TOXICO- PHARMACOLOGICAL PROPERTIES OF 'JOLOO' : A HERBAL PREPARATION USED IN THE MANAGEMENT OF TUMOURS

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Certification

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Is a record of the original research carried out

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In the Department Of Cell Biology and Genetics

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A.M. OLOYEDE

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ACKNOWLEDGEMENTS

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Joloo is a formulation extracted from seven medicinal plants used in folk medicine practice for the management and treatment of breast cancer and some other health malaise in southwestern Nigeria. The study evaluates some of its traditionally acclaimed pharmacological properties in mice and rats as well as the safety of *Joloo*.

Three toxicity levels of study which included acute, subchronic and chronic toxicity were undertaken to evaluate the extract. *Joloo* was prepared using plants in a definite ratio as prescribed in traditional practice. Fifty mice weighing between 20-32g having an average wt of 26 ± 3.5g and comprising ten mice per group were orally administered five doses (400, 800, 1200, 1600 and 2000 mg kg^{-1} b. wt.) of ethanolic extract of *Joloo* respectively for 24hrs to determine the LD_{50}. The same dosage was also repeated for intraperitoneal administration and Median lethal dose (LD_{50}) estimation was calculated from log dose/probit analysis. Subchronic toxicity was evaluated using forty rats distributed into four groups, weighing between 128-160g and comprising of ten rats per group. Doses 400-1600 mg kg^{-1} b. wt. was administered per Os, daily for 28days. Chronic toxicity was determined on another set of forty mice, ten per group and weighing between 20 and 30g. Repeated doses were administered orally for 91 days, and the control received distilled water for the entire study period. Genotoxicity was investigated using selected *Allium cepa* bulbs weighing 28-30g (60) and cytotoxicity of the extracts was tested against chicken embryo fibroblast (CEF) and African green monkey kidney cells (VERO cells) using the MTT (3-(4,5-Dimethylthiazol-2-) -2,5-diphenyltetrazolium bromide) assay at three different concentrations (1.0, 2.5 and 5mg). During pharmacological profile analysis, Analgesic study was done using the Acetic acid- induced abdominal constriction assay, formalin- induced pain test and hot plate- induced pain test. In the antipyretic study, pyrexia was induced in the rats by the administration of 10 mg^{-1}kg b. wt., of 2, 4-Dinitrophenol intraperitoneally while measurement was by inserting a clinical thermometer into their anal cavities for about 2 min. acetylsalicylic acid was used as reference drugs in rats, while water served as control. The antiplasmodial study involved two phases; the suppressive where mice were administered plant

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The LD_{50} derived from the oral administration was 2000 mg kg^{-1} b. wt. while 400 mg kg^{-1} b. wt. was observed in the ip administration. In the genotoxicity studies, *Joloo* induced macroscopic and microscopic changes causing a dose-related root growth inhibition and chromosomal aberrations in *Allium cepa*. The effect of the extract was more pronounced at 1600 mg kg^{-1} b. wt., dose while the effective concentration (EC_{50}) was at 380 mg kg^{-1} after 72 h. In cytotoxicity, *Joloo* (mixed, fractionated portions and individual constituent plant) extracts exhibited varying degree of dose-dependent cytotoxicity at all concentrations on both VERO and CEF cell lines. Thus, it is possible that *Joloo* was able to induce the release of cytochrome c from mitochondria. It may also trigger caspase independent apoptosis if some of the cytochrome c released from mitochondria accumulates in the nucleus. The oral administration of *Joloo* to rats for 28 days at 400 and 800 mg kg^{-1} b. wt. in the subchronic studies did not result in death and the body and organ weights of rats were normal as well as the levels of biochemical analytes. Histopathologic abnormalities were also not observed. Thus *Joloo* may be regarded safe especially at doses between 400 and 800 mg kg^{-1} b. wt. but not at 1600 mg kg^{-1} b. wt. at subchronic level. The repeated oral administration of *Joloo* for a period of 91 days indicated that lower doses 400 and 800 mg kg^{-1} b. wt. of *Joloo* are devoid of toxicity, whereas high dose (1600 mg kg^{-1} b. wt.) is associated with some toxicity concerns especially under prolonged usage as observed in the significant increase in biochemical analytes of the liver and kidney as well as slight alteration in the histoarchitechture of the liver, heart and the spleen. Pharmacologic studies of *Joloo* indicated that it exhibited analgesic, anti-inflammatory, antipyretic and antimalarial activities.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Introduction and Background of study

From antiquity, Africans have used medicinal plants –derived remedies in their struggle for survival. Survey has shown that 60% to 80% of the world population depends largely on herbal medicines for their healthcare needs (WHO 2002). Also on record till date, is the fact that over 15,000 compounds of significant therapeutic benefits encompassing anti-tumour, antibiotics, antimalarials, analgesics , sedatives , hypotensive tranquilizers and anti-inflammatory steroids have been isolated from about 200 plant species, most of which are from tropical origin (Busia, 2005). However the total number of natural products (Anti-infective agents) produced by plants has been estimated to be between 500,000 and 600,000, of which 160,000 have already been elucidated (Demain, 2009). It is also established that one quarter of all medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogs (Gurib-Fakim, 2006).

Joloo is a Nigerian formulation extracted from seven medicinal plants *Allium ascalonicum* Linn. (*Liliaceae: Alliaceae*), *Butyrospermum paradoxum* Gaertn (*Sepotaceae*), *Hoslunda opposita* Vahl (Lamiaceae), *Olax subscorpioidea* Olive (*Olacaceae*), *Xylopia aethiopica* Dunal A. Richard (*Annonaceae*), *Securidaca longepedunculata* Fresen (*Polygalaceae*), and *Tetrapleura tetraptera* Schum / Thonn (*Leguminosae: Mimosidae*).

The extracts from these seven plants have been used for various purposes: *Butyrospermum paradoxum* as a stimulant and carminative in Nigeria (Burkill, 2000), antihelminthic and antihypertensive (Odugbemi, 2006), *Hoslundia opposita* has antipyretic, diuretic, cholagogic,

antimalarial and anticonvulsion properties (Burkill, 1995), *Olax subscorpioidea* as an antiarthritic, antirheumatic (Burkill, 1997) and antimicrobial (Ayandele and Adebiyi, 2007). *Xylopia aethiopica* has been previously used in the treatment of cancer and ulcer in Nigeria (Burkill, 1985), while *Securidaca longepedunculata* is reported to possess antirheumatic, antipyretic and anti-inflammatory properties (Asres *et al*., 2001). The pod extract of *Tetrapleura tetraptera* is used to treat chest pain, female sterility, ulcer, convulsion and arthritis (Burkill, 1995; Odugbemi, 2006). Though these plants have been utilized individually in various ways to treat different ailments, to the best of my knowledge, there is no known report on the Pharmacological and antitumour properties of these plants when used in combination.

1.2 Statement of the problem

The World Health Organization (WHO) defined herbal medicines as herbs, herbal materials, herbal preparations, and finished herbal products, containing active ingredient parts of plant and other plant materials, or combination (Gurib-fakim, 2006). It is a practice that could be explicably or inexplicably used in diagnosing, preventing or eliminating a physical, mental or social diseases which may rely exclusively on past experience or observation handed down from generation to generation.

Joloo is traditionally used in the treatment of breast tumour, ulcer, pain, fever, and general malaise. Many synthetic drugs that are anti-tumour, anti-ulcer, anti-inflammatory, antipyretic and analgesics are readily available, however many of them are becoming less acceptable due to their serious adverse effects (Xiao *et al.,* 2006). This however necessitates the continued search for medications with minimal or no side side-effects. Ethnobotanical use of plants have often proved to be a more efficient method of drug discovery (Fawole *et al*., 2009), it has shown wide range of

therapeutic use and suitable for chronic treatment and also cheaper than synthetic drugs. Odugbemi (2006) reported that more than 80% of Nigerian population use herbal remedies in one form or the other.

1.3 Aims and Objective

This research is designed to investigate the toxicity and apoptotic properties of *Joloo* in order to define its safety profile. Thus, particular aims of this study are to evaluate the;

1. Acute, subchronic and chronic toxicity

2. Cytotoxic and apoptotic effects

3. Analgesic, antimalarial, antipyretic and anti-inflammatory profile

4. Anti-oxidant and free radical scavenging profile

5. Physico-chemical parameters

1.4 Significance of study

Medicinal plants may produce several biological activities in humans; the distinctive characteristics that make them different from synthetic drugs is the presence of more than one active compound and the active principle is frequently unknown. Thus, safety should be the overriding criterion in the selection of medicinal plants for use in healthcare systems. The rational for this study is to evaluate and ascertain the toxicokinetics and safety of *Joloo* and give scientific validity for its acclaimed therapeutic activities in the management of some health malaise. The serious quest for new drugs, new drug leads and new chemical entities from natural products and medicinal plants is a great advantage for this study, and again being in a developing country where the cost of orthodox healthcare is enormous; there is the need to solve our health problems using the resources available in our society. Consequent on this, the result from this

study will not only to validate the use of *Joloo*, but also provide an opportunity for the exploration of the rich flora of Nigeria in the management and curing of myriads of diseases. It could also serve as one of the instruments of significant novelty in the spectra of national strategies to attain the Millennium Development Goals as pertains to disease eradication.

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Operational definition of terms

Acute Toxicity	The adverse effects resulting from a single exposure to a substance
Analgesic	A medication that reduces or eliminates pain
Anti-inflammatory Agent	An agent that counteracts or suppresses the inflammatory process
Antipyretic Agent	A medication that reduces fever.
Aspartate aminotransferase (AST/GOT)	An enzyme present in the heart, liver etc and released into serum on cellular injury
Cytotoxicity	Toxic effect on cells
Haematolgy	About blood and blood producing organs
Histologic	About microscopic level of cells within an organ
Median Lethal dose (LD$_{50}$):	Administered dose of a drug capable of causing death
Mortality	The number of deaths in a given time and place
Packed cell volume:	Blood cells making up the reliable parameter for anaemia
Phytochemicals	Substances of chemical nature and often of medicinal effects present in plants
Alkaline Phosphatase (ALP)	An enzyme present in bone, liver, kidney and placenta, and if present in high amount in blood could indicate disease of any of these organs or tissues

Phlogistic	An agent capable of causing inflammation especially, but sometimes fever also.
Pyrexia	Abnormal elevation of body temperature above normal range
Alanine Amino Transferase	An enzyme which promotes transfer of amino group from glutamic acid to pyruvic acid and which if present in high amount in blood could indicate liver disease.
Sub-acute toxicity	Adverse effects falling between acute and chronic toxicity duration, usually within 14 days of drug administration
Toxicity	The poisonous effects of a drug
Writhing	A syndrome characterized by a wave of contraction of abdominal musculature followed by extension of hind limb

Abbreviations

CAT: Catalase, **DNP:** 2,4-Dinitrophenol, **GIT:** Gastrointestinal tract, **GPx:** Glutathione peroxidase, **SOD**: Superoxide dismutase. **GSH:** Glutathione, **I.P.:** Intra peritoneal, **MDA:** Malonaldehyde-bis-dimethylacetyl, **MTT:** (3-(4, 5-Dimethylthiazol-2-) -2,5-diphenyltetrazolium bromide), **NOAEL:** No Observable Adverse Effects, **OECD:** Organisation for Economic Cooperation and Development, **P.O.:** *Per Os* or Oral, **PCV:** Packed Cell Volume, **PGs:** Prostaglandins, **S.C.:** Subcutaneous, **SEM:** Standard Error of the Mean.

CHAPTER TWO

2.0 Literature Review

Animal toxicity studies are carried out to investigate and evaluate a drug's short- and long-term functional and morphological adverse effects. A major challenge in drug candidate screening and development of new chemical entity (NCE) or new biological entity (NBE) as therapeutic agents is precise determination of their human toxicity (Astashkina *et al*. 2012). Depending on the nature of a drug, its intended use, and the extent of its proposed study in clinical trials, toxicokinetic studies may comprise some or all of the following elements: acute, sub-acute or sub-chronic, chronic, cytotoxicity, carcinotoxicity and genotoxicity (Teo, 2002).

Acute toxicity testing a single dose toxicity studies mainly designed to evaluate the short –term adverse effects of a drug when administered either in a single dose or in multiple doses during a period of 24 hours (OECD, 1992). Tests are performed commonly in rats or mice. Food is often withheld the night prior to dosing. Results from acute toxicity studies provides information on: the lethal dose-50 (LD_{50}); the appropriate dosage for multiple-dose studies; the potential target organs of toxicity; the time-course of drug-induced clinical observations until 14 days; and an estimate of the safe acute doses for humans. The number of animals that die in the first 24 or 48-hours is important in the estimation of the LD_{50} (Rosenstock *et al.,* 2005)**.**

Sub-chronic toxicity study allows investigators to measure a drug's toxic potentials and pathologic effects over a longer period. These tests are undertaken not less than 14 days (Wilson *et al*., 2001; Teo *et al*., 2002). During the study, the following data are collected, as appropriate, for the specific test compound: observed effects, mortality, body weight, food and water consumption, physical examinations, haematologic and biochemical parameters, organ weights,

gross pathology, and histopathology (Raza *et al*., 2002).The route of administration during testing must be that intended for therapeutic purpose; tests may be repeated for other routes. Oral drugs can be given by gavage or incorporated in food. The test compound is administered daily at three dose levels.

Chronic toxicity studies may be defined as those involving the characterization of adverse health effects through the long-term, repeated administration of a test substance over a significant portion of the lifespan of the test animal species. In general, the term usually denotes a study conducted for longer than 3 months (Lahlou *et al*., 2008; Rao *et al*., 2001). However, depending upon the test species being used, a 24–72 h exposure period could represent a chronic study for aquatic insects between hatching and flying, a span of some months for birds, etc., or a number of years if dogs or monkeys were to be used.

The basis of any chronic study is to provide a database which will assure the public about the safety of chemicals found in the human environment, the sources usually being air, food, and water, and the results of such studies being used in safety evaluation, risk assessment, and risk management decisions (Ecobichon, 2005). The aim of long-term toxicity testing, mostly in rodents, is to assess the potential chronic toxicity of a chemical, including carcinogenicity, effects that would not be evident in subchronic studies. Pertinent to the studies is the development of a dose–effect relationship that ranges from no observed effect through minimal changes to overt toxicity and the determination of dosages at which these observations occur. These values important to regulatory agencies so as to determine safe levels of exposure stated as a maximum allowable concentration, recommended maximum levels, reference dose, virtually safe dose, tolerance or acceptable daily intake and so on (Ecobichon, 2005).

Cytotoxicity tests using specialized cells have proved very useful for harmful substances. These tests have also provided useful insight into the pathogeneses of many diseases. Cells derived from different organs or tissues, that retain some specialized functions *in vitro* or that maintain specialized structures, have also been widely used in toxicology. A number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). The most convenient, modern assays have been optimized for the use of microtiterplates (96-well format). This miniaturization allows many samples to be analyzed rapidly and simultaneously. Colorimetric and luminescence based assays allow samples to be measured directly in the plate by using a microtiterplate reader or ELISA plate reader. Cytototoxicity assays have been developed which use different parameters associated with cell death and proliferation. We chose four of these common assays to have a closer look on their comparability.

One parameter for cell death is the integrity of the cell membrane, which can be measured by the cytoplasmic enzyme activity released by damaged cells. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane (Korzeniewski and Callewaert, 1983; Yin et al 2004). The LDH activity is determined in an enzymatic test. The first step is the reduction of NAD+ to NADH/H+ by the LDH-catalyzed conversion of lactate to pyruvate. In a second step, the catalyst (diaphorase) transfers H/H+ from NADH/H+ to the tetrazolium salt 2-(4-iodophenyl) - 3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to a red formazan (Lappalainen *et al*., 1994). Another parameter used as the basis for colorimetric assays is the metabolic activity of viable cells. Tetrazolium salts are reduced only by metabolically active cells (Kigondu *et al.,* 2009). Thus, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium

bromide (MTT) can be reduced to a blue colored formazan (Kigondu *et al.*, 2009). Neutral red (3-amino-*m*-dimethylamino-2-methylphenazine hydrochloride) has been used previously for the identification of vital cells in cultures (Kviecinski *et al.,* 2008). This assay quantifies the number of viable, uninjured cells after their exposure to toxicants; it is based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red. Quantification of the dye extracted from the cells has been shown to be linear with cell numbers, both by direct cell counts and by protein determinations of cell populations (Weyermann *et al*., 2005).

In evaluation of the testes for gonadotoxicity, there are three possible routes by which spermatogenesis can be affected by toxicants (Sharpe, 1998): an alteration of the hypothalamic-pituitary function; the alteration of other functions than that of the hypothalamus, the pituitary or the reproductive system, but which leads secondarily to adverse effects on the spermatogenetic process; and, the direct disruption of cells in the testis. The latter can involve factors that affect vasculature of the testis, Leydig cells, peritubular cells, Sertoli cells, or have a direct effect on germ cells (Sharpe, 1998).

Genotoxicity is the study of adverse effects of compounds on genetic material of cells (DNA) and the further expression of these changes (Connell, 1997). Genotoxicity is important due to the fact that the induction of genetic damage may cause an increased incidence of genetic diseases in future generations and contribute to somatic cell diseases. Hence it is significantly important to detect compounds that affect the genetic material so as to prevent human exposure (Connell, 1997: Wayne and Ming-Ho, 1995). To assess the potential genotoxicity of any compound, its ability to cause chromosomal damage needs to be evaluated in multiple tests (Repetto *et al.*, 2001). Higher plants provide reliable bioassays for monitoring and testing genotoxins (Grant,

1999), with the *Allium* test being particularly sensitive and reproducible (Fiskesjo, 1985). Small mammals are also useful models for testing genotoxicity (Topashka-Ancheva *et al.*, 2003).

Metal toxicity also, has been an issue in toxicity studies. Diversity in observed toxicities of different metals likely reflects the variety of biochemical mechanisms by which they exert their effects and variability in their toxicokinetic properties (Basgel and Erdemoglu, 2006). Metals are a unique class of toxicants in that their chemical form may be changed as a result of environmental conditions, and these different physical forms may significantly affect toxicity. Many metals (essential metals) are needed (typically in very low concentrations) as cofactors for normal biochemical functions (Basgel and Erdemoglu, 2006). Excessive amounts of trace metals may occur naturally as a result of normal geological conditions such as ore formation. Many medicinal herbs and their mixtures can present a health risk due to the presence of toxic elements such as Pb, Cd, Al, Hg and other elements like Cr, which are hazardous to humans, depending on their oxidation states and present at high concentrations (Garcia *et al*., 2000; Lekouch *et al.,* 2001; Lopez *et al.*, 2000). Several effects of metals toxicity are well documented, such as effects on the neurological, cardiovascular, hematological, gastrointestinal, musculoskeletal, immunological, and epidermal systems (Basgel and Erdemoglu, 2006).

A wide range of biochemical and physiological parameters are involved in this study both during and after treatment. Extensive gross and microscopic morphological examinations are carried out so as to observe possible organs or tissues where test agent may exert effect. A spectrum of biological markers such as test of haematology and blood serum biochemistry are very important in chronic toxicity studies (Ecobichon, 2005). The core haematology tests recommended for animal toxicity and safety studies are total leukocyte (white blood cell) count, absolute

differential leukocyte count (Zawidzka, 1990), erythrocyte (red blood cell) count, evaluation of red blood cell morphology, platelet (thrombocyte) count, hemoglobin concentration, haematocrit (or packed cell volume), mean corpuscular volume, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration. (Weingand, *et al.,* 1996) while the core clinical chemistry tests recommended for animal toxicity and safety studies are glucose, urea nitrogen, creatinine, total protein, albumin, calculated globulin, calcium, sodium, potassium, total cholesterol, and appropriate hepatocellular and hepatobiliary tests (Weingand, *et al.,* 1996).

Pathology testing is an integral component of evaluation of the toxicologic potential of therapeutic agents. Results of hematology, clinical chemistry, and urinalysis tests provide information regarding the overall health status of animals, as well as target organs and general metabolic, adaptive, or toxic processes associated with exposure to test articles. Clinical pathology results assist in establishing dose-response relationships and mechanisms of toxicity and provide a link with clinical observations and anatomic pathology findings (Smith *et al*., 2002).

End points of toxicity and the severity of observed adverse health effects obtained from a range of doses study may be represented by arbitrarily determined dosage values such as the lowest-observed-adverse-effect level (LOAEL), the no-observed-adverse-effect level (NOAEL), or the no-observed-effect-level (NOEL). These values must be derived at the end of the chronic toxicity study based on the observations. It is unlikely that all three values would be obtained from a study. Usually, one of these indices might be determined with a degree of reliability in the estimated value. Either the NOAEL or NOEL can be used by regulatory bodies to establish reasonable, estimated values for the indices (Ecobichon, 2005).

Plant drugs, also known as phytomedicines or phytopharmaceuticals are plant derived medicines that contain chemical compounds that act individually or in combination on the human body to prevent disorders and to restore or maintain health (Gurib-Fakim *et al*., 2006). Herbal drugs have been broadly categorized into four groups such as indigenous herbal medicines, herbal medicines in systems, modified herbal medicines and imported products with herbal medicine base (WHO, 2003).

Standardization of plant-derived ingredients in medicinal products is becoming increasingly important as a means of ensuring a consistent supply of high-quality phyto-pharmaceutical products. It can be defined as the establishment of reproducible pharmaceutical quality by comparing a product with established reference substances and by defining minimum amounts of one or several compounds or groups of compounds. In the field of phyto-medicines, standardization only applies to extracts. Standards for active ingredients to be used in medicinal products may be found in monographs and/or pharmacopeias (Gurib-fakim, 2006). The route of administration is very important since the dose, solubility, permeability, stability, bioavailability and pharmacokinetic parameters absorption, distribution, metabolism and excretion are the important properties that affect the fate of drugs through oral route. These parameters govern the design of dosage form for the specific drug candidate. Solubility and permeability of drugs can be correlated to their absorption through gastrointestinal tract. According to biopharmaceutical classification these two are the main parameters affecting the oral bioavailability (Amidon, *et al*. 1995). The drug discovery setting as described by Lipinski *et al*. (2001) illustrates that potency; solubility and permeability comprise the triad which is responsible to get acceptable absorption from this route. Metabolic stability, which is the rate and extent to which a molecule is metabolized also play a major role in drug development. A molecule which is rapidly and

extensively metabolized is considered to have a low degree of metabolic stability (Thompson, 2000). The physical stability of the drug also contributes for the presystemic loss of drug when administered perorally. While designing a dosage form for any drug candidate, it is important to consider the solubility, permeability and presystemic loss as major contributors for the drugs having poor bioavailability could help in successful design of the suitable dosage form or delivery system which can markedly improve the drug's performance through oral route. Many

Herbal preparations have proven to contain antioxidants. The global market of antioxidants is increasing rapidly, because of the increased health risk in a constantly polluting environment. Free radicals are highly reactive molecules or chemical species containing unpaired electrons that cause oxidative stress, which is defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage" (Sies, 1997). Oxidative stress can damage lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues, resulting in membrane damage, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins and even lead to cell death induced by DNA fragmentation and lipid peroxidation (Beckman and Ames, 1998). These consequences of oxidative stress construct the molecular basis in the development of cancer, neurodegenerative disorders, cardiovascular diseases, diabetes and autoimmune disorders.

Human antioxidant defense is equipped with enzymatic scavengers like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase; hydrophilic scavengers like urate, ascorbate, glutathione and flavonoids; lipophilic radical scavengers such as tocopherols, carotenoids and ubiquinol. The defense also comprises enzymes involved in the reduction of oxidized forms of molecular antioxidants like glutathione reductase, dehydroascorbate reductase. Apart from these

scavengers, there exists cellular machinery, which maintains a reducing environment, for example regeneration of NADPH by glucose-6-phosphate dehydrogenase. Some of these agents synthesized by cell itself; however, majority including ascorbic acid, lipoic acid, polyphenols and carotenoids are derived from dietary sources. In disease conditions, the defense against ROS is weakened or damaged and the oxidant load increases. In such conditions, external supply of antioxidants is essential to countervail the deleterious consequences of oxidative stress (Liu, 2004).

Antioxidants are classified into enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants are endogenous such as enzymes, low molecular weight molecules and enzyme cofactors. The most common endogenous antioxidant systems are: superoxide dismutase, catalase, and glutathione peroxidase. Among non-enzymatic antioxidants, many are obtained form dietary sources. Dietary antioxidants can be classified into various classes (Liu, 2004), of which polyphenols is the largest class. Polyphenols consist of phenolic acids and flavonoids. The other classes of dietary antioxidants include vitamins, carotenoids, organosulfural compounds and minerals.

Antioxidant enzymes, SOD and CAT, are not consumed and have high affinity and rate of reaction with ROS. Therefore, it may be hypothesized that the enzymes afford more effective protection against acute massive oxidative insults, such as hyperoxia or inflammation. Antioxidant enzymes are more potential agents in treating severe acute insults due to oxidative stress (Christofidou-Solomidou and Muzykantov, 2006). SOD and CAT are among the most potent antioxidants known in nature. There are three types of SODs in humans namely cytosolic CuZn-SOD, mitochondrial Mn- SOD and extracellular SOD.

Superoxide dismutase is a group of enzymes that catalyze the degradation of the superoxide anion into oxygen and hydrogen peroxide at a rate that is approximately 10,000-fold when compared to the non-catalyzed reaction by a group of enzymes collectively called superoxide dismutases (SODs) (Zelko *et al.,* 2002). This conversion is beneficial because hydrogen peroxide is much less toxic to the human body than superoxide. SOD enzymes require a metallic cofactor, either copper, manganese, zinc, or iron, which determines their location within the cell (Johnson *et al.,* 2008). In humans, the copper/zinc SOD is functional within the cytosol, whereas the manganese SOD is located in the mitochondrion (Bannister *et al.,* 1987). Additionally, in the extracellular fluid there is a third form of SOD. This SOD is made functional by having zinc and copper present in its active sites (Nozik-Grayck *et al.,* 2000).

CAT occurs abundantly in the body, with the highest activity in the liver, followed by erythrocytes, then the lungs. SOD catalyzes dismutation of superoxide into oxygen and hydrogen peroxide and it is widespread in nature in eukaryotic and prokaryotic organisms (Venkat *et al*., 2006). CAT protects cells by catalyzing hydrogen peroxide decomposition into molecular oxygen and water with no free radical production. This enzyme is unique in that it undergoes a double displacement reaction even though its only substrate is hydrogen peroxide (Hiner *et al.,* 2002). In addition, CAT acts on toxic compounds such as phenols, formic acid, formaldehyde and alcohols by peroxidative reaction. Although, catalase provides a valuable service by removing hydrogen peroxide, its deficiency has little effect in the human and animal model (Muller *et al.,* 1997).

The most abundant group of protective enzymatic antioxidants is the glutathione peroxidases, a selenium dependent family of multiple isozymes. These isozymes function to further the reaction generated by superoxide dismutase by further reducing H_2O_2 into water by acting as an electron

donor. Mammalian tissues have four major types of isozymes, abbreviated as GPx (Zelko *et al.,* 2002). The first is the classical isozyme, GPx1 is found in red blood cells, the liver, lungs and kidney. Second is the isozyme GPx2 located in the gastrointestinal tract. The third isoenzyme, GPx3 is extracellular, but most abundant in the plasma of the kidney, lung, epididymus, vas deferens, placenta, seminal vesicle, heart and muscle. Last, is the phospholipid isozyme, GPx. This isozyme is widely distributed similarly to the third isozyme of the plasma. More distinctively, each isozyme functions optimally in a specific sub-cellular location. The classical isozyme is found in the cytosol, nucleus and mitochondria. Gastrointestinal tract isozyme is located in the cytosol and the nucleus , the plasma isozyme can be found in the cytosol exclusively, and the phospholipid isozyme is accumulated in the nucleus, cytosol, mitochondria and bound to membranes. Also glutathione peroxidases also reduce organic peroxides to alcohols, providing another route for eliminating toxic oxidants (Margis *et al.,* 2008).

These free radical scavenging enzymes have been found to change qualitatively and quantitatively in various tissues and cells of patients with mitochondrial diseases and elderly subjects. It is now well established that the mitochondria are the major producers and also the main targets of ROS.

Human antioxidant defense system is incomplete without dietary antioxidants. The body also takes in antioxidants from exogenous sources found in the diet. There are two broad classifications of dietary antioxidants that are based on solubility: hydrophilic and hydrophobic. Generally, cytoplasm and blood are protected by water-soluble antioxidants and cell membranes by lipid soluble antioxidants (Sies, 1997). The action of an antioxidant is dependent on concentration, radical affinity, and the proper functions of the various components in the

antioxidant system. Recently, the antioxidant components of seeds obtained from common fruits and vegetables have been studied for the purposes of cancer prevention (Parry *et al.,* 2008).

Plants have a long history of use in the treatment of cancer (Gurib-Fakim *et al.*, 2006), though many or the claims for the efficacy or such treatments should be viewed with some skepticism because cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine (Gurib-Fakim *et al*., 2006).

Cancer development is associated with increased proliferation and decreased apoptosis. Failure of apoptosis creates a permissive environment for genomic instability with accumulation of mutations that increase cell survival, block differentiation and promote angiogenesis, invasion and metastasis and later culminating in neoplastic transformation (Ashe and Berry, 2003). Cancer cells evade apoptosis by downregulation of death receptors, overexpression of antiapoptotic proteins, and or reduced expression of proapoptotic proteins and caspases (Ashe and Berry, 2003). Recent ideas of carcinogenesis suggest a series of random genetic changes leading to a selective growth advantages over healthy cells. These changes leads to the disruption of coordinated network of intercellular communication thereby causing physiological change in cellular behaviour which affect processes such as proliferation, differentiation, and apoptosis. The continuous dysregulation of cellular functions indicates that cancer is not a morphological entity, but a process in which malignancy is gradually acquired (Lewis, 2006).

Table 1: Some plants with anticancer properties are as follows;

S/N	Name of plants	Active phytochemical	Medicinal use	References
1	*Catharanthus roseus*	Vincristine and vinblastine	Leukaemia , breast cancer, Hodgkin's diseases and choriocarcinoma	Gurib-Fakim *et al.*, 2006
2	*Podophyllum peltatum*	Podophyllotoxin	Lung cancer, testicular cancer and lymphomas	Cirla and Mann, 2003
3	*Taxus brevifolia*	Paclitaxel	Breast cancer	Cirla and Mann, 2003
4	*Combretum caffrum*	Combrestatin	Tumours	Cirla and Mann, 2003
5	*Brucea antidysenterica*	Quassinoid glycosides	Human tumour cell lines	Imamura *et al.*, 1993

Table 2: Plants studied as follows;

S/N	Botanical name of plants / synonyms	Common name	Local name
1	*Allium ascalonicum*	Shallot/spring onion	Alubosa elewe
2	*Butyrospermum paradoxum/ B. parkii/ Vitellaria paradoxa*	Shea butter tree	Eso ori
3	*Hoslundia opposite*	Hoslundia	Efinrin odan

4	*Olax subscorpioidea*	Olax, stink ant forest	Egbo ifon
5	*Xylopia aethiopica*	African guinea pepper	Eru awonka, eeru, erunje
6	*Securidaca longepedunculata*	Violet tree	Egbo ipeta
7	*Tetrapleura tetraptera*	Aridan	Aidan onigun

The individual plants used for this study have been reported to possess different pharmacological properties against various ailments as reported below;

Allium ascalonicum Linn. (Liliaceae) is an annual herbaceous plant belonging to the family liliaceae and commonly propagated in West Africa and widely used as spice for food (Owoyele *et al.,* 2004). *Allium ascalonicum* are reported to be effective traditionally as being antimicrobial, antifungal, antihypertensive, anticancer, and antihyperlipidemic (Trakranrungsie *et al.*, 2008; Adeniyi and Anyiam, 2004). Shallot has also been used in combination with other medicinal plants against cholera, rheumatism and gonorrhea (Adeniyi and Anyiam, 2004). It also posses antioxidative and free radical scavenging abilities (Wrongmekiat *et al*., 2008: Leelarungrayub *et al*., 2006). Some phytochemical studies showed that it contains S-alkylcysteine sulphoxides (Adeniyi and Anyiam, 2004), flavonoids, allicin and their derivatives (Bonaccorsi *et al*., 2008). Allium vegetables have been reported to contain Diallyl sulfide (DAS) which Upregulate Bax, downregulate Bcl-2, Upregulate p53, activate caspase 3 and downregulate Bcl-2; Ajoene which Activate caspase 3, downregulate Bcl-2 and activates JNK, p38, ERK; Allicin which Activate caspases 3, 8, 9 and cleave PARP; S-allyl cysteine (SAC) which Downregulate Bcl-2 and S-

allylmercaptocysteine (SAMC) which Increase caspase 3 activity and activate JNK, all of which help in the induction of apoptosis (Martin, 2006).

The Shea tree (*Vitellaria paradoxa* C.F. Gaertn., syn. *Butyrospermum paradoxum* sub sp. *Parkii* Hepper) of Sapotaceae family is a characteristic species of the woody flora in Sudan and Guinea savanna woodland of Africa (Lovett and Haq, 2000). The species is seldom planted. The existing populations of the species result from natural regeneration (Lamien *et al.,* 2006*)*. The extracts have been found useful in the traditional treatment of several human and animal diseases. The leaves, stem bark, root bark, fruits and kernels contain varying amounts of alkaloids, steroids, tannins, saponins and flavones which may be responsible for their usefulness in traditional medicine (Ogunwande *et al.,* 2001) .Decoctions of the leaves have been used in the treatment of malaria .The seed oil is used as a fertility stimulant, and an emollient which is applied on skin and mucous membranes in scabies, ulcers and nasal stiffness. The seeds have found use in Ivory Coast with other plants for the treatment of sleeping sickness, wounds and microbial infections (Ogunwande, *et al.,* 2001). Tapondjou *et al.* (2011) reported the presence of A triterpenoid saponins 3-*O*-[β-d-apifuranosyl-(1→3)-β-d-glucopyranosyl]oleanolic acid 28-*O*-[β-d-apifuranosyl-(1→3)-β-d-xylopyranosyl-(1→4)-[α-l-rhamnopyranosyl-(1→3)]-α-l-rhamnopyranosyl-(1→2)β-d-xylopyranoside in *Butyrospermum parkii* as antitumour. The antiploriferative activity could be related to the presence of apiose units observed in it.

Securidaca longepedunculata (fresen), commonly known as violet tree (family: polygalaceae), is a shrub or a small 2−10m high plant (Adebiyi *et al.,* 2006) with sweetly scented violet flowers, alternate leaves of variable sizes and shapes and pale grey smooth bark (Adeyemi *et al.,* 2010). The flowering savannah tree which is widely distributed in tropical Africa occurs naturally in the

north western and south western parts of Nigeria. It is reported that in Nigeria and some other African countries like South Africa, the roots, stem bark, and leaves of *S. longepedunculata* are used medicinally for treating a plethora of human and animal ailments (Adebiyi *et al.,* 2006). In Africa (Ghana, Nigeria) the plant decoction is used to treat asthma and other diseases associated with smooth muscle contraction and as antinociceptive and antidepressant (Muanda *et al.,* 2010; Adebiyi *et al.,* 2006). It is used as a general remedy for several other ailments such as coughs, colds, fever, backache, toothache, sleeping sickness, venereal disease, malaria, inflammation, rheumatism, snakebite, tuberculosis, ulcers and pneumonia. *S. longepedunculata* is also used as a contraceptive (Rakuambo *et al.,* 2006). Phytochemical analysis of the root showed the presence of methyl salicylate, senegin, aglycone, presenegenin, xanthone, ergot alkaloids, Elymoclavine and dehydroelymoclavine, securidacaxanthone (Meyer *et al.,* 2008). *S. longipedunculata* also contain indole alkaloids (Costa *et al*., 1992) which has been found to be anticancer (Quetin, 1994).

Tetrapleura tetraptera (Taub) (family: Fabaceae) is a single-stemmed, robust, perennial tree of 15–20 m high, with dark green leaves and a thick, woody base and spreading branches (Ojewole, 2005). The plant is naturally distributed over a large part of tropical Africa, especially in the rain forest belt of West, Central and East Africa. The four-winged fruit has a fragrant and pungent aromatic odour, which has been attributed to its insect repellent property (Okokon *et al*., 2007). The mature pods, which constitute the fruits of the plant, are about 15 –27 cm long and 4– 5 cm wide, and dark-brown when fully ripe. The fruit consists of fleshy pulp with some small, brownish-black seeds (Ojewole and Adewunmi, 2004). In Nigeria and some other West African countries, the nutritional, molluscicidal, anticonvulsant, analgesic, antiinflammatory and antidiabetic and other ethnomedical properties of extractives from the plant's fruit have been

reported (Ojewole, 2005: Okokon *et al*., 2007). The fruit had also been described as containing a volatile oil with strong phenolic odour which makes it useful as an anti-convulsant drug (Ukpong *et al.*, 1994). Scopoletin, a hydroxycoumarin was reported present in the fruits of *T. tetraptera.* Reports abound that coumarins such as 7-hydroxycoumarin are natural substances that has shown series of antitumour activities invivo, by inhibiting the release of cyclin D1, which is overexpressed in many types of cancer (Kawaii *et al*., 2000; 2001).

Xylopia aethiopica (Dunal) A. Rich. African guinea pepper is an angiosperm belonging to the family Annonaceae. *X. aethi*opica it is an evergreen, aromatic tree, largely found in West, Central and Southern Africa (Semova *et al.,* 2001). The fruits are reported to have high nutritive and medicinal value and have been shown to have antimicrobial action against gram positive and negative bacteria (Somova *et al.,* 2001). In Nigeria, the fruits are used in cough medicines, as well as a carminative and stimulating additive to other medicines. The powdered root is employed as a dressing and in the local treatment of cancer (Asekun and Adeniyi, 2004). It is also employed as a post-partum tonic and against female sterility (Tatsadjieu *et al*., 2003). Choumessi *et al*., (2012) reported the presence ent-15-oxokaur-16-en-19-oic acid.compound as one of the cytotoxic and antiproliferating compound in ethanolic extracts of Xylopia aethiopica. The crushed seed can also be applied topically on the forehead in the treatment of headache and neuralgia; it can also be taken as a decoction or concoction or chewed and swallowed. They exert peppery effects producing lacrimination and gustatory rihinorhoea or nasal dripping (Igwe *et al.,* 2003). Phytochemical studies of *X. aethiopica* show the presence of essential oil, volatile oil, resin, arocene, a rutheroside fat, alkaloids, glycosides, saponnis, tanins, stereols, carbohydrate,

protein and free fatty acid, mucilages and acidic compounds (Igwe *et al*., 2003), kaurene derivatives and xylopic acid (Somova *et al.,* 2001).

Hoslundia opposita Vahl is a herbaceous perennial shrub (1–2 m tall) belonging to the Lamiaceae. It is distributed in tropical and subtropical open lands of Africa. In Nigeria it is known as efirin odan by the Yorubas. Various parts of *H. opposita* are used traditionally in many parts of Africa to treat gonorrhea, cystitis, coughs, fever, wounds, convulsions, sores, mental disturbances, abdominal pains, snake bites and for the relief of swellings (Prakash and van Staden, 2007). It can also be used in the treatment of epilepsy and convulsions (Risa *et al.*, 2004), malaria, convulsion and microbes (Usman *et al.,* 2010). Phytochemical analysis revealed the presence of 5, 7 dimethyl-6-methylflavone, hoslundiol, eusaphic acid and some esters. Oils like germacrene D and β-caryophyllene chemotypes were also observed (Usman *et al.,* 2010).

Olax subscorpioidea Oliv belongs to the family of Olacaeae (Victoria *et al*., 2010). It is a shrubby plant commonly found in the tropics especially Africa. *O*. *subscorpioidea* can grow to 10m or more in height (Ayandele and Adebiyi, 2007). This plant has been reported to possess membrane stabilizing and antiprotease activities (Victoria *et al*., 2010). Fai *et al.,* (2009) reported the presence of oleanolic acid in *O. subscopioidea* and Ju-Hong *et al*., (2009) explained that oleanolic acid showed potential anti-tumor promoting and cytotoxic activities, inhibiting proliferation, inducing apotosis and preventing invasion. Its ethanolic stem extract possess antimicrobial activity (Ayandele and Adebiyi, 2007), extract of the root, effective against asthma, constipation and ulcer (Victoria *et al*., 2010). Phytochemical studies showed that *O*. *subscorpioidea* contains tannins, alkaloids, glycosides, saponins, flavonoids and steroids (Ayandele and Adebiyi, 2007).

CHAPTER THREE

3.0 Methodology

The plants were sourced in August 2007 from traditional medicine practitioners in Totoro village, Abeokuta Ogun State of Nigeria. They were identified and authenticated by the Forestry Research Institute, Ibadan and the Department of Pharmacognosy, Faculty of Pharmacy, University of Lagos and voucher specimens deposited. The seven plant materials used for this study are *Butyrospermum paradoxum* seed (PCGH 437; FHI 107924), *Securidata longepunculata* bark (PCGH 439; FHI 103049), *Tetrapleura tetraptera* pod (PCGH 382; FHI107984), *Hoslundia opposita* (PCGH 322; FHI 108121) leaves, *Xylopia aethiopica* seed (PCGH 441; FHI107698), *Olax subscorpioidea* stem (PCGH 438; FHI 107986) and *Allium ascalonicum* (PCGH 440; FHI 107763)

3.1.0 Extract preparation

The total plant materials (weighing 214g) *Butyrospermum paradoxum* seed (56.34g) , *Securidata longepunculata* bark (11.26g), *Tetrapleura tetraptera* stem (33.79g), *Hoslundia opposita* leaves (22.53g), *Xylopia aethiopica* seed (45.05g), *Olax subscorpioidea* stem(11.26g) and *Allium ascalonicum*(33.79g) were made into a cocktail (Personal communication with Late Pa Fatai) to produce the desired pharmacological action. Samples were air dried, powdered, weighed and then allowed to stand in 500 mL 95% cold ethanol for 72 h. They were thereafter decanted and filtered using a muslin cloth. The extract was further evaporated to dryness using a freeze-dryer at Biochemistry Department University of Lagos. Finally, the dried extract weighed 19.2 g. The cocktail was then reconstituted to concentrations of 400, 800 and 1600 mg mL$^-$. Fresh extract of *Joloo* was always prepared when desired.

3.1.1 Fractionation of extracts

Fractionation was carried out according to Yesilada and Kupeli, (2002) and Seyfi *et al*., (2010); Freshly obtained plant materials of *Butyrospermum paradoxum* seed (11.8g) , *Securidata longepunculata* bark (2.36g), *Tetrapleura tetraptera* stem (7.08g), *Hoslundia opposita* leaves (4.72g), *Xylopia aethiopica* seed (9.44g), *Olax subscorpioidea* stem(2.36g) and *Allium ascalonicum*(7.08g) all totalling (44.8g) was dissolved in 200ml of ethanol: distilled water (9:1), the mixture was then shaken with n-hexane and extract was evaporated under reduced pressure to yield hexane fraction (2.6g). Ethanol was evaporated from the remaining extract and diluted with distilled H_2O to 200ml and further fractionated by successive solvent extraction with chloroform ($4\times$ 100ml), ethyl acetate ($2\times$100ml) and n-butanol saturated with H_2O ($3\times$100ml). Each extract was evaporated under reduced pressure to yield 'chloroform fraction (3.6g), ethylacetate fraction (1.45g), butanol fraction (12.6g) and the remaining ethanol fraction (23.5g).

3.1.2 Animals

Swiss mice and albino rats (*Rattus rattus*) of either sex weighing 20-32g and100-180g respectively, bred in the animal house of the University of Lagos were used for this study. The animals were maintained under standard environmental conditions as described by the method of Bishayee and Chanterjee (1994) according to Mbagwu *et al*., (2007). They had access to water and standard feed *ad libitum.*

3.2.0 Acute, Subchronic and Chronic toxicity studies

3.2.1 Acute Toxicity

Acute toxicity (LD_{50}) was estimated in mice (50 in each case) by intraperitoneal (i.p.) and oral (p.o.) administration of the cocktail. The animals were randomly divided into 5 groups of 10 mice each for i.p. and p.o., respectively. The control group received only distilled water p.o. while the different test groups received intraperitoneal and oral doses of 400, 800, 1200, 1600 and 2000 mg kg^{-1} b. wt., of the coctail. The general symptoms of toxicity observed within 24 h in each group were monitored and recorded. The number of mortality was recorded within 24 h for the i.p. group and 72 h for the p.o. group. The median lethal dose (LD_{50}) was estimated from the graph of percentage (%) mortality (converted to probit) against log-dose of the extract-probit 5 being 50% (Nwanjo, 2005).

3.2.2 Subchronic Toxicity

Animals weighing between 128-160g were divided into four groups of 10 rats each were administered with *Joloo* for 28 days by oral gavage using a curved blunt tipped stainless steel feeding needle. Group one (control) was administered with distilled water 10 ml kg^{-1} while groups two, three and four received *Joloo* at doses of 400, 800 and 1600 mg kg^{-1} b. wt., respectively. The weights of the animals were monitored and recorded weekly for four weeks after which animals were sacrificed. The organs and tissues were then collected, weighed and processed on day 28 for relevant assays and histopathologic analysis.

3.2.3 Chronic Toxicity

Mice of either sex, weighing 24–30 g, were housed in plastic cages (10/ cage). The animals were divided into four groups and their weights were recorded. The extract was administered daily by gavage for 91 days to different groups of rats at doses of 400 mg kg^{-1} b. wt., 800 mg kg^{-1} b. wt., and 1600 mg kg^{-1} b. wt., while the control rats received distilled water only. The weights of the animals were monitored and recorded weekly for thirteen weeks after which animals were sacrificed. The organs and tissues were then collected, weighed and processed on day 91 for relevant assays and histopathologic analysis.

3.2.4. Mortality and clinical signs

During the administration period of toxicity studies, animals were observed for general appearance, mortality and clinical signs.

3.2.5 Haematology, Relative organ weight and Necropsy

The mice were fasted for 16-19 h on the autopsy day and anesthetized with ethyl ether. All the animals were then euthanized by exsanguinations and blood samples collected from the abdominal aorta into EDTA vials for routine estimation of neutrophils, pack cell volume, red blood cells, white blood cells, lymphocytes, eosinophils, monocytes and basophils. The spleen, heart, liver, kidneys, brain and lungs were harvested and weighed to determine the absolute organ weight. The relative organ weight of each animal was then calculated based on body weight measured on the day of sacrifice as follows:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight}}{\text{Body weight of rat on sacrifice day}} \times 100$$

The organs were thereafter preserved in 10% buffered formalin for histopathologic examinations. The tissue biopsies were dehydrated and embedded in paraffin, cut into 4-5 μm sections with rotary microtome (LEICA RM 2235 Rotary Microtome), and stained with hematoxylin-eosin for photomicroscopic examination.

3.2.6 Biochemical analysis

Liver and kidney samples were dissected out (1g each) and washed immediately with ice cold saline to remove as much blood as possible. Liver homogenates (5% w/v) were prepared in cold 50mM potassium phosphate buffer (pH 7.4) using glass homogenizer in ice. The cell debris was removed by centrifugation at 5000 rpm for 15 at 4^0C using refrigerated centrifuge. The homogenate derived from each sample was used for all assays which included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol, creatinine kinase (Cknac), protein, urea and albumin. Spectrophotometer optimal sp 3000 model was used for this study at Nigerian Institute for Medical Research, Yaba.

3.2.7 Determination of lipid peroxidative indices

Lipid peroxidation as evidenced by the formation of TBARS was measured by the method of Niehaus and Samuelsson (1968) as modified by Rukumani *et al.,* (2004). 0.1 ml of tissue homogenate (Tris-HCl buffer , pH 7.5) was treated with 2ml of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobabituric acid 0.37%, 0.25N HCl and 15% TCA) and placed in a water bath for 15min, cooled and centrifuged at room temperature for 10min at 1,000 rpm . The absorbance of clear pink supernatant was measured against reference blank at 535nm.

3.2.8 Determination of reduced glutathione, superoxide dismutase, Catalase and glutathione peroxidase

3.2.8.1 Determination of reduced glutathione (Non-enzymic antioxidant)

Reduced glutathione (GSH) was determined by the method of Ellman (1959) as modified by Rukumani *et al.,* (2004). To the homogenate 10% TCA was added and centrifuged. 1.0ml of supernatant was treated with 0.5ml of Ellman's reagent (19.8mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm.

3.2.8.2. Superoxide dismutase (SOD)

Superoxide dismutase (SOD) was assayed using the technique of Kakkar *et al.,* (1984). Plant samples (600 mg) were homogenized in 5 ml of 0.25 M sucrose under cold conditions. The homogenate was centrifuged at 17,000g for 20 min at 4°C. Assay mixture contained 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml 186 mM phenazine methosulfate, 0.3 ml 300 mM nitroblue tetrazolium, 0.2 ml NADH (780 mM), 0.5 ml plant extract, and water in a total volume of 3 ml. Reaction was started by the addition NADH. After incubation at 30°C for 90 s, the reaction was stopped by the addition of 1.0 ml glacial acetic acid. The absorbance was read at 560 nm. A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazolium) reduction/min/mg protein.

3.2.8.3 Catalase (CAT)

Catalase (CAT) was assayed colorimetrically at 620 nm and expressed as μmoles of H_2O_2 consumed/min/mg protein as described by Sinha (1972) as modified by Rukumani *et al.,* (2004). The reaction mixture (1.5ml) contained 1.0 ml of 0.01M pH 7.0 phosphate buffer, 0.1 ml of

tissue homogenate and 0.4 ml of 2M H_2O_2. The reaction was stopped by the addition of 2.0ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).

3.2.8.4 Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) activity was measured by the method described by Sinha (1972) as modified by Rukumani *et al.,* (2004). Reaction mixture contained 0.2ml of 0.4M phosphate buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of tissue homogenate (homogenized in 0.4M, phosphate buffer pH 7.0), 0.2 ml glutathione, and 0.1 ml of 0.2mM hydrogen peroxide. The contents were incubated at 37^0C for 10 min. The reaction was arrested by adding 0.4 ml of 10% TCA, and then centrifuged. Supernatant was assayed for glutathione level by using Ellmans reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate), which measures the reduction of 5,50-dithio-bis (2-nitrobenzoic acid) (DTNB) by sulfhydryl groups to 2-nitro-5-mercaptobenzoic acid, which has an intense yellow color.

3.2.8.5 Total protein and Albumin

Preparation of protein reagent

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of I liter. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid (Bradford, 1976).

Protein assay (standard method)

Protein solution containing 10 to 100 pg protein in a volume up to 0.1 ml was pipetted into 12 x 100 mm test tubes. The volume in the test tube was adjusted to 0.1 ml with 0.4 phosphate buffer. Five milliliters of protein reagent was added to the test tube and the contents mixed either by inversion or vortexing. The absorbance at 595 nm was measured after 2 min and before I hr in 3 ml cuvettes against a reagent blank prepared from 0.1 ml of the 0.4m phosphate buffer and 5 ml of protein reagent (Bradford, 1976).

Microprotein assay

Protein solution containing 1 to 10 pg protein in a volume up to 0.1 ml was pipetted into 12 x 100 mm test tubes. The volume of the test tubes was adjusted to 0.1 ml with 0.4m phosphate buffer. One milliliter of protein reagent was added to the test tube and the contents mixed as in the standard method of protein assay. Absorbance at 595 nm was measured as in the standard method except in 1 ml cuvettes against a reagent blank prepared from 0.1 ml of the phosphate buffer and 1 ml of protein reagent (Bradford, 1976).

3.3.0 Genotoxicity test

3.3.1 *Allium cepa* assay

The method described by Odeigah *et al*., (1997) was adopted. Fresh and healthy looking onions with fresh primordial roots, with 15-22 mm size and a weight of 2-4 g were selected and divided into four groups of ten onions each. Onions were grown in control medium for first 24 hours:

Individual onions were put onto 15-ml-Falcon tubes, filled with control medium. The base of the onion was allowed to reach the medium surface. Tube-stands were covered with aluminum to keep the onion roots in dark during growth. They were incubated at 25±1 °C in cultivator with light cycle. After 24 hours exposition, the plants with the poorest root growth were excluded. Three concentrations of the cocktail (400, 800 and 1600 $mg\ kg^{-1}$ b. wt) and distilled water were used as control. Test solutions were changed daily and the length of roots were measured daily for seven days.

3.3.2 Maceration of the root tips and preparation for microscopy

After 96 h, some roots from each test group were harvested for cytological studies by the conventional aceto-orcein squash technique described by Fiskesjo (1985). The Root tips harvested were cut from treated and control plants at a length of 10mm and placed into10-ml-glass tube with 2 ml acetic acid/HCl solution {mixture of 45% acetic acid (9 parts) and 1M HCl (1 part)}. The root tips were then heated for 5 minutes at 50 ºC, thereafter, they were placed on a microscope-slide on a black background and the terminal tips (1-2 mm) were cut off. The rest of root material and liquid were removed from the slide. 2 drops of orcein solution was added and mixed properly with the roots by stirring and knocking with a stick of stainless steel (stirring spattle) for about 5-10 min. Cover slip was placed on the root cells. The cells were squashed by placing filter paper on the cover slip and pressing slightly with thumb. Slides were then observed by microscopy.

3.3.3. Microscopic examination and determination of mitotic index

For each root tip, the numbers of mitotic and total meristematic cells were counted in 5-8 fields of view using high power (100X) light microscope (Badria *et al*., 2001). In all, 350-500 cells were counted and cells manifesting different stages of mitosis were recorded. The mitotic index was calculated using the formula below:

Mitotic index = (P+M+A+T/Total observed cells)

Where;

P= prophase

M= metaphase

A= anaphase

T= telophase

3.4.0 Cytotoxicity assay

Cytotoxicity of the extracts was measured using the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described by Kigondu *et al.,* (2009), on African green monkey kidney cells (Vero) and chicken embryo fibroblast (CEF). The principle of cell viability determination is due to the cleaving of the MTT tetrazolium salt by mitochondrial Succinate-dehydrogenase enzyme of viable cells and converted to an insoluble purple formazan. 100 μL containing 2×10^4 of cell suspension was placed in each of 96-well plates cells incubated at $37^O C$ in 5% CO_2 for 24 h to attach. After attachment, the medium was aspirated off carefully and cell cultures were treated with the extracts at concentrations of 1mg/ml, 2.5mg/ml and 5mg/ml. Cyclosphosphamide and cells without media were used as positive and negative controls

respectively. Each sample was replicated 4 times and the cells incubated at 37° C in 5% CO_2 for 48 h. After 48 h incubation, 20 µL of the proliferation reagent MTT at concentration of 5 mg mL$^-$ ^{2} in phosphate-buffered saline (PBS, pH 7.4) was added to each well and the cells were incubated at 37 ^{o}C for 4 h in humidified atmosphere with 5% CO_2. At the end of the incubation period, the medium together with MTT was aspirated off from the wells; formazan precipitates were solubilized by addition of 100 µL dimethyl sulfoxide (DMSO) and the plate shaken gently for 5min. After 10 min at room temperature, the formazan salts were quantified by reading spectrophotometrically, the absorbance at 492 nm of each well in a microculture plate reader {Lab systems (Multiskan EX) serial RS-232 C}.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of experimental well}}{\text{Absorbance of control wells}} \times 100 \text{ (Sreeja and Sreeja, 2009)}$$

3.5.0 Gonadotoxicity Assay

Mice (male) weighing between 28 and 30g were divided into four groups of five mice. Each was administered with *Joloo* for 28 days by oral gavage using a curved blunt tipped stainless steel feeding needle. Group 1-3 were administered *Joloo* at doses of 400, 800 and 1600 mg kg-1 b. wt. respectively, the last group (control) was administered with distilled water 10 ml kg-1.

At the end of the exposure period, mice were sacrificed by cervical dislocation. The testes were excised and fixed in Bouin's fluid for 48 h. They were processed in an automatic tissue processor, and embedded in paraffin wax. Sections of 5 µm thicknesses were cut with rotary microtome (LEICA RM 2235 Rotary Microtome) by serial sectioning until the entire thickness of the testis was sectioned. The sections were stained with haematoxylin and eosin (H & E) for

light microscopy using a Leitz microscope. Test slides were compared to those of the controls to detect pathological changes associated with treatments (Orisakwe *et al.,* 2004).

3.6.0 Pharmacologic evaluation of general malaise

3.6.1 Analgesic activity

3.6.2 Acetic acid- induced abdominal constriction assay

The method adopted was as described by Mohamad *et al*., (2005). Fifty Swiss albino mice of either sex were divided into five groups (Control-1; Reference-2 and Test 3-5) of ten animals each. The control animals were given 10 mL kg^{-1} of normal saline only while the reference groups were injected with 100 mg kg^{-1} acetylsalicylic acid subcutaneously. The test animals were treated intraperitoneally (i.p.) with varying doses of 400, 800 and 1600 mg kg^{-1} of the ethanol extract respectively. Forty minutes after treatment, a dose of 10 mL kg^{-1} of 0.6% v/v acetic acid solution in normal saline was administered i.p. to all the groups to induce pain. Thereafter, the number of abdominal constrictions occurring within 5 and 15 min were counted. The fewer number of constrictions observed among the test groups compared with the constrictions recorded for the control group was considered as evidence of the presence of analgesia and was expressed as a percentage inhibition of constrictions (Amir and Kumar, 2005). Data calculation was according to the following formula:

$$\% \text{ Inhibition} = \frac{\text{Mean No. of constrictions (control)} - \text{Mean No. of constrictions (treated)}}{\text{Mean No. of constrictions (control)}} \times 100$$

3.6.3 Formalin- induced pain test

The method of Shibata *et al.,* (1979) as reported by Mbagwu *et al*., (2007) was used. Fifty Swiss mice of either sex were divided into two- control and three test treatment groups of 10 mice each. Each of the test groups were given the cocktail at a dose of 400, 800 and 1600 mg kg^{-1} b. wt, respectively while the two control groups received 10 mL kg^{-1} normal saline orally and 100 mg kg^{-1} b. wt of acetylsalicylic acid (ASA) subcutaneously for thirty minutes. 0.2mL of 1% formalin was injected subcutaneously into the right hind paw of pretreated mice to induce pain. Responses were measured five minutes after formalin injection, for the first phase and the second phase were taken 15-30 min later. The licking of the injected paw and the duration was indicative of pain.

3.6.4 Hot plate- induced pain test

The hot plate method as described by Gupta *et al*., (2007) was carried out with minor modifications. Mice were divided into five groups of ten animals each. A dose of 400, 800 and 1600 mg kg^{-1} b. wt of the cocktail was administered orally to the three test groups while 10 mL kg^{-1} of distilled water was given to the control and 2 mg kg^{-1}b. wt morphine administered subcutaneously to the reference group. Thirty mins later, the animals were dropped gently on the hot-plate set at 55±1°C. The reaction time was determined as the interval between placement of the animals on the hot plate and the moment the animal either licks its fore-paws or jumps out of the plate.

3.7.0 Anti-inflammatory activity

3.7.1 Carageenan-induced paw oedema

The induced pedal inflammation in rat hind paw by the subplantar injection of the phlogistic agent described by the method of Winter *et al*., (1962) as reported by Owoyele *et al*., (2004) with minor modifications to fit available laboratory conditions was performed. The Albino rats of either sex used for this study were fasted for 12 h but allowed access to water only. The ethanol extract 400 -1600 mg kg^{-1} b. wt was administered orally to the test groups of rat while indomethacin 10 mg kg^{-1}b. wt was given subcutaneously to the reference group. The control group received only normal saline orally. To induce paw oedema, 0.1 mL kg^{-1} of 1% Carageenan diluted in distilled water was injected into the sub-plantar region of the right hind paw 1 h after treatment. Oedema was assessed immediately after Carageenan injection at intervals of 0, 1, 2, 3, 4, 5 and 6 h using the cotton thread method described by Mbagwu *et al.,* (2007). The increase in paw swelling was measured and percentage inhibition calculated.

3.7.2 Egg albumin-induced inflammation:

Hind paw inflammation in rats was induced by injecting Egg albumin (0.1 mL kg^{-1} 1% w/v in normal saline) into the sub-plantar tissue of the right hind paw. Control animals were administered 10 mL kg^{-1} distilled water orally. The cocktail at a dose 400-1600 mg kg^{-1} b. wt and chloropheniramine (100 mg kg^{-1} b. wt) were administered to the test and reference groups 90 min before injecting egg albumin to induce inflammation. The linear paw circumference was assessed at 15 min interval over 180 min using the cotton thread method (Mbagwu *et al*., 2007).

3.8.0 Anti-pyretic activity

Anti-pyretic activity of the preparation was carried out using the methods of Berken *et al.,* (1991). Rats were weighed and randomized into five groups of ten rats per group. The baseline body temperatures of the rats were taken by inserting a clinical thermometer into their anal cavities for 2 min. The steady temperature readings obtained were recorded as the pre-treatment temperatures. Pyrexia was induced in the rats by the administration of 10 mg kg^{-1} b. wt., of 2, 4-Dinitrophenol (DNP) intraperitoneally. Hyperthermia developed 30 min later after DNP administration. Different doses of the preparation (ranging between 400 - 1600 mg kg^{-1} b. wt) were given orally, aspirin (100 mg kg^{-1} b. wt. i.p) and distilled water (10 mL kg^{-1} b. wt.) was administered orally to the treatment and control groups of animals. Rectal temperatures were obtained at 30min interval for 5 hr.

3.9.0 Antimalarial activity

3.9.1 Parasite inoculation

The chloroquine sensitive *Plasmodium berghei* was obtained from Biochemistry Department of Nigerian Institute of Medical Research, Lagos, Nigeria and maintained in mice by passaging. The inoculum consisted of 5×10^7 *P. berghei* parasitized red blood cells per ml. This was prepared by determining both the percentage parasitaemia and the red blood cell count of the donor mouse and diluting the blood with isotonic saline in proportions indicated by both determinations. Each mouse was inoculated on day 0, intraperitoneally, with 0.2 ml of infected blood containing about 1×10^7 *P. berghei* parasitized red blood cell.

3.9.2 Evaluation of schizontocidal activity on early infection (5-day test)

Schizontocidal activity of the *Joloo* was evaluated using the method described by Okokon *et al.,* (2007) Each mouse was intraperitoneally inoculated on the first day (day 0), with 0.2 ml of infected blood containing about 1×10^7 *P. berghei* parasitized erythrocytes. The animals were divided into five groups of five mice each and orally administered, shortly after inoculation with 400, 800 and 1600 mg/kg day doses of *Joloo*, chloroquine 5 mg/kg day and an equivalent volume of distilled water (negative control) for four consecutive days (days 0–3). On the fifth day (day 4), thin and thick films were made from the tail blood of each mouse and the parasitaemia level was determined by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields of the microscope. Average percentage chemosuppression was calculated as $100((A-B)/A)$, where A is the average percentage parasitaemia in the negative control group and B, average percentage parasitaemia in the test group.

3.9.3 Evaluation of schizontocidal activity on established infection (curative or Rane test)

Evaluation of curative activity of *Joloo* was done according to Okokon *et al.* (2007). The mice were injected intraperitoneally with standard inoculum of 1×10^7 *P.berghei* infected erythrocytes on the first day (day 0). Seventy-two hours later, the mice were divided into five groups of five mice each. The groups were orally administered with *Joloo* (400, 800 and 1600 mg/kg day), chloroquine (5 mg/kg) was given to the positive control group and an equal volume of distilled water to the negative control group. The treatment, control and chloroquine were administered once daily for 5 days. Thin and thick films stained with Giemsa stain were prepared from tail blood of each mouse daily for 5 days to monitor the parasitaemia level.

3.10.0 Microbiological studies

3.10.1 Microbiological Media

The antimicrobial agents used were: Amoxicillin (Reichamox Trademark) and Fluconazole (MA Holder TEVA UK Ltd). The Medium used for the cultivation includes Sabouraud Dextrose Agar, Nutrient Agar and Muller Hinton Agar.

3.10.1.1 Sample collection

Bacterial Isolation

Pure strains of Gram negative organisms *Klebsiella pneumoniae, Escherichia coli* (ATCC 12900, *Pseudomonas aeruginosa,* and *shigellia dysenteriae* were obtained from the Nigeria Institute of Medical Research Laboratory (NIMR) Yaba, Lagos. Other isolates used were obtained from the Redeemer's University Microbiology laboratory: *Salmonella typhi* (isolated from human faeces), *Proteus vulgari* (isolated from water) and *Yersinia enterocolitica* (isolated from drinks). They were maintained on Agar slants at 4^oC in the refrigerator.

3.10.2 Antimicrobial Assay: Bacteria

The Agar-well dilution method was used for this susceptibility studies. An inoculum of each bacterial test organism was transferred into test tubes containing 5 ml of enrichment media (peptone water). The broth was incubated for 2- 6 hours at 35 ^0C and adjusted until it became of standardized concentration with McFarland standard to provide an initial cell count of about 2×10^8 CFU/ml.

3.10.2.1 Inoculation of the bacterial plate

A sterile cotton swab was dipped into the adjusted suspension and used to streak all over the dried surface of a sterile Muller Hinton Agar plate. This streaking process was repeated 2 − 3 times to ensure that the test organisms were evenly distributed. The inoculum was allowed to diffuse into the agar for about 10 minutes. Four wells (6mm) were aseptically made using sterile cork borer equidistant to each other, fixed volume 0.1ml (100ul) of the plant extract at different concentrations were carefully placed into each hole while the fourth hole contained a broad spectrum antibiotic (Amoxicillin) as control. The plates were prepared in triplicates and then incubated at 37 ^{0}C for 18-24 hours. The zone of inhibition of each well was obtained by measuring the underside of the plate in two planes with a ruler calibrated in millimeter.

3.10.2.2 Fungal isolation

Fungi species used were obtained from the research laboratory of the Microbiology Department, Redeemer's university Ogun, Nigeria. The fungi (*Trichoderma* Spp, *Colletrichum gloeosoprioides*, *Aspergillus niger*, *Rhizopus* Spp) were sub cultured on Sabouraud Dextrose agar and incubated for 72 hours, after which the fungi strains were identified.

3.10.3 Antimicrobial Assay: fungi

Inoculum of the fungi was prepared by removing 2 cm^3 block of agar containing actively growing mycelium with a cork borer and placing it into 10 ml sterile distilled water and then macerated. A sterile bent glass rod was used for spreading 2ml of cell suspension into each plate on the agar surface. Four wells (6mm) were made using sterile cork borer equidistant to each other, 0.1ml of the plant formulation at different concentrations were added into the holes while the fourth

hole contained a broad spectrum fungicide (Fluconazole) as control. The plates were then incubated at 30 ^{0}C for 72 hours.

The antimicrobial activity of *Joloo* was measured as the diameter (mm) of clear zone of growth inhibition.

3.10.4 Determination of Minimum Inhibitory Concentration (MIC)

The determination of the minimum inhibitory concentration of the extract was carried out using the agar plate method as described by Adeniyi and Ayepola (2008). Serial dilutions of *Joloo* were prepared and 1ml of each dilution of the formulation was mixed with 20 ml of Mueller Hinton agar, poured into Petri dishes and allowed to set. The agar was streaked with an overnight broth culture of the microbial isolates and incubated overnight. The plates were then examined for the presence or absence of growth. In all cases the lowest concentration at which there was no growth was recorded as the MIC.

3.10.5 Physico-chemistry analysis of heavy metals

Nitric acid digestion for Atomic Absorption Spectrophotometry (AAS)

The method of Hseu (2004) was adopted. One gram of each sample was placed in a 250 ml digestion tube and 10 ml of concentrated HNO_3 was added. The sample was heated for 45 min at 90 ^{0}C, temperature was increased to 150 ^{0}C and sample was boiled for at least 8 h until a clear solution was obtained. 3×5ml Concentrated HNO_3 was added to the sample and digestion occurred until the volume was reduced to about 1 ml. The interior walls of the tube were washed down with a little distilled water and the tube was swirled throughout the digestion to keep the wall clean and prevent the loss of the sample. After cooling, 5 ml of 1 % HNO_3 was added to the

sample. The solution was filtered with Whatman No. 42 filter paper and <0.45 lm Millipore filter paper. It was then transferred quantitatively to a 25 ml volumetric flask by adding distilled water. The concentrations of Ca, Se, Cu, Mn, Fe, and Zn in the final solutions were determined by an atomic absorption spectrometer (AAS) (Schimadzu Model 7000) located in Redeemer's University, Mowe, Ogun state, Nigeria.

4.0 RESULTS

4.1 Acute toxicity studies

Mortality was not observed in the different groups of mice that received the cocktail orally, until the maximum dose of 1600 mg kg^{-1} b. wt. However, 60% mortality was recorded after 72 h for this same orally treated group at 2000 mg kg^{-1} b. wt. dosage. In addition, within the (i.p.) group, mortality rate progressed from 50% at 400 mg kg^{-1} b. wt., to 100% at 1600 and 2000 mg kg^{-1} b. wt., dose of the cocktail (Fig. 1). The LD$_{50}$ value was 400 mg kg^{-1} b. wt. (i.p.) and 2000 mg kg^{-1} b. wt. (per os).
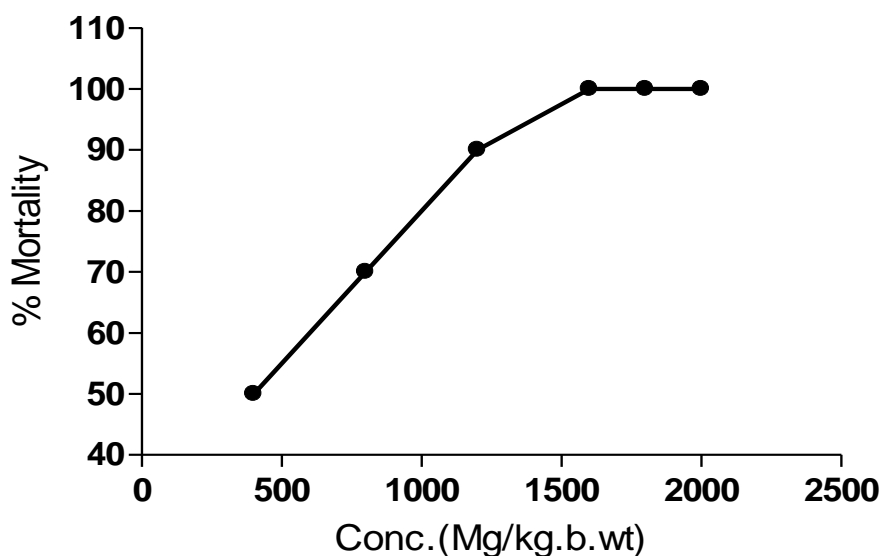
Fig.1. Percentage mortality of mice treated (I.P) with *Joloo*

I.P – intraperitoneal

4.2 Subchronic toxicity

4.2.1 Clinical signs and mortality

During the 28 days treatment period, 20% mortality was recorded among the animals administered with 1600 $mg\ kg^{-1}$ b. wt dose of *Joloo*. These animals showed signs of emaciation but, neither mortality nor any other adverse clinical manifestations were observed in the 400 and 800 $mg\ kg^{-1}$ b. wt dose test groups.

Table 3: Effect of *Joloo* on body weight of albino rats in 28 days

Dose (Mg)	Wk 1(g)	Wk 2(g)	Wk 3(g)	Wk 4(g)	Mean±SEM
Control	128.2	132.2	147.4	160.6	142.0±7.4
400	142.4	150.0	150.9	162.9	151.6±4.2
800	149.8	129.2	142.7	148.8	142.6±4.7
1600	158.8	162.3	177.2	174.7	168.3±4.5*

Data presented as Mean ± SEM. N= 10. Significantly different from control: *$p < 0.05$

4.2.2 Haematologic indices

Variable changes with no defined patterns were observed in the level of neutrophiles while the WBC on the other hand, increased with increasing doses. Graded decrease in both PCV and RBC was noticed with the increased dose levels.

Table 4: Effect of *Joloo* on haematological parameters of albino rats post 28 days treatment

Dosage/mg	Neutrophils %	PCV	RBC (10^{12}/1)	WBC (10^9/1)
Control	23.8±6.9	44±2.8	1.62±2.1	37.8±0.86
400	15.3±4.6	39.5±0.7	1.14±0.6*	47.7±1.12
800	21±5.9	39.8±2.6	0.98±0.5*	53.8±0.37
1600	17.7±2.5	38.7±0.8	0.85±1.12*	60.6±1.73

Data presented as Mean ± SEM. N= 10. Significantly different from control: *$p < 0.05$.

(Confirmed in haematology Dept. by Mr Sam in National Institute for Medical Research, Yaba)

PCV-Packed cell volume; RBC- Red blood cell; WBC-white blood cell.

4.2.3 Biochemical indices

The biochemical indices clearly showed that the effect of *Joloo* was dose-dependent and that no statistically significant adverse effects were observed in all the parameters (Table 5). Generally, the biochemical analytes were compared to the control except in ALP and C/knac where significant decrease in activity was observed at 400 and 800 mg kg^{-1} b. wt. However, alkaline phosphatase activity in the rats treated with 1600 mg kg^{-1} b. wt of *Joloo* was significantly higher than the control group.

Table 5: Effect of *Joloo* on rat liver enzymes activities and serum biochemical analytes post 28 days treatment

Dose mg kg^{-1}	ALP IU/L	ALT IU/L	AST IU/L	CHOLS mg/dl	C/knac U/l	Creatinine mgdl^{-1}
Control (10)	27.5±37.3	7.1±0.4	84.1±5.5	59.92±5.5	23.2±8.6	1.64±0.14
400	17.2±44.0 [c]	25.3±0.6	78.99±3.3	56.5±4.9	1.9±1.6 [c]	1.51±0.21
800	17.5±44.0 [c]	26.7±0.7	88.3±4.5	58.7±3.5	8.4±4.9 [c]	1.55±0.05
1600	47.8±6.70	29.4±3.2	88.8±10.3	62.6±0.8	17.3±3.7 [b]	1.84±0.03

Data presented as mean ± S.E.M (N=10); [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$ significantly different from control.

ALP-Alkaline phosphatase

ALT- Alanine transaminase

AST- Aspartate transaminase

CHOLS- Cholesterol

C/knac- Creatine kinase

Gross and Histologic Pathology of Sub-Chronic Toxicity

Plate 1; Photomicrograph of lungs of Rats after subchronic studies, all the features were conserved
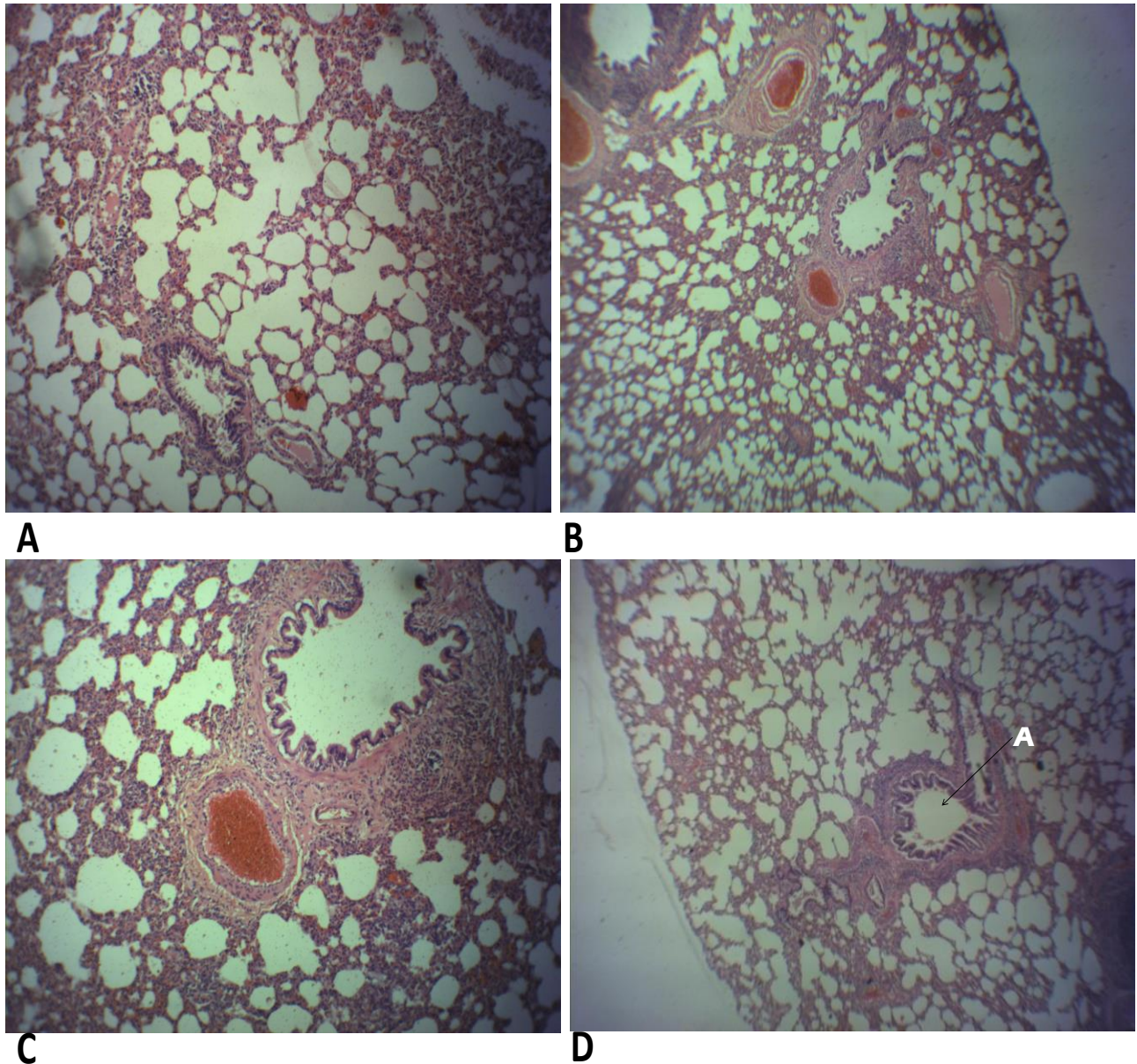
Photomicrograph of Lungs of Rats treated with - **A**; distilled water (control), **B**;400 mg kg^{-1} b.wt, **C**;800 mg kg^{-1} b.wt **D**;1600 mg kg^{-1} b.wt of '*Joloo*' for 28 days.

Stains: haematoxylin and eosin. Mag: x100.

Plate 2; Photomicrograph of liver of Rats after subchronic studies

All the livers tested were conserved and no injury was observed

A

B

C
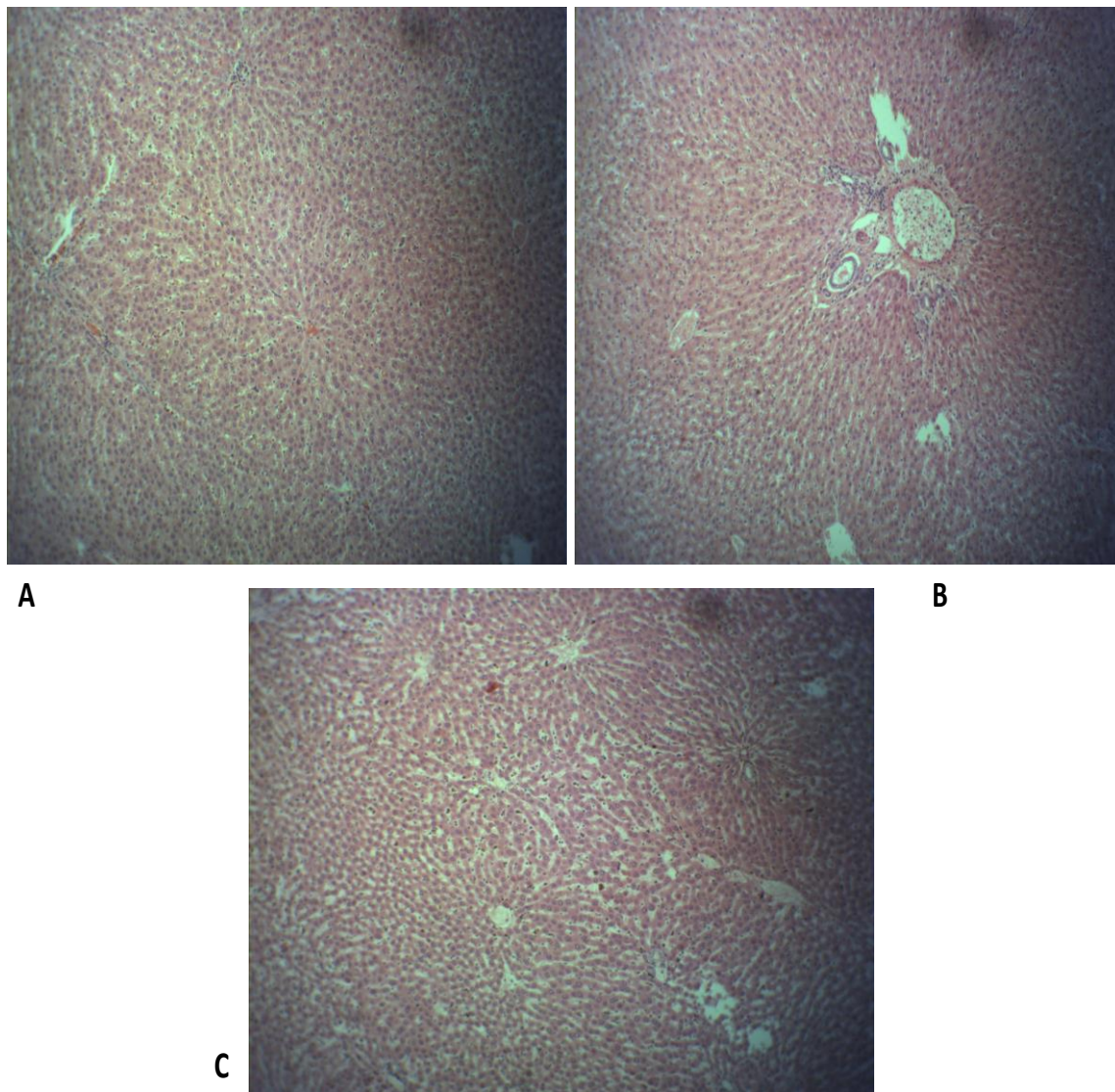
Photomicrograph of liver of Rats treated with- **A**; distilled water (control), B; 400 mg kg^{-1} b.wt, **C**; 800 mg kg^{-1} b.wt '*Joloo*' for 28 days.

Stains: haematoxylin and eosin. Mag: x100.

Plate 3; Photomicrograph of kidney of Rats after subchronic studies

Joloo did not show any aberration after 28days administration on the kidney of rats during subchronic toxicity

A   **B**
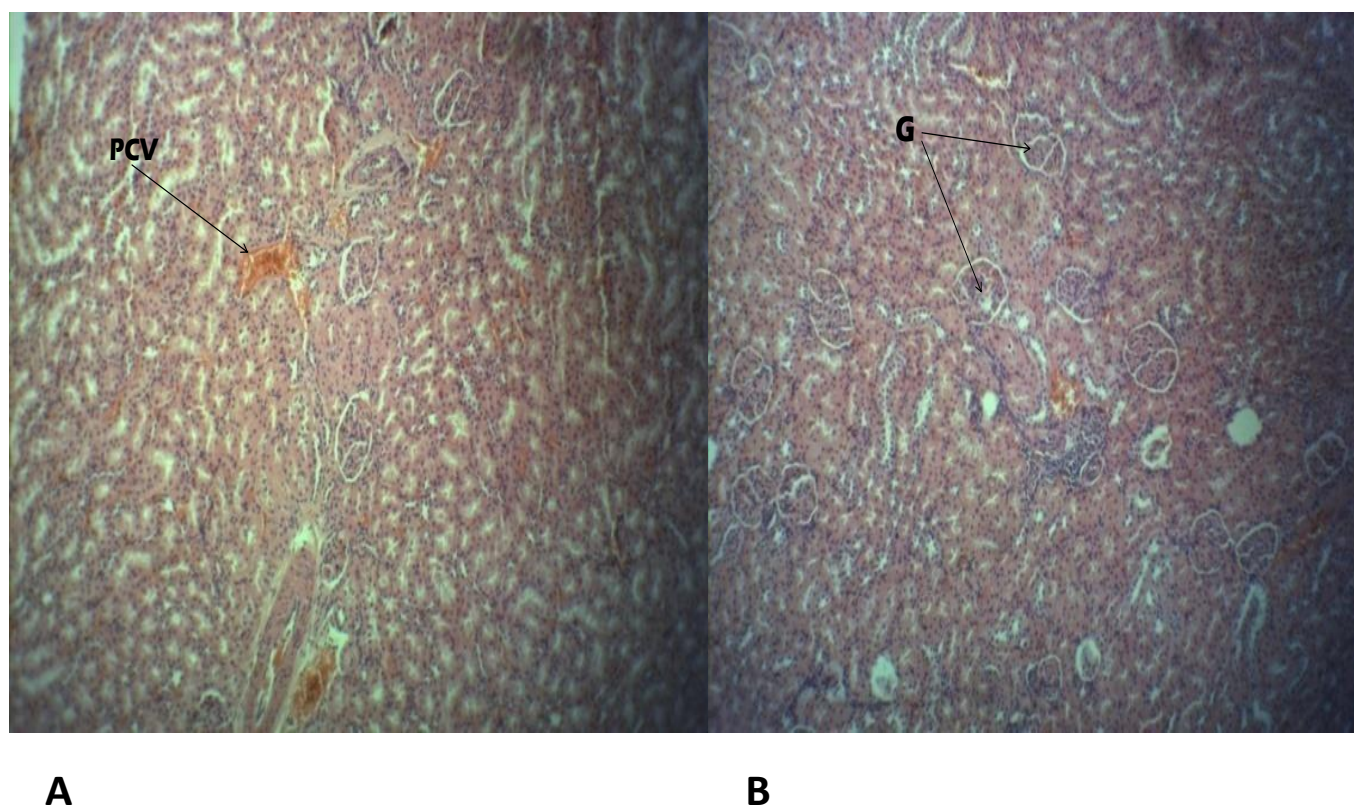
Photomicrograph of kidneys of Rats treated with- **A**; distilled water (control), **B**; 1600 mg kg^{-1} b.wt, for 28 days.

G; glomerulus, PCV; packed cell volume

Stains: haematoxylin and eosin. Mag: x100.

Plate 4; Photomicrograph of Heart of Rats after subchronic studies

Joloo did not cause any aberration after 28days administration on the heart of rats during subchronic toxicity
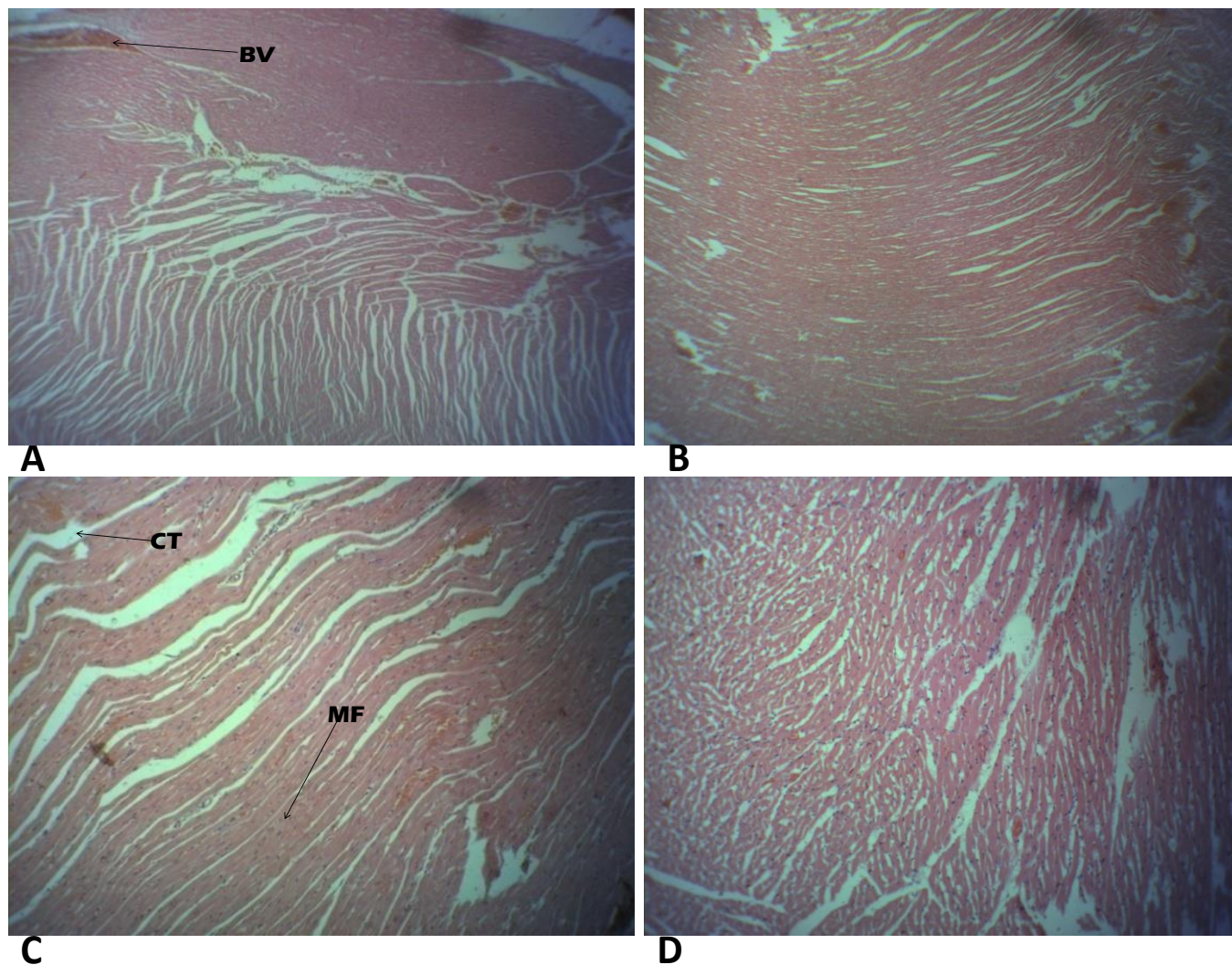
Photomicrograph of Heart of Rats treated with- **A**; distilled water (control), **B**;400 mg kg^{-1} b.wt, **C**;800 mg kg^{-1} b.wt **D**;1600 mg kg^{-1} b.wt for 28 days.

MF – muscle fibre, CT- connective tissue, BV- Blood vessel.

Stains: haematoxylin and eosin. Mag: x100.

Plate 5; Photomicrograph of Spleen of Rats after subchronic studies

Joloo did not induce any aberration after 28days administration on the heart of rats during subchronic toxicity
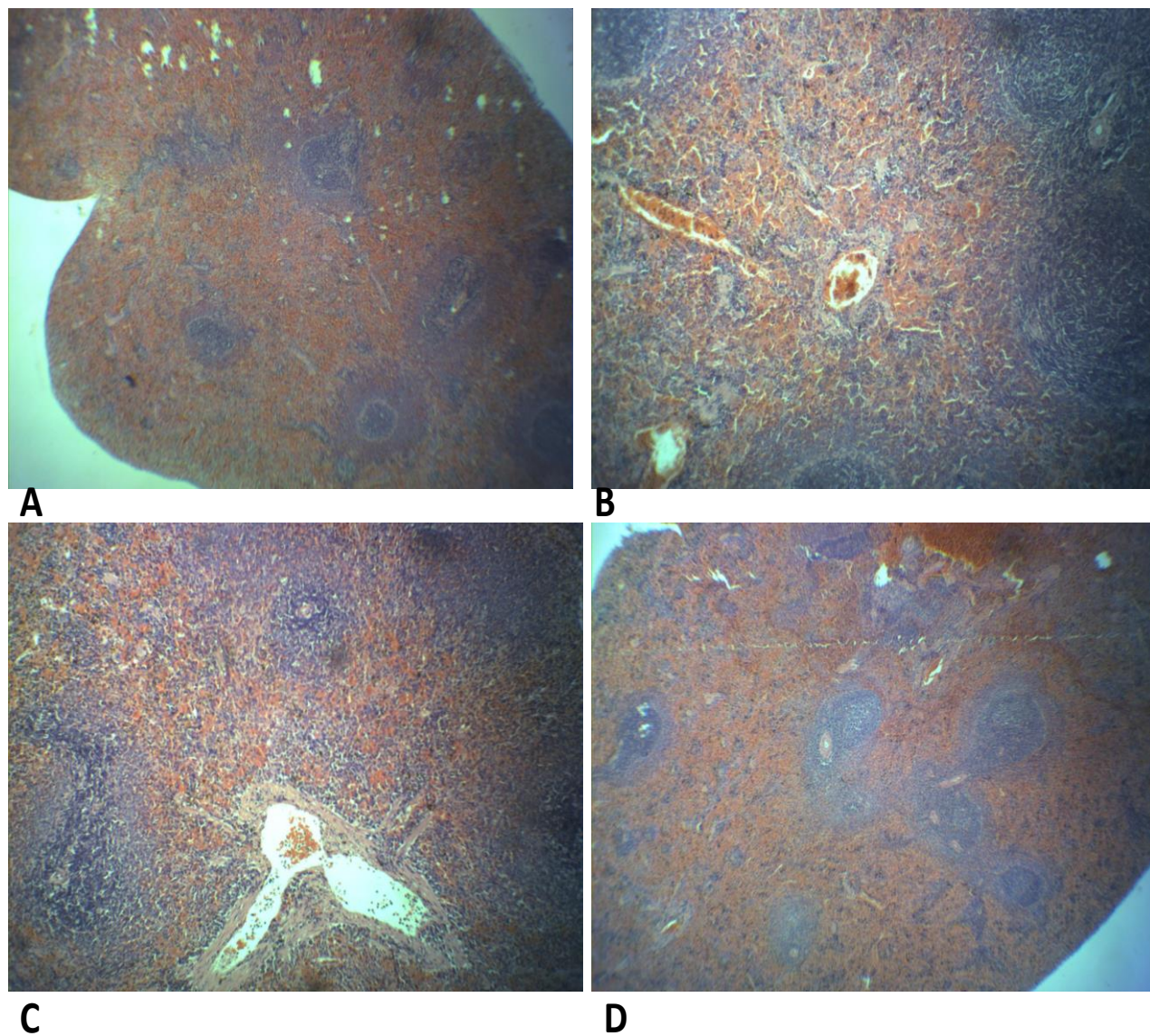
Photomicrograph of Spleen of Rats treated with- A; distilled water (control), **B**;400 mg kg^{-1} b.wt, **C**;800 mg kg^{-1} b.wt **D**;1600 mg kg^{-1} b.wt for 28 days.

Stains: haematoxylin and eosin. Mag: x100.

4.4 Chronic toxicity

4.4.1 Clinical observations

All mice survived until scheduled necropsy and showed normal growth and appeared healthy through the study. Daily general observations, ophthalmoscopy and clinical examinations revealed no treatment-related changes.

Table 6: Effects of oral administration of *Joloo* for 13 weeks on relative organ weight

Treatment	Hearts	Liver	Lungs	Brain	Spleen	Right kidney	Left kidney
control	0.13±0.03	1.45±0.09	0.54±0.14	0.32±0.02	0.10±0.03	0.17±0.05	0.16±0.04
400	0.11±0.02	1.18±0.08	0.18±0.02	0.34±0.04	0.10±0.02	0.17±0.05	0.16±0.04
800	0.13±0.04	1.20±0.07	0.21±0.05	0.37±0.04[a]	0.09±0.02	0.18±0.03	0.16±0.04
1600	0.13±0.03	1.34±0.10	0.25±0.04	0.37±0.04 [a]	0.16±0.30[b]	0.19±0.04	0.17±0.03

Values are mean±SEM (N=10); [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$ significantly different from control.

There were no significant changes in the relative weights of the heart, liver, lung and kidneys of all the animals. For other organs such as the brain and spleen, significant variations ($P < 0.05$) appeared between those of controls and treated group at 800 and 1600 mg kg^{-1} b.wt (Table 6).

Table 7: Biochemical analytes from the liver of mice treated with *Joloo* extract for 13 weeks

Dose (mg kg⁻¹ b.wt)	ALB (g/l)	ALT/GPT (U/l)	AST/GOT (U/l)	CHOL (mmol/l)	UREA (mg/dl)	PROT (g/l)
control	44.23±0.83	22.67±0.77	47.40±0.64	2.87±0.10	28.44±0.25	36.48±0.18
400	45.32±0.34	21.96±0.53	52.62±1.18[a]	2.83±0.05	23.95±0.58	33.53±0.41
800	45.81±0.26[a]	23.09±0.49	61.98±0.84[a]	2.86±0.07	25.06±0.76	33.85±0.78
1600	46.94±0.56[a]	26.00±0.69[a]	62.1±1.44[a]	2.9±0.11	26.83±0.55	38.18±0.46[a]

Values are mean±SEM (N=10); [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$significantly different from control

ALB-Albumin

ALP-Alkaline phosphatase

ALT- Alanine aminotransaminase

AST- Aspartate aminotransaminase

CHOLS- Cholesterol

PROT-Protein

Table 8: Biochemistry values from the kidney of mice treated with *Joloo* extract for 13 weeks

Dose (mg kg^{-1} b.wt)	ALB (g/l)	ALT/GPT (U/l)	AST/GOT (U/l)	CHOL (mmol/l)	UREA (mg/dl)	PROT (g/l)
control	44.96±0.30	28.92±0.30	31.46±1.05	2.84±0.06	28.94±0.34	35.42±0.50
400	47.79±0.46	21.45±0.31	56.29±1.39[a]	2.77±0.07	25.78±0.39	36.45±0.28
800	48.52±0.62[a]	22.12±0.75	66.73±1.00[a]	2.81±0.07	28.61±0.41	37.01±0.46[a]
1600	54.60±0.84[a]	24.50±0.60	71.85±1.76[b]	2.83±0.08	28.83±0.68	37.09±0.38[a]

Values are mean±SEM (N=10); [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$significantly different from control

ALB-Albumin

ALP-Alkaline phosphatase

ALT- Alanine aminotransaminase

AST- Aspartate aminotransaminase

CHOLS- Cholesterol

PROT-Protein

The biochemical analytes from the liver (Table 7) did show that ALT was significantly different from control at 1600 mg kg^{-1} b.wt while AST was significantly different from control at all doses after *Joloo* administration for 91 days. Among the biochemical indicators of kidney function tested (Table 8), ALB was significantly different from control at 1600 mg kg^{-1} b.wt dose and AST was significantly different from control at all the doses.

Table 9: Antioxidant activity in the liver of mice treated with *Joloo* extract for 13 weeks

Dose (mg kg^{-1} b.wt)	CAT(μmol/min/ mg/protein)	SOD(SOD/min /mgprotein)	MDA (nmol/ml)	GSH(μmol /ml)	GPx(μmol /ml)
control	2.96±0.07	96.72±0.90	44.36±0.67	1.58±0.44	3.81±0.11
400	2.68±0.16	93.95±0.58	36.97±0.60	1.00±0.09	4.17±0.11
800	2.77±0.13	100.38±0.94 [a]	40.25±0.89 [a]	1.03±0.08	4.38±0.16[b]
1600	3.02±0.18[a]	109.53±1.32 [a]	40.99±0.57 [a]	1.91±0.40	4.82±0.05[c]

Values are mean±SEM (N=10); [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$ significantly different from control (student's t-test)

CAT: Catalase, **SOD**: Superoxide dismutase, **MDA:** Malonaldehyde-bis-dimethylacetyl, **GSH:** Glutathione, **GPx:** Glutathione peroxidase.

Table 10: Antioxidant activity assay values from the kidney of mice treated with *Joloo* extract for 13 weeks

Dose (mg kg^{-1} b.wt)	CAT(µmol/min/mg/protein)	SOD(SOD/min/mgprotein)	MDA (nmol/ml)	GSH(µmol/ml)	GPX(µmol/ml)
control	3.3±0.17	96.97±0.69	42.74±0.27	1.09±0.04	4.38±0.11
400	3.45±0.11	95.12±0.93	37.56±0.52	1.09±0.08	4.15±0.12
800	3.59±0.18	100.95±0.82 [a]	38.00±0.51	1.10±0.13	4.65±0.10 [a]
1600	4.03±0.11[b]	105.71±1.09 [a]	40.97±0.83[a]	1.17±0.07	4.71±0.12 [b]

Values are mean±SEM (N=10); [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$significantly different from control (student's t-test)

CAT: Catalase, **SOD**: Superoxide dismutase, **MDA:** Malonaldehyde-bis-dimethylacetyl, **GSH:** Glutathione, **GPx:** Glutathione peroxidase.

Joloo exhibited a gradual dose-dependent increase in all hepatic and kidney antioxidant enzymes, CAT, at 1600 mg kg^{-1} b.wt, SOD at 800 and 1600 mg kg^{-1} b.wt and GPx significantly different ($p<0.05$) from control at both 800 and 1600 mg kg^{-1} b.wt. MDA showed a significant reduction ($p<0.05$) from control (Table 9&10).

Table 11: Haematologic values of mice treated with *Joloo* extract for 13 weeks.

Dosage(mg/kg)	PCV %	WBC mm^3	N%	L%	E	M%	B
control	28.5±0.42	2200±11.89	47.5±3.12	52.5±3.12	-	-	-
400	20.67±1.01	1633±6.80	58.7±1.74	40.0±1.77	-	2	-
800	31.5±0.58	1850±4.33	39.0±1.58	61.0 ±0.51*	-	2	-
1600	23.00±1.01	2233±8.70	37.3±0.51	62.7±0.51*	-	-	-

Values are mean±SEM (N=10/group). * Significantly different from controls ($p < 0.05$).

The result of haematologic examination in chronic test is shown in (Table 11). There were no statistically significant difference in the values of PCV, WBC, and differential leucocytes counts.

PCV- Packed cell volume, **WBC**- White blood cell, **N**- Neutrophils, **L**- Leucocytes, **E**- Eosinophils, **M**- Monocytes, **B**- Basophils.

Gross and Histologic Pathology of Chronic Toxicity

Plate 6; Photomicrograph of heart of mice treated with *Joloo* for 90-day showed mild necrosis at the highest dose tested
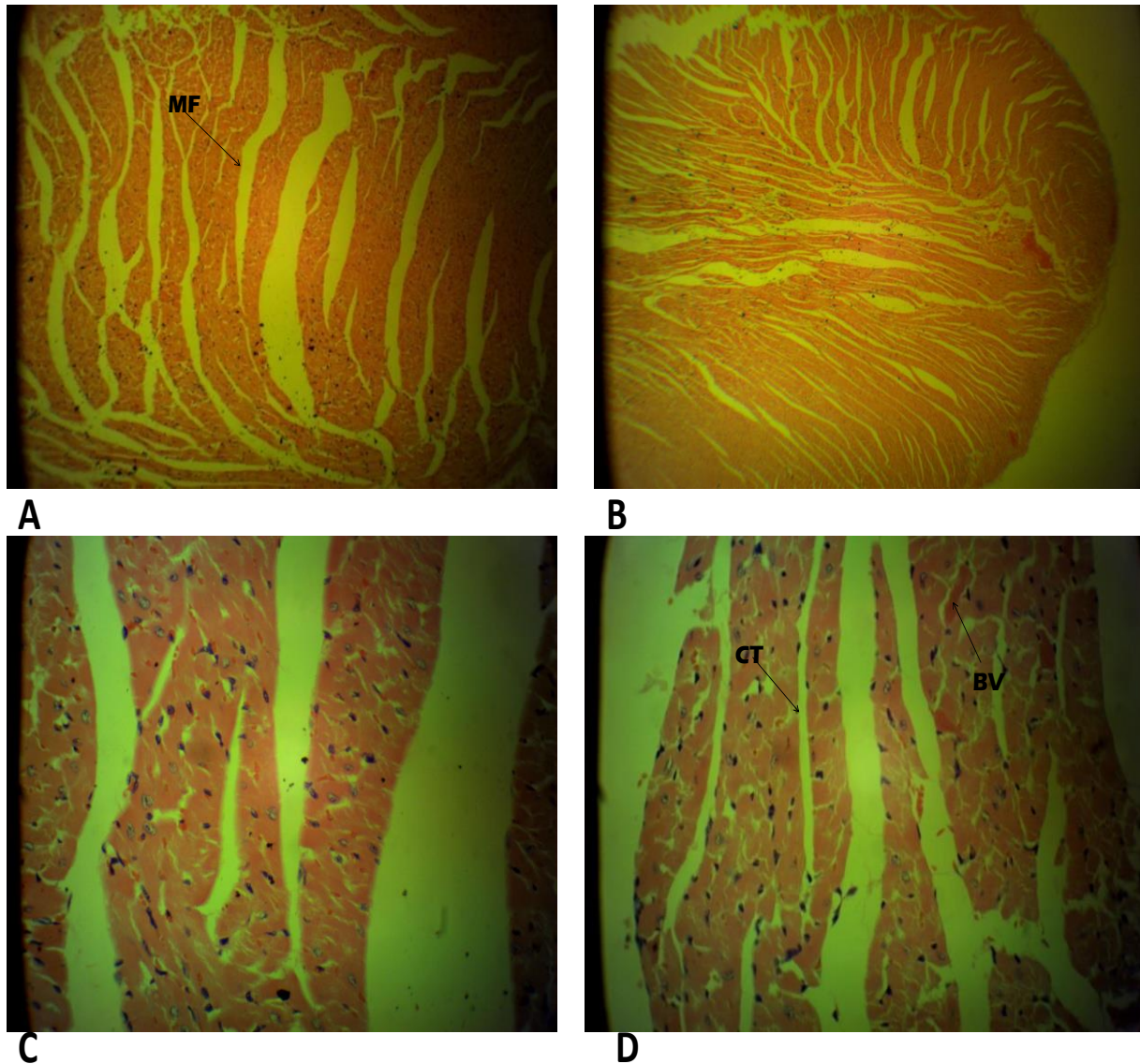
Photomicrograph of heart of mice treated with A: distilled water (control), B; 400 mg kg^{-1} b.wt, C; 800 mg kg^{-1} b.wt, D; 1600 mg kg^{-1} b.wt for 13 weeks. MF-myocardial fibre, CT- connective tissue; BV- blood vessel.

Stains: haematoxylin and eosin. Mag: x100.

Plate 7; Photomicrograph of Kidney of mice exposed to *Joloo* for 90 days did not show any significant difference from control
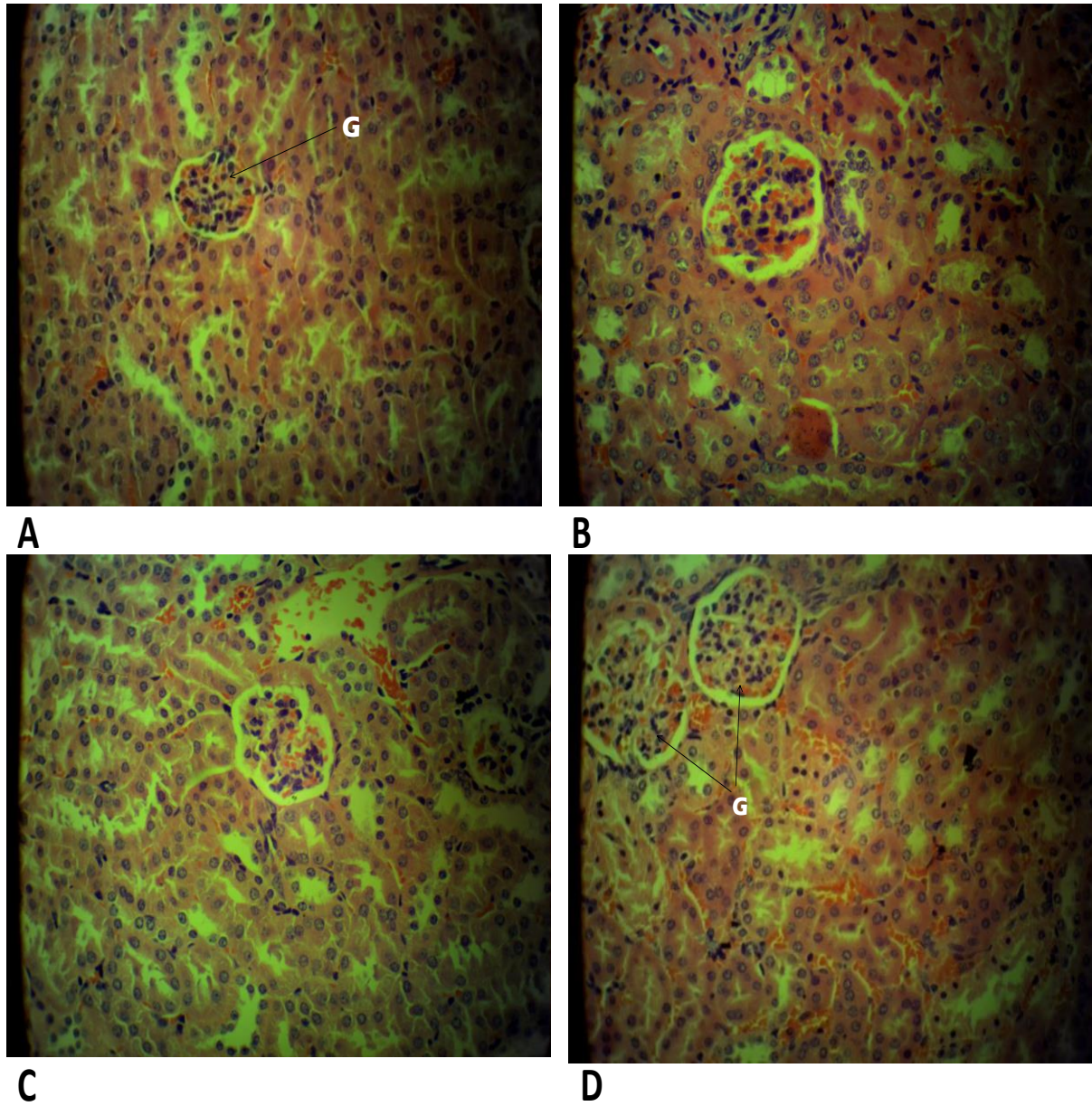
Photomicrograph of Kidney of mice treated with A; distilled water (control), B; 400 mg kg^{-1} b.wt
C; 800, D;1600 mg kg^{-1} b.wt for 13 weeks. G- Glomerulus.

Stains: haematoxylin and eosin. Mag: x100.

Plate 8; Photomicrograph of liver of mice treated with '*Joloo*' for 90-day, showing dose dependent increase in necrosis
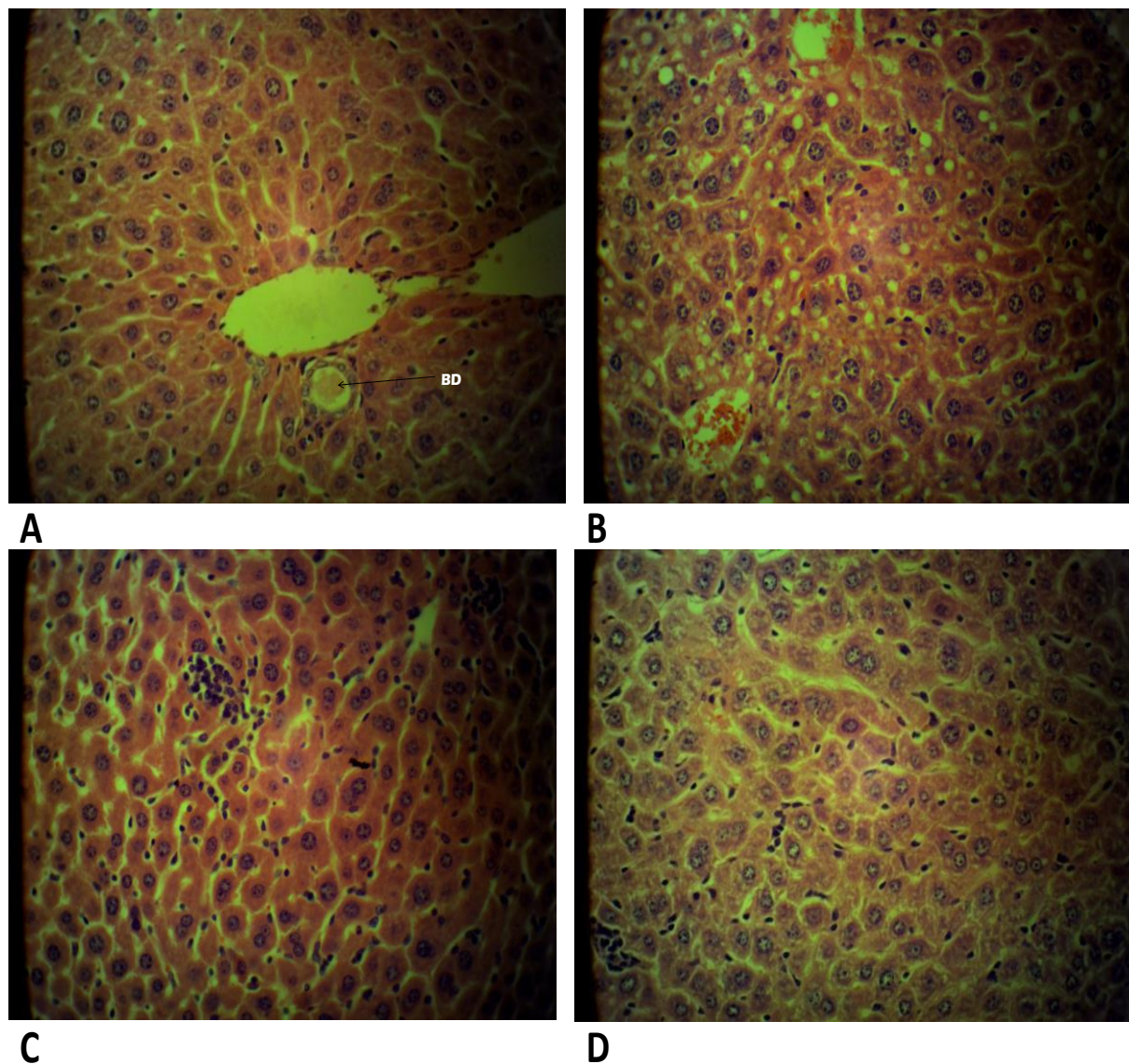
Photomicrograph of liver of mice treated with **A**; distilled water (control), **B**;400 mg kg^{-1} b.wt, **C**; 800 mg kg^{-1} b.wt, **D**; 1600 mg kg^{-1} b.wt for 13 weeks. BD- Bile duct.

Stains:haematoxylin and eosin. Mag: x100.

Plate 9; Photomicrograph of Lungs of mice exposed to *Joloo* for 90 days did not show any

significant difference from control
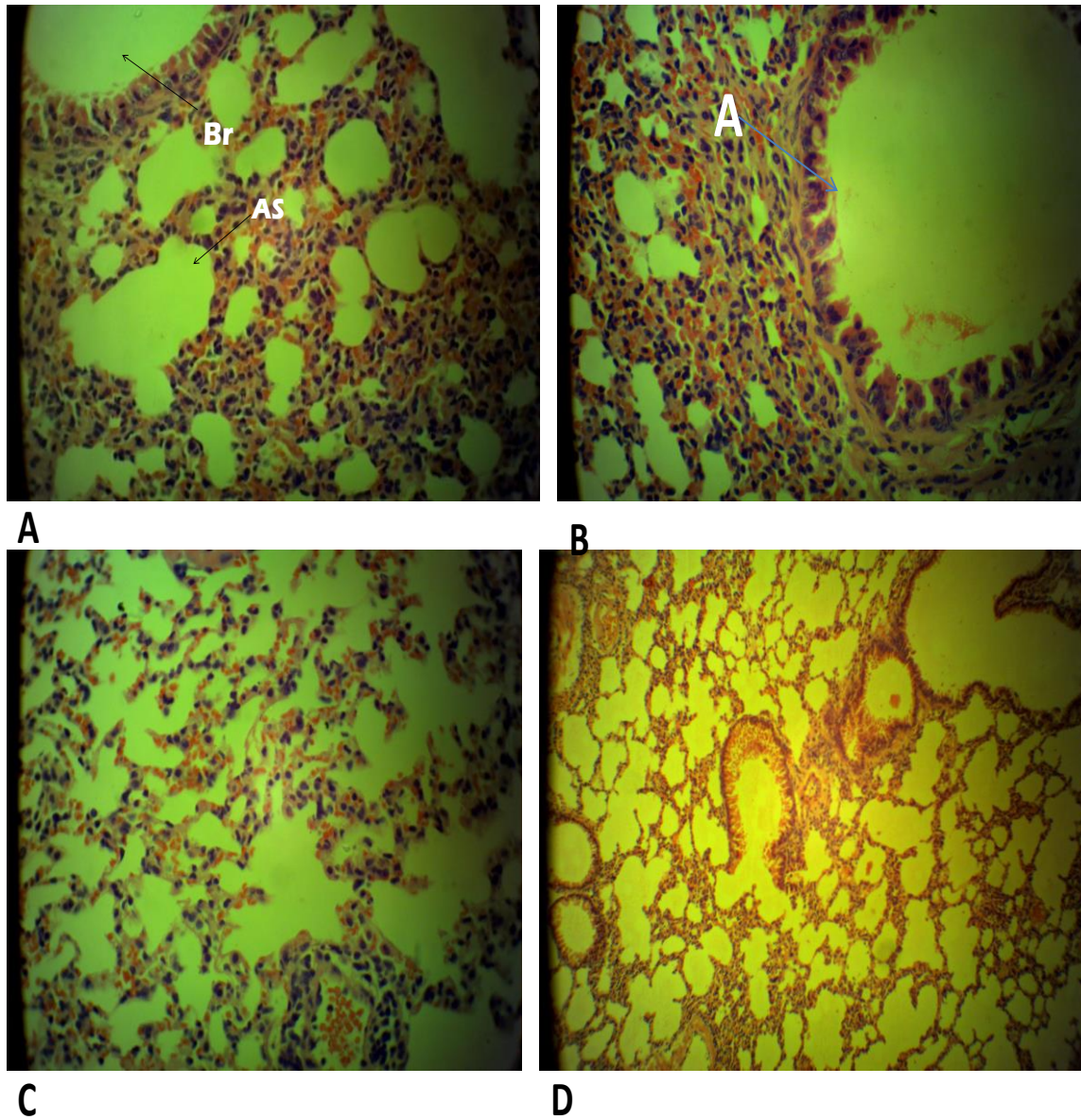
Photomicrograph of lungs of mice treated with **A**; distilled water (control), **B**; 400 mg kg^{-1} b.wt,

C; 800 mg kg^{-1} b.wt. **D**; 1600 mg kg^{-1} b.wt for 13 weeks. Br-Bronchiole; AS- Alveolar space.

Stains: haematoxylin and eosin. Mag: x100.

Plate 10; Photomicrograph of the spleen exposed to *Joloo* for 90 days showing a dose dependent

increase in necrosis from 800 to 1600 mg kg^{-1} b.wt
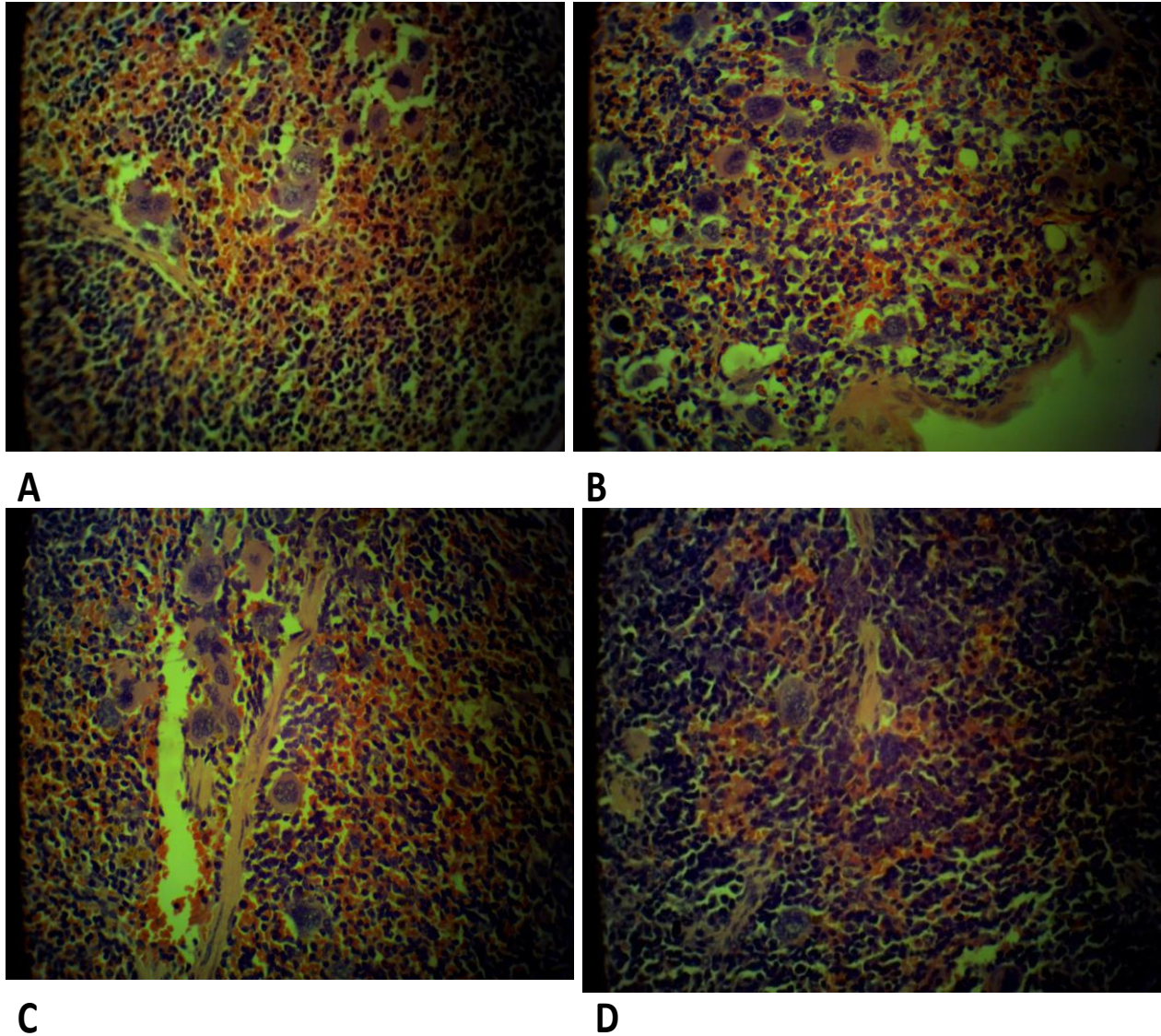
Photomicrograph of the spleen of mice treated with **A**; distilled water (control), **B**; 400 mg kg^{-1}

b.wt, **C**; 800 mg kg^{-1} b.wt. **D**; 1600 mg kg^{-1} b.wt for 13 weeks.

Stains: haematoxylin and eosin. Mag: x100.

4.4 Genotoxicity

4.4.1 Macroscopic parameter

No structural changes were observed on the *Allium cepa* root tips but the extract caused significant decrease (p<0.05) in the root length with increasing concentrations as shown in Table 12. However, a slight increase in root length was observed from day 1-7 at concentrations of 400 and 800 mg while there was growth cessation on day 4 at 1600 mg. There was 81% root growth inhibition (Table 12) at concentration of 1600 mg. The EC_{50} value was 380 mg.

4.4.2 Microscopic parameters

The microscopic analysis of the effect of the cocktail on cell division showed that it interfered with mitosis. All the concentrations of the cocktail induced chromosome aberrations which were statistically significant (p<0.05) from the control. Different types of chromosomal aberrations such as sticky chromosomes, c-mitosis, vagrant chromosomes, bridges, fragment and attached chromosomes are shown in Table 13. However, most of the aberrations occurred at anaphase stage and mostly as bridges or sticky chromosomes. At the lower concentration of 400 mg, out of the 412 cells examined, four sticky chromosomes, four bridge fragments and one attached chromosome were observed.

Table 12: Percentage root growth inhibition

Treatment	Dose (mg kg^{-1})	Root length (Mean±SEM)	Root growth inhibition (%)
Control	-	3.20±0.60	-
Joloo	400	1.50±0.30*	53.6
	800	0.90±0.20**	68.3
	1600	0.50±0.10***	81.3

*Significant, **More significant, ***Most significant

Table 13: Effect of *Joloo* on cell division

Concentration	No. of cells	Dividing cells	Mitotic index	Mean±SEM	Stickiness	C-mitosis	Vagrant	Bridges fragment	Binucleus	Multipolar anaphase	Attached	Total aberration (Mean±SEM)
Control	500	31	0.062	6.0±0.04	1	0	0	1	0	0	0	0.29±0.10
400mg	412	16	0.04	3.8±0.02	4	0	0	4	0	0	1	1.29±0.70
800mg	360	14	0.04	3.8±0.02	3	1	2	3	0	0	1	1.43±0.40*
1600mg	354	15	0.04	4.2±0.01	3	0	1	3	2	1	3	1.86±0.40**

*Significant, **More significant, ***Most significant

Plate 11; Photomicrograph of cells of *A. cepa* root tip In Genotoxicity study treated with *Joloo* (400mg) showing chromosomal aberration

Chromosome aberrations increased with higher concentrations. The highest concentration (1600 mg) produced significant aberration of 1.8±0.4** compared to the control (0.2±0.1). The extract at 400 mg concentrations had a minimal effect on the Mitotic Index (MI) in root-tip cells, but 800 and 1600 mg concentrations significantly reduced the MI (Table 13).
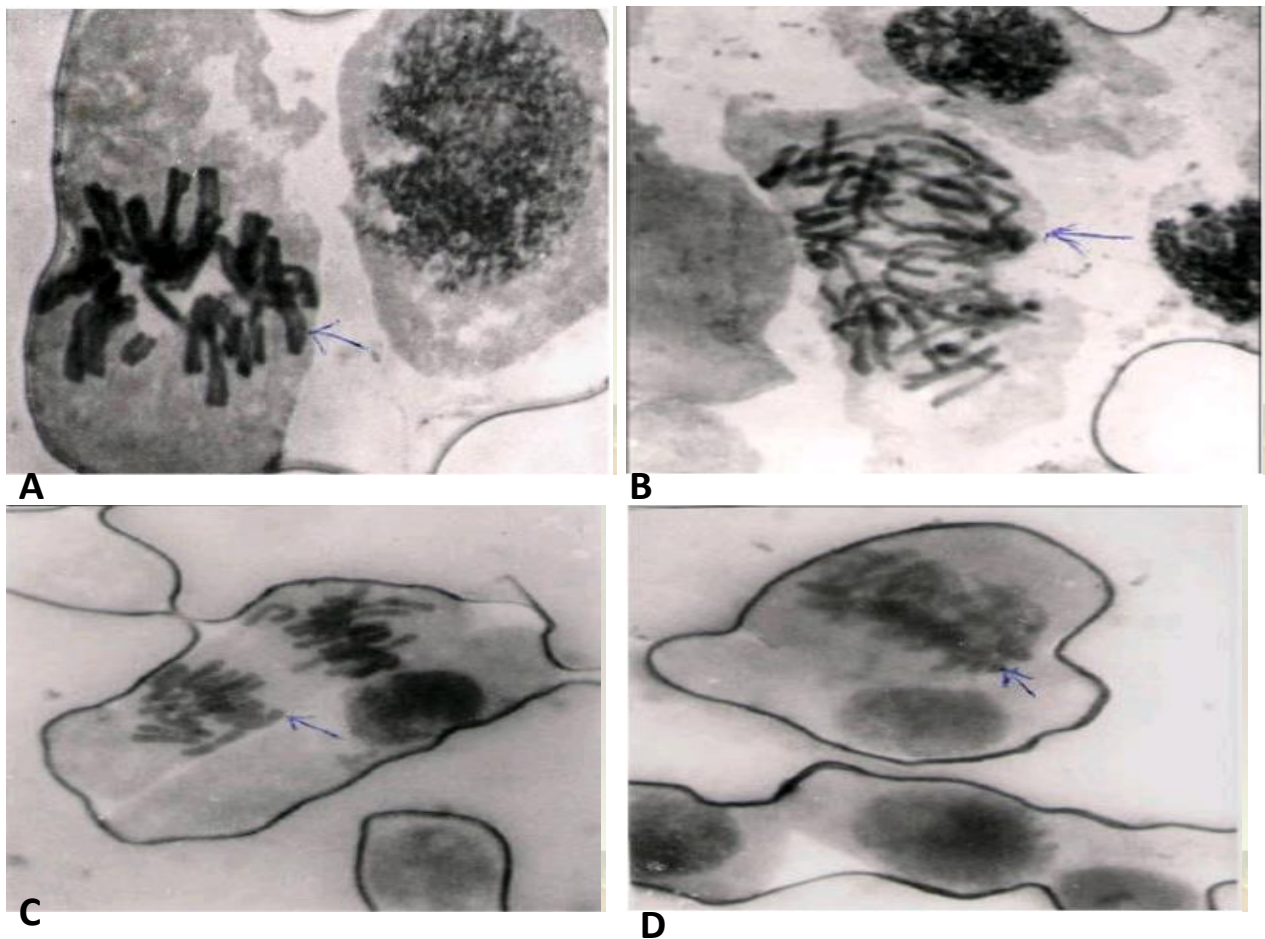
Cells of *A. cepa* root tip in Genotoxicity study treated with *Joloo* A ;(400mg), B.800mg (vagrant chromosome) C; 1600mg (anaphase with laggards) and D; 1600mg (Clump).

4.5 Cytotoxicity

Table 14: Cytotoxic activity of *Joloo* and its constituents on VERO and CEF cell-lines

Plants	VERO 492nm Treatment (mg)				CEF 492nm Treatment (mg)			
	1	2.5	5	IC $_{50}$	1	2.5	5	IC $_{50}$
Securidaca longepedunculata	0.370 ± 0.02^{b}	0.358 ± 0.02^{b}	0.287 ± 0.01^{c}	**8.5**	0.191 ± 0.02	0.176 ± 0.00	0.133 ± 0.00^{c}	**8.7**
Hoslunda opposite	0.408 ± 0.02^{a}	0.299 ± 0.02^{c}	0.278 ± 0.02^{c}	**6.5**	0.181 ± 0.01	0.161 ± 0.00	0.138 ± 0.00^{c}	**10.5**
Olax subscopoiidea	0.326 ± 0.03^{b}	0.294 ± 0.03^{c}	0.231 ± 0.01^{c}	**5.6**	0.197 ± 0.00	0.177 ± 0.00	0.164 ± 0.00	**15.3**
Xylopia aethiopia	0.380 ± 0.01^{b}	0.264 ± 0.01^{c}	0.257 ± 0.00^{c}	**5.9**	0.164 ± 0.00	0.149 ± 0.01^{b}	0.129 ± 0.00^{c}	**10.6**
Butyrospermum paraxodum	0.448 ± 0.04	0.441 ± 0.02	0.351 ± 0.02^{b}	**4.9**	0.163 ± 0.01	0.156 ± 0.01	0.134 ± 0.01^{c}	**12.5**
Tetrapleura tetraptera	0.321 ± 0.01^{b}	0.293 ± 0.03^{c}	0.282 ± 0.01^{c}	**11.7**	0.147 ± 0.01^{b}	0.143 ± 0.01^{b}	0.130 ± 0.01^{c}	**16.8**

Allium ascalonicum	0.460±0.03	0.258±0.04 [c]	0.219±0.02 [c]	**4.5**	0.252±0.016	0.210±0.01	0.182±0.01	**10.8**
Joloo Cl	0.388±0.01 [b]	0.300±0.05 [c]	0.197±0.01 [c]	**4.5**	0.141±0.01 [b]	0.127±0.01 [c]	0.124±0.01 [c]	**15.7**
Joloo Bu	0.613±0.03	0.407±0.04 [a]	0.403±0.04 [a]	**8.3**	0.149±0.01 [b]	0.131±0.00 [c]	0.125±0.01 [c]	**12.7**
Joloo E	0.493±0.02	0.348±0.04 [b]	0.225±0.02 [c]	**4.9**	0.202±0.00	0.197±0.01	0.181±0.01	**19.3**
Joloo He	0.692±0.01	0.562±0.04	0.410±0.03 [a]	**7.7**	0.156±0.01	0.137±0.01 [c]	0.135±0.01 [c]	**16.1**
Joloo W	0.447±0.01	0.296±0.06 [c]	0.201±0.02 [c]	**4.5**	0.152±0.00 [a]	0.129±0.01 [c]	0.106±0.00 [c]	**7.1**
Cyclophosphamide			0.289±0.06				0.129±0.00	**-**
Control			0.433±0.01				0.159±0.00	**-**

Data are presented as mean ±SEM for quadruplicates. Value is statistically significant compared to control (student's t- test), [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$.

Joloo - **Cl**; chloroform. **Bu**; butanol. **E**; ethylacetate. **He**; n-hexane and **W**; water fractions
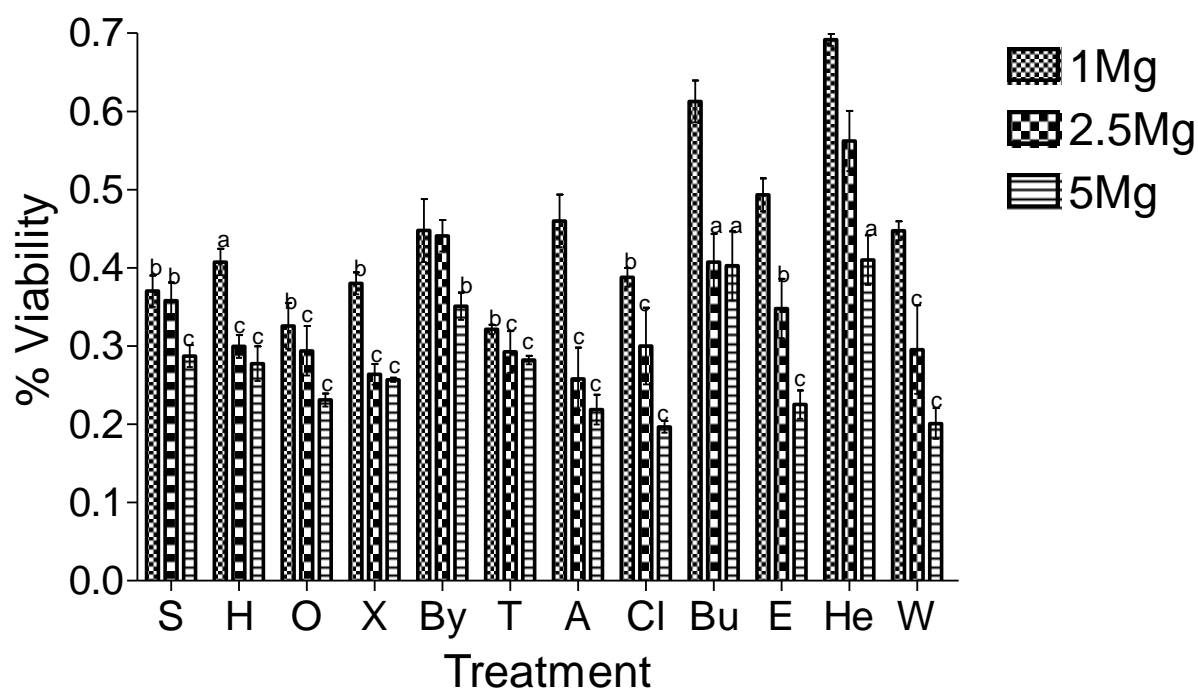
Fig. 2: Cell viability of VERO cells at different concentrations.

S; *Securidaca longepedunculata*. **H**; *Hoslunda opposite* **O**; *Olax subscopoiidea* **X**; *Xylopia aethiopica* **By**; *Butyrospermum paradoxum* **T**; *Tetrapleura tetraptera*. **A**; *Allium ascallonicum*. Others are **Cl**; chlorophorm. **Bu**; butanol, **E**; ethylacetate. **He**; hexane and **W**; water (Fractions of *Joloo*)

Data are presented as mean ±SEM for quadruplicates. Value is statistically significant compared to control (student's t- test), [a]p < 0.05; [b]p < 0.01; [c]p < 0.001.
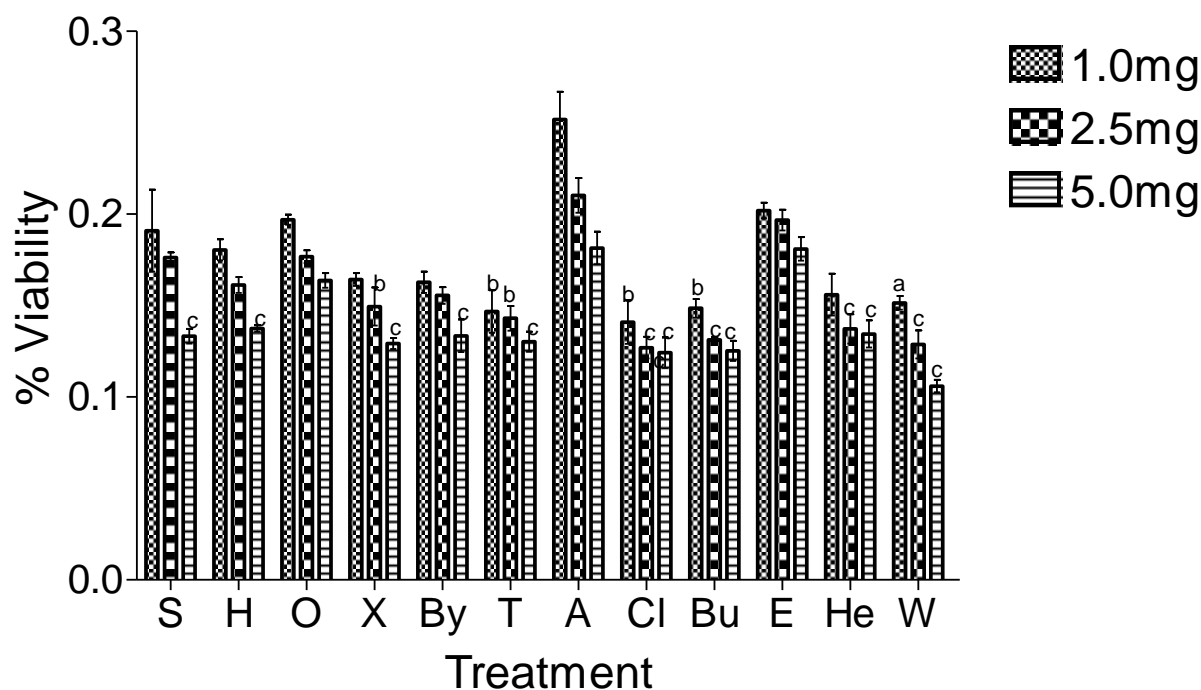
Fig. 3: Cell viability of CEF cells at different concentrations.

Data are presented as mean ±SEM for quadruplicates. Value is statistically significant compared to control (student's t- test), [a]p < 0.05; [b]p < 0.01; [c]p < 0.001

Fig. 2 and 3 shows the effect of individual plants used in formulating *Joloo* and different fractionated portions of *Joloo* on Vero and CEF cells, bars showing percentage of viable cells at concentrations 1.0, 2.5. and 5.0mg/ml, experiments were replicated in quadruplicates.

4.6 Gonadotoxicity

4.6.1 Activities of *Joloo* on Testes of mice

Plate 12: Photomicrograph of testis of mice treated orally administered with '*Joloo*' for 28 days.Germ cells increased with increase in doses. The highest dose 1600 mg kg^{-1} b. wt. showed a remarkable increase in germ cells.
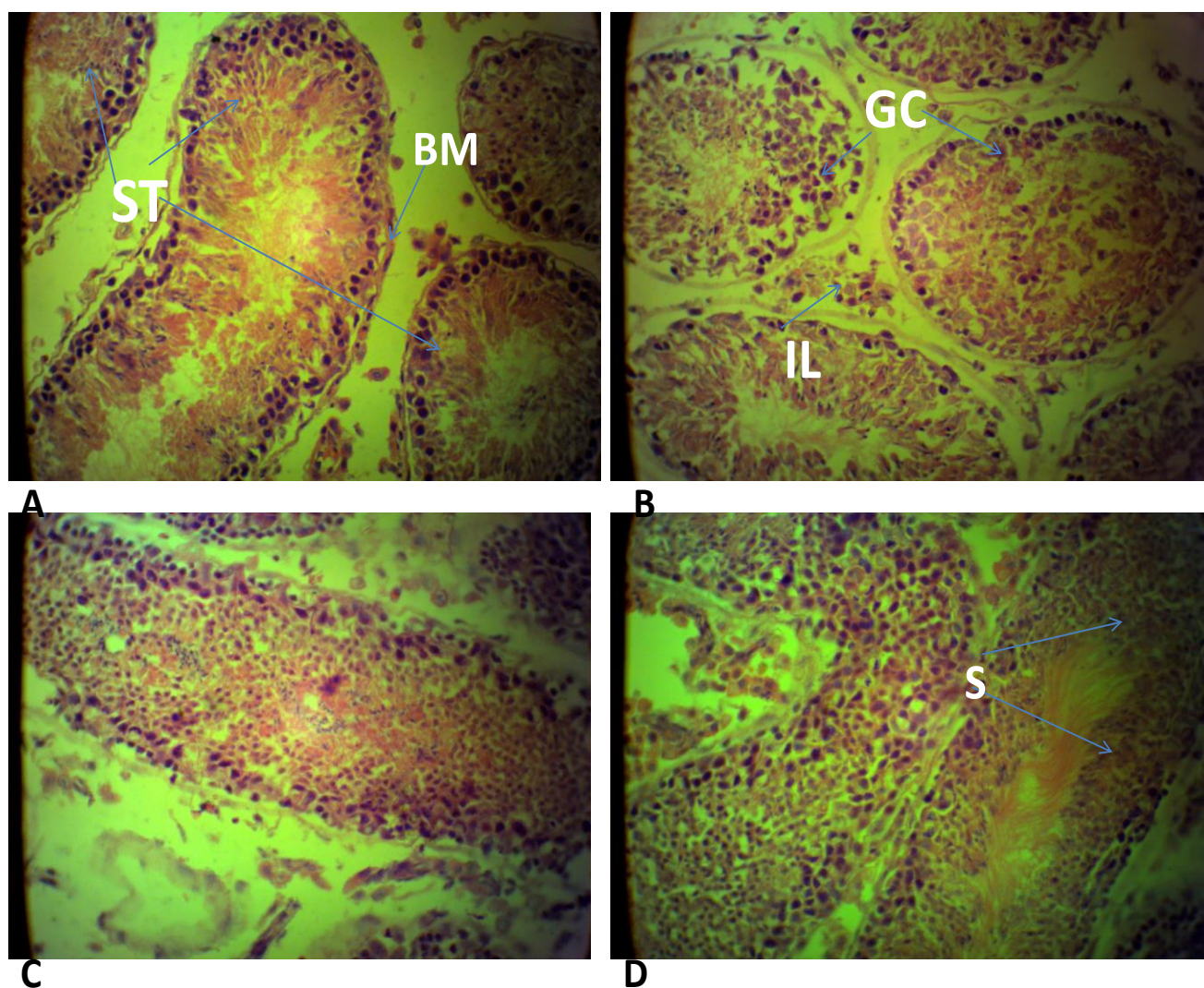
Photomicrograph of testis of mice treated with distilled water (control), *Joloo* B: 400, C: 800 and D: 1600 mg kg^{-1} b.wt for 28 days.

GM- germ cells: BM- basal membrane: IL- interstitial cells of leydig, ST-seminiferous tubule. Stains: haematoxylin and eosin. Mag: x400.

4.7 Analgesic studies

4.7.1 Mice abdominal constriction assay

The animals injected with 10 mL kg^{-1} b.wt of 0.6% acetic acid intraperitoneally presented 54.3±1.69 constrictions (n = 10) in 15 min. The group treated with the 400-1600 mg kg^{-1} b.wt cocktail exhibited dose dependent and significant, reductions in number of constrictions by 24.9, 53.6 and 74.0%, respectively (Table 15). The dosage ranges of 800 and 1600 mg kg^{-1} b.wt of the cocktail, produced significant inhibition ($p<0.05$) greater than 100 mg kg^{-1} b.wt acetylsalicylic acid which inhibited abdominal constriction by 47.1%.

Table 15: Effect of *Joloo* on acetic acid-induced constriction in mice

Treatment	Dose (mg kg^{-1} b.wt)	No. of constrictions	Inhibitions (%)
Control	-	54.3±1.69	-
Joloo	400	40.8±1.55	24.9
	800	25.2±1.33	53.6
	1600	14.1±1.72	74.0
Acetylsalicylic acid	100	28.7±1.69	47.1

Values expressed as mean±SEM: $p \leq 0.05$ significantly different from control (students t-test)

4.7.2 Formalin test

Four hundred to sixteen hundred milligram per kilogram of the cocktail demonstrated dose-related inhibitions against both phases of formalin-induced pain (Table 16). An inhibition of 38.9% was recorded at the concentration of 400 mg kg^{-1} b. wt while 800 mg kg^{-1} b.wt and 1600 mg kg^{-1} b.wt doses, produced inhibition that is (56.6 and 65.3%) higher than ASA in the first phase. Significant pain inhibition was recorded, at all the doses of the cocktail and acetylsalicylic acid (100 mg kg^{-1} b. wt) in the second phase. However, the duration of paw licking was longer among rats treated with 400 mg kg^{-} of the extract than those in the 800 and 1600 mg kg^{-1} b.wt group.

Table 16: Effect of *Joloo* on formalin-induced pain

Treatment	Dose (mg kg^{-1} b.wt)	0-5min	Inhibition %	15-30min	Inhibition %
control	-	73.2±3.89	-	64.0±1.77	-
Joloo	400	44.7±1.93	38.9	18.6±0.82	70.9
	800	31.7±2.00	56.6	4.7±1.17	77.0
	1600	25.4±3.37	65.3	6.9±1.69	89.2
Acetylsalicylic acid	100	40.5±4.37	44.7	19.0±2.49	70.3

Values expressed as mean±SEM: p<0.05 significantly different from control (student's t-test)

4.7.3 Hot plate test

Table 17 shows the reaction time of mice to hot plate-induced pain. The reaction time in mice treated with the cocktail showed a dose relationship with the dosage administered. Although the cocktail inhibited better that the control at all doses, morphine returned a higher pain tolerance time of 7.2 sec

Table 17: Effect of *Joloo* on hot-plate test

Treatment	Dose (mg kg^{-1} b.wt)	Reaction time (sec)	Inhibition (%)
Control	-	2.2 ±0.1	-
Joloo	400	2.5±0.2	13.63
	800	3.5±0.2	59.09
	1600	5.4±0.4	145.95
Morphine	2	7.2±0.5	227.30

Values expressed as mean±SEM: $p < 0.05$ significantly different from control (student's t-test)

4.8 Anti-inflammatory studies

4.8.1 Carrageenan-induced paw oedema in rats

The cocktail administered one hour before carrageenan showed a fluctuating rhythm with recourse to time in a biphasic pattern (Table 18a). Although the various doses of the cocktail (400-1600 mg kg^{-1} b.wt) inhibited inflammation, inhibition at 1600 mg kg^{-1} b.wt was very significant at the 5 and 6 h. The cocktail compared favourably with the reference drug (Indomethacin 10 mg kg^{-1} b.wt) which also produced a very significant inhibition.

4.8.7 Egg albumin-induced oedema

Egg albumin produced rapid swelling that peaked in 90 min (2.61±0.02). The swelling fluctuated over the period of the experiment (Table 18b). Oedema inhibition was significant in the rats treated with cocktail dose of 800 and 1600 mg kg^{-1} b.wt from 120 to 180 min. Rats treated with 800 and 1600 mg kg^{-1} b.wt of cocktail produced significant inflammation inhibition from 120-180 min as shown in (Table 18b). This compared with the activity of the chlorpheniramine over the same period significantly ($p < 0.05$).

Table 18a: Effect of *Joloo* on carrageenan induced rat paw oedema

Treatment (mg kg^{-1} b.wt)	0h	1h	2h	3h	4h	5h	6h
Control	2.33±0.02	2.51±0.01	2.45±0.03	2.49±0.03	2.43±0.02	2.38±0.03	2.41±0.04
400	2.41±0.01	2.53±0.02	2.26±0.10^c	2.37±0.02^a	2.27±0.02^c	2.25±0.01^b	2.23±0.02^b
800	2.62±0.02	2.65±0.01^c	2.35±0.04^a	2.52±0.02	2.30±0.02^c	2.20±0.02^c	2.21±0.03^c
1600	2.38±0.03	2.52±0.02	2.28±0.02^c	2.44±0.03	2.52±0.01^c	2.18±0.01^c	2.13±0.02^c
Indometh.	2.15±0.11	2.08±0.12^b	2.07±0.11^b	2.15±0.12^a	2.10±0.11^a	2.02±0.12^a	1.91±0.11^c

Values are Mean±SEM; (N=10); ^{a}p<0.05; ^{b}p<0.01; ^{c}p<0.001

Table 18b: Effect of *Joloo* on egg albumin-induced rat paws oedema

Treatment (mg kg^{-1} b.wt)	15min	30min	45min	60min	75min	105min	120min	135min	150min	165min	180min
Control	2.65±0.01	2.40±0.02	2.57±0.03	2.55±0.01	2.61±0.02	2.60±0.02	2.58±0.03	2.55±0.03	2.52±0.02	2.52±0.02	2.50±0.03
400	2.52±0.02^b	2.47±0.02^a	2.54±0.02	2.29±0.02^c	2.32±0.02^c	2.38±0.03	2.36±0.03^c	2.36±0.01^c	2.33±0.01^c	2.30±0.02^c	2.28±0.02^c
800	2.48±0.03	2.37±0.02	2.51±0.27	2.31±0.03^c	2.33±0.03^c	2.37±0.02^c	2.30±0.02^c	2.28±0.02^c	2.19±0.01^c	2.14±0.01^c	2.10±0.02^c
1600	2.41±0.02^b	2.39±0.02	2.32±0.02	2.29±0.02^c	2.32±0.03^c	2.41±0.02^c	2.34±0.02	2.32±0.02^c	2.22±0.02^c	2.22±0.01^c	2.10±0.02^c
Chlorphen.	2.64±0.01	2.35±0.01^c	2.58±0.02^c	2.30±0.01	2.34±0.01^c	2.41±0.02^c	2.32±0.03	2.30±0.01^c	2.21±0.02^c	2.20±0.02^c	2.14±0.02^c

Values are Mean±SEM; (N=10); ^{a}p<0.05; ^{b}p<0.01; ^{c}p<0.001 significantly different from control (student's t-test)

Joloo achieved 100% chemosuppression of parasitaemia at 1600 and 800 mg kg^{-1} b.wt, thereby comparing favourably with the chloroquine which also recorded a 100% chemosuppression. However the 400 mg kg^{-1} b.wt dose did not produce significant chemosuppression when compared to control (Table 19a).

Table 19a; Suppressive activity of *Joloo* on *P.berghei* infection in mice (5-day test)

Dose of Drug/ extract (mg kg^{-1} b.wt /day)	Parasitaemia (Mean ± SEM)	%Chemosuppression
1600	0	100
800	0	100
400	2.7±0.8	44.5
Chloroquine (5mg)	0	100
Control(0.2Ml)	4.8±1.1	0

Values are Mean ± SEM (N=5)

After established parasitaemia in 72 hours post-inoculation, the animals administered with *Joloo*, showed dose-dependent activities against parasites. The 1600 mg kg^{-1} b. wt dose significantly reduced parasitaemia. As the days of infection increased animals appeared weak and critically sick in the 1600 and 400 mg kg^{-1} b. wt doses respectively. At the 8th day three mice died in the 400 mg kg^{-1} b. wt dose group, while all the mice in the controlled group died. However 100% clearance was observed in the group administered with 5 mg kg^{-1} b. wt. Chloroquine (Table 19b)

Table 19b; Curative activity of *Joloo* on *P.berghei* infection in mice (10-day test)

Treatment mg kg⁻¹ (b. wt)	Days									
	0	1	2	3	4	5	6	7	8	9
1600	168.4±1.5	5.2±0.2	1.9±0.3 [c]	1.9±0.3 [c]	4.3±0.7 [c]	5.2±0.9 [b]	5.4±1.2 [c]	10.1±0.1 [c]	11.5±0.2	6.1±0.8
800	242.2±3.4	5.5±0.4	4.4±0.4	2.8±0.4 [b]	4.6±0.3 [c]	5.7±0.3 [b]	10.7±0.8	10.7±0.8 [c]	15.7±0.5	21.8±12
400	586.8±1.6	2.8±0.4	3.4±0.3	5.5±0.1	5.7±0.3	11.1±0.1 [b]	13.3±0.8	12.9±0.5	16.2±0.9	26.3±0.8
Chloroquine(5mg)	219.4±2.4	4.1±0.4	0	0	0	0	0	0	0	0
Control	261.6±3.6	618.6±3.8	7911.3±5.6	11378.3±10.9	26007.4±16.5	66485.4±35.4	76804.5±12.8	84576.3±23.3	All died	All died

Values are Mean±SEM; (N=5); [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$ significantly different from control (student's t-test)

4.7.8   2, 4 Dinitrophenol -induced pyrexia

The formulation showed a biphasic pattern of activity. All doses (400-1600) reduced hyperthermia dose-dependently. The activity of *Joloo* was significantly different from the control. The formulation also compared favourably with the reference drug (acetylsalicylic acid 100mg).

Table 20; Effect of *Joloo* on DNP-induced pyrexia

Treatment (mg kg^{-1} b.wt)	30min	60min	90min	120min	150min	180min	210min	240min	270min	300min
Control	39.1±0.1	39.1±0.10	39.5±0.2	39.4±0.1	38.9±0.2	38.2±0.2	38.9±0.1	39.0±0.1	39.1±0.1	38.9±0.1
400	38.2±0.3[a]	37.7±0.2[a]	38.2±0.2[a]	37.9±0.2[b]	37.8±0.2[b]	37.7±0.2[b]	37.6±0.1[b]	37.6±0.1[c]	37.6±0.1[C]	37.5±0.1[b]
800	39.4±0.2[a]	38.6±0.2[a]	39.0±0.2	38.8±0.1	38.3±0.3	38.8±0.2[b]	38.3±0.3[b]	38.1±0.2[b]	37.8±0.2[C]	37.6±0.1[C]
1600	36.5±0.3[C]	35.9±0.4[C]	36.5±0.4[C]	36.3±0.3[C]	36.6±0.2[C]	36.4±0.2[C]	37.2±0.1[C]	37.5±0.1[C]	37.5±0.1[C]	37.5±0.1[C]
ASA	38.7±0.2[b]	38.3±0.2[b]	37.7±0.2[C]	37.6±0.2[C]	38.4±0.1	37.4±0.2[b]	38.3±0.1	38.2±0.1[a]	38.1±0.9[b]	37.9±0.1[C]

Values are Mean±SEM; (N=10); [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$ significantly different from control (student's t-test)

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4.8.0 Antibacterial activities

The result of the susceptibility profile of the test organisms is shown in Table 18. *Joloo* was able to inhibit most of the bacterial test organisms with measurable zones of inhibitions. The standard (Amoxicillin) showed an average inhibition diameter of 29mm.The organism with the highest zone of inhibition was *Proteus vulgari* (20mm) and *Yersinia enterocolitica* (15mm), *Klebsiella pneumonia* was however resistant at all concentration.

At 500mg/ml, *Joloo* was able to inhibit most test organisms but with minimal zones of inhibitions. The maximum activity of *Joloo* was observed on the *Yersinia enterocolitica* with 19% (Plate 13) followed closely by *Pseudomonas aeruginosa* with 18%. The least inhibition at this concentration was observed against *Salmonella typhi*. However no inhibition was observed against *Klebsiella pneumoniae* (0%).

Table 21; Antibacterial activities of *Joloo* at different concentractions.

microorganisms	Zone of inhibition(mm)			Amoxicillin
	500mg/ml	1000mg/ml	1500mg/ml	50mg/ml
Escherichia coli	12.33± 0.33	11.67±0.88	14.33±2.85	29.09±3.0
Shigella dysenteriae	11.00±1.53	9.67±0.88	12.00±1.73	29.09±3.0
Pseudomonas aeruginosa	12.33±0.33	9.67±0.88	13.00±1.53	29.09±3.0
Salmonella typhi	9.00±1.16	8.00±1.16	10.00±1.00	29.09±3.0
Yersinia enterocolitica	13.33±0.33	13.33±0.88	15.00±1.16	29.09±3.0
klebsiella pneumoniae	0.00	0.00	0.00	29.09±3.0
Proteus vulgari	11.67±0.88	13.00±0.58	20.00±1.53	29.09±3.0

Data represent mean± SEM of zonal inhibition of bacteria

Ec-Escherichia coli, Sd- Shigella dysenteriae, Pa- Pseudomonas aeruginosa, St- Salmonella typhi , Ys- Yersinia enterocolitica, Kb-Klebsiella pneumonia, Prt-Proteus vulgari

Plate 13: *Zones of inhibition of E.coli*

Plate 14:   *Zones of inhibition on Pseudomonas aerugenosa*

Fig 4: Antifungal activity of *Joloo* at different Concentration
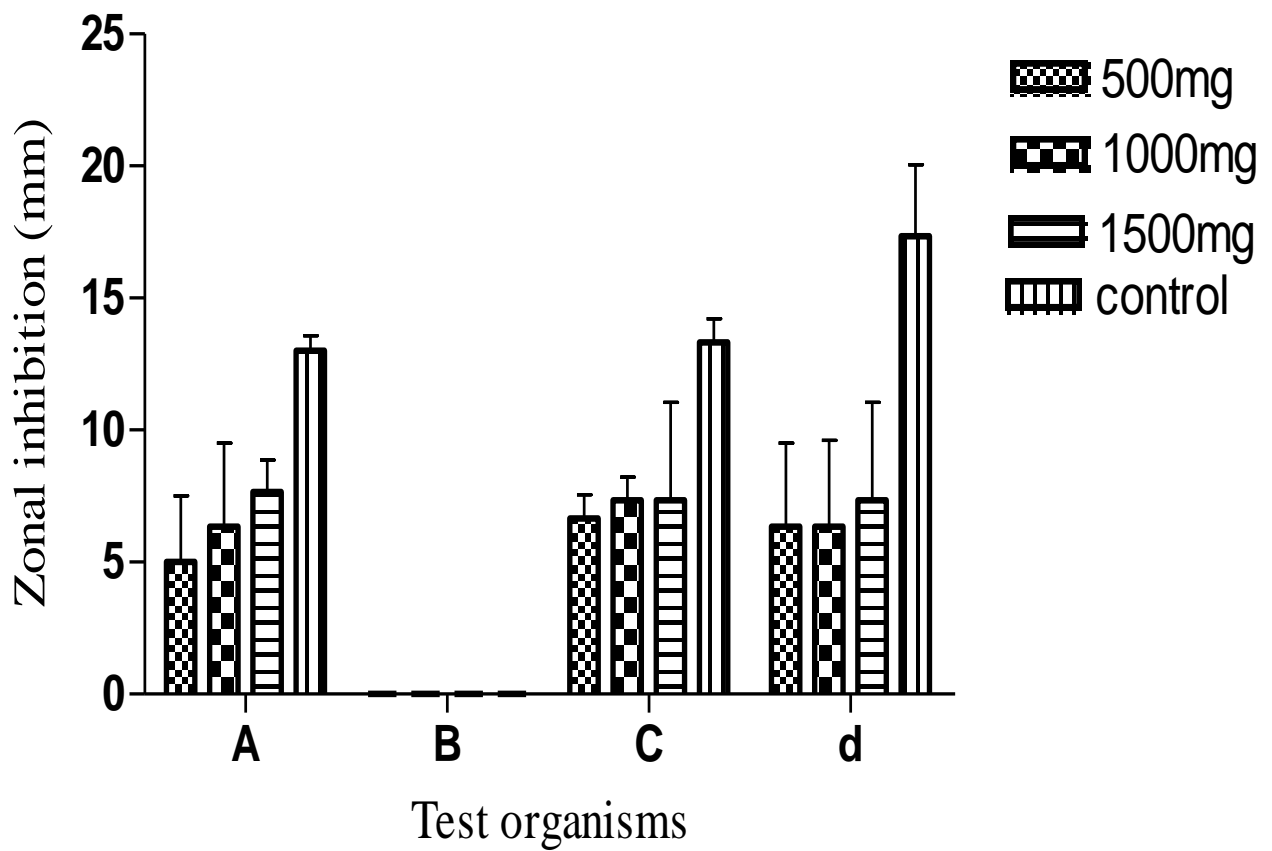
A-*Trichoderma* Spp, B-*Colletrichum gloeeosoprioides*, C- *Aspergillus niger*, D-*Rhizopus* Spp

The Susceptibility test for the fungal showed that *Joloo* was able to inhibit *Rhizopus spp*, followed by *Aspergillus niger* and *Trichoderma* spp but had no activity on colletrichum gleosoprioides.

Table 22; Physico-chemical properties of *Joloo* ppm

Extract	Ca	Se	Mn	Cu	Fe	Zn
H	6.04	2.64	-2.60	-0.36	9.16	0.25
S	21.31	1.86	-2.43	-0.36	38.53	0.31
Bu	11.77	2.97	-2.61	-0.47	25.57	0.32
O	38.50	3.47	-2.12	-0.39	36.93	0.17
T	16.26	2.77	-2.64	-0.47	26.37	0.66
X	22.86	3.72	-2.63	-0.43	30.42	0.12
A	40.01	2.93	-2.40	-0.40	25.81	0.30
Comb	7.36	2.59	-2.63	-0.52	16.17	0.46

H; *Hoslunda oppposita*, S; *Securidaca longepedunculata*, Bu; *Butyrospermom paradoxa*, O; *Olax subscorpioidea*, T; *Tetrapleura tetraptera,* X: *Xylopia aethiopica*, A; *Allium ascalonicum*, Comb; Combinned formulation '*Joloo*' Ca; calcium, Se; Selenium, Mn; Manganese, Cu; Copper, Fe; iron, Zn; Zinc. Ppm; part per million.

The various elements observed in *Joloo* in the atomic absorption spectrophotometry analysis are within recommended values and under standard regulations, no element showed threat of toxicosis.

CHAPTER FIVE

5.0 DISCUSSION

The use of botanical medicine is ancient, and plant chemicals are still the backbone of our pharmacopoeia because more than 50% of drugs used in Western pharmacopoeia are isolated from herbs or derived from modification of chemical compounds first found in plants (De Smet, 2002; Huxtable, 1990). If plants contain pharmacologically useful and active compounds, they probably may not be devoid of certain toxic substances (Huxtable, 1990).

Acute toxicity study is a useful parameter in estimating the Therapeutic Index (i.e., LD_{50}/EC_{50}) of drugs and xenobiotics (Rang *et al*., 2001). The oral LD_{50} value of the formulation in mice was 2000 mg kg^{-1} b. wt whereas that of the intraperitoneal administration was 400 mg kg^{-1} b. wt. The infusion of crude extract prepared contains many phytochemicals that could have direct access to the bloodstream intraperitoneally unlike the oral route where several barriers were encountered before absorption (Etuk *et al*., 2006). This is an indication that the cocktail may be toxic when administered intraperitoneally. Gathumbi *et al*., (2002) stated that the mode of administration of an herbal preparation is important in assessing the suitability of such preparation for therapeutic use. The high mortality recorded among the intraperitoneally treated mice in comparison with the low rate observed within the set that were orally treated, could also be attributed to the impurity of the crude formulation since medicines administered I.P have direct access to the bloodstream and must be pyrogen-free.

In the 28-days subchronic toxicity test showed absence of mortalities, adverse clinical signs, decrease in body weight as well as the lack of decrease in mean relative organ weight at doses of 400 and 800 mg kg^{-1} b. wt. This suggests that the cocktail '*Joloo*' may be relatively safe at low

doses. However, the 20% mortality recorded in the 1600 mg kg^{-1} b. wt dose group and the observed signs of emaciation and significant increase in weight may be attributed to increase in bone mass. This is because the surviving animals in this group were healthy as observed in the histopathologic studies. The Society of Toxicologic Pathology (STP) has established the evaluation of organ weights in toxicologic studies as an integral component in the assessment of pharmaceuticals, chemicals and medical devices (Sellers *et al*., 2007). Thus the histopathologic evaluation of vital organs which did not reflect any injury or damage may be suggestive that *Joloo* does not exhibit toxicity at tissue level under the studied period. Biological markers like endogenous enzymes have been shown and established to be organ-specific and can leak from a damaged or an injured organ (Kubavat and Asdaq, 2009). Hepatic function has been monitored by the evaluation of the levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspertate aminotransferase (AST) in conjunction with biochemical analytes such as cholesterol, creatine kinase and creatinine in the liver. The insignificant differences in the activity levels of the hepatic enzymes and biochemical analytes between the treated and control rats at lower doses is likely to show that *Joloo* has no notable toxicity at the sub-chronic level. Although the rats treated with 1600 mg kg^{-1} b. wt dose of *Joloo* appeared to show slight but insignificant increase in enzyme activities. *Joloo* showed an insignificant increase in antioxidants as indicated in the result of 28 day study but exhibited strong antioxidant activities during prolonged chronic studies. Histopathologic examinations did not reveal any treatment-related significant changes at all doses used as revealed by normal histo-architecture of organs studied. Haematologic analysis showed an increase in WBC while other parameters were not different from control. However, the lack of negative effect on WBC, neutrophils and PCV shows that *Joloo* does not exhibit any adverse effect on the defense mechanism of the body. Hematopoietic

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indices have been reported to be very sensitive to toxic compounds and serve as important index of physiologic and pathologic status for both animals and humans (Rosidah *et al*., 2009).

The repeated oral administration of *Joloo* for a period of 91 days in mice showed minor and insignificant variations in the body and some organs weight. The relative organ weight of the heart, liver, lungs and kidneys were comparable to control. However the brain and spleen were significantly different from control at higher doses.

It is well established that haematologic tests form the very front-line investigations on which diagnosis of various diseases is based. PCV, WBC and other haematologic parameters compared favorably to control except the lymphocytes which increased significantly. This may suggest that *Joloo* did not have any adverse effects on the general health of the animals but rather boosted their body's immunity. *Joloo* did not affect body weight; however abnormality in the histoarchitecture of organs has been regarded as reliable indication of toxicity (Singh *et al*., 2009) as characterized by mild necrosis which probably is an indication of early toxicity. The necrosis observed in the histoarchitechture of the spleen may be an indication of early immunotoxicity. The presence of pathologic necrosis in the liver and spleen is not unconnected with the fact that the liver is the main organ of biotransformation in the body (Morphosa *et al*., 2008), while the spleen filters foreign matters from the blood and involved in many metabolic disturbances (Kumar *et al.*, 2007), hence the organs may be exposed to the toxic or active agents, especially during prolonged usage and at high dose of 1600 mg kg^{-1} b.wt. There was significant increase in liver ALT and AST as well as kidney AST against control which may indicate hepatocellular damage, however, the activities of these enzymes are within range of non-toxic reference value (Levine, 1995). Moreso, the significant increase in albumen and total protein in both liver and kidney cells is an indication that no serious damage has occurred in the organs

(Cheesbrough, 2006). This is an indication that the liver and kidney might be target candidates for toxicity when exposed to prolong administration of *Joloo* at high dose of 1600mg.

Joloo exhibited significant (p< 0.05) dose-dependent increase in antioxidant enzymes and reduced lipid peroxidation during the chronic toxicity studies due to significant reduction of MDA. There was dose dependent, but insignificant increase in GSH though not significantly. Antioxidants are compounds that can delay, inhibit, or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. Oxidative stress is an imbalanced state where excessive quantities of reactive oxygen and/or nitrogen species (ROS/RNS, such as superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite) overcome endogenous antioxidant capacity, leading to oxidation of varieties of biomacromolecules, such as enzymes, proteins, DNA and lipids. Oxidative stress is important in the development of chronic degenerative diseases including coronary heart disease, cancer and aging (Jin and Russell, 2010). A significant increase in GPx was observed in the chronic studies, which implies that *Joloo* can protect cells against oxidative injury since GPx activity is not limited to H_2O_2, but covers a wide range of substrates from H_2O_2 to organic hydroperoxides (Rukkumani *et al*., 2004). The significant increase in SOD and CAT is an indication that *Joloo* can protect cells against oxidative stress, as these enzymes act as preventative antioxidant which plays important role in protection against deleterious effects of lipid peroxidation (Rukkumani *et al*., 2004). The antioxidant mechanism of *Joloo* may be attributed to one or more of the following; inhibition of oxidative enzymes like cytochrome P450, chelating and disarming oxidative properties of metal ions, scavenging or neutralizing of free radicals, interacting with oxidative cascade and preventing its expression and oxygen quenching and reducing its availability for oxidative reaction (Rukkumani *et al*., 2004). *Joloo* can therefore be regarded as a

good antioxidant. This chronic study has indicated that lower doses 400 and 800 mg kg^{-1} b. wt of *Joloo* are devoid of toxicity, whereas high dose (1600 mg kg^{-1} b. wt) is associated with some toxicity concerns especially under prolonged usage.

Genotoxic test revealed that *Joloo* induced a statistically significant ($p<0.05$) number of chromosome aberrations at high concentrations of 800 and 1600 mg and the abnormal chromosome types observed were vagrant, bridges, laggards and fragments. Rathore *et al*., (2006) showed that the extract from *Myrobalan* reduced mitotic index of *Allium* root tip cells but did not cause any chromosomal aberrations at the highest concentration. There was a dose response correlation in the growth of *Allium cepa* root in the cocktail. This gradual and significant ($p<0.05$) reduction in root growth at increasing concentration may suggest toxicity which caused the significant chromosomal aberrations and consequently resulting in a gradual and significant decline in mitotic index value. The decline in mitotic index value shows interference in the cell cycle. It has been observed that when the separation of chromosomes occurs during cell division, any chemical or toxic substance introduced into the system affects the structural integrity of the cell. Li *et al*., (2005) observed that a microtubule drug benomyl may lead to chromosome loss as a result of a defect in kinetochore-to-microtubule attachment during prometaphase, metaphase, or anaphase, if such a defect is not caught by the spindle assembly checkpoint and corrected.

The ability of the *Joloo* to reduce MI by inhibiting cell proliferation particularly at the metaphase and anaphase stage may be adjudged similar to the action of plant derived drugs such as Taxol, vinblastine and vincrestine. Vinblastine and vincristine block the process of microtubule assembly whereas Taxol stabilizes microtubule and promotes the formation of abnormal

microtubule bundles (Lewis, 2006). In either case, the mitotic spindle will be disrupted and cells cannot divide. It is also possible that the cocktail will have phytochemicals that may bind to proteins of the cell cycle, thereby influencing their activities and this would obviously affect cell division. *Joloo* exhibits genotoxic properties from this study which may be an indication that it is inhibiting the activity of one or more components of the cell cycle. Thus, this cocktail may indeed, prove valuable for managing tumors which corroborate its use in ethno medicine.

Apoptotic induction has been a continuous effort in cancer treatment. Huang *et al*., (2003) reported that reduction in cell growth and induction in cell death are two major ways to inhibit tumour growth. Apoptosis was evaluated using the MTT test, which monitors a reduction of yellow tetrazolium salt by mitochondrial dehydrogenase enzyme of metabolically active (viable) cells to purple formazan crystals. In the cytotoxic study different solvent extracts of *Joloo* caused significant apoptosis in VERO and CEF cell lines with IC_{50} ranges from 2.8-9.8 and 6.5-14.8 mg ml^{-1} respectively, so *Joloo* may have apoptotic properties. From the IC_{50} observed between treatments on the two cell lines, VERO was more susceptible to cytotoxicity than CEF cell lines. *A.ascalonicum*, water and chloroform fractions exhibited the strongest cytotoxicity on the VERO cells than others, whereas in the CEF cells water fractions and *S. longepedunculata* were the most potent. Generally *T. tetraptera* and chloroform fraction caused cell death at all the concentrations used on both cells. Apoptosis is one of the most potent defenses against cancer, since this process eliminates potentially deleterious, mutated cells (Martin, 2006). It is thought that mechanisms of apoptosis are mainly implicated in two signal pathways; the mitochondrial pathway and the cell death receptor pathway (Lui *et al*., 2006). The main occurrence in the mitochondrial pathway is the migration of cytochrome c from the mitochondria to the cytosol. Immediately cytochrome c arrives the cytosol, together with Aparf-1 activates caspase-9, which

then activates caspase-3 (Kidd, 1998). The cell death receptor pathway is characterized by binding of cell death ligands and cell death receptors and the subsequent activation of caspase-8 and 3 (Nijhawan *et al.,* 1997). All the treatments on VERO cells showed some degree of cytotoxicity especially at 2.5 and 5.0 mg ml^{-1} except *B. paradoxum* and hexane fraction that showed a very weak activity inducing apoptosis only at 5.0mg ml^{-1} concentration. This supposedly apoptotic effect of *A. ascalonicum* juxtaposed with the anti-angiogenesis result of Seyfi *et al*., (2010). Moreover, tumor growth and metastasis are angiogenesis-dependent (Hanahan, 1998). *Allium* plants have also been reported to possess anti-oxidative and anti-tumour properties (Ogra *et al.*, 2005). *S. longepedunculata, O. subscopoiidea, A. ascalonicum* and ethyl-acetate fraction did not produce any apoptotic effect on CEF implying that *Joloo* could be selective in affecting cells. This result is an indication that *Joloo* may possibly localize on tumour cells and induce the release of cytochrome c from mitochondria. It may also trigger caspase independent apoptosis if some of the cytochrome c released from mitochondria accumulates in the nucleus (Liu *et al*., 2006).

These genotoxic and antiproliferative activities observed in chromosomal aberrations of *Allium cepa* assay and CEF and VERO cytotoxicity assay respectively, may be attributed to the synergistic reaction of all the antitumour compounds in individual plants of the polyherbal '*Joloo'* which include ; diallyl sulfide (DAS) , ajoene, allicin , S-allyl cysteine (SAC) and S-allylmercaptocysteine (SAMC) in *A. ascalonicum* (Martin, 2006); triterpenoid saponins 3-*O*-[β-d-apifuranosyl-(1→3)-β-d-glucopyranosyl]oleanolic acid 28-*O*-[β-d-apifuranosyl-(1→3)-β-d-xylopyranosyl-(1→4)-[α-l-rhamnopyranosyl-(1→3)]-α-l-rhamnopyranosyl-(1→2)β-d-xylopyranoside in *Butyrospermum parkii* (Tapondjou *et al.,* 2011) ; indole alkaloids in *S. longipedunculata* (Costa *et al*., 1992); coumarins such as 7-hydroxycoumarin in *T. tetraptera*

(Kawaii *et al.*, 2000; 2001); ent-15-oxokaur-16-en-19-oic acid in *X. aethiopica* (Choumessi *et al.*, 2012); and oleanolic acid in *O. subscopioidea* (Fai *et al.*, 2009). Hence, justifies its folkloric use an antitumour agent.

The inhibitions of the two phases of formalin test by *Joloo* as well as increased the reaction time of the hot plate test observed in the analgesic study suggest that it is a centrally acting analgesic like morphine (Pastermak, 1993). The extract also inhibited the acetic acid-induced writhing in mice suggesting that the analgesic effect of the extract is also peripherally mediated (Ndebia *et al.*, 2007).

Investigations from the anti-inflammatory studies showed that the formulation reduced paw swelling as effectively as indomethacin and chlorpheniramine in a dose-dependent relationship. *Joloo* may therefore, be inhibiting mediators of acute inflammation as stated by Mossa *et al.*, (1995) and reported by Olaleye *et al.*, (2004). Many compounds have been proposed as inflammatory mediators released locally at the site of inflammation and having biological properties that cause the signs and symptoms of inflammation (Galti *et al.*, 2001).

Pyrexia could be induced by tissue damage, inflammation, infections, malignancy and other disease-states. The infected or damaged tissue serves as a pyrogenic stimulus and the pyrogens are phagocytized by the Kupffer cells, monocytes, macrophages etc. leading to the release of cytokines (cytokines e.g. interleukins and TNF-α), which increase the synthesis of prostaglandin E2 near the pre-optic hypothalamic area, thereby stimulating the hypothalamus to elevate the set point of normal body temperature (Spacer and Breder, 1994). Dinitrophenol (DNP) acts as a proton ionophore, across biological membranes. It defeats the proton gradient across mitochondrial membranes, collapsing the proton motive force that the cell uses in the production of most of its ATP chemical energy. Instead of producing ATP, the energy of the proton gradient

is lost as heat (Leftwich *et al.,* 1982), which usually result in increased level of intracellular calcium, muscle contraction and hyperthermia (Kumar *et al*., 2002) A dose-dependent significant reduction in pyrexia was observed in rats treated with *Joloo* in all the concentrations used and through-out the study. This suggests that *Joloo* possess a significant antipyretic effect in DNP-provoked hyperthermia in rats and its effect was comparable to the acetylsalicylic acid.   In general, non-steroidal anti-inflammatory drugs (NSAIDs) produce antipyretic action by inhibiting the production of prostaglandins in the hypothalamus (Binny *et al*., 2010). Since the hypothalamus regulates the body temperature, *Joloo* may have inhibited COX-2 expression and consequently inhibiting PGE_2 biosynthesis so as to reduce the elevated plasma level. Beside prostaglandin synthesis, there are several multiprocesses underlying the pathogenesis of fever. Inhibition of such mediators may cause antipyresis (Sahu *et al*., 2012). *Joloo* may also enhance the production of body own antipyretics like vasopressin and arginine (Okokon *et al*. 2011) and may have caused genetic down regulation of COX-2 expression in certain cell types. Generally antipyretics, analgesics and antiiflammatory drugs have the ability to inhibit eicosanoids like prostaglandins, prostacycline and thromboxanes which have been suggested to contribute to cancer and metastasis development, particularly in colorectal tumour (Subbaramaiah and Dannenberg, 2003), besides, Puntoni *et al*., (2008) suggested that anti-inflammation may be an important strategy in cancer management and treatment. Hence any extract that is antinflammatory, may be antipyretic and analgesic and vice versa, such may also possess antitumour properties. Since cancer cells are heterogeneous and endowed with complex, redundant, converging and diverging pathways spanning both the genetic and metabolic networks to gain growth advantages (Hsieh and Wu, 2008), a polyherbal like *Joloo* which may possibly possess multitarget facets like genotoxically causing chromosomal aberration,

cytotoxically inducing apoptosis, and pharmacologically inhibiting eicosanoids, may have better potential for the management and treatment of cancer.

It was found out that the polyherbal '*Joloo*' formula significantly reduced the parasitaemia dose-dependently in the suppressive model with 800 and 1600mg/kg doses, recording 100% chemosuppression. In the curative studies there was dose-dependent reduction in parasitaemia during the period of administration and subsequently parasitaemia gradually increased in the post administration period of observation. Studies have implicated some phytochemicals compounds like alkaloids, terpenes and their derivatives as antiplasmodial (Akpan *et al.,* 2012). The presence of these phytochemicals (as also observed in the course of this study) may have contributed to the antiplasmodial activity of the polyherbal formula.

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The histoarchitechture of the testes after 28 days oral administration of *Joloo* revealed a dose-dependent increase in germ cells in the seminiferous tubules and all parameters in the testes conserved. *Joloo* did not cause any spermatotoxic effect but indicated a high androgenic potential with dose-dependent increased hyperplasia from 400 to1600 mg kg^{-1} b. wt. This activity may be attributed to the highly   testicular antioxidant properties of *Joloo*.

The antimicrobial studies of *Joloo* showed that it inhibited the growth of almost all the bacterial test organisms with varying effectiveness. The antibacterial activity of *Joloo* increased dose-dependently. *Joloo* inhibited the growth of six out of the seven bacterial test organisms. This may be an indication of the presence of antibacterial properties in some of the constituent plants used in the preparation of *Joloo* like *S. longepedunculata* (Adebiyi *et al*., 2006), *X. aethiopica* (Tatsadjieu *et al*., 2003), *O. subscorpioidea* (Ayandele and Adebiyi, 2007), *A. ascalonicum*

(Amin *et al*., 2005) and *T. tetraptera* (NIMR, 2009). the result from this study also indicated *Proteus vulgari* as having highest sensitivity to *Joloo* with the highest zone of inhibition as demonstrated in a similar study by (Ayepola, 2009) followed by *Yersinia enterocolitica.* Gram negative bacteria are believed to have high resistance to antimicrobial agents (Ndukwe *et al.,* 2005; Ogundiya *et al.,* 2006). This could be linked to the cell walls of gram negative bacteria which have an outer phospholipid membrane with structural lipopolysaccharides components that make the cell wall impenetrable to antimicrobial compounds (Chessebrough, 2006). The highest antifungal activity was observed with *Rhizopus* spp. followed by *Trichoderma* spp. and *Aspergillus niger* with an MIC of 500mg ml^{-1}. However from this study it has been clearly shown that the formulation '*Joloo'* may have the ability to inhibit several different modes of action (inhibition of cell wall synthesis, protein synthesis, alternation of cell membrane function, inhibition of the nucleic acid synthesis and antimetabolite activities) of the Gram negative bacterial cells.

The Atomic absorption spectrophotometric (AAS) analysis of *Joloo* and its individual constituent plants showed that they all contain relevant trace elements like zinc, iron, and selenium and macro-element calcium in varying concentration, though within standard acceptable range. A growing body of evidence has indicated that many trace elements play an important role in a number of biological processes by activating or inhibiting enzymatic reactions, competing with other elements and metalloproteins for binding sites, affecting the permeability of cell membranes or through other mechanisms (Drake and Sky-Peck, 1989; Sky-Peck 1986). It is, therefore, reasonable to assume that these trace elements would exert action, directly or indirectly, on the carcinogenic process (Drake and Sky-Peck, 1989). Statistically

significant differences from the normal distribution of Fe, Cu, Zn, and Se have been reported to occur in patients with various forms of cancer (Trush and Kensler, 1991; Stevens and Nerishi, 1992). The relationship between breast cancer and these trace elements, Fe, Cu, Zn, and Se has also been investigated (Percudani *et al.,* 1981; Garofalo *et al.,* 1980; Kanias *et al.,* 1994). Selenium has been implicated as an anti-tumour and anti-oxidative, it is also effective against lukaemia, mammary and prostate tumours (Ogra *et al.,* 2005). It therefore implies that the mechanisms of pathogenesis could be mediated by direct effects of certain *Joloo* trace elements (*e.g.,* Fe, Cu) on the formation of hydroxyl free-radicals from hydrogen peroxide and superoxide via the Fenton and Haber-Weiss reactions, or influence of *Joloo* trace elements on intracellular concentration of Se, Fe, Cu, and Zn (Sunderman, 1986; Halliwell and Gutteridge, 1990). This study has revealed that the level of these trace elements in *Joloo* are within standard range devoid of toxicity, but rather capable of eliciting prophylaxic action or cure for cancer and other ailments since it is established that there is an association between oxidative stress and trace elements in patients with breast cancer (Huang *et al*., 1999).

6.0 SUMMARY OF FINDINGS AND CONTRIBUTION TO KNOWLEDGE

6.1 Summary of findings

The data derived from this study suggest that, *Joloo* does not possess acute and subchronic toxicity, so it is safe when administered orally especially at 400 and 800 mg kg^{-1} b. wt. It possesses cytotoxic properties on normal cell lines at concentrations tested indicating that it may have some apoptotic properties and doses could be manipulated for use against cancer cells. *Joloo* also produced considerable anti-inflammatory, analgesic, and antipyretic actions

confirming its pharmacological claims. The extract is safe when taken orally. However, continuous long-term use of *Joloo* at 1600 mg kg^{-1} b. wt can be toxic.

6.3 Contribution to Knowledge

This study has provided scientific basis for the use of this cocktail in the treatment of various diseases and thus making the following significant contributions to Knowledge:

1. *Joloo* is safe for short-term use preferably with 400 and 800 mg kg^{-1} b. wt doses

2. The antioxidant, anti-inflammatory, anti-pyretic, and analgesic, properties of *Joloo* justifies its traditional use as an anti-cancer formulation.

3. Portends promising drug-lead for anticancer agents due to its apoptotic properties

4. The study has partially standardized the galenical (extract cocktail)

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