*
Nicotine	31.00±1.80*	52.00±2.55*	40.00±1.58*	54.00±1.08*
Alcohol + Nicotine	31.00±1.00*	50.00±2.74*	34.00±1.80*	56.00±1.02*
Alcohol + Quercetin	88.00±3.01[a]	75.00±2.24[a]	80.00±3.54[a]	16.00±1.03[a]
Nicotine + Quercetin	88.00±2.00[b]	75.00±2.02[b]	83.00±2.55[b]	14.00±1.05[b]
Alcohol + Nicotine+ Quercetin	70.00±2.24[a,b]	76.00±1.82[a,b]	80.00±2.74[a,b]	14.00±1.00[a,b]

Values are presented as mean±SEM, n=5; significant at $^{*}p<0.05$ when compared to Control, $^{a}p<0.05$ significantly different from Alcohol, $^{b}p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly different from Alcohol+Nicotine.

4.3. Effects of quercetin on alcohol and nicotine treatment on percentage change in body weight in male Wistar rats

There was a significant increase ($p<0.05$) in body weight of alcohol treated rats when compared with the nicotine or alcohol plus nicotine treated rats. There was an insignificant increase ($p>0.05$) in the body weight of alcohol treated rats compared with the control group that received distilled water only, corn oil treated group and corn oil plus quercetin group. However, there was an insignificant differences ($p>0.05$) in body weight between groups treated with nicotine, alcohol plus nicotine, nicotine plus quercetin, nicotine plus alcohol plus quercetin treated rats. The changes in body weight were significantly reduced ($p<0.05$) in the groups treated with nicotine, alcohol plus nicotine, nicotine plus quercetin and alcohol plus nicotine plus quercetin compared with groups treated with distilled water (control), corn oil, and quercetin (Table 4.3)

Table 4.3: Effects of Quercetin on alcohol and nicotine treatment on percentage change in body weight in male Wistar rats

Group	Initial body weight (g)	Final body weight (g)	Change in body weight (%)
Control	1.94±4.00	2.20±5.48	11.82
Corn oil	1.92±4.89	2.11±7.14	9.00
Quercetin	1.90±4.47	2.06±4.00	7.77
Alcohol	1.91±4.00	2.30±4.47	16.96*
Nicotine	1.92±4.89	1.87±6.63	-2.67*
Alcohol+Nicotine	1.84±4.00	1.87±8.60	1.60*
Alcohol+Quercetin	1.92±4.06	2.10±6.12	8.57a
Nicotine+Quercetin	1.90±4.47	1.95±7.07	2.56b
Alcohol+Nicotine+ Quercetin	1.86±4.00	1.97±6.63	5.58a,b

Value are presented as mean ± SEM, n=5; significant at *$p<0.05$ when compared to Control. a$p<0.05$ significantly difficult different from Alcohol, $p<0.05$ significantly different from Nicotine, a,b$p<0.05$ significantly differently difficult from Alcohol+Nicotine.

4.4. Effects of quercetin on testicular index in male rats treated with alcohol and/or nicotine

The results in figure 4.1 showed no significant differences ($p>0.05$) in gonado-somatic index of alcohol and/or nicotine treated rats when compared with their control counterpart. Also, no significance changes were observed in the quercetin co-treated groups.

Testicular index= testicular weight/body mass x 100 (DeVlaming *et al.,* 1982).

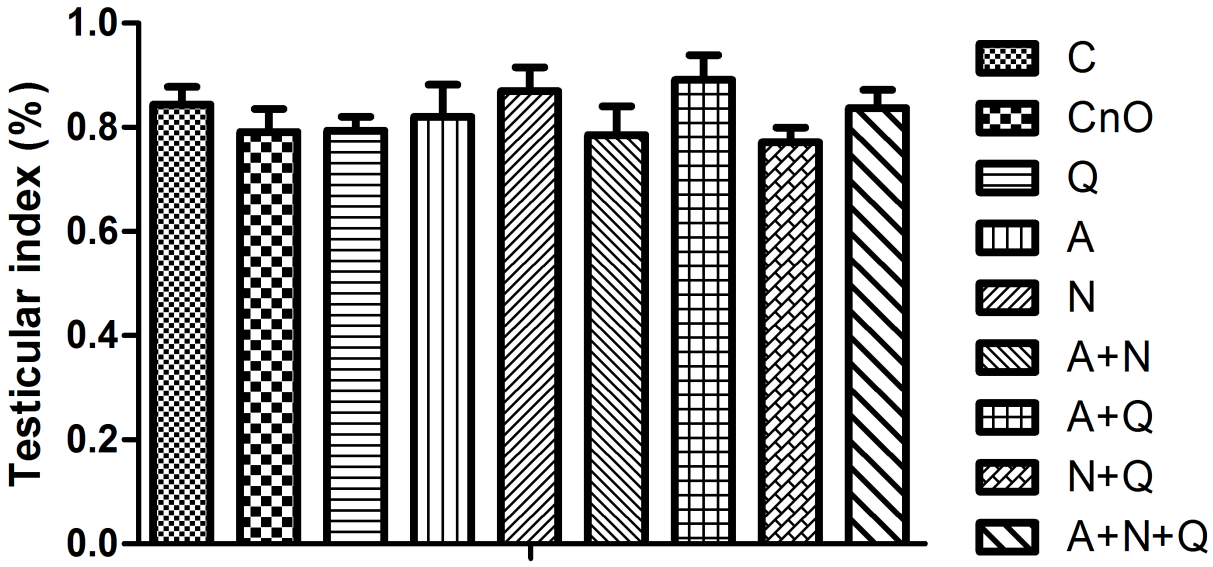


Figure 4.1: Effects of quercetin on testicular index in male rats treated with alcohol and/or nicotine. Values are expressed as mean ±SEM.

Key: C- Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.5. Effects of quercetin on epididymal index in male rats treated with alcohol and/or nicotine

The results showed an insignificant differences ($p>0.05$) in epididymal index of alcohol and/or nicotine treated rats when compared with their control counterpart. Also, no significance changes were observed in the quercetin co-treated groups (figure 4.2).

Epididymal index= epididymal weight/Body weight x 100 (Barber and Blake, 2006).

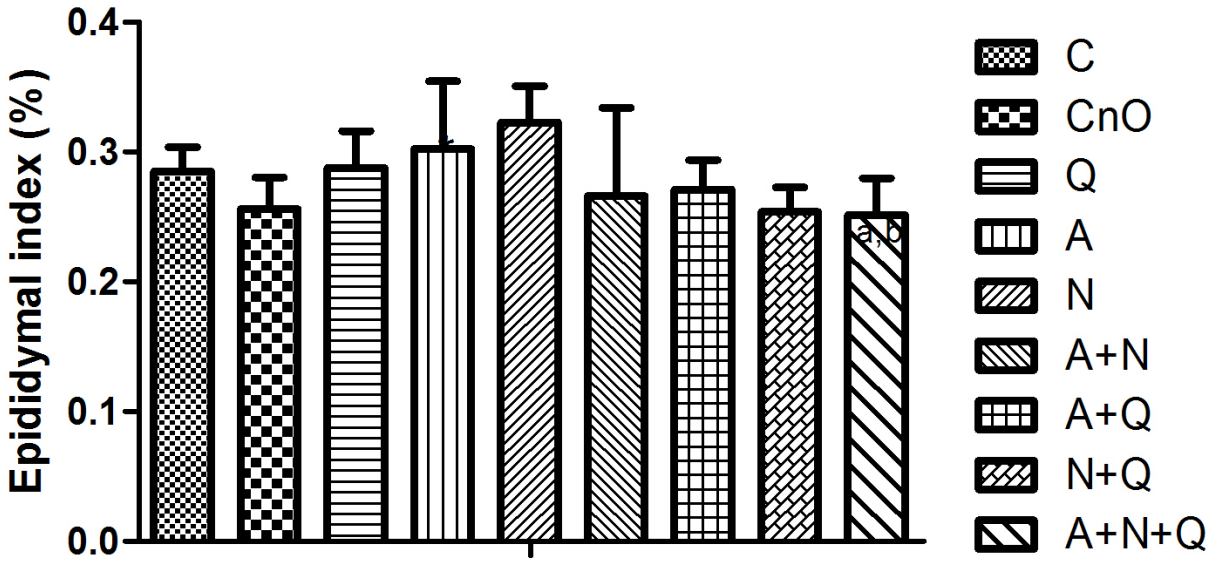


Figure 4.2: Effects of quercetin on percentage epididymal index in male rats treated with alcohol and/or nicotine. Values are expressed as mean ±SEM.

Key: C- Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.6. Effects of quercetin on mean serum lipid profile in male Wistar rats treated with alcohol and/or nicotine

There was no significant change ($p>0.05$) in the mean total cholesterol of nicotine alone treated group, alcohol plus nicotine treated group when compared with the control group. However, there was no significant increase in the mean total cholesterol in alcohol plus quercetin treated group when compared with the control group. However, there was no significant increase in alcohol alone, nicotine alone, alcohol plus quercetin and alcohol plus nicotine plus quercetin treated groups in comparison to their control counterparts. The groups treated with alcohol alone, nicotine alone, alcohol plus quercetin, alcohol plus nicotine plus quercetin also had an insignificant increase ($p>0.05$) in mean low density lipoprotein levels when compared with the control group. There was an insignificant increase ($p>0.05$) in the mean serum triglycerides and VLDL levels of corn oil, corn oil plus quercetin groups compared with the control, however, there was an insignificant difference in the serum triglyceride levels in rats treated with alcohol alone, nicotine alone, alcohol plus nicotine, alcohol plus quercetin, nicotine plus quercetin, alcohol plus nicotine plus quercetin when compared with the control group as shown in table 4.5.

Table 4.4: Effects of quercetin on lipid profile of male Wistar rats treated with alcohol and/or nicotine

Group	Total cholesterol (mmol/l)	Triglycerides (mmol/l)	HDL-cholesterol (mmol/l)	LDL-cholesterol (mg/dl)	VLDL (mmol/l)
Control	140.4 ± 11.54	45.84 ± 11.98	1966 ± 512.2	1834 ± 519	9.168 ± 2.396
Corn oil	117.8 ± 9.671	22.21 ± 51.66	1647 ± 255.4	1554 ± 253	24.45 ± 10.33
Quercetin	116.6 ± 13.20	23.30 ± 117.5	1473 ± 53.29	1403 ± 56.1	46.60 ± 23.51
Alcohol	136.8 ± 8.305	62.39 ± 15.35	2502 ± 430.9	2378 ± 431	12.48 ± 3.077
Nicotine	115.4 ± 8.419	47.11 ± 25.51	2124 ± 181.4	2018 ± 184	9.422 ± 5.103
Alcohol + Nicotine	110.6 ± 9.913	52.21 ± 11.46	1396 ± 122.0	1295 ± 128	10.44 ± 2.292
Alcohol+ Quercetin	158.2 ± 3.211	36.92 ± 11.44	2531 ± 304.5	2381 ± 303	7.385 ± 2.887
Nicotine+ Quercetin	128.4 ± 5.721	48.39 ± 18.74	2061 ± 193.5	1943 ± 200	9.677 ± 3.747
Alcohol + Nicotine + Quercetin	133.3 ± 16.06	62.39 ± 28.07	2646 ± 256.3	2525 ± 249	12.48 ± 5.614

Values are presented as mean ± S.E.M.

4.7. Effects of quercetin on sperm capacitation and acrosome reaction in alcohol and nicotine treated male Wistar rats

Administration of alcohol and/or nicotine for a period of 52 days caused a significant ($p<0.05$) increased in uncapacitated acrosome intact spermatozoa in rats when compared with their control counterparts. However, rats exposed to alcohol (3g/kg B.W) and/or nicotine (1mg/kg B.W) co-treated with 30mg/kg B.W. of quercetin had a significant ($p<0.05$) increase in number of spermatozoa that underwent capacitation and acrosome reaction compared with those treated with alcohol and/or nicotine alone treated groups (figure 4.3a and b).

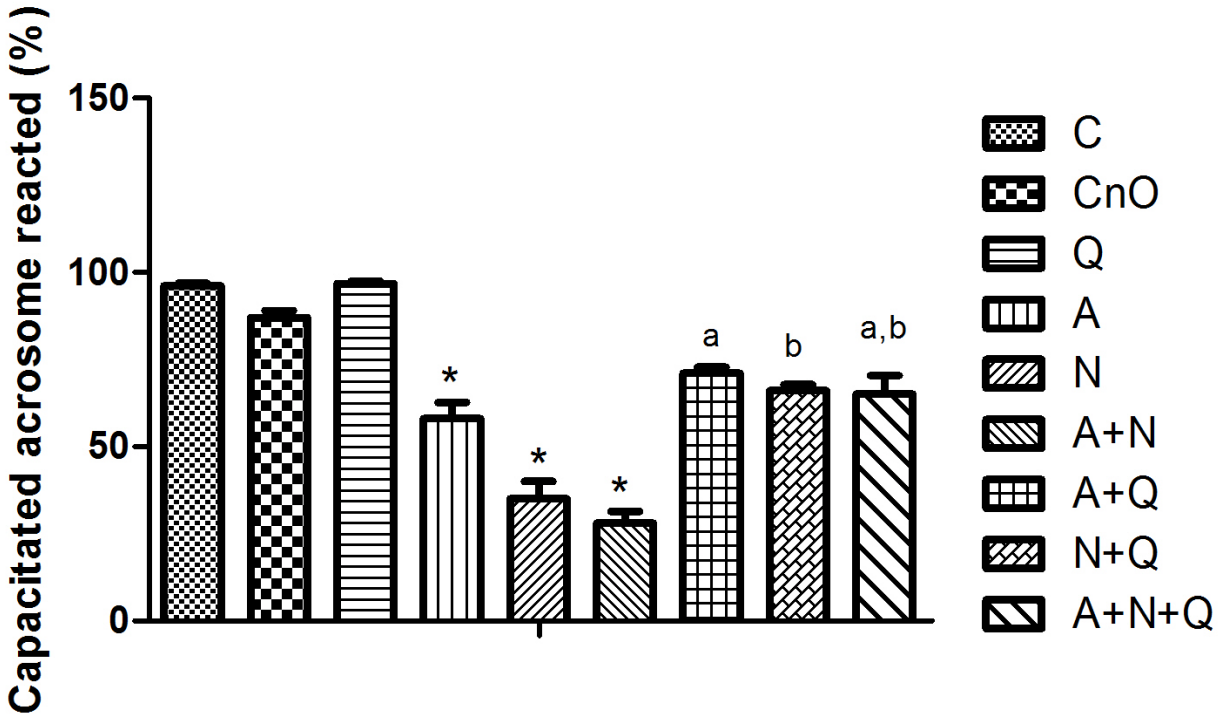


Figure 4.3a: Effects of quercetin on capacitation and acrosome reaction in male rats treated with alcohol and/or nicotine. Value are presented as mean ± SEM, n=5; significant at $^{*}p<0.05$ when compared to Control. $^{a}p<0.05$ significantly difficult different from Alcohol, $p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly differently difficult from Alcohol+Nicotine.

Key: C- Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

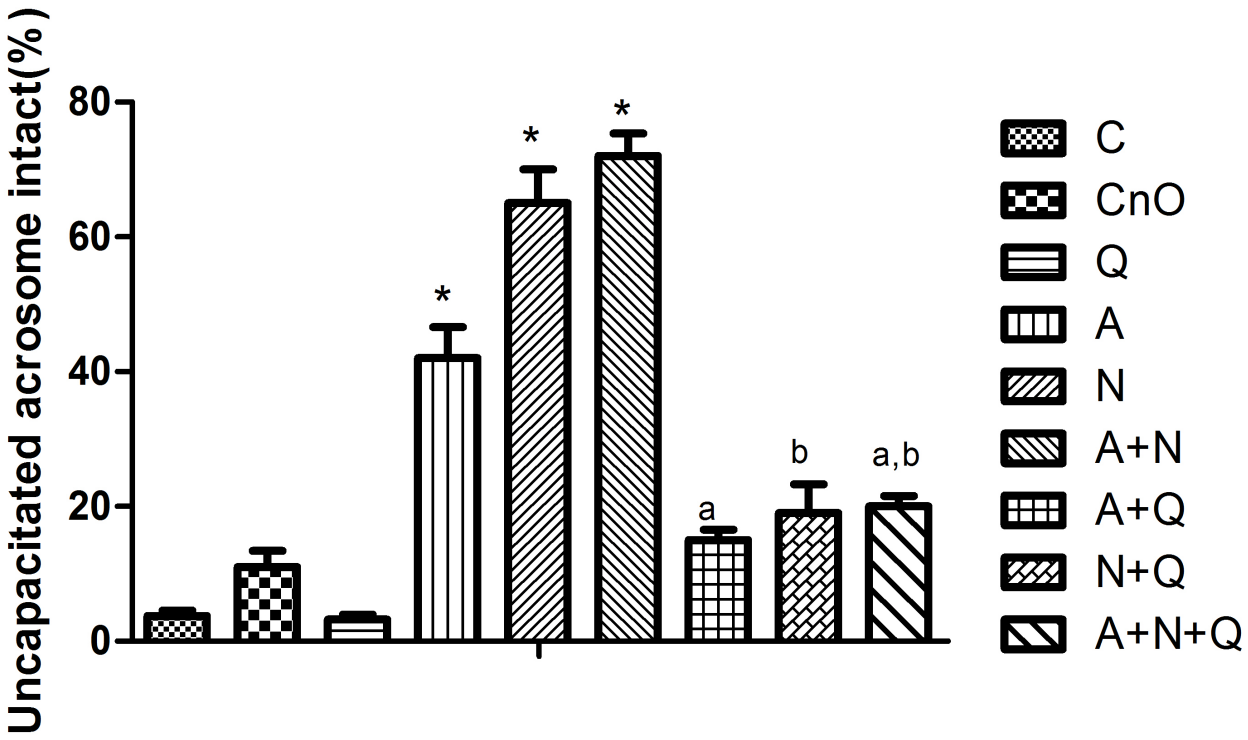


Figure 4.3b: Effects of quercetin on uncapacitation and acrosome intact in male rats treated with alcohol and/or nicotine. Value are presented as mean ± SEM, n=5; significant at $^{*}p<0.05$ when compared to Control. $^{a}p<0.05$ significantly difficult different from Alcohol, $p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly differently difficult from Alcohol+Nicotine.

Key: C- Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

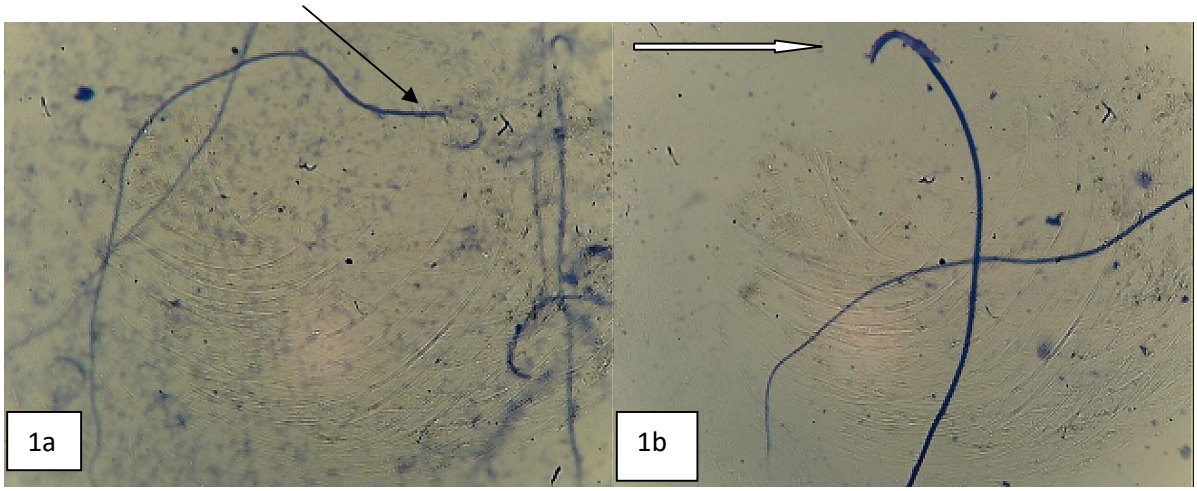


Plate 1a: Acrosome-reacted sperm cell (unstained area underwent capacitation and acrosome reaction, because the protein is not in them to pick up the stain).

Plate 1b: Uncapacitated acrosome intact (the stained area did not react because the acrosome is still intact) (X 1000 Magnification).

4.8. Effects of quercetin on mean serum testosterone level (T) on alcohol and/or nicotine treated male Wistar rats

The results in figure 4.4 showed that there were significant reductions in testosterone levels of groups treated with alcohol alone, nicotine alone, alcohol and nicotine compared to the control, corn oil, corn oil plus quercetin. However, following quercetin administration with the alcohol, nicotine, alcohol and nicotine groups, there was no significant difference in testosterone levels compared to the control, corn oil, corn oil plus quercetin groups.

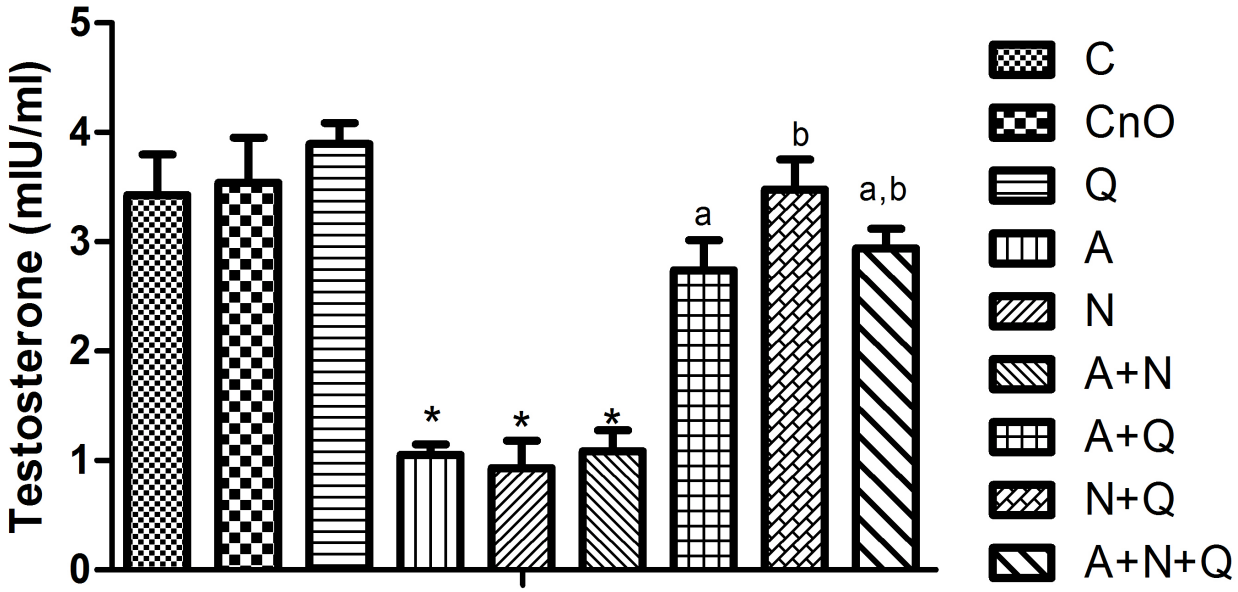


Figure 4.4: Effects of quercetin on alcohol and/or nicotine treatment on serum testosterone level in male Wistar rat. Value are presented as mean ± SEM, n=5; significant at $^{*}p<0.05$ when compared to Control. $^{a}p<0.05$ significantly difficult different from Alcohol, $p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly differently difficult from Alcohol+Nicotine.

Key: C- Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.9. Effects of quercetin on serum follicle stimulating hormone (FSH) level in alcohol and/or nicotine treated male Wistar rats

The results in figure 4.5 showed that there were significant reductions in FSH levels of groups treated with alcohol alone, nicotine alone, alcohol and nicotine compared to the control, corn oil, corn oil plus quercetin. However, following quercetin administration with the alcohol, nicotine, alcohol and nicotine groups, there was no significant difference in FSH levels compared to the control, corn oil, corn oil plus quercetin groups.

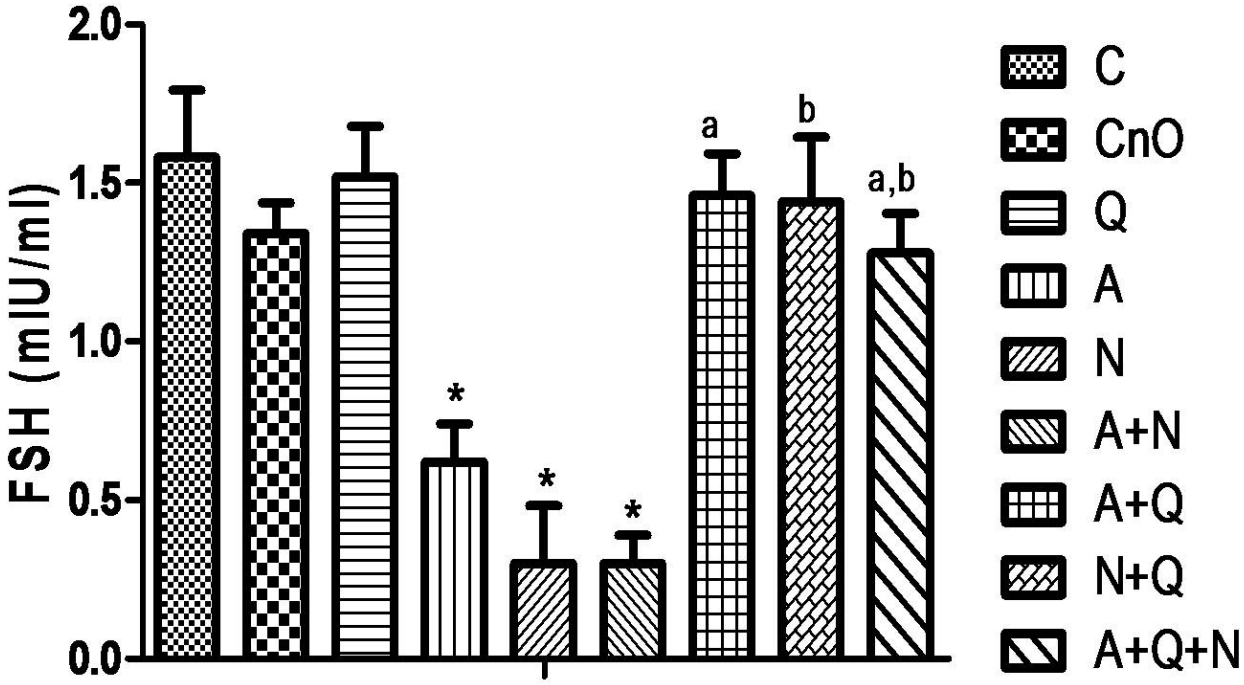


Figure 4.5: Effects of quercetin on serum FSH level in alcohol and/or nicotine treated male Wistar rats. Value are presented as mean ± SEM, n=5; significant at $^{*}p<0.05$ when compared to Control. $^{a}p<0.05$ significantly difficult different from Alcohol, $p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly differently difficult from Alcohol+Nicotine.

Key: C- Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.10. Effects of quercetin on serum luteinizing hormone (LH) level in alcohol and/or nicotine treated male Wistar rats

The results in figure 4.6 showed that there were significant reductions in LH levels of groups treated with alcohol alone, nicotine alone, alcohol and nicotine compared to the control, corn oil, corn oil plus quercetin. However, following quercetin administration with the alcohol, nicotine, alcohol and nicotine groups, there was no significant difference in LH levels compared to the control, corn oil, corn oil plus quercetin groups.

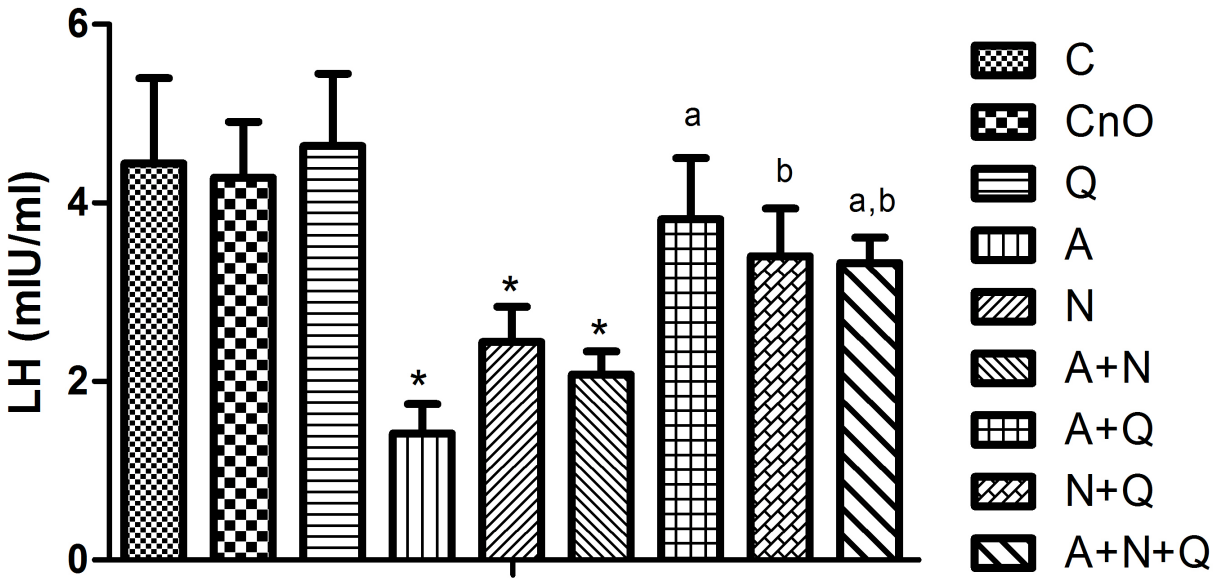


Figure 4.6: Effects of quercetin on serum LH level in alcohol and/or nicotine treated male Wistar rats. Value are presented as mean ± SEM, n=5; significant at $^{*}p<0.05$ when compared to Control. $^{a}p<0.05$ significantly difficult different from Alcohol, $p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly differently difficult from Alcohol+Nicotine.

Key: C- Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.11. Effects of quercetin on mean malondialdehyde (MDA) concentration in testicular tissues of rats treated with alcohol and/or nicotine

The results in table 4.5 showed that there were significant reductions ($p<0.05$) in mean MDA of alcohol alone, nicotine alone, alcohol and nicotine treated groups in comparison to the control, corn oil, corn oil plus quercetin groups, however, there were no significant differences ($p>0.05$) in mean MDA of groups treated with alcohol plus quercetin, nicotine plus quercetin, alcohol and nicotine plus quercetin compared to the control, corn oil, corn oil plus quercetin groups. Also, there was a significant difference ($p<0.05$) in mean MDA of alcohol treatment group compared to nicotine plus quercetin treatment group, there was also a significant increase ($p<0.05$) in mean MDA of alcohol group compared to the alcohol plus quercetin treated group, more so, there was a significant increase ($p<0.05$) in mean MDA of alcohol treated group compared to alcohol and nicotine plus quercetin treated group. There was a significant increase ($p<0.05$) in mean MDA of nicotine alone treated group compared to the nicotine plus quercetin treated group, there was also a significant increase ($p<0.05$) in mean MDA of nicotine treated group compared to alcohol plus quercetin treated group. Group treated with nicotine alone had a significant increase ($p<0.05$) in mean MDA when compared to alcohol and nicotine plus quercetin treated group, however, there was also a significant increase ($p<0.05$) in mean MDA of alcohol plus nicotine treated group compared to nicotine plus quercetin. There was a significant increase ($p<0.05$) in mean MDA of alcohol plus nicotine treated group compared to alcohol plus quercetin treated group. However, there was a significant increase ($p<0.05$) in mean MDA of alcohol plus nicotine treated group compared to alcohol and nicotine plus quercetin treated group.

Table 4.5: Effects of quercetin on malondialdehyde concentration in alcohol and/or nicotine treated male Wistar rats.

Group	Testicular MDA levels (µm/mg protein)
Control	96.0±0.98
Corn oil	93.7±1.65
Quercetin	93.1±1.00
Alcohol	142.0±3.00*
Nicotine	154.0±3.05*
Alcohol + Nicotine	165.0±3.03*
Alcohol + Quercetin	99.3±4.05a
Nicotine + Quercetin	102.0±4.00b
Alcohol + Nicotine + Quercetin	104.0±6.00 a,b

Value are presented as mean ± SEM, n=5; significant at *$p<0.05$ when compared to Control. a$p<0.05$ significantly difficult different from Alcohol, $p<0.05$ significantly different from Nicotine, a,b$p<0.05$ significantly differently difficult from Alcohol+Nicotine.

4.12. Effects of quercetin on mean testicular antioxidant status in alcohol and/or nicotine treated male Wistar rats

The results in table 4.6 showed that there were significant reductions ($p<0.05$) in mean SOD and reduced GSH levels of alcohol alone, nicotine alone, alcohol and nicotine treated groups in comparison to the control, corn oil, corn oil plus quercetin groups, however, there were no significant differences ($p>0.05$) between mean of SOD and reduced GSH groups treated with alcohol plus quercetin, nicotine plus quercetin, alcohol and nicotine plus quercetin when compared with each other. There was also no significant difference ($p>0.05$) in mean SOD and GSH of groups treated with nicotine plus alcohol, alcohol plus nicotine, alcohol and nicotine plus quercetin compared to the control, corn oil, corn oil plus quercetin groups. Also, there was a significant decrease ($p<0.05$) in mean SOD and GSH of alcohol treatment group compared to nicotine plus quercetin treatment group, there was also a significant increase ($p<0.05$) in mean SOD and GSH of alcohol group compared to the alcohol plus quercetin treated group, more so, there was a significant increase ($p<0.05$) in mean SOD and GSH of alcohol treated group compared to alcohol and nicotine plus quercetin treated group. There was a significant increase ($p<0.05$) in mean SOD and GSH of nicotine alone treated group compared to the nicotine plus quercetin treated group, there was also a significant increase ($p<0.05$) in mean SOD and GSH of nicotine treated group compared to alcohol plus quercetin treated group. Group treated with nicotine alone had a significant increase ($p<0.05$) in mean SOD and GSH when compared to alcohol and nicotine plus quercetin treated group, however, there was also a significant increase ($p<0.05$) in mean SOD and GSH of alcohol plus nicotine treated group compared to nicotine plus quercetin. There was a significant increase ($p<0.05$) in mean SOD and GSH of alcohol plus nicotine treated group compared to alcohol plus quercetin treated group. However, there was a significant increase ($p<0.05$) in mean SOD and GSH of alcohol plus nicotine treated group compared to alcohol and nicotine plus quercetin treated group.

Table 4.6: Effects of quercetin on testicular antioxidant markers in experimental male Wistar rats treated with alcohol and/or nicotine.

Group	SOD (U/mg protein)	GSH (mmol/mg protein)
Control	121.0±0.74	354.0±16.00
Corn oil	118.0±2.60	345.0±16.60
Quercetin	120.0±3.54	343.0±15.10
Alcohol	66. 8± 7.38*	184.0±27.20*
Nicotine	70.0±4.44*	192.0±27.80*
Alcohol + Nicotine	79.6±4.60*	136.0±5.52*
Alcohol + Quercetin	112.0±5.14a	320.0±12.60a
Nicotine + Quercetin	110.0±5.14b	323.0±23.60b
Alcohol + Nicotine + Quercetin	112.0±4.81a,b	315.0±9.28a,b

Value are presented as mean ± SEM, n=5; significant at *$p<0.05$ when compared to Control. a$p<0.05$ significantly difficult different from Alcohol, $p<0.05$ significantly different from Nicotine, a,b$p<0.05$ significantly differently difficult from Alcohol+Nicotine.

4.13. Effects of quercetin on serum alanine aminotransferase (ALT) levels in male rats treated with alcohol and/or nicotine

The results in figure 4.7 showed that there was no significant difference ($p>0.05$) in serum ALT level of group treated with alcohol alone, alcohol plus nicotine plus quercetin compared to the corn oil group, however, no significant differences ($p>0.05$) were observed between experimental groups in comparison with the control.

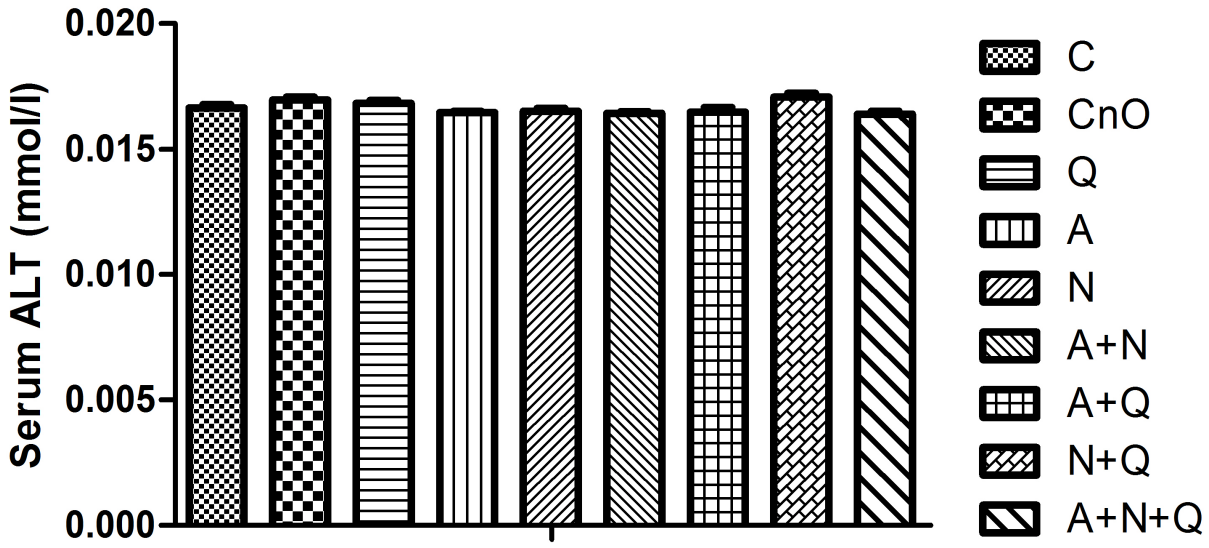


Figure 4.7: Effects of quercetin on serum ALT levels in male rats treated with alcohol and/or nicotine. Values are expressed as mean ± SEM.

Key: C- Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.14. Effects of quercetin on serum aspartate aminotransferase (AST) levels in male rats treated with alcohol and/or nicotine

The groups treated with alcohol alone, nicotine alone and alcohol plus nicotine showed an increase in serum AST levels which was not statistically significant ($p>0.05$) compared with their control groups. However, with quercetin administration there was a reduction in serum AST levels of alcohol and/or nicotine treated groups in comparison with their control counterparts.

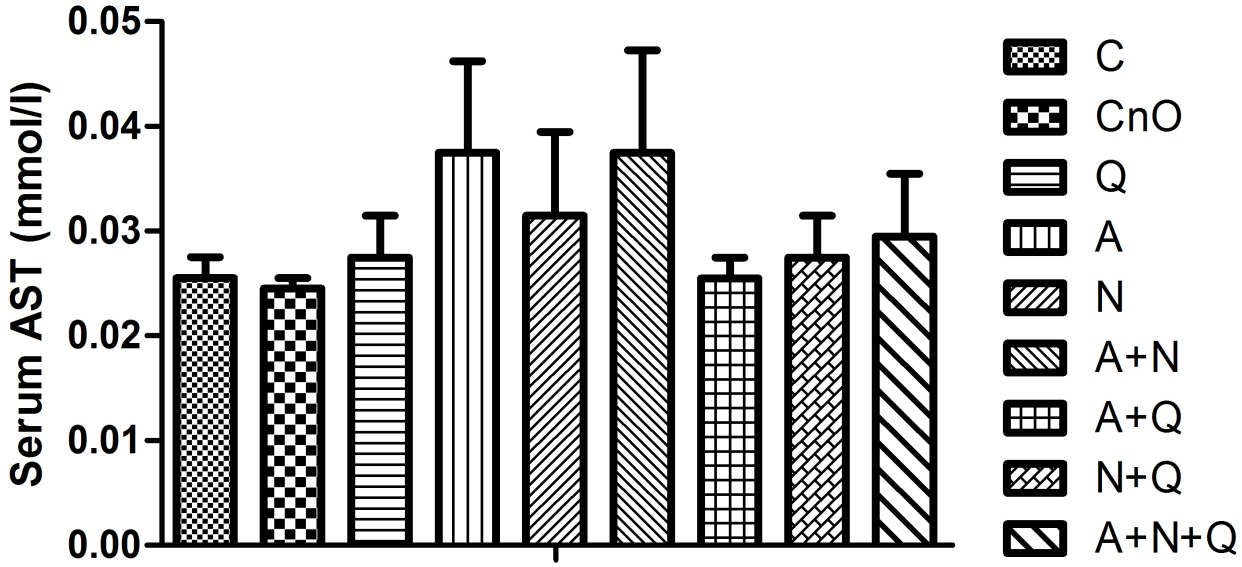


Figure 4.8: Effects of quercetin on serum AST levels in male rats treated with alcohol and/or nicotine. Values are expressed as mean ± SEM.

Key: C- Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.15. Effects of quercetin on serum alkaline phosphatase (ALP) level in male rats treated with alcohol and/or nicotine

The results in figure 4.9 showed that there was an insignificant decrease ($p>0.05$) in serum ALP level of group treated with alcohol alone compared with the control counterpart; an insignificant increase were observed in the nicotine alone, alcohol and nicotine groups compared to the control, corn oil, corn oil plus quercetin. However, following quercetin administration with the alcohol, nicotine, alcohol and nicotine groups, there was no significant difference ($p>0.05$) in serum ALP levels compared to the control, corn oil, corn oil plus quercetin groups.

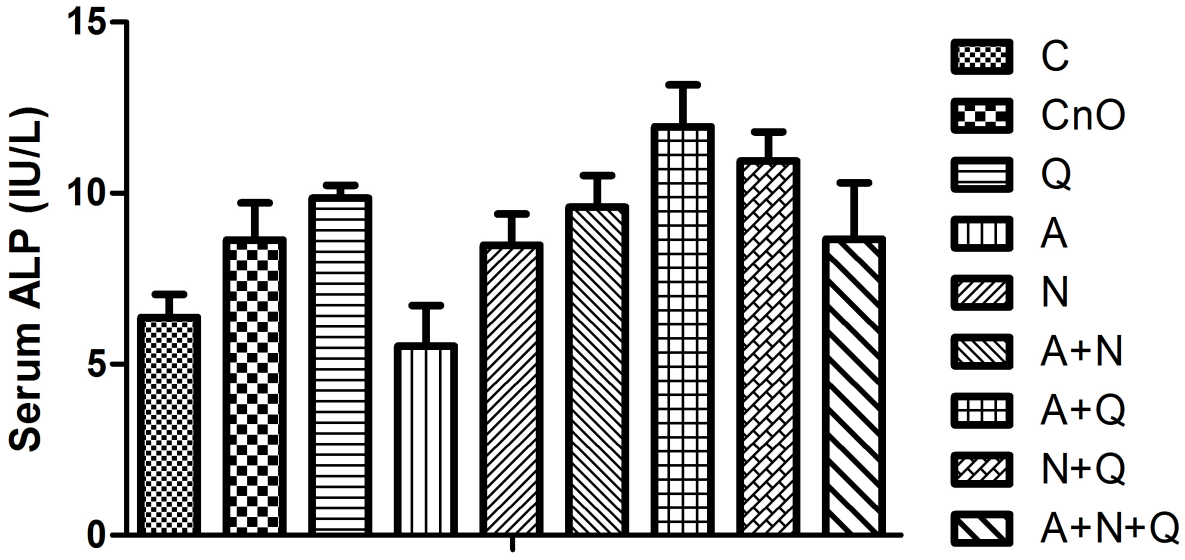


Figure 4.9: Effects of quercetin on serum ALP level in male rats treated with alcohol and/or nicotine. Values are expressed as mean ±SEM.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.16. Effects of quercetin on testicular lactate dehydrogenase (LDH) level in male Wistar rats treated with alcohol and/or nicotine

The results in figure 4.10 showed that there was no significant differences ($p>0.05$) in testicular LDH levels of groups treated with alcohol alone, nicotine alone, alcohol and nicotine compared to the control, corn oil, corn oil plus quercetin. However, following quercetin administration with the alcohol, nicotine, alcohol and nicotine groups, there was no significant difference in LDH levels compared to the control, corn oil, corn oil plus quercetin groups.

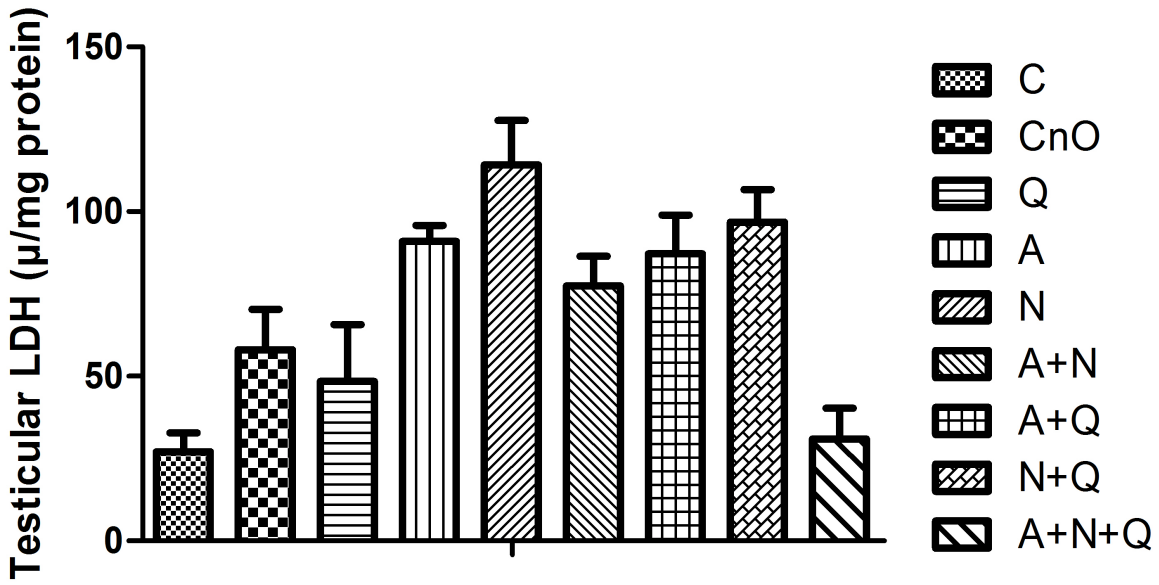


Figure 4.10: Effects of quercetin on testicular LDH level in male Wistar rats treated with alcohol and/or nicotine. Values are expressed as mean ± SEM.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.17. Effects of quercetin on serum acid phosphatase (ACP) level in male Wistar rats treated with alcohol and/or nicotine

The results in figure 4.11 showed that there was a insignificant decrease ($p<0.05$) in serum ACP level of group treated with alcohol alone compared to alcohol plus quercetin treated group, also a significant decrease was observed in alcohol treated group compared to nicotine plus quercetin treated group. However, only quercetin administration with alcohol treated group had no significant difference when compared to the control group.

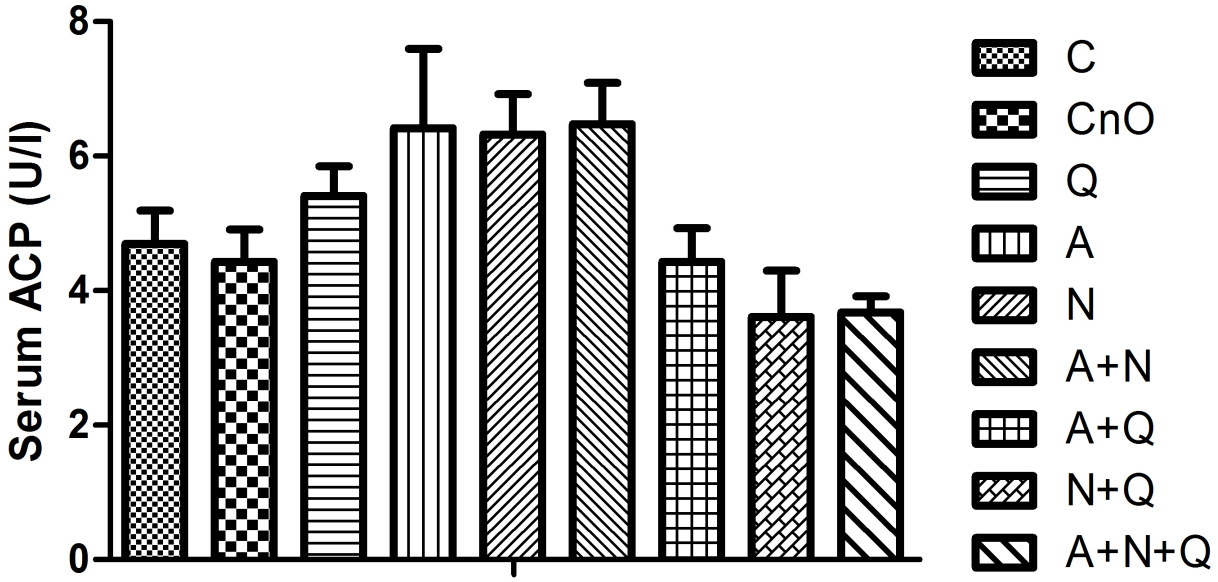


Figure 4.11: Effects of quercetin on serum ACP level in male Wistar rats treated with alcohol and/or nicotine. Values are expressed as mean ±SEM.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.18. Effects of quercetin on serum prostate specific antigen (PSA) level in male Wistar rats treated with alcohol and/or nicotine

There were no significant differences ($p<0.05$) in serum prostate specific antigen (PSA) levels in all the experimental groups when compare with the control group for a period of 52 days of treatment (figure 4.12).

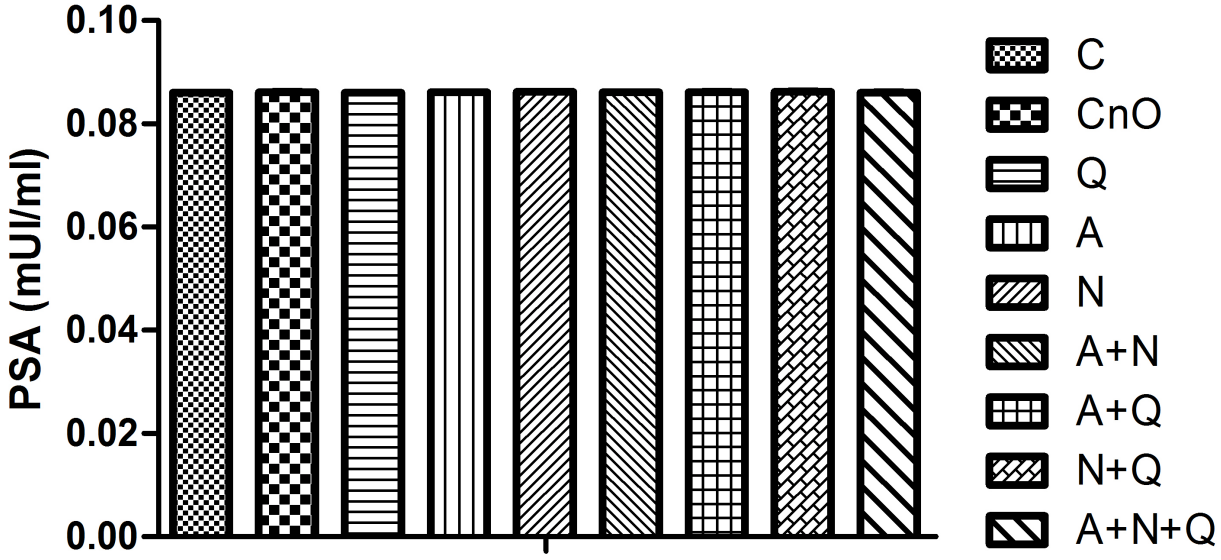


Figure 4.12: Effects of quercetin on serum PSA level of alcohol and /or nicotine treated male Wistar rats. Values are expressed as mean ± S.E.M.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.19. Effects of quercetin on testicular glucose-6-phosphate dehydrogenase (G6PDH) concentration in alcohol and/or nicotine treated male Wistar rats

The result in figure 4.13 showed that there were significant reductions ($p<0.05$) in the mean G6PDH level of groups treated with alcohol and/or nicotine when compared to the control, however, rats exposed to alcohol and/or nicotine with quercetin supplementation had an increase in the G6PDH levels when compared to the control rats.

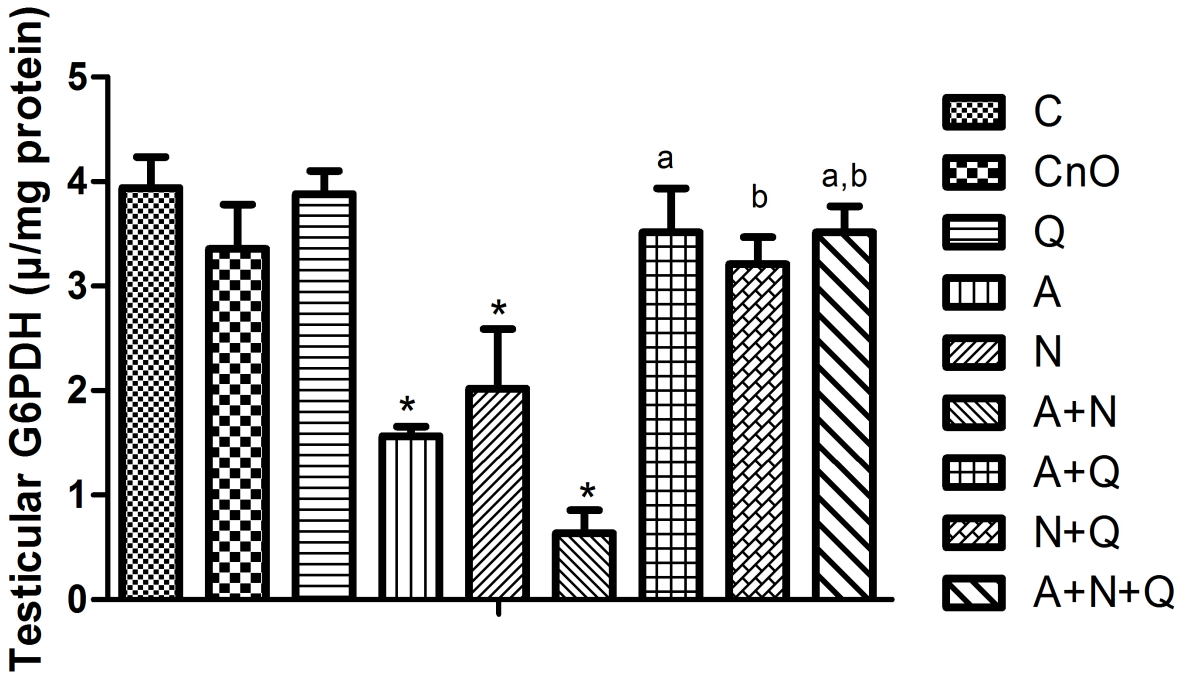


Figure 4.13: Effects of quercetin on testicular G6PDH concentration in alcohol and/or nicotine treated male Wistar rats. Values are presented as mean ± SEM $^{*}p<0.05$ considered significant compared to control, $^{a}p<0.05$ significantly different from Alcohol, $^{b}p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly different from Alcohol+Nicotine.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.20. Effects of quercetin on testicular gamma glytamyl transferase (GGT) concentration in alcohol and/or nicotine treated male Wistar rats

Administration of alcohol and/or nicotine for a period of 52 days caused a significant decrease ($p<0.05$) in the mean testicular GGT level in rats when compared with their control counterpart. In groups exposed to alcohol and or nicotine with quercetin administration showed a significant increase ($p<0.05$) in testicular GGT levels when compared with the alcohol and/or nicotine alone groups (figure 4.14).

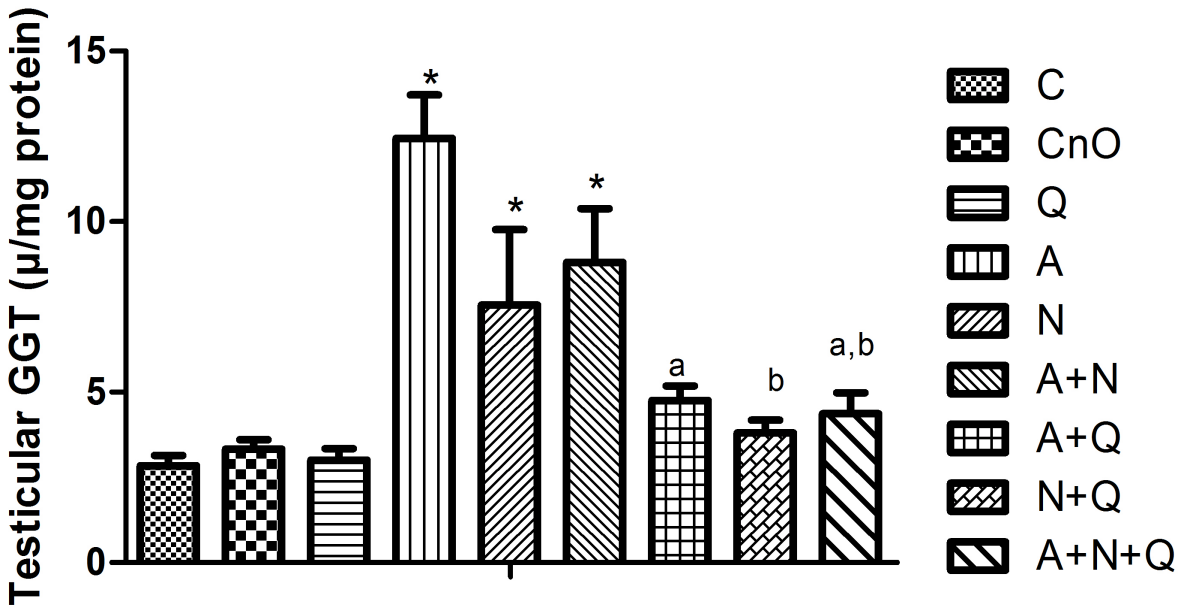


Figure 4.14: Effects of quercetin on serum GGT level in male Wistar rats treated with alcohol and/or nicotine. Values are presented as mean ± SEM $^{*}p<0.05$ considered significant compared to control, $^{a}p<0.05$ significantly different from Alcohol, $^{b}p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly different from Alcohol+Nicotine

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.21. Effects of quercetin on serum protein level in male Wistar rats treated with alcohol and/or nicotine

The results showed that there was a significant reduction ($p<0.05$) in the mean serum protein level of groups treated with alcohol and nicotine plus quercetin when compared with the control group, also a significant difference was observed in alcohol alone group compared with the alcohol and nicotine plus quercetin treated rats. Significant difference was also noted in alcohol plus quercetin compared with alcohol and nicotine plus quercetin group. The results also showed significant difference in alcohol plus nicotine treated rats compared with alcohol and nicotine plus quercetin group (figure 4.15).

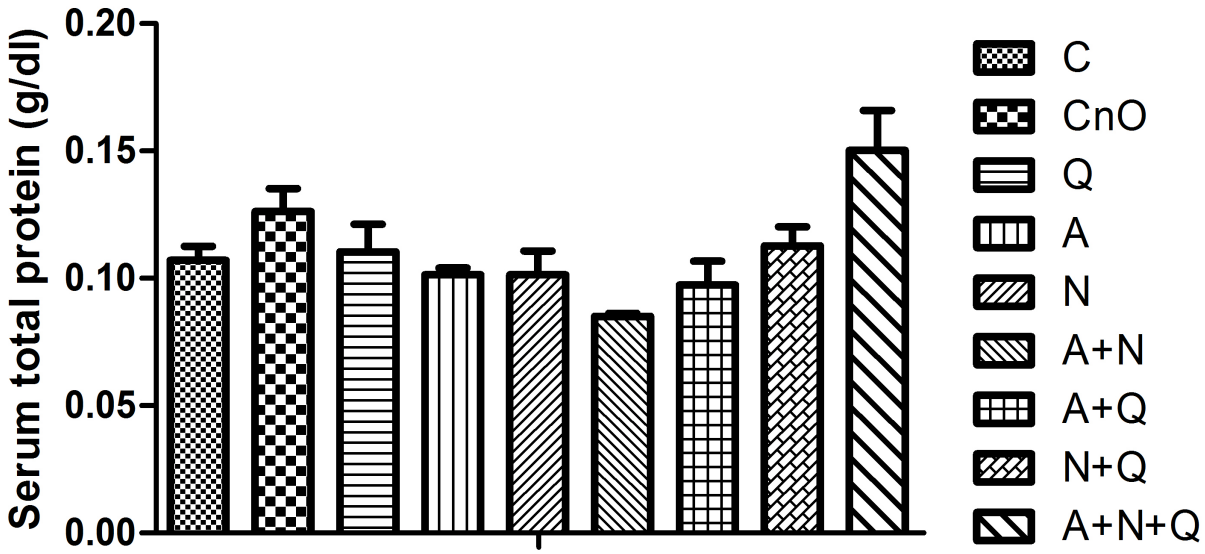


Figure 4.15: Effects of quercetin on serum protein level in male Wistar rats treated with alcohol and/or nicotine. Values are expressed as mean ± S.E.M.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.22. Effects of quercetin on serum electrolytes levels in male Wistar rats treated with alcohol and/or nicotine

The results in table 4.7 showed a significant increase ($p<0.05$) in calcium ion level of alcohol alone, alcohol plus nicotine treated rats compared with the control. There was also a significant increase in chloride ion level of alcohol alone, nicotine alone and alcohol plus nicotine treated rats compared with the control rats.

Table 4.7 Effects of quercetin on serum electrolytes concentration in male Wistar rats treated with alcohol and/or nicotine

Group	Sodium (mmol/l)	Potassium (mmol/l)	Calcium (mmol/l)	Chloride (mmol/l)
Control	440.0 ± 4.1	123.1 ± 44.92	0.483±0.05	3.008 ± 0.02
Corn oil	362.0 ± 5.4	120.2 ± 35.82	0.925± 0.17	2.960 ± 0.03
Quercetin	366.8 ± 8.5	125.0 ± 48.24	0.701±0.05	4.250 ± 0.10
Alcohol	112.0 ± 9.2	116.0 ± 37.44	1.023 ± 0.08*	4.626 ± 0.25*
Nicotine	133.8 ± 7.8	88.10 ± 46.91	0.695±0.10	4.804 ± 0.18*
Alcohol + Nicotine	111.4 ± 0.7	63.09 ± 21.86	0.960 ± 0.13*	4.464 ± 0.13*
Nicotine + Quercetin	333.8 ± 1.6	129.17 ± 20.63	0.931±0.05	4.488 ± 0.20
Alcohol + Quercetin	481.8 ± 6.5	106.79 ± 19.02	0.870 ± 0.12	4.722 ± 0.23
Nicotine + Alcohol + Quercetin	371.1 ± 8.5	113.1 ± 24.20	0.630±0.09	4.932 ± 0.22

Values are presented as mean ± S.E.M. *$p<0.05$ were considered significant with respect to control.

4.23 Effects of quercetin or alcohol and/or nicotine treatment on histology of hypothalamus of male Wistar rats

The control group, corn oil and quercetin tested groups shows normal neuronal cells and no pathological lesion, alcohol group shows mild necrosis of the neurons (black arrow) and nicotine treated shows hyalinated purkinje cells and the monolayer showed necrotized neurons (black arrow). Alcohol + Nicotine also shows hyalinated purkinje cells and necrotized neurons. Alcohol + Nicotine, Nicotine + Quercetin groups showed normal purkinje cells with normal architecture. Alcohol + Nicotine + Quercetin also shows normal hypothalamic architecture with moderate hyalinized purkinje cells.

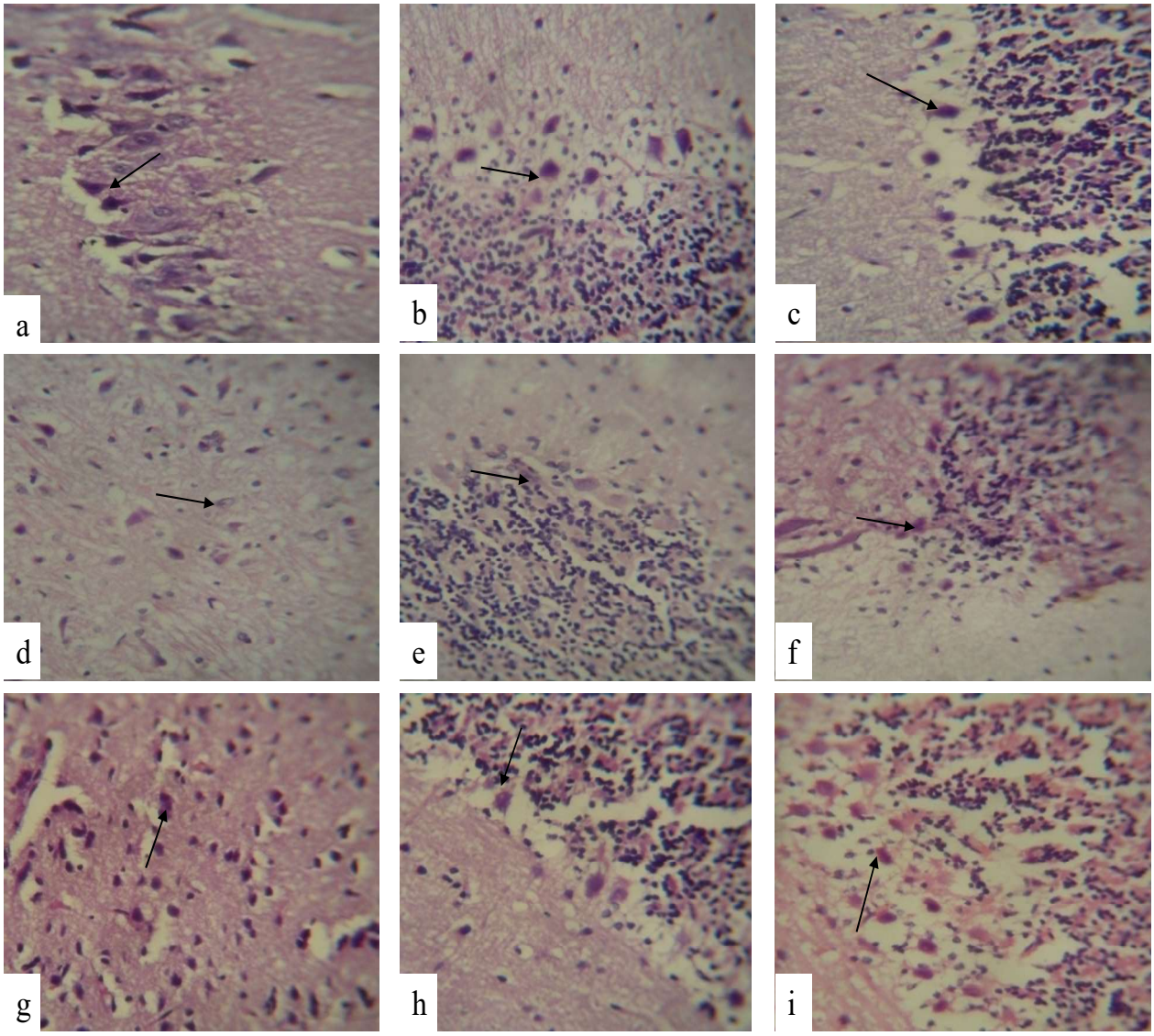


PLATE 2. Photomicrographs of hypothalamic sections from control and treated male wistar rats. Tissue sections were stained by H & E and presented at x100 magnification.

a) Control: The hypothalamus (black arrow) appears normal and shows no infiltration and no pathological lesion.

b) Corn oil: The hypothalamus (black arrow) appears normal and shows no pathological lesion

c) Quercetin: The hypothalamus (black arrow) appears normal and shows no pathological lesions.

d) Alcohol: The hypothalamus appears necrotic; mild necrosis of the neurons.

e) Nicotine: The hypothalamus (black arrow) appears hyalinated purkinje cell, and the monolayer showed necrotic neurons.

f) Alcohol + Nicotine: The hypothalamus appears necrotic with hyalinated purkinje cell and the monolayer showed necrotic neurons.

g) Alcohol + Quercetin: The hypothalamus appears normal. Purkinje cells seen.

h) Nicotine + Quercetin: Normal purkinje cells seen.

i) Alcohol + Nicotine + Quercetin: The hypothalamic architecture appear normal with moderate hyalinzed purkinje cells.

4.24. Effects of quercetin on alcohol and/or nicotine treatment on histology of pituitary gland of male Wistar rats

The control, corn oil and Quercetin treated groups showed normal appearance of pituitary cells. Alcohol treated group shows mild necrosis, nicotine group showed moderate to severe congestion (blue arrow). Alcohol + Nicotine group showed mild vascular congestion. Alcohol + Quercetin shows monolayer without lesion and normal cell appearance. Nicotine + Quercetin and Alcohol + Nicotine + Quercetin showed normal morphology of the pituitary cells.

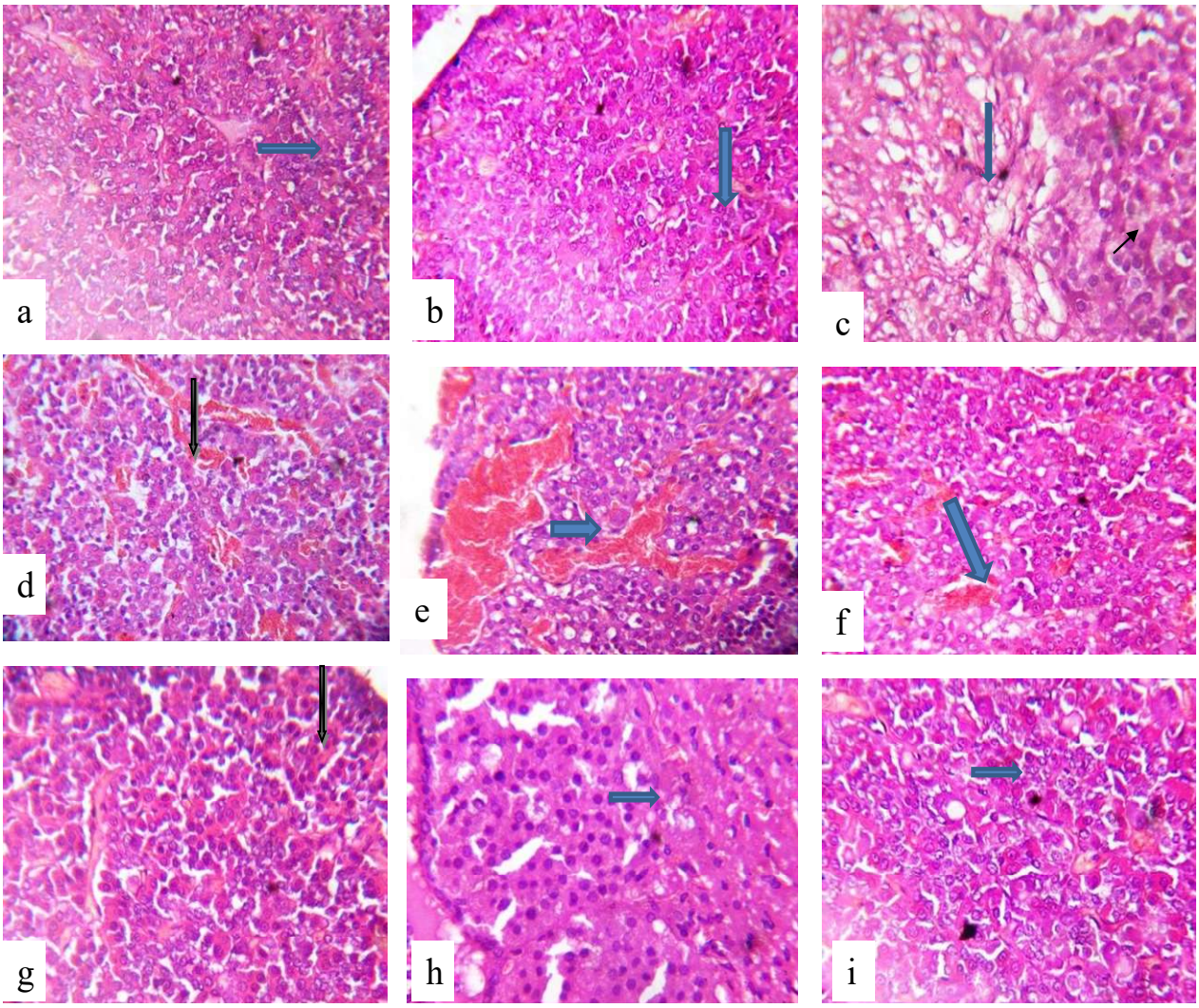


PLATE 3. Photomicrographs of pituitary sections from control and treated male wistar rats. Tissue sections were stained by H & E and presented at x100 magnification

a) Control: shows normal architecture. The pituitary cells appear normal with no pathological lesion seen (black arrow).

b) Corn oil: The pituitary cells appear normal and no pathological lesion seen (blue arrow).

c) Quercetin: The pituitary cells appear normal, normal architecture with no pathological lesion seen.

d) Alcohol: The architecture of the pituitary section shows mild necrosis.

e) Nicotine: The histology of the pituitary shows moderate to severe vascular congestion (blue arrow).

f) Alcohol + Nicotine: shows mild vascular congestion (blue arrow).

g) Alcohol + Quercetin: The morphology shows monolayer without lesion and no pathological lesion seen. The pituitary cells appear normal.

h) Nicotine + Quercetin: No pathological lesion seen, normal morphology of the pituitary cells.

i) Alcohol+Nicotine + Quercetin: No pathological lesion seen. Normal morphology of the pituitary cells.

4.25. Effects of quercetin on alcohol and/or nicotine treatment on histology of the testes of male Wistar rats

The control, corn oil and quercetin showed testicular tissue architecture with normal seminiferous tubule having germinal cells layer, there is complete maturation of sperm cells within the seminiferous tubules and the interstitial cells appeared normal. Alcohol treated groups shows poor maturation of spermatozoa and the Leydig cells appeared hyperplasic. Nicotine treated group shows appearance of sloughing of germ cells layer. Alcohol + Nicotine groups showed thickening of membrane and absence of germ cells. Alcohol + Quercetin, Nicotine + Quercetin and Alcohol + Nicotine + Quercetin showed maturation of primary to secondary spermatocytes respectively.

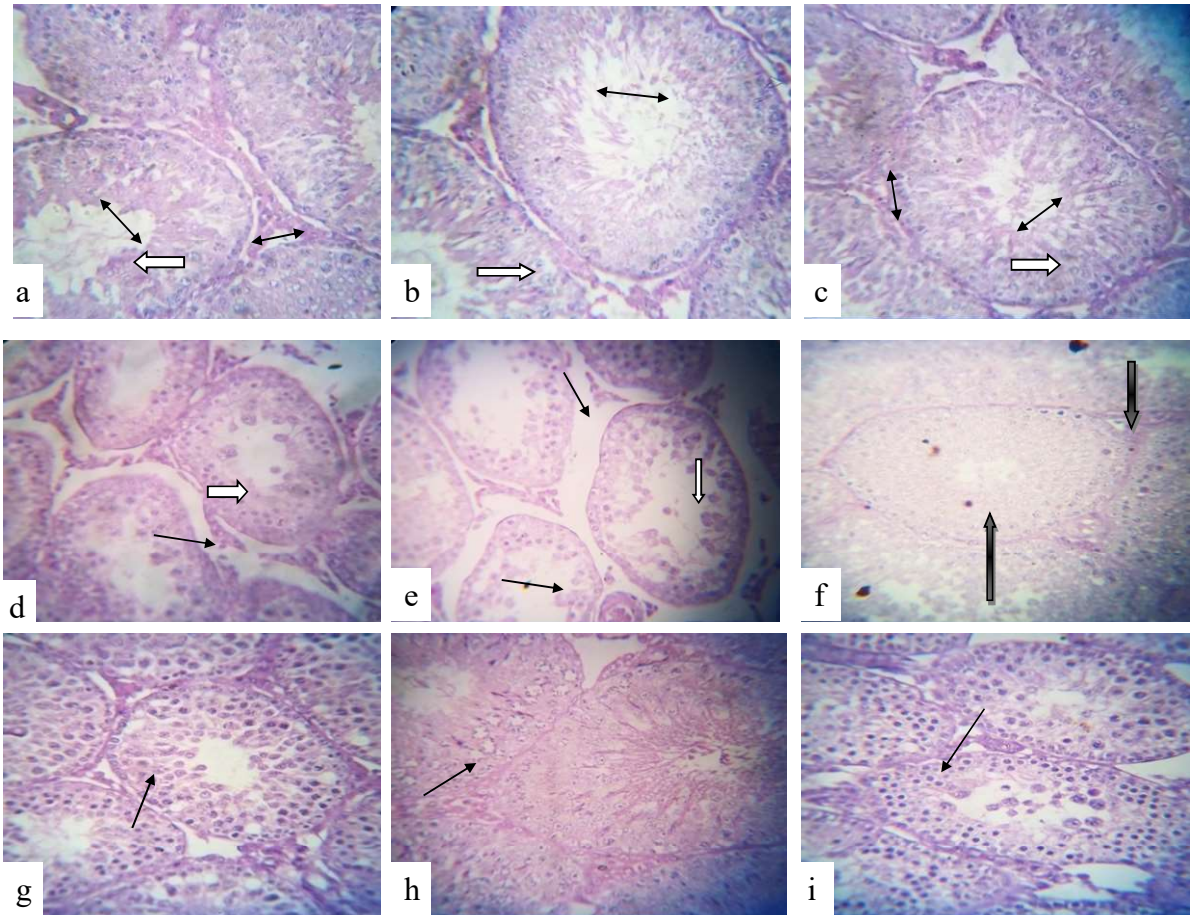


PLATE 4. Photomicrographs of testicular sections of control and treated male wistar rats. Tissues was stained by H & E and presented at x100 magnification

a) Control: Testicular section shows normal seminiferous tubule containing maturing germ cell layer. There is no pathological lesion seen and the interstitial cells appear normal.

b) Corn oil: Testicular section shows no pathological lesion with complete maturation of sperm cells within the seminiferous tubule and the interstitial cells appear normal.

c) Quercetin: The testicular section shows normal seminiferous tubules with complete maturation of sperm cells within the seminiferous tubules (white arrow). No pathological lesion seen.

d) Alcohol: Testicular section shows poor maturation of spermatozoa (short arrow) and the Leydig cells appear hyperplasic

e) Nicotine: There is poor maturation of spermatozoa and the germ cell layer appeared sloughing off the tubules

f) Alcohol + Nicotine: The testicular section shows thickening of the membrane and absence of germ cells

g) Alcohol + Quercetin: the testicular section shows normal seminiferous tubules with maturing germ cell layer. The spermatozoa cells and Leydig cells appear normal. The lumen also appear normal with maturation of primary to secondary spermatocytes

h) Nicotine + Quercetin: Testicular section shows seminiferous tubules with mature germ cell layers. There is maturation of primary to secondary spermatocytes.

i) Alcohol + Nicotine + Quercetin: Testicular section shows normal seminiferous tubules with no loss of membrane. There is maturation of spermatocytes from primary to secondary.

4.26. Effects of quercetin on alcohol and/or nicotine treatment on histology of the epididymis of male Wistar rats

The control, corn oil and quercetin groups had normal epididymal tissue architecture and with presence of spermatozoa in the lumen. Alcohol, Nicotine and Alcohol + Nicotine groups showed thickening of the lumen, interstitial hyperplasic and disruption of the membrane respectively. Alcohol + Quercetin, showed no vascular congestion and presence of spermatozoa in the lumen. Nicotine + Quercetin and Alcohol + Nicotine + Quercetin showed normal appearance of epithelial lining with no interstitial cells hyperplasic.

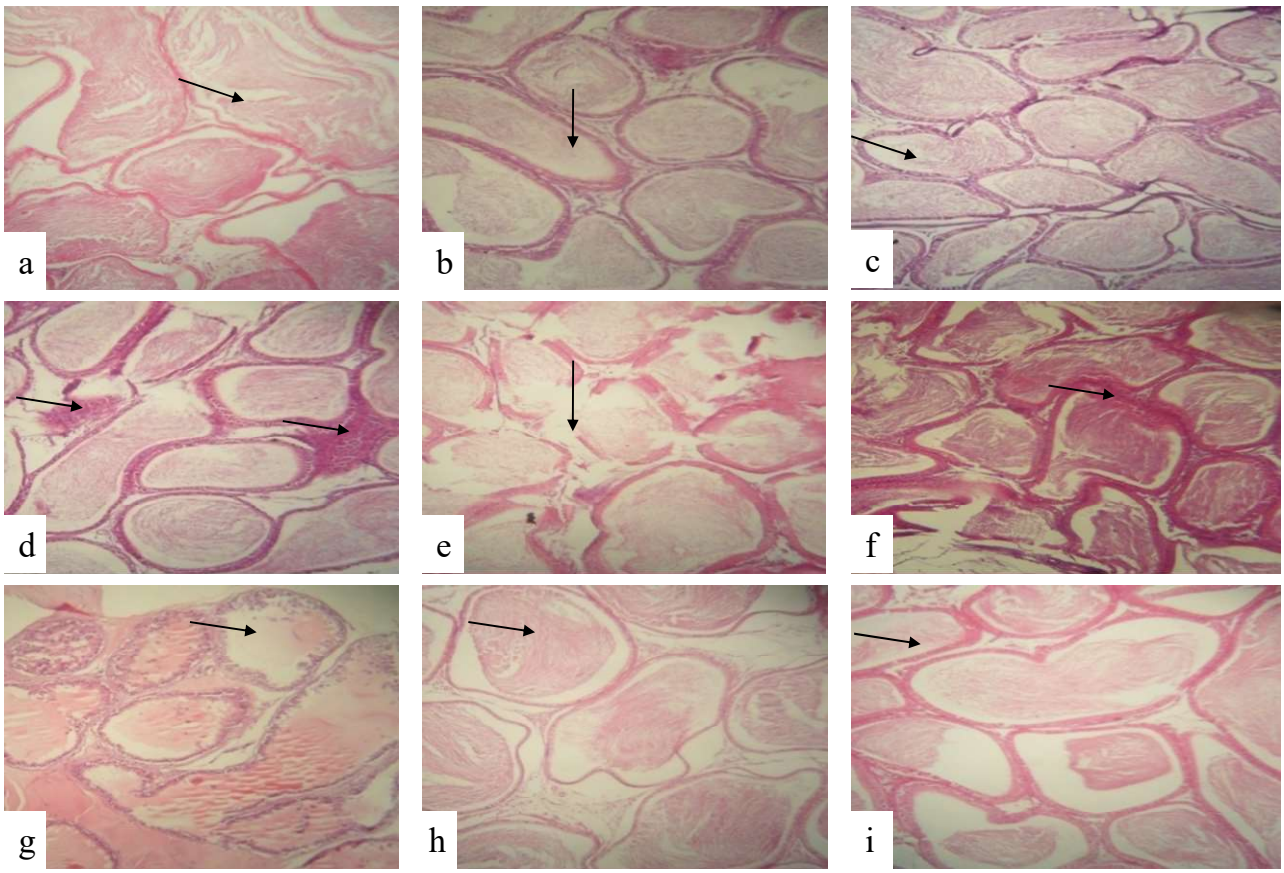


PLATE 5. Photomicrographs of epididymal sections of control and treated male wistar rats. Tissue sections were stained by H & E and presented at x100 magnification.

a) Control: shows normal epididymal duct with muscle and epithelial layer, accumulation of spermatozoa in the lumen, interstitial cells appear normal.

b) Corn oil: shows normal architecture in the of the lumen and epididymal duct with of spermatozoa.

c) Quercetin: Normal epididymal duct with no pathological lesion and presence of spermatozoa in the lumen and the interstitial cells appears normal.

d) Alcohol: shows atrophic ducts and interstitial hyperplastic and thickening of muscle layer of the epididymal. There are lack of spermatozoa in the lumen.

e) Nicotine: shows disruption of the epididymal duct and sperm cells are sloughed off the duct, and also shows pathological lesion with some lumen lacking spermatozoa.

f) Alcohol + Nicotine: shows thickening of the smooth muscle layer of the ductus epididymal and the congestion at the interstitium.

g) Alcohol + Quercetin: normal appearance of epithelial lumen with no pathological lesion, lumen contains spermatozoa.

h) Nicotine + Quercetin: normal architecture of epithelial lining with presence of spermatozoa in the lumen, no pathological lesion seen.

i) Alcohol + Nicotine + Quercetine: No pathological lesion seen, normal appearance of epithelial lining with presence of spermatozoa in lumen.

4.27. Effects of quercetin on alcohol and/or nicotine treatment on histology of prostate gland of male Wistar rats.

Control, corn oil and quercetin groups showed normal and appearance of epithelial lining.

Section for alcohol; showed intense hemorrhage. The epithelial lining in are disrupted in sections for nicotine. Sections for Alcohol + Nicotine showed interstitial hyperplasic with mild hemorrhage. There was a normal prostate tissues with no lesion from sections of Alcohol + Quercetin. Sections for nicotine + Quercetin and Alcohol + Nicotine + Quercetin had normal architecture of epithelial lining.

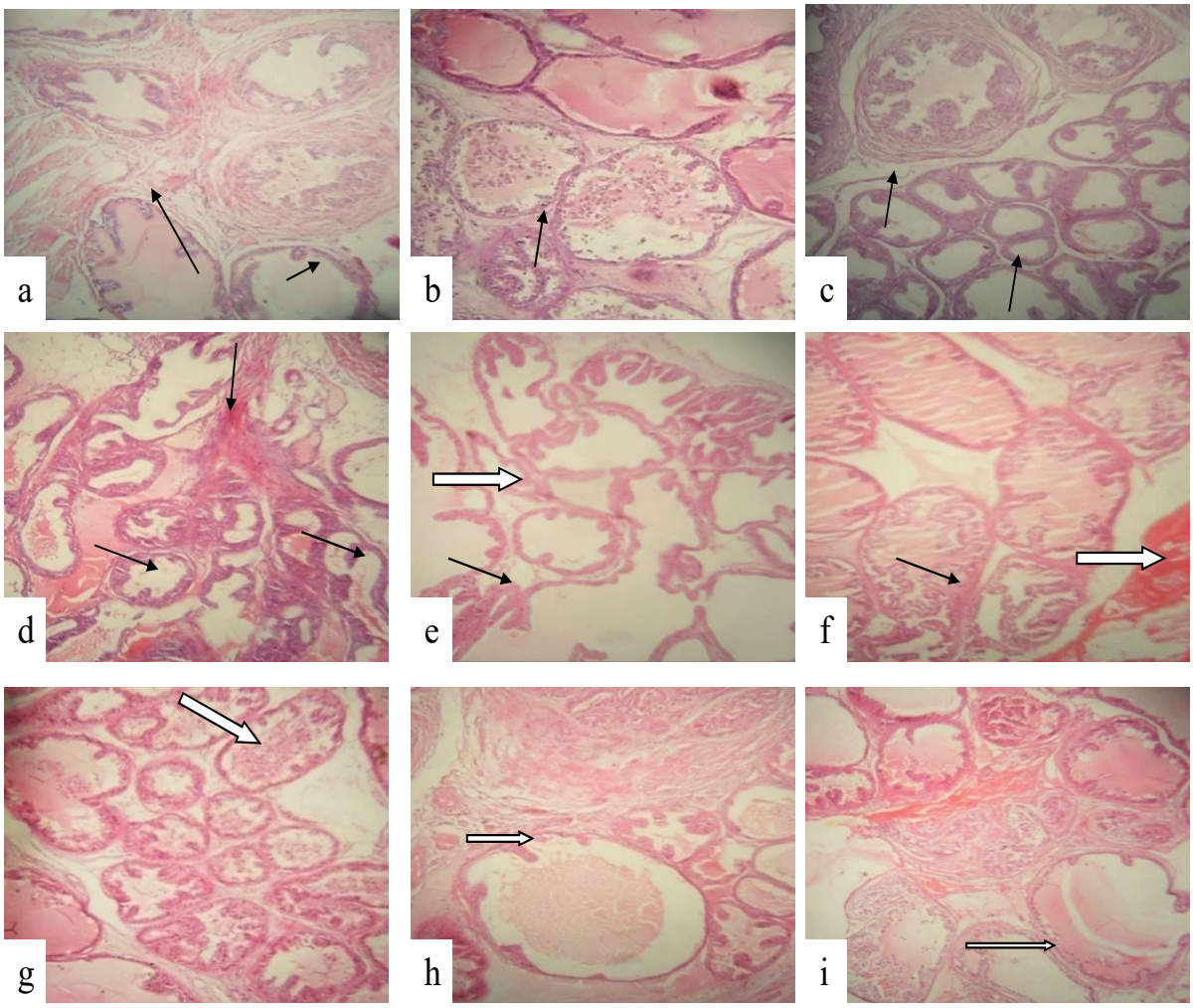


PLATE 6. Photomicrograph of prostate gland sections from control and treated male

Wistar rats. Tissue sections were stained by H & E and presented at x100 magnification.

a) Control: shows normal prostate gland with sections and corpora amylacea. The gland are also lined with normal tall columnar secretary cells

b) Corn oil: shows normal histological section of prostate gland, with normal corpora amylacea. The glands are also lined with normal tall columnar secretary cells.

c) Quercetin: shows normal prostate gland with corpora amylacea and normal cytoarchitecture and appearance of epithelial lining with tall columnar secretary cells.

d) Alcohol: shows pathological lesion with an intense hemorrhage at the interstitial space.

e) Nicotine: shows pathological lesion with disruption of epithelial lining.

f) Alcohol + Nicotine: shows intestinal hyperplasia and mild hemorrhage.

g) Alcohol + Quercetin: shows normal prostatic tissue with no pathological lesion (white arrow).

h) Nicotine + Quercetin: shows normal prostate gland containing secretions and normal architecture of epithelial lining.

i) Alcohol + Nicotine + Quercetin: shows normal prostate gland with normal architecture of epithelial lining (white arrow) and containing secretions.

4.28. Effects of quercetin on alcohol and/or nicotine treatment on histology of seminal vesicle of male Wistar rats

Sections from control, corn oil and quercetin showed normal cytoarchitecture with no pathological lesion seen. However, alcohol treatment showed mild vesicular congestion while there was necrotic muscle layer and congestion in the vesicle of nicotine, and alcohol + nicotine groups. However, groups with quercetin (alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin) showed normal architecture of the seminal vesicle with no pathological lesion.

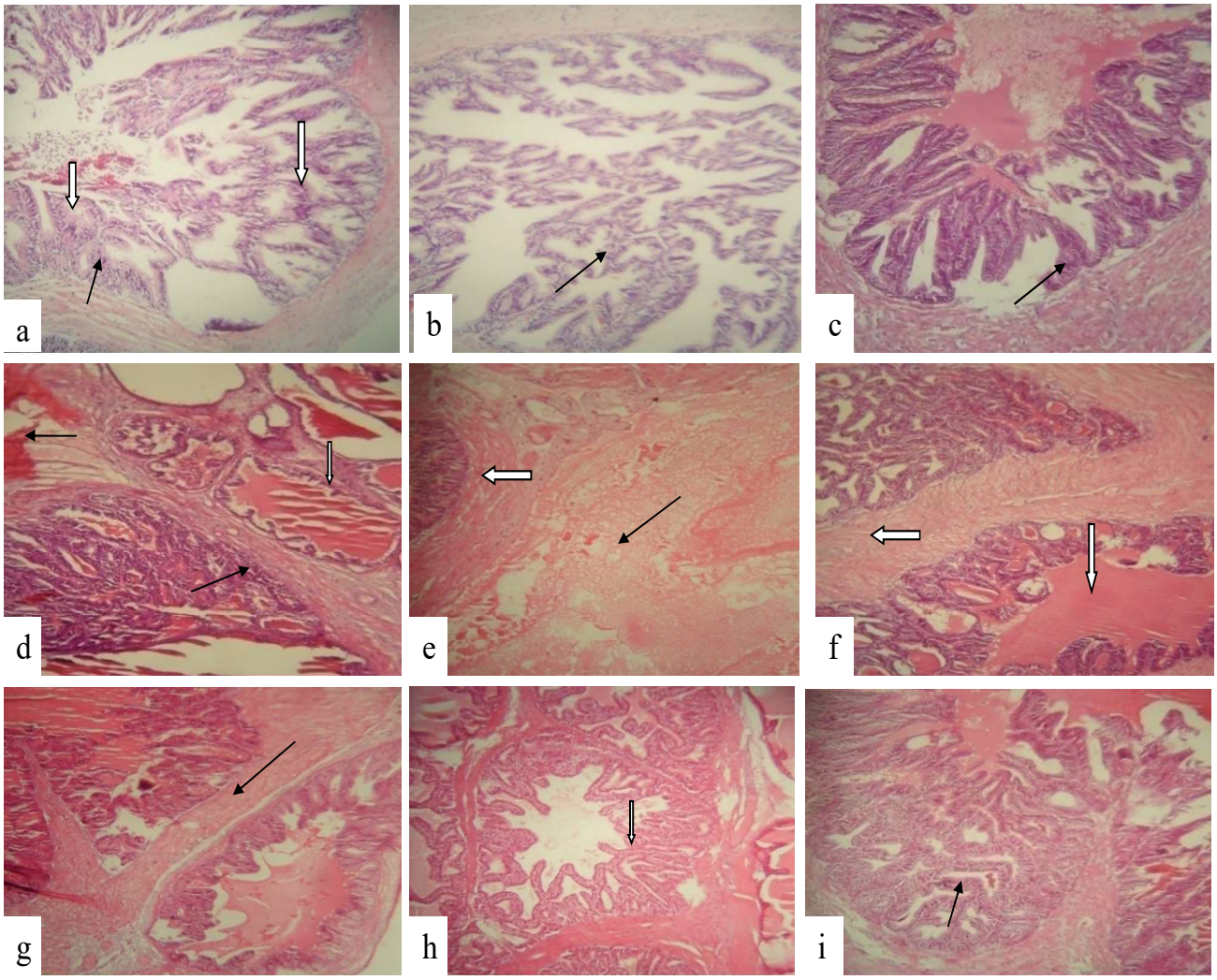


PLATE 7. Photomicrographs of seminal vesicular sections of control and treated male Wistar rats. Tissue were stained by H & E and presented at x100 magnification.

a) Control: shows normal alveolus-like mucosal folds, the internal and external muscle layer appear normal and the lumen lined by tall epithelium.

b) Corn oil: alveolus-like mucosal fold, with normal appearance of internal and external muscle layer (black arrow).

c) Quercetin: shows normal alveolus-like mucosal folds and the internal and external muscle layers appear normal and the lumen lined with tall epithelium.

d) Alcohol: shows disruption of the alveolus-like mucosal fold with mild vesicular congestion.

e) Nicotine: shows disruption of alveolus-like mucosal fold and internal and external muscle layer (black arrow) with necrotic and congestive muscle layer.

f) Alcohol + Nicotine: shows disrupted alveolus –like mucosal fold and internal and external muscle layer with vesicular congestion of the muscle layer.

g) Alcohol + Quercetin: shows normal alveolus-like mucosal folds, with normal histology and no pathological lesion seen.

h) Nicotine + Quercetin: shows normal alveolus-like mucosal folds, with normal histology (the lumen is lined by columnar epithelium) and no pathological lesion seen.

i) Alcohol + Nicotine + Quercetin: shows normal histological architecture of the seminal vesicular gland with normal alveolus-like mucosal folds, the lumen is lined with tall epithelium (black arrow).

4.33. Effects of quercetin on alcohol and/or nicotine treatment on histology of liver in male Wistar rats

Sections from control, corn oil and quercetin groups show normal architecture of the liver. Alcohol and/or nicotine groups showed mild infiltration of inflammatory cells. However, following quercetin supplementation with the alcohol and/or nicotine treated groups, there was no congestion of vessel and no infiltration of sinusoid by inflammatory cells within sinusoid with the alcohol and or nicotine treated group.

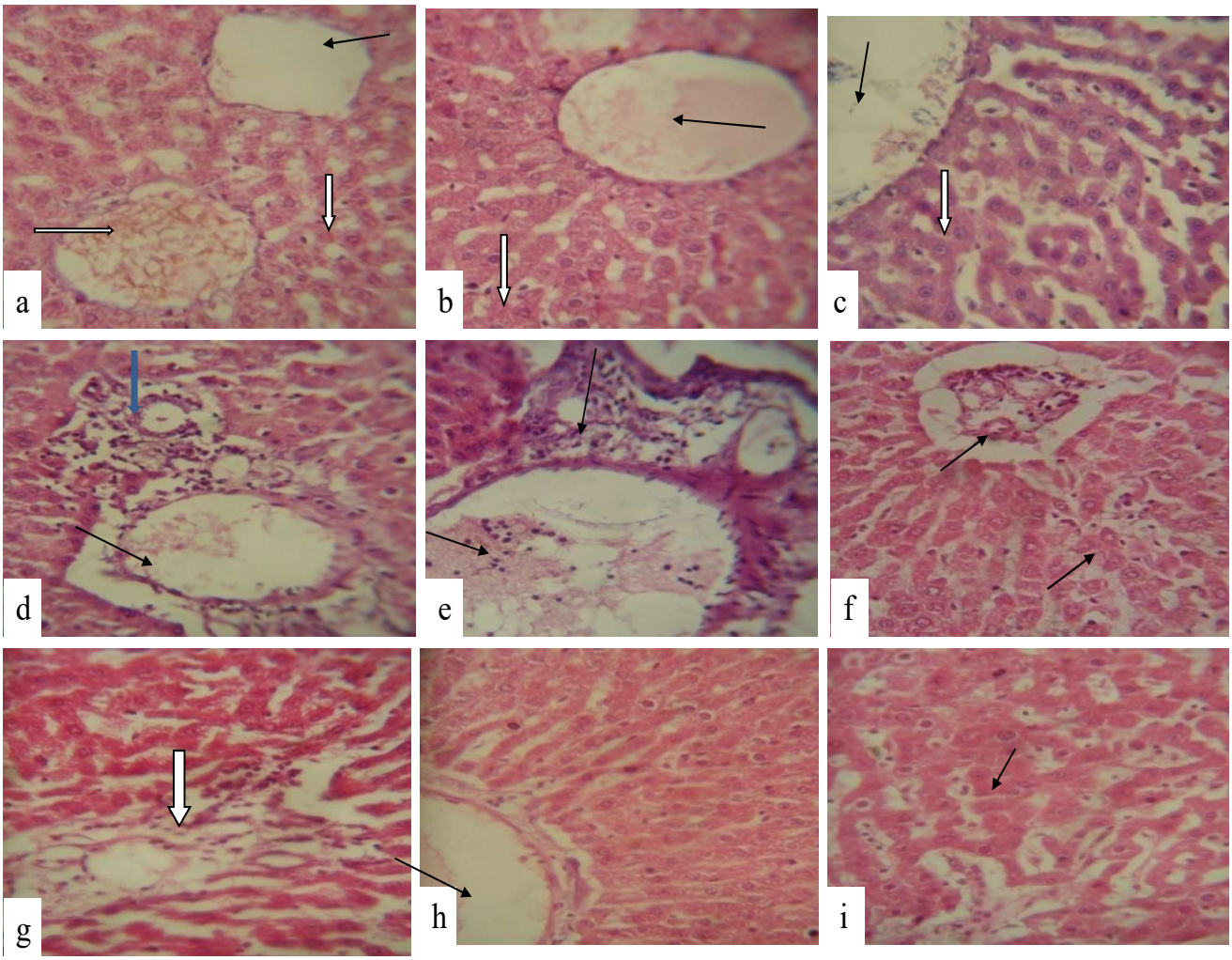


PLATE 8. Photomicrograph of liver sections of control and treated male wistar rats. Tissues were stained by H & E and presented at x100 magnification.

a) Control: shows normal architecture of the histological section with no central vacuole congestion (slender arrow) and hepatocytes appeared normal (white arrow).

b) Corned oil: shows normal histology with no central vacuole congestion and hepatocytes appeared normal (white arrow).

c) Quercetin: shows normal histology with no central vacuole congestion and the

hepatocytes appeared normal (white arrow).

d) Alcohol: shows mild periportal infiltration by inflammatory cells (slender arrow).

e) Nicotine: shows mild periportal infiltration by inflammatory cells (slender arrow).

f) Alcohol + Nicotine: shows mild infiltration by inflammatory cells (slender arrow).

g) Alcohol + Quercetin: shows no congestion of vessel and with no infiltration of sinusoid by inflammatory cells, hepatocytes appeared normal.

h) Nicotine + Quercetin: shows no congestion of vessel and with no infiltration of sinusoid by inflammatory cells, the hepatocytes appeared normal.

i) Alcohol + Nicotine +Quercetin: shows scanty inflammatory cells within the sinusoid.

4.29. Effects of quercetin on alcohol and/or nicotine treatment on testicular expression of P^{53} in male Wistar rats

Qualitatively, alcohol, nicotine, and alcohol + nicotine administration showed an intense expression of p53 at the interstitium of the testicular tissue, while the control, corn oil and quercetin treated groups had mild expression of p53. Moderate expressions of p53 were showed at the interstitial space in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin in the testicular tissue.

Quantitatively, the expression of p53 were reduced in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin with respect to the control group.

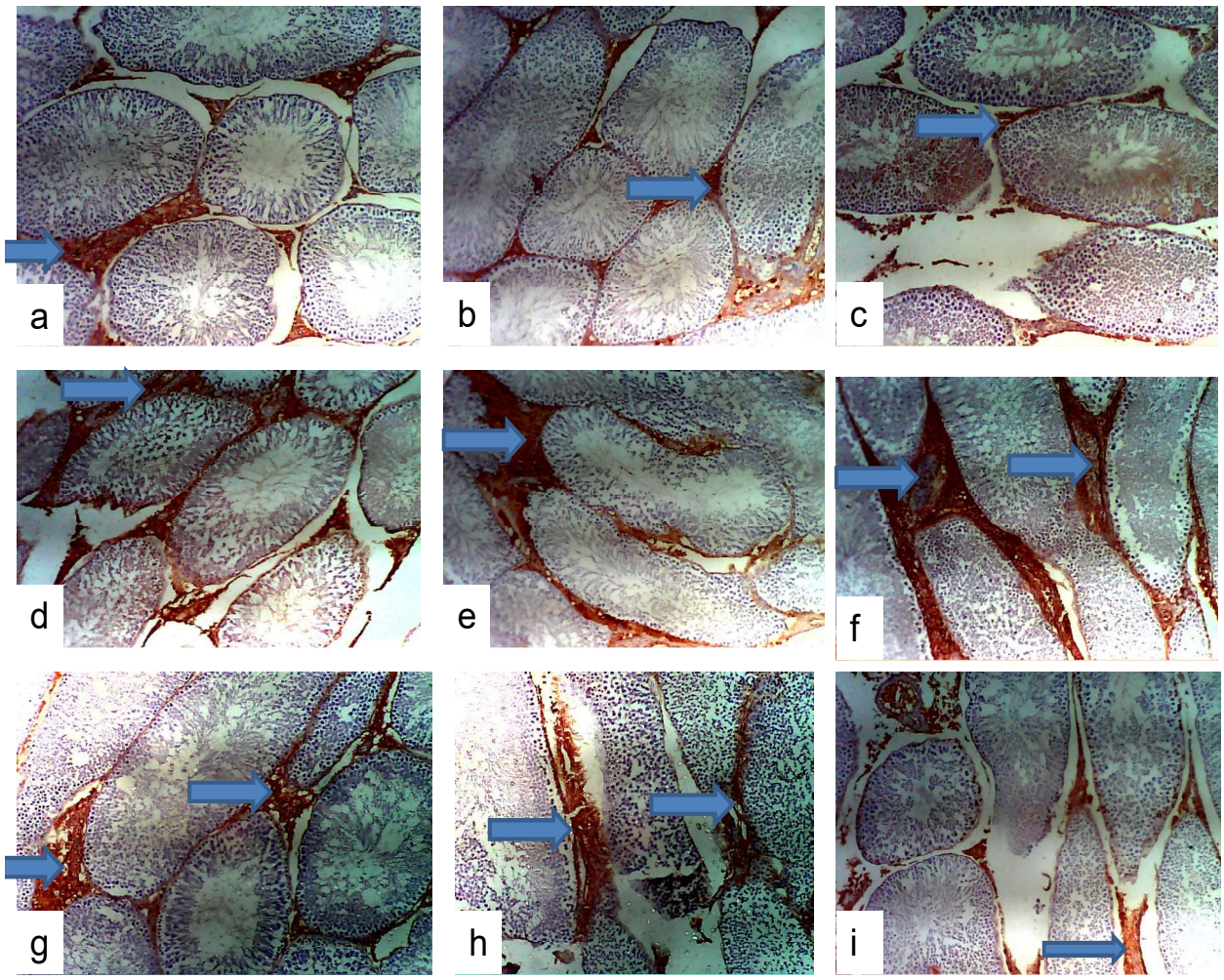


PLATE 9. Qualitative Immunohistochemistry of p53 expression in sections of control and treated male wistar rats. Tissues were stained by 3,3'-diaminobenzidine and presented at x40 magnification.

a) Control: shows a mild expression of p53 (blue arrow) in the Leydig cells of the testis in male Wistar rats.
b) Corned oil: shows mild expressions of p53 at the interstitial cells of the testis.

c) Quercetin: shows mild expression of p53 in the Leydig cells of the testicular tissue

d) Alcohol: shows an intense expression of p53 at the interstitial space of the testicular tissue (blue arrow).

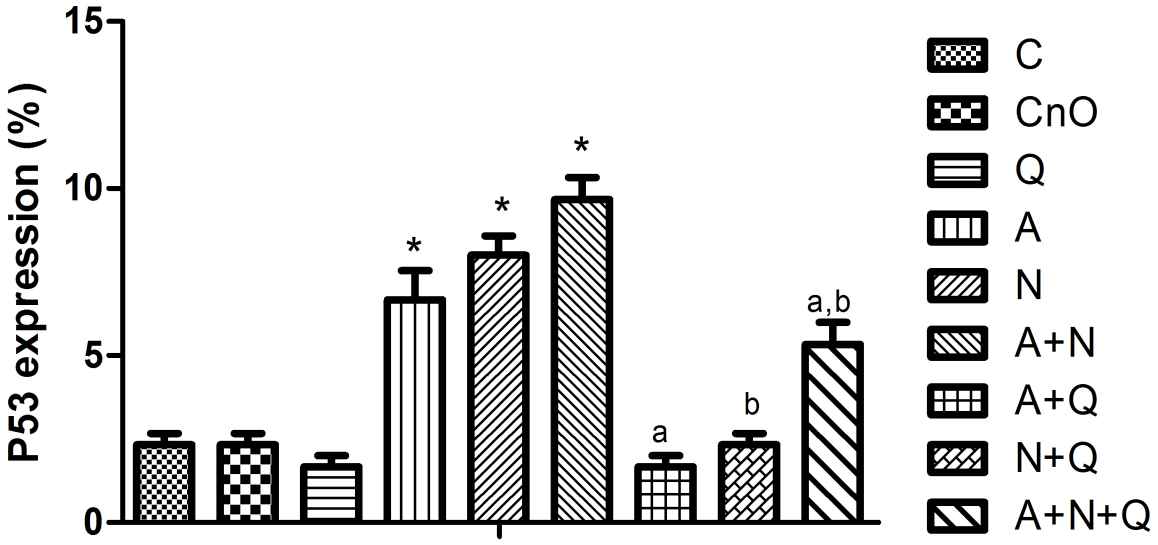
e) Nicotine: shows an intense expression of p53 at the interstitial space of the testicular tissue (blue arrow).

f) Alcohol + Nicotine: shows an intense expression of p53 at the interstitial space of the testicular tissue (blue arrow).

g) Alcohol + Quercetin: shows moderate expression of p53 at the interstitial space of the testicular tissue (blue arrow).

h) Nicotine + Quercetin: shows moderate expression of p53 at the interstitial space of the testicular tissue (blue arrow).

i) Alcohol + Nicotine +Quercetin: shows moderate expression of p53 at the interstitial space of the testicular tissue (blue arrow).



Fiqure 4.16. Quantitative immunohistochemistry of p53 expression in control and treated rats. Values are presented as mean ± SEM $^{*}p<0.05$ considered significant compared to control, $^{a}p<0.05$ significantly different from Alcohol, $^{b}p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly different from Alcohol+Nicotine.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.30. Effects of quercetin on alcohol and/or nicotine treatment on testicular expression of Bcl-2 in male Wistar rats

Qualitatively, alcohol, nicotine, and alcohol + nicotine administration showed a mild expression of Bcl-2 at the interstitial space of the testicular tissue; while the control, corn oil and quercetin treated groups had intense expression of Bcl-2. Intense expressions of Bcl-2 were showed at the interstitial space in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin in the testicular tissue.

Quantitatively, the expression of Bcl-2 were increased in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin with respect to the control group.

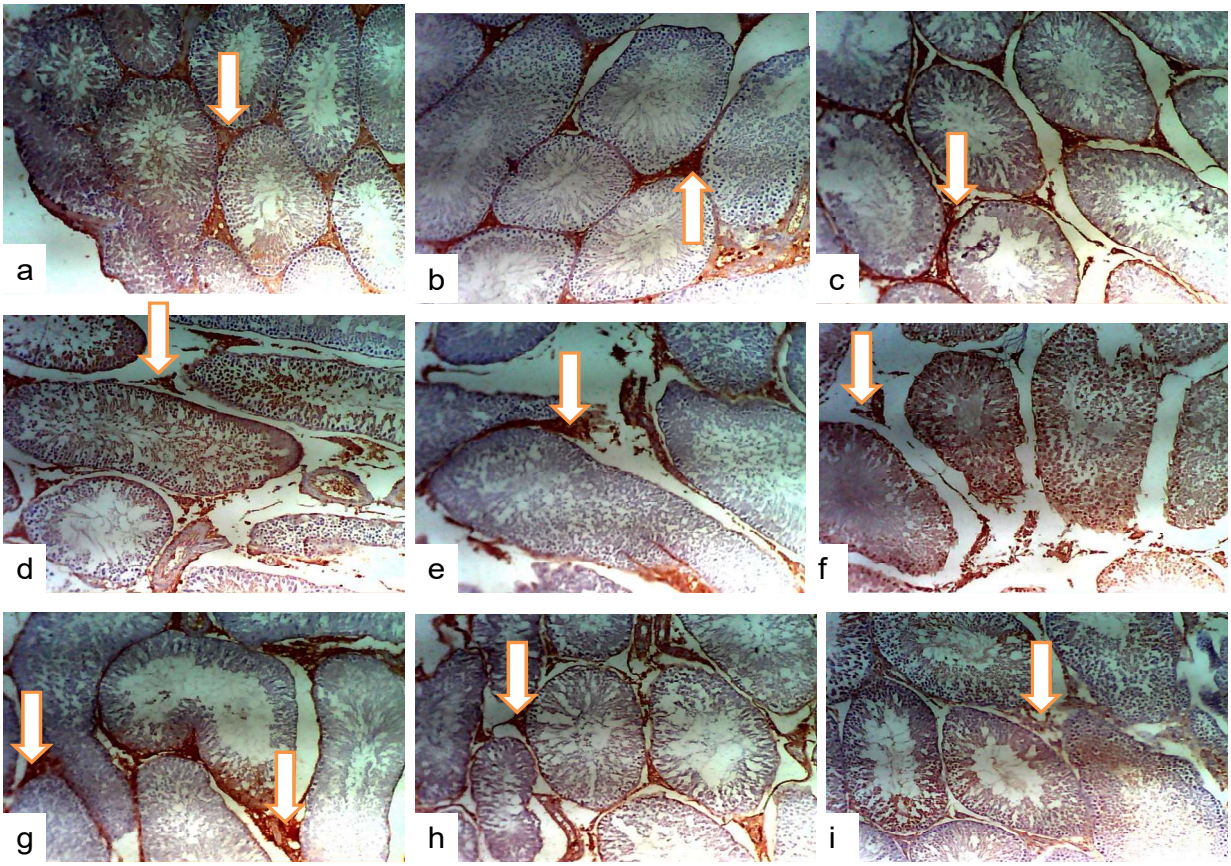


PLATE 10. Qualitative Immunohistochemistry of Bcl-2 expression in sections of control and treated male wistar rats. Tissues were stained by 3,3'-diaminobenzidine and presented at x40 magnification.

a) Control: shows an intense expression of Bcl-2 (yellow arrow) in the Leydig cells of the testis in male Wistar rats.
b) Corned oil: shows intense expressions of Bcl-2 at the interstitial cells of the testis
c) Quercetin: shows intense expression of Bcl-2 in the Leydig cells of the testicular tissue
d) Alcohol: shows a mild expression of Bcl-2 at the interstitial space of the testicular tissue (yellow arrow).

e) Nicotine: shows a mild expression of Bcl-2 at the interstitial space of the testicular tissue (yellow arrow).

f) Alcohol + Nicotine: shows a mild expression of Bcl-2 at the interstitial space of the testicular tissue (yellow arrow).

g) Alcohol + Quercetin: shows intense expression of Bcl-2 at the interstitial space of the testicular tissue (yellow arrow).

h) Nicotine + Quercetin: shows moderate expression of Bcl-2 at the interstitial space of the testicular tissue (yellow arrow).

i) Alcohol + Nicotine +Quercetin: shows moderate expression of Bcl-2 at the interstitial space of the testicular tissue (yellow arrow).

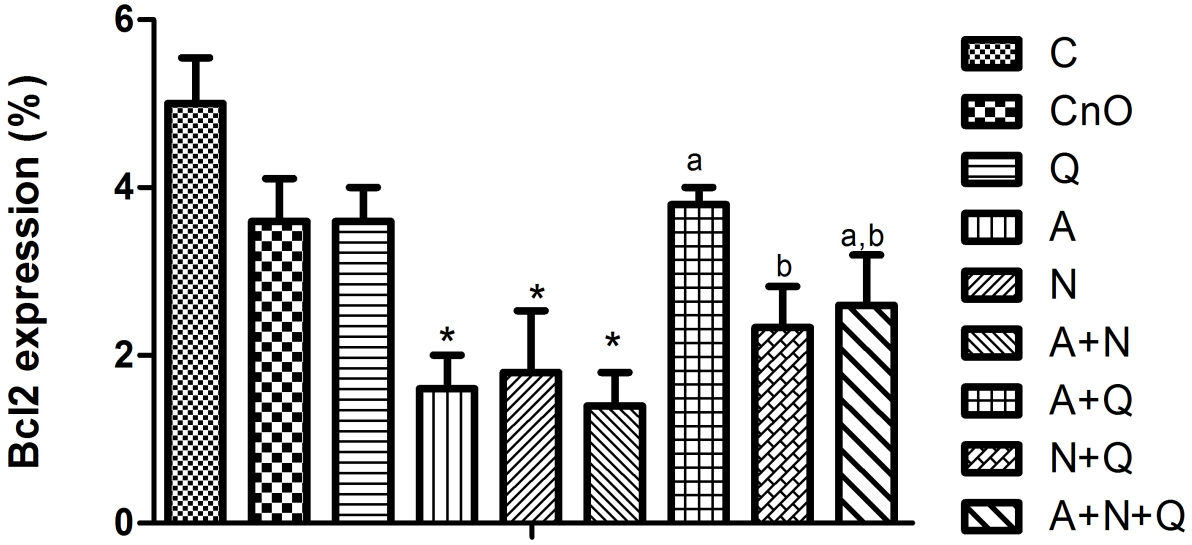


Figure 4.17. Quantitative immunohistochemistry of Bcl-2 expression in control and treated rats. Values are presented as mean ± SEM $^{*}p<0.05$ considered significant compared to control, $^{a}p<0.05$ significantly different from Alcohol, $^{b}p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly different from Alcohol+Nicotine.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.31. Effects of quercetin on alcohol and/or nicotine treatment on testicular expression of Ki67 in male Wistar rats

Qualitatively, alcohol, nicotine, and alcohol + nicotine administration showed a mild expression of Ki67 at the interstitial space of the testicular tissue; while the control, corn oil and quercetin treated groups had intense expression of Ki67. Intense expressions of Ki67 were showed at the interstitial space in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin in the testicular tissue.

Quantitatively, the expression of Ki67 were increased in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin with respect to the control group.

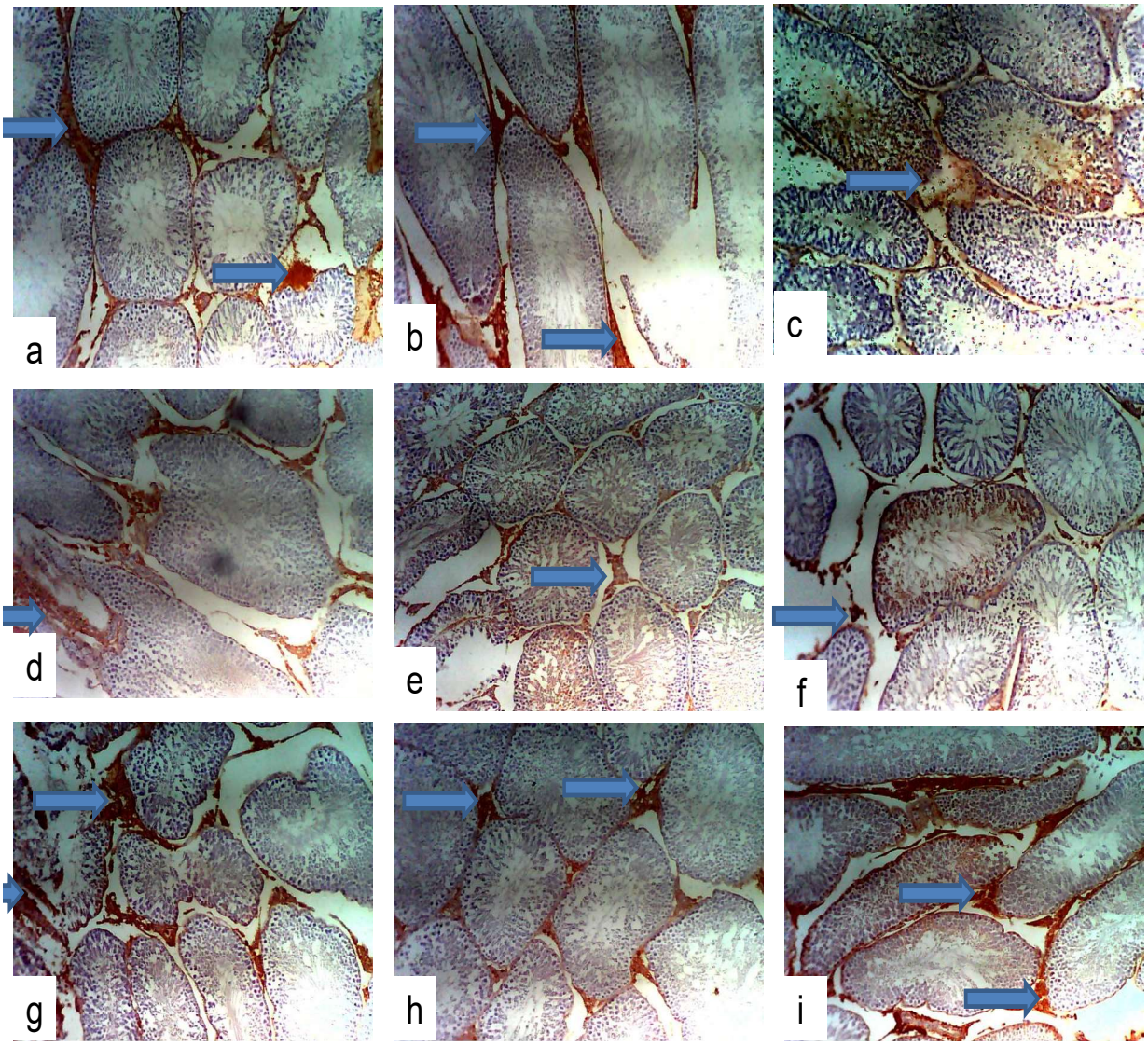


PLATE 11. Qualitative Immunohistochemistry of Ki67 expression in sections of control and treated male wistar rats. Tissues were stained by 3,3'-diaminobenzidine and presented at x40 magnification.

a) Control: shows an intense expression of Ki67 (blue arrow) in the Leydig cells of the testis in male Wistar rats.
b) Corned oil: shows intense expressions of Ki67 at the interstitial cells of the testis

c) Quercetin: shows intense expression of Ki67 in the Leydig cells of the testicular tissue

d) Alcohol: shows a mild expression of Ki67 at the interstitial space of the testicular tissue (blue arrow).

e) Nicotine: shows a mild expression of Ki67 at the interstitial space of the testicular tissue (blue arrow).

f) Alcohol + Nicotine: shows a mild expression of Ki67 at the interstitial space of the testicular tissue (blue arrow).

g) Alcohol + Quercetin: shows intense expression of Ki67 at the interstitial space of the testicular tissue (blue arrow).

h) Nicotine + Quercetin: shows intense expression of Ki67 at the interstitial space of the testicular tissue (blue arrow).

i) Alcohol + Nicotine +Quercetin: shows intense expression of Ki67 at the interstitial space of the testicular tissue (blue arrow).

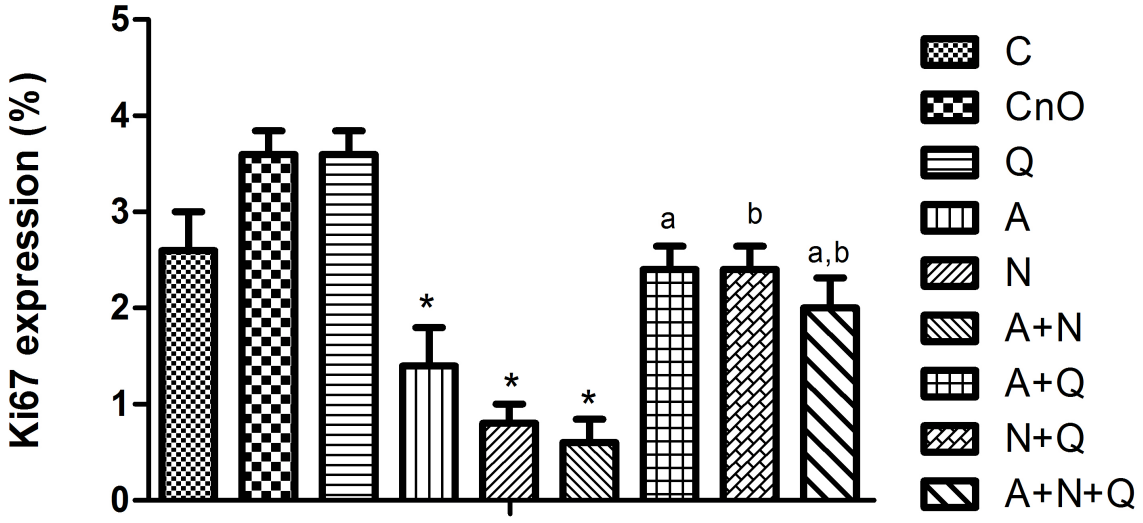


Figure 4.18. Quantitative immunohistochemistry of Ki67 expression in control and treated rats. Values are presented as mean ± SEM $^{*}p<0.05$ considered significant compared to control, $^{a}p<0.05$ significantly different from Alcohol, $^{b}p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly different from Alcohol+Nicotine.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.32. Effects of quercetin on alcohol and/or nicotine treatment on testicular expression of EGFR in male Wistar rats

Qualitatively, alcohol, nicotine, and alcohol + nicotine administration showed a mild expression of EGFR at the interstitial space of the testicular tissue; while the control, corn oil and quercetin treated groups had intense expression of EGFR. Moderate expressions of EGFR were showed at the interstitial space in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin in the testicular tissue.

Quantitatively, the expressions of EGFR were increased in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin with respect to the control group.

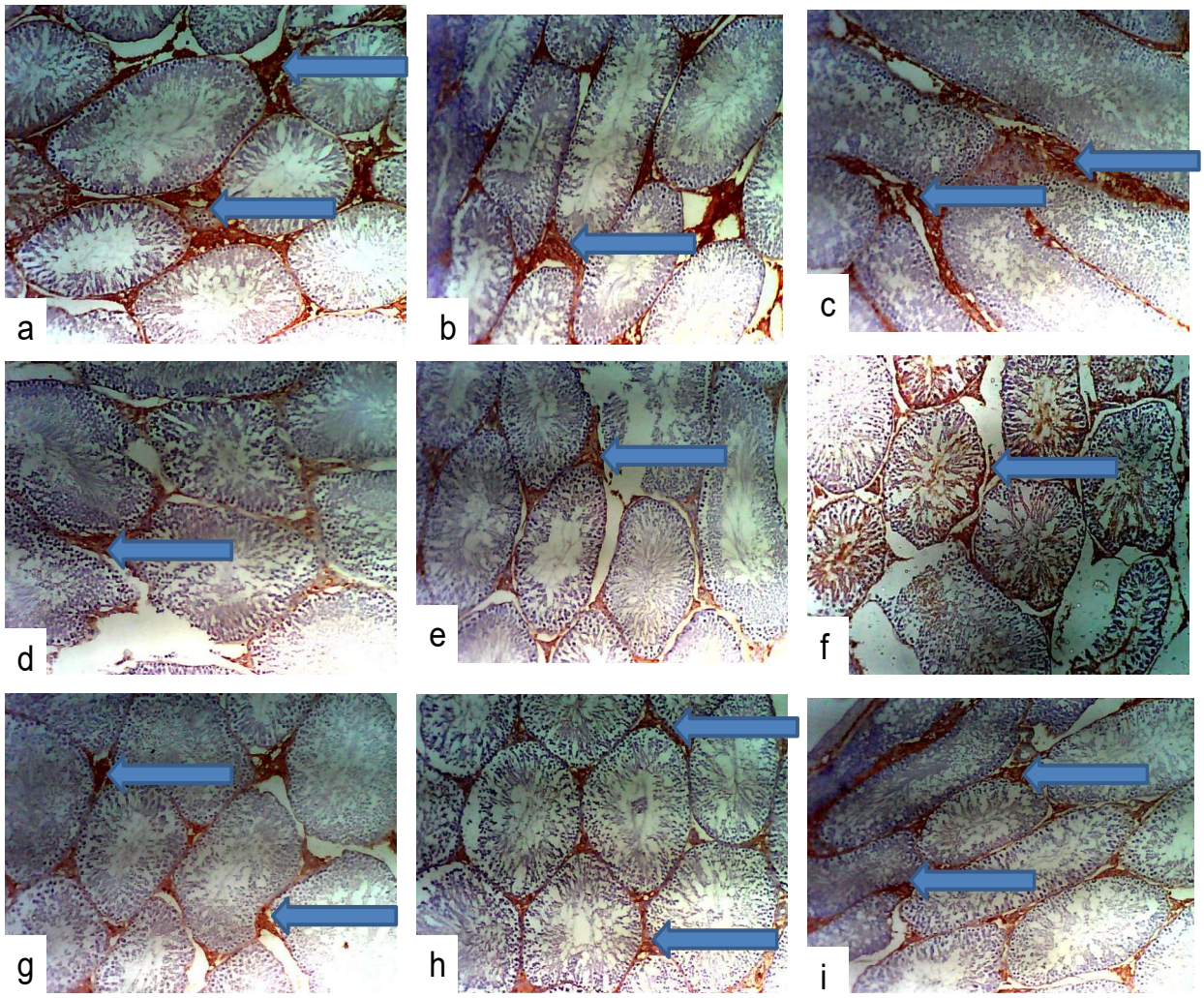


PLATE 12. Quantitative Immunohistochemistry of EGFR expression in sections of control and treated male wistar rats. Tissues were stained by 3,3'-diaminobenzidine and presented at x40 magnification.

a) Control: shows an intense expression of EGFR (blue arrow) in the Leydig cells of the testis in male Wistar rats.
b) Corned oil: shows intense expressions of EGFR at the interstitial cells of the testis
c) Quercetin: shows intense expression of EGFR in the Leydig cells of the testicular tissue
d) Alcohol: shows a mild expression of EGFR at the interstitial space of the testicular tissue (blue arrow).

e) Nicotine: shows a mild expression of EGFR at the interstitial space of the testicular tissue (blue arrow).

f) Alcohol + Nicotine: shows a mild expression of EGFR at the interstitial space of the testicular tissue (blue arrow).

g) Alcohol + Quercetin: shows moderate expression of EGFR at the interstitial space of the testicular tissue (blue arrow).

h) Nicotine + Quercetin: shows moderate expression of EGFR at the interstitial space of the testicular tissue (blue arrow).

i) Alcohol + Nicotine +Quercetin: shows moderate expression of EGFR at the interstitial space of the testicular tissue (blue arrow).

Figure 4.19. Quantitative immunohistochemistry of EGFR expression in control and treated rats. Values are presented as mean ± SEM $^{*}p<0.05$ considered significant compared to control, $^{a}p<0.05$ significantly different from Alcohol, $^{b}p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly different from Alcohol+Nicotine.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

4.33. Effects of quercetin on alcohol and/or nicotine treatment on testicular expression of CYD in male wistar rats

Qualitatively, alcohol, nicotine, and alcohol + nicotine administration showed a mild expression of CYD at the interstitial space of the testicular tissue; while the control, corn oil and quercetin treated groups had intense expression of CYD. Moderate to intense expressions of CYD were showed at the interstitial space in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin in the testicular tissue.

Quantitatively, the expressions of CYD were increased in alcohol + quercetin, nicotine + quercetin and alcohol + nicotine + quercetin with respect to the control group.

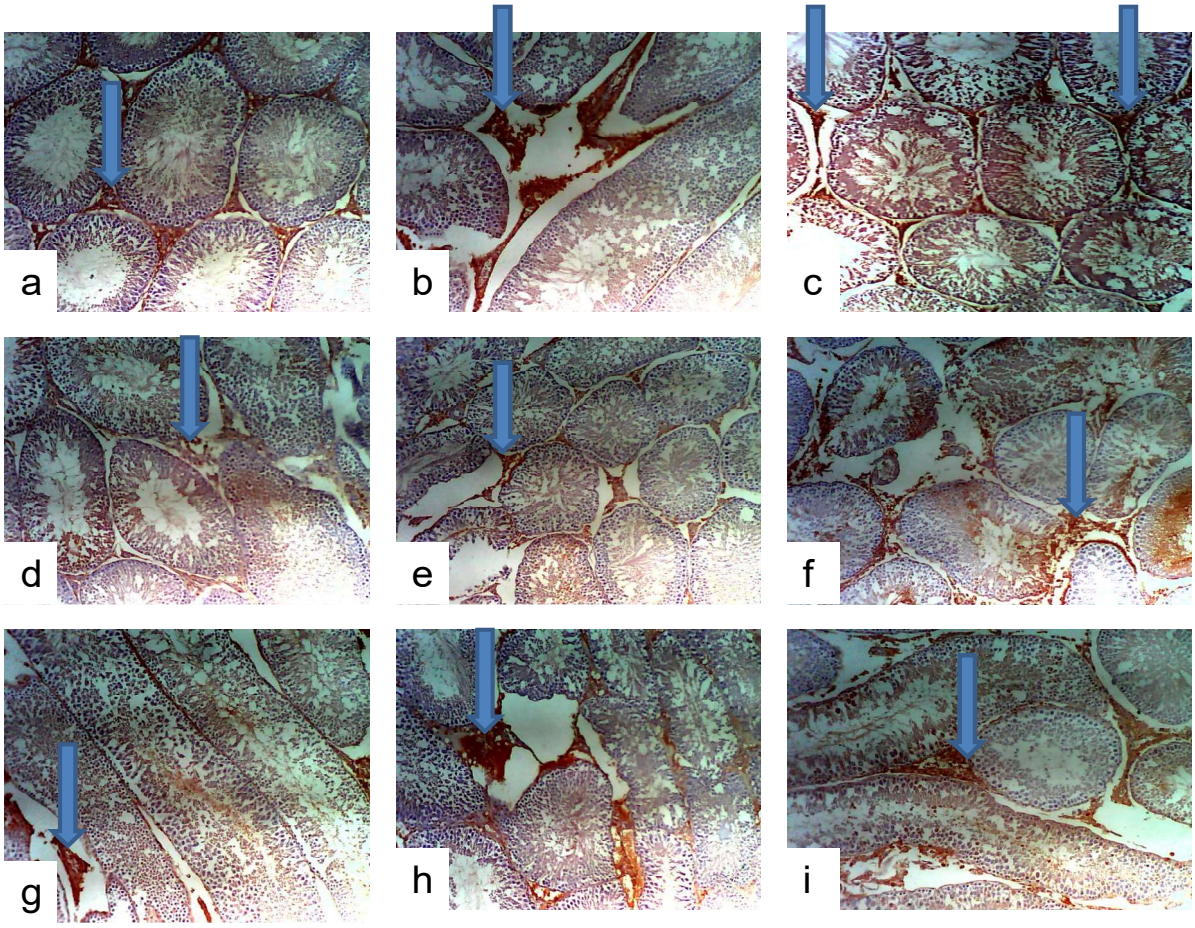


PLATE 13. Qualitative Immunohistochemistry of CYD expression in sections of control and treated male wistar rats. Tissues were stained by 3,3'-diaminobenzidine and presented at x40 magnification.

a) Control: shows an intense expression of CYD (blue arrow) in the Leydig cells of the testis in male Wistar rats.
b) Corned oil: shows intense expressions of CYD at the interstitial cells of the testis

c) Quercetin: shows intense expression of CYD in the Leydig cells of the testicular tissue

d) Alcohol: shows a mild expression of CYD at the interstitial space of the testicular tissue (blue arrow).

e) Nicotine: shows a mild expression of CYD at the interstitial space of the testicular tissue (blue arrow).

f) Alcohol + Nicotine: shows a mild expression of CYD at the interstitial space of the testicular tissue (blue arrow).

g) Alcohol + Quercetin: shows moderate expression of CYD at the interstitial space of the testicular tissue (blue arrow).

h) Nicotine + Quercetin: shows intense expression of CYD at the interstitial space of the testicular tissue (blue arrow).

i) Alcohol + Nicotine +Quercetin: shows moderate to intense expression of CYD at the interstitial space of the testicular tissue (blue arrow).

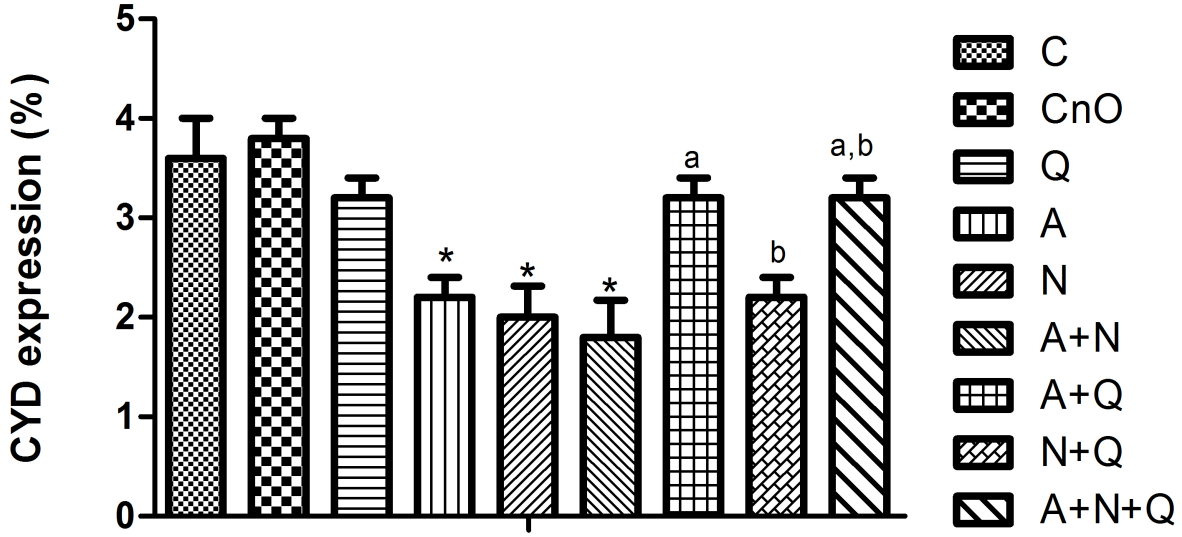


Figure 4.20. Quantitative immunohistochemistry of CYD expression in control and treated rats. Values are presented as mean ± SEM $^{*}p<0.05$ considered significant compared to control, $^{a}p<0.05$ significantly different from Alcohol, $^{b}p<0.05$ significantly different from Nicotine, $^{a,b}p<0.05$ significantly different from Alcohol+Nicotine.

Key: C-Control, CnO- Corn Oil, Q- Quercetin, A- Alcohol, N- Nicotine, A+N- Alcohol+Nicotine, A+Q- Alcohol+Quercetin, N+Q- Nicotine+Quercetin, A+N+Q- Alcohol+Nicotine+Quercetin.

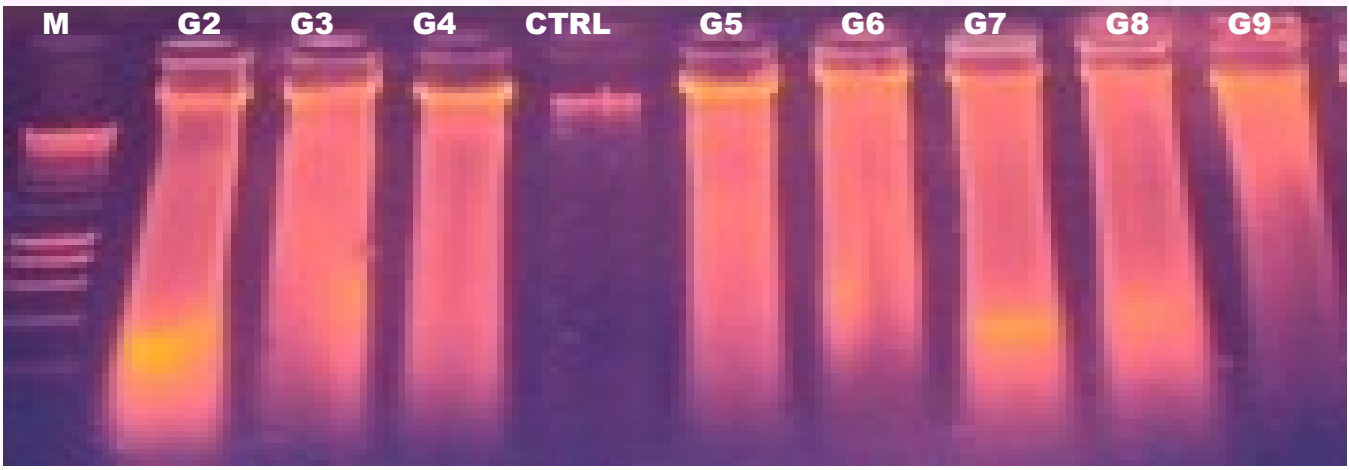


Plate 14. Agarose gel electrophoresis of chromosomal DNAs from the testicular tissue samples of experimental treated rats and control. Lane 1. 100 bp DNA ladder (Bio line); Lanes G2 – G9 = DNA samples of experimental and control (CTRL) rats.

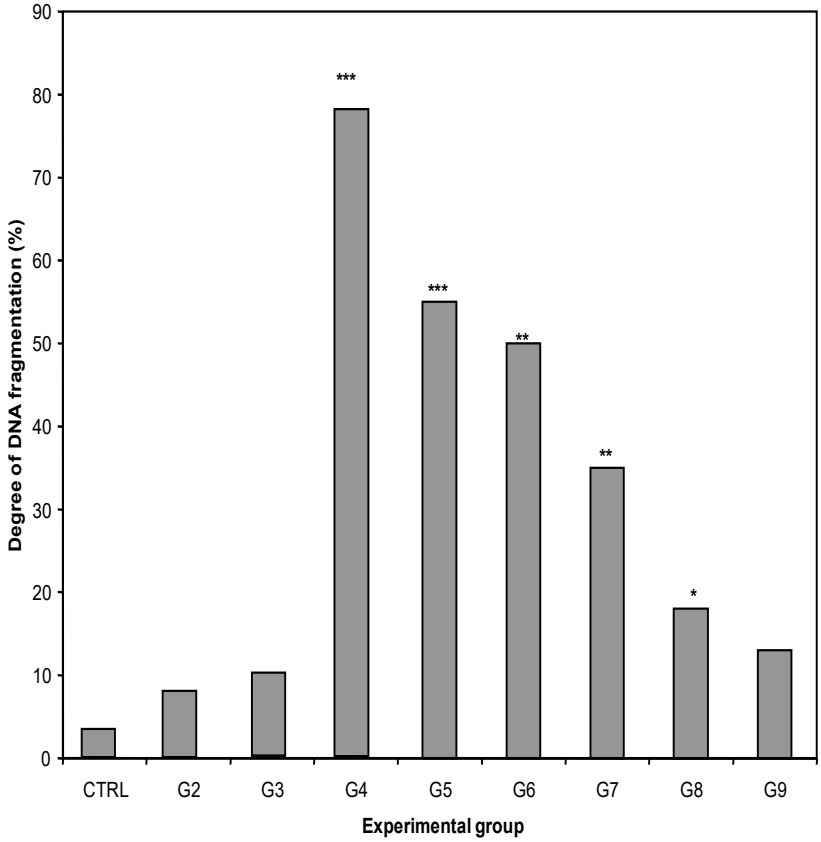


Figure 4.21. Evaluation of DNA fragmentation in experimental rats compared to the control. Disparity in DNA fragmentation was analyzed by chi square (χ^2) test. ***P<0.0001; **P<0.01; *P<0.05

Key: CTRL-Control, G2-Corn oil, G3-Quercetin, G4-Alcohol G5-Nicotine, G6-Alcohol+nicotine, G7-Nicotine+Quercetin, G8-Alcohol+Quercetin, G9-Alcohol+Nicotine+Quercetin.

CHAPTER FIVE

5.0 DISCUSSION

5.1: Effects of quercetin on body and organ weight in male Wistar rats treated with alcohol and/or nicotine

The toxicity effects of alcohol and nicotine treatment showed a marked increase caused by alcohol and decrease as a result of nicotine on body weight in rats following administration. It was observed that there was an increase in body weight of rats administered with alcohol in the results of the present study. In the groups treated with nicotine alone and alcohol plus nicotine had a marked reduction in body weight and organ weight. Study has documented that smoking cigarette is associated to reduced or decreased body weight which could be due to one of the major reasons why many adolescent smoke in order to lose body weight, (Rolls and Rolls, 1982). Rolls and Rolls, (1982) reported the dehydrating effect of alcohol and sometimes it leads to inhibition of food intake and is not in agreement with the present findings. However, there was a controversial report on whether alcohol causes an increase in body weight as reported in previous studies, but it does increase body weight in some studies (Traversy and Chaput, 2015). According to studies, the correlation between alcohol and body weight is an important and relevant topic to groups of several individuals that engage in taking alcoholic beverages and people who are willing to lose body weight or to maintain it (Rohrer *et al.,* 2005). A small decrease in body weight was found among women after consuming alcohol according to the study by Mannisto *et al.,* (1997). In the present study an increase in body weight of alcohol treated rats was observed compared to the control group and this differs from the reports by Rolls and Rolls, 1982, they showed that the role of alcohol in causing dehydration could be due to its inhibitory effects on food intake. The administration of alcohol and nicotine action on food intake has been reported and there is a correlation between these drugs (Shyngle, 2006).

However, correlation was found in nicotine treated groups thus inhibiting the feeding center thereby resulting in organ and body weight loss. Alcohol and nicotine co-administration in rats in inhibiting food and water intake could be due to their synergistic actions and thus a significant reduction was observed in body and organ weight in the results of this study which was not contrary in the reports of Shyngle, (2006). The result on reduction in body weight and organ

weight following nicotine and alcohol plus nicotine treatment in this study is in agreement with the study by Rees and Seyfoddin, (2017), but an increased weight was observed in alcoholics in a separate study (French *et al.,* 2011). The reasons why nicotine could cause a reduction in body weight have not been fully concluded, but it could be suggested that nicotine inhibits the feeding centre in the hypothalamus and at the same time stimulating the satiety centre respectively. The reduced testicular and accessory sex organ weight as observed in the treated group (nicotine), can be reported that the observed decrease may be as a result of reduced germ cells number thus inhibiting the process of spermatogenesis either directly or indirectly. Although, study by Bishop *et al.,* (2002), established that neuropeptide Y and some other important peptide hormones such as leptin could be linked to nicotine feeding related action. Physiologically, neuropeptide Y is an important and potent hormone which stimulates feeding centre and could be inhibited by nicotine plus alcohol intake. Also, neuropeptide like substances such a serotonin and dopamine could be increased by the action of nicotine and these neurotransmitters have been found to cause reduction in food intake (Turner *et al.,* 2013). However, food consumption rate by animals and neurotransmitter levels were not estimated in this study but the body weights were monitored, thus, the reduced body weight and organ weight may be as a result of protein synthesis inhibition and malabsorption rate at the level of the gastrointestinal tract according to report by MoBeler and Kampheus, (2017). Moreover, the reduced organ weight (testis, seminal vesicle, prostate, and so on) observed in the nicotine treated group could be due to the free radical generation and reduced blood perfusion and nutrients supply which may be attributed to the degenerative and necrotic changes that was caused by nicotine plus alcohol. The toxic effects of alcohol and/or nicotine were assessed by measuring the weight loss of the body weight and organ weight in these animals.

5.2: Effects of quercetin on sperm parameters and histology of the testis and accessory sex organs in male Wistar rats treated with alcohol and/or nicotine

In this study, alcohol and/or nicotine treatment caused significant reduction in sperm concentration, sperm motility, sperm viability, mature sperm and increased sperm morphology. The marked decrease in sperm profile of alcohol plus nicotine treated group could be as a result of the synergistic effect of these two drugs compared to alcohol alone, nicotine alone and the control groups respectively. Sperm variables can present vital information in respect to

reproductive toxicity (Seed *et al.,* 1996). Reports on cigarette smoking in individuals is associated with reduced sperm concentration, motility and increased sperm morphology (Kumosani *et al.,* 2008; Liu *et al*., 2010). There is a strong correlation between nicotine and abnormal retention of cytoplasmic residues in semen which shows an alteration in sperm morphology thus impairing those functions sperm profile according to the report by Mak *et al*., (2000). Spermatogenesis is the production of spermatozoa from primordial germ cell or spermatogonia. However, different toxicants or agents have been reported in literature to alter the process and phases of spermatogenesis thereby leading to infertility. However, when spermatogenesis is interrupted or altered at any phase of differentiation, be it at the phase of proliferation, growth stage, maturation and transformation respectively, it may lead to reduced sperm count, sperm motility and increased-defective sperm morphology (Sharp, 2010).

The results of sperm parameters obtained in the groups treated with alcohol, nicotine, and alcohol plus nicotine were different from groups treated with alcohol plus co-administration of quercetin, nicotine plus quercetin and alcohol plus nicotine with quercetin intervention. Thus, quercetin ameliorates the deleterious activity of alcohol and/or nicotine on sperm parameters which could be attributed to its antioxidant potential. Also, administration of alcohol and/or nicotine caused reduction in sperm content at the lumen of the seminiferous tubules and caused damage to the cytoarchitecture of the testicular histology which pose as an indication of altered spermatogenesis which led to damage to the primary and secondary spermatocytes and spermtogonia respectively as observed in this study, this could have been as a result of generation of free radicals as also reported by Chowdhury and Gupta, (2006). The findings in this study revealed that alcohol and/or nicotine treatment in experimented rats induced reproductive dysfunction and also caused series of testicular derangement including seminiferous tubules deterioration and spermatogenic cell loss, hence with quercetin intervention all of which were ameliorated. However, the modulatory role of quercetin intervention on the deleterious actions of both alcohol and nicotine is mainly due to its antioxidant properties serving as a scavenger of free radical generation.

The damaging effects of alcohol and/or nicotine in the structural and functional gonads in the male Wistar rats as well as accessory organs (epididymis, seminal vesicles, prostate gland) in the treated groups of the present study showed an alteration in the architecture of the seminiferous

tubules of the reproductive organs (testes) in the treated rats, which suggests that alcohol and/or nicotine could have crossed the testicular blood barrier thereby causing their deleterious effects on the gonads by directly suppressing the function of the Leydig cells thereby affecting testosterone secretion and also by directly altering the function of the Sertoli cells thereby affecting the process of spermatogenesis. Also, the histological section of the testis and the accessory organs in rats treated with both alcohol and nicotine showed various degree of degeneration. Histological studies indicate that the testis could be more sensitive to the effects of alcohol than the liver (Wallock-Montelius *et al*., 2007). These alterations caused by alcohol and/or nicotine were ameliorated following quercetin intervention as observed in the results of this study. The loss of gonadal, epididymal and accessory organ of reproductive system as well as the histology of the hypothalamic-pitutary axis, seminal vesicle, prostate and the epididymis could be considered as criteria for toxic agents that may cause fertility problems in experimental animals. Following Quercetin supplementation with alcohol and/or nicotine in male Wistar rats in the present study, there was a marked increase in body weight and organ weight respectively. This suggests that Quercetin ameliorated the effects of alcohol and/or nicotine by increasing body weight and organ weight through its antioxidant potential and via the stimulation of the feeding centre in the hypothalamus.

5.3: Effects of quercetin on lipid peroxidation and antioxidant enzymes in male Wistar rats treated with alcohol and/or nicotine

Antioxidant levels and lipid peroxidation were assessed in male Wistar rats treated with alcohol and/or nicotine, increase in lipid peroxidation has been reported to be associated with both active and passive smokers as well as alcohol intake in both sexes and in animals as well (Ashakumary and Vijayammal, 1996). It was also observed that there were significant decrease (*$p<0.05$) SOD and reduced glutathione peroxidase following administration of alcohol and/or nicotine for a period of 52 days in the results obtained in the present study. The endogenous antioxidant enzymes among which are superoxide dismutase (SOD) and Catalase (CAT) serve or function as scavengers by scavenging or inhibiting the generation of free radicals and thus, by removing superoxide anions and also converting them into water molecule (Husain *et al.,* 2001). An important index in measuring the extent of damage done to a tissue or cell membrane is via lipid peroxidation (generation of free radicals).

The increased lipid peroxidation which is the product of TBARS as observed in this study following administration of alcohol and/or nicotine is probably through their generation of free radical. Studies have established that chronic consumption of alcohol resulted in a significant increase in the TBARS concentration of rats' testes (Cvetkovic *et al.,* 2015). More so, a significant There have also been a correlation between semen parameters and lipid peroxidation (Ben Abdallah *et al.,* 2009), they observed a positive correlation between the levels of MDA with acrosomal abnormalities and the presence of residual cytoplasmic droplets in spermatozoa. In this present study, alcohol and/or nicotine treatment in rats showed a marked increase in MDA levels when compared to the control groups, which could have been due to peroxidation of spermatozoa polyunsaturated fatty acids (PUFA) on the cell membrane and this is in correlation with the findings by Shamsi *et al*., (2010), who reported a positive correlation between the level of MDA in blood and percentage of dead sperm and abnormal sperm morphology.

5.4: Effects of quercetin on biochemical profile in male Wistar rats treated with alcohol and/or nicotine

The results of the present study showed an increase in ALP, AST, ALT and LDH activities which are liver function indicators following alcohol and nicotine treatment in rats. The result obtained showed a significantly increased in rats treated with nicotine. The increase in activity of liver enzymes indicates alteration or damage to the liver as caused by chronic administration of alcohol and/or nicotine in male Wistar rats in this study is in agreement or alignment with the results obtained by Dhouib *et al*., (2014).

LDH being a cytoplasmic enzyme marker is an important tissue and cell damage indicator by toxic substances. Elevated level of LDH indicates loss of cell membrane functional integrity as a result of damage to the cell. Study by Zhang and Colleague revealed the changes in the activities of SDH, LDH and GGT which may be responsible for the toxic effects of toxicants on fertility assessment in male rats. More so, assessing the level of toxic agents, Zhang and Lin, 2009, also evaluated the effects of toxicants on the reproductive system of male rats assessing the activities of SDH, LDH, GGT, G6PDH and beta-glucuronidase. However in this study there was an insignificant difference in the activities of SDH, LDH, G6PDH and GGT in the experimental rats when compared with the control.

5.5: Effects of quercetin on electrolytes and protein levels in male Wistar rats treated with alcohol and/or nicotine

Homeostasis is an important physiologic process in the body, in maintaining a constant milieu and alteration in serum electrolytes level or chemistry could result in certain diseases (Cheesbrough, 1991). From the findings of the present data, there was a difference in the concentration of serum electrolytes level (Na^+, Ca^{2+}, K^+, Cl^- and HCO_3^-), more pronounced in the sodium and potassium levels in the experimental groups compared to the control. Reports suggested that nicotine/alcohol could lead to electrolytes imbalance in animal models. The decreased in serum electrolytes level obtained may be as a result of metabolic imbalance/alteration, reduced absorption and elevated losses as reported (Macallan, 1999). Administration of alcohol and/or nicotine altered serum levels of electrolytes in the present data and these are consistent with the findings of Padmavathi *et al.,* (2009). However, there was a recovery in serum electrolytes level in the quercetin co-treated with alcohol and/or nicotine when compared to the control rats. Moreover, no significant difference in protein level of alcohol and/or nicotine treated rats observed compared with the control rats.

5.6: Effects of quercetin on sperm capacitation and acrosome reaction in male Wistar rats treated with alcohol and/or nicotine

The results obtained in this study showed that alcohol and/or nicotine treated rats showed uncapacitated and intact acrosome which is about 90% of sperm cells that did not undergo capacitation in respect to the control group which could be extrapolated to the absence of pups in alcohol and nicotine treated rats that were cohabited with the untreated female rats. Whereas, following quercetin intervention with alcohol and/or nicotine treated groups showed a significant increase in the values obtained in capacitated-acrosome reacted sperm cells and this suggests the fertility success rate obtained in these groups. The results obtained also indicated that for successful fertilization, capacitation and acrosome reaction must take place and these are vital and cannot be over-emphasized. Moreover, it was observed in the results of this study that alcohol and/or nicotine treated rats had higher percentage of uncapacitated and acrosome intact sperm cells which reflect the reproductive outcome of these groups after successfully cohabiting them with untreated female Wistar rats. It was also observed that fertility profile showed a significant reduction in libido in rats treated with alcohol, nicotine and alcohol plus nicotine

respectively. The three most distinctive phases of spermatogenesis include firstly, the spermatogonia which involve a sequence or series of mitotic division and differentiation into primary spermatocytes, secondly, the spermatocytes undergo meiotic division to give rise to spermatids with an haploid number of chromosomes and thirdly, spermiogenesis which requires the transformation of spermatids into mature spermatozoa. For a successful fertilization to take place, the spermatozoa have to undergo a capacitation (an important biochemical and physiological process) and acrosome reaction in the female reproductive tract (which is the breaking down of the acrosomal sperm outer membrane) and followed by the release of acrosomal enzymes or proteins which are pivotal in facilitating the passage of sperm via the granulosal cells and will be invested on the surface of the zona pellucida in the female egg (Bedfor, 1998). The reduced libido score is associated with decreased level of testosterone which was caused by chronic administration of alcohol and/or nicotine by invading the testicular blood barrier thereby affecting the synthetic function of Leydig cells in the production or secretion of testosterone of which is a major factor affecting sexual drive, moreover, high level of testosterone is associated with energy, stamina and vitality (Mooradian *et al.,* 1987). There was a significant decrease in the size of litters produced by the untreated female rats used in mating with the experimental rats treated with alcohol and/or nicotine respectively. This could be caused also by a significant reduction in sperm motility of alcohol and nicotine treatment in rats. Low sperm count, motility and high percentage of abnormal spermatozoa level have been associated with reduced or altered fertility (Agarwal *et al.,* 2016; Van heertum and Rossi, 2017).

5.7: Effects of quercetin on apoptotic, cell proliferative markers and DNA fragmentation in male Wistar rats treated with alcohol and/or nicotine

Two principal pathways have been indicated to be involved in the process of apoptotic pathway (Fadeel and Orrenius, 2005). The first one is at the mitochondria level which is the intrinsic pathway and the second is the extrinsic pathway. The intrinsic mitochondria pathway is enhanced via stress stimuli or oxidative stress which could have either a direct effect on Bcl-2 apoptotic protein involved in stabilizing or destabilizing the membrane of the mitochondria via the proapoptotic and antiapoptotic factors (Bax and Bcl-xL). Report revealed that expression in the rodent testis of proapoptotic and preapoptotic factors (Bax, Bad, Bcl-Xl and Bcl-2) are similar in the expression of the apoptotic factors/proteins as observed in the alcohol and/or

nicotine treated groups, however, the report of Cui and Placzek, (2018), is similar to the findings of the expression of Bcl-2 family members of apoptotic protein observed in the experimented rats of the present study (alcohol and/or nicotine administration).

P^{53} is a potent indicator of apoptosis and it has been shown that P^{53} gene is an inhibitor of cell cycle progression or inducing cell apoptosis in response to DNA damage found in high concentrations in the testes (Nijs *et al.,* 2011). The reduced expression of molecular factors (P^{53}) is associated with increased DNA damage. Study by Chang *et al.,* (2010), revealed that optimal expression of P^{53} is vital for the elimination of sperm by apoptotic mechanism. P^{53} also participate in DNA repair mechanisms, studies have reported that down regulation or under expression of P^{53} may lead to increased DNA damage which was observed in alcohol and/or nicotine treated groups as compared with the control which is in agreement with findings by Vaux *et al.*, 1998. Ki67 as reported by Mac Callum and Hall, 1991, is an important molecular nuclear protein and is expressed during cell cycle phases (G1, S, G2 and M). Nicotine and/or alcohol caused an increase in expression of P^{53} and Ki67 in the interstitial cells and at the seminiferous tubule of the testicular tissue of rats. However, quercetin supplementation with alcohol and/or nicotine modulated these findings. Reports on the expression of P^{53} showed that it is confined to the spermatocytes in the seminiferous tubules of the testis, thus the expression of P^{53} was more confined in the interstitial cells (Leydig cells) and less in seminiferous tubules of the testicular organs of the experimental rats and this is in contrary to the report of (Almon *et al.,* 1993; Sjoblom and Lahdetie, 1996; Stephan *et al.,* 1996).

Bcl-2 has been identified as the first member of the growing family of genes that regulate cell death in either a positive and negative manner. Epidermal growth factor receptor was reported to play a significant role in male reproductive functions by stimulating the meiotic phase of spermatogenesis. Thus, the expression of EGFR were more prominent revealing interstitial staining with tubular staining in alcohol and treated rats, staining at reduced germ cell layer in nicotine treated rats and at the fibrosed tubule in alcohol plus nicotine treated rats. Moreover, in order to limit the population of germ cell or to control the process of spermatogenesis, testicular germ cell apoptosis is of physiological importance, therefore, the regulation of testicular germ cell apoptosis is regulated by the P^{53}-fas-signaling and Bcl-2 pathway (Woolverdge and Morrs, 2000). Thus, there is a coincide report in which increased testicular cell apoptosis is in line with

the increase expression of apoptotic factors Bcl-2 and P^{53} as observed in the present findings. However, if apoptosis is in excess or if it is not adequate enough, it may alterthe process of spermatogenesis; in the alcohol and/nicotine treated rats it was observed that apoptosis was induced by these drugs as indicated by up-regulation of the apoptotic proteins in the present findings and this juxtaposed the fertility success rate that was compromised following administration of alcohol and/or nicotine, and this was inhibited with quercetin intervention as observed in the number of litters and litter sizes delivered by the untreated female rats in the present study.

Studies have also revealed that one of the major key factors in the process of apoptosis is oxidative stress reported that oxidative stress (Buttke and Sandstrom, 1994; Dallak *et al.,* 2008), the increased expression of apoptotic protein observed in the alcohol and/or nicotine treated rats may be linked to the generation of reactive oxygen species (ROS), thus this was observed in the results of the present finding. Cyclin D is a cell cycle protein and is implicated in the control and induction of mitosis as reported by Hunter and Pines, 1994). The reduced expression (under expression/down regulation) of cyclin D induced by alcohol and/or nicotine in the present studies indicated that the drugs could cause arrest of mitosis in spermatogenesis and this evident by the decreased spermatocytes and alteration in fertility profile as observed. Meanwhile, with quercetin intervention, there was a reversal in the results (cyclin D) obtained, thus leading to up-regulation of the cell cycle protein in respect to their control counterpart.

5.8 CONCLUSION

The results of this study show that alcohol and/or nicotine alter (s) the serum biochemical parameters and reproductive hormones in the male Wistar rats, which can be biochemically antagonized by quercetin. The mechanism of alcohol and/or nicotine's effects suggested to be via an increased oxidative stress on the reproductive organs with the impairment of the hypothalamic-pituitary function in the male Wistar rats. Also, alcohol and/or nicotine caused increased expression of apoptotic factor and DNA fragmentation respectively. However, the results obtained with quercetin are likely due to its antioxidant activities-scavenging of ROS, prevention of ROS formation and inhibition of lipid peroxidation and more so, quercetin also resulted in up-regulation of proliferative factors and down regulation of apoptotic proteins.

5.9 CONTRIBUTION TO KNOWLEDGE

The outcome of the data obtained in this study revealed thus:

(1) Alcohol and nicotine administration affect fertility by decreasing spermatozoa production in the testes and epididymal spermatozoa number (count)

(2) Alcohol and nicotine-induced reproductive dysfunction occur via the induction of oxidative stress observed in the testicular tissue

(3) Exposure to alcohol and nicotine-induced apoptosis is via up-regulation of apoptosis protein (P^{53}), down regulation of anti-apoptotic protein (Bcl-2)

(4) Alcohol and nicotine exert anti-androgenic effects at the testicular axis

(5) Quercetin, contributes in ameliorating the negative outcome of alcohol and nicotine on altered reproductive functions by enhancing the process of fertility

(6) Quercetin prevents alcohol and/or nicotine-induced endocrine disruption through up regulation of antioxidant status

(7) Quercetin inhibits the induction of testicular DNA and apoptosis via down-regulation of expression of apoptotic protein, up-regulation of proliferative and cell cycle markers

Summary

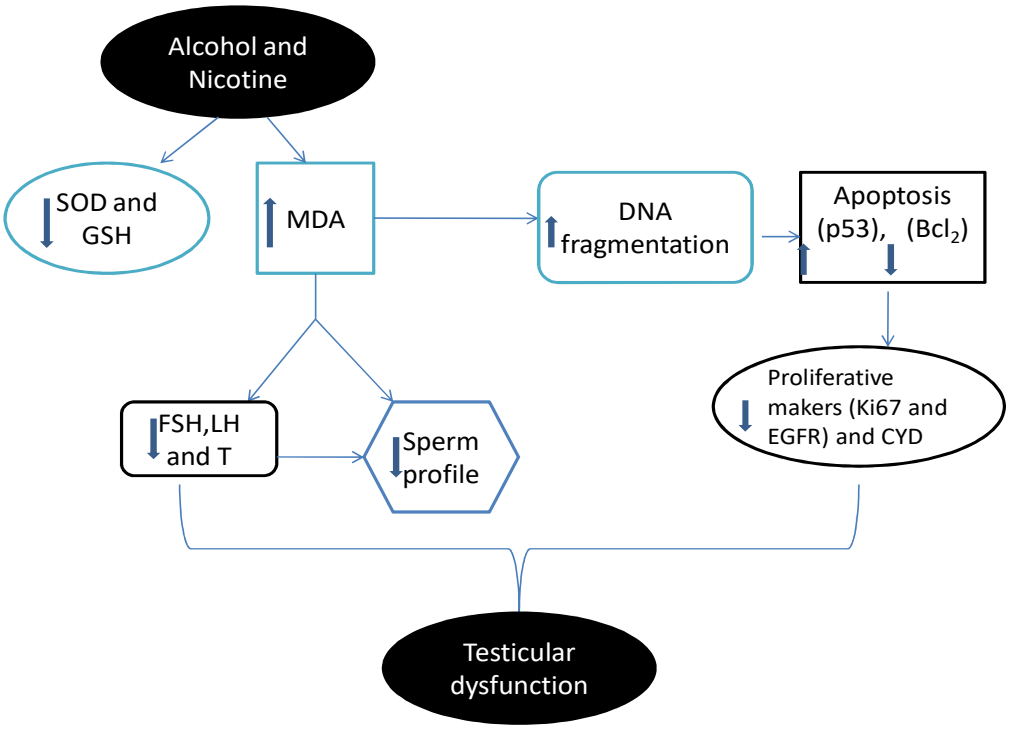


Figure 4.22: Schematic showing the effects of Alcohol and Nicotine-altered testicular functions

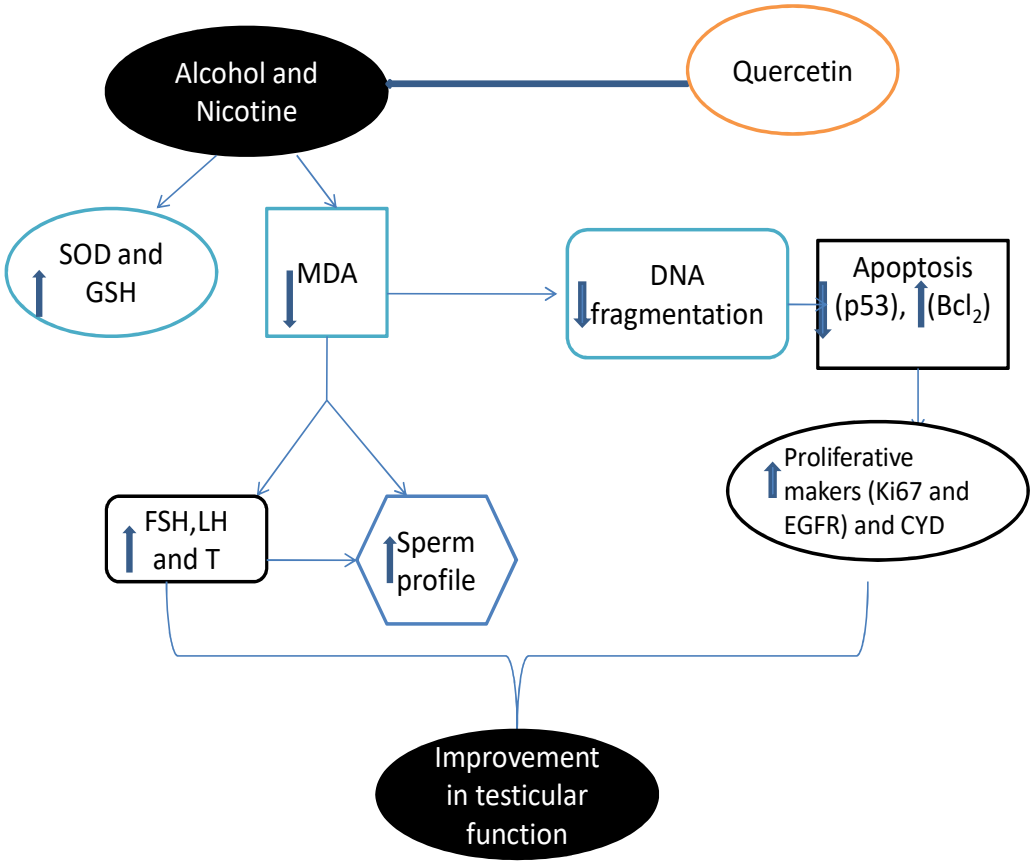


Figure 4.23: Schematic showing the preventive effects of Quercetin on Alcohol and Nicotine-altered testicular functions

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APPENDICES

ESTIMATION OF CALCIUM ION

Kit Contents: **BXC0293A**

R1 Buffer	2 x 80 ml
R2 MTB Reagent	2 x 80 ml
R4 Standard	1 x 5 ml

Test Principle:

Calcium ions react with Methylthymol Blue in an alkaline medium. The colour formed is directly proportional to the Calcium present in the sample.

Reagent Concentration:

R1 Buffer	Sodium Sulphite	76 mmol/l
	Ethanolamine	200 ml/l
	Detergent	2 g/l
R2 MTB Reagent	Methylthymol Blue 8-Hydroxyquinolone PVP	0.092 mmol/l 11 mmol/l 3 g/l
R4 Standard	Calcium	2.50 mmol/l (10 mg/dl)

Reagent Handling and Preparation:

Reagents are supplied ready to use. The reagents are stable until the expiry date stated.

Working Reagent:

A working reagent is prepared by mixing equal volumes of Buffer R1 and MTB Reagent R2. This mix will be stable for 1 day at 2-8 0C when stored in the dark and free of contamination.

Manual Procedure: Single Reagent Format

Wavelenght	Temperature	Cuvette	Measurement
612 nm (Hg 623 nm)	20-250C/ 370C	1 cm light path	Against reagent blank

Pipette into test tubes as follows:			
	Reagent Blank	**Standard**	**Sample**
Standard	---	10 µl	---
Sample	---	---	10 µl
Working Reagent	1000 µl	1000 µl	1000 µl
Read absorbance of the sample/Standard against the reagent blank after 5-50 minutes			

Calculation:

Calcium Concentration = $\frac{\text{Abs sample}}{\text{Abs standard}} \times \text{Std. conc}$

ESTIMATION OF CHLORIDE ION

Kit Contents: **BXC0282A**

R1 Reagent	2 x 60 ml
R4 Standard Low	1 x 5 ml
R4 Standard High	1 x 5 ml

Intended Use:

This test is used for quantitative determination of Chloride from Serum, Plasma or Urine.

Principle

Chloride ions in serum react with a Hg-TPTZ complex. This reaction liberates TPTZ which then reacts with a ferrous ion toform a blue Fe-TPTZ complex. Chloride concentration is proportional to the absorbance of the blue complex formed.

$Hg(TPTZ)2^{++} + 2Cl^- \rightarrow Hg(Cl)2 + 2TPTZ$

$2TPTZ + Fe^{++} \rightarrow Fe(TPTZ)2^{++}$ (blue)

Reagent Concentration

TPTZ Reagent	2,4,6-tri-(2-pyridyl)-1,3,5-triazine	1.8 mmol/l
	Ferrous sulphate	0.4 mmol/l
	Mercury nitrate	0.9 mmol/l
	Chloride	75 mmol/l
	Chloride	100 mmol/l

Reagent Handling and Preparation:

Chloride reagent and standards are ready to use. Stable up to the expiry date when stored at +15 to $+20^0$C.

Manual Procedure:

Wavelenght	Temperature	Cuvette	Measurement
610 nm (595-630nm)	$30/37^0$C	1 cm light path	Against Air or Distilled water

Pipette into test tubes as follows:		
	Sample	Standard
Standard	---	5 µl
Sample	5 µl	---
Reagent	1000 µl	1000 µl

Mix well, incubate for 5 minutes at 30 or 37^0C then measure the absorbance of the sample and standard against air. Absorbance measurements must be carried out at 30 or 37^0C due to the temperature sensitivity of the reaction product. Reagent and test are sensitive to light. Store reagent protected from light. Measure the absorbance of the sample and standards at 30/37^0C within 10 minutes.

Calibration:

1. Construct a calibration curve by plotting the absorbance values of the standards against the corresponding chloride concentrations. Do not extrapolate to zero.
2. The chloride concentration of the sample is determined from the calibration curve.

ESTIMATION OF SODIUM ION

Kit Contents: BXC0146A

R1: Colour Reagent	25x 20 ml
R4: Sodium Standard	2 x 5 ml

Intended use: The Fortress Sodium Monoliguid Kit is intended for quantitative determination of Sodium in serum.

Summary:

The reaction is based on the ability of sodium to react with specific chomogens to produce an increased absorbance with the increase in concentration of sodium ions in the sample material.

Reagent Concentration:

R1 Buffer	Tris	pH8.7
	Proclin 300	0.02%
	Chromogen	< 8 mg/l

Reagent Handling and Preparation:

R1- Colour Reagent:

Supplied ready to use. Once opened, stable for a period of 4 weeks when stored tightly copped at 28^0C.

Manual Procedure:

Wavelength	Temperature	Cuvette	Measurement
630 nm	37^0C	1 cm path	Against Reagent Blank

Pipette into test tubes as follows:		
	Blank	Standard/ Sample
Colour Reagent (R1)	1000 µl	1000 µl
	---	10 µl
Mix and incubate for 5 minutes at 37^0C. Read at 630nm against reagent blank.		

Calculation:

Sample sodium conc = (ΔA Standard) x Std Conc.

ESTIMATION OF POTASSIUM ION

Kit Contents: BXC0138A BXC0138B

R1 TPB Reagent R4 Standard	5 x 20 ml	10 x 20 ml

Test Principle:

Sodium tetraphenylboron reacts with potassium ions in an alkaline medium to produce a turbid suspension of potassium tetraphenylboron. Turbidity thus produced directly proportional to the concentration of Potassium ions in the serum tested.

Reagent Concentration:

R1 TPB Reagent	NaoH	0.6 mol/l
	Sodium Tetraphenylboron	250 mmol/l
R4 Standard	Lot Specific	

Reagent Handling and Preparation:

Reagents are supplied ready to use and will be stable to the expiry date quoted when stord at 250C without contamination of potassium and tightly capped. Once opened the reagents are stable for 28 days when stored at 25^0C.

Manual Procedure:

Wavelenght	Temperature	Cuvette	Measurement
Hg 578 nm	25 or 370C	1 cm light path	Against reagent blank

	Reagent Blank	Standard	Sample
R1 TPB Reagent	1.00 ml	1.00 ml	1.00 ml
R4 Standard	----	10 µl	----
Sample	----	----	10 µl
Mix, incubate for 3 minutes at 37^0C or 5 minutes at 25^0C. Mix again and read absorbance of standard and sample against the reagent blank.			

Calculation:

$$\text{Potassium Con} = \frac{\text{Abs Sample}}{\text{Abs Standard}} \times \text{Std Concentration}$$

ESTIMATION OF BIOCHEMICAL INDICES

ALKALINE PHOSPHATASE

Kit Contents: BXC0183A

R1	ALP Substrate	1 x 50 ml
R2	ALP Colour Reagent	2 x 125 ml
R4	ALP Standard	1 x 5 ml

Intended Use:

This kit is used for the direct colorimetric determination of alkaline phosphatase in serum.

Test Principle:

The alkaline Phosphatase acts on the AMP-buffered sodium thymolphthalein monophosphate. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen, which is measured at 590 nm.

Reagent Concentration:

R1: Substrate	Sodium Thymolphthalein Monophosphate	3.6 mM
	2-Amino-2-Methyl-l-Propapanol Buffer pH 10.2	0.2 M
	Magnesium Chloride	1.0 mM
R2: Colour Reagent	Sodium Hydroxide	0.09 M

	Sodium Carbonate	0.1 M
R4: Standard	50 U/L	

Reagent Handling and Preparation:

All reagents are ready-to-use and valid up until the expiration date on bottles if they are kept at 4^0C.

Manual Procedure:

Wavelenght	Temperature	Cuvette	Measurement
590 nm (580-630nm)	37^0C	1 cm	Against reagent blank
Pipette into test tubes as follows:			
	Reagent Blank	Standard/ Sample	
R1 Substrate	500 µl	500 µl	
Equilibrate at 37^0C for 3 minutes			
DDH20	50 µl	---	
Standard/ Sample	---	50 µl	
Incubate exactly 10 minutes at 37^0C			
	2500 µl	2500 µl	
Mix well, Read against the Reagent Blank, absorbance of Standard, Control and sample			

Calculation:

$\frac{\text{Abs of Unknown}}{\text{Abs of Standard}}$ x Value of Std. (IU/L) = Unk. (IU/L)

ESTIMATION OF LACTATE DEHYDROGENASE

UV (Pyruvate → Lactate)

Kit Contents: BXC0242A BXC0242B

R1 Buffer/Sustrate	5 x 20 ml	10 x 10 ml
R2 NADH	1 x 20 ml	2 x 10 ml

Intended Use:

This test is used for quantitative determination of Lactated Dehydrogenase from Serum.

Test Principle:

This test is for the quantitative determination of Lactate Dehydrogenase (LD) in serum and plasma. Lactate dehydrogenase catalyses the conversion of pyruvate to lactate; NADH is oxidized to NAD in the process. The rate of decrease in NADH is directly proportional to the LDH activity and is determined by measurement of the rate of absorbance change at 340 nm due to the reduction.

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LD}} \text{L-Lactate} + NAD^+$$

Reagent Concentration:

R1 Buffer/ Substrate	Tris Buffer pH7.5	50 mm0l/l
	Pyruvate	0.6 mmol/l
R2 NADH	NADH	0.18 mmol/l

Reagent Handling and Preparation:

All reagents are ready to use. Stable up to the expiry date when stored at +2 to 8^0C.

To prepare the Working reagent for Serum start procedures, mix 5 volumes of Buffer/ Substrate (R1) with 1 volume of NADH (R2). This working reagents is stable for 7 days at 2-8^{0}C or 1 day at 15-25^{0}C.

Manual Procedure: Serum Start

Wavelenghrt	Temperature	Cuvette	Measurement
340 nm (Hg 334 nm or Hg 365 nm)	25/30/37^{0}C	1 cm light path	Against Air

Pipette into cuvette	
Working reagent	1000 µl
Sample (Serum/ Plasma)	20 µl
Mix incubate at the assay temperature for 1 minute, read initial absorbance and start timer simultaneously. Read after exactly 1, 2 and 3 minutes.	

Calculation:

To calculate the LD activity using the following formulae:

25/ 30 / 37^{0}C

U/L = 8095 x ΔA 340 nm/min

U/L = 8253 x ΔA Hg 334 nm/min

U/L = 14998 x ΔA Hg 365nm/min

Manual Procedure: Substrate Start

Wavelenght	Temperature	Cuvette	Measurement
340 nm (Hg 334 nm or Hg 365 nm)	25/30/37^{0}C	1 cm light path	Against Air or Distilled water

Pipette into cuvette:	
	25/30/37^{0}C
R1 Buffer/Substrate	1000 µl
Sample (Serum/Plasma)	20 µl
Mix, incubate for 60 seconds at assay temperature and then add R2	
R2 NADH	200 µl
Mix, read initial absorbance and start timer simultaneously. Read after exactly 1, 2 and 3 minutes.	

GAMMA-GLUTAMYL TRANSFERASE

Kit Contents:	BXC0362A	BXC0362D
R1 Buffer/ Glycylglycine R2 Substrate	1 x 60 ml 1 x 12 ml	2 x 60 ml 2 x 12 ml

Intended Use:

Fortress γ-GT is intended for the quantitative determination of γ-glutamyl transferase in serum and plasma.

Test p\Principle:

The substrate L γ-glutamyl-3-carboxy-4-nitroanilide, in the presence of glycylcine is converted to 5-amino-2-nitrobenzoate by γ-GT measured at 450 nm. The increase in absorbance is proportional to γ-GT activity.

γ-GT

L γ-glutamyl-3-carboxy-4-nitroanilide + glycylglycine → L γ-glutamylglycylglycine + 5-amino-2-nitrobenzoate.

Reagent Concentration:

R1 Buffer/ Glycylglycine	Tris Buffer pH 8.25	100 mmol/l
R2 Substrate	L γ-glutamyl-3-carboxy-4-nitroanilide	2.9 mmol/l

Reagent Handling and Preparation:

All reagents are ready to use. Stable upto the expiry date when stored at 2-8^{0}C.

Preparation of Working Reagent:

Mix 5 volumes of R1 with 1 volume of R2. This working reagent is stable for 28 days at 2-8^{0}C or 7 days at 15-25^{0}C.

Manual Procedure:

Wavelenght	Temperature	Cuvette	Measurement
Hg 405 nm (400-420 nm)	25, 30 and 37^{0}C	1 cm light path	Against Air or Distilled water

Procedure: Sample start

Pipette into cuvette as follows:

	Blank	Sample
Sample	---	100µl
Working Reagent	1000 µl	1000 µl
Mix well and allow to stand for 1 minute at 25, 30 or 37^0C. Measure the absorbance of the sample per minutes for 3 minutes. (ΔAbs/min).		

Calculations:

To calculate the GGT activity, use the following formula:

U/L = 1158 x ΔA 405 nm/min

ESTIMATION OF ACID PHOSPHATASE

Kit Contents: BXC0401A BXC0401B BXC0401C

R1 Citrate Buffer	1 x 65 ml	1 x 105 ml	1 x 35 ml
R2 Substrate	6 x 10 ml	10 x 10 ml	10 x 3 ml
R3 Tartrate	3 x 10 ml	5 x 10 ml	5 x 3 ml
R4 Stabilizer	1 x 5 ml	1 x 5 ml	1 x 5 ml

Intended Use:

For the quantitative determination of Acid Phosphatase in Serum.

Test Principle:

1-naphthyl phosphate + H20 → phosphate + 1-naphthol

1-naphthol + Fast Red TR salt → azo dye

Fast Red TR salt = 4-chloro-2-methylphenyldiazonium salt.

Reagent Concentration:

R1 Buffer	Citrate Buffer	75 mmol/l
	Preservative	0.1 % w/v
R2 Substrate	1-naphthyl phosphate	10 mmol/l
	Fast Red TR salt	2.5 mmol/l
R3 Tartrate	Sodium Tartrate	135 mmol/l
R4 Stabiliser	Acetic Acid	3.0 mmol/l

Reagent Handling and Preparation:

1. Buffer: Ready to Use. Stable upto the expiry date when stored at 2-8^{0}C
2. Substrate:

(2a) For determination of Total Acid Phosphatase reconstitute 1 vial of substrate (R2) with 10 ml of buffer supplied.

(2b) For determination of non-prostatic acid phosphatase reconstitute 1 vial of substrate (R2) with 10 ml of the buffer supplied. Transfer the total contents to one vial of tartrate (R3) and mix. These will be stable for 5 days at 2-8^{0}C protected from light.

Sample: Serum

Acid Phosphatase must be stabilized by adding 1 drop of the stabilizer provided to 1 ml of serum samples. Measurement should be made within 24 hours if the sample is stored at 2-8^{0}C. If longer storage is required the sample should be frozen at -20^{0}C.

Stability:	3 days at 2-8^{0}C
	24 hours at + 15 to +25^{0}C

Manual Procedure:

Wavelenght	Temperature	Cuvette	Measurement
Hg 405 nm	30-37^{0}C	1 cm light path	Against Air or Distilled Water

Pipette into test tubes as follows (37^{0}C)		
	Macro	Micro
Sample	200 µl	100 µl
Reagent solution 2 (a) or 2 (b)	2000 µl	1000 µl
Mix thoroughly and incubate for 5 minutes at 30/37^{0}C then read the initial absorbance. Read absorbance again after 1, 2 and 3 minutes and calculate the change in absorbance per minute (ΔAbs) over the course of the reaction.		

Calculation:

Total Acid Phosphatase Activity (U/I) = ΔAbs of solution 2(a)/min x 743

Prostatic Acid Phosphatase Activity (U/I) = (ΔAbs of solution 2(a)/min- ΔAbs of solution 2(b)/min) x 743.

DETERMINATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PDH)

Kit Content: BXC0571A

R1 Buffer	2 x 50 ml
R2 NADP	1 x 2 ml
R3 Substrate	1 x 2 ml
R4 Digitonin	1 x 20 ml

Intended Use:

This kit is used for the determination of G-6-PDH in serum.

Test Principle:

Glucose-6-phosphate dehydrogenase (G6PDH) catalyses the oxidation of glucose-6-phosphate to 6-phophogluconate with a concurrent conversion of NADPH. The enzyme activity is determined by measurement of the rate of increase in NADPH concentration. The rate of increase in bsorbance at 340 nm is the measure of enzyme activity.

$$\text{G-6-P} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^{+}$$

Reagent Concentration:

R1 Buffer	Triethanolamine Buffer pH7.6	31.7 mmol/l
	EDTA	3.2 mmol/l
R2 NADP		0.34 mmol/l
R3 Substrate		0.58 mmol/l
R6 Digitonin		0.6 mmol/l

Reagent Handling and Preparation:

Buffer (R1)

Contents readyfor use. Stable up to the expiry date when stored at +2 to $+8^{0}$C.

NADP (R2)

Reconstitute the contents of R2 NADP with 2 ml of distilled water. Stable for 4 weeks at +2 to $+8^{0}$C.

Substrate (R3)

Reconstitute the contents of R3 substrate with 2 ml of distilled water. Stable for 4 weeks at +2 to $+8^{0}$C.

Digitonin (R6)

Contents ready for use. Stable up to the expiry date specified when stored at +2 to +8^{0}C.

Manual Procedure:

Wavelenght	**Temperature**	**Cuvette**	**Measurement**
340 nm (Hg 334 nm or Hg 365 nm)	37^{0}C.	1 cm light path	Against Air

Assay of G-6-PDH using Serum:

Pipette into test tubes as follows:				
	25^{0}C / 30^{0}C.		37^{0}C.	
	Macro	**Semi Micro**	**Macro**	**Semi Micro**
Buffer (R1)	2.00 ml	1.00 ml	2.00 ml	1.00 ml
NADP (R2)	0.10 ml	0.05 ml	0.10 ml	0.05 ml
Serum / Plasma	1.00 ml	0.50 ml	0.50 ml	0.25 ml
Mix, incubate for 10 minutes at 37^{0}C, then add:				
Substrate (R3)	0.05 ml	0.025 ml	0.05 ml	0.025 ml
Mix, read initial absorbance and start timer simultaneously, read again after 1, 2 and 3 minutes.				

Calculation:

To calculate the G-6-PDH activity use the following formulae:

37^{0}C

MU/ml= 841 x ΔA 340 nm/min

MU/ml= 858 x ΔA Hg 334 nm/min

MU/ml= 1514 x ΔA Hg 365 nm/min

25^{0}C/30^{0}C

MU/ml= 500 x ΔA 340 nm/min

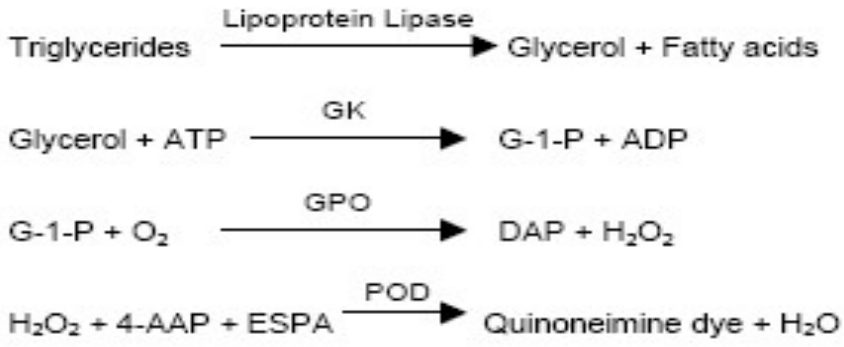
MU/ml= 510 x ΔA Hg 334 nm/min

MU/ml= 900 x ΔA Hg 365 nm/min.

EVALUATION OF TOTAL SERUM TRIGLYCERIDE (TG)

Principle and procedure:

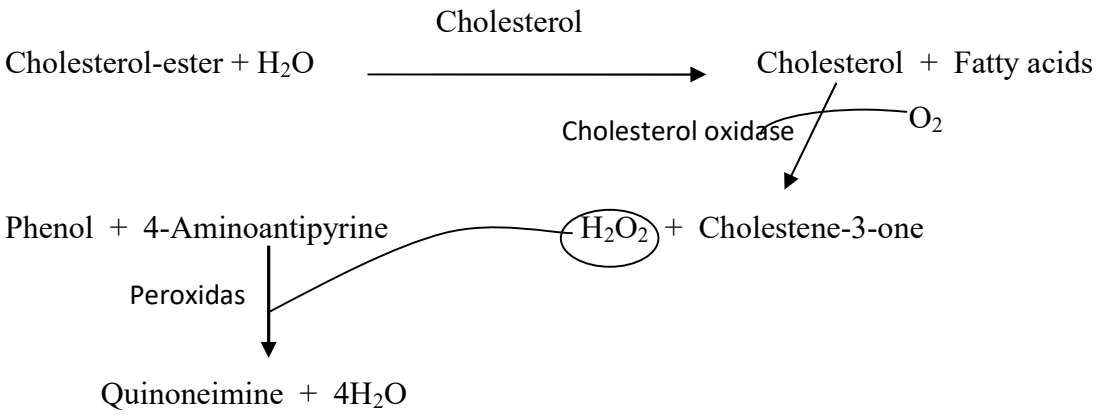
The assay (using Randox laboratories Ltd.) kit was based on enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids. The glycerol produced was then measured by coupled enzyme reactions. Triglycerides were first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. Glycerol was then phosphorylated by adenosine-5-triphosphate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine-5-diphosphate (ADP) in the reaction catalyzed by glycerol kinase (GK). G-1-P was then oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Peroxidase (POD) catalyzes the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to triglyceride concentration of the sample.



EVALUATION OF TOTAL SERUM CHOLESTEROL (TC)

Principle and procedure:

Method (using the Randox laboratories^{TM}) was based on the formation of quinoneimine from the reaction between hydrogen peroxide and 4-Aminoantipyrine in the presence of phenol. The entire reaction is presented as follows:



Absorbance is read at 546nm and the concentration of cholesterol is thereby, calculated.

Into test tubes marked for samples and reagent blank, 10ul of sample and 10ul of distilled H_2O were respectively added. Reagent solution (1ml) was added to all the tubes, then, incubated for 10min. at 25^{o}C. Absorbance was then measured at 546nm against the blank within 1 hour. Calculation of cholesterol in sample was done as follows:

Cholesterol (mg/dL) = Absorbance x 840

DETERMINATION OF HIGH DENSITY LIPOPROTEIN (HDL)

Principle and procedure:

Determination (using Randox laboratories^{TM} assay kit) is such that, very low density lipoprotein (VLDL) and low density (LDL) lipoproteins from serum or plasma are precipitated by phosphotungstate in the presence of magnesium ions. After removal by centrifugation the clear supernatant containing high density lipoproteins (HDL) is used for the determination of HDL cholesterol. Into a centrifuge tube, 100uL of a precipitating reagent made of 14mmol/L of phosphotungstic acid and 2mmol/L of magnesium chloride was added followed by 1ml of the sample (serum or plasma). This was mixed properly and allowed to stand for 10min at room temperature. Centrifugation was carried out at 1,789 xg for 2min). The supernatant of the liquid that separates out was collected for HDL test. Assay was done on test tubes marked as 'blank', 'standard' and 'sample' as required. Contents of each tube was mixed thoroughly and incubated for 5min at 37^{o}C. Absorbance (A) was then read of the samples, standard, and blank at 505nm. Calculation was carried out as follows:

$$\text{HDL-cholesterol (mg/dL)} = \frac{A_{sample}}{A_{standard}} \times \text{(Amount of Cholesterol Standard used)}$$

DETERMINATION OF LOW DENSITY LIPOPROTEIN (LDL)

Principle and procedure:

Low density lipoproteins in serum precipitates after addition of a citrate buffered solution on their isoelectronic point. After centrifugation, the very low density lipoprotein (VLDL) and the HDL remains in the supernatant and is determined with the Centronic cholesterol^{TM} reagent. From the difference of the concentration of the total cholesterol and the concentrations of the VLDL and HDL in the supernatant, the LDL-cholesterol concentration is calculated. Into a test tube, 1ml of precipitation reagent was added followed by 0.1ml of serum then, mixed properly and incubated at room temperature for 10min. The mixture is then centrifuged for 15min. at 1,800 xg. The supernatant (0.1ml) was taken and 1ml of cholesterol reagent was added. This was incubated for 10min. at 25^{o}C. Absorbance was finally read against blank at 500nm. The entire procedure was completed within 1hr.

DETERMINATION OF ASPARTATE AMINOTRANSFERASE (AST) ACTIVITY

Principle and procedure:

AST determination (using the Randox laboratories^{TM} assay kit) is based on description of Reitman and Frankel (1957). Aspartate aminotransferase (AST), also known as Serum Glutamate-oxaloacetate (SGOT) activities, catalyses the reaction:

α-Ketoglutarate + L-Aspartate $\longrightarrow$ L-Glutamate + Oxaloacetate

Oxaloacetate so formed is coupled with 2, 4-Dinitrophenyl hydrazine, (pH 7.4) to give the corresponding hydrazone, which gives brown colour in alkaline medium which can be measured colorimetrically. Buffered phosphate, aspartate and α-Ketoglutarate Substrate (0.25ml) was added to 0.05ml of serum, plus 0.05ml of distilled water, mixed properly and incubated at 37^{o}C for 30min. Solution of 2, 4-dinitrophenylhydrazine (0.25ml) was then added, mixed well and left to stand at room temperature for 20min . Then, (2.5ml) of NaOH was added, mixed and absorbance read against blank at 546nm. AST concentration was calculated as:

AST (U/I) = (Absorbance x 174) – 0.7333.

DETERMINATION OF ALANINE AMINOTRANSFERASE (ALT) ACTIVITY

Principle and procedure:

ALT determination (using the Randox laboratories^{TM} assay kit) is based on description of Reitman and Frankel (1957). ALT also known as SGPT (Serum Glutamate-pyruvate transaminase) activities, catalyses the reaction:

α-Ketoglutarate + L-Alanine $\longrightarrow$ L-Glutamate + Pyruvate

Pyruvate so formed is coupled with 2, 4-Dinitrophenyl hydrazine, (pH 7.4) to give the corresponding hydrazone, which gives brown colour in alkaline medium which can be measured colorimetrically.

Same procedure as described for AST above, applied to ALT activity determination, and was followed).

ESTIMATION OF PROSTATE SPECIFIC ANTIGEN

Summary and Explanation

Prostate Specific Antigen (PSA) is a single chain glycoprotein produced by epithelial cells of the prostate gland. PSA is useful in the management of patients with prostate cancer. The measurement of serum PSA has become the most accepted test to indicate men who are at risk of having prostate cancer and who should be examined by other tests. Using a cut-off of 4 ng/ml, 92% of men over 50 years of age with malignant prostatic tissues, 8% of healthy men and 28% of men with benign prostate hyperplasia (BPH) test positive for PSA. Three major forms of PSA exist in the serum: free PSA, bound PSA and complex PSA. Bound PSA is found in higher concentrations in patients with prostate cancer, whereas, free PSA is detected in higher concentrations in patients with BPH. If the free PSA to total PSA ratio is >25%, it is unlikely that the patient has prostate Cncer; whereas, if free PSA is <16% then prostate cancer is likely to be the cause. Serial measurement of PSA concentration in the serum is an important tool in monitoring patients with prostatic cancer and determining the potential and actual effectiveness of surgery or other therapies, or may allow for earlier discovery of residual or recurrent carcinoma after radical prostatectomy or radiotherapy. Current indications suggest that men over 50 years should be screened with digital rectal examination and PSA. Men with a high risk of

prostate cancer, such as a family history or of African heritage, should begin annual testing at age 40 years. If both are normal, the patient can be followed with annual evaluations and monitoring to determine the rate of change. Slight evaluations in PSA (4.1 ng/ml to 10.0 ng/ml) warrant a transrectal ultrasound (TRUS) to evaluate prostate volume and echogenicity of the gland. Hypo-echogenic lesions should be biopsied. Elevated PSA density (>0.15 ng/ml/cc), very high PSA (>10 ng/ml) or free-to-total PSA ratio of <16% warrants systemic biopsy.

Principle of the Test

The PSA ELISA kits is a solid phase assay based on streptavidin-biotin principle. The standards, samples and a reagent mixture of Anti-PSA-Enzyme and Biotin conjugates (conjugate reagent) are added into wells, coated with Streptavidin. PSA in the patient's serum forms a sandwich between two highly specific Anti-PSA antibodies, labeled with Biotin and HRP. Simultaneously, the biotinylated antibody is immobilized onto the well through a high affinity Streptavidin-Biotin interaction. Unbound protein and excess biotin/enzyme conjugated reagent are washed off, by washing buffer. Upon the addition of the substrate, the intensity of colour developed is directly proportional to the concentration of PSA in the samples. A standard curve is prepared relating colour intensity to the concentration of the PSA.

Materials Provided	96 Tests
1. Microwells coated with Streptavidin	12 x 8 x 1
2. PSA Standard: 6 vials (ready to use)	0.5 ml
3. Anti-PSA Conjugate Reagent: 1 bottle (ready to use)	12 ml
4. TMB Substrate: 1 bottle (ready to use)	12 ml
5. Stop Solution: 1 bottle (ready to use)	12 ml
6. 20X Wash concentrate: 1 bottle	25 ml

Assay Procedure

Prior to assay, allow reagents to stand at room temperature. Gently mix all reagents before use.

1. Place the desired number of coated strips into the holder
2. Pipette the desired number of coated strips into the holder
3. Add 100 µl of the Anti-PSA conjugate reagent into all wells. Shake the plate for (10-30) sec.
4. Cover the plate and incubate for 60 minutes at room temperature (18-26^{0}C).
5. Remove liquid from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on absorbance paper or paper towel.
6. Add 100 µl of TMB substrate into all wells.
7. Incubate for 15 minutes at room temperature.
8. Add 50 µl of stop solution into all wells. Shake the plate gently to mix the solution.
9. Read absorbance on ELISA Reader at 450 nm within 15 minutes after adding the stopping solution.

Calculation of Results

The standard curve is constructed as follows:

1. Check PSA standard value on each standard vial. This value might vary from lot to lot. Make sure you check the value on every kit.
2. To construct the standard curve, plot the absorbance for the PSA standards (vertical axis) against its concentration in ng/ml (horizontal axis) on a linear graph paper. Draw the best curve through the points.
3. Use the absorbance for controls and each unknown sample to examine the corresponding concentration of PSA from the standard curve.

Protocol for histology

The hypothalamus, pituitary, testis, prostate, seminal vesicle epididymis and the liver from each of the group were harvested and cleared of adherent tissue and immediately fixed in Bouin's fluid for six hours before transferred into 10% formalin, in order to preserve various constituents in their normal micro-anatomical positioning and prevent them from any degeneration or autolytic changes (The hypothalamus and the pituitary were fixed in formo-calcium). The following processes were then carried out sequentially.

(1) Dehydration: Water was removed from the tissue because it was mot miscible with paraffin (putting the tissue in ascending grades of alcohol did this). The tissues were put

in 50%, 70%, 90%, 95% and lastly absolute alcohol. Ascending concentration of alcohol was used to prevent sudden rush out of water from the tissues, so that the cells will not be distorted or damaged. The tissue was left for about 2 hours in each alcohol preparation.

(2) Clearing: Alcohol was removed from the tissue because it was not miscible with paraffin. The tissue was infiltrated with xylene, which replaced alcohol and was miscible with paraffin. Xylene also made the opaque tissue transparent, the tissue has passed twice into xylene and spent about two hours each time.

(3) Embedding: The tissue was placed in a molten wax at a constant temperature of 56-60^{0}C in an oven. The tissue was changed twice and spent about 2 hours each time

(4) Blocking Making: an L-shaped metal block was filled with molten paraffin wax, and tissues were placed in them immediately with forceps, the surface to be cut was facing downwards. After the paraffin has cooled off, it was then immersed in water, and was allowed to solidify; the L-metal blocks were removed.

(5) Sectioning: The section was cut extremely thin between 5µ and 15µ, using rotary microtone.

(6) Mounting of Paraffin Sections: One surface of a slide was made sticky by rubbing it with a drop of egg albumen. A section was put in the center of the slide, the section was immersed in water bath kept at temperature between 50-55^{0}C. The section became straightened and wrinkles disappeared. Water was drained off and the slide was put in an incubator at 37-40^{0}C overnight so that the section was completely fixed on the slide and became dry.

(7) Staining: The section was deparaffinized in xylene for 1 minute so that the stains can permeate. The slide was then immersed in a descending concentration of alcohol 95%, 70%, 50% for about 30 seconds each to dehydrate it, because the stains were aqueous solution of haematoxylin for about 10 minutes till the colour of the section became blue (it was checked under microscope, the nuclei were stained blue). The slide was dipped in 1% aqueous eosin for 30 seconds. The slide was rinsed in water for few seconds, so that eosin stain was washed off. The slide was immersed in 70% alcohol and 90% alcohol for 30 seconds each to dehydrate the preparation. The preparation was cleared off alcohol by dipping it in xylene for 1 minute. After then, the slide was blotted and mounted in Canada balsam under a cover slide and air bubbles were prevented from getting in. The slide was

read under the microscope and lesions were noted. A photomicrograph of the slide was then taken.

Reagents for DNA Extraction

10X TBE BUFFER

10X TBE BUFFER

3 Liters

324 g Tris base

165g Boric acid

120mls 0.5M EDTA (pH 8.0)

autoclave for 20 min

(8) 2 Liters

216g Tris base

110g Boric acid

80mls 0.5M EDTA (pH 8.0)

autoclave for 20 min

(9) 1 Liters

108g Tris base

55g Boric acid

40mls 0.5M EDTA (pH 8.0)

autoclave for 20 min

(10) TBE Buffer Recipe (Tris-Borate-EDTA)

(11) 10X TBE Buffer Preparation:

(12) Mix following components to make 1 liter 10X TBE.

Component	Amount	10X Stock Concentration	Final 1X Concentration
Tris Base	108 g	890 mM	89 mM
Boric Acid	55 g	890 mM	89 mM
EDTA (pH 8.0)	40 ml	20 mM	2 mM

5X TBE Buffer Preparation:

(13) Mix following components to make 1 liter 5X TBE.

Agarose gel electrophoresis

Materials

(14) Agarose (SeaKem), Biozym Diagnostik. Germany

(15) TBE x10 (Prepared as indicated above)

(16) Gel cast plus comb

(17) Ethidium bromide

(18) Loading buffer

Procedure

(19) Prepare 0.9% agarose gel by dissolving 0.9g of agarose powder in 100 mL of x1 TBE (prepared by 1:10 dilution of x10 TBE stock with distilled water). Boil and allow the gel to cool to 50^0C before adding 50 uL of 1 mg/mL ethidium bromide. After this, pour the ethidium bromide-stained gel into the gel casting tray with comb inserted for well creation.

(20) Load DNA sample (8 uL of DNA sample + 2 uL Loading buffer) into each well of the gel submerged in x1 TBE (pH 8.3) buffer in the electrophoretic tank.

(21) Close the circuit and run at 10V/cm for 45 min.

(22) Visualize DNA bands under UV light using a UV transilluminator. Take photograph of DNA bands using a digital camera.

(23) Extrapolate the size of DNA based on mobilities and sizes of the DNA markers co-electrophoresed with the sample.