ANTIMALARIAL AND ANTITUBERCULAR ACTIVITIES OF CRUDE METHANOL EXTRACT AND FRACTIONS OF THE BULB OF *CRINUM JAGUS* (Linn.)

BY

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ABSTRACT

Crinum jagus is a medicinal plant used traditionally to treat tuberculosis, malaria and other bacterial infections. However, there are limited documented scientific studies to substantiate the use of this plant. Due to increase in resistance to malaria and tuberculosis drugs, the need for the development of other drugs is pertinent. This study was designed to determine the pharmacological activities of extract and fractions of *Crinum jagus*.

Methanol extract of *C. jagus* obtained by soxhlet extraction was subjected to phytochemical analysis and fractionated using column chromatography. Antitubercular and antimicrobial activities of the extract and its fractions were evaluated against isolates of *Mycobacterium tuberculosis* and selected microorganisms using the disc and agar diffusion methods. Antimalarial activity was assessed *in vivo* using Rane's test in *Plasmodium berghei* infected mice (n = 80 in 10 groups) treated orally with tween 80 (control), 10, 25, 50 and 75 mg/kg of extract and its fractions at 10 mg/kg respectively, while chloroquine (10 mg/kg) and arteether (3 mg/kg) groups served as positive controls. Anti-inflammatory potential was assessed in rats using carrageenan-induced paw inflammatory model. *In vitro* antioxidant potentials were determined spectrophotometrically using 1,1-diphenyl-2-picryl hydrazyl (DPPH), hydroxyl radical scavenging activities, Total Flavonoids Contents (TFC) and Phenolic Contents (TPC) Antioxidant indices- Superoxide dismutase (SOD) and Catalase (CAT) activities and levels of Malondialdehyde (MDA) and reduced Glutathione (GSH) were determined by spectrophotometry. Aspartate (AST) and Alanine (ALT) amino transferases and Alkaline Phosphatase (ALP) were estimated spectrophotometrically. Data were analysed by Student's t test at p = 0.05.

Phytochemical analysis revealed the presence of alkaloids, flavonoids, phenols and steroids in the crude extract. The extract and its fractions (F1, F2 and F3) showed a concentration-dependent inhibition of *Mycobacterium tuberculosis*, with F1 having the lowest $IC_{50}$of : 0.22mg/mL relative to rifampicin (IC_{50} : 0.19mg/mL) and isoniazid (0.23mg/mL). The extract at 10, 25, 50, 75 mg/kg and F1, F2 and F3 at 10 mg/kg suppressed parasitaemia in *Plasmodium berghei* infected mice by 70.0, 76.0, 79.0, 87.0% and 89.0, 76.0, 78.0% respectively relative to chloroquine (100%) and arteether (89.0%). The extract at 10, 25, 50, 75 mg/kg and F1, F2 and F3 at 10 mg/kg inhibited oedema in rat paws by 26.0, 30.0, 32.0, 66.0% and 80.0, 25.0, 52.0%

respectively when compared with indomethacin (95.0%). The extract and its fractions significantly scavenged DPPH and hydroxyl radical *in vitro*. The TPC and TFC of extract, F1, F2 and F3 at 500 µg/ml were 0.310, 0.460, 0.240, 0.380 µg/mg and 0.523, 0.864, 0.396, 0.643 µg/g respectively. The extract and its fractions significantly reduced MDA level while GSH, SOD and CAT levels were increased. Activities of AST, ALT and ALP were significantly increased at 50 and 75 mg/kg body weight of extract .

Crinum jagus exhibited antitubercular, antimalarial and anti-inflammatory activities via scavenging of radicals and antioxidative mechanism. This indicates a promising potential of the plant for drug development.

Keywords: *Crinum jagus*, antituberculosis, antimalarial, antioxidant.

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CERTIFICATION

I certify that this work was carried out by KOLAWOLE ADEBOLA OLAYEMI in the Department of Biochemistry, University of Ibadan, Nigeria.

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DEDICATION

This work is dedicated to Almighty God, for giving me strength to carry out this work.

My late mother for giving me a sound education despite all odds.

My husband for loving and supporting me.

My daughter for enduring with me.

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ABBREVIATION AND SYMBOLS USED

ACTs	:	Artemisin-Combination Therapies
AFB		Acid Fast Bacillus
AIDS	:	Acquired Immune Deficiency Syndrome
ALP	:	Alkaline Phophatase
ALT	:	Alanine AminoTransferase
AST	:	Aspartate Amino Transferase
ATP	:	Adenosine Triphosphate
BC	:	Before the Birth of Christ
BCG	:	Bacillus Calmette-Guerin Vaccine
BSA	:	Bovine Serum Albumin
CD	:	Conjugated Diene
CDNB	:	1-Chloro-2, 4 Dinitrobenzene
COX	:	Cyclooxygenase
COX-2	:	Cyclooxyganease (II)
Cu^{2t}	:	Copper (II) ion
$CUSO_4$	:	Copper (II) Sulphate
DNA	:	Deoxyribonucleic Acid
DNPH	:	Dinitrophenyl Hydrazine
Do	:	Paw cirucumferenceat hour just before carrageenan injection
DPPH	:	1,1-Diphenyl-2-picrylhydrazyl
DTNB	:	5,5,-Dithiobis -2 nitrobenzioic acid
EDTA	:	Ethylene Diamine tetra Acetic Acid
ETC	:	Electron Transport Chain
F1	:	Fraction 1
F2	:	Fraction 2
F3	:	Fraction 3
F_e^{2t}	:	Iron(II) ion
F_e^{3t}	:	Iron(III) ion
F_eCl_3	:	Iron(III) Chloride
Fig	:	Figure

FP	:	Fe (11) Protoporphyrin
FRIN	:	Forestry Research Institute of Nigeria
FSC	:	Fused Silica Capillary
G-	:	Gram Negative
GAE	:	Gallic Acid Equivalent
GC-MS	:	Gas Chromatography Mass Spectroscopy
GOT	:	Glutamate Oxaloacetate Transaminase
GPT	:	Glutamate Pyruvic Transaminase
GSH	:	Reduced Glutathione
GST	:	Glutathione –S- Transferase
G^+	:	Gram Positive
H&E	:	Haematoxylinand Eosin
H_2O_2	:	Hydrogen Peroxide
Hb	:	Haemoglobin
HCl	:	Hydrochloric Acid
HDL-C	:	High Density Lipoprotein Cholestrol
HIV	:	Human ImmunoDefficiency Virus
IC_{50}	:	Fifty Percent Inhibitory Concentration
IMRAT	:	Institute for Advanced Medical Research and Training
$K_2Cr_2O_7$	:	Potassium heptaoxodichromate (V)
KH_2PO_4	:	Potassium hydrogen phosphate
KI	:	Potassium Iodide
LDH	:	Lactate Dehydrogenase
LDL	:	Low Density Lipoprotein
LDLC	:	Low Density Lipoprotein Cholestrol
LJ	:	Lowestein Jensen
LTB	:	Latent Tuberculosis Infection
Na_2HPO_4	:	Disoduim hydrogen phosphate
MBC	:	Minimum Bactericidal Concentration
MCH	:	Mean Corpuscular Haemoglobin
MCHC	:	Mean Corpuscular Haemoglobin Concentration

MCV	:	Mean Corpuscular Volume
MDA	:	Malondialdehyde
MDR	:	Multi Drug Resitant
MFC	:	Minimum Fungicidal Concentration
MIC	:	Minimum Inhibitory Concentration
MST	:	Mean Survival Time
MTB	:	*Mycobacterium tuberculosis*
NaCl	:	Sodium Chloride
NaOH	:	Sodium Hydroxide
NIAD	:	National Institute of Allergy and Infection Disease
NIST	:	National Institute of Science and Technology
NSAIDS	:	Non SteroidalAntinflammatory Drugs
O_2	:	Superoxide
OH^-	:	Hydroxyl ion
PAMPs	:	Associated Molecular Patterns
PCV	:	Packed Cell Volume
PPRs	:	Pattern Recognition Receptor
PUFAS	:	Poly Unsaturated Fatty Acids
RBC	:	Red Blood Cells
Rf	:	Retarding factors
ROS	:	Reactive Oxygen Species
RT	:	Retention Time
SOD	:	Superoxide Dismutase
TB	:	Tuberculosis
TBA	:	Thiobarbituric Acid
TC	:	Total Cholestrol
TG	:	Triglyceride
TLC	:	Thin Layer Chromatography
UNESCO	:	United Nations Educational Scientific and Cultural Organisaiton
US	:	United State
VLDL	:	Very Low Density Lipoprotein

WBC	:	White Blood Cells
WHO	:	World Health Organization
XDR	:	Extensively Drug Resistant
XDR-TB	:	Extensively Drug Resistant Tuberculosis

CHAPTER ONE

1.0 INTRODUCTION

In all countries of the world, there exists traditional knowledge related to health of humans and animals. According to World Health Organization (WHO, 1978a; WHO, 1978b) the definition of traditional medicine may be summarized as the sub-total of all knowledge or practice, whether explicable or inexplicable, used in diagonising, preventing or eliminating a physical, mental or social disease which may rely exclusively on past experience or observations handed down from generation to generation, verbally or in writing. It also comprises therapeutic practices that have been in existence often for hundreds of years before the development of modern scientific medicine and are still in use today without any documented evidence of adverse effects.

Traditional medicine practice is an important part of health care delivery system (Sheldom *et al.*, 1997). Traditional folklore medicine plays an important role in health services around the globe. About three quarters of the World's population relies on plant and its extract for health care (Premanathan *et al.*, 2000, Gabhe *et al.*, 2006). A good number of populations particularly those living in villages depend largely on herbal medicine.

Traditional medicine has expanded globally and is clearly gaining patronage, its acceptability for primary health care is not limited to poor developing countries but in countries where conventional medicine is predominantly in the national health care system (Lanfraco,1999). In developed countries such as the United States of America, majority of people (55%) combine alternative treatment with conventional medicine (Stein, 2004).

Traditional medicine as a major part of Africa socio-cultural heritage, obviously in existence for several hundred years, was once believed to be primitive and wrongly challenged with animosity, especially by foreign religions dating back to the colonial days in Africa and subsequently by the conventional or orthodox medical practitioner (Elujoba *et al.*, 2005). Traditional medicine has been brought into focus for meeting the goals of an under coverage of primary health care delivery not only in Africa but also, to various extent, in all countries of the

world. Traditional medicine is the first choice health care system for at least 80% of Africans who suffer from high fever and other common ailments.

Primary health care is the key to the development of a national health policy as defined by the Alma-Ata Declaration in 1978. It is an essential health care based on practical, scientifically sound and socially acceptable methods and technology. It is made universally acceptable to individuals and families in the community and through their full participation and at a cost that the community and the country can afford in order to maintain at every stage of their development, in the spirit of self reliance and self determination. It is the first level of contact for the individual family and the community within the national health care system, bringing health care as close as possible to where people live and work and this constitutes the first element of a continuing health care process (WHO, 1978a). A health system based on primary health care was adopted as the means of achieving the goal of health for all by the year 2000.

The WHO has since urged developing countries of the world to utilize the resources of traditional medicine for achieving the goals of primary health care.

Traditional medicine has remained the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities, and local therapy is the only means of medical treatment for such communities (Yinger and Yewhalaw, 2007). The World Health organization (WHO, 2001) reported that herbal medicine serves the health needs of about 80% of the world population, especially for millions of people in the vast rural areas of the developing countries

There are clear trends to show that the mainstream in pharmaceutical research is moving away from single target approach to combination and multiple target approaches (Wermuth, 2004). The problems with the traditional herbal medicines, especially in developing countries, have to do with the methods, preparations and standardization of herbal formulations of recent times.

Herbal medicine is the oldest form of health care known to mankind. Recorded history has it that medicinal plants have been in use for the past fifty centuries, which until the last two and half centuries were the main source of treatment of man and animal. The use of plant in medicine is older than recorded history (Osai, 1998). Medicinal plants form the basis of

traditional health care delivery system used by about 80% of the population of most developing countries who rely on traditional medicine for their health care needs (Ghani and Onaolapo, 1989).

Medicinal plants have been used over the years in the rural communities to treat infections and diseases and have had, undoubtedly, good results hence there is a level of reliance on plants as whole chemotherapeutics which involves the extraction and development of drugs from plants, and their derivatives has been carried out over the years to counter the effect of many diseases. This means that specific pants called medicinal plants are of great importance in the manufacture of drugs since the chemical component of plants are used for the production of these drugs (Houghton and Raman, 1998).

It is also known that about 60% of the global population does not have readily available access to modern drugs and medication thereby leading to an increased mortality rate. Hence the need arises to seek cheaper and available alternative to help alleviate these diseases. The use of traditional medicine and medicinal plants in most developing countries as the normative basis for the maintenance of good health has been widely observed (UNESCO, 1998). Furthermore, reliance on the use of medicinal plants in the industrialized societies have been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from the traditionally used rural herbal remedies (UNESCO, 1998).

Various organic compounds are derived from plants that are important in combating different diseases (Enwuru, 2008). Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions and to defend against attack from predators such as insects, fungi and herbivorous animals. Many of these phytochemcials have beneficial effects on long term health when consumed by human and can be used to effectively treat human diseases. At least 12,000 such compounds have been so far isolated , a number estimated to be less than 10% of the total number (Lai and Roy, 2004; Fabricant and Farnsworth, 2001). Chemical components in plants mediate their effect on the human body through a process identical to those already well understood for conventional drugs. Thus herbal medicine do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicine to be as effective as conventional medicines but also give

them the same potential to cause harmful side effect (Lai and Roy, 2004, Fabricants and Farnsworth, 2001). All plants produce chemical compounds known as phytochemical as part of their normal metabolic activities.

Phytochemicals are divided into two classes, namely :

- Primary metabolites such as sugar and fats which are found in all plants
- Secondary metabolites which are found in a smaller range and serving more specific function (Billiry and Sherman, 1998). Examples of secondary metabolites are alkaloids, polyphenols, glycosides, terpenes, steroids, tannins.

In developing countries of the World such as Nigeria, Kenya, Zimbabwe and Malawi the use of medicinal plant, is well acknowledged and established (Hedberg,1988). This is particularly so in rural areas where the services of modern hospitals may be limited and also in some circumstances when traditional medicine seems to be preferred. The main problem in the use of traditional medicine is the proof requirement that the active components contained in the medicinal plant are useful, safe and effective. The proof of pharmacological activity that are available at present are mostly based on empirical experience. The scientific poof then becomes the most important theory in order to eliminate the concern of using medicinal plant, as drug of alternative treatment.

Ethnobotany which is the study of traditional human uses of plants, is a preliminary method of research suitable for gathering information on the use of plants. The 'quack' medical knowledge handed down by the common people constitutes oral sources of information useful for scientific research, therefore science and tradition have a strong connection between them, science in fact has often traditional origin (Lentini , 2000). During the last few decades, there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world but documenting the indigenous knowledge through entnobotanical studies is important for the conservation and utilization of biological resources. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plant for the treatment of various diseases (Aziazeh *et al.*, 2003).

Traditional medical knowledge of medicinal plants and their use by indigenous culture is not only useful for conservation of cultural tradition and biodiversity but also for community health care and drug development in the present and future (Pei, 2001).

Africa is known to be richly endowed with medicinal plants, one of such plants is *Crinum jagus*. The plant is found in tropical and subtropical areas, worldwide, where for centuries, they have been used traditionally to cure ailments and diseases . The powdered bulb is taken orally with honey as a remedy for tuberculosis in Southern part of Nigeria (Idu *et al*., 2010). The bulb is eaten raw as a cure for snake bite in some parts of Nigeria (Ode *et al*., 2006). The bulb of the plant is used for the treatment of asthma cough in Western part of Nigeria and is commonly called asthma cough plant (Ogunkunle and Olopade, 2011). The warm leaf juice of the bulb of the plant with a pinch of common salt is used for ear-complaint as an emetics (Gill, 1992). Among the Binis, the decoction of the bulb of the plant is used as a vermifuge and purgative (Gill, 1992). The bulb of *Crinum jagus* is used in Southern Nigeria for memory loss and other mental symptoms associated with aging (Peter *et al*., 2004). Morphine, hamayne and lycorine have been isolated as active alkaloids of *Crinum jagus* (Banwell *et al*., 2011). Preliminary phytochemical study of the plant revealed the presence of alkaloids, flavonoids, saponins phenols, steroids (Ode *et al.*, 2010). Previous work done on the therapetuic importance of the bulb extract of the plant reported the antibacterial and antifungal activities (Adesanya *et al.*, 1992), antitumour, immunostimulating and anticonvulsant activities (Edema and Okieimen, 2002; Azikiwe and Siminilay, 2012), anti-snake venom activity (Ode and Azuzu, 2006), antimutotic activity (Nwankamma and Okoli, 2010), anti-oxidant and anti-haemorrhagic activities (Ode *et al*.,2010) as well as anti-cholinergic activity (Peter *et al*., 2004).

Figure 1 : The bulb of *Crinum jagus*

1.1 Rationale

Medicinal plants have been an integral part of ancient health practices for centuries. Today, modern medicine recognizes medicinal plants as alternative and complementary therapeutics for a host of conditions. Among the most popular uses of plants include; increase energy, disease prevention, pain relief and general health (Rica, 2010). Herbal mixtures are increasingly becoming the choice of most patients nowadays. The promise of presenting the natural quantities that promote a healthier method of healing different ailments contributed to the popularity of these medicines, especially, as these types of medicine are grown wild or can be tendered at one's home background. It is also cheaper as compared to manufactured medicines.

Herbal medicine is the alternative for commercially manufactured medicines which are already made available in the market. Herbal medicine differs from modern medicines in view of the fact that it is produced with one hundred recent content of natural plant extract believed to have medicinal values as effective as the modern drugs, less side effects and less cost. There are several herbal plants widely used to treat various illnesses. Herbal plants are scientifically studied to support the benefit claimed to contain each plant. These studies are recognized and acknowledged by the medical community to have proven effectiveness recommended to treat multitude of illnesses. Note worthy break through are being produced by researchers and there is a steady progress and indeed very promising trend towards meaningful research into the development of essential drugs from numerous local medicinal plants in the developing countries of the world. It is against this background that the bulb of *Crinum jagus* used in some parts of Nigeria for treating tuberculosis, asthma, ear pain, memory loss, snake bite, convulsion and as antibacterial, antifungal, vermifuge, purgative was investigated in this study.

The main objective was to investigate the biological activities of the crude methanol extract and fractions of the bulb of *Crinum jagus* to provide scientific evidence for the traditional use of the plant for treatment of these various diseases.

Specific objectives include the following:

- To determine the secondary metabolites present in the crude extract
- To investigate the antioxidant activity of the crude extract and fractions of the plant (*in vitro and in vivo)*

- To assess the antimicrobial activity of the crude extract and fractions of the plant.
- To evaluate the possible anti-inflammatory activity of the crude extract and fractions of the plant.
- To evaluate the antiplasmodial activity of the crude extract and fractions of the plant in *Plasmodium berghei* infected mice
- To investigate the possible inhibitory effect of the crude extract and fractions of the plant on *Mycobacterium tuberculosis* isolates.
- To evaluate the toxicological profile of the plant extract

CHAPTER TWO

LITERATURE REVIEW

2.0 The *Crinum* species

The genus *Crinum* belong to the Family Amaryllidaceae, Phyllum-Angiospermae and Subphyllum-Liliflora and it is distributed throughout the tropics and warm temperature regions of the world.Amaryllidaceae is a great widely spread family all over the world and contains about 90 genera and 1,310 species (Benson, 1970) with wide geographical distribution throughout the tropics, subtropics and warm temperature regions of the world (Mabberly, 1990). The genus *Crinum* represents an important sector in the family. The name *Crinum* originates from Greek "Krinon" which means White lily and they are commonly known as milk river or veld lilies. This genus is related to a group of mostly Southern African endemic genera constituting the tribe Amaryllidaceae (Meerow and Sinijman, 1998). Like other members of Amaryllidaceae, *Crinum* can occupy many different habitats such as seasonal dry places, ephemeral pools, rainforest, coastal areas and river blanks. Worldwide, *Crinum* comprises about 130 species distributed in Africa, America, Southern Asia and Australia, Africa lands enjoy most species and about twenty one are endemic to Southern Africa.

Crinum are perennial herbaceous plants with giant fleshy bulbs larger in stature than most other species of Amaryllidaceae. They can grow from 1-5 feet in height depending on the species and they produce a neck or pseudo stem made up of the sheathing old leaves. Worldwide, *Crinum* species have a substantial economic value as ornamentals due to their showy flowers. In addition, huge numbers are traded for traditional medicines. Several species are cultivated for medicinal purposes, (Burkhill, 1985; Ghosal *et al.*, 1985, Fennel, 2001; Tram *et al.*, 2002). Since about the 1950"s, *Crinum* species have been subjected to extensive chemical, cytological and pharmacologically evaluation of active principles (Wildman,1960). Phytochemical investigations have resulted in isolation of diverse classes of compounds and have been focused predominantly on alkaloids. Phenolics prevail among the non alkaloidal constituents (Tram *et al.*, 2002)

2.1 Chemistry of *Crinum* Species

The ease of hybridisation of *Crinum* species has afforded about160 of these species, out of which 30 have been investigated. The following ring structures are found in the alkaloidal skeleton, Crinane (5,10b- ethanophenatridine), Lycorine (pyrrolophnathridine), Galanthamine, Ryllistine and Cherylline. Structural variations occur in ring C (double bond and subtituent). Examples include 3-oxocrinine (Fig1:1) from *Crinum americanum* and palmilycorine (Fig 1:2) from *Crinum asiaticum*. Trisphaeridin (Fig 1:3) found in *Crinum americanus* was the first phenathridine type alkaloid. Zeylamine (Fig 1:4) was found in *Crinum zeylanicum* while 5 hydroxyhomolycorine (Fig 1.5) was found in *Crinum dexifum*. These compounds can be found in the bulb or fruits (Ali *et al*., 198la; Ghosa *et al*., 1981 and Tram *et al*., 2002).

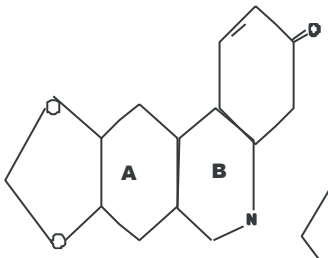


Figure 2.1: 3 (Oxocrinine)

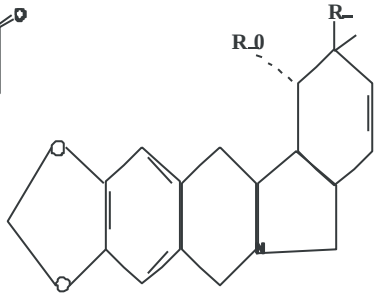


Figure 2.2 : Palmilycorine
R_1 = Palmitoyl, R = XOH

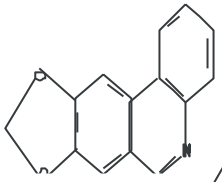


Figure 2.3 : Trisphearidin

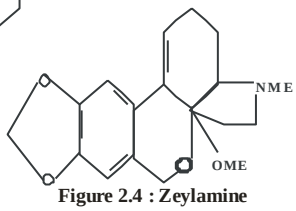


Figure 2.4 : Zeylamine

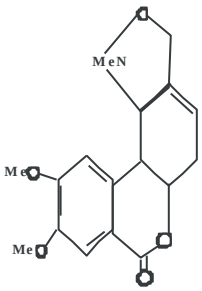


Figure 2.5 : 5 (Hydroxyhomolycorine

Figure 2: Alkaloids from Crinum species
(Ghosal et al., 1985)

The non alkaloidal compounds present in the *Crinum* species consist of the following :

(i) Long chain alkanes

(ii) Acids and Esters (examples of which are arachidonic acid, capric acid and palmitic acid from *Crinum augustum*, *Crinum americanum* and *Crinum bulbispermium*)

(iii) Alcohols and ketones (example of which are triacentanol and 5- hydroxy hexacosan - 9-one from *Crinum augustum*)

(iv) Terpenoids and sterols (examples of which are Vomifoliol and Sitosterol from *Crinum firmifolium* and *Crinum moorei*)

(v) Flavonoids (examples of which are 4-hydroxyl-7-methoxyflavan and 4-hydroxyl-7-methoxy-8-methylflavan)

(vi) Chromones (examples of which are eugenin and noreugeni from *Crinum moorei*)

(vii) Carbohydrates, an example of which is pectin from *Crinum amabile*).

Resins and polysaccharides have also been shown to be present in the *Crinum* bulbs (Ghosal *et al.*, 1985 and Tram *et al.*, 2002).

2.2 Biological and Pharmacological Activities of *Crinum* Species

Various biological activities assigned to *Crinum* species include :

(a) Antibacterial and antifungal activities of *Crinum* species

Crinamine from *Crinum jagus* possess strong antibacterial activity while lycorine, hamayne and 6-hydroxycrinamine are inactive (Adesanya *et al*., 1992)

(b) Antiparasitic activity

Augustine and lycorine from *Crinum ornatum* showed moderate antimalaria activity against *Plasmodium falciparum* but the selectivity was low compared with antimalaria control compounds (Ali *et al.*, 198la and b)

(c) Antitumour and immunostimulating activities

Lycorine and 6-hydroxycrinamine from *Crinum delagoense* were active against BL-6 mouse melanoma cells, (Hanghwitz *et al*., 1965; Onyirinka and Jackson, 1978 ; Ali *et al*., 1981b).

(d) Insecticidal activity

Crinisine from the bulb of *Crinum asiaticum* was an effective insecticide (Tram *et al*., 2002)

(e) Anti-cholinergic activity

Alkaloidal fractions from the bulb of *Crinum jagus* and *Crinum glaucum* showed inhibition of acetylcholine esterase, an activity exploited therapeutically to raise the depressed levels of acetylcholine in the brain associated with Alzheimer's disease hence the plants are used in traditional medicine in Southern Nigeria for curing memory loss and other mental symptoms associated with aging (Peter *et al*., 2004).

(f) Anti-snake venom activity

The anti-snake venom activity of the methanol extract of the bulb of *Crinum jagus* was investigated *in-vitro* and *in-vivo* against the venom of three noteable snake species. The bulb extract of *Crinum jagus* showed anti-snake venom activity and protected the injected mice from death, myonecrosis and haemorrhage induced by the effect of the snakes (Ode and Azuzu, 2006).

(g) Anti-convulsant activity

The bulb of *Crinum jagus* and *Crinum ornatum* have been shown to have anti-convulsant activity (Edema and Okiemen 2002; Azikwe *et al*., 2012). Lycorine and haemanthine from *Crinum ornatum* showed a dose dependent anti-convulsant effect (Oloyede *et al*., 2000).

Crinum jagus is a bulbous plant with spirally arranged leaves and conspicuous flowers. It is a largely showy plants with umbels of lily- like flowers. It is found in tropical and subtropical regions throughout the world (Mabbery, 1991). It belongs to the Family: Amaryllidaceae, Genus: *Crinum*, and Species: *jagus.* Its local name is Ogede Odo in Yoruba, Alubarha in Edo and Oyimbakar in Efik/Ibibio. It has a wide spread distribution in Africa, America, Southern Asian and Australia.

Crinum jagus is the largest tropical genus of Amaryllidaceae family (Ode *et al.,* 2010). *Crinum jagus* is a variable species that ocurs in Africa and its leaves may be broad in some forms whereas they are narrower or parallel in other forms. The plant may be found in swampy conditions, seasonal wet lands or in grassland (savannah). Some plant are remarkably fragnant flowered vanilla scented while others may have little or no scent/odours. Buds are enclosed in several sheathing. Morphine, hamayne, and lycorine have been isolated as active alkaloids of *Crinum jagus.* Lycorine has been shown to possess antitumour activity (Banwell *et al*., 2011

2.3 Free Radicals

Oxygen is used as part of the process for generating metabolic energy. However, a small amount of this oxygen often gets loose and produce unstable products called free radicals. By definition, a free radical is an atom e.g oxygen, nitrogen with at least one unpaired electron in the outermost shell that is capable of independent existence (Davies, 1995). Free radicals are highly reactive due to the presence of unpaired electrons. The unpaired electrons participate in various chemical reactions.

A free radical is easily formed when a covalent bond between entities is broken and one electron remains with each newly formed atom (Segal, 2005). Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). Oxygen centred free radicals contain two unpaired electrons in the outermost shell. When free radicals steal one electron from a surrounding compound or molecule a new free radical is formed in its place. In turn, the newly formed radical then looks to return to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules. Thus the chain reaction continues and can be thousands of events long (Diego-Otero *et al.*, 2009).

The electron transport chain (ETC) which is found in the inner mitochondria membrane utilizes oxygen to generate energy in form of adenosine triphosphate (ATP). Oxygen acts as the terminal electron acceptor within the electron transport chain (ETC). During exercise, oxygen consumption increases 10 to 20 fold. In turn, electron ignore escape from the electron transport chain (ETC) is further enhanced. Thus when calculated 6 to 35ml/kg/min of the total oxygen intake during exercise has the ability to form free radicals (Dekkers *et al.*, 1996). Electrons appear to escape from electron transport chain at the ubiquinone cytochrome level (Sjodin *et al.*, 1990).

Polyunsaturated fattyacids (PUFAs) are abundant in cellular membranes and in low density liporotein (LDL) (Dekker *et al.*, 1996). The polyunsaturated fatty acid maintains fluidity of cellular membranes. A free radical prefers to steal electrons from the lipid membrane of a cell initiating a free radical attack on the cells in a process known as lipid peroxidaiton (Fig 3). Reactive oxygen species target the carbon- carbon double bond and polyunsaturated fatty acids. The double bond on the carbon weakens the carbon-hydrogen allowing for easy dissociation of

the hydrogen by a free radical. A free radical will steal the single electron from the hydrogen associated with the carbon at the double bond. In turn, this leaves the carbon with an unpaired electron and hence becomes a free radical. In an effort to stabilize the carbon-centred free radical, molecular rearrangement occurs. The newly arranged molecules is called a conjugated diene (CD). The conjugated diene then very easily react with oxygen to form a proxy radical. The proxy radical steals an electron from another lipid molecule in a process called propagation. This process then continues in a chain reaction (Aly and Shahin, 2010). The lipid peroxidation is a chain reaction, which means that it consists of several interconnected steps where the products of some are the reactants of others. The first step is called initiation and in the case under discussion it involves the formation of a (carbon centred) lipid radical. This is often achieved by hydrogen abstraction from the lipid molecule (LH) by an hydroxyl radical, OH initiation.

$LH + OH \longrightarrow L + H_2O$

The most probable site of attack for the hydroxyl radical is the -CH_2-methylene group that bridges the two double bonds of the chain, simply because this will correspond to the weaker C-H bond (the radical so formed in resonance stabilized by a bis-allylic arrangement) (Ho *et al.*, 1997).

The radical L* can undergo several reaction e.g it can cross-link with other molecular oxygen dissolved in the cells membrane, yielding the peroxyl radical LOO*

Propagation

$L^* + O_2 = LOO^*$

The peroxyl radical can then abstract an hydrogen atom from another molecule of lipid (LH), forming a new lipid radical

Propagation

$LOO^* + LH = LOOH + L^*$

which can react with another oxygen and so on (Huang *et al.*, 1992) although the chain can be broken by a termination step.

Termination.

LOO* + LOO* = molecular products.

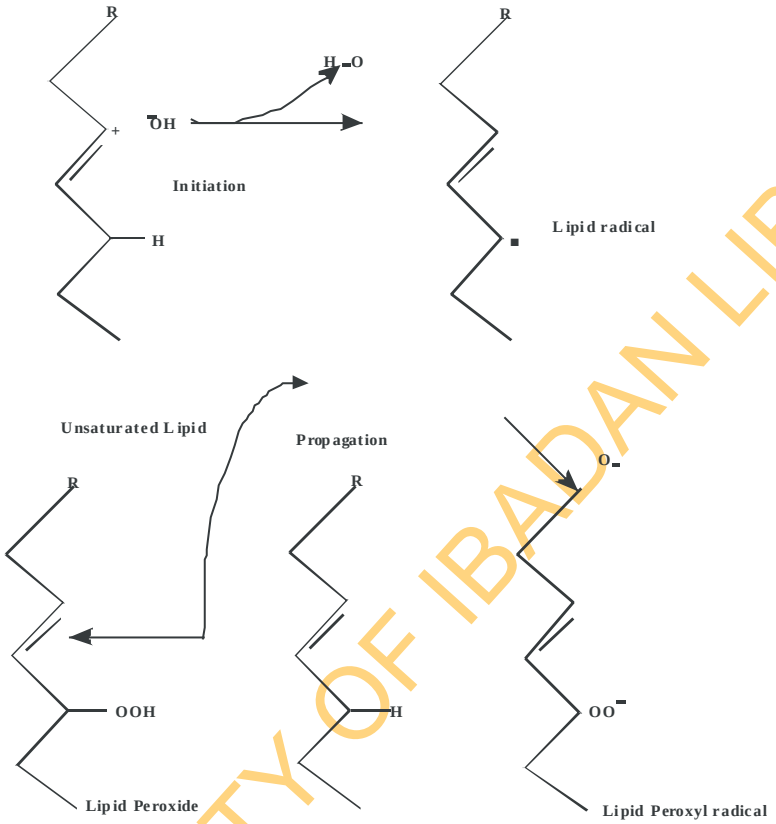


Figure 3 : The free radical mechanism of lipid peroxidation
(Halliwell and Gutteridge, 1989)

Radicals react with other molecules by:

(a) Combining their unpaired electrons to form a covalent bond as follows :

Covalent bond

$$A^* + A^* \longrightarrow A - A$$

(b) Donating their unpaired electron to another molecule or take an electron from another molecule (pairing). Thus a situation where a radical gives one electron to or takes one electron from a non-radical makes the non radical to become a radical thereby leading to a series of chain reactions hence their participation in the pathogenesis of certain disorders, examples of which are cancer, asthma, arthritis, inflammation, artherosclerosis, parkinsons (Harry 1972; Tappel, 1992). These oxygen reactive species are formed under the influence of ultraviolet or ionising radiations, in the presence of various chemicals including; transition metals and xenobiotics. Enzymes or electrons leakage in the course of metabolic pathways may result in the formation of reactive oxygen species.

2.4 Classification of Free Radicals

(i) Superoxide (O_2^-)

Superoxide **(O_2^-)** is produced by addition of a single electron to oxygen and several mechanisms exist by which superoxide can be produced *in-vivo* (Halliwell *et al.*, 1992). Several molecules, including adrenalin, flavones nucleotides, thiol compounds and glucose can be oxidized in the presence of oxygen to produce superoxide and these reactions are greatly accelerated by the presence of transition metals such as iron or copper. The electron transport chain in the inner mithochondria membrane performs the reduction of oxygen to water.During this process, free radical intermediates are generated, which are generally tightly bound to the components of the transport chain. However, there is a constant leak of few electrons into the mithochondrial matrix and this results in the formation of superoxide (Beeker *et al*., 1999). There might also be continuous production of superoxide by vascular endothelium to neutralize nitric oxide (Lius *et al.*, 2002). Superoxide has been implicated in initiating oxidation reaction associated with aging (Cotelle *et al.*, 1996). It has also been implicated in several pathophysiological process due to its transformation into more reactive species such as singlet oxygen, hydrogen peroxide and hydroxyl radical that initate lipid peroxidation and play an

important role in the induction of oxidative damage in DNA, lipid and proteins (Aurand *et al*., 1977)

(ii) **Hydrogen peroxide (H_2O_2)**

Several enzyme reactions including those catalyzed by glycolate oxidase might produce hydrogen peroxide directly (Chance *et al*., 1979). Hydrogen peroxide and oxygen are also formed when superoxide dismutase (SOD) removes superoxide. Hydrogen peroxide is not a free radical itself but is usually included under the general heading of reactive oxygen species (ROS). It is a weak oxidizing agent that might directly damage proteins and enzymes containing reactive thiol group. However, its most vital property is the ability to cross membrane freely, which superoxide generally is not capable of doing (Halliwell *et al*., 1989). Therefore, hydrogen peroxide formed in one location might diffuse a considerable distance before decomposing to yield the highly reactive hydroxyl radical which is likely to mediate most of the toxic effects ascribed to hydrogen peroxide, therefore, hydrogen peroxide act as a conduct to transmit free radical induced damage across cell compartment and between cells.

(iii) **Hydroxyl radical (OH^-)**

Hydroxyl radical (OH^-) is the final mediator of most free radical induced tissue damage (Lloyd *et al*., 1997). All of the reactive oxygen species described above exert most of their pathologic effects by giving rise to hydroxyl radical. The hydroxyl radical is short lived but the most damaging species within the body. The reason for this is that hydroxyl radical with extremely high rate constants react with almost every type of molecule found in living cells inculding sugars, amino acids, lipids and nucleotides. Hydroxyl radical (OH^-) does not persist for more than a few seconds before combining with a molecule in its immediate vicinity. Chain reaction are thus encouraged, and if hydroxyl radical attacks DNA, free radical chain propagate through the DNA and cause chemical alteration of the bases leading to mutation as well as strand breakage (Aruoma *et al*., 1989a). Hydroxyl radical generation is paradoxically the major mechanism by which malignant cells are killed during radiotherapy. Hydroxyl radical is also able to initiate the process of lipid peroxidation by abstracting a hydrogen atom and combining with it to form water, since a hydrogen atom leaves behind an unpaired electron on the carbon atom from which it was abstracted. This carbon radical then undergoes molecular rearrangement

to form a conjugated diene (CD) which then reacts with oxygen to give a peroxyl radical. The peroxyl radical abstracts hydrogen atom from an adjacent fatty acid side chain to continue the process of converting itself into a lipid peroxide (Fig 4). These are stable when pure, however *in-vivo*, their decomposition is catalyzed by copper and iron ions and complexes e.g haemethaemoglobin and cytochromes. Lipid peroxidation is thus a radical reaction.

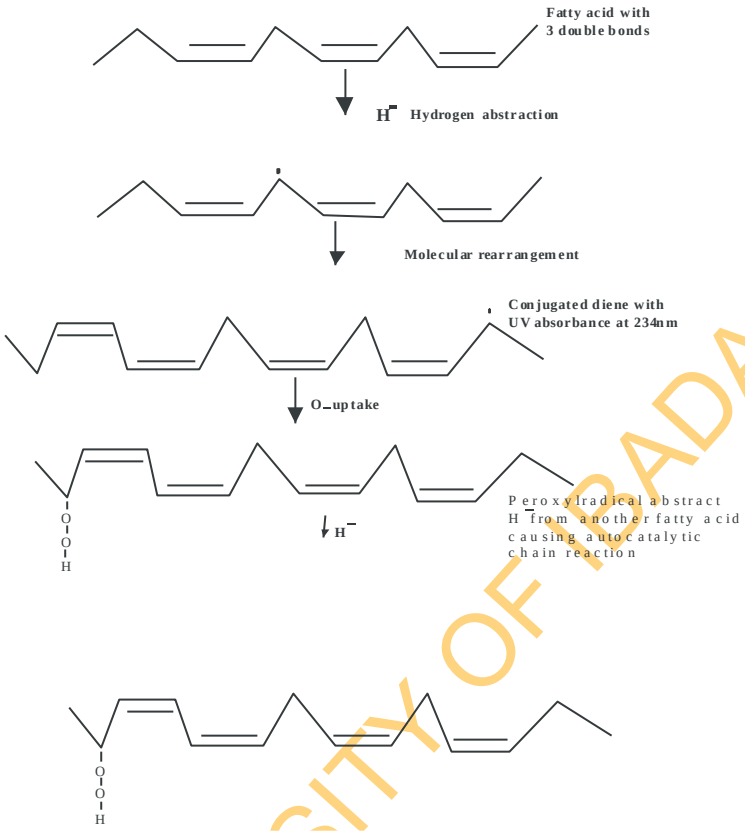


Figure 4: Hydroxyl radical initiated reaction of polyunsaturated fatty acid side chain (Hallwell and Gutteridge., 1984)

Although hydroxyl radical formation can occur in several ways, by far the most important mechanism *in-vivo* is likely to be the transition metal catalyzed decomposition of superoxide and hydrogen peroxide (Stohls, 1995).

(iv) **Transition metals**

All elements in the first row of the d-block of the periodic table are classified as transition metals. In general, they contain one or more unpaired electrons and are, therefore, themselves radicals when in the elemental state. However their key property from the point of view of free radical biology is their variable valency, which allows them to undergo reactions involving the transfer of a single electron. The most important transition metals in human diseases are iron and copper. These elements play a key role in the production of hydroxyl radical *in-vivo* (Stohls *et al.*, 1995). Copper has a full outershell but loses and gains electron very easily making itself a free radical (Halliwell *et al.*, 1985). In addition, iron has the ability to gain and lose electrons very easily. This property makes iron and copper the two common catalyst of oxidation reaction, iron is a major component of red blood cells (RBC). A possible hypothesis is that the stress encountered during oxidation may break down red blood cells releasing free iron. The release of iron can be dentrimental to cellular membrane because of the pro-oxidation effect it can have. Hydrogen peroxide can react with iron (II) or Copper (I) to generate hydroxyl radical in the following reaction, as described by Fenton, 1884.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{2+} + HO + OH$$

(v) **Alkoxyl/peroxyl radical**

Radical reactions have been shown to produce organic peroxides which decompose into alkoxyl and peroxyl radical lipids that can themselves abstract hydrogen and cause the chain reaction of lipid perioxidation when metal ions (Fe^{2+} and Cu^{2+}) are added to lipid systems. The end product of these complex metal ion-catalysed breakdown of lipid hydroperoxide include cytotoxic aldehydes as well as hydrogen carbon gases such as ethane and pentane (Halliwell, 1989).

Table 1 : Free radicals and their effects

Oxidant	Description
Superoxide	One electron state of O_2 formed in many autooxidation reactions and by the electron transport chain. Rather unreactive but can release Fe^{2+} from iron- sulphur. Protein and ferritin undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precausor for metal-catalysed OH formation.
Hydrogen peroxide (H_2O_2)	Two electron reduction state, formed by dismutation of O_2^- or by direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
Hydroxyl radical (OH)	Three electron reduction state ,formed by Fenton reaction and decomposition of peroxy nitrite. Extremely reactive, will attack most components.
Organic hydroperoxide (ROOH)	Formed by radical reactions with cellular components such as lipids and nucleobases
Alkoxyl and peroxyl radical (RO and ROO)	Oxygen centred organic radicals, Lipid forms participate in lipid proxidaiton reaction. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
Hypochlorous acid (HOCl)	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents including thiol groups, amino groups and methionine.
Peroxynitrite ($ONOO^-$)	Formed in a rapid reaction between O_2^- and $NO^\cdot$ Lipid soluble and similar in reactivity to hypochlorus acid. Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen droxide

(Sies 1983; Meyers *et al.*, 1996)

2.5 Physiological Effects of Free Radicals

Under normal conditions (at rest), the antioxidant defence system within the body can easily handle free radicals produced by the body. During times of increased oxygen flux (during exercise), there is increase in free radical production. The body counters the increase in radical production through the antioxidant defence system. When free radical induction exceeds clearance, oxidative damage occurs. Free radicals formed during chronic exercise may exceed the protective capacity of the antioxidant defence system thereby making the body more immune to disease and injury. Free radicals have been implicated in the etiology of a number of diseases such as cardiovascular disease, Parkinson disease (Tanizawa *et al.*, 1992). A free radical attack on a membrane usually damages a cell to the point that it must be removed from the immune system, if free radical formation and attack are not controlled within the muscle during exercise, a large quantity of muscle could be damaged. Damaged inside could in turn inhibit performance by the induction of fatigue.

2.6 Measurement of Free Radicals

Free radicals have a very short half-life which makes them very hard to measure in the laboratory. Multiple methods of measurement are available today, each with their own benefits and limits.

Radicals can be measured using election spin resonance and spin trapping methods. The methods are both very sophisticated and can trap even the short-lived free radical. Exogenous compounds with a high affinity for free radicals (i.e xenobiotics) are utilized in the spin techniques. The compound and radical together, form a stable entity that can be easily measured. This indirect approach has been termed finger printing (Karrison *et al.*, 1997). However this method is not 100% accurate, spin trapping collection techniques have poor sensitivity which can skew result (Acworth and Bally, 1997). A commonly used alternative approach measures markers of free radicals rather than the actual radical. These markers of oxidative stress are measured using a variety of difference assays described below

When a fatty acid is peroxidised, it is broken down into aldehyde such as thiobarbituric acids which are excreted. Thiobarbituric acids (TBAs) have been widely accepted as a general marker of free radical production. The most commonly measured TBAs is malondialdehyde

(MDA) (Karrison, 1997). The TBA test have been challenged because of its lack of specificity. The use of liquid chromatography spectrophotometric techniques help reduce these errors (Wong *et al*., 1987). In addition the test seems to mark best when applied to membrane system such as microsome (Halliwell and Chinico, 1993). Gases such as pentane and ethane are also created as lipid peroxidation occurs. These gases are expired and commonly measured during free radical research (Karrison, 1997). Karter *et al.*, (1988) have reported that serum MDA levels correlated with blood levels of creatinine kinase, an indicator of muscle damage.

Also, conjugated dienes (CD) are often measured as indicators of free radical production (Acmorth and Balley, 1997). Oxidation of unsaturated fatty acids result in the formation of conjugated diene. The conjugated diene formed are measured and provide a marker of the early stage of lipid peroxidation (Halliwell and Gutteridge, 1985). A newly developed technique for measuring free radical production shows promise in producing more valid result. The technique uses monoclonal antibodies and may prove to be the most accurate measurement of free radicals.

2.7 Oxidative Stress

Oxidative stress is defined as an imbalance between oxidants and antioxidants in favour of oxidants, potentially causing damage to cells or cellular components (Sies, 1997). Oxidative stress is caused by an imbalance between the production of reactive oxygen species and biological systems ability to readily detoxify the reactive intermediate or easily repair the resulting damage.

Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all the components of the cells including proteins, lipids and DNA. Further, some reactive oxidative species act as cellular messenger in redox signalling. Thus oxidative stress can cuse disruptions in normal mechanism of cellular signaling.

2.7.1 Oxidative stress and human diseases

In humans, oxidative stress is involved in the development of many disease or may exacerbate their symptoms. These include cancer (Halliwell, 2007), Parkinson's disease (Valko *et al.*, 2007), Alzheimers disease (Robinson, 2000)), atherosclerosis, heart failure (Singh *et al*.,

1995), myocardial infarction (Ramond *et al*., 2011) schizophrenia (Boskovic, 2011), bipolar disorder (Dean *et al*., 2011), fragile X syndrome (Diego *et al*., 2009), sickle cell diseases (Amer *et al*., 2006), lichens planus (Aly and Shahin, 2010), vitiligo (Arican and Kurutus, 2008), autism (James *et al*., 2004), chronic fatigue syndrome (Gwen *et al*., 2005).

Oxidative stress involved in neurodegenerative diseases including Lon Gehrigis disease, Parkinson disease, Alzheimer's disease, Huntinton's disease (Patel and Chi, 2011). Indirect evidence by monitoring biomarkers such as reactive oxygen species and reactive nitrogen species production, indicates oxidative damage which may be involved in pathogenesis of these diseases (Nunomura *et al*., 2005, Boskovic *et al*., 2011) while cummulative oxidative stress with disrupted mithochondrial respiration and mitochondrial damage are related with Alzheimer's, Parkinson and other neurodegenerative disease (Nys and Meirleir, 2006).

Oxidative stress is also linked to certain cardiovascular disease since oxidation of low density lipoprotein (LDL) in the vascular endothelium is a precausor to plaque formation. Oxidative stress also plays a role in the Ischemic cascade due to oxygen reperfusion injury following hypoxia. This cascade inculdes both strokes and heart attack. Oxidative stress also contributes to tissue injury following irradiation and hyperoxia as well as causes diabetes.

Oxidative stress is likely to be involved in age-related development of cancer. The reactive species produced in oxidative stress can cause direct damage to the DNA and are therefore mutagenic and it may also suppress apoptosis and promote proliferation invasiveness and metastalsis (Halliwell, 2007). Infection by *Helicobacter pylori* which increases the production of reactive oxygen and nitrogen species in human stomach is also taught to be important in the development of gastric cancer.

Oxidative stress play adual role in infectious diseases such as trypanosomiasis, malaria and tuberculosis (Ridgley *et al*., 1999; Dondog *et al*., 2003; Kondaveeti *et al*., 2012). In the process of infection, there is generation of reactive species by myloperoxidase, NADPH oxidase and nitric synthase. Some pathologies arising during infection can be attributed to oxidative stress and generation of reactive species in infection can have fatal consequences.

2.8 Antioxidants

An antioxidant is a molecule slowing or preventing the oxidation of other molecules. In their definition of the term "antioxidant", Halliwell and Gutteridge (1989) states that an antioxidant is any substance that when present in low concentration compared with that of oxidisable substrate, specifically delays or inhibits oxidation of that substrate. Oxidation is a chemical reaction that transfers electron from a substance to an oxidizing agent, oxidation reaction can produce free radicals which start chain reactions that damage cells. Antioxidants terminate these reactions by removing free radical intermediates and inhibiting other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols (Sies, 1997). Antioxidants are added as redox systems possessing higher oxidative potential than the drug that they are designed to protect or as chain inhibitors of radical induced decomposition. In general, the effect of antioxidants is to break up the chains formed during propagation process by providing a hydrogen atom or an electron to the free radical and receiving excess energy possessed by the activated molecule (Lachman, 1986). Fruits, vegetables and natural plants contain a large variety of phytochemicals which are the main source of antioxidants in the diet, that could decrease the potential stress caused by reactive oxygen species. The natural antioxidants may have free radical scavengers, reducing agents, potential complexers of prooxidant metals, quenchers of singlet oxygen (Ebadi, 2002). The antioxidant can intefere with the oxidation process by reacting with free radicals (Gupta, 2004)

2.8.1 History of antioxidants

Originally the term antioxidants was specifically used to refer to a chemical that prevented the consumption of oxygen. In the late 19th and 20th Centuries, extensive study was devoted to the use of antioxidant in important industrial processes, such as prevention of metal corrosion; the vulcanistaiton of rubber; and the polymerization of fuels in the fouling of internal combustion engines (Mail, 1947). Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats which is the causes of rancidity (German, 1999). However, it was the identification of vitamin A, C and E as antioxidant that

revolutionized the field and led to the realization of the importance of antioxidants in biochemistry of living organisms (Jacob, 1996; Knight, 1998).

The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be the one that is itself readily oxidised (Moreu and Dufraisse, 1992). Research into how vitamins prevent the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavenging reactive oxygen species before they could damage cells (Wolf, 2005).

A vast majority of complex life requires oxygen for its existence. Oxygen is a highly reactive molecule that damage living organism by producing reactive oxygen species (Davies, 1995). Consequently, organisms contain a complex network of antioxidant metabolites such as vitamin C, vitamin E, glutathione and enzymes such as catalase, superoxide dismutase, various peroxidases that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids (Sies, 1997; Vertuani *et al*., 2004). In general, antioxidant systems either prevent these reactive species from being formed or remove them before they can damage vital components of the cell (Davies, 1995; Sies , 1997). However, since reactive oxygen species do have useful function in cells, such as redox signalling, the function of antioxidant system is not to remove the oxidant entirely but instead to keep them at an optimum level (Rhee, 2006). Antioxidants are classified into two broad divisions depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water soluble antioxidants react with oxidant in the cells cytosol and the blood plasma while lipid soluble antioxidants protect cell membrane from lipid peroxidation (Sies, 1997). These compounds may be synthesized in the body or obtained from the diet (Verutani *et al*., 2004). The different antioxidants are present at a wide range of concentrations in body fluids and tissues with some (such as glutathione or ubiquinone) mostly present within cells while others (such as uric acid) are more evenly distributed. Some antioxidants are only found in a few organisms and these compounds may be important in pathogens and can be virulence factors (Miller, 1997).

2.8.2 Classification of Antioxidants

(1) **Antioxidant enzymes**

(a) **Superoxide dismutase (SOD)**

Superoxide dismutases (SOD) are a class of closely related enzymes that catalyse the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Bannister *et al*., 1987; Zelko *et al*., 2002). SOD enzymes are present in almost all aerobic cells and in the extracellular fluid (Johnson and Giulvi, 2005). SOD enzyme contain metal ion cofactor that, (depending on the isozyme) can be copper, zinc, manganese or iron. In humans, the copper/zinc SOD is present in the mithochondrion (Bannister *et al.*, 1987). There also exists a third form of SOD in extracellular fluids which contain copper and zinc in its active sites (Nozik *et al*., 2005). The mitochondrial isozyme seems to be the most biologically important of these three since mice lacking this enzyme die soon after birth (Melov *et al*., 1998). In contrast, the mice lacking copper/zinc SOD are viable but have numerous pathologies and a reduced life span while mice without the extracellualr SOD have minimal defects (Reaume *et al*., 1996; Ho *et al*., 1998). In plants SOD isozymes are present in the cytosol and mitochondria with an iron SOD found in chloroplasts that is absent form vertebrates and yeast (Van camp *et al*., 1997).

(b). **Catalase**

Catalase are enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen using either iron or manganese cofactor (Zamocky and koller, 1999; Chelikani *et al*., 2004). This protein is localized to peroxisomes in most eukaryotic cells (del Rio *et al*, 1992). Catalase is an unusual enzyme since, despite hydrogen peroxide being its only substrate, it follows a ping-pong mechanism. Its cofactor is oxidize by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate (Hiner *et al*., 2002). Despite its apparent importance in hydrogen peroxide removal, humans with genetic deficiency of catalase acatalasemia suffer few ill effects (Ogata, 1991; Mueller *et al.*, 1997).

(c) **Peroxiredoxins**

Peroxiredoxins are peroxidases that catalyse the reduction of hydrogen peroxide, organic hydroperoxides as well as peroxynitrite (Rhee *et al.*, 2005). They are divided into three classes,

namely; typical 2-cysteine peroxiredoxins, atypical 2-cysteine peroxiredoxins and 1-cysteine peroxiredoxins (Wood *et al*., 2003). These enzymes share the same basic catalytic mechanism in which a redox-active cysteine in the active site is oxidized to a sulfonic acid by the peroxide substrate (Claiborne *et al*., 1999). Over-oxidation of this cysteine residue in peroxiredoxins inactivates these enzymes, but this can be reversed by the action of sulfiredoxin (Jonsson and Lowther, 2007). Peroxiredoxins seems to be important in antioxidant metabolism as mice lacking peroxiredoxin 1 or 2 have shortened life span and suffer from haemolytic anaemia while plants use peroxiredixons to remove hydrogen peroxide generated in chloroplasts (Neuman *et al*., 2003; Lee *et al*., 2003; Dietz *et al*., 2006); according to the following chemical equation.

$$ROOH + 2GSH \longrightarrow GSSG + H_2O + ROH$$

(d) **Glutathione peroxidase and Glutathione reductase**

The glutathione system includes glutathione peroxidase and glutathione-S-transfease (Meister and Anderson, 1983). This system is found in animals, plants and microorganisms (Meister and Anderson, 1983; Creissen *et al*., 1996). Glutathione peroxidase is an enzyme containing four selenium-cofactors that catalyses the break down of hydrogen peroxide and organized hydroperoxides such as lipid hydroperoxide (Takashi *et al*., 1986).

There are at least four different glutathione perioxidase isozymes in animals (Brigelius, 1999). Glutathione peroxidase-1, is the most abundant and is a very efficient scavenger of hydrogen peroxide while glutathione-4, is the most active with lipid hydroperoxide.

Glutathione peroxidase-1 is dispensable, as mice lacking this enzyme have normal life spans (Ho *et al*., 1997) but they are hypersensitive to induced oxidative stress (de Haan *et al*., 1998). In addition, the glutathione-S-transferase show high activity with lipid peroxides (Sharma *et al*., 2004). These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism (Hayes *et al*., 2005).

(2) **The chain breaking antioxidants**

Whenever a free radical interacts with another molecule, secondary radicals may be generated that can then react with other targets to produce yet more radical species. The classic example of such a chain reaction is lipid peroxidation, the reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralized by a chain

breaking antioxidants (De Zwart *et al*., 1999). Chain breaking antioxidants are small molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable by products (Halliwell *et al*., 1993). In general, the change associated with the presence of an unpaired electron becomes dissociated over the scavenger and the resulting product will not readily accept an electron from or donate an electron to another molecule thereby preventing the further propagation of the chain reaction.

(a) **Lipid chain breaking antioxidant**

These antioxidants scavenge radicals in the membrane and lipoprotein particles and are crucial in preventing lipid peroxidation. The most important lipid phase antioxidant is probably vitamin E (Esterbavier *et al*., 1991). Vitamin E occurs in nature in eight different forms which differ greatly in their degree of biological activity. The tocopherol (α, β Y and ∂) have a chromanol ring and a phyllyl tail and differ in the number and position of the methyl groups on the ring. The tocotrienols (α ,β, Y and ∂) are structurally similar but have unsaturated tails both classes of compounds are lipid soluble and have pronounced antioxidant properties (Horwitt *et al*., 1991).

(b) **Aqueous phase chain breaking antioxidant**

These antioxidants will directly scavenge radicals present in the aqueous compartment. Quantitatively, the most important antioxidant of this type is vitamin C (Ascorbic acid) (Levine *et al*., 1991). In humans, vitamin C acts as an essential cofactor for several enzymes catalyzing the hydroxylation reaction. In most cases, it provided electrons for enzymes that requires prosthetic metal ion in a reduced form to achieve full enzymatic activity. Its best known role is as a cofactor for prolyl and lyoyl oxidases in the synthesis of collagen.

(3) **Transition metal binding protein**

Transition metal binding proteins (Ferritin, transferrin, lactoferrin and caeruloplasmin) act as a crucial component of the antioxidant defence system by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical. The main copper binding protein caeruloplasmin might also function as an antioxidant enzyme that can catalyse the oxidation of divalent ion. Fe^{2+} is the form of iron that drives the Fenton reaction and the

rapid oxidation of Fe^{2+} to the less reactive Fe^{3+} form is therefore an antioxidant effect as shown in the following equation.

$$Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$$

(4) **Dietary antioxidants**

Some antioxidants are produced within the body and others have to be obtained from dietary sources. Biological antioxidants are characterized by being readily absorbed and transported to the relevant site within the cell for optimum function and are non toxic at nutritionally relevant intakes. The main dietary antioxidants are discussed below :

(a) **Vitamin E (D- α-Tocopherol)**

As one of the eight naturally occurring vitamin E compounds that are synthesized by plants from homogentisic acid, D-α-tocopherol is a derivative of ∂ chromanol with a saturated C_{16} phytol side chain. It is an example of a phenolic antioxidant. Such molecules readily donate H from the OH group on the ring structure to free radicals which then become unreative. On donating the H, the phenolic itself becomes a relatively unreative free radical because the unpaired electron on the 'O' can be delocalized into the aromatic ring structure thereby increasing its stability (Scott, 1997). Its major biological role is to protect polyunsaturated fats and other components of cell membranes from oxidation by free radicals and is therefore primarily located within the phosphohipid bilayer of cell membrane. It is particularly effective in preventing lipid peroxidation by a series of chemical reaction involving the oxidative deterioration of polyunsasturated fatty acids (Duthie, 1993). Vegetable oils are a major source of D-α-tocopherol in food products although other plant-based food groups contain tocopherol.

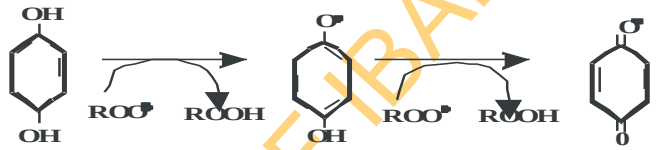


Figure 5 : Simple two steps hydrogen donation by a phenolic antioxidant to lipid radical (ROO $^{\bullet}$). The resulting unpaired electron becomes delocalised within the ring structure so that the phenolic radical is relatively unreactive before ultimately forming quimone.
(Duthie *et al*., 2000)

(b) **Vitamin C (Ascorbc acid)**

Vitamin C (L-Ascorbic acid) can be synthesized from D-glucose or D-galactose in the liver of most higher organism except primates including homosapiens, guinea pig and some species of brids (Halstead, 1993). Major sources include a wide range of vegetables and fruits. Vitamin C is one of the most important water soluble antioxidants in cells that efficiently scavenges a range of reactive oxygen species such as superoxide, hydroxyl radical, peroxyl radicals and singlet oxygen (Sies *et al.*, 1992). By efficiently trapping peroxyl radicals in the aqueous phase of the plasma or cytosol, vitamin C can protect biomembranes and low density lipoprotein from peroxidative damage, in addition to acting as an oxygen scavenger. It can also chelate trace elements such as Fe and Cu which catalyse the decomposition of hydroperoxides to initiate free radical chains. Moreover, vitamin C in chemical systems can donate a H to the tocopheroxyl radical.

(c) **Carotenoids**

Carotenoids are synthesized by photosynthetic microorganisms but not by animals. Particularly rich sources are yellow orange fruits and dark green leafy vegetables. About seven hundred naturally occurring carotenoids have been identified to date, of which about fifty can serve as precursor for vitamin A (Olsen, 1992). All carotenoids are primarily symmetrical C_{40} polyisoprenoid structures with an extensive conjugated double bond system. In general, major dietary classes of carotenoids are hydrocarbon carotenoids (carotene), oxygen containing carotenes (Xantophylls) and carotenyl fatty acid esters (Beecher and Khachik, 1992). Whether or not the primary role of carotenoids is as a biological antioxidant is a matter of debate. However, there is little doubt that because of their polyene structured feature, a number of carotenoids show strong reactions with reactive oxygen species in chemical and biochemical systems. For example β carotene has long been known to be a particularly effective scavenger of singlet oxygen (Bendich and Olsen, 1989). This ability is also shared by α carotene, lycopene, lutein and β cyrptoxanthin and appears to depend largely on physical quenching as opposed to chemical reaction. Antioxidant efficiency of certain carotenoids is also apparent in non photochemical free radical medicated reactions. For example β carotene has also been shown to inhibit peroxidation of liposomal phospholipids exposed to a range of free radical

generation including non dye induced peroxyl radicals, superoxide and transition metals (Liebler *et al*.,1997). Nutritional aspect of carotenoids have been reviewed recently by Castenmiller and West, 1998). Similar to vitamin E, carotenoids trap reactive oxygen species from chemical reaction and prevent oxidative damage.

(d) **Coenzyme Q_{10} (Ubiquinone)**

Ubiquinone is a lipohilic quinone which is similar in structure to vitamin E and which functions as an electron carrier in the mithochondria electron transport chain. However, it also protects membrane phospholipids from peroxidation (Cabrini *et al.*, 1986), and therefore has antioxidant properties. For this reason, it is not classified as a vitamin. However the ability to synthesise ubiqunone decreases with age and it has been proposed that there may be an increasing dependence on food to supply the nutrient. The major sources of Coenzyme Q are meat, vegetable oils and grain (Beyer *et al*., 1956).

(e) **Lipoic acid**

Lipoic acid is needed for mitochondrials function and it is also an antioxidant. It is made in the cell and participates as a cofactor in the conversion of carbohydrates to energy. As an antioxidant, it is both water and fat soluble it can eliminate free radicals in the water compartment of cell in a manner similar to vitamin C and it protects lipids against oxidation similar to vitamin E. Alpha-lipoic acid helps to break down sugars so that energy can be produced from them through cellular respiration. α-lipoic acid plays a truly central role in antioxidant defence, it is an extraordinarily broad spectrum antioxidant which is able to quench a wide range of free radical in both aqueous water and lipid fat chains. Moreover, it has the remarkable ability to recycle several other important antioxidant including Vitamin C and E, glutathione and coenzyme Q_{10} as well as itself. For these reason, α-lipoic acid is called the universal antioxidant. Lipoic acid is the only antioxidants that can boost the level of intracellular glutathione. Major food sources of α-lipoic acid include liver and yeast (Packer *et al.*, 1995).

(5) **Polyphenolic antioxidants**

Phytochemicals are plant chemicals that are neither vitamins nor minerals yet they have health enhancing effects. Phytochemicals help protect against cancer, cardiovascular disease. Many phytochemicals, including flavonoids, polyphenols, alkaloids, are antioxidants and protect cells

against oxidative damage and reduce risk of developing certain types of cancer. In a manner similar to vitamin E, they have the potential to act chemically as antioxidants by ready donation of electron or hydrogen from their hydroxyl moieties.

2.9 The Role of Antioxidants In Disease Treatment and Prevention

The brain is uniquely vulnerable to oxidative injury due to its high metabolic rate and elevated levels of polyunsaturated lipids, the target of lipid peroxidation (Reiter, 1995). Consequently, antioxidants are commonly used as medications to treat various forms of brain injury (Warner *et al*., 2004), sodium thiopental and propofol are used to treat reperfusion injury and traumatic brain injury (Wilson and Gelb, 2002) while the experimental drugs disfuention sodium (Lees *et al.*, 2006) and ebselen (Yamaguchi *et al*., 1998) are being applied in the treatment of stroke. These compounds appear to prevent oxidative stress in neurons and prevent apoptosis and neurological damage. Antioxidants are also being investigated as possible treatment for neuro degenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (Di Matteo and Esposito, 2003; Rao and Balachandram, 2002) and as a way to prevent noise induced hearing loss (Koke *et al.*, 2007). Targeted antioxidants may lead to better medicinal effects. Mithochondria-targeted ubiquinone, for example, may prevent damage to the liver caused by excessive alcohol.

Antioxidants can cancel out the cell damaging effects of free radicals (Sies, 1997). Furthermore, people who eat fruits and vegetables, which are good sources of antioxidants, have a lower risk of heart disease and some neurological disorders (Stanner *et al.*, 2004) and there is evidence that certain types of vegetables, and fruits in general, probably protect against a number of cancers (World Cancer Research Fund, 2007). These observations suggested that antioxidants might help prevent these conditions. There is some evidence that antioxidants assist in preventing disease such as muscular degeneration (Bartlett and Eperjesi, 2003), suppress immunity due to poor nutrition (Wintergerst *et al*., 2006). Oxidation of low density lipoprotein in the blood contributes to heart disease and initial observational studies found that people taking vitamin E supplements had a lower risk of developing heart disease (Rimm *et al*., 1993).

2.10 Infectious Disease

Worldwide infectious disease is the number one cause of death accounting for approximately one half of all deaths in tropical countries (WHO, 2004). In industrialized nations despite the progress made in understanding of microbiology and their control, incidents of epidemics due to drug resistant microorganism and the emergence of hitherto unknown disease causing microbes pose enormous public health concerns. Perhaps, it is not surprising to see these statistics in developing nations but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries such as the United State. Death from infectious disease ranked 5th in 1981, has become the 3rd leading cause of death in 1992, an increase of 58% (Pinner *et al.*, 1996, National Centre for Health Statistics, 2003). It is estimated that infectious disease is the underlying cause of death in 8% of death occurring in United State. The increases are attributed to increase in respiratory tract infection and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Furthermore, the most dramatic increase are occurring in the 25-44 years old age group (Pinner *et al.*, 1996). These negative health trends call for a renewed interest in infectious disease in the medical and pubic health communities and renewed strategies on treatment and prevention. Proposed solution that are outlined include prevention such as vaccination, improved monitoring and development of new treatments. It is this last solution that would encompass the development of new antimicrobials. Historically, plants have provided a good source of anti-infective agents. Emetine, quinine, berberine remain highly effective drugs in the fight against microbial infection. Phytomedicines derived form plants have shown great promise in the treatment of interactable infectious diseases, including the opportunistic AIDS infection.

2.11 Use of Plants as Antimicrobials

Infection caused by pathogenic bacteria and fungi are increasingly recognized as an emerging threat to public health (Walsh *et al.*, 1996; Wu, 1999). Although many drugs are available at present for the treatment of infectious diseases, their use is limited by a number of factors such as low potency, poor solubility, emergence of drug resistant strains and drug toxicity (Kaufman, 2000; Jhon 2002). The use of antibiotic is not successful always since many

of the pathogenic microbes especially bacterial and fungi have developed substantial resistance to the antimicrobial drugs (Jones 1998; Sashilkumar, 1998; Austin *et al*., 1999). This may be due to selective pressure on antibiotic sensitive organisms from the population with the consequent increase in the number of resistant ones. While such a development poses a serious threat to the public, and a great challenge to physician, most affected are the underdeveloped as well as developed countries (Jones, 1998). Decades ago, in India, typhoid could be cured with three inexpensive drugs namely cephalosporins, penicillin G and chloramphenicol but today, these drugs are largely ineffective against life threating typhoid fever. Therefore, there is an urgent need for the discovery of alternative, safe and more effective antimicrobial agents in order to control the life threatening pathogens which has brought in the attention of biologically active compounds from plant and animals sciences (Arokiyaraj *et al*., 2008; Gangadewit *et al*., 2008; Rehan., 2008; Chellaram and Edward, 2008). Plants are the oldest source of pharmacologically active compounds and have provided humankind with many medically useful compounds for centuries (Cordell, 1981). Among the available 250,000 to 500,000 species of plants on earth, only 1% to 10% of these are used as food by both human and animal species. It is possible that even more can be utilized for this purpose. Hipporcates (in the late fifth century, BC) has already mentioned 300 to 400 medicinal plants (Schulites, 1978). It is estimated that more than two thirds of the worlds population relies on plant derived drugs. Some 7,000 medicinal compounds used in the Western Pharmacopoeia are derived from plants (Caudfield, 1991). The bioactive compounds in the plants are produced as secondary metabolites. Examples include alkaloids and proteins (Chakraborty and Branther, 1999) which may be stage specific or tissue specific. Infact, there are several studies which have revealed the presence of such compounds with antimicrobial properties (Cowon, 1999). Numerous orchid species are traditionally used in herbal medicine as a remedy for microbial infection and number of other ailments. However the potential of most of the orchid species for therapeutic use is yet to be scientifically explored. Antimicrobials of plant origin have enormous therapeutic potential and they have been used since time immemorial. They have proved effective in the treatment of infectious disease, simultaneously mitigating many of the side effects which are often associated with synthetic antibiotics. Positive response of plant based drugs might lie on the structure of

the natural products which react with toxins and pathogens in such a way that less harm is done to other important molecules or turning their attention to natural products looking for new leads to develop better drugs against cancer as well as oral and microbial infections (Hoffman *et al.*, 1993; Srinivasan *et al.*, 2001). The medicinal value of plants lies in some chemical substances that produce definite physiological actions on human body. The most important of these include alkalolids, flavonoids, tannins and saponins. The phytochemical research based ethnopharmacological information is generally considered an effective approach in the discovery of new anti-infection agents from higher plant (Duraipandiyan *et al*, 2006).

2.11.1 Resistance of antimicrobial drugs

Antimicrobials is a general term given to medicines that kill or slow down the growth of microbes. Antibiotics resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic. Despite a push for new antibiotics therapies, there has been a continuous decline in the number of newly approved drugs. Antibiotics resistant therefore poses a significant problem. The increasing incidence of microbial diseases and non infectious diseases in man and therapeutics difficulties occasioned the search for new drugs. Antimicrobial resistance is a global problem that affects all countries (Chatterjee and Fleck, 2011) and it is becoming more dangerous, particularly in West African countries where high prevalence of multi drug resistant bacteria producing beta-lactamases have been reported (Aka *et al*., 1987; Keasah *et al*., 1998; Okesola *et al*., 1999; Benbachir *et al*., 2001; Kacou-Ndouba *et al*., 2001; Akoua *et al*., 2004; Akinyemi *et al*., 2005). In addition, morbidity and mortality of transmitted diseases are on the increase within the population including the young one. Antimicrobials have saved counteless lives and blunted serious complications of many feared diseases and infections. The success of antimicrobials against disease causing microbes is among modern medicine's great achievement. After more than 70 years of widespread use, evolution of disease causing microbes also has resulted in many antimicrobials loosing their effectiveness. As microbials evolve, they adapt to their environment. If something stops them from growing and spreading, such as an antimicrobial, the organisms will evolve a new mechanism to resist the antimicrobial intervention by changing the genetic structure. Changing the genetic structure ensures that the offspring of the resistant microbes also are resistant. Bacteria may be

intrinsically resistant to, greater than, or equal to the first class of antimicrobial agents or may acquire resistance by denovo mutation or via the acquisition of resistant genes from other organisms. Acquired resistance genes may enable a bacterium to:

(a) Produce enzymes that destroy the antibacterial drug

(b) Express efflux systems that prevent the drug from reaching its intracellular target.

(c) Produce an alternative metabolic pathway that by passes the action of the drug.

Acquisition of new genetic material by antimicrobial susceptible strains or bacteria may occur through conjugation, transformation or transduction with transporon which often facilitates the incorporation of multiple resistant genes into the host's genome or plasmid. Antimicrobial resistance makes it harder to eliminate infection from the body, as a result of microbes ability to survive in the presence of antimicrobials.

2.11.2 Screening methods to determine antimicrobial activity of natural products.

The emergence of new infectious diseases, the resurgence of several infections that appeared to have been controlled and the increase in bacterial resistance have created the necessity for studies directed towards the development of new antimicrobials. Considering the failure to acquire new molecules with antimicrobial properties from microorganisms, the optimization for screening methods used for the identification of antimicrobials from other natural resources is of great importance because of the failure of available antimicrobials to treat infectious diseases. Many researchers have focused on the investigation of natural products as a source of new bioactive molecules (Recio and Rios; 1989, Silver and Bostian, 1993). A variety of methods are found for this purpose since not all of them are based on the same principles and results obtained will also be profoundly influenced not only by the method selected but also by the microorganism used to carry out the test as well as the degree of solubility of each test compound (Kios *et al*., 1988; Vanden and Vlietinck, 1991). A test system should ideally be simple, rapid, reproducible and inexpensive and maximize high sample through put in order to cope with a varied number of extracts and fractions. The complexity of the bioassay must be defined by laboratory facilities, quality, and available personeel (Hostettman *et al.*, 1997; Hadacek and Greger, 2006). The currently available screening methods of antimicrobials activity of natural products fall into three groups including bioautogrpahic, agar diffusion and

agar dilution methods. The bioautographic and agar diffusion methods are known as qualitative techniques, since these methods will only give an idea of the presence or absence of substance with antimicrobial activity. On the other hand, agar dilution methods are considered quantitative assays once they determine the minimum inhibitory concentrations (Vaden and Vlietinck, 1991).

2.12 Inflammation

Inflammation is part of a complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells or irritants (Ferrero *et al*., 2007). Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate a healing process (Newman *et al*., 2000). Inflammation is the means by which the body deals with insult and injury (Suffredini, 1999; Streetz *et al*.,2001). Insult may be caused mechanically (e.g by pressure or foreign bodies), chemically (e.g by toxins, acidity, alkalinity), physically (e.g by temperature), by internal process (e.g uremia) and by microorganisms (e.g bacteria, virus, parasites). Inflammation rids the body of foreign matter and disposes off damage cells and initiate wound healing. Inflammation is not a synonym for infection, even in cases where inflammation is caused by infection. Although infection is caused by a microorganism, inflammation is one of the responses of the organism to the pathogen. However, inflammation is a stereotyped response and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen (Abbas and Litchman, 2009). Innate immunity is what is naturally present in our bodies when we are born and not the adaptive immunity which we acquire after an infection or vaccination. Innate immunity is generally non- specific while adaptive immunity is specific to one pathogen.

The word “nflammation” comes from Latin “inflammo’’ meaning I set alight, I ignite. Without inflammation, wounds and infections would never heal. Similarly, progressive destruction of the tissue would compromise the survival of the organism. However, chronic inflammation can also lead to a host of diseases such as hay fever, periondantitis, atherosclerosis, rheumatoid arthritis and even cancer e.g gall bladder (carcinoma). It is for that reason that inflammation is normally closely regulated by the body. Inflammation controlled by mast cells that are in close proximity to autonomic nerves. Mast cells are constituents of connective tissue containing large granules

that contain heparin, serotonin, bradykinin and histamine (Alexacos *et al.*,1999). These substances are released from the mast cells in response to injury and infection and by their degranulation, they control most of the process of inflammation. Mast cells are responsive to other controls for example under the influence of estrogen they release histamine. Another important pathway is known as arachidonic acid cascade which is largely controlled by eicosanoids (Bello *et al.*, 1999). Eicosanoids are local hormone that is made from 20-carbon essential fatty acid. They are short lived and can affect many aspects of physiological functions at the cellular level. Eicosanoids include all prostaglandins, thromboxane and leukotrienes. Depending on genetic as well as other factors, eiocosanoids transform or control prostaglandins, thromboxanes and leukotrienes all of which are inflammatory mediators. Eicosanoids can initiate all local inflammatory reponses. When inflammation affects a joint (such as rheumatoid), the cartilage can be damaged by neutrophils and lysosomal enzymes that enter the area. This lead to a vicious cycle of repeated injury.

2.12.1 Causes of inflammation

Causes of inflammation are as follows:

- Burns
- Chemical irritants
- Frost bite
- Toxins
- Infection by pathogens
- Immune reactions due to hypersensitivity
- Physical injury, blunt or penetrating ionising radiation
- Foreign bodies including splinters, dirt and debris
- Stress
- Trauma
- Alcohol

2.12.2 Types of inflammation

(i) **Acute inflammation**

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increase movement of plasma and leukocytes (especially granulocytes) from the blood into injured tissues. A cascade of biochemical events propagates and matures the inflammatory response involving the local vascular system, the immune system and various cells within the injured tissue. Acute inflammation starts rapidly (rapid onset) and quickly becomes severe. Signs and symptons are only present for a few weeks. Examples of disease conditions and situation which can result in acute inflammation include acute bronchitis, infected toe nail, sore throat from a cold or flu, a stratch, cut on the skin, acute appenditis, acute dermatitis, acute menigitis, acute sinutisis or a blow. Within a few seconds or minutes after tissue is injured, acute inflammation starts occurring. The damage may be a physical one or might be caused by an immune response. Three main processes occur before and during acute inflammation :

(i) Arterioles, small branches of artieries that leads to the damaged region, dilate thereby resulting in increased blood flow. The capillaries become more permeable, so fluid and blood protein can move into interstitial spaces between tissues.

(ii) Neutrophils and possibly some macrophages migrate out of the capillaries and venules (small veins that go from a capiallary to vein) and move into interstitial spaces. A neutrophil is a type of granulocyte (white blood cells). It is filled with tiny sacs which contain enzymes that digest microorganisms. Macrophages are also a type of white blood cells that ingest foreign materials for example when the skin is scratched and it is not broken, one may see a pale red line. Soon the area around the scratch goes red, this is because the arteriole have dilated and the capillaries have filled up with blood and become more permeable to move.

Acute inflammation is a short term process usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimulus (Cotran *et al*., 1998). It is characterized by five cardinal signs (Parakama and Clive, 2005). The acronyms that may be used for this is PRISH for pain, redness, immobility (loss of function), swelling and heat. The traditional names for signs of inflammation comes from Latin. Dolor (pain), Color (Heat), Rubor (Redness), Tumour (Swelling) and Function laesa (loss of function) (Ruth, 2009).

The first four classical sign were described by Celcius (30BC-38AD) (Vogel *et al.*, 2009), while loss of function was added later by Galen Porth,(2007).

The five cardinal signs of acute inflammation (PRISH)

Pain : The inflamed area is likely to be painful especially when touched. Chemicals that stimulate nerve endings are released, making the area much sensitive.

Redness : This is because the capillaries are filled up with more blood than usual.

Immobility : There may be some loss of function.

Swelling : Swelling is caused by accumulation of fluid.

Heat : More blood moves to the affected area and makes it feel hot to the touch. These five signs appear when acute inflammation occurs in the body surface whereas acute inflammation of internal organs may not result in the full set. The process of acute inflammation is initiated by cells already present in all tissue, mainly resident macrophages dendritic cells, histiocytes, kupffer cells and mastocytes. These cells present on their surface contain receptors named pattern recognition receptors (PRRs) which recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively, referred to as pathogen. Associated molecular patterns (PAMPs). At the onset of an infection, burn or other injuries these cells undergo activation (one of their PPRs recognize a PAMP) and release inflammatory mediators responsible for the clinical signs of inflammation, vasodilaton and its resulting increase blood flow causes the redness (rubor) and increased heat (calor). Increased permeability of the blood vessels result in an exudation (leakage of plasma proteins and fluids into the tissue (edema) which manifests it self as tumour. Some of the released mediators such as bradykinins, increase the sensitivity to pain (hyperalgesia, dolor). The mediator molecules also alter the blood vessels (extravasation) into the tissues. The neutrophil migrate along a chemotactic gradient created by the local cells to reach the site of injury (Cotran *et al.*, 1998). The loss of function is probably the result of a neurological reflex in response to pain. In addition to cell-derived mediators, several cellular biochemical cascade systems consisting of preformed plasma proteins act in parallel to initiate and propagate the inflammatory response. These include the complement system activated by bacteria and the coagulation and fibrinolysis systems activated by necrosis e.g a burn or a trauma.The acute inflammatory response requires a

constant stimulation to be sustained. Inflammatory mediators have short half life and are quickly degraded in the tissue. Hence, acute inflammation ceases once the stimulus has been removed.

(ii) **Chronic inflammation**

Chronic inflammation means long term inflammation which can last for several months and even years. It can result from failure to eliminate whatever was causing an acute inflammation. An auto immune response to a self antigen, the immune system attack healthy tissues, mistaking it for harmful pathogens. Examples of diseases and conditions with chronic inflammation include asthma, chronic peptic ulcer, tuberculosis, rheumatoid, arthritis, chronic periondotitis, ulcerative colitis, chronic hepatitis. Chronic inflammation leads to a progressive shift in type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. It can also manifest, histologically, by the presence of lymphocytes, macrophages and result in fibrosis and tissue necrosis. Infections, wounds and any damage to tissue would never heal without inflammation. Tissues would become more and more damaged and the body or any organ would eventually perish.

Table 2 : Comparison between acute and chronic inflammation (Graham *et al*., 1988)

	Acute	Chronic
Causative agent	Pathogens, injured tissues.	Persistent acute inflammation due to non degradable pathogens, persistent foreign bodies or autoimmune reaction.
Major cells involved	Neutrophils, mononuclear cells (monocytes, macr phages).	Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts.
Primary mediators	Vaso-active Amines, eiocosanoids.	IFN-Y and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes.
Onset	Immediate	Delayed
Duration	Few days	Up to many months
Outcomes	Resolution, abscess formation chronic inflammation	Tissue destruction, fibrosis, necrosis

2.12.3 Mediators of inflammation

Inflammatory mediators are soluble diffusible molecules that act locally at the site of tissue damage and infection and at more distant sites. In appreciating the inflammatory process, it is important to understand the role of chemical mediators. These are the substances that tend to direct inflammatory response. These inflammatory mediators come from plasma proteins or cells including mast cells, platelets, neurtrophils and monocytes. They are triggered by bacterial product or host proteins. Chemical mediators bind to specific receptors on target cells and can increase vascular permeability and neutrophil chemotaxis, stimulate smooth muscle contraction, have direct enzymatic activity, induce pain or mediate oxidative damage. Most mediators are short lived but cause harmful effects (Cotran *et al*., 1998). Chemical mediators of the inflammatory process include a variety of substances originating in the plasma and the cells of the injured tissues and possibly from the damage tissues. The mediators of inflammation include: :

(a) Plasma protein such as complement and antibodies
(b) Cytokine and chemokines such as IL-10
(c) Lipids such as prostaglandins and polyunsaturated fatty acids
(d) Vasoactive amines such as histamine and serotonin
(e) Gases such as NO and O_2
(f) Kinins such as bradykinin
(g) Neuropeptides.

Once leukocytes have arrived at a site of infection or inflammation, they release mediators which control the later accumulation and activation of other cells. However, in inflammatory reactions initiated by the immune system, the ultimate control is exerted by the antigen itself, in the same way as it controls the immune response itself. For this reason, the cellular accumulation at the site of chronic infection or in auto immune reaction (where the antigen cannot ultimately be eradicated) is quite different from that at sites where the antigenic stimulus is rapidly cleared.

There are four major plasma enzymes systems which have an important role in homeostasis and control inflammation. These are complement system, the clotting system, the fibrinolytic

system and the kinnin system. Inflammatory mediators can be divided into exogenous and endogenous mediators. Bacterial products and toxins can act as exogenous mediators of inflammation and noteable among this is endotoxin. The immune system of higher organisms has probably evolved in a veritable sea of endotoxin, so it is perhaps not surprising that this substance evokes powerful response. For example, endotoxin can trigger complement activation resulting in the formation of anaphylatoxins (C3a and C5a) which cause vasodilation and increase vascular permeability. In addition, endotoxin elicit T cell proliferation and have been described as super antigen for T cells.

Endogenous mediators of inflammation are produced from within the innate and adaptive immune system itself as well as other systems. For example, they can be derived from molecules that are normally present in the plasma in an active form such as peptide, fragments of some components of complements, coagulation and kinnin systems. Mediators of inflammatory responses are also released at the site of injury by a number of cells types that either contain them as preformed molecules within storages granules e.g histamine which can rapidly switch on the machinery requires to synthesize the mediators when they are required for example, to produce metabolites of arachidonic acid.

Monocular phagocytes (monocytes and macrophages) are central to inflammation as they produce many components which participate in or regulate the different plasma enzymes systems and hence the mediators of inflammatory response. Early phase mediators are produced by mast cells and platelets. They are especially important in acute inflammation and include mainly histamine, serotonin and other vasoactive substances. To the early phase mediators also belong chemoatractants e.g (5a and cytokines such as IL-1, IL-6 and TNF.

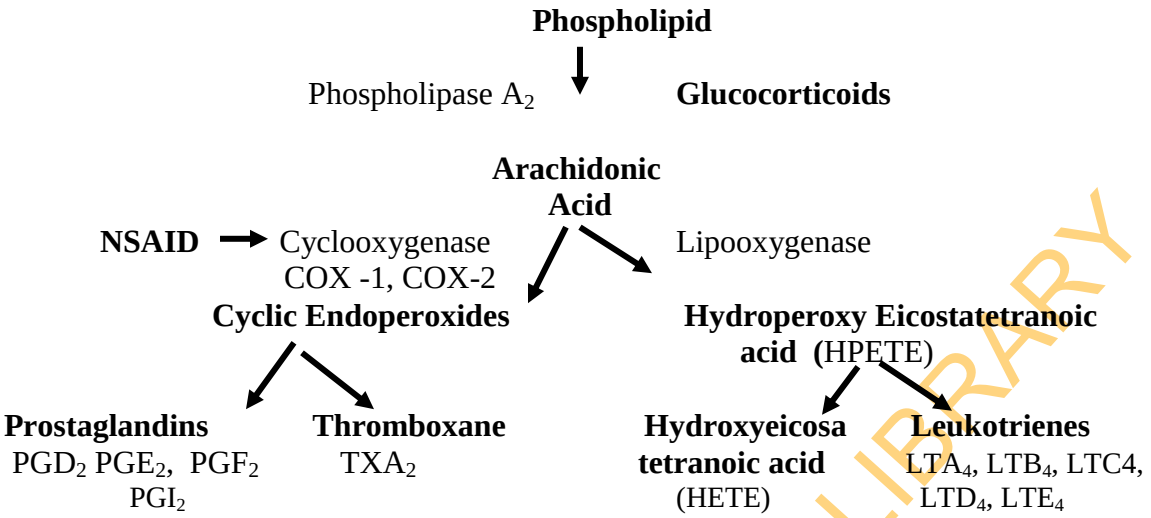
2.12.4 Antinflammatory drugs

Anti-inflammatory drugs are drugs with analgesics and antipyretic (fever reducing) effect and which have in higher doses anti-inflammatory effect. Anti-inflmamtory drugs are usually abbreviated to as non steroidal anti-inflammatory drug (NSAIDs). The term non steroidal is used to distinguish these drugs from steroids which among a broad range of other effect have a similar eicosanoid-depressing anti-inflmmatory action. NSAIDS work by reducing the production of prostaglandins. Prostaglandins are chemicals that promote inflammation, pain and

fever. They also protect the lining of the stomach and intestines from the damaging effects of acid and promote blood clotting by activating blood platelets. Prostaglandins also affect kidney function. The enzymes that produce prostaglandins are called cyclooxygenase (COX). There are two types of COX enzymes, COX-1 and COX-2. Both enzymes produce prostaglandins that promote inflammation pain and fever, however only COX-1 produces prostanglandins that activate platelets and protect the stomach and intestinal lining, NSAIDs block COX enzymes and reduce production of prostaglandins. Therefore, inflammation, pain and fever are reduced. Most prominent members of this groups of drugs are aspirin, ibruprofen and naproxes all of which are available over the counter in many areas (Stuart *et al*., 2010).

2.12.4.1 Mechanism of action of non steroidal anti-inflammatory drugs (NSAIDs)

Non sterioidal anti-inflammatory drugs (NSAIDs) act as non selective inhibitors of the enzyme cycoloxgenase (COX) which is known to have at least two distinct isoforms; COX-1 and the inducible isoform COX-2 . The NSAIDs inhibit both COX-1 and COX-2 isoenzymes. Cycloxygenase (COX) catalyses the formation of prostaglandins and thomboxane from arachidonic acid. Prostaglandins act as messenger molecules in the process of inflammation. COX-1 has clear physiological function. Its activation leads for instance to the production of prostaglandin which when released by the endothelium is antithrombogenic and when released by gastric mucosa is cytoprotecive. COX-2, discovered 6 years ago, is induced by inflammatory stimuli and cytokines in migratory and other cells. It is therefore attractive to suggest that the anti-inflammatory action of NSAIDs is due to inhibiton of COX-2, whereas the unwanted side effects such as irritation of the stomach lining are due to inhibition of COX-1. Drugs that have the highest COX-2 activity and a more favourable COX-2 : COX-1 activity ratio will have a more potent anti-inflammatory activity with fewer side effects than drugs with a less faovurably COX-2 : COX-1 activity ratio. The identification of selective inhibitors of COX-2 will, therefore, lead to an advance in therapy.



Three isoforms of cyclooxygenase have been identified, cyclooxygenase 1, cyclooxygenase 2 (COX -1 and COX-2) and cyclooxygenase 3 (or cyclooxygenase 1b). COX-1 is normally present in all tissues while COX-2 is induced by cytokines and certain serum factors.

Glucocorticoids inhibit phospholipase A_2 and thus block the production of both prostaglandins and leukotrienes, exerting a potent anti-inflammatory effect. Glucocorticoids also block the action of cyclooxygenase -2.

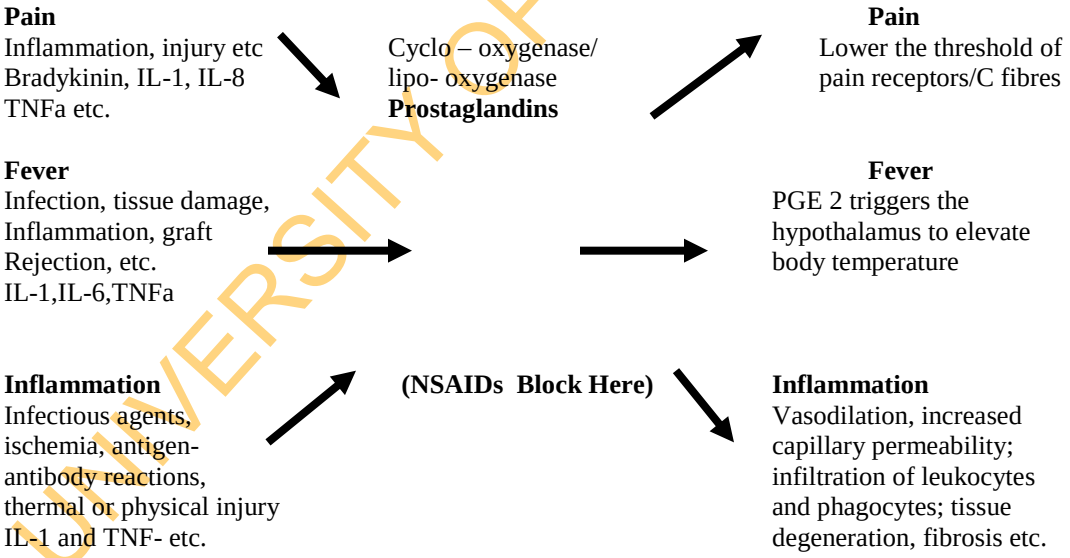


Figure 6 : Cyclooxygenase and lipooxygenase pathway to produce prostaglandins and leukotrienes respectively (Nelson and Randy, 2005)

2.13 Malaria

Malaria is a mosquito borne infectious disease of humans and other animals caused by protozoans parasites of the genus *Plasmodium.* It is one of the most common infectious diseases and an enormous public health problem. It infects between 300-500 million people every year and causes between one and three million deaths annually mostly among young children in SubSaharan Africa (Miller *et al*., 1994; More, 2002; David *et al*., 2004; Martin *et al*., 2004; Wright, 2005; WHO, 2005). It begins with a bite from an infected female mosquito which is introduced into the parasite via its saliva into the circulatory system and ultimately to the liver where they mature and reproduce. The disease causes symptoms that typically include fever and headache which in severe cases can progress to coma and death. Malaria is widespread in subtropical regions in a broad band around the equator, including much of SubSaharan Africa, Asia and the Americas. Five species of *Plasmodium* can infect and be transmitted by humans (Hardman *et al*., 2001). The vast majority of deaths are caused by *Plasmodium falciparum* while *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae* cause a generally milder form of malaria that is rarely fatal. *Plasmodium falciparum*, the most important pathogenic representative of this species is responsible for the majority of cases (Tramputz et al., 2003; Batista *et al*., 2009; Kakkilaya, 2008) The zoonotic species *Plasmodium knowlesi*, prevalent in Southeast Asia causes malaria in macaques but can also cause severe infection in humans. Malaria is prevalent in the tropical region because significant amount of rainfall, warm temperature and stagnant waters provide habitats ideal for mosquito larvae. Disease transmission can be reduced by preventing mosquito bites; by distribution of mosquito nets and insect repellants, or with mosquito control measures such as spraying insecticides and draining standing water. Malaria is typically diagnosed by microscopic examination of blood using blood films with antigen based rapid diagnostic tests. Modern techniques that use the polymerase chain reaction to detect parasite DNA have also been developed but these are not widely used in malaria endemic areas due to their high cost and complexity.

The World Health Organization (WHO) has estimated that in the year 2010, there were 216 million documented cases of malaria. In that period, between 655,000 and 1.2 million people died from the disease (roughly 2,000-3,000 per day) (Hardman *et a*l., 2001) many of whom

were children in Africa. Malaria is commonly associated with poverty and is also a major hinderance to economic development. Despite a need, no effective vaccine currently exists; although efforts to develop one are ongoing. Several medications are available to prevent malaria in travelers to malaria endemic countries (prophylaxis). A variety of antimalaria medications are available. Severe malaria is treated with intravenous or intramuscular quinine or since the mid-2000s, with the artermisinin derivative artesunate, which is superior to quinine, in both children and adults and is given in combination with a second antimalarial, such as mefloquine.

2.13.1 Causes of malaria

Malaria is caused by the protozoan parasite of the genus *Plasmodium.* They belong to the class porozoa which are characterized by giving rise to the young forms (sporozoites) usually enclosed in a cyst (Bruce- Chawatt, 1980). In humans, malaria is caused by *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax* and *Plasmodium knowlesi* (Hartman *et al*., 2010; Rijken *et al*., 2012). Among those infected, *Plasmodium falciparum* is the most commonly species identified (75%), followed by *Plasmodium vivax* (20%) (Nadjim and Behrens, 2012). *Plasmodium falciparum* accounts for the majority of death (Mueller *et al*., 2007), non falciparum species have been found to be the cause of about 14% case of severe malaria in some groups (Nadjim and Behrens, 2012). *Plasmodium vivax* proportionally is more common outside of Africa (Collins, 2012). There have been documented human infections with several species of *Plasmodium* from higher apes, however, with the exception of *Plasmodium knowlesi*, a zoonotic species that causes malaria in macaques (Rijken *et al*., 2012). These are mostly of limited public health importance (Sarkar *et al*., 2009). Rodent malaria parasites include *Plasmodium berghei, Plasmodium yoelli, Plasmodium vinckei* and *Plasmodium chabandi* (Garnham, 1966).

2.13.2 The life cycle of malaria parasite

In the life cycle of *Plasmodium,* a female anopheles mosquito (the definite host) transmits a motile infective stage called the sporozoites to a vertebrate host such as human (the secondary host), thus acting as a transmission vectors. A sporozoite travels in blood vessels to liver cells, where it reproduces asexually. Some offspring called merozoites enter red blood cells and liver

cells where they divide asexually. Other offspring develop into immature gametes or gametocytes. When a mosquito bites an infected person, gameotcytes are taken up with the blood and mature in the mosquito gut. Gametes fuse and form zygotes which develop into new sporozoites. The sporozoites migrate to the insect salivary gland where they then ready to infect a new vertebrate host. The sporozoites are injected into the skin, alongside saliva, when the mosquito takes a subsequent blood meal. This type of transmission is occasionally referred to as anterior station transfer, only female mosquitoes feed on blood, male mosquitoes feed on plant nectar and thus do not transmit the disease. The females of the Anopheles genus of mosquito prefer to feed at night. They usually start searching for a meal at dusk and will continue throughout the night until taking a meal (Collins and Barmwell, 2009). Malaria parasite can also be transmitted by blood transfusion although this is rare.

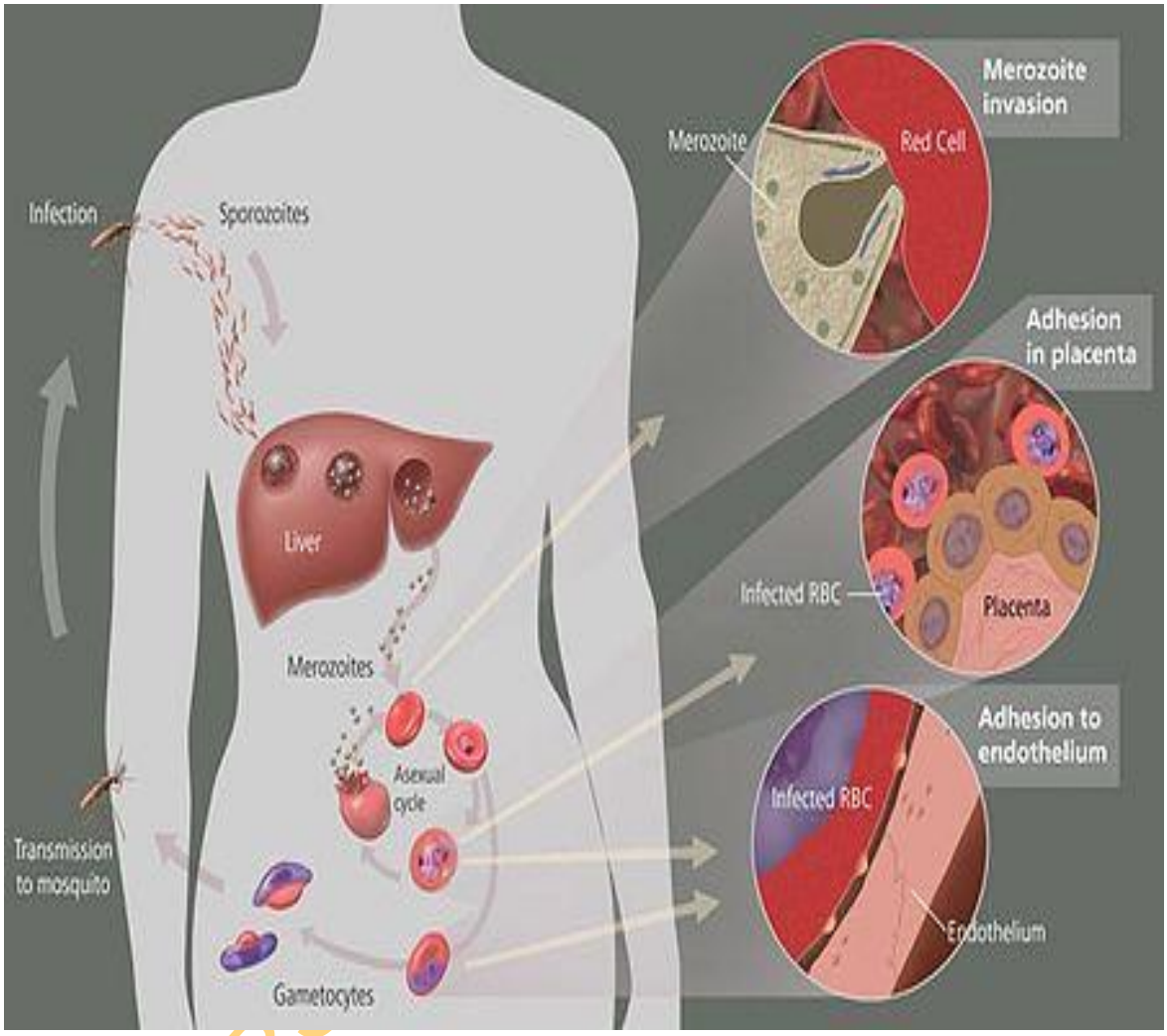


Figure 7 : Life cycle of malaria parasite
(Talman *et al*., 2004)

2.13.3 Chemotherapy of malaria

In the 1950s and 1960s, (WHO) concentrated efforts on eradication of malaria, and failure of the eradication programme led to the decision to control the disease through the use of antimalaria drugs (Bruce-Chawtt, 1980), such drugs may be used for some or all of the following: Treatment of malaria in an individual with suspected or confirmed infection, prevention of infection in individuals visiting a malaria endemic region who have no immunity (prophylaxis), routine intermittent treatment of certain groups in endemic regions (intermittent preventive therapy). Current practice in treating cases of malaria is based on the concept of combination therapy, since this offers several advantages including risk of treatment failure, reduced risk of developing resistance, enhanced convenience and reduce side effects. Prompt parasitological confirmation microscope or, alternatively, by rapid diagnostic test is recommended in all patients suspected of malaria before treatment is started. Treatment on the basis of clinical suspicion should only be considered when parasitological diagnosis is not accessible (Mayvar *et al.*, 2012). There are several families of drugs used to treat malaria as it was cheap and effective, chloroquine was the antimalaria drug for many years in most part of the world. However, resistance of *Plasmodium falciparum* to chloroquine has spread recently from Asia to Africa making the drug ineffective against the most dangerous *Plasmodium* strain in many affected regions of the world. In those areas where chloroquine is still effective, it remains the first treatment choice. Unfortunately, chloroquine resistance is associated with reduced sensitivity to other drugs such as qunine, amodiaquine (Tinto *et al.*, 2005). Some of the antimalaria drugs are used for treatment and partial, prevention (prophylaxis). Many drugs can be used for both purposes. Currently commercially available anti malaria drugs include:

- Arthemeter-lumefantrine (Therapy only and commercially named coartem)
- Artesunate-amodiaquine (Therapy only)
- Artesunate-melfoquine (Therapy only)
- Artesunate-sulfadoxine / pyrimethamine (Therapy only)
- Quinine (Therapy only)
- Chloroquine (Therapy and prophlaxis, and usefulness now reduce due to resistance).
- Cotrifazid (Therapy and prophylaxis)

- Doxycycline (Therapy and prophylaxis)
- Mefloquine (Trade name Lariam) (Therapy and prophylaxis).
- Primaquine (Therapy in *Plasmodium vivax* and *Plasmodium ovale* only).
- Proguanil (Prophylaxis only)
- Sulfadoxine-pyrimethamine (Therapy, prophylaxis in pregnant women).

2.13.4 Some selected antimalaria drugs and their mechanisms of action

(a) **Chloroquine**

Chloroquine is a derivative of 4-aminoqunolines. It was discovered in 1934 by Hans Andersug and coworkers at the Bayer laboratories who named it " Resochin" (Krafts *et al*., 2012) because it was considered toxic for human use. During the World War II. The United State of America government sponsored clinical trials for antimalaria drug development showed unequivocally that chloroquine has a significant therapeutic value as an antimalaria drug. It was introduced into clinical practice in 1947 for the prophylactic treatment of malaria (Farhurst and Wellen,2010) . Chloroquine has long been used in the treatment or prevention of malaria. After the malaria parasite *Plasmodium falciparum* started to develop widespread resistance to chloroquine (Plowe, 2005; Uhelman and Krishna, 2005), new potential uses of cheap and widely available drugs have been investigated.

Mechanism of action of chloroquine

Chloroquine exhibits outstanding activity against the asexual blood forms of all plasmodial species causing malaria in man. The drug is a blood schizontoncide that is effective not only against *Plasmodium falciparum* but also against chloroquine sensitive strain of *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale.* Chloroquine is one of the most rapid inhibitors of nucleic acid and protein synthesis in susceptible parasite strains. It acts on ring stages of the parasites. Chloroquine primarily acts by intercalating between deoxyribonucleic acid base pairs preventing sorting out, transcription and consequently block the replication process. Chloroquine is a strong inhibitor of hemozoin crystal inside the red blood cells, and the malaria parasite must degrade haemoglobin to acquire essential amino acids which the parasite requires to construct its own protein and for energy metabolism. Digestion is carried out in a vacuole of the parasitic cell. During this process, the parasite releases the toxic soluble molecule

a haeme. The haeme moiety consists of the porphyrin ring called Fe (II)-pritoporphyrin (FP). To avoid destruction by this molecule. The parasite biocyrstalises haeme to form hemozoin, a non toxic molecule. Hemozoin collects in the digestive vacuole as insoluble crystals. Chloroquine enters the red blood cell, inhabiting parasite cell and digestive vacuole by simple diffusion. Chloroquine then becomes protonated to $CQ^{2+,}$ as the digestive vacuole is known to be acidic (pH 4.7). Chloroquine cap hemozoin molecules to prevent further biocrystallisation of haeme, thus leading to heme build up. Chloroquine binds to haeme or (FP) to form the FP-chloroquine complex, this complex is highly toxic to the cells and disrupts membrane function. Action of the toxic FP-chloroquine and FP results in cell lysis and ultimately parasite cell autodigestion.

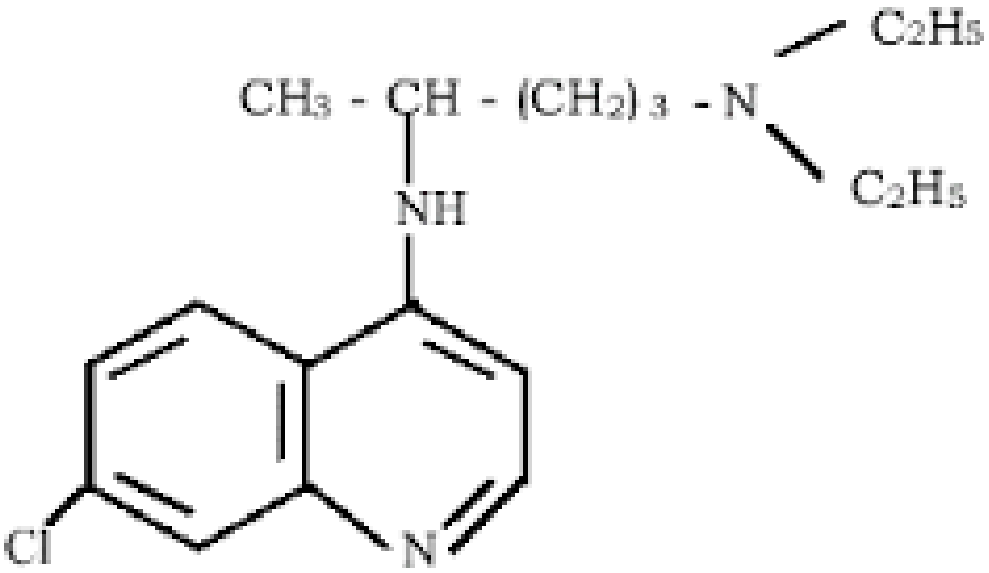


Figure 8: Chloroquine (7– cloroquinolin-4–yl) – N, N , diethyl – pentane – 1,4, diamine)

(Krafts *et al*., 2012)

(b) Artemisinin

Artemisinin (also known as Quinghaosu) and its derivative are a group of drug that possess the most rapid action of all current drug against *Plasmodium falciparum* malaria (Whitney, 1997). Treatment containing an artemisinin derivative artemisinin-combination therapies (ACTs) are now standard treatment worldwide for *Plasmodium falciparum* malaria. The compound artemisinin was isolated from the plant *Artemisia annua* (sweet worm wood), an herb employed in Chinese traditional medicine. Chemically, artemisin is a sesquiterpene lactone containing an unusual peroxide bridge. This peroxide is believed to be responsible for the drug mechanism of action. Use of the drug by itself as a monotherapy is explicitly discouraged by the WHO as there has been signs that malaria parasites are developing resistance to the drug (Rehwagen, 2006). Therapies that combine artemisinin with some other antimalaria drug are the preferred treatment for malaria and are both effective and well tolerated in patients. Artemisinin offers over 90% efficacy rates but their supply is not meeting demand (Senior, 2005). Since the WHO has recommended using ACTs as first line treatment for uncomplicated malaria in areas experiencing resistance to older medications (White, 2004). The most recent WHO treatment guidelines for malaria recommend four different ACTs. While numerous countries including most African nations have adopted the change in their official malaria treatment policies, cost remains a major barrier to ACTs implementation because it is up to twenty times as much as older medications, and ACTs remain unaffordable in many malaria endemic countries. Artemisinin can be used alone, but this leads to a high rate of recrudesence (returns of parasite) and other drugs are required to clear the body of all parasites and prevent recurrence. The WHO has recommended artemisinin combination therapies (ACTs) to be the first line therapy for *Plasmodium falciparum malaria,* world wide. Combination therapies are effective because the artemisin component kill the majority of parasites at the start of the treatment while the more slowly eliminated patner drug clear the remaining parasites (White, 2004). Several fixed dose ACTs are now available containing an artermisinin component and a patner drug which has a long half-life such as melfloquine lumefantrine, amodiaquine, piperaquine (Knidsood *et al*., 2010).

Artemisinins are not used for malaria prophylaxis (prevention) because of the extremely short activity (half-life) of the drug. To be effective, artemisinins would have to be administered multiple times each day because artemisinin itself has physical properties such as poor bioavailability that limit its effectiveness. Semisynthetic derivative of artemisinin have been developed. These include artesunate, artemether, dihydro artemisinin, artenilic acid, artenimol, artemotil.

Mechanism of action of Artemisinin

All artemisinins used today are prodrugs of the biologically active metabolite dihydro artemisinin which is active during the stage when the parasite is located inside red blood cells. Although there is no consensus regarding the mechanism through which artemisinin derivative destroys the parasites (Kappe *et al.*, 2010), several lines of evidence indicate that artemisinin exert their antimalaria action by pertubing redox homeostasis in malaria parasite. When the parasite that causes malaria infect a red blood cell, it consumes hemoglobin within its digestive vacuole, a process that generates oxidative stress (Ginsburg and Atamna, 1994).

The iron of the haeme molecule directly reduce the peroxide bond in artemisinin thereby generating high valent iron-oxospecies and resulting in a cascade of reactions that produce reactive oxygen radicals which damage the parasite and lead to its death (Cumming, 1997). A more recently described alternative mechanism is that artemisinin disrupts cellular redox cycling (Haynes *et al.*, 2011). Numerous studies have investigated the type of damage oxygen radicals may induce, for example, Pandey *et al*., (1999) have observed inhibition of digestive vacuole cysteine protease activity of malaria parasites by artermisinin. These observation were supported by *ex-vivo* experiment showing accumulation of haemoglobin in the parasites treated with artemisinin and inhibition of hemozoin formation by malaria parasites. Electron microscopic evidence linking artemisinin action to the parasite's digestive vacuole has been obtained showing that the digestive vacuole membrane suffers damage soon after parasites are exposed to artemisinin (del Pilar *et al.*, 2008). This would also be consistent with the data showing that the digestive vacuole is already established by mid-ring stage of the parasites blood cycle (Abubakar *et al.*, 2010), a stage that is sensitive to artemisinins but no other antimalarials. Mo and Shen (2005) investigated the mode of action of artemisinin using a yeast

model and they demonstrated that the drug acts on the electron transport chain, generates local reactive species and causes depolarization of the mithochondrial membrane. However, replacement of mithochondrial function in transgenic asexual stage parasites does not alter sensitivity to artemisinins (as would be predicted if mithochondrial targeting was relevant to artemisinin action), whereas atovaquinone resistance is observed which would be consistent with mithochodrial targeting of this type of malaria (Vaidya and Akhil, 2012).

2.14 Tuberculosis

Tuberculosis (TB) is a common and often deadly infectious disease caused by various strains of *Mycobacteria* usually *Mycobacterium tuberculosis* in humans (Kumar *et al.*, 2007). Tuberculosis usually attacks the lungs but can also affect other parts of the body. It is spread through the air when people who have the disease cough, sneeze or spit (Konstantines, 2010). Most infection in human result in an asymptomatic latent infection and about one in ten latent infection eventually progresses to active disease which if left untreated, kills more than 50% of its victims.

The classic symptoms are a chronic cough with blood tinged sputum, fever, night sweats and weight loss. Infection of other organs causes a wide range of symptoms. Diagnosis relies on radiology (commonly chest X –rays), a tuberculin skin test, blood tests, as well as microscopic examination and microbiological culture of body fluids. Treatment is difficult and requires long courses of multiple antibiotics. Antibiotic resistance is a growing problem in multi drug resistant tuberculosis. Prevention relies on screening programs and vaccination, usually with Bacillus Calmette-Guerin vaccine (BCG). One third of the world's population are thought to be infected with *M. tuberculosis* (Jasmer *et al*., 2002; WHO, 2010) and new infections occur at a rate of about one per second (WHO, 2007). The proportion of people who become sick with tuberculosis each year is stable or falling world wide but because of population growth, the absolute number of new cases is still increasing. In the year 2007, there were an estimated 13.7 million chronic active cases, 9.3 million new cases and 1.8 million deaths, mostly in developing countries (WHO, 2009). In addition, more people in the developed world are contracting tuberculosis because their immune systems are compromised by immunosuppressive drugs, substance abuse or AIDs. The distribution of tuberculosis is not uniform across the globe, about

80% of the population in many Asian and African countries test positive in tuberculosis tests, while only 5-10% of the US population test positive (Kumar *et al.*, 2007).

2.14.1 Causes of tuberculosis

The primary cause of TB, *Mycobacterium tuberculosis* (MTB) is a small aerobic non motile bacillus, high lipid content of this pathogen accounts for many of its unique clinical characteristic (Southwick and Frederick, 2007). It divides every 16 to 20 hours, an extremely low rate compared with other bacteria which usually divide in less than an hour (Cox, 2004). Since *Mycobacterium tuberculosis* has a cell wall but lack a phospholipid outer membrare, it is classified as a Gram-positive bacterium if a Gram stain is performed, MTB either stains very weakly or does not retain dye due to high lipid and mycolic acid content of its cells wall (Maidson,2001), MTB can withsand weak disinfectants and survive in a dry state for weeks. In nature, the bacterium can grow only within the cells of a host organism but *M. tuberculosis* can be cultured *in-vitro* (Parish and stoker, 1999). MTB retains certain stains after being treated with acidic solution and it is classified as an acid fast bacillus (AFB). (Kumar *et al.*, 2007). The most common acid fast staining technique, the Ziehl-Neelsen stain, dyes AFBs a bright red that stands out clearly against a blue background. Other ways to visualize AFBs include an auraminerhomadine stain and fluorescent microscopy. The *M. tuberculosis* complex includes four other TB-causing *Mycobacteria: M. bovis, M. africanum, M. carnetti* and *M. microti* (Van Soolingen *et al.*, 1997). *M. africanum* is not wide spread but in parts of Africa it is a significant cause of tuberculosis (Niemann *et al.*, 2002). *M. bovis* was once a common cause of tuberculosis, but the introduction of pasteurized milk has largely eliminated this as a public health problem in developed countries (Thoen, *et al.*, 2006). *M. canetti* is rare and seems to be limited to Africa, although a few cases have been seen in African emigrants (Pfyffer *et al*., 1998). *M. microti* is mostly seen in immunodeficent people, although it is possible that the prevealence of this pathogen has been under estimated (Niemann *et al*., 2000). Other known pathogenic mycobacteria include *Mycobacterium leprae, Mycobacterium kansasii*. The last two are part of the non tuberculous *mycobacteria* (NTM) group. Non tuberculous *mycobacteria* cause neither TB nor leprosy, but they do cause pulmonary diseases resembling TB (American Lung Association, 1997).

2.14.2 Pathogenesis of tuberculosis

About 90% of those infected with *Mycobacterium tuberculosis* have asymptomatic Latent TB infection (LTBI) with only a 10% life time chance that a latent infection will progress to TB disease (Kumar *et al.*, 2007). However, if untreated, the death rate for these active TB cases is more than 50% (Onyebujoh, 2006). TB infection begins when the *mycobacteria* reach the pulmonary alveoli where they invade and replicate within the endosomes of alveolar macrophages (Houben *et al.*, 2006). The primary site of infection in the lungs is called the Ghon focus and is generally located in either the upper part of the lower lobe or the lower part of the upper lobe. Bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local lymph nodes. Further spread is through the blood stream to other tissues and organs where secondary TB lesions can develop in other parts of the lung, peripheral lymph nodes, kidney, brain and bone (Herrmann and Lagrange, 2005)). All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscle and pancreas (Agarval *et al.*, 2005).

Tuberculosis is classified as one of the granulomatous inflammatory conditions. Macrophages, T-lymphocytes, B-lymphocytes and fibroblasts are among the cells that aggregate to form a granuloma with lymphocytes surrounding the infected macrophages. The granuloma functions not only to prevent dissemination of the *Mycobacteria*, but also provides a local environment for communication of cells of the immune system. Within the granuloma, T-ymphocytes secrete cytokines such as interferon gamma, which activates macrophage to destroy the bacteria with which they are infected (Kaufmann, 2002). Cytotoxic T cells can also directly destroy infected cells, by secreting perforin and granulysin (Houben *et al.*, 2006). Importantly, bacteria are not always eliminated within the granuloma, but can become dormant resulting in a latent infection. Another feature of the granulomas of human tuberculosis is the development of abnormal cell death, also called necrosis in the center of tubercles. To the naked eye this has the texture of soft white cheese and was termed caseous vecrosis (Grosset, 2003). If TB bacteria gain entry to the blood stream from an area of damaged tissue, they spread through the body and set up many foci of infection, all appearing as tiny white tubercules in the tissue . This severe form of TB disease is common in infants and the elderly and is called miliary

tuberculosis. Patients with this disseminated TB have a fatality rate near 100% if untreated. However, if treated early, the fatality rate is reduced to near 10% (Kim *et al.*, 2003).

2.14.3 Signs and symptons of tuberculosis

When the disease becomes active, 75% of the cases are pulmonary TB that is TB in the lungs. Symptons include chest pain, coughing up blood and a productive prolonged cough for more than three weeks. Systemic symptons include fever, chills, night sweats, appetite loss, weight loss, and often a tendency to fatigue very easily (WHO, 2009). In the other 25% of active cases, the infection moves from the lungs, causing other kinds of TB, collectively denoted extrapulmonary tuberculosis. This occurs more commonly in immune suppressed persons and young children. Extrapulmonary infection sites include the pleura in tuberculosis pleurisy, the central nervous systems in meningitis, the lymphatic system in scrofula of the neck, the genitourinary system in urogential tuberculosis and bones and joints in Pott's disease of the spine. An especially serious form is disseminated TB, more commonly known as miliary tuberculosis. Extrapulomonary TB may co-exist pulmonary TB as well (Centre for Disease Control and Prevention, 2000).

2.14.4 Treatment of tuberculosis

Treatment for TB uses antibiotic to destroy the bacteria. Effective TB treatment is difficult due to the unusual structure and chemical composition of the mycobacterial cell wall which makes many antibiotics ineffective and hinders the entry of drugs (Migliore *et al.*, 1966; Acharya and Goldman, 1970; Brennan and Nikaido, 1995). The two antibiotics most commonly used are rifampicin and isoniazid. However, instead of the short course of antibiotics typically used to cure other bacterial infection, TB requires much longer periods of treatment (around 6 to 24 months) to entirely eliminate *mycobacteria* from the body (Centre for Disease Control and Prevention, 2000). Latent TB treatment usually uses a single antibiotic, while active TB disease is best treated with combination of several antibiotics, to reduce the risk of the bacteria developing antibiotic resistance (O' Brien, 1994). People with latent infection are treated to prevent them from progressing to active TB disease later in life.

Drug resistant tuberculosis is transmitted in the same way as regular TB. Primary resistance occurs in persons who are infected with a resistant strain of TB. A patient with fully susceptible

TB develops secondary resistance during TB therapy because of inadequate treatment, not taking the prescribed regimen appropriately or using low quality medication (O' Brien, 1994). Drug resistant TB is a public health issue in many developing countries as treatment is longer and requires more expensive drugs. Multi drug-resistant tuberculosis (MDR-TB) is defined as resistance to the two most effective first-line TB drugs: rifampicin and isoniazid. Extensively drug-resistant TB (XDR-TB) is also resistant to three or more of the six classes of second-line drugs (Centre for Disease Control and Prevention, 2006).

2.14.5 Prevention of tuberculosis

TB prevention and control takes two parallel approaches. In the first, people with TB and their contacts are identified and then treated. Identification of infections often involves testing high-risk groups for TB. In the second approach, children are vaccinated to protect them from TB. No vaccine is available that provides reliable protection for adults. However, in tropical areas where the levels of other species of *mycobacteria* are high, exposure to non tuberculous mycobacteria gives some protection against TB (Fine *et al.*, 2001).

The WHO declared TB a global health emergency in 1993 and the Stop-TB-partnership developed a global plan to stop tuberculosis that aims to save 14 million lives between 2006 and 2015 (WHO, 2006). Since humans are the only host of *Mycobacterium tuberculosis*, eradication would be possible. This goal would be helped greatly by an effective vaccine (Martin, 2006). Many countries use Bacillus Calmette-Guerin (BCG) vaccine as part of their TB control programmes, especially for infants. According to the WHO, this is the most often used vaccine world wide with 85% of infants in 172 countries immunized in 1993 (WHO, 1995). This was the first vaccine for TB and developed at the Pasteur Institute in France between 1905 and 1921 (Bonah, 2005). The protective efficacy for preventing pulmonary TB in adolescents and adult is variable, ranging from 0 to 80% (Bannon and Finn, 1999). BCG provides some protection against severe forms of pediatric TB, but it has been shown to be unreliable against adult pulmonary TB which accounts for most of the disease burden world wide. Currently, there are more cases of TB on the planet than at any other time in history and most people agree that there is an urgent need for a newer, more effective vaccine that would prevent all forms of TB including drug resistant strains in all age groups and among people with

HIV (Sadoff, 2006). Several new vaccine to prevent TB infection are being developed. The firstrecombinant tuberculosis vaccine $rBCG_{30}$ entered clinical trials in the United State in 2004, sponsored by the National Institute of Allergy and Infectious Diseases (NIAID, 2004). A 200study showed that a DNA TB vaccine given with conventional chemotherapy can accelerate thedisappearance of bacteria as well as protect against re-infection in mice (Ha *et al*., 2005). A very promising TB vaccine, MVA85A, is currently in phase II trials in South Africa by a group led by Oxford University (Ibanga *et al*., 2006) and it is based on a genetically modified vaccine virus. Many other strategies are also being used to develop novel vaccine (Doherty and Andersen, 2005), including both subunit vaccines (fusion molecules composed of two recombinant protein delivered in an adjuvant) such as Hybrid-1, Hyvac-4 or M72, and recombinant adeno viruses such as Ad35 (Saten Serum Institute, 2009). Some of these vaccines can be effectively administered without needles making them preferable for areas where HIV is very common (Dietrich *et al*., 2006). All of these vaccines have been successfully tested in humans and are now in extended testing in TB-endemic regions. To encourage further discovery, researchers and policy makers are promoting new economic models of vaccine development including prizes, tax incentives and advance market commitments (Webber *et al*., 2001; Barder *et al*., 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.0 Source of Plant Materials

Fresh bulbs of *Crinum jagus* were collected from Omi-Adio, a town located at the surburb area in Ibadan, Oyo State of Nigeria. Samples were authenticated and identified at the Herbarium of Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State. Specimens (Voucher No: FHI-109011) were deposited at FRIN. The bulbs were chopped in order to increase the surface area of the samples. The chopped samples were air-dried. The dried samples were pulverized into powder and weighed.

3.1 Preparation of Crude Methanol Extract

The powdered bulbs were loaded into an extraction thimble, covered with cotton wool at the top. This was then extracted with boiling petroleum ether for 24 hours in a Soxhlet apparatus. The first extraction was a cleansing exercise which remove lipids and several pigments (Fenwick *et al.*, 1992). The solvent was then changed to methanol and the extraction continued for another 24 hours. The petroleum ether and methanol were recovered by simple distillation. The solvents (methanol or petroleum ether) remaining in the extract was finally removed by concentrating the extract using a rotary evaporator. The extract which was a brown semi-solid substance was transferred into a clean dry bottle, weighed and labelled as the crude methanol extract.

3.2 Experimental Animals

Male wistar rats of the albino strain weighing between 160-200g were obtained from the animal house of the Anatomy Department, College of Medicine, University of Ibadan, Ibadan.

Male swiss mice weighing between (20-30g) were obtained from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan. The animals were kept in well ventilated cages, with 12 hours light/dark cycling. Commercial rat pellet and water were given *ad libitum*. The animals were allowed to acclimatize for two weeks with their enviroment before the commencement of experiments.

3.3 Phytochemcial Screening

Phytochemcial screening of the bulb extract of *Crinum jagus* was carried out in order to determine some of the bioactive components of the plant. The extract was chemically tested for alkaloids flavonoids, proteins, carbohydrates, saponins, cardiac glycosides, tannins, phenols, steroids and reducing sugars by the method described by Edeoga *et al.*, (2005) using standard pharmacognosis procedures.

3.3.1 Qualitative test for phytochemicals and other substances

Procedures

1. **Alkaloids**

A 0.5g sample of the plant extract was stirred with 5ml of 10% hydrochloric acid on a steam bath and a drop of Drangerdoffs, Wagner and Meyer's reagent added to different portions of 1ml of extract filtrate. Appearance of yellowish brown or orange precipitate was used to check for the presence of alkaloids.

2. **Flavonoids**

A 2ml aliquot of the methanolic solution of the plant extract (1g/100ml) was dissolved in dilute NaOH and some drops of dilute HCl added. Formation of a yellow solution on addition of dilute NaOH was taken as a preliminary evidence for the presence of flavonoids, and subsequent decolourisation in the presence of dilute HCl was used as a confirmatory test for the presence of flavonoids.

3. **Saponins**

A 1ml portion of the methanolic solution of the plant extract (1g/100ml) was shaken with 5ml of distilled water. Presence of frothing was used to check for the presence of saponins

4. **Tannins**

A 0.5g sample of the plant extract was stirred with 5ml of distilled water, filtered and iron (II) chloride reagent added to the filtrate. Blue-green colouration was used to confirm the presence of tannins.

5. **Phenols**

To 5ml of the methanolic solution of the plant extract was added 3ml of 5% iron (II) chloride solution, followed by few drops of 5% potassium ferricyanide solution. The presence of a dark green precipitate was taken as a positive test for the presence of phenols.

6. **Steroids**

A 1ml portion of the methanolic solution of the plant extract was added to 1-2ml of concentrated H_2SO_4. Presence of blood red colouration confirmed the presence of steroids.

7. Protein

A 5ml of the methanolic solution of the extract (1g/100ml) was placed in a test tube. Five drops of picric acid solution was added. The formation of a precipitate was used to confirm the presence of protein.

8. **Carbohydrate (Molisch's test)**

A 5ml aliquot of the methanolic solution of the extract (1g/100ml) in a test tube was added to ten drops of 10% α-naphtol and 1ml of concentrated H_2SO_4 was poured slowly down the side of the tube to give a lower layer. A violet colour at the junction of the two layers indicates the presence of carbohydrate.

9. **Cardiac Glycosides**

A 0.5g sample of the methanolic extract was dissolved in 2ml of glacial acetic acid containing one drop of the iron (II) chloride solution followed by the addition of 1ml of concentrated H_2SO_4. A brown ring at the interface confirmed the presence of cardiac glycosides.

10. **Reducing Sugars**

The extract was first hydrolysed with hydrochloric acid by dissolving 0.25g of the extract in 25ml of 1M HCl under heating, and then filtered. To a mixture of 1ml of Fehling solution A and 1ml of Fehling solution B was added ten drops of the filtrate and boiled in a water bath for few minutes. The formation of a brick red precipitate of cuprous oxides was used to confirm the presence of reducing sugars.

3.3.2 Procedures for quantitative phytochemcial test

1. **Alkaloids determination**

This is a distillation and titrimetric procedure (Henry, 1993). A 2g portion of the sample was weighed into a 100ml beaker and 20ml of 80% absolute alcohol added to give a smooth paste. The mixture was transferred to a 50ml conical flask and more alcohol added to make up to 100ml and 1g of magnesium oxide added. The mixture was digested in a boiling water bath for 1 hour 30 minutes under a reflex air condenser, with occasional shaking. The mixture was filtered while hot through a small bucher funnel. The residue was returned to the flask and re-digested for 30 minutes with 50ml alcohol after which the alcohol was evaporated. Hot water was then added to replace the alcohol lost, when all the alcohol has been removed and 3 drops of 10% HCl was added. The whole solution was later transferred into a 250ml volumetric flask and 5ml of zinc acetate solution and of 5ml potassium ferricyanide solution were added with thorough mixing to give a homogenous solution. The flask was allowed to stand a few minutes, and the contents filtered through a dry filter paper and 10ml of the filtrate was transferred into a separating funnel and the alkaloids present were extracted by shaking vigorously with five successive portions of chloroform. The residue obtained was dissolved in 10ml hot distilled water and transferred into a Kjeldahl tube with the addition of 0.02g sucrose and 10ml conc H_2SO4 and 0.02g selenium for digestion to a colourless solution to determine the percentage nitrogen by Kjeldahl distillation method. The result obtained was converted to percentage (%) total alkaloid by multiplying with a factor 3.26 as follows;

Total alkaloids = % N x 3.26.

2. **Flavonoids determination**

A 0.50g of the sample was weighed into a 100ml beaker and 80ml of 95% ethanol added with stirring with a glass rod. The mixture was filtered through a Whatman No 1 filter paper into a 100ml volumetric flask and made up to mark with ethanol. One ml of the extract was pipetted into a 50ml volumetric flask and 4 drops of conc HCl was added via a dropping pipette, after which 0.5g of magnesium turnings was added to develop a magenta red colouration. Standard flavonoid solution, of range 0-5ppm, was prepared from 100ppm solution and treated in a

similar way with HCl and magnesium turnings like the sample. The absorbance of a resultant magenta red colouration of sample and standard solution was read on a digital Jenway V6300 spectrophotometer at a wavelength of 520nm. The percentage concentration of flavonoids was calcaulted using the formula;

$$\% \text{ Flavonoid} = \frac{\mathbf{A} \text{ sample} \times \text{average gradient factor} \times \text{dilution factor}}{\text{wt of sample} \times 10{,}000}$$

where **A** = absorbance

3. **Saponins determination**

The spectrophotometric method of Bruner (1984) was used for saponin analysis. A 1g sample was weighed into a 250ml beaker, and 100ml of isobutyl alcohol was added. The mixture was shaken on UDY shaker machine for 5 hours to ensure uniform mixing. Thereafter, the mixture was filtered through a Whatman No1 Filter paper into a 100ml beaker and 20ml of 40% saturated solution of magnesium carbonate was added. The mixture obtained with saturated magnesium carbonate was again filtered through a Whatman No1 filter paper to obtain a clear colourless solution. An aliquot of 1ml of the colourless solution was pipetted into 50ml volumetric flask and 2ml of 5% $FeCl_3$ solution was added and made up to mark with distilled water. It was allowed to stand for 30 minutes for blood red colour to develop. A 0-10ppm standard solution was treated similarly with 2ml of 5% $FeCl_3$ solution as was done for 1ml sample above. The absorbance of the red coloured sample as well as standard saponin solutions were read after colour development in a Jenway V6300 spectrophotmeter at a wave length of 480nm.

$$\% \text{ Saponin} = \frac{\mathbf{A} \text{ sample} \times \text{gradient factor} \times \text{dilution factor}}{\text{wt of sample} \times 10{,}000}$$

where **A** = absorbance

4. **Phenol determination**

About 4g of the sample was treated with 30ml of 80% acetone in a 250ml beaker at 25^0C in the dark to extract the phenolics. The mixture was transferred to a blender, and homogenised 3 times with successive addition of 30ml of 80% acetone. Alternatively, the mixture can be

centrifuged at 3000 rpm for 30minutes, each with 30ml addition of 80% acetone. The homogenised mixture was then filtered through a Whatman No1 filter paper into a 100ml volumetric flask and made up to mark with 80% aqueous acetone. An aliquot of 0.05ml of the phenolic extract was diluted to 2ml in a 10ml measuring flask, 1ml of Folin Ciolcateau reagent was added and the flask was vigorously shaken . Immediately afterwards 5ml of 20% sodium carbonate solution was pipetted into the 10ml flask and the mixture made up to 10ml, with thorough shaking. A 0-10ppm range of gallic acid standard solutions were prepared from 100ppm gallic acid standard and treated similarly like sample above. After 20 minutes, the absorbance of the sample as well as standard were read on a spectronic 2ID spectrophotmeter at a wavelength of 735nm.

The calculation of % total phenolic = $\frac{\mathbf{A} \text{ sample} \times \text{gradient factor} \times \text{dilution factor}}{\text{wt of sample} \times 10{,}000}$

where **A** = Absorbance

5. **Steroids determination**

A 0.5g of the sample was weighted into a 100ml beaker, 20ml of chloroform was later filtered through a Whatman No1 filter paper into another dry clean 100ml conical flask . The resultant residue was repeatedly treated with chloroform- methanol mixture until it is free of steroids. 1ml of filtrate was pipetted into a 30ml test tube and 5ml of alcoholic KOH was added and shaken thoroughly to obtain a homogenous mixture. The mixture was later placed in a water bath at 37-40^{0}C for 90 minutes. The mixture was cooled to room temperature and 10ml of petroleum ether was added, followed by the addition of 5ml distilled water. This was evaporated to dryness on the water bath and 6ml of the Liebermann Burchard reagent was added to the residue in dry bottle and absorbance taken at a wavelength of 620nm on a spectronic 21D spectrophotometer. Standard steroids of concentrations of 0-4mg/ml were prepared from 100mg/ml stock steroid solution and treated similarly like sample above.

The calculation of % steroid = $\frac{\mathbf{A} \text{ sample} \times \text{gradient factor} \times \text{dilution factor}}{\text{wt of sample} \times 10{,}00}$

where **A** = Absorbance

3.4 Fractionation of the Crude Extract by Column Chromatography

A modified form of classical column chromatography called the flash chromatography was employed for the fractionation of the crude methanol extract of the bulb of *Crinum jagus* in the study. A glass column was packed with silica gel (60-200 flash chromatography grade) using n-hexane under positive pressure. The bulb extract adsorbed with silica gel was packed on to the column layer and then allowed to settle. The mobile phase consisted of three solvents: hexane (non polar), ethylacetate (mid polar) and methanol (polar). The solvents were mixed in various proportions so as to achieve gentle gradient in terms of separation. The various proportions of the solvents were pushed through the bed by the application of positive pressure using the vacuum pump. Twenty one fractions were obtained.

3.5 Separation of Crude Extract of *Crinum jagus* by Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out using analytical silica gel pre-coated plates. The fractions were spotted on TLC plates using capillary tubes. The spotted plates were developed in a chamber saturated with ethylacetate / methanol : 9:1 as mobile phase. The separated spots were then visualized under ultraviolet lamp (354nm). Positive spots with the same retention factor (RF) were pooled together.

3.6 Acute Toxicity Study of the Methanol Extract of *Crinum jagus*

A total of 25 male Swiss mice of the albino strain were used for the study. They were kept in the experimental cage for two weeks for acclimatization before the experiment. The animals were fed on standard mouse cubes and allowed free access to drinking water. The mice were weighed and grouped into A, B, C, D and E with five animals per group. Groups B, C, D, and E received 50, 100, 200 and 400 mg/kg of the extracts, respectively, through oral administration while the animals in group A served as the control and received normal saline (vehicle for the stock solution of *Crinum jagus* by the same route). The animals were observed for 48 hours under room temperature. Percentage mortality was then calculated using the moving average interpolation method (Thomson and Williams, 1974).

3.7 *In vitro* Antioxidant Study on Crude Extract and Fractions of the bulb of *Crinum jagus*

3.7.1 Determination of DPPH radical scavenging activity of extract and fractions

Free radical scavenging activity was determined using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) photometric method of Mensor *et al*., (2001).

Principle.

When DPPH reacts with an antioxidant compound which can donate hydrogen, it is reduced and the change in colour from deep violet to golden light yellow is measured at 518nm spectrophotometrically.

Reagents

1. Stock solutions (10mg/ml)

A 0.1g sample of the crude methanol extract and each of the fractions of the bulb of *Crinum jagus* were dissolved separately in methanol and the volume made up to 10ml to obtain the respective stock solutions.

2. Test solutions (100µg/ml)

From the stock solution of the crude methanol extract and each of the chromatographic fraction, 1ml was dissolved in 9ml each of 70% methanol to obtain their respective test solutions. Various concentrations of the crude extract and each of the fractions, ranging between 100µg/ml-500µg/ml, were prepared. Ascorbic acid, the standard antioxidant, was prepared using the same procedure.

3. DPPH (0.3mM)

This was prepared by dissolving 0.03g of DPPH in methanol and the volume made up to 250ml.

Procedure

1ml of 0.3mM of DPPH solution was added to 1ml each of the test solutions (various concentrations of the extract and the fractions ranging from 100-500µg/ml), and this was then allowed to react at room temperature, in the dark for 30 minutes. Iml of 0.3mM of DPPH solution was added to 1ml of methanol to serve as negative control. Methanol (1ml) was added to 1ml of extract to serve as blank. The positive control is ascorbic acid solution. Absorbance at 518nm was read after 30minutes and the percentage activity was calculated as:

DPPH scavenging activity = ($\mathbf{A}_o$ – **A**s/$\mathbf{A}_o$) x 100

Ao = absorbance without sample

As = absorbance with sample .

3.7.2 Determination of Hydroxyl Radical Scavenging Activity of Extract and Fractions

The hydroxyl radical scavenging activity was determined by the photometric method of Halliwell *et al.*, (1987).

Principle

The principle is based on the competition between deoxyribose and the test extract for hydroxyl radicals generated by Fe^{3+}/ ascrobate/ EDTA/H_2O_2 system.

Reagents

1. **Deoxyribose (2.8mM)**

A 0.38g weight of deoxyribose was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

2. **Ferric chloride (200µM)**

A 0.003g weight of $FeCl_3$ was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

3. **EDTA (1.04mM)**

A 0.31g weight of EDTA was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

4. **H_2O_2 (1.0mM)**

This solution was prepared by making up 0.1ml of 30% H_2O_2 to 100ml using distilled water.

5. **Ascorbic acid (1.0mM)**

A 0.18g weight of ascorbic acid was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

6. **Thobarbituric acid (1%w/v)**

A 1.0g weight of TBA was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

7. Glacial acetic acid (20%, pH 3.5)

This solution was prepared by adding 20ml of glacial acetic acid to 80ml of distilled water and adjusting the pH to 3.5 and stored in a reagent flask.

8. 0.1M Phosphate buffer 9 (pH 7.4)

(a) A 13.61g weight of KH_2PO4 was dissolved in 1L of distilled water.

(b) A 17.4g weight of K_2HPO_4 was also dissolved in 1L of distilled water.

Solution (a) was added to (b) and the pH of the obtained solution was adjusted to 7.4

Procedure

All solutions were freshly prepared. An aliquot of 1.0ml of the reaction mixture contained 100µl of 2.8mM deoxyribose (dissolved in phosphate buffer, pH7.4), 500µl of each of the various concentrations of the extract and the fractions (100-500µg/ml), 200µl of 200µM $FeCl_3$ and 1.04µM EDTA (1:1v/v),100µl of 1.0mM H_2O_2, 100µl of 1.0mM ascorbic acid . The reaction mixture was incubated for 1 hour at 37^0C in a water bath. 1ml of TBA and 1ml of glacial acetic acid were added after incubation. The entire mixture was heated at 100^0C for 1 hour. After cooling the extent of deoxyribose degradation was measured by the thiobarbituric reaction. The absorbance was read at 532 nm. The percentage hydroxyl radical scavenging activity was calculated as:

% Hydroxyl Radical Scavenging Activity = 100 - [(**A** sample – **A** control /**A** control)] x 100

3.7.3 Determination of the reductive potential of extract and fractions

The spectrophotometric method of Oyiazu (1986) was employed in determining the reducing power of the extract and the fractions.

Principle

The reducing power of a compound is related to its electron transfer ability and the principle is based on the ability of the extract and the fractions to reduce Fe^{3+} to Fe^{2+}.

Reagents

1. Phosphate buffer (0.1M,pH 7.4)

(a) A 13.61g weight of KH_2PO_4 was dissolved in 1L of distilled water.

(b) A 17.4g weight of K_2HPO_4 was also dissolved in 1L of distilled water.

Solution (a) was added to (b) and the pH of the obtained solution was adjusted to 7.4

2. Potassium ferricyanide

This was prepared by dissolving 1g of potassium ferricyanide in distilled water and the volume was made up to 100ml.

3. Trichloroacetic acid (10% TCA)

A 10g weight of TCA was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

4. Ferrous chloride ($FeCl_3$, 1%).

A 1g weight of $FeCl_3$ was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

Procedure

An aliquot of 150µl of each of the various concentrations of the extract and the fractions (100-500µg/ml) in 1ml distilled water was mixed with 2.5ml each of phosphate buffer and potassium ferricyanide. The mixture was incubated at 50^0C for 20minutes in a water bath. A 2.5ml portion of 10% TCA was then added and the mixture was centrifuged at 1000g for 10minutes. Thereafter, 2.5ml of the supernatant was mixed with 2.5ml of distilled water and 0.5ml of $FeCl_3$. The absorbance was read at 700nm in spectrophotometer. Ascorbic acid was used as positive control.

3.7.4 Determination of the total flavonoids content of the extract and fractions.

Principle

The total favonoids content of the extract and the fractions was estimated by the aluminium chloride colorimetric method, using aluminium chloride-flavonoids complex formation as described by Chang *et al.*, (2002).

Reagents

1. 70% Methanol

70ml of absolute methanol was made up to 100ml with distilled water before storing in a reagent flask.

2. 10% Aluminium chloride

A 10g weight of aluminum chloride was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

3. 1.0 M Sodium acetate

A 9.3g weight of sodium acetate was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

Procedure

Each of the various concentrations of the plant extract and the fractions (1ml) were added to 0.1ml of 10% aluminum chloride, 0.1ml of 1M sodium acetate and 2.8ml of distilled water. The reaction mixture was incubated at room temperature for 30 minutes in a water bath. The absorbance of the reaction was read at 415nm in spectrophotometer. Quercetin as the standard was treated in a similar manner at various concentrations to prepare the standard calibration curve (Fig 9). Total flavonoid content was expressed as µg/g quercetin equivalent using the formula.

$$F = \frac{C.V}{W}$$

Where F = total content of flavonoid compound mg/g plant extract in QE

C = concentration of quercetin equivalent of extract established from the calibration curve (mg/ml)

V = volume of extract (ml)

W = weight of the pure plant extract (g)

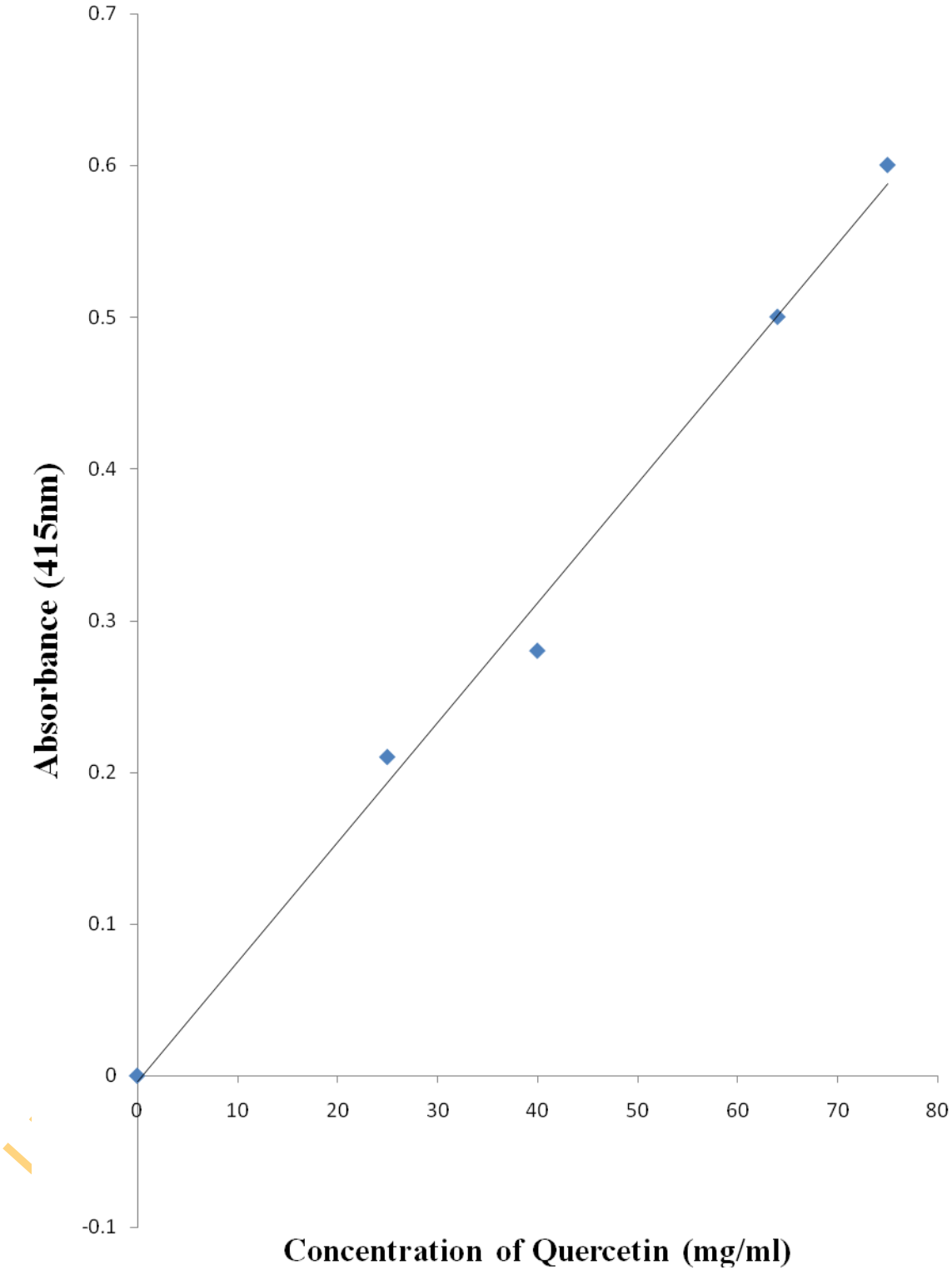


Figure 9 : Quercetin standard calibration curve

3.7.5 Determination of the total phenolic content of the extract and fractions

Principle

The assay was based on the reduction of Folin ciocalteu reagent (phosphomolybdate and pholphotungstate) by the phenolic compounds as described by McDonald *et al.*,(2001). The reduced Folin-Ciocalteu reagent is blue and thus detectable with spectrophotometer in the range of 500-750nm.

Reagents

1. 10% Folin-Ciocalteu reagent

A 10ml portion of Folin-Ciocalteu reagent was made up to 100ml with distilled water before storing in a reagent flask.

2. Gallic acid standard (0.5%w/v)

A 0.5g weight of dry gallic acid was dissolved in methanol and the volume made up to 100ml in a reagent flask.

3. 15% Sodium carbonate

A 15g weight of sodium carbonate was dissolved in distilled water and the volume made up to 100ml in a standard volumetric flask.

Procedure

Serial dilutions of 0.5mg/ml, 1.0mg/ml, 2.0mg/ml and 2.5mg/ml of gallic acid solutions were prepared from the gallic acid standard solution. Serial dilutions ranging from 0.5-2.5mg/ml were also prepared from the various concentrations of the extract and the fractions. The total phenolic content was determined by mixing 0.5ml of the various concentrations of the gallic acid, the extract and the fractions with 2.5ml of 10% Folin-Ciocalteu phenol reagent and 2.0ml of 15% sodium carbonate was added after 3 minutes. The reaction mixture was incubated at 40^0C for 30 minutes and the absorbance was read at 750nm with gallic acid standard. Total content of phenolic compounds in the plant extract (in gallic acid equivalent (GAE) was calculated using the formula:

$$P = \frac{C.V}{W}$$

Where P = total of content of phenolic compound (mg/g) plant extract in GAE

C = concentration of gallic acid of extract equivalent established from the calibration curve (mg/ml)

V = volume of extract (ml)

W = Weight of the pure plant extract (g)

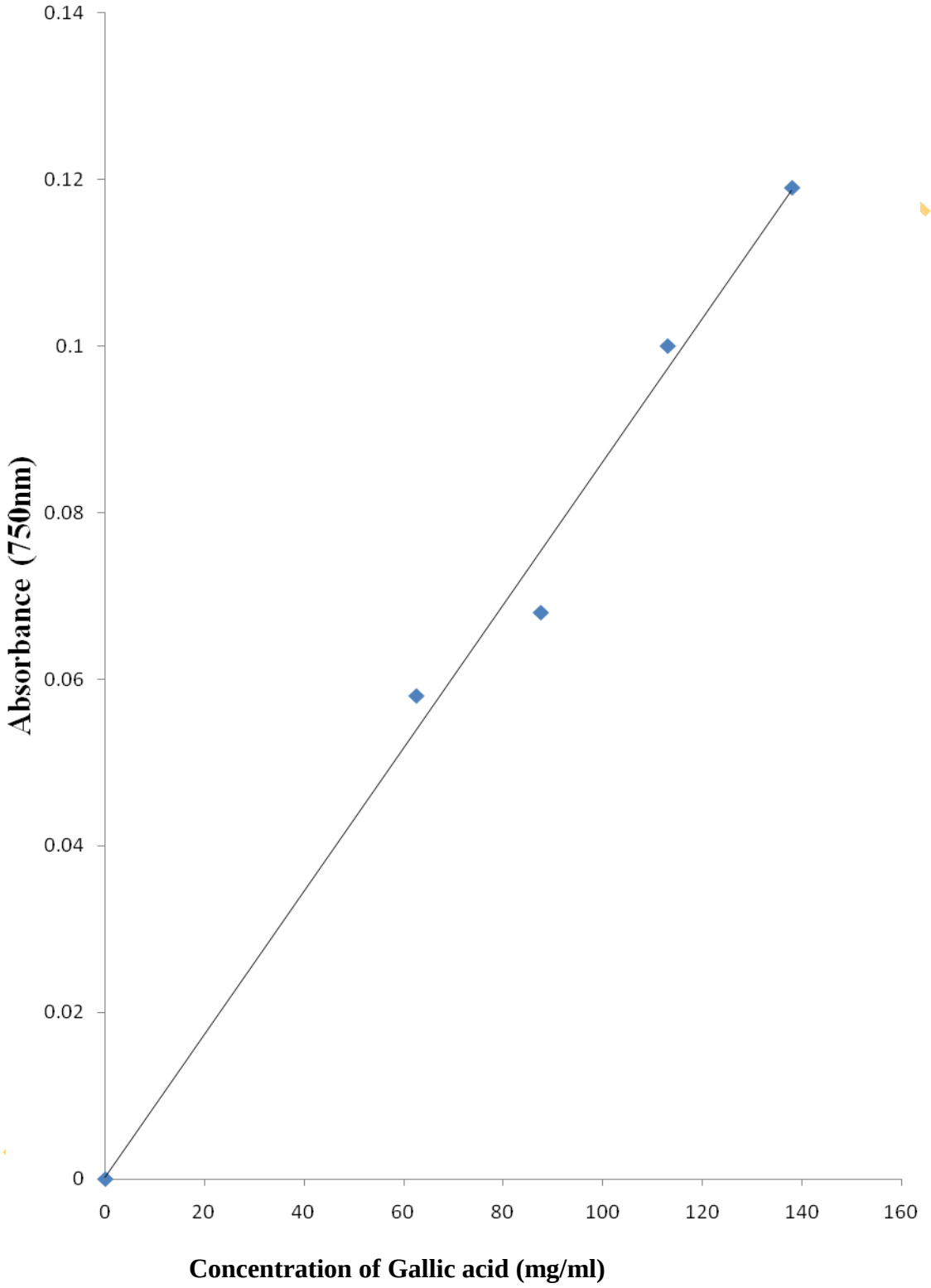


Figure 10 : Gallic acid standard calibration curve

3.8 *In vivo* Antioxidant Activity of Crude Extract and Fractions of the Bulb of *Crinum jagus* in rats

3.8.1 Administration of the crude methanol extract of the bulb of *Crinum jagus*

A total of 30 male rats were randomly distributed into five groups of six animals per group. The crude extract was dissolved in corn oil before administration to the rats using oral cannula and administration was done for 30 days. The control group A received 0.2ml corn oil (vehicle for the extract) for 30 days. Varying doses of 10, 25, 50 and 75mg/kg were administered orally to animal in groups B, C, D and E respectively for 30 days.

3.8.2 Administration of fractions of the bulb of *Crinum jagus*

A total of 35 male rats were divided in to seven groups of five animals each. The control group A received 0.2ml corn oil for 30 days. Animals in groups B and C were treated with 5 and 10mg/kg of fraction 1 respectively. Groups D and E animals were treated with 5 and 10mg/kg of fraction 2 respectively while those in groups F and G received 5 and 10mg/kg of fraction 3 respectively for 30 days through oral administration.

3.8.3 Preparation of tissue homogenate for biochemical analysis

Reagents

1. Homogenising buffer (0.1M phosphate buffer, pH 7.4)

(a) A 11.8g weight of Na_2HPO_4 was dissolved in distilled water and made up to 100ml.

(b) A 6.8g weight of KH_2PO_4 was dissolved in distilled water and made up to 500ml.

Then 800mls of (a) was mixed with 200ml of (b) above to make 1L and the pH was adjusted to 7.4

2. 1.15% Potassium chloride (KCl)

A 1.15g weight of KCl was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

Procedure

The animals were sacrificed by cervical dislocation, 24hrs after the administration of last doses of treatment. The liver and kidney samples were quickly removed and rinsed in 1.15% KCl, dried and weighed. The liver and kidney samples were homogenized in 4 volumes of phosphate buffer (0.1M, pH 7.4). The resulting homogenate were centrifuged at 10,000xg for 20minutes

to obtain the post-mitochondrial supernatant which was decanted into samples bottles and stored at 8^0C until used

3.8.4 Biochemical Assays

3.8.4.1 Protein determination

Protein concentration of the liver and kidney homogenates were estimated by the colorimetric Biuret method as described by Gornall *et al.*, (1949) using Bovine Serum Albumin (BSA) as standard. Potassium iodide was added to the Biuret reagent to prevent the precipitation of cupric ions (Cu^{2+}) to cuprous oxide.

Principle

The assay is based on the reaction of Cu^{2+} and protein under alkaline conditions to form a blue complex with maximum absorbance at 540nm. The absorbance of the complex is proportional to the protein concentration in the sample. The Biuret reagent consists of copper sulphate, potassium iodide and sodium-potassium tartarate, which acts as a stabilizer for the reagent.The procedure is usually calibrated with a BSA standard curve.

Reagents

1. **0.2M NaOH**

A 8.0g weight of NaOH was dissolved in litte distilled water and the solution made up to 1L and stored in a reagent flask

2. **Biuret reagent**

A 3.0g weight of copper sulphate ($CuSO_4.\ 5H_20$) and 5g of Na-K tartarate were dissolved in 500ml of 0.2M NaOH. 5g of KI was added to the solution and the volume made up to 1L with 0.2M NaOH.

3 . **0.9% NaCl solution (saline)**

A 0.9g weight of NaCl was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

4. **Stock Bovine Serum Albumin (BSA) solution (1mg/ml)**

A 0.1g weight of BSA was dissolved in 100ml of distilled water to give a stock solution of 1mg/ml.

Procedure

Serial dilutions of the stock BSA solution were made using normal saline. 4ml of Biuret reagent was added to 1ml of each diluted protein standard solution (BSA) and the mixture allowed to stand at room temperature for 30minutes. The absorbance of the solution were then read at 540nm in spectrophotometer and a graph of absorbance against mg BSA was then plotted.

Table 3 : Protein determination assay medium

TABLE NO	1	2	3	4	5	6	7
Stock BSA (ml)	0	0.05	0.10	0.20	0.30	0.40	0.50
Normal saline (ml)	1.0	0.95	0.90	0.80	0.70	0.60	0.50
Biuret reagent (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Amount of BSA(mg)	0	0.05	0.1	0.2	0.3	0.40	0.5
Absorbance (540nm)	0.0	0.001	0.006	0.015	0.017	0.031	0.047

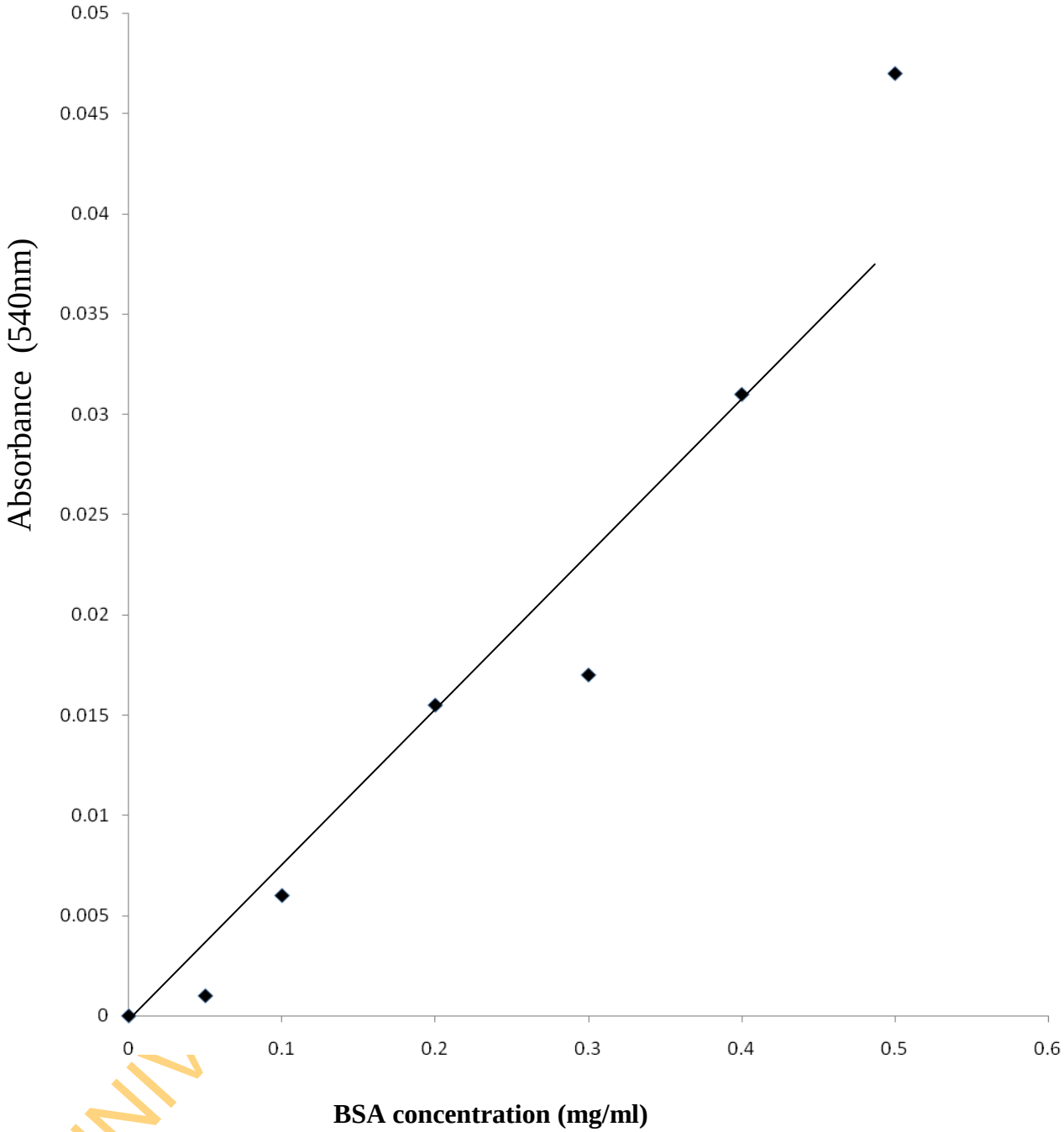


Figure 11 : Protein estimation standard curve

Estimation of protein in test samples followed a procedure identical to those employed for the standard were used except that suitable dilutions of the test samples were made. This was done to reduce the level of protein in the sample to the sensitivity range of Biuret method. 1ml of the diluted samples (0.1ml of sample with 0.9ml of distilled water) was taken and the process for protein determination as described above was repeated. The absorbance was read in a spectrophotometer at 540nm against sample blank containing 1ml of distilled water and 4ml of Biuret reagent. Protein content of samples were extrapolated from the protein standard curve and multipled by 100 to get the actual amount of protein in the sample.

3.8.4.2 Lipid peroxidation assay.

Lipid peroxidation was assessed by measuring the thiobarbituric acid reactive (TBAR) products using the spectrophotometric procedure of Vashney and Kale (1990) and expressed as micromolar of malondialdehyde (MDA)/g tissue.

Principle

Small amounts of malondialdehyde (MDA) are produced during lipid peroxidation and these are able to react with thiobarbituric acid (TBA) to generate a pink coloured complex which in acidic solution absorbs light at 532nm.

Reagents

1. 30% Trichloroacetic acid (TCA) solution

A 9g weight of TCA was dissolved in distilled water and the volume made up to 30ml.

2. 0.75% Thiobarbituric acid (TBA) solution

A 0.225g weight of TBA was dissolved in 30ml of 0.1M HCl by shaking in hot water before storing in a reagent flask.

3. 0.15M Tris KCl (pH 7.4)

A 1.12g weight of KCl and 2.36g of Tris base were separately dissolved in distilled water, the two solutions were mixed together, and the volume made up to 100ml and the pH adjusted to 7.4

Procedure

A 0.1ml portion of the sample was diluted in 0.9ml of distilled water to make a dilution of 1 in 10. An aliquot of 0.4ml of the test sample was mixed with 1.6ml Tris KCl buffer to which 0.5ml

of 30% TCA was added. A 0.5ml portion of TBA was then added and placed in water bath for 45 minutes at 80^0C. This produced pink coloured reaction mixtures which were centrifuged at 14000g for 15 minutes. The absorbance of the clear pink supernatant was then read against reference blank of distilled water at 532nm in spectrophotometer.

Calculations

$$\text{MDA (units/mg protein)} = \frac{\text{absorbance x volume of mixture}}{\text{E 532 x volume of sample x mg protein}}$$

E 532 = molar absorbitivity at 532nm = 1.56×10^6

3.8.4.3 Superoxide dismutase assay

The level of superoxide dismutase (SOD) activity was determined by the photometric method of Misra and Fridovich (1972)

Principle

The ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine at pH 10.2 makes this reaction a basis for a sample assay for SOD. Superoxide anion (O_2) generated by the xanthine oxidase reaction is known to cause the oxidation of epinephrine to adrenochrome. The yield of adrenochrome produced per superoxide anion introduced increased with increasing concentration of epinephrine.

These results led to the proposal that auto-oxidation of epinephrine proceeds by at least two distinct pathways, one of which is a free radical chain reaction involving superoxide anion radical and hence inhibitable by SOD.

Reagents

1. 0.05M Carbonate buffer (pH, 10.2)

A 14.3g weight of $Na_2CO_3.10H_2O$ and 4.2g of $NaHCO_3$ were dissolved in distilled water and made up to 1000ml mark in a litre standard volumetric flask. The solution was adjusted to pH 10.2

2 . 0.3mM Adrenaline

A 0.003g weight of epinephrine was dissolved in 50ml of distilled water and stored in a reagent flask. The solution was prepared fresh just before use.

Procedure

A 0.1ml portion of the sample was diluted in 0.9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml of carbonate buffer, 0.3ml of substrate (adrenaline) and 0.2ml of distilled water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Calculation

$$\text{Increase in absorbance per minute} = \frac{A_3 - Ao}{t}$$

where A_3 = Final absorbance

Ao = Initial absorbance

t = Time of final absorbance (150s or 2.5mins)

$$\% \text{ Inhibition} = \frac{\text{Increase in absorbance of sample/min}}{\text{Increase in absorbance of blank}} \times 100$$

$$\text{Unit of activity} = \frac{\% \text{ inhibition}}{50\%}$$

$$\text{Specific activity} = \frac{\text{Enzyme activity}}{\text{Total protein (mg)}} \times \text{dilution factor}$$

3.8.4.4 Catalase assay

Catalase activity was determined according to the colorimetric method of Sinha (1971).

Principle

This method is based on the fact that dichromate in acetic acid is reduced to chromic acid when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromic acetate so produced is measured colorimetrically at 570-610nm. Dichromate has no absorbance at this wavelength and hence its presence in the assay mixture does not interfere with the determination of chromic acetate. Catalase preparation in samples is allowed to split H_2O_2 for different periods of time. The reaction was stopped at a particular

time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically, after heating the reaction mixture.

Reagents

1. 5% Potassium heptaoxodichromate (5% $K_2Cr_2O_7$)

A 5.0g weight of K_2Cr_2O7 was dissolved in some distilled water in a 100ml volumetric flask and made up to the mark.

2. 0.2M Hydrogen peroxide (H_2O_2)

A 0.67g weight of H_2O_2 was mixed with little distilled water and then made up to 100ml mark in a standard volumetric flask and stored at 4^0C.

3. Dichromate/acetic acid solution

This solution was prepared by mixing solution of 5% K_2Cr_2O7 with glacial acetic acid and storing in a brown bottle.

4. 0 .01M Phosphate buffer (pH 7.0)

A 3.58g weight of $Na_2HP0_4.12H_2O$ and 1.19g of $NaH_2PO_4.2H_2O$ were dissolved in 900ml of distilled water. The pH was adjusted to 7.0 and distilled water added to make it up to 1litre.

Procedure

Preparation of H_2O_2 Standard Curve

Different amounts of H_2O_2 ranging from 10 to 60µmoles were taken in small test tubes and 2ml of dichromate/acetic acid was added to each. Addition of the reagents instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10minutes in a boiling water bath changed the colour of the solution to stable green due to formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3ml with distilled water and the absorbance measured with a spectrophotometer at 570nm. The concentration of the standard were plotted against absorbance.

Table 4 : Assay mixture for calibration of standard curve for catalase

TUBE NO	1	2	3	4	5	6	7
H_2O_2 (ml)	0.00	0.10	0.20	0.30	0.40	0.50	0.60
Dichromate/acetic acid (ml)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water (ml)	1.00	0.90	0.80	0.70	0.60	0.50	0.40
H_2O_2 concentration (mg)	0.00	0.67	1.34	2.01	2.68	3.35	4.02
Absorbance (540nm)	0.0	0.07	0.22	0.26	0.30	0.40	0.48

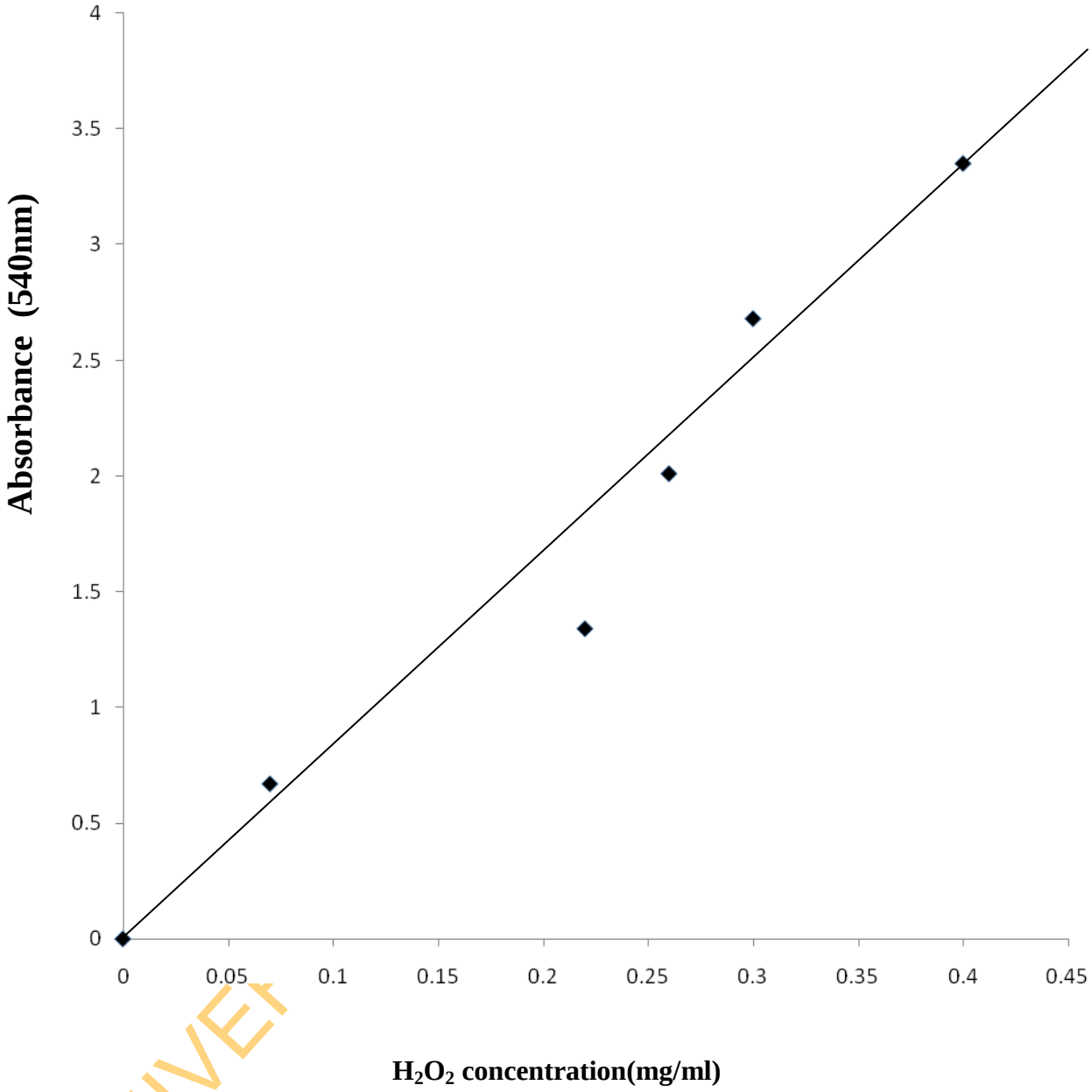


Figure 12 : Catalase standard curve

Determination of catalase activity of test samples

A 1ml portion of the sample was mixed with 49ml distilled water to give 1 in 50 dilution of the sample. The assay mixture contained 4ml of H_2O_2 solution (800µl) and 5ml of phosphate buffer in a 10ml flask. A1ml portion of properly diluted enzyme preparation (test sample) was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. A 1ml portion of the reaction mixture was withdrawn and added into 2ml dichromate/acetic acid reagent at 60 seconds intervals for 3 minutes. The H_2O_2 contents of the withdrawn sample were determined by the method described above.

Calculation

$$K = 1/t \log So/S$$

Where So = the initial concentration of H_2O_2

S = concentration of peroxide at t min (60 seconds interval)

t = time interval (1minute)

The value of K is plotted against time in minute and the velocity constant of catalse (Ko) at time zero determined by extrapolating the catalase content of the enzyme preparation expressed in terms of katalase feiahigkeit or kat "F" according to Von Euler and Josephson (1927).

$$\text{Kat F} = \frac{\text{Ko}}{\text{Mg protein/ml (µ/mg protein)}}$$

3.8.4.5 Reduced glutathione (GSH) assay

The total sulfhydryl groups, protein bound sulphydryl groups and free-sulphydryl groups (such as GSH) in biological samples can be determined using Ellman's reagent (5.5 dithiobis-(2-nitrobenzoic acid) (DTNB) as described by Jollow *et al.*,(1974)

Principle

The method is based on the development of a relatively stable (yellow) complex formed as a result of reaction between Ellman's reagent and free sulphydryl groups. The reduced form of glutathione (GSH) in most instances is the bulk of cellular non-protein sulphydryl groups. The chromophoric product, 2-nitro-5-thiobenzoic acid, resulting from the reaction of Ellman's reagent with GSH possesses a molar absorption at 412nm. The absorbance of this complex at 412nm is proportional to the level of GSH in the sample.

$GSH + R – S –S- R \longrightarrow R\text{-}S^{-} + GSS$

$R\text{-}S^{-}$ = Yellow complex

Reagents

1. Ellman's reagent (DTNB)

A 4.0mg weght of Ellman's reagent (5,5- Dithio-bis (2-nitrobenzoic acid) was dissolved in litte amount of 0.1M phosphate buffer, pH 7.4 and made up to 100ml mark in a standard volumetric flask with the same buffer. It was stored at 4^0C.

2. 0.1M Phosphate buffer (pH 7.4)

(a) A 35.81g weight of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) was dissolved in 1 litre of distilled water.

(b) A 13.61g weight of anhydrous potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 1 litre of distilled water.

(c) A portion of 4 volumes of solution (a) was added to 1 volume of solution (b) and the pH of the resulting solution adjusted to 7.4 using a pH meter.

3. 4% Sulphosalicylic acid (precipitating reagent)

A 4.0g weight of sulphosaliyclic acid was dissolved in litte quantity of distilled water and made up to 100ml mark in a standard volumetric flask with more distilled water.

4. Reduced GSH (Working standard)

A 40mg weight of reduced GSH was dissolved in 100ml of 0.1M phosphate buffer, pH 7.4 and then stored at 4^0C.

Procedure

Serial dilutions of the stock GSH were prepared as shown in table below. To each tube (in duplicates) were added appropriate volumes of phosphate buffer and then followed by the addition of 4.5ml Ellman's reagent. The absorbance of the yellow colour formed upon the addition of Ellman's reagent was read within 30mintues at 412nm using spectrophotometer. A plot of absorbance versus concentration of reduced GSH was obtained.

Table 5 : Assay mixture for calibration of glutathione standard curve

GSH Solution (ml)	PO_4 Buffer (ml)	Ellman's Reagent (ml)	GSH Conc (µg/ml)	Absorbance (412nm)
0.00	0.50	4.5	0	0.00
0.05	0.45	4.5	20	0.05
0.10	0.40	4.5	40	0.12
0.15	0.35	4.5	60	0.15
0.20	0.30	4.5	80	0.20
0.25	0.25	4.5	100	0.25
0.30	0.20	4.5	120	0.30
0.40	0.10	4.5	140	0.36

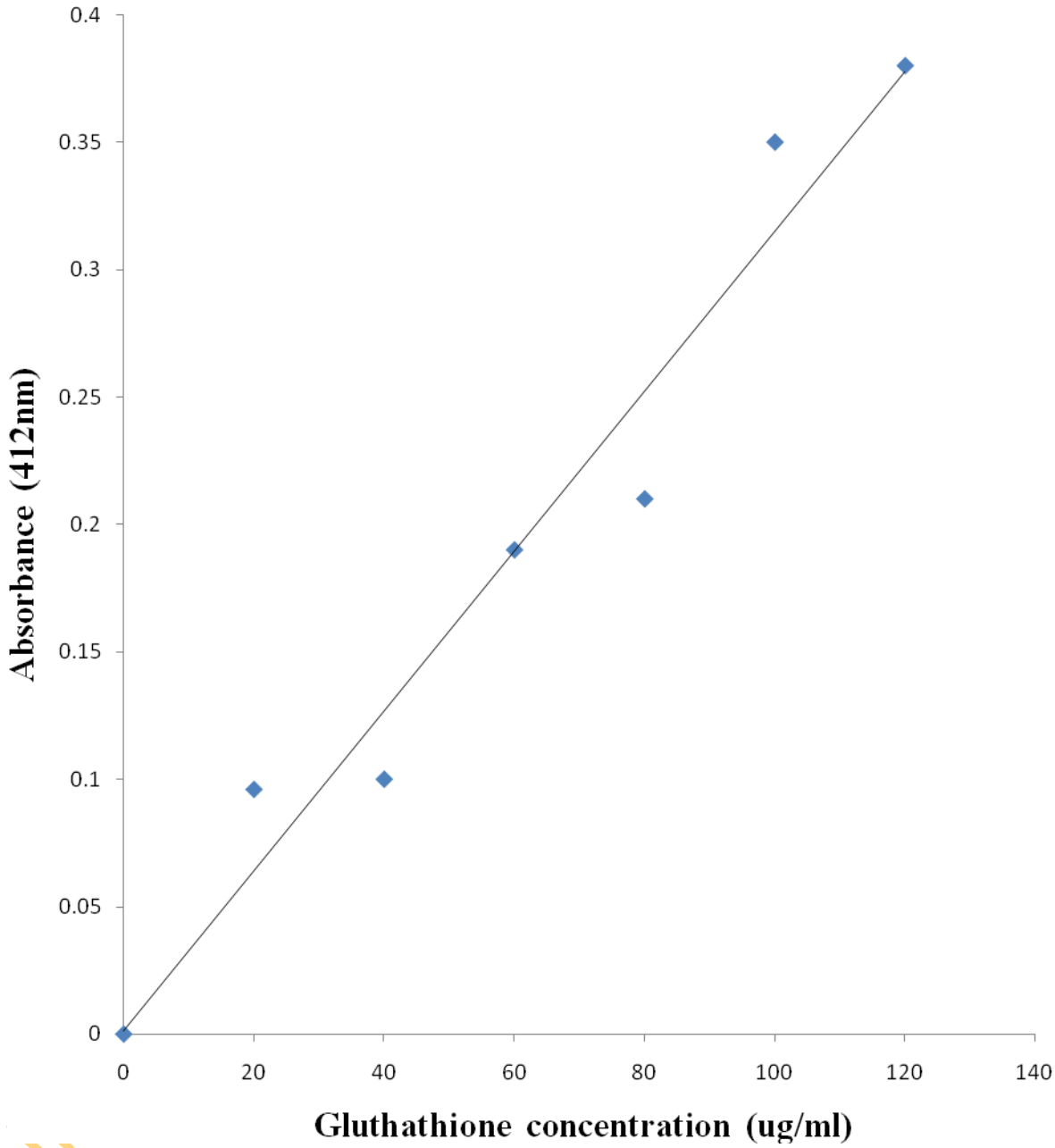


Figure 13 : Glutathione standard curve

Estimation of GSH in test sample

A 0.2ml portion of the sample was mixed with 1.8ml of distilled water to give 1 in 10 dilution. About 3ml of precipitating reagent (4% sulphosalicylic acid) was added to the diluted sample and then allowed to stand for 10minutes. The mixture was centrifuged at 3,000g for 5 minutes and 0.5ml of supertanatant added to 4ml of phosphate buffer and finally 0.5ml of Ellman's reagent was added. The optical densities were read within 30 minutes of colour development at 412nm using spectrophotometer. A blank was prepared with 0.5ml of the diluted precipitating solution (diluted twice with 0.1m phosphate buffer) and 4.5ml of Ellman's reagent. Reduced glutathione is proportional to the absorbance at 412nm.

3.8.4.6 Glutathione-S-transferase (GST) assay

Glutathione-S-transferase activity was determined according to the method of Habig *et al.*, (1977).

Principle

The assay is based on the fact that all GST demonstrate a relatively high activity with 1 chloro-2-4 dinitrobeanzene as the second substrate. Consequently, the conventional assay for GST activity utilises 1-chloro-2, 4,dinitrobenzene as susbtrate. When this substance is conjugated with reduced glutathione its absorption maximum shifts to a longer wavelength. The absorption increases at the new wavelength of 340nm provides a direct measurement of the enzymatic reaction.

Reagents

1. 1-Chloro-2,4-Dinitrobenzene (20mM)

A 30.37mg weight of 1-chloro-2,4-dinitrobenzene (CDNB) was dissolved in 1ml of ethanol and stored in a reagent flask.

2. Reduced Glutathione (0.1M)

A 30.73mg weight of reduced glutathione (GST) was dissolved in 1ml of 0.1M phosphate buffer (pH 6.5) and stored in a reagent flask.

3. Phosphate Buffer (0.1M)

This was prepared by dissolving 4.96g of dipotassium hydrogen phosphate ($K_2HPO_{4)}$ and 9.73g of potassium dihdrogen phosphate (KH_2PO_4) in little amount of distilled water and then made up to mark in 1 litre volumetric flask. The pH was adjusted to 6.5.

Procedure

The medium for the estimation was prepared as shown below and the reaction was allowed to run for 60 seconds each time before the absorbance was read against the blank at 340nm. The temperature was maintained at approximately 31^0C. The absorbance was measured using spectrophotometer.

Table 6 : Glutathione-S-transferase assay medium

Reagent	Blank	Test
Reduce GSH (0.1ml)	30µl	30µl
CDNB (20mM)	150µl	150µl
Phosphate buffer (pH 6.5)	2.82ml	2.79ml
Cytosol/Microsomes	-	30µl

Calculation

The extinction coefficient of CDBN = 9.6mn $^{-1}$ cm^{-1}

Glutathione-S- transferase activity = $\frac{\text{OD/min}}{9.6} \times \frac{1}{0.03\text{ml/mg protein}}$

= µmole/min/mg protein

3.9 Antimicrobial Studies

3.9.1 Determination of antibacterial and antifungal activities of the crude extract and fractions of the bulb of *Crinum jagus*

Antimicrobial activity was assessed by the agar diffusion method (Vollekova *et al*., 2001; Usman *et al*., 2005).

Principle

Agar diffusion method is one of the most widely used methods to determine the susceptibility of microorganisms to antimicrobial agents. The principle of this method is dependent upon the inhibition of reproduction of a microorganism on the surface of a solid medium by antimicrobial agent which diffuses into the medium by a filter paper disc or by creating a 'well' on the agar and filling the wells with different concentrations of the antimicrobial agent. Thus for an organism which is truly sensitive (susceptible) to an antimicrobial agent, a zone of inhibition will be seen around the discs or the wells.

3.9.2 Microorganisms

All the organisms used in this study were isolates obtained from Department of Pharmaceutical Microbiology, University of Ibadan. The organisms were maintained on agar slopes at 4^0C and subcultured for 24 hours before use.

Two Gram-positive bacteria : *Staphylococcus aureus, Bacillus subtilis,* four Gram-negative bacteria : *Escherichia coli, Pseudomonas aeruginosa, Salmonellae typhi, Klebsiella pneumonae* and six fungi *: Candida albicans, Candida tropicalis*, *Candida krusei, Penicillum notatum, Aspergillus niger, Aspergillus flavos* were used for the bioassay.

Table 7 : List of microorganisms used in the assessment of antimicrobial activities of the crude extract and fractions of the bulb of *Crinum jagus*

Microorganism	Type	Source
Bacteria		
1. *Staphylococcus aureus*	Gram +ve	Department of Pharmaceutical Microbiology, U.I, Ibadan.
2. *Baccillus subtilis*	Gram +ve	‘’
3. *Escherichia coli*	Gram – ve	‘’
4. *Pseudomonas aeruginosa*	Gram –ve	‘’
5. *Salmonellae typhi*	Gram –ve	‘’
6. *Klebsiella pneumonae*	Gram –ve	‘’
Fungus		
7.*Candida albicans*	Yeast	‘’
8. *Canadida tropicalis*	Mould	‘’
9. *Canadida krusei*	Mould	‘’
10. *Penicillum Notatum*	Mould	‘’
11. *Aspergillus niger*	Mould	‘’
12. *Aspergillus flavos*	Mould	‘’

3.9.3 Preparation of culture media

The culture media used for bacterial growth were nutrient broth and nutrient agar while the media used for fungal growth were tryptone soya broth and potato dextrose agar.

Eight grammes of nutrient broth and thirty grammes of tryptone soya broth powder were dissolved separately in one litre quantities of distilled water over a water bath. The homogenous solution formed in each case was then dispensed into test tubes in 5ml or 10ml quantities. All the test tubes were covered and sterilized at 121^0C for 15 minutes in an autoclave. The sterile broth tubes were kept at room temperature. Any broth in which turbidity was observed was discarded.

3.9.4 Preparation of bacterial and fungal cultures

Colonies of Gram-positive and Gram-negative bacteria kept on agar slopes were innoculated into 5ml quantities of sterile nutrient broth and incubated for 18-24 hours at 37^0C. For the fungal cultures fungal colonies kept on agar slopes were also innoculated into 5ml quantities of tryptone soya broth and the tubes were incubated at room temperature and checked for growth at 24 and 48 hours.

3.9.5 Preparation of test solutions (crude extract)

A 1g weight of the crude extract was dissolved in 5ml methanol (solvent of extraction) to obtain a stock solution of 200mg/ml. From the stock solution, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25 mg/ml of the test solutions were prepared through serial dilution into 6 test tubes. The 7^{th} and 8^{th} test tubes contained ampicillin (10mg/ml) and tiaconazole (10%w/v) and were used as positive controls while the 9^{th} tube contained methanol used as negative control of the experiment

3.9.6 Preparation of test solutions (fractions)

A 0.25mg weight of each of the fraction was dissolved in 5ml methanol to obtain a stock solution of 50µg/ml, from the stock solution, 25µg/ml; 12.5µg/ml; 6.25µg/ml; 3.125µg/ml; 1.56µg/ml and 0.78µg/ml of the test solution were prepared through serial dilutions into six test tubes. The 7^{th} and 8^{th} test tubes contained ampicillin (10µg/ml) and tiaconazole (10%w/v) and were used as positive controls of the experiment.

3.9.7 Agar diffusion test

Procedure

From the overnight culture of bacteria and fungi, 0.1ml was taken and put into 9.9mls of sterile distilled water to get a 10^{-2} of the dilution of the organism. From the diluted organism, 0.2ml was taken and asceptically poured into the sterile petridishes containing agar at about 45^0C and allowed to set. The surface of the set agar was dried in the incubator for 20 minutes at 37^0C. Equidistant wells were cut in the agar using a sterile cork borer of 8mm diameter. An aliquot of 80µl of known concentrations of the extracts and the drugs (ampicillin for bacteria isolates and tiaconazole for fungal isolates) were poured into the wells, methanol was used as negative control. The plates were allowed to stand on the bench for about 2 hours to allow the extract to diffuse properly into the agar. For bacteria the plates were incubated for 18-24 hours at 37^0C while for fungi, the plates were incubated for 48 hours at $26\text{-}28^0$C. the plates were checked for growth daily for a period of 7 days. The average diameters of three readings of the clear zone surrounding the hole was taken as the measure of the inhibitory level of the plant extract and the fractions against the bacteria and fungi tested.

3.9.8 Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the crude extract and fractions of the bulb of *Crinum jagus*

Minimum inhibitory concentration (MIC) is defined as the smallest concentration at which an antimicrobial agent will inhibit the growth of a named organism under specific condition.

Minimum bactericidal concentration (MBC) is the smallest concentration at which an antimicrobial agent will kill a named organism under specific condition.

The minimum inhibitory concentration (MIC) of the crude extract and the fractions were determined as described by Kabir *et al.*,(2005).

Procedure

For the bacteria isolates, six different concentrations of the crude extract ranging from 1.56mg/ml-50mg/ml were prepared while five different concentrations of each of the fractions ranging from 0.95µg/ml-3.125µg/ml were prepared to determine the MIC value, 2mls of each of the different concentration was added to 18mls of agar in test tubes making up the volume to

20mls and 1.0ml of standardized broth cultures contained 1.0 x 10^{-7} CFU/ml were added to each tube and then incubated at 37^0C for 18-24 hours. For comparision extract free tubes were used. Following incubation, turbidity was examined. The lowest concentration of the crude extract and the fractions that produced no visible bacterial growth (no turbidity) when compared with the control tube was regarded as minimum inhibitory concentration. MIC determination were done in triplicates.

To determine the MIC of the fungal isolates, six different concentrations of the crude extract ranging from 6.25mg/ml-200mg/ml were also prepared while five different concentrations of each of the fraction ranging from 0.195µg/ml-3.125µg/ml were also prepared. A 2mls portion of each of the different concentrations was added to 18mls of agar in test tubes making up the volume to 20mls and then asceptically poured into sterile plates. All the plates were allowed to set and then the different organism (mould and yeast) was streaked on the plates. The plates were incubated at 26^0C for 48 hours and checked for growth. The lowest concentration of the extract and the fraction that did not show growth of the organism was regarded as the minimum inhibitory concentration (MIC).

To determine the minimum bacterial concentration (MBC), all the concentrations of the extract and the fractions that did not show growth from the MIC were reprepared and poured into sterile plates and the different organisms streaked on the plates and incubated. For bacterial isolates, the plates were incubated at 37^0C for 18-24 hours while for the fungal isolates, the plates were incubated at 26^0C for 48 hours, after incubation, the plates were observed for growth. Presence of colonies served as indication of growth. The lowest concentration at which no colony was seen was taken as the minimum bactericidal concentration (MBC).

To obtain the number of colony used for the MIC determination, 0.1ml of 10^{-7} dilution of the 18 hours culture used was innoculated into molten sterile nutrient agar at 45^0C, poured and allowed to set. The surface was dried and the plates were incubated at 37^0C for 24 hours. Colonies formed in the agar were counted using the colony counter. The colony forming unit (CFU/ml) was calculated as follows:

CFU/ml = no of colonies counted x dilution factor

Volume taken for incubation

3.10 Determination of Anti-inflammatory Activities of the Crude Extract and Fractions of the Bulb of *Crinum jagus*

The crude extract and fractions of the bulb of *Crinum jagus* were screened for anti-inflammatory activity using the established Carrageenan, induced rat paw oedema test described by Winter *et al*.,1962; Perianayagam *et al*., 2006. This is the basic model for screening agents with anti-inflammatory activity.

Principle

This model is based on the principle of release of various inflammatory mediators, oedema formation due to carrageenan in the rat paw is a biphasic event. The initial phase is attributed to the release of histamine and serotonin, the second phase of oedema is due to the release of prostaglandins protease and lysosome. Injection of carrageenan into the rat paw increased tissue water and plasma protein extravasion.

Reagents

1. 0.9% NaCl solution (saline)

A 0.9g weight of NaCl was dissolved in distilled water and the volume made up to 100ml.

2. 1% carrageenan solution

A 0.1g weight of carrageenan was dissolved in 10ml of normal saline and stored in a reagent flask.

3. Indomethacin solution (10mg/ml)

A 100mg weight of indomethacin (BDH Chemichals, UK) was dissolved in 10ml of normal saline and stored in a reagent flask .

Procedure

3.10.1 Administration of the crude extract

Thirty males rats were divided into six groups of five animals each. Group A received 0.2ml normal saline and served as the control, group B received indomethacin (5mg/kg), groups C, D,

E and F received the crude extract at the doses of 10, 25, 50 and 75mg/kg, respectively. All administration were done intraperitoneally.

3.10.2 Administration of the fractions

Fourty males rats were used to test for the anti-inflammatory potential of the fractions. The animals were divided into eight groups of five animals each. Group A received 0.2ml saline and served as the control group, group B received indomethacin (5mg/kg), groups C and D received fraction 1 at the doses of 10 and 20mg/kg, groups E and F received fraction 2 at the doses of 10 and 20mg/kg while groups G and H received fraction 3 at the doses of 10 and 20mg/kg respectively. All administrations were done intraperitoneally.

3.10.3 Evaluation of the anti-inflammatory activity

Thirty minutes later, oedema was induced in the right hind paws of rats by injecting 0.1ml of 1% carrageenan into the subplantar region of the paws. Measurement of the paw size was done by wrapping a piece of cotton thread round the paw of rats and measuring the circumference on a metre rule (Bamgbose and Noamesi, 1981). Measurement was done before carrageenan injection (Do) and at intervals of one hour for 5 hours after carrageenan injection (Dt). The inhibition of oedema at the 3rd hour post carrageenan administration was calculated according to the formula.

$$\text{Percentage inhibition} = \frac{(Dt - Do)\ \text{control} - (Dt - Do)\ \text{test}}{(Dt - Do)\ \text{control}} \times 100$$

Where Dt = Linear paw circumference 3.0 hours after carrageenan injection

Do = Linear paw circumference 0.0 hours (just before carrageenan injection).

3.11 *In vivo* Antimalarial Activity of the Crude Extract and Fractions of the Bulb of *Crinum jagus* in *Plasmodium berghei* Infected Mice

The *in-vivo* antimalaria test was carried out using the method of Ryley and Peter (1970). The 4-day suppressive test is a standard test commonly used for antimalaria screening and determination of percentage inhibition of parasitaemia is the most reliable parameter. The test also relies on the ability of standard innoculum of *Plasmodium berghei* to kill the recipient

mouse within six days of inoculation, extension of survival beyond 12 days was regarded as activity.

Reagents

1. Giemsa stain (stock)

A 3.04g weght of Giemsa powder was weighed and mixed with 250ml of methanol and 250ml of glycerol. The solution was poured into a brown bottle to prevent oxidation. After proper shaking, the solution was filtered to removed the undissolved particles. Finally, it was kept in a dark cupboard for two weeks for maturation prior use.

2. 10% Giemsa stain

For thick film smear, 10% Giemsa stain was prepared by adding 1ml of Giemsa stock solution to 9ml of distilled water. For thin smear, 10% Giemsa stain was prepared by adding 1ml of Giemsa stock solution to 9ml of phosphate buffer.

3.11.1 Experimental animals

Eighty male swiss albino mice (20-30g) were used for the study.

3.11.2 Parasite innoculation

A strain of *Plasmodium berghei* (ANKA) that was chloroquine sensitive was supplied from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Nigeria. The mice, previously infected with *Plasmodium berghei* and with a high parasitaemia level, served as the donor. For this study, a donor mouse with a rising parasitaemia of 20% was sacrificed and blood samples was taken from the donor and diluted with phosphate-buffered saline such that 0.2ml injected intraperitoneally to the experimental animals contained 1×10^7 infected erythrocytes.

3.11.3 Administration of the extract and the fractions

Eighty male swiss mice were used for the study. On the first day of the experiment (Day O), the mice were infected with the parasite and randomly divided into ten groups of eight animals based on their weight. Each mouse was innoculated on Day O intraperitoneally with 0.2ml of infected blood containing 1×10^7 *Plasmodium berghei* parasitised red blood cells. Treatment was withheld for 72 hours to allow for the establishment of infection, and was commenced when parastaemia had been established. The crude extract and the fractions were dissolved in

Tween 80 (vehicle for the extract) and administered orally. Group 1 received 0.3ml Tween 80 for three consecutive days and served as the control group, Group 2 received chloroquine (10mg/kg) for three consecutive days, Group 3 received arteether (3mg/kg) for three consecutive days, Groups 4 ,5, 6 and 7 received the crude extract at the doses of 10, 25, 50 and 75mg/kg respectively for four consecutive days. Groups, 8, 9 and 10 received 10mg/kg of fractions 1, 2 and 3 respectively, for four consecutive days.

3.11.4 *In-vivo* antimalarial test

Each day starting from the day treatment commenced until the end of the experiment blood films were made form the tail of each infected mouse, stained with Giemsa stain and examined microscopically to assess the level of parasitaemia. Blood was also collected from the tip of the animal tail into capillary tube to determine the packed cell volume (PCV). The body weight of the mice were measured to observed whether the plant extract prevented weight loss that is common with increasing parasitaemia in infected mice.

3.11.5 Preparation of thin blood films

A drop of blood from the tail is placed on the edge of a clean microscope slide, spread across to a length of about 5cm and allowed to dry. The smear is then fixed with absolute methanol and stained with Giemsa stain solution for 30minutes before rinsing with running tap water and allowing the slide to dry.`

3.11.6 Determination of percentage parasiteamia

The prepared thin blood films were viewed under the microscope using a high magnification (X100) with oil immersion for intracellular stages of *Plasmodium yoelli* to estimate cells. At least, a total of 1000 cells were counted from the blood films to calculate the percentage parasitaemia.

$$\% \text{ parasitaemia} = \frac{\text{no of parasitized RBC}}{\text{no of parasitized RBC} + \text{total no of RBC}} \times 100$$

3.11.7 Determination of packed cell volume (PCV)

Packed cell volume (PCV) is a measure of the proportion of red blood cells to the whole blood. Small volume of blood was collected from the tip of animal tail (tail tip amputation) into heparinised capillary tube. The capillary tube was sealed and spun for ten minutes in

haematocrit centrifuge to separate the blood into plasma and packed cells. The percentage of packed cells was calculated using haematocrit reader.

3.12 Inhibitory Effect of the Crude Extract and the Fractions of the Bulb of *Crinum jagus* on *Mycobacterium tuberculosis* Isolates.

Anit-TB assays were performed in Lowenstein Jensen (L-J) and Middle brook 7H10 agar obtained from Sigma Chemicals, USA.

Culture technique is considered to be the gold standard and it offers the possibility of more rapid and more sensitive diagnosis of active tuberculosis and drug susceptibility.

3.12.1 Mycobacterial isolates

Mycobacterial tuberculosis strains and the reference drug susceptible strain H37Rv used as control were obtained from Tuberculosis Reference Laboratory (South-West Zone), Medical Microbiology Department, College of Medicine, University of Ibadan. The standard tuberculosis drugs to which these isolates and the strain are susceptible are rifampicin, isoniazid, streptomycin, ethambutol.

3.12.2 Preparation of Lowenstein Jensen (L-J) medium

A 37.24g weight of L-J medium was suspended in 600ml of distilled water containing 12ml glycerol (for bovine bacteria or other glycerophobic organisms, addition of glycerol is not desirable). The medium was boiled until it was completely dissolved and it was sterilized by autoclaving at 121^0C for 15 minutes. 100ml of whole egg emulsion was prepared and aseptically collected. The homogenized egg, base and Gruit mycotological supplement (if desired) were administered to the medium. It was properly mixed to obtained a uniform mixture and dispensed in 6-8ml sterile screw cap tubes. The caps were tightly closed. The medium was inspissated at 85^0C for 45minutes in a slanted position in a water bath or autoclave used for isolation and cultivation of *Mycobacterium* species.

3.12.3 Preparation of Middle brook 7H10 agar

A 19.50g weight of Middle brook 7H10 agar was dissolved in 900ml of distilled water. To this was added 5ml of glycerol supplement (80021). The mixture was heated until it was completely dissolved and autoclaved at 121^0C for 15 minutes. The mixture was cooled and 100ml of Middlebrook 7H10 supplement (81035) was added. The Middlebrook agar was then poured

into sterile petridishes and allowed to set. The plates were then sterilized by autoclaving at 121^0C for 15minutes.

3.12.4 Preparation of test solutions

A 0.01g weight of the crude extract and each of the fractions (F1, F2 and F3) were dissolved, separately in 10ml of methanol to obtain a stock solution of 1mg/ml. From the stock solution, various concentrations (0.2mg/ml; 0.4mg/ml; 0.6mg/ml; 0.8mg/ml and 1.0mg/ml) of the crude extract and each of the fraction were obtained. Stock solutions (1mg/ml) of rifampicin and isoniazid were prepared using the method described above, from which various concentrations were obtained and used for the drug susceptibility test.

3.12.5 Determination of colony forming units (CFU) on Lowenstein-Jensen (L-J) medium

The procedure followed was that described by Gupta *et al.*,(2010). The ten fold dilution of standard 1mg/ml *Mycobacterium tuberculosis* suspensions were prepared. One loopful (6µl) of the suspension was streaked on the L-J slants using 3mm external diameter loop. The crude extract and each of the fractions at concentration of 0.2mg/ml; 0.4mg/ml; 0.6mg/ml; 0.8mg/ml and 1.0mg/ml were incorporated in the medium. The medium was then incubated at 37^0C for 42 days. For comparison, extract free control slants were used. Each test was done in duplicates. After 4 weeks, the culture were examined for possible growth of *Mycobacteria tuberculosis.* Percentage inhibition was calculated by mean reduction in number of colonies on extract containing as compared to extract free controls. The percentage inhibition of extract/fractions were then plotted against the logarithm of the extract/fractions concentrations to determine the fifty percent inhibitory concentration (IC_{50}). Susceptibility testing of the isolates was also performed against standard drugs, rifampicin and isoniazid in the same batch of media for comparison of colony forming units on drug free control.

3.12.6 Determination of colony forming units (CFU) on Middle brook 7H10 agar

The procedure followed was that described by Claude *et al.*, (2012) using the disc diffusion method. The discs were separately impregnated with 20µl of the various concentrations (0.2mg/ml; 0.4mg/ml; 0.6mg/ml; 0.8mg/ml and 1.0mg/ml) of the extract, each of the fractions and the standard drugs and were left to dry for 24 hours. The culture medium (the sterile Middle

brook 7H10 agar) was placed in 90mm diameter petri dishes with quadrants. In each quadrant of the petridish was put 5.0ml of the medium. The solidified medium in the quadrants was innoculated with *M.tuberculosis,* using a swab. A rifampicin-impregnated disc was placed in the first quadrant, in the second quadrant was put isoniazid-impregnated disc, the third quadrant had the extract/fractions-impregnated disc while the fourth quadrant contained a blank disc (negative control). This was achieved for the varying concentrations of the extract, the fractions and standard drugs. The petridishes were left on safety cabinets overnight to allow diffusion of the extract, the fractions and the drugs and then sealed with a carbondioxide permeable tape. These were then incubated at 37^0C in a carbon dioxide incubation for up to 4 weeks. The susceptibility of *Mycobacterial tuberculosis* to the extract and the drugs was determined by counting the number of bacteria in each quadrant. Percentage inhibition was calculated by mean reduction in number of colonies on extract containing as compared to extract free controls. The percentage inhibition of extract/fraction were then plotted against the logarithm of the extract/fractions concentrations to determine the fifty percent inhibitory concentration (IC_{50}). Susceptibility testing of the isolates was also performed against standard drugs, rifampicin and isoniazid in the same batch of media for comparison of colony forming units on drug free control.

3.13 Toxicological Profiles of the Crude Extract of the Bulb *Crinum jagus*

3.13.1 Administration of test substance and preparation of tissue homogenate

Reagents

1. Homogenizing Buffer (0.1M phosphate buffer, pH 7.4)

(a) A 11.8g weight of disodium hydrogen phosphate (Na_2HPO_4) was dissolved in distilled water and made up to 1000ml.

(b) A 6.8g weight of potassium hydrogen phosphate (KH_2PO_4) was dissolved in distilled water and made up to 500ml.

(c) A solution of 800ml of (a) was mixed with 200ml of (b) to make 1000ml. The pH was adjusted to 7.4 and the solution was stored at 4^0C.

2. Washing Buffer (1.15% KCl)

A 11.5g weight of potassium chloride was dissolved in distilled water and made up to 100ml **1.**

3. 0.9% NaCl solution (saline)

.A 0.9g weight of NaCl was dissolved in distilled water and the volume made up to 100ml with the same and stored at 4^0C.

3.13.2 Administration of test substance

Thirty male rats were randomly distributed to five groups of six animals per group. The crude extract was dissolved in normal saline before administration to the rats using oral cannula and administration was done for 30 days. Group A animals served as the control and were given 0.2ml normal saline in place of extract. Group B were administered with 10mg/kg of the extract. Group C received 25mg/kg of the extract, Group D were administered with 50mg/kg of the extract while group E received 75mg/kg of the extract for 30days.

3.13.3 Preparation of plasma

Twenty four hours after the last treatment, the animals were sacrificed and blood samples were collected by cardiac puncture into EDTA bottles and gently rotated to allow proper mixing with the anticoagulant. The sample was centrifuged at 2000g for 10 minutes and the plasma transferred into another clean tube, stored at 4^0C and used for haematological study.

3.13.4 Preparation of serum

Blood samples were collected into plain sample bottles and were allowed to stand for 1 hour and were centrifuged at 3000g for 10minutes to obtain the serum and serum collected using a micropipette.

3.13.5 Preparation of liver and kidney homogenates

Liver and kidney tissues from rats in each group were quickly removed and washed in ice cold 1.15% KCl solution, dried and then weighed. The samples were homogenized in 4 volumes of 0.1M phoslpahte buffer (pH 7.4) and were centrifuged at 10,000g for 15 minutes to obtain the post mithochondria supernatant fractions of the liver and kidney samples. The samples were stored at 4^0C until required.

3.13.6 Biochemical Assays

3.13.6.1 Determination of Aspartate amino transferase (AST) activity

Principle

Aspartate amino transferase (AST) also referred to as glutamate oxaloacetate transaminase (GOT) catalyses the transfer of the amino group of the glutamic acid to oxaloacetate in reversible reactions. The transaminase activity is proportional to the amount of oxalate formed over a definite period of time and is measured by a reaction with 2, 4 Dinitrophenyl hydrazine (DNPH) in alkaline solution (Rietman and Frankel, 1957).

α -ketoglutarate + L-aspartate $\xrightleftharpoons{\text{AST}}$ L-glutamate + Oxaloacetate

Reagents

Randox kit was used. The kit contains the following:

	Enzyme Reagent	**Concentration**
R_1 a	DL-Aspartate	100mmol/L
R_2 (Substrate GOT)	α-Ketoglutarate	2mmol/L
Developer	2,4 Dinitrophenyl hydrazine (DNPH)	1mmol/L
GOT calibrator	Primary calibrator of pyruric	1.2mmol/L
	Sodium hydroxide	0.4N

Procedure

A 0.5ml portion of the GOT was pippeted into a test tube, mixed and incubated in water bath for 5 minutes at 37^0C. To the test tube, 100µl of the sample was added and incubated in a water bath for 60 minutes before 0.5ml of dinitrophenyl hydrazine (DNPH) was added to the mixture, mixed and allowed to stand for 20 minutes at room temperature. A 5.0ml portion of 0.4N sodium hydroxide was added to the mixture, mixed and allowed to stand for 15minutes at room temperature. The absorbance was read against a water blank at wavelength of 546nm. The standard curve for AST activity was plotted and the absorbance reading was extrapolated from the graph.

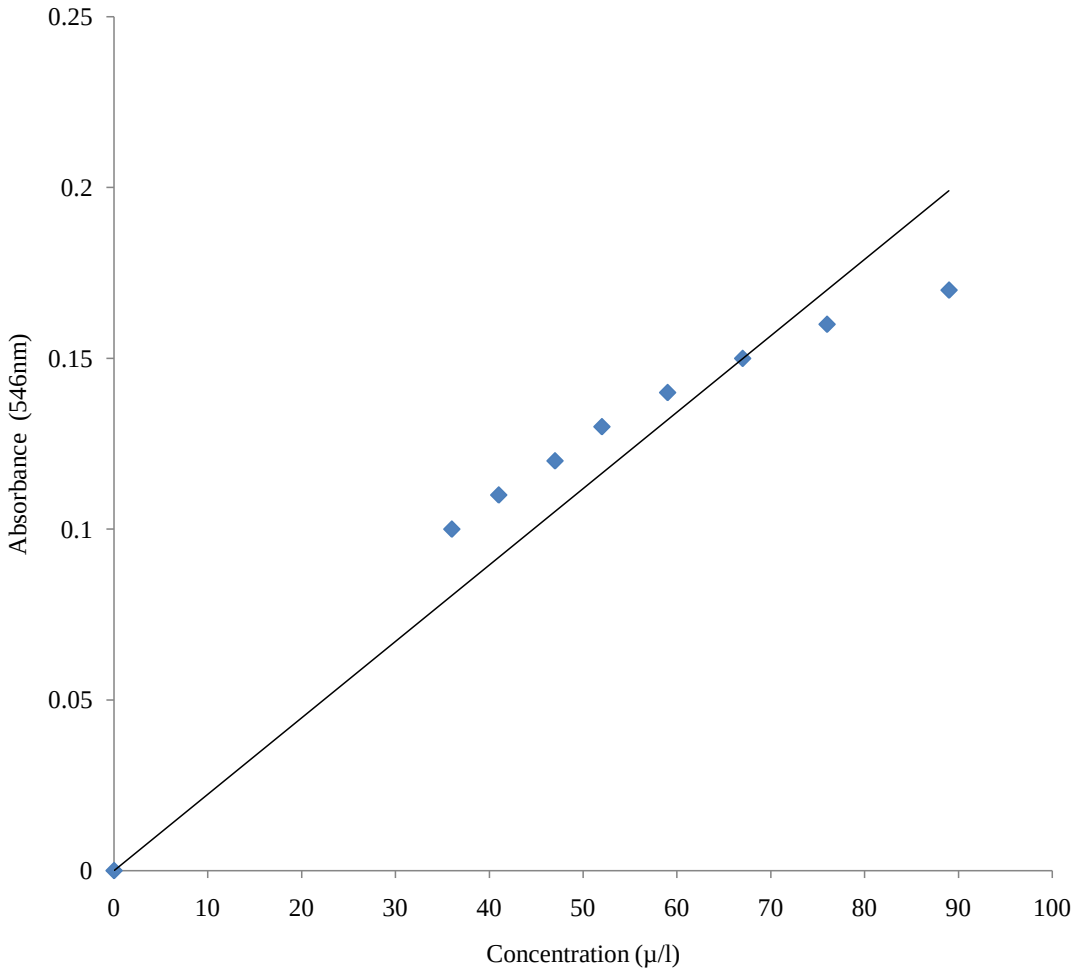


Figure 14 : AST standard curve

3.13.6.2 Determination of Alanine amino transferase (ALT) activity

Principle

Alanine amino transferase (ALT) also referred to as glutamate pyruvate transaminase (GPT) catalyses the transfer of amino group of glutamic acid to pyrurate in reversible reaction. The trasnaminase activity is proportional to the amount of pyruvate formed over a definite period of time and is measured by a reaction with 2,4 dinitrophenyl hydrazine (DNPH) in alkaline solution (Reitman and Frankel, 1957).

α-Ketoglutarate + L-alanine $\overset{\text{AST}}{\rightleftharpoons}$ L-glutamate + pyruvate

Reagents

Randox kit was used. The kit contain the following:

	Enzyme Reagent	**Concentration**
R_1 b	DL-Alanine	200mmol/L
R_2 (Substrate GPT)	α-ketoglutarate	2mmol/L
Developer	2,4 Dinitrophenyl hydrazine (DNPH)	1mmol/L
GPT Calibrator	Primary calibrator of pyruvic	1.2mmol/L
	Sodium hydroxide	0.4N

Procedure

A 0.5ml portion of the GPT was pippeted into a test tube, mixed and incubated in water bath for 5 minutes at 37^0C. To the test tube, 100µl of the sample was added and incubated in water bath for 30 minutes before 0.5ml of dinitrophenyl hydrazine (DNPH) was added to the mixture, mixed and allowed to stand for 20 minutes at room temperature. A 5.0ml portion of 0.4N sodium hydroxide was added to the mixture, mixed and allowed to stand for 15minutes at room temperature. The absorbance was read against a water blank at wavelength of 546nm. The standard drug for ALT was plotted and the absorbance reading was extrapolated from the graph.

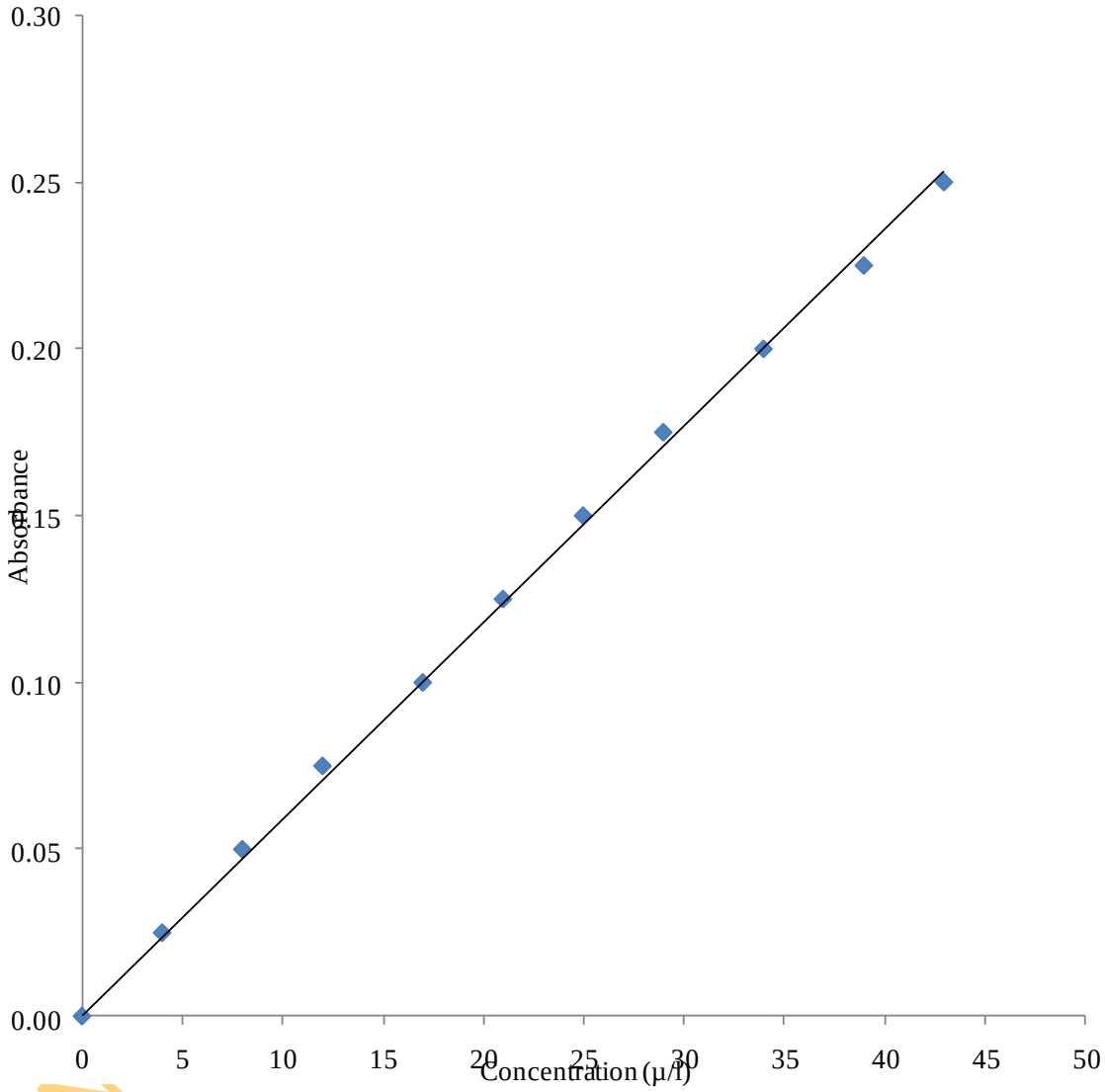


Figure 15 : ALT standard curve

3.13.6.3 Determination of Alkaline Phosphatase (ALP) activity

Principle

Serum alkaline phosphatase hydrolyses a colourless substrate of phenophtalein monophospahte giving rise to phosphoric acid and phenophtalein which at alkaline pH values turns into a pink colour that can be photometrically determined (Klein and Babson, 1960).

$$\text{p-Nitrophenyl phosphate} + H_2O \xrightarrow[\text{phosphatase}]{\text{alkaline}} \text{p-Nitrophenol} + \text{phosphate}$$

The rate of p-nitrophenol formation is proportional to the catalytic concentration of alkaline phosphatase present in the sample.

Reagents

Randox kit was used. The kit contains the following:

	Reagents
A	Chromogenic Susbtrate
B	Colour Developer (content was dissolved in 250ml of deionised water)
C	Standard Solution of alkaline phosphatase in water / ethanol equivalent to 30U/L

Procedure

An aliquot of 1.0ml of water was pippeted into two test tubes (test tube SA and ST), to each of the test tube was added 1 drop of the chromogen substrate and was mixed and then incubated at 37^0C for 5 minutes. Then to test tube SA, 0.lml of the sample was added and to test tube ST, 0.1ml of standard alkaline phosphatase was added. The test tubes were mixed and incubated at 37^0C for 20 minutes. To each of the test tubes, 5ml of the colour developer was added. Absorbance was read against a water blank at wavelength of 550nm. The activity of ALP in the sample was calculated using the formula :

$$\text{U/L of Alkaline phosphatase} = \frac{\text{SA Absorbance}}{\text{ST Absorbance}} \times 30$$

3.13.6.4 Determination of Lactate Dehydrogenase (LDH) activity

Principle

Lactate dehydrogenase (LDH) catalyses the reduction of pyruvate by NADH to lactate, according to the following reaction:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+.$$

The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of LDH present in the sample (Pesce and Kaplan, 1984).

Reagents

Randox kit was used. The kit contains the following:

	Reagents	**Concentration**
R_1	Phosphate (pH 7.8)	80mm0l/L
Buffer	Pyrurate	0.6mmo/L
R_2 substrate	NADH	0.18mm0/L

Working Reagent (WR): Mix 4 volumes of R_1 with 1 volume of R_2

Procedure

A 3.0ml portion of working reagent (WR) was pippeted into a test tube, and 100µl of the sample was added and the mixture incubated for 1 minute. The absorbance of the sample was read at 340nm at 1 minute interval for 3 minutes. The difference of absorbance and average absorbance difference was calculated per minute (DA/min). LDH activity in the sample was calculated using the formula :

U/L LDH = DA/min X 4925.

3.13.6.5 Determination of total bilirubin

Principle

Bilirubin reacts with diazotized sulfanilic acid to produce coloured azobilirubin which has an absorbance maximum at 560nm. The intensity of the colour produced is proportional to the bilirubin concentration in the sample (Kaplan *et al*., 1984; Malloy *et al*., 1937; Martinek, 1966).

Reagents

Randox kit was used. The kit contain the following:

	Reagents	Concentration
R_1	Dimethyl sulphoxide	7mmol/L
	Sulphanilic acid	30mmol/L
	Hydorchloric acid (HCl)	50mmol/L
R_2	Sodium nitrite	29mmol/L
	Bilirubin calibrator	20mg/dL

Procedure

A 1.5ml portion of reagent R_1 was pippeted into two test tubes (test tubes BL and SA). To testube SA was added 50µl of reagent R_2. To the two test tubes were added 100µl of the sample. The tubes were mixed and incubated for 5 minutes at room temperature. The absorbance was read against the blank at 555nm. The concentration of total bilirubin in the sample was calculated using the formula :

Total bilirubin (mg/dl) = 17 .1 X **A**

Where **A** = Absorbance of the sample

3.13.6.6 Determination of direct bilirubin

Principle

Bilirubin reacts with diazotised sulphanilic acid to produce coloured azobilirubin which has an absorbance maximum at 560nm. The intensity of the colour produced is proportional to the bilirubin concentration in the sample (Kaplan *et al.*,1984; Malloy *et al.*; 1973, Martinek, 1966)

Reagents

Randox kit was used. The kit contains the following:

	Reagents	Concentration
R_1	Sulphanilic acid	30mmol/L
	Hydrochloric acid (HCl)	150mmol/L
R_2	Sodium nitrite	29mmo/L
	Bilirubin calibrator	20mg/dL

Procedure

A 1.5ml portion of reagent R_1 was pippeted into two test tubes (test tube BL and SA). To test tube SA was added 50µl of reagent R_2. To the test tubes were added 100µl of the sample. The tubes were mixed and incubated for 5minutes at room temperature. The absorbance was read against the blank at 555nm. The concentration of direct bilirubin in the sample was calculated using the formula.

Direct bilirubin (mg/dl) = 17.1 X **A**

A = Absorbance of the sample

3.13.6.7 Determination of serum triglycerides

Principle

The serum triglyceride was determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide and 4-amino phenazone under the catalytic influence of peroxidase (Mc Gowan,1983).

$$\text{Triglycerides} + H_2O \xrightarrow{\text{lipase}} \text{Glycerol} + \text{Fattyacids.}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + HCl + 4H_2O$$

Where :

GK = Glycerol kinase

GPO = Glycerol-3-phosphate oxidase

POD = Peroxidase

Reagents

Randox kit was used. The kit contains the following:

(a) **Buffer**	**Concentration**
PipesBuffer	40mmol/L
4-chlorophenol	5.5mmol/L
Magnesium-ions	17.5mmo/L
(b) **Enzyme Reagent**	**Concentration**
4-aminophenazone	5.5mmol/L
ATP	1.0mmol/L
Lipases	≥150U/ml
Glycerol kinase	≥0.4U/ml
Glycerol-3-phosphate oxidase	≥1.5U/ml
Peroxidase	≥0.5U/ml
(c) **Standard**	2.29mmol/L (200mg/dl)

Working Reagent: One vial of the enzyme reagent was reconstituted with 15ml of buffer.

Procedure

An aliquot of 1000µl working reagent was added to 10µl of the sample. 1000µl of working reagent was added to 10µl of standard in another clean test tube. 1000µl of working reagent was put into another clean tube to serve as blank. Each tube was mixed and incubated at 20-25^{0}C for 10minutes. The absorbance of the sample and standard were measured against the reagent blank within one hour at 505nm. The concentration of triglyceride in the sample was calculated using the formula.

$$\text{Triglyceride conc (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200$$

3.13.6.8 Determination of serum total cholesterol

Principle

The serum cholestrol is determined after enzymatic hydrolysis and oxidation with lipases. The colorimetric indicator is quinoneimine which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under catalytic action of peroxidases (Trinder, 1969)

$$\text{Cholestrol ester} \xrightarrow[\text{esterase}]{\text{cholestrol}} \text{Cholestrol} + \text{Fatty acids}$$

$$\text{Cholestrol} + H_2O \xrightarrow[\text{Oxidase}]{\text{cholestrol}} \text{Cholestenone} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + 4 + \text{aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + H_2O_2$$

Reagents

Randox kit was used which contain the following:

Reagents	**Concentration**
Pipes buffer	80mol/L
4-aminoantipyrine	0.3mmol/L
Phenol	6mmol/L
Cholesterol esterase	≥0.15U/ml
Cholesterol oxidase	≥0.10U/ml
Peroxidase	≥0.5U/ml
Standard	5.17mmol/L (200mg/dl)

Working reagent: One vial of the enzyme reagent was reconstituted with 15ml of buffer

Procedure

An aliquot of 1000µl working reagent (WR) was added to 10µl of distilled water to serve as blank. An aliquot of 1000µl of working reagent was added to 10µl of the standard in a separate clean tube. An aliquot of 1000µl of working reagent was added to 10µl of the sample in another tube. The tubes were mixed and incubated for 10 minutes at 20-25^{0}C. The absorbance of the

sample and standard were measured against the reagent blank within one hour at 505nm. The concentration of total cholestrol in the sample was calculated using the formular.

$$\text{Total cholestrol conc (mg/dl)} = \frac{\text{Change in absorbance of sample}}{\text{Change in absorbance of standard}} \times 200$$

3.13.6.9 Determination of HDL-cholestrol

Principle

Serum HDL-cholestrol was determined by enzymatic method as described for total cholestrol determination after selective precipitation of the interfering very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) with a precipitating reagent, phosphotungstic acid and magnesium chloride. After centrifugation, the cholestrol concentration in high density lipoprotein fraction which remain in the supernatant was determined (Lopes-virella *et al.*, 1977).

Reagent

Randox kit was used which contain the following:

Reagents	**Concentration**
Phosphotungstic acid	0.55mmol/L
Magnesium chloride	25mmol/L

Reagent solution for cholestrol CHOD-PAP assay

The precipitating reagent was diluted in the ratio 4:1 with distilled water and stored at 15-25^{0}C.

Procedure

An aliquot of 500µl of diluted precipitant was added to 200µl of sample in the centrifuge tube. The tubes were mixed and allowed to stand for 10minutes at room temperature. It was centrifuged for 10minutes at 4,000 rpm. The supernatant was separated within two hours and the cholestrol content was determined by CHOD-PAP method.

$$\text{HDL conc (mg/dl)} = \frac{\text{Change in absorbance of sample}}{\text{Change in absorbance of standard}} \times 200$$

3.13.6.10 Determination of serum LDL-cholestrol

Principle

Serum LDL-cholestrol was determined using the method of Friedwald *et al*.,(1972). This method involves prior knowledge of the fasting serum total cholesterol, the HDL-cholestrol and total triglyceride concentrations based on the fact that:

1. The ratio of the mass of triglyceride to that of VLDL-cholestrol is apparently relatively constant at about 5:1 in normal subjects (Fredickson *et al*., 1967; Hatch and Lees, 1968) and in patients with all types of hyperlipoproteinemia, except the rare type 3 (Fredickson *et al.*, 1967).
2. When chylomicrons are not detectable, most of the triglyceride in the blood is contained in the very-low-density lipoprotein fraction.

The concentration of LDL-C in the sample was calculated using the formula.

$$\text{LDL-C (mg/dl)} = \text{Total cholestrol} - \frac{\text{Triglyceride}}{5} - \text{HDL-C}$$

3.14 Determination of Haematological Parameters

1. Haemoglobin concentration (Hb)

The blood haemoglobin concentration was estimated using the cyanomethaemoglobin method as described by Dacie and Lewis,(1991).

Principle

After hemolysis of red cells, haemoglobin is converted to cyanmethaemogloin by the cyanide in the diluting solution (Drabkins diluents). The chromogen formed is assayed spectrophotometrically at 540nm.

Reagent

Drabkins diluents

A 1g weight of sodium hydrogen carbonate (Hopkins and Williams Ltd. England) and 198mg of potassium ferricyanide (BDH Chemical Ltd. England) were dissolved in distilled water, and made up to 1 litre.

Procedure

A 0.02ml sample of the blood was added to 4ml of Drabkins diluents. The solution was allowed to stand for 10 minutes and the absorbance of the resultant solution was read at 540nm with the

Drabkins diluents as reference. Absorbance of haemoglobin standard of known concentration was also determined, concentration of haemoglobin was calculated as shown below.

$$\text{Hb con(g/dl)} = \frac{\text{Absorbance of test solution}}{\text{Absorbance of standard solution}} \times \text{Hb conc of standard solution} \times \text{dilution factor}$$

Dilution factor = 200, Hb concentration of standard = 0.068g/dl

2. **Packed Cell Volume (PCV)**

PCV was determined in duplicate using the microhaematocrit method described by Dacie and Lewis,(1991). This method involves filling a capillary tube with blood. One end of the tube was sealed and the tube centrifuged in a microhaematocrit centrifuge for 10 minutes. The PCV in percentage was read directly from a graphic reader.

3. **Red Blood Cell Count (RBC)**

The red blood cell count was determined using the haemocytometer method described by Dacie and Lewis,(1991).

Reagent

Hayem's solution

A 0.25g weight of mercury chloride ($HgCl_2$) and 0.25g of sodium sulphate (Na_2SO4) (Hopkins and Williams Ltd, England) were dissolved in distilled water and made up to 100ml. This solution was isotonic with blood and prevented roleaux formation and coagulation.

Procedure

Blood was drawn by means of rubber tubing attached to the end of the red cell pipette to 0.5mark on the pipette. Keeping the pipette nearly horizontal, Hayem's solution was drawn to the 101 mark with care to prevent overshoot. Holding the pipette horizontal, the rubber tubing was removed without squeezing. The pipette was then rotated for about 1 minute to ensure thorough mixing of blood and dilution of the fluid in the bulb. Diluted blood was then introduced carefully into the counting chamber with clean cover slip in place. Red cell counting was done using X40 objective of microscope. Counting was carried out in 5 sets of small squares containing 80 small squares. All red cells overlapping the top and left hand sides of a square were counted while those overlapping the bottom and right hand slide were not counted.

The total number of erythrocytes obtained was multiplied by the depth (X10), area (X5) and dilution factor (X200) hence Y-erythrocytes per millimeter in the sample would be 10,000Y.

4. **Haematocrit Indices**

Mean corpscular volume (MCV), mean corpscular haemoglobin (MCH) and mean corpscular haemoglobin concentration (MCHC) were calculated from the values of RBC, PCV and Hb as described below.

MCV provides the average volume of individual RBC and is calculated using the formula.

$$\text{MCV (pg)} = \frac{\text{PCV (\%) X10}}{\text{RBC (X } 10^6 \mu\text{L)}}$$

MCH expresses the average weight of Hb present in an erythrocyte and is calculated as follows:

$$\text{MCH (pg)} = \frac{\text{Hb (g/dl) X10}}{\text{RBC (X}10^6\ \mu\text{L)}}$$

MCHC gives the percentage of the MCV which Hb occupies and is calculated as follows:

$$\text{MCHC (g/dl)} = \frac{\text{Hb (g/dl) X100}}{\text{PCV(\%)}}$$

5. **Total and Differential White Blood Cells Count**

White cells were counted using a haemocytometer, white cell pippette and white cell diluting fluid.

Reagent

White cell diluting fluid

This contained 2% acetic acid and 2ml of methyl violet dye.

Procedure

The procedure followed was similar to that used to determine the red cell count. The ratio of dilution was 1:20. The sum of the white blood cell counts from the 4 large corner squares of haemocytometer chamber was multiplied by the depth (X10) and the dilution factor (X20) and divided by the square counted. Thus the number of leucocytes per millimeter in the original sample was 50X where X was the number of leucocytes counted in the 4 squares. Differential WBC counts in which 100 cells per slides were counted were made from Giemsa stained blood smear.

3.15 Histopathology study

The liver and kidney samples were harvested after the animals were sacrificed. The tissue was processed using an automatic tissue processor, embedded in paraffin wax and section (5µm) thickness were cut using a rotary microtome. The sections were stained by haematoxylin and eosin (H & E) method for light microscopic examination. Photomicrograph of stained sections were taken with the aid of a light camera fitted microscope.

3.16 Determination of Chemical Composition of Fraction 1 (F1)

The chemical composition of fraction 1 was determined by a combined gas chromatography mass spectrometry (GC-MS) technique (Karasek and Clement, 1988).

Principle

Gas chromotogrphy mass spectroscopy (GCMS) is a method that combines the feature of gas-liquid chromatography and mass spectroscopy to identify different volatile substances within a test sample. The GC-MS is composed of two major building blocks, the gas chromatography and mass spectrophotometer. The gas chromatography utilizes a capillary column, molecules are retained by the column and the elute from the column are ionized and the fragments detected by the mass spectrophotometer, using a charge-to-mass ratio computation to show the fragmentation pattern.

Procedure

The sample was analysed by GC-MS using a Schimadzu model QP 2010 gas chromatography system with split/splittes injection interfaced to a 5973 mass selective detector. Innowax fused silica capillary (FSC) column (30m x 0.25mm, with 0.25µm film thickness) was used with helium as carrier gas at a flowrate of 1ml/min . The GC oven temperature was kept at 80^0C (hold for 2mins) and programmed to reach 200^0C at a rate of 4^0C/min, then kept constant at 280^0C for 10 minutes being the final hold time. The split ratio was adjusted to 50:1. The injector temperature was at 250^0C. Mass spectra were recorded at 70eV. Mass range was from m/z 30 to 50V. Identification of components was achieved on the basis of their retention indices determined with a reference to a homologous series of n-alkanes and by comparison of their mass spectral fragmentation on patterns (NIST data base/ chemstation data system) with data

previously reported in literature (Adams 2001; Joulain and Konig, 1998; Mclatterfly and Staufer, 1989).

Statistical Analysis

Data were expressed as mean $\pm$ standard deviations and analysed by student t-test and one way analysis of variance (ANOVA) and $P < 0.05$ was considered to be significant.

IC_{50} was determined by regression analysis using a statistical package known as microcal origin.

CHAPTER FOUR

4.0 EXPERIMENTS AND RESULTS

Experiment 1 : Phytochemical Analysis and Fractionation of the Crude Extract of the Bulb of *Crinum jagus*

Introduction

Numerous investigations have shown that medicinal plants contain diverse classes of bioactive compounds; such as alkaloids, flavonoids, tannins and phenols which exhibit various pharmacological properties (Emam, 2010). However, in the absence of any scientific proof of their effectiveness, the validity of these remedies and their use by local communities remains restricted (Kaur and Arora, 2009). Phytochemicals and pharmacological investigations of several plants have already led to the isolation of natural drugs. However the potential of higher plants as sources of new drugs is still largely unexplored. Consequently, there is need to screen natural products for the presence of bioactive chemical constituents.

The bulb of *Crinum jagus* use in Western part of Nigeria has been shown to have biological activites such as antibacterial, antifungal, anticonvulsant, anticholinergic, anti-snake venom activities (Adesanya *et al*., 1992; Edema and Okiemen 2002; Peter *et al*., 2004; Ode *et al*., 2006).

This experiment was carried out on the bulb of *Crinum jagus* so as to gain insight into the possible phytochemicals present in them, with aim of studying their relevance in drug development and design, as well as to justify their usuage in folk medicine.

Procedure

Standard pharmacognosis procedures were used for qualitative and quantitative phytochemical screening as have been described in section 3.3 under “Materials and Methods.

A modified form of classical column chromatography called the flash chromatography was used for the fractionation of the crude extract of the bulb of *Crinum jagus*. Glass column was packed with silica gel (flash chromatography grade). The bulb extract adsorbed with silica gel was packed on to the column layer and then allowed to settle. The mobile phase consisted of three solvents; hexane, ethylacetate and methanol, mixed in various proportions. The various

proportions of the solvents were pushed through the bed by application of positive pressure using the vaccum pump. Twenty one fractions were obtained. The fractions were pooled together by thin layer chromatography (TLC) and this reduced the number of the fractions to five.

Results

Tables 8 and 9 show the phytochemicals present in the bulb extract of *Crinum jagus*. Qualitative phytochemcial screening revealed that the methanol extract of the plant contains alkaloids, flavonoids, phenols, saponins, steroids, but no traces of tannins. Quantitative phytochemcial test revealed that the extract contains high concentrations of alkaloids, phenols, saponins and appreciable amount of steroids and flavonoids. Fractionation of the crude extract by column and thin layer chromatography yielded five fractions. The biological activities of three fractions (F1, F2 and F3) out of the five fractions were tested because the yield of the other two fractions obtained were too small to be tested for any biological activity.

Conclusion

The crude methanol extract of the bulb of *Crinum jagus* contains high concentration of bioactive substances such as alkaloids, phenols, saponins, and appreciable amounts of flavonoids and steroids.

Table 8 : Phytochemcial constituents of the bulb extract of *Crinum jagus*

CONSTITUENTS	INFERENCE
Alkaloids	++
Flavonoids	++
Phenols	++
Saponins	++
Steroids	++
Tannins	-

++ indicates present

- Indicates absent

Table 9 : Relative phytochemical contents of *Crinum jagus*

PHYTOCHEMICAL	% PRESENT
Alkaloids	1.38
Phenols	0.649
Saponins	0.426
Steroids	0.076
Flavonoids	0.048

Experiment 2 : Acute Toxicity Study of the Methanol Extract of the Bulb of *Crinum jagus*

Introduction

The use of medicinal plant as therapy for disease condition is an age long practice. In regions with rich diversity of flora spread, it forms an important component of their natural wealth. Herbs and herbal formulation for the treatment of ailments have continued to receive increased attention because of the strong belief that the use of these products is safe (Farnsworth and Soejarto, 1985; Said *et al*., 2002). This assumption, to a large extent, may have influenced the indiscriminate use of plant formulations by many, particularly amongst the rural populace. The incidence of adverse effects and sometimes life-threatening conditions allegedly emanating from these herbal medicines has been reported among various ethnic groups (Elvin-Lewis, 2001; Chan, 2003). Consequently, it has become imperative to ascertain the toxicity profile of these medicinal herbs.

Pharmacological and toxicological evaluations of medicinal plants are essential for drug development. In the present study the acute toxicity of the methanol extract of the bulb of *Crinum jagus* plant was evaluated in mice to assess its safety.

Procedure

A total of twenty five swiss albino mice (25-30g) were used for the study. The animals were acclimatized for a week in clean cages and randomly divided into five groups of five animals per group. The animals were fasted for 14 hours and the groups received methanol extract orally within the range of 50-400mg/kg. The control group was given 0.2ml of normal saline orally. The animals were observed, closely, for general toxicity signs and behavior change for the first 6 hours and mortality recorded within 24 hours. LD_{50} was calculated using the moving average interpolation method of Thomson and Williams, (1974).

Results

Clinical signs for toxicity observed were decreased activity to the environment, huddling, general weakness and changes in the skin and fur. The LD_{50} value calculated by moving average interpolation method was 109.04mg/kg (Table 10).

Conculsion

This study have shown that *Crinum jagus* in very large doses have toxic properties.

Table 10 : Acute toxicity of the methanol extract of the bulb of *Crinum jagus* in mice by oral route after 24 hours of observation.

Group	Dose (mg/kg)	No of death	% Mortality
1	0	0	0
2	50	1	20
3	100	2	40
4	200	4	80
5	400	5	100

LD_{50} = 109. 04mg/kg

Confidence limit = (108.76 – 109.31) mg/kg.

Experiment 3 : *In vitro* Antioxidant Study on the Crude Extract and Fractions of the Bulb of *Crinum jagus*

Introduction

Free radical induced oxidative damage has long been thought to be the most important consequence of the aging process (Harman, 1992), such a condition is considered to be important in the development of diseases including diabetes, stroke, arteriosclerosis, cancer and cardiovascular diseases (Prior and CaO, 2000; Yamaguchi *et al.*, 2000).

Antioxidants are agents which scavenge the free radicals and stop the damage casued by them. Antioxidants are added as redox system possessing higher oxidative potential than the drug that they are designed to protect or as chain inhibitors of radicals. The effect of antioxidant is to break up the chains formed during the propagation by providing a hydrogen atom or an electron to the free radical and receiving the excess energy possessed by the activated molecule (Lachman, 1986). Recently, interest has increased considerably in finding naturally occurring antioxidants for use in food and medicinal materials to replace synthetic antioxidants which are being restricted due to their side effects such as carcinogenicity (Kumaran, 2007).

Plants contain a large variety of phytochemicals which are the main sources of antioxidants in the diet that could decrease the potential stress caused by reactive oxygen species. The natural antioxidants may have free radical scavengers, reducing agents, potential complexers of prooxidant metals and quenchers of singlet oxygen (Ebadi, 2000). The antioxidants can interfere with oxidation process by reacting with free radicals (Gupta, 2004).

This experiment was therefore carried out to evaluate the antioxidant property of the crude extract and fractions of the bulb of *Crinum jagus* as possible scavengers of free radicals using *in-vitro* and *in-vivo* models.

Procedure

DPPH free radical scavenging activities of the extract and its fraction were determined using the DPPH photometric method of Mensor *et al.*, (2001). Hydroxyl radical scavenging activities were determined by the method of Halliwel *et al*., (1987). The reducing power of the extract and the fractions of the plant were determined by the method of Oyiazu (1986). The total flavonoid content of the extract and the fractions of *Crinum jagus* bulb were assessed by the method of

Chang *et al., (*2002). Total phenolic content of the methanol extract and its fractions were estimated based on the reduction of Folin-Ciolcateu reagent (phosphomolybdate and phosphotungstate) by the phenolic compounds using the method of Mc Donald *et al.*, (2001). as earlier described in section 3.7. under 'Materials and Methods' '

Results

The DPPH radical scavenging activites of the extract and its fractions are shown in Figures 16 and 17. The methanol extracts and its fractions all scavenged DPPH in a concentration dependent manner, compared to control. The varying concentrations of the methanol extract 100, 200, 300, 400 and 500µg/ml significantly ($P < 0.05$) scavenged DPPH by 47.58%, 53.16%, 58.93%, 67.17% and 72.46% respectivley (Fig 16). Similary the same concentrations of F1, F2 and F3 significantly scavenged DPPH by (48.11%, 56.68%, 62.30%, 73.43%, 81.37%), (30.44%, 36.46%, 41.46% ,54,90%, 57.84%) and (33.57%, 40.94%, 54.51%, 63.10% and 69.83%), respectively (Fig 17). However these activities are less than that of ascorbic acid, the standard antioxidant. Highest percentage scavenging effect was obtained with F1 (81.37%) at 500µg/ml. The order of potency was: F1 > F3 > CE > F2.

The percentage hydroxyl radical (OH^-) scavenging activities of the extract and its fractions are shown in Figures 18 and 19. The methanol extract and its fractions scavenged hydroxyl radical in a concentration dependent manner compared to control. The highest percentage hydroxyl, radical scavenging activity was detected in F1 (88.32%) at 500µg/ml, the hydroxyl radical scavenging activity of F1 compared well with ascorbic acid (89.32%) . The order of potency was : F1 > F3 > CE > F2.

The reductive capabilities of the extract and its fractions are shown in Figures 20 and 21 compared to ascorbic acid, the standard antioxidant, the reducing powers of the extract and its fractions increased with increasing amount of the sample. F1 showed the highest reducing ability (0.628nm) than all the other fractions tested at 500µg/ml. However, the activity was less than the standard ascorbic acid (1.530nm). The reducing power of the extract and the fractions were in the order F1: (0.628) > CE (0.476) > F3 (0.473) > F2 (0.272).

The quantity of the total flavonoid contents of the plant extract and its fractions are shown in Figures 22 and 23. The methanol extract and its fractions (F1, F2 and F3), all contained

flavonoids in a concentration dependent manner. The varying concentrations of the methanol extract are 100, 200 ,300 ,400 and 500µg/ml while the corresponding concentrations of the total flavonoids content in quercetin equivalent are 0,195, 0.305, 0.365, 0.456 and 0.584µg/g (Fig 22). Similar concentrations of F1, F2 and F3 show the prescence of flavonoids in quercetin equivalent to be (0.216, 0.438, 0.552, 0.615, and 0.864µg/g), (0.028, 0.167, 0.260, 0.284, and 0.396µg/g) and (0.11, 0.350, 0.436, 0.582, and 0.643µg/g) respectively,
(Fig 23). F2 contained the least level of total flavonoids at its maximum concentration
(0.396µg/g) while the highest concentration of total flavonoids was obtained in F1 at 500µg/ml (0.864µg/g) quercetin equivalent (QE). The concentration and expression of the total flavonoids in the extract and its fractions are in the order: F1 > F3 > CE > F2.

The total phenolic contents of the methanol extract and its fractions (F1, F2 and F3) at different concentrations are shown in Figures 24 and 25 respectively. The methanol extract and its fractions, all contained phenolic compounds in a concentration dependent manner. The varying concentrations of the methanol extracts are 100, 200, 300, 400 and 500µg/ml while the corresponding concentration of the phenolic contents in gallic acid equivalent are 0.05, 0.128, 0.194, 0.260µg/mg and 0.356µg/mg respectively. Similarly, the same concentrations of F1, F2 and F3 showed the phenolic contents in gallic acid equivalent to be (0.09, 0.124, 0.210, 0.351 and 0.460µg/mg), (0.012, 0.030, 0.050, 0.140 and 0.240µg/mg) and (0.04, 0.082, 0.104, 0.220 and 0.380µg/mg), respectively (Figure 25). F2 contained the least phenolic compounds at its maxmium concentration while the highest concentration of total phenolic compounds was obtained in F1 at 500µg/ml (0.460µg/mg). The concentration and expression of the phenolic compounds in the methanol extract and the fractions are in the order: F1 > F3 > F2 > CE.

Conculsion

The results of these experiments indicated that the methanol extract and its fractions possess antioxidant properties as evident by their DPPH radical scavenging activity, hydroxyl radical scavenging activity and their reductive potential compared to ascorbic acid, a standard antioxidant, these activities are mostly expressed in F1. From the result obtained, it was observed that the methanol extract and its fractions contained flavonoids and phenolic compounds in a concentration-dependent manner with F1 having the highest flavonoids and phenolic contents.

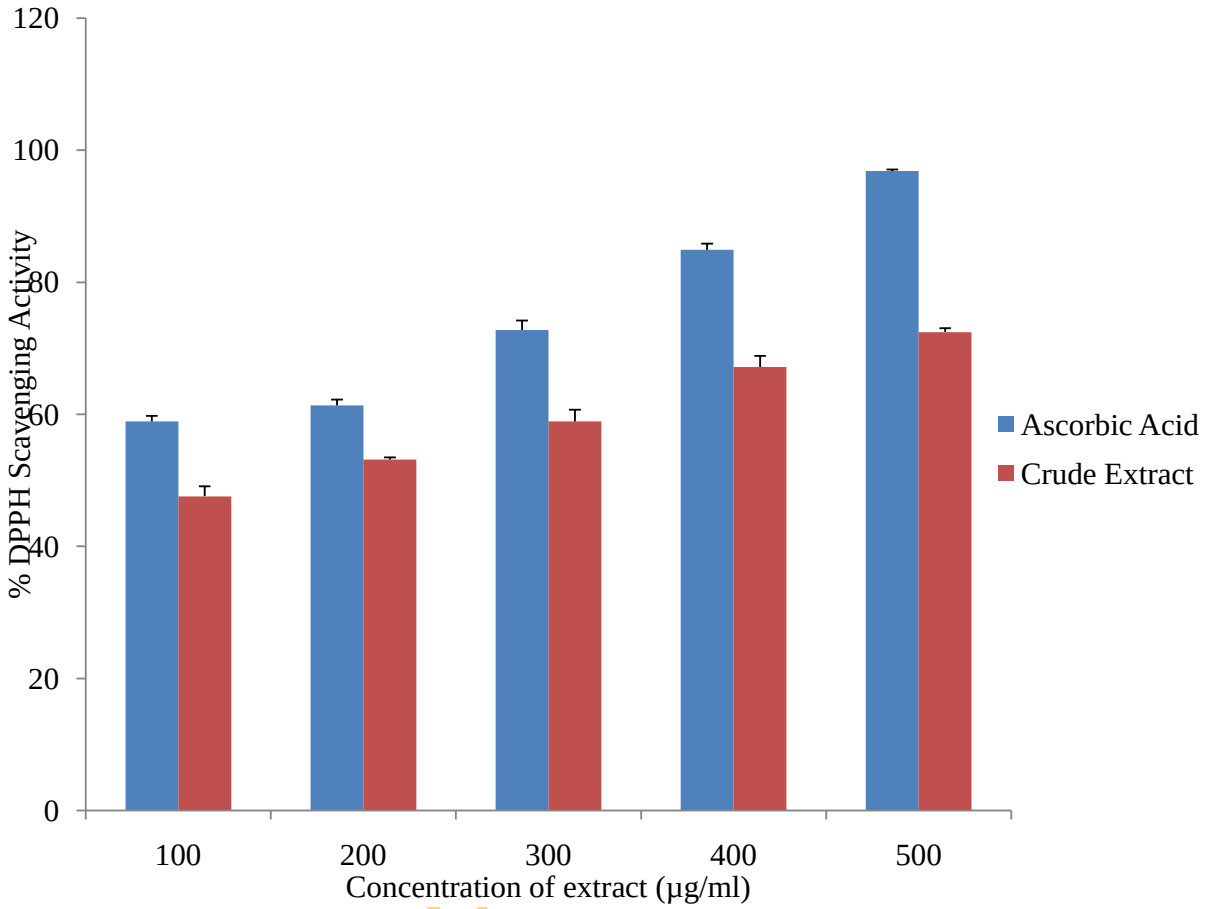


Figure 16: DPPH scavenging activity of the crude methanol extract of the bulb of *Crinum jagus*. (Ascorbic acid vs.Crude extract : $p < 0.05$)

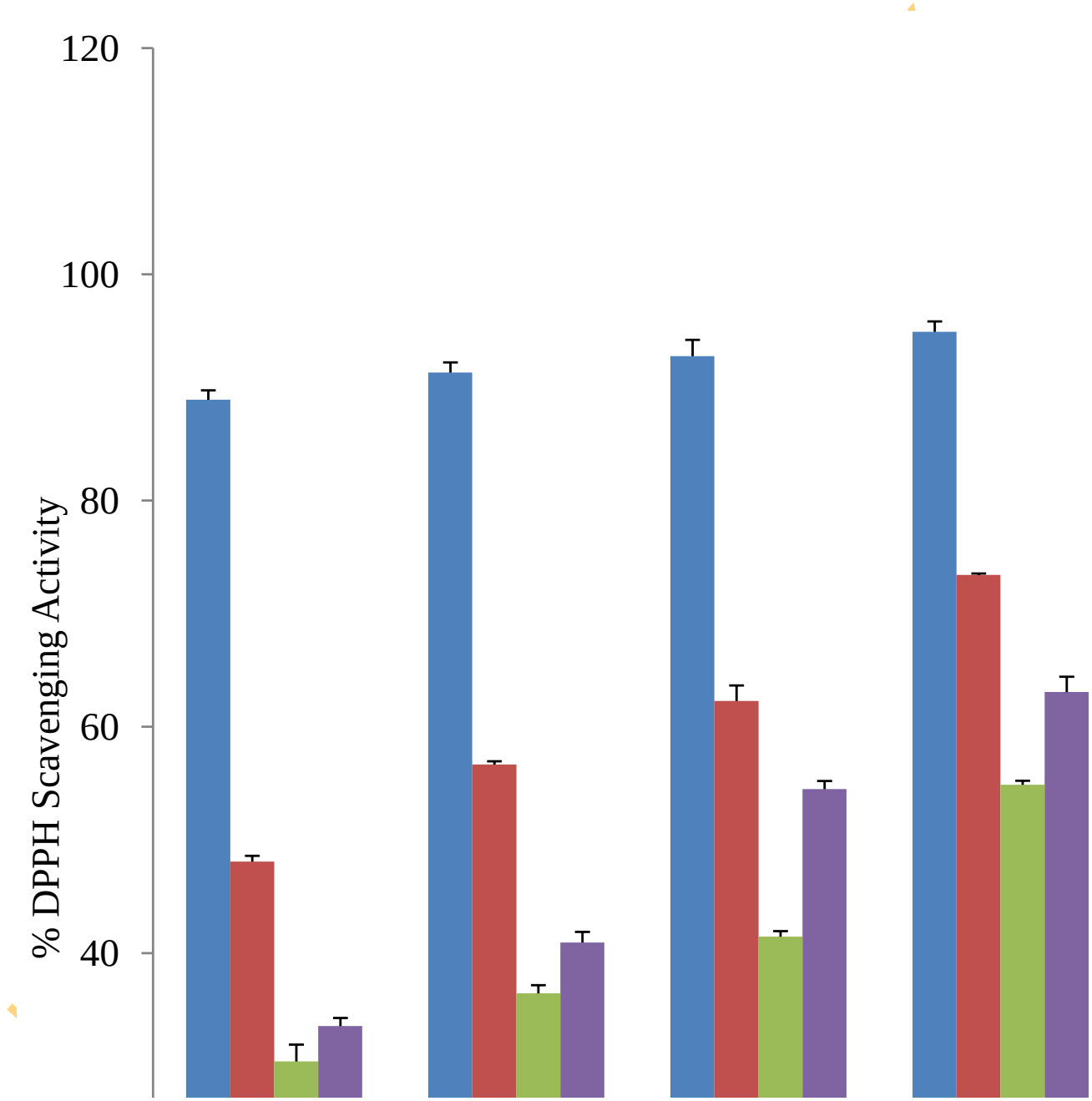


Figure 17: DPPH scavenging activities of the fractions (F) of the bulb of *Crinum jagus*.

(Ascorbic acid vs.Fractions 1,2, & 3 : $p < 0.05$)

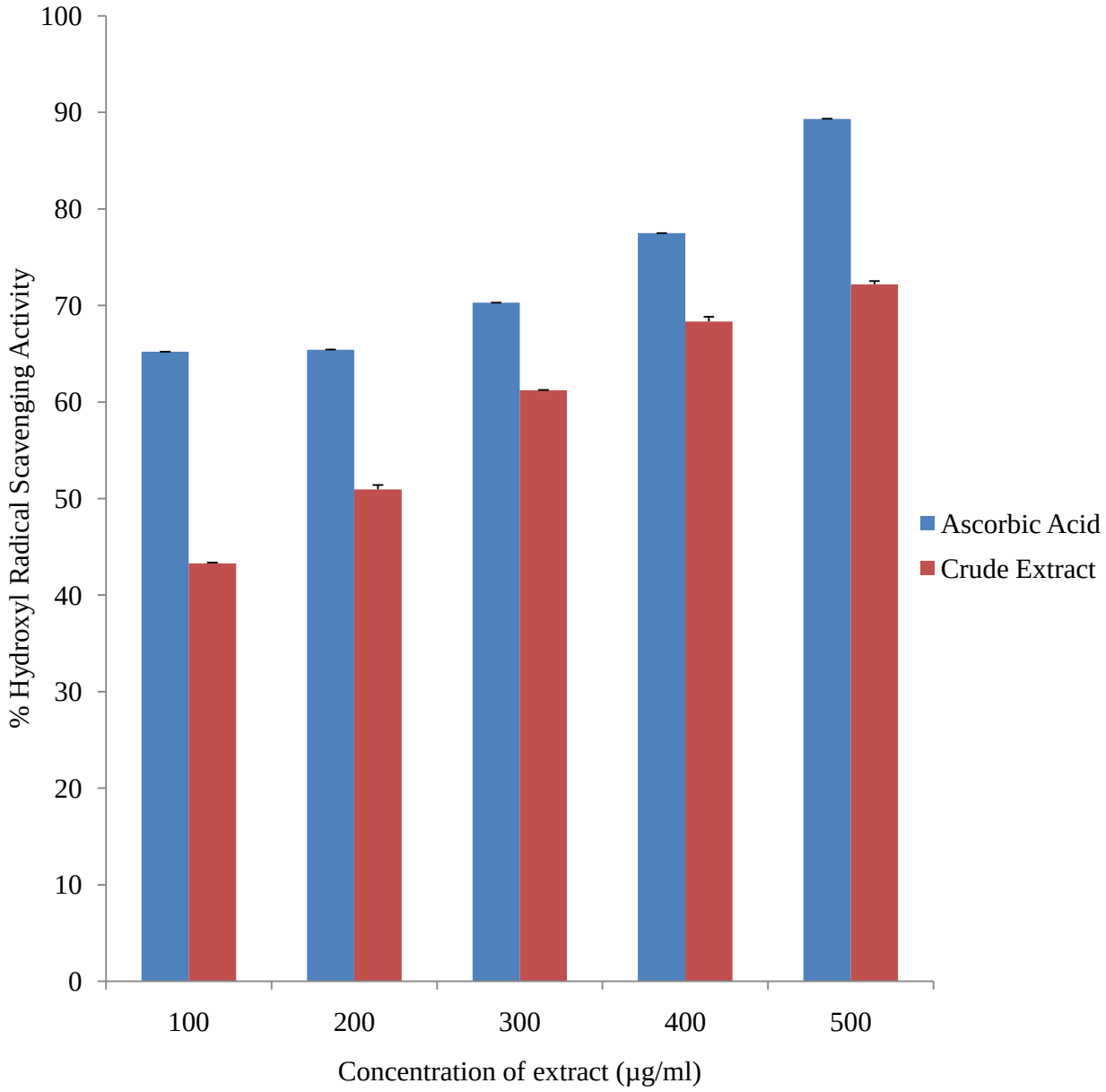


Figure 18: Hydroxyl radical scavenging activity of the crude methanol extract of the bulb of *Crinum jagus*.

(Ascorbic acid vs.Crude extract : $p < 0.05$)

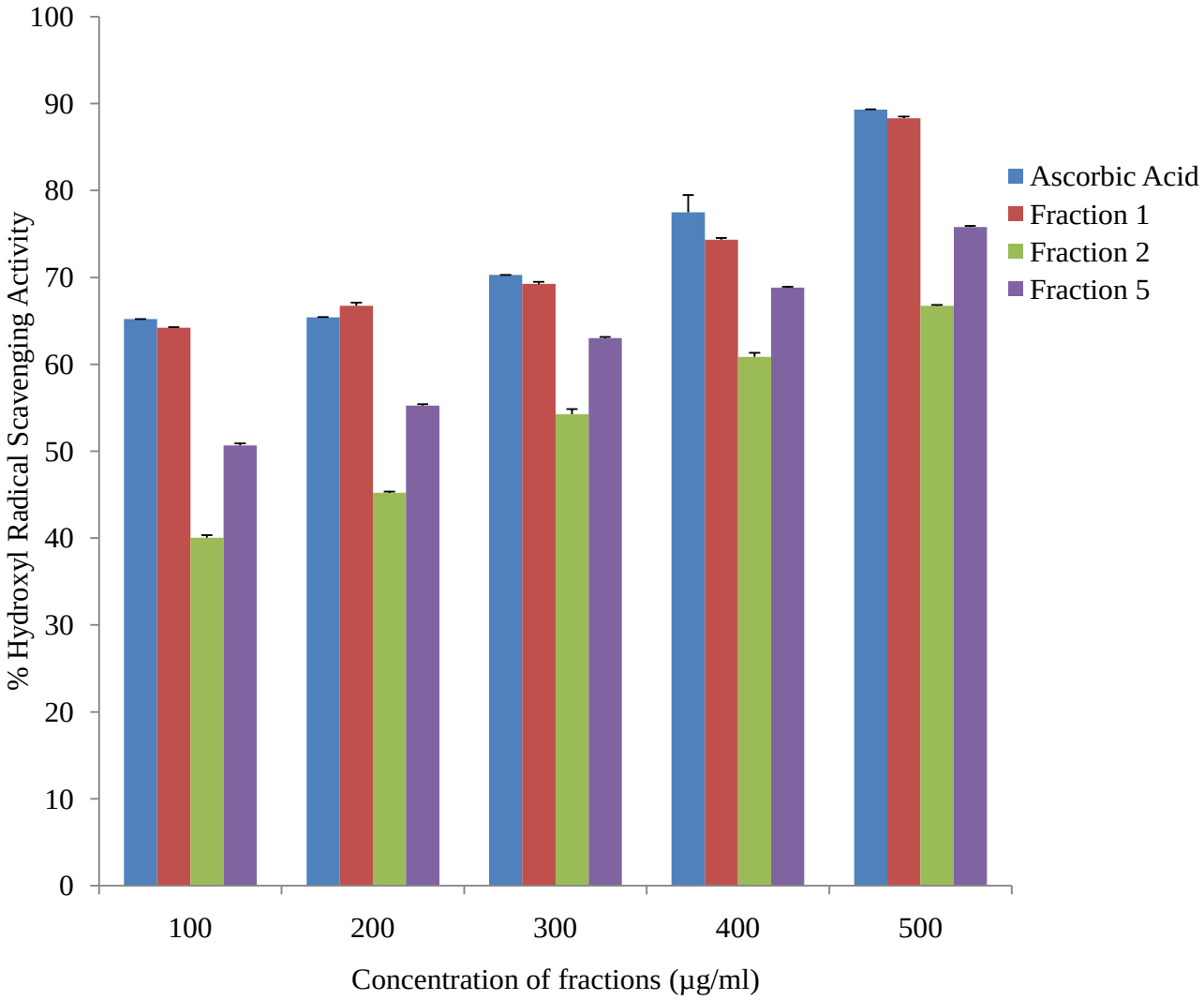


Figure 19 : Hydroxyl radical scavenging activities of the fractions (F) of the bulb of *Crinum jagus*.

(Ascorbic acid vs. Fraction 1,2, & 3 : $p < 0.05$)

(Ascorbic acid vs. Fraction 1 : $p > 0.05$)

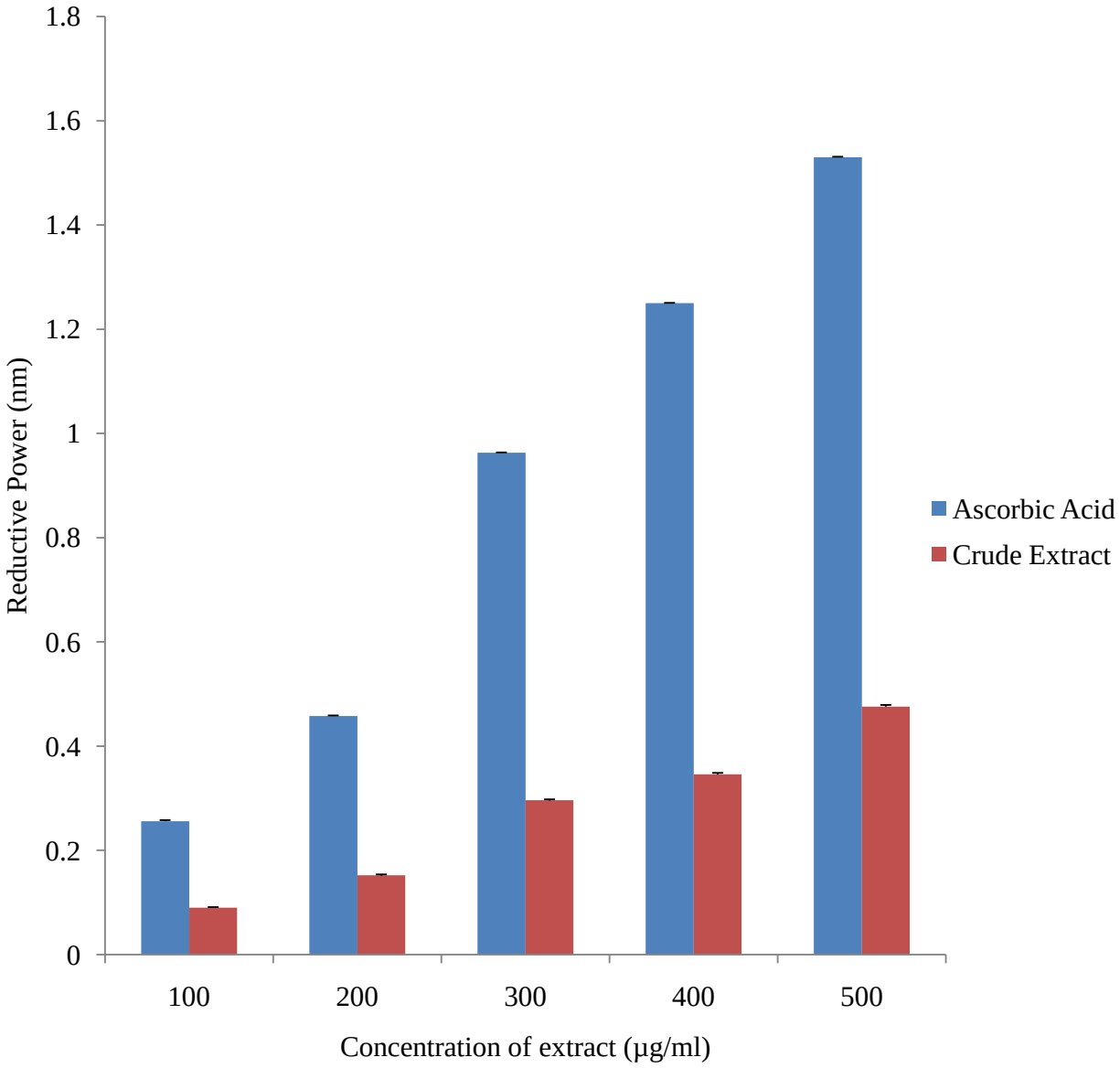


Figure 20: Reductive potential of the crude methanol extract of the bulb of *Crinum jagus*.

(Ascorbic acid vs.Crude extract : $p < 0.05$)

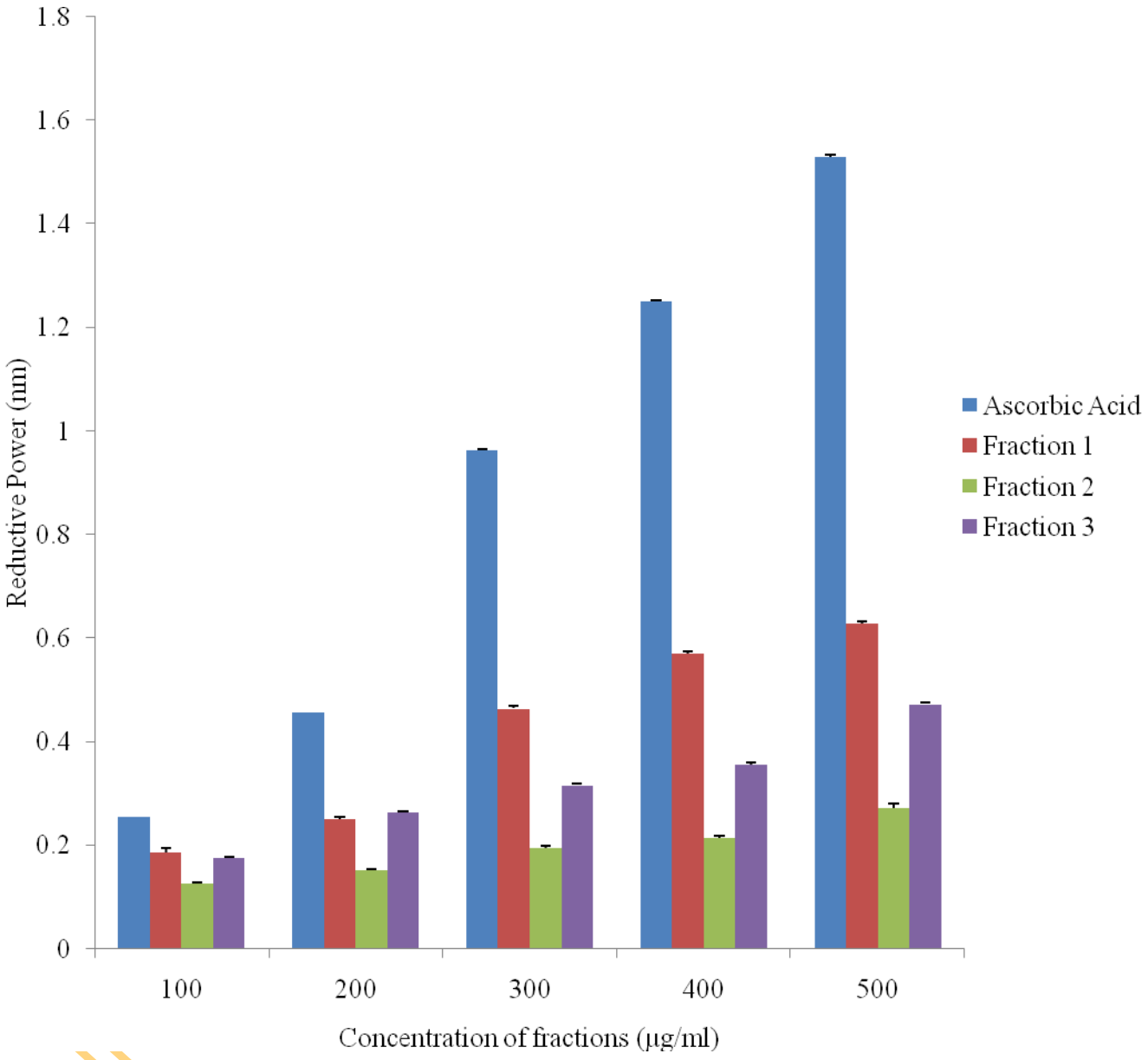


Figure 21: Reductive potentials of the fractions (F) of the bulb of *Crinum jagus.*

(Ascorbic acid vs.Fractions 1,2,& 3 : $p < 0.05$)

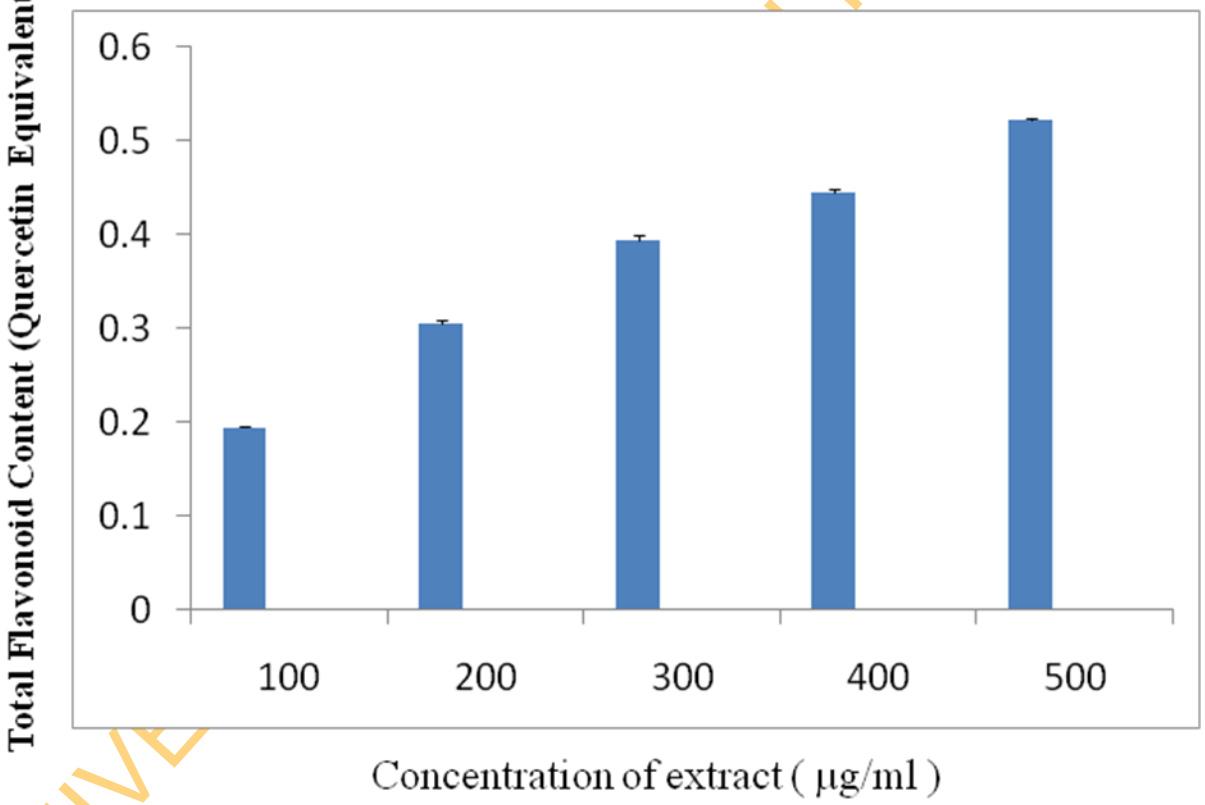


Figure 22: Total Flavonoid Content of the Crude Methanol Extract of the Bulb of *Crinum jagus*

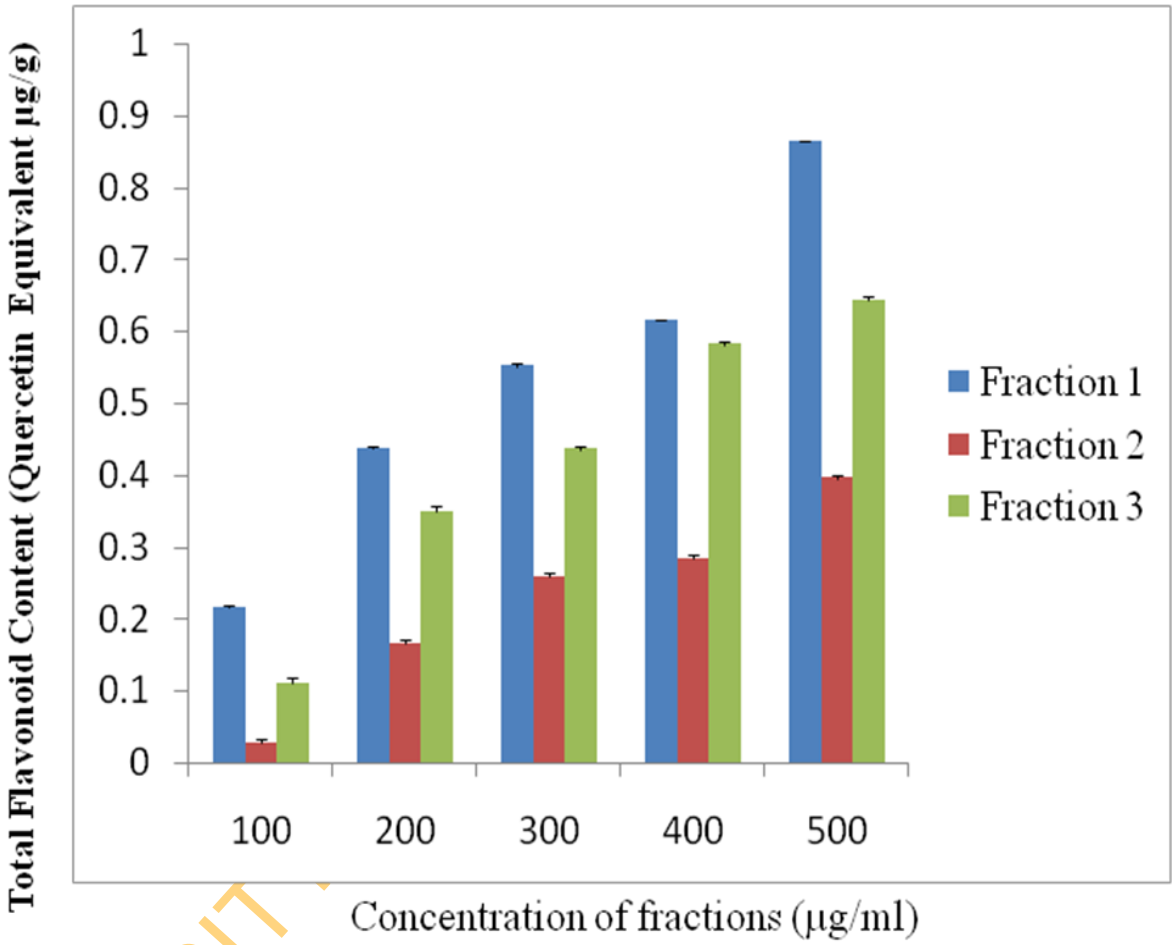


Figure 23: Total Flavonoid Content of Fractions of the Bulb of *Crinum jagus*

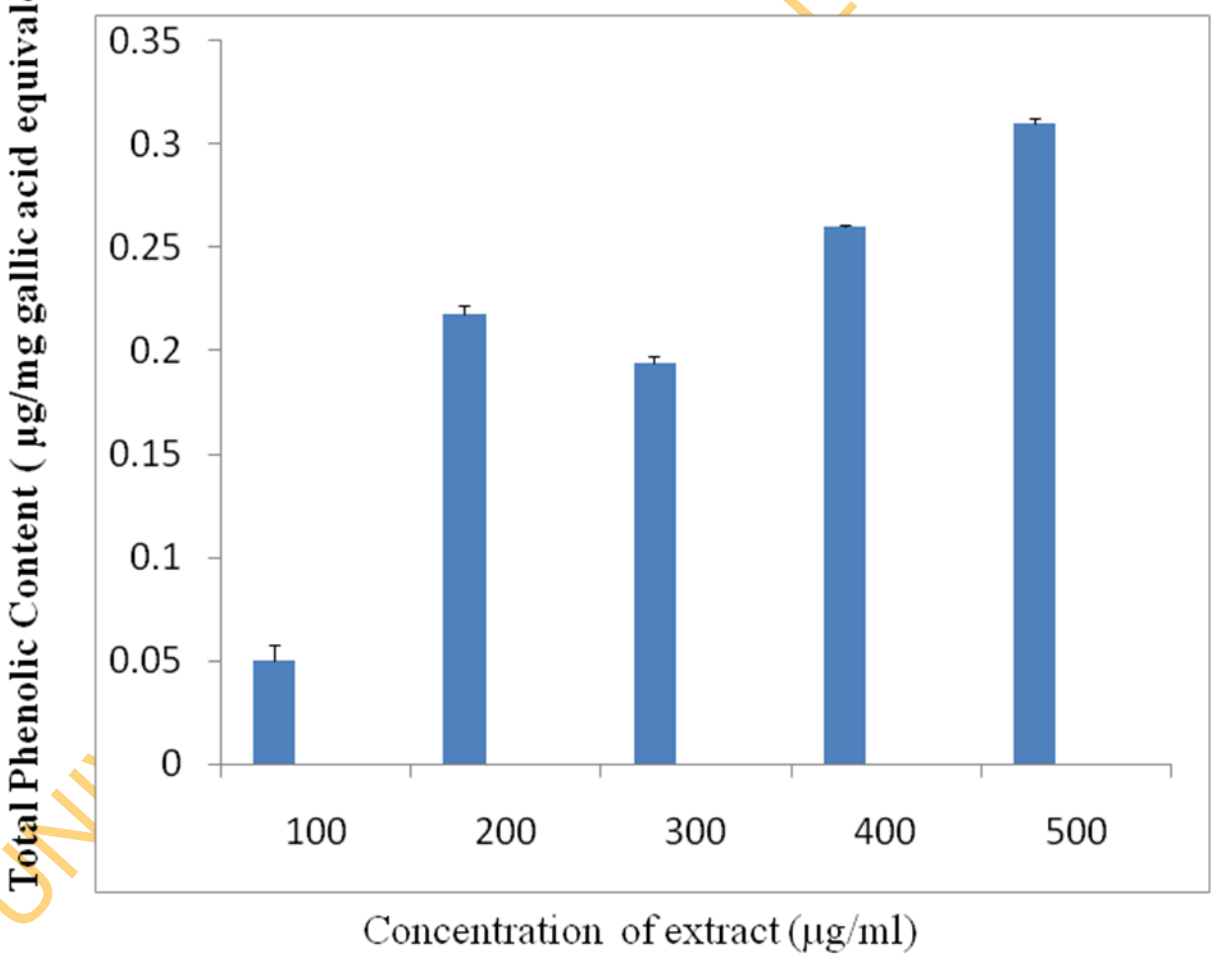


Figure 24 : Total phenolic content of the crude methanol extract of the bulb of *Crinum jagus*

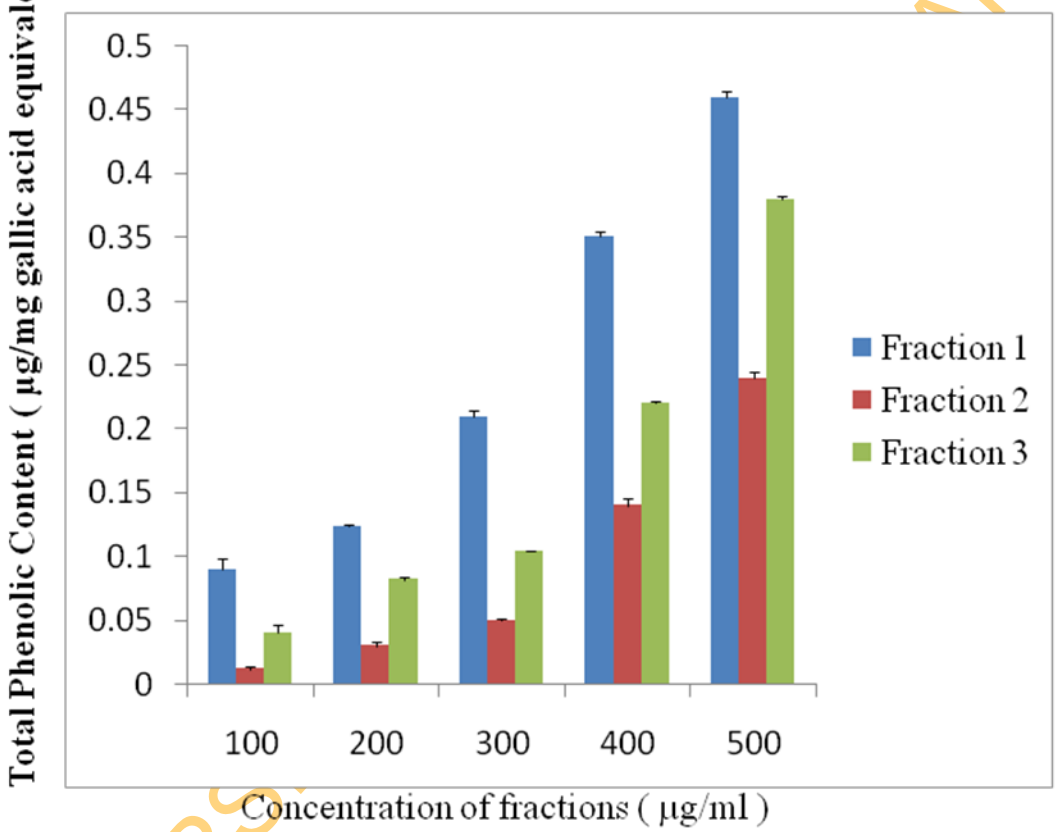


Figure 25: Total Phenolic Content of Fractions of the Bulb of *Crinum jagus*

Experiment 4 : *In vivo* Antioxidant Study on the Crude Extract and Fractions of the Bulb of *Crinum jagus*

Introduction

Antioxidants are molecules that can slow down or prevent the oxidation of other molecules. They can greatly reduce the damage due to oxidants by neutralising the free readicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohdyrates and DNA (Fang, 2002). Antioxidants can be classified into two major classes: enzymatic and non enzymatic. The enzymatic antioxidants are produced endogenously, some of which include superoxide dismutase, catalase and glutathione peroxidases. Crucial components of the antioxidant defense system in the body are cellular antioxidant enzymes: superoxide dismutase (SOD), catalase, glutathione peroxidases. which are involved in the reduction of reactive oxygen species (ROS) and peroxides produced in living organisms and thus playing a primary role in the maintenance of a balanced redox status (Michiels *et al*., 1994), hence they can serve as potential marker of susceptibility, early and reversible tissue damage and of decrease in antioxidant defense (Lester, 1994). Natural antioxidants from plant extracts provide a measure of production that slows the process of oxidative damage hence in this study the *in-vivo* antioxidant activity of the crude methanol extract and the fractions of the bulb of *Crinum jagus* was investigated.

Procedure

Thirty male Wistar strain albino rats were divided into five groups of six animals per group. Group A received 0.2ml of corn oil and served as the control. Groups B, C, D and E received 10, 25, 50 and 75 mg/kg of the crude extract separately and respectively for 30 days. For the fractions, thirty five male rats were divided into seven groups of five animals each. Group A received 0.2ml corn oil and served as the control. Groups B and C received F1 at the doses of 5 and 10mg/kg, Groups D and E received F2 at the doses of 5 and 10mg/kg while groups F and G received F3 at the doses of 5 and 10mg/kg respectively for 30 days. All administrations were done orally. Twenty four hours after the last treatment, the animals were sacrificed by cervical dislocation, the liver and kidney samples were removed, washed in ice cold 1.15% KCl, dried

and weighed. The samples were homogenized in 4 volumes of phosphate buffer (0.1M, pH 7.4). The resulting homogenate were centrifuged at 10,000g for 20 minutes to obtain the post-mithochondrial supernatant used for biochemical assays.

Protein concentration of liver and kidney homogenates were determined using Biuret method of Gornal *et al.*, (1949) as earlier described in section 3.8 under "Materials and Methods". Lipid peroxidation assay was determined by the method of Vashney and Kale (1990), superoxide was assayed based on the method of Misra and Fridovich, (1972), catalase was assayed by the method of Sinha (1971), reduced glutathione concentration was determined using the procedure of Jollow *et al.*, (1974) and glutathione-S -transferase assay was carried out using the procedure of Habig *et al.*, (1974) as decribed in section 3.8 under "Materials and Methods".

Results

The results of the effect of crude methanol extract of the bulb of *Crinum jagus* on lipid peroxidation in rat liver and kidney is presented in Figure 26. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) caused reduction that is not significant in the malondialdehyde (MDA) level in the liver while a significant ($P < 0.05$) and dose dependent reduction of MDA level was observed in the kidney compared to the control group. In the kidney, the extract significantly inhibited lipid peroxidation by 46.80, 62.23, 85.63 and 93.62% respectively. The result showed that the three fractions (F1, F2 and F3) caused significant ($P < 0.05$) reduction in hepatic and renal MDA levels compared to that of the control group after 30 days of oral administration of the fractions (Fig 27 and 28). F1 at 5 and 10mg/kg significantly inhibited lipid peroxidation in the liver by 41.01% and 49.51% respectively. F2 at 5 and 10mg/kg significantly inhibited lipid peroxidation in the liver by 15.33% and 25.00% respectively while fraction F3 at 5 and 10mg/kg inhibited lipid peroxidation in the liver by 27.91% and 31.07% respectively. In the kidney, F1, F2 and F3 at 5 and 10mg/kg significantly inhibited lipid peroxidation by (63.25% and 67.45%), (11.17% and 19.17%) and (49.10% and 61.45%), respectively, compared with the control group. F1 showed the highest inhibitory activity.

The result of the effect of thirty days oral administration of the crude extract on hepatic and renal superoxide dismutase (SOD) activity is presented in Figure 29. SOD activity in the liver was significantly ($P < 0.05$) increased in 25, 50 and 75mg/kg dose by 20.72, 23.45 and 26,00% respectively. In the kidney SOD activity was observed to increase by 34.83, 55.43, 78.65 and 87.27% respectively in all the treated groups. Results from Figures 30 and 31 shows that F1 caused significant elevation in the activity of superoxide dismutase in the liver and kidney. The two doses of F1 administered (5 and 10mg/kg) elevated superoxide distmutase activity in the liver and kidney by 37.78 and 39.01% and 52 and 84.00% respectively. The increase in SOD activity caused by administration of 10mg/kg dose of F2 in the liver was not significant while in the kidney, admnistration of 10mg/kg dose elevated SOD activity by 38.00%. The two doses of F3 (5 and 10mg/kg) elevated SOD activity in the liver and kidney by (44.44 and 64.41%) and (32.00 and 52.00%), respectively.

Figure 32 shows the result of 30 days oral administration of the crude extract of *Crinum jagus* on catalase activities in the liver and kidney. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) significantly ($P < 0.05$) increased the activity of catalase in the liver by 30.77, 40,00 88.89 and 122.20% and kidney by 29.63, 31.65, 47.06 and 103.8% respectively, compared with the control group. F1 (5mg and 10mg/kg) significantly ($P < 0.05$) increased catalase activity in the liver by (34.88 and 44.00%) and kidney by (59.09 and 90.91%), respectively, compared with the control group. In F2, catalase activity was lower than that of the control group in both liver and kidney following administration of the 5 and 10mg/kg doses for 30 days. Non significant increase in the activity of catalase was observed in the liver and kidney of F3 treated animals, relative to control group (Figures 33 and 34).

Figure 35 indicates that administration of varying concentrations of the crude methanol extract of *Crinum jagus* (10, 25, 50 and 75mg/kg), for 30 days, resulted in the significant ($P < 0.05$) increase in the level of reduced glutathione (GSH) both in the liver and kidney compared with the control group. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) significantly increased the level of reduced glutathione in the liver and kidney by (15.88, 16.58, 26,53 and 57.24%) and (19.62, 36,60, 62.26 and 100%) respectively. Figures 36 and 37 shows that treatment of animals with 5 and 10mg/kg doses of F1 significantly

increase the hepatic and renal glutathione levels by (48.35 and 64.17%) and (46.99 and 61.93%) respectively. F2 produced insignificant increase of hepatic and renal glutathione levels when compared with the control group. F3 significantly elevated glutathione levels in the liver and kidney by 47.67 and 41. 59% respectively following administration of the 10mg/kg dose.

As shown in Figure 38, thirty days of oral administration of the crude methanol extract of the bulb of *Crinum jagus* significantly increased the activity of glutathione-S-transferase (GST) both in the liver and the kidney. The varying concentrations of the crude extract 10, 25, 50 and 75mg/kg significantly increased the activity of GST in the liver and kidney by (50.00, 65.00, 100 and 150%) and (21.05, 57.89, 100 and 136.84%), respectively. From figures 39 and 40 it was indicated that the liver and kidney activites of GST were significantly elevated in the groups treated with F1 (5 and 10mg/kg) relative to control by (56.52 and 86.96%) and (49. 33 and 73.33%), respectively. F2 produced a non significant increase in hepatic and renal GST activity. With F3, the 10mg/kg significantly increased GST activity by 69.56% in the liver while in the kidney, the 5 and 10mg/kg doses significantly elevated GST activity by 48.00 and 65.33%, respectively, relative to control. Fractionation of the crude extract does not lead to the loss of biological activity but the antioxidant activity of the plant were enhanced by fractionation. F1 demonstrated the highest antioxidant activity followed by F3, F2 had the least antioxidant activity.

Conclusion

The results of these experiments indicate that the crude methanol extract and fractions of the bulb of *Crinum jagus* possess *in vivo* antioxidant activity. Treatment of rats with the crude methanol extract (10, 25, 50 and 75mg/kg) and F1, F2 and F3 (5 and 10mg/kg) resulted in a dose-dependent and significant improvement in the antioxidant status as indicated by reduction in hepatic and renal lipid peroxidaiton and simulataneous elevation in hepatic and renal supeoxide dismutase, catalase, reduced glutathione and glutathione-S-transferase relative to control.

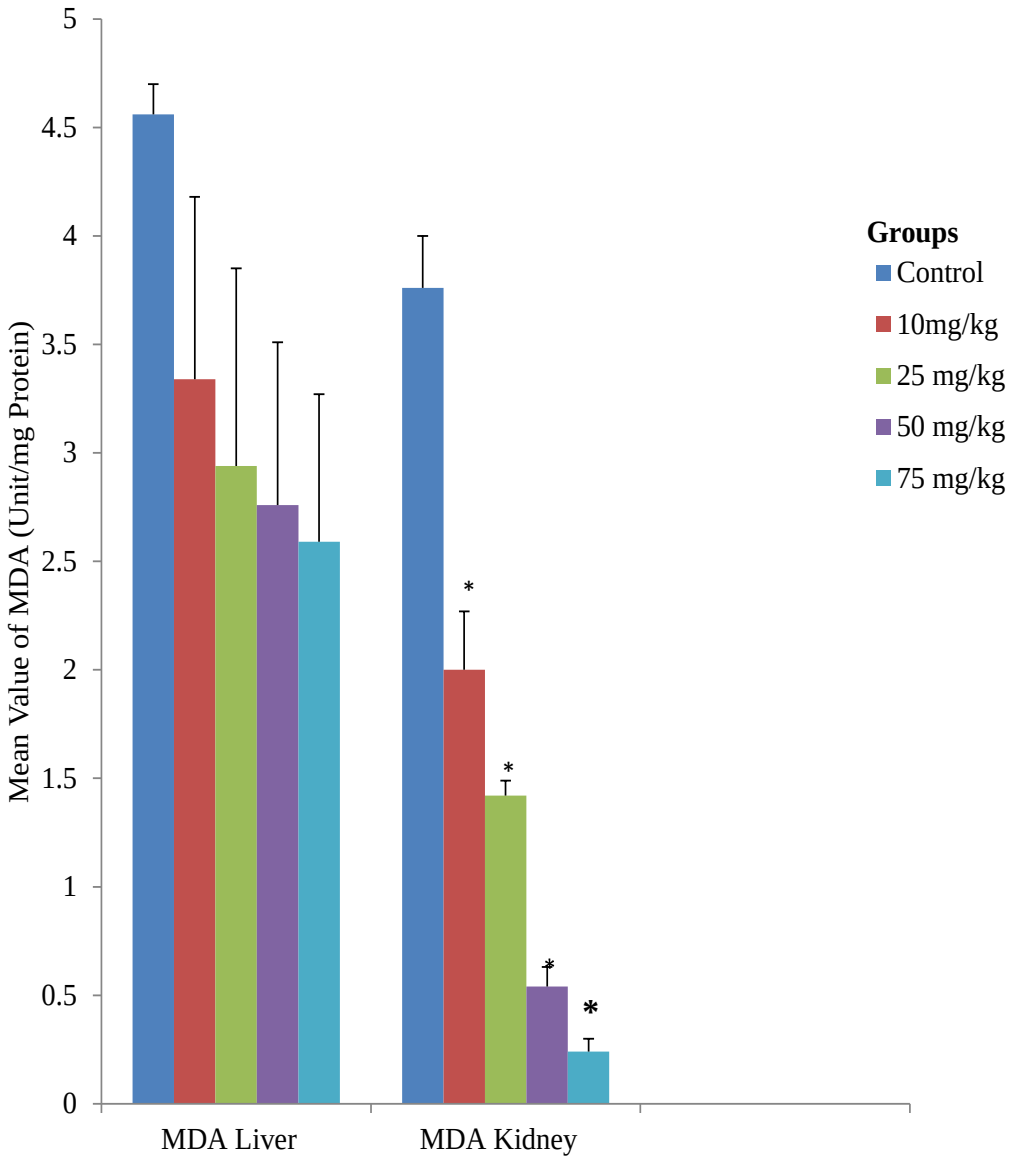


Figure 26: Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney malondialdehyde (MDA) levels in rats.

* = Significantly different from control

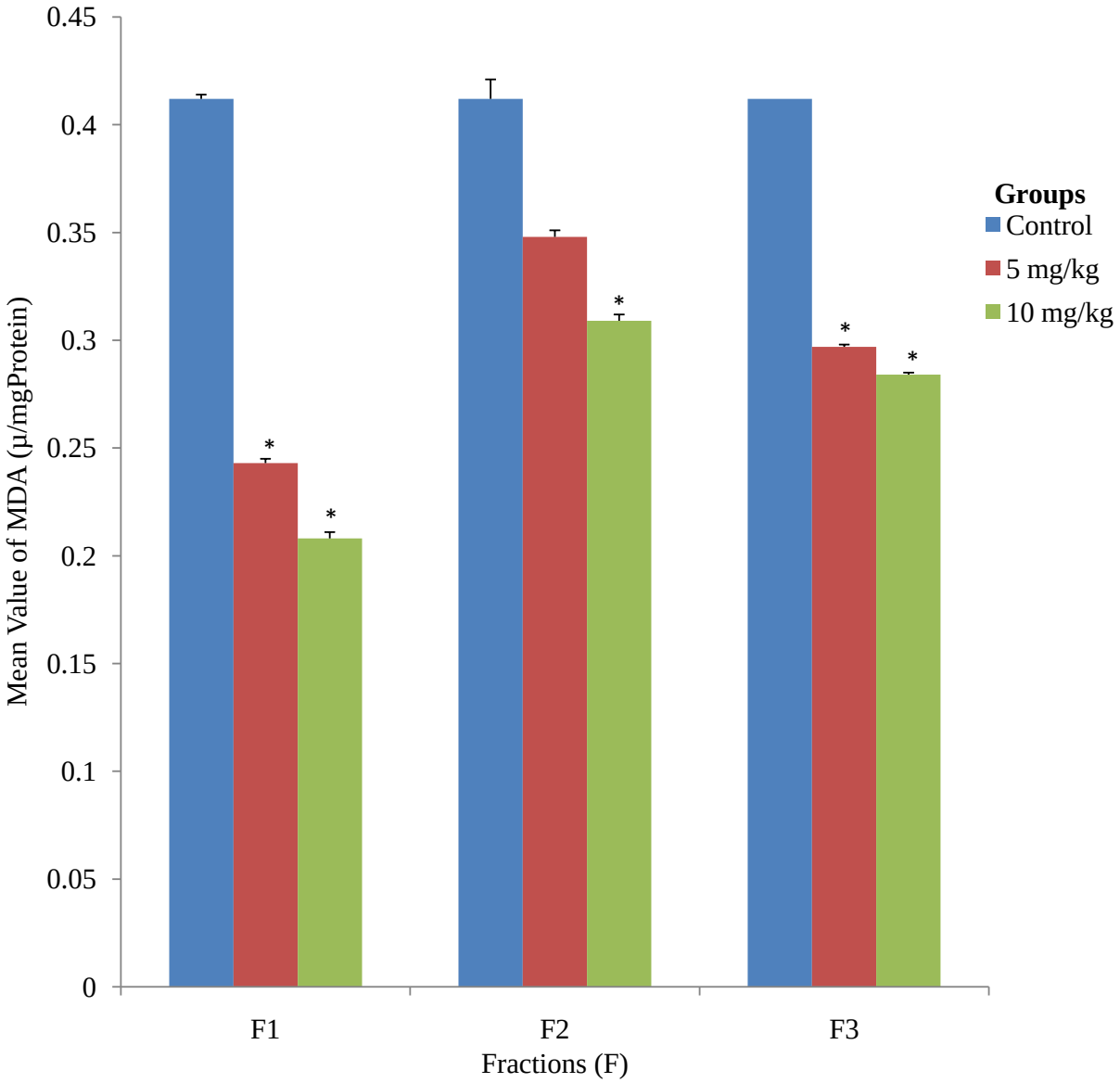


Figure 27: Effects of the fractions of the bulb of *Crinum jagus* on liver malondialdehyde (MDA) levels in rats.

* = Significantly different from control

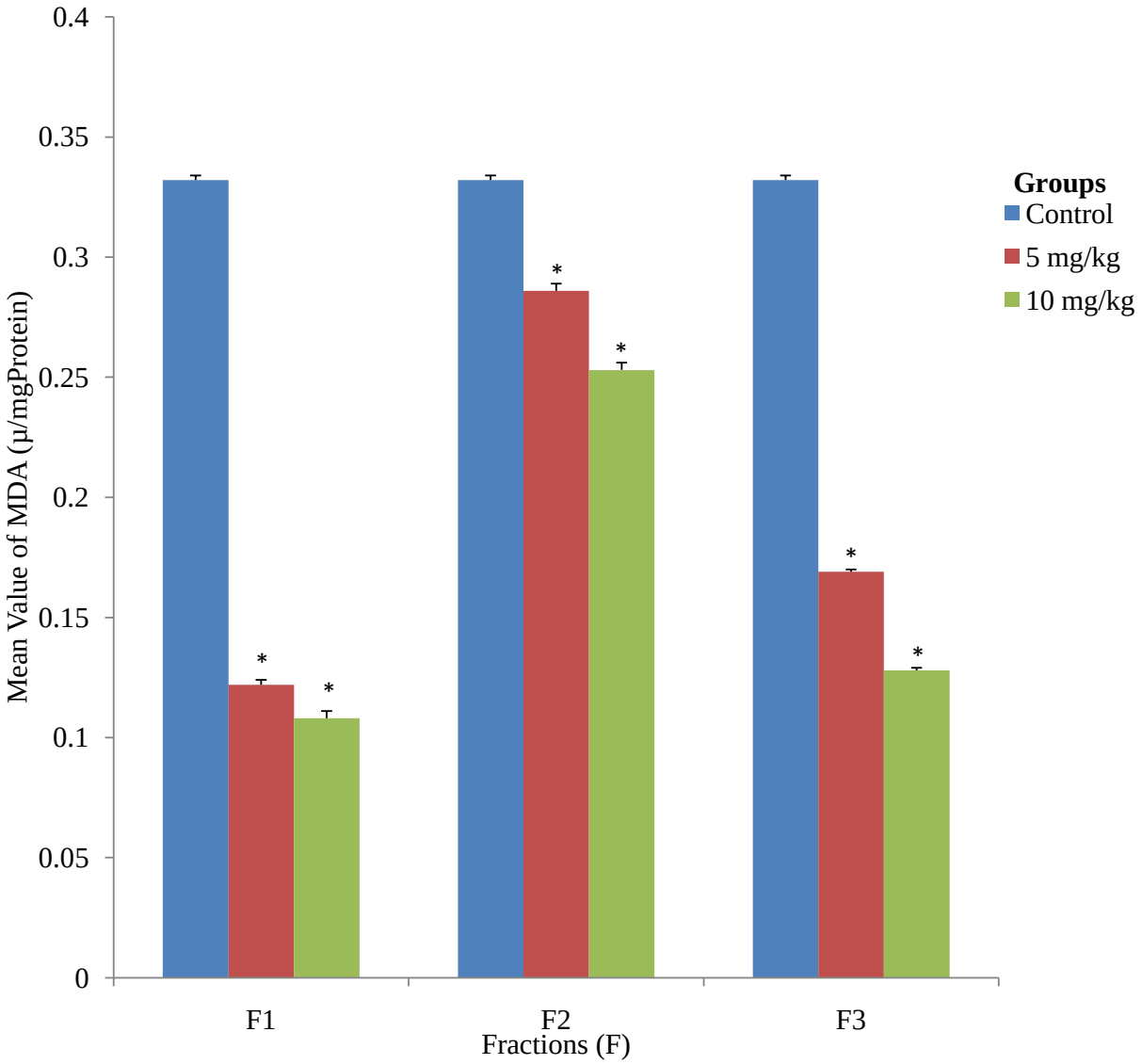


Figure 28: Effects of the fractions of the bulb of *Crinum jagus* on kidney malondialdehyde (MDA) levels in rats.

* = Significantly different from control

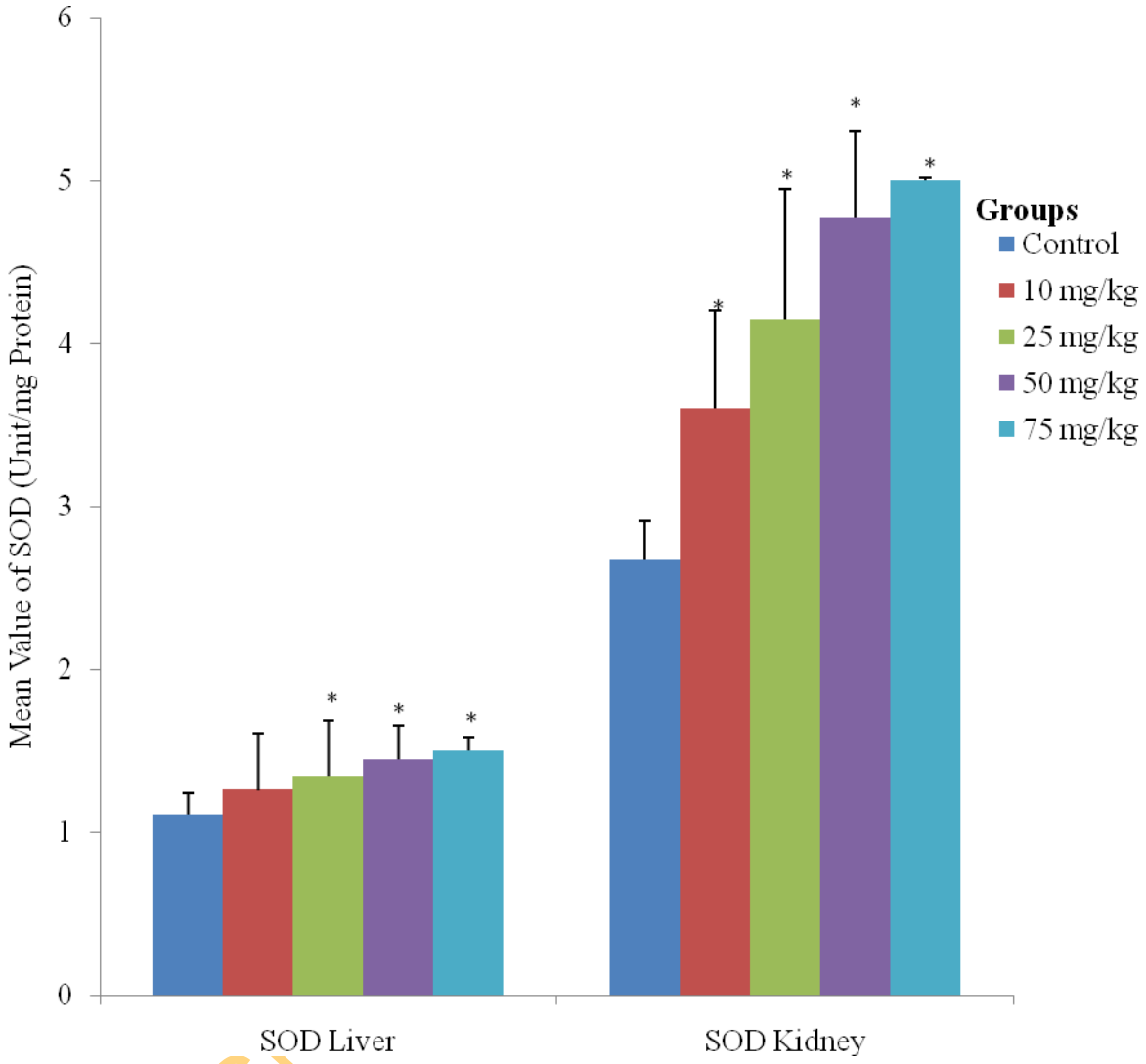


Figure 29: Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney superoxide dismutase (SOD) activities of rats.

* = Significantly different from control

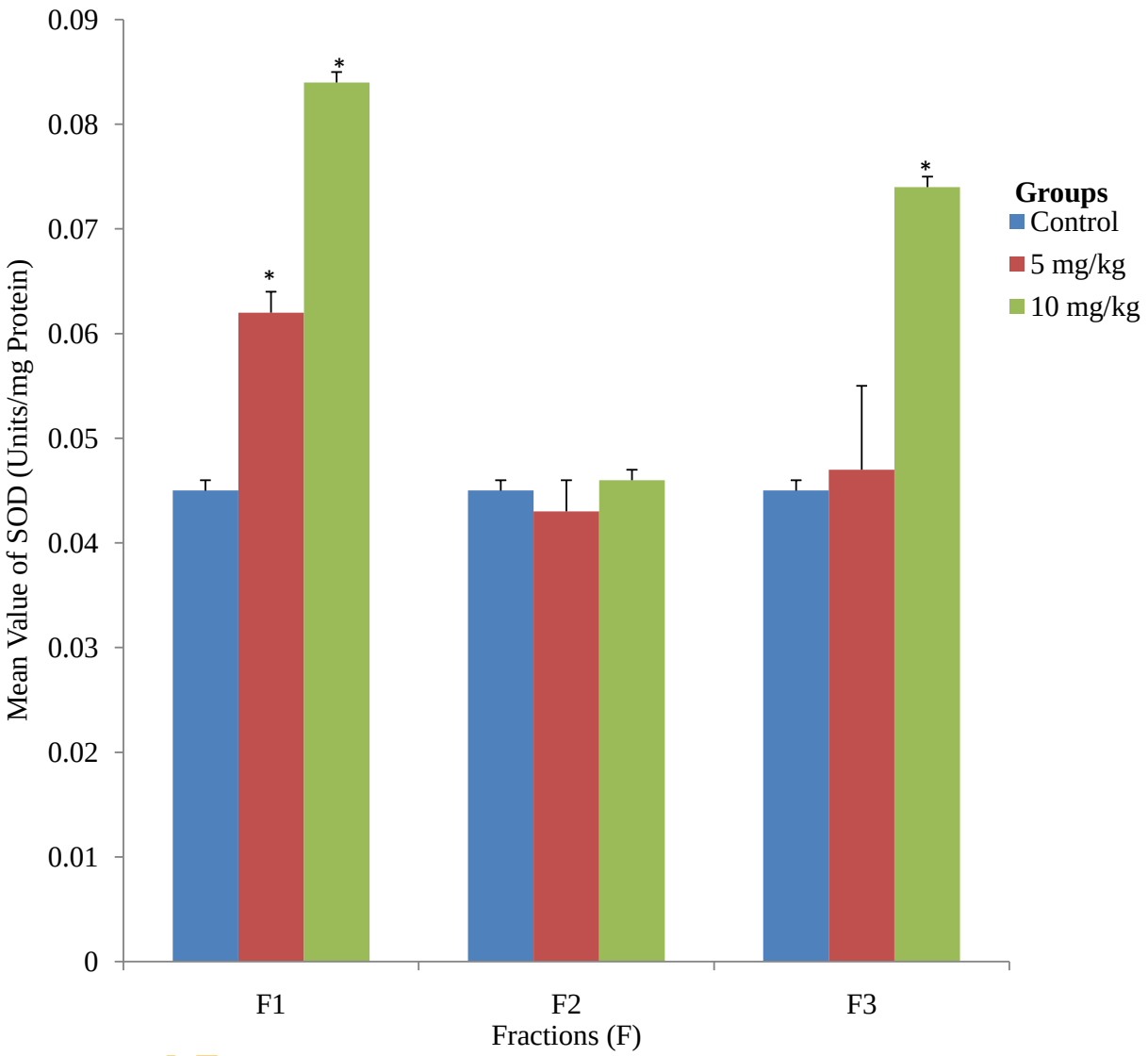


Figure 30: Effects of the fractions of the bulb of *Crinum jagus* on liver superoxide dismutase (SOD) activities of rats.

* = Significantly different from control

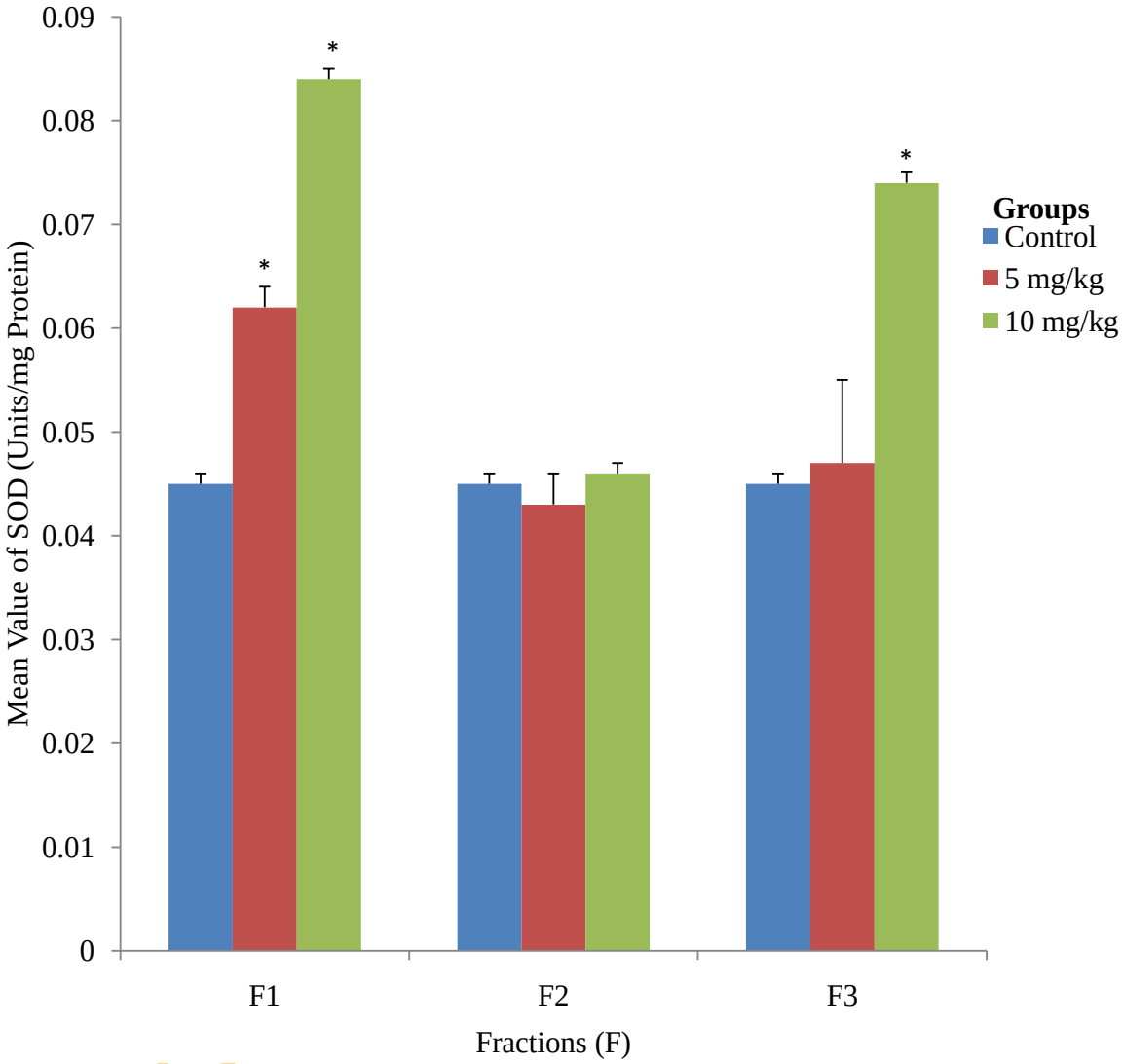


Figure 31: Effects of the fractions of the bulb of *Crinum jagus* on kidney superoxide dismutase (SOD) activities of rats.

* = Significantly different from control

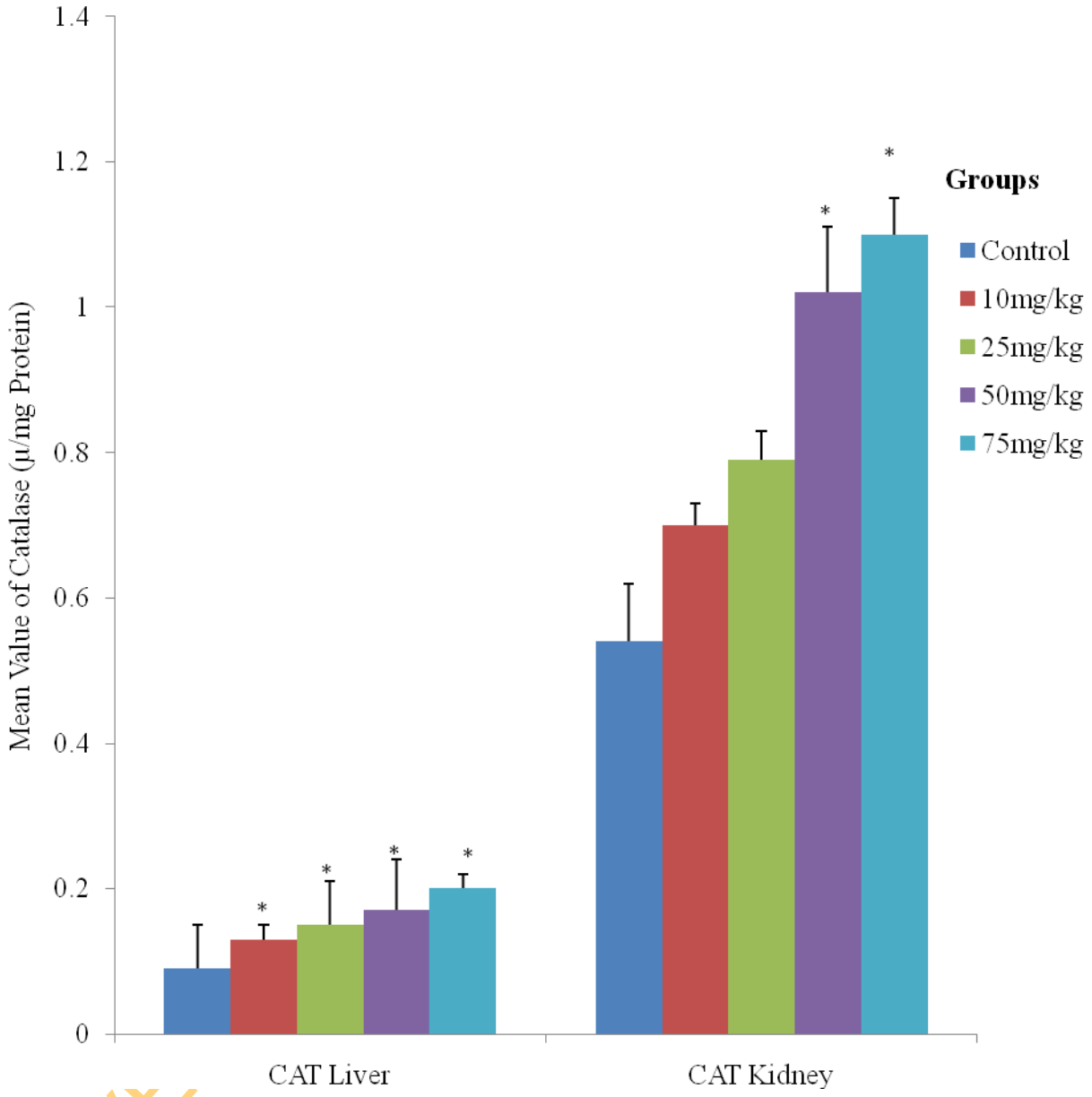


Figure 32: Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney catalase activities of rats.

* = Significantly different from control

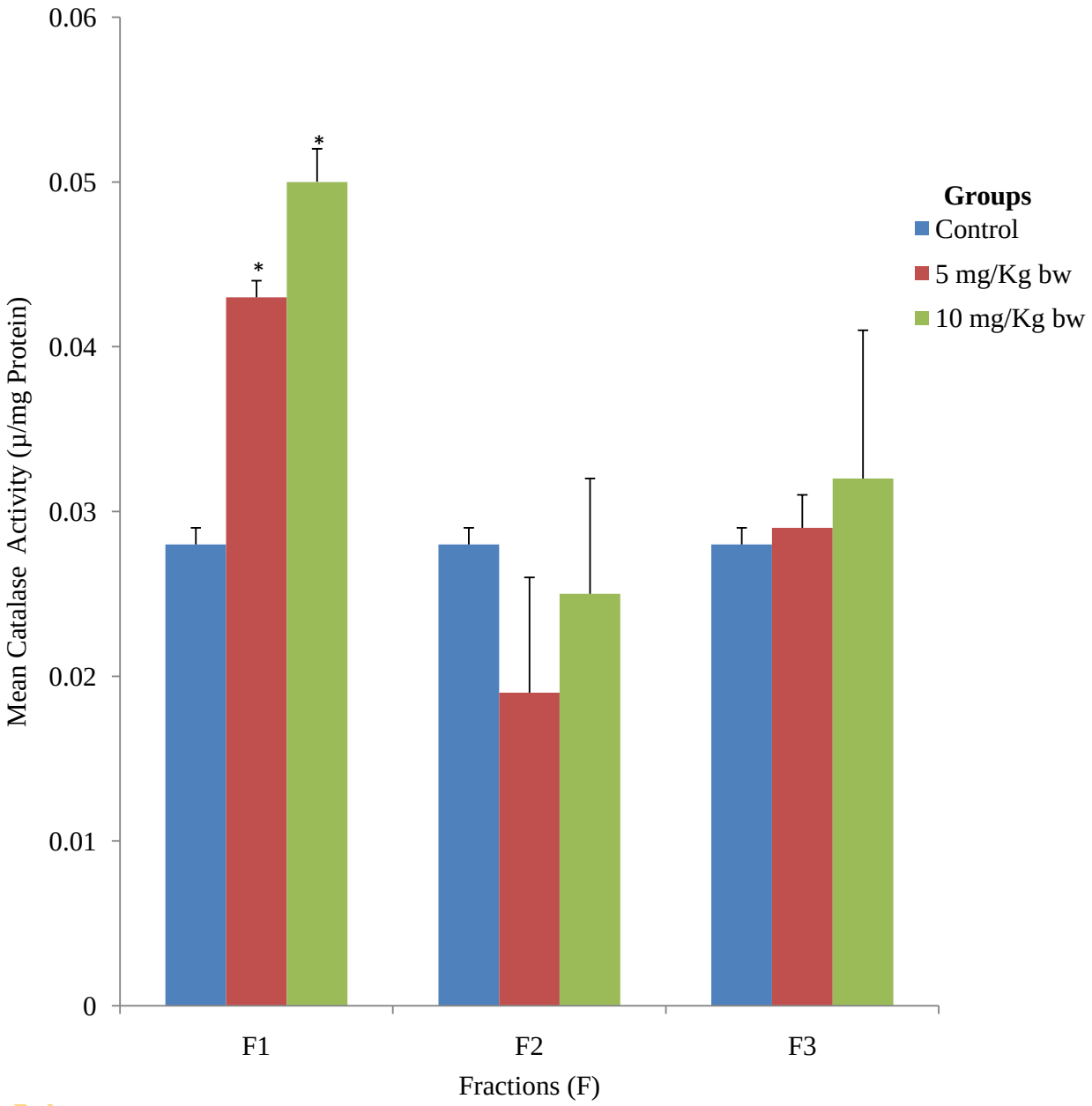


Figure 33: Effects of the fractions of the bulb of *Crinum jagus* on liver catalase activities of rats.

* = Significantly different from control

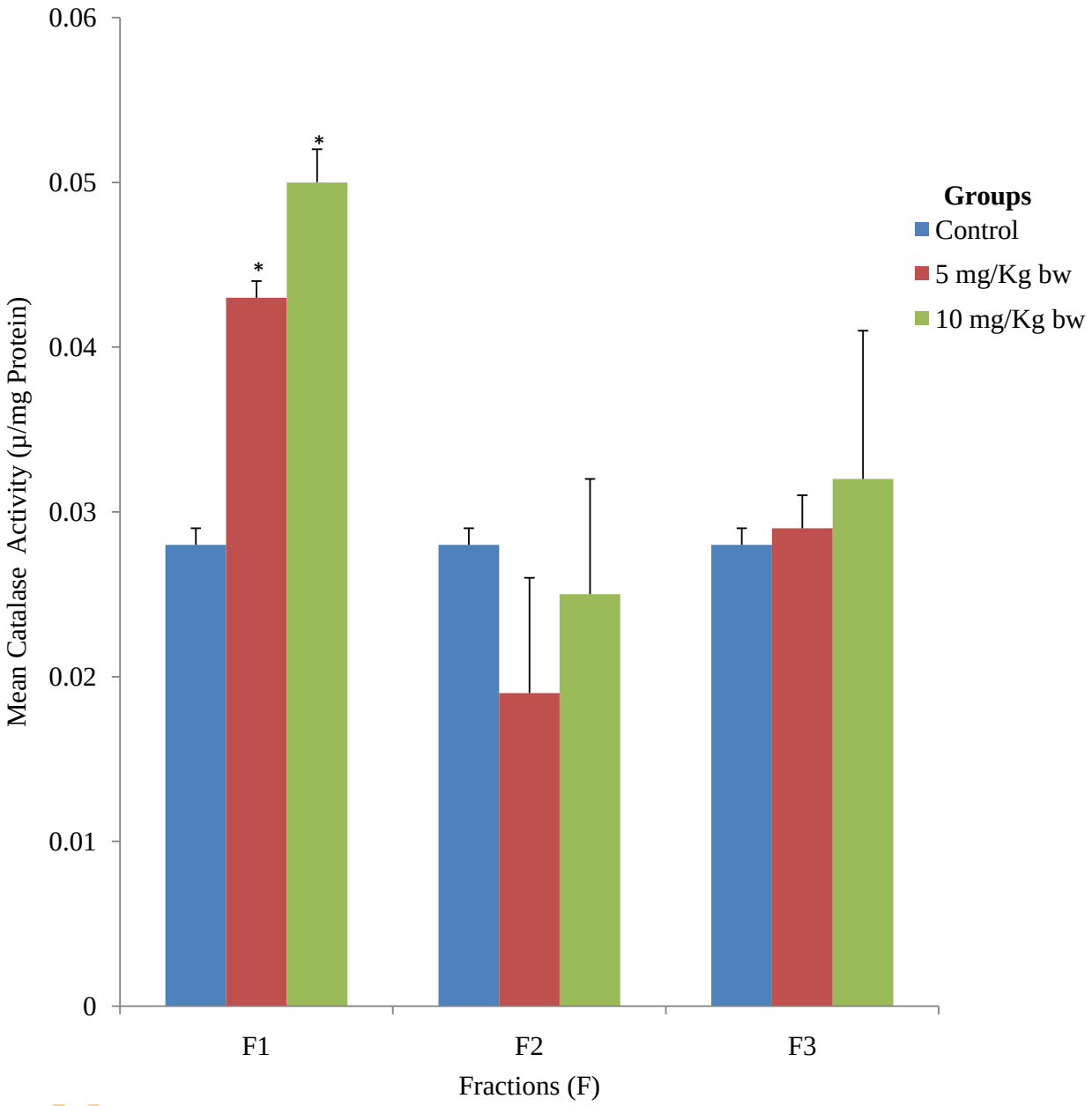


Figure 34: Effects of the fractions of the bulb of *Crinum jagus* on kidney catalase activities of rats.

* = Significantly different from control

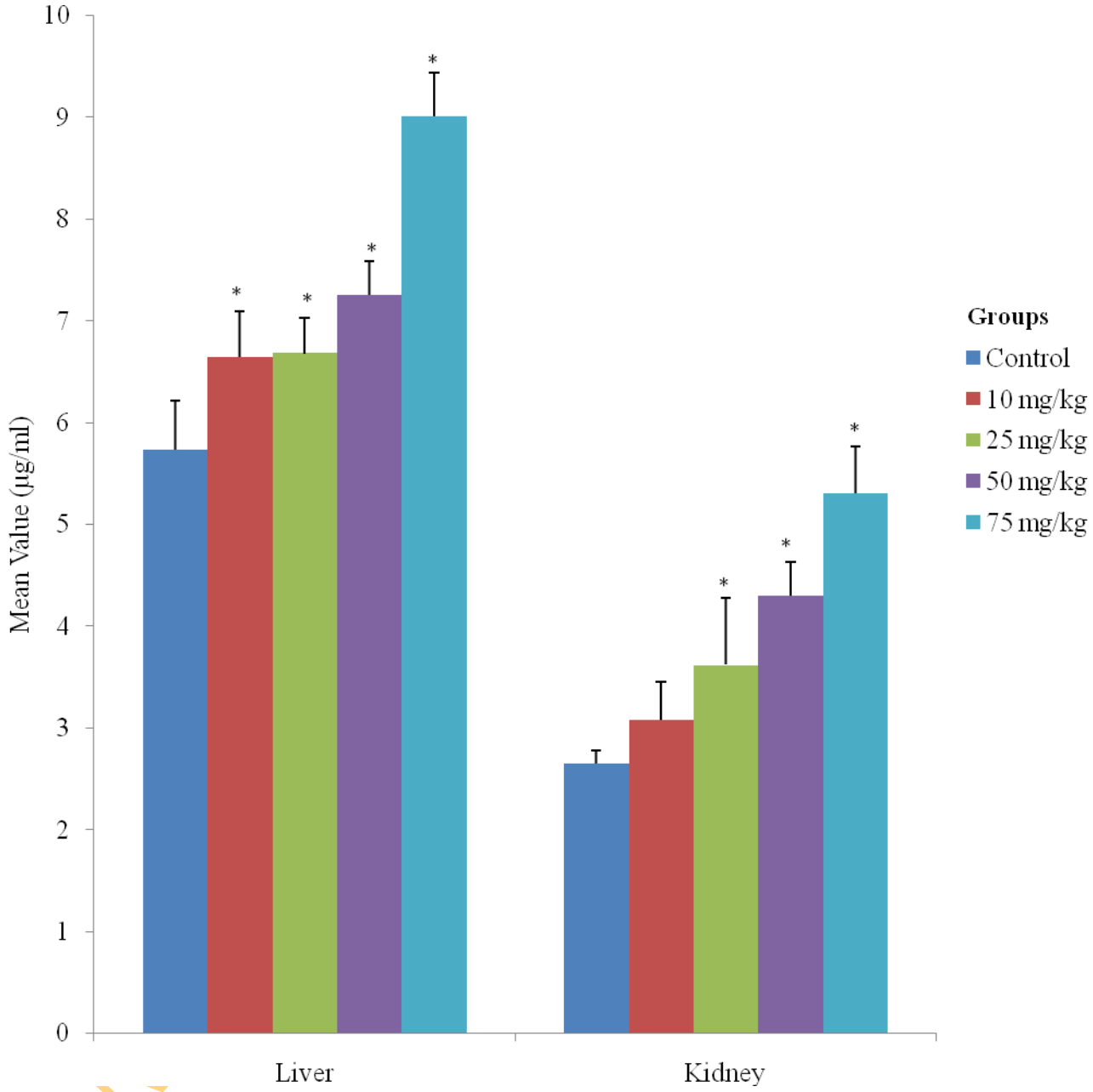


Figure 35: Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney reduced glutathione activities of rats.

* = Significantly different from control

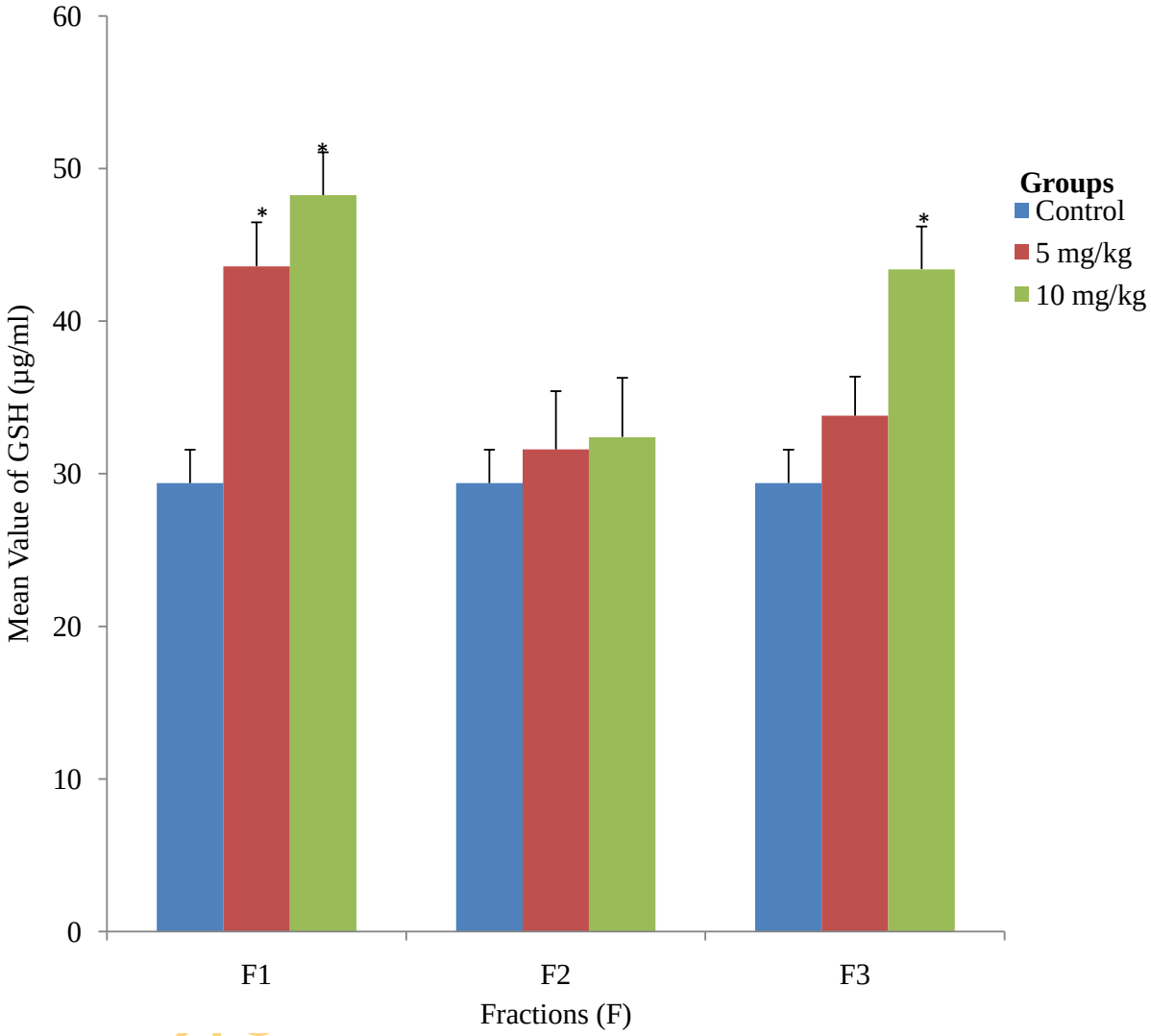


Figure 36: Effects of the fractions of the bulb of *Crinum jagus* on liver reduced glutathione (GSH) activities of rats.

* = Significantly different from control

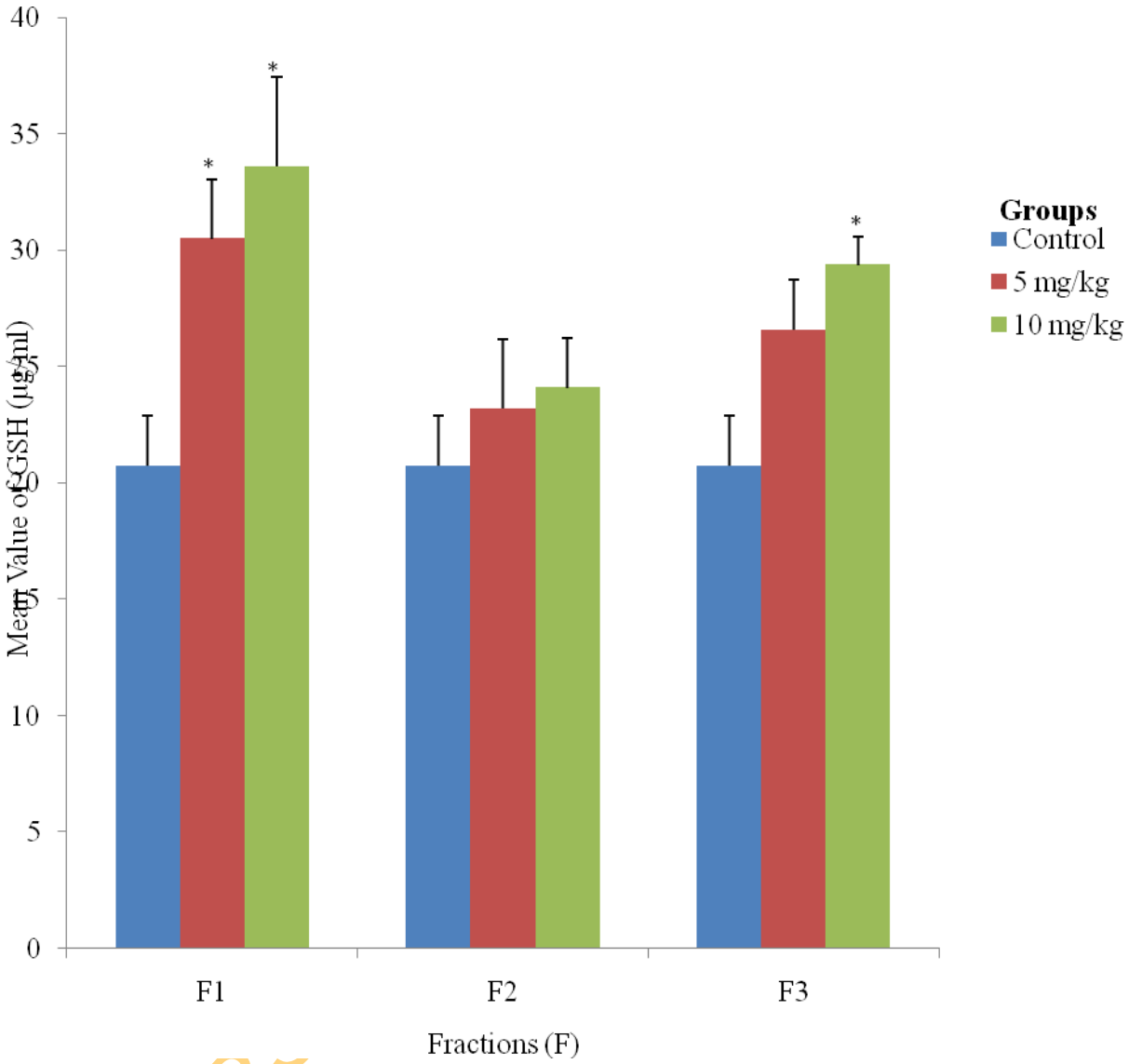


Fig 37 : Effects of the fractions of the bulb of *Crinum jagus* on kidney reduced glutathione (GSH) activities of rats.

* = Significantly different from control

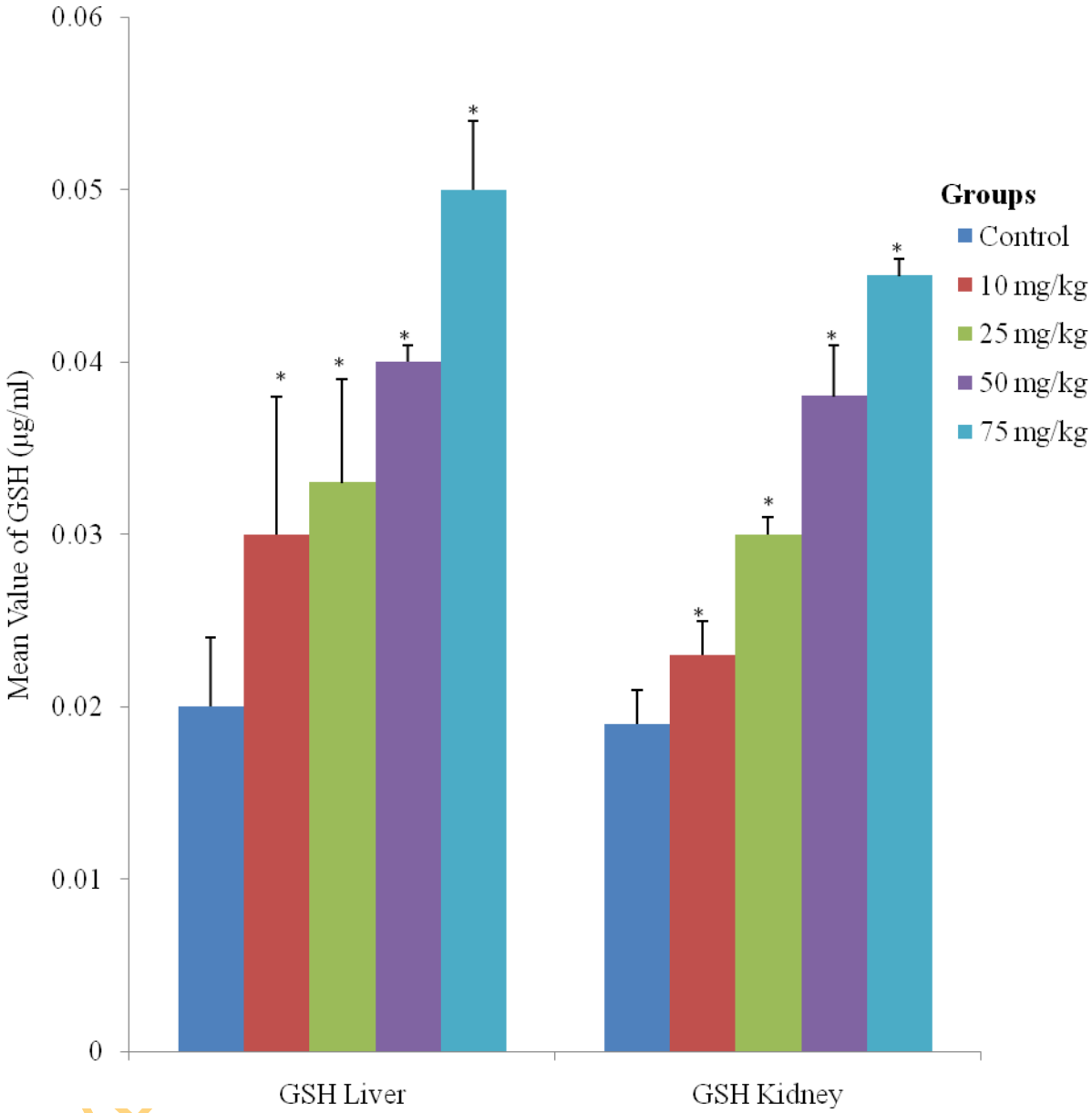


Figure 38 : Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney glutathione – S – transferase (GST) activities of rats.

* = Significantly different from control

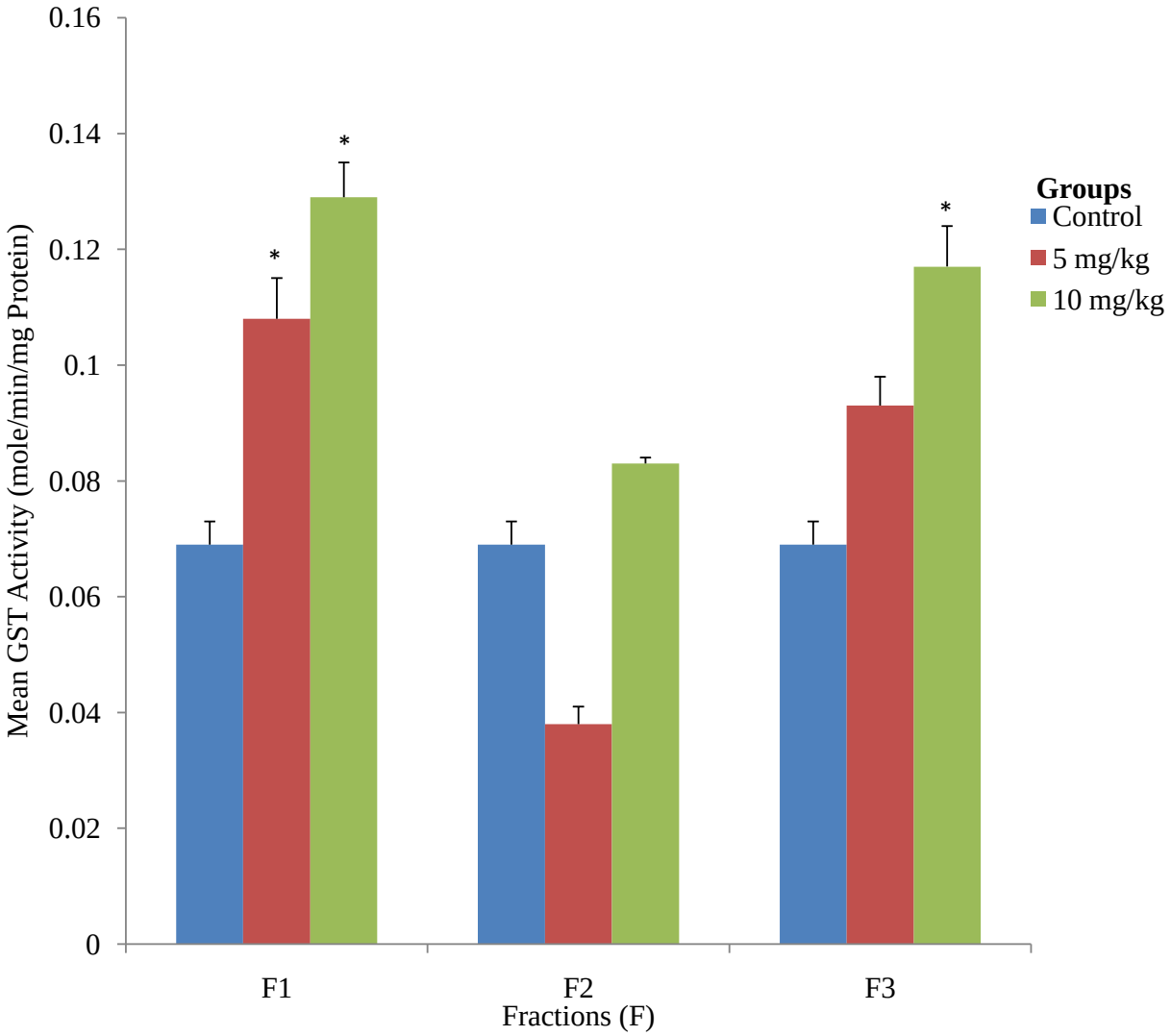


Figure 39: Effects of the fractions of the bulb of *Crinum jagus* on liver glutathione – S – transferase (GST) activities of rats.

* = Significantly different from control

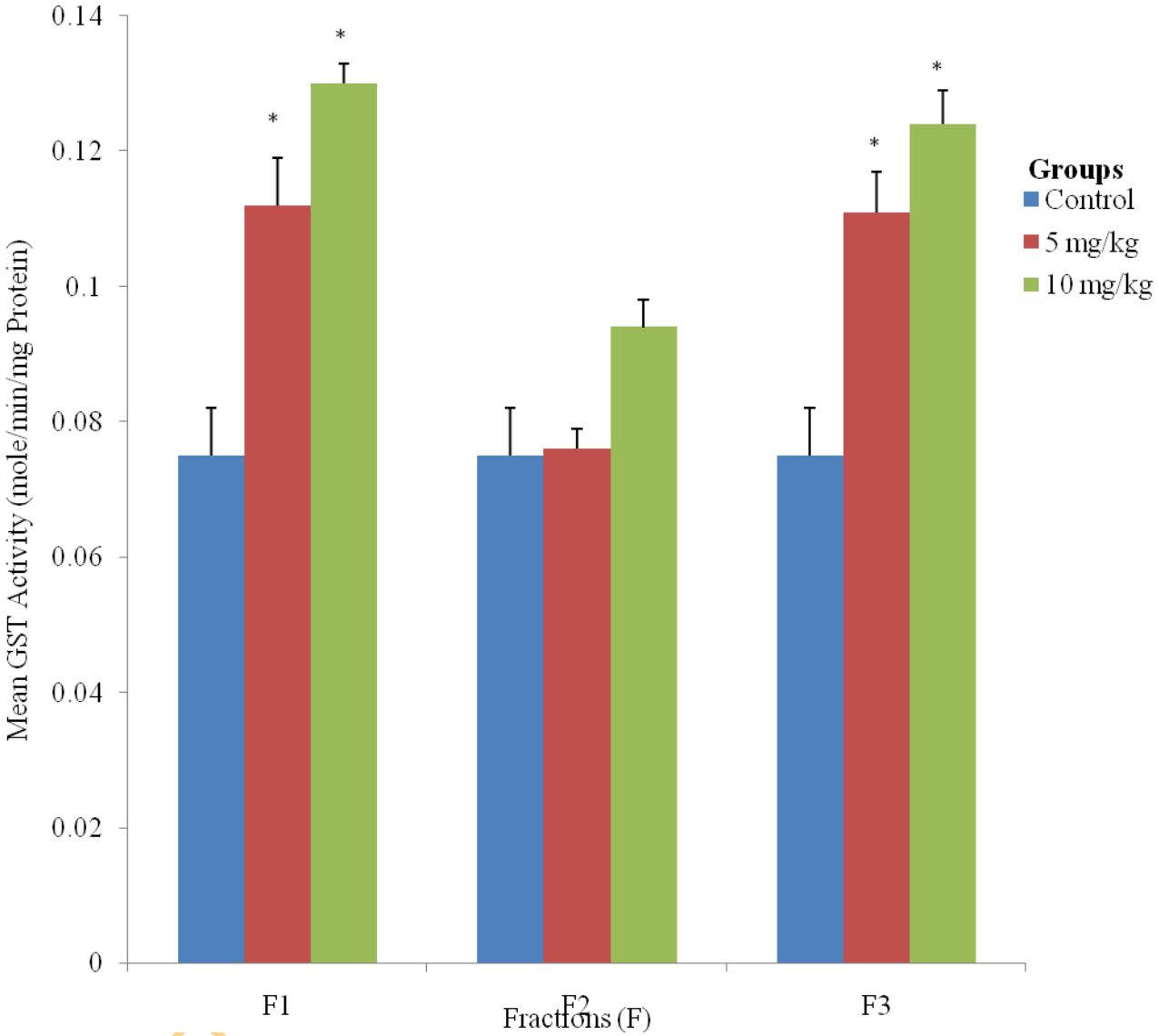


Figure 40: Effects of the fractions of the bulb of *Crinum jagus* on kidney glutathione – S – transferase activities (GST) of rats.

* = Significantly different from control

Experiment 5 : Determination of Antibacterial and Antifungal Activities of the Crude Methanol Extract and Fractions of the Bulb of *Crinum jagus*

Introduction

The expanding bacterial and fungal resistance to antibiotics has become a growing concern, worldwide (Gradam, 2000). Increasing bacterial resistance is prompting a resurgence in research of the antimicrobial role of herbs against resistance strains (Hermaiswarya *et al*.,2008; Alvanio and Alvanio 2009)). A vast number of medicinal plants have been recognised as valuable resources of natural antimicrobial compounds (Mahady, 2005). Plant derived products contain a great diversity of phytochemicals such as phenolic acids, flavonoids, alkaloids, tannins and other small compounds (Cowan, 1999). These compounds possess numerous health related effects such as antibacterial, antifungal, anticarcinogenic activities (Bedlack *et al.*, 2000). With advancement of mordern medicinal technology, it is now easier to identify specific botanical constituents and assess their potential antimicrobial activity hence in this study, the antibacterial and antifungal activities of crude methanol extract and fractions (F1, F2 and F3) of the bulb of *Crinum jagus* against several human pathogens were investigated**.**

Procedure

The antimicrobial activities of the extract/fractions were determined by measuring the inhibition zone using the agar diffusion method (Vollekova *et al*., 2001; Usman *et al*., 2005). Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the extract/fractions were also determined as described in section 3.9 under 'Materials and Methods.'

Results

Table 11 presents the result of the antimicrobial screening of the crude methanol extract of the bulb of *Crinum jagus.* The crude extract exhibited considerable level of inhibition against all the test organisms even at low concentration with the exception of *E.coli*. *Klebsiella pneumoniae* was the most susceptible bacterium of all the tested bacteria with inhibition zones ranging from 10.00 ± 0.20 to 28.00± 0.10 while *Aspergillus niger* was the most susceptible fungus with inhibition zones ranging from 10.00 ± 0.10 to 16.00 ± 0.40. The extract had a good antimicrobial activity on the *Bacillus subtilis* and *Staphylococcus aureus*, the two Gram-

positive bacteria tested. Three out of the four Gram-postive bacteria tested *Pseudomonas aeurigunosa*, *Salmonella typhi* and *Klebsiella pnenumonae* also showed appreciable antimicrobial activity. *E coli* was not inhibited by the extract with no zone of inhibition (Table 11). This is in consonance with the frequent reported cases of developed multi drug resistant to many antibiotics available in the market of which *E.coli* is the most prominent (Alonso *et al.*, 2000; Sader *et al*., 2002; Oyegade and Fasuan, 2004). The difference in the sensitivity between Gram-positive and Gram-negative bacteria could be ascribed to the difference in morphological constitution of these organisms (Nastro *et al*., 2000; Hodges, 2002). The fungal moulds *Aspergillus niger* and *Aspergillus flavos* were much sensitive to the extract than *Candida albicans*, *Candida tropicalis* and *Penicillium notatum.* The inhibition by the plant extract was less than the inhibitory action of ampicillin and tiaconazole the standard antibiotics.

Results of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the extract are presented in Table 12. The crude methanol extract demonstrated good antimicrobial activity with its MIC against Gram-positive bacteria ranging between 6.25 to 50mg/ml and that of Gram-negative bacteria ranged between 3.125 to 50mg/ml. MIC for the fungal isolates ranged between 25 to 200mg/ml. The extract showed the lowest MIC against *Klebsiella pneumonae* (3.125mg/ml). The broadest activity of the crude extract against most of the test bacteria was 12.5mg/ml as MIC. *Candida albicans* and *Candida tropicalis* had the lowest MIC value (25mg/ml) against the extract. The broadest activity of the extract against most fungi tested was MIC of 50mg/ml.

The extract also showed bactericidal and fungicidal activities on the bacteria and fungi isolates. *Klebsiella pneumonae* was the most susceptible bacteria to the plant extract, at a small concentration of 12.5mg/ml, the extract was able to kill the organism. The broadest bactericidal activity of the extract against most of the test bacteria was 25mg/ml as MBC. The extract also showed fungicidal effect on the fungi tested with *Candida albicans* and *Candida tropicalis* being the most susceptible with 50mg/ml as minimum fungicidal concentration (MFC). The broadest activity of the extract against most of the fungi tested was observed at 100mg/ml as MFC.

Tables 13, 14 and 15 represent the antimicrobial activities of each of the fractions of the bulb of *Crinum jagus* with respect of each test organism. The fractions inhibited the growth of all the test organisms, including *E. coli*, which was not inhibited by the crude extract of the plant at concentrations lower than that of the crude extract. The results of MIC determination showed that F1 is the most potent of all the three fractions. F1 had good antimicrobial activity on all the bacteria and fungi isolates tested. The MIC, MBC and MFC values of F1 for bacteria and fungi isolates were much lower than that of F2 and F3. The MIC value of F1 ranged between 0.20 to 3.125µg/ml for bacteria isolates and 0.39 to 3.125µg/ml for the fungi isolates. *Staphylococcus aureus* (a Gram-positive bacteria) was the most susceptible to F1 at MIC value of 0.20µg/ml. The yeasts: *Candida albicans*, *Candida tropicalis* and *Candida krusei* were much sensitive to F1 than the moulds with MIC values of 0.39µg/ml. *E. coli* which was not inhibited by the crude methanol extract of the plant was inhibited by F1 at MIC value of 0.39µg/ml. F1 also showed bactericidal and fungicidal activity on the bacteria and fungi isolates tested. The MBC and MFC values of F1 for bacteria and fungi were much lower than that of F2 (Table 16). F2 had a moderate antibacterial and antifungal activities against most of the test organisms. The lowest MIC value of F2 was 0.39µg/ml against two of the bacteria isolates *Staphylococcus aureus* and *Bacillus subtilis* and the lowest MIC value of 0.78µg/ml were observed against four of the fungi isolates *Candida albicans*, *Candida tropicalis*, *Candida krusei* and *Penicillum notatum*. *Staphylococcus aureus* and *Bacillus subtilis* were the most susceptible bacteria to F2, at a concentration of 0.39µg/ml as MIC which was higher than the MIC value of F1 against *Staphylococcus aureus*. Also the MBC values of F2 for the bacteria and fungi isolates were much higher than that of F1 (Table 17). F3 had a moderate antibacterial and poor antifungal activity. The lowest MIC value obtained for F3 was 0.39µg/ml against *Bacillus subtilis* and *E. coli*, for the fungi isolates, the lowest MIC value obtained was 1.56µg/ml which was much higher than the MIC values of F1 and F2. F3 also had higher MBC values of 0.78µg/ml for the bacteria isolates and MFC value of 1.56µg/ml for the fungal isolates (Table 18) hence F3 will have significant antimicrobial activity against bacteria and fungi at higher concentrations.

Conclusion

From this study, the crude methanol extract of the bulb of *Crinum jagus* demonstrated a broad-sepectrum of activity against Gram-postive and Gram-negative bacteria and fungi tested. Fractionation of the crude extract did not lead to loss of antimicrobial activity against the same group of isolates. The fractions were found to have a better and improved antimicrobial activity than the crude extract against the range of Gram-positive bacteria, Gram-negative bacteria, the fungal yeast and the moulds tested. F1 demonstrated the highest antimicrobial activity.

Table 11 : Antimicrobial activity of the crude methanol extract of the bulb of *Crinum jagus*.

Conc (mg/ml)	*Stap. aureus*	*Bacill. Subtilis*	*Esch. Coli*	*Pseud. aeuriginosa*	*Salm. typhi*	*Kleb. Pneumonae*	*Cand. albicans*	*Cand. Tropicalis*	*Cand. krusei*	*Asp. niger*	*Asp. flavos*	*Penic. Notatum*
200	18.00 ± 0.32	24.00 ± 0.01	-	20.00 ± 0.02	20.00 ± 0.20	20.00 ± 0.10	14.00 ± 0.30	12.00 ± 0.30	12.00± 0.10	16.00 ± 0.40	14.00± 0.30	12.00± 0.01
100	16.00 ± 0.20	20.00 ± 0.20	-	12.00 ± 0.40	16.00 ± 0.10	18.00 ± 0.41	10.00 ± 0.40	10.00 ± 0.20	10.00± 0.20	12.0± 0.20	12.00± 0.01	10.00± 0.10
50	14.00 ± 0.10	18.00 ± 0.06	-	10.00 ± 0.42	14.00± 0.10	16.00 ± 0.60	-	-	-	10.00 ± 0.10	10.00± 0.10	-
25	12.00 ± 0.01	16.00 ± 0.10	-	-	12.00± 0.30	14.00 ± 0.30	-	-	-	-	-	-
12.5	10.00 ± 0.12	14.00 ± 0.20	-	-	-	12.00 ± 0.22	-	-	-	-	-	-
6.25	6.00 ± 0.01	0.80 ± 0.02	-	-	-	10.00 ± 0.20	-	-	-	-	-	-
Ampicillin (10mg/ml)	50.00 ± 0.42	28.00 ± 0.10	36.00 ± 0.10	34.00 ± 0.30	24.00 ± 0.20	36.00 ± 0.20	-	-	-	-	-	-
Tiaconazole (10% w/v)	-	-	-	-	-	-	24.00 ± 0.10	20.00 ± 0.10	22.00± 0.10	24.00 ± 0.10	20.00± 0.10	22.00 ± 0.20
Methanol	-	-	-	-	-	-	-	-	-	-	-	-

Values represent diameter of zone of inhibition (mm)

- means no inhibition

Table 12 : Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentrations (MFC) of the crude methanol extract of the bulb of C*rinum jagus*

Microorganism	Type	MIC (mg/ml)	MBC/MFC (mg/ml)
Staphylococcus aureus	Bacteria (G+)	6.25	25.00
Bacillus subtilis	Bacteria (G+)	6.25	25.00
Escherichia coli	Bacteria (G-)	–	–
Pseudomonas aeruginosa	Bacteria (G-)	50.00	100
Salmonellae typhi	Bacteria (G-)	12.50	25.00
Kelbsiella pneumonae	Bacteria (G-)	3.125	12.50
Candida albicans	Yeast	25.00	50.00
Candida tropicals	Yeast	25.00	50.00
Candida krusei	Mould	50.00	200
Penicillum Notatum	Mould	50.00	200
Aspergillus niger	Mould	50.00	200
Aspergillus flavos	Mould	100	200

MIC = Minimum Inhibitory Concentration , MBC = Minimum Bactericidal Concentration , MFC = Minimum Fungicidal Concentration, G+ = Gram-positive, G - = Gram-negative.

- means no inhibition

Table 13 : Antimicrobial activity of fraction 1 (F1) of the bulb of *Crinum jagus*

Conc (µg/ml)	*Stap. Aureus*	*Bacill. subtilis*	*Esch. Coli*	*Pseud. Aeuruginosa*	*Salm. typhi*	*Kleb. pneumonae*	*Cand. albicans*	*Cand. tropicalis*	*Cand. Krusei*	*Asp Niger*	*Asp. flavos*	*Penic. Notatum*
25	20.00 ± 0.42	24.00 ± 0.32	18.00 ±0.01	20.00 ± 0.02	24.00 ± 0.30	18.00 ± 0.36	18.00 ± 0.36	16.00 ± 0.31	16.00± 0.10	16.00 ± 0.20	20.00± 0.31	14.00± 0.30
12.5	18.00 ± 0.25	18.00 ± 0.25	14.000 .20	12.00 ± 0.40	18.00 ± 0.20	16.00 ± 0.21	16.00 ± 0.43	14.00 ± 0.20	14.00± 0.30	12.00± 0.20	14.00± 0.20	12.00± 0.10
6.25	16.00 ± 0.20	12.00 ± 0.10	12.00 ± 0.12	10.00 ± 0.42	16.00 ± 0.40	14.00 ± 0.30	14.00 ±0.26	12.00 ± 0.10	12.00 ± 0.20	10.00 ± 0.10	12.00± 0.49	10.00± 0.42
3.125	14.00 ± 0.12	12.00 ± 0.41	10.00 ± 0.02	-	12.00 ± 0.10	12.00 ± 0.15	12.00±0. 31	10.00 ± 0.35	10.00± 0.24	-	10.00± 0.01	-
1.56	12.00 ± 0.11	10.00 ± 0.20	-	-	-	-	10.00 ± 0.01	-	-	-	-	-
0.78	10.00 ± 0.50	-	-	-	-	-	-	-	-	-	-	-
Ampicillin (10µg/ml)	28.00 ± 0.10	26.00 ± 0.10	28.00 ± 0.10	24.00 ± 0.20	30.00 ± 0.20	22.00 ± 0.25	-	-	-	-	-	-
Tiaconazole (10% w/v)	-	-	-	-	-	-	26.00 ± 0.10	24.00 ± 0.10	26.00± 0.20	24.00 ± 0.20	24.00± 0.15	26.00 ± 0.30
Methanol	-	-	-	-	-	-	-	-	-	-	-	-

Values represent diameter of zone of inhibition (mm)

- means no inhibition

Table 14 : Antimicrobial activity of fraction 2 (F2) of the bulb of C*rinum jagus*

Conc (µg/ml)	*Stap. Aureus*	*Bacill. subtilis*	*Esch. coli*	*Pseud. aeuriginosa*	*Salm. typhi*	*Kleb. pneumonae*	*Cand. albicans*	*Cand. tropicalis*	*Cand. Krusei*	*Asp niger*	*Asp. flavos*	*Penic. Notatum*
25	24.00 ± 0.02	24.00 ± 0.40	16.00 ±0.24	16.00 ± 0.40	16.00 ± 0.32	22.00 ± 0.36	18.00 ± 0.41	16.00 ± 0.20	18.00± 0.32	18.00 ± 0.30	16.00± 0.50	18.00± 0.20
12.5	18.00 ± 0.10	18.00 ± 0.18	12.000 .30	14.00 ± 0.50	12.00 ± 0.20	16.00 ± 0.31	14.00 ± 0.10	12.00 ± 0.10	14.00± 0.20	10.00± 0.10	12.00± 0.44	14.00± 0.13
6.25	14.00 ± 0.25	14.00 ± 0.15	10.00 ± 0.18	12.00 ± 0.30	10.00 ± 0.10	14.00 ± 0.24	12.00 ±0.26	12.00 ± 0.10	12.00 ± 0.40	-	10.00± 0.35	10.00± 0.25
3.125	12.00 ± 0.20	12.00 ± 0.20	-	10.00 ± 0.10	-	12.00 ± 0.15	10.00±0.36	10.00 ± 0.01	10.00± 0.10	-	-	-
1.56	10.00 ± 0.10	10.00 ± 0.30	-	-	-	-	-	-	-	-	-	-
0.78	10.00 ± 0.50	-	-	-	-	-	-	-	-	-	-	-
Ampicillin (10µg/ml)	28.00 ± 0.10	26.00 ± 0.10	28.00 ± 0.10	24.00 ± 0.20	30.00 ± 0.20	22.00 ± 0.25	-	-	-	-	-	-
Tiaconazole (10% w/v)	-	-	-	-	-	-	26.00 ± 0.10	24.00 ± 0.10	26.00± 0.20	24.00 ± 0.20	24.00± 0.15	26.00 ± 0.30
Methanol	-	-	-	-	-	-	-	-	-	-	-	-

Values represent diameter of zone of inhibition (mm)

- means no inhibition

Table 15 : Antimicrobial activity of fraction 3 (F3) of the bulb of *Crinum jagus*

Conc (µg/ml)	*Stap. Aureus*	*Bacill. subtilis*	*Esch. coli*	*Pseud. aeuriginosa*	*Salm. typhi*	*Kleb. pneumonae*	*Cand. albicans*	*Cand. tropicalis*	*Cand. Krusei*	*Asp niger*	*Asp. flavos*	*Penic. Notatum*
25	20.00 ± 0.02	18.00 ± 0.30	18.00 ±0.10	14.00 ± 0.25	16.00± 0.32	18.00 ± 0.10	18.00 ± 0.30	16.00 ± 0.10	14.00± 0.30	16.00 ± 0.25	14.00± 0.30	14.00± 0.20
12.5	14.00 ± 0.12	16.00 ± 0.20	16.000 .20	12.00 ± 0.20	14.00 ± 0.28	14.00 ± 0.15	14.00 ± 0.25	14.00 ± 0.15	12.00± 0.25	12.00± 0.20	14.00± 0.20	12.00± 0.16
6.25	12.00 ± 0.11	14.00 ± 0.22	14.00 ± 0.25	10.00 ± 0.20	12.00 ± 0.20	12.00 ± 0.20	12.00 ±0.10	12.00 ± 0.10	10.00 ± 0.20	10.00 ± 0.15	10.00± 0.20	10.00± 0.15
3.125	10.00 ± 0.15	12.00 ± 0.20	12.00 ± 0.20	-	10.00 ± 0.18	10.00 ± 0.18	10.00±0. 24	10.00 ± 0.12	10.00 ± 0.24	-		-
1.56	-	10.00 ± 0.15	10.0± 0.10	-	-	-	-	-	-	-	-	-
0.78	-	-	-	-	-	-	-	-	-	-	-	-
Ampicillin (10µg/ml)	28.00 ± 0.10	26.00 ± 0.10	28.00 ± 0.10	24.00 ± 0.20	30.00 ± 0.20	22.00 ± 0.25	-	-	-	-	-	-
Tiaconazole (10%w/v)	-	-	-	-	-	-	26.00 ± 0.10	24.00 ± 0.10	26.00± 0.20	24.00 ± 0.20	24.00± 0.15	26.00 ± 0.30
Methanol	-	-	-	-	-	-	-	-	-	-	-	-

Values represent diameter of zone of inhibition (mm)

- means no inhibition

Table 16 : Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of fraction 1 (F1) of the bulb of *Crinum jagus*

Microorganism	Type	MIC (µg/ml)	MBC/MFC (µg/ml)
Staphylococcus aureus	Bacteria (G+)	0.20	0.39
Bacillus subtilis	Bacteria (G+)	0.39	0.39
Escherichia coli	Bacteria (G-)	0.39	0.78
Pseudomonas aeruginosa	Bacteria (G-)	0.39	0.78
Salmonellae typhi	Bacteria (G-)	0.39	0.78
Kelbsiella pneumonae	Bacteria (G-)	0.39	0.78
Candida albicans	Yeast	0.39	0.78
Candida tropicals	Yeast	0.39	0.78
Candida krusei	Mould	0.39	0.78
Penicillum Notatum	Mould	0.78	1.56
Aspergillus niger	Mould	0.78	1.56
Aspergillus flavos	Mould	0.78	1.56

MIC = Minimum Inhibitory Concentration , MBC = Minimum Bactericidal Concentration , MFC = Minimum Fungicidal Concentration, G+ = Gram-positive, G - = Gram-negative.

Table 17 : Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of fraction 2 (F2) of the bulb of *Crinum jagus*

Microorganism	Type	MIC (µg/ml)	MBC/MFC(µg/ml)
Staphylococcus aureus	Bacteria (G+)	0.39	0.78
Bacillus subtilis	Bacteria (G+)	0.39	0.78
Escherichia coli	Bacteria (G-)	0.78	0.78
Pseudomonas aeruginosa	Bacteria (G-)	0.78	0.78
Salmonellae typhi	Bacteria (G-)	0.78	1.56
Klebsiella pneumonae	Bacteria (G-)	0.78	1.56
Candida albicans	Yeast	0.78	1.56
Candida tropicals	Yeast	0.78	1.56
Candida krusei	Mould	0.78	1.56
Penicillum Notatum	Mould	0.78	1.56
Aspergillus niger	Mould	1.56	1.56
Aspergillus flavos	Mould	1.56	1.56

MIC= Minimum inhibitory concentration, MBC = Minimum bactericidal concentration, MFC = Minimum fungicidal concentration, G + = Gram- positive, G - = Gram-negative.

Table 18 : Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of fraction 3 (F3) of the bulb of *Crinum jagus*.

Microorganism	Type	MIC(µg/ml)	MBC/MFC(µg/ml)
Staphylococcus aureus	Bacteria (G+)	0.78	1.56
Bacillus subtilis	Bacteria (G+)	0.78	0.78
Escherichia coli	Bacteria (G-)	0.78	0.78
Pseudomonas aeruginosa	Bacteria (G-)	0.78	1.56
Salmonellae typhi	Bacteria (G-)	0.78	1.56
Kelbsiella pneumonae	Bacteria (G-)	0.78	1.56
Candida albicans	Yeast	1.56	1.56
Candida tropicals	Yeast	1.56	1.56
Candida krusei	Mould	1.56	1.56
Penicillum Notatum	Mould	1.56	1.56
Aspergillus niger	Mould	1.56	1.56
Aspergillus flavos	Mould	1.56	1.56

MIC = Minimum inhibitory concentration, MBC = Minimum bactericidal concentration , MFC = Minimum fungicidal concentration , G+ = Gram-positive G - = Gram-negative.

Experiment 6: Determination of Anti-inflammatory Activity of the Crude Methanol Extract and Fractions of the Bulb Of *Crinum jagus*

Introduction

Drugs presently in use for the management of pain and inflammatory conditions are either narcotic e.g opoids or non narcotic e.g salicyclate and corticosterioids e.g hydrocortisone. All of these drugs present well known side and toxic effects. It is well documented that these non - steroidal anti-inflammatory drugs (NSAIDs) produced intestinal tract ulcer with potential internal bleeding in 10-30 % of long term users, and erosion of the stomach lining and intestinal tract in 30-50 % of cases (Hayliyar *et al*., 1992). As a result of these side effects, the use of NSAIDs is associated with 10,000 to 20,000 deaths per year. (Ament and Childers, 1997). Even the new cyclooxygenase (COX-2) inhibitor has only been reported to reduce intestinal tract damage by 50% and their toxicity to the liver and kidney is still under review. *Crinum jagus* is reported in traditional literature for the treatment of snake bite (Ode *et al.*, 2006), tuberculosis (Idu *et al.*, 2010), and asthma cough (Ogunkunle and Olopade, 2011) which are all inflammatory diseases, therefore the present research has been undertaken to investigate the anti-inflammatory activity of the crude extract and fractions of the plant.

Procedure

The antinflammatory activity was determined by carrageenan induced rat paw oedema method (Winter *et al*., 1962; Perianayagam *et al*., 2006) as described in section 3.10 under 'Materials and Methods'.

Results

Figure 41 presents the result of the anti-inflammatory activity of the crude methanol extract of the bulb of *Crinum jagus*. From the result it was observed that crude methanol extract of the plant and indomethacin significantly ($P<0.05$) reduced the paw oedema 3 hours after carrageenan injection compared with control. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) significantly ($P< 0.05$) inhibited oedema formation in the rat paw in a dose-dependent manner by 26,82%, 31.55%, 41.82% and 65.90%, respectively. The standard

drug indomethacin at 5mg/kg inhibited oedema formation by 94.82%. The extract at varying concentrations had lower potency than the indomethacin, the standard drug.

The result of the anti-inflammatory activity of the fractions of the bulb of *Crinum jagus* is presented in Figure 42. All the three fractions (F1, F2 and F3) and indomethacin significantly ($P < 0.05$) reduced paw oedema, 3 hours after carrageenan injection relative to control. At a dose level of 20mg/kg, F1, F2 and F3 significantly ($P < 0.05$) inhibited oedema formation in rats by 79.50%, 25.00% and 52.27%, respectively. The result showed that F1 and F3 were more potent as an anti-inflammatory agents than the crude extract and F2. F1 had the highest inhibitory activity (79.50%) which compared well with indomethacin the standard drug with inhibitory activity of 94.82%.

Conclusion

From the study, the crude methanol extract and fractions of *Crinum jagus* bulb was found to have impressive *in vivo* anti-inflammatory activity in experimental animals since the plant extract and its fractions significantly reduced the formation of oedema induced by carrageenan. Anti-inflammatory properties of the plant was not lost during fractionation of the crude extract but fractionation improved the anti-inflammatory activity of the plant. Two of the fractions, F1 and F3 had impressive anti-inflammatory activity.

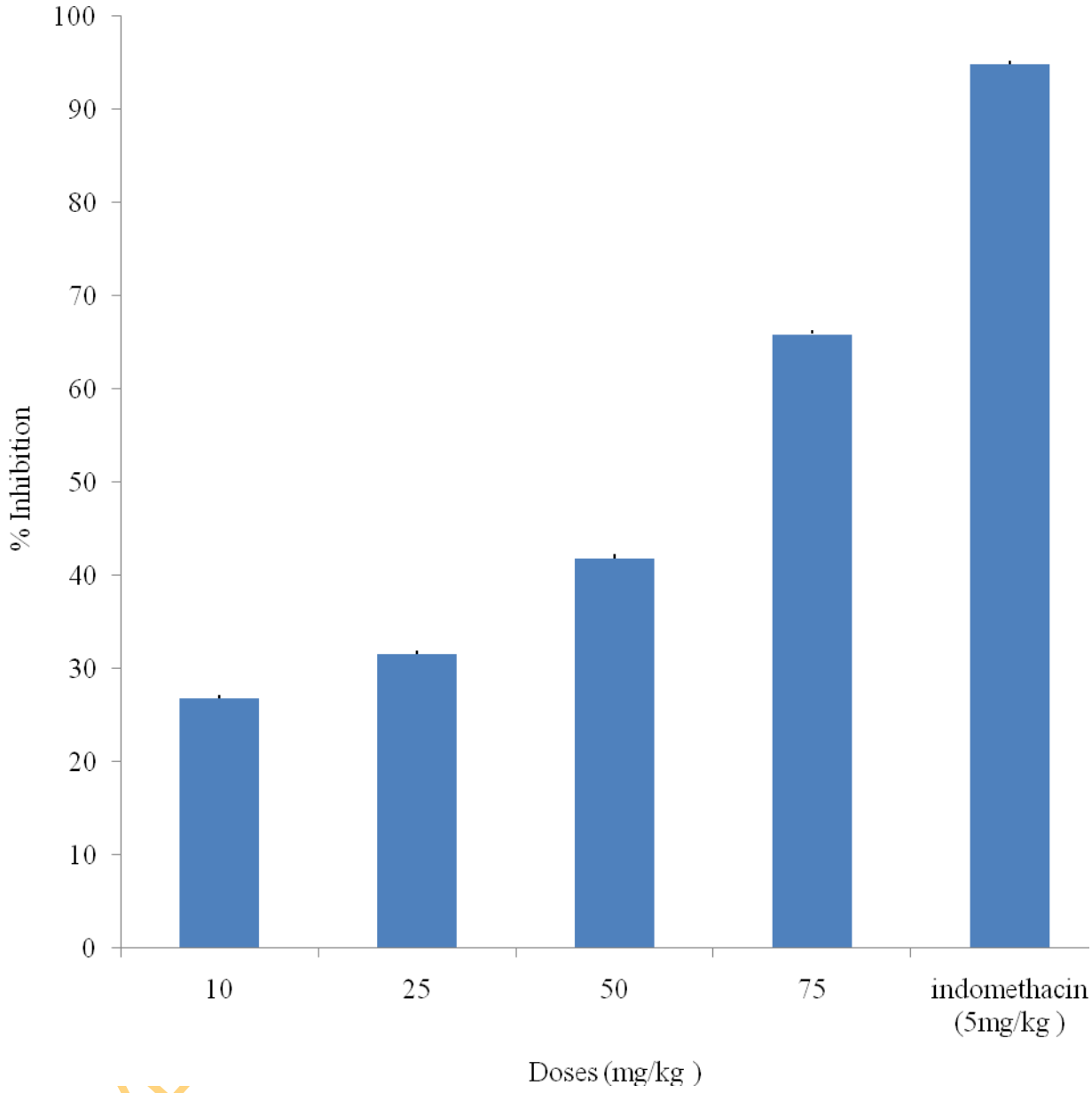


Figure 41: Anti–inflammatory activity of the crude methanol extract of the bulb of *Crinum jagus.*

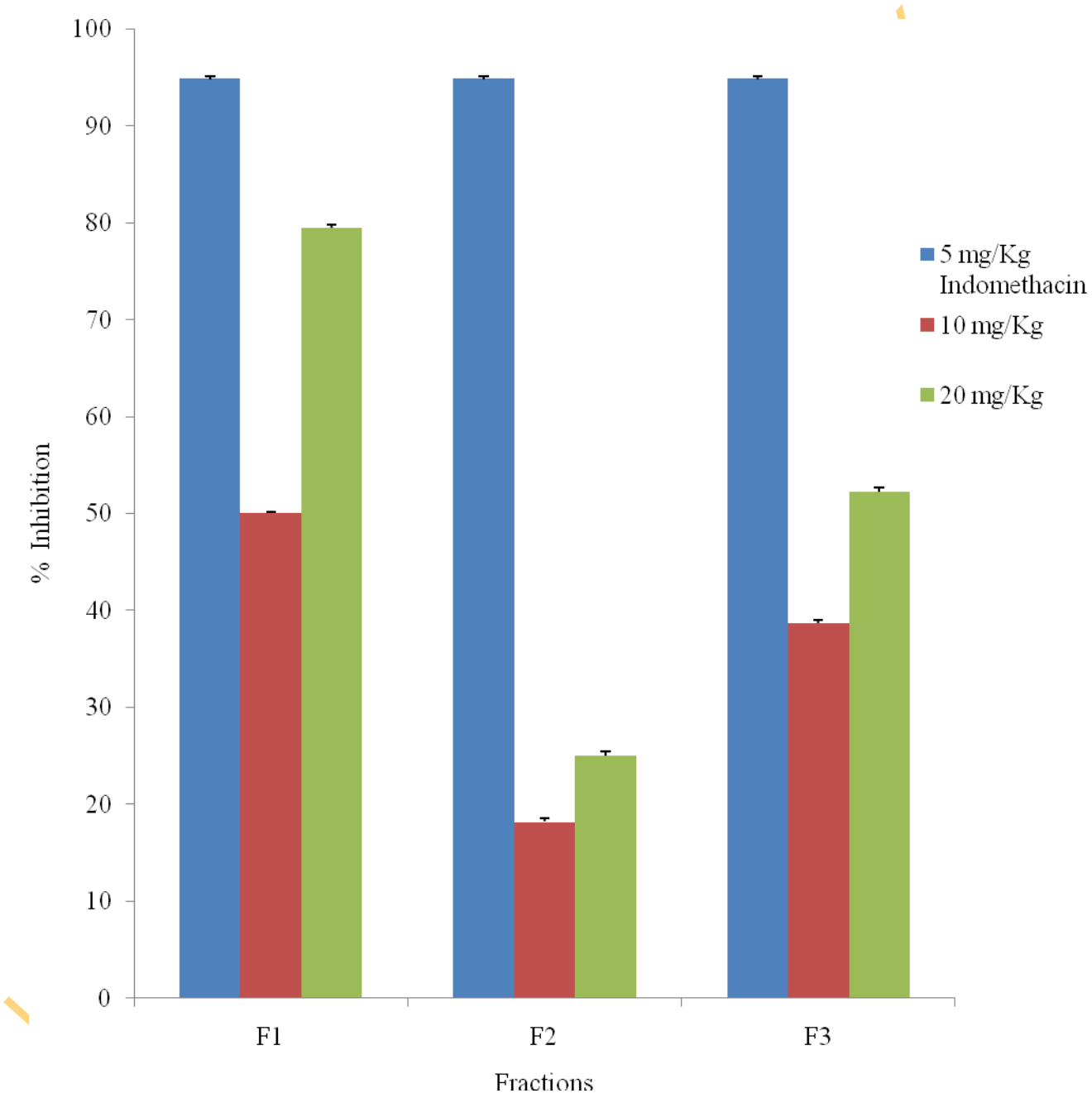


Figure 42: Anti–inflammatory activities of the fractions (F) of the bulb of *Crinum jagus*

F1 , F2 & F3 : $p > 0.05$

Experiment 7 : *In vivo* Antimalaria Activity of the Crude Methanol Extract and Fractions of the Bulb of *Crinum jagus*

Introduction

Malaria is one of the oldest recorded diseases in the world. It is estimated that more than 300 million people are infected annually and over one million deaths have been recorded in children under five years (Ramazani *et al*., 2010; Zofou *et al.*, 2011). The re-emergence of malaria in many parts of the world is due to the rapid increase of resistance of vectors to insecticides (Ridley, 2002; Zirihi *et al.*, 2005). Drug resistant strains of *Plasmodium falciparum* have been found in many endemic areas of the world and many of conventional antimalaria drugs have been associated with treatment failure. As such, there is need for continuous search for newer drugs that can retard or reverse this resistance. Since many mordern drugs such as quinine and artermisinin originate from plants, it is essential that other medicinal plants which have folkore reputation for antimalaria properties are investigated in order to establish their safety and efficacy and to determine their potential as source of new antimalaria drugs (Gester *et al.*,1994). This has led to attempts to discover other antimalaria agents mainly from plant sources.

. This study was prompted by an ethnobotanical survey that reported that *Crinum jagus* is one of the plants used traditionally for the treatment of malaria in some parts of Nigeria (Osakwe *et al.*, 2011). Therefore, in this study the *in vivo* antimalaria activity of the crude extract and fractions of the plant were investigated to give scientific proof and its potential to be developed as an antimalarial drug.

Procedure

The established infection method of Ryley and Peter, (1970) was used to evaluate the antiplasmodial activity of the crude extract and fractions of *Crinum jagus* in *Plasmodium berghei* infected mice as described in section 3.11 under 'Materials and Methods'. Blood was collected into the capillary tube to determine the packed cell volume (PCV). The body weight of the mice were measured to observe whether the plant extract prevented the weight loss that is common with increasing parastaemia in infected mice.

Results

The effect of the crude methanol extract and fractions of the bulb of *Crinum jagus* on established infection are presented in Tables 18, Figures 43 and 44. Treatment of the *Plasmodium berghei* infected mice with the extract and the fractions resulted in a daily reduction in parasitaemia level in the extract/fractions treated groups similar to that of chloroquine and arteether groups and these reductions were dose dependent. Animals in the control group which were infected but not treated showed a gradual daily increase in parasitaemia level. By day 6 post infection, there was significant reduction ($P < 0.05$) in percentage parasitaemia in the treated groups relative to the control group (tween 80), the percentage parasitaemia in the groups treated with varying concentrations of the crude extract of the plant (10, 25, 50 and 75mg/kg) were 4.99%, 4.46%, 3.95% and 2.29% respectively while the percentage parasitaemia of 2.13% and 18.72% were recorded for arteether treated and negative control groups, respectively. By day 6, there was a complete clearance of parasitaemia by chloroquine. The groups treated with 10mg/kg of F1, F2 and F3 had percentage parasitaemia of 2.00%, 4.47% and 3.50%, respectively. When treatment was withdrawn (after day 6 post infection), there was increase in the parasitaemia level in all the treated groups due to recrudesence of parasitaemia with the exception of chloroquine treated group. By day 6 post infection, there was complete clearance of parasitaemia in the chloroquine treated group. By day 6 post infection, the crude extract at different doses administered (10, 25, 50 and 75mg/kg) produced chemosuppression of 70.10%, 76.17%, 78.90% and 87.17% respectively while F1, F2 and F3 at 10mg/kg had chemosuppression of 89.3%, 76.12% and 77.70%, respectively. The standard drugs chloroquine (10mg/kg) and arteether (3mg/kg) caused 100% and 88.60% suppression respectively.

Mean survival time (MST) of 25 and 23 days respectively were observed for chloroquine and arteether treated groups compared to 19, 20, 21 and 22 days respectively observed in the groups treated with 10, 25, 50 and 75mg/kg of the crude extract. F1, F2 and F3 had a mean survival time of 25, 21 and 21 days, respectively. The untreated control groups survived for only 12.5 days (Table 19).

The packed cell volume (PCV) of parasite infected untreated animals decreased progressively until they all died while the PCV of chloroquine treated group increased progressively. During the 4 days treatment, the PCV of all the treated groups increased progressively. But when treatment was withdrawn, increase in PCV was only observed in chloroquine treated group. In arteether, crude extract and fractions treated groups, there was a decrease in PCV due to recrudesence of parasitaemia observed in the arteether, extract and the fractions treated groups (Fig 45 and 46).

Conclusion

It can be concluded that the crude methanol extract and the fractions of the bulb of *Crinum jagus* possess considerable antiplasmodial activities as shown by chemosuppression of parasitaemia and prolongation of life span of the infected mice treated with the extract and the fractions of the plant. Fractionation of the extract enhanced the antimalarial activity of the plant with F1 demonstrating the highest antiplasmodial activity.

Table 19 : Suppressive activities of the crude extract and fractions of the bulb of *Crinum jagus* on established *Plasmodium berghei* infection in mice

Treatment	Dose (mg/kg)	Average % Parasitaemia (Day 3)	Average % Parasitaemia (Day 6)	% Suppression
Crude extract	10	5.38±0.34	4.99±0.20*	70.10
Crude extract	25	5.07±0.48	4.46±0.48*	76.17
Crude extract	50	5.23±0.30	3.95±0.55*	78.90
Crude extract	75	5.40±0.30	2.29±0.20*	87.17
Fraction 1	10	5.31±0.36	2.00±0.35*	89.33
Fraction 2	10	5.38±0.31	4.47±0.51*	76.12
Fraction 3	10	5.31±0.42	3.50±0.53*	77.70
Arteether	3	5.12±0.39	2.13±0.30*	88.60
Chloroquine	10	5.22±0.38	0.00	100
Control (Tween 80)	0.3ml	5.21±0.54	18.72±1.50	-

Values are expressed as mean ± SD, n = 8, *P < 0.05

Table 20 : Mean survival time of mice treated with various doses of crude extract and fractions of the bulb of *Crinum jagus* on established *Plasmodium berghei* infection.

Treatment	Dose (mg/kg)	Mean survival time (Days)
Crude extract	10	19
Crude extract	25	20
Crude extract	50	21
Crude extract	75	22
Fraction 1	10	25
Fraction 2	10	21
Fraction 3	10	21
Arteether	3	23
Chloroquine	10	25
Control (Tween 80)	0.3ml	12.5

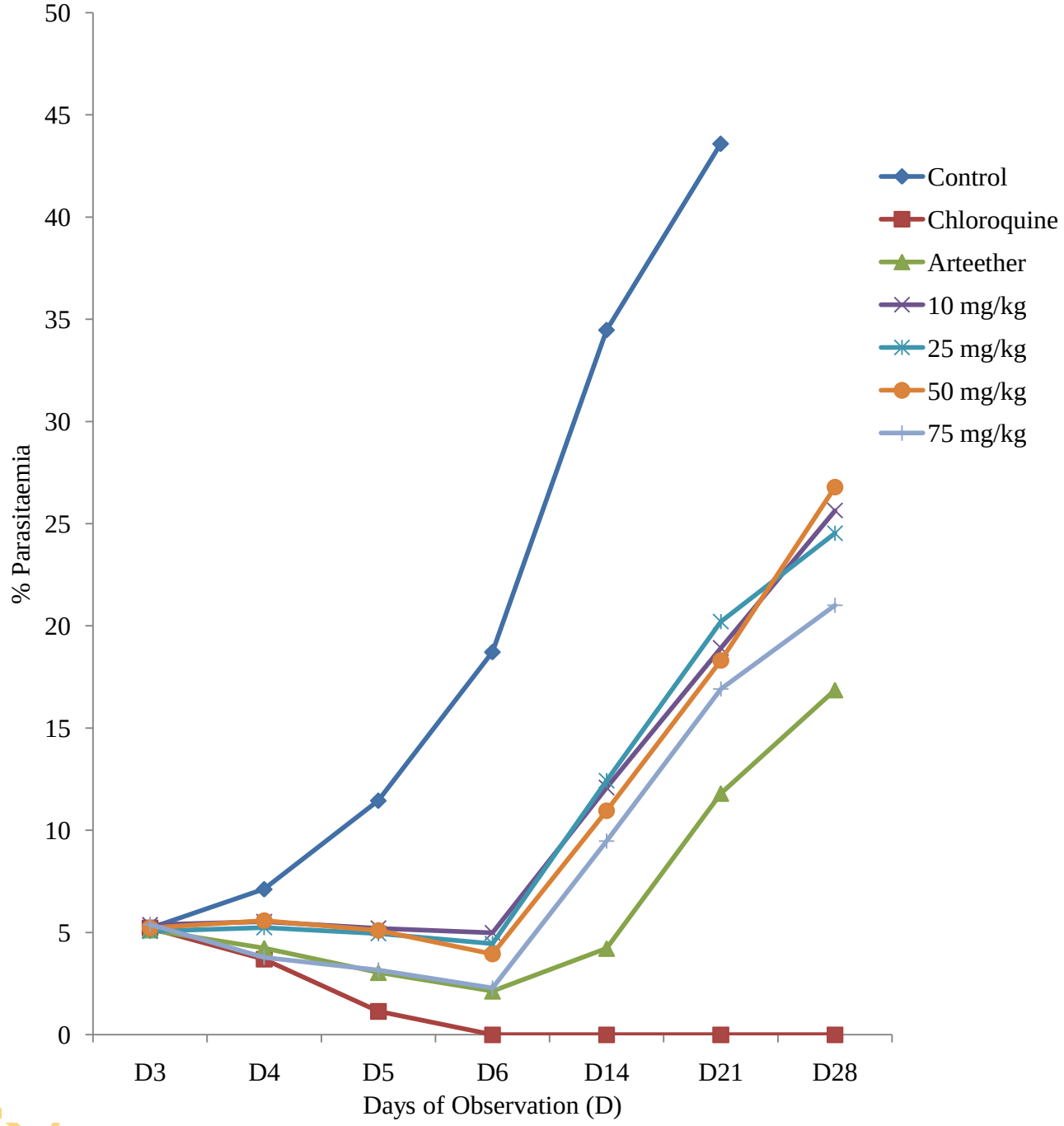


Figure 43: Antimalaria activity of the crude methanol extract of the bulb of *Crinum jagus* on established *Plasmodium berghei* infection in mice.

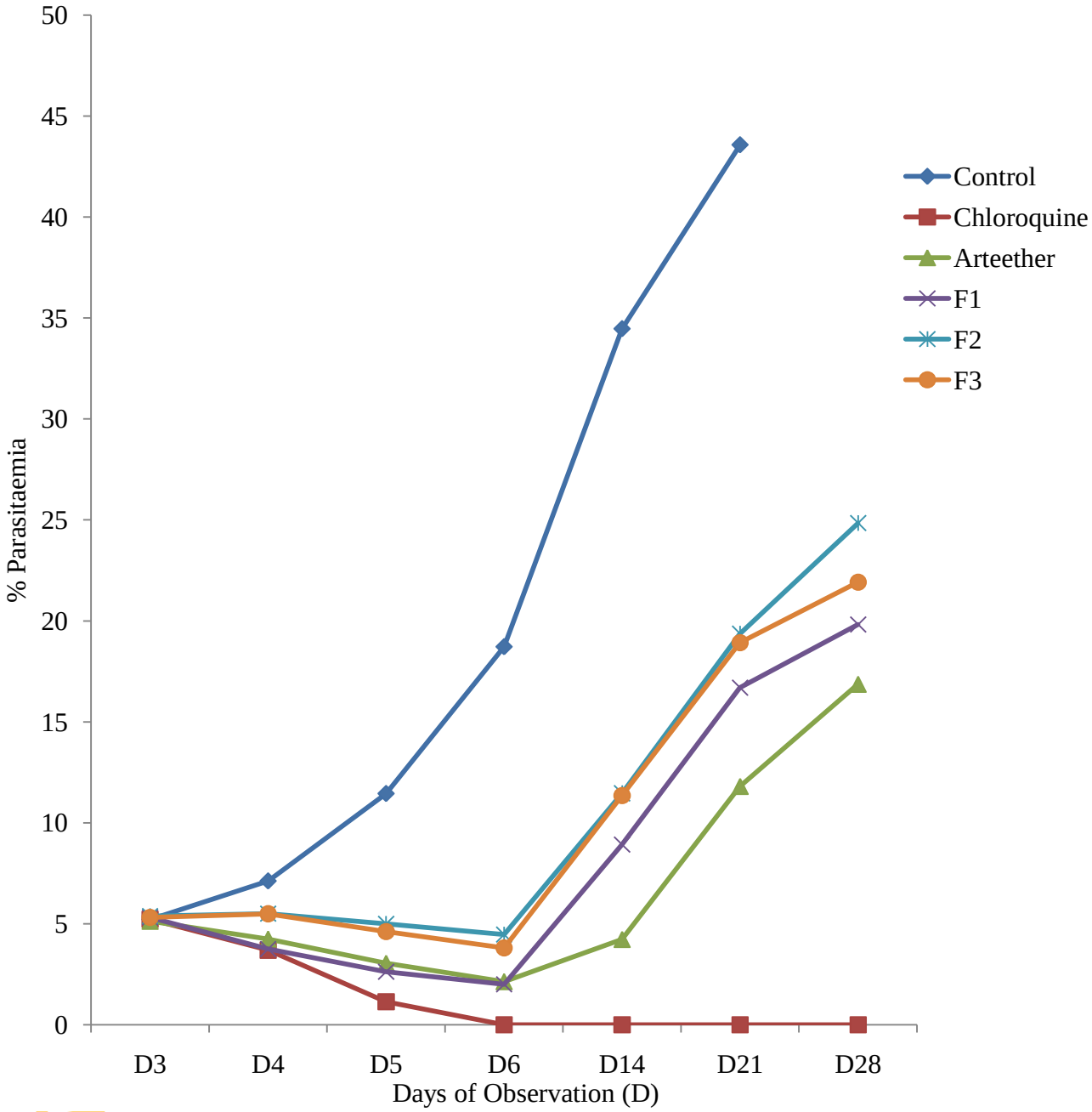


Figure 44: Antimalaria activity of the fractions (F) of the bulb of *Crinum jagus* on established *Plasmodium berghei* infection in mice.

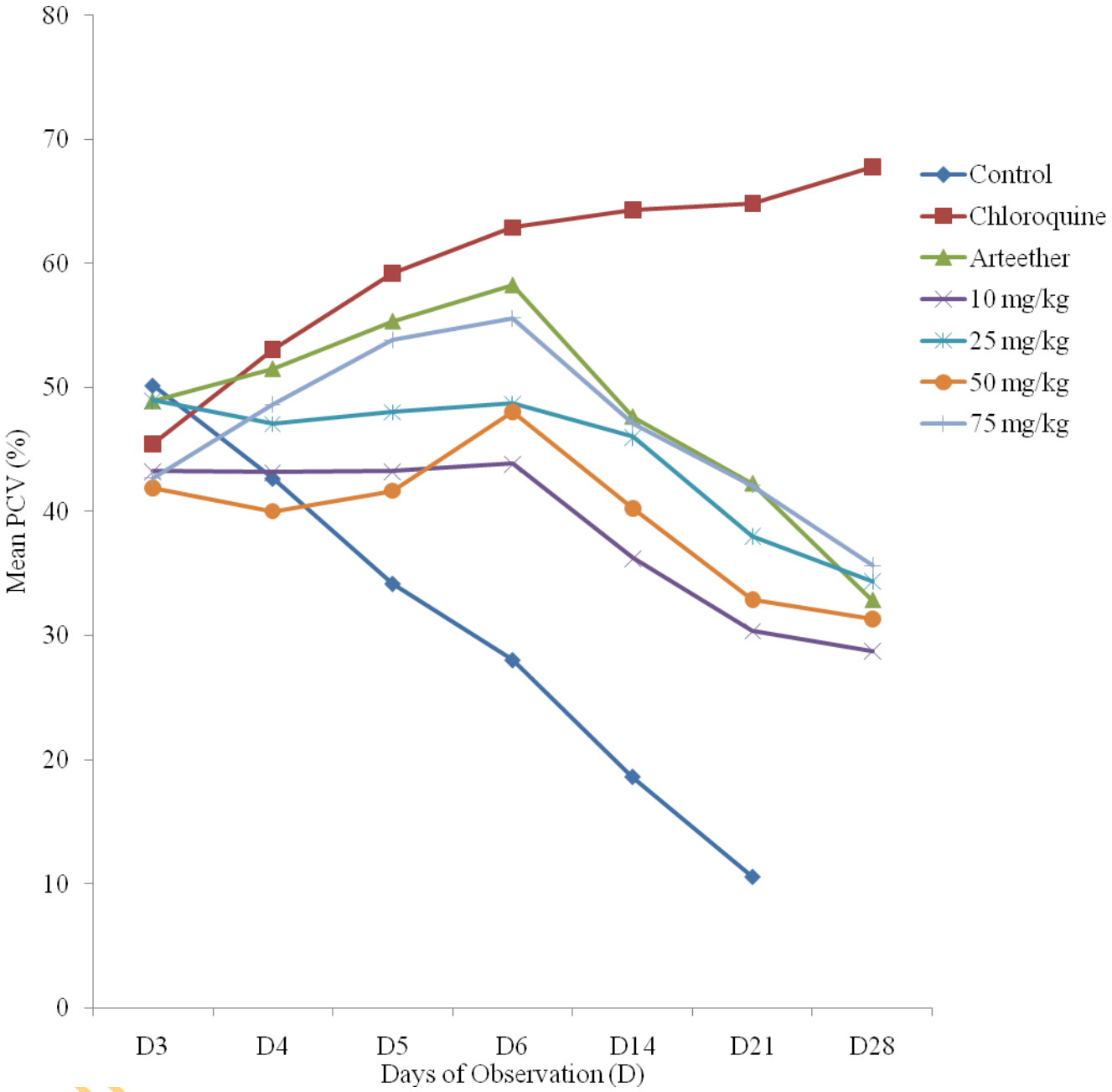


Figure 45: Effect of the crude methanol extract of the bulb of *Crinum jagus* on packed cell volume (PCV) of mice infected with *Plasmodium berghei*.

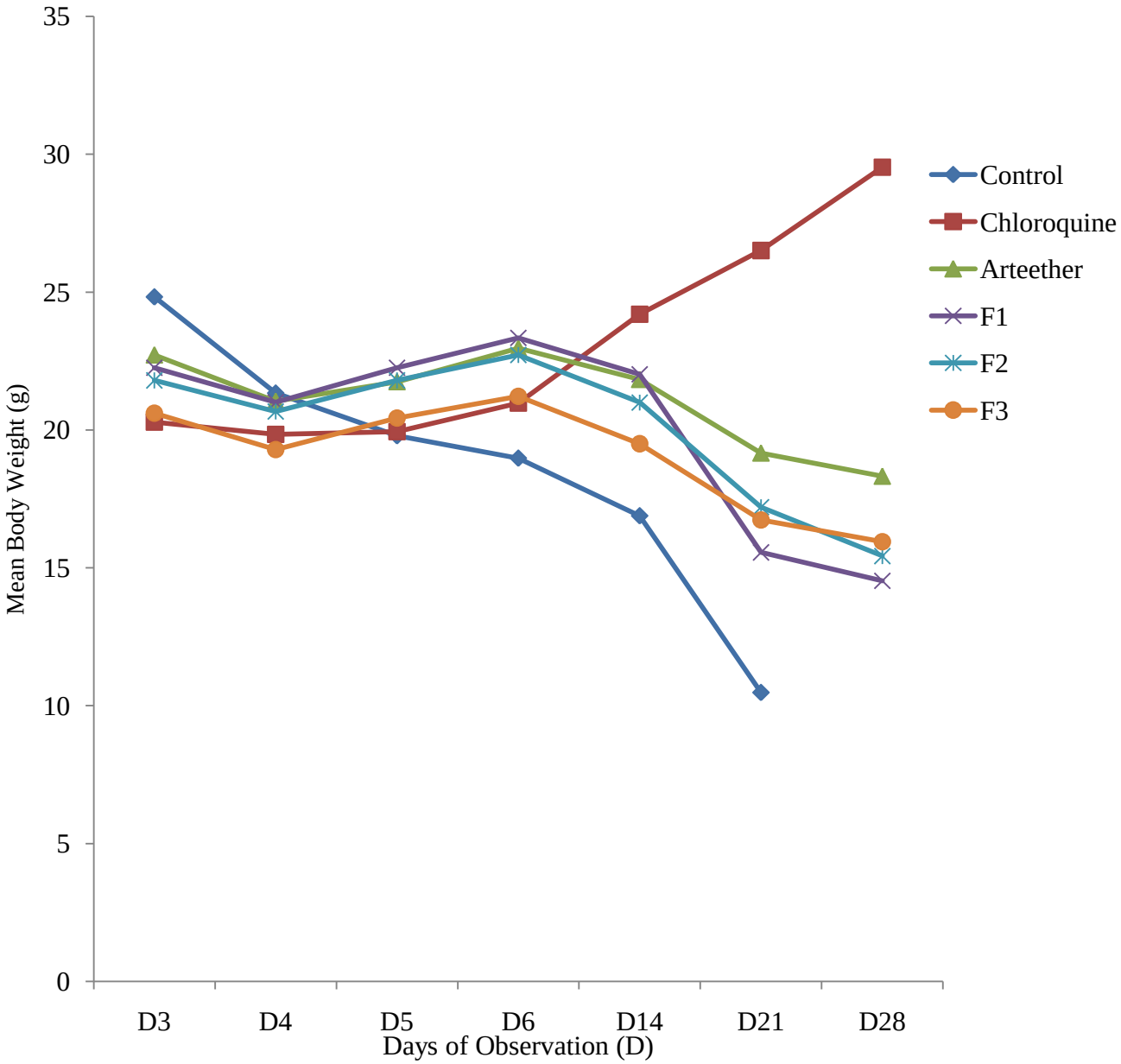


Figure 46: Effect of the fractions (F) of the bulb of *Crinum jagus* on packed cell volume (PCV) of mice infected with *Plasmodium berghei*.

Experiment 8 : Antitubercular Activity of the Crude Methanol Extract And Fractions of the Bulb of *Crinum jagus*.

Introduction

Tuberculosis (TB) remains an important public health problem world wide, accounting for eight million new cases per year. Its infectious agent, *Mycobacterum tuberculosis* kills approximately three million people every year in the world (WHO, 2007). Despite the improvement in chemotherapy, the epidemiology of tuberculosis is severely affected by the emergence of multi-drug resistance in *M. tuberculosis* strains (Leite *et al.*, 2008). For a long time, medicinal plants and herbs were used intensively in folkoric medicine for the treatment of various diseases. Recently, there has been widespread interest in drugs derived from plants. This interest stems from the belief that medicinal plants are safe and dependable as opposed to synthetic drugs that have adverse effect (Stuffness and Douros, 1982). In the past years, several reports and review articles appeared in the literature about medicinal plants and natural products with anti-mycobacterium activity (Okunade *et al*., 2004). This study was prompted by an ethnobotanical survey that reported that *Crinum jagus* is one of the plants used for the treatment of tuberculosis in Southern part of Nigeria (Idu *et al*., 2010) hence in this study the antibacterial activities of the crude methanol extract and fractions of the bulb of *Crinum jagus* against *Mycobacterium tuberculosis* isolates was investigated.

Procedure

Evaluation of antitubercular activity of the crude extract in Lowenstein Jensen (L-J) medium was carried out by the method of Gupta *et al.,* (2010). The susceptibility *of M. tuberculosis* to the extract and the drugs in Middle brook 7H10 agar was determined using the disc diffusion method described by Claude *et al.,* (2012) as described in section 3.12 under 'Materials and Methods.

Results

The results of the antitubercular activity of the crude methanol extract of the bulb of *Crinum jagus* against the three *M. tuberculosis isolates* and H37Rv strain in L.J medium and Middle brook 7H10 agar are presented in Tables 21 and 22. The results show that the crude extract at various concentrations showed a concentration dependent inhibition of the *M. tuberculosis*

isolates and H37Rv strain in both Lowenstein Jensen (L-J) and Middlebrook 7H10 media. The antitubercular activity of the extract compared well with antitubercular activity of the standard drugs (rifampicin and isoniazid) however, the potency of the extract was lowered compared to the standard drugs.

The crude extract and the standard drugs possessed marked inhibitory activity against *M. tuberculosis* with IC_{50} values ranging from 0.20 to 0.90 mg/ml. The various concentrations of the extract exhibited a significant concentration dependent inhibition of *M. tuberculosis* with IC_{50} values of 0.92, 0.92, 0.90, 0.88 mg/ml against H37Rv, MTB1, MTB2 and MTB3 respectively in L-J medium (Table 23) while IC_{50} values of 0.86, 1.45, 0.82, 0.77 mg/ml were obtained for the crude extract in Middle brook 7H10 agar. In L-J and Middle brook 7H10 media, the IC_{50} values of rifampicin against H37Rv, MTB1, MTB2 and MTB 3 were 0.20, 0.29, 0.26, 0.21 mg/ml and 0.19, 0.28, 0.21, 0.19 mg/ml respectively. Isonazid in both L-J and Middlebrook 7H10 media had the IC_{50} value of 0.25, 0.32, 0.27, 0.20 mg/ml and 0.23, 0.26, 0.22, 0.20 mg/ml respectively against H37Rv, MTB1, MTB2, MTB (Table 23). IC_{50} values of the standard drugs, rifampicin and isoniazid were found to be much less than those of the crude extract indicating that the drugs were more potent as an anti-TB agent than the extract.

Tables 25 and 26 present the result of antitubercular activity of the fractions of the bulb of *Crinum jagus* in both Lowenstein Jensen (L-J) and Middlebrook 7H10 agar. All the three fractions (F1, F2 and F3) showed a concentration dependent inhibition of *M. tuberculosis* isolates and H37Rv strain at various concentrations in both medium. The fractions were more potent than the crude extract with higher inhibition values. Of all the three fractions, F1 was the most potent with the highest inhibitory values. The inhibitory activity of F1 compared well with the standard drugs. At a concentration of 1.0mg/ml, the percentage inhibition of F1, rifampicin and isoniazid against MTB 3, was 83%, 95% and 84%, respectively.

The fractions possessed good inhibitory activity against *M. tuberculosis* with IC_{50} value ranging from 0.22 to 0.97 mg/ml. F1, F2 and F3 at various concentrations exhibited concentration dependent inhibition of *M. tuberculosis* with IC_{50} values of (0.23, 0.48, 0.32, 0.27 mg/ml), (0.41 , 0.94 , 0.78, 0.69 mg/ml) and (0.65, 0.63, 0.55, 0.48mg/ml) respectivley against H37Rv, MTB 1, MTB 2 and MTB 3 in L.J medium while IC_{50} values (0.22, 0.40,

0.32, 0.26 mg/ml), (0.42, 0.97,0.83, 0.71 mg/ml) and (0.61, 0.54, 0.54,0.46 mg/ml) were obtained for F1, F2 and F3 respectively in Middle brook 7H10 agar. F1 had the lowest IC_{50} value which compared well with the IC_{50} value of the standard drugs, rifampicin and isoniazid indicating F1 to be the most potent of all the three fractions (Table 26).

Conclusion

From this study, it can be concluded that the crude methanol extract and fractions of the bulb of *Crinum jagus* exhibited interesting anti-mycobacterial activity against *Mycobacterium tuberculosis* isolates and strains.with F1 having the highest activity which compared well with antitubercular activity of the standard drugs, rifampicin and isoniazid.

Table 21 : Antitubercular activity of the crude methanol extract of the bulb of *Crinum jagus* in Lowestein Jensen (L- J) medium

		Mean cfu on media						Percentage Inhibition (%)				
Extract/ Drug	**Isolates**	**Control**	**0.2mg/ Ml**	**0.4mg/ Ml**	**0.6mg/ Ml**	**0.8mg/ Ml**	**1.0mg/ Ml**	**0.2mg/ Ml**	**0.4mg/ ml**	**0.6mg/ ml**	**0.8mg/ ml**	**1.0mg/ ml**
Crude Extract	H37Rv	125	110	90	82	73	54	12	28	34	42	57
	MTB1	122	108	95	80	66	56	30	22	34	45	54
	MTB 2	138	121	104	88	72	61	12	25	36	48	56
	MTB 3	136	120	107	84	70	58	12	25	38	49	57
Rifampicin	H37Rv	140	62	50	36	20	12	56	64	74	86	91
	MTB1	140	86	54	45	30	20	39	61	68	79	86
	MTB 2	134	75	50	38	22	14	44	63	72	84	88
	MTB3	130	62	42	20	12	6	52	68	85	91	95
Isoniazid	H37Rv	140	72	60	46	30	20	49	57	67	79	86
	MTB1	132	112	84	75	54	40	15	36	43	59	70
	MTB2	150	84	65	52	48	36	44	57	65	68	77
	MTB3	146	70	56	40	35	24	52	62	73	76	84

MTB = *Mycobacterium tuberculosis*, cfu = colony forming unit

$$\% \text{ Inhibition} = \frac{Cc - Ct}{Cc} \times 100$$

Cc = no of colony in the control medium

Ct = no of colony in test mediu

Table 22 : Antitubercular activity of the crude methanol extract of the bulb of C*rinum jagus* in Middle brook 7H10 medium

		Mean cfu on media						Percentage Inhibition (%)				
Extract/ Drug	**Isolates**	**Control**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg/ ml**	**0.8mg /ml**	**1.0mg /ml**
Crude Extract	H37Rv	124	101	92	78	60	55	19	26	37	52	56
	MTB1	120	108	92	78	64	54	10	23	35	47	56
	MTB 2	141	122	102	84	70	59	14	27	40	50	58
	MTB 3	138	116	98	82	68	56	16	28	41	51	59
Rifampicin	H37Rv	126	58	40	31	15	5	54	68	75	88	96
	MTB1	130	74	52	40	29	14	43	60	69	78	89
	MTB 2	140	65	50	39	25	12	54	64	72	82	91
	MTB3	132	55	40	29	20	04	58	70	78	85	97
Isoniazid	H37Rv	136	68	55	40	25	15	50	60	71	82	89
	MTB1	138	105	80	62	51	38	24	42	55	63	73
	MTB2	146	80	71	60	49	31	45	52	59	66	79
	MTB3	144	68	51	39	22	16	53	65	73	85	89

MTB = *Mycobacterium tuberculosis*, cfu = colony forming unit

$$\% \text{ Inhibition} = \frac{Cc - Ct}{Cc} \times 100$$

Cc = no of colonyin the control medium

Ct = no of colony in test mediu

.

Table 23 : *In vitro* antitubercular activity of the crude methanol extract and fractions of the bulb of *Crinum jagus* against isolates and strain of *M. tuberculosis* in L-J medium.

	50% Inhibitory Concentration (mg/ml)			
	H37Rv	**MTB 1**	**MTB 2**	**MTB 3**
Crude extract	0.92	0.92	0.90	0.88
Fraction 1	0.23	0.48	0.32	0.27
Fraction 2	0.41	0.94	0.78	0.69
Fraction 3	0.65	0.63	0.55	0.48
Rifampicin	0.20	0.29	0.26	0.21
1soniazid	0.25	0.61	0.27	0.20

Table 24 : *In vitro* antitubercular activity of the crude methanol extract and fractions of the bulb of *Crinum jagus* against isolates and strain of *M. tuberculosis* in Middlebrook 7H10 medium.

	50%Inhibitory concentration (mg/ml)			
	H37Rv	**MTB 1**	**MTB 2**	**MTB 3**
Crude extract	0.86	1.45	0.82	0.77
Fraction 1	0.22	0.50	0.32	0.26
Fraction 2	0.42	0.97	0.83	0.71
Fraction 3	0.61	0.54	0.54	0.46
Rifampicin	0.19	0.28	0.21	0.19
Isoniazid	0.23	0.50	0.32	0.21

Table 25 : Antitubercular activity of the fractions of the bulb of *Crinum jagus* in Lowenstein Jensen (L-J) medium

		Mean cfu on media						Percentage Inhibition (%)				
Extract/ Drug	**Isolates**	**Control**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**
Fraction 1	H37Rv	130	70	62	46	30	22	46	52	65	77	83
	MTB 1	164	102	96	86	69	45	38	41	48	58	73
	MTB 2	118	75	58	45	40	25	36	51	62	66	79
	MTB 3	162	82	70	58	40	25	49	57	64	75	83
Fraction 2	H37Rv	128	80	74	55	41	30	38	42	57	68	77
	MTB 1	128	108	102	98	76	64	16	20	23	47	50
	MTB 2	120	101	88	80	65	54	16	18	33	46	55
	MTB 3	138	116	110	89	73	58	16	20	35	41	58
Fraction 3	H37Rv	120	95	83	74	50	39	21	31	38	58	68
	MTB 1	120	90	74	64	52	42	25	38	47	57	65
	MTB 2	122	92	68	63	45	42	25	44	48	63	66
	MTB 3	142	98	76	67	52	44	31	46	53	63	69
Rifampicin	H37Rv	140	62	50	36	20	12	56	64	74	86	91
	MTB 1	140	86	54	45	30	20	39	61	68	79	86
	MTB 2	134	75	50	38	22	14	44	63	72	84	88
	MTB 3	130	62	42	20	12	6	52	68	85	91	95
Isoniazid	H37Rv	140	72	60	46	30	20	49	57	67	79	86
	MTB 1	132	112	84	75	54	40	15	36	43	59	70
	MTB 2	150	84	65	52	48	36	44	57	65	68	77
	MTB 3	146	70	56	40	35	24	52	62	73	76	84

MTB = *Mycobacterium tuberculosis*, cfu = colony forming unit

$$\% \text{ Inhibition} = \frac{Cc - Ct}{Cc} \times 100$$

Cc = no of colony in the control medium
Ct = no of colony in test medium

Table 26 : Antitubercular activity of the fractions of the bulb of *Crinum jagus* in Middle brook 7H10 medium

		Mean cfu on media						Percentage Inhibition (%)				
Extract/Drug	**Isolates**	**Control**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**
Fraction 1	H37Rv	128	65	42	31	22	12	49	67	76	83	91
	MTB 1	158	104	94	82	65	37	34	41	48	59	76
	MTB 2	122	69	60	44	39	24	43	51	64	68	80
	MTB 3	154	78	68	52	38	22	49	59	66	75	86
Fraction 2	H37Rv	140	94	76	60	50	30	32	46	57	64	79
	MTB1	131	112	98	90	72	58	15	25	31	45	56
	MTB 2	122	95	86	72	63	50	22	29	41	48	59
	MTB3	136	102	95	77	68	50	25	30	43	50	63
Fraction 3	H37Rv	138	109	92	75	61	40	21	33	46	56	71
	MTB1	126	94	78	62	48	40	25	38	51	62	68
	MTB2	120	89	70	61	46	35	26	42	49	62	71
	MTB3	140	96	78	65	48	38	31	44	54	66	73
Rifampicin	H37Rv	126	58	40	31	15	5	54	68	75	88	96
	MTB1	130	74	52	40	29	14	43	60	69	78	89
	MTB2	140	65	50	39	25	12	54	64	72	82	91
	MTB3	132	55	40	29	20	04	58	70	78	85	97
Isoniazid	H37Rv	136	68	55	40	25	15	50	60	71	82	89
	MTB1	138	105	80	62	51	38	24	42	55	63	73
	MTB2	146	80	71	60	49	31	45	52	59	66	79
	MTB3	144	68	51	39	22	16	53	65	73	85	89

MTB = *Mycobacterium tuberculosis*, cfu = colony forming unit

$$\% \text{ Inibition} = \frac{Cc - Ct}{Cc} \times 100$$

Cc = no of colony in the control medium
Ct = no of colony in test medium

Experiment 9 : Toxicological Profiles of the Crude Methanol Extract of the Bulb of *Crinum jagus*

Introduction

The use of medicinal plants in the management of several ailments is increasing probably due to the belief that they are harmless simply because they are natural. They are also commonly used for self medication without supervision. This increase in popularity but lack of scientific studies on the safety of these plants and their phytoconstituents has raised concerns regarding toxicity and adverse effects of these remedies (Gehlot and Bohra, 2000, Saad *et al.*, 2006). Some medicinal plants must be used with caution because they can cause adverse reactions, especially if they are taken in excessive dose, or if they interact with conventional drugs (Janetzky and Morreale, 1997; Fennel *et al*., 2004). Therefore there is need to assess toxicological implications of traditionally used medicinal plants. We have among these plants, *Crinum jagus* which posses medicinal value.

Crinum jagus is a bulbous plant with spirally arranged leaves and conspicuous flowers. It is found in tropical and subtropical regions throughout the world (Mabbery, 1991). It belongs to to the family : Amaryllidaceae, genus : *Crinum,* species : *jagus.*

Crinum jagus has various therapeutic uses in herbal medicine. The powdered bulb is taken orally with honey as a remedy for tuberculosis in some parts of Nigeria (Ode *et al*., 2006). The bulb of the plant is used for the treatment of asthma cough in Western part of Nigeria and is commonly called asthma cough plant (Ogunkunle and Olopade, 2011). The warm leaf juice of the bulb of the plant with a pinch of salt is used for ear complaint as an emetics (Gill 1992). Among the Binis, the decoction of the plant is used as vermifuge and purgative (Gill, 1992). Previous work done on the therapeutic importance of the plant reported the antibacterial and antifungal activities, (Adesanya *et al*., 1992), anticonvulsant activity (Edema and Okiemen, 2002) and anti-snake venom activity of the plant (Ode *et al.,* 2010). This study was therefore designed to evaluate the sub-chronic toxicity and histopathology of the methanol extract of the bulb of the plant in

experimental animals to assess its safety or otherwise since the findings are important considering the usage of the plant by human beings.

Procedure

Thirty male rats were randomly divided into five groups of six animals each. Group A received 0.2ml normal saline and served as the control, Groups B to E received the extract at the doses of 10, 25, 50 and 75mg/kg respectively for 30 days. The extract was dissolved in normal saline before administration to the rats using oral cannula, 24 hours after the last treatment, the animals were sacrificed, blood samples were collected by cardiac puncture into plain bottles and then allowed to stand for 1 hour and were centrifuged at 3000g for 10 minutes to obtain the serum. Liver and kidney samples were removed and washed in ice cold 1.15% KCl, dried, weighed and homogenized in phosphate buffer, the homogenate was centrifuged at 10,000g for 20 minutes to obtain the supernatant used for the assay. AST and ALT were assayed using the procedures described by Rietman and Frankel (1957), ALP was assayed by the procedure of Wright *et al.*,(1972) while LDH was assayed by the method of Pesce and Kaplan, (1984). The total and direct bilirubin were determined by the methods of Kaplan *et al.*,(1984); Malloy *et al.*,1937 and Martinek, 1966). The concentrations of triglycerides (TG), total chloestrol (TC), LDC-C and HDL-C were determined by standard methods (Trinder *et al.*, 1969; Mc Gowan 1983; Albers *et a*l., 1978; Fossatti and Prencipe 1982; Demacker *et al.*, 1984; Fredickson *et al*., 1967). Haematological parameters were determined, using the standard methods as described by Dacie and Lewis, (1991). The liver and kidney samples were harvested and fixed in 10% buffered formalin for 48 hrs. The tissues were processed using an automatic tissue processor embedded in paraffin wax and section (5µm) thickness cut using a rotary microtome. The sections were stained by haematoxylin and eosin (H & E) method for light microscopy examination. Photomicrographs of relevant stained sections were taken with the aid of a camera fitted light microscope.

Results

Figure 47 shows the result of the effect of crude methanol extract of the bulb of *Crinum jagus* on the body weight of the control and the treated rats between the beginning of the study and the point of sacrifice. By the end of thirty days of treatment there was increase in the body weight of all the treated and the control rats.

The effect of crude methanol extract of the bulb of *Crinum jagus* on serum enzymes following administration of the extract for 30 days revealed that there were no significant ($P < 0.05$) differences in the serum levels of AST, ALT and ALP in the rats treated with 10 and 25 mg/kg of the extract, however, the level of AST, ALT and ALT in the serum were significantly ($P < 0.05$) elevated by administration of 50 and 75mg/kg of the extract. Both lower and higher doses of the extract did not produce any significant difference in the level of serum LDH when compared with the control group (Fig 48).

The mean liver function test values of the experimental rats is presented in Fig 49. The result of the experiment shows that there were no significant differences ($P > 0.05$) in the levels of AST, ALT and ALP following administration of the lower doses (10 and 25mg/kg) of the extract for 30 days while administration of higher doses (50 and 75mg/kg) produced significant ($P < 0.05$) elevation in the concentrations of AST, ALT and LDH relative to the control group. The higher doses (50 and 75mg/kg) of the extract does not produce any significant difference in level of liver ALP relative to the control group.

The effect of thirty days oral administration of the crude extract of the bulb of *Crinum jagus* on kidney enzymes revealed non significant differences in the concentrations of AST, ALT and ALP in the animals treated with low doses of the extract (10 and 25mg/kg) while significant increase in the levels of AST, ALT and ALP were observed with higher doses (50 and 75mg/kg). There were no significant difference in the level of LDH in the kidney in all the treated groups compared with the control group (Fig 50).

There were no significant differences in the total and conjugated bilirubin concentration in all the animals treated with lower doses of the extract (10 and 25mg/kg). Higher doses of the extract (50 and 75mg/kg) produced significant ($P < 0.05$) elevation

in the total and conjugated bilirubin levels both in the serum and the liver (Fig 51 and 52).

Figure 53 revealed that there was significant ($P < 0.05$) reduction in the serum triglyceride level when the animals were treated with high doses (50 and 75mg/kg) of the extract. Both lower and higher doses of the extract produced significant reduction in the concentration of total cholesterol in the serum while there was significant elevation in the serum HDL cholesterol in the groups treated with 25, 50 and 75mg/kg doses of the extract for thirty days relative to the control group. There were no significant differences in the serum LDL cholesterol concentration in all the treated groups compared with the control group.

The effect of 30 days oral administration of the crude methanol extract of the bulb of *Crinum jagus* on haematological parameters indicated no significant differences in the value of red blood cell count (RBC), packed cell volume (PCV) and haemoglobin (Hb) concentrations in all the treated groups relative to the control group. Also the values of mean corpuscular volume (MCV), mean corpscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were not significantly different in all the treated groups compared with the control group (Fig 54).

Fig 55 also revealed no significant differences in the values of white blood cell count (WBC), lymphocyte, neutrophils, eosinophils, monocytes and platelets in all the test groups compared with the control group after 30days of treatment with the extract.

Plates 1A, 1B and 1C show the photomicrographs obtained from the histological examination of liver sections of the different groups. As shown on the plates, the liver section of the control group showed normal hepatocyte, the central vein was normal and centrally placed (Plate 1A). The liver sections of the rats treated with lower doses of the extract (10 and 25mg/kg) showed moderate haemorrhage (Plate 1B) while exposure of the rats to high dose caused pathological changes in the liver which resulted in the enlargement of hepatocytes with multiple cytoplasimic vacuolation (fatty degeneration of hopatocytes) (Plate 1C), showing the extract to be toxic at higher dose.

Plates 2A, 2B and 2C show the photomicrographs obtained from the histological examination of kidney sections of the different groups. As seen on the plates, the kidney sections of the control showed normal cells with no visible lesions (Plate 2A). The kidney sections of the rats treated with lower doses of the extract showed moderate haemorrhage in the renal interstitium (Plate 2B) while exposure of the rats to high dose caused necrosis of the tubular epithelium with sloughing of necrosis into the lumen of the tubules and disruption of the basement membranes (Plate 2C), showing the extract to be toxic at higher dose.

Conclusion

In conclusion, toxicological evaluation of the bulb of *Crinum jagus* indicates that the plant is safe at lower doses but high doses of the plant may pose toxicological risks. Prolonged exposure for thirty days at higher doses tested (50 and 75mg/kg) resulted in the elevation of serum, liver and kidney AST, ALT and ALP. Similarly, pathological changes were observed in the liver and kidney following administration of higher doses. The plant however appears to possess beneficial effect by showing serum lipid lowering effect .

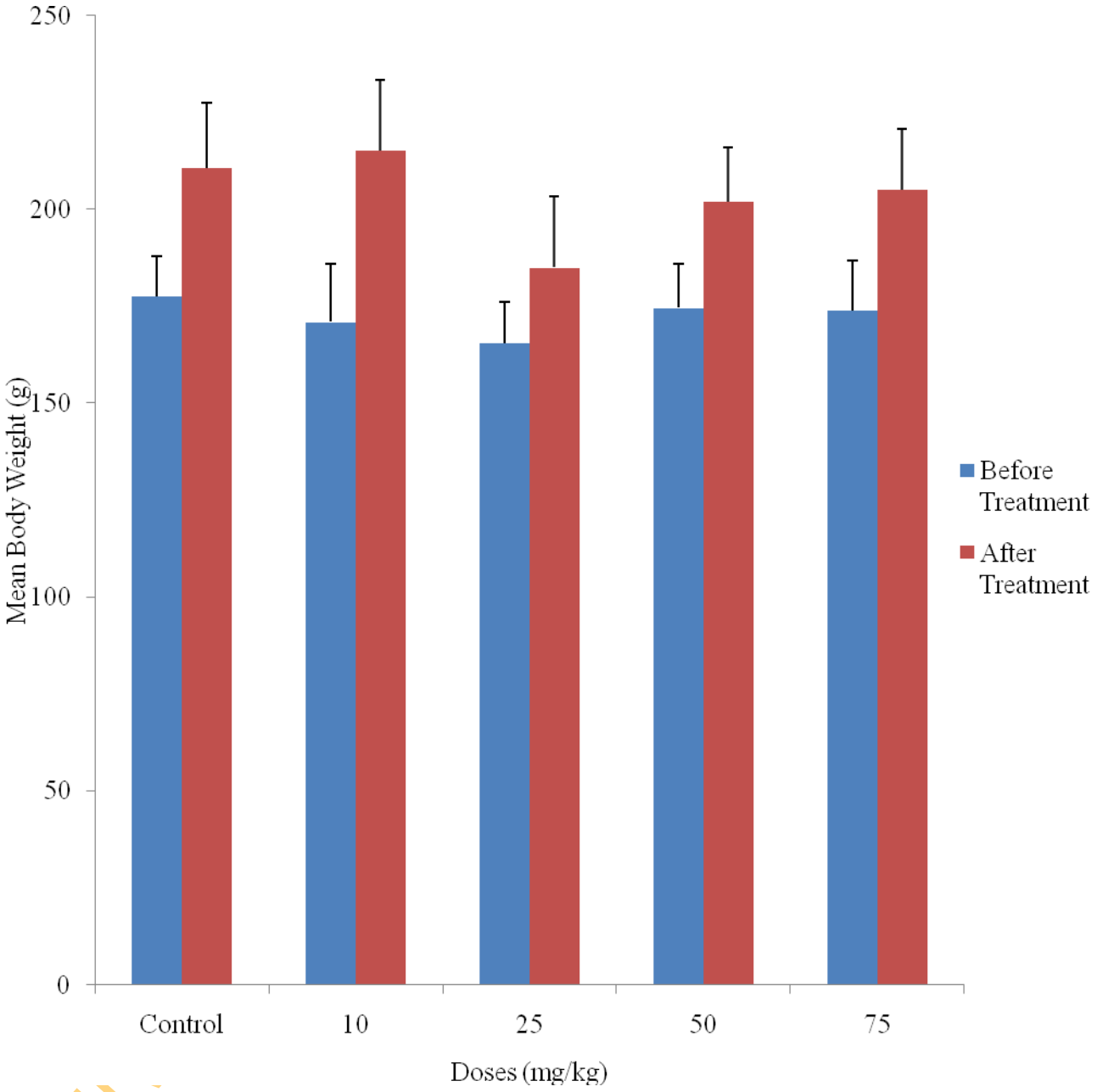


Figure 47: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on body weight of rats.

Control = 0.2ml normal saline

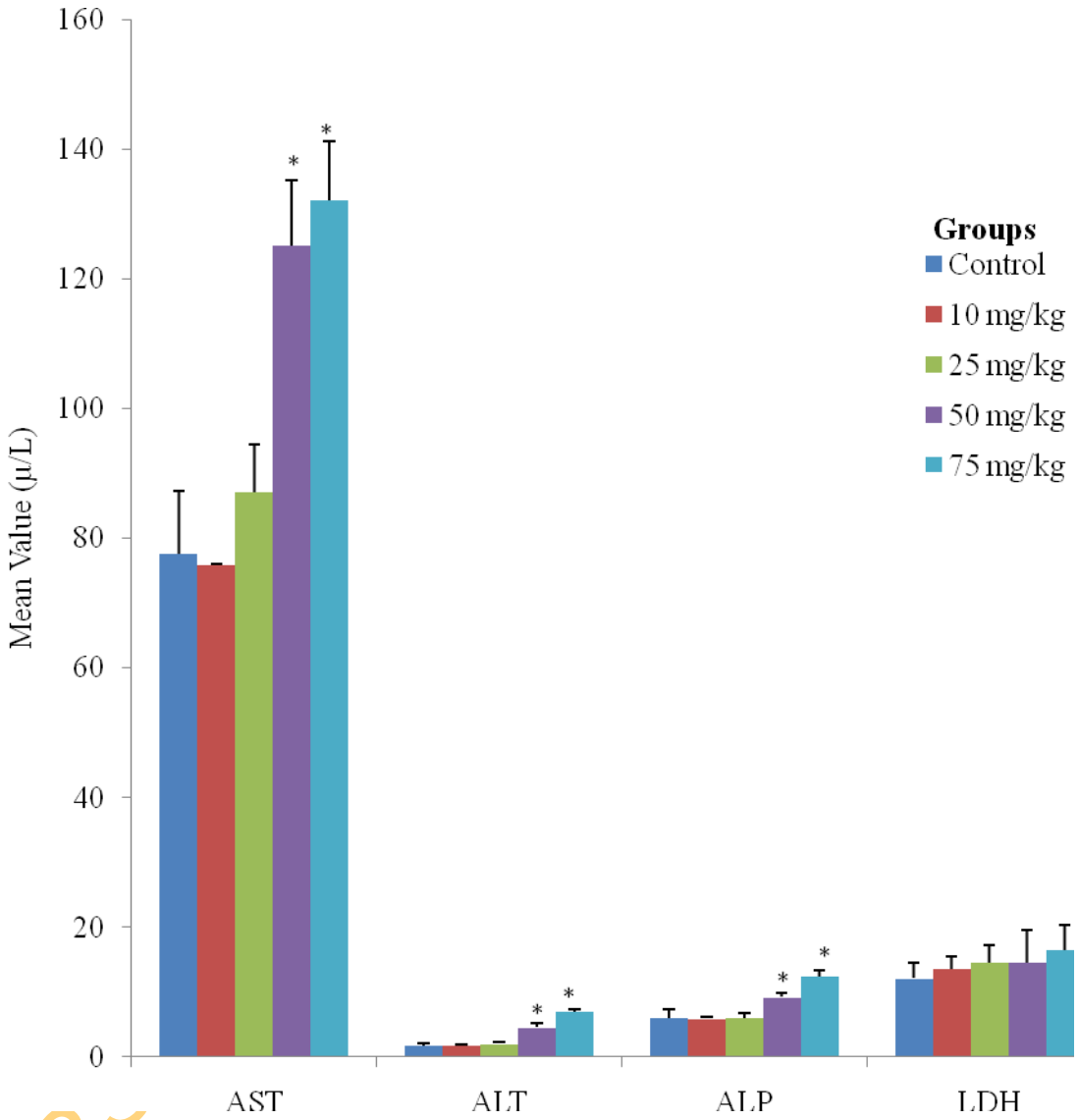


Figure 48: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on serum enzymes of rats.

* = Significantly different from control

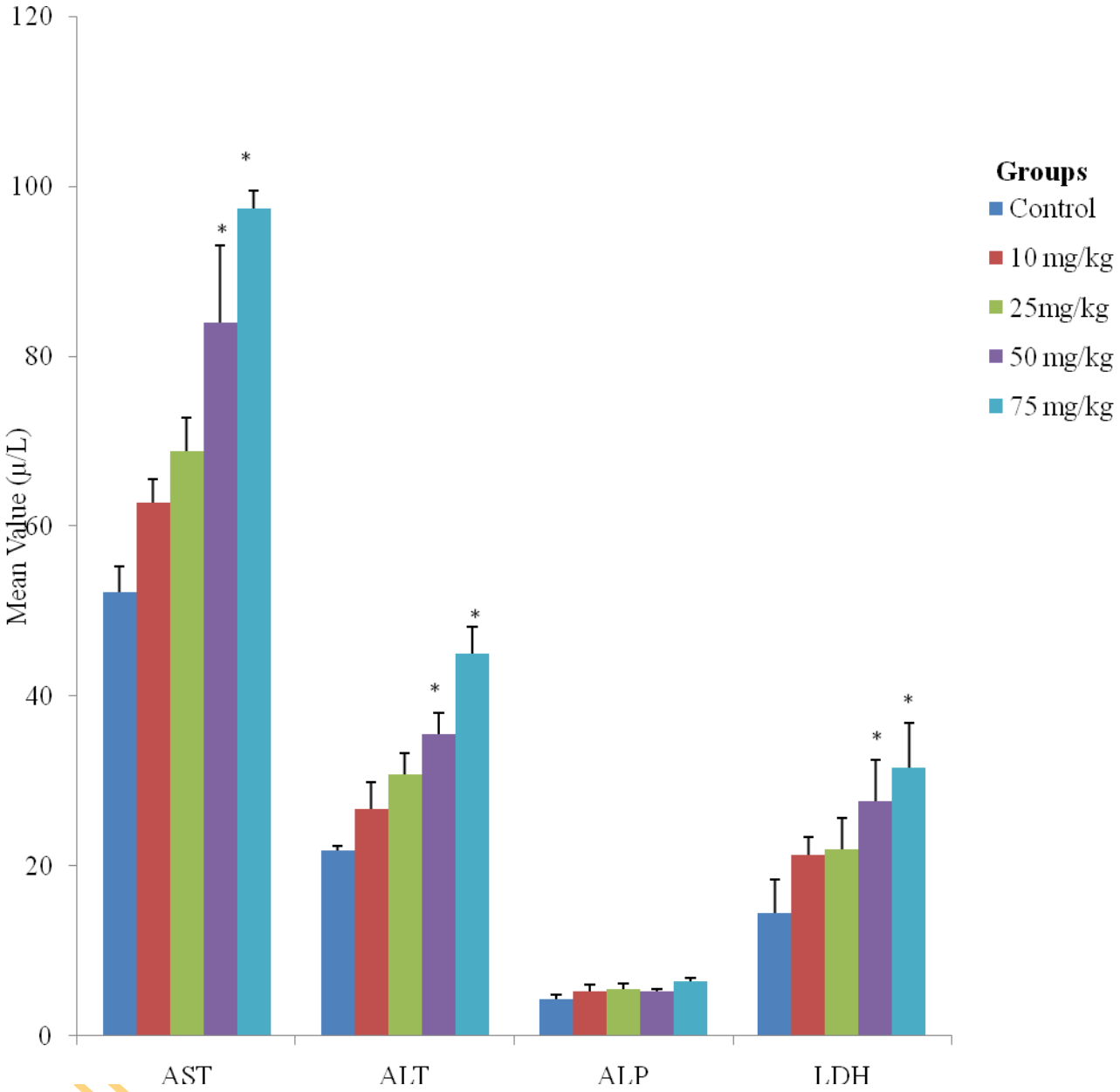


Figure 49: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on liver enzymes of rats.

* = Significantly different from control

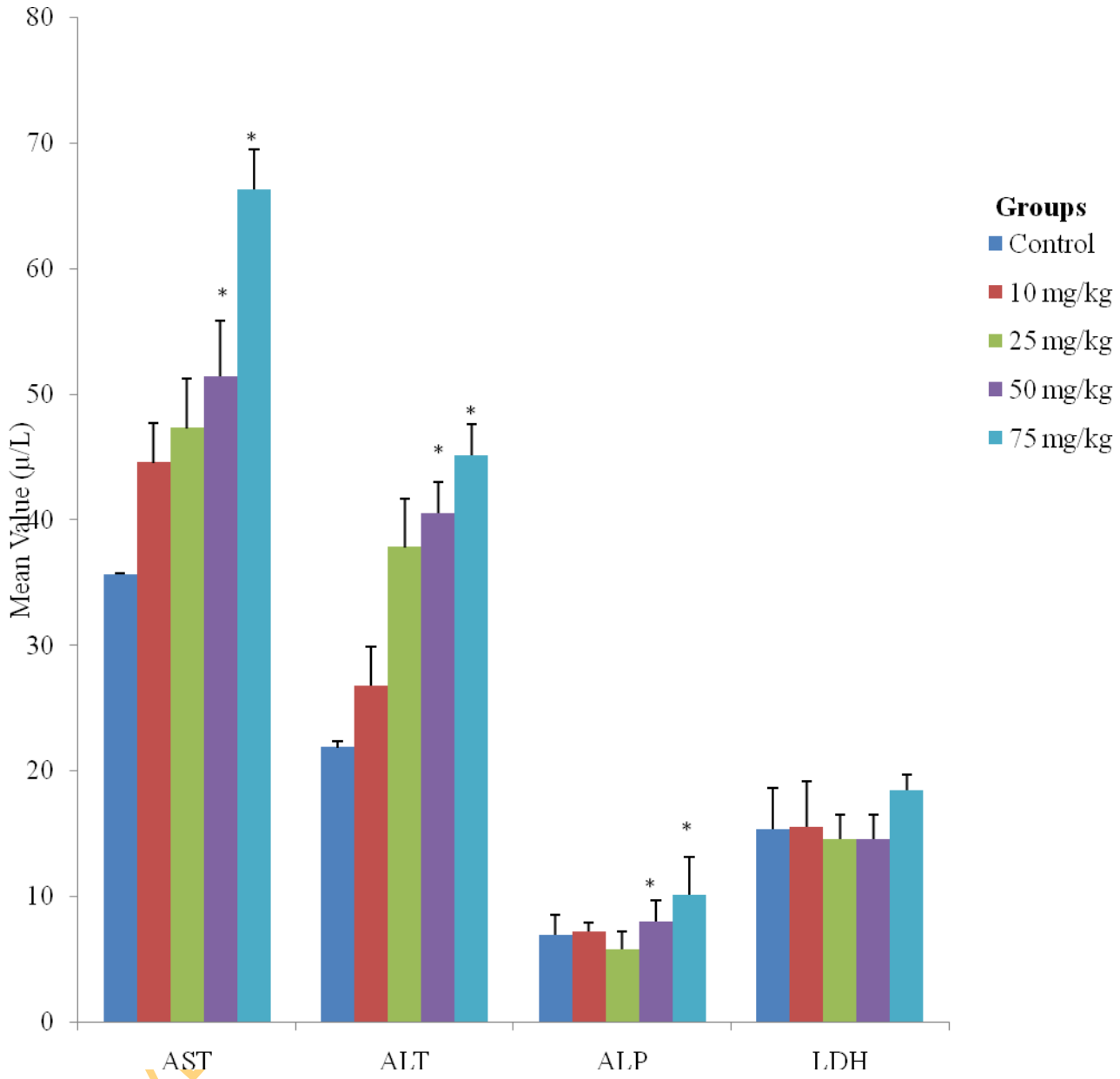


Figure 50: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on kidney enzymes of rats.

* = Significantly different from control

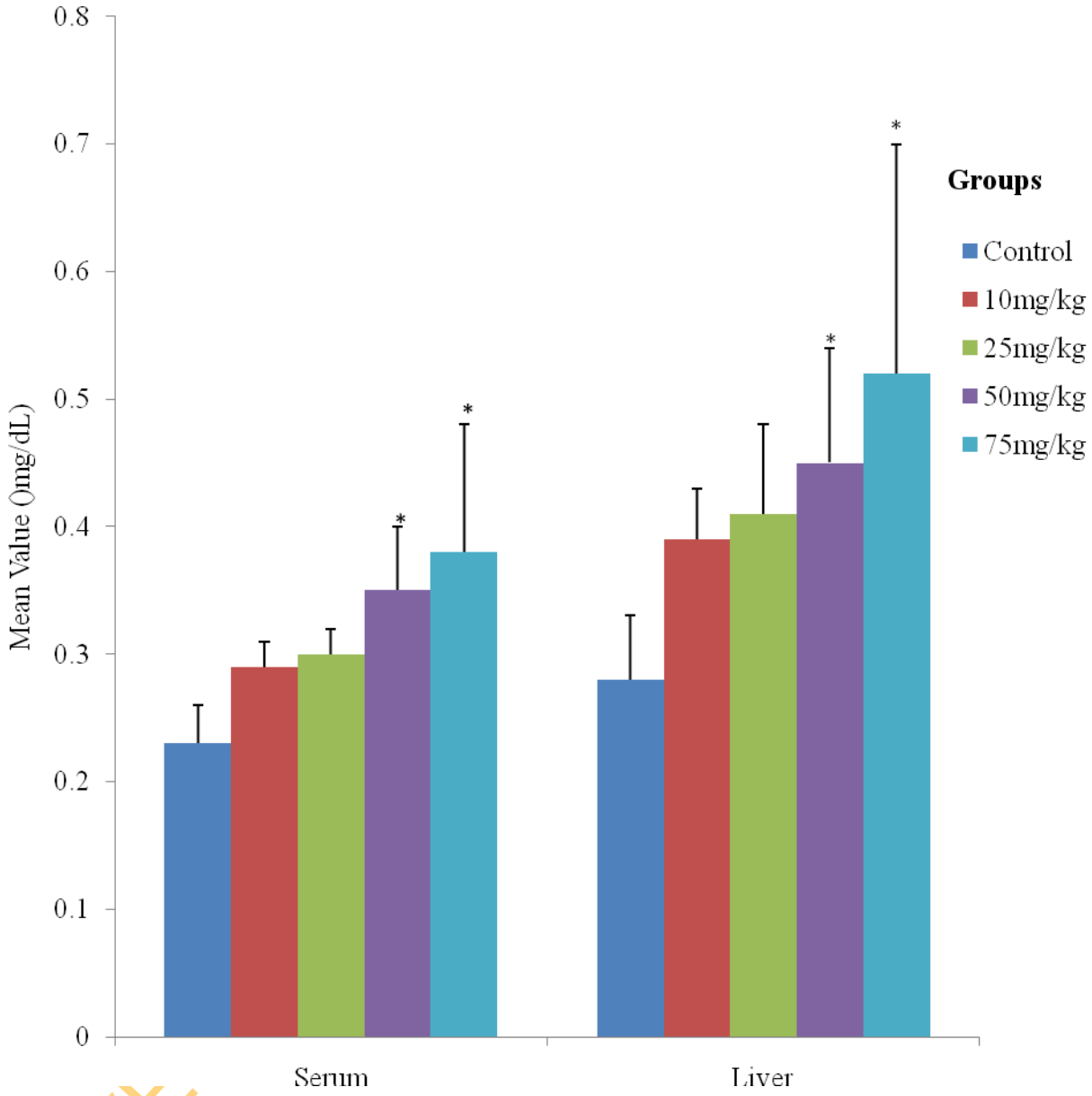


Figure 51: Effect of 30 days oral admineistration of crude methanol extract of the bulb of *Crinum jagus* on serum and liver direct bilirubin of rats.

* = Significantly different from control

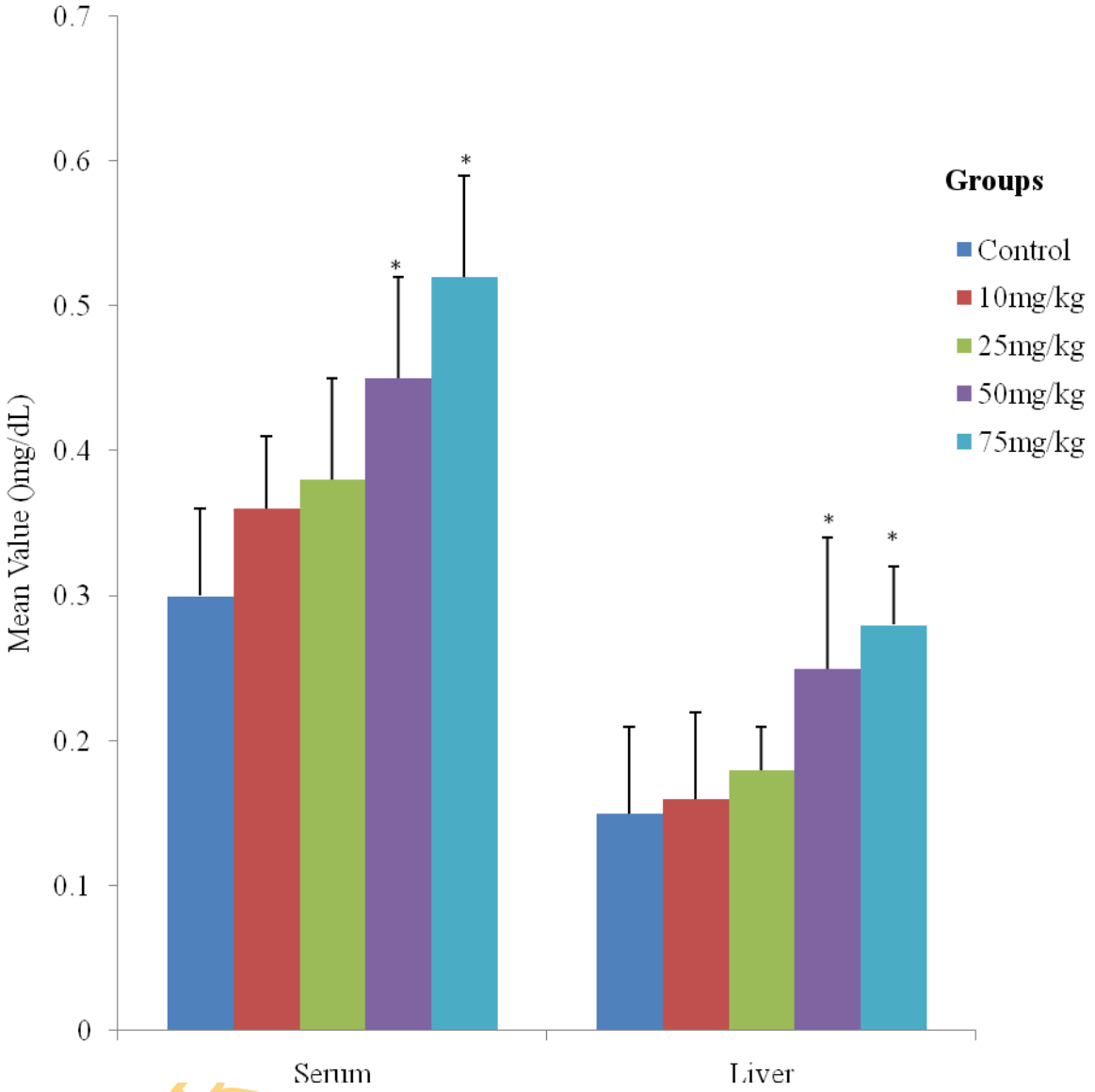


Figure 52: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on serum and liver total bilirubin of rats.

* = Significantly different from control

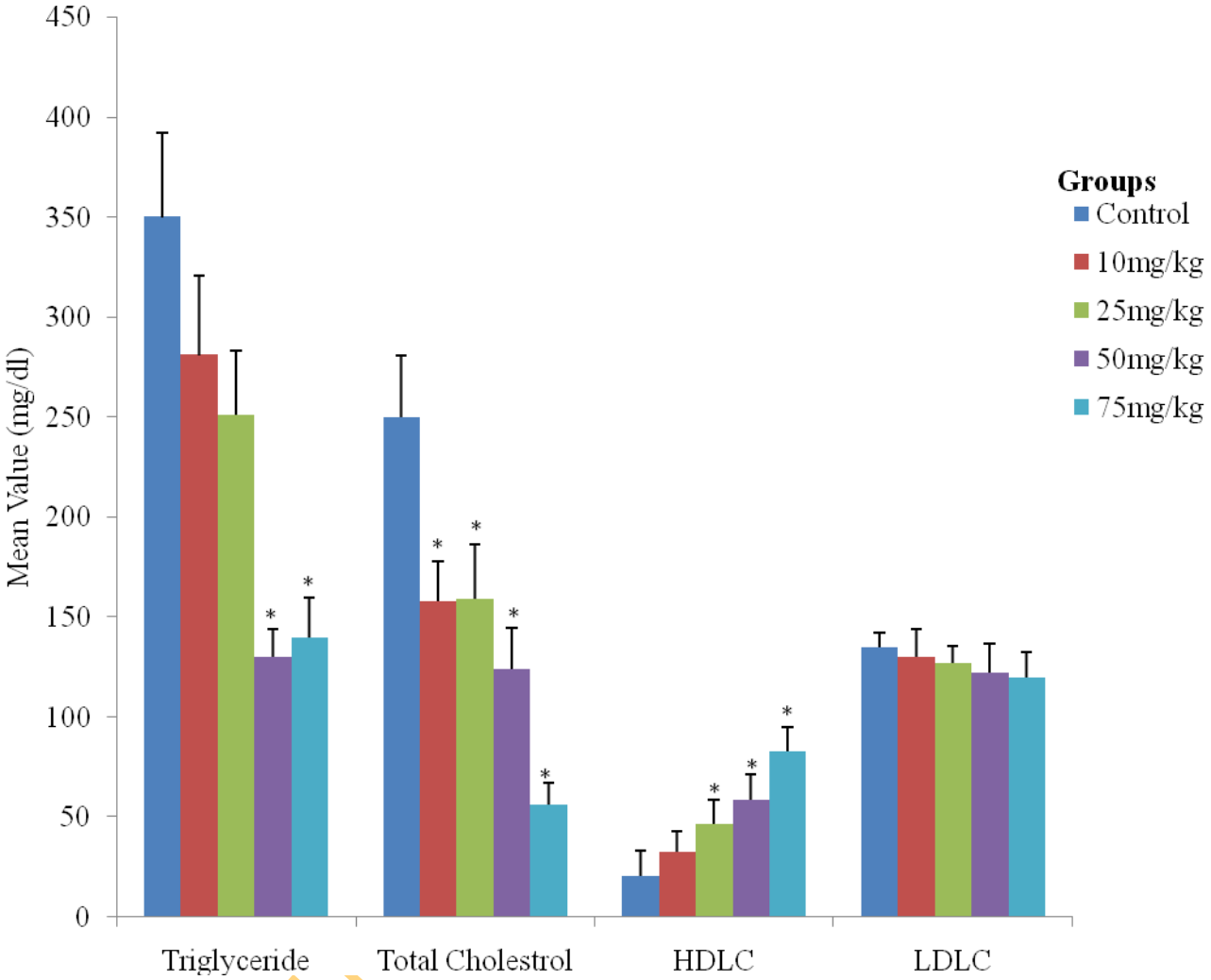


Figure 53: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on serum lipid profile of rats.

* = Significantly different from control

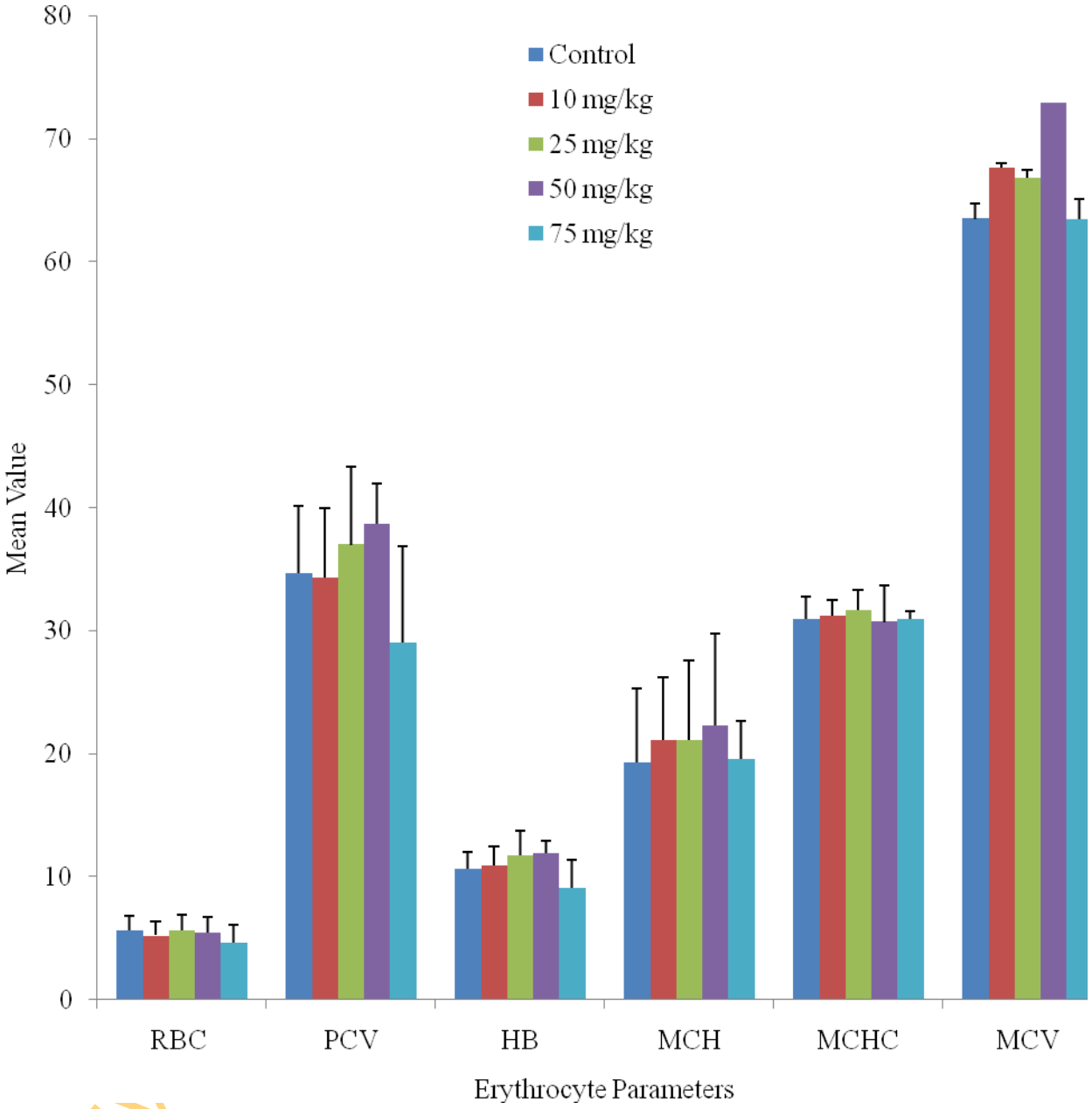


Figure 54: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on erythrocyte parameters of rats

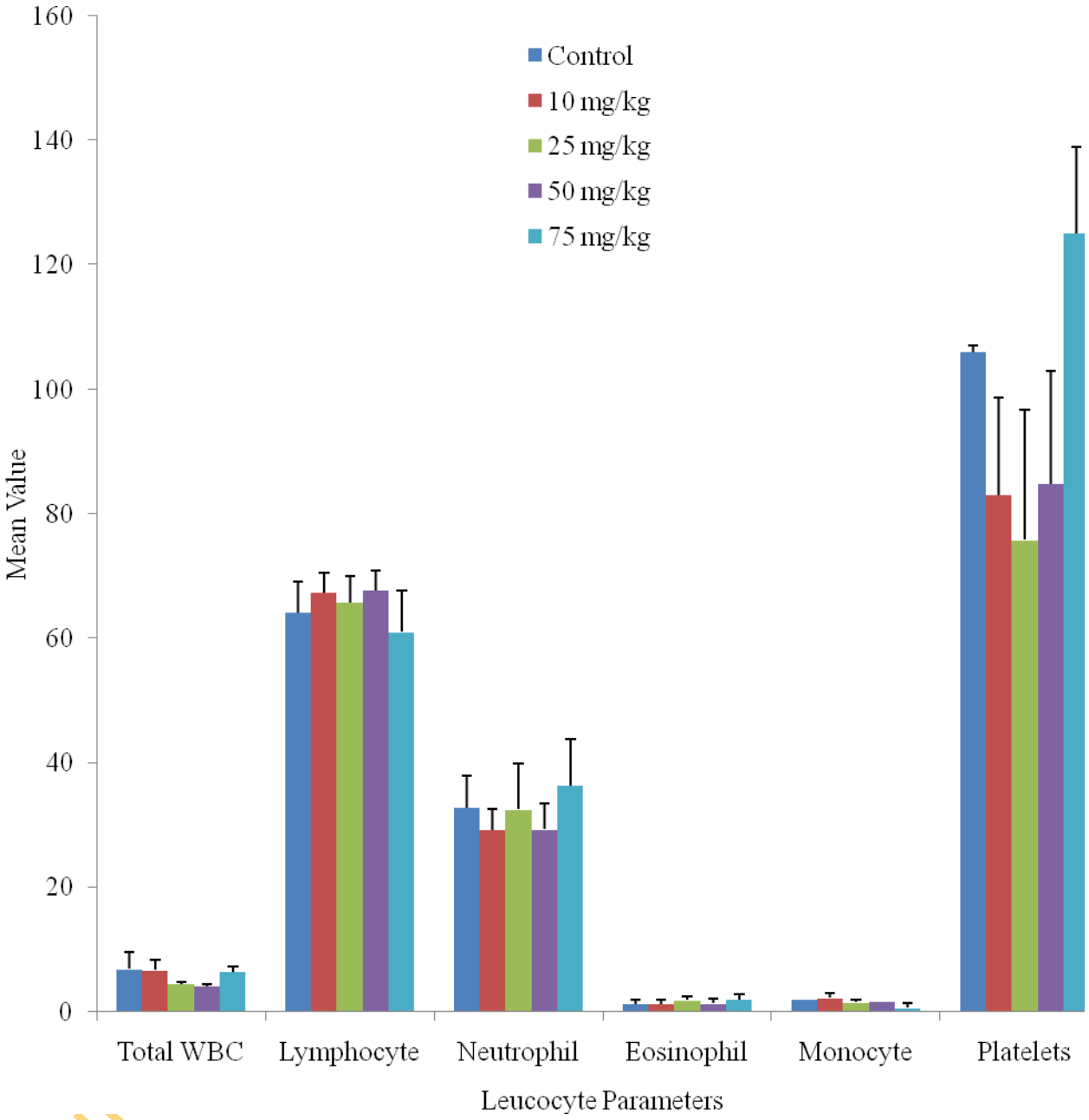


Figure 55: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on leucocyte parameters of rats.

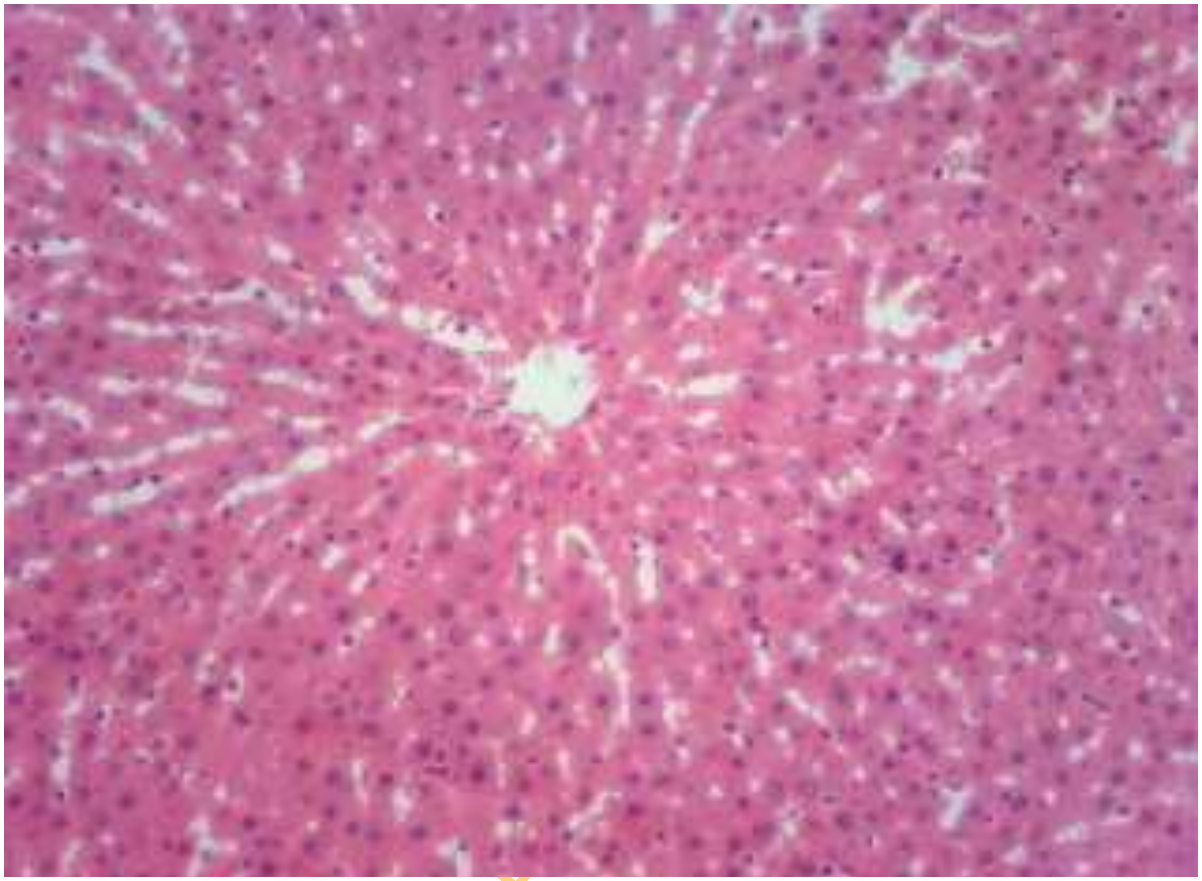


Plate 1A: Photomicrograph of the normal section of the liver of rats in the control group (X160).

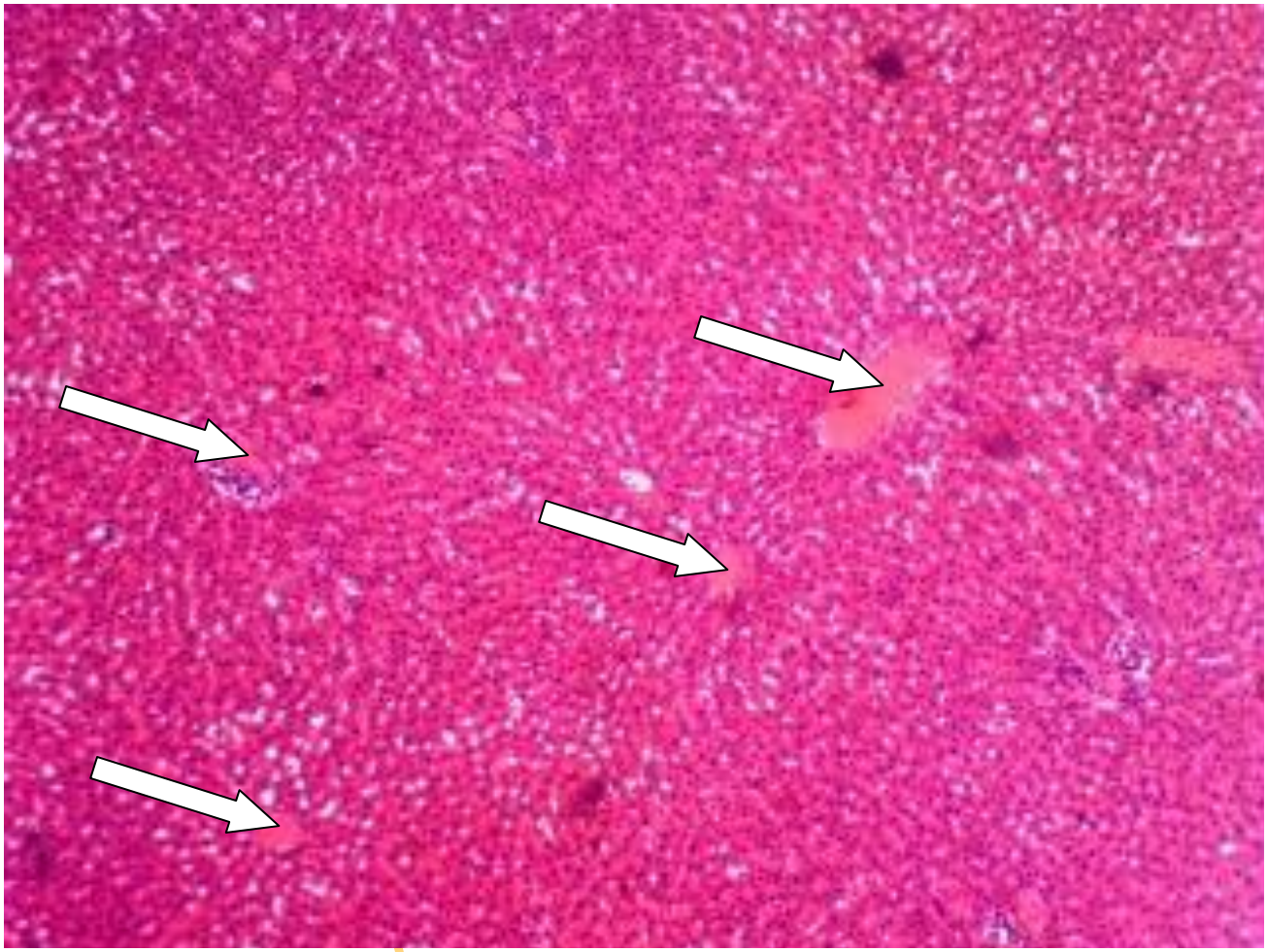


Plate 1B: Photomicrograph of the liver showing moderate haemorrhage in the renal interstitum of rats treated with low doses (10 and 25mg/kg) of *Crinum jagus* (X64).

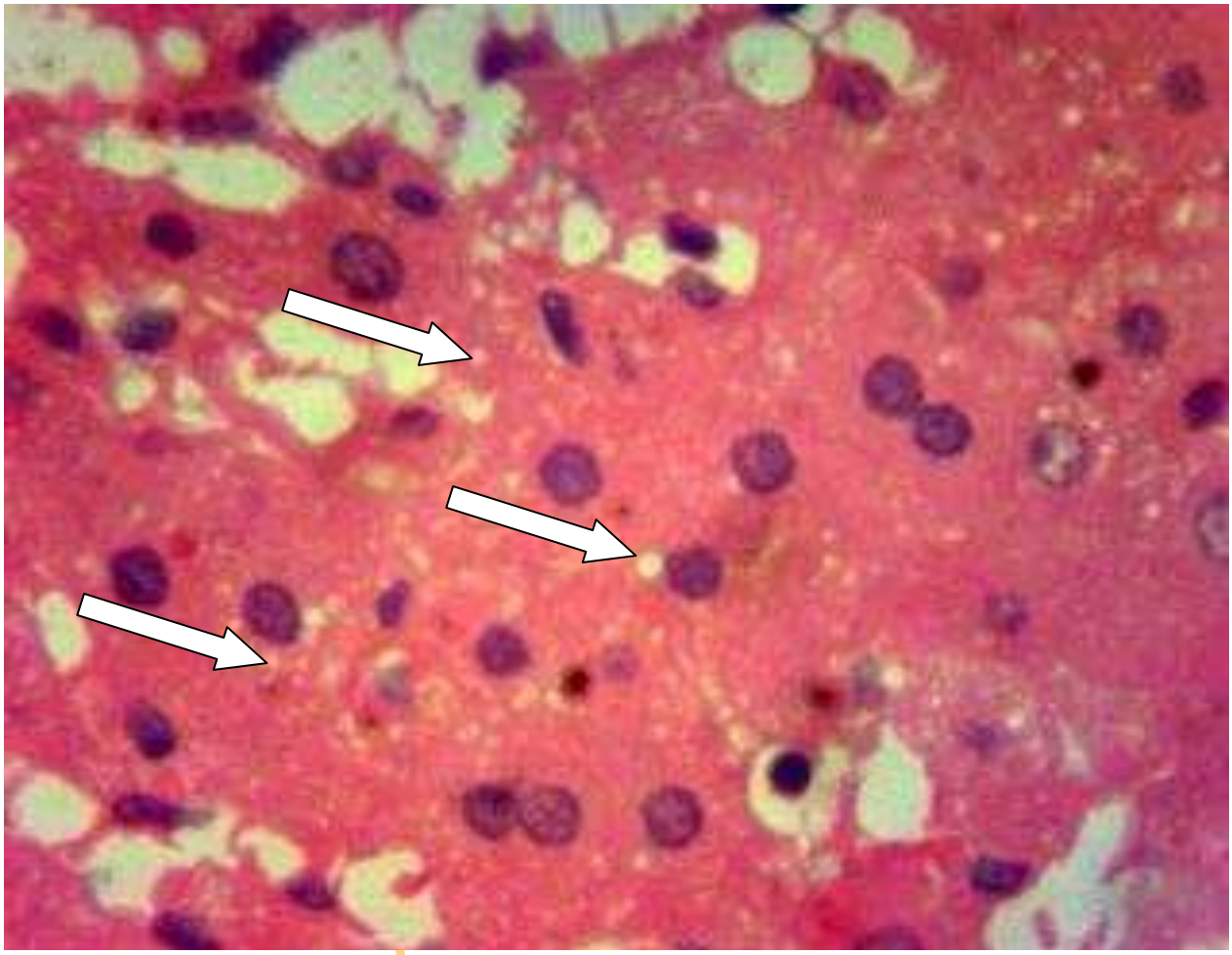


Plate 1C: Photomicrograph of the liver showing enlargement of the hepatocytes with multiple cytoplasmic vacuolations (fatty degenerations) of hepatocytes of rats treated with high doses (50 and 75mg/kg) of *Crinum jagus* (X640).

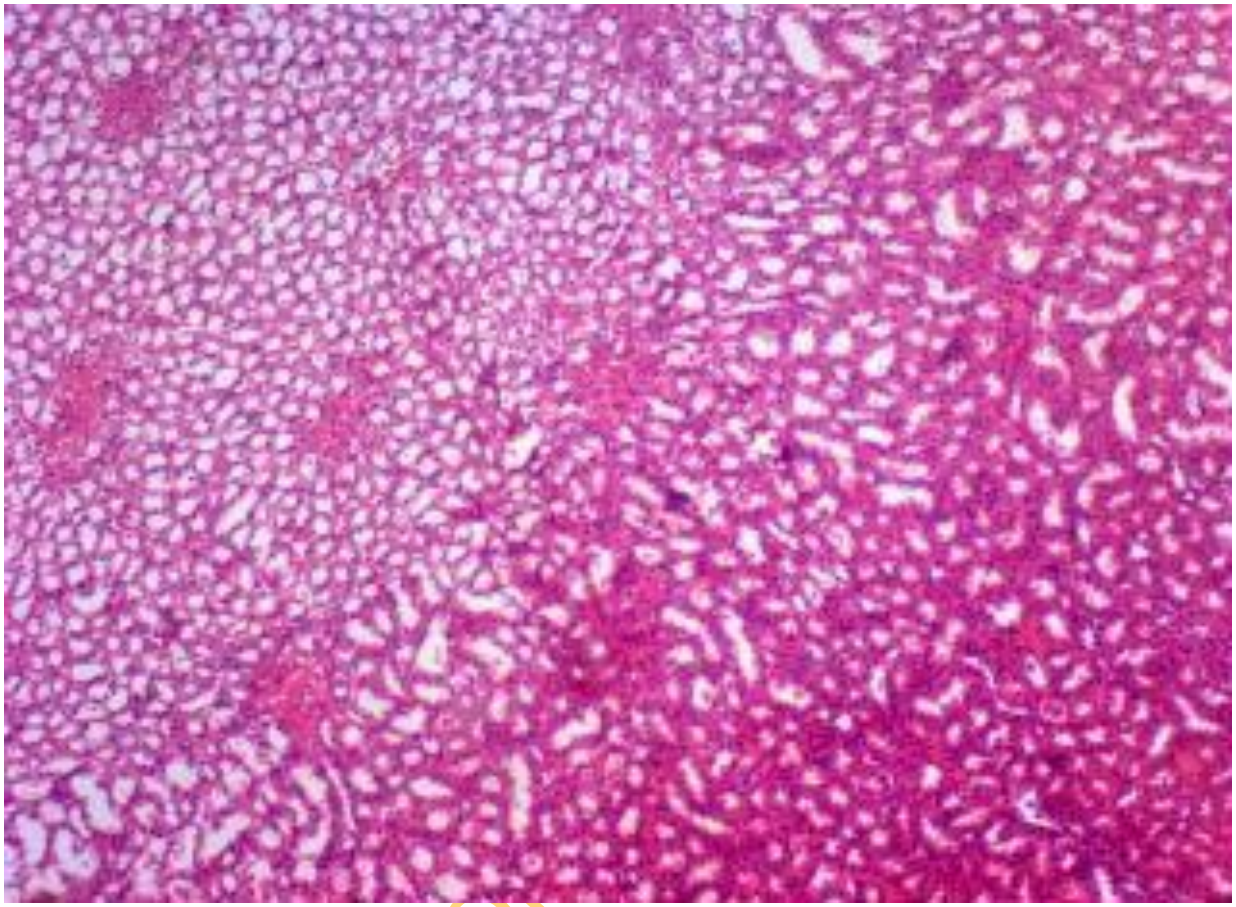


Plate 2A: Photomicrograph of the normal section of the kidney of rats in the control group (X160).

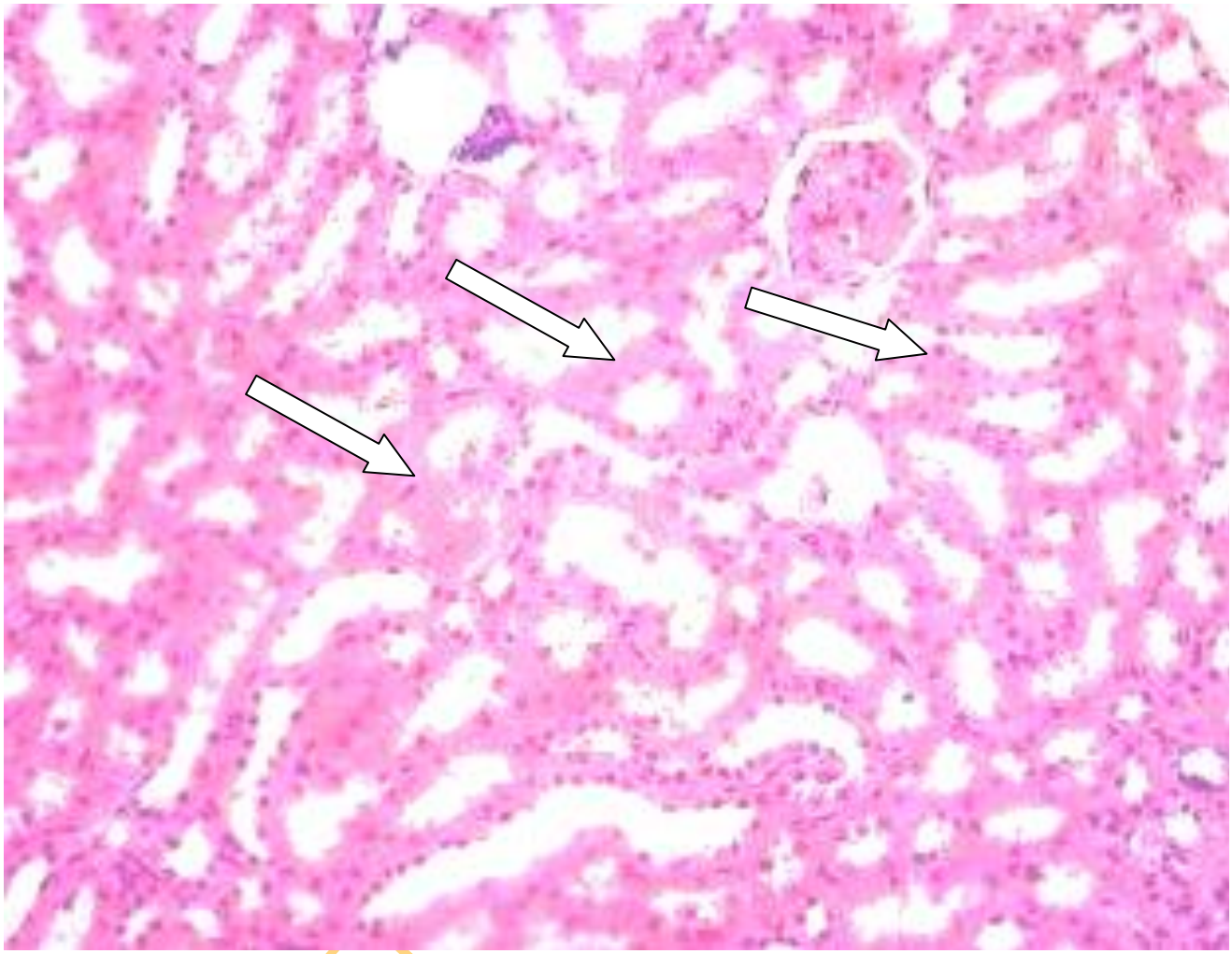


Plate 2B: Photomicrograph of the kidney showing moderate locally haemorrhage in the renal interstitium of rats treated with low doses (10 and 25mg/kg) of *Crinum jagus* (X64).

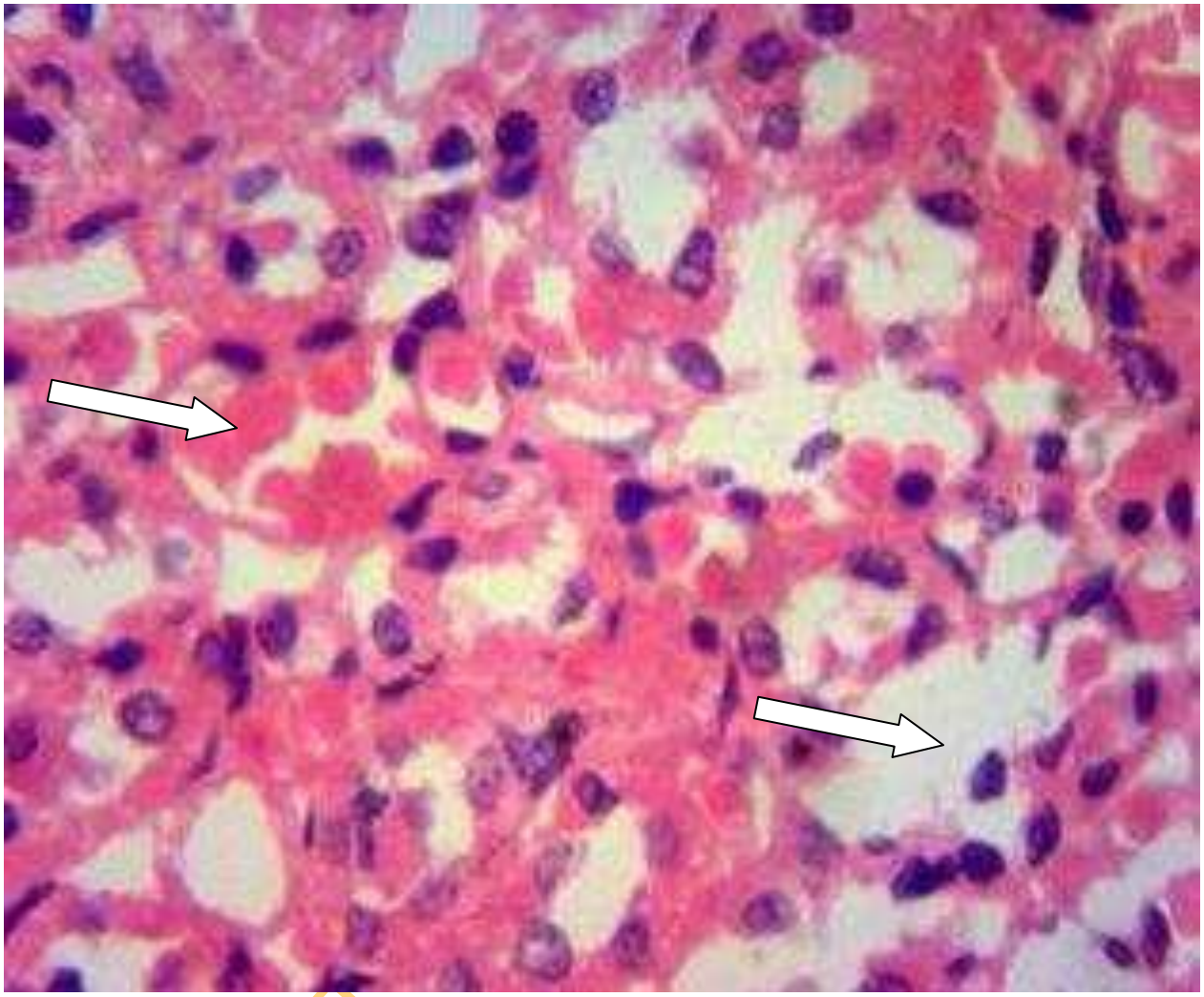


Plate 2C: Photomicrograph of the kidney showing necrosis of the tubular epithelium with sloughing of the necrotics cells into the lumen of the tubules and distruption of the basement membrane of rats treated with high doses (50 and 75mg/kg) of *Crinum jagus.* Some of the tubules denuded and dilated (X160).

Experiment 10 : Determination of Bioactive Compounds in Fraction 1 (F1) of the Bulb of *Crinum jagus* By Gas Chromatography Mass Spectroscopy (GC-MS) Technique

Introduction

Gas chromatography mass spectroscopy (GC-MS) is a method that combines the features of gas liquid chromatography and mass spectroscopy to identify different substances within a test sample. Applications of GC-MS include drug detection, environmental analysis and identification of unknown samples. In the present study, the crude methanol extract and fractions of the bulb of *Crinum jagus* was evaluated for five biological activities. For all the biological activities tested, F1 was the most potent. F1 was therefore analyzed by GC-MS to determine the bioactive compounds in the potent fraction.

Procedure

F1 was analysed by GC-MS using a schimadzu model QP2010 chromatogram, system with split/splitless injector interfaced to a 5973 mass selective detector. Innowax fused silica capillary (FSC) column (30m x 0.25mm with 0.25µm film thickness) was used with helium as carrier gas at a flow rate of 1ml/min. The GC oven temperature was kept at 8^0C (hold for 2mins) and programmed to reach 200^0C at a rate of 4^0C/ minute, then kept constant at 28^0C for 10 minutes being the final hold time. Mass range was from m/z 30 to 50V. Identification of components was achieved on the basis of their retention indices determined with a reference to a homologous series of n-alkanes and by comparison of their mass spectral fragmentation patterns (NIST Data base/ chemstation data system) with data previously reported in literature (Mclafferty and Staufer, 1989, Joulain and Konig, 1998, Adams, 2010).

Results

GC-MS analysis of F1 of the bulb of *Crinum jagus* clearly showed the presence of ten compounds. The active principles with their retention time (RT) and concentration (peak area %) are presented in Table 27. The GC-MS chromatogram of the ten peaks of the compounds detected is shown in Appendix 4. The total number of compounds identified

in the fraction, the GC-MS retention time (RT) and percentage peak of the individual compounds revealed that 5-hydroxymethyl furfural (66.70%) was found as the major component in the fraction while the nine minor compounds in the fractions are 2,3,diyhydro-3,5-dihydroy-6-methyl-4(H)-pyra-4-one (7.58%), 1,2-Hydroxyethyl-4-methyl piperazine (1.68%), E–beta-Farnese (2.80%), Germacrene D, 1-methyl-5-methylene–8 (1-methylethyl)-1,6 cyclodecadiene (1.68%), 1-methyl, 1-4-(5 -methyl-1-methylene-4-hexenylcycloxygenase (1.63%), β-sesquiphellandrene (6.84%), Palmitic acid (2.95%), 5, 6, 8, 9 tetrahydrobenz (A) anthracen-11-(10H)-one (2.88%) 1, 1, biphenyl –2 formly- 4,5,6 trimethoxy–carbaldehyde (5.25%).

Conclusion

In the present study ten chemical constituents have been identified from F1 of the bulb of *Crinum jagus* by gas chromatography mass spectrometry (GC-MS) analysis.

Table 27 : Phyto-components identified in the Fraction 1 (F1) of the bulb of *Crinum jagus*

Peak No	Name of Compound	RetentionTime (minutes)	% Composition
1	2,3,dihydro, 3,5-dihydroxy-6-methyl-4 (H)- pyra-4-one	7.881	7.58
2	5-hydroxymethyl furfural	9.553	66.70
3	1,2 hydroxyethyl-4-methyl piperazine	10.818	1.68
4	E-beta-Farnese	12.170	2.80
5	Germacrene D,1-methyl-5 methylene -8-(1-methylethyl)-1,6 cyclodecadiene	12.756	1.68
6	1-methyl-4-(5methyl-1-methylene-4-hexenylcylooxygenase	12.986	1.63
7	β-sesquiphellandrene	13.227	6.84
8	Palmitic acid	19.927	2.95
9	5,6,8,9 tetrahydrobene (A) anthracen -11 (10H) - one	28.502	2.88
10	1,1,biphenyl,2-formly-4,5,6 trimethoxy carbaldehyde	28.792	5.25
		Total	**100.00**

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CHAPTER FIVE

5.1 DISCUSSION

Plants have been used as sources of medicine by virtually all cultures for several thousand years (Baguar, 1995, Abu-Rabia, 2005). Traditional and folklore medicine play an important role in health services around the globe. About three quarters of the World's population relies on plants and its extract for health care (Premanathan *et a*l., 2000; Gabhe *et al.*, 2006). A large number of people particularly those living in the villages depend largely on herbal remedies. Its acceptability for primary health care is not limited to poor developing countries but in countries where conventional medicine is predominantly in the national health care system (Lafranco, 1999). Therefore, plants constitute the main medicinal source for the treatment of diseases. WHO (2001) reported that herbal medicine serve the health needs of about 80% of the world population, especially for million of people in the vast rural areas of the developing countries. Traditional medicine has remained the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities and local therapy is the only means of medical treatment for such communities (Yinger and Yewhalaw, 2007). Plants have the ability to synthesise a wide variety of chemical compounds that are used to perform important biological functions. Many of these phytochemicals have beneficial effects on long term health when consumed by humans and can be used effectively to treat human diseases. At least, 12,000 such compounds have been isolated so far, a number estimated to be less than 10% of the total (Lai and Roy, 2004, Tapsell *et al.*, 2006). Chemical compounds in plants mediate their effects on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs thus herbal medicine do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicine to be as effective as conventional medicine.

Medicinal plants have pharmacological activities of possible therapeutic use. Many of the pharmaceuticals currently available to physicians have a long history of use as herbal

remedies, including aspirin. Careful and planned investigation of plants are needed in order to develop new drugs that meet the criteria of normal treatment. Ethnobotany is preliminary method of research, suitable for gathering information on the use of plants. The "quack" medical knowledge handed down by the common people constitutes sources of information useful for scientific research. Many plant utilized exclusively in popular tradition, when exposed under scientific examination have been found to be useful for different sectors in the industry (Lentini, 2000). Noteworthy break-through are being produced by researchers and there is a steady progress and indeed very promising trend towards meaningful research into the development of essential drugs from numerous local medicinal plants in the developing countries of the world. Africa is known to be richly endowed with medicinal plants (Winifred, 2011) and one of such plant is *Crinum jagus*.

Crinum jagus is a bulbous plant with spirally arranged leaves and conspicious flower, it belongs to the family; Amaryllidaceae, genus : *Crinum*, species : *jagus*. Its local name is Ogede Odo in Yoruba, Alubarha in Edo, Oyimmbaker in Efik/ Ibibio. It is the largest tropical genus of Amaryllidaceae family (Ode *et al.,* 2010) and the plant may be found in swampy conditions, seasonal wet lands or grassland savannah. In Nigeria ethnomedicine, the use of *Crinum jagus* for various health conditions such as antitumour action (Burkill 1985); antibacterial, antifungal and antiviral actions (Adesanya *et al*., 1992); immunostimulating action (Fennel *et al*., 2001); anticonvulsant action (Edema and Okiemen, 2002); anticholinergic action (Peter *et al*., 2004); anti-tuberculosis action (Idu *et al*., 2010); antioxidant action (Ode *et a*l., 2010); anti-asthmatic action (Ogunkunle and Olopade, 2011); and antimalaria action (Osakwe *et al*., 2011) has been documented. Despite, the reported folkoric uses there are limited scientific claims to substantiate some of these claims. Thus effort in this project was devoted to evaluating the biological activities of *Crinum jagus* to determine and validate the folkoric claims. The biological activities investigated were, antioxidant, antimicrobial, anti-inflammatory, antimalaria and anti-tuberculosis properties of the plant. The toxicological profile of the plant was also investigated to ascertain the safety of the plant.

Antioxidant Activity

During the process of oxygen utilization, in a normal physiological and metabolic process, approximately five percent of oxygen gets univalently reduced to oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals. All these radicals known as reactive oxygen species (ROS) exert oxidative stress towards the cells of human body rendering each cell to face about 10,000 oxidative hits per second (Mondal, 2006). Oxidative stress has been recognized to have a pathological role in many types of chronic diseases such as diabetes, heart disease and cancer. Oxidative stress occurs when the formation of the free radicals increases (Elmastas *et al.*, 2006). In oxidative stress, the balance between the formation of reactive oxygen species and amount of antioxidants is destroyed. Oxidative stress causes damage to cell components such as proteins, lipids and nucleic acid (Rahim *et al.*, 2005; Wright *et al.*, 2006; Gladine *et al.*, 2007) and eventually leads to cell death (Naziroglu *et al.*, 2004; Emekli-Alturfan *et al.*, 2009). Recently, interest has increased considerably in finding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants which are being restricted due to their side effects such as carcinogenicity (Kumaran, 2007). Antioxidants from natural resources possess multifacetedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance (Shriwaikar, 2006). Food industry uses natural antioxidants as a replacement of conventional synthetic antioxidants (Govindaragan, 2003). In this study, the antioxidant activity of the crude methanol extract and fractions of the bulb of *Crinum jagus* was assessed using the *in-vitro* and *in-vivo* models.

***In vitro* Antioxidant Activity**

In vitro antioxidant capacity of the plant was studied by analyzing five biochemical parameters which are indicative of antioxidant potential of the plant. The studied biochemical parameters were DPPH radical scavenging activity, hydroxyl radical scavenging activity, reductive potential, total flavonoid and total phenolic contents of the plant.

DPPH assay is one of the most widely used method for screening antioxidant activity (Nanjo *et al.*, 1996). Diphenylpicrylhydrazyl (DPPH) is a free radical that is stable at room temperature. It produces a purple or violet colour in methanol. On reduction in the presence of an antioxidant, a yellow solution is produced. The percentage DPPH radical scavenging activity of an antioxidant is a measure of its ability to donate a proton to the DPPH free radical to yield diphenylpicrylhydrazine which is more stable giving rise to yellow colour. In this study, the crude methanol extract and the fractions of the bulb of *Crinum jagus*, all scavenged DPPH in a concentration-dependent manner (Figures 16 and 17). At 500µg/ml the crude extract, F1, F2 and F3 scavenged DPPH radicals by 72.46%, 81.37%, 57.83% and 69.83%, respectively. Highest percentage scavenging effect was obtained with F1 , though the DPPH scavenging activity of extract and the fractions were less than that of ascorbic acid (the standard antioxidant). The study showed that the crude extract and the fractions have proton donating ability and could serve as free radical scavengers acting possibly as primary antioxidants. This observation is in line with the findings of Ode *et al*., (2010) who reported the DPPH scavenging effect of the crude methanol extract of the bulb of *Crinum jagus.*

Hydroxyl radical is the final mediator of most tissue damage (Lloyd *et al.*, 1997). All reactive oxygen species exert most of their pathological effects by giving rise to hydroxyl radical because hydroxyl radical react with almost every type of molecules found in living cells including sugars, amino acids, lipids and nucleotides. Hydroxyl radical formation can occur in several ways. It can be generated by univalent reduction of oxygen (Han *et al*., 2003). The most important mechanism by which it is generated *in-vivo* is likely to be transition metal catalyzed decomposition of superoxide and hydrogen peroxide (Stohls *et al*., 1995). The percentage hydroxyl radical scavenging activities of the crude extract and its fractions are presented in Figures 18 and 19. The crude extract and its fractions scavenged hydroxyl radical in a concentration dependent manner. The crude extract, F1, F2 and F3 at 500µg/ml scavenged hydroxyl radical by 71.20%, 88.32% ,66.74%, 75.81%, respectively. The highest percentage hydroxyl radical

scavenging activity was detected in F1 (88.32%) at 500µg/ml. The hydroxyl radical scavenging activity of F1 compared well with that of ascorbic (89.32%) at 500µg/ml.

Hydroxyl radical induces various injuries to the surrounding organs and play a vital role in some clinical disorders. Any compound, natural or synthetic with antioxidant activity might contribute towards the total or partial elevation of this damage. Therefore, removing hydroxyl radical is one of the effective defence of a living body against diseases (Jer Min *et al.*,1995). This study showed that the crude extract and fractions of the bulb of *Crinum jagus* possess antioxidant activity which could exert a beneficial action against pathological alteration caused by the presence of hydroxyl radicals.

Reducing power of a compound serves as a significant indicator of its potential antioxidant activity (Gulcin and Oktay, 2003). The reducing ability of a compound depends on the existence of reductones (antioxidant) which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom (Meir *et al.*, 1997 *)*. The reducing power of the extract and the fractions increased with increasing amount of the sample. The conversion of Fe^{3+} into Fe^{2+} in the presence of the extract and the fractions was used to determine the reducing ability of the plant. F1 showed the highest reducing ability. The antioxidant activity of the crude extract and the fractions might partially be as a result of its reducing ability.

The concentrations of total flavonoid content of the plant extract and their fractions are shown in Figures 22 and 23 while that of the total phenolic content are presented in Figures 24 and 25. At a concentration of 500µg/ml, the total flavonoid content of the crude extract, F1, F2 and F3 were 0.584, 0.864, 0.396 and .0643µg/g quercetin equivalent respectively. The total phenolic content of crude extract and the fractions at 500µg/ml were 0.356, 0.460 ,0.240 and 0.380µg/mg garllic acid equivalent respectively. The highest concentration of total flavonoid and total phenolic were obtained from F1. Phenolics are ever present secondary metabolite in plants and possess wide range of therapeutic uses. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. Total phenolic assay by using Folin-Ciolcateu reagent is a simple,

convenient and reproducible method. It is employed in studying phenolic antioxidants (Huang *et al*., 2005).

Flavonoids are groups of naturally occurring compounds widely distributed as secondary metabolites in plant kingdom and have been reported to possess antioxidant and anti-radical properties (Nakayoma and Yamada, 1995). The protective effect of many flavonoids and other phenolic compounds in leaves are considered to be related to various antioxidant contained in them (Shahidi *et al*., 1992), while a strong correlation between total phenolic content and anti-oxidant activity has been reported by Velioglu *et al.*, (1998) and Javanmardi *et al.*, (2003). The presence of phenolic and flavonoid contents in the crude extract and fractions of the bulb of *Crinum jagus* may have contributed directly to the antioxidant activity of the plant by neutralizing the free radicals. The preliminary phytochemical screening of the extract showed the presence of phenols and flavonoids.

***In vivo* Antioxidant Activity**

Antioxidants can be classified into two major classes. The non enzymatic and enzymatic antioxidants. The enzymatic antioxidants include superoxide, catalase, and glutathione peroxidases. Crucial components of antioxidant defence system in the body are cellular antioxidant system which are involved in the reduction of reactive oxygen species (ROS) and peroxides as well as in the detoxification of certain compounds of exogenous origin, thus playing a primary role in the maintenance of a balanced redox status (Michiels *et al*., 1994) hence they can serve as potential marker of susceptibility, early and reversible tissue damage and of decrease in antioxidant defence (Lester, 1994). In the present study, the *in vivo* antioxidant capacity of the crude extract and the fractions of the plant was evaluated by studying the effect of thirty days oral administration of the extract and fractions of the plant on hepatic and renal malondialdehyde (Lipid peroxidation), superoxide dismutase, catalase and glutathione levels in experimental animals.

Malondialdehyde (MDA) is the major oxidative product of peroxidised polyunsaturated fatty acid and increase in malondialdehyde content is an important

indicator of lipid peroxidation . The present study assessed the effect of thirty days oral administration of the crude extract and fractions of the bulb of *Crinum jagus* on malondialdehyde concentrations in the liver and kidney of rats. Thirty days oral administration of the crude methanol extract of the bulb of *Crinum jagus* (10, 25, 50 and 75 mg/kg) caused no significant reduction in malondialdehyde (MDA) level in the liver while a significant ($P < 0.05$) and dose dependent reduction of MDA level was observed in the kidney compared to the control group. Fractionation of the crude extract does not cause loss of biological activity but rather improved the antioxidant activity of the plant. The three fractions (F1, F2 and F3) at 5 and 10mg/kg caused significant reduction ($P < 0.05$) in the hepatic and renal MDA levels relative to the control group. F1 showed the highest inhibitory activity. Significant reduction of MDA levels by the crude extract and fractions of the bulb of *Crinum jagus* indicate that the plant has protective potential against free radical induced lipid peroxidation both in liver and kidney by promoting cell membrane stability.

Superoxide dismutase and catalase are endogenous protective factors, they act by scavenging free radicals in the defense against oxidative cell injury. The enzyme are the major enzymes that catalyse the inactivation of reactive oxygen species derived from redox process of xenobiotics in the liver (Malchin and Benedich, 1987). In the present study, thirty days of oral administration of the crude methanol extract of the bulb of *Crinum jagus* resulted in significant ($P < 0.05$) increase in superoxide dismutase activity in the liver in the 25, 50 and 75mg/kg treated groups compared with the control group. The kidney also demonstrated a significant($P < 0.05$) and dose dependent elevation of superoxide dismutase activity in all the treated groups. The two doses of F1 (5 and 10mg/kg) administered for 30 days produced significant elevation of superoxide dismutase both in the liver and kidney. With F3, significant ($P < 0.05$) elevation of superoxide activity was only observed with the highest dose (10mg/kg) both in the liver and kidney. F2 caused an insignificant elevation of SOD activity in the liver at 10mg/kg while in the kidney, administration of F2 elevated superoxide activity. The observed increase in SOD activity suggests that the crude methanol extract and fractions of the

bulb of *Crinum jagus* has an efficient protective mechanism in response to reactive oxygen species (ROS).

Catalase is an antioxidant enzyme distributed in all animal tissues including red blood cells and liver. Catalase is considered as most important hydrogen peroxide removing enzyme and also a key component of antioxidative defense system. Catalase is a tetametric haemoprotein that undergoes alternate divalent oxidation and reduction at its active site in the presence of hydrogen peroxide. It is known that hydrogen peroxide can be destroyed by catalase which removes it when present in high concentration (Cascado *et al.*, 1995). The present study reveals that catalase activity was significantly increased in a dose dependent manner after thirty days of oral administration of 10, 25, 50 and 75mg/kg of the crude extract of the bulb of *Crinum jagus*. The two doses of F1 administered (5 and 10mg/kg) produced a significant elevation of catalase activity in the liver and kidney. F2 and F3 did not produced significant elevation of catalase activity in the liver and kidney after administration of 10mg/kg dose for thirty days. The antioxidant activity of the crude extract and fractions of the bulb of *Crinum jagus* may be related to their increased catalase activity in the liver and kidney resulting in the protection of the tissue by catalase.

Glutathione is a major non protein thiol in living organisms, it plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. The level of reduced glutathione (GSH) consumption is a measure of non enzymic antioxidant and cellular redox status of cells of higher animals (Chance and Boveris, 1979). Result from this study showed that oral administration of different doses of the crude methanol extract of the bulb of *Crinum jagus* increased the level of reduced glutathione in the liver and kidney relative to control. The liver and kidney glutathione levels were significantly elevated in the groups treated with F1 and F3 compared with the control group with a more pronounced effect with F1. F3 only produced significant elevation of reduced glutathione levels in the liver and kidney at higher doses. F1 demonstrated significant elevation of hepatic and renal glutathione when both low and high doses of F1 (5 and 10mg/kg) were administered. F2

did not produce significant elevation of hepatic and renal glutathione levels when compared with the control group. The result from this study showed that the crude methanol extract and fractions of the bulb of *Crinum jagus* increased the overall redox status in the liver and kidney as indicated by significant increase in the level of reduced glutathione in both liver and kidney compared with the control group.

Glutathione-S-transferase (GST) is a family of multi functional isoenzymes found in all eukaryotes catalyzing both glutathione dependent and reduction reaction (Rajurkar *et al.*, 2003). One major function of GST is to catalyse biotransformation of xenobiotics including drug detoxification in the mercapturic acid pathway leading to elimination of toxic compound (Hayes and Pulford, 1995) and also acting as antioxidant enzyme (Adaramoye and Adeyemi, 2006). The result from this finding shows that administration of the varying concentration of the crude methanol extract as well as F1 and F3 of the bulb of *Crinum jagus* significantly ($P < 0.05$) increased the activity of GST relative to control in both liver and kidney. F2 produced a non significant increase in hepatic and renal GST activity. Studies have shown that moderate oxidative stress may result in an induction of GST while severe oxidative stress may result in its decrease with concomitant depletion of GSH (Aniya and Nauto, 1993) which is a measure of the overall redox status of the cells in the blood. Increase in the level of GST activity, following thirty days of oral administration of the crude methanol extract and fractions of the bulb of *Crinum jagus*, is an indication that the plant plays an important role as a scavenger of free radicals. As the crude extract and fractions of the bulb of *Crinum jagus* improved the SOD, catalase, glutathione levels and reduced lipid peroxidation in the liver and kidney. It can be concluded that the crude extract and fractions of the bulb of *Crinum jagus* possesses *in-vivo* antioxidant activity and can be employed in protecting tissue from oxidative stress with F1 showing the highest antioxidant activity.

Antimicrobial Activity

The use of plant-derived compounds to treat infections is an age-long practice in many parts of the world, especially in developing countries where there is dependence on traditional medicine for a variety of diseases (Shiba *et al.*, 2005; Gangoue-Pieboji *et al.*,

2006). Even though, pharmaceutical industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, action must be taken to reduce this problem, for example to control the use of antibiotics, develop research to better understand the genetic mechanisms of resistance and to continue studies to develop new drugs, either synthetic or natural. The interest in plants with antimicrobial properties was revived as a result of current problems associated with the use of antibiotics (Shiota *et al.*, 2004; Abu-Shanab *et al.*, 2004). The use of plant extracts and phytochemicals for antimicrobial properties can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Sousa *et al.*, 1991; Shapoval *et al.*, 1994; Izzo *et al.*, 1995).

Crinum jagus is a known medicinal plant widely used in Africa as an antimicrobial agent (Adesanya *et al.*,1992). In the present study, the antibacterial and antifungal activities of the crude methanol extract and fractions of the bulb of *Crinum jagus* against several human pathogenic bacteria and fungi was investigated to provide scientific justification for the traditional use of the plant for the treatment of infectious diseases.

The results of the antimicrobial screening of the crude methanol extract and fractions of the bulb of *Crinum jagus* are presented in Tables 11, 12, 13, 14 and 15. The crude extract exhibited considerable level of inhibition against all the test organisms, with the exception of *E.coli*. This is in consonance with the frequently reported cases of development of multi-drug resistance to many antibiotics by bacteria of which *E.coli* is the most prominent (Alonso *et al.*, 2000; Sader *et al.*, 2002; Fasuan, 2004). *Klebsiella prenumonae* was the most susceptible bacterium to the extract, of all the tested bacteria with inhibition zones ranging from 10.00-28.00mm while *Aspergillus niger* was the most susceptible fungus with inhibition zones ranging from 10.00-16.00mm. The fractions (F1, F2 and F3) inhibited the growth of all the test organisms including *E. coli* which was not inhibited by the crude extract.

The result of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the crude extract and fractions of the plant are presented in Tables 12, 16, 17 and 18. The crude extract demonstrated considerable antimicrobial activity with its MIC against Gram-positive bacteria ranging between 6.25-50mg/ml and that of Gram-negative bacteria ranged between 3.125-50mg/ml. MIC for the fungal isolates ranged between 25-200mg/ml. The extract showed the lowest MIC against *Klebsiella pneumonae* (3.125mg/ml). The broadest activity of the crude extract against most of the test bacteria was MIC of 12.5mg/ml. *Candida albicans* and *Candida tropicalis* had the lowest MIC value (25mg/ml) against the extract. The broadest activity of the extract against most fungi tested was MIC of 50mg/ml. The extract also showed bactericidal and fungicidal activities on the bacteria and fungi isolates. The broadest bactericidal activity of the extract against most of the test bacteria was MBC of 25mg/ml. *Klebsiella prenumonae* was the most susceptible bacteria to the plant extract. *Candida albicans and Candida tropicalis* were the most susceptible fungi to the extract with MFC value of 50mg/ml. The broadest activity of the extract against most of the fungi tested was observed at MFC. of 100mg/ml. The results of MIC determination showed that F1 wass the most potent of all three fractions tested. The MIC, MBC and MFC values of F1 for bacteria and fungi isolates were much lower than that of F2 and F3. The MIC value of F1 ranged between 0.20-3.125µg/ml for bacteria isolates and 0.39-3.125µg/ml for the fungi isolates. *Staphylococcus aureus* was the most susceptible bacteria to F1 at MIC value of 0.20µg/ml. *Candida albicans, Candida tropicals* and *Candida krusei* were much sensitive to F1 than the moulds with MIC values of 0.39µg/ml. *E.coli* which was not inhibited by the crude extract was inhibited by F1 at MIC value of 0.39µg/ml. The MBC and MFC value of F1 for bacteria and fungi were much lower than that of F2. F2 had moderate antibacterial and antifungal activities against the test organisms. The lowest MIC value of F2 was 0.39µg/ml against two of the bacteria and the lowest MIC value of 0.78µg/ml were observed against four of the fungi isolates. Also the MBC value of F2 for bacteria and fungi isolates were higher than that of F1, the lowest MIC value obtained for F3 was

0.39µg/ml against *Bacillus subtilis* and *E.coli* while for the fungi isolates, the lowest MIC value obtained was 1.56 µg/ml that is much higher than the MIC values of F1 and F2.

The broad spectrum antimicrobial activities of the plant extract may possibly be due to the identified phytochemicals in the plants such as alkaloids, flavonoids, saponins and phenols. These classes of compounds are known to have curative activity against several pathogens and therefore could suggest the use of the plant as a health remedy in folklore medicine (Hassan *et al.*, 2004; Usman and Osuji, 2007). Fractionation of the extract did not lead to the loss of antimicrobial activity against the same group of isolates rather the fractions were found to have a better and improved antimicrobial activity than the extract against the range of Gram-positive bacteria and Gram-negative bacteria, the fungal yeast and the moulds tested. F1 demonstrated the highest antimicrobial activity. This observation is in line with the findings of Adesanya *et al*., (1992) who reported that some alkaloids present in *Crinum jagus* possess antibacterial and antifungal activities.

Ability of an antimicrobial agent to inhibit the growth of bacterial (bacteriostatic) or to destroy the bacteria (bactericidal) are the two main properties searched for in a potential antimicrobial agent since each has its own importance. Bacteriostatic activity may be useful in mild bacteria infection in which the bacteriostatic agent will inhibit the growth of the microorganisms while the defence mechanism will take over to get rid of the offending organisms. From the study, it was suspected that the activity of F1 may be bactericidal in nature while the crude extract, F2 and F3 were bacteriostatic. The results are encouraging enough to pursue bioactivity guided fractionation and structural elucidation of F1, the most potent fraction as possible antimicrobial agent. These findings lend credence to the traditional use of *Crinum jagus* for the treatment of microbial infections.

Inflammatory Activity

Medicinal plants have been used as a form of therapy for the relief of pain throughout history (Almieda *et a*l., 2001). The treatment of rheumatic disorder is an area in which the practitioners and traditional medicine enjoy patronage (Akah and Mwabie, 1994). Natural products in general and medicinal plants in particular are believed to be

important sources of new chemical substance with potential therapeutic efficacy. The study of plant species traditionally used as pain killers is seen as fruitful research strategy in the search of new analgesic and anti-inflammatory drugs. However, for many of the plants in use, the real efficacy and the relevant active principles are unknown. Consequently, experimental studies aimed at demonstrating the pharmacological properties of these plants and identifying the relevant active principles are needed (Sosa *et al.*, 2002*). Crinum jagus* is reported in traditional literature for the treatment of snake bite (Ode *et al*., 2006), tuberculosis (Idu *et al*., 2010) and asthma cough (Ogunkunle and Olapade, 2011) which are all inflammatory disease. Therefore, the present research was undertaken to investigate the antiinflammatory activity of the crude extract and fractions of the plant using the carragenan induced rat paw oedema method (Winter *et al.*, 1962). The results of this study are presented in Figures 41 and 42. From the results it was observed that the crude methanol extract, the fractions of the plant and indomethacin significantly ($P < 0.05$) reduced the paw oedema 3 hours after carrageenan injection relative to the control group. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) significantly ($P < 0.05$) inhibited oedema formation in the rat paw by 26.82%, 31.55%, 41.82 and 65.90%, respectively. At a dose level of 20mg/kg, F1, F2 and F3 significantly ($P < 0.05$) inhibited oedema formation in rat paw by 79.50%, 25.00% and 52.27%, respectively. The standard drug indomethacin at 5mg/kg inhibited oedema formation by 94.82%. The result showed that F1 and F3 were more potent as anti-inflammatory agent than the crude extract and F2 with F1 having the highest inhibitory activity (79.50%). This study has shown that the crude methanol extract and fractions of the bulb of *Crinum jagus* possess a significant anti-oedematogenic effect on paw oedema induced by carrageenan. Development of oedema induced by carrageenan is commonly correlated with early exudative stage of inflammation (Ozaki, 1990, Silva *et al.*, 2005). Carrageenan oedema is a multimediated phenomenon that liberates diversity of mediators. It is believed to be biphasic, the first phase (1hr) involves the release of serotonin and histamine while the second phase (over 1h) is mediated by prostaglandins, the cycloxygenase products and the continuity between the two phases is provided by

kinins (Silva *et al*., 2005; Perianayamgam *et al*., 2006). Since carrageenan induced inflammation model is a significant productive test for anti-inflammatory agents acting by the mediators of acute inflammation (Mossai *et al*., 1995; Sawadogo *et al*., 2006), the result of this study indicates that *Crinum jagus* can be effective in acute inflammatory disorders. The study has thus provided some justification for the folkoric use of the plant in several communities for conditions such as snake bite, tuberculosis and asthma cough. F1, due to its high and potent anti-inflammatory property will be chosen for further studies in which the active principle from the fraction will be isolated and this will help in understanding the mechanisms of the antinflammatory activity of *Crinum jagus*.

Antimalaria activity

Medicinal plants may provide antimalarial drugs directly as in the case of quinine from cinchona bark or they may supply template molecules on which to base further new structures by organic synthesis. The folkoric use of *Crinum jagus* for the treatment of malaria has been reported in literature (Osakwe *et al*., 2011). In this study the *in vivo* antimalaria activity of the crude extract and fractions of the plant were investigated. The result of the *in-vivo* evaluation of the crude extract and fractions of the bulb of *Crinum jagus* on established infection are presented in Table 18, Figures 43 and 44. Treatment of *Plasmodium berghei* infected mice with the extract and the fractions produced a dose dependent chemosuppression activity. Percentage suppression of parasitaemia was observed to increase as extract concentration increased. By day 6 post infection, there was significant ($P < 0.05$) reduction in percentage parasitaemia in the treated groups relative to the control group. The percentage parasitaemia in the groups treated with varying concentrations of the crude extract of the plant (10, 25, 50 and 75mg/kg) were 4.99%, 4.46%, 3.95% and 2.29%, respectively, while the percentage parasitaemia of 2.13% and 18.72% were observed for arteether treated and negative control groups respectively. By day 6 post infection, there was a complete clearance of parasitaemia by chloroquine. The groups treated with 10mg/kg of F1, F2 and F3 had percentage parasitaemia of 2.00%, 4.47% and 3.50% respectively. After four days treatment, the varying concentrations of the plant extract (10, 25, 50 and 75mg/kg) produced

chemosuppression of 70.10%, 76/17%, 78.90% and 87.17%, respectively. F1, F2 and F3 at 10mg/kg had chemosuppression of 89.33%, 76.12% and 77.70% respectively while chloroquine (10mg/kg) and arteether (3mg/kg) caused 100% and 88.60% suppression, respectively.

Mean survival time (MST) of 25 and 23 days respectively were observed for chloroquine and arteether treated groups compared to 19, 20, 21 and 22 days respectively observed for the groups treated with 10, 25, 50 and 75mg/kg of the crude extract. Fractions F1, F2 and F3 at a dose level of 10mg/kg had a mean survival time of 25, 21 and 21 days respectively. The untreated control groups survived only for 12.5 days (Table 19)

During the 4 days treatment, the packed cell volume (PCV) of the parasite infected untreated animals decreased progressively until they all died while the PCV of chloroquine treated group increased progressively. The PCV of the treated groups increased during the 4 days treatment, but when treatment was withdrawn, increase in PCV was only observed in chloroquine treated group. However in arteether, crude extract and fractions treated groups, there was a decrease in PCV due to recrudesence of parasitaemia observed in the arteether, extract and the fractions treated groups (Fig 45 and 46). The crude methanol extract of the bulb of *Crinum jagus* have been reported to contain some phytochemcial compounds like alkaloids, flavonoids, saponins, steroids, glycosides, terpenes (Ode *et al.*, 2010). The presence of these phytochemicals in this plant might be responsible for the antimalaria activity exhibited by them. Phytochemical compounds such as terpenoids are commonly implicated in the antiprotozoal and antiplasmodial activity of many plants (Philipson *et al.*, 1991; Francoise *et al*., 1996; Ghosal *et al*., 1996; Asase *et al*., 2010). An example of common terpenoids is artemisinin, the main active ingredient in the traditional Chinese antimalaria qinghaosu. Flavonoids showed significant antiparasitic activities against different strains of malaria parasites, trypanosome and Leishmania (Kim *et al.*, 2004; Monbrison *et al*., 2006; Tasdemir *et al*., 2006). Flavonoids are reported to chelate with nucleic acid base pairing of the parasite (Lui *et al.*, 1992). These chemical compounds may be acting singly or in

synergy with one another to exert the observed antimalaria activity of *Crinum jagus*. The plant *Crinum jagus* was observed to show antimalaria activity by its percentage chemosuppression compared to that of chloroquine, the standard antimalaria drug. The activity might be attributed to the presence of alkaloids or flavonoids which have been identified in this work or even a combined action of more than one metabolite. However, the active compounds, known to give this observed activity need to be identified. The antimalaria activity of the plant might also results from its antioxidant property. Reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, hydrogen peroxide which do not cause any damage under physiological conditions but at high concentration can cause cellular damage are produced during *Plasmodium* infection (Farombi *et al*., 2003), the extract and its fractions were able to reverse these conditions by increasing the activity of superoxide, catalase and decreasing lipid peroxide produced in the *Plasmodium i*nfection, thereby further confirming the antioxidant activity of the plant. This study has however established the rationale for traditional use of this plant as remedy for malaria infection and its potential development as an antimalarial agent .

Antitubercular Activity

Tuberculosis is a highly infectious disease with about one third of the world's population estimated to be infected (Agarwal, 2004). It kills about three million people per year world wide. It is a major health problem for developing countries. This problem has become serious as *Mycobacterium tuberculosis* developed resistance against both the first line and second line drugs. Due to this, there is emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tuberculosis*. Natural products are proven template for the development of new scaffolds of drugs (Butler, 2005; Chin *et al.*, 2006) and they have received considerable attention as potential anti-TB agents (Pauli *et al*., 2005). Antimycobacterial activity from hexane extract of *Adhatoda vasica* has been reported by Ignacimuthu and Shanmugam, (2010). There are also reports that antimycobacterial activity is noted in Aloe vera and garlic when tested against H37Rv (Jain, 1993; Ratnakar and Murthy, 1996; Reynolds and Dweck, 1999). Gupta *et al.*, (2010*)* reported antituberculosis activity from *Acalyphia indica* and *Allium cepa* extracts

against *Mycobacterium tuberculosis*. There are reports from folklore which claim that the bulb of *Crinum jagus* are used in Southern part of Nigeria for treatment of TB infection hence in this study the antibacterial activity of extract of the bulb of *Crinum jagus* against *M .tuberculosis*, the aetiological agent of TB was investigated.

The results of antitubercular activity of the crude methanol extract and fractions of the bulb of *Crinum jagus* are presented in Tables 21, 22, 23 and 24. The crude extract at various concentrations showed a concentration-dependent inhibition of the *M. tuberculosis* isolates and H37Rv strain in both Lowenstein Jensen (L-J) and Middlebrook 7H10 media. All the three fractions (F1, F2 and F3) inhibited *M. tuberculosis* isolates and H37Rv strain in a concentration dependent manner in both L-J and Middlebrook 7H10 media. The fractions were more potent than the crude extract with higher inhibition values. F1 was the most potent with the highest inhibitory value (83%) which compared well with the inhibitory activity of the standard drugs. At a concentration of 1.0mg/ml, the percentage inhibition of F1, rifampicin and isoniazid against MTB 3 (the most susceptible isolate) was 83%, 95% and 84%, respectively. F1 had the lowest IC_{50} value which compared well with IC_{50} value of the standard drugs. The IC_{50} value of F1, rifampicin and isoniazid against MTB 3 the most susceptible isolate) in L-J medium were 0.27, 0.21 and 0.20mg/ml respectively while the IC_{50} value of F1, rifampicin and isoniazid against MTB 3 in Middle brook 7H10 medium were 0.22, 0.19 and 0.20mg/ml respectively (Tables 23 and 24).

The antimycobacterial activity showed that bulb of *Crinum jagus* has the potential to cure tuberculosis and is a promise for future therapeutic interventions. The results from the study provide for the first time, the scientific evidence for the traditional use of *Crinum jagus* for the treatment of tuberculosis. The results assume significance and throw some light on the basis of the use of the plant in our traditional systems of medicine and in folklore. Of all the antitubercular drugs in various stages of clinical evaluation, a diarylquiniline-based drug (TMC 207) has been found to be an inhibitor of the FO subunit of the mycobacterial adenosine triphosphate (ATP) synthase proton pump (Andries *et al.*, 2005; Koul *et al*., 2007) which is a novel mechanism of action against

M. tuberculosis (Haagsma *et al.,* 2009). Therefore further detailed phytochemical screening and bioactivity studies need to be carried out using crude solvent extracts as well as further purified constituents to ascertain the active antitubercular ingredient of *Crinum jagus* to comprehend their role in antituberculosis activity and develop suitable drugs so that the most deadly disease in the world can be combated. The present study also could pave the way towards possibility to obtain anti-mycobacterial moieties against other mycobacterial species.

Toxicological Study

The use of plants for healing purposes is very common in developing countries especially in the rural areas. This is probably due to the perceived beneficial and lower side-effect profile of natural products that are extracted from plants (Leonardo *et al*., 2000). However, most medicinal plants are used indiscriminately without knowing their possible adverse effect. Over the past decades, several reports in both developed and developing countries have indicated adverse effects allegedly arising from the use of medicinal plants (Elvin Lewis, 2001). Some of these effects include abortion of pregnancy, dizziness, vomiting, diarrhea, abdominal pain, fast heart beat, death, ulcer and loss of appetite (Gessler, *et al*., 1995). These effects could be attributed to the presence of phytotoxic compounds in the plant extracts and lack of actual dosage necessary for the treatment of diseases (Azaizeh *et al.,*2003). In order to minimize this draw back, there is need for thorough scientific investigation on the toxicological effect of these plants at different doses. The world health organization (WHO) has recommended that traditional plants used for the treatment of disease warrant further evaluation of their toxicological properties.

Crinum jagus has various therapeutic uses. These include treatment of microbial infections, convulsion, snake bite, tuberculosis, asthma (Adesanya *et al*., 1992; Edema and Okiemen, 2002; Ode *et al*., 2006; Ogunkunle and Olopade 2011). However, there was no information in the scientific literature on the toxic effect of *Crinum jagus* on the haematological and biochemichal parameters in rats. Therefore, the present study was undertaken to assess the possible toxicological effects of the bulb of *Crinum jagus* extract

using haematology, serum chemistry, liver and kidney functional indices in the animal model.

The result of this study showed that the treated rats were able to maintain growth irrespective of the number of days the extract was administered to them. The study therefore, suggests that the crude methanol extract of the bulb of *Crinum jagus* did not interfere with the body weight of the treated rats (Fig 47). This revealed the potential of this plant in controlling muscle wasting.

Tissue enzyme assay can indicate tissue cellular damage long before structural damage is revealed by some other conventional technique (Akanji, 1986). The effect of crude methanol extract of the bulb of *Crinum jagus* on serum liver and kidney enzymes following administration for thirty days revealed that there were non significant ($P > 0.05$) differences in the serum, liver and kidney levels of AST, ALT and ALP in the rats treated with 10 and 25mg/kg of the extract however, the levels of AST, ALT and ALP in the serum, liver and kidney were significantly ($P < 0.05$) elevated by administration of 50 and 75mg/kg of the extract when compared with the control group (Figures 48, 49 and
50). AST, ALT and ALP are marker enzymes for liver function and integrity (Jens and Hanne, 2003). Liver damage is associated with elevated levels of these enzymes. ALT is a more specific enzyme of damage and known to increase when there is liver cell damage and it has been employed as a tool for measuring hepatic necrosis (Bush,1991). It is located in the liver and increase in its concentration suggests that there is liver damage which can cause enzyme leakage from liver into blood stream. ALP is a marker enzyme for plasma membrane and endoplasmic reticulum (Wright and Plummer 1974; Shajahan *et al.*, 2004) hence it is employed to assess the integrity of plasma membrane (Akanji *et al.*, 1993), elevation of ALP will therefore result in the leakage of the enzyme from the tissues into the serum (Sahjaham *et al.*, 2004, Aboyade *et al.*, 2009). Such loss from tissue may adversely affect adequate transportation of ions across the membrane (Akanji *et al.*, 1993) and other metabolic processes such as the synthesis of nuclear proteins, nucleic acid and phospholipid where the enzyme is involved (Ramalingam and

Vimaladevi, 2002). In serum, ALP may be considered as an indicator of cholestasis which may result from intracellular hepatic canaliculli obstruction associated with inflammation (Etuk and Muhammad, 2010). Significant ($P < 0.05$) elevation of lactate dehydrogenase was seen only with the administration of 50 and 75mg/kg for thirty days in the liver and this may have consequential effect on glycolytic pathway.

At the dosage level of 10 and 25mg/kg, there were no significant ($P < 0.05$) differences in the total and conjugated bilirubin concentrations relative to the control group. However, higher doses of the extract (50 and 75mg/kg) produced significant ($P < 0.05$) elevation in total and conjugated bilirubin levels both in the serum and liver (Fig 51 and 52) and this could be attributed to mild liver malfunction, obstruction of the common bile or hepatic duct. When the liver is not filtering normally, bilirubin builds up in the blood which in turns results into a damaged liver (cirrhosis).

Elevated plasma triglyceride concentration contribute directly to increased risk of cardiovascular diseases and such elevation is associated with obesity, metabolic syndrome, type 2 diabetic mellitus which predispose to cardiovascular diseases (Hodes *et al.*, 1999). The present study revealed that there was significant ($P < 0.05$) reduction in the serum triglyceride level when the animals were treated with high doses of the extract (50 and 75mg/kg).

High density lipoprotein and low density lipoprotein are the two main groups of plasma lipoprotein that are involved in the lipid metabolism, exchange of cholesterol, cholesterol esters and triglycerides between tissues (Gordon and Riffund, 1989; Svirdin, 1999; Mc Namara, 1999). Studies have demonstrated an inverse relationship between HDL cholesterol and incidence of cardio vascular disease (Maron, 2000). In the present study, HDL cholesterol for the groups treated with 25, 50 and 75mg/kg doses of the extract for thirty days showed significant elevation compared to the control group while there was no significant difference in the serum LDL cholesterol in all the treated groups relative to the control group. Abnormal blood lipids, particularly total cholesterol predispose individual to arterosclerosis and cardiovascular disease (Ginsberg 1994; Glew *et al.*, 2001; Chrysohoou *et al.*, 2004; Nwanjo and Oze, 2007). From this study,

administration of the crude extract of the bulb of *Crinum jagus* for thirty days resulted in significant reduction in the concentration of serum cholesterol in all the treated groups relative to the control group (Fig 53). The significantly ($P < 0.05$) lower cholesterol may have contributed to the observed significant high serum HDL cholesterol in the animal cause about 30% of blood cholesterol is carried inform of HDL cholesterol, HDL cholesterol can remove cholesterol from antheroma within the arteries and transport it back to the liver for its excretion, thus high level of HLD cholesterol protect against cardiovascular diseases (Kwiterovich, 2000). The observed significant increase in HDL cholesterol concentration upon administration of the extract for thirty days indicates that the extract have HDL cholesterol boosting effect. The extract did not have any significant effect on LDL cholesterol which transport cholesterol to the arteries where they can be retained in artheria proteoglycan starting the formation of plaques hence LDL cholesterol posses a risk of cardiovascular disease when it invades endothelium and become oxidized, oxidized form is more easily retained by proteoglycans thus increase of LDL cholesterol is associated with artherosclerosis, heart attack, stroke (Growell and Otwa, 2004). From the study it could be suggested that the extract may aid in the prevention of cardiovascular diseases.

Decrease or increase in cell counts and depletion of plasma constituent or their elevation beyond the reference range could demonstrate haematoxicity. In the present study, there were no ($P > 0.05$) significant difference in the haematological parameters of all the treated animals relative to the control group (Figures 54 and 55). There were non obvious haemolytic changes in the plasma of the extract treated rats on RBC, haemoglobin, PCV, MCV, MCH an MCHC. These indices are well known to determine the haemolytic damage on red blood cells. The absence of changes on these indices suggests that the extract does not possess toxic substances that can cause an anaemic condition in rats. The extract did not produce any effect on white blood cells and its functional indices. The result from this finding shows that the extract is not haematoxic.

Histopathological examination of tissues of the animals exposed to a toxicant may furnish additional information on the drug . Exposure of the rats to high doses of the

extract (50 and 75mg/kg) caused pathological changes in the liver which resulted in the enlargement of hepatocytes (fatty degeneration of hepatocytes) (Plate 1C). Exposure of the rats to high doses (50 and 75mg/kg) caused necrosis of the tubular epithelium with sloughing into the lumen and distruption of the basement membranes in the kidney (Plate 2C). The pathological changes observed with high doses of the extract (50 and 75mg/kg) is an indication of toxicity. The toxicological evaluation of the plant indicates that the plant is safe at lower doses but higher doses pose toxicological risks as evident by the elevation of serum, liver and kidney enzymes and pathological changes observed in the liver and kidney following administration of extract for thirty days.

GC-MS analysis of F1 of the bulb of *Crinum jagus* revealed the presence of ten bioactive compounds. The GC-MS retention time (RT) and percentage peak of the individual compounds revealed that 5-hydroxyl methyl furfural (5-HMF) was found as the major component in the fraction. 5-HMF is an organic compound derived from dehydration of certain sugars (Andrea *et al*., 2011; Robert *et al*., 2013). This yellow melting solid is highly water soluble. The molecule consists of a furan ring containing aldehyde and alcohol functional groups. *In vitro* antioxidant and antiproliferative activities of 5-HMF has been reported (Zhou *et al.*, 2013). Consequently, the antioxidant activity of the extract and fractions of *Crinum jagus* may be attributable to the presence of bioactive compounds such as 5-HMFS. The presence of various bioactive compounds in the fraction justifies the traditional use of the plant for the treatment of various ailments hence it can be concluded that *Crinum jagus* is of phytopharmaceutical importance.

In conculsion, from the various findings obtained in this study, the extract and fractions of the bulb of *Crinum jagus* posesss antioxidant, antimicrobial, anti-inflammatory, antimalaria and anti-mycobacterial properties. These properties may be due to the presence of phytochemicals such as alkaloids, flavonoids, saponins, phenols, steroids which are present in the plant. These classes of compounds are known to have curative activities and therefore could suggest the use of the plant traditionally for the treatment of various illnesses. Most of these activities are highly expressed in F1 and this

may suggest the major constituents of the bioactive compounds present in the plant are highly concentrated in the fraction. This study provides scientific evidence for the use of the plant in ethnomedicine in the treatment of various health conditions and this may find useful pharmaceutical applications in the design of lead drug that can be used for the treatment of free radical mediated diseases, antimicrobial infections, anti-inflammatory diseases, malaria and tuberculosis.

5.2 CONTRIBUTIONS TO KNOWLEDGE.

In this thesis, evidence has been presented to show that:

1. The crude methanol extract of the bulb of *Crinum jagus* contain alkaloids, flavonoids, saponins, phenols and steroids.
2 The crude extract and fractions of the bulb of *Crinum jagus* possess antitubercular, antimicrobial, anti-inflammatory, antimalarial and antioxidant activities with the fractions being more potent than the crude extract.
3. In Nigeria ethnomedicine, the antitubercular, anti-inflammatory and antimalarial activities of the plant is being reported for the first time.
4. Most of these biological activities of *Crinum jagus* are highly expressed in F1 and this may suggest the major constituents of the bioactive compounds present in the plant are highly concentrated in the fraction.
5. The antitubercular and antimalarial activities of F1 was comparable with the standard reference drugs.
6. The bulb of *Crinum jagus* contain flavonoid and phenolic compounds in a concentration dependent manner with F1 having the highest flavonoid and phenolic content.
7. *Crinum jagus* is safe when used at lower dose but administration of high dose of the plant was found to be toxic.
8. *Crinum jagus* is a potential valuable source of antitubercular, antimicrobial, anti-inflammatory, antimalarial and antioxidant agents.
9. This study provides some scientific justification for the folkoric use of *Crinum jagus* as a health remedy in folklore medicine.
10. Gas chromatography mass spectrometry (GC-MS) analysis of F1 revealed the presence of ten chemical components. The presence of various bioactive compounds in the fraction justifies the traditional use of the plant for the treatment of various ailments hence *Crinum jagus* is of phytopharmacological importance.

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APPENDICES

Appendix 1

Anti-inflammatory activity of the crude methanol extract of the bulb of *Crinum jagus*

		Paw Circumference (cm)						
Groups	**Treatment**	**0hr**	**1hr**	**2hrs**	**3hrs**	**4hrs**	**5hrs**	**Percentage Inhibition**
A (Control)	0.2ml saline	2.50± 0.17	3.14± 0.29	3.26± 0.25	3.38± 0.19	3.50± 0.14	3.60± 0.09	_____
B	5mg/kgbw indomethacin	2.58± 0.29	2.68± 0.26	2.76± 0.30	2.52± 0.29	2.38± 0.27	2.22± 0.10	94.82%
C	10mg/kgbw extract	2.64± 0.24	3.42± 0.29	3.62± 0.12	3.72± 0.19	3.92± 0.13	3.94± 0.10	26.82%
D	25mg/kgbw extract	2.64± 0.24	3.26± 0.22	3.48± 0.12	3.26± 0.26	3.00± 0.28	2.80± 0.28	29.55%
E	50mg/kgbw extract	2.60±. 0.17	3.84± 0.31	3.50± 0.26	3.20± 0.23	3.08± 0.17	3.00± 0.29	31.82%
F	75mg/kgbw extract	2.78+ 0.20	3.48+ 0.29	3.26+ 0.24	3.08+ 0.15	2.66+ 0.24	2.48+ 0.27	65.90%

The values expressed as mean ± SD (n- = 5)

The percentage inhibition was calculated using the formular :

$$\% \text{ inhibition} = \frac{\textbf{(Dt-Do) control} - \textbf{(Dt-Do) test}}{\textbf{Dt – Do) control}} \times 100$$

Where **Dt** = Linear paw circumference 3 hours after Carrageenan injection

Do = Linear paw circumference at 0 hour (just before Carrageenan injection)

Appendix 2

Anti-inflammatory activity of the chromatographic fractions of the bulb of *Crinum jagus*

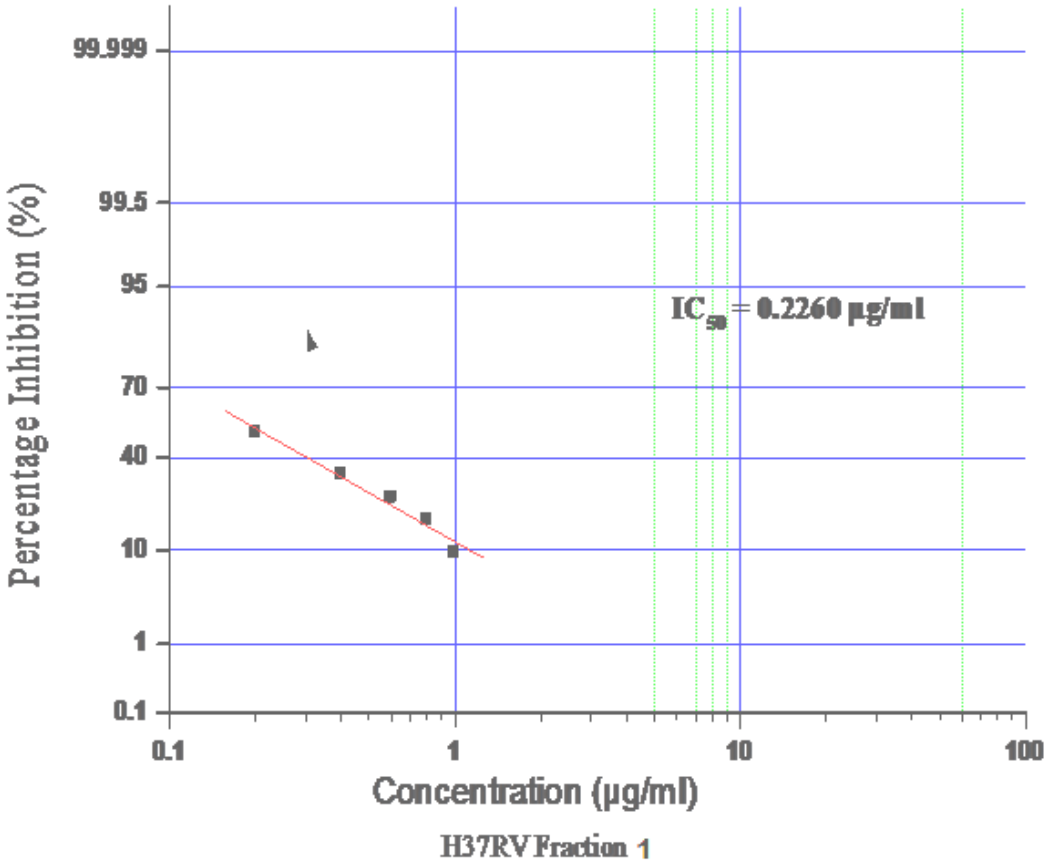
		Paw Circumference (cm)						
Groups	**Treatment**	**0hr**	**1hr**	**2hrs**	**3hrs**	**4hrs**	**5hrs**	**Percentage Inhibition**
A (Control)	0.2ml saline	2.50± 0.17	3.14± 0.29	3.26± 0.25	3.38± 0.19	3.50± 0.14	3.60+ 0.09	_____
B	5mg/kgbw indomethacin	2.58± 0.29	2.68± 0.26	2.76± 0.36	2.52± 0.29	2.38± 0.27	2.22+ 0.10	94.82%
C	10mg/kgbw F1	2.36± 2.42	3.20± 0.14	2.92± 0.15	2.80± 0.14	2.72± 0.07	2.66± 0.33	50.00%
D	20mg/kgbw F1	2.80± 0.08	3.00± 0.32	2.84± 0.30	2.62± 0.34	2.54± 0.29	1.76± 0.24	79.50%
E	10mg/kgbw F2	2.72± 0.17	3.66± 0.30	3.42± 0.35	3.38± 0.33	3.36± 0.36	3.64± 0.33	18.18%
F	20 mg/kgbw F2	2.54± 0.14	3.36± 0.21	3.62± 0.13	3.62± 0.15	3.66± 0.15	3.72± 0.12	25.00%
G	10mg/kgbw F3	2.54± 0.14	3.56+ 0.17	3.38± 0.17	3.08± 0.12	2.86± 0.10	2.54± 0.10	38.64%
H	20mg/kgbw F3	2.44± 0.19	3.14± 0.19	3.08± 0.20	2.86± 0.17	2.68± 0.12	2.64± 0.10	52.27%

The values expressed as mean ± SD (n- = 5)

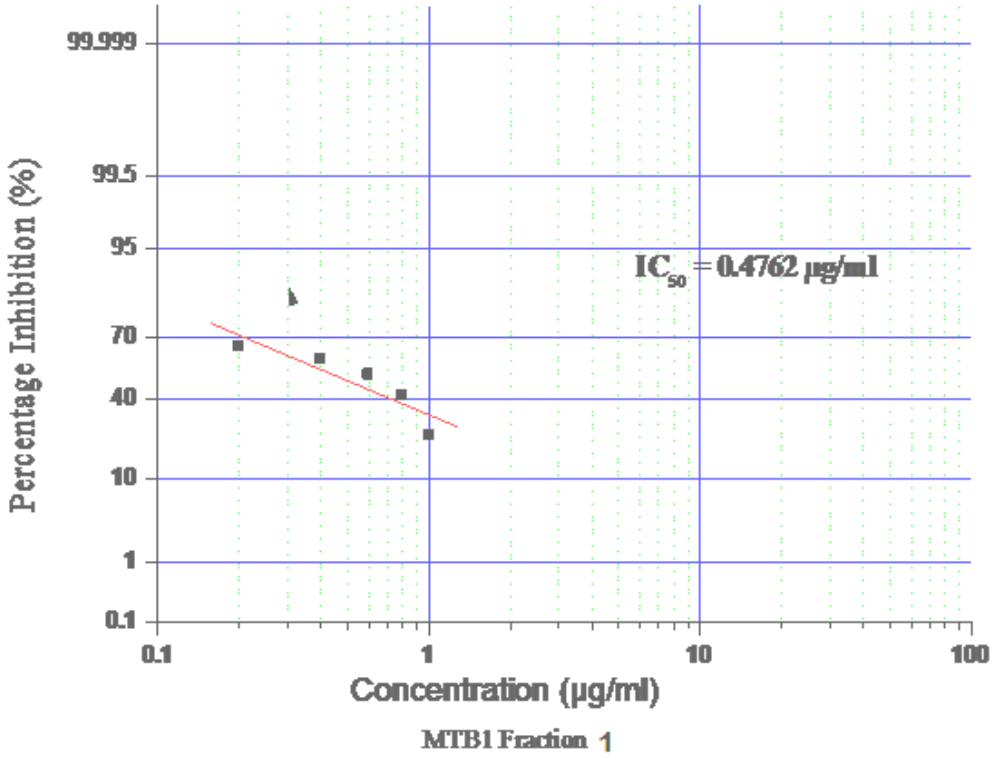
Appendix 3

In-vitro **antituberculosis activity of fraction 1 (F1) of the bulb of** ***Crinum jagus*** **against isolates and strain of** ***M.tuberculosis***

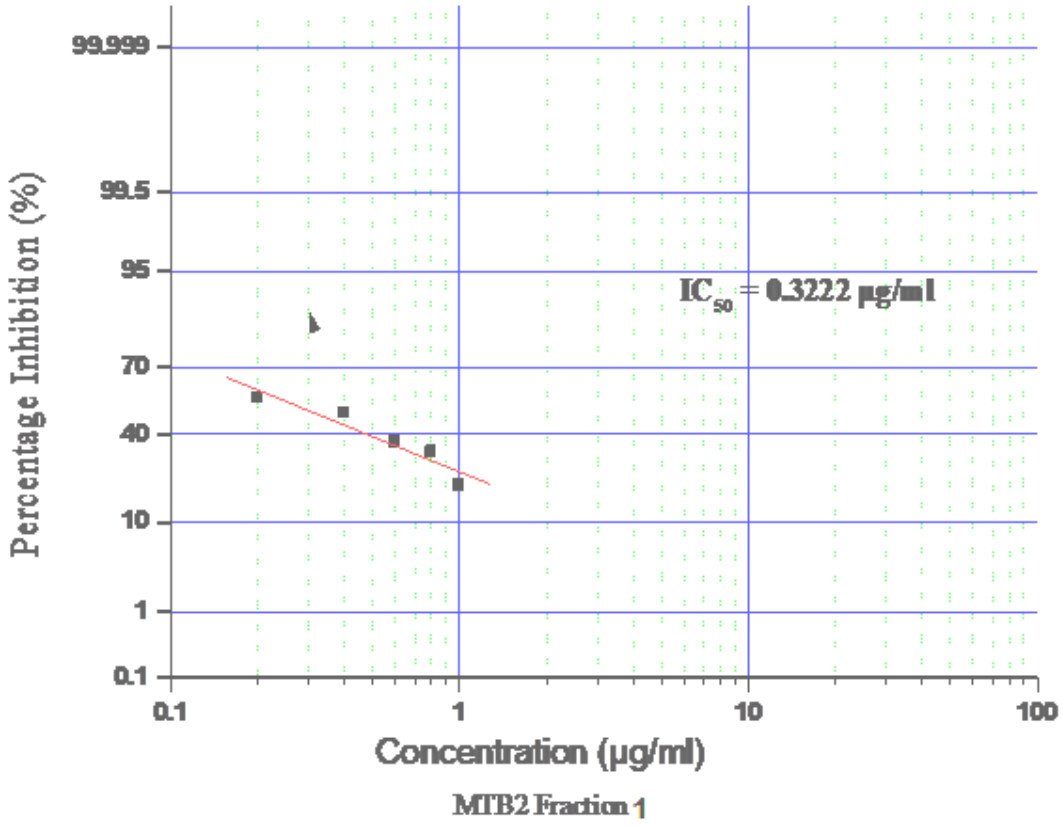
Determination of 50% inhibitory concentration (IC_{50})



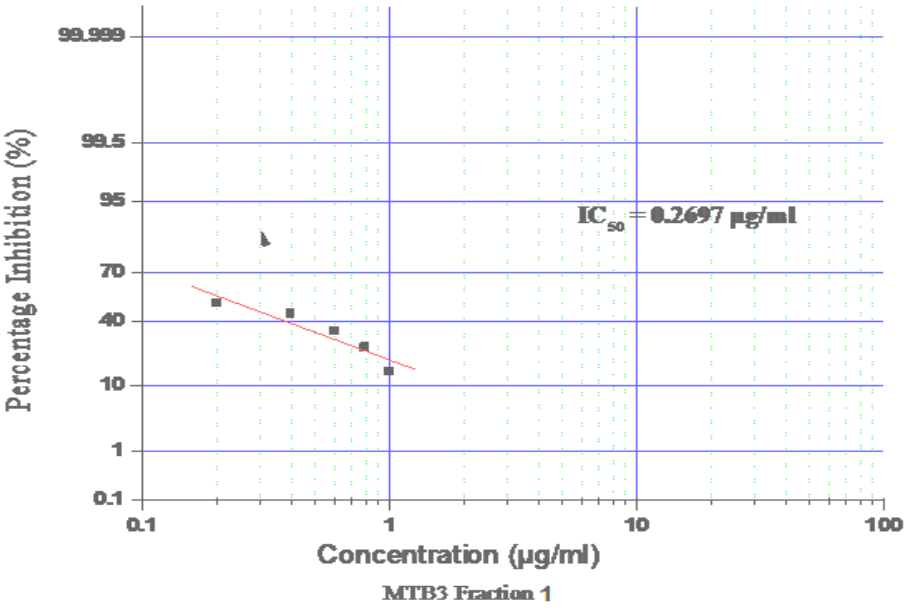
Determination of 50% inhibitory concentration (IC_{50})



Determination of 50% inhibitory concentration (IC_{50})



Determination of 50% inhibitory concentration (IC_{50})



CHAPTER ONE

1.0 INTRODUCTION

In all countries of the world, there exists traditional knowledge related to health of humans and animals. According to World Health Organization (WHO, 1978a; WHO, 1978b) the definition of traditional medicine may be summarized as the sub-total of all knowledge or practice, whether explicable or inexplicable, used in diagonising, preventing or eliminating a physical, mental or social disease which may rely exclusively on past experience or observations handed down from generation to generation, verbally or in writing. It also comprises therapeutic practices that have been in existence often for hundreds of years before the development of modern scientific medicine and are still in use today without any documented evidence of adverse effects.

Traditional medicine practice is an important part of health care delivery system (Sheldom *et al.*, 1997). Traditional folklore medicine plays an important role in health services around the globe. About three quarters of the World's population relies on plant and its extract for health care (Premanathan *et al.*, 2000, Gabhe *et al.*, 2006). A good number of populations particularly those living in villages depend largely on herbal medicine.

Traditional medicine has expanded globally and is clearly gaining patronage, its acceptability for primary health care is not limited to poor developing countries but in countries where conventional medicine is predominantly in the national health care system (Lanfraco,1999). In developed countries such as the United States of America, majority of people (55%) combine alternative treatment with conventional medicine (Stein, 2004).

Traditional medicine as a major part of Africa socio-cultural heritage, obviously in existence for several hundred years, was once believed to be primitive and wrongly challenged with animosity, especially by foreign religions dating back to the colonial days in Africa and subsequently by the conventional or orthodox medical practitioner (Elujoba *et al.*, 2005). Traditional medicine has been brought into focus for meeting the goals of an under coverage of primary health care delivery not only in Africa but also, to various extent, in all countries of the

world. Traditional medicine is the first choice health care system for at least 80% of Africans who suffer from high fever and other common ailments.

Primary health care is the key to the development of a national health policy as defined by the Alma-Ata Declaration in 1978. It is an essential health care based on practical, scientifically sound and socially acceptable methods and technology. It is made universally acceptable to individuals and families in the community and through their full participation and at a cost that the community and the country can afford in order to maintain at every stage of their development, in the spirit of self reliance and self determination. It is the first level of contact for the individual family and the community within the national health care system, bringing health care as close as possible to where people live and work and this constitutes the first element of a continuing health care process (WHO, 1978a). A health system based on primary health care was adopted as the means of achieving the goal of health for all by the year 2000.

The WHO has since urged developing countries of the world to utilize the resources of traditional medicine for achieving the goals of primary health care.

Traditional medicine has remained the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities, and local therapy is the only means of medical treatment for such communities (Yinger and Yewhalaw, 2007). The World Health organization (WHO, 2001) reported that herbal medicine serves the health needs of about 80% of the world population, especially for millions of people in the vast rural areas of the developing countries

There are clear trends to show that the mainstream in pharmaceutical research is moving away from single target approach to combination and multiple target approaches (Wermuth, 2004). The problems with the traditional herbal medicines, especially in developing countries, have to do with the methods, preparations and standardization of herbal formulations of recent times.

Herbal medicine is the oldest form of health care known to mankind. Recorded history has it that medicinal plants have been in use for the past fifty centuries, which until the last two and half centuries were the main source of treatment of man and animal. The use of plant in medicine is older than recorded history (Osai, 1998). Medicinal plants form the basis of

traditional health care delivery system used by about 80% of the population of most developing countries who rely on traditional medicine for their health care needs (Ghani and Onaolapo, 1989).

Medicinal plants have been used over the years in the rural communities to treat infections and diseases and have had, undoubtedly, good results hence there is a level of reliance on plants as whole chemotherapeutics which involves the extraction and development of drugs from plants, and their derivatives has been carried out over the years to counter the effect of many diseases. This means that specific pants called medicinal plants are of great importance in the manufacture of drugs since the chemical component of plants are used for the production of these drugs (Houghton and Raman, 1998).

It is also known that about 60% of the global population does not have readily available access to modern drugs and medication thereby leading to an increased mortality rate. Hence the need arises to seek cheaper and available alternative to help alleviate these diseases. The use of traditional medicine and medicinal plants in most developing countries as the normative basis for the maintenance of good health has been widely observed (UNESCO, 1998). Furthermore, reliance on the use of medicinal plants in the industrialized societies have been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from the traditionally used rural herbal remedies (UNESCO, 1998).

Various organic compounds are derived from plants that are important in combating different diseases (Enwuru, 2008). Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions and to defend against attack from predators such as insects, fungi and herbivorous animals. Many of these phytochemcials have beneficial effects on long term health when consumed by human and can be used to effectively treat human diseases. At least 12,000 such compounds have been so far isolated , a number estimated to be less than 10% of the total number (Lai and Roy, 2004; Fabricant and Farnsworth, 2001). Chemical components in plants mediate their effect on the human body through a process identical to those already well understood for conventional drugs. Thus herbal medicine do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicine to be as effective as conventional medicines but also give

them the same potential to cause harmful side effect (Lai and Roy, 2004, Fabricants and Farnsworth, 2001). All plants produce chemical compounds known as phytochemical as part of their normal metabolic activities.

Phytochemicals are divided into two classes, namely :

- Primary metabolites such as sugar and fats which are found in all plants
- Secondary metabolites which are found in a smaller range and serving more specific function (Billiry and Sherman, 1998). Examples of secondary metabolites are alkaloids, polyphenols, glycosides, terpenes, steroids, tannins.

In developing countries of the World such as Nigeria, Kenya, Zimbabwe and Malawi the use of medicinal plant, is well acknowledged and established (Hedberg,1988). This is particularly so in rural areas where the services of modern hospitals may be limited and also in some circumstances when traditional medicine seems to be preferred. The main problem in the use of traditional medicine is the proof requirement that the active components contained in the medicinal plant are useful, safe and effective. The proof of pharmacological activity that are available at present are mostly based on empirical experience. The scientific poof then becomes the most important theory in order to eliminate the concern of using medicinal plant, as drug of alternative treatment.

Ethnobotany which is the study of traditional human uses of plants, is a preliminary method of research suitable for gathering information on the use of plants. The 'quack' medical knowledge handed down by the common people constitutes oral sources of information useful for scientific research, therefore science and tradition have a strong connection between them, science in fact has often traditional origin (Lentini , 2000). During the last few decades, there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world but documenting the indigenous knowledge through entnobotanical studies is important for the conservation and utilization of biological resources. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plant for the treatment of various diseases (Aziazeh *et al.*, 2003).

Traditional medical knowledge of medicinal plants and their use by indigenous culture is not only useful for conservation of cultural tradition and biodiversity but also for community health care and drug development in the present and future (Pei, 2001).

Africa is known to be richly endowed with medicinal plants, one of such plants is *Crinum jagus*. The plant is found in tropical and subtropical areas, worldwide, where for centuries, they have been used traditionally to cure ailments and diseases . The powdered bulb is taken orally with honey as a remedy for tuberculosis in Southern part of Nigeria (Idu *et al*., 2010). The bulb is eaten raw as a cure for snake bite in some parts of Nigeria (Ode *et al*., 2006). The bulb of the plant is used for the treatment of asthma cough in Western part of Nigeria and is commonly called asthma cough plant (Ogunkunle and Olopade, 2011). The warm leaf juice of the bulb of the plant with a pinch of common salt is used for ear-complaint as an emetics (Gill, 1992). Among the Binis, the decoction of the bulb of the plant is used as a vermifuge and purgative (Gill, 1992). The bulb of *Crinum jagus* is used in Southern Nigeria for memory loss and other mental symptoms associated with aging (Peter *et al*., 2004). Morphine, hamayne and lycorine have been isolated as active alkaloids of *Crinum jagus* (Banwell *et al*., 2011). Preliminary phytochemical study of the plant revealed the presence of alkaloids, flavonoids, saponins phenols, steroids (Ode *et al.*, 2010). Previous work done on the therapetuic importance of the bulb extract of the plant reported the antibacterial and antifungal activities (Adesanya *et al.*, 1992), antitumour, immunostimulating and anticonvulsant activities (Edema and Okieimen, 2002; Azikiwe and Siminilay, 2012), anti-snake venom activity (Ode and Azuzu, 2006), antimutotic activity (Nwankamma and Okoli, 2010), anti-oxidant and anti-haemorrhagic activities (Ode *et al*.,2010) as well as anti-cholinergic activity (Peter *et al*., 2004).

Figure 1 : The bulb of *Crinum jagus*

1.1 Rationale

Medicinal plants have been an integral part of ancient health practices for centuries. Today, modern medicine recognizes medicinal plants as alternative and complementary therapeutics for a host of conditions. Among the most popular uses of plants include; increase energy, disease prevention, pain relief and general health (Rica, 2010). Herbal mixtures are increasingly becoming the choice of most patients nowadays. The promise of presenting the natural quantities that promote a healthier method of healing different ailments contributed to the popularity of these medicines, especially, as these types of medicine are grown wild or can be tendered at one's home background. It is also cheaper as compared to manufactured medicines.

Herbal medicine is the alternative for commercially manufactured medicines which are already made available in the market. Herbal medicine differs from modern medicines in view of the fact that it is produced with one hundred recent content of natural plant extract believed to have medicinal values as effective as the modern drugs, less side effects and less cost. There are several herbal plants widely used to treat various illnesses. Herbal plants are scientifically studied to support the benefit claimed to contain each plant. These studies are recognized and acknowledged by the medical community to have proven effectiveness recommended to treat multitude of illnesses. Note worthy break through are being produced by researchers and there is a steady progress and indeed very promising trend towards meaningful research into the development of essential drugs from numerous local medicinal plants in the developing countries of the world. It is against this background that the bulb of *Crinum jagus* used in some parts of Nigeria for treating tuberculosis, asthma, ear pain, memory loss, snake bite, convulsion and as antibacterial, antifungal, vermifuge, purgative was investigated in this study.

The main objective was to investigate the biological activities of the crude methanol extract and fractions of the bulb of *Crinum jagus* to provide scientific evidence for the traditional use of the plant for treatment of these various diseases.

Specific objectives include the following:

- To determine the secondary metabolites present in the crude extract
- To investigate the antioxidant activity of the crude extract and fractions of the plant (*in vitro and in vivo)*

- To assess the antimicrobial activity of the crude extract and fractions of the plant.
- To evaluate the possible anti-inflammatory activity of the crude extract and fractions of the plant.
- To evaluate the antiplasmodial activity of the crude extract and fractions of the plant in *Plasmodium berghei* infected mice
- To investigate the possible inhibitory effect of the crude extract and fractions of the plant on *Mycobacterium tuberculosis* isolates.
- To evaluate the toxicological profile of the plant extract

CHAPTER TWO

LITERATURE REVIEW

2.0 The *Crinum* species

The genus *Crinum* belong to the Family Amaryllidaceae, Phyllum-Angiospermae and Subphyllum-Liliflora and it is distributed throughout the tropics and warm temperature regions of the world.Amaryllidaceae is a great widely spread family all over the world and contains about 90 genera and 1,310 species (Benson, 1970) with wide geographical distribution throughout the tropics, subtropics and warm temperature regions of the world (Mabberly, 1990). The genus *Crinum* represents an important sector in the family. The name *Crinum* originates from Greek "Krinon" which means White lily and they are commonly known as milk river or veld lilies. This genus is related to a group of mostly Southern African endemic genera constituting the tribe Amaryllidaceae (Meerow and Sinijman, 1998). Like other members of Amaryllidaceae, *Crinum* can occupy many different habitats such as seasonal dry places, ephemeral pools, rainforest, coastal areas and river blanks. Worldwide, *Crinum* comprises about 130 species distributed in Africa, America, Southern Asia and Australia, Africa lands enjoy most species and about twenty one are endemic to Southern Africa.

Crinum are perennial herbaceous plants with giant fleshy bulbs larger in stature than most other species of Amaryllidaceae. They can grow from 1-5 feet in height depending on the species and they produce a neck or pseudo stem made up of the sheathing old leaves. Worldwide, *Crinum* species have a substantial economic value as ornamentals due to their showy flowers. In addition, huge numbers are traded for traditional medicines. Several species are cultivated for medicinal purposes, (Burkhill, 1985; Ghosal *et al.*, 1985, Fennel, 2001; Tram *et al.*, 2002). Since about the 1950"s, *Crinum* species have been subjected to extensive chemical, cytological and pharmacologically evaluation of active principles (Wildman,1960). Phytochemical investigations have resulted in isolation of diverse classes of compounds and have been focused predominantly on alkaloids. Phenolics prevail among the non alkaloidal constituents (Tram *et al.*, 2002)

2.1 Chemistry of *Crinum* Species

The ease of hybridisation of *Crinum* species has afforded about160 of these species, out of which 30 have been investigated. The following ring structures are found in the alkaloidal skeleton, Crinane (5,10b- ethanophenatridine), Lycorine (pyrrolophnathridine), Galanthamine, Ryllistine and Cherylline. Structural variations occur in ring C (double bond and subtituent). Examples include 3-oxocrinine (Fig1:1) from *Crinum americanum* and palmilycorine (Fig 1:2) from *Crinum asiaticum*. Trisphaeridin (Fig 1:3) found in *Crinum americanus* was the first phenathridine type alkaloid. Zeylamine (Fig 1:4) was found in *Crinum zeylanicum* while 5 hydroxyhomolycorine (Fig 1.5) was found in *Crinum dexifum*. These compounds can be found in the bulb or fruits (Ali *et al*., 198la; Ghosa *et al*., 1981 and Tram *et al*., 2002).

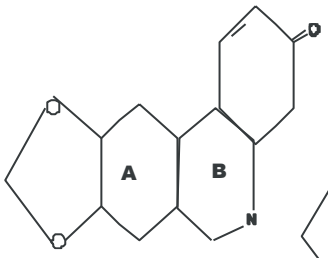


Figure 2.1: 3 (Oxocrinine)

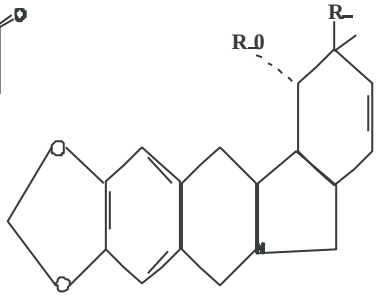


Figure 2.2 : Palmilycorine
R_1 = Palmitoyl, R = XOH

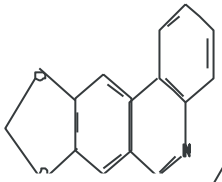


Figure 2.3 : Trisphearidin

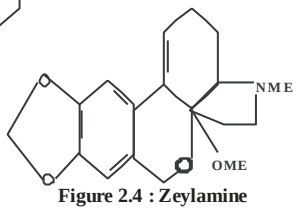


Figure 2.4 : Zeylamine

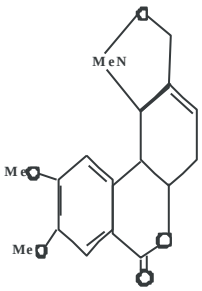


Figure 2.5 : 5 (Hydroxyhomolycorine

Figure 2: Alkaloids from Crinum species
(Ghosal et al., 1985)

The non alkaloidal compounds present in the *Crinum* species consist of the following :

(i) Long chain alkanes

(ii) Acids and Esters (examples of which are arachidonic acid, capric acid and palmitic acid from *Crinum augustum*, *Crinum americanum* and *Crinum bulbispermium*)

(iii) Alcohols and ketones (example of which are triacentanol and 5- hydroxy hexacosan - 9-one from *Crinum augustum*)

(iv) Terpenoids and sterols (examples of which are Vomifoliol and Sitosterol from *Crinum firmifolium* and *Crinum moorei*)

(v) Flavonoids (examples of which are 4-hydroxyl-7-methoxyflavan and 4-hydroxyl-7-methoxy-8-methylflavan)

(vi) Chromones (examples of which are eugenin and noreugeni from *Crinum moorei*)

(vii) Carbohydrates, an example of which is pectin from *Crinum amabile*).

Resins and polysaccharides have also been shown to be present in the *Crinum* bulbs (Ghosal *et al.*, 1985 and Tram *et al.*, 2002).

2.2 Biological and Pharmacological Activities of *Crinum* Species

Various biological activities assigned to *Crinum* species include :

(a) Antibacterial and antifungal activities of *Crinum* species

Crinamine from *Crinum jagus* possess strong antibacterial activity while lycorine, hamayne and 6-hydroxycrinamine are inactive (Adesanya *et al*., 1992)

(b) Antiparasitic activity

Augustine and lycorine from *Crinum ornatum* showed moderate antimalaria activity against *Plasmodium falciparum* but the selectivity was low compared with antimalaria control compounds (Ali *et al.*, 198la and b)

(c) Antitumour and immunostimulating activities

Lycorine and 6-hydroxycrinamine from *Crinum delagoense* were active against BL-6 mouse melanoma cells, (Hanghwitz *et al*., 1965; Onyirinka and Jackson, 1978 ; Ali *et al*., 1981b).

(d) Insecticidal activity

Crinisine from the bulb of *Crinum asiaticum* was an effective insecticide (Tram *et al*., 2002)

(e) Anti-cholinergic activity

Alkaloidal fractions from the bulb of *Crinum jagus* and *Crinum glaucum* showed inhibition of acetylcholine esterase, an activity exploited therapeutically to raise the depressed levels of acetylcholine in the brain associated with Alzheimer's disease hence the plants are used in traditional medicine in Southern Nigeria for curing memory loss and other mental symptoms associated with aging (Peter *et al*., 2004).

(f) Anti-snake venom activity

The anti-snake venom activity of the methanol extract of the bulb of *Crinum jagus* was investigated *in-vitro* and *in-vivo* against the venom of three noteable snake species. The bulb extract of *Crinum jagus* showed anti-snake venom activity and protected the injected mice from death, myonecrosis and haemorrhage induced by the effect of the snakes (Ode and Azuzu, 2006).

(g) Anti-convulsant activity

The bulb of *Crinum jagus* and *Crinum ornatum* have been shown to have anti-convulsant activity (Edema and Okiemen 2002; Azikwe *et al*., 2012). Lycorine and haemanthine from *Crinum ornatum* showed a dose dependent anti-convulsant effect (Oloyede *et al*., 2000).

Crinum jagus is a bulbous plant with spirally arranged leaves and conspicuous flowers. It is a largely showy plants with umbels of lily- like flowers. It is found in tropical and subtropical regions throughout the world (Mabbery, 1991). It belongs to the Family: Amaryllidaceae, Genus: *Crinum*, and Species: *jagus.* Its local name is Ogede Odo in Yoruba, Alubarha in Edo and Oyimbakar in Efik/Ibibio. It has a wide spread distribution in Africa, America, Southern Asian and Australia.

Crinum jagus is the largest tropical genus of Amaryllidaceae family (Ode *et al.,* 2010). *Crinum jagus* is a variable species that ocurs in Africa and its leaves may be broad in some forms whereas they are narrower or parallel in other forms. The plant may be found in swampy conditions, seasonal wet lands or in grassland (savannah). Some plant are remarkably fragnant flowered vanilla scented while others may have little or no scent/odours. Buds are enclosed in several sheathing. Morphine, hamayne, and lycorine have been isolated as active alkaloids of *Crinum jagus.* Lycorine has been shown to possess antitumour activity (Banwell *et al*., 2011

2.3 Free Radicals

Oxygen is used as part of the process for generating metabolic energy. However, a small amount of this oxygen often gets loose and produce unstable products called free radicals. By definition, a free radical is an atom e.g oxygen, nitrogen with at least one unpaired electron in the outermost shell that is capable of independent existence (Davies, 1995). Free radicals are highly reactive due to the presence of unpaired electrons. The unpaired electrons participate in various chemical reactions.

A free radical is easily formed when a covalent bond between entities is broken and one electron remains with each newly formed atom (Segal, 2005). Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). Oxygen centred free radicals contain two unpaired electrons in the outermost shell. When free radicals steal one electron from a surrounding compound or molecule a new free radical is formed in its place. In turn, the newly formed radical then looks to return to its ground state by stealing electrons with antiparallel spins from cellular structures or molecules. Thus the chain reaction continues and can be thousands of events long (Diego-Otero *et al.*, 2009).

The electron transport chain (ETC) which is found in the inner mitochondria membrane utilizes oxygen to generate energy in form of adenosine triphosphate (ATP). Oxygen acts as the terminal electron acceptor within the electron transport chain (ETC). During exercise, oxygen consumption increases 10 to 20 fold. In turn, electron ignore escape from the electron transport chain (ETC) is further enhanced. Thus when calculated 6 to 35ml/kg/min of the total oxygen intake during exercise has the ability to form free radicals (Dekkers *et al.*, 1996). Electrons appear to escape from electron transport chain at the ubiquinone cytochrome level (Sjodin *et al.*, 1990).

Polyunsaturated fattyacids (PUFAs) are abundant in cellular membranes and in low density liporotein (LDL) (Dekker *et al.*, 1996). The polyunsaturated fatty acid maintains fluidity of cellular membranes. A free radical prefers to steal electrons from the lipid membrane of a cell initiating a free radical attack on the cells in a process known as lipid peroxidaiton (Fig 3). Reactive oxygen species target the carbon- carbon double bond and polyunsaturated fatty acids. The double bond on the carbon weakens the carbon-hydrogen allowing for easy dissociation of

the hydrogen by a free radical. A free radical will steal the single electron from the hydrogen associated with the carbon at the double bond. In turn, this leaves the carbon with an unpaired electron and hence becomes a free radical. In an effort to stabilize the carbon-centred free radical, molecular rearrangement occurs. The newly arranged molecules is called a conjugated diene (CD). The conjugated diene then very easily react with oxygen to form a proxy radical. The proxy radical steals an electron from another lipid molecule in a process called propagation. This process then continues in a chain reaction (Aly and Shahin, 2010). The lipid peroxidation is a chain reaction, which means that it consists of several interconnected steps where the products of some are the reactants of others. The first step is called initiation and in the case under discussion it involves the formation of a (carbon centred) lipid radical. This is often achieved by hydrogen abstraction from the lipid molecule (LH) by an hydroxyl radical, OH initiation.

$LH + OH \longrightarrow L + H_2O$

The most probable site of attack for the hydroxyl radical is the -CH_2-methylene group that bridges the two double bonds of the chain, simply because this will correspond to the weaker C-H bond (the radical so formed in resonance stabilized by a bis-allylic arrangement) (Ho *et al.*, 1997).

The radical L* can undergo several reaction e.g it can cross-link with other molecular oxygen dissolved in the cells membrane, yielding the peroxyl radical LOO*

Propagation

$L^* + O_2 = LOO^*$

The peroxyl radical can then abstract an hydrogen atom from another molecule of lipid (LH), forming a new lipid radical

Propagation

$LOO^* + LH = LOOH + L^*$

which can react with another oxygen and so on (Huang *et al.*, 1992) although the chain can be broken by a termination step.

Termination.

LOO* + LOO* = molecular products.

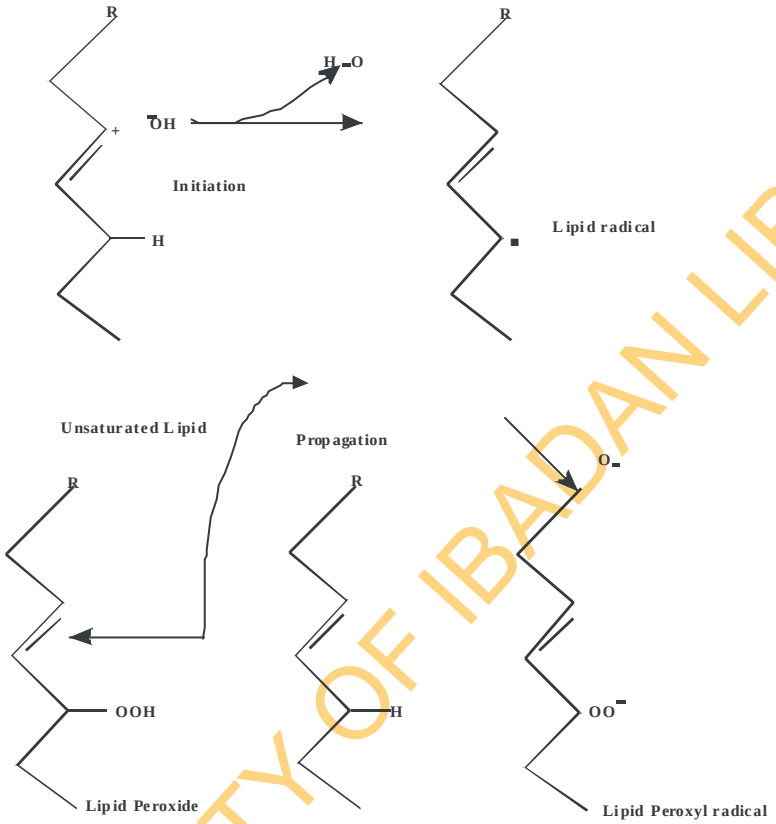


Figure 3 : The free radical mechanism of lipid peroxidation
(Halliwell and Gutteridge, 1989)

Radicals react with other molecules by:

(a) Combining their unpaired electrons to form a covalent bond as follows :

Covalent bond

$$A^* + A^* \longrightarrow A - A$$

(b) Donating their unpaired electron to another molecule or take an electron from another molecule (pairing). Thus a situation where a radical gives one electron to or takes one electron from a non-radical makes the non radical to become a radical thereby leading to a series of chain reactions hence their participation in the pathogenesis of certain disorders, examples of which are cancer, asthma, arthritis, inflammation, artherosclerosis, parkinsons (Harry 1972; Tappel, 1992). These oxygen reactive species are formed under the influence of ultraviolet or ionising radiations, in the presence of various chemicals including; transition metals and xenobiotics. Enzymes or electrons leakage in the course of metabolic pathways may result in the formation of reactive oxygen species.

2.4 Classification of Free Radicals

(i) Superoxide (O_2^-)

Superoxide **(O_2^-)** is produced by addition of a single electron to oxygen and several mechanisms exist by which superoxide can be produced *in-vivo* (Halliwell *et al.*, 1992). Several molecules, including adrenalin, flavones nucleotides, thiol compounds and glucose can be oxidized in the presence of oxygen to produce superoxide and these reactions are greatly accelerated by the presence of transition metals such as iron or copper. The electron transport chain in the inner mithochondria membrane performs the reduction of oxygen to water.During this process, free radical intermediates are generated, which are generally tightly bound to the components of the transport chain. However, there is a constant leak of few electrons into the mithochondrial matrix and this results in the formation of superoxide (Beeker *et al*., 1999). There might also be continuous production of superoxide by vascular endothelium to neutralize nitric oxide (Lius *et al.*, 2002). Superoxide has been implicated in initiating oxidation reaction associated with aging (Cotelle *et al.*, 1996). It has also been implicated in several pathophysiological process due to its transformation into more reactive species such as singlet oxygen, hydrogen peroxide and hydroxyl radical that initate lipid peroxidation and play an

important role in the induction of oxidative damage in DNA, lipid and proteins (Aurand *et al*., 1977)

(ii) **Hydrogen peroxide (H_2O_2)**

Several enzyme reactions including those catalyzed by glycolate oxidase might produce hydrogen peroxide directly (Chance *et al*., 1979). Hydrogen peroxide and oxygen are also formed when superoxide dismutase (SOD) removes superoxide. Hydrogen peroxide is not a free radical itself but is usually included under the general heading of reactive oxygen species (ROS). It is a weak oxidizing agent that might directly damage proteins and enzymes containing reactive thiol group. However, its most vital property is the ability to cross membrane freely, which superoxide generally is not capable of doing (Halliwell *et al*., 1989). Therefore, hydrogen peroxide formed in one location might diffuse a considerable distance before decomposing to yield the highly reactive hydroxyl radical which is likely to mediate most of the toxic effects ascribed to hydrogen peroxide, therefore, hydrogen peroxide act as a conduct to transmit free radical induced damage across cell compartment and between cells.

(iii) **Hydroxyl radical (OH^-)**

Hydroxyl radical (OH^-) is the final mediator of most free radical induced tissue damage (Lloyd *et al*., 1997). All of the reactive oxygen species described above exert most of their pathologic effects by giving rise to hydroxyl radical. The hydroxyl radical is short lived but the most damaging species within the body. The reason for this is that hydroxyl radical with extremely high rate constants react with almost every type of molecule found in living cells inculding sugars, amino acids, lipids and nucleotides. Hydroxyl radical (OH^-) does not persist for more than a few seconds before combining with a molecule in its immediate vicinity. Chain reaction are thus encouraged, and if hydroxyl radical attacks DNA, free radical chain propagate through the DNA and cause chemical alteration of the bases leading to mutation as well as strand breakage (Aruoma *et al*., 1989a). Hydroxyl radical generation is paradoxically the major mechanism by which malignant cells are killed during radiotherapy. Hydroxyl radical is also able to initiate the process of lipid peroxidation by abstracting a hydrogen atom and combining with it to form water, since a hydrogen atom leaves behind an unpaired electron on the carbon atom from which it was abstracted. This carbon radical then undergoes molecular rearrangement

to form a conjugated diene (CD) which then reacts with oxygen to give a peroxyl radical. The peroxyl radical abstracts hydrogen atom from an adjacent fatty acid side chain to continue the process of converting itself into a lipid peroxide (Fig 4). These are stable when pure, however *in-vivo*, their decomposition is catalyzed by copper and iron ions and complexes e.g haemethaemoglobin and cytochromes. Lipid peroxidation is thus a radical reaction.

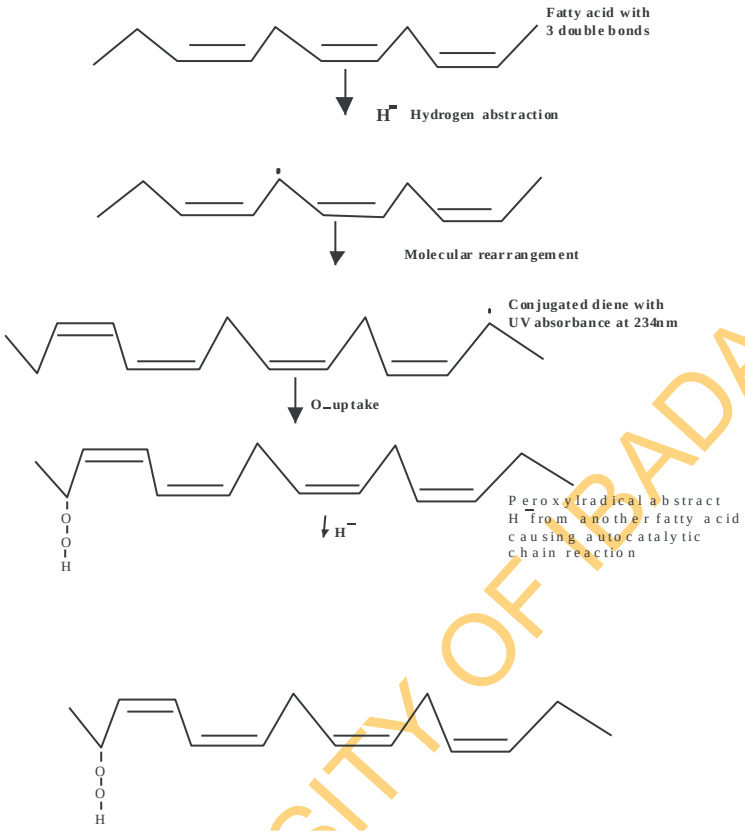


Figure 4: Hydroxyl radical initiated reaction of polyunsaturated fatty acid side chain (Hallwell and Gutteridge., 1984)

Although hydroxyl radical formation can occur in several ways, by far the most important mechanism *in-vivo* is likely to be the transition metal catalyzed decomposition of superoxide and hydrogen peroxide (Stohls, 1995).

(iv) **Transition metals**

All elements in the first row of the d-block of the periodic table are classified as transition metals. In general, they contain one or more unpaired electrons and are, therefore, themselves radicals when in the elemental state. However their key property from the point of view of free radical biology is their variable valency, which allows them to undergo reactions involving the transfer of a single electron. The most important transition metals in human diseases are iron and copper. These elements play a key role in the production of hydroxyl radical *in-vivo* (Stohls *et al.*, 1995). Copper has a full outershell but loses and gains electron very easily making itself a free radical (Halliwell *et al.*, 1985). In addition, iron has the ability to gain and lose electrons very easily. This property makes iron and copper the two common catalyst of oxidation reaction, iron is a major component of red blood cells (RBC). A possible hypothesis is that the stress encountered during oxidation may break down red blood cells releasing free iron. The release of iron can be dentrimental to cellular membrane because of the pro-oxidation effect it can have. Hydrogen peroxide can react with iron (II) or Copper (I) to generate hydroxyl radical in the following reaction, as described by Fenton, 1884.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{2+} + HO + OH$$

(v) **Alkoxyl/peroxyl radical**

Radical reactions have been shown to produce organic peroxides which decompose into alkoxyl and peroxyl radical lipids that can themselves abstract hydrogen and cause the chain reaction of lipid perioxidation when metal ions (Fe^{2+} and Cu^{2+}) are added to lipid systems. The end product of these complex metal ion-catalysed breakdown of lipid hydroperoxide include cytotoxic aldehydes as well as hydrogen carbon gases such as ethane and pentane (Halliwell, 1989).

Table 1 : Free radicals and their effects

Oxidant	Description
Superoxide	One electron state of O_2 formed in many autooxidation reactions and by the electron transport chain. Rather unreactive but can release Fe^{2+} from iron- sulphur. Protein and ferritin undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precausor for metal-catalysed OH formation.
Hydrogen peroxide (H_2O_2)	Two electron reduction state, formed by dismutation of O_2^- or by direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
Hydroxyl radical (OH)	Three electron reduction state ,formed by Fenton reaction and decomposition of peroxy nitrite. Extremely reactive, will attack most components.
Organic hydroperoxide (ROOH)	Formed by radical reactions with cellular components such as lipids and nucleobases
Alkoxyl and peroxyl radical (RO and ROO)	Oxygen centred organic radicals, Lipid forms participate in lipid proxidaiton reaction. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
Hypochlorous acid (HOCl)	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents including thiol groups, amino groups and methionine.
Peroxynitrite ($ONOO^-$)	Formed in a rapid reaction between O_2^- and $NO^\cdot$ Lipid soluble and similar in reactivity to hypochlorus acid. Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen droxide

(Sies 1983; Meyers *et al.*, 1996)

2.5 Physiological Effects of Free Radicals

Under normal conditions (at rest), the antioxidant defence system within the body can easily handle free radicals produced by the body. During times of increased oxygen flux (during exercise), there is increase in free radical production. The body counters the increase in radical production through the antioxidant defence system. When free radical induction exceeds clearance, oxidative damage occurs. Free radicals formed during chronic exercise may exceed the protective capacity of the antioxidant defence system thereby making the body more immune to disease and injury. Free radicals have been implicated in the etiology of a number of diseases such as cardiovascular disease, Parkinson disease (Tanizawa *et al.*, 1992). A free radical attack on a membrane usually damages a cell to the point that it must be removed from the immune system, if free radical formation and attack are not controlled within the muscle during exercise, a large quantity of muscle could be damaged. Damaged inside could in turn inhibit performance by the induction of fatigue.

2.6 Measurement of Free Radicals

Free radicals have a very short half-life which makes them very hard to measure in the laboratory. Multiple methods of measurement are available today, each with their own benefits and limits.

Radicals can be measured using election spin resonance and spin trapping methods. The methods are both very sophisticated and can trap even the short-lived free radical. Exogenous compounds with a high affinity for free radicals (i.e xenobiotics) are utilized in the spin techniques. The compound and radical together, form a stable entity that can be easily measured. This indirect approach has been termed finger printing (Karrison *et al.*, 1997). However this method is not 100% accurate, spin trapping collection techniques have poor sensitivity which can skew result (Acworth and Bally, 1997). A commonly used alternative approach measures markers of free radicals rather than the actual radical. These markers of oxidative stress are measured using a variety of difference assays described below

When a fatty acid is peroxidised, it is broken down into aldehyde such as thiobarbituric acids which are excreted. Thiobarbituric acids (TBAs) have been widely accepted as a general marker of free radical production. The most commonly measured TBAs is malondialdehyde

(MDA) (Karrison, 1997). The TBA test have been challenged because of its lack of specificity. The use of liquid chromatography spectrophotometric techniques help reduce these errors (Wong *et al*., 1987). In addition the test seems to mark best when applied to membrane system such as microsome (Halliwell and Chinico, 1993). Gases such as pentane and ethane are also created as lipid peroxidation occurs. These gases are expired and commonly measured during free radical research (Karrison, 1997). Karter *et al.*, (1988) have reported that serum MDA levels correlated with blood levels of creatinine kinase, an indicator of muscle damage.

Also, conjugated dienes (CD) are often measured as indicators of free radical production (Acmorth and Balley, 1997). Oxidation of unsaturated fatty acids result in the formation of conjugated diene. The conjugated diene formed are measured and provide a marker of the early stage of lipid peroxidation (Halliwell and Gutteridge, 1985). A newly developed technique for measuring free radical production shows promise in producing more valid result. The technique uses monoclonal antibodies and may prove to be the most accurate measurement of free radicals.

2.7 Oxidative Stress

Oxidative stress is defined as an imbalance between oxidants and antioxidants in favour of oxidants, potentially causing damage to cells or cellular components (Sies, 1997). Oxidative stress is caused by an imbalance between the production of reactive oxygen species and biological systems ability to readily detoxify the reactive intermediate or easily repair the resulting damage.

Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all the components of the cells including proteins, lipids and DNA. Further, some reactive oxidative species act as cellular messenger in redox signalling. Thus oxidative stress can cuse disruptions in normal mechanism of cellular signaling.

2.7.1 Oxidative stress and human diseases

In humans, oxidative stress is involved in the development of many disease or may exacerbate their symptoms. These include cancer (Halliwell, 2007), Parkinson's disease (Valko *et al.*, 2007), Alzheimers disease (Robinson, 2000)), atherosclerosis, heart failure (Singh *et al*.,

1995), myocardial infarction (Ramond *et al*., 2011) schizophrenia (Boskovic, 2011), bipolar disorder (Dean *et al*., 2011), fragile X syndrome (Diego *et al*., 2009), sickle cell diseases (Amer *et al*., 2006), lichens planus (Aly and Shahin, 2010), vitiligo (Arican and Kurutus, 2008), autism (James *et al*., 2004), chronic fatigue syndrome (Gwen *et al*., 2005).

Oxidative stress involved in neurodegenerative diseases including Lon Gehrigis disease, Parkinson disease, Alzheimer's disease, Huntinton's disease (Patel and Chi, 2011). Indirect evidence by monitoring biomarkers such as reactive oxygen species and reactive nitrogen species production, indicates oxidative damage which may be involved in pathogenesis of these diseases (Nunomura *et al*., 2005, Boskovic *et al*., 2011) while cummulative oxidative stress with disrupted mithochondrial respiration and mitochondrial damage are related with Alzheimer's, Parkinson and other neurodegenerative disease (Nys and Meirleir, 2006).

Oxidative stress is also linked to certain cardiovascular disease since oxidation of low density lipoprotein (LDL) in the vascular endothelium is a precausor to plaque formation. Oxidative stress also plays a role in the Ischemic cascade due to oxygen reperfusion injury following hypoxia. This cascade inculdes both strokes and heart attack. Oxidative stress also contributes to tissue injury following irradiation and hyperoxia as well as causes diabetes.

Oxidative stress is likely to be involved in age-related development of cancer. The reactive species produced in oxidative stress can cause direct damage to the DNA and are therefore mutagenic and it may also suppress apoptosis and promote proliferation invasiveness and metastalsis (Halliwell, 2007). Infection by *Helicobacter pylori* which increases the production of reactive oxygen and nitrogen species in human stomach is also taught to be important in the development of gastric cancer.

Oxidative stress play adual role in infectious diseases such as trypanosomiasis, malaria and tuberculosis (Ridgley *et al*., 1999; Dondog *et al*., 2003; Kondaveeti *et al*., 2012). In the process of infection, there is generation of reactive species by myloperoxidase, NADPH oxidase and nitric synthase. Some pathologies arising during infection can be attributed to oxidative stress and generation of reactive species in infection can have fatal consequences.

2.8 Antioxidants

An antioxidant is a molecule slowing or preventing the oxidation of other molecules. In their definition of the term "antioxidant", Halliwell and Gutteridge (1989) states that an antioxidant is any substance that when present in low concentration compared with that of oxidisable substrate, specifically delays or inhibits oxidation of that substrate. Oxidation is a chemical reaction that transfers electron from a substance to an oxidizing agent, oxidation reaction can produce free radicals which start chain reactions that damage cells. Antioxidants terminate these reactions by removing free radical intermediates and inhibiting other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols (Sies, 1997). Antioxidants are added as redox systems possessing higher oxidative potential than the drug that they are designed to protect or as chain inhibitors of radical induced decomposition. In general, the effect of antioxidants is to break up the chains formed during propagation process by providing a hydrogen atom or an electron to the free radical and receiving excess energy possessed by the activated molecule (Lachman, 1986). Fruits, vegetables and natural plants contain a large variety of phytochemicals which are the main source of antioxidants in the diet, that could decrease the potential stress caused by reactive oxygen species. The natural antioxidants may have free radical scavengers, reducing agents, potential complexers of prooxidant metals, quenchers of singlet oxygen (Ebadi, 2002). The antioxidant can intefere with the oxidation process by reacting with free radicals (Gupta, 2004)

2.8.1 History of antioxidants

Originally the term antioxidants was specifically used to refer to a chemical that prevented the consumption of oxygen. In the late 19th and 20th Centuries, extensive study was devoted to the use of antioxidant in important industrial processes, such as prevention of metal corrosion; the vulcanistaiton of rubber; and the polymerization of fuels in the fouling of internal combustion engines (Mail, 1947). Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats which is the causes of rancidity (German, 1999). However, it was the identification of vitamin A, C and E as antioxidant that

revolutionized the field and led to the realization of the importance of antioxidants in biochemistry of living organisms (Jacob, 1996; Knight, 1998).

The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be the one that is itself readily oxidised (Moreu and Dufraisse, 1992). Research into how vitamins prevent the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavenging reactive oxygen species before they could damage cells (Wolf, 2005).

A vast majority of complex life requires oxygen for its existence. Oxygen is a highly reactive molecule that damage living organism by producing reactive oxygen species (Davies, 1995). Consequently, organisms contain a complex network of antioxidant metabolites such as vitamin C, vitamin E, glutathione and enzymes such as catalase, superoxide dismutase, various peroxidases that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids (Sies, 1997; Vertuani *et al*., 2004). In general, antioxidant systems either prevent these reactive species from being formed or remove them before they can damage vital components of the cell (Davies, 1995; Sies , 1997). However, since reactive oxygen species do have useful function in cells, such as redox signalling, the function of antioxidant system is not to remove the oxidant entirely but instead to keep them at an optimum level (Rhee, 2006). Antioxidants are classified into two broad divisions depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water soluble antioxidants react with oxidant in the cells cytosol and the blood plasma while lipid soluble antioxidants protect cell membrane from lipid peroxidation (Sies, 1997). These compounds may be synthesized in the body or obtained from the diet (Verutani *et al*., 2004). The different antioxidants are present at a wide range of concentrations in body fluids and tissues with some (such as glutathione or ubiquinone) mostly present within cells while others (such as uric acid) are more evenly distributed. Some antioxidants are only found in a few organisms and these compounds may be important in pathogens and can be virulence factors (Miller, 1997).

2.8.2 Classification of Antioxidants

(1) **Antioxidant enzymes**

(a) **Superoxide dismutase (SOD)**

Superoxide dismutases (SOD) are a class of closely related enzymes that catalyse the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Bannister *et al*., 1987; Zelko *et al*., 2002). SOD enzymes are present in almost all aerobic cells and in the extracellular fluid (Johnson and Giulvi, 2005). SOD enzyme contain metal ion cofactor that, (depending on the isozyme) can be copper, zinc, manganese or iron. In humans, the copper/zinc SOD is present in the mithochondrion (Bannister *et al.*, 1987). There also exists a third form of SOD in extracellular fluids which contain copper and zinc in its active sites (Nozik *et al*., 2005). The mitochondrial isozyme seems to be the most biologically important of these three since mice lacking this enzyme die soon after birth (Melov *et al*., 1998). In contrast, the mice lacking copper/zinc SOD are viable but have numerous pathologies and a reduced life span while mice without the extracellualr SOD have minimal defects (Reaume *et al*., 1996; Ho *et al*., 1998). In plants SOD isozymes are present in the cytosol and mitochondria with an iron SOD found in chloroplasts that is absent form vertebrates and yeast (Van camp *et al*., 1997).

(b). **Catalase**

Catalase are enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen using either iron or manganese cofactor (Zamocky and koller, 1999; Chelikani *et al*., 2004). This protein is localized to peroxisomes in most eukaryotic cells (del Rio *et al*, 1992). Catalase is an unusual enzyme since, despite hydrogen peroxide being its only substrate, it follows a ping-pong mechanism. Its cofactor is oxidize by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate (Hiner *et al*., 2002). Despite its apparent importance in hydrogen peroxide removal, humans with genetic deficiency of catalase acatalasemia suffer few ill effects (Ogata, 1991; Mueller *et al.*, 1997).

(c) **Peroxiredoxins**

Peroxiredoxins are peroxidases that catalyse the reduction of hydrogen peroxide, organic hydroperoxides as well as peroxynitrite (Rhee *et al.*, 2005). They are divided into three classes,

namely; typical 2-cysteine peroxiredoxins, atypical 2-cysteine peroxiredoxins and 1-cysteine peroxiredoxins (Wood *et al*., 2003). These enzymes share the same basic catalytic mechanism in which a redox-active cysteine in the active site is oxidized to a sulfonic acid by the peroxide substrate (Claiborne *et al*., 1999). Over-oxidation of this cysteine residue in peroxiredoxins inactivates these enzymes, but this can be reversed by the action of sulfiredoxin (Jonsson and Lowther, 2007). Peroxiredoxins seems to be important in antioxidant metabolism as mice lacking peroxiredoxin 1 or 2 have shortened life span and suffer from haemolytic anaemia while plants use peroxiredixons to remove hydrogen peroxide generated in chloroplasts (Neuman *et al*., 2003; Lee *et al*., 2003; Dietz *et al*., 2006); according to the following chemical equation.

$$ROOH + 2GSH \longrightarrow GSSG + H_2O + ROH$$

(d) **Glutathione peroxidase and Glutathione reductase**

The glutathione system includes glutathione peroxidase and glutathione-S-transfease (Meister and Anderson, 1983). This system is found in animals, plants and microorganisms (Meister and Anderson, 1983; Creissen *et al*., 1996). Glutathione peroxidase is an enzyme containing four selenium-cofactors that catalyses the break down of hydrogen peroxide and organized hydroperoxides such as lipid hydroperoxide (Takashi *et al*., 1986).

There are at least four different glutathione perioxidase isozymes in animals (Brigelius, 1999). Glutathione peroxidase-1, is the most abundant and is a very efficient scavenger of hydrogen peroxide while glutathione-4, is the most active with lipid hydroperoxide.

Glutathione peroxidase-1 is dispensable, as mice lacking this enzyme have normal life spans (Ho *et al*., 1997) but they are hypersensitive to induced oxidative stress (de Haan *et al*., 1998). In addition, the glutathione-S-transferase show high activity with lipid peroxides (Sharma *et al*., 2004). These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism (Hayes *et al*., 2005).

(2) **The chain breaking antioxidants**

Whenever a free radical interacts with another molecule, secondary radicals may be generated that can then react with other targets to produce yet more radical species. The classic example of such a chain reaction is lipid peroxidation, the reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralized by a chain

breaking antioxidants (De Zwart *et al*., 1999). Chain breaking antioxidants are small molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable by products (Halliwell *et al*., 1993). In general, the change associated with the presence of an unpaired electron becomes dissociated over the scavenger and the resulting product will not readily accept an electron from or donate an electron to another molecule thereby preventing the further propagation of the chain reaction.

(a) **Lipid chain breaking antioxidant**

These antioxidants scavenge radicals in the membrane and lipoprotein particles and are crucial in preventing lipid peroxidation. The most important lipid phase antioxidant is probably vitamin E (Esterbavier *et al*., 1991). Vitamin E occurs in nature in eight different forms which differ greatly in their degree of biological activity. The tocopherol (α, β Y and ∂) have a chromanol ring and a phyllyl tail and differ in the number and position of the methyl groups on the ring. The tocotrienols (α ,β, Y and ∂) are structurally similar but have unsaturated tails both classes of compounds are lipid soluble and have pronounced antioxidant properties (Horwitt *et al*., 1991).

(b) **Aqueous phase chain breaking antioxidant**

These antioxidants will directly scavenge radicals present in the aqueous compartment. Quantitatively, the most important antioxidant of this type is vitamin C (Ascorbic acid) (Levine *et al*., 1991). In humans, vitamin C acts as an essential cofactor for several enzymes catalyzing the hydroxylation reaction. In most cases, it provided electrons for enzymes that requires prosthetic metal ion in a reduced form to achieve full enzymatic activity. Its best known role is as a cofactor for prolyl and lyoyl oxidases in the synthesis of collagen.

(3) **Transition metal binding protein**

Transition metal binding proteins (Ferritin, transferrin, lactoferrin and caeruloplasmin) act as a crucial component of the antioxidant defence system by sequestering iron and copper so that they are not available to drive the formation of the hydroxyl radical. The main copper binding protein caeruloplasmin might also function as an antioxidant enzyme that can catalyse the oxidation of divalent ion. Fe^{2+} is the form of iron that drives the Fenton reaction and the

rapid oxidation of Fe^{2+} to the less reactive Fe^{3+} form is therefore an antioxidant effect as shown in the following equation.

$$Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O$$

(4) **Dietary antioxidants**

Some antioxidants are produced within the body and others have to be obtained from dietary sources. Biological antioxidants are characterized by being readily absorbed and transported to the relevant site within the cell for optimum function and are non toxic at nutritionally relevant intakes. The main dietary antioxidants are discussed below :

(a) **Vitamin E (D- α-Tocopherol)**

As one of the eight naturally occurring vitamin E compounds that are synthesized by plants from homogentisic acid, D-α-tocopherol is a derivative of ∂ chromanol with a saturated C_{16} phytol side chain. It is an example of a phenolic antioxidant. Such molecules readily donate H from the OH group on the ring structure to free radicals which then become unreative. On donating the H, the phenolic itself becomes a relatively unreative free radical because the unpaired electron on the 'O' can be delocalized into the aromatic ring structure thereby increasing its stability (Scott, 1997). Its major biological role is to protect polyunsaturated fats and other components of cell membranes from oxidation by free radicals and is therefore primarily located within the phosphohipid bilayer of cell membrane. It is particularly effective in preventing lipid peroxidation by a series of chemical reaction involving the oxidative deterioration of polyunsasturated fatty acids (Duthie, 1993). Vegetable oils are a major source of D-α-tocopherol in food products although other plant-based food groups contain tocopherol.

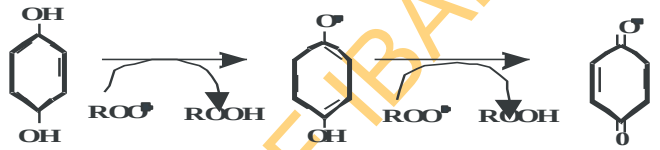


Figure 5 : Simple two steps hydrogen donation by a phenolic antioxidant to lipid radical (ROO $^{\bullet}$). The resulting unpaired electron becomes delocalised within the ring structure so that the phenolic radical is relatively unreactive before ultimately forming quimone.
(Duthie *et al*., 2000)

(b) **Vitamin C (Ascorbc acid)**

Vitamin C (L-Ascorbic acid) can be synthesized from D-glucose or D-galactose in the liver of most higher organism except primates including homosapiens, guinea pig and some species of brids (Halstead, 1993). Major sources include a wide range of vegetables and fruits. Vitamin C is one of the most important water soluble antioxidants in cells that efficiently scavenges a range of reactive oxygen species such as superoxide, hydroxyl radical, peroxyl radicals and singlet oxygen (Sies *et al.*, 1992). By efficiently trapping peroxyl radicals in the aqueous phase of the plasma or cytosol, vitamin C can protect biomembranes and low density lipoprotein from peroxidative damage, in addition to acting as an oxygen scavenger. It can also chelate trace elements such as Fe and Cu which catalyse the decomposition of hydroperoxides to initiate free radical chains. Moreover, vitamin C in chemical systems can donate a H to the tocopheroxyl radical.

(c) **Carotenoids**

Carotenoids are synthesized by photosynthetic microorganisms but not by animals. Particularly rich sources are yellow orange fruits and dark green leafy vegetables. About seven hundred naturally occurring carotenoids have been identified to date, of which about fifty can serve as precursor for vitamin A (Olsen, 1992). All carotenoids are primarily symmetrical C_{40} polyisoprenoid structures with an extensive conjugated double bond system. In general, major dietary classes of carotenoids are hydrocarbon carotenoids (carotene), oxygen containing carotenes (Xantophylls) and carotenyl fatty acid esters (Beecher and Khachik, 1992). Whether or not the primary role of carotenoids is as a biological antioxidant is a matter of debate. However, there is little doubt that because of their polyene structured feature, a number of carotenoids show strong reactions with reactive oxygen species in chemical and biochemical systems. For example β carotene has long been known to be a particularly effective scavenger of singlet oxygen (Bendich and Olsen, 1989). This ability is also shared by α carotene, lycopene, lutein and β cyrptoxanthin and appears to depend largely on physical quenching as opposed to chemical reaction. Antioxidant efficiency of certain carotenoids is also apparent in non photochemical free radical medicated reactions. For example β carotene has also been shown to inhibit peroxidation of liposomal phospholipids exposed to a range of free radical

generation including non dye induced peroxyl radicals, superoxide and transition metals (Liebler *et al*.,1997). Nutritional aspect of carotenoids have been reviewed recently by Castenmiller and West, 1998). Similar to vitamin E, carotenoids trap reactive oxygen species from chemical reaction and prevent oxidative damage.

(d) **Coenzyme Q_{10} (Ubiquinone)**

Ubiquinone is a lipohilic quinone which is similar in structure to vitamin E and which functions as an electron carrier in the mithochondria electron transport chain. However, it also protects membrane phospholipids from peroxidation (Cabrini *et al.*, 1986), and therefore has antioxidant properties. For this reason, it is not classified as a vitamin. However the ability to synthesise ubiqunone decreases with age and it has been proposed that there may be an increasing dependence on food to supply the nutrient. The major sources of Coenzyme Q are meat, vegetable oils and grain (Beyer *et al*., 1956).

(e) **Lipoic acid**

Lipoic acid is needed for mitochondrials function and it is also an antioxidant. It is made in the cell and participates as a cofactor in the conversion of carbohydrates to energy. As an antioxidant, it is both water and fat soluble it can eliminate free radicals in the water compartment of cell in a manner similar to vitamin C and it protects lipids against oxidation similar to vitamin E. Alpha-lipoic acid helps to break down sugars so that energy can be produced from them through cellular respiration. α-lipoic acid plays a truly central role in antioxidant defence, it is an extraordinarily broad spectrum antioxidant which is able to quench a wide range of free radical in both aqueous water and lipid fat chains. Moreover, it has the remarkable ability to recycle several other important antioxidant including Vitamin C and E, glutathione and coenzyme Q_{10} as well as itself. For these reason, α-lipoic acid is called the universal antioxidant. Lipoic acid is the only antioxidants that can boost the level of intracellular glutathione. Major food sources of α-lipoic acid include liver and yeast (Packer *et al.*, 1995).

(5) **Polyphenolic antioxidants**

Phytochemicals are plant chemicals that are neither vitamins nor minerals yet they have health enhancing effects. Phytochemicals help protect against cancer, cardiovascular disease. Many phytochemicals, including flavonoids, polyphenols, alkaloids, are antioxidants and protect cells

against oxidative damage and reduce risk of developing certain types of cancer. In a manner similar to vitamin E, they have the potential to act chemically as antioxidants by ready donation of electron or hydrogen from their hydroxyl moieties.

2.9 The Role of Antioxidants In Disease Treatment and Prevention

The brain is uniquely vulnerable to oxidative injury due to its high metabolic rate and elevated levels of polyunsaturated lipids, the target of lipid peroxidation (Reiter, 1995). Consequently, antioxidants are commonly used as medications to treat various forms of brain injury (Warner *et al*., 2004), sodium thiopental and propofol are used to treat reperfusion injury and traumatic brain injury (Wilson and Gelb, 2002) while the experimental drugs disfuention sodium (Lees *et al.*, 2006) and ebselen (Yamaguchi *et al*., 1998) are being applied in the treatment of stroke. These compounds appear to prevent oxidative stress in neurons and prevent apoptosis and neurological damage. Antioxidants are also being investigated as possible treatment for neuro degenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (Di Matteo and Esposito, 2003; Rao and Balachandram, 2002) and as a way to prevent noise induced hearing loss (Koke *et al.*, 2007). Targeted antioxidants may lead to better medicinal effects. Mithochondria-targeted ubiquinone, for example, may prevent damage to the liver caused by excessive alcohol.

Antioxidants can cancel out the cell damaging effects of free radicals (Sies, 1997). Furthermore, people who eat fruits and vegetables, which are good sources of antioxidants, have a lower risk of heart disease and some neurological disorders (Stanner *et al.*, 2004) and there is evidence that certain types of vegetables, and fruits in general, probably protect against a number of cancers (World Cancer Research Fund, 2007). These observations suggested that antioxidants might help prevent these conditions. There is some evidence that antioxidants assist in preventing disease such as muscular degeneration (Bartlett and Eperjesi, 2003), suppress immunity due to poor nutrition (Wintergerst *et al*., 2006). Oxidation of low density lipoprotein in the blood contributes to heart disease and initial observational studies found that people taking vitamin E supplements had a lower risk of developing heart disease (Rimm *et al*., 1993).

2.10 Infectious Disease

Worldwide infectious disease is the number one cause of death accounting for approximately one half of all deaths in tropical countries (WHO, 2004). In industrialized nations despite the progress made in understanding of microbiology and their control, incidents of epidemics due to drug resistant microorganism and the emergence of hitherto unknown disease causing microbes pose enormous public health concerns. Perhaps, it is not surprising to see these statistics in developing nations but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries such as the United State. Death from infectious disease ranked 5th in 1981, has become the 3rd leading cause of death in 1992, an increase of 58% (Pinner *et al.*, 1996, National Centre for Health Statistics, 2003). It is estimated that infectious disease is the underlying cause of death in 8% of death occurring in United State. The increases are attributed to increase in respiratory tract infection and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosicomial and community acquired infections. Furthermore, the most dramatic increase are occurring in the 25-44 years old age group (Pinner *et al.*, 1996). These negative health trends call for a renewed interest in infectious disease in the medical and pubic health communities and renewed strategies on treatment and prevention. Proposed solution that are outlined include prevention such as vaccination, improved monitoring and development of new treatments. It is this last solution that would encompass the development of new antimicrobials. Historically, plants have provided a good source of anti-infective agents. Emetine, quinine, berberine remain highly effective drugs in the fight against microbial infection. Phytomedicines derived form plants have shown great promise in the treatment of interactable infectious diseases, including the opportunistic AIDS infection.

2.11 Use of Plants as Antimicrobials

Infection caused by pathogenic bacteria and fungi are increasingly recognized as an emerging threat to public health (Walsh *et al.*, 1996; Wu, 1999). Although many drugs are available at present for the treatment of infectious diseases, their use is limited by a number of factors such as low potency, poor solubility, emergence of drug resistant strains and drug toxicity (Kaufman, 2000; Jhon 2002). The use of antibiotic is not successful always since many

of the pathogenic microbes especially bacterial and fungi have developed substantial resistance to the antimicrobial drugs (Jones 1998; Sashilkumar, 1998; Austin *et al*., 1999). This may be due to selective pressure on antibiotic sensitive organisms from the population with the consequent increase in the number of resistant ones. While such a development poses a serious threat to the public, and a great challenge to physician, most affected are the underdeveloped as well as developed countries (Jones, 1998). Decades ago, in India, typhoid could be cured with three inexpensive drugs namely cephalosporins, penicillin G and chloramphenicol but today, these drugs are largely ineffective against life threating typhoid fever. Therefore, there is an urgent need for the discovery of alternative, safe and more effective antimicrobial agents in order to control the life threatening pathogens which has brought in the attention of biologically active compounds from plant and animals sciences (Arokiyaraj *et al*., 2008; Gangadewit *et al*., 2008; Rehan., 2008; Chellaram and Edward, 2008). Plants are the oldest source of pharmacologically active compounds and have provided humankind with many medically useful compounds for centuries (Cordell, 1981). Among the available 250,000 to 500,000 species of plants on earth, only 1% to 10% of these are used as food by both human and animal species. It is possible that even more can be utilized for this purpose. Hipporcates (in the late fifth century, BC) has already mentioned 300 to 400 medicinal plants (Schulites, 1978). It is estimated that more than two thirds of the worlds population relies on plant derived drugs. Some 7,000 medicinal compounds used in the Western Pharmacopoeia are derived from plants (Caudfield, 1991). The bioactive compounds in the plants are produced as secondary metabolites. Examples include alkaloids and proteins (Chakraborty and Branther, 1999) which may be stage specific or tissue specific. Infact, there are several studies which have revealed the presence of such compounds with antimicrobial properties (Cowon, 1999). Numerous orchid species are traditionally used in herbal medicine as a remedy for microbial infection and number of other ailments. However the potential of most of the orchid species for therapeutic use is yet to be scientifically explored. Antimicrobials of plant origin have enormous therapeutic potential and they have been used since time immemorial. They have proved effective in the treatment of infectious disease, simultaneously mitigating many of the side effects which are often associated with synthetic antibiotics. Positive response of plant based drugs might lie on the structure of

the natural products which react with toxins and pathogens in such a way that less harm is done to other important molecules or turning their attention to natural products looking for new leads to develop better drugs against cancer as well as oral and microbial infections (Hoffman *et al.*, 1993; Srinivasan *et al.*, 2001). The medicinal value of plants lies in some chemical substances that produce definite physiological actions on human body. The most important of these include alkalolids, flavonoids, tannins and saponins. The phytochemical research based ethnopharmacological information is generally considered an effective approach in the discovery of new anti-infection agents from higher plant (Duraipandiyan *et al*, 2006).

2.11.1 Resistance of antimicrobial drugs

Antimicrobials is a general term given to medicines that kill or slow down the growth of microbes. Antibiotics resistance is a type of drug resistance where a microorganism is able to survive exposure to an antibiotic. Despite a push for new antibiotics therapies, there has been a continuous decline in the number of newly approved drugs. Antibiotics resistant therefore poses a significant problem. The increasing incidence of microbial diseases and non infectious diseases in man and therapeutics difficulties occasioned the search for new drugs. Antimicrobial resistance is a global problem that affects all countries (Chatterjee and Fleck, 2011) and it is becoming more dangerous, particularly in West African countries where high prevalence of multi drug resistant bacteria producing beta-lactamases have been reported (Aka *et al*., 1987; Keasah *et al*., 1998; Okesola *et al*., 1999; Benbachir *et al*., 2001; Kacou-Ndouba *et al*., 2001; Akoua *et al*., 2004; Akinyemi *et al*., 2005). In addition, morbidity and mortality of transmitted diseases are on the increase within the population including the young one. Antimicrobials have saved counteless lives and blunted serious complications of many feared diseases and infections. The success of antimicrobials against disease causing microbes is among modern medicine's great achievement. After more than 70 years of widespread use, evolution of disease causing microbes also has resulted in many antimicrobials loosing their effectiveness. As microbials evolve, they adapt to their environment. If something stops them from growing and spreading, such as an antimicrobial, the organisms will evolve a new mechanism to resist the antimicrobial intervention by changing the genetic structure. Changing the genetic structure ensures that the offspring of the resistant microbes also are resistant. Bacteria may be

intrinsically resistant to, greater than, or equal to the first class of antimicrobial agents or may acquire resistance by denovo mutation or via the acquisition of resistant genes from other organisms. Acquired resistance genes may enable a bacterium to:

(a) Produce enzymes that destroy the antibacterial drug

(b) Express efflux systems that prevent the drug from reaching its intracellular target.

(c) Produce an alternative metabolic pathway that by passes the action of the drug.

Acquisition of new genetic material by antimicrobial susceptible strains or bacteria may occur through conjugation, transformation or transduction with transporon which often facilitates the incorporation of multiple resistant genes into the host's genome or plasmid. Antimicrobial resistance makes it harder to eliminate infection from the body, as a result of microbes ability to survive in the presence of antimicrobials.

2.11.2 Screening methods to determine antimicrobial activity of natural products.

The emergence of new infectious diseases, the resurgence of several infections that appeared to have been controlled and the increase in bacterial resistance have created the necessity for studies directed towards the development of new antimicrobials. Considering the failure to acquire new molecules with antimicrobial properties from microorganisms, the optimization for screening methods used for the identification of antimicrobials from other natural resources is of great importance because of the failure of available antimicrobials to treat infectious diseases. Many researchers have focused on the investigation of natural products as a source of new bioactive molecules (Recio and Rios; 1989, Silver and Bostian, 1993). A variety of methods are found for this purpose since not all of them are based on the same principles and results obtained will also be profoundly influenced not only by the method selected but also by the microorganism used to carry out the test as well as the degree of solubility of each test compound (Kios *et al*., 1988; Vanden and Vlietinck, 1991). A test system should ideally be simple, rapid, reproducible and inexpensive and maximize high sample through put in order to cope with a varied number of extracts and fractions. The complexity of the bioassay must be defined by laboratory facilities, quality, and available personeel (Hostettman *et al.*, 1997; Hadacek and Greger, 2006). The currently available screening methods of antimicrobials activity of natural products fall into three groups including bioautogrpahic, agar diffusion and

agar dilution methods. The bioautographic and agar diffusion methods are known as qualitative techniques, since these methods will only give an idea of the presence or absence of substance with antimicrobial activity. On the other hand, agar dilution methods are considered quantitative assays once they determine the minimum inhibitory concentrations (Vaden and Vlietinck, 1991).

2.12 Inflammation

Inflammation is part of a complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells or irritants (Ferrero *et al*., 2007). Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate a healing process (Newman *et al*., 2000). Inflammation is the means by which the body deals with insult and injury (Suffredini, 1999; Streetz *et al*.,2001). Insult may be caused mechanically (e.g by pressure or foreign bodies), chemically (e.g by toxins, acidity, alkalinity), physically (e.g by temperature), by internal process (e.g uremia) and by microorganisms (e.g bacteria, virus, parasites). Inflammation rids the body of foreign matter and disposes off damage cells and initiate wound healing. Inflammation is not a synonym for infection, even in cases where inflammation is caused by infection. Although infection is caused by a microorganism, inflammation is one of the responses of the organism to the pathogen. However, inflammation is a stereotyped response and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen (Abbas and Litchman, 2009). Innate immunity is what is naturally present in our bodies when we are born and not the adaptive immunity which we acquire after an infection or vaccination. Innate immunity is generally non- specific while adaptive immunity is specific to one pathogen.

The word “nflammation” comes from Latin “inflammo’’ meaning I set alight, I ignite. Without inflammation, wounds and infections would never heal. Similarly, progressive destruction of the tissue would compromise the survival of the organism. However, chronic inflammation can also lead to a host of diseases such as hay fever, periondantitis, atherosclerosis, rheumatoid arthritis and even cancer e.g gall bladder (carcinoma). It is for that reason that inflammation is normally closely regulated by the body. Inflammation controlled by mast cells that are in close proximity to autonomic nerves. Mast cells are constituents of connective tissue containing large granules

that contain heparin, serotonin, bradykinin and histamine (Alexacos *et al.*,1999). These substances are released from the mast cells in response to injury and infection and by their degranulation, they control most of the process of inflammation. Mast cells are responsive to other controls for example under the influence of estrogen they release histamine. Another important pathway is known as arachidonic acid cascade which is largely controlled by eicosanoids (Bello *et al.*, 1999). Eicosanoids are local hormone that is made from 20-carbon essential fatty acid. They are short lived and can affect many aspects of physiological functions at the cellular level. Eicosanoids include all prostaglandins, thromboxane and leukotrienes. Depending on genetic as well as other factors, eiocosanoids transform or control prostaglandins, thromboxanes and leukotrienes all of which are inflammatory mediators. Eicosanoids can initiate all local inflammatory reponses. When inflammation affects a joint (such as rheumatoid), the cartilage can be damaged by neutrophils and lysosomal enzymes that enter the area. This lead to a vicious cycle of repeated injury.

2.12.1 Causes of inflammation

Causes of inflammation are as follows:

- Burns
- Chemical irritants
- Frost bite
- Toxins
- Infection by pathogens
- Immune reactions due to hypersensitivity
- Physical injury, blunt or penetrating ionising radiation
- Foreign bodies including splinters, dirt and debris
- Stress
- Trauma
- Alcohol

2.12.2 Types of inflammation

(i) **Acute inflammation**

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increase movement of plasma and leukocytes (especially granulocytes) from the blood into injured tissues. A cascade of biochemical events propagates and matures the inflammatory response involving the local vascular system, the immune system and various cells within the injured tissue. Acute inflammation starts rapidly (rapid onset) and quickly becomes severe. Signs and symptons are only present for a few weeks. Examples of disease conditions and situation which can result in acute inflammation include acute bronchitis, infected toe nail, sore throat from a cold or flu, a stratch, cut on the skin, acute appenditis, acute dermatitis, acute menigitis, acute sinutisis or a blow. Within a few seconds or minutes after tissue is injured, acute inflammation starts occurring. The damage may be a physical one or might be caused by an immune response. Three main processes occur before and during acute inflammation :

(i) Arterioles, small branches of artieries that leads to the damaged region, dilate thereby resulting in increased blood flow. The capillaries become more permeable, so fluid and blood protein can move into interstitial spaces between tissues.

(ii) Neutrophils and possibly some macrophages migrate out of the capillaries and venules (small veins that go from a capiallary to vein) and move into interstitial spaces. A neutrophil is a type of granulocyte (white blood cells). It is filled with tiny sacs which contain enzymes that digest microorganisms. Macrophages are also a type of white blood cells that ingest foreign materials for example when the skin is scratched and it is not broken, one may see a pale red line. Soon the area around the scratch goes red, this is because the arteriole have dilated and the capillaries have filled up with blood and become more permeable to move.

Acute inflammation is a short term process usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimulus (Cotran *et al*., 1998). It is characterized by five cardinal signs (Parakama and Clive, 2005). The acronyms that may be used for this is PRISH for pain, redness, immobility (loss of function), swelling and heat. The traditional names for signs of inflammation comes from Latin. Dolor (pain), Color (Heat), Rubor (Redness), Tumour (Swelling) and Function laesa (loss of function) (Ruth, 2009).

The first four classical sign were described by Celcius (30BC-38AD) (Vogel *et al.*, 2009), while loss of function was added later by Galen Porth,(2007).

The five cardinal signs of acute inflammation (PRISH)

Pain : The inflamed area is likely to be painful especially when touched. Chemicals that stimulate nerve endings are released, making the area much sensitive.

Redness : This is because the capillaries are filled up with more blood than usual.

Immobility : There may be some loss of function.

Swelling : Swelling is caused by accumulation of fluid.

Heat : More blood moves to the affected area and makes it feel hot to the touch. These five signs appear when acute inflammation occurs in the body surface whereas acute inflammation of internal organs may not result in the full set. The process of acute inflammation is initiated by cells already present in all tissue, mainly resident macrophages dendritic cells, histiocytes, kupffer cells and mastocytes. These cells present on their surface contain receptors named pattern recognition receptors (PRRs) which recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively, referred to as pathogen. Associated molecular patterns (PAMPs). At the onset of an infection, burn or other injuries these cells undergo activation (one of their PPRs recognize a PAMP) and release inflammatory mediators responsible for the clinical signs of inflammation, vasodilaton and its resulting increase blood flow causes the redness (rubor) and increased heat (calor). Increased permeability of the blood vessels result in an exudation (leakage of plasma proteins and fluids into the tissue (edema) which manifests it self as tumour. Some of the released mediators such as bradykinins, increase the sensitivity to pain (hyperalgesia, dolor). The mediator molecules also alter the blood vessels (extravasation) into the tissues. The neutrophil migrate along a chemotactic gradient created by the local cells to reach the site of injury (Cotran *et al.*, 1998). The loss of function is probably the result of a neurological reflex in response to pain. In addition to cell-derived mediators, several cellular biochemical cascade systems consisting of preformed plasma proteins act in parallel to initiate and propagate the inflammatory response. These include the complement system activated by bacteria and the coagulation and fibrinolysis systems activated by necrosis e.g a burn or a trauma.The acute inflammatory response requires a

constant stimulation to be sustained. Inflammatory mediators have short half life and are quickly degraded in the tissue. Hence, acute inflammation ceases once the stimulus has been removed.

(ii) **Chronic inflammation**

Chronic inflammation means long term inflammation which can last for several months and even years. It can result from failure to eliminate whatever was causing an acute inflammation. An auto immune response to a self antigen, the immune system attack healthy tissues, mistaking it for harmful pathogens. Examples of diseases and conditions with chronic inflammation include asthma, chronic peptic ulcer, tuberculosis, rheumatoid, arthritis, chronic periondotitis, ulcerative colitis, chronic hepatitis. Chronic inflammation leads to a progressive shift in type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. It can also manifest, histologically, by the presence of lymphocytes, macrophages and result in fibrosis and tissue necrosis. Infections, wounds and any damage to tissue would never heal without inflammation. Tissues would become more and more damaged and the body or any organ would eventually perish.

Table 2 : Comparison between acute and chronic inflammation (Graham *et al*., 1988)

	Acute	Chronic
Causative agent	Pathogens, injured tissues.	Persistent acute inflammation due to non degradable pathogens, persistent foreign bodies or autoimmune reaction.
Major cells involved	Neutrophils, mononuclear cells (monocytes, macr phages).	Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts.
Primary mediators	Vaso-active Amines, eiocosanoids.	IFN-Y and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes.
Onset	Immediate	Delayed
Duration	Few days	Up to many months
Outcomes	Resolution, abscess formation chronic inflammation	Tissue destruction, fibrosis, necrosis

2.12.3 Mediators of inflammation

Inflammatory mediators are soluble diffusible molecules that act locally at the site of tissue damage and infection and at more distant sites. In appreciating the inflammatory process, it is important to understand the role of chemical mediators. These are the substances that tend to direct inflammatory response. These inflammatory mediators come from plasma proteins or cells including mast cells, platelets, neurtrophils and monocytes. They are triggered by bacterial product or host proteins. Chemical mediators bind to specific receptors on target cells and can increase vascular permeability and neutrophil chemotaxis, stimulate smooth muscle contraction, have direct enzymatic activity, induce pain or mediate oxidative damage. Most mediators are short lived but cause harmful effects (Cotran *et al*., 1998). Chemical mediators of the inflammatory process include a variety of substances originating in the plasma and the cells of the injured tissues and possibly from the damage tissues. The mediators of inflammation include: :

(a) Plasma protein such as complement and antibodies
(b) Cytokine and chemokines such as IL-10
(c) Lipids such as prostaglandins and polyunsaturated fatty acids
(d) Vasoactive amines such as histamine and serotonin
(e) Gases such as NO and O_2
(f) Kinins such as bradykinin
(g) Neuropeptides.

Once leukocytes have arrived at a site of infection or inflammation, they release mediators which control the later accumulation and activation of other cells. However, in inflammatory reactions initiated by the immune system, the ultimate control is exerted by the antigen itself, in the same way as it controls the immune response itself. For this reason, the cellular accumulation at the site of chronic infection or in auto immune reaction (where the antigen cannot ultimately be eradicated) is quite different from that at sites where the antigenic stimulus is rapidly cleared.

There are four major plasma enzymes systems which have an important role in homeostasis and control inflammation. These are complement system, the clotting system, the fibrinolytic

system and the kinnin system. Inflammatory mediators can be divided into exogenous and endogenous mediators. Bacterial products and toxins can act as exogenous mediators of inflammation and noteable among this is endotoxin. The immune system of higher organisms has probably evolved in a veritable sea of endotoxin, so it is perhaps not surprising that this substance evokes powerful response. For example, endotoxin can trigger complement activation resulting in the formation of anaphylatoxins (C3a and C5a) which cause vasodilation and increase vascular permeability. In addition, endotoxin elicit T cell proliferation and have been described as super antigen for T cells.

Endogenous mediators of inflammation are produced from within the innate and adaptive immune system itself as well as other systems. For example, they can be derived from molecules that are normally present in the plasma in an active form such as peptide, fragments of some components of complements, coagulation and kinnin systems. Mediators of inflammatory responses are also released at the site of injury by a number of cells types that either contain them as preformed molecules within storages granules e.g histamine which can rapidly switch on the machinery requires to synthesize the mediators when they are required for example, to produce metabolites of arachidonic acid.

Monocular phagocytes (monocytes and macrophages) are central to inflammation as they produce many components which participate in or regulate the different plasma enzymes systems and hence the mediators of inflammatory response. Early phase mediators are produced by mast cells and platelets. They are especially important in acute inflammation and include mainly histamine, serotonin and other vasoactive substances. To the early phase mediators also belong chemoatractants e.g (5a and cytokines such as IL-1, IL-6 and TNF.

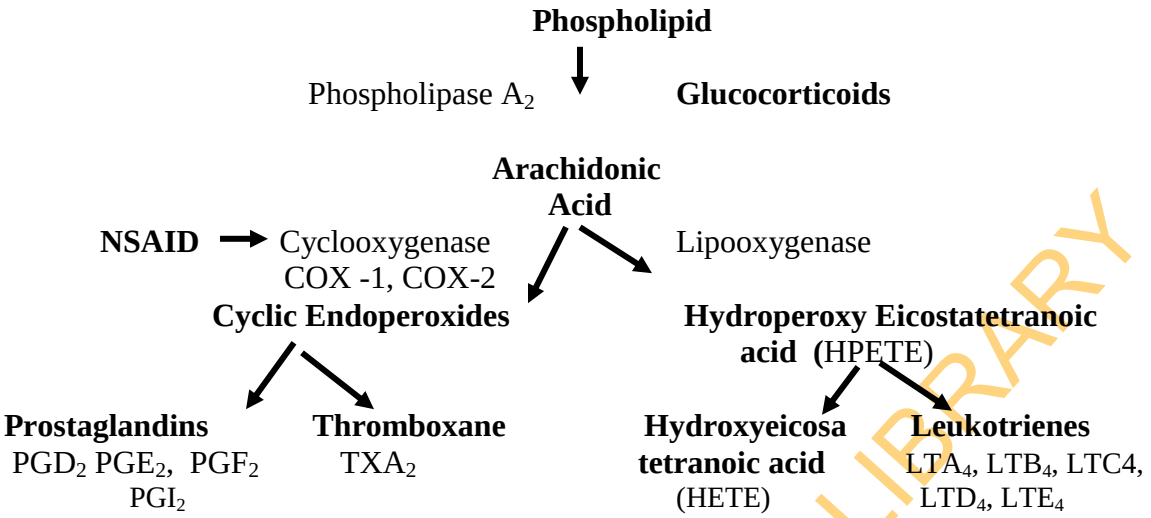
2.12.4 Antinflammatory drugs

Anti-inflammatory drugs are drugs with analgesics and antipyretic (fever reducing) effect and which have in higher doses anti-inflammatory effect. Anti-inflmamtory drugs are usually abbreviated to as non steroidal anti-inflammatory drug (NSAIDs). The term non steroidal is used to distinguish these drugs from steroids which among a broad range of other effect have a similar eicosanoid-depressing anti-inflmmatory action. NSAIDS work by reducing the production of prostaglandins. Prostaglandins are chemicals that promote inflammation, pain and

fever. They also protect the lining of the stomach and intestines from the damaging effects of acid and promote blood clotting by activating blood platelets. Prostaglandins also affect kidney function. The enzymes that produce prostaglandins are called cyclooxygenase (COX). There are two types of COX enzymes, COX-1 and COX-2. Both enzymes produce prostaglandins that promote inflammation pain and fever, however only COX-1 produces prostanglandins that activate platelets and protect the stomach and intestinal lining, NSAIDs block COX enzymes and reduce production of prostaglandins. Therefore, inflammation, pain and fever are reduced. Most prominent members of this groups of drugs are aspirin, ibruprofen and naproxes all of which are available over the counter in many areas (Stuart *et al*., 2010).

2.12.4.1 Mechanism of action of non steroidal anti-inflammatory drugs (NSAIDs)

Non sterioidal anti-inflammatory drugs (NSAIDs) act as non selective inhibitors of the enzyme cycoloxgenase (COX) which is known to have at least two distinct isoforms; COX-1 and the inducible isoform COX-2 . The NSAIDs inhibit both COX-1 and COX-2 isoenzymes. Cycloxygenase (COX) catalyses the formation of prostaglandins and thomboxane from arachidonic acid. Prostaglandins act as messenger molecules in the process of inflammation. COX-1 has clear physiological function. Its activation leads for instance to the production of prostaglandin which when released by the endothelium is antithrombogenic and when released by gastric mucosa is cytoprotecive. COX-2, discovered 6 years ago, is induced by inflammatory stimuli and cytokines in migratory and other cells. It is therefore attractive to suggest that the anti-inflammatory action of NSAIDs is due to inhibiton of COX-2, whereas the unwanted side effects such as irritation of the stomach lining are due to inhibition of COX-1. Drugs that have the highest COX-2 activity and a more favourable COX-2 : COX-1 activity ratio will have a more potent anti-inflammatory activity with fewer side effects than drugs with a less faovurably COX-2 : COX-1 activity ratio. The identification of selective inhibitors of COX-2 will, therefore, lead to an advance in therapy.



Three isoforms of cyclooxygenase have been identified, cyclooxygenase 1, cyclooxygenase 2 (COX -1 and COX-2) and cyclooxygenase 3 (or cyclooxygenase 1b). COX-1 is normally present in all tissues while COX-2 is induced by cytokines and certain serum factors.

Glucocorticoids inhibit phospholipase A_2 and thus block the production of both prostaglandins and leukotrienes, exerting a potent anti-inflammatory effect. Glucocorticoids also block the action of cyclooxygenase -2.

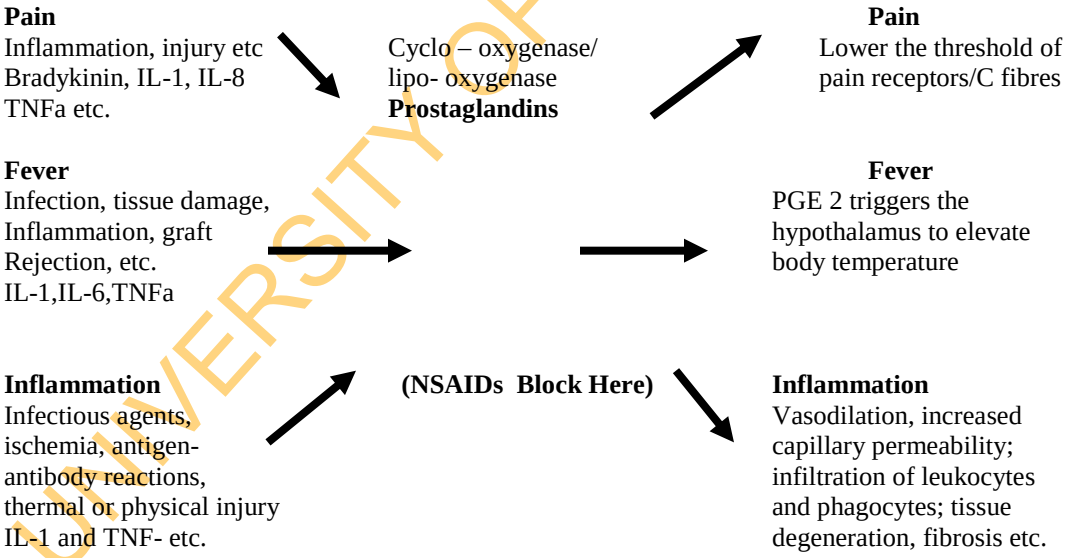


Figure 6 : Cyclooxygenase and lipooxygenase pathway to produce prostaglandins and leukotrienes respectively (Nelson and Randy, 2005)

2.13 Malaria

Malaria is a mosquito borne infectious disease of humans and other animals caused by protozoans parasites of the genus *Plasmodium.* It is one of the most common infectious diseases and an enormous public health problem. It infects between 300-500 million people every year and causes between one and three million deaths annually mostly among young children in SubSaharan Africa (Miller *et al*., 1994; More, 2002; David *et al*., 2004; Martin *et al*., 2004; Wright, 2005; WHO, 2005). It begins with a bite from an infected female mosquito which is introduced into the parasite via its saliva into the circulatory system and ultimately to the liver where they mature and reproduce. The disease causes symptoms that typically include fever and headache which in severe cases can progress to coma and death. Malaria is widespread in subtropical regions in a broad band around the equator, including much of SubSaharan Africa, Asia and the Americas. Five species of *Plasmodium* can infect and be transmitted by humans (Hardman *et al*., 2001). The vast majority of deaths are caused by *Plasmodium falciparum* while *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae* cause a generally milder form of malaria that is rarely fatal. *Plasmodium falciparum*, the most important pathogenic representative of this species is responsible for the majority of cases (Tramputz et al., 2003; Batista *et al*., 2009; Kakkilaya, 2008) The zoonotic species *Plasmodium knowlesi*, prevalent in Southeast Asia causes malaria in macaques but can also cause severe infection in humans. Malaria is prevalent in the tropical region because significant amount of rainfall, warm temperature and stagnant waters provide habitats ideal for mosquito larvae. Disease transmission can be reduced by preventing mosquito bites; by distribution of mosquito nets and insect repellants, or with mosquito control measures such as spraying insecticides and draining standing water. Malaria is typically diagnosed by microscopic examination of blood using blood films with antigen based rapid diagnostic tests. Modern techniques that use the polymerase chain reaction to detect parasite DNA have also been developed but these are not widely used in malaria endemic areas due to their high cost and complexity.

The World Health Organization (WHO) has estimated that in the year 2010, there were 216 million documented cases of malaria. In that period, between 655,000 and 1.2 million people died from the disease (roughly 2,000-3,000 per day) (Hardman *et a*l., 2001) many of whom

were children in Africa. Malaria is commonly associated with poverty and is also a major hinderance to economic development. Despite a need, no effective vaccine currently exists; although efforts to develop one are ongoing. Several medications are available to prevent malaria in travelers to malaria endemic countries (prophylaxis). A variety of antimalaria medications are available. Severe malaria is treated with intravenous or intramuscular quinine or since the mid-2000s, with the artermisinin derivative artesunate, which is superior to quinine, in both children and adults and is given in combination with a second antimalarial, such as mefloquine.

2.13.1 Causes of malaria

Malaria is caused by the protozoan parasite of the genus *Plasmodium.* They belong to the class porozoa which are characterized by giving rise to the young forms (sporozoites) usually enclosed in a cyst (Bruce- Chawatt, 1980). In humans, malaria is caused by *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax* and *Plasmodium knowlesi* (Hartman *et al*., 2010; Rijken *et al*., 2012). Among those infected, *Plasmodium falciparum* is the most commonly species identified (75%), followed by *Plasmodium vivax* (20%) (Nadjim and Behrens, 2012). *Plasmodium falciparum* accounts for the majority of death (Mueller *et al*., 2007), non falciparum species have been found to be the cause of about 14% case of severe malaria in some groups (Nadjim and Behrens, 2012). *Plasmodium vivax* proportionally is more common outside of Africa (Collins, 2012). There have been documented human infections with several species of *Plasmodium* from higher apes, however, with the exception of *Plasmodium knowlesi*, a zoonotic species that causes malaria in macaques (Rijken *et al*., 2012). These are mostly of limited public health importance (Sarkar *et al*., 2009). Rodent malaria parasites include *Plasmodium berghei, Plasmodium yoelli, Plasmodium vinckei* and *Plasmodium chabandi* (Garnham, 1966).

2.13.2 The life cycle of malaria parasite

In the life cycle of *Plasmodium,* a female anopheles mosquito (the definite host) transmits a motile infective stage called the sporozoites to a vertebrate host such as human (the secondary host), thus acting as a transmission vectors. A sporozoite travels in blood vessels to liver cells, where it reproduces asexually. Some offspring called merozoites enter red blood cells and liver

cells where they divide asexually. Other offspring develop into immature gametes or gametocytes. When a mosquito bites an infected person, gameotcytes are taken up with the blood and mature in the mosquito gut. Gametes fuse and form zygotes which develop into new sporozoites. The sporozoites migrate to the insect salivary gland where they then ready to infect a new vertebrate host. The sporozoites are injected into the skin, alongside saliva, when the mosquito takes a subsequent blood meal. This type of transmission is occasionally referred to as anterior station transfer, only female mosquitoes feed on blood, male mosquitoes feed on plant nectar and thus do not transmit the disease. The females of the Anopheles genus of mosquito prefer to feed at night. They usually start searching for a meal at dusk and will continue throughout the night until taking a meal (Collins and Barmwell, 2009). Malaria parasite can also be transmitted by blood transfusion although this is rare.

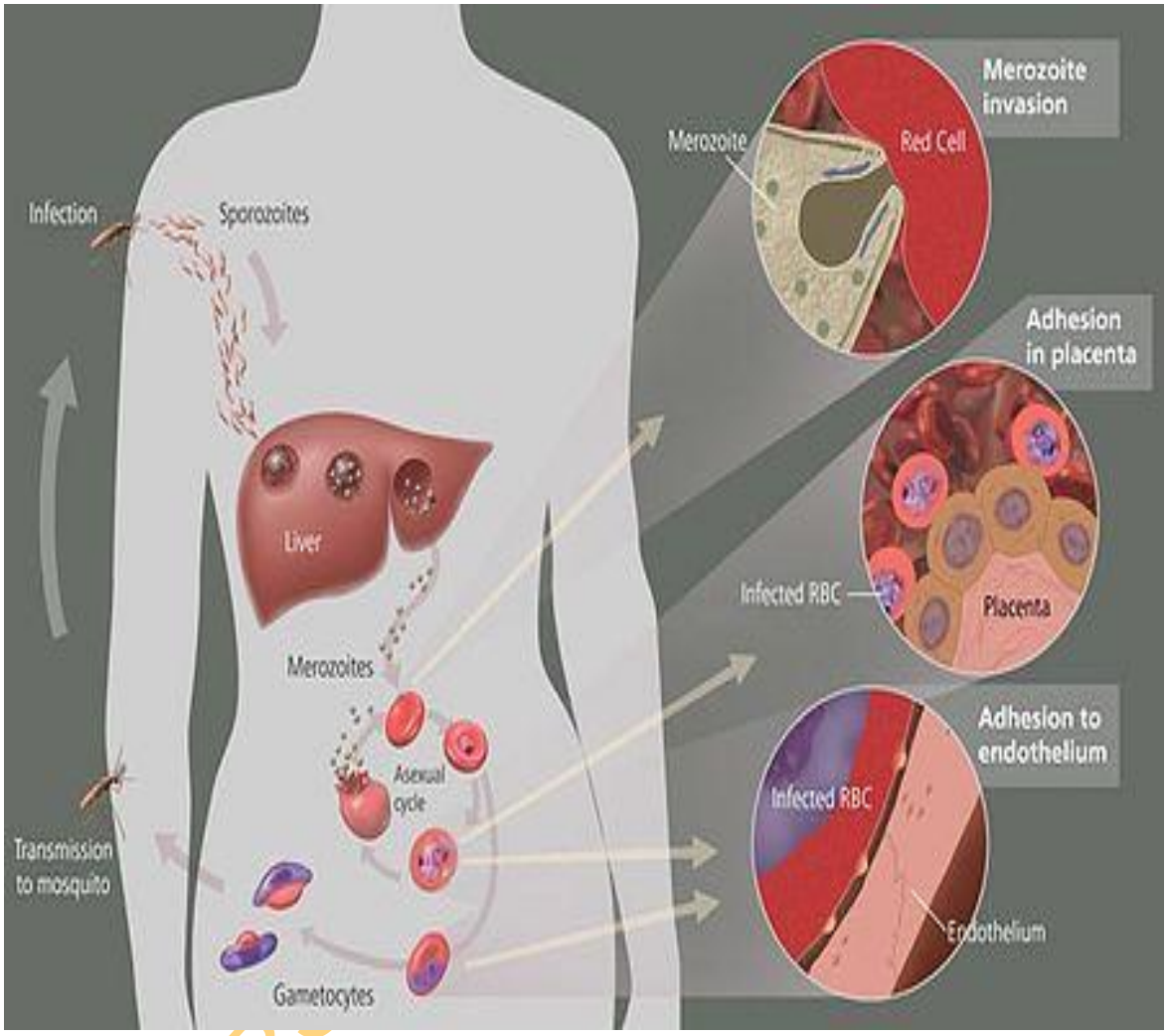


Figure 7 : Life cycle of malaria parasite
(Talman *et al*., 2004)

2.13.3 Chemotherapy of malaria

In the 1950s and 1960s, (WHO) concentrated efforts on eradication of malaria, and failure of the eradication programme led to the decision to control the disease through the use of antimalaria drugs (Bruce-Chawtt, 1980), such drugs may be used for some or all of the following: Treatment of malaria in an individual with suspected or confirmed infection, prevention of infection in individuals visiting a malaria endemic region who have no immunity (prophylaxis), routine intermittent treatment of certain groups in endemic regions (intermittent preventive therapy). Current practice in treating cases of malaria is based on the concept of combination therapy, since this offers several advantages including risk of treatment failure, reduced risk of developing resistance, enhanced convenience and reduce side effects. Prompt parasitological confirmation microscope or, alternatively, by rapid diagnostic test is recommended in all patients suspected of malaria before treatment is started. Treatment on the basis of clinical suspicion should only be considered when parasitological diagnosis is not accessible (Mayvar *et al.*, 2012). There are several families of drugs used to treat malaria as it was cheap and effective, chloroquine was the antimalaria drug for many years in most part of the world. However, resistance of *Plasmodium falciparum* to chloroquine has spread recently from Asia to Africa making the drug ineffective against the most dangerous *Plasmodium* strain in many affected regions of the world. In those areas where chloroquine is still effective, it remains the first treatment choice. Unfortunately, chloroquine resistance is associated with reduced sensitivity to other drugs such as qunine, amodiaquine (Tinto *et al.*, 2005). Some of the antimalaria drugs are used for treatment and partial, prevention (prophylaxis). Many drugs can be used for both purposes. Currently commercially available anti malaria drugs include:

- Arthemeter-lumefantrine (Therapy only and commercially named coartem)
- Artesunate-amodiaquine (Therapy only)
- Artesunate-melfoquine (Therapy only)
- Artesunate-sulfadoxine / pyrimethamine (Therapy only)
- Quinine (Therapy only)
- Chloroquine (Therapy and prophlaxis, and usefulness now reduce due to resistance).
- Cotrifazid (Therapy and prophylaxis)

- Doxycycline (Therapy and prophylaxis)
- Mefloquine (Trade name Lariam) (Therapy and prophylaxis).
- Primaquine (Therapy in *Plasmodium vivax* and *Plasmodium ovale* only).
- Proguanil (Prophylaxis only)
- Sulfadoxine-pyrimethamine (Therapy, prophylaxis in pregnant women).

2.13.4 Some selected antimalaria drugs and their mechanisms of action

(a) **Chloroquine**

Chloroquine is a derivative of 4-aminoqunolines. It was discovered in 1934 by Hans Andersug and coworkers at the Bayer laboratories who named it " Resochin" (Krafts *et al*., 2012) because it was considered toxic for human use. During the World War II. The United State of America government sponsored clinical trials for antimalaria drug development showed unequivocally that chloroquine has a significant therapeutic value as an antimalaria drug. It was introduced into clinical practice in 1947 for the prophylactic treatment of malaria (Farhurst and Wellen,2010) . Chloroquine has long been used in the treatment or prevention of malaria. After the malaria parasite *Plasmodium falciparum* started to develop widespread resistance to chloroquine (Plowe, 2005; Uhelman and Krishna, 2005), new potential uses of cheap and widely available drugs have been investigated.

Mechanism of action of chloroquine

Chloroquine exhibits outstanding activity against the asexual blood forms of all plasmodial species causing malaria in man. The drug is a blood schizontoncide that is effective not only against *Plasmodium falciparum* but also against chloroquine sensitive strain of *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale.* Chloroquine is one of the most rapid inhibitors of nucleic acid and protein synthesis in susceptible parasite strains. It acts on ring stages of the parasites. Chloroquine primarily acts by intercalating between deoxyribonucleic acid base pairs preventing sorting out, transcription and consequently block the replication process. Chloroquine is a strong inhibitor of hemozoin crystal inside the red blood cells, and the malaria parasite must degrade haemoglobin to acquire essential amino acids which the parasite requires to construct its own protein and for energy metabolism. Digestion is carried out in a vacuole of the parasitic cell. During this process, the parasite releases the toxic soluble molecule

a haeme. The haeme moiety consists of the porphyrin ring called Fe (II)-pritoporphyrin (FP). To avoid destruction by this molecule. The parasite biocyrstalises haeme to form hemozoin, a non toxic molecule. Hemozoin collects in the digestive vacuole as insoluble crystals. Chloroquine enters the red blood cell, inhabiting parasite cell and digestive vacuole by simple diffusion. Chloroquine then becomes protonated to $CQ^{2+,}$ as the digestive vacuole is known to be acidic (pH 4.7). Chloroquine cap hemozoin molecules to prevent further biocrystallisation of haeme, thus leading to heme build up. Chloroquine binds to haeme or (FP) to form the FP-chloroquine complex, this complex is highly toxic to the cells and disrupts membrane function. Action of the toxic FP-chloroquine and FP results in cell lysis and ultimately parasite cell autodigestion.

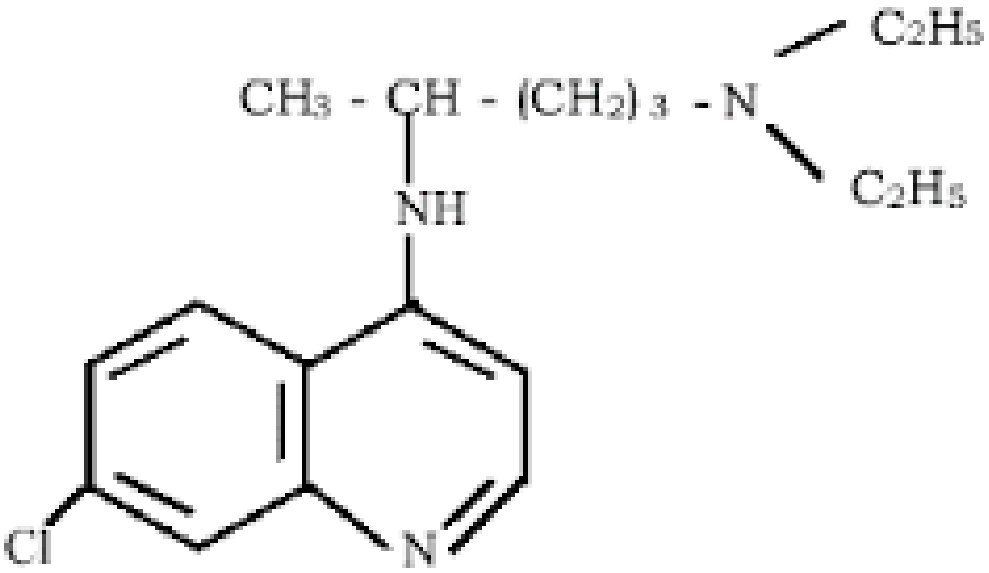


Figure 8: Chloroquine (7– cloroquinolin-4–yl) – N, N , diethyl – pentane – 1,4, diamine)

(Krafts *et al*., 2012)

(b) Artemisinin

Artemisinin (also known as Quinghaosu) and its derivative are a group of drug that possess the most rapid action of all current drug against *Plasmodium falciparum* malaria (Whitney, 1997). Treatment containing an artemisinin derivative artemisinin-combination therapies (ACTs) are now standard treatment worldwide for *Plasmodium falciparum* malaria. The compound artemisinin was isolated from the plant *Artemisia annua* (sweet worm wood), an herb employed in Chinese traditional medicine. Chemically, artemisin is a sesquiterpene lactone containing an unusual peroxide bridge. This peroxide is believed to be responsible for the drug mechanism of action. Use of the drug by itself as a monotherapy is explicitly discouraged by the WHO as there has been signs that malaria parasites are developing resistance to the drug (Rehwagen, 2006). Therapies that combine artemisinin with some other antimalaria drug are the preferred treatment for malaria and are both effective and well tolerated in patients. Artemisinin offers over 90% efficacy rates but their supply is not meeting demand (Senior, 2005). Since the WHO has recommended using ACTs as first line treatment for uncomplicated malaria in areas experiencing resistance to older medications (White, 2004). The most recent WHO treatment guidelines for malaria recommend four different ACTs. While numerous countries including most African nations have adopted the change in their official malaria treatment policies, cost remains a major barrier to ACTs implementation because it is up to twenty times as much as older medications, and ACTs remain unaffordable in many malaria endemic countries. Artemisinin can be used alone, but this leads to a high rate of recrudesence (returns of parasite) and other drugs are required to clear the body of all parasites and prevent recurrence. The WHO has recommended artemisinin combination therapies (ACTs) to be the first line therapy for *Plasmodium falciparum malaria,* world wide. Combination therapies are effective because the artemisin component kill the majority of parasites at the start of the treatment while the more slowly eliminated patner drug clear the remaining parasites (White, 2004). Several fixed dose ACTs are now available containing an artermisinin component and a patner drug which has a long half-life such as melfloquine lumefantrine, amodiaquine, piperaquine (Knidsood *et al*., 2010).

Artemisinins are not used for malaria prophylaxis (prevention) because of the extremely short activity (half-life) of the drug. To be effective, artemisinins would have to be administered multiple times each day because artemisinin itself has physical properties such as poor bioavailability that limit its effectiveness. Semisynthetic derivative of artemisinin have been developed. These include artesunate, artemether, dihydro artemisinin, artenilic acid, artenimol, artemotil.

Mechanism of action of Artemisinin

All artemisinins used today are prodrugs of the biologically active metabolite dihydro artemisinin which is active during the stage when the parasite is located inside red blood cells. Although there is no consensus regarding the mechanism through which artemisinin derivative destroys the parasites (Kappe *et al.*, 2010), several lines of evidence indicate that artemisinin exert their antimalaria action by pertubing redox homeostasis in malaria parasite. When the parasite that causes malaria infect a red blood cell, it consumes hemoglobin within its digestive vacuole, a process that generates oxidative stress (Ginsburg and Atamna, 1994).

The iron of the haeme molecule directly reduce the peroxide bond in artemisinin thereby generating high valent iron-oxospecies and resulting in a cascade of reactions that produce reactive oxygen radicals which damage the parasite and lead to its death (Cumming, 1997). A more recently described alternative mechanism is that artemisinin disrupts cellular redox cycling (Haynes *et al.*, 2011). Numerous studies have investigated the type of damage oxygen radicals may induce, for example, Pandey *et al*., (1999) have observed inhibition of digestive vacuole cysteine protease activity of malaria parasites by artermisinin. These observation were supported by *ex-vivo* experiment showing accumulation of haemoglobin in the parasites treated with artemisinin and inhibition of hemozoin formation by malaria parasites. Electron microscopic evidence linking artemisinin action to the parasite's digestive vacuole has been obtained showing that the digestive vacuole membrane suffers damage soon after parasites are exposed to artemisinin (del Pilar *et al.*, 2008). This would also be consistent with the data showing that the digestive vacuole is already established by mid-ring stage of the parasites blood cycle (Abubakar *et al.*, 2010), a stage that is sensitive to artemisinins but no other antimalarials. Mo and Shen (2005) investigated the mode of action of artemisinin using a yeast

model and they demonstrated that the drug acts on the electron transport chain, generates local reactive species and causes depolarization of the mithochondrial membrane. However, replacement of mithochondrial function in transgenic asexual stage parasites does not alter sensitivity to artemisinins (as would be predicted if mithochondrial targeting was relevant to artemisinin action), whereas atovaquinone resistance is observed which would be consistent with mithochodrial targeting of this type of malaria (Vaidya and Akhil, 2012).

2.14 Tuberculosis

Tuberculosis (TB) is a common and often deadly infectious disease caused by various strains of *Mycobacteria* usually *Mycobacterium tuberculosis* in humans (Kumar *et al.*, 2007). Tuberculosis usually attacks the lungs but can also affect other parts of the body. It is spread through the air when people who have the disease cough, sneeze or spit (Konstantines, 2010). Most infection in human result in an asymptomatic latent infection and about one in ten latent infection eventually progresses to active disease which if left untreated, kills more than 50% of its victims.

The classic symptoms are a chronic cough with blood tinged sputum, fever, night sweats and weight loss. Infection of other organs causes a wide range of symptoms. Diagnosis relies on radiology (commonly chest X –rays), a tuberculin skin test, blood tests, as well as microscopic examination and microbiological culture of body fluids. Treatment is difficult and requires long courses of multiple antibiotics. Antibiotic resistance is a growing problem in multi drug resistant tuberculosis. Prevention relies on screening programs and vaccination, usually with Bacillus Calmette-Guerin vaccine (BCG). One third of the world's population are thought to be infected with *M. tuberculosis* (Jasmer *et al*., 2002; WHO, 2010) and new infections occur at a rate of about one per second (WHO, 2007). The proportion of people who become sick with tuberculosis each year is stable or falling world wide but because of population growth, the absolute number of new cases is still increasing. In the year 2007, there were an estimated 13.7 million chronic active cases, 9.3 million new cases and 1.8 million deaths, mostly in developing countries (WHO, 2009). In addition, more people in the developed world are contracting tuberculosis because their immune systems are compromised by immunosuppressive drugs, substance abuse or AIDs. The distribution of tuberculosis is not uniform across the globe, about

80% of the population in many Asian and African countries test positive in tuberculosis tests, while only 5-10% of the US population test positive (Kumar *et al.*, 2007).

2.14.1 Causes of tuberculosis

The primary cause of TB, *Mycobacterium tuberculosis* (MTB) is a small aerobic non motile bacillus, high lipid content of this pathogen accounts for many of its unique clinical characteristic (Southwick and Frederick, 2007). It divides every 16 to 20 hours, an extremely low rate compared with other bacteria which usually divide in less than an hour (Cox, 2004). Since *Mycobacterium tuberculosis* has a cell wall but lack a phospholipid outer membrare, it is classified as a Gram-positive bacterium if a Gram stain is performed, MTB either stains very weakly or does not retain dye due to high lipid and mycolic acid content of its cells wall (Maidson,2001), MTB can withsand weak disinfectants and survive in a dry state for weeks. In nature, the bacterium can grow only within the cells of a host organism but *M. tuberculosis* can be cultured *in-vitro* (Parish and stoker, 1999). MTB retains certain stains after being treated with acidic solution and it is classified as an acid fast bacillus (AFB). (Kumar *et al.*, 2007). The most common acid fast staining technique, the Ziehl-Neelsen stain, dyes AFBs a bright red that stands out clearly against a blue background. Other ways to visualize AFBs include an auraminerhomadine stain and fluorescent microscopy. The *M. tuberculosis* complex includes four other TB-causing *Mycobacteria: M. bovis, M. africanum, M. carnetti* and *M. microti* (Van Soolingen *et al.*, 1997). *M. africanum* is not wide spread but in parts of Africa it is a significant cause of tuberculosis (Niemann *et al.*, 2002). *M. bovis* was once a common cause of tuberculosis, but the introduction of pasteurized milk has largely eliminated this as a public health problem in developed countries (Thoen, *et al.*, 2006). *M. canetti* is rare and seems to be limited to Africa, although a few cases have been seen in African emigrants (Pfyffer *et al*., 1998). *M. microti* is mostly seen in immunodeficent people, although it is possible that the prevealence of this pathogen has been under estimated (Niemann *et al*., 2000). Other known pathogenic mycobacteria include *Mycobacterium leprae, Mycobacterium kansasii*. The last two are part of the non tuberculous *mycobacteria* (NTM) group. Non tuberculous *mycobacteria* cause neither TB nor leprosy, but they do cause pulmonary diseases resembling TB (American Lung Association, 1997).

2.14.2 Pathogenesis of tuberculosis

About 90% of those infected with *Mycobacterium tuberculosis* have asymptomatic Latent TB infection (LTBI) with only a 10% life time chance that a latent infection will progress to TB disease (Kumar *et al.*, 2007). However, if untreated, the death rate for these active TB cases is more than 50% (Onyebujoh, 2006). TB infection begins when the *mycobacteria* reach the pulmonary alveoli where they invade and replicate within the endosomes of alveolar macrophages (Houben *et al.*, 2006). The primary site of infection in the lungs is called the Ghon focus and is generally located in either the upper part of the lower lobe or the lower part of the upper lobe. Bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local lymph nodes. Further spread is through the blood stream to other tissues and organs where secondary TB lesions can develop in other parts of the lung, peripheral lymph nodes, kidney, brain and bone (Herrmann and Lagrange, 2005)). All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscle and pancreas (Agarval *et al.*, 2005).

Tuberculosis is classified as one of the granulomatous inflammatory conditions. Macrophages, T-lymphocytes, B-lymphocytes and fibroblasts are among the cells that aggregate to form a granuloma with lymphocytes surrounding the infected macrophages. The granuloma functions not only to prevent dissemination of the *Mycobacteria*, but also provides a local environment for communication of cells of the immune system. Within the granuloma, T-ymphocytes secrete cytokines such as interferon gamma, which activates macrophage to destroy the bacteria with which they are infected (Kaufmann, 2002). Cytotoxic T cells can also directly destroy infected cells, by secreting perforin and granulysin (Houben *et al.*, 2006). Importantly, bacteria are not always eliminated within the granuloma, but can become dormant resulting in a latent infection. Another feature of the granulomas of human tuberculosis is the development of abnormal cell death, also called necrosis in the center of tubercles. To the naked eye this has the texture of soft white cheese and was termed caseous vecrosis (Grosset, 2003). If TB bacteria gain entry to the blood stream from an area of damaged tissue, they spread through the body and set up many foci of infection, all appearing as tiny white tubercules in the tissue . This severe form of TB disease is common in infants and the elderly and is called miliary

tuberculosis. Patients with this disseminated TB have a fatality rate near 100% if untreated. However, if treated early, the fatality rate is reduced to near 10% (Kim *et al.*, 2003).

2.14.3 Signs and symptons of tuberculosis

When the disease becomes active, 75% of the cases are pulmonary TB that is TB in the lungs. Symptons include chest pain, coughing up blood and a productive prolonged cough for more than three weeks. Systemic symptons include fever, chills, night sweats, appetite loss, weight loss, and often a tendency to fatigue very easily (WHO, 2009). In the other 25% of active cases, the infection moves from the lungs, causing other kinds of TB, collectively denoted extrapulmonary tuberculosis. This occurs more commonly in immune suppressed persons and young children. Extrapulmonary infection sites include the pleura in tuberculosis pleurisy, the central nervous systems in meningitis, the lymphatic system in scrofula of the neck, the genitourinary system in urogential tuberculosis and bones and joints in Pott's disease of the spine. An especially serious form is disseminated TB, more commonly known as miliary tuberculosis. Extrapulomonary TB may co-exist pulmonary TB as well (Centre for Disease Control and Prevention, 2000).

2.14.4 Treatment of tuberculosis

Treatment for TB uses antibiotic to destroy the bacteria. Effective TB treatment is difficult due to the unusual structure and chemical composition of the mycobacterial cell wall which makes many antibiotics ineffective and hinders the entry of drugs (Migliore *et al.*, 1966; Acharya and Goldman, 1970; Brennan and Nikaido, 1995). The two antibiotics most commonly used are rifampicin and isoniazid. However, instead of the short course of antibiotics typically used to cure other bacterial infection, TB requires much longer periods of treatment (around 6 to 24 months) to entirely eliminate *mycobacteria* from the body (Centre for Disease Control and Prevention, 2000). Latent TB treatment usually uses a single antibiotic, while active TB disease is best treated with combination of several antibiotics, to reduce the risk of the bacteria developing antibiotic resistance (O' Brien, 1994). People with latent infection are treated to prevent them from progressing to active TB disease later in life.

Drug resistant tuberculosis is transmitted in the same way as regular TB. Primary resistance occurs in persons who are infected with a resistant strain of TB. A patient with fully susceptible

TB develops secondary resistance during TB therapy because of inadequate treatment, not taking the prescribed regimen appropriately or using low quality medication (O' Brien, 1994). Drug resistant TB is a public health issue in many developing countries as treatment is longer and requires more expensive drugs. Multi drug-resistant tuberculosis (MDR-TB) is defined as resistance to the two most effective first-line TB drugs: rifampicin and isoniazid. Extensively drug-resistant TB (XDR-TB) is also resistant to three or more of the six classes of second-line drugs (Centre for Disease Control and Prevention, 2006).

2.14.5 Prevention of tuberculosis

TB prevention and control takes two parallel approaches. In the first, people with TB and their contacts are identified and then treated. Identification of infections often involves testing high-risk groups for TB. In the second approach, children are vaccinated to protect them from TB. No vaccine is available that provides reliable protection for adults. However, in tropical areas where the levels of other species of *mycobacteria* are high, exposure to non tuberculous mycobacteria gives some protection against TB (Fine *et al.*, 2001).

The WHO declared TB a global health emergency in 1993 and the Stop-TB-partnership developed a global plan to stop tuberculosis that aims to save 14 million lives between 2006 and 2015 (WHO, 2006). Since humans are the only host of *Mycobacterium tuberculosis*, eradication would be possible. This goal would be helped greatly by an effective vaccine (Martin, 2006). Many countries use Bacillus Calmette-Guerin (BCG) vaccine as part of their TB control programmes, especially for infants. According to the WHO, this is the most often used vaccine world wide with 85% of infants in 172 countries immunized in 1993 (WHO, 1995). This was the first vaccine for TB and developed at the Pasteur Institute in France between 1905 and 1921 (Bonah, 2005). The protective efficacy for preventing pulmonary TB in adolescents and adult is variable, ranging from 0 to 80% (Bannon and Finn, 1999). BCG provides some protection against severe forms of pediatric TB, but it has been shown to be unreliable against adult pulmonary TB which accounts for most of the disease burden world wide. Currently, there are more cases of TB on the planet than at any other time in history and most people agree that there is an urgent need for a newer, more effective vaccine that would prevent all forms of TB including drug resistant strains in all age groups and among people with

HIV (Sadoff, 2006). Several new vaccine to prevent TB infection are being developed. The firstrecombinant tuberculosis vaccine $rBCG_{30}$ entered clinical trials in the United State in 2004, sponsored by the National Institute of Allergy and Infectious Diseases (NIAID, 2004). A 200study showed that a DNA TB vaccine given with conventional chemotherapy can accelerate thedisappearance of bacteria as well as protect against re-infection in mice (Ha *et al*., 2005). A very promising TB vaccine, MVA85A, is currently in phase II trials in South Africa by a group led by Oxford University (Ibanga *et al*., 2006) and it is based on a genetically modified vaccine virus. Many other strategies are also being used to develop novel vaccine (Doherty and Andersen, 2005), including both subunit vaccines (fusion molecules composed of two recombinant protein delivered in an adjuvant) such as Hybrid-1, Hyvac-4 or M72, and recombinant adeno viruses such as Ad35 (Saten Serum Institute, 2009). Some of these vaccines can be effectively administered without needles making them preferable for areas where HIV is very common (Dietrich *et al*., 2006). All of these vaccines have been successfully tested in humans and are now in extended testing in TB-endemic regions. To encourage further discovery, researchers and policy makers are promoting new economic models of vaccine development including prizes, tax incentives and advance market commitments (Webber *et al*., 2001; Barder *et al*., 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.0 Source of Plant Materials

Fresh bulbs of *Crinum jagus* were collected from Omi-Adio, a town located at the surburb area in Ibadan, Oyo State of Nigeria. Samples were authenticated and identified at the Herbarium of Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo State. Specimens (Voucher No: FHI-109011) were deposited at FRIN. The bulbs were chopped in order to increase the surface area of the samples. The chopped samples were air-dried. The dried samples were pulverized into powder and weighed.

3.1 Preparation of Crude Methanol Extract

The powdered bulbs were loaded into an extraction thimble, covered with cotton wool at the top. This was then extracted with boiling petroleum ether for 24 hours in a Soxhlet apparatus. The first extraction was a cleansing exercise which remove lipids and several pigments (Fenwick *et al.*, 1992). The solvent was then changed to methanol and the extraction continued for another 24 hours. The petroleum ether and methanol were recovered by simple distillation. The solvents (methanol or petroleum ether) remaining in the extract was finally removed by concentrating the extract using a rotary evaporator. The extract which was a brown semi-solid substance was transferred into a clean dry bottle, weighed and labelled as the crude methanol extract.

3.2 Experimental Animals

Male wistar rats of the albino strain weighing between 160-200g were obtained from the animal house of the Anatomy Department, College of Medicine, University of Ibadan, Ibadan.

Male swiss mice weighing between (20-30g) were obtained from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan. The animals were kept in well ventilated cages, with 12 hours light/dark cycling. Commercial rat pellet and water were given *ad libitum*. The animals were allowed to acclimatize for two weeks with their enviroment before the commencement of experiments.

3.3 Phytochemcial Screening

Phytochemcial screening of the bulb extract of *Crinum jagus* was carried out in order to determine some of the bioactive components of the plant. The extract was chemically tested for alkaloids flavonoids, proteins, carbohydrates, saponins, cardiac glycosides, tannins, phenols, steroids and reducing sugars by the method described by Edeoga *et al.*, (2005) using standard pharmacognosis procedures.

3.3.1 Qualitative test for phytochemicals and other substances

Procedures

1. **Alkaloids**

A 0.5g sample of the plant extract was stirred with 5ml of 10% hydrochloric acid on a steam bath and a drop of Drangerdoffs, Wagner and Meyer's reagent added to different portions of 1ml of extract filtrate. Appearance of yellowish brown or orange precipitate was used to check for the presence of alkaloids.

2. **Flavonoids**

A 2ml aliquot of the methanolic solution of the plant extract (1g/100ml) was dissolved in dilute NaOH and some drops of dilute HCl added. Formation of a yellow solution on addition of dilute NaOH was taken as a preliminary evidence for the presence of flavonoids, and subsequent decolourisation in the presence of dilute HCl was used as a confirmatory test for the presence of flavonoids.

3. **Saponins**

A 1ml portion of the methanolic solution of the plant extract (1g/100ml) was shaken with 5ml of distilled water. Presence of frothing was used to check for the presence of saponins

4. **Tannins**

A 0.5g sample of the plant extract was stirred with 5ml of distilled water, filtered and iron (II) chloride reagent added to the filtrate. Blue-green colouration was used to confirm the presence of tannins.

5. **Phenols**

To 5ml of the methanolic solution of the plant extract was added 3ml of 5% iron (II) chloride solution, followed by few drops of 5% potassium ferricyanide solution. The presence of a dark green precipitate was taken as a positive test for the presence of phenols.

6. **Steroids**

A 1ml portion of the methanolic solution of the plant extract was added to 1-2ml of concentrated H_2SO_4. Presence of blood red colouration confirmed the presence of steroids.

7. Protein

A 5ml of the methanolic solution of the extract (1g/100ml) was placed in a test tube. Five drops of picric acid solution was added. The formation of a precipitate was used to confirm the presence of protein.

8. **Carbohydrate (Molisch's test)**

A 5ml aliquot of the methanolic solution of the extract (1g/100ml) in a test tube was added to ten drops of 10% α-naphtol and 1ml of concentrated H_2SO_4 was poured slowly down the side of the tube to give a lower layer. A violet colour at the junction of the two layers indicates the presence of carbohydrate.

9. **Cardiac Glycosides**

A 0.5g sample of the methanolic extract was dissolved in 2ml of glacial acetic acid containing one drop of the iron (II) chloride solution followed by the addition of 1ml of concentrated H_2SO_4. A brown ring at the interface confirmed the presence of cardiac glycosides.

10. **Reducing Sugars**

The extract was first hydrolysed with hydrochloric acid by dissolving 0.25g of the extract in 25ml of 1M HCl under heating, and then filtered. To a mixture of 1ml of Fehling solution A and 1ml of Fehling solution B was added ten drops of the filtrate and boiled in a water bath for few minutes. The formation of a brick red precipitate of cuprous oxides was used to confirm the presence of reducing sugars.

3.3.2 Procedures for quantitative phytochemcial test

1. **Alkaloids determination**

This is a distillation and titrimetric procedure (Henry, 1993). A 2g portion of the sample was weighed into a 100ml beaker and 20ml of 80% absolute alcohol added to give a smooth paste. The mixture was transferred to a 50ml conical flask and more alcohol added to make up to 100ml and 1g of magnesium oxide added. The mixture was digested in a boiling water bath for 1 hour 30 minutes under a reflex air condenser, with occasional shaking. The mixture was filtered while hot through a small bucher funnel. The residue was returned to the flask and re-digested for 30 minutes with 50ml alcohol after which the alcohol was evaporated. Hot water was then added to replace the alcohol lost, when all the alcohol has been removed and 3 drops of 10% HCl was added. The whole solution was later transferred into a 250ml volumetric flask and 5ml of zinc acetate solution and of 5ml potassium ferricyanide solution were added with thorough mixing to give a homogenous solution. The flask was allowed to stand a few minutes, and the contents filtered through a dry filter paper and 10ml of the filtrate was transferred into a separating funnel and the alkaloids present were extracted by shaking vigorously with five successive portions of chloroform. The residue obtained was dissolved in 10ml hot distilled water and transferred into a Kjeldahl tube with the addition of 0.02g sucrose and 10ml conc H_2SO4 and 0.02g selenium for digestion to a colourless solution to determine the percentage nitrogen by Kjeldahl distillation method. The result obtained was converted to percentage (%) total alkaloid by multiplying with a factor 3.26 as follows;

Total alkaloids = % N x 3.26.

2. **Flavonoids determination**

A 0.50g of the sample was weighed into a 100ml beaker and 80ml of 95% ethanol added with stirring with a glass rod. The mixture was filtered through a Whatman No 1 filter paper into a 100ml volumetric flask and made up to mark with ethanol. One ml of the extract was pipetted into a 50ml volumetric flask and 4 drops of conc HCl was added via a dropping pipette, after which 0.5g of magnesium turnings was added to develop a magenta red colouration. Standard flavonoid solution, of range 0-5ppm, was prepared from 100ppm solution and treated in a

similar way with HCl and magnesium turnings like the sample. The absorbance of a resultant magenta red colouration of sample and standard solution was read on a digital Jenway V6300 spectrophotometer at a wavelength of 520nm. The percentage concentration of flavonoids was calcaulted using the formula;

$$\% \text{ Flavonoid} = \frac{\mathbf{A} \text{ sample} \times \text{average gradient factor} \times \text{dilution factor}}{\text{wt of sample} \times 10{,}000}$$

where **A** = absorbance

3. **Saponins determination**

The spectrophotometric method of Bruner (1984) was used for saponin analysis. A 1g sample was weighed into a 250ml beaker, and 100ml of isobutyl alcohol was added. The mixture was shaken on UDY shaker machine for 5 hours to ensure uniform mixing. Thereafter, the mixture was filtered through a Whatman No1 Filter paper into a 100ml beaker and 20ml of 40% saturated solution of magnesium carbonate was added. The mixture obtained with saturated magnesium carbonate was again filtered through a Whatman No1 filter paper to obtain a clear colourless solution. An aliquot of 1ml of the colourless solution was pipetted into 50ml volumetric flask and 2ml of 5% $FeCl_3$ solution was added and made up to mark with distilled water. It was allowed to stand for 30 minutes for blood red colour to develop. A 0-10ppm standard solution was treated similarly with 2ml of 5% $FeCl_3$ solution as was done for 1ml sample above. The absorbance of the red coloured sample as well as standard saponin solutions were read after colour development in a Jenway V6300 spectrophotmeter at a wave length of 480nm.

$$\% \text{ Saponin} = \frac{\mathbf{A} \text{ sample} \times \text{gradient factor} \times \text{dilution factor}}{\text{wt of sample} \times 10{,}000}$$

where **A** = absorbance

4. **Phenol determination**

About 4g of the sample was treated with 30ml of 80% acetone in a 250ml beaker at 25^0C in the dark to extract the phenolics. The mixture was transferred to a blender, and homogenised 3 times with successive addition of 30ml of 80% acetone. Alternatively, the mixture can be

centrifuged at 3000 rpm for 30minutes, each with 30ml addition of 80% acetone. The homogenised mixture was then filtered through a Whatman No1 filter paper into a 100ml volumetric flask and made up to mark with 80% aqueous acetone. An aliquot of 0.05ml of the phenolic extract was diluted to 2ml in a 10ml measuring flask, 1ml of Folin Ciolcateau reagent was added and the flask was vigorously shaken . Immediately afterwards 5ml of 20% sodium carbonate solution was pipetted into the 10ml flask and the mixture made up to 10ml, with thorough shaking. A 0-10ppm range of gallic acid standard solutions were prepared from 100ppm gallic acid standard and treated similarly like sample above. After 20 minutes, the absorbance of the sample as well as standard were read on a spectronic 2ID spectrophotmeter at a wavelength of 735nm.

The calculation of % total phenolic = $\frac{\mathbf{A} \text{ sample} \times \text{gradient factor} \times \text{dilution factor}}{\text{wt of sample} \times 10{,}000}$

where **A** = Absorbance

5. **Steroids determination**

A 0.5g of the sample was weighted into a 100ml beaker, 20ml of chloroform was later filtered through a Whatman No1 filter paper into another dry clean 100ml conical flask . The resultant residue was repeatedly treated with chloroform- methanol mixture until it is free of steroids. 1ml of filtrate was pipetted into a 30ml test tube and 5ml of alcoholic KOH was added and shaken thoroughly to obtain a homogenous mixture. The mixture was later placed in a water bath at 37-40^{0}C for 90 minutes. The mixture was cooled to room temperature and 10ml of petroleum ether was added, followed by the addition of 5ml distilled water. This was evaporated to dryness on the water bath and 6ml of the Liebermann Burchard reagent was added to the residue in dry bottle and absorbance taken at a wavelength of 620nm on a spectronic 21D spectrophotometer. Standard steroids of concentrations of 0-4mg/ml were prepared from 100mg/ml stock steroid solution and treated similarly like sample above.

The calculation of % steroid = $\frac{\mathbf{A} \text{ sample} \times \text{gradient factor} \times \text{dilution factor}}{\text{wt of sample} \times 10{,}00}$

where **A** = Absorbance

3.4 Fractionation of the Crude Extract by Column Chromatography

A modified form of classical column chromatography called the flash chromatography was employed for the fractionation of the crude methanol extract of the bulb of *Crinum jagus* in the study. A glass column was packed with silica gel (60-200 flash chromatography grade) using n-hexane under positive pressure. The bulb extract adsorbed with silica gel was packed on to the column layer and then allowed to settle. The mobile phase consisted of three solvents: hexane (non polar), ethylacetate (mid polar) and methanol (polar). The solvents were mixed in various proportions so as to achieve gentle gradient in terms of separation. The various proportions of the solvents were pushed through the bed by the application of positive pressure using the vacuum pump. Twenty one fractions were obtained.

3.5 Separation of Crude Extract of *Crinum jagus* by Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out using analytical silica gel pre-coated plates. The fractions were spotted on TLC plates using capillary tubes. The spotted plates were developed in a chamber saturated with ethylacetate / methanol : 9:1 as mobile phase. The separated spots were then visualized under ultraviolet lamp (354nm). Positive spots with the same retention factor (RF) were pooled together.

3.6 Acute Toxicity Study of the Methanol Extract of *Crinum jagus*

A total of 25 male Swiss mice of the albino strain were used for the study. They were kept in the experimental cage for two weeks for acclimatization before the experiment. The animals were fed on standard mouse cubes and allowed free access to drinking water. The mice were weighed and grouped into A, B, C, D and E with five animals per group. Groups B, C, D, and E received 50, 100, 200 and 400 mg/kg of the extracts, respectively, through oral administration while the animals in group A served as the control and received normal saline (vehicle for the stock solution of *Crinum jagus* by the same route). The animals were observed for 48 hours under room temperature. Percentage mortality was then calculated using the moving average interpolation method (Thomson and Williams, 1974).

3.7 *In vitro* Antioxidant Study on Crude Extract and Fractions of the bulb of *Crinum jagus*

3.7.1 Determination of DPPH radical scavenging activity of extract and fractions

Free radical scavenging activity was determined using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) photometric method of Mensor *et al*., (2001).

Principle.

When DPPH reacts with an antioxidant compound which can donate hydrogen, it is reduced and the change in colour from deep violet to golden light yellow is measured at 518nm spectrophotometrically.

Reagents

1. Stock solutions (10mg/ml)

A 0.1g sample of the crude methanol extract and each of the fractions of the bulb of *Crinum jagus* were dissolved separately in methanol and the volume made up to 10ml to obtain the respective stock solutions.

2. Test solutions (100µg/ml)

From the stock solution of the crude methanol extract and each of the chromatographic fraction, 1ml was dissolved in 9ml each of 70% methanol to obtain their respective test solutions. Various concentrations of the crude extract and each of the fractions, ranging between 100µg/ml-500µg/ml, were prepared. Ascorbic acid, the standard antioxidant, was prepared using the same procedure.

3. DPPH (0.3mM)

This was prepared by dissolving 0.03g of DPPH in methanol and the volume made up to 250ml.

Procedure

1ml of 0.3mM of DPPH solution was added to 1ml each of the test solutions (various concentrations of the extract and the fractions ranging from 100-500µg/ml), and this was then allowed to react at room temperature, in the dark for 30 minutes. Iml of 0.3mM of DPPH solution was added to 1ml of methanol to serve as negative control. Methanol (1ml) was added to 1ml of extract to serve as blank. The positive control is ascorbic acid solution. Absorbance at 518nm was read after 30minutes and the percentage activity was calculated as:

DPPH scavenging activity = ($\mathbf{A}_o$ – **A**s/$\mathbf{A}_o$) x 100

Ao = absorbance without sample

As = absorbance with sample .

3.7.2 Determination of Hydroxyl Radical Scavenging Activity of Extract and Fractions

The hydroxyl radical scavenging activity was determined by the photometric method of Halliwell *et al.*, (1987).

Principle

The principle is based on the competition between deoxyribose and the test extract for hydroxyl radicals generated by Fe^{3+}/ ascrobate/ EDTA/H_2O_2 system.

Reagents

1. **Deoxyribose (2.8mM)**

A 0.38g weight of deoxyribose was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

2. **Ferric chloride (200µM)**

A 0.003g weight of $FeCl_3$ was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

3. **EDTA (1.04mM)**

A 0.31g weight of EDTA was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

4. **H_2O_2 (1.0mM)**

This solution was prepared by making up 0.1ml of 30% H_2O_2 to 100ml using distilled water.

5. **Ascorbic acid (1.0mM)**

A 0.18g weight of ascorbic acid was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

6. **Thobarbituric acid (1%w/v)**

A 1.0g weight of TBA was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

7. Glacial acetic acid (20%, pH 3.5)

This solution was prepared by adding 20ml of glacial acetic acid to 80ml of distilled water and adjusting the pH to 3.5 and stored in a reagent flask.

8. 0.1M Phosphate buffer 9 (pH 7.4)

(a) A 13.61g weight of KH_2PO4 was dissolved in 1L of distilled water.

(b) A 17.4g weight of K_2HPO_4 was also dissolved in 1L of distilled water.

Solution (a) was added to (b) and the pH of the obtained solution was adjusted to 7.4

Procedure

All solutions were freshly prepared. An aliquot of 1.0ml of the reaction mixture contained 100µl of 2.8mM deoxyribose (dissolved in phosphate buffer, pH7.4), 500µl of each of the various concentrations of the extract and the fractions (100-500µg/ml), 200µl of 200µM $FeCl_3$ and 1.04µM EDTA (1:1v/v),100µl of 1.0mM H_2O_2, 100µl of 1.0mM ascorbic acid . The reaction mixture was incubated for 1 hour at 37^0C in a water bath. 1ml of TBA and 1ml of glacial acetic acid were added after incubation. The entire mixture was heated at 100^0C for 1 hour. After cooling the extent of deoxyribose degradation was measured by the thiobarbituric reaction. The absorbance was read at 532 nm. The percentage hydroxyl radical scavenging activity was calculated as:

% Hydroxyl Radical Scavenging Activity = 100 - [(**A** sample – **A** control /**A** control)] x 100

3.7.3 Determination of the reductive potential of extract and fractions

The spectrophotometric method of Oyiazu (1986) was employed in determining the reducing power of the extract and the fractions.

Principle

The reducing power of a compound is related to its electron transfer ability and the principle is based on the ability of the extract and the fractions to reduce Fe^{3+} to Fe^{2+}.

Reagents

1. Phosphate buffer (0.1M,pH 7.4)

(a) A 13.61g weight of KH_2PO_4 was dissolved in 1L of distilled water.

(b) A 17.4g weight of K_2HPO_4 was also dissolved in 1L of distilled water.

Solution (a) was added to (b) and the pH of the obtained solution was adjusted to 7.4

2. Potassium ferricyanide

This was prepared by dissolving 1g of potassium ferricyanide in distilled water and the volume was made up to 100ml.

3. Trichloroacetic acid (10% TCA)

A 10g weight of TCA was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

4. Ferrous chloride ($FeCl_3$, 1%).

A 1g weight of $FeCl_3$ was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

Procedure

An aliquot of 150µl of each of the various concentrations of the extract and the fractions (100-500µg/ml) in 1ml distilled water was mixed with 2.5ml each of phosphate buffer and potassium ferricyanide. The mixture was incubated at 50^0C for 20minutes in a water bath. A 2.5ml portion of 10% TCA was then added and the mixture was centrifuged at 1000g for 10minutes. Thereafter, 2.5ml of the supernatant was mixed with 2.5ml of distilled water and 0.5ml of $FeCl_3$. The absorbance was read at 700nm in spectrophotometer. Ascorbic acid was used as positive control.

3.7.4 Determination of the total flavonoids content of the extract and fractions.

Principle

The total favonoids content of the extract and the fractions was estimated by the aluminium chloride colorimetric method, using aluminium chloride-flavonoids complex formation as described by Chang *et al.*, (2002).

Reagents

1. 70% Methanol

70ml of absolute methanol was made up to 100ml with distilled water before storing in a reagent flask.

2. 10% Aluminium chloride

A 10g weight of aluminum chloride was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

3. 1.0 M Sodium acetate

A 9.3g weight of sodium acetate was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

Procedure

Each of the various concentrations of the plant extract and the fractions (1ml) were added to 0.1ml of 10% aluminum chloride, 0.1ml of 1M sodium acetate and 2.8ml of distilled water. The reaction mixture was incubated at room temperature for 30 minutes in a water bath. The absorbance of the reaction was read at 415nm in spectrophotometer. Quercetin as the standard was treated in a similar manner at various concentrations to prepare the standard calibration curve (Fig 9). Total flavonoid content was expressed as µg/g quercetin equivalent using the formula.

$$F = \frac{C.V}{W}$$

Where F = total content of flavonoid compound mg/g plant extract in QE

C = concentration of quercetin equivalent of extract established from the calibration curve (mg/ml)

V = volume of extract (ml)

W = weight of the pure plant extract (g)

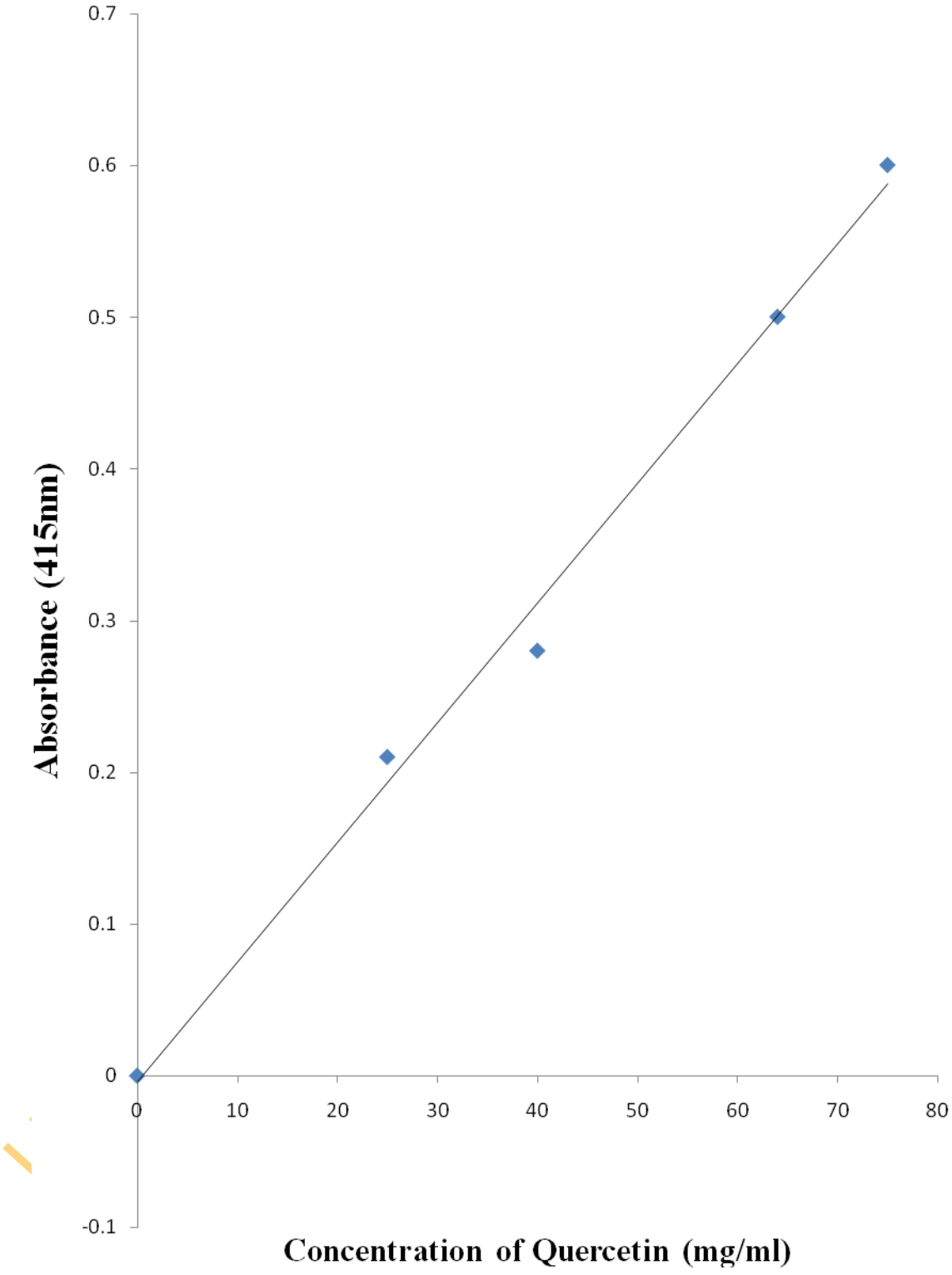


Figure 9 : Quercetin standard calibration curve

3.7.5 Determination of the total phenolic content of the extract and fractions

Principle

The assay was based on the reduction of Folin ciocalteu reagent (phosphomolybdate and pholphotungstate) by the phenolic compounds as described by McDonald *et al.*,(2001). The reduced Folin-Ciocalteu reagent is blue and thus detectable with spectrophotometer in the range of 500-750nm.

Reagents

1. 10% Folin-Ciocalteu reagent

A 10ml portion of Folin-Ciocalteu reagent was made up to 100ml with distilled water before storing in a reagent flask.

2. Gallic acid standard (0.5%w/v)

A 0.5g weight of dry gallic acid was dissolved in methanol and the volume made up to 100ml in a reagent flask.

3. 15% Sodium carbonate

A 15g weight of sodium carbonate was dissolved in distilled water and the volume made up to 100ml in a standard volumetric flask.

Procedure

Serial dilutions of 0.5mg/ml, 1.0mg/ml, 2.0mg/ml and 2.5mg/ml of gallic acid solutions were prepared from the gallic acid standard solution. Serial dilutions ranging from 0.5-2.5mg/ml were also prepared from the various concentrations of the extract and the fractions. The total phenolic content was determined by mixing 0.5ml of the various concentrations of the gallic acid, the extract and the fractions with 2.5ml of 10% Folin-Ciocalteu phenol reagent and 2.0ml of 15% sodium carbonate was added after 3 minutes. The reaction mixture was incubated at 40^0C for 30 minutes and the absorbance was read at 750nm with gallic acid standard. Total content of phenolic compounds in the plant extract (in gallic acid equivalent (GAE) was calculated using the formula:

$$P = \frac{C.V}{W}$$

Where P = total of content of phenolic compound (mg/g) plant extract in GAE

C = concentration of gallic acid of extract equivalent established from the calibration curve (mg/ml)

V = volume of extract (ml)

W = Weight of the pure plant extract (g)

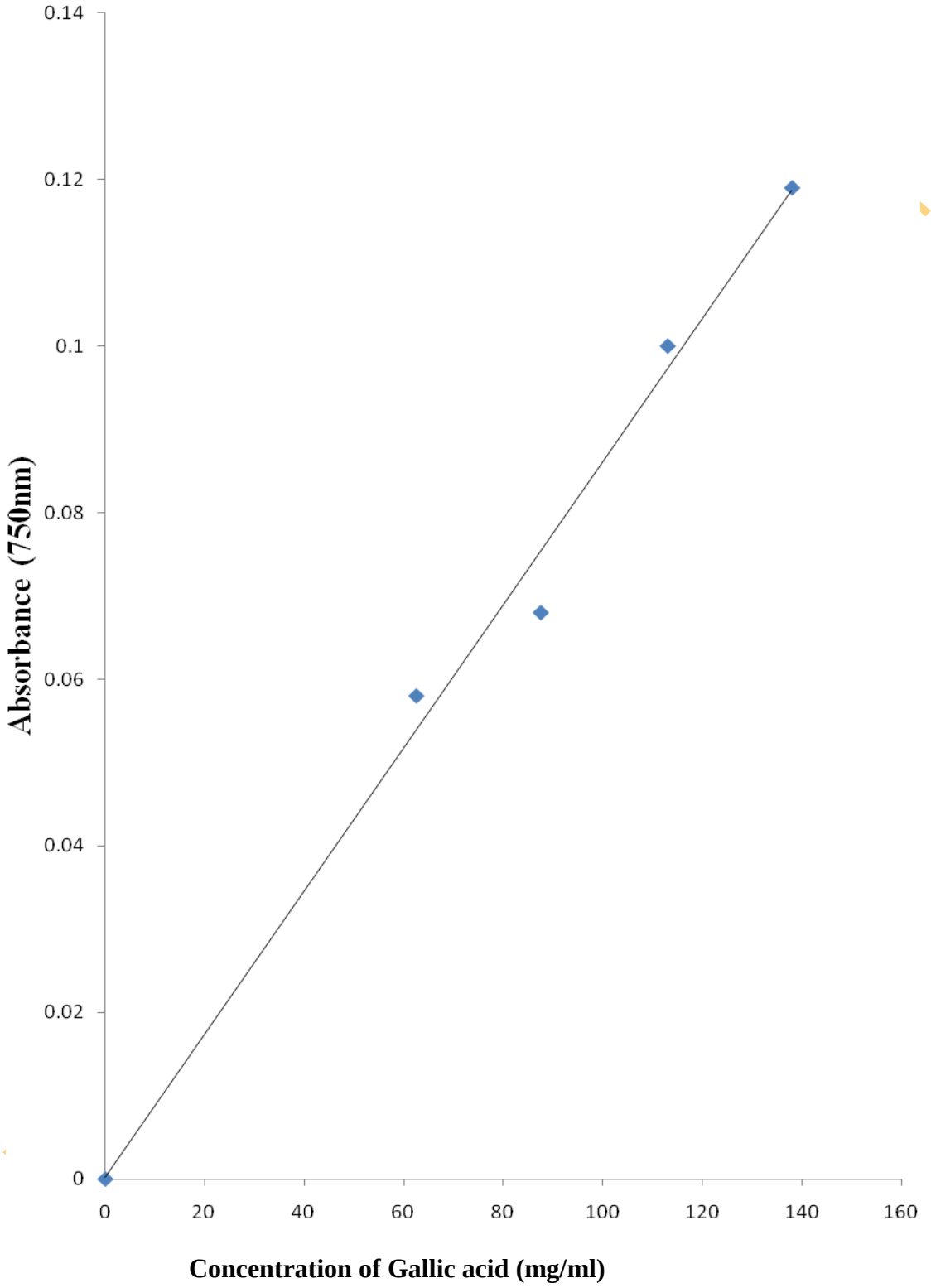


Figure 10 : Gallic acid standard calibration curve

3.8 *In vivo* Antioxidant Activity of Crude Extract and Fractions of the Bulb of *Crinum jagus* in rats

3.8.1 Administration of the crude methanol extract of the bulb of *Crinum jagus*

A total of 30 male rats were randomly distributed into five groups of six animals per group. The crude extract was dissolved in corn oil before administration to the rats using oral cannula and administration was done for 30 days. The control group A received 0.2ml corn oil (vehicle for the extract) for 30 days. Varying doses of 10, 25, 50 and 75mg/kg were administered orally to animal in groups B, C, D and E respectively for 30 days.

3.8.2 Administration of fractions of the bulb of *Crinum jagus*

A total of 35 male rats were divided in to seven groups of five animals each. The control group A received 0.2ml corn oil for 30 days. Animals in groups B and C were treated with 5 and 10mg/kg of fraction 1 respectively. Groups D and E animals were treated with 5 and 10mg/kg of fraction 2 respectively while those in groups F and G received 5 and 10mg/kg of fraction 3 respectively for 30 days through oral administration.

3.8.3 Preparation of tissue homogenate for biochemical analysis

Reagents

1. Homogenising buffer (0.1M phosphate buffer, pH 7.4)

(a) A 11.8g weight of Na_2HPO_4 was dissolved in distilled water and made up to 100ml.

(b) A 6.8g weight of KH_2PO_4 was dissolved in distilled water and made up to 500ml.

Then 800mls of (a) was mixed with 200ml of (b) above to make 1L and the pH was adjusted to 7.4

2. 1.15% Potassium chloride (KCl)

A 1.15g weight of KCl was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

Procedure

The animals were sacrificed by cervical dislocation, 24hrs after the administration of last doses of treatment. The liver and kidney samples were quickly removed and rinsed in 1.15% KCl, dried and weighed. The liver and kidney samples were homogenized in 4 volumes of phosphate buffer (0.1M, pH 7.4). The resulting homogenate were centrifuged at 10,000xg for 20minutes

to obtain the post-mitochondrial supernatant which was decanted into samples bottles and stored at 8^0C until used

3.8.4 Biochemical Assays

3.8.4.1 Protein determination

Protein concentration of the liver and kidney homogenates were estimated by the colorimetric Biuret method as described by Gornall *et al.*, (1949) using Bovine Serum Albumin (BSA) as standard. Potassium iodide was added to the Biuret reagent to prevent the precipitation of cupric ions (Cu^{2+}) to cuprous oxide.

Principle

The assay is based on the reaction of Cu^{2+} and protein under alkaline conditions to form a blue complex with maximum absorbance at 540nm. The absorbance of the complex is proportional to the protein concentration in the sample. The Biuret reagent consists of copper sulphate, potassium iodide and sodium-potassium tartarate, which acts as a stabilizer for the reagent.The procedure is usually calibrated with a BSA standard curve.

Reagents

1. **0.2M NaOH**

A 8.0g weight of NaOH was dissolved in litte distilled water and the solution made up to 1L and stored in a reagent flask

2. **Biuret reagent**

A 3.0g weight of copper sulphate ($CuSO_4.\ 5H_20$) and 5g of Na-K tartarate were dissolved in 500ml of 0.2M NaOH. 5g of KI was added to the solution and the volume made up to 1L with 0.2M NaOH.

3 . **0.9% NaCl solution (saline)**

A 0.9g weight of NaCl was dissolved in distilled water and made up to 100ml before storing in a reagent flask.

4. **Stock Bovine Serum Albumin (BSA) solution (1mg/ml)**

A 0.1g weight of BSA was dissolved in 100ml of distilled water to give a stock solution of 1mg/ml.

Procedure

Serial dilutions of the stock BSA solution were made using normal saline. 4ml of Biuret reagent was added to 1ml of each diluted protein standard solution (BSA) and the mixture allowed to stand at room temperature for 30minutes. The absorbance of the solution were then read at 540nm in spectrophotometer and a graph of absorbance against mg BSA was then plotted.

Table 3 : Protein determination assay medium

TABLE NO	1	2	3	4	5	6	7
Stock BSA (ml)	0	0.05	0.10	0.20	0.30	0.40	0.50
Normal saline (ml)	1.0	0.95	0.90	0.80	0.70	0.60	0.50
Biuret reagent (ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Amount of BSA(mg)	0	0.05	0.1	0.2	0.3	0.40	0.5
Absorbance (540nm)	0.0	0.001	0.006	0.015	0.017	0.031	0.047

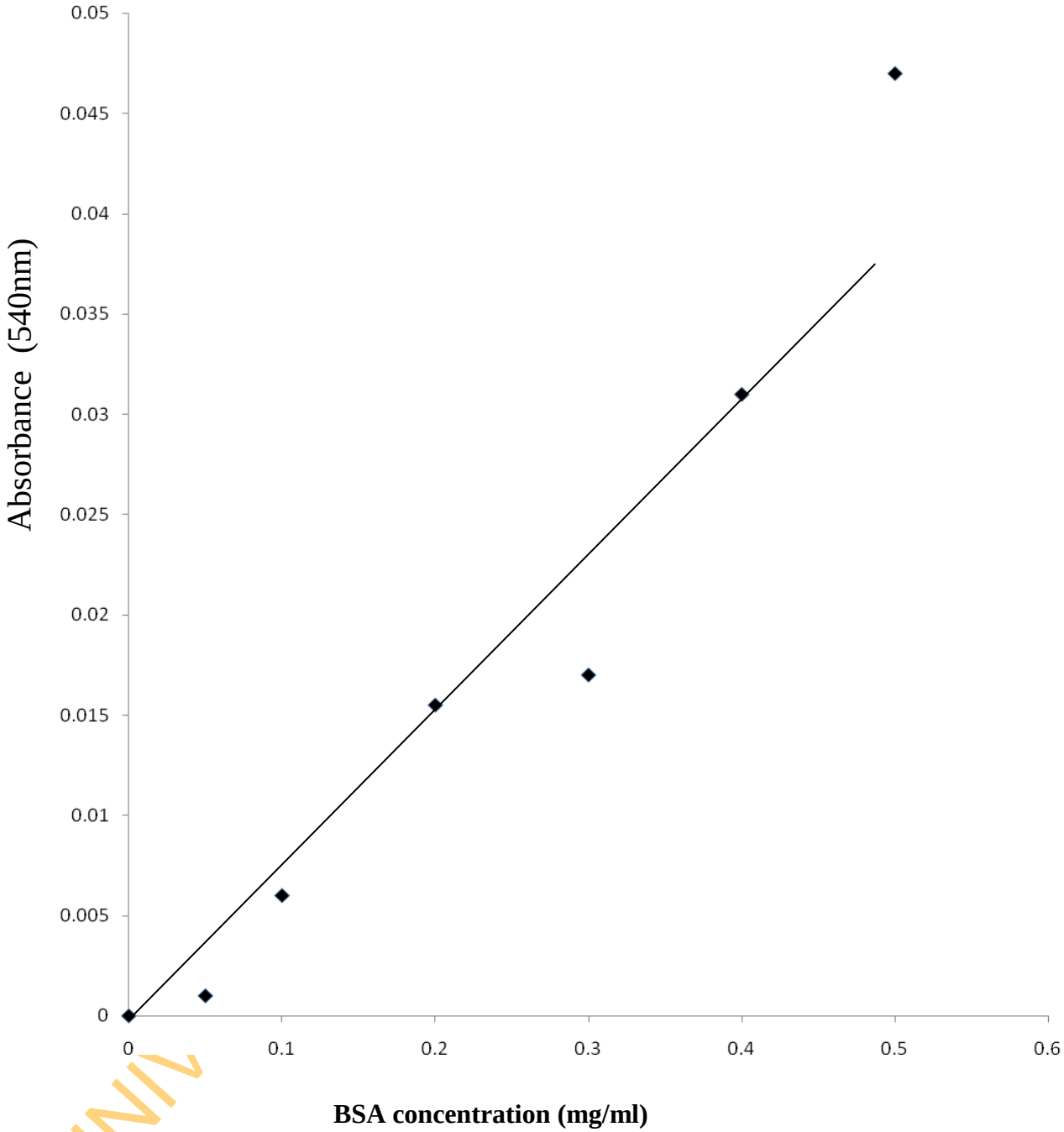


Figure 11 : Protein estimation standard curve

Estimation of protein in test samples followed a procedure identical to those employed for the standard were used except that suitable dilutions of the test samples were made. This was done to reduce the level of protein in the sample to the sensitivity range of Biuret method. 1ml of the diluted samples (0.1ml of sample with 0.9ml of distilled water) was taken and the process for protein determination as described above was repeated. The absorbance was read in a spectrophotometer at 540nm against sample blank containing 1ml of distilled water and 4ml of Biuret reagent. Protein content of samples were extrapolated from the protein standard curve and multipled by 100 to get the actual amount of protein in the sample.

3.8.4.2 Lipid peroxidation assay.

Lipid peroxidation was assessed by measuring the thiobarbituric acid reactive (TBAR) products using the spectrophotometric procedure of Vashney and Kale (1990) and expressed as micromolar of malondialdehyde (MDA)/g tissue.

Principle

Small amounts of malondialdehyde (MDA) are produced during lipid peroxidation and these are able to react with thiobarbituric acid (TBA) to generate a pink coloured complex which in acidic solution absorbs light at 532nm.

Reagents

1. 30% Trichloroacetic acid (TCA) solution

A 9g weight of TCA was dissolved in distilled water and the volume made up to 30ml.

2. 0.75% Thiobarbituric acid (TBA) solution

A 0.225g weight of TBA was dissolved in 30ml of 0.1M HCl by shaking in hot water before storing in a reagent flask.

3. 0.15M Tris KCl (pH 7.4)

A 1.12g weight of KCl and 2.36g of Tris base were separately dissolved in distilled water, the two solutions were mixed together, and the volume made up to 100ml and the pH adjusted to 7.4

Procedure

A 0.1ml portion of the sample was diluted in 0.9ml of distilled water to make a dilution of 1 in 10. An aliquot of 0.4ml of the test sample was mixed with 1.6ml Tris KCl buffer to which 0.5ml

of 30% TCA was added. A 0.5ml portion of TBA was then added and placed in water bath for 45 minutes at 80^0C. This produced pink coloured reaction mixtures which were centrifuged at 14000g for 15 minutes. The absorbance of the clear pink supernatant was then read against reference blank of distilled water at 532nm in spectrophotometer.

Calculations

$$\text{MDA (units/mg protein)} = \frac{\text{absorbance x volume of mixture}}{\text{E 532 x volume of sample x mg protein}}$$

E 532 = molar absorbitivity at 532nm = 1.56×10^6

3.8.4.3 Superoxide dismutase assay

The level of superoxide dismutase (SOD) activity was determined by the photometric method of Misra and Fridovich (1972)

Principle

The ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine at pH 10.2 makes this reaction a basis for a sample assay for SOD. Superoxide anion (O_2) generated by the xanthine oxidase reaction is known to cause the oxidation of epinephrine to adrenochrome. The yield of adrenochrome produced per superoxide anion introduced increased with increasing concentration of epinephrine.

These results led to the proposal that auto-oxidation of epinephrine proceeds by at least two distinct pathways, one of which is a free radical chain reaction involving superoxide anion radical and hence inhibitable by SOD.

Reagents

1. 0.05M Carbonate buffer (pH, 10.2)

A 14.3g weight of $Na_2CO_3.10H_2O$ and 4.2g of $NaHCO_3$ were dissolved in distilled water and made up to 1000ml mark in a litre standard volumetric flask. The solution was adjusted to pH 10.2

2 . 0.3mM Adrenaline

A 0.003g weight of epinephrine was dissolved in 50ml of distilled water and stored in a reagent flask. The solution was prepared fresh just before use.

Procedure

A 0.1ml portion of the sample was diluted in 0.9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml of carbonate buffer, 0.3ml of substrate (adrenaline) and 0.2ml of distilled water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Calculation

$$\text{Increase in absorbance per minute} = \frac{A_3 - Ao}{t}$$

where A_3 = Final absorbance

Ao = Initial absorbance

t = Time of final absorbance (150s or 2.5mins)

$$\% \text{ Inhibition} = \frac{\text{Increase in absorbance of sample/min}}{\text{Increase in absorbance of blank}} \times 100$$

$$\text{Unit of activity} = \frac{\% \text{ inhibition}}{50\%}$$

$$\text{Specific activity} = \frac{\text{Enzyme activity}}{\text{Total protein (mg)}} \times \text{dilution factor}$$

3.8.4.4 Catalase assay

Catalase activity was determined according to the colorimetric method of Sinha (1971).

Principle

This method is based on the fact that dichromate in acetic acid is reduced to chromic acid when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromic acetate so produced is measured colorimetrically at 570-610nm. Dichromate has no absorbance at this wavelength and hence its presence in the assay mixture does not interfere with the determination of chromic acetate. Catalase preparation in samples is allowed to split H_2O_2 for different periods of time. The reaction was stopped at a particular

time by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically, after heating the reaction mixture.

Reagents

1. 5% Potassium heptaoxodichromate (5% $K_2Cr_2O_7$)

A 5.0g weight of K_2Cr_2O7 was dissolved in some distilled water in a 100ml volumetric flask and made up to the mark.

2. 0.2M Hydrogen peroxide (H_2O_2)

A 0.67g weight of H_2O_2 was mixed with little distilled water and then made up to 100ml mark in a standard volumetric flask and stored at 4^0C.

3. Dichromate/acetic acid solution

This solution was prepared by mixing solution of 5% K_2Cr_2O7 with glacial acetic acid and storing in a brown bottle.

4. 0 .01M Phosphate buffer (pH 7.0)

A 3.58g weight of $Na_2HP0_4.12H_2O$ and 1.19g of $NaH_2PO_4.2H_2O$ were dissolved in 900ml of distilled water. The pH was adjusted to 7.0 and distilled water added to make it up to 1litre.

Procedure

Preparation of H_2O_2 Standard Curve

Different amounts of H_2O_2 ranging from 10 to 60µmoles were taken in small test tubes and 2ml of dichromate/acetic acid was added to each. Addition of the reagents instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10minutes in a boiling water bath changed the colour of the solution to stable green due to formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3ml with distilled water and the absorbance measured with a spectrophotometer at 570nm. The concentration of the standard were plotted against absorbance.

Table 4 : Assay mixture for calibration of standard curve for catalase

TUBE NO	1	2	3	4	5	6	7
H_2O_2 (ml)	0.00	0.10	0.20	0.30	0.40	0.50	0.60
Dichromate/acetic acid (ml)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water (ml)	1.00	0.90	0.80	0.70	0.60	0.50	0.40
H_2O_2 concentration (mg)	0.00	0.67	1.34	2.01	2.68	3.35	4.02
Absorbance (540nm)	0.0	0.07	0.22	0.26	0.30	0.40	0.48

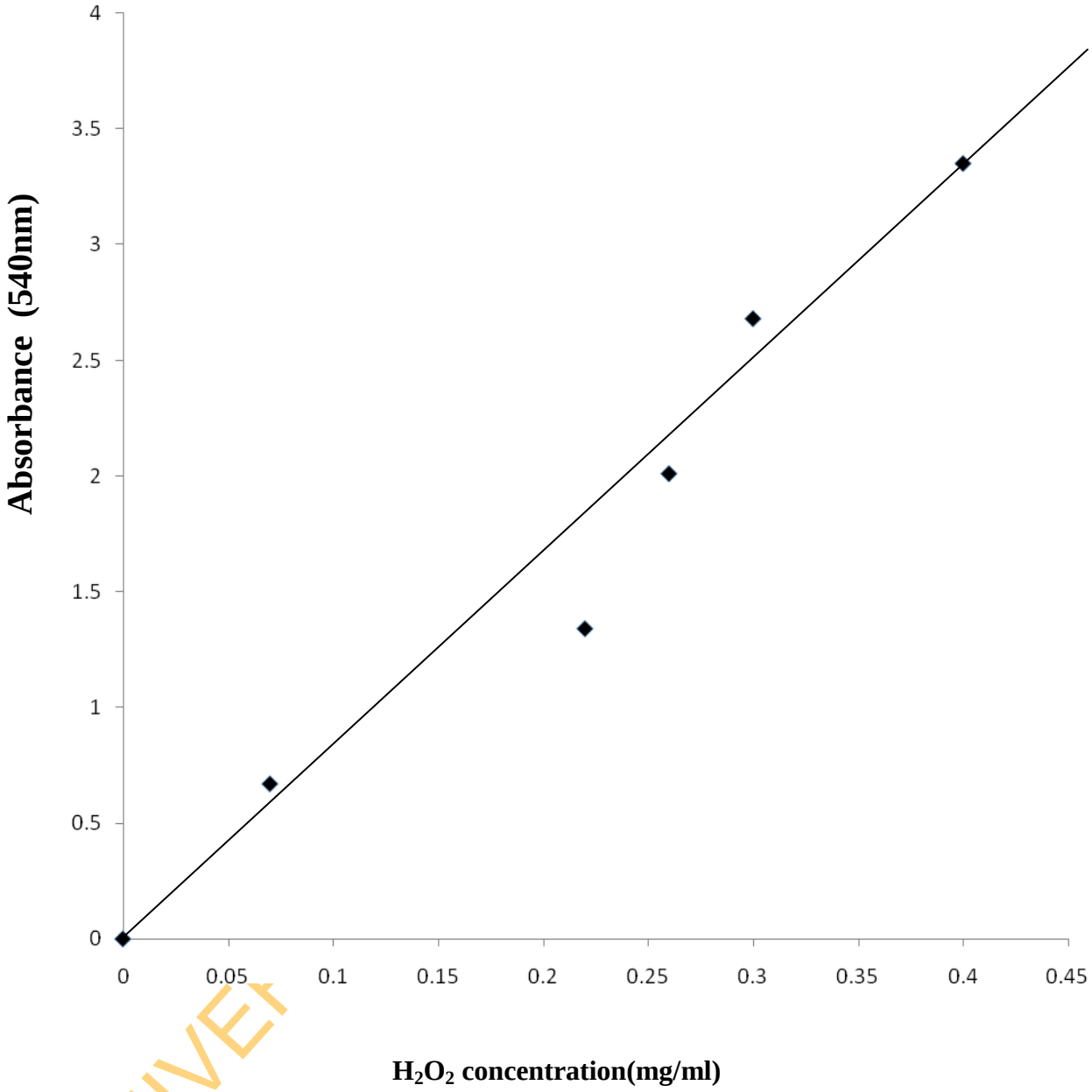


Figure 12 : Catalase standard curve

Determination of catalase activity of test samples

A 1ml portion of the sample was mixed with 49ml distilled water to give 1 in 50 dilution of the sample. The assay mixture contained 4ml of H_2O_2 solution (800µl) and 5ml of phosphate buffer in a 10ml flask. A1ml portion of properly diluted enzyme preparation (test sample) was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. A 1ml portion of the reaction mixture was withdrawn and added into 2ml dichromate/acetic acid reagent at 60 seconds intervals for 3 minutes. The H_2O_2 contents of the withdrawn sample were determined by the method described above.

Calculation

$$K = 1/t \log So/S$$

Where So = the initial concentration of H_2O_2

S = concentration of peroxide at t min (60 seconds interval)

t = time interval (1minute)

The value of K is plotted against time in minute and the velocity constant of catalse (Ko) at time zero determined by extrapolating the catalase content of the enzyme preparation expressed in terms of katalase feiahigkeit or kat "F" according to Von Euler and Josephson (1927).

$$\text{Kat F} = \frac{\text{Ko}}{\text{Mg protein/ml (µ/mg protein)}}$$

3.8.4.5 Reduced glutathione (GSH) assay

The total sulfhydryl groups, protein bound sulphydryl groups and free-sulphydryl groups (such as GSH) in biological samples can be determined using Ellman's reagent (5.5 dithiobis-(2-nitrobenzoic acid) (DTNB) as described by Jollow *et al.*,(1974)

Principle

The method is based on the development of a relatively stable (yellow) complex formed as a result of reaction between Ellman's reagent and free sulphydryl groups. The reduced form of glutathione (GSH) in most instances is the bulk of cellular non-protein sulphydryl groups. The chromophoric product, 2-nitro-5-thiobenzoic acid, resulting from the reaction of Ellman's reagent with GSH possesses a molar absorption at 412nm. The absorbance of this complex at 412nm is proportional to the level of GSH in the sample.

$GSH + R – S –S- R \longrightarrow R\text{-}S^{-} + GSS$

$R\text{-}S^{-}$ = Yellow complex

Reagents

1. Ellman's reagent (DTNB)

A 4.0mg weght of Ellman's reagent (5,5- Dithio-bis (2-nitrobenzoic acid) was dissolved in litte amount of 0.1M phosphate buffer, pH 7.4 and made up to 100ml mark in a standard volumetric flask with the same buffer. It was stored at 4^0C.

2. 0.1M Phosphate buffer (pH 7.4)

(a) A 35.81g weight of disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) was dissolved in 1 litre of distilled water.

(b) A 13.61g weight of anhydrous potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 1 litre of distilled water.

(c) A portion of 4 volumes of solution (a) was added to 1 volume of solution (b) and the pH of the resulting solution adjusted to 7.4 using a pH meter.

3. 4% Sulphosalicylic acid (precipitating reagent)

A 4.0g weight of sulphosaliyclic acid was dissolved in litte quantity of distilled water and made up to 100ml mark in a standard volumetric flask with more distilled water.

4. Reduced GSH (Working standard)

A 40mg weight of reduced GSH was dissolved in 100ml of 0.1M phosphate buffer, pH 7.4 and then stored at 4^0C.

Procedure

Serial dilutions of the stock GSH were prepared as shown in table below. To each tube (in duplicates) were added appropriate volumes of phosphate buffer and then followed by the addition of 4.5ml Ellman's reagent. The absorbance of the yellow colour formed upon the addition of Ellman's reagent was read within 30mintues at 412nm using spectrophotometer. A plot of absorbance versus concentration of reduced GSH was obtained.

Table 5 : Assay mixture for calibration of glutathione standard curve

GSH Solution (ml)	PO_4 Buffer (ml)	Ellman's Reagent (ml)	GSH Conc (µg/ml)	Absorbance (412nm)
0.00	0.50	4.5	0	0.00
0.05	0.45	4.5	20	0.05
0.10	0.40	4.5	40	0.12
0.15	0.35	4.5	60	0.15
0.20	0.30	4.5	80	0.20
0.25	0.25	4.5	100	0.25
0.30	0.20	4.5	120	0.30
0.40	0.10	4.5	140	0.36

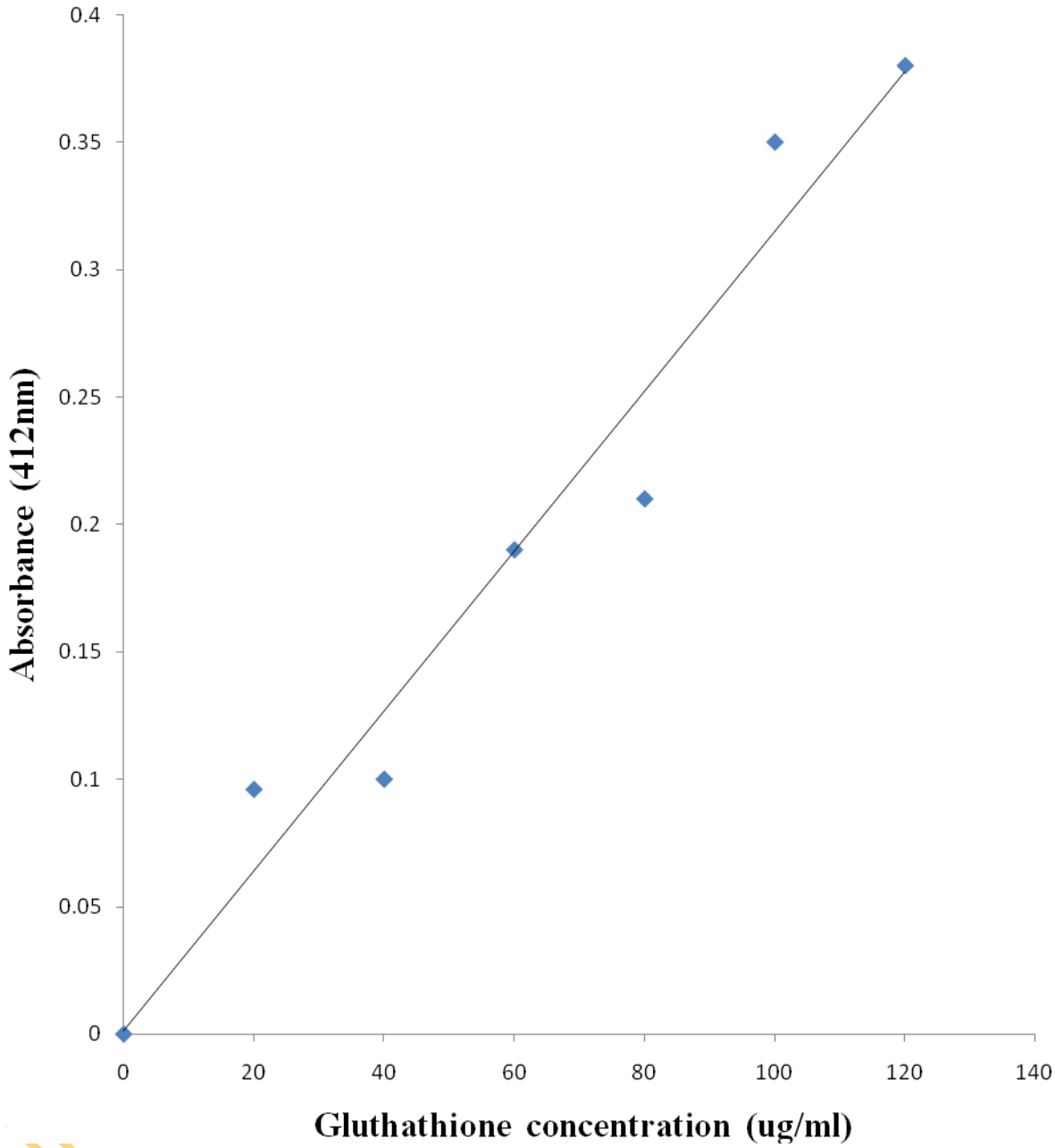


Figure 13 : Glutathione standard curve

Estimation of GSH in test sample

A 0.2ml portion of the sample was mixed with 1.8ml of distilled water to give 1 in 10 dilution. About 3ml of precipitating reagent (4% sulphosalicylic acid) was added to the diluted sample and then allowed to stand for 10minutes. The mixture was centrifuged at 3,000g for 5 minutes and 0.5ml of supertanatant added to 4ml of phosphate buffer and finally 0.5ml of Ellman's reagent was added. The optical densities were read within 30 minutes of colour development at 412nm using spectrophotometer. A blank was prepared with 0.5ml of the diluted precipitating solution (diluted twice with 0.1m phosphate buffer) and 4.5ml of Ellman's reagent. Reduced glutathione is proportional to the absorbance at 412nm.

3.8.4.6 Glutathione-S-transferase (GST) assay

Glutathione-S-transferase activity was determined according to the method of Habig *et al.*, (1977).

Principle

The assay is based on the fact that all GST demonstrate a relatively high activity with 1 chloro-2-4 dinitrobeanzene as the second substrate. Consequently, the conventional assay for GST activity utilises 1-chloro-2, 4,dinitrobenzene as susbtrate. When this substance is conjugated with reduced glutathione its absorption maximum shifts to a longer wavelength. The absorption increases at the new wavelength of 340nm provides a direct measurement of the enzymatic reaction.

Reagents

1. 1-Chloro-2,4-Dinitrobenzene (20mM)

A 30.37mg weight of 1-chloro-2,4-dinitrobenzene (CDNB) was dissolved in 1ml of ethanol and stored in a reagent flask.

2. Reduced Glutathione (0.1M)

A 30.73mg weight of reduced glutathione (GST) was dissolved in 1ml of 0.1M phosphate buffer (pH 6.5) and stored in a reagent flask.

3. Phosphate Buffer (0.1M)

This was prepared by dissolving 4.96g of dipotassium hydrogen phosphate ($K_2HPO_{4)}$ and 9.73g of potassium dihdrogen phosphate (KH_2PO_4) in little amount of distilled water and then made up to mark in 1 litre volumetric flask. The pH was adjusted to 6.5.

Procedure

The medium for the estimation was prepared as shown below and the reaction was allowed to run for 60 seconds each time before the absorbance was read against the blank at 340nm. The temperature was maintained at approximately 31^0C. The absorbance was measured using spectrophotometer.

Table 6 : Glutathione-S-transferase assay medium

Reagent	Blank	Test
Reduce GSH (0.1ml)	30µl	30µl
CDNB (20mM)	150µl	150µl
Phosphate buffer (pH 6.5)	2.82ml	2.79ml
Cytosol/Microsomes	-	30µl

Calculation

The extinction coefficient of CDBN = 9.6mn $^{-1}$ cm^{-1}

Glutathione-S- transferase activity = $\frac{\text{OD/min}}{9.6} \times \frac{1}{0.03\text{ml/mg protein}}$

= µmole/min/mg protein

3.9 Antimicrobial Studies

3.9.1 Determination of antibacterial and antifungal activities of the crude extract and fractions of the bulb of *Crinum jagus*

Antimicrobial activity was assessed by the agar diffusion method (Vollekova *et al*., 2001; Usman *et al*., 2005).

Principle

Agar diffusion method is one of the most widely used methods to determine the susceptibility of microorganisms to antimicrobial agents. The principle of this method is dependent upon the inhibition of reproduction of a microorganism on the surface of a solid medium by antimicrobial agent which diffuses into the medium by a filter paper disc or by creating a 'well' on the agar and filling the wells with different concentrations of the antimicrobial agent. Thus for an organism which is truly sensitive (susceptible) to an antimicrobial agent, a zone of inhibition will be seen around the discs or the wells.

3.9.2 Microorganisms

All the organisms used in this study were isolates obtained from Department of Pharmaceutical Microbiology, University of Ibadan. The organisms were maintained on agar slopes at 4^0C and subcultured for 24 hours before use.

Two Gram-positive bacteria : *Staphylococcus aureus, Bacillus subtilis,* four Gram-negative bacteria : *Escherichia coli, Pseudomonas aeruginosa, Salmonellae typhi, Klebsiella pneumonae* and six fungi *: Candida albicans, Candida tropicalis*, *Candida krusei, Penicillum notatum, Aspergillus niger, Aspergillus flavos* were used for the bioassay.

Table 7 : List of microorganisms used in the assessment of antimicrobial activities of the crude extract and fractions of the bulb of *Crinum jagus*

Microorganism	Type	Source
Bacteria		
1. *Staphylococcus aureus*	Gram +ve	Department of Pharmaceutical Microbiology, U.I, Ibadan.
2. *Baccillus subtilis*	Gram +ve	‘’
3. *Escherichia coli*	Gram – ve	‘’
4. *Pseudomonas aeruginosa*	Gram –ve	‘’
5. *Salmonellae typhi*	Gram –ve	‘’
6. *Klebsiella pneumonae*	Gram –ve	‘’
Fungus		
7.*Candida albicans*	Yeast	‘’
8. *Canadida tropicalis*	Mould	‘’
9. *Canadida krusei*	Mould	‘’
10. *Penicillum Notatum*	Mould	‘’
11. *Aspergillus niger*	Mould	‘’
12. *Aspergillus flavos*	Mould	‘’

3.9.3 Preparation of culture media

The culture media used for bacterial growth were nutrient broth and nutrient agar while the media used for fungal growth were tryptone soya broth and potato dextrose agar.

Eight grammes of nutrient broth and thirty grammes of tryptone soya broth powder were dissolved separately in one litre quantities of distilled water over a water bath. The homogenous solution formed in each case was then dispensed into test tubes in 5ml or 10ml quantities. All the test tubes were covered and sterilized at 121^0C for 15 minutes in an autoclave. The sterile broth tubes were kept at room temperature. Any broth in which turbidity was observed was discarded.

3.9.4 Preparation of bacterial and fungal cultures

Colonies of Gram-positive and Gram-negative bacteria kept on agar slopes were innoculated into 5ml quantities of sterile nutrient broth and incubated for 18-24 hours at 37^0C. For the fungal cultures fungal colonies kept on agar slopes were also innoculated into 5ml quantities of tryptone soya broth and the tubes were incubated at room temperature and checked for growth at 24 and 48 hours.

3.9.5 Preparation of test solutions (crude extract)

A 1g weight of the crude extract was dissolved in 5ml methanol (solvent of extraction) to obtain a stock solution of 200mg/ml. From the stock solution, 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25 mg/ml of the test solutions were prepared through serial dilution into 6 test tubes. The 7^{th} and 8^{th} test tubes contained ampicillin (10mg/ml) and tiaconazole (10%w/v) and were used as positive controls while the 9^{th} tube contained methanol used as negative control of the experiment

3.9.6 Preparation of test solutions (fractions)

A 0.25mg weight of each of the fraction was dissolved in 5ml methanol to obtain a stock solution of 50µg/ml, from the stock solution, 25µg/ml; 12.5µg/ml; 6.25µg/ml; 3.125µg/ml; 1.56µg/ml and 0.78µg/ml of the test solution were prepared through serial dilutions into six test tubes. The 7^{th} and 8^{th} test tubes contained ampicillin (10µg/ml) and tiaconazole (10%w/v) and were used as positive controls of the experiment.

3.9.7 Agar diffusion test

Procedure

From the overnight culture of bacteria and fungi, 0.1ml was taken and put into 9.9mls of sterile distilled water to get a 10^{-2} of the dilution of the organism. From the diluted organism, 0.2ml was taken and asceptically poured into the sterile petridishes containing agar at about 45^0C and allowed to set. The surface of the set agar was dried in the incubator for 20 minutes at 37^0C. Equidistant wells were cut in the agar using a sterile cork borer of 8mm diameter. An aliquot of 80µl of known concentrations of the extracts and the drugs (ampicillin for bacteria isolates and tiaconazole for fungal isolates) were poured into the wells, methanol was used as negative control. The plates were allowed to stand on the bench for about 2 hours to allow the extract to diffuse properly into the agar. For bacteria the plates were incubated for 18-24 hours at 37^0C while for fungi, the plates were incubated for 48 hours at $26\text{-}28^0$C. the plates were checked for growth daily for a period of 7 days. The average diameters of three readings of the clear zone surrounding the hole was taken as the measure of the inhibitory level of the plant extract and the fractions against the bacteria and fungi tested.

3.9.8 Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the crude extract and fractions of the bulb of *Crinum jagus*

Minimum inhibitory concentration (MIC) is defined as the smallest concentration at which an antimicrobial agent will inhibit the growth of a named organism under specific condition.

Minimum bactericidal concentration (MBC) is the smallest concentration at which an antimicrobial agent will kill a named organism under specific condition.

The minimum inhibitory concentration (MIC) of the crude extract and the fractions were determined as described by Kabir *et al.*,(2005).

Procedure

For the bacteria isolates, six different concentrations of the crude extract ranging from 1.56mg/ml-50mg/ml were prepared while five different concentrations of each of the fractions ranging from 0.95µg/ml-3.125µg/ml were prepared to determine the MIC value, 2mls of each of the different concentration was added to 18mls of agar in test tubes making up the volume to

20mls and 1.0ml of standardized broth cultures contained 1.0 x 10^{-7} CFU/ml were added to each tube and then incubated at 37^0C for 18-24 hours. For comparision extract free tubes were used. Following incubation, turbidity was examined. The lowest concentration of the crude extract and the fractions that produced no visible bacterial growth (no turbidity) when compared with the control tube was regarded as minimum inhibitory concentration. MIC determination were done in triplicates.

To determine the MIC of the fungal isolates, six different concentrations of the crude extract ranging from 6.25mg/ml-200mg/ml were also prepared while five different concentrations of each of the fraction ranging from 0.195µg/ml-3.125µg/ml were also prepared. A 2mls portion of each of the different concentrations was added to 18mls of agar in test tubes making up the volume to 20mls and then asceptically poured into sterile plates. All the plates were allowed to set and then the different organism (mould and yeast) was streaked on the plates. The plates were incubated at 26^0C for 48 hours and checked for growth. The lowest concentration of the extract and the fraction that did not show growth of the organism was regarded as the minimum inhibitory concentration (MIC).

To determine the minimum bacterial concentration (MBC), all the concentrations of the extract and the fractions that did not show growth from the MIC were reprepared and poured into sterile plates and the different organisms streaked on the plates and incubated. For bacterial isolates, the plates were incubated at 37^0C for 18-24 hours while for the fungal isolates, the plates were incubated at 26^0C for 48 hours, after incubation, the plates were observed for growth. Presence of colonies served as indication of growth. The lowest concentration at which no colony was seen was taken as the minimum bactericidal concentration (MBC).

To obtain the number of colony used for the MIC determination, 0.1ml of 10^{-7} dilution of the 18 hours culture used was innoculated into molten sterile nutrient agar at 45^0C, poured and allowed to set. The surface was dried and the plates were incubated at 37^0C for 24 hours. Colonies formed in the agar were counted using the colony counter. The colony forming unit (CFU/ml) was calculated as follows:

CFU/ml = no of colonies counted x dilution factor

Volume taken for incubation

3.10 Determination of Anti-inflammatory Activities of the Crude Extract and Fractions of the Bulb of *Crinum jagus*

The crude extract and fractions of the bulb of *Crinum jagus* were screened for anti-inflammatory activity using the established Carrageenan, induced rat paw oedema test described by Winter *et al*.,1962; Perianayagam *et al*., 2006. This is the basic model for screening agents with anti-inflammatory activity.

Principle

This model is based on the principle of release of various inflammatory mediators, oedema formation due to carrageenan in the rat paw is a biphasic event. The initial phase is attributed to the release of histamine and serotonin, the second phase of oedema is due to the release of prostaglandins protease and lysosome. Injection of carrageenan into the rat paw increased tissue water and plasma protein extravasion.

Reagents

1. 0.9% NaCl solution (saline)

A 0.9g weight of NaCl was dissolved in distilled water and the volume made up to 100ml.

2. 1% carrageenan solution

A 0.1g weight of carrageenan was dissolved in 10ml of normal saline and stored in a reagent flask.

3. Indomethacin solution (10mg/ml)

A 100mg weight of indomethacin (BDH Chemichals, UK) was dissolved in 10ml of normal saline and stored in a reagent flask .

Procedure

3.10.1 Administration of the crude extract

Thirty males rats were divided into six groups of five animals each. Group A received 0.2ml normal saline and served as the control, group B received indomethacin (5mg/kg), groups C, D,

E and F received the crude extract at the doses of 10, 25, 50 and 75mg/kg, respectively. All administration were done intraperitoneally.

3.10.2 Administration of the fractions

Fourty males rats were used to test for the anti-inflammatory potential of the fractions. The animals were divided into eight groups of five animals each. Group A received 0.2ml saline and served as the control group, group B received indomethacin (5mg/kg), groups C and D received fraction 1 at the doses of 10 and 20mg/kg, groups E and F received fraction 2 at the doses of 10 and 20mg/kg while groups G and H received fraction 3 at the doses of 10 and 20mg/kg respectively. All administrations were done intraperitoneally.

3.10.3 Evaluation of the anti-inflammatory activity

Thirty minutes later, oedema was induced in the right hind paws of rats by injecting 0.1ml of 1% carrageenan into the subplantar region of the paws. Measurement of the paw size was done by wrapping a piece of cotton thread round the paw of rats and measuring the circumference on a metre rule (Bamgbose and Noamesi, 1981). Measurement was done before carrageenan injection (Do) and at intervals of one hour for 5 hours after carrageenan injection (Dt). The inhibition of oedema at the 3rd hour post carrageenan administration was calculated according to the formula.

$$\text{Percentage inhibition} = \frac{(Dt - Do)\ \text{control} - (Dt - Do)\ \text{test}}{(Dt - Do)\ \text{control}} \times 100$$

Where Dt = Linear paw circumference 3.0 hours after carrageenan injection

Do = Linear paw circumference 0.0 hours (just before carrageenan injection).

3.11 *In vivo* Antimalarial Activity of the Crude Extract and Fractions of the Bulb of *Crinum jagus* in *Plasmodium berghei* Infected Mice

The *in-vivo* antimalaria test was carried out using the method of Ryley and Peter (1970). The 4-day suppressive test is a standard test commonly used for antimalaria screening and determination of percentage inhibition of parasitaemia is the most reliable parameter. The test also relies on the ability of standard innoculum of *Plasmodium berghei* to kill the recipient

mouse within six days of inoculation, extension of survival beyond 12 days was regarded as activity.

Reagents

1. Giemsa stain (stock)

A 3.04g weght of Giemsa powder was weighed and mixed with 250ml of methanol and 250ml of glycerol. The solution was poured into a brown bottle to prevent oxidation. After proper shaking, the solution was filtered to removed the undissolved particles. Finally, it was kept in a dark cupboard for two weeks for maturation prior use.

2. 10% Giemsa stain

For thick film smear, 10% Giemsa stain was prepared by adding 1ml of Giemsa stock solution to 9ml of distilled water. For thin smear, 10% Giemsa stain was prepared by adding 1ml of Giemsa stock solution to 9ml of phosphate buffer.

3.11.1 Experimental animals

Eighty male swiss albino mice (20-30g) were used for the study.

3.11.2 Parasite innoculation

A strain of *Plasmodium berghei* (ANKA) that was chloroquine sensitive was supplied from the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Nigeria. The mice, previously infected with *Plasmodium berghei* and with a high parasitaemia level, served as the donor. For this study, a donor mouse with a rising parasitaemia of 20% was sacrificed and blood samples was taken from the donor and diluted with phosphate-buffered saline such that 0.2ml injected intraperitoneally to the experimental animals contained 1×10^7 infected erythrocytes.

3.11.3 Administration of the extract and the fractions

Eighty male swiss mice were used for the study. On the first day of the experiment (Day O), the mice were infected with the parasite and randomly divided into ten groups of eight animals based on their weight. Each mouse was innoculated on Day O intraperitoneally with 0.2ml of infected blood containing 1×10^7 *Plasmodium berghei* parasitised red blood cells. Treatment was withheld for 72 hours to allow for the establishment of infection, and was commenced when parastaemia had been established. The crude extract and the fractions were dissolved in

Tween 80 (vehicle for the extract) and administered orally. Group 1 received 0.3ml Tween 80 for three consecutive days and served as the control group, Group 2 received chloroquine (10mg/kg) for three consecutive days, Group 3 received arteether (3mg/kg) for three consecutive days, Groups 4 ,5, 6 and 7 received the crude extract at the doses of 10, 25, 50 and 75mg/kg respectively for four consecutive days. Groups, 8, 9 and 10 received 10mg/kg of fractions 1, 2 and 3 respectively, for four consecutive days.

3.11.4 *In-vivo* antimalarial test

Each day starting from the day treatment commenced until the end of the experiment blood films were made form the tail of each infected mouse, stained with Giemsa stain and examined microscopically to assess the level of parasitaemia. Blood was also collected from the tip of the animal tail into capillary tube to determine the packed cell volume (PCV). The body weight of the mice were measured to observed whether the plant extract prevented weight loss that is common with increasing parasitaemia in infected mice.

3.11.5 Preparation of thin blood films

A drop of blood from the tail is placed on the edge of a clean microscope slide, spread across to a length of about 5cm and allowed to dry. The smear is then fixed with absolute methanol and stained with Giemsa stain solution for 30minutes before rinsing with running tap water and allowing the slide to dry.`

3.11.6 Determination of percentage parasiteamia

The prepared thin blood films were viewed under the microscope using a high magnification (X100) with oil immersion for intracellular stages of *Plasmodium yoelli* to estimate cells. At least, a total of 1000 cells were counted from the blood films to calculate the percentage parasitaemia.

$$\% \text{ parasitaemia} = \frac{\text{no of parasitized RBC}}{\text{no of parasitized RBC} + \text{total no of RBC}} \times 100$$

3.11.7 Determination of packed cell volume (PCV)

Packed cell volume (PCV) is a measure of the proportion of red blood cells to the whole blood. Small volume of blood was collected from the tip of animal tail (tail tip amputation) into heparinised capillary tube. The capillary tube was sealed and spun for ten minutes in

haematocrit centrifuge to separate the blood into plasma and packed cells. The percentage of packed cells was calculated using haematocrit reader.

3.12 Inhibitory Effect of the Crude Extract and the Fractions of the Bulb of *Crinum jagus* on *Mycobacterium tuberculosis* Isolates.

Anit-TB assays were performed in Lowenstein Jensen (L-J) and Middle brook 7H10 agar obtained from Sigma Chemicals, USA.

Culture technique is considered to be the gold standard and it offers the possibility of more rapid and more sensitive diagnosis of active tuberculosis and drug susceptibility.

3.12.1 Mycobacterial isolates

Mycobacterial tuberculosis strains and the reference drug susceptible strain H37Rv used as control were obtained from Tuberculosis Reference Laboratory (South-West Zone), Medical Microbiology Department, College of Medicine, University of Ibadan. The standard tuberculosis drugs to which these isolates and the strain are susceptible are rifampicin, isoniazid, streptomycin, ethambutol.

3.12.2 Preparation of Lowenstein Jensen (L-J) medium

A 37.24g weight of L-J medium was suspended in 600ml of distilled water containing 12ml glycerol (for bovine bacteria or other glycerophobic organisms, addition of glycerol is not desirable). The medium was boiled until it was completely dissolved and it was sterilized by autoclaving at 121^0C for 15 minutes. 100ml of whole egg emulsion was prepared and aseptically collected. The homogenized egg, base and Gruit mycotological supplement (if desired) were administered to the medium. It was properly mixed to obtained a uniform mixture and dispensed in 6-8ml sterile screw cap tubes. The caps were tightly closed. The medium was inspissated at 85^0C for 45minutes in a slanted position in a water bath or autoclave used for isolation and cultivation of *Mycobacterium* species.

3.12.3 Preparation of Middle brook 7H10 agar

A 19.50g weight of Middle brook 7H10 agar was dissolved in 900ml of distilled water. To this was added 5ml of glycerol supplement (80021). The mixture was heated until it was completely dissolved and autoclaved at 121^0C for 15 minutes. The mixture was cooled and 100ml of Middlebrook 7H10 supplement (81035) was added. The Middlebrook agar was then poured

into sterile petridishes and allowed to set. The plates were then sterilized by autoclaving at 121^0C for 15minutes.

3.12.4 Preparation of test solutions

A 0.01g weight of the crude extract and each of the fractions (F1, F2 and F3) were dissolved, separately in 10ml of methanol to obtain a stock solution of 1mg/ml. From the stock solution, various concentrations (0.2mg/ml; 0.4mg/ml; 0.6mg/ml; 0.8mg/ml and 1.0mg/ml) of the crude extract and each of the fraction were obtained. Stock solutions (1mg/ml) of rifampicin and isoniazid were prepared using the method described above, from which various concentrations were obtained and used for the drug susceptibility test.

3.12.5 Determination of colony forming units (CFU) on Lowenstein-Jensen (L-J) medium

The procedure followed was that described by Gupta *et al.*,(2010). The ten fold dilution of standard 1mg/ml *Mycobacterium tuberculosis* suspensions were prepared. One loopful (6µl) of the suspension was streaked on the L-J slants using 3mm external diameter loop. The crude extract and each of the fractions at concentration of 0.2mg/ml; 0.4mg/ml; 0.6mg/ml; 0.8mg/ml and 1.0mg/ml were incorporated in the medium. The medium was then incubated at 37^0C for 42 days. For comparison, extract free control slants were used. Each test was done in duplicates. After 4 weeks, the culture were examined for possible growth of *Mycobacteria tuberculosis.* Percentage inhibition was calculated by mean reduction in number of colonies on extract containing as compared to extract free controls. The percentage inhibition of extract/fractions were then plotted against the logarithm of the extract/fractions concentrations to determine the fifty percent inhibitory concentration (IC_{50}). Susceptibility testing of the isolates was also performed against standard drugs, rifampicin and isoniazid in the same batch of media for comparison of colony forming units on drug free control.

3.12.6 Determination of colony forming units (CFU) on Middle brook 7H10 agar

The procedure followed was that described by Claude *et al.*, (2012) using the disc diffusion method. The discs were separately impregnated with 20µl of the various concentrations (0.2mg/ml; 0.4mg/ml; 0.6mg/ml; 0.8mg/ml and 1.0mg/ml) of the extract, each of the fractions and the standard drugs and were left to dry for 24 hours. The culture medium (the sterile Middle

brook 7H10 agar) was placed in 90mm diameter petri dishes with quadrants. In each quadrant of the petridish was put 5.0ml of the medium. The solidified medium in the quadrants was innoculated with *M.tuberculosis,* using a swab. A rifampicin-impregnated disc was placed in the first quadrant, in the second quadrant was put isoniazid-impregnated disc, the third quadrant had the extract/fractions-impregnated disc while the fourth quadrant contained a blank disc (negative control). This was achieved for the varying concentrations of the extract, the fractions and standard drugs. The petridishes were left on safety cabinets overnight to allow diffusion of the extract, the fractions and the drugs and then sealed with a carbondioxide permeable tape. These were then incubated at 37^0C in a carbon dioxide incubation for up to 4 weeks. The susceptibility of *Mycobacterial tuberculosis* to the extract and the drugs was determined by counting the number of bacteria in each quadrant. Percentage inhibition was calculated by mean reduction in number of colonies on extract containing as compared to extract free controls. The percentage inhibition of extract/fraction were then plotted against the logarithm of the extract/fractions concentrations to determine the fifty percent inhibitory concentration (IC_{50}). Susceptibility testing of the isolates was also performed against standard drugs, rifampicin and isoniazid in the same batch of media for comparison of colony forming units on drug free control.

3.13 Toxicological Profiles of the Crude Extract of the Bulb *Crinum jagus*

3.13.1 Administration of test substance and preparation of tissue homogenate

Reagents

1. Homogenizing Buffer (0.1M phosphate buffer, pH 7.4)

(a) A 11.8g weight of disodium hydrogen phosphate (Na_2HPO_4) was dissolved in distilled water and made up to 1000ml.

(b) A 6.8g weight of potassium hydrogen phosphate (KH_2PO_4) was dissolved in distilled water and made up to 500ml.

(c) A solution of 800ml of (a) was mixed with 200ml of (b) to make 1000ml. The pH was adjusted to 7.4 and the solution was stored at 4^0C.

2. Washing Buffer (1.15% KCl)

A 11.5g weight of potassium chloride was dissolved in distilled water and made up to 100ml **1.**

3. 0.9% NaCl solution (saline)

.A 0.9g weight of NaCl was dissolved in distilled water and the volume made up to 100ml with the same and stored at 4^0C.

3.13.2 Administration of test substance

Thirty male rats were randomly distributed to five groups of six animals per group. The crude extract was dissolved in normal saline before administration to the rats using oral cannula and administration was done for 30 days. Group A animals served as the control and were given 0.2ml normal saline in place of extract. Group B were administered with 10mg/kg of the extract. Group C received 25mg/kg of the extract, Group D were administered with 50mg/kg of the extract while group E received 75mg/kg of the extract for 30days.

3.13.3 Preparation of plasma

Twenty four hours after the last treatment, the animals were sacrificed and blood samples were collected by cardiac puncture into EDTA bottles and gently rotated to allow proper mixing with the anticoagulant. The sample was centrifuged at 2000g for 10 minutes and the plasma transferred into another clean tube, stored at 4^0C and used for haematological study.

3.13.4 Preparation of serum

Blood samples were collected into plain sample bottles and were allowed to stand for 1 hour and were centrifuged at 3000g for 10minutes to obtain the serum and serum collected using a micropipette.

3.13.5 Preparation of liver and kidney homogenates

Liver and kidney tissues from rats in each group were quickly removed and washed in ice cold 1.15% KCl solution, dried and then weighed. The samples were homogenized in 4 volumes of 0.1M phoslpahte buffer (pH 7.4) and were centrifuged at 10,000g for 15 minutes to obtain the post mithochondria supernatant fractions of the liver and kidney samples. The samples were stored at 4^0C until required.

3.13.6 Biochemical Assays

3.13.6.1 Determination of Aspartate amino transferase (AST) activity

Principle

Aspartate amino transferase (AST) also referred to as glutamate oxaloacetate transaminase (GOT) catalyses the transfer of the amino group of the glutamic acid to oxaloacetate in reversible reactions. The transaminase activity is proportional to the amount of oxalate formed over a definite period of time and is measured by a reaction with 2, 4 Dinitrophenyl hydrazine (DNPH) in alkaline solution (Rietman and Frankel, 1957).

α -ketoglutarate + L-aspartate $\xrightleftharpoons{\text{AST}}$ L-glutamate + Oxaloacetate

Reagents

Randox kit was used. The kit contains the following:

	Enzyme Reagent	**Concentration**
R_1 a	DL-Aspartate	100mmol/L
R_2 (Substrate GOT)	α-Ketoglutarate	2mmol/L
Developer	2,4 Dinitrophenyl hydrazine (DNPH)	1mmol/L
GOT calibrator	Primary calibrator of pyruric	1.2mmol/L
	Sodium hydroxide	0.4N

Procedure

A 0.5ml portion of the GOT was pippeted into a test tube, mixed and incubated in water bath for 5 minutes at 37^0C. To the test tube, 100µl of the sample was added and incubated in a water bath for 60 minutes before 0.5ml of dinitrophenyl hydrazine (DNPH) was added to the mixture, mixed and allowed to stand for 20 minutes at room temperature. A 5.0ml portion of 0.4N sodium hydroxide was added to the mixture, mixed and allowed to stand for 15minutes at room temperature. The absorbance was read against a water blank at wavelength of 546nm. The standard curve for AST activity was plotted and the absorbance reading was extrapolated from the graph.

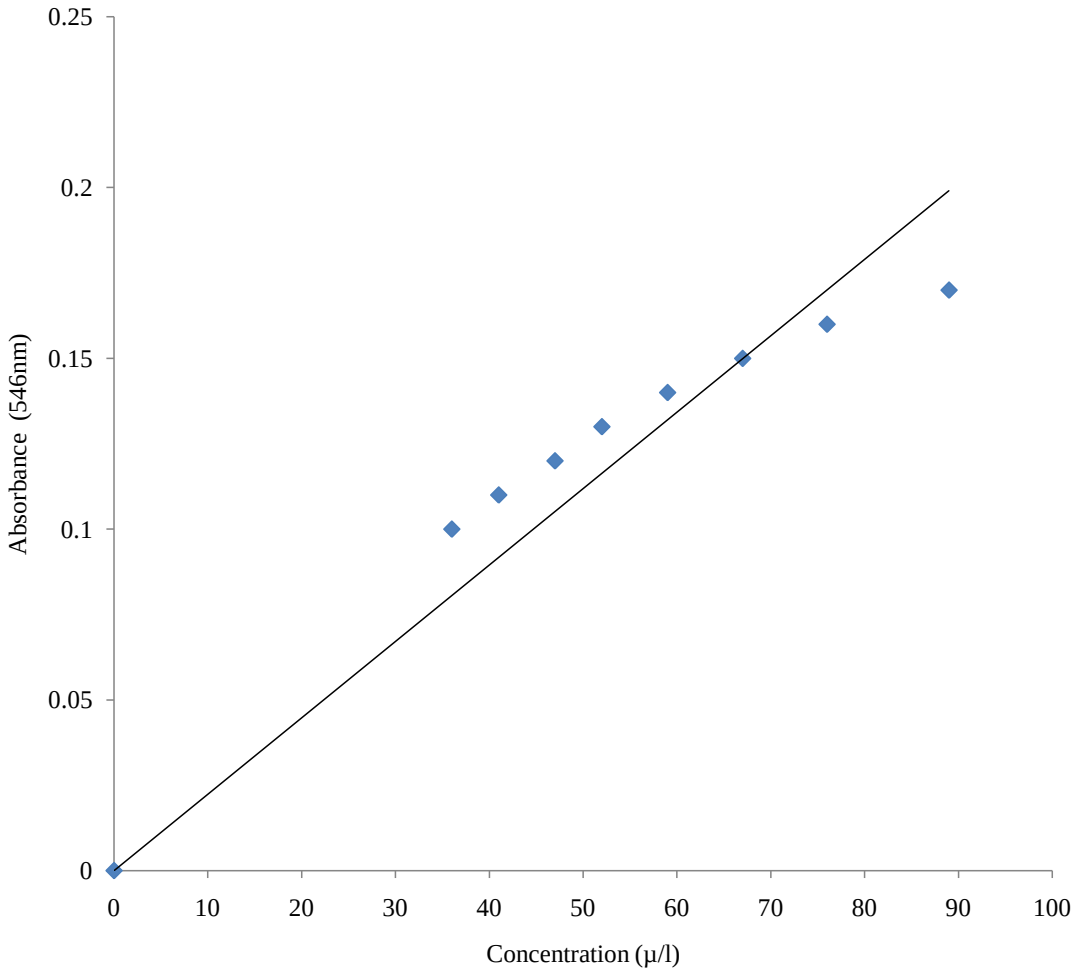


Figure 14 : AST standard curve

3.13.6.2 Determination of Alanine amino transferase (ALT) activity

Principle

Alanine amino transferase (ALT) also referred to as glutamate pyruvate transaminase (GPT) catalyses the transfer of amino group of glutamic acid to pyrurate in reversible reaction. The trasnaminase activity is proportional to the amount of pyruvate formed over a definite period of time and is measured by a reaction with 2,4 dinitrophenyl hydrazine (DNPH) in alkaline solution (Reitman and Frankel, 1957).

α-Ketoglutarate + L-alanine $\overset{\text{AST}}{\rightleftharpoons}$ L-glutamate + pyruvate

Reagents

Randox kit was used. The kit contain the following:

	Enzyme Reagent	**Concentration**
R_1 b	DL-Alanine	200mmol/L
R_2 (Substrate GPT)	α-ketoglutarate	2mmol/L
Developer	2,4 Dinitrophenyl hydrazine (DNPH)	1mmol/L
GPT Calibrator	Primary calibrator of pyruvic	1.2mmol/L
	Sodium hydroxide	0.4N

Procedure

A 0.5ml portion of the GPT was pippeted into a test tube, mixed and incubated in water bath for 5 minutes at 37^0C. To the test tube, 100µl of the sample was added and incubated in water bath for 30 minutes before 0.5ml of dinitrophenyl hydrazine (DNPH) was added to the mixture, mixed and allowed to stand for 20 minutes at room temperature. A 5.0ml portion of 0.4N sodium hydroxide was added to the mixture, mixed and allowed to stand for 15minutes at room temperature. The absorbance was read against a water blank at wavelength of 546nm. The standard drug for ALT was plotted and the absorbance reading was extrapolated from the graph.

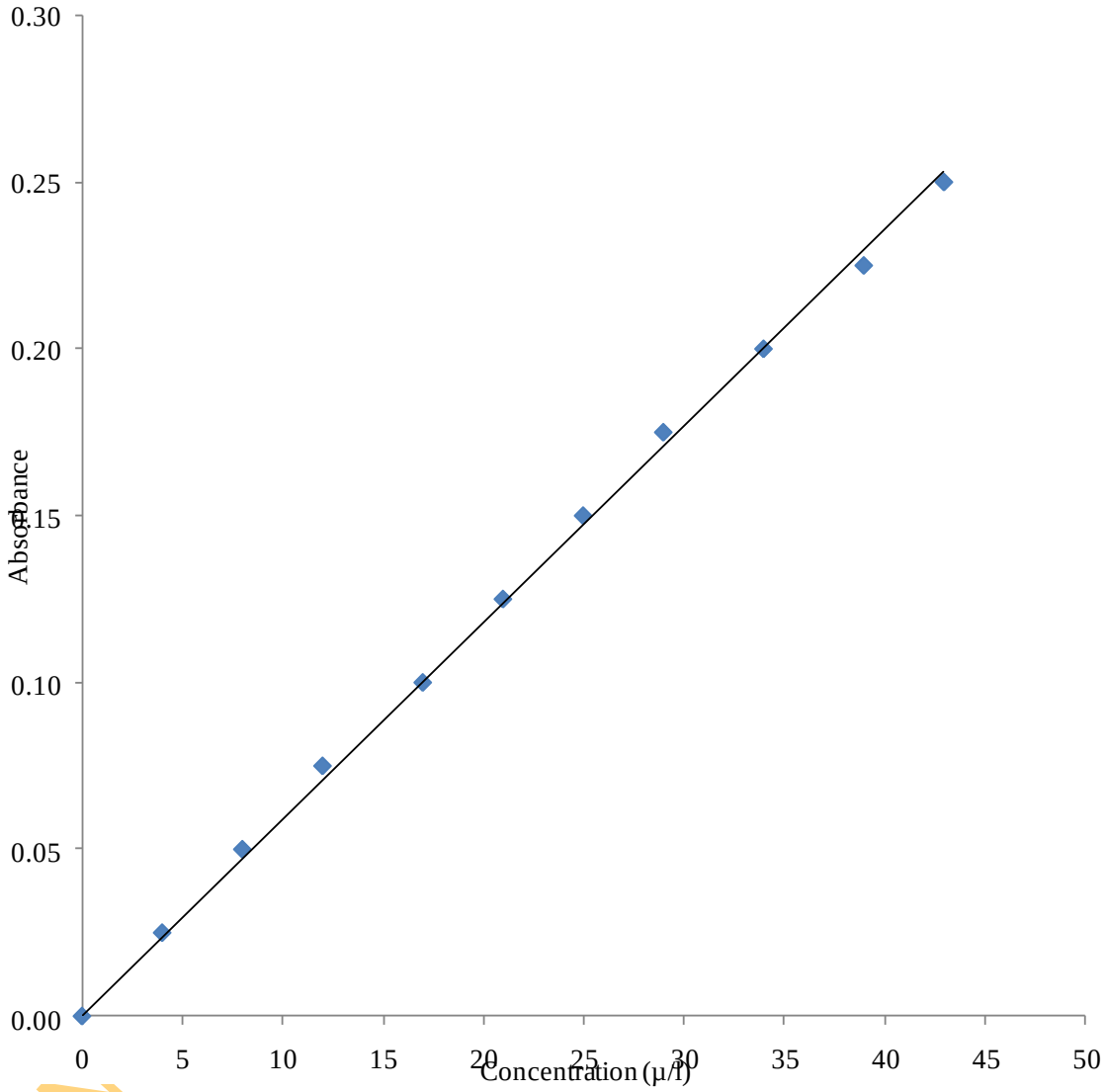


Figure 15 : ALT standard curve

3.13.6.3 Determination of Alkaline Phosphatase (ALP) activity

Principle

Serum alkaline phosphatase hydrolyses a colourless substrate of phenophtalein monophospahte giving rise to phosphoric acid and phenophtalein which at alkaline pH values turns into a pink colour that can be photometrically determined (Klein and Babson, 1960).

$$\text{p-Nitrophenyl phosphate} + H_2O \xrightarrow[\text{phosphatase}]{\text{alkaline}} \text{p-Nitrophenol} + \text{phosphate}$$

The rate of p-nitrophenol formation is proportional to the catalytic concentration of alkaline phosphatase present in the sample.

Reagents

Randox kit was used. The kit contains the following:

	Reagents
A	Chromogenic Susbtrate
B	Colour Developer (content was dissolved in 250ml of deionised water)
C	Standard Solution of alkaline phosphatase in water / ethanol equivalent to 30U/L

Procedure

An aliquot of 1.0ml of water was pippeted into two test tubes (test tube SA and ST), to each of the test tube was added 1 drop of the chromogen substrate and was mixed and then incubated at 37^0C for 5 minutes. Then to test tube SA, 0.lml of the sample was added and to test tube ST, 0.1ml of standard alkaline phosphatase was added. The test tubes were mixed and incubated at 37^0C for 20 minutes. To each of the test tubes, 5ml of the colour developer was added. Absorbance was read against a water blank at wavelength of 550nm. The activity of ALP in the sample was calculated using the formula :

$$\text{U/L of Alkaline phosphatase} = \frac{\text{SA Absorbance}}{\text{ST Absorbance}} \times 30$$

3.13.6.4 Determination of Lactate Dehydrogenase (LDH) activity

Principle

Lactate dehydrogenase (LDH) catalyses the reduction of pyruvate by NADH to lactate, according to the following reaction:

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+.$$

The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of LDH present in the sample (Pesce and Kaplan, 1984).

Reagents

Randox kit was used. The kit contains the following:

	Reagents	**Concentration**
R_1	Phosphate (pH 7.8)	80mm0l/L
Buffer	Pyrurate	0.6mmo/L
R_2 substrate	NADH	0.18mm0/L

Working Reagent (WR): Mix 4 volumes of R_1 with 1 volume of R_2

Procedure

A 3.0ml portion of working reagent (WR) was pippeted into a test tube, and 100µl of the sample was added and the mixture incubated for 1 minute. The absorbance of the sample was read at 340nm at 1 minute interval for 3 minutes. The difference of absorbance and average absorbance difference was calculated per minute (DA/min). LDH activity in the sample was calculated using the formula :

U/L LDH = DA/min X 4925.

3.13.6.5 Determination of total bilirubin

Principle

Bilirubin reacts with diazotized sulfanilic acid to produce coloured azobilirubin which has an absorbance maximum at 560nm. The intensity of the colour produced is proportional to the bilirubin concentration in the sample (Kaplan *et al*., 1984; Malloy *et al*., 1937; Martinek, 1966).

Reagents

Randox kit was used. The kit contain the following:

	Reagents	Concentration
R_1	Dimethyl sulphoxide	7mmol/L
	Sulphanilic acid	30mmol/L
	Hydorchloric acid (HCl)	50mmol/L
R_2	Sodium nitrite	29mmol/L
	Bilirubin calibrator	20mg/dL

Procedure

A 1.5ml portion of reagent R_1 was pippeted into two test tubes (test tubes BL and SA). To testube SA was added 50µl of reagent R_2. To the two test tubes were added 100µl of the sample. The tubes were mixed and incubated for 5 minutes at room temperature. The absorbance was read against the blank at 555nm. The concentration of total bilirubin in the sample was calculated using the formula :

Total bilirubin (mg/dl) = 17 .1 X **A**

Where **A** = Absorbance of the sample

3.13.6.6 Determination of direct bilirubin

Principle

Bilirubin reacts with diazotised sulphanilic acid to produce coloured azobilirubin which has an absorbance maximum at 560nm. The intensity of the colour produced is proportional to the bilirubin concentration in the sample (Kaplan *et al.*,1984; Malloy *et al.*; 1973, Martinek, 1966)

Reagents

Randox kit was used. The kit contains the following:

	Reagents	Concentration
R_1	Sulphanilic acid	30mmol/L
	Hydrochloric acid (HCl)	150mmol/L
R_2	Sodium nitrite	29mmo/L
	Bilirubin calibrator	20mg/dL

Procedure

A 1.5ml portion of reagent R_1 was pippeted into two test tubes (test tube BL and SA). To test tube SA was added 50µl of reagent R_2. To the test tubes were added 100µl of the sample. The tubes were mixed and incubated for 5minutes at room temperature. The absorbance was read against the blank at 555nm. The concentration of direct bilirubin in the sample was calculated using the formula.

Direct bilirubin (mg/dl) = 17.1 X **A**

A = Absorbance of the sample

3.13.6.7 Determination of serum triglycerides

Principle

The serum triglyceride was determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide and 4-amino phenazone under the catalytic influence of peroxidase (Mc Gowan,1983).

$$\text{Triglycerides} + H_2O \xrightarrow{\text{lipase}} \text{Glycerol} + \text{Fattyacids.}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + HCl + 4H_2O$$

Where :

GK = Glycerol kinase

GPO = Glycerol-3-phosphate oxidase

POD = Peroxidase

Reagents

Randox kit was used. The kit contains the following:

(a) **Buffer**	**Concentration**
PipesBuffer	40mmol/L
4-chlorophenol	5.5mmol/L
Magnesium-ions	17.5mmo/L
(b) **Enzyme Reagent**	**Concentration**
4-aminophenazone	5.5mmol/L
ATP	1.0mmol/L
Lipases	≥150U/ml
Glycerol kinase	≥0.4U/ml
Glycerol-3-phosphate oxidase	≥1.5U/ml
Peroxidase	≥0.5U/ml
(c) **Standard**	2.29mmol/L (200mg/dl)

Working Reagent: One vial of the enzyme reagent was reconstituted with 15ml of buffer.

Procedure

An aliquot of 1000µl working reagent was added to 10µl of the sample. 1000µl of working reagent was added to 10µl of standard in another clean test tube. 1000µl of working reagent was put into another clean tube to serve as blank. Each tube was mixed and incubated at 20-25^{0}C for 10minutes. The absorbance of the sample and standard were measured against the reagent blank within one hour at 505nm. The concentration of triglyceride in the sample was calculated using the formula.

$$\text{Triglyceride conc (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200$$

3.13.6.8 Determination of serum total cholesterol

Principle

The serum cholestrol is determined after enzymatic hydrolysis and oxidation with lipases. The colorimetric indicator is quinoneimine which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under catalytic action of peroxidases (Trinder, 1969)

$$\text{Cholestrol ester} \xrightarrow[\text{esterase}]{\text{cholestrol}} \text{Cholestrol} + \text{Fatty acids}$$

$$\text{Cholestrol} + H_2O \xrightarrow[\text{Oxidase}]{\text{cholestrol}} \text{Cholestenone} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + 4 + \text{aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + H_2O_2$$

Reagents

Randox kit was used which contain the following:

Reagents	**Concentration**
Pipes buffer	80mol/L
4-aminoantipyrine	0.3mmol/L
Phenol	6mmol/L
Cholesterol esterase	≥0.15U/ml
Cholesterol oxidase	≥0.10U/ml
Peroxidase	≥0.5U/ml
Standard	5.17mmol/L (200mg/dl)

Working reagent: One vial of the enzyme reagent was reconstituted with 15ml of buffer

Procedure

An aliquot of 1000µl working reagent (WR) was added to 10µl of distilled water to serve as blank. An aliquot of 1000µl of working reagent was added to 10µl of the standard in a separate clean tube. An aliquot of 1000µl of working reagent was added to 10µl of the sample in another tube. The tubes were mixed and incubated for 10 minutes at 20-25^{0}C. The absorbance of the

sample and standard were measured against the reagent blank within one hour at 505nm. The concentration of total cholestrol in the sample was calculated using the formular.

$$\text{Total cholestrol conc (mg/dl)} = \frac{\text{Change in absorbance of sample}}{\text{Change in absorbance of standard}} \times 200$$

3.13.6.9 Determination of HDL-cholestrol

Principle

Serum HDL-cholestrol was determined by enzymatic method as described for total cholestrol determination after selective precipitation of the interfering very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) with a precipitating reagent, phosphotungstic acid and magnesium chloride. After centrifugation, the cholestrol concentration in high density lipoprotein fraction which remain in the supernatant was determined (Lopes-virella *et al.*, 1977).

Reagent

Randox kit was used which contain the following:

Reagents	**Concentration**
Phosphotungstic acid	0.55mmol/L
Magnesium chloride	25mmol/L

Reagent solution for cholestrol CHOD-PAP assay

The precipitating reagent was diluted in the ratio 4:1 with distilled water and stored at 15-25^{0}C.

Procedure

An aliquot of 500µl of diluted precipitant was added to 200µl of sample in the centrifuge tube. The tubes were mixed and allowed to stand for 10minutes at room temperature. It was centrifuged for 10minutes at 4,000 rpm. The supernatant was separated within two hours and the cholestrol content was determined by CHOD-PAP method.

$$\text{HDL conc (mg/dl)} = \frac{\text{Change in absorbance of sample}}{\text{Change in absorbance of standard}} \times 200$$

3.13.6.10 Determination of serum LDL-cholestrol

Principle

Serum LDL-cholestrol was determined using the method of Friedwald *et al*.,(1972). This method involves prior knowledge of the fasting serum total cholesterol, the HDL-cholestrol and total triglyceride concentrations based on the fact that:

1. The ratio of the mass of triglyceride to that of VLDL-cholestrol is apparently relatively constant at about 5:1 in normal subjects (Fredickson *et al*., 1967; Hatch and Lees, 1968) and in patients with all types of hyperlipoproteinemia, except the rare type 3 (Fredickson *et al.*, 1967).
2. When chylomicrons are not detectable, most of the triglyceride in the blood is contained in the very-low-density lipoprotein fraction.

The concentration of LDL-C in the sample was calculated using the formula.

$$\text{LDL-C (mg/dl)} = \text{Total cholestrol} - \frac{\text{Triglyceride}}{5} - \text{HDL-C}$$

3.14 Determination of Haematological Parameters

1. Haemoglobin concentration (Hb)

The blood haemoglobin concentration was estimated using the cyanomethaemoglobin method as described by Dacie and Lewis,(1991).

Principle

After hemolysis of red cells, haemoglobin is converted to cyanmethaemogloin by the cyanide in the diluting solution (Drabkins diluents). The chromogen formed is assayed spectrophotometrically at 540nm.

Reagent

Drabkins diluents

A 1g weight of sodium hydrogen carbonate (Hopkins and Williams Ltd. England) and 198mg of potassium ferricyanide (BDH Chemical Ltd. England) were dissolved in distilled water, and made up to 1 litre.

Procedure

A 0.02ml sample of the blood was added to 4ml of Drabkins diluents. The solution was allowed to stand for 10 minutes and the absorbance of the resultant solution was read at 540nm with the

Drabkins diluents as reference. Absorbance of haemoglobin standard of known concentration was also determined, concentration of haemoglobin was calculated as shown below.

$$\text{Hb con(g/dl)} = \frac{\text{Absorbance of test solution}}{\text{Absorbance of standard solution}} \times \text{Hb conc of standard solution} \times \text{dilution factor}$$

Dilution factor = 200, Hb concentration of standard = 0.068g/dl

2. **Packed Cell Volume (PCV)**

PCV was determined in duplicate using the microhaematocrit method described by Dacie and Lewis,(1991). This method involves filling a capillary tube with blood. One end of the tube was sealed and the tube centrifuged in a microhaematocrit centrifuge for 10 minutes. The PCV in percentage was read directly from a graphic reader.

3. **Red Blood Cell Count (RBC)**

The red blood cell count was determined using the haemocytometer method described by Dacie and Lewis,(1991).

Reagent

Hayem's solution

A 0.25g weight of mercury chloride ($HgCl_2$) and 0.25g of sodium sulphate (Na_2SO4) (Hopkins and Williams Ltd, England) were dissolved in distilled water and made up to 100ml. This solution was isotonic with blood and prevented roleaux formation and coagulation.

Procedure

Blood was drawn by means of rubber tubing attached to the end of the red cell pipette to 0.5mark on the pipette. Keeping the pipette nearly horizontal, Hayem's solution was drawn to the 101 mark with care to prevent overshoot. Holding the pipette horizontal, the rubber tubing was removed without squeezing. The pipette was then rotated for about 1 minute to ensure thorough mixing of blood and dilution of the fluid in the bulb. Diluted blood was then introduced carefully into the counting chamber with clean cover slip in place. Red cell counting was done using X40 objective of microscope. Counting was carried out in 5 sets of small squares containing 80 small squares. All red cells overlapping the top and left hand sides of a square were counted while those overlapping the bottom and right hand slide were not counted.

The total number of erythrocytes obtained was multiplied by the depth (X10), area (X5) and dilution factor (X200) hence Y-erythrocytes per millimeter in the sample would be 10,000Y.

4. **Haematocrit Indices**

Mean corpscular volume (MCV), mean corpscular haemoglobin (MCH) and mean corpscular haemoglobin concentration (MCHC) were calculated from the values of RBC, PCV and Hb as described below.

MCV provides the average volume of individual RBC and is calculated using the formula.

$$\text{MCV (pg)} = \frac{\text{PCV (\%) X10}}{\text{RBC (X } 10^6 \mu\text{L)}}$$

MCH expresses the average weight of Hb present in an erythrocyte and is calculated as follows:

$$\text{MCH (pg)} = \frac{\text{Hb (g/dl) X10}}{\text{RBC (X}10^6\ \mu\text{L)}}$$

MCHC gives the percentage of the MCV which Hb occupies and is calculated as follows:

$$\text{MCHC (g/dl)} = \frac{\text{Hb (g/dl) X100}}{\text{PCV(\%)}}$$

5. **Total and Differential White Blood Cells Count**

White cells were counted using a haemocytometer, white cell pippette and white cell diluting fluid.

Reagent

White cell diluting fluid

This contained 2% acetic acid and 2ml of methyl violet dye.

Procedure

The procedure followed was similar to that used to determine the red cell count. The ratio of dilution was 1:20. The sum of the white blood cell counts from the 4 large corner squares of haemocytometer chamber was multiplied by the depth (X10) and the dilution factor (X20) and divided by the square counted. Thus the number of leucocytes per millimeter in the original sample was 50X where X was the number of leucocytes counted in the 4 squares. Differential WBC counts in which 100 cells per slides were counted were made from Giemsa stained blood smear.

3.15 Histopathology study

The liver and kidney samples were harvested after the animals were sacrificed. The tissue was processed using an automatic tissue processor, embedded in paraffin wax and section (5µm) thickness were cut using a rotary microtome. The sections were stained by haematoxylin and eosin (H & E) method for light microscopic examination. Photomicrograph of stained sections were taken with the aid of a light camera fitted microscope.

3.16 Determination of Chemical Composition of Fraction 1 (F1)

The chemical composition of fraction 1 was determined by a combined gas chromatography mass spectrometry (GC-MS) technique (Karasek and Clement, 1988).

Principle

Gas chromotogrphy mass spectroscopy (GCMS) is a method that combines the feature of gas-liquid chromatography and mass spectroscopy to identify different volatile substances within a test sample. The GC-MS is composed of two major building blocks, the gas chromatography and mass spectrophotometer. The gas chromatography utilizes a capillary column, molecules are retained by the column and the elute from the column are ionized and the fragments detected by the mass spectrophotometer, using a charge-to-mass ratio computation to show the fragmentation pattern.

Procedure

The sample was analysed by GC-MS using a Schimadzu model QP 2010 gas chromatography system with split/splittes injection interfaced to a 5973 mass selective detector. Innowax fused silica capillary (FSC) column (30m x 0.25mm, with 0.25µm film thickness) was used with helium as carrier gas at a flowrate of 1ml/min . The GC oven temperature was kept at 80^0C (hold for 2mins) and programmed to reach 200^0C at a rate of 4^0C/min, then kept constant at 280^0C for 10 minutes being the final hold time. The split ratio was adjusted to 50:1. The injector temperature was at 250^0C. Mass spectra were recorded at 70eV. Mass range was from m/z 30 to 50V. Identification of components was achieved on the basis of their retention indices determined with a reference to a homologous series of n-alkanes and by comparison of their mass spectral fragmentation on patterns (NIST data base/ chemstation data system) with data

previously reported in literature (Adams 2001; Joulain and Konig, 1998; Mclatterfly and Staufer, 1989).

Statistical Analysis

Data were expressed as mean $\pm$ standard deviations and analysed by student t-test and one way analysis of variance (ANOVA) and $P < 0.05$ was considered to be significant.

IC_{50} was determined by regression analysis using a statistical package known as microcal origin.

CHAPTER FOUR

4.0 EXPERIMENTS AND RESULTS

Experiment 1 : Phytochemical Analysis and Fractionation of the Crude Extract of the Bulb of *Crinum jagus*

Introduction

Numerous investigations have shown that medicinal plants contain diverse classes of bioactive compounds; such as alkaloids, flavonoids, tannins and phenols which exhibit various pharmacological properties (Emam, 2010). However, in the absence of any scientific proof of their effectiveness, the validity of these remedies and their use by local communities remains restricted (Kaur and Arora, 2009). Phytochemicals and pharmacological investigations of several plants have already led to the isolation of natural drugs. However the potential of higher plants as sources of new drugs is still largely unexplored. Consequently, there is need to screen natural products for the presence of bioactive chemical constituents.

The bulb of *Crinum jagus* use in Western part of Nigeria has been shown to have biological activites such as antibacterial, antifungal, anticonvulsant, anticholinergic, anti-snake venom activities (Adesanya *et al*., 1992; Edema and Okiemen 2002; Peter *et al*., 2004; Ode *et al*., 2006).

This experiment was carried out on the bulb of *Crinum jagus* so as to gain insight into the possible phytochemicals present in them, with aim of studying their relevance in drug development and design, as well as to justify their usuage in folk medicine.

Procedure

Standard pharmacognosis procedures were used for qualitative and quantitative phytochemical screening as have been described in section 3.3 under “Materials and Methods.

A modified form of classical column chromatography called the flash chromatography was used for the fractionation of the crude extract of the bulb of *Crinum jagus*. Glass column was packed with silica gel (flash chromatography grade). The bulb extract adsorbed with silica gel was packed on to the column layer and then allowed to settle. The mobile phase consisted of three solvents; hexane, ethylacetate and methanol, mixed in various proportions. The various

proportions of the solvents were pushed through the bed by application of positive pressure using the vaccum pump. Twenty one fractions were obtained. The fractions were pooled together by thin layer chromatography (TLC) and this reduced the number of the fractions to five.

Results

Tables 8 and 9 show the phytochemicals present in the bulb extract of *Crinum jagus*. Qualitative phytochemcial screening revealed that the methanol extract of the plant contains alkaloids, flavonoids, phenols, saponins, steroids, but no traces of tannins. Quantitative phytochemcial test revealed that the extract contains high concentrations of alkaloids, phenols, saponins and appreciable amount of steroids and flavonoids. Fractionation of the crude extract by column and thin layer chromatography yielded five fractions. The biological activities of three fractions (F1, F2 and F3) out of the five fractions were tested because the yield of the other two fractions obtained were too small to be tested for any biological activity.

Conclusion

The crude methanol extract of the bulb of *Crinum jagus* contains high concentration of bioactive substances such as alkaloids, phenols, saponins, and appreciable amounts of flavonoids and steroids.

Table 8 : Phytochemcial constituents of the bulb extract of *Crinum jagus*

CONSTITUENTS	INFERENCE
Alkaloids	++
Flavonoids	++
Phenols	++
Saponins	++
Steroids	++
Tannins	-

++ indicates present

- Indicates absent

Table 9 : Relative phytochemical contents of *Crinum jagus*

PHYTOCHEMICAL	% PRESENT
Alkaloids	1.38
Phenols	0.649
Saponins	0.426
Steroids	0.076
Flavonoids	0.048

Experiment 2 : Acute Toxicity Study of the Methanol Extract of the Bulb of *Crinum jagus*

Introduction

The use of medicinal plant as therapy for disease condition is an age long practice. In regions with rich diversity of flora spread, it forms an important component of their natural wealth. Herbs and herbal formulation for the treatment of ailments have continued to receive increased attention because of the strong belief that the use of these products is safe (Farnsworth and Soejarto, 1985; Said *et al*., 2002). This assumption, to a large extent, may have influenced the indiscriminate use of plant formulations by many, particularly amongst the rural populace. The incidence of adverse effects and sometimes life-threatening conditions allegedly emanating from these herbal medicines has been reported among various ethnic groups (Elvin-Lewis, 2001; Chan, 2003). Consequently, it has become imperative to ascertain the toxicity profile of these medicinal herbs.

Pharmacological and toxicological evaluations of medicinal plants are essential for drug development. In the present study the acute toxicity of the methanol extract of the bulb of *Crinum jagus* plant was evaluated in mice to assess its safety.

Procedure

A total of twenty five swiss albino mice (25-30g) were used for the study. The animals were acclimatized for a week in clean cages and randomly divided into five groups of five animals per group. The animals were fasted for 14 hours and the groups received methanol extract orally within the range of 50-400mg/kg. The control group was given 0.2ml of normal saline orally. The animals were observed, closely, for general toxicity signs and behavior change for the first 6 hours and mortality recorded within 24 hours. LD_{50} was calculated using the moving average interpolation method of Thomson and Williams, (1974).

Results

Clinical signs for toxicity observed were decreased activity to the environment, huddling, general weakness and changes in the skin and fur. The LD_{50} value calculated by moving average interpolation method was 109.04mg/kg (Table 10).

Conculsion

This study have shown that *Crinum jagus* in very large doses have toxic properties.

Table 10 : Acute toxicity of the methanol extract of the bulb of *Crinum jagus* in mice by oral route after 24 hours of observation.

Group	Dose (mg/kg)	No of death	% Mortality
1	0	0	0
2	50	1	20
3	100	2	40
4	200	4	80
5	400	5	100

LD_{50} = 109. 04mg/kg

Confidence limit = (108.76 – 109.31) mg/kg.

Experiment 3 : *In vitro* Antioxidant Study on the Crude Extract and Fractions of the Bulb of *Crinum jagus*

Introduction

Free radical induced oxidative damage has long been thought to be the most important consequence of the aging process (Harman, 1992), such a condition is considered to be important in the development of diseases including diabetes, stroke, arteriosclerosis, cancer and cardiovascular diseases (Prior and CaO, 2000; Yamaguchi *et al.*, 2000).

Antioxidants are agents which scavenge the free radicals and stop the damage casued by them. Antioxidants are added as redox system possessing higher oxidative potential than the drug that they are designed to protect or as chain inhibitors of radicals. The effect of antioxidant is to break up the chains formed during the propagation by providing a hydrogen atom or an electron to the free radical and receiving the excess energy possessed by the activated molecule (Lachman, 1986). Recently, interest has increased considerably in finding naturally occurring antioxidants for use in food and medicinal materials to replace synthetic antioxidants which are being restricted due to their side effects such as carcinogenicity (Kumaran, 2007).

Plants contain a large variety of phytochemicals which are the main sources of antioxidants in the diet that could decrease the potential stress caused by reactive oxygen species. The natural antioxidants may have free radical scavengers, reducing agents, potential complexers of prooxidant metals and quenchers of singlet oxygen (Ebadi, 2000). The antioxidants can interfere with oxidation process by reacting with free radicals (Gupta, 2004).

This experiment was therefore carried out to evaluate the antioxidant property of the crude extract and fractions of the bulb of *Crinum jagus* as possible scavengers of free radicals using *in-vitro* and *in-vivo* models.

Procedure

DPPH free radical scavenging activities of the extract and its fraction were determined using the DPPH photometric method of Mensor *et al.*, (2001). Hydroxyl radical scavenging activities were determined by the method of Halliwel *et al*., (1987). The reducing power of the extract and the fractions of the plant were determined by the method of Oyiazu (1986). The total flavonoid content of the extract and the fractions of *Crinum jagus* bulb were assessed by the method of

Chang *et al., (*2002). Total phenolic content of the methanol extract and its fractions were estimated based on the reduction of Folin-Ciolcateu reagent (phosphomolybdate and phosphotungstate) by the phenolic compounds using the method of Mc Donald *et al.*, (2001). as earlier described in section 3.7. under 'Materials and Methods' '

Results

The DPPH radical scavenging activites of the extract and its fractions are shown in Figures 16 and 17. The methanol extracts and its fractions all scavenged DPPH in a concentration dependent manner, compared to control. The varying concentrations of the methanol extract 100, 200, 300, 400 and 500µg/ml significantly ($P < 0.05$) scavenged DPPH by 47.58%, 53.16%, 58.93%, 67.17% and 72.46% respectivley (Fig 16). Similary the same concentrations of F1, F2 and F3 significantly scavenged DPPH by (48.11%, 56.68%, 62.30%, 73.43%, 81.37%), (30.44%, 36.46%, 41.46% ,54,90%, 57.84%) and (33.57%, 40.94%, 54.51%, 63.10% and 69.83%), respectively (Fig 17). However these activities are less than that of ascorbic acid, the standard antioxidant. Highest percentage scavenging effect was obtained with F1 (81.37%) at 500µg/ml. The order of potency was: F1 > F3 > CE > F2.

The percentage hydroxyl radical (OH^-) scavenging activities of the extract and its fractions are shown in Figures 18 and 19. The methanol extract and its fractions scavenged hydroxyl radical in a concentration dependent manner compared to control. The highest percentage hydroxyl, radical scavenging activity was detected in F1 (88.32%) at 500µg/ml, the hydroxyl radical scavenging activity of F1 compared well with ascorbic acid (89.32%) . The order of potency was : F1 > F3 > CE > F2.

The reductive capabilities of the extract and its fractions are shown in Figures 20 and 21 compared to ascorbic acid, the standard antioxidant, the reducing powers of the extract and its fractions increased with increasing amount of the sample. F1 showed the highest reducing ability (0.628nm) than all the other fractions tested at 500µg/ml. However, the activity was less than the standard ascorbic acid (1.530nm). The reducing power of the extract and the fractions were in the order F1: (0.628) > CE (0.476) > F3 (0.473) > F2 (0.272).

The quantity of the total flavonoid contents of the plant extract and its fractions are shown in Figures 22 and 23. The methanol extract and its fractions (F1, F2 and F3), all contained

flavonoids in a concentration dependent manner. The varying concentrations of the methanol extract are 100, 200 ,300 ,400 and 500µg/ml while the corresponding concentrations of the total flavonoids content in quercetin equivalent are 0,195, 0.305, 0.365, 0.456 and 0.584µg/g (Fig 22). Similar concentrations of F1, F2 and F3 show the prescence of flavonoids in quercetin equivalent to be (0.216, 0.438, 0.552, 0.615, and 0.864µg/g), (0.028, 0.167, 0.260, 0.284, and 0.396µg/g) and (0.11, 0.350, 0.436, 0.582, and 0.643µg/g) respectively,
(Fig 23). F2 contained the least level of total flavonoids at its maximum concentration
(0.396µg/g) while the highest concentration of total flavonoids was obtained in F1 at 500µg/ml (0.864µg/g) quercetin equivalent (QE). The concentration and expression of the total flavonoids in the extract and its fractions are in the order: F1 > F3 > CE > F2.

The total phenolic contents of the methanol extract and its fractions (F1, F2 and F3) at different concentrations are shown in Figures 24 and 25 respectively. The methanol extract and its fractions, all contained phenolic compounds in a concentration dependent manner. The varying concentrations of the methanol extracts are 100, 200, 300, 400 and 500µg/ml while the corresponding concentration of the phenolic contents in gallic acid equivalent are 0.05, 0.128, 0.194, 0.260µg/mg and 0.356µg/mg respectively. Similarly, the same concentrations of F1, F2 and F3 showed the phenolic contents in gallic acid equivalent to be (0.09, 0.124, 0.210, 0.351 and 0.460µg/mg), (0.012, 0.030, 0.050, 0.140 and 0.240µg/mg) and (0.04, 0.082, 0.104, 0.220 and 0.380µg/mg), respectively (Figure 25). F2 contained the least phenolic compounds at its maxmium concentration while the highest concentration of total phenolic compounds was obtained in F1 at 500µg/ml (0.460µg/mg). The concentration and expression of the phenolic compounds in the methanol extract and the fractions are in the order: F1 > F3 > F2 > CE.

Conculsion

The results of these experiments indicated that the methanol extract and its fractions possess antioxidant properties as evident by their DPPH radical scavenging activity, hydroxyl radical scavenging activity and their reductive potential compared to ascorbic acid, a standard antioxidant, these activities are mostly expressed in F1. From the result obtained, it was observed that the methanol extract and its fractions contained flavonoids and phenolic compounds in a concentration-dependent manner with F1 having the highest flavonoids and phenolic contents.

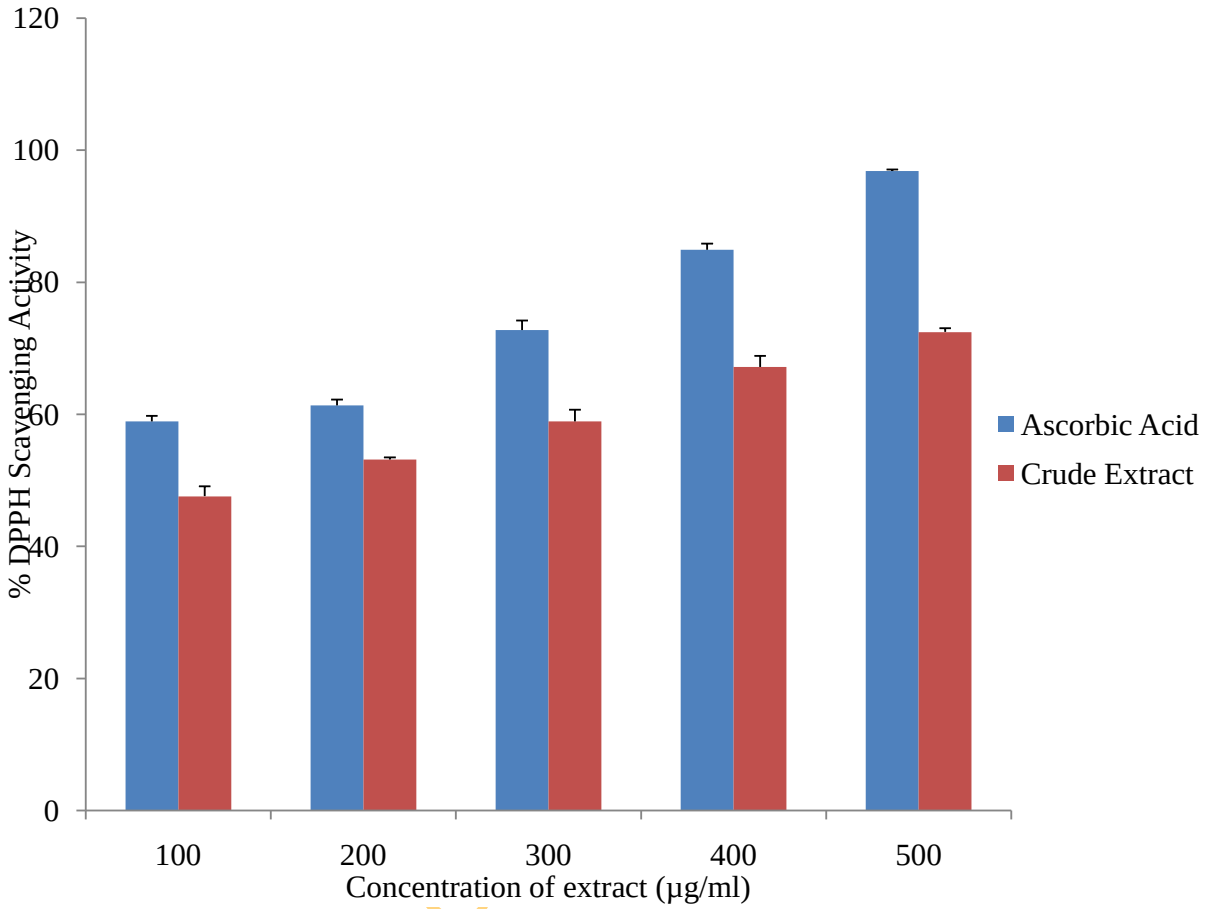


Figure 16: DPPH scavenging activity of the crude methanol extract of the bulb of *Crinum jagus*. (Ascorbic acid vs.Crude extract : $p < 0.05$)

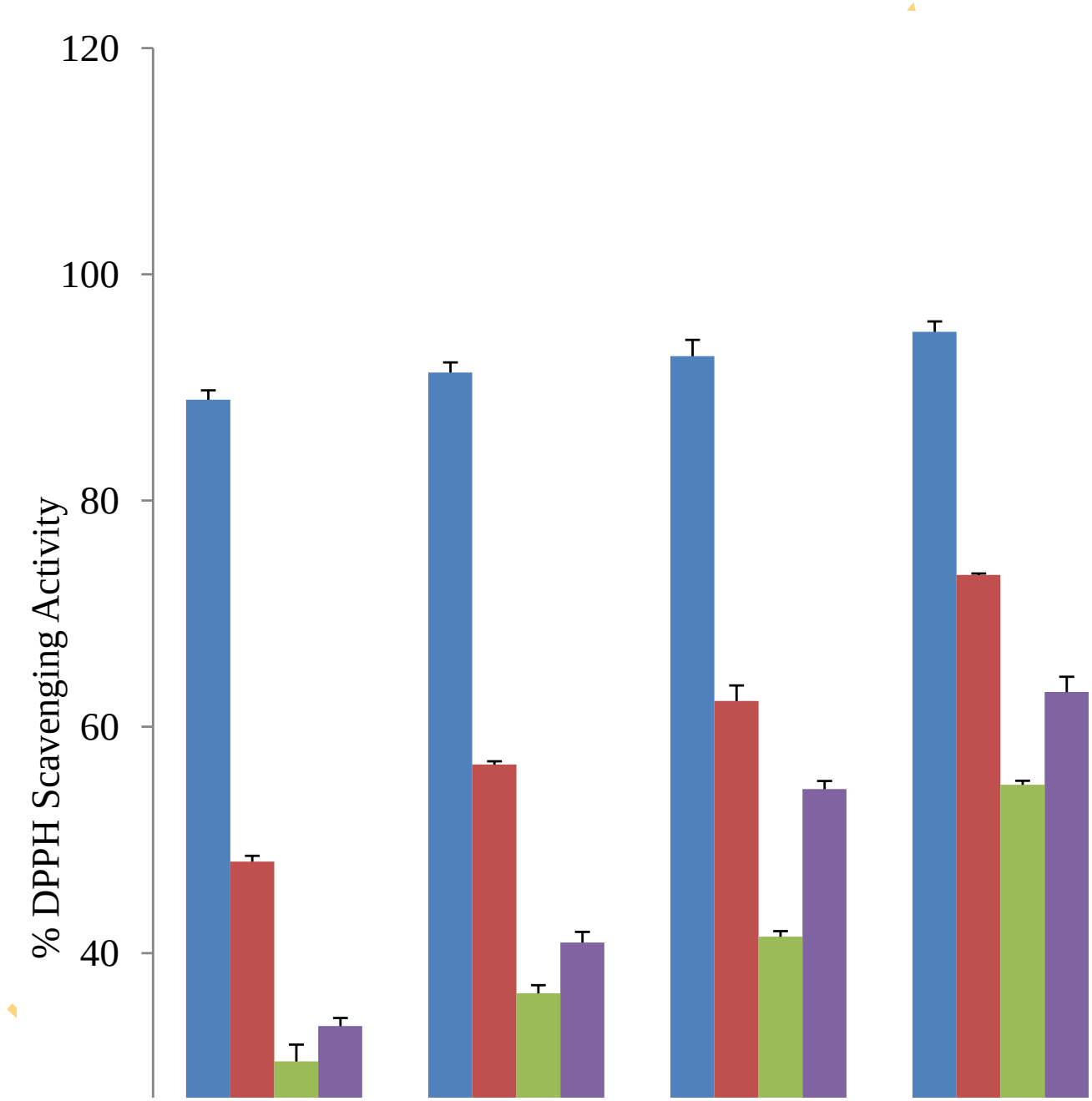


Figure 17: DPPH scavenging activities of the fractions (F) of the bulb of *Crinum jagus*.

(Ascorbic acid vs.Fractions 1,2, & 3 : $p < 0.05$)

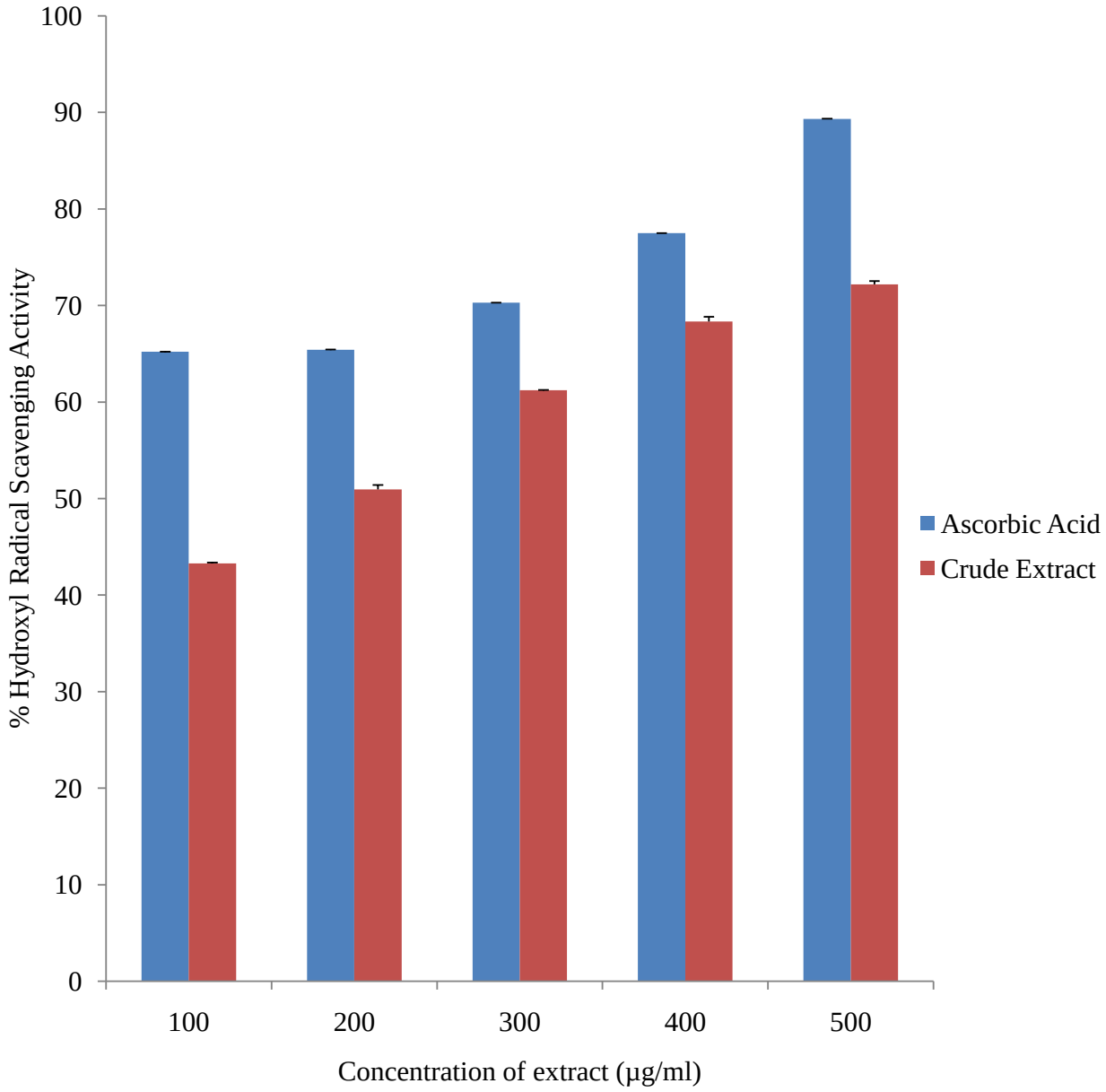


Figure 18: Hydroxyl radical scavenging activity of the crude methanol extract of the bulb of *Crinum jagus*.

(Ascorbic acid vs.Crude extract : $p < 0.05$)

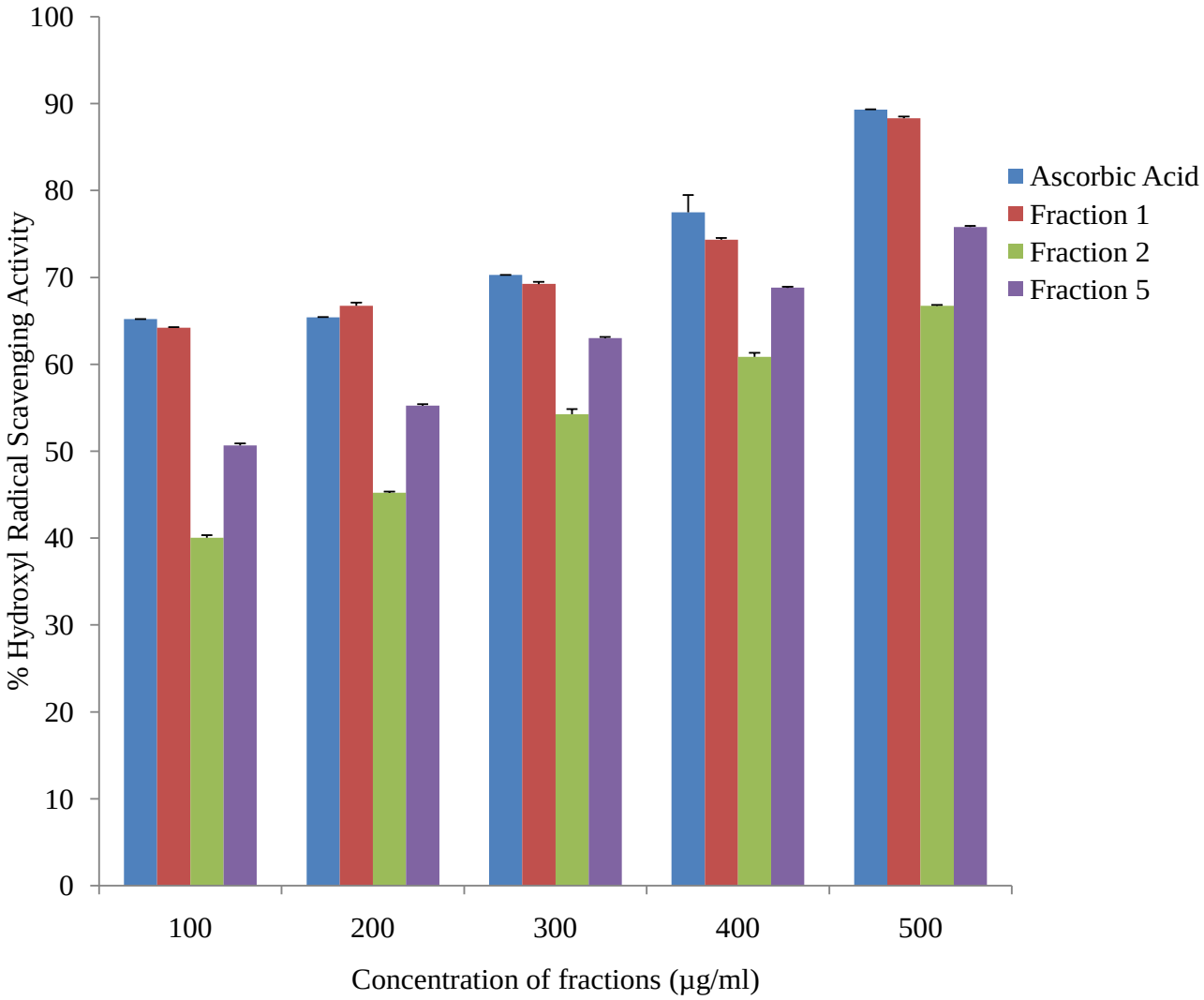


Figure 19 : Hydroxyl radical scavenging activities of the fractions (F) of the bulb of *Crinum jagus*.

(Ascorbic acid vs. Fraction 1,2, & 3 : $p < 0.05$)

(Ascorbic acid vs. Fraction 1 : $p > 0.05$)

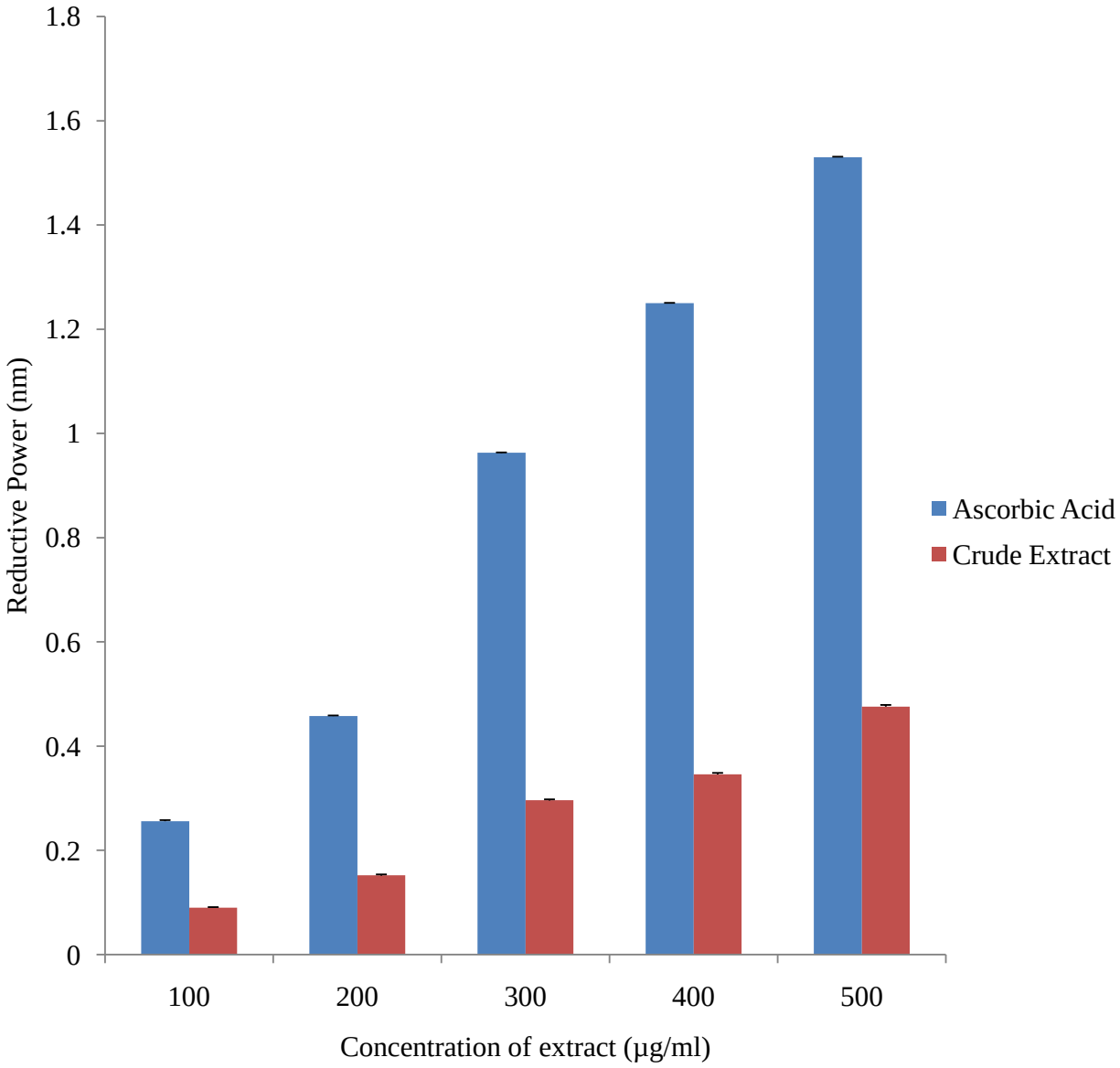


Figure 20: Reductive potential of the crude methanol extract of the bulb of *Crinum jagus*.

(Ascorbic acid vs.Crude extract : $p < 0.05$)

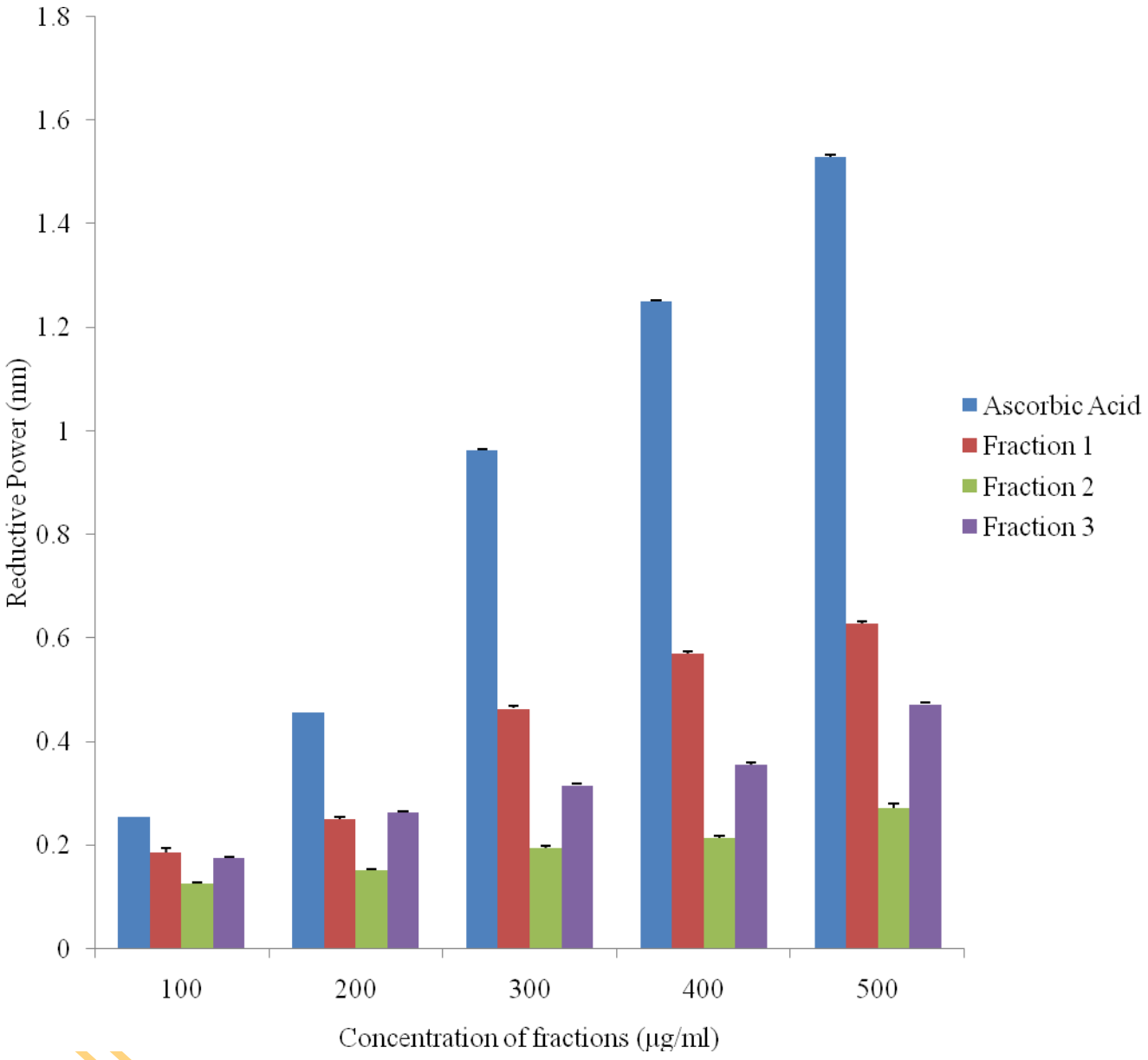


Figure 21: Reductive potentials of the fractions (F) of the bulb of *Crinum jagus.*

(Ascorbic acid vs.Fractions 1,2,& 3 : $p < 0.05$)

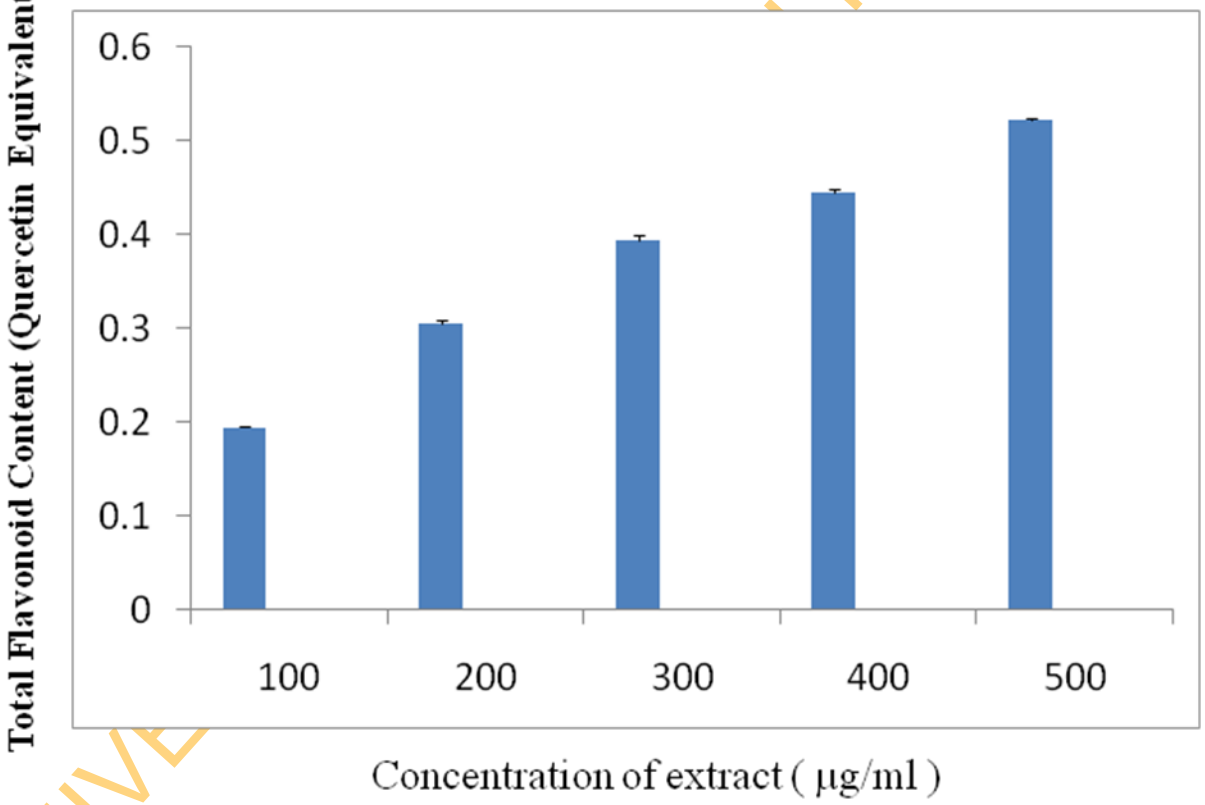


Figure 22: Total Flavonoid Content of the Crude Methanol Extract of the Bulb of *Crinum jagus*

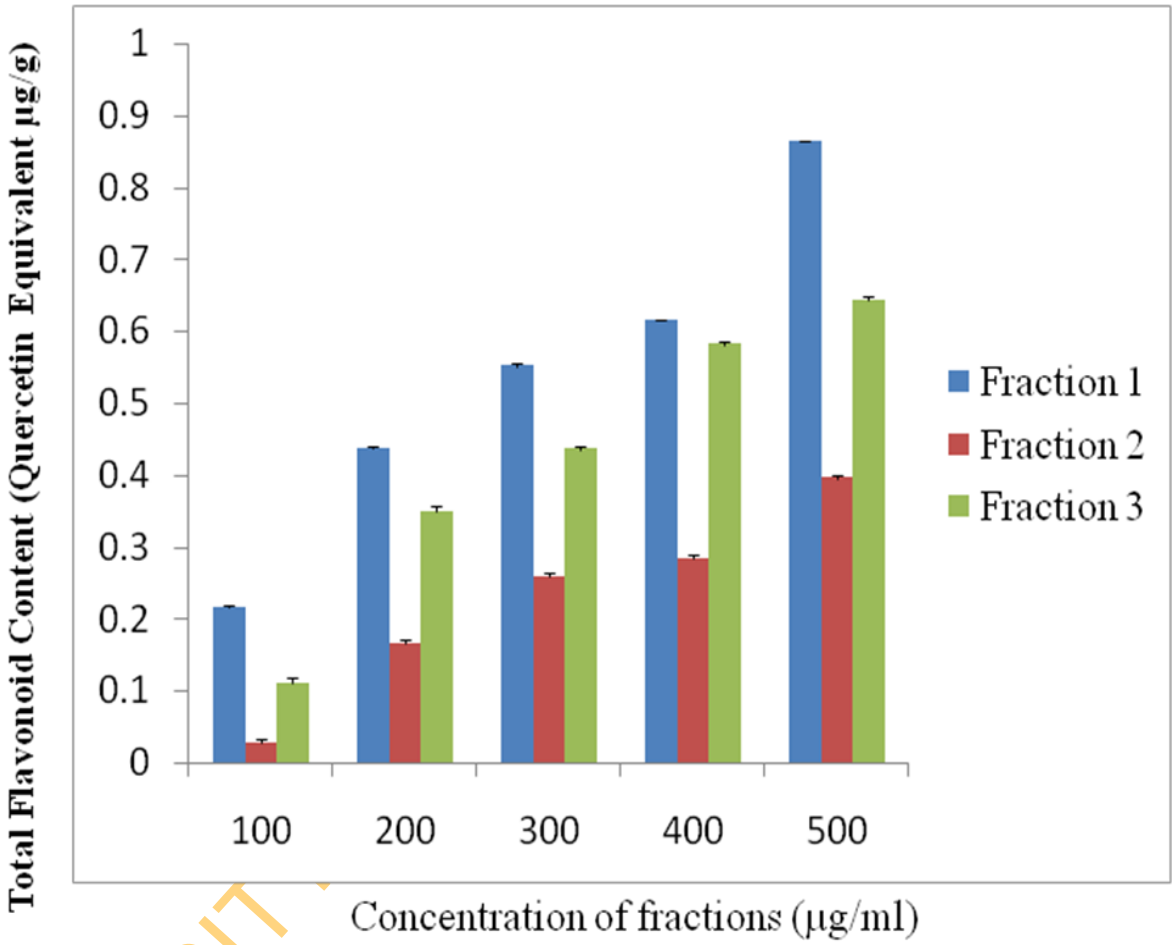


Figure 23: Total Flavonoid Content of Fractions of the Bulb of *Crinum jagus*

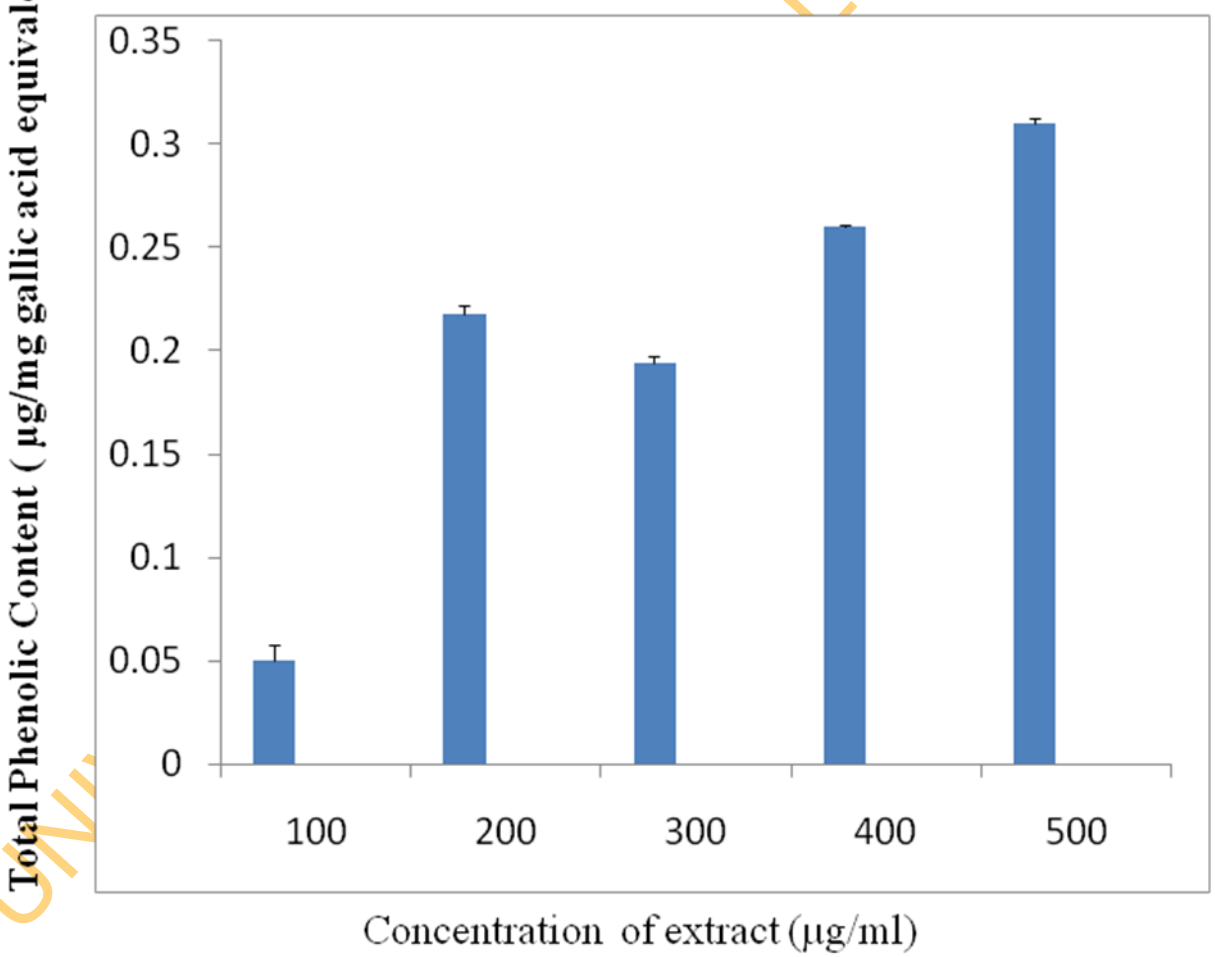


Figure 24 : Total phenolic content of the crude methanol extract of the bulb of *Crinum jagus*

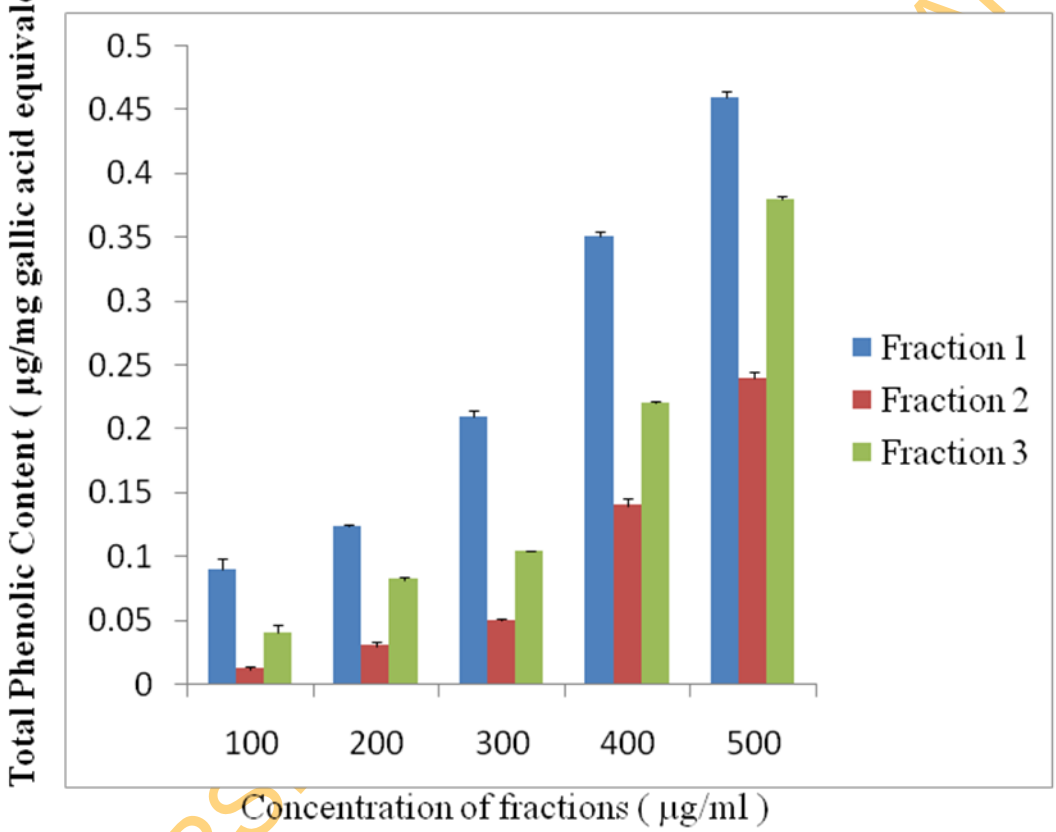


Figure 25: Total Phenolic Content of Fractions of the Bulb of *Crinum jagus*

Experiment 4 : *In vivo* Antioxidant Study on the Crude Extract and Fractions of the Bulb of *Crinum jagus*

Introduction

Antioxidants are molecules that can slow down or prevent the oxidation of other molecules. They can greatly reduce the damage due to oxidants by neutralising the free readicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohdyrates and DNA (Fang, 2002). Antioxidants can be classified into two major classes: enzymatic and non enzymatic. The enzymatic antioxidants are produced endogenously, some of which include superoxide dismutase, catalase and glutathione peroxidases. Crucial components of the antioxidant defense system in the body are cellular antioxidant enzymes: superoxide dismutase (SOD), catalase, glutathione peroxidases. which are involved in the reduction of reactive oxygen species (ROS) and peroxides produced in living organisms and thus playing a primary role in the maintenance of a balanced redox status (Michiels *et al*., 1994), hence they can serve as potential marker of susceptibility, early and reversible tissue damage and of decrease in antioxidant defense (Lester, 1994). Natural antioxidants from plant extracts provide a measure of production that slows the process of oxidative damage hence in this study the *in-vivo* antioxidant activity of the crude methanol extract and the fractions of the bulb of *Crinum jagus* was investigated.

Procedure

Thirty male Wistar strain albino rats were divided into five groups of six animals per group. Group A received 0.2ml of corn oil and served as the control. Groups B, C, D and E received 10, 25, 50 and 75 mg/kg of the crude extract separately and respectively for 30 days. For the fractions, thirty five male rats were divided into seven groups of five animals each. Group A received 0.2ml corn oil and served as the control. Groups B and C received F1 at the doses of 5 and 10mg/kg, Groups D and E received F2 at the doses of 5 and 10mg/kg while groups F and G received F3 at the doses of 5 and 10mg/kg respectively for 30 days. All administrations were done orally. Twenty four hours after the last treatment, the animals were sacrificed by cervical dislocation, the liver and kidney samples were removed, washed in ice cold 1.15% KCl, dried

and weighed. The samples were homogenized in 4 volumes of phosphate buffer (0.1M, pH 7.4). The resulting homogenate were centrifuged at 10,000g for 20 minutes to obtain the post-mithochondrial supernatant used for biochemical assays.

Protein concentration of liver and kidney homogenates were determined using Biuret method of Gornal *et al.*, (1949) as earlier described in section 3.8 under "Materials and Methods". Lipid peroxidation assay was determined by the method of Vashney and Kale (1990), superoxide was assayed based on the method of Misra and Fridovich, (1972), catalase was assayed by the method of Sinha (1971), reduced glutathione concentration was determined using the procedure of Jollow *et al.*, (1974) and glutathione-S -transferase assay was carried out using the procedure of Habig *et al.*, (1974) as decribed in section 3.8 under "Materials and Methods".

Results

The results of the effect of crude methanol extract of the bulb of *Crinum jagus* on lipid peroxidation in rat liver and kidney is presented in Figure 26. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) caused reduction that is not significant in the malondialdehyde (MDA) level in the liver while a significant ($P < 0.05$) and dose dependent reduction of MDA level was observed in the kidney compared to the control group. In the kidney, the extract significantly inhibited lipid peroxidation by 46.80, 62.23, 85.63 and 93.62% respectively. The result showed that the three fractions (F1, F2 and F3) caused significant ($P < 0.05$) reduction in hepatic and renal MDA levels compared to that of the control group after 30 days of oral administration of the fractions (Fig 27 and 28). F1 at 5 and 10mg/kg significantly inhibited lipid peroxidation in the liver by 41.01% and 49.51% respectively. F2 at 5 and 10mg/kg significantly inhibited lipid peroxidation in the liver by 15.33% and 25.00% respectively while fraction F3 at 5 and 10mg/kg inhibited lipid peroxidation in the liver by 27.91% and 31.07% respectively. In the kidney, F1, F2 and F3 at 5 and 10mg/kg significantly inhibited lipid peroxidation by (63.25% and 67.45%), (11.17% and 19.17%) and (49.10% and 61.45%), respectively, compared with the control group. F1 showed the highest inhibitory activity.

The result of the effect of thirty days oral administration of the crude extract on hepatic and renal superoxide dismutase (SOD) activity is presented in Figure 29. SOD activity in the liver was significantly ($P < 0.05$) increased in 25, 50 and 75mg/kg dose by 20.72, 23.45 and 26,00% respectively. In the kidney SOD activity was observed to increase by 34.83, 55.43, 78.65 and 87.27% respectively in all the treated groups. Results from Figures 30 and 31 shows that F1 caused significant elevation in the activity of superoxide dismutase in the liver and kidney. The two doses of F1 administered (5 and 10mg/kg) elevated superoxide distmutase activity in the liver and kidney by 37.78 and 39.01% and 52 and 84.00% respectively. The increase in SOD activity caused by administration of 10mg/kg dose of F2 in the liver was not significant while in the kidney, admnistration of 10mg/kg dose elevated SOD activity by 38.00%. The two doses of F3 (5 and 10mg/kg) elevated SOD activity in the liver and kidney by (44.44 and 64.41%) and (32.00 and 52.00%), respectively.

Figure 32 shows the result of 30 days oral administration of the crude extract of *Crinum jagus* on catalase activities in the liver and kidney. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) significantly ($P < 0.05$) increased the activity of catalase in the liver by 30.77, 40,00 88.89 and 122.20% and kidney by 29.63, 31.65, 47.06 and 103.8% respectively, compared with the control group. F1 (5mg and 10mg/kg) significantly ($P < 0.05$) increased catalase activity in the liver by (34.88 and 44.00%) and kidney by (59.09 and 90.91%), respectively, compared with the control group. In F2, catalase activity was lower than that of the control group in both liver and kidney following administration of the 5 and 10mg/kg doses for 30 days. Non significant increase in the activity of catalase was observed in the liver and kidney of F3 treated animals, relative to control group (Figures 33 and 34).

Figure 35 indicates that administration of varying concentrations of the crude methanol extract of *Crinum jagus* (10, 25, 50 and 75mg/kg), for 30 days, resulted in the significant ($P < 0.05$) increase in the level of reduced glutathione (GSH) both in the liver and kidney compared with the control group. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) significantly increased the level of reduced glutathione in the liver and kidney by (15.88, 16.58, 26,53 and 57.24%) and (19.62, 36,60, 62.26 and 100%) respectively. Figures 36 and 37 shows that treatment of animals with 5 and 10mg/kg doses of F1 significantly

increase the hepatic and renal glutathione levels by (48.35 and 64.17%) and (46.99 and 61.93%) respectively. F2 produced insignificant increase of hepatic and renal glutathione levels when compared with the control group. F3 significantly elevated glutathione levels in the liver and kidney by 47.67 and 41. 59% respectively following administration of the 10mg/kg dose.

As shown in Figure 38, thirty days of oral administration of the crude methanol extract of the bulb of *Crinum jagus* significantly increased the activity of glutathione-S-transferase (GST) both in the liver and the kidney. The varying concentrations of the crude extract 10, 25, 50 and 75mg/kg significantly increased the activity of GST in the liver and kidney by (50.00, 65.00, 100 and 150%) and (21.05, 57.89, 100 and 136.84%), respectively. From figures 39 and 40 it was indicated that the liver and kidney activites of GST were significantly elevated in the groups treated with F1 (5 and 10mg/kg) relative to control by (56.52 and 86.96%) and (49. 33 and 73.33%), respectively. F2 produced a non significant increase in hepatic and renal GST activity. With F3, the 10mg/kg significantly increased GST activity by 69.56% in the liver while in the kidney, the 5 and 10mg/kg doses significantly elevated GST activity by 48.00 and 65.33%, respectively, relative to control. Fractionation of the crude extract does not lead to the loss of biological activity but the antioxidant activity of the plant were enhanced by fractionation. F1 demonstrated the highest antioxidant activity followed by F3, F2 had the least antioxidant activity.

Conclusion

The results of these experiments indicate that the crude methanol extract and fractions of the bulb of *Crinum jagus* possess *in vivo* antioxidant activity. Treatment of rats with the crude methanol extract (10, 25, 50 and 75mg/kg) and F1, F2 and F3 (5 and 10mg/kg) resulted in a dose-dependent and significant improvement in the antioxidant status as indicated by reduction in hepatic and renal lipid peroxidaiton and simulataneous elevation in hepatic and renal supeoxide dismutase, catalase, reduced glutathione and glutathione-S-transferase relative to control.

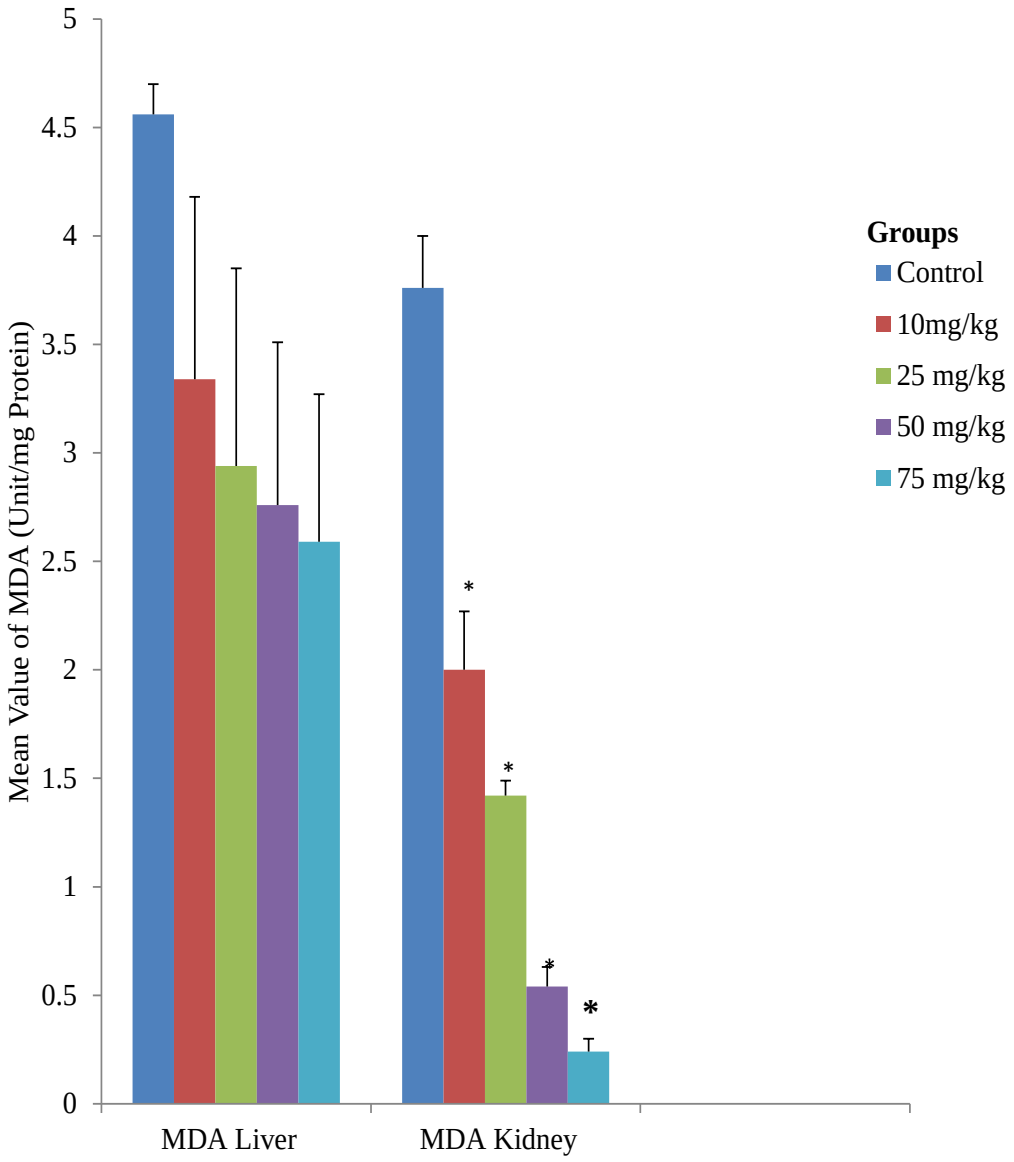


Figure 26: Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney malondialdehyde (MDA) levels in rats.

* = Significantly different from control

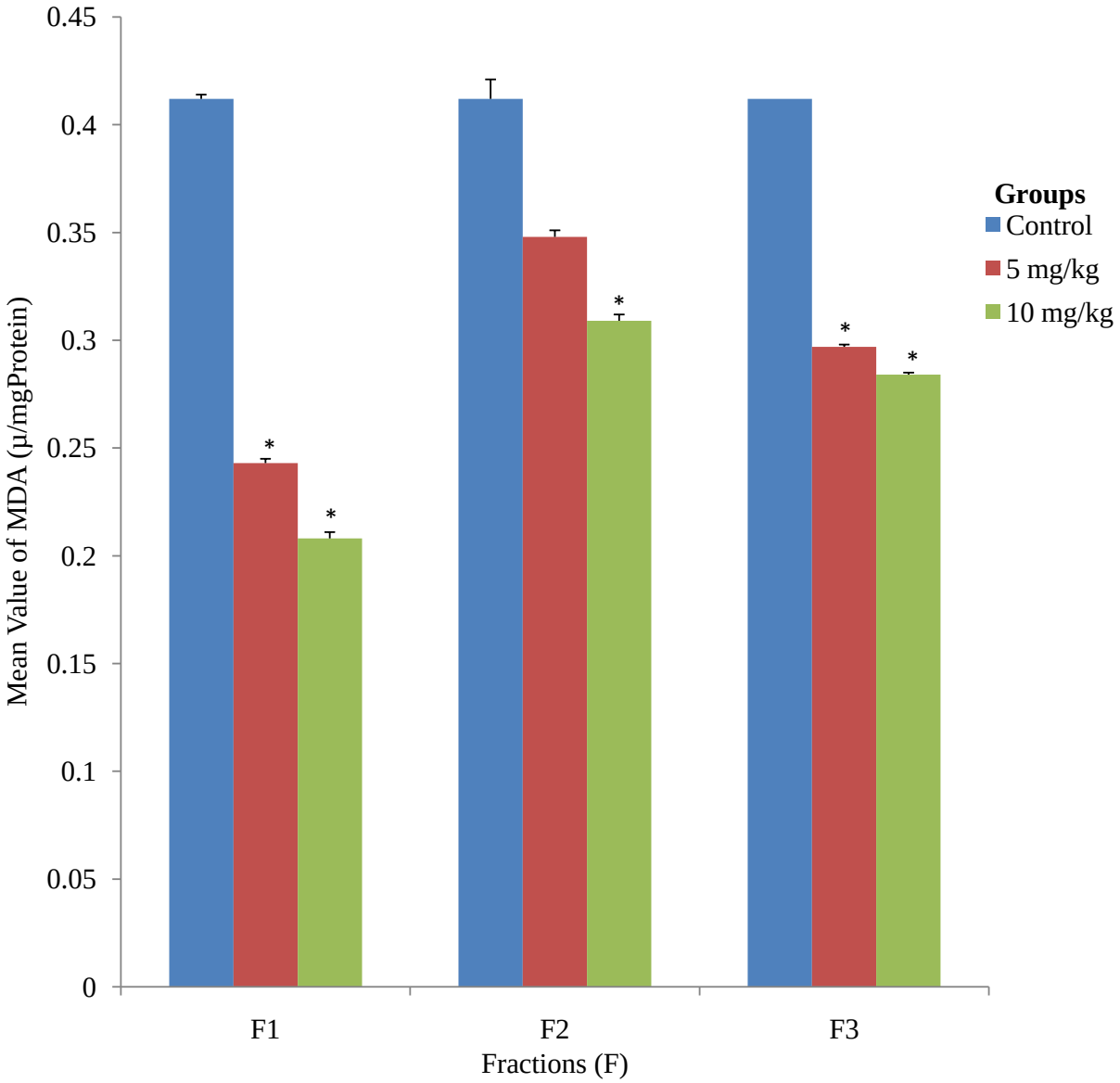


Figure 27: Effects of the fractions of the bulb of *Crinum jagus* on liver malondialdehyde (MDA) levels in rats.

* = Significantly different from control

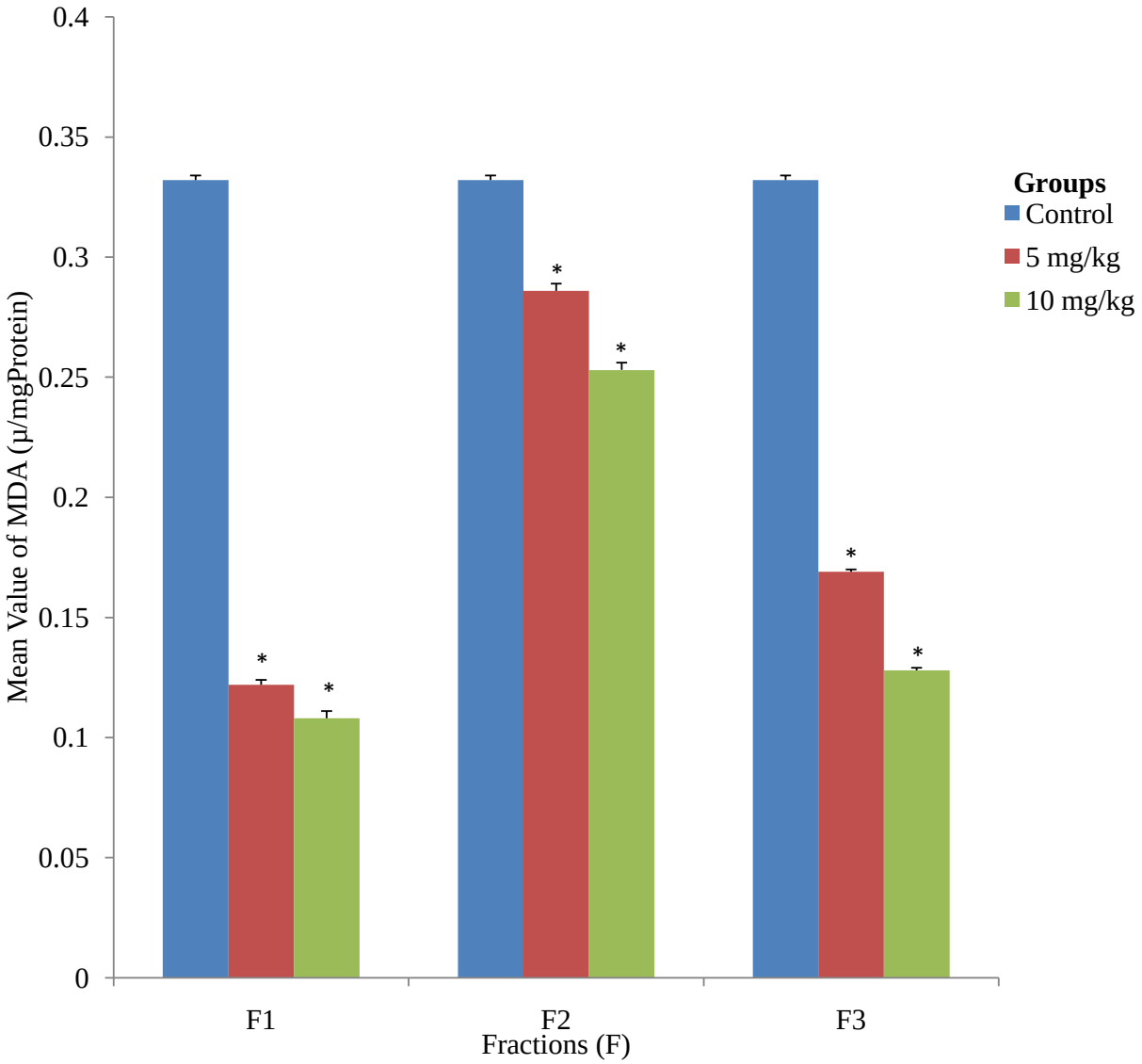


Figure 28: Effects of the fractions of the bulb of *Crinum jagus* on kidney malondialdehyde (MDA) levels in rats.

* = Significantly different from control

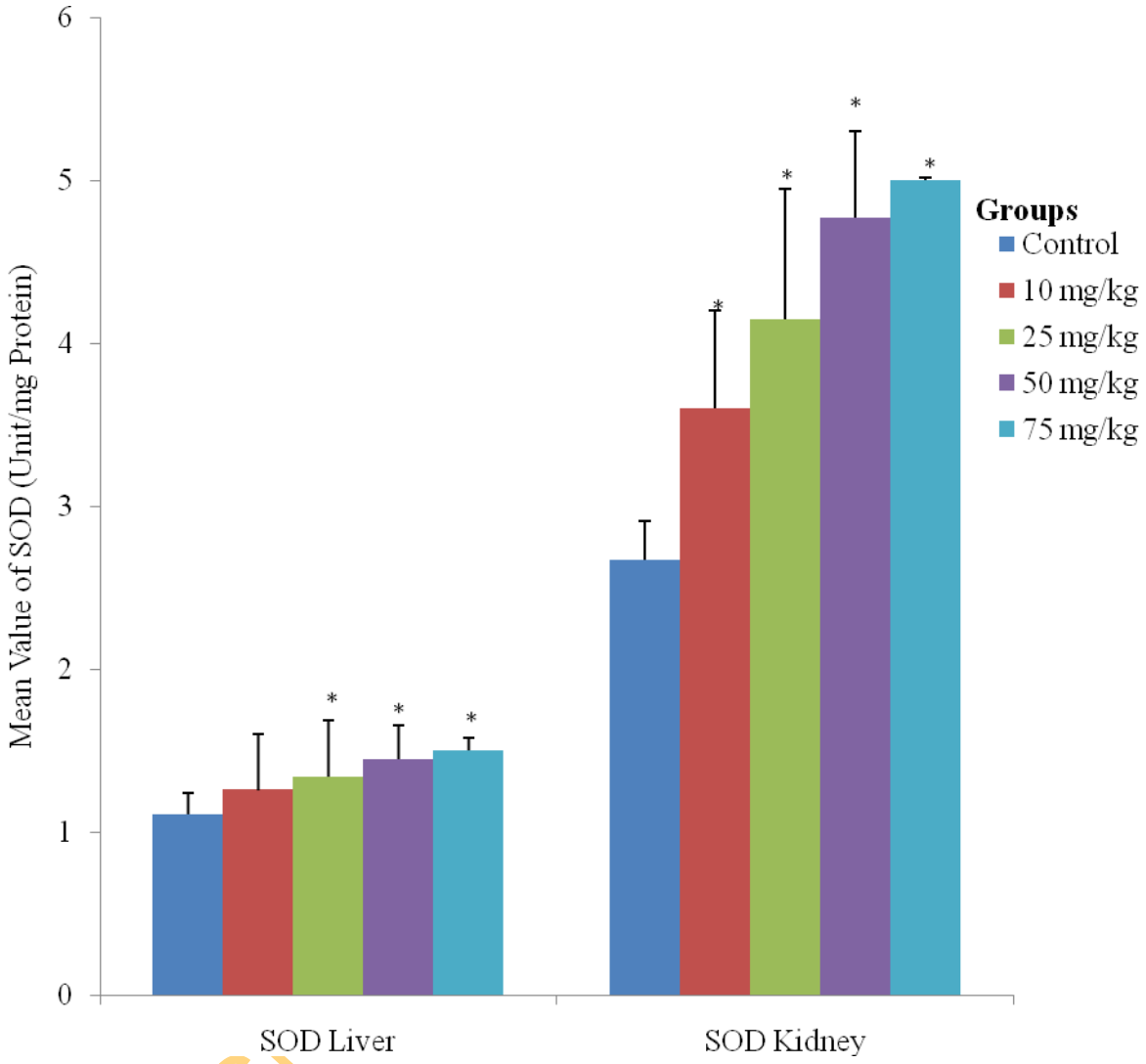


Figure 29: Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney superoxide dismutase (SOD) activities of rats.

* = Significantly different from control

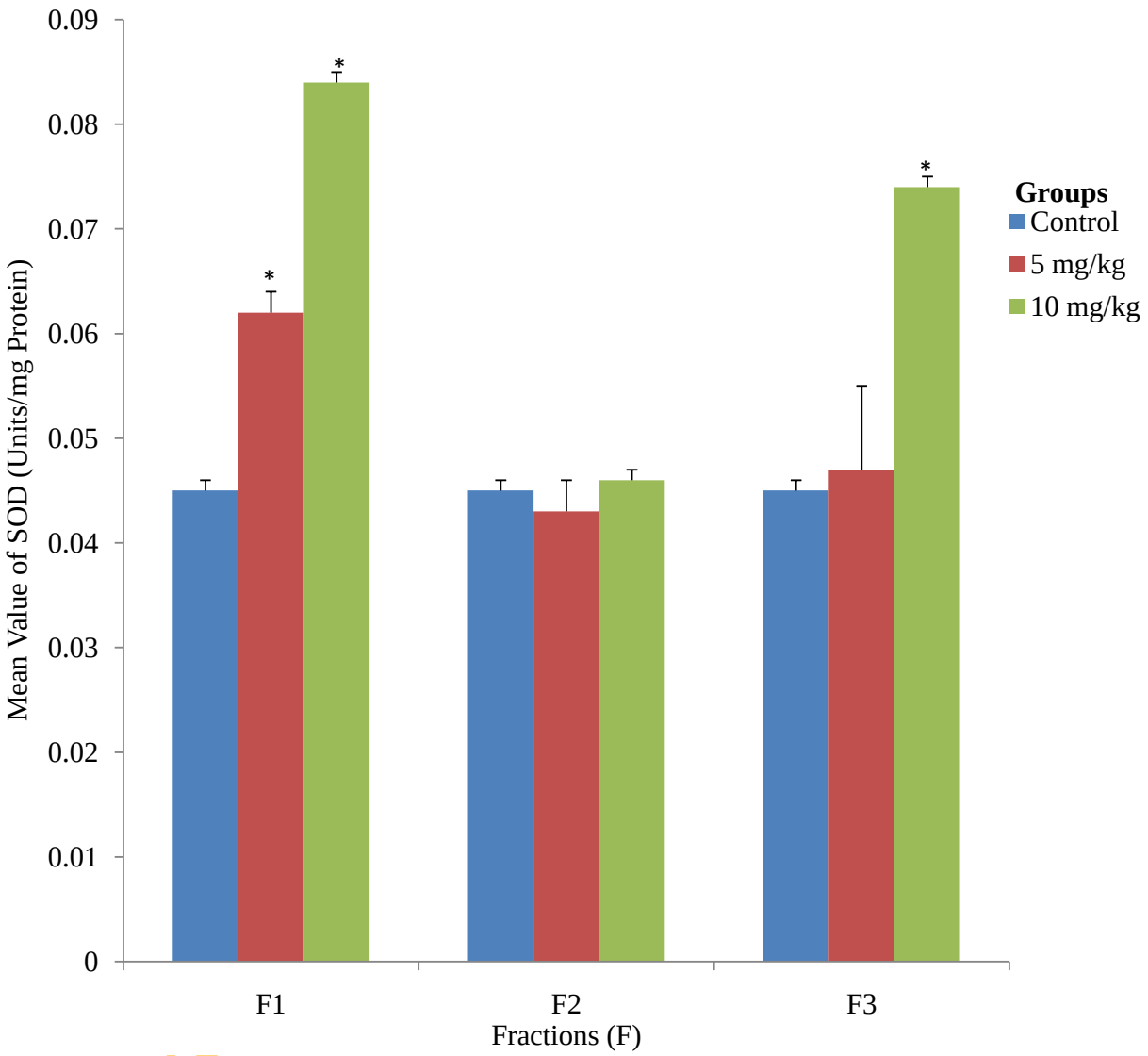


Figure 30: Effects of the fractions of the bulb of *Crinum jagus* on liver superoxide dismutase (SOD) activities of rats.

* = Significantly different from control

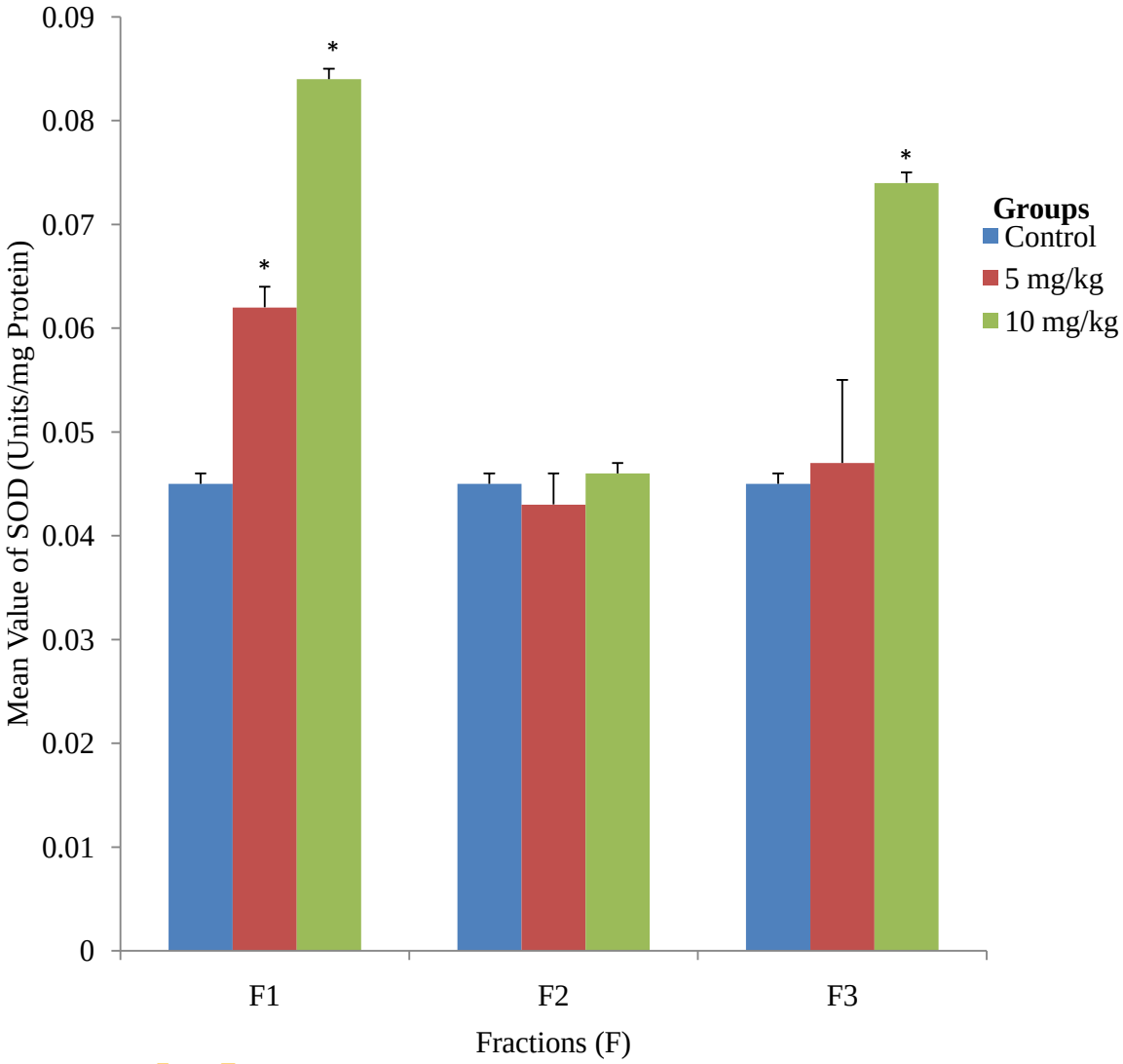


Figure 31: Effects of the fractions of the bulb of *Crinum jagus* on kidney superoxide dismutase (SOD) activities of rats.

* = Significantly different from control

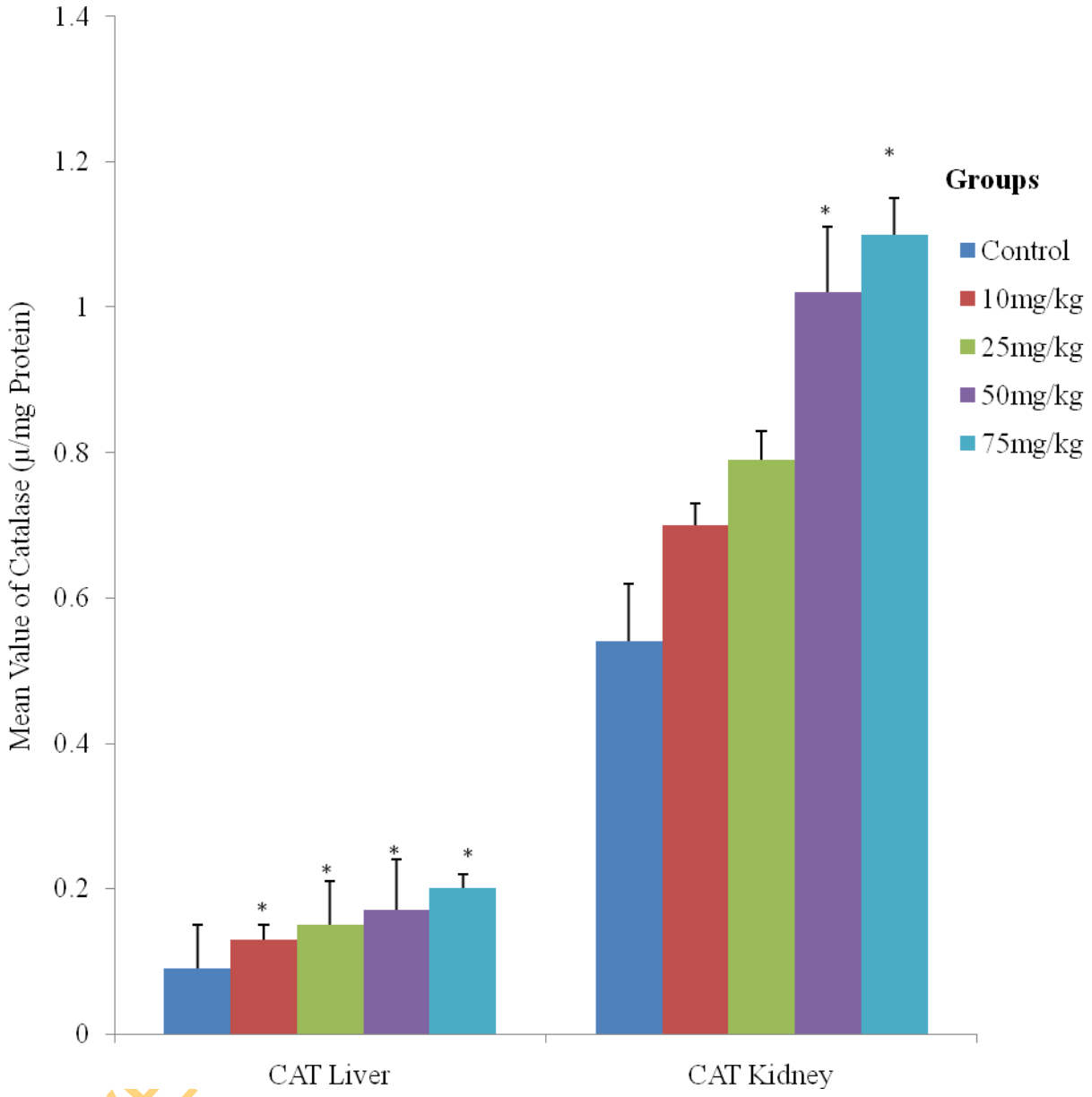


Figure 32: Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney catalase activities of rats.

* = Significantly different from control

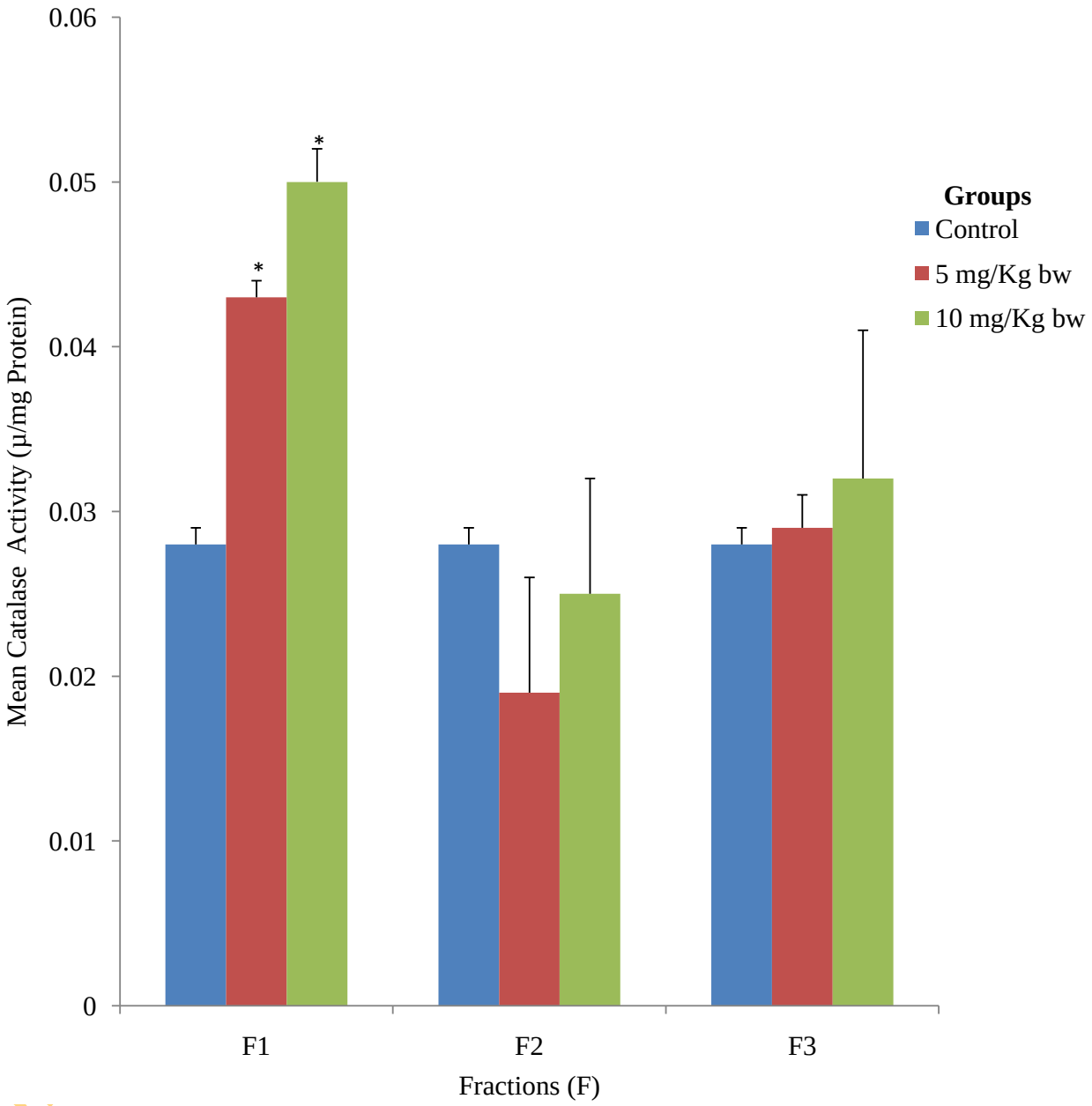


Figure 33: Effects of the fractions of the bulb of *Crinum jagus* on liver catalase activities of rats.

* = Significantly different from control

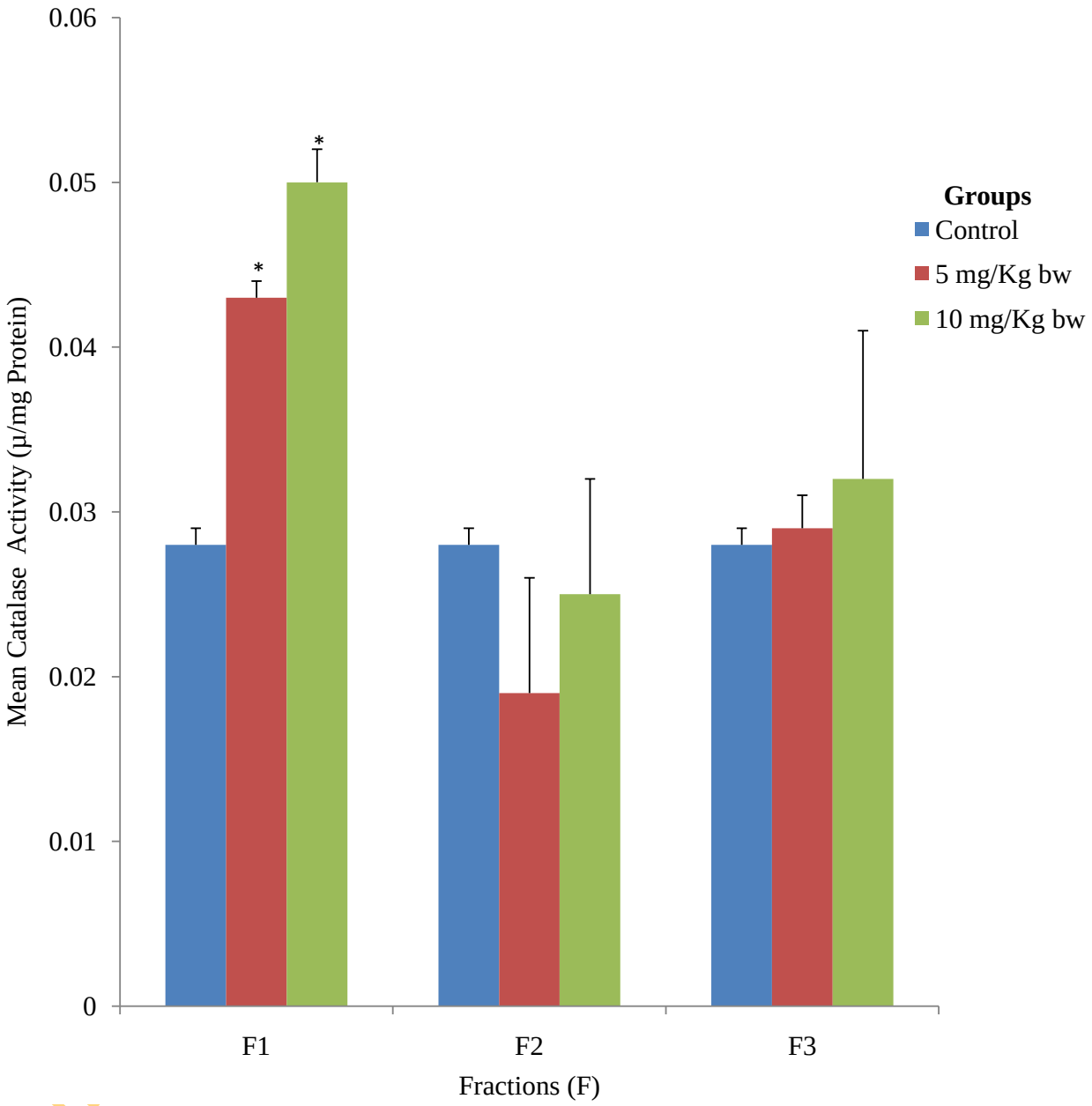


Figure 34: Effects of the fractions of the bulb of *Crinum jagus* on kidney catalase activities of rats.

* = Significantly different from control

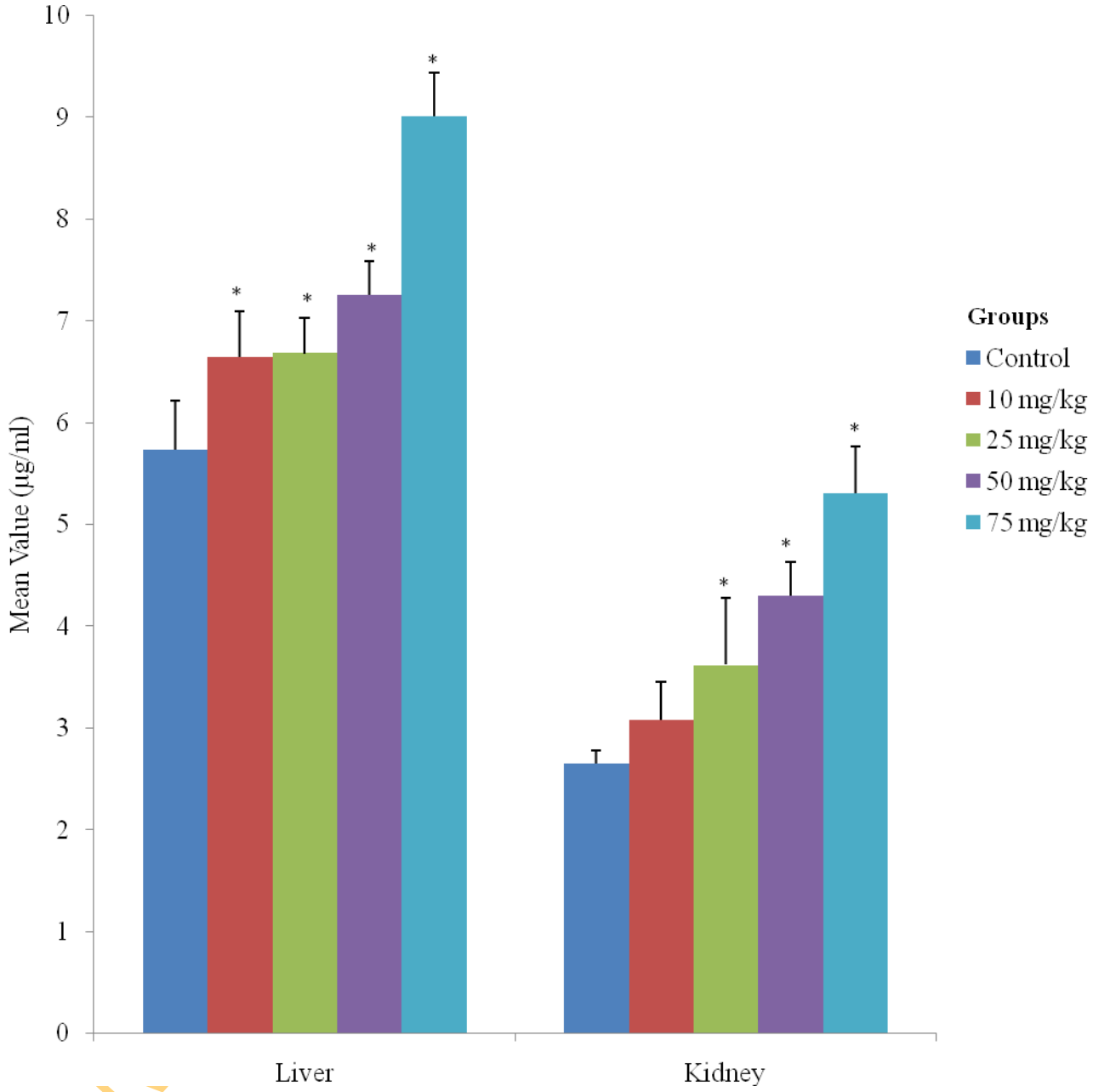


Figure 35: Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney reduced glutathione activities of rats.

* = Significantly different from control

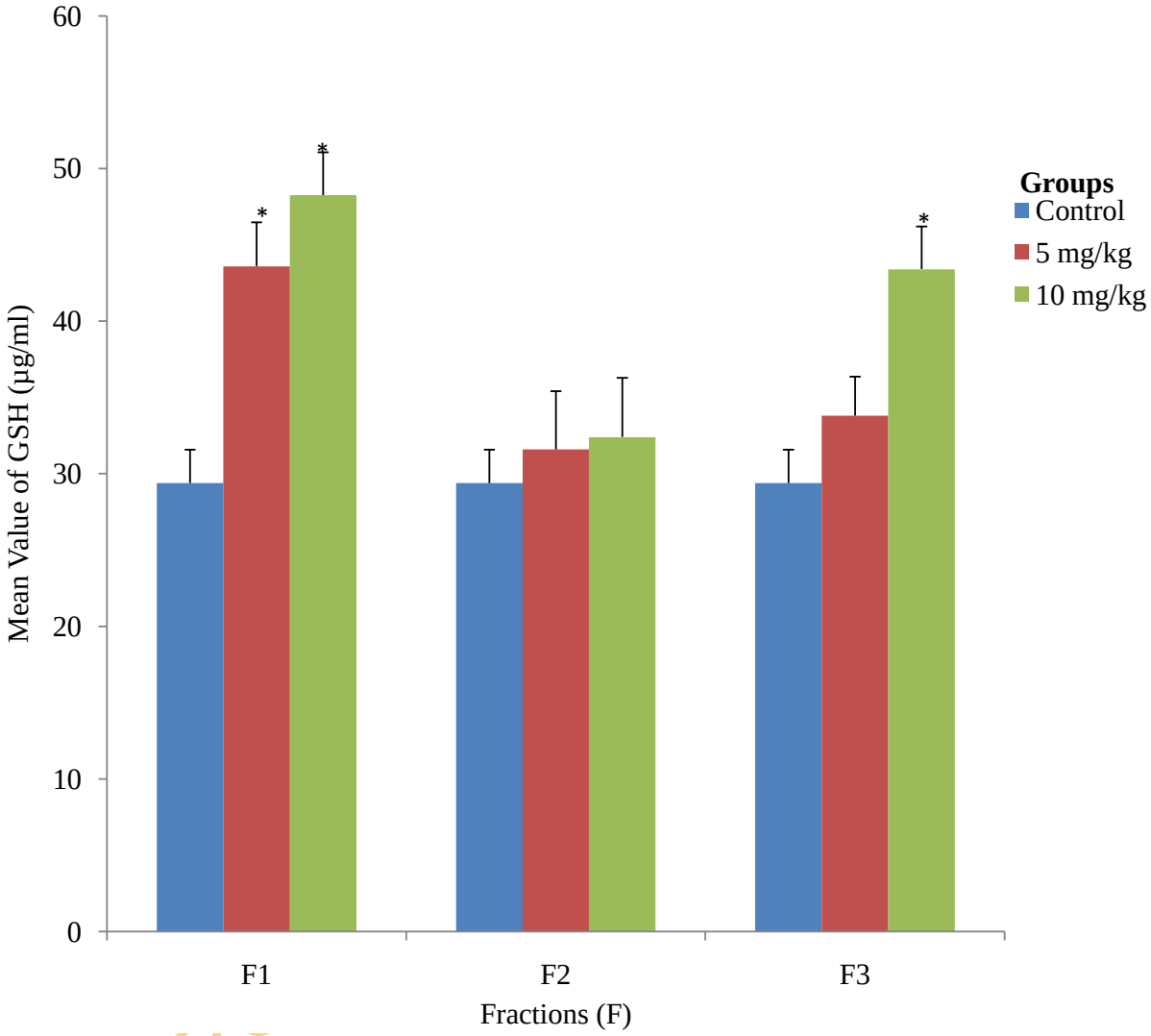


Figure 36: Effects of the fractions of the bulb of *Crinum jagus* on liver reduced glutathione (GSH) activities of rats.

* = Significantly different from control

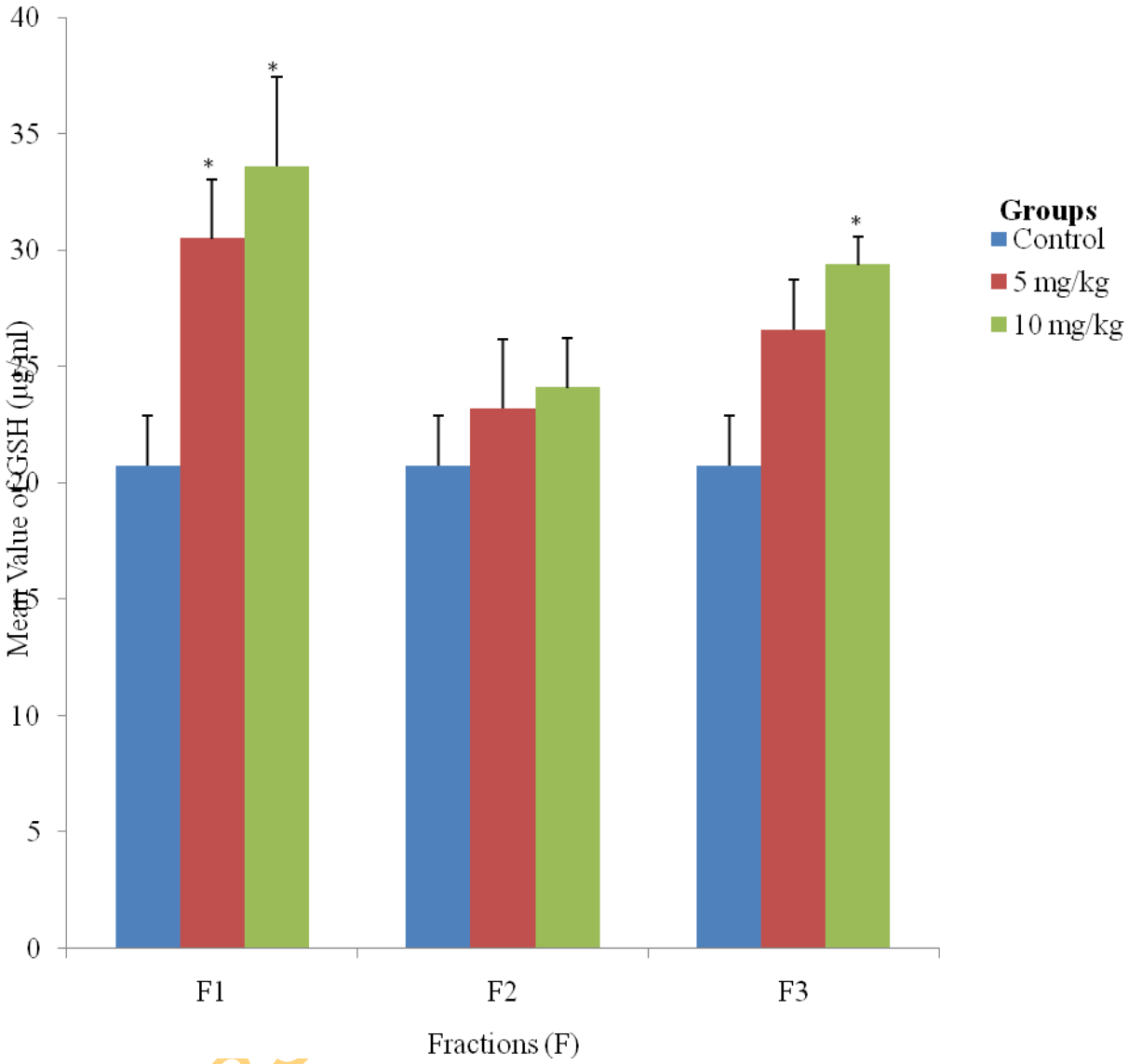


Fig 37 : Effects of the fractions of the bulb of *Crinum jagus* on kidney reduced glutathione (GSH) activities of rats.

* = Significantly different from control

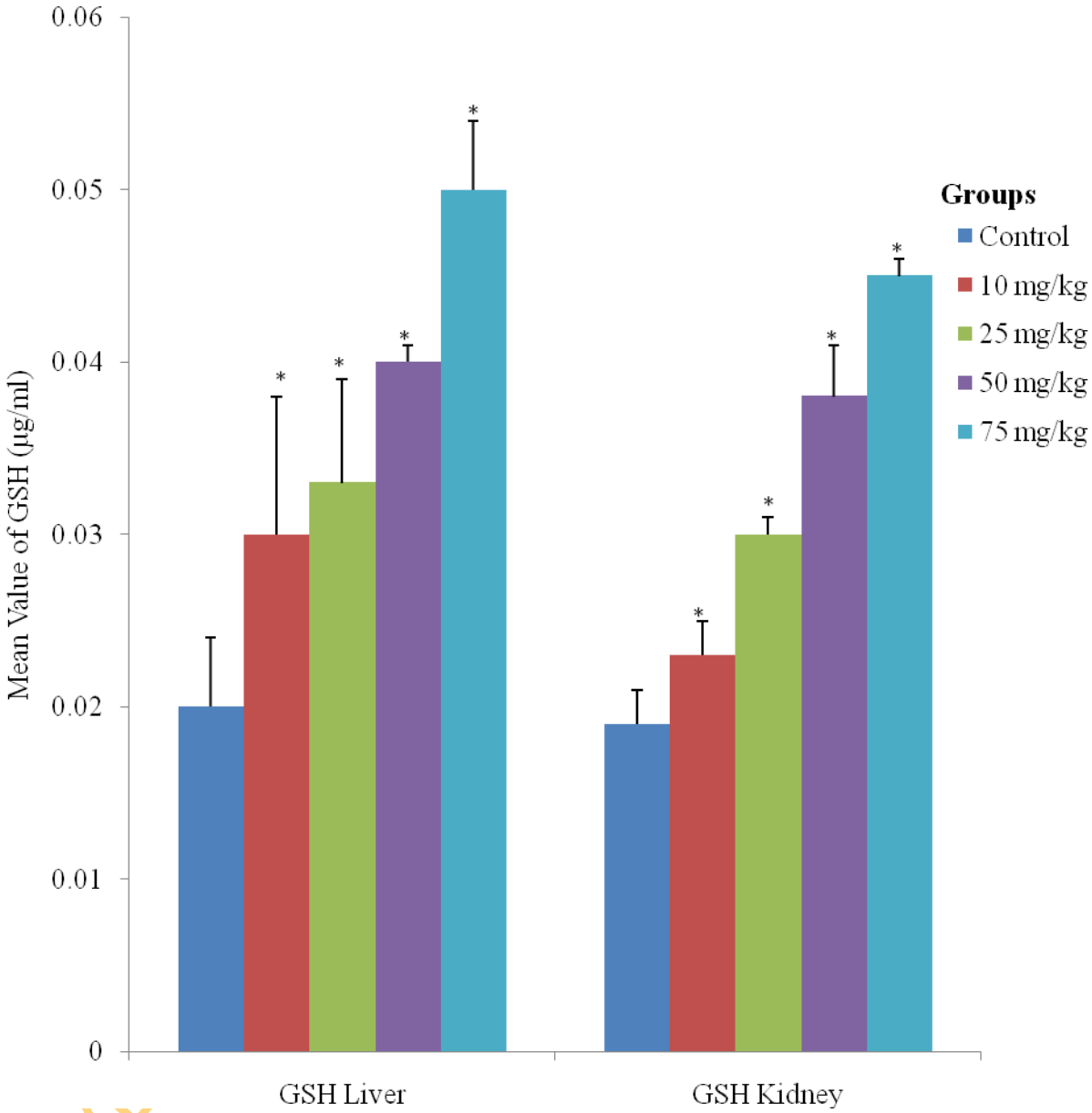


Figure 38 : Effect of crude methanol extract of the bulb of *Crinum jagus* on liver and kidney glutathione – S – transferase (GST) activities of rats.

* = Significantly different from control

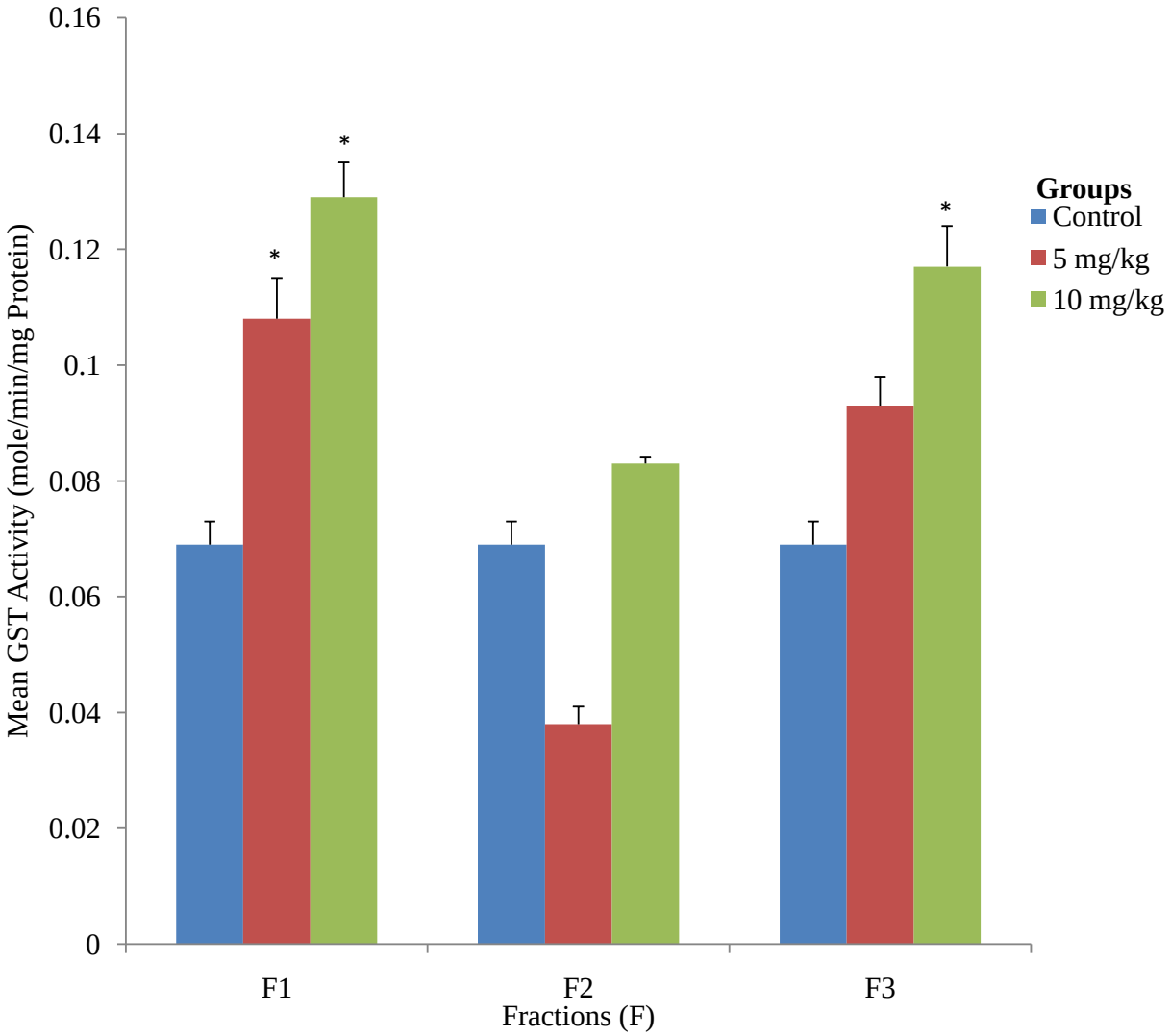


Figure 39: Effects of the fractions of the bulb of *Crinum jagus* on liver glutathione – S – transferase (GST) activities of rats.

* = Significantly different from control

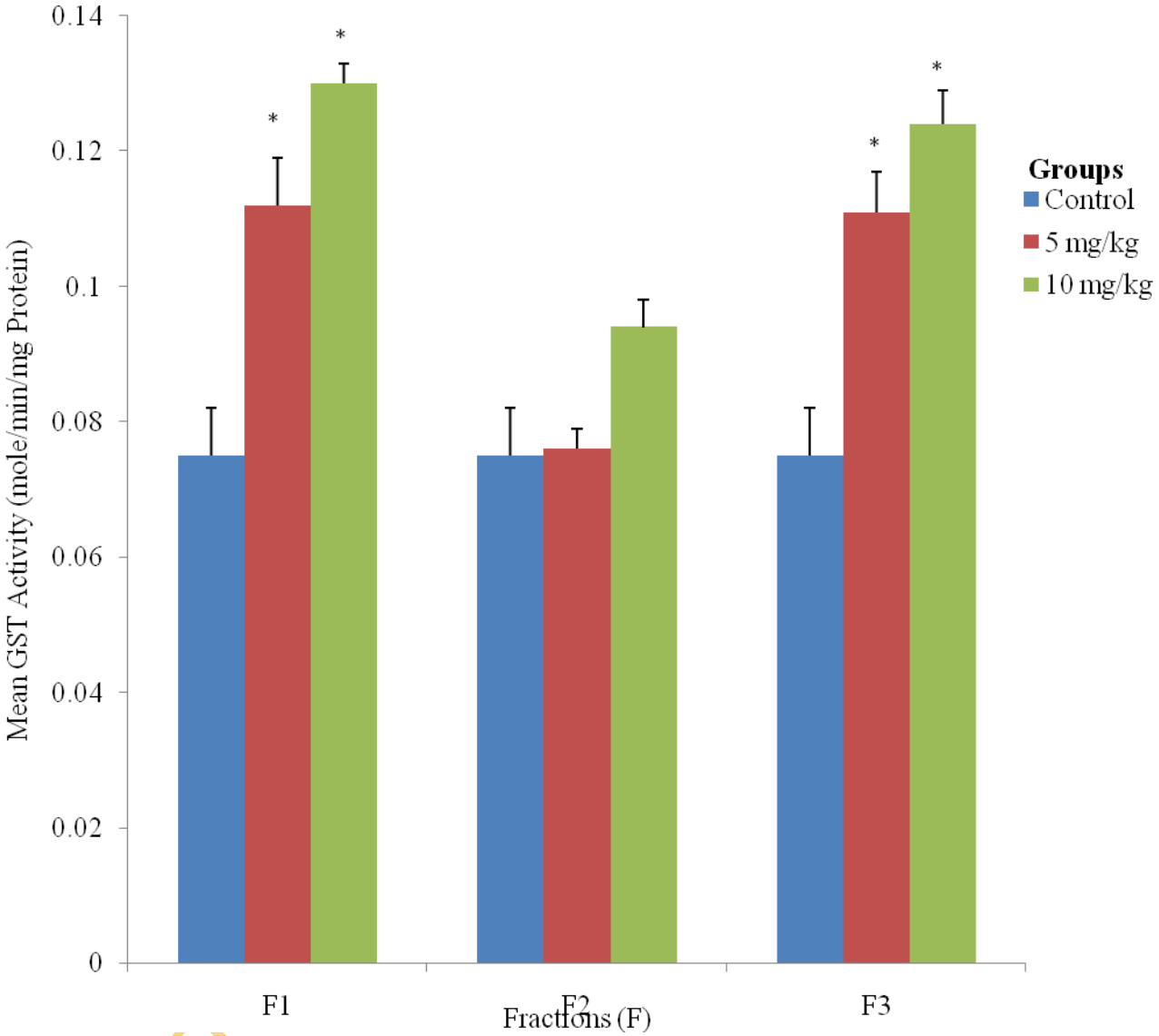


Figure 40: Effects of the fractions of the bulb of *Crinum jagus* on kidney glutathione – S – transferase activities (GST) of rats.

* = Significantly different from control

Experiment 5 : Determination of Antibacterial and Antifungal Activities of the Crude Methanol Extract and Fractions of the Bulb of *Crinum jagus*

Introduction

The expanding bacterial and fungal resistance to antibiotics has become a growing concern, worldwide (Gradam, 2000). Increasing bacterial resistance is prompting a resurgence in research of the antimicrobial role of herbs against resistance strains (Hermaiswarya *et al*.,2008; Alvanio and Alvanio 2009)). A vast number of medicinal plants have been recognised as valuable resources of natural antimicrobial compounds (Mahady, 2005). Plant derived products contain a great diversity of phytochemicals such as phenolic acids, flavonoids, alkaloids, tannins and other small compounds (Cowan, 1999). These compounds possess numerous health related effects such as antibacterial, antifungal, anticarcinogenic activities (Bedlack *et al.*, 2000). With advancement of mordern medicinal technology, it is now easier to identify specific botanical constituents and assess their potential antimicrobial activity hence in this study, the antibacterial and antifungal activities of crude methanol extract and fractions (F1, F2 and F3) of the bulb of *Crinum jagus* against several human pathogens were investigated**.**

Procedure

The antimicrobial activities of the extract/fractions were determined by measuring the inhibition zone using the agar diffusion method (Vollekova *et al*., 2001; Usman *et al*., 2005). Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the extract/fractions were also determined as described in section 3.9 under 'Materials and Methods.'

Results

Table 11 presents the result of the antimicrobial screening of the crude methanol extract of the bulb of *Crinum jagus.* The crude extract exhibited considerable level of inhibition against all the test organisms even at low concentration with the exception of *E.coli*. *Klebsiella pneumoniae* was the most susceptible bacterium of all the tested bacteria with inhibition zones ranging from 10.00 ± 0.20 to 28.00± 0.10 while *Aspergillus niger* was the most susceptible fungus with inhibition zones ranging from 10.00 ± 0.10 to 16.00 ± 0.40. The extract had a good antimicrobial activity on the *Bacillus subtilis* and *Staphylococcus aureus*, the two Gram-

positive bacteria tested. Three out of the four Gram-postive bacteria tested *Pseudomonas aeurigunosa*, *Salmonella typhi* and *Klebsiella pnenumonae* also showed appreciable antimicrobial activity. *E coli* was not inhibited by the extract with no zone of inhibition (Table 11). This is in consonance with the frequent reported cases of developed multi drug resistant to many antibiotics available in the market of which *E.coli* is the most prominent (Alonso *et al.*, 2000; Sader *et al*., 2002; Oyegade and Fasuan, 2004). The difference in the sensitivity between Gram-positive and Gram-negative bacteria could be ascribed to the difference in morphological constitution of these organisms (Nastro *et al*., 2000; Hodges, 2002). The fungal moulds *Aspergillus niger* and *Aspergillus flavos* were much sensitive to the extract than *Candida albicans*, *Candida tropicalis* and *Penicillium notatum.* The inhibition by the plant extract was less than the inhibitory action of ampicillin and tiaconazole the standard antibiotics.

Results of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the extract are presented in Table 12. The crude methanol extract demonstrated good antimicrobial activity with its MIC against Gram-positive bacteria ranging between 6.25 to 50mg/ml and that of Gram-negative bacteria ranged between 3.125 to 50mg/ml. MIC for the fungal isolates ranged between 25 to 200mg/ml. The extract showed the lowest MIC against *Klebsiella pneumonae* (3.125mg/ml). The broadest activity of the crude extract against most of the test bacteria was 12.5mg/ml as MIC. *Candida albicans* and *Candida tropicalis* had the lowest MIC value (25mg/ml) against the extract. The broadest activity of the extract against most fungi tested was MIC of 50mg/ml.

The extract also showed bactericidal and fungicidal activities on the bacteria and fungi isolates. *Klebsiella pneumonae* was the most susceptible bacteria to the plant extract, at a small concentration of 12.5mg/ml, the extract was able to kill the organism. The broadest bactericidal activity of the extract against most of the test bacteria was 25mg/ml as MBC. The extract also showed fungicidal effect on the fungi tested with *Candida albicans* and *Candida tropicalis* being the most susceptible with 50mg/ml as minimum fungicidal concentration (MFC). The broadest activity of the extract against most of the fungi tested was observed at 100mg/ml as MFC.

Tables 13, 14 and 15 represent the antimicrobial activities of each of the fractions of the bulb of *Crinum jagus* with respect of each test organism. The fractions inhibited the growth of all the test organisms, including *E. coli*, which was not inhibited by the crude extract of the plant at concentrations lower than that of the crude extract. The results of MIC determination showed that F1 is the most potent of all the three fractions. F1 had good antimicrobial activity on all the bacteria and fungi isolates tested. The MIC, MBC and MFC values of F1 for bacteria and fungi isolates were much lower than that of F2 and F3. The MIC value of F1 ranged between 0.20 to 3.125µg/ml for bacteria isolates and 0.39 to 3.125µg/ml for the fungi isolates. *Staphylococcus aureus* (a Gram-positive bacteria) was the most susceptible to F1 at MIC value of 0.20µg/ml. The yeasts: *Candida albicans*, *Candida tropicalis* and *Candida krusei* were much sensitive to F1 than the moulds with MIC values of 0.39µg/ml. *E. coli* which was not inhibited by the crude methanol extract of the plant was inhibited by F1 at MIC value of 0.39µg/ml. F1 also showed bactericidal and fungicidal activity on the bacteria and fungi isolates tested. The MBC and MFC values of F1 for bacteria and fungi were much lower than that of F2 (Table 16). F2 had a moderate antibacterial and antifungal activities against most of the test organisms. The lowest MIC value of F2 was 0.39µg/ml against two of the bacteria isolates *Staphylococcus aureus* and *Bacillus subtilis* and the lowest MIC value of 0.78µg/ml were observed against four of the fungi isolates *Candida albicans*, *Candida tropicalis*, *Candida krusei* and *Penicillum notatum*. *Staphylococcus aureus* and *Bacillus subtilis* were the most susceptible bacteria to F2, at a concentration of 0.39µg/ml as MIC which was higher than the MIC value of F1 against *Staphylococcus aureus*. Also the MBC values of F2 for the bacteria and fungi isolates were much higher than that of F1 (Table 17). F3 had a moderate antibacterial and poor antifungal activity. The lowest MIC value obtained for F3 was 0.39µg/ml against *Bacillus subtilis* and *E. coli*, for the fungi isolates, the lowest MIC value obtained was 1.56µg/ml which was much higher than the MIC values of F1 and F2. F3 also had higher MBC values of 0.78µg/ml for the bacteria isolates and MFC value of 1.56µg/ml for the fungal isolates (Table 18) hence F3 will have significant antimicrobial activity against bacteria and fungi at higher concentrations.

Conclusion

From this study, the crude methanol extract of the bulb of *Crinum jagus* demonstrated a broad-sepectrum of activity against Gram-postive and Gram-negative bacteria and fungi tested. Fractionation of the crude extract did not lead to loss of antimicrobial activity against the same group of isolates. The fractions were found to have a better and improved antimicrobial activity than the crude extract against the range of Gram-positive bacteria, Gram-negative bacteria, the fungal yeast and the moulds tested. F1 demonstrated the highest antimicrobial activity.

Table 11 : Antimicrobial activity of the crude methanol extract of the bulb of *Crinum jagus*.

Conc (mg/ml)	*Stap. aureus*	*Bacill. Subtilis*	*Esch. Coli*	*Pseud. aeuriginosa*	*Salm. typhi*	*Kleb. Pneumonae*	*Cand. albicans*	*Cand. Tropicalis*	*Cand. krusei*	*Asp. niger*	*Asp. flavos*	*Penic. Notatum*
200	18.00 ± 0.32	24.00 ± 0.01	-	20.00 ± 0.02	20.00 ± 0.20	20.00 ± 0.10	14.00 ± 0.30	12.00 ± 0.30	12.00± 0.10	16.00 ± 0.40	14.00± 0.30	12.00± 0.01
100	16.00 ± 0.20	20.00 ± 0.20	-	12.00 ± 0.40	16.00 ± 0.10	18.00 ± 0.41	10.00 ± 0.40	10.00 ± 0.20	10.00± 0.20	12.0± 0.20	12.00± 0.01	10.00± 0.10
50	14.00 ± 0.10	18.00 ± 0.06	-	10.00 ± 0.42	14.00± 0.10	16.00 ± 0.60	-	-	-	10.00 ± 0.10	10.00± 0.10	-
25	12.00 ± 0.01	16.00 ± 0.10	-	-	12.00± 0.30	14.00 ± 0.30	-	-	-	-	-	-
12.5	10.00 ± 0.12	14.00 ± 0.20	-	-	-	12.00 ± 0.22	-	-	-	-	-	-
6.25	6.00 ± 0.01	0.80 ± 0.02	-	-	-	10.00 ± 0.20	-	-	-	-	-	-
Ampicillin (10mg/ml)	50.00 ± 0.42	28.00 ± 0.10	36.00 ± 0.10	34.00 ± 0.30	24.00 ± 0.20	36.00 ± 0.20	-	-	-	-	-	-
Tiaconazole (10% w/v)	-	-	-	-	-	-	24.00 ± 0.10	20.00 ± 0.10	22.00± 0.10	24.00 ± 0.10	20.00± 0.10	22.00 ± 0.20
Methanol	-	-	-	-	-	-	-	-	-	-	-	-

Values represent diameter of zone of inhibition (mm)

- means no inhibition

Table 12 : Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentrations (MFC) of the crude methanol extract of the bulb of C*rinum jagus*

Microorganism	Type	MIC (mg/ml)	MBC/MFC (mg/ml)
Staphylococcus aureus	Bacteria (G+)	6.25	25.00
Bacillus subtilis	Bacteria (G+)	6.25	25.00
Escherichia coli	Bacteria (G-)	–	–
Pseudomonas aeruginosa	Bacteria (G-)	50.00	100
Salmonellae typhi	Bacteria (G-)	12.50	25.00
Kelbsiella pneumonae	Bacteria (G-)	3.125	12.50
Candida albicans	Yeast	25.00	50.00
Candida tropicals	Yeast	25.00	50.00
Candida krusei	Mould	50.00	200
Penicillum Notatum	Mould	50.00	200
Aspergillus niger	Mould	50.00	200
Aspergillus flavos	Mould	100	200

MIC = Minimum Inhibitory Concentration , MBC = Minimum Bactericidal Concentration , MFC = Minimum Fungicidal Concentration, G+ = Gram-positive, G - = Gram-negative.

- means no inhibition

Table 13 : Antimicrobial activity of fraction 1 (F1) of the bulb of *Crinum jagus*

Conc (µg/ml)	*Stap. Aureus*	*Bacill. subtilis*	*Esch. Coli*	*Pseud. Aeuruginosa*	*Salm. typhi*	*Kleb. pneumonae*	*Cand. albicans*	*Cand. tropicalis*	*Cand. Krusei*	*Asp Niger*	*Asp. flavos*	*Penic. Notatum*
25	20.00 ± 0.42	24.00 ± 0.32	18.00 ±0.01	20.00 ± 0.02	24.00 ± 0.30	18.00 ± 0.36	18.00 ± 0.36	16.00 ± 0.31	16.00± 0.10	16.00 ± 0.20	20.00± 0.31	14.00± 0.30
12.5	18.00 ± 0.25	18.00 ± 0.25	14.000 .20	12.00 ± 0.40	18.00 ± 0.20	16.00 ± 0.21	16.00 ± 0.43	14.00 ± 0.20	14.00± 0.30	12.00± 0.20	14.00± 0.20	12.00± 0.10
6.25	16.00 ± 0.20	12.00 ± 0.10	12.00 ± 0.12	10.00 ± 0.42	16.00 ± 0.40	14.00 ± 0.30	14.00 ±0.26	12.00 ± 0.10	12.00 ± 0.20	10.00 ± 0.10	12.00± 0.49	10.00± 0.42
3.125	14.00 ± 0.12	12.00 ± 0.41	10.00 ± 0.02	-	12.00 ± 0.10	12.00 ± 0.15	12.00±0. 31	10.00 ± 0.35	10.00± 0.24	-	10.00± 0.01	-
1.56	12.00 ± 0.11	10.00 ± 0.20	-	-	-	-	10.00 ± 0.01	-	-	-	-	-
0.78	10.00 ± 0.50	-	-	-	-	-	-	-	-	-	-	-
Ampicillin (10µg/ml)	28.00 ± 0.10	26.00 ± 0.10	28.00 ± 0.10	24.00 ± 0.20	30.00 ± 0.20	22.00 ± 0.25	-	-	-	-	-	-
Tiaconazole (10% w/v)	-	-	-	-	-	-	26.00 ± 0.10	24.00 ± 0.10	26.00± 0.20	24.00 ± 0.20	24.00± 0.15	26.00 ± 0.30
Methanol	-	-	-	-	-	-	-	-	-	-	-	-

Values represent diameter of zone of inhibition (mm)

- means no inhibition

Table 14 : Antimicrobial activity of fraction 2 (F2) of the bulb of C*rinum jagus*

Conc (µg/ml)	*Stap. Aureus*	*Bacill. subtilis*	*Esch. coli*	*Pseud. aeuriginosa*	*Salm. typhi*	*Kleb. pneumonae*	*Cand. albicans*	*Cand. tropicalis*	*Cand. Krusei*	*Asp niger*	*Asp. flavos*	*Penic. Notatum*
25	24.00 ± 0.02	24.00 ± 0.40	16.00 ±0.24	16.00 ± 0.40	16.00 ± 0.32	22.00 ± 0.36	18.00 ± 0.41	16.00 ± 0.20	18.00± 0.32	18.00 ± 0.30	16.00± 0.50	18.00± 0.20
12.5	18.00 ± 0.10	18.00 ± 0.18	12.000 .30	14.00 ± 0.50	12.00 ± 0.20	16.00 ± 0.31	14.00 ± 0.10	12.00 ± 0.10	14.00± 0.20	10.00± 0.10	12.00± 0.44	14.00± 0.13
6.25	14.00 ± 0.25	14.00 ± 0.15	10.00 ± 0.18	12.00 ± 0.30	10.00 ± 0.10	14.00 ± 0.24	12.00 ±0.26	12.00 ± 0.10	12.00 ± 0.40	-	10.00± 0.35	10.00± 0.25
3.125	12.00 ± 0.20	12.00 ± 0.20	-	10.00 ± 0.10	-	12.00 ± 0.15	10.00±0.36	10.00 ± 0.01	10.00± 0.10	-	-	-
1.56	10.00 ± 0.10	10.00 ± 0.30	-	-	-	-	-	-	-	-	-	-
0.78	10.00 ± 0.50	-	-	-	-	-	-	-	-	-	-	-
Ampicillin (10µg/ml)	28.00 ± 0.10	26.00 ± 0.10	28.00 ± 0.10	24.00 ± 0.20	30.00 ± 0.20	22.00 ± 0.25	-	-	-	-	-	-
Tiaconazole (10% w/v)	-	-	-	-	-	-	26.00 ± 0.10	24.00 ± 0.10	26.00± 0.20	24.00 ± 0.20	24.00± 0.15	26.00 ± 0.30
Methanol	-	-	-	-	-	-	-	-	-	-	-	-

Values represent diameter of zone of inhibition (mm)

- means no inhibition

Table 15 : Antimicrobial activity of fraction 3 (F3) of the bulb of *Crinum jagus*

Conc (µg/ml)	*Stap. Aureus*	*Bacill. subtilis*	*Esch. coli*	*Pseud. aeuriginosa*	*Salm. typhi*	*Kleb. pneumonae*	*Cand. albicans*	*Cand. tropicalis*	*Cand. Krusei*	*Asp niger*	*Asp. flavos*	*Penic. Notatum*
25	20.00 ± 0.02	18.00 ± 0.30	18.00 ±0.10	14.00 ± 0.25	16.00± 0.32	18.00 ± 0.10	18.00 ± 0.30	16.00 ± 0.10	14.00± 0.30	16.00 ± 0.25	14.00± 0.30	14.00± 0.20
12.5	14.00 ± 0.12	16.00 ± 0.20	16.000 .20	12.00 ± 0.20	14.00 ± 0.28	14.00 ± 0.15	14.00 ± 0.25	14.00 ± 0.15	12.00± 0.25	12.00± 0.20	14.00± 0.20	12.00± 0.16
6.25	12.00 ± 0.11	14.00 ± 0.22	14.00 ± 0.25	10.00 ± 0.20	12.00 ± 0.20	12.00 ± 0.20	12.00 ±0.10	12.00 ± 0.10	10.00 ± 0.20	10.00 ± 0.15	10.00± 0.20	10.00± 0.15
3.125	10.00 ± 0.15	12.00 ± 0.20	12.00 ± 0.20	-	10.00 ± 0.18	10.00 ± 0.18	10.00±0. 24	10.00 ± 0.12	10.00 ± 0.24	-		-
1.56	-	10.00 ± 0.15	10.0± 0.10	-	-	-	-	-	-	-	-	-
0.78	-	-	-	-	-	-	-	-	-	-	-	-
Ampicillin (10µg/ml)	28.00 ± 0.10	26.00 ± 0.10	28.00 ± 0.10	24.00 ± 0.20	30.00 ± 0.20	22.00 ± 0.25	-	-	-	-	-	-
Tiaconazole (10%w/v)	-	-	-	-	-	-	26.00 ± 0.10	24.00 ± 0.10	26.00± 0.20	24.00 ± 0.20	24.00± 0.15	26.00 ± 0.30
Methanol	-	-	-	-	-	-	-	-	-	-	-	-

Values represent diameter of zone of inhibition (mm)

- means no inhibition

Table 16 : Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of fraction 1 (F1) of the bulb of *Crinum jagus*

Microorganism	Type	MIC (µg/ml)	MBC/MFC (µg/ml)
Staphylococcus aureus	Bacteria (G+)	0.20	0.39
Bacillus subtilis	Bacteria (G+)	0.39	0.39
Escherichia coli	Bacteria (G-)	0.39	0.78
Pseudomonas aeruginosa	Bacteria (G-)	0.39	0.78
Salmonellae typhi	Bacteria (G-)	0.39	0.78
Kelbsiella pneumonae	Bacteria (G-)	0.39	0.78
Candida albicans	Yeast	0.39	0.78
Candida tropicals	Yeast	0.39	0.78
Candida krusei	Mould	0.39	0.78
Penicillum Notatum	Mould	0.78	1.56
Aspergillus niger	Mould	0.78	1.56
Aspergillus flavos	Mould	0.78	1.56

MIC = Minimum Inhibitory Concentration , MBC = Minimum Bactericidal Concentration , MFC = Minimum Fungicidal Concentration, G+ = Gram-positive, G - = Gram-negative.

Table 17 : Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of fraction 2 (F2) of the bulb of *Crinum jagus*

Microorganism	Type	MIC (µg/ml)	MBC/MFC(µg/ml)
Staphylococcus aureus	Bacteria (G+)	0.39	0.78
Bacillus subtilis	Bacteria (G+)	0.39	0.78
Escherichia coli	Bacteria (G-)	0.78	0.78
Pseudomonas aeruginosa	Bacteria (G-)	0.78	0.78
Salmonellae typhi	Bacteria (G-)	0.78	1.56
Klebsiella pneumonae	Bacteria (G-)	0.78	1.56
Candida albicans	Yeast	0.78	1.56
Candida tropicals	Yeast	0.78	1.56
Candida krusei	Mould	0.78	1.56
Penicillum Notatum	Mould	0.78	1.56
Aspergillus niger	Mould	1.56	1.56
Aspergillus flavos	Mould	1.56	1.56

MIC= Minimum inhibitory concentration, MBC = Minimum bactericidal concentration, MFC = Minimum fungicidal concentration, G + = Gram- positive, G - = Gram-negative.

Table 18 : Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of fraction 3 (F3) of the bulb of *Crinum jagus*.

Microorganism	Type	MIC(µg/ml)	MBC/MFC(µg/ml)
Staphylococcus aureus	Bacteria (G+)	0.78	1.56
Bacillus subtilis	Bacteria (G+)	0.78	0.78
Escherichia coli	Bacteria (G-)	0.78	0.78
Pseudomonas aeruginosa	Bacteria (G-)	0.78	1.56
Salmonellae typhi	Bacteria (G-)	0.78	1.56
Kelbsiella pneumonae	Bacteria (G-)	0.78	1.56
Candida albicans	Yeast	1.56	1.56
Candida tropicals	Yeast	1.56	1.56
Candida krusei	Mould	1.56	1.56
Penicillum Notatum	Mould	1.56	1.56
Aspergillus niger	Mould	1.56	1.56
Aspergillus flavos	Mould	1.56	1.56

MIC = Minimum inhibitory concentration, MBC = Minimum bactericidal concentration , MFC = Minimum fungicidal concentration , G+ = Gram-positive G - = Gram-negative.

Experiment 6: Determination of Anti-inflammatory Activity of the Crude Methanol Extract and Fractions of the Bulb Of *Crinum jagus*

Introduction

Drugs presently in use for the management of pain and inflammatory conditions are either narcotic e.g opoids or non narcotic e.g salicyclate and corticosterioids e.g hydrocortisone. All of these drugs present well known side and toxic effects. It is well documented that these non - steroidal anti-inflammatory drugs (NSAIDs) produced intestinal tract ulcer with potential internal bleeding in 10-30 % of long term users, and erosion of the stomach lining and intestinal tract in 30-50 % of cases (Hayliyar *et al*., 1992). As a result of these side effects, the use of NSAIDs is associated with 10,000 to 20,000 deaths per year. (Ament and Childers, 1997). Even the new cyclooxygenase (COX-2) inhibitor has only been reported to reduce intestinal tract damage by 50% and their toxicity to the liver and kidney is still under review. *Crinum jagus* is reported in traditional literature for the treatment of snake bite (Ode *et al.*, 2006), tuberculosis (Idu *et al.*, 2010), and asthma cough (Ogunkunle and Olopade, 2011) which are all inflammatory diseases, therefore the present research has been undertaken to investigate the anti-inflammatory activity of the crude extract and fractions of the plant.

Procedure

The antinflammatory activity was determined by carrageenan induced rat paw oedema method (Winter *et al*., 1962; Perianayagam *et al*., 2006) as described in section 3.10 under 'Materials and Methods'.

Results

Figure 41 presents the result of the anti-inflammatory activity of the crude methanol extract of the bulb of *Crinum jagus*. From the result it was observed that crude methanol extract of the plant and indomethacin significantly ($P<0.05$) reduced the paw oedema 3 hours after carrageenan injection compared with control. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) significantly ($P< 0.05$) inhibited oedema formation in the rat paw in a dose-dependent manner by 26,82%, 31.55%, 41.82% and 65.90%, respectively. The standard

drug indomethacin at 5mg/kg inhibited oedema formation by 94.82%. The extract at varying concentrations had lower potency than the indomethacin, the standard drug.

The result of the anti-inflammatory activity of the fractions of the bulb of *Crinum jagus* is presented in Figure 42. All the three fractions (F1, F2 and F3) and indomethacin significantly ($P < 0.05$) reduced paw oedema, 3 hours after carrageenan injection relative to control. At a dose level of 20mg/kg, F1, F2 and F3 significantly ($P < 0.05$) inhibited oedema formation in rats by 79.50%, 25.00% and 52.27%, respectively. The result showed that F1 and F3 were more potent as an anti-inflammatory agents than the crude extract and F2. F1 had the highest inhibitory activity (79.50%) which compared well with indomethacin the standard drug with inhibitory activity of 94.82%.

Conclusion

From the study, the crude methanol extract and fractions of *Crinum jagus* bulb was found to have impressive *in vivo* anti-inflammatory activity in experimental animals since the plant extract and its fractions significantly reduced the formation of oedema induced by carrageenan. Anti-inflammatory properties of the plant was not lost during fractionation of the crude extract but fractionation improved the anti-inflammatory activity of the plant. Two of the fractions, F1 and F3 had impressive anti-inflammatory activity.

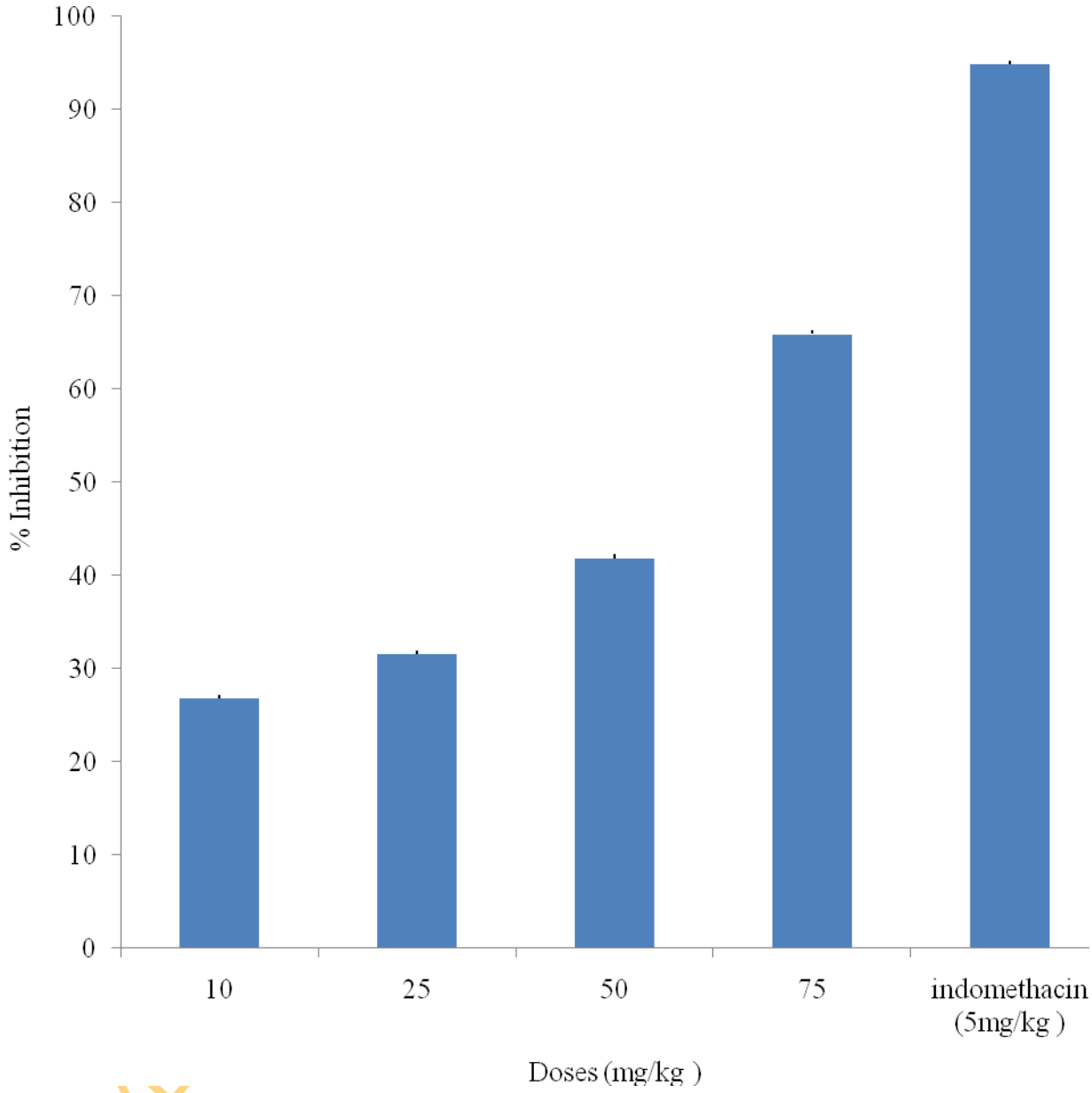


Figure 41: Anti–inflammatory activity of the crude methanol extract of the bulb of *Crinum jagus.*

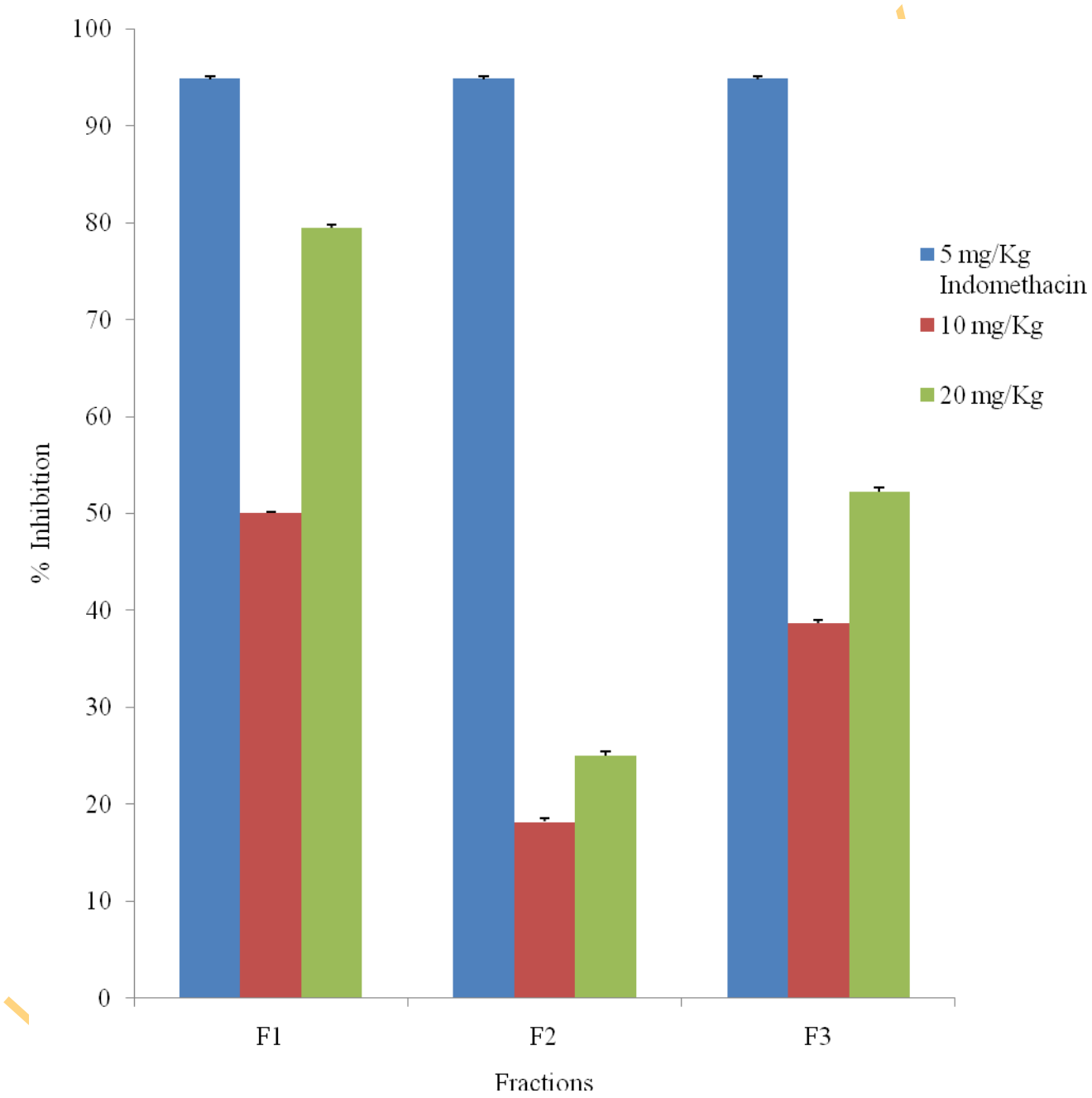


Figure 42: Anti–inflammatory activities of the fractions (F) of the bulb of *Crinum jagus*

F1 , F2 & F3 : $p > 0.05$

Experiment 7 : *In vivo* Antimalaria Activity of the Crude Methanol Extract and Fractions of the Bulb of *Crinum jagus*

Introduction

Malaria is one of the oldest recorded diseases in the world. It is estimated that more than 300 million people are infected annually and over one million deaths have been recorded in children under five years (Ramazani *et al*., 2010; Zofou *et al.*, 2011). The re-emergence of malaria in many parts of the world is due to the rapid increase of resistance of vectors to insecticides (Ridley, 2002; Zirihi *et al.*, 2005). Drug resistant strains of *Plasmodium falciparum* have been found in many endemic areas of the world and many of conventional antimalaria drugs have been associated with treatment failure. As such, there is need for continuous search for newer drugs that can retard or reverse this resistance. Since many mordern drugs such as quinine and artermisinin originate from plants, it is essential that other medicinal plants which have folkore reputation for antimalaria properties are investigated in order to establish their safety and efficacy and to determine their potential as source of new antimalaria drugs (Gester *et al.*,1994). This has led to attempts to discover other antimalaria agents mainly from plant sources.

. This study was prompted by an ethnobotanical survey that reported that *Crinum jagus* is one of the plants used traditionally for the treatment of malaria in some parts of Nigeria (Osakwe *et al.*, 2011). Therefore, in this study the *in vivo* antimalaria activity of the crude extract and fractions of the plant were investigated to give scientific proof and its potential to be developed as an antimalarial drug.

Procedure

The established infection method of Ryley and Peter, (1970) was used to evaluate the antiplasmodial activity of the crude extract and fractions of *Crinum jagus* in *Plasmodium berghei* infected mice as described in section 3.11 under 'Materials and Methods'. Blood was collected into the capillary tube to determine the packed cell volume (PCV). The body weight of the mice were measured to observe whether the plant extract prevented the weight loss that is common with increasing parastaemia in infected mice.

Results

The effect of the crude methanol extract and fractions of the bulb of *Crinum jagus* on established infection are presented in Tables 18, Figures 43 and 44. Treatment of the *Plasmodium berghei* infected mice with the extract and the fractions resulted in a daily reduction in parasitaemia level in the extract/fractions treated groups similar to that of chloroquine and arteether groups and these reductions were dose dependent. Animals in the control group which were infected but not treated showed a gradual daily increase in parasitaemia level. By day 6 post infection, there was significant reduction ($P < 0.05$) in percentage parasitaemia in the treated groups relative to the control group (tween 80), the percentage parasitaemia in the groups treated with varying concentrations of the crude extract of the plant (10, 25, 50 and 75mg/kg) were 4.99%, 4.46%, 3.95% and 2.29% respectively while the percentage parasitaemia of 2.13% and 18.72% were recorded for arteether treated and negative control groups, respectively. By day 6, there was a complete clearance of parasitaemia by chloroquine. The groups treated with 10mg/kg of F1, F2 and F3 had percentage parasitaemia of 2.00%, 4.47% and 3.50%, respectively. When treatment was withdrawn (after day 6 post infection), there was increase in the parasitaemia level in all the treated groups due to recrudesence of parasitaemia with the exception of chloroquine treated group. By day 6 post infection, there was complete clearance of parasitaemia in the chloroquine treated group. By day 6 post infection, the crude extract at different doses administered (10, 25, 50 and 75mg/kg) produced chemosuppression of 70.10%, 76.17%, 78.90% and 87.17% respectively while F1, F2 and F3 at 10mg/kg had chemosuppression of 89.3%, 76.12% and 77.70%, respectively. The standard drugs chloroquine (10mg/kg) and arteether (3mg/kg) caused 100% and 88.60% suppression respectively.

Mean survival time (MST) of 25 and 23 days respectively were observed for chloroquine and arteether treated groups compared to 19, 20, 21 and 22 days respectively observed in the groups treated with 10, 25, 50 and 75mg/kg of the crude extract. F1, F2 and F3 had a mean survival time of 25, 21 and 21 days, respectively. The untreated control groups survived for only 12.5 days (Table 19).

The packed cell volume (PCV) of parasite infected untreated animals decreased progressively until they all died while the PCV of chloroquine treated group increased progressively. During the 4 days treatment, the PCV of all the treated groups increased progressively. But when treatment was withdrawn, increase in PCV was only observed in chloroquine treated group. In arteether, crude extract and fractions treated groups, there was a decrease in PCV due to recrudesence of parasitaemia observed in the arteether, extract and the fractions treated groups (Fig 45 and 46).

Conclusion

It can be concluded that the crude methanol extract and the fractions of the bulb of *Crinum jagus* possess considerable antiplasmodial activities as shown by chemosuppression of parasitaemia and prolongation of life span of the infected mice treated with the extract and the fractions of the plant. Fractionation of the extract enhanced the antimalarial activity of the plant with F1 demonstrating the highest antiplasmodial activity.

Table 19 : Suppressive activities of the crude extract and fractions of the bulb of *Crinum jagus* on established *Plasmodium berghei* infection in mice

Treatment	Dose (mg/kg)	Average % Parasitaemia (Day 3)	Average % Parasitaemia (Day 6)	% Suppression
Crude extract	10	5.38±0.34	4.99±0.20*	70.10
Crude extract	25	5.07±0.48	4.46±0.48*	76.17
Crude extract	50	5.23±0.30	3.95±0.55*	78.90
Crude extract	75	5.40±0.30	2.29±0.20*	87.17
Fraction 1	10	5.31±0.36	2.00±0.35*	89.33
Fraction 2	10	5.38±0.31	4.47±0.51*	76.12
Fraction 3	10	5.31±0.42	3.50±0.53*	77.70
Arteether	3	5.12±0.39	2.13±0.30*	88.60
Chloroquine	10	5.22±0.38	0.00	100
Control (Tween 80)	0.3ml	5.21±0.54	18.72±1.50	-

Values are expressed as mean ± SD, n = 8, *P < 0.05

Table 20 : Mean survival time of mice treated with various doses of crude extract and fractions of the bulb of *Crinum jagus* on established *Plasmodium berghei* infection.

Treatment	Dose (mg/kg)	Mean survival time (Days)
Crude extract	10	19
Crude extract	25	20
Crude extract	50	21
Crude extract	75	22
Fraction 1	10	25
Fraction 2	10	21
Fraction 3	10	21
Arteether	3	23
Chloroquine	10	25
Control (Tween 80)	0.3ml	12.5

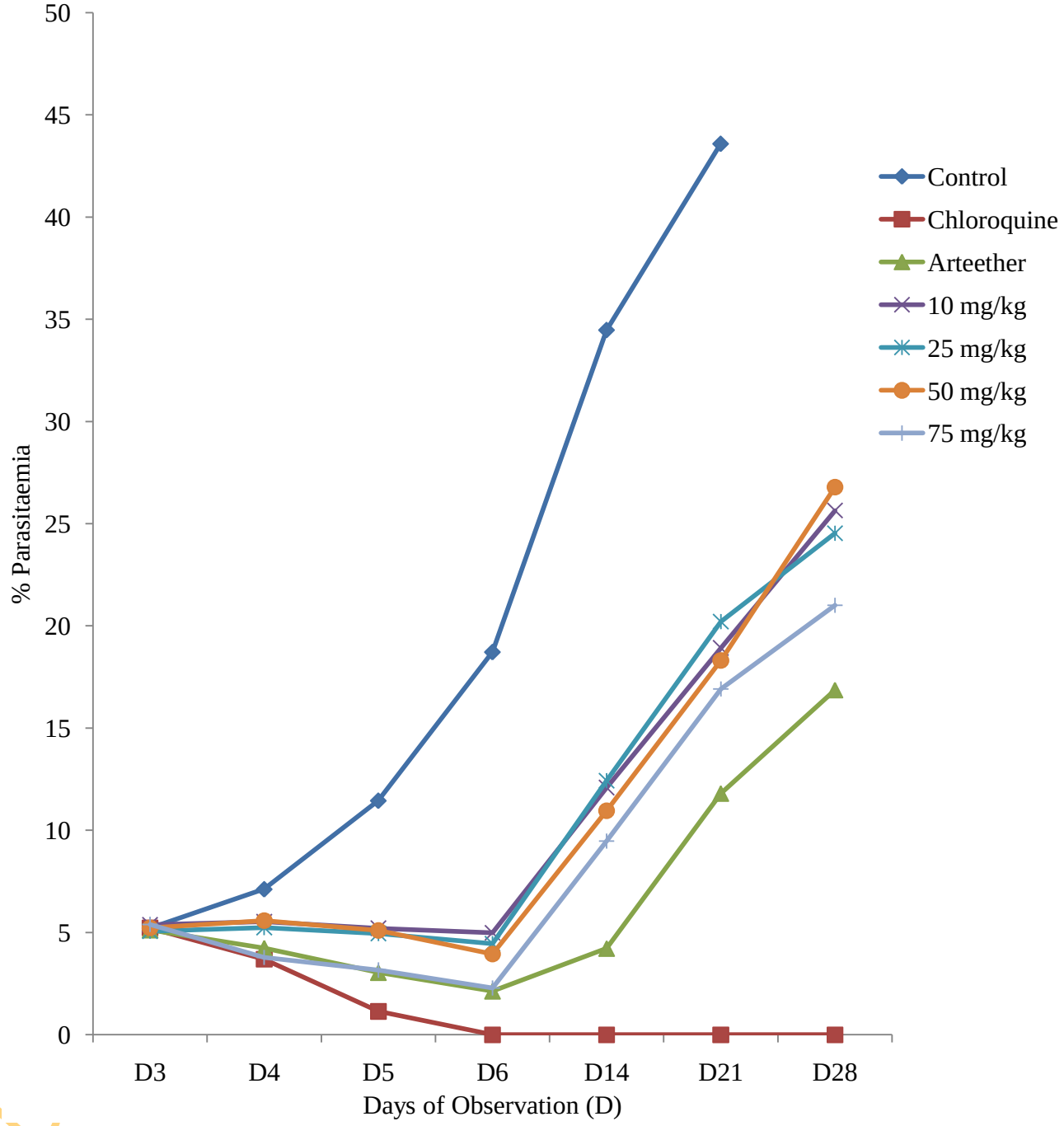


Figure 43: Antimalaria activity of the crude methanol extract of the bulb of *Crinum jagus* on established *Plasmodium berghei* infection in mice.

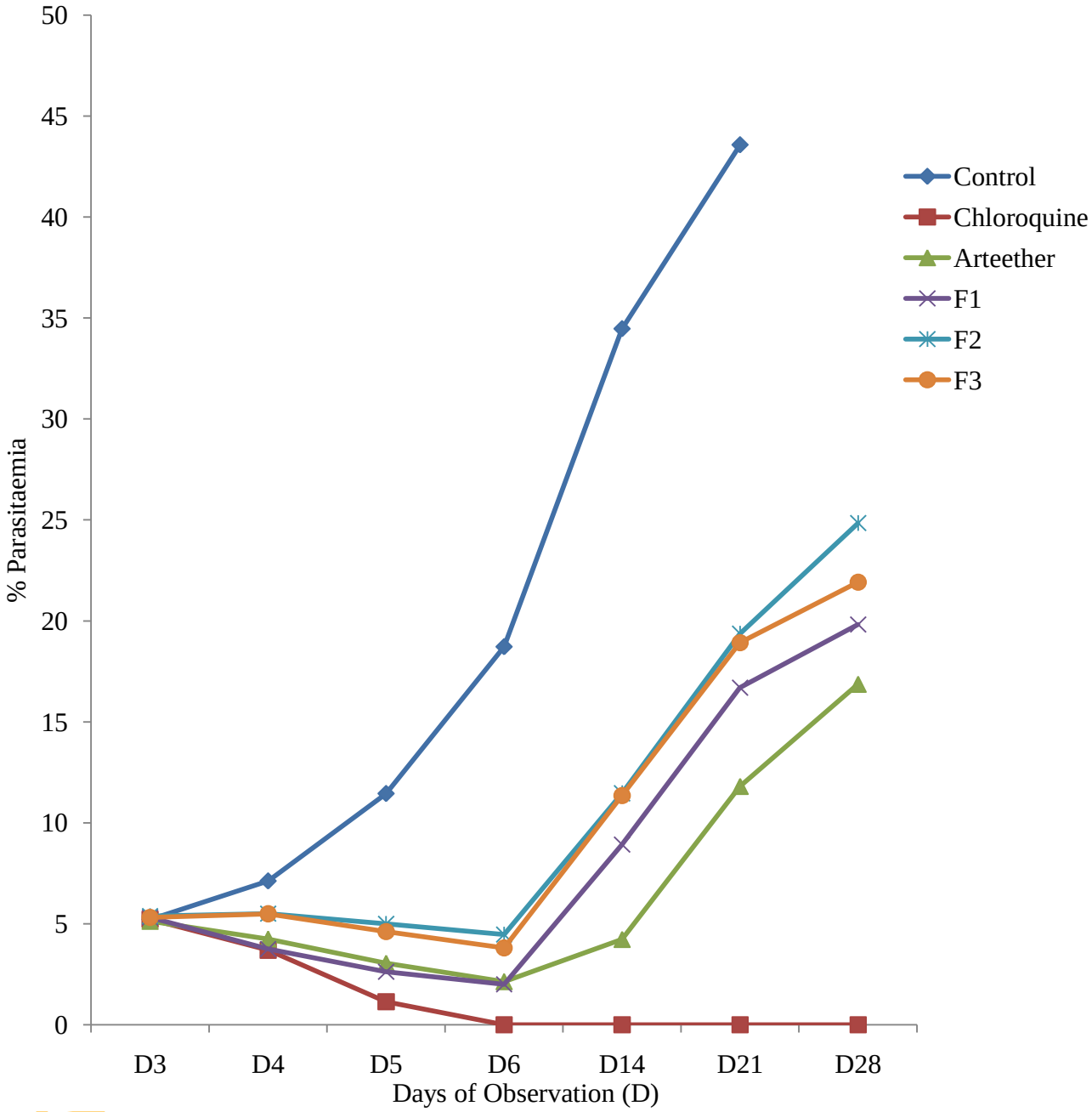


Figure 44: Antimalaria activity of the fractions (F) of the bulb of *Crinum jagus* on established *Plasmodium berghei* infection in mice.

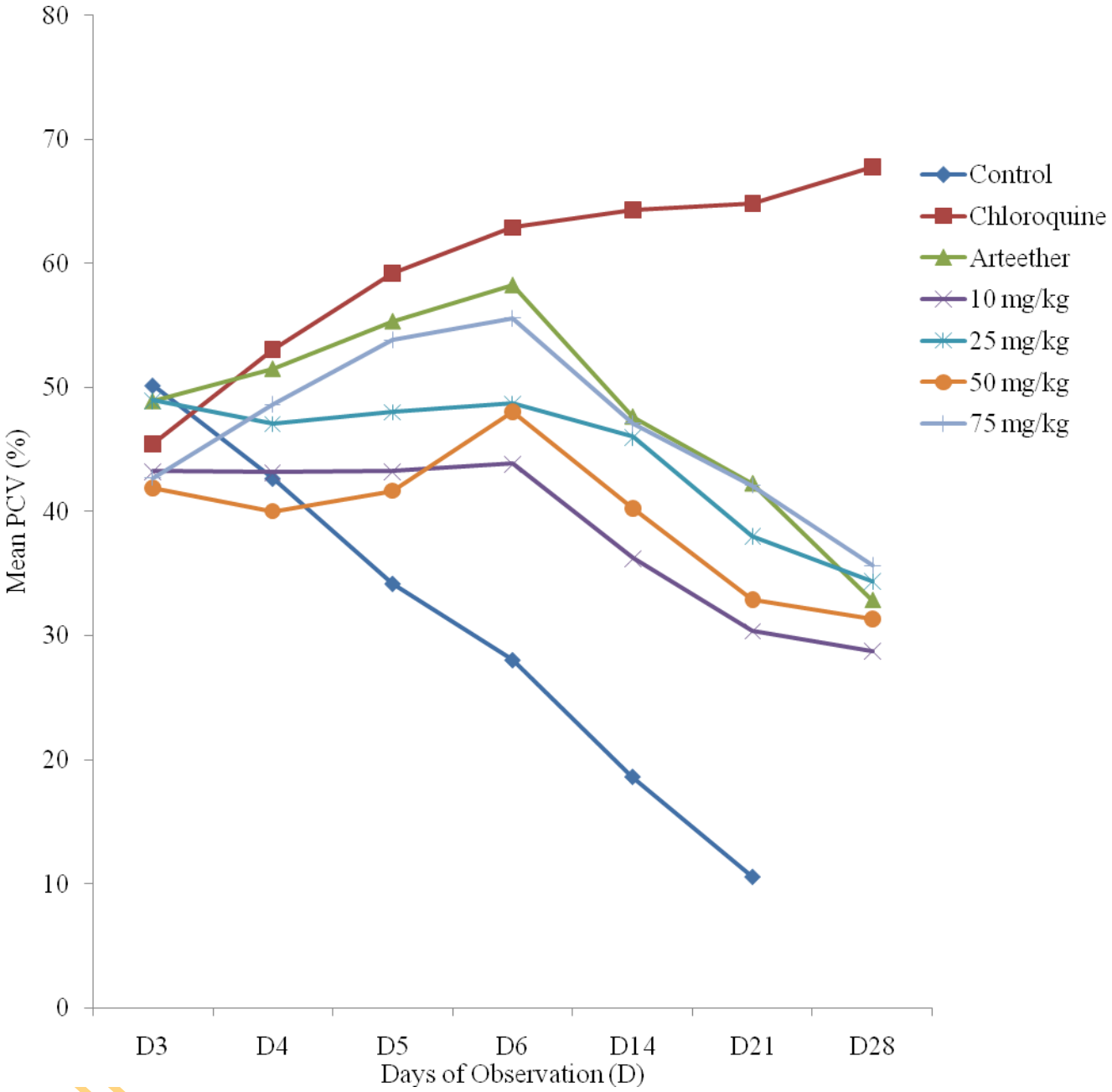


Figure 45: Effect of the crude methanol extract of the bulb of *Crinum jagus* on packed cell volume (PCV) of mice infected with *Plasmodium berghei*.

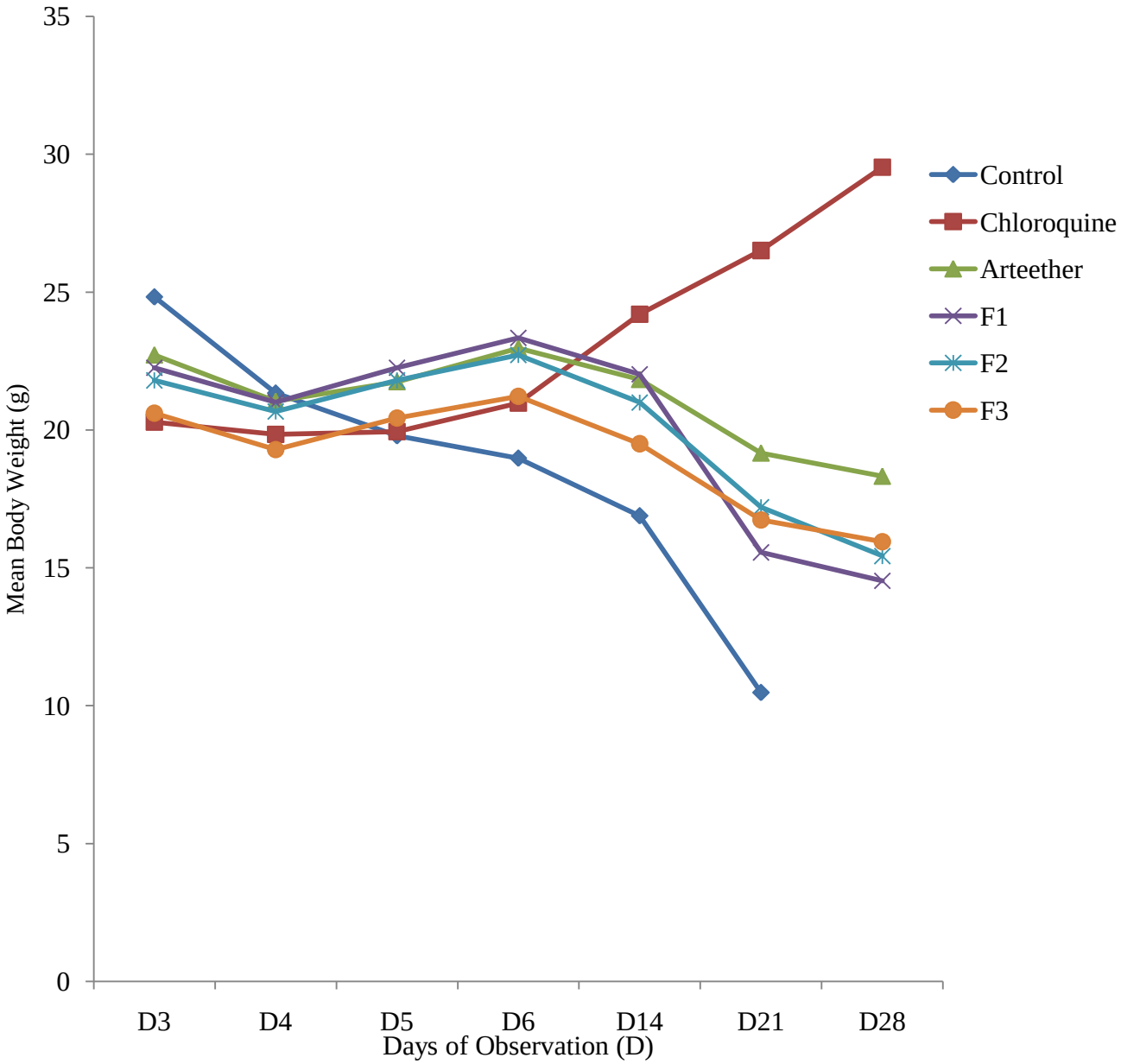


Figure 46: Effect of the fractions (F) of the bulb of *Crinum jagus* on packed cell volume (PCV) of mice infected with *Plasmodium berghei*.

Experiment 8 : Antitubercular Activity of the Crude Methanol Extract And Fractions of the Bulb of *Crinum jagus*.

Introduction

Tuberculosis (TB) remains an important public health problem world wide, accounting for eight million new cases per year. Its infectious agent, *Mycobacterum tuberculosis* kills approximately three million people every year in the world (WHO, 2007). Despite the improvement in chemotherapy, the epidemiology of tuberculosis is severely affected by the emergence of multi-drug resistance in *M. tuberculosis* strains (Leite *et al.*, 2008). For a long time, medicinal plants and herbs were used intensively in folkoric medicine for the treatment of various diseases. Recently, there has been widespread interest in drugs derived from plants. This interest stems from the belief that medicinal plants are safe and dependable as opposed to synthetic drugs that have adverse effect (Stuffness and Douros, 1982). In the past years, several reports and review articles appeared in the literature about medicinal plants and natural products with anti-mycobacterium activity (Okunade *et al*., 2004). This study was prompted by an ethnobotanical survey that reported that *Crinum jagus* is one of the plants used for the treatment of tuberculosis in Southern part of Nigeria (Idu *et al*., 2010) hence in this study the antibacterial activities of the crude methanol extract and fractions of the bulb of *Crinum jagus* against *Mycobacterium tuberculosis* isolates was investigated.

Procedure

Evaluation of antitubercular activity of the crude extract in Lowenstein Jensen (L-J) medium was carried out by the method of Gupta *et al.,* (2010). The susceptibility *of M. tuberculosis* to the extract and the drugs in Middle brook 7H10 agar was determined using the disc diffusion method described by Claude *et al.,* (2012) as described in section 3.12 under 'Materials and Methods.

Results

The results of the antitubercular activity of the crude methanol extract of the bulb of *Crinum jagus* against the three *M. tuberculosis isolates* and H37Rv strain in L.J medium and Middle brook 7H10 agar are presented in Tables 21 and 22. The results show that the crude extract at various concentrations showed a concentration dependent inhibition of the *M. tuberculosis*

isolates and H37Rv strain in both Lowenstein Jensen (L-J) and Middlebrook 7H10 media. The antitubercular activity of the extract compared well with antitubercular activity of the standard drugs (rifampicin and isoniazid) however, the potency of the extract was lowered compared to the standard drugs.

The crude extract and the standard drugs possessed marked inhibitory activity against *M. tuberculosis* with IC_{50} values ranging from 0.20 to 0.90 mg/ml. The various concentrations of the extract exhibited a significant concentration dependent inhibition of *M. tuberculosis* with IC_{50} values of 0.92, 0.92, 0.90, 0.88 mg/ml against H37Rv, MTB1, MTB2 and MTB3 respectively in L-J medium (Table 23) while IC_{50} values of 0.86, 1.45, 0.82, 0.77 mg/ml were obtained for the crude extract in Middle brook 7H10 agar. In L-J and Middle brook 7H10 media, the IC_{50} values of rifampicin against H37Rv, MTB1, MTB2 and MTB 3 were 0.20, 0.29, 0.26, 0.21 mg/ml and 0.19, 0.28, 0.21, 0.19 mg/ml respectively. Isonazid in both L-J and Middlebrook 7H10 media had the IC_{50} value of 0.25, 0.32, 0.27, 0.20 mg/ml and 0.23, 0.26, 0.22, 0.20 mg/ml respectively against H37Rv, MTB1, MTB2, MTB (Table 23). IC_{50} values of the standard drugs, rifampicin and isoniazid were found to be much less than those of the crude extract indicating that the drugs were more potent as an anti-TB agent than the extract.

Tables 25 and 26 present the result of antitubercular activity of the fractions of the bulb of *Crinum jagus* in both Lowenstein Jensen (L-J) and Middlebrook 7H10 agar. All the three fractions (F1, F2 and F3) showed a concentration dependent inhibition of *M. tuberculosis* isolates and H37Rv strain at various concentrations in both medium. The fractions were more potent than the crude extract with higher inhibition values. Of all the three fractions, F1 was the most potent with the highest inhibitory values. The inhibitory activity of F1 compared well with the standard drugs. At a concentration of 1.0mg/ml, the percentage inhibition of F1, rifampicin and isoniazid against MTB 3, was 83%, 95% and 84%, respectively.

The fractions possessed good inhibitory activity against *M. tuberculosis* with IC_{50} value ranging from 0.22 to 0.97 mg/ml. F1, F2 and F3 at various concentrations exhibited concentration dependent inhibition of *M. tuberculosis* with IC_{50} values of (0.23, 0.48, 0.32, 0.27 mg/ml), (0.41 , 0.94 , 0.78, 0.69 mg/ml) and (0.65, 0.63, 0.55, 0.48mg/ml) respectivley against H37Rv, MTB 1, MTB 2 and MTB 3 in L.J medium while IC_{50} values (0.22, 0.40,

0.32, 0.26 mg/ml), (0.42, 0.97,0.83, 0.71 mg/ml) and (0.61, 0.54, 0.54,0.46 mg/ml) were obtained for F1, F2 and F3 respectively in Middle brook 7H10 agar. F1 had the lowest IC_{50} value which compared well with the IC_{50} value of the standard drugs, rifampicin and isoniazid indicating F1 to be the most potent of all the three fractions (Table 26).

Conclusion

From this study, it can be concluded that the crude methanol extract and fractions of the bulb of *Crinum jagus* exhibited interesting anti-mycobacterial activity against *Mycobacterium tuberculosis* isolates and strains.with F1 having the highest activity which compared well with antitubercular activity of the standard drugs, rifampicin and isoniazid.

Table 21 : Antitubercular activity of the crude methanol extract of the bulb of *Crinum jagus* in Lowestein Jensen (L- J) medium

		Mean cfu on media						Percentage Inhibition (%)				
Extract/ Drug	**Isolates**	**Control**	**0.2mg/ Ml**	**0.4mg/ Ml**	**0.6mg/ Ml**	**0.8mg/ Ml**	**1.0mg/ Ml**	**0.2mg/ Ml**	**0.4mg/ ml**	**0.6mg/ ml**	**0.8mg/ ml**	**1.0mg/ ml**
Crude Extract	H37Rv	125	110	90	82	73	54	12	28	34	42	57
	MTB1	122	108	95	80	66	56	30	22	34	45	54
	MTB 2	138	121	104	88	72	61	12	25	36	48	56
	MTB 3	136	120	107	84	70	58	12	25	38	49	57
Rifampicin	H37Rv	140	62	50	36	20	12	56	64	74	86	91
	MTB1	140	86	54	45	30	20	39	61	68	79	86
	MTB 2	134	75	50	38	22	14	44	63	72	84	88
	MTB3	130	62	42	20	12	6	52	68	85	91	95
Isoniazid	H37Rv	140	72	60	46	30	20	49	57	67	79	86
	MTB1	132	112	84	75	54	40	15	36	43	59	70
	MTB2	150	84	65	52	48	36	44	57	65	68	77
	MTB3	146	70	56	40	35	24	52	62	73	76	84

MTB = *Mycobacterium tuberculosis*, cfu = colony forming unit

$$\% \text{ Inhibition} = \frac{Cc - Ct}{Cc} \times 100$$

Cc = no of colony in the control medium

Ct = no of colony in test mediu

Table 22 : Antitubercular activity of the crude methanol extract of the bulb of C*rinum jagus* in Middle brook 7H10 medium

		Mean cfu on media						Percentage Inhibition (%)				
Extract/ Drug	**Isolates**	**Control**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg/ ml**	**0.8mg /ml**	**1.0mg /ml**
Crude Extract	H37Rv	124	101	92	78	60	55	19	26	37	52	56
	MTB1	120	108	92	78	64	54	10	23	35	47	56
	MTB 2	141	122	102	84	70	59	14	27	40	50	58
	MTB 3	138	116	98	82	68	56	16	28	41	51	59
Rifampicin	H37Rv	126	58	40	31	15	5	54	68	75	88	96
	MTB1	130	74	52	40	29	14	43	60	69	78	89
	MTB 2	140	65	50	39	25	12	54	64	72	82	91
	MTB3	132	55	40	29	20	04	58	70	78	85	97
Isoniazid	H37Rv	136	68	55	40	25	15	50	60	71	82	89
	MTB1	138	105	80	62	51	38	24	42	55	63	73
	MTB2	146	80	71	60	49	31	45	52	59	66	79
	MTB3	144	68	51	39	22	16	53	65	73	85	89

MTB = *Mycobacterium tuberculosis*, cfu = colony forming unit

$$\% \text{ Inhibition} = \frac{Cc - Ct}{Cc} \times 100$$

Cc = no of colonyin the control medium

Ct = no of colony in test mediu

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Table 23 : *In vitro* antitubercular activity of the crude methanol extract and fractions of the bulb of *Crinum jagus* against isolates and strain of *M. tuberculosis* in L-J medium.

	50% Inhibitory Concentration (mg/ml)			
	H37Rv	**MTB 1**	**MTB 2**	**MTB 3**
Crude extract	0.92	0.92	0.90	0.88
Fraction 1	0.23	0.48	0.32	0.27
Fraction 2	0.41	0.94	0.78	0.69
Fraction 3	0.65	0.63	0.55	0.48
Rifampicin	0.20	0.29	0.26	0.21
1soniazid	0.25	0.61	0.27	0.20

Table 24 : *In vitro* antitubercular activity of the crude methanol extract and fractions of the bulb of *Crinum jagus* against isolates and strain of *M. tuberculosis* in Middlebrook 7H10 medium.

	50%Inhibitory concentration (mg/ml)			
	H37Rv	**MTB 1**	**MTB 2**	**MTB 3**
Crude extract	0.86	1.45	0.82	0.77
Fraction 1	0.22	0.50	0.32	0.26
Fraction 2	0.42	0.97	0.83	0.71
Fraction 3	0.61	0.54	0.54	0.46
Rifampicin	0.19	0.28	0.21	0.19
Isoniazid	0.23	0.50	0.32	0.21

Table 25 : Antitubercular activity of the fractions of the bulb of *Crinum jagus* in Lowenstein Jensen (L-J) medium

		Mean cfu on media						Percentage Inhibition (%)				
Extract/ Drug	**Isolates**	**Control**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**
Fraction 1	H37Rv	130	70	62	46	30	22	46	52	65	77	83
	MTB 1	164	102	96	86	69	45	38	41	48	58	73
	MTB 2	118	75	58	45	40	25	36	51	62	66	79
	MTB 3	162	82	70	58	40	25	49	57	64	75	83
Fraction 2	H37Rv	128	80	74	55	41	30	38	42	57	68	77
	MTB 1	128	108	102	98	76	64	16	20	23	47	50
	MTB 2	120	101	88	80	65	54	16	18	33	46	55
	MTB 3	138	116	110	89	73	58	16	20	35	41	58
Fraction 3	H37Rv	120	95	83	74	50	39	21	31	38	58	68
	MTB 1	120	90	74	64	52	42	25	38	47	57	65
	MTB 2	122	92	68	63	45	42	25	44	48	63	66
	MTB 3	142	98	76	67	52	44	31	46	53	63	69
Rifampicin	H37Rv	140	62	50	36	20	12	56	64	74	86	91
	MTB 1	140	86	54	45	30	20	39	61	68	79	86
	MTB 2	134	75	50	38	22	14	44	63	72	84	88
	MTB 3	130	62	42	20	12	6	52	68	85	91	95
Isoniazid	H37Rv	140	72	60	46	30	20	49	57	67	79	86
	MTB 1	132	112	84	75	54	40	15	36	43	59	70
	MTB 2	150	84	65	52	48	36	44	57	65	68	77
	MTB 3	146	70	56	40	35	24	52	62	73	76	84

MTB = *Mycobacterium tuberculosis*, cfu = colony forming unit

$$\% \text{ Inhibition} = \frac{Cc - Ct}{Cc} \times 100$$

Cc = no of colony in the control medium
Ct = no of colony in test medium

Table 26 : Antitubercular activity of the fractions of the bulb of *Crinum jagus* in Middle brook 7H10 medium

		Mean cfu on media						Percentage Inhibition (%)				
Extract/Drug	**Isolates**	**Control**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**	**0.2mg /ml**	**0.4mg /ml**	**0.6mg /ml**	**0.8mg /ml**	**1.0mg /ml**
Fraction 1	H37Rv	128	65	42	31	22	12	49	67	76	83	91
	MTB 1	158	104	94	82	65	37	34	41	48	59	76
	MTB 2	122	69	60	44	39	24	43	51	64	68	80
	MTB 3	154	78	68	52	38	22	49	59	66	75	86
Fraction 2	H37Rv	140	94	76	60	50	30	32	46	57	64	79
	MTB1	131	112	98	90	72	58	15	25	31	45	56
	MTB 2	122	95	86	72	63	50	22	29	41	48	59
	MTB3	136	102	95	77	68	50	25	30	43	50	63
Fraction 3	H37Rv	138	109	92	75	61	40	21	33	46	56	71
	MTB1	126	94	78	62	48	40	25	38	51	62	68
	MTB2	120	89	70	61	46	35	26	42	49	62	71
	MTB3	140	96	78	65	48	38	31	44	54	66	73
Rifampicin	H37Rv	126	58	40	31	15	5	54	68	75	88	96
	MTB1	130	74	52	40	29	14	43	60	69	78	89
	MTB2	140	65	50	39	25	12	54	64	72	82	91
	MTB3	132	55	40	29	20	04	58	70	78	85	97
Isoniazid	H37Rv	136	68	55	40	25	15	50	60	71	82	89
	MTB1	138	105	80	62	51	38	24	42	55	63	73
	MTB2	146	80	71	60	49	31	45	52	59	66	79
	MTB3	144	68	51	39	22	16	53	65	73	85	89

MTB = *Mycobacterium tuberculosis*, cfu = colony forming unit

$$\% \text{ Inibition} = \frac{Cc - Ct}{Cc} \times 100$$

Cc = no of colony in the control medium
Ct = no of colony in test medium

Experiment 9 : Toxicological Profiles of the Crude Methanol Extract of the Bulb of *Crinum jagus*

Introduction

The use of medicinal plants in the management of several ailments is increasing probably due to the belief that they are harmless simply because they are natural. They are also commonly used for self medication without supervision. This increase in popularity but lack of scientific studies on the safety of these plants and their phytoconstituents has raised concerns regarding toxicity and adverse effects of these remedies (Gehlot and Bohra, 2000, Saad *et al.*, 2006). Some medicinal plants must be used with caution because they can cause adverse reactions, especially if they are taken in excessive dose, or if they interact with conventional drugs (Janetzky and Morreale, 1997; Fennel *et al*., 2004). Therefore there is need to assess toxicological implications of traditionally used medicinal plants. We have among these plants, *Crinum jagus* which posses medicinal value.

Crinum jagus is a bulbous plant with spirally arranged leaves and conspicuous flowers. It is found in tropical and subtropical regions throughout the world (Mabbery, 1991). It belongs to to the family : Amaryllidaceae, genus : *Crinum,* species : *jagus.*

Crinum jagus has various therapeutic uses in herbal medicine. The powdered bulb is taken orally with honey as a remedy for tuberculosis in some parts of Nigeria (Ode *et al*., 2006). The bulb of the plant is used for the treatment of asthma cough in Western part of Nigeria and is commonly called asthma cough plant (Ogunkunle and Olopade, 2011). The warm leaf juice of the bulb of the plant with a pinch of salt is used for ear complaint as an emetics (Gill 1992). Among the Binis, the decoction of the plant is used as vermifuge and purgative (Gill, 1992). Previous work done on the therapeutic importance of the plant reported the antibacterial and antifungal activities, (Adesanya *et al*., 1992), anticonvulsant activity (Edema and Okiemen, 2002) and anti-snake venom activity of the plant (Ode *et al.,* 2010). This study was therefore designed to evaluate the sub-chronic toxicity and histopathology of the methanol extract of the bulb of the plant in

experimental animals to assess its safety or otherwise since the findings are important considering the usage of the plant by human beings.

Procedure

Thirty male rats were randomly divided into five groups of six animals each. Group A received 0.2ml normal saline and served as the control, Groups B to E received the extract at the doses of 10, 25, 50 and 75mg/kg respectively for 30 days. The extract was dissolved in normal saline before administration to the rats using oral cannula, 24 hours after the last treatment, the animals were sacrificed, blood samples were collected by cardiac puncture into plain bottles and then allowed to stand for 1 hour and were centrifuged at 3000g for 10 minutes to obtain the serum. Liver and kidney samples were removed and washed in ice cold 1.15% KCl, dried, weighed and homogenized in phosphate buffer, the homogenate was centrifuged at 10,000g for 20 minutes to obtain the supernatant used for the assay. AST and ALT were assayed using the procedures described by Rietman and Frankel (1957), ALP was assayed by the procedure of Wright *et al.*,(1972) while LDH was assayed by the method of Pesce and Kaplan, (1984). The total and direct bilirubin were determined by the methods of Kaplan *et al.*,(1984); Malloy *et al.*,1937 and Martinek, 1966). The concentrations of triglycerides (TG), total chloestrol (TC), LDC-C and HDL-C were determined by standard methods (Trinder *et al.*, 1969; Mc Gowan 1983; Albers *et a*l., 1978; Fossatti and Prencipe 1982; Demacker *et al.*, 1984; Fredickson *et al*., 1967). Haematological parameters were determined, using the standard methods as described by Dacie and Lewis, (1991). The liver and kidney samples were harvested and fixed in 10% buffered formalin for 48 hrs. The tissues were processed using an automatic tissue processor embedded in paraffin wax and section (5µm) thickness cut using a rotary microtome. The sections were stained by haematoxylin and eosin (H & E) method for light microscopy examination. Photomicrographs of relevant stained sections were taken with the aid of a camera fitted light microscope.

Results

Figure 47 shows the result of the effect of crude methanol extract of the bulb of *Crinum jagus* on the body weight of the control and the treated rats between the beginning of the study and the point of sacrifice. By the end of thirty days of treatment there was increase in the body weight of all the treated and the control rats.

The effect of crude methanol extract of the bulb of *Crinum jagus* on serum enzymes following administration of the extract for 30 days revealed that there were no significant ($P < 0.05$) differences in the serum levels of AST, ALT and ALP in the rats treated with 10 and 25 mg/kg of the extract, however, the level of AST, ALT and ALT in the serum were significantly ($P < 0.05$) elevated by administration of 50 and 75mg/kg of the extract. Both lower and higher doses of the extract did not produce any significant difference in the level of serum LDH when compared with the control group (Fig 48).

The mean liver function test values of the experimental rats is presented in Fig 49. The result of the experiment shows that there were no significant differences ($P > 0.05$) in the levels of AST, ALT and ALP following administration of the lower doses (10 and 25mg/kg) of the extract for 30 days while administration of higher doses (50 and 75mg/kg) produced significant ($P < 0.05$) elevation in the concentrations of AST, ALT and LDH relative to the control group. The higher doses (50 and 75mg/kg) of the extract does not produce any significant difference in level of liver ALP relative to the control group.

The effect of thirty days oral administration of the crude extract of the bulb of *Crinum jagus* on kidney enzymes revealed non significant differences in the concentrations of AST, ALT and ALP in the animals treated with low doses of the extract (10 and 25mg/kg) while significant increase in the levels of AST, ALT and ALP were observed with higher doses (50 and 75mg/kg). There were no significant difference in the level of LDH in the kidney in all the treated groups compared with the control group (Fig 50).

There were no significant differences in the total and conjugated bilirubin concentration in all the animals treated with lower doses of the extract (10 and 25mg/kg). Higher doses of the extract (50 and 75mg/kg) produced significant ($P < 0.05$) elevation

in the total and conjugated bilirubin levels both in the serum and the liver (Fig 51 and 52).

Figure 53 revealed that there was significant ($P < 0.05$) reduction in the serum triglyceride level when the animals were treated with high doses (50 and 75mg/kg) of the extract. Both lower and higher doses of the extract produced significant reduction in the concentration of total cholesterol in the serum while there was significant elevation in the serum HDL cholesterol in the groups treated with 25, 50 and 75mg/kg doses of the extract for thirty days relative to the control group. There were no significant differences in the serum LDL cholesterol concentration in all the treated groups compared with the control group.

The effect of 30 days oral administration of the crude methanol extract of the bulb of *Crinum jagus* on haematological parameters indicated no significant differences in the value of red blood cell count (RBC), packed cell volume (PCV) and haemoglobin (Hb) concentrations in all the treated groups relative to the control group. Also the values of mean corpuscular volume (MCV), mean corpscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were not significantly different in all the treated groups compared with the control group (Fig 54).

Fig 55 also revealed no significant differences in the values of white blood cell count (WBC), lymphocyte, neutrophils, eosinophils, monocytes and platelets in all the test groups compared with the control group after 30days of treatment with the extract.

Plates 1A, 1B and 1C show the photomicrographs obtained from the histological examination of liver sections of the different groups. As shown on the plates, the liver section of the control group showed normal hepatocyte, the central vein was normal and centrally placed (Plate 1A). The liver sections of the rats treated with lower doses of the extract (10 and 25mg/kg) showed moderate haemorrhage (Plate 1B) while exposure of the rats to high dose caused pathological changes in the liver which resulted in the enlargement of hepatocytes with multiple cytoplasimic vacuolation (fatty degeneration of hopatocytes) (Plate 1C), showing the extract to be toxic at higher dose.

Plates 2A, 2B and 2C show the photomicrographs obtained from the histological examination of kidney sections of the different groups. As seen on the plates, the kidney sections of the control showed normal cells with no visible lesions (Plate 2A). The kidney sections of the rats treated with lower doses of the extract showed moderate haemorrhage in the renal interstitium (Plate 2B) while exposure of the rats to high dose caused necrosis of the tubular epithelium with sloughing of necrosis into the lumen of the tubules and disruption of the basement membranes (Plate 2C), showing the extract to be toxic at higher dose.

Conclusion

In conclusion, toxicological evaluation of the bulb of *Crinum jagus* indicates that the plant is safe at lower doses but high doses of the plant may pose toxicological risks. Prolonged exposure for thirty days at higher doses tested (50 and 75mg/kg) resulted in the elevation of serum, liver and kidney AST, ALT and ALP. Similarly, pathological changes were observed in the liver and kidney following administration of higher doses. The plant however appears to possess beneficial effect by showing serum lipid lowering effect .

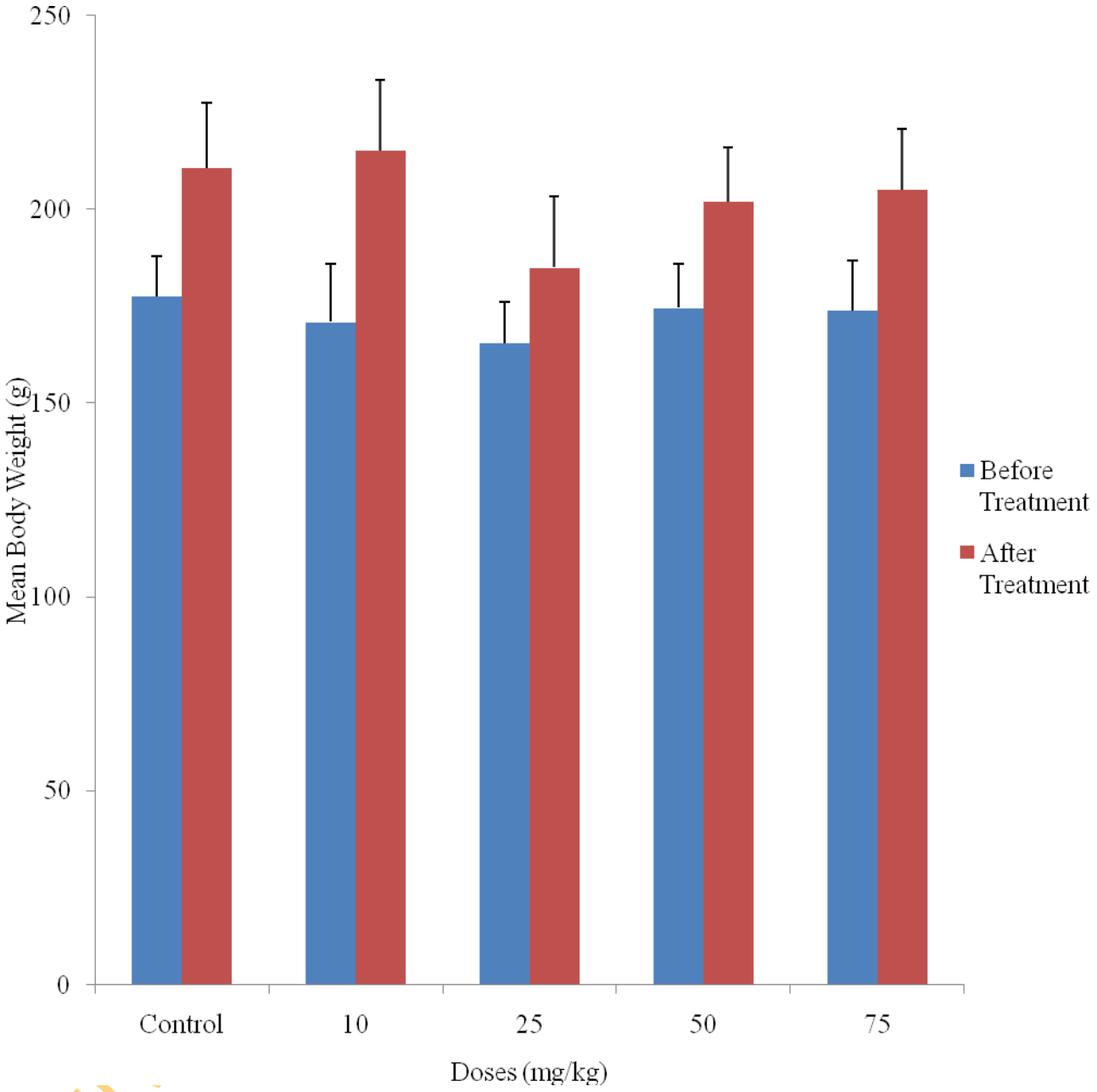

Figure 47: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on body weight of rats.

Control = 0.2ml normal saline

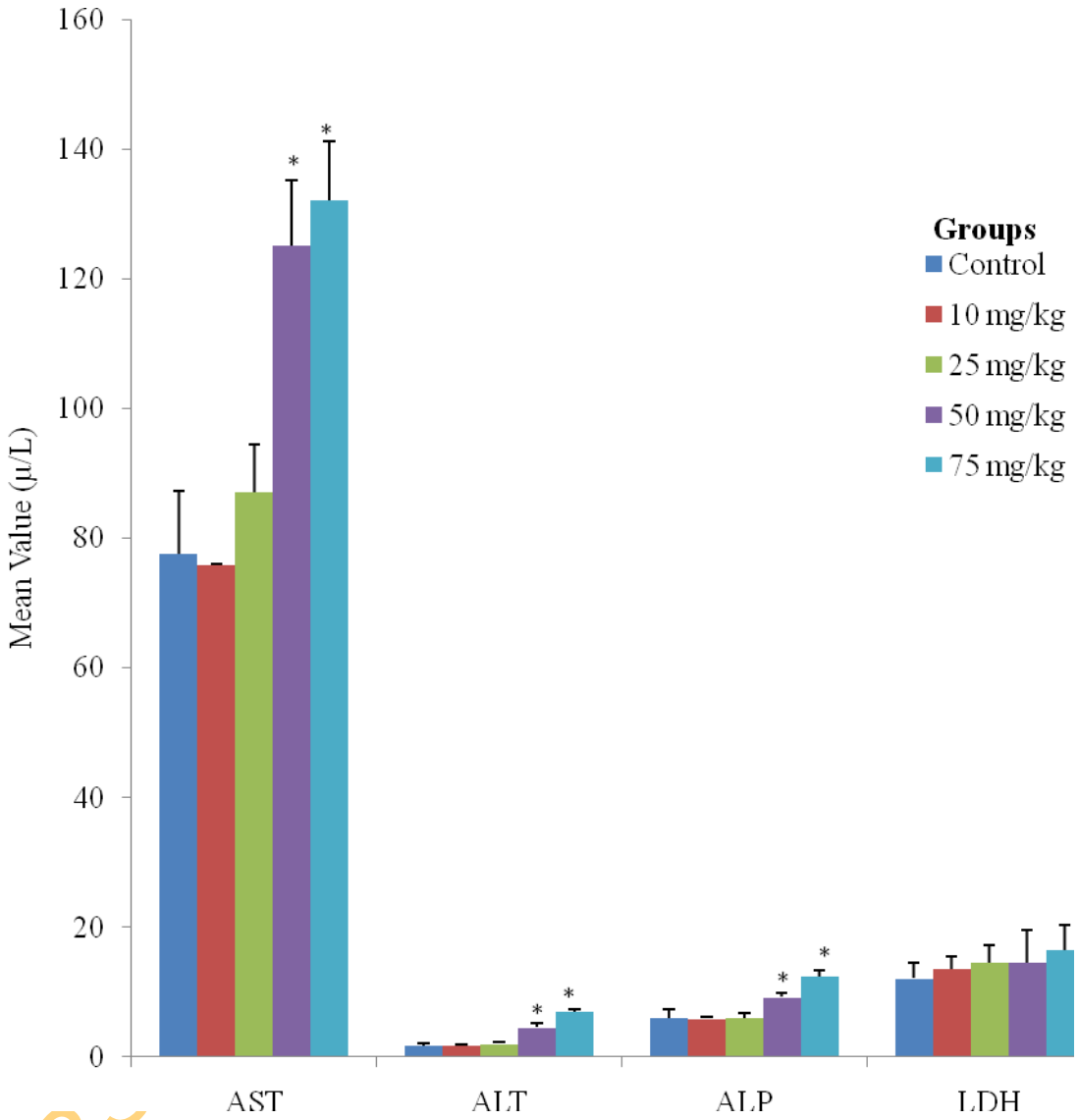

Figure 48: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on serum enzymes of rats.

* = Significantly different from control

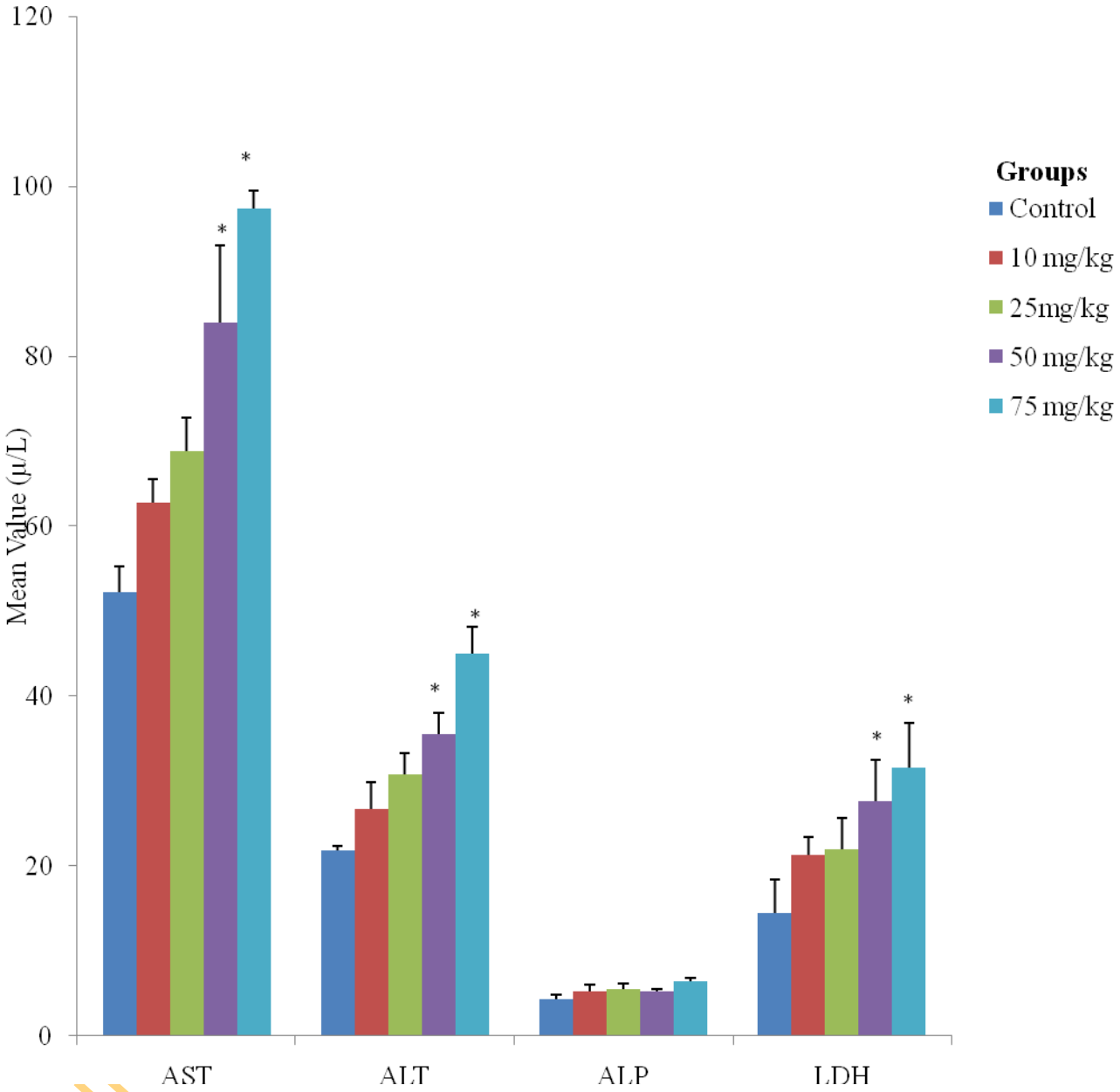

Figure 49: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on liver enzymes of rats.

* = Significantly different from control

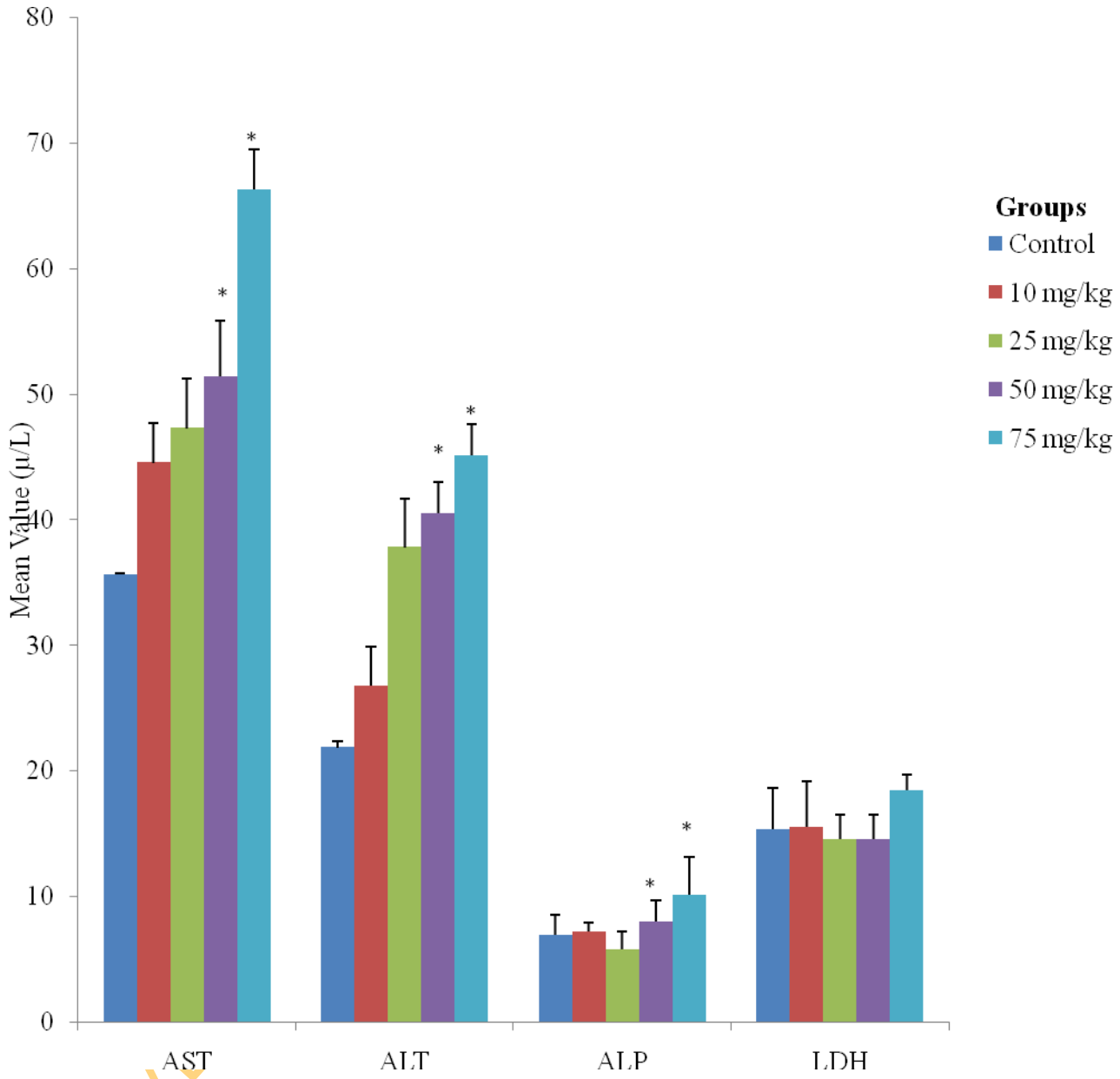

Figure 50: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on kidney enzymes of rats.

* = Significantly different from control

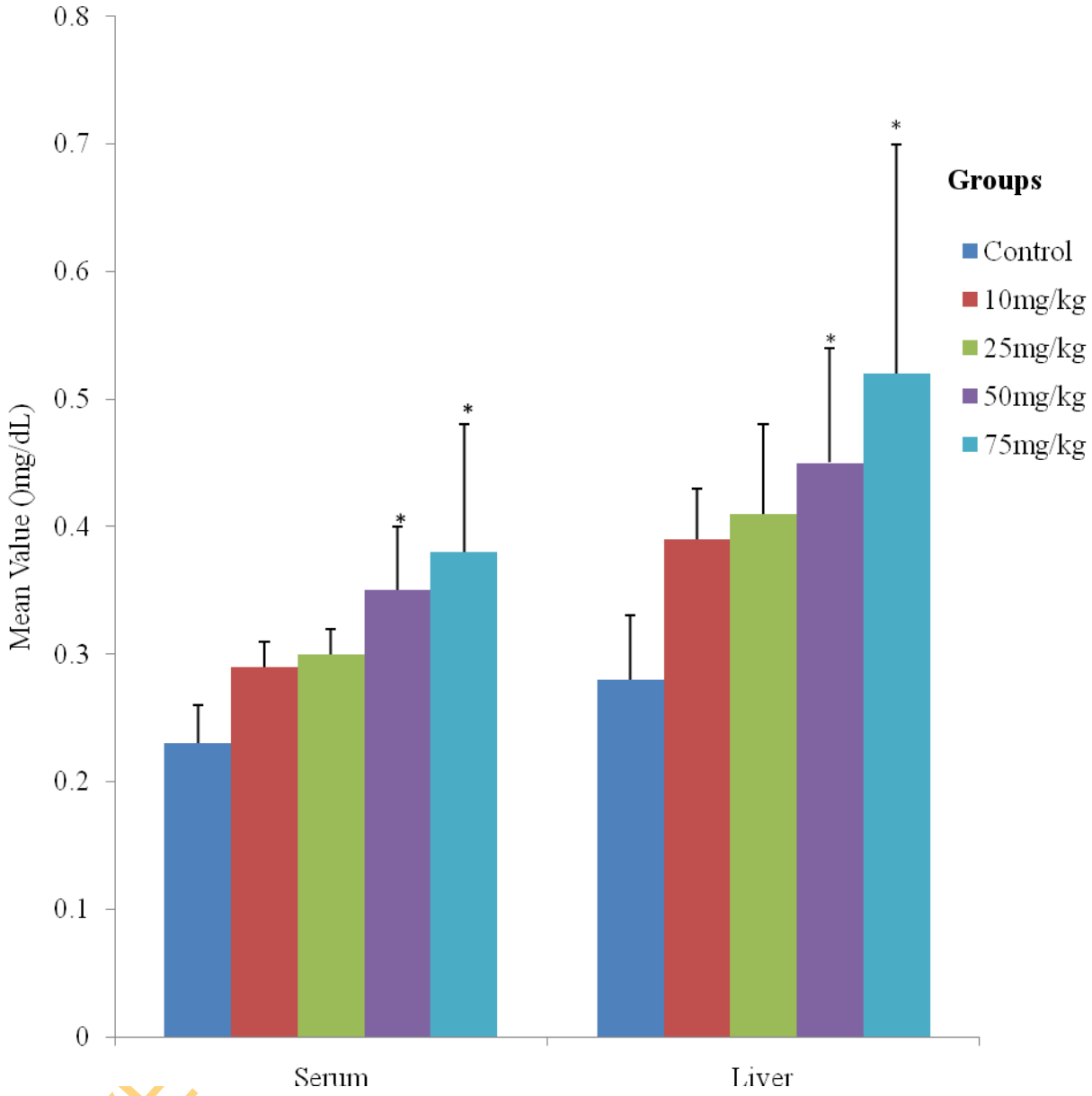

Figure 51: Effect of 30 days oral admineistration of crude methanol extract of the bulb of *Crinum jagus* on serum and liver direct bilirubin of rats.

* = Significantly different from control

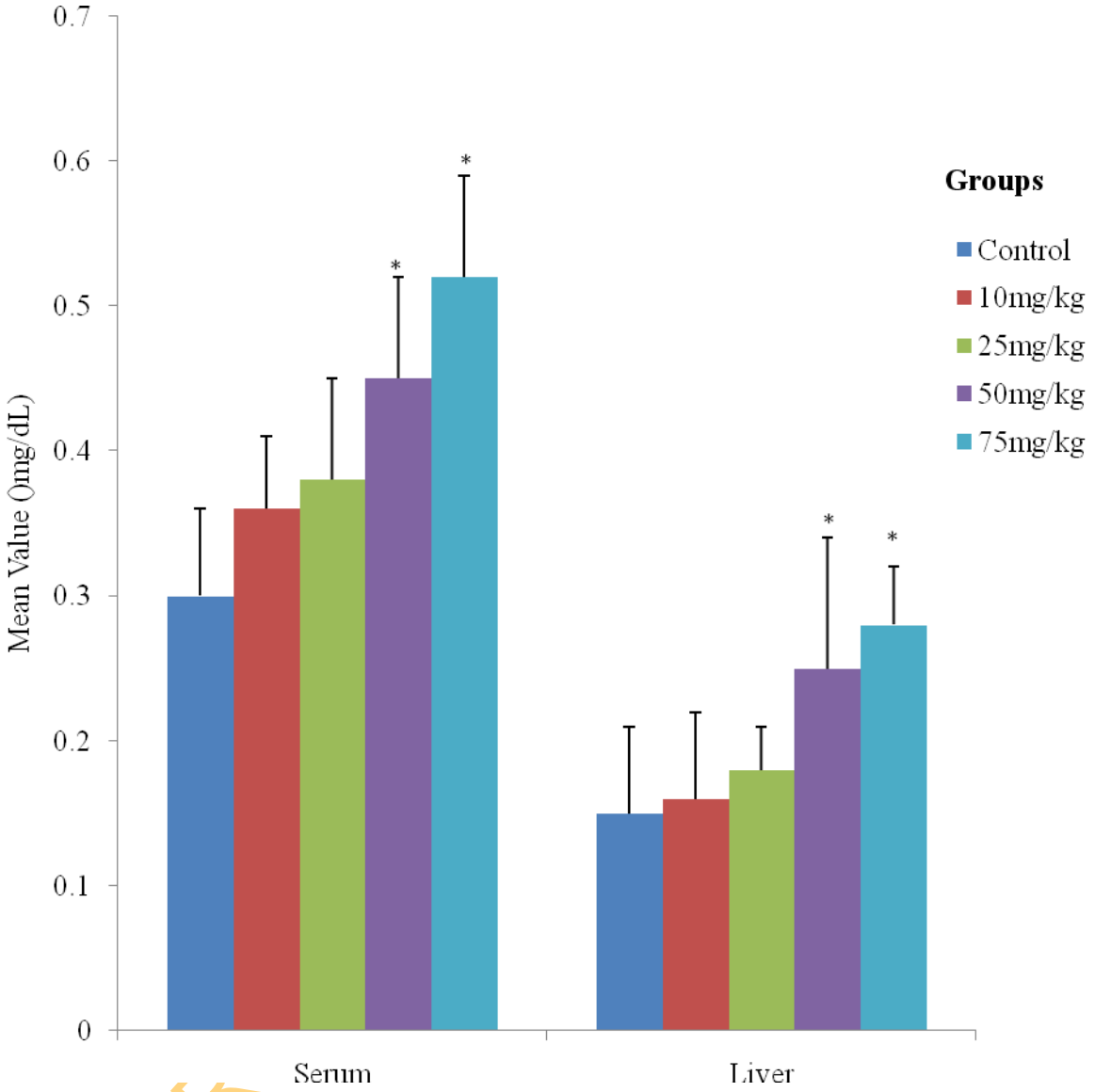

Figure 52: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on serum and liver total bilirubin of rats.

* = Significantly different from control

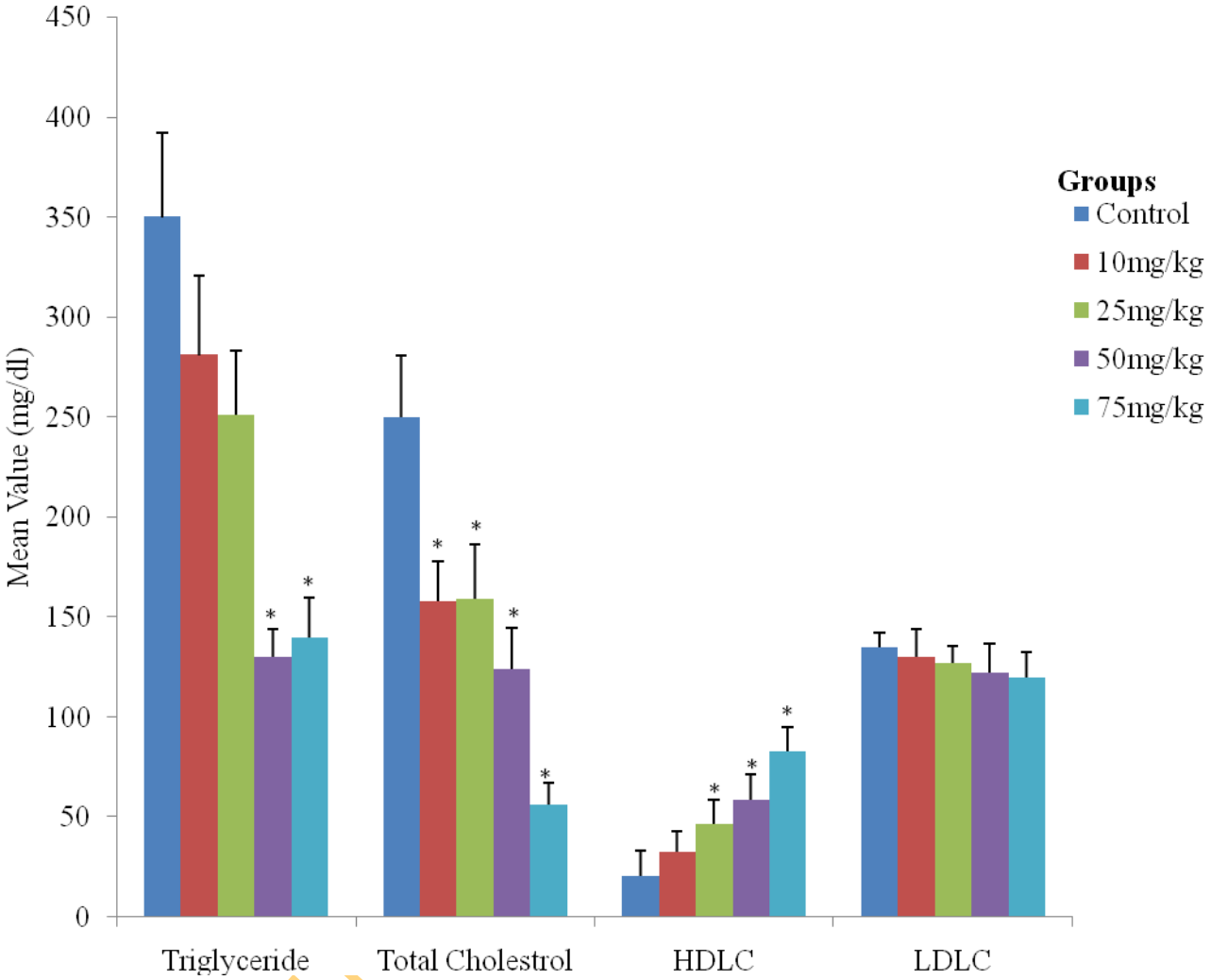

Figure 53: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on serum lipid profile of rats.

* = Significantly different from control

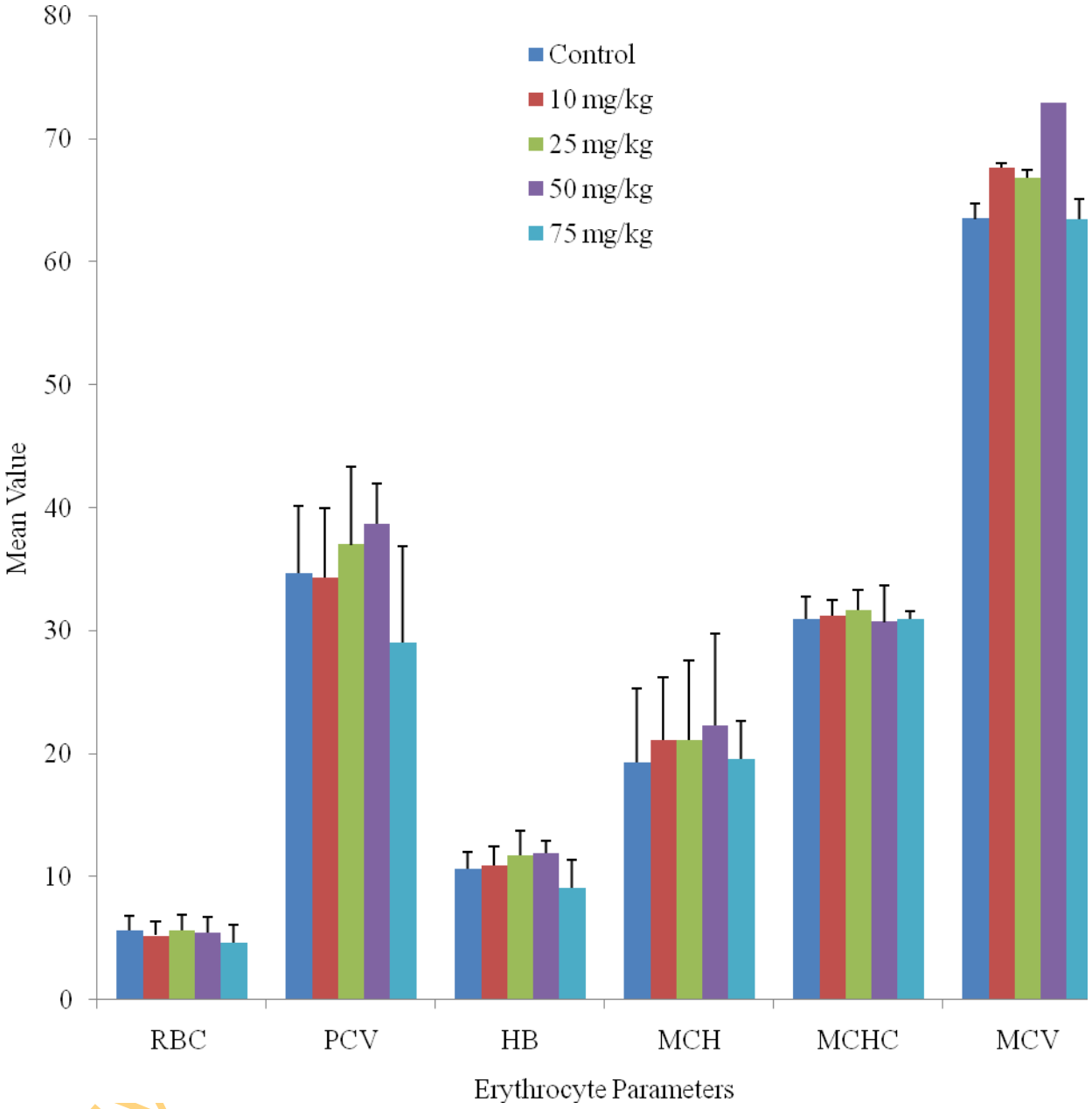

Figure 54: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on erythrocyte parameters of rats

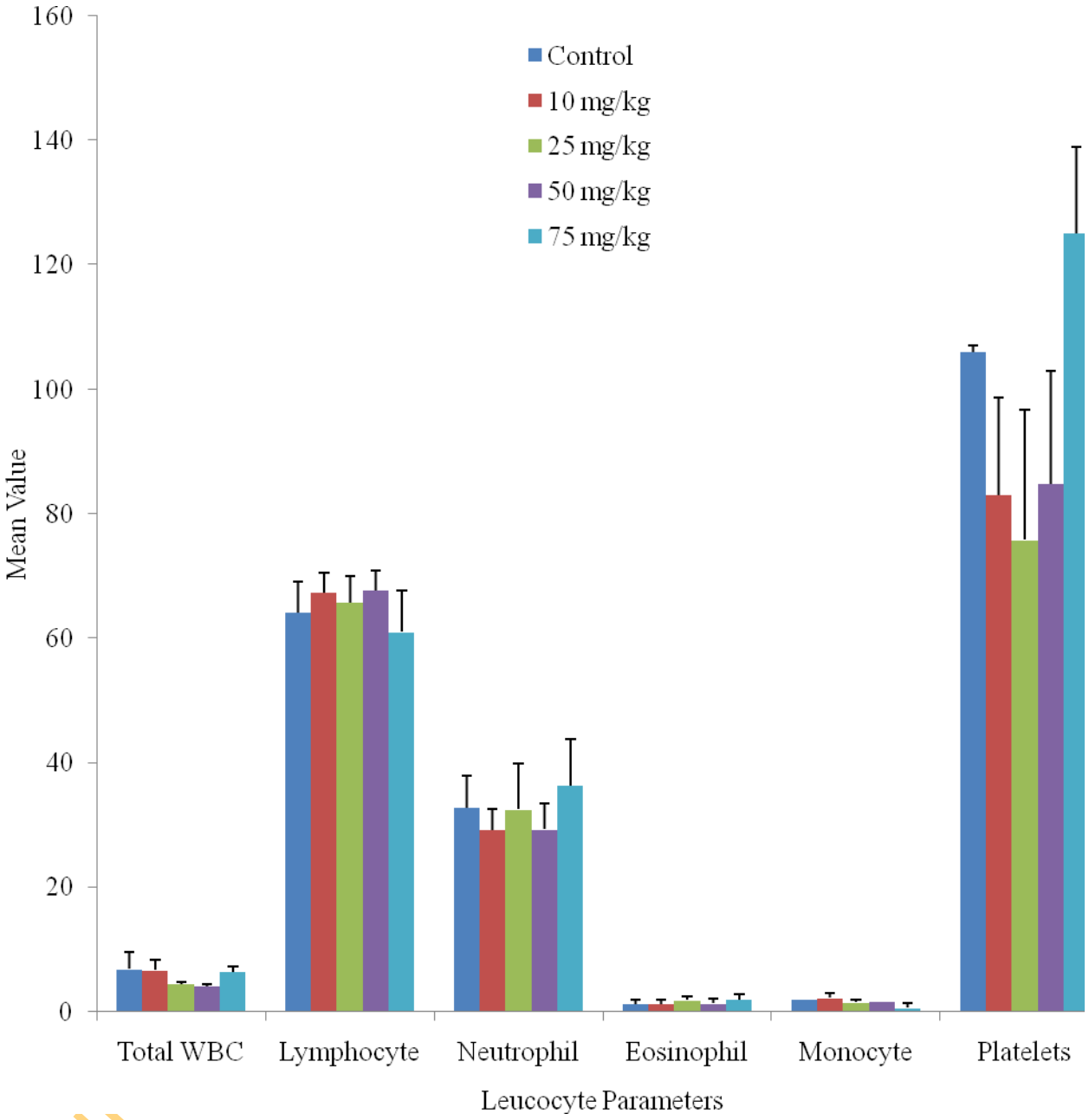

Figure 55: Effect of 30 days oral administration of crude methanol extract of the bulb of *Crinum jagus* on leucocyte parameters of rats.

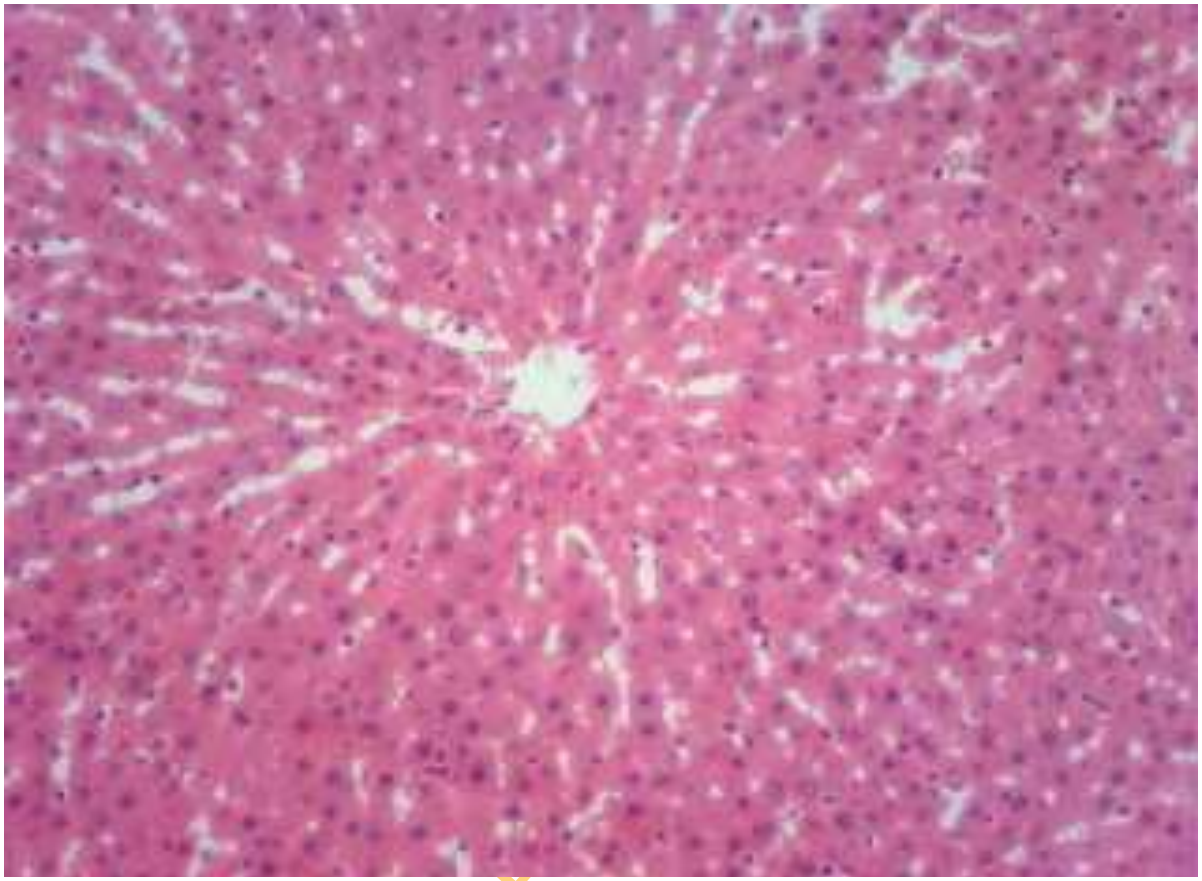

Plate 1A: Photomicrograph of the normal section of the liver of rats in the control group (X160).

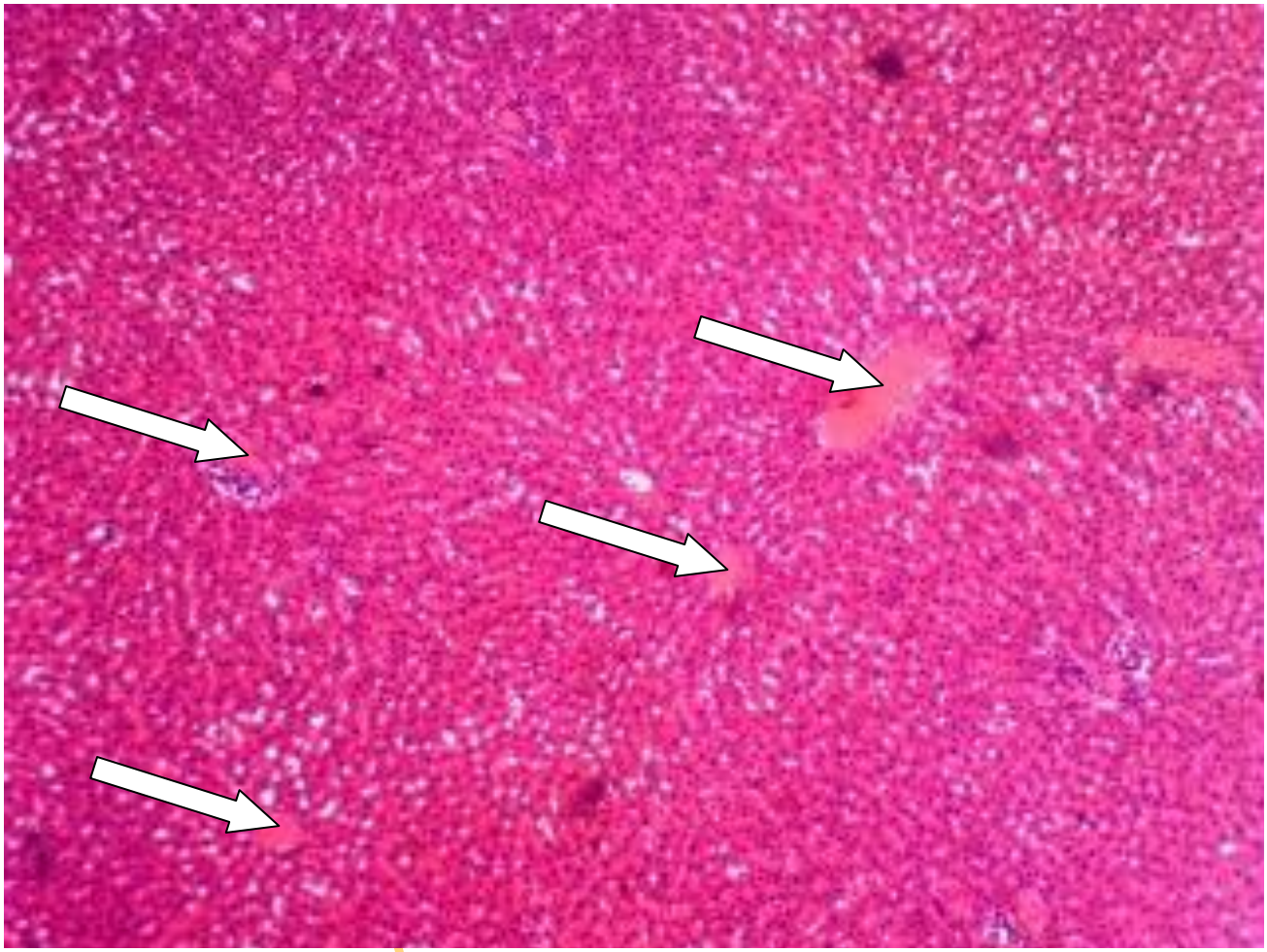

Plate 1B: Photomicrograph of the liver showing moderate haemorrhage in the renal interstitum of rats treated with low doses (10 and 25mg/kg) of *Crinum jagus* (X64).

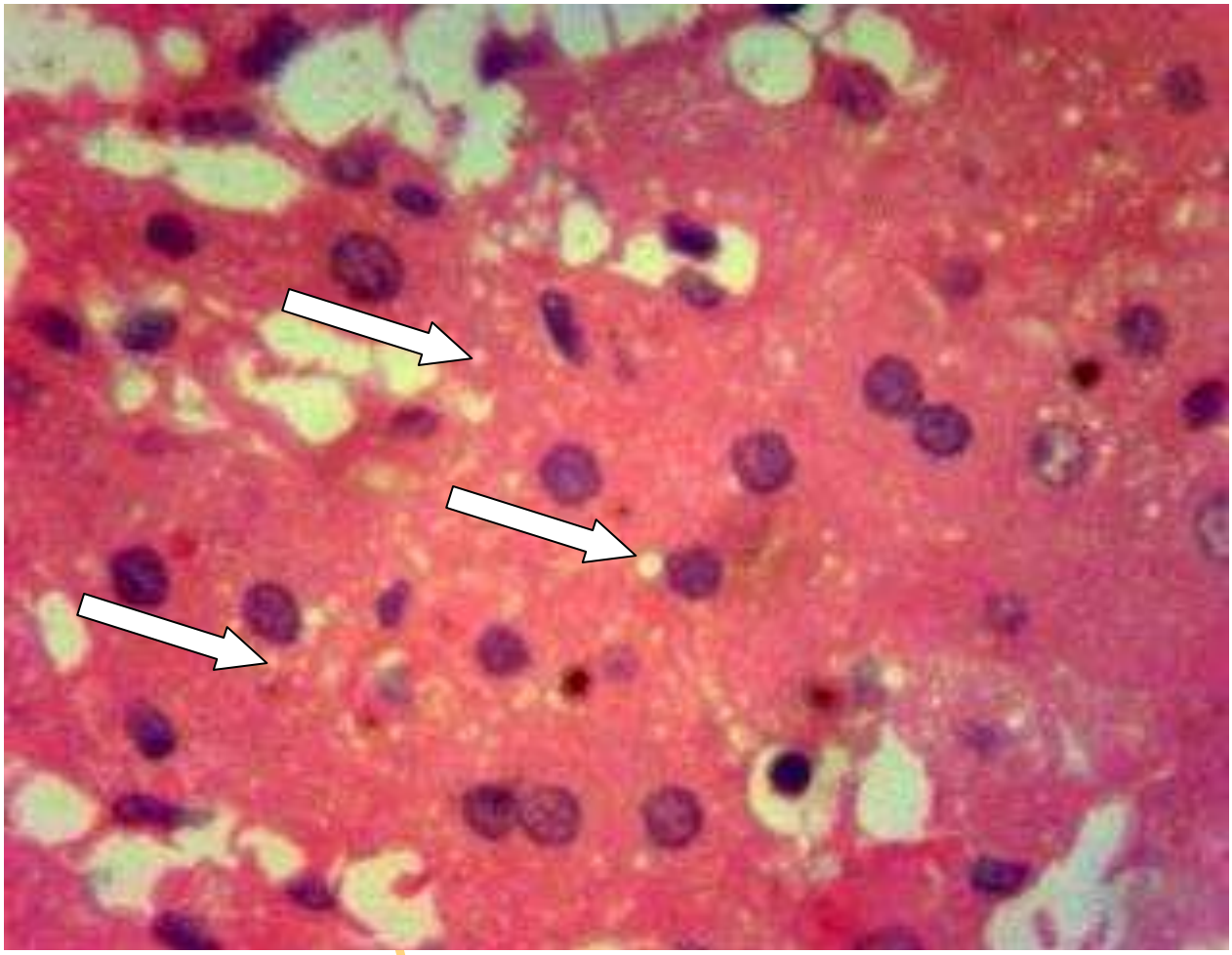

Plate 1C: Photomicrograph of the liver showing enlargement of the hepatocytes with multiple cytoplasmic vacuolations (fatty degenerations) of hepatocytes of rats treated with high doses (50 and 75mg/kg) of *Crinum jagus* (X640).

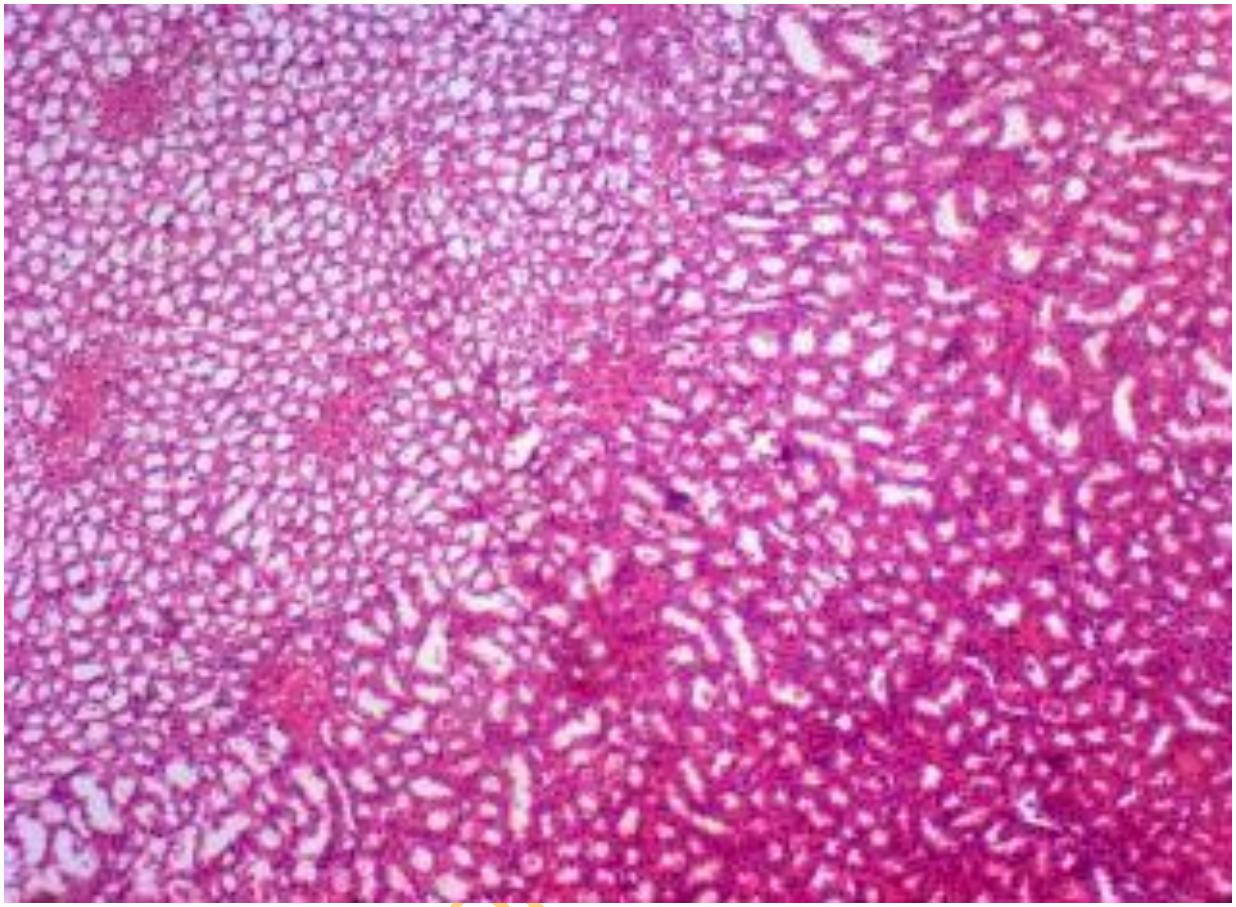

Plate 2A: Photomicrograph of the normal section of the kidney of rats in the control group (X160).

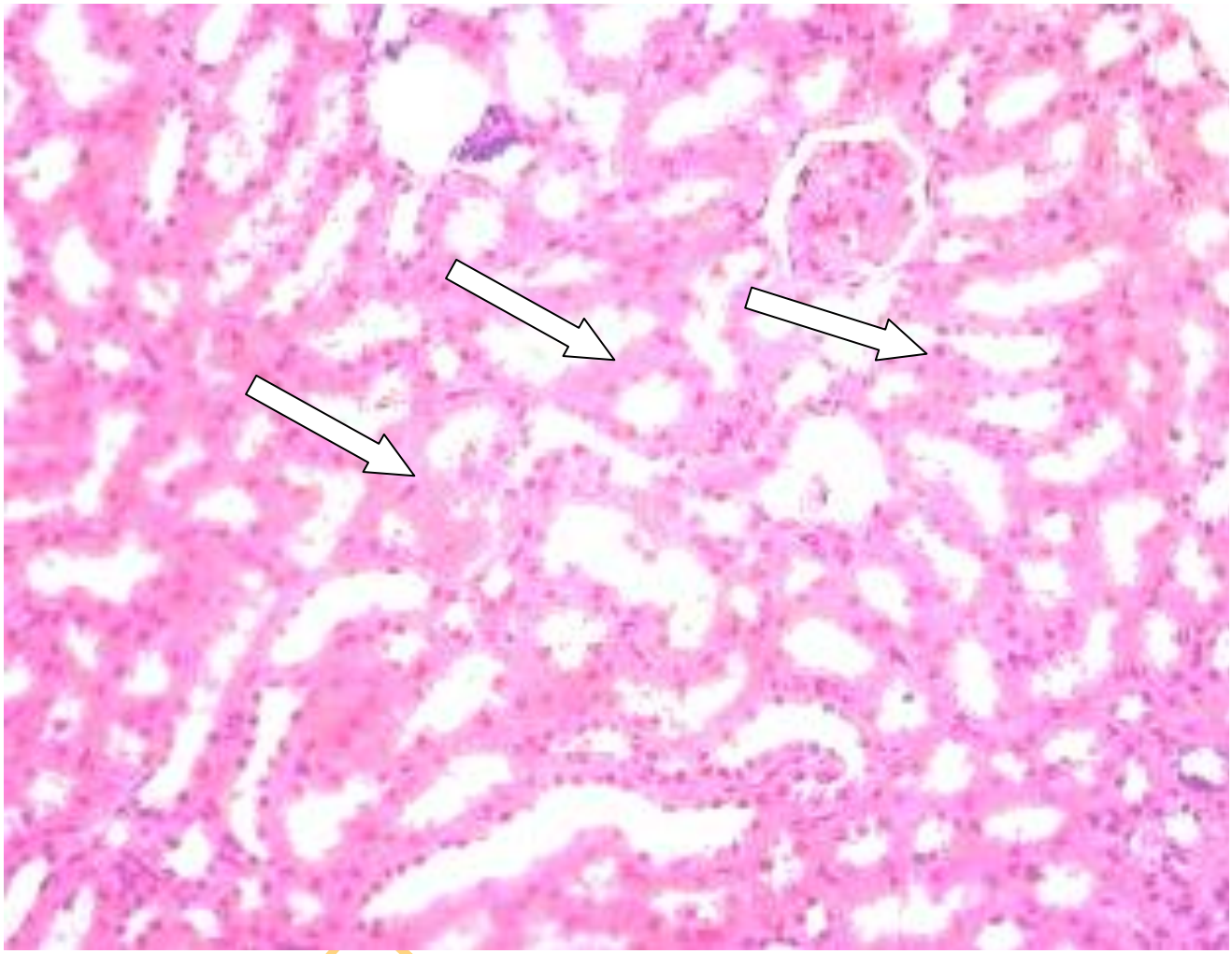

Plate 2B: Photomicrograph of the kidney showing moderate locally haemorrhage in the renal interstitium of rats treated with low doses (10 and 25mg/kg) of *Crinum jagus* (X64).

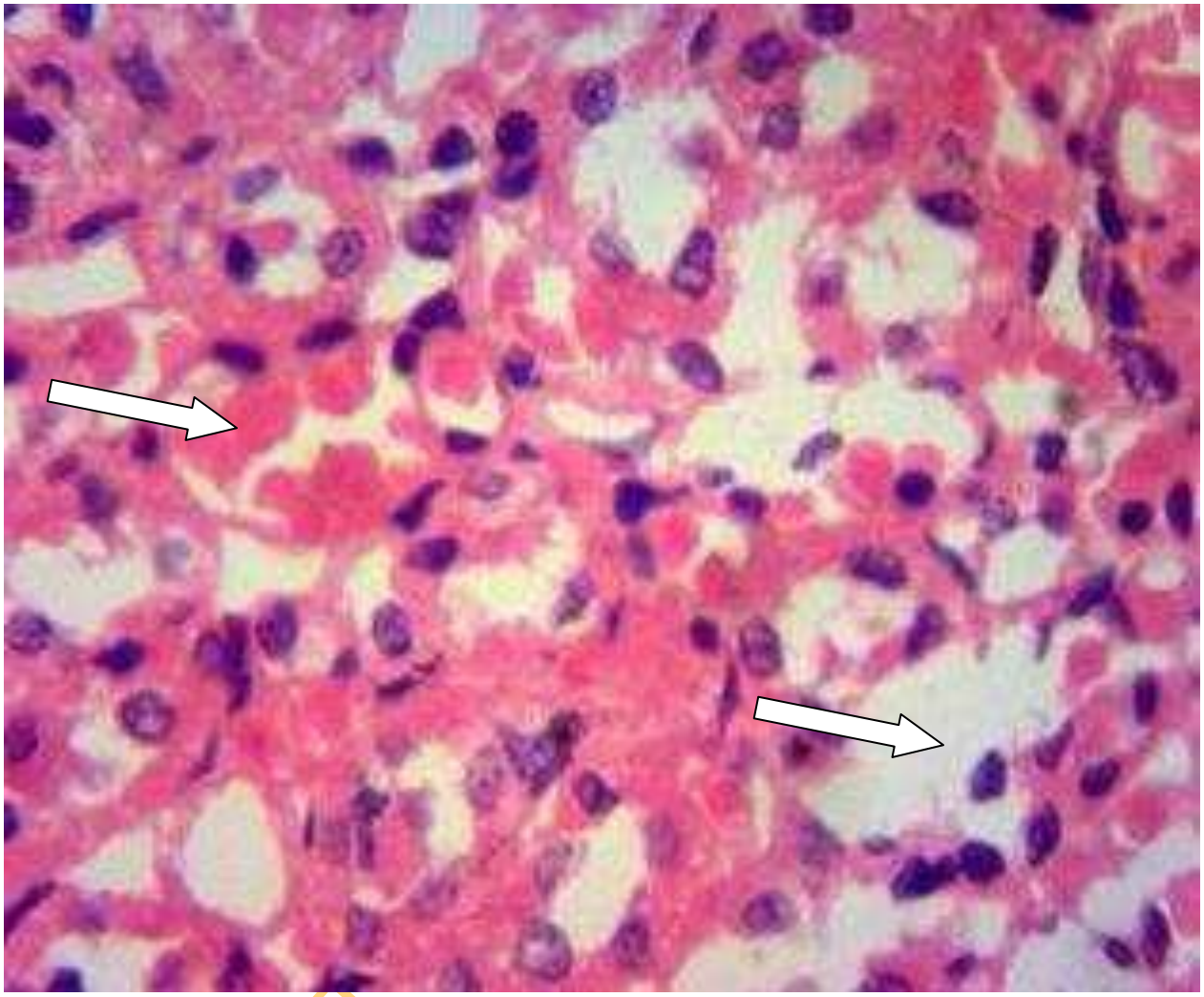

Plate 2C: Photomicrograph of the kidney showing necrosis of the tubular epithelium with sloughing of the necrotics cells into the lumen of the tubules and distruption of the basement membrane of rats treated with high doses (50 and 75mg/kg) of *Crinum jagus.* Some of the tubules denuded and dilated (X160).

Experiment 10 : Determination of Bioactive Compounds in Fraction 1 (F1) of the Bulb of *Crinum jagus* By Gas Chromatography Mass Spectroscopy (GC-MS) Technique

Introduction

Gas chromatography mass spectroscopy (GC-MS) is a method that combines the features of gas liquid chromatography and mass spectroscopy to identify different substances within a test sample. Applications of GC-MS include drug detection, environmental analysis and identification of unknown samples. In the present study, the crude methanol extract and fractions of the bulb of *Crinum jagus* was evaluated for five biological activities. For all the biological activities tested, F1 was the most potent. F1 was therefore analyzed by GC-MS to determine the bioactive compounds in the potent fraction.

Procedure

F1 was analysed by GC-MS using a schimadzu model QP2010 chromatogram, system with split/splitless injector interfaced to a 5973 mass selective detector. Innowax fused silica capillary (FSC) column (30m x 0.25mm with 0.25µm film thickness) was used with helium as carrier gas at a flow rate of 1ml/min. The GC oven temperature was kept at 8^0C (hold for 2mins) and programmed to reach 200^0C at a rate of 4^0C/ minute, then kept constant at 28^0C for 10 minutes being the final hold time. Mass range was from m/z 30 to 50V. Identification of components was achieved on the basis of their retention indices determined with a reference to a homologous series of n-alkanes and by comparison of their mass spectral fragmentation patterns (NIST Data base/ chemstation data system) with data previously reported in literature (Mclafferty and Staufer, 1989, Joulain and Konig, 1998, Adams, 2010).

Results

GC-MS analysis of F1 of the bulb of *Crinum jagus* clearly showed the presence of ten compounds. The active principles with their retention time (RT) and concentration (peak area %) are presented in Table 27. The GC-MS chromatogram of the ten peaks of the compounds detected is shown in Appendix 4. The total number of compounds identified

in the fraction, the GC-MS retention time (RT) and percentage peak of the individual compounds revealed that 5-hydroxymethyl furfural (66.70%) was found as the major component in the fraction while the nine minor compounds in the fractions are 2,3,diyhydro-3,5-dihydroy-6-methyl-4(H)-pyra-4-one (7.58%), 1,2-Hydroxyethyl-4-methyl piperazine (1.68%), E–beta-Farnese (2.80%), Germacrene D, 1-methyl-5-methylene–8 (1-methylethyl)-1,6 cyclodecadiene (1.68%), 1-methyl, 1-4-(5 -methyl-1-methylene-4-hexenylcycloxygenase (1.63%), β-sesquiphellandrene (6.84%), Palmitic acid (2.95%), 5, 6, 8, 9 tetrahydrobenz (A) anthracen-11-(10H)-one (2.88%) 1, 1, biphenyl –2 formly- 4,5,6 trimethoxy–carbaldehyde (5.25%).

Conclusion

In the present study ten chemical constituents have been identified from F1 of the bulb of *Crinum jagus* by gas chromatography mass spectrometry (GC-MS) analysis.

Table 27 : Phyto-components identified in the Fraction 1 (F1) of the bulb of *Crinum jagus*

Peak No	Name of Compound	RetentionTime (minutes)	% Composition
1	2,3,dihydro, 3,5-dihydroxy-6-methyl-4 (H)- pyra-4-one	7.881	7.58
2	5-hydroxymethyl furfural	9.553	66.70
3	1,2 hydroxyethyl-4-methyl piperazine	10.818	1.68
4	E-beta-Farnese	12.170	2.80
5	Germacrene D,1-methyl-5 methylene -8-(1-methylethyl)-1,6 cyclodecadiene	12.756	1.68
6	1-methyl-4-(5methyl-1-methylene-4-hexenylcylooxygenase	12.986	1.63
7	β-sesquiphellandrene	13.227	6.84
8	Palmitic acid	19.927	2.95
9	5,6,8,9 tetrahydrobene (A) anthracen -11 (10H) - one	28.502	2.88
10	1,1,biphenyl,2-formly-4,5,6 trimethoxy carbaldehyde	28.792	5.25
		Total	**100.00**

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CHAPTER FIVE

5.1 DISCUSSION

Plants have been used as sources of medicine by virtually all cultures for several thousand years (Baguar, 1995, Abu-Rabia, 2005). Traditional and folklore medicine play an important role in health services around the globe. About three quarters of the World's population relies on plants and its extract for health care (Premanathan *et a*l., 2000; Gabhe *et al.*, 2006). A large number of people particularly those living in the villages depend largely on herbal remedies. Its acceptability for primary health care is not limited to poor developing countries but in countries where conventional medicine is predominantly in the national health care system (Lafranco, 1999). Therefore, plants constitute the main medicinal source for the treatment of diseases. WHO (2001) reported that herbal medicine serve the health needs of about 80% of the world population, especially for million of people in the vast rural areas of the developing countries. Traditional medicine has remained the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities and local therapy is the only means of medical treatment for such communities (Yinger and Yewhalaw, 2007). Plants have the ability to synthesise a wide variety of chemical compounds that are used to perform important biological functions. Many of these phytochemicals have beneficial effects on long term health when consumed by humans and can be used effectively to treat human diseases. At least, 12,000 such compounds have been isolated so far, a number estimated to be less than 10% of the total (Lai and Roy, 2004, Tapsell *et al.*, 2006). Chemical compounds in plants mediate their effects on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs thus herbal medicine do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicine to be as effective as conventional medicine.

Medicinal plants have pharmacological activities of possible therapeutic use. Many of the pharmaceuticals currently available to physicians have a long history of use as herbal

remedies, including aspirin. Careful and planned investigation of plants are needed in order to develop new drugs that meet the criteria of normal treatment. Ethnobotany is preliminary method of research, suitable for gathering information on the use of plants. The "quack" medical knowledge handed down by the common people constitutes sources of information useful for scientific research. Many plant utilized exclusively in popular tradition, when exposed under scientific examination have been found to be useful for different sectors in the industry (Lentini, 2000). Noteworthy break-through are being produced by researchers and there is a steady progress and indeed very promising trend towards meaningful research into the development of essential drugs from numerous local medicinal plants in the developing countries of the world. Africa is known to be richly endowed with medicinal plants (Winifred, 2011) and one of such plant is *Crinum jagus*.

Crinum jagus is a bulbous plant with spirally arranged leaves and conspicious flower, it belongs to the family; Amaryllidaceae, genus : *Crinum*, species : *jagus*. Its local name is Ogede Odo in Yoruba, Alubarha in Edo, Oyimmbaker in Efik/ Ibibio. It is the largest tropical genus of Amaryllidaceae family (Ode *et al.,* 2010) and the plant may be found in swampy conditions, seasonal wet lands or grassland savannah. In Nigeria ethnomedicine, the use of *Crinum jagus* for various health conditions such as antitumour action (Burkill 1985); antibacterial, antifungal and antiviral actions (Adesanya *et al*., 1992); immunostimulating action (Fennel *et al*., 2001); anticonvulsant action (Edema and Okiemen, 2002); anticholinergic action (Peter *et al*., 2004); anti-tuberculosis action (Idu *et al*., 2010); antioxidant action (Ode *et a*l., 2010); anti-asthmatic action (Ogunkunle and Olopade, 2011); and antimalaria action (Osakwe *et al*., 2011) has been documented. Despite, the reported folkoric uses there are limited scientific claims to substantiate some of these claims. Thus effort in this project was devoted to evaluating the biological activities of *Crinum jagus* to determine and validate the folkoric claims. The biological activities investigated were, antioxidant, antimicrobial, anti-inflammatory, antimalaria and anti-tuberculosis properties of the plant. The toxicological profile of the plant was also investigated to ascertain the safety of the plant.

Antioxidant Activity

During the process of oxygen utilization, in a normal physiological and metabolic process, approximately five percent of oxygen gets univalently reduced to oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals. All these radicals known as reactive oxygen species (ROS) exert oxidative stress towards the cells of human body rendering each cell to face about 10,000 oxidative hits per second (Mondal, 2006). Oxidative stress has been recognized to have a pathological role in many types of chronic diseases such as diabetes, heart disease and cancer. Oxidative stress occurs when the formation of the free radicals increases (Elmastas *et al.*, 2006). In oxidative stress, the balance between the formation of reactive oxygen species and amount of antioxidants is destroyed. Oxidative stress causes damage to cell components such as proteins, lipids and nucleic acid (Rahim *et al.*, 2005; Wright *et al.*, 2006; Gladine *et al.*, 2007) and eventually leads to cell death (Naziroglu *et al.*, 2004; Emekli-Alturfan *et al.*, 2009). Recently, interest has increased considerably in finding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants which are being restricted due to their side effects such as carcinogenicity (Kumaran, 2007). Antioxidants from natural resources possess multifacetedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance (Shriwaikar, 2006). Food industry uses natural antioxidants as a replacement of conventional synthetic antioxidants (Govindaragan, 2003). In this study, the antioxidant activity of the crude methanol extract and fractions of the bulb of *Crinum jagus* was assessed using the *in-vitro* and *in-vivo* models.

***In vitro* Antioxidant Activity**

In vitro antioxidant capacity of the plant was studied by analyzing five biochemical parameters which are indicative of antioxidant potential of the plant. The studied biochemical parameters were DPPH radical scavenging activity, hydroxyl radical scavenging activity, reductive potential, total flavonoid and total phenolic contents of the plant.

DPPH assay is one of the most widely used method for screening antioxidant activity (Nanjo *et al.*, 1996). Diphenylpicrylhydrazyl (DPPH) is a free radical that is stable at room temperature. It produces a purple or violet colour in methanol. On reduction in the presence of an antioxidant, a yellow solution is produced. The percentage DPPH radical scavenging activity of an antioxidant is a measure of its ability to donate a proton to the DPPH free radical to yield diphenylpicrylhydrazine which is more stable giving rise to yellow colour. In this study, the crude methanol extract and the fractions of the bulb of *Crinum jagus*, all scavenged DPPH in a concentration-dependent manner (Figures 16 and 17). At 500µg/ml the crude extract, F1, F2 and F3 scavenged DPPH radicals by 72.46%, 81.37%, 57.83% and 69.83%, respectively. Highest percentage scavenging effect was obtained with F1 , though the DPPH scavenging activity of extract and the fractions were less than that of ascorbic acid (the standard antioxidant). The study showed that the crude extract and the fractions have proton donating ability and could serve as free radical scavengers acting possibly as primary antioxidants. This observation is in line with the findings of Ode *et al*., (2010) who reported the DPPH scavenging effect of the crude methanol extract of the bulb of *Crinum jagus.*

Hydroxyl radical is the final mediator of most tissue damage (Lloyd *et al.*, 1997). All reactive oxygen species exert most of their pathological effects by giving rise to hydroxyl radical because hydroxyl radical react with almost every type of molecules found in living cells including sugars, amino acids, lipids and nucleotides. Hydroxyl radical formation can occur in several ways. It can be generated by univalent reduction of oxygen (Han *et al*., 2003). The most important mechanism by which it is generated *in-vivo* is likely to be transition metal catalyzed decomposition of superoxide and hydrogen peroxide (Stohls *et al*., 1995). The percentage hydroxyl radical scavenging activities of the crude extract and its fractions are presented in Figures 18 and 19. The crude extract and its fractions scavenged hydroxyl radical in a concentration dependent manner. The crude extract, F1, F2 and F3 at 500µg/ml scavenged hydroxyl radical by 71.20%, 88.32% ,66.74%, 75.81%, respectively. The highest percentage hydroxyl radical

scavenging activity was detected in F1 (88.32%) at 500µg/ml. The hydroxyl radical scavenging activity of F1 compared well with that of ascorbic (89.32%) at 500µg/ml.

Hydroxyl radical induces various injuries to the surrounding organs and play a vital role in some clinical disorders. Any compound, natural or synthetic with antioxidant activity might contribute towards the total or partial elevation of this damage. Therefore, removing hydroxyl radical is one of the effective defence of a living body against diseases (Jer Min *et al.*,1995). This study showed that the crude extract and fractions of the bulb of *Crinum jagus* possess antioxidant activity which could exert a beneficial action against pathological alteration caused by the presence of hydroxyl radicals.

Reducing power of a compound serves as a significant indicator of its potential antioxidant activity (Gulcin and Oktay, 2003). The reducing ability of a compound depends on the existence of reductones (antioxidant) which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom (Meir *et al.*, 1997 *)*. The reducing power of the extract and the fractions increased with increasing amount of the sample. The conversion of Fe^{3+} into Fe^{2+} in the presence of the extract and the fractions was used to determine the reducing ability of the plant. F1 showed the highest reducing ability. The antioxidant activity of the crude extract and the fractions might partially be as a result of its reducing ability.

The concentrations of total flavonoid content of the plant extract and their fractions are shown in Figures 22 and 23 while that of the total phenolic content are presented in Figures 24 and 25. At a concentration of 500µg/ml, the total flavonoid content of the crude extract, F1, F2 and F3 were 0.584, 0.864, 0.396 and .0643µg/g quercetin equivalent respectively. The total phenolic content of crude extract and the fractions at 500µg/ml were 0.356, 0.460 ,0.240 and 0.380µg/mg garllic acid equivalent respectively. The highest concentration of total flavonoid and total phenolic were obtained from F1. Phenolics are ever present secondary metabolite in plants and possess wide range of therapeutic uses. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. Total phenolic assay by using Folin-Ciolcateu reagent is a simple,

convenient and reproducible method. It is employed in studying phenolic antioxidants (Huang *et al*., 2005).

Flavonoids are groups of naturally occurring compounds widely distributed as secondary metabolites in plant kingdom and have been reported to possess antioxidant and anti-radical properties (Nakayoma and Yamada, 1995). The protective effect of many flavonoids and other phenolic compounds in leaves are considered to be related to various antioxidant contained in them (Shahidi *et al*., 1992), while a strong correlation between total phenolic content and anti-oxidant activity has been reported by Velioglu *et al.*, (1998) and Javanmardi *et al.*, (2003). The presence of phenolic and flavonoid contents in the crude extract and fractions of the bulb of *Crinum jagus* may have contributed directly to the antioxidant activity of the plant by neutralizing the free radicals. The preliminary phytochemical screening of the extract showed the presence of phenols and flavonoids.

***In vivo* Antioxidant Activity**

Antioxidants can be classified into two major classes. The non enzymatic and enzymatic antioxidants. The enzymatic antioxidants include superoxide, catalase, and glutathione peroxidases. Crucial components of antioxidant defence system in the body are cellular antioxidant system which are involved in the reduction of reactive oxygen species (ROS) and peroxides as well as in the detoxification of certain compounds of exogenous origin, thus playing a primary role in the maintenance of a balanced redox status (Michiels *et al*., 1994) hence they can serve as potential marker of susceptibility, early and reversible tissue damage and of decrease in antioxidant defence (Lester, 1994). In the present study, the *in vivo* antioxidant capacity of the crude extract and the fractions of the plant was evaluated by studying the effect of thirty days oral administration of the extract and fractions of the plant on hepatic and renal malondialdehyde (Lipid peroxidation), superoxide dismutase, catalase and glutathione levels in experimental animals.

Malondialdehyde (MDA) is the major oxidative product of peroxidised polyunsaturated fatty acid and increase in malondialdehyde content is an important

indicator of lipid peroxidation . The present study assessed the effect of thirty days oral administration of the crude extract and fractions of the bulb of *Crinum jagus* on malondialdehyde concentrations in the liver and kidney of rats. Thirty days oral administration of the crude methanol extract of the bulb of *Crinum jagus* (10, 25, 50 and 75 mg/kg) caused no significant reduction in malondialdehyde (MDA) level in the liver while a significant ($P < 0.05$) and dose dependent reduction of MDA level was observed in the kidney compared to the control group. Fractionation of the crude extract does not cause loss of biological activity but rather improved the antioxidant activity of the plant. The three fractions (F1, F2 and F3) at 5 and 10mg/kg caused significant reduction ($P < 0.05$) in the hepatic and renal MDA levels relative to the control group. F1 showed the highest inhibitory activity. Significant reduction of MDA levels by the crude extract and fractions of the bulb of *Crinum jagus* indicate that the plant has protective potential against free radical induced lipid peroxidation both in liver and kidney by promoting cell membrane stability.

Superoxide dismutase and catalase are endogenous protective factors, they act by scavenging free radicals in the defense against oxidative cell injury. The enzyme are the major enzymes that catalyse the inactivation of reactive oxygen species derived from redox process of xenobiotics in the liver (Malchin and Benedich, 1987). In the present study, thirty days of oral administration of the crude methanol extract of the bulb of *Crinum jagus* resulted in significant ($P < 0.05$) increase in superoxide dismutase activity in the liver in the 25, 50 and 75mg/kg treated groups compared with the control group. The kidney also demonstrated a significant($P < 0.05$) and dose dependent elevation of superoxide dismutase activity in all the treated groups. The two doses of F1 (5 and 10mg/kg) administered for 30 days produced significant elevation of superoxide dismutase both in the liver and kidney. With F3, significant ($P < 0.05$) elevation of superoxide activity was only observed with the highest dose (10mg/kg) both in the liver and kidney. F2 caused an insignificant elevation of SOD activity in the liver at 10mg/kg while in the kidney, administration of F2 elevated superoxide activity. The observed increase in SOD activity suggests that the crude methanol extract and fractions of the

bulb of *Crinum jagus* has an efficient protective mechanism in response to reactive oxygen species (ROS).

Catalase is an antioxidant enzyme distributed in all animal tissues including red blood cells and liver. Catalase is considered as most important hydrogen peroxide removing enzyme and also a key component of antioxidative defense system. Catalase is a tetametric haemoprotein that undergoes alternate divalent oxidation and reduction at its active site in the presence of hydrogen peroxide. It is known that hydrogen peroxide can be destroyed by catalase which removes it when present in high concentration (Cascado *et al.*, 1995). The present study reveals that catalase activity was significantly increased in a dose dependent manner after thirty days of oral administration of 10, 25, 50 and 75mg/kg of the crude extract of the bulb of *Crinum jagus*. The two doses of F1 administered (5 and 10mg/kg) produced a significant elevation of catalase activity in the liver and kidney. F2 and F3 did not produced significant elevation of catalase activity in the liver and kidney after administration of 10mg/kg dose for thirty days. The antioxidant activity of the crude extract and fractions of the bulb of *Crinum jagus* may be related to their increased catalase activity in the liver and kidney resulting in the protection of the tissue by catalase.

Glutathione is a major non protein thiol in living organisms, it plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. The level of reduced glutathione (GSH) consumption is a measure of non enzymic antioxidant and cellular redox status of cells of higher animals (Chance and Boveris, 1979). Result from this study showed that oral administration of different doses of the crude methanol extract of the bulb of *Crinum jagus* increased the level of reduced glutathione in the liver and kidney relative to control. The liver and kidney glutathione levels were significantly elevated in the groups treated with F1 and F3 compared with the control group with a more pronounced effect with F1. F3 only produced significant elevation of reduced glutathione levels in the liver and kidney at higher doses. F1 demonstrated significant elevation of hepatic and renal glutathione when both low and high doses of F1 (5 and 10mg/kg) were administered. F2

did not produce significant elevation of hepatic and renal glutathione levels when compared with the control group. The result from this study showed that the crude methanol extract and fractions of the bulb of *Crinum jagus* increased the overall redox status in the liver and kidney as indicated by significant increase in the level of reduced glutathione in both liver and kidney compared with the control group.

Glutathione-S-transferase (GST) is a family of multi functional isoenzymes found in all eukaryotes catalyzing both glutathione dependent and reduction reaction (Rajurkar *et al.*, 2003). One major function of GST is to catalyse biotransformation of xenobiotics including drug detoxification in the mercapturic acid pathway leading to elimination of toxic compound (Hayes and Pulford, 1995) and also acting as antioxidant enzyme (Adaramoye and Adeyemi, 2006). The result from this finding shows that administration of the varying concentration of the crude methanol extract as well as F1 and F3 of the bulb of *Crinum jagus* significantly ($P < 0.05$) increased the activity of GST relative to control in both liver and kidney. F2 produced a non significant increase in hepatic and renal GST activity. Studies have shown that moderate oxidative stress may result in an induction of GST while severe oxidative stress may result in its decrease with concomitant depletion of GSH (Aniya and Nauto, 1993) which is a measure of the overall redox status of the cells in the blood. Increase in the level of GST activity, following thirty days of oral administration of the crude methanol extract and fractions of the bulb of *Crinum jagus*, is an indication that the plant plays an important role as a scavenger of free radicals. As the crude extract and fractions of the bulb of *Crinum jagus* improved the SOD, catalase, glutathione levels and reduced lipid peroxidation in the liver and kidney. It can be concluded that the crude extract and fractions of the bulb of *Crinum jagus* possesses *in-vivo* antioxidant activity and can be employed in protecting tissue from oxidative stress with F1 showing the highest antioxidant activity.

Antimicrobial Activity

The use of plant-derived compounds to treat infections is an age-long practice in many parts of the world, especially in developing countries where there is dependence on traditional medicine for a variety of diseases (Shiba *et al.*, 2005; Gangoue-Pieboji *et al.*,

2006). Even though, pharmaceutical industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, action must be taken to reduce this problem, for example to control the use of antibiotics, develop research to better understand the genetic mechanisms of resistance and to continue studies to develop new drugs, either synthetic or natural. The interest in plants with antimicrobial properties was revived as a result of current problems associated with the use of antibiotics (Shiota *et al.*, 2004; Abu-Shanab *et al.*, 2004). The use of plant extracts and phytochemicals for antimicrobial properties can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Sousa *et al.*, 1991; Shapoval *et al.*, 1994; Izzo *et al.*, 1995).

Crinum jagus is a known medicinal plant widely used in Africa as an antimicrobial agent (Adesanya *et al.*,1992). In the present study, the antibacterial and antifungal activities of the crude methanol extract and fractions of the bulb of *Crinum jagus* against several human pathogenic bacteria and fungi was investigated to provide scientific justification for the traditional use of the plant for the treatment of infectious diseases.

The results of the antimicrobial screening of the crude methanol extract and fractions of the bulb of *Crinum jagus* are presented in Tables 11, 12, 13, 14 and 15. The crude extract exhibited considerable level of inhibition against all the test organisms, with the exception of *E.coli*. This is in consonance with the frequently reported cases of development of multi-drug resistance to many antibiotics by bacteria of which *E.coli* is the most prominent (Alonso *et al.*, 2000; Sader *et al.*, 2002; Fasuan, 2004). *Klebsiella prenumonae* was the most susceptible bacterium to the extract, of all the tested bacteria with inhibition zones ranging from 10.00-28.00mm while *Aspergillus niger* was the most susceptible fungus with inhibition zones ranging from 10.00-16.00mm. The fractions (F1, F2 and F3) inhibited the growth of all the test organisms including *E. coli* which was not inhibited by the crude extract.

The result of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the crude extract and fractions of the plant are presented in Tables 12, 16, 17 and 18. The crude extract demonstrated considerable antimicrobial activity with its MIC against Gram-positive bacteria ranging between 6.25-50mg/ml and that of Gram-negative bacteria ranged between 3.125-50mg/ml. MIC for the fungal isolates ranged between 25-200mg/ml. The extract showed the lowest MIC against *Klebsiella pneumonae* (3.125mg/ml). The broadest activity of the crude extract against most of the test bacteria was MIC of 12.5mg/ml. *Candida albicans* and *Candida tropicalis* had the lowest MIC value (25mg/ml) against the extract. The broadest activity of the extract against most fungi tested was MIC of 50mg/ml. The extract also showed bactericidal and fungicidal activities on the bacteria and fungi isolates. The broadest bactericidal activity of the extract against most of the test bacteria was MBC of 25mg/ml. *Klebsiella prenumonae* was the most susceptible bacteria to the plant extract. *Candida albicans and Candida tropicalis* were the most susceptible fungi to the extract with MFC value of 50mg/ml. The broadest activity of the extract against most of the fungi tested was observed at MFC. of 100mg/ml. The results of MIC determination showed that F1 wass the most potent of all three fractions tested. The MIC, MBC and MFC values of F1 for bacteria and fungi isolates were much lower than that of F2 and F3. The MIC value of F1 ranged between 0.20-3.125µg/ml for bacteria isolates and 0.39-3.125µg/ml for the fungi isolates. *Staphylococcus aureus* was the most susceptible bacteria to F1 at MIC value of 0.20µg/ml. *Candida albicans, Candida tropicals* and *Candida krusei* were much sensitive to F1 than the moulds with MIC values of 0.39µg/ml. *E.coli* which was not inhibited by the crude extract was inhibited by F1 at MIC value of 0.39µg/ml. The MBC and MFC value of F1 for bacteria and fungi were much lower than that of F2. F2 had moderate antibacterial and antifungal activities against the test organisms. The lowest MIC value of F2 was 0.39µg/ml against two of the bacteria and the lowest MIC value of 0.78µg/ml were observed against four of the fungi isolates. Also the MBC value of F2 for bacteria and fungi isolates were higher than that of F1, the lowest MIC value obtained for F3 was

0.39µg/ml against *Bacillus subtilis* and *E.coli* while for the fungi isolates, the lowest MIC value obtained was 1.56 µg/ml that is much higher than the MIC values of F1 and F2.

The broad spectrum antimicrobial activities of the plant extract may possibly be due to the identified phytochemicals in the plants such as alkaloids, flavonoids, saponins and phenols. These classes of compounds are known to have curative activity against several pathogens and therefore could suggest the use of the plant as a health remedy in folklore medicine (Hassan *et al.*, 2004; Usman and Osuji, 2007). Fractionation of the extract did not lead to the loss of antimicrobial activity against the same group of isolates rather the fractions were found to have a better and improved antimicrobial activity than the extract against the range of Gram-positive bacteria and Gram-negative bacteria, the fungal yeast and the moulds tested. F1 demonstrated the highest antimicrobial activity. This observation is in line with the findings of Adesanya *et al*., (1992) who reported that some alkaloids present in *Crinum jagus* possess antibacterial and antifungal activities.

Ability of an antimicrobial agent to inhibit the growth of bacterial (bacteriostatic) or to destroy the bacteria (bactericidal) are the two main properties searched for in a potential antimicrobial agent since each has its own importance. Bacteriostatic activity may be useful in mild bacteria infection in which the bacteriostatic agent will inhibit the growth of the microorganisms while the defence mechanism will take over to get rid of the offending organisms. From the study, it was suspected that the activity of F1 may be bactericidal in nature while the crude extract, F2 and F3 were bacteriostatic. The results are encouraging enough to pursue bioactivity guided fractionation and structural elucidation of F1, the most potent fraction as possible antimicrobial agent. These findings lend credence to the traditional use of *Crinum jagus* for the treatment of microbial infections.

Inflammatory Activity

Medicinal plants have been used as a form of therapy for the relief of pain throughout history (Almieda *et a*l., 2001). The treatment of rheumatic disorder is an area in which the practitioners and traditional medicine enjoy patronage (Akah and Mwabie, 1994). Natural products in general and medicinal plants in particular are believed to be

important sources of new chemical substance with potential therapeutic efficacy. The study of plant species traditionally used as pain killers is seen as fruitful research strategy in the search of new analgesic and anti-inflammatory drugs. However, for many of the plants in use, the real efficacy and the relevant active principles are unknown. Consequently, experimental studies aimed at demonstrating the pharmacological properties of these plants and identifying the relevant active principles are needed (Sosa *et al.*, 2002*). Crinum jagus* is reported in traditional literature for the treatment of snake bite (Ode *et al*., 2006), tuberculosis (Idu *et al*., 2010) and asthma cough (Ogunkunle and Olapade, 2011) which are all inflammatory disease. Therefore, the present research was undertaken to investigate the antiinflammatory activity of the crude extract and fractions of the plant using the carragenan induced rat paw oedema method (Winter *et al.*, 1962). The results of this study are presented in Figures 41 and 42. From the results it was observed that the crude methanol extract, the fractions of the plant and indomethacin significantly ($P < 0.05$) reduced the paw oedema 3 hours after carrageenan injection relative to the control group. The varying concentrations of the crude extract (10, 25, 50 and 75mg/kg) significantly ($P < 0.05$) inhibited oedema formation in the rat paw by 26.82%, 31.55%, 41.82 and 65.90%, respectively. At a dose level of 20mg/kg, F1, F2 and F3 significantly ($P < 0.05$) inhibited oedema formation in rat paw by 79.50%, 25.00% and 52.27%, respectively. The standard drug indomethacin at 5mg/kg inhibited oedema formation by 94.82%. The result showed that F1 and F3 were more potent as anti-inflammatory agent than the crude extract and F2 with F1 having the highest inhibitory activity (79.50%). This study has shown that the crude methanol extract and fractions of the bulb of *Crinum jagus* possess a significant anti-oedematogenic effect on paw oedema induced by carrageenan. Development of oedema induced by carrageenan is commonly correlated with early exudative stage of inflammation (Ozaki, 1990, Silva *et al.*, 2005). Carrageenan oedema is a multimediated phenomenon that liberates diversity of mediators. It is believed to be biphasic, the first phase (1hr) involves the release of serotonin and histamine while the second phase (over 1h) is mediated by prostaglandins, the cycloxygenase products and the continuity between the two phases is provided by

kinins (Silva *et al*., 2005; Perianayamgam *et al*., 2006). Since carrageenan induced inflammation model is a significant productive test for anti-inflammatory agents acting by the mediators of acute inflammation (Mossai *et al*., 1995; Sawadogo *et al*., 2006), the result of this study indicates that *Crinum jagus* can be effective in acute inflammatory disorders. The study has thus provided some justification for the folkoric use of the plant in several communities for conditions such as snake bite, tuberculosis and asthma cough. F1, due to its high and potent anti-inflammatory property will be chosen for further studies in which the active principle from the fraction will be isolated and this will help in understanding the mechanisms of the antinflammatory activity of *Crinum jagus*.

Antimalaria activity

Medicinal plants may provide antimalarial drugs directly as in the case of quinine from cinchona bark or they may supply template molecules on which to base further new structures by organic synthesis. The folkoric use of *Crinum jagus* for the treatment of malaria has been reported in literature (Osakwe *et al*., 2011). In this study the *in vivo* antimalaria activity of the crude extract and fractions of the plant were investigated. The result of the *in-vivo* evaluation of the crude extract and fractions of the bulb of *Crinum jagus* on established infection are presented in Table 18, Figures 43 and 44. Treatment of *Plasmodium berghei* infected mice with the extract and the fractions produced a dose dependent chemosuppression activity. Percentage suppression of parasitaemia was observed to increase as extract concentration increased. By day 6 post infection, there was significant ($P < 0.05$) reduction in percentage parasitaemia in the treated groups relative to the control group. The percentage parasitaemia in the groups treated with varying concentrations of the crude extract of the plant (10, 25, 50 and 75mg/kg) were 4.99%, 4.46%, 3.95% and 2.29%, respectively, while the percentage parasitaemia of 2.13% and 18.72% were observed for arteether treated and negative control groups respectively. By day 6 post infection, there was a complete clearance of parasitaemia by chloroquine. The groups treated with 10mg/kg of F1, F2 and F3 had percentage parasitaemia of 2.00%, 4.47% and 3.50% respectively. After four days treatment, the varying concentrations of the plant extract (10, 25, 50 and 75mg/kg) produced

chemosuppression of 70.10%, 76/17%, 78.90% and 87.17%, respectively. F1, F2 and F3 at 10mg/kg had chemosuppression of 89.33%, 76.12% and 77.70% respectively while chloroquine (10mg/kg) and arteether (3mg/kg) caused 100% and 88.60% suppression, respectively.

Mean survival time (MST) of 25 and 23 days respectively were observed for chloroquine and arteether treated groups compared to 19, 20, 21 and 22 days respectively observed for the groups treated with 10, 25, 50 and 75mg/kg of the crude extract. Fractions F1, F2 and F3 at a dose level of 10mg/kg had a mean survival time of 25, 21 and 21 days respectively. The untreated control groups survived only for 12.5 days (Table 19)

During the 4 days treatment, the packed cell volume (PCV) of the parasite infected untreated animals decreased progressively until they all died while the PCV of chloroquine treated group increased progressively. The PCV of the treated groups increased during the 4 days treatment, but when treatment was withdrawn, increase in PCV was only observed in chloroquine treated group. However in arteether, crude extract and fractions treated groups, there was a decrease in PCV due to recrudesence of parasitaemia observed in the arteether, extract and the fractions treated groups (Fig 45 and 46). The crude methanol extract of the bulb of *Crinum jagus* have been reported to contain some phytochemcial compounds like alkaloids, flavonoids, saponins, steroids, glycosides, terpenes (Ode *et al.*, 2010). The presence of these phytochemicals in this plant might be responsible for the antimalaria activity exhibited by them. Phytochemical compounds such as terpenoids are commonly implicated in the antiprotozoal and antiplasmodial activity of many plants (Philipson *et al.*, 1991; Francoise *et al*., 1996; Ghosal *et al*., 1996; Asase *et al*., 2010). An example of common terpenoids is artemisinin, the main active ingredient in the traditional Chinese antimalaria qinghaosu. Flavonoids showed significant antiparasitic activities against different strains of malaria parasites, trypanosome and Leishmania (Kim *et al.*, 2004; Monbrison *et al*., 2006; Tasdemir *et al*., 2006). Flavonoids are reported to chelate with nucleic acid base pairing of the parasite (Lui *et al.*, 1992). These chemical compounds may be acting singly or in

synergy with one another to exert the observed antimalaria activity of *Crinum jagus*. The plant *Crinum jagus* was observed to show antimalaria activity by its percentage chemosuppression compared to that of chloroquine, the standard antimalaria drug. The activity might be attributed to the presence of alkaloids or flavonoids which have been identified in this work or even a combined action of more than one metabolite. However, the active compounds, known to give this observed activity need to be identified. The antimalaria activity of the plant might also results from its antioxidant property. Reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, hydrogen peroxide which do not cause any damage under physiological conditions but at high concentration can cause cellular damage are produced during *Plasmodium* infection (Farombi *et al*., 2003), the extract and its fractions were able to reverse these conditions by increasing the activity of superoxide, catalase and decreasing lipid peroxide produced in the *Plasmodium i*nfection, thereby further confirming the antioxidant activity of the plant. This study has however established the rationale for traditional use of this plant as remedy for malaria infection and its potential development as an antimalarial agent .

Antitubercular Activity

Tuberculosis is a highly infectious disease with about one third of the world's population estimated to be infected (Agarwal, 2004). It kills about three million people per year world wide. It is a major health problem for developing countries. This problem has become serious as *Mycobacterium tuberculosis* developed resistance against both the first line and second line drugs. Due to this, there is emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tuberculosis*. Natural products are proven template for the development of new scaffolds of drugs (Butler, 2005; Chin *et al.*, 2006) and they have received considerable attention as potential anti-TB agents (Pauli *et al*., 2005). Antimycobacterial activity from hexane extract of *Adhatoda vasica* has been reported by Ignacimuthu and Shanmugam, (2010). There are also reports that antimycobacterial activity is noted in Aloe vera and garlic when tested against H37Rv (Jain, 1993; Ratnakar and Murthy, 1996; Reynolds and Dweck, 1999). Gupta *et al.*, (2010*)* reported antituberculosis activity from *Acalyphia indica* and *Allium cepa* extracts

against *Mycobacterium tuberculosis*. There are reports from folklore which claim that the bulb of *Crinum jagus* are used in Southern part of Nigeria for treatment of TB infection hence in this study the antibacterial activity of extract of the bulb of *Crinum jagus* against *M .tuberculosis*, the aetiological agent of TB was investigated.

The results of antitubercular activity of the crude methanol extract and fractions of the bulb of *Crinum jagus* are presented in Tables 21, 22, 23 and 24. The crude extract at various concentrations showed a concentration-dependent inhibition of the *M. tuberculosis* isolates and H37Rv strain in both Lowenstein Jensen (L-J) and Middlebrook 7H10 media. All the three fractions (F1, F2 and F3) inhibited *M. tuberculosis* isolates and H37Rv strain in a concentration dependent manner in both L-J and Middlebrook 7H10 media. The fractions were more potent than the crude extract with higher inhibition values. F1 was the most potent with the highest inhibitory value (83%) which compared well with the inhibitory activity of the standard drugs. At a concentration of 1.0mg/ml, the percentage inhibition of F1, rifampicin and isoniazid against MTB 3 (the most susceptible isolate) was 83%, 95% and 84%, respectively. F1 had the lowest IC_{50} value which compared well with IC_{50} value of the standard drugs. The IC_{50} value of F1, rifampicin and isoniazid against MTB 3 the most susceptible isolate) in L-J medium were 0.27, 0.21 and 0.20mg/ml respectively while the IC_{50} value of F1, rifampicin and isoniazid against MTB 3 in Middle brook 7H10 medium were 0.22, 0.19 and 0.20mg/ml respectively (Tables 23 and 24).

The antimycobacterial activity showed that bulb of *Crinum jagus* has the potential to cure tuberculosis and is a promise for future therapeutic interventions. The results from the study provide for the first time, the scientific evidence for the traditional use of *Crinum jagus* for the treatment of tuberculosis. The results assume significance and throw some light on the basis of the use of the plant in our traditional systems of medicine and in folklore. Of all the antitubercular drugs in various stages of clinical evaluation, a diarylquiniline-based drug (TMC 207) has been found to be an inhibitor of the FO subunit of the mycobacterial adenosine triphosphate (ATP) synthase proton pump (Andries *et al.*, 2005; Koul *et al*., 2007) which is a novel mechanism of action against

M. tuberculosis (Haagsma *et al.,* 2009). Therefore further detailed phytochemical screening and bioactivity studies need to be carried out using crude solvent extracts as well as further purified constituents to ascertain the active antitubercular ingredient of *Crinum jagus* to comprehend their role in antituberculosis activity and develop suitable drugs so that the most deadly disease in the world can be combated. The present study also could pave the way towards possibility to obtain anti-mycobacterial moieties against other mycobacterial species.

Toxicological Study

The use of plants for healing purposes is very common in developing countries especially in the rural areas. This is probably due to the perceived beneficial and lower side-effect profile of natural products that are extracted from plants (Leonardo *et al*., 2000). However, most medicinal plants are used indiscriminately without knowing their possible adverse effect. Over the past decades, several reports in both developed and developing countries have indicated adverse effects allegedly arising from the use of medicinal plants (Elvin Lewis, 2001). Some of these effects include abortion of pregnancy, dizziness, vomiting, diarrhea, abdominal pain, fast heart beat, death, ulcer and loss of appetite (Gessler, *et al*., 1995). These effects could be attributed to the presence of phytotoxic compounds in the plant extracts and lack of actual dosage necessary for the treatment of diseases (Azaizeh *et al.,*2003). In order to minimize this draw back, there is need for thorough scientific investigation on the toxicological effect of these plants at different doses. The world health organization (WHO) has recommended that traditional plants used for the treatment of disease warrant further evaluation of their toxicological properties.

Crinum jagus has various therapeutic uses. These include treatment of microbial infections, convulsion, snake bite, tuberculosis, asthma (Adesanya *et al*., 1992; Edema and Okiemen, 2002; Ode *et al*., 2006; Ogunkunle and Olopade 2011). However, there was no information in the scientific literature on the toxic effect of *Crinum jagus* on the haematological and biochemichal parameters in rats. Therefore, the present study was undertaken to assess the possible toxicological effects of the bulb of *Crinum jagus* extract

using haematology, serum chemistry, liver and kidney functional indices in the animal model.

The result of this study showed that the treated rats were able to maintain growth irrespective of the number of days the extract was administered to them. The study therefore, suggests that the crude methanol extract of the bulb of *Crinum jagus* did not interfere with the body weight of the treated rats (Fig 47). This revealed the potential of this plant in controlling muscle wasting.

Tissue enzyme assay can indicate tissue cellular damage long before structural damage is revealed by some other conventional technique (Akanji, 1986). The effect of crude methanol extract of the bulb of *Crinum jagus* on serum liver and kidney enzymes following administration for thirty days revealed that there were non significant ($P > 0.05$) differences in the serum, liver and kidney levels of AST, ALT and ALP in the rats treated with 10 and 25mg/kg of the extract however, the levels of AST, ALT and ALP in the serum, liver and kidney were significantly ($P < 0.05$) elevated by administration of 50 and 75mg/kg of the extract when compared with the control group (Figures 48, 49 and
50). AST, ALT and ALP are marker enzymes for liver function and integrity (Jens and Hanne, 2003). Liver damage is associated with elevated levels of these enzymes. ALT is a more specific enzyme of damage and known to increase when there is liver cell damage and it has been employed as a tool for measuring hepatic necrosis (Bush,1991). It is located in the liver and increase in its concentration suggests that there is liver damage which can cause enzyme leakage from liver into blood stream. ALP is a marker enzyme for plasma membrane and endoplasmic reticulum (Wright and Plummer 1974; Shajahan *et al.*, 2004) hence it is employed to assess the integrity of plasma membrane (Akanji *et al.*, 1993), elevation of ALP will therefore result in the leakage of the enzyme from the tissues into the serum (Sahjaham *et al.*, 2004, Aboyade *et al.*, 2009). Such loss from tissue may adversely affect adequate transportation of ions across the membrane (Akanji *et al.*, 1993) and other metabolic processes such as the synthesis of nuclear proteins, nucleic acid and phospholipid where the enzyme is involved (Ramalingam and

Vimaladevi, 2002). In serum, ALP may be considered as an indicator of cholestasis which may result from intracellular hepatic canaliculli obstruction associated with inflammation (Etuk and Muhammad, 2010). Significant ($P < 0.05$) elevation of lactate dehydrogenase was seen only with the administration of 50 and 75mg/kg for thirty days in the liver and this may have consequential effect on glycolytic pathway.

At the dosage level of 10 and 25mg/kg, there were no significant ($P < 0.05$) differences in the total and conjugated bilirubin concentrations relative to the control group. However, higher doses of the extract (50 and 75mg/kg) produced significant ($P < 0.05$) elevation in total and conjugated bilirubin levels both in the serum and liver (Fig 51 and 52) and this could be attributed to mild liver malfunction, obstruction of the common bile or hepatic duct. When the liver is not filtering normally, bilirubin builds up in the blood which in turns results into a damaged liver (cirrhosis).

Elevated plasma triglyceride concentration contribute directly to increased risk of cardiovascular diseases and such elevation is associated with obesity, metabolic syndrome, type 2 diabetic mellitus which predispose to cardiovascular diseases (Hodes *et al.*, 1999). The present study revealed that there was significant ($P < 0.05$) reduction in the serum triglyceride level when the animals were treated with high doses of the extract (50 and 75mg/kg).

High density lipoprotein and low density lipoprotein are the two main groups of plasma lipoprotein that are involved in the lipid metabolism, exchange of cholesterol, cholesterol esters and triglycerides between tissues (Gordon and Riffund, 1989; Svirdin, 1999; Mc Namara, 1999). Studies have demonstrated an inverse relationship between HDL cholesterol and incidence of cardio vascular disease (Maron, 2000). In the present study, HDL cholesterol for the groups treated with 25, 50 and 75mg/kg doses of the extract for thirty days showed significant elevation compared to the control group while there was no significant difference in the serum LDL cholesterol in all the treated groups relative to the control group. Abnormal blood lipids, particularly total cholesterol predispose individual to arterosclerosis and cardiovascular disease (Ginsberg 1994; Glew *et al.*, 2001; Chrysohoou *et al.*, 2004; Nwanjo and Oze, 2007). From this study,

administration of the crude extract of the bulb of *Crinum jagus* for thirty days resulted in significant reduction in the concentration of serum cholesterol in all the treated groups relative to the control group (Fig 53). The significantly ($P < 0.05$) lower cholesterol may have contributed to the observed significant high serum HDL cholesterol in the animal cause about 30% of blood cholesterol is carried inform of HDL cholesterol, HDL cholesterol can remove cholesterol from antheroma within the arteries and transport it back to the liver for its excretion, thus high level of HLD cholesterol protect against cardiovascular diseases (Kwiterovich, 2000). The observed significant increase in HDL cholesterol concentration upon administration of the extract for thirty days indicates that the extract have HDL cholesterol boosting effect. The extract did not have any significant effect on LDL cholesterol which transport cholesterol to the arteries where they can be retained in artheria proteoglycan starting the formation of plaques hence LDL cholesterol posses a risk of cardiovascular disease when it invades endothelium and become oxidized, oxidized form is more easily retained by proteoglycans thus increase of LDL cholesterol is associated with artherosclerosis, heart attack, stroke (Growell and Otwa, 2004). From the study it could be suggested that the extract may aid in the prevention of cardiovascular diseases.

Decrease or increase in cell counts and depletion of plasma constituent or their elevation beyond the reference range could demonstrate haematoxicity. In the present study, there were no ($P > 0.05$) significant difference in the haematological parameters of all the treated animals relative to the control group (Figures 54 and 55). There were non obvious haemolytic changes in the plasma of the extract treated rats on RBC, haemoglobin, PCV, MCV, MCH an MCHC. These indices are well known to determine the haemolytic damage on red blood cells. The absence of changes on these indices suggests that the extract does not possess toxic substances that can cause an anaemic condition in rats. The extract did not produce any effect on white blood cells and its functional indices. The result from this finding shows that the extract is not haematoxic.

Histopathological examination of tissues of the animals exposed to a toxicant may furnish additional information on the drug . Exposure of the rats to high doses of the

extract (50 and 75mg/kg) caused pathological changes in the liver which resulted in the enlargement of hepatocytes (fatty degeneration of hepatocytes) (Plate 1C). Exposure of the rats to high doses (50 and 75mg/kg) caused necrosis of the tubular epithelium with sloughing into the lumen and distruption of the basement membranes in the kidney (Plate 2C). The pathological changes observed with high doses of the extract (50 and 75mg/kg) is an indication of toxicity. The toxicological evaluation of the plant indicates that the plant is safe at lower doses but higher doses pose toxicological risks as evident by the elevation of serum, liver and kidney enzymes and pathological changes observed in the liver and kidney following administration of extract for thirty days.

GC-MS analysis of F1 of the bulb of *Crinum jagus* revealed the presence of ten bioactive compounds. The GC-MS retention time (RT) and percentage peak of the individual compounds revealed that 5-hydroxyl methyl furfural (5-HMF) was found as the major component in the fraction. 5-HMF is an organic compound derived from dehydration of certain sugars (Andrea *et al*., 2011; Robert *et al*., 2013). This yellow melting solid is highly water soluble. The molecule consists of a furan ring containing aldehyde and alcohol functional groups. *In vitro* antioxidant and antiproliferative activities of 5-HMF has been reported (Zhou *et al.*, 2013). Consequently, the antioxidant activity of the extract and fractions of *Crinum jagus* may be attributable to the presence of bioactive compounds such as 5-HMFS. The presence of various bioactive compounds in the fraction justifies the traditional use of the plant for the treatment of various ailments hence it can be concluded that *Crinum jagus* is of phytopharmaceutical importance.

In conculsion, from the various findings obtained in this study, the extract and fractions of the bulb of *Crinum jagus* posesss antioxidant, antimicrobial, anti-inflammatory, antimalaria and anti-mycobacterial properties. These properties may be due to the presence of phytochemicals such as alkaloids, flavonoids, saponins, phenols, steroids which are present in the plant. These classes of compounds are known to have curative activities and therefore could suggest the use of the plant traditionally for the treatment of various illnesses. Most of these activities are highly expressed in F1 and this

may suggest the major constituents of the bioactive compounds present in the plant are highly concentrated in the fraction. This study provides scientific evidence for the use of the plant in ethnomedicine in the treatment of various health conditions and this may find useful pharmaceutical applications in the design of lead drug that can be used for the treatment of free radical mediated diseases, antimicrobial infections, anti-inflammatory diseases, malaria and tuberculosis.

5.2 CONTRIBUTIONS TO KNOWLEDGE.

In this thesis, evidence has been presented to show that:

1. The crude methanol extract of the bulb of *Crinum jagus* contain alkaloids, flavonoids, saponins, phenols and steroids.
2 The crude extract and fractions of the bulb of *Crinum jagus* possess antitubercular, antimicrobial, anti-inflammatory, antimalarial and antioxidant activities with the fractions being more potent than the crude extract.
3. In Nigeria ethnomedicine, the antitubercular, anti-inflammatory and antimalarial activities of the plant is being reported for the first time.
4. Most of these biological activities of *Crinum jagus* are highly expressed in F1 and this may suggest the major constituents of the bioactive compounds present in the plant are highly concentrated in the fraction.
5. The antitubercular and antimalarial activities of F1 was comparable with the standard reference drugs.
6. The bulb of *Crinum jagus* contain flavonoid and phenolic compounds in a concentration dependent manner with F1 having the highest flavonoid and phenolic content.
7. *Crinum jagus* is safe when used at lower dose but administration of high dose of the plant was found to be toxic.
8. *Crinum jagus* is a potential valuable source of antitubercular, antimicrobial, anti-inflammatory, antimalarial and antioxidant agents.
9. This study provides some scientific justification for the folkoric use of *Crinum jagus* as a health remedy in folklore medicine.
10. Gas chromatography mass spectrometry (GC-MS) analysis of F1 revealed the presence of ten chemical components. The presence of various bioactive compounds in the fraction justifies the traditional use of the plant for the treatment of various ailments hence *Crinum jagus* is of phytopharmacological importance.

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APPENDICES

Appendix 1

Anti-inflammatory activity of the crude methanol extract of the bulb of *Crinum jagus*

		Paw Circumference (cm)						
Groups	**Treatment**	**0hr**	**1hr**	**2hrs**	**3hrs**	**4hrs**	**5hrs**	**Percentage Inhibition**
A (Control)	0.2ml saline	2.50± 0.17	3.14± 0.29	3.26± 0.25	3.38± 0.19	3.50± 0.14	3.60± 0.09	_____
B	5mg/kgbw indomethacin	2.58± 0.29	2.68± 0.26	2.76± 0.30	2.52± 0.29	2.38± 0.27	2.22± 0.10	94.82%
C	10mg/kgbw extract	2.64± 0.24	3.42± 0.29	3.62± 0.12	3.72± 0.19	3.92± 0.13	3.94± 0.10	26.82%
D	25mg/kgbw extract	2.64± 0.24	3.26± 0.22	3.48± 0.12	3.26± 0.26	3.00± 0.28	2.80± 0.28	29.55%
E	50mg/kgbw extract	2.60±. 0.17	3.84± 0.31	3.50± 0.26	3.20± 0.23	3.08± 0.17	3.00± 0.29	31.82%
F	75mg/kgbw extract	2.78+ 0.20	3.48+ 0.29	3.26+ 0.24	3.08+ 0.15	2.66+ 0.24	2.48+ 0.27	65.90%

The values expressed as mean ± SD (n- = 5)

The percentage inhibition was calculated using the formular :

$$\% \text{ inhibition} = \frac{\textbf{(Dt-Do) control} - \textbf{(Dt-Do) test}}{\textbf{Dt – Do) control}} \times 100$$

Where **Dt** = Linear paw circumference 3 hours after Carrageenan injection

Do = Linear paw circumference at 0 hour (just before Carrageenan injection)

Appendix 2

Anti-inflammatory activity of the chromatographic fractions of the bulb of *Crinum jagus*

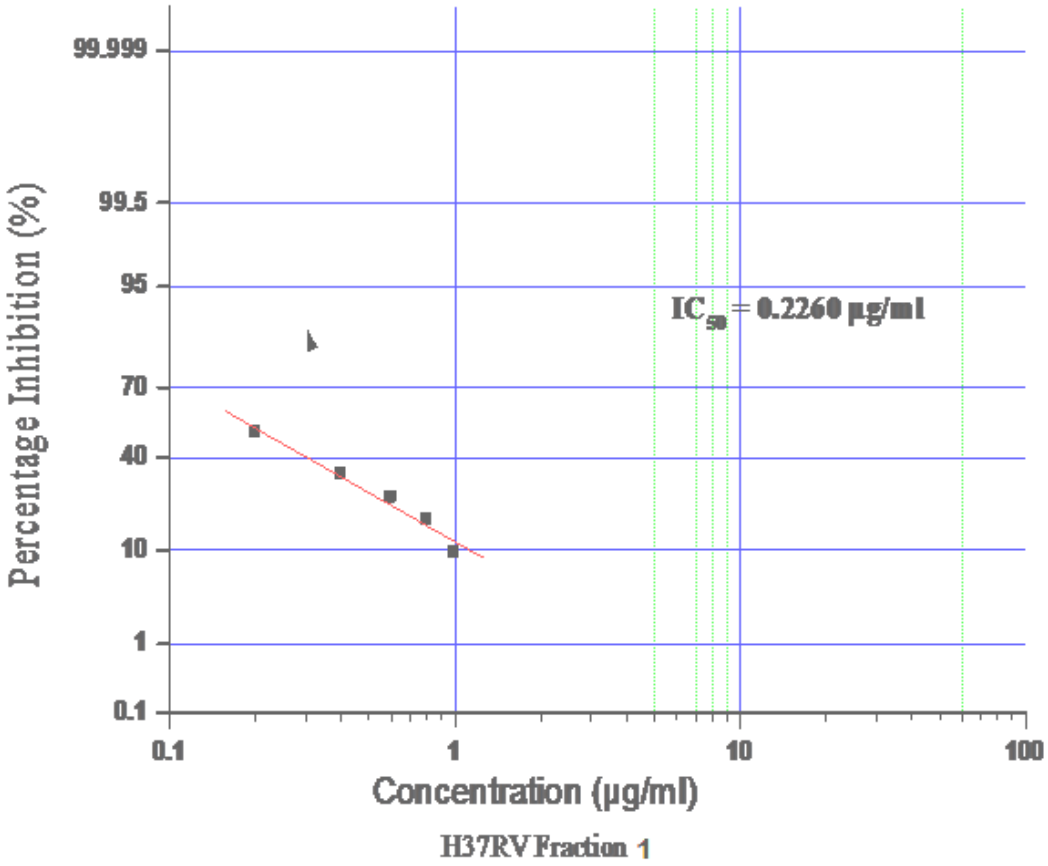
		Paw Circumference (cm)						
Groups	**Treatment**	**0hr**	**1hr**	**2hrs**	**3hrs**	**4hrs**	**5hrs**	**Percentage Inhibition**
A (Control)	0.2ml saline	2.50± 0.17	3.14± 0.29	3.26± 0.25	3.38± 0.19	3.50± 0.14	3.60+ 0.09	_____
B	5mg/kgbw indomethacin	2.58± 0.29	2.68± 0.26	2.76± 0.36	2.52± 0.29	2.38± 0.27	2.22+ 0.10	94.82%
C	10mg/kgbw F1	2.36± 2.42	3.20± 0.14	2.92± 0.15	2.80± 0.14	2.72± 0.07	2.66± 0.33	50.00%
D	20mg/kgbw F1	2.80± 0.08	3.00± 0.32	2.84± 0.30	2.62± 0.34	2.54± 0.29	1.76± 0.24	79.50%
E	10mg/kgbw F2	2.72± 0.17	3.66± 0.30	3.42± 0.35	3.38± 0.33	3.36± 0.36	3.64± 0.33	18.18%
F	20 mg/kgbw F2	2.54± 0.14	3.36± 0.21	3.62± 0.13	3.62± 0.15	3.66± 0.15	3.72± 0.12	25.00%
G	10mg/kgbw F3	2.54± 0.14	3.56+ 0.17	3.38± 0.17	3.08± 0.12	2.86± 0.10	2.54± 0.10	38.64%
H	20mg/kgbw F3	2.44± 0.19	3.14± 0.19	3.08± 0.20	2.86± 0.17	2.68± 0.12	2.64± 0.10	52.27%

The values expressed as mean ± SD (n- = 5)

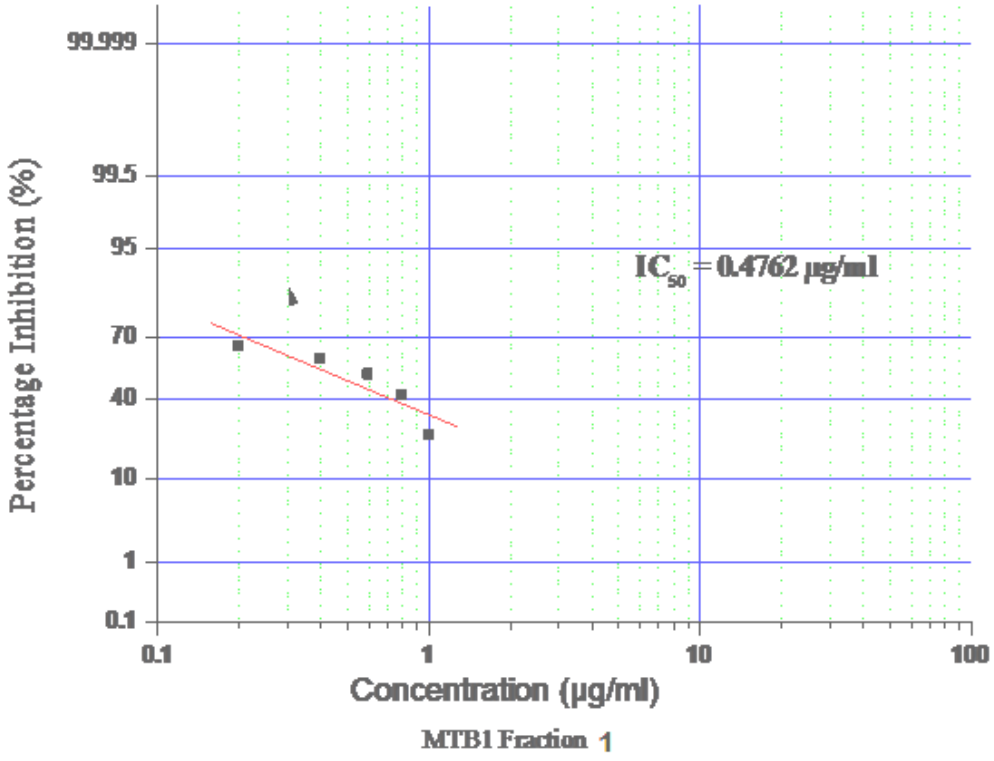
Appendix 3

In-vitro **antituberculosis activity of fraction 1 (F1) of the bulb of** ***Crinum jagus*** **against isolates and strain of** ***M.tuberculosis***

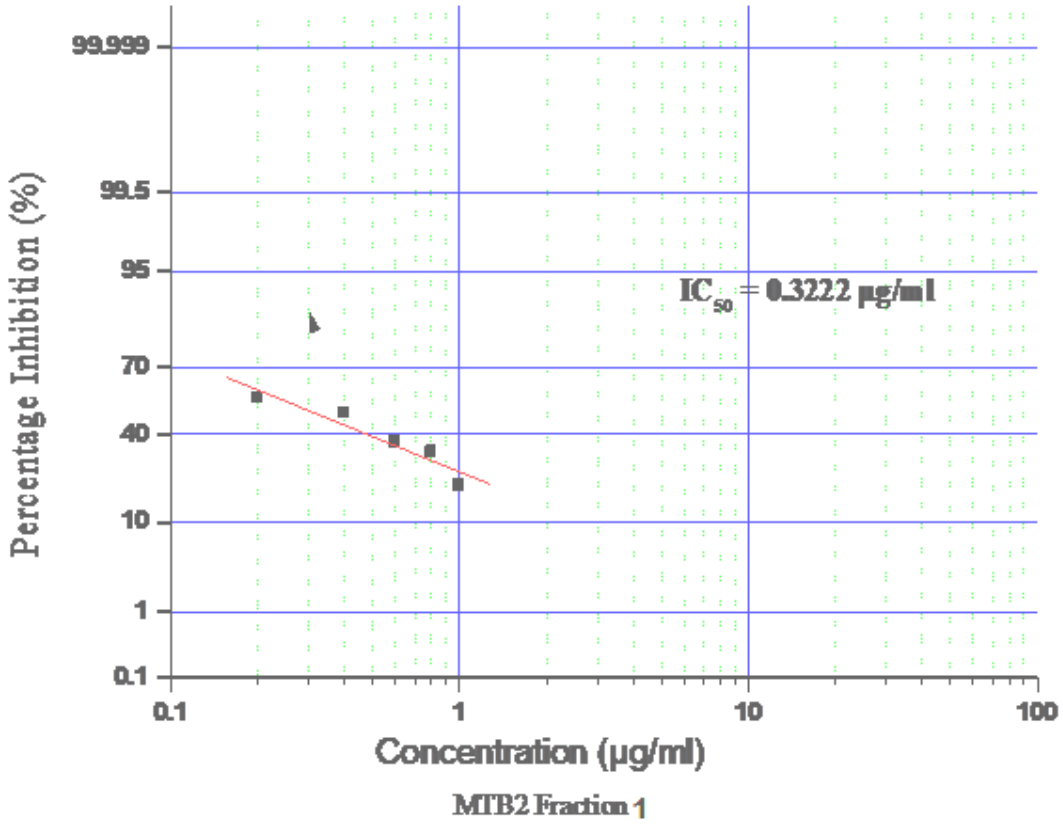
Determination of 50% inhibitory concentration (IC_{50})



Determination of 50% inhibitory concentration (IC_{50})



Determination of 50% inhibitory concentration (IC_{50})



Determination of 50% inhibitory concentration (IC_{50})