EFFECTS OF ETHANOL EXTRACT OF STEM BARK OF *ALSTONIA BOONEI* ON THE REPRODUCTIVE PARAMETERS OF ALBINO RATS.

BY

OZE, GABRIEL OBASI, BSc. (Nigeria), MSc., (Benin.)

(Reg. NO. 20054585608)

A THESIS SUBMITTED TO THE POST GRADUATE SCHOOL FEDERAL UNIVERSITY OF TECHNOLOGY, OWERRI

IN PARTIAL FULFILMENT OF THE EQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (Ph.D) IN BIOCHEMISTRY

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CERTIFICATION

This is to certify that this study on "Biochemical effects of the stem bark extract of *Alstonia boonei* (De wild) on the reproductive parameters of albino rats" was carried out by Oze, Gabriel Obasi (Reg. No. 20054585608) in the Department of Biochemistry, Federal University of Technology, Owerri, Nigeria and has been approved by the following.

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DEDICATION.

To God, Almighty. The Unquestionable One.

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

LIST OF PLATES

ALSTONIA BOONEI PLANT

Abstract

The effect of ethanol stem bark extract of *Alstonia boonei* (ABE) was studied on some reproductive parameters of Winstar albino rats. The objective was to determine if the crude ethanol extract of *A. boonei*, which is widely used in folkmedicine as antipsychotic, antimalarial and labour – inducing agent, portend any health risk to the users. The phytochemical identification of the constituents was carried out. Under controlled experimental protocols , the extract was evaluated for acute and sub-acute toxicity, biometric and biochemical determination of serum sex hormones, kidney and liver functions , as well as lipid peroxidation and haematological studies. The tests were carried out at a lower dose of 50/100mg/kg and a higher dose of 200/300mg/kg as the case may be. The extract was given by oral intubation for 2 and 4 weeks respectively. The results showed that the LD_{50} of the crude extract was 562mg/kg. The extract contained alkaloids, flavonoids, tannins and cardiac glycosides. The extract significantly ($p<0.05$) reduced the weight of the rats. The serum levels of estrogen and progesterone were significantly ($p<0.05$) elevated at the higher dose of the extract. There was no difference in the morphology and number of litters between the control and test groups. The serum testosterone concentration was reduced significantly($p<0.05$) . The sperm characteristics were more affected adversely in the 2weeks segment. The concentration of VMA in the anterior pituitary gland was raised significantly ($p<0.05$) in a dose dependent pattern. The results also showed that the kidney and liver functions were compromised. The lipid and haematological indices were adversely altered. The extract exhibited pro-oxidant properties. The histopathological findings supported the biochemical outcome. It is concluded that the crude ethanol extract of *Alstonia boonei* may have deleterious effects on reproductive parameters; and associated organs, especially at higher doses and on prolonged usage.

CHAPTER ONE

1.0 INTRODUCTION

1.1 INSIGHT

Researches have shown that ancient civilizations thrived on Traditional Medicinal Products (TMP). It is also true that these traditional medicinal products have out-lived the civilizations that birthed them. A study carried out in 1992 by the Collaborative Research Team from the Association for the Promotion of Traditional Medicine (PROMETRA), the University of Tulane, New-Orleans, and the Morehouse School of Medicine, Georgia, Atlanta, USA, revealed that 80% of the world still depend partly or wholly on traditional medicine [TM] (Dabra *et al.,* 2005). It is also on record that the most civilized and industrialized nation of the world, USA, spent approximately 17 billion US Dollars in 2000 on traditional medicine-based drugs; in a world where an average of sixty billion dollars is spent annually on TM preparations (WHO, 2008).

In the mid-15th century, the Spanish invaders of the civilization of the American-Indians, Aztec and Maya, met an advanced form of TM practice; and were encouraged to preserve some of the practices. The cinchona tree which yielded the miraculous quinine and subsequently chloroquine and other anti-malaria alkaloids, was obtained from the Amazon forest of South America. Owing to its great medicinal values, the British government developed the plant and similar ones in the Kew Botanic Garden, England. Quinine remains one of the most potent antimalarials today. In similar manner, the popular non-steriodal anti-inflammatory agent (NSAIA), aspirine, was harvested from salicin, an extract from the stem bark of the white willow plant. Some natural products have served as precurssors or templates for orthodox medicines. (Choi and Hwang, 2005). Historical antecedents show that the standard antihypertensive drug, reserpine, was obtained from the root extract of the *Rauwolfia vomitoria* found in the forest

of East Africa and some other parts of the world. The muscle relaxant, physostigmine (Eserine), was obtained from the forest of Nigeria as an alkaloid from the Calabar bean, *Physostigma venenosum*, whose local Efik name is Esere. The First WHO Congress on TM was held in Benjing, China, in November 2008.The congress gave rise to the Benjing Declaration which mandated member nations to increase the role of TM in their healthcare systems (Nyka, 2008).Similarly, the World Health Assembly (WHA) recognizes TM and reaffirmed the call for its integration into biomedicine in the search for the cure of HIV/AIDS ,and related diseases (WHA, 56.31,2003). About 35-40% of clinically used drugs are wholly or partly derived from natural products (Akerele, 1993). and the global market for TM is about 60 billion US dollars annually (Calixto, 2000; Kronenberg, 2001).

Further, Gutman (2004) quoted the report of the Canadian survey which showed that the dependence of the populace on natural drugs had increased to 81% between 1992 – 1997. The highest percentage of users being the urban dwellers with good income. A similar study in the United States predicted that the number of alternative drug users would continue to rise in coming years. The fear borne out of the foregoing led to the integration of orthodox and alternative medicines in the emerging science of Complementary Medicine. As at 1997, more than 30 colleges of Medicine in the United States had incorporated alternative medicine into their academic curricula. Moreover, the US health insurers have included it in their insurance risk schedule. (Matthew, 1999, Calixto, 2000) Furthermore, the giant pharmaceutical companies in that country are buying up nutriceutical, herbal and the natural product-based manufacturing facilities as a way of positioning themselves to face the future challenges, and to ensure that they remain relevant in the years to come.

Generally, biomolecular agents are empirically employed in folkmedicine without the scientific study of their therapeutics, mechanism of action and

possible adverse or toxic effects (Ahmed *et al.*, 2001). This is inimical to the health management of any nation, and negates the principles of rational drug use and application. In recent years, the health policies of Nigeria have shifted to accommodate complementary and alternative medicine (CAM) in line with the global trend and WHO recommendations (WHO, 2003).

1.2 Historical Background:

The more one goes into the study of natural medicinal products, the greater the chances of one discovering new medicaments for combating man's diseases as well as improving the health and well-being of the individual and the society in general. Most natural medicinal products are biochemicals. They represent indispensable tools for the development of new drugs in both the developing and industrialized nations (Bonati, 1980; Krogsgard *et al.*,1984; Naranjo, 1995; WHO, 1998; Choi & Hwang, 2003). The World Health Organization (2006) reported that about 70% of the population in developing countries still depend on natural medicinal plants. In 2007 the WHA recommended a combination therapy of artemisinine with locally researched drug agents to combat malaria which has become resistant to existing antimalarial agents in endemic countries.

The wisdom in this recommendation could be seen from the understanding that many civilizations have grown and survived on plant-based drugs. Conversely, plant-based drugs have outlived these civilizations and cultures (Schultes, 1978); such as those of the ancient Chinese, Indians, Aztecs, Greeks, Mayans, and the Egyptians, (Ahmed *et al.*, 2001). The great Chinese physician, Chou Kung, wrote the Thya in 1110 B.C. The book described a wide range of biomolecular compounds used as drugs. The same is true of Hippocrates (The Father of Medicine) and Dioscorides in Greece; then Susruta in India (Brain and Ross, 1978). In Central America, the ancient Aztecs are known to have documented the plant-derived drugs of their civilization. These were preserved

by the Spanish invader in the mid-15th century, and have contributed in no small measure to the growth and development of modern medicine. The 4-amino qunolines are products of this region of the world. The anti-oxidant-rich Noni Juice (NAFDAC No. 01-5106) is obtained from *Morinda citrifolia* which is native to the Polynesian Island of Tahiti, South Pacific. It was discovered and characterized in 1977 by the eminent biochemist, Dr. Raph Heinicke. The product is currently a major export commodity of Tahiti with annual national income running into millions of dollars. It is also a product of the Amazon region. Hence, Farnsworth, the great pharmacognocist, sees plants as the sleeping giant of drug development, (Farnsworth, 1984).

Benzoic acid was the first chemical to be isolated from plant in 1560 (Farnsworth, 1984). This was followed two-hundred and twenty-four years later by the opiate, morphine, from the dried latex of *Papaver somniferum* (Brain and Rose, 1978). Then in 1918, the first chemically pure alkaloid, ergotamine, was isolated from *Claviceps purpurea*; a fungus which grows on rye.

In 1938, Hoffman and Stoll partially synthesized ergometrine, a derivative of the ergot alkaloid. This opened the way for the industrial production of the drug (Stadler and Giger, 1984). The year 1954 saw the opening of an exciting new chapter in the medicinal chemistry and pharmacology of ergot alkaloids with the synthesis of bromocryptine, a dopamine receptor agonist. However, some of the biochemical parameters are still elusive.

After more than forty-decades from the isolation of benzoic acid, only about 5000 fully characterized chemicals have followed from the estimated 250,000 plant species known to exist on this planet (Schultes, 1978; Fairbaine, 1980; Ernt, 2001). Of this number, slightly more than 100 plant-derived drugs, which have been fully characterized biochemically, phytochemically and pharmacologically; are clinically in common use throughout the world (Grifo, 1996, Ernst 2001). This obviously depicts the slow pace of development of new

drugs from plants sources; while at the same time emphasizing the inadequacy of man's knowledge of the therapeutic potentials inherent in the plant kingdom. It is a sorry situation to note that the United States in 2000 alone spent 17.0 billion dollars on plant-based prescription drugs. Unlike China, which has about 1500 herbal pharmaceutical factories and more than 700 patented plant-based products (Snodgrass, 2001), no pharmaceutical firm in the US appears to run a well established research unit aimed at discovering new drugs from plant sources. Ironically too, the western world in recent years have stepped up their patronage for natural products against orthodox ones (Choi & Hwang, 2003).

In realization of these limitations the World Health Organization (WHO) in its First Internationl Congress on TM initiated the Benjing Declaration of November 2008, which called on member nations to increase the role of TM in their healthcare systems (Nyka, 2008). Previously the World Health Assembly in its 41st meeting held in Geneva, in May 1988, endorsed the Chiang Mai Declaration of March, 1988 (WHA: 41.19), calling on member states to initiate comprehensive programmes for the identification, evaluation, preparation, cultivation and conservation of medicinal plants used in folk medicine (Akerele, 1980).

1.2.0 Medicinal Plants in Drug Research and Development, Problems and Prospects:-

1.2.1 Problems:

At this stage, one common fact emerges: that there are problems and prospects associated with the research and development of new drugs from plant sources. Prominent in the list is the inability of the researcher to reproduce an initially interesting biological activity in some cases (Wagner and Horhamar, 1970; Farnsworth, 1984; Asuzu and Chineme; 1988, Gbile & Adesina 2004). This constitutes the sad experience of many researchers and a major frustration when studying plant extracts for biochemical and pharmacological activities.

(Yoshihira, *et al.*,1978; Galeff 1980, Nityanand & Anand, 1984; Levin *et al.*, 1988). It is difficult to identify where the problem emanates unless the researcher has absolute control of the botanical, biological and chemical aspects of the research (Farnsworth, 1984). Further, the measure of difficulty can be judged by the fact that the National Cancer Institute (N.C.I) programme of the United Sates only yielded four anti cancer agents after more than twenty years of existence and screening more than 40,000 plant products (Brain and Ross, 1978; Farnsworth 1984, Matthew, 1999, Elisabetsky & Costa-Campos, 2006).

Plant glycosides present special problems in drug research (Wagner and Horhammar, 1970; Farnsworth, 1980). The isolation of this class of plant constituent involves complicated procedures, and often their structural elucidation is difficult to the extent that phytochemical work on it is often given up (Fairbaine, 1980). Furthermore, most plant glycosides are labile and metabolically unstable, and may undergo rapid hydrolysis either enzymatically or spontaneously during the process of isolation and fractionation (Razdan, 1983). This can partly explain the frustration of biochemists in natural products research.

The instability of some plant products in the course of extraction and storage is an inevitable problem (Fairbaine, 1980) even for specialized chemists (Galeff, 1980). This problem can partly be overcome by employing rapid extraction procedures (such as the use of high performance liquid chromatography with electrochemical detectors) and identification processes and by storing the extracts at low temperature so as to slow the process of metabolic breakdown. They may also be preserved in organic solvents that are known not to react with the constituents of the extract. The cost in terms of time and resources is so much that even multinational drug manufacturers are reluctant to invest in medicinal plant research. The assumption is that there is greater economic advantage in purchasing the patency of already discovered drugs.

The unfortunate truth about the whole process is that the rural dwellers who harbour some of these plants in their forests could hardly afford the cost of the drugs emanating from them (Ampofo and Johnson-Romauld, 1978, WHO, 2008). The situation is more pathetic with the understanding that the developing countries are the cradle of a reasonable percentage of orthodox plant-derived therapeutic biochemicals (Schulter, 1978. Okujagu, 2005). It has been reported that some haematological problems including anaemia are associated with the unscientific ingestion of plant materials as therapeutic agents (Okonkwo *et al.*, 2004, Adebayo, 2005). In 1998, Professor Wambebe, former Director General of the Institute of Pharmaceutical Research, Abuja, raised the fear that some of our medicinal plants have gone into extinction because we have been slow to researching on them.

1.2.2 Prospects:

As was noted earlier, the use of plant-based medicinal products as therapeutic agents is as old as man himself and has outlived the civilizations that birthed them. Some domestic pets such as dogs seek out the appropriate plants to eat when they are sick!

The period between 1804 (when morphine was isolated) and the present day, witnessed a surge, catalysed by the boundless curiosity to discover the medicinal values harboured by the estimated 250,000-500,000 species of plants on earth (Matthew, 1999). The United States N.C.I. alone screened over 40,000 plant products in search of anticancer property. Not this alone, she spent over 17.0 billion dollars in 2000 alone on plant-based prescription drugs; while in Great Britain more than 500 remedies in current use are derived from about 5000 medicinal plants (Fairbaine, 1980). It is of particular interest to note that about 35-40% of all prescription drugs in the industrialized countries contain active principles derived from plants (Atta-ur- Rahman *et al.*, 1984; Ernst, 2001). At the same time about 75% of the population of developing countries

depend on medicinals of plant origin (Ampofo and Johnson-Romauld, 1978), and more than 60% of the population of industrialized nations patronize herbal products (Snodgrass 2001): Stop to think of the current stocking of supermarkets with herbal toilet soaps and tooth-pastes, including the popular Unilever Close-up herbal toothpaste! Furthermore,Xechem Nigeria Ltd (A phytopharmaceutical company based in Lagos, Nigeria) is currently marketing Nicosan(TM) for the treatment of sickle cell disease.Furthermore, the GDP Ayurvedic University, New Brunswick, New Jersey, USA, signed an agreement with Dr. Lious Obyo Nelson (formerly of The Raw Material Development and Research Council .Nigeria; and the US patent holder of the antidiabetic drug derived from Vernonia amygdalina) on Tuesday, 3rd February 2009, at the Sheraton Hotels and Towers, Abuja, in the presence of the representatives of the Nigerian government; for the production and marketing of the phytomedicine.Therefore, the cardinal role played by phytotherapy in the health- care system of any nation should be given due merit.

Scientific investigations, particularly in this century, have resulted in the identification and characterization of a growing number of active constituents of plants, many of which are routinely used in modern medicine e.g reserpine, quinine and the related 4-aminoquinolines, atropine, morphine, digoxine, artemisinine etc.

Vinblastine, vincristine, vincamin, and camtothecin are interesting alkaloids obtained from *Catharantus roseus* (Elizabetsky & Costa-Campos, 2006). These alkaloids are of special interest because of the circumstance surrounding their discovery. *C. roseus* was selected for screening based mainly on the folkloric claim of hypoglycemic property, but its extracts turned out to possess antitumour activity. Further, they were simultaneously discovered in 1958 by Eli Lilly Company, U.S.A and a group of scientists: Nobel, Beer and Cutts, in

Canada. They turned out to be the only antitumour agents which emerged from the more than twenty years labour of the N.C.I.

Perhaps of equal importance is the fact that plant derived products have extensively served as lead-substances for the discovery and synthesis of new drugs. Besides, they have often been employed as templates for the total or partial synthesis of new agents e.g the structural modification and chemical synthesis of a number of quinine, artemisinine. Ergot and morphine derivatives in attempts to reduce their adverse effects improve potency, combat resistance and create new markets. Etorphine is one of the ethnomorphinans reputed to be more than 1000 times active than morphine itself (Rapoport, 1984; Witkop and Brossi, 1984). Equally interesting is the isolation of dermorphine from a specie of South American frog belonging to the genus *phyllomedusa*. The analgesic activity of this agent is also claimed to be 1000 times that of morphine (Rapoport, 1984).

Tropane (Anatoxin-A) commonly called “very fast death factor” (VFDF) is derived from the fresh water blue-green algae called *Anabaena flosaquae*. Tropane is 30 times more active than carbachol, and it is one of the most potent nicotinic acetylcholine receptor agonist known . The persistence and potency of tropane is partly accounted for by the fact that it is not a choline ester, and is therefore neither sensitive to acetylcholinesterase (AChE) nor to pseudocholinesterase hydrolysis (Witkop and Brossi, 1984). Like the Amazon arrow poison (Curare), Tropane may be chemically modified to yield life saving drugs.

Nabilone is marketed in Canada as an antiemetic and antinausea agent. It is the first clinically employed and legally marketed derivative of cannabinol (Tetrahydrocannabinol [THC]). Nabitan and Naboctate have similarly been developed from the same source for use as antiglaucoma drugs (Razdan, 1984).

The discovery of saponin in the plant kingdom has revolutionized the steroid-based industrial preparations such as cortisone, oral contraceptives, steroid hormones, confectionaries and some brewery products (Claus *et al.*, 1974; Udeala, 1984). The economic importance of this discovery is better appreciated when it is recalled that prior to this discovery, more than 40 cattles were slaughtered and the adrenal-steroid extracted for the synthesis of few tablets of cortisone (Sofowora, 1984). In recent times, about 300,000 pounds of licorice saponin -extract alone are imported annually into the United States for pharmaceutical and allied uses (Claus *et al.*, 1974). Medicinal plants have in recent times assumed a bridal position with the discovery that some of them contain secondary metabolites with antioxidant properties (Lands *et al.*, 1995; Tsai *et al.*, 2000, Kent *et al.*, 2003; Bounom, 2000; Afolabi *et al.*, 2007).

1.3 Medicinal Plants in Drug Research and Development: The Folkloric Approach:

Among the various approaches adopted for the development of plant-based drugs, folkloric approach is the most popular. This is so because it has yielded more clinically important drugs than any other approach (Brain and Ross, 1978; Farnsworth, 1984, Olajide and Awe, 2000). Among others, quinine, digoxine, tubocurarine were selected based on this approach (Brain and Ross, 1978). However, occasions have arisen where folklore claims have either proved false or yielded drugs with different pharmacologic properties. This was the case with C. *roseus* considered earlier.It is in recognition of the immense success of folkloric approach to the development of plant-based drugs that *Alstonia boonei* was selected.

1.4 Basic Pharmacological Profile of Some of The Known Constituents of *Alstonia boonei* and Related Plant Species:

Oigiangbe *et al.*, (2007) reported the insecticidal activity of *A. boonei* against the laeva of *Maruca Fabricus* (Cow-pea pest). The report indicated that the aqeuaose stem bark extract exhibited chemo-toxic action against the laeva. Some other studies demonstrated the activity spectrum ranging from, anthelmintic, (Wesche *et al.*, 1990, Wright *et al.*, 1993); bacteriostatic, anti diuresis, anti hypertensive, anti-malarial (Iwu, 1993, Foster *et al.*, 1990). The leaf extract was reported to possess anti-inflammatory, antimicrobial and anti-rheumatoid activities (Foster *et al.*, 1990).

In 2005, Fasola and Egunyomi showed the presence of cardiac glycosides and saponins in *A. boonei* and *Azaridicta indica* stem bark extracts. A similar report was published on the flavonoid components of *Alstonia scholaris* (Singh & Singh, 2005). The pesticidal effect of *A. boonei* root and stem bark extract have been reported (Sadek, 2003).

The medicinal profiles of other Alstonia species have been worked on. There are more than 60 botanically characterized species, out of which *A. boonei* stands out in medicinal values.

1.5 Ethnomedical Uses:

There was consistency in the claims of Alhaji Busali Odia (Ukhu village, Ekpoma, Edo State), Nze Oparaji (Aboh Mbaise, Imo State) and Stephen Omosowa (Benin) in separate interviews regarding the tradomedical uses of *A. boonei*. The extract of the stem bark in water or local gin is essentially used for the treatment of malaria and mental illness. The extract of the leaves and roots serve similar purposes as the stem bark. The method of preparation could be varied to make it suitable for use in the treatment of jaundice, as antivenom against snake bite, as arrow poison or as analgesic and anti-inflammatory

preparation in rheumatoid arthritis, Visceral and menstrual pains. It could also be prepared to as a tonic for penil erection! A *boonei* is a sacred medicinal plant among the Baka pygmies of Central Africa. (Bethi, 2004). The extract of the stem bark is listed as an anti-malaria agent in the African .Pharmacopoeia (Olajide *et al.,* 2007)

1.6 Biochemical and Pytochemical Profile of *A. boonie*:

There is little biochemical and toxicological report on this specie of Alstonia. Elisabetsky and Costa-campos (2006) reported the presence of indole alkanoids, porphyrines, triterpenoids, echitamidins and Alstovenines; from which the following compounds have been isolated and characteriszed: alstonine, echitamine, reserpine, chlorogenine, ditamine, and venenatinine.

This specie was among the more than 540 Nigerian medicinal plants phytochemically screened by Odebiyi and Sofowora (1978) and Sofowora (1984). They did show that the roots, stems and leaves of *Alstonia boonei* specie so far screened contained extracts suspected to be saponins, flavonoids, alkaloids, steroids, terpenoids and tannins. These results corroborate the findings of Onanga *et al.,* (1999) and Oyedemi (2005) in the screening of *A. boonei.* They showed that the stem bark extract of the plant contained saponins, flavonoids and cardiac glycosides, but did not show the presence of tannins.

1.7 Toxicity of Medicinal Plant Extracts:

The fact remains that any crude medicinal extract poses a potential health risk because of non-standardization and the presence of multiple biochemical substances, some of which are potent toxins (Boston, 1998). There have been reported cases of injury, deformity or death arising from the use, misuse, abuse, contamination and/or adulteration of medicinal herb extracts (De-Smat *et al.,* 1997, Ernst, 2001, 2002, Snodgrass, 2001; Raji *et al.,* 2005). Baliga *et al.,*.,

(2004) showed that the aqueous and methanol extracts of A. *scholaris* were cytotoxic at 240mg/kg in rats and mice. The foregoing underscores the need for co-ordinated biochemical studies on these commonly used medicinal plants such as *A. boonei*. Some of the adverse effects of *A. boonei* on the kidneys, liver and blood have been reported (Oze *et al.*, 2006, 2010).

The preliminary work further showed that the alcoholic extract of this plant had sedative effect on rats, nephrotoxic, hepato-toxic, lactogenic, and spasmolytic activities in rodents. Prolongation of barbiturate-induced sleeping time in rats and anticonvulsant property at doses ranging from 120mg/kg of crude extract and above are also known.

It has been reported that aqueous *Ocimum gratissimum* leaf extract causes some functional damage to critical organs such as the kidney and liver when taken in varying doses (Effraim *et al.,* 2003). *Myristica fragrans* (nutmeg) has been shown to have similar effects on the organs, causing degeneration, necrosis with desquamation of tubular epithelial cells and congestion of renal blood vessels as well as degeneration of myocardial tissues in albino rats (Olaleye *et al.,*2006). Furthermore, Ajagbonna and Onyeyili (2003) revealed that *Rhaptopetalum coriaceum* stem bark extract produced vascular congestion, hepatic and renal necrosis in rats. There is paucity of published work on the effect of *A. boonei* on the organs, especially the reproductive organ. This makes this study essential and imperative.

1.8 Reproductive Toxicity of Plant Extracts:

Some antimalarial and antimicrobial agents have been reported to have adverse effects on reproductive indices. For instance, the antisteroidogenic and antifertility actions of quinine, and chloroquine (Alkaloids from the stem bark of the Cinchona plant) have been documented (Meisel *et al.,* 1993). Neuruleptic

drugs adversely cause breast enlargement and stimulate lactation because of their action on the catecholamine in the CNS.

The induction of reversible male infertility and teratogenicity in experimental animals and humans resulting from treatment with medicinal plants which are claimed to posses' antimalarial properties have also drawn the attention of researchers. The antisteroidogenic and antifertility activities of extracts of *Carica papaya*, *Quassia amara* and *Azaridicta indica* have also been reported (Raju *et al.,* 2006).

With the renewed efforts in the development of alternative antimalarial drugs due to the challenges posed by the chloroquine resistant strains of malaria parasites, the scientific evaluation of *A. boonei* stem bark extract, which is a common antimalaria agent in folkmedicine becomes necessary; In the absence of adequate information on the reproductive toxicity of *Alstonia boonei*, whose extract is globally employed in mental illness and malaria chemotherapy in local settings, the present work serves useful purpose to supply some of the information. Further, no literature was found with the histopathologic study on the reproductive organs of animals treated with *A.boonei.* This study wil help to bridge the information gap especially in rats.

The crude extracts of *A. boonei* are extensively used in folkmedicine by a reasonable percentage of the population especially the developing nations. But there is dearth of organized scientific information about their beneficial or deleterious effects, as well as it's and measure of safety. In the light of the foregoing, this work attempts to provide some scientific information on some aspects of the biochemical and acute toxicity parameters of the extract.

1.9 Research Question: In the course of local treatment of some of these ailments using *A.boonei* crude extract, can some of the functions of the reproductive organs be compromised under controlled experimental setting?

1.10 Aim and Objectives:

1.10.1 Overall Aim:

➢ To investigate the biochemical and acute toxicity effects of the ethanol extract of the stem bark of *A. boonei* on some reproductive potential of Wistar albino rats. The tissues to be used in the studies include those of the uterus, testes, pituitary, liver, kidney and blood.

1.10.2 Specific Objectives:

1. To identify the phytochemical constituents of ethanol extract of the stem bark of *Alstonia boonei*.
2. To determine the serum levels of some reproductive hormones using rats.
3. To study the possible effects of dose or duration of the extract on the reproductive parameters.
4. To determine some possible effects of the extract on the foetus.
5. To study the effects of the extract on the histology of some of the affected organs/tissues.

1.10.3 Justification:

The crude extracts of *A. boonei* are extensively used in folkmedicine especially in the developing nations. But there is inadequate scientific information about the adverse or toxic effects of the crude extract, especially on reproductive parameters. This study should contribute to generate information in this area.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 THE PLANT: *ALSTONIA BOONEI*

Alstonia is named after Dr. Charles Alston (1685-1760), a professor of botany at Edinburgh University, Scotland, from 1716-1760. Alstonia is a widespread genus of evergreen trees and shrubs from dogbane family (Apocynaceae). Altsonia consists of about 40-60 species (Gandhi and Vinayak, 1990). It is native to tropical and subtropical Africa, Central America, South East Asia, Australia and New South Wales, England, and the Oceana; with most of the species in the Malaysian region (forster, *et al.,* 1990; Kade, 1998). The present study is conducted on *A. boonei* (De Wild). *Alstonia boonei* is a deciduous tree that grows up to 30m tall, 1.2m in diameter and 3m in circumference, with brown bark which exudes white copious latex when slashed.

Some Trade Names - Alstonia, Cheeswood, Pattern wood, stool wood, Australian quinine.

Taxonomy (Kade, 1998)

Kingdom	Plantae	Plant
Subkingdom	Tracheobionta	Vascular Plants
Super Division	Spermatophyte	Seed bearing plant
Division	Magnoliophyta	Flower bearing plant
Class	Magnoliopsida	Dicotyledon
Subclass	Asteridae	
Order	Gentianeles	

Family	Apocynaceae	Dogbane
Genus	Alstonia	
Specie	Boonei	
Current name	Alstonia boonei	
Authority	De-Wild	Initial Identity

2.1.1 Ecology

Alstonia boonei is found in dry, peripheral, semi-evergreen Guinea-Congolian tropical forest and transitional rain forest. Else where, it occurs in similar habitats and in swamps and riverine forests, (Okafor, 2000). *Alstonia boonei* requires large amounts of light and colonizes gaps in the forest. It has plenty of natural regeneration in young secondary forest.

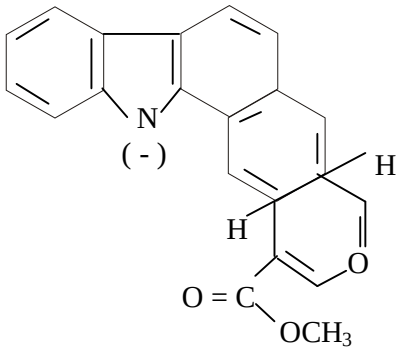
In West Africa, *Alstonia boonei* is a tree of the wet forest. It can tolerate a wide range of sites from rocky hillsides to seasonal swamps. In general, it prefers damp situation, but grows satisfactorily on well-drained slopes. (Katende, 1995).

2.1.2 Phytochemical Constituents of *Alstonia boonei* Stem Bark Extract:

Results from a number of phytochemical studies showed that *A. boonei* contains indole alkaloids such as alstonine, porphyrines and astonidine, which are said to have antipsychotic and anxiolytic properties (Costa-Campos *et al.*, 2004a). The bark of *Alstonia boonei* was also reported to contain echitamine derivatives and boonein. The triterpenes, beta-amyrin and lupenol-amyrin, are also found in the bark. (Kucera *et al.,* 1972; Raji *et al.*, 2007).

2.1.3 Medicinal Chemistry:

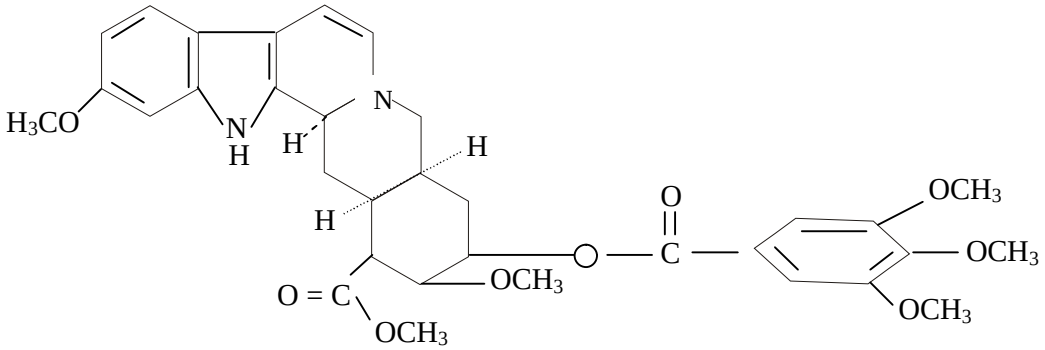
The main psychoactive constituent of *A. boonei* stem bark is alstonine. (Elizabetsky & Costa-campos, 2006). Alstonine is an indole alkaloid whose structure is closely related to standard psychoactive compounds such as reserpine and α - yohimbine. Their structural relationships are shown below:



Alstonine

(Elizabetsky & Costa – Campos, 2006)

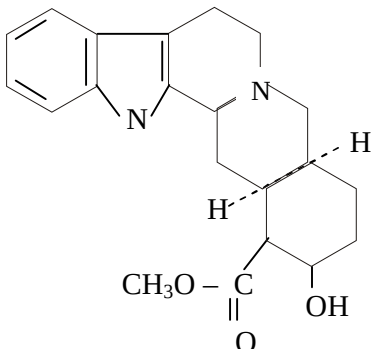
Fig. 2.1a: Structure of alstonine.



(Katzung *et.al*., 2009)

Reserpine

Fig.2.1b: The structure of reserpine.



α - Yohimbine

(Olaniyi, 2000)

Fig.2.1c: Structure of alpha-yohimbine.

Reserpine is an obsolete neuroleptic agent. Alpha-yohimbine was popular as a sex stimulant. Alstonine exhibits neuroleptic properties (Elizabetsky & Costa-Campos, 2006).

Alstonine is one of the important members of the indole alkaloids found in *A. boonei.* Most of the psycchoactions are attributed to alstonine (Cano *et al.,* 2003). According to Maureen *et al.,* (2000), alkaloids are large group of nitrogenous bases found in plants. They are usually present in the leaves, bark and seeds of plants.

William (1990), observed that more than 25% of known alkaloids possess interesting pharmacological properties; the indole alkaloids making up the greater percentage.

The alkaloids *of Alstonia boonei* belong to the indole group. The indole alkaloid, alstonine, found in the plant is represented by the chemical formula: 3,4,5,6,16,17-hexadehydro-16- (Methoxy carbonyl) - 19 α - methyl -20 -α - oxynoimbanium; [$C_{22}H_{22}$ N $_2O_3$] (Olaniyi,1990).

2.1.4 Economic Uses of *A. boonei*:

2.1.4.1 Timber:

The sapwood of *A. boonei,* which has little or no difference to the heartwood, is used in the making of some export items; items such as stools, carvings, domestic utensils, toys, masks, canoes, horns, boxes and woodwool for packing of bananas. The well-known Ashanti stools of Ghana are made from it. (Katenda,1995).

2.1.4.2 Latex:

The latex of *A. boonei* gives an inferior resinous coagulate which has been used to adulterate rubber. The latex is toxic to delicate tissues, especially the eyes (William, 1990).

2.1.5 Ethnomedical Uses of *Alstonoa Boonei* Extract:

The infusion of the root or stem bark extract is taken internally or used to bathe as a remedy for dizziness asthma and impotence (Asanga, 2005). *Alstonia boonei* is among the natural collections of the Baka pygmies of Central Africa for treating hernia, typhoid fever, snakebites and malaria. Similar uses are also known in other African countries such as Equatorial Guinea (Bitsindou, 1996), Congo-Brazzaville, (Diafouka, 1997); Nigeria (Inyang, 2003), Senegal and Togo (Richel, 1995) and the Democratic Republic of Congo (Magilu *et al.*, 1996). The anti pyretic property of the plant extract may explain its frequent use for malaria and typhoid fever (Oliver-Bever, 1986; Olajide *et al.*, 2000). These two diseases are characterized by high fever, which appears to be one of the important symptoms for their diagnosis (Betti, 2002a; 2004).

The use of *A. boonei* extract in septicemia, hernia and snakebites may be linked to the analgesic (Olajide *et al.*, 2000) and anti-inflammatory (Kweifio-Okai *et al.*, 1995; Olajide *et al.*, 2000, Raji, *et al.*, 2000) properties of the plant. The stem bark of *A. boonei* has widespread uses in Ghana to assuage toothaches and to induce placenta discharge (Bluementtal *et al.*, 1998). This agrees with the oxytocic effect of the extract reported by Hobbs (1992). In Sierra Leone; it is used as an anthelminthic agent. In Cote d'ivoire, the leaves, pulped to a paste, are topically applied to treat oedema and the leaf-sap for cleansing of sores (Burkill, 1994). It is used in Burkina Faso as a decoction to cleanse suppurating sores and exposed fractures. In Nigeria, it is also used for the management of sores and ulcers; while in Cameroon and Liberia, it is used against snakebite and arrow poison (Murray, 1995). These uses futher suggest that *A. boonei* may possess antimicrobial, analgesic and antinflammatory properties.

The stem bark is also used as an astringent, (Obute 2000). Dalziel (1957, 1961), Oliver and Bever (1986) and Iwu (1993) reported the popular use of the extract

to treat malaria; and is listed in the African pharmacopoeia as an anti-malaria drug (Ojewole, 1984). Asuzu and Anaga (1991) reported the use of the stem bark extract for treating ailments such as painful micturition, menstrual pain, and rheumatoid conditions. Oluka, (2006) claims the use of the aqueous extract of the stem bark against malaria, stomach ache and mental illness at Mbaise, Nigeria.

2.1.6 Ethno-pharmacological Profile of *A. boonei*:

2.1.6.1 Schizophrenic Syndrome:

Alstonine is an important constituent of *A. boonei*. The neuroleptic properties of *A. boonei* are associated with the alkaloid. Elisabetsky & Costa-Compos (2006) reported their experience in Western Nigeria as follows: "A piece of root about 20cm long and 5cm in diameter was added to 2.5L of water and boiled until the liquid turned reddish brown. Initial dose for an adult was one cup daily in severe cases of madness, and the dose is lowered as the restoration process progresses. Similarly, the aqueous leaf extract has sedative effects. If the patient arrived in a very agitated state, one teaspoon of the powdered root is prepared in 20ml alcohol and taken in a single dose to cause almost 3 hours of deep sleep." A decoction with the bark of *A. boonei* is used extensively in Ghana to treat longstanding hypertention (Carol and Kofi, 2005). There have also been reports of the plant extract in anxiogenesis and as an abortifacient (Elizabesky and Costa-campos, 2006).

2.1.6.2 Anxiogenic Syndrome:

Symptoms of anxiety are very common and are thought to be predisposing factors to mental disorders, including mania, schizophrenia, depression, neurosis etc. Antipsychotic therapy reduces anxiety in addition to alleviating psychosis (Bourin *et al.,*, 2001). Alstonine possess clear neuroleptic and

anxiolytic activities mediated by 5- HT receptors; indicative of atypical antipsychotic mechanism (Odini, 2005; Costa- Campos, 2006).

Further, other pharmacological actions reported include diuresis (Kucera *et.al,* 1972); hypertention (Baliga *et al.,* 2004), and lipid peroxdation (Olaleye *et al.,* 2004).

Alstonine seems to antagonize the glutamate neurotransmitter in a manner consistent with its beneficial effects in schizophrenia.It lacks the neurological side-effects associated with classical anti-psychotics, a considerable advantage for long term usage. The lack of direct effect on the catecholamine neuronal pathway suggests lack of significant extra-pyramidal side- effects, a major drawback of classical antipsychotic agents .These properties make alstonine atypical as a neuroleptic agent (Elizabetsky and Costa-campos, 2006).

2.1.6.3 Action on Clones of Plasmodium Falciparum:

A closely related *Alstonia congensis* was reported by Awe and Opeke (1990) to be effective against *Plasmodium berghei berghei* in mice. The extract of *A. boonei* significantly inhibited the growth of W-2 and D-6 clones of *Plasmodium falciparium* in-vitro(Wesche *et .al*, 1990).

2.1.6.4 Male Anti-Fertility Properties:

Raji *et al.,*. (2005) reported that the methanol extract of *Alstonia boonei* stem bark inhibited sperm viability, motility and lowered sperm counts in rats. The reproductive parameters returned to normal after 12 weeks of therapy with the plant extract,and concluded that the plant extract should have reversible anti-fertlity property in male rats .Oze *et al.,* .(2007) made a similar observation.

2.1.6.5 Anti-inflammatory, and Analgesic Properties:

Studies showed that *A. boonei* stem bark extract inhibited carrageenan- induced paw-oedema, cotton pellet granuloma, and exhibited an anti-arthritic activity in

rats (Sadebe, 2002). Olajide *et al.,* (2002) found that vascular permeability induced by acetic acid in the peritoneum of mice was also inhibited. Furthermore, *A. boonei* extract produced marked analgesic activity by reducing acetic acid-induced writhing, as well as the early and late phases of paw licking in mice. Kweifo-Okai *et al.,* (1995) also reported that the extract of the plant attenuated serum hyaluronidase and blood granulocytes towards non-arthritic levels, and corrected moderate anaemia of adjuvant arthritis.

2.1.6.6 Diuretic Activity:

Kucera *et al.,* (1972) and Raji *et al.,* (2005) reported the net loss of electrolyte, water and weight in the presence of aqueous extract of the stem bark of *A. boonei* in rats. The effects of the extract on serum electrolyes were alsoreported by Oze *et al.,* (2006).

2.1.6.7 Aphrodisiac Activity:

Lewis and Elvin-Lewis (1977) reported its aphrodisiac activity, causing sexual stimulation and excitement in males; and prolongation of penil erection and delayed orgasm; acting as a general tonic and mild stimulant for the nerves.

2.1.6.8 Hyperlipidaemic and Pro-oxidant Properties:

The extract of *Alstonia boonei* increased the total cholesterol level in experimental animals which may suggest its hyperlipidaemic property (Baliga *et al.,* 2004).Its weak antioxidant activity was reported by Olaleye *et al.,* (2004); and they attributed this to its indole alkaloid content.The pro-oxidant activity of its extract was also published by Genesh *et al.,(*2005). Similar findings were made by Oze *et al.,* (2009).

2.1.7 Some Adverse Effects of Extracts of Alstonia Species as Pharmacological Agents:

The work of Raji *et al.*,(2005) suggested that the extract was capable of reducing serum testosterone level as well as having deleterious effect on testicular androgen secretory function, due to the observation of visible lesions in the testes of the experimental animals.

Genesh *et al.*, (2005) further demonstrated that high dose of the extract, time-dependently elevated lipid peroxidation and reduced the serum level of the endogenous antioxidant, glutathione (GSH). Further, high dose of the crude extract of *A. scholaris* raised the serum liver marker enzymes, creatinine, urea, glucose and amonium ion (Baliga *et al.,* 2004).Similarly, we have reported the nephrotoxic effects of *A. boonei* extracts (Oze *et al.*,2006).

2.2 THE GONADS:

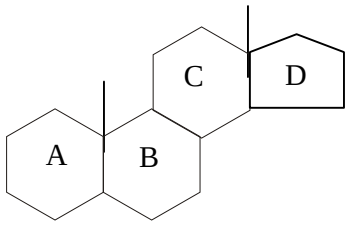
The gonads are bifunctional organs that produce germ cells and the sex hormones. These two functions are closely correlated in the sense that high local concentration of the sex hormones is required for germ cell development. The ovaries produce ova and the steroid hormones, estrogen and progesterone (Skibola *et al.,* 2004). The testes produce spermatozoa and testosterone. The gonadal hormones act by nuclear mechanism similar to that employed by the adrenal steroid hormones.

2.2.1 Reproductive Hormones and Gonadal Functions:

The testes, ovaries and adrenal glands synthesize the male and female sex steroids responsible for the establishment and maintenance of sexual function. The testes are located in the scrotum and contain a network of tubules for the production and transport of sperm, and a system of leydig cells that contain the biosynthetic enzymes that contribute to the production of male sex hormones.

The male sex hormones are collectively known as androgens (Griffin and Wilson, 1992).

Human ovaries are oval-shaped, and lie on either side of the ureters: each is approximately 2-5cm in length and weighs between 10-20g (Carr, 1992). Functionally, the ovaries produce ova and the female sex hormones-estrogens and progesterone. The production of the male and female sex hormones are under the regulatory control of the hypothalamic-pituitary axis (HTPA) (Tietz, 2001; Waugh & Grant, 2006).



Steriod Nucleus

Fig.2.2: The steroid backbone.

Fig 2.2 MALE REPRODUCTIVE SYSTEM:

2.3.1 Biochemistry of Testosterone:

Testosterone is the primary sex hormone of the male reproductive system and is involved with spermatogenesis, sexual differentiation and maturation (Griffin and Wilson, 1992). It also affects various aspects of secondary sexual development such as deepening of the voice, increase of muscle mass, hair growth and sex drive. In males, its secretion increases during puberty. Other androgens secreted by the gonads include androstenedione and dehydroepiadosterone (DHEA); while the adrenal glands also secret DHEA,

DHEA- Sulphate (DHEA-S), rostendione and androstenediol. These can be metabolized to testosterone and dihydrotestosterone (DHT) in target tissues.

In males, testosterone is secreted by the leydig cells of the testes. Females produce about 5% to 10% as much testosterone as do the males; out of which 50% is derived from the peripheral conversion of androstenedione, 25% from the ovaries and 25% from the adrenal glands, (except at the mid point of the menstrual cycle, where the ovarian contribution increases to 10%-15%).

Adrenal androgens are synthesized by the zona fasciculata and zona reticularis from their main precursor 17α-hydroxyl pregnenolone. There are two pathways for the synthesis of androgens from pregnenolone: One involves the transformation of pregnenolone to 17-hydroxyl pregnenolone, to dehydroepiandrosterone and then to androstenedione and to testosterone. The other pathway leads from pregnenolone to progesterone and then to androstenedione and testosterone. Testosterone is reduced to dihydrotesterone (DHT) by 5α-reductase that is found in the prostate, skin (particularly genital skin), and seminal vesicles (Tietz, 2001).

2.3.2 Metabolism of Testosterone:

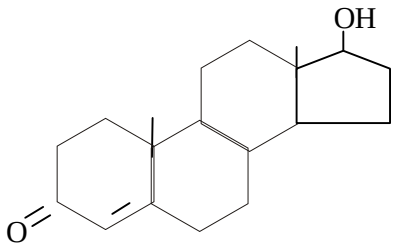


Fig.2.3: The structure of testosterone.

Etiocholanolone, androsterone and epiandrosterone are the main metabolites of androstenedione and testosterone and are produced by reduction of the double bond between their C-4 and C-5-carbon atoms and the keto group at the C-3 carbon atom.

The 17-ketosteroids are metabolic precurssors secreted by the adrenal glands, the testes and to some extent the ovaries. In males, approximately one third of the total urinary 17 – ketosteroids represent metabolites of testosterone secreted by the testes, whereas most of the remaining two thirds are derived from the steroids produced by the adrenal glands (Tietz, 2001; Murray *et al.*,., 2000).

2.3.3 Transport of testosterone:

Testosterone and dihydrotestosterone circulate in plasma as either their free form or bound to sex hormone – binding globulin (SHBG), β - globulin or to albumin. In males 2 % - 3% of testosterone circulates in the free form, 65% is bound to SHBG and the rest is loosely bound to albumin. In females, 80% of their circulating testosterone is bound to SHBG, 19% to albumin, and 1% circulates in the free form.

2.3.4 Factors That Affect Testosterone and Sperm Characteristics:

Among the factors that effect testosterone level and sperm characteristics, drugs and chemicals have been studied to have adverse effects on them. A study on the reproductive dysfunction of gasoline inhalation in albino rats showed that inhalation of gasoline caused a significant reduction in the serum levels of testosterone (Ugwuoke *et al.*,., 2005).

Onyenekwe *et al.* (2003), in their study on the effect of gossypol, a male contraceptive device reported that gossypol administration in male albino rats caused reduced serum concentration of testosterone level and induced azospermia and necrospermia. *Garcinia Kola* seed extract has been reported to cause marked reduction in serum testosterone level, induced arrest of

spermatogenesis and resulted in degeneration of spermatozoa (Braide *et al.,* 2003). The cholinergic stimulant, *nadiracetam* has been shown to decrease plasma testosterone and cause atrophy of the testicular seminiferous tubule (Shimomura *et al.,*, 1999). Chronic exposure to formaldehyde has been shown to impair leydig cell function and inhibit steroidogenesis, resulting to decline in the serum testosterone level (Majunder & Kumar, 1995).

Season, environment, nutrition, breed, age, emotion and testis size have been shown to affect semen quantity (Colenbrander and Kempi, 1990). The quality and quantity of sperm cells are essential factors in male fertility.During fetal development, if the testes fails to descend into the scrotum, the higher temperature of the abdominal cavity or inguinal canal may cause sperm degeneration. Chloroquine, a potent antimalarial drug, has also been found to reduce the fertilizing capacity of epididymal sperm in rats (Adeeko and Dasa, 1998).

2.3.5 Antifertility Effect of *A. boonei* on Male Reproduction Functions:

It is true that *decoction* of the stem bark of *Alstonia boonei* is given after childbirth to help the delivery of the placenta .This is based on its uterogenic and oxytocic property as reported by Hobbs (1992). The reproductive characteristics of male rats treated with methanolic extract of *Alstonia boonei* stem bark extract was reported by Raji *et al.,*, (2005). This appears to be the only published work on the specie in this area of study. They reported that the extract possess reversible antifertility effect on male rats.

2.4 THE FEMALE REPRODUCTIVE SYSTEM:

The female reproductive system consists of those structures within the female body that are designed to procreate and nourish new life. The system includes the ovaries, fallopian tubes, the breast, uterus, cervix and vagina.

The reproductive process begins in the ovaries. The ovaries are small, egg-shaped glands located in the lower abdomen. These glands control the cyclic functions of the reproductive system-ovulation and menstruation. Ovulation is the monthly production and release of a mature egg (occasionally two or more eggs). This process and other reproductive processes are regulated primarily by two hormones, estrogen and progesterone, which are also secreted by the ovaries. Estrogen regulates the secondary sex traits of hair growth, breast development, ovulation, and menstruation. Progesterone is responsible for preparing the uterus for pregnancy and contributing to the enlargement of the breast during pregnancy, and milk production and secretion post-partum.

The released egg travels through the fallopian tubes, which extend from each ovary into the uterus. If a sperm fertilizes it, it implants in the uterus and develop into a fetus (Charles *et al.,* 2001). Some plant extracts have been observed to inhibit the processes of nidation in rats (Gebrie *et al.,* 2005).

2.4.1 The Uterus:

The uterus is a pear shaped hollow organ that is normally about the size of a lemon fruit. It is a highly muscular organ, located between the bladder, and proximal rectum, and connected to the abdominal wall by ligament. The uterus encloses and supports the developing embryo called the fetus and expels the mature fetus in the process of parturition. The wall of the uterus consists of a lining, the endometrium and a coat of smooth muscles called the myometrium. The interior of the uterus is normally quite small and enlarges only during pregnancy. The narrow passage of the lower end of the uterus is the cervical canal. The cervix of the uterus leads to the body exterior prosterior to the urethra and anterior to the rectum. Although the uterus is open at one end, the interior of the uterus is protected from the entry of foreign materials because the cervix is narrow and usually filled with thick mucus. (Charles *et al.,* 2001). The

development of the uterus and its associated structures are regulated by the estrogen and progesterone (Pinaud *et al.,.,* 1991). A disruption of the physiological concentrations of these hormones portends danger for the foetus and other supporting structures (Pamir *et al.,.,* 2006). Further, some herbal extracts have been shown to affect the uterus and foetus adversely.

2.4.2 Estrogen:

Estrogens are responsible for implantation, development and maintenance of the female sex organs and female secondary sex characteristics. This partly explains why the plasma concentration of these hormones rise in pregnancy. In the normal non-pregnant female, estrogens are secreted in major quantities by the ovaries only, although minute amounts are also secreted by the adrenal cortices (Car *et al.,.,* 1992). Only three estrogens are present in significant quantities in the plasma of the female: β-estradiol, esterone and estroil. The principal estrogen secreted by the ovaries is β-estradiol. Small amount of estrone are also secreted, and usually forms the target for the studying of the estrogen level in animals. But most of this is formed in the peripheral tissues from androgens secreted by the adrenal cortices and by the ovarian theca cells. Estriol has weak hormonal activity when compared with the parent compounds, being an oxidative product of β-estradiol and estrone, in the liver (Tietz, 2001).

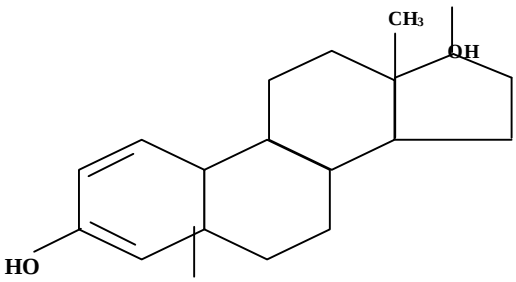


Fig.2.4: Chemical structure of 17-β-estradiol.

It is a C_{18} steroid hormone with a phenolic A ring. It has a molecular weight of 272g. It is the most natural estrogen, produced mainly by the ovary, and placenta; and in a smaller amount by the adrenal cortex, and the male testes.The phenolic group of ring A and the hydroxyl radical at C_{17} are essential for biological activity.Substitution at other positions in the molecule diminishes feminizing potency (Car *et al.*, 1992).

Estrogen secreted into the blood stream have 98% of it bound to sex-hormone-binding-globulin (SHBG). It is also bound to other serum proteins such as albumin (1-2%). A fraction circulates as free hormone. Estrogenic activity is effected via estradiol-receptor which trigger the appropriate response at the nuclear target sites. These sites include the follicles, uterus, virgina, urethra, hypothalamus, pituitary and to a lesser extent liver and skin.

In pregnant women with normal menstrual cycles, estradiol follows a cyclic, biphasic pattern with the highest found immediately prior to ovulation. The rising concentration is understood to exert a negative feedback at the level of the pituitary where it influences the gonadotropins, follicle stimulating hormone (FSH), leutinizing hormone (LH), which are essential for follicular stimulation and ovulation, respectively. Following ovulation, the levels fall rapidly until the luteal cells become active. This is followed by a secondary gentle rise and plateau of phase of estradiol. During pregnancy, serum estradiol level rises considerably, to well above the pre-ovulatory peak levels, and are sustained throughout pregnancy.

Measurement of serum estrogenis is a valuable index in evaluating menstrual dysfunction such as delayed puberty, primary and secondary amenorrhea. Estradiol levels have been reported to be high in patients with feminizing syndromes and testicular tumors. Serum determination of estradiol is useful for induction of ovulation following treatment with ovulation enhancing agents such as clomiphene citrate, LH- releasing hormone (LH-RH), or exogenous gonadotropins. During ovarian hyperstimulahtion for in-vitro fertilization (IVF), serum estradiol concentrations are usually monitored daily for optimal timing of human chorionic gonadotropin (HCG) administration and oocyte collection.

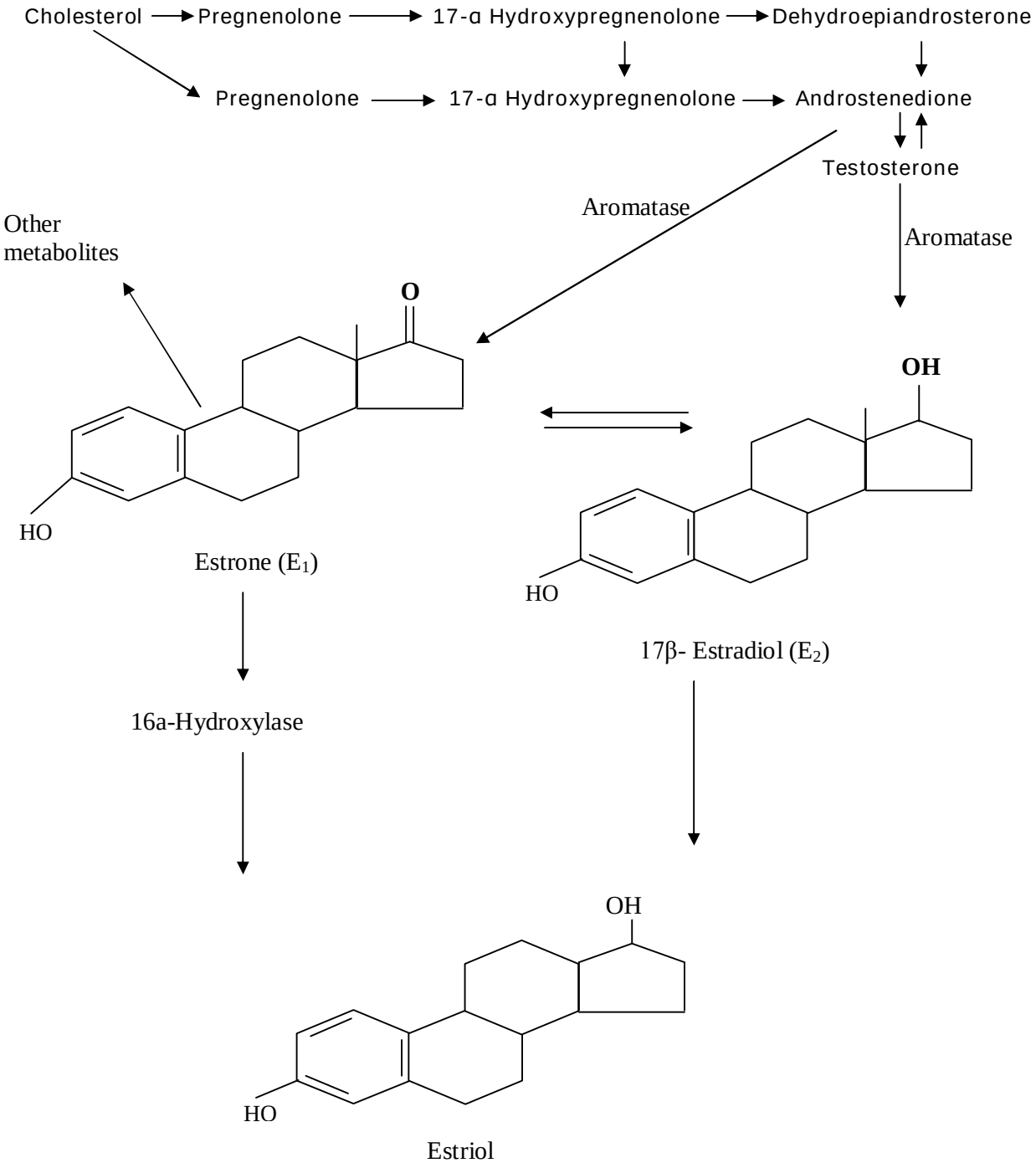


Figure 2.5 Biogenesis of Estrogen

The normal human ovary produces all the sex steroids: estrogen, progesterone, and androgens. However, estradiol and progesterone are the primary secretary products. Unlike the testis, the ovary possesses a highly active aromatase system which rapidly converts androgen, to estrogens (Murray *et al.,* 2006).

Estrogens are formed by the aromatization of androgens in a complex process that involves three hydroxylation steps. The aromatase is a multi-enzyme system which requires the participation of mixed function oxidase, Cytochrome P_{450}. and NADPH Estradiol is formed if the substrate of the enzyme complex is testosterone, whereas estrone results from the aromatization of androstenedione (Tietz, 2001).

In the normal course of estrogen metabolism, estradiol is believed to form a reversible redox system with estrone. Estrone is then metabolized along two alternative pathways: the 2-hydroxylation pathway leads to the formation of cateched estrogens and the 16 α-hydroxylation pathway leads predominantly to the formation of estriol (Carr *et al.,.,* 1992). Evidence indicates that in non-pregnant women, the 2-hydroxylation pathway is quantitatively more important than the 16α-pathway. The direction of estradiol metabolism is dependent on the pathophysiological state of the system.

2.4.3 Transport of Estrogen:

Estrogen in the blood binds mainly with plasma albumin and specific estrogen-binding globulins. The binding with plasma albumin is loose and rapidly released to the target tissues (Murray *et al.,.,* 2006). As such, circulating estradiol is largely unconjugated. It is strongly bound to sex-hormone-binding-globulin (SHBG) but loosely bound to albumin. Only 2-3% of total estradiol circulates in the biologically active free form. In contrast, estrone and estrone-sulfate circulate bound almost exclusively to albumin (Montgomery *et al.,*1990; Tietz, 2001).

2.4.4 Functions of Estrogen:

The principal function of estrogen is to cause cellular proliferation and growth of the tissues of the sex organs, and other tissues related to reproduction. During parturition, estrogens are secreted only in minute quantities. In puberty, the quantity of estrogen secreted under the influence of the pituitary gonadotropic hormones increases 20 –folds or more; the ovaries, fallopian tubes, uterus, and vagina increase in size during this period. Besides, the external genitalia are enlarged with deposition of fat in the mons veneris, labia majora and the labia minora. These physiological changes are made possible by the enhanced estrogen and progesterone secretion.

In addition, estrogen changes the cells of the vaginal epithelium from cuboidal into stratified form, which makes it considerably more resistant to trauma and infection than pre- pubertal epithelium. Estrogen causes marked proliferation of the endometrial stroma and greatly increases the development of the endometrial glands (Paruir *et al.,* 2006) that will later aid in nourishing the implanted ovum. These cytological changes form the basis of the light microscopic examination of vaginal smear as indices of fertilization in rats (Yen *et al.,* 1992).

Other actions of estrogen include its effect on the mucosal lining of the fallopian tubes. They cause the glandular tissues to proliferate and the number of ciliated epithelial cells that line the fallopian tubes to increase. Further, the activity of the cilia is considerably enhanced. (They help to propel the fertilized ovum toward the uterus). Estrogens cause the development of the stroma tissues of the breast, growth of extensive ductile system and deposition of fat in the breast (Guyton, 1996).

Indeed, Estrogen initiates breast enlargement and lactation .It causes enhanced osteoblastic activity; leading to early union of the epiphysis with the shaft of the long bones. It also raises antidiuretic hormone (ADH) activity leading to water

retention as well as the basal metabolic rates, thus leading to increase in total body protein.

2.4.5 Biotransformation and Elimination:

Like other steroids, the liver is the primary site for the inactivation of estrogens. The main biochemical reactions are hydroxylation, oxidation, reduction and methylation. The hormone is eliminated from the body by conjugation with glucuronic or sulfuric acid, which imparts polarity to steroids and makes them readily excretable through the kidney.

2.4.6 Progesterone:

Progesterone is also a steroid hormone. It is by far the most important progestin. However, small quantity of 17-α-hydroxyprogesterone is also secreted along with progesterone and have essentially the same effects. Progesterone is secreted mainly by the corpus luteum and it is especially important in preparing the uterus for the implantation of the gamete and in maintaining pregnancy. In conjunction with estrogen, progesterone regulates the accessory organs during menstrual cycle (Adashi *et al.*, 1992; Yen *et al.*, 1992). In pregnancy, the placenta becomes the major source of the hormone. Minor sources are the adrenal cortex in both sexes and the testes in males.

2.4.7 Biochemistry of Progesterone:

The structural formula of progesterone shows that it contains a keto group at C-3, and a double bond between C-4 and C-5; both structural characteristics are essential for progestational activity.

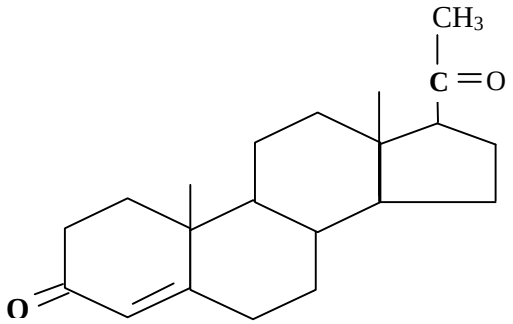


Fig. 2.6 The structure of Progesterone

The biosynthesis of progesterone in ovarian tissues is believed to follow the same pathway as it does in the adrenal cortex (Adashi *et al.,* 1992). Progesterone is synthesized mainly from cholesterol and to some extent from acetyl coenzyme, a molecule which can combine to form the appropriate steroid nucleus.

In luteal tissue, however, Low Density Lipoprotein (LDL) cholesterol is thought to serve as the preferred precursor. LH and FSH regulate initiation and control of luteal secretion of progesterone. (Carr *et al.,* 1992; Yen *et al.*,1992).

2.4.8 Transport of Progesterone:

No specific plasma binding protein has been identified for progesterone, unlike cortisol which binds to cortisol binding globulin (CBG) and estrogen which binds to SHBG. The percentage of unbound progesterone (2-10%) remains constant throughout the normal menstrual cycle. The synthesis of progesterone peaks during the luteal phase and the third trimester of pregnancy, the serum level can rise as much as 30 and 300mg/day respectively.

2.4.9 Functions of Progesterone:

The most important function of progesterone is to promote secretory changes in the endometrium during the latter half of the menstrual cycle. Under physiological concentrations, progesterone decreases the frequency and intensity of uterine contractions, thereby helping to prevent expulsion of the fertilized ovum. Progesterone also promotes secretory changes in the mucosal lining of the fallopian tubes. These mucous secretions are necessary for preservation of the fertilized ovum as it traverses the fallopian tube before nidation.

Progesterone promotes development of the tubules and alveoli of the breast, causing the alveolar cells to proliferate, enlarge and become secretory in nature. At higher concentrations it enhances the activity of antidiuretic hormone, thereby leading to enhanced salt and water reabsorption from the distal tubules of the kidney. The formal condition leads to breast enlargement, while the latter leads to weight gain.

2.4.10 Biotransformation and Elimination:

The liver actively metabolizes progesterone to pregnanedione, pregnanolones and pregnanediols. Pregnanediol is the major metabolite. The compound undergoes reduction and conjugation. About 10% of the parent compound is excreted via the urine unchanged.

2.4.11 Regulation of Ovarian Hormones:

At the beginning of the menstrual cycle, ovarian follicles are undeveloped and the plasma concentration of estradiol is low. LH and FSH secretion increase. LH and FSH together cause growth and maturation of the follicles. By the seventh day of the cycle, one follicle becomes especially sensitive to FSH and matures while the rest atrophy. LH then stimulates estradiol secretion, the

plasma concentration of which rises steadily. This stimulates the regeneration of the endometrium. The dormant follicle develops rapidly as the plasma estradiol concentration rises. This triggers a surge of LH release via the anterior pituitary gland in the course of ovulation.

The high concentration of the LH after ovulation stimulates the granulocytes of the ruptured follicle to lutenize and to form the corpus luteum. The corpus luteum synthesizes and secretes progesterone and estradiol. However, progesterone is the principal hormone of the luteal phase. The progesterone prepares the endometrium for the implantation and development of the fertilized ovum.

In females, plasma progesterone concentration remains relatively constant throughout the follicular phase of the menstrual cycle. The concentration then increases rapidly following ovulation and remains elevated for 4-6 days and decreases to the initial level 24 hours before the onset of menstruation. In pregnancy, placental progesterone production rises steadily to levels of 10 to 20 times those of the luteal phase peak.

Progesterone measurements are thus performed to determine ovulation as well as to characterize luteal phase defects. Monitoring of progesterone therapy and early stage pregnancy evaluations are some of the uses of plasma progesterone determination.

2.5 TOXICITY STUDIES ON FEMALE REPRODUCTIVE ORGAN:

Some drugs and chemicals have been studied and found to adverse affect on the uterus and related structures. The thalidomide episode of the 1940s and 50s has remained a classic reference point for the eminent dangers of chemicals on the female reproductive system (Rang *et al.*, 2000). The contagen which was the offending drug that caused the phocomelia was withdrawn from the market in

the 1960s, but the bitter lesson of that experience underscores the vital importance of toxicity studies on possible drug candidates on the female reproductive organ. Kayode *et al.*, (2007) reported the adverse effect of the methanolic extract of *Aspillia africana* leaf on the estrous cycle and uterine tissues of rats in which they showed that the extract caused reduction in uterine weight and prevented implantation of the fertilized ovum. Fukuda *et al.*, (2006) reported the likely oxytocic effect of *Huntera umbellate* on isolated rat uterus. The effect was traced to the alkaloidal component of the plant extract.

The studies on the chloroform extract of *Carica papaya* seed on the estrous cycle and fertility in female albino rats showed that the extract caused mild histological changes and significant reduction in serum estrogen (Raji *et al.*, 2005a, 2005b). Cherian (2000), demonstrated that the extract of the latex of *C. papaya* caused cytological disruption of the isolated gravid and non-gravid uteri.

Studies on the possible mechanism for the anti- fertility effect of root extract of *Rumex stendelii* showed that the extract stimulated the uterine smooth muscle thereby preventing the implantation of the ovum. The extract also decreased the serum levels of estrogen and progesterone in the rats (Endalt *et al.*, 2005).

Furthermore, Neeru and Sharma (2007) also documented the anti implantation effect of *Hibiscus rosa-sinensis* root extract on uterine epithelium. Azadey *et al.*,., (2007) also demonstrated the anti-fertility effects of *physalis alkekengi* alcoholic extract in female rats. The work further revealed that the extract reduced the number of implantation sites in the uterus and also caused a decrease in serum estrogen and progesterone levels.

Phytoestrogenic extracts may have pro-hormonal or anti-hormonal effects on mammalian system. The resultant effect is multifaceted ranging from

histological and biometric changes, teratogenicity and abortifacience; as well as alteration in the implantation sites. The abnormal elaboration of these hormones is bound to have adverse effects on the organs. Furthermore, it is established that hormonal balance is essential for implantation and proper development of the product of conception (William, 1999; Guyton, 2003).

2.6 Brain Cells and The Hypothalamic Pituitary Axis

The brain and spinal cord are enveloped within three meninges or membranes called the dura mater, arachnoid mater and pia mater. Their names imply their qualities: the dura is the strong, tough outer layer; the arachnoid is a delicate layer between the dura mater and the pia delicate skin that contains blood vessels. (Waugh and Grant, 2006).

The brain is part of the nervous system. It is the most complex organ in the body. It is made up of neuronal networks which regulates the activities of the body. Many chemical substances encountered either as drugs or in the environment may damage the nervous system. Neuronal metabolism may be disturbed directly or result to damage of other organs such as the liver or the kidney. The outcome depends on the extent of damage, toxicity of the substance, the dose and the duration of exposure.

2.6.1 Reproductive Activities and the Hypothalamic-Pituitary Axis (HTPA):

The hypothalamic-pituitary axis refers to the neuronal network which links the hypothalamus, the pituitary gland and the periphery.It regulates the primitive functions of the body such as water balance and sexual drive.The hypothalamus regulates the activities of the pituitary gland. The gland is a small, grey coloured, oval shaped structure attached to the base of the brain(Saladin and Porth,1998).It is made up of two lobes,the anterior and posterior lobes.The hormones of the HTPA are synthesized in the neuronal cell-bodies within the

hypothalamus. They are transported through the axon and stored in the lobes until signals elicit release.The clinical studies on this pathway is paucy(Gerendai, 2004) .

The anterior pituitary gland (APG) releases follicle stimulating hormone which is secreted by the gonadotropes of pituitary gonaldal axis. The FSH stimulates the development of eggs and follicles (vesicles) that contain them. In the testis, FSH stimulates the biosynthesis of sperm. In the female, LH stimulates the release of eggs (ovulation). After luteum (yellow body), the LH stimulate the corpus luteum to produce progesterone. In males, the LH is sometimes called interstitial cell-stimlating hormone (ICSH). LH/ICSH stimulates the testis to secret testosterone.

2.6.2 Prolactin (PRL):

It is secreted by the lactotropes (mammatropes). The size and number of lactotropes increases during pregnancy. The lactotropes secret prolactin during childbirth and during baby nursing. PRL stimulates mik synthesis in the mammary gland. In male, PRL makes the testis more sensitive to LH. Thus indirectly enhancing testosterone secretion (Saladin and Porth, 1998). The posterior pituitary gland secrets antidiuretic hormone (ADH) and oxytocin (OT). OT stimulates milk secretion and contraction of the uterine muscle during parturition.

2.6.3 Vanilmandelic Acid (VMA) and Homovanilic Acid (HVA):

Dopamine (DA), noradrenalin (NA) and Adrenalin (Adr) are the sympathetic neurotransmitters of the HTPA (Rosano *et al.,* 2006). The plasma concentration of these neurotransmitters correlates their cellular and CNS acivities. The catecholammes and DA in particular is known to regulate hormonal activities via this pathway (Geredai & Halasz, 2001). Vanilmandelic acid and homovanilic acid (HVA) are the stable metabolites of the catecholamines.

Homovanilic acid is the predominant metabolites of DA, while V-MA is their common stable metabolic product (White *et.al,* 1978; Rosano *et al.,* 2006).

The assay of these metabolites is used as the confirmatory clinical diagnosis of phaechromocytoma and neuroblasomas (Wybenga & Pilleggi 1996; Tietz, 2007). Phaechromocytoma and neuroblastoma are catecholamines-producing tumors in the adult and paediatrics respectively. The laboratory analysis of the plasma or tissue levels of VMA also provides useful information about the activities of the reproductive hormonesof the HTPA, and some sexual characteristics in man and rodents (Fukuda *et al.,* 1984; Card *et al.,* 1993). Elevated tissue levels of these metabolites are taken as indication of exacerbated activities of the catecholamnes (Rosano *et al.,*2006).

2.7 THE KIDNEY:

In man, the kidneys are beans-shaped excretory organs about 11mm long, 6cm wide, 3cm thick, and weighs about 130g each. It is about the size of a tightly clenched fist. It is embedded in and held in position by a mass of fat. Each of the two kidneys lies on each side of the vertebral column behind the peritoneum and below the diaphragm. They extend from the level of the 12^{th} thoracic vertebra to the 3^{rd} lumber vertebra, receiving some protection from the rib cage. The gross structure of the kidney consists of a fibrous capsule, the cortex, the medulla, the helium and the renal pelvis with each performing its functions. The microscopic structure of the kidney consists of the nephrons and the collecting tubules (Waugh and Grant, 2001).

The nephron is the kidney's primary functional unit with each kidney having about 1 million nephrons. The nephron contains a filtering system known as the glomerulus; and a tubule, through which the filtered liquid passes. Each glomerulus has a capillary network surrounded by a membrane called the

Bowman's capsule. Urine formed within the kidney passes from the renal papillae into the minor calyces. From the latter, urine moves into the major calyces, collects in the renal pelvis and exits the kidney through the ureter.

2.7.1 Functions:

The most important function of the kidney is the formation and excretion of urine, which takes place through ultra filteration, selective re-absorption and secretion in the kidney tubule. The kidneys have the function of excreting waste products of metabolism and play the essential homeostatic role of maintenance of the interior of the body, adjusting the excretory rate of water and different plasma constituents (Nsirim, 1999; Baker *et al.,* 2001).

The kidney also performs limited gluconeogenic functions using substrates such as free fatty acids, lactate and glycerol in the presence of a catalytic enzyme such as glycerol kinase and carboxykinase (Murray *et al.,*, 2000). The kidney also functions as an important endocrine organ producing such hormones as renin, calcitriol and erythropoietin, maintenance of osmoregulation and electrolyte balance (Waugh and Grant, 2006).

Kidney disorders are many. They include acute pyelonephritis, chronic pyelonephritis, acute renal failure ; acute tubular necrosis, which could be ischaemic or nephrotic, The latter is caused by toxic chemicals e.g. carbon tetrachloride, pesticides, mercuric compounds, drugs and crude natural products, paracetamol overdose, amino glycosides, endogenous substances etc.

Pyelonephrritis, which is an infectious and inflammatory disease, that usually begins in the renal pelvis but extends progressively into the renal parenchyma. It can result from bacteria infection especially the colon bacillus. At this stage it is acute and later becomes chronic as a result of repeated attacks of acute

pyelonephritis with scar tissue formation and this may lead to concurrent hypertension and then to and renal failure (Nwanjo, 2003).

Nephrotic Syndrome:- a condition in which the wall of the glomeruli is damaged and protein passes through the damaged wall into the urine, leading to edema and protinuria. Acute and chronic renal failure may occur after acute and chronic glomerulonephritis. They may cause death due to uncontrolled diabetes mellitus especially in the case of early onset insulin dependent diabetes mellitus. This is called diabetic kidney (Guyton, 1996).

2.7.2 Clinical Chemistry and Some Renal Diseases:

The effects of renal disease depend on the proportion of glomeruli to tubules affected and number of nephrons involved. The consequences of renal disease may be easier to understand by considering hypothetical individual nephrons (Mayne, 1994).

In the reduced glomerular filteration rate (GFR) with normal tubular function:-

- Total amounts of urea and creatinine excreted are affected by the GFR. If the rate of filteration fails to balance that of production, plasma concentration will rise.
- A large proportion of the reduced amount of filtered sodium is reabsorbed by isosmotic mechanism; less than usual is available for exchange with hydrogen and potassium distally. This results to reduction of H^+ secretion and since bicarbonate can only be reclaimed if hydrogen ion is secreted, its concentration will fall. There will also be reduction of potassium secretion in distal tubule causing potassium retension.
- Systemic aldosteronic secretion will be maximal such that any sodium reaching the distal tubule will be almost completely reabsorbed in exchange for K^+ and H^+ causing low urinary sodium.

Thus the clinical syndrome in vein or serum from the affected nephrons will be:

- High urea and creatinine concentrations.
- Low bicarbonate concentration with high pH .
- Hyperkalaemia.
- Hyperuricaemia and hyperphosphataemia.
- In reduced tubular with normal glomerular functions, tubules cannot secrete H^+ and therefore cannot reabsorb bicarbonate normally nor acidify the urine.
- The response to aldosterone and therefore for exchange mechanisms involving reabsorption of sodium are impaired.
- Normal urea and creatinine concentration (normal glomerular function).
- Due to proximal or tubular failure, there is low bicarbonate concentration with low pH and hypokalaemia. (Mayne, 1994).

2.7.3 Investigations of Renal Function:

Renal function test refers to a group of biochemical and laboratory investigations useful confirming that the kidney is diseased, indicating the causes of the diseases and the extent of kidney damage or failure and also assessing the progness in the treatment of the disease (Nwanjo, 2005).

Practical examination of kidney status in renal disease include examining the nephron functions of glomerular filteration rate (GFR), the secretory capacity of particular endogenous and exogenous compounds and; the kidneys reabsoption capacity for water and electrolytes as manifested by the urine concentrating ability of the kidneys (Tietz, 1996).

Clearance test is the valued test for renal function. The clearance of a substance from plasma is the value of plasma which a given volume of urine clears of that substance. This definition is based on the premise that estimation of the glomerular filteration rate can be made by measuring the urinary excretion of a

substance, which is completely filtered from the blood by the glomeruli and is not reabsorbed, secreted or metabolized by the renal tubules. The concept could be represented by the equation:-

$$\text{Clearance} = \frac{\text{Urinary excretion of substance (ml/minute)}}{\text{Plasma concentration of the substance}} \times \text{Urine volume}$$

This involves the inulin, urea and creatinine. Inulin does not occur naturally in blood and has to be experimentally infused into blood for the assessment, making it difficult to assess a true plasma concentration. Thus the use of such substances like urea and creatinine which are naturally present in the blood and so are present in the glomerular filtrate, are preferred. (Mayne, 1994).

Urea clearance, though feasible is rarely used because plasma concentration is more variable than creatinine. Moreso, because a little urea diffuses back from the tubules with water. From the foregoing, it follows that neither cretainine nor urea clearance measures the true glomerular filtration rate. However, they both estimate it to such an extent as to give a satisfactory diagnostic index, hence the routine use of creatinine clearance tests for assessment of kidney functions. Furthermore, the use of urea-creatinine ratio is also valuable in this regard.

However measurement of body electrolytes can help in assessing the kidney's tubular function. The kidney maintains electrolyte balance; therefore any imbalance in the electrolyte level is an indication of kidney dysfunction (Nsirim, 1999).

2.7.4 Serum Urea and Creatinine Measurement:

The estimation of serum urea and cretinine concentration in plasma and serum are some of the tests for renal functions because any excess of urea and

creatinine in the ciurculation is eliminated from the blood stream by the kidneys and passed out in urine under normal condition. This means that excess in blood indicates kidney dysfunction (Nwanjo *et al.*, 2005).

A marked increase in serum urea is indicative of damaged renal function. The causes of renal failure leading to increase in urea levels are the diseases or conditions that cause a fall in the glomerular filtration rate. Conversely, a decrease in urea level may be found in pregnancy, malnutrition and Acquired Immune Deficiency Syndrome (AIDS), severe liver disease and water overload. Notwithstanding, slight increases in urea (not more than three times the upper limit of the reference range) may occur when there is dehydration, diuretic therapy, gastrointestinal blood loss and any condition associated with increased protein catabolism.

High level of creatinine is associated with the conditions that cause low glomerular filtration rate. Increased plasma creatinine level may be caused by strenuous exercise and the effect of drugs, but these increases are normally insignificant, making creatinine estimation in blood a more specific test for renal function (Cheesbrough, 1998).

2.7.5 Serum Electrolytes:

Electrolytes are compounds, which dissociate in solution to form electrtically charged particles or ions. Positively charged ions are called cations (sodium and potassium) and negatively charged ions are called anions (chloride and bicarbonate). The electrolytes found in the body fluids and most frequently measured in the laboratory are sodium, potassium chloride and bicarbonate (Cheesbrough, 1998).

Sodium is the main regulator of osmotic pressure in body fluids and its metabolism is regulated by adrenocortical steroids with the exception of

androgen. It combines with chloride and bicarbonate to regulate acid base balance. It is the major extracellular cation and makes nerve conduction possible, passage of nutrients into cells as wellas maintenance of water balance, and neuromuscular activity by its movement into cells allowing potassium to shift out of the cell (depolarization). Sodium level can decrease in cases like diarrhoes, ketoacidosis, kidney disease, osmotic dilution, abnormal anti diuretic hormone, use of diuretics; and can increase in Cushings syndrome, dehydration, osmotic diuresis etc.

Potassium is the most abundant intracellular cation and regulates water and acid base balance of the body alongside with sodium. It also regulates heart beat reactions and energy metabolism etc. The principal organ of excretion of potassium is the kidney. It is filtered by the glomerulus and secreted by the tubules (Mayne, 1994). Low level of potassium can be observed in cases like disrrhoea, hyperaldosteronism, renal tubular acidosis, diuretic use etc. Its level can also increase in cases such as haemolysis, metabolic or respiratory acidosis, and renal failure.

Chloride is the chief extra-cellular anion present in large quantities in serum, exerting important influence on acid base balance, osmotic pressure regulation and water balance. Increase in its level can be seen in dehydration, renal tubular acidosis, acute renal failure, respiratory alkalosis etc. While its decrease can be seen in cases associated with excessive sweating, prolonged vomiting, adrenocortical deficiency, diuretics therapy (Tietz, 1996)

Bicarbonate is the most abundant buffering system operating in cells and fluids. It is important in regulating acid base balance of the body. Its level can be affected by variety of respiratory events and can rise in cases of respiratory alkalosis and vice versa. Decreased bicarbonate levels can be caused by

increased utilization of bicarbonate (metabolic acidosis) or very occasionally by loss of carbon dioxide due to hyperventilation (respiratory alkaloids) (Hardman *et al.,* 1996.

2.8 THE LIVER:

The liver is the largest internal organ of the body and weighs approximately one fifth of the body weight in an adult. In neonates, it accounts for one tenth of the body weight. The liver is located in the upper right quadrant of the abdomen, just below the diaphragm. A thick capsule of connective tissue called glissons capsule covers the entire surface of the liver. The liver is divided into a large right lobe and a smaller left lobe. Each lobe is further divided into lobules. These hepatic lobules are the functional units of the liver. Each of the lobules consists of a hexagonal row of hepatic cells called hepatocytes. The most prominent cell in the liver is the hepatocytes, which accounts for 8% of the liver volume. The hepatocytes secret bile into the channels and also perform a variety of metabolic functions. Between each row of hepatocytes are small cavities called sinusoids. Each sinusoid is lined with kupffer cells, phagocytic cells that extract nutrients and eliminate wasters, dead cells, bacteria, and debris from the blood as it flows through sinusoids. The main function of the sinusoid is to destroy old or defective red cells, remove bacteria etc. Blood enters the liver through the hepatic artery and through the portal vein, which carries nutrient ladders blood from guts.

Approximately 1.5L of blood enter the liver each minute making it one of the vital organs in the body. Seventy-five percent of the blood flowing to the, liver comes through the portal vein. The remaining 25% is oxygenated blood that comes from the hepatic artery.

Under the liver, there is the gall bladder, which is connected to the liver by ducts known as biliary tracts. The liver therefore consists of four systems: (1) hepatocytes, (2) biliary (3) circulatory and (4) endothelial systems (Gitnick *et al.*,1992).

2.8.1 Functions:

The liver performs the following functions:

- Biosynthesis and transport of bile pigments.
- Site for lipid metabolism and conjugation of bile pigments.
- Metabolism of xenobiotics.
- The site for the metabolism of carbohydrates, proteins, and amino acids.
- Storage site for glycogen, fatty acids, vitamins etc.
- The liver synthesizes about 50 gram of protein each day primarily in the form of albumin.
- Digested fat is converted to triacylglycerol, cholesterol etc in the intestine which is further converted to glycerol and fatty acids in the liver.
- Blood coagulation factors such as prothrombin, fibrinogen etc are synthezied by the liver.
- It conserves small amount of vitamin C,D,E and K. (Baker *et al.*, 2001).

The liver cells may be damaged due to mechanical trauma, microbial infection or metabolic disorders. Viz:

2.8.1.1 Cholestasis:- This can be intrahepatic or extrahepatic. It is associated with abnormal bilirubin metabolism. Alkaline phosphatase activity is raised in this condition and serves as the hepatocellular marke for the disease.

2.8.1.2 Acute hepatitis: The biochemical findings in acute hepatitis are predominatly those of cell membrane damage with an increase in plasma alanine amino trasnferase (AST) activity. There may be a superimposed cholestatic picture and in severe cases impaired prothrombin synthesis.

2.8.1.3 Viral hepatitis:- The term describes three types of viral infections (Hepatitis A, B and C) in which the clinical features of the acute form are similar but with different incubation period.

Plasma transaminase activities are very high from the onset of symptoms and peak about four days later. In the early onset, there is often a cholestatic element with pale stools due to reduced intestinal bilirubin, and dark urine due to rise in plasma unconjugated bilirubin arising from impaired hepatocellular conjugation.

2.8.1.4 Acute alcoholic hepatitis:- Occurs in heavy drinkers, usualy after a period of increased alcohol intake. Although the clinical features mimic acute viral hepatitis, the plasma transminase activities and bilirubin concentration are not usually as markedly elevated as in viral hepatitis

2.8.1.5 Chronic hepatitis: The finding of persistent, but slightly raised plasma transaminase activities sometimes without chronic or recurrent symptoms of liver disease. It may be the only abnormal biochemical finding of persistent, but slightly raised plasma transaminase activities without clinical signs or symptoms and without a significant change in their activities over the years.

2.8.1.6 Liver cirrhosis: In the early stages there may be abnormal clinical finding; drug phase of active cellular necrosis, plasma AST and ALT activities rise slightly. In advanced cases, the clinical finding may be reduced functioning cell mass.

2.8.1.7 Hepatocellular failure: Liver damage is severe enough to cause obvious clinical signs of impaired hepatic function, advanced cirrhosis which may follow an overdose of liver toxins such as paracetamol

The clinical findings include raised AST and ALT, as well as jaundice is progressive. In the final stage, the number of hepatocytes are reduced leading to low transaminase plasma activities.

2.8.1.8 Hepatic infiltration or invasion: - Invasion of the liver by secondary carcinoma or fatty infiltration may be associated with abnormal clinical findings such as raised plasma AST activity. ALT may be raised and both may become rapidly raised if hepatocellular carcinoma develops (Mayne, 1994).

2.8.2 Bilirubin:

Erythrocytes at the end of their lifespan, which is normally 120 days, are removed from the recticuloendothelial system (RES). The protopophrin ring of the heam group of heamoglobin is opened up and the resulting bilirubin-iron complex (choleglobin) is formed. The protein and iron are then removed. The former is catabolised to amino acids, which enter into new protein synthesis while the latter is used for the synthesis of new heamoglobin and is retained in the RES in the form of heamosiderin and ferritin.

After the removal of the protein and iron, the remaining green pigment, biliverdin, is reduced by the tissue enzymes to a yellow pigment, bilirubin. Bilirubin, a water insoluble substance, enters the plasma and is bound to plasma albumin, which on passing into the parenchymal cells of the liver is conjugated principally with glucuronic acid to form the water soluble mono and diglucuronides. This conjugation takes place under the influence of unridyl diphosphoglucoronyl transferase.

Conjugated bilirubin may enter the circulation and is then also bound to albumin but principally is transported out of the liver cell into the bile canaliculi and excreted in the bile. The two conjugated forms of bilirubin are present in the blood and are responsible for normal level of up to 10mmol/L. After passing into the gall bladder, the bilirubin glucuronides may be re-oxidised to biliverdin, thus giving rise to green colour of the bile of the gall bladder.

From the gall bladder, the bilirubin passes the bile duct into the small intestine where it is ultimately reduced by bacterial enzymes to a colourless compound, stercobilin. Small amount may be reabsorbed and passes via the portal vein to the liver where it is re-excreted in the bile. The urinary excretory product is known as urobilinogen which can be oxidised to urobilin in the urine (Baker *et al.*, 2001).

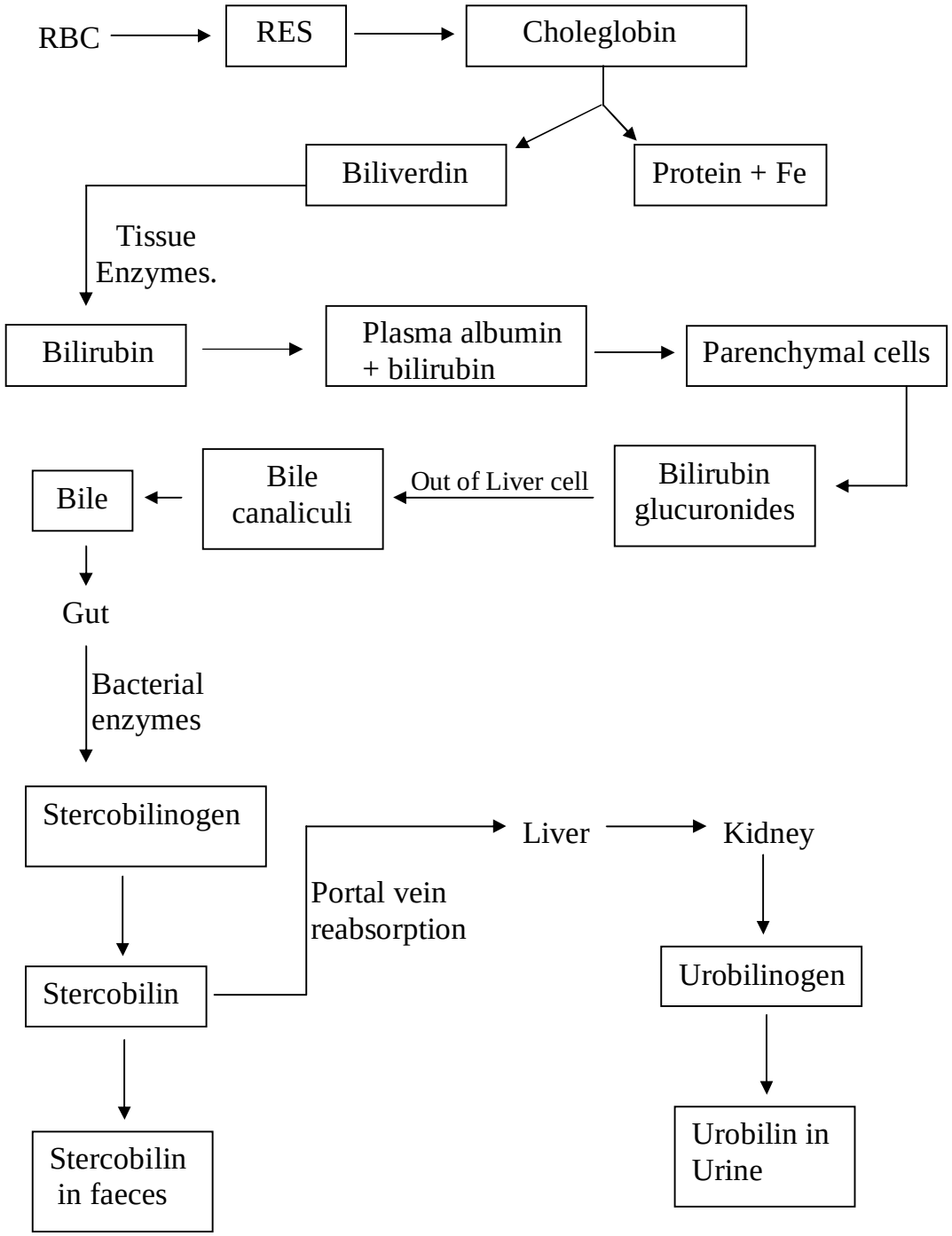


Figure 9: Schematic representation of bilirubin biosynthesis (Baker *et.al.*., 2001).

2.8.2.1 Factors Affecting Bilirubin Metabolism:

A number of factors such as inherited and acquired diseases have been shown to affect one or more of the steps involved in the production, uptake, storage, metabolism and excretion of bilirubin.

2.8.2.2 **Bilirubinaemia**:

It is frequently a direct result of these disturbing factors. Hyperbilirubinaemia is defined as excess bilirubin in the blood which clinically manifestes as jaundice, a condition in some newborn.

2.8.2.3 Conjugated Hyperbilirubinaemia:

Conjugated bilirubin is the water-soluble form of bilirubin, which is frequently secreted from the hepatocytes (where the conjugation process occurred) into the bile.

In hepatobiliary diseases of various causes, bilirubin uptake, storage and excretion are impaired to varying degrees. Conjugated and unconjugated bilirubin is retained in these hepatic disorders and wide range of abnormal serum concentrations of each form may be observed (Tietz *et al.*, 1996).

Conjugated bilirubin associated disorders such as Dubin-Johnson and Rotor syndromes, which cause chronic idiopathic and chronic familial jaundice respectively, can cause conjugated hyperbilirubinaemia. These conditions are caused by defective excretion of bilirubin from the cells after conjugation. While Dubin Johnson syndrome is characterized by porphyria and deposition of black pigment in liver, rotor syndrome is a variant of Dubin Johnson syndrome and there is no deposition of black pigment in the liver (Hoffrand *et al.*, 1997).

2.8.2.4 Unconjugated Hyperbilirubinaemia

The amount of unconjugated bilirubin may rise in blood following various conditions that are not actually hepatic in origin. It can be physiological as a result of excessive breakdown of red blood cells following diseases such as lassaemia major, glucose-6-phosphate dehydrogenase deficiency or may occur due to ineffective erythropoesis commonly seen in newborn during the first few days of their life. In some cases, the liver is not able to cope with the rapid rate of breakdown of the erythrocytes. This condition is known as physiological jaundice. It is identified with a yellow colouration of the skin and mucus membrane of patients. (Nwanjo, 2003).

Haemolytic anaemia as a result of hereditary spherocytosis, hereditary elliptocytosis, autoimmune complex disease, blood transfusion incompatibility etc can also cause unconjugated hyperbilirubinaemia (Hoffrand *et al.,* 1997). Unconjugated hyperbilirubinaemia may occurs as hereditary abnormalities as found in Crigler Najjiar syndrome caused as a result of inability of the liver to remove bilirubin from the blood stream due to congenital deficiency of uridine diphosphoglucuronyl transferase, the bilirubin glucuronidation enzyme. Another hereditary abnormality is Gilbert's syndrome in which there is failure of uptake of bilirubin by the liver cell from the circulating plasma (Nwanjo, 2003).

2.8.3 Enzymes Reflecting Hepatocellular Injury

Any injury to the liver that results in cytolysis or cellular necrosis causes the liberation of various enzymes. The measurement of these hepatic enzymes in serum is used to assess the extent of liver damage and to differentiate hepatocellular injury from obstructive disease. The most commonly assayed ones are alanine and aspartate transaminase (ALT and AST) and alkaline phosphatase (ALP). The activities of the gamaglutamyl transaminase (GGT),

catalase, glutathione peroxidase (GSH-peroxidase) and GSH-reductase have been successfully exploited for the same purpose.

2.8.3.1 Aspartate andAlanine Amino Transaminases:

The amino transaminase catalyses chemical transfer of an amino group from a donor molecule to an acceptor molecule, hence the name Amino Transaminase. Alanine amino transaminase (ALT) is found largely in the liver. It is released into the blood stream as a result of hepatocellular injury. It therefore serves as a fairly specific index of liver injury (Tietz, 2006).

Aspartate amino transaminase (AST) on the other hand is found in a number of tissues including the liver, heart, muscle, kidney and brain. It is released into the serum when any of these tissues is damaged or injured due to mechanical trauma, microbial infection or disease (Henry, 1994).

The precise levels of these enzymes do not correlate well with the extent of liver damage. Thus exact levels of AST and ALT cannot be used alone to determine the degree of liver injury or disease. This is because other conditions such as hepatitis A may elevate plasma AST and ALT levels (Nsirim, 1999).

The highest levels of ALT and AST are found with disorders that cause death of numerous liver cells. This may occur in conditions of viral hepatitis A or B, and pronounced liver damage (when the liver is deprived of fresh blood bringing oxygen and nutrients). Mild to moderate elevations of these liver enzymes can be seen in fatty liver caused by alcohol abuse, diabetes mellitus etc. Abnormal levels can also be caused by some medications.

2.8.3.2 Alkaline Phosphatase (ALP)

Alkaline phosphatase is an enzyme that removes 5-phosphate groups from DNA and RNA as well as from nucleotides and proteins. It can be found in various organs and tissues of the body.

It is a membrane-bound enzyme, which becomes elevated in response to increased intracellular concentrations of bile acids. An elevation of the serum level also occur in an intrahepatic obstruction, infiltration disease such as amyloidosis or neoplasia, granulomatous hepatitis, certain drug reactions and other chronic cholestatic conditions.

The brain and spinal cord are enveloped within three meninges or membranes called the dura mater, arachnoid mater and pia mater. Their names imply their qualities: the dura is the strong, tough outer layer; the arachnoid is a delicate layer between the dura mater and pia delicate skin and contains blood vessels. (Waugh and Grant, 2001; Nwanjo, 2003).

2.9 THE HEART AND LIPID PROFILE

The heart is a roughly cone-shaped hollow muscular organ. It is about 10cm long and weighs between 225-310g in man. It lies in the thoracic cavity of the mediastenum between the lungs. It lies obliquely, a little more to the left than the right of the thoraxic cavity, and presents a base above and an apex below.

The heart is composed of three layers of tissues, viz; pericardium, myocardium and endocardium. The pericardium consists of two sacs i.e. the outer sac, which consists of fibrous tissue, and the inner sac made up of a continuous double layer of serous membrane. The myocardium is composed of specialized cardiac muscle found only in the heart, while the endocardium forms the lining of the myocardium and the heart valves. Interiorly, the heart is divided into a left and

right side by the septum, a partition consisting of myocardium covered by endocardium.

After birth, blood cannot cross the septum from one side to the other. Each side is divided by an arterioventricular valve into an upper chamber (the atrium) and a lower chamber (the ventricle). Double folds of endocardium strengthened by a little fibrous tissue form the arterioventricular valves. The valves are called the tricuspid and bicuspid valves and are between the atria and ventricles. Press valves open and close passively according to changes in pressure in the chambers.

When the ventricles contract blood (systolic movement), the pressure in the ventricles rises above that in the atria and the valves snap shut preventing backward flow of blood. The valves are prevented from opening upwards into the atria by tendinous cords called chordae tendinae (Waugh and Grant, 2006). Oxygenated blood leaves the heart through arteries except the pulmonary artery while deoxygenated blood enters the heart through veins except the pulmonary veins.

2.9.1 Functions:

2.9.1.1 Blood Circulation: - Contractions of the heart generate blood pressure, which is responsible for blood circulation through the body.

2.9.1.2 Routing Blood: - The heart separates the pulmonary and systemic circulations and ensures better oxygenation of blood flowing to the tissues.

2.9.1.3 Ensuring One-Way Blood Flow: - The valves of the heart ensure a one-way flow of blood through the heart and blood vessels. (Seeley *et al.*,2002)

2.9.2 Lipids:

Lipids are chemically diversified group of organic compounds of biological origin. They include fats and substances of fat-like nature. The common identity

is their insolubility in polar solvents such as water. They are soluble in non-polar solvents Galton and Krone (1991) explained that their water insolubility is as a result of their long chain hydrocarbon with little electrical charge; thus their dispersion in water is never achieved.

Lipids perform diverse functions in living organism and these functions depend on the particular lipid. They serve as energy reservoir. The storage capacity of fat is virtually unlimited. In eukaryotic cells, triglycerides (fatty acid of glycerol) form separate phase of microscopic, oily droplets in the aqueous cytosol, serving as depots of metabolic fuel in specialized cells called adipocytes. Adepocytes store large amounts of triglycerides as fat droplets, which nearly fill the cell (Lehninger *et al.,* 1993).

Lipids form part of the cell biomembranes and mitochondria. They also form insulating and protective coating in the subcutaneous tissues and around organs such as the kidney. Cholesterol is an important animal lipid. It is an assential component of the membranes of every human cell. It is required for tissue repair; growth and cell replication of specialized cell of the gonads, adrenals and skin.

2.9.3 Cholesterol:

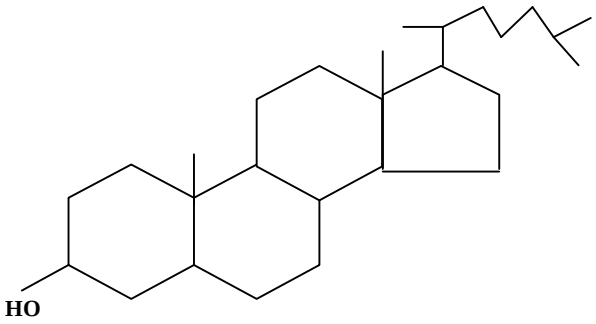


Fig.2.8: structure of cholesterol

Cholesterol is the major sterol in the human body. It is a structural component of cell membrane and plasma lipoproteins. It is also the precursor for many important physiological steroids such as bile salts and steroid hormones. It exists in the body in both free and esterifird forms. Most cholesterol in human plasma are esterified. (Szabo, 1979; Mayne, 1994.)

After the conversion of cholesterol into micelles, they are hydrolysed by the pancreatic enzyme cholesteryl esterase and then absorbed by diffusion from the micelles into the mucosal cells where much of it is subsequently reconverted into cholesteryl esters. Its absorption occurs mostly in the ileum (Montgomery *et al.*, 1999).

Lipids undergo a number of metabolic reactions in the body. Excesses after utilization and storage are excreted. Cholesterol is excreted in the faeces. It is delivered from the liver into the intestinal in the bile and additional amounts are delivered from the sloughed intestine mucosal cells. Moreover, some dietary cholesterol is excreted without being absorbed. Some in the bowel are acted on by intestinal bacteria and enzymes and converted to other neutral sterols before excretion in the faeces.

2.9.4 Lipoproteins and Lipid Transport

The principal lipids in the human serum are fatty acids, triglycerides, cholesterol and phospholipids. Their esters are relatively insoluble in water and are circulated freely in the blood stream.

While most of the free fatty acids are carried by albumin, the other lipids including cholesterol is circulated bound to proteins called apoprotains or apolipoproteins and transported as large molecular complexes (Nduka, 1995).

Galton and Krone (1991) defined the lipoproteins as complex aggregates of lipids and protein molecules which are sufficiently stable to form particles for

circulation in the plasma. They are therefore the major transport media for lipids, combining hydrophobic fats with hydrophilic compounds as apoproteins and phospholipids.

Lipoprotein particles consist of an outer shell of phospholipids and cholesterol in which various peptide components (apolipoproteins) are embebbed. The core of the particles carries the lipid load. The proteins found in lipoproteins vary considerably in their molecular characteristics but all tend to form helical structures that exhibit hydrophobic as well as hydrophilic surfaces that enable them function in solubilizing the lipids and to interact with enzymes and receptors that control lipid metabolism (Nduka, 1999).

The classification of plasma lipoproteins depends on their physicochemical properties such as charge and density. The greater the lipid- protein ratio in the complex, the lower its density. Lipids are less dense than proteins, therefore as the property of lipids in the lipoproteins increase, the density of the complex decreases and vise – versa.

The classification of the lipoproteins therefore depends on;

i. The density of the lipoproteins: When plasma or serum is subjected to centrifugation, the different classes of lipoproteins separate with the least dense on the uppermost layer while the heaviest settle at the bottom.

ii. The charge carried by the lipoprotein: When the serums sample is subjected to electrophoresis with agraose or cellulose acetate as support matrix, the chylomirons remain at the origin because they carry no charge. The lipoprotein bands that appear in before the beta (ß) band are called pre - ß lipoprotein. This corresponds to the very low density lipoprotein (VLDL). The next band which migrates in the beta position is called ß lipoprotein and corresponds to low density

lipoprotein (LDL). The lipoprotein that migrates farthest toward the anode in the alpha (α) position is called α – lipoprotein and this corresponds to high density lipoprotein (HDL).

The five main classes of lipoproteins involved in lipid transport in their order of increasing densities are: Chylomicrons, very low density lipoproteins (VLDL), intermediated density lipoproteins (IDL), Low density lipoproteins (LDL) and high density lipoproteins (HDL). The chlomicrons are derived from exogenous (intestinal) absorption of triglycerides. They carry triglycerides from the intestine to the blood stream, then to all cells.

VLDL (pre -β -lipoprotein) has density less than 1.006. They are mainly synthesized in the liver, although a small amount is produced in the intestinal mucosa. Dietary fat is broken down to fatty acids and partly glycerides in the small intestine by the action of pancreatic and intestinal lipases. Endogenous fat which is synthesized in the liver from glucose and fatty acids is secreted into the blood stream as VLDL. VLDL is 90% lipid and 10% protein. Their function is to transport endogenous lipids (mainly triglyceride) to cells.

IDL are normally transient lipoproteins formed during the conversion of VLDL to LDL. They are usually undetectable in normal plasma but seen in pathological lipoprotein metabolism.

LDL (β-lipoprotein) have a density between 1.006 and 1.063. LDL is the final stage in VLDL catabolism. VLDL circulating in the blood are gradually cleared by the action of lipolytic enzymes (lipoprotein lipase and hepatic lipase) and then converted through IDL into LDL.
LDL transport cholesterol from the liver to the cells. HDL (α-lipoproteins) have density between 1.063 and 1.21. They are synthesized primarily by the liver cells and then by the intestinal cells as nascent HDL. They are disc-shaped.

They are then modified in the plasma by association with other apolipoproteins and particularly the enzyme lecithin – cholesterol acyltransferase (LCAT) which converts them into spherical particles. They are further modified during which the small HDL_3 particles pick up surface components of VLDL such as phospholipids and apolipoproteins C and E. The small HDL_3 particles transforms into the larger HDL_2 particle. HDL transports cholesterol from cells to the liver.

2.9.5 Mechanism of Lipoprotein Atherogenicity

Atherogenicity is the development of plague on the intima of blood vessels as a result of the deposition of substances, particularly lipids on the vessels. LDL has been sufficiently established as important precussor of atherosclerosis (Igwe, 2005). High levels of LDL and low levels of HDL lead to increased infilteration of lipids into the vascular bed, where they predispose to oxidation than the circulating lipids. There are two mechanisms by which atherogenicity develops: Hyperlipidaemic plasma rich in LDL passes into the intima of blood vessels by the process of insudation (a low grade inflammatory edema). (Goldberger, 1990). Damage to endothelium is the primary event in the genesis of lesion. The exact location of a plague is determined by the lesion. The Endothelium can synthesize and secrete several growth factors including platelet derived growth factor. According to Goldberger (1990), the first response to minor endothelium cell injury is the attachment of monocytes which migrate subendothelially, turn into microphages and accumulate lipids. Both endothelial cells and macrophages secrete growth factors which affect smooth muscle cells and fibroblasts.

Loss and damage to endothelium also result in platelet attachment. The interaction of these events leads to atherosclerosis. The cause of initial injury and elevated LDL and VLDL is not clearly understood. However, Dale and foreman (1989). Suggest the possibility of altered rations of cholesterol to

lipoproteins in plasma, hence increasing the chances of injury. Another hypothesis believe that LDL oxidized by macrophages damage endothelial cells.

Murray *et al.,* (2006) defined lipid peroxidation as a chain reaction, which provides continuous supply of free radicals that initiate further per oxidation. This process causes damage to tissues. Anti-oxidants (especially vitamin C, E and carotenoids) break the reactive oxygen species chain, hence protect LDL against oxidation.

2.9.6 Laboratory Investigation and Management of Hyperlipidaemia

Plasma lipids most commonly determined in clinical chemistry are cholesterol and triglyceride. Both lipids are implicated as risk factors in the development of coronary heart disease. Elevated plasma concentrations of total cholesterol, LDL-cholesterol and/or triglycerides with reduced HDL-cholesterol are considered to be factors that predispose to atherosclerosis and myocardial infarction (Kaplan and Szabo, 1979). Other risk factors include hypertension, obesity, smoking, high caffeine intake, stress, and lack of exercise. Low serum triglyceride is suggestive of intravascular lipolysis and enhanced formation of HDL and vice-versa. Assay of plasma total cholesterol concentrations do not distinguish between esterified and unestrified forms. HDL mobilize lipids to the liver. Therefore increased HDL cholesterol and reduced LDL cholesterol is antiatherogenic.

Hyperlipidaemia is diagnosed if there is a clinical reason or the plasma has lipidaemic appearance, (Mayne, 1994). The knowledge of the lipoprotein particles that are present in the serum as well as clinical and family history are pre-requisites for measuring the total serum cholesterol and triglyceride concentrations. Examination of fasting serum, that has stood overnight can give

a clear insight of the disorder. Cream layer on a blood sample, opalescence and a clear sample suggests increased serum chylomicron, VLDL and LDL respectively. The distinction of increased LDL with VLDL from VLDL remnants is complex. However, electrophoresis and centrifugation of the serum separate the various lipoprotein classes.

2.10 HAEMATOLOGICAL PROFILE

Most plant preparations used in the treatment of diseases cause anaemia as side effect. Anaemias resulting from plant extract treatment is usually caused either by the destruction of blood cells or by the inhibition of blood cell formation in the bone marrow. Sometime, both cell destruction and bone marrow failure are seen. (Okonkwo *et al.,* 2004)

Anaemia is the most common red cell disorder (Ogunbode *et al.,* 1992, Cheesbrough, 2000). It occurs when the concentration of hemoglobin (hb) falls below the normal range for a person's age, gender and environment, resulting in the reduction in the oxygen carring capacity of the blood. The normal hb range of an adult male is 130 – 180g/l and 120 – 150g/l for females. Packed Cell Volume (PCV) ranges from 0.4-0.54 and 0.36-0.46l/l in males and females respectively (Cheesbrough, 2000). Reduction in haemoglobin is usually accompanied by a fall in red cell count and packed cell volume. (Hoffbrand *et al.,* 2001). Leonard and Zadok (1986) reposted that the hb, PCV and WBC ranges of normal rats are 133-174g/l, 384-456l/l and 5.4-23.6x10^9/l respectively.

Anaemia is common because many conditions can lead to it, viz; Excessive red cell destruction, folate and vitamin B_{12} deficiency, iron deficiency, thalassaemia, bone marrow failure and acute blood loss are among the conditions that can lead to anaemia.

Classification of anaemia is mainly based on the value of the red cell indices in such investigation. The microcytic hypochromic anaemia describes a condition in which the mean cell Haemoglobin (MCH) is less than 27 picograms, and with a variation of the normal red cell. Normocytic normochromic anaemia has MCV value of 80-95 fentolitres and MCH greater than 26 picograms, with the retention of normal red cell colour. Any anaemia with MCV greater than 95 fentolitres is macrocytic.

In this work, the possibility of *Alstonia boonei* stem bark extract to cause anaemia is investigated.

2.10.1 Laboratory Investigation of Anaemia

The normal adult bone marrow is able to produce different specialized cell types – red cells (erythrocytes), white cells (leucocytes) and platelets (thrombocytes). In haemolytic anaemia, the normal marrow is able to produce greater than the normal RBC. This may lead to reticulocytosis in which primitive immature red cells are produced.

A set of complementary tests called full blood count is carried out in the investigation of anaemia (Baker *et al.,* 2000). In general the full blood count consist of measurement of the values of the following parameters: heamoglobin, packed cell volume, red cell count, calculation of red cell indices, total and differential white blood cell count and platelet count. Reticulocyte count is used to assess the rate of red cell production and hence bone marrow function.

2.10.1.1 Heamoglobin (Hb)

Haemoglobin is the main transporter of oxygen and carbon di oxide in the blood. As blood circulates is the body, oxygen dissociates from heamoglobin and is delivered to body cells. Each heamoglobin molecule contains four linked

polypeptide (globin) chains which in an adult consist of two alpha chains (containing 141 amino acids), and two beta polypeptide chains combined with an iron containing porphyrin pigment called heam. Heam is the oxygen carrying part of the haemoglobin molecule. The level of heamoglobin in blood falls below the normal range in anaemia. As with packed cell volume (PCV), heamoglobin is an important determinant of anaemia in man.

2.10.1.2 Packed cell volume (PCV)

Packed cell volume (PCV), also called haematocrit is defined as the portion of whole blood occupied by the cells. It can also be said to be the measurement of the percentage (%) of cells in whole blood. The reference range for PCV in adult male is 40-54% and 36-46% in female. (Cheesbrough, 2000). PCV has a direct relationship with Hb, therefore it could be used in the investigation of anaemia when it is not possible to measure Hb.

2.10.1.3 Red Blood cell count

Red blood cell (RBC) count is the number of RBC in a particular volume of blood. Red cell count is important in the investigation of anaemia because it has a direct relationship with heamogolobin concentration in the blood, therefore a reduction in the number of red cells will also cause a reduction in heamoglobin level and vice verse.

2.10.1.4 RBC indices (Absolute values)

The RBC indices are: mean cell volume (MCV), mean cell haeemoglobin (MCH) and mean cell heamoglobin concentration (MCHC). They are also called absolute values because they are calculated from red cell count, haemoglobin and packed cell volume, and the information obtained from them provide valuable guide to the classification of anaemia. RBC indices also suggest the nature of the primary defect as well as indicating the underlying abnormality before progression of the anaemia

2.10.1.5 Mean Cell Volume (MCV)

This is the average volume of a single RBC. It is calculated from the packed cell volume (PCV) and RBC count.

$$MCV = \frac{PCV}{RBC} \text{ (fl)}$$

2.10.1.6 Mean Cell Haemogolobin (MCH)

This is the amount of heamoglobin in picograms (pg) in an average RBC. It is calculated from haemoglobin, Hb and RBC count:

$$MCH = \frac{Hb}{RBC} \text{ (pg)}$$

2.10.1.7 Mean Cell Haemoglobin Concentration (MCHC)

This refers to the amount of haemoglobin in grams per liter (mg/L) in one packed cells. It can also be measured in percentage.

$$MCHC = \frac{Hb}{PCV} \text{ (mg/L)} \quad \text{or} \quad \frac{Hb}{pcv} \times 100 \text{ (\%)}$$

2.10.1.8 White Blood Cell (WBC) Counts

The white blood cells (WBC) fight infections, defend the body against invasion by foreign organisms and produce antibodies in the immune response. The types of WBC are; neutrophils, easinophils, basophils, lymphocytes, and monocytes. The first three are known as granulocytes because they have granules, while the last two do not have granules and are therefore called agranulocytes.

The identification of the different cell types is possible by microscopic examination of anticoagulated blood films stained with a romanowsky stain. This technique is called “differential WBC count”. The total number of white blood cells can also be counted without identifying the individual cell types using the “total white blood cell count” technique. This technique employs the use of a weak acidic solution (Turks solution) that lyses the red cells and platelets so that only the white cells would be left to be counted.

Apart from the defensive nature of the white blood cells, their measurement in peripheral blood helps to distinguish ‘pure anaemia’ from pantocytopenia (a drop in red cells, granulocytes and platelets) which suggests a more general marrow defect. The number of white blood cells are reduced in anaemia suggesting there is a defect in the production of blood by the bone marrow. The normal range of WBC in humans is $4.0\text{-}11.0 \times 10^{9}$/L.

2.10.1.9 Reticulocyte count

Reticulocuytes are immature RBC. They contain resident ribonucleic acid (RNA) and ribosome. They are differentiated from mature red cells in romanowsky stained blood films by their blue-grey colour (polychromasia) and large size. Reticulocytes are normally present in small numbers in the blood (2%). Increased numbers in the peripheral blood can be found in anaemia due to red cell destruction, when there is effective erythropoiesis. Since the number of the reticulocytes increase with increased erythropietic activity, their count therefore helps in the assessment of bone marrow activity as well as in monitoring the erythropoietic response of an anaemic patient following treatment. (Cheesbrough, 2000).

2.11 LIPID PEROXIDATION AND ANTIOXIDANTS

Lipid peroxidation is the oxidative deterioration of polysaturated lipids as a result of the attack of free radicals. It is one of the reactions set into motion as a consequence of the formation of free radicals in cells and tissues. The mechanism by which free radicals induce cell injury have recently been reviewed. They include;

i Reaction with nucleic acids, nucleotides, polysaccharides, proteins and non-protein thiols,

ii Convalent binding to membrane components (protein, lipids, enzymes, receptors and transport systems) and

iii Initiation of lipid peroxidation.

The production of free radicals triggers off a proliferate network of multifarious disturbances (Halliwell, 1991). Lipid peroxidation is commonly divided into three phases: initiation, propagation and termination. The initiation step is the interaction of free radicals with polyenoic fatty acids of membrane phospholipids. Such attack occurs at the allylic hydrogen on the carbon atom between two doube bonds because of the relatively low bond dissociation energy (75 Kcal/mol) which renders these hydrogen particularly susceptible to attack.

2.11.1 Antioxidants and Free Radicals

The principles of antioxidation form the basis for the application of some chemicals in food preservation e.g. Butylated hydroxyanisole (BHA), Butylated hydrooxytoluene (BHT) and propylgalate. These act as free radical scavengers to prevent the spoilage of food via oxidative process. However, some of these chemicals have been reported to have adverse health effects on rats. BHT is particularly said to cause aderse foetal development (Gebelein, 2003).

The body contains some natural antioxidants which act as free radical scavengers. e.g. Glutathione, catalase, superoxide dismutase.

2.11.2 Antioxidants and Free Radicals Mechanism

The chain begins with a fat molecule (RH) reacting with O_2 molecule to form a part of free radical, a high-energy specie that reacts rapidly.

$$R - H + O_2 \rightarrow R^{\cdot} + H O O^{\cdot}$$

Alkyl radical Peroxy radical

(Free Radicals)

The alkyl free radicals reacts with another molecule of O_2 in the medium:

$$R^{\cdot} + O_2 \rightarrow R^{\cdot} - O - O^{\cdot}$$

Peroxyradials

The peroxyradical ($ROO^{\cdot}$) reacts with a fat molecule to form a hydroperoxide (ROOH), and the alkyl free radical repeats the cycle:

$$ROO + R\text{-}H \rightarrow ROOH + R^{\cdot}$$

Hydroperoxide alkyl free radical

The unstable ROOH spontaneously separates from the free radicals which perpetuate the chain.

$$ROOH \longrightarrow R^{\cdot} + HOO^{-}$$

peroxy radical

A single reaction between a fat molecule, for instance, and molecular O_2 triggers off the chain, creating free radicals in the process. The free radicals sustain the reaction chain. This mechanism has been postulated as the as the underlying cause of a number of disease conditions in man e.g. oxidative stress.

Molecules called radicals trap can stop the reactive chain. They are appropriately called ANTIOXIDANTS. There are two broad classes of antioxidants: The enzymatic and non- enzymatic antioxidants. The enzymatic antioxidants are systemic enzymes that are capable of arresting the progression of the chain of free radical reaction. They include superoxide dismutase, catalase, glutathione (GSH) reductase, GSH peroxidase, GSH transpepetidase, monoamine oxidase (MAO).

The non-enzymatic antioxidants are compounds which as a result of their unique chemical properties are capable of arresting the progression of the free radical chain reaction. Some of them are endogenous compounds which sometimes are called natural antioxidants e.g. Glutathione, and β-carotenoids. The non-enzymatic antioxidant also include retinol (vitamin A), Ascorbic Acid (vitamin C) and α-tocopherol (Vitamin E), flavonoids.

The general mechanism is represented by the equation below:

$$A - H + R^{\cdot} \rightarrow R - H + A^{+}$$

Consider an antioxidant (A - H) reacts with an alkyl free radical ($R^{\cdot}$) to reform a stable R-H, leaving a relative unreactive radical A^{+}. This reaction is capable of stopping the progression of oxidation of the fat molecule.

2.11.3 Pro-Oxidants

Some compounds promote the progression of free radicals reactions in the body. They are called pro-oxidants. Examples of such compounds include polycyclic aromatic hydrocarbons (PAH), Bromobenzene, natural products such as aflatoxin -B. Some non-free radicals can generate free radicals in biological environment e.g. peroxynitrites ($ONOO^{-}$), hypochlorus acid (HOCl), H_2O_2 Ozone (O_3), Singlet Oxygen (O). The free radical and the non-free radical species make up the Reactive Oxygen Species.

2.11.4 Free Radical and Reactive Oxygen Species (ROS)

Free radicals are molecular fragments containing one or more unpaired electron in an atomic or molecular orbital (Halliwell & Gutteridge, 1999); and capable of independent existence. Radicals derived from oxygen are important to the body. Molecular oxygen (dioxygen) is a radical. The addition of an electron to dioxygen gives a superoxide radical (O_2^-) (Miller et al., 1990).

ROS are free radicals with highly reactive oxidizing potential. The major source of ROS in the body is the mitochondrial electron transport chain (ETC) generates O_2^-, H_2O_2 & HO^-. Electron Transport Chain is the main source of ATP in the mammalian cell. In the process of Electron Transport Chain, some electrons leak out from the mitochondria to generate oxygen free radicals, O_2^-. The O_2^- is generated from complex I & III of the respiratory chain. In its atomic form the oxygen is highly charged to cross the inner surface of the mitochondria (Valko *et al.,*. 2006). Hydroxyl has very high reactivity with a short half-life. The redox state of the cell is dependent on iron / copper redox couple. This process ensures that no free radicals arising from the serum survives within the cell. However, under conditions of oxidative stress, iron-sulphur (4Fe – 4 S) is established. The released Fe^{2+} can participate in the fenton reaction giving rise to HO^- radicals: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + OH^-$ (Fenton Reaction).

2.11.4.1 Generation of free radicals

The free radicals may be produced by the body in the following processes:

- During the normal oxidation of food.
- As a result of leakage in the electron transport chain in mitochondria. About 1-4% of oxygen taken up in the body is converted as free radicals.

- Some enzymes such as xanthine oxidase and aldehyde oxidase form superoxide anion radical or hydrogen peroxide.

- NADPH Oxidase in the inflammatory cells (neutrophils, eosinophiles, monocytes and mocrophages) produce superoxide anion by a process of respiratory burst during phagocytosis

- Macrophages also produce NO from arginine by the enzyme nitric oxide synthase. This is also an important antibacterial mechanism.

- Peroxidation is also generated by the activity of lipo – oxygenase in platelets and leucocytes. (Vassudevan, 2005).

2.11.5 Anti Oxidants as Free Radical Scavenger

Antioxidants are free radical scavengers. According to Cano *et al.*,, (2003) indole alkaloids possess this property. Oxidation reactions can produce free radicals, which initiate chain reactions that damage cells (Valko *et al.*, 2007). Antioxidants terminate these chain reactions by removing free radical intermediates and inhibiting other oxidation reactions. As a result, antioxidants are often reducing agents e.g. thiols or polyphenols (Vertuani *et al.*, 2004).

Although oxidation reactions are crucial for life, they can also be damaging. Hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione (GSH), vitamine C, (Ascorbate) vitamin A (Reinol) E (α-Tocopherol); as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Sustained low levels of antioxidants, or inhibition of the antioxidant enzymes gives rise to oxidative stress which may damage or kill the cells (Davies, 1995).

2.11.6 Ascorbic Acid (Vitamin C)

Ascorbic acid or “vitamin C” is a monosaccharide found in both animals and plants. It cannot be synthesized in humans and must be obtained from the diet. Some other animals are able to produce this compound in-vivo (Linster & Van Schaftingen, 2007).

In cells, it is maintained in its reduced form by reacting with glutathione. (Menster, 1994). Ascorbic acid is a reducing agent and can reduce and thereby neutralize reactive oxygen species (ROS) such as hydrogen peroxide (Padayatty *et al.,.,* 2003). In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the antioxidant enzyme, ascorbate peroxidase, a property that is of particular importance in stress resistance in plants. Its deficiency causes scurvy in humans. (Shigeoka *et al.,* 2002).

2.11.6.1 Functions

In humans, vitamin C. is a highly effective antioxidant, acting to lessen oxidative stress, a substrate for ascorbate peroxidase, as well as an enzyme cofactor for the biosynthesis of many important biochemicals. Vitamin C acts as an electron donor for some different enzymes (Peterkotsky, 1991). Three of these enzyme systems take part in post translational modification of collagen. Vitamin C participate as a co-enzyme in collagen hydroxylation. It react with the hydroxyl groups of the amino acids proline or lysine in collagen molecule (via proline hydroxylase and lysyl hydroxylase), thereby allowing the collagen molecule to assume its triple helix structure; thus making ascorbate essential to the development and maintenance of tissues, blood vessels, and cartilage; wound heading and scar formation. As a co-enzyme, ascorbic acid is necessary for synthesis of carmtine. Carnitine is essential for the transport of fatty acids into mitochondria for β-oxidation (Levine, *et al.,* 1992).

2.11.7 Vitamin E (α-Tocopherol)

Vitamin E is the collective name for a group of eight related tocopherols and tocotrienols, which are fat – soluble antioxidants (Herrera and Barbas, 2001.)

Of these – tocopherol has been most studied as an antioxidant. It also has the highest bioavailability, with the body preferentially absorbing and metabolizing this form (Brigelius and Traba 1999). The – tocopherol form is the most important – lipid soluble antioxidant and protects cell membranes against oxidative damage by free radical reactions.(Herrera & Barbas, 2001).

The oxidized – tocopheroxy radical may be recycled back to the active form by reduction.

Another measureable end product that is used as indicator of lipid peroxidation is malondialdelyde (MDA). This compound can be formed as a result of the fission of the cyclic endoperoxides. It is a lipid peroxidative product commonly used as a bio-marker of lipid peroxidation (Symons *et al.*,1987).

2.11.8 Oxidative Stress

The occurrence of reactive oxygen species known as pro-oxidants is an attribute of normal cells. The steady – state formation of pro-oxidants is countered by the neutralizing action of antioxidants: enzymatic or non – enzymatic. Oxidative stress results from imbalance in favour of the pro-oxidants (Helmut *et al.*, 1991; Ogugua & Eze, 2001). The existence and development of cells in an oxygen containing environment would not be possible without the presence of defence systems that include enzymes and non – enzymatic antioxidant components. Aerobic life is characterized by a steady formation of proxidants balanced by a similar rate of elimination by antioxidants to maintain homeostasis. There is a requirement for continuous regeneration of antioxidant capacity and if this is not met, oxidative damage accumulates resulting in pathophysiologic events which is commonly termed oxidative stress. (Sies, 1991).

Many of the pro-oxidants generated by metabolism of endogenus compounds and xenobiotics are free radicals. The study of these provides a new field of interest in biological science and medicine. Such studies have helped to explain a number of pathophysiological phenomena which were hitherto unknown. for instance in cases involving inflammation, ageing, carcinogenesis, drug-induced, toxicity and immnuo suppression. (Helmut *et al.*, 1991).

2.11.9 Damage done by oxidative stress

Oxidative stress can produce metabolic stress, which may lead to DNA strand breakage, rises in intracellular free Ca^{2+}, damage to membrane transport proteins and other specific proteins; and peroxidation of lipids. The damage can be direct, indirect or both. Thus an excessive rise in Ca^{2+} can activate proteases (which may attack the cytoskeleton) and muscles (fragmenting DNA), protein kinases, thiol containing proteins (such as thioredoxin), and some cell surface receptors that perturb cell metabolism. (Helmut *et al.*, 1991).

CHAPTER THREE

3.0 MATERIALS AND METHODS.

3.1 MATERIALS

3.1.1. Chemicals and Reagents:

The chemicals and reagents used in this work were of analytical grade. They were purchased from H1-TECH Diagnostics Ltd Nigeria, Kenten Nigeria Ltd., Okey Surgicals Ltd., and used without further purification.

3.1.2 Plant Materials:

The stem bark of *Alstonia boonei* was collected and will be identified by a Plant Taxonomist, Dr. C. Okeke of the Department of Plant Science and Biotechnology, Evan Enwerem University, Owerri. A voucher specimen is deposited for reference in the Plant Science and Biotechnology Herbarium, Evan Enwerem University, Owerri.

3.1.3 Preparation of Plant Materials:

The extract was prepared according to World Health Organization (1983) Protocol for the preparation of alcoholic extract of medicinal plants. The stem bark of *Alstonia boonei* was collected and cut into pieces, and dried to constant weight. The dried stem bark was pulverized using Thomas Contact Mill (Pye, Unicam, Cambridge). It was weighed using a scale (Ohaus, England).

3.1.4 Animals

Albino mice were used for the toxicity studies and Winstar albino rats for the main studies. The animals were obtained from the Department of Pharmacology, University of Port Harcourt, Michael Okpara University of Agriculture, Umudike, or College of Medicine Animal House, Evan Enwerem

University (EEU), Owerri. Rats weighing about 150-200g were housed in ventilated stainless steel cages in the Animal House of the College of Medicine, Evan Enwerem University for 2 weeks to acclimatize under standard laboratory animal conditions. Feed and water were provided *ad libitum*. The cages were cleaned daily using aqueous ethanol; with daily changes of feed and water. Five rats were randomly assigned to each experimental group.

3.2 METHODS

3.2.1 Extraction Procedure

About Four hundred grammes (400g) of the powdered stem bark was soaked in 7.75L of 70% ethanol (v/v) and left to macerate at room temperature for 24 hours in a stainless steel extractor. The mixture was stirred at intervals during this period. The solvent containing the extract was slowly drained and filtered. The filtrate was evaporated under reduced pressure at 45°C using rotatory evaporator, (Laborato, 2000, China). The extract was recovered with aliquots of 70% ethanol. The recovered extract was concentrated to dryness in an oven (Accumax, India) at 50°C. The residue was weighed to obtain 30.55 grammes representing the yield of 3.06%. This was constituted to the required doses using normal saline.

3.2.2 Phytochemical Identification

Phytochemical identification of the extract was carried out according to the methods of Wall *et al.,* (1952), and sofowora (1993) to screen for alkaloids, tannins, steroids, cardiac glycosides, flavonoids, phlobatannins, saponins, cyanogenic glycosides and reducing sugars. And according to the official method of analysis of The Association of Official Analytical Chemists (AOAC) and the procedures as reported by Trease & Evans (1983).

3.2.2.1 Test for Glycosides

About five mililitres of 10% aq sulfuric acid was added to about 0.5g of the extract. The solution was boiled for 10 minutes and filtered. Few milliliters of 20% sodium hydroxide (NaOH) solution was added until the solution was neutral or slightly alkaline. Two mililitres of the resulting filtrate was added to 2ml of well mixed Fellings' solutions A and B. The appearance of green, light-brown to red precipitate was positive indication for the presence of reducing sugars derived from the hydrolysis of the glycosidic bond of the glycoside.

3.2.2.2 Test for Saponins

3.2.2.3 Method 1, Frothing Test

About 0.5g of the extract was added to 10mls of distilled water and shaken vigorously. The appearance of persistent froth, even on warming, was taken as preliminary indication for the presence of saponins

3.2.2.4 Method 2, Blood Agar Test for Hemolysis

About 1.0 cm diameter filter paper disc was soaked in the solution of the extract and placed on freely prepared 7% blood agar. The set up was allowed to incubate at room temperature for about 6 hours. The occurrence of hemolysis around the region of the drug material is confirmatory evidence for the presence of saponin (Stahl, 1973)

3.2.8.5 Test for Cardiac Glycoside

About 10ml of distilled water was added to a sample of the extract. 10ml of 10% lead-acetate solution was added. The resulting precipitate was removed by filtration. The filtrate was extracted twice with chloroform. The chloroform solution was dried with anhydrous sodium sulfate and filtered. The chloroform was evaporated in a water bath, leaving a residue.

About 2.0ml of freshly prepared methanolic solution of 3.5 -dinitrobenzoic acid and sodium hydroxide solution were added successively to the residue. Colour change of the solution to red-violet within 5 minutes indicates the presence of butanolic ring of cardiac glycoside (Stahl, 1973).

3.2.2.6 Test for Flavonoids

About 5ml 0.1M sodium hydroxide solution and 5ml 0.1M hydrochloric acid solution were successively added to 5ml solution of the extract. The formation of yellow precipitate with the acid was an indication for the presence of flavonoids.

3.2.2.7 Borntrager's Test for Anthracene Derivatives

3.2.2.8 Test for Anthraquinones

About 0.5g of the extract was shaken with 10ml benzene and filtered. 5ml 10% aq ammonium hydroxide solution was added to the filtrate and shaken.

The presence of anthraquinones is indicated by the formation of pink, red or violet colour in the ammoniacal phase.

3.2.2.8.1 Test for Mixed Anthraqinones

To about 0.5g of the extract was added to 10ml 1% aq. hydrochloric acid solution and boiled. It was filtered hot. 5ml benzene and 2.5ml 10 ammonium hydroxide (NH_4Oh) solution were added to the filtrate. Positive indication is the same as for anthraquinones.

3.2.2.8.2 Test for Tannins

10mls of distilled water was added to about 0.5g of the extract and stirred. 10ml 10% aq. Lead acetate solution was added to the solution. The appearance of copious precipitate was positive indication for the presence of tannins.

3.2.2.8.3 Test for Alkaloids

3.2.2.8.3.1 Method 1; Mayer's Working Solution

About 0.5g of the extract was heated with 5mls of 1% aq. hydrochloric acid solution in a water bath and filtered. Few drops of Mayer's reagent was added to 1ml of the filtrate. The formation of trurbidity or precipitate was a preliminary indication for the presence of alkaloids (Stahl. 1973).

3.2.2.8.3.2 Method 2, Dragendorff's Working Solution

The above process was repeated with Dragendorff's reagent. The positive indications are the same.

3.2.2.8.3.3 Method 3, Confirmation Test for Alkaloid

Few millilitres of the sample were spotted on silica gel TLC plates and developed in different solvent systems. The chromatograms were allowed to dry and then sprayed with freshly prepared Dragendorff's spray reagent (Stahl.1973: Wagner *et al.,* 1984). Confirmatory evidence is shown by the development of an orange or brownish coloured spot against a pale yellow background (Farnsworth and Euler, 1962).

3.2.3 Acute Toxicity Test:

3.2.3.1 Lethal Dose (LD_{50})

This was conducted based on the technique of Turner (1965) as adapted by Alui & Nwude (1982). Thirty five mice divided into 7 groups of 5 mice each were administered (i.p.) with graded doses of the extract as follows: 0.0, 100, 200, 300, 400, 500 and 600mg/kg body weight (bw) respectively. The control received 1mg/kg bw normal saline. The animals were observed for 24 hours for adverse or acute toxic reactions or death.

3.2.3.2 Acute Toxicity Profile

The mice were observed empirically and scored (+ / -) for adverse behavioural changes involving sedation, aggression, irritability, algesia, anorexia, writhing, pruritus, unco-ordinated movement or death.

3.2.3.3 Sub-acute Toxicity Study

The technique of Kluwe (1981) was adapted: Twenty non pregnant female rats weighing about 100-150g were orally administered the extract at the dose of 0.0, 100, 200 and 300mg/kg with 5 rats in each group. The control was given 1.0mg/kg normal saline. The rats were weighed every three days for a period of 15 days.

3.2.3.4. Foetal Toxicity Study:

The method of Ramox *et al.,*. (2000) as adapted by Vasudeva & Sharma (2007) was used. Twenty female rats (150-200g) of about seven-days pregnancy were divided into 4 groups of 5 rats each. Group 1 received 1.0mg/kg normal saline (NS) and served as control. Groups II, III and IV received 100, 200, and 300mg/kg of the extract respectively. The extract was administered for the remaining period of the pregnancy.

The weight and number of foetus were taken. The fetuses in each group were observed for possible biometric and phenotypical changes.

3.2.3.5 Protocol for Male Fertility Studies

The procedure of Raji *et al.* (2005) was adopted. Thirty male rats weighing 150-250g were divided into six groups of 5 rats each. The drug extracts were administered following the order below:

2 weeks segment

Group 1 received 1.0ml/kg bw / day normal saline (NS)

Group 2 received 50mg/kg/day ethanol extract of A. boonei (ABE)

Group 3 got 200mg/kg/day

4 weeks segment

Group 4 receive 1.0ml/kg/day

Group 5 obtained 50mg/kg/day

Group 6 received 200mg/kg/day

All drugs were given by oral intubation for 2 and 4 weeks respectively.

The initial and final body weights of the animals were taken. At the end of the 2^{nd} and 4^{th} weeks the animals were sacrificed by chloroform anaesthesia after 12 hours of deprivation from feed and respective drug treatments. The testes, penile organ, and pituitary gland were dissected out and processed histologically for each group where appropriate. The sperm count, viability, motility and morphology were determined. The caudal epididymis was dissected out. About 1.5mm incision was made on it, and the sperm was aspirated onto the microscope slide. The sperm motility was assessed by calculating motile spermatozoa per unit area, expressed as percentage motility. The sperm counts were taken using haemocytometer, expressed as million per millimeter of general suspension. The viability of the sperm was assessed using Eosin/Nigrosin stain (Raji *et al.*, 2003).

3.2.3.5.1 Neurochemical study

The anterior pituitary gland of the control and test groups in both segments were dissected out. The tissues were homogenized in sodium phosphate buffer (pH 7.0), filtered, and the filtrate was analysed for homovanillic acid (HVA).

3.2.3.5 .2 Collection of spermatozoa (Blazak *et. al.*, 1993, cheesbrough, 2000)

24 hours after the last doses of respective treatments were given, and the caudal epididymis, testes, seminal vescicle, pituitary gland and penile organ were dissected out; an incision (about 1.0mm) was made in the caudal epiddymis.

Sperm fluid was aspirated into a blender containing sodium bicarbonate-formalin diluting fluid within one hour of collection and counted using haemocytomerter.

3.2.3.5 .3 Spermatozoa Microscopy Principle (Adelman And Cahil, 1936)

The sample was examined within an hour of collection, [No reduction of activity was expected within three hours of emission. Light reduction occurs after six hours and usually complete cassation of motility after twelve hours of emission (Ojiegbe, 2005). The microscopy yielded the motility count, viability count, total sperm count, and sperm morphology. (Aladakatta *et al.* 2001).

3.2.3.5 .4 Estimation of the Percentage Motility of Spermatozoa Principle

The motility of spermatozoa means direct progress in approximately a straight line and this is an important property of spermatozoa. More than 50% of spermatozoa are expected to be motile within 60 minutes of ejaculation in cells. The motility is sustained for several hours.

Procedure

A drop (10 – 15μl) of well mixed liquefied sperm was placed on a slide and covered with a 20x20mm cover glass. The 10x objective was used to focus the specimen. The condenser iris was sufficiently close to give good contrast. The 40x objective eye-piece was used too examine several field to assess motility. The total count of 100 spermatozoa was made and noted; out of the 100 the nature and degree of motility were recorded, taking note of the number that were sluggish or non-motility were recorded.

Nb; Normal Motility

Over 50% of spermatozoa are expected to be motile within 6o minutes of ejaculation. The spermatozoa remain motile for several hours (Cheesbrough, 2000).

3.2.3.5 .5 Estimation of The Percentage Viability of Spermatozoa

Principle

This is usually done when more than 60% of spermatozoa are non-motile. Eosin preparation was used to assess whether the spermatozoa were viable or non-viable. 75% or more should be viable or unstained by eosin in normal spermatozoa. A large proportion of non-motile, non-viable spermatozoa may indicate structural defect.

Processes

A drop (10 - 15μl) of sperm was mixed with a drop of 0.5% eosin solution in a slide. After two minutes, the preparation was examined microscopically. The 10x objective was used to focus the specimen and the 40x objective was used to count the sperms in the field to obtain percentage of viable and non-viable spermatozoa.

The value of spermatozoa which remained unstained are viable, while non-viable spermatozoa stained.

3.2.3.5 .6 Estimation of Total Count of Spermatozoa (Freund and Carol, 1964)

Composition of the dilution fluid:

Sodium Carbonate	5g
Formaline	1ml
Distilled water	99ml

In Normal cases, the number of spermatozoa per ml of semen is 20×10^6 counts

Procedure

A 1 in 20 dilution of the semen using the dilution fluid was made in a graduated tube. A Pasteur pipette was used to fill an improved Neubauler chamber with well-diluted semen (Freund and Carol, 1964). The 10x objective with the

condenser iris sufficiently close to give good contrast was used to count the number of spermatozoa in an area of 2 square millimeters (12 large squares). The number of spermatozoa in 2ml of fluid was calculated by multiplying the number counted by 100,000.

3.2.3.5 .7 Examination of The Morphology of Spermatozoa (Hernmavathia Rahman, 1993)

Morphology implies the presence of not less than 60 percent normal shade sperm cells, abnormal shapes above 40% are indicative of serious derangement of spermatogenesis (Ojiegbe, 2005). On appropriate staining, the nucleus of the cell is expected to be dark blue; the cytoplasm of the head blue; and pink for the middle piece and tail.

Normal Spermatozoa

A normal spermatozoa measures 50-70μm in length. Each consists of an oval shaped head (with acrosomal cap), which measures 3-5x2-3μm, a short middle piece and a long thin tail (at least 45μm in length). In normal semen, at least 50% of spermatozoa should show normal morphology. Most specimens contain not more than 20% abnormal forms

Abnormal Spermatozoa

Head

Greatly increased or decreased in size.

- Abnormal shaped and tapering head (Pyriform)
- Acrosomal cap absent or abnormally large
- Nucleus Contains vacuoles
- Additional residual body i.e. cytoplasmic droplet. A typical distribution of chromatin vacuoles or double heads

Middle piece

- Absent or markedly increase in size. Appears divided (bifurcated), angle where it meets tail swollen.

Tail

- Absent or markedly reduced in length
- Bent or coiled tail, rudimentary or absent (Cheesbrough, 2000).

Procedure

A thin smear of the liquefied well-mixed fluid was made on a side while wet, the smear was fixed with 95% v/v ethanol for 5 minutes and allowed to dry. The smear was washed with sodium-bicarbonate and formalin solution to remove any mucus, which may be present. The smear was rinsed with several changes of water. It was covered with dilute (1 in 20) carbon fuchsine and allowed to stain for 3 minutes. It was then washed with water. Counterstaining was done by covering the smear with dilute (1 in 20) loefflers methylene blue for 20 minutes. The stain was washed off with water, drained and allowed to air-dry

Spermatozoa was observed using 40 x objectve. The 100x objective was used to confirm abnormalities. A count of 100 spermatozoa was made and the percentage showing normal morphology and the percentage that appeared abnormal were estimated.

3.2.3.5 .8 Protocols for female fertility studies

The method of Skibola *et al.*, (2004) was adapted. Thirty female albino rats (Wistar strain) with 3 regular cycles were housed with healthy adult male rats (Wistar strain) in the ratio of 3:1 per cage, under controlled laboratory conditions.

Prior to co-habitation, the female rats were separated for 1 month to forestall pre-existing pregnancy before the drug-extract regimen. The female rats were divided into 6 groups of 5 rats per cage and were given the drug-extract by oral intubation at the seventh day pregnancy as follows:

Group 2 normal saline 1.0ml/kg/day for 2 weeks

Group 2 50mg/kg/day for 2 weeks

Group 3 200mg/kg/day for 2 weeks

Group 4 normal saline 1.0ml/kg/day for 4 weeks

Group 5 50mg/kg/day for 4 weeks

Group 6 200mg/kg/day for 4 weeks

Vaginal smear was taken for each rat and examined between 9-10 am daily under stereomicrope (Leica, 2000) for spermatozoa or leucocytes which served as index for mating and pregnancy (Marcondes *et al.T* 2002). The female rats were sacrificed after 12 hours of feed deprivation under chloroform anaestesia at the end of the respective study periods. The uteri were dissected out by laparotomy. The mean number of fetuses were recorded for each group. The fetuses were collectively weighed for each group after removal of appendages. The uteris were stored in formaline for histological studies.

3.2.3.6 Experiment Design for Kidney, Liver, Blood and Lipid Profile Studies:

The rats were assigned randomly into six groups of five rats each. Animals in groups II and III received 50 and 200mg/kg extract respectively for two weeks. Groups V and VI were treated with 50 and 200mg/kg extract for four weeks respectively. Groups I and IV received 1.0 ml/kg normal saline in addition to the feed and water for two and four weeks respectively. They served as controls for the respective segments. All drugs were administered by oral intubation.

3.2.3.7 Experimental Design for Determination Lipid Peroxidation

After two weeks of acclimatization, the animals were weighed and given ear tags for individual identification. Twenty rats of mixed sexes were randomly assigned to four groups of five animals and kept in different compartments of the cage. Group 1: received only normal saline, and serve as control for two weeks. Group II: received paracetamol (750mg/kg to induce hepatotoxicity).

Group ll: received paracetamol (750mg/kg) before receiving the extract of *Alsonia boonei* (50mg/kg) for two weeks. Group iv: received paracetamol (750mg/kg) before receiving the extract of *Alstonia boonei* (200mg/kg) for two weeks. Route of administration was oral by intubation for all the groups.

3.2.4. Preparation of Serum:

About 5ml of blood sample was collected by cardiac puncture from chloroform/ ether anaesthetized animals using sterile syringes. The blood was collected into vials and allowed to clot for 30 minutes. It was centrifuged for 10 minutes at 2500rpm using Wisperfuge Centrifuge (Model 1384, Janson, Holland) at room temperature. The serum obtained was used for the analyses/ assay.

3..2.5. Determination of serum concentrations

3.2.5.1.Determination of serum concentrations of sex hormones

The serum concentrations of estrogen, progesterone and testosterone were determined with automated electrochemiluminescence immunoassay (ECLIA) techniques using Acurex (EISA System, USA) and Elecsys autoanalyser (Model 1010, product of Roche Mannheinn, Germany).

3.2.5.2 Determination of Sperm Characteristics:

The methods of Freund & Carol (1964), Chesbrough (2000) and Adelman & Cahil (1936) was used to determine the sperm count, motility, morphology and viability respectively. The percentage morphology was assessed based on the difference between the intact sperm cell count and the deformed ones.

3.2.5.3 Determination of Tissue Concentration of Vanilmandelic Acid (VMA)

The tissue of the pituitary gland was homogenized in phosphate buffer (pH 7.0) and analysed [using Biosystem VMA Test Kit (Costa Brava, Barcellona, Spain), and Biochromatic Photometer (Model 1904, Product of Awareness Technology Inc., USA)] for VMA concentration.

3.2.5.4 Determination of Serum Renal Electrolytes and Metabolites:

The serum concentrations of Na^+, K^+. HCO_3^- , Cl^- ions, were determined using electrolyte autoanalyser, Humalyte (Product of Human, Germany); while the urea and creatinine concentrations were determined using Automatic Chemistry Analyser (Product of Awareness Technology Inc., USA). The urea/creatinine ratio were calculated.

3.2.5.5 Assay of Serum Liver Marker Enzymes:

The assay of liver marker enzymes, Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP) were carried out ; as well as determine the serum concentrations of bilirubin (Total, conjugated and unconjugated) using the biochemical multiple autoanalyser, Humana 80 (Product of Human, Germany) attached to an electrochemical device and computer.

3.2.5.6 Determination of Serum lipid and lipoprotein concentrations:

The concentrations of serum triacyglycerol, total cholesterol, and high density lipoprotein cholesterol were determined using Vitros Chemistry Autoanalyser (Model DT 6011, product of Johnson Inc., USA).

3.2.5.7 Determination of serum lipid peroxidation product and some non-enzymatic antioxidant concentrations:

The serum concentration of lipid peroxidation product, malondialdehyde

(MDA), and non-enzymatic antioxidants, ascorbic acid and α -tocopherol were estimated using the methods of Albro *et al.*, (1956), Omaye *et.al* (1979) and Essein (1995) respectively. Paracetamol – induced hepatoxicity was used as the positive control.

3.2.5.8 Determination of Haematological Parameters:

Haematologic parameters, RBC count, Hb, PCV, MCV, MCH. MCHC and reliculocytes as well as total WBC were estimated using Haematology Autoanalyser, Sysmex, (Model KX - 21 N, Sysmex Inc., USA).

In all the cases normal saline was used as baseline control.

3.2.6 Histological Preparation/Staining:

The processing followed the procedure of Drury and Wallington (1980) as modified by John *et al.,* (1990): Paraffin sections were dewaxed through two changes of xylene for 3 minutes each. The section were passed through two changes of descending grades of absolute ethanol (95% and 70%) for 1 minute respectively, and rinsed in running water for 5 minutes. The section were stained with haematoxyline, rinsed and differentiated in 1% acid alcohol. They were blued in running tap water and counter stained with eosin and rinsed with water accordingly.

The sections were passed through ascending grades of ethanol (75%, 95%). They were transferred to two changes of xylene for 2 minutes and mounted in DPX and covered with a cover slip. The sections were dried in the oven. The histological processing was further carried out using automated Histokinetic Tissue Processor II (Miles , USA). The serial sections were processed using Spencer's cryostat and Hertz Rotary microtome (5μ thickness) (Model 1010 – SMT- 011, England). The microscopic examination were carried out using Olympus stereo-microscope with digital photomicrographic camera (Leica,

DM500, Lasez, Switzerland) attached to one of the eye- piece and the image projected to a computer from which clear images were visualized and interpreted by a histopathologist.

Each experimental procedure and data analyses were repeated for at least three times, as the case may be, and the reproducible results used for the final analyses.

3.2.7 Statistical Analyses:

Data obtained were analysed with student's t-test and one way ANOVA using statistical software packages (SSPS, [version 15 and 17] and statsoft). Inferences were drawn at 95% confidence level.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS.

4.1 RESULTS

4.1.1 Phytochemical Composition

The result of the qualitative phytochemical screening of *A. boonei* stem bark extract is presented in Table 1. The result showed that the stem bark contains alkaloids, tannins, flavonoids, saponins, cardiac glycosides, phlobatannins and reducing sugars.

The acute toxicity results show the toxicity signs observed following the administration of the extract, and the calculated LD_{50} which are presented in Tables 2 and 3 respectively. The result shows that there was no mortality in the control group and the group which received 200mg/kg of the extract. Mortality occurred in groups that received 300, 400, 500 and 600mg/kg for24 hours .100% mortality was recorded in group 6 (600mg/kg) within 1 hour. Mortality occurred in all the groups within 24 hours.

The animals showed signs of toxicity (Table 2) such as dizziness, insensitivty to pain, loss of appetite, itching, unco-ordinated movements and death. Table 3 shows that the LD_{50} was 562mg/kg.

TABLE 1: RESULT OF THE PHYTOCHEMICAL SCREENING OF STEM BARK EXTRACT OF *ALSTONIA BOONEI*

Test	Result
Tannins	+++
Alkaloids	+++
Saponins	+
Cardiac glycosides	++
Steroids	+
Flavonoids	++
Reducing sugar	+
Cyanogenic glycosides	+

Key:

+++ **=** high.

++ = moderate.

+ = trace.

- = not detected.

TABLE 4.2: ACUTE TOXICITY STUDIES

Group	Dose (mg/kg)	Sedation	Aggression	Irritability	Analgesia	Anorexia	Writhing	Uncordinated Movement	Pruritus	Death
1	0.0	-	-	-	-	-	-	-	-	0
2	200	-	-	-	+	-	-	-	-	0
3	300	+	-	-	+	-	-	-	-	2
4	400	+	-	+	+	+	-	+	-	3
5	500	+	-	+	+	+	-	+	-	3
6	600	+	-	+	+	+	+	+	-	6

Key:

+ = present.

- = absent.

TABLE 4.3: LD_{50} OF THE EXTRACT OF *A. BOONEI* IN MICE (n=6)

Group	Dose (mg/kg)	Dose Difference (a)	Death (d)	Mean Death (d/n)	Dose Difference x Mean Death (a x d/n)
1 (saline control)	0.0	-	Nil	-	-
2	100	100	0	0	0
3	200	100	0	0	0
4	300	100	2	0.3	30
5	400	100	3	0.5	50
6	500	100	3	0.5	50
7	600	100	6	1.0	100

$$LD_{50} = \text{Least dose that killed all the animals} - \frac{\sum \text{Dose diff. x Mean}}{\text{No of animals in group}}$$

$$= 600 - \frac{(\sum [a \times d/n])}{n}$$

$$= 600 - \frac{(30 + 50 + 50 + 100)}{6}$$

$$\approx 562 mg/kg.$$

(Aliu & Nwude, 1982)

4.1.2 THE EFFECT ON FEMALE REPRODUCTIVE PARAMETERS

4.1.2.1 The Effects of *A. boonei* Stem Bark Extract on the Serum Concentration of Reproductive Hormones:

Figure 4.1 depicts that the three doses of the extract, 100, 200 and 300mg/kg retarded the growth of the female rats in 15days of treatment. The mean weight gain in the control was consistent over the period with a percentage gain of 85.6 ± 3.5%.The weight gain depreciated in the test groups over the period by 40.2 ± 5.1%, 22.7 ± 3.1% and 4.7 ± 0.7% for 100, 200, and 300mg/kg bw respectively. This represents a significant ($p< 0.05$) growth retardation of the rats in the test groups.

The ABE had significant ($p<0.05$) effects on serum estrogen concentration at 200mg/kg bw for the 2 and 4 wks segments. The highest values were 60.6 and 84.6pg/ml respectively, against a control value of 42.1pg/ml. The increase was not significant at 50 and 100mg/kg for 2wks.But rose from a control value of 43.16pg/ml to 55.6pg/ml by the 4^{th} wk. ($p<0.05$) (Fig.4.3)

The administration of *A. boonei* stem bark extract had significant effect on the serum progesterone concentration. At two weeks after administration, the highest concentration of progesterone, 0.78ng/ml was recorded from the female rats which received 200 mg/kg of *A. boonei* for 2wks. This value was significantly different from the 0.43mg/ml recorded for rats in the control. There was no significant difference observed at 50 and 100mg/kg ($p > 0.05$) compared with the control (Fig.4.4).

At four weeks, the increase in the concentration of progesterone was significant (1.37ng/ml) at 200mg/kg but not at 50mg/kg (0.57ng/ml) compared with the control value of 0.45ng/ml. (Fig.4.4). The histological results show that ABE has adverse effects on the uterine tissues (Plates. 4.1-4.5, Table 4.4a).

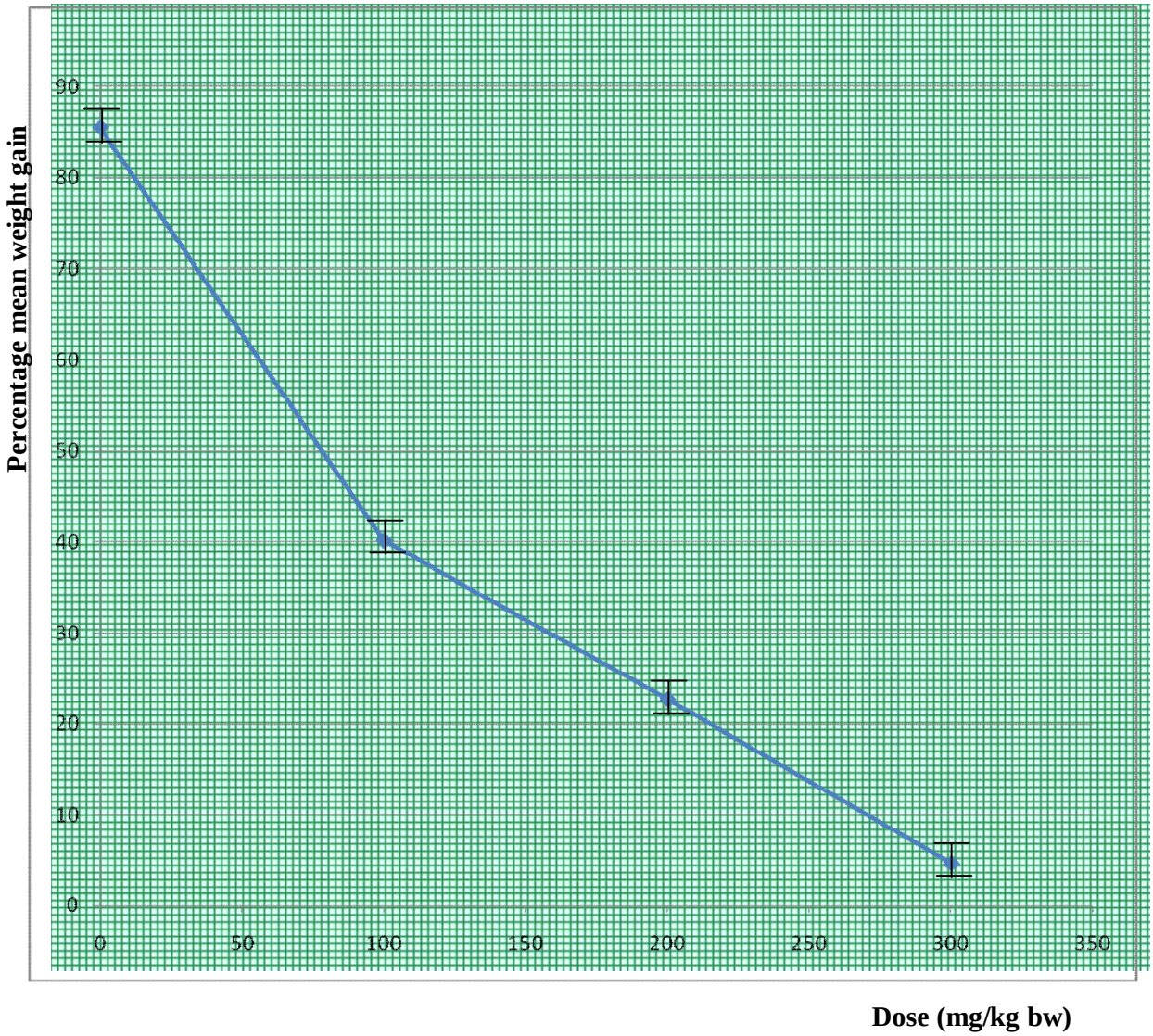


Fig 4.1: Plot of negative mean weight gain against dose on female rats treated with *A. boonei* (n = 5).

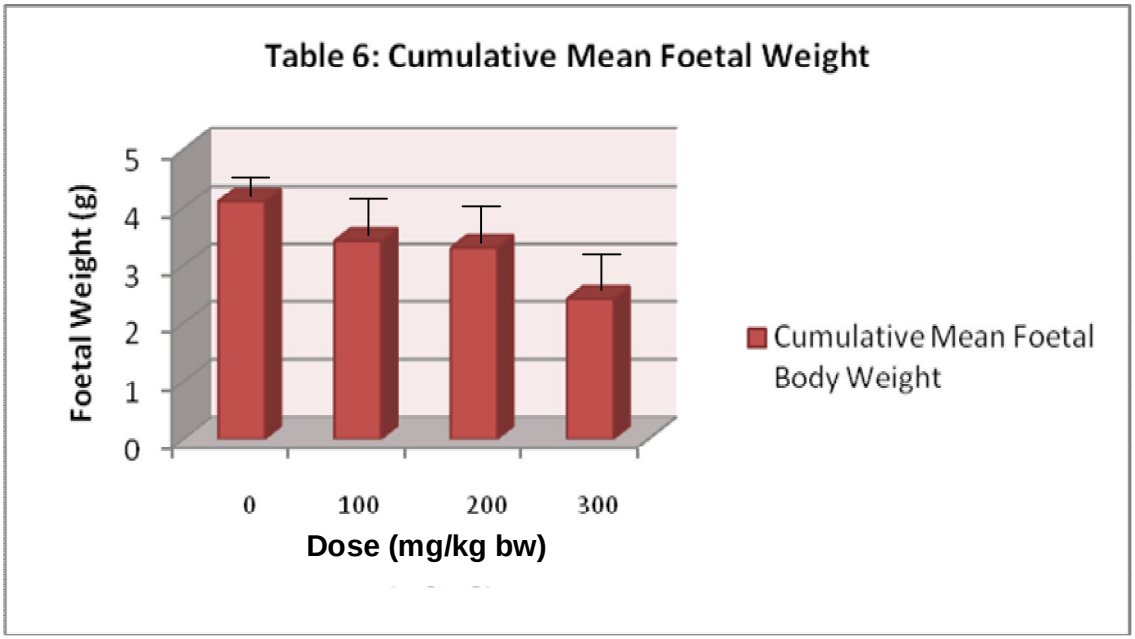


Fig 4.2: Mean cummulative foetal weight values against the dose of ABE

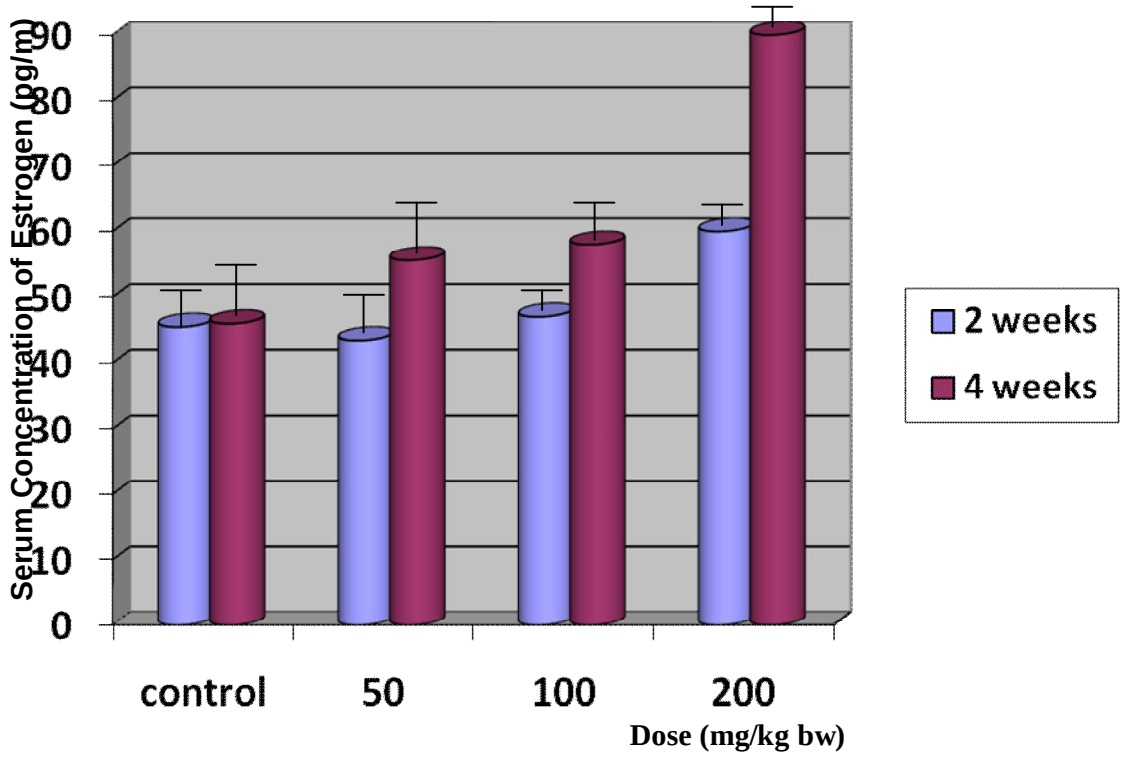


Fig. 4.3: Serum concentration of estrogen against dose after administration of *A. boonei* stem bark extract.

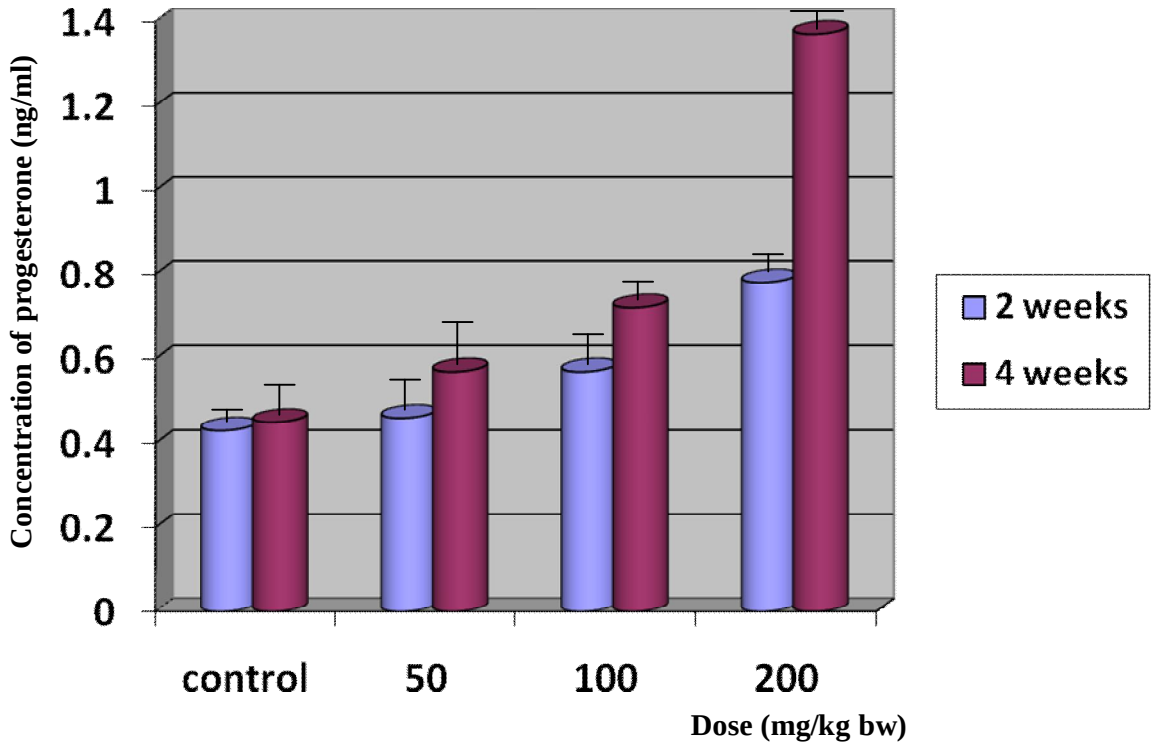


Fig.4.4: Serum Concentration of Progesterone against dose after administration of *A. boonei* stem bark extract.

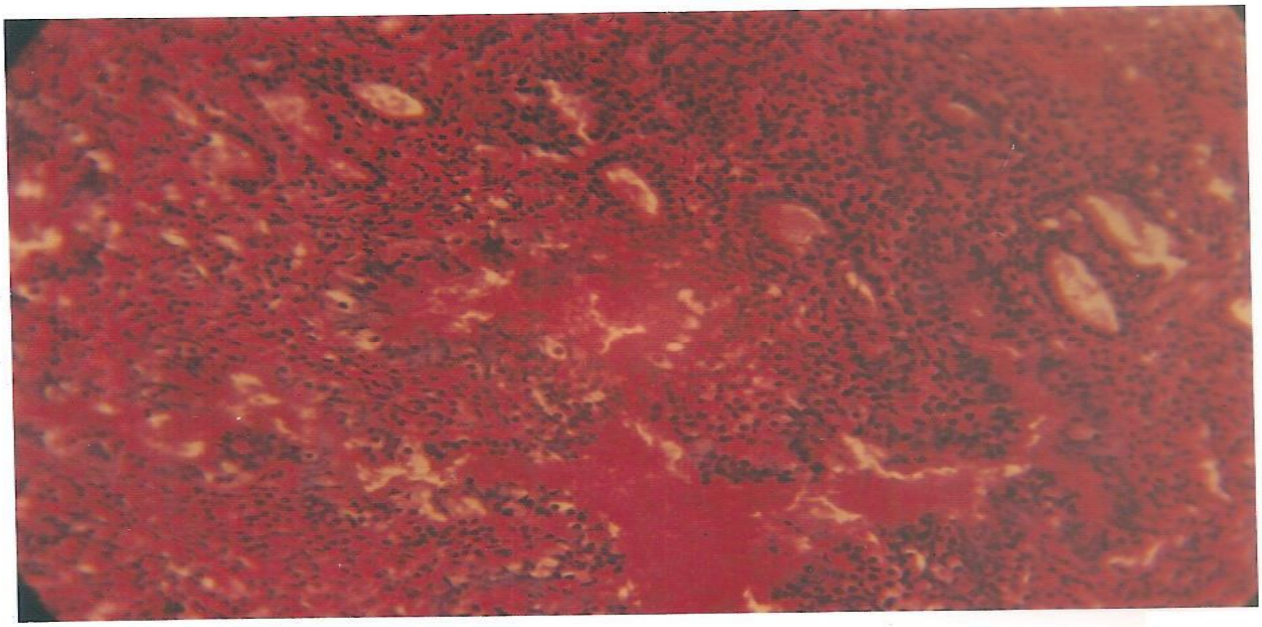


Plate 4.1: Photomicrograph of the uterus of rats used as control. It presents a normal structure of the uterus with endometrium having large epithelial cells with nuclei.

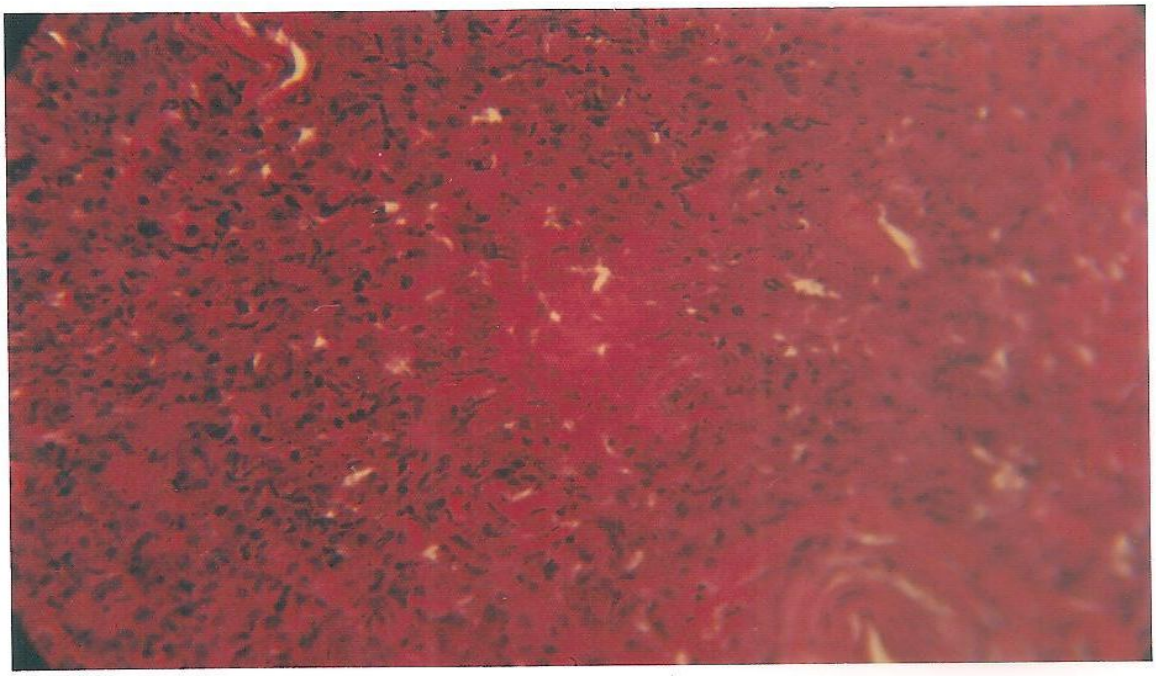


Plate 4.2 Photomicrograph of the uterus of rats treated with 50mg/kg of *Alstonia boonei* for 2 weeks. The endometrial glands are still intact, there are no significant histological changes.

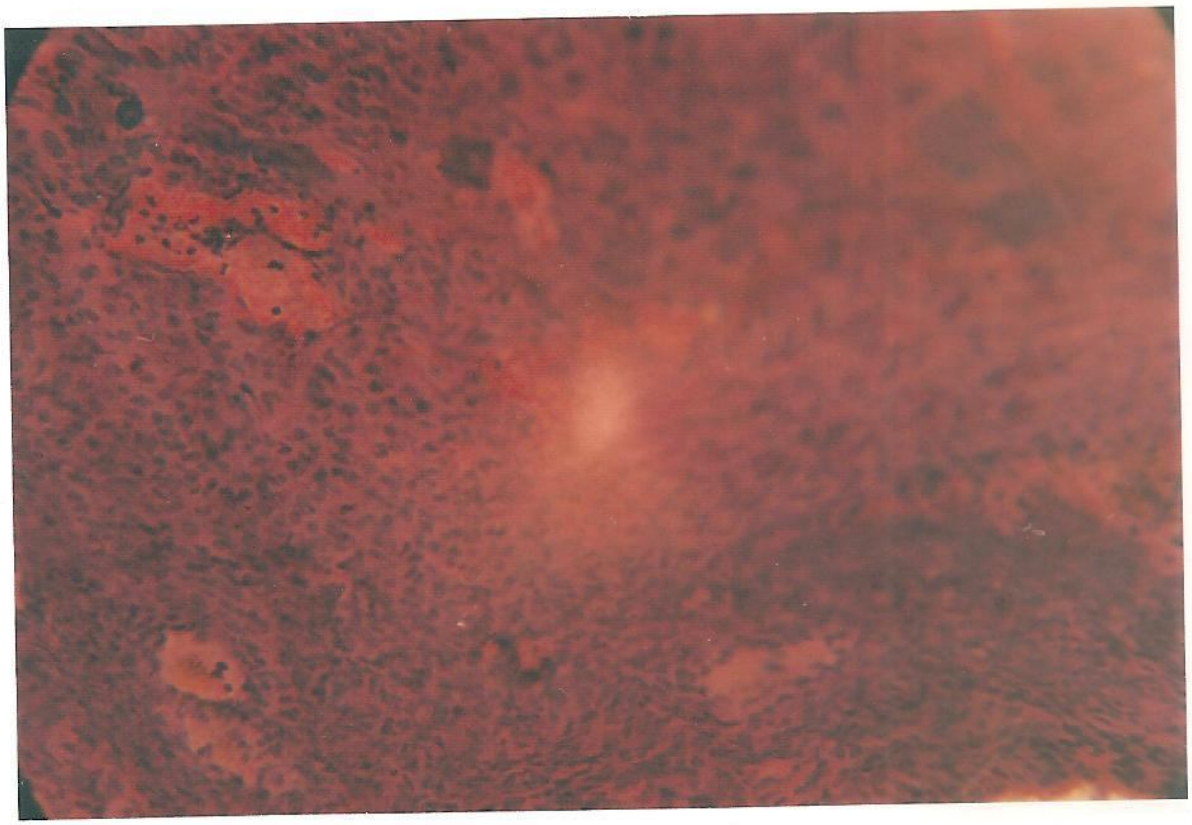


Plate 4.3: Photomicrograph of the uterus of rats treated with 200mg/kg of *Alstonia boonei* for 2 weeks. Irregular endometrial glands are present and the endometrial stroma are slightly disaggregated.

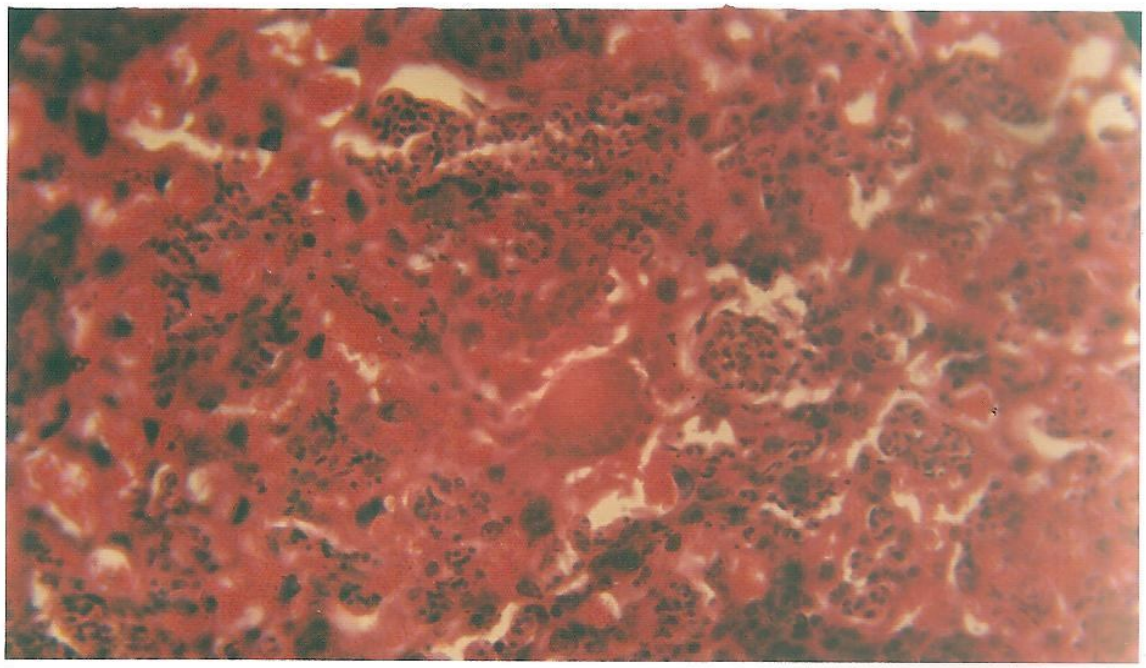


Plate 4.4: Photomicrograph of the uterus of rats treated with 50mg/kg of *Alstonia boonei* for four weeks. The tissue shows a cellular and pale stroma and the presence of pinkish deposits (haemorrhage). There are cellular infiltration of the glands, and distortion of the histology of the uterus.

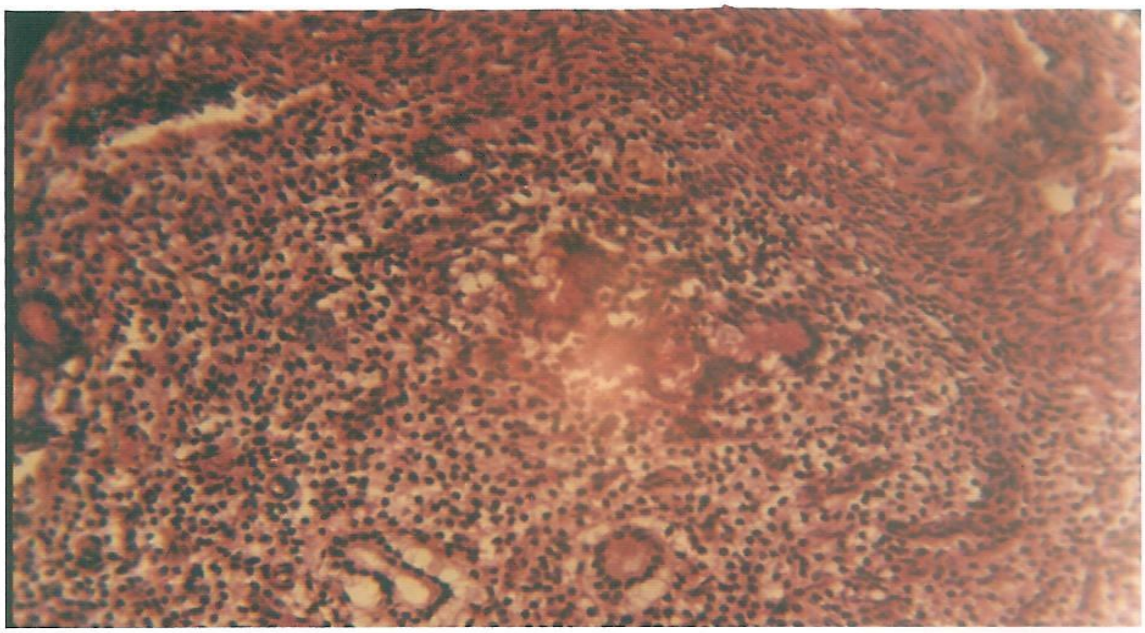


Plate 4.5: Photomicrograph of the uterus of rats treated with 200mg/kg of *Alstonia boonei* for four weeks show proliferation of both glandular and tissue stroma. The stroma appeared to be pale and oedematous. There is also the presence of pinkish deposits (haemorrhage).

Table 4.4a: Summary Histological Results on Uterine Tissue Architecture.

2 WEEKS SEGMENT:

Control	No histological changes.
50mg/kg	No significant histological changes.
200mg/kg	No significant histological changes.

4 WEEKS SEGMENT:

Control	No histological changes
50mg/kg	• Cystic spaces. • Stroma pale and edematous. • Large nuclei, with prominent nucleolus. • Hyalinization. • Uterine glands filled with secretory deposits. • Amorphous pink deposits – haemorrhage.
200mg/kg	• Stroma cellular. • Few cystic spaces. • Proliferation of stroma tissue and glandsular elements. • Thickened columnar epithelial lining. • Cystically dilated glands. • Hyalinized materials. • Pinkish Deposits.

4.3 MALE REPRODUCTIVE PARAMETERS:

In Fig 4.5, the effect of the extract on the rat serum testosterone concentration is shown. There was a transient dose, but not time, dependent reduction in the serum testosterone in the rats. The concentration fell from 1.2 $\pm$ 0.16 ng/ml in the control to 1.0 $\pm$ 0.25 ng/ml at 50mg/kg bw and 1.10 $\pm$ 0.20ng/ml at 200mg/kg bw respectively at the end of 2 weeks of treatment with the extract. For the 4 weeks segment, it fell from 1.46 $\pm$ 0.11 ng/ml in the control, to 1.44 $\pm$ 0.23ng/ml at 50mg/kg bw and 1.38 $\pm$ 0.25 ng/ml at 200mg/kg respectively. These changes in serum hormonal concentrations were not significant ($p>0.05$), except for the 50mg/kg bw at 2 weeks.

Figs. 4.6 – 4.10 show the pattern of the sperm characteristics: The sperm count reduced progressively from 88.8 $\pm$ 3.03 millions/ml in the control to 41.4 $\pm$ 2.41 millions/ml at 200mg/kg at the end of 4 weeks of extract administration. The percentage motility fell from 75.0 $\pm$ 2.55% in the control, to 56.0 $\pm$ 3.10% at 200mg/kg in the 4^{th} week. The percentage viability was also attenuated when the control value of 87.4 $\pm$ 1.95% is compared with 71.4 $\pm$ 3.27% at 200mg/kg seen in the 4^{th} week. The reductions were statistically significant ($p<0.05$) in the above parameters.

The morphology (Fig. 4.8 & 4.9) of the spermatozoa was also affected by the treatment of the extract. The percentage of normal (N) spermatozoa fell from 84.6 $\pm$ 3.85% in the control to 63.2 $\pm$ 5.4% at 200mg/kg by the end of the 4^{th} week of treatment. The percentage difference between the control and the test was also significant ($p<0.05$). The photomicrographs of the histological presentations are shown below, (Plates 4.6 – 4.10).

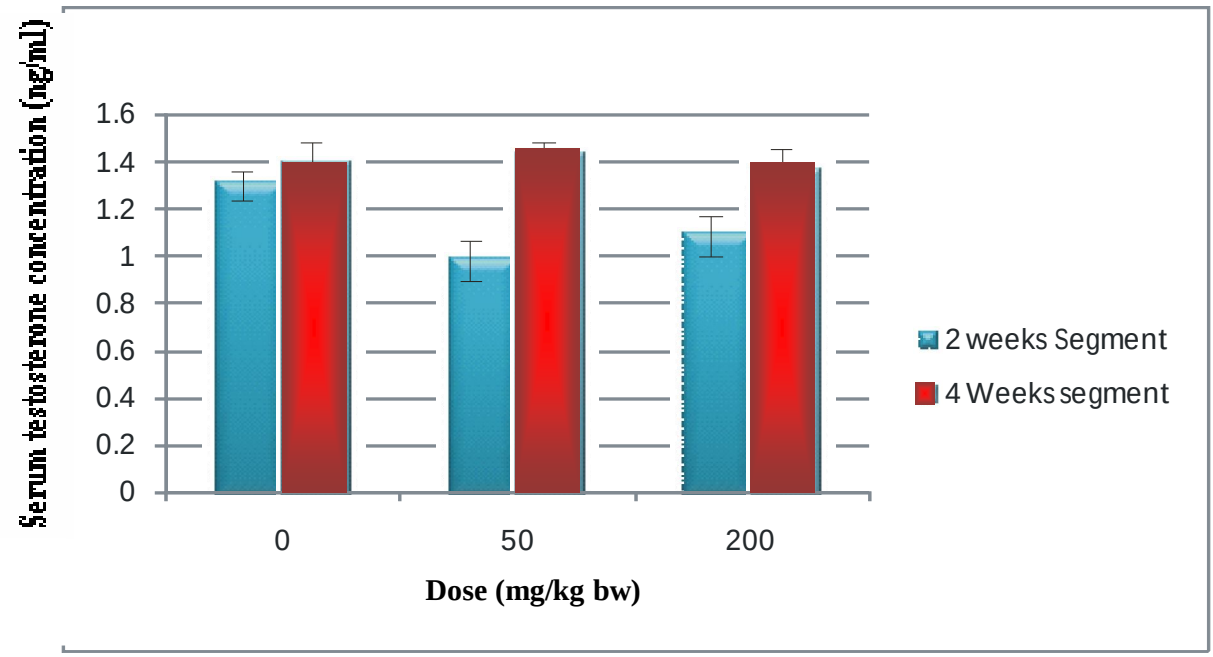


Fig 4.5: Concentration of Serum Testosterone in male rats treated with *A. boonei* extracts against the dose (n = 5).

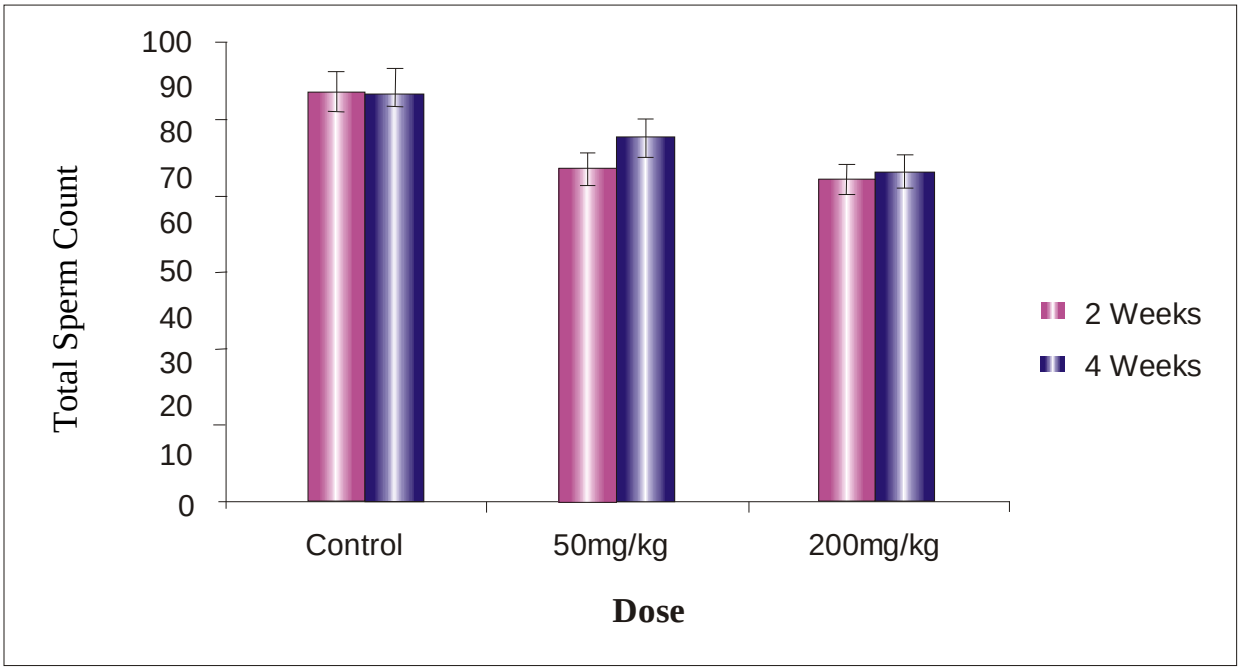


Fig 4.6: Total sperm count of rats treated with *A. boonei* stem extract at 2 weeks and 4 weeks (n=5).

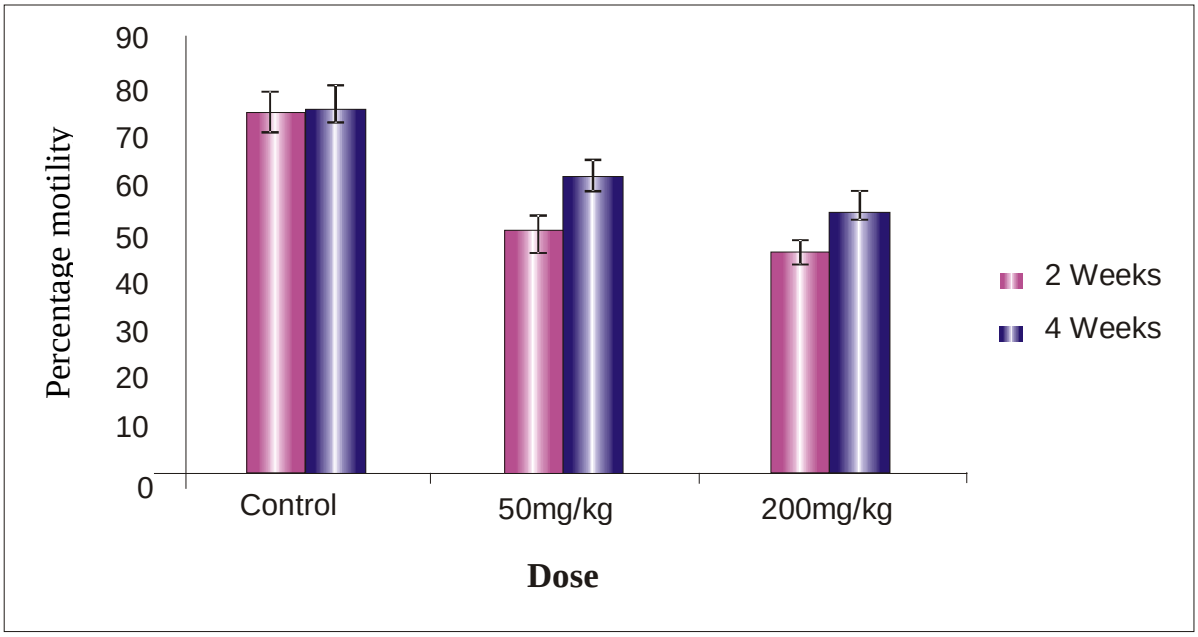


Fig . 4.7 : Percentage sperm motility of rats treated with *A. boonei* stem extract for 2 weeks and 4 weeks (n = 5).

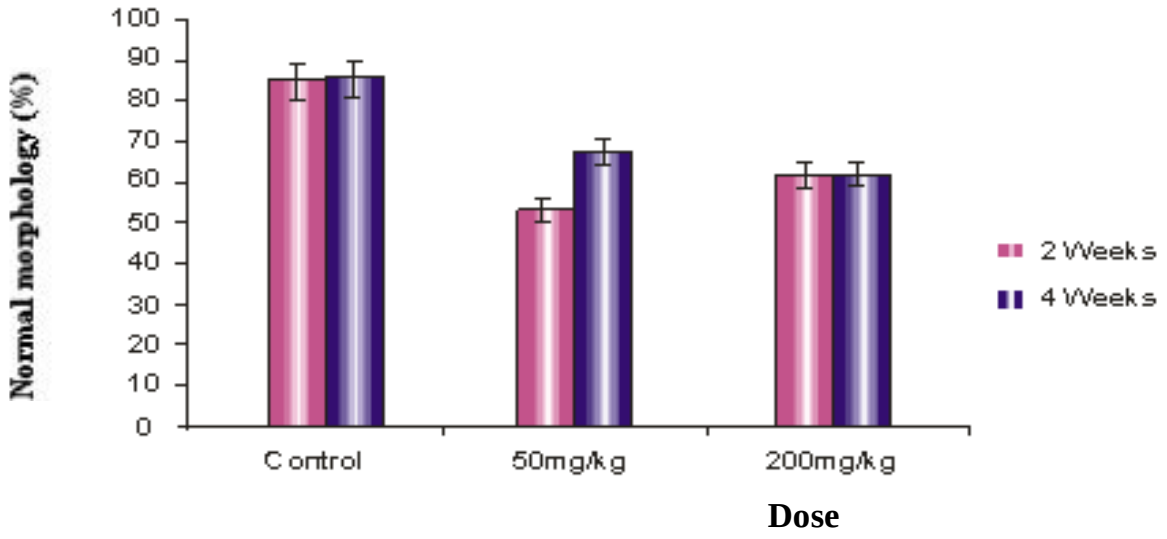


Fig. 4.8 : Normal sperm morphology of rats treated with *A. boonei* stem extract for 2 weeks and 4 weeks (n = 5).

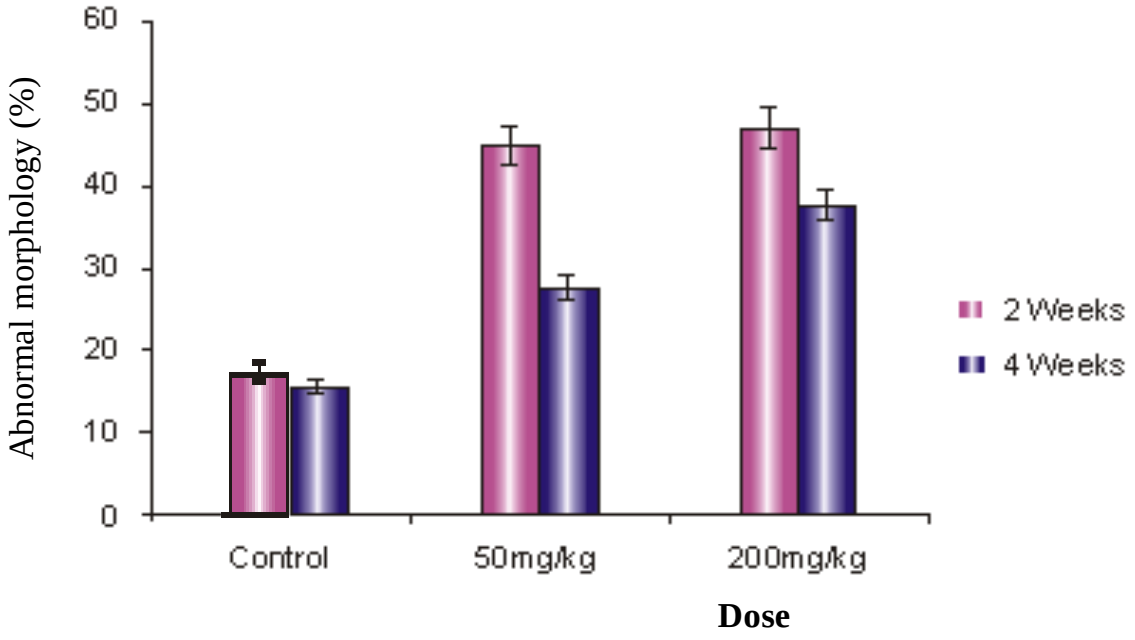


Fig. 4.9: Abnormal sperm morphology of rats treated with *A. boonei* stem extract for 2 weeks and 4 weeks (n=5).

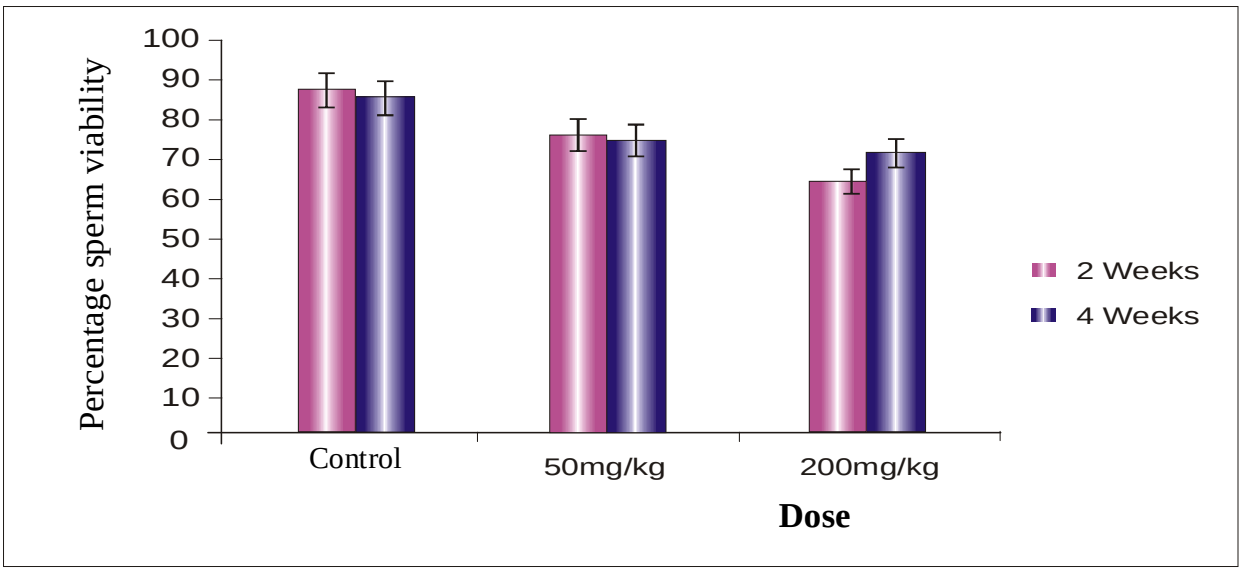


Fig. 4.10: Percentage sperm viability of rats treated with *A. boonei* stem extract for 2 weeks and 4 weeks (n=5).

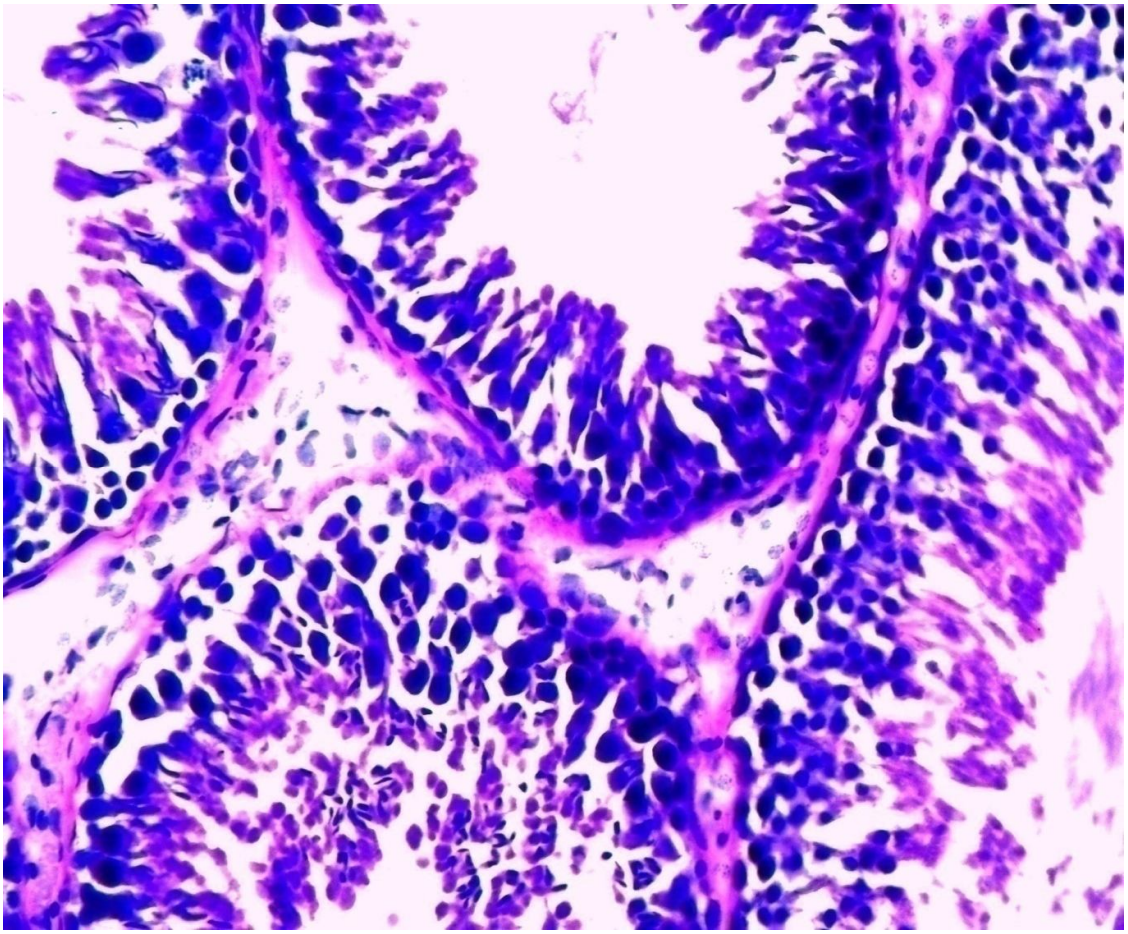


Plate 4.6: Control: Photomicrograph of Rat Testicular Cells. Intact testicular cells.

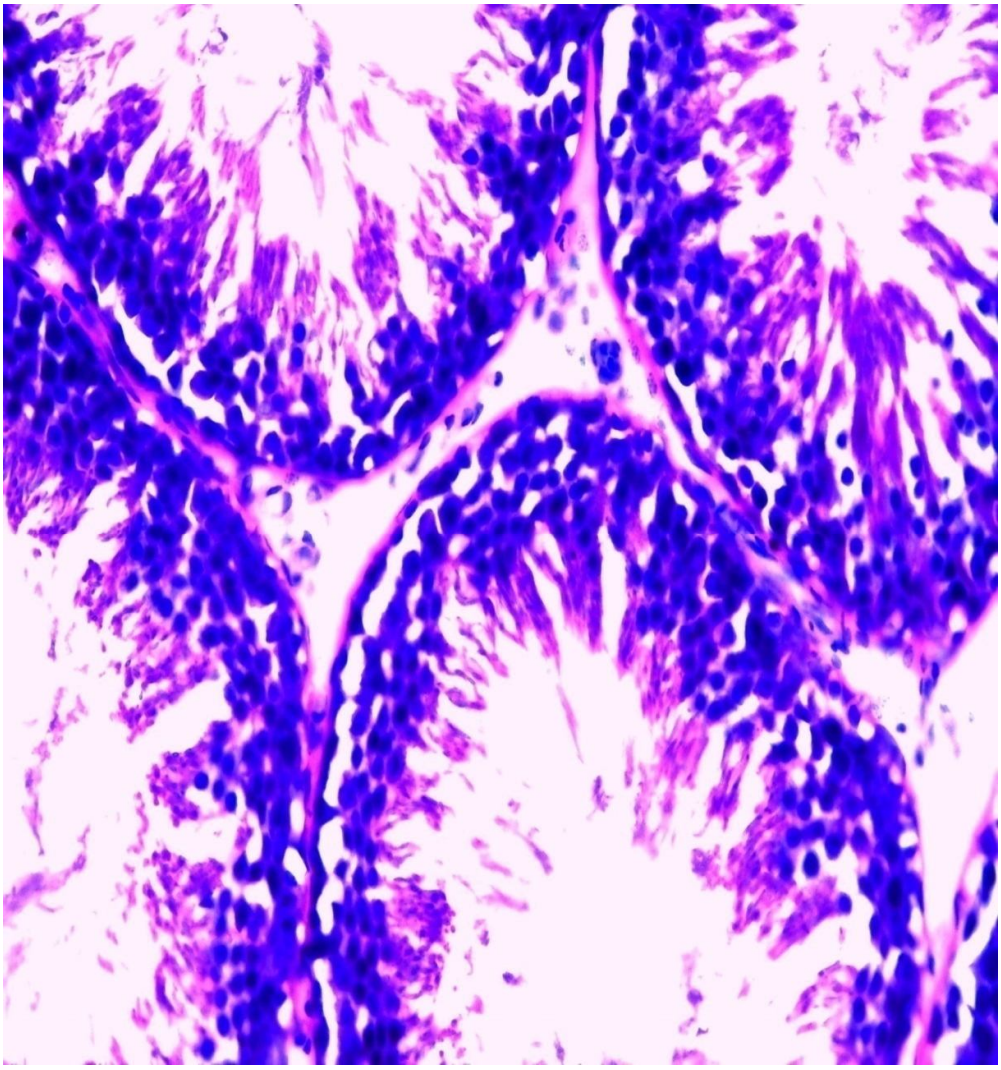


Plate 4.7: Photomicrograph of Rat Testicular Cells (50mg/kg x 2wks) Mild Enlargement of somniferous tubule.

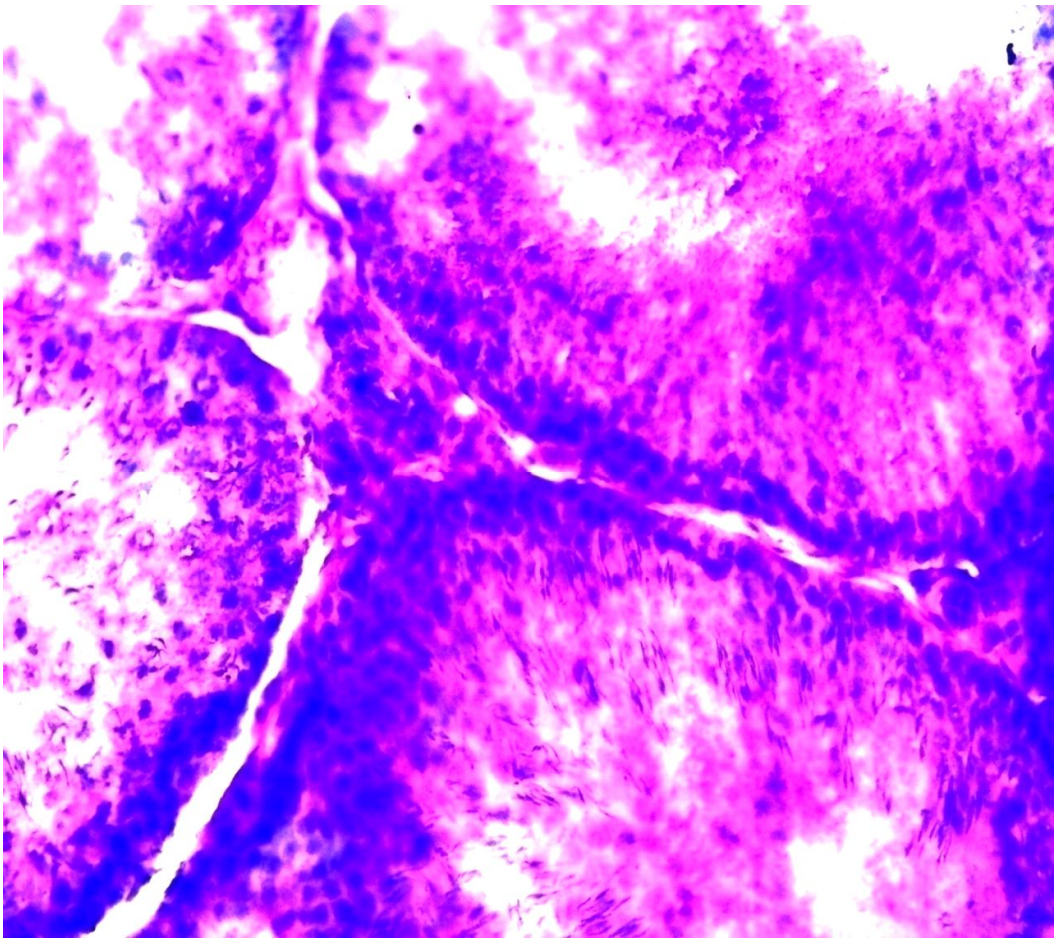


Plate 4.8 : Photomicrograph of Rat Testicular Cells. 200mg x 2wks. Enlargement of somniferous tubule. Mild edema. Congestion of connective tissue stroma.

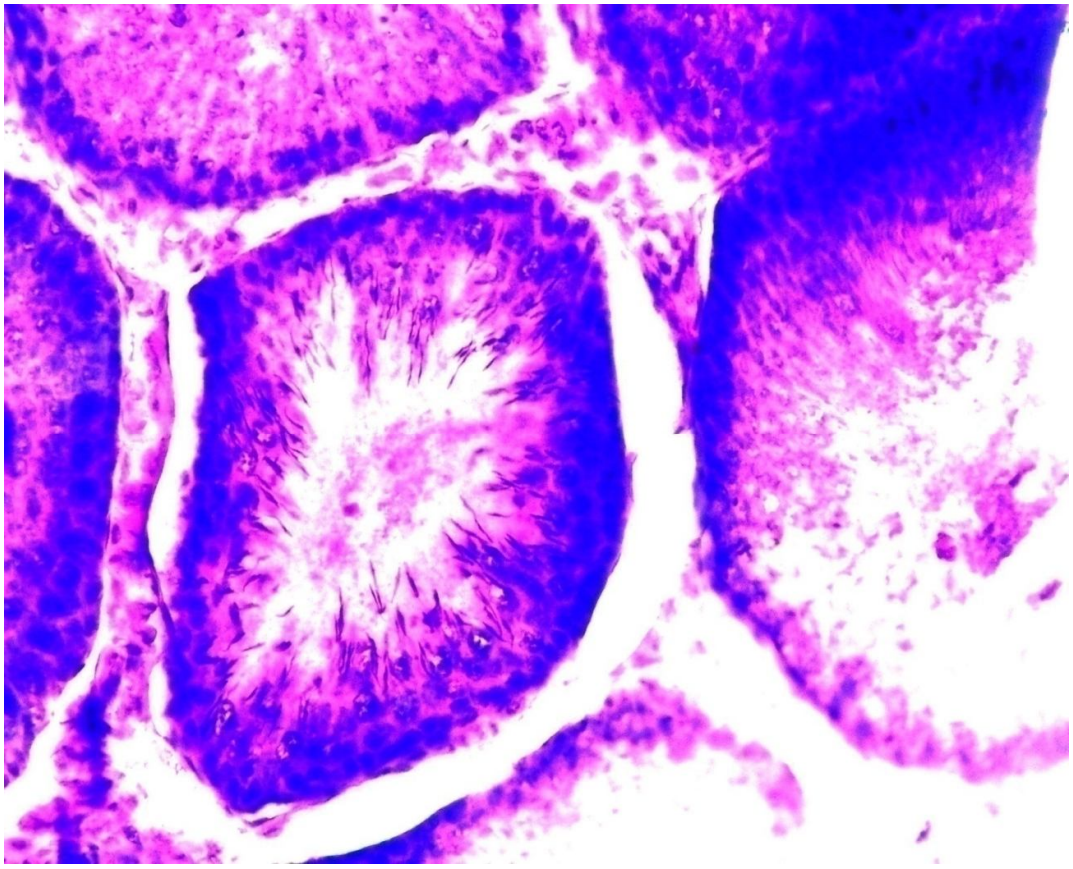


Plate 4.9: Photomicrograph of Rat Testicular Cells 50mg/kg x 4wks. Moderate architectural changes.

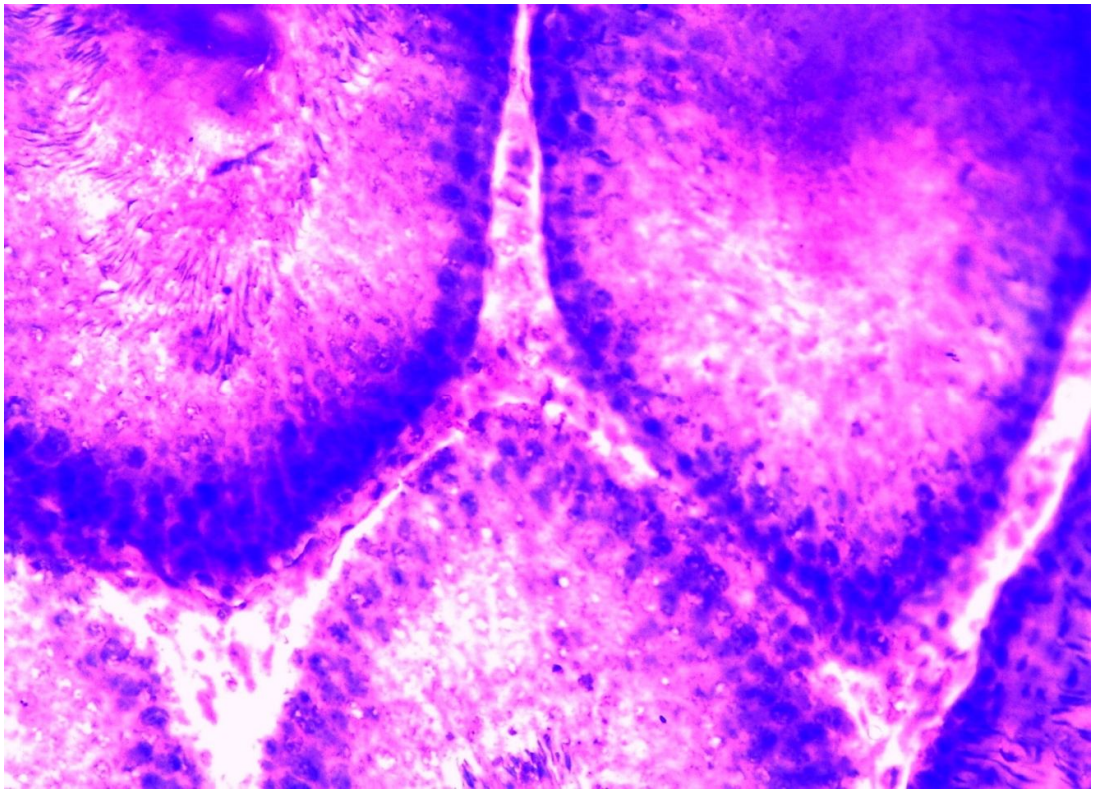


Plate 4.10: Photomicrograph of Rat Testicular Cells 200mg/kg x 4wks. Mild architectural changes.

Plate 4.11: Control. Photomicrograph of rat penile tissue. Penil tissue intact.

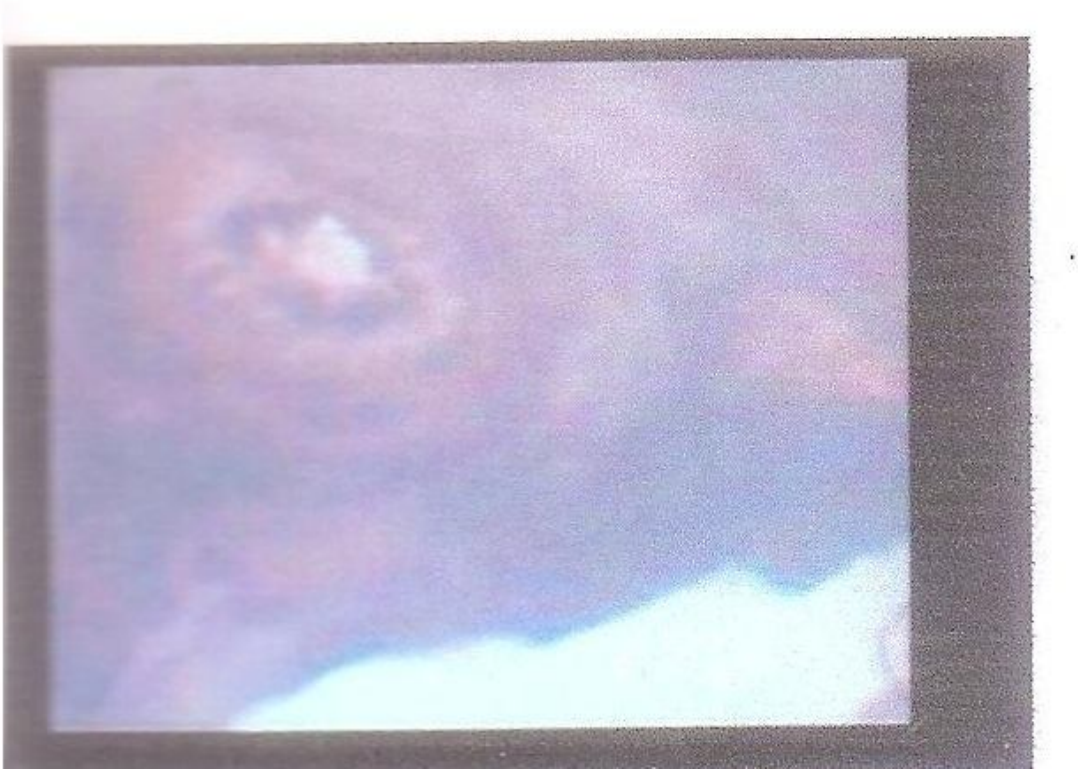


Plate 4.12 : Photomicrograph of past penile tissue. 200mg/kg x 4wks. No Change.

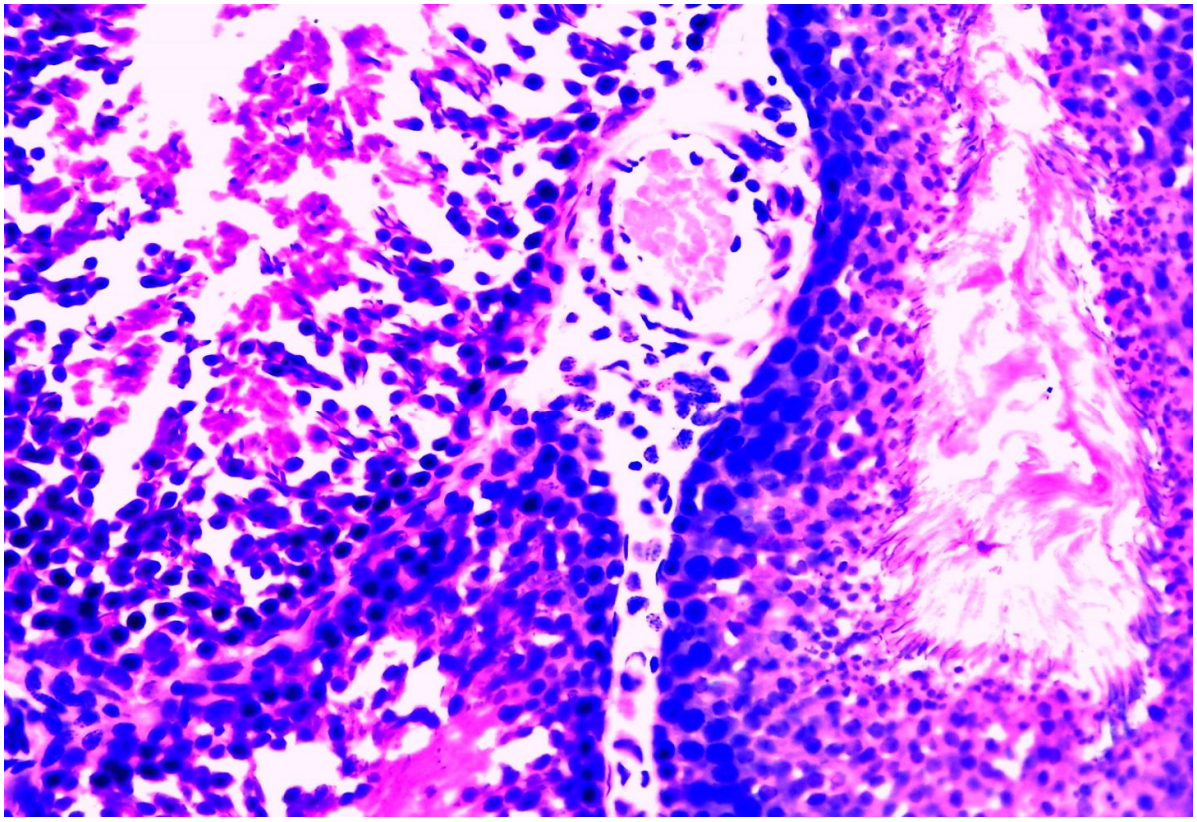


Plate 4.13: Control. Photomicrograph of rat epididymis. Epididymal tissue Intact.

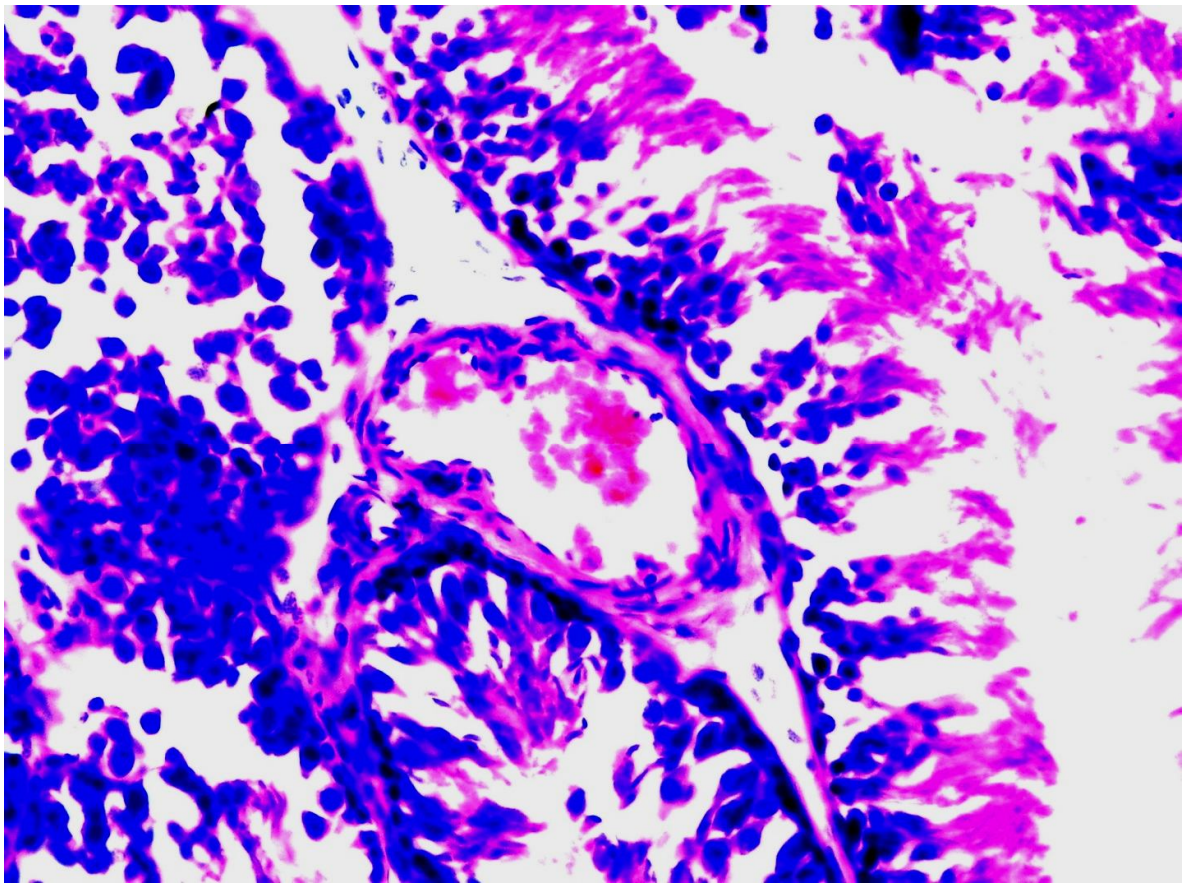


Plate 4.14 : Photomicrograph of rat epididymis. 200mg/kg x 4weeks.
No visible change.

4.1.4 VANILMANDELIC ACID CONCENTRATION IN PITUITARY GLAND OF RATS TREATED WITH EXTRACT OF *A. BOONEI* (n=5):

There was significant difference ($p < 0.05$) in the concentration of vanilmandelic acid in the brain tissue of rats treated with extract of *A. boonei* as observed on Fig. 4.11. At two weeks of treatment, the highest observed mean value of 3.32mg/ml was recorded from rats treated with 200mg/kg bw. The difference was significant ($p < 0.05$) when compared the 2.10 been in the control and mean from rats treated with 50mg/kg bw. The rats in the control segment showed the least mean concentration (2.10mg/ml).

At 4 weeks of treatment, the increase in the seum concentration of VMA was observed to increase in the same order as in the second week with the highest mean concentration of 4.07mg/ml observed from rats that received 200mg/kg bw of the extract of *A. boonei.* The photomicrographs of the histological presentations are shown below, (Plates 4.15-4.19).

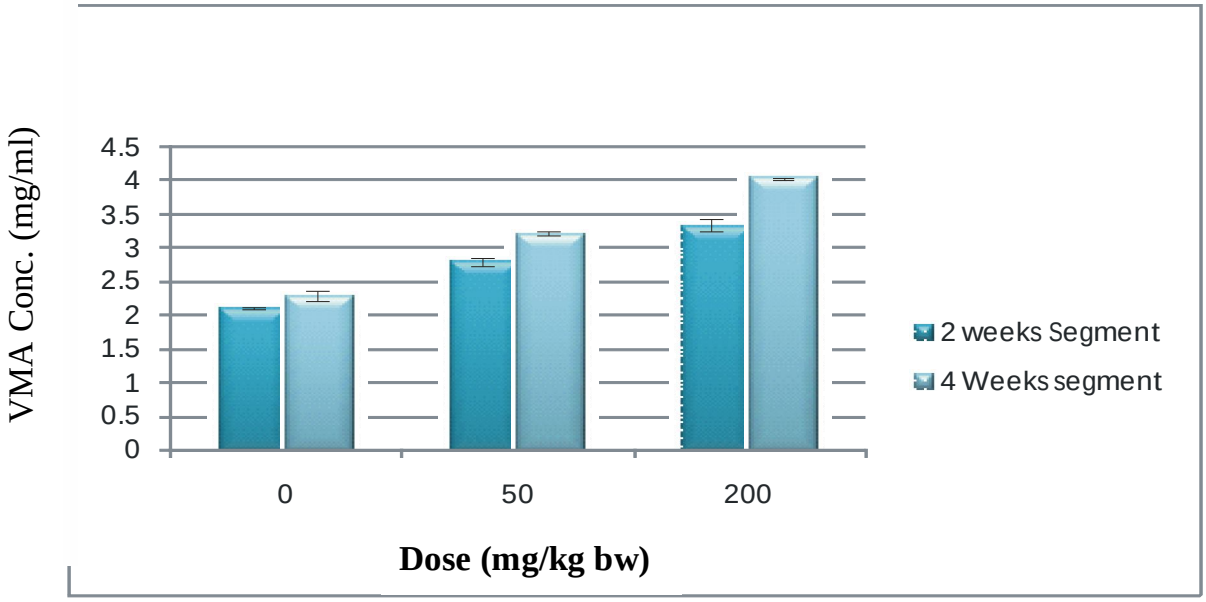


Fig. 4.11 Concentration of vanilmandelic acid in rat brain tissue following ABE administration.

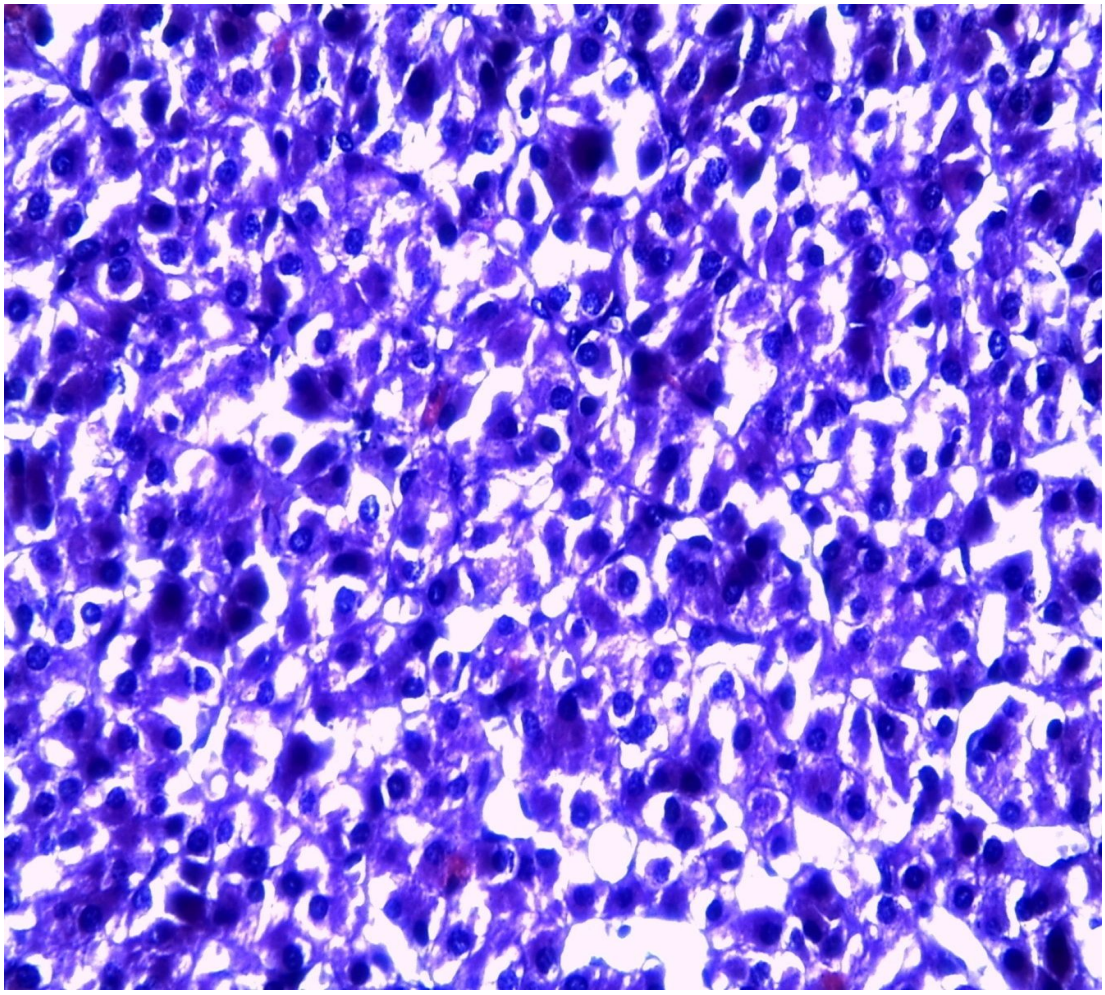


Plate 4.15: Control. Photomicrograph of male rat pituitary tissue. Intact regular Cells.

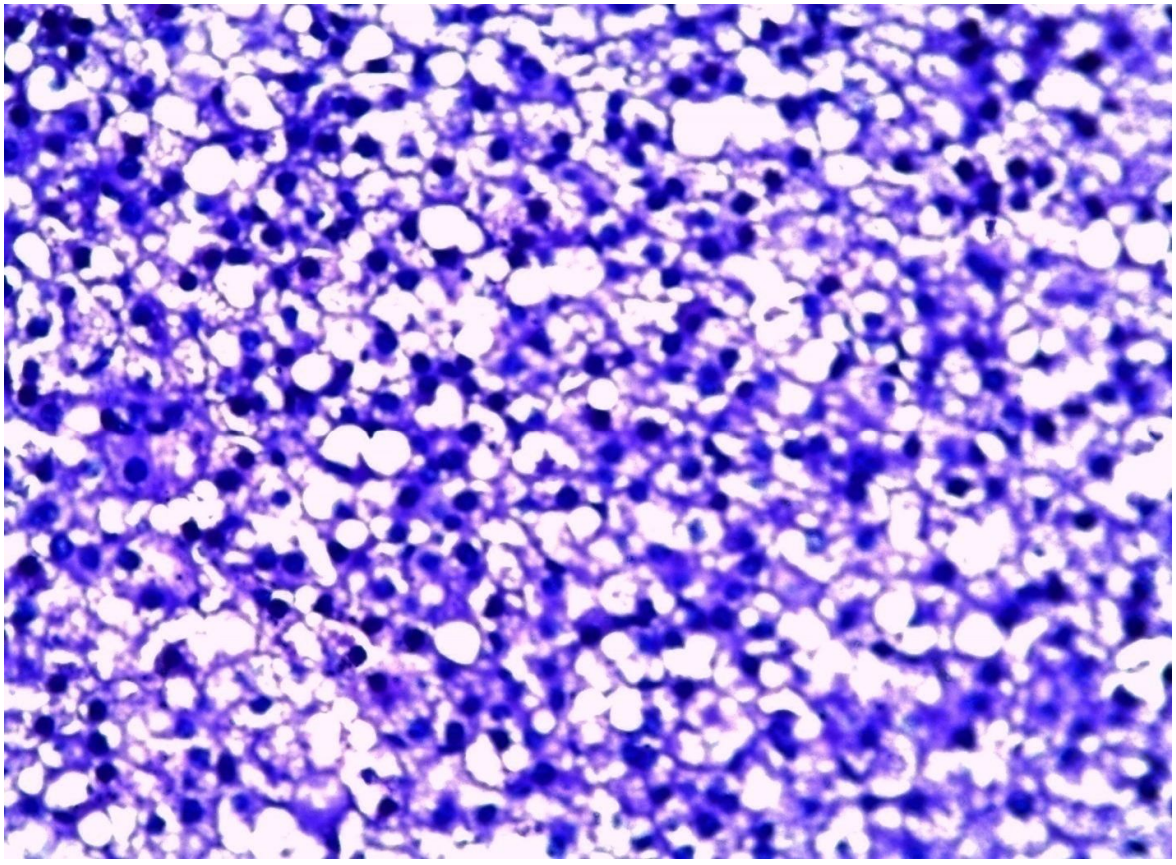


Plate 4.16: Photomicrograph of male rat pituitary tissue. 50mg/kg x 2wks. No visible cellular changes.

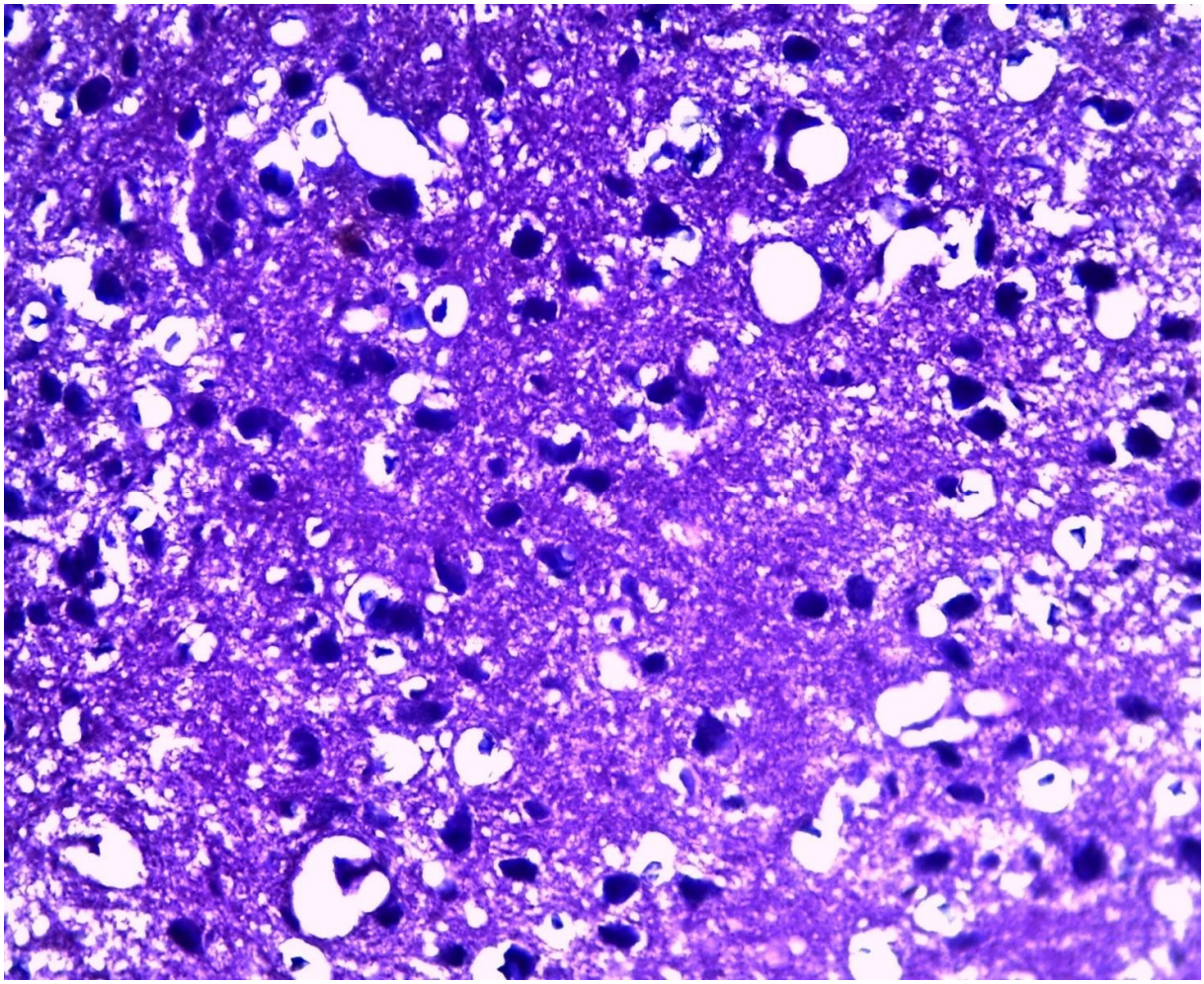


Plate 4.17: Photomicrograph of male rat pituitary tissue. 200mg/kg x 2wks. Cystically dilated spaces. Distortions of regular cellular architecture.

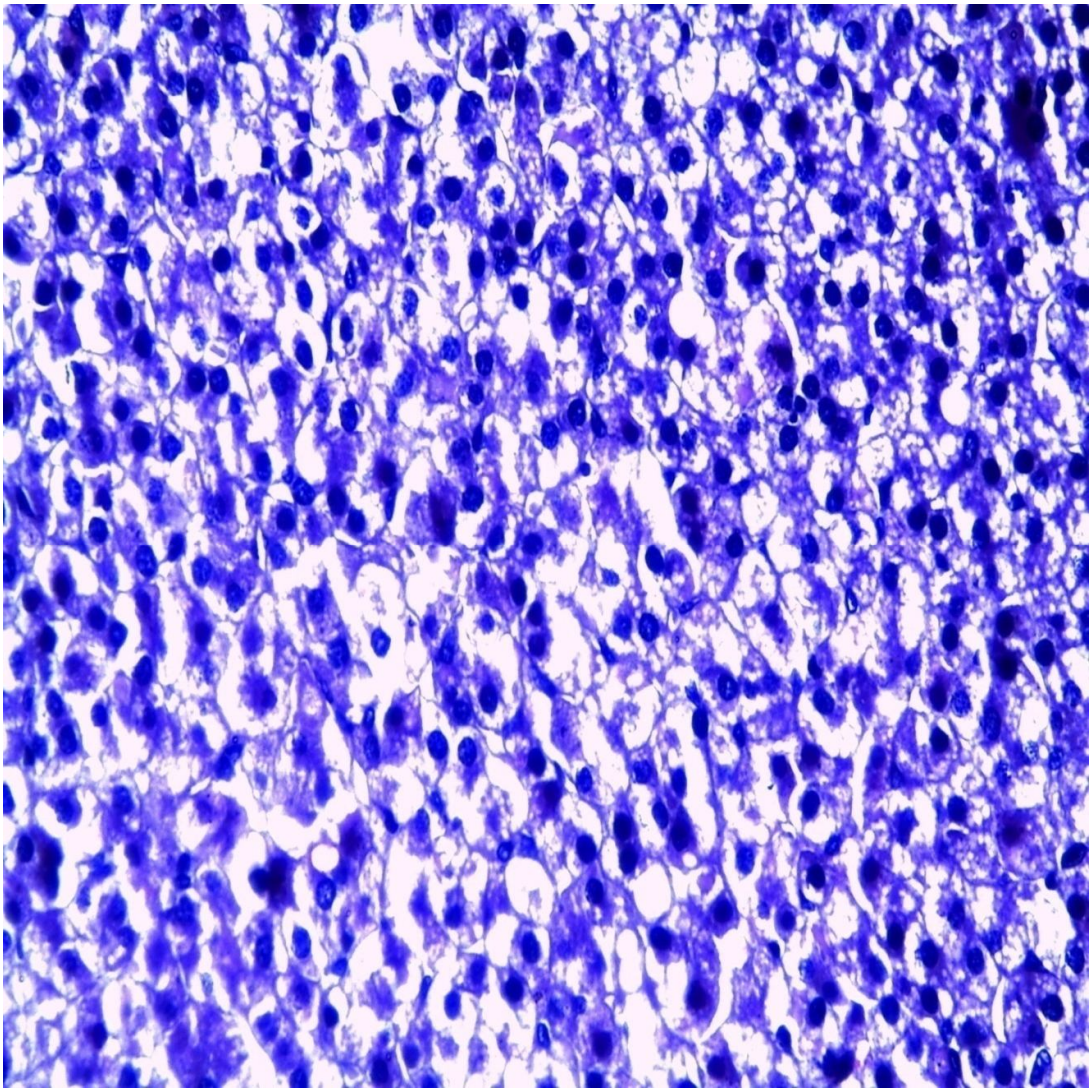


Plate 4.18: Photomicrograph of male rat pituitary tissue. 50mg/kg x 4wks. Crystically dialated spaces. Mild proliferation of tissue stroma.

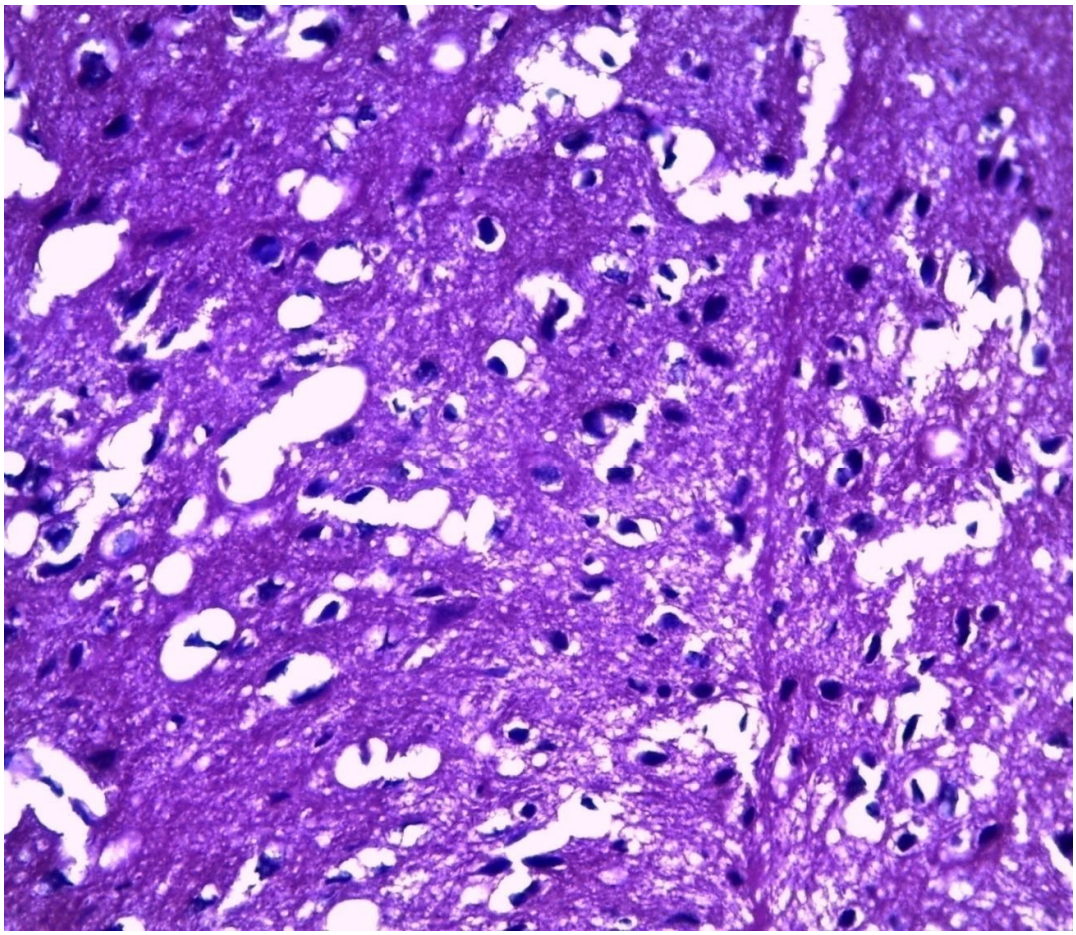


Plate 4.19: Photomicrograph of male rat pituitary tissue. 200mg/kg x4wks. Cystically dilated spaces. Proliferation of tissue stroma. Slightly enlarged cells.

4.1.5 EFFECT OF ABE ON SERUM GLUCOSE CONCENTRATION

The extract elevated the serum concentration of glucose in a dose dependent pattern (Fig.4.12).The rise was not significant ($p > 0.05$) at 100, 200 and 300mg/kg.

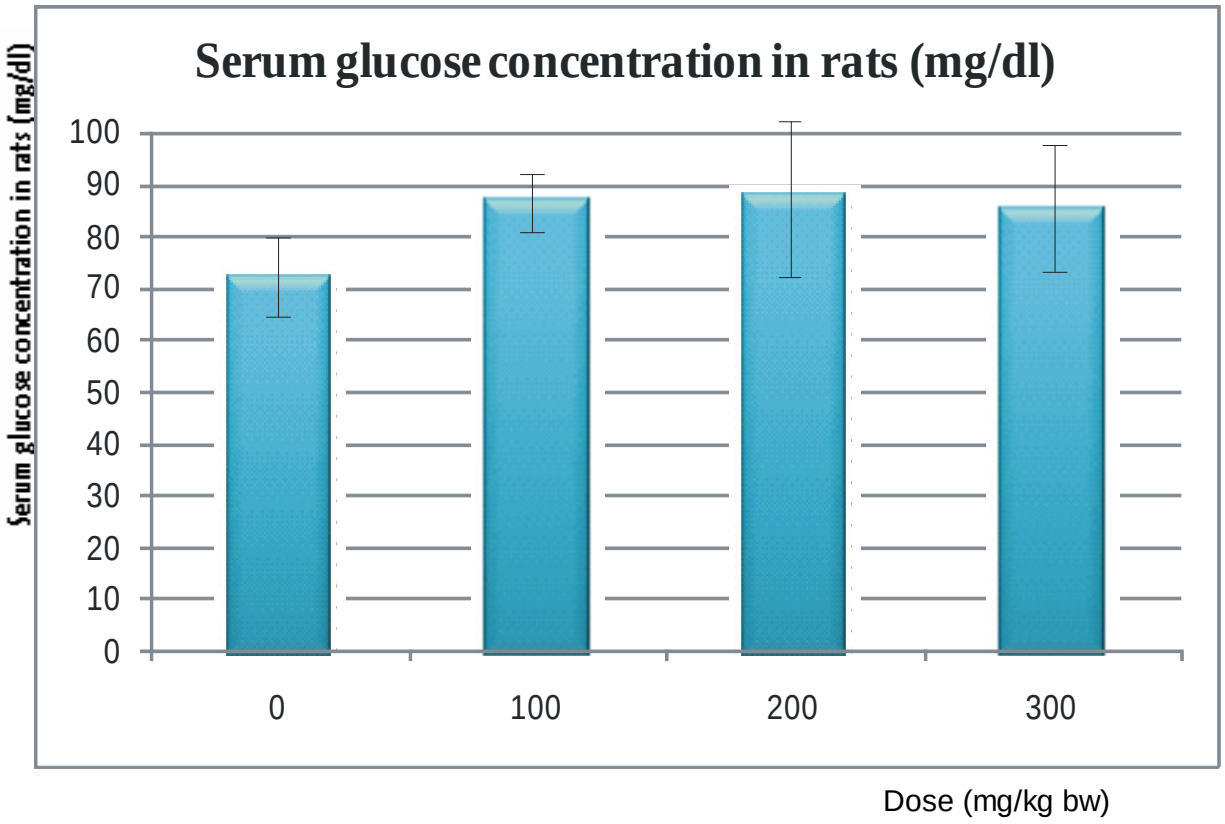


Fig. 4.12: Serum glucose level of rats treated with extract of *A. boonei.* The extract elevated serum glucose ($p > 0.05$) (n=5).

4.1.6 KIDNEY FUNCTION TEST:

4.1.6.1 The Effects of *A. boonei* Stem Bark Extract on the Renal Parameters:

The serum urea level was elevated following the administration of *A. boonei* stem bark extract. At two weeks, rats which received 200mg/kg, bw had the highest mean value (20.56mg/dl) of urea which was significantly different ($p < 0.05$) from the least mean value (12.64mg/dl) observed from the control. There was also a significant difference ($p < 0.05$) between the mean serum urea concentration observed from rats treated with 50mg/kg (15.2mg/dl) and those which received 200mg/kg. Again at 4 weeks, the rats which received 200mg/kg exhibited gave the highest mean value of serum urea of 33.2 mg/dl which was significantly different ($p < 0.05$) from the 28.24 mg/dl mean value obtained from the 50mg/kg group (Fig 4.13).

Treatment with 50mg/kg ABE produced no significant ($p>0.05$) effect on serum creatinine concentration at two weeks. The observed means were 0.30 mg/dl, 0.30mg/dl and 0.27mg/dl from rats which received 200mg/kg, 50mg/kg and control respectively. At 4 weeks, the serum creatinine was elevated significantly ($p<0.05$) in rats treated with 50mg/kg of the *A. boonei* extract. The mean value (0.44mg/dl) of creatinine observed from rats given 200mg/kg showed a significant difference ($p < 0.05$) from the control (Fig 4.14).

The serum Sodium concentration was not significantly affected in the rats at two weeks after *A. boonei* stem bark extract administration; however, the highest mean sodium concentration (50.0 mmol/l) was seen in rats in the control group while the least mean sodium level (48.2 mmol/L) was observed from rats which received 200mg/kg for two weeks (Fig 4.15). In the 4^{th} week, rats given 50 mg/kg bw of the *A. boonei* stem bark extract, gave the lowest mean serum sodium concentration (45.3 mmol/L) which was not significantly different ($p > 0.05$) from the 48.24 mmol/L observed from rats which received 200mg/kg. (Fig. 4.15).

Conversely, rats in the 2 weeks segment which received 50mg/kg of ABE gave (2.3 mmol/L) mean serum value of potassium, which was not significantly different when compared with 2.13 mmol/L observed in the control. The rats which received 200mg/kg bw for 2 weeks showed serum value of 2.8mmol/L (Fig 4.16). By the fourth week, at 200mg/kg, bw, the serum potassium value was 3.3mmol/L, which was significantly different ($p<0.05$) from the control.

The bicarbonate level in the rats was significantly raised ($p < 0.05$) by the extract of ABE in the 2^{nd} and 4^{th} weeks (Fig. 4.17). At 2 weeks, the least (4.9 mmol/L) mean value was observed from the control while the highest (8.8mmol/L) mean value was observed from rats given 50mg/kg bw. The pattern of the result was similar in the 4^{th} week where the serum bicarbonate concentration were 6.5 and 6.2mmol/L for 50 and 200mg/kg against a control value of 4.7mmol/L (Fig. 4.17).

The chloride ion concentration observed from rats which received 50mg/kg was highest with 36.56 mmol/L but not significantly different from the 36.54 mmol/L mean observed from rats given 200mg/kg. Both were not significantly different ($p > 0.05$) from the control. The rats given 200mg/kg for 4 weeks had the highest mean value of chloride ion with 41.2 mmol/L, which was significantly different ($p < 0.05$) from the control (Fig 4.18). The urea/creatininine ratio was also significantly raised ($p < 0.05$) (Fig. 4.19).

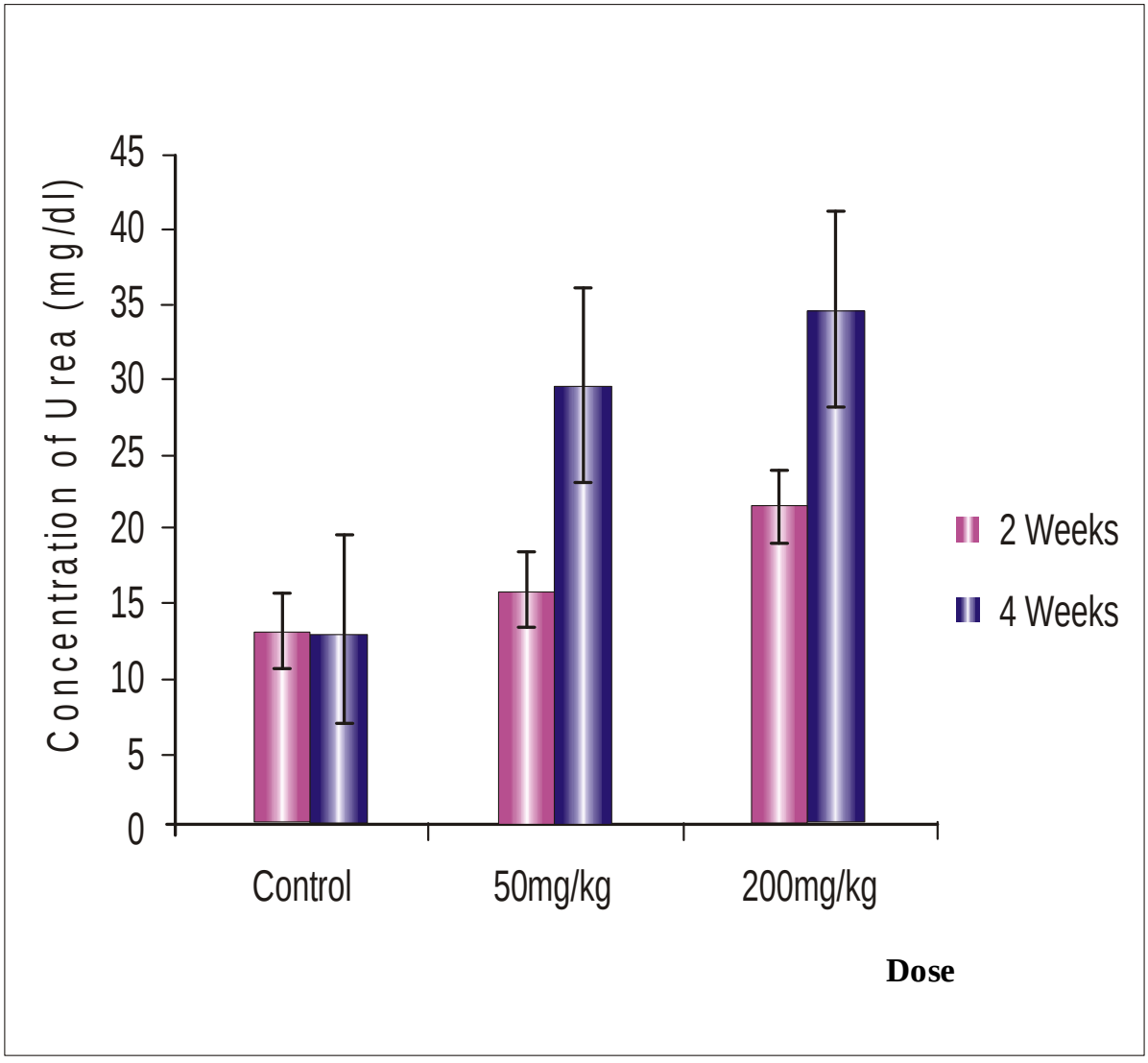


Fig.4.13: Serum urea concentration in rats treated with extract of *A. boonei.* The serum urea concentration was raised dose and time dependently.

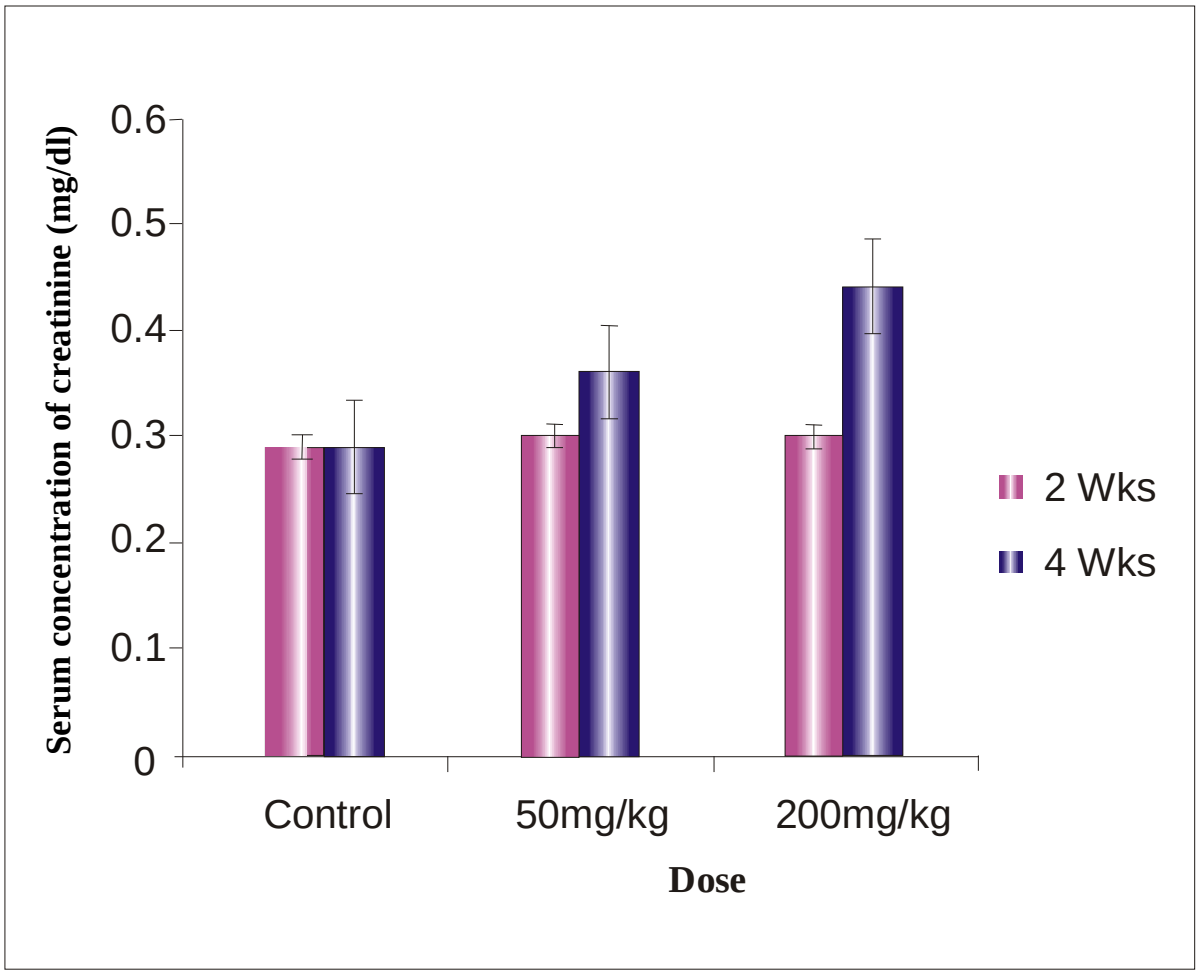


Fig. 4.14: Serum creatinine concentration in rats treated with extract of *A. boonei* stem bark. The concentration of creatinine increased significantly ($p<0.05$) at the higher dose.

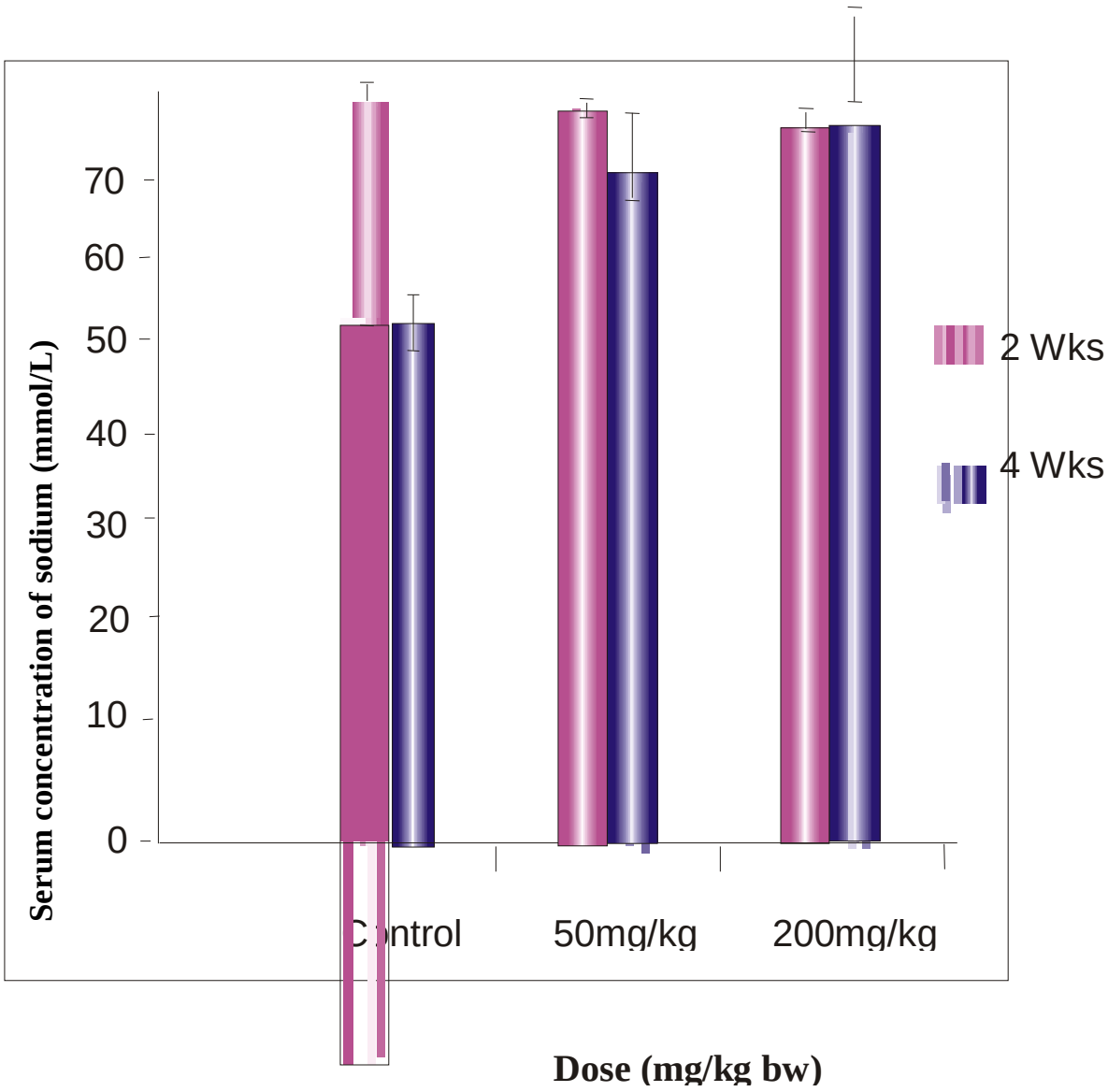


Fig.44: Serum Sodium concentration of rats treated with extract of *A. boonei*

The extract reduced the serum concentration of sodium non-significantly ($p>0.05$).

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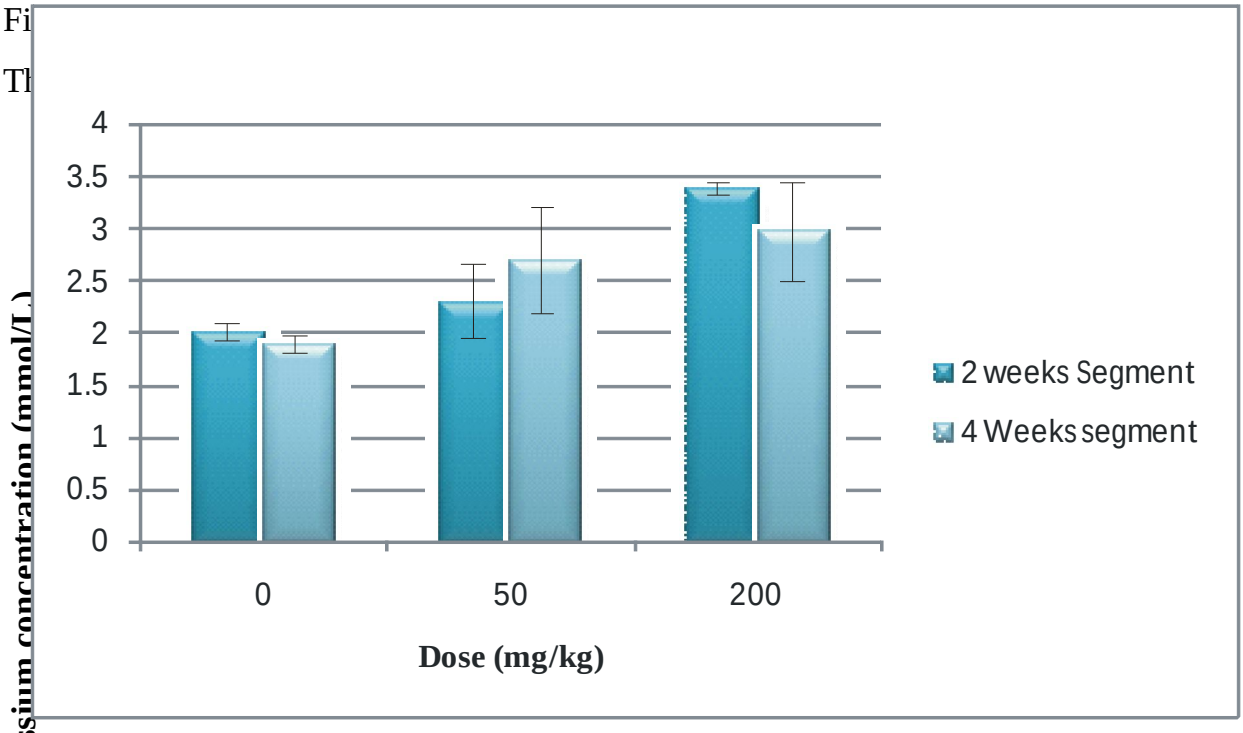

Serum potassium concentration (mmol/L)

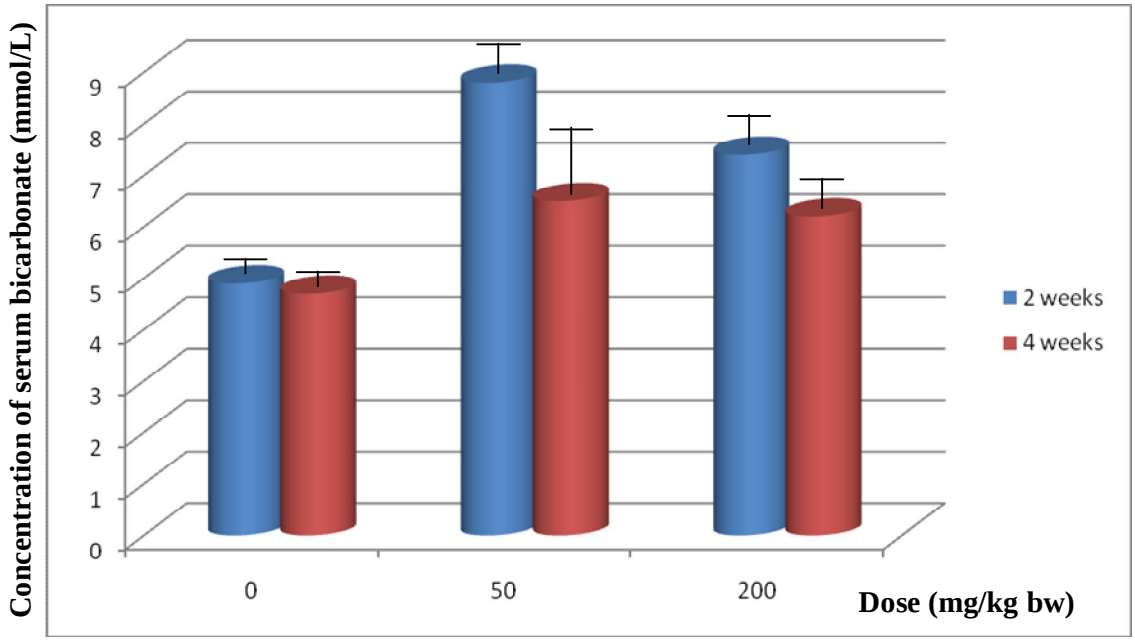

Fig.4.17: Serum bicarbonate concentration of rats treated with extract of *A. boonei*. The serum bicarbonate concentration was reduced significantly ($p<0.05$) ($n = 5$).

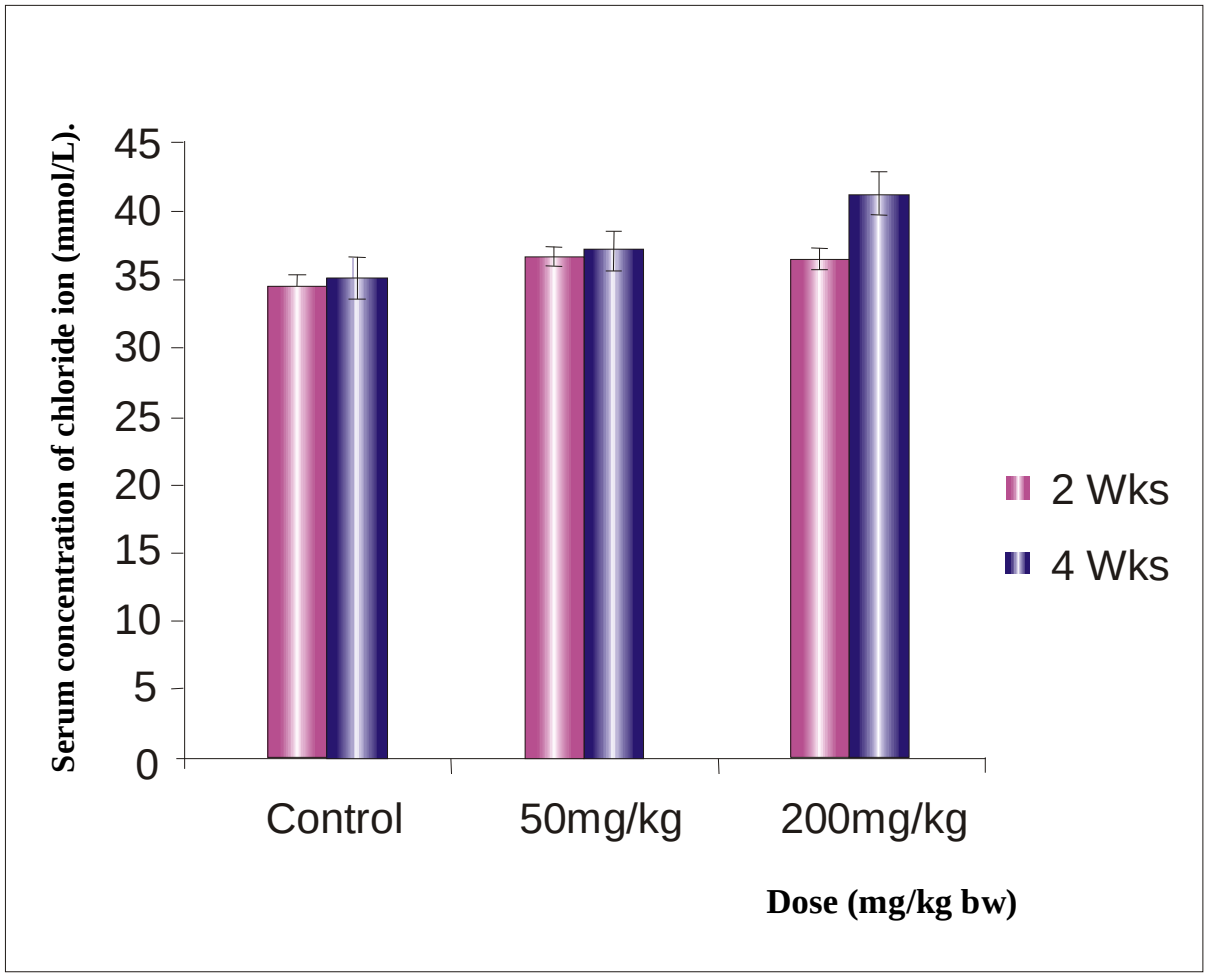

Fig. 4.18: Serum chloride concentration of rats treated with extract of *A. boonei*. The level was increased ($p>0.05$) ($n = 5$).

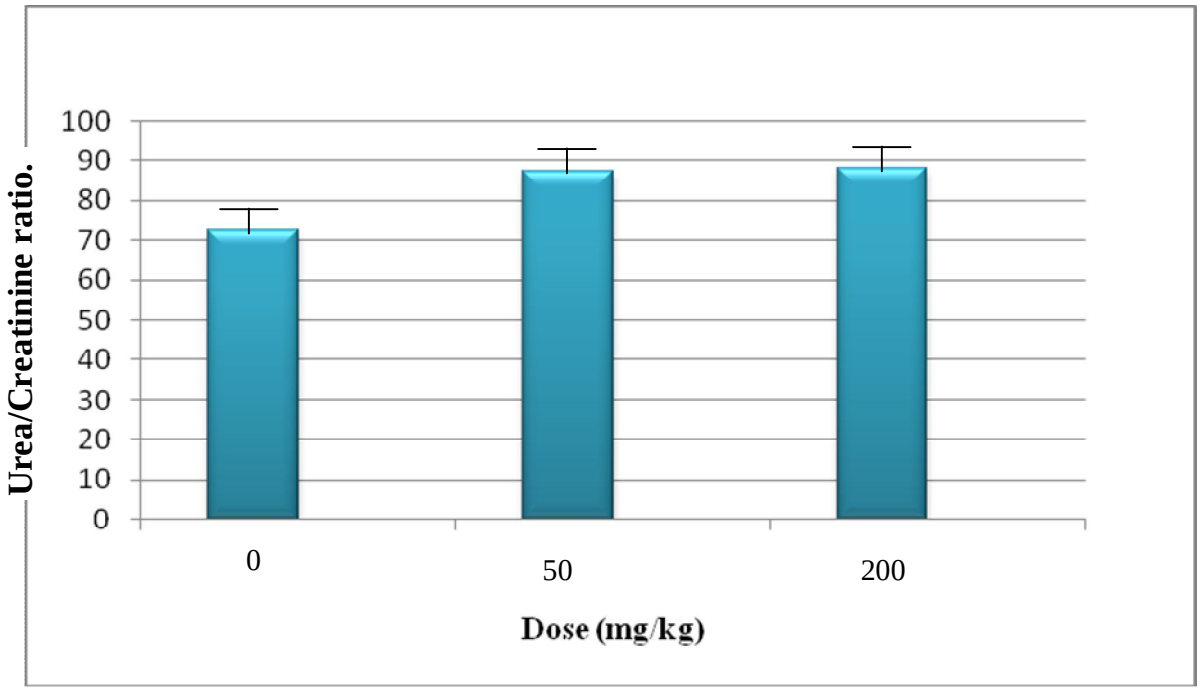

Fig 4.19: The urea/creatinine ratio was also elevated at both doses and duration.

Photomicrograph of Kidney Cells

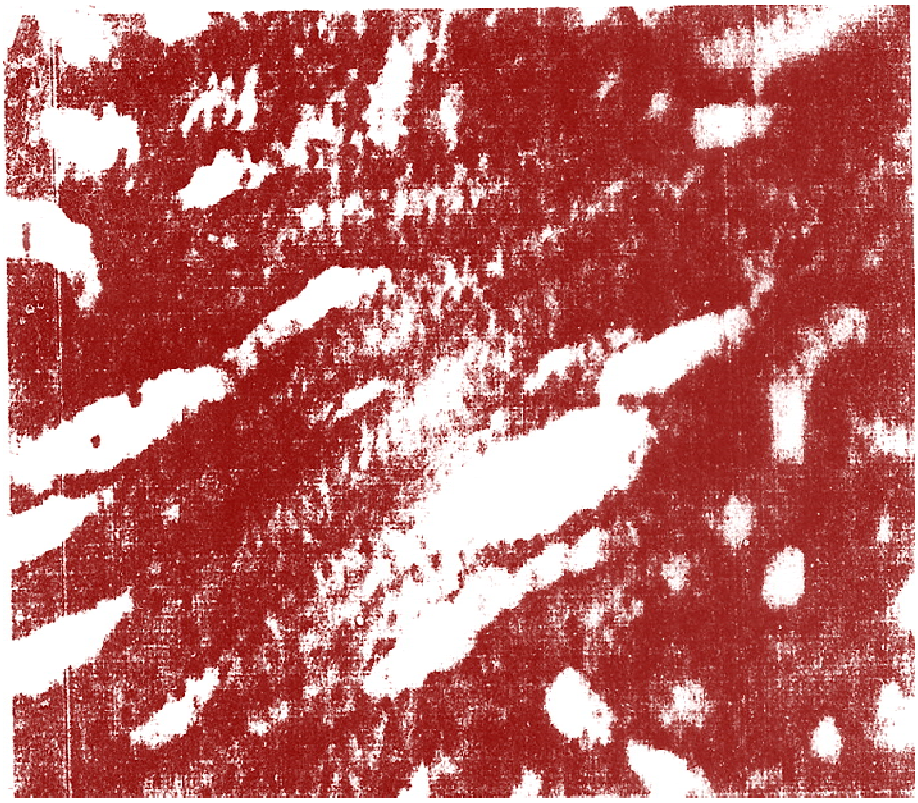

Plate 4.20: Control. Photomicrograph of renal tissue. Normal cellular presentation.

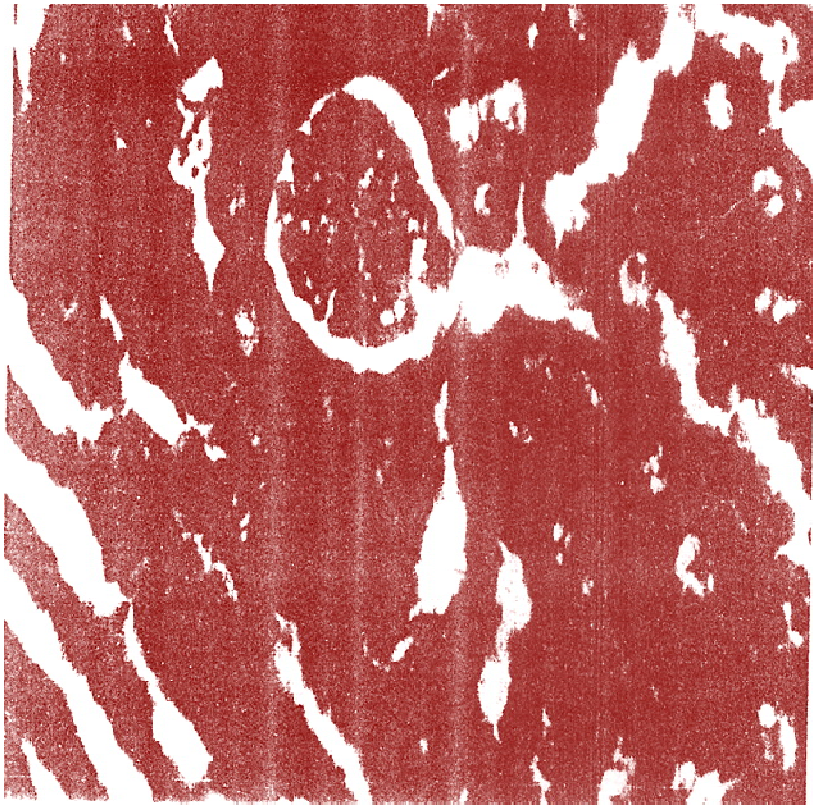

Plate 4.21: Photomicograph of renal tissue treated with 50mg/kg x 2wks. Mild Oedema.

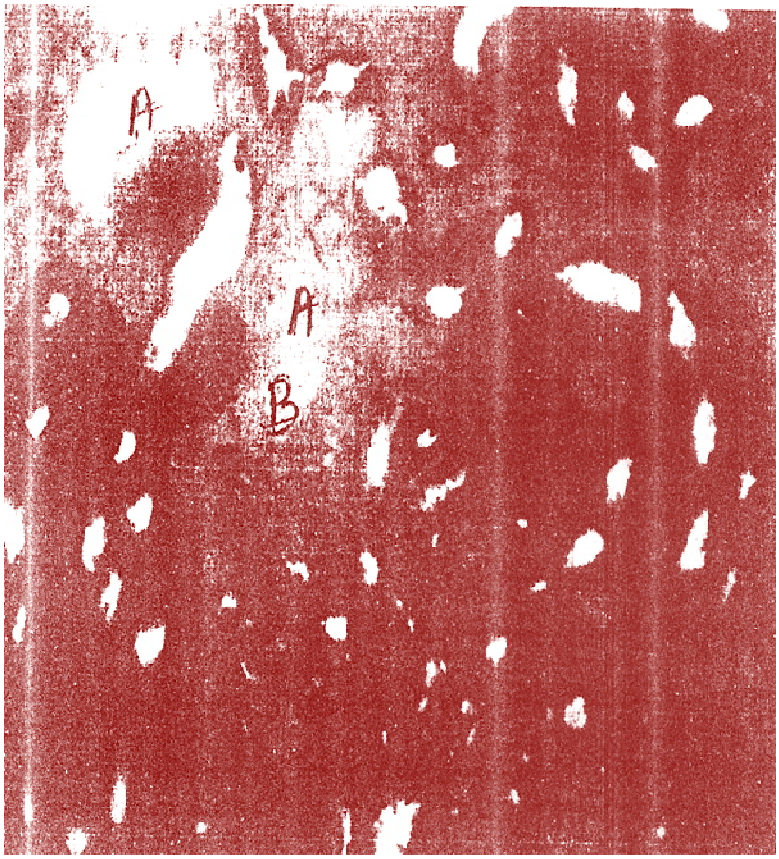

Plate 4.22: Photomicograph of renal tissue treated with 200mg/kg x 2wks.

A. Mild Oedema.
B. Moderate hyalinization.
C. Moderate increase in size of endothelial and mesothelial cells.

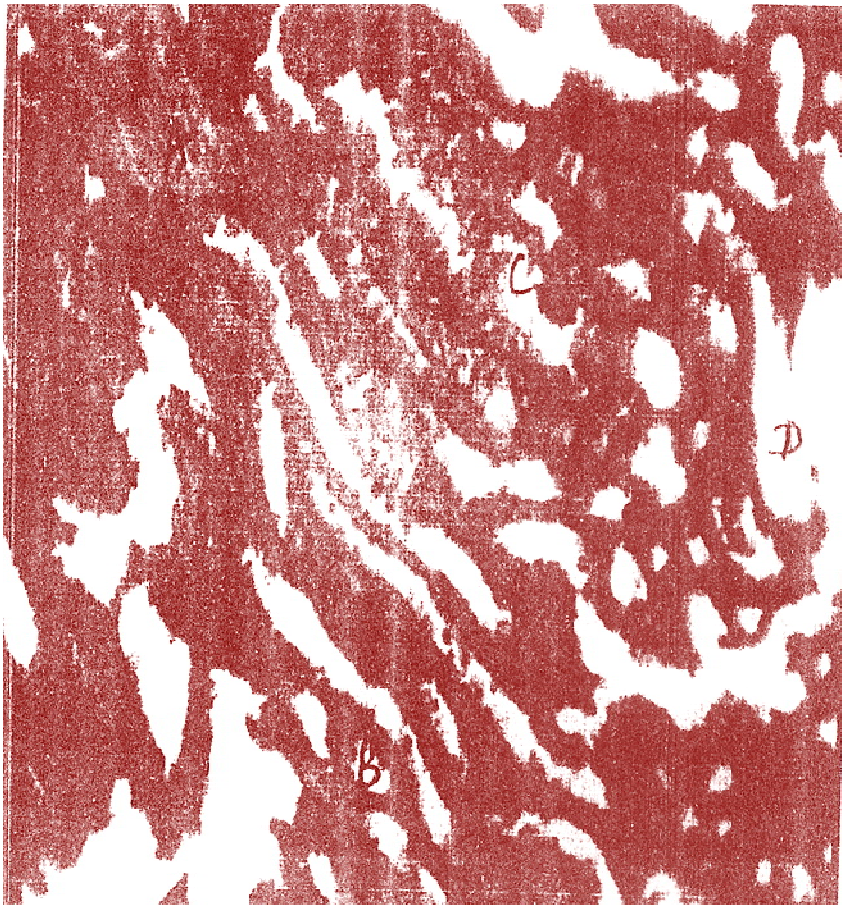

Plate 4.23: Photomicograph of renal tissue treated with 50mg/kg x 4wks.

A. Oedema.
B. Moderate Vacuolation of the Cytoplasm.
C. Moderate Shrunken Glomeruli.
D. Moderate Thickening of the Basement Membrane of Bowman's Capsule.

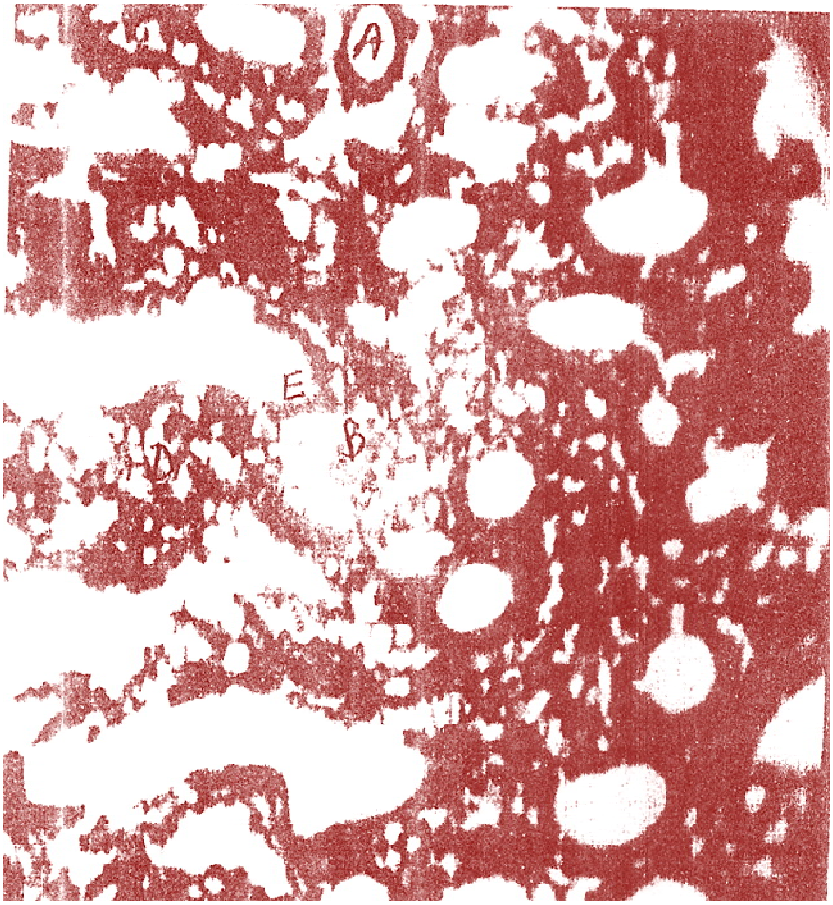

Plate 4.24 : Photomicograph of renal tissue treated with 200mg/kg x 4wks.

A. Enlarged lobule
B. Infiltration of glamerular tuft with eosinophilic material.
C. Foci necrosis and disruprion of the capillary loops.
D. Shrunken glomerular
E. Vacuolated cytoplasm
F. Thickening of the basement membrane of Bowman’s capsule.
G. Increase in number and size of the endothetlial and mesoglial cells.

Table 4.4b: Summary of Histological Presentations of Renal Tissues Treated with Ethanol Extract of *A. boonei*

2 WEEKS SEGMENT:

0.0mg/kg (control)	Normal Cellular Architecture.
50mg/kg	Mild Oedema observed.
200mg/kg	• Mild Oedema. • Moderate hyalinization. • Moderate increase in the size of endothelial and mesothelial cells.

4 WEEKS SEGMENT:

0.0mg/kg (control)	Normal Cellular Architecture.
50mg/kg	• Oedema. • Moderate vacuolation of the cytoplasm. • Shrunken glomeruli. • Thickening of the basement membrane of Bowman's capsule.
200mg/kg	• Elongated lobule, infilteration of glomeruli tuft with eosinophillic material. • Focal necrosis and disruption of capillary loop. • Shrunken glomeruli (severe). • Vacuolated cytoplasm. • Thickened basement membrane of Bowman's capsule.

4.1.7 LIVER FUNCTION TEST:

4.1.7.1 Effects of *A. boonei* Stem Bark Extract Treatment on Liver Function:

Alstonia boonei treatment significantly affected the liver marker enzymes. The plasma concentratons of AST, ALT and ALP at two weeks increased with increase in the dose of the extract.

With 200mg/kg, bw the serum concentration of AST recorded was 28.0 IU/L in 2 weeks, which was significantly different ($p<0.05$) from the least value of AST (8.60 IU/L) recorded from the control. The 28.0 IU/L was the highest and was also significantly different from the 15.0 IU/L recorded from rats which received 50 mg/kg for two weeks (Fig 4.20). At 4 weeks of ABE treatment, the serum concentration of AST was elevated to 70.41U/L at 200mg/kg bw. This was significantly different from the 26.6 IU/L concentration of AST recorded from rats which received 50mg/kg *A. boonei* stem bark extract and from the control (Fig 4.20).

The highest level of ALT (16.8 IU/L) recorded at two weeks was caused by the administration of with 200mg/kg bw of the extract. This value was significantly different ($p<0.05$) from the 7.2 IU/L record from the control. Similarly, the 15.6 IU/L recorded from rats which received 50mg/kg of *A. boonei* stem bark extract was significantly different from the highest ALT in two weeks (Fig 4.21).

In a similar trend, the rats which received 200mg/kg for four weeks gave the highest (29.2 IU/L) concentration of ALT which was significantly different from the 26.6 IU/L recorded from rats which received 50mg/kg (Fig 4.21).

At two weeks and four weeks after *A. boonei* administration, the serum ALP was elevated to 91.0 ± U/L at 200mg/kg. These values were significantly different from the value obtained from rats treated with 50 mg/kg bw ABE which were 69.2 IU/L and 88.4 IU/L for two weeks and four weeks respectively (Fig4.23).

On the other hand, the concentration of total bilirubin (TB) in rats which received *A. boonei* stem bark extract were not significantly different ($p > 0.05$) from the control at 50mg/kg. However, the value was significantly different ($p < 0.05$) at 200mg/kg by the 4^{th} week (Fig. 4.23). The highest value was 0.91mg/dl against a control value of 0.73mg/dl observed from rats given 200mg/kg of the extract (Fig 4.23).

The mean serum concentration of 0.4mg/dl conjugated bilirubin (CB) was obtained from rats which received 200mg/kg of *A. boonei* stem bark extract against a control value of 0.37 mg/dl at 4 weeks. But this was not statistically significant ($p>0.05$) (Fig 4.24). The pattern was similar for the two weeks segments with 0.38mg/dl against a conrol value of 0.39mg/dl for rats which received 200mg/kg (Fig 4.24).

The serum concentration of unconjugated bilirubin (UB) in the rats was significantly increased ($p<0.05$) by the extract at the 4^{th} week from a control value of 0.36mg/dl to 0.61mg/dl. At 2 weeks, 0.38mg/dl was obtained for 200mg/kg bw of extract, against a control value of 0.37mg/dl. There was no significant difference in the serum concentration of UB in rats which received 50mg/kg bw for both segments (0.35 and 0.36mg/dl) in 2 weeks (Fig 4.25). The photomicrographs of the control liver cells and the treated ones are shown below in plates 4.25-4.29, Table 4.4c.

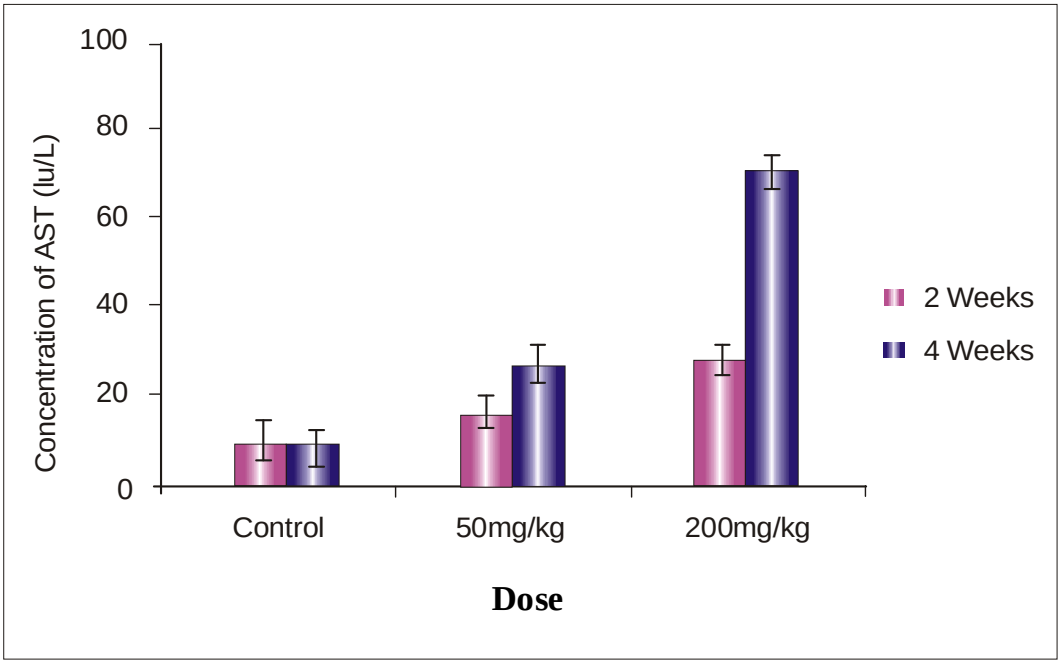

Fig 4.20: Serum aspartate transaminase activity of rats treated with *A. boonei*.The enzyme level increased significantly ($p<0.05$) at 200mg/kg by the 4th week.

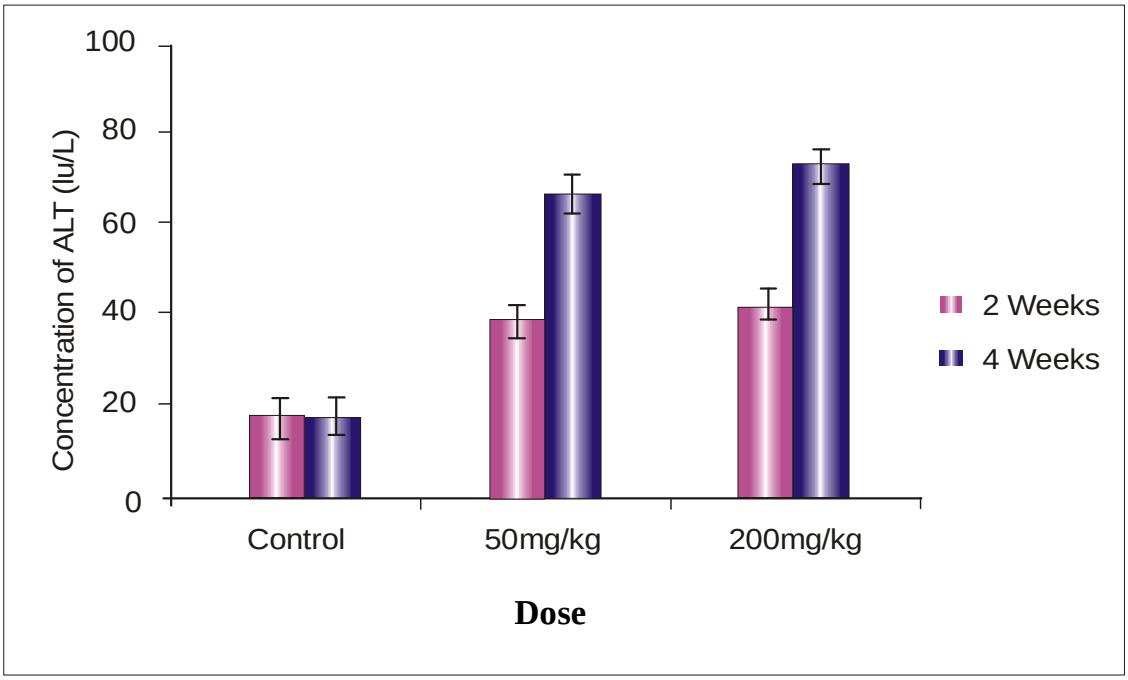

Fig. 4.21: Serum alanine transaminase activity of rats treated with extract of *A. boonei*. The enzyme level was significantly ($p<0.05$) elevated at both doses and period.

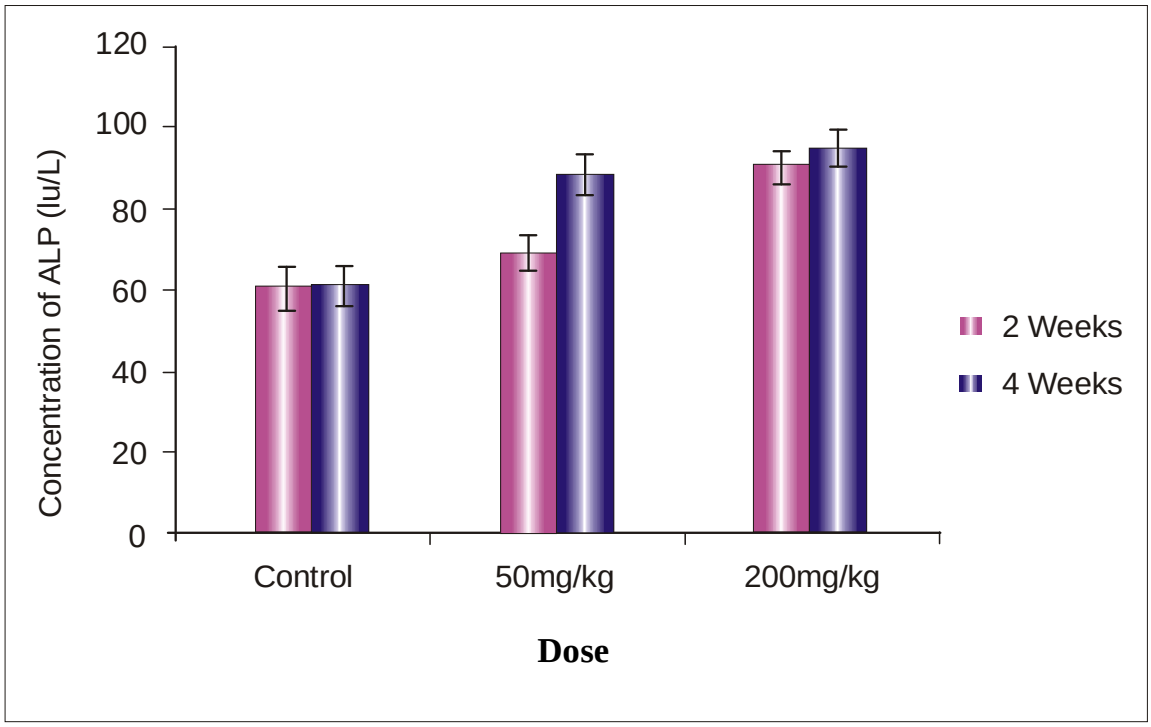

Fig. 4.22: Serum alkaline phosphatase activity of rats treated with extract of *A. boonei*. The enzyme activity rose significantly in both doses and period of administration.

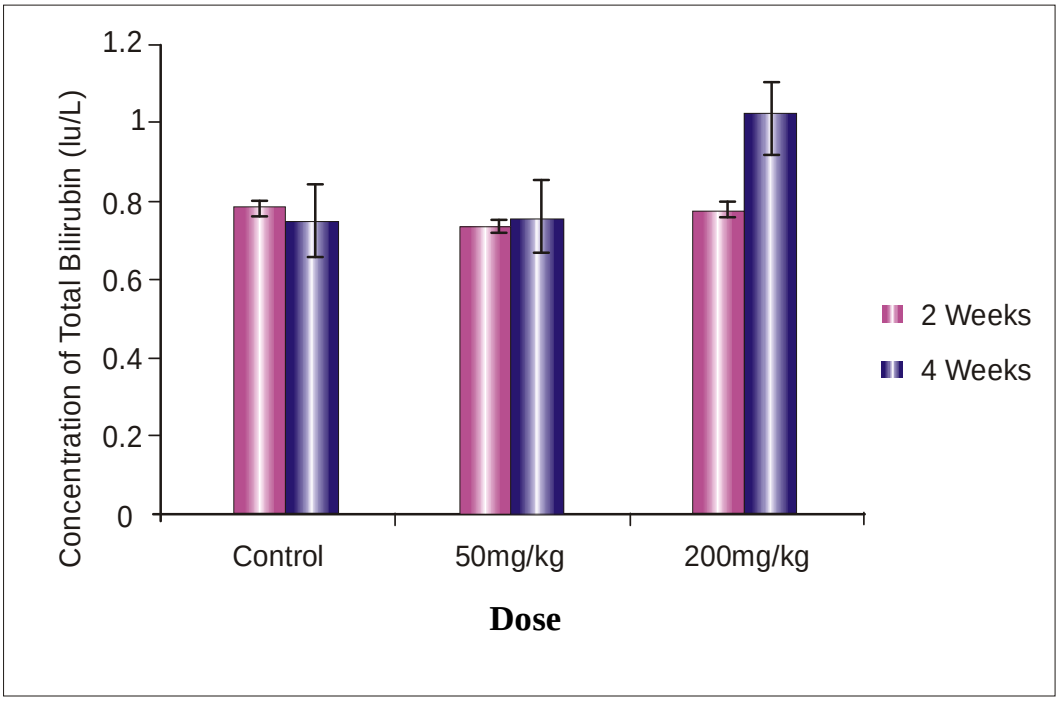

Fig. 4.23: Serum total bilirubin concentration of rats treated with extract of *A. boonei.* Total bilirubin concentration was elevated at higher dose and 4th wk period.

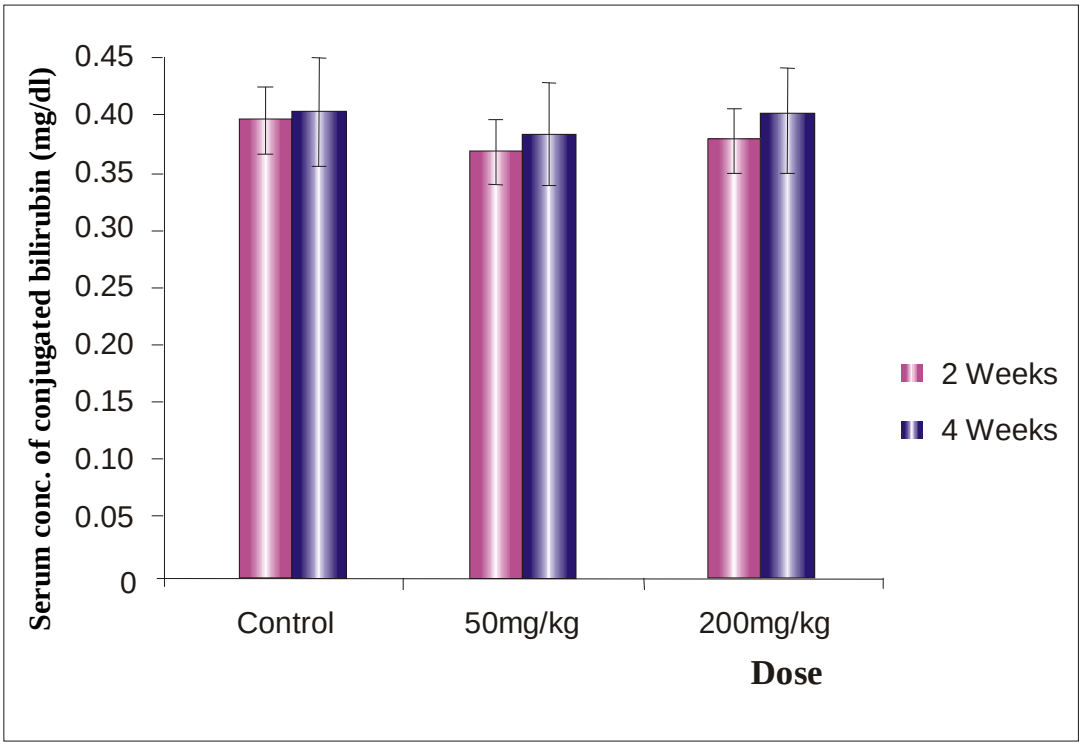

Fig.4.24: Serum conjugated bilirubin concentration of rats treated with *A. boonei.* There was no significantly ($p>0.05$) change in the serum concentration of conjugated bilirubin.

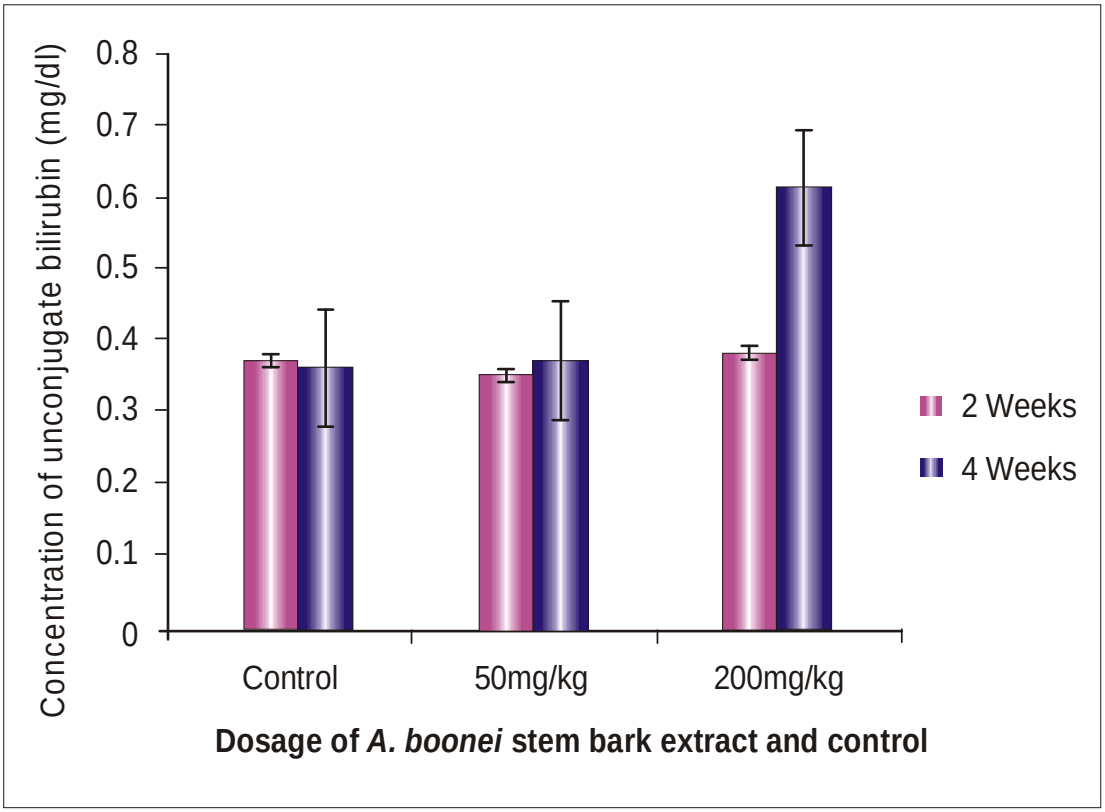

Fig 4.25: Concentration of serum unconjugated bilirubin in rats treated with extract of *A.boonei.* The level rose at higher concentration of the extract in the 4th week.

Photomicrograph of Liver Cells

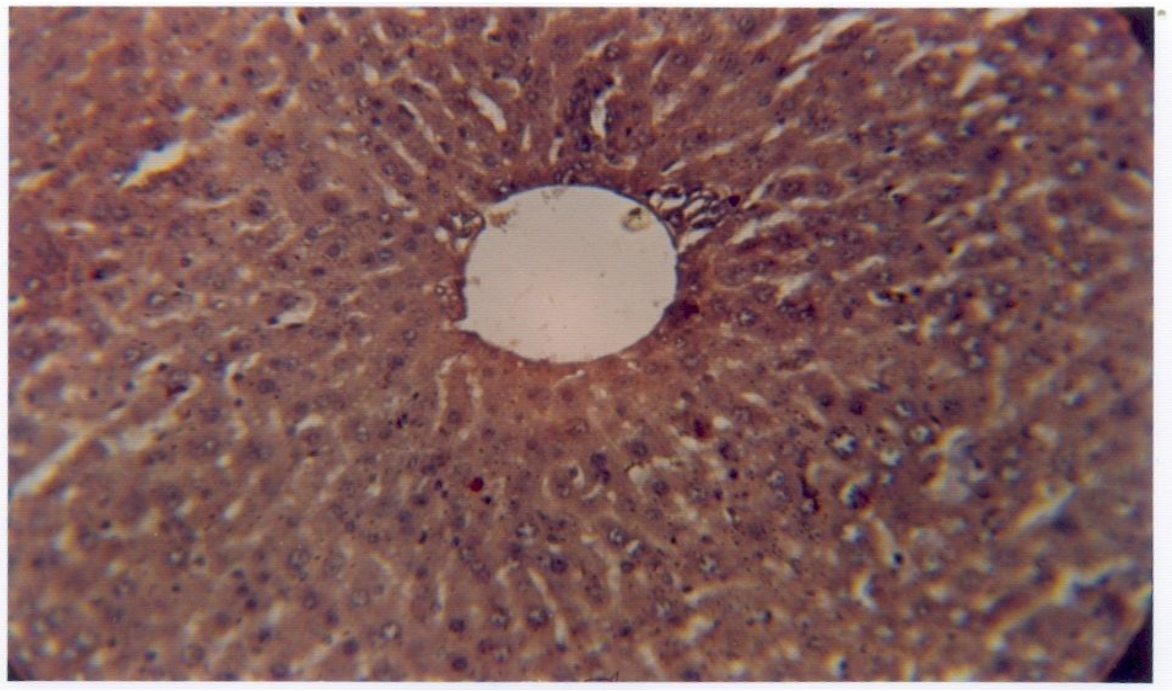

Plate 4.25: Photomicrograph of liver cell treated with *A. boonei* extract.
Control: Normal hepatocytes.

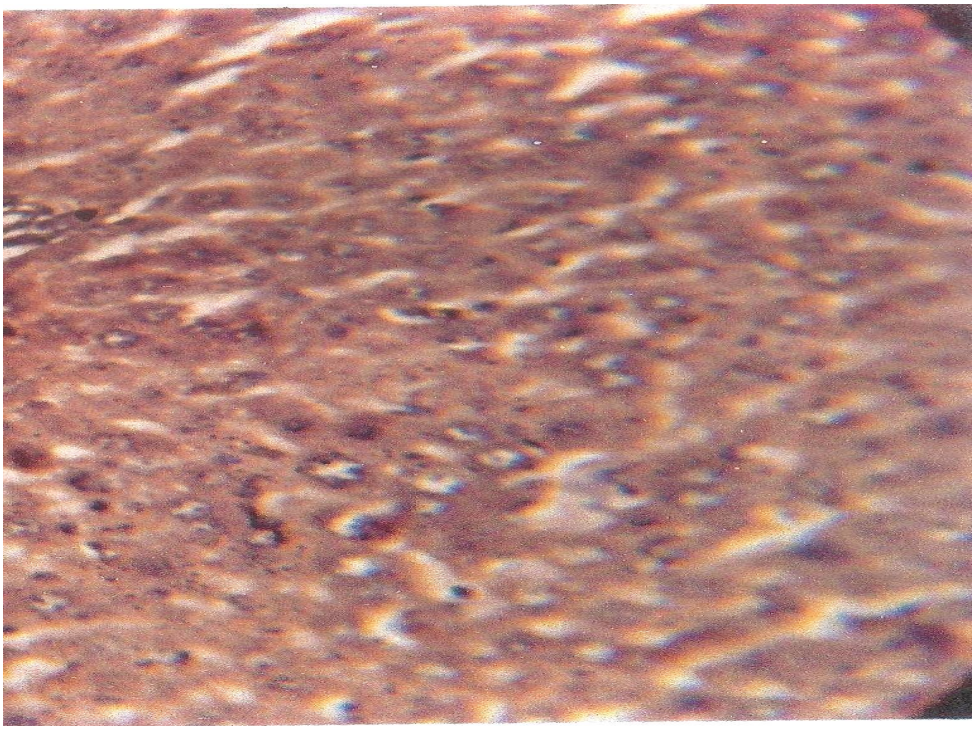

Plate 4.26 : Photomicrograph of liver cell treated with *A. boonei* extract. 50mg/kg x 2wks.

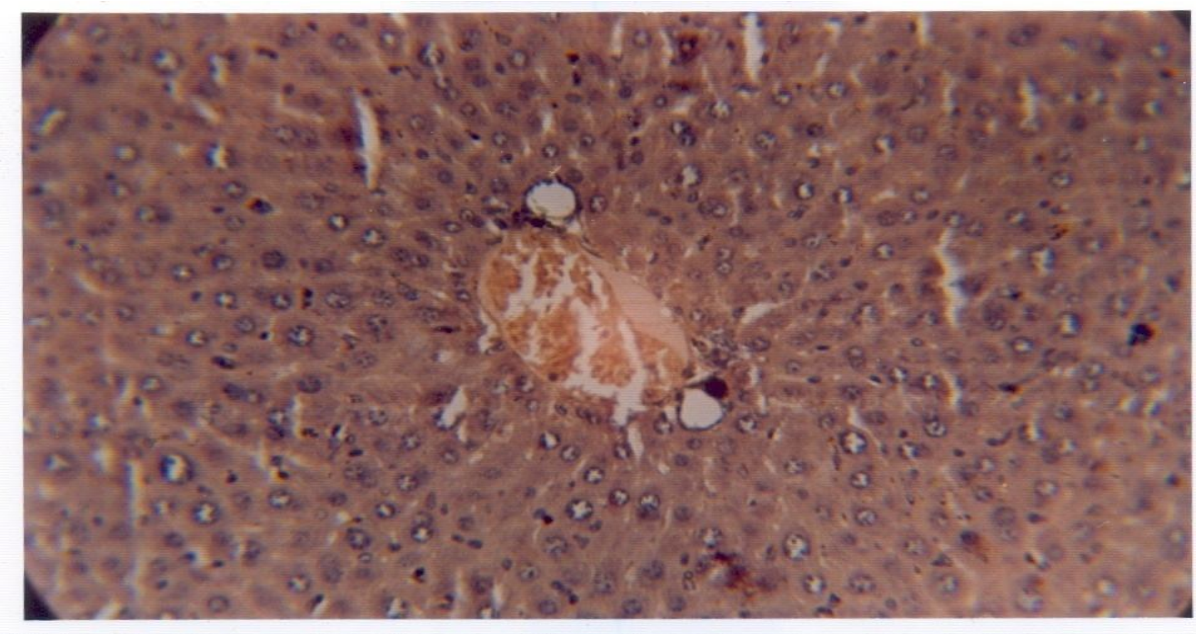

Plate 4.28: Photomicrograph of liver cell treated with *A. boonei* extract. 200mg/kg x 2wks.

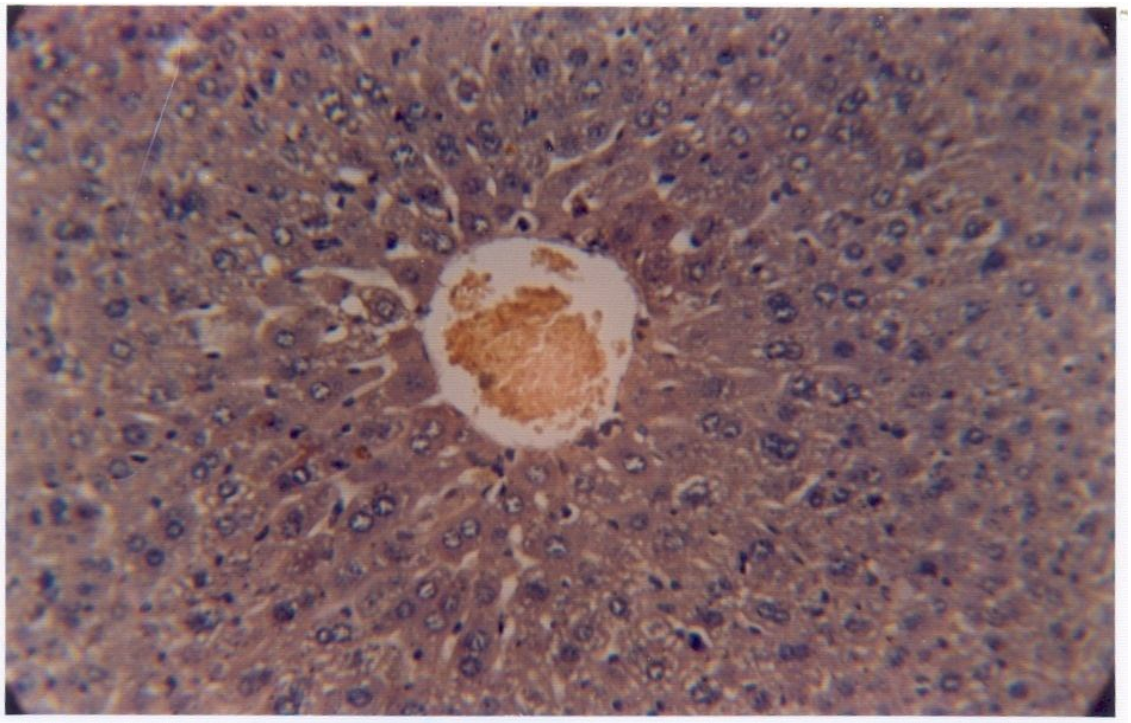

Plate 4.29 : Photomicrograph of Liver cell treated with *A. boonei* extract. 50mg/kg x 4wks.

- Few Normal hepatocytes.
- Large Nucleolus.
- Moderate fatty changes.

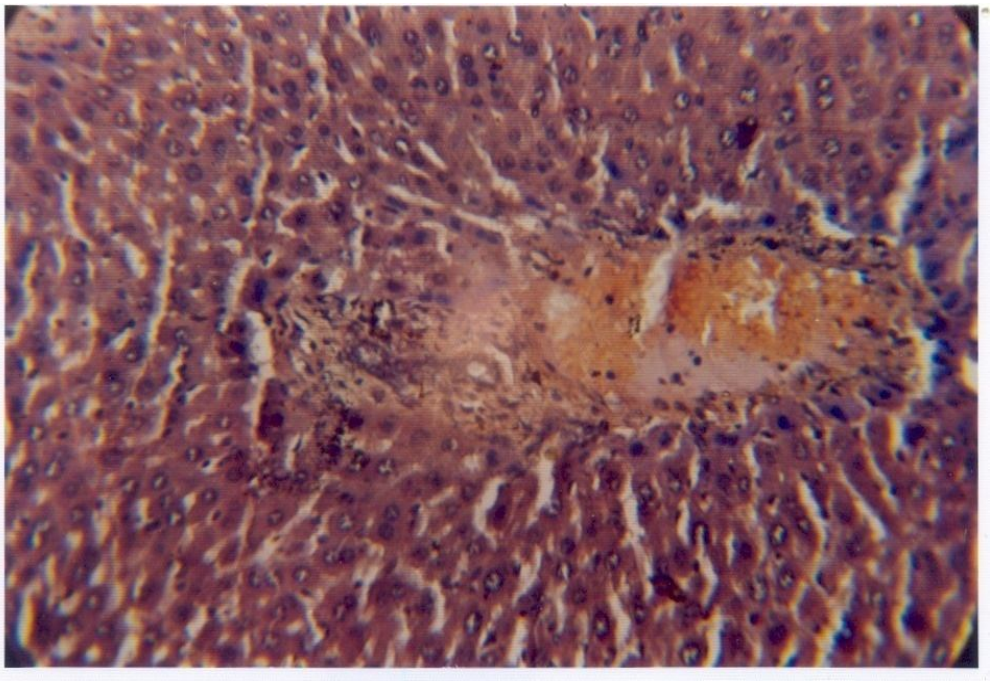

Plate 4.30: Photomicrograph of liver cell treated with *A. boonei* extract. 200mg/kg x 4wks.

- Few hepatocytes with large thick walls.
- Large nucleolus.
- Swollen Hepatocytes.
- Fatty infilteration.
- Hepatocellular necrosis.

Table 4.4c: Summary of Histological Presentations of Liver Tissues Treated with Ethanol Extract of *A. boonei* for 4wks.

2 WEEKS SEGMENT:

0.0mg/kg bw (control)	Normal hepatocytes.
50mg/kg bw	No visible changes
200mg/kg bw	Mild fatty infiltration

4 WEEKS SEGMENT:

0.0mg/kg bw (control)	Normal hepatocytes.
50mg/kg bw	• Mild hepatocellular changes. • Large Nucleolus. • Moderate fatty changes.
200mg/kg bw	• Few hepatocytes with large thick walls. • Large nucleolus. • Swollen Hepatocytes. • Fatty infilteration. • Hepatocellular necrosis.

4.1.8 LIPID PROFILE ANALYSIS:

4.8.1 Effects of *A. boonei* stem bark extract on the lipid profile in Rats:

The total cholesterol (TC) concentration was elevated from a control value of 1.1.2 to 299.0mg/dl by the 4th week at 200mg/kg in the treated group. The difference was significant ($p<0.05$) (Fig. 4.26).

The serum triglyceride (Tg) concentration was significantly increased ($p<0.05$) from the control value of 87.8mg/dl to a respective mean values of 187.1 and 190.7mg/dl by the 4th week at 50 and 200mg/kg bw (Fig. 4.27).

For HDL-C, the highest mean serum concentration was 34.4 and 30.7mg/dl observed for rats treated with 50 and 200mg/kg, bw respectively, against the control value of 32.3mg/dl by the 4th week. The diference was not significant. For the 2 weeks segment, the result was 34.3 and 32.5mg/dl for 50 and 200mg/kg, respectively, against the control of 33.3mg/dl. The difference was not statistically significant ($p>0.05$) (Fig. 4.28).

The LDL-C serum concentration was significantly accentuated ($p<0.05$) from the control value of 78.0mg/dl to 188.2mg/dl by the 4th week at 50mg/kg; and 201.6mg/dl for 200mg/kg at the same period. The serum concentration changes of LDL-C were not significant by the 2nd week at both doses ($p>0.05$), but rose significantly ($p < 0.05$) by the 4th week at both doses.(Fig. 4.29).

The ratio of the atherogenic risk predictor indices (TC/HDL-C) was raised beyond 3.5 at 200mg/kg bw at 4 weeks. This may indicate a rise in risk of coronary artery disease (CAD) at this dose and duration.

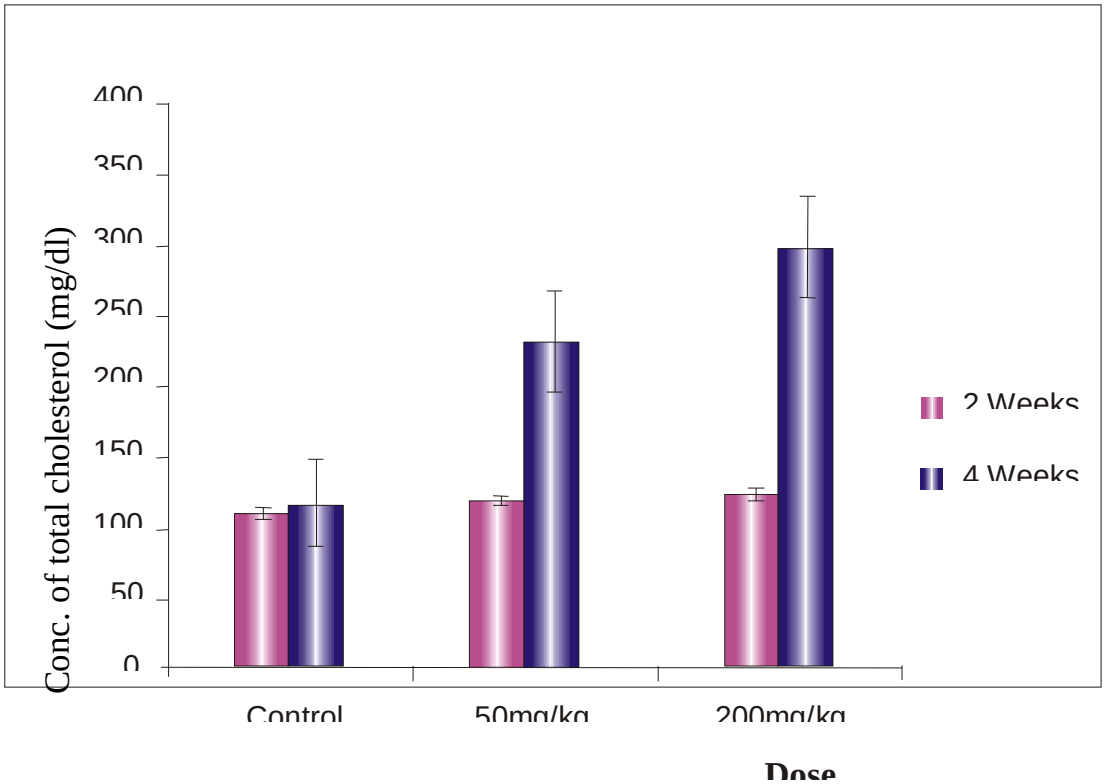

Fig.4.26: Serum total cholesterol concentration in rats treated with extract of *A. boonei.* Total cholesterol was elevated significantly by the 4th wk.

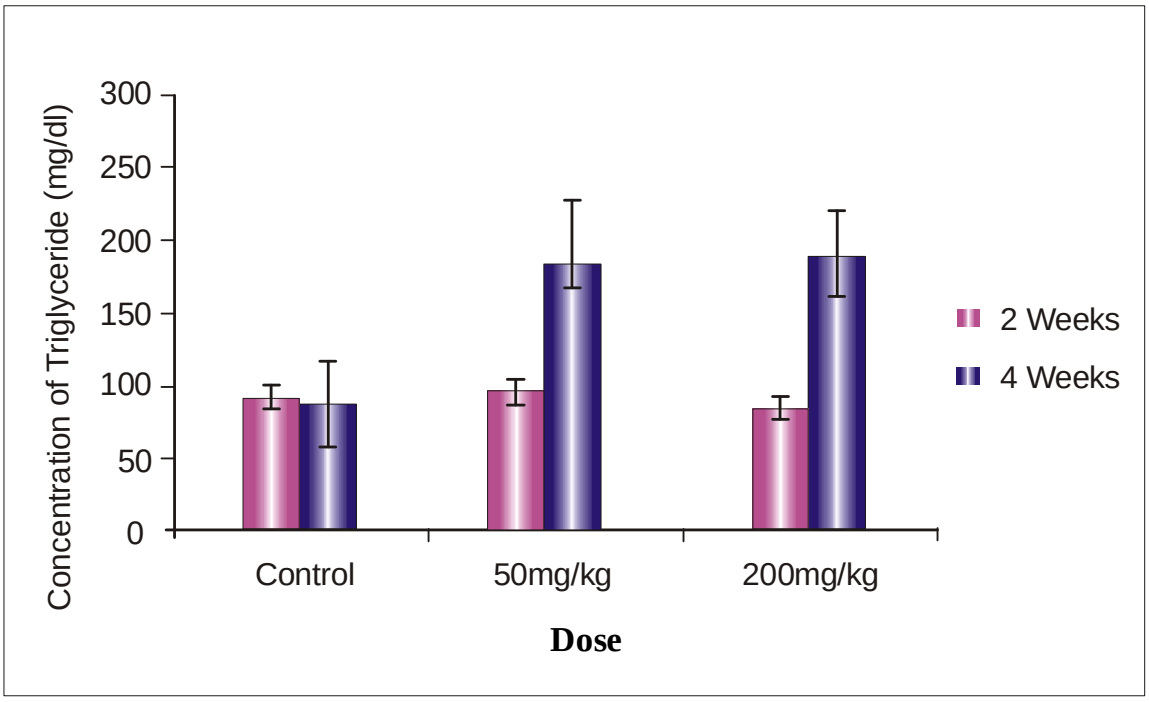

Fig. 4.27: Serum triglyceride concentration of rats treated with extract of *A. boonei*. The level was raised significantly.

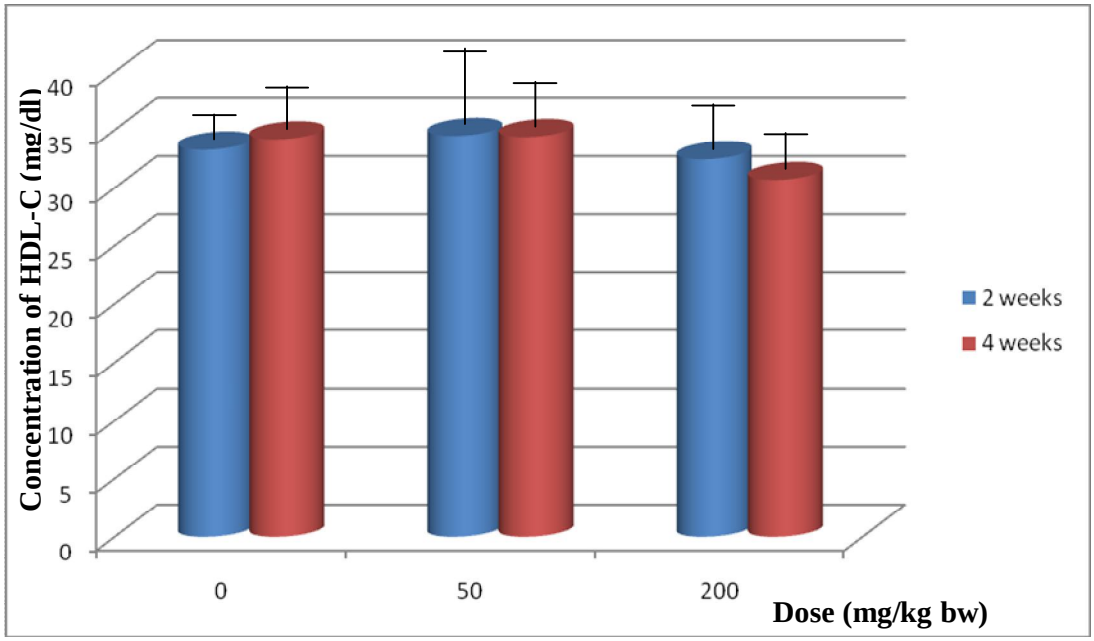

Fig. 4.28: Serum high density lipoprotein cholestorol concentration in rats treated with extract of *A. boonei.*

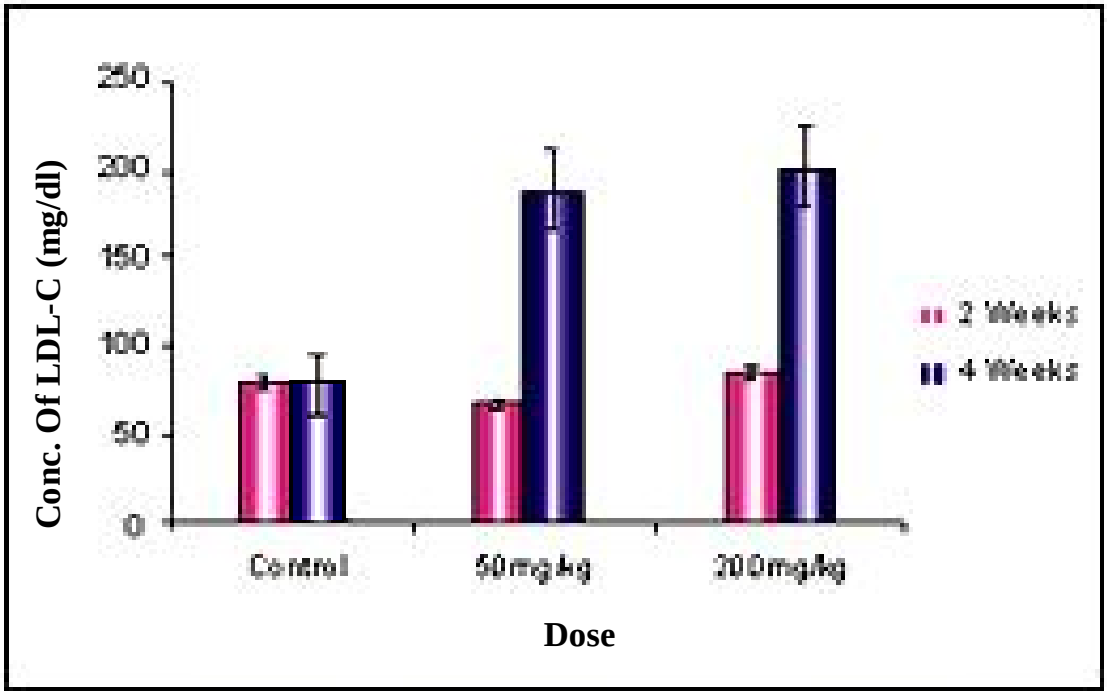

Fig. 4.29: Serum low-density lipoprotein cholesterol concentration in rats treated with extract of *A. boonei*. LDL-C concentration increased significantly ($p < 0.05$) by the extract at 200mg/kg.

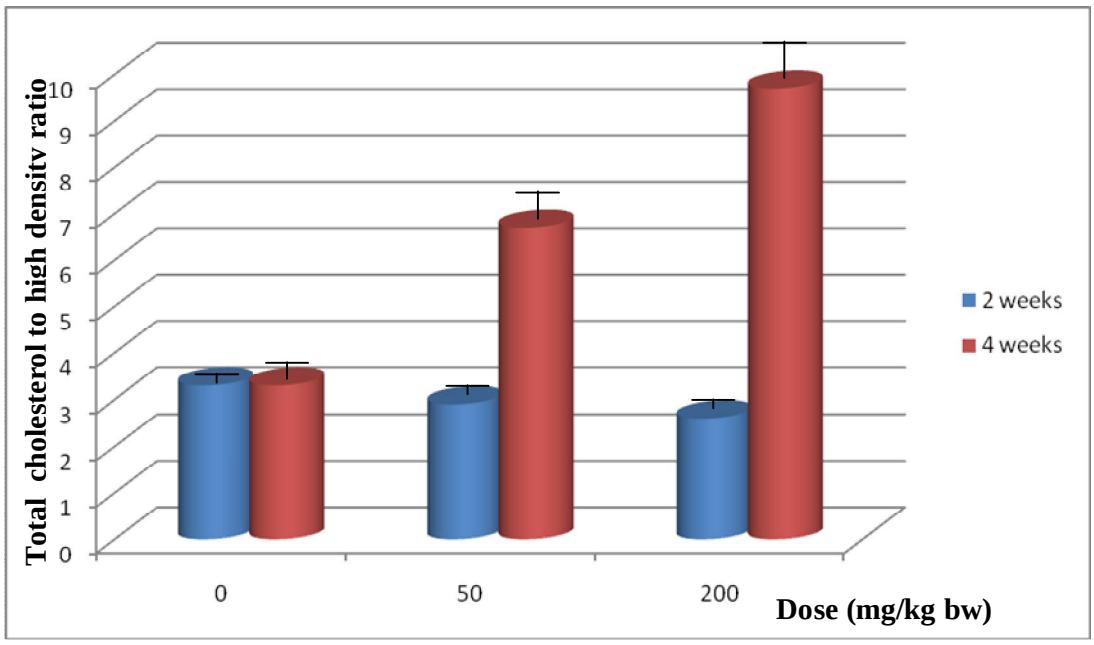

Fig. 4.30: Serum total to high-density cholesterol concentration ratio in rats treated with extract of *A. boonei.*

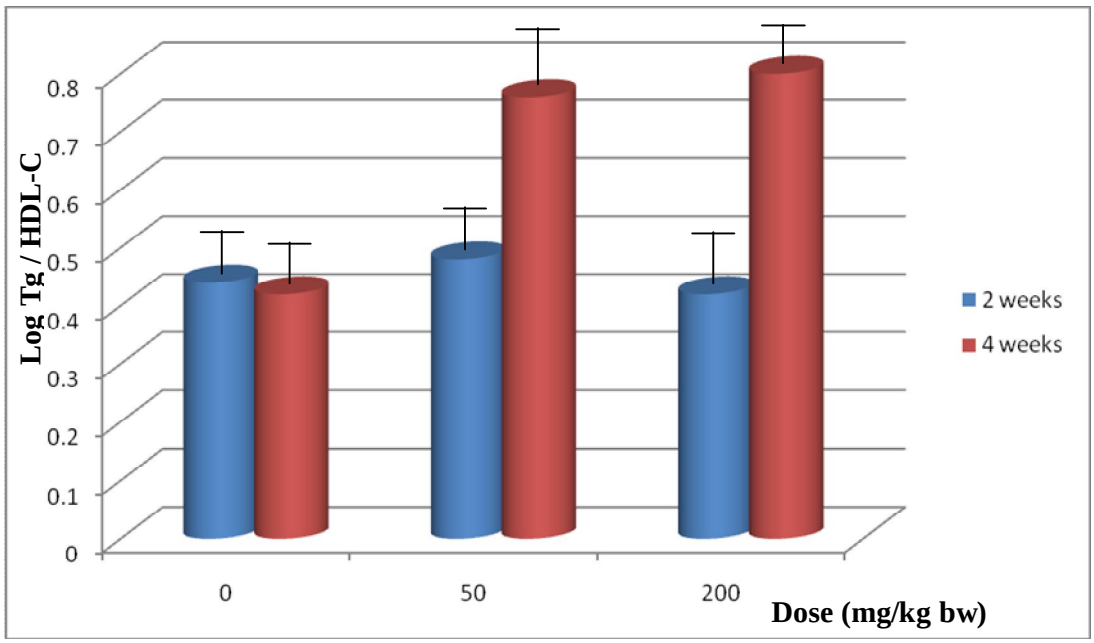

Fig. 4.31: Log Serum concentration of Triglyceride to high-density lipoprotein cholesterol in rats treated with extract of *A. boonei.*

4.1.9. OXIDATIVE PROFILE:

4.1.9.1 Effect of ABE on Paracetamol-induced Hepatotoxicity:

The concentration of the anti-oxidant vitamin, ascorbate was decreased by the 50 and 200mg/kg bw of ABE. The decrease was significant ($p<0.05$) at 0.09mg/ml at 200mg/kg against a control mean of 0.10mg/ml at the end of 2 weeks (Fig 4.32).

At 2 weeks, the mean concentration of α-Tocopherol was 0.21 IU/L in the control against the mean test values of 0.19 IU/L at 50mg/kg bw and 0.11 IU/L at 200mg/kg bw by the end of 2 weeks. The fall in plasma concentration of α-Tocopherol was significant ($p<0.05$). A similar pattern was observed in the 4 weeks segment (Fig 4.33).

The mean serum concentration of malondiadehyde at the end of 2 weeks was 0.23mg/ml at 50mg/kg and 0.15 at 200mg/kg against a mean control value of 0.07mg/ml. The rise in malondialdchyde followed a similar pattern in the 4^{th} week. The increases were significant, ($p<0.05$) and tended to be dose and time dependent (Fig 4.34).

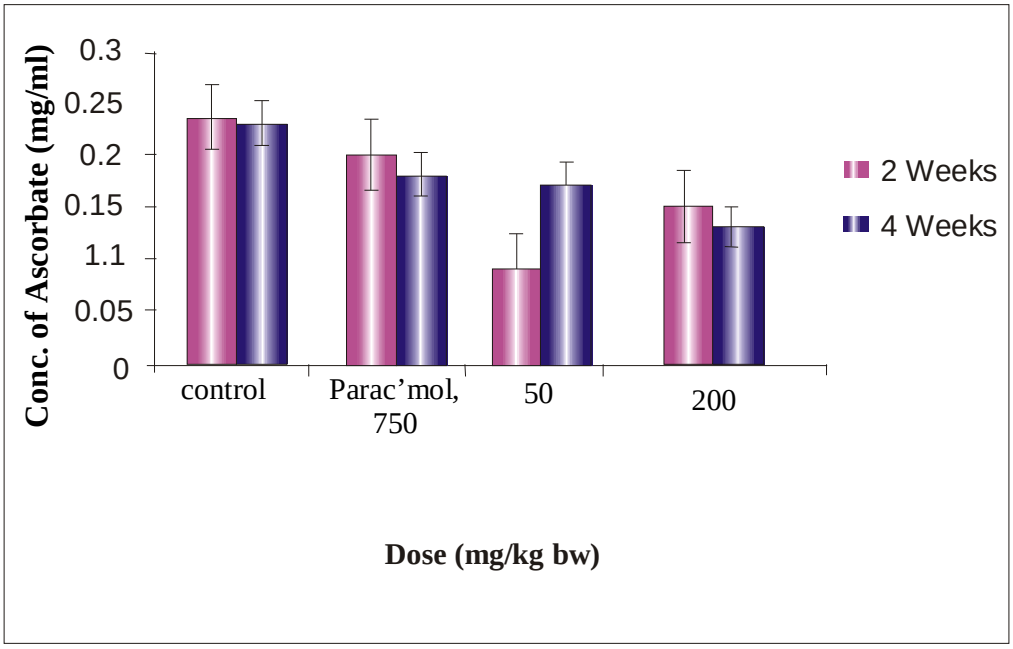

Fig. 4.32: The effect of extract on serum ascorbate in paracetamol-induced hepatotoxicity in rats.

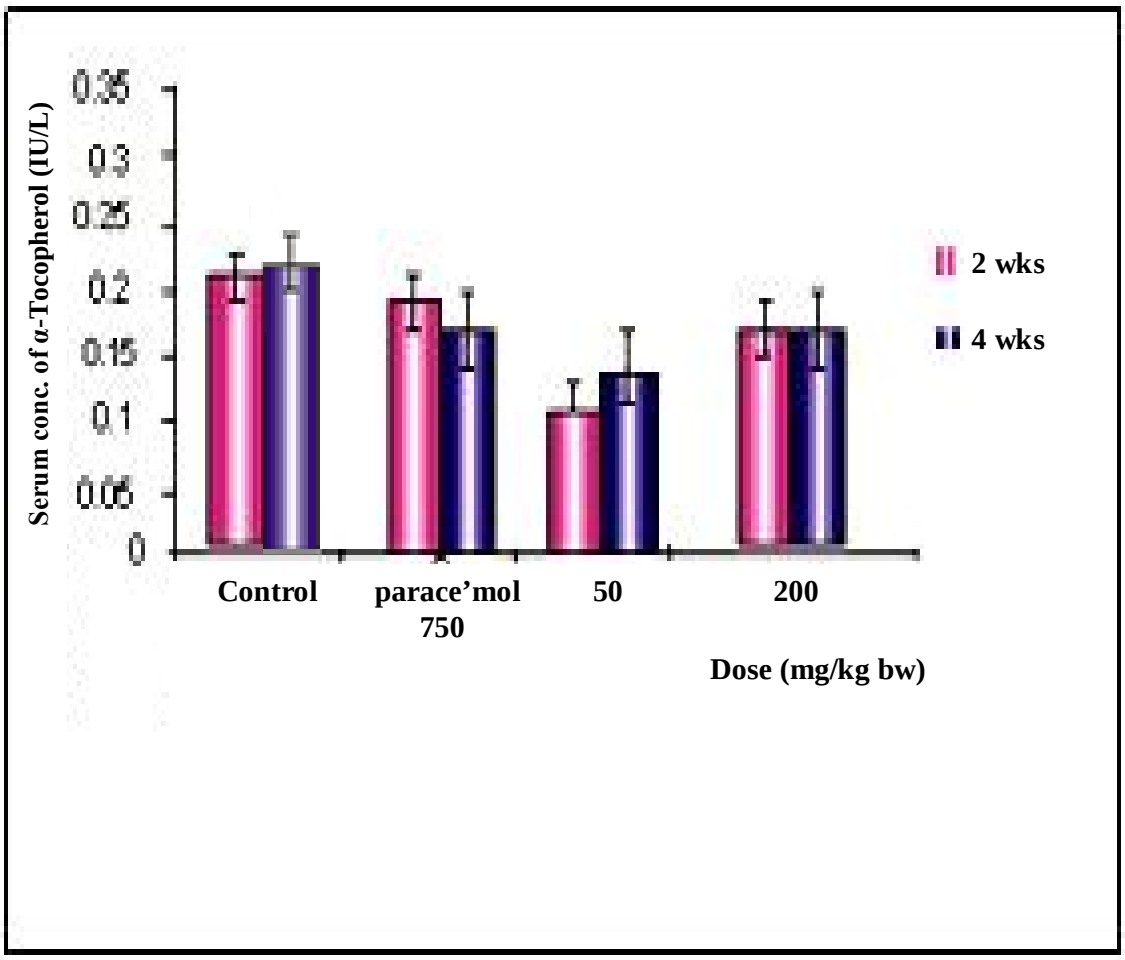

Fig. 4.33: The effect of extract on serum α-Tocopherol in paracetamol-induced hepatotoxicity in rats. The serum level of α-tocopherol was significantly reduced ($p < 0.05$) ($n = 5$).

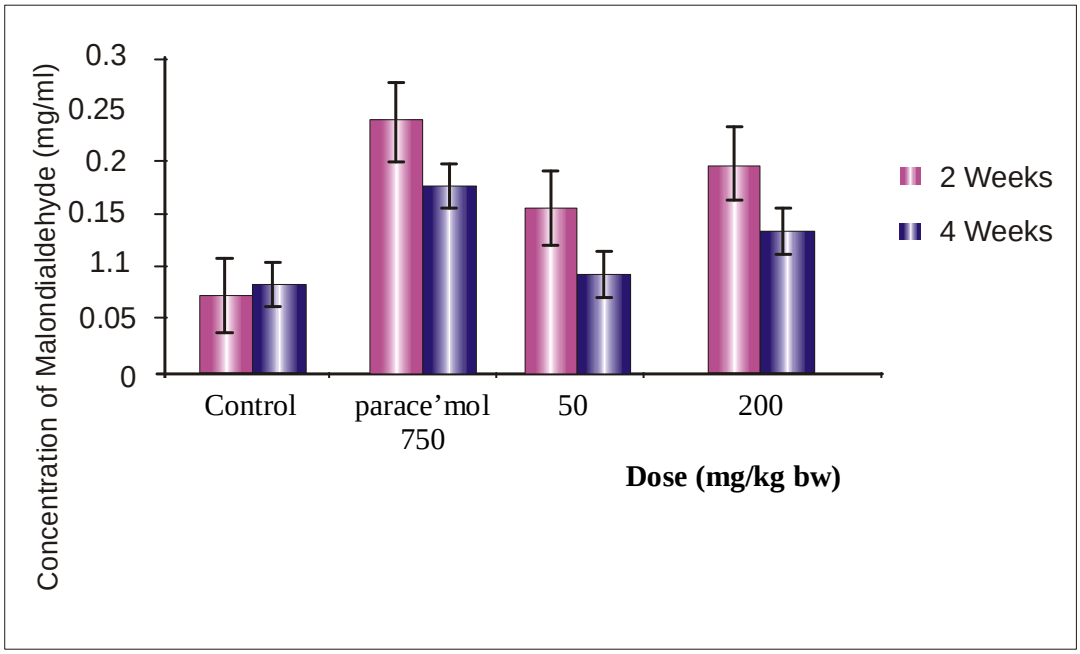

Fig.4.34: The effect of extract on serum Malondialdehyde in paracetamol-induced hepatotoxicity in rats. The serum MDA was significantly raised by both doses.

4.1.10 HAEMATOLOGICAL PROFILE:

4.1.10.1 The Effects of ABE on Haematological Profile:

There was no significant difference ($p>0.05$) at 2 weeks between the mean RBC in the rats in the control segment and those in the 50mg/kg segment whose means were 8.0 x 10^{12}/L and 7.7 x 10^{12}/L respectively. At 4 weeks, mean values were observed for the controls (8.0 and 8.2 x 10^{12}/L). But the RBC value was significantly ($p<0.05$) reduced to 6.7 x 10 1^{2}/L at 200mg/kg bw (Fig 4.35).

The Hb mean values did not show any significant difference ($p>0.05$) at 2weeks. The control, and 50mg/kg treated rats gave serum values of 16.4g/L, and 15.7g/L mean values respectively. However, the Hb was significantly lowered ($p<0.05$) at 200mg/kg by the 4^{th} week (Fig 4.36).

The PCV was significantly reduced ($p<0.05$) from a control value of 0.38L/L to 0.30L/L at 50 mg/kg in the 2 weeks segment. ABE did not affect the PCV in this segment. However, the PCV reduction was significant by the 4^{th} week at 200mg/kg bw (0.24L/L) (Fig 4.37).

At 2 weeks, the rats treated with 50 and 200mg/kg did not show any significant difference ($p>0.05$) in the MCV when compared with the control. This pattern was also observed in the 4 weeks segment, except at 200mg/kg which significantly lowered the MCV from a control value of 57.6 to 47.8 Fl. (Fig 4.38).

The pattern of results for the MCHC were similar to MCH where the control serum concentration was 37.7mg/dl, against a test value of 38.9mg/dl, at 200mg/kg; in 4 weeks; the test being significantly different ($p<0.05$) from the control (Fig 4.39 & 4.40).

For Reticulocytes, the highest observed mean serum value (2.5%) at 2 weeks was obtained for rats which received 50mg/kg. The difference was significant ($p<0.05$) when compared with the control value of 1.7%. Within the same period, there was a non-significant rise at a dose of 200mg/kg (1.5%). However, in the 4 weeks segment, there was a significant fall ($p<0.05$) with 200mg/kg (1.5%) (Fig. 4.41).

The total serum WBC was not significantly reduced ($p>0.05$) after 2 weeks treatment with ABE with a control value of 12.4 and $11.1x10^{9}$/L at 200mg/kg bw. The pattern of reduction of the WBC was similar in the 4th week segment where both doses significantly reduced the serum WBC concentrations from a control value of $12.6x10^{9}$/L to $8.8x10^{9}$/L and $9.2x10^{9}$/L for 50 and 200mg/kg respectively (Fig.4.42).

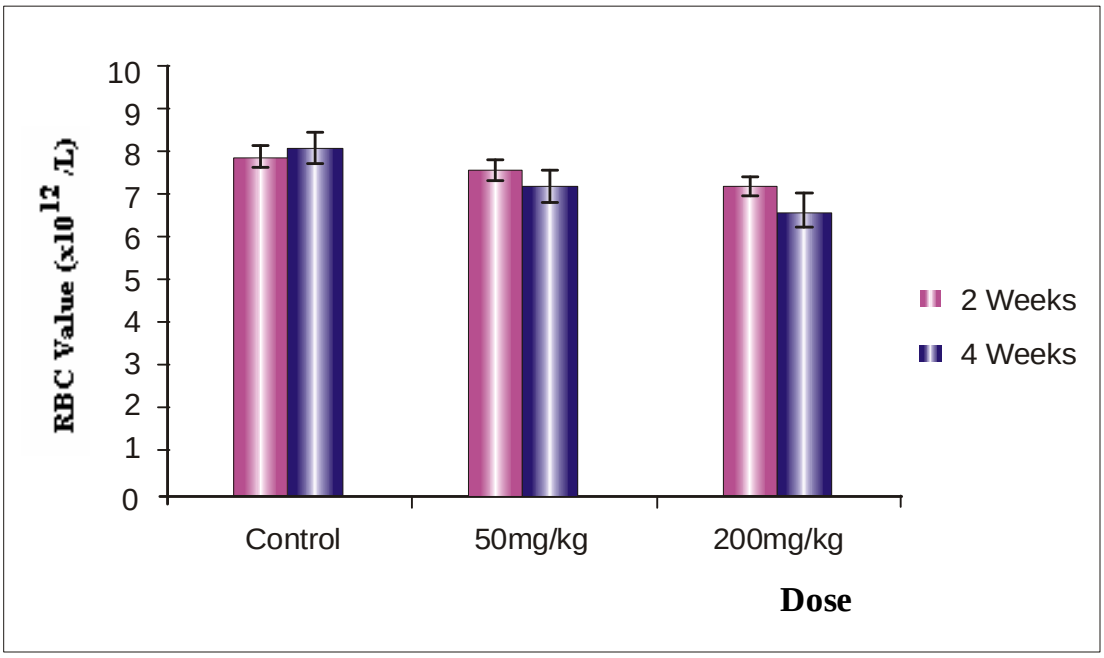

Fig. 4.35: Serum RBC value of rats treated with extract of *A. boonei.* The RBC count was reduced significantly at 200mg/kg extract.

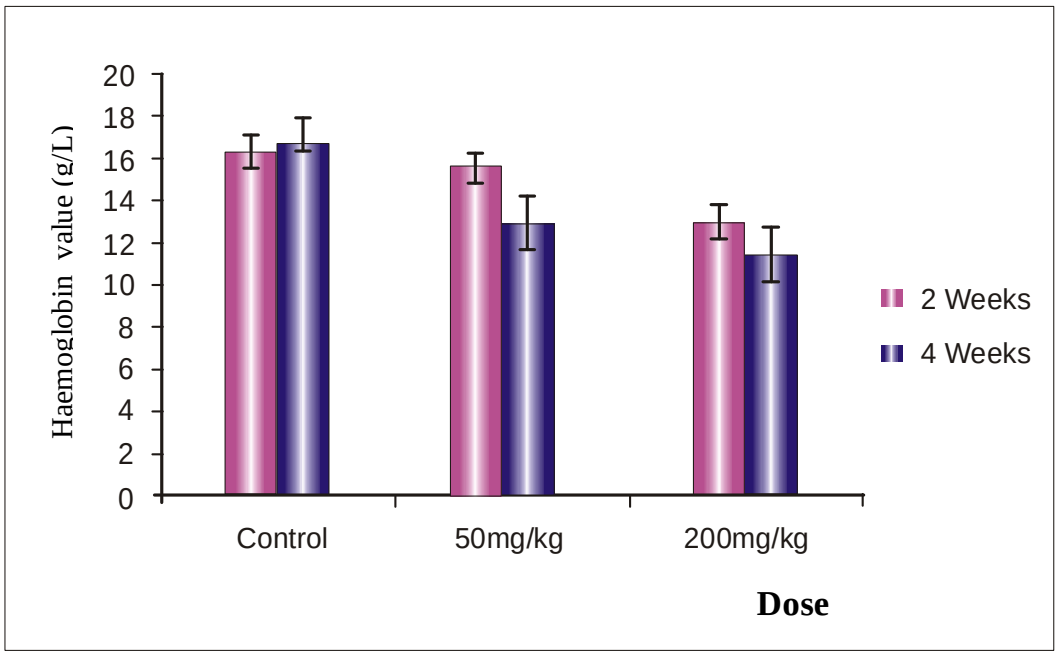

Fig. 4.36: Serum Haemoglobin value of rats treated with extract of *A. boonei.* Haemoglobin value was significantly reduced.

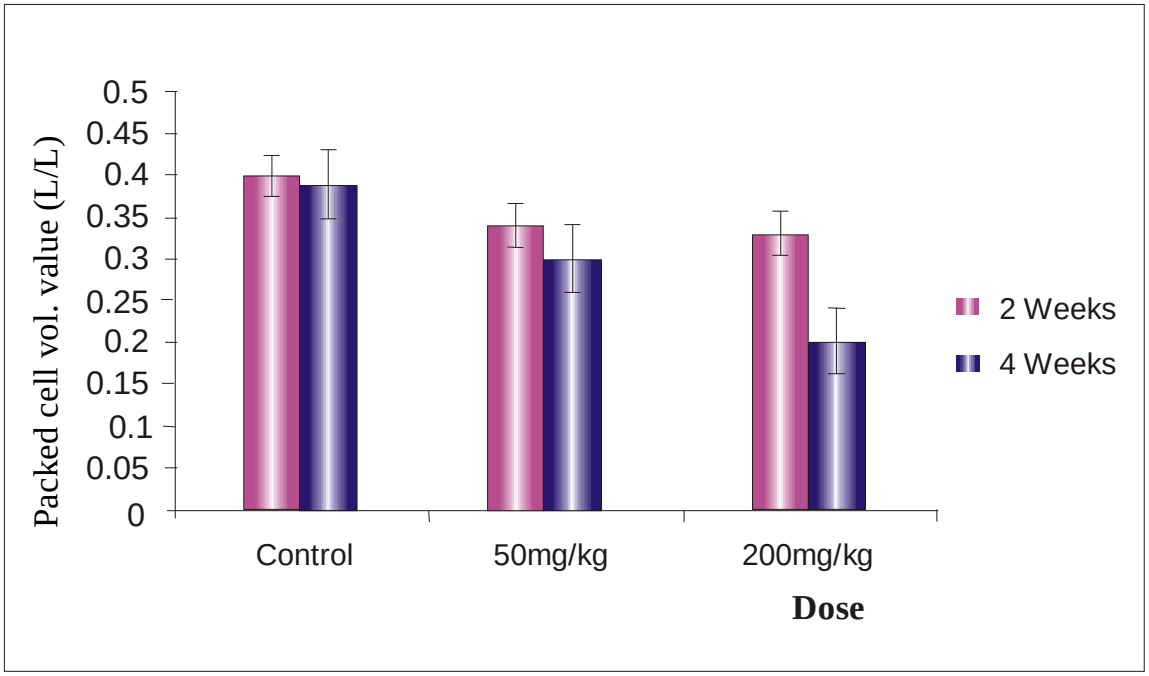

Fig. 4.37: Serum Packed cell volume (PCV)value of rats treated with extract of *A. boonei.* PCV value was significantly reduced at 4th week.

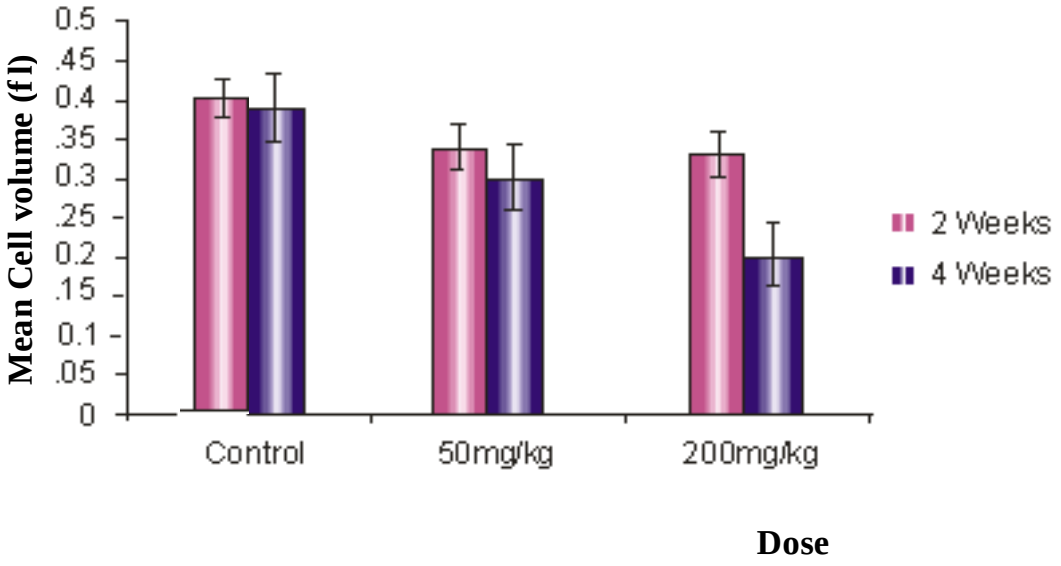

Fig. 4.38: Serum Mean Cell Volume (MCV) value of rats treated with extract of *A. boonei.* MCV value was significantly reduced at 4th week.

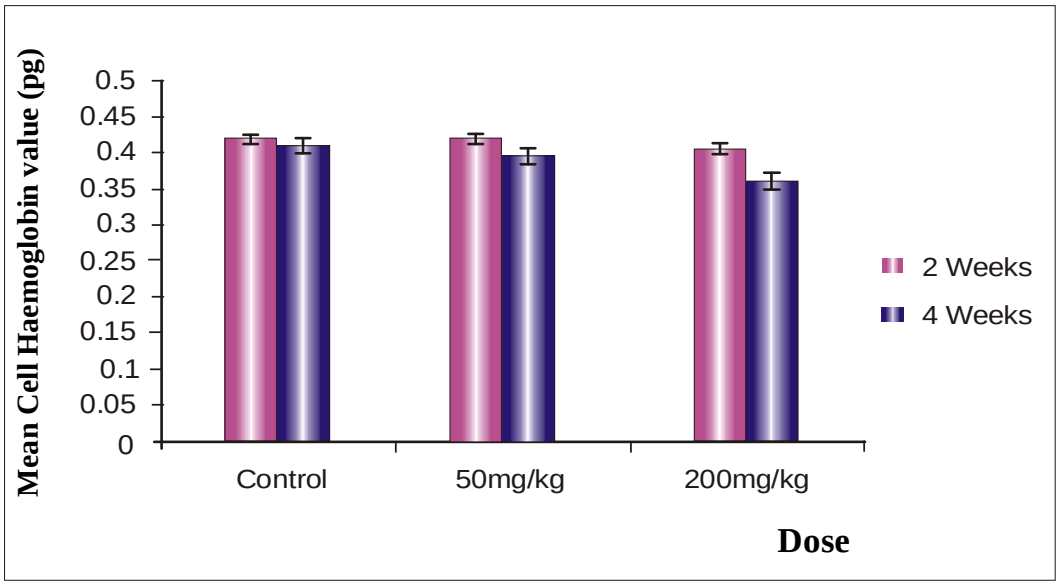

Fig. 4.39: Serum mean cell haemoglobin (MCH) value of rats treated with extract of *A. boonei*. MCH value was slightly reduced at 4th week.

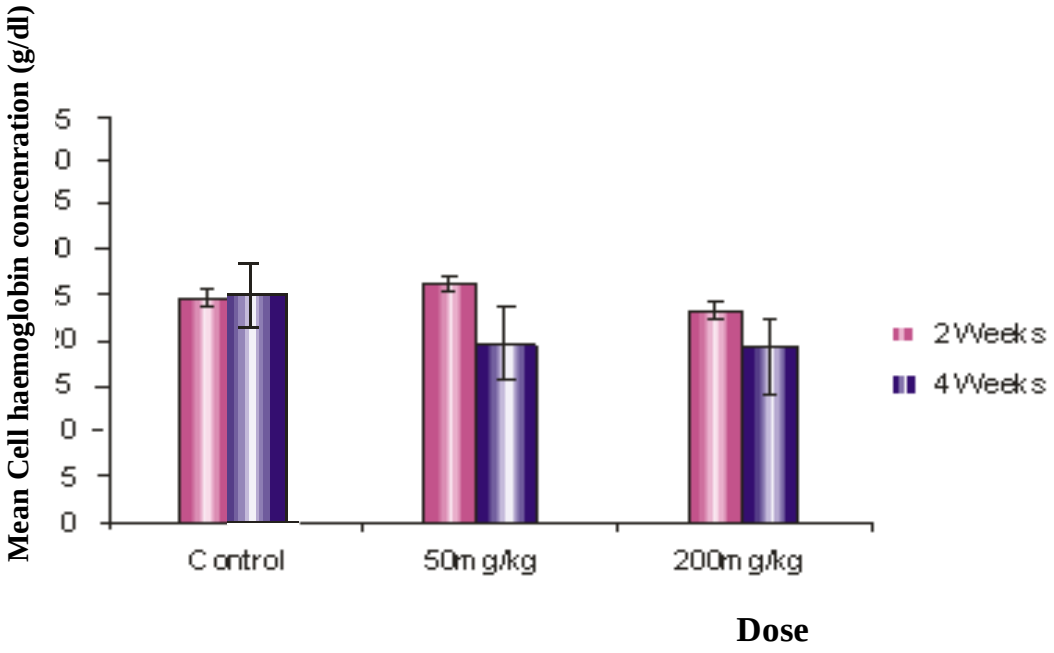

Fig. 4.40: Serum mean cell haemoglobin concentration (MCHC) value of rats treated with extract of *A. boonei.* MCHC value was slightly reduced at 4th week.

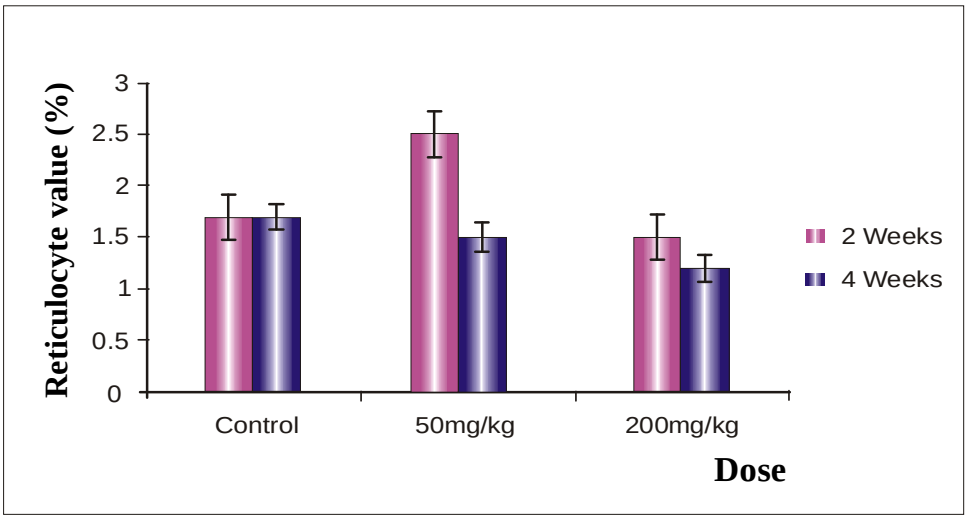

Fig. 4.41: Serum Reticulocyte value of rats treated with extract of *A. boonei.* Reticulocyte value was significantly reduced at 4th week.

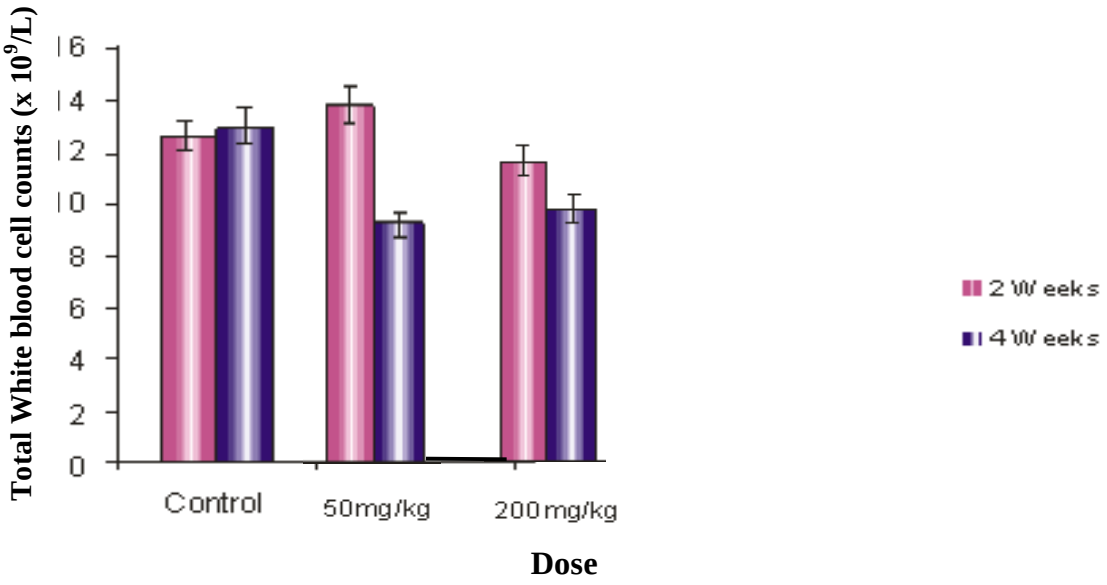

Fig 4.42: Serum white blod cel counts (WBC) value of rats treated with extract of *A. boonei*. WBC value was significantly reduced by 4th week.

4.2 DISCUSIONS

There does not seem to exist any scientific study on the biochemical effects of ethanol extract of *Alstonia boonei* stem back (ABE) on reproductive parameters in rats.This work is an attempt to contribute in this area in view of the wide spread application of ABE in folkmedicine.

Phytochemical Identification and Biometric Measurement

The result of the qualitative phytochemical composition of ABE is presented in table 4.1. It showed that ABE contains alkaloids, tannins, flavonoids, saponins, cardiac glycosides, phlobatannins, reducing sugars, and steroids. Anthraquinones were not detected .The results agree with the work of Odebiyi & Sofowora (1978); who identified saponins, flavonoids, alkaloids, steroids, terpenoids and tannins in the plant extract. Iwu (1993) and Odeyemi (2005) corroborated these reports.

The acute toxicity test showing the toxicity profile and the calculated LD_{50} (Aliu & Nwude, 1982) are presented on tables 4.2 and 4.3. The LD_{50} of the crude extract was 562 mg/kg body weight (bw). As a crude extract the value seems low when compared with the effective dose. But this is the case with drug substances which have narrow therapeutic index such as the cytotoxic anatoxin-A (Witkop & Brossi, 1984); the hepatotoxic dermorphine (Wagner & Horhammar, 1970) and the digitalis- derived cardiac glycosides (Schild, 1980).

The toxicity profile also showed some behavioural parameters which are regarded as preliminary indices of systemic toxicity in animals (Katzung *et al.,* 2009).At the dose of 400mg/kg bw, 62% of the toxic parameters were present in the mice treated with ABE; and 75% at the dose of 600mg/kg bw, under which 100% of the animals died (Table 4.2).These are initial evidence that ABE may exhibit unwanted biochemical effects, especially at higher dose levels.

Figure 4.1 showed the effect of ABE on the body weights of non-pregnant female rats exposed to various doses for 15 days.The results indicated that the growth of the rats was retarded significantly ($p<0.05$) in the treated groups by the end of the study period (Fig 4.1). Kluwe (1981) and Poore and Fowden (2002) ascerted that weight loss or gain in animals were an indication of systemic toxicity. The weight loss may be attributed to the alteration of hormonal balance by the extract which acts on the anterior pituitary gland.The gland stores, among others, growth and sex steroid hormornes which regulate growth, development, metabolic functions and sexual characteristics (Poore and Fowden, 2002; Waugh and Grant, 2006). This outcome may be an indication that ABE may have deleterious effects on some organs in the rats.

Female Reproductive Parameters

The results showed that ABE has adverse biochemical effects on female reproductive parameters in rats.The mean foetal weight was significantly ($p<0.05$) reduced when compared with the control (Fig. 4.2). But the morphology and number of litters were not altered. Further, ABE dose dependently elevated the rat serum estrogen and progesterone concentrations (Fig.4.3 and 4.4).This was also reflected in the histological presentations (Plates 4.1 -4.5,Table 4.4a) which contained vacuolations and distortions of the uterine architecture, especially at 200mg/kg bw (Plates. 4.3-4.5).The photomicrograph of the control showed the intact uterine cells (Plate 4.1).

There is a physiological plasma threshold concentration of estrogen and progesterone that support fertility, nidation and development of foetus (Waugh and Grant, 2006).The alteration of this threshold leads to hormonal imbalance and adverse effects on the foetus (Pinaud *et al.*, 1991; Enan *et al.*,1996; Vasudervan and Sreekumaris, 2007).

Foetal malformation have been observed in rats treated with fansidar at the early stages of pregnancy.This was attributed to the pyrimethamine component which is a

folic acid antagonist.The same is true of rats treated with the extract of *Rumex studelii* (Gebrie *et al.,* 2005). But the mechanism of its teratogenic effect is unknown.

The effect on foetal body weight may be an indication that ABE has deleterious effect on the rat foetus. This may be based on the report of Kluwe (1981) which stated that weight gain or loss in animals is an indication of systematic toxicity. Poore and Fowden (2002) also demonstrated that the hypothalamic pituitary axis (HTPA) was functional in the course of foetal development, and that its hormones were known to affect the growth and development of foetal tissues and organs.They further illustrated that the interference with the axis have adverse effect on foetal weight, as well as increased risk of cardiovascular and metabolic disorders in adult life. Pamir *et al.,* (2006) reported that adverse increase in serum progesterone concentration may give rise to teratogenicity and musculanization of the foetus.

At physiologic concentration, progesterone induces synthesis of egg white proteins such as ovalbumin and ovumucoid (Gebrie *et al.,*2005; Pamir *et al.,* 2006). Similarly, estrogen induces creatine kinase (Huang *et al.,*. 1987), but causes adverse proliferation of uterine cells, and embryonic defects at high serum concentration (Maccio *et al.,*.2008). Paoletti *et al.,.,* (2001) and Tanriverdi *et al.,* (2003) previously reported that high plasma estrogen concentration interferes with immune functions, and may have a role to play in the pathophysiology of autoimmune diseases. Kayode *et al.,*. (2007) found out that elevated serum estrogen caused the distortions of the uterine cells in rats treated with *Aspillia africana* leaf extract.Gunin *et al.,* (2005) reported the induction of morphogenetic alteration of uterine tissues as a result of high serum estrogen concentration, and cautioned that the condition may predispose to cancer of the reproductive organs. They further suggested that estrogen may be associated with the acetylation of histone. They went further to show that the inhibitors of histone de-acetylase such as trichostan-A and sodium butyrate produced similar proliferation of mammary and endometrial cells as did high serum concentration of estrogen. This

may serve as a possible explanation for the observed biochemical effects of proliferation and distortions of uterine cells observed in this study. Furthermore, the anti- convulsant, sodium valproate, was found to antagonise histone deacetylase and caused teratogenicity and carcinogenicity in a similar way as trichostan-A. From the foregoing, it is suspected that ABE may indirectly predispose to teratogenicity and carcinogenesis at high doses and prolonged usage because of its effects in increasing serum estrogen and progesterone concentrations at high doses.

Male Reproductive Parameters

Spermatozoa possess two vital features: motility and fertility. The quantity and quality of the sperm are determined by the sperm-count, viability and morphology of the sperm. Some plant extracts such as *Occimum sanctum, Amarantus spinosus, Carica papaya* and *Spirulina plantensis* have been reported to affect spermatic qualities (Murugavel *et a* ,.1989; Chio and Hwang, 2005) and consequently testicular function. Drugs or chemicals which affect testicular function often do so by their effect on the quantity and quality of sperm cells (Orisakwe *et al.,*2003).The implication is that the administration of these extracts may cause variations in sperm count, motility, viability, morphology, and invariably affect their fertility potentials (Aladakatti *et al.*, 2000).

The present study evaluated the reproductive effects caused by the stem bark extract of *Alstonia boonei* at two dose levels of 50 and 200 mg/kg bw, and for two and four weeks duration.The results from the four weeks segment showed that there were retardation in growth of the rats, which may imply possible organ toxicity (Kluwe, 1981; Laumann *et al.,* 1995).

The serum testosterone concentration increased marginally when the outcome in the test groups were compared with the respective controls, in the 2^{nd} and 4^{th} week segments at 200mg/kg bw but decreased by 50 mg/kg bw in the 2 weeks segment (Fig. 4.5). Peripheral testosterone concentrations are known to affect mainly testicular

secretary function (Jubiz *et al.*,1974). It has also been observed that delayed spermiation impairs testosterone function (Jewel *et al.*, 1998) and cause decrease in sperm count. It has been reasoned that some plant extracts may cause testosterone depletion at the target sites while sparing the serum concentration to a large extent. This is the possible pharmacokinetic mechanism of a tissue , rather than plasma bound drugs extract. But this conclusion cannot be drawn for ABE at this stage. However, it may serve a useful purpose to explain the present observation in this study where the sperm characteristics were significantly affected without a corresponding effect on the serum testosterone concentration.

Moreover, testosterone is essential for steroidogenesis, maturation and survival of spermatocytes (Dyson and Orgebein, 1973). These are indeed cellular rather than plasma bound drugs. Following this observation, the inhibition of serum testosterone by ABE at 50mg/kg bw in 2weeks, may have a fundamental effect on the sperm parameters.

Fifty and 200mg/kg bw of ABE in two weeks study period significantly ($p<0.05$) reduced the sperm count, percentage motility, viability, and percentage normal morphology (Fig.4.6-4.7). These agree with the work of Raji et al., (2005) who previously reported the inhibition of the sperm characteristics at 50mg/kg of the pant extract, and a possible reversal of the effect at higher doses and duration of the extract. For the four weeks study segment, ABE significantly decreased the sperm parameters, but to a lesser extent than the 2 weeks segment (Fig. 4.6-4.8). Raji *et al.*, (2005) also reported the restoration of full spermatic function after the 12^{th} week of study in the presence of the plant extract. Sexana *et al.*,, (1980), Lohiya *et al.*, (1994), as well as Raji *et al.*,. (2003) reported similar observations.Working with niferacetam, Shinmura *et al.*,. (2004), observed that the reduced spermatic activities returned to normal by the 4^{th} week of the drug treatment. These findings are supported by the histological presentations (Fig.4.6-4.14) in the current study where there were derangements of the

testicular cyto-architecture especially at 50mg/kg bw for two weeks, and apparent cell recovery by the 4 week. Nonetheless, the derangement of the sperm cell morphology found in this study may have implications for fertility or foetal defects.

Further, the decreased serum concentration of testosterone at 50mg/kg bw may imply reversible damage to testicular cells. The return of the serum testosterone concentration at 20mg/kg towards the baseline value could imply possible recovery, replacement of damaged testicular cells, resistance or possible adaptation by the existing testicular cells to the drug action. However, the extract does not possess testosterogenic activity at higher doses.

Some species of Alstonia such as *A. scholaris* and *A. spectabilis* have been shown to inhibit the release of nitric oxide (NO) in cell culture (Choi and Hwang, 2005). Nitric oxide is known to be a potent vasodilator and smooth muscle relaxant. Its inhibition would lead to vasoconstriction, hypertrophy, ischemia, and possible necrosis of the reproductive cells.The result obtained in this study may not be unconnected with such metabolic interference in NO release.This action could have deleterious effect on the spermatocytes and reproductive characteristics of the male rats. However, if ABE is found to inhibit NO like the other Alstonia species mentioned above, it may serve as possible explanation for its local use to enhance penile erection and sexual stimulation, similar to Pfizer's sildenafil.

In view of the adverse biochemical and histological effects on male reproductive parameters, it is suspected that may induce ABE induced testicular damage at lower dose and duration.The reduction in sperm-count, attenuation of motility and the adverse changes in the cyto-architecture of the gem cells may negatively impact on fertility in the male rats. However, there are reports where the natural agents are selectively toxic to the mammalian tissues while sparing the rodents due to their more efficient xenobiotic biotransformation system (Laumann *et al.,* 1995) relative to humans. As such the susceptibility of man to the toxic propensities of such natural agents should be higher.This is open to further investigations.

Effect of ABE on Pituitary Vanilmandelic Acid Concentration

It has been established that the Hypothalamic Pituitary Axis (HTPA) releases hormones which regulate various functions in some parts of the body. These hormones are biosynthesized in the neuronal cell bodies within the hypothalamus in the mid-brain. They are transported through the vessel and stored in the anterior or posterior lobe of the pituitary gland until neuronal signals trigger demand (Saladin and Porth, 1996). Among others, the prolactin and gonadotropin releasing hormones are part of this axis. The former releases lactotropes, while the latter releases follicle stimulating hormones (FSH) and leitenizing hormone (LH). The hormones regulate reproductive activities in males and females (Waugh and Grant 2006).

It is also known that classical and atypical neuroleptics cause enlargement of the pituitary size and stimulate lactotropes in man and rodents (Asplund *et al*., 1982; Murali, 2006; Elisabetsky and Costa Campos, 2006). Pariante & Paola (2006) posited that the pituitary enlargement may be due to proliferation of adrenocorticotropic hormone (ACTH) producing cells and related ones in the gland. There is also emerging evidence linking psychosis and antipsychotic therapy with metabolic abnormalities (Pariante & Paola, 2006). Besides, the neurochemical basis of most antipsychotic therapy is based on the metabolic regulation of the catecholamines; with VMA or HVA serving as a bio-marker for neurotoxicity.

One of the local uses of *Alstonia boonei* extract is as an antipsychotic agent. In this study, ethanolic extract of *A. boonei* significantly ($p < 0.05$) elevated the tissue concentration of vanilmandelic acid (VMA) in the anterior pituitary gland at a dose of 50 and 200mg/kg bw in 2 and 4 weeks period of treatment (Fig.4.11). This observation is in tandem with the histological vacuolation and distortions of the pituitary cells seen in the photomicrographs (Plates 4.15-4.19).Vanilmandelic acid (VMA) is a metabolic end-product of the catecholamines. It is a serum and tissue specific marker for the assay of the catecholamines, especially noradrenaline and

adrenaline. The increased cellular concentration of VMA may be a reflection of elevated concentration of the catecholamines which were denied access to their receptors by the plant extract. Under this condition, the catecholamines became vulnerable to the metabolic activities of monoamine oxidase (MAO) and catechol-o-methyl transference (COMT). This suggestion follows because the plant extract is extensively used to treat mental illness in folkmedicine. The antipsychotic action of the plant extract has been reported by Elizabetsky and Costa-campos (2006). Most of the classical antipsychotic drugs act by blocking post–synaptic catecholamine receptors in the CNS, the pituitary gland being a component of the CNS.

The alkaloid, alstonine, which has been identified in the *A.boonei* extract, is structurally related to known drugs with some antipsychotic effects, such as reserpine and α-yohimbine. Reserpine acts by depleting the neuronal store of the catecholamines, exposing them as substrates to MAO and COMT. Alpha-yohimbine acts as a pre-synaptic sympathetic α_2 - receptor blocker. This event abolishes the neuronal regulatory process, which involves feedback, and re-uptake mechanism associated with the catecholamine, and makes them candidates for enzymatic metabolism, thereby giving rise to elevated concentration of the metabolite, VMA.

Haddad and Wieck (2004) also showed that the blockade of catecholamine receptors stimulate proliferation of lactotropes and cell of the pituitary. Gebrie *et al.,.,* (2005) and Pamir *et al.*, (2006) reported the histologic damage to the uterine tissues as a result of high concentration of HTPA-mediated sex hormones, estrogen and progesterone. Hence the histologic distortions of the pituitary tissues may be associated with elevated levels of the catecholamines metabolite (VMA) and related hormones due to treatment with ABE.

It is suspected that the distortions of the pituitary cells may be due to the damaging effects of the plant extract. Caution should be exercised in its application, especially as an antipsychotic agent.

Effect on ABE on kidney Function

The therapeutic importance of the extract of the stem bark of *A. boonei* in folk medicine have been documented (Iwu, 1993, Raji *et al.*, 2005, and Elisabestky and Costa campos, 2006). Notwithstanding, there is paucity of information regarding the possible adverse effect or toxicity of the plant extract in spite of its wide spread use in folk medicine practice. The current study showed that the ethanolic extract of the stem bark could be potentially nephrotoxic, especially when the dose is high and the duration of use extended.

The administration of the extract resulted in the loss of functional capacity of the kidney. This can be measured by the dye excretion tests, concentration and dilution test as well as method for examination of blood concentration of excretory products and electrolyte constituents, to demonstrate the presence or absence of active lesion in the kidney and assess the normal functioning capacity of different parts of the functioning unit-nephron (Panda, 1999). However, urea, creatinine and electrolytes (Na^+, K^+, HCO_3^-, Cl^-) are the most sensitive biochemical markers employed in the diagnosis of renal damage because urea and creatinine are excreted through the kidney while electrolytes are reabsorbed and excreted in the tubules. In cellular damage, there will be retention of urea and creatinine in the blood and non re-absorption or non-excretion of electrolytes by the tubules (Guyton, 1996).

From Fig. 4.13 however, the stem bark of *Alstonia boonei* caused dose and duration dependent increase in serum urea with significant increase ($p < 0.05$) observed. Also creatinine showed a significant increase ($p<0.05$) at 50 and 200mg/kg bw after four weeks only at 200mg/kg dose (Fig. 4.14). Urea and creatinine are waste products of protein metabolism that need to be excreted by the kidney, therefore marked increase in serum urea and creatinine are indications of functional damage to the kidney (Panda, 1999).

Serum urea concentration can be increased by many other factors such as dehydration, antidiuretic drugs, diet etc which are not associated with kidney damage. Creatinine is therefore more specific to kidney since kidney damage is the only significant factor that increases serum creatinine concentration (Cheesbrough, 1998). Therefore the significant increase in urea and creatinine at high dose and longer duration showed that the kidney was adversely affected by the extract. But the adverse effect was prominent when high dose of the extract was given for longer period. The significant increase in urea/creatinine ratio is also a measure of renal toxicity (Fig 4.19).

The final profile of Na^+ concentration after four weeks in which sodium decreased significantly at 200mg/kg may be associated with the diuretic property of the plant which may have affected the functional capacity of the nephron especially the tubular function, thereby impairing the response to aldosterone and therefore the exchange mechanisms involving re-absorption of sodium (Yakubu et al., 2005) (Fig 4.15).

Potassium ions play important role in nerve impulses propagation. The Na^+ pump maintains the intracellular K^+ concentration of 140m M/L as against the extracellular K^+ concentration of 5Mm/L (Tietz et al., 1994). The increase in potassium observed after four weeks of extract administration was significant ($p<0.05$) at 50mg/kg and 200mg/kg dose relative to the control (Fig. 4.16). This result may be an indication that the extract affect the Na^+ pump . Hyperkalaemia is a dangerous condition because of its effect on the cardiovascular system, but it rarely occurs unless renal function is depressed (Blaustein and Hamyln 1991). This may be possible, with adverse effect on the pump that maintains the homeostasis of the extracellular electrolyte concentration (Zilva *et al.*, 1991). However, increase in potassium observed after four weeks is an indication of reduced glomerular filtration rate of the kidney (Mayne, 1994) which may be due to prolonged administration of the extract and corresponding impairment of potassium excretion.

The increase in serum bicarbonate by the extract (Fig. 4.17) is a reflection of likely compromise of glumerula filtration process due to possible decrease in glomerula filtration associated with damage of the renal cells by the extract. Chloride increased significantly ($p<0.05$) at 200mg/kg doses after four weeks when compared with the control (Fig 4.18). Increase in serum chloride can be seen in dehydration, renal tubular acidosis, and acute renal failure (Waugh and Grant, 2006). The increase in chloride seen here may therefore be attributed to dehydration associated with the diuretic action of ABE which was observed in the course of the study.

Histologically the rat kidneys treated for 2 weeks showed mild oedema, hyalinization and increase in size of endothelial and mesothelial cells. Glomerular and pyelonephritis were observed for rats treated for 4 weeks , the kidney showed infiltration of the glomerular tuft, enlarged lobule, vacuolated cytoplasm, oedema, glomerular degeneration, possibly due to severe necrosis. Necrosis is one of the end causes of inflammatory responses (Kumar *et al.,* 2001) giving rise to possible kidney dysfunction and failure. (Plates 4.20 -4.24, table 4.4b). This result may not be surprising since the kidney is the primary organ of excretion and might have been exposed to the necrotic principle present in the extract.

It is therefore speculated that ABE may be nephrotoxic, especially at high doses and on chronic application.

The Effect of ABE on Liver Function

The beneficial uses of the extract of *Alstonia boonei* have been reported (Iwu 1993, Raji *et al.*, 2005, Elizabersky and Costa-campos, 2006). However, this study showed that the extract could be potentially hepatotoxic especially when the administration is prolonged; and more severe when the dose is high, in rats.

The administration of the extract resulted in significant ($p<0.05$) weight loss in the rats treated with 200mg/kg of the extract for four weeks. Kucera *et al.*, (1972) and Iwu

(1993) reported the diuretic and hypotensive actions of the plant extract. The diuretic activity may result in loss of water and electrolytes (especially sodium) and consequently loss of body weight. The present study showed significant ($p<0.05$) elevation of plasma hepatospecific marker- enzymes.The serum aspartate aminotransaminase (AST) activity increased after two weeks at 200mg/kg bw and after four weeks at both doses (Fig.4.20). Alanine aminotransaminase (ALT) activity increased significantly ($p<0.05$) too (Fig.4.21). This outcome is an indication of hepatotoxicity.

Experimental studies however have shown that while membrane changes are sufficient to allow passage of intracellular enzymes to the extracellular compartment, cell damage or cell death exacerbates membrane permeability causing some cytosolic enzymes to spill into the sinusoids and from there to the peripheral blood (Adamsel *et al.,* 2001). The measurement of enzyme activities in plasma or serum provides useful information in studying necrotic changes occurring in some tissues and organs especially the liver. Apartate amino transminase AST is a diagnostic marker in man and animals; with ALT being more specific to the liver (Woodman, 1999).

However, in this study, increase in AST and ALT after four weeks may indicate that prolonged administration of the extract could cause liver damage. Nevertheless, alkaline phosphatase (ALP) can be increased also by liver damage especially when the problem is due to obstructive jaundice, as well as by other factors such as bone disease. But increase in AST and ALT are not seen in bone disease (Eastham, 1995), as such the increase in ALP found here is also from the liver.

Biochemical alterations in bilirubin concentration after two and four weeks were observed but none was significant ($p>0.05$). In liver disease associated with some degree of hepatic damage, serum bilirubin concentration, AST and ALT activities are elevated even before clinical symptoms of the disease appear, especially in jaundice (Yakubu *et al.,* 2005).

The possible damage to the liver by *A. boonei* extract has not been previously reported, moreso in rats, and with the histological changes. The mechanism and the possible constituent responsible for the toxicity are therefore not yet established. However, echitamine, an alkaloid found in the extract of the stem bark of *Alstonia boonei* had earlier been reported by Baliga *et al.,* (2004) as the main cause of high AST, ALT and ALP in rats and mice treated with the extract of *Alstonia scholaris*. Since echitamine is a common constituent of the stem bark of the Alstonia speices, the increase in the enzymatic activities of the hepatospecific markers seen here may be associated with the presence of echitamine.

The result of the current investigation indicates that *Alstonia boonei* caused deleterious effect to the rat liver. Swollen hepatocytes, large thick wall and fatty infiltrations were observed in the liver necropsy sections in the test groups (Plates 4.25-4.29 table 4.4c). The lesions observed might have occurred due to the presence of necrolytic metabolites in the plant extract, The liver of the control group was normal (Plate 4.25).

The component of the plant extract that may be responsible for the observed histological changes have not been identified. However the presence of tissue damaging alkaloids or saponins in the extracts might have contributed to such histopatholigcal observations (Ajagonna and Onyeyili, 2002, 2003). In a similar study, servere necrosis was reported in the liver of animals treated with Crotalaria specie containing tissue necrotic alkaloids (Nuhu *et al.*, 2000).

These alterations in the functional indices of the liver and their values which do not compare favourably with the baseline controls are clear indications of the adverse effects of the extract on the parameters evaluated. The present study has shown that repeated and long term administration of the extract could be deleterious to the basic functions of the liver, and the effect could be more severe when high dose of extract is given.

The Effect of ABE on Lipid and Haematological Profile:

Atherogenicity with subsequent cardiovascular manifestations is one of the major causes of death and morbidity in the world (Raju and Binda, 2005). Various studies indicate that high serum cholesterol levels are strongly related to coronary atherosclerosis and increased risk of coronary atery diseases (CAD). Clinical studies have also shown that lowering levels of serum cholesterol using diets or drugs decreases the risk of incidence of coronary heart disease (Treasure, *et al.,* 1995).

Increased LDL cholesterol with decreased HDL cholesterol usually increases the serum total cholesterol. This is because the plasma clearance of cholesterol is often impaired in the presence of low HDL-C. In recent times,triacyglycerols concentrations has also been found to increase with increase in plasma cholesterol.

Atherogenicity therefore develops when LDL cholesterol, triacylglycerols and total cholesterol are elevated relative to plasma HDL-C. Elevated HDL-cholesterol improves the transportation of cholesterol from the plasma to the liver for biotransformation and excretion, thereby preventing atheroma formation and blood vessel occlusion (Ojiako and Nwanjo, 2005a).

The administration of 50 and 200mg/kg ABE produced hyperlipidaemic and atherogenic effect after two weeks of treatment. The ABE significantly ($p < 0.05$) increased the ratio of total cholesterol to HDL-C (atherogenic risk predictor indices) to undesirable values, and also increased the log Tg/HDL-C in the rats (Fig. 4.30-4.31). The use of these indices as atherogenic risk predictors were adopted by Ojiako and Nwanjo (2005b & 2009). The significant increase increase in these ratios is a possible indication that ABE may increase the risk of coronary artery disease (CAD) at the tested doses and duration.

Saponin, one of the constituents of the plant extract has been reported to be responsible for some of the pharmacological activities observed in the plant extract.

Saponin is a pro-oxidant and can generate free radicals (Paula, 2001). Antioxidants prevent the oxidative modification of lipoproteins before their incorporation into the fatty streaks of the arterial wall. Studies have shown that oxidation of lipids increases their deposition on arterial walls, hence atherosclerosis and atherogenicity. However, the exact mechanism by which antioxidants lower blood cholesterol is not properly established. Galton and Krone (1991) suggested that it could be by promoting the stimulation of cholesterol excretion in the faeces via its biotransformation to bile acids. However, Baliga *et al.*, (2004) in a study of *Alstonia scholaris* extract (which also contains saponin) reported that saponin increased plasma cholesterol. The study also showed that it exhibited a dose and time dependent cholesterol peroxidative effect.

The blood is the vital fluid that transports gases and nutrients to the tissues of the body. The biochemistry of the blood is directly linked to the functional capacity of the blood. The functional capacity of the blood is associated with the status of the blood parameters. The status of the blood parameters are indices of health and disease conditions such as anemia. The blood is also host to foreign bodies, including drugs and drug products.

The ABE are widely employed in traditional medicine practice where they are claimed to alleviate a wide range of ailments. Irrespective of their target organs, the extracts are conveyed to their sites of action via the blood stream. This makes it imperative to study the effect of this extract on blood parameters.

The 200mg/kg bw ABE at the 4^{th} week significantly decreased some heamatological parameters especially haemoglobin, WBC and reticulocytes. (Fig. 4.35-4.42).

Decreased haemoglobin value is associated with weight loss as fall in serum reticulocytes indicates depression in the formation of new blood cells. Costa-campos, *et.al*, (1999) and Baliga (2004) reported that echitamine alkaloid from *A. boonei*

reduced the total WBC count dose dependently as seen in this study (Fig. 4.42). The reduction of WBC count may lead to compromise of systemic immunity, and possible predisposition to opportunistic diseases and infections. It is suspected that *A. boonei* stem extract caused hyperlipidaemic effect at the tested doses. Further studies are suggested to confirm the validity or otherwise of these findings.

Effect of ABE on Lipid Peroxidation:

The oxidative break down of polyunsaturated fatty acids (PUFA) predisposes to oxidative stress and stress-related conditions (Halliwell, 1991). This condition can be induced under paracetamol hepatotoxicity (George and Nmoka, 2003). The development could be monitored by the assay of plasma malondialdehyde (MDA) which is a serum specific oxidative marker. The attenuation of the plasma concentration is an indication of antioxidant actively and vice- versa. Oxidative stress can also be evaluated by measuring changes in the serum concentration of the antioxidant vitamins such as retinol, ascorbate and alpha- tocopherol. The increase of the serum concentrations of these vitamins is taken as possible indication of antioxidant effect and vice-versa (Hojo *et al.*, 2001; George and Nmoka, 2003; Nwanjo *et al.*, 2007).

Akimoladum *et al.,* (2007) demonstrated that the aqueous extract of the plant stem bark possess low antioxidant activity. In a related study, Oze et al., (2010) showed that the ethanol extract of the plant may have pro-oxidant effect because it significantly elevated serum MDA concentration, and attenuated serum ascorbate and α-tocopherol concentrations in rats. Orisakwe *et al.,* (2010) observed that the risk of toxicity is more apparent with crude natural products.

Figures 4.32 & 4.33 further showed that ABE at the doses of 50 and 200mg/kg attenuated the serum concentrations of the antioxidant vitamins C and E in the test groups when compared with the controls. The results suggest the enhancement of

oxidative indices by the extract. This may be a possible indication that the extract posses pro-oxidant effect.

The mechanism for the pro-oxidant potential is unknown. However, the phytochemical studies revealed the presence of phenolic compounds, alkaloids (Iwu, 1993, Afolabi *et al*., 2007), cardiac glycosides and saponins (Akimoladun *et al*., 2007). Saponins and phenols are known to have potent oxidizing potentials; while the cardiac glycosides are known to have narrow therapeutic index, which makes them toxic and sometimes lethal; with little variation in doses.

It is speculated that ABE may possess pro-oxidant and possibe cytotoxic properties. This agrees with the folkloric use of the extract as arrow-poison, as anthelmint, as antimicrobial agent, and in the management of supporative sores and wound healing.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

From the foregoing, the ethanol extract of *A. boonei* may have deleterious biochemical effects on the reproductive parameters of albino rats. This outcome may be a possible indication of the toxic effects on the human users of the plant extract. The study also showed that long term users of the extract stand greater health risks. This finding also underscores the need for caution and the rational use of the plant extract because of its suspected toxicity.

5.2 RECOMMENDATIONS

On the basis of the results, the following are recommended:

(a) Pregnat women should be adviced to keep away from the plant extract, especially in the early stages of pregnancy, in other to avoid possible defects on the foetus.

(b) Men who have fertility problems should be cautious if they must use the extract.

(c) It will be wise for girls at the age of puberty to avoid high dose of the extract because of possible damage to the uterine cells.

(d) Individuals with compromised liver or kidney function should seek professional medical advice and avoid taking the plant extract, especially at high dosage.

(e) Stress prone individuals should apply caution if they must use the extract.

(f) Some aspects of health education of the folkmedicine practitioners and users may be necessary to avert potential dangers of irrational application of the extract in traditional healthcare management.

(g) A short term application of the extract should be adopted to forestall adverse biochemical effects on reproductive and other organs.

(h) The doses should be standardized because of the untoward effects associated with higher doses in this study.

(i) Greater and collaborative scientific research should be put in the study of the plant extracts because of the apparent prospects of its chemical components.

(j) There is the need for the government to finance institutional researches in natural products (especially *Alstonia boonei*) because of their inherent advantages.

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APPENDIX

APPENDIX I

Calculation of Dose of Extract

Concentration of stock solution of extract = 100 mg/ml

Dose to be given = 50 mg/kg or 50 mg/1000g

For a rat weighing 200g:

50mg/kg bw

50mg/1000g bw

1000g rat will receive 50mg of extract.

1.0g rat will receive 50/1000 mg.

Therefore,

200g will receive (50/1000) x 200mg = 10mg.

Question:

In what volume of 100mg/ml extracts do we have 10mg?

100mg is contained in 1.0ml.

1mg will be contained in (1/100)ml

10mg will be contained in (1/100) x 10ml = 0.1ml of extract.

The same procedure applies for 200mg/kg body weight.

APPENDIX II

Material and Reagents

During the course of the study the following materials were used:

- Wistar albino rats
- Stainless steel cage/wooden cage
- Saw dust
- Grower feed
- Alstonia boonei stem bark extract
- Elctric weighing scale (Mettler PN 163)
- Weight scale
- Thomas contact mill (pye, unicam Cambridge, England)
- 70% ethanol
- Soxhlet extractor(standard model, India)
- Oven (Accumax, India)
- Measuring cylinders and beakers
- Cannula, syringes and needles
- Centrifuge test tubes and racks Stirrer (magnetic bar)
- Rotatory evaporator (model – laborato, 4000, England)
- Feed and water container
- Laboratory coat and gloves
- Centrifuge (Wisperfuge model 1384, Holland)
- Pipettes, masking tape and cotton wool
- Refrigerator, spectrophotometer and water bath
- Lighter, tripod stand and burnsen burner
- Acurex (EISA System, USA)
- Elecsys Autoanalyser (model 1010, Roche Mannheinn, Germany)
- Biosystem VMA Test kit

- Biochromaic photometer (1904, Awareness Technology Inc., USA)
- Humalyte (Human, Germany)
- Automatic Chemisry Analyser (Awareness Trechnology Inc., USA)
- Humana80 (Human, Germany)
- Vitros Chemistry Autoanalyser (DT6011, Johnson Inc., USA)
- Sysmex Autoanalyser (KX-21N, Sysmex Inc., USA)
- Automated Histokinetic Tissue Procesor II (Miles, USA)
- Spencer's Cryostat (England)
- Hertz Rotary Microtome (1010-SMT-011, England)
- Rotary Evaporator (Laborato, China)
- Olympus Stereo Microscope
- Digital photomicrographic camera (Leica, DM500, Switzerland).

APPENDIX III

Preparation of Reagents for phytochemical studies.

Anisaldehyde-Sulphuric Acid Reagents (AS)

(Stahl, 1973; Wagner et. al. 1984).

0.5ml Anisaldehyde is mixed with 10ml glacial acetic acid, followed 85ml methanol and 5ml concentrated sulphuric acid, in that order. The reagent has limited stability, and is no longer usable when the colour has turned to red-voilet.

Anisaldehyde-Phosphomolybdic Acid Reagent (AP) (Stahl, 1973).

1.0g Phosphomolybdic acid is dissolved in 10ml anisaldehydesulphuric acid.

Kedde Reagent (Stahl, 1973)

0.1g 3,5-dinitrobenzoic acid is dissolved in 10ml methanol (must always be freshly prepared).

Dragendorff Reagent

(Stahl, 1973; Wagner et. al. 1984).

Stock solution: 2.6g basic bismuth carbonate and 7.0g sodium iodide are boiled with 25ml gacial acetic acid for a few minutes. After 12 hours, the precipitate sodium acetate crystals are filtered off. Of the clear brown filtrate, 20ml is mixed with some ethyl acetate and 0.5ml water, stable for some time in brown bottles.

Spray solution: of the stock solution, 0.5ml is mixed with 12ml ethyl acetate and 5ml glacial acetic acid.

Mayer's Reagent (Stahl, 1973).

1.35g mercury (II) chloride and 5.0g potassium iodide are dissolved in 30ml water and then brought to 100ml with water.

Blood Agar:

(Stahl, 1973; Wagner et. al. 1984).

(a) Nutrient agar is heated to about 80^{o}C on a water bath with stiring.

(b) After cooling the agar solution in (a) to about 40 ^{o}C, 7ml of serum free defibrinated blood is added to 93ml toagar solution and shaken. About 50ml of the blood-agar suspension is immediately cast in an agar plate in which it solidifies (to be freshly prepared before use).

APPENDIX IV

	Body Weight of female rats (g) n=5						Percentage Mean Weight gain(g) (n=5)
Dose (mg/kg)	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	
0.0	118	156	180	202	212	219	85.6 ± 3.5
100	117	142	174	175	167	164	40.2 ± 5.1
200	127	148	162	153	150	133	22.7 ± 3.6
300	97	137	160	133	128	119	4.7 ± 0.7

Dose (mg/kg)	1	2	3	4	5	Cum. mean foetal weight (n=5)
0.0	4.97	3.72	4.53	4.22	3.13	4.11
100	3.81	3.11	3.62	3.90	2.73	3.43
200	3.52	3.72	3.60	2.12	3.59	3.31
300	2.83	3.0	3.11	-	3.14	2.42

Serum testosterone concentration (ng/ml)

2 Weeks Segment

Dose (mg/kg)	(Mean ± SD) (n = 5)
0.0	1.32 ± 0.16
50	1.0 ± 0.23
200	1.10 ± 0.20

4 Weeks Segment

Dose (mg/kg)	(Mean ± SD) (n = 5)
0.0	1.40 ± 0.11
50	1.44 ± 0.23
200	1.38 ± 0.25

Serum vanilmandelic acid concentration in rats (mg/ml) (n = 5)

2 Weeks Segment

Dose (mg/kg)	
0.0	2.10 ± 0.01
50	2.83 ± 0.06
200	3.32 ± 0.08

4 Weeks Segment

Dose (mg/kg)	
0.0	2.3 ± 0.06
50	3.21 ± 0.02
200	4.07 ± 0.01

Ratio of Urea to Creatinine

2 weeks segment

Dose (mg/kg)	Urea / Creatinine ratio
0.0	46.81
50	50.67
200	68.53

Weeks Segment

Dose (mg/kg)	Urea / Creatinine ratio
0.0	43.03
50	78.44
200	75.45

Potassium

2 Weeks Segment

Dose (mg/kg)	Serum Potassium Concentration (mmol/L)
0.0	2.13 ± 0.09
50	2.3 ± 0.38
200	4.4 ± 0.07

4 Weeks Segment

Dose (mg/kg)	Serum Potassium Concentration (mmol/L)
0.0	1.9 ± 0.08
50	2.7 ± 0.53
200	3.0 ± 0.49

Bicarbonate

2 weeks segment

Dose (mg/kg)	Serum bicarbonate Concentration
0.0	4.9 ± 0.27
50	8.8 ± 0.77
200	7.4 ± 0.74

4 weeks segment

Dose (mg/kg)	Serum bicarbonate Concentration
0.0	4.5 ± 0.26
50	6.5 ± 1.4
200	6.2 ± 0.8

Serum Chloride

2 weeks segment

Dose (mg/kg)	
0.0	34.4 ± 0.3
50	36.6 ± 0.45
200	36.5 ± 0.5

Table IV.15

4 weeks segment

Dose (mg/kg)	
0.0	36.4 ± 0.3
50	37.0 ± 3.3
200	41.3 ± 2.2

Serum glucose concentration in rats (mg/dl)

Dose (mg/kg)	
0.0	72.4 ± 3.7
100	87.3 ± 2.7
200	88.2 ± 7.3
300	86.0 ± 5.9

LIVER

Aspartate Aminotransaminase

2 weeks segment

Dose (mg/kg)	AST (IU/L)
0.0	8.6 ± 1.82
50	15 ± 9.3
200	28.0 ± 0.62

4 weeks segment

Dose (mg/kg)	AST (IU/L)
0.0	8.4 ± 1.14
50	26.6 ± 2.47
200	70.4 ± 1.18

Alanine Aminotransaminase

2 weeks segment

Dose (mg/kg)	ALT (IU/L)
0.0	7.2 ± 1.67
50	15.6 ± 0.64
200	16.8 ± 0.66

4 weeks segment

Dose (mg/kg)	ALT (IU/L)
0.0	7.0 ± 1.58
50	26.6 ± 0.73
200	29.2 ± 0.89

Alkaline Phosphatase

2 weeks segment

Dose (mg/kg)	ALP (IU/L)
0.0	61.0 ± 1.58
50	69 ± 2.39
200	91.0 ± 2.96

4 weeks segment

Dose (mg/kg)	ALP (IU/L)
0.0	61.4 ± 1.67
50	88.4 ± 2.7
200	94.8 ± 0.56

Total Bilirubin

2 weeks segment

Dose (mg/kg)	TB (mg/dl)
0.0	0.77 ± 0.03
50	0.72 ± 0.03
200	0.76 ± 0.12

4 weeks segment

Dose (mg/kg)	TB (mg/dl)
0.0	0.73 ± 0.07
50	0.74 ± 0.03
200	1.0 ± 0.12

Conjugated Bilirubin

2 Weeks Segment

Dose (mg/kg)	Concentration of Conjugated Bilirubin
0.0	0.39 ± 0.02
50	0.37 ± 0.02
200	0.38 ± 0.10

4 Weeks Segment

Dose (mg/kg)	Concentration of Conjugated Bilirubin
0.0	0.37 ± 0.01
50	0.38 ± 0.01
200	0.40 ± 0.22

Unconjugated Bilirubin

2 weeks segment

Dose (mg/kg)	UB (mg/dl)
0.0	0.37 ± 0.01
50	0.35 ± 0.04
200	0.38 ± 0.04

4 weeks segment

Dose (mg/kg)	UB (mg/dl)
0.0	0.36 ± 0.02
50	0.37 ± 0.01
200	0.61 ± 0.06

Blood Parameters

RBC

2 weeks segment

Dose (mg/kg)	RBC (x 10^{12}/L)
0.0	8.0 ± 0.4
50	7.7 ± 0.3
200	7.3 ± 0.4

4 weeks segment

Dose (mg/kg)	RBC (x 10^{12}/L)
0.0	8.2 ± 0.4
50	7.3 ± 0.3
200	6.7 ± 0.2

Haemoglobin

2 weeks segment

Dose (mg/kg)	Hb (g/L)
0.0	16.4 ± 1.5
50	15.7 ± 1.2
200	13.1 ± 1.1

4 weeks segment

Dose (mg/kg)	Hb (g/L)
0.0	16.8 ± 1.3
50	13.0 ± 0.1
200	11.5 ± 0.5

Packed Cell Volume

2 weeks segment

Dose (mg/kg)	PCV (L/L)
0.0	0.42 ± 0.05
50	0.34 ± 0.04
200	0.33 ± 0.04

4 weeks segment

Dose (mg/kg)	PCV (L/L)
0.0	0.39 ± 0.04
50	0.3 ± 0.02
200	0.24 ± 0.03

MCV

2 weeks segment

Dose (mg/kg)	MCV(fl)
0.0	53.4 ± 5.5
50	53.4 ± 5.6
200	51.7 ± 1.0

4 weeks segment

Dose (mg/kg)	MCV(fl)
0.0	52.8 ± 2.5
50	52.8 ± 3.1
200	49.8 ± 3.3

Mean Cell Haemoglobin

2 weeks segment

Dose (mg/kg)	MCH (pg)
0.0	21.3 ± 1.1
50	21.2 ± 2.0
200	20.5 ± 0.7

4 weeks segment MCH

Dose (mg/kg)	MCH (pg)
0.0	20.8 ± 0.7
50	20.0 ± 1.0
200	18.3 ± 0.7

Mean Cell Haemoglobin Concentration

2 weeks segment

Dose (mg/kg)	MCHC (g/dl)
0.0	38.4 ± 6.4
50	38.6 ± 1.2
200	38.2 ± 2.7

4 weeks segment

Dose (mg/kg)	MCHC (g/dl)
0.0	38.9 ± 5.3
50	37.7 ± 5.8
200	37.5± 1.7

Recticulocytes

2 weeks segment

Dose (mg/kg)	Retics (%)
0.0	1.7 ± 0.5
50	2.5 ± 0.5
200	1.5 ± 0.05

4 weeks segment

Dose (mg/kg)	Retics (%)
0.0	1.7 ± 0.04
50	1.5 ± 0.04
200	1.2 ± 0.04

White Blood Cell

2 weeks segment

Dose (mg/kg)	Total WBC (counts x 10^9/L)
0.0	12.6 ± 1.7
50	13.9 ± 1.8
200	10.2 ± 1.2

4 weeks segment

Dose (mg/kg)	Total WBC (counts x 10^9/L)
0.0	13.0 ± 0.3
50	11.7 ± 1.3
200	6.8 ± 0.8

Mean Serum Estrogen Concentration (n=5) (pg/ml)

Duration of treatment(weeks)	Control	50	100	200
2	42.1±1.76	43.5±1.59	48.9±2.1	60.6±0.57
4	43.1±1.33	55.6±1.96	61.7±2.8	84.6±3.32

Mean Serum Progesterone Concentration (ng/ml)

Duration Of treatment (weeks)	Control	50	100	200
2	0.43 ± 0.02	0.46 ± 0.03	0.57 ± 0.08	0.78 ± 0.06
4	0.45 ± 0.03	0.57 ±0.64	0.72 ± 0.09	1.37 ± 0.12

Mean Serum Lipid Profile (n= 5) (Mg/dl)

2 WEEKS SEGMENT				
DOSE	**TC**	**Tg**	**HDL-C**	**TC/HDL-C**
0.0 (control)	111.1 ± 0.26	92.8 ± 0.32	33.3 ± 2.2	3.3 ± 0.01
50	125.4 ± 3.2	97.1 ± 0.32	32.49 ± 7.2	2.9 ± 0.01
200	124.4 ± 3.2	84.9 ± 2.4	34.32 ± 4.7	2.6 ± 0.01

4 WEEKS SEGMENT				
DOSE	TC	Tg	HDL-C	TC/HDL-C
0.0 (control)	88.4 ± 3.27	87.8 ± 1.3	32.3 ± 0.3	3.5 ± 0.07
50	241.5 ± 2.7	187.1± 3.7	34.4 ± 0.67	6.7 ± 0.01
200	262.3 ± 3.3	190.7 ± 3.0	30.7 ± 0.92	9.7 ± 0.08

APPENDIX V

Reagent for semen analysis of reagents for determination of sperm characteristics.

Sperm Diluting Fluid:

Sodium bicarbonate	5gm
Formalin	$1cm^3$
Distilled water	$100cm^3$
$1cm^3$ of semen	$9cm^3$

Dissolve 0.1g of eosin in 20ml of fresh physiological saline

5% v/v Ethanol

Calculate fuchsine

Dilute loefflers methylene blue.

APPENDIX VI

Raw Data Of Serum Testosterone Level (mg/ml) For Each Rat In A Group And Mean (mg/ml) For Each Group At 2 Weeks And 4 Weeks.

Rat at (2wks)	Control (2wks)	50mg/kg (2wks)	200mg/kg (2wks)	Rat at (4wks)	Control (4wks)	50mg/kg (4wks)	200mg/kg (4wks)
	Group A	Group B	Group C		Group D	Group E	Group F
R_1	1.2	0.8	1.2	R_1	1.5	1.4	1.2
R_2	1.0	1.3	0.8	R_2	1.5	1.8	1.3
R_3	1.4	0.9	1.0	R_3	1.6	1.5	1.4
R_4	1.3	1.2	1.3	R_4	1.3	1.2	1.8
R_5	1.1	0.8	1.2	R_5	1.4	1.3	1.2
X ± S.D	1.2±0.16	1.0±0.23	1.1±0.20		1.4±0.11	1.44±0.23	1.38±0.25

APPENDIX VII

RAW DATA OF SPERM MOTILITY (%) FOR EACH RAT IN A GROUP AND MEAN VALUE OF SPERM MOTILITY (%) FOR EACH GROUP AT 2WEEKS AND 4WEEKS

Rat at (2wks)	Control (2wks)	50mg/kg (2wks)	200mg/kg (2wks)	Rat at (4wks)	Control (4wks)	50mg/kg (4wks)	200mg/kg (4wks)
	Group A	Group B	Group C		Group D	Group E	Group F
R_1	84	35	3.5	R_1	62	10	30
R_2	80	42	2.5	R_2	60	14	22
R_3	79	48	4.0	R_3	64	15	28
R_4	82	38	3.5	R_4	60	12	25
R_5	85	40	3.8	R_5	64	15	25
TOTAL	**410**	**203**	**16.8**		**310**	**66**	**130**
X ± S.D	82.0±2.55	40.6±4.88	3.36±0.61		61.4±1.67	13.2±2.17	26±3.10

APPENDIX VIII

RAW DATA OF SPERM VIABILITY (%) FOR EACH RAT IN A GROUP AND MEAN VALUE OF SPERM VIABILITY (%) FOR EACH GROUP AT 2WEEKS AND 4WEEKS

Rat at (2wks)	Control (2wks) Group A	50mg/kg (2wks) Group B	200mg/kg (2wks) Group C	Rat at (4wks)	Control (4wks) Group D	50mg/kg (4wks) Group E	200mg/kg (4wks) Group F
R_1	85	50	3.0	R_1	62	46	50
R_2	86	48	2.2	R_2	60	48	54
R_3	90	46	2.0	R_3	64	44	54
R_4	88	52	2.5	R_4	60	48	48
R_5	88	54	2.4	R_5	62	40	56
TOTAL	**437**	**250**	**12.1**		**308**	**223**	**262**
X ± S.D	87.4±1.95	50±3.16	2.42±0.38		61.6±1.67	44.6±2.97	52.4±3.27

APPENDIX IX

RAW DATA OF TOTAL SPERM COUNT (million/ml) FOR EACH RAT IN A GROUP AND MEAN VALUE OF TOTAL SPERM COUNT (million/ml) FOR EACH GROUP AT 2WEEKS AND 4WEEKS

Rat at (2wks)	Control (2wks) Group A	50mg/kg (2wks) Group B	200mg/kg (2wks) Group C	Rat at (4wks)	Control (4wks) Group D	50mg/kg (4wks) Group E	200mg/kg (4wks) Group F
R_1	70	50	50	R_1	56	50	48
R_2	72	54	48	R_2	55	52	52
R_3	75	55	46	R_3	54	48	54
R_4	78	52	52	R_4	52	46	53
R_5	74	50	53	R_5	57	50	50
TOTAL	**369**	**261**	**249**		**274**	**246**	**257**
X ± S.D	73.8±3.03	52.2±2.86	49.8±2.86		54.8±1.92	40±2.65	57.4±2.41

APPENDIX X

RAW DATA OF MORPHOLOGY (NORMAL AND ABNORMAL) (%) FOR EACH RAT IN A GROUP AND MEAN VALUE OF MORPHOLOGY (%) FOR EACH GROUP AT 2WEEKS

Rat at (2wks)	Control at (2wks) A		50mg/kg (2wks) B		200mg/kg (2wks) C	
	N	AB	N	AB	N	AB
R_1	60	40	25	75	40	60
R_2	65	35	45	55	45	55
R_3	62	38	30	40	40	60
R_4	70	30	35	48	48	52
R_5	66	34	40	42	42	58
TOTAL	**323**	**177**	**177**	**325**	**215**	**285**
X ± S.D	64.6±3.85	35.4±3.85	35±7.91	65.0±7.91	43.0±3.46	57±3.46

APPENDIX XI

RAW DATA OF MORPHOLOGY (NORMAL AND ABNORMAL) (%) FOR EACH RAT IN A GROUP AND MEAN VALUE OF MORPHOLOGY (%) FOR EACH GROUP AT 4 WEEKS

Rat at (4wks)	Control at (4wks) A		50mg/kg (4wks) B		200mg/kg (4wks) C	
	N	AB	N	AB	N	AB
R_1	80	20	40	60	20	80
R_2	86	24	50	50	24	76
R_3	70	30	55	45	30	70
R_4	74	26	38	62	28	72
R_5	68	32	30	70	34	66
TOTAL	**378**	**132**	**213**	**287**	**136**	**364**
X ± 5.0	70±7.40	26.4±4.77	42.6±9.93	57.4±9.94	27.2±5.40	72.6±5.27

APPENDIX XII

REAGENT USED IN LIPID PER OXIDATION TEST

Malondialdehyde:

1. Thiobabituric acid (TBA) 0.67% was prepared by dissolving `0.67g of TBA in 60ml of deionzed H_2O. The volume was then made up to 100ml of deionized H_2O.

2. Trichloroacetic acid (TCA) 20% Thus reagent was prepared by dissolving 20g% of trichloroacetic in 100ml of deionized H_2O.

Malondialdehy curve: prepare by using 1,1,3,3
Tetraethoxypropane (TEP). Hydrochloroactric acid
Butanol

Vitamin C Reagent:

i. Trichloroacetic acid crystals (10percent W/V)

ii. 2 4-Dinitropheny hydrazine reagent-crstalline compound (2.0gm) was dissolved in 100ml 0f 9.0N sulphuric acid (75ml of water Plus 25ml of concentrated sulpuric acid). The solution was filtered and Stored in brown bottle in the refrigerator. A portion was always refiltered before use.

iii. Thiourea solution: Thiourea (10gm) was dissolved in 100ml of 50 percent ethanol and refrigerated.

iv. Cupric sulfate solution (1.5 percent) $CUSO_4$: 5HO (1.5gm) was dissolved and diluted to 100ml.

v. Combined colour reagent. A mixture of 5ml of 2,4-dinitrophenylhydrazme reagent, 0.1ml of cupric sulfate solution, and 0.1ml og thiourea solution were freshly prepared on the day of

use.

vi. Sulpuric acid (85 percent).
Concentrated sulphuric acid (180ml) was added to 20ml of distilled Water, mixed,cooled and stored in a glass – stoppered bottle in the Refrigerator.

VITAMIN E REAGENT:

i. Absolute ethanol (aldehyde – free)

ii. Xylene

iii. 2,2-Dipyridyl, 1.20g/l in n-propanol ie 0.12g of 2,2 dipyridyl was dissolved in 100ml of n-propanol.

iv. Ferric chloride solution, $fecl_3.6\ H_2O$/L (1.2g) was dissolved in 100ml of ethanol and kept in a brown bottle.

v. Standard solution of D-1-2- Tocopherol (10mg/L in ethanol). This was prepared from 30mg/ml solution of D-L-2 tocopherol.

APPENDIX XIII

Formular for the calculation of result of plasma vitamin E measured with spectrophotometer.

$$\text{Vitamin E} = \frac{\text{Reading at 520nm} - \text{Reading at 460nm} \times 10 \times 0.29}{\text{Reading of standard 520nm}}$$

APPENDIX XIV

Formular for the calculation of malondialdehyde standard curve using standard dilution and Optical density.

1. 100 dilution = 15nm (2.46ng MDA/ml) given therefore 0.1M = (5nm(246g MDA/ml)

1:25 dilution = 0.24 = $\frac{2.46}{0.10} \times \frac{0.24}{1}$ 5.90ma MDA/ml

APPENDIX XV

Statistical terms and formular

Mean x = $\frac{\sum x}{N}$

Where: $\sum$ = Summation

X = value of observation

N = of value of observation.

SD = $\sqrt{\sum \frac{(x-x)2}{n-1}}$

Where: SD = Standard Deviation

$\sum$ = Summation

X = mean.

T – test

T calculated = (Xt – xc)

$$\frac{(nt--1)St2 + (nc-1)}{nt + nc}$$

Nt = Sample size of test

n_c = Sample size of control

St = Standard deviation of test

Sc = Standard deviation of control

Hypothesis: Ho ×1 = x_2 = Null hypothesis

H_1x 1 = X_2 = alternative hypothesis level of significance = (at. 0.05 ie 95%)

Critical region T 2/2

T tabulated = 2.22.8

Degree of freedom = n_1+n_2-2

Where n_1 = number of samples in group Ho if T calculated = T tabulated (there is statistically significant difference between the groups compared). But accept Ho if other wise no statistically significant difference between the groups compared).

APPENDIX XVI

RAW DATA OF ANTIOXIDANT VITAMINS (mg/dl) AND MALONDIALDEHYDE LEVELS (nmol/ml) OF DIFFERENT GROUPS

GROUP 1

S/N	VITAMIN C	VITAMIN E	MDA
1	1.30	1.17	0.12
2	1.00	1.09	0.89
3	1.40	0.98	0.64
4	0.98	0.93	0.10
5	1.50	1.20	0.08
Mean±SD	1.24±0.21	1.07±0.10	0.37±0.34

GROUP 2

S/N	VITAMIN C	VITAMIN E	MDA
1	0.35	0.60	1.07
2	0.40	0.56	0.14
3	0.36	0.49	1.06
4	0.48	0.56	0.45
5	0.42	0.64	0.91
Mean±SD	0.38±0.03	0.57±0.03	0.73±0.37

GROUP 3

S/N	VITAMIN C	VITAMIN E	MDA
1	0.42	0.67	0.29
2	1.90	1.02	0.96
3	1.47	0.77	0.11
4	0.41	0.82	0.78
5	0.99	1.00	0.10
Mean±SD	0.73±0.03	0.86±0.13	0.45±0.36

GROUP 4

S/N	VITAMIN C	VITAMIN E	MDA
1	0.69	0.88	0.64
2	1.20	0.83	0.12
3	1.00	0.77	0.10
4	0.96	1.20	0.97
5	1.78	0.64	0.36
Mean±SD	1.13±0.37	0.86±0.19	0.44±0.33

APPENDIX XVII

VALUES FOR MALONDIALDEHYDE STANDARD CURVE

Standard dilutions	Optical density	Concentration of MDA
1:25	0.24	5.90
1:50	0.17	4.18
1:75	0.09	2.71
1:100	0.10	2.46
1:150	0.07	1.72
1:200	0.06	1.48
1:400	0.04	0.98
1:500	0.05	1.23
1:800	0.03	0.74
1:1000	0.05	0.98