METHANOL EXTRACT OF *Parquetina nigrescens* (AFZEL.) BULLOCK LEAF AND SQUALENE AMELIORATE ARSENIC TRIOXIDE-INDUCED REPRODUCTIVE TOXICITY IN MALE WISTAR RATS

BY

Oore-oluwapo Iretioluwa ADEDOTUN
MATRIC NO.: 154395
B.Sc. Physiology (Ekpoma), M.Sc. Physiology (Ibadan)

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ABSTRACT

In Nigeria, the concentration of arsenic in ground water, which is the major source of drinking water, is high. Arsenic causes male reproductive dysfunction by inducing oxidative stress. *Parquetina nigrescens* leaf, reported to have antioxidant property, is used in African traditional medicine to improve sexual performance, which is strongly related to gonadal functions. This study was designed to investigate the effects of Methanol Extract of *Parquetina nigrescens* Leaf (MEPL) on gonadal functions in arsenic trioxide-treated (As_2O_3) male Wistar rats.

Parquetina nigrescens plant was collected within the premises of the University of Ibadan and authenticated at Forestry Research Institute of Nigeria (FHI: 109785). The MEPL was obtained by soxhlet extraction and concentrated. An aliquot of MEPL was subjected to gas chromatography mass spectrometry for identification of its constituents. Analytical grade squalene was used for the study. Fifty male Wistar rats (150-180 g), divided into ten groups (n=5), were treated orally for 54 days as follows: group 1 (distilled water, 3 mL/kg), group 2 (3 mg/kg As_2O_3), group 3 (250 mg/kg MEPL), group 4 (500 mg/kg MEPL), group 5 (1000 mg/kg MEPL), group 6 (100 mg/kg squalene), group 7 (As_2O_3+250 mg/kg MEPL), group 8 (As_2O_3+500 mg/kg MEPL), group 9 (As_2O_3+1000 mg/kg MEPL) and group 10 (As_2O_3+100 mg/kg squalene). The animals were anaesthetised using sodium thiopental, prior to collection of blood, testes and epididymes. Epididymal sperm analysis was done using computer-aided sperm analyser; testicular malondialdehyde, glutathione peroxidase, superoxide dismutase, catalase and 17-beta hydroxysteroid dehydrogenase (17β-HSD) were assayed spectrophotometrically; serum testosterone and 8-oxo-2-deoxyguanosine (8-OHdG) were assayed using ELISA. Testicular Bax and Bcl-2 expression were assessed by immunohistochemistry. Testicular and epididymal tissues were examined microscopically. Data were analysed using ANOVA at $\alpha_{0.05}$.

Twenty-four compounds including squalene (4.0%) were identified in MEPL. Sperm motility increased in As_2O_3+250 mg/kg MEPL, As_2O_3+500 mg/kg MEPL and As_2O_3+squalene treated groups when compared with As_2O_3 (91.2±3.3, 86.3±2.8, 88.1±2.1 against 70.9±4.5%). Testicular malondialdehyde (2.1±0.6; 1.6±9.5; 0.5±0.3 vs. 6.5±1.0 $x10^{-3}$nM/mg tissue) and 8–OHdG (2.1±0.1, 1.1±0.2, 2.1±0.1 against 4.6±0.7 ng/mL) decreased, while activities of glutathione peroxidase (1.4±0.0, 1.3±0.0, 1.4±0.0 vs.1.2±0.0 $x10^{-3}$U/mg protein) and 17β-HSD (268.5±39.0, 268.4±40.1, 261.0±18.4 against 123.9±16.1%) increased in As_2O_3+250 mg/kg MEPL, As_2O_3+500 mg/kg MEPL and As_2O_3+squalene compared with As_2O_3. Superoxide dismutase activity decreased significantly in As_2O_3+500 mg/kg MEPL but increased in As_2O_3+squalene when compared with As_2O_3 (50.4±1.1, 71.1±8.60 against 50.7±9.0 U/mg) while catalase level increased significantly in As_2O_3+500 mg/kg MEPL compared with As_2O_3 (9.6±1.3, 7.1±0.2 μ/mg tissue). Testosterone level increased in As_2O_3+250 mg/kg MEPL and As_2O_3+500 mg/kg MEPL compared with As_2O_3 (1.0±0.1, 1.0±0.1 vs. 0.5±0.1nmol/L). Expression of Bcl-2 increased, while Bax expression reduced in As_2O_3+250 mg/kg MEPL, As_2O_3+500 mg/kg MEPL and As_2O_3+squalene compared with As_2O_3. Atrophy of seminiferous tubules, depletion of germ cell layers and absence of spermatozoa were observed in As_2O_3 group, but not in groups co-treated with MEPL or squalene.

Methanol Extract of *Parquetina nigrescens* leaf and squalene ameliorated arsenic trioxide-induced gonadal toxicity *via* the prevention of cell death and oxidative stress in male rats.

Keywords: *Parquetina nigrescens*, Squalene, Testicular toxicity, Arsenic trioxide, Apoptosis

Word count: 487

DEDICATION

This work is dedicated to my God Who has made the seemingly impossible things to become possible and to my parents, Chief B. M. and Mrs. S. O. A. Adedotun who both took my Ph.D. programme as if they were the ones who will be awarded the degree.

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In Yoruba language, there is a proverb that goes thus; “Egungunnla ni o ma n gbeyin igbale”. This literarily means that the biggest mascurade comes out last from the ritual forest. In this case, my parents, Chief B. M. and Mrs. S. O. A. Adedotun are my biggest mascurades, my idols and my “super” support that carried my burden on their heads for more than thirty years of my life. I say a big thank you to both of you. You will enjoy your rewards for the good works you have done in my life and that of my siblings in Jesus name.

May God bless you all and raise help for you all at every point of your life when you require help. May you all eat the good fruits of your labour and enjoy the peace of God in all aspects of life.

CERTIFICATION

I certify that this work tittled "**Methanol extract of *Parquetina nigrescens* (Afzel.) Bullock leaf and squalene ameliorate arsenic trioxide-induced reproductive toxicity in male Wistar rats"** was carried out by Oore-oluwapo Iretioluwa ADEDOTUN in the Department of Physiology, College of Medicine, University of Ibadan.

…….

Supervisor

Professor Y. Raji,

B.Sc., M.Sc., Ph.D. (Ibadan); F. Med. Ed. (Philad., U.S.A.)

Department of Physiology,

Faculty of Basic Medical Sciences,

College of Medicine,

University of Ibadan, Nigeria.

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ABRRIVIATIONS

17β-HSD	17β-Hydroxysteroid Dehydrogenase
8-OHdG	8 –hydroxy -2’ –deoxyguanosine
AAE	Ascorbic Acid Equivalent
$ABTS^{+}$	Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AdoMet	S-adenyl Methisonine
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
APL	Acute Promyolocytic Leukemia
As	Arsenic
As_2O_3	Arsenic trioxide
As(V)	Arsenic acid
AST	Aspartate Aminotransferase
ATSDR	Agency for Toxic Substances and Disease Registry
ATP	Adenosine-5’-Triphosphate
BAX	B-cell lymphoma Associated X
Bcl-2	B-cell Lymphoma 2
BTB	Blood Testes Barrier
cGMP	cyclic Guanosine Monophosphate
CoA	Acetyl Coenzyme A
DMA	Dimethylarsenous
DMA(III)	Dimethylarsinous Acid
$DMAs^{V}$	Dimethylarsinic Acid
DMPS	Dimercapapto-1-Propanesulfonate
DMPS	2,3-dimercapapto-1-propanesulfonate
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl hydrate
EDTA	Ethylenediaminetetraacetic Acid
EFSA	European Food Safety Authority
FIC	Ferrous Ion-Chelating
FRIN	Forestry Research Institute of Nigeria
FSH	Follicle Stimulating Hormone

GC-MS	Gas Chromatography-Mass Spectrometry
GnRH	Gonadotropin Releasing Hormone
GPR54	G Protein-coupled receptor 54
GPx	Glutathione Peroxidase
GSSG	Oxidized Glutathione
Hb	Haemoglobin
HMG CoA	3-hydroxy-3-methylglutaryl Coenzyme A
HMGR	3-hydroxy-3-methylglutaryl Coenzyme A Reductase
IARC	International Agency for Research on Cancer
IC_{50}	50 % inhibition
INSL3	Insulin-Like Factor 3
IPP	Delta 3-isopentenyl diphosphate
JNK	Jun N-terminsl Kinase
LD_{50}	Median Lethal Dose
LH	Luteinizing Hormone
MDA	Malondialdehyde
MEPL	Methanol Extract of *Parquetina nigrescens* Leaf
MMA	Monomethyl Arsenic Acid
MMA (III)	Monomethylarsonous Acid
$MSMAs^{V}$	Monosodium Methanearsonate
NER	Nucleotide Excision Repair
NF-κB	Transcription Factor Nuclear Factor κB
NIST	Nigerian Institute of Standards and Technology
NPY	Neuropeptide Y
NRC	National Research Council
p53	Tumor Suppressor Protein
PCV	Packed Cell Volume
Ppb	Part per billion
Ppm	Part per million
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
SAM	S-Adenosyl Methiomine

SOD	Superoxide Dismutase
SQ	Squalene
STAR	Steroid Regulatory Protein
TAC	Total Antioxidant Capacity
TBARS	Thiobarbituric Acid Reactive Substance
US EPA	United State Enviromnental Protection Agency
UV	Ultraviolet
WBC	White Blood Cell
WHO	World Health Organization

CHAPTER 1

1.0 INTRODUCTION

1.1 Background of the study

Infertility is a global world problem (Ojiyi *et al*., 2012). In some African cultures, it is believed that men cannot be infertile (Ojiyi *et al*., 2012). However, the true picture is that African fertility clinics are usually flooded by as many men as women (Abarikwu, 2013). Epidemiology research indicates that males are to be blamed for forty to fifty percent of infertility instances in Nigeria, although, reasons vary from one locality to another (Uadia and Emokpae, 2015). In a fertility clinic in south eastern part of Nigeria where some couples presented for infertility, about 44% of the male patners were infertile as indicated by their sperm analysis (Ugboma *et al*., 2012). In North America, Europe and Australia, infertility rate is about 15% while male infertility accounts for 6-9% of the total infertility (Agarwal *et al*., 2015). Infertility in males may arise from; genetic problems, systemic disorders, risky life style such as smoking (Friedman and Dull, 2012), male accessory gland infection (Friedman and Dull, 2012) and exposure to environmental toxicants (Sharma, 2017). Prominent among these environmental toxicants is arsenic.

Arsenic exhibits the qualities of metals and non-metals, it is therefore called metalloid. It is a major pollutant of drinking water (Mazumder *et al*., 1988). Sources of environmental arsenic pollution are smelting of nonferrous metals, mining and burning (Mazumder *et al*., 1988). It is used for production of colourless glass, preservation of wood and manufacture of electronics (NRC, 1999). In Africa and Europe, the regular medium of having contact with arsenic is through ground water containing about 0.02 –1760 $\mu g.L^{-1}$ and surface water containing about ten thousand microgram per litre (Ahoule *et al*., 2015). In Asian countries like Bangladesh, water concentration is as high as 2,500 -3,200 $\mu g.L^{-1}$ (Katsoyianis *et al*., 2013). Contact with arsenic above 0.001 ppm or 10 $\mu g/m^3$ for 8 hours (ATSDR, 2007) is linked with high risk of male reproductive organ damage which includes reduction in

number of spermatogonia, destruction of seminiferous tubules, atrophy of interstitial cells and Sertoli cells which eventually lead to reduction in testicular weight, decreased testosterone concentration, decreased sperm count and motility, increased oxidative stress markers and genotoxicity (Ali *et al*., 2013). Arsenic acts through; induction of oxidative stress (OS), inhibition of testicular androgenic enzymes (3β-HSD and 17β-HSD), inhibition of enzymes necessary for sperm motility such as cystine-rich secretory protein, induction of apoptosis, (Jimi *et al*., 2004) and oxidation of sperm DNA (Yamanaka *et al*., 2001).

Arsenic displays significant impact in male infertility. In a study conducted in an infertility clinic in China, a mutual relationship was found between the presence of arsenic metabolite, dimethylarsenous (DMA) in urine of participants and low sperm concentration as well as between arsenic content in semen and ratio of living sperm and the wholeness of acrosome (Xu *et al*., 2012). In Taiwan, it was discovered that men who resided in communities where contact with arsenic was high (> 50 ppb) showed a low free testosterone concentration than those who resided in locations where contact with arsenic was low (< 50 ppb) (Hsieh *et al*., 2008).

Oxidative stress (OS) is identified as a major method associated with sperm cytotoxic effects of arsenic (Singh *et al*., 2010). Mechanisms associated with arsenic action such as mutation induction are all directly or indirectly driven by OS (Ercal *et al*., 2001). Another common component of arsenic mechanism that has been reported is inflammation (Singh *et al*., 2010). Evidences suggest that arsenic, in addition to causing OS brings about increased production of inflammatory mediators leading to organ damage (Singh *et al*., 2010). It is therefore imperative that efforts to cure or prevent arsenic-induced damage to the reproductive system should first consider how OS could be removed, alleviated or prevented.

One of the drugs used in the treatment of arsenic-induced male reproductive damage is 2,3-dimercapapto-1-propanesulfonate (DMPS), a chelator which chelates arsenic and makes it available for excretion in urine (Flora *et al*., 2007). However, its side effect is that other metals in the body like Sodium, Calcium, Zinc, Cobalt, Magnesium and Molybdenum needed in trace amount are usually also excreted along with arsenic thereby leaving the body deficient of these metals. Antioxidants are also used for treating arsenic-

induced OS (Pace *et al*., 2017). The body is enriched with antioxidant enzymes produced within itself such as glutathione peroxidase and catalase both of which participate in methylation of arsenic and makes it available for urination. However, the higher the exposure to arsenic, the higher the rate of depletion of these antioxidant enzymes (Wu *et al*., 2001), making the individual become more vulnerable to the oxidative stress.

The means through which people provide solution to the problems of infertility is partialy influenced with worth and culture of the geographical location they live in (Inhorn, 2003). *Parquetina nigrescens* is normally used in Africa for curing male infertility (Iwu, 1993) and it may be a common herb with capacity for management of arsenic-induced reproductive toxicity. *Parquetina nigrescens* (Afzel.) Bullock (Periplocaceae) is a climber plant which grows in secondary forests of West Africa, it grows well in tropical weather. It is commonly called African parquetina (Erinoso and Aworinde, 2012), while its local names in Nigeria are Kwànkwánín tsa tsumbe in Hausa and Ogbo in Yoruba (Awobajo and Olatunji-Bello, 2010). The plant is employed in West and East Africa in treatment of series of ailments ranging from insanity, diarrhoea, gonorrhea and worm infections among others (Iwu, 1993). The decoction of *Parquetina nigrescens* root is used for the treatment of stomach pain as well as snake bites (Alvarez, 2012). Attie people in Ivory Coast bathe with extract of the leaf to combat general body pain (Datte *et al*., 1996). The concoction of *Parquetina nigrescens* prepared with water is orally administered to people by traditional medicinal practitioners in Ilorin, Nigeria for curing fever, headache and pain (Alvarez, 2012). It has been implied that methanol concoction of *Parquetina nigrescens* has antioxidant effect (Aderibigbe *et al*., 2011; Ayoola *et al*., 2011). The leaf concoction is usually taken in the evening as aphrodisiac in East Africa (Odetola *et al*., 2006; Owoyele *et al*., 2009). It is also used to enhance parturition (Datte *et al*., 1996). Some other reports on *Parquetina nigrescens* include its cardiotonic and sympathomimetic effects (Datte and Ziegler, 2001), uterotonic effects (Datte *et al*., 1996), haematological effects, antidiabetic and haematinic impacts (Saba *et al*., 2010), hypoglycaemic effect (Awobajo and Bello, 2010) as well as antimicrobial and gastroprotective properties (Odetola *et al*., 2006).

Squalene (2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all E)-) is a triterpene found among the important lipids available in *Parquetina nigrescens*. It was first isolated from the liver oil of Shark (*Squalus spp.)*, hence its name squalene (Tsujimoto,

1906). It is endogenously produced in all plants and animals and plays an essential role in cholesterol production, a precursor of steroid hormones, carotenes as well as oil soluble vitamins (Spanova and Daum, 2011). It is also known to stimulate metabolism by participating in oxidative processes in almost all tissues (Spanova and Daum, 2011). Scientifically, squalene is known to protect against the harzadous impact of contact with gamma radiation (Ibrahim *et al*., 2012), myocardial infarction and photoaging in women (Cho *et al*., 2010) by eliciting antioxidant effects (Farvin *et al*., 2004). Currently, there are ongoing researches on possible effects of squalene on reproductive organ functions in experimental animals (Ibrahim *et al*., 2012).

1.2 Statement of the problem

In Nigeria, men contributes about 40 – 50% to the prevalence of infertility (Uadia and Emokpae, 2015; Omokanye *et al*., 2016). In Western countries, male infertility accounts for 6 - 9% of the total infertility which is 15% (Agarwal *et al*., 2015).

World Health organization and Agency for Toxic Substances and Disease Registry (ATSDR) gave the limit for arsenic in drinking water to be 0.01 mg/L (Garba, 2012) and 0.050 part per million (ATSDR, 2007) respectively. However, in Ugbe Akoko area of Ondo state; arsenic concentration was found to be between 0.500 -3.700 ppm in 13 wells out of 20 wells (Afolabi *et al*., 2011). In Ibadan, concentration in water during raining season is usually as high as 1.03-3.06 mg/L (Izah and Srivastav, 2015).

1.3 Justification of the study

It has been established that arsenic acts by the production of free radical, blocking enzymes responsible for male reproductive hormone formation, inhibiting steroidogenesis (Alli *et al*., 2013), causing mitochondrially induced apoptosis (Jimi *et al*., 2004) and oxidating spermatozoal deoxyribonucleic acid, usually indicated by production of 8- oxo-2'-deoxyguanosine (Yamanaka *et al*., 2001). Since the primary mechanism of arsenic action is by OS induction, it is imperative to investigate the possible effect of exogenous antioxidants from plant on arsenic-induced male reproductive toxicity.

It has been suggested that the leaf of *Parquetina nigrescens* brings about cellular antioxidant effect (Aderibigbe *et al*., 2011; Ayoola *et al*., 2011). Moreover, squalene which is of interest in the methanol extract of *Parquetina nigrescens* leaf has been reported to

stimulate metabolism by participating in oxidation processes in almost all tissues (Ibrahim *et al*., 2012) thereby preventing OS. Farvin *et al*. (2004) also suggested that Squalene possesses antioxidant properties. The suggested antioxidant effects of *Parquetina nigrescens* and squalene are yet to be established in arsenic trioxide-induced male reproductive toxicity. Thus, this work was therefore designed to determine the influence of methanol extract of *Parquetina nigrescens* leaf and squalene on arsenic-induced reproductive toxicity in male Wistar rats.

1.4 Aim of the study

The aim of this work was to investigate the effects of MEPL as well as its constituent, squalene on the male reproductive system in arsenic trioxide-induced reproductive toxicity in male Wistar rats.

1.5 Specific objectives

- To determine the acute toxicity of MEPL in Wistar rats.
- To identify the compounds present in MEPL and to determine the antioxidant ability of MEPL and squalene.
- To determine the reproductive effects of MEPL on arsenic-induced reproductive toxicity in male Wistar rats.
- To compare the reproductive effects of MEPL with squalene and to determine their mechanisms of action in arsenic-induced male reproductive toxicity.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 The male reproductive system

The male reproductive system is composed of a pair of testes which is the primary male sex organ and other accessory organs like rete-testes, epididymis, vas deferens, ampulla, prostate gland, seminal vesicles, urethra, scrotum and the penis.

The testes

The functions of the testes are spermatogenesis and steroidogenesis. These occur in morphologically and functionally two different compartments. These are the tubular compartments and the interstitial compartments. The functions of the testes are regulated by the pituitary gland, hypothalamus and in turn controlled by local autocrine and paracrine hormones of the testes (Nieschlag *et al*., 2010).

Tubular compartment

The testis is made up of two-hundred and fifty to three-hundred lobules separated by septa of connective tissue; each one holds between one to three extremely long-winded seminiferous tubules, each having a length between 30-80 cm and the entire tubules total about 600. When considering an average length of about 60 cm for each seminiferous tubule, the addition of all the length per testis is about 360 m and in both testes, it equals about 720 m, the length of a football field. The tubular compartment constitutes about 60-80 % of the testicular volume and it is the site for spermatogenesis. Present in this compartment are the spermatogonia and some other cells (Nieschlag *et al*., 2010).

Interstitial compartment

This represent 12-15% of the testicular volume in human, it consist of the Leydig which was talked about by Franz Leydig in 1850, blood and lymphatic vessels, etc. The Leydig

cells makes up about 10-20 % of the interstitial compartment and it is the most important in this compartment because, it secretes androgen as well as insulin-like factor 3 (INSL3).

Sertoli cells

Sertoli cells were talked about in1865 by Enrico Sertoli; they lie in the germinal epithelium right on the basal membrane and goes deep down into the lumen of the seminiferous tubules. There are about 25 $x10^6$ Sertoli cells/g of testis (Raleigh *et al*., 2004). The cytoplasmic content of each cell which is the same as that of a typical cell suits the fuctions that they perform.

Sertoli cells provide support for the germinal epithelium all through the period of morphological and physiological differentiation as well as coordinate spermatogenic processes topographically and functionally, each cell communicating with a given number of spermatogonia and sperm. Also, they secrete proteins, growth factors, prostaglandins and cytokines. One spermatogenic cycle lasts about sixteen days in man (Amann, 2008). Sertoli cells join with one another to build tight junctions that eventually form the blood testes barrier (BTB) which serves to separate blood from seminiferous tubules. The BTB also prevents large and middle sized molecules from gaining access into the seminiferous tubules while permitting entry of nutritive substances and hormones that are necessary for spermatogenesis. The integrity of the BTB can however be compromised by viral infection or trauma, this brings about the entry of sperms into the blood and thus activating the immune system against them. The result of this is usually the production of antibodies against them which destroy the germ cells and cause sterility (Franca *et al*., 1998).

Germinal cells

Spermatogenesis refers to the various processes that bring about matured spermatozoa formation from stem cells (Amann, 2008). The stem cells produce the germinal cells organized in four spermatogenic stages namely:

Spermatogoniogenesis

Meiotic division of spermatocytes resulting in haploid germ cells (spermatids)

Spermiogenesis

Spermiation

There are two classes of spermatogonia namely;

Type A spermatogonia
Type B spermatogonia

Type A is differentiated both functionally as well as physiologically into two types namely Ad (dark) spermatogonia which do not proliferate but represent the testicular stem cells that can undergo mitosis division and Ap (pale) spermatogonia which divides to renew themselves and give rise to two B spermatogonia (Ehmcke and Schlatt, 2006).

The type B spermatogonia gives rise to preleptone spermatocytes which then commence DNA synthesis at the beginning of meiotic division (Alastalo *et al*., 1998). Secondary spermatocytes containing half the number of chromosomal set in two separate arrangements arises from the first meiotic divison. Spermatids emanates from second meiotic division, and undergo series of structural changes which leads to emergence of differentiated elongated spermatids and spermatozoa. Spermiogenesis have 4 different stages namely golgi, cap, acrosomal and maturation stages (Amann, 2008).

Rete testis

At the apex of each seminiferous tubule, the twisted tiny tubes join with a straight small tube having small width which passes into mediastinum and join together to give a conjuction of thin walled channels called rete testis.

Spermatozoa maturation in the epididymis

After spermatozoa production in the seminiferous tubules, it takes some days to pass through the epididymis. Spermatozoa develop the capability for motility after spending about 24 hours within the epididymis, although several inhibitory proteins prevent final motility until after ejaculation (Ehmcke *et al*., 2005).

Vas deferens

At the caudal pole of testis, epididymis turns sharply upon itself and passes as vas deferens without any definite demarcation.

Function of the seminal vesicles

Each one lined with secretory epithelium is a tortuous, loculated tube which secretes large quantity of fructose, citric acid, prostaglandins and fibrinogen. Immediately vas deferens empties sperm, the pair of seminal vesicles then gives off its contents to ejaculatory duct.

All other components of seminal fluid serves as nutrient for the spermatozoa, prostaglandins counter female cervical mucus for reception of spermatozoa movement, thus aiding fertilization (Ehmcke *et al*., 2005).

Function of the prostate gland

This produces and releases a light, milky fluid composed of profibrinolysin, phosphate ion, calcium, citrate ion and a clotting enzyme. It is capsule-like in nature and gives off its contents at the same time with the vas deferens during emission, so its content adds more to bulk of the emitted semen. Prostatic fluid is slightly alkaline in nature and therefore, neutralizes the acidic fluid emitted by the vas deferens, this helps in successful fertilization of the ovum by reducing the acidity of the fluids in the female cervix to between 6.0 and 6.5, thus enhancing sperm motility (Amann, 2008).

Urethra

The urethra is firstly made up of an internal urethra that passes through the penis and continues as the second part called the external urethra. Mucus glands exist throughout its entire length.

Penis

The male genitalia is known as the Penis. It serves as a passage for Urethra and opens outward. It is made up of three erectile tissue masses namely; a paired corpora cavernosa and an unpaired corpus spongiosum. The latter surrounds the urethra and ends outwardly to give rise to the glans penis.

2.2 Hormonal control of the testicular functions

Steroidogenic and gametogenic functions of testis are under the control of gonadotropins being secreted by pituitary gonadotropes. Activities of these hormones are controlled by hypothalamic gonadotropin hormone-releasing hormone (GnRH) usually secreted at regular intervals. Its secretion is however caused by a biological transducer found on the superficial layer of GnRH neurons and triggered by kisspeptin-54, a peptide expressed by neurons located in the different hypothalamic nuclei. Pituitary function is also regulated by negative feed back mechanism from gonadal steroid and peptides (Amann, 2008).

2.2.1 Secretion of gonadotropin hormone releasing hormone

Gonadotropin hormone releasing hormone is produced as well as released by hypothalamus and released in discrete pulses into the portal blood in such a manner that its release pattern also determines the mode of LH and FSH secretion. GnRH serves as a major cause of release for both gonadotropins, while altering its rate of occurence gives rise to prioritizing their release (Hayes and Crowley 1998). Kisspeptin/ GPR54 system brings about the continuous tonic inhibition of peripheral steroids on secretion as well as pulsatile release of GnRH. Testosterone or its metabolite, dihydrotestosterone (DHT) are the peripheral steroids that decreases the frequency of GnRH pulsatility and also stops the secretion of gonadotropins through reverse information from the hypothalamus and pituitary gland respectively and this is very vital to developing male fertility control regimen (Hayes and Crowley 1998).

2.3 Heavy metals as causes of male reproductive toxicity.

In 1970, researchers' accertained an increase in rate of male infertility due to a significant drop in sperm quality (Pasqualotto *et al*., 2003). There are so many predisposing factors that bring about increased infertility (Multigner *et al*., 2002). Research suggests that men are exposed to many work-related substances which may affect their fertility. In the 20^{th} century, the swift shift to industrialization and agriculture due to technology had to do with exposure to various substances like metals, pesticides, estrogen-like substances, chlorinated compounds, etc., that are harzadous to humans including the male reproductive system. There are several external causes of infertility among which is coming in contact with substances like arsenic, lead, mercury, cadmium, etc related to occupation.

2.3.1 Arsenic

Arsenic as an element occurs in the physical environment normally and as such is an environmental pollutant that is rated very high among the causes of cancer by (IARC, 1989). It exists as organic and inorganic arsenic. The former one is in form of molecules that have at least one carbon atom and hydrogen an example is 4-hydroxy-3-nitrobenzenearsonic acid marketed by Pfizer with Roxarsone as the brand name, it was effective for use as a chicken feed supplement until it was banned. Inorganic arsenic is arsenic with no carbon atoms but combines with atleast one of oxygen, chlorine or sulfur and it is classed into two namely arsenite and arsenate (Hughes *et al*., 2011). Organic types

are found in all sorts of fish and other marine animals at very low levels and are generally considered as none-toxic (ASTDR, 2007). However, arsenosugars and arsenolipid toxicity are still under investigation (Schmeisser *et al*., 2006).

It exhibits the qualities of metal as well as non-metal, its relative atomic number is 74.922, its boiling point is 612^{o} C while its melting point is 817^{o} C. Scientist often refers to arsenic as a metal because it exhibit toxicity like heavy metals (Jaishankar *et al*., 2014). Arsenic naturally exist everywhere. The compounds of arsenic may occur as powdered, form crystalline form, amorphous form or vitreous form (Walker and Fosbury, 2009).

(a) Mode of exposure to arsenic

The mode of coming in contact with arsenic could be through inhalation.The dose range of inhalation in work places was between 0.03-0.05 mg/m^3, however, residents who lives in the environment where arsenic is in the air also inhale some quantity (ASTDR, 2007). Arsenic in the air could be in the form of dust or arsenic trioxide vapour. Also, communities around smelters either inhale or come in contact with arsenic from contaminated soil.

Pollution by arsenic is largely contributed to by the activities of man. Arsenic is available in underground water within and outside the USA, because of its ubiquitous nature, it is also present in food, thus, it is ingested orally in these regions of the world. It is present within the range of about 1 µg/L to several hundreds of µg/L in underground water (Nordstrom, 2002) while, the estimated daily consumption in food is about 0.13-0.56 µg/kg/day (EFSA, 2009).

(b) Gastrointestinal absorption of arsenic

Absorption of arsenic may be from food, water and beverages and its bioavailability will depend largely on its solubility and on the type of matrix in which it was ingested. A common indicator of absorption rate in both animal and human study is the quantification of excreted arsenic in both urine and feces within a few days after ingestion. For example, it was documented that about 81 to 86 % of different dosages of sodium arsenate orally administered to Mouse was excreted in urine. In human study about 62.3 ± 4.0 % of the administered dosage was recovered in the subjects's urine within seven days while only very little quantity was recovered in the subject's feces (Hughes *et al*., 1994).

Ingested inorganic arsenic has a biological half-life of about ten hours while that of methylated arsenic is about 30 hours. About 50 to 80% is excreted in about 3 days.

(c) Uses of arsenic trioxide

Medicinal uses

Intravenous injection of ten milligram per kilogram arsenic trioxide for twentyeight to sixty days was introduced to be a treatment by the Chinese in 1992 for acute promyolocytic leukemia (APL) (Scheindlin, 2005). Further research has proven this same usefulness of arsenic trioxide in clininal trials without any significant difference in white blood cell count.

In 1786, a medicine comprising of one percent potassium arsenite and goes by the name Fowler's solution was employed as curative measure to treat malaria, asthma, Psoriasis, eczema, syphilis, chorea and leukemia (Scheindlin, 2005).

As pigments

Arsenic was used as pigments e.g., paris green or copper acetoarsenate, in toys, candles, fabrics and wallpaper industries, however these products became unintended sources of arsenic poisoning (Scheindlin, 2005). It is also used as pigment in the production of eye shadows. Arsenic is employed in the production of glass and semiconductors till date (ASTDR, 2007).

As pesticides

Paris green and lead arsenate were used as pesticides to control potato beetles and apple and cherry orchards respectively but were both banned in the USA in 1988 because of their toxicity to farm workers and the level of their retention in those fruit thereby posing a public health threat to consumers (Cullen, 2008). However, its use as pesticides in some case still exist in the USA, for example, chromate copper arsenate is being used till date as wood preservatives for woods used for none-residential purposes (US EPA, 2008). Those which have pentavalent states are also in use as pesticides but are less toxic because they are not easily absorbed therefore, they pass through little metabolism (Cohen *et al*., 2006).

(d) Toxicity of arsenic

The major source of human arsenic toxicity is from contaminated drinking water. The international agency for research on cancer (IARC) identifies inorganic arsenic compounds as group 1 carcinogens (Martinez *et al*., 2012), however, organic arsenic compounds such as arsenobetaine are nearly non-toxic (Cullen and Reimer, 1989).

Trivalent arsenic exhibits its toxicity by acting in diverse ways such as inhibition of pyruvate dehydrogenase, reducing the creation of acetyl co A (CoA) from pyruvate as well as reducing the activity of citric acid cycle leading to significantly low ATP production (Bergquist *et al*., 2009). It can also inhibit cellular absorption of glucose and gluconeogenesis. It was reported that arsenic prevents gluthathione production which functions to preserve cells from oxidative damage. Pentavalent arsenic is not as poisonous as trivalent arsenic because only little portion of it can cross cell membrane barrier and be immediately converted to the trivalent state (Cullen and Reimer, 1989).

The LD_{50} of arsenic trioxide in rat model was found to be 4.5 mg/kg and 14.98 mg/kg by different researchers, while in human it was 1 mg/kg (Buchet *et al*., 1980; Saxena *et al*., 2009), the half life in rat model was also stated as 60 days because it binds to erythrocytes while in humans, it is between 2-10 days because, it is excreted faster and therefore has a shorter half life. The half life of organic arsenic is reported to be about 18 hours (Buchet *et al*., 1980).

(e) Metabolism of arsenic

It is believed that inorganic arsenic is methylated by the endogenous enzymes namely S-adenylmethisonine (AdoMet) and its co-factor, glutathione. Arsenic methylation is said to be localized in cytosol mainly of liver but to a lesser extent, in kidney and lungs as well. Methylation also occur sequentially (Styblo *et al*., 1996). However, another report from an investigation done by Healy *et al*., (1998) indicated that all tissues can methylate arsenite but testis has a greater capacity to form monomethyl arsenic acid (MMA), followed by the kidney, liver and the lung.

(f) Mode of action of arsenic

Interaction with sulphydryl group: Arsenic binds with sulphydryl group of enzymes and then brings about oxidation of pyruvate thereby causing pathological effects in the body;

this can be reversed by dosing an individual poisioned with arsenic with a lot of monothiols, which was later found to be ineffective (Cohen *et al*., 1931).

Interaction with Phosphate: Other research works have reported that arsenic acts by interacting with phosphate *in vitro,* but little or nothing is known about its *in vivo* interaction. In various biochemical reactions, arsenate can take the place of phosphate (Winski and Carter, 1998). However, in reactions where arsenate substitutes phosphorous, it is usually more unstable than the latter and therefore easily dissociates. In both humans and rabbits, it has been observed that arsenate distrupts the formation of adenosine-5'-triphosphate (ATP) *in vitro* during glycolysis and oxidative phosphorylation by hydrolyzing glucose-1-phosphate, thus forming arsenate anhydrides through a mechanism called ''arsenolysis'' (Winski and Carter, 1998).

Formation of reactive oxygen species: The ROS created by arsenic plays negatives roles in DNA repair inhibition, genotoxicity, distruption of signal transduction as well as distruption of cell proliferation (Hughes *et al*., 2011). Indications that arsenic forms ROS *in vivo* includes increase in lipid peroxidation level, rise in 8 –OH-2-dG, increase in number of genes which are triggered due to stress and loss of defenses against oxidant (Shi *et al*., 2004).

Genotoxicity: Arsenite has been termed comutagenic because it promotes mutation effects of ultraviolet radiation (UV), methyl methansulfonate as well as N-methyl-N nitrosourea in mammalian cell. Arsenic induces effects which includes; oxidative DNA damage, deletion mutations, exchange of sister chromatid, DNA strand breaks, deviation from normal in chromosomes and micronuclei (Basu *et al*., 2001; Rossman, 2003).

Altered DNA repair: The modification arsenic impact on DNA which needs to be repaired is possibly because of its comutagenic influence which gives rise to its genotoxicity (Rossman, 2003). It was reported that arsenic achieved alteration in DNA repair by indirectly inducing ROS or by altering cell signaling process which then changed gene expression. Certain enzymes involved in nucleotide repair are also stoped when arsenic encounter zinc finger motifs of proteins whose function is to bind to cysteinyl residues (Rossman, 2003; Ding *et al*., 2009).

The method through which arsenic inhibits the enzymes involved in nucleotide repair are either by replacing zinc or by causing inactivation of sulfhydryl groups of cysteine.

Signal transduction: Natural occurences in cells are organized and controled by signal transduction sequence of reaction. Arsenic can change this pathway, thus promting activation of transcription factors or inhibition of regulatory proteins as the case may be which will then attach to DNA and control gene transfer (Druwe and Vaillancourt, 2010). Arsenic is popular for its ability to cause cancer, but it is also used to cure acute promyolocytic leukemia because of its ability to increase proteins that cause apoptosis while downregulating proteins that prevent apoptosis, and causing the leukemic cells to go through apoptosis whilest the subject undergo a state of remission to the cancer.

Altered DNA methylation: Gene transcribing is controlled by DNA methylation, therefore, altered DNA methylation could lead to distortion in cell growth, giving rise to tumor or adenocarcinoma. Arsenic toxicity has been implicated in altered DNA methylation. Present in cells are proteins that regulates it division or generally, cell cycle, one of such is tumor suppressor protein p53. Arsenic is known to prevent expression of this protein thus, leading to cancer growth. This has been scientifically proven (Chanda *et al*., 2006).

Its possible means of action to alter DNA methylation is not fully understood, but there are suggestions that since S-adenosylmethiomine (SAM), which gives off methyl to both DNA and arsenic is shunted towards arsenic methlation in case of exposure, the DNA now having insufficient or even lack methyl for its methylation becomes hypomethylated (Hughes *et al*., 2011).

(g) Treatment of arsenic toxicity

Results from clinical trials have shown that chelators can be used to stop deterioration of symptoms arising from chronic arsenic toxicity. An example of such chelator is 2,3-dimercapto-1-propanesulfonate (DMPS) which was reported to have significantly improved chronic arsenicosis (Guha, 2003). Also, Guha *et al*. (2003) reported that DMPS raised urinary excretion of arsenic by seven-fold in patients who were exposed to arsenic toxicity.

Coming from the nutritional angle, Guha (2003) suggested that proteinous food may help to get rid of inorganic arsenic by increasing its methylation and therefore, advised that people who are exposed to arsenic toxicity should increase their consumption of both animal and plant protein.

Antioxidants have beneficial effects in treatment of arsenic toxicity; moreover, antioxidants from herbal source have been tested in West Bengal where a lot of people have contact with copious amount of arsenic. Curcumin capsules from turmeric was administered to confirmed subjects who had prominent DNA damage and exhausted antioxidant for three months, the oucome was a reduced DNA damage, suppressed ROS and higher antioxidant activity in plasma (Biswas *et al*., 2010).

(h) Effects of arsenic on blood analysis and liver enzymes

Yasmin *et al*. (2011) stated that the administration of 3 mg/kg arsenic trioxide to Swiss Albino Mice for 15 days did not significantly affect any of the blood parameters. In humans, acute, intermediate and chronic exposure to arsenic has been associated with anaemia and leukopenia due to direct haemolysis, cytotoxicity of blood cells or suppression of erythropoiesis (Armstrong *et al*., 1984; Franzblau and Lilis, 1989). Arsenic trioxide was found to cause a rise in serum ALT, AST and ALP (Yasmin *et al*., 2011).

(i) Effects of arsenic on antioxidant enzymes

Studies on human subjects have shown that increased arsenic concentration in plasma leads to increased reactive oxidants and thus lowers endogenous antioxidants (Wu *et al*., 2001). Arsenic is known to compromise mitochondrial membrane integrity leading to uncontrolled formation of superoxide anion radical that brings about a cascade mechanism of free radical formation and at the same time depletes cellular antioxidant defense system (Cohen *et al*., 2006). In both whole animal and tissue studies, arsenic toxicity has led to rise in ROS formation (Flora *et al*., 2007).

Mammalian cell is enriched with different antioxidant defense system, SOD and catalase happens to be the initial stage of defense. The SOD dismutates superoxide into O_2 and H_2O_2 and catalase catalyses the breakdown of H_2O_2 to H_2O and O_2 (Jomova *et al*., 2011). The second line of cellular defense systems are the thiol-based antioxidants (Manna *et al*., 2008).

(j) Effects of arsenic on male reproductive system

The impacts of arsenic poison on male reproduction cannot be overemphasized; several works documented the disadvantages of arsenic in man and animal models, while some have gone extra mile to profound solutions to some of these toxic effects in males. Arsenic is reported to induce gonadal dysfunction through reduction of testosterone synthesis, apoptosis and necrosis (Shen *et al*., 2013). In some epidemiological cases, arsenic was reported to cause male infertility through induction of low sperm quality and erectile dysfunction (Hsieh *et al*., 2008). Several research works have demonstrated that arsenic accumulates in testis and other accessory organs (Pant *et al*., 2001). Another scientist, Souza *et al*. (2016) claimed that the administration of sodium arsenite to Wistar rats brought about reduction in spermatid number per gram of testis as well as decreased sperm production, shortage of sperm count recovered from the caudal epididymis was also reported.

Alli *et al*. (2013) showed that 3 and 4 mg of arsenic trioxide brought about a reduction of sperm parameters and testosterone while LH level significantly increased relative to their control. The treatment of rabbits with it was reported to have brought about a fall in sperm profile serum LH, FSH and testosterone levels while the co administration of arsenic and 100 mg/kg of Vitamin E ameliorated all of these effects (Zubair *et al*., 2014). Also, dosing Swiss albino rats with three milligram per kilogram $NaAsO_2$ orally for forty days led to destruction of testicular histoarchitecture. Khan *et al*. (2013) said administration of 69.6 mgL^{-1} of sodium arsenite to Wistar rats for 28 days lead to significant rise in sperm DNA damage, significant decrease in testicular levels of 3 and 17β-HSD and testicular androgen level, the proportion of leydig cells that underwent apoptosis also significantly increased.

The probable mode of action of arsenic in causing low sperm quality is tought to be the binding of arsenic to thiol-rich protamines within nuclear chromatins of spermatozoa and in the flagellum of sperm (Uckun *et al*., 2002). Its mechanism for causing shortage in serum gonadotropins as well as testosterone has also been proposed to be the elevation in coticosterone levels which invariably inhibits the adequate production of the gonadotrophins as well as testosterone (Biswas *et al*., 1994; Hardy *et al*., 2005). Also, cholesterol metabolism is very important in the synthesis of testosterone, Moreover, several enzymes participates in sequential steps of testosterone synthesis from cholesterol,

some of those enzymes are acute steroid regulatory protein (STAR) and the dehydrogenases. It has been proposed that low testosterone level in experimental animals exposed to arsenic may be due to a reduction in the quantity of enzymes involved in synthesis of testosterone from cholesterol, thereby making cholesterol available in a significantly high amount while testosterone concentration will be significantly decreased. This was observed and reported by Chinoy *et al*. (2004).

Rat model seems appropriate for study of arsenic activity *in vivo* because in comparison with mice, rabbits and hamsters in which inorganic arsenic is eliminated from blood swiftly, rat erythrocytes retain same as dimethyl arsonous (DMA) (Marafante *et al*., 1982).

2.4 *Parquetina nigrescens*

Parquetina nigrescens is a perennial plant mostly seen in tropical zones in West and East Africa. Its leaf is ten to fifteen centimeters in length, six to eight centimeters in width and has got fine and lengthy twig (Fig. 2.1). It is called eweogbo and Kwankwantsasube in Yoruba and Hausa language respectivly. Commonly, it is known as Africa Parquetina. Its leaf is usually employed in treatment of diseases affecting the liver, gonorrhea, rickets and asthma by traditional healers in Nigeria (Imaga *et al*., 2008). Lots of studies have been done with the leaf of *Parquetina nigrescens*, using different solvents for its extraction process. Some of these experiments are highlighted below.

2.4.1 Proximate analysis, phytochemical screening and lethal dose

The proximate analysis carried out by Imaga *et al.*, (2010) was found to contain the following; 41% of protein, 14% of carbohydrate, 11% of lipid, 11% of ash, 21% of fibre and 2% of moisture. The report on the phytochemical constituents showed existence of alkaloids, flavonoids, glycosides, cardiac glycosides, tannins, saponins, anthraquinones, phlobatannins and oil. (Ayoola *et al*., 2011).

The LD_{50} of *Parquetina nigrescens* leaf's methanol extract was discovered to be 4.5 g/kg (Aderibigbe *et al.*, 2011).

2.4.2 Effect of *parquetina nigrescens* on haematological profile

It was reported that aqueous extract of *Parquetina nigrescens* leaf corrected the reduction in RBC count, haemoglobin concentration, haematocrit, mean corpuscular haemoglobin concentration as well as severe thrombocytopenia that was observed in alloxan-induced

diabetic Wistar rats, but leucopenia was observed in the extract alone group (Saba *et al*., 2010). *Parquetina nigrescens* is a constituent of a commercial herbal preparation known as Jubi formula for the treatment of anaemia in Nigeria (Erah *et al*., 2003) and this has been scientifically proven to improve haematocrit and haemoglobin concentration in anaemic Wistar rats. Agbor and Odetola (2005) also reported that the administration of aqueous extract of *Parquetina nigrescens* to haemorrhage-induced anaemic rats improved red blood cell count, haemoglobin as well as haematocrit concentrations.

In another experiment, Omoboyowa *et al*. (2016) reported that both ethanol and chloroform extract of *Parquetina nigrescens* leaf caused significant increase in packed cell volume, haemoglobin concentration, RBC count, WBC count, and differential white blood cell count in normal Wistar rats after 21 day of administration.

2.4.3 Effect of *Parquetina nigrescens* leaf extract on blood glucose level

Parquetina nigrescens (1000 mg/kg) reportedly reduced glucose level found in the blood of rats treated with alloxan to induce diabetes but it was normal in the group treated with the extract only on comparing with the control group (Saba *et al.,* 2010). A similar result was generated from Sprague Dawley rats loaded with glucose and treated with either aqueous or methanol extract of *Parquetina nigrescens* leaf (Awobojo and Olatunji-Bello, 2010).

2.4.4 Effect of *Parquetina nigrescens* on gastrointestinal system

It was reported that decoction of *Parquetina nigrescens* leaf significantly reduced gastric acid secretion (Odetola *et al.,* 2006). It was also observed to increase gastric wall mucus secretion. It also reduced non-protein sulphydryl group. The ulcerogenic activity of the plant was also conducted and discovered not to produce ulcer in the gastric mucosa when compared with the control, but indomethacin produced ulcer significantly (Owoyele *et al.,* 2009). In an experiment in which the methanol extract was used to treat ulcerated rats, the dosages decreased the level of peroxidation of lipids in stomach. It also increased protein concentration, catalase activity and SOD activity (Aderibigbe *et al.,* 2011).

2.4.5 Antioxidant effect of *Parquetina nigrescens*

Flavonoid was extracted from the plant using methanol and 100µg concentration had a strong antioxidant effect. 50 mg of different extract of the leaf also retarded lipid peroxidation in liver mitochondrion that was induced by 200 µM ascorbate/20 µM Fe^{2+}. Also 50 mg /kg of the crude flavonoid extract caused no significant difference in SOD and Catalase level in the group given 50 mg/kg + CCl_4 (Ayoola *et al.*, 2011).

2.4.6 Analgesic, anti-inflammatory and antipyretic effects of *Parquetina nigrescens* leaf extract

Hot plate latency test: The aqueous extract brought about pain relief by increasing the latency period (seconds) of the time it took the animals to react when placed on hot plate, although the group given indomethacin showed the highest latency period (Owoyele *et al*., 2009).

Carrageenan-induced paw oedema: paw oedema formation due to injection of Carrageenan was reduced after 3 and 5 hours of oral administration of the extract as well as prevented granuloma tissue formation. It also inhibited arthritic limb compared with the control in a pattern that is similar to that of indomethacin.

2.4.7 Antiasmathic effect of aqueous extract of *Parquetina nigrescens*

Aquous extract of *Parquetina nigrescens* brought about a fall in the contractile effect of $C_5H_9N_3$ as well as carbachol on trachea of guinea-pig. The doses ranged from 0.024-1.536 mg/ml. 400 and 800 mg/kg of the extract showed protective effect against histamine and antigen-induced broncho spasm when compared with the standard drug, astemizole (Terlarbi *et al*., 2000).

2.4.8 Uterotonic effect of *Parquetina nigrescens* leaf extract

Hydromethanol extract of *Parquetina nigrescens* was able to cause spasm of excised myometrium of pregnant rat. It was then suggested that the extract has influence similar to that of oxytocin characterized by an extracellular influx of calcium (Datte *et al*., 1996).

2.4.9 Effect on body weight and organ weight

Study on impact of *Parquetina nigrescens* on weight of the body and of organ is not numerous, however, it was observed in a study with aqueous extract of the leaf that all the groups given *Parquetina nigrescens* had a considerable weight loss, while the control

group had a considerable weight gain (Saba *et al*., 2010). The liver weight and the kidney weight were also reduced (Saba *et al*., 2010).

2.4.10 Effect on reproductive system

Hydromethanol extract of *Parquetina nigrescens* was reported to cause spasm of excised myometrium of pregnant rat (Datte *et al*., 2006). Oyelowo *et al*. (2012) reported that aqueous root extract of *Parquetina nigrescens* enhanced male reproductive behavior in Wistar rats, it was also suggested that it improved serum testosterone concentration while a reduction in sperm analysis was observed in the same study.

The above experimental studies have proven that *Parquetina nigrescens* have so many health benefits which may be explored.

Fig. 2.1. *Parquetina nigrescens* leaves sample from the University of Ibadan premises (FHI No: 109785).

2.5 SQUALENE

2.5.1 Brief history and source of squalene

Squalene, naturally occurring oil was uncovered in 1906 in Japan (Tsujimoto, 1906). Squalene is a triterpene (Fig. 2.2), its name is derived from the scientific name of shark (*Squalus spp*.), because the liver oil of shark happens to be the richest in squalene (Mitrea-Vasilescu *et al*., 2014). It is present in reasonable amount in olive oil being its second largest source, peanut, soyabean and palm oil (Popa *et al*., 2014).

There is also advancement in the technology employed in the production of squalene so much so that micro-organisms such as yeast (Saccharomyces, Torulaspora delbrueckii, Pseudomonas, Candida, algae Euglena e.t.c.) and prokaryotes are now also used as sources mainly because they have massive or fast growth usually in a laboratory controlled environment and production by them is faster (Socaciu *et al*., 1995). For example, Chang *et al*. (2008) isolated a new yeast strain characterized by genetic analysis as belonging to the genus pseudozyma sp. from sea environment and which produced a higher concentration of squalene and more polyunsaturated fatty acids. This strain was then considered good for commercial production of squalene. The advantages of producing squalene by yeast fermentation consist in the possibility to carry out the process at large scale, with a high yield in order to obtain squalene of high purity in controlled conditions.

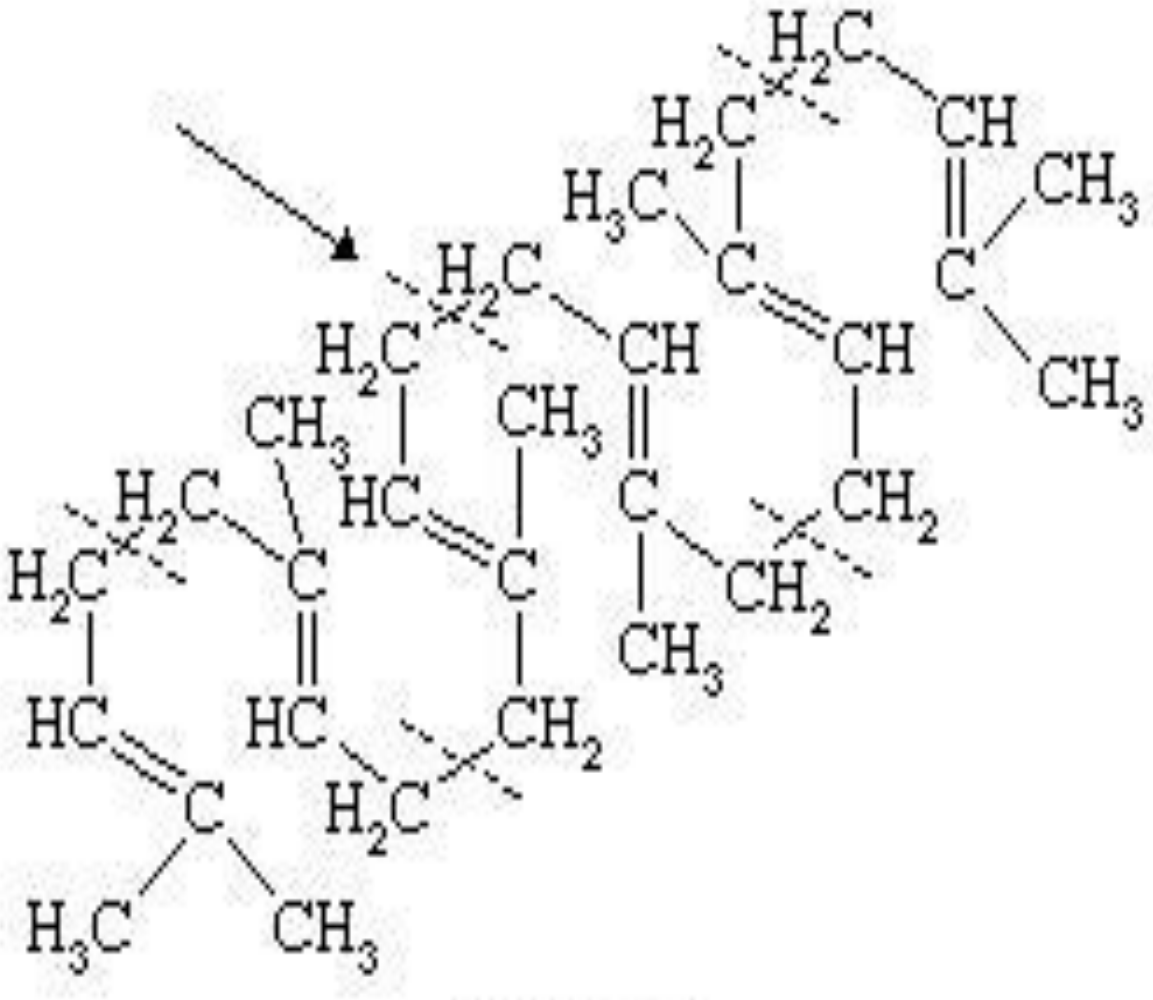


Fig. 2.2. Chemical structure of Squalene (Gregory and Kelly, 1999).

2.5.2 Synthesis

Endogenous Production of Squalene

Although the synthetic pathway for the production of squalene is the same as that for the production of cholesterol, squalene must first be produced because cholesterol is subsequently produced from squalene. Squalene is produced in mammalian cells within the cytosol by an initial synthesis of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) from acetyl CoA in the presence of acetyl CoA thiolase (niacin), HMG CoA is further reduced to mevalonate by the enzyme HMG CoA reductase (HMGR) (Fig. 2.3). The rate limiting factor of production of squalene is the regulation of HMGR.

Mevalonate is subjected to three stages of phosphorylation under the influence of magnesium dependent enzymes before it is further decarboxylated to form delta 3-isopentenyl diphosphate (IPP). Subsequently, condensation with another IPP molecule yields 15-carbon farnesyl diphosphate. Squalene synthase combines two molecules of farnesyl diphosphate in the presence of niacin to form squalene. Cholesterol can then be further produced from squalene when necessary (Tansey and Shechter, 2000).

Squalene is produced in sebaceous glands in large amount and carried by small and very small density lipoproteins. The squalene concentration in lipids of human skin is around five hundred microgram per gram while for adipose tissue, it is about 300 µg/g (Tsimidou, 2010).

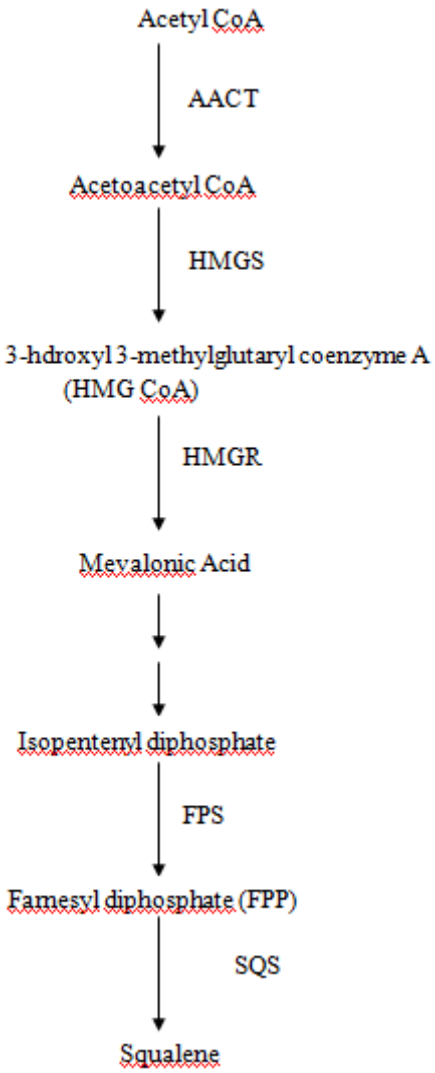


Fig. 2. 3. Squalene synthesis through formation of mevalonic acid in mammalian cell. AACT (acetoacetyl-CoA thiolase, FPS (FPP synthase), HMGS (HMG-CoA synthase), HMGR (HMG-CoA reductase) and SQS (squalene synthase) (Tansey and Shechter, 2000)

2.5.3 Biological activity

It is essential in cholesterol production needed for synthesis of steroid hormones, carotenes as well as oil soluble vitamins (Spanova and Daum, 2011).

Cardioprotective Activity: Through epidemiological research, it was observed that countries where high amount of olive oil were consumed had far significantly less number of cases of cardiovascular health diseases than in countries where little of it were consumed (Keys , 1995). This observation was then ascribed to squalene in it, hence squalene is known to have a cardioprotective effect (Newmark, 1997).

Antioxidant Activity: The susceptibility of squalene to peroxidation is very low, hence, it acts at the skin level in the capacity of quenching singlet oxygen. (Kim *et al*., 2003).The chemical structure of squalene as an isoprenoid hydrocarbon having six double bonds gives it the ability to function as a strong antioxidant and natural antibiotics, it is also highly reactive, giving off oxygen which on reaching the cells, helps to intensify cellular metabolism thereby enhancing functions of certain body parts.

On the contrast, Warleta *et al*. (2010) reported that, at high concentration, squalene showed no antioxidant effect against free radicals in an *in vitro* assay, while other researchers concluded that the antioxidant activity of squalene is very low (Psomiadou and Tsimidou, 1999). Invariably, Warleta *et al*., (2010) concluded that squalene's ability to function as antioxidant was dependent on whether it can do one or all of the following; increase glutathione concentration in cells (Das *et al*., 2003) or deregulate antioxidant systems in tumor cells (Klaunig and Kamendulis, 2004). Of great importance are the differences in its uptake by cells, its utilization and its accumulation (Das *et al*., 2008).

Antitumor and anticancer activity: It has also been proposed based on preclinical experiments using rat models that squalene has some antitumor and anticancer effects (Popa *et al*., 2014). The mode of action by which squalene act against tumor formation may be that it inhibits the catalytic activity of related enzymes involved in xenobiotic metabolism and subsequently inhibits farnesylation of Ras oncoproteins.

2.5.4 Uses of squalene

Squalene is used for production of cosmetics because of its softening and soothing nature on the skin as well as its easy absorption into the skin, it also possess other characteristics such as spreadability, light consistency, non-greasy texture and antibacterial properties (Huang *et al*., 2009). Also, drug producers use it majorly because of its odourless and colourless characteristics. The antibacterial and low melting point quality that it possess enables its inclusion in admixtures used in preparing cooling formation for local treatment of burns (Ogawa and Doi, 1996). In Sweden and Norway fishermen traditionally used this oil to cure wounds and respiratory tract conditions (Tjan, 2001). Squalene in conjuction with lecithin, tween 80 and span 85 is used to prepare emulsion as an adjuvant for vaccine delivery e.g., influenza vaccine (FLUAD, Chiron, Italy). (Moroti *et al*., 2012).

2.5.5 Experimental evidences of biological activities

In a study, meat-type male chicken were treated with squalene for two weeks, this led to rise in spermatozoa number, semen volume as well as increased serum testosterone concentration. It was also observed that it increased fertility and brought about sexual and aggressive behavior (Li *et al*., 2010).

2.5.6 Toxicity

In animal experiments conducted in rats and dogs exposed to squalene for three months, no side-effects appreciable sign of toxicity was reported in biochemical as well as liver function test (Kamimura *et al*., 1989).

2.6 Review of antioxidants

Antioxidants are materials which avert or setback addition of oxygen to oxidizable substrates. They scavenge or inhibit ROS production as well as catalyse detoxifying enzymes (Halliwell, 2007). Dietary plants are said to contain natural antioxidants to which the ablility to prevent cancer have been attributed (Bennett *et al*., 2012). Natural antioxidants may be gotten from fruits, vegetables, nuts, seeds, flowers, leaves, bark and roots (Sadowska-Bartosz and Bartosz, 2014). Reports from various works revealed that some polyphenolic constituents in plants have greater efficiency in laboratory experiments than vitamins E and C and may therefore improve greatly one's health because of the protection they offer against peroxidative damages brought about by reactive molecule (Sin *et al*., 2013). Some of these antioxidants are discussed:

Vitamin E

Vitamin E also known as α-Tocopherol is one of the four fat-soluble vitamins, its biological potency can not be overestimated and is membrane bound and used by cells (Jiang, 2014). It majorly protects against lipid peroxidation (Pryor, 2000).

Vitamin C

The research on benefits of vitamin C, a chain breaking antioxidant on spermatozoa development and maintenance have become extensive since 1998. Several works have been carried out with it to prove its effectiveness as an antioxidant against free radicals. It is reported to protect against H_2O_2-induced DNA damage Hughes *et al.* (1998). It was also reported to enhance sperm motility during an *in vivo* study and it also brought very low the ROS production induced by H_2O_2 and subsequently, DNA damage in both normal and and abnormal spermatozoa samples (Verma and Kanwar, 1998).

Gluthatione

Several researches on GSH have shown that it could possiblly be a therapeutic drug for infertilty patients due to its popular antioxidant qualities. Glutathione (GSH) is found within cells and is a non-enzymatic antioxidant (Valko, 2006). It is saddled with the responsibility of regulating redox status possibly by scavenging free radicals and it serves as a basis for GPx and GST to act upon when free radical producing compounds are been detoxified, it may also be a cofactor in metabolism of drugs. Report suggests that GSH protects spermatozoa from ROS-induced DNA damage and improved spermatozoa profile (Lopez *et al.,* 1998).

Selenium

Iwanier and Zachara (1995) and Scott *et al*. (1998) engaged selenium in an experiment for 3 months on subfertile men and claimed that it had no effect. Other authors have proposed that when selenium is combined with another drug such as vitamin E, it leads to improved sperm profile (Safarinejad and Safarinejad, 2009).

Superoxide dismutase

It is uanimously agreed that SOD has benefits over lipid peroxidation *in vitro*: several works have reported the ability of SOD to boost sperm profile (Lewin and Lavon. 1997).

Glutathione peroxidase

Glutathione peroxidase (GPx) is available within the testes, matrix of mitochondrial in a spermatozoa as well as seminal plasma (Pfeifer *et al*., 2001). It brings about a fall in volume of hydrogen peroxide and peroxides of phospholipids (Galecka *et al*., 2008) and participates in production of oxidized glutathione (GSSG) (Huang and Sultatos, 1993). GSH is then formed from GSSR by GSR.

Catalase

Catalase participates enormously in the elimination of H_2O_2 (Spolarics and Wu, 1997). When GPx has been exhausted during the detoxification of H_2O_2, then catalase takes over (Spolarics and Wu, 1997; De Bleser *et al*., 1999).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Plant collection and identification

Fresh leaves of *Parquetina nigrescens* were hand picked within the premises of University of Ibadan, Ibadan. They were authenticated at the Forestry Research Institute of Nigeria (FRIN) and given FHI number - 109785.

3.2 Method of extraction

The leaves were left to dry at room temperature for about six weeks and blended to powdery form with Daewoo electrical blender (DBL-819, Posco Daewoo Corporation, Korea). *Parquetina nigrescens* powder weighed 1100 g, and using absolute methanol, soxhlet extraction method was employed for the extraction process (Aderibigbe *et al*., 2011). The methanol extract of *Parquetina nigrescens* leaf (MEPL) was concentrated using rotary evaporator at 40°C and thereafter, freeze dried. The methanol extract of *Parquetina nigrescens* leaf weighing 104 g was obtained, and was stored at room temperature throughout the experiment.

3.3. Phytochemical study of methanol extract of *Parquetina nigrescens* leaf

This was done according to the method described by Walls *et al*. (1952, 1954).

3.3.1 Test for alkaloids

Five hundred (500) mg was taken from MEPL and dissolved in methanol to give 5 mL, and mixed with 5 mL of 1% hydrochloric acid in a petri dish over a water bath. 1 mL was then dispensed into a test-tube plus few drops of Mayer's reagents. The appearance of fluorescent brownish precipitate showed the presence of alkaloids (Walls *et al*., 1952, 1954).

3.3.2 Test for saponin

Small quantity of MEPL was dispensed and distilled water was added to it. It was then warmed over a burner and the appearance of froth persistently indicated that saponin was present (Walls *et al*., 1952, 1954).

3.3.3 Test for tannin

The presence or absence of tannin was established as described by the method of Trease and Evans (1989). Five (5) g of MEPL was stired with 10 mL of distilled water and filtered. Few drops of ferric chloride reagent was added to the filtrate. The appearance of blue-black or blue-green precipitate was taken as evidence for the presence of tannins.

3.3.4 Test for anthraquinone

The MEPL (5 g) was separated into a test tube while 10 mL of benzene was poured into it and vigorously mixed and filtered. To the filtrate, 10% ammonia solution (five millilitre) was mixed, and development of pink colour showed the prescence of anthraquinones (Walls *et al*., 1952, 1954).

3.3.5 Test for flavonoids

To 3 mL of MEPL, a piece of magnesium ribbon and 1 mL of concentrated HCl was added. Development of red colour indicated presence of flavonoids (Kumar *et al*., 2007).

3.3.6 Test for cardiac glycosides

Two (2) mL of MEPL filtrate was added to 1 mL of glacial acetic acid containing 1-2 drops of 2 % ferric chloride ($FeCl_3$) solution. The mixture was immediately transferred into a test-tube containing 2 mL of concentrated hydrochloric acid. The appearance of a brown ring at the interface indicates the presence of a deoxy sugar, characteristic of cardenolides. A violet ring appeared below the brown ring Parekh and Chanda (2007).

3.4 *In vitro* antioxidant study

3.4.1 2,2-diphenyl-1-picrylhydrazyl hydrate assay

Principle

A stable 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) was used to determine the radical scavenging ability of methanol extract of *P.nigrescens* leaf. Diphenyl-1-picrylhydrazyl hydrate, a deep violet colored substance that reduce on reaction with an antioxidant

compound that can donate hydrogen and have its colour changed to light yellow that is then measured spectrophotometrically at 517 nm (Blois, 1958).

Procedure

This test was done as described by Brand-Williams *et al*. (1995). To 1 mL of different concentrations (10, 5, 2.5, 1.25, 0.625, 0.3125 mg/ml) of MEPL, squalene or standard (vitamin C) was added 1 mL of 0.3 mM DPPH in methanol. This was mixed and incubated in the dark for 30 minutes and thereafter, the absorbance was read at 517 nm against a DPPH control. The percent of inhibition wad calculated as follows:

$I\% = [(A_{blank}-A_{sample})/A_{blank}] \times 100$

Where:

A_{blank} = Absorbance of the control reaction

A_{sample} = Absorbance of the test compound

Sample concentration providing 50 % inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration.

3.4.2 Determination of total flavonoid content

Standard quercetin with varying concentration 0.1, 0.2, 0.3, 0.4 and 0.5mg/ml was used as standard in comparison to the sample extract or squalene. This was carried out based on the aluminum chloride colorimetric assay method according to Zhilen *et al.* (1999) as described by Miliauskas *et al.* (2004).

Procedure

To 0.1 mL of extract, squalene or standard was added 0.4 mL of distilled water. This was followed by the addition 0.1mL of 5% sodium nitrite. After 5minutes, 0.1 mL of 10 % aluminum chloride and 0.2 mL of sodium hydroxide was added and the volume was made up to 2.5 mL with distilled water. The absorbance at 510 nm was measured against the blank. The total flavonoid content of the plant, expressed as mg quercetin equivalents per gram of the plant extract was calculated as:

$$X = q * \frac{V}{w}$$

X = Total content of flavonoid compound in quercetin equivalent

q= concentration of quercetin established from the standard curve

V= volume of extract (ml)

w= weight of the MEPL obtained.

3.4.3 Determination of total phenol content

This was done according to the method of Singleton and Joseph (1965) as described by Gulcin *et al*. (2003), using an oxidizing reagent known as folinciocalteu's phenol reagent.

Procedure

A mixture of the sample (0.1 mL) and water (0.9 mL) was prepared and 0.2 mL of folinciocalteu's phenol reagent was added, the mixture was voltexed. After 5 minutes, 1.0 mL of 7 % (w/w) Na_2CO_3 solution was added. The mixture was then made up to 2.5 mL with distilled water before being incubated for 90 minutes at room temperature. Its absorbance was then measured against blank (1 mL of distilled water) at 750 nm. 0.1 mg/mL of Gallic acid was used as standard.

3.4.4 Metal chelating assay

The ferrous ion-chelating (FIC) assay was done according to the method of Singh and Rajini (2004). Solution of 2 mM $FeCl_2 \cdot 4H_2O$ and 5 mM ferrozine were diluted 20 times. An aliquot (1 mL) of different concentrations of MEPL or squalene was mixed with 1mL $FeCl_2 \cdot 4H_2O$ and incubated for 5 minutes. To the mixture, 1 mL of ferrozine was added, shaked vigorously and incubated for ten minutes. The absorbance was then measured at 562 nm..

The percentage inhibition of ferrozine–Fe^{+2} complex formations was calculated by using the formula:

Chelating effect % = $[(A_{control}-A_{sample})/A_{control}] \times 100$

Where:

$A_{control}$= Absorbance of control sample

A_{sample} = Absorbance of a tested samples.

3.4.5 Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid radical scavenging activity

The free radical scavenging activity of MEPL was determined by the ABTS radical cation decolourisation assay (Re *et al*., 1999). The ABTS radical cation ($ABTS^+$) was produced by reacting 7 mM ABTS stock solution with 7mM potassium persulphate in a ratio of 2:1 and kept in the dark at room temperature for 16 hours before use.

Prior to use, the stock solution was diluted with 70% ethanol to an absorbance of 0.75 ± 0.05, to give ABTS working solution. About 2 mL of ABTS working solution was mixed with 50 µL of different concentrations of MEPL, squalene or trolox which served as the standard and the absorbance was measured after 20 minutes at 734 nm.

3.4.6 Determination of total antioxidant capacity

Principle

The principle of this method is centered on the reduction of molybdenum (VI) to molybdenum (V) by the sample and the subsequent formation of a green phosphate/molybdenum (V) complex at an acidic pH (Prieto *et al.,* 1999).

Procedure

To 0.1 mL of the MEPL, squalene or standard solutions of ascorbic acid (20, 40, 60, 80, 100µg/ml) was added 1.0 mL of the reagent solution (0.6M sulphuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). The tubes containing the reacting mixture were incubated in a water bath at 95^{o} C for 90 minutes. The mixture was cooled to room temperature, and its absorbance was measured at 695 nm against blank. The total antioxidant activities of the MEPL and squalene were expressed as an ascorbic acid equivalent (AAE).

3.5 Gas chromatography-mass spectrometry

A Perkin Elmer Gas chromatography-mass spectrometry (GC–MS) was used at the Central Laboratory, University of Lagos. The procedure was followed step by step as described by Karasek and Clement (1988).

Two (2.0) g of the MEPL was dissolved in 4.0 mL of Absolute methanol. The mixture was centrifuged for 10 minutes at 5,000 g; 0.05 mL of the volatile supernatant was transferred to Varian 450 Gas Chromatograph coupled to a Varian 240−MS ion−trap mass spectrometer (VF-5 MS Column) with injector and oven temperature at 250 °C and 200 °C. The heating rate of GC−MS was programmed at 100 °C/minute to 250 °C at 10 °C/minute. The injection was performed in the split ratio of 200 and the volume was 10.0 µL. The flow of carrier gases was constant at 1.0 mL/minutes during the run of volatile MSC (0.50 mL) solution (Rajeswari *et al.,* 2012).

The identification of chemical compounds present in MEPL was carried out by similarity searches, and mass spectra data in the National Institute of Standards and Technology (NIST) MS Search 2.0 Library. The chemical components in the MEPL were quantified with Mass Spectrometry workstation software.

3.6 Chemicals

Squalene, hydrogen peroxide and hydrochloric acid (HCL) were bought from Aldrich Chemical Co., Saint Louis, USA. The following were bought from Fine Chem. PVT. Ltd. India; Arsenic trioxide, tween 80, trichloroacetic acid, thiobarbituric acid, adrenaline, hydrogen peroxide, tetraoxosulphate VI, pricric acid, formaline, ethanol, bovine serum albumin, pyrogallol, sodium citrate, ethylenediaminetetraacetic acid (EDTA), potassium permanganate, sodium hydroxide and 5,5' ditho (bis) nitrobenzoic acid.

3.7 Experimental animals

Eighty-six Wistar rats; 6 females and 80 males (150 – 180 g) were used. They were obtained from the Central Animal House, College of Medicine, University of Ibadan, Ibadan. They had free access to rat feed and drinking water *ad libitum*. All animals were distributed into groups and acclimatized for two weeks. All experiments on animals were carried out with the approval of University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC) and the number - UI-ACUREC/18/0067 was assigned.

3.7.1 LD_{50} test

The LD_{50} was done using limit test as described by Organization for Economic Co−operation and Development (OECD) guideline 423 (2001). Six healthy and non-pregnant adult female rats were used for acute toxicity test. They were grouped into two, each group consisting of three animals. The first group was administered 2,000 mg/kg bw of MEPL and observed for fourteen days. The second group was also administered the same dosage of MEPL and observed for 14 days.

3.7.2 Experimental design

Study 1

Forty (40) male Wistar rats weighing 150 -180 g were used to study the effects of methanol extract of *Parquetina nigrescens* leaf on sperm profile, testicular oxidative status and histology in arsenic trioxide-induced reproductive toxicity in Wistar rats. They were

divided into groups and treated for 54 days as follows group 1 (distilled water, 3 mL/kg), group 2 (3 mg/kg As_2O_3), group 3 (250 mg/kg MEPL), group 4 (500 mg/kg MEPL), group 5 (1000 mg/kg MEPL), group 6 (As_2O_3+250 mg/kg MEPL), group 7 (As_2O_3+500 mg/kg MEPL) and group 8 (As_2O_3+1000 mg/kg MEPL) (Table 3.1).

Table 3.1: Experimental grouping for effects of methanol extract of *Parquetina nigrescens* leaf on sperm profile, testicular oxidative status and testicular histology in arsenic trioxide-induced reproductive toxicity in Wistar rats

S/No	Groups	No. of animals	Duration (days)
i	Control (distilled water)	5	54
ii	3 mg/kg As_2O_3	5	54
iii	250 mg/kg MEPL	5	54
iv	500 mg/kg MEPL	5	54
v	1000 mg/kg MEPL	5	54
vi	250 mg/kg MEPL + As_2O_3	5	54
Vii	500 mg/kg MEPL + As_2O_3	5	54
viii	1000 mg/kg MEPL + As_2O_3	5	54

Study 2

Forty (40) male Wistar rats (150-180 g) were grouped into 8 (n = 5) and used to study the effects of methanol extract of *Parquetina nigrescens* leaf and squalene on sperm profile, testicular redox status, hormones, apoptotic markers testicular and epididymal histology in arsenic trioxide-induced testicular toxicity in male Wistar rat. They were treated for 54 days as follows; group 1 (distilled water, 3 mL/kg), group 2 (3 mg/kg As_2O_3), group 3 (250 mg/kg MEPL), group 4 (500 mg/kg MEPL), group 5 (100 mg/kg squalene), group 6 (As_2O_3+250 mg/kg MEPL), group 7 (As_2O_3+500 mg/kg MEPL), group 8 (As_2O_3+100 mg/kg squalene) (Table 3.2).

Table 3.2: Experimental grouping for study 2: Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on sperm profile, testicular redox status, hormones and apoptotic markers in arsenic trioxide-induced testicular toxicity in male Wistar rats

s/no	Groups	No. of animals	Duration (days)
i	Control (distilled water)	5	54
ii	3 mgkg As_2O_3	5	54
iii	250 mg/kg MEPL	5	54
iv	500 mg/kg MEPL	5	54
v	100 mg/kg SQ	5	54
vi	250 mg/kg MEPL + As_2O_3	5	54
Vii	500 mg/kg MEPL + As_2O_3	5	54
Viii	100 mg/kg SQ + As_2O_3	5	54

The administration of MEPL, arsenic trioxide, and squalene were carried out once daily through oral route for fifty four (54) days. The dosage of Arsenic trioxide used was adopted from Alli *et al*. (2013). Tween 80 (10 %) was used to dissolve MEPL.

3.7.3 Sacrifice and collection of organs

After 54 days of oral treatment with MEPL and arsenic trioxide, all animals were anaesthetized using intraperitoneal injection of sodium thiopental (40 mg/kg) (Shittu *et al*., 2018) and blood was collected by cardiac puncture into plain sample bottles and EDTA bottles. Testis, epididymis as well as liver were harvested, weighed and preserved in bouine's fluid and 10 % formaline respectively until needed for further laboratory studies.

3.8 Blood indices

3.8.1 Determination of packed cell volume and hemoglobin concentration

Microhematocrit method was used to determine packed cell volume (PCV) (Bull *et al*., 2000). A heparinized capillary tube was held at right angle to the blood in the EDTA bottle and was allowed to fill to ¾ of its volume by capillary action. The capillary tube was sealed with plasticine placed at the blood filled end and centrifuged at 12, 000 rpm for 5 minutes. The capillary tube was then placed in the groove of a haematocrit reader and was read off from the scale in percentage.

The haemoglobin concentration in grams per deciliter (g/dL) of blood was determined by multiplying packed cell volume with 0.34 and this gives one third of the value of PCV as the value of haemoglobin concentration.

3.8.2 Red blood cell count

Red blood cell (RBC) count was done microscopically using the Haemocytometer apparatus (Math *et al*., 2016). The tip of a diluting pipette for red blood cells marked 101 was applied to the blood in the EDTA bottle. The pipette was positioned horizontally and by gentle suction, blood was sucked to 0.5 mark, it was used to draw Hayem's RBC diluting fluid from a petri dish up to the 101 mark and with the finger on the tip of the pipette, it was swirled for a few seconds thus diluting the blood 1:200. About two to three drops was blown out until the mixture reduced to the 100 mark and the pipette was swirled again for about 2 minutes. About 2-3 drops was then used to fill a clean coupled and dry Neubauer's counting chamber. It was placed under a microscope and all the red blood cells in 5 selected squares of the thoma ruling were counted under x10 and x40 magnification.

The number of red blood cells (RBC) counted was multiplied by 10, 000 and expressed as 10^6/µL.

3.8.3 White blood cell count and platelet count

A white blood cell (WBC) count was done microscopically, using a haemocytometer and a standard microscope system (kessel, 1998). A WBC pipette was dipped into the blood in EDTA bottle and was withdrawn by suction up to 0.5 mark. It was also dipped into a petri dish containing Turks solution and by suction was drawn up to the 11 mark. The pipette was rotated between the fingers to ensure thorough mixing of both blood and diluting fluid. The already arranged counting chamber was filled appropriately with blood and allowed to stand for about 10 minutes and thereafter placed under a light microscope and x10 magnification was used to focus on it while counting was done using x40. White blood cells were counted in four large squares and the total number was multiplied by 200, while platelets were counted in the depressed small squares on the same chamber.

3.8.4 Differential white blood cell count: preparation of slide

Few drops of blood were placed on the end side of a frosted end microscope slide. A Thin film of blood smear was made by placing another glass slide at an angle of 45 degree to the blood containing slide and moved sidewise. The spreader was held firmly and moved to the other end in a straight line with same force and pressure. Few drops of leishman's stain were spread over the surface of the smear and left on the working table for 2-5 minutes. Few drops of distilled water was poured over the slide and left for 10 minutes. The slide was rinsed and kept adide to dry.

3.8.5 Identification of differential white blood cells

Using high power magnification, focus was directed to one end of the prepared blood slide where the cells were well distributed, the slide was moved in a fashion to avoid passing over the same cells twice and each time a type of WBC was encountered, it was identified and recorded and this was done until 100 cells were counted and recorded. The percentage was then determined.

3.9 Biochemical assay

Testis and liver (1 g each) were weighed and the liver was rinsed with 1.15% of KCl and blotted with filter paper. Each one was then homogenized with 5 ml of phosphate buffer saline (pH 7.4) using Teflon homogenizer and centrifuged at 10,000 rpm for 10 minutes. The obtained supernatant was used for the estimation of malondialdehyde (MDA) concentration, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), alkaline phosphatase (ALP), alanine aminotransferase (ALT) as well as aspartate aminotransferase (AST) activities.

3.9.1 Malondialdehyde

Malondialdehyde (MDA) is an organic compound indicating the extent of peroxidation reaction. When MDA is present in any sample under test for peroxidation, it reacts with thiobarbituric acid, one of the reagents for the test, to give pink colouration that can be absorbed at 535 nm.

The reagents used were trichloroacetic acid-tiobarbituric acid-hydrochloric acid (TCA-TBA-HCl) stock which was composed of 15 g of trichloroacetic acid, 0.375 g of thiobarbituric acid and 0.25N of hydrochloric acid. The procedure designed by Rice-Evans *et al*. (1986) was adopted.

Calculation

The MDA concentration of samples was calculated using the formular:

$$\text{TBARS activity} = \frac{\text{O.D x Vb x 1000}}{\text{A x V x 1 x Y}}$$

Where O.D = absorbance of sample test at 535nm

Vb = volume of the reaction

A= molar estimation coefficient of product = $1.56 \times 10^5 m$

V= volume of tissue extract used

Y= mass of tissue in mg in the volume of serum

1= height path (cm)

3.9.2 Superoxide dismutase

Superoxide dismutase (SOD) activity was carried out according to the method described by Misra and Fridovich (1972). Having set the spectrophotometer at 420 nm, it was zeroed with a blank made up of 3.0 mL of distilled water. Distilled water or samples (0.2 mL) was added to appropriately labelled test tube, followed by 2.5 mL of the carbonate buffer and incubated at room temperature. Adrenaline solution of 0.3mM concentration (0.3mL) was added to the reference and each of the test solutions, then mixed by inversion and read using the spectrophotometer.

Calculation

$$\text{Inhibition} = \frac{(O.D_{Ref} - O.D_{Test})}{O.D_{Ref}} \times 100$$

1 unit of SOD activity is taken as the amount of SOD required to cause 50% inhibition of the auto-oxidation of adrenaline to adrenochrome per minute.

So, the enzyme activity can be calculated as follows:

$$\text{Units/mg wet tissue} = \frac{\%\ \text{inhibition}}{50 \times y}$$

Where,

y= mg of tissue in the volume of the sample used.

3.9.3 Catalase

Catalase activity was determined according to the procedure described Sinha (1972). The reagents used were 30 nM H_20_2, distilled water, 6 M of H_2SO_4, 0.05 M KPO_4 buffer (pH of 7.4) and 7 mL of 0.01 M $KMnO_4$. Test tubes were labeled for samples, blank and standard, 0.5 ml of sample was dispensed into the appropriate test tubes, this was followed by the addition of 5.0 mL of 30 nM H_2O_2, blank and standard, 0.5 mL of distilled water was dispensed into the test tube for blank. The mixture was mixed by inversion and made to stand for 3 minutes. Thereafter, 1.0ml of 6M H_2SO_4 was added to all the test tubes.7.0 mL of 0.01M KMnO4 dispensed into the test-tubes containing samples while 5.5 mL of 0.05M phosphate buffer was added to the standard.The mixture was then thoroughly mixed by inversion and absorbance was read at 480 nm against distilled water after 30 second and after 60 seconds.

Calculation

The formular employed was:

Kc log = So/S_3 x 203/T, Where;

Kc log is the log of catalase.

So = absorbance of standard at zero time,

S3 = absorbance of standard at 3 minutes.

T = time in seconds.

The inverse log of Kc was determined and this gave the value of catalase.

3.9.4. Glutathione peroxidase assay

Glutathione peroxidase activity was determined according to the method of Rotruck *et al*. (1973). The reagents used were; 0.0325g of 10mM sodium azide (NaN_3) dissolved in 50 mL of distilled water, 0.0123 g of 4 mM reduced glutathione (GSH) dissolved in 10 mL of phosphate buffer, 28 µL of 2.5 mM hydrogen peroxide (H_2O_2) dissolved in 100 mL of distilled water, 2 g of 10% trichloroacetic acid (TCA) dissolved in 20 mL of distilled water, 5.23 g of 0.3M Dipotassium hydrogen orthophosphate (K_2HPO_4) dissolved in 100 mL of distilled water, 0.04 g of 5'-5'-dithiobis-(2-dinitrobenzoic acid) (DTNB) dissolved in 100 mL of phosphate buffer and lastly, 0.992 g of dipotassium phosphate (K_2HPO_4) and 1.946 g of monopotassium phosphate (KH_2PO_4).

Procedure

The following reagents were dispensed and mixed thoroughly in an appropriately labelled test-tube; 500 µL of phosphate buffer, 100 µL of sodium azide, 200 µL of reduced glutathione, 100 µL of hydrogen peroxide, 500 µL of sample and 600 µL of distilled water. The whole reaction mixture was incubated at 37°C for 3 minutes after which 0.5ml of TCA was added and thereafter centrifuged at 3000 rpm for 5 minutes. To 1 mL of the supernatant, 2 mL of dipotassium phosphate and 1 mL of 5'-5'-dithiobis-(2-dinitrobenzoic acid) were added and the absorbance was read at 412 nm against a blank.

Calculation

Glutathione peroxidise activity was observed by plotting the standard curve and the concentration of the remaining GSH was extrapolated from the curve.

$$\text{GSH consumed} = 245.84 - \text{GSH remaining}$$

$$\text{Glutathione peroxidase activity} = \frac{\text{GSH consumed}}{\text{mg protein}}$$

3.9.5 Aspartate aminotransferase

The activitiy of aspartate amino transferase (AST) was assayed by the colorimetic method described by Reitman and Frankel (1957) using randox diagnostic kit. The liver supernatant (0.1 mL) and solution 1 provided along with the kit (L-aspartate + phosphate buffer + L-oxoglutarate) were pipetted into a test tube. Then 0.5 mL of distilled water and the solution I were pipetted into another tube as blank. The content of each tube was well mixed and incubated for 30 minutes at 37 °C. Then 0.5 mL of solution 2 also included in the kit (2, 4, dinitrophenylhydrazine + NaOH) was added to each tube. The tubes content was mixed and allowed to stand for 20 minutes at 25 °C. Then five millilitres (5.0 mL) NaOH was added to each tube and mixed. The absorbance was read at 540 nm against the blank after 5 minutes.

3.9.6 Alanine amino transferase

Alanine transaminase was measured based on the colorimetric method of Reitman and Frankel (1963) using a Randox diagnostic kit. To 0.1 mL liver supernatant sample and 0.5 mL of solution 1 provided along with the kit (DL-alanine + α-oxoglutarate + phosphate buffer) were pipetted into a test tube. The blank tube contained 0.1 mL distilled water and 0.5 mL solutions 1. Each tube was well mixed and allowed to incubate for 30 minutes at 37 °C. Then 0.5 mL of solution 2 (containing 2, 4 dinitrophenyl hydrazine + NaOH) was added to the tube, mixed and incubated for 20 minutes at 25 °C. Five millilitres (5.0 mL) NaOH was added. The content of the tube was mixed, and the absorbance read at 546 nm against the blank after 5 minutes.

3.9.7 Alkaline phosphatase

Alkaline phosphatase assay was done by colorimetric method described by DGKC (1972). Alkaline phosphatase substrate (0.5 mL) was dispensed into sample and equilibrated at 37^0C for 3 minutes. Fifty (50) µL of standard and liver supernatants and blank (deionized water) were dispensed into appropriate test tubes, mixed and incubated for exactly ten minutes at 37^0C. This was repeated a second time. An average of 2.5 millilitre of ALP colour developer was included. The absorbance of sample was obtained at 590 nanometer and documented. The ALP activity in the liver was calculated.

3.10 Sperm analysis

3.10.1 Sperm count, motility and kinetics

The caudal epididymis was cut and washed in two millilitre of PBS (pH 7.4). A Neubauer's counting chamber was prepared and filled appropriately with the sperm sample. The sperm count, motility and kinetics were analyzed with the aid of computer assisted sperm analyzer (JH-6004 CASA) (WHO, 2010).

3.10.2 Sperm viability

Sperm viability was accessed microscopically according to the method described by WHO (2010). Spermatozoa from caudal epididymis was squeezed onto a microscope slide and a thin smear was made. Two drops of eosine-nigrosine stain were added to the smear in order to improve the contrast between the sperm head and the background, thus making observation very easy. The slide was allowed to air dry and using x10 and x40 objectives, 100 spermatozoa were counted in selected fields. The numbers of viable and non-viable spermatozoa were noted and percentage viability and non-viability of sperm were determined.

3.10.3 Sperm morphology

With a clean slide, a thin smear of epididymal fluid was made, fixed in ethanol and then left to dry. Sodium bicarbonate-formalin was used to wash it and water was used to rinse more than once, then diluted (1:20) carbon fuchsin was spread over it and staining was allowed for 3 minutes and then counterstained with dilute Loeffler's methylene blue. Water was used to wash away excess stain and allowed to dry. The slide was then viewed microscopically. Abnormalities (such as tapering head, bent, coiled or double tail) were

confirmed using x100 objectives. One hundred spermatozoa were identified and percentage of spermatozoa showing normal and abnormal morphology was estimated.

3.11 Evaluation of sperm chromatin condensation abnormality using acidic Aniline blue stain

The principle which enables one to detect spermatozoa deoxyribonucleic acid damage was explained by Hammadeh *et al*. (2001) and the procedure described by Wong *et al*. (2008) and Park *et al*. (2011) was adopted. Sperm smear was made on frosted end glass slide and allowed to dry, it was then fixed in 4 % formalin for 5 minutes at room temperature, rinsed in water and stained with 5% aniline blue in 4% acetic acid (pH 3.5) solution for 5 minutes. This was followed by rinsing the slides in water and counter-stained with 0.5 % eosin for 1 minute. Thereafter, they were rinsed, air dried and viewed under light microscope. 300 spermatozoa were counted in different fields of each slide using ×1,000 magnification. Immature sperm cells stained dark blue by the eosin counterstain. The percentage of abnormal sperm chromatin condensation was calculated as the ratio of the number of dark-blue sperm to the total number of sperm analyzed.

3.12 Hormone assay

Blood sample obtained by cardiac puncture from ventricles of each rat using a 5 mL syringe into plain sample bottles was centrifuged at 3000 rpm for 15 minutes. Serum was removed from the centrifuged blood sample into plain sample bottles using a Pasture pipette, iced and kept at -20 ^{o}Cfor further studies. Determination of the serum concentration for testosterone, FSH and LH were done by using enzyme-linked immunosorbent assay (ELISA). The ELISA kit used was bought from Calbiotech kits (Inc. California). The procedure was carried out accordisng to the instruction given by the kit provider.

Principle for Testosterone

Testosterone enzyme immunoassay is based on the principle of competitive binding between testosterone in the test specimen and testosterone haptoglobin related protein for a constant amount of mouse antitestosterone site on the microwell plate. The enzyme activity was indicated by a change in color after the addition of TMB.

Principle for FSH and LH

Both FSH and LH are determined based on the same principle. The assay utilizes a high affinity and specificity monoclonal antibody directed against a distinct antigenic determinant on the intact hormone (FSH or LH) molecule. The test sample was allowed to react simultaneously with the two antibodies resulting in the hormone molecules being sandwiched between the solid phase and enzyme linked antibodies. TMB-Substrate is added and incubated, resulting in the development of a blue color. The color development is stopped with the addition of stopping reagent, changing the color to yellow. The concentration of the hormone (FSH or LH) is directly proportional to the color intensity of the test sample. Absorbance is measured spectrophotometrically at 450 nm.

Procedure

The assay procedure for testosterone, FSH and LH are the same. The microplate wells were brought to room temperature. Using a pipette with new disposable tips, 0.025 mL (25µl) of calibrator, control serum, and samples were dispensed into appropriate wells.The first six wells were filled with testosterone in protein based buffer that was provided together with the kit. They had the concentrations: 0, 5, 10, 25, 50 and 100 mIU/mL.The next well was filled with the control serum samples. Conjugate (0.100 mL (100 µL)) was pipette into each well except the blank, thereafter, the microplate was covered and gently swirled for 20-30 seconds to mix and then incubated at room temperature for 60 minutes.The content of the microplate was decanted and blotted with absorbent paper. Washing solution (0.3 mL (300 µL)) was added to each microplate and it was decanted using the wash bottles and blotted, the washing procedure was repeated for three more times. This was followed by the addition of 0.10 mL of TMB substrate into each well at timed interval and incubation of the plate for 25-30 minutes at room temperature.0.15 mL of stopping reagent was added into each well at the same time interval, gently mixed for 5 to 10 seconds and read off in a microplate reader at 450 nm within 20 minutes after addition of the stopping reagent.The optical density, graph and hormone concentration was printed out by a printer connected to the ELISA microplate reader.

3.13 Histology of the tissues

The conventional method of haematoxylin and eosine (H&E) staining techenique was used for tissue histology. The collected liver and epididymis tissue were fixed in 10% formalin while testis was fixed in Bouine's fluid for minimum of five hours before the commencement of tissue processing which involved the following five stages: dehydration with different percentages of alcohol; clearing using xylene; embedding in paraffin wax; trimming; nicking and sectioning with a microtome and lastly, staining of the tissues with Harris-haematoxiline and counterstained with eosine. The slide was cleared in xylene and mounted with DPX. Thereafter, pictures of the processed tissue slide were taken so as to observe morphological changes.

3.14 17 β –Hydroxysteroid dehydrogenase assay

The assay for 17β –hydroxysteroid dehydrogenase (17 β-HSD) was done spectrophotometrcally as described by Jarabak *et al*. (1962). Testicular tissue was homogenized in 15 % spectroscopic grade glycerol containing 5 mmol potassium phosphate and 1 mmol EDTA at a tissue concentration of 100 mg/mL. The homogenized mixture was centrifuged at 10,000 rpm for 30 mins. The supernatant (1 mL) of the supernatant was mixed with 1 mL of 440 µmol sodium pyrophosphate buffer (pH 10.2), 40 µL of ethanol containing 0.3 µmol of testosterone and 960 µL of 25% BSA making the incubation mixture a total of 3 mL. this was followed by the addition of 1.1 µmol of NAD to the mixture. The enzyme activity was then measured in a spectrophotometer against blank at a wave length of 340 nm. One unit of enzyme activity is equivalent to a change in absorbance of 0.001/min at 340 nm.

3.15 8-Hydroxydeoxyguanosine

The 8-Hydroxydeoxyguanosine (8-OHdG) test using ELISA kit employed Competitive-ELISA (Elabscience ELISA kit). To determine the optical density, change in colouration was measured with spectrophotometer and compared with standard curve. The samples used were serum and testicular tissue. Blood sample was allowed to clot for 2 hours at room temperature before centrifuging for 15 minutes at 1000×g. The supernatant was decanted and used for assay. The testicular homogenate was centrifuged for 20 minutes at 1000×g under the temperature of 2–8 °C and the clear supernate was decanted and kept at -20 °C until needed for the assay.

Procedure

All reagents and samples were brought to room temperature and centrifuged again after thawing before the assay. All the reagents were thoroughly mixed by gently swirling. Standard, blank, or sample (50 μL) were added to designated wells. Immediately, 50 μL of Biotinylated Detection Ab working solution was added to each well, the plate was covered with a Plate sealer, gently swirled to ensure thorough mixing and incubated for 45 minutes at 37 °C. Each well was aspirated and washed four times with 350 μL of wash buffer and properly decanted by inverting the plate and patting it against thick clean absorbent paper. Next, 100 μL of HRP Conjugate working solution was added to each well, the plate was covered with a new plate sealer and Incubated for 30 minutes at 37° C. after that, the aspiration/wash process was then repeated for five times. Thereafter, 90μL of Substrate Solution was added to each well, the plate was then covered and Incubated in the dark for about 15 minutes at 37° C. When apparent gradient (colour change) appeared in standard wells, the reaction was terminated. This was followed by the addition of 50 μL of Stop Solution to each well and immediately, the colour turned yellow.The microplate reader was opened ahead, preheated and all testing parameters were set. The optical density (OD) of each well was then determined at once, using a microplate reader set to 450 nm. The concentration of 8-OHdG in the samples was then determined by comparing the OD of the samples to the standard curve.

3.16 Immunohistochemistry

Immunohistochemistryhas to do with employing staining techniques to "unhide" antigenic sites on tissues embedded in paraffin wax by ensuring that specific antibodies bind to them. The methods used include the exposure of paraffin embedded sections to high heat in a microwave oven while bathed in various solutions with controlled pH.

Procedure

A section of the paraffin wax embedded tissue was trimmed nicked and sectioned using a microtome. It was then deparaffinized by placing it in citrate buffer, pH 6.0, microwaved at 750 W for 20 minutes, and then cooled. The pretreated deparaffinized tissue section was incubated in 3% (v/v) H_2O_2 in phosphate buffered saline at room temperature for 10 minutes and then washed again. Thereafter, the section was incubated in blocking solution

for 10 minutes, primary antibody already diluted in blocking solution was added to it and then incubated overnight at 28 ^{0}C. The sample was washed once again phosphate buffer saline, and the section was incubated with peroxidase labeled polymer. It was conjugated by a secondary antibody for 30 minutes followed by washing the sections with PBS.Application of substrate solution (DAB or other suitable peroxidase substrate) was also done. The sample was washed thoroughly under running tap water and counterstained the samples in Mayer's hematoxylin and then dehydrated. It was then mounted. The photomicrograph of the slides were taken.

3.17 Digital Image Analysis/Quantification Using ImageJ

The photomicrograph images were trained by selecting a Region of Interest (ROI) through a rectangular tool in IHC tool box of imageJ software (version 1.49, National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). The Read Model button of the tool box was set to read the default model H-DAB.txt for brown colour detection. Once the colour detection model (for example, the H-DAB. txt) was read by pressing the colour button on the main panel. The quantification consists of counting the positive nuclei and oval fitted nuclei segmentation (Jie *et al*., 2014).

3.18 Statistical analysis

Data from each group were expressed as Mean $\pm$ standard error of mean (Mean $\pm$SEM). The data collected were analysed using one way analysis of variance followed by Waller-Duncan's post-hoc test. The p-value was set at less than 0.05 to determine the level of significance.

CHAPTER 4

4.0 RESULTS AND DISCUSSION

4.1 Phytochemical constituents of methanol extract of *Parquetina nigrescens* leaf

The following plant phytochemicals; alkaloids, tannins, saponins, flavonoids and anthraquinones were present in methanol extract of *Parquetina nigrescens* leaf (Table 4.1). Phytosterol, glycosides and cardial glycosides were absent in MEPL.

The presence of alkaloids, tannins, saponins, flavonoids as well as anthraquinnones are consistent with the report of Ayoola *et al*. (2011). The presence of flavonoid in MEPL as revealed by phytochemical screening was also confirmed by the result of the *in vitro* antioxidant assay. Flavonoids are antioxidant and anti-inflammatory substances which prevents oxidative stress (Verma *et al*., 1976).

Tannins were also found to be present in MEPL. Tannins are of two types, the first is the condensed and the second type is the hydrolysable which are composed of monosaccharide core. The latter is less stable and has the potential to cause toxicity (Bernhoft, 2010). In an *in vitro* experiment, motility of human spermatozoa exposed to tannic acid remained unchanged (Taitzoglou *et al*., 2001). Tannins are used as astringents in case of diarrhoea and can also hasten wound healing. They ameliorate inflammed mucus membrane (Leal *et al*., 2015).

Saponins were also found to be one of the phytochemical components of MEPL. They have been said to possess anti-neoplastic influences and immune modulatory influence (Bernhoft, 2010). Saponins are known to have adverse effects on fertility, however, ginseng saponin extracts was documented to increase sperm motility, plasma androgen and gonadotropin levels, (Chen *et al*., 1998).

Alkaloids are of different types, examples are; pyrrolizidin alkaloids, tropane alkaloids, etc. However, the type that is accredited with male reproductive effects is named

methylxantine alkaloids. In rodents, it's low to moderate intake have shown reduced sperm production and atrophy of the testis (Bernhoft, 2010). Alkaloids have been reported to impair fertility, arrest spermatogenesis at the spermatid level and reduced the number of seminiferous tubules (Cooke *et al*., 1978).

Anthraquinones are usually present in plants used in malaria treatment which are also known for their anti-fertility effect, however. They have antioxidant and anti-inflamatory effects which protects against certain diseases (Raji *et al*., 2005; Dave and Ledwani, 2012).

Table 4.1. Phytochemical constituents of methanol extract of *Parquetina nigrescens* leaf

Phytochemical Constituent	Present or Absent
Flavonoids	++
Tannins	++
Saponins	++
Alkaloids	++
Anthraquinones	+
Phytostetrol	-
Glycosides	-
Cardial glycosides	-

Key:

++ Presence of detectable quantity

+ Suspected presence

- Absent

4.2 Chemical compounds present in methanol extract of *Parquetina nigrescens* leaf

The identification of chemical compounds present in methanol extract of *Parquetina nigrescens* leaf are shown in Table 4.2.

The GC-MS revealed 24 compounds in MEPL, however, the ones present in significant amount were; Caryophyllene; 2H-1-Benzopyran, 7-methoxy-2,2-dimethyl-; 6-Methylbenzo[4,5]imidazo[1,2-c]quinazoline; n-Hexadecanoic acid; Phytol; 9,17-Octadecadienal (Z)-; 2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all E)- (squalene).

Caryophyllene also known as beta caryophyllene (BCP) is present in numerous herbs employed for various reasons (Calleja *et al*, 2013). It gained the approval of US FDA to be used as additive as well as flavour in food because of its spicy odour and low toxicity (Adams *et al*., 2011). The receptor of BCP is known as cannabinoid receptor type- 2 (CB2) (Calleja *et al*, 2013). Experiments have shown that when BCP was administered orally, it reduced pain caused by high temperature (Klauke *et al*., 2014). Earlier experiment by Fernandes *et al*., (2007) suggested that BCP elicited anti-inflammatory and cytoprotective effects. Hayate *et al*. (2016) also reported that BCP elicits neuroprotection effects in rat. The antioxidant effects of BCP has also been documented by Calleja *et al*. (2013) who demonstrated its protective ability on rat's liver against carbon tetrachloride-induced fibrosis.

The 2H-1-Benzopyran, 7-methoxy-2,2-dimethyl- is also known as Precocene 1 or Precocene I (Halpin *et al*., 1984). It was reported that 300 mg/kg single i.p administration of Precocene 1 to rat led to hepatic damage which was evidenced by severe centrolobular necrosis and increased level of plasma glutamic pyruvic transaminase (Halpin *et al*., 1984). Also, it has been documented that the mode of action of Precocene 1 in inducing hepatic damage is by causing a time and dose dependent depletion of liver glutathione (GSH) activity. It was also discovered that Precocene 1 had an insecticidal effect on *Coptotermes formosanus* and *Locusta migratory* (Mao *et al*., 2010).

The chromatogram with the highest percentage, 6-Methyl-benzo[4,5]imidazo[1,2-c]quinazoline (24.87%) has got only a handful information on it, however, its derivatives

have been reported to possess antibacterial effects against gram negative bacteria and also antifungal activity (Nandwana *et al*., 2018).

The n-Hexadecanoic acid is a fatty acid otherwise called palmitic acid. It is present in living things in greater amount than other types of fatty acid (Gunstone *et al*., 2007). Its percentage is highest in palm oil but is also present in meat, cheese, cocoa butter, soya bean oil and sun flower oil. The n-Hexadecanoic acid is employed in production of toiletries, cosmetics and convenience food, its ester, palmitate ester is used as a carrier medium for long-acting anti-psychotic drug, paliperidone. Palmitic acid has been implicated in hypothalamic insulin resistance in rats fed 20% of palmitic acid and 80% of carbohydrate by altering the central nervous control of insulin secretion and by suppressing signals emanating from insulin and leptin meant to inhibit appetitte (Benoit *et al*., 2009). It was also reported to boost metastasis of human oral tumor cell (Pascual *et al*., 2017).

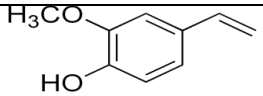
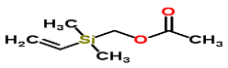
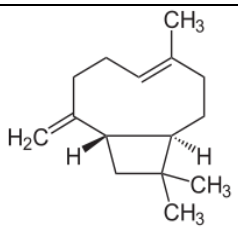
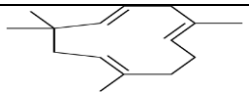
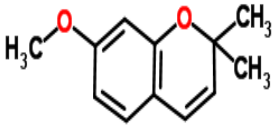
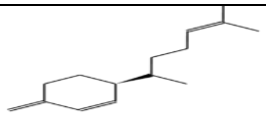
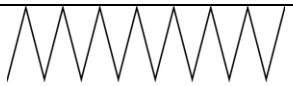
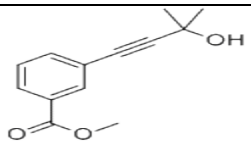
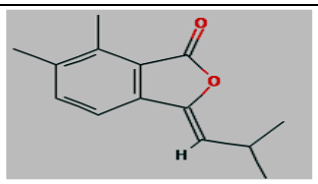
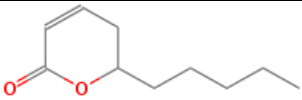
Phytol is an acyclic diterpene and a by-product of chlorophyll metabolism. It belongs to the group of branched-chain unsaturated alcohols (McGinty *et al*., 2010). Consumed plant materials by ruminants as well as diverse non-human primates are fermented in the hindgut to give off significant amount of phytol (Moser *et al*., 2013). Phytol can be changed to its acidic form and kept away in fats. It is a precursor for producing synthetic forms of Vitamins E and K (Thomas, 2007). It is also used to add sweet smell to cosmetics and all kinds of soap. Report indicates that Phytol prevents maturity of *Staphylococcus aureus* (Inoue *et al*., 2005) and also blocks the teratogenic effects of retinol in mice model (Arnhold *et al*., 2002). In an experiment, it was reported that graded doses of phytol inhibited or delayed nociceptive response in mice models made to undergo formalin test and acetic acid test without depressing locomotive activity or causing any sedative effect (Santos *et al*., 2013). It was also observed that it had *in vitro* antioxidant effects (Santos *et al*., 2013). In some other experiments, the anti-inflammatory and antioxidant activities of phytol were evaluated and confirmed (Silva *et al*., 2014). Also, effects of phytol against mycobacterium tuberculosis have been proven (Saikia *et al*., 2010). Phytol is also reported to be toxic to malignant cells, moreover, its mode of activity was discovered to be by inducing apoptosis and autophagy of human gastric adenocarcinoma cells. It also inhibited autophagy and suppressed reactive oxygen species accumulation in normal cells (Song and

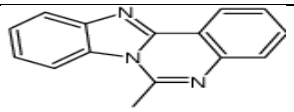
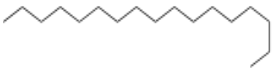
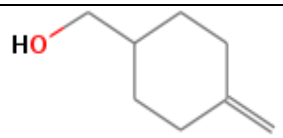
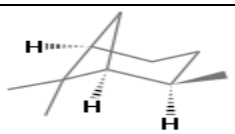
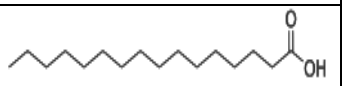
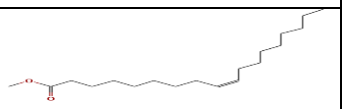
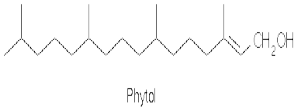
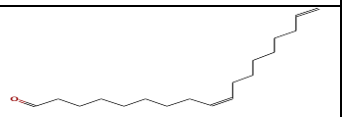
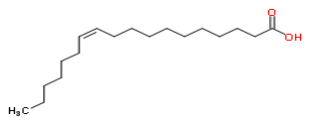
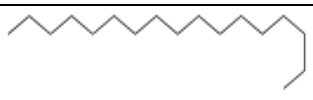
Cho, 2015). Phytol was also demonstrated to show an anticonvulsant effect against pilocarpine-induced seizure that was more potent than diazepam (Costa *et al*., 2012).

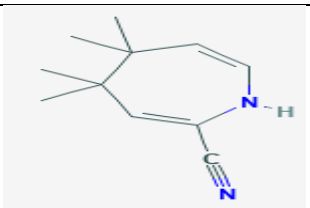
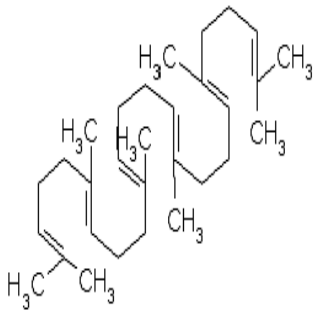
2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all E)- (squalene) is a triterpene with the formula $C_{30}H_{50}$. It was discovered in 1906 by the Japanese researcher Dr. Mitsumaru Tsujimoto (Tsujimoto, 1906). Squalene was also identified in vegetal oils such as olive oil, soybean oil, grape seed oil, hazelnuts oil, peanuts oil, corn oil, palm oil and amaranth seed oil (Popa *et al*., 2014). A yeast strain belonging to the genus *Pseudozyma sp*. was discovered to be a producer of squalene and it gained favour for the commercial production of squalene because production in controlled conditions was possible (Socaciu *et al*., 1995).

Its traditional use include wound healing and for curing various respiratory tract conditions (Tijan, 2001). Squalene is an important component of human sebum and is used in cosmetic formulations. It is used as an adjuvant in vaccines (Moroti *et al*., 2012). It is an intermediate metabolite in cholesterol synthesis in humans (Spanova and Dum, 2011). It is not particularly susceptible to peroxidation and it is stable against peroxide radical's attack (Kim et al., 2003). Experiments have shown that squalene ameliorates hypercholesteromia and coronary heart disease, it has also been suggested to possess anticancer and cytoprotective activity (Popa *et al*., 2014) and promote testosterone concentration in rats (Liu *et al*., 2009). Certain research have also been carried out on squalene in poultry birds and in boars (Li *et al*., 2010; Zhang *et al*., 2008).

Table 4.2. Chemical compounds present in methanol extract of *Parquetina nigrescens* leaf

Pk #	RT	Area %	Chemical Formula	Library/ID	Chemical structure
1	9.024	3.09	$C_9H_{10}O_2$	2-Methoxy-4-vinylphenol	
2	10.185	1.14	$C_7H_{14}O$	Vinyldimethyl(acetoxymethyl)silane	
3	10.609	7.08	$C_{15}H_{24}$	Caryophyllene	
4	11.106	0.96	$C_{15}H_{24}$	.alpha.-Caryophyllene	
5	11.255	17.70	$C_{12}H_{14}O_2$	2H-1-Benzopyran, 7-methoxy-2,2-dimethyl-	
6	12.068	2.16	$C_{15}H_{24}$	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)- 6-methylene-,[S-(R*,S*)]-	
7	12.995	1.55	$C_{16}H_{34}$	Hexadecane	
8	13.166	1.12	$C_{13}H_{14}O_3$	Benzoic acid, 3-(3-hydroxy-3-methyl-1-butynyl)-	
9	14.059	3.08	$C_{14}H_{16}O_2$	3-Isobutylidene-6,7-dimethyl-3H-isobenzofuran-1- one	
10	14.282	1.90	$C_{10}H_{16}O_2$	2H-Pyran-2-one, 5,6-dihydro-6-pentyl-	

11	14.580	24.87	$C_{15}H_{11}N_3$	6-Methyl-benzo[4,5]imidazo[1,2-c]quinazoline	
12	15.364	1.60	$C_{18}H_{38}$	Octadecane	
13	15.461	1.00	$C_8H_{14}O$	Cyclohexanemethanol, 4-methylene-	
14	15.810	1.73	$C_{10}H_{18}$	Bicyclo[3.1.1]heptane,2,6,6-trimethyl-,[1S-(1.alpha.,2.beta.)]-	
15	16.737	1.50	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester	
16	17.263	6.52	$C_{16}H_{32}O_2$	n-Hexadecanoic acid	
17	17.486	1.32	$C_{20}H_{42}$	Eicosane	
18	18.511	1.18	$C_{19}H_{36}O_2$	9-Octadecenoic acid (Z)-, methyl ester	
19	18.654	6.63	$C_{20}H_{40}O$	Phytol	
20	19.014	6.06	$C_{18}H_{32}O$	9,17-Octadecadienal, (Z)-	
21	19.186	1.55	$C_{18}H_{34}O_2$	cis-Vaccenic acid	
22	19.421	1.16	$C_{18}H_{38}$	Octadecane	

23	19.901	1.09	$C_{11}H_{16}N_2$	4,4,5,5-Tetramethyl-4,5-dihydro-1H-azepine-2-carbonitrile	
24	29.806	4.01	$C_{24}H_{38}$,$C_{30}H_{50}$	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all E)-	

The GC-MS study was done at the central research laboratory, University of Lagos.

4.3 Results of *in vitro* antioxidant assay

4.3.1 2,2-diphenyl-1-picrylhydrazyl scavenging activity of methanol extract of *Parquetina nigrescens* leaf and squalene

It was observed that both MEPL and squalene scavenged 2,2-diphenyl-1-picrylhydrazyl. The MEPL showed a lower IC_{50} ($p < 0.05$) (Figure 4.1) when compared with squalene.

Diphenyl-1-picrylhydrazyl, (DPPH), a stable free radical, provides opportunity of evaluating the potential of a substance to function as a scavenger or as H^+ donor (Kedare and singh, 2011). The observed low IC_{50} of MEPL and high IC_{50} of squalene implies that little quantity of MEPL can scavenge DPPH while high quantity of squalene will be required to scavenge DPPH. The capacity of MEPL to scavenge free radicals corresponds with the report of Ayoola *et al*. (2011) which revealed the scavenging activities of flavonoid extract, methanol extract and aqueous extract of *Parquetina nigrescens* leaf. Squalene also demonstrated scavenging activity against DPPH free radical but more of it was required to scavenge the free radical with respect to MEPL. This is similar to the report of Psomiadou and Ysimidou (1999).

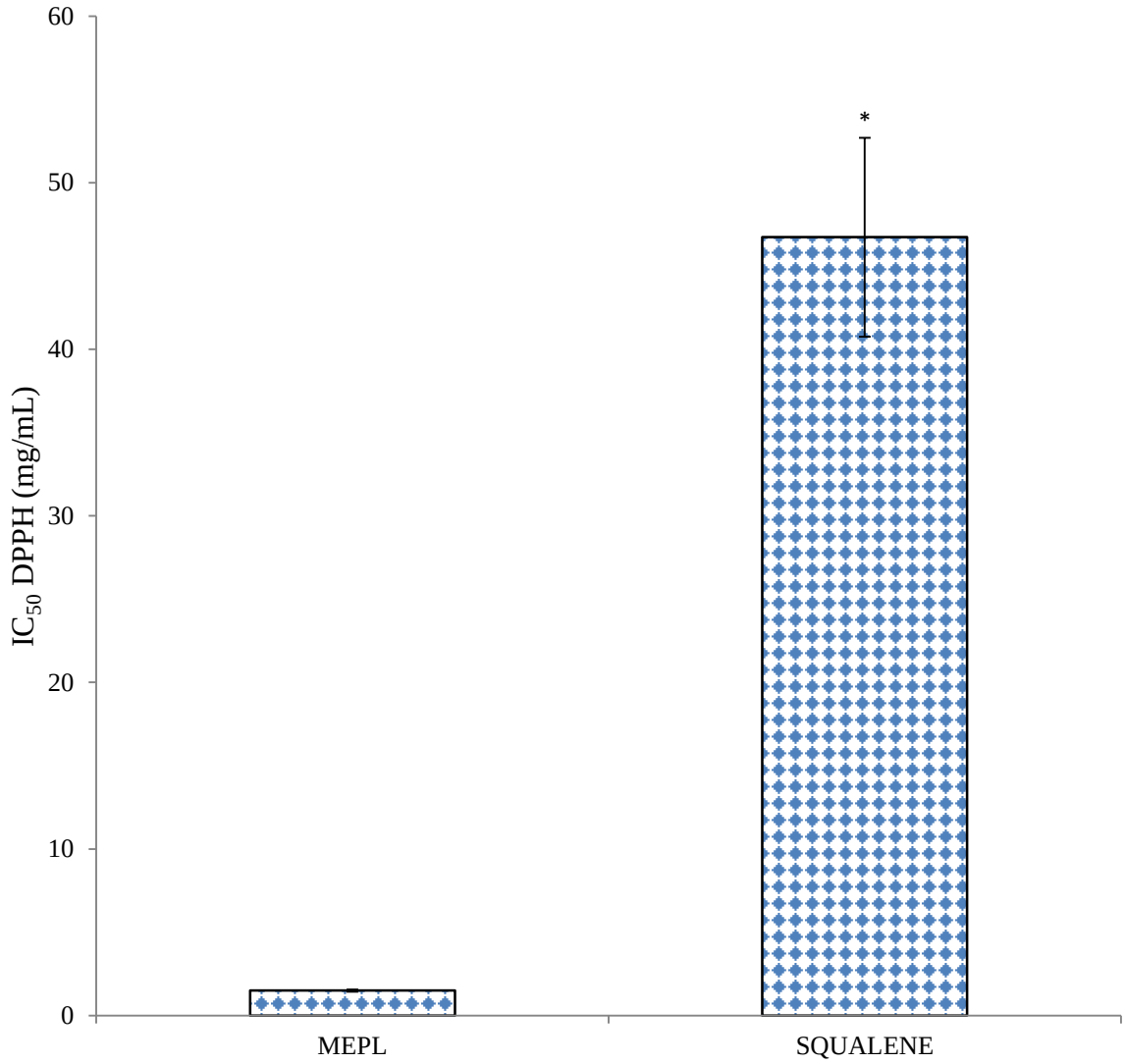


Fig. 4.1. IC_{50} of MEPL and squalene required to scavenge DPPH.

Columns are presented as mean ± SEM, n =5

$^{*}p < 0.05$ as compared with MEPL

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

4.3.2 Flavonoid content in methanol extract of *Parquetina nigrescens* leaf and squalene

Flavonoid concentration in MEPL was significantly higher ($p < 0.05$) than squalene (Figure 4.2).

Flavonoids are known to enhance spermatozoa number, percentage motility, viability as well as improve testicular histology (Khaki *et al*., 2010). They also improve serum testosterone concentration and luteinizing hormone concentration (Khaki *et al*., 2010). *In vitro* antioxidant assay carried out in this study showed that both MEPL and squalene have flavonoids, however, the result suggests higher reactive oxygen specie scavenging activities in MEPL as compared to squalene.

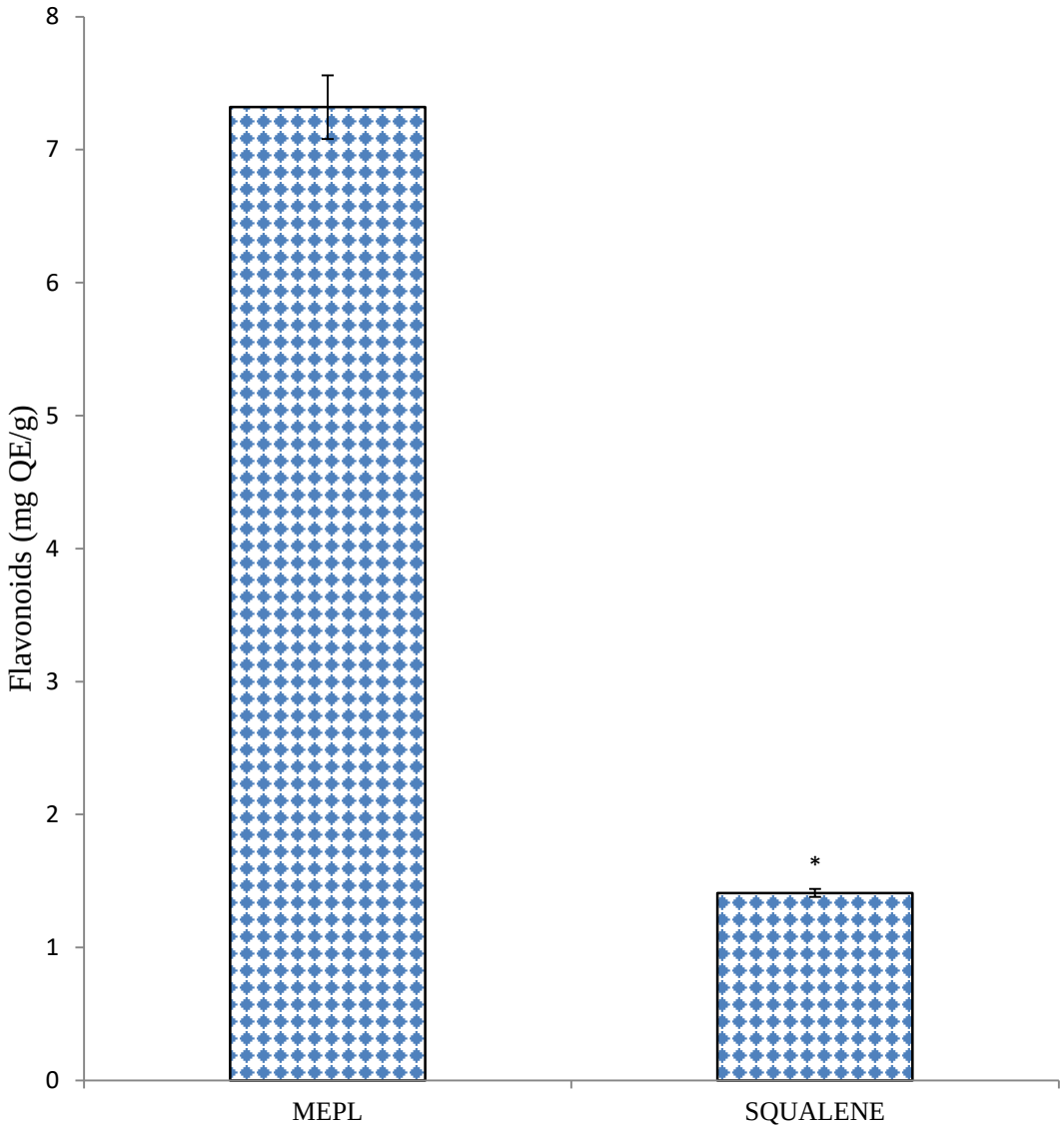


Fig. 4.2. Concentration of flavonoids in MEPL and squalene

Columns represent mean ± SEM, n = 5

*p < 0.05 as compared with MEPL

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

4.3.3 Quantity of phenol in methanol extract of *Parquetina nigrescens* leaf and squalene

It was observed that the quantity of phenol in methanol extract of *Parquetina nigrescens* leaf was significantly higher than that of squalene (Figure 4.3).

In this experiment, it was observed that the phenol content of MEPL was more than that of squalene. Plants that belong to the same family with *Parquetina nigrescens* have shown high content of phenol. Example of such is *Mondia whitei* which reportedly has very high polyphenol content (Bouba *et al*., 2010). Phenols are beneficial to health in several ways which includes; eliminating ROS, defending and regenerating other antioxidants derived from diet as well as chelating pro-oxidant metals. The presence of phenol in MEPL and squalene may therefore strengthen their plasma antioxidant capacities against free radicals. It also suppresses cell death and decreases markers of inflammation (Cicerale *et al*., 2009).

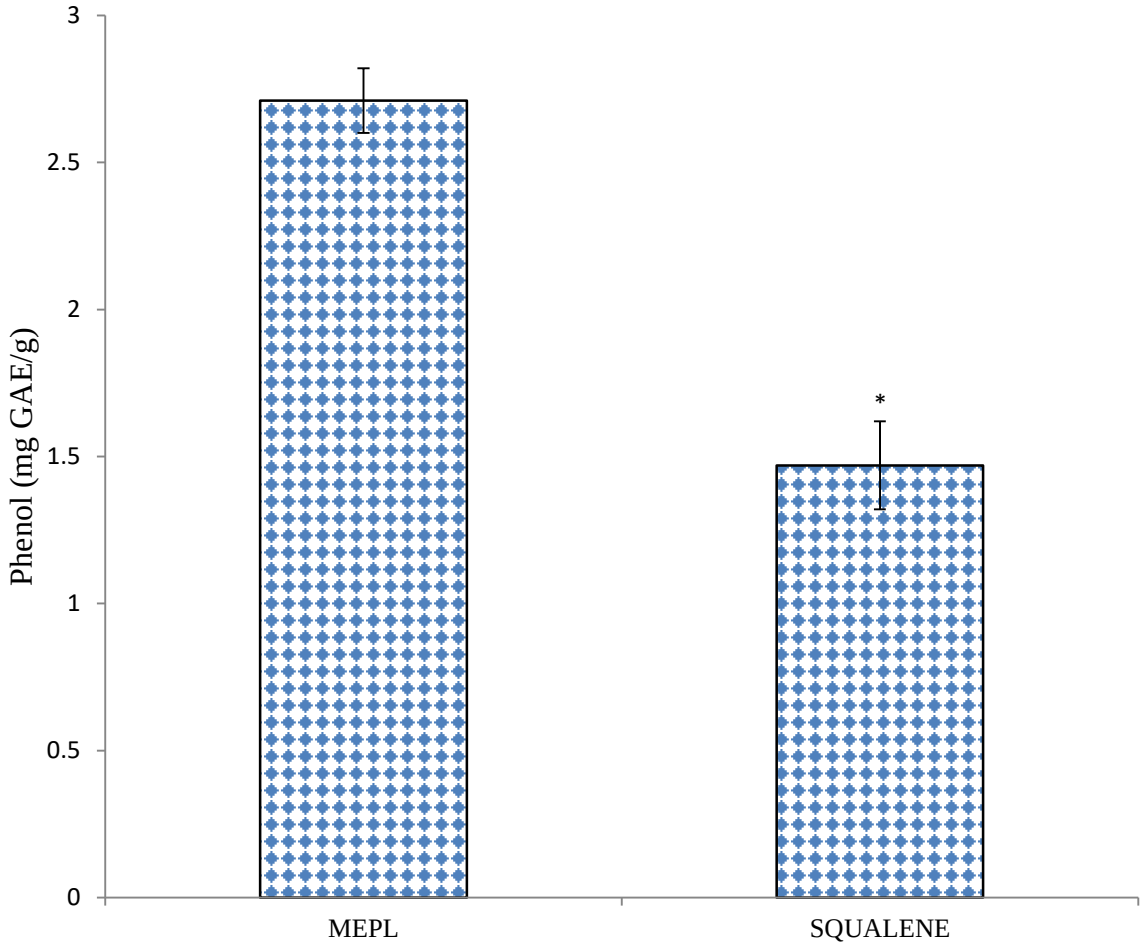


Fig. 4.3. Quantity of phenol in MEPL and squalene

Columns represent mean ± SEM, n = 5

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf.

4.3.4 *In vitro* metal chelating activity of methanol extract of *Parquetina nigrescen*s leaf and squalene

The IC_{50} of MEPL (1.43 ± 0.02 µg/mL) required to chelate metal was lesser ($p < 0.05$) when compared with that of squalene (5.55 ± 0.90 µg/mL) (Figure 4.4).

It was observed that both MEPL and squalene have metal chelating effect. A lower quantity of MEPL was required to achieve metal chelating effects, this suggests that MEPL may be a potent metal chelating agent, although, the amount (IC_{50}) of squalene needed to achieve metal chelating effects was higher. Metal chelators help to prevent heavy metal-induced toxicity in the body by chelating them and make them available for excretion (Flora *et al*., 2007). Thus, MEPL and squalene may likely chelate heavy metals and therefore prevent oxidative stress.

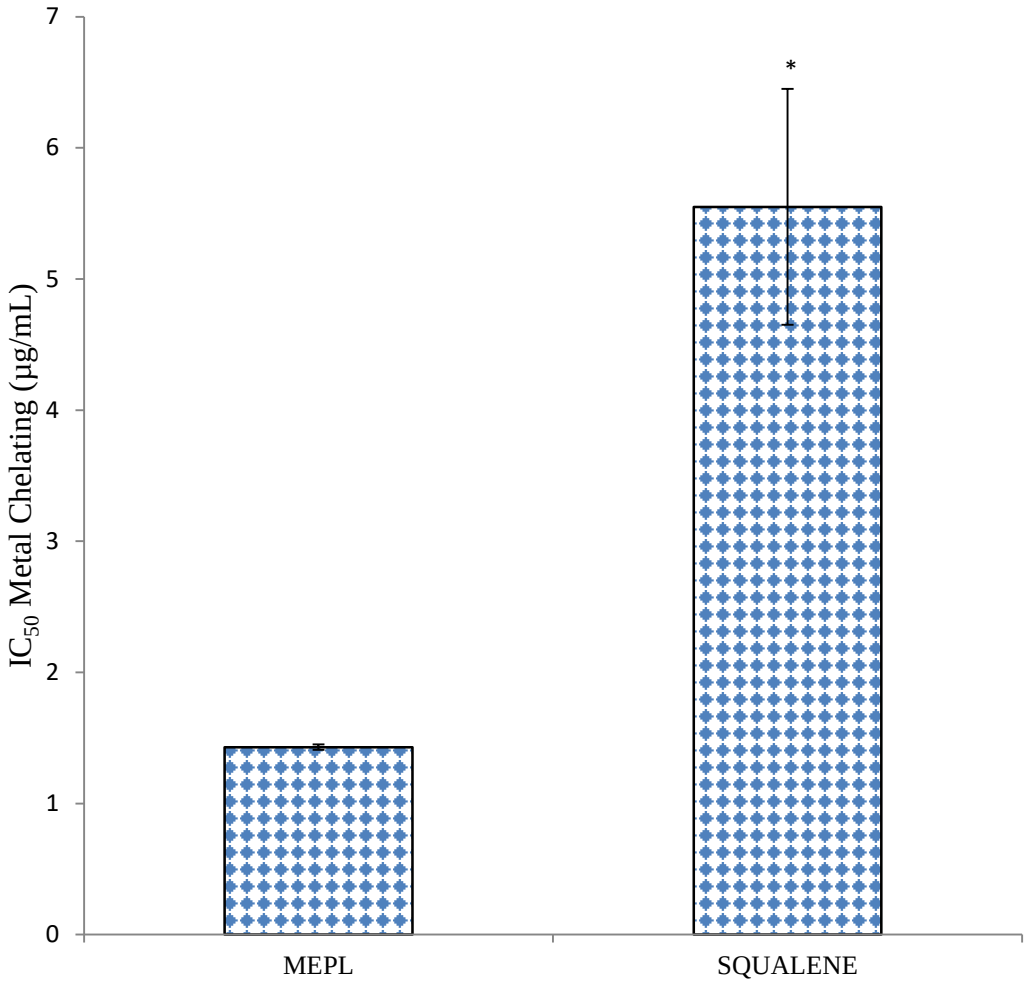


Fig.4.4. *In vitro* metal chelating activity of MEPL and squalene

Columns represent mean ± SEM, n =5

*p < 0.05 as compared with MEPL

MEPL= Methanol Extract of *Parquetina nigrescens* Leaf

4.3.5 2,2'-azino-bis(3- ethylbenzothiazoline-6-sulfonic acid) scavenging ability of methanol extract of *Parquetina nigrescens* leaf and squalene

It was observed that the IC_{50} of MEPL (10.04 ± 0.02 μg/mL) needed to scavenge 2,2'-azino-bis(3- ethylbenzothiazoline-6-sulfonic acid) was similar to that of squalene (9.64 ± 0.11 μg/mL) (Figure 4.5).

2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^+$) upon introduction of potassium persulphate becomes a radical cation and like the DPPH free radical, $ABTS^+$ is employed in the confirmation of claimed or proposed antioxidant capacity of extracts (Re *et al*., 1999). This study showed that MEPL elicited antioxidant ability against $ABTS^+$. Squalene was also observed to have demonstrated similar antioxidant capacity against $ABTS^+$ as did MEPL. The ability of the two substances to scavenge the free radical cation may indicate that they both have antioxidant capacities as earlier stated in this work.

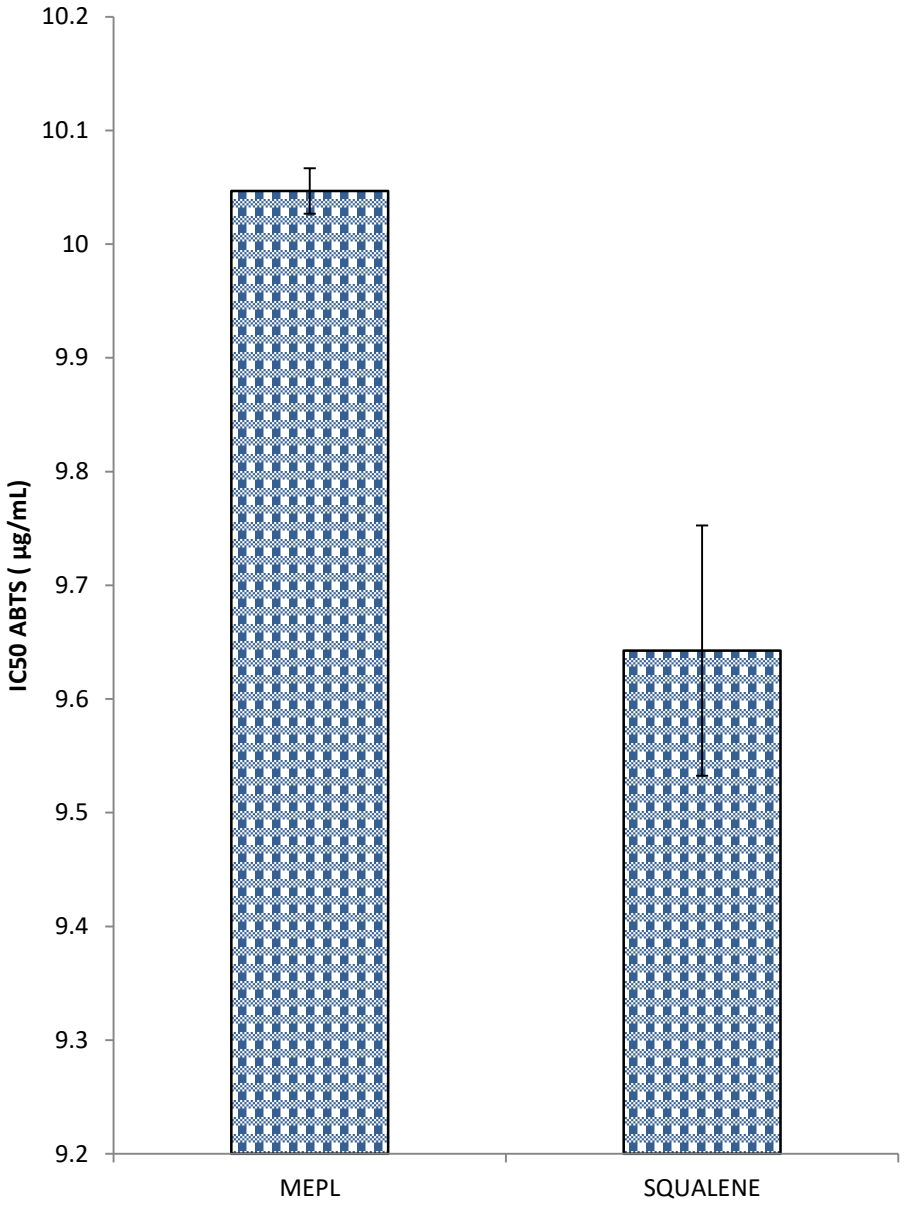


Fig.4.5. *In vitro* $ABTS^{+}$ scavenging ability of MEPL and squalene

Columns represent mean ± SEM, n =5

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

4.3.6 Total antioxidant capacity of methanol extract of *Parquetina nigrescens* leaf and squalene

The total antioxidant capacity of MEPL (7.4 ± 1.35 mg AAE/g) was significantly higher ($p < 0.05$) than that of squalene1 (13 ± 0.12 mg AAE/g) (Figure 4.6).

Total antioxidant capacity (TAC) assay evaluates the combined effects of low molecular weight, chain breaking antioxidants against oxidative stress as well as the complex interaction between the antioxidant themselves (Woodford and Whitehead, 1998). In this study, MEPL and squalene expressed TAC, although, it was higher in MEPL. There is a dearth in literature on TAC in *Parquetina nigrescens*. This could be due to the variations in results generated from several different methods of estimating TAC. There are suggestions burtressing the fact that low molecular weight antioxidant that shows a high activity using one method may not show any activity when another method is used while an antioxidant that has low activity *in vivo* may show very high activity in an *in vitro* study.

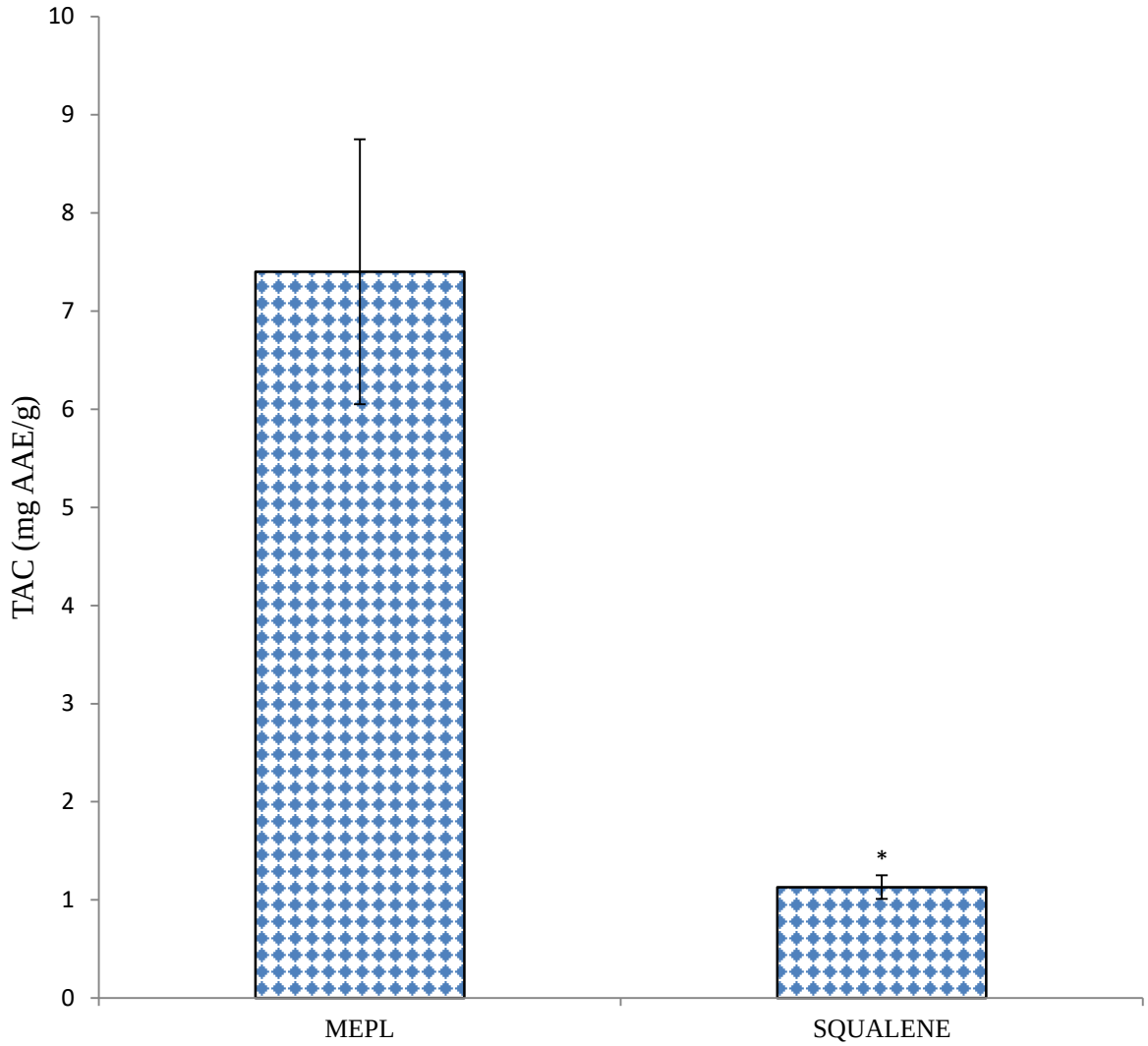


Fig. 4.6. Total antioxidant capacity (TAC) of MEPL and squalene.

Columns represent mean ± SEM, n = 5

*$p < 0.05$ as compared with MEPL

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

4.4 Result of acute toxicity and LD_{50} of methanol extract of *Parquetina nigrescens leaf*

The result of acute toxicity showed that 2000 mg/kg MEPL did not cause mortality after 24 hours and no signs and symptoms of toxicity after 14 days was observed in the female Wistar rats used (Table 4.3).

In this study, the LD_{50} of MEPL was greater than 5000 mg/kg bw using limit test in OECD 423 (2001). Aderibigbe *et al*. (2011) used Miller and Tainter's method (1944) and reported that LD_{50} of MEPL was 4500 mg/kg. Also, Awobajo *et al*. (2010) reported that the LD_{50} of aqueous extract of *Parquetina nigrescens* to be 12.60 g/kg and 13.10 g/kg using the methods described by Thompson (1947) and Finney (1952). Such wide variation in result is possible because of the differences in methodology used. This result indicates that the consumption of MEPL is safe and this informed the choice of the dosages (250, 500 and 1000 mg/kg) choosen in this work.

Table 4.3. Result of LD_{50} test of methanol extract of *Parquetina nigrescens* leaf

	No of animals used	Dosage of MEPL	No of dead animals after 24 hours	Sign of toxicity after 14 days
Step 1	3	2000 mg/kg	0	0
Step 2	3	2000 mg/kg	0	0

4.5 Effect of methanol extract of *Parquetina nigrescens* leaf on blood profile in arsenic trioxide-treated Wistar rats

4.5.1 Effect of methanol extract of *Parquetina nigrescens* leaf on packed cell volume and red blood cell count in arsenic trioxide-treated Wistar rats

The packed cell volume reduced ($p < 0.05$) significantly in the group administered with 1,000 mg/kg MEPL when compared with control (Figure 4.7). Likewise, the red blood cell count decreased ($p < 0.05$) in 1000 mg/kg MEPL when compared with control group (Figure 4.8).

It was observed that 1000 mg/kg of MEPL brought about a reduction in packed cell voulme (PCV) as well as red blood cell (RBC) count. This contradicts Saba *et al*. (2010) where treatment with1000 mg/kg aqueous extract of *Parquetina nigrescens* for four weeks did not cause any change in both PCV and RBC. Also, the result of this study contradicts the report of Omoboyowa *et al*. (2016) in which administration of 50 and 100 mg/kg *Parquetina nigrescens* within 21 days caused marked increase in both PCV and RBC and Agbor and Odetola (2001) in which 400, 800 as well as 1600 mg/kg extract of *Parquetina nigrescens* leaf for four weeks caused dose dependent increase in PCV as well as RBC. The variance of this study from previous studies could be due to difference in dosage or length of period of administration. In this study, the long period of administration being 54 days may have contributed to the effect of 1000 mg/kg MEPL to cause decrease PCV and RBC. The leaf of *Parquetina nigrescens* contains phytochemicals such as tannins and saponins both of which were reportedly discovered in this study. *In vitro* exposure to these two phytochemicals was reported to cause hemolysis and eryptosis of RBC (Bissinger *et al*., 2014). It is therefore possible that the long time exposure of the experimental animals to MEPL at a high dosage of 1000 mg/kg increased blood levels of saponin and tannin in the extracellular fluid which may have had an adverse effect on the PCV and RBC count.

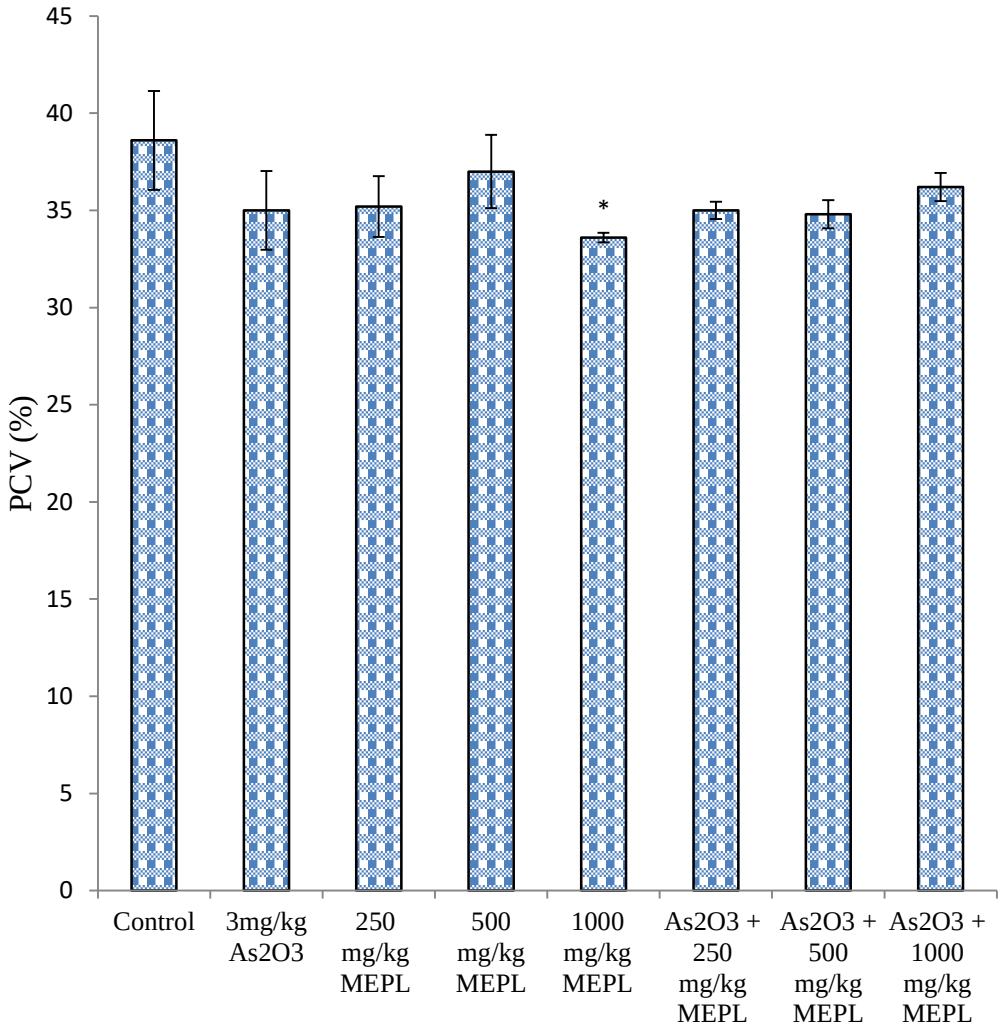


Fig.4.7. Effect of MEPL on packed cell volume in arsenic trioxide-treated Wistar rats

Columns represent mean ± SEM, n = 5.

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

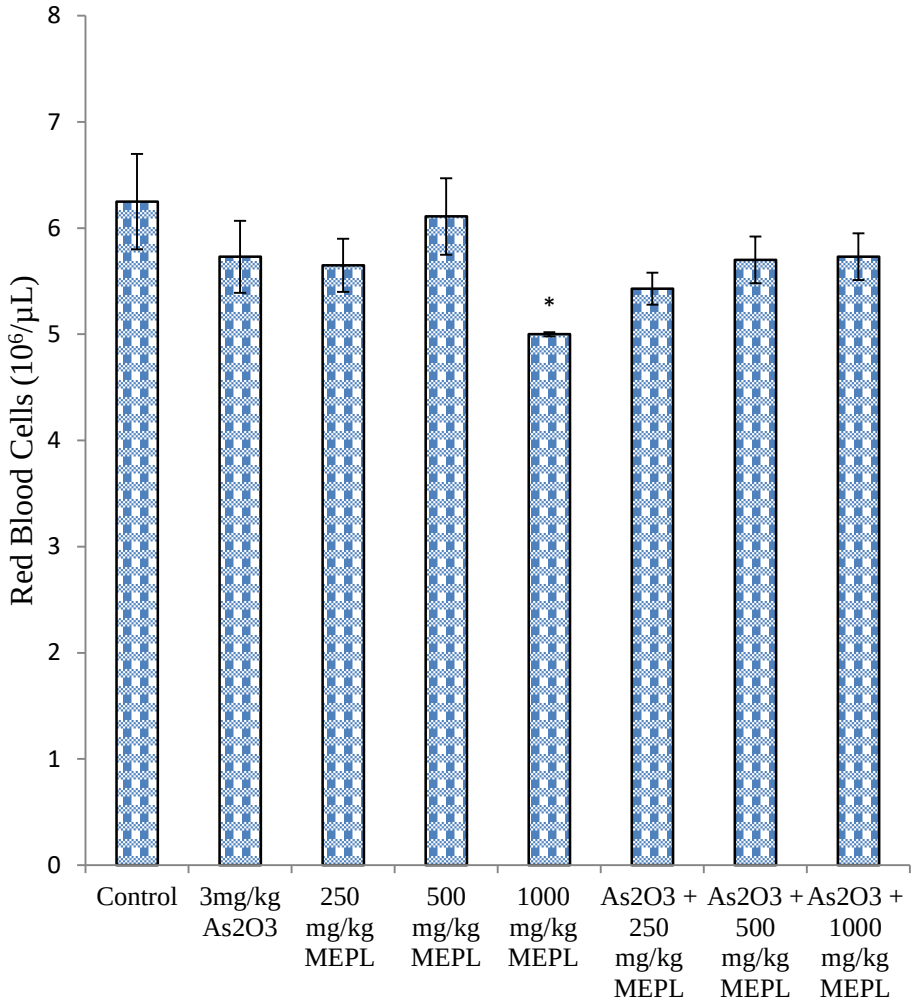


Fig. 4.8. Effect of MEPL on red blood cell count in arsenic trioxide-treated Wistar rats.

Columns represent mean ± SEM, n = 5.

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.5.2 Effect of methanol extract of *Parquetina nigrescens* leaf on haemoglobin concentration in arsenic trioxide-treated Wistar rats

There was no significant difference in haemoglobin concentration of arsenic trioxide group when compared with control. There was also no significant difference in the haemoglobin concentrations of groups treated As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + 1000 mg/kg MEPL when compared with control group and with arsenic trioxide only group (Figure 4.9).

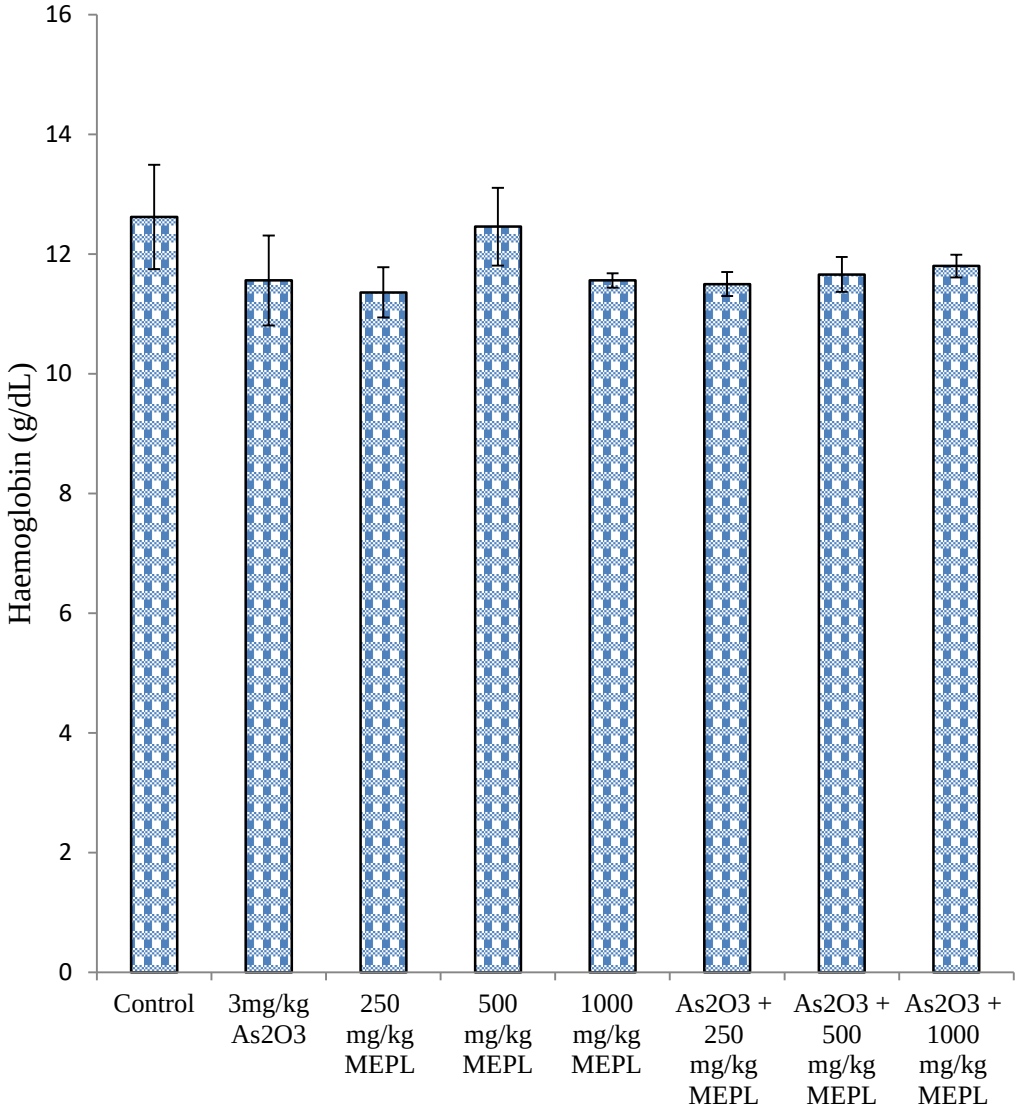


Fig.4.9. Effect of MEPL on haemoglobin concentration in arsenic trioxide-treated Wistar rats.

Columns represent mean ± SEM, n = 5.

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.5.3 Effect of methanol extract of *Parquetina nigrescens* leaf on white blood cell count and platelet count in arsenic trioxide-treated Wistar rats

The white blood cell count reduced ($p < 0.05$) in As_2O_3 only group when compared with control group. Also, an increase ($p < 0.05$) was observed in the groups administered with As_2O_3 + 250 mg/kg, As_2O_3 + 500 mg/kg and As_2O_3 +1000 mg/kg MEPL when compared with As_2O_3 alone group (Figure 4.10).

Platelet count significantly increased in 250 mg/kg MEPL, 500 mg/kg MEPL and 1000 mg/kg MEPL groups when compared with control group ($p < 0.05$). Also, an increase in platelet count was observed in As_2O_3 + 250 mg/kg MEPL and As_2O_3 + 500 mg/kg MEPL when compared with control and with As_2O_3 only group ($p < 0.05$) (Figure 4.11).

In this study, it was observed that there was a decreased WBC count in 3 mg/kg of arsenic only. This agrees with Kannan *et al*. (2001). Previous work done by other researchers revealed that exposure of experimental animals to arsenic causes leucopenia (Kyle and Pearse, 1965). The mechanism through which arsenic cause decreased WBC count may be through its capacity to cause apoptosis (Rousselot *et al*., 1999). However, it was observed that the effects of arsenic on WBC count was ameliorated by 250, 500 and 1000 mg/kg MEPL. Since increase in WBC count was not observed in MEPL only treated groups, but in the groups co-treated with MEPL and arsenic, the increase may likely be based on stimulation of granulopoietin in reaction to the reduced cells respectively (Saba *et al*., 2010).

The observed increase in platelet count in all the groups administered MEPL only and in the groups co-administered As_2O_3 and MEPL might have been triggered by the consistent administration of MEPL for a period of 54 days. This showed that MEPL may facilitate thrombopocytosis and may bring about quick hemostatic response to blood vessel damage. The normal range of values for platelete count was given as 150-460 x 10^3/mL by Johnson-Delaney (1996) and this shows that the increament in platelet count in this experiment was not detrimental to the health of the animals

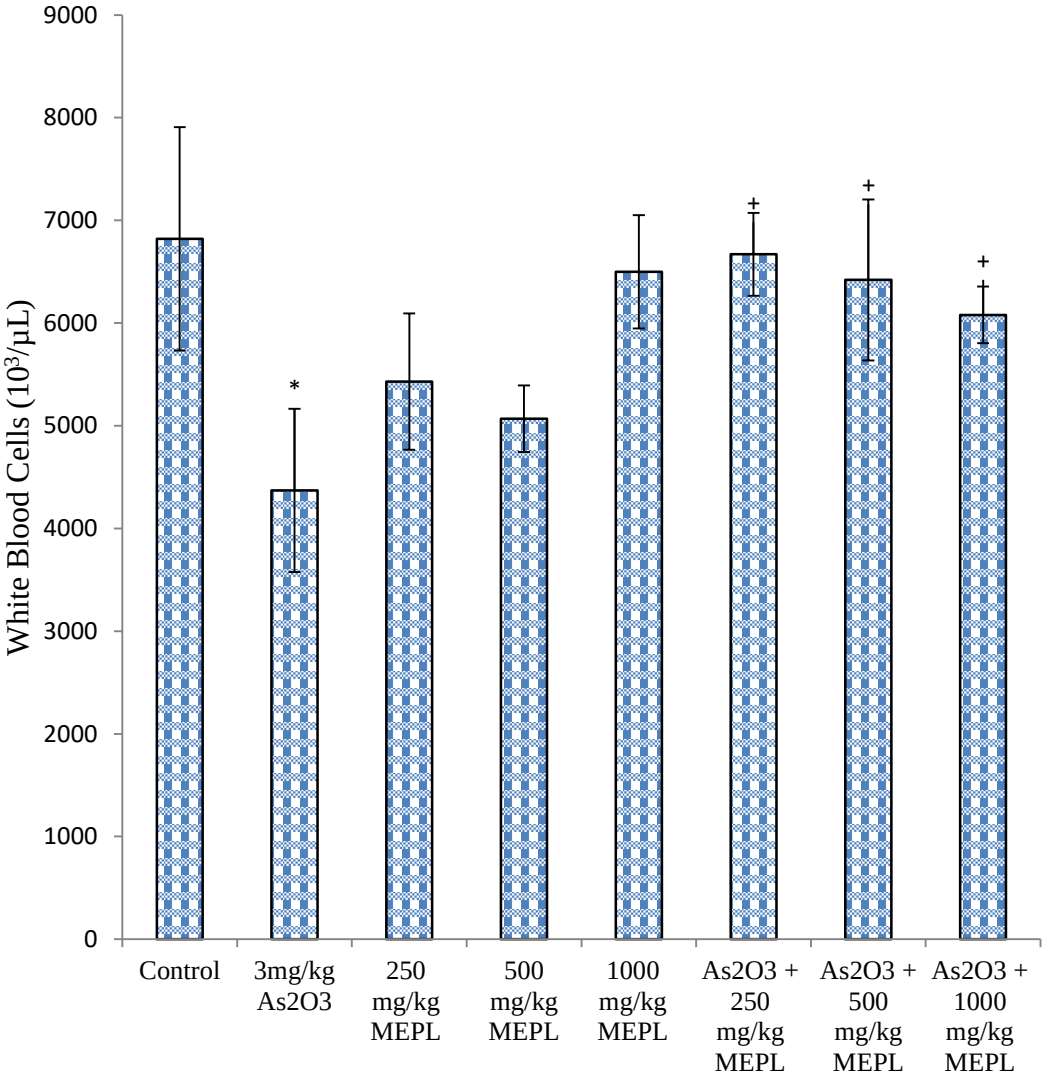


Fig. 4.10. Effect of MEPL on white blood cell count in arsenic trioxide-treated Wistar rats

Columns represent mean ± SEM, n = 5.

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

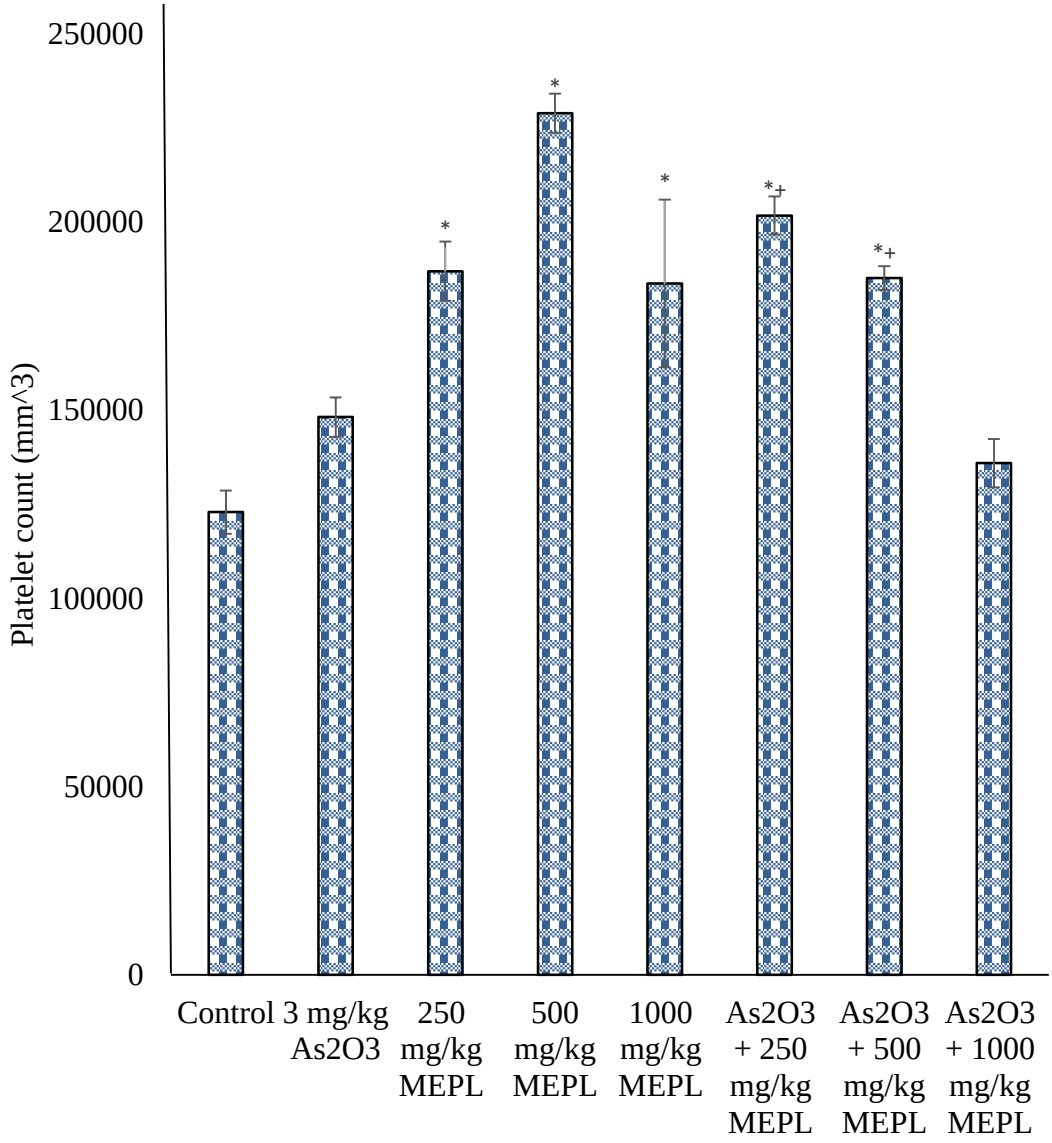


Fig. 4.11. Effect of MEPL on platelet count in arsenic trioxide-treated Wistar rats

Columns represent mean ± SEM, n = 5.

*, + $p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.5.4 Effect of methanol extract of *Parquetina nigrescens* leaf on neutrophil count in arsenic trioxide-treated Wistar rats

Neutrophil count increased ($p < 0.05$) in 500 mg/kg MEPL group when compared with control group (Figure 4.12).

The increase in neutrophil count in 500 mg/kg MEPL is in agreement with the report of Omoboyowa *et al*. (2016) in which ethanol and chloroform extract of *Parquetina nigrescens* leaf increased neutrophil count in Wistar rats. Neutrophils are known to phagocytose bacteria and fungi as well as produce enzymes, reactive oxygen species and cytokines (Malech *et al*., 2014). This result suggests that a dose of 500 mg/kg MEPL positively influences phagocytosis and first line of defence against infection.

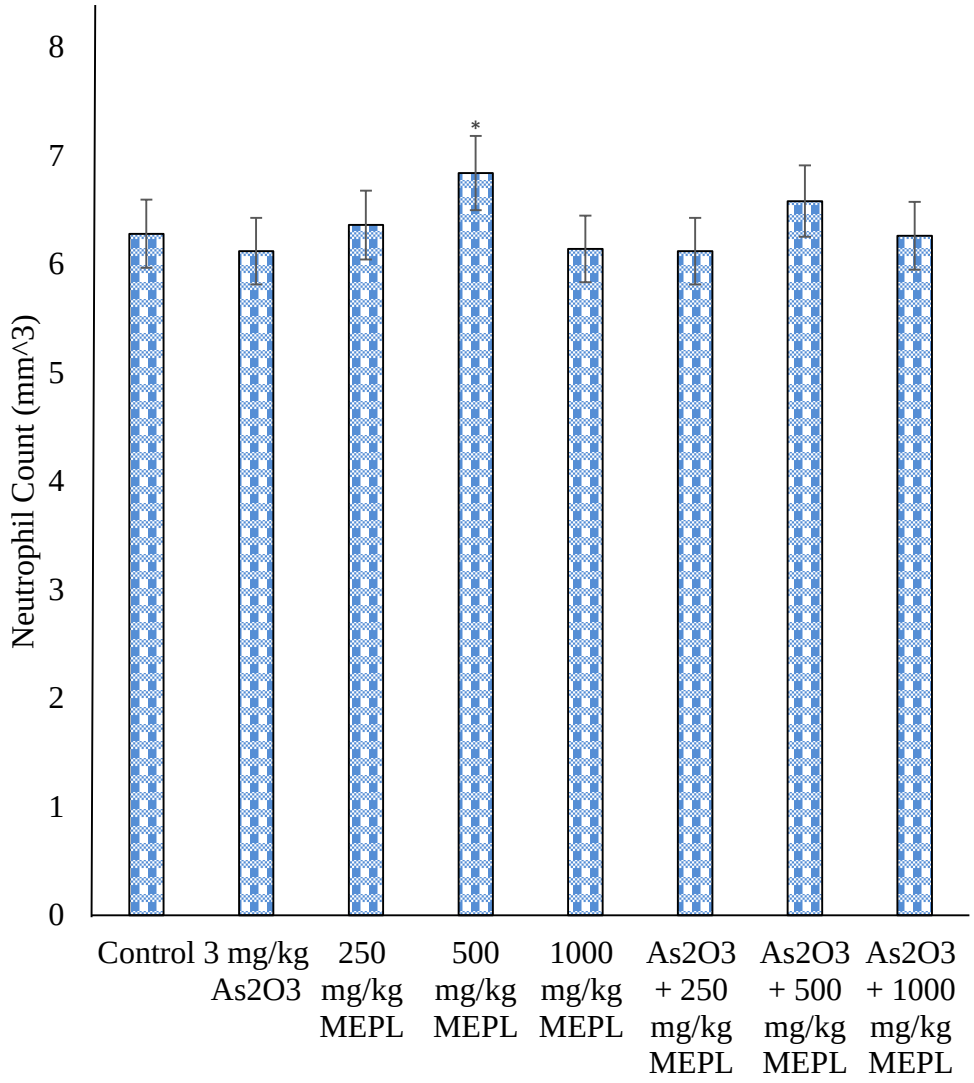


Fig. 4.12. Effect of MEPL on neutrophil count in arsenic trioxide-treated Wistar rats

Columns represent mean ± SEM, n = 5.

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.5.5 Effect of methanol extract of *Parquetina nigrescens* leaf on lymphocyte count in arsenic trioxide-treated Wistar rats

A reduction ($p < 0.05$) in lymphocyte count was seen in 500 mg/kg MEPL group when compared with control group (Figure 4.13).

The reduction of lymphocyte count observed in 500 mg/kg MEPL contradicts the report of Omoboyowa *et al*. (2016) in which ethanol extract of *Parquetina nigrescens* leaf increased lymphocyte count. Lymphocytes synthesize and liberate the γ-globulin of the plasma (Trowell, 1947). It is possible that the extract may not be able to play a role in acquired immunity since there is significant decrease in lymphocyte count.

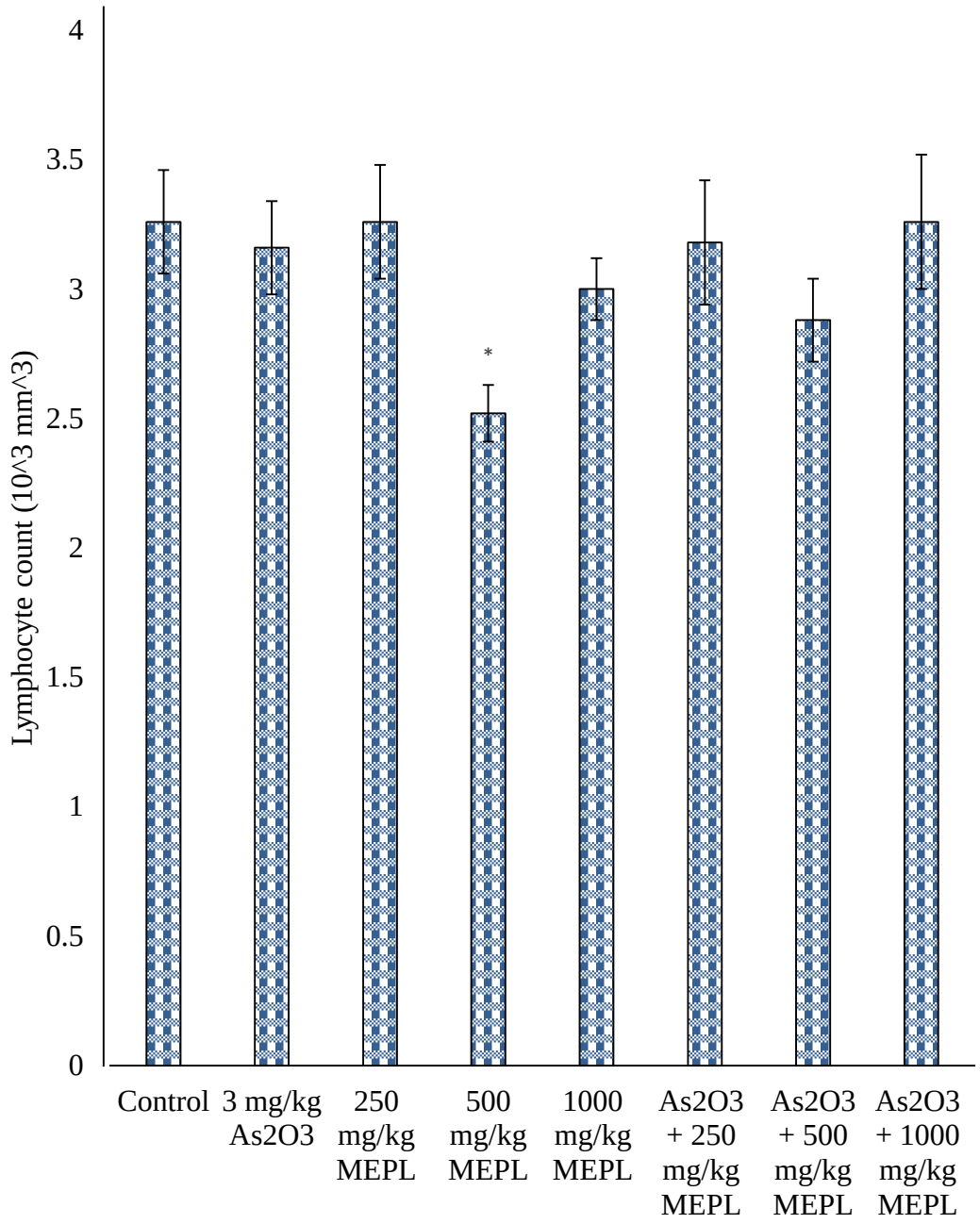


Fig. 4.13. Effect of MEPL on lymphocyte count in arsenic trioxide-treated Wistar rats.

Columns represent mean ± SEM, n = 5.

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.5.6 Effect of methanol extract of *Parquetina nigrescens* leaf on monocyte count in arsenic trioxide-treated Wistar rats

There was a significant decrease in monocyte count of groups treated with As_2O_3 + 250 mg/kg and As_2O_3 + 250 MEPL mg/kg when compared with the control and As_2O_3 only group (Figure 4.14).

Despite the fact that there was no significant changes in monocyte count of arsenic trioxide group only, the co-administration of arsenic trioxide and 250 or 500 mg/kg MEPL decreased monocyte count. This may be the fall out of decrease in WBC count caused by arsenic trioxide as observed in this same experiment, the three dosages of MEPL were able to ameliorate the decrease in other differential WBC components but 250 and 500 mg/kg could not. The arsenic trioxide + 1000 mg/kg MEPL group however did not show any difference from the control group. This indicated that 1000 mg/kg MEPL was able to maintain monocyte count despite exposure to arsenic trioxide.

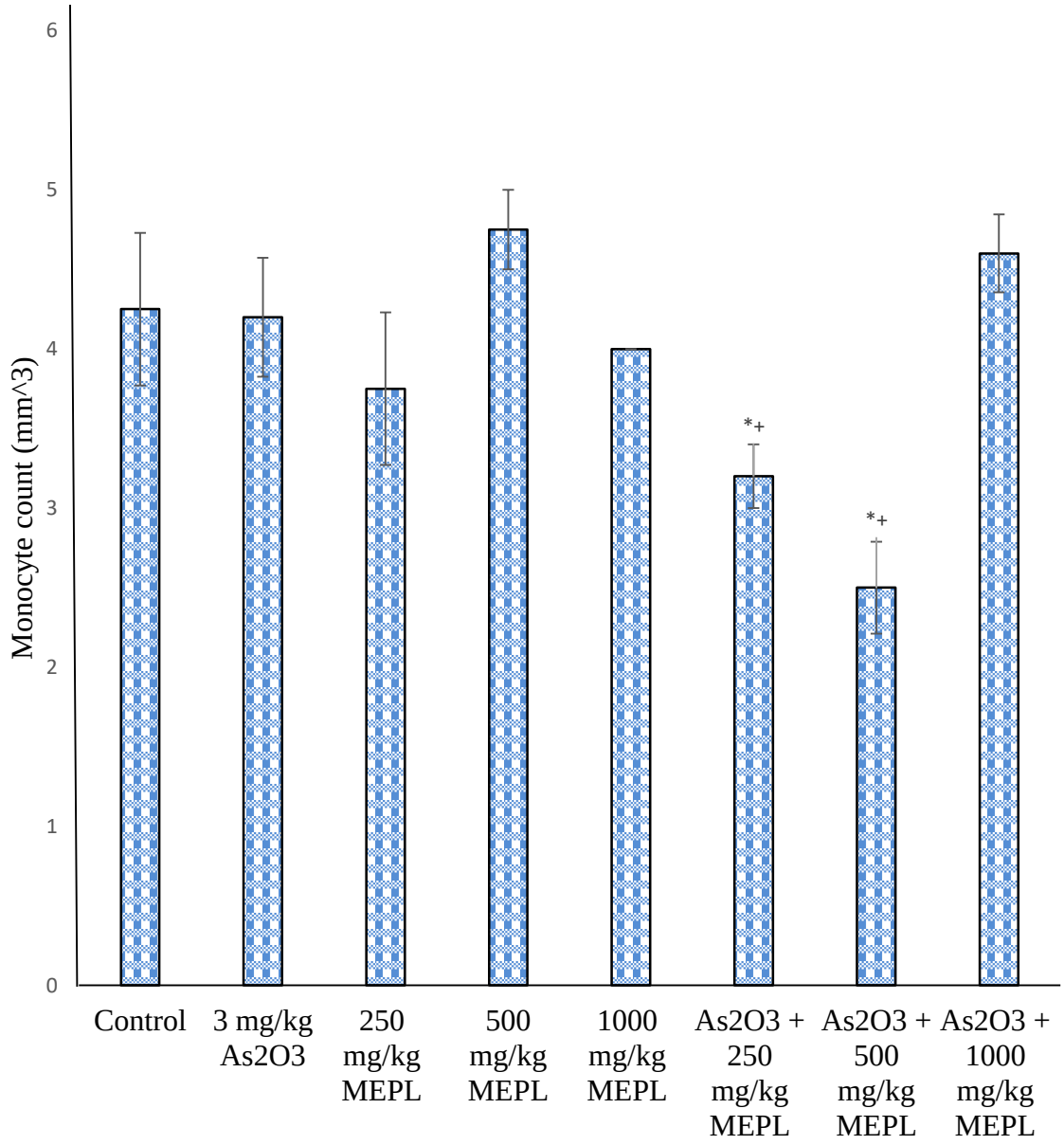


Fig. 4.14. Effect of MEPL on monocyte count in arsenic trioxide-treated Wistar rats.

Columns represent mean ± SEM, n = 5.

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.5.7 Effect of methanol extract of *Parquetina nigrescens* leaf on eosinophil count in arsenic trioxide-treated Wistar rats

There was a significant increase in eosinophil count of groups treated with As_2O_3 + 500 mg/kg MEPL when compared with As_2O_3 group (Figure 4.15).

The increase in eosinophil count observed in the group treated with As_2O_3 + 500 mg/kg showed that MEPL could help to improve the differential white blood cell count with respect to eosinophil. This assertion was based on the observed increase in WBC count in this same group, indicating that one of the ways MEPL increased WBC count was to increase eosinophil count.

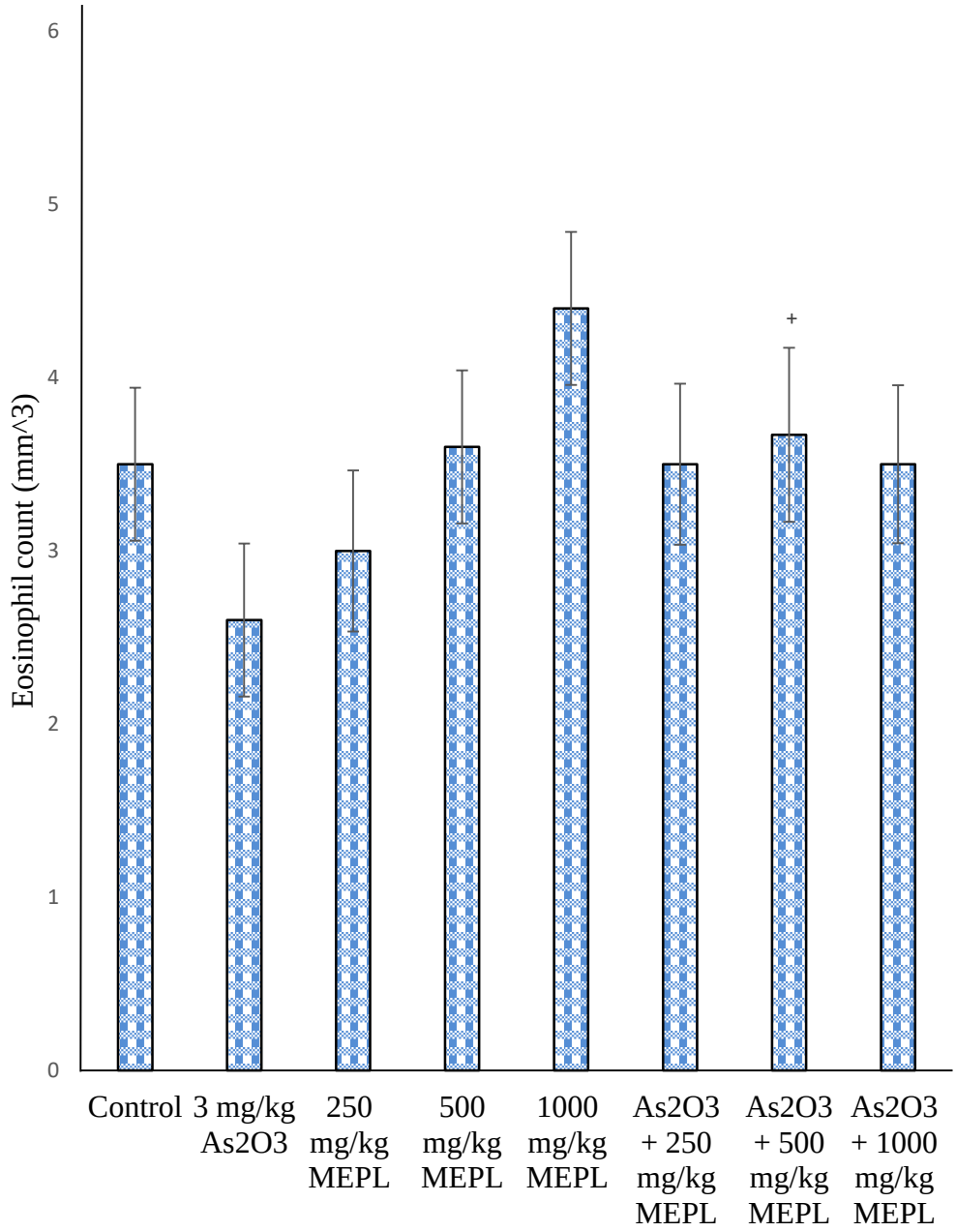


Fig. 4.15. Effect of MEPL on eosinophil count in arsenic trioxide-treated Wistar rats.

Columns represent mean ± SEM, n = 5.

$^{+}p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.6 Effect of methanol extract of *Parquetina nigrescens* leaf on liver oxidative status in arsenic trioxide-treated Wistar rats

4.6.1 Effect of methanol extract of *Parquetina nigrescens* leaf on liver malondialdehyde concentration in arsenic trioxide-treated Wistar rats

Malondialdehyde concentration significantly increased in As_2O_3 group when compared with control group ($p < 0.05$). There was also a significant decrease in MDA concentrations of groups co-administered with As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + 1000 mg/kg MEPL when compared with As_2O_3 alone group($p < 0.05$) (Figure 4.16).

In this study, the rise in liver MDA concentration seen in 3 mg/kg arsenic group only conforms to the report of Abu El-Saad *et al*. (2016) and Klibet *et al.* (2016). The measurement of MDA concentration is a means of evaluating lipid peroxidation level caused by oxidative stress. This result suggests a marked increase in free radicals in the liver which might have been mediated by arsenic treatment. It was however observed that all three dosages of MEPL ameliorated the lipid peroxidation effect of arsenic trioxide in the liver. The *Parquetina nigrescens* leaf has some phytochemical constituents such as phenol and flavonoids that participate in scavenging of free radicals. These phytochemicals might have mopped off the lipid peroxidative effects of arsenic in the liver cells which eventually lead to decreased MDA concentration in arsenic groups treated with all the three doses of MEPL.

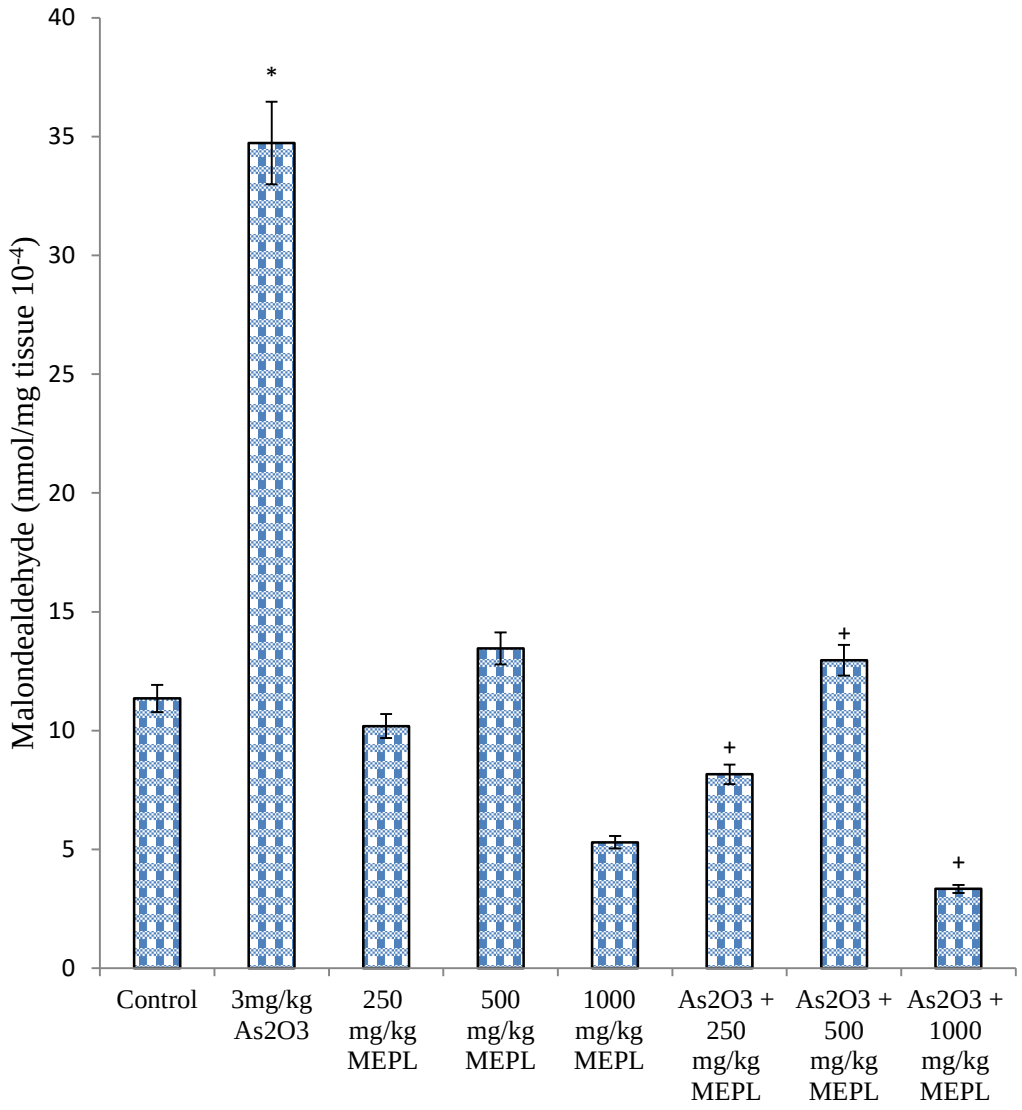


Fig. 4.16. Effect of MEPL on liver malondialdehyde concentration in arsenic trioxide-treated rats.

Columns represent mean ± SEM, n = 5.

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.6.2 Effects of methanol extract of *Parquetina nigrescens* leaf on liver superoxide dismutase activity in arsenic trioxide-treated Wistar rats

Superoxide dismutase activity decreased significantly in As_2O_3 group while an increase was observed in 250 mg/kg MEPL as well as 500 mg/kg MEPL ($p < 0.05$) when compared with control. The groups administered with As_2O_3 + 250 mg/kg MEPL and As_2O_3 + 1000 mg/kg MEPL showed an increase ($p < 0.05$) when compared with As_2O_3 only and control group respectively (Figure 4.17).

Arsenic trioxide caused a decrease in liver SOD activity of arsenic only treated group. Arsenic has been implicated in the alteration of SOD expression as it generates large amount of free radicals which can overwhelm the antioxidant defense mechanism (El-Demerdash *et al*., 2009). It was however observed that SOD activity in groups treated with arsenic and co-administered with 250 or 1000 mg/kg MEPL improved. This agrees with Ayoola *et al*. (2011) who suggested that flavonoid extract of MEPL induced production of SOD. This statement could be viewed as true because, the administration of 250 and 500 mg/kg MEPL alone brought about an increase in SOD activity in this study.

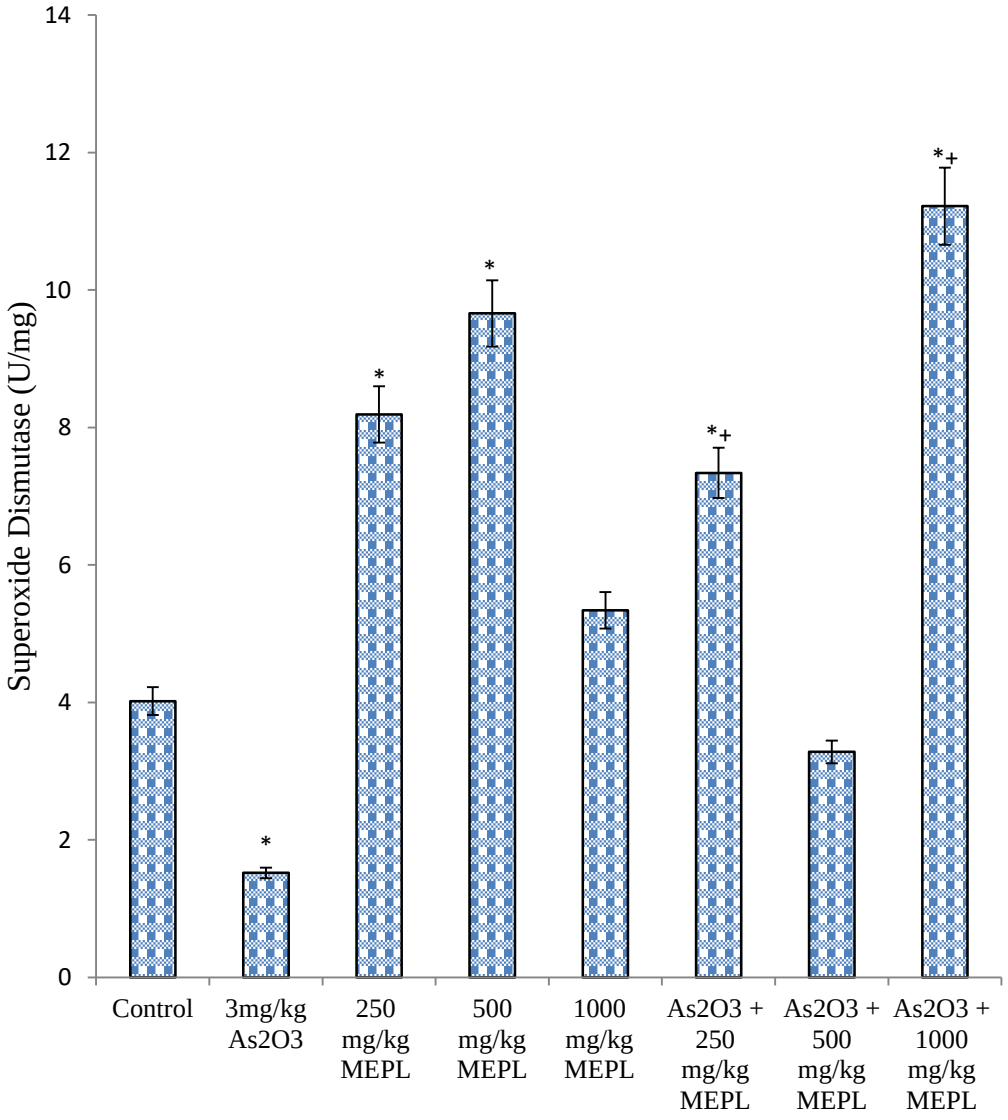


Fig.4.17. Effect of MEPL on liver SOD activity of arsenic trioxide-treated Wistar rats.

Columns represent mean ± SEM, n = 5.

*, $^{+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.6.3 Effect of methanol extract of *Parquetina nigrescens* leaf on liver catalase activity in arsenic trioxide-treated Wistar rats

An increase in liver catalase activity was observed in As_2O_3 + 250 mg/kg MEPL when compared with control group and As_2O_3 only group ($p < 0.05$) (Figure 4.18).

The amount of catalase was high only in arsenic group co-administered 250 mg/kg MEPL, the presence of arsenic could have led to the expression of catalase in liver cells in copious amount in order to keep the LPO activity of arsenic under check.

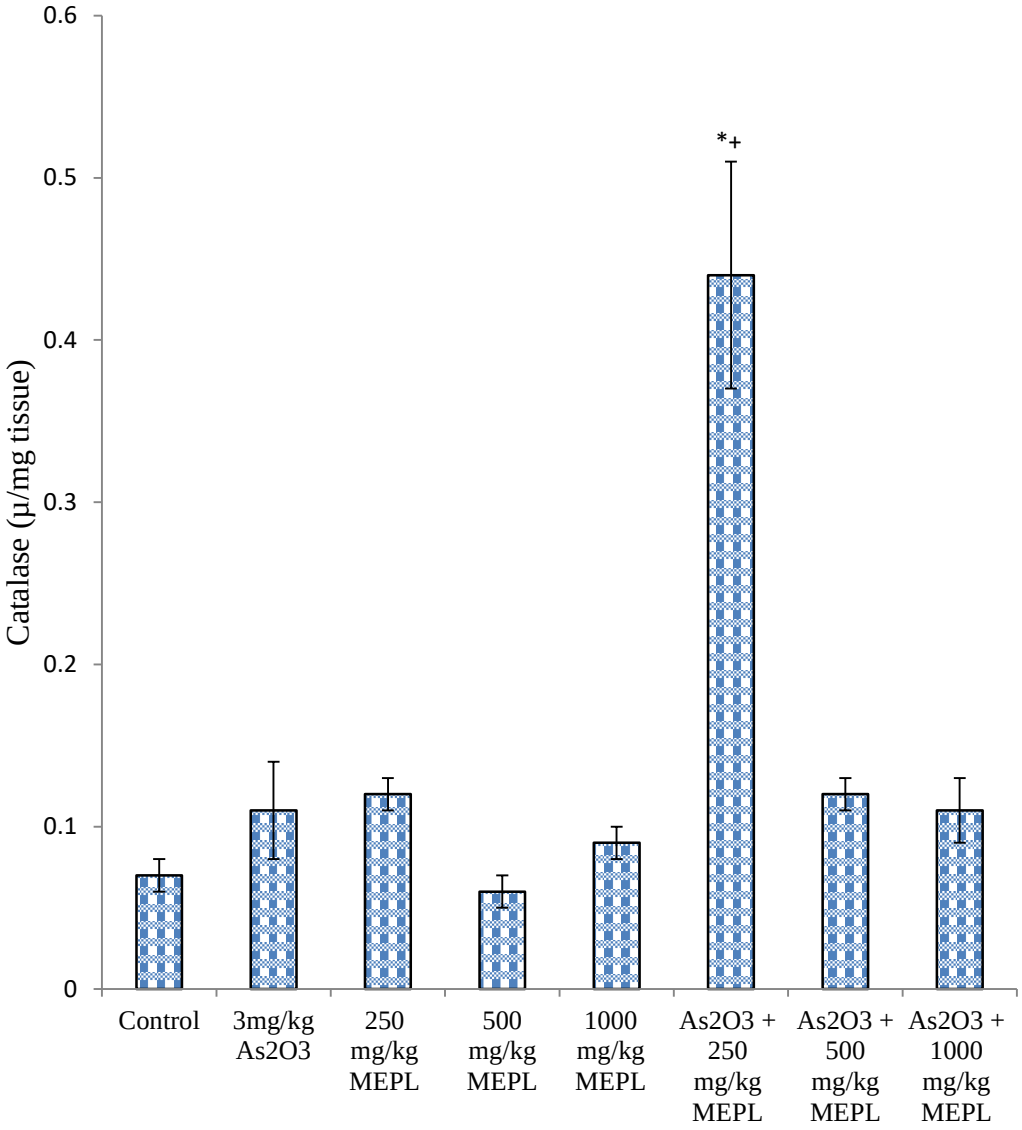


Fig. 4.18. Effect of MEPL on liver catalase activity in arsenic trioxide treated rats.

Columns represent mean ± SEM, n = 5.

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.7 Effect of methanol extract of *Parquetina nigrescens* leaf on liver aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities in arsenic trioxide-treated Wistar rats

There was an observed increase in liver AST in As_2O_3 group and in 500 mg/kg MEPL group in relations to control group ($p < 0.05$). Also, AST decreased in groups administered As_2O_3 + 500 mg/kg MEPL or As_2O_3 +1000 mg/kg MEPL when compared with As_2O_3 group ($p < 0.05$) (Figure 4.19).

Liver Alanine Aminotransferase (ALT) activity increased ($p < 0.05$) in As_2O_3 only group when compared with control group while, a decrease ($p < 0.05$) was seen in the groups administered As_2O_3 + 500 mg/kg MEPL and As_2O_3 +1000 mg/kg MEPL when compared with As_2O_3 only group (Figure 4.20).

Alkaline Phosphatase (ALP) activity increased ($p < 0.05$) significantly in As_2O_3 group and in 250 mg/kg MEPL when compared with control group. Also, an increase ($p < 0.05$) was observed in groups treated with As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + 1000 mg/kg MEPL ($p < 0.05$) when compared with control group (Figure 4.21).

The liver carries out an important function in metabolism as most drugs orally administered undergo first pass metabolism in the liver (Malcom, 1972), it was therefore deemed fit to evaluate the influence of both arsenic and MEPL administration on liver. The AST, ALT and ALP are produced by liver in large amount; they serve as indicator of hepatic cell injury (Klaassen and Watkin, 1984). In this study, increase in the three enzymes was seen in the arsenic trioxide treated alone. This agrees with Messarah *et al*. (2012). The decrease in AST and ALT in the groups co-administered arsenic and 500 or 1000 mg/kg MEPL suggests that it has hepatoprotective effect.

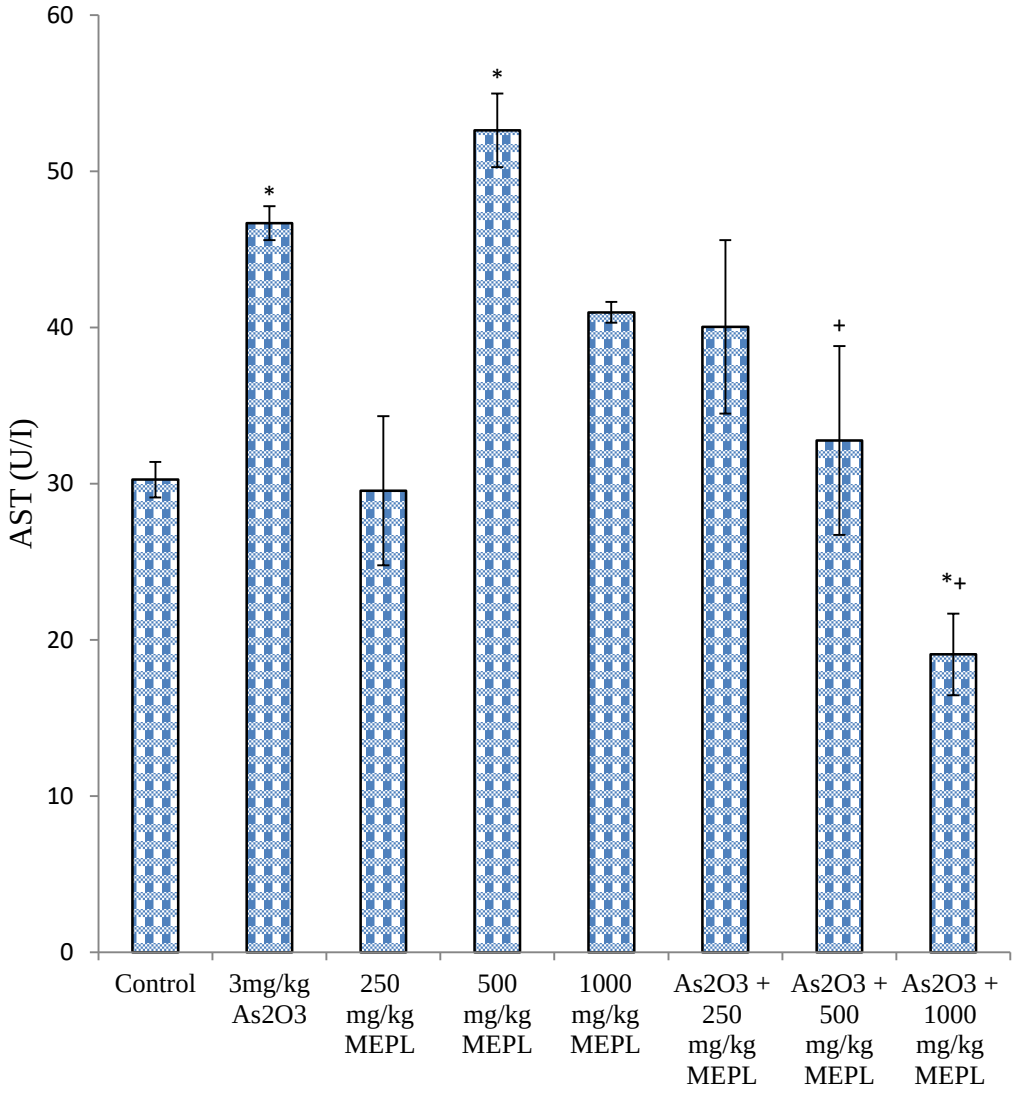


Fig. 4.19. Effects of MEPL on liver aspartate aminotransferase activity in arsenic trioxide-treated Wistar rats.

Columns represent mean ± SEM, n = 5.

$^{*, +}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

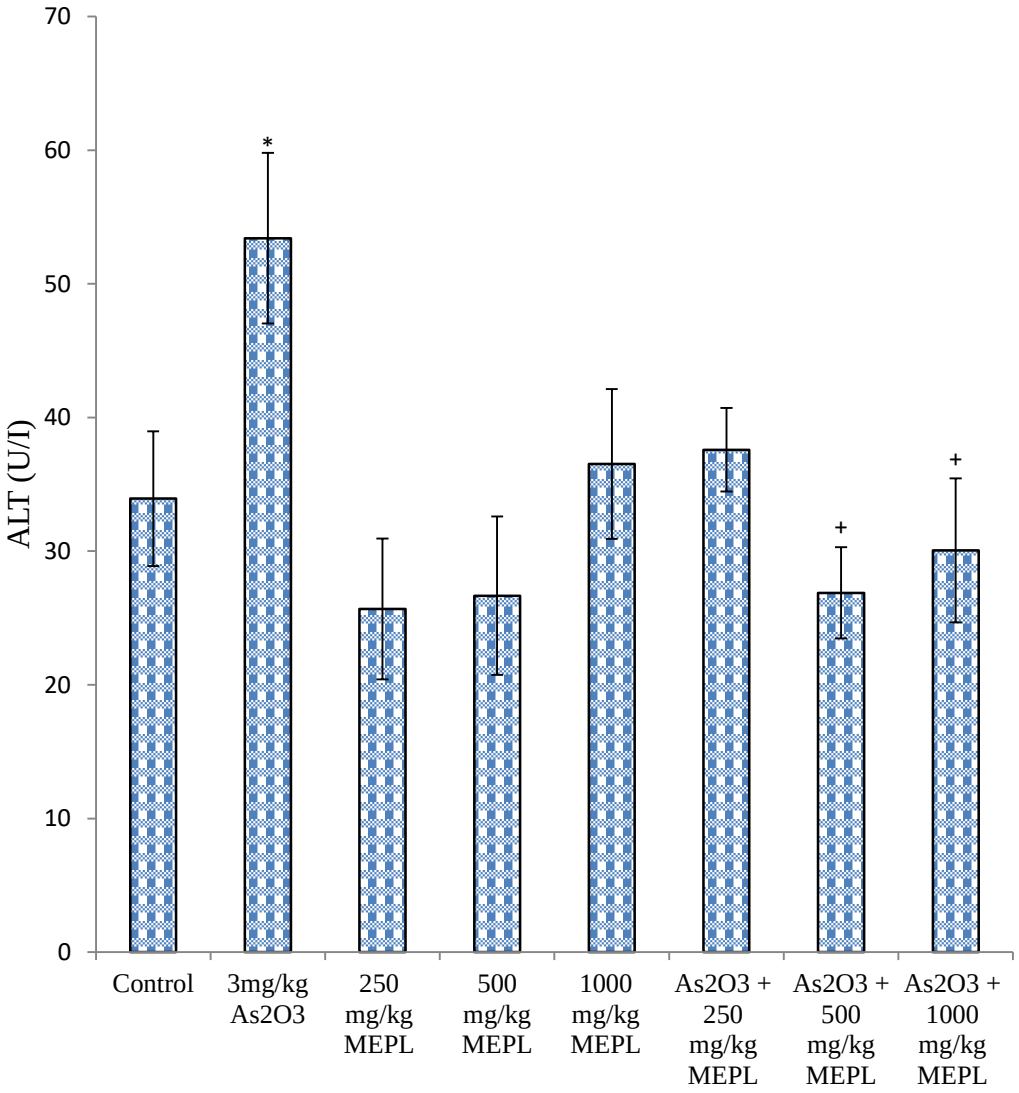


Fig. 4.20. Effects of MEPL on liver alanine aminotransferase activity in arsenic trioxide-treated Wistar rats.

Columns represent mean ± SEM, n = 5.

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

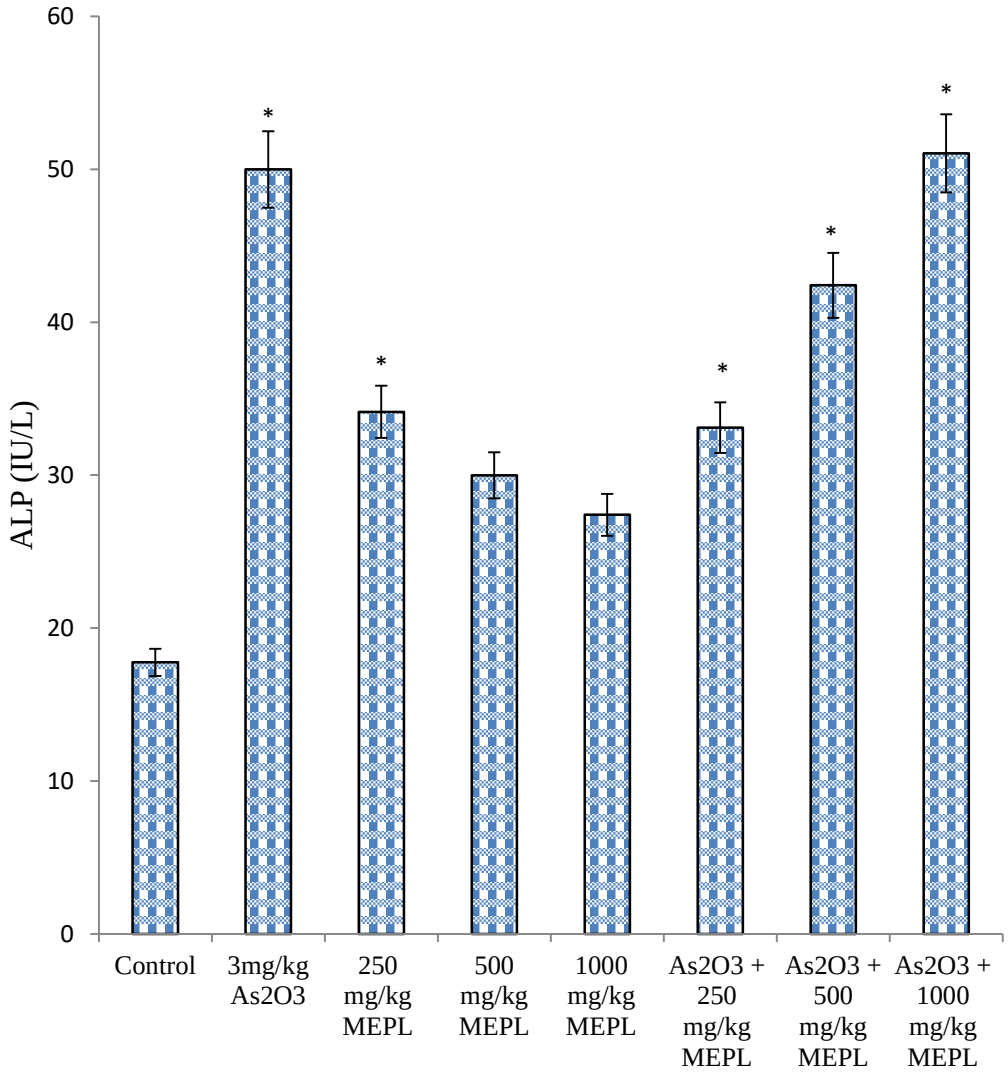


Fig. 4.21. Effects of MEPL on liver alkaline phosphatase activity in arsenic trioxide treated Wistar rats.

Columns represent mean ± SEM, n = 5.

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.8 Effect of methanol extract of *Parquetina nigrescens* leaf on sperm characteristics in arsenic trioxide-induced reproductive toxicity in male Wistar rats

4.8.1 Effect of methanol extract of *Parquetina nigrescens* leaf on sperm count in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Sperm count significantly reduced in As_2O_3 group alone when compared with control group ($p < 0.05$). Sperm count significantly increased in the group administered As_2O_3 + 500 mg/kg MEPL when compared with As_2O_3 group alone ($p < 0.05$). Also a significant increase was observed in the groups treated with As_2O_3 + 250 mg/kg MEPL and As_2O_3 + 1000 mg/kg MEPL when compared with control and with As_2O_3 group only respectively ($p < 0.05$) (Figure 4.22).

A reduction in sperm count was observed in arsenic alone. This supports Chang *et al*. (2007). Record has it that arsenic is implicated in causing oxidative stress in the testes, thereby, compromising sperm membrane integrity and depression of testicular spermatogenic functions and consequently low sperm count (Hayakawa *et al*., 2005). However, 250, 500 and 1000 mg/kg MEPL were able to improve sperm count in arsenic trioxide treated groups. This suggests that MEPL has protective effects on the spermatogenic functions of the testis which may be accorded to its tendency to combat the oxidative stress induced by arsenic in the testes as reported by Hayakawa *et al*. (2005). Interestingly, the groups co-administered with arsenic and 250 or 1000 mg/kg MEPL revealed an increament in sperm count above control group. This effect might have been stimulated by arsenic-induced decreased sperm count since the MEPL groups alone did not show any difference in number of spermatozoa.

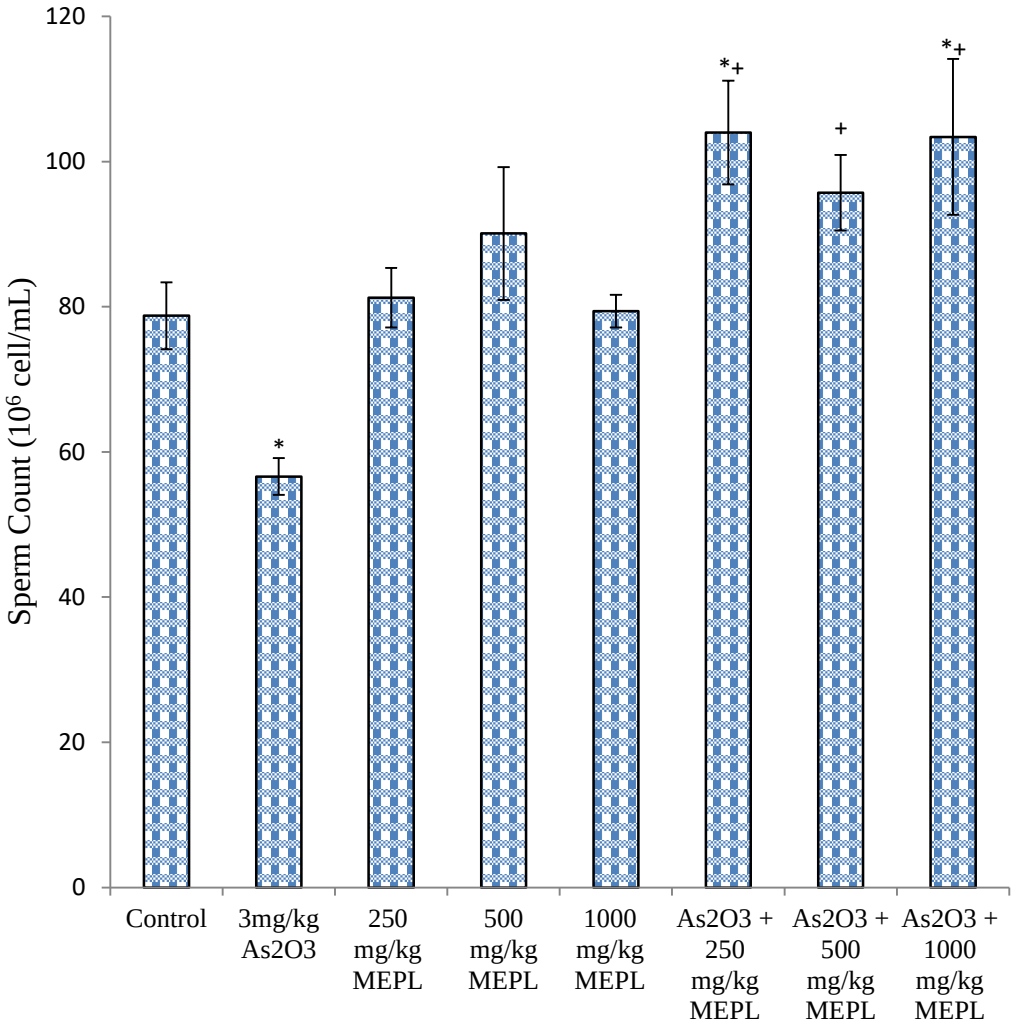


Fig. 4.22. Effects of MEPL on sperm count in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.8.2 Effect of methanol extract of *Parquetina nigrescens* leaf on sperm motility, progressive sperm motility and sperm viability in arsenic trioxide-induced reproductive toxicity in male Wistar rats

A significant reduction in sperm motility was observed in As_2O_3 only group when compared with control group ($p < 0.05$) (Figure 4.23).

A decrease in progressive sperm motility was observed in As_2O_3 only when compared with control group ($p < 0.05$) (Figure 4.24).

A significant reduction was observed in sperm viability of As_2O_3 group and in As_2O_3 + 1000 mg/kg MEPL when compared with control group ($p < 0.05$). Also, sperm viability increased ($p < 0.05$) sperm viability was observed in As_2O_3 + 250 mg/kg MEPL and As_2O_3 + 500 mg/kg MEPL when compared with As_2O_3 only ($p < 0.05$) (Figure 4.25).

Reduced spermatozoa movement, progressive motility as well as sperm viability were observed in arsenic trioxide group; this is in consonance with the works of Momeni and Eskandari (2016). Arsenic binds with thiols in sulphydryl groups of proteins in the flagellum and nuclear chromatin of sperm which contains large amount of thiols, thus providing binding sites for arsenic and leading to decrease in synthesis of enzymes required for motility. This decreases motility of sperms (Chang *et al*., 2007). The groups treated with As_2O_3 + 250 mg/kg MEPL and As_2O_3 + 500 mg/kg MEPL showed improved sperm viability. This may indicate that MEPL protect spermatozoa from toxicity of arsenic trioxide.

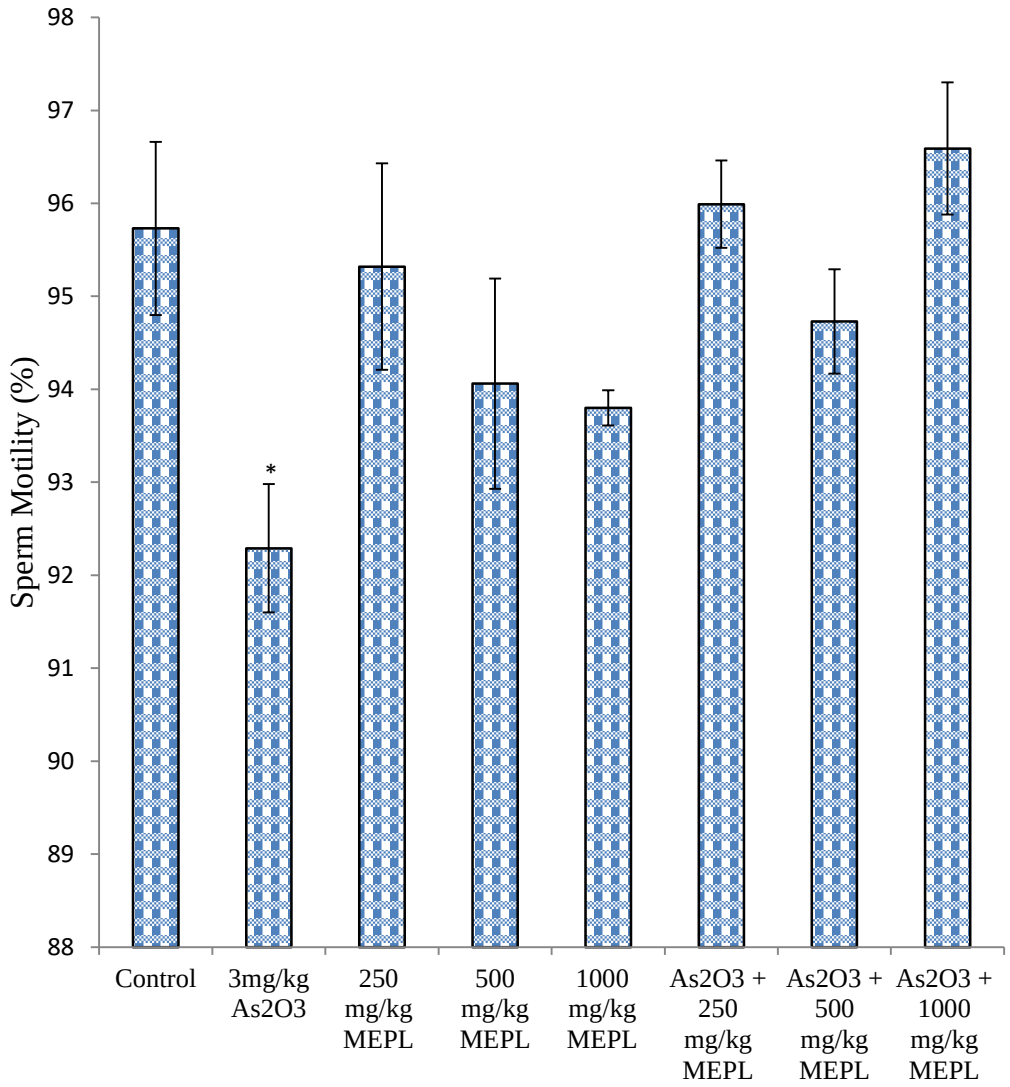


Fig. 4.23. Effects of MEPL on sperm motility in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

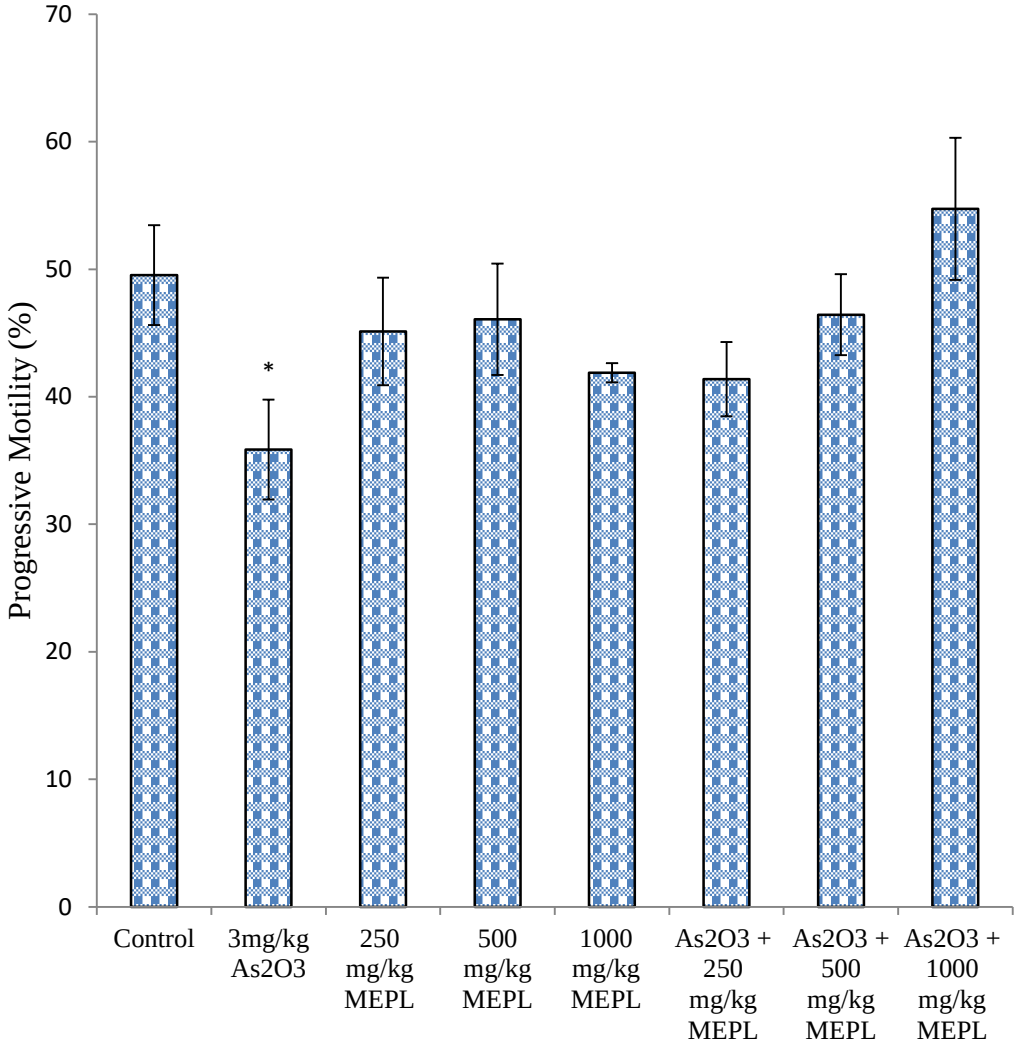


Fig. 4.24. Effects of MEPL on progressive sperm motility in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

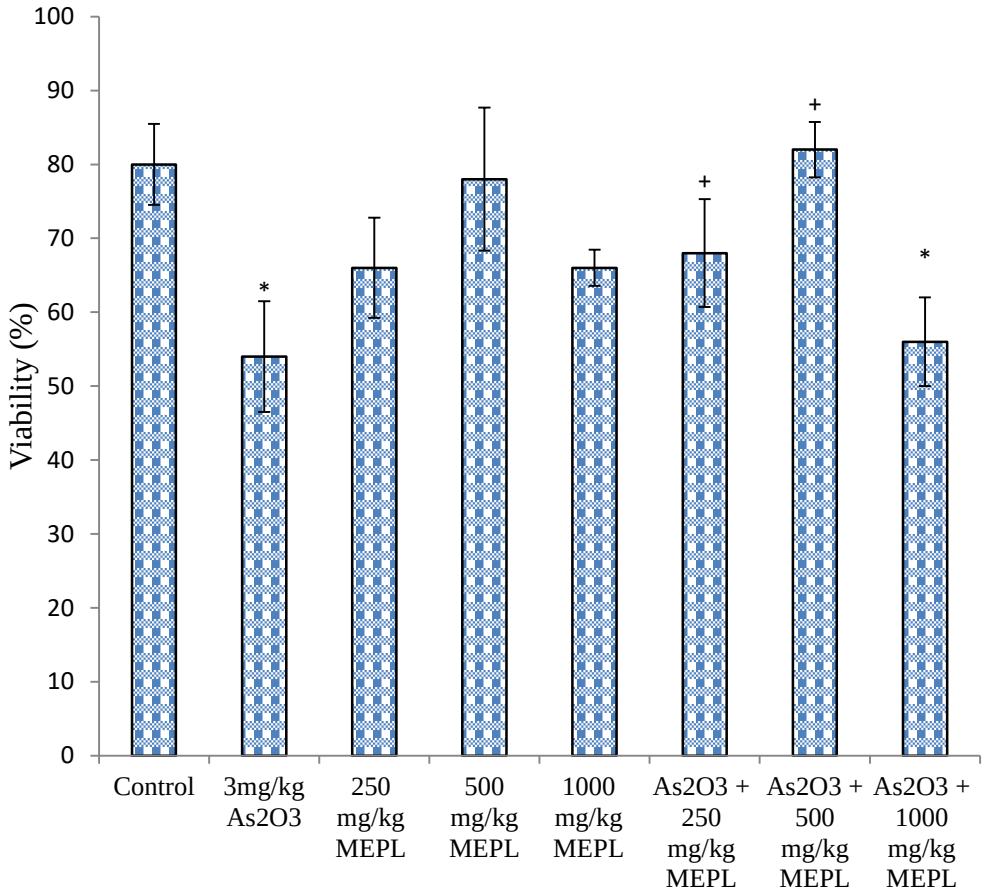


Fig. 4.25. Effects of MEPL on sperm viability in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.8.3 Effect of methanol extract of *Parquetina nigrescens* leaf on sperm normal morphology in arsenic trioxide-induced reproductive toxicity in male Wistar rats

The percentage of normal sperm morphology reduced significantly in rats administered As_2O_3 alone when compared with control group, while the groups administered As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + 1000 mg/kg MEPL showed increase ($p < 0.05$) in percentage of normal sperm morphology when compared with As_2O_3 only (Figure 4.26).

A decrease in percentage of normal morphology of spermatozoa was noticed in arsenic group alone. This is similar to the report of Momeni and Eskandari (2016). Arsenic has been reported to decrease spermatogenic functions of the testis by reducing synthesis and release of luteinizing gonadotropins as well as testosterone. Although these hormones were not assayed in this phase of the study but in subsequent phase, a similar result in the hormones level was derived. It was however observed that normal sperm morphology in the groups co-treated with arsenic and 250, 500 or 1000 mg/kg MEPL was restored. MEPL may therefore be attributed with a spermatogenic protective ability.

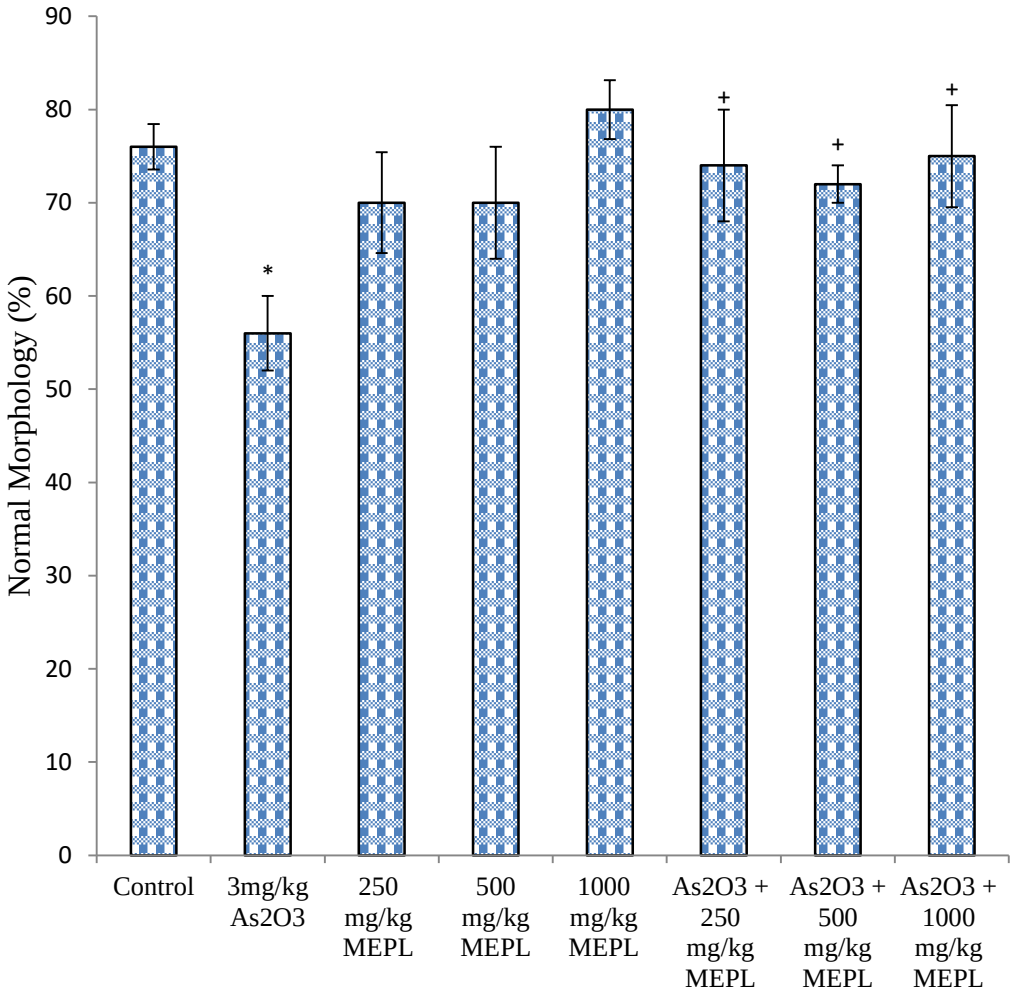


Fig. 4.26. Effects of MEPL on normal sperm morphology in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n =5

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.9 Effect of methanol extract of *Parquetina nigrescens* leaf on testicular oxidative status in arsenic trioxide-induced reproductive toxicity in male Wistar rats

4.9.1 Effect of methanol extract of *Parquetina nigrescens* leaf on testicular malondialdehyde concentration in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Testicular MDA concentration significantly increased in As_2O_3 group when compared with control group ($p < 0.05$), while a significant reduction was observed in As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL or As_2O_3 + 1000 mg/kg MEPL when compared with As_2O_3 group only ($p < 0.05$) (Figure 4.27).

Methanol extract of *Parquetina nigrescens* leaf extract has been credited with free radical scavenging activity which in turn reduces oxidative stress as demonstrated in this study. Testicular MDA concentration reduced in arsenic groups co-administered the three dosages of MEPL. The reduction in MDA concentration might also have led to the improvement in sperm viability of groups co-treated with arsenic trioxide and MEPL.

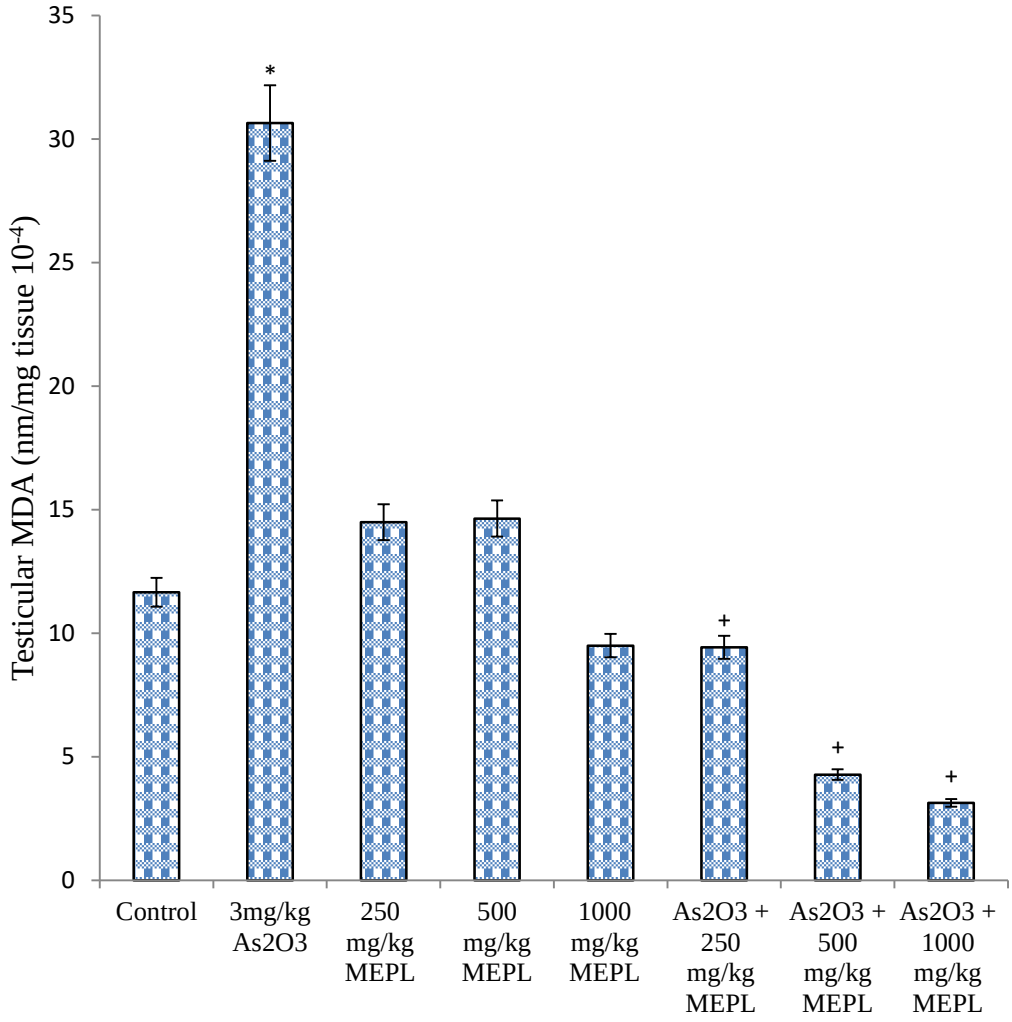


Fig. 4.27. Effects of MEPL on testicular malondialdehyde in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5.

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.9.2 Effect of methanol extract of *Parquetina nigrescens* leaf on testicular superoxide dismutase activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Testicular SOD activity significantly increased in 1000 mg/kg MEPL when compared with control group ($p < 0.05$), it equally increased significantly in As_2O_3 + 250 mg/kg MEPL and As_2O_3 + 500 mg/kg MEPL when compared with control group and As_2O_3 group respectively ($p < 0.05$) (Figure 4.28).

In this study, an increase was observed in testicular SOD of groups treated with 1000 mg/kg MEPL and this effect was also seen in groups co-treated with arsenic trioxde and 250 or 500 mg/kg MEPL. The 1000 mg/kg MEPL may have caused the preservation of endogenous SOD in the animals due to its own antioxidant properties, thereby making more SOD available in the assay or MEPL itself might have stimulated the production of SOD in the group treated with 1000 mg/kg MEPL or in the groups co-treated with arsenic trioxide and 250 or 500 mg/kg MEPL.

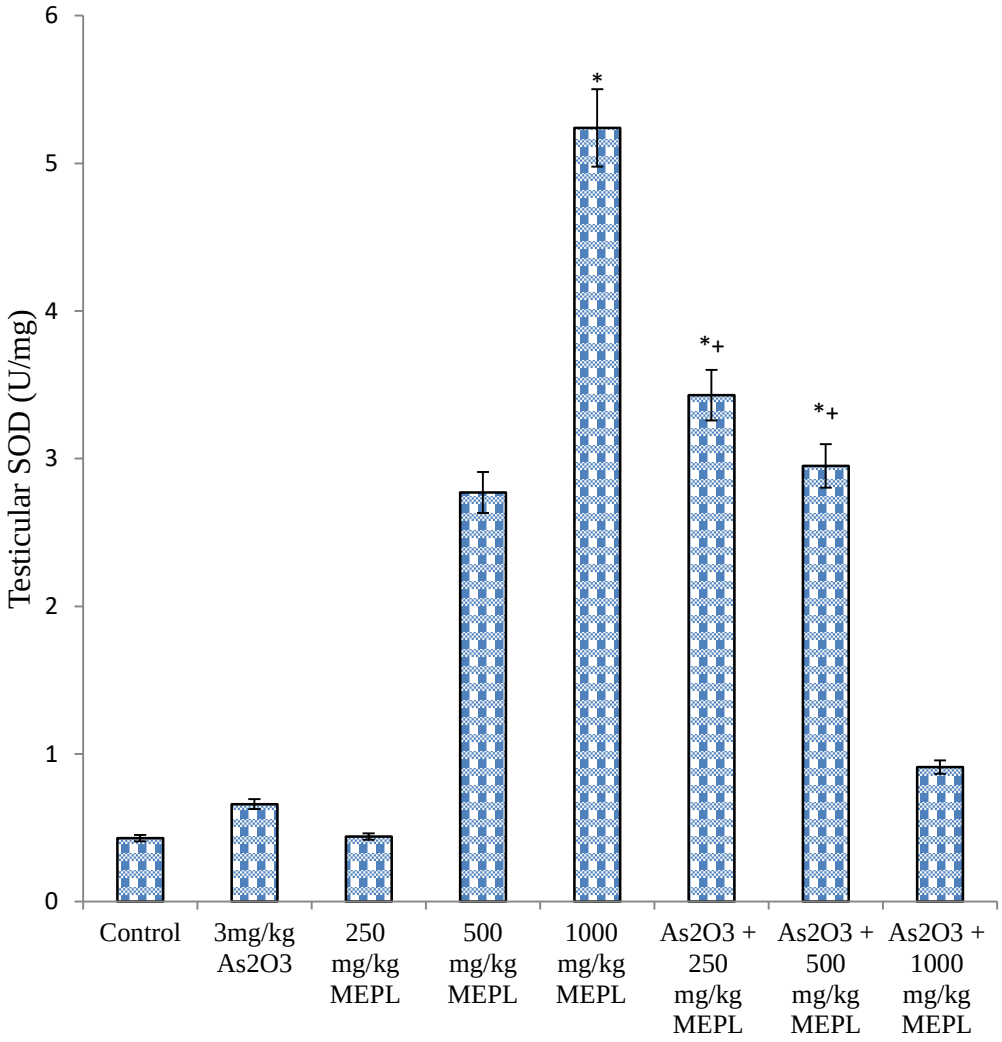


Fig.4.28. Effects of MEPL on normal testicular SOD activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5.

*, $^{+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.9.3 Effect of methanol extract of *Parquetina nigrescens* leaf on testicular catalase activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

There was no significant difference in catalase activity of As_2O_3 only group when compared with control group. There was also no significant difference in As_2O_3 + 250 mg/kg, As_2O_3 + 500 mg/kg and As_2O_3 + 1000 mg/kg when compared with control or with As_2O_3 only group (Figure 4.29).

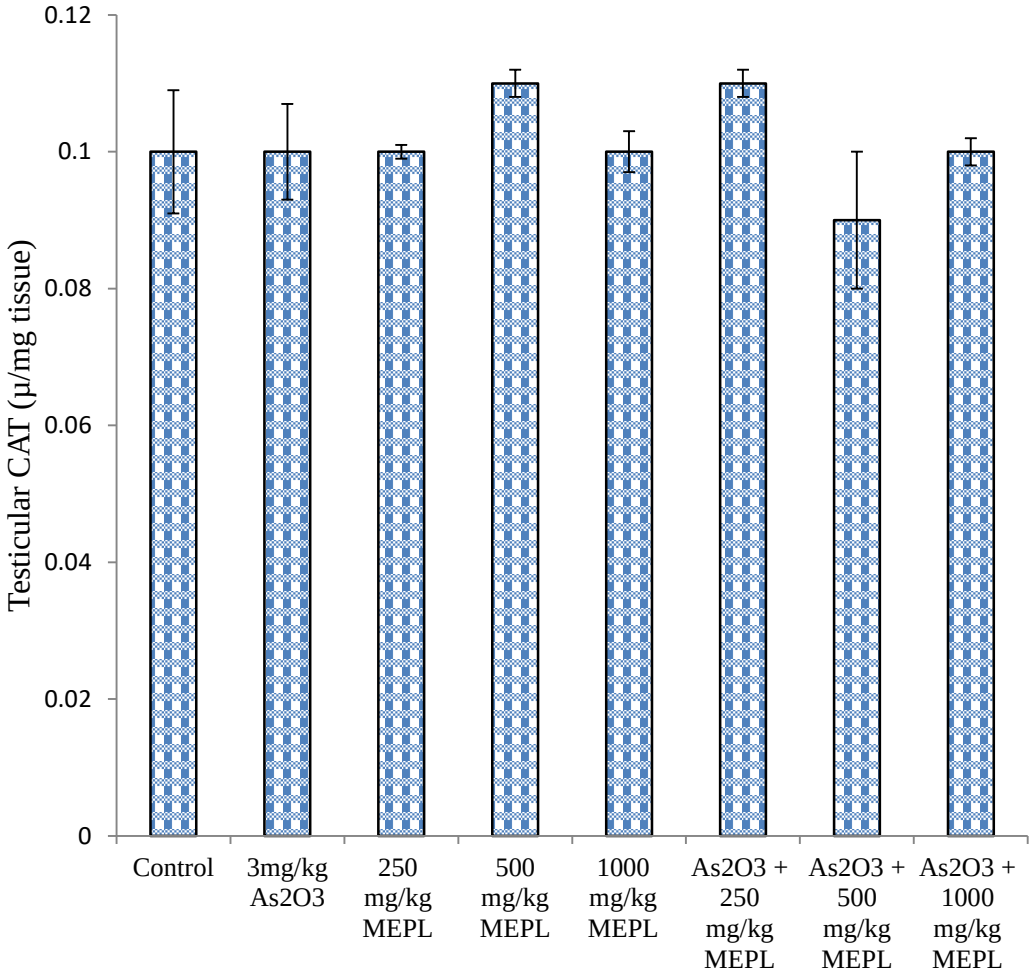


Fig.4.29. Effects of MEPL on normal testicular catalase activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

4.10 Effect of methanol extract of *Parquetina nigrescens* leaf on testicular histology in arsenic trioxide-treated Wistar rat

The histology of the As_2O_3 group showed atrophy of seminiferous tubules, empty seminiferous tubule lumen and absence of germ cell layers, but the interstitial spaces appeared normal when compared with the control which showed a normal histoarchitecture of seminiferous tubules. The histology of all the other experimental groups appeared normal and comparable with the control group (Plate 4.1).

In this study, arsenic trioxide disrupted the histoarchitecture of testis. This was demonstrated by the absence of matured spermatozoa within the seminiferous tubule lumen, widened lumen, and absence of germ cell layers with the presence of only a few spermatogonia. This supports Sarkar *et al*. (2008). As observed earlier in this study, arsenic brought about a marked rise in MDA as well as a concomitant fall in SOD in the testicular tissue, thereby, exposing the germ cells and the spermatozoa to oxidative damage which might have lead to their death. However, the groups treated with As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 +1000 mg/kg MEPL had their germ cells protected from arsenic-induced damage. This may be due to the antioxidant contained in MEPL.

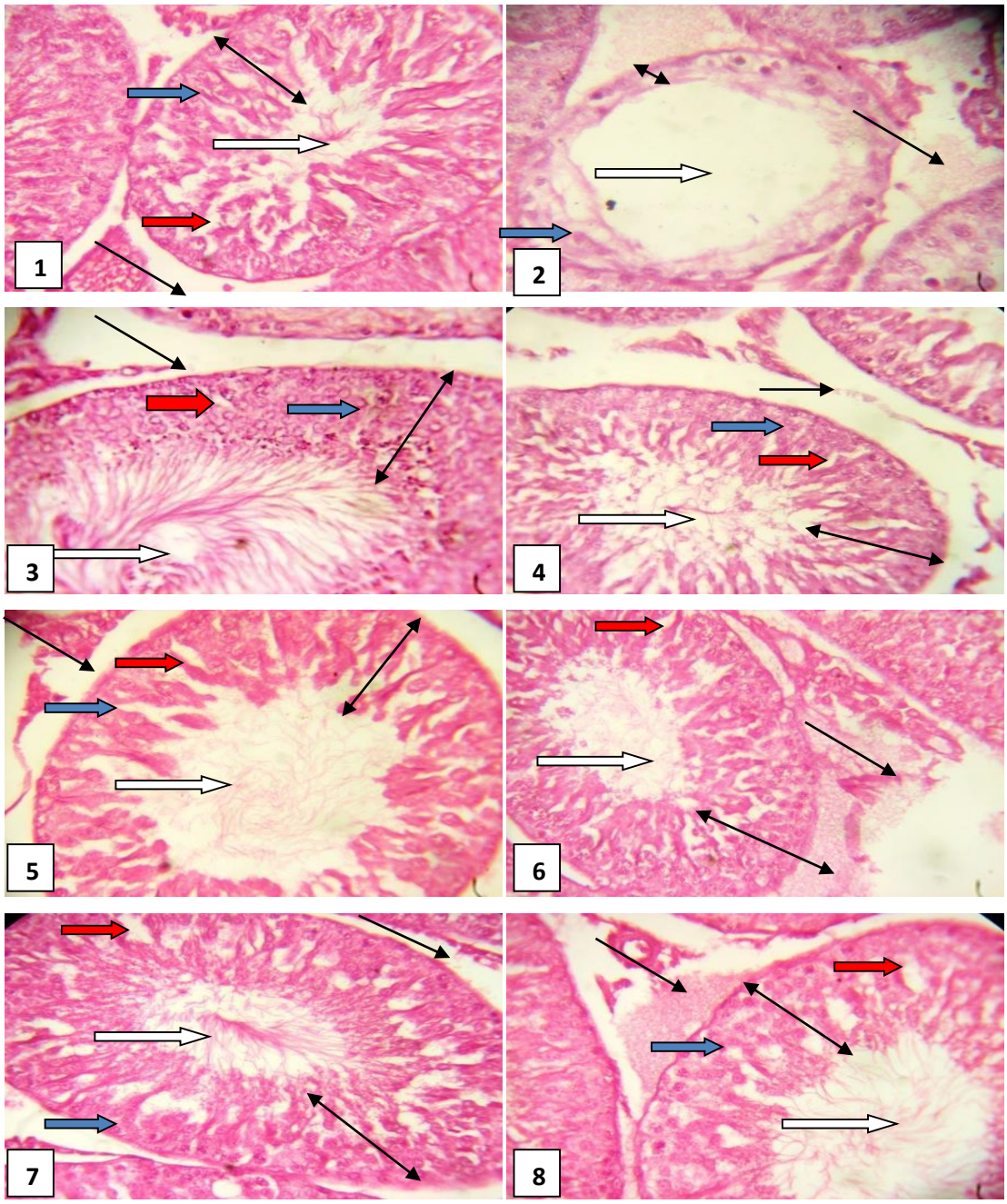


Plate 4.1. Photomicrograph of testicular sections from Wistar rats treated with As_2O_3 and MEPL stained by H&E and presented at x400. **(1)** Control, **(2)** As_2O_3, **(3)** 250 mg/kg MEPL , **(4)** 500 mg/kg MEPL, **(5)** 1000 mg/kg MEPL, **(6)** As_2O_3 + 250 mg/kg MEPL, **(7)** As_2O_3 + 500 mg/kg MEPL, **(8)** As_2O_3 + 1000 mg/kg MEPL. (White arrow indicates the seminiferous tubule lumen, spanned arrow indicates width of germ cell layer, blue arrow indicates normal spermatogonial cells, red arrow indicates normal Sertoli cells and slender arrow indicates the interstitial space).

4.11 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on 17 β-hydroxysteroid dehydrogenase activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Testicular tissue 17β-hydroxysteroid dehydrogenase (17β HSD) activity reduced significantly in As_2O_3 group when compared with control group ($p < 0.05$). Also, the groups administered with As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + SQ showed significant increase in 17β HSD when compared with As_2O_3 group ($p < 0.05$) (Figure 4.30).

The 17 β- HSD is an enzyme which takes part in converting androstenedione to testosterone during androgen production in the Leydig cells. The availability of this enzyme will determine the rate and quantity of male steroid hormone production. In this study, a reduced testicular concentration of 17 β- HSD was seen in the arsenic trioxide group. This supports Chang *et al*. (2007) and Khan *et al*. (2013). Arsenic accumulates in different organs like testes (Healy *et al*., 1998). The presence of arsenic in testes may have interfered with the steroidogenic enzymes in the Leydig cells particularly 17 β- HSD by binding with the thiol group of the enzyme, thus resulting to its depletion. However, the administration of 250 or 500 mg/kg MEPL as well as 100 mg/kg squalene to the animals treated with arsenic trioxide improved the testicular concentration of 17 β- HSD. This may be supported by the results from TAC buttressed by the DPPH scavenging effects of both MEPL and squalene as shown in this study. These properties might have helped to preserve the thiol groups of the enzyme 17 β- HSD from being bound by arsenic trioxide and thus made it available for measurement by spectrophotometry.

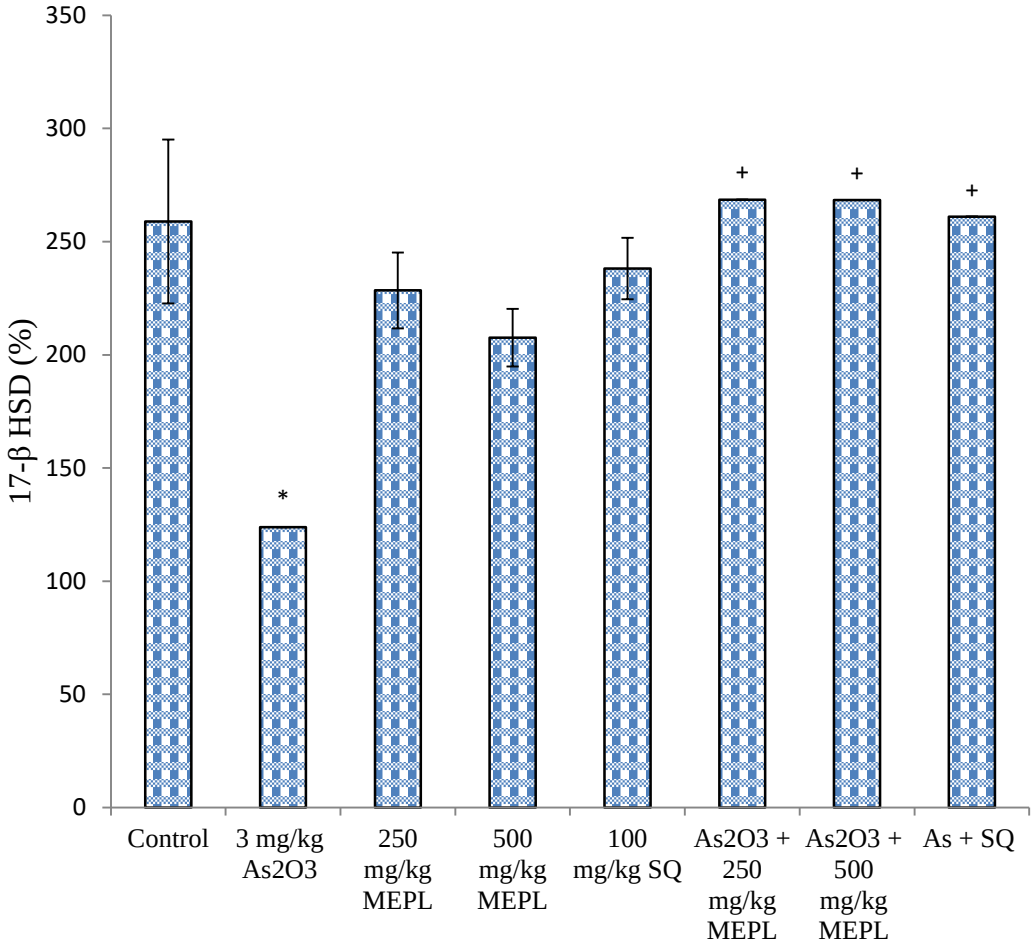


Fig. 4.30. Effects of MEPL and squalene on testicular 17-β hydroxysteroid dehydrogenase activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5.

*, $^{+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

*SQ = Squalene

4.12 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on Sperm chromatin condensation abnormality and 8-oxo-2'deoxyguanosine (8-OHdG) concentration in Arsenic trioxide-induced reproductive toxicity in male Wistar rats

4.12.1 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on sperm chromatin condensation abnormality in arsenic trioxide-induced reproductive toxicity in male Wistar rats

The percentage of sperm chromatin condensation abnormality significantly increased in As_2O_3 only group and 250 mg/kg MEPL when compared with control group ($p < 0.05$). A significant decrease was observed in groups administered As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + SQ when compared with As_2O_3 only ($p < 0.05$) (Figure 4.31).

Sperm chromatin condensation abnormality is indicated by greater histone number in relations to protamine in the chromatin (Oliva, 2006). This work shows that percentage of spermatozoa chromatin condensation abnormality was highest in arsenic group. This corroborates Mukhopadhyay *et al.* (2013). Arsenic is credited with high tendencies to induce DNA strand break by ROS produced during its methylation (Wang *et al*., 2006). The groups co-treated with arsenic trioxide and 250, 500 mg/kg MEPL and squalene elicited improvement in sperm chromatin. This also corroborates the result obtained in sperm morphology in which MEPL and squalene were able to protect spermatozoa chromatin from structural damage. This shows that both MEPL and squalene has a potential competence to stop or reduce formation of ROS which eventually cause sperm DNA damage and subsequently, abnormal morphology.

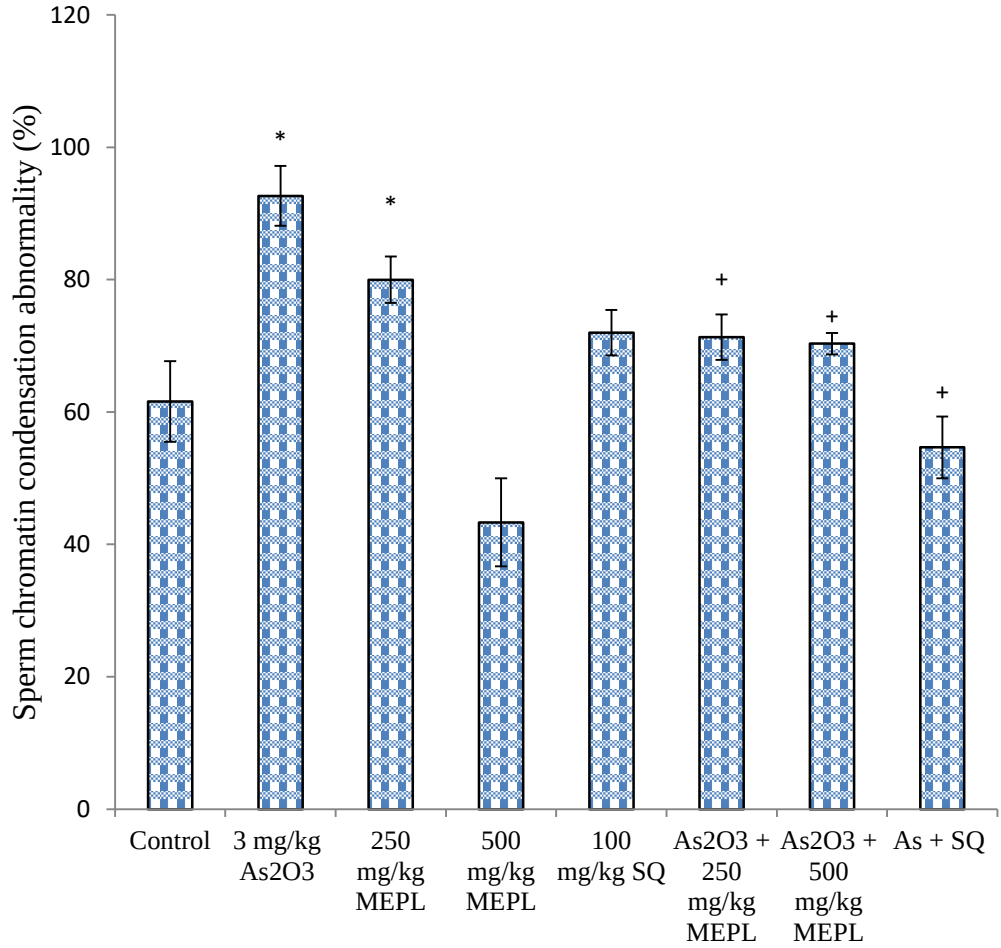


Fig. 4.31. Effects of MEPL on sperm chromatin condensation abnormality in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5.

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.12.2 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on serum concentration and testicular concentration of 8-oxo-2'deoxyguanosine in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Serum 8-OHdG increased significantly in As_2O_3 group when compared with control group ($p < 0.05$). The groups administered with As_2O_3 + 250 mg/kg MEPL , As_2O_3 + 500 mg/kg MEPL and As_2O_3 + 100 mg/kg squalene showed a significant reduction in serum concentration of 8-OHdG when compared with control group and the As_2O_3 group ($p < 0.05$) (Figure 4.32).

Testicular 8-OHdG concentration significantly increased in As_2O_3 only group when compared with control group. The groups treated with As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + 100 mg/kg squalene showed a significant reduction in testicular tissue concentration of 8-OHdG when compared with control group and with As_2O_3 only group ($p < 0.05$) (Figure 4.33).

8-oxo-2'deoxyguanosine (8- OHdG) is a sensitive biological indicator to assess gravity of DNA injury and repair that is usually derived when hydroxyl radical (HO^-) produced adjacent to cellular or mitochondrial DNA interacts with neucleobases (e.g. guanine) of DNA strands. Increased serum and testicular concentration of 8-oxo-2'deoxyguanosine was seen in arsenic group, this is in consonance with a scientific result by Celino *et al*. (2009). Elevation of 8- OHdG in arsenic group is seen as evidence that arsenic causes increased presence of ROS and DNA oxidative injury and consequently apoptosis (Nomoto *et al*., 2002). Observation was made that 250 and 500 mg/kg MEPL and 100 mg/kg squalene were able to reduce the expression of 8- OHdG in both the serum and testicular tissues. This study thus demonstrates strong antioxidant capacity of both MEPL and squalene against ROS, thus maintaining DNA integrity. This result supports the presence of phenol in the two substances and their activities against $ABTS^+$ and DPPH as earlier mentioned in this study.

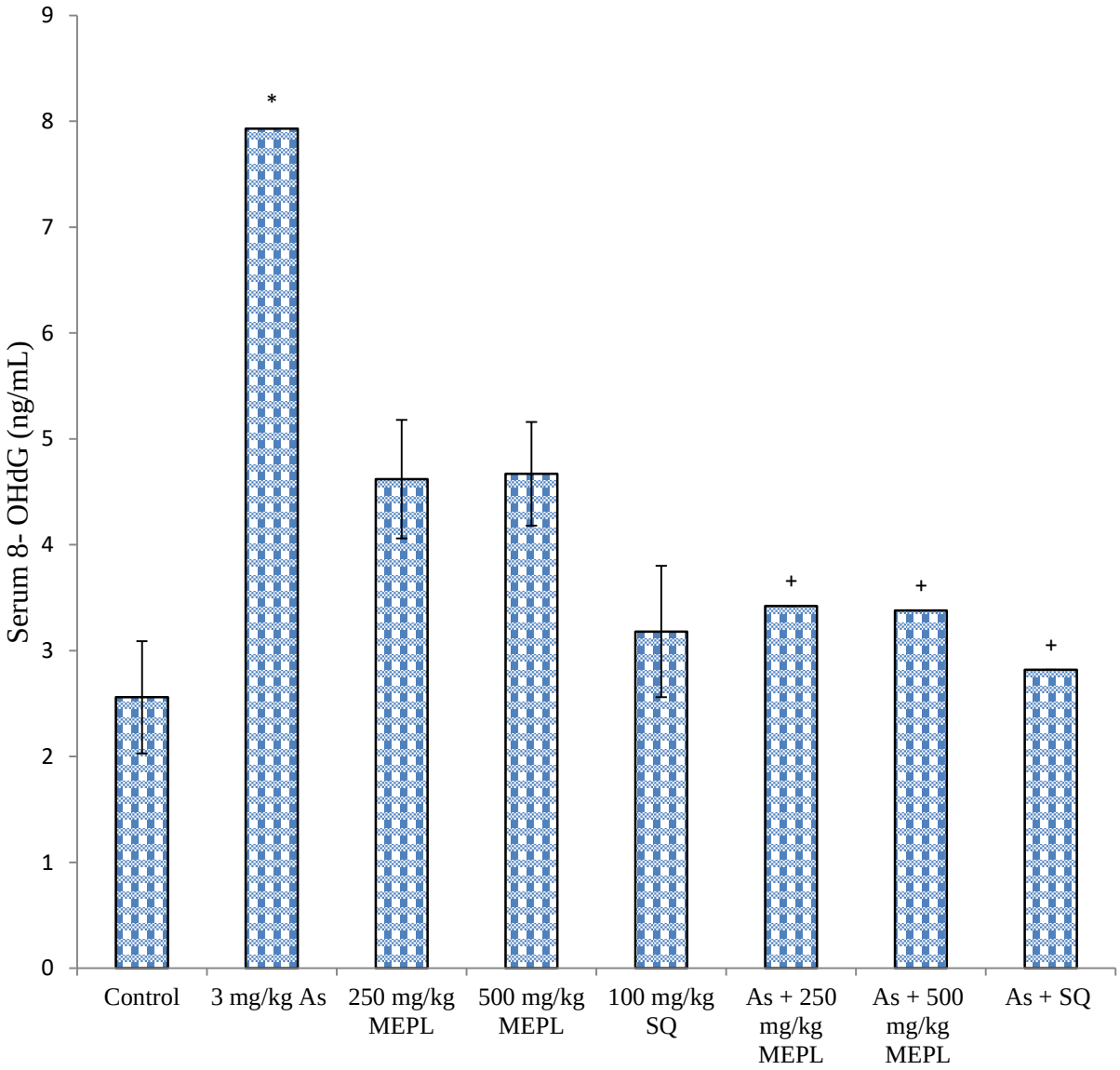


Fig. 4.32. Effects of MEPL and squalene on serum concentration of 8-OHdG in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5.

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

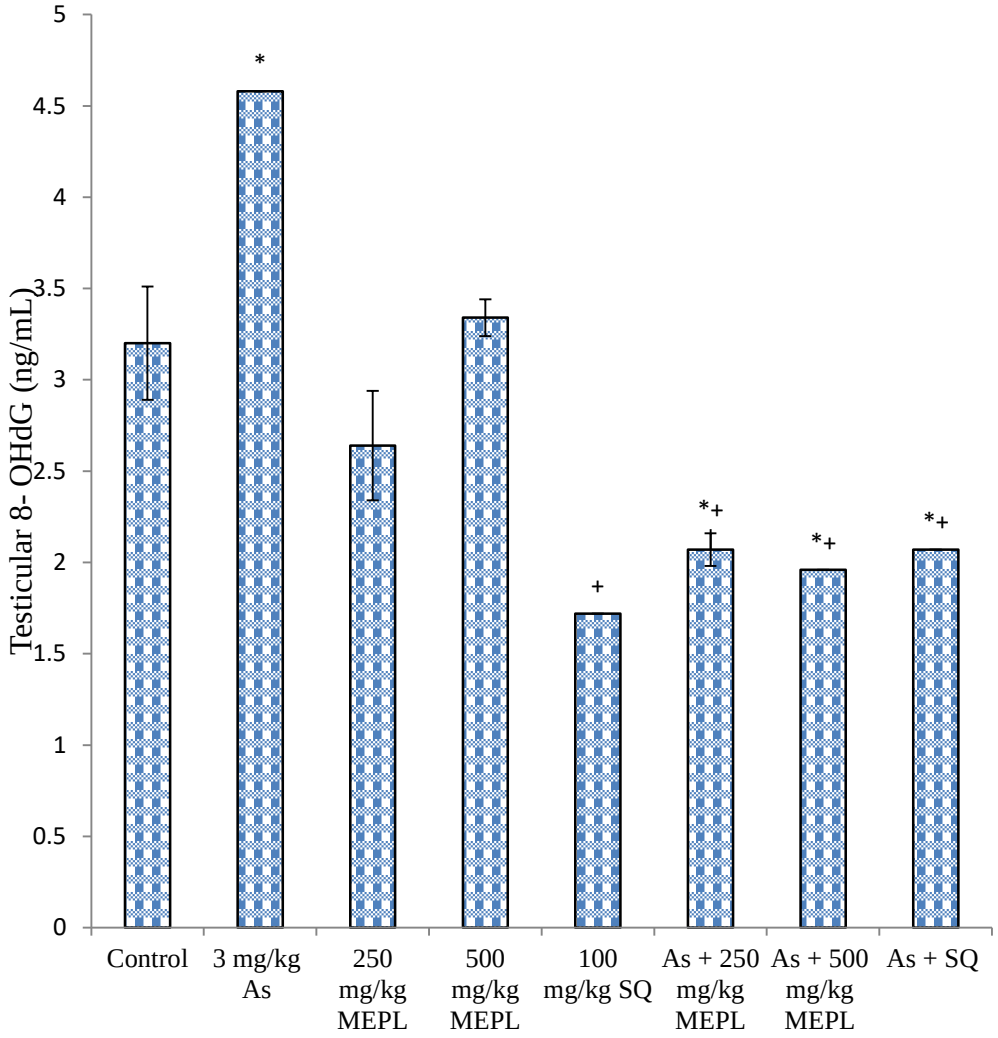


Fig.4.33 Effects of MEPL and squalene on testicular concentration of testicular 8-OHdG level in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5.

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.13 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on testicular apoptotic factor and antiapoptotic factor expression in arsenic trioxide-induced reproductive toxicity in male Wistar rats

4.13.1 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on testicular Bax gene expression and Bcl-2 gene expression in arsenic trioxide-induced reproductive toxicity in male Wistar rats

It was observed that expression of Bax increased in As_2O_3 group when compared with control group. The groups treated with As_2O_3 +250 mg/kg MEPL and As_2O_3 + SQ showed less expression of Bax when compared with As_2O_3 group only (Plate 4.2).

The group treated with As_2O_3 alone showed no Bcl-2 expression when compared with control group. A high Bcl-2 expression was seen in groups treated with As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + SQ when compared with As_2O_3 alone group (Plate 4.3).

In this study, Bax gene was expressed more in arsenic group while Bcl-2 was not expressed in the same group, this supports Khan *et al*. (2013) in which sodium arsenite caused continous expression of Bax gene and reduced expression of Bcl-2. Bax gene is a mitochondrial enzyme located within the transmembrane space and regulates the release of another cytosolic enzyme, caspase -9. Gupta *et al*. (2003) explained that apoptosis induced by arsenic involves a chain of events which includes excessive generation of ROS and DNA damage with the attending evidence of increased production of 8- OHdG and DNA damage. The alternate reduction of Bax expression and increase in Bcl-2 expression in groups co-treated with arsenic trioxide and 250 mg/kg MEPL, 500 mg/kg MEPL or squalene suggests that the substances were able to prevent the events that lead to the expression of Bax which are mainly increased ROS generation and DNA damage. This result is supported with the observed change in results of sperm chromatin condensation, serum and testicular concentration of 8-OHdG already mentioned earlier.

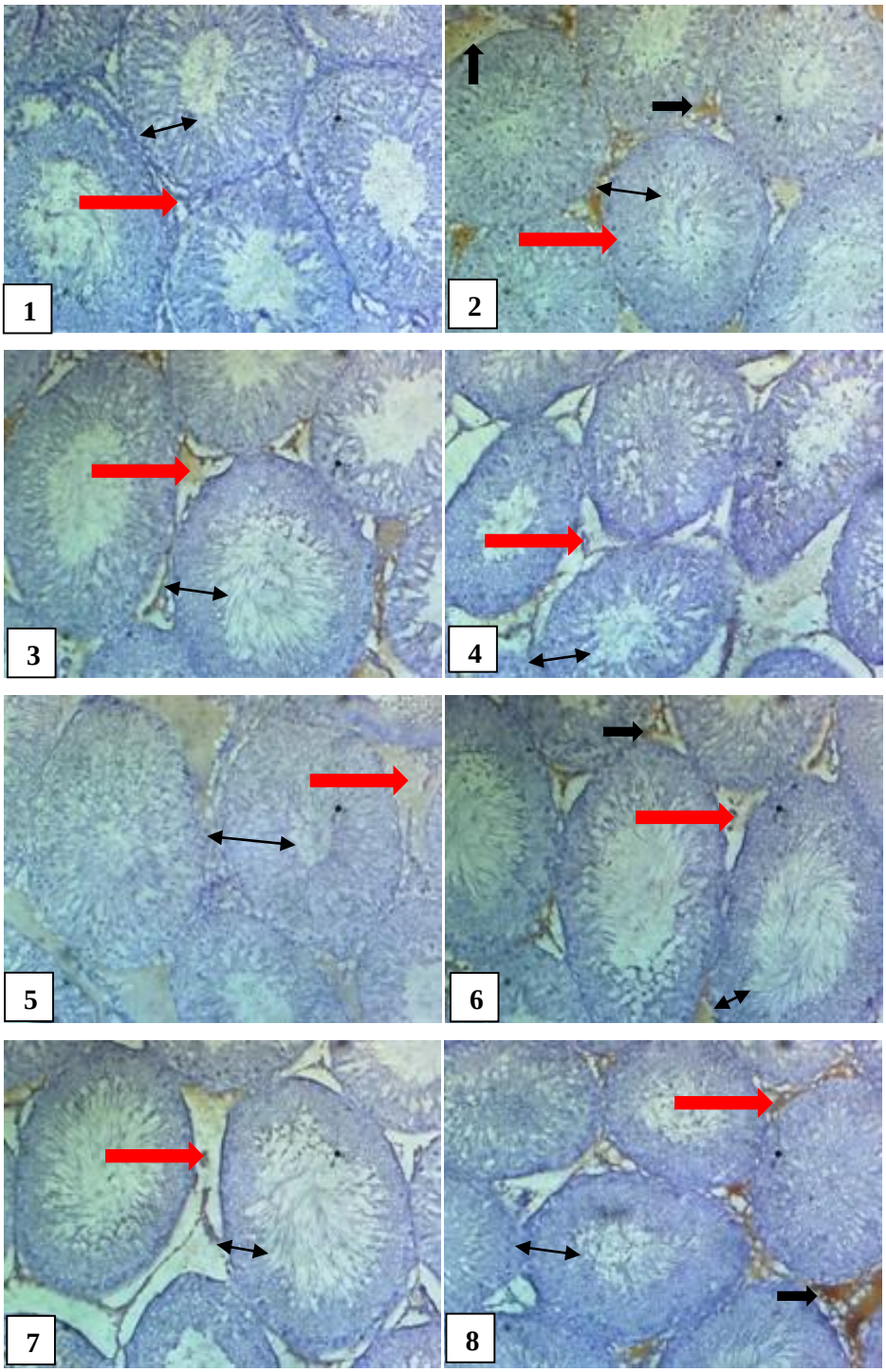


Plate 4.2. Photomicrograph of testicular immunohistochemistry showing Bax expression (x200) **(1)** Control, **(2)** As_2O_3, **(3)** 250 mg/kg MEPL, **(4)** 500 mg/kg MEPL, **(5)** 100 mg/kg SQ, **(6)** As_2O_3 + 250 mg/kg MEPL, **(7)** As_2O_3 + 500 mg/kg MEPL, **(8)** As_2O_3 arsenic + 100 mg/kg SQ. (Black arrow indicates the expression of Bax protein which appears brown in colour, spanned arrow indicate width of germ cell layer, red arrow indicate the interstitial space)

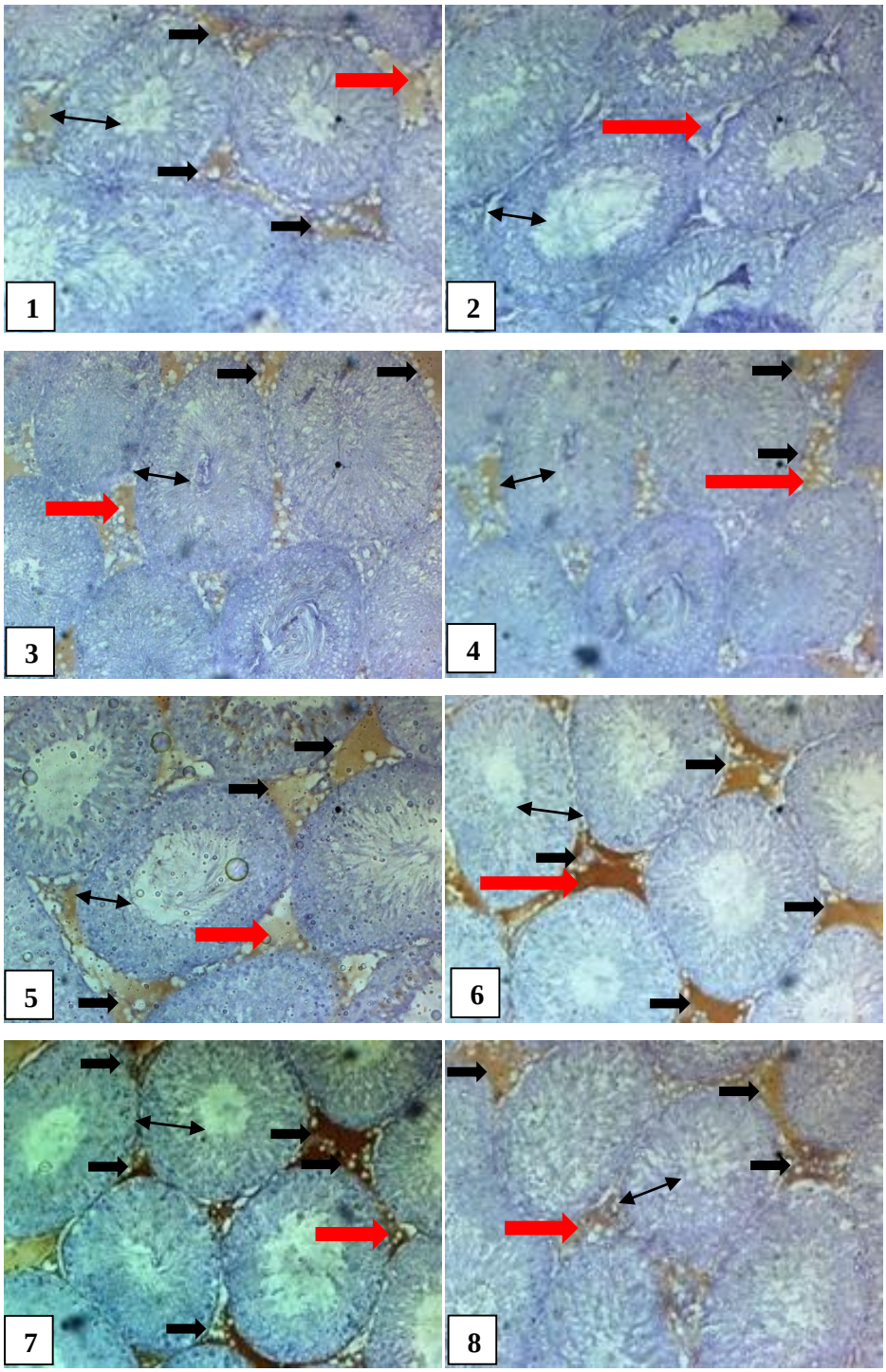


Plate 4.3: Photomicrograph of testicular immunohistochemistry showing Bcl-2 expression (x 200) **(1)** Control, **(2)** As_2O_3 **(3)** 250 mg/kg MEPL , **(4)** 500 mg/kg MEPL, **(5)** 100 mg/kg SQ, **(6)** As_2O_3+ 250 mg/kg MEPL, **(7)** As_2O_3 + 500 mg/kg MEPL , **(8)** As_2O_3 + 100 mg/kg SQ. (Black arrow indicates the expression of Bcl-2 protein, spanned arrow indicates width of germ cell layers and red arrows indicates interstitial cells).

4.13.2 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on Quantification of testicular Bax gene expression in arsenic trioxide-induced reproductive toxicity in male Wistar rats

There was an observed expression of Bax gene in As_2O_3 only group. There was no appearance of Bax gene expression in the control group while As_2O_3 + 250 mg/kg MEPL and As_2O_3 + SQ showed less expression of Bax gene when compared with As_2O_3 only group (Figure 4.34).

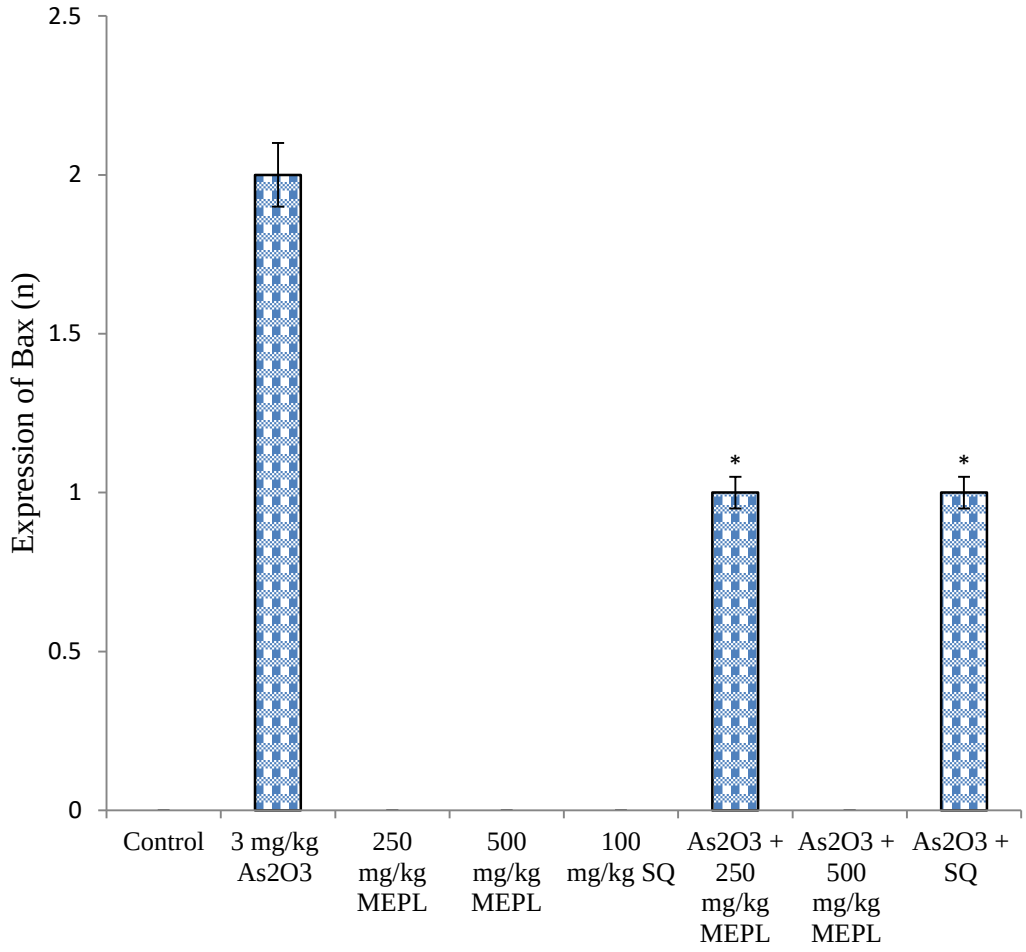


Fig. 4.34. Quantification of Bax gene expression in male Wistar rats exposed to As_2O_3-induced reproductive toxicity and treated with methanol extract of *Parquetina nigrescens* and squalene

Columns represent mean ± SEM, n = 5

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.13.3 Quantification of Bcl-2 gene expression in effects of methanol extract of *Parquetina nigrescens* leaf and squalene on testicular Bcl-2 expression in arsenic trioxide- reproductive toxicity in male Wistar rats

There was no Bcl-2 gene expression in As_2O_3 only group when compared with control ($p < 0.05$). The expression of Bcl-2 gene in the groups administered with As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + SQ showed increased Bcl-2 when compared with As_2O_3 group only (Figure 4.35).

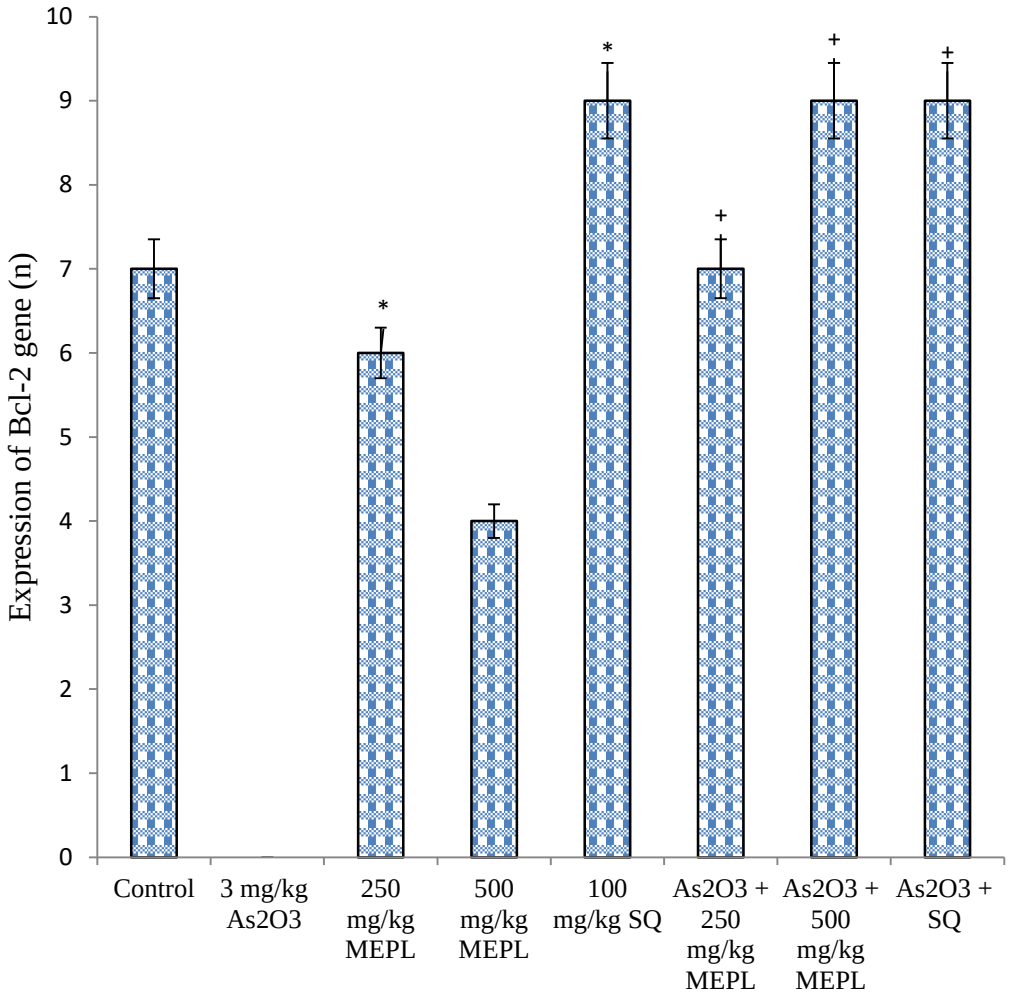


Fig. 4.35. Quantification of Bcl-2 gene expression in male Wistar rats exposed to arsenic trioxide-induced reproductive toxicity and treated with MEPL and squalene

Columns represent mean ± SEM, n = 5

*, $^{+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.14 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on sperm characteristics in arsenic trioxide-induced reproductive toxicity in male Wistar rats

4.14.1 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on sperm count in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Sperm count reduced ($p < 0.05$) in As_2O_3 only group when compared with control group (Figure 4.36).

Further in this study, sperm analysis was repeated so as to relate the activity of squalene with that of MEPL in arsenic treated rats. It was observed that arsenic brought about a reduction in sperm count in arsenic group only but this reduction was not exhibited in arsenic groups co-administered 250, 500 mg/kg MEPL or squalene. Squalene is a triterpene with proven antioxidant capacity (Warleta *et al*., 2010), which might have been responsible for its ability to prevent reduced sperm count induced by arsenic treatment thus making it exhibit similar effects with MEPL as earlier noted in this study.

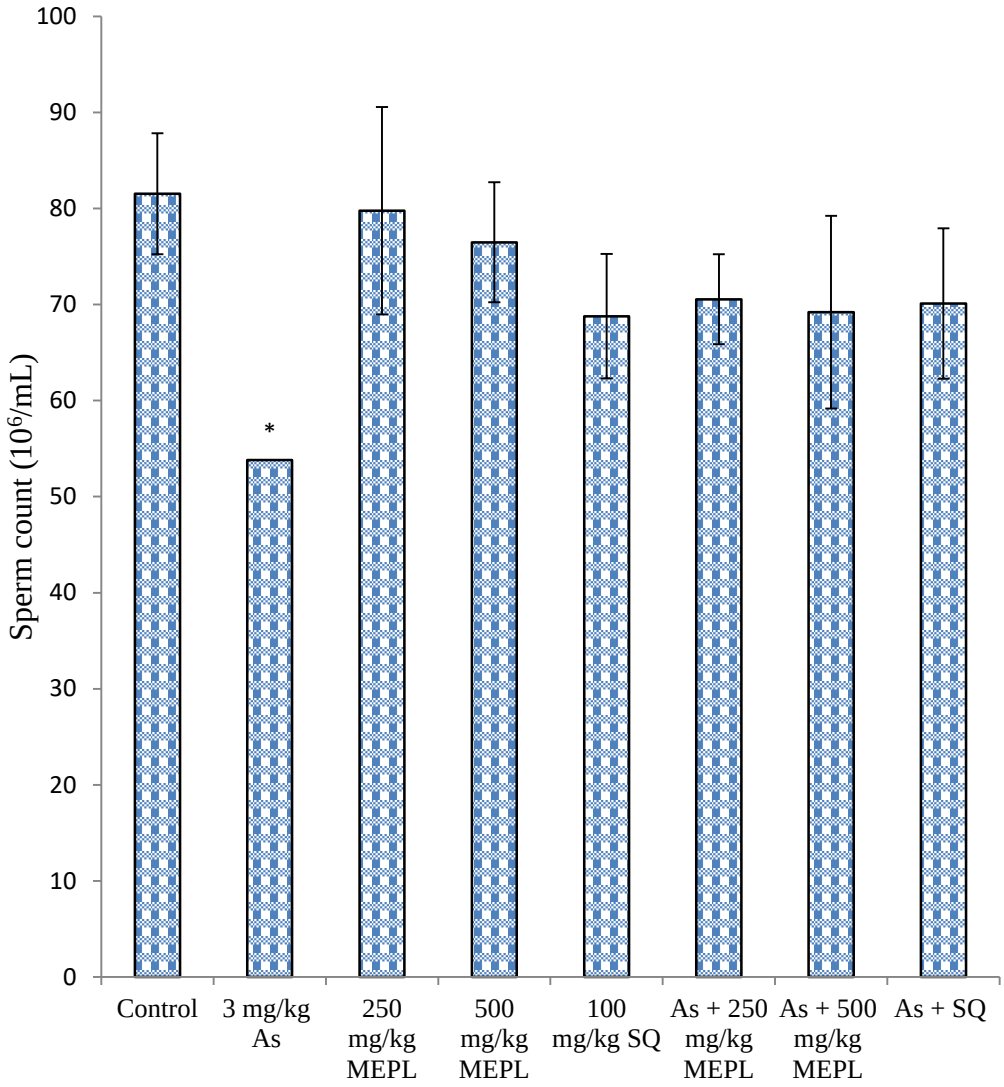


Fig. 4.36. Effects of MEPL and squalene on sperm count in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.14.2 Effects of methanol extract of *Parquetina nigrescens* and squalene on sperm motility, progressive sperm motility and sperm viability in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Sperm motility decreased ($p < 0.05$) in As_2O_3 group when compared with control group while it increased ($p < 0.05$) in groups administered As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL or As_2O_3 +SQ when compared with As_2O_3 group only (Figure 4.37).

A reduction ($p < 0.05$) in sperm progressive motility was observed in As_2O_3 only group when compared with control group. Also, an increase ($p < 0.05$) in progressive sperm motility was observed in As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + SQ when compared with As_2O_3 only group (Figure 4.38).

Sperm viability decreased ($p < 0.05$) in As_2O_3 only group and in the group treated with As_2O_3 + SQ when compared with control group (figure 4.39).

The result of sperm motility, progressive sperm motility as well as sperm viability also showed that both MEPL and squalene ameliorated the effects of arsenic. The observed effect of squalene on motility agrees with Zhang *et al*. (2008) in which 20 and 40 mg/kg of squalene caused an increase in the motility of boar sperm. It was noticed earlier in this study that MEPL reverted the impact of arsenic on spermatozoa motility as well as viability possibly by preventing arsenic from binding to thiol groups on sperms and thus reducing oxidative stress which damages mitochondria and in turn leads to cellular ATP depletion that affects sperm motion kinetics (Chang *et al*., 2007). The ability of squalene to ameliorate the anti-motility effects of arsenic trioxide on sperms may suggest that it also possess the ability to reduce oxidative stress like MEPL thus protecting spermatozoa.

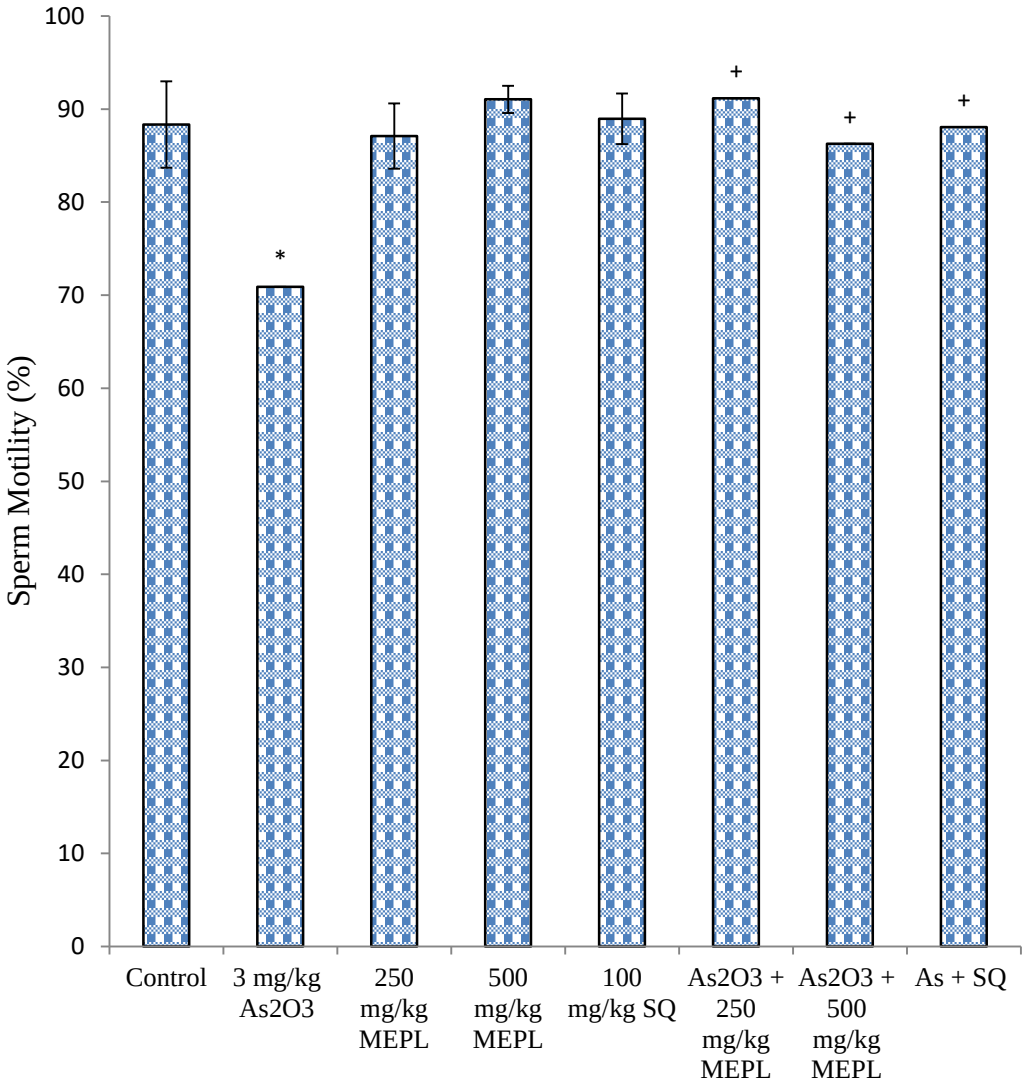


Fig. 4.37. Effects of MEPL on sperm motility in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

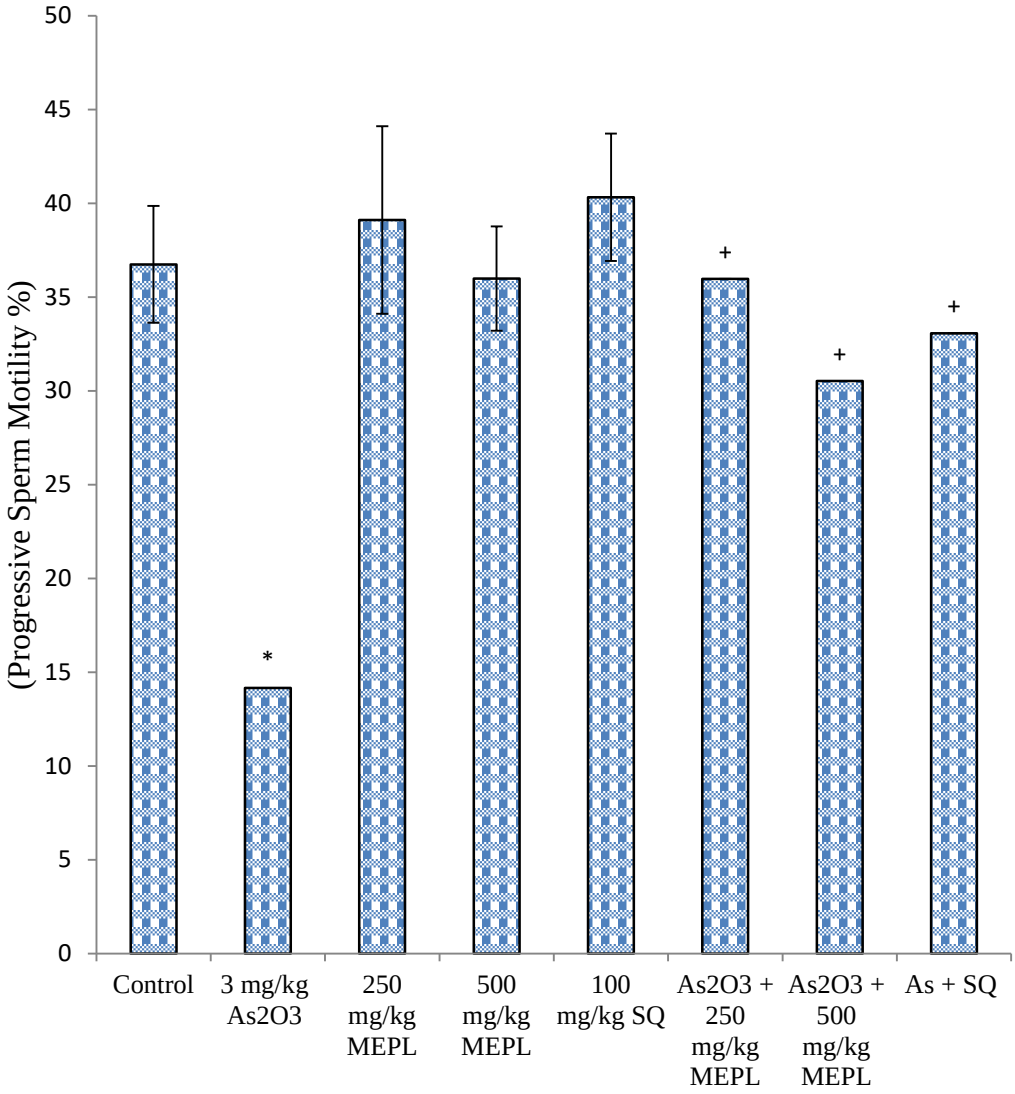


Fig. 4.38. Effects of MEPL and squalene on sperm progressive motility in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol extract of *Parquetina nigrescens* Leaf

As_2O_3 =Arsenic trioxide

SQ = Squalene

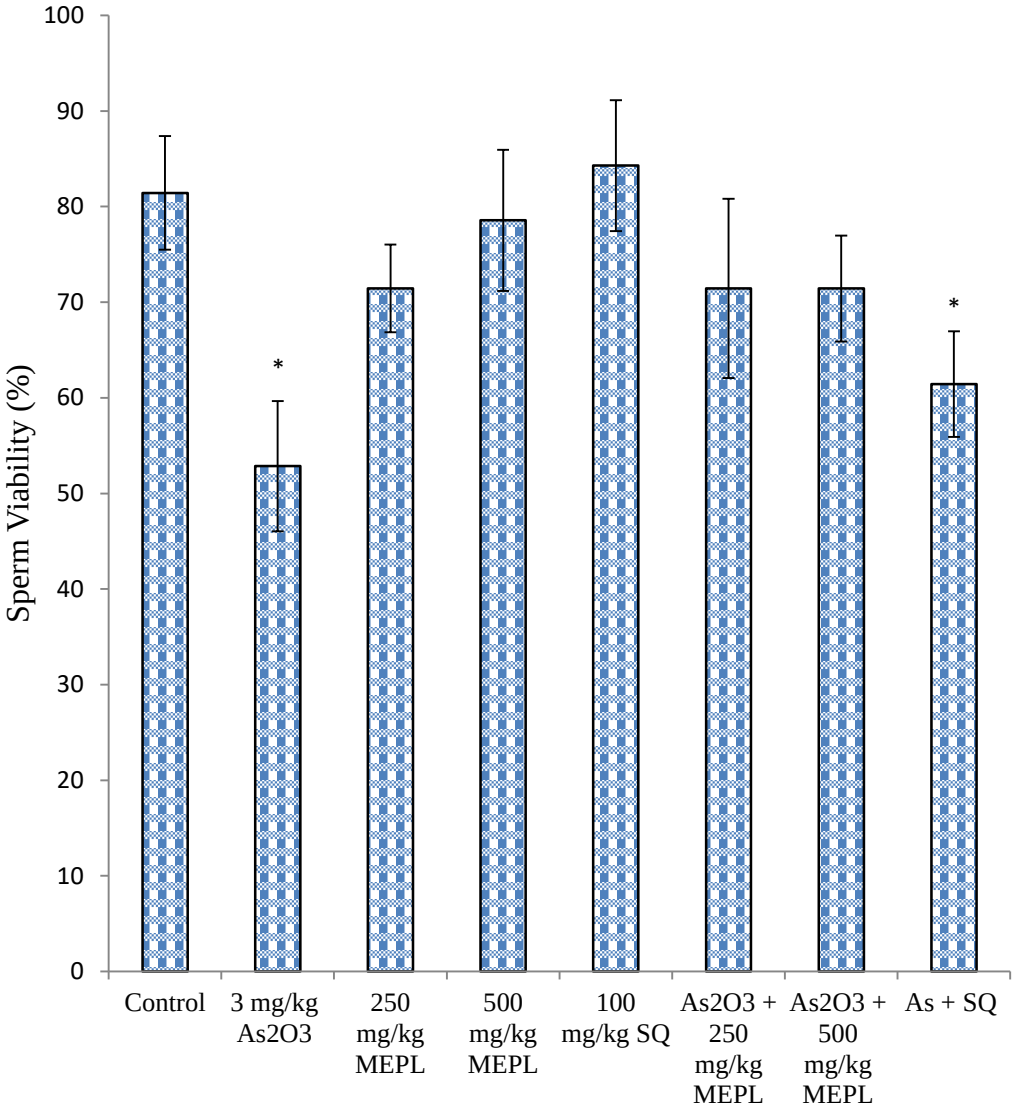


Fig. 4.39. Effects of MEPL and squalene on sperm viability in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

*$p < 0.05$ as compared with Control

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.14.3 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on sperm morphology in arsenic trioxide-induced reproductive toxicity in male Wistar rats

An increase ($p < 0.05$) was observed in abnormal sperm morphology of As_2O_3 only group when compared with control group. Also, a decrease ($p < 0.05$) was observed in abnormal morphology in groups administered As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + SQ when compared with As_2O_3 only group (Figure 4.40).

Arsenic caused a rise in percentage of abnormal sperm morphology. This is similar to an earlier observation in this study. Interestingly, MEPL and squalene, were able to ameliorate this observed effect of arsenic on sperm morphology. Momeni and Eskandari (2016) attributed the reduction in percentage of normal morphology in sodium arsenite-treated mice to a decrease in production as well as expression of LH, FSH and testosterone which may ultimately have affected spermatogenesis. The result of hormone assay in this study interestingly showed that in serum concentrations of the three aforementioned hormones in arsenic group decreased and squalene ameliorated the decrease in FSH and LH, though it was unable to improve testosterone concentration. The improvement observed in the gonadotropins may have been responsible for MEPL and squalene ability to protect sperm morphology.

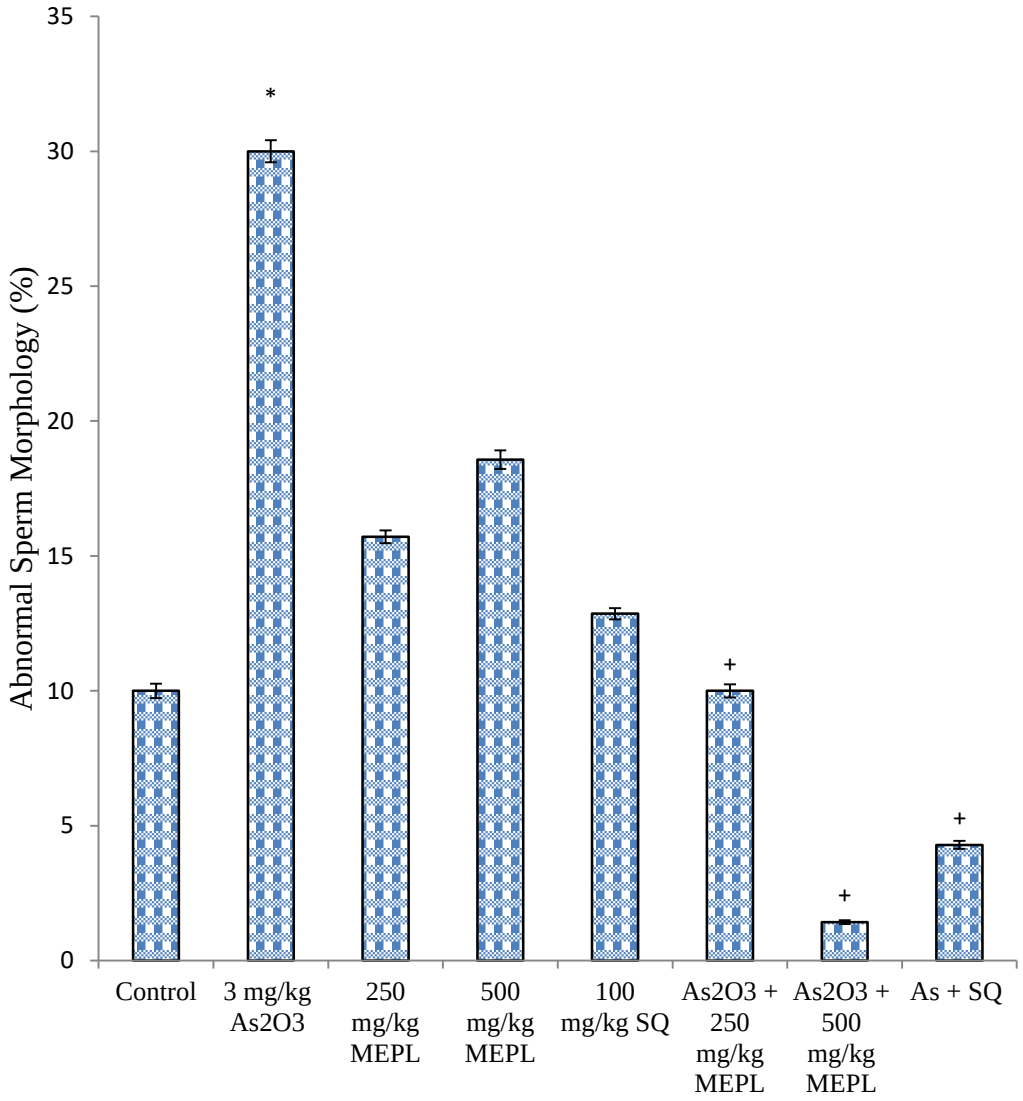


Fig. 4.40. Effects of MEPL and squalene on sperm morphology in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.14.4 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on sperm kinetics in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Average path velocity and straight line velocity showed reduction ($p < 0.05$) in As_2O_3 only group when compared with control. Also, an increase ($p < 0.05$) was observed in average path velocity in the group treated with As_2O_3 + 500 mg/kg MEPL when compared with As_2O_3 only group (Table 4.4).

Amplitude of lateral head reduced significantly ($p < 0.05$) in As_2O_3 only when compared with control group (Table 4.4).

Percent of line moving reduced ($p < 0.05$) significantly in As_2O_3 only group when compared with control group (Table 4.4).

In all four indices of sperm kinetics that were measured namely VAP, VSL, ALH and percent of line moving (LIN), a decrease in arsenic group was observed. These results further corroborate the harmful influence of arsenic already observed on sperm count, motility, viability as well as morphology because of its already established tendencies to bring about oxidative stress. As a result of antioxidative effect of MEPL, the group treated with arsenic and 500 mg/kg MEPL showed improved VAP. Both MEPL and squalene prevented the toxic effects of arsenic on all other spermatogenic indicies. It may therefore be suggested that both MEPL and squalene through their antioxidative capacities were capable of protecting the spermatozoa from oxidative stress by arsenic.

Table 4.4. Effects of methanol extract of *Parquetina nigrescens* and squalene on sperm kinetics in arsenic trioxide-induced reproductive toxicity in male Wistar rats

	Average Path Velocity (μm/s)	Straight Line Velocity (μm/s)	Amplitude of Lateral Head (μm)	Percent of Line Moving (%)
Control	16.17 ± 0.31	7.65 ± 0.44	0.82 ± 0.02	34.75 ± 3.83
As_2O_3 (3 mg/kg)	12.63 ± 1.30*	6.09 ± 0.50*	0.64 ± 0.07*	23.27 ± 2.32*
MEPL (250 mg/kg)	15.74 ± 0.76	7.77 ± 0.57	0.77 ± 0.04	35.59 ± 4.87
MEPL (500 mg/kg)	15.99 ± 0.61	7.02 ± 0.4	0.81 ± 0.04	31.01 ± 2.88
SQ (100 mg/kg)	16.22 ± 0.58	7.52 ± 0.44	0.82 ± 0.03	32.72 ± 2.94
As_2O_3 + MEPL (250 mg/kg)	14.60 ± 0.56	6.93 ± 0.26	0.71 ± 0.03	32.31 ± 2.15
As_2O_3 + MEPL (500 mg/kg)	15.21 ± 1.09^{+}	7.00 ± 0.36	0.76 ± 0.06	28.18 ± 3.05
As_2O_3+ SQ	14.96 ± 0.92	6.86 ± 0.39	0.75 ± 0.05	29.48 ± 2.91

Values are expressed as mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.15 Effects of methanol extract of *Parquetina nigrescens* and squalene on serum hormone concentration in arsenic trioxide-induced reproductive toxicity in male Wistar rats

4.15.1 Effects of methanol extract of *Parquetina nigrescens* and squalene on serum concentration of testosterone in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Serum testosterone concentration decreased ($p < 0.05$) in As_2O_3 group and in As_2O_3 + SQ when compared with control group. Serum testosterone concentration increased ($p < 0.05$) in As_2O_3 + 250 mg/kg MEPL and As_2O_3 + 500 mg/kg MEPL when compared with control group (Figure 4. 41).

Decreased serum testosterone concentration was observed in arsenic trioxide group. This agrees with the studies by Alli *et al*. (2013) and Sumedha and Miltonprabu (2014). The ability of arsenic to reduce LH concentration and consequently, 17 β- HSD concentration may have contributed to this observed decrease. Arsenic may have also acted directly on the testes to cause a decreased testosterone secretion. However, 250 and 500 mg/kg MEPL were able to revert the effect of arsenic trioxide on testosterone concentration. This may be owned to the ability of MEPL to decrease oxidative stress both within the testicular tissue as earlier observed in this study and in the HPT axis. Squalene was unable to ameliorate the low level of testosterone induced by arsenic possibly because its total antioxidant capacity and DPPH scavenging activity is lower to that of MEPL. Since squalene was able to improve LH and 17 β- HSD, it may be deduced that the inability of squalene to improve the low level of testosterone is localized to the testes.

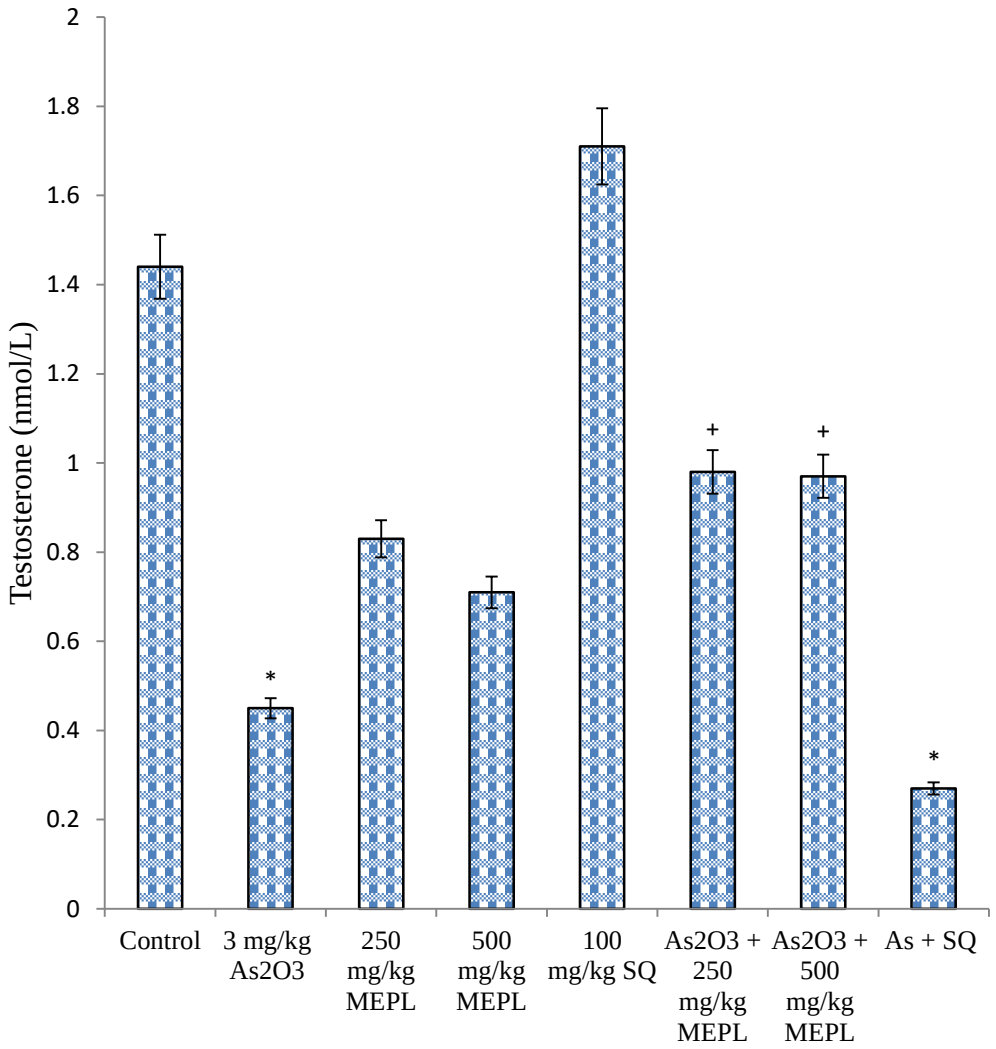


Fig. 4.41. Effects of MEPL and squalene on serum testosterone concentration in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.15.2 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on serum concentrations of follicle stimulating hormone and luteinizing hormone in arsenic trioxide-induced reproductive toxicity in male Wistar rats

The serum concentration of FSH decreased ($p < 0.05$) in As_2O_3 only group and in As_2O_3 + 250 mg/kg MEPL when compared with control group, while an increase was observed in As_2O_3 + SQ when compared with As_2O_3 only group (Figure 4.42).

Serum concentration of LH reduced ($p < 0.05$) in As_2O_3 only group when compared with control group. Also, a rise ($p < 0.05$) was observed in As_2O_3 + 250 mg/kg MEPL and As_2O_3 + SQ when compared with control group and with As_2O_3 (Figure 4.43).

In this study, reduced LH as well as FSH was seen in arsenic group. This supports Jana *et al*., (2006). This may be an indication that arsenic inhibits the secretory activities of the gonadotrophin cells of the anterior pituitary gland. It may be suggested that the capacity of arsenic to bring about oxidative stress may be accountable for decreased serum concentrations of LH and FSH. It has been reported that the production of steroidogenic enzymes such as 17 β- HSD is dependent on the levels of gonadotropins, therefore, the low level of this enzyme as observed in this study may also have been caused by reduced LH and FSH observed in arsenic group. The groups co-treated with arsenic and 250 mg/kg MEPL or squalene noticeably improved serum LH and FSH concentration. It thus appeared that MEPL and squalene protects the Hypothalamo-Pituitary-Testicular (HPT) axis possibly through their ability to diminish oxidative stress. The effects of these substances on LH and FSH concentration may also have been responsible for the improvement observed in 17 β- HSD level in this study.

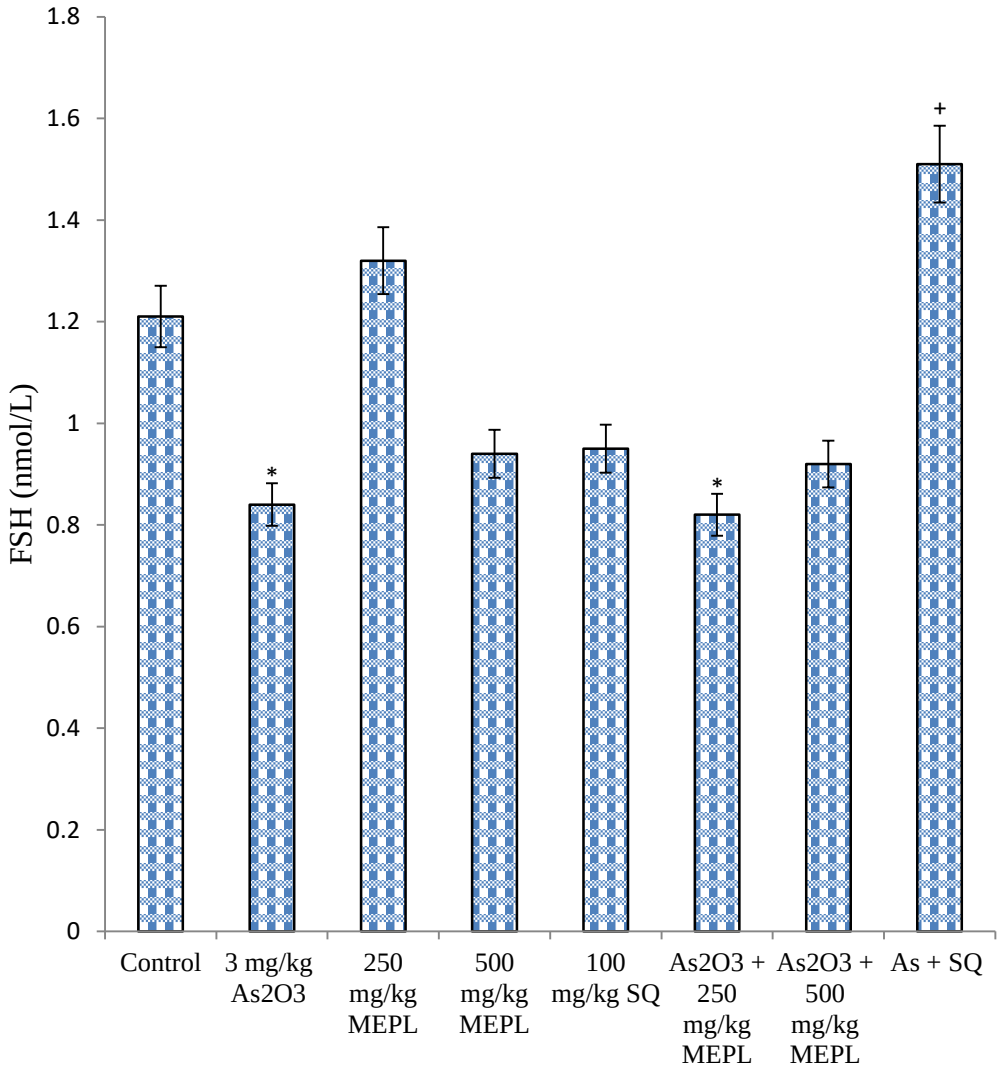


Fig. 4.42. Effects of MEPL and squalene on serum concentration of FSH in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

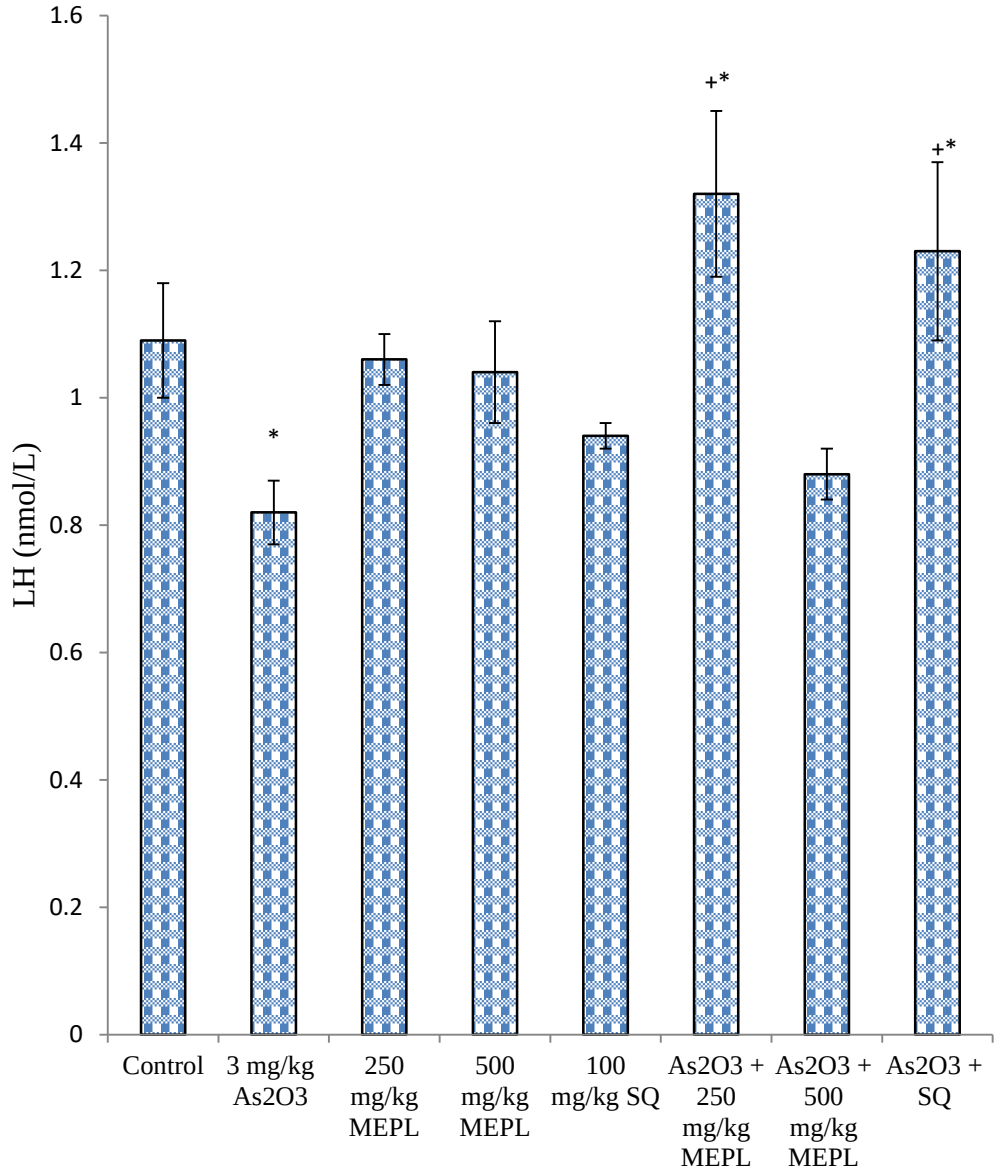


Fig. 4.43. Effects of MEPL and squalene on serum concentration of luteinizing hormone in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with Control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.16 Effects of methanol extract of *Parquetina nigrescens* and squalene on testicular redox status in arsenic trioxide-induced reproductive toxicity in male Wistar rats

4.16.1 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on testicular concentration of malondialdehyde in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Malondialdehyde concentration increased ($p < 0.05$) in As_2O_3 only group when compared with control group. Also, a decrease ($p < 0.05$) was seen in testicular MDA concentration in As_2O_3 + 250 mg/kg MEPL, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + SQ when compared with As_2O_3 only group (Figure 4.44).

With regards to the redox status of the testicular tissues, it was observed that MDA concentration increased in testes of arsenic trioxide-treated group, thus reiterating the earlier observation made on MDA concentration. Interestingly, just like MEPL did, squalene ameliorated the oxidative stress created by arsenic in the group treated with arsenic and squalene. There are assertions that squalene's vulnerability to peroxidation is very low and seems to perform as competent supressor of singlet oxygen and blocks the likelihood of lipid peroxidation, thus, defending cells from OS (Kohno *et al.*, 1995). It may therefore be suggested that squalene was able to ameliorate increased MDA concentration caused by arsenic because of its singlet oxygen quenching property.

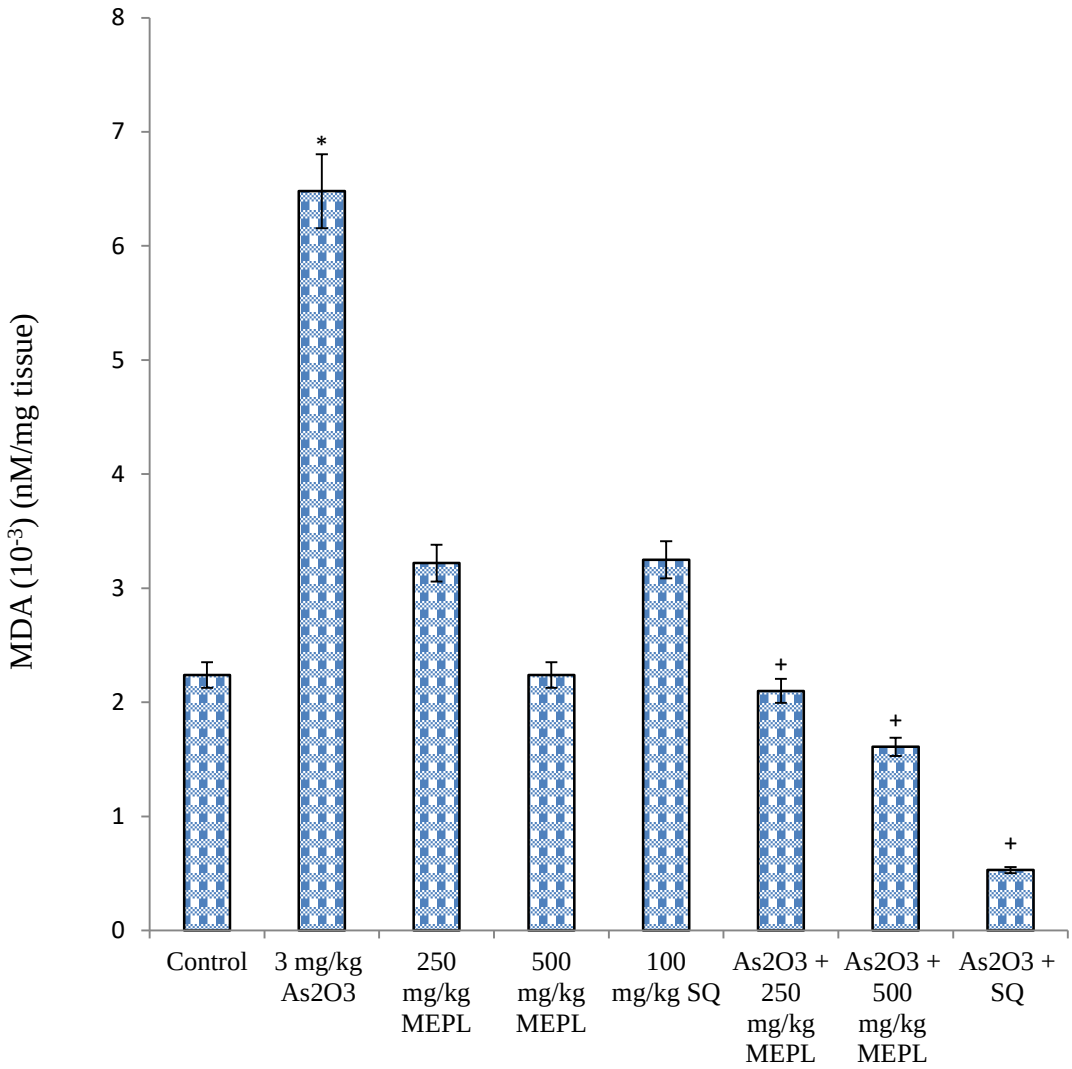


Fig 4.44. Effects of MEPL and squalene on testicular concentration of malondialdehyde in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*,+}p < 0.05$ as compared with control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.16.2 Effects of methanol extract of *Parquetina nigrescens* and squalene on testicular glutathione peroxidase in arsenic trioxide-induced reproductive toxicity in male Wistar rats

The glutathione peroxidase activitiy reduced ($p < 0.05$) in As_2O_3 only group when compared with control group while it increased ($p < 0.05$) in groups treated with As_2O_3 + 250, As_2O_3 + 500 mg/kg MEPL and As_2O_3 + SQ when compared with As_2O_3 only group (Figure 4.45).

Glutathione Peroxidase (GPx) is another antioxidant enzyme present in the testes. A number of antioxidant enzymes are present within the testes but the GPx predominates. In this study, arsenic trioxide was observed to decrease GPx activity. This is similar to the report of Wang *et al*. (2006) in which the administration of 30 mg/kg arsenic trioxide to pigs for 76 days reduced the GPx activity. Studies have shown that GPx detoxifies H_2O_2 only when it is present in low concentration, whereas catalase detoxifies H_2O_2 when GPx pathway reaches saturation with the substrate (De Bleser *et al*., 1999). It may therefore be inffered that the fall in GPx activity noticed in the arsenic group was based on high concentration of H_2O_2 and concomitant saturation of the antioxidant enzyme. It is however worthy of note that the groups co-treated with arsenic and 250, 500 mg/kg MEPL or squalene showed an increase in testicular GPx activity. Squalene was recorded to be much stronger scavenger of hydroxyl radicals than endogenous reduced glutathione (GSH) (Das *et al*., 2003). Therefore, MEPL and squalene possess the ability to maintain GPx activity level within the testes in spite of the presence of free radicals. This may be owned to the fact that both interventions are good radical scavengers as earlier seen.

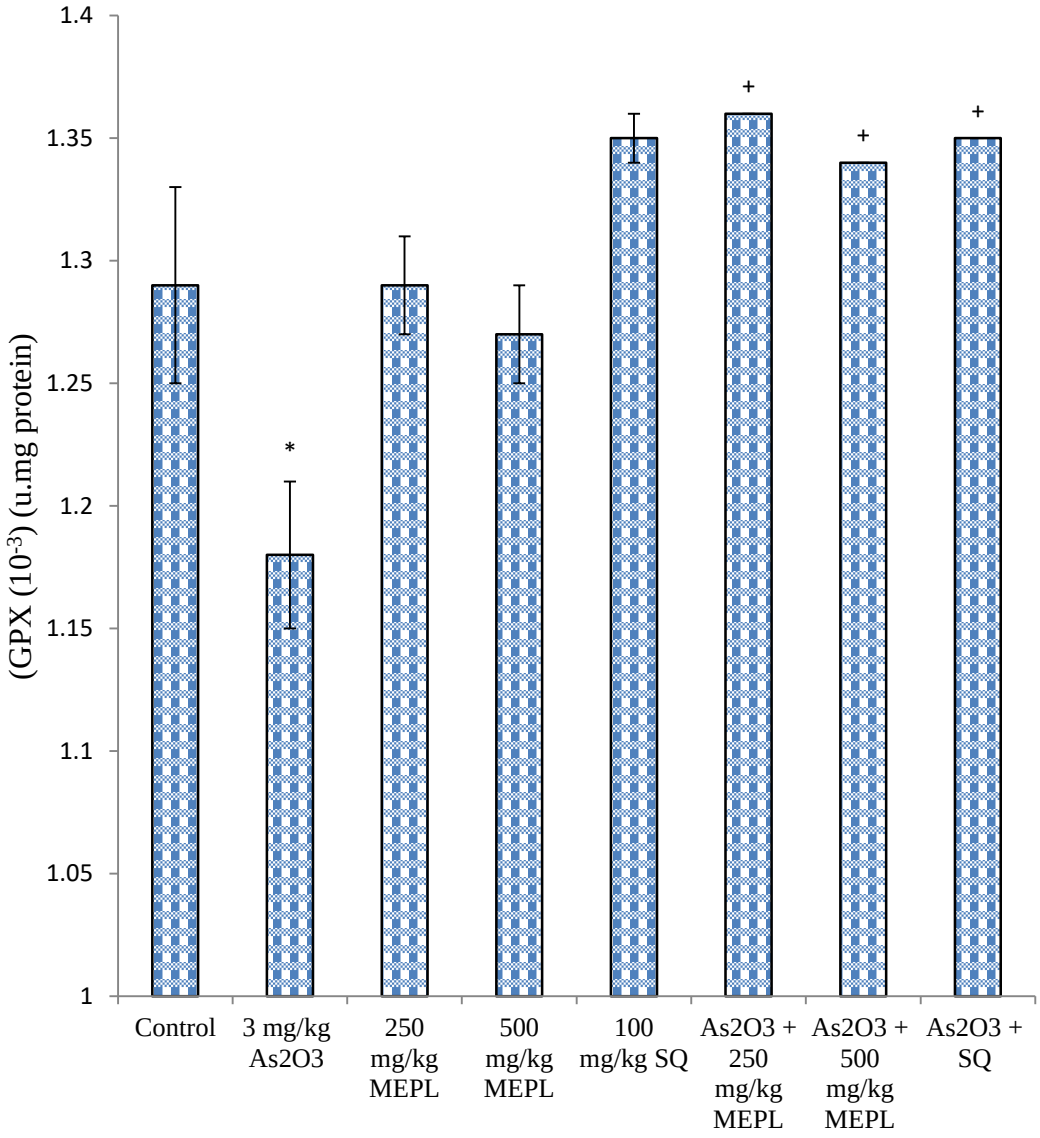


Fig. 4.45. Effects of MEPL and squalene on testicular GPX activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

*, $^{+}p < 0.05$ as compared with control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.16.3 Effects of methanol extract of *Parquetina nigrescens* and squalene on testicular superoxide dismutase activities in arsenic trioxide-induced reproductive toxicity in male Wistar rats

A decrease ($p < 0.05$) was observed in SOD activity in As_2O_3 only group and As_2O_3 + 500 mg/kg MEPL ($p < 0.05$) when compared with control group. Also, As_2O_3 + SQ showed an increase ($p < 0.05$) when compared with As_2O_3 only group (Figure 4.46).

In this current work, arsenic was observed to cause reduced testicular SOD in groups administered arsenic trioxide only and the group co-treated with arsenic trioxide and 500 mg/kg MEPL. This corroborates Banik *et al*. (2014) in which administration of 3 mg/kg arsenic trioxide to rats for 28 days led to a decrease in prostatic SOD activity. Arsenic is known to disturb pro-oxidant-antioxidant balance, thereby, exposing a cell to oxidative damage (Khan *et al*., 2013). Squalene was however able to ameliorate the decreased SOD activity induced by arsenic administration in arsenic group. Squalene reportedly caused an increase in SOD activity after gamma-irradiation on rats reduced it (Ahmed and Abdel-Magied, 2011). Squalene is reported to be a strong antioxidant because of its capacity to exchange electron and not undergo molecular distruption (Güneş, 2013). The *in vitro* antioxidant capacity of squalene has also been demonstrated in this study.

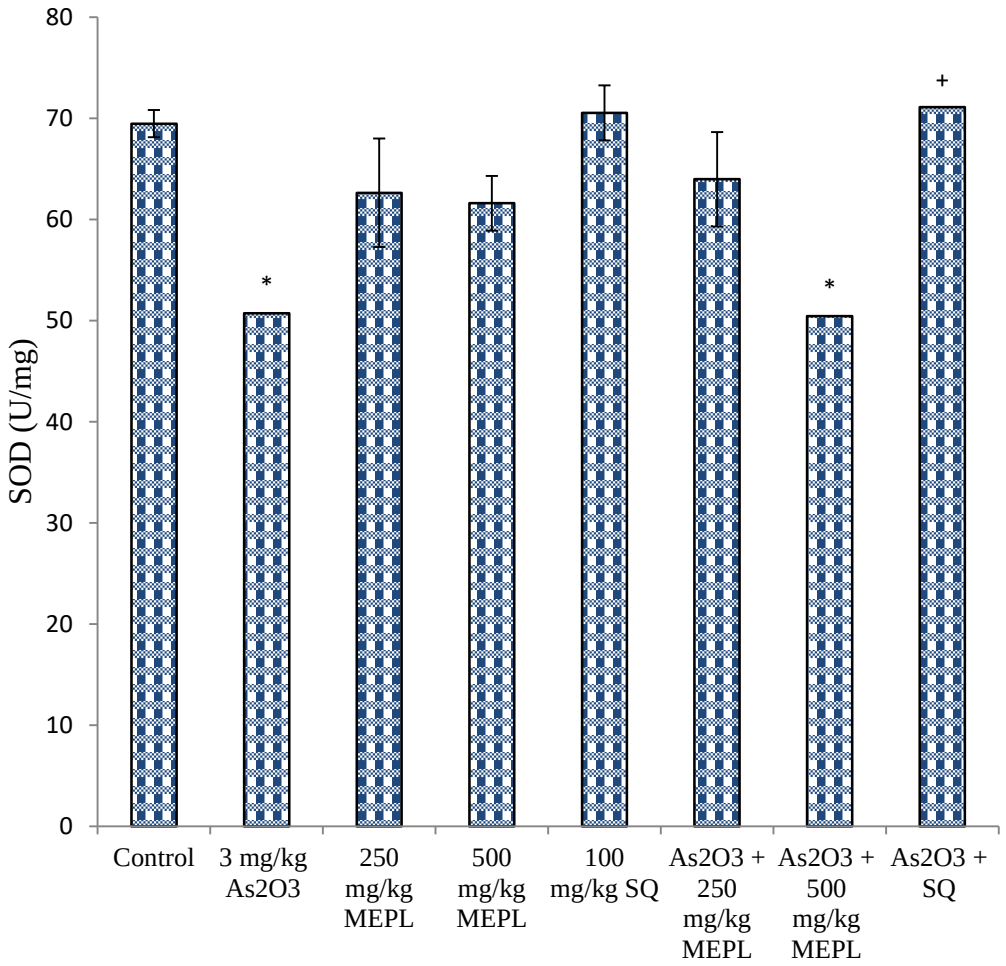


Fig. 4.46. Effects of MEPL and squalene on testicular SOD activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{*, +}p < 0.05$ as compared with control and As_2O_3 respectively

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.16.4 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on testicular catalase activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Catalase activity increased ($p < 0.05$) in As_2O_3 + 500 mg/kg MEPL when compared with As_2O_3 only group (Figure 4.47).

In this current study, a rise in catalase of the groups administered arsenic plus 500 mg/kg MEPL was observed. Catalase is ladened with detoxifying hydrogen peroxide in the body; it does this along with GPx. Catalase role comes into play when GPx pathway reaches saturation with substrate (De Bleser *et al*., 1999). The rise observed in catalase therefore may be based on gradual saturation of GPx which was equally measured in this study.

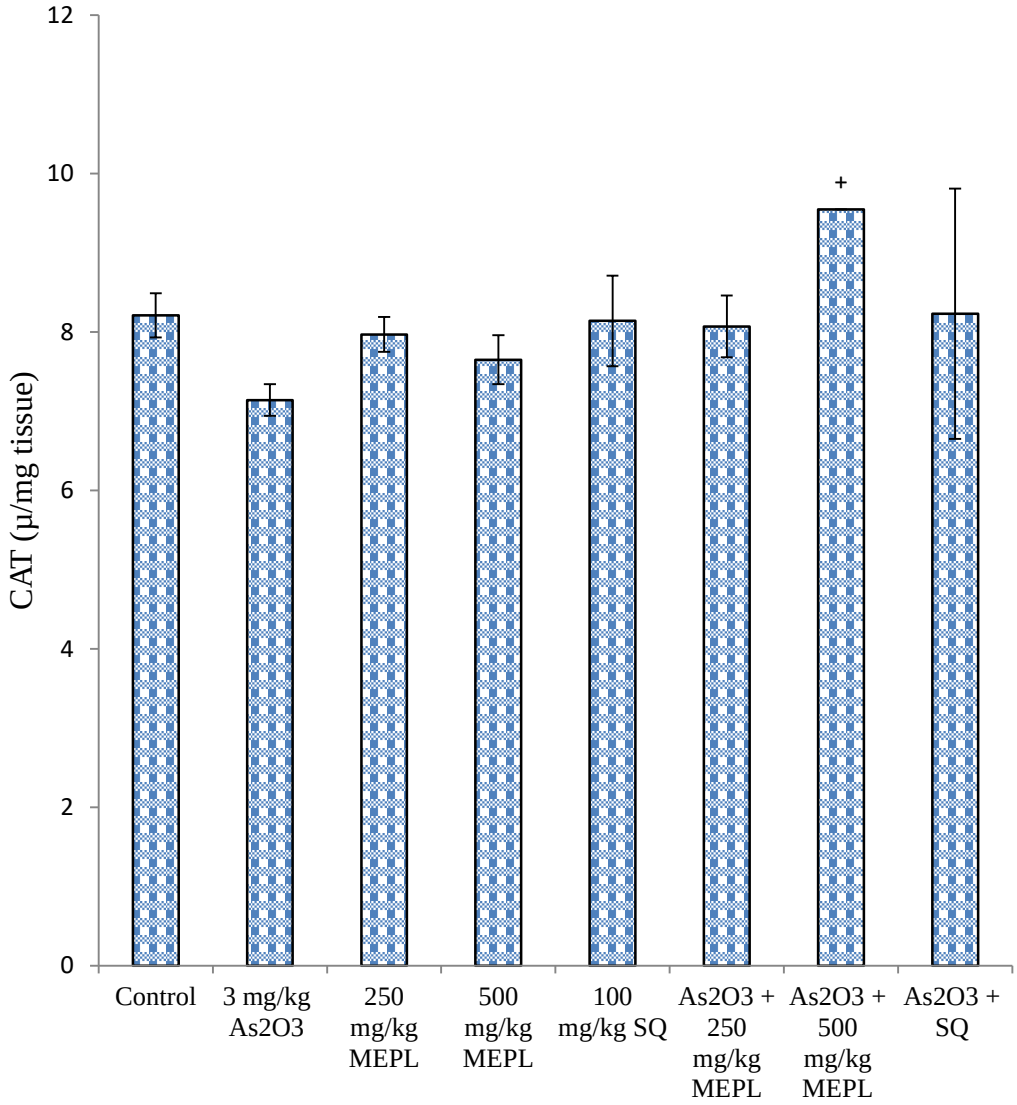


Fig. 4.47. Effects of methanol extract of MEPL and squalene on testicular catalse activity in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Columns represent mean ± SEM, n = 5

$^{+}p < 0.05$ as compared with As_2O_3

MEPL = Methanol Extract of *Parquetina nigrescens* Leaf

As_2O_3 = Arsenic trioxide

SQ = Squalene

4.17 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on testicular histology in arsenic trioxide-induced reproductive toxicity in male Wistar rats

Testicular histology of the As_2O_3 group showed atrophy of seminiferous tubules, empty seminiferous tubule lumen and sloughed germ cells, but the interstitial spaces appeared normal when compared with the control group which showed a normal histoarchitecture of seminiferous tubules. The histology of all the other experimental groups showed the presence of spermatozoa within the seminiferous tubule lumen, presence of different cells in various stages of development and uncompromised seminiferous tuble architecture, they are also comparable with the control group (Plate 4.4).

In this current study, the histoarchitecture of testis treated with arsenic showed atrophied seminiferous tubules, widened seminiferous tubule lumen, absence of germ cell layers in some tubules and sloughing of immature spermatids into the tubular lumen. This supports Khan *et al*. (2013). The increased MDA concentration noticed in the arsenic trioxide group which indicates increased lipid peroxidation and of course, increased presence of reactive oxygen species and hydroxyradicals might have caused disruption in testicular morphology. In like manner, the decreased level of FSH may have also contributed to the distorted morphology of the testes treated with arsenic because, FSH is important in the maturation of spermatogonia into spermatozoa. Since intracellular antioxidant enzymes remains in the fore front of protection against oxidative damage in cells, the observed increase in GPx activity in groups administered arsenic and MEPL or squalene proved the abilities of these substances to protect cells and hence maintain a normal histoarchitecture of testis as was evidently observed in the histology of all other experimental groups.

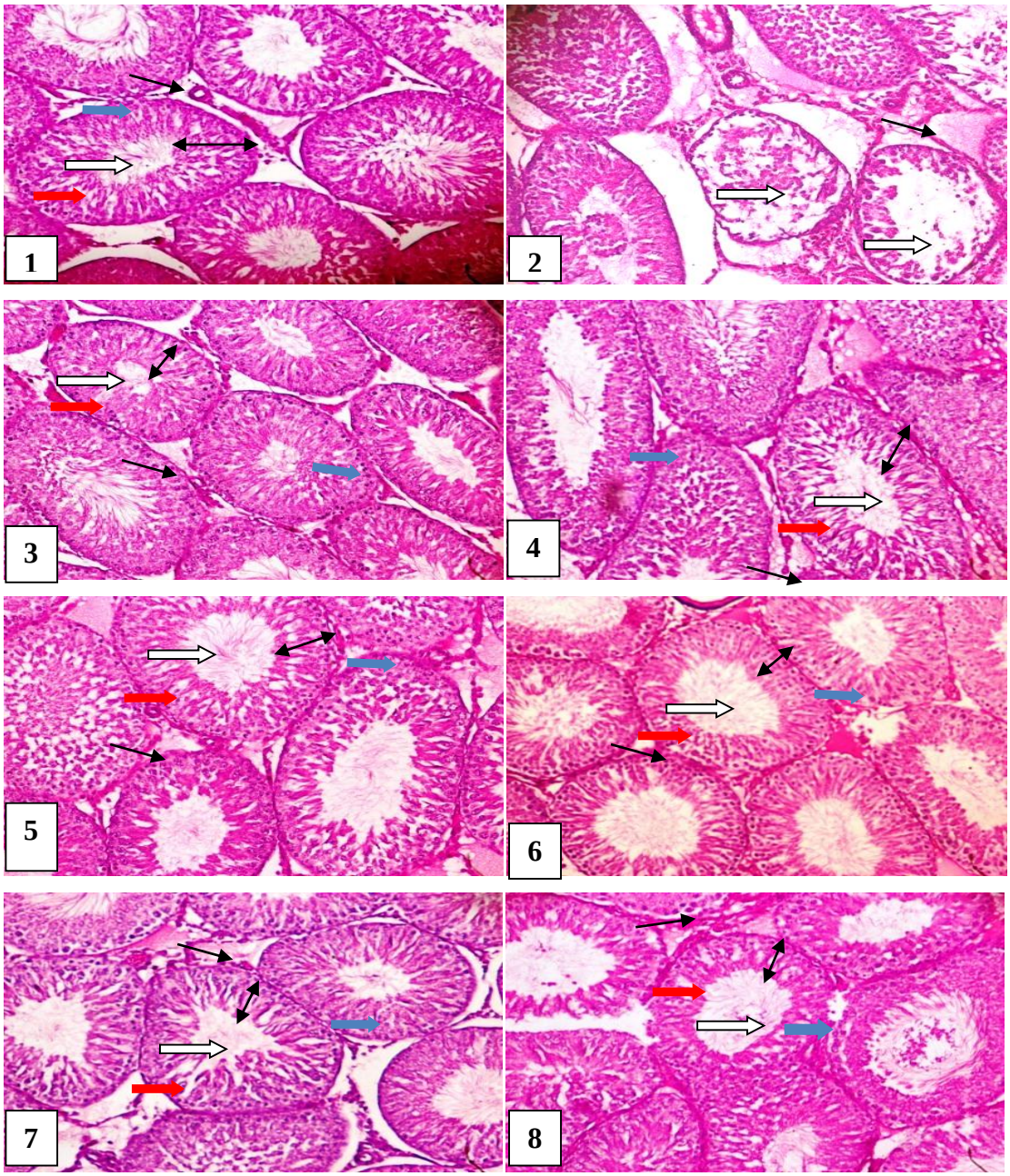


Plate 4.4. Photomicrograph of testicular sections from Wistar rats exposed to arsenic trioxide-induced reproductive toxicity and treated with either MEPL or squalene, stained by H&E and presented at x200. **(1)** Control, **(2)** As_2O_3, **(3)** 250 mg/kg MEPL , **(4)** 500 mg/kg MEPL, **(5)** 100 mg/kg squalene, **(6)** As_2O_3 + 250 mg/kg MEPL, **(7)** As_2O_3 + 500 mg/kg MEPL , **(8)** As_2O_3 + 100 mg/kg squalene. (White arrow indicates the seminiferous tubule lumen, spanned arrow indicates width of germ cell layer, blue arrow indicates normal spermatogonia, red arrow indicates normal Sertoli cells and slender arrow indicates interstitial space (Plate 4.4).

4.18 Effects of methanol extract of *Parquetina nigrescens* leaf and squalene on epididymal histology in arsenic trioxide-induced reproductive toxicity in male Wistar rats

The photomicrograph of epididymis in As_2O_3 group revealed a lot of empty ducts with lack of spermatozoa storage, In like manner, a few ducts in squalene group and in As_2O_3 + SQ appeared not well filled with spermatozoa. The groups treated with As_2O_3 + 250 mg/kg MEPL and As_2O_3 + 500 mg/kg MEPL had normal epididymal morphology with storage of spermatozoa within their ducts, normal smooth muscle layers and normal interstitial spaces that showed normal connective tissues which can be compared with the control group (Plate 4.5).

In this study, the histology of epididymis in the arsenic treated group showed several empty ducts with lack of spermatozoa storage. This observation may be a development upon the empty seminiferous tubule lumen, absence of developed sperms and germ cell layers in the testes which may mean that there were little or no spermatozoa in the seminiferous tubules to be transported to the epididymis. Also, arsenic accumulation within the epididymal duct (Workings *et al*., 1985) could result to sperm death due to increased ROS generation. The MEPL and squalene protected spermatozoa in the groups administered with arsenic trioxide and 250 mg/kg MEPL, 500 mg/kg MEPL or squalene within the testes and subsequently within the epididymis, thus normal epididymal morphology was seen in these groups.

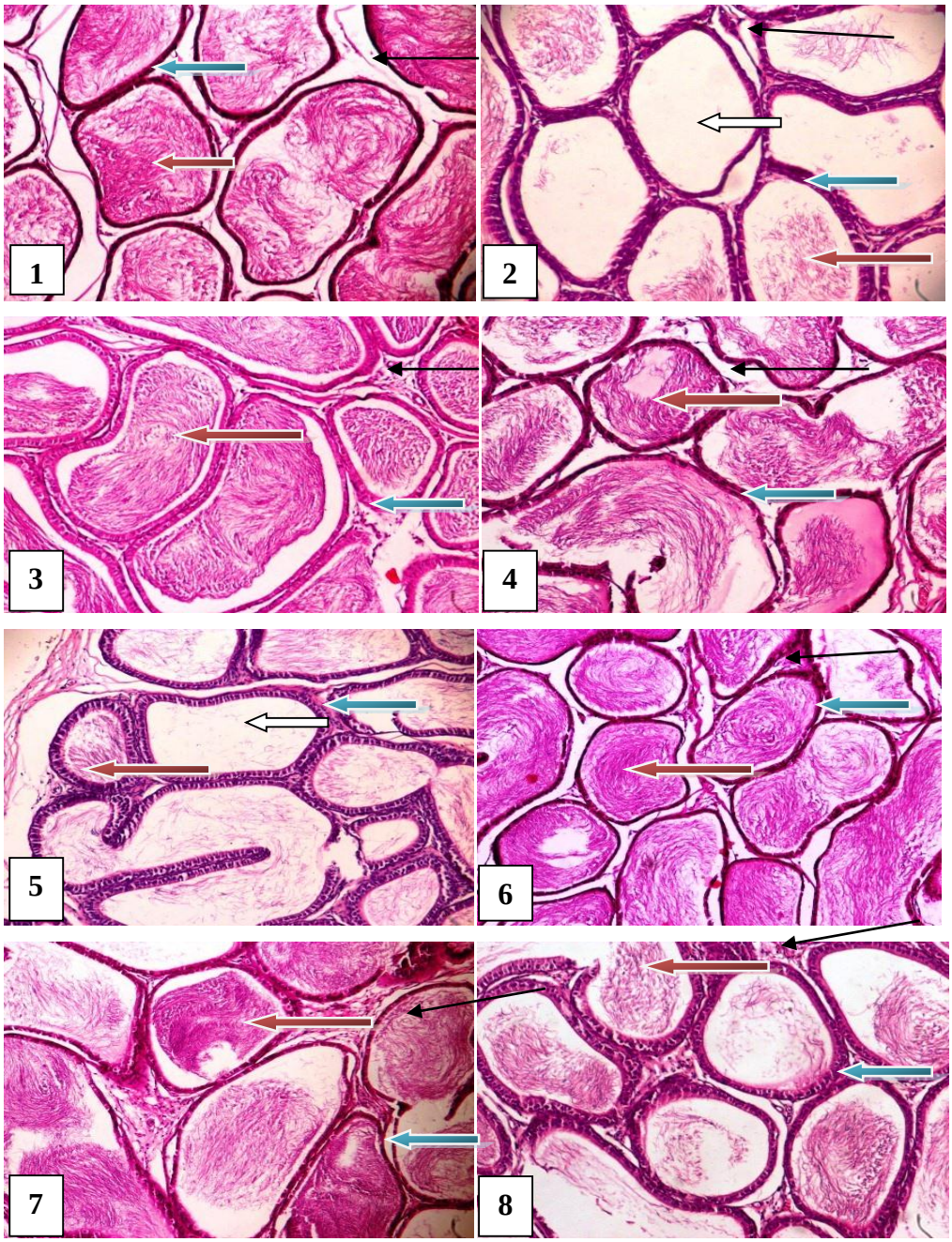


Plate 4.5. Photomicrograph of epididymal section from Wistar rats exposed to arsenic trioxide-induced reproductive toxicity and treated with either MEPL or squalene, stained with H&E and presented at x200. **(1)** Control, **(2)** As_2O_3, **(3)** 250 mg/kg MEPL, **(4)** 500 mg/kg MEPL, **(5)** 100 mg/kg SQ, **(6)** As_2O_3 + 250 mg/kg MEPL, **(7)** As_2O_3 + 500 mg/kg MEPL , **(8)** As_2O_3 + 100 mg/kg SQ. (White arrow indicates lack of spermatozoa storage within lumen, blue arrow indicates normal epididymal ducts with normal smooth muscle layer and epithelial layers, red arrow indicates presence of spermatozoa within the lumen and slender arrow indicates interstitial spaces).

CHAPTER 5

5.0 SUMMARY AND CONCLUSION

5.1 Summary

The problem of infertility in Nigeria is on the increase. Epidemiological research has provided statistics to proof that men's contribution to infertility is as much as that of women, this of course is against the African culture which always blame women for infertility and expect them to find solutions on their own. Prominent among the causes of male infertility is exposure to arsenic. Arsenic is present in underground water at high concentration in some parts of Nigeria and it causes male reproductive dysfunction by inducing oxidative stress. *Parquetina nigrescens* (Afzel) Bullock leaf is a common herb used in folkloric medicine for the management of male infertility. Report by other researchers suggested the antioxidant capacity of the leaf. Present in the chemical constituents of *Parquetina nigrescens* leaf is squalene, a compound of interest in this study. This study was designed to determine the possible effects of *Parquetina nigrescens* leaf and squalene on arsenic trioxide-induced reproductive toxicity in male Wistar rat.

Further in this study literature was reviewed on the male reproductive system and its regulation by the gonadotropins synthesized and secreted by the anterior pituitary gland. The means of exposure to arsenic, its metabolism, its mechanism of action, its toxicity as well as treatment in the case of poisoning was also reviewed. In like manner, the various research carried out on *Parquetina nigrescens* leaf and squalene were looked into. A review on the endogenous antioxidant enzymes was also considered.

The study was carried out in two phases. Phase one was done in order to be able to establish whether or not *Parquetina nigrescens* leaf has any effect on the male reproductive system of Wistar rats treated with arsenic. To achieve this, the leaf of *Parquetina nigrescens* was extracted using methanol as a solvent and freeze dried so, methanol extract of *Parquetina nigrescens* leaf (MEPL) was derived and its acute toxicity was determined. The MEPL was orally co-administered with arsenic trioxide for fifty-four (54) days to 40 male Wistar

rats grouped into 8 (n=5): group 1 (distilled water, 3 mL/kg), group 2 (3 mg/kg As_2O_3), group 3 (250 mg/kg MEPL), group 4 (500 mg/kg MEPL), group 5 (1000 mg/kg MEPL), group 6 (As_2O_3+250 mg/kg MEPL), group 7 (As_2O_3+500 mg/kg MEPL) and group 8 (As_2O_3+1000 mg/kg MEPL). An aliquot of MEPL was subjected to GC-MS and squalene among other compounds was present in it. *In vitro* antioxidant assay was also carried out on both MEPL and squalene. In the second phase of this work, squalene was introduced while the 1000 mg/kg MEPL group was eliminated, so the groups were named as follows: group 1 (distilled water, 3 mL/kg), group 2 (3 mg/kg As_2O_3), group 3 (250 mg/kg MEPL), group 4 (500 mg/kg MEPL), group 5 (100 mg/kg squalene), group 6 (As_2O_3+250 mg/kg MEPL), group 7 (As_2O_3+500 mg/kg MEPL), group 8 (As_2O_3+100 mg/kg squalene). The animals were anaesthetised using sodium thiopental, prior to collection of blood, testes and epididymes. Epididymal sperm analysis was done using computer-aided sperm analyser; testicular malondialdehyde, glutathione peroxidase, superoxide dismutase, catalase and 17-beta hydroxysteroid dehydrogenase (17β-HSD) were assayed spectrophotometrically, serum testosterone and 8-oxo-2-deoxyguanosine (8-OHdG) were assayed using ELISA. Testicular Bax and Bcl-2 expression were assessed by immunohistochemistry. Testicular and epididymal tissues were examined microscopically. Data were analysed using ANOVA and p value was set at less than 0.05.

Both MEPL and squalene were able to ameliorate the adverse effects of arsenic trioxide on sperm count, sperm motility, progressive sperm motility, sperm morphology, Bcl-2 gene expression, glutathione activity and improved 17-β HSD while reductions in testicular and serum MDA, 8-OHdG concentration sperm chromatin condensation abnormality, Bax gene expression were observed. The MEPL was able to improve testosterone concentration while squalene did not improve it but it improved FSH and LH concentration. The testicular histology and epididymal histology also improved upon the administration of MEPL and squalene.

5.2 Findings

The LD_{50} of MEPL was found to be greater than 5 g/kg. The GC-MS study revealed the presence of twenty-four compounds in MEPL, squalene was one of them and was chosen as the compound of interest. *In vitro* and *in vivo* antioxidant assay was carried out on MEPL and squalene and it was discovered that they both had antioxidant capacity but

MEPL exhibited higher *in vitro* capacity, while they both exhibited *in vivo* antioxidant capacity. The MEPL exhibited positive effects on the reproductive system function in arsenic trioxide-induced reproductive toxicity, hence it was further decided to find out its constituents and to compare the effects of both MEPL and squalene with each other. It was discovered that MEPL and squalene both ameliorated arsenic trioxide-induced reproductive toxicity in male Wistar rats.

5.3. Conclusion

This work revealed that phytochemical constituents of methanol extract of *Parquetina nigrescens* leaf has antioxidant effects and in-like manner, the *in vitro* antioxidant assay showed that MEPL and squalene have potent antioxidant abilities. Furthermore, MEPL and squalene through the mechanism of reduction of oxidative stress improved male reproductive functions by ameliorating testicular oxidative damage; improving sperm quality, testosterone concentration, 17β-hydroxysteroid dehydrogenase activity; down-regulating Bax; up-regulating Bcl-2; reducing 8-OHdG activity and maintaining normal histoarchitecture in testes and epididymes in arsenic-induced reproductive toxicity in male Wistar rats. This study therefore suggests that methanol extract of *Parquetina nigrescens* leaf and squalene have antioxidant capabilities that might be explored in the treatment of male infertility.

5.4. Contribution to knowledge

- This study has shown the presence of Phenol and flavonoids in MEPL and has proved its metal chelating ability.
- MEPL and squalene improved 17β-hydroxysteroid dehydrogenase in arsenic-treated Wistar rats.
- MEPL and squalene ameliorated oxidative stress by decreasing MDA concentration, 8-oxo-2’deoxyguanosine concentration in serum and testicular tissue while it improved glutathione activity.
- MEPL and squalene prevented apoptotic cell death of spermatozoa by down regulating Bax gene expression while up regulating Bcl-2 gene expression in the testes of arsenic treated Wistar rats.
- MEPL and squalene improved sperm count, motility and morphology in arsenic treated Wistar rats most likely by reducing oxidative stress in the testes

- MEPL improved testosterone level and luteinizing hormone level in arsenic treated Wistar rats.
- MEPL and squalene protected the histoarchitecture of testis and epididymis in arsenic treated Wistar rats.

5.5 Suggestions for further studies

Further studies will be carried out to:

1. Compare the effects of MEPL observed in this study with a standard exogenous antioxidant in arsenic-induced male reproductive toxicity in Wistar rats.
2. Quantify arsenic and other trace metals in the testes as well as epididymis and to compare the effects of MEPL observed in this study with a standard metal chelating drug in arsenic induced male reproductive toxicity.
3. Examine the anti-inflammatory potential of MEPL and squalene on arsenic trioxide-induced reproductive toxicity.

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